From Stem cells to Organoids and Gastruloids: How we can Model Human Development and disease with in vitro model systems

Some of the most important questions in biology relate to how we are formed and develop. For example, how is it that a single fertilised egg is able, in a relatively short space of time, to produce so many different cell types, tissues and organs, and position them so precisely within the body? How do cells communicate with on another in order to instruct the correct location of organs and tissues relative to one another? Why does human development occasionally go wrong, producing birth defects and pathological conditions? The answers to these questions are vital in uncovering the mechanisms behind disease states, opening up treatment possibilities involving regenerative medicine or developing better medicines and pharmaceuticals. Critically, if one knows how something should *be*, it is more straightforward to uncover *why* something is going wrong.

We currently make use of a number of experimental techniques to tease apart these questions, that traditionally use embryos from animals such as the fruit fly, frog, chicken, zebrafish and mouse. In terms of mammalian development, the mouse has proven exceptionally useful as a model system. However it has a number of limitations: 1) Since biological systems are buffered and multiple genes may have similar functions, it is not always possible to discount any possible effects of redundancy between genes when assessing the effect of mutant phenotypes; 2) Mechanical forces which have been shown to play an important role in development are very difficult to assess; 3) There is a great deal of expense involved in maintaining mouse lines (and generating new ones with specific traits) and it is difficult and technically challenging to experimentally manipulate the mouse embryo at the early stages. In addition to this, there are a number of ethical considerations to take into account when using mice and their embryos (although a number of initiatives are currently underway to dramatically reduce or replace the numbers of mice and other animals involved in experiments and refine their use[*]). Although many developmental processes and patterning events are conserved throughout diverse animal species, how sure are we that what we see in the mouse (and other model systems) recapitulates human development? What is needed is a fully tractable system (i.e. easy to control experimentally) that is cheaper than *in vivo* work, ethically responsible and one which can be utilised to ask specific questions regarding developmental processes and differentiation which can eventually be targeted to understanding human development and disease.

The promise of Stem Cell Biology in understanding Development

Embryonic stem cells (ESCs) offer an alternative and, arguably, a parallel route to dissecting the development of embryos, delineating the processes and mechanisms utilised physiologically. ESCs are a self-renewing, pluripotent population of cells which if left in the embryo, would ultimately give rise to all the tissues and organs of the embryo proper (they were first isolated from mouse blastocysts by Evans and Kaufmann in the 1980s[1]). The pluripotent, self-renewing trait of ESCs is essential during development and useful experimentally as it prevents premature exhaustion of the cells during sequential fate specification *in vivo*, and allows us to culture them indefinitely in the laboratory. Careful experimentation and genetic

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[*] *For example, the National Centre for the Replacement, Refinement and reduction of animals in research (NC3Rs).*

analyses has determined many of the genes and signals involved in patterning the early embryo, and it is this well grounded understanding that allows us to guide ESCs towards specific tissue types and cell fates by applying signals or modifying cells' gene expression in culture. For example, neural tissues can be generated by applying retinoic acid or inhibiting Bone Morphogenetic Protein (BMP) signalling, and beating cardiomyocytes can be enriched through application of BMP, ActivinA and Vascular Endothelial Growth Factor (VEGF). It is often the case that by using a tissue-culture based approach, new insights can be gained regarding the signals involved in cell fate specification. Examples of this include our recent work on dissecting the role of Wnt/β-Catenin and Fibroblast growth factor (FGF) signalling in generating a population of cells that serves as a pool for generating the body axis[2], or how cells resolve binary fate decisions depending on the signalling environment they're exposed to and the time in which they *see* these signals[3].

ESCs are, when compared to *in vivo* studies in the mouse, exceptionally easy to manipulate (genetically and chemically), reduce the necessity for animal experimentation and are orders of magnitude less expensive than keeping mice in the laboratory. However, there are a few disadvantages which have the potential to compromise our complete reliance on ESCs with respect to *in vivo* work. One of which, which will addressed hereon, involves the topographical differences between a 2D layer of cells grown on a plate versus the 3D nature of the whole embryo; the 3D spatial organisation in the embryo between cells and tissues is not replicated on a tissue culture plate. This limitation can to some extent be solved through culturing cells as 3D aggregates known as *embryoid bodies* (**Fig. 1**). To generate EBs, cells (typically in their thousands) are plated in little droplets on the inside lid of a tissue culture dish and inverted so they form aggregates at the bottom of a droplet through gravity (**Fig. 1**). Cells grown in this way produce many cell types associated with the three embryonic germ layers over time, and can even form spontaneously beating regions as cardiac precursors are generated. Unfortunately, EBs are highly disorganised[4,5] and fail to produce structures with any similarity to the embryo. Their usefulness is therefore limited to broad questions on the signals required for differentiation of various cell types, as well as generating precursor populations for further differentiation protocols.

The Organoid Revolution: From mini-brains to intestinal spheroids

Early studies using 3D structures, in parallel with EB work made use of artificial scaffolds and matrices to provide support for growing tissues in what is known as mechanically-supported air-liquid interface cultures. When skin or oesophageal primary keratinocytes grown in this way, and upon contact with the air-liquid interface (**Fig. 1**), they spontaneously differentiate and form self-organised, stratified tissues. However, within the last decade, there has been somewhat of a revolution in what can be accomplished in this field with the rise of *organoids* (**Fig. 2**). Generally speaking, cellular material (such as mouse or human ESCs, tissue fragments, primary cells etc.) is grown in 3D, and over time, can form structures very similar in the patterning and, sometimes, function to their in vivo counterpart[6] following the same developmental progression as the embryo. Brain (mini-brains)[7], optic cup[8] and gut organoids[9] have been produced to name a few, and they have the very real potential to be powerful model systems for probing both how organs develop normally and how pathologies and disease states can effect their development (**Fig. 2**).

One of the most important examples of this came from the Hongjun Song and Guo-li Ming's research groups in modelling the effect of the Zika virus on human brain development[10] (**Fig. 2**). The Zika virus, declared by

the World Health Organisation as a Public Health Emergency of International Concern, is particularly problematic for pregnant women, as it is passed onto the developing foetus resulting in developmental defects and neurological disorders such as microcephaly. By generating brain organoids (that mimic key aspects of human cortical development) from human induced pluripotency stem cells (iPSCs; cells from adult organisms that have been essentially *reprogrammed* to their embryonic state) and exposing them to the Zika virus, they were able to determine the neural cells the virus preferentially targeted and the how microcephaly may be established[10] (**Fig. 2**).

As well as modelling the progression of disease states, organoids may have uses in regenerative medicine. Using cellular material from patients through generating iPSCs, a patient's own cells can be guided towards the required cellular lineage or fate, and the organoids that are formed from this tissue source can be transplanted back into the patient, such as in the use of liver oganoids to treat liver cirrhosis[11]. This has the added benefit of avoiding tissue rejection associated with transplants from other individuals and abrogates the necessity for a lifetime of immunosuppressant drugs to counteract organ rejection.

Gastruloids: Embryonic organoids

Organoids have significant advantages over 2D techniques in terms of studying the spatial organisation and development of organs and tissues, significantly so when compared with EB culture. However, with the exception of the Zika virus study mentioned above[10], there are major issues in their reproducibility. For example, under the same experimental conditions there is significant variation between the observed outcomes and the frequency of these outcomes are low[12]. If organoids are to be used as model systems for disease states or understanding development, as well as being used clinically for drug screening or for regenerative medicine, it is essential that they are *reliable, reproducible and quantifiable*. Advances need to be made so that organoid systems can be precisely controlled and manipulated with minimal experimental variation.

With this in mind, our laboratory in Cambridge developed a new, highly reproducible and tractable tissue-culture technique using mouse ESCs to study early mammalian development and axial patterning[6,13-15] (**Fig. 2**). Building on earlier observations with the P19 embryonal carcinoma cell line[5] and the suspension culture techniques used by other groups[16], we aggregated small numbers of mouse ESCs in non-adherent conditions to form spherical structures on the same scale as the early mouse embryo at the blastocyst stage; this is in contrast to most other organoid systems or EBs where larger numbers of cells are typically used[6]. Over time, and under appropriate signalling conditions, these spherical aggregates, or, rather *embryonic organoids*, begin to show something remarkable by undergoing many of the morphological and patterning events of the early embryo. Firstly, they spontaneously break symmetry (as determined by the polarised expression of a gene known as Brachyury), alter their morphology by elongating and extending their axis similar to axial extension in during embryo development, and generate the three orthogonal axes similar to those found in the embryo (anteroposterior [head-tail], dorsoventral [back-front] and bilateral asymmetry [left-right]). Furthermore, they undertake a process similar to *gastrulation* in the embryo, generating cell types that correspond to the three germ layers[13,15,17]; it is the combination of these traits that gave rise to the name of these embryonic organoids: *Gastruloids*. A striking aspect of *Gastruloids* is the finding that they can form a structure similar to one that forms only in mammalian embryos: the *node*[15]. The node is an important

signalling centre in mammalian embryos and is important for identifying which side of the embryo is left or right, and the disruption of the left-right patterning causes severe birth defects usually leading to chronic heart disease[18]. Presently, the only way to study the effect of left-right axis disruption is through animal models, so the Gastruloid system offers an attractive window into studying this process cheaply and without using animals.

Interestingly, it is also the differences between *Gastruloids* and the embryo which have already provided an alternative interpretation of the function of certain embryonic tissues. The anterior visceral endoderm (AVE), a tissue in the embryo which helps constrain signals which confer posterior identity on the early embryo, is absent in the *Gastruloids*, yet *Gastruloids* are still able to spontaneously activate posterior markers in a polarised fashion determining an anteroposterior axis[15]. This lead to a completely new hypothesis that the AVE in the embryo ensures that the spontaneous activation of posterior markers occurs only at the posterior region of the embryo and not in random places, facilitating the proper placement of the primitive streak (site of gastrulation) and the future link with the mother (the allantois which will eventually form the umbilical cord)[6,15].

In vitro human development and disease modelling: An ethical minefield which Gastruloids may overcome

Despite the above examples, a burning issue still lingers: these model systems don't fully equate to human development. Even though many signalling pathways and patterning mechanisms have been conserved throughout evolution, other animals may do things differently to the human embryo, and disease-modelling using material from tissues, organoids and embryos that are different to that of human origin may give rise to errors in our understanding which will impact the effectiveness of treatment. An approach more dedicated to human development will eventually be required to fully appreciate how we develop. However, there are limits on what is permissible regarding experimentation on human embryos and for how long scientists can culture human embryos in culture. Presently, human embryos can only be cultured for a limited period of time, either for up to fourteen days or until the *primitive streak* structure forms which signifies the start of Gastrulation[19]. Unfortunately, many of the questions regarding the development of our organs and how we are patterned occur after this point. Human ESCs have however been obtained, but this also has important ethical issues which need to be addressed, since human embryos need to be destroyed to obtain them. It is at this point that organoids, and in our view, *Gastruloids*, have the potential to overcome this barrier in an ethically acceptable manner, and (in terms of regenerative medicine and tailored treatments), in ways more tailored to specific disease states in patients. By generating *Gastruloids* from current stocks of human ESCs or from human iPSCs we will be able to limit the requirement for further human embryos to study early human development (in the case of the former) and also take starting material directly from patients to study their disease in a genetic background identical to the patient's or provide source material for possible regenerative medicine (in the case of the latter).

On a final note, it is important for us to keep in mind potential problems which may arise in generating embryonic structures in culture from human ESCs/iPSCs as recently pointed out by Aach *et al.* (2017). As Gastruloids and organoids have the potential to form later embryonic structures without progressing through a primitive streak, it may arise that we have created a structure that has *neurulated*, generating something that has the potential to feel pain or develop sentience[19] (hence this is one of the reasons for stopping

human embryo work at the 14-day/primitive streak stage); current guidelines regarding the use of human embryos cannot apply in this case since the primitive streak hasn't formed. Research and bioethical communities may need to find new ways to solve these ethical problems, and it may be the case that new legislation should be drawn up to reflect both our new scientific understanding based on what our experiments tell us about development, and our changing perspectives on what is ethically acceptable[19].

Summary

It is clear that there are many methods to study development, generating structures that can be used both as a substitute for the embryo in research, and for regenerative medicine and modelling disease progression. However, it is essential that the gap is bridged between what we are able to accomplish through our understanding of well thought out experiments and how we translate our knowledge to the public without overselling what we hope to achieve which would otherwise provide a false picture of what is actually possible[12]. Additionally, the work using these model systems may also instruct us as to how far we can ethically take research into our own development using human embryos, ESCs, or iPSCs. Still, as great progress has been achieved in a relatively short space of time, it will be certainly of interest to see what new developments will arise in the future, and how this highly evolving field can translate its findings for regenerative medicine and modelling diseases.

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Glossary:

Blastocyst: A structure of the early mammalian embryo that contains the inner cell mass (which will go on to form the embryo) and the trophoblast (provides nutrients to the embryo and will develop the placenta)

Brachyury: A transcription factor essential for Gastrulation; up-regulated in the primitive streak in the posterior of the embryo

Directed Differentiation: The experimental process of chemically guiding cells through the specific fates a cell would undergo *in vivo* to reach the desired cell type

Embryoid Body: Three dimensional aggregates of ESCs that can differentiate into derivatives of the three germ layers, albeit in a disorganised, uncoordinated manner

Embryonic stem cells: Population of pluripotent cells from the inner-cell mass of the blastocyst that give rise to the embryo proper

Gastrulation: The process where the cells of the single layered epiblast undergo a process of reorganisation to generate a trilaminar structure made up of the three germ layers: ectoderm (neural tissues, skin), mesoderm (musculature, heart, bones, connective tissues), endoderm (parts of the gut, lungs and other internal organs).

Gastruloids: Embryonic organoids; small aggregates of mouse ES cells that undergo many of the patterning events and morphological changes of the gastrulating embryo, and generate derivatives of the three germ layers (see *Gastrulation*).
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iPSCs: Induced Pluripotent Stem Cells; a pluripotent population of cells that have been 'reprogrammed' from adult stem cells

Organoid: Groups of stem cells (either adult or embryonic) or clumps of dissected tissue that go on to form a structure in culture that has many of the properties of the organ of origin (e.g. morphology…)

Potency: Stem cells can be pluripotent, such as embryonic SCs which give rise to all embryonic tissues, multipotent in the case of adult SCs, which produce specific cell lineages or unipotent, which will give rise to just one lineage. This is in contrast to the fertilised embryo which is 'multipotent' which will not only produce the embryo and its tissues, but will generate the placenta and extraembryonic regions.

Primitive Streak: A structure found in higher vertebrates that marks the point of Gastrulation. It is an important landmark in the culture of human embryos, as it defines that latest possible time at which they can be cultured in vitro

Stem cells: Cells which are able to self renew and give rise to more differentiated cell types with a potency to differentiated determined by the type of stem cell (e.g. embryonic, adult or iPSCs).

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Figure legends:

Fig. 1. Figure showing brief description of mechanically supported culture, EB culture and Gastruloid culture

Fig. 2. An array of different organ types have been generated using organoid culture techniques. Examples include (clockwise from top left): Minibrains[7]; Gastric organoids[20] (comparison between embryonic day 18.5 mouse stomach and day-34 hGOs [top panel], and xxxxx); Forebrain[10] with examples of the effect of exposure to the Zika virus; optic cups[8], kidney organoids[21]; embryonic organoids (Gastruloids)[13-15,17]. Our thanks to the authors and publishers for permission to reprint images.

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Websites:

https://www.nc3rs.org.uk/establishment-left-right-asymmetry-mammalian-development

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