"Exploring the Potential of the Physio-Mechanical Environment and Circadian Timing in Adult Progenitor Cell Differentiation"

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Abstract

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Optimising cell/tissue constructs so that they can be successfully accepted and integrated within a host body is an essential consideration in modern tissue engineering. To do this, adult progenitor cells are frequently utilised, but there are many aspects of their environment *in vivo* that are not completely understood. There is evidence to suggest that circadian rhythms and daily circadian temporal cues have substantial effects on progenitor cell activation and differentiation, and that the mammalian circadian rhythm may be influenced by the cell's mechanical environment.

Therefore, the hypothesis presented was that the physio-mechanical environment influences the differentiation capacity of progenitor cells and this may be mediated, at least in part, by the influence of the circadian rhythm. Moreover, it was also hypothesised that the responses of the progenitor cells, as a result of changes in the physio-mechanical environment or in the progenitor cells' circadian rhythms, may differ depending on the anatomical source that the cells were derived from.

To investigate this, a unique mechanical stretch paradigm was designed to subject human adult progenitor cells derived from bone marrow, dental pulp and subcutaneous adipose tissue to varying levels of mechanical stimulation. This set up was then modified to determine how further altering the progenitor cells' physio-mechanical environment affected their response to rhythmic mechanical stretch, in terms of their differentiation capacity and circadian rhythm, including alterations to the protein substrate and the supplementation of bioactive synthetic peptides.

The research presented here demonstrates that progenitor cells respond very differently to the same signals, depending on the cells' origin of derivation. This is likely to be possible due to the fact that the cell types differ in their signal transduction mechanisms; this is necessary as the cells will be exposed to different signals depending on the anatomical location of derivation, developmental origin and level of maturity. One way in which the progenitor cells are shown to differ includes that of their circadian rhythms. Cyclical uniaxial stretch is here presented as a novel methodology to synchronise the cellular circadian rhythm with the ability to even synchronise more primitive cells that did not respond to chemical entrainment.

It is here proposed that alterations in the cellular mechano-environment directly lead to changes in the circadian rhythm, which in turn affects the differentiation capacity of progenitor cells. Any alterations in progenitor cell function then have profound implications on tissue function, proposing a mechanism for how various pathologies become established. These findings also suggest that incorporating the temporal circadian information of different human adult progenitor cells will have profound implications in optimising tissue engineering approaches and progenitor cell therapies.

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1. Rogers, E. H., Fawcett, S. A., Pekovic-Vaughan, V., & Hunt, J. A. (2017). Comparing Circadian Dynamics in Primary Derived Stem Cells from Different Sources of Human Adult Tissue. *Stem cells international*, *2017*.
2. Rogers, E. H., Pekovic-Vaughan, V., & Hunt, J. A. (2018). Mechanical stretch and chronotherapeutic techniques for progenitor cell transplantation and biomaterials. *BioMedicine*, *8*(3).
3. Rogers, E. H., Hunt, J. A., & Pekovic-Vaughan, V. (2018). Adult stem cell maintenance and tissue regeneration around the clock: do impaired stem cell clocks drive age-associated tissue degeneration?. *Biogerontology*, 1-21.

These publications have been attached in the Appendix.

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However difficult life may seem, there is always something you can do and succeed at (SH).

List of Abbreviations

ADSC	Adipose-Derived Stem/Progenitor Cell
ASC	Adult Stem/Progenitor Cell
ANOVA	Analysis Of Variance
ARNTL/Bmal1	Aryl Hydrocarbon Receptor Nuclear Translocator-Like
BMSC	Bone Marrow Derived Mesenchymal Stem/Progenitor Cell
Cry	Cryptochrome
CXCL12	Chemokine C-X-C Motif Ligand
CXCR4	Chemokine C-X-C Motif Receptor 4
DPSC	Dental Pulp Derived Mesenchymal-Like Stem/Progenitor Cell
ESC	Embryonic Stem Cell
EPL	Expressed Protein Ligation
FAK	Focal Adhesion Kinase
GFAP	Glial Fibrillary Acidic Protein
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GPCR	G-Protein Coupled Receptors
HSC	Hematopoietic Stem/Progenitor Cell
hADSC	human ADSC
hBMSC	human BMSC
KO	Knockout
NPL	Native Peptide Ligation
NC	Negative Control
NR1D1/Rev-ErbAα	Nuclear Receptor Subfamily 1 Group D Member 1
OCN	Osteocalcin
Per1/2	Period Homolog 1/2
PPARγ	Peroxisome Proliferator-Activated Receptor γ
PTS	Protein Trans-Splicing
ROS	Reactive Oxygen Species
ROCK	Rho Kinase
SC	Scrambled siRNA Control
siRNA	Short Interfering RNA
SMCs	Smooth Muscle Cells

SPPS	Solid Phase Peptise Synthesis
Sox2	SRY (sex determining region Y)-Box 2
Sox9	SRY (sex determining region Y)-Box 9
SACC	Stretch-Activated Cation Channel
SCN	Suprachiasmatic Nucleus
UT	Untreated Control

Chapter One

Introduction

1 Introduction

1.1 Adult Progenitor Cells

Pioneering experiments in stem/progenitor cell research are often credited to Canadian scientists, Becker, Till & McCulloch, in 1961-1963, who were the first to carry out clonal colony formation assays on hematopoietic stem/progenitor cells (HSCs) derived from murine bone marrow[1], indicating their multipotency. Since then, stem/progenitor cell research has progressed exponentially, with scientific breakthroughs occurring on an incredibly frequent basis. Stem cells are characterized by their unique capacity to both self-renew through cell division and to differentiate into an impressive range of tissue-specific cells in response to endogenous or external stimuli. All tissues rely on this regeneration process which is vital for maintenance and repair processes. It is these regenerative and homeostatic roles that tissue-specific stem cells routinely carry out to maintain and repair tissues, which provide their value in terms of their ability to be enhanced, stimulated, increased in numbers and localised in response to disease. Indeed, adult stem/progenitor cells are being used to treat an ever-increasing array of conditions, varying from heart disease to leukaemia. But in order for this to become a therapeutic reality, the processes that underlie these abilities must be fully uncovered and the factors that regulate self-renewal, proliferation and differentiation must be fully understood, as this regeneration process is vital for the repair and replenishment of many vital tissues.

Adult stem/progenitor cell (ASC) classification has become highly complex, since the original terms ‘haematopoietic’ and ‘mesenchymal’ covered the main types of these pluripotent cells. They now tend to be broadly classified based on their tissue of origin and differentiation potential, and one should perhaps increasingly replace the use of the term adult ‘stem cells’ in favour of adult ‘progenitor cells’, as they are somatic cells. Adult progenitor cells can be derived from seemingly all parts of the body, with high numbers found in stem cell niches. Progenitor cells have been derived and utilised from cord and peripheral blood, blood vessels and bone marrow (mesenchymal and hematopoietic stem cells)[1–3], the brain (neural stem cells)[4], skin (epidermal stem

cells)[5], skeletal muscle (muscle satellite cells/myogenic stem cells)[6], teeth (dental pulp stem cells)[7], heart (cardiac stem cells)[8], gut (intestinal stem cells)[9], liver (hepatic stem cells)[10], ovarian epithelium (ovarian stem cells)[11], breast (mammary stem cells)[12], hair follicle germ and bulge (epidermal stem cells)[13] and testis (testicular stem cells)[14].

ASCs are multipotent and are, by that definition, limited to differentiating into different cell types from their tissue of origin. They divide either symmetrically, to produce two identical cells which self-renew, proliferate and expand in number following injury or during development, or asymmetrically to produce one identical progenitor cell and one committed daughter cell, which maintain progeny population (Figure 1.1). The form of division that occurs depends on developmental and environmental signals received. It has been suggested that most ASCs have the ability to switch between asymmetric and symmetric division methods, and that the balance between the two is often disrupted in disease states[15].

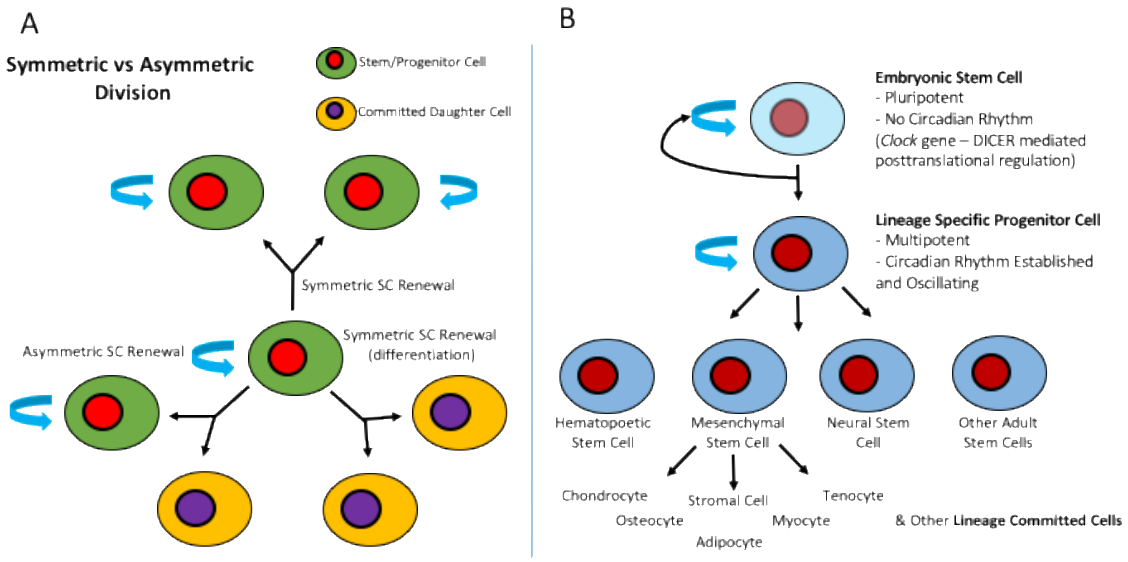

Figure 1.1. Stem/Progenitor Cell Division. (A) Adult stem cells are capable of dividing either symmetrically, to produce two identical stem cells or two identical daughter cells, or asymmetrically, to produce one identical stem cell and one committed daughter cell. (B) The hierarchy of stem cell division. Modified from Rogers et al. (2018)[16].

When comparing embryonic stem cells to ASCs, embryonic stem cells (ESCs) are pluripotent, meaning that they are capable of differentiating into any one of the three germ layers; endoderm, mesoderm or ectoderm. Since their discovery, there has been a great interest in the use of ESCs in regenerative medicine and tissue engineering,

due to their pluripotent differentiation capabilities. However, this pluripotency makes it difficult to direct their differentiation in a reliable, long term and reproducible manner. Furthermore, many *in vivo* studies have shown that ESCs, following implantation, can spontaneously differentiate and form a type of tumour called a teratoma[17]. ASCs do not demonstrate these limitations in *in vivo* models, and so there is a substantial interest in using ASCs in regenerative medicine. However, ASCs are more committed than ESCs, and so they have a more limited differentiation potential. Nevertheless, ASCs such as bone marrow derived mesenchymal stem cells (BMSCs), hematopoietic stem cells (HSCs) derived from bone marrow and cord blood, and adipose-derived stem cells (ADSCs) are all attractive targets as they still have varied differentiation potentials and are able to differentiate into a variety of cell types. For example, BMSCs are able to differentiate into bone, cartilage, fat, tendon, muscle, and marrow stroma[18], and HSCs are able to differentiate into both myeloid (including monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, dendritic cells, and megakaryocytes or platelets) and lymphoid (T cells, B cells, and natural killer cells) lineages of blood cells. These ASCs are also advantageous as they present an ease of harvest, isolation and expansion *in vitro*, when compared to ESCs. The impressive multi-lineage differentiation potential of ASCs is made possible by the broad combination of chemical, biological and physical signals present in the stem and progenitor cell niches, which direct and control their fate.

1.2 Human Adult Progenitor Cell Sources

Traditionally, the majority of progenitor cell research has focused on BMSCs, but recently, there has been an increasing drive to investigate the use of *Oct4* expressing dental pulp derived mesenchymal-like stem cells (DPSCs) in tissue engineering, as the differentiation capacity of mesenchymal stem cells (MSCs) may be restricted by their tissue of origin. These newly discovered cells offer a strong alternative to BMSCs, as they have been shown to have an even more widely varying differentiation potential than BMSCs. DPSCs have also been shown to have a faster proliferation rate as well as the potential to differentiate into not only mesenchymal cell types, but also into

neurogenic cells[19]. Their anatomical source location in the mouth also offers attractive advantages in terms of their accessibility, as tooth dental pulp is much more easily reached than bone marrow.

DPSCs are theorised to be able to differentiate into neural cells as they originate from the early migrating neural crest cells during embryogenesis[19], when migrating neural crest cells are known to differentiate into a wide variety of cell types, including neuronal cells. Dental pulp therefore consists of ecto-mesenchymal elements, containing neural crest-derived cells that exhibit both multipotency and plasticity[20]. This retained plasticity is a result of the progenitor cells' residency within the dental pulp, which is extremely protected from external stimuli in a "sealed niche", and the embryonic tissues found there remain undifferentiated within the jaws until the only organogenesis event which occurs after birth.

Another, more accessible source of adult stem cells that is currently being investigated is from adipose tissue (ADSCs); adipose tissue offers an abundant source of MSCs which can be obtained in large quantities under local anaesthetic with minimal patient discomfort, in contrast to bone marrow isolation. Dental pulp isolation, however, provides a less abundant MSC source than ADSCs, but similarly can be accessed more easily than BMSCs from wisdom teeth and exfoliated deciduous teeth. ADSCs and DPSCs show further similarity in that both types of progenitor cell can differentiate into functional odontoblastic-like cells upon appropriate stimulation[21] and both cells from either location are capable of growing self-assembled new teeth with high success rates[22], implicating both DPSCs and ADSCs in regenerative dentistry. Both DPSCs and ADSCs are more favourable to BMSCs as they also result in much lower site morbidity.

All three sources of MSCs have been investigated in terms of their differentiation capacities; for example, Davies et al. (2015) found that DPSCs exhibited the highest potential to produce mineralised matrix, but ADSCs and BMSCs showed enhanced dentinogenic and mineral volume[23]. Stanko et al. (2014) found that these three cell types showed no differences in terms of cell morphology or MSC surface marker expression, with all of the investigated progenitor cell types exhibiting fibroblastic morphologies[24]. However, they did find significant differences regarding the

expression of several pluripotency genes; BMSCs and ADSCs produced similar protein levels of several pluripotency markers but the DPSCs showed significant differences in the amount of protein products observed, including a lower expression of *Oct3/4*. These differences were hypothesised to reflect the mixed embryonic stem cell origin of DPSCs, which was also reflected in a higher level of expression of *Goosecoid*, a gene implicated in craniofacial and rib development in embryogenesis[25]. Furthermore, DPSCs were also demonstrated to have higher levels of *Sox2* expression than BMSCs and ADSCs, a key pluripotency factor that has been implicated in neuronal differentiation, again emphasising the role of DPSCs in neurogenesis[24].

1.3 The Role of Mechanical Stimulation and Stretch on ASC Differentiation

MSCs have been previously shown to be highly mechanosensitive, and therefore this may present the ideal method to non-invasively steer their differentiation, as mechanical forces have the capability of influencing progenitor cell behaviour. In the body, various tissues are exposed to varying amounts of mechanical force, which then influences their formation and functionality; for example, in the body, MSCs found within adipose tissue (ADSCs), will be exposed to vastly different levels of external force than those found within the bone marrow (BMSCs), teeth (DPSCs), tendon or muscle. This loading is vital in the maintenance of such tissue; when there is an increase in load, for example, during exercise, then this results in increases in bone and muscle mass[26], but decreases in load, as experienced in space flight or following injuries such as spinal cord damage, results in decreases[27].

There are many different types of force that may be applied to MSCs *in vivo* and *in vitro*. In the bone marrow alone, MSCs may be subject to extrinsic stresses such as tension, compression and fluid-induced shear stress, as well as intrinsic stresses such as substrate stiffness, and these are all thought to have individual roles on the different differentiation pathways that an MSC may differentiate down. However, the optimum conditions to control this lineage specific differentiation of MSCs remains unknown, and the optimum loading magnitude, duration, frequency and force type for each differentiation pathway remain unspecified. In a review by Smith and Reilly

(2012), the authors examine how each of following types of force may effect MSC maintenance and differentiation: stretching (tensile stress), hydrostatic pressure or platen abutament (compressive stress), fluid flow (shear stress), ultrasound, high frequency low magnitude displacement (vibration) and direct cell membrane magnetic stimuli, in both 2D and 3D culture[28].

There are several proposed methods that may explain how the extracellular mechanical stimuli may be converted into biochemical signals, which ultimately lead to the cellular changes seen post-stimulation. One such mechanotransduction mechanism proposes that cell membrane mechanoreceptors, including integrins, g-protein coupled receptors (GPCRs) and stretch activated ion channels signal these changes in mechanical stimuli into the cell. Regarding integrins, it is thought that the force pulls on an integrin-ligand bond, which is then transferred across the cell membrane and alters the cytoskeletal structure. For stretch activated ion channels and GPCRs, it is thought that the stretch or external force leads to deformation of the plasma membrane, which results in an ion flux into and out of the cell through the receptors[29]. Indeed, when MSCs are strained in the presence of the stretch-activated cation channel (SACC) blocker, gadolinium chloride ($GdCl_3$), there is a reduction in the otherwise observed induction of collagen I expression[30], suggesting a role for these channels in the transduction of mechanical stimulation. In the case of fluid flow, it is also thought that the glycocalyx, a GAG-proteoglycan rich layer that surrounds the cell membrane, may create drag force when fluid passes over, which again results in plasma membrane deformation[31,32]. A final proposed mechanotransduction mechanism, again relevant to fluid flow, insinuates the primary cilium as a mechanosensor, as they have been shown to bend under fluid flow and contain various signalling receptors[33]. However, this review will be mostly focusing on the effect of mechanical stretch or tensile stress in adult MSCs.

In vitro, mechanical stretch is usually applied using a mechanical stretch system available commercially, e.g. the “Flexercell” (Flexcell International Corp.), or a custom-built device designed to deliver uniaxial mechanical strain at varying frequencies and magnitudes. The usage of one such machine can be seen in a protocol made available by Kurpinski and Song (2007)[34]. Uniaxial strain is typically selected over equiaxial strain

as it is thought to better mimic the type of mechanical strain exhibited by MSCs in the body (Figure 1.2). For example, research by Park et al. (2004) directly compared the effects of uniaxial vs equiaxial strain in MSCs to find that the different modes of stretch do induce different responses. Cyclic equiaxial stretch is here shown to downregulate the smooth muscle differentiation markers SM α-actin and SM-22α and causes decreases α-actin in stress fibres. In contrast, cyclic uniaxial strain transiently increases the levels of SM α-actin and SM-22α, suggesting that this method better mimics the type of mechanical strain experienced in MSCs and smooth muscle cells (SMCs) and may promote the differentiation of MSCs into SMCs[35]. As is the case in this study, along with many others, silicone is usually the selected material used as a substrate to study the behaviour of cells under mechanical stimulation. It is selected due to its biological inertness, translucency, compliance and manipulability, and can be coated in fibronectin to allow for good cell adhesion and proliferation. Flexible silicone substrates can also be used to study the effects of mechanical stretch in 3D; research by O'Caerbhaill et al. (2007) shows that it can be constructed in tubular form to form 'pseudovessels', whereby cells can be subjected to a combination of pulsatile flow, radial distension and shear stress. Here, the MSCs are shown to be mechanosensitive, and reorientate parallel to the direction of flow and adapt their morphology in response to the stretch and other forces that they are subjected to[36].

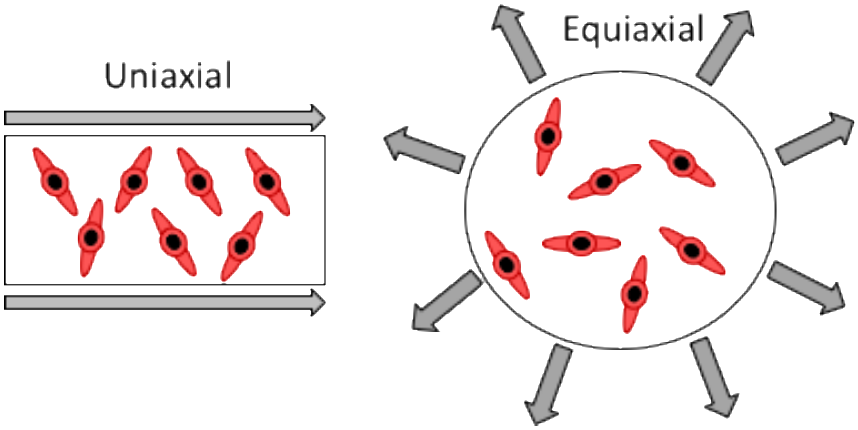

Figure 1.2. Comparison of uniaxial and equiaxial strain. Modified from Rogers et al. (2018)[37].

The effect of cyclic uniaxial strain on the morphology of cells cultured on elastic substrates has been studied in great detail. Stretching results in alterations in cell orientation and the cells tend to align perpendicularly to the load axis, in order to lessen the strain on their cell bodies and decrease the stretch of cytoskeletal elements and intercellular filaments[38,39]. Mechanical stretch can also be investigated in conjunction with the addition of chemical agents, so that their effects can be compared with and without the addition of such chemicals, and with and without stretch. For example, Rashidi et al. (2012) combine stretch with growth factor treatment and found a significant upregulation of smooth muscle cell specific markers and the alignment of cells perpendicular to the strain direction during loading time. They also observed cell elongation and F-actin fibres alignment and reorganisation[40]. The degree of morphological alteration can be seen by these changes in cell orientation and alignment, and both depend on strain amplitude[41], rate and duration[42].

Parankakh et al. (2017) sought to investigate the effects of the duration on cyclic stretch on cytoskeletal reorganisation and morphology of human BMSCs (hBMSCs) in a stepwise manner, in order to closely study morphological and phenotypic changes, as the two often occur simultaneously. The researchers found that different durations of stretch did influence the resulting changes observed[43]. Morita et al. (2013) also sought to define the optimum conditions for the cyclic stretching of hBMSCs. Here, the authors used a 2D inhomogeneous membrane strain field by removing one side holes of an elastic chamber in a commonly used uniaxial stretching device and found the axial strain threshold of hBMSCs was 4.4 ± 0.3 %[44]. Once the optimum parameters for cyclic cellular stretch are defined, this will offer a non-invasive methodology by which human BMSCs may be controlled and manipulated, so that they can be optimised for tissue engineering technologies.

1.4 The Effect of Tensile Strain on Progenitor Cell Proliferation and Differentiation

1.4.1 Proliferation

The effects that radial distention, or mechanical stretch, can bring about have been investigated in a plethora of studies, with each varying the parameters in different ways. For example, in rat MSCs isolated from bone marrow and subjected to cyclical equiaxuial stretch, the OD values of rat MSCs increase in a time-dependent and magnitude-dependent manner after being exposed to 2-8% stretch within 15-60 min at a frequency of 1 Hz. The expression of *c-fos* in these cells is also significantly higher when the cells are stretched (1 Hz, 8% strain and 60 min), when compared to static controls. Taken together, this suggests that mechanical stretch could regulate cell growth and proliferation[45]. In human cells, fibronectin coated silicone chambers have again been utilised to stretch hMSCs, and here it has also been documented that short time strain application does affect proliferation, however the protein coating of fibronectin was not found to influence MSC proliferation in any way. The authors here looked at the modulation of stretch magnitude frequency and duration and found that a frequency of 1Hz was most effective at stimulating human MSC proliferation. At a frequency of 1Hz and durations of 15, 30, 60 min, 5% strain was found to significantly increase MSC proliferation. Proliferation was also enhanced at 10% strain, 1Hz for 15 and 30 min durations, but proliferation was decreased at 60 min. At 1Hz, 15% strain, proliferation was reduced following 15 min durations, but increased following 30 and 60 min durations. Long-time strain applications (12 and 24h) were found to block the proliferation[46]. Taken together, both of these studies highlight how crucial getting the strain application conditions are in getting the optimum result, and that mechanical strain does have the potential to influence MSC proliferation.

The mechanisms that underlie the transduction of mechanical stretch information onto cellular proliferation have been investigated. In a follow up study using the same rat MSCs as mentioned previously, it has been reported that although no change in the expression of total extracellular signal-regulated kinase 1/2 (t-ERK1/2) is observed

at the protein level, the level of phosphorylation of ERK1/2 (p-ERK1/2) is increased after stretch. When rat MSCs are treated with inhibitors of ERK1/2 activity, however, there is a suppression of stretch-induced increase in p-ERK1/2 and the mRNA expression of *c-fos*, along with an abolition of the increase in stretch-induced proliferation, suggesting that ERK1/2 is crucial in the stretch-induced effect on proliferation in rat MSCs[47].

1.4.2 Myogenesis

In a study by Ghazanfari et al. (2009), the authors found data that suggested not only that cyclic strain enhanced proliferation, in agreement with the studies above, but also that the cyclic strain lead to increases in smooth muscle α-actin, reoriented actin fibres and also led to the differentiation of human MSCs into SMCs, without the addition of growth factors[48], highlighting the notion that mechanical stress can be used to enhance smooth muscle myogenesis[49]. This can also be observed in skeletal muscle myogenesis, which is expected as both fetal and adult skeletal muscle is constantly subjected to biomechanical forces in the body. One study utilised a 10% uniaxial strain at a frequency of 1 Hz on human MSCs cultured on collagen-coated silicone substrates and found that following loading, there was a rearrangement of cells and initiation of myogenic differentiation, as determined by levels of MyoD and MyoG mRNA levels, indicating that cyclic strain may be used to differentiate progenitor cells myogenically[50].

Cardiomyocytes are also subject to cyclic strain in the body, as induced by the rhythmic beating of the heart. When rat BMSCs are subjected to cyclic strain application, it has been shown that this may be sufficient to induce cardiomyogenic differentiation in itself, as can be confirmed by the induction of cardiomyocyte-related markers[51]. This too suggests that mechanical stimulation could be a novel mechanism to control adult progenitor cell differentiation.

1.4.3 Osteogenesis

Another part of the body in which adult progenitor cells reside and are subjected to varying amounts of mechanical conditioning is in the bone, where BMSCs are

subjected to external forces such as tension, compression and fluid-induced shear stress. Mechanical stimulation is so crucial in bone formation and maintenance that "distraction osteogenesis" has been defined; which is an active process of bone regeneration that occurs under mechanical stimulation. This process has been mimicked experimentally, whereby rat MSCs have been subjected to short periods of cyclical mechanical strain (40 min, 2000 microstrains). Following this, it can be observed that mechanical strain promotes ALP activity, as is characteristic of the initiation of bone formation, and enhances bone marker genes *Cbfa1* and *Ets-1*, showing that mechanical strain may act as a stimulator of osteogenic differentiation[52,53]. Kearney et al. (2010) also document an increased expression of osteogenic markers following cyclic tensile mechanical strain of 2.5% at 0.17 Hz for 1–14 days; the osteogenic markers Cbfα1, collagen type I, osteocalcin, and BMP2 are temporally expressed following mechanical stretch. However, this strain-induced increase in BMP2 can be reduced by the inhibitors of the kinases, ERK, p38, and PI3 kinase. The authors here found that long term application of strain reduced the proliferative capacity of MSCs, supporting the notion that although short-term strains may increase proliferation, long-term strains do not appear to[30]. It has also been shown that both osteoblastogenesis and osteoclastogenesis are influenced by mechanical stimulation, demonstrating how far reaching the effects of mechanical load are in bone formation and maintenance[54,55].

There are many signalling pathways that have been implicated regarding how mechanical stimulation effects osteogenesis, for example, one study shows the onset of osteogenic differentiation following mechanical stimulation is thought to be dependent on ERK1/2-Runx2 signalling[54]. Another study investigating human BMSCs reports an induction of FosB, a member of the AP-1 family of transcription factors that regulate osteogenic differentiation and bone formation, following stretch in a stretch- and time-dependent manner[56]. The p38MAPK-osterix pathway has also been implicated; intermittent stretching has been found to promote the expression of osterix mRNA, along with ALP, collagen I and osteocalcin, and the protein levels of osterix and phosphorylated p38MAPK are elevated following stretch. When osterix is silenced, a reduction in the levels of ALP, collagen I and osteocalcin mRNA are also

observed, showing that this pathway may also have an important role in stretch-induced osteogenesis[57]. A further factor that has been implicated in osteogenic differentiation following tensile strain, without the addition of osteogenic supplements, is BMP-2[58]. Interestingly, even adipose derived MSCs (ADSCs) can be induced to express increased levels of BMP-2 and *Runx2* following cyclic tensile strain of 6 hours, underlining the suggestion that cyclic tensile stretch may modulate osteogenic differentiation, via the BMP-2 signalling pathway[59].

In direct contrast to the above notion that mechanical stretch results in increased osteogenesis in MSC derived from differing sources, this does not appear to be the case in all MSCs. MSCs derived from tooth dental pulp (DPSCs) appear to show the opposite, and instead exhibit increased levels of proliferation and decreased osteogenic potential following uniaxial mechanical stretch[60]. Indeed, when human DPSCs are exposed to cyclic tensile stretch, the expression of osteogenic marker genes and proteins including BMP-2, osteocalcin and ALP are reduced, along with the odontogenic marker genes and proteins DSPP, DSP and BSP, suggesting that cyclic tensile stretch inhibits both osteogenic and odontogenic differentiation in DPSCs[61]. The differences in the response of the MSCs depending on their tissue of origin may be due to the fact that DPSCs reside within a unique niche in the body, where they are subject to extreme mechanical stresses by jaw movement, occlusal forces and hydrostatic pressures. DPSCs are one of the few progenitor cell niches to also experience thermal shock and extreme temperature fluctuations, and so are likely to respond differently to this form of stimulation.

Apart from in these unique DPSCs, the effect of mechanical stimulation is profound on the osteogenic differentiation capabilities of MSCs, having such a strong impact that it forces even adipose derived progenitor cells (ADSCs) to undergo osteogenesis and inhibits their adipogenic differentiation potential, even when cultured in the presence of adipogenic medium[62].

1.4.4 Adipogenesis

The theory that mechanical stimulation inhibits adipogenesis has been widely explored in scientific research. Initially investigated in C2C12 myoblasts, the myoblast-to-adipocyte differentiation is found to be significantly inhibited by cyclic mechanical stretch (20% elongation), which is seen alongside an enhanced expression of Wnt10b mRNA. By inhibiting Wnt signalling with a Wnt ligand, such as sFRP-2, this inhibition of adipogenesis is abolished, showing that mechanical stretch may inhibit adipogenesis through Wnt signalling[63]. Normally, when MSCs are cultured in adipogenic medium, they express peroxisome proliferator-activated receptor γ (PPARγ) and adiponectin mRNA and protein and accumulate intracellular lipids. However, when mechanical strain is applied to the MSCs for 6 h per day for 5 days, the expression of PPARγ and adiponectin is inhibited, and the decrease seen in active and total β-catenin typically exhibited during adipogenesis is prevented. Mechanical strain is also thought to inactivate glycogen synthase kinase-3β, suggesting that stretch transmits anti-adipogenic signals via this pathway, by stimulating a durable β-catenin signal[64]. Another signalling pathway that is affected by mechanical stretch and has been implicated in the commitment of MSCs towards adipocytes is the BMP pathway, as it is thought that stretching causes a downregulation of BMP4 induction of MSC adipogenesis. When MSCs are pretreated with BMP4 and then subjected to tensile stretch conditions (10% strain, 0.25 Hz, 120 min/day), it was found that the stretch supressed BMP4 induction of MSC adipogenesis and downregulated PPARγ, C/EBPα and aP2 adipogenic transcription markers and lipid accumulation. Here, it was found that the cell stretch did not affect BMP4-inducted activation in Smad or p38, as it would normally signal through Smad1/5/8 and p38MAPK, but instead the tensile stretch caused significant ERK1/2 phosphorylation. When ERK signalling is blocked, the stretch suppression of BMP4-induced MSC adipogenesis was significantly deteriorated, suggesting that stretch suppresses BMP4-induced adipogenesis by upregulating ERK[65]. However, this may be specific to only BMP4-induced adipogenesis, as Li et al. (2015) reported that mechanical stretch did indeed upregulate levels of phosphorylated Smad2, along with PPARγ-2, adiponectin and C/EBPα. Here, the authors found that pretreating MSCs with TGFβ1/Smad2 pathway antagonists

suppressed this increase in Smad2 phosphorylation, whereas pretreatment with TGFβ1/Smad2 signalling agonists facilitated the inhibitory effect of stretch on the adipogenic differentiation markers, suggesting that the anti-adipogenic effects of stretch are mediated in some way by the activation of the TGFβ1/Smad2 signalling pathway[66].

1.4.5 Tenogenesis

Another way that human adult MSCs are thought to possess clinically useful tissue-regenerative properties is for the process of tendon tissue engineering, whereby they may be used to generate tenocytes for use in cell therapy. One way that this could be controlled and utilised is by using mechanical stretch techniques. At low-magnitude stretch, MSCs express osteogenesis differentiation marker genes, in agreement to the section above, but, interestingly, when stretched using high-magnitude stretch for long periods, the tendon and ligament related genes are instead upregulated. For example, after being stretched at 10% magnitude for 48h, the expression of tenogenesis markers type I collagen, type III collagen, and tenascin-C are significantly increased[67]. This research is supported by findings from Morita et al. (2013), who show that a cyclic uniaxial stretch magnitude of 10% is the most efficient magnitude for inducing the differentiation of human BMSCs into tenocytes[68].

In the generation of tendon- or ligament-like tissue, MSC-seeded 3D collagen gels are frequently utilised under static or dynamic tension, the latter of which leads to enhanced tendinous tissue development. Cyclic stretching has been found to be beneficial to this 3D system as it allows for the expression of the tendon marker scleraxis to be maintained, where it would have normally dropped off in expression, and there are vast changes in matrix deposition and remodelling activity under dynamic loading conditions. Furthermore, differentiation regulation of MMPs can be observed, with little change in collagen mRNA levels, giving insight to the mechanisms of tenogenesis following mechanical stimulation of MSCs[69].

The pathways that underlie how mechanical stretch impacts tenogenesis have also been investigated. RhoA/ROCK and FAK have been found to regulate the mechanical-stretch induced realignment of human MSCs through cytoskeletal organization; both

RhoA/ROCK and cytoskeletal organization have been found to be essential in the phosphorylation of FAK at Tyr397 after being subjected to mechanical stimulation. This process can also be blocked by inhibiting either RhoA/ROCK, cytoskeletal organisation or FAK, implicating that these three are all vital components of the signalling network that senses mechanical stretch and then drives the tenogenic differentiation of human MSCs[70]. Furthermore, when calcium signalling is disrupted in human MSCs, by blocking stretch activated calcium channels (SACC) with galolinium, before and whilst they are subjected cyclic uniaxial tensile stretching, almost all tenogenic differentiation marker expression enhancement and ECM production is lost, suggesting that SACC also act as a mechanosensor in the strain-induced human MSC tenogenesis[71].

1.4.6 Angiogenesis

The final major differentiation process which has been investigated in MSCs following mechanical stretch is regarding angiogenesis. Mechanical stretch has been found to increase the angiogenic capacity of MSCs via VEGFA induction, as well as increasing the survivability of MSCs under nutrient deprivation. The proposed mechanism by which both of these changes is thought to occur is via the activation and manipulation of NFκB; when subjected to stretch, there is an increase of nuclear localization of NFκB activity p65, which coincides with the increase in VEGFA expression and apoptosis resistance. When NFκB activity is inhibited, these pro-angiogenesis and anti-apoptosis functions are blocked, highlighting the significance of NFκB in the pro-angiogenic response following mechanical stretch[72].

There are a vast number of ways by which mechanical stretch can influence the many different differentiation pathways MSCs are capable of, and the implications for increasing the therapeutic potential of MSCs are extremely exciting. Whether stretch is applied as a preconditioning technique or loading throughout, for long or short durations and at different magnitudes, there are many ways it can be utilised, which will only become clearer in the years to come.

1.4.7 Other Effects on Progenitor Cells Brought About By Mechanical Strain

In agreement with the above study, work by Kearney et al. (2008) has also showed that mechanical strain can have wide reaching effects on the maintenance of MSCs; here the authors also reported that cyclic uniaxial stretch affects the apoptosis of MSCs. However, the authors instead reported that strains of 7.5% or greater, over a duration of three days, lead to an induction of apoptosis, with maximal apoptosis occurring at 10% of strain[73]. High magnitude levels of mechanical strain are also thought to negatively impact MSCs as extreme magnitude stretch leads to oxygen free radical disequilibrium; when BMSCs from children are loaded with cyclic tensile strain, >12% magnitude stretch is found to enhance reactive oxygen species (ROS) synthesis, decrease the activity of superoxide dismutase and increase levels of malondialdehyde, in a time and magnitude dependent manner[74]. Another cellular function impacted by the mechanical microenvironment is the intracellular calcium dynamics, as calcium oscillations can be effected by external mechanical cues; prolonged mechanical stretch leads to intracellular calcium oscillations in human MSCs, as mediated by the cytoskeletal support, actomyosin contractility and phospholipase C (PLC) activity, showing another way by which the mechanical environment can regulate cellular functions[75].

Cyclic substrate deformation can also affect MSCs in terms of their matrix structure and formation. For example, equibiaxial cyclic strain (3%, 0.25 Hz) has been found to increase matrix mineralisation as well as inhibiting proliferation; here the strain was found to activate ERK1/2 and p38 MAPK pathways. When ERK1/2 was inhibited, this lead to an attenuation of calcium deposition, suggesting that strain-induced mineralisation is mediated by ERK1/2 signalling[76]. Furthermore, an increase in total collagen synthesis can be observed when MSCs are subjected to stretch conditions[77]. Research by Heo et al. (2015) found that short term dynamic loading causes increases in chromatin condensation, mediated by actomyosin based cellular contractility and the activity of the histone-lysine N-methyltransferase EZH2. These changes then stiffened the MSC nucleus, making it less deformable when subjected to stretch conditions. The authors also identified ATP release and calcium signalling induced by

mechanical stretch as the mediators of this condensation process. Following being subjected to stretch, it was also found that the cells retained a 'mechanical memory', whereby the cells exhibited higher amounts of chromatin condensation that persisted for longer times, when subjected to increasing numbers of loading events and strain levels, which may be a mechanism by which the MSCs sensitize themselves to future loading events[78]. Collectively, these studies emphasise just how far reaching the effects of mechanical stretch can be on MSC physiology and maintenance and show how tensile loading can affect not only cellular differentiation, but also affect apoptosis, ROS, calcium oscillations and matrix mineralisation.

1.5 Mechanical Stretch and Biomaterials: Physiological Loading and the Role of the Circadian Rhythm

Unsurprisingly, the effects that mechanical loading can have on a biomaterial seeded and optimised with human MSCs are widespread and well documented. There are an enumerable amount of existing materials, with new materials being created and optimised all of the time, and each of these may be impacted and improved by subjecting the material, and cells within, to mechanical load, or by preconditioning the cells with mechanical strain. For example, stem cell-collagen sponge constructs experience an increase in stiffness following mechanical stimulation. Two weeks of *in vitro* mechanical stimulation was here found to increase collagen type I and type III gene expression and an increase in linear stiffness and linear modulus[79]. Long term dynamic compression of MSC-seeded hydrogel constructs initiated after chondrogenesis induction has also been found to enhance matrix distribution and the mechanical properties of MSC-seeded constructs[80], showing again how mechanically stimulating cells in constructs can improve function. Electrospun fibres, which are advantageous in connective tissue regeneration in terms of their durability, deformation capacity and effects on cell adhesion, orientation and gene expression, experience an increase in cell alignment when subjected to cyclic loads, suggesting that cell morphology within a construct is also influenced by the mechanical environment[81].

However, this is not the only physiological process that is affected by the mechanoenvironment of MSCs following implantation into biomaterials. Research by Mengatto et al. (2011) explored osseointegration implant failure, to see what impacted the establishment of dental and orthopaedic implant association with bone tissue. To do this, the authors used a vitamin D deficiency model of implant failure in rats and evaluated changes in gene expression using whole genome microarray analyses. KEGG analysis was utilised and it was found that 103 genes were significantly modulated by implant placement and vitamin D deficiency, with the highest z-scores assigned to components of the circadian rhythm pathway, including neuronal PAS domain 2 (NPAS2) and period homolog 2 (Per2). Furthermore, NPAS2 and Aryl hydrocarbon receptor nuclear translocator-like (ARNTL/Bmal1) were found to be upregulated, and Per2 showed a complementary expression pattern. This study suggests that the circadian rhythm may have a key role in the establishment of osseointegration under vitamin D regulation[82].

The circadian rhythm is an important evolutionarily conserved cellular mechanism which are a subset of biological rhythms, which have a period, i.e. the time taken to complete one cycle, of approximately 24 hours. The foundation of circadian rhythmicity research is often dated back to the work done by Colin Pittendrigh and Jurgen Aschoff. These pioneers are thought to have defined the basis of circadian entrainment. Pittendrigh (1960) showed that deviation from the 24-hour cycle provides a mechanism for alignment for the internal time-keeping system, allowing the rhythm to be "reset" where necessary[83]. One example where this can be clearly demonstrated is the method by which we use light signals to reset our circadian rhythm following long distance travel, as this re-synchronisation, or entrainment, allows for a mechanism by which we counter the phenomenon of 'jet lag'. This information is relayed from the photo-sensitive cells in the retina to the suprachiasmatic nucleus (SCN) in the hypothalamus via the retino-hypothalamic tract, where the SCN acts as a master pacemaker to synchronise all periphery clocks. The molecular mechanism used to generate self-sustained circadian rhythms is conserved at all levels and relies on a network of auto-regulatory feedback loops of transcription and translation drive circadian expression patterns of the core clock components[84]. In

mammals, this is carried out by the primary feedback loop by the basic-helix-loop-helix transcription factors CLOCK and BMAL, which form the positive arm of the molecular clock. When these two proteins heterodimerise, they are able to bind to *cis*-regulatory enhancer sequences called E-boxes on target gene promoters, and so initiate transcription[85,86]. Target genes include *Period* (*Per1, Per2* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*), which themselves heterodimerise and translocate to the nucleus to affect gene expression. However, unlike the CLOCK:BMAL complex, they have a negative feedback effect, and repress their own transcription by inhibiting the CLOCK:BMAL complex[87,88]. The CLOCK:BMAL heterodimers induce a stabilising regulatory loop by activating the transcription of retinoic acid-related orphan nuclear receptors, REV-ERB and ROR. These bind to retinoic acid-related orphan receptor response elements (ROREs), which are present in *Bmal* gene promoter. REV-ERBs repress transcription of *Bmal*, whereas RORs activate it [89]. These auto-regulatory loops constitute a molecular clock machinery and take approximately 24 hours to complete a cycle (Figure 1.3).

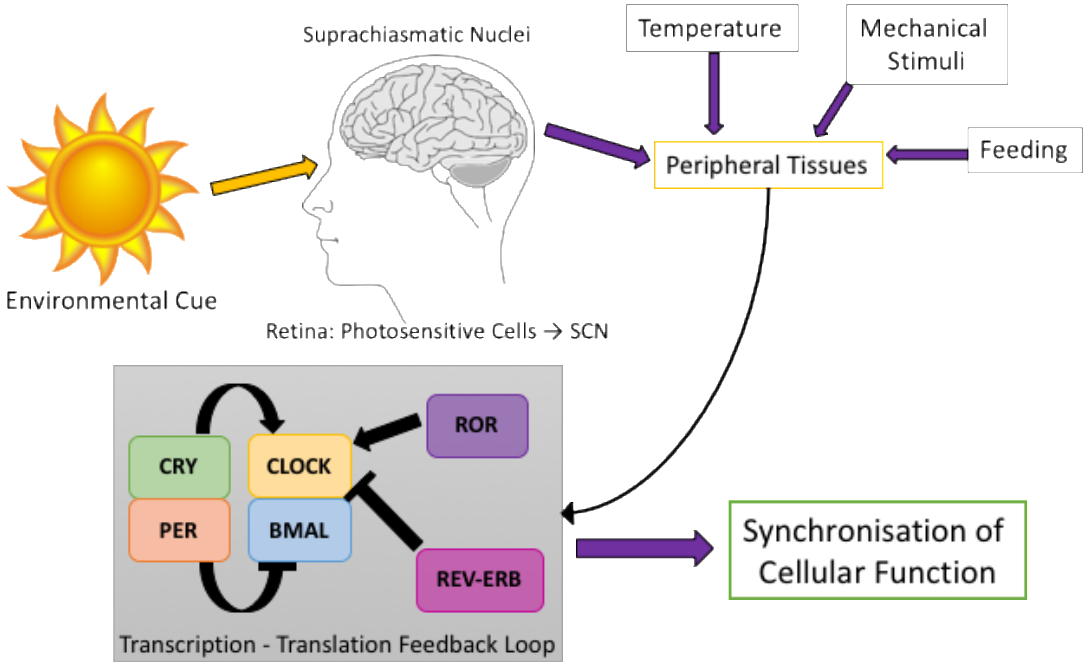

Figure 1.3. *Summary Schematic of the Circadian Rhythm. Light enters the brain through the retina and is relayed from the photosensitive cells to the SCN. The SCN then signals to a number of peripheral tissues and cells all over the body where it feeds into the molecular clock auto-regulatory feedback loop, which act on target genes and lead to the synchronisation of cellular function. Modified by Rogers et al. (2018)*[37].

Similarly to Mengatto et al.'s work, Hassan et al. (2017) also found that the circadian rhythm may influence the fate of certain biomaterials. In this instance, the circadian rhythm of BMSCs was found to be induced by titanium-based biomaterials with complex surface modifications (Ti biomaterials). When human MSCs were cultured on Ti biomaterials, it was found that *Per1* expression was suppressed, whereas NPAS1 was upregulated. BMSCs were then harvested *Npas2* knockout mice, it was found that this did not rescue the Ti biomaterial-induced reduction of *Per1* expression, and did not affect *Per2, Per3, Bmal1* or *Clock* expression, suggesting that the Ti biomaterial-induced increase in NPAS1 expression was independent of the changes in circadian component expression. The authors also found that vitamin D supplementation significantly increased *Per1* expression in BMSCs[90]. Taken together, both of these studies suggest that the circadian rhythm of BMSCs may influence the integration of Ti biomaterials into bone, and therefore should be taken into account in future biomaterial research.

The circadian clock has also been implicated in regenerative medicine as it has a profound effect on the wound healing response. For example, skin wounds in mice heal faster in the active period than they do in the rest period due to circadian regulation of the cytoskeletal protein, actin, which is involved in cell migration in fibroblasts[91]. As a result, human burn patients who incurred their injuries in the night, i.e. rest period, heal more slowly than those incurred in the day, i.e. active period. This research highlights the importance of the circadian regulation of the cytoskeleton on wound healing responses and underlines the importance of the circadian rhythm in regenerative medicine.

A recent study has demonstrated the importance of cell-matrix interactions for stem cell clocks and discovered that the mammary epithelial clock is regulated by the mechanical stiffness of the cellular microenvironment[92]. The authors demonstrated that genetic disruption of clocks compromises the self-renewal ability of the mammary epithelial stem cells, underlining the key link between clock genes and mammary stem cell function. With age, there is an increase in tissue stiffening, which is shown to suppress the amplitude of the mammary clock oscillations *in vitro* through the tension sensing cell-matrix adhesion molecule, vinculin, and the Rho/ROCK signalling pathway.

This signalling pathway of extracellular mechano-stiffness is then transduced into the cell to regulate the activity of core clock machinery. In this study, the authors also investigated whether the circadian rhythm of old cells can be restored by subjecting them to a less stiff matrix. ROCK inhibitors were here used to inhibit the ROCK pathway, which has been implicated in matrix stiffness, to soften the cell matrix elasticity, and do find that this is sufficient to improve cellular function and reset the circadian clock in old cells[92]. Therefore, it was concluded that the increase in tissue stiffening seen with ageing is thought to suppress the mammary clock *in vivo*, providing a mechanism for how ageing may disturb the mammary epithelial stem cell clock, through the circadian rhythm. This study underlines the hypothesis that the mechanical environment is sufficient in itself to impact the circadian rhythm in ASCs.

1.6 Role of the Circadian Rhythm on Adult Progenitor Cell Proliferation and Differentiation

A new field of investigation has shown that the clock genes can directly influence ASC and progenitor cell activation and differentiation, within their tissue-specific niches. For example, disrupting the clock gene *Bmal1* leads to increased adipogenesis, and thus the attenuation of Bmal1 expression *in vitro* in pre-adipocytes leads to a down-regulation of the Wnt signalling pathway and increased adipogenesis[93]. The involvement of circadian clocks in the regulation of adult stem cell activation is not only niche-specific but can also act at the cell population level. Janich and colleagues showed that the circadian clock has a differential role in regulating the activation of co-existing epidermal stem/progenitor cell populations. The authors found that the genes regulating stem cell niche dormancy, activation and differentiation contained several putative BMAL1/CLOCK-binding sites. Deleting *Bmal1* leads to circadian arrhythmia, decreased expression of Wnt related genes and TGF-β inhibitors, and causes progressive accumulation of dormant stem cells. Deleting *Per1/2*, conversely, results in progressive depletion of dormant stem cells[94]. A subsequent study showed that the stem cells responded differently to differentiation cues at certain times of the day. Interestingly, different proliferation-related or differentiation-related genes were expressed at different times of the day; for example, DNA replication and cell division

related pathways were highly expressed in the dark phase, as opposed to in the light phase, when differentiation pathways dominated[95]. The role of the circadian rhythm, on epidermal progenitor cells is further discussed below. Collectively, these studies demonstrate that the mechanical environment not only has the potential to entrain ASCs, but that the circadian rhythm itself may have effects on ASC activation and predisposition to differentiate.

The circadian rhythm has also been shown to have extensive and varying roles on the different lineages that adult progenitor cells may differentiate into, even after the original activation and commitment of precursor cells. This has been concisely reviewed by Weger et al. (2017)[96], but a summary can also be found below.

1.6.1 Hematopoietic Progenitor Cells

Hematopoietic stem/progenitor cells (HSCs) are vital precursors to a number of hematopoietic cells, including erythrocytes and platelets in the blood, and granulocytes, T cells, B cells, natural killer (NK) cells and dendritic cells in the immune system. These progenitor cells in the adult reside mostly in bone marrow niches, but HSCs are mobile and therefore can be stimulated to exit the bone marrow and move out into the circulation (egression), before returning to hematopoietic tissue (homing)[97,98]. The process of egression and homing is vital in immune surveillance and homeostasis[99]. This mobilization behaviour is regulated by the chemokine CXCL12 (Chemokine C-X-C Motif Ligand 12) and it's receptor CXCR4 (Chemokine C-X-C Motif Receptor 4). CXCL12 is regulated by rhythmic β3 adrenergic signals from the sympathetic nervous system and influences both behaviours by sending a retention signal to the bone marrow stromal cells, ensuring they remain in the bone marrow, or by acting as an attractant to encourage their return to the bone marrow niche[98,100].

It is these egression and homing behaviours that are thought to be regulated by the circadian rhythm, as was first investigated by studies dating back to the 1980s[101,102]. Here, diurnal variations in the activation and circulation of HSCs could be observed in human blood even at this early date, and HSC mobilization was later confirmed to be under regulation by the circadian rhythm[103], with the highest numbers of HSCs in the blood found when resting[104]. This is influenced by the rhythmic expression of β3

adrenergic signals and the circadian pattern of CXCL12 expression. To further validate this point, when *Bmal1* expression is knocked out in mice, this observable diurnal variations in HSC release is also lost, highlighting the role of the circadian rhythm in HSC mobilization[103]. The circadian expression of CXCL12 via β3 adrenergic receptor activation has then also been implicated in a number of other HSC processes, including neutrophil turn-over and corticosterone levels[96], emphasising the vital role the circadian rhythm has in many varying physiological behaviours.

1.6.2 Mesenchymal Progenitor Cells and Precursors

1.6.2.1 Bone

The effects of the circadian rhythm on the mesenchymal differentiation has also been investigated. The formation of bone tissue is reliant on monocyte derived bone-resorbing osteoclasts and BMSC derived bone-forming osteoblasts, that incidentally also give rise to osteocytes, which have vital roles in bone remodelling. Interestingly, the BMSCs of *Bmal1* knockout (KO) mice have been demonstrated to have decreased osteogenic differentiation, when exposed to stimulation by osteogenic conditions, resulting in a low bone mass phenotype[105]. However, these findings are disputed by an earlier study, which reports that an increase in osteoblast activity can be observed in *Bmal1* KO mice and other clock gene deficient mice (*Per1/2* and *Cry1/2* double KO mice)[106]. Here, the authors explain the observed increases in osteoblast proliferation, despite the seemingly similar phenotype being observed by the inhibition of antagonistic, opposing genes, by suggesting that the decreases of clock gene expressions lead to a relief in the inhibition of *c-Myc* expression. These findings have been supported by further studies that have also demonstrated that an inhibition of *Per2/Cry2* in both single and combined KO mice, which here also led to an increase in bone formation[107]. To this end, in the single KOs, this increase was attributed to increased osteoblast activity when *Per2* was singly knocked out and decreased osteoclast activity for *Cry2* single KO mice.

1.6.2.2 Adipose

Regarding the formation of adipose tissue, the circadian rhythm has also been shown to have profound roles on adipose tissue metabolism and differentiation, which is unsurprising when it is considered that disruptions in the circadian clock lead to increased metabolic disorders and obesity. Adipose tissue consists of pre-adipocytes and adipocytes (fat cells), the former of which act as progenitor cells and differentiate to become adipocytes, when exposed to the appropriate stimuli. Adipose tissue *in vivo* exists either as brown or white tissue; brown adipose is vital for homeostatic thermogenesis, whereas white fat is involved in the storage of energy[108]. Interestingly, it has been found that greater than 20% of adipose tissue genes oscillate in a circadian manner in mice[109,110]. However, seemingly in contrast, many circadian clock genes have been found to not exhibit rhythmic circadian expression in adipocytes and preadipocyte precursors. For example, although *Per2*, *Rev-ErbA* and *Dpb* were indeed shown to exhibit circadian rhythmicity, the expression patterns of *Per1*, *Cry* and *Bmal1* were found to not be circadian in pre-adipocytes. Despite this, the clock genes in human derived adipose stem/progenitor cells (hADSCs) have been proven to be entrain able by chemical means; for example, traditional circadian synchronisation methods such as serum shock and exposure to dexamethasone have been demonstrated to be sufficient to elicit circadian rhythmicity in the clock genes[111]. Furthermore, lithium chloride, which is used as an agent to extend the period of many clock genes, was found to bring about the predicted response here too in ADSCs, whereby it did lengthen the period of clock genes[111]. Notably, lithium chloride has also been demonstrated to have significant roles in inhibiting adipogenesis[112]. The chemokines released by adipose tissue (“adipokines”) have also been shown to be rhythmic in both their expression and their secretion, and similarly, adipokines are also thought to have a potential role in adipogenesis[113]. Taken together, these findings implicate a significant relationship between the circadian rhythm and adipogenic differentiation, although the precise mechanism behind this link has yet to be elucidated.

However, although the precise molecular mechanism has not been underpinned, the roles of various circadian clock genes on adipogenesis have been explored. For example, *Per3* is thought to have a substantial role in adipogenesis, with its knockout leading to an inhibition of the adipogenic transcription factor *PPARγ*[114]. In adipocytes, *Bmal1* is also thought to regulate both adipogenic differentiation and lipogenesis, with its knock out in mice leading to high increases in brown fat[115], and therefore Bmal1 has also been implicated in brown adipogenesis. Similarly, in mesenchymal precursors and committed progenitor cells that would give rise to brown adipocytes, *Bmal1* inhibits adipogenic lineage commitment and terminal differentiation. Moreover, when *Bmal1* is overexpressed in brown pre-adipocytes, this leads to an inhibition of their terminal differentiation, and when it is knocked down, an increased differentiation rate can here be observed[116]. This *Bmal1*-mediated regulation of adipogenesis is thought to involve signalling components of the TGF-β, Wnt and BMP pathways, which may be regulated by *Bmal1* directly[93,116]. A further clock gene emphasised in the regulation of adipogenic differentiation is *Rev-ErbA*, as it is thought to promote the terminal differentiation of adipocytes. *Rev-ErbA* is a member of the subsidiary loop of circadian rhythm gene interaction and is a direct target of *Bmal1*. Therefore, when *Bmal1* is knocked down in mice, this also leads to a decrease in *Rev-ErbA* expression alongside the increase in adipogenesis.

The evidence of circadian regulation on both adipogenic and osteogenic differentiation is substantial, and it is clear that the clock genes have a significant influence on both of these processes. However, the exact molecular pathways by which these genes influence mesenchymal differentiation have yet to be understood.

1.6.2.3 Skeletal Muscle Satellite Cells

The skeletal muscle is a distinct tissue in that it retains its impressive regenerative capacity throughout the majority of adult life, as it is frequently necessary to replace muscle tissue upon injury or damage. Skeletal muscle is composed of myofibres (multinucleated cells) and satellite cells, which are the progenitor cells of skeletal muscle tissue. Satellite cells have a high regenerative capacity, which may be

stimulated by signals feeding into the satellite cells' environment/niche, and are located between the muscle sarcolemma and the basal lamina of myofibres[117].

Bmal1 has also been implicated in myogenesis; the differentiation of primary myoblasts *ex vivo* has been found to be implicated when isolated from *Bmal1* KO mice[118]. Moreover, when *Bmal1* is overexpressed in C2C12 myoblasts, their myogenic differentiation is promoted, via components of the Wnt signalling pathway. The myogenic differentiation promoting factor, MyoD, is also thought to be under regulation by *Bmal1*[119], with the overexpression of MyoD in *Bmal1* KO cells enhancing the expression of myosin heavy chain, a marker of muscle differentiation, although the fusion of the cells was not promoted[118]. It was here implicated that the Wnt pathway is required for full differentiation[120]. However, *Bmal1* is indeed theorised to be required for full muscle regeneration after injury, by stimulating the expansion of the satellite cells. Interestingly however, whether the role of *Bmal1* on myogenesis is a clock or non-clock related gene function has yet to be shown, as no rhythmicity has here yet been demonstrated.

1.6.3 Epidermal Progenitor Cells

Another tissue that the body is dependent on for its extensive regeneration capability over an adult lifespan is the epidermis, where the skin and hair both renew constantly throughout life to maintain homeostasis and to repair following injury. The skin is a complex organ that is composed of several varying layers, such as the epidermis, i.e. the outermost layer of squamous epithelium consisting of interfollicular epidermis, which in turn is made out of mainly keratinocytes, and the pilosebaceous unit, which contains sebaceous glands and hair follicles. In both regions, in the interfollicular epidermis and pilosebaceous units, niches of epidermal stem/progenitor cells can be found.

1.6.3.1 Skin

A functional circadian rhythm has been reported in the skin[121–124]. Here, the highly proliferative epidermal progenitor cells are located in the skin's basal layer. Upon appropriate stimulation, the cells exit the cell cycle and the progeny then differentiate

into keratinocytes as they traverse up to the skin surface, where they form a protective barrier[125]. The proliferation of epidermal progenitor cells is thought to be under the influence of the circadian rhythm[121,126]. To investigate this, by replicating the epidermis *in vivo* conditions *in vitro* using low calcium conditions, the transcriptome of normal human epidermal keratinocytes has been demonstrated to be organised into five circadian waves of expression, which is in phase with the clock genes' mRNA expression[127]. In this way, the expression of epidermal progenitor cell differentiation genes are expressed in the late night to early morning period, which is when the induction of differentiation markers by TGF-β and Ca^{2+} signalling is also higher. Interestingly, when the clock gene expressions' are manipulated, premature differentiation of the cells is demonstrated *in vitro*, and defective transplantation can be observed *in vivo.* In contrast, pathways linked to proliferation and DNA replication and repair are found to peak in activity in the afternoon and evening. The segregation of the differentiation and proliferation processes is theorised to be separated to minimise DNA damage by reactive oxygen species (ROS), as ROS levels change throughout the day and are produced by metabolic processes[126]. Therefore, by being expressed at a temporally different time to DNA replication and repair, this acts as a protective mechanism. By looking at the relative levels of free and bound NADH in the basal cells of mouse adult skin, as indications of glycolysis and oxidative phosphorylation, it can be seen that glycolysis follows a diurnal oscillation and peaks in the night, when the mice are most active. However, this rhythmic expression is lost in *Bmal1* KO mice[128]. Furthermore, glycolysis levels peak in the same phase as the proliferating cells in S-phase: S-phase proliferation occurs at the same time as higher levels of glycolysis and lower levels of oxidative phosphorylation, which is similarly theorised to be to avoid the higher levels of ROS produced by higher oxidative phosphorylation. This highlights the role of the circadian rhythm in temporal segregation as a protective mechanism.

1.6.3.2 Hair

Hair is produced in the pilosebaceous units located in the skin by hair follicles, with the process of hair growth being cyclical; with phases of growth (anagen phase), rest

(telogen) and degeneration (catagen)[129]. The epidermal progenitor cells responsible for the production of growth are located in the bulge of the hair follicles, which give rise to the cell types of the lower portion of the follicle, and in the upper part of the hair follicle, where they give rise to the epidermal cells of the upper part of the hair follicle. Here, epidermal progenitor cells undergo cycles of activation and dormancy[125], as is modulated by the circadian clock, albeit with a period longer than 24 h[130]. The influence of the circadian clock in hair growth can be seen in the hair follicles of *Bmal1* KO mice, which experience a delay in anagen progression[126]. However, the overall duration of the hair growth cycle does remain unaltered in these mice. This delay in anagen is thought to be due to a potential block in the cell cycle of these cells, but the exact mechanism has yet to be elucidated; *Bmal1* KO in the interfollicular epidermis and hair follicle keratinocytes does not lead to such a delay, and therefore this delay is hypothesized to be due to signalling by the systemic factors from neighbouring cell types. The involvement of the cell cycle in this process has been further demonstrated where the circadian rhythm is disrupted in the epidermis and upper hair follicle using tissue specific KOs. Here, this leads to an increase in the amount of S phase cells in both of these tissues[126]. These observed changes in the rhythmicity of cell proliferation of the epidermal cells does not however lead to any changes in the normal hair growth cycle, and *Bmal1* KO mice do not exhibit any observable changes in epidermal thickness, despite changes in the proliferation rhythms. Therefore, a compensatory mechanism is thought to maintain normal hair growth.

Regarding the epidermal progenitor cells located in the hair follicle bulge progenitor cell compartment, the circadian rhythm has also been proven to regulate organ specific progenitor cell proliferation and differentiation[94]. For example, in Per1-luciferase reporter mice, cells located in the same bulge at the telogen phase of hair growth have been demonstrated to have half of the progenitor cell population exhibiting high levels of Per1 reporter activity, with the other half showing low levels of reporter activity. Following transcriptome analysis of these two coexisting "clock^{high}" and "clock^{low}" epidermal progenitor cell populations, it was found that the two had differential expressions of the circadian core clock genes, along with several genes involved in progenitor cell homeostasis, including genes related to the Wnt and

TGF signalling pathways, both of which have been shown to have crucial roles on bulge progenitor cell hair cycle regulation, proliferation and differentiation[94]. Interestingly, bulge stem cells are unique in terms of their heterogeneity; for example, only a subset of the cells are activated by the required stimuli at any one time in the hair cycle, while the other cells remain dormant[130,131]. This heterogeneity in the bulge progenitor cells' ability to respond to activating stimuli is therefore reflected in a correlating heterogeneity in the circadian clock and expression of signalling pathway components. Here, $clock^{high}$ progenitor cells were found to be more prone to activation and proliferation and expressed increased levels of the Wnt signalling pathway components and TBFβ antagonists when compared to $clock^{low}$ progenitor cells. Furthermore, *Bmal1* KO in mice leads to circadian arrhythmia of epidermal cells and keeps them in the $clock^{low}$ state, with the corresponding signalling pathway suggesting a dormant state[94]. Collectively, these findings insinuate a further role of the circadian rhythm in splitting the epidermal progenitor cells into coexisting populations that differ in their state of readiness; either poised to be activated or remaining dormant, allowing for subsets of the cells to be stimulated and others not when exposed to the same conditions.

In summary, these findings demonstrate how the circadian clock is able to influence and pattern the way hair bulge progenitor cells respond to activating signals and their resulting proliferative state. However, as no mice models subjected to circadian disruption have strong epidermal phenotypes, this suggests that this may be compensated by mechanisms not related to the circadian rhythm.

1.6.4 Neural Progenitor Cells

Regarding neurogenesis, the process by which neurons are produced from neural precursor cells that continues in adult life, the circadian rhythm has been implicated in this differentiation process too. There are two main neurogenic regions in the adult brain – the sub ventricular zone (SVZ) in the lateral ventricle and the sub granular zone (SGZ) in the dentate gyrus in the hippocampus, both of which contain a number of different neural stem/progenitor cells[132,133].

The circadian rhythm has been demonstrated to influence adult neurogenesis in the mammalian hippocampus[134,135], as can be observed in rodents that undergo jet leg and a resulting misalignment of their circadian rhythms, as they experience a reduced level of adult neurogenesis as a consequence[136,137]. Furthermore, clock gene expression has been reported in the neurogenic regions of both the SGZ and SVZ. By isolating SGZ and SVZ spheres, it can be demonstrated that the circadian rhythm emerges during neurogenic differentiation and influences neurogenesis by affecting neurosphere growth, proliferation and fate commitment[138,139]. Moreover, the circadian rhythm has been shown to influence these processes by restricting the growth and expansion of neural precursors and regulating neural progenitor cell cycle entry[140]. Both *Bmal1* and *Per2* KO mice have been shown to have increased numbers of actively proliferating cells and, crucially, these mice lack the circadian gating of entry into the cell cycle[140]. The mechanisms that underlie the role of the circadian rhythm on controlling neurogenic progenitor cell proliferation and cell cycle entry in the neurogenic niches have yet to be fully understood, but once they have been uncovered, this will have profound implications in brain diseases such as depression or Alzheimer's disease, as any deterioration in neural function will have severe consequences on the adult brain.

1.6.5 Embryonic Stem Cells and the Development of the Circadian Clock

The findings of the above experiments interesting elude to the notion that the onset of differentiation and the state of cellular differentiation is an instrumental factor in the development of the circadian rhythm. As detailed previously, it has been shown by research from Wu et al. (2007) that differentiated adipocytes are more readily responsive to clock synchronisation than undifferentiated pre-adipocyte precursors; interestingly, here, the less differentiated preadipocytes do not have a fully functioning circadian clock when compared to the more mature, differentiated adipocytes[111]. Furthermore, several clock genes, including *Bmal1*, have been implicated in the regulation of adipogenic differentiation[141]. Similarly, in neurogenic precursors, the circadian rhythm has been found to emerge during neurogenic differentiation[138,139]. These results imply that the onset of the circadian rhythm is

something that occurs during the differentiation process. Therefore, it is logical that completely undifferentiated stem cells will not have an established oscillating circadian rhythm, as has indeed been demonstrated by Yagita et al. (2010). In this study, undifferentiated murine ESCs do not appear to have a functioning circadian rhythm, but this ability is gained upon differentiation and can then be reversed when the cells are artificially de-differentiated[142].

1.7 Overall Aims and Objectives

The hypothesis of the research presented here was based upon taking the existing knowledge presented in the literature reviewed. Where it has been demonstrated that the mammalian circadian rhythm has an influencing role on the differentiation of adult progenitor cells, as it was hoped that this relationship could be further understood and the mechanisms that underlie this process could therefore be identified. Furthermore, the evidence presented in previous research demonstrated that mechanical stimulation, such as cyclical tensile mechanical stretch, could also lead to profound effects on adult progenitor cell proliferation and differentiation. To this end, this research sought to further elucidate and detail how both of these processes regulate adult progenitor cell differentiation, as it was also theorised that there may be interplay between the two phenomena; the hypothesis presented here was that the physio-mechanical environment may influence the differentiation capacity of progenitor cells, and this may be mediated, at least in part, by the influence of the circadian rhythm. Moreover, it was also hypothesised that the responses' of the progenitor cells, as a result of changes in the physio-mechanical environment or in the progenitor cells' circadian rhythms, may differ depending on the anatomical source that the cells were derived from. Ultimately, it was proposed that the physio-mechanical environment affects the circadian clock of adult progenitor cells, which then affects their differentiation capacity.

1.7.1 Aims

In order to address this hypothesis and to determine how both the progenitor cells' mechanical environment and their circadian rhythm affect the cells' capacity for differentiation, and to potentially determine if there is any relationship or feedback between the circadian rhythm and differentiation response, the following aims were proposed:

1) Firstly, the circadian dynamics in three types of primary human adult progenitor cell were to be analysed, to determine if the circadian rhythm was functioning in these cells, and if so, whether the cell types differed in their circadian responses based on their differing anatomical locations of origin. It was also aimed to explore if the three cell types were able to be synchronised in a circadian manner in similar ways or not, and to see if mechanical stimulation could be utilised in a circadian manner to sufficiently entrain the circadian rhythms of the cells. If so, this would imply that the mechanical environment does have a relationship with and does influence the circadian rhythm of adult progenitor cells directly. Here, rhythmic uniaxial mechanical stretch was selected as a method to mechanical stimulate the ASCs.

2) If mechanical stretch was determined to be effective at influencing the circadian rhythm of adult progenitor cells, the natural next aim in the research was to see how manipulating the mechanical environment of the cells further, here by altering their protein substrate, then affected the progenitor cells, with behaviours such as the circadian response to stretch and differentiation capacity of the ASCs both being examined.

3) If the findings of first and second aims implicated that mechanical stretch influences the differentiation of adult progenitor cells via the circadian rhythm, the next aim in this research was to see how disrupting the circadian clock impacted the response of the ASCs to mechanical stretch and the resulting changes in differential potential, to see if these resulting changes in progenitor

behaviour were dependent on the circadian rhythm components or not. This would then allow for the determination of the nature of the relationship between the circadian rhythm and cellular response to mechanical stimulation.

4) In order to elucidate how this research could be applied for therapeutic benefit, the last aim was to determine how synthetic bioactive peptides affected the physiology, growth and behaviour, the circadian rhythm, and also the cellular response to mechanical stretch of adult progenitor cells. It was hypothesised that the addition of such peptides would influence each of these processes, and the underlying mechanism was sought to be understood.

1.7.2 Clinical Applications

This last aim is particularly relevant as it has far reaching implications in terms of the application of this research. As many of the biomaterials under investigation in regenerative medicine are composed of synthetic materials and may also involve the utilisation of things such as synthetic peptides, it is vital that we further our understanding in this area, so that the resulting cellular responses can be predicted. Biomaterials are an exciting constantly evolving advancement in medical areas such as musculoskeletal, orthopaedic and orthodontic research, especially given the ageing population of society and the increased need for interventional surgical procedures to restore mobility such as hip and knee replacements, the utilisation of which are increasing exponentially with increasing longevity. The materials that are chosen to design implants out of will be selected on a number of parameters, including how progenitor cells that may be seeded into the scaffolds respond to that material and how it will stimulate their expansion and differentiation, so that the biomaterial may be fully integrated into the host tissue upon implantation. Therefore, as the circadian rhythm has been implicated in implant osseointegration, elucidating how different synthetic materials influence the circadian rhythm and differentiation capacity of ASCs, as underlined in this last aim, and how natural protein substrates influence both

the circadian and differentiation responses of adult progenitor cells, as under lined in the second aim, are of utmost importance if this is to be successful.

The research outlined in this project also has implications in terms of the treatment of disorders brought about by disruptions in the circadian rhythm; for example, some alterations in the circadian rhythm lead to an increased rate of metabolic disorders. Furthermore, circadian disruption can lead to other disorders brought about by impaired tissue development; this is thought to be due to impaired activity of adult progenitor cells within tissue specific niches, due to alterations in their circadian rhythm. It has also been well documented that there is an age-associated decline in the function of the circadian rhythm, which coincides with a deterioration of tissue function and reduced differentiation capacity of adult progenitor cells with age; these negative changes in regeneration could be as a result of the age-associated deterioration of the circadian rhythm.

In this regard, the research here presented seeks to determine the molecular mechanisms that influence the progenitor cells when their circadian rhythm is altered; for example, by disrupting circadian components to examine the resulting consequences in adult progenitor behaviour and physiology, in terms of their growth and cell number, differentiation capabilities and response to stretch, as outlined in the second aim. If it can be identified how these negative changes occur, then treatment can be designed to reverse the negative effects following circadian disruption. Therefore, once further elucidated, understanding how the circadian rhythm and the cellular physio-mechanical environment influences progenitor cell differentiation will lead to significant developments in both age-related diseases and tissue engineering.

Chapter Two

Materials and Methods

2 Materials and Methods

2.1 Isolation of Adipose-Derived Stromal Cells

Human lipoaspirate was harvested during a lipofilling procedure after breast tumour removal. Lipoaspirate (5 g) was washed by addition of serum-free DMEM medium (Gibco, UK) and centrifuged for 3 min at 500. The washed fat layer was moved to a new tube to which a digestion solution containing 10 mL of DMEM medium (Gibco, UK) and 20 µL of collagenase type 2 (Sigma, UK) was added. This was placed on a roller mixer for 30 min at 37°C. 10 mL of DMEM medium containing 10% FBS and penicillin/streptomycin (Sigma, UK) was added and the tube was spun for 5 min at 1000. The oil layer was removed and discarded, and undigested fat fraction was placed into a T25 falcon flask, with the addition of complete growth DMEM medium as above. The remaining supernatant was discarded and the pellet was resuspended and placed in a separate T25 flask, with the addition of complete growth DMEM medium. Both of these fractions were cultured until confluency and used at passage 2. All experiments were carried out with 3 independent samples of ADSCs.

2.2 Cell Culture of BMSCs and DPSCs

Primary human BMSCs and DPSCs were obtained commercially (Lonza and BioEden Limited, respectively) and expanded in Dulbecco's Modified Eagle Medium (GlutaMAX; Gibco, UK) supplemented with 1% penicillin/streptomycin and 10% FBS and incubated at 37°C in 5% CO2. Both cell types were previously validated using flow cytometry of known DPSC and BMSC markers by other members of the Clinical Engineering group. Cells were seeded throughout at a seeding density of 5,000-6,000 cells per cm^2, trypsinised upon reaching 70–80% confluency and used before reaching passage 7. All experiments were carried out with 3 independent samples of BMSCs and DPSCs.

2.3 Clock Synchronisation with Dexamethasone

Cells were seeded into 6-well plates in complete growth medium as above until confluency. The cells were synchronised using 100 nM dexamethasone and total RNA collected using TRI-reagent (Sigma) every four hours over a circadian cycle at the following time points after synchronisation (h): 16, 20, 24, 28, 32, 36, 40, 44, 48, and 52, as detailed in Figure 2.1. The cells were exposed to the dexamethasone for 1 h and then incubated in complete growth medium until sample collection. Three independent samples were collected for each condition. This synchronisation method has been previously validated in ASCs by Wu et al. (2007)[152]. Negative controls received no dexamethasone but were exposed to the same media changes and condition.

2.4 Clock Synchronisation with Serum Shock

Cells were seeded into 6-well plates in complete growth medium as above until confluency. The cells were synchronised using 50% horse serum and total RNA collected using TRI-reagent (Sigma) every four hours over a circadian cycle at the following time points after synchronisation (h): 16, 20, 24, 28, 32, and 36, as detailed in Figure 2.1. The cells were exposed to 50% horse serum for 2 h and then incubated in serum free medium until sample collection. Three independent samples were collected for each condition. This synchronisation method has been previously validated in ASCs by Huang et al. (2009)[153].

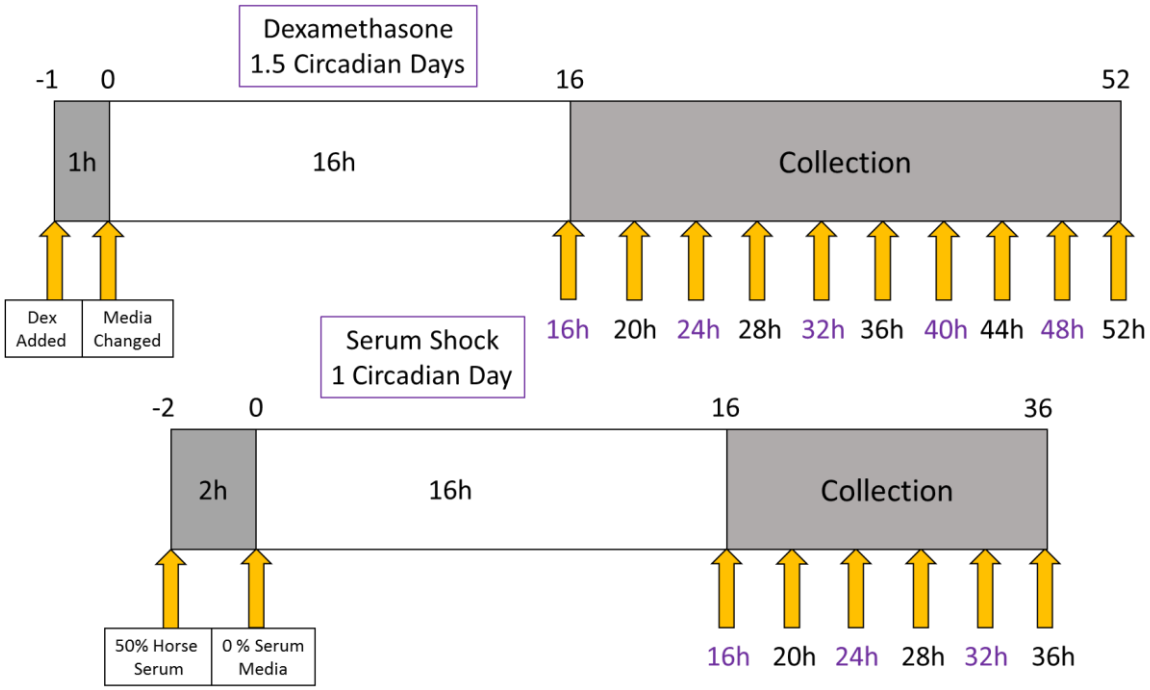

Figure 2.1. Synchronisation of Human Adult Progenitor Cells by Chemical Stimulation. Schematic diagram outlining the experimental time course undertaken to stimulate the circadian rhythm of adult human BMSCs, DPSCs and ADSCs by dexamethasone or serum shock. Cells were exposed to either dexamethasone or 50% horse serum for either 1 h or 2 h, respectively, before a 16 h latency period. Following this time, samples were collected over either 1.5 circadian day, from 16 h to 52 h (dexamethasone) or 1 circadian day, from 16 h to 36 h (serum shock).

2.5 Mechanical Stimulation

Flexible silicone chambers were coated in fibronectin, unless otherwise specified in protein substrate experiments, for 1 h before cells were seeded into the chambers and allowed at least 24 h to settle and attach. The adherence of the cells to the chambers was checked by visualisation with light microscopy. Once confluent, the chambers were loaded into a unique uniaxial stretch rig and stretched for 3 consecutive days in a rhythmic manner (frequency 1 Hz, 6.66% stretch, 12 h ON/12 h OFF) (Figure 2.2). After day 3 of rhythmic stimulation, cells were allowed to rest and total RNA was collected using TRI-reagent (Sigma) at the following times over 1 or 1.5 circadian days following mechanical stimulation (h): 16, 20, 24, 28, 32, 36, 40, 44, 48, and 52. Three independent samples were collected for each condition.

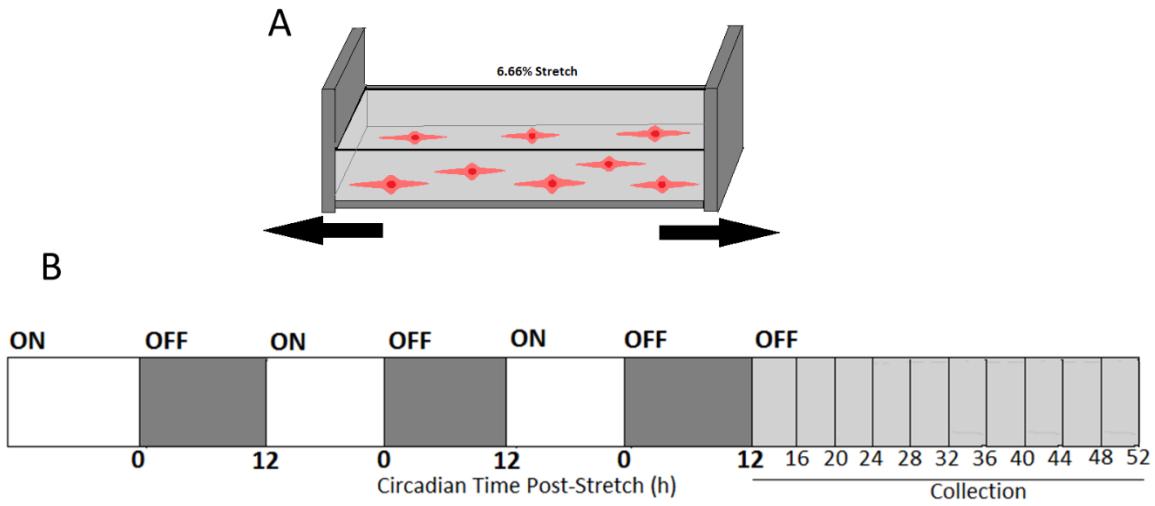

Figure 2.2. Mechanical Stretch Apparatus Set Up and Experimental Time Course. (A) Schematic diagram showing the set-up of the mechanical stretch apparatus; cells were seeded into silicone chambers and stretched for 3 days using a unique uniaxial stretch rig with off-set cams (1 Hz, 6.66% stretch, 12h ON (stretch), 12h OFF (rest)). (B) Schematic time course diagram outlining the schedule of the 12h ON:12h OFF (stretch:rest) regime followed by sample collection every 4 h between 16 h - 52 h.

2.6 Clock Synchronisation with Temperature

Cells were seeded into 6-well plates in complete growth medium as above and grown until confluency at 37.0°C. The cells were synchronised by being exposed to two different temperatures (one incubator programmed at 35.5°C and another at 38.5°C) for 12 hours each over 3 consecutive days in a rhythmic manner (12 h 35.5°C/12h 38.5°C) (Figure 2.3). After 3 days of fluctuating temperature, the cells were incubated in a constant temperature of 37.0°C and total RNA collected using TRI-reagent (Sigma) at the following times over 1 circadian day following temperature entrainment (h): 16, 20, 24, 28, 32 and 36. Three independent samples were collected for each condition.

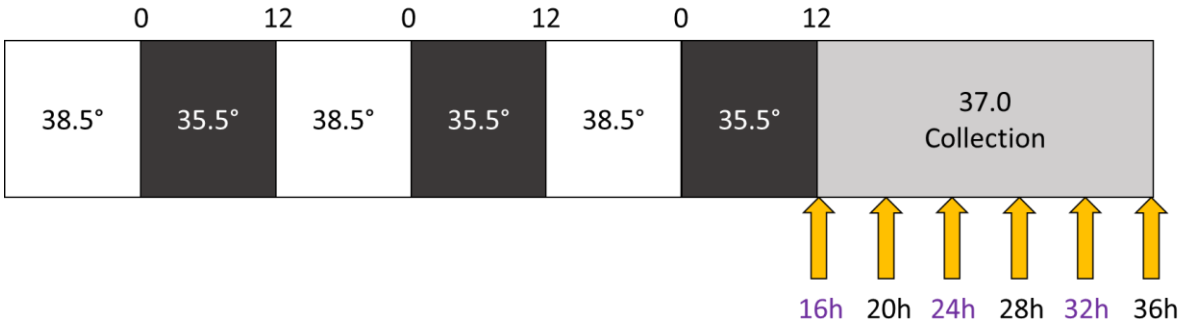

Figure 2.3. Experimental Time Course used to Syncrhonise Human Adult Progenitor Cells by Thermal Stimulation. *Schematic diagram outlining the set-up of thermal entrainment; human BMSCs and DPSCs were exposed to two varying temperatures (35.5°C and 38.5°C: difference of 3°C) for 12 h at a time over three circadian days. After this time, cells were returned to 37.0°C and samples collected every 4 h from 16 h to 36 h.*

2.7 Quantitative RT-qPCR

Total RNA was extracted using TRI-reagent (Sigma) as per the manufacturer's specifications. Total RNA was determined using nanodrop spectroscopy before cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen), with Oligo dT at 50°C for 40 min in a 20 µl reaction. Real-time RT-PCR was carried out on cDNA samples with SYBR Green PCR Supermix (Bio-Rad) using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad) under the following cycling conditions: 95°C for 3 min; 40 cycles of 95°C for 10 s; and 60°C for 30 s. Each condition comprised of 3 independent biological samples and was then analysed in duplicates for the qPCR.

Results were normalised relative to a housekeeping gene *GAPDH* expression as this gene had the most consistent level of expression throughout, when compared to *β-actin* and *18S* ribosomal RNA which were also tested. Primers were designed against the following genes: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), aryl hydrocarbon receptor nuclear translocator-like (*ARNTL* or *Bmal1*), period 2 (*Per2*), period 1 (*Per1*), nuclear receptor subfamily 1 group D member 1 (*NR1D1* or *Rev-ErbAα*), and SRY (sex determining region Y)-Box 2 (*Sox2*) and 9 (*Sox9*) (primer sequences are listed in Table 2.1). These primer sequences were validated by Otway et al. (2011)[243]. In later experiments (Chapters 4-6) where stem cell marker and differentiation gene expression was investigated, primers were also designed against

the following genes: osteocalcin (*OCN*), peroxisome proliferator-activated receptor γ (*PPARγ*) and glial fibrillary acidic protein (*GFAP*). These primer sequences were previously validated by other members of the Clinical Engineering lab group. Prior to use, melt curve analysis of all primers' efficiency was also undertaken.

Table 2.1 qPCR primer sequences (human).

Gene	Forward (5' to 3')	Reverse (5' to 3')
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG
Bmal1	TGCCTCGTCGCAATTGG	ACCCTGATTTCCCCGTTCA
Per2	GTCCAGCCCCCACCTTTC	GGGAAGGAATAACTGGGTAGCA
Per1	CTCAGTGGCTGTCTCCTTCC	GAGCCAGGAGCTCAGAGAAG
Rev-ErbAα	CTTCAATGCCAACCATGCAT	CCTGATTTTCCCAGCGATGT
Sox2	AAGGAGCACCCGGATTAT	TGCGAGTAGGACATGCTG
Sox9	GACTTCCGCGACGTGGAC	GTTGGGCGGCAGGTACTG
OCN	AGCGAGGTAGTGAAGAGAC	GAAAGCCGATGTGGTCAG
PPARγ	GACCACTCCCACTCCTTTG	GTGAATGGAATGTCTTCGTG
GFAP	AGAAGCTCCAGGATGAAACC	AGCGACTCAATCTTCCTCTC

2.8 Statistical Analyses

Data was expressed as the mean ± standard error and all variables comprised of at least three independent replicates. Statistical analyses were performed, following the determination of normal distribution, using one-way analysis of variance (ANOVA), with Tukey HSD post hoc, or the independent t-test at a confidence level of 95% (SPSS 24 Software). In order to determine circadian gene rhythmicity and its significance, cosinor periodogram analysis was used, made available online by the Refinetti circadian biology group at Boise State University (http://www.circadian.org/softwar.html). P values ≤0.05 were considered statistically significant.

2.9 Protein Substrate Experiments

2.9.1 Spin Coating of Silicone

13 mm circular glass coverslips were coated in Wacker SilGel® 612 silicone using a Laurell Spin Coater at 600 RPM for 30 s and allowed to dry in a fume cupboard overnight. Once dried, the silicone coated coverslips were placed into standard tissue culture 24 well plates and sterilised using UV, before being used in tissue culture. The silicone coverslips were then coated in the following protein substrates: rat tail collagen type I, human fibronectin, human albumin or negative control (PBS with no protein addition), at three different dilutions: 1:10, 1:100 and 1:1000. $8x10^3$ BMSCs or DPSCs were then seeded into each well and samples were collected after days one (D1), two (D2) and five (D5); coverslips were here transferred into a new, clean 24 well plate before being subjected to CyQUANT analysis. Three independent samples were collected for each condition. The adherence of the cells to the protein coated silicone was also checked using visualisation by light microscopy, to ensure that the cells had successfully adhered following cell seeding.

2.9.2 CyQUANT Analysis

The CyQUANT analysis was carried out as per the manufacturer's instructions (ThermoFisher Scientific); media was removed from the wells and the coverslips were transferred into clean 24 well plates. Samples were then placed at -80°C until all time points were collected, and on the day of analysis, removed from the freezer and allowed to thaw at RT. The CyQUANT mix was prepared as stipulated in the manufacturer's instructions. 500 µl of the CyQUANT buffer/GR solution was then added to each sample, and samples incubated for 5-10 mins at RT, protected from light. 200 µl of each sample was then transferred into black 96 well plates and read using a FLx800 plate reader at 485/528 nm. A standard curve was also prepared using known cell numbers of each cell type: DPSCs and BMSCs, for calibration and conversion of fluorescence to cell number of unknown samples.

2.9.3 Optimisation of the Protein Substrate

To investigate the effect of different protein substrates on basal levels of gene expression, standard tissue culture plastic 24 well plates were coated in either fibronectin at a dilution of 1:100 (F1:100), 1:10 (F1:10) or collagen type I at 1:100 (C1:100). The cells were incubated at 37.0°C at 5% CO_2 for three days, before total RNA was extracted and PCR analysis carried out as above.

To investigate the effect of protein substrate on mechanical stretch, the above stretch protocol was followed, with only the type of protein (human derived fibronectin or rat tail collagen type I) and dilution altered as follows: fibronectin diluted 1:100 (F1:100), 1:10 (F1:10) and collagen type I diluted 1:100 (C1:100). Samples were collected immediately at the end of the three days using TRI-reagent (Sigma) in the same way as above stipulated.

For both experiments, the adherence of the cells to the protein coated silicone was checked using visualisation by light microscopy, to ensure that the cells had successfully adhered following cell seeding and three independent biological samples were collected for each condition.

2.10 Gene Expression Manipulation of Circadian Clock Genes by siRNA

Gene interference experiments were carried out initially using FlexiTube siRNA kits for *Bmal1* and *Rev-ErbAα* (FlexiTube GeneSolution, Qiagen) as per the manufacturer's instructions, to determine the most effective siRNA at knocking down the target gene's expression level in both BMSCs and DPSCs. Once the most effective siRNA was determined, higher quantities of this siRNA were then purchased (FlexiTube siRNA) and utilised throughout. siRNA were prepared at 5µM and resuspended in RNAse-free water. Dharmafect (Dharmacon) was used as the transfection reagent, with negative controls containing either no siRNA or ALLStars negative control siRNA (Qiagen). The siRNA/Dharmafect mix was added to normal growth media containing no antibiotics,

with the final siRNA concentration used in cell culture being 20nM. Three independent biological replicates were carried out for each condition.

For the initial basal experiments, cells were cultured as per normal tissue culture procedure and mRNA was collected 48 h and 4 days after the addition of siRNA using TRI-reagent (Sigma). For experiments where the cells were subjected to mechanical stretch, cells were seeded into the silicone chambers 24 h before the siRNA was added. The cells were then incubated for a further 24h before being subjected to either the mechanical stretch (frequency 1 Hz, 6.66% stretch, 12 h ON/12 h OFF) or static conditions. Here, cells were collected immediately upon the completion of the three day regime and mRNA collected using TRI-reagent (Sigma).

2.11 Optimisation of the Mechanical Stretch Set Up Using Bioactive Peptides

Three bioactive synthetic peptides were selected: EPL 001, EPL 140 and EPL 142 at varying concentrations: 0.0, 0.15, 1.5, 15 and 150 µM.

Table 2: Synthetic Bioactive Peptides

Peptide	Length	Molecular Weight
EPL 001	14mer	1618.940
EPL 140	14mer	1633.911
EPL 142	14mer	1618.640

In the initial optimisation experiments, DPSCs and BMSCs were seeded at varying seeding densities in standard tissue culture 24 well plates: 2,500, 5,000, 10,000 and 20,000 cells/well. Cells were allowed 12 h to adhere before the supplementation of each peptide type and concentration. Images were taken using a light microscope (NIKON Diaphot Inverted Tissue Culture Microscope) (20 frames per variable) and samples were collected at 1 d, 2 d and 7 d post-supplementation. Samples were collected for CyQUANT Analysis as detailed above in *2.9.2 CyQUANT Analysis*. Images were taken on a NIKON Diaphot Inverted Tissue Culture Microscope (20x magnification, 20+ frames per variable) for each condition at each time point, and analysed using ImageJ software to determine cell number/frame.

Mechanical stretch experiments were carried out as previously described. Cells were seeded into flexible silicone chambers and allowed 24 h to adhere. Following this, in the experimental conditions, peptides EPL 001 at 0.15 µM or EPL 140 at 15 µM were supplemented into the growth media. Negative Controls received no supplementation. Cells were then incubated for 12 h before being subjected to either static or stretch (6.66% stretch, 1 Hz frequency, 12h:12h ON:OFF) conditions for 3 d. RNA was collected immediately upon completion of this regime and processed as detailed above.

Chapter Three

“Synchronising the Circadian Rhythm in Human Adult Progenitor Cells using Various Entrainment Mechanisms, Including Mechanical Stretch”

3 "Synchronising the Circadian Rhythm in Human Adult Progenitor Cells using Various Entrainment Mechanisms, Including Mechanical Stretch"

3.1 Introduction

3.1.1 Entrainment of the Circadian Rhythm

The internal circadian clock has a period of approximately, but not exactly, 24 hours. Therefore, it must be frequently reset by external cues, known as *Zeitgebers*. The most potent of these is a daily light cue, which entrains the clock in the SCN in mammals through reticulo-hypothalamic signalling mechanisms. Light enters through the retina, where it is transduced to the brain through the retino-hypothalamic tract. The SCN relays this information to the rest of the brain and peripheral tissue clocks via diffusible signals and neuroendocrine factors[143], allowing for a mechanism by which we adapt to phenomena such as 'jet lag'.

A further environmental cue by which humans adapt to their surroundings in order to entrain their own internal circadian rhythm is by thermal stimulation. The mechanism by which temperature inputs are transduced to the brain involves the detection of thermal signals: from the external environment through skin receptors and from the core body itself through hypothalamic receptors. This information is relayed to the hypothalamic regions in the brain, which act as thermoregulatory centres, and in turn maintain a relatively constant body temperature. However, this set point does indeed fluctuate over the course of 24 h, resulting in circadian oscillations of body temperature, as orchestrated by the SCN. As the SCN is continuously entrained by light signals, it is then able to synchronise circadian body temperature cycles to compliment these environmental light-dark cycles, resulting in the stipulated entrainment mechanism by temperature[144,145].

The ability of the mammalian core body temperature to oscillate in a circadian manner and therefore act as a *Zeitgeber* was initially demonstrated by subjecting cultured fibroblasts to circadian fluctuations in temperature. This was found to be sufficient to

sustain circadian rhythmicity, and abnormal temperatures cycles were shown to cause a decoupling of peripheral oscillators from the SCN[144]. Furthermore, temperature cycles have been shown to function as systemic cues that phase entrain individual oscillators in cultured cells and tissue explants. Recent findings have isolated genes with accumulation cycles that are thought to be driven by such temperature rhythms, including cold-inducible RNA-binding protein (*Cirp*), which oscillates in expression in cells synchronized by body temperature, but not in those kept at a constant temperature[146].

In addition to light and temperature, another important timing signal which has the capacity to entrain daily rhythms is via systemic factors such as growth factors and hormones. Glucocorticoids, which are a class of steroid hormones that bind to the glucocorticoid receptor (GR) present on almost every vertebrate cell surface, have been implicated in establishing circadian rhythms. The release of glucocorticoids from the pituitary gland is thought to be one way by which the SCN synchronises peripheral clocks. The contribution of the pituitary-adrenal axis in the rhythmicity of the whole animal has been studied in panels of zebrafish mutants by Dickmeis et al. (2007), where it was found that mutants with decreased level of corticotrope pituitary cells exhibit attenuated cell-proliferation cycles, with the circadian clock gene expressions remaining unaffected[147]. As corticotrope deficiency is related to reduced cortisol level, the authors implicate glucocorticoids as an integral part of a signalling pathway that systemically regulates circadian rhythmicity. Furthermore, it has also been reported that dexamethasone, a synthetic glucocorticoid hormone, induces circadian gene expression in cultured rat-1 fibroblasts and transiently changes the phase of circadian clock gene expression of peripheral oscillators, including kidney, liver and heart tissue[148]. Indeed, it has been previously evidenced that along with glucocorticoid hormones, serum shock, cAMP, protein kinase C, and Ca^{2+} can all be used to induce rhythmic circadian gene expression in Rat-1 cells[149,150], suggesting that the SCN master pacemaker may exploit and signal the release of many chemical cues to synchronise peripheral oscillators *in vivo*.

3.1.2 Circadian Synchronisation of Progenitor Cells

Glucocorticoids have also been implicated in synchronising peripheral circadian rhythms in human and murine adult progenitor cells. The core circadian clock genes, *Per1, Per2, Bmal1, Cry1, Clock,* and *Rev-Erbα*, were first characterised as actively cycling in mouse HSCs using a combination of high-speed flow cytometric cell sorting and qPCR[151]. Both murine and human primary BMSCs have also been reported to express the core circadian transcriptional machinery, and when exposed to dexamethasone, these too showed rhythmic oscillating mRNA expression of *Bmal1, Per3, Albumin D Binding Protein (Dbp), Rev-Erbα* and *Rev-Erbβ* [152]. Furthermore, circadian oscillations were also elicited when BMSCs and ADSCs were exposed to serum shock and cAMP analogs *in vitro*[153]*,* showing that adult progenitor cells can be synchronised using chemical means.

The clock genes have also been shown to oscillate in both murine adipose tissue and human subcutaneous adipose-derived progenitor cells. Here, cells were synchronised *in vitro* with the synthetic glucocorticoid, dexamethasone, the *PPAR-γ2* agonist, rosiglitazone, or 30% foetal bovine serum and total RNA collected every four hours over a 48 hour period. The authors reported that differentiated adipocytes were more readily responsive to clock synchronisation than undifferentiated pre-adipocyte precursors, but the period of clock gene oscillations were longer in differentiated adipocytes, validating the use of ADSCs as in *in vitro* adult stem cell model for the analysis of circadian rhythms[111].

Interestingly, unlike adult stem cells such as BMSCs and ADSCs, which are capable of circadian synchronisation by hormonal and growth factor signals such as dexamethasone[152], cAMP analogs and serum shock[153], embryonic stem (ES) cells lack a ~24h circadian rhythm and do not display the core TTFL required for circadian clock gene expression. However, upon differentiation, ES cells in culture can gain a molecular circadian rhythm, which can also be reversed when the cells are reprogrammed with the addition of *Oct3/4*, *Sox2, Klf4,* and *c-Myc*[142], as is consistent with the above notion that differentiated adipocytes are more responsive to circadian entrainment than undifferentiated pre-adipocyte precursors. This therefore implies

that more primitive cells do not have the required circadian components and ability to synchronise, and may acquire this as the cells mature.

3.1.3 Mechanical Stimulation as an Entraining Mechanism

A recently discovered entrainment mechanism for the circadian clock that requires fundamental research is by mechanical stimulation, whereby cells in different tissues in the body are subjected to very different levels of mechanical strain. Mechanical vibrations have the capability of resetting the clock in *Drosophila melanogaster*; it has been demonstrated that rhythmic mechanical stimulation of the chordotonal organs can synchronise the *Drosophila* circadian clock. *Drosophila* were here exposed to 12 hour:12 hour cycles of vibration and silence, and it was found that this was sufficient to entrain and synchronise their behaviour and daily locomotor activity. In order for this to occur, the Drosophila required both a functional clock and functional chordotonal organs, as the mechanosensory input pathway to the fly's circadian clock required signalling from the chordotonal organs in order to synchronise their circadian rhythm. *Drosophila* with loss of function mutations in their Period gene did not exhibit this ability of synchronisation through vibrations, highlighting the importance of functional clock systems for mechanical entrainment[154].

Previous extensive work in MSCs has demonstrated significant effects that mechanical forces *in vitro* can exert on their proliferation and differentiation properties. For example, Tirkkonen et al. (2011) used vibrational loading to cause the differentiation of human ADSCs towards bone-forming cells and inhibition of adipogenesis[155]. The authors found that the hADSCs cultured in osteogenic conditions were sensitive to vibrational loading and their osteogenic differentiation was enhanced with high frequency vibration. It has also been recently published that a mechanical environment of the epithelial progenitor cell niche within the mammalian mammary tissue controls the amplitude of the molecular clock oscillations, which is altered upon environmental (e.g. ageing) and genetic clock disruption[156].

The circadian rhythm of adult progenitor cells has also been investigated in response to the mechano-chemical stiffness of the cellular microenvironment, as circadian clock genes have been previously linked to mammary progenitor cell function. Notably, it

has recently been published that the mechanical environment of the epithelial progenitor cell niche within mammary tissue controls the amplitude of molecular clock oscillations, which can be altered upon environmental and genetic clock disruption. Moreover, cell-matrix interactions do indeed play a key role in regulating circadian biology, and tissue stiffening is now thought to suppress the mammary circadian clock *in vivo*, where the mammary clock may be controlled by the periductal extracellular matrix. Mechanistically, vinculin, a tension sensing cell-matric adhesion molecule, and the Rho/ROCK pathway, which transmits extracellular matrix stiffness signals into cells, are both thought to influence the regulation of the circadian clock. Furthermore, by disrupting the circadian clock of mammary progenitor cells, this leads to disruptions in the self-renewal capacity of the mammary epithelia, again highlighting the key roles of the mechano-environment and the circadian clocks of the epithelial stem cell niche in progenitor cell function[92].

Collectively, these recent studies demonstrate the exciting capabilities and effects that mechanical stimulation and the cellular mechano-environment can have on adult progenitor cell function and maintenance, with as far reaching effects as the synchronisation of the circadian rhythm, and implicate that mechanical stimulation in itself may be sufficient to entrain the circadian rhythm of ASCs.

3.1.4 Aims and Hypothesis

Hypothesis: Human adult mesenchymal progenitor cells sourced from different anatomical locations exhibit different circadian dynamics and respond to temporal cues differently. The more primitive adult progenitor cells such as DPSCs would be less responsive to circadian synchronisation cues compared to BMSCs or ADSCs.

In addition to well-established chemical synchronisation methodologies using a synthetic glucocorticoid, dexamethasone, and serum shock, both mechanical and thermal stimulation was also used in a circadian paradigm to investigate whether different human adult progenitor cells could be entrained using other environmental cues, such as mechanical stretch and temperature stimuli. For synchronisation using

temperature, 35.5°C and 38.5°C were selected as both are ±1.5°C the normal physiological value of 37.0°C, and the cells were exposed to each temperature for 12 h per day for a period of three days, before being restored to 37.0°C and samples collected.

The applied method investigating mechanical stimulation, using a uniaxial stretch rig composed of flexible silicone substrates at 6.66% radial distension and frequency of 1 Hz, was selected as these parameters have previously found to be within physiological range. Published data has previously shown that a uniaxial strain between 5-15% with a frequency of 1 Hz is preferable for MSCs and shows positive effects on proliferation and collagen synthesis[157]. Furthermore, O'Caerbhaill et al. (2008) found that radial distensions of 5% and frequencies of 1Hz caused mechanosensitive effects including cell reorientation parallel with direction of flow and adapted morphologies, highlighting that there is a significant cytoskeletal restructuring in these mechanically stimulated MSCs compared to static controls[158].

This chapter presents the research conducted to determine which of these entrainment mechanisms, by chemical, thermal or mechanical means, was the most effective and appropriate in each of the three types of mesenchymal progenitor cells, derived from bone marrow, dental pulp and adipose tissue. If viable, this mechanical stretch paradigm could offer a synchronisation method that excludes the need to use chemical or thermal approaches to synchronise the circadian clock, which would be hugely advantageous in tissue engineering and regenerative medicine. In this research, the circadian differences in adult stem cells derived from different human adult tissues with respect to their expression of several core clock genes, stabilising clock genes and stem cell markers in both dynamic and static conditions have been determined and compared. The results reveal differential circadian gene expression patterns in human adult stem cells derived from different tissue sources upon glucocorticoid synchronisation. It is also shown that rhythmic mechanical stimulation has the ability to entrain some human stem cells, which provides a novel clock synchronisation approach independent of chemical or temperature cues, both of which were also investigated. Such a clock synchronisation protocol may prove more advantageous in future tissue engineering applications, leading to significant

developments in both age-related diseases and tissue engineering and synchronising stem cell therapies.

3.2 Results

3.2.1 Research Section One: Human ASCs Derived from Different Sources Show Differential Circadian Clock Gene Expression Profiles

Asynchronous cultures of human primary BMSCs, ADSCs and DPSCs were grown to confluency in complete growth medium. RNA was extracted and a PCR "clock panel" of genes was used to analyse their relative mRNA expression levels. *GAPDH* was selected to be the housekeeping gene as it had the most consistent expression in the different samples, when compared to *β-actin* and *18S* ribosomal RNA, and the expression of the experimental genes were then normalised to the expression of GAPDH throughout. As expected, the different human ASCs did exhibit significantly varying amounts of clock gene expression, with the BMSCs and DPSCs showing antiphasic expressions of *Bmal1*, a component of the positive arm of the molecular clock, and *Per2*, a component of the negative arm (Figure 3.1). Interestingly, *Bmal1* showed significantly increased expression in DPSCs when compared to BMSCs and ADSCs, and the opposite was true for *Per2*, which was significantly higher in BMSCs than ADSCs and DPSCs. Furthermore, *Per1* and *Rev-ErbA* were also both significantly higher in DPSCs than BMSCs and ADSCs, in addition to *Bmal1*. In contrast, for *Bmal1*, *Per1* and *Rev-ErbA* there was no significant difference in expression levels between BMSCs and ADSCs, with the two types of progenitor cell only significantly differing in *Per2* expression.

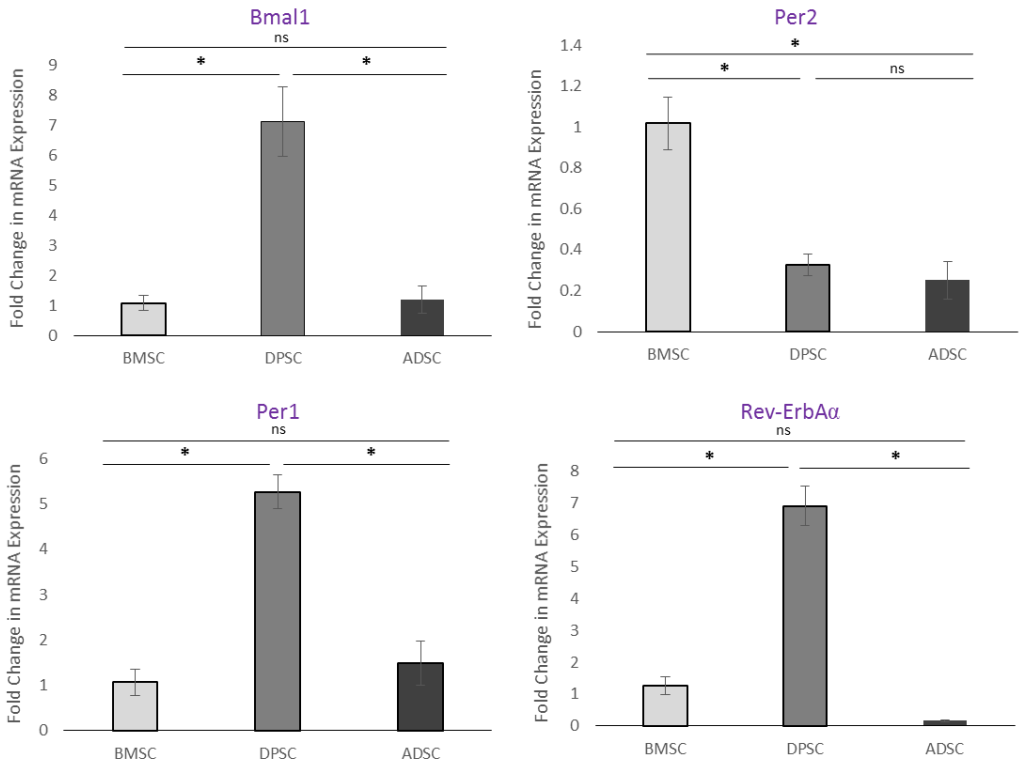

Figure 3.1. Clock gene expression in unsynchronised adult stem cells. *Quantitative RT-PCR analyses comparing the relative mRNA expression levels of core clock genes in mesenchymal-like adult stem cells derived from human bone marrow, dental pulp and adipose tissue. Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (one-way ANOVA).*

3.2.2 Research Section Two: ASC Synchronisation using Chemical Stimulation – Dexamethasone

The three human adult progenitor cell types were allowed to grow to confluency until being synchronised chemically either using dexamethasone, a synthetic glucocorticoid, or serum shock. For the former, the cells were exposed to 100nM dexamethasone for a period of 1 h, after which their media was replaced with complete growth medium. RNA samples were initially taken at 20 h or 32 h post synchronisation. As expected from the two antiphasic genes, the expressions of *Bmal1* and *Per2* did indeed peak and trough at opposite circadian times (Figure 3.2). For example, in ADSCs and DPSCs, *Bmal1* expression was much higher at 32 h than 20 h and *Per2* was much higher at 20 h than 32 h. Interestingly, the opposite appeared to be observed for BMSCs, which showed higher *Bmal1* expression and lower *Per2* expression at 20 h than 32 h, respectively. The mRNA expression of a component of

the stabilising loop in circadian machinery, *Rev-ErbAα*, was also examined. It was observed that *Rev-ErbAα* expression showed different peak/trough expressions at the two circadian time points and notably appeared to peak at 32 h in all three cell types, with *Per2* in BMSCs but with *Bmal1* in ADSCs and DPSCs.

As these two time points were picked at random 12 h apart, a broader time course was examined to determine when the real circadian peaks/troughs of gene expression were, and also to compare the different amplitudes of the changes in gene expression between the three cell types (Figure 3.3). Following exposure to 100 nM dexamethasone for 1 h and a 16 h incubation period to allow the cells to become sufficiently synchronised, samples were collected every 4 hours after synchronisation from 16 h to 52 h, as detailed above in Figure 2.1.

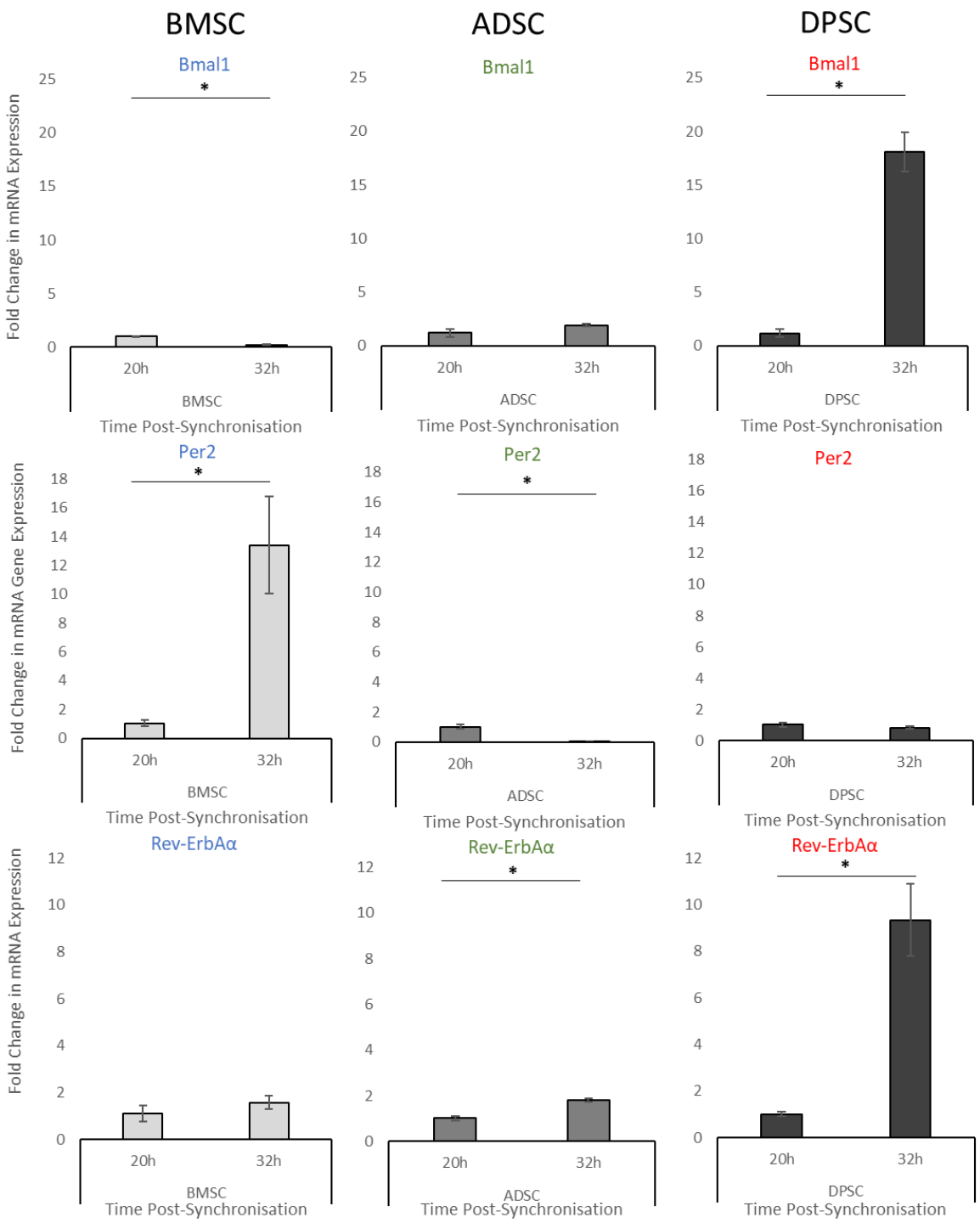

Figure 3.2 Clock gene expression in synchronised human adult stem cells at two opposite circadian phases. *Quantitative RT-PCR analyses showing the expression levels of clock genes after synchronisation with dexamethasone at two opposite circadian phases 12 h apart (20 h vs 32 h). Data are expressed as the mean of ΔCt ±SEM relative to the 20 h time point and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments, *:p<0.05 (independent t-test).*

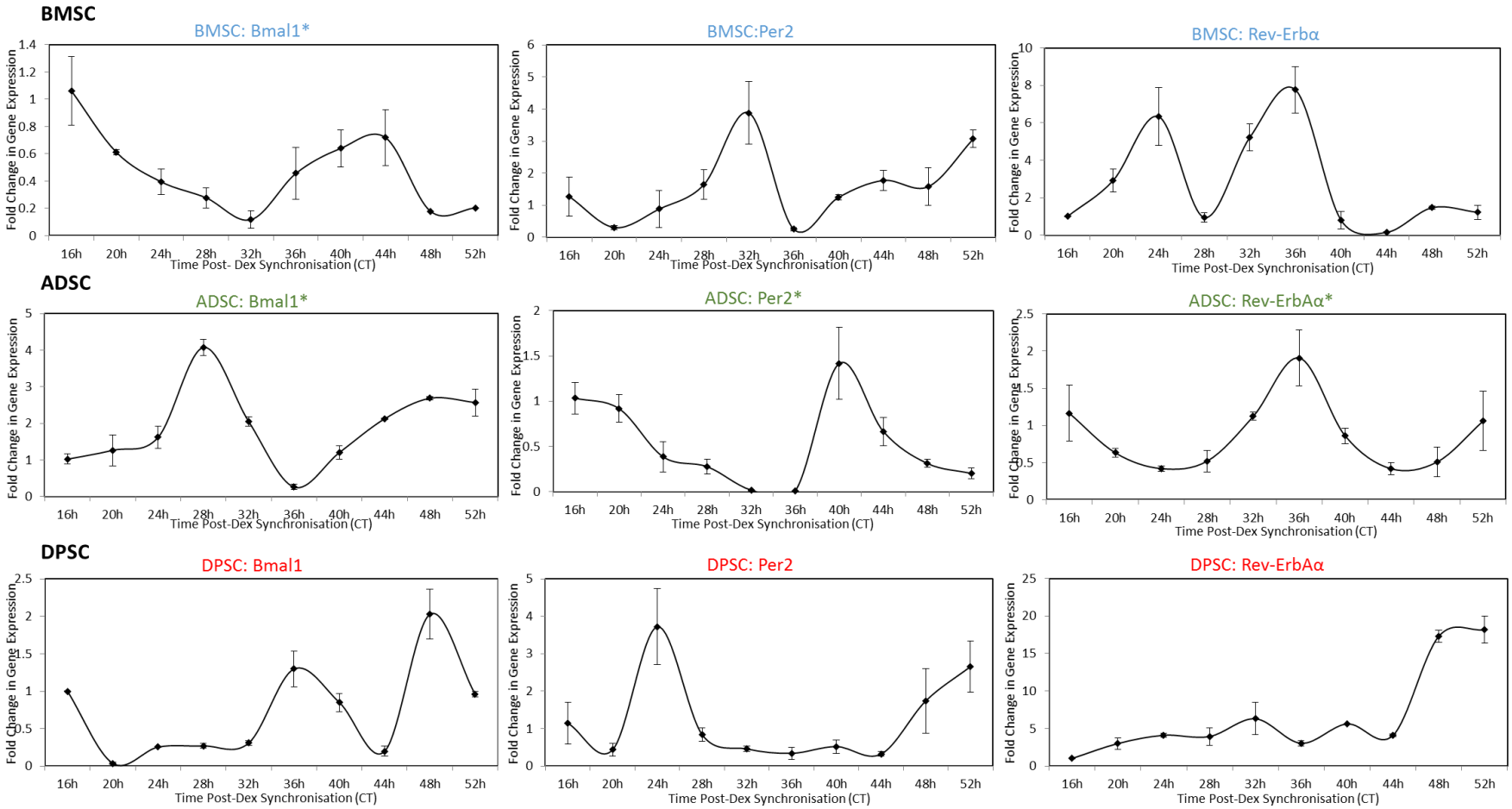

Figure 3.3 Circadian rhythm dynamics in human adult stem cells following synchronisation with dexamethasone. *Quantitative RT-PCR analyses showing temporal expression profiles of clock genes collected every four hours between 16 h - 52 h following synchronisation with dexamethasone. Data, are expressed as the mean of ΔCt ±SEM relative to 16 h timepoint and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments.*

Both the ADSCs and BMSCs showed robust oscillations of clock gene expressions as is a characteristic pattern of synchronised cells. For example, in BMSCs, *Bmal1* troughed and *Per2* peaked together at 32 h post dexamethasone synchronisation. In contrast, in ADSCs, *Bmal1* peaked and *Per2* troughed around 16-20 h, showing a clear antiphasic relationship. The more primitive DPSCs, however, did not exhibit oscillating expressions of the circadian clock genes, and no clear patterns of gene expression could be observed. When the clock gene expressions were analysed using cosinor software analysis (Table 3.1), *Bmal1* was found to show significant circadian rhythmicity in both BMSCs (n = 3, p = 0.007) and ADSCs (n = 3, p = 0.006), as well as Per2 (n = 3, p = 0.021) and *Rev-ErbAα* (n = 3, p = 0.001) in ADSCs. No clock genes were reported to have significant circadian rhythmicity in DPSCs.

Table 3.1. Cosinor analysis of circadian clock rhythmicity in human BMSCs, ADSCs and DPSCs following dexamethasone synchronisation.

Cell	Gene	Period (h)	P value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
BMSC	***Bmal1****	23.9	0.007	-76	0.331	0.451	76.2
	Per2	20.2	0.076	-261	1.099	1.597	52.1
	Rev-erbAα	26.0	0.391	-287	1.853	3.158	23.6
ADSC	***Bmal1****	20.0	0.006	-289	1.264	1.885	77.4
	Per2*	23.4	0.021	-98	0.502	0.486	66.6
	Rev-erbAα*	21.8	0.001	-314	0.611	0.914	85.1
DPSC	*Bmal1*	20.0	0.571	-9	0.325	0.720	14.9
	Per2	26.0	0.101	-176	1.089	1.071	47.9
	Rev-erbAα	26.0	0.055	-266	4.952	4.938	56.2

(* denotes significance according to the Cosinor analysis software)

Control samples, which received the same treatment without the addition of 100 nM dexamethasone, showed a dampened response to synchronisation with a substantially decreased amplitude (Figure 3.4).

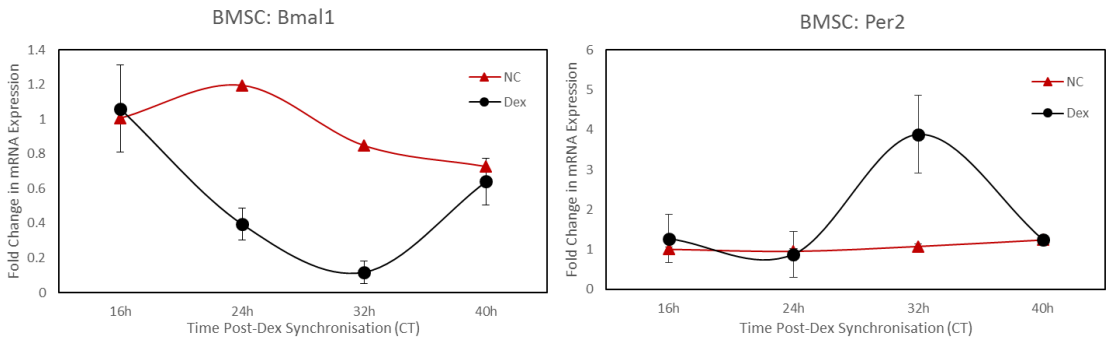

Figure 3.4 Comparison of temporal clock gene expression between unsynchronised and dexamethasone-synchronised BMSCs. *Quantitative RT-PCR analyses overlays of temporal clock gene expression in dexamethasone synchronised and unsynchronised (negative control) samples (NC). Data is expressed as the mean of ΔCt±SEM calculated from the Ct value of each gene relative to the 16h time point and normalised against the housekeeping gene GAPDH. Bars represent means ± SEM of 2-3 independent experiments.*

In order to see how this synchronisation by dexamethasone may affect other non-clock ASC related genes, the expression pattern of a progenitor cell pluripotency marker, *Sox2*, and a progenitor cell early differentiation marker, *Sox9*, were also examined to see if either of these followed a circadian gene expression pattern. To this end, the temporal expression of *Sox2* and *Sox9* was recorded every four hours over an entire circadian cycle (Figure 3.5) under the same synchronisation methodology and collection procedure (Figure 2.1).

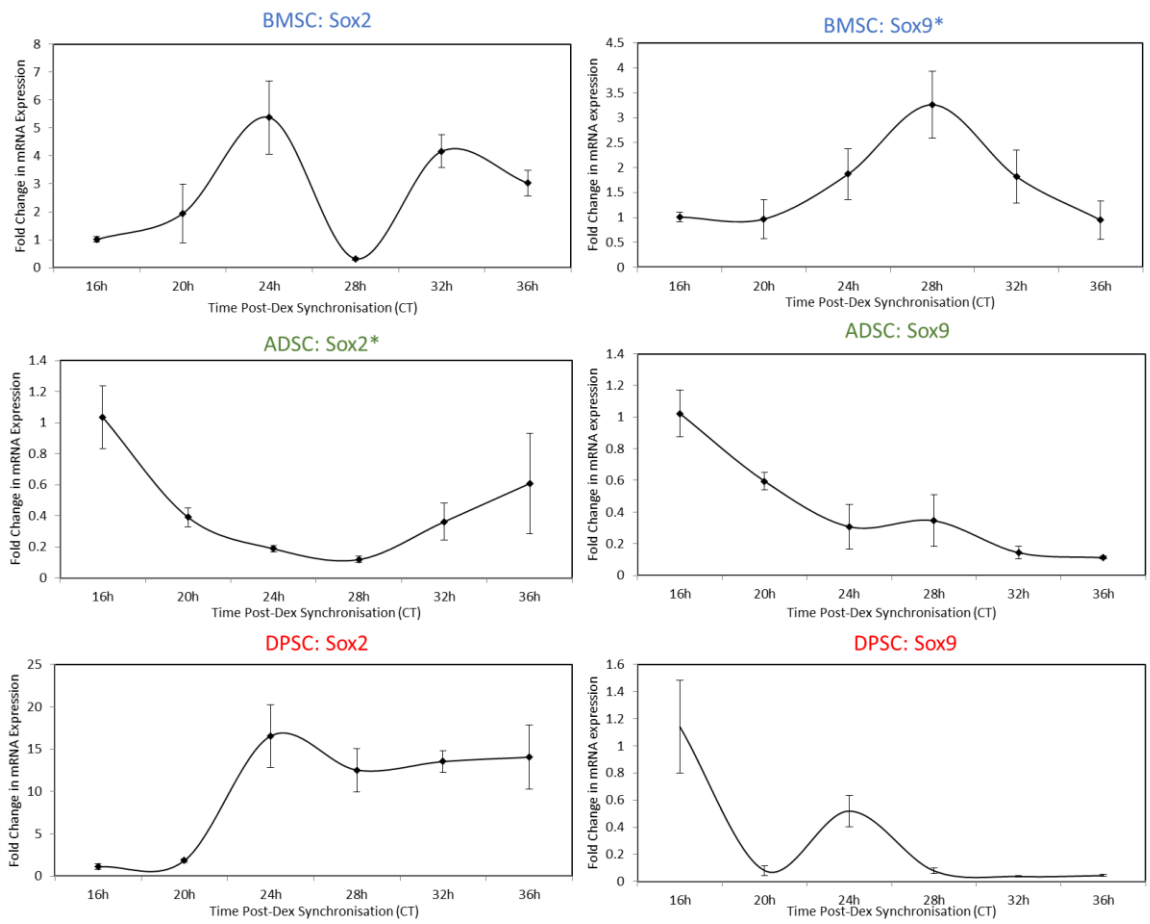

Figure 3.5. **Sox2 *and* Sox9 *gene expression dynamics in human adult progenitor cells following synchronisation with dexamethasone.*** *Quantitative RT-PCR analyses showing temporal expression profiles of the pluripotency marker,* Sox2, *and progenitor cell differentiation marker,* Sox9, *collected every four hours between 16 h - 36 h following synchronisation with dexamethasone. Data, are expressed as the mean of ΔCt ±SEM relative to 16 h timepoint and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments.*

When analysed using the cosinor software to determine the significance of circadian rhythmicity (Table 3.2), it was determined that *Sox2,* the pluripotency marker, significantly oscillated in a circadian manner in ADSCs (n = 3, p = 0.009) and oscillated in DPSCs just short of significance in DPSCs (n = 3, p = 0.07). *Sox9*, the differentiation marker, was demonstrated to significantly oscillate in BMSCs (n = 3, p = 0.02). Strikingly, it was found that *Sox2* showed a very similar pattern and amplitude of gene expression to that of *Rev-ErbAα* (Figure 3.6).

Table 3.2. Cosinor analysis of Sox2 *and* Sox9 *rhythmicity in human BMSCs, ADSCs and DPSCs following dexamethasone synchronisation.*

Gene	Cell	Period (h)	P value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
Sox2	BMSC	26.0	0.658	-218	1.114	2.348	19.0
	ADSC*	21.8	0.009	-348	0.371	0.354	74.3
	DPSC	26.0	0.074	-261	6.463	7.800	52.5
Sox9	**BMSC***	20.0	0.022	-216	1.105	1.794	92.1
	ADSC	21.7	0.438	-30	0.531	0.462	34.5
	DPSC	26.0	0.668	-49	0.213	0.223	15.1

(* denotes significance according to the Cosinor analysis software)

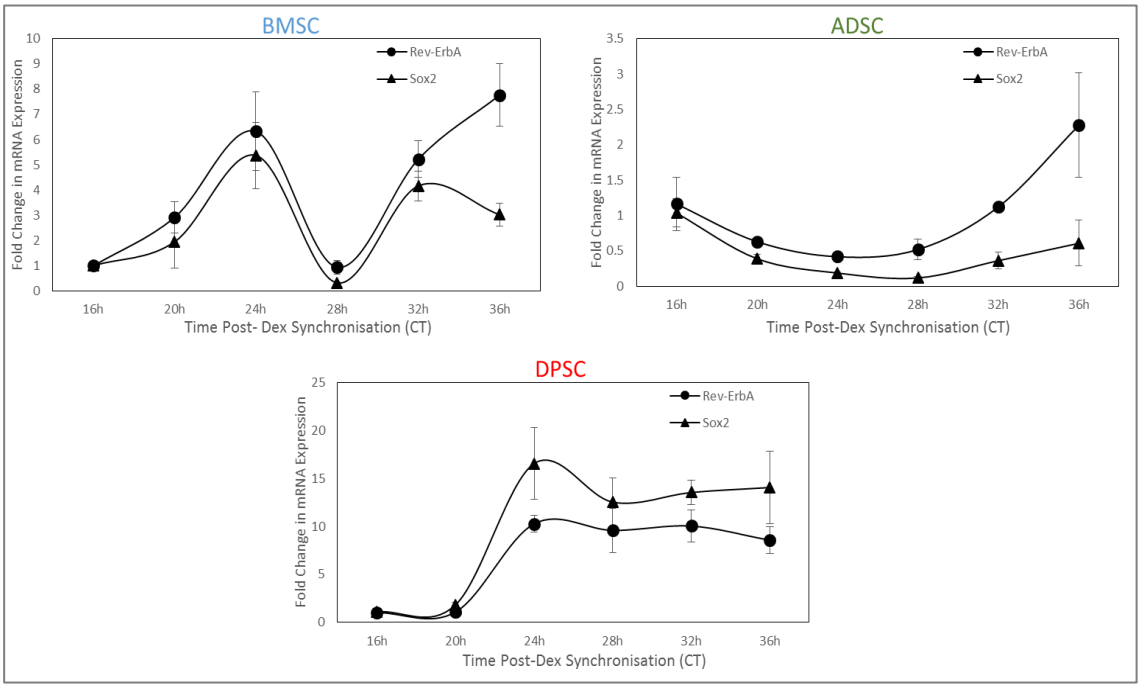

Figure 3.6. Comparison of the cyclical expression of Rev-ErbA ***and*** Sox2 ***following synchronisation by dexamethasone.*** *Quantitative RT-PCR analyses overlays of* Rev-ErbA *and* Sox2 *temporal gene expressions over 1 circadian cycle (16h to 36h) following synchronisation with dexamethasone. Data is expressed as the mean of ΔCt±SEM calculated from the Ct value of each gene relative to the 16h time point and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments.*

3.2.3 Research Section Three: ASC Synchronisation using Chemical Stimulation – Serum Shock

For synchronisation using serum shock, the three different ASCs from different anatomical sources were again allowed to grow to confluency before their growth medium was removed and replaced with media with 50% horse serum for 2 h. After this time, the 50% horse serum medium was removed and replaced by serum free medium. The cells were incubated for 16 h to allow the effects of the serum shock to occur and the cells to be synchronised. RNA samples were initially taken at 20 h or 32 h post synchronisation (Figure 3.7).

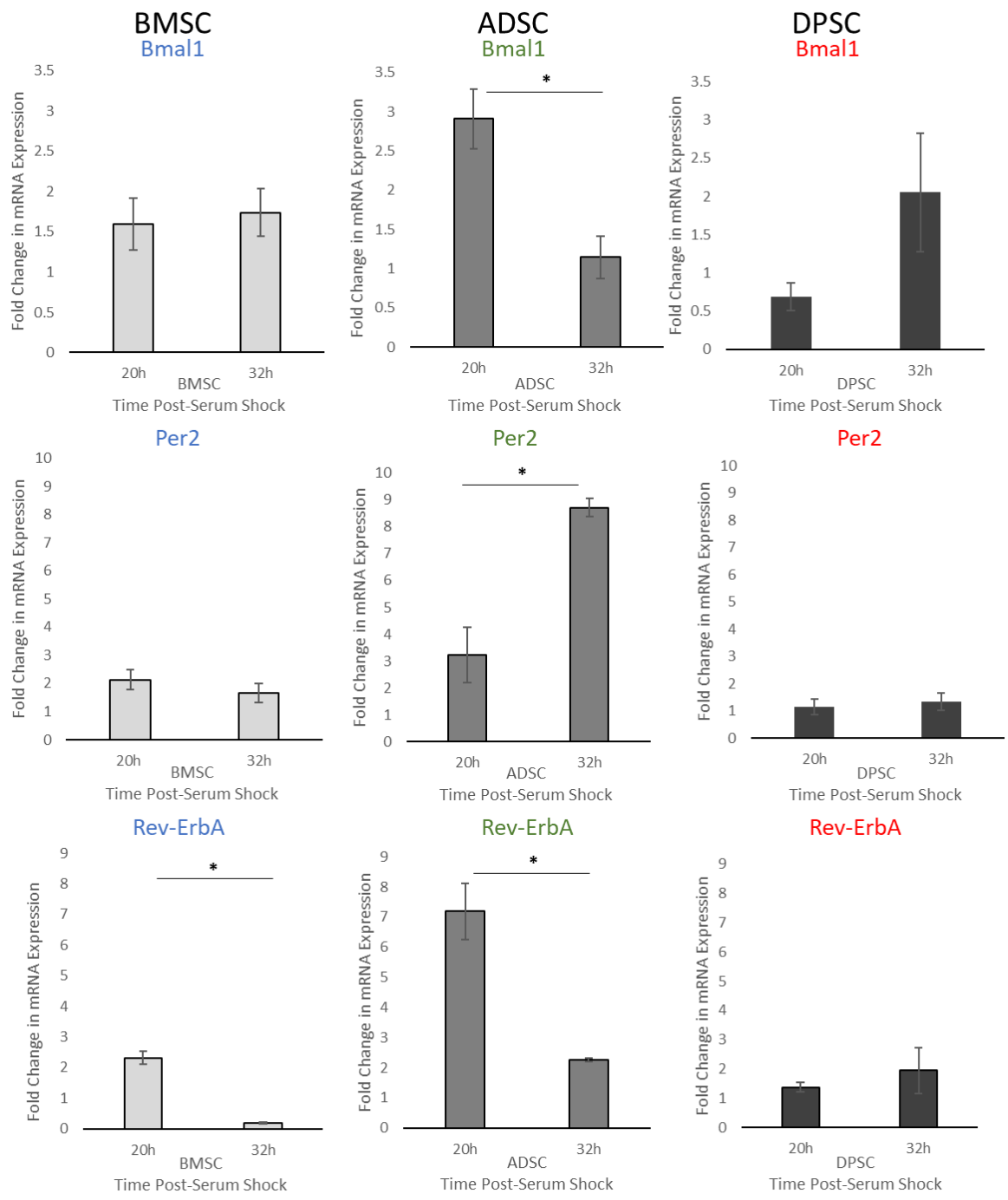

Figure 3.7 Clock gene expression in synchronised human adult stem cells at two opposite circadian phases. *Quantitative RT-PCR analyses showing the expression levels of clock genes after synchronisation with serum shock at two opposite circadian phases 12 h apart (20 h vs 32 h). Data are expressed as the mean of ΔCt ±SEM relative to the 20 h time point and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments, *:p<0.05 (independent t-test).*

In BMSCs, despite no significant differences being observed, *Bmal1* and *Per2* still showed antiphasic expression, with *Bmal1* peaking at 32 h and troughing at 20h, and *Per2* peaking at 20 h and troughing at 32 h. These observations are in direct contrast to synchronisation by dexamethasone, where *Bmal1* peaked at 20 h and *Per2* at 32 h. This was also the case for ADSCs, whereby *Bmal1* and *Per2* were expressed antiphasically but at the opposite time points compared to when synchronised with dexamethasone; *Bmal1* peaked at 32 h following exposure to dexamethasone but at 20 h following serum shock, and *Per2* peaked at 20 h following dexamethasone but at 32 h following serum shock. Similarly, *Rev-ErbAα* was also found to show the opposite expression pattern when compared to dexamethasone, peaking at 20 h in both BMSCs and ADSCs following serum shock, where it had peaked at 32 h following dexamethasone. No significant changes in the clock genes expression between the two time points were observed in DPSCs.

In order to gain a finer temporal resolution over an entire circadian cycle, the clock gene expressions were analysed over an extended time course from 16 h to 36 h, with samples being collected every four hours (Figure 3.8), as described in Figure 2.1. It was observed in BMSCs that *Bmal1* was at its lowest expression at 24 h, when *Per2* was highest in expression, demonstrating again the antiphasic relationship of the two clock genes. Similarly, in ADSCs, the expression of *Bmal1* was highest at 20 h, when the expression of *Per2* was low, and the expression of *Per2* was highest at 32 h when the expression of *Bmal1* was low. Surprisingly, no significant circadian rhythmicity was detected in any clock gene when analysed by cosinor software (Table 3.3), however, this may be due to the shorter time course utilised here, due to a restricted sample size.

Table 3.3. Cosinor analysis of circadian clock rhythmicity in human BMSCs, ADSCs and DPSCs following serum shock synchronisation.

Cell	Gene	Period (h)	P value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
BMSC	*Bmal1*	26.0	0.328	-256	0.397	1.551	52.4
	Per2	20.0	0.255	-139	0.556	1.993	59.8
	Rev-erbA	23.8	0.113	-62	0.892	0.853	76.7
	Sox2	20.0	0.531	-65	0.596	1.492	39.7
	Sox9	20.0	0.511	-71	0.276	1.316	38.1
ADSC	*Bmal1*	20.4	0.141	-88	0.825	1.600	73.0
	Per2	26.0	0.400	-253	0.683	3.686	51.3
	Rev-erbA	20.0	0.520	-66	2.035	2.628	38.8
	Sox2	26.0	0.348	-234	5.913	7.666	50.6
	Sox9	24.8	0.515	-242	2.858	4.287	35.8
DPSC	*Bmal1*	26.0	0.198	-278	1.006	1.397	66.1
	Per2	20.0	0.737	-114	0.328	1.398	18.5
	Rev-erbA	26.0	0.267	-128	0.866	1.576	58.6
	Sox2	25.9	0.119	-19	0.406	0.630	75.8
	Sox9	22.2	0.202	-64	0.459	0.762	65.6

(* denotes significance according to the Cosinor analysis software)

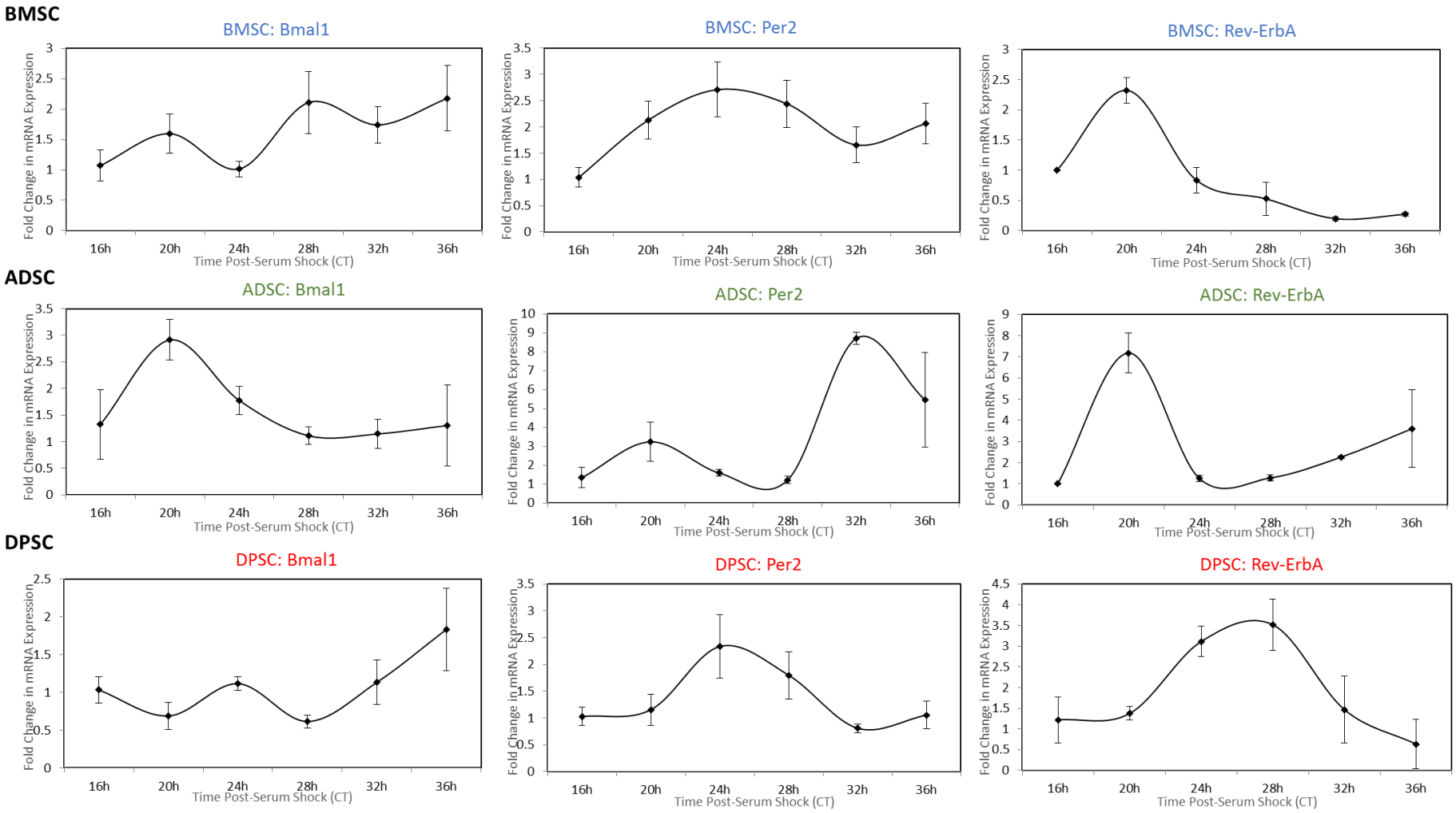

Figure 3.8. Circadian rhythm dynamics in human adult stem cells following synchronisation with serum shock. *Quantitative RT-PCR analyses showing temporal expression profiles of clock genes collected every four hours between 16 h - 36 h following synchronisation with serum shock. Data, are expressed as the mean of ΔCt ±SEM relative to 16 h timepoint and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments.*

Again, in order to see if circadian synchronisation by serum shock was sufficient to affect the expression of other non-circadian progenitor cell genes, the expression pattern and circadian rhythmicity was examined in the pluripotency marker, *Sox2*, and differentiation marker, *Sox9*. To this end, the temporal expression of both *Sox2* and *Sox9* was determined every four hours over an entire circadian cycle under the same serum shock synchronisation conditions (Figure 3.9). No significant circadian rhythmicity could be demonstrated for either gene in any of the three cell types. However, the temporal expression profiles of *Sox2* and *Sox9* appeared to show very similar patterns of expression for each cell type.

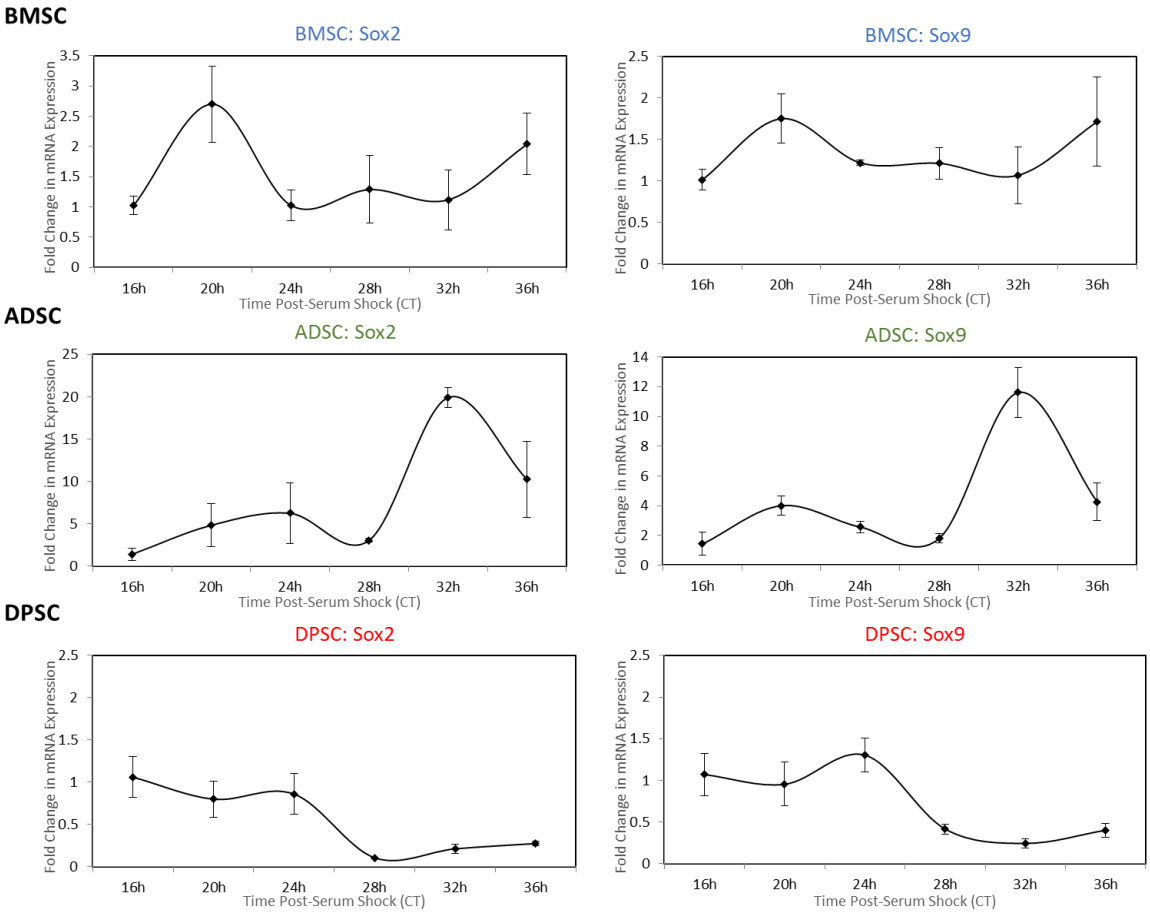

Figure 3.9. Sox2 and Sox9 gene expression dynamics in human adult progenitor cells following synchronisation with serum shock *Quantitative RT-PCR analyses showing temporal expression profiles of the pluripotency marker,* Sox2*, and progenitor cell differentiation marker,* Sox9*, collected every four hours between 16 h - 36 h following synchronisation with serum shock. Data, are expressed as the mean of ΔCt ±SEM relative to 16 h timepoint and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments.*

3.2.4 Research Section Four: ASC Synchronisation using Mechanical Stimulation

As the DPSCs had not been amenable to synchronisation by either form of chemical stimulation, it was hypothesised that, as the cells are encapsulated in a tight niche in the tooth but are still subjected to a substantial amount of mechanical stimulation, they could potentially be entrained using mechanical means, if not chemical. Therefore, both BMSCs, used as a control here as the cells had previously shown that they had the ability to be entrained in a circadian manner, and DPSCs were seeded onto fibronectin coated, flexible silicone chambers (Figure 2.2) and subjected to 3 days of rhythmic mechanical stretch, undergoing 12 h of cyclical stretching followed by 12 h of relaxation for three days. The adherence of both cell types to the fibronectin coated silicone chambers can be seen below, 24 h after being seeded into the chambers (Figure 3.10).

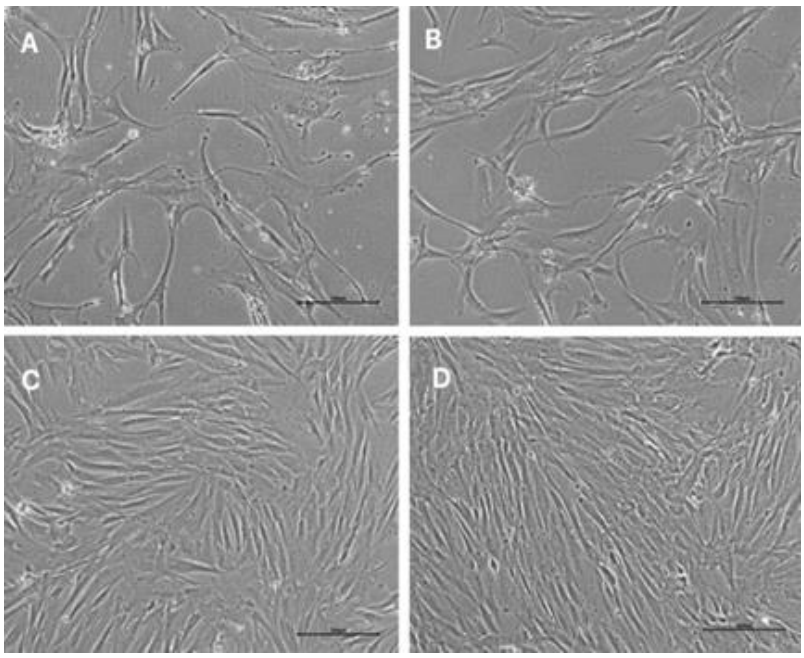

Figure 3.10 The morphology of human primary BMSCs (A-B) and DPSCs (C-D) 24h after being seeded into fibronectin coated silicone chambers.

Following stretch, the DPSCs exhibited a changed morphology and showed a degree of alignment, reorienting parallel to the direction of the mechanical stretch, as can be seen in Figure 3.11.

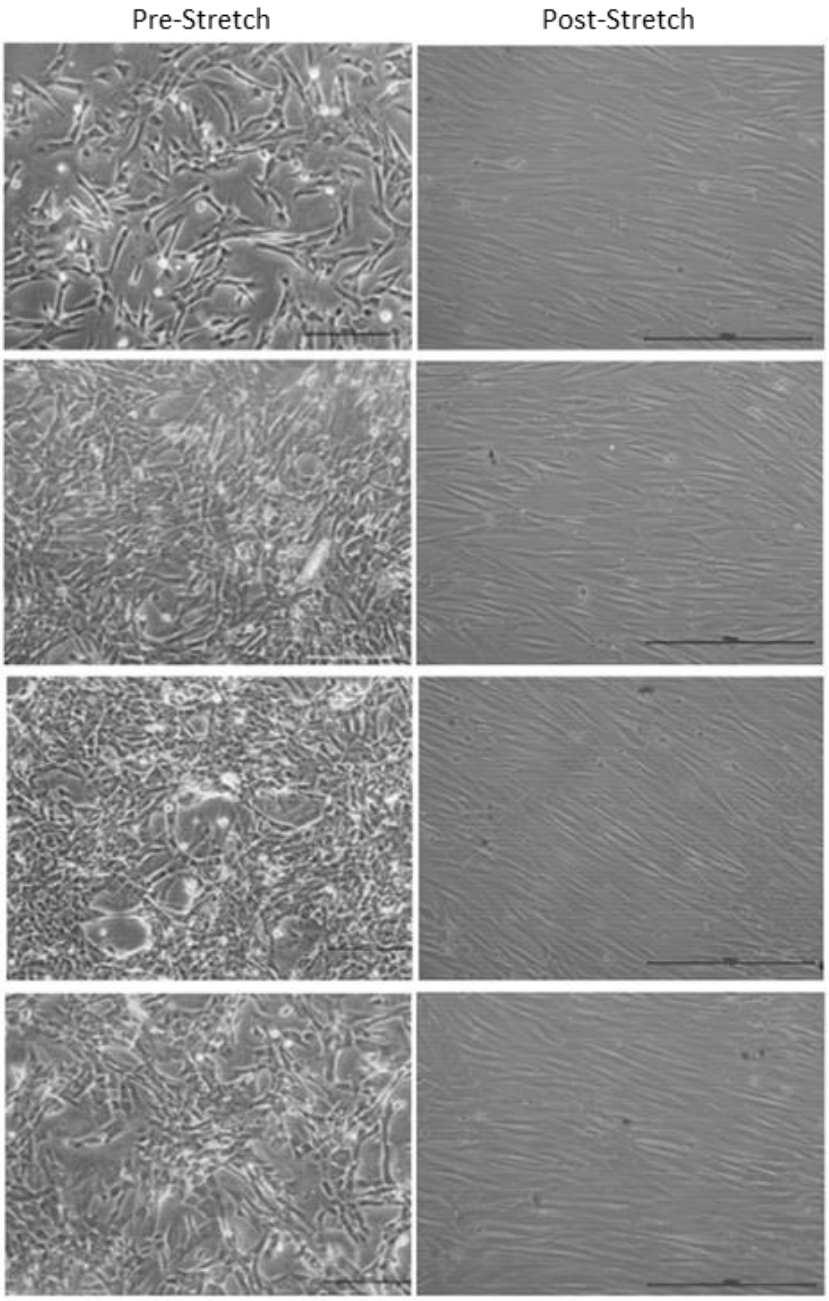

Figure 3.11. DPSCs before and after being subjected to rhythmic mechanical stretch (3 days; 12h ON/12h OFF, 6.66% stretch, 1Hz).

After the completion of this stretching regime, the cells were kept in static conditions and samples collected at 20 h and 32 h after their last exposure to the stretch, and the clock gene expressions were analysed (Figure 3.12). Here, in striking contrast to

synchronising by chemical agents, the clock gene levels in DPSCs peaked and troughed together in the characteristic antiphasic manner for *Bmal1* and *Per2*; in both BMSCs and DPSCs, *Bmal1* peaked in expression at 20 h, in contrast to *Per2*, which peaked in expression at the opposite circadian time point, being much higher in expression at 32 h, compared to 20 h. In contrast, *Rev-ErbAα* peaked at opposite time points when comparing the two cell types, peaking at 20 h in BMSCs and 32 h in BMSCs, although none of these differences were significant.

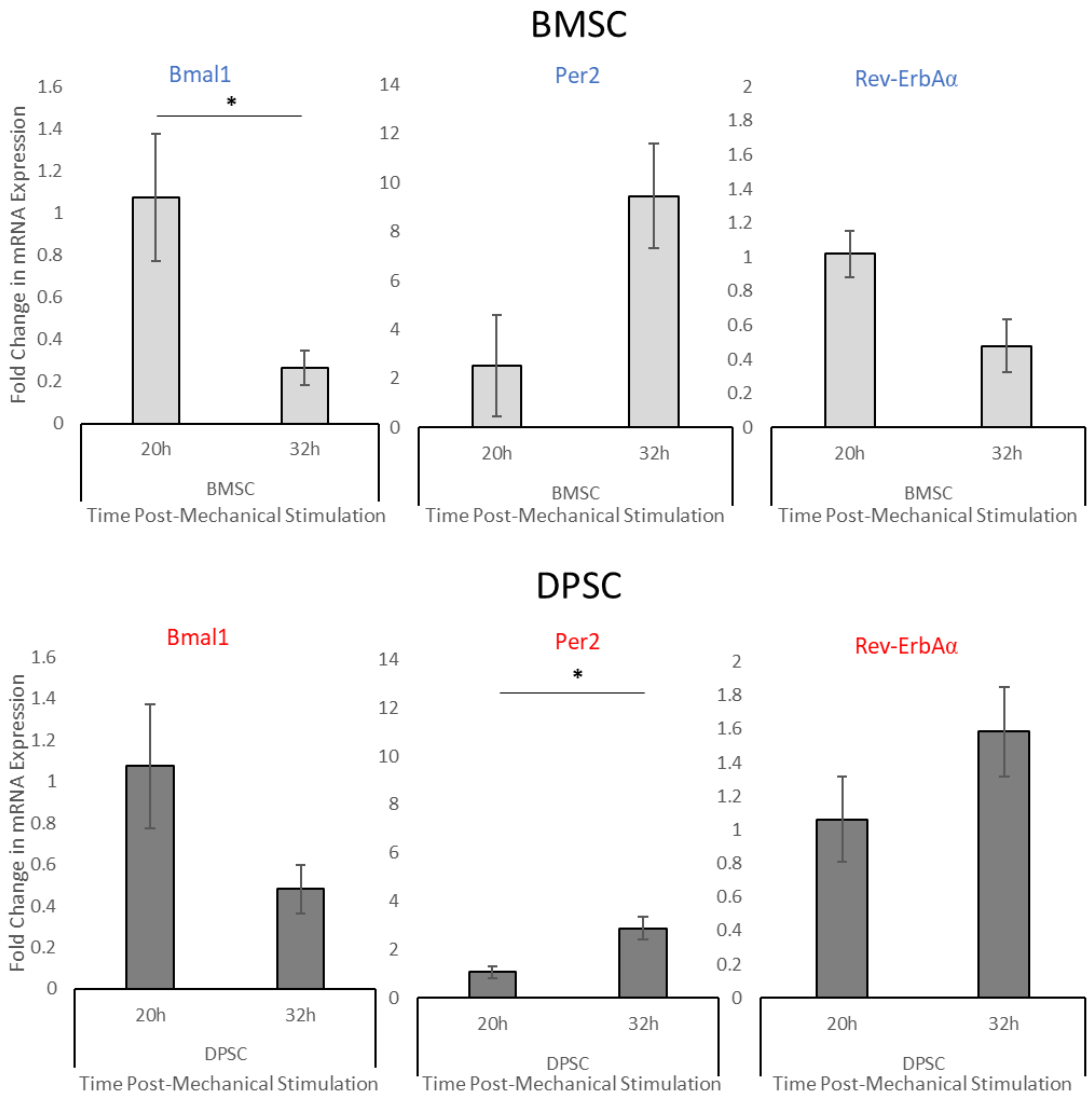

Figure 3.12 Clock gene expression in human adult stem cells following rhythmic mechanical stimulation paradigm. *Quantitative RT-PCR analyses showing the expression levels of clock genes at two opposite circadian phases (20 h vs 32 h) following rhythmic mechanical stimulation protocol. Data, are expressed as the mean of ΔCt ±SEM relative to the 20 h time point and normalised against the housekeeping gene GAPDH. Bars represent means ± SEM of 3 independent experiments, *:p<0.05 (independent t-test).*

With the aim of examining the temporal gene expression profiles over 1.5 circadian days, the clock genes expression levels were next analysed every four hours from 16 h to 52 h post mechanical stimulation (Figure 2.2B). From these longer circadian time courses, it was evident that DPSCs can be entrained in a circadian manner using rhythmic mechanical stimulation. Oscillating expressions of the core clock genes could be seen, which were surprisingly as robust in the DPSCs as in the BMSCs. In the DPSCs, *Bmal1* expression exhibited significant circadian rhythmicity when analysed by cosinor circadian software (Table 3.4) and was at its lowest expression at 32 h, which was the circadian time point when *Per2* was at its highest (Figure 3.13). This data shows that although the DPSCs could not be synchronised using chemical means, they could be entrained mechanically. Regarding the previously mentioned progenitor cell specific markers *Sox*2 and *Sox9*, time courses were also determined for their levels and patterns of gene expression over a circadian time course of 16 h to 36 h (Figure 3.14). Here, the expression of *Sox2* appeared to exhibit alternating levels of expression in the two cell types – for example, *Sox2* was at its lowest level of expression at 36 h in BMSCs when it was high in expression in the DPSCs. Furthermore, when analysed by cosinor software, the circadian expression of *Sox2* was only just short of significance ($n = 3$, $p = 0.087$). For *Sox9*, the gene expression oscillations appeared to show similar patterns of expression but did not reach significant circadian rhythmicity in either cell type (Table 3.4).

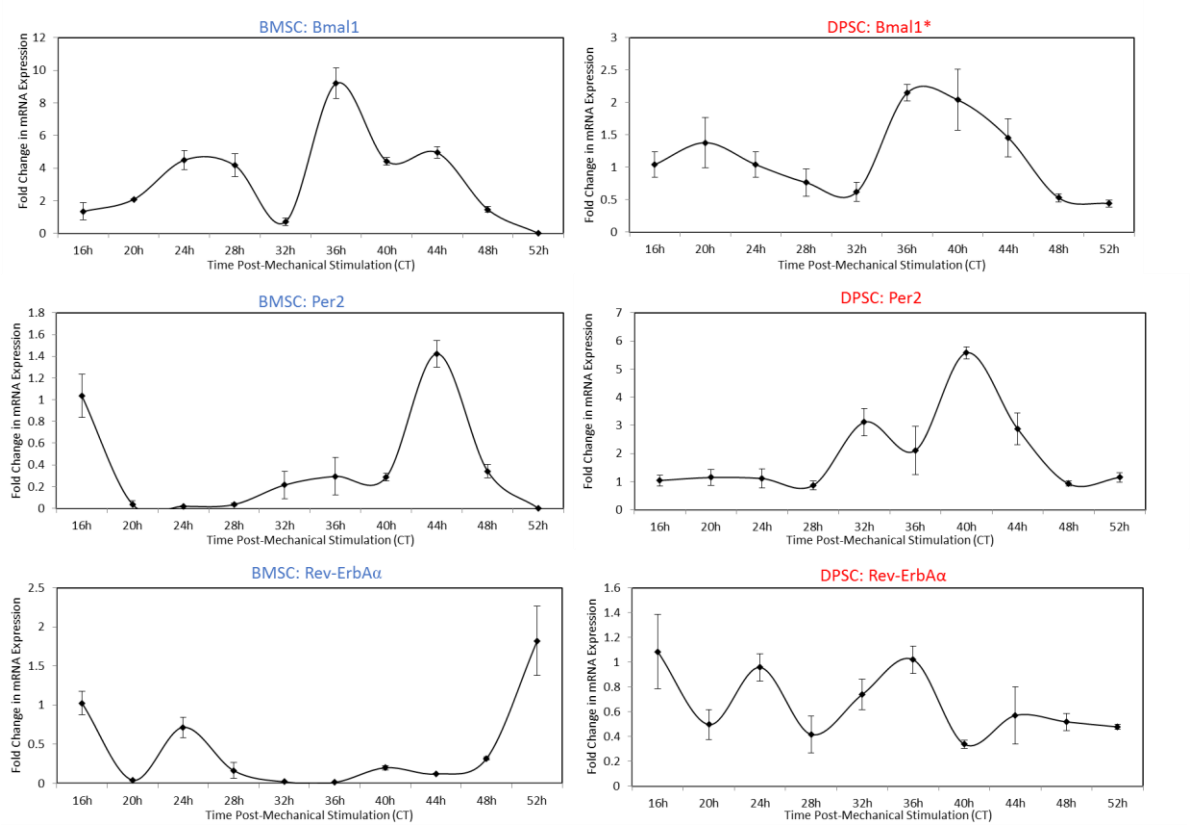

Figure 3.13. Circadian gene dynamics in human adult stem cells after rhythmic mechanical stimulation protocol. *Quantitative RT-PCR analyses showing temporal expression profiles of clock genes following 3 days of rhythmic mechanical stimulation. Data are expressed as the mean +/- of ΔCt ±SEM relative to 16 h timepoint and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments.*

Table 3.4. Cosinor analysis of circadian clock rhythmicity in human BMSCs and DPSCs following synchronisation by rhythmic mechanical stretch.

Cell	Gene	Period (h)	P value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
BMSC	*Bmal1*	26.0	0.571	-346	1.754	3.568	21.6
	Per2	26.0	0.123	-51	0.428	0.343	45.0
	Rev-erbA	26.0	0.559	-171	0.316	0.397	15.4
	Sox2	26.0	0.541	-61	0.328	0.279	41.2
	Sox9	22.2	0.149	-302	0.488	0.747	71.9
DPSC	***Bmal1****	20.0	0.020	-135	0.664	1.146	67.0
	Per2	26.0	0.132	-354	1.362	2.179	43.9
	Rev-erbA	21.2	0.505	-49	0.155	0.669	17.8
	Sox2	26.0	0.087	-311	9.926	11.858	55.7
	Sox9	20.0	0.765	-243	0.028	0.112	16.4

(* denotes significance according to the Cosinor analysis software)

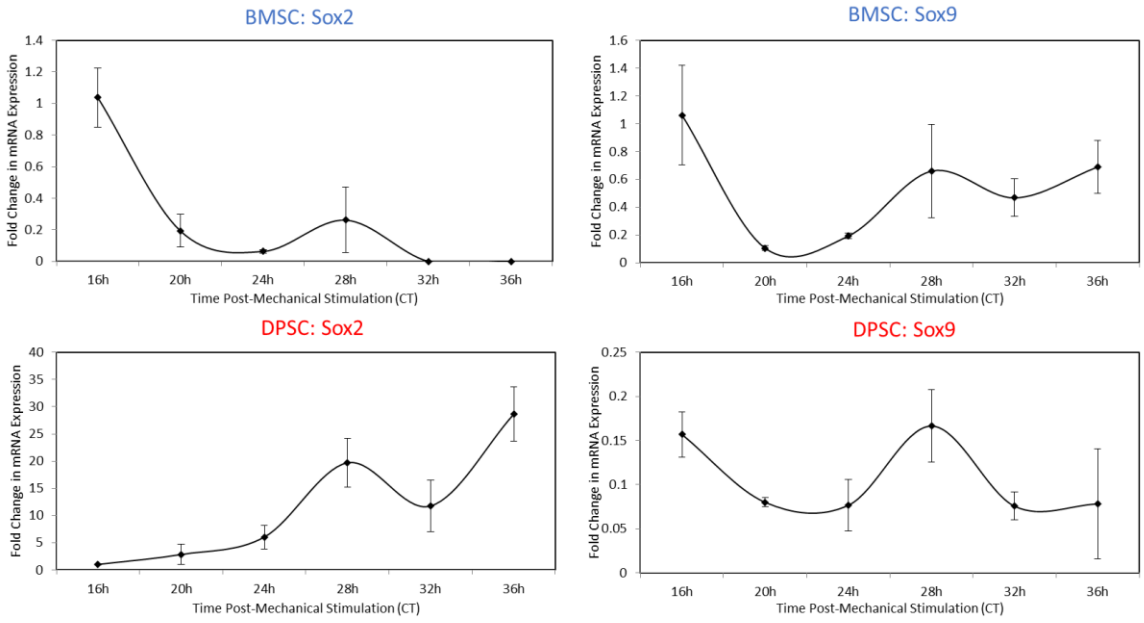

Figure 3.14. Sox2 and Sox9 gene expression dynamics in human adult progenitor cells following synchronisation by mechanical stretch. *Quantitative RT-PCR analyses showing temporal expression profiles of the pluripotency marker, Sox2, and progenitor cell differentiation marker, Sox9, collected every four hours between 16 h - 36 h following synchronisation with dexamethasone. Data, are expressed as the mean of ΔCt ±SEM relative to 16 h timepoint and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments.*

3.2.5 Research Section Five: ASC Synchronisation using Temperature Entrainment

In the body, DPSCs are subject to extreme fluctuations in temperature and 'thermal shock', due to their anatomical location in the jaw. Therefore, it was hypothesised that these cells might have the ability to respond to thermal stimulation in a rhythmic manner. BMSCs and DPSCs were exposed to three days of temperature fluctuation, whereby they were incubated at two different temperatures for 12 h each time. The temperatures of 35.5° and 38.5° were selected (1.5± 37.0° normal growth temperature). Following the three days, the cells were placed back at 37.0° and, as with the other entrainment experiments, samples were initially collected at 20 h and 32 h post-thermal stimulation (Figure 3.15). Following analysis by PCR, it was determined that *Rev-ErbA*α exhibited significantly higher levels of expression at 32 h than at 20 h ($n = 3$, $p \leq 0.05$) and *Bmal1* appeared to be higher in expression at 20 h than 32 h ($n = 3$, $p = 0.08$) in BMSCs, but there were no significant differences in *Per2* expression. In the DPSCs, similar patterns of gene expressions were seen as with the BMSCs, with the genes peaking in expression at all of the same time points, but no changes of any significance could be determined.

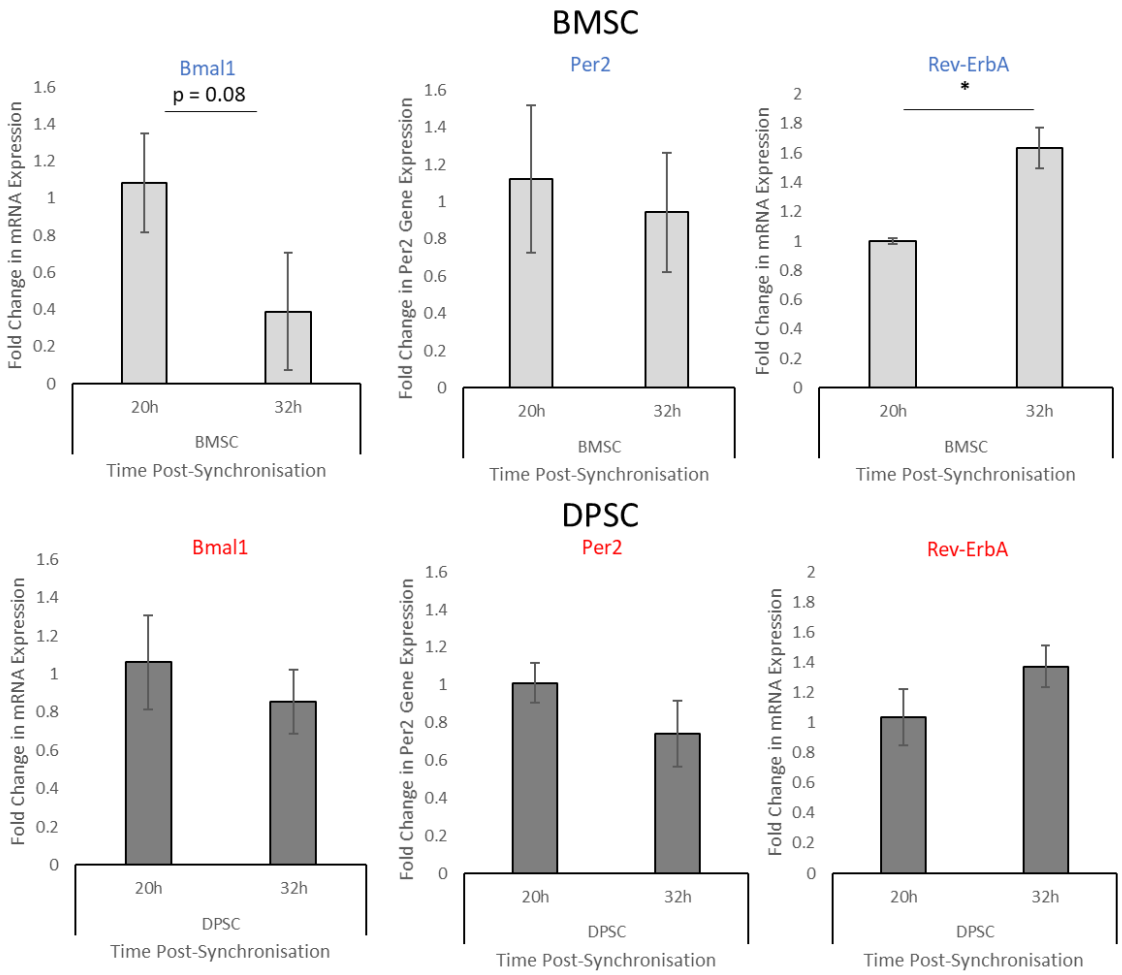

***Figure 3.15 Clock gene expression in synchronised human adult stem cells at two opposite circadian phases**. Quantitative RT-PCR analyses showing the expression levels of clock genes after synchronisation by temperature at two opposite circadian phases 12 h apart (20 h vs 32 h). Data are expressed as the mean of ΔCt ±SEM relative to the 20 h time point and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments, *:p<0.05 (independent t-test).*

When the time courses were extended, in order to fully elucidate the temporal changes in gene expression over a longer, more detailed, time period, fluctuations in gene expression could be observed. For example, *Bmal1* peaked at 24 h following temperature entrainment in BMSCs, and *Rev-ErbAα* peaked at 36 h in both BMSCs and DPSCs (Figure 3.16). As *Rev-ErbAα* peaked at the same time point for both cell types, this is a strong suggestion that the thermal stimulation was sufficient enough to alter the expression levels and temporal profiles of the clock genes in itself. As the gene expression peaked in the two cell types at the same time, this suggests that the thermal signals could be transducing into and effecting the cells and in similar ways.

Unfortunately, no significant circadian rhythmicity could be obtained for any of the three clock genes in either cells types when analysed using cosinor software, with the oscillating expression of *Bmal1* (n = 3, p = 0.057) and *Per2* (n = 3, p = 0.09) just falling short of significance in BMSCs (Table 3.5).

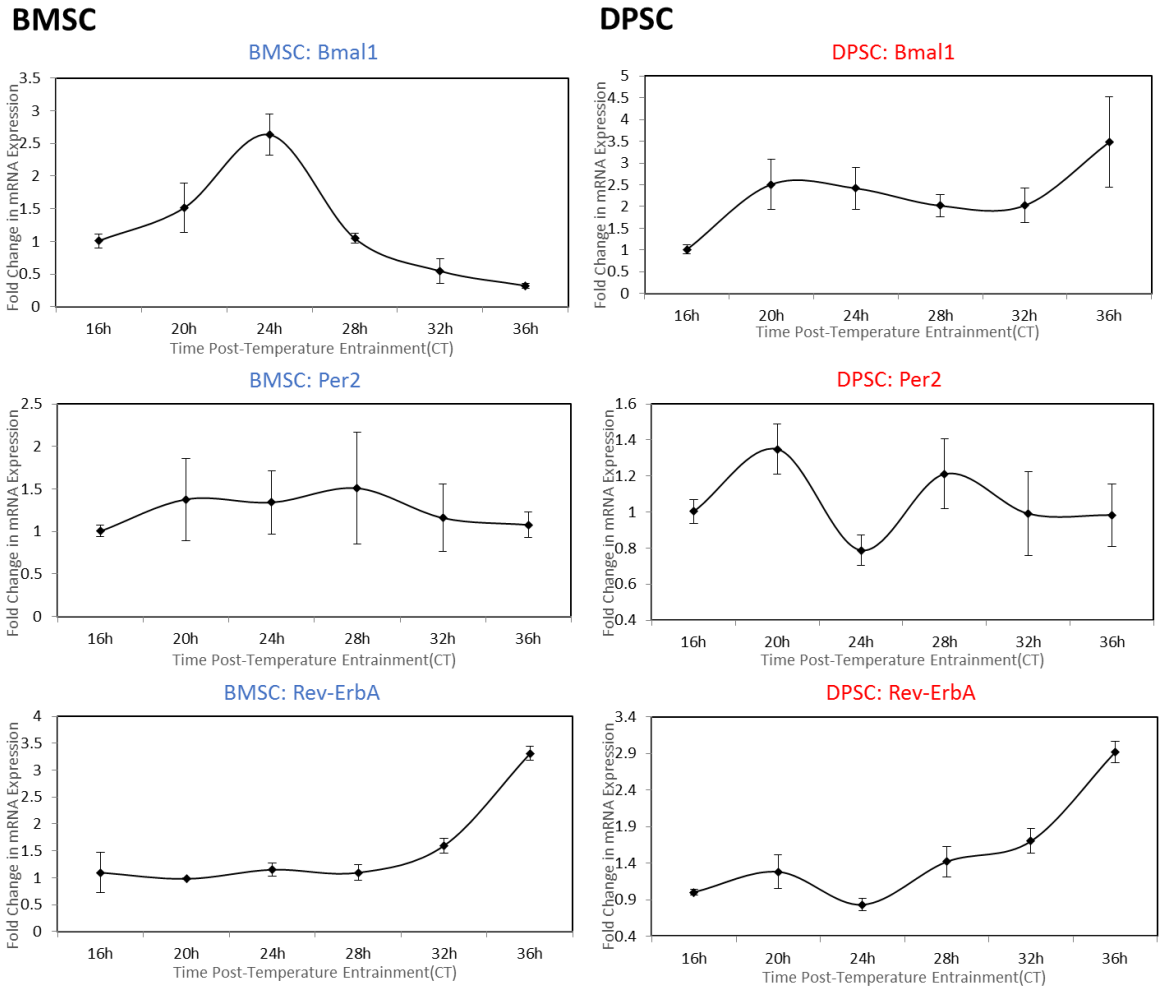

Figure 3.16. Circadian rhythm dynamics in human adult stem cells following synchronisation with temperature. *Quantitative RT-PCR analyses showing temporal expression profiles of clock genes collected every four hours between 16 h - 36 h following synchronisation with temperature. Data, are expressed as the mean of ΔCt ±SEM relative to 16 h timepoint and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments.*

Table 3.5. Cosinor analysis of circadian clock rhythmicity in human BMSCs and DPSCs following temperature entrainment.

Cell	Gene	Period (h)	P value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
DPSC	*Bmal1*	26.0	0.868	-231	0.297	2.245	8.9
	Per2	26.0	0.930	-58	0.053	1.054	4.7
	Rev-erbA	26.0	0.191	-269	0.787	1.569	66.8
	Sox2*	26.0	0.020	-208	0.470	1.433	92.6
	Sox9	26.0	0.260	-266	0.291	1.496	59.2
BMSC	*Bmal1*	22.0	0.057	-115	0.994	1.235	85.1
	Per2	26.0	0.093	-132	0.238	1.225	79.5
	Rev-erbA	26.0	0.232	-275	0.918	1.595	62.3
	Sox2	26.0	0.186	-254	0.406	1.257	67.5
	Sox9	20.0	0.124	-178	1.336	1.786	75.1

(* denotes significance according to the Cosinor analysis software)

For the progenitor cell differentiation marker *Sox9,* the level of gene expression was at its highest level at 28 h in BMSCs, shortly after the peak in *Bmal1* expression at 24 h. This suggests that the rise in *Sox9* may be as a result of this prior increase in *Bmal1* (Figure 3.17), potentially purporting that *Bmal1* may regulate the expression of progenitor cell differentiation markers in some way. Similarly, the expression of the pluripotency cell marker *Sox2* also definitively peaked in gene expression at 28 h in DPSCs (Figure 3.17), again suggesting that there may be some regulation of progenitor cell pluripotency markers gene expression by the clock genes. This was further supported when the expression of *Sox2* in DPSCs was analysed using cosinor data analysis software, where it was shown to exhibit significant circadian rhythmicity (n = 3, p = 0.02) (Table 3.5).

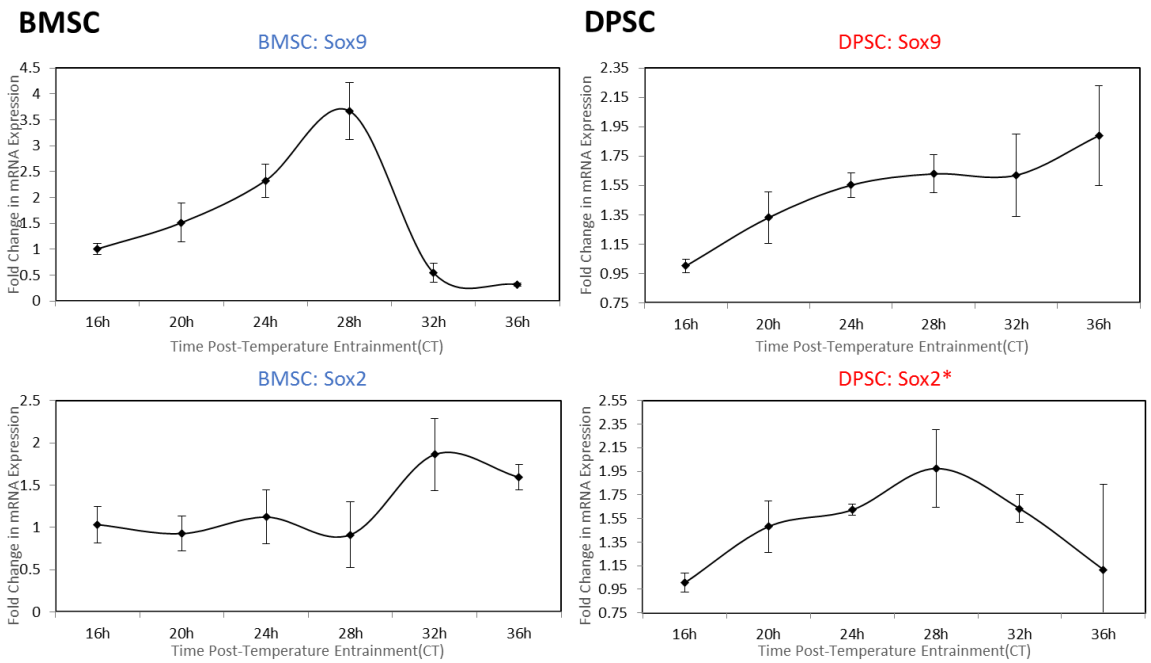

Figure 3.17. **Sox2 *and* Sox9 *gene expression dynamics in human adult progenitor cells following synchronisation with temperature.*** *Quantitative RT-PCR analyses showing temporal expression profiles of the pluripotency marker,* Sox2, *and progenitor cell differentiation marker,* Sox9, *collected every four hours between 16 h - 36 h following synchronisation with temperature. Data, are expressed as the mean of ΔCt ±SEM relative to 16 h timepoint and normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent experiments.*

3.3 Conclusions

In this research, it has been shown that human progenitor cells derived from different human adult anatomical locations did exhibit different levels and temporal expression patterns of core clock genes, stabilising loop genes and stem cell markers, as was determined by comparing the basal levels of gene expression in the three types of mesenchymal progenitor cells. It was also demonstrated and evidenced that the adult progenitor cells from different sources did indeed respond to circadian synchronising signals very differently; for instance, the BMSCs appeared to synchronise more readily in response to chemical stimulation than mechanical, but the DPSCs were much more responsive to entrainment by mechanical means.

It has been experimentally evidenced that the more primitive human progenitor cells, such as DPSCs, have a different profile of the molecular circadian rhythm, in terms of both the relative levels of clock gene expression and the oscillating temporal patterns

of gene expression, after synchronisation by chemical means (i.e. dexamethasone exposure or serum shock). It appears that the DPSCs are much less responsive to the dexamethasone or serum shock synchronisation, which may be due to less developed circadian components which relay these signals. Indeed, their relative unresponsiveness to dexamethasone may be due to their early developmental origin from the migrating neural crest cells and their resulting ecto-mesenchymal composition. Therefore, upon DPSCs differentiation, it is possible that they will gain responsiveness to circadian synchronisation and/or undergo maturation of the apparatus necessary for circadian gene oscillations. This is consistent with research by Yagita et al. (2010), who showed that embryonic stem cells do not have the capacity for circadian synchronisation by chemical means using forskolin, but upon differentiation and maturation, this ability can be gained[142].

In contrast, our results confirm previous findings that the more mature MSCs derived from bone marrow and adipose tissue did exhibit robust clock rhythms that were responsive to glucocorticoid synchronisation and showed clear antiphasic relationships of the positive and negative arms of core TTFLs. For example, Wu et al. (2007) showed similarly oscillating expressions of clock genes in cultures of murine and human BMSCs in response to dexamethasone[152] whilst Huang et al. (2009) demonstrated that human BMSCs and ADSCs have circadian oscillations induced by serum shock[153].

As the DPSCs could not be synchronised by chemical means, a novel synchronising mechanism to which they may respond was sought. Circadian mechanosensory entrainment has been previously investigated by Simoni et al. (2014), who found that 12h:12h cycles of vibration and silence, respectively, were sufficient to synchronise the daily locomotor activity of *Drosophila melanogaster*[159]. Moreover, it has recently been published that the mechanical environment of the epithelial stem cell niche within the mammalian mammary tissue controls the amplitude of the molecular clock oscillations[92]. Therefore, rhythmic mechanical stretch was utilised as an entraining factor for adult human stem cells using a uniaxial mechanical stretch apparatus. It was observed that the DPSCs can be entrained by rhythmic mechanical stretch and appeared more responsive than the BMSCs; the *Bmal1* gene oscillations induced by

rhythmic mechanical stimulation in the DPSCs resembled the temporal clock gene expression patterns seen in BMSCs in response to dexamethasone. This may be due to the fact that *in vivo*, DPSCs experience significant mechanical stimulation in the form of jaw movement, occlusion forces and hydrostatic pressures, and are one of the few stem cell niches to also experience thermal shock, and extreme temperature fluctuations, and so are much more likely to respond to this form of stimulation.

When the expression profile of the pluripotency marker *Sox2* was compared to the profile of the stabilising loop gene *Rev-ErbAα*, a strikingly similar pattern was observed following synchronisation with dexamethasone in BMSCs and ADSCs. In contrast, the rhythmic mechanical stimulation in DPSCs was able to induce the cyclical expression of the core clock genes as well as a pluripotency marker *Sox2*. Indeed, *Sox2* was found to show rhythmic expression in ADSCs following dexamethasone synchronisation and in DPSCs following exposure to dexamethasone, temperature oscillations and mechanical stimulation. These results therefore suggest a novel regulation of the *Sox2* gene which may be under both circadian and mechanical control in different progenitor cell types. Similarly to that of *Sox2*, *Sox9* also showed interesting changes in gene expression after synchronisation. For example, when ADSCs were exposed to entrainment by dexamethasone, *Sox9* exhibited statistically significantly rhythmic patterns in gene expression, when analysed using the circadian cosinor software. These finding are consistent with that of Honda et al. (2013), who showed that SOX9 mRNA showed significant circadian expression after being exposed to modulation by light-dark conditions. Here, the authors theorised that the synthesis of cartilage matrix macro-molecules and the expression of major matrix-related genes may indeed by controlled by cell-autonomous clocks, depending on the *in vivo* tissue location[160]. This is consistent with our hypothesis that circadian dynamics differ depending on the cellular anatomical location, and that various progenitor cell markers are under circadian influence.

As DPSCs are exposed to extreme fluctuations of temperature and thermal shock in the body, and rhythmic circadian fluctuations in temperature have been previously shown to synchronise the circadian rhythm of fibroblasts[144], we next theorised that if the cells could not be synchronised using chemical means, then potentially they could

be entrained using temperature alone. Here, both DPSCs and BMSCs were exposed to 12 h of 35.5°C and 38.5°C each per day, for a period of three days. After this time, the cells were restored to 37.0°C and samples collected every four hours for an entire circadian day. As a result of this, it was found that the two types of progenitor cells did show similar levels of clock gene expression, with the circadian clock genes peaking and troughing at similar time points. Furthermore, the progenitor cell marker gene *Sox2* exhibited significantly rhythmic expression in DPSCs, showing that these cells were amenable to synchronisation by thermal means. This data demonstrates that both the DPSCs and BMSCs respond to oscillations in temperature in similar ways, and therefore offers a novel and reliable way of synchronising the circadian rhythm of different types of adult progenitor cells, without the need for any chemical addition.

The circadian clock in mammals has been extensively shown to have a key regulatory role on various tissue systems, including musculoskeletal tissues. It is therefore vital that such temporal regulation be taken into account when optimising and integrating cellular and tissue constructs into the body, if one hopes for more successful tissue engineering strategies. For instance, it has previously been shown that the circadian regulation is significantly involved in the establishment of osseointegration under vitamin D regulation. Here, KEGG pathway analysis showed the potential association of the circadian rhythm with the success of implant osseointegration[82]. The circadian rhythm has also been shown to regulate coexisting populations of epidermal stem cells at opposite phases of the clock, which are differentially prone to activation by external stimulation[94]. Furthermore, research indicates that epidermal stem cells differ in their responsiveness to proliferation- and differentiation-like cues over a 24 h cycle[127], which may have vast implications in tissue engineering, where one cellular state may be favoured to encourage successful implant integration.

Overall, these findings suggest that the mechanism of entraining progenitor cell clocks by using each unique progenitor cell optimal synchronisation method offers an insightful way in which progenitor cells can be 'primed' to respond in the desired tissue engineering applications. With this in mind, mechanical entrainment of human adult progenitor cells allows for a non-invasive means by which the circadian clock in human adult progenitor cells can be directed and controlled whilst maintaining their

appropriate clock timing, thus avoiding the need for additional exogenous chemical or thermal stimuli.

Chapter Four

“The Role of Cell-Substrate Interaction in Adult Progenitor Cell Differentiation and Circadian Rhythm, in a Mechanical Stretch Paradigm”

4 "The Role of Cell-Substrate Interaction in Adult Progenitor Cell Differentiation and Circadian Rhythm, in a Mechanical Stretch Paradigm"

4.1 Introduction

4.1.1 The Extracellular Matrix

Traditionally, progenitor cell differentiation has been attributed to both genetic and molecular mediators, including growth factors and transcription factors, and therefore, cocktails of various growth factors have been utilised to trigger lineage specific control of stem cell fate. However, recently there has been substantial evidence to suggest that numerous environmental factors affect the overall state of progenitor cell activity, including that of the "solid-state" environment, i.e. the extracellular matrix (ECM). Particular interest has focused especially on the role of ECM ligands and cell surface receptor interactions, and other mechanobiological concepts, such as cell generated cell forces. Cell generated physical forces result from myosin bundles sliding along actin filaments which is then transduced using various adhesive proteins that link the extracellular and intracellular environments.

The ECM is predominantly composed of a number of proteins, including the three major structural proteins: fibronectin, collagen and laminin, and a number of other proteins including elastin, glycosaminoglycans, fibrillin, proteoglycans and tenascin. Cells sense and rely on ECM resistance, which relays a number of cues to the cells, by structural[161], biochemical[162] and mechanical[163] means; mechano-sensitive pathways transfer these biophysical cues into biochemical signals, which then influence progenitor cell fate. In this way, progenitor cells differentiate into mature cells when exposed to these intrinsic properties of the ECM, such as matrix elasticity, structure and composition, as the specific distribution and assembly pattern differs throughout the body[164].

In the body, the matrix is initially secreted by cells and then assembled by integrins into a fibrillar network. The integrins attach to very specific cell-binding regions of the

matrix, i.e. the Arginine-Glycine-Aspartic acid (RGD) on fibronectin. Once bound, clusters containing multiple bound integrins are pulled apart and the matrix proteins are unwound to reveal self-association domains. These domains then allow for homotypic-binding, e.g. fibronectin-fibronectin binding[165]. These fibrillary bundles permit the matrix to present discrete adhesive patches to cells in a controlled manner, when the binding domain is cell accessible. The cells themselves rely on ECM resistance; cells pull against the matrix and detect the resistance to deformation, and this information is then relayed via mechanically sensitive proteins.

It is therefore unsurprising that there is a significant drive to better engineer artificial ECMs through both molecular and physical interactions, in order to optimise cell behaviour. To this end, there is an increasing desire to fully elucidate and understand the basic mechanisms of cell-matrix interactions and cell signalling, to fully recapitulate the stem cell microenvironment. To do this, biomaterial approaches are being utilised to study the stem cell niche, together with microfabrication and microfluidic technologies, as they allow this complex, evolving system to be reconstructed. Both 2D and 3D biomaterial approaches are being explored, as 2D allows for a well-controlled analysis of stem cell biology and 3D better represents the *in vivo* conditions. It is also worth noting that most studies investigating this area of the role of physical cues on stem cell biology have taken place using adult progenitor cell populations, such as MSCs which tend to be heterogenous cell populations with reduced plasticity.

4.1.2 *In Vivo* Exposure of Cells to Mechanical Stimulation

In the body, cells are exposed to a substantial amount of mechanical stimulation through phenomena such as gravity, blood flow, interstitial fluid flow, muscle forces and many more physical processes. This mechanical stimulation, and the resulting interaction between cells and mechanical factors, is vital to the function of tissues and organs. Furthermore, mechanical forces have been shown to influence developmental processes and have also been implicated in stem cell fate and lineage determination. This was initially demonstrated by Glücksmann (1942), who investigated the role of mechanical stimuli such as compression and tensile stress in chick rudiments[166]. It was

here evidenced that static compression favoured cartilaginous tissue development, whereas tensile stress resulted in bone tissue formation, first proving that the mechanical environment has a strong influence during development.

Cells respond to this input of mechanical stimulation from the ECM to alter their own mechanical properties by remodelling their own cytoskeletal networks in a dynamic fashion. Therefore, the cellular response is not only determined by the input of mechanical signals, but also by how the cells respond and the coupling of the stimuli to mechanosensitive changes in ECM interaction, cytoskeletal organisation and the production of cellular force, all of which can influence stem cell function and differentiation[167]. As a result of this, the concept of "mechanobiology" has therefore rapidly increased in interest, in order to fully understand the response of stem and progenitor cells to physical loading, and the biophysical mechanisms and biochemical signal pathways that underline these interactions and their effect on differentiation[168]. The individual factors that respond to mechanical stimulation and the response of stem cells to individual physical stimuli such as compression, shear stress and cell stretch are still being investigated.

4.1.3 Progenitor Cell-ECM Interaction

The ECM molecules that influence progenitor cell regulation are currently being investigated, in order to define the factors that influence the progenitor cell niche, so that the cell's microenvironment may be understood and therefore optimised and reconstructed. However, there are many aspects of this tightly controlled environment that are still not yet understood. Biomaterial approaches have been utilised to attempt to define these specific factors, and the effects of the different identities, concentrations and patterns of ECM molecules are currently being explored. The method by which the ECM influences cell function is thought to occur via many physical mechanisms including, but not limited to: ECM elasticity, ECM geometry (at the micro- and nanoscale) and the mechanotransduction of signals from the ECM to the cells. Furthermore, the rigidity of the tissue as determined by the ECM structure, i.e. whether it is densely or loosely packed, along with the different densities of individual exposed ECM components and ECM geometries, i.e. fibre length,

diameter and crosslinking, in addition to surface topographies, all result in changes in cell signalling and adhesion. This many influencing factors make it difficult to study each component in isolation and in combination.

The potent influence of matrix mechanical properties on progenitor cell fate has been substantially investigated. As cells attach to substrates, they exert contractile forces which then result in tensile stresses in the cytoskeleton. These forces, combined with the mechanical elasticity or stiffness of the ECM, can have major influences on cell function and behaviours, including cell migration[169], proliferation[170] and apoptosis[171]. Substrate stiffness has also been linked to the differentiation of various types of progenitor cell. For example, when human MSCs were exposed to polyacrylamide gels of varying stiffness that corresponded to brain, bone and muscle, it was found that the MSCs assumed morphological and gene expression patterns consistent with differentiation that was specific to the tissue that the stiffness corresponded to[167], showing that elasticity-driven differentiation is possible in the absence of any specific growth factors. In addition, when adult neural progenitor cells were cultured on stiff matrices, they gave rise primarily to glial cells, whereas when they were cultured on softer substrates that more closely mimics soft brain tissue, neurons were the more dominant cell type[172]. Furthermore, when mouse mammary epithelial cells were grown on soft collagen substrates, they showed increased differentiation when compared to tissue culture plastic[173]. Regarding proliferation, it has also been observed that the rate of adult skeletal muscle progenitor cell proliferation increased as substrate stiffness increases. Finally, it has also been evidenced that ECM elasticity has a crucial role in the bone marrow niche, allowing for the maintenance of BMSCs over a long period[174]. However, it must be considered that multiple tissues may have similar elasticities, and therefore, it may be difficult to distinguish progenitor cell differentiation using a single set of mechanical parameters.

Matrix stiffness is theorised to affect progenitor cell differentiation via intracellular signalling through mechanotransducers such as Rho kinase (ROCK) and focal adhesion kinase (FAK), which are then thought to affect processes such as osteogenesis. For example, when MSCs were cultured on hydrogels of varying elasticities in osteogenic medium, osteogenesis was found to increase on stiffer matrices, where an increase in

the kinase activities of ROCK, FAK and ERK1/2 was also observed. When FAK and ROCK were inhibited, decreases of the osteogenic marker gene expressions were observed. Furthermore, it has also been shown that FAK influences osteogenesis through ERK1/2 but ROCK affects both FAK and ERK1/2. In addition, a2-integrin has been shown to be upregulated in stiffer matrices during osteogenic induction, and it's knockdown results in a decrease of osteogenesis through ROCK, FAK and ERK1/2[175]. Collectively, this provides a molecular mechanism by which matrix stiffness affects the differentiation of MSC into osteogenesis, via a2-integrin-mediated osteogenesis.

The ECM does not only influence cell behaviour through matrix stiffness; it can also cause changes in cell shape, which then in turn can influence progenitor cell signalling and fate determination[176]. One notable demonstration of the effect of cell shape on cellular differentiation utilised single MSCs cultured on micropatterned ECM islands, to control the size of the cell attachment area. It was evidenced that MSCs cultured on small islands adhered poorly, exhibited a rounded morphology and gained an adipogenic phenotype, whereas cells cultured on larger islands appeared spread out, adherent, acquired increased focal adhesions and gained an osteogenic phenotype[177]. This research showed that physical control of cell shape alone was sufficient in itself to regulate cell signalling and behaviour. The matrix architecture has been evidenced to cause cell shape induced changes to the cytoskeletal organisation and signalling, and it is these signals that then influenced stem cell specification and function.

The ability of cells to gain increases in tension through substrate stiffness and/or specific cell shape via surface modifications is thought to be regulated by myosin motor proteins, which gain traction forces against the matrix[178]. By blocking this process using pharmacological inhibitors, differentiation itself can be blocked[167]. A variety of mechanisms have been proposed for this process, including via force sensitive protein conformational changes[179,180], which occur in focal adhesions[181] or within the matrix itself[182], changes in Rho activity[183] and stretch activated ion channels[184]. The role of the extracellular matrix and its properties on progenitor cells has been concisely summarised in several reviews[185–187].

4.1.4 The Composition of the ECM Influences Progenitor Cell Function and Fate

Elasticity driven differentiation is dependent on several other factors; this form of mechanical regulation is coupled to the type of matrix ligand present. To this end, the composition of the matrix can regulate cells directly via differential integrin binding to exposed binding sites. For example, progenitor cell expression of osteoblastic markers is sensitive to the type of matrix ligand present[188]. Here, osteoblasts can be induced to express the osteogenic marker *Runx2* only when both specific matrix elasticity and collagen I or fibronectin being coupled to the surface are present. Even when the same matrix stiffness is provided, if the MSCs are cultured on laminin or collagen IV coupled surfaces, the cells do not undergo an initiation of differentiation in this way[189]. This notion that the presence or absence of ECM binding domains can influence differentiation, even steering MSCs towards an osteoblastic lineage has also been reported by Martino et al. (2009)[190]. This underlines that a combination of the appropriate stimuli must be taken into account to fully regulate the microenvironment, in order to influence cell behaviour.

A further form of mechanical stimulation that is influenced by the composition of the ECM is through cyclic strain. MSCs grown on various protein coated substrates which are then subjected to 5% or 10% cyclic uniaxial stretch have been shown to exhibit myogenic phenotypes, as has been determined by the expression of smooth muscle actin and other myogenic factors[191–193]. Here, the magnitude of strain is reported to be an important factor in mechanical stretch driven differentiation, as 1% and 15% stretches failed to induce myogenesis[193]. However, magnitude was not the only important factor that influenced the progenitor cells ability to undergo stretch induced differentiation; the strain induced myogenesis was dependent on which protein was coated onto the flexible membranes prior to cell attachment, and not coating the membranes resulted in a loss of this observed myogenic phenotype.

Taken collectively, these studies highlight the importance of matrix composition and the type of protein substrate on the cellular response to mechanical stimulation and progenitor cell differentiation.

4.1.5 The Influence of the ECM on Circadian Rhythms

As the circadian rhythm has been found to have a potential relationship with several ECM genes, it can be theorised that the ECM may have a regulatory role on the circadian rhythm. In a study by Mengatto et al. (2011), a vitamin D deficiency induced model of implant failure implicated the circadian rhythm in implant failure and concluded that the circadian rhythm and cartilage ECM may both be involved in osseointegration[82], suggesting a potential relationship or cross-talk between the ECM and circadian rhythm. A further study supports this potential relationship by reporting that Sox9 and other major matrix-related genes, including collagen type II and aggrecan, show circadian gene expression, and suggests that the synthesis of cartilage matrix is controlled by cell-autonomous clocks[160]. This research suggests that the circadian rhythm may have a role in ECM regulation, suggesting potential feedback mechanisms between the two.

This has supported by work by Yang et al. (2017), who investigated the role of the cellular mechano-environment on the circadian clock. Here, the authors do indeed report that the breast epithelial clock is influenced by ECM stiffness in primary cell culture and find that the tension sensing cell-matrix adhesion molecule, vinculin, and the Rho/ROCK pathway, which involved in transmitting the biophysical cues of ECM stiffness to the cells, are involved in the regulation of the circadian clock. It was determined that by culturing MECs under different mechano-chemical environments, for example, comparing 2D and 3D culture and culturing cells on alginate gels of different stiffnesses, that the ECM does influence the mammary clock, with a stiffer ECM matrix results in a dampening of the circadian clock. Furthermore, it was also evidenced that the circadian clock is required for progenitor cell function in mammary tissue and these clocks must be functional for the self-renewal capacity of progenitor cells[156]. This exciting data underlines the importance of both the ECM and the circadian rhythm on adult progenitor cell function and suggests a regulatory role for the ECM on the circadian rhythm.

4.1.6 Aims and Hypothesis

The above study by Yang et al. (2017) reports that the composition of the ECM does not influence clock activity in mammary tissue and finds that MECs cultured on 2D-substrata coated with different ECM proteins, including laminin, collagen and fibronectin, results in no differences in clock activity[156]. As the stiffness of the ECM was shown to affect the circadian clock, we hoped to further explore the response of different ECM components on the circadian rhythm and further examined the effect of coating substrates with fibronectin, collagen I and albumin. In order to fully understand the effect of ECM composition on the circadian rhythm of human adult progenitor cells, we aimed to see how ECM composition effected the circadian clock when subjected to either static conditions or in a mechanically active environment, whereby the cells were subjected to cyclic uniaxial strain.

These stretch experiments were designed as such as the previously mentioned research by Gong and Niklason (2008) demonstrated that the initiation of stretch induced differentiation is dependent on the coating protein to which the cells are attached[191]. To this end, we aimed to determine whether coating the flexible silicone substrates utilised in our previous stretch set ups in different ECM proteins could influence the cellular response to stretch. Therefore, the silicone chambers were coated in fibronectin, albumin and collagen I to determine if the stretch-induced cellular responses in progenitor cell differentiation and circadian rhythm were influenced by ECM composition.

It was hypothesised that the induction of stretch induced differentiation and the progenitor cells' circadian rhythm would be influenced by differences in ECM composition and density. Human adult BMSCs and DPSCs were subjected to substrates coated in different ECM proteins, including fibronectin, albumin and collagen I, at three different densities, in order to determine which protein type and concentration was most favourable to each cell type in terms of adhesion and proliferation. Once identified, BMSCs and DPSCs were then cultured on three different protein coatings and the response of several differentiation markers and circadian genes was analysed, to see the effect of ECM composition on progenitor cell function in isolation. Finally,

both cell types were then subjected to cyclic uniaxial strain (6.66% stretch, 1 Hz, 12 h/day) and again their responses were analysed in terms of their differentiation capabilities and circadian clock dynamics, to see how changing the composition of the ECM affects the progenitor cells' response to stretch and to further optimise the stretch set up. The identification of the ideal conditions for progenitor cell cyclic stretch may prove beneficial in future tissue engineering applications, allowing for further developments to be made in the treatment of both age-related diseases and in synchronising progenitor cell therapies.

4.2 Results

4.2.1 Research Section One: The Effect of Altering the Protein Substrate Coated onto Silicone on Progenitor Cell Number/Adhesion and Proliferation

13 mm glass coverslips were first covered in silicone using a Laurell Spin Coater and sterilised using UV, before being coated in three different types of protein substrate, rat tail collagen type I, human fibronectin or human albumin, at three different dilutions, 1:10, 1:100 or 1:1000. Negative controls were treated with PBS alone. $8x10^3$ DPSCs or BMSCs were then seeded onto the coated coverslips and samples were collected at three different time points: day one (D1), day two (D2) and day 5 (D5). Following CyQUANT analysis, the fluorescence of each sample was determined and converted into cell number using a standard curve that was quantified using known cell numbers (Figure 4.1).

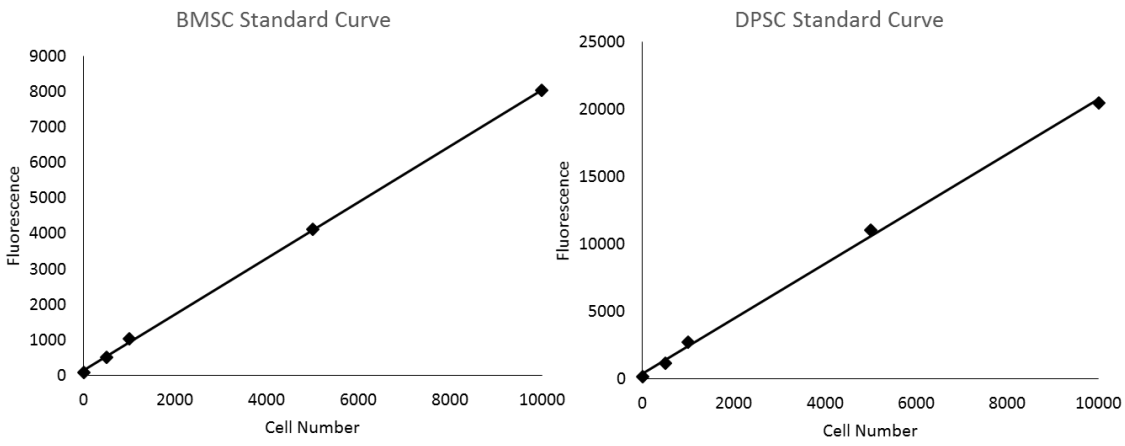

Figure 4.1 BMSC and DPSC CyQUANT Standard Curves. Known cell numbers were used to plot standard curves against their level of fluorescence (n=3).

The resulting cell numbers were then compared and statistically analysed using two-way ANOVA with Tukey's HSD post hoc tests. In BMSCs, simple main effects analysis showed that there was a statistically significant difference in mean cell number between days ($p < 0.01$) and there was statistically significant differences between protein substrates ($p \leq 0.01$) when analysed using two-way ANOVA and looking at the tests between-subjects effects. In DPSCs, there was a statistically significant difference in mean cell number between days ($p < 0.01$) and there was also statistically significant differences in mean cell number between protein substrates ($p < 0.01$). Using multiple comparisons, we could also determine that, in BMSCs, there was a statistically significant difference between D1 vs D5 ($p \leq 0.01$) and D2 vs D5 ($p \leq 0.01$) but not between D1 vs D2. In DPSCs, there was a statistically significant difference between D1 vs D5 ($p \leq 0.01$) and D2 vs D5 ($p \leq 0.01$) but not between D1 vs D2.

When comparing the overall data across all three time points and comparing each protein type and dilution, fibronectin with a dilution 1:10 was found to be statistically different compared to the uncoated negative controls in both cell types. To this end, F1:10 was found to show statistically significant increases when compared to UC in BMSCs ($p = 0.003$) (Figure 4.2). Furthermore, in DPSCs, F1:10, C1:10 and C1:100 all elicited statically significant increases in cell number, when compared to UC negative controls ($p = 0.000086$, $p = 0.025$ and $p = 0.001$ respectively) (Figure 4.3). In both cell types, coating the silicone in albumin tended to show negative effects in cell number, when compared to both the other protein substrates and uncoated negative control.

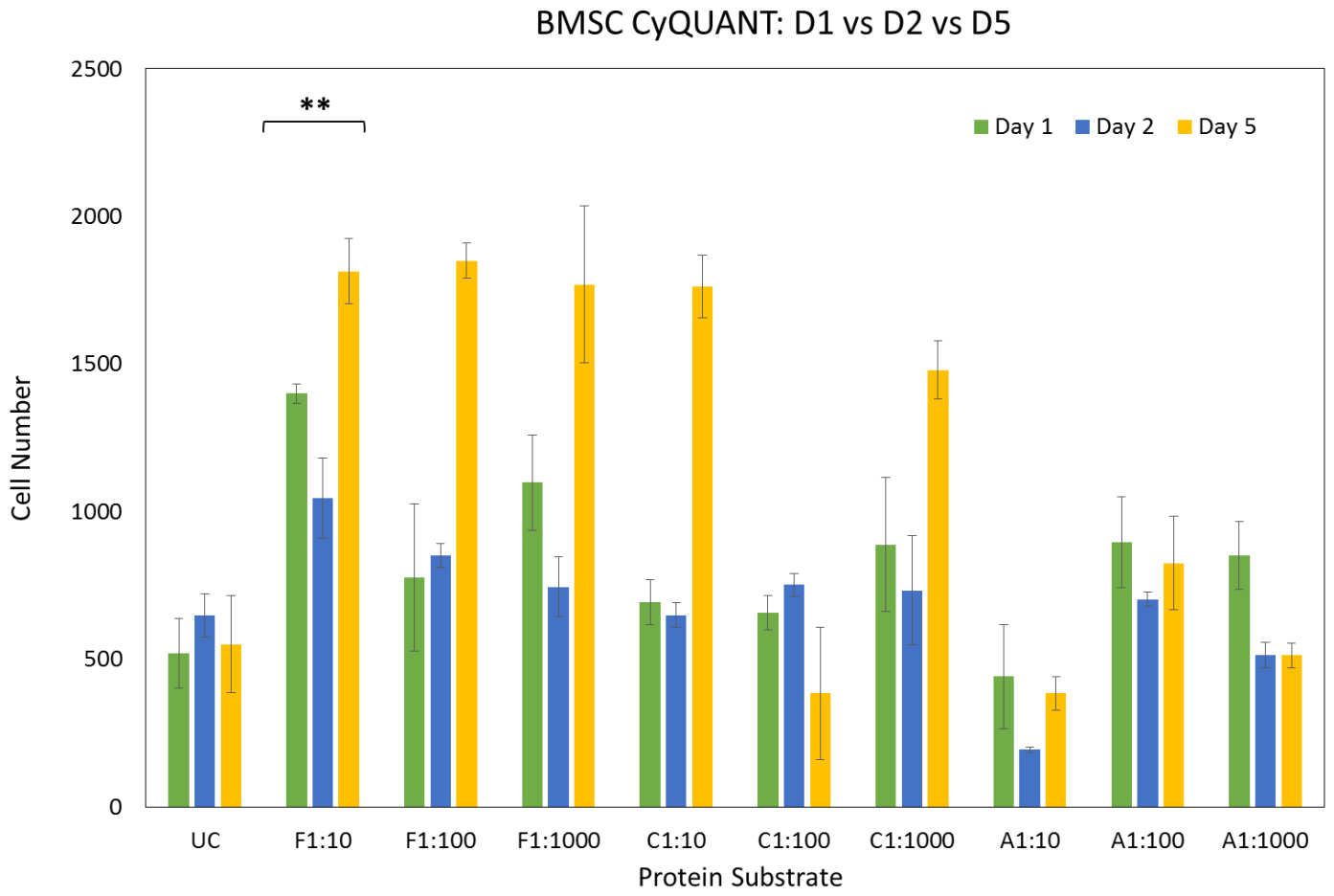

Figure 4.2: Coating silcione coverslips in different types and concentrations of ECM protein substrates lead to differences in BMSC adhesion and growth. *BMSCs cultured on different protein substrates give rise to differences in cell number when anaylsed using CyQUANT analysis over three time points: day 1, day 2 and day 5. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples, *:p<0.05, **:p<0.01 (two-way ANOVA with Tukey's HSD post hoc tests; groups compared to uncoated control (UC)).*

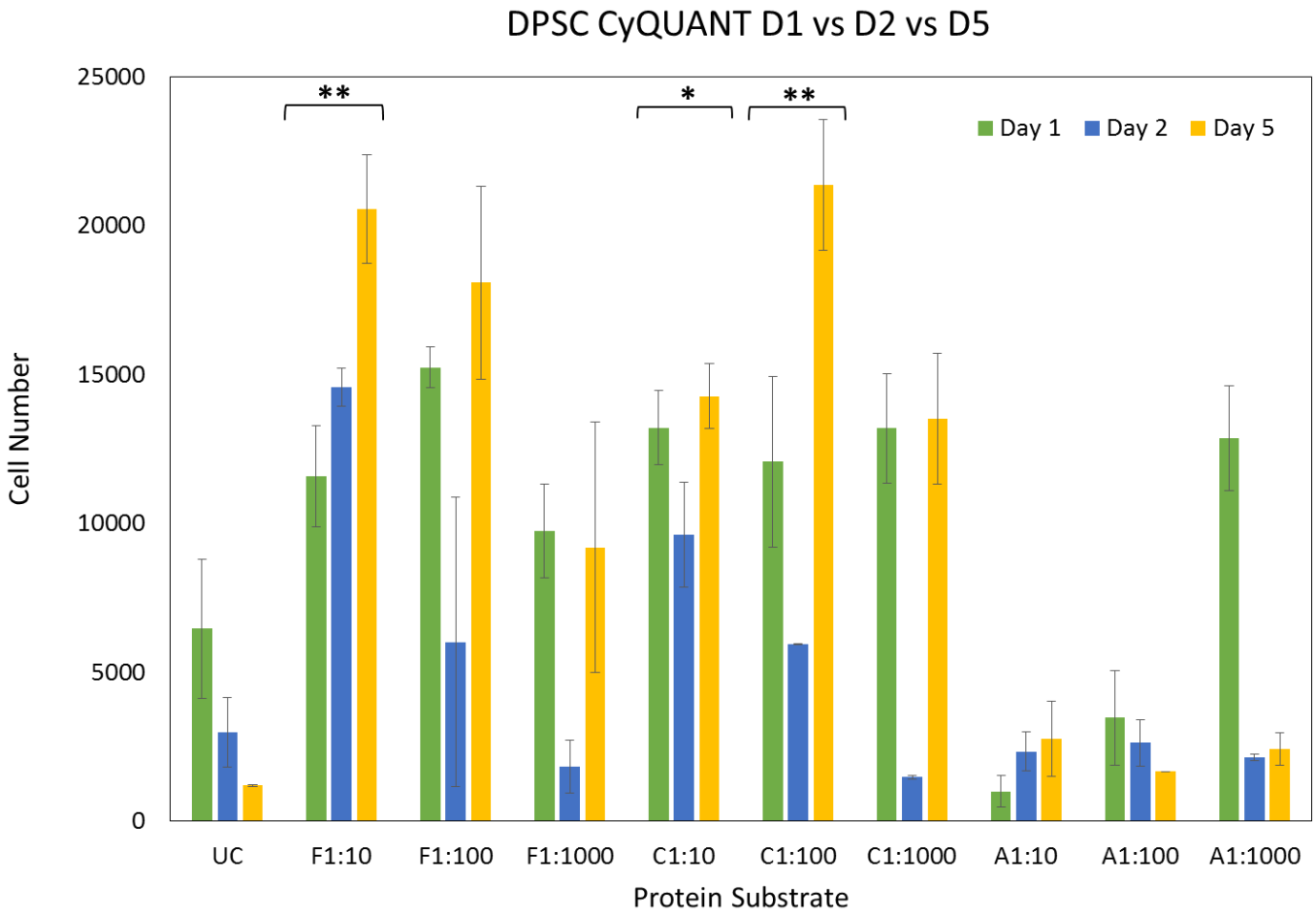

Figure 4.3: Coating silcione coverslips in different types and concentrations of ECM protein substrates lead to differences in DPSC adhesion and growth. *DPSCs cultured on different protein substrates give rise to differences in cell number when anaylsed using CyQUANT analysis over three time points: day 1, day 2 and day 5. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples, *:p<0.05, **:p<0.01 (two-way ANOVA with Tukey's HSD post hoc tests; groups compared to uncoated control (UC)).*

4.2.2 Research Section Two: Different Protein Substrates Elicit Differential Effects on Progenitor Cells Circadian Rhythm and Differentiation Capacity

As in the above experiment, both coatings in F1:10 and C1:100 resulted in highly statistically significant increases in cell number ($p \leq 0.01$), it was then investigated to determine if these coatings alone could influence the circadian rhythm and/or differentiation capability of human adult progenitor cells. As the previous mechanical stretch experimental set ups included coatings in F1:100, this was also investigated as a control, to determine whether these different protein substrates could lead to differences in the cellular response to stretch, therefore optimising the experimental set up. Initially, BMSCs and DPSCs were cultured in standard tissue culture plastic that had either been coated in F1:100, F1:10 or C1:100, with all other normal growth conditions maintained, in static conditions.

4.2.2.1 BMSCs: Differentiation Marker Expression

The response of four major progenitor cell differentiation markers to the different substrates was first investigated at basal level, to see if changing the composition of the protein substrate had any effects on cellular differentiation predisposition (Figure 4.4). The following genes were selected to represent four of the major MSC differentiation lineages: *OCN* (osteogenesis), *PPARγ* (adipogenesis), *Sox9* (chrondrogenesis) and *GFAP* (neurogenesis). It was observed that the level of *OCN* did significantly differ between each of the different protein coatings, being highest in expression in C1:100 and lowest in F1:100. For *PPARγ*, however, no significant differences could be observed, just falling short of statistical significance in C1:100 compared to F1:100, with the levels of the adipogenic marker mRNA expression being highest in C1:100. In contrast, *Sox9* was found to be statistically higher in both C1:100 and F1:10 than in F1:100. Finally, *GFAP*, a marker of neurogenesis, was found to be statistically higher in expression in F1:100 than in C1:100, with no expression being detected in F1:10. These results demonstrate that the alterations in the protein substrate lead to differences in progenitor cell differentiation marker expression.

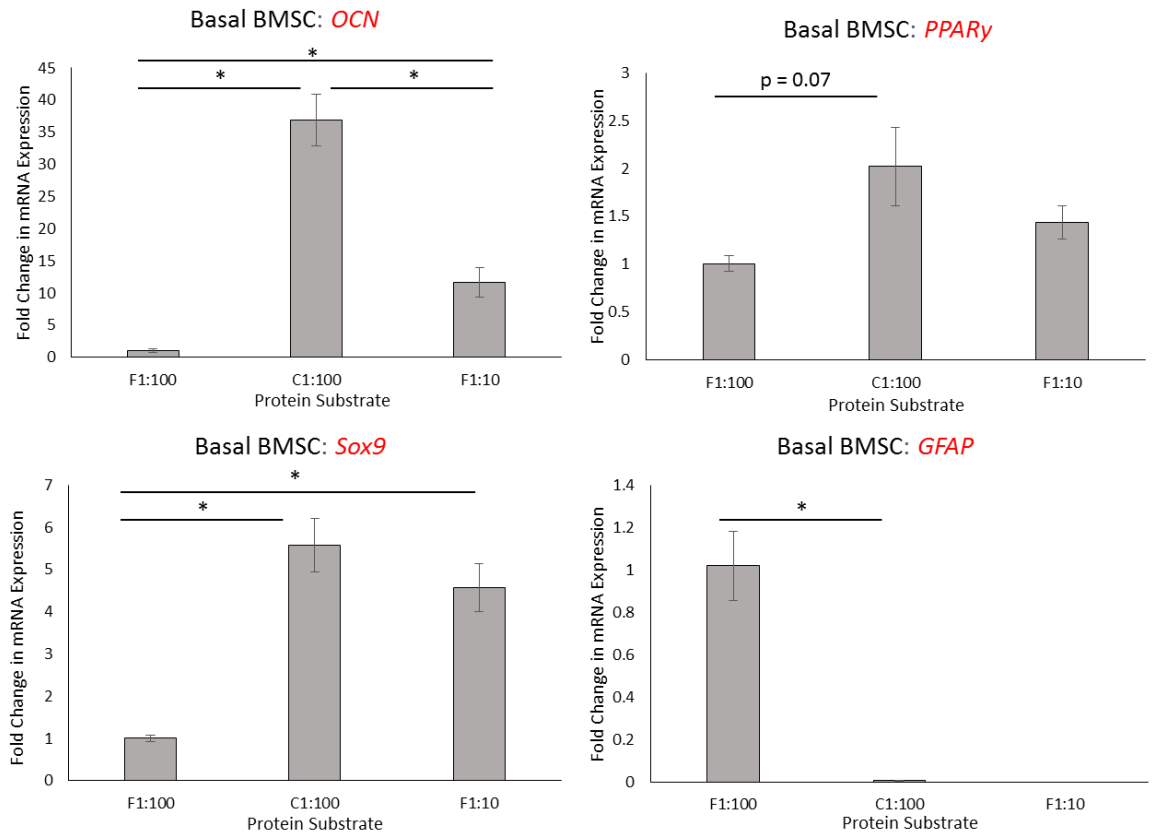

Figure 4.4: Differentiation marker gene expression in BMSCs when cultured on different protein substrates. *Quantitative RT-PCR analyses comparing the relative mRNA expression levels of differentiation marker genes (OCN, PPARγ, Sox9 and GFAP) in mesenchymal-like adult stem cells derived from human bone marrow. Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (n=3, one-way ANOVA).*

4.2.2.2 DPSCs: Differentiation Marker Expression

The level of mesenchymal progenitor cell differentiation marker expression was also investigated in DPSCs (Figure 4.5), to see if the protein substrates affected the two cell types in similar ways or not. In direct contrast to the BMSCs, however, which exhibited significantly differing levels of *OCN* expression between all three of the protein substrates, in DPSCs, no significant differences could be observed between F1:100, C1:100 or F1:10. Similarly, no significant changes could be seen in *PPARγ* expression between the three protein substrates. This is comparable to the BMSCs, which also showed no significant differences in the adipogenesis marker expression. For *Sox9*, in DPSCs the increased expression in C1:100 that was also observed in BMSCs was

present in both cell types. However, this increase was not observed in F1:10; instead, the expression of *Sox9* was significantly higher in C1:100 than either F1:100 or F1:10. Another difference could be demonstrated between the two cell types in terms of *GFAP* expression. In DPSCs, *GFAP* was expressed in all conditions, with the significantly highest expression in C1:100. These results support the above conclusion that changes to the protein substrate composition and concentration are sufficient to bring about changes in progenitor cell differentiation capacity, showing that protein substrate alone can be used to steer differentiation. Furthermore, the data also demonstrates that these changes differ between the progenitor cells, depending on their anatomical location of origin.

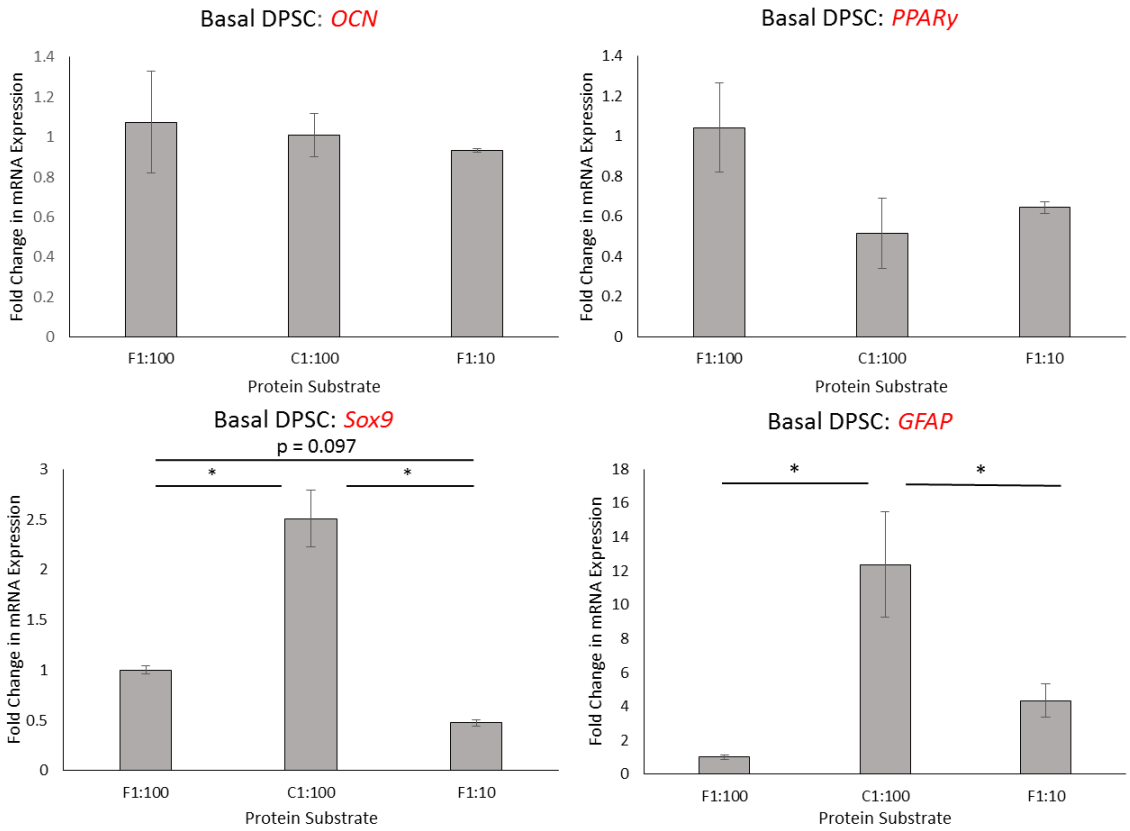

Figure 4.5: Differentiation marker gene expression in DPSCs when cultured on different protein substrates. *Quantitative RT-PCR analyses comparing the relative mRNA expression levels of differentiation marker genes (OCN, PPARγ, Sox9 and GFAP) in mesenchymal-like adult stem cells derived from human dental pulp. Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (one-way ANOVA).*

4.2.2.3 Circadian Rhythm

Next, the level of expression of the core circadian clock genes was examined to see if changing the protein substrate has any influence on the progenitor cells' circadian rhythm. Again, the two progenitor cell types differed in their patterns of gene expression brought about by the changes in protein substrate (Figure 4.6). For example, *Bmal1* expression was highest in F1:100 in BMSCs, but in C1:100 in DPSCs. Similarly, there were significant increases in *Per2* expression in DPSCs when cultured in F1:10 conditions than either F1:100 or C1:100, but no significant changes in *Per2* expression could be observed in BMSCs. Finally, for *Rev-ErbAα*, this gene appeared to peak in expression at F1:10 in DPSCs, but was significantly increased in expression in F1:100 when compared to both C1:100 and F1:10 in BMSCs.

Interestingly, several genes appeared to show similar responses to the changes in protein substrate in some differentiation markers and clock genes. For example, both *Bmal1* and *Sox9* showed similar patterns of expression when subjected to the three different protein coatings; both genes were highest in C1:100 when compared to F1:100 and F1:10, where they showed decreased expression. This may suggest that there could be potential interactions between some differentiation markers and clock genes, indicating that the clock genes may influence progenitor cell activation and differentiation by acting on these genes directly.

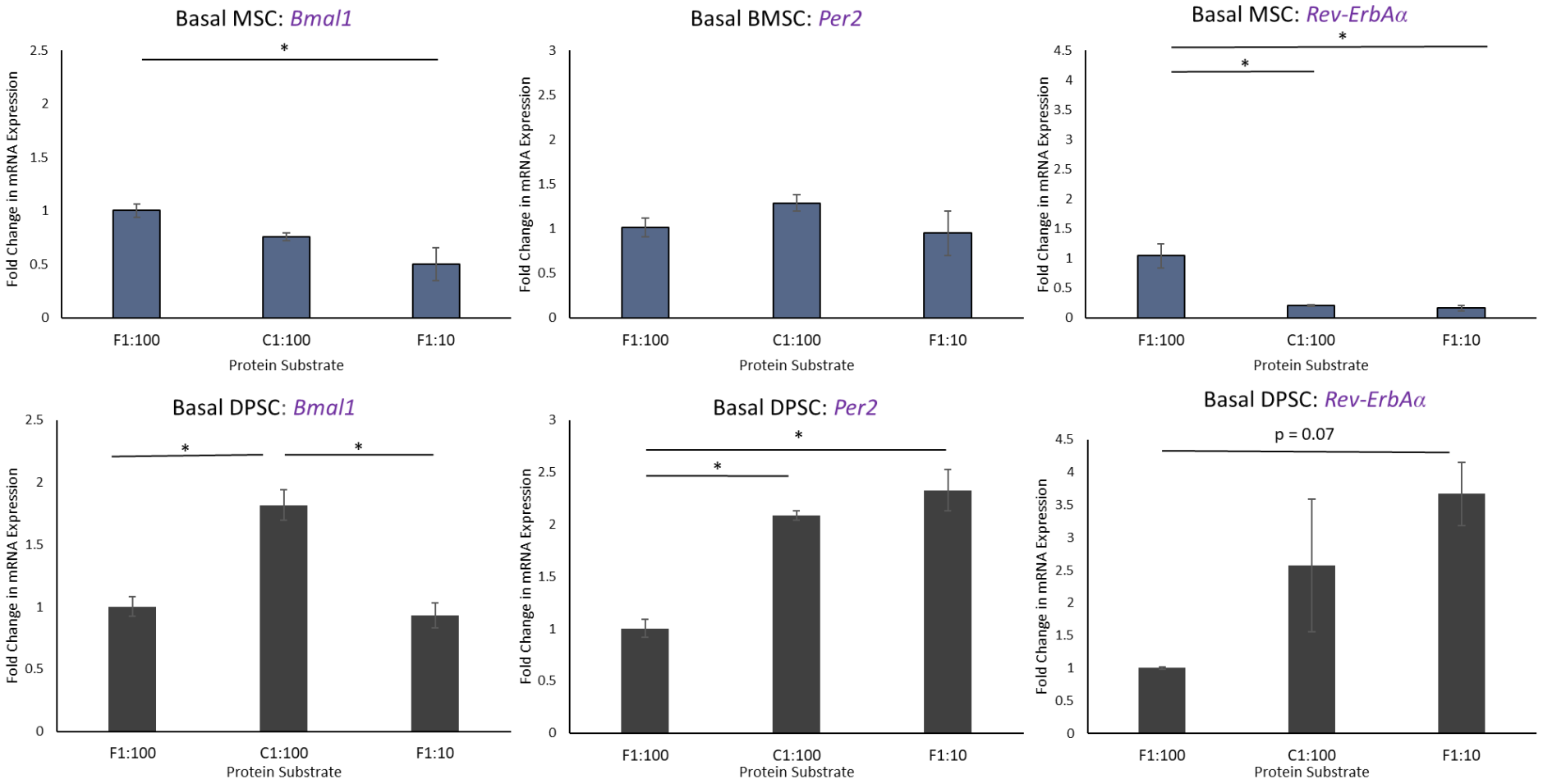

Figure 4.6 Clock gene expression in adult stem cells cultured on different protein substrates. *Quantitative RT-PCR analyses comparing the relative mRNA expression levels of core clock genes in mesenchymal-like adult stem cells derived from human bone marrow (BMSC) and dental pulp (DPSC) when cultured on different protein substrates (F1:100, F1:10 or C1:100). Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (one-way ANOVA).*

4.2.3 Research Section Three: Changes in the Protein Substrate Influences Progenitor Cell Response to Mechanical Stretch

As the above changes in protein substrate composition and dilution lead to significant changes in differentiation marker and circadian clock gene expression, it was next investigated to see if changing the protein substrate would affect the progenitor cells response to mechanical stretch. To this end, the silicone stretch chambers were coated in either F1:100, C1:100 or F1:10 for 1 h before the cells were seeded. Following the three day stretch protocol, which was carried out as previously stipulated (6.66% stretch, 1 Hz, 12h/day), samples were collected immediately after the third day and changes in gene expression determined. Control samples (static) were seeded into the same chambers and subjected to the same conditions with no stretch. The adherence of BMSCs to the different protein substrates in the silicone chambers following mechanical stretch can be seen below (Figure 4.7).

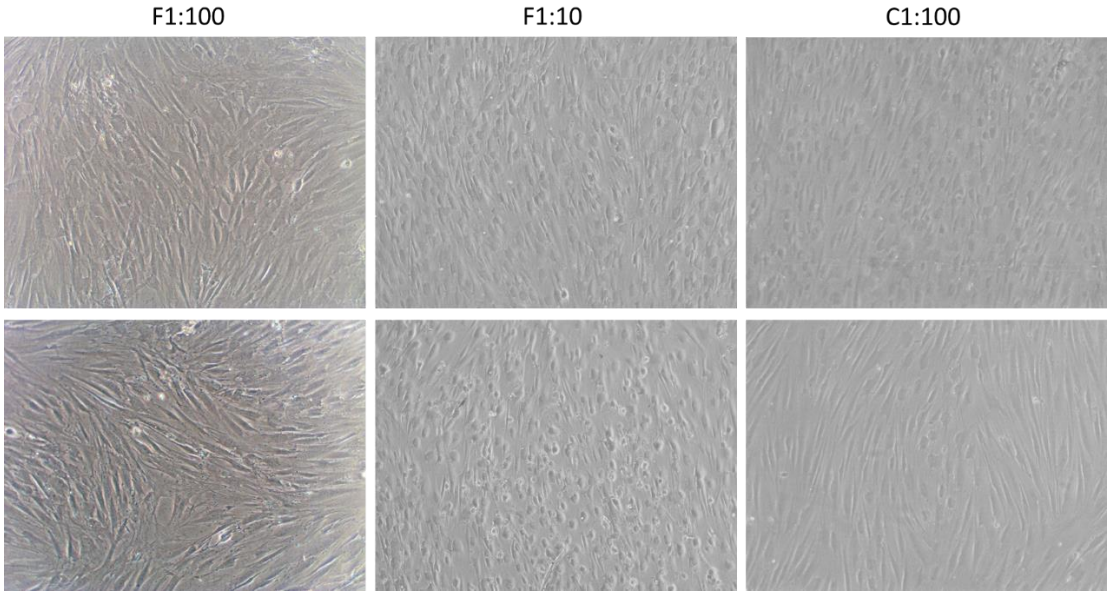

Figure 4.7 Adherence and morphology of BMSCs when seeded into and mechanically stretched in silicone chambers coated with different protein substrates. *Silicone chambers were coated in fibronectin at 1:10, fibronectin at 1:100 and collagen type I at 1:100 for 1 h prior to being seeded with BMSCs. Cells were allowed 24h to attach and then stretched mechanically for 3 days (6.66% stretch, 1 Hz, 12h ON/12h OFF).*

4.2.3.1 BMSCs: Differentiation Marker Expression in Response to Stretch

The response of the progenitor cell differentiation markers *OCN*, *PPARγ*, *Sox9* and *GFAP* were then investigated to see how their response to stretch differed depending on the protein substrate coating they were subjected to, and several interesting changes could be observed in BMSCs (Figure 4.8). For example, for *OCN*, expression was significantly higher in static conditions than stretch for F1:10, and appeared higher in static for C1:100, although this was not statistically significant. Similarly, *PPARγ* appeared higher in expression in static conditions than stretch, for F1:10 and C1:100. This trend could again be observed for the chondrogenesis marker, *Sox9*, which showed statistically significant increases expression in static conditions than stretch for F1:10. This increase could also be observed at C1:100, although it did not achieve a statistically significant result. For all three genes, *OCN*, *PPARγ* and *Sox9*, there was no difference in expression in static or stretch conditions for F1:100.

The neurogenesis marker, *GFAP*, could not be detected in either stretch or static for F1:10 or in the static condition for F1:100. However, when the chambers were coated with C1:100, there was a significant increase in *GFAP* expression when exposed to mechanical stretch.

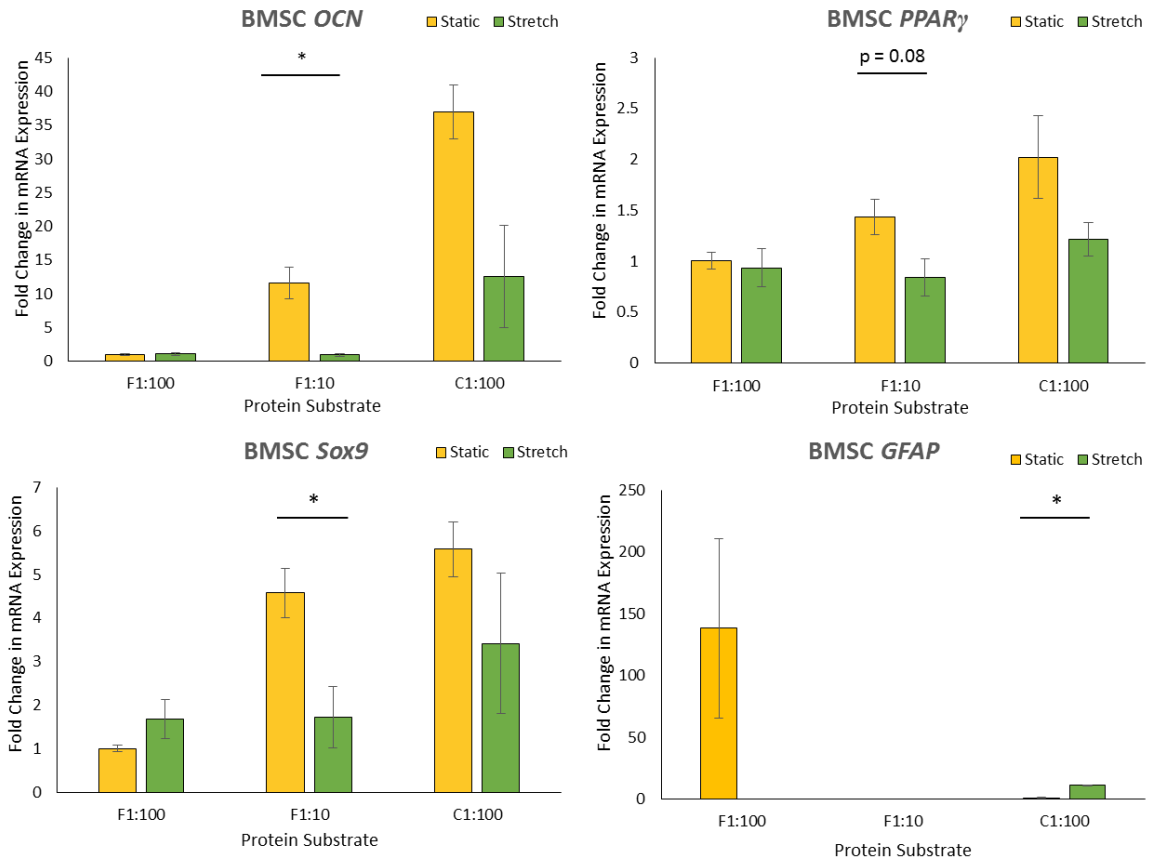

Figure 4.8: Differentiation marker gene expression in BMSCs when cultured on different protein substrates in stretch and static conditions. *Quantitative RT-PCR analyses comparing the relative mRNA expression levels of differentiation marker genes (OCN, PPARγ, Sox9 and GFAP) in mesenchymal-like adult stem cells derived from human bone marrow exposed to stretch (6.66%, 1 Hz, 12 h/day) or static conditions. Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (independent t-test, n=3).*

4.2.3.2 DPSCs: Differentiation Marker Expression in Response to Stretch

The response of DPSCs to stretch or static conditions when cultured on the different protein substrates was also investigated, in terms of the differentiation markers expression (Figure 4.9). In direct contrast to BMSCs, the level of *OCN* expression was significantly increased in F1:10 when subjected to stretch conditions, with no significant differences between stretch and static conditions for C1:100 and F1:100. No significant differences could be observed for *PPARγ* either for any of the three protein substrates, with only F1:100 appearing to show an increase in expression in the static condition. For *Sox9*, there were no significant differences in C1:100, and only

a slight increase in static conditions in F1:100. For F1:10, however, *Sox9* was significantly increased in response to stretch. Unlike with BMSCs, *GFAP* was expressed in each condition. For F1:10 and C1:100, the expression of *GFAP* was significantly higher in static conditions than stretch, and no significant changes could be observed between the two in F1:100.

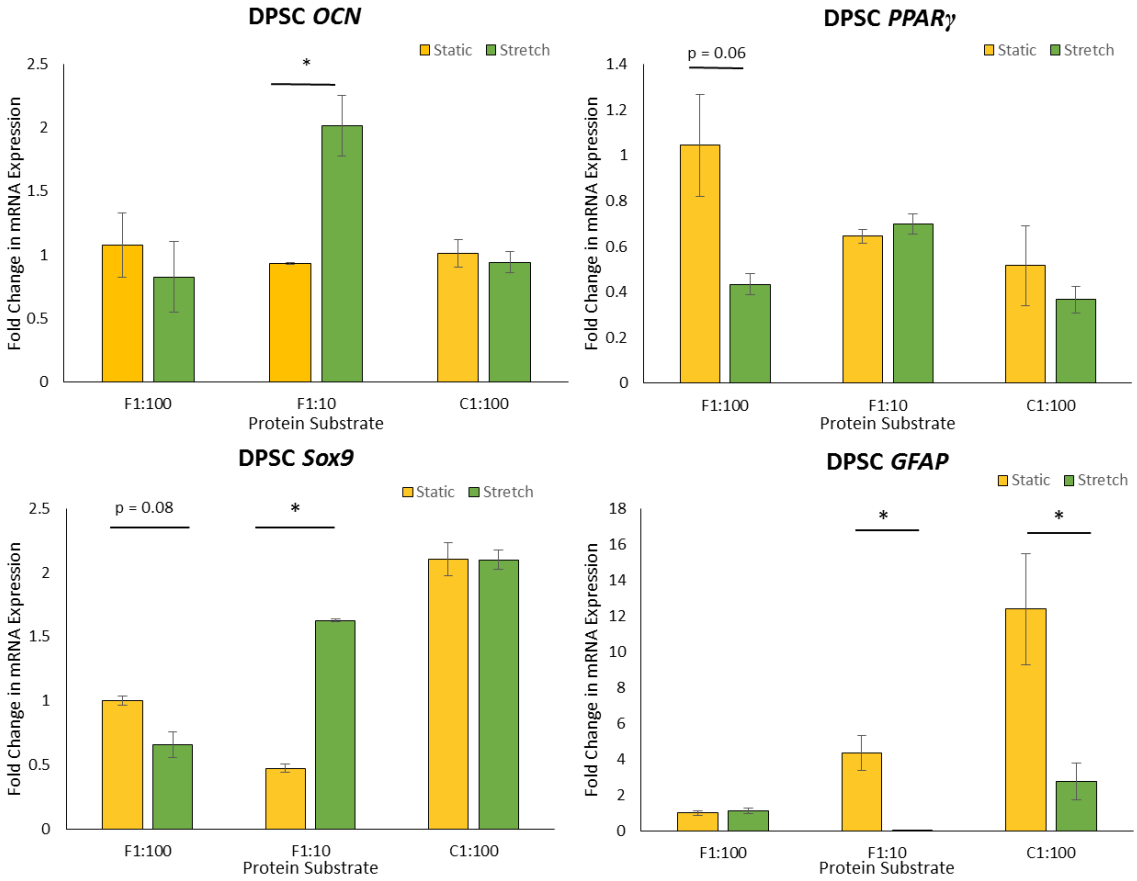

Figure 4.9: Differentiation marker gene expression in DPSCs when cultured on different protein substrates in stretch and static conditions. *Quantitative RT-PCR analyses comparing the relative mRNA expression levels of differentiation marker genes (OCN, PPARγ, Sox9 and GFAP) in mesenchymal-like adult stem cells derived from human dental pulp exposed to stretch (6.66%, 1 Hz, 12 h/day) or static conditions. Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (independent t-test).*

4.2.3.3 Circadian Rhythm

The response of the circadian rhythm to the protein substrates when subjected to stretch or static conditions was also investigated (Figure 4.10). In BMSCs, there was a decrease in *Bmal1* expression when exposed to stretch in all three conditions; this change was significant in both F1:100 and C1:100. However, in contrast, in DPSCs an increase could be seen in *Bmal1* when exposed to stretch; this change was significant in F1:10 and C1:100, showing once again how the two cell types differ in response to stretch. In both cell types, no significant changes could be observed in *Per2* expression between stretch and static in any of the protein substrate conditions. BMSCs and DPSCs showed similarities in the response of *Rev-ErbAα* to stretch - *Rev-ErbAα* was increased in both cell types in F1:10 when exposed to stretch conditions. However, the overall expression patterns differed between the cell types, with the highest levels of expression in BMSCs being with F1:100 and lowest with F1:10, unlike in DPSCs, where it was highest at F1:10 and lowest at F1:100.

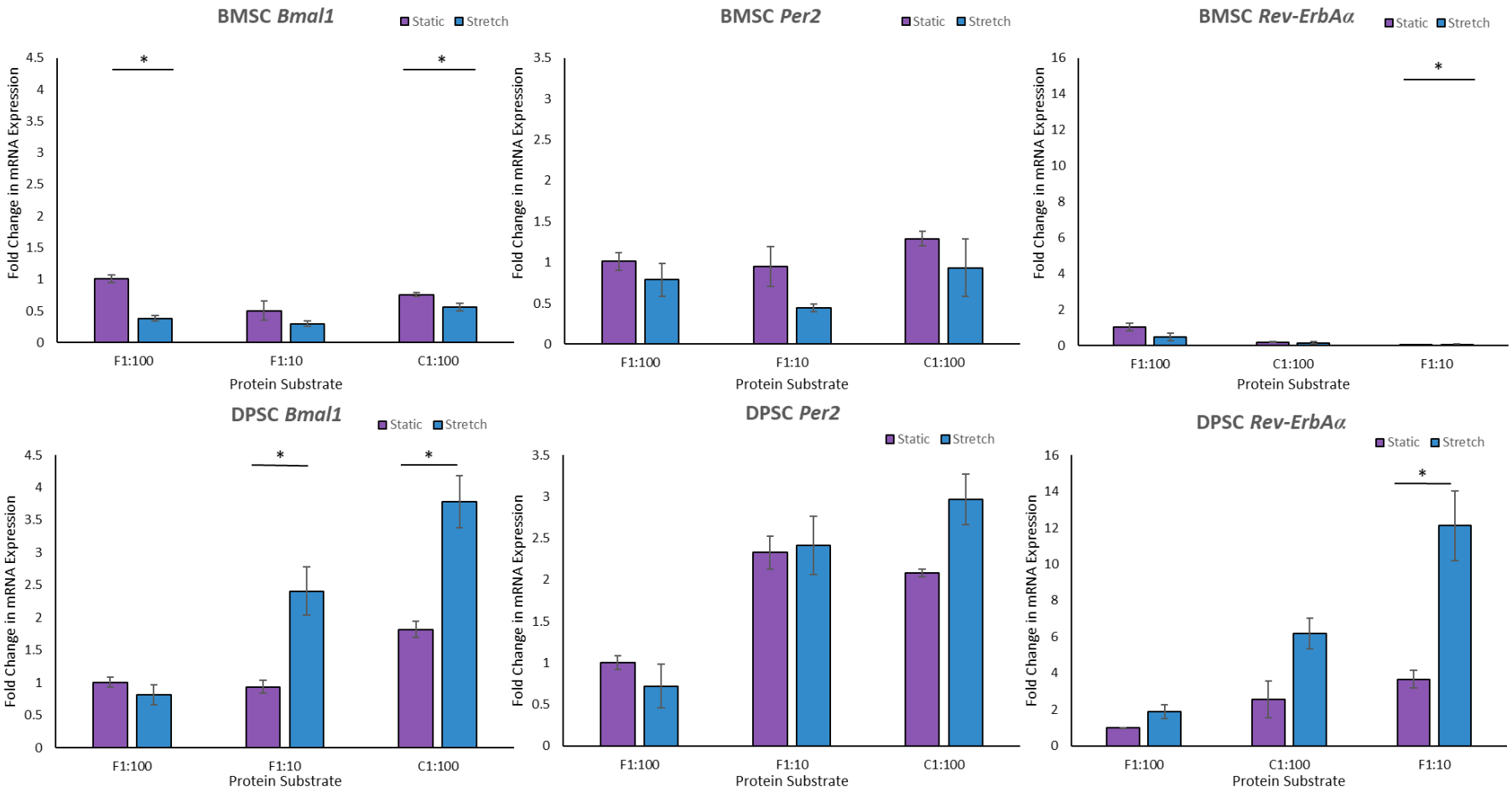

Figure 4.10 Clock gene expression in BMSCs and DPSCs when cultured on different protein substrates in stretch and static conditions. *Quantitative RT-PCR analyses comparing the relative mRNA expression levels of circadian clock genes in mesenchymal-like adult stem cells derived from human bone marrow and dental pulp exposed to stretch (6.66%, 1 Hz, 12 h/day) or static conditions. Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (independent t-test).*

4.3 Conclusions

The results of this chapter demonstrate that the cellular mechano-environment, in terms of composition of the ECM and the different protein substrates that the cells are cultured on, does influence the early induction of differentiation marker gene expressions in progenitor cells. The initial experiments investigating an array of different protein substrate conditions on progenitor cell adhesion and proliferation show that the two different types of progenitor cells respond to certain substrates preferentially. In this way, the presented data suggests that BMSCs respond positively to the highest density of fibronectin (F1:10) and DPSCs respond preferentially to both this high density of fibronectin (F1:10) but also to a lower concentration of collagen type I (C1:100).

In order to see how these two protein substrate conditions influenced progenitor cell differentiation and circadian rhythm, the cells were again cultured on these two protein substrates (F1:10 and C1:100, and F1:100 control) and their gene expression analysed, to fully quantify the cellular response to changes in the ECM composition. Here, it was reported that both DPSCs and BMSCs did indeed exhibit significant differences in cellular differentiation marker gene and core circadian clock gene expressions, with slight differences being observed between the cell types. This again shows that the two cell types do show varying responses depending on the protein substrate, depending on their anatomical location. This data is in direct contrast to research by Yang et al. (2017), who reported that changing the matrix composition does not affect the circadian rhythm[156]. However, we believe that this is the case as here, as different concentrations and protein densities were first investigated and optimised, before the cells were subjected to the different conditions. This data suggests that the ECM composition and density must be taken into account in combination to fully elicit the desired response.

Finally, the effect of the protein substrate composition was investigated in a mechanically active environment, to see if alterations in the ECM composition could bring about differences in the cellular response to stretch. Flexible silicone chambers were pre-coated in either F1:100, F1:10 or C1:100 protein coatings, that the cells were

then cultured on before being subjected to the previously described stretch regime (6.66% stretch, 1 Hz, 12 h/day). It was again demonstrated that coating the substrates in different ECM protein conditions did lead to significant changes in gene expression following exposure to stretch or static conditions. This supports findings by Gong and Nikhalson (2008), who also reported that the induction of differentiation markers following stretch was dependent on the protein to which the cells were attached[191]. The circadian clock genes were also influenced by the protein substrate that the cells were cultured upon, further highlighting that the composition of the ECM does affect the circadian rhythm of progenitor cells.

Significant differences could also be observed in the stretch directed differentiation response between the two cell types, depending on their anatomical location. For example, in BMSCs, both *OCN* and *Sox9* tended to be suppressed following mechanical stretch, showing that the differentiation response of BMSCs favoured static conditions. However, the DPSCs tended to show an increase in the differentiation marker gene expression following mechanical stretch. This is contradicted by findings by Hata et al. (2013), who found that mechanical stretch inhibits the osteogenic differentiation in DPSCs and instead increased their proliferation[60], but is in agreement with data by Tabatabaei et al. (2014), who also reported that mechanical stretch resulted in an increased expression of osteogenic markers in DPSCs[194]. Human mesenchymal progenitor cells derived from bone marrow and dental pulp were selected in this experiment due to their restricted lineage potential and ease of accessibility, along with the fact that physical factors appear to have a strong influence on MSC populations. However, it must be noted that the responses of these cell types may be due to the culture conditions selecting for pre-existing subpopulations, which can then influence cell fate itself. A further consideration regarding the use of adult progenitor cells in such research is regarding the difficulties faced in their isolation and characterization, as with progenitor cells such as MSCs, this process can be fairly taxing, and a combination of both positive and negative selection markers must be utilised for robust identification.

Collectively, this research highlights the importance of the mechano-environment, in particular the composition and density of the ECM proteins that the cells are exposed

to, on progenitor cell behaviour and fate. However, *in vivo* many intrinsic cues are presented in combination, and therefore studying these cues in isolation may be used as an indication only, as further characterization is required. Furthermore, the presented findings are based on 2D planar substrates, and therefore the results may be different when investigated in a more clinically relevant 3D context. To this end, cells cultured on rigid polystyrene tissue culture plastic in liquid media is a very different environment to that experienced in the body, where they are associated with anchored molecules, surrounding cell surfaces and a precisely modelled ECM. However, 2D culture systems are beneficial in order to utilise a simplified approach to deconstructing the niche and therefore investigating individual niche components in isolation, as was the case in the presented research.

A further consideration that must be taken into account in this area of research is that, in most studies investigating ECM-based stimuli, the majority of cues (e.g. ECM elasticity, structure or composition) do not appear to be sufficient to promote full differentiation to mature cells, and instead only results in differentiation to precursors. For example, MSCs grown on gel elasticities corresponding to muscle express early muscle markers and become spindle shaped[189], but do not fuse together[195]. Therefore, it must be considered that a combination of varying factors are presented in combination in the body, and these factors in isolation may not be sufficiently representative.

Even when studying an individual interaction such as simple mechanical loading, this results in complex physical changes including stress, strain, fluid flow, pressure and other biophysical processes such as osmotic pressure. Such changes to the microenvironment can then lead to further changes, for example, altering the structure of ECM proteins and the activity of soluble factors. In this way, it appears difficult to precisely isolate the specific effects of mechanical force from the indirect effects that are experienced as a result of each form of mechanical stimulation.

Despite all of the considerations we are here faced with, our research highlights the important implications of the mechano-environment on progenitor cell fate and function, in terms of matrix composition, density and response to tensile strain, which warrants further investigation and full characterisation. This has significant

implications in the fields of regenerative medicine and tissue engineering applications, as mechanical regulation can also be a source of dysfunction in ageing, injury and disease. To this end, alterations in the organisation of ECM components result in significant changes in the matrix stiffness, which then goes on to contribute to pathogenesis; abnormally rigid microenvironments are frequently seen in damaged or diseased tissue. To this end, diseased tissue often overexpress ECM components, causing an increase in stiffness and then a resulting inhibition of cell contractility. For example, myocardial infarctions can result in the formation of rigid scar tissue in the heart muscle, which can then go on to inhibit myogenesis from MSCs[196]. This is a result of the increased rigidity of the matrix causing a dissipation of cell-generated forces within the cell[167], altering the conformation of the proteins that connect the matrix and the cytoskeleton.

Another negative change associated with an increased stiffness of the ECM is that it results in a dampening of the circadian clock[156], which has been associated with a plethora of negative consequences, including problems such as metabolic disorders, as a functioning circadian clock is vital in tissue homeostasis and function. For example, the circadian rhythm also becomes dampened with age, which is thought to be related to the increases in tissue stiffness experienced with age and has been associated with the deterioration in progenitor cell function seen with age. Our research is therefore instrumental in uncovering more knowledge regarding the effect of matrix composition and tensile stretch on the circadian rhythm, which could then be used to reverse such negative age-related changes in progenitor cell function. It could also be instrumental in the development of new therapies which utilise the circadian rhythm to improve tissue function and viability.

Chapter 5

“The Interaction of Circadian Rhythm Genes on Progenitor Cell Differentiation Markers”

5 "The Interaction of Circadian Rhythm Genes on Progenitor Cell Differentiation Markers"

5.1 Introduction

5.1.1 The Circadian Rhythm in Adult Progenitor Cells' Function and Activation

Recent research has reported that circadian clock genes can directly influence adult progenitor cell activation and differentiation, within their tissue-specific niches[125,197,198]. There has been a substantial amount of evidence recently that suggests that not only do adult progenitor cells express the functional core circadian machinery, but that the circadian system has an important role in a variety of tissue homeostatic functions, for example, influencing the activation of stem and progenitor cells[96]. Circulating HSCs and their progenitors in the bloodstream have been shown to exhibit robust circadian oscillations in light-dark entrained animals. It has been experimentally evidenced that the release of HSCs into the bloodstream is cyclical, along with the rhythmic expression of *Cxcl12*, both of which are regulated by the core molecular clock through rhythmic noradrenaline secretion. This implies that the clock driven release of HSCs is stimulated by the CNS during the animal's resting phase, and can promote the tissue regeneration and regulate the function of a hematopoietic progenitor cell niche[103]. Any disturbances in this temporal coordination has been implied to lead to a variety of pathologies including premature ageing and cancer; indeed, recent studies have implicated circadian rhythm disruption in increased susceptibility of cancer development in all human key organ systems[199].

Recent data has demonstrated that the involvement of circadian clocks in the regulation of adult progenitor cell activation is not only cell-specific, but, remarkably, can also act at the cell population level. In a study by Benitah and colleagues (2011), it was shown that the circadian clock may have a role in regulating the activation of coexisting epidermal progenitor cell populations, which are in different phases, in order to balance hair growth and renewal. The authors noted that the genes regulating

niche dormancy, activation and differentiation contained several putative Bmal/Clock-binding sites, as revealed by promoter analysis. These key epidermal homeostasis genes included Wnt signalling factors, TGF-β regulators and modulators of Bmp and Notch signalling. Chromatin immunoprecipitation (ChIP) confirmed the binding of Bmal1/Clock to these promoters in adult tail epidermis, and that the binding of Bmal1 was circadian. Therefore, it was implied that the circadian clock creates populations that are more or less likely to become activated in response to external factors, by modulating the expression of progenitor cell regulatory genes in an oscillatory manner. The deletion of *Bmal1* led to circadian arrhythmia, decreased expression of Wnt related genes and TGF-β inhibitors, and caused progressive accumulation of dormant progenitor cells and premature epidermal ageing. Deleting *Per1/2*, conversely, resulted in progressive depletion of dormant progenitor cells[200], which may have implications in cancer.

A subsequent study using human keratinocyte progenitors showed that these cells responded better to several differentiation cues at certain times of the day. As expected, different proliferation and differentiation genes were expressed at different times of the day; for example, in the evening, DNA replication and cell division pathways were highly represented, as opposed to the morning, when differentiation pathways predominated[127]. The circadian clock coordinated the activities of glycolysis and oxidative phosphorylation with DNA synthesis in proliferating progenitor cells, potentially as a protective mechanism to prevent genotoxicity. It is highly likely that disruption of this clock-controlled mechanism in progenitor cells may contribute to progenitor cell dysfunction and may have long-term consequences on tissue homeostasis, ageing and cancer. More detail demonstrating the links between the role of peripheral tissue rhythms to tissue-specific functions, as well as the circadian clock disruptions caused in various tissues, including liver, pancreas, adipose, muscle, brain, intestine, hematopoietic and immune systems, skin and cartilage, has been reviewed by Janich et al. (2014)[201]. Further work in hair follicle cycling has shown that prominent daily mitotic rhythms are generated by peripheral circadian clock within epithelial matrix cells, which results in the hair growing faster in the morning than evening and therefore being exposed to higher exposure to genotoxic stress at certain times of the

day. Researchers exposed wild-type mice to γ-radiation in the morning (mitotic peak) versus the evening (when there is minimal hair loss), and interestingly reported that the diurnal radio-protective effect is lost in circadian mutant mice. The circadian clock was demonstrated to coordinate genotoxic stress responses with cell cycle progression by influencing the Cdc2/Cyclin B-mediated G2/M checkpoint[125].

5.1.2 The Circadian Clock Does Not Oscillate in ESCs

In direct contrast, the circadian transcriptional machinery does not seem to oscillate in ESCs, when analysed using real-time bioluminescent imaging systems[142]. However, upon differentiation *in vitro*, a molecular oscillation of the circadian clock becomes strongly induced, which can be reversed if the differentiated cells are reprogrammed using the four pluripotency factors Oct3/4, Sox2, Klf4 and c-Myc, whereby the circadian oscillations become inactivated. This suggests that the formation of the circadian oscillator is dependent on an intrinsic program that occurs during cellular differentiation[202]. When ESCs are maintained in a pluripotent state in culture, it has been demonstrated that they express a self-sustained rhythm in glucose uptake that is not coincident with clock gene oscillation and this rhythm is paralleled by glucose transporter mRNA expression, showing that circadian rhythms in metabolism emerge earlier than clock gene expression rhythms. When the cells become differentiated however, circadian patterns of clock genes can be observed, and the glucose utilization rhythm is enhanced in amplitude, providing the earliest evidence of a circadian clock in differentiated stem cells[127].

Experiments carried out by Lu et al. (2016) have demonstrated that when *Clock* is knocked out entirely using CRISPR/CAS9-mediated genetic editing techniques in mouse ESCs, there is no influence on the cells' pluripotent state, but they do exhibit a decreased proliferation rate and increased apoptosis. Interestingly, the clock gene rhythms failed to develop in these mESCs after they spontaneously differentiated, suggesting that there is no compensational factor for *Clock*, which may be critical in normal mESC differentiation[203]. These findings have been supported by research in mouse embryonic hearts and ESCs which suggested that there is a crucial role in the post transcriptional regulation of *Clock* in circadian rhythm development in

differentiating stem cells. Indeed, the appearance of CLOCK protein during ESC differentiation coincides with the emergence of molecular clock oscillations and Dicer/Dgcr8-mediated posttranscriptional suppression of CLOCK protein[204], highlighting the importance of *Clock* in the establishment of circadian clock oscillations during stem cell differentiation. Collectively, these studies emphasise that the differentiation process is an important feature in the development of the circadian clock.

5.1.3 The Molecular Clock Exerts Essential Regulation on Stem Cell Differentiation Fate

As such, the circadian rhythm has been implicated in the progenitor cell process of differentiation. To this end, the clock genes have been investigated in human subcutaneous adipose-derived progenitor cells; differentiated adipocytes are more readily responsive to clock synchronisation than undifferentiated pre-adipocyte precursors, but the period of clock gene oscillations have been found to be longer in differentiated adipocytes[111]. This shows that the differentiation state of stem or progenitor cells is an important feature in the function of the circadian clock, suggesting a potential relationship between the processes of differentiation and the circadian rhythm, explaining why ESCs do not have this ability to oscillate in a circadian manner.

5.1.3.1 Adipogenesis

The mammalian circadian rhythm has been extensively linked to progenitor cell differentiation. For example, it is well established that *Bmal1* is involved in the regulation of adipogenesis and lipid metabolism in mature adipocytes. When 3T3-L1 cells undergo adipogenic differentiation, the level of *Bmal1* mRNA increases and it is highly expressed in differentiated adipocytes. Furthermore, MEFs from *Bmal1*-deficient mice and *Bmal1* knock-down in 3T3-L1 cells by siRNA interference fail to differentiate into adipocytes. Interestingly, when BMAL1 is overexpressed with adenovirus gene transfer, this ability is restored and cells can accumulate cellular lipids and express adipocyte-related genes, such as *PPARγ2*. The promotor activity of these adipogenic genes is stimulated in a BMAL1-dependent manner, and the expression of

adipogenic factors *PPARγ2* and adipocyte fatty acid binding protein (*AP2*) also show clear circadian rhythms in adipose tissue[115]. Taken together, these results suggest that BMAL1 is an important factor in adipogenesis regulation.

More recently, it has been shown that conversely BMAL1 disruption actually leads to increased adipogenesis, adipocyte hypertrophy and obesity in global *Bmal1* KO mice. Here, it has been uncovered that the attenuation of *Bmal1* function leads to down-regulation of genes in the canonical Wnt signalling pathway, which are known to suppress adipogenesis. The promoters of several of these genes, including *Wnt10a, β-catenin, Fzd5, Dvl2* and *TCF3* displayed BMAL1 occupancy. As a result of this, when *Bmal1* was knocked down, Wnt signalling pathway was attenuated, and the opposite was true for BMAL1 overexpression. Stabilising β-catenin through Wnt ligand or GSK-3β inhibition ameliorated the decreased Wnt signalling and inhibition of adipogenesis induced by *Bmal1* knock down. Taken together, this study offered a mechanistic link between circadian disruption and altered adipogenesis with development of obesity[93]. These differences in the two studies may be due to the use of different cell types, as *Bmal1* may have different roles depending on the anatomical location and cell type.

Another clock gene implied in adipose cell differentiation is *Rev-ErbAα* (NR1D1), which has been shown to be a key regulator of brown adipose tissue development. As *Rev-ErbAα* promotes brown adipogenesis, genetic ablation of *Rev-ErbAα* impairs embryonic and neonatal brown fat formation in mice, by disrupting brown adipocyte lineage commitment and terminal differentiation. By activating REV-ERBα activity, brown adipocyte differentiation is promoted, as REV-ERBα represses key components of the TGF-β cascade, which in turn inhibits brown fat development[116]. This shows that the clock genes have profound effects on adipogenesis and the formation of fat tissue.

5.1.3.2 Neurogenesis

Adult neurogenesis, whereby new neurons and glia are generated, is also thought to be regulated by the circadian rhythm. For example, when neurosphere cultures prepared from the dentate gyrus in the brain are isolated from mPer1::luc mice, it was apparent that circadian *mPer1* gene oscillations can be observed in neurospheres where initial neurogenesis was induced using culture medium, but not in those

maintained in an undifferentiated progenitor cell state. It has also been observed when examining neuropheres from *Bmal1* KO- and *Cry1/2* KO mice, a genetic model of circadian disruption, that circadian rhythms are not required for neurosphere induction *in vitro*. However, the absence of these clock components does slow neurosphere growth, restricts neuronal fate commitment and increases cell death[138]. Quiescent neural progenitor cells (QNPs) in the subgranular zone (SGZ) of the adult hippocampus also express components of the molecular clock and proliferate in a rhythmic manner. Here, the clock proteins PER2 and BMAL1 are essential for the control of neurogenesis, once its initiation has been established; the circadian clock is crucial in timing the entry and exit of the QNPs into the cell cycle and, without these two components, the quiescent state achieved during neuronal differentiation is delayed[140].

mPer2 is also expressed from proliferating neural progenitor cells (NPCs) to mature neurons in the dentate gyrus, and *mPer2* is also thought to have a functional link underlying adult hippocampal neurogenesis by influencing the early cellular events that lead to post-mitotic granule cell production[205]. In the lateral subventricular zone (SVZ), the area in the brain where NPCs persist and postnatal neurogenesis occurs, the expression pattern of clock genes changes following the onset of differentiation and *Bmal1* begins to oscillate endogenously. If *Clock* or *Bmal1* are silenced using RNA interference, the percentage of neuronal marker (Map2)-positive cells decreases and the expression level of neurogenic transcription factors including NeuroD1 also decrease, further suggesting that the molecular clock is involved in neuronal differentiation[206]. Furthermore, a study recently published by Akle et al. (2017) shows that all neurogenic niches studied in an adult diurnal vertebrate, the zebrafish, including the dorsal telencephalon, habenula, preoptic area, hypothalamus, and cerebellum, show circadian modulation of cell cycle progression, that involves the use of both niche-specific and systemic factors[207], emphasising the role of the circadian rhythm on adult progenitor cell differentiation.

5.1.3.3 Osteogenesis

The circadian clock has also been implicated in bone homeostasis; *Bmal1* KO mice display low bone mass phenotypes which reduces further over their lifespan. These mice have decreases in cortical and trabecular bone volume and a lower bone mineral density when visualised using micro-computed tomography. BMAL1 reduction in vivo has also been shown to result in a decreased number of active osteocytes and osteoblasts, and when BMSCs were isolated, they had a reduced osteoblastic differentiation capacity, which likely contributed to the observed reduction in osteoblast and osteocyte numbers[105].

Another clock gene implicated in BMSC proliferation and osteogenesis is *Rev-Erbα*. Here, it has been demonstrated that *Rev-Erbα* expression decreased during osteogenesis, and by overexpressing it, BMSC cell proliferation and osteogenesis was inhibited, which may be partially reversed by activating Wnt/β-catenin signalling via exogenous Wnt3a protein treatment. This suggests that increased *Rev-Erbα* could promote BMSC ageing and negatively regulate osteogenesis with age[208], implicating the clock genes in the process of bone formation.

5.1.3.4 Chondrogenesis

The circadian rhythm has also been linked to chondrogenic differentiation; it has been documented that *Bmal1* is disrupted in human osteoarthritic cartilage and aged mouse cartilage. By ablating *Bmal1* expression in mouse chondrocytes, this led to progressive degeneration of articular cartilage, most likely as a result of a number of molecular pathways that *Bmal1* targets. For example, *Bmal1* ablation led to a reduction of phosphorylated SMAD2/3 and NFATc2 transcription factor, an increase in phosphorylated SMAD1/5, and reduced the expression of matrix related genes *Sox9*, *Acan*, and *Col2a1*, linking the circadian rhythm to the maintenance and repair of cartilage[209].

Furthermore, in a study by Honda et al. (2013), several chondrogenic genes were shown to exhibit statistically significant circadian patterns of mRNA expression, when rats were exposed to 12:12 h light-dark conditions[160]. For example, *type II collagen*,

aggrecan and *Sox9*, a master transcription factor in chondrogenesis, were all shown to demonstrate a circadian phase of expression, suggesting that cartilage matrix synthesis is controlled by cell-autonomous clocks, further supporting the notion that the circadian rhythm may have a regulatory role on cellular differentiation.

5.1.3.5 Myogenesis

Important roles for *Bmal1* have been demonstrated regarding myogenesis too. *Bmal1* is highly expressed in skeletal muscle and is thought to regulate myogenic differentiation via direct transcriptional activation of canonical Wnt signalling pathway components. As the master regulator of myogenesis, MyoD, has a circadian rhythm in its mRNA and protein levels and is a direct target of CLOCK and BMAL1. It has therefore been reported that *ClockΔ19* and *Bmal1*KO mice show extreme reductions in the maximal force of their skeletal muscle. These clock mutant mice also show reductions in mitochondrial volume and the remaining mitochondria have aberrant morphologies and increased respiration uncoupling, demonstrating the importance of CLOCK and BMAL1 in skeletal muscle structure and function[119]. Similarly, when *Bmal1* is knocked down in myoblasts, this leads to a reduction in total muscle mass, impaired myogenic differentiation of myoblasts and decreased expression of key myogenic regulatory factors including Myf5, Mrf4 and Myogenin as well as MHC3. Overexpression of *Bmal1* in C2C12 myoblasts conversely leads to accelerated myogenesis and attenuation of Wnt signalling, indicating that Bmal1 is required for myoblast differentiation. Direct association of *Bmal1* with canonical Wnt pathway gene promoters has been observed and consistently several Wnt signalling components show intrinsic circadian oscillations[118]. *Bmal1* can also influence skeletal muscle through influencing its regeneration processes*; Bmal1* KO mice display significantly lower satellite cell expansion, which leads to defective regenerative responses, and these mice exhibit a nearly non-existent induction of Pax7, a satellite cell marker. The satellite cell-derived myoblasts that are isolated from *Bmal1* KO mice demonstrate reduced growth and proliferation ex vivo, underscoring the role of *Bmal1* in muscle repair and preserving muscle mass[120].

5.1.3.6 Angiogenesis

Several circadian factors have also been implicated in angiogenesis. During angiogenesis, *Per2* is thought to be a key factor in maintaining endothelial progenitor cell (EPL) function. PER2 is abundantly expressed in early EPCs, and EPCs from *Per2*−/− mice demonstrate impaired proliferation, adhesion, migration and tube formation, due to inhibitions in PI3k/Akt/FoxO signalling and C-X-C chemokine receptor type 4 (CXCR4) protein level. The negative changes in proliferation rate of these EPCs can be blocked by activating PI3K/Akt/FoxO signalling. Indeed, PER2 and CXCR4 directly interact in WT EPCs, showing that Per2 is essential in maintaining early EPC function[210]. In humans, circulating progenitor cells and their proangiogenic activity also exhibit circadian variations. Both show unfavourable profiles in the morning which coincides with prevalence of cardiovascular events at this time, which is influenced by the endogenous circadian clock[211]. When *Bmal1* is conditionally deleted in endothelium and hematopoietic cells, responses to microvascular and macrovascular injury are exaggerated, highlighting the importance of circadian rhythms in maintaining vascular homeostasis[212].

5.1.3.7 Other Specialised Tissue Generation

As well as influencing all of these different forms of differentiation, in the cardiac muscle, circadian rhythms are also thought to determine cardiac metabolism and outcomes of ischemic stress. Moreover, although undifferentiated human ESCs do not possess functional rhythms as previously stated, they do start to progressively oscillate spontaneously following directed cardiac differentiation. Another cardiac process that the circadian clock has been implicated in is to do with the cardiac cellular stress response, as a number of clock-controlled output genes have been identified as an oscillatory network of stress-related genes, including some oscillators known to play an important role in human heart physiology, including PLN, KCNE4, TSPO, CAV1 and RGS2, showing the importance of circadian outputs in cardiovascular function[213]. Stem cell antigen 1-positive (SCA1+) cells, which are also present in the heart, have also been shown to possess a molecular clock and exhibit circadian oscillations that controls downstream cellular functions[214].

Along with cardiac tissue, the circadian rhythm has also been implicated in intestinal physiology. Intestinal stem/progenitor cells (ISCs) are critical components in how the intestine regenerates in order to replace dying cells. The Period transcription factor has been discovered to be a critical factor in intestinal regeneration and hundreds of transcripts that are regulated by the circadian clock have been uncovered within intestinal stem cells, including those involved in stress response and regeneration pathways. Furthermore, disruption of the clock component *Per* has been shown to lead to arrhythmic ISC divisions, demonstrating how diverse the role of the circadian rhythm and molecular clock components are in the progenitor cells of different peripheral tissues[215].

5.1.4 Aims and Hypothesis

The existing published peer reviewed knowledge base supports the theory that the cellular circadian rhythm is influenced by the differentiation state of the cell, as is seen by the research into the circadian rhythm of ESCs by Yagita et al. (2010). In this study, undifferentiated ESCs do not appear to have a functioning circadian rhythm, but this ability is gained upon differentiation and can then be reversed when the cells are artificially de-differentiated[142]. Furthermore, research by Wu et al. (2007) similarly demonstrated that the less differentiated cells do not have a fully functioning circadian clock when compared to mature cells, as is shown when comparing differentiated adipocytes vs undifferentiated pre-adipocyte precursors[111]. Collectively, these studies highlight the role of the state of cellular differentiation on the circadian rhythm. However, it has also been proven that the circadian rhythm itself has a regulatory role in progenitor cell differentiation, suggesting a feedback mechanism between the two processes. For example, the circadian clock genes have been implicated in various forms of differentiation, with disruptions in their expression causing wide spread problems in progenitor cell function and fate.

For these reasons, it was hypothesised that the circadian rhythm has a direct influence on the expression of several progenitor cell differentiation markers. This notion was further supported by work by Honda et al. (2013) who showed that the chondrogenesis differentiation marker *Sox9* showed circadian mRNA expression[160]. In

this chapter, we aimed to see how knocking down the expression of *Bmal1* and *Rev-ErbAα* using siRNA affected the expression of the differentiation markers of the major differentiation lineages: *OCN* (osteogenesis), *PPARγ* (adipogenesis) and *Sox9* (chondrogenesis) in two types of mesenchymal progenitor cell, BMSCs and DPSCs. This was investigated initially in both cell types in normal growth conditions and then also using the same mechanical stretch set up as previously described (6.66% stretch, 1 Hz, 12 h/day), to see if knocking down either of these clock genes brought about any differences in the progenitor cells' differentiation response to stretch. *Bmal1* and *Rev-ErbAα* were selected to be knocked down in expression as they had demonstrated similar patterns of gene expression to the differentiation markers in the previous chapter, in response to stretch and/or changes in protein substrate. The identification of the molecular mechanism by which the circadian rhythm influences stem and progenitor cell differentiation will allow for the design of treatments to rectify pathologies with dysfunctional circadian rhythms and therefore dysfunctional differentiation processes. Once the appropriate genes and their effects have been identified, therapies can be designed to reverse any conditions where there have been changes in circadian gene expression that have caused phenotypes as a result of impaired differentiation.

5.2 Results

5.2.1 Research Section One: The Effect of *Bmal1* and *Rev-ErbAα* Knockdown by siRNA on the Differentiation Capacity of Adult Progenitor Cells

In order to further elucidate the relationship between the circadian rhythm and the differentiation processes in adult progenitor cells, siRNAs were utilised to target the clock genes *Bmal1* and *Rev-ErbAα,* in order to knock down and reduce their relative levels of gene expression. *Bmal1* and *Rev-ErbAα* were selected, as both genes showed corresponding patterns of gene expression to several differentiation markers when previously exposed to different protein substrates in stretch and static conditions. As

these genes responded similarly to different stimuli, it was theorised that there may be some interaction between the circadian genes and differentiation markers.

First, a selection of siRNAs were trialled and optimised to see which of a commercial panel of siRNA (FlexiTube, QIAGEN) was most effective at knocking the gene expression down in each gene in both progenitor cell types: BMSCs and DPSCs.

5.2.1.1 BMSCs

BMSCs were cultured in normal growth conditions in tissue culture polystyrene plates, before being subjected to the siRNA according to the manufacturer's instructions (QIAGEN). Samples were collected both after 48 h and day 4 following the addition of the siRNA, at which time total RNA was extracted (TRI-Reagent) and qPCR undertaken to analyse relative levels of mRNA expression (Figure 5.1).

For *Bmal1*, of the four types of siRNA tested, B2 and B4 showed substantial decreases in *Bmal1* expression after 48 h when compared to the scrambled (SC) negative control (one-way ANOVA, B2 $p=0.057$, B4 $p\leq0.05$, $n=3$). After four days, however, no significant changes could be determined, with B1 and B2 showing the largest decreases in expression. At day 4, the levels of B3 and B4 resulted in a similar level of *Bmal1* expression to both the scrambled (SC) and untreated (UT) negative controls, suggesting that the 48 h decrease in *Bmal1* expression in B4 was an initial change only, and not long-term. For this reason, B2 was selected as being the best siRNA to interfere with *Bmal1* expression in BMSCs, as it gave a 0.292-fold reduction in *Bmal1* expression at 48 h and 0.294-fold reduction at day 4.

Regarding *Rev-ErbA*α, both R1 and R6 showed statistically significant decreases in *Rev-ErbA*α expression at both 48 h and day four after the application of the siRNA, when compared to the scrambled negative control. At day 4, R5 also showed significant decreases in *Rev-ErbA*α expression as well as R1 and R6 (one-way ANOVA, $p\leq0.05$, $n=3$). In this instance, R1 was selected as the most suitable siRNA to interfere with *Rev-ErbA*α expression as it led to the largest decreases in gene expression in BMSCs, with a 0.245-fold reduction in expression at 48h and 0.251-fold reduction at day 4.

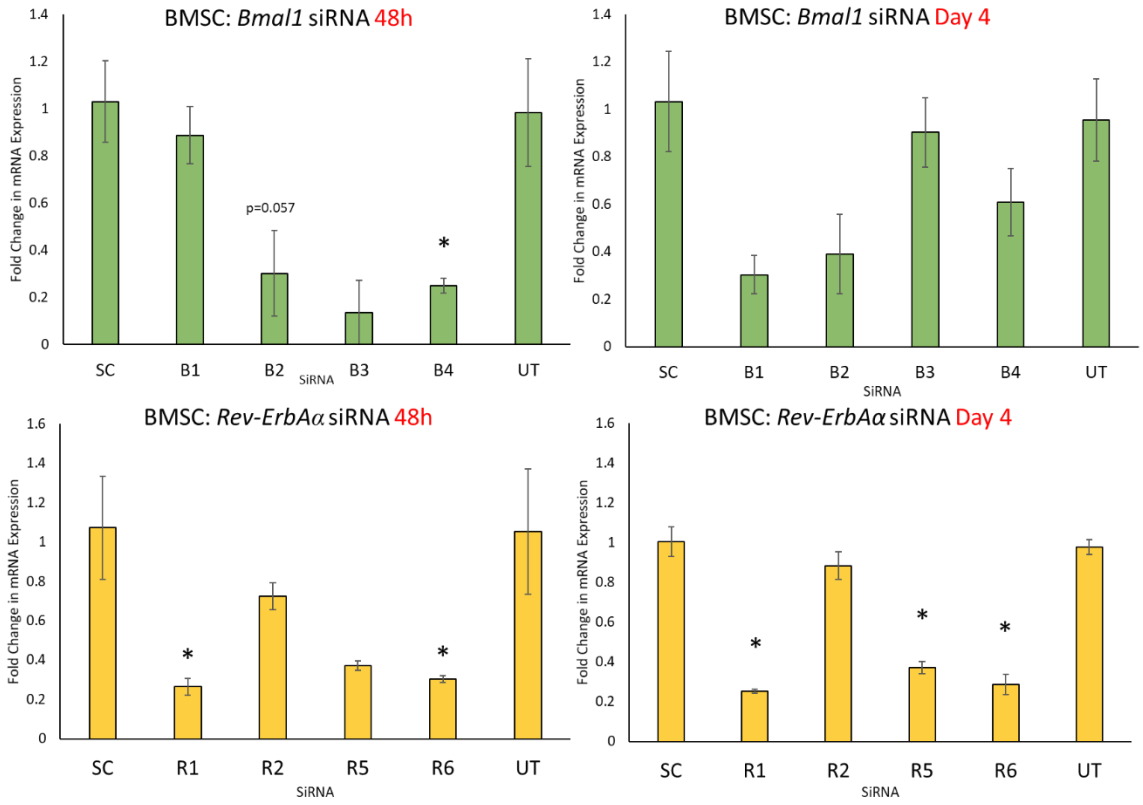

*Figure 5.1 The Effect of Various Bmal1 and Rev-ErbA siRNA on Their Target Genes in BMSCs. BMSCs were cultured in standard tissue culture conditions before being subjected to treatment with various siRNAs targeting Bmal1 and Rev-ErbA to determine which siRNA were the most effective at knocking down their target gene's relative level of expression. Control samples that contained scrambled siRNA (SC) and untreated cells (UT) were also included for comparison. Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

These two siRNAs (B2 and R1) were then utilised to investigate how knocking down both circadian genes effected the expression of three progenitor cell differentiation markers: *OCN* (osteogenesis), *PPARγ* (adipogenesis) and *Sox9* (chondrogenesis) (Figure 5.2). It was demonstrated that by knocking down *Rev-ErbAα* expression using siRNA (R1), statistically significant increases in *OCN* expression could be observed at day 4, when compared to the scrambled (SC) negative control (one-way ANOVA, $p \leq 0.05$, n=3), suggesting that *Rev-ErbAα* has a negative role on the regulation of *OCN*. Regarding *PPARγ*, it was observed that knocking down *Bmal1* led to increases in *PPARγ* gene expression, whereas knocking down *Rev-ErbAα* led to decreases. However, these

changes were not statistically significant, and similarly, no significant differences could be found for *Sox9* expression following RNA interference.

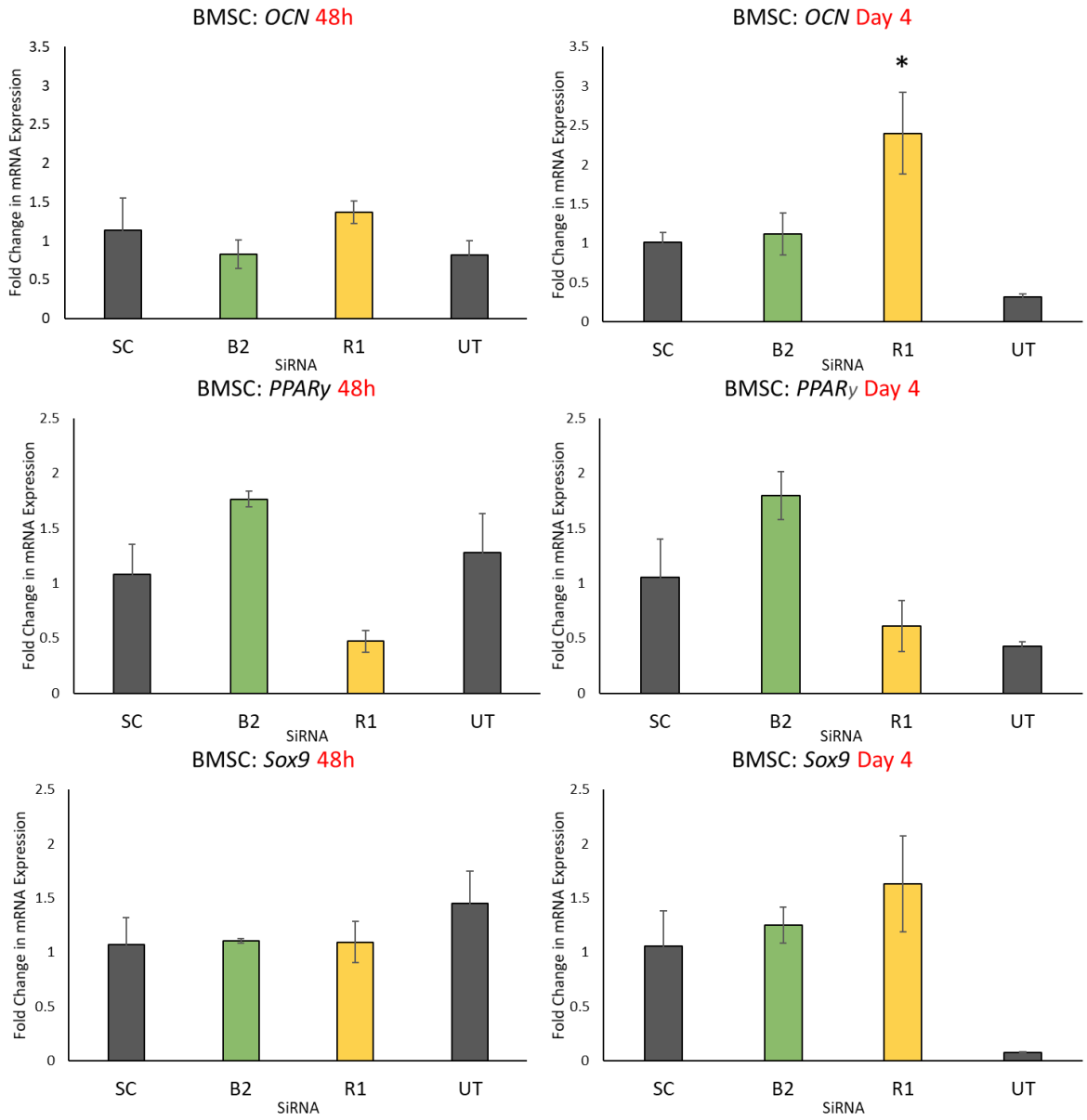

Figure 5.2 The Effect of Bmal1 (B2) and Rev-ErbA (R1) siRNA on Differentiation Marker Expression in BMSCs. *BMSCs were cultured in standard tissue culture conditions before being subjected to treatment with siRNA targeting Bmal1 (B2) and Rev-ErbA (R1) to determine the effect of clock gene knockdown on several differentiation marker gene's relative level of expression (OCN, Sox9 and PPARγ). Control samples that contained scrambled siRNA (SC) and untreated cells (UT) were also included for comparison. Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

5.2.1.2 DPSCs

DPSCs were also cultured in normal growth conditions in tissue culture polystyrene plates before the addition of siRNA was undertaken according to the manufacturer's conditions (QIAGEN). Samples were collected both after 48 h and day 4 following the addition of the siRNA at which time total RNA was extracted (TRI-Reagent) and qPCR undertaken to analyse relative levels of mRNA expression (Figure 5.3).

For *Bmal1*, both B1 and B2 showed decreases in *Bmal1* expression, with B2 decreasing by a 0.583-fold reduction in gene expression at 48 h and a 0.379-fold reduction at day 4. Unfortunately, neither of these changes reached statistical significance, but B2 was selected as the most effective siRNA as it led to the most substantial decreases in expression. For *Rev-ErbA*α, R1, R5 and R6 siRNAs all resulted in significantly significant decreases in *Rev-ErbA*α expression after both 48 h and four days. By day 4, R2 also resulted in a significant decrease in expression. R1 was selected as the most efficient siRNA as it resulted in the largest fold changes: a 0.279-fold reduction in gene expression at 48 h and a 0.076-fold reduction at day 4.

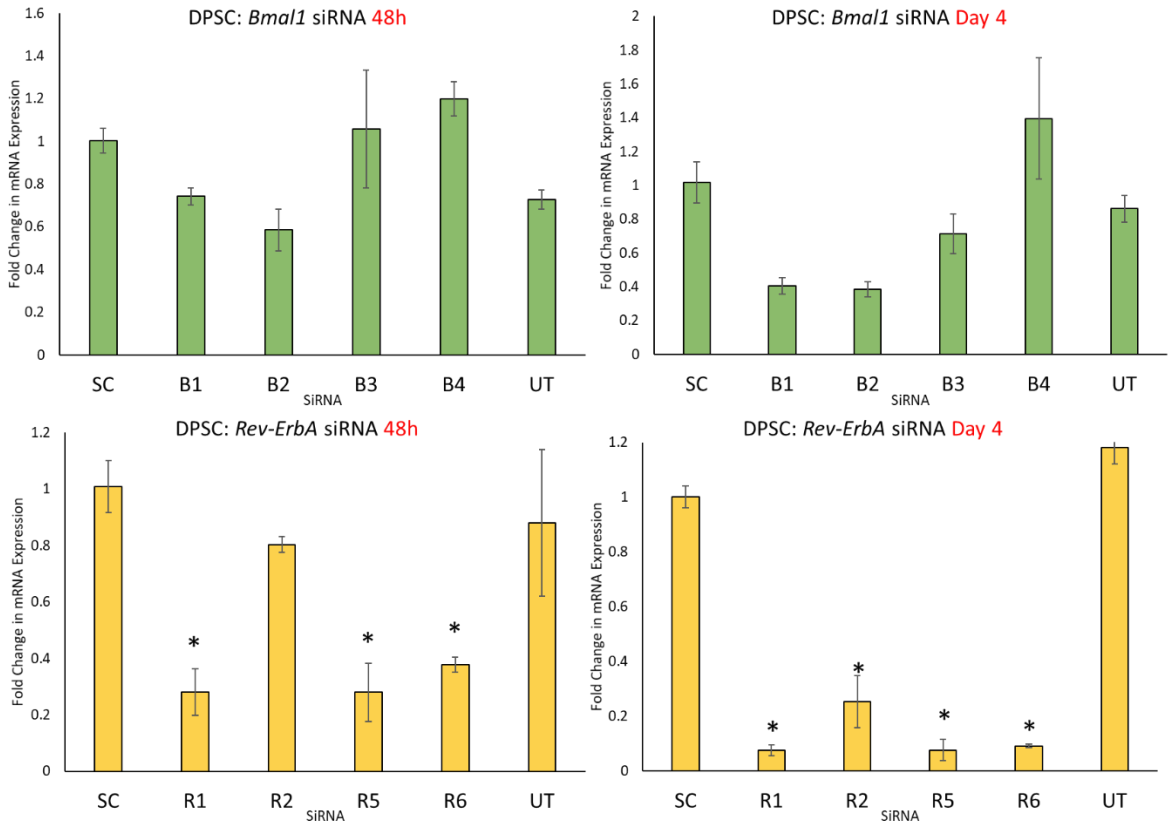

*Figure 5.3 The Effect of Various Bmal1 and Rev-ErbA siRNA on Their Target Genes in DPSCs. DPSCs were cultured in standard tissue culture conditions before being subjected to treatment with various siRNAs targeting Bmal1 and Rev-ErbA to determine which siRNA were the most effective at knocking down their target gene's relative level of expression. Control samples that contained scrambled siRNA (SC) and untreated cells (UT) were also included for comparison. Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

The effect of both the *Bmal1* and the *Rev-ErbAα* knock down on progenitor cell differentiation marker expression was then also investigated (Figure 5.4). It was found that by knocking *Rev-ErbAα* expression down, a significant initial increase in *OCN* could be observed at 48 h when compared to the scrambled control, but a significant decrease was observed at day 4 following the addition of *Rev-ErbAα* siRNA. This suggests that *Rev-ErbAα* may have an initial repression effect on *OCN* expression, but this is not long-term, and potentially an alternative compensatory mechanism may take effect.

Regarding *PPARγ, Bmal1* and *Rev-ErbAα* appeared to have opposing effects. For example, at both 48 h and day 4, *Rev-ErbAα* knock down led to a significant decrease

in *PPARγ* expression when compared to the scrambled control, insinuating that *Rev-ErbAα* has a positive effect on *PPARγ* expression. However, by day 4, *Bmal1* knock down brought about an increase in *PPARγ* expression when compared to the scrambled control. In direct contrast, this suggests that *Bmal1* has a negative role on *PPARγ* expression. Taken together, this data suggests that *PPARγ* expression and potentially various aspects of adipogenesis are strongly regulated by the circadian genes.

When the response of *Sox9* expression is analysed following *Bmal1* and *Rev-ErbAα* RNA interference, a strong initial increase in expression is seen when either of the genes is knocked down; both *Bmal1* and *Rev-ErbAα* siRNA led to a statistically significant increases in *Sox9* expression after 48 h, when compared to the scrambled control. However, this significant increase was lost by day 4, suggesting that an alternative mechanism had been stimulated in compensation, potentially by affecting the activation of the Wnt signalling pathway, as *Sox9* has been previously shown to affect Wnt signalling by promoting beta-catenin phosphorylation in the nucleus[244]. This data suggests that there is a circadian regulation of *Sox9* expression but that this mechanism can be compensated for in the absence of the necessary circadian components or may involve regulation by other clock genes in the absence of *Bmal1* and *Rev-ErbAα*.

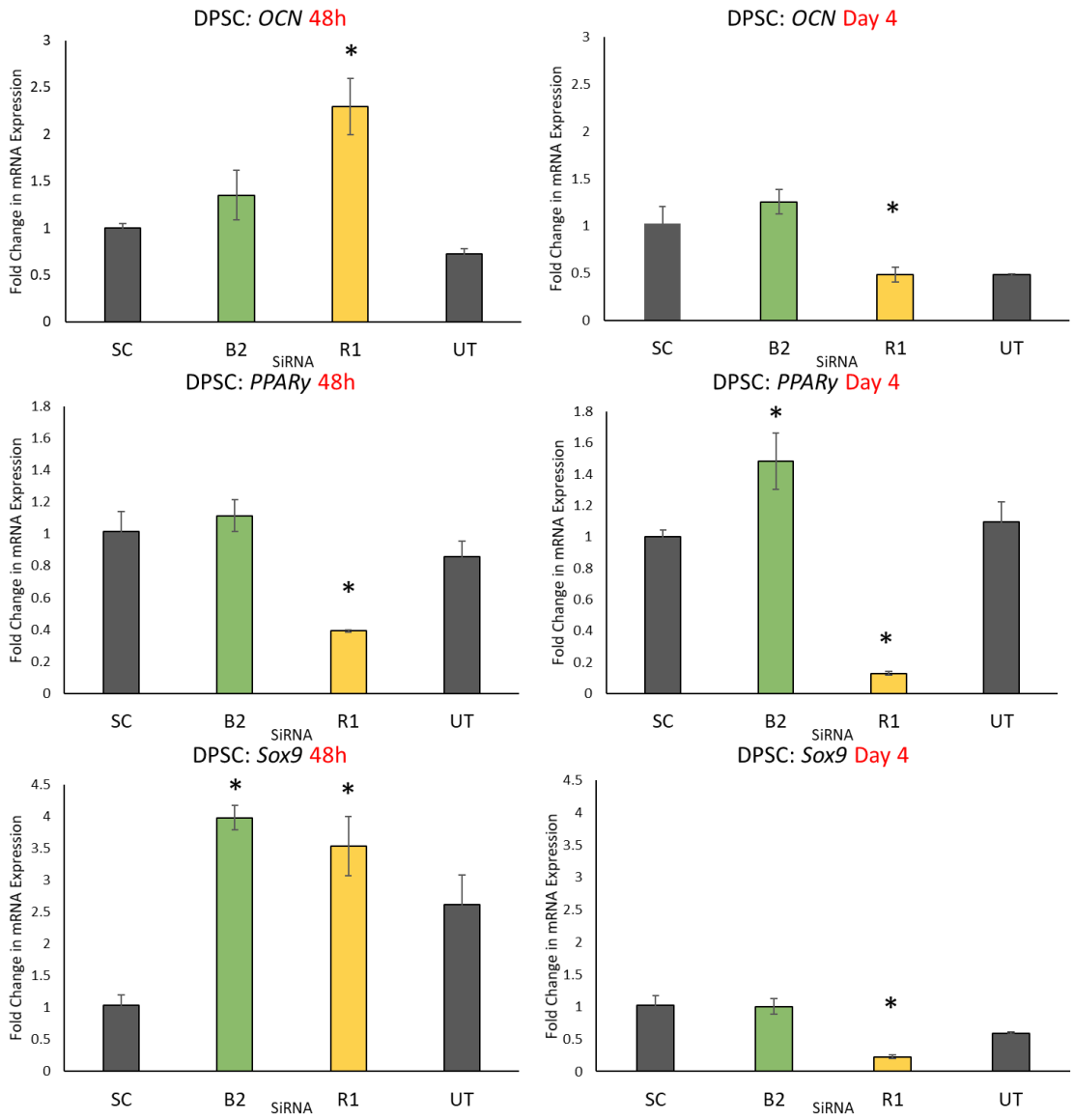

Figure 5.4 The Effect of Bmal1 (B2) and Rev-ErbA (R1) siRNA on Differentiation Marker Expression in DPSCs. *DPSCs were cultured in standard tissue culture conditions before being subjected to treatment with siRNA targeting Bmal1 (B2) and Rev-ErbA (R1) to determine the effect of clock gene knockdown on several differentiation marker gene's relative level of expression (OCN, Sox9 and PPARγ). Control samples that contained scrambled siRNA (SC) and untreated cells (UT) were also included for comparison. Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05 (one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

5.2.2 Research Section Two: The Effect of *Bmal1* and *Rev-ErbA* Clock Gene Knockdown by SiRNA on the Human Adult Progenitor Cell Response to Stretch

In order to further elucidate the role of the circadian rhythm on the adult progenitor cells' response to stretch, *Bmal1* and *Rev-ErbA*α were then knocked down using RNA interference techniques prior to both BMSCs and DPSCs undergoing the previously investigated mechanical strain paradigm. Both types of progenitor cell were exposed to siRNA targeting either *Bmal1* or *Rev-ErbA*α for 24 h before being stretched uniaxially for three days (6.66% stretch, 1 Hz, 12 h/day). After the three days, total RNA was collected and the expression of several progenitor differentiation marker was quantified, including markers for osteogenesis (*OCN*), chondrogenesis (*Sox9*) and adipogenesis (*PPAR*γ).

5.2.2.1 BMSCs

First, the level of mRNA expression was quantified for both *Bmal1* and *Rev-ErbA*α, to ensure that both genes were sufficiently knocked down, and to see how this was affected by mechanical stretch in BMSCs (Figure 5.5). It was here found that *Bmal1* knockdown (B2) did result in a significant decrease in *Bmal1* expression in both stretch and static conditions when compared to the scrambled negative control (SC) ($p \leq 0.05$, n=3, one-way ANOVA with Tukey post-hoc). Interestingly, *Rev-ErbA*α knockdown (R1) also resulted in a highly significantly decrease in *Bmal1* gene expression in static conditions, suggesting that *Rev-ErbA*α has a regulatory role on *Bmal1* expression ($p \leq 0.01$, n=3, one-way ANOVA with Tukey post-hoc). When subjected to the three-day mechanical stretch regime, it was found that *Bmal1* expression was highly significantly increased following mechanical stretch when compared to static in all three conditions: SC, B2 and R1 ($p \leq 0.01$, n=3, independent t-test), suggesting that mechanical stimulation upregulates the expression of *Bmal1* in BMSCs.

When the expression of *Rev-ErbA*α was knocked down using siRNA (R1), this did indeed result in highly significant reductions in *Rev-ErbA*α when exposed to both stretch and static conditions, when compared to scrambled (SC) negative controls

(p≤0.01, n=3, one-way ANOVA with Tukey post-hoc). Furthermore, when *Bmal1* was knocked down (B2), this also led to highly significant decreases in *Rev-ErbAα* expression when compared to SC controls; however, this decrease was still significantly higher in expression than when *Rev-ErbA* itself was knocked down (R1) (p≤0.05, n=3, one-way ANOVA with Tukey post-hoc). Similarly to *Bmal1*, *Rev-ErbAα* gene expression appeared to also be increased following mechanical stretch (Figure 5.5) and this increase was found to be highly significant in the control samples and significant following *Rev-ErbAα* knockdown (p≤0.05, n=3, independent t-test). This suggests that *Rev-ErbAα* also appears to be upregulated in response to mechanical stretch.

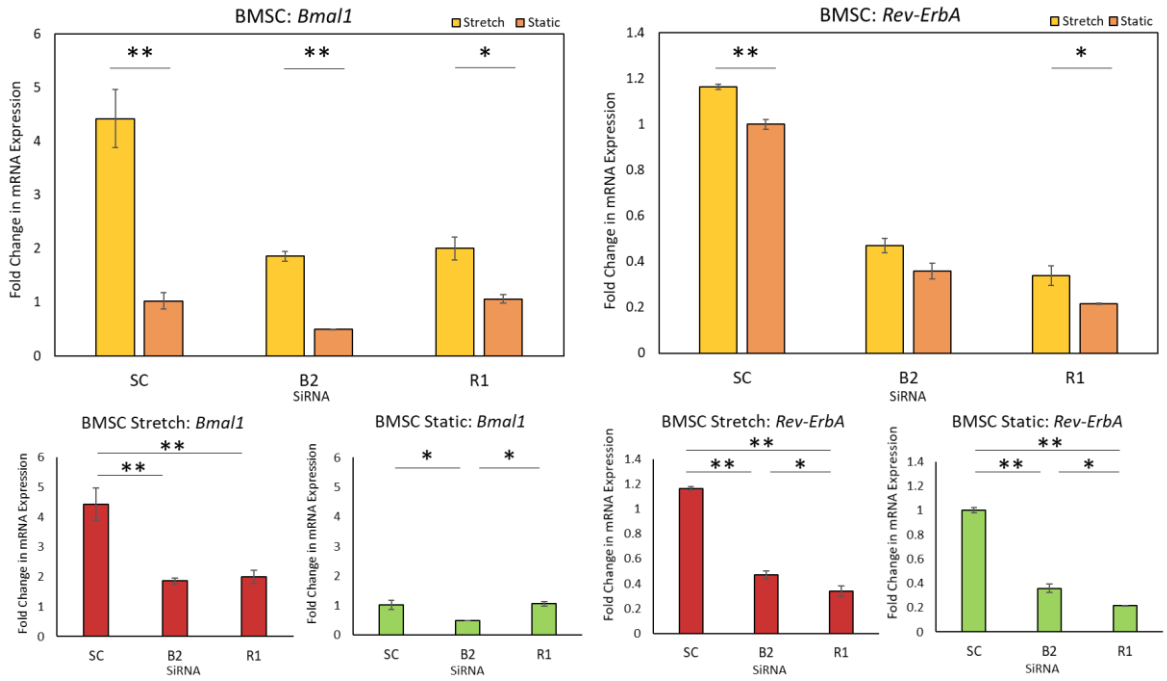

Figure 5.5 The Effect of Bmal1 and Rev-ErbAα siRNA on Their Target Genes Exposed to Mechanical Stretch and Static Conditions in BMSCs*. BMSCs were treated with siRNA targeting Bmal1 (B2) and Rev-ErbAα (R1) before being subjected to stretch (6.66%, 1Hz, 12h/day) or static conditions for three days, to determine how effective the siRNA were at knocking down the levels of gene expression in both stretch and static conditions (C-D), and to see if Bmal1 and Rev-ErbAα gene expression was affected by mechanical strain vs static conditions within the treatment conditions (A-B). Experimental samples were compared to control samples that contained scrambled siRNA (SC). Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05, **: p<0.01 (A-B: independent t-test, n=3; C-D: one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

BMSCs also showed significant responses to mechanical stretch in terms of the expression of their differentiation markers (Figure 5.6). For example, *OCN* was increased in expression in all three conditions (SC, B2 and R1) when exposed to mechanical stimulation compared to static conditions ($p \leq 0.05$, n=3, independent t-test) (Figure 5.6A). Furthermore, *OCN* expression was demonstrated to be highly significantly reduced in expression following both *Bmal1* and *Rev-ErbA*α knockdown in stretch and static conditions when compared to the scrambled control, with this decrease being significantly lower in R1 than in B2 in static conditions ($p \leq 0.05$, n=3, one-way ANOVA with Tukey post-hoc) (Figure 5.6B).

Sox9, a master transcription factor involved in chondrogenesis, was similarly found to be significantly upregulated when exposed to mechanical stretch in all three conditions ($p \leq 0.05$, n=3, independent t-test) (Figure 5.6C). The level of *Sox9* expression was found to be decreased when exposed to either B2 or R1 when compared to SC, with *Rev-ErbA*α knockdown resulting in highly significant decreases in *Sox9* when compared to either B2 or SC ($p \leq 0.01$, n=3, one-way ANOVA with Tukey post-hoc) (Figure 5.6D), suggesting that *Rev-ErbA*α has a positive role on *Sox9* regulation.

Finally, *PPAR*γ, a marker of adipogenesis, was found to be highly significantly increased in expression when exposed to static compared to stretch conditions in scrambled controls suggesting that stretch inhibits adipogenesis. However, no such significant increase could be observed when *Bmal1* was knocked down and *Rev-ErbA*α knockdown, interestingly, lead to a significant decrease in *PPAR*γ expression when exposed to static conditions, when compared to stretch, suggesting that the inhibitory response of *PPAR*γ as a result of stretch had been removed by inhibiting the clock genes (Figure 5.6E). To this end, the expression of *PPAR*γ was found to be highest when *Bmal1* was knocked down in static conditions and lowest in the scrambled negative controls when subjected to mechanical stretch (Figure 5.6F). This data demonstrates that the clock genes have a significant role on the differentiation potential of adult progenitor cells and their response to stretch.

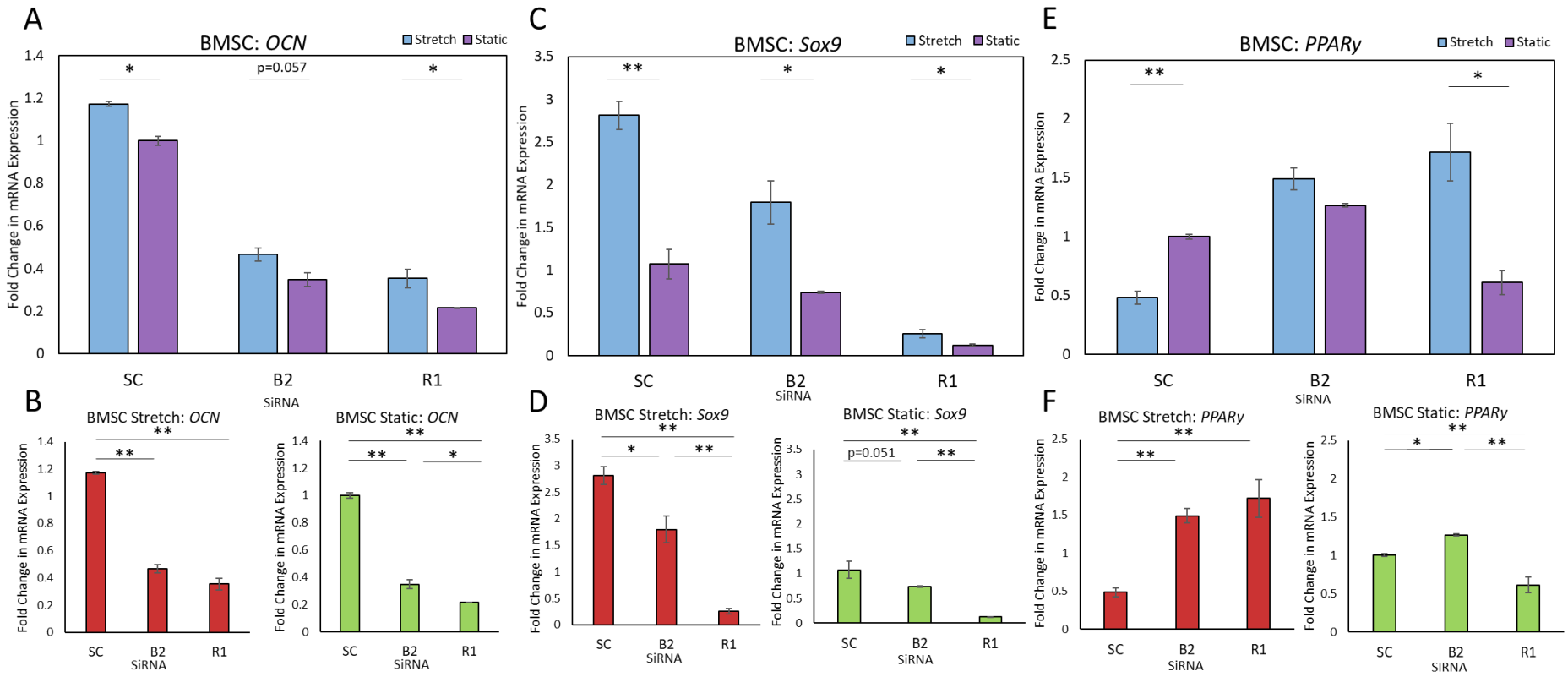

Figure 5.6 The Effect of Bmal1 and Rev-ErbAα siRNA on Differentiation Marker Gene Expression in BMSCs Exposed to Mechanical Stretch and Static Conditions. *BMSCs were treated with siRNA targeting Bmal1 (B2) and Rev-ErbAα (R1) before being subjected to stretch (6.66%, 1Hz, 12h/day) or static conditions for three days, to determine the effect of knocking down clock gene expression on differentiation marker expression (OCN, Sox9 and PPARγ) in both stretch and static conditions (C-D), and to see if differentiation marker gene expression was affected by mechanical strain vs static conditions within the treatment conditions (A-B). Experimental samples were compared to control samples that contained scrambled siRNA (SC). Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05, **: p<0.01 (A-B: independent t-test, n=3; C-D: one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

5.2.2.2 DPSCs

As with the BMSCs, the first thing to be quantified in DPSCs was the response of both targets genes following *Bmal1* and *Rev-ErbA*α knockdown by siRNA. Regarding *Bmal1*, it was found that in static conditions, *Bmal1* was highly significantly decreased following *Bmal1* siRNA when compared to SC and R1 treatment ($p \leq 0.01$, n=3, one-way ANOVA with Tukey post-hoc). In contrast, however, when exposed to mechanical stretch, a significant upregulation in *Bmal1* was observed following *Rev-ErbA*α knockdown (R1) when compared to both SC and B2 conditions (Figure 5.7). In all three conditions, *Bmal1* expression was found to be significantly higher in static conditions when compared to stretch, in direct contrast to the stretch-induced upregulation seen in BMSCs ($p \leq 0.05$, n=3, independent t-test).

Rev-ErbAα expression was highly significantly decreased when DPSCs were exposed to either B2 or R1 siRNA when compared to SC controls in both static and stretch conditions, as was consistent in BMSCs ($p \leq 0.01$, n=3, one-way ANOVA with Tukey post-hoc) (Figure 5.7). However, no significant response could be observed when stretch and static conditions were compared for both R1 and B2, with only the SC control condition resulting in a stretch-induced reduction in *Rev-ErbA*α expression (Figure 5.7).

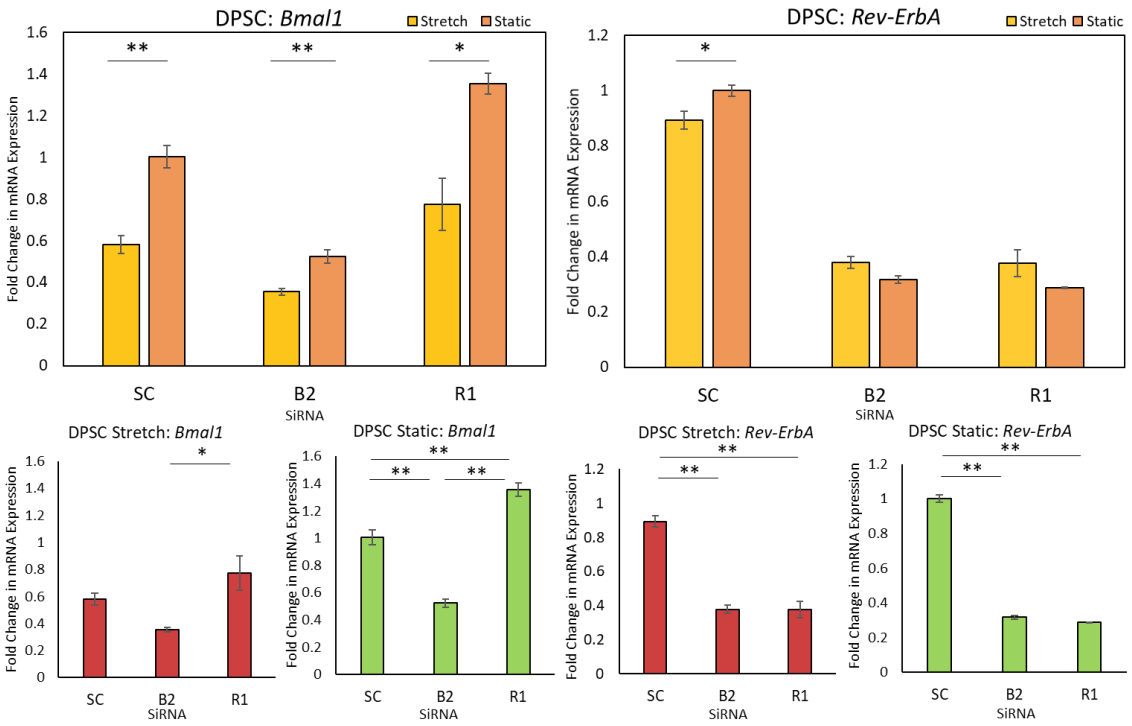

Figure 5.7 The Effect of Bmal1 and Rev-ErbAα siRNA on Their Target Genes Exposed to Mechanical Stretch and Static Conditions in DPSCs. *DPSCs were treated with siRNA targeting Bmal1 (B2) and Rev-ErbAα (R1) before being subjected to stretch (6.66%, 1Hz, 12h/day) or static conditions for three days, to determine how effective the siRNA were at knocking down the levels of gene expression in both stretch and static conditions (C-D), and to see if Bmal1 and Rev-ErbAα gene expression was affected by mechanical strain vs static conditions within the treatment conditions (A-B). Experimental samples were compared to control samples that contained scrambled siRNA (SC). Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05, **: p<0.01 (A-B: independent t-test, n=3; C-D: one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

Next, the response of the progenitor cell differentiation genes' relative levels of expression were analysed in DPSCs following exposure to *Bmal1* and *Rev-ErbAα* siRNA in stretch or static conditions (Figure 5.8). For the osteogenic differentiation marker, *OCN*, it was surprisingly observed that *OCN* showed a significantly reduced level of expression following mechanical stretch in all three treatments, when compared to static conditions ($p \leq 0.05$, n=3, independent t-test) (Figure 5.8A), again in direct contrast to the stretch-induced upregulation seen in BMSCs. However, no significant differences in *OCN* expression could be demonstrated between the three treatments (SC, B2 and R1) (Figure 5.8B), suggesting that *OCN* expression was not impacted by circadian clock gene interference in DPSCs.

Sox9 appeared to be similarly down-regulated following stretch; in the scrambled controls (SC) and *Bmal1* knockdown conditions (B2), the expression of *Sox9* was significantly lowered following stretch, with no significant difference being observed following *Rev-ErbA*α knockdown (R1) in stretch or static conditions ($p \leq 0.05$, n=3, independent t-test) (Figure 5.8C). However, significant changes could be observed between the treatment conditions. Following three days of mechanical stretch, both *Bmal1* and *Rev-ErbA*α siRNA led to highly significant decreases in *Sox9* expression, when compared to the scrambled control. Again, in static conditions, a significant decrease could also be observed in both B2 and R1, with R1 being significantly lower than B2 again ($p \leq 0.05$, n=3, one-way ANOVA with Tukey post-hoc) (Figure 5.8D). This shows, in opposition to *OCN*, that *Sox9* expression was impacted by the expression levels of the clock genes.

The response of *PPAR*γ to mechanical stretch was similar to *Sox9* in that it was also down-regulated following stretch when *Bmal1* siRNA was applied, but no significant changes between stretch and static could be seen in scrambled controls or following *Rev-ErbA*α knockdown ($p \leq 0.05$, n=3, independent t-test) (Figure 5.8E). However, no significant changes could be observed between the treatment conditions; no changes could be seen between SC, B2 and R1 in either stretch or static conditions ($p \leq 0.05$, n=3, one-way ANOVA with Tukey post-hoc) (Figure 5.8F). This shows that the clock genes *Bmal1* and *Rev-ErbA*α had no effect on *PPAR*γ expression in DPSCs.

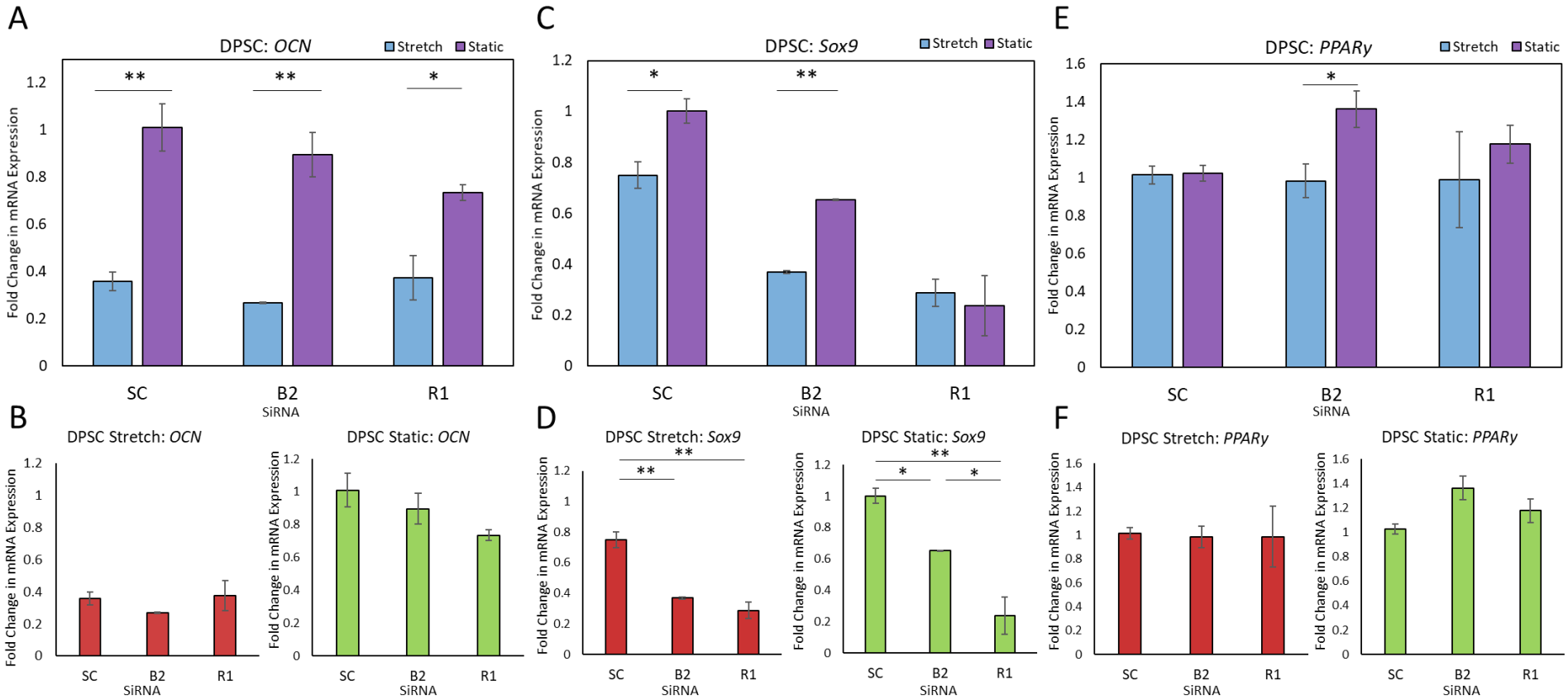

Figure 5.8 The Effect of Bmal1 and Rev-ErbAα siRNA on Differentiation Marker Gene Expression in DPSCs Exposed to Mechanical Stretch and Static Conditions. *DPSCs were treated with siRNA targeting Bmal1 (B2) and Rev-ErbAα (R1) before being subjected to stretch (6.66%, 1Hz, 12h/day) or static conditions for three days, to determine the effect of knocking down clock gene expression on differentiation marker expression (OCN, Sox9 and PPARγ) in both stretch and static conditions (C-D), and to see if differentiation marker gene expression was affected by mechanical strain vs static conditions within the treatment conditions (A-B). Experimental samples were compared to control samples that contained scrambled siRNA (SC). Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05, **: p<0.01 (A-B: independent t-test, n=3; C-D: one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

5.3 Conclusions

In this research, it has been experimentally evidenced that the induction of progenitor cell differentiation markers are influenced by the circadian rhythm and core clock genes. By decreasing the expression of two circadian genes, *Bmal1* and *Rev-ErbA*α, using siRNA techniques of gene interference, significant changes in the expression of differentiation markers of osteogenesis, adipogenesis and chondrogenesis could be observed at basal levels. For example, by knocking down the expression of *Rev-ErbA*α, increases in *OCN* and decreases in *PPAR*γ could be seen in both BMSCs and DPSCs. By knocking down the expression of *Bmal1*, increases in *PPAR*γ could be observed in both cell types, suggesting that *Bmal1* has a negative regulatory role on *PPAR*γ expression. This is supported by research by Guo et al. (2012), who showed that *Bmal1* disruption leads to increased adipogenesis, adipocyte hypertrophy and obesity in mice, supporting the notion that *Bmal1* is a critical negative regulator of adipocyte development[93].

However, differences could be observed between the two cell types' response to mechanical stretch. For example, *Bmal1* was increased in all three treatment conditions (SC, B2 and R1) following stretch when compared to static conditions in BMSCs, but a stretch-induced reduction in *Bmal1* could be observed for all three in DPSCs, showing that the two cell types differed in their response to stretch. In the BMSCs, *Bmal1* expression was decreased in both B2 and R1 when compared to SC following stretch, but *Bmal1* was only decreased in B2 in static conditions. In DPSCs, however, the decrease in *Bmal1* could be observed in B2 as with the BMSCs, but, in direct contrast, was instead increased in R1 following either stretch or static conditions, showing that the regulatory roles that *Bmal1* and *Rev-ErbA*α have on each other may differ between the cell types. *Rev-ErbA*α was also increased in all three conditions after being subjected to mechanical stretch in BMSCs and was also increased after stretch in the SC condition in DPSCs; no significant changes could be observed in *Rev-ErbA*α expression when comparing stretch and static conditions for B2 and R1 in DPSCs. In both DPSCs and BMSCs however, *Rev-ErbA*α expression was decreased in B2 and R1 when compared to SC negative controls in both stretch and

static conditions, showing that the *Rev-ErbA*α knockdown decreased the expression of both *Bmal1* and *Rev-ErbA*α in both cell types. These findings indicate that the two cell types differ in the signalling pathways activated by mechanical stretch and that knocking out certain genes in one cell type may have a different effect on another cell type and their changes in gene expression.

It was also discovered that knocking down these two circadian genes does have profound effects on the differentiation capacity of adult progenitor cells, which vary depending on the anatomical location of the cell. To this end, when the osteogenic response of both BMSCs and DPSCs was investigated after stretch and static conditions by looking at the expression of *OCN*, substantial differences can be observed between the cell types. For example, *OCN* is upregulated following mechanical stretch in BMSCs in all three conditions (SC, B2 and R1), but the opposite is true for DPSCs, where *OCN* was higher in expression in static conditions for all three treatments. This shows that the same treatment and conditions can lead to very different effects depending on the anatomical location of the progenitor cell. This difference in the cell types' differentiation responses is likely to be due to the cues that they are exposed to in their contrasting niches and their predispositions to differentiate down different lineages For example, *in vivo*, DPSCs are exposed to a vast amount of mechanical stimulation in their niche in the jaws, in constrast to BMSCs which are exposed to very little mechanical stimulation, which offers an explanation for these different responses. This data is supported by various studies that show that although mechanical stretch appears to increase the osteogenic potential of BMSCs[52], the opposite appears to be true for DPSCs, where mechanical stretch has been shown to inhibit osteogenic differentiation[60].

When comparing the different treatment conditions, the expression of *OCN* was decreased when either *Bmal1* or *Rev-ErbA*α was knocked down in both stretch and static conditions when compared to SC controls. This decrease was greatest when the cells were treated with siRNA targeting *Rev-ErbA*α, showing that *Rev-ErbA*α might have a role in mediating the stretch-induced upregulation of *OCN*, and that this upregulation of *OCN* following stretch may be blocked in the absence of *Rev-ErbA*α. This data is supported by data by Samsa et al. (2016) who showed that reductions in

Bmal1 lead to a reduced osteoblastic differentiation capacity[105] but is disputed by research by He et al. (2014), who showed that increased levels of *Rev-ErbA*α lead to an inhibition of osteogenesis in mouse BMSCs[208]. In the DPSCs, however, no significant changes could be observed between the different siRNA treatment groups, further showing that this stretch-induced change in *OCN* in response to stretch observed in BMSCs is absent in DPSCs, validating the theory that the differentiation response to stretch depends on the anatomical source of the progenitor cell and is mediated by circadian clock genes.

BMSCs and DPSCs also differed in the response of *Sox9* expression to stretch. In BMSCs, *Sox9* was increased in expression in stretch conditions when compared to static in all three treatments (SC, B2 and R1), but was higher in expression in static conditions for SC and B2 in DPSCs, with no significant changes after R1 application between stretch and static conditions, highlighting again that the differentiation response following stretch differed between the cell types. *Sox9* in BMSCs showed a similar pattern of gene expression to *OCN* when its expression was compared between the treatment groups; both *OCN* and *Sox9* were decreased in expression when *Bmal1* or *Rev-ErbA*α were knocked down compared to SC in BMSCs, with R1 decreasing even further when compared to B2, in both stretch and static conditions. This pattern was consistent in DPSCs; *Sox9* was also lower in expression in DPSCs when *Bmal1* or *Rev-ErbA*α was knocked down, with R1 resulting in a larger decrease than B2. This suggests that mechanical stretch is here resulting in a stronger progenitor cell differentiation response, with this induction being lessened when the clock genes' expression is disrupted, suggesting that their differentiation potential is being mediated by the circadian rhythm. This data is supported by research by Honda et al. (2013), who showed that *Sox9* and various other matrix related genes do exhibit circadian rhythmicity[160], supporting the idea that the clock genes have a regulatory role on *Sox9* expression and the induction of chondrogenesis, and by research by Dudek et al. (2016), who recently showed that reductions in *Bmal1* lead to reductions in *Sox9* and other matrix-related genes expression[209].

The adipogenic response was also investigated to see if mechanical stretch and/or the clock genes had any impact on the progenitor cells' differentiation to adipogenesis. In

BMSCs, *PPARγ* expression was decreased following stretch in the SC controls, showing that mechanical stretch has an inhibitory effect on adipogenesis, as was expected. However, for R1 and B2, *PPARγ* expression was higher following mechanical stretch, suggesting that this inhibition of adipogenesis in response to stretch may be mediated via the clock genes, and by knocking the clock genes down, this repression of adipogenesis is removed in BMSCs. When the three types of siRNA treatment were compared, it was found that *PPARγ* expression was higher in both stretch and static conditions when *Bmal1* was knocked down, when compared to SC negative controls, showing that blocking *Bmal1* expression leads to an increase in *PPARγ* expression. This suggests that *Bmal1* has a negative role on *PPARγ* expression, further demonstrating that the circadian clock does impact progenitor cells' adipogenic differentiation capacity, as had been previously suggested earlier. This is consistent with data by Guo et al. (2012), who demonstrated that *Bmal1* is a critical negative regulator of adipogenesis and also reported that *Bmal1* disruption leads to increased adipogenesis[93]. In DPSCs, *PPARγ* was found to be increased in static conditions after *Bmal1* knockdown, with no significant changes being observed between stretch and static conditions for SC and R1. Furthermore, no significant changes could be observed between the three treatment groups in either stretch or static conditions, in DPSCs, showing again that mechanical stretch impacts different molecular pathways between the two cell types, which may be due to the varying levels of mechanical stimulation that the cells are exposed to *in vivo* within their niches. For example, in the body, DPSCs experience significant mechanical stimulation in the form of occlusion forces, jaw movement, and hydrostatic pressures and are one of the few progenitor cell niches to experience thermal shock and extreme temperature fluctuations, and therefore are more likely to respond to this form of stimulation. Furthermore, DPSCs and BMSCs differ in their level of maturity; for example, DPSCs originate from an earlier developmental time point than BMSCs, and therefore may have to respond to the same signal in a different way, leading to the establishment of different signalling pathway responses, as seen here.

5.3.1 Summary

It is not a surprise that disruptions to the circadian rhythm lead to a plethora of pathologies, when the clock genes have such a strong effect on the gene expression of various other genes and processes, as has been detailed in this chapter. Here, it has been experimentally evidenced that the induction of progenitor cell differentiation markers are indeed influenced by the circadian rhythm and core clock genes.

By identifying these molecular mechanisms, therapies can be designed to reverse these negative changes and prevent phenomena such as pre-mature ageing, which can be seen when the circadian rhythm is disrupted and therefore the natural differentiation processes are negatively impacted. As there is a dampening of the circadian clock seen with age, alongside many other negative changes such as increased adipogenesis and decreased osteogenesis experienced with age, perhaps this can be countered by restoring the circadian rhythm back to its proper function. Furthermore, if the circadian rhythm can be altered in cells being utilised for tissue engineering, this has extreme implications for the field of regenerative medicine, whereby cells may be poised and readied for the desired differentiation pathway using the circadian rhythm, to improve things such as implant acceptance and integration. This exciting field promises many fascinating breakthroughs to come, to improve both tissue viability and cellular engineering.

Chapter 6

“The Role of EPL Bioactive Peptides on Adult Progenitor Cell Differentiation, Circadian Rhythm and Response to Mechanical Stretch”

6 "The Role of EPL Bioactive Peptides on Adult Progenitor Cell Differentiation, Circadian Rhythm and Response to Mechanical Stretch"

6.1 Introduction

6.1.1 Natural and Synthetic Materials

As previously mentioned, the field of biomaterial innovation has recently exploded in response to the increased requirement for tissue engineering and regenerative medicine techniques in an ageing population. Materials are being utilised for a plethora of applications, with the biggest commercial successes being the hip joint replacements and vascular grafts.

There are two main classes of organic material used in modern tissue engineering, the first of which involves naturally occurring bioactive molecules, for example, using native long chain ECM proteins such as fibronectin and laminin, as was previously covered in "The Role of Cell-Substrate Interaction in Adult Progenitor Cell Differentiation and Circadian Rhythm, in a Mechanical Stretch Paradigm". The other source of material frequently utilised is derived from biologically active synthetic materials, for example, short peptide sequences, which will be covered in this chapter. Both of these classes of material attempt to recapitulate the cell's immediate environment and the biomechanical signals that the cells receive. As biomimetic materials aim to direct new tissue formation and elicit specific cellular responses, this can be mediated by biomolecular recognition, which can be manipulated by altering the material's design parameters[216]. In this way, materials can therefore then provide the relevant biological cues for cell-matrix interactions, render the surface of a biomaterial as cell adhesive[217], and can also incorporate specific peptide sequences into the materials, that can make the material degradable by the cells' specific protease enzymes[218].

In vivo, the ECM proteins themselves not only have a direct role on cell behaviour and function, but they also serve as a method by which other signalling molecules may

interact with the surrounding cells[219]. For example, the ECM sequesters and presents a variety of molecules including growth factors, which bind to the ECM components such as fibronectin and heparan sulfate proteoglycans[220], until they are released from this tether either enzymatically or through dissociation. If a biomaterial is aiming to mimic the key features of the ECM, this should therefore include the ability of a material to present and sequester adhesion molecules, and the display and release of growth factors. Therefore, the material should be able to form *in situ* without damaging the residing cells or altering the biomolecules present. Furthermore, cells should be able to remodel and eventually replace the matrices, which normally occurs enzymatically, for example via matrix metalloproteinases (MMPs), as the cells do with the natural ECM[221]. In this way, any material designed, whether biologically derived and biologically produced materials, e.g. collagen or fibrin, or synthetic materials, should aim to mimic biological functionality.

When selecting the type of material to design a construct with, several factors must be taken into account. For example, when modifying the surface of a material, short peptides tend to be advantageous over long chains of native ECM proteins, as the ECM proteins are more likely to fold randomly upon adsorption to the biomaterial surface, in such a way that the receptor binding sites are not always sterically available. Short peptide sequences, however, are relatively stable throughout the modification process, with nearly all modified peptides being available for cell binding. In addition, short peptide sequences can be synthesised on large scales in laboratories, making their production more economic[216].

6.1.2 Protein Engineering and Expressed Protein Ligation (EPL)

Developing the relevant specific synthetic peptides to fulfil the criteria of certain biomaterials requires direct chemical manipulation of protein molecules and precise alteration of the covalent structure of a protein using synthetic chemistry. Peptide ligation involves the incorporation of unnatural amino acids into proteins, through both chemical and biosynthetic strategies, and allows fully unprotected polypeptide building blocks to be selectively joined together, to create the desired target protein molecule. Ligation can be carried out either chemically, involving the incorporation of

unique functionalities at the amino or carboxyl termini of the peptide segments[222], or enzymatically, through reverse proteolysis techniques[223,224]. This process allows for a selected region of a long protein sequence to be chemically manipulated, in a method analogous to synthetic peptides, and enables proteins to be site-specifically modified.

There are several forms of protein ligation that are most commonly used in material design today: Native Peptide Ligation (NPL), Protein *Trans*-Splicing (PTS) and Expressed Peptide Ligation (EPL). NPL is used to ligate two synthetic peptide fragments together in a series of controlled steps[225]. First, a transthioesterification reaction is carried out; this is a chemoselective reaction between a peptide fragment containing an amino-terminal cysteine residue and a peptide fragment containing a αthioester group. Next, a rapid intramolecular S to N acyl shift is utilised to generate an amide bond[225–227]. Reactive functionalities can be incorporated into the peptide fragments during Solid Phase Peptise Synthesis (SPPS), which can be used to make small proteins and protein domains, up to lengths of 100 amino acids – chains can then be linked together and ligated to form the short synthetic peptides.

Expressed protein ligation, the technique of utmost relevance to this chapter, is an extension of the NCL method[225] and allows for a target protein to be constructed from a series of recombinant and synthetic building blocks[228,229]. This approach of peptide ligation is a protein semi-synthesis technique which allows the assembly of a desired protein from smaller unprotected pieces, such as recombinant proteins and synthetic peptides, under native chemical ligation conditions[230]. Therefore, this approach combines both the extended size range of recombinant polypeptides with the structural flexibility of synthetic peptides, thereby allowing for "chemical mutagenesis" on extremely large protein systems[231]. EPL is based on N-terminal leader sequence removal from precursor fusion proteins and differs from NPL in that NPL involves the ligation of two synthetic parts, whereas EPL required one or more of the peptides to be recombinant.

EPL was developed from PTS, which is another intein-based approach. To this end, protein splicing is a post-translational process that involves a precursor protein, which undergoes a series of intramolecular reorganisation, and results in the specific removal of an internal region, i.e. the intein, and the ligation of the two remaining

flanking sequences, i.e. the exteins[232]. The first step in the protein splicing process entails an N to S (or N to O) acyl shift, whereby the N-extein unit is translocated to the sidechain SH or OH group of a conserved Cys/Ser/Thr residue, which is located at the intein amino terminus[233,234]. Next, a transesterification step ensures a complete N-extein unit is transferred +1 position to a second conserved Cys/Ser/Thr residue at the intein-C-extein boundary. The next step involves a cyclization step, in which a conserved asparagine residue located at the carboxyl terminus of the intein is used to resolve the branched intermediate. In this reaction, the intein is excised as a carboxy-terminal succinimide derivative. Finally, an amide bond is then formed between the two exteins, following a second S to N (or O to N) acyl shift[233,235,236].

To generate recombinant protein αthioester derivatives, the proteins expressed as in-frame amino-terminal fusions to one of the engineered inteins can be cleaved in an intermolecular transthioesterification reaction via thiols[237]. These stages that make up EPL have been summarised below in Figure 6.1. The peptide sequences that then contain an amino-terminal cysteine residue can hence be ligated to the carboxyl termini of recombinant αthioester proteins via an EPL reaction[228,229,238]. EPL differs from PTS in that EPL is a chemical process, and therefore can be performed under a wide selection of reaction conditions, whereas PTS must be performed under specific conditions that are compatible with protein folding, as part of PTS involves the reconstitution of a split intein-synthetic fragment, which must include part of the intein[236]. EPL therefore offers a powerful approach to the chemical modification of proteins and has already been utilised in a wide range of protein systems, as it allows for a variety of non-coded groups to be precisely and site-specifically introduced.

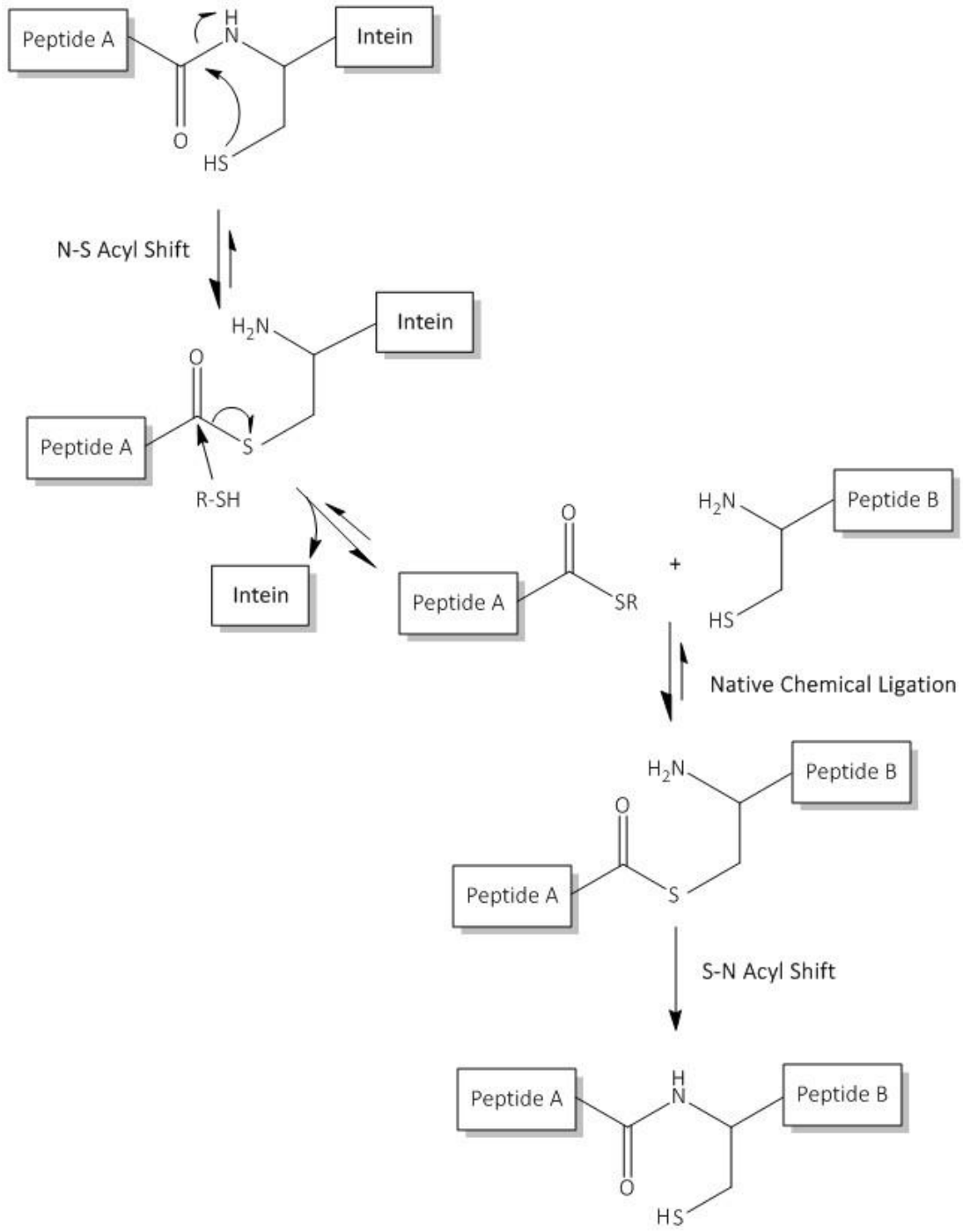

Figure 6.1: Expressed Protein Ligation (EPL). *EPL involves the semisynthetic chemical ligation of synthetic and recmbinant polypeptides; Peptide A consists of an intein fusion, which can be cleaved from the peptide with a variety of thiols, in order to produce the corresponding α-thioester derivative. Peptide B, however, contains a N-Cys that can be produced recombinantly, and both protein building blocks, Peptides A and B, can be alternatively synthesised by SPPS containing an appropriate linker*[239].

6.1.3 Aims and Hypothesis

Modular synthetic peptides, as described above, have been demonstrated to have an influential role on the biological activity of adult progenitor cells. For example, when soluble modular peptides synthesised using SPPS are added to hMSC growth medium containing osteogenic supplements, a positive influence on the osteogenic differentiation of hMSCs can be observed, when compared to cells grown in osteogenic media alone in the absence of the soluble modular peptides[240].

As the effect of naturally occurring long chain ECM proteins was previously investigated in "The Role of Cell-Substrate Interaction in Adult Progenitor Cell Differentiation and Circadian Rhythm, in a Mechanical Stretch Paradigm", where these proteins were experimentally evidenced to have a significant effect on adult progenitor cell differentiation, circadian rhythm and response to mechanical stretch, it was next hypothesised that synthetic peptides might also have a significant influence on the different types of progenitor cell. To this end, it was theorized that supplementing the normal growth media of hBMSCs and hDPSCs would lead to significant effects in the cellular behaviours and cellular responses previously investigated, which may differ between the two cell types depending on their native anatomical location. Therefore, three types of biologically active EPL peptides of differing chain lengths were utilised, to see how this impacted the two types of human adult progenitor cell.

In this chapter, we therefore aimed to see how three EPL peptides of varying chain lengths, EPL 001, EPL 140 and EPL 142, affected progenitor cell adhesion, viability and growth. Initially, the two different cell types, BMSCs and DPSCs, were cultured at varying cell densities and exposed to each of the three peptides at varying concentrations, to see which combination of parameters had positive effects on cell number, as analysed using CyQUANT and image analysis. Once identified, we next aimed to see how the identified peptide type and concentration, that had brought significant effects about when cultured at basal level, would affect the adult progenitor cells' response to mechanical stretch, in terms of the differentiation markers induced and circadian genes influenced. The effect of these synthetic

bioactive peptides and their effect on progenitor cells' response to stretch will then allow for the previously investigated mechanical stretch set up to be further optimised, allowing for advances to be made in the investigation of progenitor cell pre-treatment before being used to optimise biomaterials, as the effect of EPL peptides on either the response mechanical stretch or the circadian rhythm has not been investigated to date. Once the optimum parameters to poise a progenitor cell to commit to the desired differentiation lineage have been elucidated, biomaterials can be further improved to ensure the maximum chances of acceptation and integration when implanted within the body, allowing for significant advances in both tissue engineering and regenerative medicine.

6.2 Results

6.2.1 Quantification of the Effect of Bioactive EPL Peptides on Cell Number and Proliferation

6.2.1.1 CyQUANT Analysis

Primary adult progenitor cells derived from human bone marrow and tooth dental pulp were seeded at four different seeding densities (2500, 5000, 10,000 and 20,000 cells/2cm^2 well) in standard tissue culture plastic plates and allowed 12 h to fully attach, before bioactive peptides were supplemented into the growth media solution. Three different peptides, EPL 001, EPL 140 and EPL 142, were utilised at four different concentrations: 0.15 μM, 1.5 μM, 15 μM and 150 μM, along with control samples which received no peptide supplementation (0) at four the four different densities. The cells were cultured in these conditions for 7 days, at which time the samples underwent CyQUANT analysis according to the manufacturer's instructions (Thermo Fisher Scientific) to determine the differences in cell numbers after 7 days in these different treatment conditions. The fluorescence reading was converted into cell numbers using previously determined standard curves (Figure 4.1) for each cell type.

The differences in BMSC number following treatment with bioactive peptides for 7 days can be seen in Figure 6.2. Surprisingly, no significant differences in cell number

could be observed between any of the treatment conditions when compared to the untreated control for the samples seeded at 5000, 10,000 and 20,000 cells/2cm^2 well, and for 2500 cells/2cm^2, only the addition of EPL 142 at 150 μM (142 150) led to significant increases in cell number when compared to the untreated control (0) (one-way ANOVA with Tukey post-hoc, n = 3, $p \leq 0.05$). In contrast, several significant differences could be observed for DPSCs (Figure 6.3) at 20,000 cells/2cm^2 well. Here, EPL 140 at 1.5 (140 1.5) and 150 μM (140 150) and EPL 142 at 0.15 (142 0.15), 1.5 (142 1.5), 15 (142 15) and 150 (142 150) μM all lead to significant increases in cell number when compared to the untreated controls. However, no significant differences could be observed between the different peptide treatment conditions at 2500, 5000 or 10,000 cells/2cm^2 well (one-way ANOVA with Tukey post-hoc, n = 3, $p \leq 0.05$).

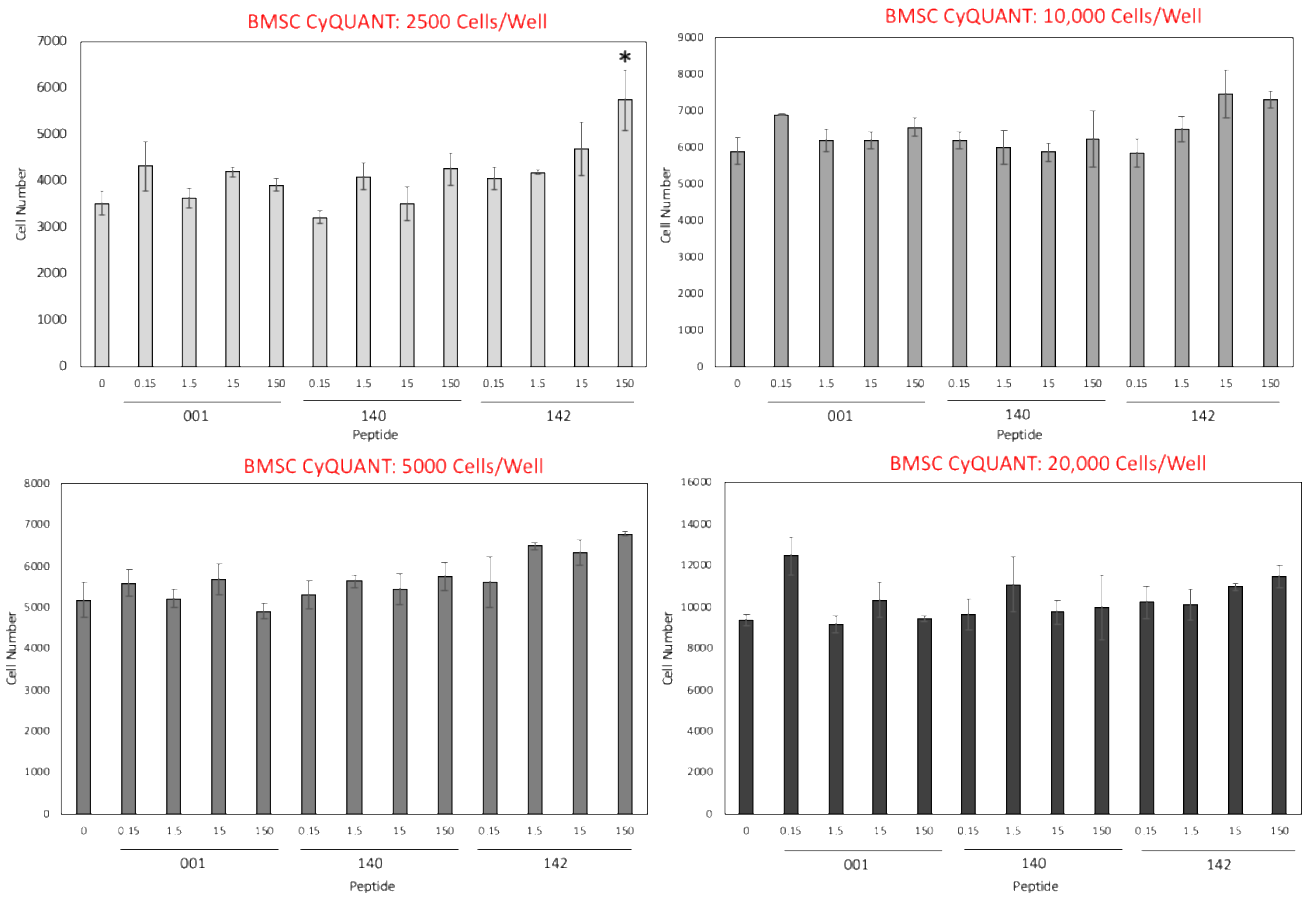

Figure 6.2: Different types and concentrations of EPL peptides lead to differences in BMSC cell number. *BMSCs cultured with different EPL peptide types (EPL 001, EPL 140 and EPL 142) and concentrations (0.15, 1.5, 15 and 150 µM) give rise to differences in cell number when anaylsed using CyQUANT analysis after 7 days. BMSCs were initially seeded at 2500, 5000, 10,000 and 20,000 cells/2cm^2 well. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples, *:p<0.05(two-way ANOVA with Tukey's HSD post hoc tests; groups compared to untreated control (0)).*

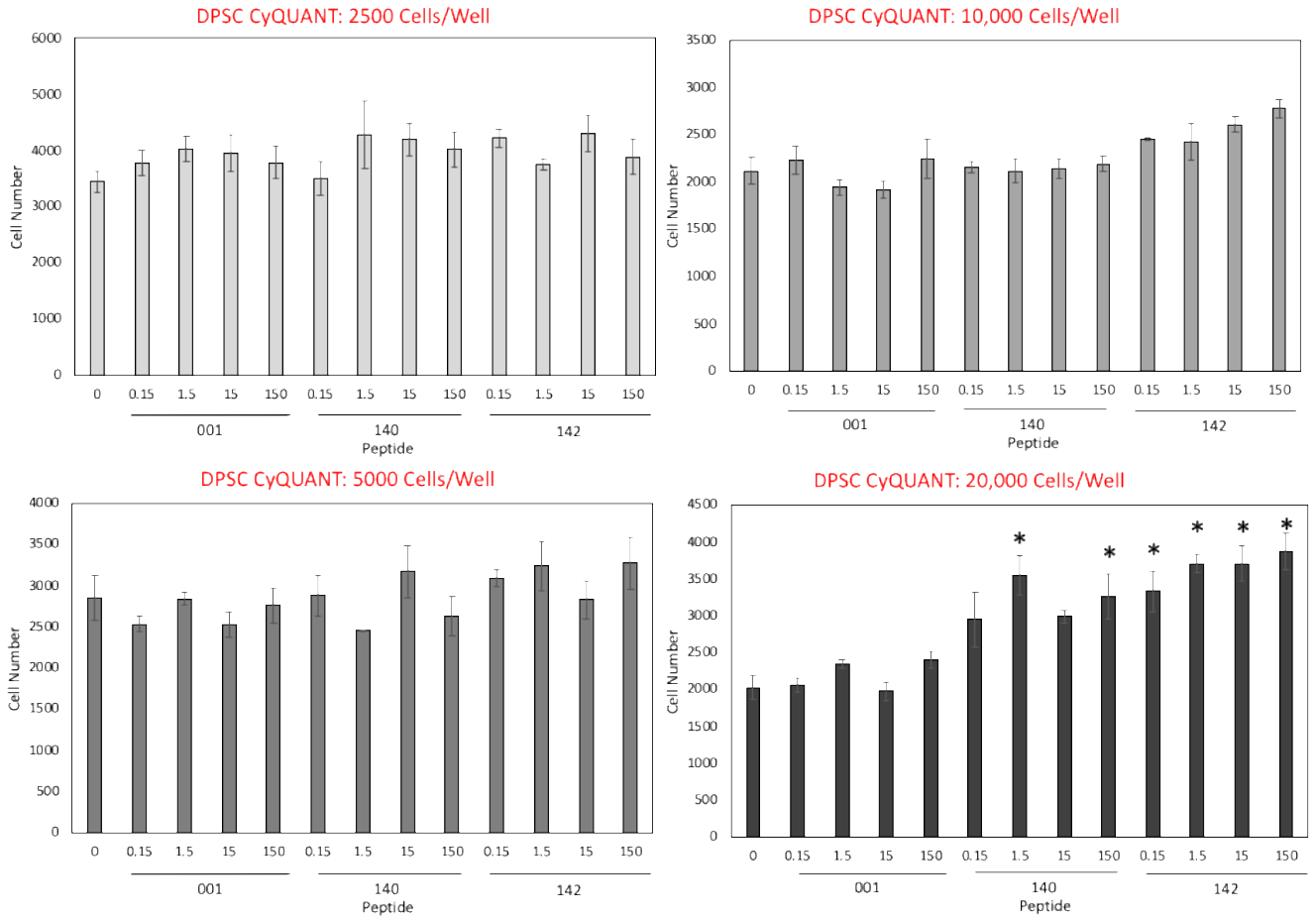

Figure 6.3: Different types and concentrations of EPL peptides lead to differences in DPSC cell number. *DPSCs cultured with different EPL peptide types (EPL 001, EPL 140 and EPL 142) and concentrations (0.15, 1.5, 15 and 150 μM) give rise to differences in cell number when anaylsed using CyQUANT analysis after 7 days. DPSCs were initially seeded at 2500, 5000, 10,000 and 20,000 cells/2cm^2 well. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples, *:p<0.05(two-way ANOVA with Tukey's HSD post hoc tests; groups compared to untreated control (0)).*

6.2.1.2 Image Analysis

It was hypothesised that the lack of significant differences from the above experiments may have been due to the effects of the peptides being short-term and fast acting, and therefore any differences may have been missed by day 7, or due to the CyQUANT analysis not being sufficiently sensitive to detect these changes. Therefore, the experiment was repeated by calculating the cell number/image frame using image analysis to determine changes in cell number/density (20 image frames/condition) at three different time points: day 1 (D1), day 2 (D2) and day 7 (D7).

6.2.1.2.1 BMSCs

For the BMSCs, a number of both positive and negative changes could be observed following the addition of the three different EPL peptides. For example, at Day 1 (D1) (Figure 6.4), the samples that were seeded at 2500 cells/2cm^2 well experienced many significant increases in cell number, including significant increases for all concentrations of EPL 001 (0.15 μM, 1.5 μM, 15 μM and 150 μM), 0.15 μM, 1.5 μM and 15 μM of EPL 140 and 0.15 μM, 15 μM and 150 μM of EPL 142. Similarly, when the seeding density was high, for example where 10,000 and 20,000 cells/2cm^2 well cells were seeded, all of the significant changes that were observed following peptide addition were positive changes. In this way, EPL 001 at 15 μM and all four concentrations of both EPL 140 and EPL 142 led to significant increases in cell number at 10,000 cells cells/2cm^2 well when compared to the untreated controls. For 20,000 cells/2cm^2 well, all four concentrations of both EPL 001 and EPL 140 and both 0.15 and 1.5 μM of EPL 142 led to significant increases in cell number when compared to the untreated controls. However, for the samples that were seeded at 5000 cells/2cm^2 well, significant decreases in cell number could be observed for EPL 001 at 0.15 and 15 μM, and EPL 142 at 15 and 150 μM, with only significant increases in cell number being observed for EPL 140 at 15 μM (one-way ANOVA with Tukey post-hoc, n = 3, 20 frames/condition, $p \leq 0.05$). This shows that the initial response observed in BMSCs following the addition of bioactive peptides is heavily dependent on cell density, peptide type and peptide concentration.

By Day 2 (D1), these initial changes in cell number could be observed even more clearly (Figure 6.5). By this time, the bioactive peptides were having a substantial positive effect on cell number, with all three peptides at each of the four concentrations leading to significantly increased cell numbers when compared to the untreated controls for samples seeded at 2500 cells/2cm^2 well. For 5000 cells/2cm^2 well, decreases at this time point could only be observed for EPL 001 at 0.15 μM and EPL 142 at 150 μM and the increase observed at D1 for EPL 140 at 15 μM was maintained at D2. Where 10,000 cells/2cm^2 well were initially seeded, EPL 001 lead to increases when applied at concentrations of 15 and 150 μM, EPL 140 at 0.15, 1.5 and 15 μM and all four concentrations for EPL 142. Regarding the highest density of cells, i.e. cells cultured following an initial seeding density of 20,000 cells/2cm^2 well, increases were observed for EPL 001 at 1.5 and 150 μM and EPL 140 at 0.15, 1.5 and 15 μM, and decreases observed for EPL 142 at 15 μM (one-way ANOVA with Tukey post-hoc, n = 3, 20 frames/condition, $p \leq 0.05$), further demonstrating how much an impact cell seeding density, peptide type and peptide concentration has on BMSC cell number and proliferation.

Finally, the samples were analysed for changes in cell number by Day 7 (D7) (Figure 6.6). For the samples seeded with an initial density of 2500 cells/2cm^2 well, increases in cell number could be observed for peptides EPL 001 at 1.5 and 15 μM, EPL 140 at all concentrations and EPL 142 at 0.15, 1.5 and 15 μM. Similarly, EPL 001 at 1.5, 15 and 150 μM and EPL 140 at all concentrations led to significant increases in cell number for 5000 cells/2cm^2 well, but, in contrast, no increases could be observed for EPL 142, with EPL 142 at 150 μM leading to significant decreases in cell number. The effects of the three peptides on the BMSCs seeded at 10,000 cells/2cm^2 well were substantially different to the previous two seeded at lower densities. Here, EPL 001 at 1.5 μM and EPL 140 at 15 μM led to significant decreases in cell number, with all four concentrations of EPL 142 bringing about significant increases in cell number. For 20,000 cells/2cm^2 well, a more similar trend to the first two seeding densities could be observed, with significant increases in cell number being observed after the application of all four concentrations of EPL 001 and EPL 140, and significant decreases following EPL 142 at 15 and 150 μM (one-way ANOVA with Tukey post-hoc, n = 3, 20

frames/condition, $p \leq 0.05$). The effects on cell number observed following the application of the different types and concentrations of peptide across the three time points together can be seen in Figure 6.7.

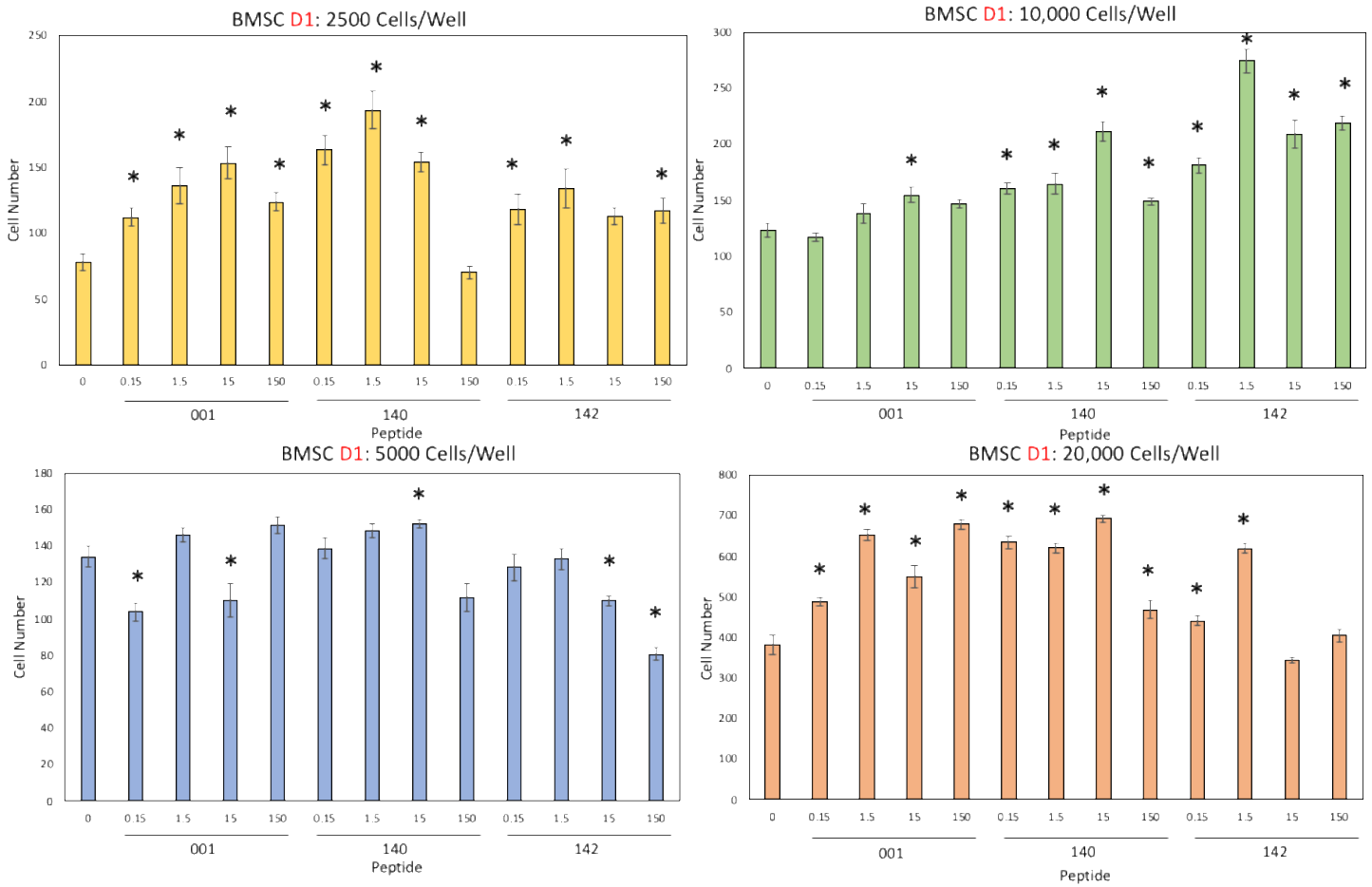

Figure 6.4: Different Types and Concentrations of EPL Peptides Lead to Differences in BMSC Cell Number After 24 H (D1). *BMSCs cultured with different EPL peptide types (EPL 001, EPL 140 and EPL 142) and concentrations (0.15, 1.5, 15 and 150 µM) give rise to differences in cell number per image frame when anaylsed using image analysis after 1 day. BMSCs were initially seeded at 2500, 5000, 10,000 and 20,000 cells/2cm2 well. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples, *:p<0.05(two-way ANOVA with Tukey's HSD post hoc tests; groups compared to untreated control (0).*

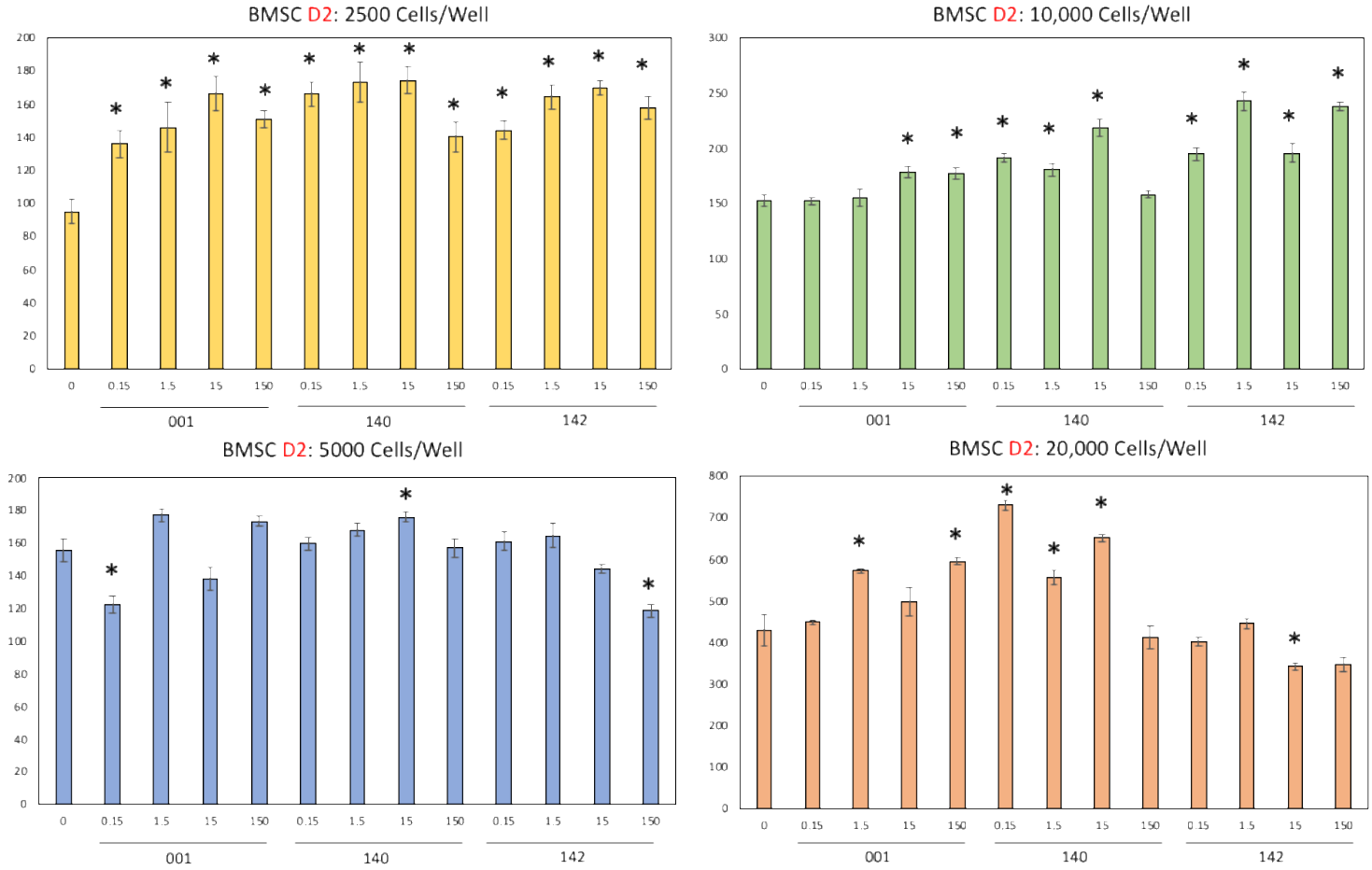

Figure 6.5: Different Types and Concentrations of EPL Peptides Lead to Differences in BMSC Cell Number After 2 Days (D2). *BMSCs cultured with different EPL peptide types (EPL 001, EPL 140 and EPL 142) and concentrations (0.15, 1.5, 15 and 150 μM) give rise to differences in cell number per image frame when anaylsed using image analysis after 2 days. BMSCs were initially seeded at 2500, 5000, 10,000 and 20,000 cells/2cm2 well. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples, *:p<0.05(two-way ANOVA with Tukey's HSD post hoc tests; groups compared to untreated control (0)).*

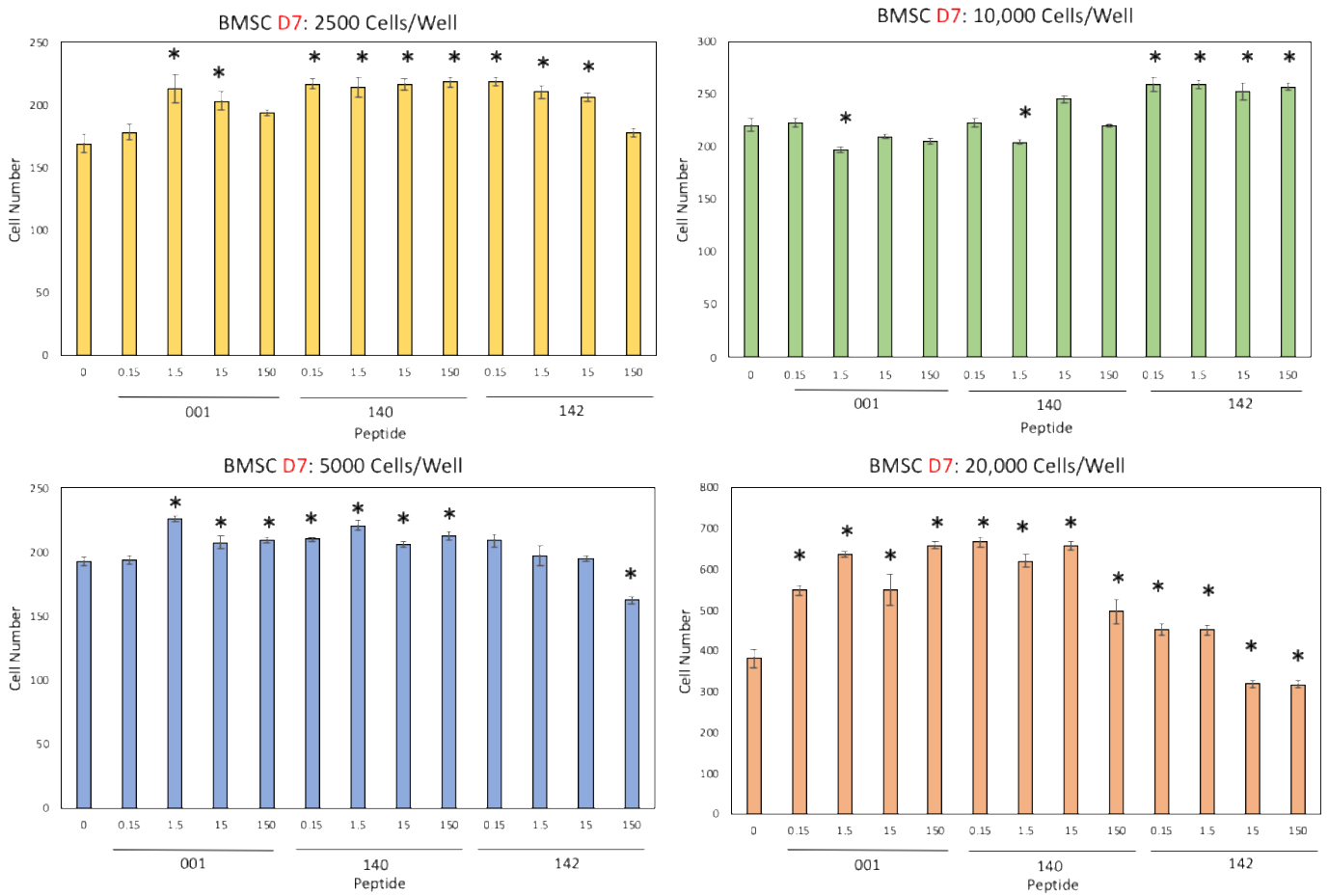

Figure 6.6: Different Types and Concentrations of EPL Peptides Lead to Differences in BMSC Cell Number After 7 Days (D7). *BMSCs cultured with different EPL peptide types (EPL 001, EPL 140 and EPL 142) and concentrations (0.15, 1.5, 15 and 150 μM) give rise to differences in cell number per image frame when anaylsed using image analysis after 7 days. BMSCs were initially seeded at 2500, 5000, 10,000 and 20,000 cells/2cm^2 well. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples, *:p<0.05(two-way ANOVA with Tukey's HSD post hoc tests; groups compared to untreated control (0)).*

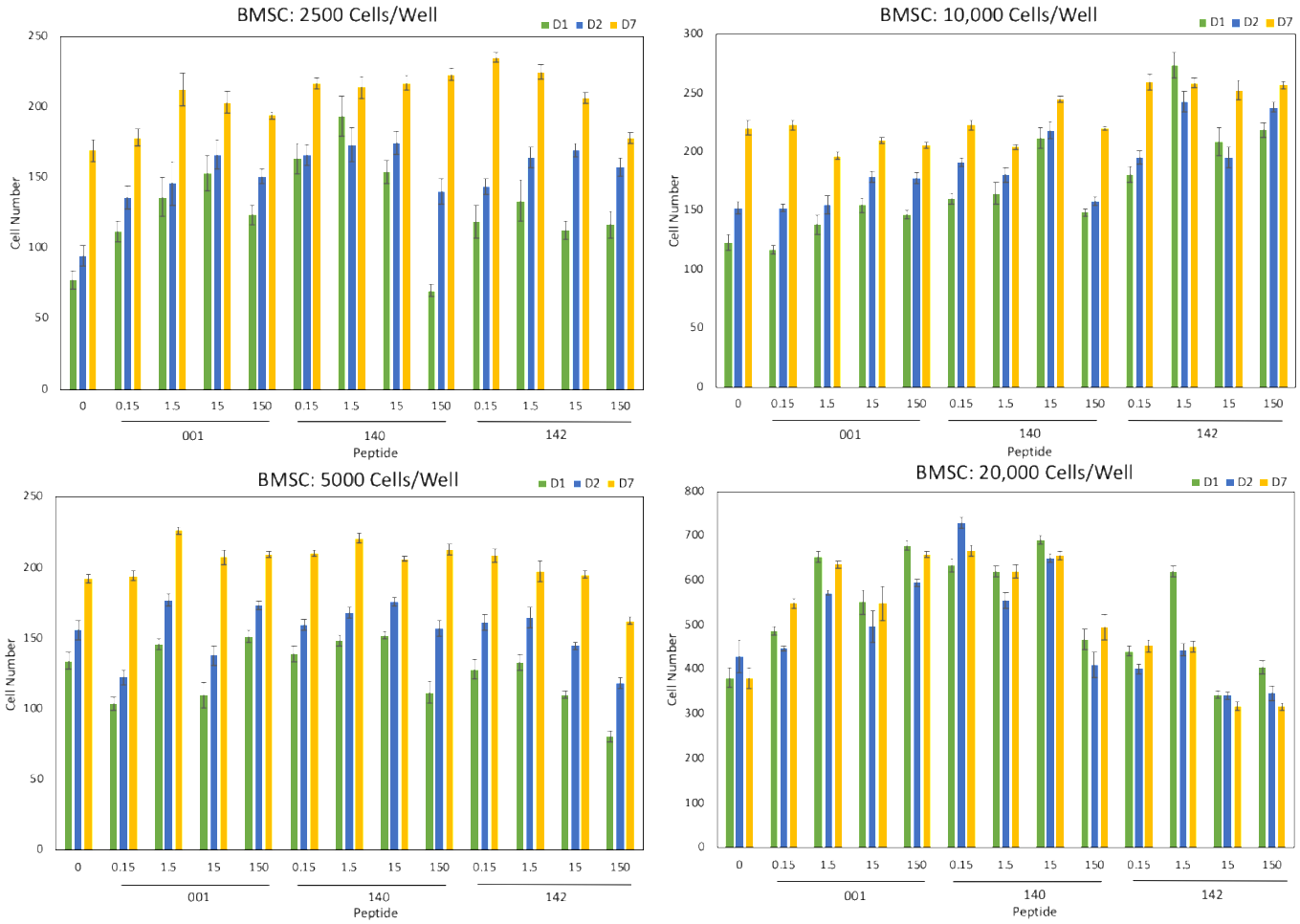

Figure 6.7: Different Types and Concentrations of EPL Peptides Lead to Differences in BMSC Cell Number Over a 7 Day Long Time Course. *BMSCs cultured with different EPL peptide types (EPL 001, EPL 140 and EPL 142) and concentrations (0.15, 1.5, 15 and 150 µM) give rise to differences in cell number per image frame when anaylsed using image analysis after 24h (D1), 2 days (D2) and 7 days (D7).. BMSCs were initially seeded at 2500, 5000, 10,000 and 20,000 cells/2cm^2 well. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples.*

6.2.1.2.2 DPSCs

The response of the DPSCs to the different types and concentrations of bioactive EPL peptides at different cell number densities was also investigated. Again, a number of both positive and negative effects on cell number could be observed, with the peptides tending to have a more negative effect on DPSC cell number than was observed in BMSCs.

After being cultured in the different peptide treatments for 1 day (D1) (Figure 6.8), the samples originally seeded at 2500 cells/2cm^2 well demonstrated significant increases in cell number only when exposed to EPL 001 at 1.5 μM, but significant decreases following the addition of EPL 001 at 150 μM, EPL 140 at 15 and 150 μM and EPL 142 at 1.5, 15 and 150 μM, when compared to the untreated controls, showing that the positive effects following peptide addition were only elicited following very specific peptide types and concentrations. For samples seeded at 5000 cells/2cm^2 well, only negative changes in cell number could be observed. For example, when any of the four concentrations of EPL 001, EPL 140 at 15 μM or EPL 142 at 1.5, 15 and 150 μM, these resulted in negative changes in cell number when compared to the untreated controls. Regarding samples seeded at 10,000 cells/2cm^2 well, EPL 001 at 1.5 μM and EPL 140 at 1.5, 15 and 150 μM led to significant increases in cell number when compared to untreated controls, in contrast to all four concentrations of EPL 142, which all led to significant decreases. Furthermore, only decreases in cell number could be observed following peptide supplementation to the samples containing the highest seeding densities of 20,000 cells/2cm^2 well; here, EPL 140 at 1.5, 15 and 150 μM and all four concentrations of EPL 142 all led to significant decreases in cell number when compared to untreated controls (one-way ANOVA with Tukey post-hoc, n = 3, 20 frames/condition, $p \leq 0.05$). These findings underline a predominantly negative initial response in DPSCs to bioactive EPL peptide supplementation, except at in very specific conditions, depending on peptide type, peptide concentration and initial cell seeding density.

By Day 2 (D2) (Figure 6.9), overall less significant changes could be observed when compared to the untreated controls. For the samples seeded at 2500 cells/2cm^2 well,

only negative changes could be observed for this later time point. For example, EPL 001 at 0.15 and 150 μM, and all four concentrations of both EPL 140 and EPL 142 led to significant decreases in cell number when compared to untreated controls. In contrast, where the samples were initially seeded with 5000 cells/2cm^2 well, increases in cell number could be demonstrated following the addition of EPL 140 at 1.5 and 150 μM, and EPL 142 at 0.15 μM. Interestingly, for this condition, no negative responses were elicited following peptide supplementation, in contrast to all three other cell seeding density conditions, which only experienced significant decreases in cell number with no increases. In line with this, when the samples were initially seeded with 10,000 cells/2cm^2 well, significant decreases could be observed following the addition of EPL 001 at 0.15, 15 and 150 μM or EPL 142 at 1.5 and 150 μM, and when seeded initially with 20,000 cells/2cm^2 well, significant decreases could be seen following the addition of EPL 142 at all concentrations (one-way ANOVA with Tukey post-hoc, n = 3, 20 frames/condition, $p \leq 0.05$). This highlights that the positive bioactive response following peptide supplementation may only be experienced at the ideal cell densities and times following peptide addition.

By Day 7 (D7) (Figure 6.10), further differences could be observed between the treatment groups. For 2500 cells/2cm^2 well, significant decreases in cell number were observed for EPL 001 at 0.15 and 150 μM, EPL 140 at 0.15, 1.5 and 15 μM and EPL 142 at 1.5, 15 and 150 μM, with no significant increases being demonstrated. This decrease in cell number following the addition of EPL 142 at 1.5, 15 and 150 μM was also observed for 5000 cells/2cm^2 well, where the previous increases in cell number observed at D1 and D2 could no longer be seen by D7. For 10,000 cells/2cm^2 well, further significant decreases could be evidenced following the addition of EPL 001 at 0.15 μM, EPL 140 at 0.15, 1.5 and 15 μM and all four concentrations of EPL 142 when compared to the untreated controls. In contrast to these three lower cell density's responses to the peptides, a significant increase in cell number could be observed for one condition when seeded at 20,000 cells/2cm^2 well and exposed to EPL 142 at 0.15 μM. However, a significant decrease could also be observed following the addition of EPL 140 at 0.15 μM, with no other significant changes being observed between the

peptide treatment conditions (one-way ANOVA with Tukey post-hoc, n = 3, 20 frames/condition, $p \leq 0.05$).

This data demonstrates the fast-acting nature of these EPL peptides, which appeared to lead to more significant differences in cell number at D1 and D2 following peptides addition than at D7, when there were less differences in cell number when compared to the untreated controls. Interestingly, where the differences in cell number appeared mostly positive following peptide supplementation in the BMSCs, the opposite was true for DPSCs, which exhibited mostly negative differences in cell number following peptide supplementation. Again, the effects on cell number observed in DPSCs following the application of the different types and concentrations of peptide across the three time points together can be seen in Figure 6.11.

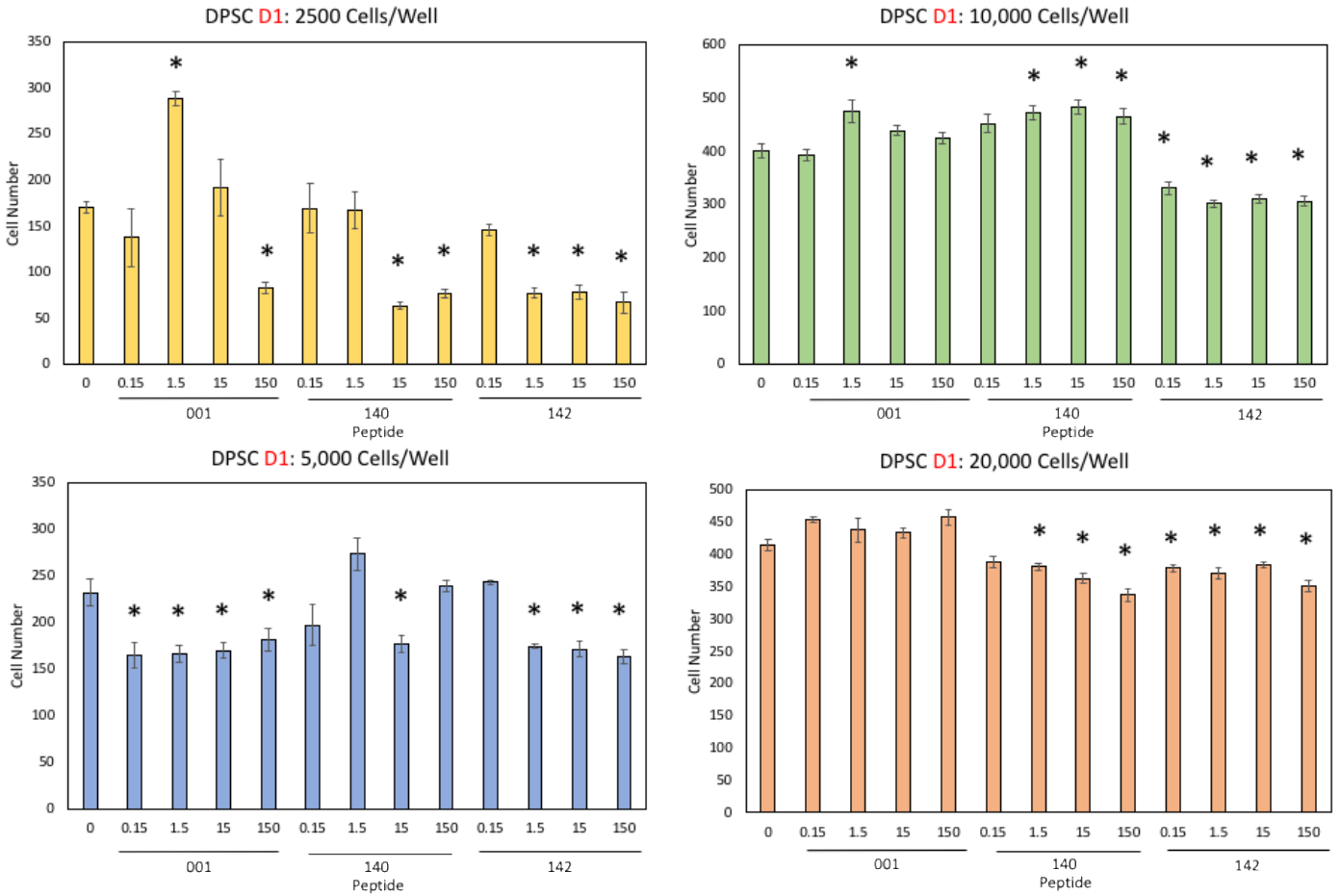

Figure 6.8: Different Types and Concentrations of EPL Peptides Lead to Differences in DPSC Cell Number After 24 H (D1). *DPSCs cultured with different EPL peptide types (EPL 001, EPL 140 and EPL 142) and concentrations (0.15, 1.5, 15 and 150 μM) give rise to differences in cell number per image frame when anaylsed using image analysis after 1 day. DPSCs were initially seeded at 2500, 5000, 10,000 and 20,000 cells/2cm2 well. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples, *:p<0.05(two-way ANOVA with Tukey's HSD post hoc tests; groups compared to untreated control (0)).*

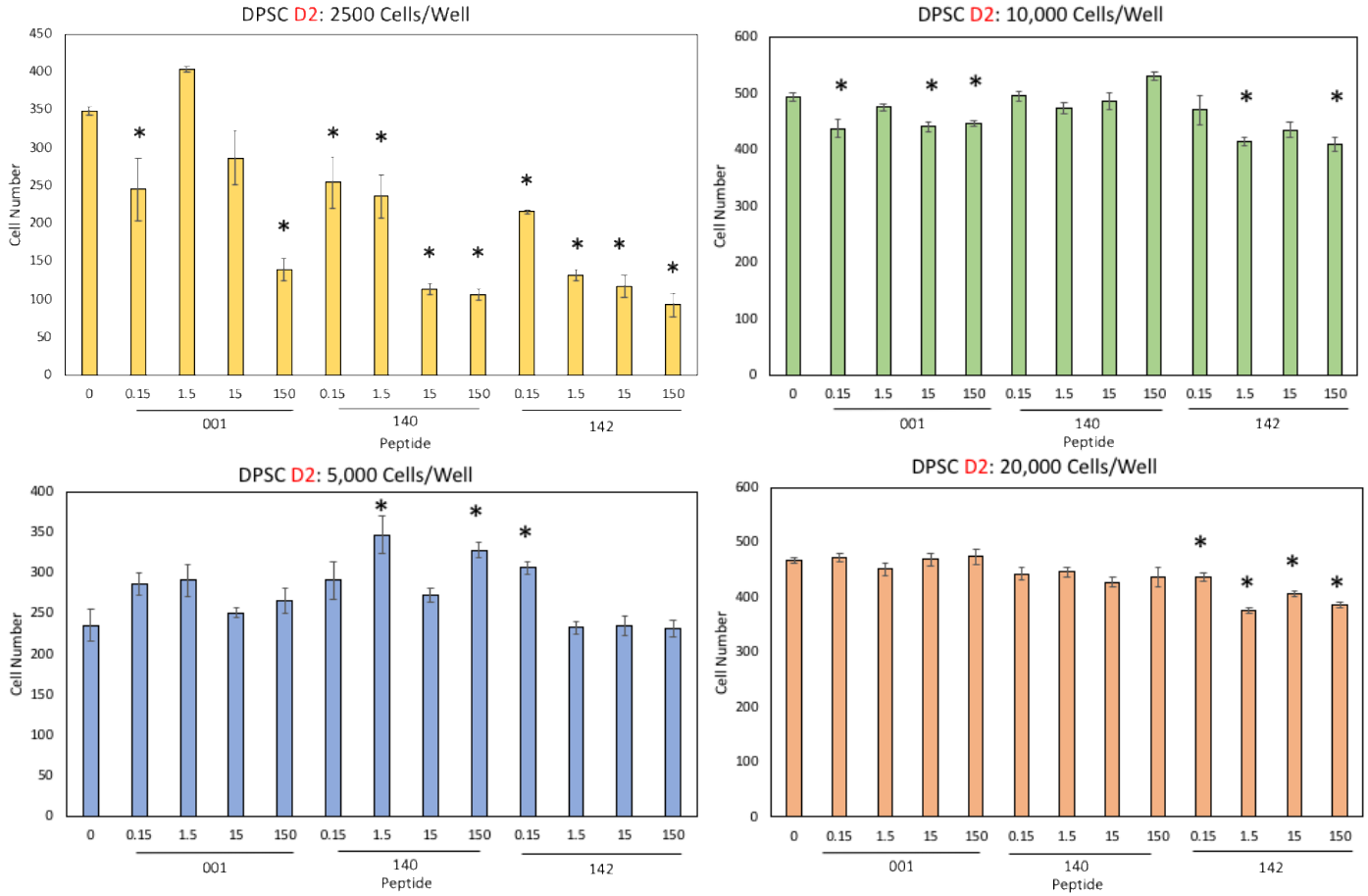

Figure 6.9: Different Types and Concentrations of EPL Peptides Lead to Differences in DPSC Cell Number After 2 Days (D2). *DPSCs cultured with different EPL peptide types (EPL 001, EPL 140 and EPL 142) and concentrations (0.15, 1.5, 15 and 150 μM) give rise to differences in cell number per image frame when anaylsed using image analysis after 2 days. DPSCs were initially seeded at 2500, 5000, 10,000 and 20,000 cells/2cm2 well. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples, *:p<0.05(two-way ANOVA with Tukey's HSD post hoc tests; groups compared to untreated control (0)).*

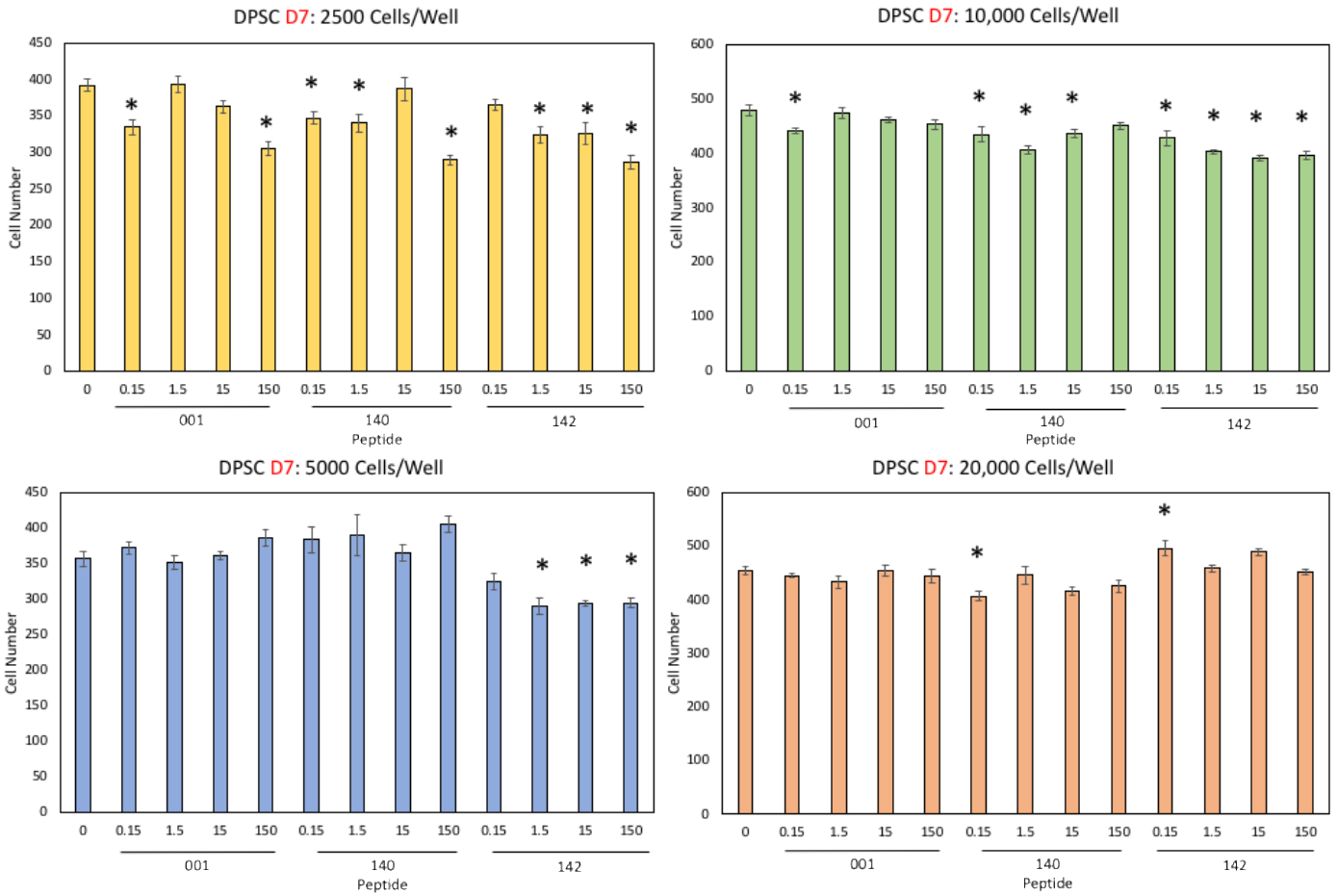

Figure 6.10: Different Types and Concentrations of EPL Peptides Lead to Differences in DPSC Cell Number After 7 Days (D7). *DPSCs cultured with different EPL peptide types (EPL 001, EPL 140 and EPL 142) and concentrations (0.15, 1.5, 15 and 150 µM) give rise to differences in cell number per image frame when anaylsed using image analysis after 7 days. DPSCs were initially seeded at 2500, 5000, 10,000 and 20,000 cells/2cm2 well. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples, *:p<0.05(two-way ANOVA with Tukey's HSD post hoc tests; groups compared to untreated control (0)).*

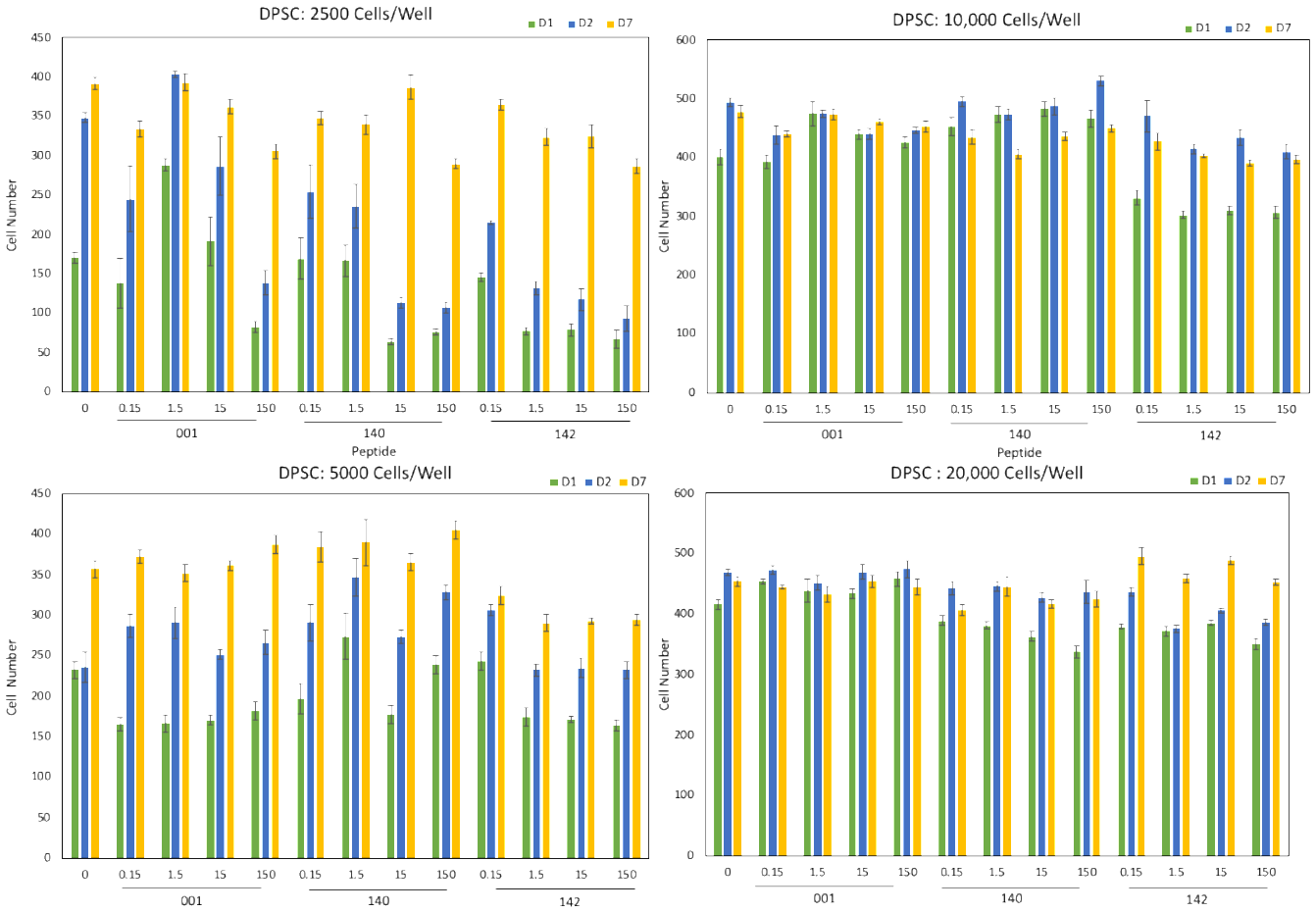

Figure 6.11: Different Types And Concentrations of EPL Peptides Lead to Differences In DPSC Cell Number Over a 7 Day Long Time Course. *DPSCs cultured with different EPL peptide types (EPL 001, EPL 140 and EPL 142) and concentrations (0.15, 1.5, 15 and 150 µM) give rise to differences in cell number per image frame when anaylsed using image analysis after 24h (D1), 2 days (D2) and 7 days (D7). DPSCs were initially seeded at 2500, 5000, 10,000 and 20,000 cells/2cm2 well. Data is expressed as the mean cell number ±SEM. Bars represent means ±SEM of 3 independent samples.*

6.2.2 Using Bioactive Peptides to Optimise the Adult Progenitor Cells' Response to Mechanical Stretch

Next, two peptides were selected that were determined to have a positive or negative effect on adult progenitor cell number, as detailed above, were selected to be used in the previously described three-day mechanical stretch regime, to see if the use of bioactive peptides could further optimise this system, in terms of the induction of differentiation lineages and/or progenitor cell circadian rhythm. One peptide that appeared to evoke positive responses was determined to be EPL 140 at 15 μM (140 15) and one that tended to evoke negative responses was EPL 001 at 0.15 μM (001 0.15).

BMSCs were seeded into fibronectin coated silicone chambers and allowed 12 h to attach before the addition of each peptide at the desired concentration. The peptides were allowed 24 h to begin to act before the onset of the mechanical stretch regime for 3 days (6.66% stretch, 1 Hz, 12 h/day), after which time total RNA was detected and relative gene expression levels determined for differentiation marker and circadian clock genes by RT-qPCR.

6.2.2.1 BMSCs

The addition of bioactive peptides to the mechanical stretch regime led to substantial changes in circadian clock gene expression (Figure 6.12). The expression of all three clock genes, *Bmal1, Per2* and *Rev-ErbAα,* appeared to be higher following exposure to stretch in all three treatment conditions, untreated (UT), EPL 140 at 15 μM (140 15) and EPL 001 at 0.15 μM (001 0.15). This increase in clock gene expression was statistically significantly increased in stretch conditions following the addition of 140 15 for both *Bmal1* and *Rev-ErbAα* when compared to static conditions, showing that this peptide supplementation increased the upregulation of the clock genes following mechanical stretch (independent t-test, $n = 3$, $p \geq 0.05$).

This was further confirmed when the peptide treatments were compared within the stretch and static conditions. For example, following exposure to mechanical stretch, *Bmal1* expression was significantly higher following the addition of 140 15 when

compared to UT (one-way ANOVA with Tukey post-hoc, n = 3, $p \geq 0.01$) or 001 0.15 (one-way ANOVA with Tukey post-hoc, n = 3, $p \geq 0.05$), and following static conditions, *Bmal1* expression was significantly higher following the addition of 001 0.15 when compared to UT (one-way ANOVA with Tukey post-hoc, n = 3, $p \geq 0.05$). However, no significant differences could be observed in *Per2* and *Rev-ErbA*α levels of expressions between the treatment groups for either stretch of static conditions (one-way ANOVA with Tukey post-hoc, n = 3, $p \geq 0.05$).

With regard to the three adult progenitor cell differentiation markers for osteogenesis (*OCN*), chondrogenesis (*Sox9*) and adipogenesis (*PPAR*γ) further changes could be observed following the addition of the bioactive peptides into the mechanical stretch regime (Figure 6.13). For each of the treatment groups, when comparing between stretch and static conditions, no significant differences could be observed for either *OCN* or *PPAR*γ expressions (independent t-test, n = 3, $p \geq 0.05$). However, *Sox9* expression was significantly increased following stretch when exposed to UT and 140 15 treatments in comparison to the static controls (independent t-test, n = 3, $p \geq 0.05$), and this increase was highly significant following the addition of 140 15 (independent t-test, n = 3, $p \geq 0.01$). In contrast, no differences could be observed between the stretch and static conditions following the addition of 001 0.15, suggesting that the stretch-induced upregulation of *Sox9* as seen in the other two conditions had been blocked following the addition of this peptide. When comparing the treatment conditions themselves in either stretch or static conditions, no significant changes in differentiation marker expression could be observed for any of the treatments when compared to the untreated controls (one-way ANOVA with Tukey post-hoc, n = 3, $p \geq 0.05$), showing that although there was a difference in the stretch induced response of *Sox9* when compared to the static condition, there was actually no real difference in the relative levels of marker expressions between the treatment groups themselves.

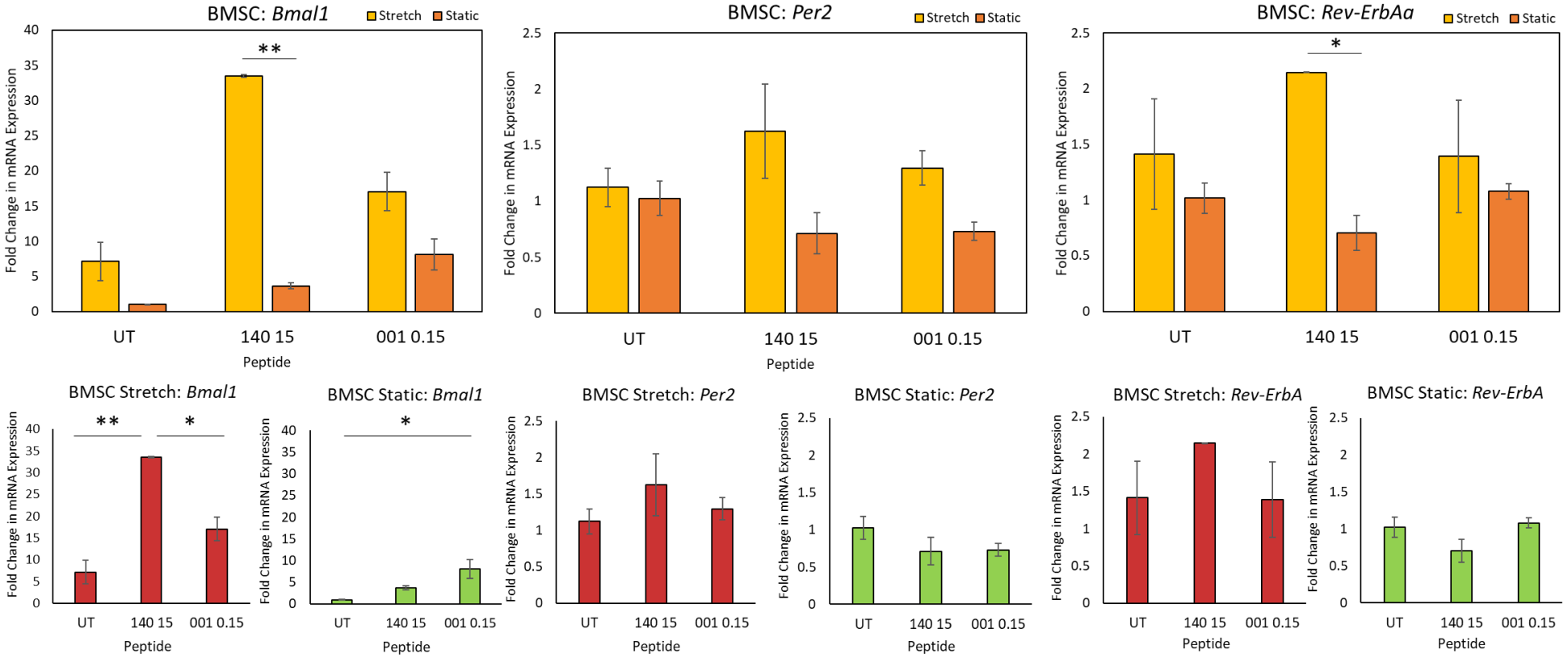

Figure 6.12 The Effect of EPL 140 at 15 µM and EPL 001 at 0.15 µM on Core Circadian Clock Genes Exposed to Mechanical Stretch and Static Conditions in BMSCs. *BMSCs were treated with two peptides, EPL 140 at 15 µM (150 15) and EPL 001 at 0.15 µM (001 0.15) before being subjected to stretch (6.66%, 1Hz, 12h/day) or static conditions for three days, to see if Bmal1, Per2 and Rev-ErbAα gene expression was affected by mechanical strain vs static conditions within the treatment conditions (A) and to determine how the effect of the peptides impacted the levels of clock gene expression in both stretch and static conditions (B). Experimental samples were compared to control samples untreated BMSCs (UT). Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05, **: p<0.01 (A-B: independent t-test, n=3; C-D: one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

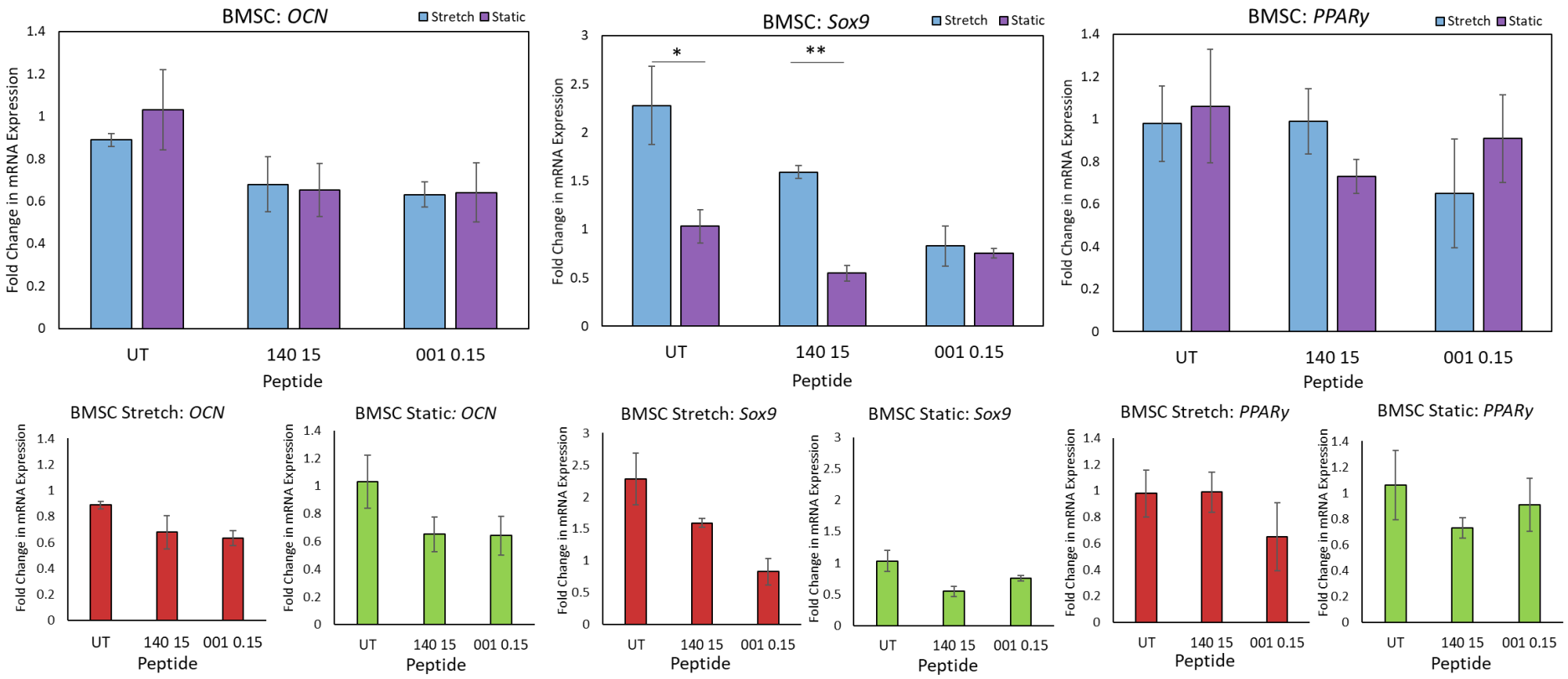

Figure 6.13 The Effect of EPL 140 at 15 µM and EPL 001 at 0.15 µM on Progenitor Cell Differentiation Marker Genes Exposed to Mechanical Stretch and Static Conditions in BMSCs. *BMSCs were treated with two peptides, EPL 140 at 15 µM (150 15) and EPL 001 at 0.15 µM (001 0.15) before being subjected to stretch (6.66%, 1Hz, 12h/day) or static conditions for three days, to see if OCN (osteogenesis), Sox9 (chondrogenesis) or PPARγ (adipogenesis) gene expression was affected by mechanical strain vs static conditions within the treatment conditions (A) and to determine how the peptides impacted the levels of differentiation marker gene expression in both stretch and static conditions (B). Experimental samples were compared to control samples untreated BMSCs (UT). Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05, **: p<0.01 (A-B: independent t-test, n=3; C-D: one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

6.2.2.2 DPSCs

As with the BMSCs, the clock genes in the DPSCs appeared to all be upregulated following exposure to mechanical stretch following the addition of the different peptide conditions (Figure 6.14). To this end, *Bmal1* expression was upregulated in each of the three conditions, UT, 140 15 and 001 0.15, in stretch conditions when compared to static (independent t-test, n = 3, $p \geq 0.05$). Furthermore, when comparing the effect of the individual peptide treatments between each other within static conditions, the expression of *Bmal1* was significantly higher in both 001 0.15 and 140 15 when compared to UT (one-way ANOVA, n = 3, $p \geq 0.05$). *Per2* expression was also higher for 140 15 when compared to UT when comparing the three treatments in static conditions (one-way ANOVA, n = 3, $p \geq 0.05$). *Per2* was also significantly upregulated following stretch for UT, but this significant level of stretch-induced increase in expression appeared to be lost following the addition of the two peptides (independent t-test, n = 3, $p \geq 0.05$). However, the opposite was true for *Rev-ErbA*; a non-significant increase in *Rev-ErbA* was observed for UT following mechanical stretch when compared to static, but this stretch-induced increase did reach statistical significance for both 140 14 and 001 0.15 when compared to static conditions (independent t-test, n = 3, $p \geq 0.05$).

Regarding the induction of the expression of the three differentiation marker gene's expressions, several interesting changes could be seen in DPSCs here too (Figure 6.15). For example, with *OCN*, although no significant changes could be observed between the stretch and static conditions for UT and 140 15, a significant increase in *OCN* could be observed in the stretch condition compared to the static when 001 15 was supplemented (independent t-test, n = 3, $p \geq 0.05$). Furthermore, in static conditions, when the expression of the different peptide treatments were compared, there was a significant decrease in *OCN* expression in both 140 15 and 001 0.15 when compared to UT (one-way ANOVA, n = 3, $p \geq 0.05$).

The expression of *Sox9* appeared higher in all three treatment groups when exposed to mechanical stretch in comparison to static conditions, with this difference obtaining a significant statistical result in UT (independent t-test, n = 3, $p \geq 0.05$) and 001 0.15

(independent t-test, n = 3, $p \geq 0.01$). However, no significant differences could be observed between the groups when compared within either stretch or static conditions. Regarding the adipogenic marker *PPARγ*, there was an increased level of expression in static conditions in the UT condition, as is typically expected and previously noted. However, with the addition of 001 0.15 peptide supplementation, the reverse could be observed; *PPARγ* expression was significantly higher in the stretch condition when compared to the static condition in 001 0.15 (independent t-test, n = 3, $p \geq 0.05$). Furthermore, within the static condition, the expression of *PPARγ* was significantly reduced in 001 0.15 when compared to either UT (independent t-test, n = 3, $p \geq 0.01$) or 140 15 (independent t-test, n = 3, $p \geq 0.05$), suggesting that the addition of EPL 001 at 0.15 μM has an inhibitory effect on the initiation of adipogenesis.

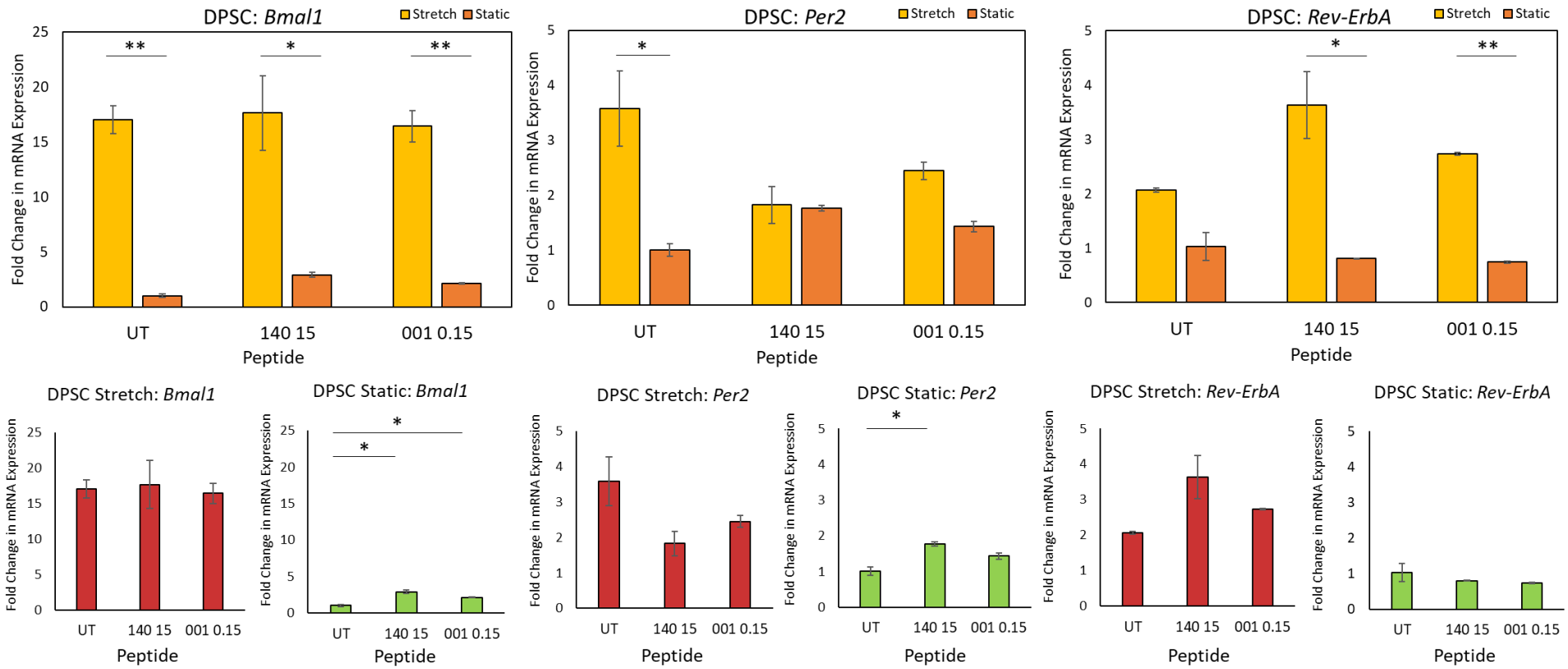

Figure 6.14 The Effect of EPL 140 at 15 µM and EPL 001 at 0.15 µM on Core Circadian Clock Genes Exposed to Mechanical Stretch and Static Conditions in BMSCs. *BMSCs were treated with two peptides, EPL 140 at 15 µM (150 15) and EPL 001 at 0.15 µM (001 0.15) before being subjected to stretch (6.66%, 1Hz, 12h/day) or static conditions for three days, to see if Bmal1, Per2 and Rev-ErbA α gene expression was affected by mechanical strain vs static conditions within the treatment conditions (A) and to determine how the effect of the peptides impacted the levels of clock gene expression in both stretch and static conditions (B). Experimental samples were compared to control samples untreated BMSCs (UT). Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05, **: p<0.01 (A-B: independent t-test, n=3; C-D: one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

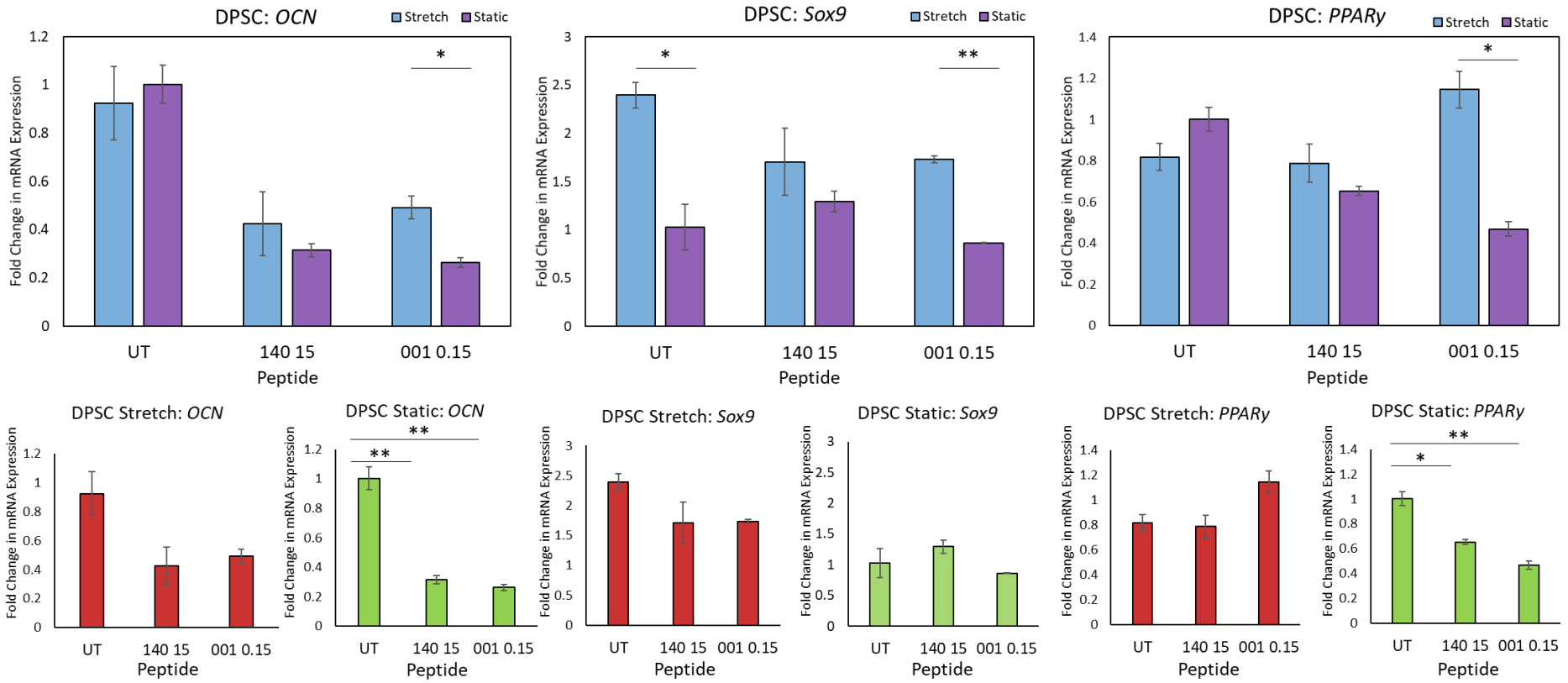

Figure 6.15 The Effect of EPL 140 at 15 µM and EPL 001 at 0.15 µM on Progenitor Cell Differentiation Marker Genes Exposed to Mechanical Stretch and Static Conditions in BMSCs. *BMSCs were treated with two peptides, EPL 140 at 15 µM (150 15) and EPL 001 at 0.15 µM (001 0.15) before being subjected to stretch (6.66%, 1Hz, 12h/day) or static conditions for three days, to see if OCN (osteogenesis), Sox9 (chondrogenesis) or PPARγ (adipogenesis) gene expression was affected by mechanical strain vs static conditions within the treatment conditions (A) and to determine how the peptides impacted the levels of differentiation marker gene expression in both stretch and static conditions (B). Experimental samples were compared to control samples untreated BMSCs (UT). Data is expressed as the mean of ΔCt ±SEM normalised against the housekeeping gene GAPDH. Bars represent means ±SEM of 3 independent samples, *:p<0.05, **: p<0.01 (A-B: independent t-test, n=3; C-D: one-way ANOVA with Tukey post-hoc, n=3, statistical significance determined by comparison to SC).*

6.3 Conclusions

The data collected in this chapter highlight the multifaceted approach to which we must appreciate progenitor cell biology, as it exposes a small insight into how many varying factors of the cellular microenvironment influence the resulting cell response. Here, peptides at one concentration led to very different effects on cell number when applied to cells initially seeded at one density versus the other; for example, at day 2, EPL 142 at 150 μM led to increases in cell numbers when applied to BMSCs initially seeded at 2,500, 10,000 and 20,000 cells/2cm^2 well, but led to decreases in cell number when applied to BMSCs seeded at 5,000 cells/2cm^2 well, when compared to untreated controls. Similar differences could be observed throughout the data points, highlighting how influential factors such as cell density, time following application, peptide type and concentration and the anatomical location from which the cells were isolated from, i.e. BMSC vs DPSC, with each factor impacting cell number in different ways. This underlines the necessity for a combinatorial approach to progenitor cell biology, with a need for each of these factors to be studied both in isolation and in combination.

The varying factors that influenced cell number here include peptide type. For example, EPL 142 tended to have a negative effect on DPSC cell number, with at least three of the concentrations of EPL 142 leading to negative changes in cell number in all but three cases in DPSCs, out of the twelve conditions. In contrast, EPL 140 appeared to have positive effects on BMSC cell number, with at least one concentration of EPL 140 bringing about positive changes on BMSC cell number in all but one case. A further factor that led to differing results was that of the peptide concentration. For example, in the DPSCs, EPL 001 at 0.15 μM led to positive changes in cell number when compared to the untreated control at day 2, whereas at 1.5 μM, this led to negative changes in cell number, highlighting the importance of peptide concentration on progenitor cell number. A further critical factor that influenced the resulting progenitor cell numbers was the density that the progenitor cells were seeded at. For example, peptide supplementation after 2 days in DPSCs led to very different results on cell number depending on the initial seeding density; where

samples were seeded at 2,500, 10,000 and 20,000 cells/2cm^2 well, the only significant changes that were reported following peptide supplementation were positive changes. However, where 5000 cells/2cm^2 well were initially seeded, the only resulting significant changes were negative changes in cell number. This further demonstrates that even if all other conditions are kept constant, cell seeding density is sufficient to lead to different increases in cell number. Furthermore, the length of time following the addition of the peptides also had a significant effect on the cell response to peptide supplementation. For example, after one day in BMSCs, both EPL 001 at 0.15 and 1.5 μM, and EPL 142 at 15 and 150 μM led to negative changes in cell number in cells seeded at 5000 cells/2cm^2 well, but 24 h later at day 2, only EPL 001 at 0.15 μM and EPL 142 at 150 μM led to negative changes, with EPL 001 at 1.5 μM and EPL 142 at 15 μM no longer significantly influencing cell number. This shows that the effects of some of the peptides only appear to be fast acting, initial changes, that may be lost as time progresses, as by this time, the cells appear to be able to compensate for such negative changes.

A final factor that influenced the results following peptide supplementation was cell type, as the DPSCs and BMSCs responded to the same conditions very differently. In general, the DPSCs appeared to respond much more negatively to the peptide supplementation than the BMSCs, with many significant decreases in cell number being observed across the varying conditions. In contrast, the BMSCs tended to respond positively to the peptide supplementation. These differences may be as a result of the different anatomical locations that the progenitor cells arise from. For example, the DPSCs have an earlier developmental origin to the BMSCs and originate from the migrating neural crest cells in development. Therefore, as the cells are established at different developmental time points, it may be necessary that they respond to the same signals in different ways, and therefore have differing mechanisms of signal transduction. Furthermore, *in vivo*, the DPSCs reside within the tight niche of the tooth dental pulp, which is less likely to be exposed to chemical signals in the body than the BMSCs, which reside in the bone marrow tissue. Therefore, it is logical that the DPSCs are less likely to be exposed to such signals, and so this negative response to the peptide would be much more rarely experienced. The

BMSCs, however, may be exposed to similar signals *in vivo,* necessitating the positive response to peptide supplementation.

Further differences depending on cell type could be observed in terms of the progenitor cell response to mechanical stretch, when supplemented with synthetic peptides, especially in terms of the expression of core circadian clock genes. In both BMSCs and DPSCs, *Bmal1* expression was highly induced following the addition of EPL 001 at 0.15 μM in static conditions. However, this induction was also brought about by EPL 140 at 15 μM in DPSCs in static conditions, but not in BMSCs, and EPL 140 at 15 μM also led to an upregulation of *Bmal1* in BMSCs following mechanical stretch, when compared to EPL 001 at 0.15 μM and untreated conditions, which was absent in DPSCs. Furthermore, no differences in *Per2* expression could be observed in BMSCs exposed to stretch or static conditions between the different peptide conditions, but in DPSCs, *Per2* was significantly higher following the addition of EPL 140 at 15 μM in static conditions. *Per2* was also upregulated following mechanical stretch in the untreated conditions in DPSCs when compared to static conditions, but this upregulation was lost when either of the two peptides were added, suggesting that the synthetic peptides blocked this stretch-induced upregulation of *Per2* expression. Here, the opposite could be observed for *Rev-ErbA*α expression; *Rev-ErbA*α was highly upregulated following mechanical stretch, and this increase was stronger when either of the peptides were supplemented.

Further differences between the cell types could be observed in the induction of several progenitor cell early differentiation markers following mechanical stretch. For example, in BMSCs, *Sox9* was upregulated following stretch in both untreated and EPL 140 at 15 supplementation conditions when compared to static, but this upregulation was not observed for EPL 001 at 0.15, with no significant differences being observed between stretch and static. In DPSCs however, no significant changes could be observed in *Sox9* expression between the different treatment conditions. Furthermore, no significant differences could be observed between the treatment groups in BMSCs in terms of *OCN* and *PPAR*γ expression, but significant differences could indeed be observed in DPSCs, showing that the chondrogenic lineage may have been stimulated in BMSCs following mechanical stretch and peptide supplementation,

whereas these same stimuli led to the osteogenesis and adipogenesis pathways being stimulated, either positively or negatively, in DPSCs. To this end, when EPL 001 at 0.15 was supplemented to the DPSCs prior to undergoing mechanical stretch, a stretch-induced upregulation in *OCN* could be observed in DPSCs when compared to static conditions. This upregulation was not observed in untreated cells or cells treated with EPL 140 at 15. Furthermore, when comparing the treatment groups in static conditions alone, both EPL 140 at 15 and EPL 001 at 0.15 led to significant decreases in *OCN* expression. Interestingly, significant changes could also be observed in *PPARγ* expression in DPSCs, which could not be observed in BMSCs. As expected, in the untreated samples *PPARγ* expression was downregulated following mechanical stretch, when compared to static conditions. However, when the EPL 001 peptide was supplemented at 0.15, instead a stretch-induced upregulation of *PPARγ* could be observed, in direct contrast to the untreated condition. Furthermore, when the treatment conditions were compared, EPL 001 at 0.15 led to a significant decrease in *PPARγ* expression when compared to the untreated condition, suggesting that the addition of this peptide supplementation has an inhibitory role on adipogenesis in DPSCs.

The evidence here presented that the addition of a synthetic peptide accentuated the stretch-induced upregulation of *OCN* is supported in literature. For example, Lee et al. (2010) also reported that the addition of soluble peptides resulted in an enhancement of the osteogenic response induced by osteogenic media, whereby progenitor cells showed an increased osteogenesis following peptide supplementation in osteogenic media, when compared to cells cultured in osteogenic media alone[240]. Interestingly, the authors here also reported that the effects of the investigated modular soluble peptides were more pronounced following surface immobilization, as opposed to simply added as soluble components to the hMSC culture medium, as presented in this chapter; surface immobilization to HA-coated substrates here enhanced the effect of the peptides, potentially as a result of the increased exposure time of the cells to the peptides. Therefore, it would be interesting to next examine how surface immobilisation would influence the effect of the peptides investigated in this chapter

in future experiments, to see how this influences the cellular response already observed, and to see if this can be accentuated further.

As previously stipulated here, a number of factors have been identified that lead to different cell responses depending on the combination in which they are presented. Therefore, it becomes immensely difficult to study each factor individually, as each must be considered in combination with other conditions, to fully observe the different responses that can be evoked. One review by Mannello and Tonti (2007) attempted to disseminate some of the factors that lead to the "optimal" conditions for stem and progenitor cell culture medium, by looking at factors such as conditioned media, feeder layers, feeder-free media; medium with FCS, human serum or enriched plasma; serum-free, serum replacement nonconditioned or ad hoc formulas, and find that different combinations a crucial for different cultures[241]. For example, this may depend on embryonic vs adult stem cells and whether more minimal or substantial manipulation is occurring, and the authors conclude that the true potential of these exciting cell types will only be realised through continued effort to increase basic scientific understanding at all levels and the development of methods to achieve the desired phenotype. Similarly, here we have observed a further number of factors that may influence progenitor cell growth and behaviour, and have aimed to further identify how these environmental stimuli influence the progenitor cells, so that their behaviour can be more carefully manipulated in a controlled manner in future experiments.

6.3.1 Summary

As there is no existing literature examining the role of bioactive peptide supplementation on the mechanical stimulation of adult progenitor cells, this research offers a novel insight into the influence that these developing technologies may have on the progenitor cells within their physico-mechanical environments *in vivo.* These initial findings will therefore provide a basic understanding of how these novel peptide technologies may affect progenitor cells when utilised in translational and clinical research.

Chapter 7

Discussion

7 Discussion

This research sought to investigate the role of the circadian rhythm on the differentiation capacity of adult progenitor cells, to understand any relationship between the two processes and identify the underlying mechanisms. Furthermore, it was hoped that the effect of mechanical stimulation on these processes and their interplay could also be elucidated. Therefore, the overall hypothesis of this research was that the physio-mechanical environment may influence the differentiation capacity of adult progenitor cells, and that this may be mediated, at least in part, by the influence of the circadian rhythm. It was also hypothesised that adult progenitor cell responses, whether this be to changes in the circadian rhythm or physio-mechanical environment, differ depending on the anatomical source from which they were derived.

To address this, the first aim of this project was to investigate the circadian dynamics in three types of primary human adult progenitor cells and determine whether these cells had functioning circadian rhythms or not, and if so, how their circadian rhythms could be entrained. Moreover, it was also aimed to determine if mechanical stimulation could be utilised as a novel method of cellular synchronisation or not. In the first experimental chapter, it was demonstrated that human adult progenitor cells derived from different anatomical locations, BMSCs, ADSCs and DPSCs, did indeed express different levels and different patterns of circadian genes and progenitor cell marker genes. When subjected to different entraining stimuli, for example, by chemical, thermal or mechanical means, it was also proven that the different cell types also responded to different entraining stimuli differently, and the cells with a more primitive developmental origin were found to be less responsive to circadian stimulation. For example, here, DPSCs were found to be unresponsive to established chemical synchronisation methods by dexamethasone and serum shock, whereas the more mature cells, BMSCs and ADSCs exhibited robust circadian rhythms and were amenable to chemical synchronisation. This is supported by research from Yagita et al. (2010)[142], who showed that undifferentiated cells do not have established circadian rhythms, Huang et al. (2009)[153], who showed that both ADSCs and BMSCs can be

synchronised in a circadian manner using serum shock, and Wu et al. (2007, 2008), who showed that ADSCs and BMSCs can be synchronised using dexamethasone and serum shock[152], and also that undifferentiated pre-adipocytes have a less well established circadian clock compared to differentiated adipocytes[111]. However, it was found that all three mesenchymal progenitor cell types could be synchronised by mechanical entrainment. The presented methodology of using cyclic uniaxial stretch to synchronise cells had not been investigated in this manner before, and interestingly, even the DPSCs could be synchronised in this manner, potentially due to the mechanical stimulation they are exposed to in the tooth dental pulp. Mechanical entrainment is highly advantageous in terms of its ability to synchronise less mature cells, as well as being a non-invasive method of circadian synchronisation, as there is no need for chemical addition or thermal manipulation.

Therefore, these results demonstrated that the mechanical environment does have a relationship with and does influence the circadian rhythm of adult progenitor cells directly. As it has been shown that progenitor cells are more likely to differentiate or proliferate depending on the circadian phase that they are in[200], this has profound implications in terms of offering a novel, non-invasive method of synchronising progenitor cells so that they may be ideally poised for differentiation, for example, before being implanted into a host body in a biomaterial, so that the biomaterial may be in a state of readiness for differentiation. In this way, if the cells seeded into a biomaterial differentiate into the desired cell types as soon as possible, the biomaterial is much more likely to become integrated and function within the host body and therefore have a higher chance of acceptance and recovery.

As the finding of this first experimental chapter demonstrated that the mechanical environment can influence the circadian rhythm of progenitor cells, it was next aimed to see how manipulating the physio-mechanical environment would then affect the progenitor cells, with behaviours such as the circadian dynamics, differentiation response and response to stretch, in terms of circadian and progenitor cell gene expression levels and patterns, being examined. Initially, it was demonstrated that changing the physio-mechanical environment, via alterations in the protein substrate, did influence the adhesion and the proliferation of human progenitor cells, which was

influenced by both the protein coating type and concentration, and the anatomical location from which the progenitor cells were derived. Furthermore, different differentiation and circadian marker gene expression levels were seen depending on the protein substrate, i.e. fibronectin vs collagen vs albumin at different concentrations, and cell type, i.e. BMSCs vs DPSCs. This disputes findings by Yang et al. (2017)[242], who, in contrast, stated that the matrix composition did not affect the circadian rhythm. It was also demonstrated in the second experimental chapter of this research that differences in the protein substrate coating led to differences in the progenitor cells' response to mechanical stretch, as is supported by research by Gong and Nikhalson (2008)[191], who found that the induction of differentiation markers following stretch were dependent on the protein substrate. Here, it was demonstrated that the differentiation response was more likely to be reduced in static conditions, whereas stretch induced the expression of differentiation markers in DPSCs, as is supported by Tabatataei et al. (2014)[194], who also demonstrated that mechanical stimulation led to an increase in osteogenesis in DPSCs. It was also demonstrated in this work that the different protein substrate conditions led to differences in the progenitor cells' circadian gene expressions.

Therefore, these results demonstrated that the differentiation response, circadian rhythm and the response to stretch of progenitor cells can be manipulated by changes in the physio-mechanical environment. However, *in vivo*, the progenitor cells will be exposed to a plethora of stimuli in combination, and therefore, the representiveness of such experiments is questionable, whereby 2D environments are utilised to study variables in isolation. This is justifiable, however, as it allows us to investigate individual components. There are many applications of this research that can be exemplified when researching potential therapies for a number of different pathologies, as these normally involve a rigid matrix which is detrimental to the cells. For example, in diseased or damaged tissue, overexpressed ECM components can be frequently found, which lead to an increase in stiffness and therefore an inhibition of cell contractility. Increases in stiffness have been associated with a dampening of the circadian clock, which can then lead to a number of further pathologies, including reduced progenitor cell function. Therefore, if therapies are to be designed to treat

these pathologies, the mechanisms that underlie them and the relationships that regulate them must be fully understood, as is in this chapter. It is therefore hoped that if the physio-mechanical environment can be manipulated accordingly, these pathologies may be reversed.

As the findings of the first and second experimental chapters implicated that mechanical stretch influences the differentiation capacity of adult progenitor cells, which may be influenced by the circadian rhythm, the next aim was to see how disrupting the circadian clock impacts the response of the progenitor cells to mechanical stretch and the resulting changes in differential potential, to determine if this was dependent on the circadian rhythm or not. In the third experimental chapter presented here, it was found that the induction of the progenitor cells' differentiation markers were indeed influenced by the circadian rhythm, and the knock down of the clock genes did alter the expression of different osteogenic, adipogenic and chondrogenic marker gene expressions. For example, when *Rev-ErbA*α was knocked down, this led to an increase in osteogenic marker expression and a decrease in adipogenic marker expression in both DPSCs and BMSCs. In contrast, when *Bmal1* was knocked down, an increase in adipogenic marker expression could be seen in both cell types, as is supported by Guo et al. (2012)[93], who demonstrated that Bmal1 has an inhibitory role on adipogenesis and that disruptions in Bmal1 expression leads to increases in adipogenesis. However, the response of the two cell types to disrupted clock gene expression differed significantly when exposed to mechanical stretch. Here, the circadian gene knock downs led to different responses depending on whether the cells were exposed to stretch or static conditions, which genes were knocked down and the anatomical location from which the cells were derived. To this end, the two cell types differed in the signalling pathways that were activated in response to mechanical stretch, as the knock downs led to different effects in one cell type versus the other. However, in both instances, it could be established that disruptions in circadian gene expressions altered the expressions of several differentiation markers, and several stretch-induced differentiation responses were lessened by clock disruption, as is supported by research by Honda at al. (2013)[160] and Dudek et al. (2016)[209].

Here, it was demonstrated that mechanical stretch impacts different molecular pathways in the two cell types, which differed in their level of maturity and the anatomical location from which they were derived, and it can therefore be shown how one stimuli effects one cell type may have profoundly different responses in the other.

In order to elucidate how this research could be applied for therapeutic benefit, the final aim of this research was to determine how synthetic bioactive peptides effected the physiology, growth, behaviour, circadian rhythm, and also the cellular response to mechanical stretch of adult progenitor cells, as it was hypothesised that the addition of such synthetic peptides would influence each of these processes. When the synthetic bioactive peptides were supplemented into the progenitor cells' culture, it was indeed found that different responses in cell adhesion and proliferation could be examined based on cell density, time following peptide supplementation, peptide type and concentration and progenitor cell type, and changing any of these variables led to differences in the resulting cell number, as each variable was found to influence the cells in different ways. This highlights the need for a combinatorial approach, as it was shown that changing one variable led to different responses based on the other variables, and therefore certain changes may not always have the same effect. In general, the DPSCs responded much more negatively to peptide supplementation than the BMSCs, with many decreases in cell number being observed following supplementation. This was theorised to be as a result of one of the following features of the DPSCs:

- Developmental origin – as the BMSCs and DPSCs originate from different developmental time points, it could be theorised that the two cell types must respond to the same signals in different ways
- Signal Transduction Pathways – as the BMSCs and DPSCs have been shown to differ in the signalling pathways responsive to stretch, this could also be the case following peptide supplementation
- Anatomical Location – as well as DPSCs responding negatively to the addition of peptides, DPSCs were previously shown in the first experimental chapter to also be unresponsive to chemical synchronisation, and therefore do not appear responsive to supplements. This may be due to the location of their

niche in the body, as DPSCs are restricted to a tight niche and so may not be exposed to this kind of stimulation *in vivo*, and therefore have no need for the signal transduction mechanisms in response to supplementation. BMSCs, however, may be exposed to this kind of stimulation in their niches.

The two cell types were also found to demonstrate different responses as a result of peptide supplementation prior to mechanical stretch, in terms of both clock gene and differentiation marker expressions. These responses differed on the peptide type and concentration, stretch versus static conditions and progenitor cell type. Interestingly, increases in differentiation marker gene expressions could be seen following the supplementation of peptides that had previously resulted in decreases in cell number. Therefore, these decreases in proliferation may be explained by an increase in differentiation response.

This research highlights the combinatorial approach that should be utilised to investigate progenitor cell research, as these highly responsive cells respond extremely sensitively depending on the specific combination of variables that they are exposed to, and the factors selected must be done so with extreme consideration. The presented research in this final chapter is novel, as there is no existing literature that examines the role of bioactive synthetic peptides in human adult progenitor cells exposed to mechanical stretch, and therefore offers an insight into the role of synthetic supplements on progenitor cells within their physio-mechanical environments *in vivo*, which will have profound implications in the advanced materials and technologies currently being implemented in modern clinical engineering and regenerative medicine.

7.1 Overall Conclusions

Overall, the research presented here highlights that progenitor cells respond very differently to different signals (e.g. circadian synchronisation, response to mechanical stretch, response to different materials and physio-mechanical environments), which is likely to be due to the fact that the cell types differ in their signal transduction

mechanisms, and therefore the same stimuli can have varying effects on the expression of different genes, depending on the anatomical location of derivation, origin and level of maturity of the cells. This is highly relevant as the circadian phase and responsiveness to synchronisation will determine how the progenitor cells respond to both differentiation signals and their success within a biomaterial. Cyclic uniaxial stretch is here presented as a novel methodology to synchronise these rhythms, and therefore offers a way by which different variables may be investigated in a mechanically active and circadian manner. Furthermore, it was here demonstrated that BMSCs are more likely to differentiate in a static environment, in contrast to DPSCs, which are more likely to differentiate in a mechanically active stretch environment. As predicted, it was also found that circadian genes do influence the expression of several differentiation markers.

Ultimately, this research presents a mechanism by which differences in the mechano-environment lead to the detrimental effects seen in diseased, damaged or aged tissue, as can be seen in Figure 7.1. It is proposed that differences in the mechano-environment (for example, increased ECM stiffness seen with age) lead to a dampening of the circadian clock, which then causes a decrease in adult progenitor cell ability and differentiation capacity, resulting in a reduction of tissue function and associated pathologies. Therefore, if the mechanical properties of such environments can be manipulated, or the circadian rhythm may be stimulated, this may offer a way by which this reduction in progenitor cell differentiation and tissue function may be rescued.

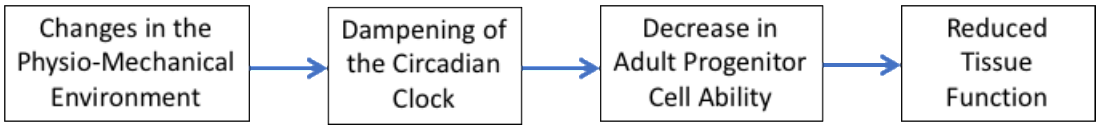

Figure 7.1. Mechanism by which differences in the mechano-environment lead to the detrimental effects seen in diseased, damaged or aged tissue.

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Appendix

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Research Article

Comparing Circadian Dynamics in Primary Derived Stem Cells from Different Sources of Human Adult Tissue

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Optimising cell/tissue constructs so that they can be successfully accepted and integrated within a host body is essential in modern tissue engineering. To do this, adult stem cells are frequently utilised, but there are many aspects of their environment *in vivo* that are not completely understood. There is evidence to suggest that circadian rhythms and daily circadian temporal cues have substantial effects on stem cell activation, cell cycle, and differentiation. It was hypothesised that the circadian rhythm in human adult stem cells differs depending on the source of tissue and that different entraining signals exert differential effects depending on the anatomical source. Dexamethasone and rhythmic mechanical stretch were used to synchronise stem cells derived from the bone marrow, tooth dental pulp, and abdominal subcutaneous adipose tissue, and it was experimentally evidenced that these different stem cells differed in their circadian clock properties in response to different synchronisation mechanisms. The more primitive dental pulp-derived stem cells did not respond as well to the chemical synchronisation but showed temporal clock gene oscillations following rhythmic mechanical stretch, suggesting that incorporating temporal circadian information of different human adult stem cells will have profound implications in optimising tissue engineering approaches and stem cell therapies.

1. Introduction

Without adult stem cells (ASCs), mammalian tissues would not be able to maintain their natural homeostasis, as these cells play a vital part in the replenishment and repair processes, whereby old or damaged cells become replaced. Stem cells are characterized by their extraordinary abilities to self-renew through cell division over a long period of time and to be able to differentiate and give rise to organ- or tissue-specific cells in response to internal or external stimuli. It is these homeostatic and regenerative abilities that allow them to regularly repair and replace damaged or injured tissues. *In vivo*, a combination of chemical, biological, and physical cues present in the stem cell niche contribute to the direction of mesenchymal stem cell (MSC) fate, which allows them to have such a broad multilineage differentiation potential.

Recently, there has been a drive to investigate the use of Oct-4 expressing dental pulp-derived mesenchymal-like stem cells (DPSCs) in tissue engineering, as the differentiation capacity of MSCs may be restricted by their tissue of origin. These newly discovered cells offer an alternative to bone marrow-derived MSCs (BMSCs), as they have been shown to have a more potent differentiation potential than BMSCs. DPSCs have been shown to have a faster proliferation rate as well as the potential to differentiate into not only several mesenchymal cell types but also neurogenic cells, as well as being highly advantageous in terms of accessibility [1]. DPSCs are able to differentiate into neural cells as they originate from the migrating neural crest cells during embryogenesis. Dental pulp therefore consists of ectomesenchymal elements, containing neural crest-derived cells that exhibit both plasticity and multipotency [2]. The stem cells that reside within the dental pulp are extremely protected from external stimuli in their "sealed niche." The embryonic tissues found there remain undifferentiated within the jaws until the only organogenesis event which occurs after birth.

Another, more accessible source of adult stem cells that is currently being investigated is from the adipose tissue (ADSCs); adipose tissue offers an abundant source of MSCs which can be obtained in large quantities with minimal patient discomfort. Both DPSCs and ADSCs are more favourable to BMSCs as they also result in much lower site morbidity. All three have been investigated in terms of their differentiation capacities; for example, Davies et al. [3] found that DPSCs exhibited the highest potential to produce a mineralised matrix, but ADSCs and BMSCs showed enhanced dentinogenic and mineral volume. Stanko et al. [4] found that these three cell types showed no differences in terms of cell morphology or MSC surface marker expression. However, they did find significant differences regarding the expression of several pluripotency genes; BMSCs and ADSCs produced similar protein levels of several pluripotency markers but the DPSCs showed significant differences in the amount of protein products observed, including a lower expression of Oct3/4. These differences were hypothesised to reflect the mixed embryonic stem cell origin of DPSCs.

One temporal cue recently discovered to regulate mesenchymal stem cell potential and differentiation capacity is governed by the circadian rhythm [5–7]. The mammalian circadian rhythms are orchestrated by a hierarchy of self-sustained tissue oscillators. The suprachiasmatic nucleus (SCN) in the anterior hypothalamus of the brain coordinates a number of peripheral tissue oscillators to regulate a coherent rhythm of a multitude of outputs regulating metabolism, physiology, and behaviour [8]. The molecular mechanisms that regulate the circadian clockwork are evolutionarily conserved and cell-autonomous, whereby a network of autoregulatory transcriptional-translational feedback loops drive circadian expression patterns of the core clock components [9]. The primary transcriptional-translational feedback loop (TTFL) is controlled by the basic helix-loop-helix transcription factors CLOCK and BMAL1 (i.e., ARNTL). When these two proteins heterodimerize, they are able to bind to *cis*-regulatory enhancer elements within target core clock genes as well as many clock-controlled genes (CCGs) [10, 11]. Core clock genes include *period (Per1, Per2*, and *Per3)* and *cryptochrome* (*Cry1* and *Cry2*); these two proteins also heterodimerize and repress their own transcription by negatively regulating the CLOCK:BMAL1 complex [12, 13]. The CLOCK:-BMAL1 heterodimers also regulate the transcription of retinoic acid-related orphan nuclear receptors, REV-ERBs (i.e., NR1D1 and NR1D2) and RORs, which form part of the stabilising loop. These are known to bind to retinoic acid-related orphan receptor response elements (ROREs), which are present in the *Bmal1* (i.e., *ARNTL*) promoter. REV-ERBs repress transcription of *Bmal1*, whereas RORs activate the transcription [14]. Both positive and negative autoregulatory loops constitute a circadian molecular clock and take approximately 24 hours to complete.

A recent field of investigation has shown that the clock genes can directly influence ASC and progenitor cell activation and differentiation, within their tissue-specific niches. For example, disrupting the clock gene *Bmal1* leads to increased adipogenesis, and thus the attenuation of Bmal1 expression *in vitro* in preadipocytes leads to a downregulation of the Wnt signalling pathway and increased adipogenesis [15]. However, in mature adipocytes, it has been found that BMAL1 is highly expressed in differentiated cells; when BMAL1 was knocked-down by RNA interference, the mature 3T3-L1 cells were only able to accumulate minimum amounts of lipid droplets in the cells. Furthermore, adenovirus-mediated BMAL1 expression resulted in the induction of several factors involved in lipogenesis, many of which showed clear circadian rhythm in mice adipose tissue [16]. This conflicting research shows that the circadian rhythm has a clear role in cell differentiation and may have differential roles in the different stages of differentiation, but this still requires extensive further research.

The involvement of circadian clocks in the regulation of adult stem cell activation is not only niche-specific but can also act at the cell population level. Janich and colleagues showed that the circadian clock has a differential role in regulating the activation of coexisting epidermal stem cell populations. The authors found that the genes regulating stem cell niche dormancy, activation, and differentiation contained several putative BMAL1/CLOCK-binding sites. Deleting *Bmal1* leads to circadian arrhythmia, decreased expression of Wnt-related genes, and TGF-β inhibitors and causes progressive accumulation of dormant stem cells. Deleting *Per1/2*, conversely, results in progressive depletion of dormant stem cells [5]. A subsequent study showed that the stem cells responded differently to differentiation cues at certain times of the day. Interestingly, different proliferation-related or differentiation-related genes were expressed at different times of the day; for example, DNA replication and cell division related pathways were highly expressed in the dark phase, as opposed to in the light phase, when differentiation pathways were more highly expressed [6].

The endogenous mammalian circadian clock has a period of approximately 24 hours, which is reset daily by external cues, known as zeitgebers. The most potent of these is a daily light cue, which entrains the clock in the SCN in mammals through reticulo-hypothalamic signalling mechanisms. The SCN relays this information to peripheral tissue clocks via diffusible neuro-endocrine signals [17]. Another important timing signal which has the capacity to entrain daily rhythms is via systemic factors such as growth factors and hormones. Glucocorticoids, which are a class of steroid hormones that bind to the glucocorticoid receptor (GR) present in almost every vertebrate cell, have been implicated in synchronising peripheral circadian rhythms. A recently discovered entrainment mechanism for the clock that requires fundamental research is by mechanical stimulation, whereby cells in different tissues in the body are subjected to very different levels of mechanical strain. Mechanical vibrations have the capability of resetting the clock in *Drosophila melanogaster*; it has been demonstrated that rhythmic mechanical stimulation of the chordotonal organs can synchronise the *Drosophila* circadian clock. *Drosophila* with loss of function mutations in their period gene did not exhibit this ability of synchronization through vibrations, highlighting

the importance of functional clock systems for mechanical entrainment [18].

Previous extensive work in MSCs has demonstrated significant effects that mechanical forces *in vitro* can exert on their proliferation and differentiation properties. For example, Tirkkonen et al. [19] used vibrational loading to cause the differentiation of human ADSCs towards bone-forming cells and inhibition of adipogenesis. The authors found that the hADSCs cultured in osteogenic conditions were sensitive to vibrational loading, and their osteogenic differentiation was enhanced with high-frequency vibration. It has also been recently published that the mechanical environment of the epithelial stem cell niche within the mammalian mammary tissue controls the amplitude of the molecular clock oscillations, which is altered upon environmental (e.g., ageing) and genetic clock disruption [7].

Interestingly, unlike adult stem cells such as BMSCs and ADSCs, which are capable of circadian synchronization by hormonal and growth factor signals such as dexamethasone [20] and serum shock [21]. Embryonic stem (ES) cells lack a ~24 h circadian rhythm and do not display the core TTFL required for circadian clock gene expression. However, upon differentiation, ES cells in culture can gain a molecular circadian rhythm, which can also be reversed when the cells are reprogrammed with the addition of *Oct3/4*, *Sox2, Klf4*, and *c-Myc* [22]. However, investigating the circadian rhythm in embryonic cells presents many challenges and the way by which we undertake our experiments may result in a disruption of endogenous oscillation(s) [23]; therefore, this lack of circadian rhythm in embryonic stem cells may not be as strict as it previously appeared.

It was hypothesised here that adult mesenchymal stem cells from different human tissue sources at different anatomical locations within the body may exhibit different circadian dynamics and respond to temporal cues differently and that the more primitive adult stem cells such as DPSCs would be less responsive to circadian synchronisation cues compared to BMSCs or ADSCs. In addition to well-established chemical synchronisation using a synthetic glucocorticoid, dexamethasone [24], mechanical stimulation was also used in a circadian paradigm to investigate whether different human adult stem cells could be entrained using mechanical cues. The applied method investigating mechanical stimulation, using a uniaxial stretch rig composed of flexible silicone substrates at 6.66% radial distension and frequency of 1 Hz, was selected as these parameters have previously found to be within physiological range. Published data has previously shown that a uniaxial strain between 5–15% with a frequency of 1 Hz is preferable for MSCs and shows positive effects on proliferation and collagen synthesis [25]. Furthermore, O'Cearbhaill et al. [26] found that radial distensions of 5% and frequencies of 1 Hz caused mechanosensitive effects including cell reorientation parallel with the direction of flow and adapted morphologies, highlighting that there is a significant cytoskeletal restructuring in these mechanically stimulated MSCs compared to static controls.

If viable, this mechanical stretch paradigm could offer a synchronisation method that excludes the need to use chemical or thermal approaches to synchronise the circadian clock, which would be hugely advantageous in tissue engineering and regenerative medicine. In this research, the circadian differences in adult stem cells derived from different human adult tissues with respect to their expression of several core clock genes, stabilising clock genes and stem cell markers in both dynamic and static conditions have been determined and compared. The results reveal differential circadian gene expression patterns in human adult stem cells derived from different tissue sources upon glucocorticoid synchronisation. It is also shown that rhythmic mechanical stimulation has the ability to entrain some human stem cells, which provides a novel clock synchronisation approach independent of chemical or temperature cues. Such a clock synchronisation protocol may prove more advantageous in future tissue engineering applications, leading to significant developments in both age-related diseases and tissue engineering and synchronising stem cell therapies.

2. Materials and Methods

2.1. Isolation of Adipose-Derived Stromal Cells. Human lipoaspirate was harvested during a lipofilling procedure after breast tumour removal. Lipoaspirate (5 g) was washed by addition of serum-free DMEM medium (Gibco, UK) and centrifuged for 3 min at 500*g*. The washed fat layer was moved to a new tube to which a digestion solution containing 10 mL of DMEM medium (Gibco, UK) and 20 μL of collagenase type 2 (Sigma, UK) was added. This was placed on a roller mixer for 30 min at 37°C. 10 mL of DMEM medium containing 10% FBS and penicillin/streptomycin (Sigma, UK) was added and the tube was spun for 5 min at 1000*g*. The oil layer was removed and discarded, and undigested fat fraction was placed into a T25 falcon flask, with the addition of complete growth DMEM medium as above. The remaining supernatant was discarded and the pellet was resuspended and placed in a separate T25 flask, with the addition of complete growth DMEM medium. Both of these fractions were cultured until confluency and used at passage 2.

2.2. Cell Culture of BMSCs and DPSCs. Primary human BMSCs and DPSCs were obtained commercially (Lonza and BioEden Limited, resp.) and expanded in Dulbecco's Modified Eagle Medium (GlutaMAX; Gibco, UK) supplemented with 1% penicillin/streptomycin and 10% FBS and incubated at 37°C in 5% CO2. Cells were trypsinised upon reaching 70–80% confluency and used before reaching passage 7.

2.3. Clock Synchronisation with Dexamethasone. Cells were seeded into 6-well plates in complete growth medium as above until confluency. They were synchronised using 100 nM dexamethasone and total RNA collected every four hours over a circadian cycle at the following time points after synchronisation (h): 16, 20, 24, 28, 32, 36, 40, 44, 48, and 52. The cells were exposed to the dexamethasone for 1 h and then incubated in complete growth medium until sample

Table 1: qPCR primer sequences (human).

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG
Bmal1	TGCCTCGTCGCAATTGG	ACCCTGATTTCCCCGTTCA
Per2	GTCCAGCCCCCACCTTTC	GGGAAGGAATAACTGGGTAGCA
Per1	CTCAGTGGCTGTCTCCTTCC	GAGCCAGGAGCTCAGAGAAG
Rev-ErbAα	CTTCAATGCCAACCATGCAT	CCTGATTTTCCCAGCGATGT
Sox2	GAGAGAAAGAAAGGGAGAGAAG	GAGAGAGGCAAACTGGAATC

collection. Negative controls received no dexamethasone but were exposed to the same media changes and conditions.

2.4. Mechanical Stimulation. Flexible silicone chambers were coated in fibronectin for 1 h before cells were seeded into the chambers and allowed at least 24 h to settle and attach. Once confluent, the chambers were loaded into a unique uniaxial stretch rig and stretched for 3 consecutive days in a rhythmic manner (frequency 1 Hz, 6.66% stretch, 12 h ON/12 h OFF). After day 3 of rhythmic stimulation, cells were allowed to rest and were collected at the following times over 1.5 circadian days following mechanical stimulation (h): 16, 20, 24, 28, 32, 36, 40, 44, 48, and 52.

2.5. Quantitative RT-qPCR. Total RNA was extracted using TRI-reagent (Sigma) as per the manufacturer's specifications. Total RNA was determined using nanodrop spectroscopy before cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen), with Oligo dT at 50°C for 40 min in a 20 μl reaction. Real-time RT-PCR was carried out on cDNA samples with SYBR Green PCR Supermix (Bio-Rad) using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad) under the following cycling conditions: 95°C for 3 min; 40 cycles of 95°C for 10 s; and 60°C for 30 s. Results were normalised relative to a housekeeping gene *GAPDH* expression. Primers were designed against the following genes: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), aryl hydrocarbon receptor nuclear translocator-like (*ARNTL* or *Bmal1*), period 2 (*Per2*), period 1 (*Per1*), nuclear receptor subfamily 1 group D member 1 (*NR1D1* or *Rev-ErbAα*), and SRY (sex determining region Y)-Box 2 (*Sox2*) (primer sequences are listed in Table 1).

2.6. Statistical Analyses. Data were expressed as the mean ± standard error. Statistical analyses were performed, following the determination of normal distribution, using one-way analysis of variance (ANOVA), with Tukey HSD post hoc, or the independent *t*-test at a confidence level of 95% (SPSS 24 Software). In order to determine circadian gene rhythmicity and its significance, cosinor periodogram analysis was used, made available online by the Refinetti circadian biology group at Boise State University (http://www.circadian.org/softwar.html). p values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Human Stem Cells Derived from Different Sources of Adult Tissue Show Differential Circadian Clock Gene Expression Profiles. Asynchronous cultures of BMSCs, ADSCs, and DPSCs were allowed to grow to confluency in complete growth medium, before their RNA was extracted and a PCR "clock panel" of genes was used to analyse their relative mRNA expression. As expected, different human adult stem cells did exhibit significantly varying amounts of clock gene expression, with the BMSCs and DPSCs appearing to show contrasting expressions of *Bmal1*, a component of the positive arm of the molecular clock, and *Per2*, a component of the negative arm (Figure 1). Interestingly, *Bmal1* was significantly higher in DPSCs compared to BMSCs and ADSCs, whilst *Per2* was significantly higher in BMSCs compared to DPSCs and ADSCs. *Per1* and *Rev-ErbA* were also both significantly higher in DPSCs than ADSCs and BMSCs. In contrast, *Bmal1*, *Per1*, and *Rev-ErbA* were not significantly different in expression levels between BMSCs and ADSCs.

3.2. Glucocorticoid Stimulation Using Dexamethasone Leads to Circadian Synchronisation in BMSCs and ADSCs but Not DPSCs. Next, the three human adult stem cell types were grown to confluency and clock synchronised using synthetic glucocorticoid, 100 nM dexamethasone for a period of 1 h, after which, their media was replaced with complete growth medium. RNA samples were initially taken at two opposite circadian phases at either 20 h or 32 h post synchronisation to examine any initial differences at opposite phases. The antiphasic relationship of *Bmal1* and *Per2* could be clearly seen with the two genes peaking and troughing at opposite circadian times, respectively. For example, in ADSCs and DPSCs, *Bmal1* expression was much higher at 32 h than at 20 h whilst *Per2* was much higher at 20 h compared to 32 h (Figure 2). Interestingly, the opposite appeared to be observed for the BMSCs, which showed higher *Bmal1* and lower *Per2* expression at 20 h versus 32 h, respectively. The mRNA expression of a component of the stabilising loop in circadian machinery was also examined. It was found that *Rev-ErbAα* expression showed temporally different expressions at the two circadian time points in different human adult stem cell types and interestingly appeared to peak at 32 h similarly to *Per2* in BMSCs, whilst at the same circadian

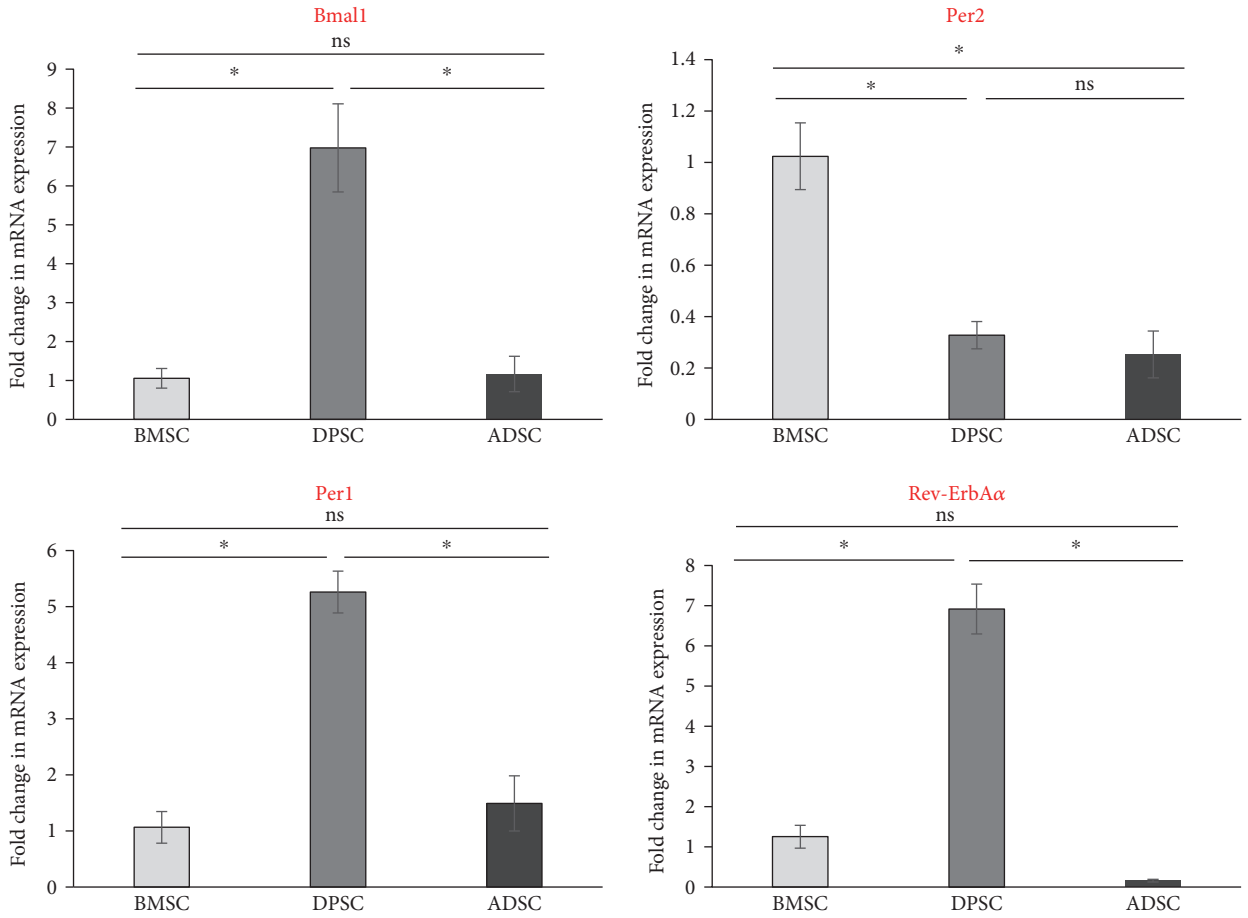

FIGURE 1: Clock gene expression in unsynchronised adult stem cells. Quantitative RT-PCR analyses comparing the relative mRNA expression levels of core clock genes in mesenchymal-like adult stem cells derived from human bone marrow, dental pulp, and adipose tissue. Data is expressed as the mean of ΔCt ± SEM normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent samples, $^{*}p < 0.05$ (one-way ANOVA).

time point, it peaked similar to *Bmal1* in the ADSCs and DPSCs.

As significant differences were observed between the two initial time points, temporal clock gene expression profiles over 1.5 circadian days were then compared in order to get a clearer understanding of the clock gene expression, omitting the first 0.5 circadian day (0–15 h) in order to exclude the transient effects of dexamethasone, as published previously [7]. Samples were collected every four hours starting with 16 h postdexamethasone synchronisation and ending at 52 h, in order to more closely investigate circadian gene dynamics between the three different human adult stem cell types (Figure 3). Both the ADSCs and BMSCs showed robust oscillations of clock gene expressions which is a characteristic antiphasic temporal pattern of synchronised cells reported previously. For example, in the BMSCs, *Bmal1* troughed whilst *Per2* peaked at 32 h postdexamethasone synchronisation. In contrast, in ADSCs, *Bmal1* peaked and *Per2* troughed around 16–20 h, showing a clear antiphasic relationship. On the other hand, the more primitive DPSCs, however, did not exhibit oscillating expressions of the circadian clock genes, and no clear temporal patterns of clock gene expression could be observed following dexamethasone synchronisation.

When analysed by cosinor periodogram (Table 2), *Bmal1* expression was shown to exhibit significant circadian rhythmicity in BMSCs ($p = 0.007$), whilst *Per2* was just short of significance ($p = 0.076$), and all three circadian clock genes (*Bmal1, Per2*, and *Rev-ErbAα*) showed circadian rhythmicity in ADSCs ($p = 0.006$, $p = 0.021$, and $p = 0.001$, resp.). However, no core clock genes were found to exhibit significant circadian rhythmicity in DPSCs, except for *Rev-ErbAα* which nearly reached significance ($p = 0.055$).

In order to confirm cell synchronisation by dexamethasone and exclude the possibility that changing the cell culture growth medium itself following dexamethasone synchronisation had any synchronising effects on cells, unsynchronised BMSCs were used as a negative control and collected temporally having received no stimulation other than the same media changes as the above time-course experiment. As expected, no circadian rhythm of core clock genes *Bmal1* or *Per2* was observed in unsynchronised BMSCs (Supplementary Fig. 1 available online at https://doi.org/10.1155/2017/2057168).

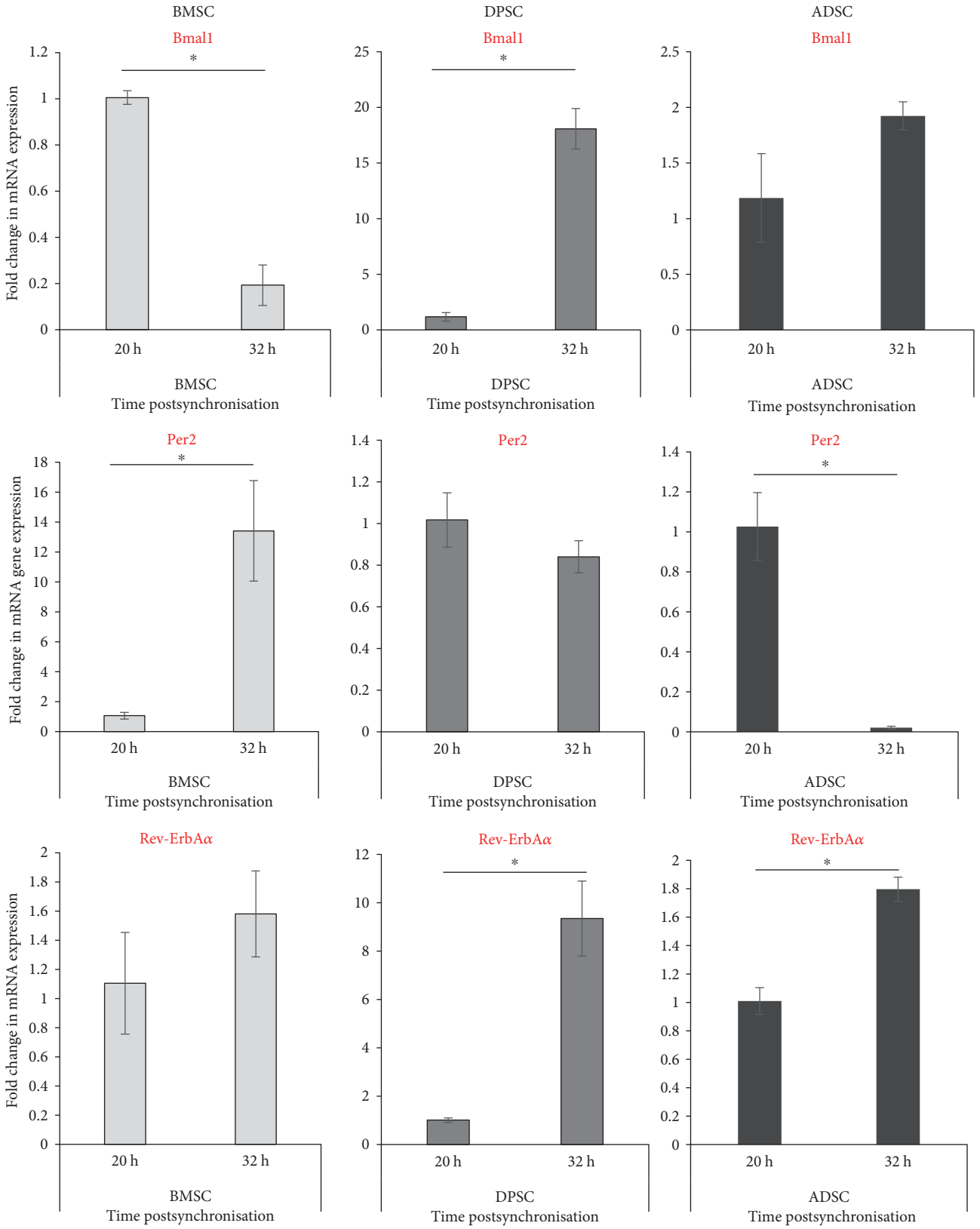

Figure 2: Clock gene expression in synchronised human adult stem cells at two opposite circadian phases. Quantitative RT-PCR analyses showing the expression levels of clock genes after synchronisation with dexamethasone at two opposite circadian phases 12 h apart (20 h versus 32 h). Data are expressed as the mean of ΔCt ± SEM relative to the 20 h time point and normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent experiments, $^{*}p < 0.05$ (independent *t*-test).

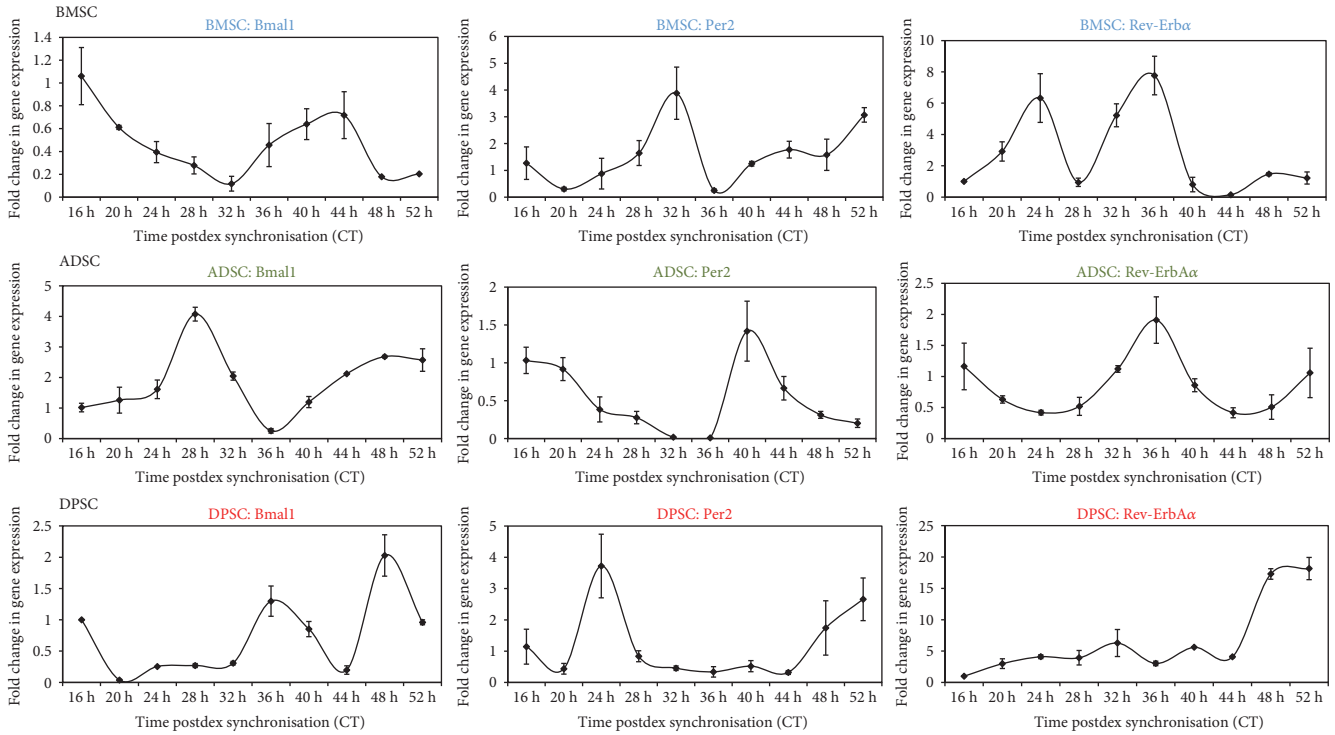

Figure 3: Circadian rhythm dynamics in human adult stem cells following synchronisation with dexamethasone. Quantitative RT-PCR analyses showing temporal expression profiles of clock genes collected every four hours between 16 h–52 h following synchronisation with dexamethasone. Data are expressed as the mean of ΔCt ± SEM relative to 16 h time point and normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent experiments.

Table 2: Cosinor analysis of circadian clock rhythmicity in human BMSCs, ADSCs, and DPSCs following dexamethasone synchronisation.

Cell	Gene	Period (h)	*p* value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
BMSC	Bmal1*	23.9	0.007	−76	0.331	0.451	76.2
	Per2	20.2	0.076	−261	1.099	1.597	52.1
	Rev-erbA	26.0	0.391	−287	1.853	3.158	23.6
ADSC	Bmal1*	20.0	0.006	−289	1.264	1.885	77.4
	Per2*	23.4	0.021	−98	0.502	0.486	66.6
	Rev-erbA*	21.8	0.001	−314	0.611	0.914	85.1
DPSC	Bmal1	20.0	0.571	−9	0.325	0.720	14.9
	Per2	26.0	0.101	−176	1.089	1.071	47.9
	Rev-erbA	26.0	0.055	−266	4.952	4.938	56.2

*Significance according to the cosinor analysis software.

3.3. DPSCs Can Be Entrained by Rhythmic Mechanical Stretch Synchronisation. In order to find a novel method of synchronising the DPSCs, it was hypothesised that as these stem cells are encapsulated in a tight niche in the tooth but are still subjected to a substantial amount of mechanical stimulation, they could potentially be entrained using mechanical means, if not chemical. Therefore, both BMSCs, used as a positive control here as these cells were previously reported to respond to mechanical stretch, and DPSCs were seeded onto fibronectin-coated, flexible silicone chambers (Figure 4(a)) and subjected to 3 days of rhythmic cyclical mechanical stretch, undergoing 12 h of cyclical stretching followed by 12 h of relaxation. After this regime was completed, samples were collected in the absence of mechanical stimulation at two opposite temporal phases at either 20 h or 32 h following their last exposure to the stretch (omitting the first 12 h to exclude any transient effects) and the clock gene expressions were analysed (Figure 4(b)), in a similar manner to the experimental design following dexamethasone exposure. Here, in striking contrast to synchronisation with dexamethasone, the clock gene levels in DPSCs appeared to peak and trough in a characteristic antiphasic manner for *Bmal1* and *Per2,* respectively; in both BMSCs and DPSCs *Bmal1* peaked in expression at 20 h, in contrast to *Per2* which

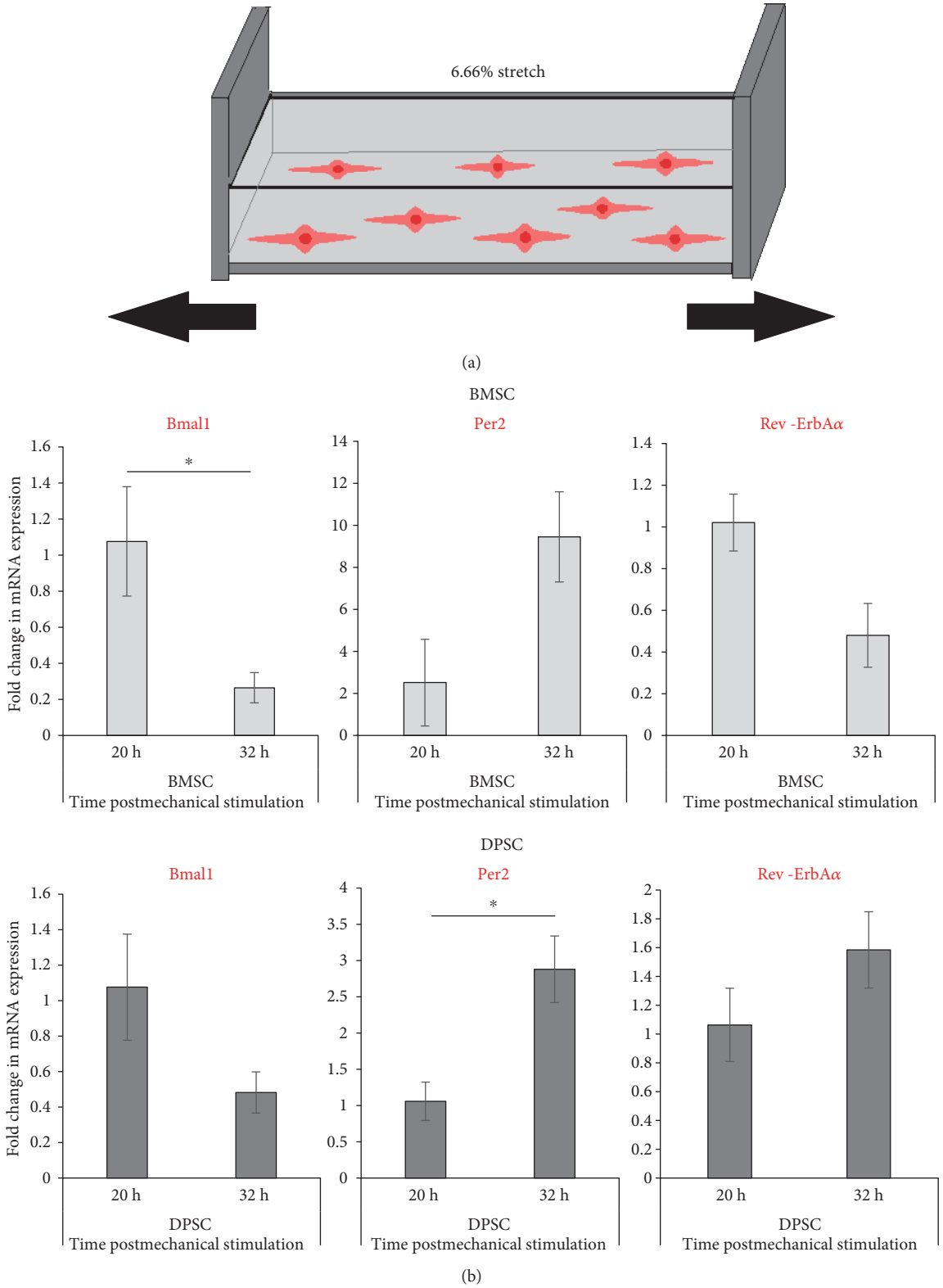

Figure 4: Clock gene expression in human adult stem cells following mechanical rhythmic stimulation paradigm. (a) Schematic diagram showing the setup of the mechanical stretch apparatus; cells were seeded into silicone chambers and stretched for 3 days using a unique uniaxial stretch rig with offset cams (1 Hz, 6.66% stretch, 12 h ON (stretch), 12 h OFF (rest)). (b) Quantitative RT-PCR analyses showing the expression levels of clock genes at two opposite circadian phases (20 h versus 32 h) following rhythmic mechanical stimulation protocol. Data are expressed as the mean of ΔCt ± SEM relative to the 20 h time point and normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent experiments, $^{*}p < 0.05$ (independent *t*-test).

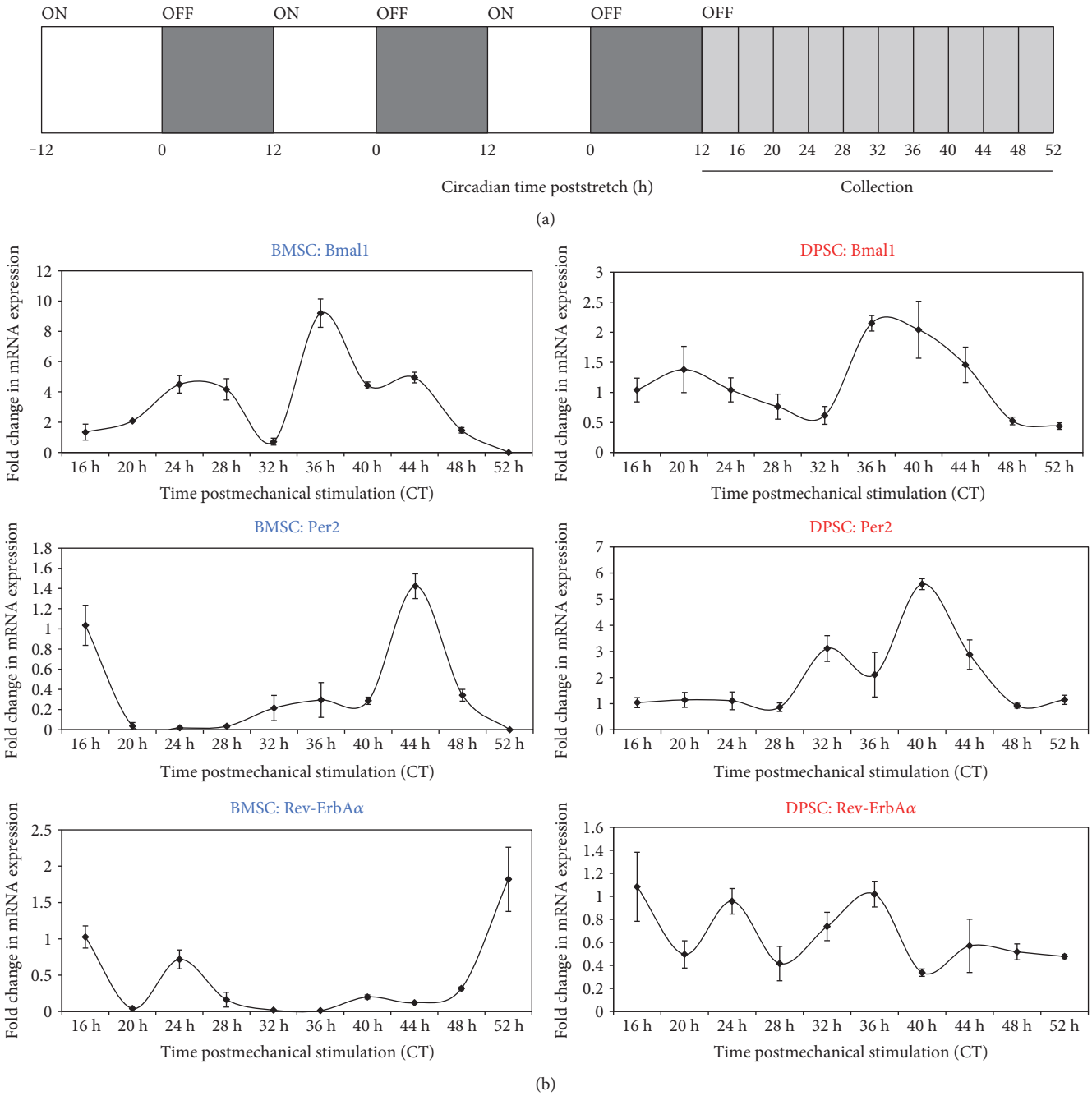

Figure 5: Circadian gene dynamics in human adult stem cells after rhythmic mechanical stimulation protocol. (a) Schematic diagram outlining the schedule of the 12 h ON : 12 h OFF (stretch : rest) regime followed by sample collection every 4 h between 16 h–52 h. (b) Quantitative RT-PCR analyses showing temporal expression profiles of clock genes following 3 days of rhythmic mechanical stimulation. Data are expressed as the mean +/− of ΔCt ± SEM relative to 16 h time point and normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent experiments.

peaked at the opposite circadian time point, being much higher in expression at 32 h, compared to 20 h.

Again, samples were then collected over a longer time course to further investigate these significant differences in gene expression and gain a finer temporal resolution over a 1.5 circadian cycle, the clock gene expressions were analysed every four hours 16 h to 52 h postrhythmic mechanical stimulation (Figure 5(a)). From these longer circadian mechanical time courses, it was evident that both DPSCs and BMSCs could produce oscillating expression of core clock genes using rhythmic mechanical stimulation. In the DPSCs, *Bmal1* expression was clearly at its trough at 32 h, which was the circadian time point at which *Per2* appeared to initially peak (Figure 5(b)). When analysed by cosinor periodogram (Table 3), *Bmal1* expression was shown to exhibit significant circadian rhythmicity in DPSCs ($p = 0.02$).

Table 3: Cosinor analysis of circadian clock rhythmicity in human BMSCs and DPSCs following synchronisation by rhythmic mechanical stretch.

Cell	Gene	Period (h)	p value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
BMSC	Bmal1	26.0	0.571	−346	1.754	3.568	21.6
	Per2	26.0	0.123	−51	0.428	0.343	45.0
	Rev-erbA	26.0	0.559	−171	0.316	0.397	15.4
DPSC	Bmal1*	20.0	0.020	−135	0.664	1.146	67.0
	Per2	26.0	0.132	−354	1.362	2.179	43.9
	Rev-erbA	21.2	0.505	−49	0.155	0.669	17.8

*Significance according to the cosinor analysis software.

Surprisingly, no circadian clock genes were found to show significant circadian rhythmicity in BMSCs.

3.4. The Pluripotency Marker Sox2 Shows Initial Cyclical Gene Expression following Glucocorticoid and Mechanical Synchronisation. Using both synchronisation mechanisms, it was next determined if any pluripotency stem cell markers displayed an initial circadian gene expression pattern following either glucocorticoid or rhythmic mechanical stimulation. To this end, *Sox2* was analysed and its temporal expression determined every four hours over one circadian day under the same synchronisation conditions as described above in the three stem cell types (Figure 6). *Sox2* showed initial cyclical expression in both BMSCs and ADSCs but not DPSCs following dexamethasone synchronisation. Strikingly, it was found that *Sox2* showed a very similar pattern of gene expression to that of *Rev-ErbAα* (Supplementary Fig. 2).

When analysed by cosinor periodogram (Table 4), *Sox2* expression was shown to exhibit significant initial circadian rhythmicity in ADSCs ($p = 0.007$) when exposed to dexamethasone synchronisation, whilst it was just short of significance in DPSCs following dexamethasone ($p = 0.074$) and cyclical mechanical stretch ($p = 0.087$). However, no significant circadian rhythmicity in *Sox2* could be observed in BMSCs, despite it showing a similar pattern.

4. Discussion

In this research, it has been shown that human stem cells derived from different human adult tissues did exhibit different levels and temporal expression patterns of core clock genes, stabilising loop genes and stem cell markers. It was also observed that the adult stem cells from different sources did indeed respond to circadian synchronising signals very differently as predicted; for instance, the BMSCs appeared to synchronise more readily in response to chemical stimulation than mechanical stimulation, but the DPSCs were much more responsive to entrainment by mechanical means. It has been experimentally evidenced that the more primitive human stem cells such as DPSCs have a different profile of the molecular circadian rhythm, in terms of both the relative levels of clock gene expression and the oscillating temporal patterns of gene expression after synchronisation by chemical means (i.e., dexamethasone exposure). It appears that the DPSCs are much less responsive to the dexamethasone synchronisation, which may be due to less developed circadian components which relay these signals. Indeed, their relative unresponsiveness to dexamethasone may be due to their early developmental origin from the migrating neural crest cells and their resulting ectomesenchymal composition. Therefore, upon DPSC differentiation, it is possible that they will gain responsiveness to circadian synchronisation and/or undergo maturation of the apparatus necessary for circadian gene oscillations. This is consistent with the research by Yagita et al. showed that embryonic stem cells do not have the capacity for circadian synchronisation by chemical means using forskolin; but upon differentiation and maturation, this ability can be gained [22]. In contrast, our results confirm previous findings that the more mature MSCs derived from the bone marrow and adipose tissue did exhibit robust clock rhythms that were responsive to glucocorticoid synchronisation and showed clear antiphasic relationships of the positive and negative arms of core TTFLs. For example, Wu et al. showed similarly oscillating expressions of clock genes in cultures of murine and human BMSCs in response to dexamethasone [19] whilst Huang et al. demonstrated that human BMSCs and ADSCs have circadian oscillations induced by serum shock [20].

As the DPSCs could not be synchronised by chemical means, a novel synchronising mechanism to which they may respond was sought. Circadian mechanosensory entrainment has been previously investigated by Simoni et al., who found that 12 h : 12 h cycles of vibration and silence, respectively, were sufficient to synchronise the daily locomotor activity of *Drosophila melanogaster* [18]. Moreover, it has recently been published that a mechanical environment of the epithelial stem cell niche within the mammalian mammary tissue controls the amplitude of the molecular clock oscillations [7]. Therefore, rhythmic mechanical stretch was utilised as an entraining factor for adult human stem cells using a uniaxial mechanical stretch apparatus. Following the stretch entrainment and subsequent analysis by cosinor periodogram, differences were observed in the phasing and period of the clock genes in the same stem cell types, highlighting how the entrainment mechanisms lead to different effects. It was observed that the DPSCs can be entrained by rhythmic mechanical stretch and appeared more responsive than the BMSCs; the *Bmal1* gene oscillations induced by rhythmic mechanical stimulation in the DPSCs resembled the temporal clock gene

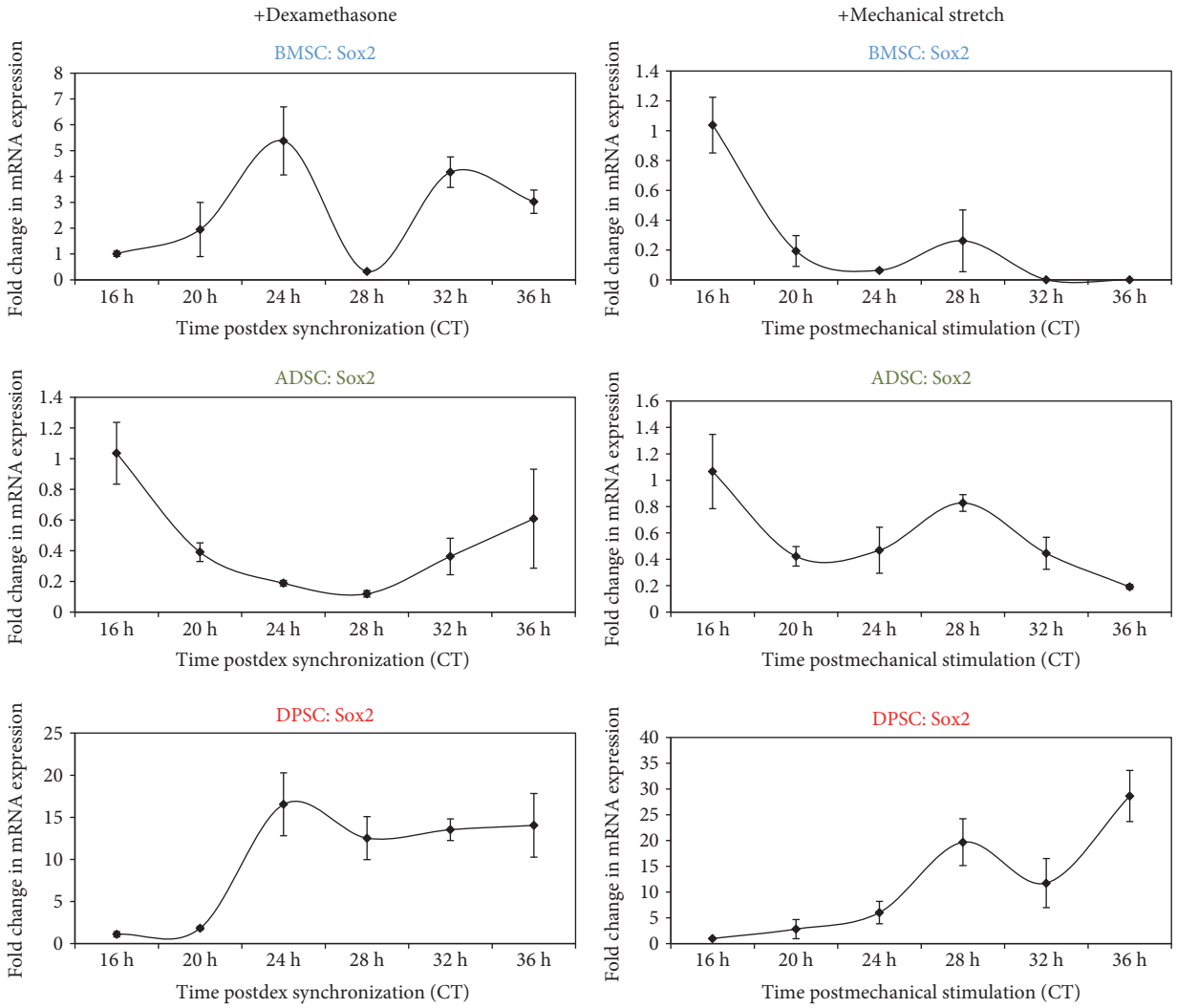

Figure 6: Human pluripotency marker SOX2 shows cyclical temporal gene expression in adult stem cells initially following dexamethasone or rhythmic mechanical stimulation. Quantitative RT-PCR analyses showing temporal expression profile of the pluripotency marker *Sox2* synchronisation with dexamethasone or rhythmic mechanical stimulation and collected every four hours over 1 circadian cycle (16 h–36 h). Data are expressed as the mean of ΔCt ± SEM relative to 16 h time point and normalised against the housekeeping gene *GAPDH*. Bars represent means ± SEM of 3 independent experiments.

Table 4: Cosinor analysis of pluripotency marker *Sox2* rhythmicity in human BMSCs, ADSCs, and DPSCs following synchronisation by dexamethasone and rhythmic mechanical stretch.

	Cell	Period (h)	*p* value	Acrophase (°)	Amplitude	Mesor	Robustness (%)
Dex	BMSC	26.0	0.658	−218	1.114	2.348	19.0
	ADSC*	21.8	0.009	−348	0.371	0.354	74.3
	DPSC	26.0	0.074	−261	6.463	7.800	52.5
Stretch	BMSC	26.0	0.541	−61	0.328	0.279	41.2
	ADSC	26.0	0.863	−107	0.119	0.569	9.3
	DPSC	26.0	0.087	−311	9.926	11.858	55.7

*Significance according to the cosinor analysis software.

expression patterns seen in BMSCs in response to dexamethasone. This may be due to the fact that *in vivo*, DPSCs experience significant mechanical stimulation in the form of jaw movement, occlusion forces, and hydrostatic pressures and are one of the few stem cell niches to also experience thermal shock and extreme temperature fluctuations and so are much more likely to respond to this form of stimulation. Moreover, when the initial expression profile of the

pluripotency marker *Sox2* was compared to the profile of the stabilising loop gene *Rev-ErbAα*, a strikingly similar pattern was observed following synchronisation with dexamethasone in BMSCs and ADSCs. In contrast, the rhythmic mechanical stimulation in DPSCs was able to induce the cyclical expression of the core clock genes as well as a pluripotency marker *Sox2*. These results therefore suggest a novel regulation of the *Sox2* gene which may be under both circadian and mechanical controls in different stem cell types.

The circadian clock in mammals has been extensively shown to have a key regulatory role on various tissue systems, including musculoskeletal tissues. It is therefore vital that such temporal regulation be taken into account when optimising and integrating any cellular/tissue constructs into the body, if one hopes for more successful tissue engineering strategies. For instance, it has previously been shown that the circadian regulation is significantly involved in the establishment of osseointegration under vitamin D regulation. Here, KEGG pathway analysis showed the potential association of the circadian rhythm with the success of implant osseointegration [27]. The circadian rhythm has also been shown to regulate coexisting populations of epidermal stem cells at opposite phases of the clock, which are differentially prone to activation by external stimulation [5]. Furthermore, research indicates that epidermal stem cells differ in their responsiveness to proliferation- and differentiation-like cues over a 24 h cycle [6], which may have vast implications in tissue engineering, where one cellular state may be favoured to encourage successful implant integration.

Overall, these findings suggest that the mechanism of entraining stem cell clocks by using each unique stem cell optimal synchronisation method offers an insightful way in which stem cells can be 'primed' to respond to the desired tissue engineering applications. With this in mind, mechanical entrainment of human adult stem cells allows for a noninvasive means by which the circadian clock in human adult stem cells can be directed and controlled whilst maintaining their appropriate clock timing, thus avoiding the need for additional exogenous chemical or thermal stimuli.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Review article

Mechanical stretch and chronotherapeutic techniques for progenitor cell transplantation and biomaterials

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ABSTRACT

In the body, mesenchymal progenitor cells are subjected to a substantial amount external force from different mechanical stresses, each potentially influences their behaviour and maintenance differentially. Tensile stress, or compression loading are just two of these forces, and here we examine the role of cyclical or dynamic mechanical loading on progenitor cell proliferation and differentiation, as well as on other cellular processes including cell morphology, apoptosis and matrix mineralisation. Moreover, we also examine how mechanical stretch can be used to optimise and ready biomaterials before their implantation, and examine the role of the circadian rhythm, the body's innate time keeping system, on biomaterial delivery and acceptance. Finally, we also investigate the effect of mechanical stretch on the circadian rhythm of progenitor cells, as research suggests that mechanical stimulation may be sufficient in itself to synchronise the circadian rhythm of human adult progenitor cells alone, and has also been linked to progenitor cell function. If proven correct, this could offer a novel, non- intrusive method by which human adult progenitor cells may be activated or preconditioned, being readied for differentiation, so that they may be more successfully integrated within a host body, thereby improving tissue engineering techniques and the efficacy of cellular therapies.

1. Introduction: human mesenchymal progenitor cell response to stretch

Precisely defining and controlling the fabrication of cell/tissue constructs so that they can be successfully delivered and integrated within a patient, is an essential consideration in cellular therapies. Adult progenitor cells are the current first choice, but there are many aspects of their environmental requirements that are still not completely understood. Mesenchymal stem cells (MSCs), which are multipotent progenitor cells [1], are frequently selected in order to replace and restore the function of deteriorated or damaged tissue. This is because of their ability to be pre-differentiated and seeded into biomaterials before their implantation, in order to ensure optimum delivery and integration of a specific cell type. MSCs are advantageous as they have the capacity to differentiate into a number of cell types including, but not necessarily limited to: fat, bone, tendon, muscle, skin, neural, cartilage, dentinogenic, marrow stroma and vascular cell types [2]. Therefore, one of the key goals in the fields of tissue engineering and regenerative medicine is to optimise the conditions for the MSCs, so that differentiation can be predetermined and terminally lineage specific upon providing appropriate stimulation. The most frequently utilised method of cellular MSC differentiation involves adding chemical agents, which can be supplemented into media, and allowing the cells to differentiate slowly over many weeks; ideally up to a month. However, although this is quite practical *in vitro*, it is not the case *in vivo*. Researchers and tissue engineers are therefore striving for a method to optimise the culture conditions so that constructs can be readied *in vitro*, before being implanted *in vivo*, where they should integrate rapidly and start to form the desired target tissue.

One stimulus which may fulfil both requisites is mechanical stimulation. MSCs have been previously shown to be highly mechanosensitive and therefore mechanical stimulation may present the ideal method to non-invasively steer their differentiation, as mechanical forces have the capability of influencing progenitor cell behaviour. In the body, various tissues are exposed to varying amounts of mechanical force, which then influences their

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formation and functionality; for example, in the body, MSCs found within adipose tissue (ADSCs), will be exposed to vastly different levels of external force compared to those found within the bone marrow (BM-MSCs), teeth (DPSCs), tendon or muscle. This mechanical physiological loading is vital in the maintenance of such tissue; for example, exercise, whereby there is an increase in physical perturbation in terms of load, the magnitude, and the frequency. This results in increases in bone and muscle mass [3], but decreases in physical loads, as experienced in the extreme case of space travel or following injury, that effects physical movement for example, spinal cord damage, tissues like bone and muscle will be lost and decrease [4].

There are many different types of force that may be applied to MSCs *in vivo* and *in vitro*. In the bone marrow alone, MSCs may be subjected to extrinsic stresses such as tension, compression and fluid movement induced shear stress, as well as intrinsic stresses such as substrate, extracellular matrix stiffness, and these are all thought to have individually significant potential and roles on the different differentiation pathways that an MSC may go down. However, the optimum conditions to control and reproducibly define lineage specific differentiation of MSCs remains unknown, and the optimum loading magnitude, duration, frequency and force type for different lineage specific differentiation pathway remain unspecified. In a review by Smith and Reilly (2012), the authors examine how each of following types of force may effect MSC maintenance and differentiation: stretching (tensile stress), hydrostatic pressure or platen abutament (compressive stress), fluid flow (shear stress), ultrasound, high frequency low magnitude displacement (vibration) and direct cell membrane magnetic stimuli, in both 2D and 3D culture systems [5].

There are several proposed methods that may explain how extracellular mechanical stimuli is converted into biochemical signals, which ultimately leads to the cellular changes seen post-stimulation. One such mechanotrandsuction mechanism implies cell membrane mechanoreceptors, including integrins, g- protein coupled receptors (GCPRs) and stretch activated ion channels as vital components of this signal transduction. Regarding integrins, it is thought that the mechanical force pulls on an integrin-ligand bond, which is then transferred across the cell membrane and alters the cytoskeletal structure. For stretch activated ion channels and GPCRs, it is theorised that the stretch or external force leads to deformation of the plasma membrane, which results in ion flux into and out of the cell through the receptors [6]. Indeed, when MSCs are strained in the presence of the stretch-activated cation channel (SACC) blocker, gadolinium chloride ($GdCl_3$), there is a reduction in the otherwise observed induction of collagen I expression [7], suggesting a role for these channels in the transduction of mechanical stimulation. In the case of fluid flow, it is also thought that the glycocalyx, a GAG-proteoglycan rich layer that surrounds the cell membrane, may create drag force when fluid passes over, which again results in plasma membrane deformation [8, 9]. A final proposed mechanotransuction mechanism, again relevant to fluid flow, insinuates the primary cilium as being a mechanosensor, as they have been shown to bend under fluid flow and contain various signalling receptors [10]. However, this review principally focuses on the effect of mechanical stretch or tensile stress in adult MSCs.

In vitro, mechanical stretch is usually applied using a mechanical stretch system available "off the shelf" commercially or utilising a custom-built device made to deliver uniaxial mechanical loading at varying frequencies and magnitudes. The application of one such custom system is demonstrated by Kurpinski and Song (2007) [11]. Uniaxial strain is typically selected over eqiaxial strain (Fig. 1) as it is thought to better mimic the type of mechanical strain exhibited by MSCs in the body. For example, research by Park *et al*. (2004) directly compare the effects of uniaxial *vs* eqiaxial strain in MSCs and find that the different modes induce different responses. Cyclic eqiaxial stretch is here shown to downregulate the smooth muscle differentiation markers SM α-actin and SM-22α, and decreases α-actin in stress fibres. In contrast, cyclic uniaxial strain transiently increases the levels of SM α-actin and SM-22α, suggesting that this method better mimics the type of mechanical strain experienced in MSCs and smooth muscle cells (SMCs) and may promote the differentiation of MSCs into SMCs [12].

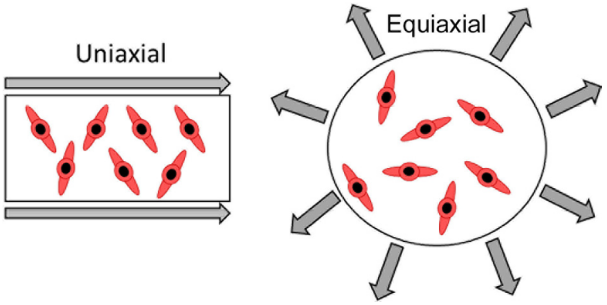

Fig. 1 - Comparison of uniaxial and equiaxial strain.

As is this case in this study, along with many others, silicone is usually the selected material used as a substrate to study the behaviour of cells under mechanical stimulation. It is selected due to its biological inertness, translucency, compliance and manipulability, and can be coated in fibronectin to allow for good cell adhesion and proliferation. Flexible silicone substrates can also be used to study the effects of mechanical stretch in 3D; research by O'Caerbhaill *et al*. (2007) shows that it can be constructed as a tube to form 'pseudovessels', whereby cells can be subjected to a combination of pulsatile flow, radial distension and shear stress. Here, the MSCs are shown to be mechanosensitive, and reorientate parallel to the direction of flow and adapt their morphology in response to the stretch and other forces that they are subjected to [13].

The effect of cyclic uniaxial strain on elastic substrates on the morphology of cultured cells has been studied in great detail. Stretching results in alterations in cell orientation and the cells tend to align perpendicularly to the load axis, in order to lessen the strain on their cell bodies and decrease the stretch of cytoskeletal elements and intercellular filaments [14, 15]. Mechanical stretch can also be investigated in conjunction with the addition of chemical agents, so that their effects can be compared with and without their addition, and with and without stretch. For example, Rashidi *et al*. (2012) combined stretch with growth factor treatment, and found a significant up-regulation of smooth muscle cell specific markers and alignment of cells perpendicular to the strain direction during loading time. They also observed cell elongation and F-actin fibres alignment and reorganisation [16]. The degree of morphological alteration can be seen by these changes in cell orientation and alignment, and both depend on strain amplitude [17], rate and duration [18]. Parankakh *et al*. (2017) sought to investigate the effects of different durations of cyclic stretch on cytoskeletal reorganisation and morphology of human MSCs in a stepwise manner, in order to closely study

morphological and phenotypic changes, as the two often occur simultaneously. The researchers found that different durations of stretch did influence the resulting changes observed [19]. Morita *et al*. (2013) also sought to define the optimum conditions for the cyclic stretching of human MSCs. Here, the authors used a 2D inhomogeneous membrane strain field, achieved by removing holes in one side of an elastic chamber, in a commonly used uniaxial stretching device and found the axial strain threshold of hBMSCs was 4.4 ± 0.3 % [20]. Once the optimum parameters for cyclic cellular stretch are defined, this will offer a non-invasive methodology by which human MSCs may be controlled and manipulated, so that they can be optimised for tissue engineering technologies.

2. The effect of tensile strain on progenitor cell proliferation and differentiation

2.1. Proliferation

The effects that radial distention, or mechanical stretch, can bring about on human adult progenitor cell proliferation have been investigated in a plethora of studies, with each varying the parameters in different ways. For example, in rat MSCs isolated from bone marrow and subjected to cyclical eqiaxial stretch, the OD values of rat MSCs increase in a time-dependent and magnitude-dependent manner after being exposed to 2-8% stretch, for within 15-60 min, at a frequency of 1 Hz, suggesting that cell proliferation increases following short term stretch. The expression of c-fos, a regulator of cell proliferation, in these cells is also significantly higher when the cells are stretched (1 Hz, 8% strain and 60 min) compared to static controls; this suggests that mechanical stretch alone could influence cell growth and proliferation [21]. In human cells, fibronectin coated silicone chambers have been utilised to stretch human MSCs, and here it was also documented that the short time application of strain did affect proliferation. Protein coating using fibronectin, however, did not influence MSC proliferation. The authors also looked at the modulation of stretch magnitude, frequency and duration, and found that a frequency of 1 Hz was most effective at stimulating human MSC proliferation. At a frequency of 1 Hz and durations of 15, 30, 60 min, 5% strain was found to significantly increase MSC proliferation. Proliferation was also enhanced at 10% strain, 1 Hz for 15 and 30 min durations, but proliferation decreased at 60 min. At 1 Hz, 15% strain, proliferation was reduced following 15 min durations, but increased following 30 and 60 min durations. Long-time strain applications (12 and 24 h) were found to block proliferation [22]. Collectively, both of these studies highlight how crucial getting strain application conditions optimal are in getting the desired result, and demonstrate that mechanical strain does have the potential to influence MSC proliferation.

The mechanisms that underlie the transduction of mechanical stretch information onto cellular proliferation have been investigated. In a follow up study using the same rat MSCs as mentioned previously, it has been reported that although no change in the expression of total extracellular signal- regulated kinase 1/2 (total ERK1/2) at the protein level was observed, the phosphorylation of ERK1/2 was increased after stretch. When rat MSCs are treated with inhibitors of ERK1/2 activity, there was a suppression of stretch-induced increase in phosphorylated ERK1/2 and mRNA expression of c-fos, along with an abolition of the increase in stretch-induced proliferation, suggesting that ERK1/2 is crucial in the stretch-induced proliferation of rat MSCs [23].

2.2. Myogenesis

In a study by Ghazanfari *et al*. (2009), the authors found data that suggested that cyclic strain not only enhanced proliferation, in agreement with the studies above, but also that cyclic strain lead to increases in smooth muscle α-actin, reoriented actin fibres and led to the differentiation of human MSCs into SMCs, without the addition of growth factors [24], implying that mechanical stress can be used to enhance smooth muscle myogenesis [25]. This can also be observed in skeletal muscle myogenesis, which is logical as both foetal and adult skeletal muscle are constantly subjected to biomechanical forces in the body. One study used a 10% uniaxial strain at 1 Hz on human MSCs cultured on collagen-coated silicone substrates and found that, following loading, there was a rearrangement of cells and initiation of myogenic differentiation, as determined by levels of MyoD and MyoG mRNA levels, both of which are key factors in myogenesis, indicating that cyclic strain may be used to differentiate progenitor cells myogenically [26].

Cardiomyocytes are also subject to cyclic strain in the body, as induced by the rhythmic beating of the heart. When rat BM-MSCs were subjected to cyclic strain application, it has been shown that this may be sufficient to induce cardiomyogenic differentiation in itself, as can be confirmed by the induction of cardiomyocyte-related markers [27]. This too suggests that mechanical stimulation could be a novel mechanism to control adult progenitor cell differentiation.

2.3. Osteogenesis

Another part of the body in which adult progenitor cells reside and are subjected to varying amounts of mechanical conditioning is in bone; as previously mentioned, BM-MSCs are subjected to external forces such as tension, compression and fluid-induced shear stress. Mechanical stimulation is so crucial in bone formation and maintenance that "distraction osteogenesis" has been created; an active process for bone regeneration using mechanical stimuli. This process has been mimicked experimentally, whereby rat MSCs are subjected to short periods of cyclica mechanical strain (40 min, 2000 microstrains). Following this, it can be observed that mechanical strain promotes ALP activity, as is vital in the initiation of bone formation, and enhances bone marker genes Cbfa1 and Ets-1 expression, showing that mechanical strain may act as a stimulator of osteogenic differentiation [28, 29]. Kearney *et al*. (2010) also document an increased expression of osteogenic markers following cyclic tensile mechanical strain of 2.5% at 0.17 Hz for 1–14 days; the osteogenic markers Cbfα1, collagen type I, osteocalcin, and BMP2 are temporally expressed. However, this strain-induced increase in BMP2 can be reduced by the inhibitors of the kinases, ERK, p38, and PI3 kinase. The authors also found that this long term application of strain reduced the proliferative capacity of MSCs, supporting the notion that although short-term strains may increase proliferation, long-term strains do not appear to [7]. It has also been shown that both osteoblastogenesis and osteoclastogenesis are influenced by mechanical stimulation, showing how far reaching the effects of mechanical load are in bone formation and maintenance [30, 31].

There are many signalling pathways that have been implicated regarding how mechanical stimulation effects osteogenesis, for example, one study shows that the onset of osteogenic differ-

entiation following mechanical stimulation may be dependent on ERK1/2-Runx2 signalling [30]. Another study investigating human BM-MSCs following stretch, reports an induction of FosB, a member of the AP-1 family of transcription factors which regulate osteogenic differentiation and bone formation, in a time- and stretch-dependent manner [32]. The p38MAPK-osterix pathway has also been implicated; intermittent stretching has been found to promote the expression of osterix mRNA, along with ALP, collagen type I and osteocalcin, and the protein levels of osterix and phosphorylated p38MAPK were elevated following stretch. When osterix was silenced, a reduction in the levels of ALP, collagen I and osteocalcin mRNA were also observed, showing that this pathway may have an important role in stretch-induced osteogenesis [33]. A further factor that has been implicated in osteogenic differentiation following tensile strain, without the addition of osteogenic supplements, is BMP-234. Interestingly, even adipose derived MSCs (ADSCs) can be induced to express increased levels of BMP-2 and Runx2 following cyclic tensile strain of 6 hours, underlining the suggestion that cyclic tensile stretch may modulate osteogenic differentiation, *via* the BMP-2 signalling pathway [35].

In direct contrast to the above notion that mechanical stretch results in increased osteogenesis, this does not appear to be the case in all MSCs. MSCs derived from tooth dental pulp (DPSCs) appear to show the opposite, and instead exhibit increased levels of proliferation and decreased osteogenic potential following uniaxial mechanical stretch [36]. Indeed, when human DPSCs were exposed to cyclic tensile stretch, the expression of osteogenic marker genes and proteins including BMP-2, osteocalcin and ALP were reduced, along with the odontogenic marker genes and proteins DSPP, DSP and BSP, suggesting that cyclic tensile stretch inhibits both osteogenic and odontogenic differentiation in DPSCs [37]. The differences in the response of the MSCs depending on their tissue of origin may be due to the fact that DPSCs reside within a unique niche in the body, where they are subjected to extreme mechanical stresses by jaw movement, occlusal forces and hydrostatic pressures, and are one of the few progenitor cell niches to also experience thermal shock and extreme temperature fluctuations, and so are likely to respond differently to mechanical stimulation. Apart from in these unique DPSCs, the effect of mechanical stimulation on the osteogenic differentiation capabilities of MSCs is profound, even having such a strong impact that it forces even adipose derived progenitor cells (ADSCs) to undergo osteogenesis and inhibits their adipogenic differentiation potential, even when cultured in the presence of adipogenic medium [38].

2.4. Adipogenesis

The theory that mechanical stimulation inhibits adipogenesis has been widely explored in scientific research. Initially investigated in C2C12 myoblasts, myoblast-to-adipocyte differentiation was found to be significantly inhibited by cyclic mechanical stretch (20% elongation), which was seen alongside an enhanced expression of Wnt10b mRNA. By inhibiting Wnt signalling with a Wnt ligand, sFRP-2, this inhibition of adipogenesis was abolished, showing that mechanical stretch may inhibit adipogenesis through Wnt signalling [39]. Normally, when MSCs are cultured in adipogenic medium, they express peroxisome proliferator-activated receptor γ (PPARγ) and adiponectin mRNA and protein, and accumulate intracellular lipids. However, when mechanical strain was applied to the MSCs for 6 h per day for 5 days, the expression of PPARγ and adiponectin was inhibited and the decrease seen in active and total β-catenin typically exhibited during adipogenesis was prevented. Mechanical strain was also thought to inactivate glycogen synthase kinase-3β, suggesting that stretch transmits anti-adipogenic signals *via* this pathway, by stimulating a durable β-catenin signal [40]. Another signalling pathway that is affected by mechanical stretch and has been implicated in the commitment of MSCs towards adipocytes is the BMP pathway, as it is thought that stretching causes a downregulation of BMP4 induced MSC adipogenesis. When MSCs were pretreated with BMP4 and then subjected to tensile stretch conditions (10% strain, 0.25 Hz, 120 min/day), it was found that the stretch supressed BMP4 induction of MSC adipogenesis and downregulated PPARγ, C/EBPα and aP2 adipogenic transcription markers, and lipid accumulation. Here, it was found that the cellular stretch did not affect BMP4-inducted activation in Smad or p38, as which this pathway would normally signal through Smad 1/5/8 and p38MAPK, but instead the tensile stretch caused significant ERK1/2 phosphorylation. When ERK signalling is blocked, the stretch suppression of BMP4-induced MSC adipogenesis was significantly deteriorated, suggesting that stretch suppresses BMP4-induced adipogenesis *via* upregulating ERK [41].

However, this may be specific to only BMP4-induced adipogenesis, as Li *et al*. (2015) reported that mechanical stretch did indeed upregulate levels of phosphorylated Smad2, along with PPARγ-2, adiponectin and C/EBPα. Here, the authors found that by pretreating MSCs with TGFβ1/Smad2 pathway antagonists suppressed this increase in Smad2 phosphorylation, whereas pretreatment with TGFβ1/Smad2 signalling agonists facilitated the inhibitory effect of stretch on the adipogenic differentiation markers, suggesting that the anti-adipogenic effects of stretch are mediated in some way by the activation of the TGFβ1/Smad2 signalling pathway [42].

2.5. Tenogenesis

Another way that human adult MSCs are thought to possess clinically useful tissue-regenerative properties is for the process of tendon tissue engineering, whereby they may be used to generate tenocytes for use in cell therapy. One way that this could be controlled and utilised is by using mechanical stretch techniques. At low-magnitude stretch, MSCs express osteogenesis differentiation marker genes, in agreement to the section above, but, interestingly, when stretched using high- magnitude stretch for long periods, the tendon and ligament related genes are instead upregulated. For example, after being stretched at 10% magnitude for 48 h, the expression of tenogenesis markers type I collagen, type III collagen, and tenascin-C are significantly increased [43]. This research is supported by findings from Morita *et al*. (2013), who demonstrated that a cyclic uniaxial stretch magnitude of 10% was the most efficient magnitude for inducing the differentiation of human BM-MSCs into tenocytes [44].

In the generation of tendon- or ligament-like tissue, MSC-seeded 3D collagen gels are frequently utilised under static or dynamic tension, the latter of which leads to enhanced tendinous tissue development. Cyclic stretching has been found to be beneficial to this 3D system as it allows for the expression of the tendon marker scleraxis to be maintained, where it would have normally dropped off in expression, and there are vast changes in matrix deposition and remodelling activity under dynamic loading conditions. Furthermore, differential regulation of MMPs can be observed, with little change in collagen mRNA levels, giving

insight to the mechanisms of tenogenesis following mechanical stimulation of MSCs [45].

The pathways that underlie how mechanical stretch impacts tenogenesis have also been investigated. Following mechanical stimulation, RhoA/ROCK and FAK have been found to regulate the mechanical- stretch induced realignment of human MSCs through cytoskeletal organization. Furthermore, after being subjected to mechanical stimulation, both RhoA/ROCK and cytoskeletal organization have been found to be essential in the phosphorylation of FAK at Tyr397. This phosphorylation process can be blocked by inhibiting either RhoA/ROCK, cytoskeletal organisation or FAK, implicating that these three are all vital components in the signalling network that senses mechanical stretch and then drives the tenogenic differentiation of human MSCs46. Moreover, when calcium signalling is disrupted in human MSCs, by blocking stretch activated calcium channels (SACC) with gadolinium, before and whilst they are subjected cyclic uniaxial tensile stretching, almost all tenogenic differentiation marker expression enhancement and ECM production is lost, suggesting that SACC also act as a mechanosensor in the strain-induced model of human MSC tenogenesis [47].

2.6. Angiogenesis

The final major differentiation process which has been investigated in MSCs following mechanical stretch is regarding angiogenesis. Mechanical stretch has been found to increase the angiogenic capacity of MSCs *via* VEGFA induction, as well as increasing the survivability of MSCs under nutrient deprivation. The proposed mechanism by which both of these changes is thought to occur is *via* the activation and manipulation of NFκB; when subjected to stretch, there is an increase of nuclear localization of NFκB activity p65, which coincides with the increase in VEGFA expression and apoptosis resistance. When NFκB activity is inhibited, these pro-angiogenesis and anti-apoptosis functions are blocked, highlighting the significance of NFκB in the pro-angiogenic response following mechanical stretch48.

There are clearly a vast number of ways by which mechanical stretch can influence the many different differentiation pathways that MSCs are capable of, and the implications for increasing the therapeutic potential of MSCs are extremely exciting. Whether stretch is applied as a preconditioning technique or loading throughout implantation, for long or short durations and at high or low magnitudes and frequencies, there are clearly many ways it can be utilised, which will only become further understood in the years to come.

3. Other effects brought about by mechanical strain

In agreement with the above study, which demonstrated that stretch can have anti-apoptotic effects on human progenitor cells, work by Kearney *et al*. (2008) has also showed that mechanical strain can have wide reaching effects on the maintenance of MSCs; here the authors also reported that cyclic uniaxial stretch affects the apoptosis of MSCs. However, the authors instead reported that strains of 7.5% or greater, over a duration of three days, lead to an induction of apoptosis, with maximal apoptosis occurring at 10% of strain [49]. High levels of mechanical strain are thought to negatively impact MSCs as extreme stretch leads to oxygen free radical disequilibrium; when BM-MSCs from children were loaded with cyclic tensile strain, >12% magnitude stretch was found to enhance reactive oxygen species (ROS) synthesis, decrease the activity of superoxide dismutase and increase levels of malondialdehyde, in a time and magnitude dependent manner [50].

Another cellular function impacted by the mechanical microenvironment is intracellular calcium dynamics, as calcium oscillations can be effected by external mechanical cues. Prolonged mechanical stretch leads to intracellular calcium oscillations in human MSCs, as mediated by the cytoskeletal support, actomyosin contractility and phospholipase C (PLC) activity, showing another way by which the mechanical environment can regulate cellular functions [51].

Cyclic substrate deformation can also affect MSCs in terms of the matrix structure and formation of the cells. For example, equibiaxial cyclic strain (3%, 0.25 Hz) has been found to increase matrix mineralisation as well as inhibiting proliferation; here the strain was found to activate ERK1/2 and p38 MAPK pathways. When ERK1/2 was inhibited, this lead to an attenuation of calcium deposition, suggesting that strain-induced mineralisation was mediated by ERK1/2 signalling [52]. Furthermore, an increase in total collagen synthesis has been observed when MSCs were subjected to stretch conditions [53]. Research by Heo *et al*. (2015) found that short term dynamic loading causes increases in chromatin condensation, mediated by acto-myosin based cellular contractility and the activity of the histone-lysine N-methyltransferase EZH2. These changes stiffened the MSC nucleus, making it less deformable when subjected to stretch conditions. The authors identified ATP release and calcium signalling induced by mechanical stretch as the mediators of this condensation process. Following being subjected to stretch, it was also found that the cells retained a 'mechanical memory', whereby the cells exhibited higher amounts of chromatin condensation that persisted for longer times, when subjected to increasing numbers of loading events and strain levels, which may be a mechanism by which the MSCs sensitize themselves to future loading events [54]. Collectively, these studies emphasise just how far reaching the effects of mechanical stretch can be on MSC physiology and maintenance, and show how tensile loading can affect not only cellular differentiation, but also affect apoptosis, ROS, calcium oscillations and matrix mineralisation. A summary of the effects on cellular processes brought about by mechanical strain can be found below (Fig. 2).

4. Mechanical stretch and biomaterials: physiological loading and the role of the circadian rhythm

Unsurprisingly, the effects that mechanical loading can have on a biomaterial seeded and optimised with human MSCs are widespread and well documented. There are an enumerable amount of new materials being created and optimised constantly, and each of these may be impacted and improved by subjecting the material, and cells within, to mechanical load, or by preconditioning the cells with mechanical strain. For example, stem cell-collagen sponge constructs increase in stiffness following mechanical stimulation. Two weeks of *in vitro* mechanical stimulation was here found to increase collagen type I and type III gene expression and an increase in linear stiffness and linear modulus [55]. Long term dynamic compression of MSC-seeded hydrogel constructs initiated after chondrogenesis induction has also been found to enhance

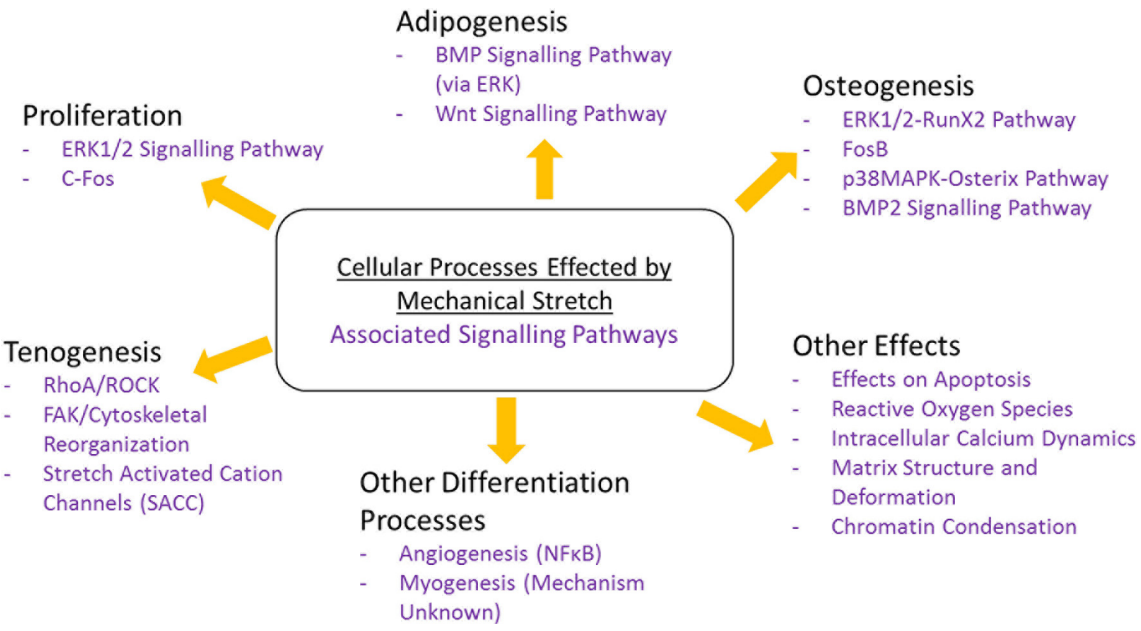

Fig. 2 - Summary of the common changes seen in adult stem cells following mechanical stretch.

matrix distribution and the mechanical properties of MSC- seeded constructs [56], showing again how mechanically stimulating cells in constructs can improve function. Electrospun fibres, which are advantageous in connective tissue regeneration in terms of their durability, deformation capacity and effects on cell adhesion, orientation and gene expression, experience an increase in cell alignment when subjected to cyclic loads, suggesting that cell morphology within a construct is also influenced by the mechanical environment [57].

However, this is not the only physiological process that is effected by the mechanoenvironment of MSCs following implantation into biomaterials. Research by Mengatto *et al.* (2011) explored osseointegration implant failure, to see what impacted the establishment of dental and orthopaedic implant association with bone tissue. To do this, the authors used a vitamin D deficiency model of implant failure in rats and evaluated changes in gene expression using whole genome microarray analyses. KEGG analysis was utilised and it was found that 103 genes were significantly modulated by implant placement and vitamin D deficiency, with the highest z-scores assigned to components of the circadian rhythm pathway, including neuronal PAS domain 2 (NPAS2) and period homolog 2 (Per2). Furthermore, NPAS2 and Aryl hydrocarbon receptor nuclear translocator-like (ARNTL/Bmal1) were found to be upregulated, and Per2 showed a complementary expression pattern following the vitamin D model. This study suggests that the circadian rhythm may have a key role in the establishment of osseointegration under vitamin D regulation [58].

The circadian rhythms are important evolutionarily conserved cellular mechanisms which are a subset of biological rhythms, they have a period, *i.e.* the time taken to complete one cycle, of approximately 24 hours. The foundation of circadian rhythmicity research is often dated back to the work done by Colin Pittendrigh and Jurgen Aschoff. These pioneers are thought to have defined the basis of circadian entrainment. Pittendrigh (1960) showed that deviation from the 24-hour cycle provides a mechanism for alignment for the internal time-keeping system, allowing the rhythm to be "reset" where necessary [59]. The molecular mechanism used to generate self-sustained circadian rhythms rely on a network of auto-regulatory feedback loops of transcription and translation to drive circadian expression patterns of the core clock components [60]. In mammals, this is carried out by the primary feedback loop by the basic-helix-loop-helix transcription factors CLOCK and BMAL, which form the positive arm of the molecular clock. When these two proteins heterodimerise, they are able to bind to cis-regulatory enhancer sequences called E-boxes on target gene promoters, and so initiate transcription [61, 62]. Target genes include Period (Per1, Per2 and Per3) and Cryptochrome (Cry1 and Cry2), which themselves heterodimerise and translocate to the nucleus to affect gene expression. However, unlike the CLOCK:BMAL complex, they have a negative feedback effect, and repress their own transcription by inhibiting the CLOCK:BMAL complex [63, 64]. The CLOCK:BMAL heterodimers induce a stabilising regulatory loop by activating the transcription of retinoic acid-related orphan nuclear receptors, REV-ERB and ROR. These bind to retinoic acid-related orphan receptor response elements (ROREs), which are present in Bmal gene promoter. REV-ERBs repress transcription of Bmal, whereas RORs activate it [65]. These auto- regulatory loops constitute a molecular clock machinery and take approximately 24 hours to complete a cycle (Fig. 3).

Similarly to Mengatto *et al.*'s work, Hassan *et al.* (2017) also found that the circadian rhythm may influence the fate of certain biomaterials. In this instance, the circadian rhythm of BM-MSCs was found to be induced by Titanium (Ti) -based biomaterials with complex surface modifications (Ti biomaterials). When human MSCs were cultured on Ti biomaterials, it was found that Per1 expression was suppressed, whereas NPAS1 was upregulated. BM-MSCs were then harvested Npas2 knockout mice, it was found that this did not rescue the Ti biomaterial-induced reduction of Per1 expression, and did not affect Per2, Per3, Bmal1 or Clock

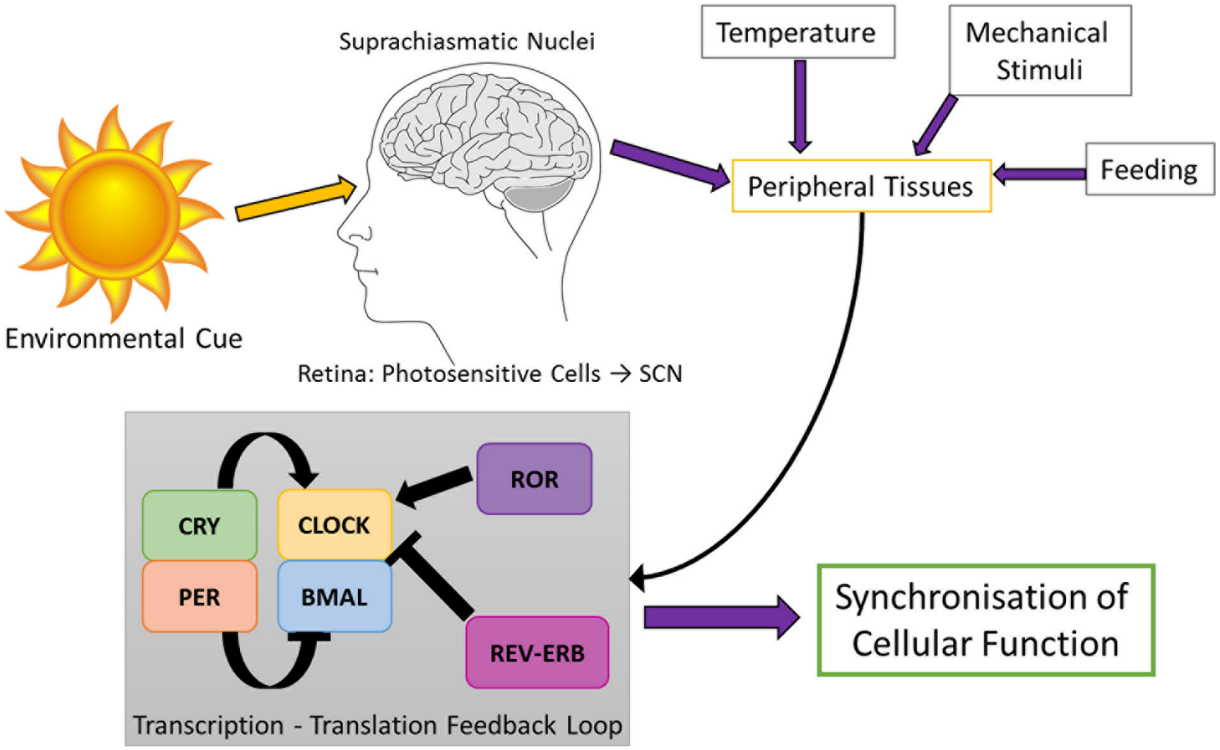

Fig. 3 - Summary Schematic of the Circadian Rhythm. Light enters the brain through the retina and is relayed from the photosensitive cells to the SCN. The SCN then signals to a number of peripheral tissues and cells all over the body where it feeds into the molecular clock autoregulatory feedback loop, which act on target genes and lead to the synchronisation of cellular function.

expression, suggesting that the Ti biomaterial-induced increase in NPAS1 expression was independent of the changes in circadian component expression. The authors also found that vitamin D supplementation significantly increased Per1 expression in BM-MSCs66. Taken together, both of these studies suggest that the circadian rhythm of BM-MSCs may influence the integration of Ti biomaterials into bone, and therefore should be taken into account in future biomaterial research.

5. Using the cellular mechanoenvironment to control the circadian rhythm of adult progenitor cells

The idea that mechanical stimulation could be used to direct and control the circadian rhythm was first investigated by Simoni *et al*. (2014). Drosophila melanogaster were here exposed to 12 hour: 12 hour cycles of vibration and silence, and it was found that this was sufficient to entrain and synchronise their behaviour and daily locomotor activity. In order for this to occur, the Drosophila required both a functional clock and functional chordotonal organs, as the mechanosensory input pathway to the fly's circadian clock required signalling from the chordotonal organs in order to synchronise their circadian rhythm [67]. This exciting research then led to other findings from our own group; it was published that different human adult progenitor cells have peripheral circadian rhythms of their own and the cells are capable of being synchronised on a molecular level by a number of different means, including both by chemical and mechanical stimulation. Human progenitor cells derived from tooth dental pulp, subcutaneous adipose and bone marrow were all exposed to synchronisation by dexamethasone, a synthetic glucocorticoid, and it was found that the more mature ADSCs and BM-MSCs could be readily synchronised in response to this treatment. The more primitive DPSCs, however, were less able to respond to this form of entrainment, which could again be due to their unique developmental origin. Next, the different progenitor cells were exposed to cyclic tensile stretch of 12 hour: 12 hour cycles of stretch and relaxation (6.66% stretch, 1 Hz, 12 h/day) for three days. This was found to be sufficient to entrain the progenitor cells of different tissue sources, including the DPSCs, showing the diverse functionality that mechanical stretch has to offer [68]. This offers a novel, non-intrusive methodology by which the circadian rhythm of progenitor cells can be poised and made ready for implantation.

The circadian rhythm of adult progenitor cells has also been investigated in response to the mechano- chemical stiffness of the cellular microenvironment, as circadian clock genes have been previously linked to mammary progenitor cell function. Notably, it has recently been published that the mechanical environment of the epithelial progenitor cell niche within mammary tissue controls the amplitude of molecular clock oscillations, which can be altered upon environmental and genetic clock disruption. Moreover, cell-matrix interactions do indeed play a key role in regulating circadian biology, and tissue stiffening is now thought to suppress the mammary circadian clock *in vivo*, where the mammary clock may be controlled by the periductal extracellular matrix. Mechanistically, vinculin, a tension sensing cell-matrxc adhesion molecule, and the Rho/ROCK pathway, which transmits

extracellular matrix stiffness signals into cells, are both thought to influence the regulation of the circadian clock. Furthermore, by disrupting the circadian clock of mammary progenitor cells, this leads to disruptions in the self-renewal capacity of the mammary epithelia, again highlighting the key roles of the mechano-environment and the circadian clocks of the epithelial stem cell niche in progenitor cell function [69].

6. Conclusion

In this review, we have explored the broad ranging effects that mechanical strain can have on adult progenitor cell activation and maintenance. It has been shown that tensile stretch has the capacity to influence not only adult progenitor cell proliferation and differentiation into a number of lineages relevant to cell based tissue engineering and regenerative medicine today, but they can also have profound effects on progenitor cell homeostasis, the optimisation of biomaterials and even the circadian rhythm. Now is an extremely exciting time for mechano-biology, which will only continue to grow in terms of the knowledge base, along with realising the potential impacts and significance that have already been experimentally evidenced with respect to controlling and defining progenitors cells, the implications for future regenerative medicine and tissue engineering are extremely positive and optimistic looking.

Conflicts of interest

The authors declare no conflict of interest.

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REVIEW ARTICLE

Adult stem cell maintenance and tissue regeneration around the clock: do impaired stem cell clocks drive age-associated tissue degeneration?

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Abstract Human adult stem cell research is a highly prolific area in modern tissue engineering as these cells have significant potential to provide future cellular therapies for the world's increasingly aged population. Cellular therapies require a smart biomaterial to deliver and localise the cell population; protecting and guiding the stem cells toward predetermined lineage-specific pathways. The cells, in turn, can provide protection to biomaterials and increase its longevity. The right combination of stem cells and biomaterials can significantly increase the therapeutic efficacy. Adult stem cells are utilised to target many changes that negatively impact tissue functions with age. Understanding the underlying mechanisms that lead to changes brought about by the ageing process is imperative as ageing leads to many detrimental effects on stem cell activation, maintenance and differentiation. The circadian clock is an evolutionarily conserved timing mechanism that coordinates physiology, metabolism and behavior with the 24 h solar day to provide temporal tissue homeostasis with the external environment. Circadian rhythms deteriorate with age at both the behavioural and molecular levels, leading to age-associated changes in downstream rhythmic tissue physiology in humans and rodent models. In this review, we highlight recent advances in our knowledge of the role of circadian clocks in adult stem cell maintenance, driven by both cell-autonomous and tissue-specific factors, and the mechanisms by which they co-opt various cellular signaling pathways to impose temporal control on stem cell function. Future research investigating pharmacological and lifestyle interventions by which circadian rhythms within adult stem niches can be manipulated will provide avenues for temporally guided cellular therapies and smart biomaterials to ameliorate age-related tissue deterioration and reduce the burden of chronic disease.

Keywords Adult stem cells · Circadian rhythms · Aging · Proliferation · Differentiation · Mechanical stimulation

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An introduction to adult stem cells

Pioneering experiments in stem cell research are often credited to Canadian scientists Till and McCulloch in 1961–1963, who were the first to carry out clonal colony formation assays on hematopoietic stem cells (HSCs) in murine bone marrow, indicating their

multipotency (Becker et al. 1963). Since then, stem cell research has progressed exponentially, with numerous scientific breakthroughs. In this review, we will introduce some of the well-studied as well as emerging mechanisms regulating adult stem cell function and how these become affected during ageing. Recently, there has been an increased interest in several research areas pertaining to stem cell function in 3D environments including the role circadian clocks play in stem cell physiology (temporal regulation) as well as the role of mechanotransduction processes in modulating stem cell function (spatial regulation). This review aims to bring together developments in these exciting research fields and reveal important areas for novel research to be conducted. Further insights into these spatio-temporal mechanisms will allow better understanding of environmental cues that regulate stem cell physiology in vivo and aid future design of new stem cell therapies for age-related diseases.

Stem cells are characterized by their extraordinary capacity to both self-renew through cell divisions and to differentiate into a wide range of tissue-specific cells in response to endogenous or external stimuli. It is these regenerative roles that stem cells within each tissue niche carry out to repair tissues following injury/disease and maintain tissues throughout lifecourse. Indeed, adult stem cells (ASCs), which can be more appropriately referred to as adult progenitor cells, are being used to treat an ever-increasing array of human conditions, varying from heart disease to leukaemia. But in order for ASCs to become a therapeutic reality, these tissue regenerative processes must fully be understood, and the environmental factors that regulate this regeneration process fully uncovered, which is vital for the repair and replenishment of most tissues in the body.

Adult stem cell (ASC) classification has become highly complex since the original terms 'haematopoietic' and 'mesenchymal' covered the main types of these pluripotent cells. Currently, they tend to be classified based on their tissue of origin and differentiation potential, and one should perhaps increasingly replace the use of the term adult 'stem cells' in favour of adult 'progenitor cells'. Adult progenitor cells, being somatic stem cells, can be derived from all parts of the body, where they are found in tissue-specific stem cell niches. Progenitor cells have been derived and utilised from cord and peripheral blood, blood vessels and bone marrow (mesenchymal and hematopoietic stem cells), the brain (neural stem cells), skin (epidermal stem cells), skeletal muscle (muscle satellite cells/myogenic stem cells), teeth (dental pulp stem cells), heart (cardiac stem cells), gut (intestinal stem cells), liver (hepatic stem cells), ovarian epithelium (ovarian stem cells), breast (mammary stem cells) and testis (testicular stem cells).

ASCs are multipotent and are, by that definition, limited to giving rise to different cell types from their tissue of origin. They divide either symmetrically to produce two identical cells which self-renew, proliferate and expand in number, or asymmetrically to produce one identical stem cell and one committed daughter cell which maintain progeny population (Fig. 1). The form of division that occurs depends on developmental and environmental signals. It has been suggested that most ASCs have the ability to switch between asymmetric and symmetric division models, and that the balance between the two is often altered in disease states (Morrison and Kimble 2006).

When comparing embryonic stem cells to ASCs, embryonic stem cells (ESCs) are pluripotent, meaning that they are capable of differentiating into any one of the three germ layers; endoderm, mesoderm or ectoderm. Since their discovery, there has been a great interest in their use in regenerative medicine and tissue engineering, due to their pluripotent differentiation capabilities. However, the pluripotency of ESCs makes it difficult to direct their differentiation in a reliable, long term and reproducible manner. Furthermore, many in vivo studies have shown embryonic stem cells, following implantation, can spontaneously differentiate and form a type of tumour called a teratoma (Thomson et al. 1998). ASCs do not demonstrate these limitations in in vivo models, and so there is a substantial interest in using ASCs in regenerative medicine. However, ASCs are more committed than ESCs, and so they have a more limited differentiation potential. Nevertheless, ASCs such as bone marrow derived mesenchymal stem cells (BM-MSCs), hematopoietic stem cells (HSCs) from bone marrow and cord blood, and adipose-derived stem cells (ADSCs) are all attractive targets as they still have varied differentiation potentials and are able to differentiate into a variety of cell types. For example, BM-MSCs are able to differentiate into bone, cartilage, fat, tendon, muscle, and marrow

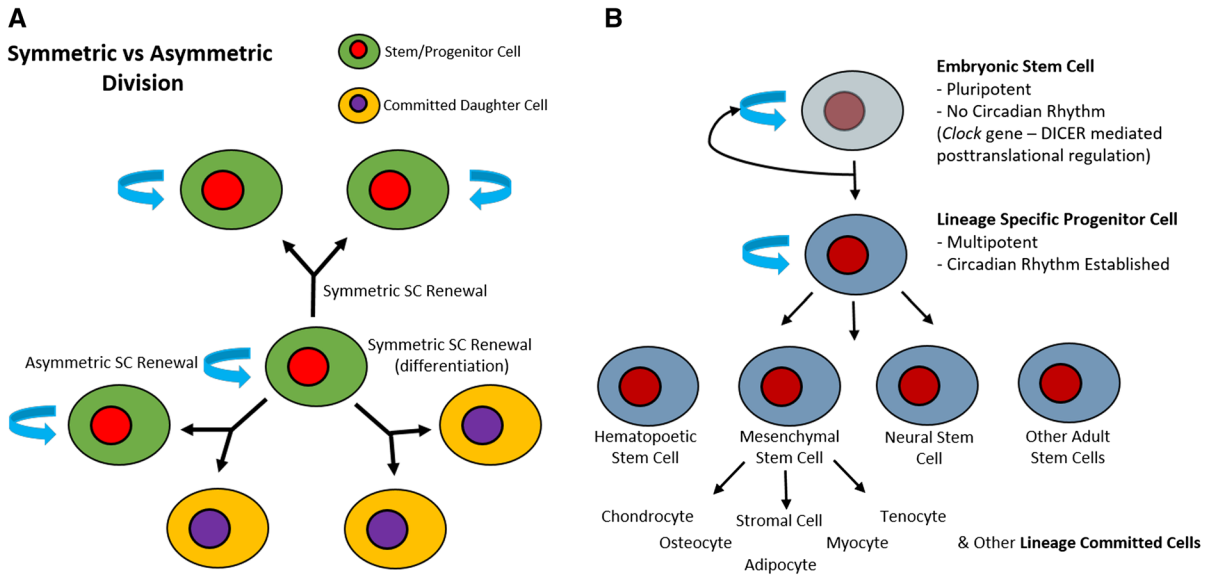

Fig. 1 Stem Cell Division. **a** Adult stem cells are capable of dividing either symmetrically, to produce two identical stem cells or two identical daughter cells, or asymmetrically, to produce one identical stem cell and one committed daughter cell. **b** The hierarchy of stem cell division

stroma (Pittenger et al. 1999), and HSCs are able to differentiate into both myeloid (including monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, dendritic cells, and megakaryocytes or platelets) and lymphoid (T cells, B cells, and natural killer cells) lineages of blood cells. These ASCs are also advantageous as they present an ease of harvest, isolation and expansion in vitro, when compared to embryonic stem cells. The impressive multi-lineage differentiation potential of ASCs is made possible by the broad combination of chemical, biological and physical signals present in their stem cell niches, which direct and control their fate.

A brief introduction to the body's pacemaker: the circadian clock

An important evolutionarily conserved mechanism that becomes altered with age is the circadian clock, the body's innate time-keeping system. Circadian rhythms are a subset of biological rhythms, which have a period of approximately 24 h. The foundation of circadian rhythmicity research is often dated back to the work done by Colin Pittendrigh and Jurgen Aschoff. These pioneers have defined the basis of circadian entrainment. Pittendrigh (1960) showed that deviation from the 24 h cycle provides a mechanism for alignment for the internal time-keeping system, allowing the rhythm to be "reset" where necessary (Pittendrigh 1960). In line with this anticipatory daily role of the circadian clocks, there are a number of mammals living in extreme conditions without a functioning circadian clock, which can be restored upon appropriate environmental stimuli. For example, indigenous Arctic reindeer do not express circadian rhythms during the periods of constant sunlight in the summer or constant darkness in winter months in the Arctic (Lu et al. 2010). Similarly, free-living Arctic ground squirrels do not express circadian rhythms during hibernation in the winter but do exhibit robust daily circadian body temperature oscillations over 24 h during their active season (Williams et al. 2011, 2017). In this way, such mammals are able to restablish circadian rhythmicity coincident with emergence to the surface and the resumption of surface activity (Williams et al. 2012).

The mammalian circadian rhythms are orchestrated by a hierarchy of oscillators. The master clock located within the suprachiasmatic nucleus (SCN) in the anterior hypothalamus of the brain, coordinates a number of independent central nervous system (CNS) and peripheral tissue oscillators, acting as a pacemaker to regulate a coherent rhythm at the level of the whole

organism (Yamazaki et al. 2000). The input pathway for the master pacemaker encompasses the light information that enters the retina through retinal photoreceptors, which is relayed to and entrains the SCN, which, in turn, sends neuro-endocrine signals that result in synchronisation of peripheral tissue clocks. The molecular mechanism that generates cell-autonomous, self-sustained circadian rhythms is governed by a network of auto-regulatory feedback loops of transcription/translation that drive circadian expression patterns of genes, proteins and metabolites within each tissue (Reppert and Weaver 2002). In mammals, this is carried out by the primary feedback loop which includes the basic-helix-loop-helix transcription factors CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL (also known as ARNTL, Aryl hydrocarbon Receptor Nuclear Translocator-Like protein 1), which form the positive arm of the molecular clock. When these two proteins heterodimerise, they are able to bind to *cis*-regulatory enhancer sequences called E-boxes on target gene promoters, and initiate transcription of numerous genes (King et al. 1997; Gekakis et al. 1998). Target genes include core clock components such as *Period* (*Per1, Per2* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) as well as numerous clock-controlled genes (CCGs). PER and CRY proteins then heterodimerise and translocate to the nucleus. They form a negative arm of the feedback loop, and repress target gene expression, including their own transcription, by inhibiting the CLOCK:-BMAL complex (Kume et al. 1999; Shearman et al. 2000). The CLOCK:BMAL heterodimer also induces a stabilising regulatory loop by activating the transcription of Retinoic Acid-Related (RAR) orphan nuclear receptors, *Rev-erb* (also known as *Nr1d1, Nuclear Receptor subfamily 1, group D, member 1*) and *Ror* (also known as *Nr1f1, Nuclear Receptor subfamily 1, group F, member 1*). These bind to retinoic acid-related orphan receptor response elements (ROREs), which are present in many clock-controlled gene promoters as well as the core clock gene *Bmal*. REV-ERB represses transcription of *Bmal*, whereas ROR activates it (Guillaumond et al. 2005). These auto-regulatory loops constitute a molecular clock machinery and take approximately 24 h to complete a cycle. A diagram depicting the circadian system organisation in mammals and the components of the molecular clock are depicted in Fig. 2.

There has been a number of recent exciting studies documenting how ageing affects the molecular clock in several tissues (Kondratov et al. 2006). Mouse models of genetic clock disruption show premature ageing in many tissues. For example, circadian disruption of *Bmal1* led to muscle loss and sarcopenia (Andrews et al. 2010), disrupted cartilage formation (Dudek et al. 2016), bone loss and other features of premature ageing (Kondratov et al. 2006). This showed that genetic disruption of the circadian clock not only leads to circadian arrhythmia, but also degenerative changes in many tissues that are associated with advanced age. Future work will reveal how much of tissue degeneration resulting from *Bmal1* deficiency is caused by impaired *Bmal1*-dependent stem cell homeostasis, which may be responsible for some of the age-related changes seen in various tissue systems.

The link between circadian disruption and adult stem cell function during ageing has been investigated recently by comparing the clock dynamics in epidermal and muscle stem cells isolated from adult and old mice (Solanas et al. 2017). Unexpectedly, the authors demonstrated that adult stem cells from aged mice ($\geq$ 18 months) retained circadian rhythms and that the core clock machinery of the epidermal and muscle stem cells remained robustly rhythmic. However, it was shown that there was an extensive reprogramming of the oscillating transcriptome in the aged stem cells, switching from homeostatic genes to genes involved in tissue-specific stresses, for example DNA damage or autophagy. It was concluded that physiological tissue ageing was associated with the rewiring of stem cell's diurnally timed functions, rather than with the arrhythmia. The former is hypothesized to be switched on in older organisms in order to adapt to metabolic cues and tissue-specific age-related traits. This age-associated rewiring of the oscillatory diurnal transcriptome was significantly rescued in old mice by long-term caloric restriction (CR), known to reverse the effects of ageing. As CR diet is known to uncouple the cycling of peripheral tissues from the SCN, this rewiring may be driven by the molecular changes in the SCN.

The role of circadian clock genes in stem cell function has also been investigated in $Clock^{\Delta 19}$ mutant mouse model with inactive BMAL1/CLOCK transcriptional complex (Yang et al. 2017). It was shown that mammospheres arising from mammary adult stem

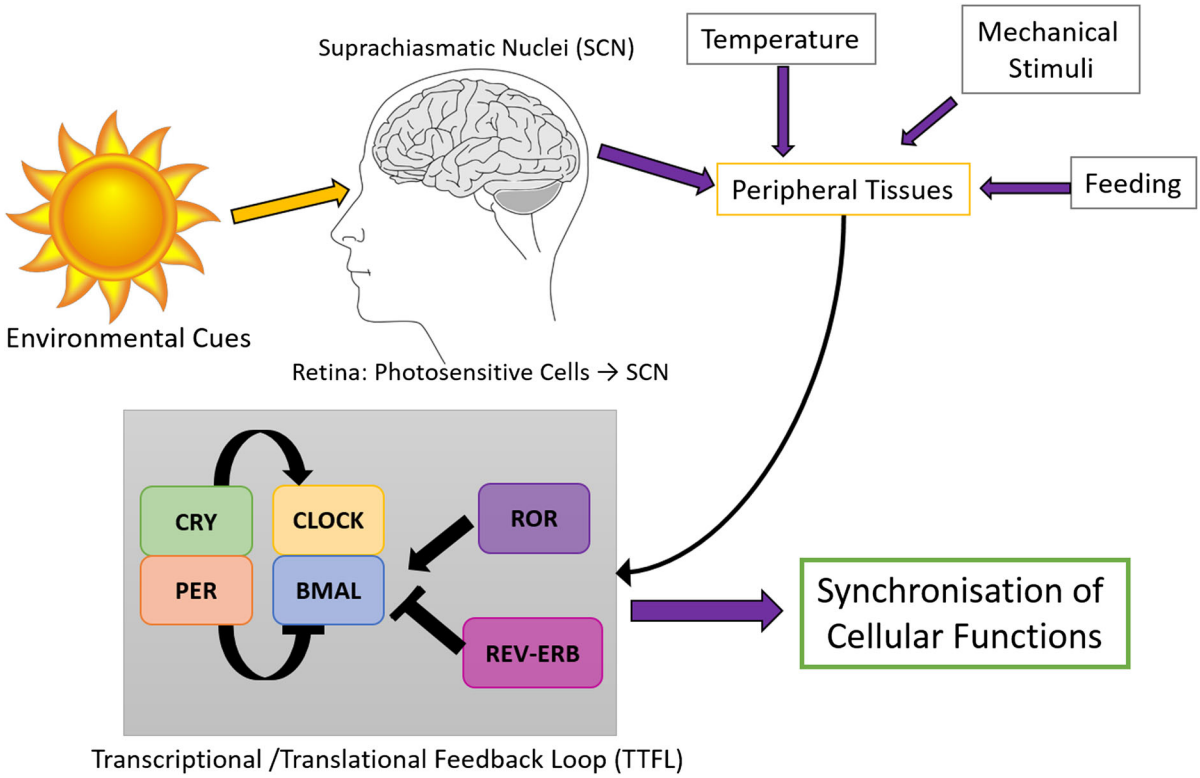

Fig. 2 The circadian system organisation and the molecular clock. Light enters the retina via photoreceptor cells and is transduced to the 'master pacemaker', the suprachiasmatic nucleus (SCN), in the anterior hypothalamus of the brain. The SCN relays signals to the 'peripheral tissue clocks' throughout the body. At a molecular level, circadian clocks are regulated via primary and stabilising feedback loops, which regulate core clock genes within each loop and numerous target genes in each tissue (clock-controlled genes, CCGs), leading to synchronisation of tissue-specific cellular functions (modified from Rogers et al. (2018) with persmission from BioMedicine)

cell progenitors demonstrated rhythmic PER2::luc oscillations, which were dampened in the mutant mice. Importantly, the ability of the *Clock*$^{\Delta 19}$ cells to form mammospheres was considerably reduced, showing that mice containing genetically disrupted clocks have impaired stem cell function and renewal. This further supports the hypothesis that the age-associated changes seen in mouse models with genetically disrupted clocks may be driven by the clock dysfunction within tissue-specific stem cells.

The implications of ageing on stem cell self-renewal, proliferation and differentiation

One physiological process that has been implicated in the loss of stem cell homeostasis with age, and is under the control of circadian rhythms, relates to tissue regeneration. Despite the promising advances made using ASCs in tissue engineering and regenerative medicine, challenges remain due to diminished tissue regeneration seen with ageing when either utilising stem cells from old donors or treating an older patient. Upon ageing, ASCs exhibit reduced proliferation and differentiation which are thought to contribute to tissue deterioration, due to a decline in cell function and/or decreased numbers with age (Boyle et al. 2007). As ASCs have key role in the maintenance of several organs and tissues, any decrease in their number and/or function can seriously compromise the maintenance of tissues and contribute to a number of age-related phenotypes. For example, studies have shown that cell-extrinsic changes contribute to a decline in stem cell abilities to repair damaged tissues with age in muscle progenitor cells (Conboy et al. 2005).

The age-associated phenotype in ASCs has predominantly been studied in human mesenchymal stem cells (MSCs). Morphologically, old MSCs appear larger and more flattened when they reach approximately 40 population doublings and stop proliferating (Bruder et al. 1997). These age-related changes are associated with cellular senescence in vitro. The cells do not only appear larger in size, but show increased spreading and display more podia (Mauney et al. 2004), as well as produce more actin stress fibres (Stenderup et al. 2001). Furthermore, with each passage, the MSCs experience a shortening of their telomeres. As MSCs from younger donors have longer telomeres, they can expand over many population doublings in vitro, proliferate faster and express pluripotency markers such as *Oct-4*, *Nanog*, *Rex-1*, *SSEA-3*, *SSEA-4*, *Tra-1-60*, and *Tra-1-81* (Guillot et al. 2007). Furthermore, chronological age has been shown to influence the proliferation rate of ASCs in rodents (Fafián-Labora et al. 2015). MSCs isolated from older donors vary in their expression of proliferation marker Ki67, with the reduction in Ki67 corresponding to lower proliferation rates whilst increases seen in self-renewal marker CD117 correspond to higher cell numbers.

Moreover, ASCs harvested from older donors show that the frequency of MSCs in bone marrow is significantly lower than in young donors (Tokalov et al. 2007). Using methods such as flow cytometry to determine the proportions of cells from different cell lineages within bone marrow isolated from rats of different ages, it has been demonstrated that bone marrow consists of three main populations of nucleated cells; polynuclear cells (PNCs), megakaryocytic cells (MKCs) and mononuclear cells (MNCs), and the proportions of these populations differs with age. During ageing, an increase in PNCs, a decrease in MNCs and a limited change in the relative number of MKCs was observed. Within the CD90 + MNC population, the number of MSCs significantly decreased with age due to a decrease in the maximal lifespan of these cells.

Upon appropriate stimulation, MSCs give rise to a number of different mesenchymal cell types, most frequently undergoing osteogenesis, adipogenesis, chondrogenesis or myogenesis. These distinct cellular fates are defined by their particular patterns of gene expression. When MSCs differentiate, they switch from one pattern of gene expression to another; the lineage is determined by the activation of phenotype-specific transcription factors, such as the adipocyte specific PPAR-γ2 (Tontonoz et al. 1994) or the osteoblast specific RUNX2/CBFA-1 (Ducy et al. 1997). Interestingly, it has been shown that despite increased markers of senescence in MSCs isolated from older animals, aged MSCs and ADSCs retain their differentiation potential into particular cell fates such as into Schwann cells (Mantovani et al. 2012). Similarly, it was documented that the endothelial differentiation potential of MSCs does not change with age. However, research by Fafián-Labora et al. (2015) showed, in contrast, that MSCs isolated from older rats exhibited a significantly lower differentiation potential than those from younger rats, when induced to differentiate into the osteogenic, chrondrogenic or adipogenic cell fates (Fafián-Labora et al. 2015). The authors also reported that the MSCs isolated from the older group of rats exhibited significantly lower amounts of *Nanog*, implicating that MSCs from older donors have a lower pluripotency potential. It was also demonstrated that the metabolic profiles significantly differed between these different age groups. The authors carried out iTRAQ analysis to compare global profile of proteins from MSCs of rat bone marrow at different ages and discovered increases in metabolic proteins including lactate dehydrogenase in MSCs from adult rats, which suggested increased rates of glycolysis. This was further supported by differences observed in the pentose phosphate pathway activity, whereby decreases in glucose 6 phosphate dehydrogenase activity were seen in the pre-pubertal group whilst increases were observed in the adult group.

Furthermore, a study by Han et al. (2012) sought to ameliorate these age-related changes seen in MSCs to improve decreased proliferative capacity and myogenic differentiation potential with age (Han et al. 2012). To combat this, the authors ectopically expressed the pluripotency marker *Nanog* using lentiviral transduction in BM-MSCs from neonatal and adult donors. They discovered that *Nanog* re-expression did indeed ameliorate reductions in proliferation and myogenic differentiation with age. Several signalling pathways that mediate these changes were identified, including the PPAR signalling pathway which was significantly altered in BM-MSCs upon *Nanog* expression, with both adipogenic genes *CEBPα* and *PPARγ* becoming downregulated. The

differentiation of BM-MSCs into smooth muscle cells was also enhanced by *Nanog* expression, as demonstrated by increased contractility, myogenic function and an increased expression of smooth muscle cell markers such as smoothelin, SM22 and caldesmon (Han et al. 2012). This research suggested that the ectopic expression of *Nanog* may rescue age-mediated decline in BM-MSC functions, which could allow for the use of BM-MSCs from older donors in regenerative medicine.

Osteogenic progenitors also show a reduced capacity for self-renewal in vivo with age (Bellows et al. 2003). Indeed, there is an increased number of adipocytes in the old bone marrow and a decreased number of bone-forming osteoblasts, accompanied by reductions in bone mass. This reduction in osteogenic potential and acquired adipogenic potential with age is referred to as an 'adipogenic switch' (Ross et al. 2000). Research carried out by Moerman et al. (2004) showed that bone marrow aspirates of old mice gave rise to fewer osteoblastic colonies and more adipocytic colonies, when compared to adult mice. From this, they concluded that ageing alters the differentiation potential of MSCs, leading to cells being more likely adipogenic than osteogenic. Using a PPAR-γ2 agonist, rosiglitazone, the authors demonstrated that sensitivity to PPAR-γ is increased in the old bone marrow, and that TGF-β/BMP signalling pathways are altered with age (Moerman et al. 2004). In addition, Gharibi et al. (2014) sought to ameliorate the reductions in osteogenesis seen in vitro in MSCs with age (Gharibi et al. 2014). To do this, the authors demonstrated that blocking AKT/mTOR pathway prevented the development of this age-related phenotype, maintained the MSC morphology and enhanced proliferation capacity, matching those seen in early passage MSCs. MSCs cultured with inhibitors of AKT and/or mTOR also maintained their osteogenic potential. It was speculated that these effects may be influenced by the expression of pluripotency genes such as *Nanog* and *Oct-4* and due to a reduction in reactive oxygen species production (Gharibi et al. 2014). Other research has shown that growing BM-MSCs on certain biomaterials preserved age-compromised functions, maintained the differentiation potential and enhanced proliferation capacity. BM-MSCs were grown on denatured collagen matrices which significantly influenced the protective stress responses and proliferation capacity ex vivo, reduced the rate of morphological alterations and increased the osteogenic potential of old BM-MSCs (Mantovani et al. 2012). Overall, understanding the mechanisms that underlie the ageing processes within ASCs is vital both in terms of ameliorating the age-related tissue deterioration, but also for selecting the appropriate biological age of stem cell donors and designing future stem cell therapies for older patients.

Circadian clocks as a novel mechanism for temporal control of stem cell function

A recent field of investigation has shown that the clock genes can directly influence adult stem cell activation and differentiation, within their tissue-specific niches (Brown 2014; Aguilar-Arnal and Sassone-Corsi 2011; Plikus et al. 2015). The core circadian clock genes, *Per1, Per2, Bmal1, Cry1, Clock,* and *Rev-erbα*, were first characterised as actively cycling in mouse HSCs using a combination of cell sorting by high-speed flow cytometry and gene expression analyses (Tsinkalovsky et al. 2005). The circadian clock genes are well documented to be expressed in an oscillatory manner in murine adipose tissue, following which they were also investigated in human subcutaneous adipose-derived stem cells (Wu et al. 2007). Here, cells were synchronised in vitro with synthetic glucocorticoid dexamethasone, PPAR- γ2 agonist rosiglitazone, or 30% foetal bovine serum, and total RNA collected every 4 h over a 48 h period. The authors reported that differentiated adipocytes were more readily responsive to clock synchronisation than undifferentiated pre-adipocyte precursors, but the period of clock gene oscillations were longer in differentiated adipocytes, validating the use of ADSCs as in in vitro adult stem cell model for the analysis of circadian rhythms (Wu et al. 2007). In MSCs, Wu et al. (2008) reported the presence of the core circadian transcriptional machinery in both murine and human primary BM-MSCs, and when exposed to the synchronising effects of the synthetic glucocorticoid, dexamethasone, BM-MSCs showed oscillating expression of the mRNAs encoding *Bmal1, Per3, Albumin D Binding Protein (Dbp), Rev-erbα* and *Rev-erbβ* (Huang et al. 2009). Furthermore, circadian oscillations were also elicited when BM-MSCs and ADSCs were exposed to serum shock and cAMP analogs (Huang et al. 2009). The circadian mechanisms that have been recently used to

Table 1 The Circadian Synchronisation Mechanisms investigated in Adult Stem Cells

Entrainment mechanism	References	Adult stem cell type
cAMP agonists (forskolin)	Huang et al. (2009)	BM-MSCs, ADSCs
Glucocorticoids (dexamethazone)	Wu et al. (2007, 2008)	BM-MSCs
Growth factors (serum shock)	Huang et al. (2009)	BM-MSCs, ADSCss
Temperature	O'Neill and Reddy (2011)	RBCs
Mechanical stretch	Simoni et al. (2014)	*Drosophila Melanogaster*
	Rogers et al. (2017)	BM-MSCs, ADSCs, DPSCs
Biomaterials	Mengatto et al. (2011)	Mice
	Hassan et al. (2017)	BM-MSCs

synchronise adult stem cells in vitro can be found in Table 1, along with rhythmic temperature cycles, which have been used to synchronise human red blood cells (RBCs) (O'Neill and Reddy 2011).

There has been a substantial amount of evidence that suggests that not only do ASCs express the functional core circadian machinery, but that circadian clocks have important role in a variety of tissue homeostatic functions thus influencing the activation of stem and progenitor cells (Gimble et al. 2009; Weger et al. 2017). Circulating HSCs and their progenitors in the bloodstream have been shown to exhibit robust circadian oscillations in light/dark entrained animals (Méndez-Ferrer et al. 2008). Here, it has been experimentally evidenced that the release of HSCs into the bloodstream is cyclical, along with the rhythmic expression of *C-X-C motif chemokine 12 (Cxcl12)*, both of which are regulated by the core molecular clock through rhythmic noradrenaline secretion. This implies that the clock-driven release of HSCs is stimulated by the CNS during the animal's resting phase and can promote the tissue regeneration and regulate the function of a hematopoietic stem cell niche.

Consequently, any disturbances in this temporal coordination have been implicated in a variety of pathologies including premature ageing and cancer. Indeed, recent studies have implicated circadian rhythm disruption in an increased susceptibility to cancer development in all major organ systems (Fu and Kettner 2013). Research by Puram et al. (2016) utilised a series of in vivo RNA interference screens to identify which transcription factors influenced a murine model of acute myeloid leukaemia (AML) (Puram et al. 2016). It was demonstrated that *Clock* and *Bmal1* were required for the growth of AML cells both in vitro and in vivo, and that the disruption of canonical circadian components led to anti-leukemic effects. RNA interference screens were also used to examine the effects of DNA damage and ageing on the maintenance of HSCs (Wang et al. 2016). It was discovered that *Per2* is activated in lymphoid-biased HSCs (as opposed to myeloid-biased HSCs) and, in hematopoietic cells, wherein it stimulates DNA damage responses and p53-dependent apoptosis. Therefore, *Per2* has been identified as a negative regulator of lymphoid-biased HSCs, immune function and lymphopoiesis. Another core clock gene that has been implied in cancer stem cells is *Per3*. *Per3* has been shown to have an important role in colorectal cancer, being downregulated in colorectal cancer stem cells (Zhang et al. 2017). As *Per3* has been reported to have a critical role in colorectal cancer stem cells pluripotency, it may be a promising gene for targeting cancer stem cells. Furthermore, circadian rhythms have been linked to tumour cell proliferation by regulating iron metabolism; iron is essential for DNA synthesis and therefore critical in the enhanced rates of proliferation seen in tumours (Okazaki et al. 2016). Circadian variations in DNA synthesis and proliferation are seen in tumour cells, and 24-h rhythms can be observed in iron regulatory protein 2 (IRP2). IRP2 regulates the 24 h rhythm in *transferrin receptor 1* mRNA post-transcriptionally, and *Irp2* is promoted by BMAL1:-CLOCK heterodimers, demonstrating a role for the

circadian clock in tumour cell stem proliferation by regulating iron metabolism.

The circadian rhythm regulates adult stem cell activity at a multi-cellular level

Recent data has demonstrated that the involvement of circadian clocks in the regulation of adult stem cell activity is not only cell-specific, but, remarkably, can also act at the cell population level. In a study by Janich et al. (2011), it was shown that the circadian clock may have a role in regulating the activation of coexisting epidermal stem cell populations, which are in different phases, in order to balance hair growth and renewal (Janich et al. 2011). The authors noted that the genes regulating niche dormancy, activation and differentiation contained several putative BMAL1/CLOCK-binding sites, as revealed by gene promoter analysis. These key epidermal homeostasis genes included WNT signalling factors, TGF-β regulators and modulators of BMP and NOTCH signalling pathways. Chromatin immunoprecipitation (ChIP) assays confirmed the binding of BMAL1/CLOCK to these gene promoters in adult tail epidermis, and that the binding of BMAL1 to these target gene promoters was rhythmic. Therefore, the molecular clock generates cell populations that show heterogeneous responses to external factors, by modulating the expression of stem cell regulatory genes in an oscillatory manner. Furthermore, the deletion of *Bmal1* led to circadian arrhythmia, decreased expression of WNT-related genes and TGF-β inhibitors, and caused a progressive accumulation of dormant stem cells and premature epidermal ageing. In contrast, deleting the negative clock loop components *Per1/2*, resulted in progressive depletion of dormant stem cells, which may have implications for cancer.

A subsequent study using human keratinocyte progenitors showed that these cells responded better to several differentiation cues at certain times of the day (Janich et al. 2013). Interestingly, genes encoding key proliferation or differentiation proteins were expressed at different times of the day. For example, DNA replication and cell division pathways were highly represented in the evening, whilst differentiation pathways predominated in the morning. Moreover, the circadian clock coordinated the activities of glycolysis and oxidative phosphorylation with DNA synthesis in proliferating stem cells, most likely as a protective mechanism to prevent genotoxicity. Disruption of this clock-controlled mechanism in stem cells may therefore contribute to stem cell dysfunction and have long-term consequences for tissue homeostasis. Further work in hair follicle cycling has shown that prominent daily mitotic rhythms are generated by peripheral circadian clock within epithelial matrix cells (Plikus et al. 2013), which results in the hair growing faster in the morning than evening and therefore being exposed to higher exposure to genotoxic stress at certain times of the day. Researchers exposed wild-type mice to γ-radiation in the morning (mitotic peak) versus the evening (when there is minimal hair loss), and reported that the diurnal radioprotective effect is lost in clock mutant mice. The circadian clock was demonstrated to coordinate genotoxic stress responses with cell cycle progression by influencing the Cdc2/Cyclin B-mediated G2/M checkpoint. Further research demonstrating the links between various mammalian peripheral tissue clocks and downstream effects on tissue-specific functions, including liver, pancreas, adipose, skeletal muscle, brain, intestine, hematopoietic and immune system, skin and cartilage, has been reviewed in detail (Janich et al. 2014).

In contrast to ASCs, the circadian transcriptional machinery does not seem to oscillate in ESCs, when analysed using real-time bioluminescent imaging systems (Yagita et al. 2010). However, upon differentiation in vitro, a molecular clock oscillations become strongly induced, which can be reversed if the differentiated cells are reprogrammed into induced pluripotent stem cells (IPSCs) using the four pluripotency factors *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* (Paulose et al. 2012). This suggests that formation of the circadian molecular oscillator is dependent on an intrinsic program that occurs during cellular stem cell differentiation. When ESCs are maintained in a pluripotent state in culture, it has been discovered that they express a self-sustained rhythm in glucose uptake that is not coincident with clock gene oscillations, and this rhythm is paralleled by rhythmic glucose transporter mRNA expression, indicating that circadian rhythms in metabolism emerge earlier than clock gene expression rhythms (Paulose et al. 2012). When stem cells become differentiated, however, circadian patterns in clock gene expression can be observed, and the glucose utilization rhythm becomes

enhanced in amplitude, providing the evidence of a circadian clock in differentiated stem cells. Further experiments carried out by Lu et al. (2016) have demonstrated that when *Clock* is knocked out entirely using CRISPR/CAS9-mediated genetic editing techniques in mouse ESCs, there was no influence on the cellular pluripotent state, but mESCs did exhibit a decreased proliferation rate and an increased apoptosis. Interestingly, clock gene rhythms failed to develop in these mESCs following differentiation, suggesting that *Clock* may be critical in mESC differentiation (Lu et al. 2016). These findings have been supported by research in mouse embryonic hearts and ESCs which demonstrated a role for the posttranscriptional regulation of *Clock* in development of molecular clock oscillations in differentiating stem cells. Indeed, the appearance of CLOCK protein during ESC differentiation coincides with the emergence of molecular clock oscillations and *Dicer/Dgcr8*-mediated post-transcriptional regulation of CLOCK protein (Umemura et al. 2017), highlighting the importance of CLOCK in the establishment of circadian clock oscillations during embryonic stem cell differentiation.

Mechanistically, it has been shown that expressing both transcription factor *c-Myc* and ablation of DNA methyltransferase 1 (*Dnmt1*) leads to disruption in the establishment of the molecular clock oscillations in differentiating mouse ESCs. It has been reported that, when there is a failure of clock oscillation development, an increase in *Kpna2* (*Importin-α2*) and altered subcellular PERIOD protein localization can be observed; therefore differentiation-coupled transcription of specific genes may regulate circadian clock development in mouse ESCs (Umemura et al. 2014). Furthermore, ESCs have immature mitochondria, are reliant on glycolysis for fuel and have no discernible rhythms, compared to differentiated stem cells which have mature mitochondria, acquire oxidative respiration and show clear rhythms, linking redox regulation to stem cell differentiation and the development of circadian clocks. Indeed, clear redox oscillations can be imaged in proliferating epidermal stem cells, demonstrating the role for circadian clocks in regulating metabolism in adult stem cells (Stringari et al. 2015). The adult stem cell clock coordinates the activities of DNA synthesis with glycolysis and oxidative phosphorylation, whereby increased glycolysis is found during the night, along with a higher proportion of stem cells in S phase (when DNA synthesis occurs) (Stringari et al. 2015). This temporal segregation of metabolic processes from stem cell proliferation is thought to act as a protective mechanism against genotoxicity.

The molecular clock exerts essential regulation of stem cell differentiation fate

Adipogenesis

In addition to their role in stem cell activation, circadian rhythms have also been extensively linked to stem cell proliferation and differentiation. It is well established that *Bmal1* is involved in the regulation of adipogenesis and lipid metabolism in mature adipocytes. When 3T3-L1 cells are subjected to adipogenic differentiation, the level of *Bmal1* mRNA increases and it is highly expressed in differentiated adipocytes (Guo et al. 2012). Furthermore, mouse embryonic fibroblasts (MEFs) from *Bmal1*-deficient mice as well as 3T3-L1 cells with *Bmal1* knock-down fail to differentiate into adipocytes. Interestingly, when BMAL1 is overexpressed with adenoviral gene transfer, this ability is restored and cells can accumulate lipids and express adipocyte-related genes, such as *PPARγ2*. The promotor activity of these adipogenic genes is stimulated in a BMAL1-dependent manner, and the expression of adipogenic factors PPARγ2 and adipocyte fatty acid binding protein (AP2) show clear circadian rhythms in murine adipose tissue (Guo et al. 2012). Taken together, these results suggest that BMAL1 is an important factor in adipogenesis regulation.

More recently, it has been shown that *Bmal1* disruption in vivo actually leads to increased adipogenesis, adipocyte hypertrophy and obesity in global *Bmal1* KO mice (Shimba et al. 2005). Here, it has been uncovered that *Bmal1* deletion leads to down-regulation of genes in the canonical WNT signalling pathway, which are known to suppress adipogenesis. The gene promoters of several of these WNT-regulated genes, including *Wnt10a*, *β-catenin*, *Fzd5*, *Dvl2* and *Tcf3* displayed BMAL1 occupancy. Similarly, *Bmal1* knock down led to attenuation of WNT signalling pathway, whilst BMAL1 overexpression led to opposite effects. Stabilising β-catenin through WNT ligand administration or GSK-3β inhibition

ameliorated the decreased WNT signalling and rescued inhibition of adipogenesis induced by *Bmal1* knock-down. Taken together, this study offered a mechanistic links between *Bmal1* disruption, altered adipogenesis and development of obesity in mice (Shimba et al. 2005). Another clock gene implied in adipose cell differentiation is *Rev-erbα (NR1D1)*, which has been shown to be a key regulator of brown adipose tissue development (Nam et al. 2015). As *Rev-erbα* promotes brown adipogenesis, genetic ablation of *Rev-erbα* impairs embryonic and neonatal brown fat formation in mice, by disrupting brown adipocyte lineage commitment and terminal differentiation. By pharmacologically activating REV-ERBα activity, brown adipocyte differentiation can be promoted, as REV-ERBα represses key components of the TGF-β cascade, which, in turn, inhibits brown fat development.

Neurogenesis

Adult neurogenesis, which generates both new neurons and glia, is regulated by circadian rhythms. When neurosphere cultures prepared from the dentate gyrus in the brain are isolated from *mPer1::luc* clock reporter mice, it was apparent that circadian *mPer1* gene rhythms can be observed in neurospheres where neurogenesis was induced, but not in those neurospheres maintained in the stem cell state (Malik et al. 2015). In addition, neurospheres used from both *Bmal1 KO* as well as *Cry1/2* KO mice, another genetic model of circadian disruption, showed that circadian rhythms are not required for neurosphere induction in vitro. However, the absence of these clock components did restrict neurosphere growth, neuronal fate commitment and increased cell death. Quiescent neural progenitor cells (QNPs) in the subgranular zone (SGZ) of the adult hippocampus also express components of the molecular clock and proliferate in a rhythmic manner (Bouchard-Cannon et al. 2013). Here, the clock proteins PER2 and BMAL1 are essential for the control of neurogenesis. The circadian clock is crucial in timing the entry and exit of the QNPs into the cell cycle and, without these clock components, the quiescent state achieved during neuronal differentiation is delayed.

Moreover, *mPer2* is also expressed by neural stem/progenitor cells (NPCs) differentiating to mature neurons in the dentate gyrus. It has been postulated that *mPer2* provides a functional link by influencing the early cellular events that lead to post-mitotic granule cell production underlying adult hippocampal neurogenesis (Borgs et al. 2009). In the lateral subventricular zone (SVZ), the area in the brain where NPCs persist and postnatal neurogenesis occurs, the expression pattern of clock genes changes following the onset of differentiation, and *Bmal1* begins to oscillate endogenously. If *Clock* or *Bmal1* were silenced using RNA interference, the percentage of neuronal marker Map2-positive cells decreased as well as the expression level of neurogenic transcription factors such as NeuroD1 (Kimiwada et al. 2009). A study recently published by Akle et al. (2017) showed that all neurogenic niches studied in an adult diurnal vertebrate, the zebrafish, including the dorsal telencephalon, habenula, preoptic area, hypothalamus and cerebellum, showed circadian modulation of cell cycle progression that involved the use of both niche-specific and systemic factors (Akle et al. 2017).

Osteogenesis

The circadian clock has also been implicated in bone homeostasis. *Bmal1 KO* mice display low bone mass phenotype which worsens over lifespan. These mice have decreases in cortical and trabecular bone volume and a lower bone mineral density when visualised using micro-computed tomography (Samsa et al. 2016). *Bmal1* deficiency in vivo has been shown to result in a decreased number of active osteocytes and osteoblasts, and isolated BM-MSCs displayed a reduced osteoblastic differentiation capacity, likely contributing to the observed reduction in osteoblast and osteocyte numbers (Samsa et al. 2016). Another clock gene implicated in BM-MSC proliferation and osteogenesis is *Rev-erbα*. It has been demonstrated that *Rev-erbα* expression decreases during osteogenesis. *Rev-erbα* overexpression led to inhibition of BM-MSC proliferation and osteogenic differentiation, which were partially rescued by activating Wnt/β-catenin signalling via exogenous Wnt3a ligand administration (He et al. 2014). This suggests that increased *Rev-erbα* levels could promote BM-MSC ageing and negatively regulate osteogenesis with age, which warrants further investigation.

Chrondrogenesis

It has also been documented that *Bmal1* expression is decreased in human cartilage from osteoarthritic patients and aged mouse cartilage (Dudek et al. 2016). By ablating *Bmal1* expression in mouse chondrocytes specifically, this led to progressive degeneration of articular cartilage, most likely as a result of a number of altered molecular pathways that *Bmal1* targets. Indeed, *Bmal1* ablation in cartilage led to altered TGFβ pathway associated with an altered ratio in levels of phosphorylated SMAD2/3 versus phosphorylated SMAD1/5. Moreover, altered NFATc2 transcription factor pathway was observed, together with reduced expression of matrix related genes *Sox9*, *Acan*, and *Col2a1,* linking the circadian clock to the maintenance and repair of cartilage.

Myogenesis

Important role for *Bmal1* has been demonstrated regarding myogenesis. *Bmal1* Is highly expressed in skeletal muscle and is thought to regulate myogenic differentiation via direct transcriptional activation of canonical Wnt signalling pathway components (Chatterjee et al. 2013, 2015). Moreover, it has been shown that a master regulator of myogenesis, MyoD, displays cyclical mRNA and protein levels, and is a direct target of CLOCK and BMAL1. *Bmal1* KO and $Clock^{\Delta 19}$ mouse models show reductions in the total muscle mass and maximal force including reduced mitochondrial volume, demonstrating the importance of CLOCK and BMAL1 in skeletal muscle structure and function (Andrews et al. 2010). Similarly, when *Bmal1* is knocked down in myoblasts, this led to impaired myogenic differentiation and decreased expression of key myogenic regulatory factors including *Myf5*, *Mrf4* and *Myogenin* as well as *Myosin Heavy Chain 3 (MHC3)*. Overexpression of *Bmal1* in C2C12 myoblasts conversely led to accelerated myogenesis and attenuation of Wnt signalling, indicating that *Bmal1* is required for myoblast differentiation (Chatterjee et al. 2013) Direct binding of BMAL1 to gene promoters coding for canonical Wnt pathway genes has been demonstrated in line with circadian oscillations of several Wnt pathway components (Chatterjee et al. 2015). *Bmal1* can also influence skeletal muscle function through influencing its regeneration process (Sun et al. 2014). *Bmal1 KO* mice display significantly lower satellite cell expansion, which leads to defective regenerative responses. These mice exhibit a nearly non-existent induction of Pax7, a satellite cell marker. The satellite cell-derived myoblasts isolated from *Bmal1 KO* mice demonstrate reduced growth and proliferation ex vivo, underscoring the role of *Bmal1* in both muscle maintenance and repair (Sun et al. 2014).

Angiogenesis

Circadian clock protein PER2 is thought to be a key factor in maintaining endothelial progenitor cell (EPL) function during angiogenesis. PER2 is abundantly expressed in early EPCs, whilst EPCs from $Per2^{-/-}$ - mice demonstrate impaired proliferation, adhesion, migration and tube formation due to inhibitions of both PI3K/Akt/FoxO signalling and protein levels of C-X-C chemokine receptor type 4 (CXCR4). Interestingly, the negative proliferation effects of *Per2* deficiency can be rescued by activating PI3K/Akt/FoxO signalling. Indeed, PER2 and CXCR4 directly interact in EPCs, showing that PER2 is essential in maintaining early EPC function (Al Mheid et al. 2014). In humans, circulating progenitor cells and their proangiogenic activity also exhibit circadian variations. They show unfavourable profiles in the morning which coincides with prevalence of cardiovascular events, directly influenced by the endogenous circadian clock (Bhatwadekar et al. 2017). When *Bmal1* is conditionally deleted in endothelium and hematopoietic cells specifically, cellular responses to microvascular and macrovascular injuries are exaggerated (Dierickx et al. 2017), highlighting the importance of circadian rhythms in maintaining vascular homeostasis.

Other tissue-specific cell differentiation

In the cardiac muscle, circadian rhythms regulate cardiac physiology and metabolism and are known to determine outcomes of ischemic stress (Van Laake et al. 2018). Human ESCs progressively oscillate following directed cardiac differentiation. Furthermore, a number of clock-controlled output genes in the heart have been identified as an oscillatory network of stress-related genes, including genes known to play an important role in human heart physiology, including *Pln*, *Kcne4*, *Tspo*, *Cav1* and *Rgs2* (Dierickx et al.

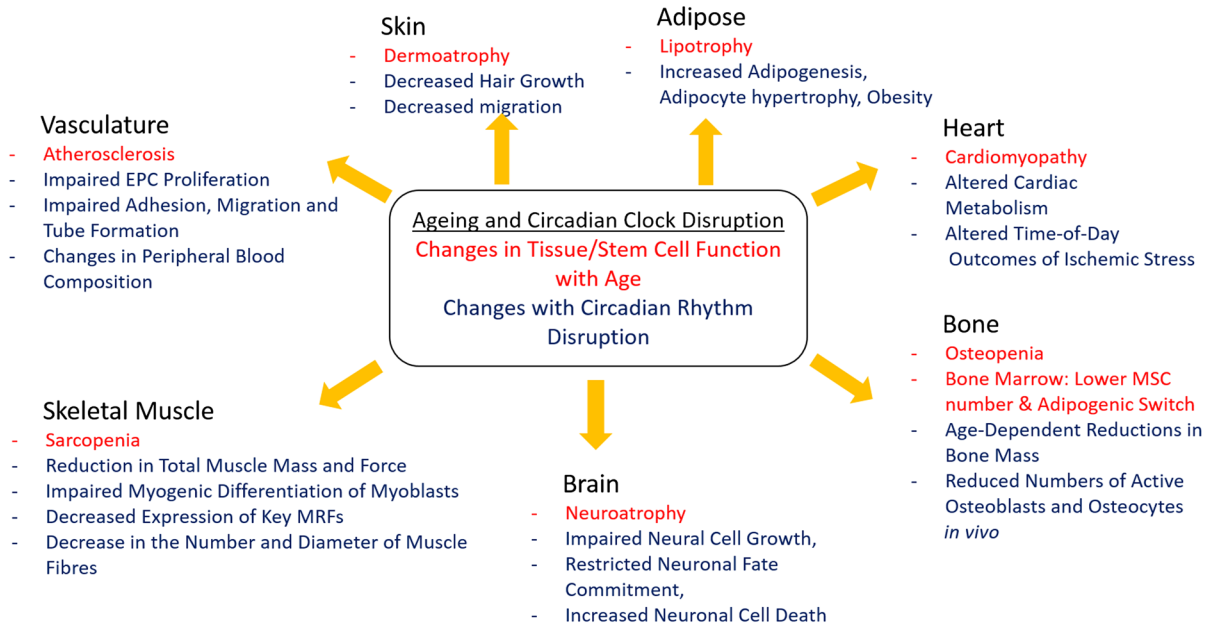

Fig. 3 Summary of the Common Changes seen with Ageing and Circadian Clock Disruption

2017). Stem cell antigen 1-positive (SCA1+) cells, which can be detected in the heart, have been shown to possess a molecular clock and exhibit circadian oscillations that control downstream cellular functions (Du Pré et al. 2017). These findings demonstrate the importance of circadian regulation in cardiovascular functions.

Intestinal stem cells (ISCs) are critical in determining how the intestine regenerates in order to replace dying cells. The PER transcription factor has been discovered to be essential in intestinal regeneration, and numerous gene transcripts that are regulated by the circadian clock have been uncovered within intestinal stem cells, including those involved in stress response and regeneration pathways (Karpowicz et al. 2013). Disruption of clock component *Per* has been shown to lead to arrhythmic ISC divisions, demonstrating how diverse roles in stem cells are played by the components of the molecular clock in different peripheral tissues (Karpowicz et al. 2013). A summary of tissue phenotypes associated with disrupted circadian rhythms which are commonly found in various tissue systems affected during ageing is shown in Fig. 3.

The impact of the circadian rhythms on stem cell homeostasis and cell cycle progression

Research by Boucher et al. (2016) has demonstrated the effects of the clock genes on MSC differentiation, migration and cell cycle regulation. They showed that knock down of *Clock* or *Per2* led to inhibition of adipocyte differentiation, while osteoblastic differentiation was unaffected. Cell migration was decreased in *Per2* KD cells together with altered hMSC cell cycle stage distribution. This was in line with observed changes in cyclin expression profiles including significant decreases in several cell cycle regulators p19, p27, Cyclin B1 and Cyclin D1 protein levels (Boucher et al. 2016). This is further evidence that the circadian clock in stem cells is important in maintaining their function and properties.

Further research examined the role of the clock in cell cycle regulation of hair follicle stem cells (HFSCs) that reside within the bulge (Zagni et al. 2017). One of the major regulators of the PI3K/AKT pathway, PTEN, has a critical role in controlling HFSC number and size, and maintaining the pluripotent state of stem cells. PTEN and BMAL1 show functional links; when *Pten* is deregulated, this causes constitutive activation of BMAL1, and BMAL1 is involved in the maintenance of the PTEN-induced

Table 2 Signalling Pathways Controlled by the Circadian Clock relevant to Adult Stem Cell Function

Signalling pathways	Role in adult stem cells	References
WNT	Differentiation	Janich et al. (2011), Guo et al. (2012), Chatterjee et al. (2013), Chatterjee et al. (2015)
NOTCH	Self-renewal	Zhang et al. (2017) and Janich et al. (2011)
		Several factors bound by *Bmal1*
p53	Apoptosis	Yagita et al. (2010)
		Activated by *Per2*
Cyclin B	Cell cycle	Plikus et al. (2013), Boucher et al. (2016)
PI3K/AKT	Homeostasis/ proliferation	Sun et al. (2014), Zagni et al. (2017)
TGF-β	Homeostasis/ proliferation	Moerman et al. (2004), Janich et al. (2011) and Plikus et al. (2013)
BMP	Proliferation	Janich et al. (2011)
Rho/ROCK	Homeostasis	Yang et al. (2017)
ROS/NRF2	Homeostasis	Kondratov et al. (2006), Kondratov et al. (2009) and Pekovic-Vaughan et al. (2014)

phenotype. Short-term and long-term *Pten* depletion leads to BMAL1 activation, which contributes to HFSC accumulation.

Another important aspect of circadian clock regulation of stem cells relates to the 'clock-gated' cell division cycles. Using 3D murine organoids, researchers have demonstrated Wnt-mediated intercellular coupling between the cell cycle and the circadian clock (Matsu-ura et al. 2016). The molecular clock 'gates' the existence of cell populations with heterogeneous cell cycle times generating self-synchronised, 12-h cell division cycles. It is thought that an intercellular signal linking circadian and cell division cycles is established by differentiated cells (Matsu-ura et al. 2016). A summary of the signalling pathways that affect stem cell activation, proliferation and differentiation, which are under circadian control, can be found in Table 2.

Furthermore, circadian clock is implicated in several cellular protective mechanisms that are important for stem cell homeostasis such as antioxidant stress responses (Kondratov et al. 2009). Reactive oxygen species (ROS) are produced as a by-product of cellular metabolism and serve as signalling molecules. However, increased ROS production and/or clearance results in oxidative stress, leading to oxidative damage to major macromolecules including proteins, lipids and DNA, which is associated with tissue degeneration with ageing (Paul et al. 2014; Dickinson and Chang 2011). Importantly, genetic disruption of *Bmal1* in mice leads to altered redox homeostasis with increased accumulation of ROS in several tissues (Kondratov et al. 2006), which can be partially ameliorated with administration of glutathione precursor *N*-acetyl cysteine (NAC) (Kondratov et al. 2009). Moreover, $Clock^{\Delta 19}$ mice show diminished levels of antioxidant transcription factor *Nrf2* and its target antioxidant genes leading to an increased accumulation of protein oxidative damage in the lungs with ageing (Pekovic-Vaughan et al. 2014). CLOCK and BMAL1 heterodimer were found to positively regulate *Nrf2* transcription by temporal control of the E-box element in the *Nrf2* gene promoter. NRF2 protein, in turn, drives the rhythmic transcription of antioxidant genes via temporal binding to antioxidant response elements (AREs) in several gene promoters encoding enzymes involved in glutathione synthesis and utilisation. The lack of NRF2-mediated antioxidant defence provides a potential mechanism for the premature onset of age-related pathologies seen in the absence of *Bmal1* in mice (Pekovic-Vaughan et al. 2014).

The effects of circadian rhythms on stem cell biomaterials and tissue engineering

In regenerative medicine, there is a great need to generate mature functional tissues in vitro, especially given our increasingly ageing society. Therefore, it is

essential to discover effective means to direct differentiation of stem cells into tissue-specific cells in a controlled manner, which requires appropriate biochemical and biophysical signals within cellular microenvironments. Biomaterials provide a 3D environment equipped with biological, mechanical and chemical cues, which can stimulate cells to proliferate, differentiate, secrete extracellular matrix and form functional tissues (Hwang et al. 2008; Burdick and Vunjak-Novakovic 2008; Singh and Elisseeff 2010; Lutolf et al. 2009; Dawson et al. 2008). Intelligent biomaterials that can mimic the required physical and biochemical environments and provide the necessary signals for stem cell activation and differentiation have recently undergone intense research. Many types of biomaterials have been developed, including the use of natural materials, which consist of extracellular matrix components such as collagen, fibrinogen, hyaluronic acid, glycosaminoglycans (GAGs), laminin and hydroxyapatite (HA), as well as synthetic materials, such as polymers, ceramics and metals. There has also been a drive to utilise surface modifications of biomaterials, including chemical and biological modifications, so that the differentiation of stem cells may be selectively steered down particular cellular fates. Several articles have reviewed in detail the application of biomaterials in stem cell differentiation (Dawson et al. 2008; Hwang et al. 2008; Burdick and Vunjak-Novakovic 2008; Singh and Elisseeff 2010; Lutolf et al. 2009).

The circadian rhythms have recently been implied in dental and orthopedic implant integration. In order to investigate causes of failure in osseo-integration, an implant failure model under vitamin D deficiency was utilised to identify crucial gene networks that underpin this process (Mengatto et al. 2011). Following genome-wide transcriptomic and bioinformatic molecular pathway analyses, it was reported that the circadian rhythm pathway was among the top molecular pathway affected. *Npas2* and *Bmal1* were upregulated around the implant and diminished by vitamin D deficiency, whereas the *Per2* expression pattern showed the opposite trend. It was thus concluded that the circadian rhythms, along with the extracellular matrix, may be involved in osseo-integration establishment under vitamin D regulation.

In a recent follow up study by Hassan et al. (2017), the circadian rhythm of BM-MSCs was induced using titanium-based biomaterials with complex surface modifications (Hassan et al. 2017). When cultured on such materials, BM-MSCs supressed *PER1* expression and upregulated *NPAS2*. When further investigated using *Npas2* KO mice, the titanium biomaterial-induced suppression of *Per1* was not rescued and the expression of other clock genes *Per2, Per3, Bmal1* and *Clock* was not affected, suggesting that the altered expression of *Per1* was independent of *Npas2*. The authors concluded that titanium-based biomaterials can influence BM-MSC circadian rhythms, and altered BM-MSC rhythms may be important factor in determining the rate of titanium-based biomaterial integration into bone. This exciting area of research examining how materials influence the stem cell clock warrants further research, and the authors of this review are optimistic that it will be a very prosperous area of research to come.

The circadian clock has recently been implicated in regenerative medicine as it has been shown to have a profound effect on the wound healing response in mice and humans (Hoyle et al. 2017). Skin wounds in mice healed faster in the active period than those incurred in the rest period ex vivo and in vivo. Using a proteome-wide screen for rhythmically expressed proteins in fibroblast cells, the authors uncovered a circadian regulation of actin, a cytoskeletal protein involved in cell migration, which is an important aspect in the wound-healing response of fibroblasts and keratinocytes (Hoyle et al. 2017). Analysis of a database of human burn injuries showed that human burns patients who incurred their injuries in the night, i.e. rest period, healed more slowly than those incurred in the day, i.e. active period. This research highlights the importance of the circadian regulation of the cellular cytoskeleton in modulating wound healing responses and underlines the importance of the circadian rhythms in regenerative medicine.

The effects of the rhythmic mechanical cues on stem cell clocks

The circadian clock has a period of approximately, but not exactly, 24 h. Therefore, it must be reset daily by external cues, known as *Zeitgebers*. The most common of these cues is light which, in mammals, entrains rhythms in the SCN through retino-hypothalamic tract. The SCN relays this temporal information to the rest of the brain and peripheral tissue clocks via

diffusible signals and neuroendocrine factors (Silver et al. 1996). As the mammalian core body temperature itself oscillates in a circadian manner, this too can act as a *Zeitgeber*, as was initially shown by subjecting cultured fibroblasts to rhythmic temperature oscillations (Brown et al. 2002). This temperature entrainment was demonstrated to be sufficient to sustain circadian rhythmicity in vivo, and abnormal temperatures cycles were reported to cause decoupling of peripheral oscillators from the SCN (Brown et al. 2002). Glucocorticoids, which are a class of steroid hormones that bind to the glucocorticoid receptor (GR) present on almost every vertebrate cell surface (except the SCN), have also been implicated in synchronising peripheral circadian rhythms in human and murine ASCs (Balsalobre et al. 2000; Wu et al. 2008). Huang et al. (2009) have demonstrated that human stem cells have circadian oscillations that can be induced by serum shock and cAMP analogues in vitro (Huang et al. 2009), showing that stem cells can be synchronised using hormones and growth factors.

One emerging entrainment mechanism of circadian clocks is mechanical stimulation. Indeed, in the body, different cells in various anatomical locations are subjected to varying amounts of mechanical strain. Published data has demonstrated that a uniaxial strain between 5 and 15% with a frequency of 1 Hz has notable effects on MSCs both on proliferation and collagen synthesis (Sun et al. 2016). Furthermore, O'Caerbhaill et al. (2008) highlighted that radial distensions of 5% and frequencies of 1 Hz caused mechanosensitive effects on stem cells including cell reorientation parallel with direction of flow and altered cellular morphologies, highlighting that there is a significant cytoskeletal restructuring in mechanically stimulated MSCs compared to static cells.

Recent research has also demonstrated that mechanical vibrations have the capability of resetting the clock in *Drosophila melanogaster*. It has been shown that rhythmic mechanical stimulation of the chordotonal organs can synchronize the *Drosophila* circadian clock (Simoni et al. 2014). Loss-of-function mutation in the *Period* gene led to impaired ability of mechanical synchronization, highlighting the importance of a functional clock system for mechanical entrainment. Research from our own group has recently demonstrated that cyclical mechanical stretch can be used to synchronise circadian rhythms of human ASCs (Rogers et al. 2017). Here, primary human ASCs from bone marrow, dental pulp and adipose tissues were synchronised using a novel mechanical cell stretch paradigm (12 h ON: 12 h OFF cyclical uniaxial stretch for three days) and the expression of core clock genes analysed over two circadian cycles in the absence of rhythmic stimulation. Rhythmic mechanical stimulation was sufficient to synchronise circadian rhythms in distinct ASCs, with differential propensity for mechanical synchronisation displayed by ASCs derived from distinct human tissue locations. Interestingly, mechanical strain has also been shown to inhibit adipogenesis in MSCs by stimulating a robust β-catenin signal (Sen et al. 2008), which is in line with the previous reports showing that *Bmal1* influences adipogenesis through β-catenin pathway. Whether BMAL1 has a role in regulating mechano-transduction pathways to influence adipogenesis is currently unknown and warrants further investigation. A summary of the circadian synchronising mechanisms that have been investigated in ASCs is presented in Table 1.

A recent study has demonstrated the importance of cell–matrix interactions for stem cell clocks and discovered that the mammary epithelial clock is regulated by the mechanical stiffness of the cellular microenvironment (Yang et al. 2017). The authors demonstrated that genetic disruption of clocks compromises the self-renewal ability of the mammary epithelial stem cells, underlining the key link between clock genes and mammary stem cell function. Interestingly, the authors demonstrated a functional link between tissue matrix stiffness with age and the amplitude changes in clock oscillations. Increased tissue stiffening with age was shown to suppress the amplitude of the mammary clock oscillations through the tension sensing cell–matrix adhesion molecule, vinculin, and the Rho/ROCK signalling pathway. This mechano-transduction signalling pathway is, in turn, transduced into the cell to regulate the activity of core clock machinery. In this study, the authors also investigated whether the circadian rhythms in mammary tissues isolated from old mice can be restored by administering drugs to alter matrix stiffness. ROCK pathway inhibitors were used, implicated in matrix stiffness regulation, which led to improved amplitude of clock gene oscillations in mammary tissues from old mice (Yang et al. 2017). Therefore, it was concluded that tissue stiffening seen with ageing is

thought to suppress the mammary clock in vivo, providing a mechanism of how ageing disturbs the mammary epithelial stem cell clock through altering the stem cell niche.

Summary

The field of circadian stem cell biology is a dynamic area, which has provided us critical insights into the temporal control of stem cell function and maintenance. Whilst these are exciting developments, there is much fundamental research that is still needed as our understanding in this crucial area continues. Key aspects to build on over the next few years include the bi-directional effects of the biomaterials and biomechanical factors on stem cell clocks. Future research assessing the role of epigenetic factors on stem cell clocks, which are well documented to influence both circadian rhythms and ASC function, will be of great importance to the application of circadian biology to stem cell research. In summary, the invaluable knowledge pertaining to temporal regulation of stem cell physiology and metabolism by tissue-specific circadian clocks will provide novel insights into the dynamic processes that change during stem cell ageing, and allow appropriate optimisation of smart biomaterials and mechanical cues essential for steering stem cells into particular cell fates. Such critical findings are essential for future design of novel cellular therapies to ameliorate and/or slow down a number of age-related diseases and begin to tackle their prevention.

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