# The Source of Enteric Nervous System Progenitor Cells Present in Aganglionic Gut in Hirschsprung's Disease

**Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master of Philosophy by**

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# Abstract


## The source of Enteric Nervous System Progenitor Cells present in Aganglionic Gut in Hirschsprung's disease – George Stephen Bethell

The enteric nervous system develops predominantly from vagal neural crest cells which proliferate and differentiate into enteric neurons and glia whilst migrating to the distal gut. Failure of this process for unknown reasons results in Hirschsprung's disease (HSCR) which is characterised by an absence of enteric ganglia in a variable length of distal gut. This causes life threatening bowel obstruction and requires surgical intervention to remove the affected bowel. Following surgery 10-30% of patients suffer from long term constipation or faecal incontinence which can worsen with time.

Our group has previously shown that enteric nervous system progenitor cells (ENSPC) can be isolated from ganglionic HSCR gut which then differentiate into enteric neurons and glia *in vitro*. Most importantly, these ENSPC form clusters of cells known as neurospheres which when implanted into *ex vivo* aganglionic mouse gut, restore normal patterns of contractility. More recently and quite surprisingly, ENSPC have also been obtained from aganglionic HSCR gut and behave similarly to ganglionic ENSPC *in vitro*. The aim of this thesis is to add understanding to this finding by determining the source of ENSPC in aganglionic HSCR gut.

The first results chapter focuses on the optimum medium which should be used to culture neurospheres. It was found that a horse serum based medium promotes the formation of neurospheres under adherent conditions more effectively than a more complex medium containing various growth factors.

The next section tests the hypothesis that ENSPC are located within thickened nerve trunks in aganglionic gut. Using samples of aganglionic HSCR gut from patients with all variants of HSCR it was possible to correlate the presence of thickened nerve trunks and the ability to obtain ENSPC as aganglionic gut from total colonic and total intestinal HSCR doesn't contain thickened nerve trunks. It was found that it was not possible to obtain ENSPC from aganglionic HSCR gut where thickened nerve trunks were absent. This suggests that ENSPC are associated with the thickened nerve trunks in aganglionic gut.

Subsequently, experiments were aimed at determining whether aganglionic ENSPC are of neural crest lineage. Fluorescence activated cell sorting was used to obtain a sub-population of P75 positive neural crest derived cells from freshly dissociated aganglionic HSCR gut. After 6 days in culture these cells differentiated into neurons whereas this was not the case in the P75 negative sub-population. Finally immunohistochemistry was used to look at the structure of thickened nerve trunks in aganglionic gut and the expression of P75 is correlated to the possible cellular sources of aganglionic ENSPC.

When combined, work in this thesis narrows down the possibilities of the cellular origin of ENSPC of which the most likely source is cells of Schwann cell lineage located within thickened nerve trunks. These findings direct further work and make the potential of *in vivo* ENSPC stimulation and a medical treatment for HSCR more possible.

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# Abbreviations

| | |
|---|---|
| DAPI | 4',6-diamidino-2-phenylindole |
| ASCL1 | Achaete scute homolog 1 |
| ANOVA | Analysis of variance |
| BMP4 | Bone morphogenic factor 4 |
| DNA | Deoxyribonucleic acid |
| DMEM | Dulbecco’s modified Eagle medium |
| EDNRB | Endothelin B receptor |
| ECE1 | Endothelin converting enzyme |
| EDN3 | Endothelin-3 |
| ENS | Enteric nervous system |
| ENSPC | Enteric nervous system progenitor cells |
| ENCC | Enteric neural crest cells |
| FGF-10 | Fibroblast growth factor 10 |
| FACS | Fluorescence activated cell sorting |
| GI | Gastrointestinal |
| GDNF | Glial cell line derived neurotrophic factor |
| GFRα1-4 | Glycosyl-phosphatidyl-inositol linked cell surface glycoproteins |

| | |
|---|---|
| HSCR | Hirschsprung's disease |
| IBB | Immunofluorescence blocking buffer |
| KIF26A | Kinesin superfamily protein 26A |
| NCC | Neural crest cells |
| Tuj | Neuron specific class III ß tubulin |
| NTN | Neurturin |
| NOS | Nitric oxide synthase |
| PBS | Phosphate buffered saline |
| PHOX2B | Paired-like homeobox 2b |
| PTEN | Phosphatase and tensin homolog |
| PGP9.5 | Protein gene product 9.5 |
| RET | Receptor tyrosine kinase |
| S100 | S100 Calcium binding protein B |
| SMA | Smooth muscle actin |
| SOX10 | SRY-related HMG-box |
| SEM | Standard error of mean |
| ZFHX1B | Zinc finger homeobox 1b |

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# Chapter 1 – Background and introduction

## 1.1 Overview

This chapter starts by introducing Hirschsprung's disease (HSCR). HSCR is a congenital disorder where the enteric nervous system (ENS) has failed to form in a variable length of distal gut resulting in tonic contraction in the affected, otherwise known as aganglionic, gut. This results in a functional obstruction of the gut and dilation of the proximal section, known as megacolon. Most commonly, HSCR is suspected if a neonate fails to pass meconium within 24 hours of birth or has distension of the abdomen. Diagnosis is confirmed by taking suction biopsies from the rectum and the absence of ganglia is looked for. If HSCR is diagnosed the definitive treatment is surgical intervention to remove the aganglionic gut in early life. Many patients experience lifelong morbidity.

As well as an overview of HSCR, this introduction also covers the function and development of the ENS. Finally, work to date, exploring stem cell adjunctive therapies for HSCR, is described leading onto the aims of this thesis.

## 1.2 Hirschsprung's Disease

### 1.2.1 Introduction

HSCR is a congenital disorder resulting in aganglionosis of a variable length of distal gut with an incidence of 1 in 5000 live births and occurs with a male to female ratio of 4:1. Incidence varies between ethnic groups with HSCR being rarer in Hispanics (1 in 10,000) and relatively more common in Asians with an incidence of 2.8 per 10,000 live births.(Amiel et al. 2008)

It was initially suspected that genetics had a role in HSCR for a few reasons. Firstly, there was an increased incidence in siblings than observed in the general population; secondly, the unbalanced sex ratio; thirdly, the association of HSCR with other congenital abnormalities and also many reports of mouse models of colonic aganglionosis showing Mendelian patterns of inheritance.(Tam & Garcia-Barcelo 2009)

HSCR is an isolated disorder in 70% of cases however it is associated with other congenital abnormalities and termed syndromic HSCR in the remaining 30% of cases.(Borrego et al. 2013) In instances where HSCR occurs as an isolated disorder it can be split into sporadic disease and familial disease.

### 1.2.2 Familial and sporadic HSCR

Familial HSCR accounts for 20% of cases, has a complex pattern of inheritance and displays low, sex dependent penetrance. Sporadic HSCR accounts for the remaining patients who have the isolated disorder. It is well reported that there is variability in the HSCR phenotype despite similar genotypes. In total there are nine different genes that are associated with the HSCR phenotype.(Tam & Garcia-Barcelo 2009) These are the genes for receptor tyrosine kinase (RET), glial cell line derived neurotrophic factor (GDNF), neurturin (NTN), paired-like homeobox 2b (PHOX2B), endothelin B receptor (EDNRB), endothelin-3 (EDN3), endothelin converting enzyme (ECE1), SRY-related HMG-box (SOX10) and zinc finger homeobox 1b (ZFHX1B).(Kenny, Tam & Garcia-Barcelo 2010)

The first gene that is linked with HSCR was initially discovered in the late 1980's. This discovery came about through mouse work and through genotyping patients with HSCR. It was found that the HSCR phenotype was present in mice with disruption of the *RET* gene on chromosome 10, it was also found that the *RET* gene mutations were present in families of HSCR patients. Mutations to the *RET* gene account for 50% of familial HSCR cases and 35% of sporadic HSCR.(Tam & Garcia-Barcelo 2009) RET is a receptor found on enteric nervous system progenitor cells (ENSPC), it is vital for normal development of the ENS and is discussed in more detail later in this chapter.

The *GDNF* gene was first suspected as being involved in HSCR as it is the factor that is detected by the RET receptor in the ENS. GDNF has several co-ligands one of which is NTN. Work with mice found a similar phenotype between mice with knockout of RET and other mice with a knockout of GDNF. There have however been no reports of HSCR patients with an isolated mutation to GDNF or NTN. There have been very small numbers of HSCR patients reported with a GDNF mutation alongside a RET mutation and just one case report of a NTN mutation alongside a RET mutation; this was in a familial case.(Kenny, Tam & Garcia-Barcelo 2010; Tam & Garcia-Barcelo 2009) There has also been a study where genotyping of patients with lower gastrointestinal tract motility disorders such as slow transit constipation however there were no mutations to GDNF found in these patients.(Chen et al. 2002)

*PHOX2B* encodes for a surface protein expressed by cells of the ENS which is essential for RET expression. When homozygous disruption of the *PHOX2B* gene occurs in mice the ENS fails to develop.(Tam & Garcia-Barcelo 2009) In a separate study it was reported that mouse embryos with mutation to the *PHOX2B* gene fail to express *RET*, this is expected due to what is known about the significance of *RET*.(Pattyn et al. 1999) There have been reports of the presence of *PHOX2B* mutation in syndromic HSCR in humans and there is one study which has found the association of this gene to non-syndromic HSCR. This study found that a single nucleotide polymorphism of *PHOX2B* is associated with HSCR in a study of 91 patients. It is thought that this is in the presence of a *RET* mutation too however

this was not looked into and is required to give definitive evidence as to whether an isolated *PHOX2B* mutation can cause HSCR.(Garcia-Barcelo et al. 2003)

The involvement of the *EDNRB* gene was first demonstrated in mice. Knock outs of this gene gave rise to the HSCR phenotype. In humans, mutations of the *EDNRB* gene are found in both isolated and syndromic HSCR, with the syndrome being Shah-Waardenburg syndrome. (Kenny, Tam & Garcia-Barcelo 2010) The mutation was found to be more penetrant when it was homozygous however this was sex dependent. It has also been shown that a heterozygous disruption gives rise to isolated HSCR whereas homozygous disruption results in Shah-Waardenburg syndrome. The *EDNRB* gene mutation is usually inherited from unaffected parents and results in short segment HSCR. This mutation is only responsible for about 5% of cases of HSCR.(Tam & Garcia-Barcelo 2009)

Disruption of the *EDN3* gene in mice results in a similar phenotype to disruption of the *EDNRB* gene. This can be expected due to what is known about the signalling pathway which includes the receptor and peptide encoded for by these genes and is discussed further on in this chapter. Very few mutations to the *EDN3* gene have been found in patients with HSCR. When they have been found heterozygous mutations have given rise to isolated disease whereas homozygous mutations have resulted in Shah-Waardenburg syndrome. This is similar to what is seen in mutations to the *EDNRB* gene. A gene closely associated with *EDN3* is *ECE1* as this

encodes for an enzyme which converts *EDN3* into its active form. Evidence from mouse models show that a knockout of this gene results in the HSCR phenotype however this has only ever been associated in HSCR once. This patient had syndromic HSCR.(Tam & Garcia-Barcelo 2009)

*SOX10* is a transcriptional regulator involved in the development of cell lineage, the functions of *SOX10* are discussed further on in this chapter. In a mouse model with heterozygous knock out of the *SOX10* gene the phenotype was very similar to Shah-Waardenburg syndrome in humans. In humans a heterozygous mutation results in Shah-Waardenburg syndrome, showing that this gene is associated with syndromic HSCR. There have however been no reports of *SOX10* mutations in isolated HSCR.(Kenny, Tam & Garcia-Barcelo 2010; Tam & Garcia-Barcelo 2009)

*ZFHX1B* encodes for a protein that is essential for formation of vagal neural crest cells. There are no known mouse studies of this gene. It has been shown that mutations to the *ZFHX1B* gene are present in Mowat Wilson syndrome, a syndrome resulting in craniofacial deformities, epilepsy, mental retardation and HSCR. (Kenny, Tam & Garcia-Barcelo 2010) There have been no reports of *ZFHX1B* being involved in isolated HSCR.

### 1.2.3 Syndromic HSCR

HSCR is associated with chromosomal anomalies or congenital abnormality in 30% of cases. Of the chromosomal abnormalities trisomy 21, also known as Down syndrome, is most commonly associated with HSCR. This has led to work looking at the over expression of genes on chromosome 21, related to the development of the ENS however there have been limited data produced from these studies so far. It is known however that the *RET* hypomorphic allele, which causes HSCR, is more common in patients with trisomy 21.(Amiel et al. 2008)

HSCR is associated with other neurocristopathies, this is congenital disease due to failure of migration, proliferation, differentiation or survival of neural crest cells (NCC). This is predictable due to the development of the ENS and will be discussed later in this chapter. It is particularly important to promptly identify multiple endocrine neoplasia syndrome type 2B associated with HSCR, which is one of the neurocristopathies, by genetic screening due to the risk that medullary thyroid carcinoma poses.(Kenny, Tam & Garcia-Barcelo 2010)

### 1.2.4 Classification

HSCR is classified on the length of gut affected by the disease. Short segment disease does not extend proximal to the rectosigmoid junction of the large intestine, this accounts for over 80% of cases.(Obermayr et al. 2013) Long segment disease extends proximally beyond the rectosigmoid junction and has an incidence of 15-20%. In total colonic disease the whole of the large intestine is affected, this accounts for 5% of HSCR. A very rare variant of HSCR is total intestinal aganglionosis which extends at least to the duodenum and has even been reported to extend to the oesophagus, this is seen in around 1 in 500,000 live births.(Bergeron, Silversides & Pilon 2013; Ruttenstock & Puri 2009) Interestingly, the male to female gender bias in short segment disease is 4:1, in long segment disease it is 2:1 and it is lost in total colonic and total intestinal aganglionosis.(Borrego et al. 2013; Kenny, Tam & Garcia-Barcelo 2010)

### 1.2.5 History

The first observation of the disease may have been reported in 1691 by a Dutch anatomist called Frederick Ruysh.(Cass 1986) He described a case where a five year old presented with abdominal pain and did not respond to the “usual treatment” of the time which was not documented. She subsequently died and on post mortem a megacolon was seen. Although there a many unknowns regarding this case there is a suspicion that this is the first documented case of HSCR.

There were various similar reports over the years until in 1901 Tittel, an Austrian physician reported the first histological study of one of these patients. He reported “sparse development of the plexuses throughout the colon, but normal findings in the ileum”.(Tittel 1901)

In 1904 Harald Hirschsprung wrote a chapter in a textbook dedicated to congenital dilation of the colon and described 10 patients who had “congenital dilation of the colon”. Five of the ten patients died before 12 months of life. In all of the patients who underwent post mortem, dilated and hypertrophied gut was described. It was also confirmed that there was no obvious mechanical obstruction however there was little thought at this point into the aetiology of the disease. It was however recognised as a congenital abnormality.(Holschneider 2008)

In 1920 a case report was produced detailing the absence of nerve ganglia in the sigmoid colon but presence of ganglia in more proximal colon in two brothers. This triggered thought into the aetiology of the disease and many trials using rodents took place to show that the megacolon was in fact due to the absence of the ENS in distal colon, giving the first understanding of the pathological process which caused HSCR.(Holschneider 2008)

In 1948 Swenson discovered, by use of a barium enema and fluoroscopy in patients with HSCR, that there was spasm in a section of gut in these patients. This defined the level of obstruction and added further information to the process causing megacolon in HSCR patients.(Holschneider 2008; Swenson 1999) In the same year the abnormal histological findings were correlated to the abnormal physiological findings in the affected gut and HSCR was recognised histopathologically as the absence of ENS ganglia. (Zuelzer & Wilson 1948)

The subsequent key events in the history of HSCR involve the evolution of surgical techniques used to treat the disease. This is discussed in detail in the management section further on in this chapter.

### 1.2.6 Pathology

HSCR is caused by failure of development of the ENS, causing aganglionosis, in a variable length of distal gut. There may be one pathophysiology for all cases of HSCR or different pathophysiologies depending on the length of aganglionosis present. Current thought is that functional enteric neural crest cells (ENCC) have failed to migrate along the full length of the gut. Another theory has come about from previous work from our group looking at aganglionic gut from HSCR patients.(Wilkinson et al. 2013) This is that the ENCC have migrated but have failed to differentiate into functional ganglia. This is discussed in more detail later on in this chapter.

In HSCR, the affected length of gut causes a functional obstruction of the contents of the gastrointestinal (GI) tract as the lack of inhibitory innervation results in tonic contraction of the smooth muscle in the two muscular layers of the gut. The result of this is visible at surgery as the proximal section of gut is dilated due to the obstruction and is sometimes described as 'megacolon'.

In the aganglionic segment of gut in HSCR there is an absence of myenteric and submucosal ganglia and presence of hypertrophied extrinsic nerve trunks which originate from the pelvic plexus in short and long segment HSCR.(Okamoto, Satani & Kuwata 1982) In long segment HSCR there are a reduced number of thickened trunks observed compared to short segment HSCR on histology for reasons

unknown at this point in time.(Watanabe et al. 1998) In total colonic disease there is a total absence of thickened nerve trunks in aganglionic gut, this is also the case in total intestinal aganglionosis. Subsequently, interstitial cells of Cajal are also usually absent from the muscular layers in aganglionic total colonic and total intestinal HSCR gut but are present in the same layers in short and long segment HSCR gut.(Solari, Piotrowska & Puri 2003)

This raises questions on the pathophysiology behind total colonic and total intestinal disease as there are major histological differences in these patients compared to short and long segment disease in regards to the thickened nerve trunks.

Very little is known about the formation, presence and function of thickened nerve trunks. One unpublished theory that might explain the presence of these thickened trunks in aganglionic gut in short and long segment HSCR is that the ENS normally inhibits the growth of the extrinsic nerve trunks within the myenteric plexus and submucosal plexus. Therefore when the ENS is not present there is overgrowth of these nerves.

### 1.2.7 Clinical presentation

The typical presentation of HSCR is when a neonate fails to pass meconium within the first 24 hours of life. The neonate can also develop life threatening bowel obstruction with necrotising enterocolitis due to the build of faeces and bacterial overgrowth within the gut. It is also possible, particularly with short segment disease, for a patient to present later on in childhood or very rarely in their early teenage years with chronic constipation.(Arshad, Powell & Tighe 2012)

Antenatal screening is currently not used to screen for HSCR. It would be possible to genotype a foetus antenatally however this involves invasive tests which would only be carried out on suspicion of some other congenital abnormality. It is not possible to detect HSCR on a foetal ultrasound scan. Even if it was possible to identify HSCR genetically the extent of aganglionosis and prognostic factors such as likely response to surgery would be unknown and hence there is little argument regarding a benefit to discovering HSCR antenatally.

### 1.2.8 Investigations

If HSCR is suspected rectal suction biopsies from 2cm above the dentate line are taken, an adequate biopsy must include the submucosal tissue layer, which in normally innervated gut would include the submucosal plexus. Histopathological staining then takes place to attempt to identify an increased activity of acetylcholinesterase which is present within the thickened nerve trunks within the muscular layers in HSCR.(de Arruda Lourencao et al. 2013) The presence or absence of ganglia is also assessed using haematoxylin and eosin staining. This is particularly important as it will differentiate total colonic and total intestinal HSCR from normally innervated gut.(De Lorijn et al. 2005) The limitation of this investigation is that it is superficial and does not take tissue from below the submucosal layer of the rectum.

To confirm the diagnosis several full thickness biopsies, including both muscular layers of the gut, are taken under general anaesthesia at the time of surgery for HSCR. The biopsy is sent fresh to the laboratory during the procedure and acetylcholinesterase staining takes place, as with the suction biopsies. Once a definite diagnosis is given the level of the aganglionosis can be determined as the several biopsies at various lengths along the dilated gut will reveal this information. Once this is known surgery can continue as the aganglionic gut to be resected has been determined histologically.(De Lorijn et al. 2005)

### 1.2.9 Treatment

Treatment for short and long segment disease consists of removal of the affected length of gut by one of three techniques. In total colonic disease there are various approaches to treatment however most commonly treatment involves a total colectomy with the formation of a distal ileostomy or the formation of an ileal pouch. Outcomes are poor for these patients and the majority experience lifelong morbidity.(Moore 2012) In more extensive aganglionosis a jejunostomy is usually formed and the patient must rely on a small gut transplant for any chance of survival beyond the neonatal period.(Bond & Reyes 2004) The aim of any definitive surgical intervention is to remove the dysfunctional gut which includes the aganglionic gut and the transition zone, leaving only ganglionic gut (Diagram 1.1).

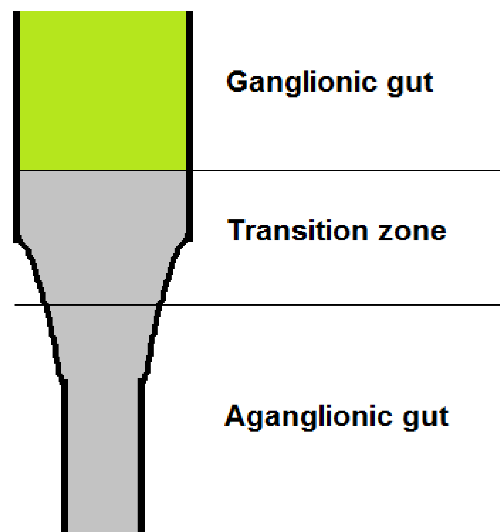


**Diagram 1.1. Schematic showing gut resected in definitive surgery for HSCR.** Areas shaded in grey show gut resected and areas in green shows gut spared during surgery.

The three surgical techniques for HSCR are the Swenson, the Duhamel and the Soave procedures.

### 1.2.9.1 Swenson procedure

The Swenson procedure was the first definitive treatment for HSCR and was first described in 1948.(Swenson & Bill 1948) After the level of aganglionosis is determined, the distal colon is mobilised by laparotomy and resection down to the anal canal takes place. The aganglionic section of colon is then removed and the ganglionic colon is anastomosed to the anal canal. This procedure is considered as very invasive and carries a large risk to nearby urogenital structures including the autonomic plexus. The internal anal sphincter is also removed in this procedure. Due to the risks that this procedure carried other techniques were developed.

Recently however this technique has been revisited and adapted so that a full-thickness dissection occurs via the anus. This isn't common practice but the outcomes from one centre report that the technique is as desirable as the other more modern techniques.(Levitt et al. 2013)

### 1.2.9.2 Duhamel procedure

The Duhamel technique was originally described in 1956.(Duhamel 1956) The advantages of this procedure over the Swenson procedure was that the internal

anal sphincter was left intact which was thought to give a lower risk of long term complications. This was accomplished by commencing the full thickness dissection a few centimetres above the dentate line. The resection via the abdominal route was also only into the rectal pouch hence avoiding trauma to the urogenital organs. The aganglionic gut is resected and an anastomosis takes place between the aganglionic colon and the remaining few centimetres of rectum.

Since 1956 the main modification to this technique has been the introduction of a stapler to form a longitudinal anastomosis via the anus at operation.(Steichen, Spigland & Nunez 1987)

**1.2.9.3 Soave procedure**

In 1964 Soave developed a technique for treating HSCR that involved a more superficial dissection and was undertaken by a transanal approach.(Soave 1964) The dissection started a few centimetres above the dentate line and was only as deep as the submucosal layer of the rectum. The dissection then continues moving proximally for another 3-4cm before the dissection becomes full thickness. The aganglionic section of gut is then removed via the transabdominal route and resected. This technique has the advantage of leaving an aganglionic muscular cuff in which the pulled through ganglionic gut sits on top of. Therefore there is less risk of damage to the organs of the pelvis. Originally the anastomosis between the

rectum and the ganglionic colon was delayed for 10 days before taking place via a subsequent laparotomy.

Later on in 1964 Boley modified the Soave technique and reported the use of a primary anastomosis instead of delaying it as Soave had originally described.(Boley 1964) This has led to the Soave procedure becoming commonly known as the Soave-Boley procedure.

In 1996 Saltzman reported a transanal pull through of the aganglionic gut with primary anastomosis.(Saltzman et al. 1996) The major advantage of this modification was a shorter operating time as the transanal dissection can occur at the same time as the biopsies are obtained with laparotomy. A shorter stay postoperatively was also reported, although there were no suggestions as to why this was the case.

Finally in 1999 a laparoscopic assisted transanal pull through with primary anastomosis was described.(Georgeson et al. 1999) This carried the same advantages as Saltzman had reported but with the additional benefits that laparoscopic surgery brings.

#### 1.2.9.4 Current practice

In 2011 every paediatric surgeon in the UK and Ireland who operated on children with HSCR was surveyed.(Bradnock & Walker 2011) There were 64 respondents in total from 21 of the 26 tertiary paediatric surgery centres. Only 40 of the respondents routinely performed definitive surgery for HSCR giving a median of 5 (range 1.5-16) procedures per year per consultant. 35 out of the 40 consultants aim to operate on a neonate at less than 3 months of age. The Duhamel procedure was the technique of choice for 18 of the 40 surgeons with the Soave-Boley technique being favoured for the other 22 surgeons. No surgeons favoured using the historical Swenson technique. Remarkably only 18 of the 40 surgeons used a laparoscopic assisted approach.

The authors of the paper reporting this survey and compared their work to a similar study(Huddart 1998) which was published eleven years before theirs. The major changes that occurred over the eleven year time period was the increase in the number of surgeons willing to operate on neonates younger than 3 months (46% v 88%). The amount of paediatric surgeons operating on HSCR patients also increased from 41% in 1998 to 85% in 2011. The use of laparoscopy and the Soave-Boley technique has also increased over this time period.

Some surgeons are only seeing a handful of these patients per year as there are a large proportion of surgeons operating on patients with HSCR. There have therefore

been suggestions that fewer surgeons should operate on these patients and that subspecialisation should occur in all units. The main thought behind this is that there are bound to be better outcomes for the patients however there is currently no long term outcome data available to support this.(Bradnock & Walker 2011)

### 1.2.10 Complications

Short term complications for all surgical HSCR procedures include enterocolitis, anastomotic leak and strictures. Most worryingly however are the long term complications that can result in lifelong morbidity.

The limitation of current surgery is that the internal anal sphincter, which is supplied by the ENS, is not removed and is anastomosed to the ganglionic gut. This results in the aganglionic internal anal sphincter failing to relax and contract as it should do in normally innervated gut. Therefore, between 10% and 30% of patients suffer from lifelong morbidity due to either faecal incontinence or chronic constipation.(Wilkinson, Edgar & Kenny 2012) There is also evidence which shows that complications worsen as the patient grows older.(Jarvi et al. 2010) Chronic constipation can be improved with regular Botulinum toxin injections targeted at the internal anal sphincter. Unfortunately, results vary from patient to patient and more work is needed to understand who benefits best from this treatment.(Koivusalo, Pakarinen & Rintala 2009) For the patients who suffer from faecal incontinence there are various strategies which are used. These include

changes to diet to avoid certain foods such as fatty foods, the usage of laxatives (to treat pseudo diarrhoea), rectal washouts and also anti-diarrhoeal medication.(Catto-Smith, Trajanovska & Taylor 2007) Some patients, particularly those with anastomotic strictures require reoperation on the muscular cuff left with the Soave procedure.(Dickie et al. 2014) Despite attempts to treat these complications, 10% of these patients require a permanent colostomy due to the severity of their symptoms.(Baillie et al. 1999)

## 1.3 The Enteric Nervous System

### 1.3.1 Introduction

The ENS is an extensive network of neurons and glia which are present within the GI tract and innervate the gut. Its main function is to control the motility of ingested food through the gut along with the control of absorption and secretion, of nutrients and electrolytes.

### 1.3.2 Structure of the ENS

In humans there are approximately 400-600 million neurons within the ENS which group together forming ganglia. These ganglia are located and confined to two separate layers of the gut wall, the myenteric plexus and the submucosal plexus.(Obermayr et al. 2013) Along with connections between the ganglia in each plexus enteric nerves extend into and through the muscular layers to connect the two nervous layers. In addition to this nerves also extend to the mucosal layer.(Gulbransen & Sharkey 2012)

### 1.3.3 Function of the ENS

The ENS has several important functions. Firstly, it innervates the smooth, circular and longitudinal muscle of the GI tract from the oesophagus through to the internal anal sphincter therefore controlling, through reflex pathways, peristalsis of

digesting food along the GI tract. The ENS is also responsible for controlling both water and electrolyte secretion and absorption, along with blood flow, throughout the GI tract.

Smooth muscle sphincters along the GI tract are also controlled by the ENS, including the internal anal sphincter which is vital for continence and normal defecation. The ENS has both sympathetic and parasympathetic innervation from extrinsic pelvic nerves. When the sympathetic pathway is activated passage of the contents of the GI tract is restricted by inhibition of the muscular layers. Blood supply, fluid secretion and electrolyte secretion is also reduced. With increased sympathetic innervation the sphincters of the tract which are controlled by the ENS contract. Parasympathetic innervation produces opposite consequences to sympathetic innervation.(Furness 2008)

In terms of differing functions between the two layers it is thought that the myenteric plexus (located as a layer between the longitudinal and circular muscle) has a larger role in motility than the submucosal plexus which oversees absorption and secretion.(Hall & Guyton 2011; Jiang, Liu & Gershon 2003)

### 1.3.4 Development of the Enteric Nervous System

The ENS is formed from NCC which have migrated into the foregut, as they are migrating they are also proliferating and to some extent differentiating. Migration continues to the distal hindgut in order to colonise the entire gut with ENSPC which give rise to the mature ENS.

#### 1.3.4.1 Migration and proliferation of Neural Crest Cells

There are five different populations of NCC within the developing embryo; these are the cranial, trunk, cardiac, vagal and sacral NCC. The populations relevant to the ENS are the vagal and sacral NCC. Ablation studies of NCC in chicks have shown that the majority of the contribution to the ENS is by the vagal crest, which is formed adjacent to somites one to seven.(Bergeron, Silversides & Pilon 2013; Wallace & Burns 2005) In addition to the colonisation by vagal NCC, there is also migration in the caudal to rostral direction of sacral NCC, which are found adjacent to somites 28 onwards. This has been shown using a quail-chick chimera system and sacral NCC were found to only colonise the gut beyond the umbilical portion. (Obermayr et al. 2013) It has been shown that these sacral NCC rely on contact with the vagal NCC before the sacral NCC spread rostrally within the gut.(Sasselli, Pachnis & Burns 2012) Therefore sacral cells cannot contribute to the development of the ENS without the presence of vagal NCC in the distal gut. Furthermore, the axons of extrinsic neurons migrate into the distal hindgut from the pelvic plexus with the

sacral NCC but the signalling pathways involved with this are unknown.(Erickson et al. 2012; Wang et al. 2011)

Once the vagal and sacral NCC migrate from the hindbrain into the foregut they are termed ENCC. These ENCC colonise the gut as far as and including the internal anal sphincter travelling in the rostral caudal direction within the mesenchyme.(Bergeron, Silversides & Pilon 2013) These ENCC migrate through the gut in cords of connected cells forming a migratory wave front.(Conner et al. 2003) Studies using mice embryos have shown that the migration to the caecum is a continuous process of advancing cells arranged in cords provided there is a sufficient number of ENCC present. If only a small number of these cells are in the gut then there is an arrest in migration.(Druckenbrod & Epstein 2005) At the caecum a different pattern of migration is seen, the wave front becomes static for around 8-12 hours during which some ENCC advance as isolated cells into the proximal colon. The isolated cells then form groups so that there are a number of isolated small populations of cells. Strands then form between these groups and migration continues as a continuous wave to the distal colon, colonising the full length of the gut.

In mouse studies, this unique process in the caecum has been shown to be due to specific signalling of the caecal mesenchyme. This signalling occurs by the release of various factors which act upon the ENCC. Fibroblast growth factor 10 (FGF-10) is

responsible for the growth of caecal epithelium, the superficially layer of the caecum, which is the section of gut between the ileum and ascending colon. It is unique to the caecum.(Burns, Pasricha & Young 2004; Druckenbrod & Epstein 2005) Bone morphogenic factor 4 (BMP4) has been shown to stimulate the development of enteric neurons as well as reduce the size of the progenitor pool.(Chalazonitis 2004; Druckenbrod & Epstein 2005) There are also increased levels of GDNF and EDN3 released from the caecal mesenchymal cells. GDNF is a chemo-attractant for ENCC whilst EDN3 inhibits the actions of GDNF. This may explain how the population is arrested in their migration within the caecum. The presence of GDNF prevents ENCC continuing their migration as they have done up to the caecum. The increase of EDN3 signalling, 8-12 hours later, may inhibit the stasis of these cells within the caecum and hence allow the continuation of migration into and beyond the proximal colon.(Druckenbrod & Epstein 2005)

Another source of isolated advancing cells in the colon is from ENCC that have crossed the mesentery from the small intestine.(Druckenbrod & Epstein 2005) In humans and mice the mid small intestine and proximal colon are transiently juxtaposed during normal gut development. This happens during the rotation and physiological herniation of the mid gut and takes place between weeks 4 and 8 of gestation in humans. The mid gut herniates into the proximal umbilical coelom and then rotates $90^{o}$ anticlockwise around the vitelline duct. The herniated mid gut then returns to the abdominal cavity whilst rotating a further $180^{o}$ anticlockwise. The gut now lies in the correct orientation after a total of $270^{o}$ rotation. Following this

embryological process in mice it has been found that ENCC are present in the mesentery between these two sections of gut.(Coventry et al. 1994; Obermayr et al. 2013) It has been shown that a substantial proportion of ENCC in the colon are from this anatomical shortcut from the small gut. It has been shows that the GDNF-RET signalling pathway has a role to play in this process.(Nishiyama et al. 2012) This work suggests that the rotation of the mid gut is not only required to give rise to normal, mature anatomy in this region but to also allow colonisation of the distal gut tube with ENCC in order to give rise to the ENS.

In addition to longitudinal migration there must also be movement of ENCC centripetally. In mice, rats and chicks ENCC migrate once they are present in the superficial layer (which will become the myenteric plexus) into the deeper, submucosal plexus so that both layers are innervated. This is shown to be regulated by Netrin which is a protein which guides axon development.(Jiang, Liu & Gershon 2003)

The colonisation of the entire gut is a relatively lengthy process and takes seven weeks in humans. NCC enter the proximal gut at week five of gestation, the now termed ENCC reach the terminal ileum at week seven and finally reach the distal rectum at week twelve of gestation.(Fu et al. 2004) In mice this process takes around 5 days and starts with NCC entering the foregut at day 9 of gestation, the ENCC reach the caecal region at day 11.5 and reach the distal rectum by day 14.5 of

gestation.(Young et al. 1998) The average gestation of a mouse is 20 days. As discussed, the whole process is regulated by cell surface molecules expressed by the migrating ENCC along with factors secreted by the mesenchymal cells.(Obermayr et al. 2013) As well as migrating, the cells are also proliferating and differentiating into neurons and glial cells. This is a continuous process, which starts as soon as NCC enter the foregut.(Anderson et al. 2007)

#### 1.3.4.2 GDNF-RET pathway

The most important signalling pathway within the development of the ENS is the GDNF-RET pathway. GDNF is produced by mesenchymal cells within the developing gut and RET is a tyrosine kinase receptor which is expressed by ENCC.(Obermayr et al. 2013) The interaction between the RET receptors and GDNF is regulated by glycosyl-phosphatidyl-inositol linked cell surface glycoproteins (GFRα1-4).(Barlow, de Graaff & Pachnis 2003) In studies using mouse models where knockout of the RET, GDNF and GFRα1 have occurred total aganglionosis of the gut results. Whereas hypomorphic mutation results in partial aganglionosis in mice, which can be used as a model of HSCR.(Barlow, de Graaff & Pachnis 2003; Cacalano et al. 1998; Moore et al. 1996; Pichel et al. 1996)

Signalling between GDNF and RET is essential for survival, proliferation, migration and differentiation of ENCC. GDNF is expressed by the mesenchyme from the foregut to the caecum. The concentration of GDNF within different sections along

the gut vary over time producing a concentration gradient in the caudal rostral direction. This keeps the ENCC moving towards the distal gut and prevents stasis except for in the caecum as mentioned previously. Despite this it is thought that GDNF does not have a role in attracting NCC to the foregut in the first place as experiments in RET knockout mice found that these cells die as they reach the foregut instead of before. Therefore GDNF-RET interaction is exclusive to ENCC.(Natarajan et al. 2002; Young et al. 2001)

There are numerous transcription factors but most notably SOX10 and PHOX2B that are needed for the expression of RET, therefore failure of production of these factors results in failure of the GDNF-RET pathway.(Southard-Smith, Kos & Pavan 1998)

As well as positive regulators of RET there are two negative regulators which are required to avoid over expression of RET and the resultant hyperganglionosis. These regulators, Sprouty 2 and the Kinesin superfamily protein 26A (KIF26A), have an important role in determining the total number of neurons.(Taketomi et al. 2005; Zhou et al. 2009) This has been shown in a mouse model. Mice with knock outs of the genes responsible for these factors develop up to 50% more neurons in the gut. This hyperganglionosis also results in megacolon, as already mentioned a dilation of the gut due to the accumulation of gut contents proximal to an obstruction, and oesophageal achalasia. Oesophageal achalasia is a disorder of motility where the

primary problem in that the lower oesophageal sphincter fails to relax along with absence of peristalsis. This is thought to be caused by an imbalance to the complex excitatory and inhibitory mechanisms within the ENS due to the hyperganglionosis.(Taketomi et al. 2005)

Another cause of hyperganglionosis is the deletion of the phosphatase and tensin homolog (PTEN) gene which is responsible for a phosphatase which controls cell growth, proliferation and death. In a mouse model involving the deletion of this gene, megacolon and intestinal pseudo obstruction occurred which resulted in death at around two to three weeks after birth.(Puig et al. 2009) This is most likely due to failure of normal neural cell apoptosis during development and hence the result is hyperganglionosis. It is also known that there is crosstalk between the PTEN and the RET pathway.(Zbuk & Eng 2007)

It is clear that pathology occurs with hyperganglionosis as well aganglionosis, hence this has to be considered when developing any ENS stem cell therapy to ensure that hyperganglionosis does not occur.

#### 1.3.4.3 Differentiation of enteric neural crest cells

It has been shown in mouse models that neurogenesis occurs within 24 hours of ENCC entering the foregut. This process of differentiation into neurons and glial

cells continues into the postnatal period. In mice, this process stops at around 3 months of life and does not normally continue into the adult period. Neurogenesis has however been shown to continue in the adult period as a response to ENS injury and is regulated by 5-hydroxytryptamine 4 receptors.(Laranjeira et al. 2011; Obermayr et al. 2013)

Many of the chemical factors which influence ENCC migration also affect cellular differentiation. There are various molecules produced by the gut mesenchyme that promote the differentiation of the ENCC, to note these are GDNF, neurotrophin 3 and bone morphogenetic proteins including BMP4.(Obermayr et al. 2013) There is also one factor that inhibits the differentiation of ENCC as shown in mouse studies. This is EDN3 and its function is to prevent premature differentiation of ENCC whilst they are still migrating in order to preserve the progenitor pool.(Wu et al. 1999)

There are also transcription factors expressed by ENCC that have a role in the differentiation of ENCC within the primitive gut. SOX10 inhibits ENCC differentiation and is expressed by all NCC as they migrate towards the foregut. Once the cells enter the foregut PHOX2B and Achaete scute homolog 1 (ASCL1) are also expressed. The expression of these two factors once the NCC have entered the gut causes inhibition of SOX10. Therefore an enhanced expression of SOX10 prevents the differentiation of NCC and a reduced expression of SOX10 results in premature

neurogenesis and gliogeneis.(Obermayr et al. 2013; Sasselli, Pachnis & Burns 2012) To note, SOX10 is also expressed by mature Schwann cells.

#### 1.3.4.4 EDN3-EDNRB pathway

As stated, NCC start to differentiate into mature neurons and glial cells within 24 hours of entering the foregut in mice. This is regulated by EDN3 which is a signalling factor produced by gut mesenchyme that interacts directly with the EDNRB on ENCC. In mice with a knock-out of the gene coding for EDNRB an increased rate of differentiation of the ENCC is seen. This reduces the amount of immature progenitor cells present in the gut resulting in arrest in migration and hence aganglionosis is seen in the distal gut.(Obermayr et al. 2013) The distal gut is only affected as this pathway is required for terminal ENCC migration and not the initial attraction or migration within the foregut and midgut.(Lee, Levorse & Shin 2003)

## 1.4 A stem cell adjunctive therapy to HSCR

### 1.4.1 Introduction

Despite the development of many surgical options for patients with HSCR, current treatment is associated with lifelong morbidity for many patients.(Baillie et al. 1999; Jarvi et al. 2010; Wilkinson, Edgar & Kenny 2012) Therefore, research is needed which looks at the prevention of long term constipation and faecal incontinence which many HSCR patients suffer from.

Work undertaken by our group previously has explored the feasibility of generating an ENS *in vivo* with the use of ENSPC to reinstate normal patterns of contractility within the gut of patients with HSCR. It is thought that his would prevent long term postoperative morbidity. Mouse and human tissue has been used previously and is detailed in this section.

To avoid immunological rejection issues autologous implantation is considered the best approach. It is also thought that a stem cell therapy would best function as an adjunctive therapy to surgery for the reason that it would be very difficult to colonise the entire gut with progenitor cells however it would be possible to colonise a small but functionally important area, such as the internal anal sphincter.

### 1.4.2 Characteristics of ENSPC obtained from embryonic mouse

In recent years, ENSPC were isolated from embryonic mouse gut. These cells were shown to differentiate *in vitro* into enteric neurons and glia and form neurospheres. A neurosphere is a self-adherent cluster of neural stem cells, progenitor cells and their progeny which were originally cultured from cells derived from the central nervous system before groups such as ours have been able to culture enteric neurospheres.(Uchida et al. 2000) In addition, these enteric neurospheres were implanted into *ex vivo* mouse gut. Following time in culture proliferation, migration and differentiation of ENSPC into neurons and glia was observed within the *ex vivo* gut.(Almond et al. 2007) Most importantly, when these neurospheres were implanted into *ex vivo* aganglionic mouse gut, high frequency contractions were restored which showed the ability of ENSPC to develop a functional ENS *in vitro*.(Lindley et al. 2008)

The next step was to translate this work to experiments using human enteric neurospheres containing ENSPC.

### 1.4.3 Characteristics of ENSPC obtained from human neonatal gut

Cells were obtained from human neonatal gut and neurospheres were generated in culture. As with the embryonic mouse neurospheres, human neurosphere transplantation into *ex vivo* mouse gut allowed ENSPC migration and differentiation

into neurons and glia.(Almond et al. 2007) Most importantly human neurospheres were also shown to restore normal patterns of smooth muscle contractility in aganglionic *ex vivo* mouse gut by forming functional synapses to the native smooth muscle.(Lindley et al. 2008) These findings were also identical in neurospheres formed from cells obtained from ganglionic HSCR gut.

The next step involved developing a sufficient quantity of neurospheres as the number of primary neurospheres that were cultured was relatively low. This involved dissociating primary neurospheres and allowing secondary neurospheres to form. This was then repeated to produce tertiary neurospheres. This resulted in exponential growth from ten primary neurospheres to around 300 tertiary neurospheres. Each generation of neurospheres were found to have similar characteristics in terms of size and neuronal marker immunoreactivity using PGP9.5. This is important as it shows that it is possible to generate a large number of neurospheres which is required for a stem cell therapy for HSCR using neurosphere implantation.(Lindley et al. 2009)

It was assumed from previous experiments that neurospheres contained progenitor cells and provided an environment for progenitor cell proliferation. However it was not known how the cells behaved within the neurosphere formation. Further experiments found that ENSPC rapidly proliferate on the surface of the neurosphere before moving towards the centre. After 8 days in the centre these progenitor cells

started to show a neural phenotype. Notch signalling was inhibited in cells within neurospheres by inhibiting a notch signalling peptide which resulted in a reduction in proliferation to 55% compared to the controls. Differentiation subsequently increased, this was identified by increased expression of the neuron markers Tuj and NOS. It was concluded that Notch signalling was responsible for progenitor cell proliferation and inhibition of progenitor cell differentiation in the periphery of the neurosphere.(Theocharatos et al. 2013) This is significant as a failure of notch signalling in an embryo may be result in a reduced progenitor pool and premature development of the ENS before cells have migrated to the distal hindgut causing HSCR. Another implication of this finding is that it may be possible to control ENSPC within neurospheres once implanted into aganglionic gut by inhibiting Notch signalling. This would stimulate the differentiation of cells and limit proliferation which is required for development of the ENS. Most importantly, differentiated cells are safer compared to un-differentiated progenitor cells in terms of their ability to form tumours. This is a concern with any stem cell therapy and could be marginalised with *in vivo* Notch signalling inhibition.

### 1.4.4 Human ENSPC from aganglionic HSCR gut

Most recently, and quite remarkably, neurospheres have been cultured from cells obtained from aganglionic short and long segment HSCR gut. These aganglionic neurospheres were then compared to neurospheres formed from cells obtained from ganglionic HSCR gut. It was found that there is a similar proportion of smooth muscle, neural crest derived cells and mature neurons in both types of neurosphere

over four time points. Neurospheres from aganglionic gut were also implanted into aganglionic, *ex vivo* mouse gut and normal patterns of contractility were restored.(Wilkinson et al. 2013)

This finding has important implications. Firstly, aganglionic gut obtained at pull through procedure can be used as a source of ENSPC which can be cultured into neurospheres and implanted into the remaining aganglionic internal anal sphincter. This avoids the requirement to resect ganglionic gut in HSCR patients to use as a source of ENSPC for stem cell therapy. However, most interestingly this work raises a new question which is whether ENSPC can be stimulated within the aganglionic HSCR gut *in vivo* to proliferate and differentiate into a functional ENS. In order to explore this further the molecular cues which trigger ENSPC proliferation and differentiation must be understood. Ultimately, further work in this area could lead to a therapeutic treatment for HSCR and surgical intervention could be avoided all together.

In order to explore the use of aganglionic ENSPC for stem cell therapy for HSCR the source of these cells needs to be understood, predominantly for safety reasons and also to improve the efficacy of a stem cell therapy. There are three likely possibilities. Firstly, it may be that ENCC have migrated to the distal gut during development but have failed to differentiate into a functional ENS. Hence, they have remained in an undifferentiated state until exposed to culture conditions.

Secondly, that a cell type located within the thickened nerve trunks which is the only neuronal tissue in aganglionic HSCR gut, acts as ENSPC. Lastly, it may be possible that ENSPC obtained from aganglionic HSCR gut are of non neural crest lineage and when these cells are exposed to culture conditions they are able to differentiate into enteric neurons and glia.

As well as understanding the source and cellular origin of ENSPC in aganglionic gut the reason why these cells have failed to form an ENS when they are present in the tissue must also be answered in order to understand how these cells can be stimulated *in vivo*.

## 1.5 Aims of the thesis

Overall the aim of this thesis is to confirm the finding that it is possible to obtain ENSPC from aganglionic HSCR gut as well as determining the source of these cells. This can be further broken down into the aims of each results chapter:

1. To determine which culture media is optimal for culturing human enteric neurospheres in terms of neurosphere yield and ENSPC yield. This work is required as the group has previously used two different types of media which are both capable of culturing neurospheres. There has however been no formal work to determine which media is best suited for this duty.
2. To determine whether ENSPC are located within the thickened nerve trunks in aganglionic HSCR gut. This is a possible source of ENSPC and therefore these trunks should be investigated further.
3. To determine whether ENSPC from aganglionic HSCR gut are of neural crest origin. If this can be determined then it will be possible to rule out a non neural source of ENSPC such as interstitial cells of Cajal which may have the ability to reprogram and act as ENSPC.
4. To understand the structure and cell types associated with thickened nerve trunks within aganglionic HSCR gut compared to ganglia in ganglionic HSCR gut. This is required to determine which cell types within thickened nerve trunks may be the source of ENSPC in aganglionic HSCR gut.

# Chapter 2 – Comparison of neurosphere development in different culture media

## 2.1 Overview

Our group has used two different media to culture neurospheres from ganglionic and aganglionic HSCR gut samples. However, there have been no formal experiments to determine which medium is optimal. It was found in this chapter that both media give rise to a similar number of ENSPC, however the horse serum based medium favoured the formation of neurospheres. Therefore, only horse serum medium will be used to culture neurospheres from human gut in subsequent experiments. This information will facilitate progress towards future neurosphere preparation for implantation into the gut of HSCR patients.

## 2.2 Introduction

Previous neurosphere culture by our group has relied on a relatively complex medium known as neurosphere medium. The contents of this are detailed in the methods section of this chapter although to note this contains epidermal growth factor, fibroblast growth factor and fetal bovine serum. This medium, used together with non adherent conditions was successful at culturing neurospheres containing ENSPC and their enteric neurons and glia progeny.(Almond et al. 2007; Lindley et al. 2008; Lindley et al. 2009) Alongside the work with neurospheres, cells from aganglionic gut were also cultured with the aim of growing smooth muscle cells to produce artificial sphincters for neurosphere implantation to test for restoration of function. These cells were grown in medium without added growth factors but containing horse serum rather than fetal bovine serum and using adherent dishes. Remarkably, in this horse serum medium neurospheres developed in cultures of aganglionic HSCR gut as discussed in Chapter 1. The subsequent experiments to compare the characteristics of neurospheres from ganglionic and aganglionic HSCR gut also used the horse serum medium however no formal experiments took place to determine whether there was any difference between horse serum medium and neurosphere medium in their abilities to culture neurospheres from cells isolated from human gut.

## 2.3 Aims

We wanted to determine whether there was a difference between neurosphere yield, the proportion of ENSPC to other cells and also neuronal development in cultures using either the horse serum medium or neurosphere medium. This will achieved by culturing cells from the same patients in the two different media and performing immunocytochemistry staining with NCC and neuronal markers at four different time points.

## 2.4 Methods

### 2.4.1 Buffers and media

#### 2.4.1.1 Horse serum medium

DMEM high glucose (4.5% w/v) was supplemented with 20% v/v medium 199 (Gibco®), 7% v/v heat inactivated horse serum (Gibco®), 100 Units/ml penicillin and 100 µg/ml streptomycin.

#### 2.4.1.2 Neurosphere medium

DMEM low (1% w/v) glucose (Gibco®, Life Technologies, Paisley, UK) was supplemented with 2% v/v chicken embryo extract (Sera Laboratories, West Sussex, UK), 1% v/v fetal calf serum (Sigma-Aldrich, Dorset, UK), 2mM final concentration L-glutamine (Gibco®), 100 Units/ml penicillin, 100 µg/ml streptomycin, 0.05mM final concentration mercaptoethanol (Gibco®), 20ng/ml final concentration fibroblast growth factor II (Source Bioscience, Nottinghamshire, UK) and 20ng/ml final concentration epidermal growth factor (Sigma-Aldrich).

#### 2.4.1.3 Immunofluorescence Blocking Buffer

PBS ($CaCl_2$ / $MgCl_2$ free, Gibco®) was supplemented with 20% v/v Goat serum (Gibco®), 0.1% w/v NaN3 (Sigma-Aldrich) and 1% w/v bovine serum albumin (Sigma-Aldrich).

## 2.4.2 Cell isolation, culture and immunofluorescence

### 2.4.2.1 Post natal human samples

Ethical approval from North West 3 Research Ethics Committee (Ref:10/H1002/77) was obtained to remove $1cm^2$ of gut from paediatric patients undergoing stoma closure. The author obtained consent himself for the majority of samples from the patients' parents and senior surgeons obtained consent where the author was not available. Parents were provided with information leaflets about the research which were individualised to those with HSCR and then those without HSCR (Appendix 1). The sample was transported under sterile conditions, wrapped in saline soaked gauze, to the universities stem cell laboratory at room temperature.

### 2.4.2.2 Details of samples used

| Breakdown of samples | | | |
|---|---|---|---|
| **Patient ID** | **Pathology** | **Age at operation** | **Gestation at birth** |
| C011 | Necrotising enterocolitis | 2 months | 32 weeks |
| C012 | Anorectal malformation | 9 months | 38 weeks |
| C013 | Necrotising enterocolitis | 11 months | 40 weeks |
| C014 | Anorectal malformation | 10 months | 34 weeks |

**Table 2.1. Details of samples used in experiments in this chapter**

#### 2.4.2.3 Isolation of cells

The sample was removed from the saline gauze and washed twice in 10ml PBS under sterile conditions in a 90mm petri dish (Sterilin, Newport, UK). A final wash of PBS supplemented with Gentamycin at a concentration of 50 µg/ml took place. A dissecting microscope (M165FC, Leica Microsystems, Bucks, UK) was then used to separate the mucosa and sub mucosa from the specimen along with removal of any blood vessels and any adipose tissue from the serosal surface (Diagram 2.2). The remaining tissue, which is the muscular layers, was then manually chopped into $1mm^2$ pieces and transferred to a 15ml centrifuge tube (E1415-0200, Starlab, Hertfordshire, UK). The sample was centrifuged at 150g for 5 minutes in order to remove the PBS/Gentamycin solution. 2ml of Dispase (Gibco®) and 2ml of Collagenase IV (Gibco®), both at a concentration of 1% (w/v), were added to the sample and left in a water bath for 1 hour at $37^oC$. Every 15 minutes the sample was gently triturated using a 5ml glass pipette. After 1 hour the sample is centrifuged at 150g for 5 minutes and fresh enzymes were added. This cycle would be repeated 2-3 times depending on the appearance of the sample when 10µl was taken and viewed under an inverted microscope, (Nikon TMS-F, Nikon UK, Surrey, UK). Once there are no visible pieces remaining, centrifugation produced a pellet of cells which was re-suspended in PBS and passed through a 100µm cell strainer (Falcon®, BD Biosciences, UK). Following centrifugation a final pellet was re-suspended in Horse serum medium. 10µl of the sample was then taken and added to 10µl 0.4% (w/v) Trypan blue, before counting cells using a haemocytometer. Between $2\text{-}4 \times 10^7$ cells were then plated on 60mm adherent dishes (Nalge Nunc, New York, USA) with 6ml

of Horse serum medium or Neurosphere medium supplemented with 0.5µg/ml amphotericin B (Fungizone®, Gibco®) and 50µg/ml Gentamicin (Gibco®). The cultures were kept in an incubator at 37°C with 5% $CO_2$ added to air.

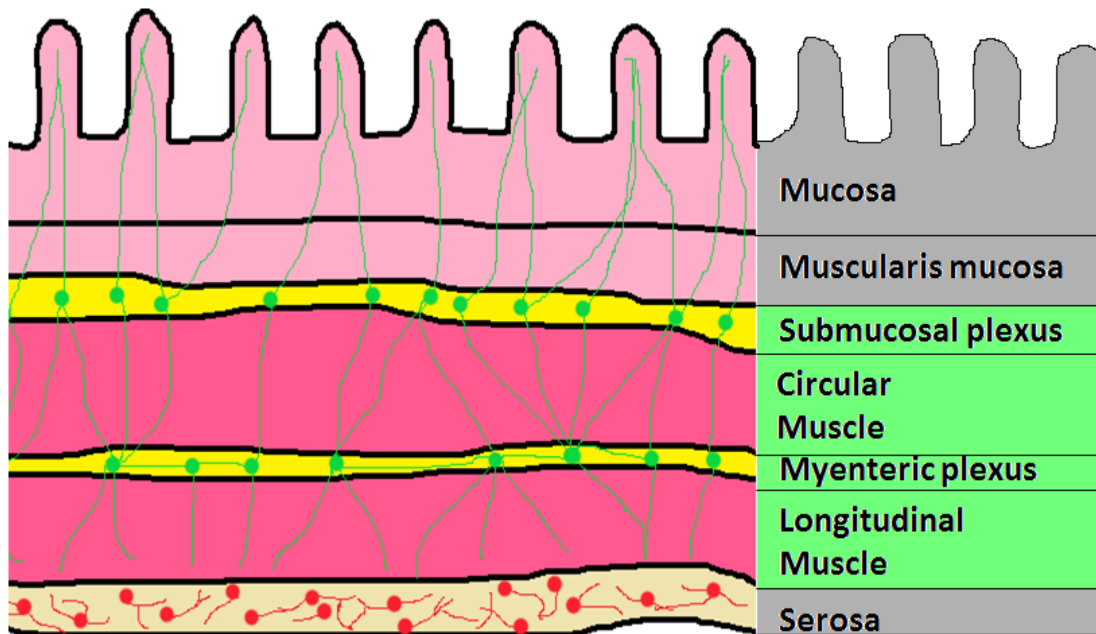


**Diagram 2.2. Structure of gut tissue.** The ganglia of the ENS are shown as green circles located in the submucosal plexus and the myenteric plexus. Nerve fibres connect ganglia within each layer, connect to ganglia in the other layer, innervate the circular and longitudinal muscle layers and innervate the mucosal layer. Blood vessels are depicted as red circles in the serosal layer. The layers marked in grey on the right of the diagram are dissected and discarded from human samples and the layers marked in green are kept and dissociated for tissue culture in this thesis. Adapted from Gulbransen and Sharkley 2012.

#### 2.4.2.4 Refreshing culture media

Twice a week the cultures were removed from the incubator in order to refresh the media. Firstly, the dish was swirled to move any debris into the centre of the dish, 3ml of media was then removed from the sides of the dish using a 1ml pipette. This media was then replaced with 3ml of fresh media at 37$^{o}$C. Amphotericin B and Gentamycin, in the concentrations above, were added to the media when feeding occurred during the first 7 days.

#### 2.4.2.5 Immunofluorescence of cells in culture using chamber well slides

At isolation, before a cell suspension has been cultured, the volume of the suspension to contain 20,000 cells, was calculated. Alternatively, cells in culture had their supernatant removed by centrifugation at 150g for 5 minutes in a 15ml centrifuge tube. The pellet produced was then re-suspended in 1ml of 0.05% (w/v) Trypsin (Sigma-Aldrich) and added to the culture dish along with a further 2ml of Trypsin. After 10 minutes at 37$^{o}$C in an incubator gentle manual dissociation occurred using a 1ml pipette. The suspension was then centrifuged to produce a pellet which was then re-suspended in 1ml of media. A cell count took place and the volume needed to give 20,000 cells was calculated. The remaining cells were re-cultured on a fresh 60mm adherent dish.

200µl of PBS containing 5% (v/v) Fibronectin (Merck Millipore) was added to each chamber of the adherent chamber well slide (Thermo Scientific). This was then

incubated at $37^oC$ for 1 hour before the PBS containing Fibronectin was removed and a brief PBS wash took place. 20,000 cells were then re-suspended in 500µl of the same medium as they were in when in culture and added to each well. The cells were then incubated overnight at $37^oC$ with 5% $CO_2$. The following day a PBS wash took place and 200µl of 4% (w/v) paraformaldehyde was added to each well to fix the cells. Following a PBS wash, 200µl of immunofluorescence blocking buffer (IBB) was added to each well and left for 1 hour. Primary antibodies were then diluted in IBB to a concentration specified in table 2.3 and added to each well. At least one well for a control received IBB alone. The slide was then left overnight refrigerated at 2-$4^oC$. A PBS wash took place and secondary antibodies also diluted in IBB then added to the concentration specified in table 2.4. The slide was then left at room temperature in the dark for 1 hour. A final PBS wash took place and the chamber was separated from the glass slide using the tool supplied with the chamber well slides. The slide was left until dry and a drop of ProLong® Gold antifade reagent with DAPI (Life Technologies) was placed over each well on the slide before covering with a cover slip (Thermo scientific). The slide was then left at room temperature in the dark overnight to dry before viewing and imaging.

#### 2.4.2.6 Primary antibodies

| Primary antibodies | | | |
|---|---|---|---|
| **Antibody** | **Supplier** | **Concentration** | **Host** |
| P75 (ab3125) | Abcam | 1:1000 | Mouse Monoclonal IgG1 |
| NOS | Abcam | 1:200 | Rabbit Polyclonal IgG |
| Tuj (MMS-435P) | Convance | 1:500 | Mouse Monoclonal IgG2a |

**Table 2.3. Primary antibodies used in experiments in this chapter**

P75 was used as a neural crest marker as this binds to nerve growth factor receptors on neural crest cells and their differentiated progeny. This will therefore identify ENSPC, enteric neurons and glial in the cells obtained from human gut. This has been used previously by our group and others and dual stains with both neuronal and glial markers. (Almond et al. 2007; Lindley et al. 2009)

#### 2.4.2.7 Secondary antibodies

| Secondary antibodies | | | |
|---|---|---|---|
| **Antibody** | **Supplier** | **Concentration** | **Host** |
| Alexa Fluor 488 - Anti-mouse | Life Technologies | 1:1000 | Goat IgG |
| Alexa Fluor 594 - Anti-rabbit | Life Technologies | 1:1000 | Goat IgG |

**Table 2.4. Secondary antibodies used in experiments in this chapter**

### 2.4.2.8 Immunofluorescence of neurosphere sections

Neurospheres were removed from culture and placed in a Peel-A-Way® mould (Polysciences Europe, Eppelheim, Germany). The neurospheres were then washed using PBS and fixed using 4% (w/v) paraformaldehyde for 15 minutes. The neurospheres are then embedded in 500µl of Shandon Cryomatrix (Thermo Scientific) and frozen to -80$^{o}$C. After a minimum of 3 hours at -80$^{o}$C sections were cut at 7µm with a MX35 microtome blade and a HM505N cryostat (both Thermo Scientific). Sections were mounted onto Superfrost® Plus microscope slides (Thermo Scientific) and washed once with PBS. A hydrophobic PAP marker pen (DAKO, Cambridgeshire, UK) was then used to delineate the neurospheres from unused sections of the slide. IBB was then added as described in the immunofluorescence of cells in culture using chamber well slides section and the same protocol was used from this point on to produce neurosphere section slides.

### 2.4.2.9 Analysis of immunofluorescence

Slides were viewed using a Leica DM IRB Inverted microscope (Leica microsystems). Images were captured using Leica Application Suite V4.3 (Leica Microsystems). Manual random sampling of rows of cells occurred using the 20x objective and the number of fluorescing cells and nuclei identified by DAPI were counted. Each row contained between 100-300 cells and the mean of the three rows was taken to give a percentage of cells expressing the antigen in the sample. A limitation of this approach is that it is vulnerable to observer bias however this was favoured over

the use of computer imaging software due to the cost and complexity of these systems. Adobe Photoshop CS3 (Adobe Inc., California, USA) was used for merging images.

#### 2.4.2.10 Phase contrast images of cultures

Images of cultures were obtained using a Leica DMRB Upright microscope with a Leica DFC420C camera and captured using Leica Application Suite V4.3.

#### 2.4.2.11 Statistical analysis

GraphPad Prism version 5 (GraphPad Software Corporation, California, USA) was used to create graphs and perform statistical analyses. Two-way ANOVA was performed unless otherwise specified.

## 2.5 Results

### 2.5.1 Morphology of cells in culture

Cells were successfully isolated from all the samples from all four patients without HSCR undergoing stoma closure. Cells were photographed at days 15, 30 and 45 in culture in the two media (Figure 2.5).

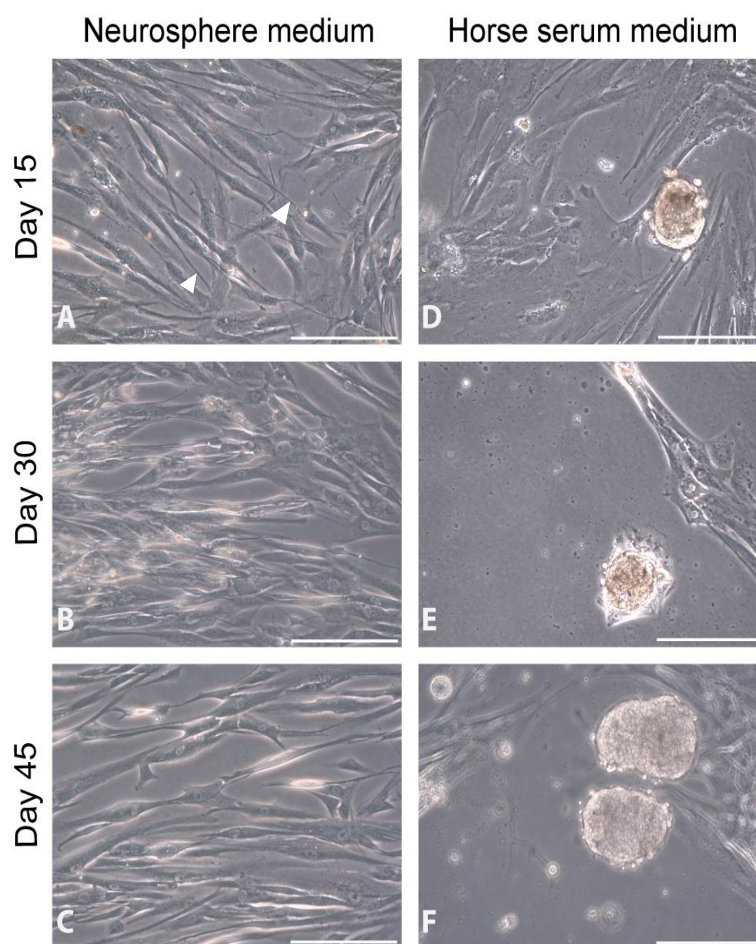


**Figure 2.5. Representative phase contrast images of human ENS and smooth muscle cells cultured in neurosphere medium or horse serum medium.** A-C: cells in neurosphere medium; D-F: cells in horse serum medium; A and D: after 15 days in culture; B and E: after 30 days in culture; C and F after 45 days in culture. Arrows in A highlight neurite like processes. Scale bars represent 200µm.

It is evident in the neurosphere medium that the morphology of the cells in culture do not vary between each time point (Figure 2.5 A-C). In the horse serum medium, the size of the neurosphere like structures appears to increase along with the number of them as time in culture increases (Figure 2.5 D-F). This is consistent with previous work completed by our group.(Almond et al. 2007)

There are obvious differences between the morphologies of the cells in the neurosphere medium compared to the horse serum media. In the neurosphere medium, cells are flat and elongated or spindle-shaped and there are also some neurite like processes present in the culture (see arrows in Figure 2.5 A). However, in the horse serum medium there are neurosphere like structures that have budded off from the flat cells on the surface of the dish. Initially they are attached to the flat cells on the surface of the dish (Figure 2.5 D) but then detach and are suspended in the medium (Figure 2.5 F). It should also be noted that the numbers of cells visible on the surface of the dish decreases as the number of neurospheres increases.

### 2.5.2 P75 expression by cells in both media

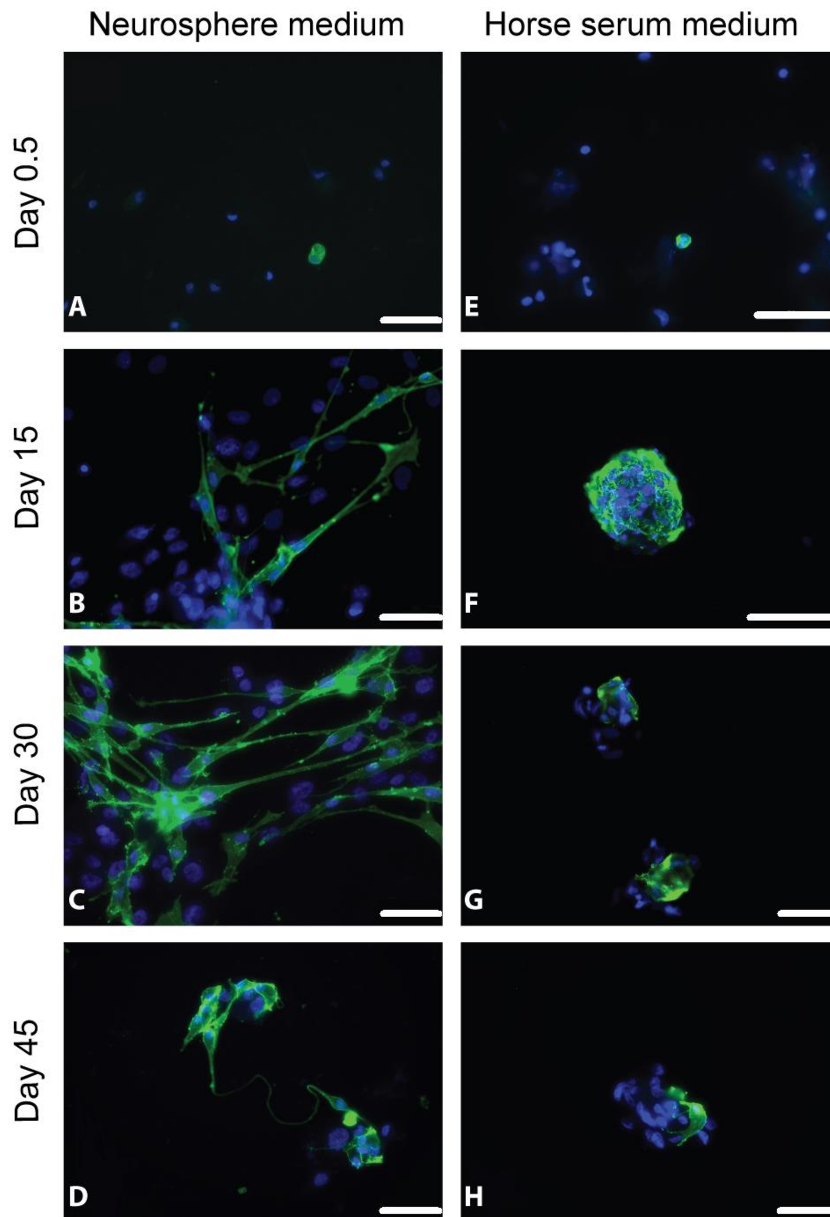


**Figure 2.6. Representative immunocytochemistry images of human ENS and smooth muscle cells cultured in neurosphere medium or horse serum medium labelled with the neural crest cell marker P75.** Cell nuclei are labelled blue with DAPI and P75 cell surface receptors are labelled green. A-D: cells in neurosphere medium; E-H: cells in horse serum medium; A and E: after overnight culture; B and F: after 15 days in culture; C and G: after 30 days in culture; D and H: after 45 days in culture. Scale bars represent 50µm.

After overnight culture cells expressing P75 within the isolated cells have not formed neurosphere like structures or elongated cells bearing the morphology of neurons (Figure 2.6 A and E). The cells cultured in the neurosphere medium expressing P75 are flat with long neurite projections to other expressing cells (Figure 2.6 B-D). After 45 days in neurosphere medium the cells appear more concentrated despite being seeded in equal number on the chamber slide, the cells expressing P75 have not formed neurospheres and long neurites are visible (Figure 2.6 D). The cells in horse serum media have formed dense clusters containing cells expressing P75, these appear to be early neurospheres (Figure 2.6 E-G).

## 2.5.3 Percentage of cells expressing P75 in culture in both media

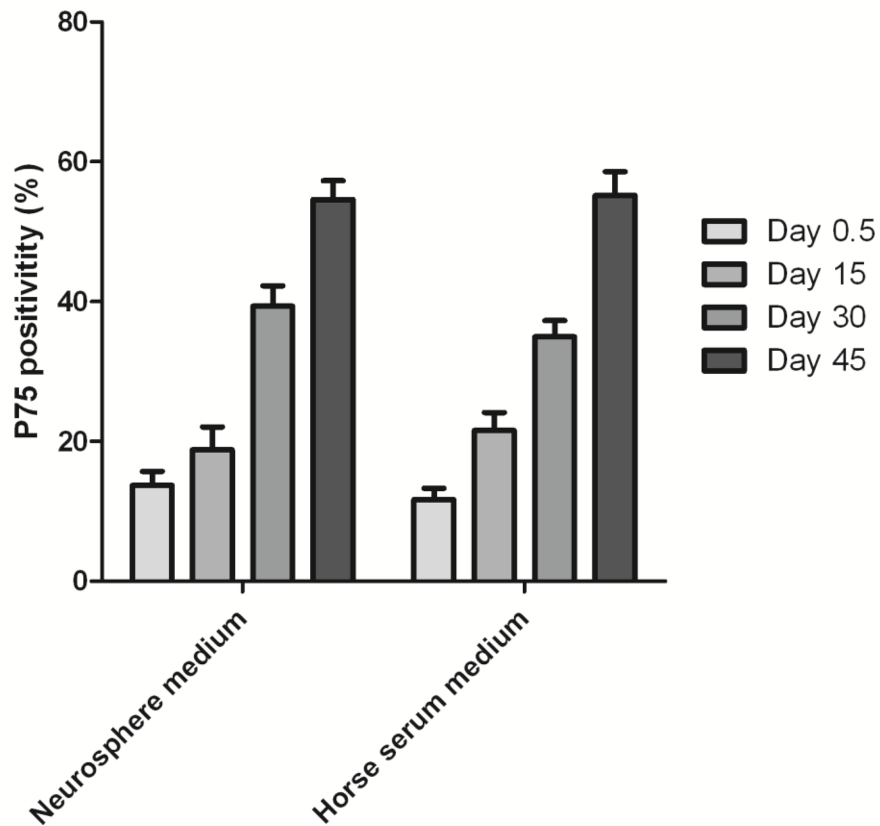


**Figure 2.7. Time course of expression of the neural crest marker P75.** There is a significant difference (P<0.0001) in percentage positivity between number of days in culture however there is no significant difference (P=0.470) between the two media. Error bars represent standard error of mean (SEM), n=4. A two-way ANOVA was performed.

There is no significant difference in the number of cells expressing P75 between the two media (Figure 2.7). Both conditions appear to promote culture of neural crest derived cells as the expression of P75 increases over time.

### 2.5.4 Number of cells present in culture at different time points

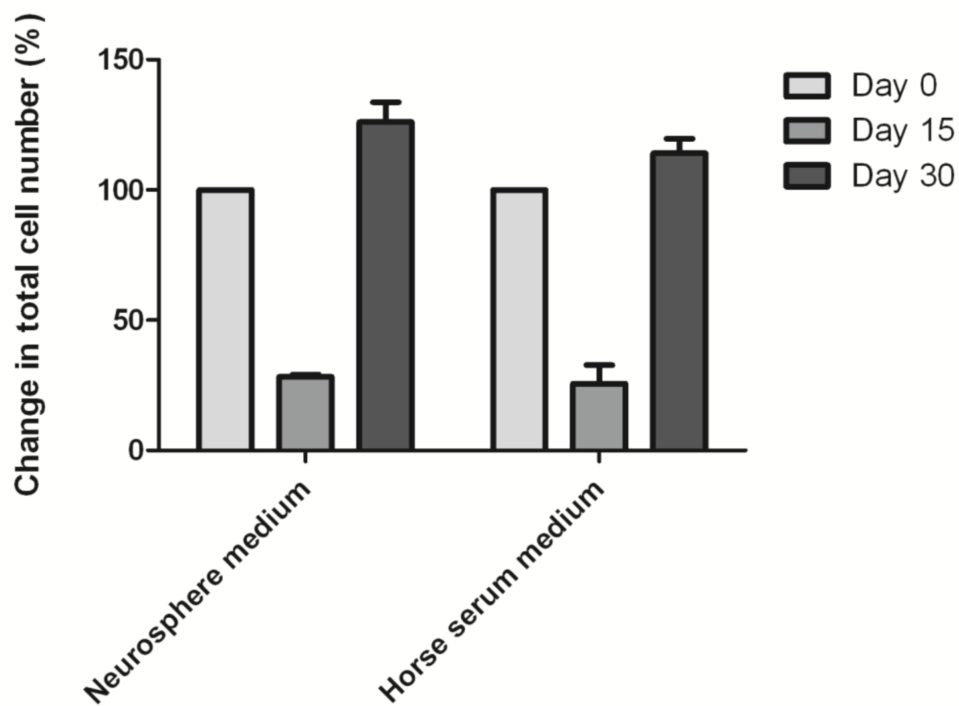


**Figure 2.8. Percentage change in total cell numbers in culture between passages.** Each passage occurred every 15 days. Note a percentage change is shown rather than total numbers as the initial number of cells dissociated varies substantially. There is a significant difference (P <0.0001) in the change in total number of cells in culture over time however there is no significant difference (P=0.275) between the two media. Error bars represent SEM, n=4. A two-way ANOVA was performed.

The change in number of cells since the last passage was calculated instead of total numbers as there was large variation in the number of cells isolated mainly due to the quality of sample obtained. Note the day 30 data relates to the change from day 15. Data for the change in numbers between day 30 and day 45 have not been included as unfortunately culture contamination meant that some of the cultures

were discarded. During the first 15 days in culture there is a similar reduction in the number of living cells present in the two culture conditions however between day 15 and day 30 there was a mean increase in the number of cells present in both media (Figure 2.8). There is a moderately large SEM however there is no statistical significance between the two media. Combined with the data on percentage of cells expressing P75 (Figure 2.7) this shows that the mean number of these cells present is similar in each culture.

## 2.5.5 P75 expression within neurospheres in horse serum medium

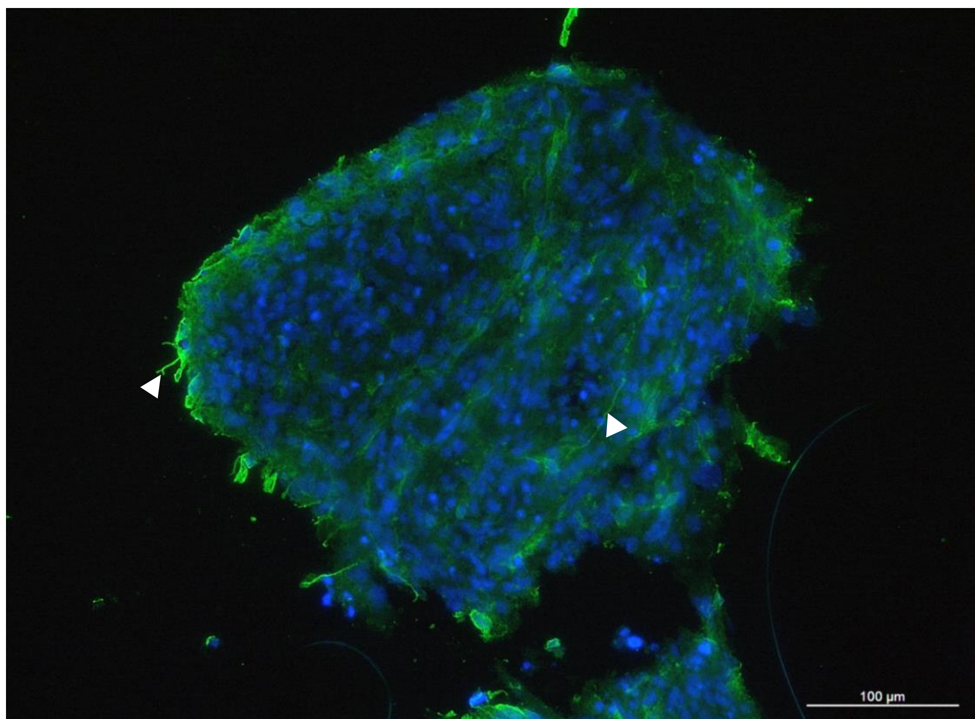


**Figure 2.9. Immunohistochemistry of a representative section of a neurosphere removed from horse serum media culture stained with the NCC marker P75.** Cell nuclei are labelled blue with DAPI and P75 cell surface receptors are labelled green. Arrows highlight neurite like processes. Scale bar represents 100µm.

The neurosphere like structures seen in horse serum media are neurospheres as they are shown to contain cells which label with P75 which has been previously shown to identify ENSPC and their neuronal and glial progeny (Figure 2.9). There are fine neurite processes present emerging from the periphery of the neurosphere and also within the neurosphere too (see arrows in Figure 2.9).

### 2.5.6 ENSPC differentiation in both media

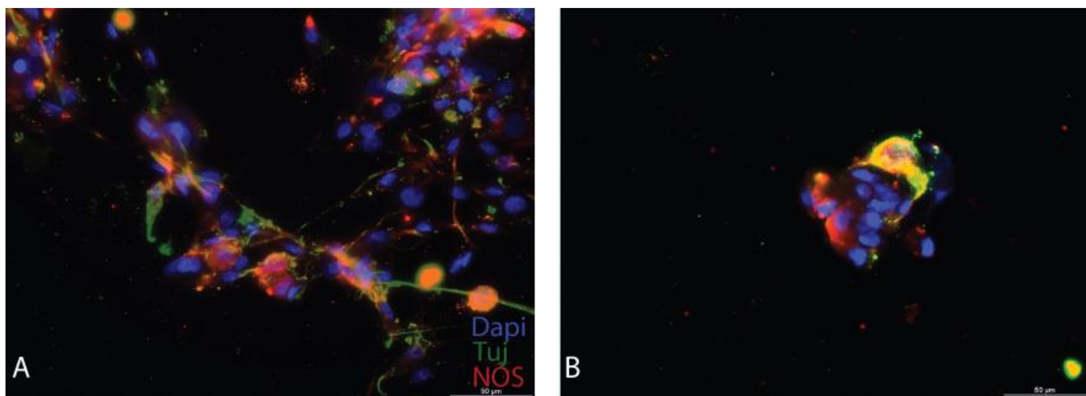


**Figure 2.10. Representative immunocytochemistry images of human ENS and smooth muscle cells cultured for 45 days in neurosphere medium or horse serum medium labelled with the immature and mature neuronal markers Tuj and NOS respectively.** Cell nuclei are labelled blue with DAPI, cells expressing Tuj are green and cells expressing NOS are red. A: neurosphere medium; B horse serum medium. Scale bars represent 50µm.

Although previous experience by our group with P75 has shown that the majority of cells dual label with P75 and neuronal markers after time in culture, it is not clear so far in these experiments whether cells in both conditions were differentiating as well as proliferating. Therefore neuronal markers were also used to look for the

presence of neurons in the samples. Neuronal cells in neurosphere medium appear elongated and fine neurites are visible (Figure 2.10 A) whereas neuronal cells in horse serum medium are clustered into what appears to be an early neurosphere (Figure 2.10 B). Most importantly in both media, there are not only immature neurons labelled with Tuj but also mature neurons labelled with NOS.

## 2.6 Discussion

In the literature, ENS and other neurospheres have mainly been derived and cultured under non adherent condition.(Almond et al. 2007; Lindley et al. 2008; Reynolds & Weiss 1992) However, it has been reported that a greater yield of neurospheres can be achieved using adherent culture dishes in CNS neurospheres.(Konagaya et al. 2011, 2013) As the use of autologous neurospheres as a therapy for HSCR is being explored, adherent culture conditions have been favoured by our group over recent years.(Wilkinson, Edgar & Kenny 2012) Additionally, an advantage of using adherent conditions is that there is no need to repeatedly passage neurospheres to prevent them adhering to the dish surface which has been required in previous work by our group. (Almond et al. 2007) The work in this chapter has therefore sought the optimal medium to culture ENS neurospheres under adherent conditions by comparing the two media previously used by our group.

### 2.6.1 Both media produce a similar number of ENSPC

It is clear from the mean percentage of P75 expression and the mean number of cells in both culture conditions at different time points that each medium produces a similar proportion and total number of neural crest derived cells. It has been well documented that ENS neurons and glia both express the NCC marker P75, consistent with the notion that this NCC marker also identifies ENSPC.(Joseph et al. 2011; Lindley et al. 2009) Here it is evident that both media promote differentiation

of ENSPC into neurons which is an essential property of any medium to culture cells for autologous stem cell therapies in HSCR.(Wilkinson, Edgar & Kenny 2012) Therefore, in terms of culture of ENSPC it would seem that there is no difference between the two media.

### 2.6.2 Horse serum medium promotes neurosphere formation

The main difference between the two media is the morphology of the cells cultured in them. After 12 hours in culture the appearance of cells expressing P75 is similar suggesting that either these cells are yet to recover from the stresses of tissue dissociation or these cells are ENSPC prior to differentiation. Following 15 days in culture it is clear that horse serum medium promotes the formation of neurospheres containing ENSPC whereas neurosphere medium results in a flat appearance of the cells with long neurite projections. These findings sound paradoxical as neurosphere medium does optimally promote neurosphere development, however our work has used adherent conditions as opposed to non adherent conditions where neurosphere generation has been successful.(Almond et al. 2007; Lindley et al. 2009) As mentioned, there is evidence for the use of adherent conditions to generate neurospheres but in order to confirm these findings with ENS neurospheres further study could compare the two media under adherent and non adherent conditions.(Konagaya et al. 2011, 2013)

In the horse serum medium where neurospheres formed, phase contrast imaging showed that neurospheres began to form on the surface of the dish before enlarging in size and finally budding off to become suspended in the medium. This raises the question of whether one single ENSPC proliferates to form the neurosphere or if multiple cells adhere to form the structure together. Some study has already taken place to answer this question which found that ENS neurospheres are multipotent but this doesn't address whether all neural crest derived cells in a neurosphere originate from one ENSPC.(Almond et al. 2007) In order to understand the formation of ENS neurospheres further work should look at these neurospheres forming possibly by utilising live cell imaging with immunofluorescence of ENSPC with P75 antibodies. It was also noticed that neurospheres were greater in size after a longer time in horse serum medium cultures, this would suggest that more cells are present however it is not clear as to whether there is any advantage in having larger neurospheres when considering autologous implantation. It is logical that an increased number of cells will have greater potential in the number of ENS neurons and ganglia that are formed however as a neurosphere becomes larger questions are raised about the ability of oxygen and nutrients to diffuse to the centre of these structures. If diffusion is not possible past a certain size then it is possible that central cells apoptose leaving a hollow structure.

### 2.6.3 Presence of non neural crest derived cells within neurospheres

In both media it is clear that there are non neural crest derived cells present. The majority of these cells in primary ENS neurospheres have been shown to be smooth

muscle cells in previous work from our group.(Wilkinson et al. 2013) The ratio of neural crest derived cells to smooth muscle cells increases from generation to generation of neurosphere because the culture conditions favour cells of the neural crest lineage.(Lindley et al. 2009) It is not clear however whether smooth muscle cells apoptose or neural crest derived cells proliferate at a much greater rate than other cells. It is also unknown what role, if any, these smooth muscle cells have within the ENS neurosphere. A clonal NCC can proliferate and differentiate into cells expressing smooth muscle actin as well as cells expressing neuronal markers, which may explain the origin of smooth muscles cells in ENS neurospheres.(Morrison et al. 2000) A possibility for future work would be to attempt to culture purified neurospheres as it would be safer to implant one cell type instead of many.

### 2.6.4 Further optimisation of media

The major differences in the horse serum medium is the larger amount of serum and glucose present however it is not clear why this promotes the formation of neurospheres. It is clear that the components of the neurosphere medium are effective at culturing ENSPC. The question has to be asked as to whether some of these components should be added to the horse serum medium. However to undertake experiments to determine whether there is an advantage to developing a new medium a large number of cells and time will be required as many components at multiple concentrations will have to be tried.

It should also be noted that neurosphere medium has more components meaning that it is more expensive and leaves more room for variability between batches depending on where the components have been sourced from and when, which is another reason why horse serum medium is favourable. Before human implantation of ENS neurospheres can take place a serum free medium will have to be developed due to cross species infection risk which represents a challenge for the future.

## 2.7 Conclusion

It is clear that horse serum medium with adherent conditions should be used for all future work involving the culture of human ENS neurospheres before human trials. There may be some advantage in adding components such as growth factors to the horse serum medium to create a new medium however these are areas for future work and are outside of the work achievable in this thesis. It must also be considered that there are advantages to using a simpler medium which will be easier to adapt to serum free, as there is hope that this work will culminate in clinical trials in the future.

# Chapter 3 – Thickened nerve trunks as a source of ENSPC in aganglionic HSCR gut

## 3.1 Overview

This chapter confirms the previous observation that it is possible to isolate ENSPC from aganglionic short and long segment HSCR gut where there are thickened nerve trunks present. However it is demonstrated here that it is not possible to obtain ENSPC from aganglionic total colonic and total intestinal HSCR gut in which there are no thickened nerve trunks. The implication of this finding is discussed.

## 3.2 Introduction

As described in chapter one, preliminary work from our group has shown that ENSPC can be isolated from aganglionic gut in short and long segment HSCR. These ENSPC then form mature neurons and ganglia of the ENS. There is however no data from patients with the rarer total colonic and total intestinal variants of HSCR. This information would be interesting as it is well documented that thickened nerve trunks are present within aganglionic gut in patients with short and long segment disease but these trunks are absent in total colonic and total intestinal disease.(Kobayashi, O'Briain & Puri 1994b; Solari, Piotrowska & Puri 2003). Thus, observations on aganglionic total colonic and total intestinal HSCR gut tissue may aid our identification of the source of ENSPC in aganglionic gut. Thickened nerve trunks are hypertrophied extrinsic nerves originating from the pelvic plexus.

Normally, extrinsic nerve fibres consist of bundles of axons which are encased by Schwann cells. There are many of these bundles in nerves which are contained within fibrous layers, which are the endoneurium, epineurium and perineurium (Diagram 3.1). Immunohistochemistry of tissue sections of aganglionic HSCR gut using the NCC marker P75 by others and by our group have demonstrated high levels of immunoreactivity in cells at the periphery of the nerve fibre, which in a normal nerve would be the fibrous perineurium (Figure 3.2).(Kobayashi, O'Briain & Puri 1994b; Wilkinson et al. 2013)

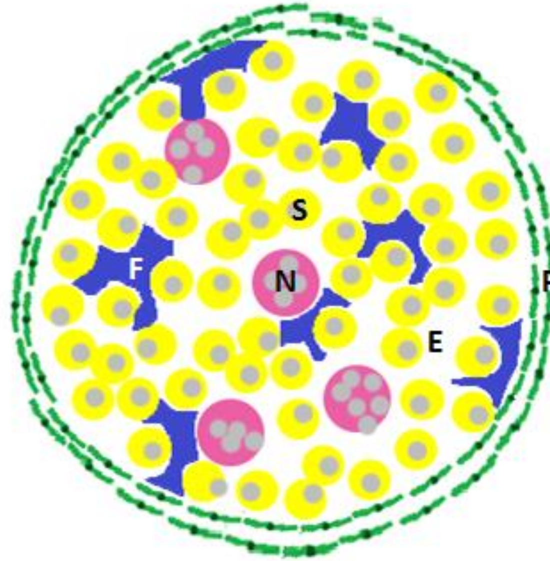


**Diagram 3.1. Structure of a typical extrinsic nerve.** The nerve is encased by the perineurium (P) consisting of perineurial cells. Axons (grey circles) are wrapped by myelinated Schwann cells (S) and non-myelinated Schwann cells (N). Within the endoneurium (E) are endoneurial fibroblasts (F). Adapted from Joseph et al 2004.

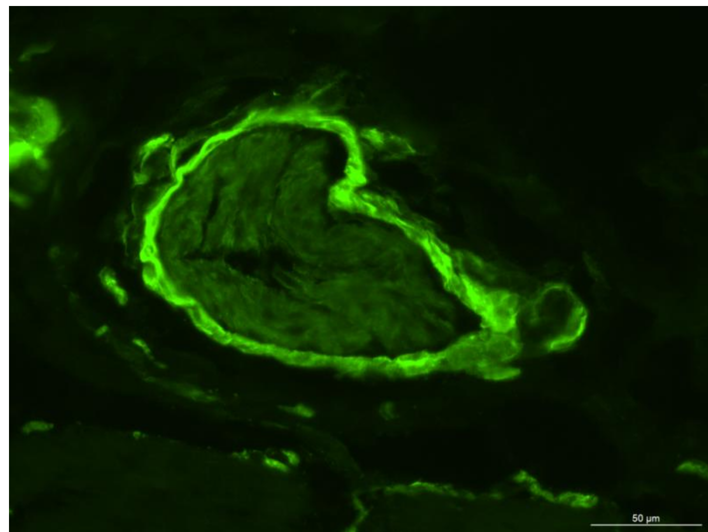


**Figure 3.2. Thickened nerve trunk expressing P75 in aganglionic short segment HSCR gut.** The most intense expression is at the periphery of the nerve trunk but there is lower levels of expression within the trunk corresponding to the location of endoneurial compartments. Scale bar represents 50μm.

The hypothesis in this chapter is that the source of ENSPC obtained from aganglionic gut are the thickened nerve trunks. This is proposed as ENSPC *in vitro* express the NCC marker along with the thickened nerve trunks *in vivo*. As it is known that total colonic and total intestinal aganglionic gut do not have thickened nerve trunks observations on this tissue presents the possibility of testing this hypothesis.(Kobayashi, O'Briain & Puri 1994b; Solari, Piotrowska & Puri 2003) To this end, aganglionic gut samples were obtained from patients with different variants of HSCR, as confirmed by a consultant histopathologist, in order to attempt to isolate and culture ENSPC. The success or failure of this is then correlated with the presence or absence of thickened nerve trunks.

## 3.3 Aim

To establish whether thickened nerve trunks are the source of ENSPC expressing the NCC marker P75 in aganglionic HSCR gut.

## 3.4 Methods

Cells from the muscular layers of aganglionic HSCR gut were dissociated and cultured with horse serum medium as described in chapter 2. It should be noted that samples from patients with total colonic and total intestinal disease were obtained at the site of stoma formation which was proximal jejunum in total intestinal disease and distal ileum in total colonic disease.

### 3.4.1 Hospital histology reports

Histology reports were produced by the Histopathology department at Alder Hey Children's Hospital where the samples were obtained and accessed electronically. The presence or absence of thickened nerve trunks, along with the extent of disease, was noted.

### 3.4.2 Details of aganglionic samples used

| Breakdown of samples | |
|---|---|
| **Patient ID** | **Pathology** |
| H020 | HSCR – total colonic |
| H021 | HSCR – short segment |
| H022 | HSCR – short segment |
| H023 | HSCR – short segment |
| H024 | HSCR – long segment |
| H025 | HSCR – total intestinal |
| H027 | HSCR – short segment |
| H028 | HSCR – short segment |
| H029 | HSCR – short segment |
| H030 | HSCR – short segment |
| H031 | HSCR – short segment |
| H032 | HSCR – short segment |
| H033 | HSCR – short segment |
| H034 | HSCR – short segment |
| H035 | HSCR – short segment |

**Table 3.3. Details of samples used in experiments in this chapter**

| Summary of samples obtained | |
|---|---|
| **Pathology** | **Number of samples** |
| HSCR – short segment | 12 |
| HSCR – long segment | 1 |
| HSCR – total colonic | 1 |
| HSCR – total intestinal | 1 |

**Table 3.4. Summary of samples used in experiments in this chapter**

### 3.4.3 Immunofluorescence using chamber well slides

This took place as described in section 2.4.2.5. The primary antibody used was P75 (Abcam) and the secondary was Alexa Fluor goat anti-mouse 488 (Life Technologies) at the concentration specified in section 2.4.2.6 and 2.4.2.7. Negative controls consisting of immunocytochemistry with Alexa Fluor 488 secondary antibodies but no primary antibodies was performed for all samples.

## 3.5 Results

### 3.5.1 Histopathology reports on aganglionic samples obtained

Histopathology reports found aganglionosis in all samples along with a presence or absence of thickened nerve trunks as detailed in table 3.5. Results from all levels of aganglionic biopsies was looked at to determine thickened nerve trunk presence.

| Pathology reports for samples used | | |
|---|---|---|
| **Patient ID** | **Pathology** | **Thickened nerve trunks?** |
| H020 | HSCR – total colonic | No |
| H021 | HSCR – short segment | Yes |
| H022 | HSCR – short segment | Yes |
| H023 | HSCR – short segment | Yes |
| H024 | HSCR – long segment | Few |
| H025 | HSCR – total intestinal | No |
| H027 | HSCR – short segment | Yes |
| H028 | HSCR – short segment | Yes |
| H029 | HSCR – short segment | Yes |
| H030 | HSCR – short segment | Yes |
| H031 | HSCR – short segment | Yes |
| H032 | HSCR – short segment | Yes |
| H033 | HSCR – short segment | Yes |
| H034 | HSCR – short segment | Yes |
| H035 | HSCR – short segment | Yes |

**Table 3.5. Details of the extent of HSCR and presence of thickened nerve trunks in the aganglionic gut sample as determined by a consultant histopathologist for each patient**

### 3.5.2 P75 expression by cells obtained from aganglionic HSCR gut after 0.5 days in culture

Cells were successfully obtained from all samples detailed above and immunocytochemistry was performed after overnight culture (Figure 3.6). In terms of cell yield, there was no difference between samples noticed.

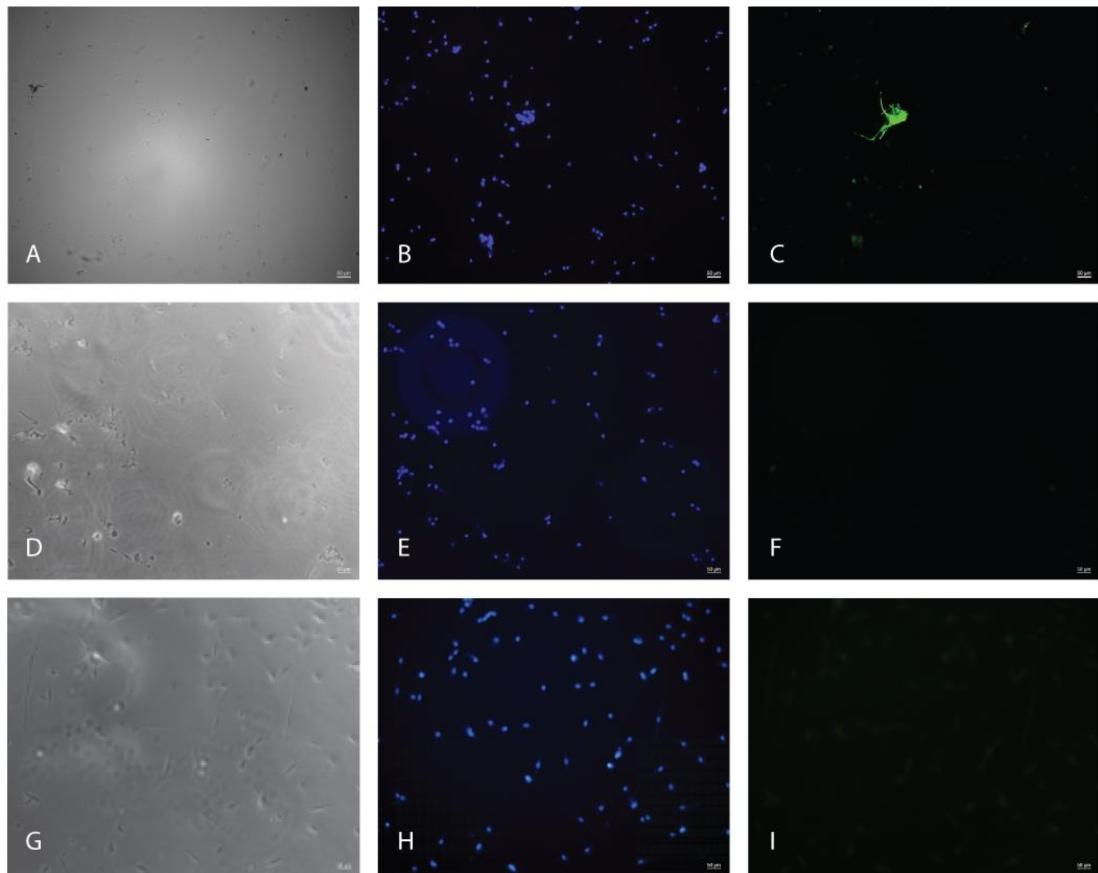


**Figure 3.6. Phase contrast and immunocytochemistry of cells from aganglionic HSCR gut after overnight culture stained with P75 antibodies.** A-C: short segment HSCR; D-F: total colonic HSCR; G-I: negative control consisting of short segment HSCR gut with only secondary antibodies. A, D, G: phase contrast; B, E, H: cell nuclei identified by DAPI (blue); C, F, I; neural crest derived cells expressing P75 (green). Scale bars represent 50µm.

Cells were present in both samples as shown by phase contrast imaging and identification of cell nuclei with DAPI (Figure 3.6 A and D, B and E). However P75 positive neural crest derived cells were only present in aganglionic gut from short and long segment HSCR (Figure 3.6 C) but such cells were not detected from the samples from patients with total colonic or total intestinal HSCR (Figure 3.6 F). Also, the specificity of the secondary antibody is shown confirming the positive finding (Figure 3.6 I). The percentage of P75 expression and the number of samples tested in shown in Figure 3.8.

### 3.5.3 P75 expression by cells obtained from aganglionic HSCR gut after 10 days in culture

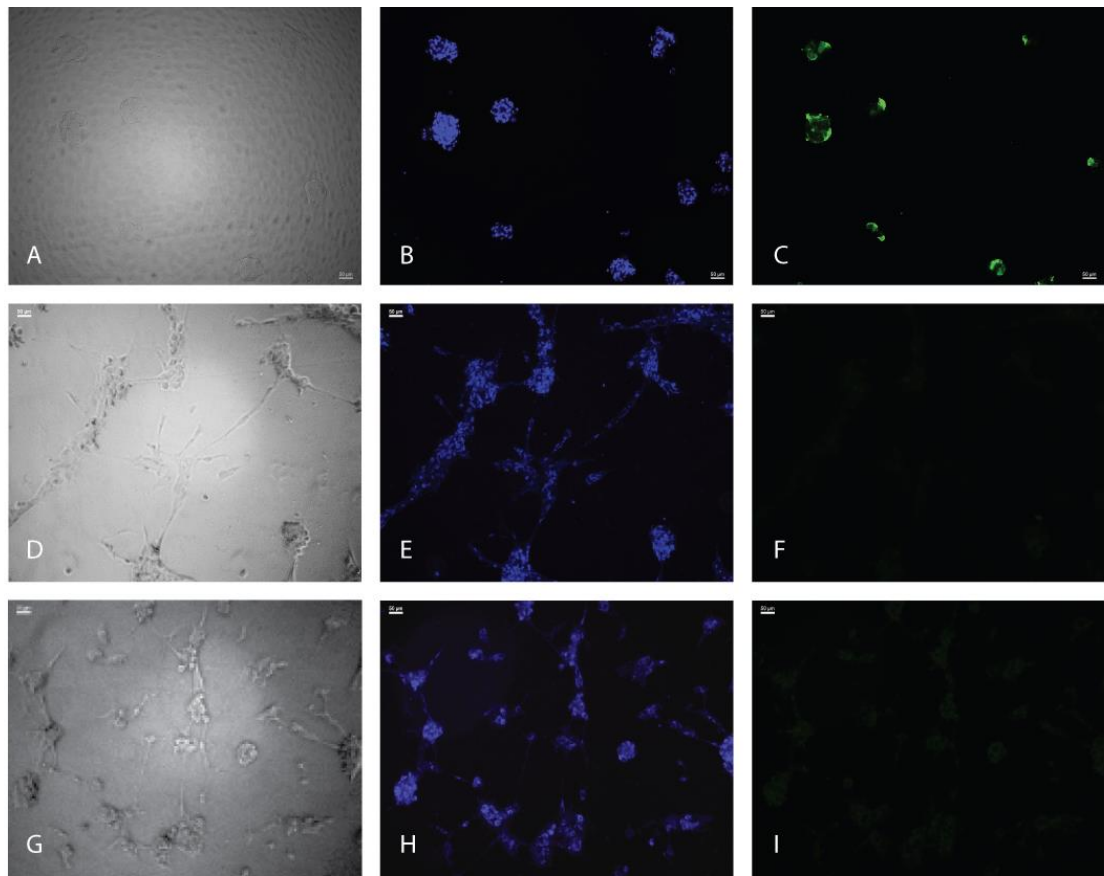


**Figure 3.7. Phase contrast and immunocytochemistry of cells from aganglionic gut after 10 days in culture stained with P75 antibodies.** A-C: short segment HSCR; D-F: total colonic HSCR; G-I: negative control consisting of short segment HSCR gut with only secondary antibodies. A, D, G: phase contrast; B, E, H: cell nuclei identified by DAPI (blue); C, F, I; neural crest derived cells expressing P75 (green). Scale bars represent 50µm.

After 10 days culture the presence of cells was again confirmed with phase contrast imaging and identification of cell nuclei (Figure 3.7 A and D, B and E). Cells expressing P75 were only identified in aganglionic short and long segment HSCR gut

(Figure 3.7 C) and there was no expression of this marker detected amongst cells obtained from aganglionic total colonic or total intestinal HSCR gut (Figure 3.7 F). As at day 0, a negative control was performed which confirms the reliability of the method and the results (Figure 3.7 I). It should also be noted that the percentage of cells expressing P75 increased after time in culture, this is quantified in Figure 3.8.

### 3.5.4 Percentage of P75 expression in aganglionic samples

The percentage of cells expressing P75 for each variant of HSCR at each time point was calculated (Table 3.8).

| Mean P75 expression at day 0.5 and day 10 amongst the different extents of HSCR | | | | |
|---|---|---|---|---|
| **Pathology** | **Number of patients** | **Thickened nerve trunks?** | **P75 expression day 0.5** | **P75 expression day 10** |
| Short segment | 12 | Yes | 10.0% +/- 1.9 SEM | 22.7% +/- 2.9 SEM |
| Long segment | 1 | Few | 11.1% | 23.1% |
| Total colonic | 1 | No | 0% | 0% |
| Total intestinal | 1 | No | 0% | 0% |

**Figure 3.8. Mean expression of P75, thickened nerve trunk presence and number of samples tested for each variant of HSCR within the aganglionic gut.** The mean is shown along with the SEM for short segment samples. In the other variants of HSCR there is one sample of each so a mean cannot be calculated.

Figure 3.6, 3.7 and 3.8 show that it was possible to isolate and culture cells expressing P75 without fail from aganglionic short and long segment HSCR gut whereas it was not possible to do this from aganglionic total colonic or total intestinal HSCR gut. As well as observing this link the percentage of cells expressing P75 in culture from aganglionic short and long segment gut increased over time in keeping with previous work by the group.(Wilkinson et al. 2013)

## 3.6 Discussion

### 3.6.1 Aganglionic ENSPC are associated with thickened nerve trunks

It was possible to identify P75 positive neural crest derived cells in the dissociates obtained from aganglionic gut in short and long segment HSCR where also thickened nerve trunks were present. However this was not achievable in samples from the 2 patients with total colonic and total intestinal HSCR where these thickened nerve trunks were absent. As our group has previously shown that mature enteric neurons and glia *in vitro* express P75, this fits with the hypothesis that the ENSPC which can be isolated from aganglionic gut in short and long segment disease are derived from the thickened nerve trunks.(Wilkinson et al. 2013) The correlation between the presence of thickened nerve trunks in short and long segment HSCR and the absence of these in total colonic and total intestinal HSCR in our patients is consistent with reports from other groups.(Kobayashi, O'Briain & Puri 1994b; Solari, Piotrowska & Puri 2003)

In the aganglionic gut obtained from the patient with long segment HSCR, few thickened nerve trunks were seen on histology. There is an obvious limitation with this classification which relies on the observations of the histopathologist and no quantitative evidence. However, in this sample a similar percentage of cells expressing P75 compared to aganglionic gut from short segment HSCR was observed at both time points. With only one patient it is not possible to draw a conclusion from this result and repeats with more samples are required. A method

of quantifying the number of thickened nerve trunks present should also be considered as this would allow the observation of a dose response relationship between the number of thickened nerve trunks and the percentage of P75 expression in the tissue. This would ultimately strengthen the results in this chapter.

### 3.6.2 P75 expression increases with time in culture

It has also been shown in this experiment that if ENSPC were not identified in the single cell suspensions at isolation they are also not present after up to 10 days in culture. This may dismiss the possibility that when ENSPC are present in culture they are derived from non neural crest derived cells, such as reprogrammed mesenchymal cells, which are displaying a neural phenotype after time in culture.(Mareschi et al. 2006) It should also be noted that where cells expressing P75 were isolated, the percentage of cells expressing this marker increased after 10 days in culture. This is likely due to the conditions used favouring the survival and proliferation of cells of NCC lineage whereas cells of non NCC lineage fail to survive in these conditions. This is in keeping with previous work by our group and is promising as it shows that when these cells are placed in the culture conditions used the percentage of these cells increases.(Almond et al. 2007; Lindley et al. 2008; Lindley et al. 2009) This is important when considering the culture and implantation of human ENSPC into HSCR patients.(Wilkinson, Edgar & Kenny 2012)

### 3.6.3 Limitations

The limitation of this work is that there was only one sample from each of the total colonic and total intestinal variants of HSCR which was investigated. This problem comes with any work looking at very rare disease and in the paediatric centre where our samples are obtained around one case of this disease is seen per year. Future work should therefore aim to repeat this work to confirm these findings. One way to increase the availability of these rare samples would be to create a national network where consent was applicable nationwide and human samples could be obtained.

A further limitation of this study is that the site the aganglionic sample is taken from differs between patients depending on the extent of disease. There is little known about differences between proximal and distal aganglionic gut in total colonic and total intestinal patients. However, biopsies were obtained from multiple levels of the gut in these patients including the rectum which was reported in both cases as aganglionic with absent thickened nerve trunks.

### 3.6.4 Aganglionic short and long segment HSCR gut can be used for stem cell retrieval

Previously it was observed in six patients with short segment HSCR and one long segment HSCR that ENSPC were obtainable from the aganglionic gut.(Wilkinson et

al. 2013) This has now been repeated successfully with a further twelve short segment and one long segment aganglionic HSCR samples. This is consistent with work which found that ENSPC can arise from a niche external to the ENS ganglia.(Liu et al. 2009) This added evidence strengthens the possibility that autologous ENSPC implantation will be achievable using the aganglionic gut removed at operation for HSCR patients with short and long segment disease. This would of course allow any stem cell treatment to be much more practical and involve less cell number expansion before implantation compared to if ganglionic gut was required for ENSPC culture. Unfortunately in the rarer variants of disease ganglionic bowel will need to be sourced to obtain the progenitor cells.

### 3.6.5 Revisiting the source of ENSPC obtainable from ganglionic HSCR gut

If in aganglionic HSCR gut ENSPC are obtained from thickened nerve trunks it is not clear why ENSPC from ganglionic gut have similar characteristics as described previously.(Lindley et al. 2008; Lindley et al. 2009) This may be that the ENSPC are sourced from non-hypertrophied extrinsic nerves in ganglionic gut however the number of cells obtained from aganglionic gut should be greater than ganglionic gut, which is not the case. The similarities observed between ganglionic and aganglionic ENSPC may also lead towards glial including Schwann cells as a source of ENSPC as glia are present within ganglia of the ENS and Schwann cells are present

within extrinsic nerves.(Joseph et al. 2011; Kalcheim & Rohrer 2014; Laranjeira et al. 2011)

### 3.6.6 Revisiting the aetiology of HSCR

The aetiology of HSCR is unknown however from small mammal studies it can be said that the most likely cause is failure of migration of ENCC.(Obermayr et al. 2013) If ENSPC that can be obtained from aganglionic HSCR gut are present within the thickened nerve trunks it seems more likely that ENCC have failed to migrate from the vagal neural crest to the distal gut. It is less likely that ENCC have migrated to the distal gut and failed to differentiate into enteric neurons and glia otherwise we should be able to obtain ENSPC from aganglionic gut where thickened nerve trunks are absent. However, this cannot be ruled out as there is work which shows that for sacral NCC to enter the hind gut extrinsic nerve fibres must be present.(Erickson et al. 2012) There is also the possibility that different variants of HSCR have different aetiologies which is suggested by the histopathological differences seen in the aganglionic tissue.

### 3.6.7 ENSPC from aganglionic HSCR may be of Schwann cell lineage

Cells of Schwann cell lineage are a possible source of ENSPC in aganglionic HSCR gut from within thickened nerve trunks. It has been put forward by two groups that ENSPC are most likely of glial origin when obtained from post natal gut and

Schwann cells are a type of glial cell in the peripheral nervous system.(Joseph et al. 2011; Laranjeira et al. 2011) In addition it has also been possible to generate peripheral nervous system neurospheres from Schwann cells obtained for mouse and rat sciatic nerve.(Martin et al. 2012) Most significantly migrating NCC have been shown to dual label with SOX10 and PHOX2B showing that in the autonomic nervous system Schwann cells precursors also have the ability to undergo neurogenesis and hence have neuronal progenitor characteristics.(Kalcheim & Rohrer 2014) However, it has been shown that sacral NCC migrate into the hindgut with extrinsic nerve fibres so it is possible that ENSPC from aganglionic gut are NCC that have failed to differentiate and remain in progenitor form.(Erickson et al. 2012) This theory coincides with the finding that it is only possible to obtain ENSPC from aganglionic gut where thickened nerve trunks have formed.

To fully explore the possibility of Schwann cells as a source of ENSPC further work should look at the characterisation of aganglionic HSCR tissue sections with a glial marker and expression of this marker should be looked for amongst cells isolated from aganglionic gut. The hypothesis would be that P75 positive cells co stain with a glial marker such as S100.(Hirose, Sano & Hizawa 1986) This could be evaluated using fluorescence activated cell sorting and is an area for further work.

### 3.6.8 The potential of a medical treatment for HSCR

This work also raises the question as to why ENSPC fail to leave the thickened nerve trunks *in vivo* and develop into ganglia of the ENS. If this can be understood there is the possibility of therapeutic stimulation of these cells *in vivo* in HSCR patients to differentiate into a ENS and restore functionality in the aganglionic gut. This would treat HSCR without the need for surgical intervention which has substantial benefits for patients.

## 3.7 Conclusion

The work in this chapter has raised several key questions that should be a focus of further work. Most importantly the cell which is the source of ENSPC needs to be identified in order to develop an autologous stem cell transplant for HSCR patients which is as safe and efficacious as possible. Of equal importance the possibility of stimulating ENSPC *in vivo* to differentiate into a functional ENS should be explored to exclude the need of surgical intervention in these patients.

# Chapter 4 – Are ENSPC obtained from aganglionic HSCR gut of neural crest lineage?

## 4.1 Overview

This chapter utilises fluorescence activated cell sorting (FACS) to show that neural crest derived cells obtained from aganglionic gut are the source of enteric neurons *in vitro* and are therefore ENSPC. This not only adds to our understanding of the origin of ENSPC that can be isolated from aganglionic HSCR gut but also raises questions on whether neural crest derived cells can be stimulated in situ to proliferate and differentiate into enteric neurons and glia in HSCR patients.

## 4.2 Introduction

It is clear from work by us and others that the thickened nerve trunks present in aganglionic HSCR gut contain cells of neural crest origin.(Kobayashi et al. 1994; Kobayashi, O'Briain & Puri 1994a; Wilkinson et al. 2013) Our group has shown that cells expressing markers for enteric neurons and glia cultured from aganglionic HSCR gut dual stain with the NCC marker, P75.(Wilkinson et al. 2013) Also, results in chapter 3 show that it was not possible to obtain ENSPC from aganglionic gut where thickened nerve trunks are absent and these results suggest that ENSPC are sourced from the thickened nerve trunks in aganglionic gut. Taking this into account, it is logical to assume that cells expressing the P75 NCC marker in culture are the same cells which label with P75 in the thickened nerve trunks within tissue sections in aganglionic gut, however there is a possibility that these cells derive from reprogramming of other isolated cells. These cells could be reprogrammed mesenchymal cells for example that behave as ENSPC *in vitro*.(Mareschi et al. 2006) Subsequently, there is evidence which shows that mature interstitial cells of Cajal have the ability to act as fibroblast like cell progenitors and therefore it must be considered whether they also have the capability to function as neural progenitors.(Huizinga & White 2008)

In regards to a therapy for HSCR, it could be argued that it is irrelevant to know which cells are ENSPC as long as they are obtainable from aganglionic HSCR gut and differentiate into mature enteric neurons and glia. However, if more is understood

about the origin and characteristics of ENSPC it is more likely that it will be possible to understand how to control these cells *in vivo* post transplantation so that a functional ENS is generated without uncontrolled proliferation and tumour formation. Also, with further information about these cells, it is more likely that it will be possible to stimulate ENSPC *in vivo* to proliferate and differentiate in aganglionic gut without the need for stem cell retrieval. With this information, there is the possibility of developing a therapeutic treatment for HSCR and hence surgical intervention could be avoided.

It is hypothesised that a sub-population of cells expressing the P75 NCC marker separated immediately from other cells dissociated from aganglionic gut in HSCR patients will differentiate in culture into enteric neurons.

FACS has been used previously to sort cells obtained from rat sciatic nerve and normoganglionic embryonic mouse gut with the neural crest marker, P75, however to our knowledge this marker has not been used to sort human cells.(Bixby et al. 2002) It is also unclear whether the sorted cells will survive the sorting process to be cultured when the duration between surgical resection of the sample to culture of the sorted cells is around 24 hours. Therefore, part of the results section details the development of the technique used.

## 4.3 Aims

The first aim is to develop a technique that will allow FACS of freshly dissociated cells that will give two sub-populations of cells that can be cultured. Once this is achieved it can be used to determine whether ENSPC are primarily of neural crest lineage or not.

## 4.4 Methods

### 4.4.1 Details of samples used (in chronological order)

| Breakdown of samples used | | |
|---|---|---|
| **Patient ID** | **Pathology** | **Ganglionic, Aganglionic or Both gut used** |
| H029 | HSCR – short segment | Aganglionic |
| H026 | HSCR – total colonic | Ganglionic |
| H030 | HSCR – short segment | Both |
| H030 | HSCR – short segment | Both |
| C015 | Anorectal malformation | Ganglionic |
| C016 | Anorectal malformation | Ganglionic |
| C017 | Anorectal malformation | Ganglionic |
| C018 | Anorectal malformation | Ganglionic |
| C016 | Anorectal malformation | Ganglionic |
| H031 | HSCR – short segment | Both |
| H032 | HSCR – short segment | Both |
| C018 | Anorectal malformation | Both |
| H033 | HSCR – short segment | Both |
| H034 | HSCR – short segment | Both |
| H035 | HSCR – short segment | Both |

**Table 4.1. Details of samples used for experiments in this chapter.** Gut samples from 15 patients were used in total. 9 of these patients had HSCR and 6 had anorectal malformations. Where applicable the innervation status of the samples used, as confirmed by a consultant histopathologist, is also specified.

### 4.4.2 Obtaining cultured cells

To develop the labelling and sorting technique human gut cells which included ENSPC which had already been cultured for around 10 days were used. A pellet of cells was obtained using the technique for obtaining a cell pellet for immunocytochemistry in section 2.4.2.5 however 0.05% (w/v) Trypsin was replaced with 0.2% Collagenase IV (Gibco®) as P75 labelling and detection with FACS was unsuccessful when Trypsin was used. This is likely to Trypsin damaging the cell surface receptors.

### 4.4.3 Tissue dissociation

It was found that it was not possible to label and identify a separate subpopulation of NCC using the tissue dissociation technique used in other chapters of this thesis (section 2.4.2.3). This problem was likely due to the combination of the enzymes and time spent in the enzymes damaging or removing the P75 cell surface receptor from NCC. A technique published by another group was therefore modified and tested which allowed the identification of a sub-population of NCC after tissue dissociation.(Bixby et al. 2002) Thus the technique as described in section 2.4.2.3 was modified as follows: 1% w/v Dispase was removed from the protocol leaving 1% w/v Collagenase IV as the only enzyme used. Tissue was left in the enzyme solution for 90 minutes with trituration through a 5ml pipette ten times every 15 minutes. This is a reduced duration compared to the original tissue dissociation method

where tissue was left in enzyme solution for between 2 and 3 hours depending on dissociation progress as assessed using an inverted microscope and a 10µl sample.

### 4.4.4 Labelling of the NCC sub-population

The cell suspension was transferred to ice-cold FACS buffer (PBS supplemented with 1% w/v bovine serum albumin, penicillin (100units/ml) and 0.1% w/v streptomycin). After 20 min, 200,000 cell aliquots were re-suspended in 200µl FACS buffer containing 1:1000 mouse anti normal mouse IgG antibody (Santa Cruz) for the negative controls or 5 million cells in 1:1000 mouse anti-P75 antibody (Abcam) for FACS selection. After 30 minutes on ice, 800µl of ice-cold FACS buffer was added and cell aliquots were centrifuged and re-suspended in 200µl FACS buffer with 1:1000 Alexa Fluor 488 goat anti-mouse secondary antibody (Life technologies). After 45 minutes on ice, 800µl of ice-cold FACS buffer was added and the cells were pelleted, re-suspended in 1ml FACS buffer and passed through a 70µm cell strainer.

### 4.4.5 Immunocytochemistry

The effectiveness of cell labelling was evaluated by also undertaking immunocytochemistry on 20,000 cells taken from the sample. The technique is as described in section 2.4.2.5 using P75 primary antibodies and Alexa Flour 488 secondary antibodies where cells are cultured overnight before fixing.

### 4.4.6 Labelling of dead cells

Where mentioned in the results section dual staining of cells for FACS took place with Sytox Orange (Life Technologies) dead cell stain. Following labelling of the NCC sub-population as detailed above 1µl of Sytox Orange was added to the labelled cells. The cells were left for 30 minutes before they were ready for FACS.

### 4.4.7 Florescence activated cell analysis

Florescence activated cell analysis took place using FACScalibur (BD Biosciences).

### 4.4.8 Florescence activated cell sorting

Fluorescence activated cell sorting took place using FACSaria (BD Biosciences). Cells were sorted into 5ml polystyrene round bottom tubes (BD Biosciences) that contained 1ml horse serum medium (DMEM high glucose (4.5% w/v) supplemented with 20% v/v medium 199 (Gibco®), 7% v/v heat inactivated horse serum (Gibco®), 100 Units/ml penicillin and 100 µg/ml streptomycin). Filled tubes were then stored on ice until the sort had finished.

### 4.4.9 Culture of sorted cells

Cells were initially cultured on adherent 24 well plates (Nalge Nunc, New York, USA) in densities of 100,000, 200,000 and 500,000 in horse serum medium. However the cells did not survive.

Subsequently, 8 well chamber slides (Thermo Scientific) were prepared by coating with 10µg/cm$^2$ Poly-D Lysine and 1µg/cm$^2$ Laminin before washing in $H_2O$ and allowing to air dry. 100,000 cells from the P75 positive and negative sorted subpopulations were plated separately in each chamber in 500µl horse serum medium and incubated at 37°C with 5% $CO_2$ for 6 days. Immunocytochemistry was performed as previously described with Tuj (Covance, Maidenhead, UK) antibodies diluted 1:500 in IBB.

### 4.4.10 FACS software

Flowing Software (Flowingsoftware.com) was used to count the number of events and produce graphs.

## 4.5 Results

### 4.5.1 Overview of samples used to develop method and test hypothesis

| Patient ID | Pathology | FACS immediately post tissue dissociation? | Ganglionic (G), Aganglionic (Ag) or Both (B) | Successful FACS analysis with separate P75 population? | Successful sub-population culture? |
|---|---|---|---|---|---|
| H029 | HSCR – short segment | No | Ag | Yes | Not attempted |
| H026 | HSCR – total colonic | No | G | Yes | Not attempted |
| H030 | HSCR – short segment | Yes | B | No | Not attempted |
| H030 | HSCR – short segment | No | B | Yes | No |
| C015 | Anorectal malformation | Yes | G | Yes | Not attempted |
| C016 | Anorectal malformation | Yes | G | Yes | Not attempted |
| C017 | Anorectal malformation | Yes | G | Yes | Not attempted |
| C018 | Anorectal malformation | Yes | G | Yes | Not attempted |

| Patient ID | Pathology | FACS immediately post tissue dissociation? | Ganglionic (G), Aganglionic (Ag) or Both (B) | Successful FACS analysis with separate P75 population? | Successful sub-population culture? |
|---|---|---|---|---|---|
| C016 | Anorectal malformation | No | G | Yes | No |
| H031 | HSCR – short segment | Yes | B | Yes | No |
| H032 | HSCR – short segment | Yes | B | Yes | No |
| C018 | Anorectal malformation | No | B | Yes | No |
| H033 | HSCR – short segment | Yes | B | Yes | Yes |
| H034 | HSCR – short segment | Yes | B | Yes | Yes |
| H035 | HSCR – short segment | Yes | B | Yes | Yes |

**Table 4.2. Table showing the results of experiments on each sample, underlining whether it was possible to identify a P75 positive population and whether it was possible to culture the sub-populations.** Green rows indicate successful P75 positive cell identification and culture of sub-populations. Amber rows indicate successful P75 positive cell identification without an attempt to culture the sub-populations. Red rows indicate failure of P75 positive cell identification or failure of culture of sub-populations.

### 4.5.2 Identification of P75 expression amongst cells taken from culture using FACS

Initially, cells which had been obtained from short segment aganglionic HSCR gut (as specified in Table 4.2) were taken after 10 days in culture and prepared for FACS then analysed (Figure 4.3).

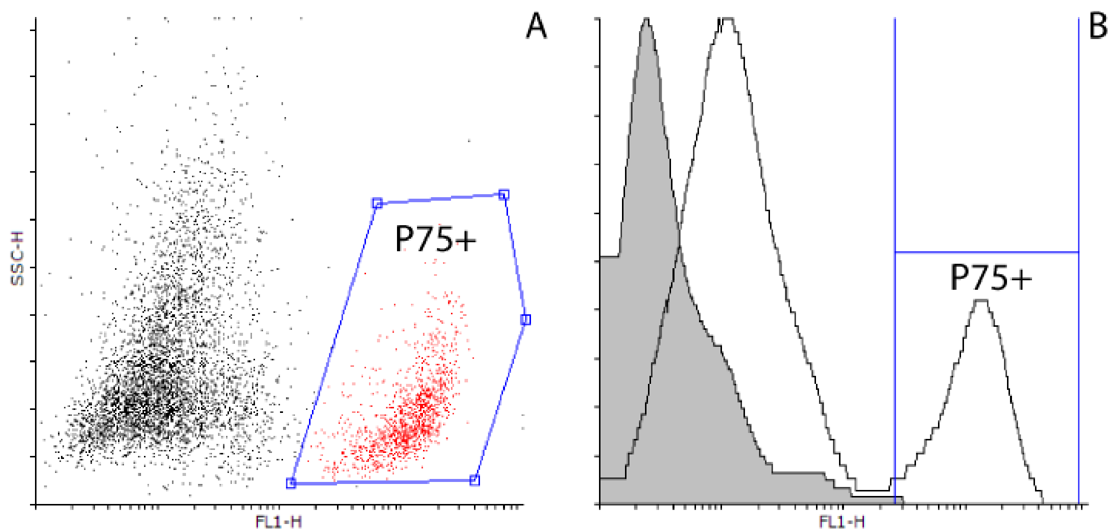


**Figure 4.3. FACS analysis of cells cultured from short segment aganglionic HSCR gut labelled with P75.** A: represents a dot plot showing the separate population of labelled cells. B: represents a overlaid histogram showing the separate population of labelled cells. The grey peak is the negative control using isotype primary antibodies and the clear peak is cells labelled with the P75 antibodies. The population of P75 labelled cells is 21.5% of the total cells present.

It was possible to identify a population of cells labelled with the NCC marker P75 in previously cultured cells from short segment aganglionic HSCR gut (Figure 4.3 A and

B). The percentage of cells expressing P75 is consistent with the number identified using immunocytochemistry after the same duration in culture in chapter 3.

### 4.5.3 Evaluation of the P75 antibody when used with FACS

Following the results of the first experiment there was concern the positive finding could be dead cells binding to the P75 antibody due to changes in membrane permeability of dead cells which is well documented. Therefore to check that the P75 antibody was not binding to dead cells Sytox Orange dead cell stain was used with cultured cells.

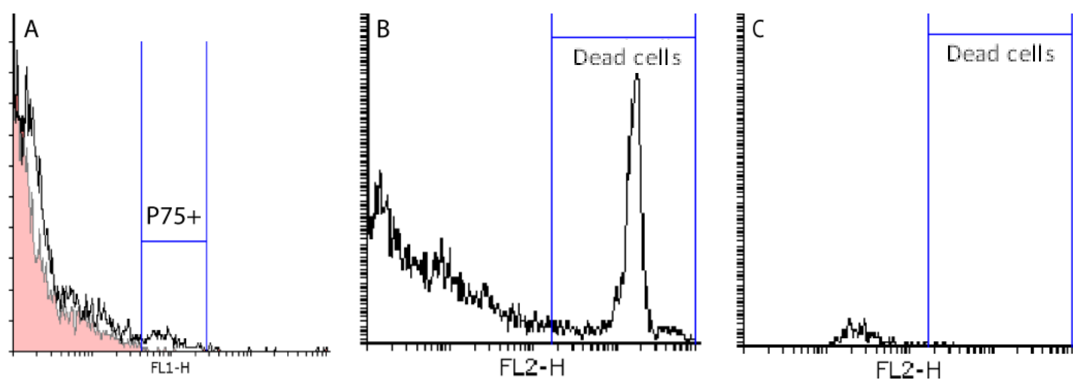


**Figure 4.4. FACS analysis of cells cultured from total colonic ganglionic HSCR gut labelled with P75 and Sytox orange.** A: represents a histogram showing cells labelled with P75. The red peak is the negative control using isotype primary antibodies and the clear peak is cells labelled with the P75 antibodies. The population of P75 labelled cells is 18.1% of the total cells present. B: represents a histogram showing cells labelled as dead with Sytox orange. The population of cells labelled as dead is 21.9% of the total cells present. C: represents a histogram of only cells labelled with P75 showing cells also labelled as dead with Sytox orange. The majority of cells are alive.

The vast majority of cells labelled with P75 were alive (Figure 4.4 C). This finding combined with the use an isotype primary antibody control is evidence that neural crest derived cells are being labelled specifically, and can be maintained alive for culture.

### 4.5.4 Attempts to identify freshly dissociated cells expressing P75 using FACS

Identification of a P75 labelled population was then attempted on freshly dissociated cells from short segment aganglionic HSCR gut. The original tissue dissociation technique detailed in section 2.4.2.3 using 1% (w/v) Collagenase IV and 1% (w/v) Dispase was used.

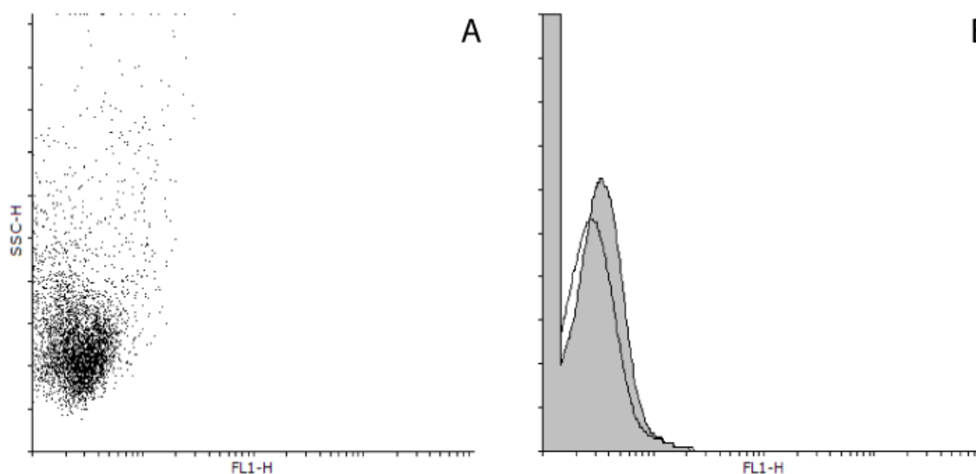


**Figure 4.5. FACS analysis of cells freshly dissociated from short segment aganglionic HSCR gut labelled with P75.** A: represents a dot plot showing no separate population of labelled cells. B: represents a histogram showing no separate population of labelled cells. The grey peak is the negative control using isotype primary antibodies and the clear peak is cells exposed to P75 antibodies.

It was not possible to label and identify a population of cells expressing P75 in freshly dissociated cells (Figure 4.5 A and B). Immunocytochemistry was performed on a sample of the cells which did identify cells expressing P75 at a percentage consistent with findings in chapter 3 (not shown).

### 4.5.5 Identification and sorting of freshly dissociated cells expressing P75 using FACS with culture and characterisation of sub-populations

As this technique works with cultured cells it is likely that the effects of the dissociation process are preventing the P75 antibody binding to the cell surface receptors on neural crest derived cells. The tissue dissociation was therefore modified as described in the methods section in an attempt to retain the structure of the cell surface receptors and allow labelling of freshly dissociated cells. This technique was successful and sub-populations of cells were cultured and immunocytochemistry was performed to characterise the sub-populations.

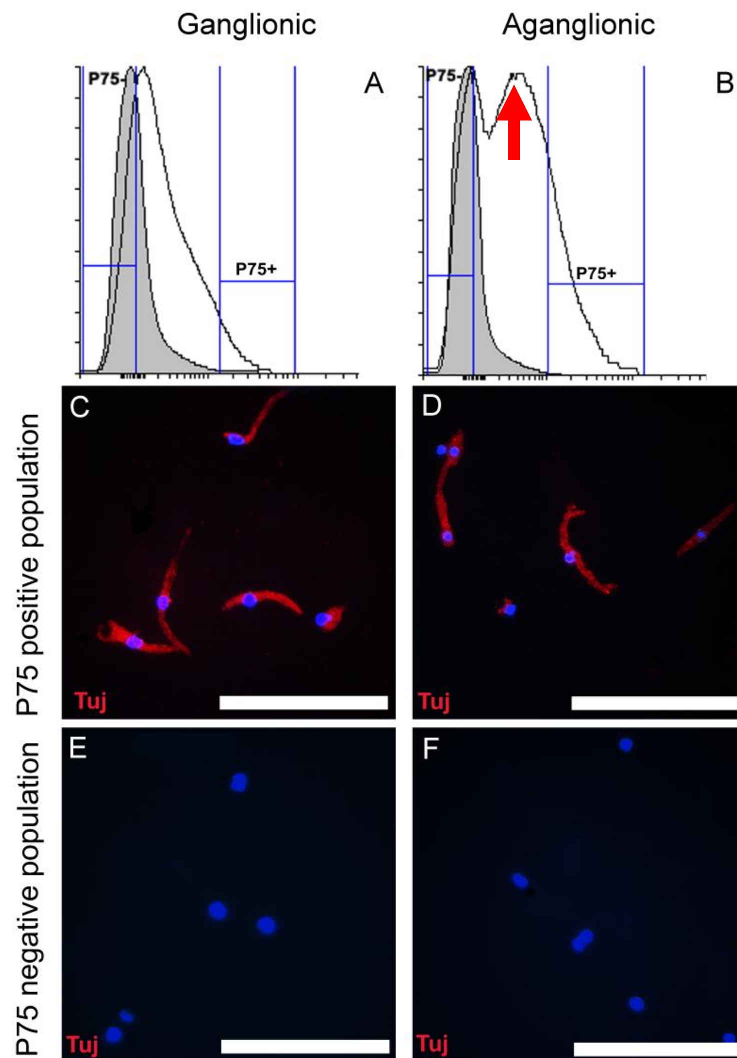


**Figure 4.6. Fluorescence activated cell sorting of cells obtained from ganglionic and aganglionic HSCR gut immediately after tissue dissociation and characterisation of each population following 6 days in culture.** A and B: histograms of the FACS displaying P75 positive and negative selected subpopulations. The grey peaks are the negative control using isotype primary antibodies and the clear peak are cells labelled with the P75 antibodies. The arrow in B highlights a second peak of P75 expression. C and D: Tuj immunofluorescence staining of the P75 positive cell subpopulations from ganglionic and aganglionic bowel respectively after 6 days culture (note that the majority of cells are Tuj positive). E and F: Tuj immunofluorescence staining of the P75 negative cell subpopulations from ganglionic and aganglionic bowel respectively (note the absence of Tuj positive cells).

It was possible to label, identify and sort cells expressing P75 from cells freshly dissociated from ganglionic and aganglionic short segment HSCR gut (Figure 4.6 A and B). The histogram from aganglionic gut differs from ganglionic gut in the way that there is a second peak of cells expressing P75 in the aganglionic sample but not to such an intensity in terms of florescence as the majority of positively sorted cells which are identified on the histograms (Figure 4.6 A-B, see arrow for second peak on B).

Initially, it was attempted to culture the two sub-populations of cells on adherent 24 well plates as described in the methods section but neither population survived. It is thought that this was due to the limited cell population size as trophic factors secreted from cells would be much more dilute compared to culture conditions used previously. The failure of survival may also have been due to the trauma that the cells experienced in the 24 hour period between tissue dissociation and culture with this FACS method. Therefore, laminin and poly-d-lysine coated chamber slides were used (see methods) for culturing sorted cells. The positively sorted cells survived but there were fewer cells from the negatively sorted sub-population which survived in culture for 6 days. This is most likely due to the culture conditions favouring cells of neural crest lineage. In order to show this is the case and that the negative population is not just cell debris, this population was stained with the dead cell stain, Sytox orange, immediately after sorting (Figure 4.7).

In both the ganglionic and aganglionic HSCR gut the positively stained sub-populations expressed the neuronal marker Tuj after 6 days in culture (Figure 4.6 C and D). The mean percentage of cells expressing Tuj are 89.3% and 85.4% (n=3) in ganglionic and aganglionic gut respectively. In both the ganglionic and aganglionic HSCR gut the negatively stained sub-populations did not express the neuronal marker Tuj after 6 days in culture (Figure 4.6 E and F). A negative control consisting of staining with only the secondary antibody was carried out for all sub-populations (not shown).

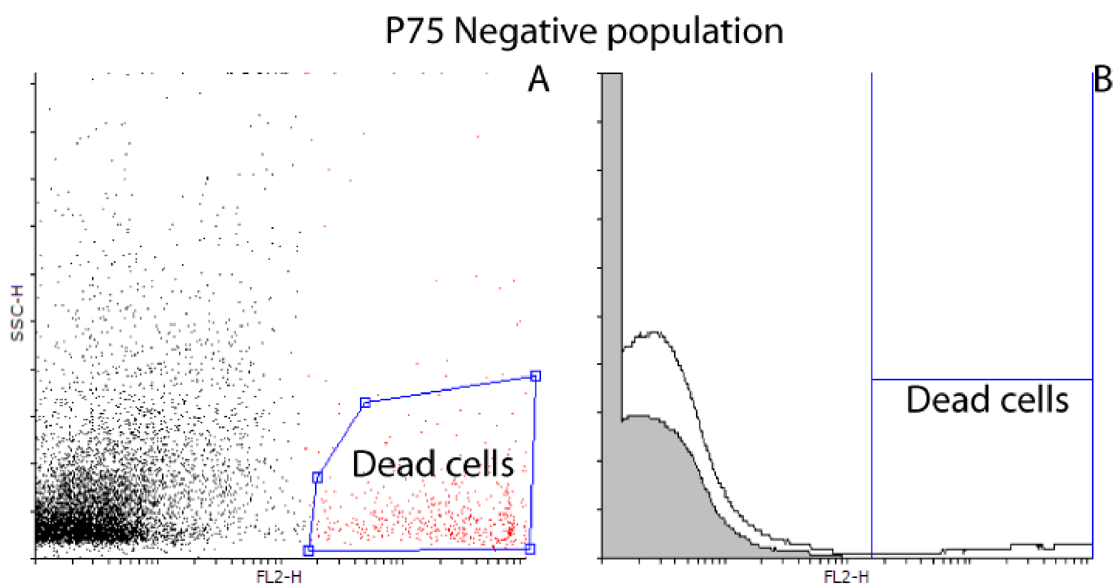


**Figure 4.7. FACS analysis of the P75 negative sub-population of cells immediately after sorting from freshly dissociated cells from short segment aganglionic HSCR gut labelled with Sytox orange, dead cell stain.** A: represents a dot plot showing the separate population of cells labelled as dead. B: represents a histogram showing the separate population of cells labelled as dead. The grey peak represents a control consisting of unlabelled cells whereas the white peak represents cells labelled with the dead cell stain, Sytox orange. 5.6% of cells labelled as dead.

Of the cells sorted as not expressing P75 only 5.6% of the cells were dead immediately after sorting (Figure 4.7 A and B). Once the cells were seeded onto a 8 well chamber slide for culture the majority of cells appeared dead and did not adhere to the slide hence the majority of cells fail to take to culture conditions. A much larger proportion of the cells in the P75 positive sub population survive in the culture conditions used.

## 4.6 Discussion

### 4.6.1 ENSPC obtained from aganglionic HSCR gut are of neural crest lineage

It has been shown that cells of neural crest lineage separated by FACS from other cells immediately after dissociation from aganglionic short segment HSCR gut can differentiate *in vitro* into neurons. This shows that P75 positive neural crest derived cells in aganglionic gut are partially or wholly responsible for ENSPC and the mature enteric neurons and glia which have been cultured from aganglionic short segment HSCR gut previously.(Wilkinson et al. 2013) Moreover, the fact that it was not possible to derive neurons from the population of cells not expressing P75 on isolation suggests that there is no non neural crest lineage source of cultured ENSPC.

### 4.6.2 The source of ENSPC in ganglionic HSCR gut

This finding questions the source of ENSPC which can be obtained from ganglionic HSCR gut. Previously it was thought that these cells are sourced from the ganglia, as this is the main source of neural cells in ganglionic gut.(Almond et al. 2007) However, now ENSPC have now been shown to be present in both ganglionic and aganglionic gut and also ENSPC cultured from each segment behave the same *in vitro* and upon implantation into *ex vivo* aganglionic mouse gut, it is entirely possible that ENSPC obtainable from ganglionic HSCR gut are extra-

ganglionic.(Wilkinson et al. 2013) It is therefore feasible that cells expressing P75 associated with the extrinsic nerves are the source of ENSPC in ganglionic HSCR gut.

### 4.6.3 The cellular origin of ENSPC in HSCR gut

As cells of non neural crest lineage did not give rise to neurons we can consider the cellular origins of ENSPC in HSCR gut. It is possible that cells of Schwann cell lineage are the source of ENSPC in aganglionic HSCR gut and either these cells of Schwann cell lineage, associated with extrinsic nerves or other glial cells associated with the ganglia, are the source in ganglionic HSCR gut. As discussed in chapter 3 it has been found that Schwann cell precursors have dual roles as functional Schwann cells and neuronal progenitors in the developing parasympathetic nervous system, which may also be the case in HSCR gut.(Kalcheim & Rohrer 2014) Subsequently, it is known that glial cells have a role in neurogenesis *in vitro* and also described by one group *in vivo*.(Joseph et al. 2011; Laranjeira et al. 2011) A possible non glial source of ENSPC are neuron cell bodies, which are present in ganglia but absent in extrinsic nerves which only contain the axon.(Topp & Boyd 2006) This seems less likely though due to common characteristics of ENSPC from ganglionic and aganglionic gut.(Wilkinson et al. 2013) The other well documented NCC derivative in extrinsic nerve trunks are fibroblasts within the endoneurium, however little is known about the ability of these cells to undergo neurogenesis.(Joseph et al. 2004) The migration of sacral NCC during development into the hindgut with extrinsic fibres has been described.(Erickson et al. 2012) This is another possibility as to a source of ENSPC in

aganglionic gut as NCC may be left in an un-differentiated state from development within the gut.

To explore these possibilities further we could use the experience gained using FACS with human gut cells in this chapter. Small changes to the method could take place which allow separation of a population of cells expressing a glial marker, such as S100.(Hirose, Sano & Hizawa 1986) This would give an isolated sub-population of glial cells which could then be cultured and characterised in a similar way to experiments in this chapter to determine whether glial, and therefore Schwann cells, can behave as ENSPC.

### 4.6.4 Differences in FACS histograms between ganglionic and aganglionic HSCR gut cells labelled with P75

Most interestingly there is a key difference in the FACS histogram of cells obtained from ganglionic HSCR gut to aganglionic HSCR gut when labelled with P75. The second peak seen in the aganglionic sample shows that there are two populations of cells labelling with different intensities of fluorescence suggesting that two different types of neural crest cell are present. This is consistent with characterisation of ganglionic compared to aganglionic HSCR gut where there is intense expression of P75 in a ring surrounding thickened nerve trunks in aganglionic gut which is not present around ganglia.(Kobayashi, O'Briain & Puri 1994b) It is therefore suggestive that the second peak seen in aganglionic gut with

FACS is from cells associated with the ring of intense P75 expression surrounding thickened nerve trunks on immunohistochemistry. Further characterisation of these nerve trunks is required however to confirm this finding. Further FACS could separate the two different peaks into independent sub-populations and the ability to differentiate into enteric neurons could then be tested. This would provide evidence for the cell type which are acting as ENSPC in aganglionic gut. Again the similarities between ENSPC from both ganglionic and aganglionic HSCR gut question whether the cells with increased expression do have a role.(Wilkinson et al. 2013)

### 4.6.5 Implications of findings in this chapter

This work has several implications. Firstly, it has been shown that neural crest derived cells are the source of ENSPC obtained from aganglionic HSCR gut. This will allow further work to explore how NCC can be stimulated to differentiate and proliferate into an ENS *in vivo* in HSCR patients. This information could be used to develop a therapeutic treatment for HSCR. This would be even more promising if the exact cell type could be established. Secondly, it should be established whether neurospheres can be cultured from isolated cells of neural crest origin and if so whether they have the same characteristics in terms of restoring contractility in *ex vivo* aganglionic mouse gut as un-purified enteric neurospheres. Now that a cell sorting technique has been developed this work could take place with the hypothesis that a purified neural crest cell neurosphere will be more effective in terms of restoring contractility as a neurosphere containing other cell types. Even, if this is not the case there are likely to be major safety advantages to transplanted

one lineage of cells by excluding other cells such as smooth muscle cells when considering a future stem cell therapy for HSCR.(Wilkinson, Edgar & Kenny 2012)

Finally, the finding that neural crest derived cells alone are responsible for the ENSPC obtained from HSCR gut make it likely that in normal human development NCC are the only progenitor for the ENS. Although this has been shown in several mouse studies this adds further evidence to human ENS development and can be used to direct further work exploring the aetiology of HSCR.(Obermayr et al. 2013)

## 4.7 Conclusion

This chapter has found that ENSPC are neural crest derived cells when obtained from both ganglionic and aganglionic HSCR gut. This adds understanding to the previous findings that ENSPC can be isolated from aganglionic HSCR gut and questions the source of ENSPC in ganglionic HSCR gut. This work raises several pertinent questions, but most importantly further work should look to identify the cell type which behaves as ENSPC in aganglionic HSCR gut. Currently it is felt that cells of Schwann cell lineage are responsible, however to develop a safe and efficacious stem cell therapy, along with the possibility of a therapeutic treatment for HSCR, this information needs to be known.

# Chapter 5 – Characterisation of ganglionic and aganglionic HSCR gut

## 5.1 Overview

This chapter builds on previous work characterising aganglionic HSCR gut and in particular, the thickened nerve trunks. Full thickness gut was obtained from HSCR patients and labelled with antibody markers for NCC, neurons, glia and surrounding muscular tissue to detail the histology in both ganglionic and aganglionic HSCR gut. Findings in this chapter add understanding to work carried out in chapters 3 and 4 *in vitro* where it was found that ENSPC from aganglionic gut are neural crest derived cells associated with the thickened nerve trunks.

## 5.2 Introduction

As detailed in chapters 3 and 4 it has been shown that it is possible to isolate from short and long segment aganglionic HSCR gut ENSPC cells which express the neural crest maker P75. As cells in the thickened nerve trunks in the aganglionic gut also express P75, along with the association of not being able to obtain ENSPC from aganglionic gut where thickened nerve trunks are absent, it is hypothesised that these cells within the trunks have progenitor properties. In order to determine which cell has the progenitor properties of the cells present within the thickened nerve trunks, further characterisation is needed.

Electron microscopy has previously been used to characterise thickened nerve trunks in aganglionic gut compared to intrinsic and extrinsic nerves in ganglionic gut.(Baumgarten, Holstein & Stelzner 1973) It was observed that Schwann cells, axons and large collagen filled spaces were contained within the endoneurium of these nerves. The Schwann cells seen in this study were of immature appearance, as the cytoplasm contained few organelles, compared to mature Schwann cells. It was also stated that the Schwann cells in thickened nerve trunks bear the appearance of un-differentiated Schwann cell precursors in the developing peripheral nervous system. In terms of the axons, there were more present per Schwann cell in the thickened nerve trunks compared to extrinsic nerves in ganglionic gut and the axons were hypertrophied as they contained large fluid filled vesicles. The presence of these is attributed to the fact that the axons are blind

ending and fail to synapse. Finally, it was reported that the thickened nerve trunks were encased abnormally in multiple layers of coherent perineurial cells.

There has been some more recent work published characterising histopathologically aganglionic HSCR gut, however the majority of this work focuses on the finding of the thickened nerve trunks and their relation to other cells such as Interstitial cells of Cajal without focus on the internal structure of the trunks.(Matsuda et al. 2006; Nemeth et al. 2001; Solari, Piotrowska & Puri 2003) One study has reported the presence of a thick ring of cells surrounding the thickened nerve trunks which labelled with P75 antibodies, possibly consisting of perineurial cells.(Kobayashi, O'Briain & Puri 1994b) This study found a similar appearance in mesenteric nerves and therefore concluded that thickened nerve trunks in aganglionic gut are of extrinsic origin. This finding is consistent with previous work by our group in which this thick ring of cells expressing P75 was also detected.(Wilkinson et al. 2013) It is unclear why thickened nerve trunks are present in aganglionic gut and why they differ in appearance to normal extrinsic nerves. With antibody staining, paying particular attention to the varying intensities of P75 expression and associated neuronal plus glia expression it is hoped that more can be understood about the internal structures of these nerves but most importantly potential sources of ENSPC from aganglionic gut can be identified.

ENSPC obtained from ganglionic and aganglionic HSCR gut have similar characteristics, in that they differentiate into enteric neurons and glia *in vitro* and can restore contractility in aganglionic *ex vivo* mouse gut when transplanted, this therefore suggests that they are from the same source. It is hoped that by characterising ganglionic gut as well as aganglionic gut we can come closer to understanding the similarities that both groups of cells share. In order to do this, ganglia and extrinsic nerves in ganglionic gut must be compared to thickened nerve trunks within aganglionic gut.

## 5.3 Aim

The aim of the experiments in this chapter is to characterise using immunofluorescence both ganglionic and aganglionic HSCR gut focussing on the thickened nerve trunks. In order to do this a variety of markers associated with the thickened nerve trunks which identify neurons, glia and NCC will be used. The surrounding smooth muscle will also be identified to determine how this relates structurally to nerve fibres.

## 5.4 Methods

### 5.4.1 Immunohistochemistry

Human full thickness gut tissue was obtained as described in section 2.4.2.1. Tissue for immunohistochemistry was immersed in 4% (w/v) paraformaldehyde for 2-3 hours to fix the tissue. The tissue was then rinsed twice with PBS and placed overnight in 20% (w/v) sucrose before dissection took place to orientate the tissue planes. Tissue was then placed in Peel-A-Way moulds (Polysciences Europe, Eppelheim, Germany), embedded in clear Shandon Cryomatrix (Thermo Scientific) and left at -80$^{o}$C overnight. 7µm sections of tissue were then cut using a MX35 microtome blade and HM505N cryostat (both Thermo Scientific) at -22$^{o}$C. Tissue was mounted on Superfrost Plus microscope slides (Thermo Scientific) and stored at -80$^{o}$C.

Antibody staining, slide preparation and imaging then took place as described for immunocytochemistry in section 2.4.2.5. The antibodies used are described below.

### 5.4.2 Primary antibodies

| Primary antibodies | | | | |
|---|---|---|---|---|
| **Antibody** | **Supplier** | **Concentration** | **Host** | **Target** |
| P75 (ab3125) | Abcam | 1:1000 | Mouse Monoclonal IgG1 | NCC (Morrison et al. 2000) |
| Calretinin (ab702) | Abcam | 1:250 | Rabbit Polyclonal IgG | ENS cells (Lindley et al. 2009) |
| S100 (ab868) | Abcam | 1:500 | Rabbit Polyclonal IgG | Glia (Almond et al. 2007) |
| Tyrosine Hydroxylase (ab112) | Abcam | 1:500 | Rabbit Polyclonal IgG | Early Dopaminergic neurons (Almond et al. 2007) |
| Laminin (l9393) | Sigma-Aldrich | 1:500 | Rabbit Polyclonal IgG | Connective tissue (Kostrominova, 2011) |
| SMA (ab7817) | Abcam | 1:200 | Mouse Monoclonal IgG2a | Smooth muscle (Lindley et al. 2009) |

**Table 5.1. Primary antibodies used in experiments in this chapter**

### 5.4.3 Secondary antibodies

| Secondary antibodies | | | |
|---|---|---|---|
| **Antibody** | **Supplier** | **Concentration** | **Host** |
| Alexa Fluor 488 - Anti-mouse | Life Technologies | 1:1000 | Goat IgG |
| Alexa Fluor 594 - Anti-rabbit | Life Technologies | 1:1000 | Goat IgG |

**Table 5.2. Secondary antibodies used in experiments in this chapter**

## 5.5 Results

### 5.5.1 Expression of P75 and calretinin in aganglionic HSCR gut

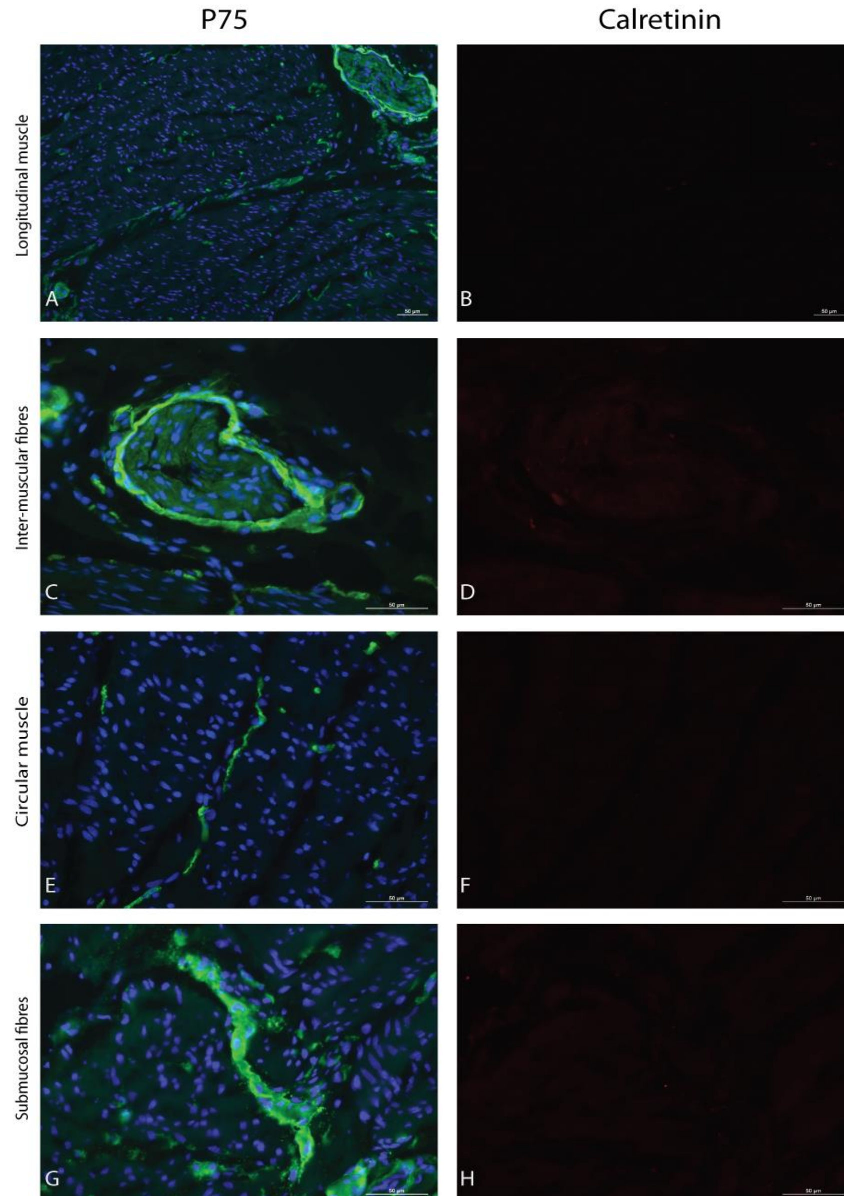


**Figure 5.3. Immunohistochemistry of full thickness aganglionic short segment HSCR gut labelled with P75 and Calretinin.** A and B: longitudinal smooth muscle layer; C and D: extrinsic nerve fibres in the inter-muscular layers; E and F: circular smooth muscle layer; G and H: extrinsic nerve fibres in the submucosal layer. A,C,E,G are labelled with DAPI (blue) and P75 (green). B,D,F,H are labelled with Calretinin (red). Scale bars represent 50µm.

It is evident that there is a ring of cells expressing high levels of P75, surrounding a thickened nerve trunk with some expression from within the structure (Figure 5.3 C). Within the muscular layers in aganglionic HSCR tissue there is some expression of P75 within neurite like structures (Figure 5.3 A and E). Calretinin staining, which identifies enteric neurons confirms the innervation status of the tissue (Figure 5.3 B,D,F,H).

## 5.5.2 Expression of P75 and calretinin in ganglionic HSCR gut

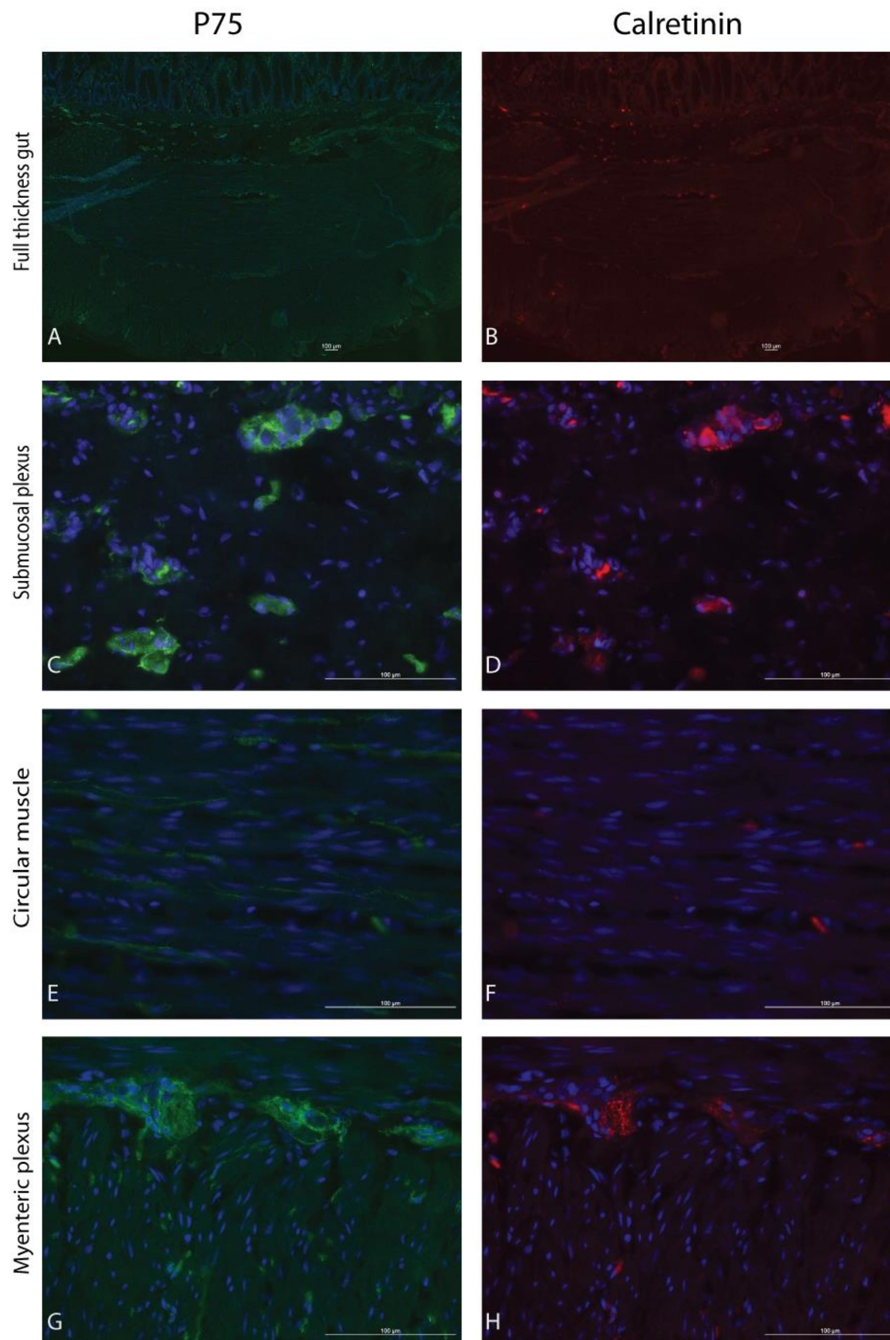


**Figure 5.4. Immunohistochemistry of full thickness ganglionic short segment HSCR gut labelled with P75 and Calretinin.** A and B: full thickness gut; C and D: submucosal plexus; E and F: circular smooth muscle layer; G and H: myenteric plexus. A,C,E,G are labelled with DAPI (blue) and P75 (green). B,D,F,H are labelled with DAPI (blue) and Calretinin (red). Scale bars represent 50µm.

In ganglionic HSCR gut the ganglia express P75 however there is one intensity of expression throughout the ganglia and certainly no surrounding ring (Figure 5.4 C and G). Within the muscular layers there is some expression of P75 running in in the muscular fibres (Figure 5.4 A and G). Calretinin staining, which identifies enteric neurons confirms the innervation status of the tissue (Figure 5.4 B,D,F,H). Ganglionic HSCR gut was also compared to gut obtained from patients without HSCR, there was no differences observed between ganglia or nerve distribution (not shown).

## 5.5.3 Expression of P75 and S100 in aganglionic HSCR gut

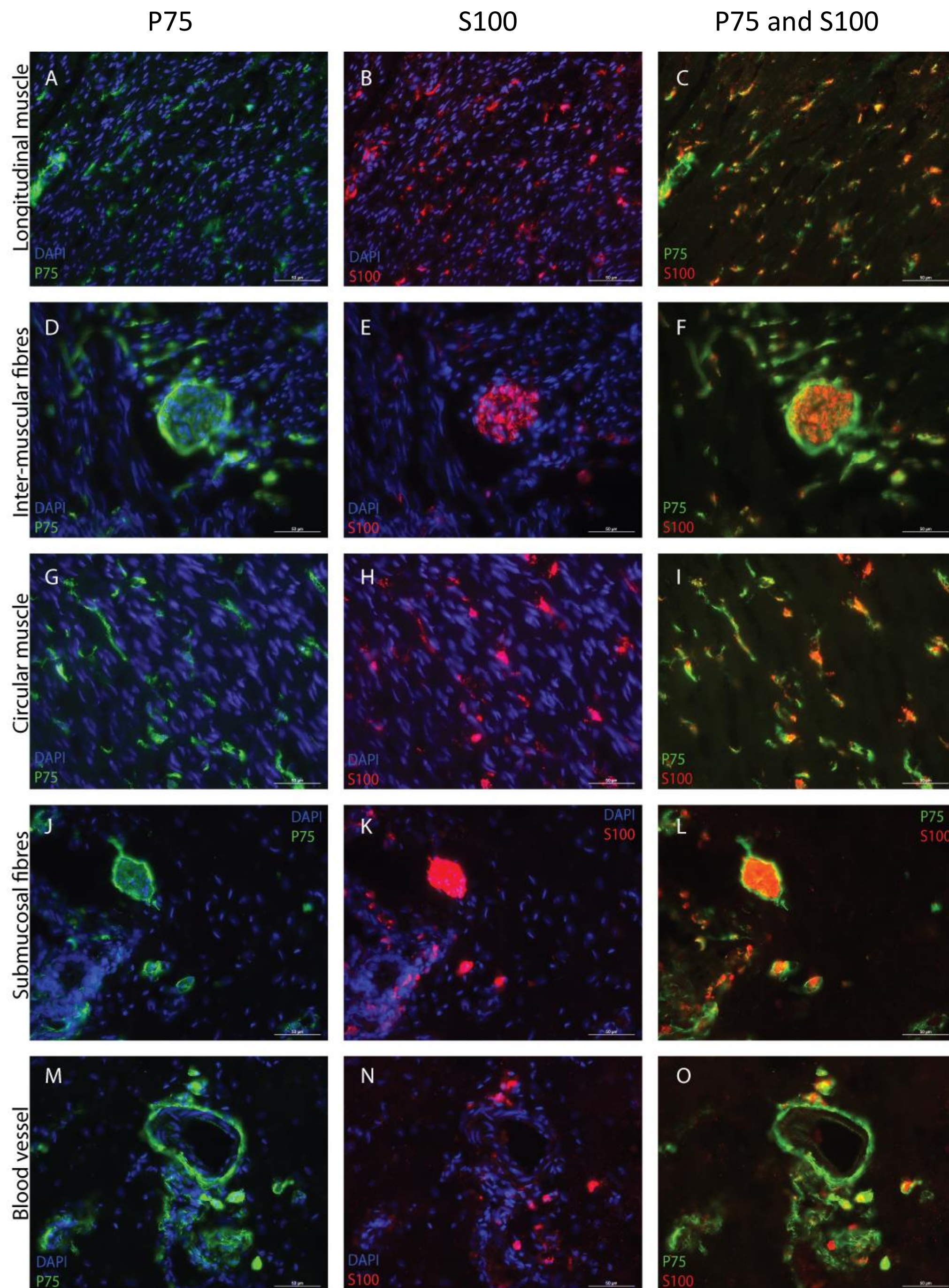


**Figure 5.5. Immunohistochemistry of full thickness aganglionic short segment HSCR gut labelled with P75 and S100.** A-C: Longitudinal muscle layer; D-F: Inter-muscular layer fibres; G-I: Circular muscle layer; J-L: Submucosal fibres; M-O: Blood vessel within submucosal layer. A,D,G,J,M: are labelled with DAPI (blue) and P75 (green); B,E,H,K,N: are labelled with DAPI (blue) and S100 (red); C,F,I,L,O: are labelled with P75 (green) and S100 (red). Scale bars represent 50µm.

When aganglionic tissue was dual labelled with P75 and S100, a glial marker, it appears that the centre of thickened nerve trunks dual stain however the ring intensely expressing P75 does not express S100 (Figure 5.5 F and L). Many of the fibres expressing P75 in the muscular layers also express S100, showing that most of fibres are associated with glial cells (Figure 5.5 C and I). Finally, a blood vessel can be identified in the aganglionic gut showing the clear difference in appearance between this and the thickened nerve trunks (Figure 5.5 M-O).

### 5.5.4 Expression of P75 and calretinin in ganglionic HSCR gut

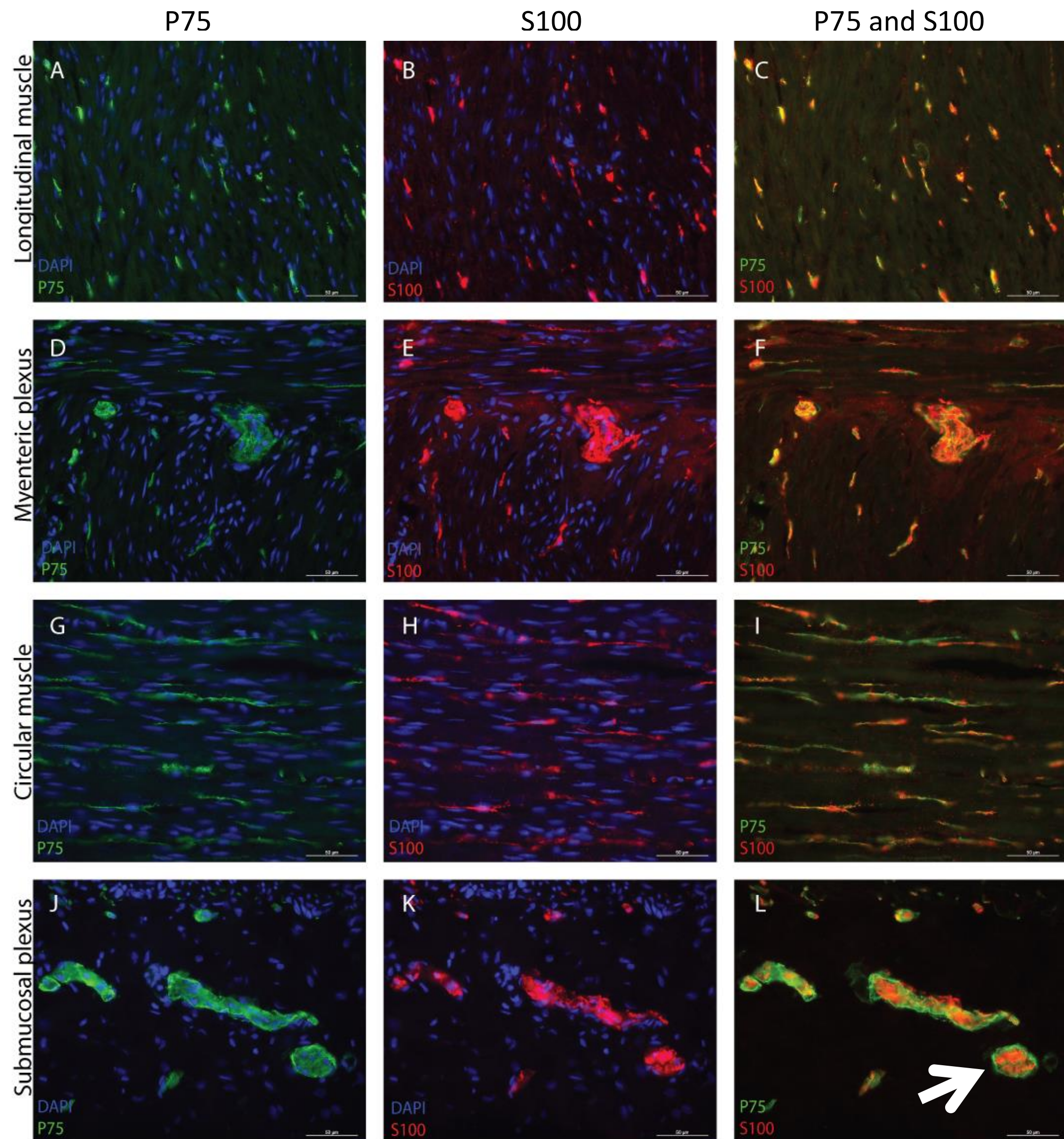


**Figure 5.6. Immunohistochemistry of full thickness ganglionic short segment HSCR gut labelled with P75 and S100.** A-C: Longitudinal muscle layer; D-F: Myenteric plexus; G-I: Circular muscle layer; J-L: Submucosal plexus. A,D,G,J: are labelled with DAPI (blue) and P75 (green); B,E,H,K: are labelled with DAPI (blue) and S100 (red); C,F,I,L: are labelled with P75 (green) and S100 (red). Arrow in L shows identifies an extrinsic nerve like fibre. Scale bars represent 50µm.

When ganglia are dual stained with P75 and S100 there is expression of both markers throughout (Figure 5.6 F and L). Structures with the appearance of extrinsic nerves can also be identified in the ganglionic gut (Figure 5.6 L see arrow). Many of the fibres expressing P75 in the muscular layers also express S100, identifying the presence of glial cells (Figure 5.6 C and I). Ganglionic HSCR gut was also compared using the S100 glial marker to gut obtained from patients without HSCR, there were no differences observed (not shown).

## 5.5.5 Expression of P75 and tyrosine hydroxylase in HSCR gut

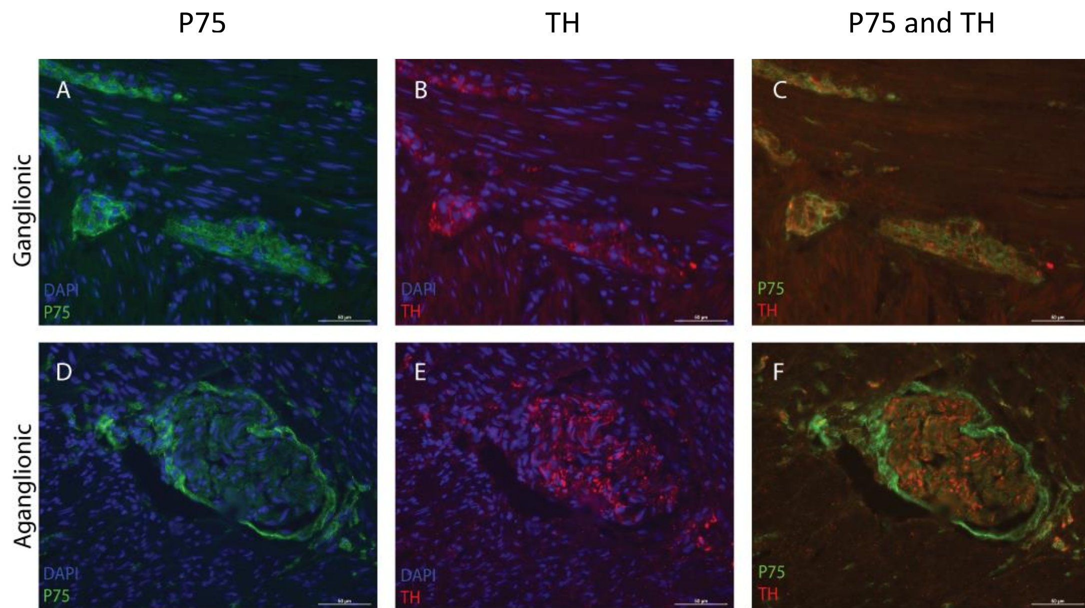


**Figure 5.7. Immunohistochemistry of full thickness ganglionic and aganglionic short segment HSCR gut labelled with P75 and Tyrosine Hydroxylase.** A-C: ganglionic gut; D-F: aganglionic gut. A,D: are labelled with DAPI (blue) and P75 (green); B,E: are labelled with DAPI (blue) and TH (red); C,F: are labelled with P75 (green) and TH (red). Scale bars represent 50µm.

In ganglia the dopaminergic neuronal marker, tyrosine hydroxylase (TH), dual labels with P75 in a similar pattern to S100 in that there is expression of both markers throughout the ganglia (Figure 5.7 C). In contrast, when aganglionic tissue is dual stained, the centre of the thickened nerve trunks expresses both P75 and TH however the ring intensely expressing P75 does not label with TH (Figure 5.7 F).

## 5.5.6 Expression of P75 and laminin in HSCR gut

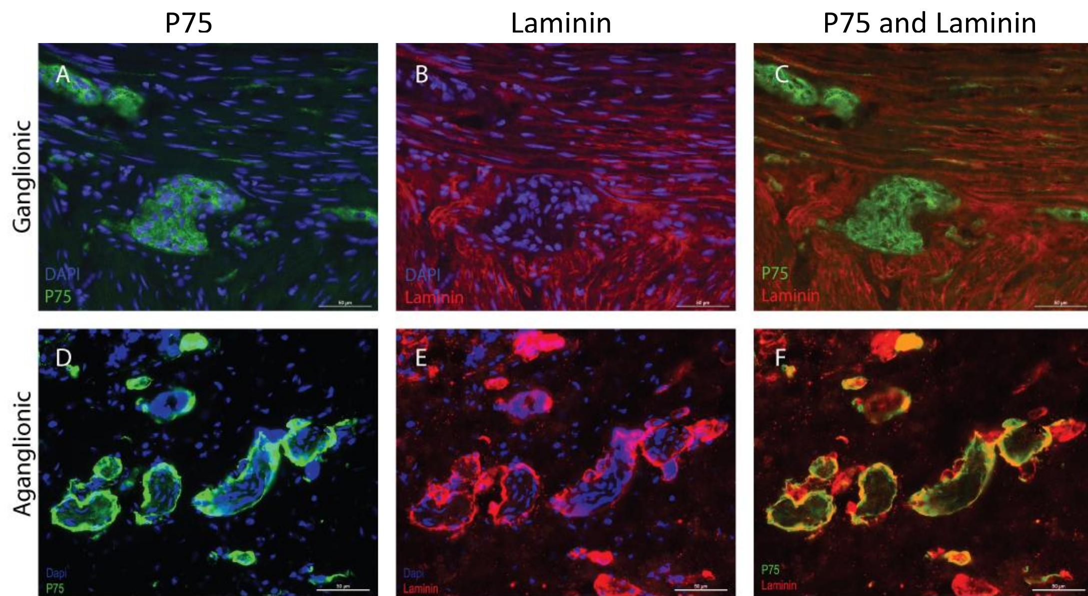


**Figure 5.8. Immunohistochemistry of full thickness ganglionic and aganglionic short segment HSCR gut labelled with P75 and Laminin.** A-C: ganglionic gut; D-F: aganglionic gut. A,D: are labelled with DAPI (blue) and P75 (green); B,E: are labelled with DAPI (blue) and Laminin (red); C,F: are labelled with P75 (green) and Laminin (red). Scale bars represent 50µm.

Antibodies to laminin were used to identify the basement membranes associated with the nerve structures. In ganglionic gut there was no expression of this marker surrounding ganglia (Figure 5.8 C). However, when thickened nerve trunks were dual stained with P75 and laminin there was expression of both markers in close relationship to the ring surrounding the trunks. The laminin however was expressed very marginally externally to the P75 expression (Figure 5.8 F).

### 5.5.7 Expression of smooth muscle actin and S100 in HSCR gut

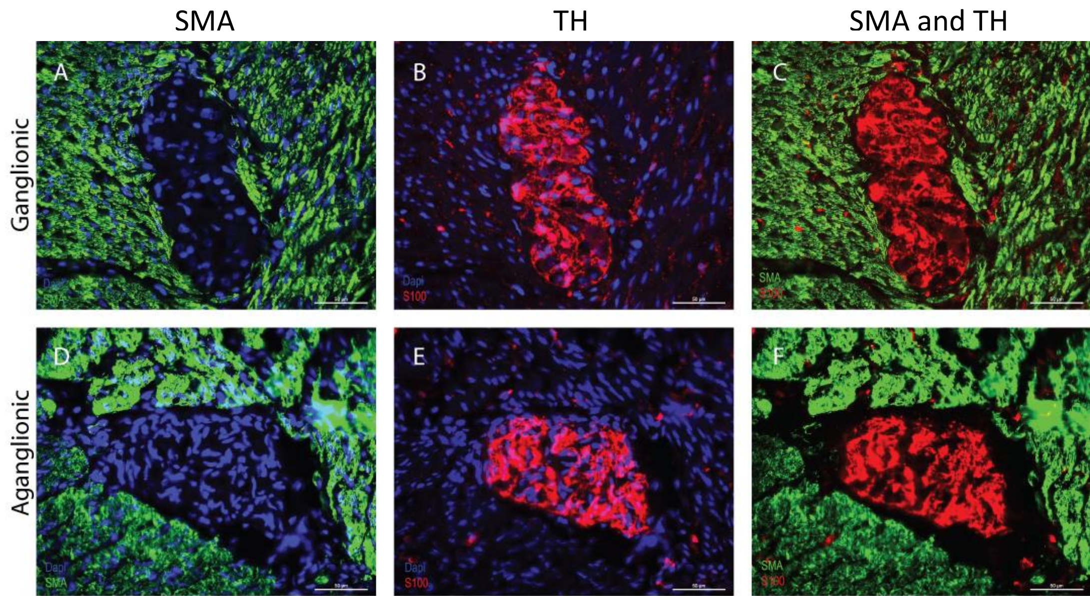


**Figure 5.9. Immunohistochemistry of full thickness ganglionic and aganglionic short segment HSCR gut labelled with Smooth Muscle Actin and S100.** A-C: ganglionic gut; D-F: aganglionic gut. A,D: are labelled with DAPI (blue) and SMA (green); B,E: are labelled with DAPI (blue) and S100 (red); C,F: are labelled with SMA (green) and S100 (red). Scale bars represent 50µm.

Lastly, to identify the relationship of the smooth muscle to neuronal structures, tissue from both ganglionic and aganglionic gut was dual stained with S100 and smooth muscle actin (SMA). The ganglia appeared to have a close relationship with the smooth muscle in the ganglionic gut and there was is no layer between the two tissue types (Figure 5.9 C). However in aganglionic gut there is a space between the two tissue types which contains cell nuclei (Figure 5.9 E and F). When compared with figure 5.5, where dual labelling has taken place with P75 and S100 it would appear that this space is occupied by the ring intensely expressing P75.

## 5.6 Discussion

### 5.6.1 A ring of intense P75 expression surrounds thickened nerve trunks

The thickened nerve trunks identified in both the submucosal and inter-muscular layers of aganglionic gut were of a similar appearance in terms of P75 expression to those described in the literature in that there was a ring of intense P75 expression on the periphery of the structure.(Kobayashi, O'Briain & Puri 1994b). Additionally, axons expressing the dopaminergic neuronal marker tyrosine hydroxylase and endoneurial P75 positive glia are shown to be deep to the intense ring of P75 expression. There is some evidence which shows that the fibroblastic cells of the perineurium surrounding extrinsic nerves consists of neural crest derived cells however more recently lineage tracing has shown that this is not the case.(Hirose, Sano & Hizawa 1986; Joseph et al. 2004) Also, using electron microscopy multiple layers of perineurial cells can be observed surrounding thickened nerve trunks compared to normal extrinsic nerves.(Baumgarten, Holstein & Stelzner 1973) This evidence suggests that the thickened rings surrounding the thickened nerve trunks are unusual. It is unlikely that these cells are perineurial cells as found in normal extrinsic nerves as they express the neural crest marker P75, are thickened compared to normal perineurium and express high levels of P75 compared to endoneurial tissue within the nerve. On the contrary, the expression of laminin just outside the thickened nerve trunks appears to identify basement membranes which are in keeping with the structure of a surrounding perineurial layer.

It may also be possible that the increased expression is actually ENCC which migrated into the gut from the sacral NCC contribution with extrinsic nerves during the embryological period, but have failed to differentiate into an ENS.(Erickson et al. 2012) However it has been reported that this ring of intense P75 expression is also seen in mesenteric nerves which are peripheral nerves and therefore this possibility would appear less likely.(Kobayashi, O'Briain & Puri 1994b)

### 5.6.2 The cellular source of aganglionic ENSPC

The hypothesis that cells of Schwann cell lineage are the source of ENSPC in aganglionic gut is supported by the expression of the glial marker, S100, and weak P75 expression within the thickened nerve trunks. The intense ring of P75 expression does not label with S100 however and therefore does not these contain Schwann cells. It is therefore possible that cells of this thickened surrounding layer around the nerve trunks are the source of ENSPC. However it seems more probable from studies showing the ability of glial and Schwann cells to undergo neurogenesis that this is not the case.(Joseph et al. 2011; Laranjeira et al. 2011) Additionally, as ENSPC from both ganglionic and aganglionic gut behave the same and S100 is also expressed within ganglia it is more likely that glial cells are a source of ENSPC from both ganglionic and aganglionic HSCR gut. It may also be possibly that ENSPC in ganglionic gut are obtained from cells of Schwann cell lineage within the extrinsic fibres and not the ganglia.

### 5.6.3 The significance of thickened nerve trunks

It has been shown that thickened nerve trunks in aganglionic HSCR gut are blind ending extrinsic nerves but it is not clear why they are present in this gut.(Tam & Boyd 1990) In the central nervous system it has been found that blind ending nerves which fail to synapse atrophy instead of hypertrophy as in the aganglionic gut.(Hefti et al. 1986) It seems logical that the extrinsic nerves increase in size as they seek synapses with the ENS however this has not been shown and contrasts the behaviour of central neurons in similar circumstances.

### 5.6.4 Nerve fibres are present in muscular layers of ganglionic and aganglionic HSCR gut

Previous work has found, as expected, that nerve fibres are present within the muscular layers in non HSCR gut and ganglionic HSCR gut which appear to connect the two layers of the ENS, this was also seen in this study.(Kobayashi, O'Briain & Puri 1994b; Matsuda et al. 2006) In terms of the aganglionic gut there was disparity in the literature regarding the presence of nerve fibres in this tissue. One study reported the absence of these fibres within the muscular layers(Kobayashi, O'Briain & Puri 1994b) whereas another group observed the presence of these neurites in the aganglionic gut.(Matsuda et al. 2006) It was also found in this study that nerve fibres were present within the aganglionic gut in the muscular layers. These fibres are likely of extrinsic origin and if functional are likely responsible for the tonic smooth muscle contraction seen in HSCR. It is unclear as to whether these fibres are

identical in ganglionic and aganglionic gut however if they are it would seem that dysfunction seen in HSCR is solely due to the absence of enteric ganglia.

### 5.6.5 Further work with more extensive HSCR variants

Unfortunately tissue from HSCR affecting the whole of the colon or beyond was not available in our study. It has previously been reported that that interstitial cells of Cajal are present in the muscular layers of aganglionic gut in short segment HSCR however these cells are absent in the same layers of aganglionic total colonic HSCR gut.(Solari, Piotrowska & Puri 2003) It is likely from the findings in chapter 3 detailing the absence of neural crest derived cells amongst cells isolated from aganglionic total colonic HSCR gut that there is also an absence of neurites in the muscular layers in this gut too. If this hypothesis is true there is further evidence to support different aetiologies in the more extensive forms of HSCR. This is another area where further work is required.

## 5.7 Conclusion

Work in this chapter has added to a limited number of studies which have characterised aganglionic HSCR gut. Of particular importance, the presence of Schwann cells and neurons has been identified in the thickened nerve trunks which are of neural crest origin and support the hypothesis that cells of Schwann cell lineage are the source of ENSPC. There are still many unanswered questions surround the thickened nerve trunks including their development and function as well as new questions about the aetiology of the different extensities of HSCR.

# Chapter 6 – Overall discussion, areas for further work and conclusion

## 6.1 Overview

This final chapter brings the work carried out in this thesis together in one final discussion along with review of the aims of this thesis and new developments in the field are addressed. The key areas focussed on are the cellular origin of ENSPC in aganglionic gut, the appearance of the thickened nerve trunks and the aetiology of HSCR. Finally, areas for further work are identified and the clinical implications of work in this thesis when considering a stem cell therapy for HSCR are stated.

## 6.2 Introduction

As stated in chapter 1, the aim of work carried out in this thesis is to confirm and add understanding to the observation that ENSPC can be isolated from short and long segment aganglionic HSCR gut. Specifically, the objective has been to locate the cellular origin of ENSPC within aganglionic gut so more can be understood about how these cells can be controlled, predominantly for safety and efficacy reasons, before work can progress to human stem cell therapy trials. Not only has the original observation been confirmed, but it has also been shown that the ENSPC from aganglionic HSCR gut are neural crest derived and the findings in chapter 3 and chapter 5 suggest that these cells are located within thickened nerve trunks which are pathognomonic of the aganglionic gut in short and long segment disease.

In addition, chapter 2 identified that horse serum medium is favourable to neurosphere medium under adherent conditions for culturing neurospheres. In order to explore the possibility of transplantation of neurospheres in HSCR patients they must be first cultured, therefore horse serum medium was the only media used beyond chapter 2 of this thesis. Furthermore, horse serum medium contains fewer components than neurosphere medium, which has been used by our group previously.(Almond et al. 2007; Lindley et al. 2008; Lindley et al. 2009) This is likely to be advantageous if this work progresses to human trials, where adaption to serum free media is required for safety reasons, as there will be fewer components to replace.

## 6.3 The cellular source of ENSPC in aganglionic HSCR gut

Using samples of aganglionic HSCR gut from patients with all variants of HSCR it was possible to correlate the presence of thickened nerve trunks and the ability to obtain ENSPC, as aganglionic gut from total colonic and total intestinal doesn't contain thickened nerve trunks. However, only two patients with total colonic or total intestinal disease were sampled. Subsequently, using cell sorting it was also found that P75 positive neural crest derived cells obtained from aganglionic HSCR gut differentiate into neurons indicating that ENSPC are of neural crest origin, whereas the P75 negative cells did not do this.

In terms of the cellular origin of ENSPC, it has been shown that enteric glia can form functional neurons, *in vitro*, although the ability of these cells to generate neurons *in vivo* has been questioned.(Joseph et al. 2011; Laranjeira et al. 2011) Most interestingly, it has very recently been discovered that the progenitors of Schwann cells can also act as neuronal progenitors in the developing parasympathetic nervous system.(Kalcheim & Rohrer 2014) With this information highlighting glia and specifically the Schwann cell lineage as neuronal progenitors it is highly likely that Schwann cells located in thickened nerve trunks in aganglionic HSCR gut are the source of aganglionic ENSPC. If this is the case then further efforts in this area should look at the control of cells of Schwann cell lineage to differentiate into neurons which if discovered could open the doors to therapeutic treatment for HSCR.

## 6.4 Appearance of thickened nerve trunks

Thickened nerve trunks present in aganglionic gut of short and long segment HSCR are extrinsic, blind ending nerves originating from the pelvic plexus.(Tam & Boyd 1990) Another group described an intense ring of P75 neural crest cell marker expression surrounding these nerves along with weakened expression internally.(Kobayashi, O'Briain & Puri 1994b) This thickened ring corresponds to an observation that thickened nerve trunks have multiple layers of perineurium compared to other extrinsic nerves which have much fewer.(Baumgarten, Holstein & Stelzner 1973) It was found in this thesis that there was expression of the neuronal and glia markers, tyrosine hydroxylase and S100 respectively, from within the trunk where there was also expression of the neural crest marker P75. However, surrounding this core there is a ring of intense P75 expression which does not express any of the other markers used, including neuronal and glial markers. In normal extrinsic nerves the perineurium has been shown using lineage tracing to not be of NCC lineage, therefore what we and others have seen is not normal perineurium surrounding a thickened nerve trunk as normal perineurium does not express P75.(Joseph et al. 2004) The identity of the cells expressing P75 in this thickened ring is unclear. However, it is entirely possible that these cells are ENSPC as we show that neural crest derived cells from aganglionic HSCR gut differentiate into neurons.

Of particular interest, it was found that there are two populations of cells expressing P75 identified by FACS on the aganglionic sample whereas there was just one population in the ganglionic sample. In the aganglionic sample, one of populations expressed P75 with more intensity than the other, which may correspond to the cells of the ring of intense P75 expression seen in the thickened nerve trunks. In regards to this, there was no effort made to select only one of these populations using cell sorting and this is an area for further work in order to narrow down further the source of ENSPC in aganglionic gut.

Using S100 and TH antibodies glia and neurons were identified in the core of the thickened nerve trunks. As these nerve trunks are of extrinsic origin the glia identified are Schwann cells. This corresponds to an observation of thickened nerve trunks using electron microscopy which found Schwann cells in these trunks, but most importantly, on ultramorphology they were of immature appearance, similar to those found in the developing peripheral nervous system.(Baumgarten, Holstein & Stelzner 1973) This makes it more likely when combined with the finding that Schwann cells precursors act as neuronal progenitors in the developing parasympathetic nervous system that these Schwann cells are the source of ENSPC from aganglionic gut.(Kalcheim & Rohrer 2014)

## 6.5 Aetiology of HSCR

Work in this thesis has raised several questions about the aetiology of HSCR. It has been shown in small mammal studies that the HSCR phenotype occurs due to failure of migration of vagal NCC to the distal gut.(Obermayr et al. 2013) Work in this thesis suggests that one type of ENSPC are located within thickened nerve trunks in aganglionic HSCR gut however it is unknown whether these are ENCC or other neural crest derived cells such as Schwann cells. There is evidence to suggest that the sacral contribution of NCC to the ENS travel into the gut via the extrinsic fibres.(Erickson et al. 2012) If these cells fail to differentiate and remain as progenitors then it is entirely possible that these are the ENSPC we are able to obtain from aganglionic HSCR gut. If this is the case, then the aetiology of HSCR may include failure of proliferation of sacral NCC with or without failure of migration too.

Although it is thought extrinsic nerves hypertrophy as a response to failing to synapse it is unclear why thickened nerve trunks are present in aganglionic short and long segment HSCR gut.(Hefti et al. 1986) Most interestingly it must be considered why these trunks are absent in aganglionic total colonic and total intestinal HSCR gut. This would suggest that the aetiology of the more extensive disease differs from short and long segment disease. It is possible that the more extensive forms are the result of more extensive NCC disease as the ENS and extrinsic nerves in the gut have failed to form. On the other hand total colonic and

total intestinal HSCR may be due to disease of smooth muscle in the gut which is unfavourable to neuronal development as both nervous systems are affected. It is already known that short segment HSCR can be caused by an extrinsic defect consisting of failure of the EDN3-EDNRB signalling pathway as EDN3 is not produced by gut mesenchyme.(Obermayr et al. 2013) This theory is supported by an observation that interstitial cells of Cajal are absent in aganglionic gut in total colonic and total intestinal HSCR but present in the aganglionic gut in short and long segment disease suggesting abnormalities in the smooth muscle in the more extensive forms of HSCR.(Solari, Piotrowska & Puri 2003)

## 6.6 Final conclusion

In order to develop a stem cell therapy for HSCR the cellular identity of ENSPC obtainable from aganglionic gut needs to be established for safety and efficacy reasons. Results in this thesis showing that ENSPC are of neural crest origin located within thickened nerve trunks, combined with evidence showing that Schwann cells have an ability to act as neuronal precursors, makes it likely that cells of Schwann cell lineage are the ENSPC we have isolated from aganglionic gut.(Joseph et al. 2011; Kalcheim & Rohrer 2014; Laranjeira et al. 2011) Other neural crest derived cells expressing P75 in thickened nerve trunks are neurons, which can be ruled out as ENSPC as only axons and not cell bodies are present in extrinsic nerves, and abnormal surrounding perineurial cells which may have a role however little is known about this abnormal tissue.

Now that it has been established that ENSPC are located within aganglionic gut in short and long segment HSCR there are two possibilities in terms of translating this work to human HSCR therapy. Firstly, aganglionic gut obtained at pull through procedure could be used to obtain ENSPC. Aganglionic neurospheres containing ENSPC and their differentiated neurons and glia can then be cultured and implanted into the remaining aganglionic internal anal sphincter which is thought to cause the long term complications after HSCR surgery. It is then likely that cells within neurospheres will proliferate and differentiate to form a functional ENS and therefore a functional sphincter. There is a risk with any stem cell therapy that uncontrolled proliferation of immature cells will result in tumour formation, this risk could be minimalised if cell sorting provided a population of mature neurons and glia that form neurospheres without the addition of immature ENSPC too. However, if the endogenous ENSPC in the thickened nerve trunks of short and long segment HSCR aganglionic gut can be stimulated to proliferate and differentiate in situ then it possible that HSCR patients can avoid surgery and undergo medical treatment to stimulate development of a functional ENS.

## 6.7 Future work

### 6.7.1 Are cells of Schwann cell lineage the source of ENSPC in aganglionic HSCR gut?

To explore this cell sorting could be used with similar methodology as used in chapter 4. However, if a surface receptor glial marker is used which is similar to S100 then an isolated sub-population of Schwann cells could be characterised after time in culture and the expression of mature neuronal markers could be searched for. This would give a definitive answer regarding the cellular origin of ENSPC in aganglionic gut. The possible barriers to undertaking this work is the challenge of finding a cell surface receptor marker that can be used with FACS successfully and the challenges of culturing a small yield of primary cells that is inevitable from such a minimal cell sub-population.

### 6.7.2 Which chemical factors stimulate the differentiation of ENSPC?

In order to identify therapeutic agents which may have the potential to stimulate the development of an ENS in aganglionic gut *in vivo* a variety of different agents must be tested *in vitro*. There are some agents that seem more likely including those which stimulate $5HT_4$ receptor activation, which has been shown to allow enteric neural regeneration in guinea pigs.(Matsuyoshi et al. 2010) Additionally, inhibition of the Notch pathway in human ENSPC neurospheres has been shown to decrease ENSPC proliferation whilst increasing differentiation *in vitro*.(Theocharatos

et al. 2013) Nevertheless, to comprehensively undertake this work high throughput screening may be required using a library of chemical agents. This has been well documented in the stem cell field and would efficiently identify chemicals which can be further tested and potentially trialled in mouse models of HSCR.(Gupta et al. 2009)

### 6.7.3 Suicide gene incorporation into ENSPC and other cells within neurospheres

A key concern with any stem cell therapy is the risk of losing control of a cell type after implantation resulting in tumour formation.(Wilkinson, Edgar & Kenny 2012) In order to reduce the risk to patients of treatment for what is a benign disease there should be a method to control cells post implantation. To achieve this a suicide gene could be incorporated into the DNA of all cells before implantation which would induce apoptosis by production of the p53 protein for example.(Malecki 2012) If there is any concern about uncontrolled cell proliferation a known therapeutic agent which activates the gene can be administered. To develop this technique, *in vitro* experimentation is required. A potential problem with this idea is, genetic changes in cancerous cells may reduce or abolish the effectiveness of the suicide gene. In terms of the method itself careful consideration is required to ensure that all implanted cells within a neurosphere are incorporated with the gene.

### 6.7.4 Implantation of human aganglionic neurospheres into a mouse model of HSCR

It has been shown that florescence labelled mouse enteric neurospheres can be transplanted into postnatal mouse gut which then form ganglia and display similar electrophysiological functional characteristics to ENS ganglia.(Hotta et al. 2013) However, to our knowledge, there have been no studies with a mouse model of HSCR such as $EDNRB^{-/-}$ mice and transplantation of enteric neurospheres to assess the ability to restore normal functionality in aganglionic gut.(Erickson et al. 2012) In particular, aganglionic human neurospheres should be transplanted into the aganglionic mouse gut and observation should take place short term, to test the efficacy of the treatment and long term, to identify any signs of tumour formation. If a stem cell therapy for HSCR is shown to be safe and efficacious in mice then the logical progression will be to human clinical trials.

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# Appendix 1 – Patient information sheets

# Information for Parents of children with Hirschsprung's disease

Your child is being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.
Thank you for reading this.

## Study Title

Enteric Nervous System Stem Cell Behaviour in Mature Muscle Models

## What is the purpose of the study?

Your child has Hirschsprung's disease. In this condition nerve cells are missing from the rectum. Current treatment is removal of the affected segment of bowel but even after this many children have problems with constipation or soiling. We hope to understand how to grow nerve cells to allow us to replace them, so as to improve the treatment for children with Hirschsprung's disease in future.

## Why has your child been chosen?

Because your child is going to have an operation involving removal of a section of bowel. This would normally be disposed of at the end of the operation but we would like to use a small piece of this tissue to study the nerves and muscle within it.

## Does your child have to take part?

No. It is up to you to decide whether or not you wish your child to take part. If you do decide that your child may take part you will be given this information sheet to keep and be asked to sign a consent form. Should you not want us to use the specimen in this way, there will be no effect on the way in which we treat your child. At the end of our tests, the specimen will be disposed of in the normal way.

## What will happen to my child if I do take part?

Your child is going to have an operation in which surgery is performed on the bowel. During this surgery a small amount of bowel tissue will be removed and routinely sent for tests. We would like you to help us by letting us use two small pieces of this tissue (approximately two square centimetres) to grow the nerve cells and muscle cells that are contained in the bowel wall.

No extra tissue would be removed and this procedure would not affect our ability to do the routine tests on the bowel segment. It will have no effect on your child.

## What are the possible disadvantages and risks of taking part?
There are no additional disadvantages or risks of taking part.

## What are the possible benefits to my child of taking part?
It is likely that the main benefits of your child taking part will be to future children born with Hirschsprung's disease rather than you directly. However, the information that we discover may have direct benefits to your child should we be able to isolate stem cells, as in future it may be possible to transplant them.

## Will my child's participation in this study be kept confidential?
Yes. Your child's name will not be disclosed outside of the hospital.

## What will happen to the results of the study?
We will publish the results of this work in medical and scientific journals and if you would like to know our eventual results please inform us.

## Who is organising and funding the research?
This study is organised and run by Mr Simon Kenny, Consultant Paediatric Surgeon, Alder Hey Children's Hospital, Liverpool and Mr David Wilkinson, Clinical Research Fellow, University of Liverpool. The research has been kindly funded by the British Association of Paediatric Surgeons and the Royal College of Surgeons of England.

## Who has reviewed the study?
This study has been reviewed by a panel of experts, on behalf of the British Association of Paediatric Surgeons and the Royal College of Surgeons of England, who believe that it is of the highest standard. It has also been approved by the Liverpool Research Ethics Committee.

## Contact for further information:

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L69 3GE

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If you have any complaints or concerns about your child's involvement you can contact the Patient Advice Liason Service (PALS):
PALS Office
Alder Hey Children's NHS Foundation Trust
Eaton Road
Liverpool
L12 2AP

Tel: 0151 252 5374/5161 Email: PALS@alderhey.nhs.uk

# Information for Parents of children without Hirschsprung's disease

Your child is being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

## What is the purpose of the study?

Some children have a condition called Hirschsprung's disease. In this condition nerve cells are missing from part of the bowel. Current treatment is removal of the affected segment of bowel but even after this many children have problems with constipation or soiling. We hope to understand how to grow nerve cells to allow us to replace them, so as to improve the treatment for children with Hirschsprung's disease in future.

## Why has your child been chosen?

Because your child is going to have an operation involving removal of a section of bowel. This would normally be disposed of at the end of the operation but we would like to use a small piece of this tissue to study the nerves and muscle within it.

## Does my child have to take part?

No. It is up to you to decide whether or not you wish your child to take part. If you do decide that your child may take part you will be given this information sheet to keep and be asked to sign a consent form. Should you not want us to use the specimen in this way, there will be no effect on the way in which we treat your child. At the end of our tests, the specimen will be disposed of in the normal way.

## What will happen to my child if they do take part?

Your child is going to have an operation in which surgery is performed on the bowel. During this surgery a small amount of bowel tissue will be removed and routinely sent for tests. We would like you to help us by letting us use two small pieces of this tissue (approximately two square centimetres) to grow the nerve cells and muscle cells that are contained in the bowel wall.

No extra tissue would be removed and this procedure would not affect our ability to do the routine tests on the bowel segment. It will have no effect on your child.

## What are the possible disadvantages and risks of taking part?

There are no additional disadvantages or risks of taking part.

## What are the possible benefits of taking part?

There will no direct benefits to your child in taking part other than the knowledge that they are helping others.

## Will my child's participation in this study be kept confidential?

Yes. Your child's name will not be disclosed outside of the hospital.

## What will happen to the results of the study?

We will publish the results of this work in medical and scientific journals and if you would like to know our eventual results please inform us.

## Who is organising and funding the research?

This study is organised and run by Mr Simon Kenny, Consultant Paediatric Surgeon, Alder Hey Children's Hospital, Liverpool and Mr David Wilkinson, Clinical Research Fellow, University of Liverpool. The research has been kindly funded by the British Association of Paediatric Surgeons and the Royal College of Surgeons of England.

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If you have any complaints or concerns about your child's involvement you can contact the Patient Advice Liason Service (PALS):

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