Characteristics of Enteric Neural Crest Stem Cells and their Therapeutic Potential on Hirschsprung's Disease

BAO, Lihua

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy
in
Anatomy

The Chinese University of Hong Kong
August 2010

Thesis/Assessment Committee

Professor CHO Yu Peng (Chair)

Professor CHAN Wood Yee (Thesis Supervisor)

Professor NG Tzi Bun (Committee Member)

Professor YUNG Ken Kin Lam (External Examiner)

Abstract

Abstract of thesis entitled:
Characteristics of Enteric Neural Crest Stem Cells and their Therapeutic Potential on Hirschsprung's Disease
Submitted by **BAO LIHUA**
For the degree of Doctor in Philosophy
At The Chinese University of Hong Kong in July, 2010

Hirschsprung's disease (HSCR) is caused by the absence of the enteric neural crest-derived neurons at the distal region of the gut. Cell-based therapy using stem cells or progenitors gives the potential to supplement these missing enteric neurons in the gut. Enteric neural crest stem cells isolated from the human or rodent gut can give rise to neurons and glia after they are transplanted into the recipient guts of the mouse or rat. However, numbers of issues are unresolved about the basic biology of the enteric nervous system, the characteristics of the stem cells isolated from the enteric nervous system and the biological significance of these cells in prenatal and postnatal periods. In this study, the characteristics and therapeutic potential on HSCR of the enteric neural crest stem cells were explored.

For the purpose of developing an effective therapeutic strategy for HSCR, the enteric neural crest stem cells were investigated firstly which were isolated from the E14.5 mouse embryonic gut, cultured as neurospheres and characterized by multiple immunofluorescence and reverse transcription-PCR, population doubling time, frequency of forming secondary neurospheres and limited dilution assay. In the differentiation culture medium, several types of cells were induced to form from the neurospheres derived from single cells. Hence the putative enteric neural crest stem cells, which were isolated from the embryonic mouse gut tube and cultured as neurospheres for many passages *ex vivo* with the demonstrated capacity of proliferation, self-renewal and differentiation, showed properties of stem cells.

Their potential applications in the transplantation experiments were shown by transplantation of the neurospheres isolated to the gut tube maintained in an

organotypic culture or to the descending colon of neonates at postnatal day 7. The development of the enteric neural crest stem cells from the neurospheres was found to be compatible to endogenous enteric neural crest-derived cells in the recipient gut as evidenced by the formation of interconnected cellular networks of donor stem cells and endogenous neural crest-derived cells. The enteric neural crest stem cells also possess the potential to compensate the loss of enteric neural crest-derived cells *ex vivo* and *in vivo* in recipient prenatal and postnatal guts.

In addition to the above, a recombination organotypic gut culture *ex vivo* showed that the colonization of enteric neural crest-derived cells in the recipient gut was influenced not only by the genotypes of enteric neural crest-derived cells themselves but also the microenvironment of the gut through which enteric neural crest-derived cells migrated. For instance, the developmental stage of the recipient gut and also the presence of endogenous enteric neural crest-derived cells along the migratory pathway of neural crest-derived cells both affected the extent of the migration and colonization of exogenous enteric neural crest-derived cells and stem cells. The gradual maturation and differentiation of the neighboring structures, such as the smooth muscle layer, during the time period of the enteric neural crest cells migration, might also suggest that these neighboring tissues may have a role in regulating the neural crest-derived cells migration.

In conclusion, enteric neural crest stem cells isolated from the embryonic mouse gut tube showed properties of stem cells, and had the potential to compensate missing enteric neural crest-derived cells both *ex vivo* and *in vivo*. However, the colonization of enteric neural crest-derived cells in the developing gut was affected cell-autonomously and also by the microenvironment of the gut and the presence of existing enteric neural crest-derived cells.

摘要

題目名稱：腸神經脊幹細胞的特性及其治療先天性巨結腸的應用潛能

暴麗華與二零一零年七月在香港中文大學呈交博士論文

先天性巨結腸是由於結腸腸壁內缺乏神經脊來源的腸神經細胞而引起的消化系統發育畸形。利用幹（祖）細胞替代療法為補償缺失的腸神經元帶來希望。從嚙齒目動物或人腸壁內分離得到的腸神經脊幹細胞移植入大鼠或小鼠腸壁後可以提供腸神經元和神經膠質細胞。但是腸神經系統以及從腸神經系統分離得到的幹細胞的特性，幹細胞移植入出生前後腸壁後的生物學特徵尚未完全清楚。本實驗的目的是進一步明確腸神經脊幹細胞的特性以及其在治療先天性巨結腸症中的應用潛能。

本實驗首先研究分離培養得到的腸神經脊幹細胞的特性。從胚齡14.5天的小鼠腸內分離培養神經球樣的腸神經脊幹細胞，通過細胞免疫化學染色，RT-PCR，細胞倍增時間，次代神經球形成率以及誘導細胞分化等方法驗證這些細胞具有多次傳代並始終保持增殖和分化能力的幹細胞特性，是腸神經脊幹細胞。

移植細胞於胚胎腸壁內的體外實驗和通過剖腹術將細胞移植于新生小鼠降結腸的體外實驗，表明外源性的腸神經脊幹細胞能在出生前和出生後的受體腸內補充缺失的腸神經細胞，並與已存在的內源性的腸神經脊源性的細胞相容形成相互連接的細胞網路。

除此之外，腸段連接並體外培養實驗發現腸神經脊源性的細胞在受體腸段內遷移不僅受腸神經脊源性細胞自身的基因型影響而且與移行的腸神經脊源性細胞的周圍微環境有關，如不同發育階段的受體腸段及在遷移中遇到已存在的內源性腸神經脊源性細胞都將影響外源性腸神

經脊源性的細胞及幹細胞的遷移。腸神經脊源性的細胞周圍逐漸成熟並分化的的結構如平滑肌細胞的發育，提示周圍組織可能影響腸神經脊源性的細胞遷移。

綜上所述，從胚胎小鼠腸內分離培養得到腸神經脊幹細胞具有幹細胞特性，能夠補充缺失的腸神經脊源性的細胞。而腸神經脊源性的細胞在腸內的遷移受細胞自身特性、細胞遷移所在的微環境以及已存在的腸神經脊源性的細胞的影響。

Acknowledgements

I would like to express my gratitude to all those who helped me during the writing of this thesis.

First of all, I would like to extend my sincere gratitude to my supervisor, Prof. Wood Yee CHAN, for his patient instruction, insightful criticism, expert guidance, invaluable suggestion and encouragement. Without his consistent and illuminating instruction, this thesis could not have reached its present form. His persistence and unfeigned interest on the research and perfect presentation skill will benefit all my life. I also thank him for encouraging and supporting me to attend many international and local conferences, which undoubted extend my knowledge and experience.

Secondly, I would like to express my heartfelt gratitude to Dr. Alex CHEUNG and Dr. WANG Xia for their advices and discussion on my project. My sincere thanks are also given to Ms. Typhoon and Ms. Gigi for their great technical assistance and help. I also owe much to all my labmates Ms. SHANG Na, Ms. CHEN Jielin, Ms. LI Mi and Dr. Emily TSANG for giving me this lucky unforgettable experience.

I am also great indebted to Prof. Sun On CHAN, Prof. Yu Peng CHO and Prof. ZHAO Hui and all labmates in their labs for the discussion and communication in the journal clubs.

My deepest gratitude goes to Ms. Jenny HOU, Ms. Corinna AU, and Ms. Jean KUNG for their kindly instructions and assistance. I would like to thank all the staff and colleagues in the School of Biomedical Sciences, the Chinese University of Hong Kong, for their helps and supports.

Last my thanks would go to my beloved family for their loving considerations and great confidence in me and supporting me without a word of complaint all through these years.

Table of Contents

Abbreviations

CAMs	**cell adhesion molecules**
CNS	**central nervous system**
Dom	**Dominant megacolon**
ECM	**extracellular matrix**
ENCSCs	**enteric neural crest stem cells**
ENS	**enteric nervous system**
GFP	**green fluorescent protein**
HSC	**hematopoietic stem cell**
HSCR	**Hirschsprung's disease**
iPS cells	**induced pluripotent stem cells**
MP	**myenteric plexus**
NC	**neural crest**
NCCs	**neural crest cells**
NCSCs	**neural crest stem cells**
PFA	**paraformaldehyde**
PNS	**peripheral nervous system**
SMP	**submucosal plexus**
SVZ	**subventricular zone**

Chapter one

General Introduction

1.1 Stem cells

Since the beginning of stem cell research in mid 1800's, more and more researchers and institutes have made efforts to explore the stem cells using varieties of techniques. In the last decade, a remarkable number of papers have been published in this fast-paced field. Particularly the discovery of induced pluripotent stem (iPS) cells in 2006 (Takahashi and Yamanaka, 2006) added more excitements and also hopes for much more wider applications of stem cells in the future. Stem cells with properties of expanding *ex vivo* and giving rise to cells of the three germ layers make them promising tools for disease modeling, studies in toxicology and pharmacology and stem cell–targeted therapies.

1.1.1 History of stem cell research

The history of stem cell research had a humble beginning in the mid 1800's when scientists began to try to fertilize eggs from mammals in a laboratory setting. The first attempt was documented in the year of 1878. At that time, scientists were experimenting only on animals but not humans. Now stem cell research is embroiled in a controversy over the use of human embryonic stem cells for research (Viegas, 2003).

In 1945 the first real stem cells were discovered when it was found that some cells generated blood cells (Owen, 1945). After a French medical researcher, Jean Dausset,

made a critical medical discovery about the human immune system – the first human histocompatibility antigen in 1958, the bone marrow transplant in humans was attempted on a larger scale (Mathe, 1968). It was not until the 1960's that physicians knew enough about HLA (human leukocyte antigen) compatibility to perform transplants between siblings who were not identical twins (Kerry *et al.*, 2004a). In the year of 2002 the number of blood and marrow transplants has increased to at least 40,000 in patients with lethal human diseases (Kerry *et al.*, 2004a) according to the IBMTR/ABMTR (International Bone Marrow Transplant Registry/Autologous Blood and Marrow Transplant Registry). In 2006, a total 50,417 first hematopoietic stem cell transplantations were reported including 21,516 allogeneic (46%) and 28,901 autologous (57%) trials (Gratwohl *et al.*, 2010). Adult stem cells also have shown great promises in other areas. These cells have shown the potential to form many different kinds of cell types and tissues, including functional hepatocyte-like (liver) cells (Schwartz *et al.*, 2002). Such cells might be useful in repairing organs ravaged by diseases.

In 1998, James Thomson at University of Wisconsin isolated human embryonic cells from the inner cell mass of early embryos-human blastocysts, and developed the first embryonic stem (ES) cell lines (Thomson *et al.*, 1998). In the same year, John Gearhart of Johns Hopkins University derived germ cells from cells in fetal gonadal tissues (primordial germ cells) (Shamblott *et al.*, 1998).

Pluripotent stem cells have provided high hopes for cell replacement therapies because of their ability to self-renew and their potential to form all cell lineages in the body (Thomson *et al.*, 1998). Human embryos are the main source for producing human pluripotent stem cells that are genetically unmodified so far. There are alternative methods for producing pluripotent stem cells, such as fusion of fibroblasts

with embryonic stem cells (Cowan *et al.*, 2005; Tada *et al.*, 2001) or somatic cell nuclear transfer which involves replacing the genetic material of unfertilized or newly fertilized eggs with that from an adult cell of a patient and then forcing the cell to divide to create an early-stage embryo (Egli *et al.*, 2007; Wakayama *et al.*, 2001). However the therapeutic application of either approach has been experiencing both ethical and technical difficulties (Jaenisch, 2004). Reprogramming human somatic cells into induced pluripotent stem (iPS) cells without the need of embryos or eggs will solve the technical and ethical problems.

Induced pluripotent stem cells were first generated from mouse cells by introducing four factors---*Oct3/4, Sox2, c-Myc* and *Klf4* by Shinya Yamanaka's team at Kyoto University in 2006 (Takahashi and Yamanaka, 2006). In 2007 with the same factors: *OCT3/4, SOX2, KLF4* and *C-MYC*, adult human dermal fibroblasts were reprogrammed into iPS cells by the same team (Takahashi *et al.*, 2007). Meanwhile another grouping of four factors: *OCT4*, *SOX2*, *NANOG* and *LIN28* were also sufficient to reprogram human somatic cells to pluripotent stem cells that exhibited the essential characteristics of embryonic stem cells (Yu *et al.*, 2007).

The above described is a milestone in creating iPS from adult human cells, but the created cells might be prone to form tumors. To overcome the danger caused by viral transfection, Konrad Hochedlinger and his Harvard University research team successfully used non-integrating adenoviruses transiently expressing OCT4, SOX2, KLF4, and C-MYC in the skin and liver cells of mice, resulting in cells identical to embryonic stem cells (Stadtfeld *et al.*, 2008). Yamanaka demonstrated that reprogramming could be accomplished via plasmid without any virus transfection system at all, although at very low efficiencies (Okita *et al.*, 2008). In May 2009, a team of scientists generated human iPS cells by direct delivery of proteins, thus

eliminating the need for viruses or genetic modification (Kim *et al.*, 2009). The group of Sheng Ding in La Jolla, California, had also shown that the generation of mouse iPS cells was possible using a similar methodology: a repeated treatment of the cells with certain proteins channeled into the cells via poly-arginine anchors was sufficient to induce pluripotency (Zhou *et al.*, 2009). Expression of induced pluripotency genes could also be increased by treating somatic cells with FGF2 under low oxygen conditions (Page *et al.*, 2009).

1.1.2 What are stem cells?

To precisely define the term stem cells has been a difficult task for researchers. The earliest stem cells recognized experimentally in the blood showed that the multi-lineage reconstitution by a self-renewing cell was a cardinal feature of a stem cell. Some stem cells have a greater capacity of self-renewal and multi-lineage differentiation than others. Smith (2001) adopted this definition in an article in the Annual Review of Cell Development Biology: "Stem cells are defined functionally as cells that have the capacity to self-renew as well as the ability to generate differentiated cells" (Kelly, 2007).

Because of their relative rarity and lack of definitive markers, stem cells have traditionally been characterized on the basis of functional criteria. This characterization is dependent on the putative stem cells acting or performing in a manner that can be measured so as to recognize their existence and quantify their behavior under different experimental paradigms. Thus, the discovery of a population of stem cells in a given tissue is usually contingent on the development of *ex vivo* culture conditions enabling a rigorous characterization. To meet these criteria, a putative stem cell, in culture, must demonstrate the ability to (1) proliferate, (2)

self-renew over an extended period of time, and (3) generate a large number of progeny that can differentiate into the primary cell types of the tissue from which it is obtained (Potten and Loeffler, 1990).

Stem cells can be classified into four broad categories, based on their plasticity or how versatile they may be in their development (Kelly, 2007):

1. Totipotent stem cells are found only in early embryo, zygote, and descendants of the first two cell divisions. Each cell can form a complete organism (e.g., identical twins) as well as trophoblastic cells of the placenta.

2. Pluripotent stem cells exist in the undifferentiated inner cell mass of the blastocyst and can form any of the over 200 different cell types arising from the three germ layers found in the body.

3. Multipotent stem cells are derived from fetal tissues, cord blood, and adult stem cells and classified according to their ability to differentiate into limited types of cells depending on their location. Although their ability to differentiate is more limited than pluripotent stem cells, they already have a good record of success in cell-based therapies.

4. Unipotent stem cells can produce only a single cell lineage. Unipotent stem cell found in adult tissues such as skin stem cells and spermatogenic stem cells which provide functional and structural components to a body tissue or organ. Comparing with other types of stem cells, unipotent stem cells have the lowest differentiation potential but still have self-renewal property (http://www.stemcellresources.org/celltalk_lesson_2.html).

Stem cells may also be classified according to their origin such as embryonic stem cells (ESCs), embryonic germ stem cells (EGSCs), and adult stem cells (or somatic stem cells) (Kelly, 2007). Embryonic stem cells are isolated from the inner

cell mass of the blastocyst, while embryonic germ stem cells are similar to embryonic stem cells except they are collected from the fetus later in development. The cells come from a region known as the gonadal ridge which will later develop into the sex organs. Because the cells are farther along in the development process, they are slightly limited in their ability to give rise to other organs of the body. Adult stem cells (somatic stem cells) originate in a mature organism and help maintain and repair the tissues in which they are found.

1.1.3 Progenitor cells

Like stem cells, progenitor cells have a capacity to differentiate into a specific type of cells. In contrast to stem cells, however, they are already far more specific: they are pushed to differentiate into their "target" cells. The most important difference between stem cells and progenitor cells is that stem cells can replicate indefinitely, whereas progenitor cells can only self-renew for a limited number of cycles and often unipotent, meaning that, progenitor cells would exhibit limited proliferative capacity when compared with stem cells.

Nonetheless, controversy remains because embryonic stem cells are true stem cells in that they are pluripotent and show unlimited capacity for self-renewal. In contrast, many cells termed adult stem cells which are undifferentiated cells located in a differentiated tissue or organ would be better defined as progenitor cells, as their capacities for unlimited self-renewal and plasticity have not been comprehensively demonstrated. The concept is still evolving because new evidence has suggested that postnatal stem cells may not be limited to the narrow range of differentiation as previously thought. For example, hematopoietic stem cells may be able to transdifferentiate into non-blood lineages such as cardiomyocytes (Orlic *et al.*, 2001).

Type-C amplifying progenitors of the subventricular zone (SVZ) had function as stem cells *ex vivo*, after exposure to high concentrations of EGF (Doetsch *et al.*, 2002). This shows that progenitor cells retain stem-cell properties. Also, manipulations in the culture of differentiated or partially differentiated neural cells could induce dedifferentiation into a stem-cell state (Brewer, 1999; Kondo and Raff, 2000; Raff *et al.*, 1983). Despite the difficulty in defining progenitors, the term is frequently used in research (Seaberg and van der Kooy, 2003) (See also http://en.wikipedia.org/wiki/Progenitor_cell).

1.1.4 Stem cell niche

The molecular mechanisms governing stem cells fate are complex. *In vivo*, the stem cell niche creates a specialized microenvironment that regulates stem cell survival, self-renewal and differentiation (Discher *et al.*, 2009). The niche consists of soluble and surface-bound signalling factors, cell-cell contacts, stem cell niche support cells, extracellular matrix (ECM) and local mechanical microenvironment (Peerani and Zandstra, 2010). These components are combined with systemic (Carlson *et al.*, 2008; Conboy *et al.*, 2005) and neural (Katayama *et al.*, 2006; Spiegel *et al.*, 2008) inputs that either directly or indirectly regulate physio-chemical cues, such as oxygen, metabolites, and hormones (Peerani and Zandstra, 2010).

The stem cell niche, often represented as a static microenvironment into which stem and progenitor cells are nested, is in fact spatially and temporally dynamic, actively integrating long-term developmental signals with short-term cyclic and injury-mediated regenerative responses (Johnston, 2009).

Prospective alteration of the properties of stem cell niches may provide new therapeutic strategies by interrupting disease processes or accelerating regenerative

healing.

1.1.5 Applications

Stem cells including ES, iPS and other somatic stem cells can provide useful platforms for disease modeling, toxicology and pharmacology studies and stem cell–targeted therapies.

1. Disease modeling

Using iPS cells from patients of certain disease to generate tissue or cells carrying disordered phenotype as a unique platform, scientists are able to study the disease mechanisms and potential treatment strategy for this disease. For example, related studies on spinal muscular atrophy (SMA) model from patient's iPS cells have been ongoing now (Ebert *et al.*, 2009).

2. Toxicology and pharmacology studies

The currently available cellular models for preclinical testing consist of primary and immortalized cells. These cellular models display limitations in terms of available amount and similarities to their *in vivo* counterparts, respectively. Human ES from the patients may provide a physiological model for any human cell types. Revolutionized drug discovery has potential to be done in desired amounts for earliest steps of drug development, notably for pharmacological, metabolic and toxicity evaluation (Kiskinis and Eggan, 2010). This new generation of model may contribute to reduce, refine or replace animal testing and decrease drug attrition.

3. Stem cell–targeted therapies

With the advancement on the technique and knowledge of stem cells, preclinical and clinical research based on stem cell–targeted therapies is underway for many diseases, including cancers, heart failure, neural degenerative diseases, diabetes,

autoimmune disorders and some inherited genetic hematological disorders such as congenital immunodeficiency disorders – hemoglobinopathies (Lindvall and Kokaia, 2010; Muench, 2005; Nagy *et al.*, 2005).

1.2 Enteric nervous system

Enteric nervous system (ENS) is the largest and the most complex part of the peripheral nervous system (PNS) (Gershon and Erde, 1981). It is composed of an integrative neural network organized mainly in two plexuses. One is located in the myenteric region between the longitudinal and circular muscle layers called myenteric plexus (MP) or Auerbach's plexus, and another lies within the submucosa on the luminal side of the circular muscle named submucosal plexus (SMP) or Meissner's plexus.

These plexuses contain as many neurons as the spinal cord and the functional and chemical diversity of enteric neurons resembles that of the central nervous system (CNS) (Benarroch, 2007). Although functions of the gut are clearly influenced by the output of central nervous system nuclei via the sympathetic and parasympathetic branches of the autonomic nervous system, basic functions of the gut such as peristalsis, secretion and blood flow are primarily regulated by the intrinsic network of the enteric ganglia. Therefore, the enteric nervous system has also been named a second brain (Gershon, 1999). About 80–100 million enteric neurons can be classified into functionally distinct subpopulations, including intrinsic primary neurons, interneurons, motor neurons, secretomotor and vasomotor neurons (Furness *et al.*, 2003).

1.3 Hirschsprung's disease

Harald Hirschsprung, a Danish pediatrician, presented the well-known and concise description of congenital megacolon through two unrelated boys who died from chronic severe constipation with abdominal distension resulting in congenital megacolon at the Society of Pediatrics in Berlin in 1886 and published his presentation in 1888 (Amiel *et al.*, 2008; Hirschsprung, 1888; Holschneider and Puri, 2008). In the 1940s, the cause of this disorder was found related to the absence of intramural ganglion cells of the myenteric (Auerbach) and submucosal (Meissner) plexuses downstream of the dilated part of the colon (Whitehouse and Kernohan, 1948). Later, a simple and reliable diagnostic confirmation from rectal suction biopsies was established using histochemical staining for acetylcholinesterase (AchE) (Meier-Ruge *et al.*, 1972). In 1948, Ovar Swenson and Bill performed a successful life-saving proximal colostomy that relieved obstructive symptoms (Swenson, 1996). The first comprehensive description of the pathophysiology, clinical symptoms, diagnosis and therapy of Hirschsprung's disease was outlined in 1970 by Theodor Ehrenpreis, Professor of Pediatric Surgery at the Karolinska Institute of Sweden, in a booklet entitled "Hirschsprung's Disease".

In 1974, the term neurocristopathy was proposed by Bolande for syndromes or tumors involving neural crest cells (Bolande, 1974). Hirschsprung's Disease resulted from an anomaly of the enteric nervous system derived from the neural crest is therefore regarded as a neurocristopathy (Bolande, 1974; Iwashita *et al.*, 2003; Le Douarin and Kalcheim, 1999).

1.3.1 Definition

Hirschsprung's disease (HSCR, known as congenital aganglionic megacolon) is a congenital malformation characterized by the congenital absence of intrinsic ganglion cells in the submucosal (Meissner) and myenteric (Auerbach) plexuses of the gastrointestinal tract (Whitehouse and Kernohan, 1948). Up to date, the cause of HSCR is regarded as the failure of the cranial-caudal migration of vagal neural crest-derived cells in the hindgut between the 5^{th} and 12^{th} week of gestation to form the enteric nervous system and is therefore regarded as a neurocristopathy (Bolande, 1974; Taraviras and Pachnis, 1999).

1.3.2 Epidemiology

The incidence of HSCR is estimated at 1/5000 newborns. However, the incidence varies significantly among ethnic groups (1.0, 1.5, 2.1, and 2.8 per 10000 live births in Hispanics, Caucasian-Americans, African-Americans, and Asians, respectively) (Amiel *et al.*, 2008; Torfs, 1998). There is also a sex bias with a preponderance of affected males and a sex ratio of 4/1 in males and females (Badner *et al.*, 1990).

1.3.3 Etiology

With increasing numbers of studies on the enteric nervous system, increasing numbers of genes, molecules and signalling pathways have been found and identified interfering with the enteric nervous system development. RET/GDNF/GFRα1 signalling pathway, EDN3-EDNRB signalling pathway, molecules such as transcription factors *Phox2b*, *Sox10*, *Pax3*, *Mash1*, and *Hox11L1* (Newgreen and Young, 2002a), molecules such as SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptor) proteins (Vohra *et al.*, 2006), β-integrin

(Nagy *et al.*, 2009), BBS (Bardet-Biedl syndrome) proteins (Tobin *et al.*, 2008), electrical activity, neurotransmitter release (Hao *et al.*, 2010) and the development of the vascular endothelium in the gut (Nagy *et al.*, 2009) all have effects on the enteric nervous system development (more will be discussed in the Section 3.1.2 of Chapter 3). Deletion or mutation of one or more of these genes or affecting their normal expression and function may interfere with the enteric nervous system development and cause HSCR-like pathologies.

1.3.4 Clinical features and diagnosis

Symptoms of Hirschsprung's disease usually show up in very young children, but sometimes they do not appear until the patient becomes a teenager or an adult. The symptoms are a little different at different ages.

In most cases, the diagnosis of HSCR is made in the newborn period (Torfs, 1998) due to intestinal obstruction with the following features:

(1) delayed passage of meconium (>24 h after birth);

(2) abdominal distension that is relieved by rectal stimulation or enemas;

(3) vomiting; and

(4) neonatal enterocolitis.

Some patients are diagnosed later in infancy or in adulthood with severe constipation, chronic abdominal distension, vomiting and failure to thrive (Amiel *et al.*, 2008; Parc *et al.*, 1984).

According to the length of the aganglionic segment, the patients are diagnosed with the short-segment form (S-HSCR, approximately 80% of cases) when the aganglionic segment does not extend beyond the upper sigmoid colon, and with the long-segment form (L-HSCR, 20% of cases) when aganglionosis extends proximal to

the sigmoid colon (Amiel *et al.*, 2008). There are also four variants: (1) total colonic aganglionosis; (2) total intestinal HSCR; (3) ultra-short segment HSCR involving the distal rectum below the pelvic floor and the anus; (4) suspended HSCR, a controversial condition, where a portion of the colon is aganglionic above a normal distal segment (Amiel *et al.*, 2008).

Suction rectal biopsy remains the golden standard for confirming the diagnosis in most cases demonstrating an increased acetylcholinesterase activity (Amiel *et al.*, 2008; Kurer *et al.*, 1986). Nonetheless, full thickness rectal biopsy is another method to reach the diagnosis. Furthermore, seromuscular biopsies will be needed at operation to define the proximal limit of the aganglionic segment.

1.3.5 Treatment and prognosis

At present, therapeutic strategies for HSCR and indeed enteric neuropathies in general remain limited to surgery. The underlying principle is to resect the abnormal bowel and then release the tonic contraction of the internal anal sphincter. With laparoscopic and trans-anal pull-through techniques, the patient can get a scarless HSCR surgery.

Fistula or stenosis of anastomosis and enterocolitis are the main short term complications (Hackam *et al.*, 1998). Long term complications include chronic constipation (10–15%) and soiling (Moore *et al.*, 1996; Yanchar and Soucy, 1999). Mortality has been below 6% since the 1980s and may be related to short term complications or caused by the associated malformations (Yanchar and Soucy, 1999). However, the treatment of children with total colonic aganglionosis remains hazardous.

1.3.6 Frontiers in the treatment of Hirschsprung's disease

Despite considerable surgical expertise accumulated over several decades in HSCR, many of the children with this condition are presented with substantial post-operative morbidity and have severe functional abnormalities ranging from severe constipation to feces incontinence. The long-term outcome is not satisfactory even into the adult life, highlighting the need to develop new therapies. In the last decade the tremendous advances in molecular biology and genetics have significantly enhanced the understanding of the development and function of the enteric nervous system. These have also facilitated not only the acquisition of knowledge of the pathogenesis of enteric neuropathies but have also coupled with the equivalent progress in the field of stem cell biology and the identification of novel experimental tools and targets for therapies (Thapar, 2007).

A number of investigations have suggested that therapeutic strategies based on the replacement of missing or malfunctioning enteric ganglia could play an important role in restoring functionality of the aganglionic bowel (Thapar, 2009).

1.4 *DOM*---mouse model for human Hirschsprung's disease

Dominant megacolon (symbol *Dom*), a semidominant mutation in the mouse, was described first in 1984 (Lane and Liu, 1984). *Dom* is arisen spontaneously in a mouse colony. In $Sox10^{Dom/+}$, *Sox10* transcript levels were reduced leading to enteric hypo- or aganglionosis and spotted pigmentation, the severity of which varies with the genetic background. The majority of $Sox10^{Dom/Dom}$ embryos die before embryonic day 13 (E13) (Lane and Liu, 1984). However, on a C57BL/6J x C3H/HheOuJ hybrid

genetic background, very few *Sox10*$^{Dom/Dom}$ embryos develop to term and newborn *Sox10*$^{Dom/Dom}$ pups die within a few hours after birth (Herbarth *et al.*, 1998). Mutation of the gene encoding the SRY-related transcription factor Sox10 on chromosome 15 in mice was reported as the cause of defect (Herbarth *et al.*, 1998). An A-to-T transversion at position 32 of the open reading frame (ORF) causes a substitution of glutamate by valine, whereas the insertion of an additional G after position 579 results in an altered reading frame, which leaves the first 193 amino acids of Sox10 including the high mobility group (HMG) domain intact, but replaces the remaining 273 residues by a divergent carboxyl terminus of 99 unrelated amino acids (Herbarth *et al.*, 1998). The frame shift mutation after position 579 completely abolishes the ability of the synergistically enhanced activity of *Tst-1/Oct6/SCIP* and *Pax3*. In contrast, the glutamate-to-valine substitution mutant synergistically enhances the transcriptional activity of *Tst-1/Oct6/SCIP* to the same extent as *Sox10*$^{+/+}$. Histochemical study of *Dom* mouse (in a hybrid background C57BL/6J x C3HeB/FeJLe-a/a) found that dysganglionosis including aganglionosis, hypoganglionosis and unclassified dysganglionosis was present in 79% of *Sox10*$^{Dom/+}$ mice cases (Brizzolara *et al.*, 2004). Hence *Dom* represents an important mouse model for human HSCR for further experimental studies.

1.5 Objectives of the present study

With the increasing amount of scientific discoveries in the stem cell research and in the development of the enteric nervous system, the applicability of stem cell therapeutic in neuropathies become higher and higher. In this thesis, aims are set to characterize the enteric neural crest stem cells isolated and expanded from E14.5 mouse embryos, to clarify some environmental influences which may affect the migration and colonization of exogenous enteric neural crest-derived cells, and finally to explore the therapeutic potential of the enteric neural crest stem cells in prenatal and postnatal *Dom* mice.

The criteria for putative stem cells are their ability to proliferate, to self-renew over an extended period of time, and to generate a large number of progeny that can differentiate into the primary cell types of tissues from which it is obtained. To use enteric neural crest stem cells in the experiments of transplantation to be carried out later and to subsequently explore the therapeutic potential of neural crest stem cells, enteric neural crest stem cells were first derived as neurospheres from the embryonic gut at E14.5 and then characterized as described in the following Chapter 2.

The migration and colonization of enteric neural crest-derived cells in the gut are affected by many genes and molecules. In *Dom* mice, the rostral-to-caudal migration and colonization of vagal enteric neural crest-derived cells was delayed in the $Sox10^{Dom/+}$ mouse embryos. When these enteric neural crest-derived cells failed to colonize the terminal hindgut, this region would become hypo- or aganglionic, resulting in a condition like Hirschsprung's Disease in humans. Further there is a possibility of cell therapy to repair the abnormal region of megacolon with abnormal enteric neurons or without enteric neurons. However, some issues remain unclear

about the basic biology of the enteric nervous system that has implications for cell therapy to generate or replace enteric neurons. Therefore, Chapter 3 was designed to study the development of colonic aganglionosis and some factors of influencing the colonization of exogenous enteric neural crest-derived cells.

Lastly, in Chapter 4, the therapeutic potential of the enteric neural crest stem cells was examined by transplanting the GFP-labelled enteric neural crest stem cells as neurospheres into the prenatal and postnatal gut. Then Chapter 5 was a discussion Chapter on the observations made in this study.

Chapter two

Enteric neural crest stem cells

2.1 Introduction

The enteric nervous system (ENS) has received more and more attention from developmental biologists, mainly due to insights into many developmental processes gained following analyses of the ENS in mice with spontaneous or targeted mutations. Such studies have made known some of the major signalling pathways involved in the ENS development, although without doubt, much remains to be elucidated. The focus on the ENS development has also led to the investigation of stem cells as a replacement therapy for treatment of ENS defects, where numbers of enteric neurons are reduced or absent.

2.1.1 Enteric neural crest-derived cells development and enteric neural crest stem cells

All of the neurons and glial cells comprising the ENS are derived from the neural crest (NC) which is located on the dorsal aspect of the neural tube. Numerous experimental strategies have been used to trace neural crest cell (NCC) lineages as they migrate to and within the gut such as (i) microsurgical ablation of segments of the neural tube in order to remove pre-migratory neural crest cells residing within the neural tube, and subsequent examination of embryos for loss of cell types (Burns *et al.*, 2000; Peters-van der Sanden *et al.*, 1993; Yntema and Hammond, 1954); (ii) interspecies grafting in avian embryos, where segments of the neural tube from quail

embryos are microsurgically transplanted into chick embryos and quail cells, can subsequently be detected within the developing chick using anti-quail cell antibodies (Delalande *et al.*, 2008; Le Douarin and Teillet, 1973); and (iii) microinjection of cell tracers, such as the fluorescent dye, DiI or replication-deficient retroviruses to directly label neural crest cells (Serbedzija *et al.*, 1991; Shepherd *et al.*, 2001). Using these methods, two specific regions of the neural crest have been shown to form the ENS: the vagal neural crest, which contributes the majority of ENS neurons and glia cells along the entire length of the gut, and the sacral neural crest, which contributes a smaller number of cells mainly restricted to the hindgut (Le Douarin and Kalcheim, 1999). In the mouse, the gut is colonized by vagal neural crest-derived cells at E14 (Embryonic day 14) (Young *et al.*, 1998). A delayed entry of sacral neural crest cells into the hindgut has been reported in the mouse (Kapur, 2000).

Stemple and Anderson first coined the term neural crest stem cells (NCSCs) for self-renewing, oligopotent cells isolated from rat neural tube explants and immunoselected by an anti-low affinity nerve growth factor receptor ($p75^{LNTR}$) antibody (Stemple and Anderson, 1992). Several different methods have been used to identify and select stem cells. Through prospective flow cytometry of cell suspensions derived from different tissues or culturing dissociated tissues in conditions that favor the formation of neurosphere-like bodies (NLB) (Almond *et al.*, 2007; Bondurand *et al.*, 2003; Schafer *et al.*, 2003), neural crest stem cells have been located and studied.

Cells with similar properties of early neural crest stem cells have been isolated at post-migratory phases of the neural crest development, from the rat embryonic sciatic nerve and gut, again by expression of p75 (Bixby *et al.*, 2002; Bondurand *et al.*, 2003). The cells with properties similar to the neural crest stem cells isolated from the enteric nervous system of gut are enteric neural crest stem cells (ENCSCs). Recently

adult neural crest stem cells have been isolated from rat dorsal root ganglia (Li *et al.*, 2007). Neural crest stem cells are also found in the skin named as epidermal neural crest stem cells (EPI-NCSCs) or skin-derived precursors (SKPs) (Sieber-Blum *et al.*, 2004; Toma *et al.*, 2001; Wong *et al.*, 2006), in a cardiac side population (SP) (Tomita *et al.*, 2005), in the mouse cornea (Yoshida *et al.*, 2006; Yoshida *et al.*, 2005), in the teeth (Miura *et al.*, 2003) and in the bulge area of cultured human hair follicles (Yu *et al.*, 2010). Mesenchymal stem cells (MSCs), a heterogeneous subset of stromal stem cells in adult bone marrow, have been reported originated from at least two developmental origins, one of which is the neural crest (Morikawa *et al.*, 2009). The identification of NCSCs in accessible adult tissues provides a new potential source for autologous cell therapy after nerve injury or disease (Nagoshi *et al.*, 2008).

Neural crest stem cells could be isolated at their pre-migratory phase from the neural tube explants or at their post-migratory phase when they have already arrived at their final locations such as gut, skin, heart, cornea, teeth and hair follicles. Neural crest stem cells are able to generate an astonishingly diverse array of cell types during vertebrate development. These include bone; tendons; neurons; glia; melanocytes; and connective, endocrine, and adipose tissue (Crane and Trainor, 2006) , whereas neural stem cells are harbored in the embryonic central nervous system and throughout life mainly in the subventricular zone (SVZ) as well as the subgranular zone of central nervous system (Garzon-Muvdi and Quinones-Hinojosa, 2010). In general, neural stem cells can differentiate into the three major CNS lineages: astrocytes, oligodendrocytes, and neurons (Ahmed, 2009).

Although neural crest stem cells can be identified using available neural crest cell markers, specific isolation of a pure population of neural crest stem cell remains a problem. In a study of enteric neural crest stem cells, it was shown that only 1-2% of

E14.5 rat gut cells were strongly $p75^+$ and α4-integrin$^+$ (Iwashita *et al.*, 2003), suggesting that only a small number of enteric cells expressed neural crest stem cell markers. When these cells were plated clonally, only some 60% of those double positive cells formed colonies, and of these, only 80% generated mixed neuron/glia/myofibroblasts colonies. This finding illustrates nicely the complexity of these stem cell studies, in which even after fluorescence-activated cell sorting "purified" cells remain a mixture and are simply enriched for stem cells. Studies examining early neural crest cell primary cultures have shown that the majority of NCCs that grow out from the cultures, for example mouse E9 neural tube explants, are $Sox10^+$ and $p75^+$ (Hari *et al.*, 2002), yet *in vivo* studies of lineage markers suggest that these cells are a mixture of cell types, including, for example, melanocyte precursors (Wilson *et al.*, 2004). In conclusion, when stem cells studies are considered, one has to bear in mind that the stem cell population under study may be contaminated by other cell types because, to date, no specific methods for isolating pure stem cells from embryonic or adult structures have been identified.

2.1.2 Neural crest stem cells related markers and their potential applications

Although there are several neural crest stem cell markers available for identifying the neural crest stem cells, a difficulty in the study of neural crest stem cells with multiple, restricted or partially restricted developmental potentials is the lack of markers that unambiguously label a single cell type or cells at a single stage of their development (Young *et al.*, 2003). Nevertheless, the molecules described below have gradually emerged as cell markers of neural crest stem cells mostly found in the

developing gastrointestinal tract.

p75, low affinity neurotrophin receptor, a transmembrane glycoprotein, is considered as a standard marker for neural crest cells (Bixby *et al.*, 2002; Lo and Anderson, 1995). Expressed as a surface molecule, it binds, although with different affinities, the neurotrophin nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (Friedman and Greene, 1999). In the postnatal intestine, this marker localizes in the submucosal and myenteric plexuses (Bixby *et al.*, 2002), suggesting that differentiated enteric glial cells also express p75 (Young *et al.*, 2003). However, using a cell sorter to fractionate the population with the strongest p75 expression, it was possible to select and enrich multipotential enteric neural crest stem cells (Kruger *et al.*, 2002).

Nestin is an intermediate filament type VI protein and is used as a marker to analyze neural precursors in the developing central nervous system (Frederiksen and McKay, 1988; Lendahl *et al.*, 1990). It has been identified in neural crest-derived cells in the rat embryonic gut (Chalazonitis *et al.*, 1998) and the human fetal gut (Rauch *et al.*, 2006; Vanderwinden *et al.*, 2002). In the central nervous system, uncommitted neural precursor cells and committed neuroblasts and glioblasts express nestin, which is down-regulated in post-mitotic neurons (Frederiksen and McKay, 1988), but up-regulated in astrocytes after injuries (Julien, 1999). Nestin-immunoreactive cells in intact gut preparations from postnatal rodents have not yet been identified, possibly because they represent only a very small population of cells. The expression of nestin by cells of intact myenteric ganglia demonstrated by RT-PCR is nevertheless indicative of the existence of a population of uncommitted neural progenitors in the myenteric plexus of postnatal rats during the first two weeks of postnatal life (Silva *et al.*, 2008). However, nestin cannot be used as an exclusive marker because other cell

types, such as endothelial cells, $CD34^+$ fibroblast-like cells and pericytes, are also positive for nestin (Ehrmann *et al.*, 2005; Rauch *et al.*, 2006).

Sox genes carry a single HMG (high mobility group) domain that binds to the DNA in a sequence-specific manner (Kamachi *et al.*, 2000). When the HMG domain binds to the DNA, it causes a bending of the DNA molecule, suggesting that it functions as an architectural element in the assembly of nucleoprotein structures (Prior and Walter, 1996). More than 20 *Sox* genes have been identified in vertebrates, and they are classified into seven groups, Groups A-G, according to mutual similarities in their HMG domains (Wegner, 1999). This high-mobility group gene family plays a critical role in the formation of tissues and organs during embryonic development, and is widely distributed in the animal kingdom.

Among all the sox genes, **Sox10** (group E), the early neural crest marker found in the embryonic gut, is encoded by SRY (sex determining region-Y) box 10. The protein product of this gene with DNA binding domains works as a transcriptional regulator and is active in neural crest cells (Mollaaghababa and Pavan, 2003). Sox10 expresses not only on glial cells but also on undifferentiated enteric neural crest stem cells. It seems to be crucial for the maintenance of the stem cell state (Kim *et al.*, 2003) by inhibiting neuronal and glial differentiation of multipotent enteric neural stem cells (Bondurand *et al.*, 2006). At later stage of ENS development, Sox10 is expressed in the glial cell lineage, and is proposed to function in cell fate specification and glial cell differentiation (Kelsh, 2006). Therefore, it is likely to be a good marker for undifferentiated neural crest stem cells in early development of enteric nervous system.

Sox2 is one Sox gene in group B of Sox gene family (Pevny and LovellBadge, 1997; Wenger and Markwalder, 1999). Sox2, with its target site in the regulatory

region of the delta-crystalline gene, is located just adjacent to the binding site of delta EF3, which is in turn essential for transactivation by Sox2 (Kamachi *et al.*, 1995). Sox2 seems to utilize distinct partners in a cell type-specific manner. For instance, in embryonic stem cells (ES) or embryonic carcinoma (EC) cells, Sox2 interacts with Octamer Transcription Factor 3 (OCT3) protein on binding to the enhancers of FGF4 (Yuan *et al.*, 1995) and undifferentiated embryonic cell transcription factor 1 (UTF1) genes (Nishimoto *et al.*, 1999). Within the central nervous system, highly proliferative neural progenitors and neural stem cells can be identified by their expression of Sox2. Moreover, Sox2 functions to maintain cells in a neural progenitor state (Episkopou, 2005). In the gut, microarray experiments have identified *Sox2* as a previously undescribed ENS-expressed gene (Heanue and Pachnis, 2006). The identification of *Sox2* may represent expression within a population of ENS stem cells present in the gut. Sox2 expression is detectable in the ENS from E11.5 to at least postnatal day 4. It is possible that, as in the central nervous system, Sox2 is expressed within the neural stem cell population that can be expanded *ex vivo*. If so, resources that are used to enrich neural stem cells from the central nervous system, such as the Sox2-EGFP mouse (Ellis *et al.*, 2004), could also be applied in future studies of the ENS stem cell population. Li (Li *et al.*, 2007) also detected several neural stem cell-specific genes including *Sox2* in the adult dorsal root ganglia-derived neurospheres.

2.1.3 Objective of this chapter

To use enteric neural crest stem cells in the experiments of transplantation to be carried out later in my study and to subsequently explore the therapeutic potential of neural crest stem cells, in this chapter, enteric neural crest stem cells were first isolated and cultured as neurospheres from the embryonic gut at E14.5 and then characterized

with following methods:

a. Detection of stem cell markers (p75, nestin, Sox2 and Sox10) expression with immunohistochemical staining and reverse-transcription-PCR (RT-PCR);
b. Determination of the proliferation property as indicated by the expression of proliferation marker PH3 following immunohistochemical staining, the population doubling time, the frequency of forming secondary neurospheres and results in the limited dilution assay;
c. Examination of the differentiation property as indicated by the expression of several differentiated cell type markers such as Tuj1, GFAP and SMA and also different subtype neuronal markers such as TH and SYP, following immunohistochemical staining.

By using the aforementioned methods, the cells cultured *ex vivo* as neurospheres after isolated from the E14.5 mouse embryonic gut were shown to possess properties of neural crest stem cells.

2.2 Materials and Methods

2.2.1 Mouse embryonic enteric neural crest stem cell culture

A. Dissection of mouse embryos

In biological safety cabinet (SterilGARD, class II type A/B3 biological safety cabinet, the baker company), a 60 mm Petri dish (Falcon 353002, Becton Dickinson, USA) was filled with Ca^{++} and Mg^{++}-free HBSS (Hank's balanced salt solution without calcium and magnesium, Sigma, H2387). The medium was used to temporarily sustain the survival of the embryos after they were removed from the mother. Another two 60 mm Petri dish (Falcon, 353002, Becton Dickinson, USA) were filled with just enough DPBS (Dulbecco's phosphate buffered saline, Invitrogen, 14190). These dishes were then placed on ice. After the Petri dishes with medium had been prepared, the dissection of the mouse could be carried out.

The timed pregnant ICR mouse at E14.5 was sacrificed by cervical dislocation. The sacrificed mouse was placed on its back and wet the lower abdomen with 70% alcohol. Using a pair of scissors and forceps, the uterus was exposed. The deciduas in the uterus were exposed by dissecting the uterus open with scissors. Then the deciduas were isolated and transferred to the 60 mm Petri dish filled with HBSS using a pair of blunt forceps.

B. Dissection of the embryonic gut

Under a dissection microscope, the embryos were extracted from the deciduas with forceps. After dissecting open the yolk sac and amniotic sac, the embryo was exposed from its embryonic membranes. Using forceps, the umbilical vessels and

vitelline artery were cut distal to the embryos. During dissection, care had to be taken not to damage the embryos, particularly the posterior ends of the gut. The embryos were lifted by grasping them gently by the head or neck and placed into another 60 mm Petri dish with DPBS.

E14.5 ICR mouse gut segments extending from the stomach to the hindgut were dissected in DPBS using fine forceps (F.S.T) as described below. The head and the posterior end of the embryo were cut off. It was turned to its back with the thorax and abdomen facing upward, while the forelimb and hindlimb buds were spread out. One pair of forceps was placed near the shoulders, and another forceps grabbed the top of the thoracic cavity contents and pulled in a posterior direction to pull out all the visceral organs including the gut tube in the abdomen. The gut tube could then be cleared from all attached tissues and placed into sterile Ca^{++} and Mg^{++}-free HBSS on ice while awaiting dissociation into single cells.

C. Dissociation of the gut tube into single cells for culture

Ten gut tubes were digested in 1ml Ca^{++} and Mg^{++}-free HBSS with 0.025% trypsin (Sigma, T9935) and 1% collagenase IV (Worthington Biochemicals, LS004186) in a 37°C water bath for 4 minutes and then triturated into single cells with a flame-polished glass pipette. 2 ml quench solution [15 µl of DNase I (Sigma D4527, 5 mg/ml in Ca^{++} and Mg^{++}-free HBSS) in 2 ml of fluorescence-activated cell sorting (FACS) solution] were added into the medium to stop the enzymatic reactions. The mixture was spun down at 1000 rpm (193g, Allegra X-22R centrifuge, Beckman coulter) for 4 minutes. The pellet was re-suspended in the self-renewal medium (Bixby *et al.*, 2002) (Appendix I) and filtered through a nylon mesh (40 microns, High fashion group, Hong Kong). The filtered single cells were plated on to 60 mm tissue culture

Petri dishes (Falcon, 351007) at a density of $1x10^6$ cells per dish for non-adherent neurosphere cultures. Small clusters of cell spheres (neurospheres) were formed in the culture two days later. Cultures were then re-plated once to another tissue culture dish two days later after primary culture without refreshing the culture medium and then half of the culture medium was refreshed every two days afterwards.

D. Subculture of neurospheres

0.025% trypsin/EDTA solution (Gibco) was first pre-warmed to 37°C. The expanded neurospheres were collected in a centrifuge tube (Corning, 430791) after 5 to 7 days culture, spun down at 1000 rpm (193g) for 4 minutes, and re-suspended in the HBSS without calcium and magnesium and spun down at 1000 rpm (193g) again. The HBSS was removed and 1 ml pre-warmed 0.025% trypsin/EDTA in HBSS without calcium and magnesium solution was added into the tube. The pellet was re-suspended and incubated in a 37°C water bath for 15 minutes.

The mixture was triturated up and down 10 times using syringe with a 21-gauge needle, and 1 ml ice-cold quench solution [10% chicken embryonic extract (US Biological, C3999) in FACS solution] was added. The mixture was again triturated up and down 10 times again and spun down at 1000 rpm (193g) for 4 minutes at 4°C.

The dissociated cells were re-suspended in 1 to 2 ml self-renewal medium (Appendix I) and counted with a hematocytometer (Fisher, USA). Cells were plated at a low cell density of $3\text{-}5x10^5$ cells per 60 mm Petri dishes (falcon, 351007) (Widera *et al.*, 2009) and half of the self-renewal medium was refreshed every two days. The neurospheres were subcultured into single cells again after 5 to 7 days.

2.2.2 Population doubling time

0.5-1x10^5 cells dissociated from non-adherent neurospheres were cultured in a 35 mm Petri dish containing 1.5 ml self-renewal medium. The cell number was determined every 24 hours by counting with a hematocytometer (Fisher, USA). When the cell number was counted, neurospheres were treated for 10-15 minutes with 0.025% trypsin/EDTA at 37 ℃ and centrifuged at 1000 rpm (193g, AllegraTM X-22R Centrifuge, BECKMAN, USA) for 4 minutes. The cell numbers were determined in triplicate. Population doubling time was calculated using the algorithm provided by http://www.doubling-time.com.

2.2.3 Limited dilution assay

After enzymatic and mechanical dissociation of the neurospheres, the single cells were diluted in the self-renewal medium to obtain theoretically 1 cell per 100 µl, and 100 µl of the medium containing cells at this cell density were subsequently added to each well of a 96-well flat-bottom plate (costar, 3599, USA). The presence of one single cell in a well was confirmed with phase-contrast microscopy and photography with a Nikon inverted microscope after 24 hours and 48 hours. The 96-well plates were scored 7–10 days later for the number of neurospheres derived from single cells.

2.2.4 Frequency of forming secondary neurospheres

After enzymatic and mechanical dissociation of the neurospheres, single cells were diluted to 500 cells/1.5 ml medium in one 35 mm Petri dish (Corning, 430165) (Louis *et al.*, 2008). Each 35 mm Petri dish (Corning, 430165) was examined for newly formed neurospheres using an inverted microscope equipped with phase contrast 5 to 7 days later.

2.2.5 GFP-labelled and unlabelled cells co-culture

Using the same method described in Section 2.2.1, GFP-labelled enteric neural crest stem cells were isolated and cultured from EGFP-mouse embryos (C57BL/6-Tg(CAG-EGFP)1Osb/J, The Jackson Laboratory). The transgenic mouse carries an "enhanced" GFP (EGFP) cDNA under the control of a chicken beta-actin promoter and cytomegalovirus enhancer that drive the expression of EGFP in all tissues, except erythrocytes and hairs, making the whole animal appear green under excitation light from 395 nm to 475 nm or UV (ultraviolet ray) light (http://jaxmice.jax.org/strain/003291.html). Unlabelled enteric neural crest stem cells were also isolated and cultured from normal, non-GFP-labelled embryos according to the same methods described in Section 2.2.1. The single cell suspension of enteric neural crest stem cells was obtained by enzymatic digestion of neurospheres (see Section 2.2.1D). The GFP-labelled single cells were mixed in a 1:1 ratio with unlabelled single cells and co-cultured at $1x10^5$ cells per 55 mm Petri dish in 3 ml mouse self-renewal medium (Appendix I). The numbers of GFP^+, GFP^- and chimeric GFP^+/GFP^- neurospheres were counted after 6 days (Louis *et al.*, 2008).

2.2.6 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

All cells from the entire gut tube and enteric neural crest stem cells from different passages were harvested and stored at -80℃ until extraction of RNA. The total RNA of the gut tube and cultured neurospheres were extracted with SV RNA Isolation System (Promega) following the instruction of the kit, and then DNase I (Deoxyribonuclease I, Amplification Grade, Invitrogen, 18068-01) containing solution was used to treat the extracted RNA. The components of the DNase I

solutions are listed below:

Extracted RNA sample	x μl (<1 μg)
DNase I buffer 10x	1μl
DNase I (1Unit/μl)	1 μl
EDPC treated H_2O	(8-x) μl
Total	10 μl

The mixture was incubated at 24℃ for 15 minutes, and 1 μl 25mM EDTA was added before the mixture was incubated at 65℃ for another 10 minutes.

Then using DNase I treated RNA as the template, the cDNA was synthesized with oligo (dT) from the SuperScript™ III First-strand cDNA Synthesis System (Invitrogen, 18180-051) as the primer at 50℃ for 50 minutes. The cDNA synthesis was terminated at 85℃ for five minutes. The resulting cDNA was then used for PCR amplification. The PCR reaction mixture was put at 94℃ for 5 minutes first to initially denature the cDNA. Each PCR thermocycle included: 94℃ for 30 seconds for cycle denaturation, melting temperature (Tm) (refer to the Table 1 below for primer sequences and Tm) for 30 seconds for primer annealing and 72℃ for 30 seconds for primer extension and a total of 30 cycles were performed. Then the reaction products were extended at 72℃ for 7 minutes for final extension.

The PCR products were separated by 1% UltraPure™ Agarose gel (Invitrogen, 15510-027) electrophoresis and detected by gel red (Biotium, 41003) staining.

Table1 Primer sequences and Tm for each gene

Gene	3' primer	5'primer	Tm
β-Actin	taaagacctctatgccaacacag	cacgatggaggggccggactcatc	62℃

p75	cctgtccgtcggtctccaat	tgcagatcgaggtccataaaa	60℃
Sox2	aaaccgtgatgccgacta	gaagcgcctaacgtaccact	58℃
Nestin	ctaccaggagcgcgtggc	tccacagccagctggaactt	62℃
Tuj1	aggagggggagatgtatgaagat	acagaggtggctaaaatggggag	60℃
Gfap	cacgaacgagtccctagagc	ccttctgacacggatttggt	60℃
Sma	ctgacagaggcaccactgaa	gaaggaatagccacgctcag	60℃

2.2.7 Cytospin preparations

Cells or neurospheres were washed in ice-cold PBS twice and suspended in ice-cold DPBS at cell density of not more than $5x10^5$ cells/ml. All samples were sure to be kept on ice. The slides and filters were placed to appropriate slots in the cytospin machine (Universal 320R) with the cardboard filters facing the center of the cytospin. Each pair of the filter and slide was settled with each other and that the hole in the filter was in a proper position so that the cells would be able to reach the slide. 50-100 μl of each sample was quickly added into the appropriate wells of the cytospin machine. The lid of the cytospin was shut and the slides were spun at a speed of 23g (Universial 320R) for 2 minutes. The filters were removed from their slides without contacting the smears on the slides. The cells were fixed in the 4% paraformaldehyde (PFA) for 15 minutes, and washed in PBS 5 minutes for three times. Then the cells or neurospheres were dried in air and stored at -20℃ or stained by immunohistochemical staining.

2.2.8 Preparation of paraffin sections and cryosections

Mouse embryos were dissected out in PBS and fixed in 4% PFA overnight at 4 ℃

and rinsed in PBS 15 minutes for three times.

For paraffin sectioning, the embryos were processed through a dehydration series of graded alcohol from 70%, 80%, 95%, absolute alcohol (twice) and xylene (twice) and then incubated in paraffin wax twice. The treatment time for each dehydration solution depended on the stage of embryos. Then the processed embryos were embedded in an appropriated orientation. The paraffin-embedded embryos were sectioned at 5 μm using a microtome and sections were mounted onto histological glass slides (Superfrost® Plus slide, Menzel-Glaser) and dried overnight for immunostaining or stored at room temperature until use.

For cryosectioning, the embryos were dehydrated by a graded series of 10%, 20% and 30% sucrose solution (USB, 119849) at least each for 8 hours to make sure that the sucrose infiltrated into the all tissues of the embryos. The embryos were transferred into embedding molds fabricated from household aluminum foil. The molds were then filled with Optimal Cutting Temperature compound (OCT, Tissue-Tek). The molds were submerged into isopentane which was kept cool with liquid nitrogen. After the OCT was frozen, the embedded embryos were stored at -20℃. The cryosections of 10 μm were cut using a cryostat (Thermo) and mounted onto histological glass slides (Superfrost® Plus slide, Menzel-Glaser) and stored at -20℃ after being dried overnight.

2.2.9 Immunohistochemical staining

2.2.9.1 Whole-mount immunohistochemical staining

The entire gut tubes were dissected out with as little attaching tissues as possible and then fixed in 4% PFA overnight at 4℃. The preparations were washed with PBS

three times for 15 minutes each time. Then the preparations were treated with the blocking solution [PBS with 10% normal horse serum (Vector, s-2000) and 1% Triton X-100 (USB, 22686)] for 1 hour. After removing the blocking solution without washing the preparations, the preparations were then incubated overnight at 4℃ with the primary antibodies diluted in the blocking solution (PBS with 10% normal horse serum and 1% Triton X-100). The blocking solution without any primary antibodies was used for the negative control preparations. All of the preparations were then washed three times with PBS for 15 minutes each time followed by incubation with the secondary antibodies (Molecular Probes) tagged with different Alexa fluor® fluorochrome at room temperature for 1 hour in the dark. The subsequent steps were also performed in the dark. Finally the preparations were washed three times with PBS for 15 minutes each time. After all the incubations, the preparations were mounted with the mounting solution (DAPI: glycerol 1:3) on the histological glass slides and covered with a coverslip. These whole-mount preparations were imaged with an Olympus FV1000-ZCD laser confocal microscope. The primary and secondary antibodies used in the study are listed in Table 2.

2.2.9.2 Immunohistochemical staining on sections and cytospin preparations

The paraffin sections on slides were warmed at 49℃ in an oven for 20 minutes to make sure that the slides were thoroughly dried before going to the next step. The sections were dewaxed in xylene three times each for 5 minutes and hydrated through a series of alcohol of decreasing concentrations (100% to 95% to 80% to 70% and then tap water twice, each step for 5 minutes). The sections were washed in PBS three times each for 5 minutes.

For the cyrosections and cytospin preparations, immunochemical staining started from being washed in PBS three times each for 5 minutes. The sections on the slides were circled with a PAP-pen and blocked in 10% normal horse serum and 1% triton X-100 in PBS at room temperature for 1 hour. The encircled sections and neurosphere were incubated at 4℃ overnight in PBS with primary antibodies from different species diluted in 10% normal horse serum. The blocking solution without any primary antibodies was used for the negative control preparations. The sections were washed in PBS three times each for 5 minutes, and then incubated with secondary antibodies tagged with different Alexa fluor® fluorochrome diluted in 10% normal horse serum in PBS for 1 hour at room temperature (reaction in the dark from this step onward). The sections were washed with PBS three times each for 5 minutes and were mounted with a mounting solution (DAPI: glycerol=1:3). The immunostained sections were examined under a fluorescence microscope or an Olympus FV1000-ZCD laser confocal microscope.

2.2.9.3 Immunocytochemical staining on coverslips

The neurospheres cultured on the Poly-D-Lysine (PDL, Sigma, P7886) coated coverslips in the differentiation medium contain 2.5% FBS (fetal bovine serum, Gibco, 26140) in the DMEM/F12 (Gibco, 11330-032) for 4 days were fixed in 4% PFA for 15-30 minutes at room temperature. The preparations on the coverslips were rinsed three times with PBS each for 5 minutes. They were stored in PBS at 4°C for several days or stored at -20°C after air-dried before staining. The preparations were incubated in 0.1% Triton X-100 in PBS for 15-30 minutes at room temperature for permeabilization. Then they were rinsed three times each for 5 minutes in PBS, before the cells were blocked in 10% normal horse serum in PBS for 1 hour at room

temperature. The primary antibodies were diluted to the appropriate concentration using 10% normal horse serum and an appropriate volume of the antibody solution, which was sufficient to cover the entire coverslip (e.g. 50-100 µl per 13 mm coverslip), was added onto the coverslip. The coverslip together with the antibody solution was then covered with a piece of parafilm with an appropriate size. The preparations were incubated in the primary antibody/antibodies at 4°C overnight and rinsed in PBS three times each for 15 minutes. The blocking solution without any primary antibodies was used for the negative control preparations. Then the preparations were incubated with the secondary antibody/antibodies tagged with different Alexa fluor® fluorochrome for 1 hour at room temperature. The secondary antibodies were diluted in 10% normal horse serum in PBS. From this step onward, the preparations had to be kept away from light. It had to be sure that a correct isotype-specific secondary antibody for each primary antibody had been chosen. The preparations were rinsed in PBS for three times each for 10 minutes in the dark. Blank microscopy glass slides were prepared by putting labels for each corresponding coverslip. A drop of mounting solution (DAPI: glycerol=1:3) was added onto the surface of each microscopy slide and then each coverslip was picked up with a forceps and placed to the mounting medium with the cell-side facing the mounting solution. The cells were examined using a fluorescence microscope or an Olympus FV1000-ZCD laser confocal microscope.

Table 2 Primary and secondary antibodies for immunohistochemical staining

Primary antibody (antiserum)	**Host**	**Source**	**Dilution of primary antibody**	**Dilution of secondary antibody**
GFAP (Anti-Glial Fibrillary Acidic Protein)	Rabbit	Covance (MMS-435P)	1:150	1:300

Hu (HuC/HuD neuronal protein)	Mouse	Invitrogen (A21271)	1:150	1:150
nestin (Anti-Nestin, clone rat-401)	Mouse	Chemicon (MAB353)	1:100	1:300
p75 (Anti-Nerve Growth Factor Receptor)	Rabbit	Chemicon (AB1554)	1:300	1:300
PH3 [Anti-phospho-Histone H3 (Ser10), clone RR002]	Mouse	Upstate (RR002)	1:1000	1:300
SMA (Monoclonal Anti-α-Smooth Muscle Actin antibody)	Mouse	Sigma (A2574)	1:300	1:300
Sox10 (N-20)	Goat	Santa Cruz (sc17342)	1:30	1:150
Sox2 (Y-17)	Goat	Santa Cruz (sc17320)	1:100	1:300
TH (Anti-Tyrosine Hydroxylase)	Rabbit	Chemicon (AB1554)	1:100	1:300
Tuj1 (Neuronal Class III ß-Tubulin)	Mouse	Covance (MMS-435P)	1:150	1:300

Secondary antibody	**Source**
Donkey anti-mouse Alexa 555, 488	Molecular Probes
Donkey anti-rabbit Alexa 555, 488	Molecular Probes
Donkey anti-goat Alexa 555, 633, 488	Molecular Probes

2.3 Results

2.3.1 Neurospheres derived from the mouse embryonic gut at E14.5

According to the isolation and culture methods for the neural crest stem cells (Section 2.2.1), the entire gut was firstly dissected out from the E14.5 mouse embryo (Fig. 2.1A) and digested into single cells (Fig 2.1B), which were then cultured in tissue culture dishes. Two days after plating, small spherical clusters appeared (arrows in Fig. 2.1C1), and flattened and elongated mesenchymal cells grew in a monolayer attached to the bottom of the dish (Fig. 2.1C1). These small spherical clusters were referred to as neurospheres hereafter, as in the line with the notion that free-floating structures derived from putative neural stem cells of the tissues in the central nervous system *ex vivo* are called neurospheres (Bez *et al.*, 2003). These neurospheres were then re-plated into another tissue culture dish (Fig. 2.1C2) and another two days later half of the self-renewal medium was refreshed. Four and six days after first plating bigger neurospheres appeared to suspend in the medium (Fig. 2.1D and E). At the sixth day, the neurospheres were subcultured at passage 2 (Fig. 2.1F1). Two days after subculture, small neurospheres started to form (Fig. 2.1F2). The neurospheres became bigger after 4 days (Fig. 2.1F3) and 6 days (Fig. 2.1F4). The average diameter of neurospheres at day 6 was about 85.79 ± 30.17 μm (n=214). Then a new subculture cycle (passage 3) began. In all assays mentioned below, neurospheres at passages 3-30 were used.

2.3.2 Characterization

2.3.2.1 Proliferation

2.3.2.1.1 Proliferation and morphology of neurospheres

Neurospheres developed from the guts of E14.5 embryos were subcultured (passage) repeatedly and they were easily maintained and expanded in culture for up to at least 30 passages.

The stem cells and progenitor cells are capable of proliferating to form neurospheres (Reynolds and Rietze, 2005), but progenitor cells do not exhibit long-term self-renewal capacity. Only cultures containing stem cells can be expanded continuously, whereas cultures only with progenitor cells gradually die out. It is commonly accepted that seven can be used as the minimal number of passages to demonstrate self-renewal and proliferation over an extended period of time (Louis *et al.*, 2008). In this study, the neurosphere culture was kept until the 30^{th} passage with continuous expansion and renewal. The morphology of neurospheres remained similar regardless of the numbers of passages and days following subculture (Fig. 2.2A-D).

2.3.2.1.2 Population doubling time

The population doubling time of enteric neural crest stem cells was 49.4 ± 10.02 hours (n=6) (Fig. 2.3C). The doubling time of cells was increased with passages, suggesting that progenitor cells within the neurospheres became less proliferative with the increasing number of passages.

2.3.2.1.3 Frequency of forming secondary neurospheres

The frequency of forming secondary neurospheres was 5.13% to 22.96% of the total number of dissociated single cells plated.

Table 3 Frequency of forming secondary neurospheres at different passage

Passage2	Passage3	Passage4	Passage5	Passage6	Passage7	Passage8	Passage9	Passage10	Passage11
22 96 %	12 59%	12 93 %	10 06 %	6 43 %	5 13 %	4 67 %	8 35 %	7 79 %	12 %

2.3.2.1.4 Limited dilution assay

In the limited dilution assay, 11.93% to 26.67% of dissociated single cells generated neurospheres.

Table 4 Limited dilution assay

Passage2	Passage3	Passage4	Passage5	Passage6	Passage 9
21.14%	11.93%	15.43 %	18.78 %	26.67 %	17.58%

2.3.2.2 Expression of stem cell and differentiation markers in the neurospheres

The protein expression of neural crest stem cell markers p75, nestin and Sox10 was examined by immunohistochemical staining (Fig. 2.4). Expression of PH3 (Phosphorylation of Histone H3, a mitotic marker of late G2 phase), a proliferation marker which are highly selective for proliferating (mitotic) cells (Hendzel *et al.*, 1997), was also examined using immunohistochemical staining. Within neurospheres, most of the cells were nestin and p75 double immunoreactive, and p75, Sox10 and PH3 triple immunoreactive, indicating that within the neurospheres, many cells expressing stem cell markers p75, nestin, and Sox10 and the mitotic marker PH3

were most probably stem cells. The percentage of cells expressing stem cell makers were analyzed by staining the single cells dissociated from the neurospheres (Fig. 2.6A): 81.11 ± 6.98% (n=5) of cells were anti-Sox10 immunoreactive; 95.36 ± 2.18% (n=8) were p75 immunoreactive; 81.06 ± 9.41% (n=6) were nestin immunoreactive.

In some neurospheres, a few cells expressed the differentiated cell markers: Tuj1, GFAP and SMA proteins (Fig. 2.5). Tuj1 is commonly regarded as a neuronal marker in developmental neurobiology and stem cell research, while coexpression of Tuj1 with the glia antigen GFAP has been observed in astrocytes (Draberova *et al.*, 2008; Eng, 1985). This may be interpreted as neuronal or glial cells undergoing "transdifferentiation" phenomena by changing their phenotypes/antigenic profile or presumed phenotypic markers being not specific enough to distinguish between different cell types during some, or all, developmental stages (Eng, 1985). SMA is a common marker in the study of smooth muscle differentiation and expressed by myofibroblasts (Darby *et al.*, 1990; Skalli *et al.*, 1986). Neural crest stem cells from sciatic nerve and gut could give rise to neurons, glia cells and myofibroblasts (Bixby *et al.*, 2002; Morrison *et al.*, 1999). However the percentage of the differentiated cells in the neurospheres was not an analysis in this study.

Additionally the RNA expression of several markers was shown by reverse transcription (RT)-PCR (Fig. 2.6B). The stem cell markers *p75, nestin,* and *Sox2* were strongly expressed in all the passages examined indicating the presence of putative stem cells in the neurospheres from passage 1 to 30. Results from RT-PCR also showed the expression of *Gfap* and *Tuj1* in the neurospheres at all passages. These observations were consistent with the immunostaining results above where expression of markers of differentiated neurons and glia cells was detected within the neurospheres. However *Sma* expression was too low to be detected in almost all the

passages examined except primary cells (cells in passage 1) from the E14.5 guts, implicating that few cells in the neurospheres differentiated into myofibroblasts.

2.3.2.3 Co-culture of GFP-labelled and unlabelled cells

Normal, unlabelled cells and genetically GFP-labelled cells from EGFP mouse transgenic embryos constitutively expressing enhanced green fluorescent protein were mixed in a 1:1 ratio and plated at $1x10^5$ cells per 1.5 ml in 55 mm Petri dishes. The numbers of GFP^-, GFP^+ and chimeric GFP^-/GFP^+ neurospheres 6 days later were scored. These co-culture experiments using GFP-labelled and unlabelled cells revealed that approximately 87.25 ± 3.95% (n=511, passage 3) of neurospheres were derived from a single cell (i.e. clonal descendants, meaning that cells of the neurospheres examined were either all GFP-labelled or all GFP negative(Fig. 2.7A and B); in contrast, 12.75 ± 3.95% of neurospheres were chimeric with GFP-labelled cells and unlabelled cells (Fig. 2.7A), indicating that these neurospheres were not derived from a single cells (i.e. not clonally derived).

2.3.2.4 Induced differentiation

The neurospheres derived from one single cell growing at a very low cell density (i.e. clonal density of 500 cells/ 1.5 ml in a 35 mm Petri dish) were then cultured in the differentiation medium containing 2.5% fetal bovine serum on the poly-D-lysine coated coverslips for 4 days. After fixation and immunohistochemical staining, it was found that all of the cells within one neurosphere were either GFAP immunoreactive or negative, and some of them were positive for both Tuj1 and GFAP (Fig. 2.8A1-A3). TH (tyrosine hydroxylase) (Fig. 2.8B1-B3) and SYP (synaptophysin) (Fig. 2.8C1-C3) immunoreactivities could also be detected in some induced neurospheres. SYP

immunoreactivity was also co-localized with Tuj1 immunoreactivity (Fig. 2.8C1-C3). GFP^+ neurospheres cultured from the E14.5 guts of EGFP mouse also could also be induced in the differentiation medium. Some cells were Hu immunoreactive within neurospheres (Fig. 2.9A2). Cells could be Tuj1 or GFAP positive (Fig. 2.9B2 and C2). Also a few of cells express smooth muscle actin (SMA) (Fig. 2.9D2).

2.3.3 Sox2 as a stem cell marker in the enteric neural crest stem cells

2.3.3.1 Sox2 expression in the neurospheres

Immunostaining using specific antibodies to Sox2, p75 and PH3 showed that the cells within the neurospheres were almost all triple immunoreactive (Fig. 2.10A1-A4). These Sox2 immunoactive cells also expressed stem cell marker nestin together with p75 (Fig. 2.10B1-B4). 89.31±11.11% of the cells in the neurospheres (n=3) were Sox2 immunoreactive. RT-PCR results on different passages neurospheres showed that *Sox2* expressed in neurospheres at passages 1 to 30 (Fig. 2.6B).

2.3.3.2 Sox2 expression in the embryonic gut from E10.0 to E14.5

At E9.5-10.0 neural crest-derived cells enter the foregut mesenchyme, and thereafter they named enteric neural crest-derived cells (ENCCs) (Newgreen and Young, 2002b). At E10.0, neural crest-derived cells had already migrated from the neural crest to the mesenchymal regions on two sides of the neural tube. Most of them expressed neural crest specific marker p75 and also stem cell marker Sox2 (Fig. 2.11A1-A3 and B1-B3). Within the neural tube, Sox2 expression was stronger than the migrating neural crest cells on two sides of the neural tube. It has been reported that

highly proliferative neural progenitors and neural stem cells can be identified by their expression of Sox2 (Graham *et al.*, 2003). Microarray experiments have also identified Sox2 as a gene product expressed in the enteric nervous system (Heanue and Pachnis, 2006). In the mesenchyme of the foregut and midgut, Tuj1 immunoreactive neurons were Sox2 negative, while some Tuj1 negative but p75 positive enteric neural crest-derived cells were Sox2 positive (Fig. 2.11C1-C3, D1-D3 and E1-E3). In the epithelium of the foregut from the esophagus to the stomach/intestine boundary, Sox2 expressed strongly (Fig. 2.11C1-C3 and D1-D3), implicating that it may play roles in region-specific differentiation and morphogenesis of the endoderm through epithelial-mesenchymal interactions during the development of the gut and lung epithelia (Ishii *et al.*, 1998).

At E11.5, the enteric neural crest-derived cells, which expressed specific marker p75 in the gut, had already entered the gut and were colonizing it in a rostral-to-caudal manner (Fig. 2.12A1-A3). The migration front had migrated to the region of proximal hindgut. Some neural crest-derived cells crossed the cecum and also expressed Tuj1 or Sox2 (Fig. 2.12A1-A3). In the midgut, differentiated Tuj1 positive neurons exhibited a much weaker expression of the stem cell marker Sox2 (pointed by thick arrows in Fig. 2.12B1-B3) as compared with undifferentiated neural crest-derived cells, which were $p75^{+}/Tuj1^{-}$ (pointed by thin arrows in Fig. 2.12B1-B3). However, some of the undifferentiated $p75^{+}/Sox2^{+}$ neural crest-derived cells were mitotic as indicated by the expression of PH3, a mitotic marker of late G2 phase, in the midgut at E11.5 (Fig. 2.12C1-C3 and D1-D3).

In the midgut of E12.5, there was a higher density of enteric neural crest-derived cells ($p75^{+}$) than the density in the same region of the gut at E11.5 (Fig. 2.13A1-A3). Many of them were immunoreactive to both neural crest-derived cells specific

marker p75 and stem cell marker Sox2 (Fig. 2.13B1). The differentiated neurons expressed Tuj1 but the expression of Sox2 became much weaker (Fig. 2.13B2). At the migratory wave front of neural crest-derived cells in the hindgut, many neural crest-derived cells remained undifferentiated and expressed p75 and Sox2 (thin arrows in Fig. 2.13C1-C3) with a few of them differentiated into $Tuj1^+$ neurons. Those neurons expressed a low level of Sox2 (thick arrows in Fig. 2.13C1-C3).

At E13.5, the migratory wave front moved more caudally to the region near the distal end of the hindgut. Most of the cells in the hindgut were $p75^+$ (Fig. 2.14A1). Some of them differentiated into neurons and expressed Tuj1 (Fig. 2.14A2). Some of them expressed Sox2 (Fig. 2.14A3). In the midgut and hindgut, the density of the enteric neural crest-derived cells was higher than those at the previous stages, and many of them were $p75^+$ and/or $Sox2^+$ (Fig. 2.14B1 and C1). When cells underwent differentiation into $Tuj1^+$ neurons, the expression of Sox2 became weak or down-regulated (thick arrows in Fig. 2.14B2 and C2).

At E14.5, enteric neural crest-derived cells had colonized the entire gut and the enteric nervous system network was formed densely in the gut (Fig. 2.15A). More differentiated neurons which were $Tuj1^+/Sox2^-$ were found (Fig. 2.15B).

2.4 Discussion

In this chapter, the culture and characterization of enteric neural crest stem cells isolated from the gut at E14.5 were described and also the expression of Sox2 in neural crest-derived cells of the gut at different developmental stage was examined.

2.4.1 Culture of enteric neural crest stem cells as neurospheres

Neurospheres are floating three-dimensional aggregated cell structures obtained by exposing dissociated embryonic or adult cells of central nervous system to growth factors (Reynolds *et al.*, 1992; Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Weiss *et al.*, 1996). Neurospheres are easy to prepare and to maintain in large numbers and their 3D structure creates a niche that allows the modeling of a dynamic changing environment (Campos, 2004). Moreover, neurospheres have proved to be useful to tackle difficult issues, like distinguishing between stem cell maintenance/renewal and committed progenitor proliferation (Campos, 2004; Molofsky *et al.*, 2003).

Upon passages, growth factor-responsive cells of neurospheres can theoretically be passaged indefinitely, and in practice for at least seven passages with little change in the proliferation or differentiation potential of cells from early to late passages (Gritti *et al.*, 1999; Reynolds and Weiss, 1996; Vescovi *et al.*, 1999).

In the neural crest stem cells studies, several different methods have been used to identify and select neural crest stem cells, which include isolating cells through prospective flow cytometry from cell suspensions derived from different tissues or growing dissociated neural crest-derived tissues in conditions that are easy to form neurosphere-like bodies (Almond *et al.*, 2007). Neural crest stem cells from the

enteric nervous system named enteric neural crest stem cells could also be isolated from the embryonic gut (Bixby *et al.*, 2002; Fu *et al.*, 2004a), postnatal gut myenteric ganglia (Silva *et al.*, 2008) or adult gut mucosa (Metzger *et al.*, 2009).

In this chapter, enteric neural crest stem cells isolated from the embryonic gut at E14.5 could form neurospheres in the medium containing growth factors such as bFGF (basic fibroblast growth factor), human IGF (human insulin-like growth factor 1) and nutrition supplement chick embryo extract, N2 and B27. These neurospheres formed after enzymatic dissociation into single cell suspension and were passaged for more 30 passages. The population doubling time of enteric neural crest stem cells from E14.5 was 49.4 ± 10.02 hours (n=6) (plated at 0.5-1x10^5 cells/1.5 ml medium in 35 mm Petri dish). In the study of palatal neural crest related stem cells (pNC-SCs), the population doubling time was determined as 65.43h (plated at 1x10^5/1ml) (Widera *et al.*, 2009). The population doubling time of human neural crest cell (hNCC) lines is 40-48 hours (Thomas *et al.*, 2008).

More than 30 passages of the neurospheres culture from the enteric cells at E14.5 indicated that the neurospheres contained proliferative and self-renewal enteric neural crest stem cells. This kind of culture system also made it feasible to analyze the cellular activity of the enteric neural crest stem cells *ex vivo*.

2.4.2 Cellular characteristics of enteric neural crest stem cells within neurospheres

The neurospheres from the central nervous system contain stem cells, progenitors and differentiated cells (Campos, 2004). The neurospheres from the embryonic mouse spinal cord are heterogeneous spheroid structures in the presence of FGF2 and EGF. For example, they contain more than 90% nestin positive cells, 76% RC2 positive

cells (radial glia), 95% brain lipid-binding protein (BLBP) positive cells and 63% glial high affinity glutamate transporter (GLAST) positive cells, a small percentage (<1%) of β-III-tubulin and O4 immunoreactive cells, 2.1% of S100b positive cells, and virtually no GFAP positive cells (Deleyrolle *et al.*, 2006).

In the present study, the cellular composition of the neurospheres cultured from the embryonic gut was analyzed by immunohistochemical staining and there were 81.11 ± 6.98% Sox10 positive cells, 95.36 ± 2.18% p75 positive cells, 81.06 ± 9.41% nestin positive cells and 89.1 ± 11.11% Sox2 positive cells. According to the immunohistochemical staining result, there should be lots of neural crest stem cells in the neurospheres. In addition, multiple immunostaining of the neurospheres in cytospin preparations showed that about 80% of cells within the neurospheres were p75 and nestin double positive and p75, Sox10 and PH3 triple positive, meaning that the most of the cells in the neurospheres might be neural crest stem cells. Results of RT-PCR on the mRNA expression in the neurospheres also showed that the stem cell marker *Nestin* and *p75* were both expressed in all passages. The mRNA of the differentiated cell type markers such as *Gfap* and *Tuj-1* were also expressed in all passages, albeit at lower levels than those of stem cell markers, but *Sma* mRNA was too low to be detected in almost all the passages except primary cells (cells in passage 1) from the E14.5 guts. All of these results indicated the heterogeneity of the cellular composition within the neurospheres with the majority of cells expressing stem cell makers (e.g. nestin, p75, Sox2, Sox10) and neural crest cell markers (e.g. p75, Sox10). Though there were neurons and glia within the neurospheres, the percentage of these differentiating or differentiated cells was rather low and cells expressing stem cell markers were very much higher.

It was shown that only 1-2% of cells of the rat gut tube at E14.5 were strongly

$p75^+$ and α4-integrin$^+$ (Iwashita *et al.*, 2003), suggesting that only a very small number of enteric cells within the embryonic gut tube express stem cell markers. In the present study, the expression of various stem cell markers together with the extended period of survival of neurospheres in over 30 passages suggests that enteric neural crest stem cells have been isolated and enriched from the embryonic gut at E14.5 and that these enteric neural crest stem cells could be kept in the stem cell state for an extended period of time *ex vivo*.

2.4.3 Proliferation and self renewal properties of the enteric neural crest stem cells within neurospheres

In the mammalian nervous system, neural stem cells are often studied using a culture system referred to as the neurosphere assay (NSA) to estimate the stem cell frequency (Pevny and Rao, 2003). When a population of neurospheres from the embryonic or adult primary central nervous system tissue was dissociated into a single cell suspension and plated at a clonal density [i.e. a low density which allows each cell cluster (neurosphere) to be generated from one single parent cell], 2.4% of viable cells formed secondary neurospheres (Reynolds and Rietze, 2005). This is generally interpreted as that 2.4% of the plated cells were stem cells.

Rodney and colleagues reported that neurosphere assay overestimated this frequency using mathematical modeling and neural colony-forming cell assay (NCFCA) could discriminate stem cells from progenitor cells on the basis of their proliferative potential (Louis *et al.*, 2008; Reynolds and Rietze, 2005). Using their mathematical modeling, it was calculated that the neural stem cell frequency was 0.16%, which was lower than that predicted by the conventional neurosphere assay and the NCFCA (2500-7500 E14.5 striatal cells/35 mm dish) for estimating stem cell

frequency (frequency of colonies which were bigger than 2 mm in diameter) to be 0.07% of total cells (Louis *et al.*, 2008), because in the assay the progenitor cells were excluded. However, the neurosphere assay remains the most frequently adopted method to enrich, expand, and even calculate the frequency of neural stem cells (Pevny and Rao, 2003).

Another factor, cell density, has been identified as one of the factors that influence embryonic stem cell behavior and differentiation, e.g. initial plating densities affect optimal differentiation into adipocytes and osteocytes by human mesenchymal stem cells (McBeath *et al.*, 2004). Neural stem cell proliferation is also increased when cells are grown at a high density in the presence of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) (Morshead and van der Kooy, 2004), because of an unknown factor which is produced by cells when plated at high density. This unknown factor may be produced via cell-cell interactions and crosstalk between cell surface receptors. For example, the level of β1 integrin expressed by the cells conditions the number of secondary neurospheres formed (Campos *et al.*, 2004).

Most authors use low cell densities for neurosphere formation assays (rather than clonal analysis), ranging from 5000 to 50 000 cells/ml (Hitoshi *et al.*, 2002; Morshead and van der Kooy, 2004; Tropepe *et al.*, 1999). In this study, the frequency of enteric neural crest stem cell forming secondary neurospheres was 5.13% - 22.96% (planted at a low cell density: 500 cells/1.5 ml medium in 35 mm dish). The frequency of palatal neural crest-related stem cells (pNC-SCs) forming secondary neurospheres was 1.8% (Widera *et al.*, 2009). While highly enriched rodent skin precursors (SKPs) from whisker follicles isolated by microdissection showed a sphere-forming frequency of up to 24.4% (Hunt *et al.*, 2008).

In the present study, when cells of neurospheres were cultured in the limited

dilution assay (1 cell/100 μl in 96 well plate), 11.93% - 26.67% of dissociated single cells from the enteric neural crest stem cell neurospheres generated secondary neurospheres. This observation may also reflect the percentage of stem cells in the neurospheres. It is possible that this analysis might overestimate the percentage of stem cells compared with the methods of the mathematical modeling and neural colony-forming cell assay (NCFCA) because of the possible inclusion of progenitor cells in the assay.

Co-culture of GFP-labelled and unlabelled cells revealed that approximately 87.25 ± 3.95% of colonies in the neurospheres were derived from single cells; in contrast to 12.75 ± 3.95% of chimeric neurospheres containing both GFP-labelled cells and unlabelled cells (plated at $1x10^5$ cells/55 mm Petri dish in 3 ml medium). Hence the clonal nature of the neurospheres depends very much on the plating density (Louis *et al.*, 2008).

2.4.4 Differentiation property of the neurospheres

In the discussion above, self-renewal over an extended period time has already been indicated in the neurosphere culture. The results of the present study also demonstrate that enteric neural crest stem cells within the neurosphere could also give rise to at least three different cell lineages, namely neurons, glia, and myofibroblasts. In another study when cells of neurospheres were plated clonally, some 60% of cells showed double immunoreactivity, and of these, 80% generated mixed neuron/glia/myofibroblasts colonies (Iwashita *et al.*, 2003).

When neurospheres derived from one single cell growing at a very low cell density were induced to differentiate, most of the cells within the neurospheres became Tuj1 or GFAP immunoreactive. TH neuron and SYP [synaptophysin, which is

present on the membrane of neuronal presynaptic vesicles (Ozcelik *et al.*, 1990)] neurons could be detected in some induced neurospheres. GFP^+ neurospheres derived from the guts of the EGFP mouse at E14.5 could also be induced to undergo differentiation as evident by the presence of Hu positive cells in the induced neurospheres. Hu family consists of RNA binding proteins which recognize a nuclear antigen present in all neurons but not in other tissues and are expressed in early post-mitotic neurons (Akamatsu *et al.*, 1999; Dalmau *et al.*, 1992). In addition a few of cells expressed smooth muscle actin (SMA). All these observations implicate that cells within the neurospheres are capable to form multiple lineages if they are induced under proper conditions.

Hence at a clonal density, a single parental cell can give rise to a neurosphere which is a heterogeneous spheroid structure. The expression of differentiated cell type specific markers in the cells within the neurosphere further indicates that a single cell which grows into a neurosphere is capable to proliferate and generate a large number of progenies of different lineages.

2.4.5 Sox2 as an enteric neural crest stem cell marker

Several stem cell markers had been used in the present study to characterize the stem cell properties of neurospheres derived from the embryonic gut. These markers included Sox10, p75, nestin and Sox2. Sox10, an early neural crest cell marker, is expressed in undifferentiated enteric neural crest stem cells (Bondurand *et al.*, 2006). p75, the low affinity neurotrophin receptor, has been considered a standard and specific marker for neural crest-derived cells in the gut (Bixby *et al.*, 2002; Lo and Anderson, 1995). Nestin has been used as a marker to analyze neural precursors in the developing central nervous system (Frederiksen and McKay, 1988; Lendahl *et al.*,

1990) and identified in neural crest-derived cells in the rat embryonic gut (Chalazonitis *et al.*, 1998) and human fetal gut (Rauch *et al.*, 2006; Vanderwinden *et al.*, 2002). In the gut, microarray experiments have already identified *Sox2* as a gene expressing in the enteric nervous system (Heanue and Pachnis, 2006). It is therefore possible that, as in the central nervous system, Sox2 is expressed within the neural crest stem cell population of the enteric nervous system.

In the neurospheres developed from the embryonic gut, most cells exhibited nestin and p75 double immunoreactivity, and some also expressed p75, Sox10 and PH3. Additionally the RNA expression of several markers using RT-PCR showed that the stem cell markers *p75* and *Nestin* were strongly expressed in all passages examined indicating the presence of putative stem cells in the neurospheres at passages 1-30. Sox2 immunoreactive cells were also found to express stem cell markers nestin and p75 simultaneously. Results of RT-PCR at different passages of neurospheres showed that *Sox2* was expressed in the neurospheres with *p75* and *Nestin* expression. In mouse embryos or gut tubes at E10.0 to E14.5, upon immunohistochemical staining, both pre-enteric neural crest cells (neural crest cells prior to entry to the gut) and enteric neural crest-derived cells (neural crest-derived cells inside the gut tube) showed Sox2 and p75 double immunoreactivity. These cells did not express differentiated cell type markers such as Tuj1. When cells differentiated into Tuj1 positive neurons, the expression of Sox2 and also p75 became weak or down-regulated. All of these observations indicate that Sox2 can also be a good marker for neural crest stem cells in the gut.

2.5 Summary

The results of this chapter are summarized as follows:

Enteric neural crest stem cells could be cultured as neurospheres from the mouse embryonic gut at E14.5. The cells within these neurospheres had the capacity to proliferate and could be cultured *ex vivo* for more than 30 passages. The frequency of enteric neural crest stem cells to form secondary neurospheres was 5.13% - 22.96% (planted at low cell densities: 500 cells/1.5 ml medium in 35 mm dish). When neurospheres were dissociated and cultured in the limited dilution assay (1 cell/100 µl in one well of 96-well plate), 11.93% - 26.67% of dissociated single cells formed neurospheres. Co-culture of GFP-labelled and unlabelled cells revealed that approximately 87.25 ± 3.95% of colonies or neurospheres were derived from single cells when plated at $1x10^5$ cells/3 ml medium in 55 mm dish.

Neurospheres derived from the embryonic gut contained 81.11 ± 6.98% Sox10 positive cells, 95.36 ± 2.18% p75 positive cells, 81.06 ± 9.41% nestin positive cells and 89.31 ± 11.11% Sox2 positive cells. Immunostaining on cytospin preparations of neurospheres showed that most of the cells within the neurospheres were p75 and nestin double positive and some are p75, Sox10 and PH3 triple positive. The mRNA expression of stem cell marker *Nestin*, *p75* and *Sox2* were detected in all passages examined. The mRNA of the differentiated cell type such as *Gfap* and *Tuj-1* were expressed in all passages but *Sma* expression was too low to be detected in the all the passages except primary cells (cells in passage 1) from the guts at E14.5.

The neurospheres could be induced to differentiate into GFAP positive, Tuj1 and GFAP double positive or Tuj1 and GFAP double negative cells of non neuron and non glia cells in the differentiation medium containing 2.5% FBS. TH and SYP positive

neurons could be detected in some induced neurospheres. In the induced GFP^+ neurospheres, some cells were Hu positive or most of the cells were Tuj1, GFAP or SMA positive.

Sox2 could be an enteric neural crest stem cell marker because Sox2 was expressed together with the expression of the stem cell markers nestin and p75 in the neurospheres, and using immunohistochemical staining, the pre-enteric neural crest cells and enteric neural crest-derived cells were both p75 and Sox2 double positive except in Tuj1 positive neurons.

Hence these putative enteric neural crest stem cells, which were isolated from the mouse gut tube at E14.5 and cultured as neurospheres for many passages *ex vivo* with the demonstrated capacity of proliferation, self-renewal and differentiation, show properties of stem cells, and their potential applications in the transplantation experiments would be further explored in the following chapters.

2.6 Figures and Legends

Fig 2.1 Neurospheres developed from the cells dissociated from the mouse embryonic gut at E14.5

(A) Drawing of an E14.5 embryo isolated from the timed pregnant mouse. For neurosphere culture, cells are to be isolated in DPBS, using fine forceps, from the entire gut tube staring from the stomach to the hindgut of the embryo at this stage.

(B) Single cells immediately after plated to an un-coated tissue culture Petri dishes. The single cells were dissociated from the gut tube of mouse embryos at E14.5 with a mixture of 0.025% trypsin and 1% collagenase IV.

(C1) and (C2) 2 days after plating, small clusters of neurospheres are found (white arrows in C1), and the flattened and elongated mesenchymal cells grow into a monolayer attached to the bottom of the culture dish (C1). At this time point of the culture, these small clusters or neurospheres are re-plated to another uncoated tissue culture dish (white arrows in C2).

(D) 4 days after first plating, half of the culture medium was refreshed and the neurospheres were re-plated again by transferring them to another dish. The neurospheres are found bigger (white arrows) now.

(E) 6 days after first plating, the neurospheres suspended in the medium were bigger (white arrows) and there are fewer mesenchymal cells attached to the bottom of the dish (white arrow heads).

(F1) to (F4) A new passage (subculture) cycle from single cell suspension to neurospheres: Single cells (F1) were obtained by subculture and cultured with half of self-renewal medium refreshed every 2 days. 2 days after subculture, small neurospheres start to form (white arrows in F2). The neurospheres become bigger and bigger on day 4 (F3) and day 6 (F4) after subculture. The diameter of neurospheres on day 6 is on average 85.79 ± 30.17 μm (F4) (n=214).

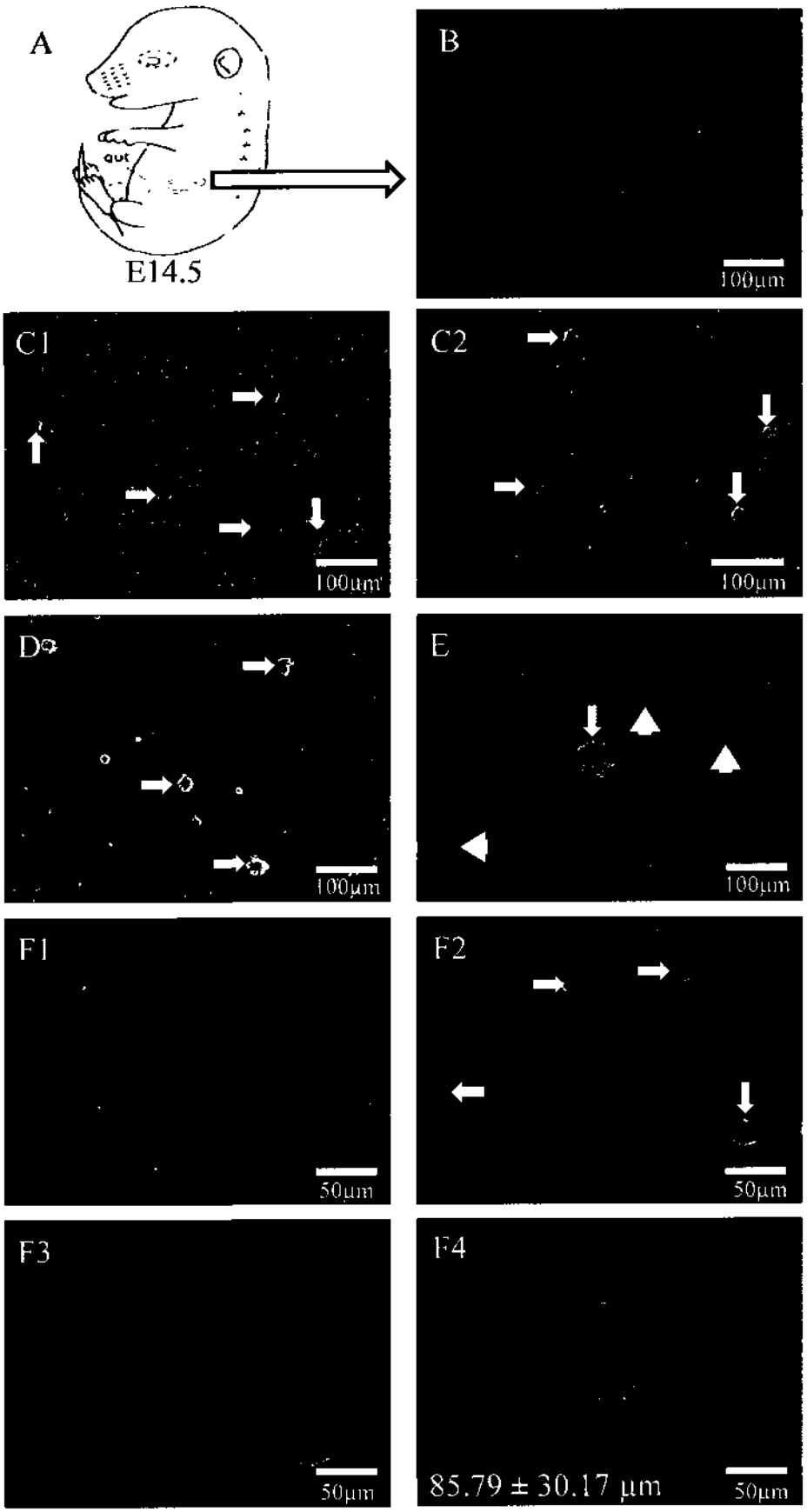

A
aut
E14.5
B
100μm
C1
100μm
C2
100μm
D
100μm
E
100μm
F1
50μm
F2
50μm
F3
50μm
F4
85.79 ± 30.17 μm
50μm

Fig 2.2 Morphology of neurospheres derived from the gut of E14.5 embryos

(A), (B), (C), (D) show the morphology of neurospheres all on day 6 of passage 7 (p7), passage 11 (p11), passage 13 (p13) and passage 22 (p22), respectively.

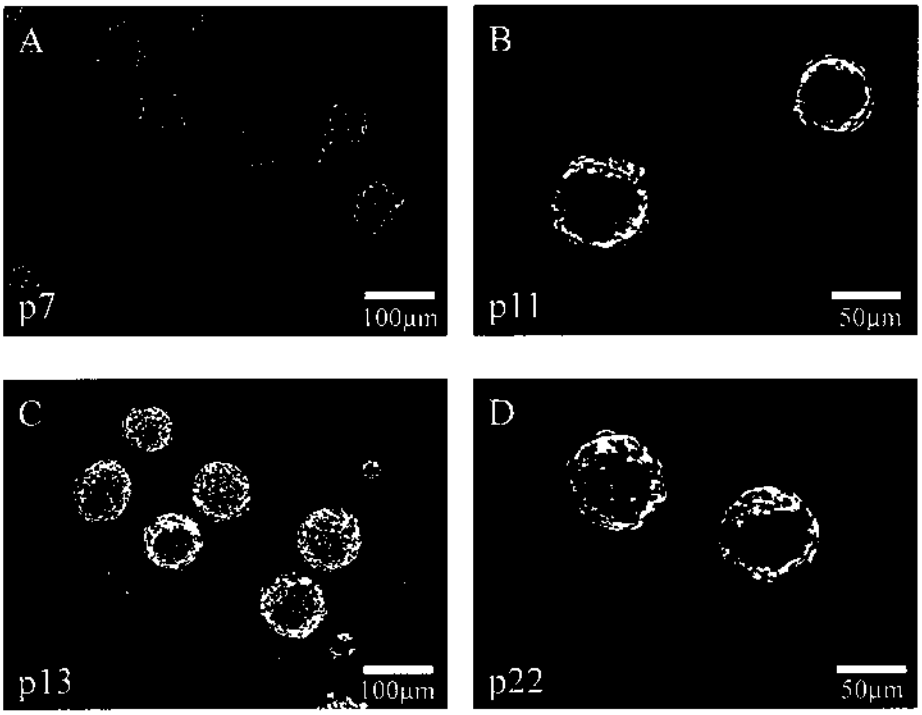

A
p7
100μm
B
p11
50μm
C
p13
100μm
D
p22
50μm

Fig 2.3 Population doubling time

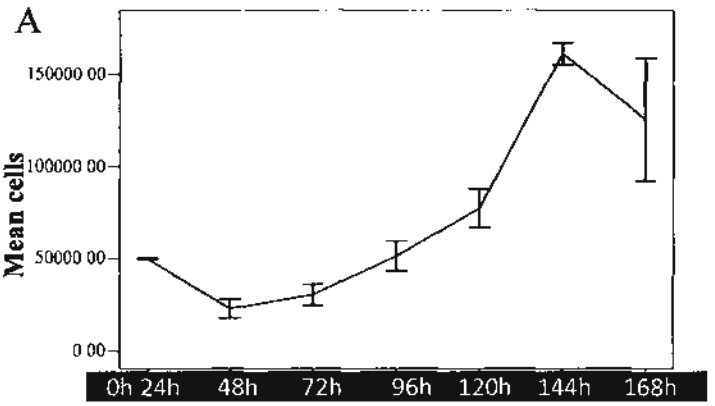
Cell proliferation within neurospheres derived from the gut of E14.5 embryos was analyzed by counting the total cell number after neurospheres were dissociated into single cells at different time points. These cells proliferate *ex vivo* with an average population doubling time of 49.4±10.02 hours.

(A) A typical example of cell numbers of the passage 3 at different time points (n=3 at each time point).

(B) Calculation of the population doubling time using the passage 3 and the algorithm provided by http://www.doubling-time.com as an example. X-axis is the time point of culture and Y-axis shows cell numbers counted at the time points. Using the algorithm on the website, the population doubling time can be calculated.

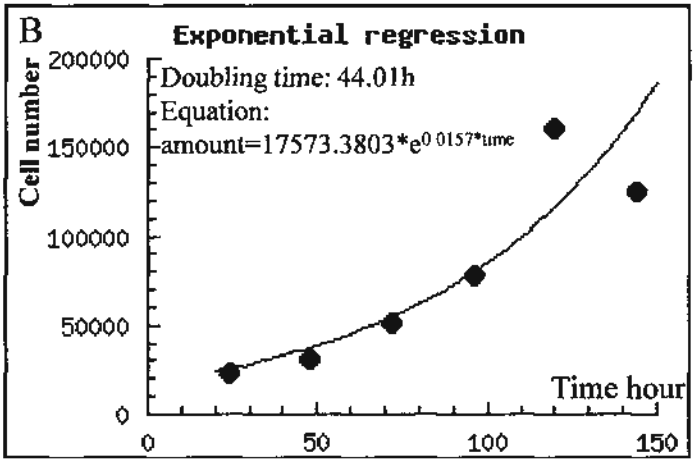
(C) The population doubling time of different passages. The average population doubling time of the enteric neural crest stem cells is 49.4±10.02 hours (n=6).

A



Error bars:+/- 1.00SD
Growth curve

B



C

Passage	Doubling time
p3	44.01 h
p4	44.16 h
p6	38.03 h
p7	49.86 h
P8	53.72 h
p10	66.63 h
Average doubling time	49.4±10.02 h

Fig 2.4 Expression of stem cell markers and mitotic marker in neurospheres

(A1) to (A4) Most of the cells (about 80 %) of neurospheres show both nestin (A1) and p75 (A2) immunoreactivity in cytospin preparations. DAPI staining (A3) is a nuclear staining while (A4) shows the merged image of (A1) to (A3).

(B1) to (B4) Many of the cells (about 80%) of neurospheres also contain p75 (B1), PH3 (B2) and Sox10 (B3) triple immunoreactive cells. (B4) shows the merged image of (B1) to (B3).

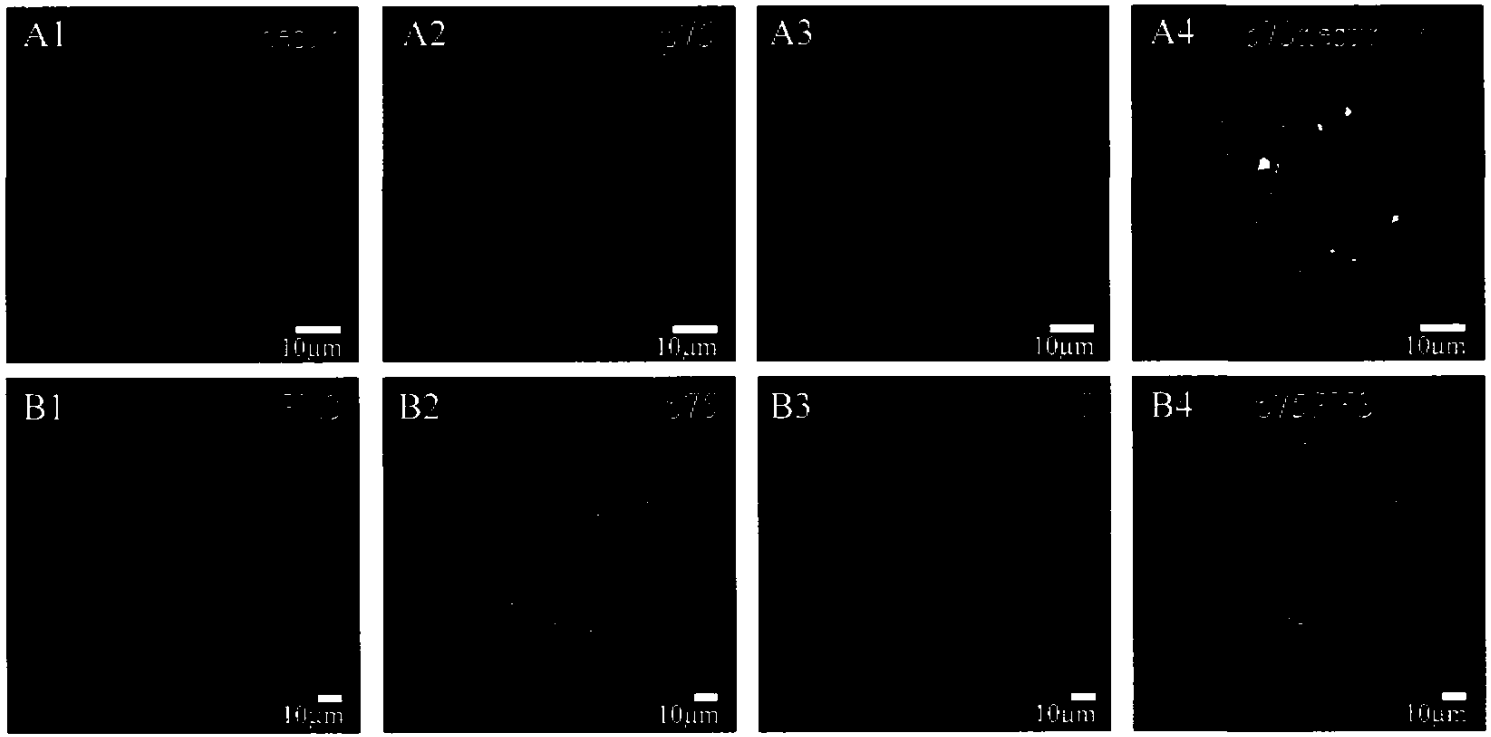

A1
10μm
A2
10μm
A3
10μm
A4
10μm
B1
10μm
B2
10μm
B3
10μm
B4
10μm

Fig 2.5 Expression of differentiated cell markers in neurospheres

(A1) to (A3) Few cells are found to express Tuj1 (arrow) meaning that there are differentiated neurons in the neurosphere in the cytospin preparation.

(B1) to (B3) Few GFAP immunoreactive cells (arrow) are found in the neurospheres in the cytospin preparation.

(C1) to (C3) shows a neurosphere containing SMA immunoreactive positive cells in the cytospin preparation.

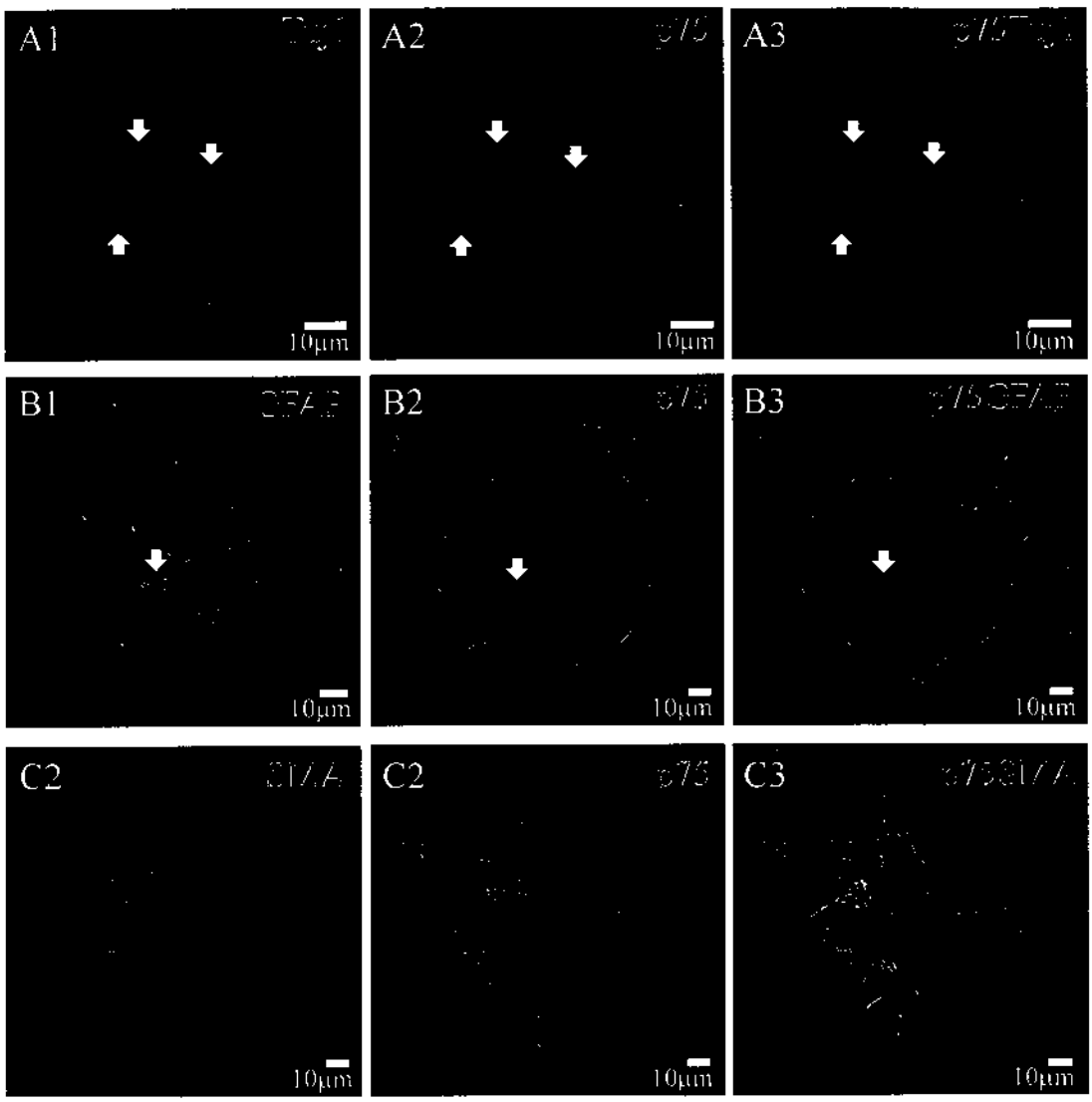
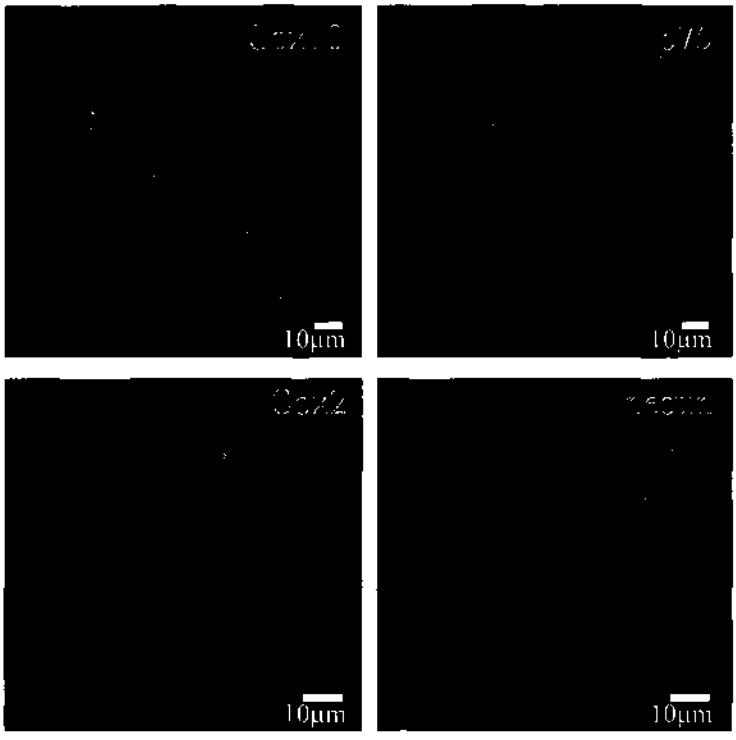

A1
A2
A3
10μm
10μm
10μm
B1
B2
B3
10μm
10μm
10μm
C2
C2
C3
10μm
10μm
10μm

Fig 2.6 Expression of different markers in cells dissociated from neurospheres *ex vivo* by immunohistochemical staining and reverse-transcription PCR (RT-PCR)

(A) The cells dissociated from the neurospheres were prepared by cytospin and stained for Sox10, p75, Sox2 and nestin. 81.11 ± 6.98% cells are Sox10 positive. 95.36 ± 2.18% of the cells express p75. 89.31 ± 11.11% cells are Sox2 positive. 81.06 ± 9.41% cells are nestin positive.

(B) RT-PCR shows presence of the bands for the stem cells markers *Nestin, Sox2* and also neural crest marker *p75*, indicating that cells of neurospheres express both stem cell and neural crest cell markers throughout all the passages examined. In addition, cells of the neurospheres also express the differentiated cell makers *Tuj1* and *Gfap*, indicating some of the cells have already exhibited phenotypes of neurons and glia. Expression of *Sma* was too low to be detected in almost all the passages examined except primary cells (cells in passage 1) from the E14.5 guts, implicating that few cells in the neurospheres differentiated into myofibroblasts.

A


10μm
10μm
10μm
10μm

B

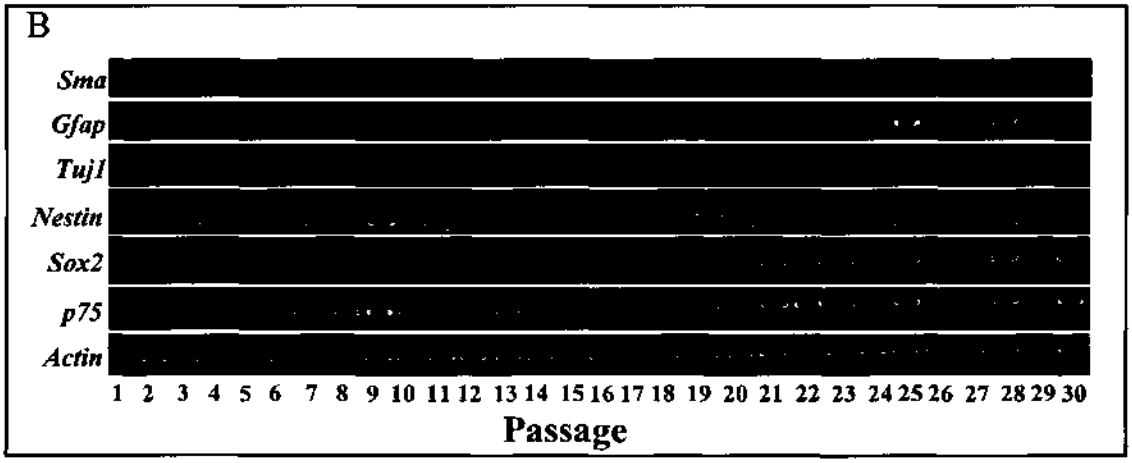
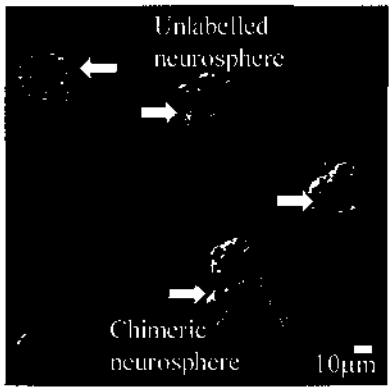

Sma
Gfap
Tuj1
Nestin
Sox2
p75
Actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Passage

Fig 2.7 Co-culture of GFP-labelled and unlabelled cells

(A) Non-chimeric unlabelled neurospheres are shown with white arrows and chimeric neurospheres containing a mixture of GFP^- and GFP^+ cells are shown with a yellow arrow.

(B) Non-chimeric unlabelled neurosphere and non-chimeric GFP-labelled neurosphere are shown with a white arrow and a green arrow respectively.

A


Unlabelled
neurosphere
Chimeric
neurosphere
10µm

B

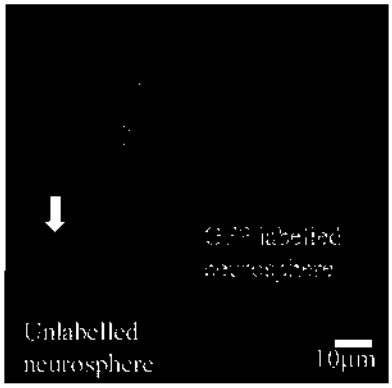

Unlabelled
neurosphere
10µm

Fig 2.8 Induced differentiation of cells within neurospheres

Neurospheres could be induced to differentiate in the differentiation medium. The neurospheres derived from one single cell at a very low cell density (i.e. 500 cells/1.5 ml medium in 35 mm Petri dish) were cultured with the differentiation medium containing 2.5% FBS on the poly-D-lysine coated cover slips for 4 days.

(A1) to (A3) GFAP immunoreactive cells (green arrows), cells double positive for both Tuj1 and GFAP (yellow arrows) or negative for both Tuj1 and GFAP (white arrow) are found within one neurosphere. (A3) is a merged image of (A1) and (A2).

(B1) to (B3) TH (tyrosine hydroxylase) immunoreactivity (arrow in B1) can be detected in some induced neurospheres. DAPI only stains the nuclei (B2). (B3) is a merged image of (B1) and (B2).

(C1) to (C3) SYP (synaptophysin) immunoreactivity (C2) can be detected in some induced neurospheres and it is co-localized with Tuj1 immunoreactivity (C1 and C3). (C3) is a merged image of (C1) and (C2).

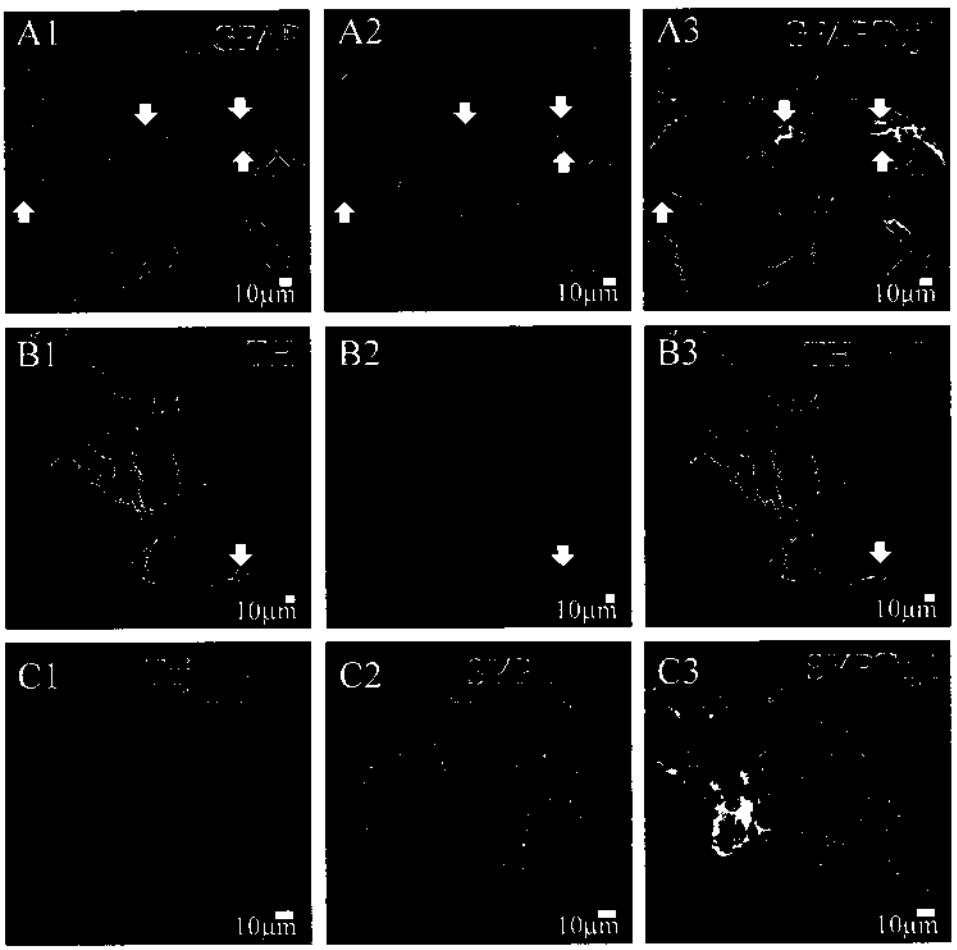

A1
A2
A3
B1
B2
B3
C1
C2
C3
10μm

Fig 2.9 Expression of differentiated cell markers within GFP^+ neurospheres derived from the E14.5 guts of C57BL/6-Tg(CAG-EGFP)1Osb/J mice in the differentiation medium

Upon induced differentiation, the neurospheres become flattened and the cells are elongated. The images on the third (far right, start counting from the far left) column are merged images of the first and second (two left) columns

(A1) to (A3) Within one neurosphere, some cells are Hu positive.

(B1) to (B3) In this differentiated neurosphere, many of the cells are Tuj1 positive.

(C1) to (C3) In one neurosphere, most of the cells differentiate into GFAP positive cells.

(D1) to (D3) In another neurosphere, some cells express smooth muscle actin (SMA).

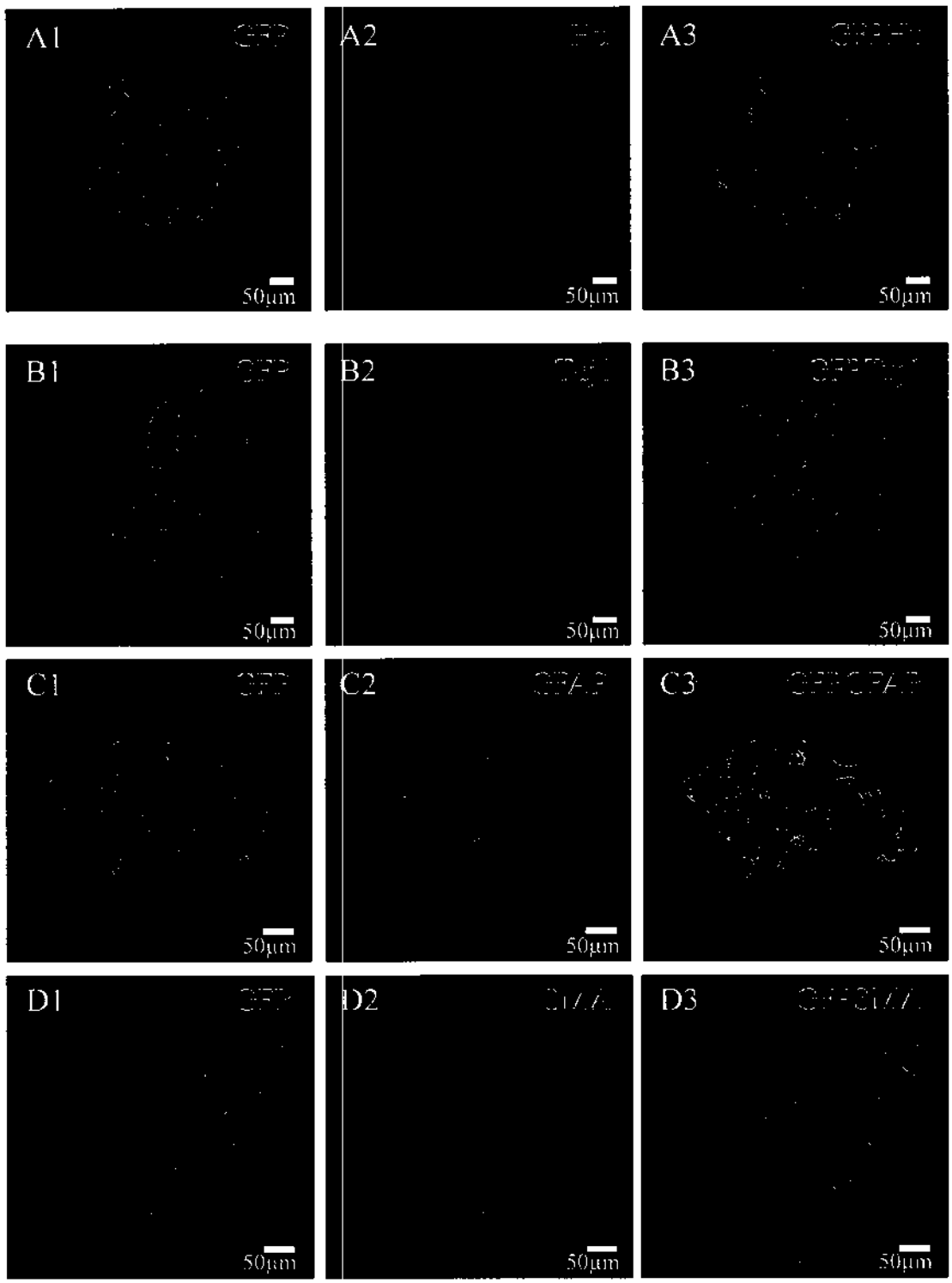

A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3
50μm

Fig 2.10 Immunohistochemical staining for neural crest stem cell marker p75, mitotic marker PH3 and stem cell marker Sox2

(A1) to (A4) The cells within neurospheres are almost all triple immunoreactive for p75 (A1), PH3 (A2) and Sox2 (A3). (A4) is the merged image of (A1) to (A3).

(B1) to (B4) Sox2 (B3) immunoreactive cells also express the stem cell marker nestin (B2) and p75 (B1). (B4) is the merged image of (B1) to (B3).

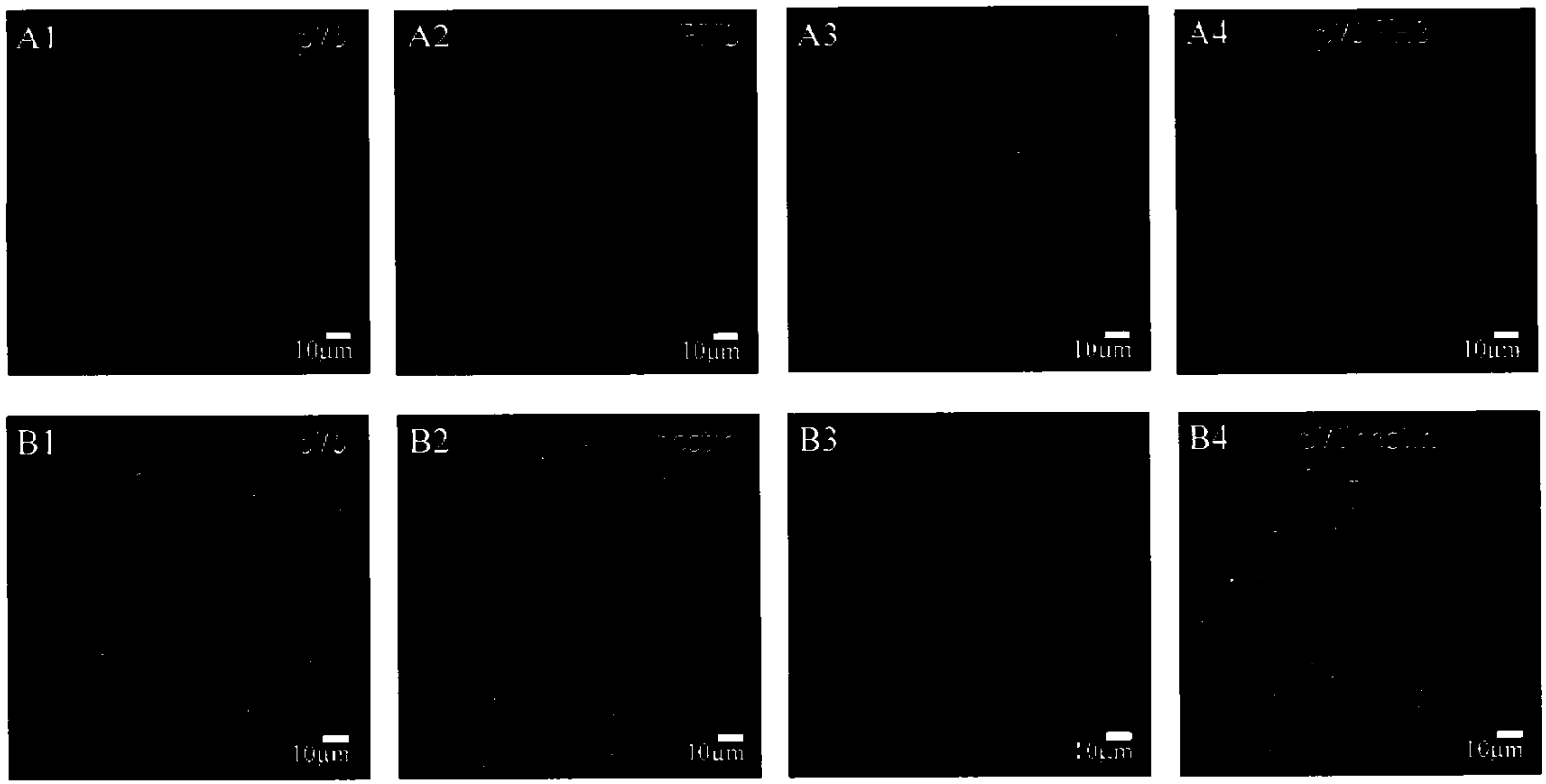

A1
10μm
A2
10μm
A3
10μm
A4
10μm
B1
10μm
B2
10μm
B3
10μm
B4
10μm

Fig 2.11 Expression of neural crest and stem cell markers as well as neuron specific markers in cross sections of mouse embryos at E10.0

The images of the third column (far right, start counting from the far left) are merged images of the first and second (two left) columns.

(A1) to (A3) Neural crest stem cells which migrated from the neural tube to the regions on two sides of the neural tube are p75 (neural crest cell specific marker) and Sox2 (stem cell marker) double immunoreactive in (A1). $Tuj1^+$ neurons are not found on the side of the neural tube (A2).

(B1) to (B3) High magnification of the corresponding boxed areas in (A1) to (A3) shows that early migrating neural crest-derived cells all express p75 and Sox2 (B1) and $Tuj1^+$ neurons are not found on side of the neural tube (B2).

(C1) to (C3) A cross section through the foregut shows the boxed areas magnified in the corresponding (D1) to (D3).

(D1) to (D3) High magnification shows that some neural crest-derived cells within the gut mesenchyme are $p75^+/Sox2^+$ (yellow arrows in D1). Tuj1 immunoreactivity in the foregut mesenchyme is not co-localized with Sox2 immunoreactivity (white arrows in D2 and D3).

(E1) to (E3) In the midgut, some neural crest-derived cells still express p75 and Sox2 (yellow arrows in E1), but $Sox2^+$ cells are $Tuj1^-$ (white arrows in E2 and E3).

(F1) to (F3) In the hindgut, neural crest-derived cells have not yet colonized this gut segment, and hence no p75, Sox2 and Tuj1 immunoreactive cells are found in the mesenchyme of the hindgut.

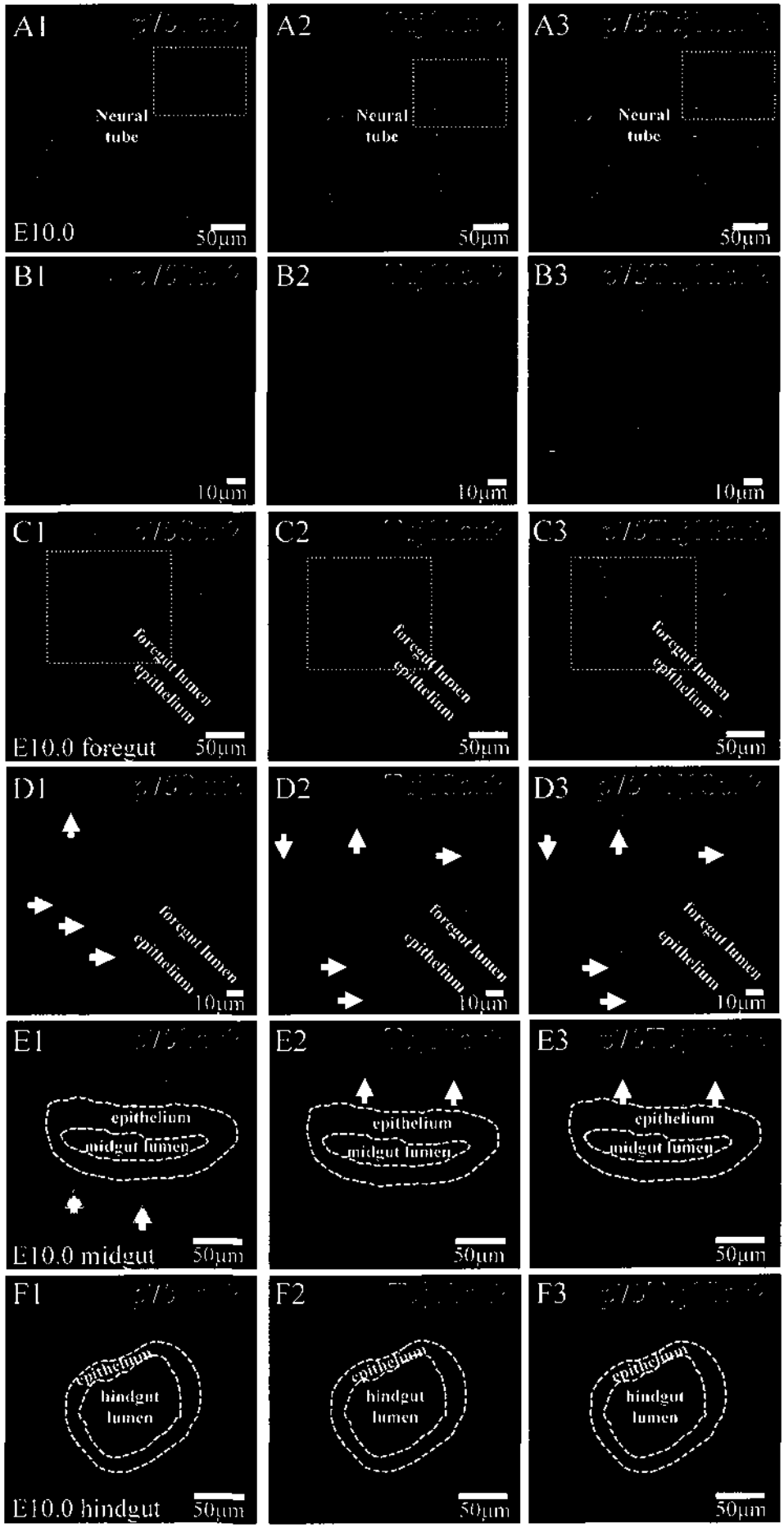

A1
Neural tube
E10.0
50μm
A2
Neural tube
50μm
A3
Neural tube
50μm
B1
10μm
B2
10μm
B3
10μm
C1
foregut lumen
epithelium
E10.0 foregut
50μm
C2
foregut lumen
epithelium
50μm
C3
foregut lumen
epithelium
50μm
D1
foregut lumen
epithelium
10μm
D2
foregut lumen
epithelium
10μm
D3
foregut lumen
epithelium
10μm
E1
epithelium
midgut lumen
E10.0 midgut
50μm
E2
epithelium
midgut lumen
50μm
E3
epithelium
midgut lumen
50μm
F1
epithelium
hindgut lumen
E10.0 hindgut
50μm
F2
epithelium
hindgut lumen
50μm
F3
epithelium
hindgut lumen
50μm

Fig 2.12 Expression of neural crest and stem cell markers as well as neuron specific marker in whole mount preparations and cross sections of the gut at E11.5

The image of the third column (far right, start counting from the far left) are merged images of the first and second (two left) columns except panels (A1), (A2) and (A3).

(A1) to (A3) In the whole mount preparations of the gut, enteric neural crest-derived cells, which are marked by the specific marker p75 (A1), have already migrated to regions near the cecum and many of them have crossed the cecum. Note that many of the p75 immunoreactive neural crest-derived cells are also Tuj1 positive (A2) or Sox2 positive (A3).

(B1) to (B3) High magnification of the midgut shows that Sox2 is expressed strongly with the neural crest cell marker p75 (thin arrows), but when cells become differentiated into neurons and express Tuj1, both p75 and Sox2 expressions are down-regulated (thick arrows).

(C1) to (C3) A cross section of the midgut at the same stage shows that some neural crest-derived cells as marked by p75 immunoreactivity reside in the myenteric plexus (C1) and also express Sox2 (C1) and PH3 (C2 and C3).

(D1) to (D3) High magnification of the boxed area in (C1) demonstrates some neural crest-derived cells ($p75^+$ cells) in the myenteric layer are Sox2 immunoreactive (D1) but only a portion of $Sox2^+$ cells are mitotic as indicated by PH3 immunoreactivity (thick arrows in D1 to D3).

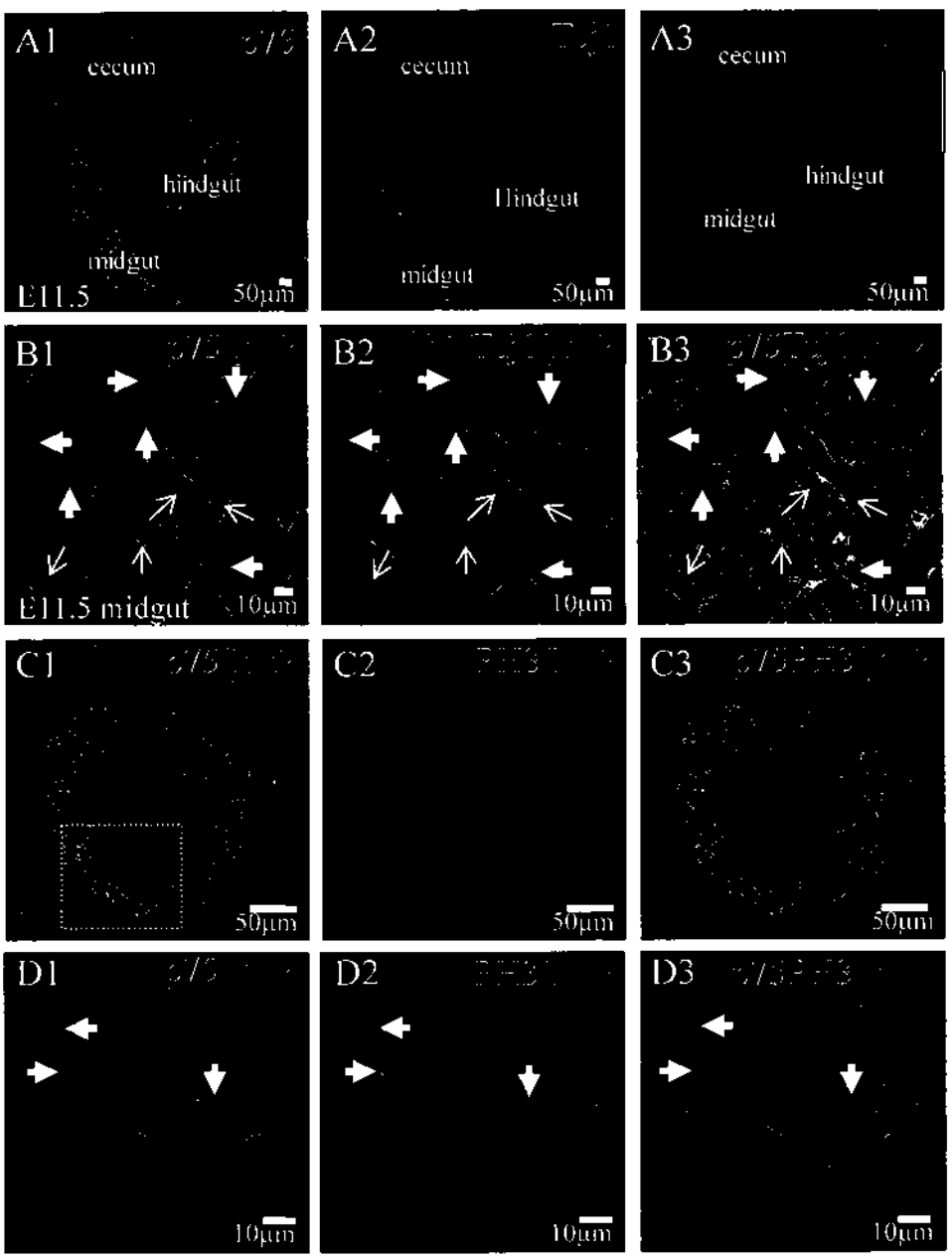

A1
cecum
hindgut
midgut
E11.5
50μm
A2
cecum
midgut
50μm
A3
cecum
hindgut
midgut
50μm
B1
E11.5 midgut
10μm
B2
10μm
B3
10μm
C1
50μm
C2
50μm
C3
50μm
D1
10μm
D2
10μm
D3
10μm

Fig 2.13 Expression of neural crest and stem cell markers as well as neuron specific marker in whole mount preparations of the gut at E12.5

The images of the third column (far right, start counting from the far left) are merged images of the first and second (two left) columns except panels (A1), (A2) and (A3).

(A1) to (A3) At this stage, in the whole mount preparation, the enteric neural crest stem cells, which are $p75^+$ (A1) and/or $Sox2^+$ (A3), have already colonized the hindgut. Some of the neural crest stem cells have become differentiated into neurons, which are immunoreactive to Tuj1 (A2). Note that the enteric neural crest stem cell density in the midgut at E12.5 is higher than those at earlier stages.

(B1) to (B3) High magnification of the whole mount preparations of the midgut shows the differentiated neurons which are Tuj1 immunoreactive (thick arrows in B2), but they weakly express p75 (thick arrows in B1) and do not express Sox2 (thick arrows in B1 to B3).

(C1) to (C3) In the migratory wave front of neural crest-derived cells in the hindgut, neurons expressing Tuj1 (thick arrows in C2) are found to express weak p75 (arrows in C1 and C3) but not to express Sox 2 (arrows in C1 to C3). $p75^+/Sox2^+$ cells (thin arrows in C1) are not Tuj1 positive (thin arrows in C2 and C3).

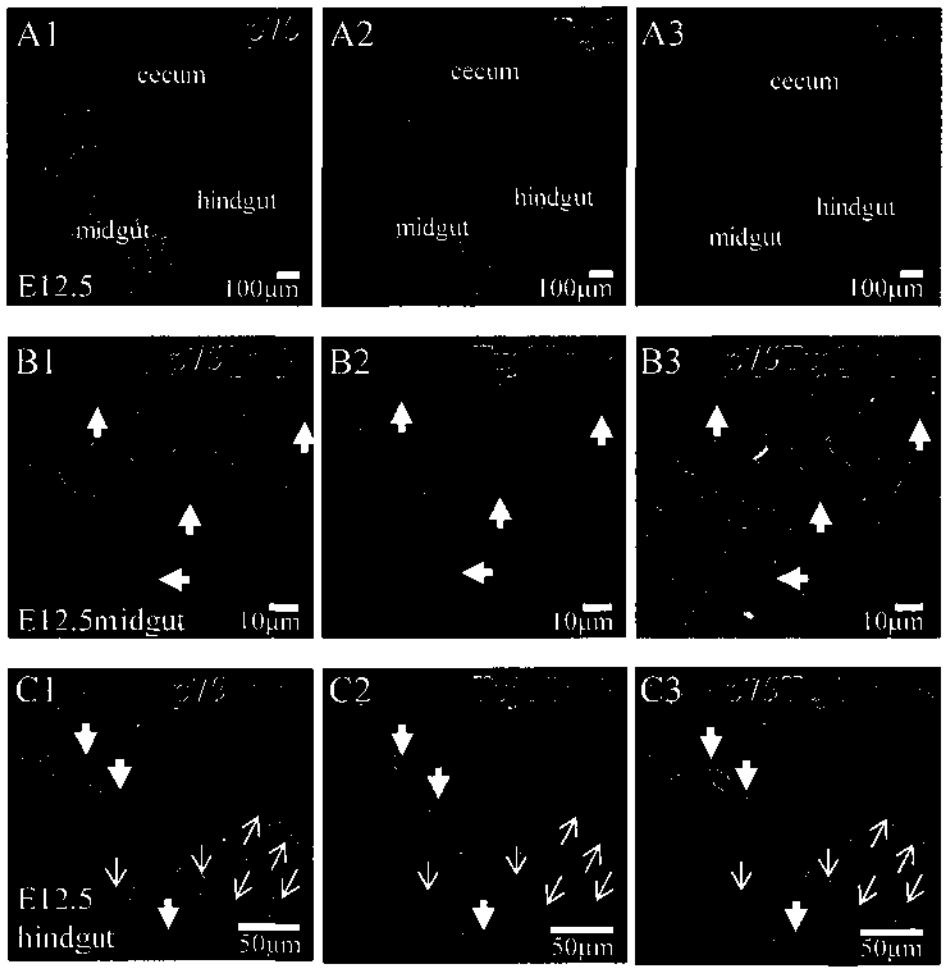

A1
cecum
hindgut
midgut
E12.5
100μm
A2
cecum
hindgut
midgut
100μm
A3
cecum
hindgut
midgut
100μm
B1
E12.5midgut
10μm
B2
10μm
B3
10μm
C1
E12.5
hindgut
50μm
C2
50μm
C3
50μm

Fig 2.14 Expression of neural crest and stem cell markers as well as neuron specific marker in whole mount preparations of the gut at E13.5

The images of the third column (far right, start counting from the far left) are merged images of the first and second (two left) columns except panels (A1), (A2) and (A3).

(A1) to (A3) The migratory wave front of the neural crest-derived cells at this stage is near the distal (terminal) end of the hindgut. In the midgut and hindgut, a higher density of neural crest-derived cells is found, all of which are $p75^+$ (A1). Some of them are differentiated neurons with Tuj1 immunoreactivity (A2). The Sox2 immunoreactivity in the midgut is high but low in the hindgut (A3).

(B1) to (B3) In the midgut, most of the neural crest stem cells are $p75^+$ and $Sox2^+$ (B1) and some differentiated neurons, which are Tuj1 positive (arrows in B2), down-regulate the expression of Sox2 (arrows in B1 to B3).

(C1) to (C3) In the hindgut, many of the cells are still undifferentiated with the expression of p75 and Sox2 (C1), while the differentiated $Tuj1^+$ neurons express low levels of Sox2 (arrows in C1 to C3).

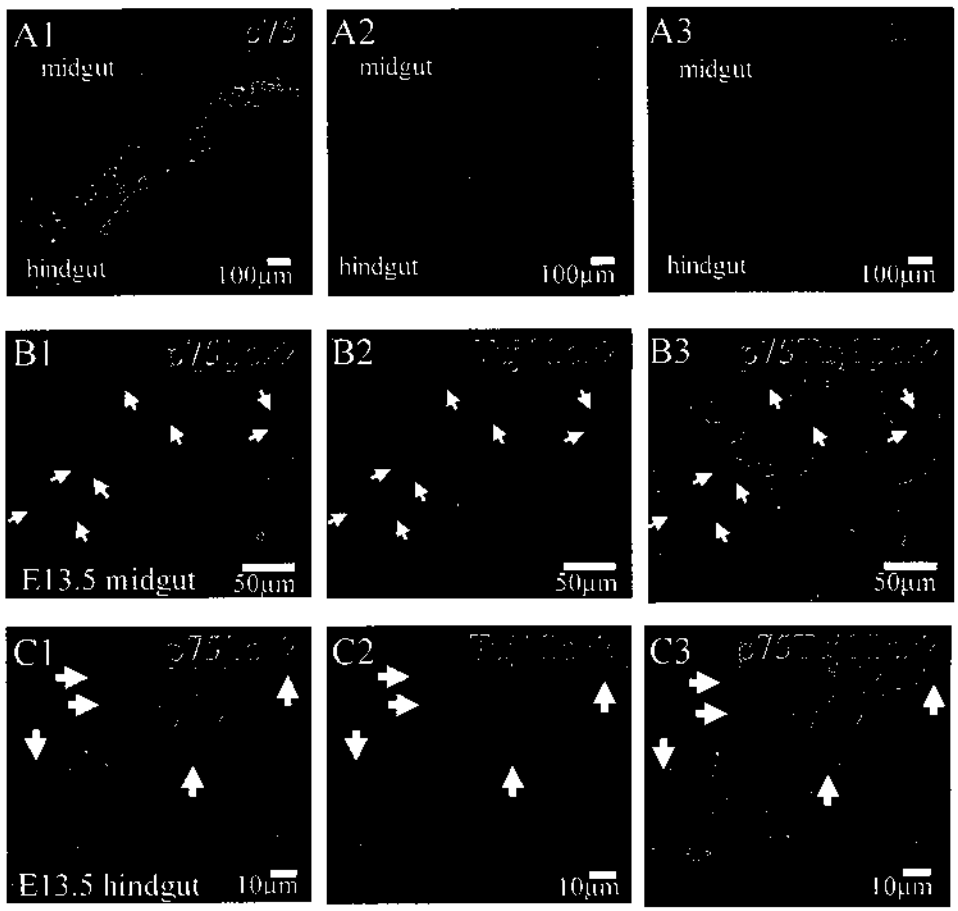
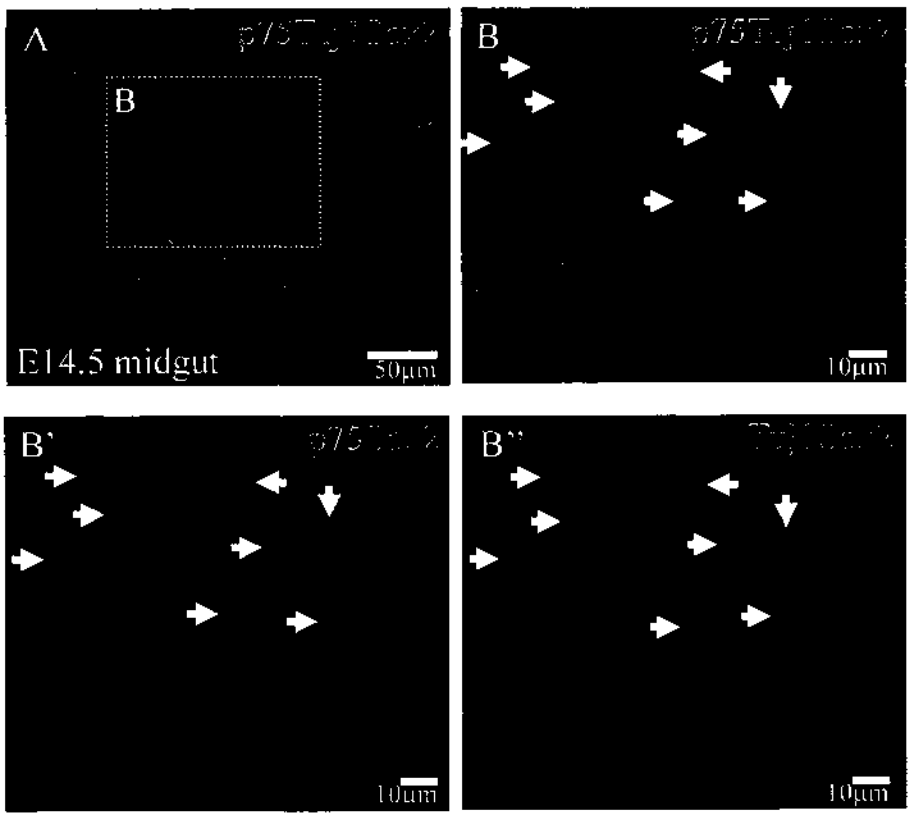

A1
midgut
hindgut
100μm
A2
midgut
hindgut
100μm
A3
midgut
hindgut
100μm
B1
E13.5 midgut
50μm
B2
50μm
B3
50μm
C1
E13.5 hindgut
10μm
C2
10μm
C3
10μm

Fig 2.15 Expression of neural crest and stem cell markers as well as neuron specific marker in whole mount preparations of the gut at E14.5

(A) The enteric nervous system network has already been formed densely in the E14.5 midgut, in which cells express p75, Tuj1 and Sox2.

(B) Higher magnification of the boxed area in (A), which is also the merged image of (B') and (B"), shows more Tuj1 immunoreactive neurons (arrows in B"), which are Sox2 negative (arrows in B' and B"), than at earlier stages. Some of these neurons still express p75 (arrows in B').


A
B
E14.5 midgut
50μm
B
10μm
B'
10μm
B''
10μm

Chapter three

Factors affecting colonization of the gut by enteric neural crest-derived cells

3.1 Introduction

3.1.1 The migration of neural crest cells from neural tube to gut tube

Neural crest cell (NCC) ablation studies (Burns *et al.*, 2000; Peters-van der Sanden *et al.*, 1993; Yntema and Hammond, 1954), quail-chick interspecies grafting (Burns and Le Douarin, 2001; Le Douarin and Teillet, 1973; Le Douarin and Teillet, 1974) and other cell-tracing experiments (Epstein *et al.*, 1994) demonstrate the migration of vagal neural crest cells. Vagal neural crest cells that emigrate from the level of the neural tube adjacent to somite 1 to 7, enter the foregut around E9.5, migrate and colonize the entire gut in a rostral-to-caudal manner in mice (Kapur *et al.*, 1992; Wallace and Burns, 2005). A contribution from the sacral neural crest to the post-umbilical gut has also been demonstrated in the chick and mouse (Burns and Le Douarin, 1998; Le Douarin and Teillet, 1973; Pomeranz and Gershon, 1990; Pomeranz *et al.*, 1991; Serbedzija *et al.*, 1991), although the contribution and the phenotypes that arise from these cells have yet to be fully elucidated in mammals (Wallace and Burns, 2005).

Besides the primary migration wave, i.e. the rostral-to-caudal migration of vagal neural crest-derived cells in the gut tube, a secondary migration wave of neural

crest-derived cells also takes place across the radius of the developing gut in mice and chicks (Burns and Le Douarin, 1998; Kapur *et al.*, 1992; McKeown *et al.*, 2001; Pham *et al.*, 1991; Young *et al.*, 1998). In the small and large intestines of mice and the small intestines of chicks, neurons and glia initially form ganglion plexuses in the myenteric region, and the submucosal plexus develops later (Burns and Le Douarin, 1998; McKeown *et al.*, 2001; Pham *et al.*, 1991; Young *et al.*, 1998). In contrast, the submucosal plexus develops before the myenteric plexus in the large intestine of chicks (Burns and Douarin, 1998). Secondary migration of neural crest-derived cells and the spatio-temporal development of the annular structures and ganglion plexuses have not yet been studied in humans (Fu *et al.*, 2004b).

Neural crest cells are heterogeneous,meaning that after neural crest cells emigrate from the neural tube and enter the pre-enteric migration pathway, some of them start to differentiate during the migration period and some maintain the abilities to self renew, proliferate and differentiate, which are all properties of stem cells. The post-migratory neural crest cells in the gut tube also contain undifferentiated, differentiating and differentiated neural crest-derived stem cells. It was shown that only 1-2% of cells of the rat gut tube at E14.5 were strongly $p75^+$ and α4-integrin$^+$ suggesting that only a very small number of enteric cells within the embryonic gut tube express stem cell markers. When cells of neurospheres were plated clonally, some 60% of cells showed p75 and α4-integrin double immunoreactivity, and of these, 80% generated mixed neuron/glia/myofibroblasts colonies (Iwashita *et al.*, 2003).

3.1.2 Genes and molecules involved in the development of the enteric nervous system

Over the last decades, a number of genes which are summarized in the Table 5

have been identified playing important roles in the development of the enteric nervous system.

Table 5 Genes involved in the morphogenesis and differentiation of the enteric nervous system (Holschneider and Puri, 2008)

Gene	Human Chromosomal assignment	Function
RET	10q11.2	Tyrosine kinase receptor
GDNF	5p12–13.1	Glial cell-derived neurotropic factor
NTN	19q13.3	Neurturin, RET ligand
GFRα1	10q26	GDNF family receptor alpha 1
EDNRB	13q22	Endothelin receptor-B
EDN-3	20q13 2–13.3	Endothelin-3
ECE-1	1p36.1	Endothelin-converting enzyme
SOX 10	22q13.1	Sry/HMG box transcription factor
PHOX2B	4p12	Paired-like homeobox 2b
PAX3	2q35	Paired box gene 3
ZFHX1B (SIP1)	2q22	Smad-interacting protein-1
MASH1	12	Mammalian achaete-scute homologue 1
IHH	2q33-q35	Indian hedgehog
SHH	7q36	Sonic hedgehog
HOX11L1	2p13.1	*Enx*, enteric neuron homeobox

RET/GDNF/GFRα1 signalling pathway

This signalling pathway has been shown to promote survival of neurons, mitosis of neuronal progenitor cells and differentiation of neurons and neurite extension by *ex vivo* and *in vivo* assays (Montgomery *et al.*, 1999; Newgreen and Young, 2002a; Taraviras and Pachnis, 1999).

RET (REarranged during Transfection) protein is a 1114 amino acid transmembrane receptor with a cadherin-like extracellular domain, a cysteine-rich region and an intracellular tyrosine kinase domain (Ceccherini *et al.*, 1993). The RET proto-oncogene has been demonstrated to be a major gene causing Hirschsprung's disease (Edery *et al.*, 1994; Kusafuka and Puri, 1997a; Kusafuka and Puri, 1997b; lou *et al.*, 1993; Romeo *et al.*, 1994). The RET receptor is the signalling component of receptor complexes with four ligands: glial derived neurotropic factor (GDNF), neurturin (NTN), artemin (ATM) and persephin (PSP) (Newgreen and Young, 2002a; Newgreen and Young, 2002b). The complete receptor complex includes the RET receptor tyrosine kinase and a glycosyl phosphatidyl inositol-anchored binding component (GFRα1, GFRα2, GFRα3, and GFRα4) (Jing *et al.*, 1996; Jing *et al.*, 1997; Newgreen and Young, 2002b). Through the receptor complex RET/GFRα-1, RET mediates the expression of phosphorylating focal adhesion kinase (FAK), paxillin and $p130^{Cas}$ which are associated with focal adhesions and believed to play a role in integrin-mediated intracellular signalling (Murakami *et al.*, 1999). RET also increases the expression of integrins thereby enhancing the invasive and adhesion to extracellular matrix (ECM) proteins by GDNF and focal adhesions by pancreatic cancer cells (Funahashi *et al.*, 2005). *In vivo* the absence of GDNF/GFRα1-mediated signalling leads to the failure of enteric nervous system development, whereas the absence of NTN/GFRα2-mediated signalling leads to more subtle abnormalities in enteric nervous system development (Newgreen and Young, 2002b). Hirschsprung-associated RET mutation impair pro-apoptotic activity of RET which is inhibited in the presence of its ligand glial cell line-derived neurotrophic factor (GDNF) (Bordeaux *et al.*, 2000). Mutations of RET account for 50% of familial and 15-20% of sporadic cases of Hirschsprung's disease (Kusafuka and Puri, 1997b;

Martucciello *et al.*, 2000). RET knockout mice exhibit total intestinal aganglionosis and renal agenesis (Schuchardt *et al.*, 1994). Over-expression of RET in sacral neural crest cells increased their ENS developmental potential such that larger numbers of cells entered the gut earlier in development in heterotopic quail–chick grafting studies (Delalande *et al.*, 2008).

GDNF, known as a major survival factor for many types of neurons, is a transforming growth factor-β (TGF-β) related 211 residue protein, proteolytically cleaved to a 134 residue mature protein. The development of the enteric nervous system is dependent upon the action of GDNF, which stimulates the proliferation and survival of neural crest precursor cells in the embryonic gut (Holschneider and Puri, 2008). when the enteric neural crest-derived cells are colonizing the midgut, GDNF expression is unregulated in the more posterior cecum anlage (Natarajan *et al.*, 2002). It has been reported that GDNF is the ligand of RET (Durbec *et al.*, 1996a). To activate RET, GDNF needs the presence of a glycosyl phosphatidyl inositol (GPI)-linked co-receptor GFRα1 (Jing *et al.*, 1996). Mice carrying the homozygous null mutation in GDNF demonstrate the lack of kidneys and enteric nervous system, confirming the crucial role of GDNF in the development of the enteric nervous system (Pichel *et al.*, 1996; Sanchez *et al.*, 1996). Although a causative role for GDNF mutations in some patients with Hirschsprung's disease has been suggested, the occurrence of such cases is uncommon, and it is more likely that the GDNF mutations are involved in modulation of the Hirschsprung's disease phenotype via its interaction with other susceptibility loci such as RET (Amiel *et al.*, 2008; Angrist *et al.*, 1995).

EDN3–EDNRB signalling pathway

Endothelin 3 (EDN3) is a peptide in a secreted-peptide family and a functional

ligand for the 7-transmembrane G-protein coupled receptors, endothelin receptor-B (EDNRB) (Burns and Thapar, 2006). EDNRB is expressed primarily by migrating enteric neural crest-derived cells while EDN3 is expressed in the midgut and hindgut mesoderm during early phases of enteric neural crest cell migration and at high levels in the cecum and proximal colon as enteric neural crest-derived cells are colonizing terminal gut regions (Barlow *et al.*, 2003; Heanue and Pachnis, 2007; Leibl *et al.*, 1999). EDN3–EDNRB expression pattern and the *Edn3* and *Ednrb* mutant mice with delayed enteric neural crest-derived cells migration (Barlow *et al.*, 2003; Lee *et al.*, 2003; Ro *et al.*, 2006) could explain that EDN3–EDNRB signalling is involved in regulating the normal migration of enteric neural crest-derived cells (Heanue and Pachnis, 2007). Conditional deletion studies have identified a critical time window between E10 and E12.5 for EDN3–EDNRB signalling during enteric neural crest-derived cells migration (Shin *et al.*, 1999). EDN3–EDNRB signalling also has a role in maintaining enteric progenitors in a proliferative state, because enteric neural crest cell numbers are reduced in *Edn3* mutant mice (Barlow *et al.*, 2003; Bondurand *et al.*, 2006) and enteric neural crest-derived cells differentiation is inhibited in the presence of EDN3 (Bondurand *et al.*, 2006; Hearn *et al.*, 1998; Nagy and Goldstein, 2006; Wu *et al.*, 1999). Between RET/GDNF/GFRα1 Signalling and EDN3–EDNRB signalling, there is interaction which is extremely important during the development of the enteric nervous system. *Ex vivo* studies show that EDN3 enhances the GDNF-induced proliferative effect and decreases the differentiation of neurons induced by GDNF (Barlow *et al.*, 2003; Hearn *et al.*, 1998; Wu *et al.*, 1999). Protein kinase A (PKA) is likely to be an important component in the interactions between RET- or EDNRB-mediating signalling, as the effects of both GNDF and EDN3 on the proliferation and migration of neural crest cells are mediated by PKA (Barlow *et al.*,

2003). *EDNRB* and *EDN3* mutant cases are associated with Waardenburg syndrome, which is characterized by colonic aganglionosis, pigmentation defects and deafness (Amiel and Lyonnet, 2001; Brooks *et al.*, 2005) in human and mice models carrying mutations in *Edn3*, *Ednrb* or *Ece1* (endothelin converting enzyme which converts an inactive precursor form of EDN3 into an active form) also exhibit aganglionosis in addition to pigmentation defects (Baynash *et al.*, 1994; Hosoda *et al.*, 1994; Yanagisawa *et al.*, 1998).

Transcription factors

PHOX2B

PHOX2B is a paired box homeodomain transcription factor expressed by all developing autonomic neurons, including enteric neurons (Brunet and Pattyn, 2002; Pattyn *et al.*, 1997) and can regulate the expression of *Ret* (Lo *et al.*, 1998; Morin *et al.*, 1997; Pattyn *et al.*, 1999). Mice and zebrafish lack *Phox2b* display aganglionosis due to the absence of RET in the enteric neural crest-derived cells (Pattyn *et al.*, 1997).

SOX10

Haploinsufficiency for SOX10, a sex-determining region Y (SRY)-related high mobility group (HMG)-box transcription factor, is associated with Hirschsprung's disease with Waardenburg syndrome (Amiel and Lyonnet, 2001; Brooks *et al.*, 2005), and accounts for <5% of Hirschsprung's disease cases (McCallion *et al.*, 2003). Mice and zebrafish carrying mutant *Sox10* exhibit colonic aganglionosis and hypopigmentation similar to the phenotypes of Hirschsprung's disease (Dutton *et al.*, 2001; Herbarth *et al.*, 1998; Southard-Smith *et al.*, 1998). The protein product of this gene has DNA binding domains and is active in neural crest derived cells as a transcriptional regulator (Mollaaghababa and Pavan, 2003). SOX10 is initially

required for the survival of undifferentiated enteric neural crest-derived cells and later required for glial fate acquisition (Britsch *et al.*, 2001; Kapur, 1999; Kim *et al.*, 2003; Paratore *et al.*, 2002; Paratore *et al.*, 2001; Southard-Smith *et al.*, 1998). SOX10 regulates the expression of a number of different genes alone or together with other transcription factors such that SOX10 is required for the induction of *Phox2b* and SOX10 together with PAX3 activate *Ret* (Newgreen and Young, 2002a). As well as positively regulating the promoter of the *P0* gene in the neural crest derived Schwann cells, SOX10 also activates the microphthalmia-associated transcription factor (MITF) promoter and this is increased further by PAX3 (Bondurand *et al.*, 2000; Peirano and Wegner, 2000; Potterf *et al.*, 2000). Recently, Schizophrenia 1 (Disc1) is found as a potent regulator of foxd3 and sox10 expression, cranial neural crest migration and specification in the zebrafish (Drerup *et al.*, 2009). Normal enteric neural crest-derived cells development also requires *Zfhx1b* (Zinc Finger HomeoboX 1b; also known as ZEB2, Zinc finger E-box Binding homeobox 2 or SIP1, Smad Interacting Protein 1) and *Sox10* to coordinate and balance interactions (Stanchina *et al.*, 2010).

PAX3

PAX3 is a member of the paired-box-containing family of transcription factors (Goulding *et al.*, 1991). In embryonic mice, Pax3 is expressed by many neural crest derived cells, including enteric neuron and melanocyte precursors (Goulding *et al.*, 1991; Lang *et al.*, 2000). Homozygous mutant *PAX3* in humans and mice are lethal with neural tube and cardiac defects and an absence of enteric neurons from the gut segment caudal to the stomach (Ayme and Philip, 1995; Lang *et al.*, 2000). Heterozygous mice have no obvious enteric neuronal defects (Lang *et al.*, 2000). PAX3 interacts with SOX10 to initiate RET expression and PAX3 is essential for

initiating RET expression (Newgreen and Young, 2002a).

MASH1 (mammalian achaete-scute homologue 1)

Mash1 encodes a transcription factor that belongs to the basic helix-loop-helix (bHLH) family. In embryonic mice, it is expressed transiently during embryogenesis in the central nervous system and in enteric neural crest-derived cells (Durbec *et al.*, 1996b; Guillemot and Joyner, 1993; Guillemot *et al.*, 1993; Johnson *et al.*, 1990; Lo *et al.*, 1991). MASH1 is required for the differentiation of neural crest cells but not for migration as evident by the fact that in *Mash1*$^{-/-}$ mice neural crest cells could migrate to their correct locations but fail to differentiate into neurons (Guillemot *et al.*, 1993; Sommer *et al.*, 1995). MASH1 activated *Ret* indirectly via the action of PHOX2A (Hirsch *et al.*, 1998; Lo *et al.*, 1998).

HOX11L1

HOX11L1, also known as *TLX2*, *Ncx*, or *Enx*, belongs to a family of orphan homeobox genes with two other members, *TLX1* and *TLX3*, and encodes a protein of 285 amino acids which is specifically expressed in several tissues derived from neural crest cells at the end of their migration (Borghini *et al.*, 2006). HOX11L1 is expressed in the neurons of the developing enteric nervous system and in other neural crest derived neurons (Newgreen and Young, 2002a). Mice with mutation of the *Hox11L1* gene develop an INDB (intestinal neuronal dysplasia type B, a human congenital disorder with orphan pathological phenotype and controversial clinical significance)-like condition—that is megacolon with enteric nervous system hyperplasia in the colon and hypoplasia in the ileum (Hatano *et al.*, 1997; Shirasawa *et al.*, 1997), followed by death of some of these neurons (Hatano *et al.*, 1997). At present, the finding of PHOX2B–*HOX11L1* promoter interactions suggests a

physiological role in the transcription-factor cascade underlying the differentiation of neuronal lineages of the autonomic nervous system during human embryogenesis (Borghini *et al.*, 2006). However, although some of the regulatory elements for this gene have been defined (Iitsuka *et al.*, 1999), there is not much information on the genes that it regulates or on the transcriptional targets of *HOX11L1* (Newgreen and Young, 2002a).

ZFHX1B (SIP1)

ZFHX1B (Zinc Finger HomeoboX 1b; also known as ZEB2, Zinc finger E-box Binding homeobox 2 or SIP1, Smad Interacting Protein 1) belongs to the Zfhx1 family proteins in vertebrates (Stanchina *et al.*, 2010). Studies on mice have shown that ZFHX1B is a Smad1-, 2-, 3-, and 5-interacting protein (Verschueren *et al.*, 1999). The Smad proteins are central for the transduction of TGF-β (including BMP-4) signals to the nucleus (Massague, 1998). Though there is no significant abnormal phenotype in *Zfhx1b* heterozygous guts but removal of one *Zfhx1b* allele in *Sox10* heterozygous animal results in much more severe phenotypes than that of single mutants, indicating a strong genetic interaction between *Sox10* and *Zfhx1b* during enteric nervous system development (Stanchina *et al.*, 2010).

Other factors

There are other influencing factors affecting the development of the enteric nervous system. Copy number variations (CNVs) in the coding region of RET, EDN3, GDNF and ZFHXIB (HSCR related genes) are not a common molecular cause of Hirschsprung's disease, at least in the Spanish population but further studies are required to determine the presence of copy number variations affecting non-coding regulatory regions, as well as other candidate genes (Nunez-Torres *et al.*, 2009).

Indian hedgehog (IHH) and Sonic hedgehog (SHH) expressed in the gut endoderm (Bitgood and McMahon, 1995; Echelard *et al.*, 1993) may influence the enteric nervous system development. IHH and SHH bind to the transmembrane protein-Patched (Ptc), inhibit Ptc, derepress the cell surface molecule–Smoothened (Smo) signalling, activate the transcription factor Gli1, and also induce expression of the TGF-β family member and bone morphogenetic protein 4 (BMP4) (Marigo *et al.*, 1996; Narita *et al.*, 1998; Newgreen and Young, 2002a; Roberts *et al.*, 1995; Roberts *et al.*, 1998). SHH also controls the proliferation and differentiation of neural crest cells and modulates the responsiveness of neural crest cells toward GDNF inductions (Fu *et al.*, 2004a).

SNARE [soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptor] proteins-dependent exocytosis is important for the migration of precursors of the enteric nervous system and neurite extension (Vohra *et al.*, 2006). Electrical activity and neurotransmitter release (Hao *et al.*, 2010) have effects on the migration of the enteric nervous system development. Endothelial cells provide a substrate for the migration of enteric neural crest-derived cells via an interaction between β1-integrins on the enteric neural crest cell surface and extracellular matrix proteins expressed by the intestinal vasculature (Nagy *et al.*, 2009). BBS (Bardet-Biedl syndrome) proteins modulate neural crest cells migration, contributing to craniofacial morphogenesis and development of the enteric nervous system which can explain the association of Hirschsprung's disease with BBS (Tobin *et al.*, 2008).

3.1.3 Objective of this chapter

The rostral-to-caudal migration and colonization of the gut by vagal enteric neural crest-derived cells is delayed in the *Sox10*$^{Dom/+}$ mouse embryos (Kapur *et al.*,

1996; Wang, 2006). When these enteric neural crest-derived cells fail to colonize the terminal hindgut, the aganglionic region becomes non-functional and results in a condition like Hirschsprung's disease in humans. Among all the treatment strategies, cell therapy is one of the possible paradigms to repair the abnormal region of megacolon in which abnormal or absence of enteric neurons are found. However, before I can move on to explore cell therapy as a mean of recruiting aganglionosis; I have to first understand the normal migration of enteric neural crest-derived cells within the hindgut and the interactions of migrating enteric neural crest-derived cells with the microenvironment through which the enteric neural crest-derived cells migrate in normal and mutant embryos. To achieve these, standard double or triple immunohistochemical staining and recombination organotypic culture which combined a donor gut segment carrying donor neural crest-derived cells with a recipient gut segment were carried out for this chapter as listed below:

a. To examine the spatio-temporal pattern of enteric neural crest-derived cells colonization within the *Dom* embryonic gut from E11.5 to E14.5 using p75 and Sox10 as the neural crest cell markers in whole-mount immunohistochemical staining.
b. To detection the muscle layer differentiation pattern in the embryonic gut from E11.5 to E15.5 using smooth muscle actin (SMA) as the marker for early differentiating muscle cells in immunohistochemical staining on cryo-sections, with the purpose of finding the possible role of muscle differentiation in neural crest cell migration in the gut.
c. To compare, in the organotypic recombination culture, the colonization of the hindgut by enteric neural crest-derived cells. In the organotypic culture, a segment of the hindgut from the $Sox10^{+/+}$ and $Sox10^{Dom/+}$ embryos was used as the donor

while another segment of the hindgut isolated from the $Sox10^{+/+}$, $Sox10^{Dom/+}$ and $Sox10^{Dom/Dom}$ embryos was used as the recipient for the donor neural crest-derived cells. Using immunohistochemical staining on whole mount preparations of the recombined gut tissues, the migration of donor neural crest-derived cells in the recipient hindgut segment could be revealed.

d. Also in the organotypic recombination culture, to find out and compare different colonization pattern of donor neural crest-derived cells in the aganglionic recipient segment of the midgut and hindgut at different developmental stages from E11.5 to E15.5 by whole mount immunohistochemical staining in order to determine the influences of the developmental stages of the recipient gut segment on neural crest cell migration.

e. Then compare the colonization of donor neural crest-derived cells in the ganglionic and aganglionic recipient gut segments to determine the influences of an enteric nervous system on neural crest cell migration.

Through the experiments mentioned above, the cell-autonomous effects of neural crest-derived cells themselves and also the non-cell autonomous influences from the surrounding microenvironment through which neural crest-derived cells migrated, including differentiation of the neighboring muscle layers, developmental age of the gut and the presence of an existing enteric nervous system, on migration could be better understood.

3.2 Materials and Methods

3.2.1 *Dom* mouse Genotyping by polymerase chain reaction (PCR)

For *Dom* adult mouse genotyping, a length of 0.5 cm from the tail was cut, and for *Dom* embryonic mouse genotyping, the tissues from visceral yolk sac, tail or limb were used. The PCR homogenizing buffer was prepared by mixing: 25 µl of 1M KCl, 5 µl of 1M Tris-HCl pH7.5 (Invitrogen, 15567-027), 0.5 µl of 10mg/ml gelatin (BDH, 440454B), 0.225 µl of 0.45% Nodinet P-40 (Sigma, N-3516), 0.225 µl of 0.45% Tween 20 (USB, 20605), 16.55 µl of autoclaved H_2O and 2.5 µl of 20 mg/ml Proteinase K (BDH, added last). 25 µl or 50 µl homogenizing lysis buffer was used to digest the tissues from embryos or adult tails respectively. The tissues were incubated with the lysis buffer in a 1.5 ml Eppendorf tube at 57℃ for 8 hours or overnight with thorough mixing in a Thermomixer comfort (Eppendorf) until tissue clumps were not observed. The crude lysate was incubated at 99℃ for 30 minutes to stop the action of Proteinase K. The lysate was centrifuged at the maximum speed for a few minutes at room temperature in a microcentrifuge (Epppendorf).

The 0.5 µl supernatant was added to a 14.5 µl PCR reaction mixture. The 14.5 µl PCR reaction mixture was composed of 9.58 µl of autoclaved MiliQ water, 3 µl of 5x GoTaQ PCR buffer (Promega), 0.9 µl of 25mM $MgCl_2$ (Promega), 0.3 µl of 10mM dNTPs, 0.3 µl of 10µM forward primer, 0.3 µl of 10µM reverse primer and 0.12 µl of 5u/µl Taq polymerase (Promega). The D15MIT71 primers were used to amplify the polymorphic microsatellite sequence which is a linkage marker for the *Dom* allele (Kapur *et al.*, 1996). The sequences of the primers were synthesized as follows: forward primers: 5'-TAA TGA CAG TGC CAA ATC TTG G-3'; reverse primer: 5'-

CCC AAC TCA TAT GTA TTA TCC TGC-3'. The PCR reactions were run with the following cycles: 94℃ for 5 min for initial denaturation, 29 amplification cycles each including 94℃ for 30 seconds for cycle denaturation, 57℃ for 30 seconds for primer annealing and 72℃ for 30 seconds for primer extension and finally at 72℃ for 7 minutes for final extension.

The PCR products were separated by 3% Ultra Pure™ Agarose gel (Invitrogen, 15510-027) electrophoresis and detected by gel red (Biotium, 41003) staining. Wild type ($Sox10^{+/+}$) sample showed one band at 132 base pairs. Homozygous ($Sox10^{Dom/Dom}$) sample had one single band at 118 base pairs, and heterozygous ($Sox10^{Dom/+}$) sample showed one band at 132 base pairs and one band at 118 base pairs.

3.2.2 Organotypic recombination gut culture

3.2.2.1 Organotypic gut culture

The semi-floating organotypic gut culture protocol followed the method established by Dr Wang Liang in our laboratory (Wang, 2006). An autoclaved 4% Ultra Pure™ Agarose (Invitrogen, 15510-027) block was prepared in double distilled water and transferred to the inner well of the organ culture dish (Falcon, 353037). DMEM/F12 (Gibco, 12400) was added to the surface of the agarose block and the medium together with the agarose block were incubated in the incubator supplied with 5% CO_2 at 37℃ to equilibrate the agarose block. Before the gut segment was cultured, the agarose block was cut into a square 1cm^2 by cutting away the peripheral part of the agarose. Then 2 ml DMEM/F12 with 10% FBS (Gibco, 26140) was added to the inner well of the dish.

The gut segment was dissected from the embryo in the DMEM/F12 without sodium bicarbonate in the biological safety cabinet (SterilGARD, class II type A/B3 biological safety cabinet, the baker company) and then transferred to the surface of agarose block. The gut segments were held to the agarose block by sterilized tungsten needles which pierced through the mesentery (the connective tissue beside the gut) at two ends of the gut. Then more medium was carefully added to the inner well of the culture dish and some autoclaved distilled water was added in the outer trough of the culture dish. The individual dishes were then placed in the incubator supplied with 5% CO_2 at 37℃ for up to 3 days depending on design of the experiments. The medium in the inner well of the dish was replaced by the freshly prepared medium every day and the positions of the tungsten needles were adjusted frequently following the elongation of the gut so that the needles were always pinned at the two ends of the gut segment to keep it straight and suspended in the medium.

3.2.2.2 Recombination of gut segments in organotypic culture

For recombination of gut segments from different embryos, a segment of the proximal hindgut plus a short segment of the midgut (approximate 1.5 mm) (i.e. the whole gut segment extending from the midgut 0.5 mm anterior the cecum to the middle of the hindgut) and the caudal half of the hindgut approximate 1 mm (from the middle of hindgut to the distal end of the hindgut) were dissected and placed on the surface of the pre-equilibrated agarose block. The two segments were placed in a good position so that the caudal end of the proximal hindgut segment was closely apposed to the rostral end of the distal hindgut segment (Fig. 3.6A). Sterilized tungsten needles were used to hold the two recombined gut segments in place by piercing through the

mesentery next to the tissue junction of the caudal end of the proximal hindgut and the rostral end of the distal hindgut. The recombined gut segments were incubated with 5% CO_2 at 37℃ for several hours and the junction was checked. If there was still a gap between two segments, the positions of the tungsten needles were re-adjusted to re-appose the junction more closely. One or two days later, if the recombination was successful and the two gut segments had already fused together, the tungsten needles near the junction could be removed so that the needles could not hinder the elongation of the gut segments. Three days after culture, the recombined gut was harvested by washed once with PBS and fixed in the 4% PFA overnight. The next day, the fixed gut segment was washed with PBS 20 minutes for three times and stored in PBS at 4℃ for whole-mount immunohistochemical staining (see Section 2.2.9.1 for the procedure of Whole-mount immunohistochemical staining).

3.2.3 Cryo-sectioning of embryonic gut and the recombined gut

The guts from E11.5 to E15.5 of *Sox10*$^{+/+}$ and C57 mice were dissected according to Section 2.2.1B of Chapter 2 and fixed in the 4% PFA overnight. After rinsed in PBS 15 minutes for three times, the guts were dehydrated by a graded series of 10%, 20% and 30% sucrose solution each for one to two hours. The mesentery between midgut and hindgut of E11.5 was not separated and whole gut was embedded in OCT with several hairs as marks on a piece of parafilm. The midgut and hindgut of E12.5 to E15.5 were separated and embedded respectively in the OCT. Serial cross sections of the embryonic gut were mounted onto histological glass slides and stored at -20℃ after being dried overnight. Immunohistochemical staining was followed the method described in Section 2.2.9.2 of Chapter 2.

After whole-mount immunohistochemical staining and imaging, the recombined gut was dehydrated by a graded series of 10%, 20% and 30% sucrose solution, oriented by several hairs on a piece of parafilm and embedded in the OCT. The orientation of the gut and hairs were fixed in the frozen OCT after the parafilm was transferred into -20℃ refrigerator slowly and steadily. The cryosections of 10 μm were cut serially using a cryostat (Thermo) and mounted onto histological glass slides and stored at -20℃ after being dried overnight in darkness. After rinsed in PBS 5 minutes 3 times, the cryosections on the slides were mounted with a mounting solution (DAPI: glycerol=1:3).

3.2.4 Animal crosses for *Dom* mutants

Dom mutant mouse line had been maintained on a C57BL/6J x C3HeB/FeJLe-a/a background from Jackson Laboratory. F1 mice displaying a ventral belly spot and white feet were selected for B6C3Fe-a/a F1 ($Sox10^{Dom/+}$) and were mated with B6C3HeB F1 to get $Sox10^{Dom/+}$.

All mice studied in this study were derived from $Sox10^{Dom/+}$ x $Sox10^{Dom/+}$ crosses and were divided into $Sox10^{+/+}$, $Sox10^{Dom/+}$ and $Sox10^{Dom/Dom}$ according to *Sox10* mutation genotyping.

3.2.5 Quantification and statistical analyses

The recombined segments of E11.5 $Sox10^{+/+}$ and $Sox10^{Dom/+}$ donor proximal hindguts with the E11.5 *Dom* recipient hindguts was immunohistochemical stained for SOX10 and Hu according to the method of Section 2.2.9.1 in Chapter 2. A series of images of the recipient segments were acquired with an Olympus FV1000-ZCD laser confocal microscope. The number of the enteric neural crest-derived cells

($Sox10^+$ and $SOX10^-/Hu^+$ cells) in the recipient segments was counted in the serial images. Each recombination group had 5 preparations. Statistical analyses of the cell numbers in recipients were conducted with SPSS 13.0 for windows software using a one-way analysis of variance (ANOVA) followed by Turkey HSD Post Hoc test. A significant difference was accepted when the probability levels of $P < 0.05$.

3.3 Results

3.3.1 Spatio-temporal colonization of the *Dom* embryonic gut by enteric neural crest-derived cells

The whole length of the guts of *Dom* embryos from the same litter at E11.5 to E14.5 were dissected out and stained with p75 and Sox10 antibodies by whole-mount immunohistochemical staining. The genotypes of the individual guts were identified by PCR (see Section 3.2.1). Whole-mount preparations of the guts following immunohistochemical staining were imaged with an Olympus FV1000-ZCD laser confocal microscope to detect the colonization of *Dom* embryonic guts by enteric neural crest-derived cells.

As reported by Kapur (Kapur *et al.*, 1996), at E11.5, enteric neural crest-derived cells expressing p75 and Sox10 had colonized the midgut and the proximal hindgut just posterior to the cecum in $Sox10^{+/+}$ embryos (Fig. 3.1A1-A4). In $Sox10^{Dom/+}$ embryos, the enteric neural crest-derived cells had colonized the midgut anterior to the cecum at E11.5 (Fig. 3.1B1-B4). However, in E11.5 $Sox10^{Dom/Dom}$ embryos (Fig. 3.1C1-C4), there were only several enteric neural crest-derived cells appeared in the stomach but not in any other regions of the gut.

At E12.5, the enteric neural crest-derived cells colonized the anterior half of the hindgut in $Sox10^{+/+}$ embryos (Fig. 3.2A1-A4). However in $Sox10^{Dom/+}$ embryos, the enteric neural crest-derived cells just colonized the cecum sparsely in same litter (Fig. 3.2B1-B4). In the $Sox10^{Dom/Dom}$ E12.5 mice, there were no p75 or Sox10 positive enteric neural crest-derived cells except the p75 positive processes extending into the stomach from the outside of the gut (Fig. 3.2C1-C4).

At E13.5, the enteric neural crest-derived cells colonized almost the whole gut

except the distal end in *Sox10*$^{+/+}$ embryos (Fig. 3.3A1-A4). In *Sox10*$^{Dom/+}$ from the same litter, enteric neural crest-derived cells had not fully colonized the midgut but only colonized the region entirely anterior to the cecum (Fig. 3.3B1-B4).

The enteric neural crest-derived cells colonized the entire gut at E14.5 in *Sox10*$^{+/+}$ embryos (Fig. 3.4A1-A4), while the posterior half of the hindgut in *Sox10*$^{Dom/+}$ gut from the same litter had not been colonized completely in varying degrees (Fig. 3.4B1-B4 and C1-C4). No p75 or Sox10 positive enteric neural crest-derived cells were found in the *Sox10*$^{Dom/Dom}$ mice after E12.5.

The migration of endogenous *Sox10*$^{Dom/+}$ enteric neural crest cells in *Sox10*$^{Dom/+}$ gut was delayed as compared with the migration of endogenous *Sox10*$^{+/+}$ enteric neural crest cells (normal) in *Sox10*$^{+/+}$ gut from E11.5.

3.3.2 Differentiation of muscle layers in the gut tube from E11.5 to E15.5

The differentiation of muscle layers in the gut tube was examined following immunohistochemical staining with an antiserum to smooth muscle actin (SMA, smooth muscle marker) on the frozen sections of the midgut and the hindgut from E11.5 to E15.5. At E11.5, SMA was expressed in the vitelline artery only but not expressed in the midgut and hindgut (Fig. 3.5A). At E12.5, the midgut exhibited some SMA immunoreactive cells in the gut mesenchyme (Fig. 3.5B1), which were presumably forming the muscle layer of the midgut, but in the hindgut no SMA immunoreactive cells were found (Fig. 3.5B2). After E13.5, the SMA positive muscle layer became more discernible in the midgut and the hindgut (Fig. 3.5C1-C2). In addition, SMA positive cells formed two distinctive muscle layers: the longitudinal muscle and circular muscle layers at E14.5 and E15.5 (Fig. 3.5D1-D2 and E1-E2).

3.3.3 Colonization of the recipient gut segment by donor enteric neural crest-derived cells

3.3.3.1 At E11.5, colonization of the recipient gut segment by enteric neural crest-derived cells was cell autonomous, minimally influenced by the migratory environment of the *Dom* hindgut

According to the method described in Section 3.2.2.2, the caudal end of a proximal half of the hindgut (plus a short segment of the midgut, see the line drawing in Fig. 3.6A) was recombined (or closely apposed) with the rostral end of the distal half of another hindgut. The recombined segments were maintained in organotypic culture for 3 days (signed by +3d). The donor segments, which had already been colonized by vagal neural crest-derived cells and served as a donor of enteric neural crest-derived cells, were isolated from *Sox10*$^{+/+}$ (Fig. 3.6B-D) or *Sox10*$^{Dom/+}$ embryos (Fig. 3.6E-G). The recipient segments, which were the distal halves of the hindgut without any colonization of enteric neural crest-derived cells yet at E11.5 (see Fig. 3.1) and received enteric neural crest-derived cells from the donor, were dissected from *Sox10*$^{+/+}$, *Sox10*$^{Dom/+}$ and *Sox10*$^{Dom/Dom}$ embryos. Three days after culture, the donor and recipient segments attached to each other, and enteric neural crest-derived cells from the donor segment crossed the junction to reach the recipient segment. With whole-mount immunohistochemical staining, enteric neural crest-derived cells were marked by the neural crest cell marker Sox10 and the neuronal marker Hu (Fig. 3.7A-F).

Enteric neural crest-derived cells (Sox10^{+} and SOX10^{-}/Hu^{+} cells) from the *Sox10*$^{+/+}$ donors migrated into the recipient segments and colonized the whole distal

hindgut of *Sox10*$^{+/+}$ (Fig. 3.7A), *Sox10*$^{Dom/+}$ (Fig. 3.7B), or *Sox10*$^{Dom/Dom}$ (Fig. 3.7C). The numbers of enteric neural crest-derived cells (Sox10^{+} and Sox10^{-}/Hu^{+}) found in these three types of recipient hindgut segments (i.e. *Sox10*$^{+/+}$, *Sox10*$^{Dom/+}$ and *Sox10*$^{Dom/Dom}$) were not significantly different (n=5, One-Way-ANOVA followed Turkey HSD Post Hoc test, $p<0.05$) (groups 1, 2 and 3 in Fig. 3.7H). This indicated that *Sox10*$^{+/+}$ enteric neural crest-derived cells could migrate equally well in the all three genotypes examined. The enteric neural crest-derived cells network that formed within the recipient hindgut segments was immunohistochemical similar to the hindgut segment which was isolated from the *Sox10*$^{+/+}$ embryos at E11.5 and then cultured under the same organotypic culture conditions for three days (Fig.3.7G). However, if the donor segments were isolated from the *Sox10*$^{Dom/+}$ embryos at E11.5 and combined with recipient segments under the same organotypic culture conditions for three days, then colonization of the recipient segments of all the three genotypes (*Sox10*$^{+/+}$, *Sox10*$^{Dom/+}$ and *Sox10*$^{Dom/Dom}$ in Fig. 3.7D, E and F respectively) by the *Sox10*$^{Dom/+}$ enteric neural crest-derived cells migrated from the donor gut segments was found to be sparse and incomplete and the donor enteric neural crest-derived cells were not able to reach the distal end of the recipient hindgut segment (Fig. 3.7D-F). When the numbers of *Sox10*$^{Dom/+}$ donor enteric neural crest-derived cells (Sox10^{+} and Sox10^{-}/Hu^{+}) in the recipient hindgut segments of these three different genotypes (Groups 4, 5 and 6 in Fig.3.7H) were compared with recipient groups which received *Sox10*$^{+/+}$ enteric neural crest-derived cells from donor segments (Groups 1, 2 and 3 in Fig. 3.7H), significant differences in enteric neural crest-derived cell numbers were found between the *Sox10*$^{+/+}$ donor groups and *Sox10*$^{Dom/+}$ donor group (i.e. Group 1, 2 and 3 vs Groups 4, 5 and 6 in Fig. 3.7) (n=5, one-way ANOVA followed by Turkey HSD post hoc test, $p<0.05$). It again indicated that the genotype

of donor enteric neural crest-derived cells affected the migration within the recipient hindgut segment while the genotype of the recipient segment did not seem to have much influences on the migration of the donor enteric neural crest-derived cells.

Cross sections of the recipient gut segment showed that the enteric neural crest-derived cells from *Sox10*$^{+/+}$ donor segments were located in the mesenchyme of the *Sox10*$^{Dom/+}$ recipient segments (Fig. 3.8A1-A3). In the cross sections of the recombined gut segment, the *Sox10*$^{+/+}$ and *Sox10*$^{Dom/+}$ donor enteric neural crest-derived cells were all located in the mesenchyme of the *Sox10*$^{Dom/Dom}$ recipient segment (Fig. 3.8B1-B3 and C1-C3), which was also the location of the enteric neural crest-derived cells in the donor segment. It indicated that the enteric neural crest-derived cells migrated in the normal location in the recipient segments after crossing the junction.

3.3.3.2 Developmental stage of the recipient segment affected the colonization by enteric neural crest-derived cells

Using the tissue recombination method described in the Section 3.2.2.2, each donor segment from the midgut of a GFP-labelled (C57BL/6-Tg(CAG-EGFP)1Osb/J) mouse at E11.5 was recombined end-by-end with a recipient segments from the midgut of a *Sox10*$^{Dom/Dom}$ mouse at E11.5, E12.5, E13.5, E14.5 or E15.5 (Fig. 3.9A) and cultured for 3 days. In the recipient midgut segment of the *Sox10*$^{Dom/Dom}$ from E11.5 to E15.5, no endogenous enteric neural crest-derived cells were found.

Three days after recombination and culture, the recipients from E11.5, E12.5, E13.5, E14.5 and E15.5 were signed by E11.5+3d, E12.5+3d, E13.5+3d, E14.5+3d and E15.5+3d respectively. GFP-labelled cells migrated into the recipients in all of the

recombinations (E11.5+3d, n=5; E12.5+3d, n=11; E13.5+3d, n=7; E14.5+3d, n=5; E15.5+3d, n=3) (Fig. 3.10). In the recipients of E11.5+3d and E12.5+3d, GFP-labelled donor cells colonized a large area of the recipient midgut and formed an interconnected neural network (Fig. 3.10A1-A3 and B1-B3). However, from E13.5+3d onwards, the GFP-labelled cells migrating into the recipient midgut segment were much fewer than those found in the recipient segments of E11.5+3d and E12.5+3d (comparing C3 with A3 and B3 in Fig. 3.10). In the recipients of E14.5+3d and E15.5+3d, the colonization of GFP-labelled cells was also sparse (Fig. 3.10D1-D3 and E1-E3) similar with in the recipient of E13.5+3d.

Similarly, a donor segment from the proximal half of the hindgut (plus a small segment of midgut) of a GFP-labelled mouse at E11.5 were recombined with a recipient segments from the hindgut of a *Sox10*$^{Dom/Dom}$ mouse at E12.5 to E15.5 (Fig. 3.9B and Fig. 3.11). Three days after recombination, donor GFP-labelled enteric neural crest-derived cells from E11.5 proximal hindgut migrated into the recipient hindgut segment of the *Sox10*$^{Dom/Dom}$ mice from E12.5 to E15.5 embryos which were all aganglionic. In E11.5+3d *Sox10*$^{Dom/Dom}$ recipient segment, the colonization of donor enteric neural crest-derived cells (Fig. 3.7C) was similar to the normal distribution of enteric neural crest-derived cells in normal mouse embryos of the same developmental stages (Fig. 3.7G) as described in the Section 3.3.3.1. In the E12.5+3d recipient segment, the GFP^{+} enteric neural crest-derived cells also colonized the whole recipient (Fig. 3.11A1-A3), however, donor neural crest-derived cells was found fewer as compared with those in the E11.5+3d recipient. From E13.5+3d to E15.5+3d, similar to the observations made in the recipient midgut as described in the previous paragraph, fewer donor GFP-labelled enteric neural crest-derived cells migrated into the recipients and were not able to completely colonize the proximal

part of the recipient guts (Fig. 3.11 B1-B3, C1-C3 and D1-D3).

The age of the recipients hence influenced the colonization of donor enteric neural crest-derived cells, an observation which was in line with findings of previous reports (Druckenbrod and Epstein, 2009; Hotta *et al.*, 2009a). It may be plausible that the gradual differentiation of different tissues of the hindgut, which also include muscle cells, with age might be a cause of this reduced colonization of enteric neural crest-derived cells in more developed gut segments.

3.3.3.3 Presence of endogenous enteric neural crest-derived cells within the recipient gut segment affected the colonization of donor enteric neural crest-derived cells

Next, a donor GFP-labelled midgut segment at E11.5 was recombined with a recipient midgut from a $Sox10^{+/+}$, $Sox10^{Dom/+}$ and $Sox10^{Dom/Dom}$ mouse at E11.5 (Fig. 3.12). In $Sox10^{Dom/+}$ midguts, the extent of colonization by endogenous neural crest-derived cells varied a lot, with some midgut segments highly colonized (Fig. 3.12B2) while some barely colonized by endogenous neural crest-derived cells (Fig. 3.12C2). Fewer donor GFP-labelled cells were found in the ganglionic midgut segments (i.e. already colonized by the endogenous neural crest-derived cells) of the $Sox10^{+/+}$ (Fig. 3.12A1-A3) and $Sox10^{Dom/+}$ recipients (Fig. 3.12B1-B3) than in the aganglionic midgut segments (i.e. no endogenous neural crest-derived cells) of $Sox10^{Dom/+}$ (Fig. 3.12C1-C3) and $Sox10^{Dom/Dom}$ recipients (Fig. 3.12D1-D3). Upon careful examination of the colonization of the ganglionic segment of the $Sox10^{+/+}$ (Fig. 3.13A1-A3) and $Sox10^{Dom/+}$ recipients (Fig. 3.13B1-B3), GFP-labelled processes derived from the donor segment were found extending into the ganglionic

recipients (Fig. 3.13A1 and B1).

As described above, the migration and colonization of donor enteric neural crest-derived cells were influenced by the presence of endogenous enteric neural crest-derived cells within the recipient gut segment.

3.4 Discussion

With more studies on the enteric nervous system, increasing numbers of genes, signalling pathways and molecules have been found and identified interfering with the enteric nervous system development. RET/GDNF/GFRα1 signalling pathway, EDN3-EDNRB signalling pathway and transcription factors *Phox2b*, *Sox10*, *Pax3*, *Mash1*, *Hox11L1* (Newgreen and Young, 2002a), molecules such as SNARE proteins (Vohra *et al.*, 2006), β-integrin (Nagy *et al.*, 2009), RhoA (Rupp and Kulesa, 2007), BBS proteins (Tobin *et al.*, 2008), electrical activity, neurotransmitter release (Hao *et al.*, 2010) and the presence of the developing vascular endothelium in the gut (Nagy *et al.*, 2009) all have effects on the enteric nervous system development. Deletion or mutation of one or more of these genes affecting their normal expression and function may interfere with the enteric nervous system development and cause HSCR-like pathologies. The cellular defects are responsible for the failure of enteric neural crest-derived cells to advance within the hindgut remain poorly understood.

To study the development of colonic aganglionosis, the mouse models of megacolon such as Ret^{TGM}, $Ednrb^{flex3/flex3}$, *ls/ls*, *Sox10* and many others were used. In this study *Dom* embryos with a spontaneously *Sox10* mutation on a C57BL/6J x C3HeB/FeJLe-a/a background were used. In $Sox10^{Dom/+}$ embryos, *Sox10* transcript levels are reduced leading to enteric hypo- or aganglionosis and spotted pigmentation which vary with the genetic background (Herbarth *et al.*, 1998).

3.4.1 Colonization by enteric neural crest-derived cells in *Dom* recipient hindgut segments is predominantly cell autonomous at E11.5

The migration of endogenous enteric neural crest-derived cells in the $Sox10^{Dom/+}$ hindgut was delayed as compared with the migration in normal $Sox10^{+/+}$ embryos from E11.5 to E14.5 as shown by Kapur (Kapur *et al.*, 1996) and by Wang (Wang, 2006). By time-lapse imaging study, the migration of mutant enteric neural crest-derived cells is shown to be delayed by ~24 hours behind those in same age-matched control gut between E10.5 to E11.5 (Druckenbrod and Epstein, 2009).

The delayed migration in the $Sox10^{Dom/+}$ embryonic gut is caused by the mutant enteric neural crest-derived cells themselves at E11.5. In the previous study of my laboratory colleague (Wang, 2006), the recombination of the midgut segment with hindgut segments of various genotypes in organotypic culture demonstrates that the microenvironment of the hindgut of the $Sox10^{Dom/+}$ and $Sox10^{Dom/Dom}$ embryos can not affect the migration of donor enteric neural crest-derived cells from the $Sox10^{+/+}$ midgut. However the migration of donor $Sox10^{Dom/+}$ enteric neural crest-derived cells in the hindgut of three genotypes is all defective. In the present study using the proximal hindgut recombined with the distal hindgut, the enteric neural crest-derived cells from the $Sox10^{+/+}$ proximal hindgut could colonize the whole length of the distal hindgut, and no significant difference has been found among all the recipients of different genotypes. It indicates that the genotypes of the recipient have minimal effect on the migration of donor enteric neural crest-derived cells. However, $Sox10^{Dom/+}$ donor enteric neural crest-derived cells from the proximal hindgut could only partially colonize the recipients of all three genotypes forming a sparse and incomplete network of cells, which is significantly different from the cellular network

formed by *Sox10*$^{+/+}$ donors. These observations are consistent with our previous findings (Wang, 2006) and support the idea that the defect of enteric colonization by the enteric neural crest-derived cells is not related to the microenvironment provided by the *Sox10* mutant gut wall (Kapur, 1999). In *Ret* or *Ednrb* mutant mice (Bondurand *et al.*, 2003; Kruger *et al.*, 2003; Natarajan *et al.*, 1999), enteric neural crest-derived cells from wild type embryos could also colonize the aganglionic gut of *Ret* or *Ednrb* mutant embryos. Montage comparing the *in situ* advance of normal and mutant enteric neural crest-derived cells in the colon has found that the mutant enteric neural crest-derived cells following a rostral-caudal trajectory have a reduced migratory speed and show a greater variability of direction (Druckenbrod and Epstein, 2009). Therefore, it is plausible that the migratory ability of the mutant enteric neural crest-derived cells is compromised inducing abnormal colonization of the hindgut.

3.4.2 Developmental stage of the gut tube affects the colonization of the aganglionic midgut and hindgut by enteric neural crest-derived cells

The gastrointestinal tract is formed from a primitive gut tube comprising endoderm, which gives rise to the inner epithelial lining of the gut and the splanchnic mesoderm, which gives rise to the smooth muscle (Roberts, 2000). In the development of the gut, the mesenchyme with distinct smooth muscle cells and muscle layers cannot be detected in the midgut and hindgut at E11.5. From E12.5 onwards, smooth muscle cells gradually become more differentiated in the midgut, and the muscle layers become more and more distinct with the developmental stages. At E13.5, the differentiated smooth muscle cells can be found in the hindgut and the muscle layers are more differentiated at later stages.

During the development of the mouse (McKeown *et al.*, 2001) and human (Fu *et al.*, 2004b; Wallace and Burns, 2005), enteric neural crest-derived cells colonize the entire length of the gut before distinct muscle layers have formed (Hotta *et al.*, 2009a). In present study, midgut segments isolated from mouse embryos genetically labelled with GFP were used as donors of enteric neural crest-derived cells. Midgut segments of $Sox10^{Dom/Dom}$ embryos at E11.5 to 15.5 where enteric neural crest-derived cells were completely absent were used as recipients of enteric neural crest-derived cells from the donor segments. In the midgut recipients at E11.5+3d and E12.5+3d, GFP-labelled enteric neural crest-derived cells colonized a large area and formed a network. However, in midgut recipients of E13.5+3d to E15.5+3d, cells colonizing the recipients were fewer and sparser than in midgut recipients of E11.5+3d and E12.5+3d. In hindgut recipients of E13.5+3d to E15.5+3d, similar to the colonization of the midgut recipients, GFP-labelled enteric neural crest-derived cells migrated into the hindgut recipients but were not able to completely colonize the proximal part of the recipients. The formation of the incomplete network of enteric neural crest-derived cells from E13.5 onwards coincides with the time at which the muscle layers of the gut tube start to differentiate. These observation are also consistent with the reports on *Ret* and *Ednrb* mutant mice where older recipient colon is less permissive for enteric neural crest-derived cells migration than younger colon (Druckenbrod and Epstein, 2009; Hotta *et al.*, 2009a).

The nonpermissive environment for migration at advanced stages might also be associated with changes in laminin expression because laminin is sparsely distributed as punctate deposits in E13.5 wavefronts but increased at E14.5 (Druckenbrod and Epstein, 2009). The migration of avian enteric neural crest-derived cells also responds to the extracellular matrix such as laminin, fibronectin and β-integrin while the

migration of enteric neural crest-derived cells on the smooth muscle has been found to be minimal (Nagy *et al.*, 2009). In zebrafish, enteric precursors to the posterior end of the intestine have migrated reaching the end of the intestine by 66 hpf (hours post fertilization) while the circular smooth muscle begins differentiating until 69 hpf when the smmhc (smooth muscle myosin heavy chain), the first markers of smooth muscle development is translated (Olden *et al.*, 2008). In mice, the circular muscle layer begins differentiating at E12 with organization of smooth muscle actin (Wu *et al.*, 2000). However, the relationship between the differentiation of the muscle layers and the migration of enteric neural crest-derived cells however awaits further investigations.

3.4.3 Presence of endogenous enteric neural crest-derived cells within the recipient gut affects the colonization by donor enteric neural crest-derived cells

It was found in this study that donor GFP-labelled enteric neural crest-derived cells from E11.5 GFP-labelled embryos were fewer in the ganglionic segments of the *Sox10*$^{+/+}$ and *Sox10*$^{Dom/+}$ midgut than in the aganglionic *Sox10*$^{Dom/+}$ and *Sox10*$^{Dom/Dom}$ midgut segments of *Dom* recipients of E11.5+3d. Similar observations have also been made in the study where enteric neural crest-derived cells have failed to enter most of the *Ret*$^{+/+}$ hindgut recipients at E14.5 to E16.5 where the hindgut has already been completely colonized by endogenous neural crest-derived cells (Hotta *et al.*, 2009a), and in quail-chick chimeric study, quail enteric neural crest-derived cells cannot colonize segments of embryonic chick gut containing pre-existing enteric neural crest-derived cells (Meijers *et al.*, 1992).

GFP-labelled processes were found in the ganglionic recipients of *Sox10*$^{Dom/+}$

and *Sox10*$^{+/+}$ midgut segments extending from the donor gut segments. This is inconsistent with the result that neurite of dorsal root ganglia co-cultured with the presumptive aganglionic *ls/ls* bowel but no cell bodies entered the abnormal zone (Jacobscohen *et al.*, 1987).

It has been reported that neural crest cells cultured *ex vivo* move away from each other, disperse quickly (Davis and Trinkaus, 1981) and demonstrate contact inhibition of locomotion both *in vivo* and *ex vivo*. Wnt planar cell polarity (PCP, or non-canonical) pathway is required for neural crest cells migration in Xenopus and zebrafish embryos (De Calisto *et al.*, 2005; Matthews *et al.*, 2008). Inhibition of non-canonical Wnt signalling abolishes both contact inhibition of locomotion and the directionality of neural crest cells migration, and RhoA (One member of the Rho family of GTPases) as downstream molecule affected the PCP in contact inhibition of locomotion (Carmona-Fontaine *et al.*, 2008). RhoA has a role to control the temporal dynamics of neural crest cell–cell interactions and cell movement to ensure accurate neural crest cell navigation and collective migration (Rupp and Kulesa, 2007). The influence of endogenous enteric neural crest-derived cells on the migration of the donor/exogenous enteric neural crest-derived cells might be related with this contact inhibition property of neural crest cells.

3.5 Summary

The results of this chapter are summarized as follows:

In the *Sox10*$^{+/+}$ embryos, enteric neural crest-derived cells completely colonized the whole gut tube in a rostral-to-caudal manner by E14.5 while in the *Sox10*$^{Dom/+}$ embryos, the migration of enteric neural crest-derived cells was much delayed and they were not able to colonize the whole length of the gut tube at E14.5. In the *Sox10*$^{Dom/Dom}$ embryos, no enteric neural crest-derived cells were found along the whole length of the gut tube.

During the development of the gut, the smooth muscle cells within the gut mesenchyme remained undifferentiated until E12.5, at which stage differentiating muscle cells expressing smooth muscle actin were observed in the midgut, and a muscle layer was gradually formed. In the hindgut, from E13.5 onwards, differentiated smooth muscle cells were also found and the muscle layers were differentiated gradually at later stages.

Using the proximal segment of the hindgut as the donor of enteric neural crest-derived cells to recombine with the distal half of the hindgut of another embryo serving as the recipient in an organotypic culture, the donor enteric neural crest-derived cells from the *Sox10*$^{+/+}$ hindgut segment colonized the whole distal half of the hindgut regardless of the genotype of the recipient. However, when a donor *Sox10*$^{Dom/+}$ hindgut segment was used, donor enteric neural crest-derived cells were not able to fully colonize the recipient hindgut of all three genotypes examined and a sparse and incomplete network of enteric neural crest-derived cells was formed, which was significantly different from the *Sox10*$^{+/+}$ donor enteric neural crest-derived cells. Hence it appeared that the genotype of donor neural crest-derived cells affected their

migration within the recipient while the genotype of the recipient did not influence the neural crest cell migration to a discernible extent.

Midguts from GFP-labelled embryos at E11.5 were also used as donor segments and other midgut segments from *Sox10*$^{Dom/Dom}$ embryos at E11.5 to E15.5 without endogenous enteric neural crest-derived cells were used as recipient segments in the recombination organotypic culture. In E11.5+3d and E12.5+3d recipient segments, GFP-labelled enteric neural crest-derived cells colonized a large area of the midgut and formed an interconnected network. However, in E13.5+3d to E15.5+3d recipients, donor neural crest-derived cells colonizing the recipients were fewer and unable to form a dense network as compared with the interconnected network in recipient segments of E11.5+3d and E12.5+3d. When hindgut segments at E13.5 to E15.5 were used as recipients of neural crest-derived cells from donor hindgut segments, similar to the colonization of the midgut, GFP-labelled enteric neural crest-derived cells migrated into the recipients but did not completely colonize the recipients. Hence it seemed that the developmental stage of the recipient affected the migration of enteric neural crest-derived cells within the gut tube. The stage of the recipient beyond which a morphologically normal network of neural crest-derived cells could not form was E13.5, which was also the stage at which muscle cells started to form distinct layers within the gut mesenchyme.

The presence of endogenous neural crest-derived cells within the gut tube also affected the migration of donor enteric neural crest-derived cells. Donor enteric neural crest-derived cells from E11.5 GFP-labelled embryos were able to better colonize the aganglionic midgut recipient from *Sox10*$^{Dom/+}$ or *Sox10*$^{Dom/Dom}$ embryos as compared with to colonize the ganglionic midgut recipient from *Sox10*$^{+/+}$ and *Sox10*$^{Dom/+}$ mutant embryos at E11.5 by the same donor cells which formed a sparse and incomplete

network. However, GFP-labelled processes were found extending from the donor segment to the ganglionic recipient segment of $Sox10^{Dom/+}$ or $Sox10^{+/+}$ embryos.

The colonization of enteric neural crest-derived cells in the developing gut was hence affected by their genotypes, the developmental stage of the microenvironment through they migrated and the presence of the endogenous enteric neural crest-derived cells. During cell-based therapy for the aganglionic colon, these factors need to be considered so as to obtain good therapeutic effects.

3.6 Figures and Legends

Fig 3.1 Confocal photomicrographs after whole-mount immunohistochemical staining for p75 and Sox10 showing the distribution of enteric neural crest-derived cells in the offspring of *Sox10*$^{Dom/+}$ x *Sox10*$^{Dom/+}$ mutant mice at E11.5

(A1) to (A4) (A2) to (A4) were the magnified images of (A1). In *Sox10*$^{+/+}$ embryos, most of enteric neural crest-derived cells are p75 and/or Sox10 positive, also seen in references (Anderson *et al.*, 2006; Young *et al.*, 2005). The immunoreactive cells are found in the midgut and the cecum, and a few of them are also found in the proximal hindgut just posterior to the cecum. These images are representative images of 6 preparations.

(B1) to (B4) In *Sox10*$^{Dom/+}$ embryos, enteric neural crest-derived cells (p75^{+}/Sox10^{+} double immunoreactive) are present in the midgut anterior to the cecum (n=4).

(C1) to (C4) Only a few enteric neural crest-derived cells (p75^{+}/Sox10^{+}) are found in the stomach of *Sox10*$^{Dom/Dom}$ embryos and no enteric neural crest-derived cells are present in the midgut and hindgut (n=3).

E11.5 Sox10Dom/Dom
E11.5 Sox10Dom/+
E11.5 Sox10+/+
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
stomach
midgut
hindgut
cecum
100μm

Fig 3.2 Confocal photomicrograph on whole mount preparations of the *Dom* embryonic gut at E12.5

(A1) to (A4) In the *Sox10*$^{+/+}$ embryos, enteric neural crest-derived cells (p75^{+} or Sox10^{+}) migrate to the middle of the hindgut (arrow shows the enteric neural crest-derived cells in the wavefront of migration) (n=4).

(B1) to (B4) In *Sox10*$^{Dom/+}$ embryos, most of the enteric neural crest-derived cells (p75^{+} or Sox10^{+}) are found in the midgut with only a few of them migrated to the cecum (arrow shows p75^{+} enteric neural crest-derived cells in the wavefront of migration) (n=4).

(C1) to (C4) In the *Sox10*$^{Dom/Dom}$ embryos, no p75 or Sox10 immunoreactive cells are found along the entire length of the gut tube except that some p75 immunoreactive processes are observed extending into the stomach from the outside (n=1).

E12.5 Sox10Dom/Dom
E12.5 Sox10Dom/+
E12.5 Sox10+/+
C1
B1
A1
stomach
midgut
hindgut
caecum
midgut
hindgut
caecum
midgut
hindgut
C2
B2
A2
midgut
hindgut
caecum
hindgut
midgut
hindgut
C3
B3
A3
hindgut
hindgut
hindgut
C4
B4
A4
hindgut
hindgut
hindgut

Fig 3.3 Confocal photomicrograph on whole mount preparations of the *Dom* embryonic gut at E13.5

(A1) to (A4) In *Sox10*$^{+/+}$ embryos, enteric neural crest-derived cells (p75^{+} or Sox10^{+}) are found almost along the whole gut (arrow shows the enteric neural crest-derived cells in the wavefront of migration) except the distal end of the hindgut (n=1). (A2) to (A4) are magnified views of the boxed area in (A1).

(B1) to (B4) In *Sox10*$^{Dom/+}$ embryos from the same litter, enteric neural crest-derived cells (p75^{+} or Sox10^{+}) are observed in the midgut (arrow shows the enteric neural crest-derived cells in the wavefront of migration) anterior to the cecum and the colonization of the midgut is not completed (n=3). (B2) to (B4) are magnified views of the boxed area in B1.

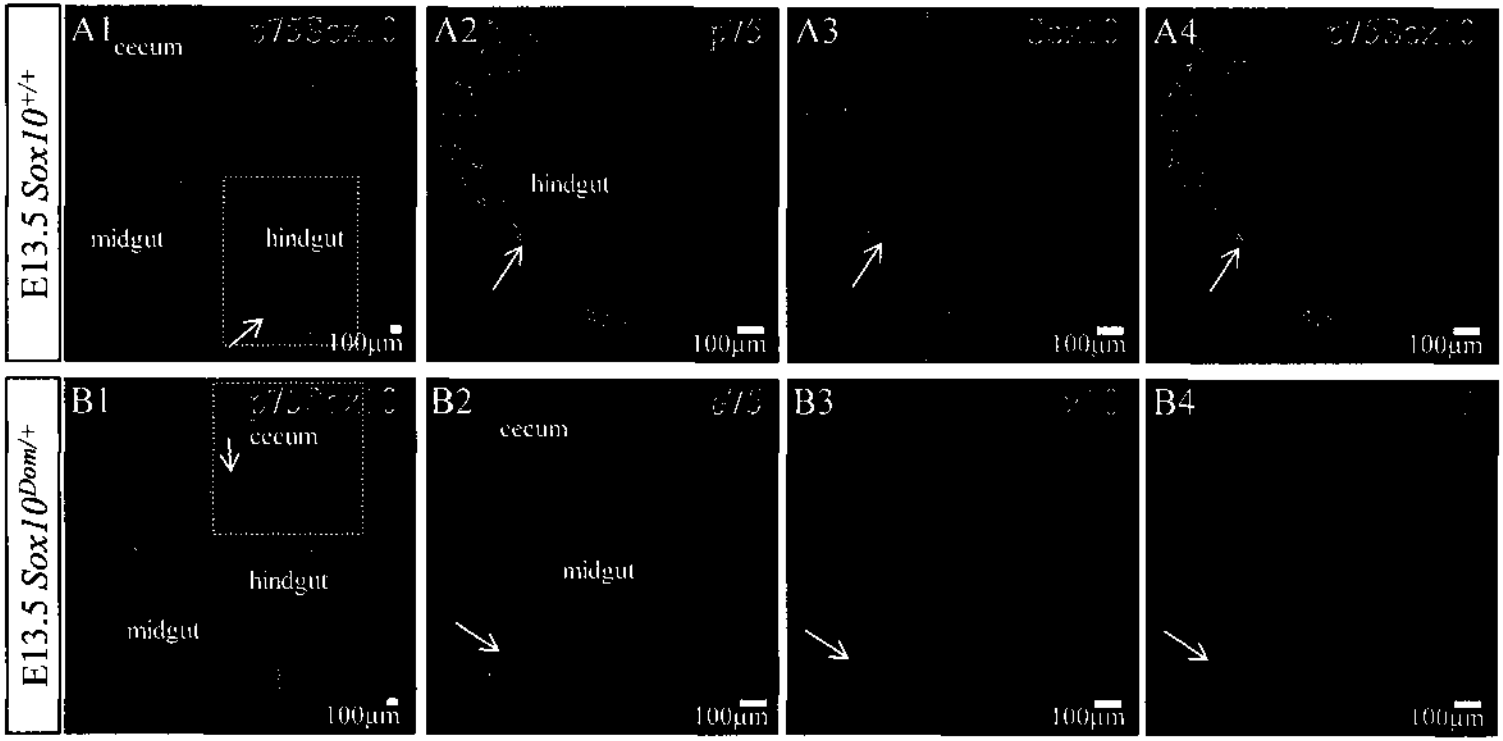

E13.5 Sox10+/+
E13.5 Sox10Dom/+
A1
cecum
midgut
hindgut
100μm
A2
hindgut
100μm
A3
100μm
A4
100μm
B1
cecum
hindgut
midgut
100μm
B2
cecum
midgut
100μm
B3
100μm
B4
100μm

Fig 3.4 Confocal photomicrograph on whole mount preparations of the *Dom* embryonic gut at E14.5

(A1) to (A4) In *Sox10*$^{+/+}$ embryos, enteric neural crest-derived cells (p75^{+} or Sox10^{+}) are found throughout the whole length of the gut tube (n=3). (A2) to (A4) are magnified views of the boxed area in (A1).

(B1) to (B4) and (C1) to (C4) In *Sox10*$^{Dom/+}$ embryos from the same litter, the most caudal enteric neural crest-derived cells (p75^{+} or Sox10^{+}) are found in the middle of the hindgut (n=2) such as arrows indicated in (B1) to (B4) or only anterior to the cecum (n=3) such as arrows indicated in (C1) to (C4). The p75^{+} or Sox10^{+} cells in the end of the hindgut in (B2) to (B4) are enteric neural crest-derived cells from sacral neural crest along caudal-to-rostral migration pathway in the hindgut. (B2) to (B4) and (C2) to (C4) are magnified views of the boxed areas in (B1) and (C1) respectively.

E14.5 Sox10+/+
E14.5 Sox10Dom/+
E14.5 Sox10Dom/+
A1
hindgut
100μm
B1
midgut
hindgut
100μm
C1
cecum
midgut
hindgut
100μm
A2
hindgut
100μm
B2
hindgut
100μm
C2
midgut
100μm
A3
100μm
B3
100μm
C3
100μm
A4
100μm
B4
100μm
C4
100μm

Fig 3.5 Differentiation of muscle layers in the gut tube from E11.5 to E15.5

Cross sections are cut through the middle of the midgut (A, B1-E1) and/or the middle of the hindgut (A, B2-E2) at E11.5 to E15.5.

At E11.5 (A), the SMA was expressed in the vitelline artery (pointed by arrow) only but not expressed in the midgut and hindgut.

At E12.5 (B1) and (B2), in the midgut (B1) there are some SMA immunoreactive cells in the mesenchyme and more SMA immunoreactive cells in the vitelline artery (arrow in B1). However, in the hindgut a layer of muscle could not be observed (B2).

At E13.5 (C1) and (C2), the SMA immunoreactive muscle layer becomes discernible in the midgut (C1) and hindgut (C2).

At E14.5 (D1) and (D2), the longitudinal muscle layer (LM) and circular muscle layers (CM) are distinct with a lot of SMA immunoreactive cells in the midgut (D1) and in the hindgut (D2).

At E15.5 (E1) and (E2), the longitudinal muscle layer (LM) and circular muscle layers (CM) are more distinct in all of the midgut (E1) and in the hindgut (E2).

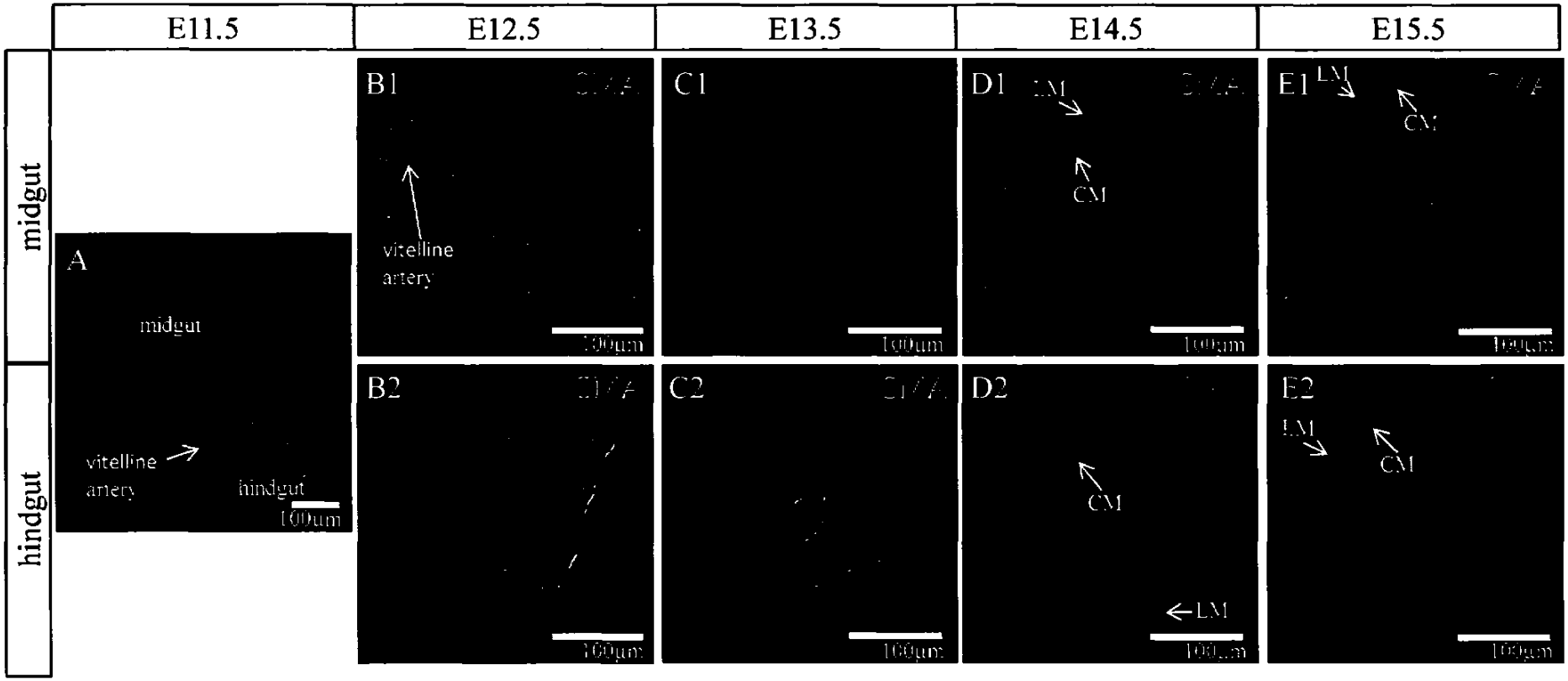
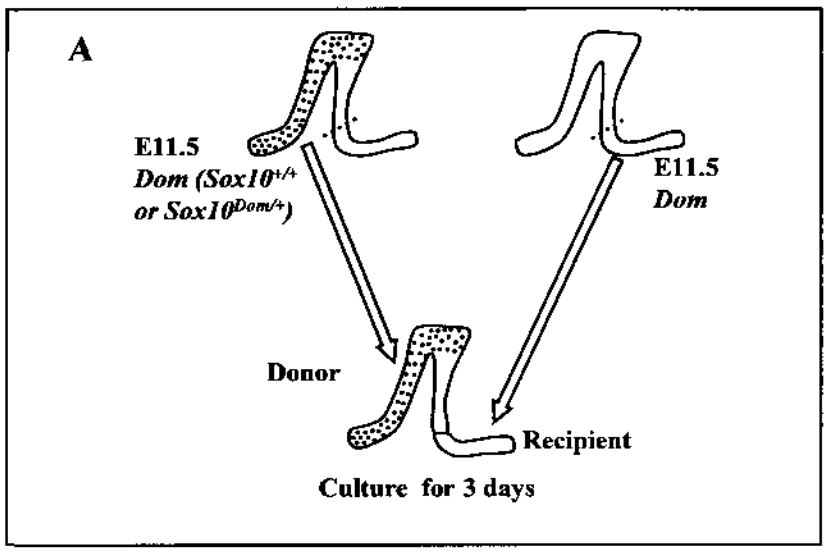

E11.5
E12.5
E13.5
E14.5
E15.5
midgut
hindgut
A
midgut
vitelline artery
hindgut
100μm
B1
vitelline artery
100μm
C1
100μm
D1
LM
CM
100μm
E1
LM
CM
100μm
B2
100μm
C2
100μm
D2
CM
LM
100μm
E2
LM
CM
100μm

Fig 3.6 Recombination of donor and recipient gut segments in organotypic culture

The caudal end of a proximal half of the hindgut together with a short segment of the midgut (donor) from E11.5 *Sox10*$^{+/+}$ embryos was recombined (closely apposed) with the rostral end of the distal half of another hindgut segment (recipient) from *Dom* embryos also at E11.5 of different genotypes (line drawing with the dotted line being the junction of recombination. Recombination and culture protocols are described in Section 3.2.2.2). Note that enteric neural crest-derived cells (dots in the line drawing) have already colonized the midgut segment and the cecum of the donor segment. The recombined segments were maintained in organotypic culture for 3 days. The recombinations include the following:

Donor segments were isolated from *Sox10*$^{+/+}$ (B-D) or from *Sox10*$^{Dom/+}$ (E-G) embryos and the recipient segments were dissected from *Sox10*$^{+/+}$ (B, E), *Sox10*$^{Dom/+}$ (C, F) and *Sox10*$^{Dom/Dom}$ (D, G) embryos. Two segments connected and attached well to each other after 3 days in culture. The junctions between the donor and recipient segments are indicated by arrows.

Hindgut recombination between E11.5 *Dom* (*Sox10*$^{+/+}$ *or* *Sox10*$^{Dom/+}$) *and* E11.5 *Dom* in organotypic culture



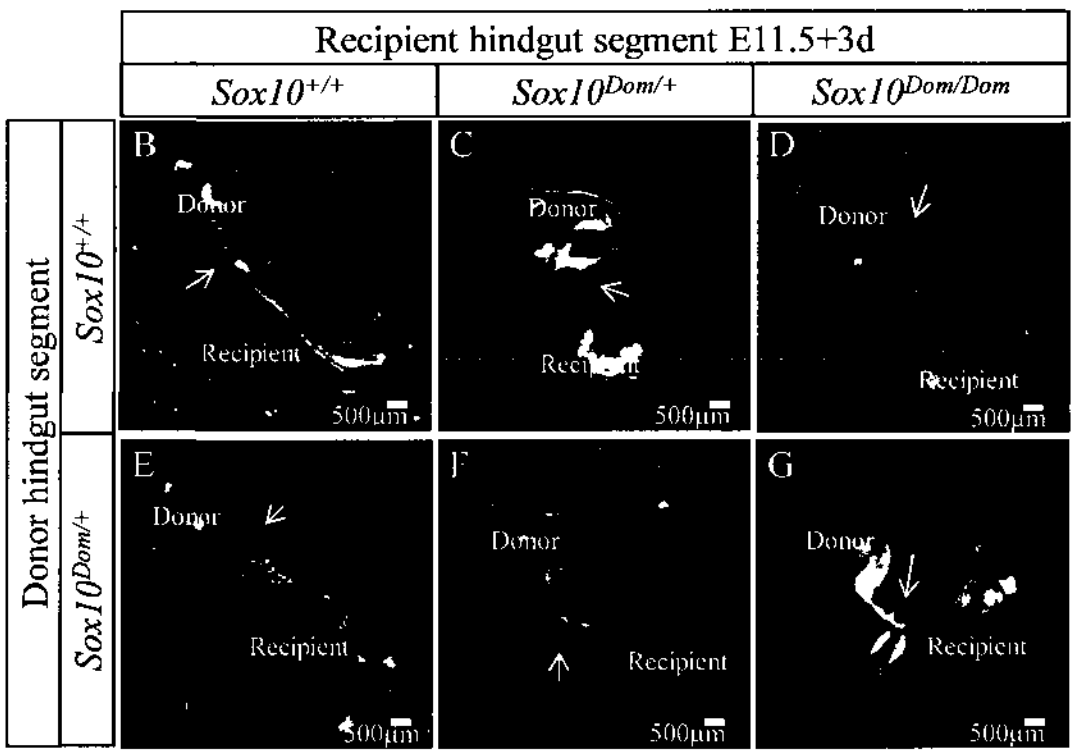


Fig 3.7 Colonization of the recipient *Dom* gut segment at E11.5 by enteric neural crest-derived cells

With immunohistochemical staining on whole mount preparations, the enteric neural crest-derived cells colonized the recipient segments were marked by the neural crest marker Sox10 and neuronal marker Hu. The enteric neural crest-derived cells from the $Sox10^{+/+}$ donor segments migrate into the recipient segments and colonize the whole distal hindgut of $Sox10^{+/+}$ (A), $Sox10^{Dom/+}$ (B) and $Sox10^{Dom/Dom}$ (C) embryos after 3 days in organotypic culture. The distribution of neural crest-derived cells within the recipient hindgut segments is similar to that of neural crest-derived cells within the $Sox10^{+/+}$ E11.5+3d hindgut, which has been cultured under the same organotypic culture conditions from E11.5 for 3 days (G).

The enteric neural crest-derived cells from $Sox10^{Dom/+}$ donor hindgut segments also migrate into the recipient hindgut segments of $Sox10^{+/+}$ (D), $Sox10^{Dom/+}$ (E) and $Sox10^{Dom/Dom}$ (F) embryos, but the distribution of these $Sox10^{Dom/+}$ donor enteric neural crest-derived cells is sparse and incomplete when compared with the distribution of the $Sox10^{+/-}$ donor enteric neural crest-derived cells (A, B and C).

The junction between the donor and recipient segments is indicated by an arrow. The small photo in the lower left corner of the image of each recipient segment images shows the $Sox10^{+}$ or Hu^{+} enteric neural crest-derived cells in the recipient segments.

The numbers of enteric neural crest-derived cells ($Sox10^{+}$ and $Sox10^{-}/Hu^{+}$) in the recipient segments were analyzed by one-way ANOVA followed by Turkey HSD post hoc test. There was no significant difference between groups A, B and C (i.e. bars a, b and c in H) and between groups D, E, and F (i.e. bars d, e and f in H), but there were significant differences between groups A and C; groups B and E; groups C and F ($n=5$, $p<0.05$). It indicates that the genotype of donor enteric neural crest-derived cells affects the migration in the recipient hindgut segments while the genotype of the recipient hindgut segments exhibits minimal effects on enteric neural crest-derived cells migration.

In (H), y-axon means the mean number of the enteric neural crest-derived cells in every recipient. a corresponds to group A; b corresponds to group B; c corresponds to group C; d corresponds to group D; e corresponds to group E; f corresponds to group F.

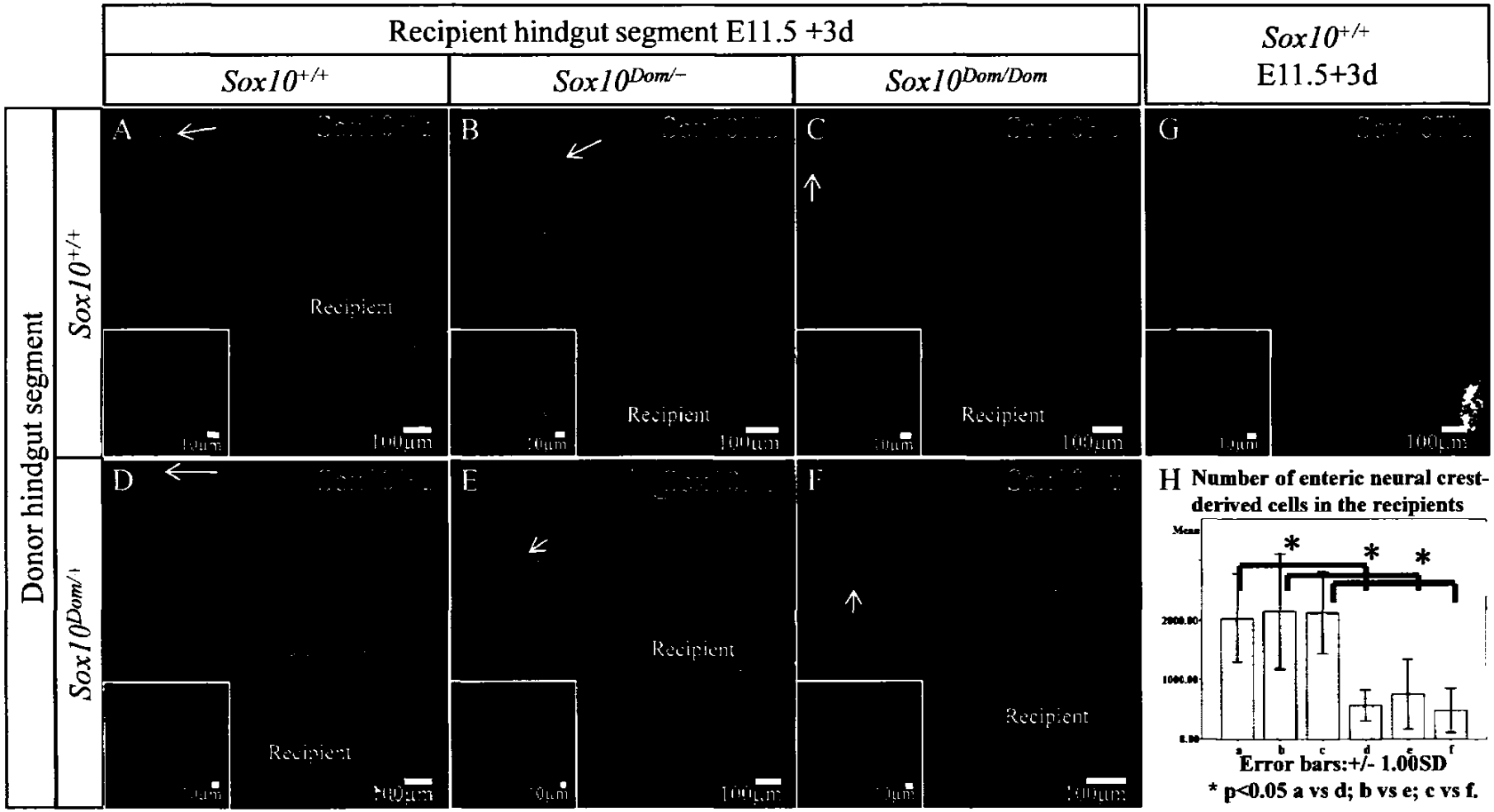

Recipient hindgut segment E11.5 +3d
Sox10+/+
Sox10Dom/–
Sox10Dom/Dom
Sox10+/+ E11.5+3d
Donor hindgut segment
Sox10+/+
Sox10Dom/+
A
B
C
D
E
F
G
Recipient
100µm
H Number of enteric neural crest-derived cells in the recipients
Mean
2000.00
1000.00
0.00
a
b
c
d
e
f
Error bars:+/- 1.00SD
* p<0.05 a vs d; b vs e; c vs f.

Fig 3.8 Sections showing the locations of donor enteric neural crest-derived cells in recipient gut segments

In the diagrammatic representation of the recombination of the proximal hindgut with the distal half of the hindgut (Inset of (A1), (B1) and (C1)), the black dots represent enteric neural crest-derived cells in the donor segment at the time of recombination, and the grey dots represent enteric neural crest-derived cells in the recombined segments after cultured for 3 days. The red line indicates the location where the section was made. The white dotted line in the section outlines the gut lumen.

(A1) to (A3) In the cross section of the *Sox10*$^{Dom/+}$ recipient segment (the red line in the diagrammatic representation in the inset of A1), Sox10 immunoreactive donor cells (A1) and Hu immunoreactive donor cells (A2) from the *Sox10*$^{+/+}$ donor segment are found in the mesenchyme of the recipient segment. (A3) is the merged image of (A1) and (A2). All the cell nuclei in the sections are stained by DAPI in (A1) to (A3).

(B1) to (B3) In the longitudinal section through the junction (arrows) of the *Sox10*$^{+/+}$ donor segment and *Sox10*$^{Dom/+}$ recipient segment (the section was cut through the gut segment along the line indicated by the red line in the diagrammatic representation in the inset of B1), Sox10 immunoreactive donor cells (B1) and Hu immunoreactive donor cells (B2) are found in the mesenchyme of the *Sox10*$^{+/+}$ donor segment and *Sox10*$^{Dom/+}$ recipient segment because hair was used to mark the junction and orientation when the gut segment was embedded and sectioned (B3). (B3) is the merged image of (B1) and (B2).

(C1) to (C3) In the longitudinal section through the junction (arrows) of the *Sox10*$^{Dom/+}$ donor segment and *Sox10*$^{Dom/Dom}$ recipient segment (the section was cut through the gut segment along the line indicated by the red line in the diagrammatic representation in the inset of C1), Sox10 immunoreactive donor cells (C1) and Hu immunoreactive donor cells (C2) are found in the mesenchyme of the *Sox10*$^{Dom/+}$ donor segment and *Sox10*$^{Dom/Dom}$ recipient segment. (C3) is the merged image of (C1) and (C2).

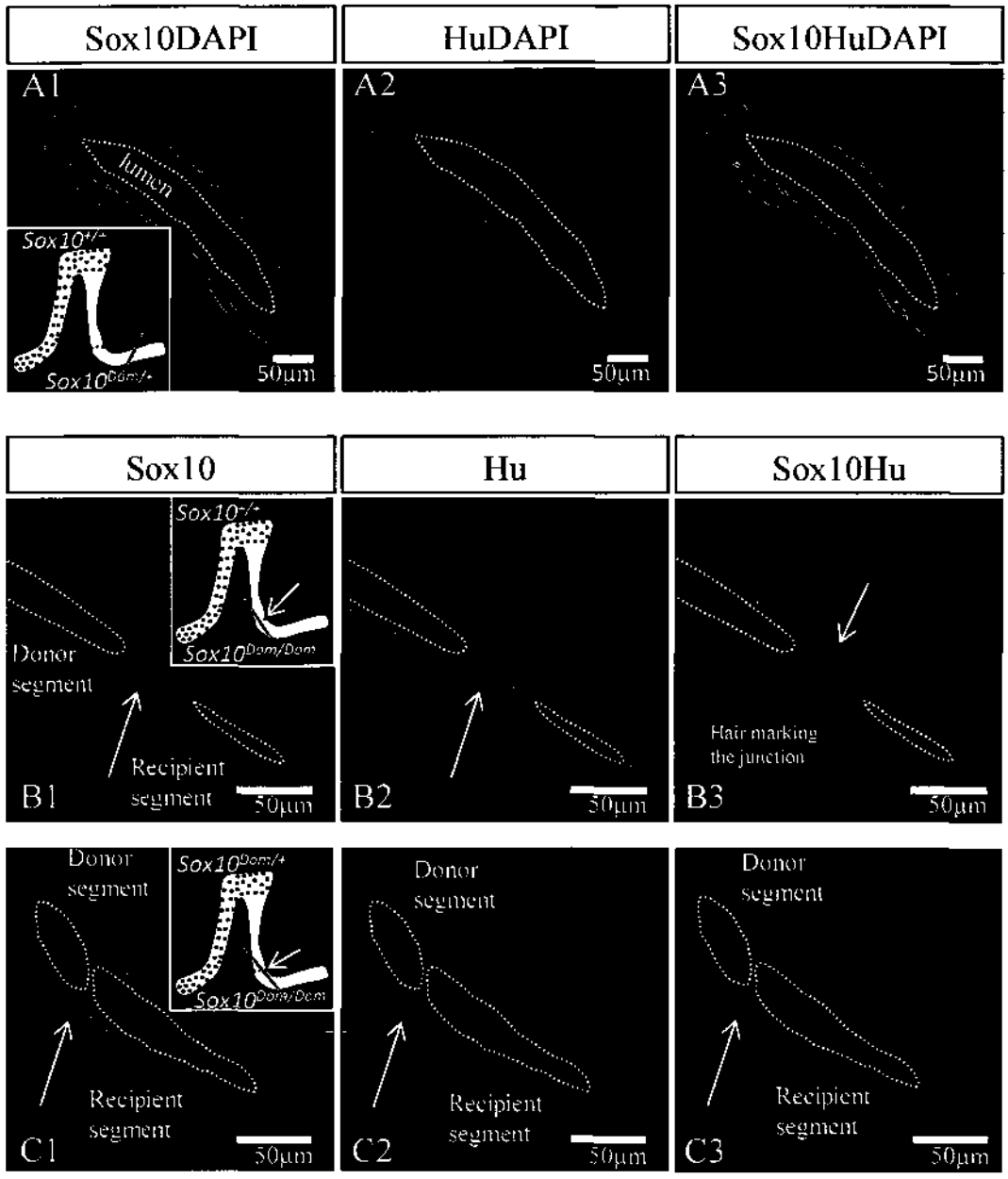

Sox10DAPI
HuDAPI
Sox10HuDAPI
A1
A2
A3
lumen
Sox10+/-
Sox10Dom/+
Sox10Dom/Dom
50μm
Sox10
Hu
Sox10Hu
Donor segment
Recipient segment
Hair marking the junction
B1
B2
B3
C1
C2
C3

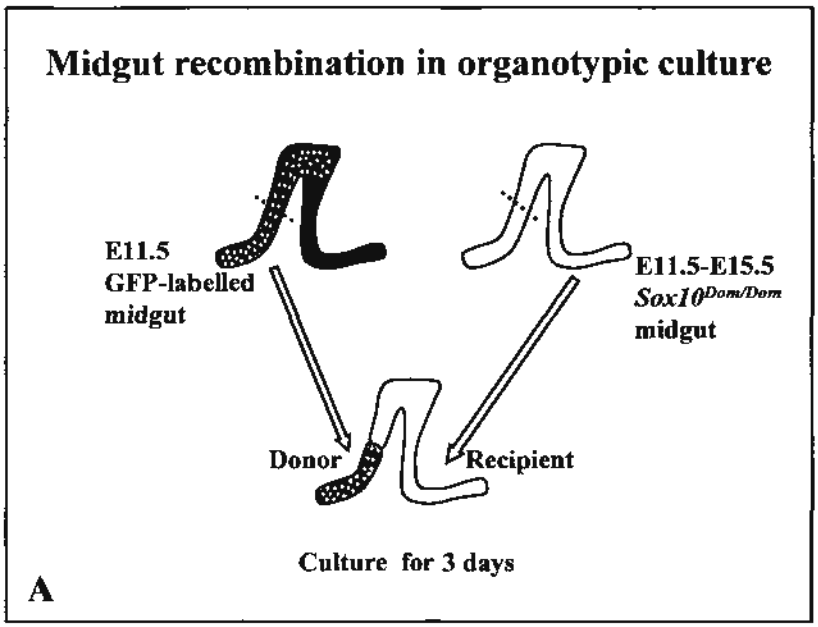
Fig 3.9 Diagramatic representations of the recombination of a GFP-labelled donor segment with a *Dom* recipient gut segment in the organotypic culture

(A) Midgut recombination in the organotypic culture:

The caudal end of an E11.5 GFP-labelled midgut segment is recombined with the rostral end of a $Sox10^{Dom/Dom}$ midgut segment at E11.5 to E15.5 (for recombination and culture protocols are described in Section 3.2.2.2). The recombined segments are to be maintained in the organotypic culture for 3 days before fixation for whole mount immunohistochemical staining.

(B) Hindgut recombination in the organotypic culture:

The caudal end of an E11.5 GFP-labelled hindgut segment is recombined with the rostral end of a $Sox10^{Dom/Dom}$ hindgut segment at E12.5 to E15.5 (for recombination and culture protocols, see Section 3.2.2.2). The recombined segments are to be maintained in the organotypic culture for 3 days before fixation for whole mount immunohistochemical staining.


Midgut recombination in organotypic culture
E11.5
GFP-labelled
midgut
E11.5-E15.5
Sox10Dom/Dom
midgut
Donor
Recipient
Culture for 3 days
A

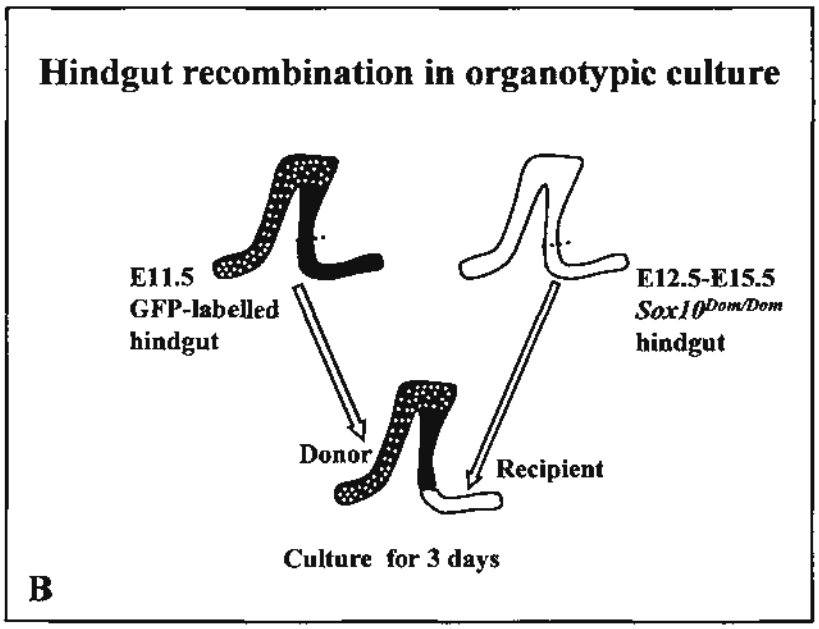

Hindgut recombination in organotypic culture
E11.5
GFP-labelled
hindgut
E12.5-E15.5
Sox10Dom/Dom
hindgut
Donor
Recipient
Culture for 3 days
B

Fig 3.10 Colonization by donor enteric neural crest-derived cells from the midgut of E11.5 EGFP mice in the recipient midgut segments of homozygous (*Sox10*$^{Dom/Dom}$) embryos from E11.5 to E15.5 in organotypic culture

The midgut of E11.5 EGFP mice is recombined with the midgut segment of *Sox10*$^{Dom/Dom}$ embryos from E11.5 to E15.5 where no enteric neural crest-derived cells are found. After culture for 3 days and whole mount immunostained, in all of the recipient E11.5+3d (A1-A3, n=5), E12.5 E11.5+3d (B1-B3, n=11), E13.5 E11.5+3d (C1-C3, n=7), E14.5 E11.5+3d (D1-D3, n=5) or E15.5 E11.5+3d (E1-E3, n=3), GFP-labelled enteric neural crest-derived cells from the midgut of E11.5 EGFP mice embryos are found. Tuj1 immunoreactivity shows that some of the GFP-labelled cells can differentiate into neurons, which is one of the derivatives of neural crest cells.

In the recipients at E11.5+3d (A1-A3) and E12.5+3d (B1-B3), the donor GFP-labelled enteric neural crest-derived cells are found to migrate into the recipients and colonize a large area of the midgut.

In the recipients at E13.5+3d (C1-C3), E14.5+3d (D1-D3) and E15.5+3d (E1-E3), GFP-labelled enteric neural crest-derived cells which migrate into the recipients are fewer than in recipient segments of E11.5+3d and E12.5+3d. There is only very sparse colonization of GFP-labelled enteric neural crest-derived cells in recipients from E13.5+3d to E15.5+3d.

The junctions between the donor and recipient segments are indicated by arrows.

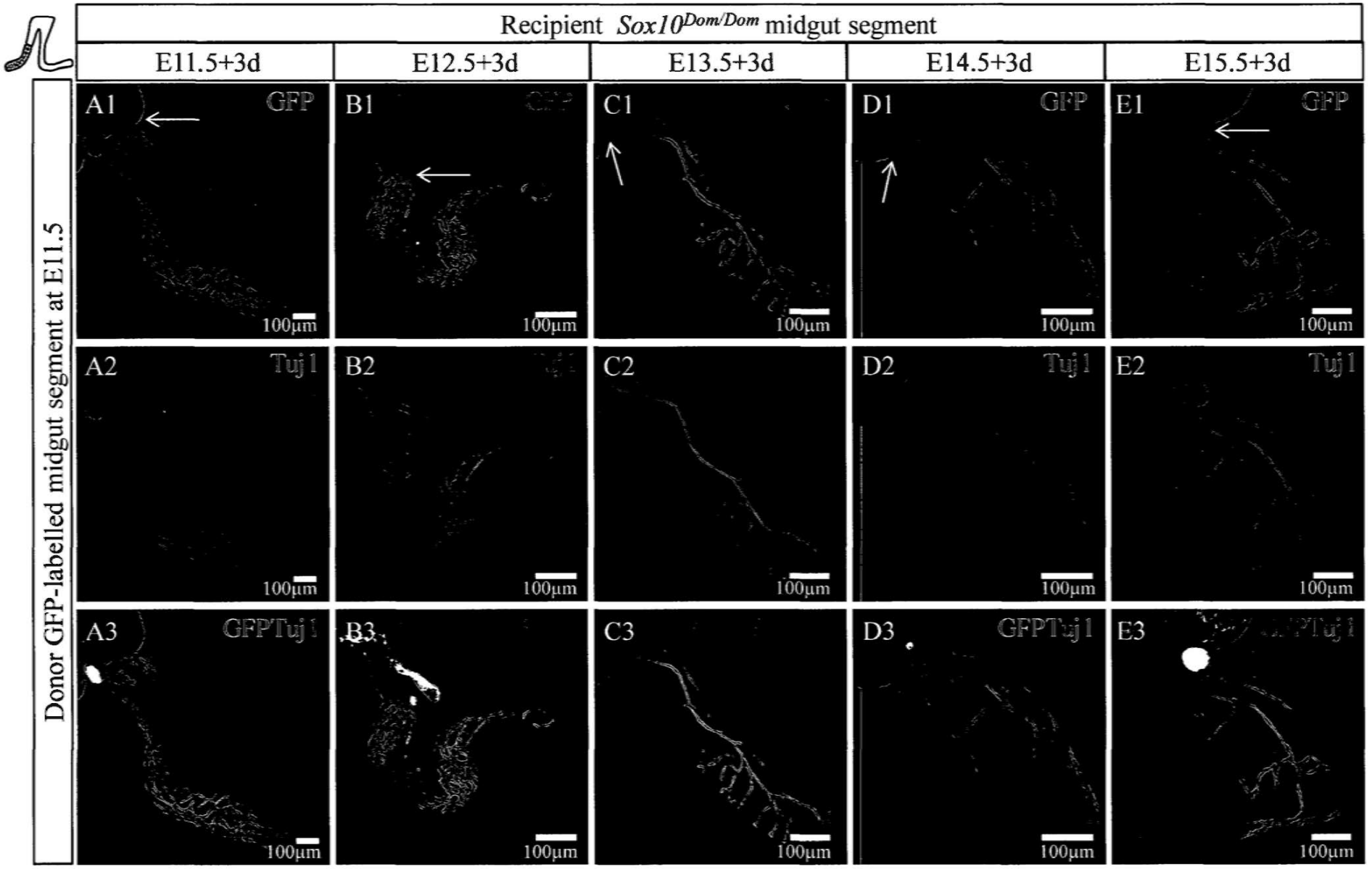

Recipient Sox10Dom/Dom midgut segment
E11.5+3d
E12.5+3d
E13.5+3d
E14.5+3d
E15.5+3d
Donor GFP-labelled midgut segment at E11.5
A1
GFP
B1
C1
D1
E1
A2
Tuj1
B2
C2
D2
E2
A3
GFPTuj1
B3
C3
D3
E3
100μm

Fig 3.11 Colonization by donor enteric neural crest-derived cells from the hindgut of E11.5 EGFP mice in the recipient hindgut segments of homozygous (*Sox10$^{Dom/Dom}$*) embryos from E12.5 to E15.5 in organotypic culture

The proximal hindgut of E11.5 EGFP mice is recombined with the aganglionic segment of the hindgut of *Sox10$^{Dom/Dom}$* embryos from E12.5 to E15.5. Whole mount preparations of the recombined segments are immunohistochemically stained after 3 days in culture. Donor GFP-labelled enteric neural crest-derived cells from the proximal hindgut of E11.5 EGFP mice are found to colonize the recipients in all of these recombination segments (E12.5+3d, n=4; E13.5+3d, n=3; E14.5+3d, n=3; E15.5+3d, n=2).

In E12.5+3d recipient segments (A1-A3), donor GFP-labelled enteric neural crest-derived cells are found to colonize the whole recipient segments and form a network of interconnecting cells. Tuj1 immunoreactivity shows that some of the GFP-labelled cells are capable to differentiate into neurons, which are one type of neural crest cell derivatives.

In E13.5+3d (B1-B3), E14.5+3d (C1-C3) and E15.5+3d (D1-D3) recipients, similar colonization pattern of donor GFP-labelled enteric neural crest-derived cells in the recipient segments is observed. However, within these recipient segments, sparse neural networks of cells are seen.

The junctions between the donor and recipient segments are indicated by arrows.

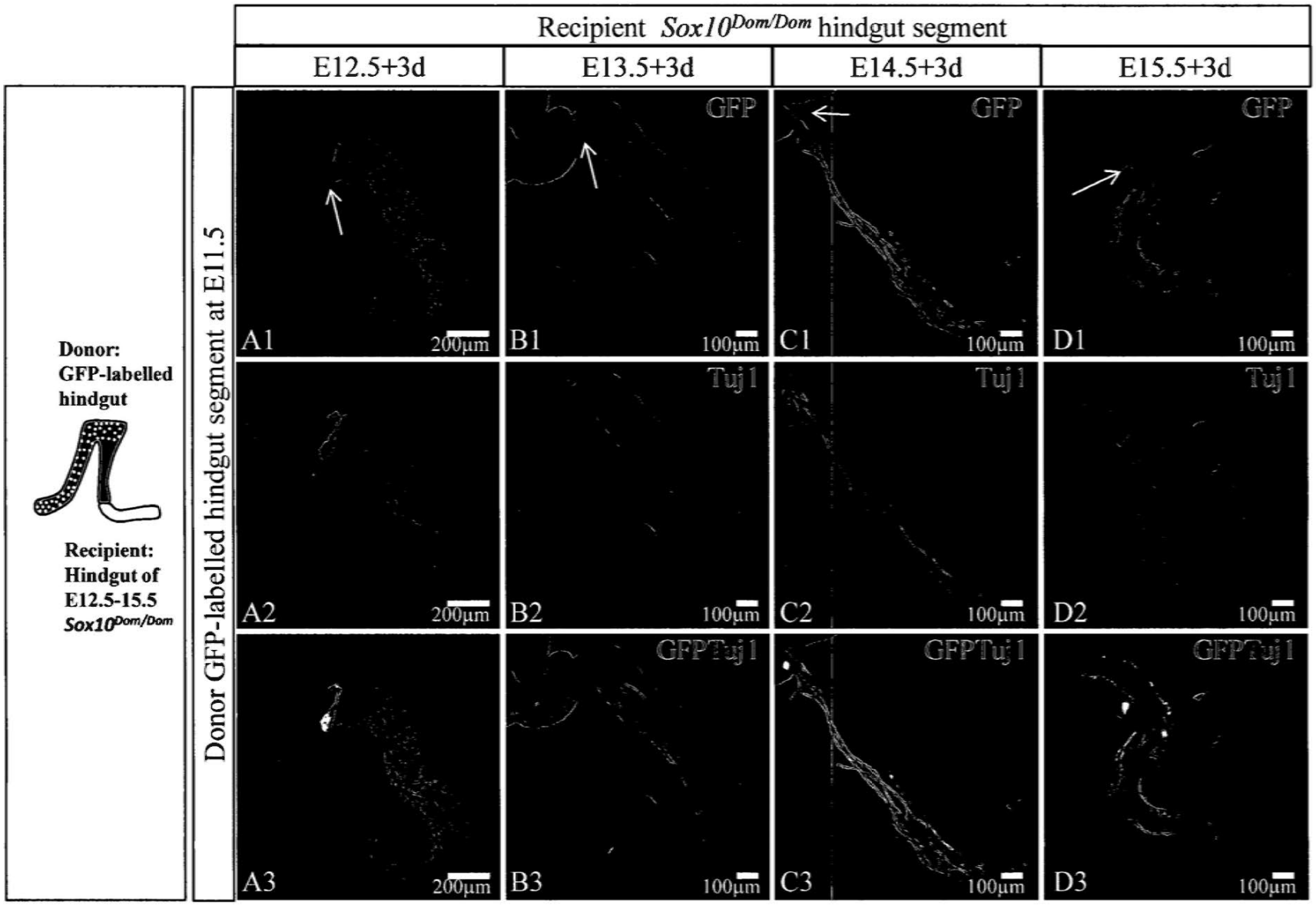

Recipient Sox10Dom/Dom hindgut segment
E12.5+3d
E13.5+3d
E14.5+3d
E15.5+3d
Donor GFP-labelled hindgut segment at E11.5
Donor:
GFP-labelled
hindgut
Recipient:
Hindgut of
E12.5-15.5
Sox10Dom/Dom
GFP
Tuj 1
GFPTuj 1
A1
B1
C1
D1
A2
B2
C2
D2
A3
B3
C3
D3
200μm
100μm

Fig 3.12 Presence of enteric neural crest cell within the recipient midgut segment at E11.5 affects the colonization of donor enteric neural crest-derived cells

In the recombination organotypic culture, a midgut segment at E11.5 is recombined with another midgut segment end-to-end. After 3 days in culture, the whole preparations of the recombined segments are immunohistochemically stained for Tuj1, which is a general marker for differentiating neurons.

In the recipient segment of the E11.5+3d *Sox10*$^{+/+}$ midgut (A1-A3), endogenous neural crest-derived cells have differentiated into neurons, become Tuj1 immunoreactive (but GFP negative) and are found throughout the whole recipient segment, while donor GFP-labelled neural crest-derived cells (green fluorescent cells) are found to migrate only a limited distance from the donor segment and only few GFP-labelled cells are observed in the proximal part of the recipient segment (n=5).

Similarly in the ganglionic recipient segment of the E11.5+3d *Sox10*$^{Dom/+}$ midgut (B1-B2), Tuj1 immunoreactive endogenous neural crest-derived cells are found throughout the entire length of the segment and only few donor GFP-labelled neural crest-derived cells (green fluorescent cells) are observed in the proximal part of the recipient close to the recombination junction (n=8).

However when aganglionic segments of the midgut of the E11.5 *Sox10*$^{Dom/+}$ (n=4) (C1-C3) or *Sox10*$^{Dom/Dom}$ (n=5) (D1-D3) embryos are used as recipient segments, no endogenous neural crest-derived cells (GFP^{-}/Tuj1^{+}) are found in these E11.5+3d recipients (C3 and D3), but many donor GFP-labelled neural crest-derived cells (C1 and D1), which are also Tuj1 immunoreactive because neural crest cells (C2 and D2) can differentiate into neurons, are observed to migrate quite a long distance away in these E11.5+3d recipient and form a neural network of interconnecting cells (C3 and D3).

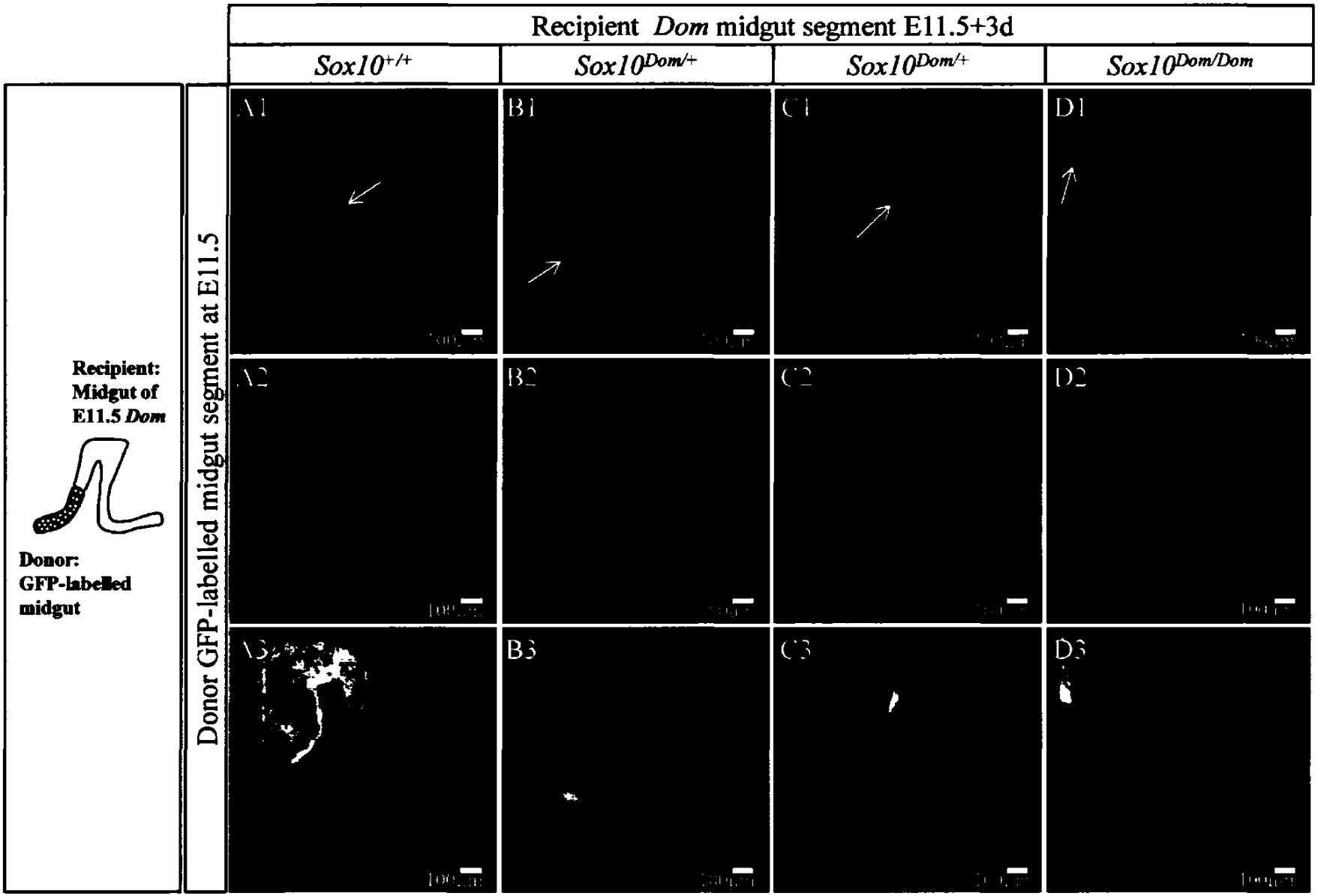
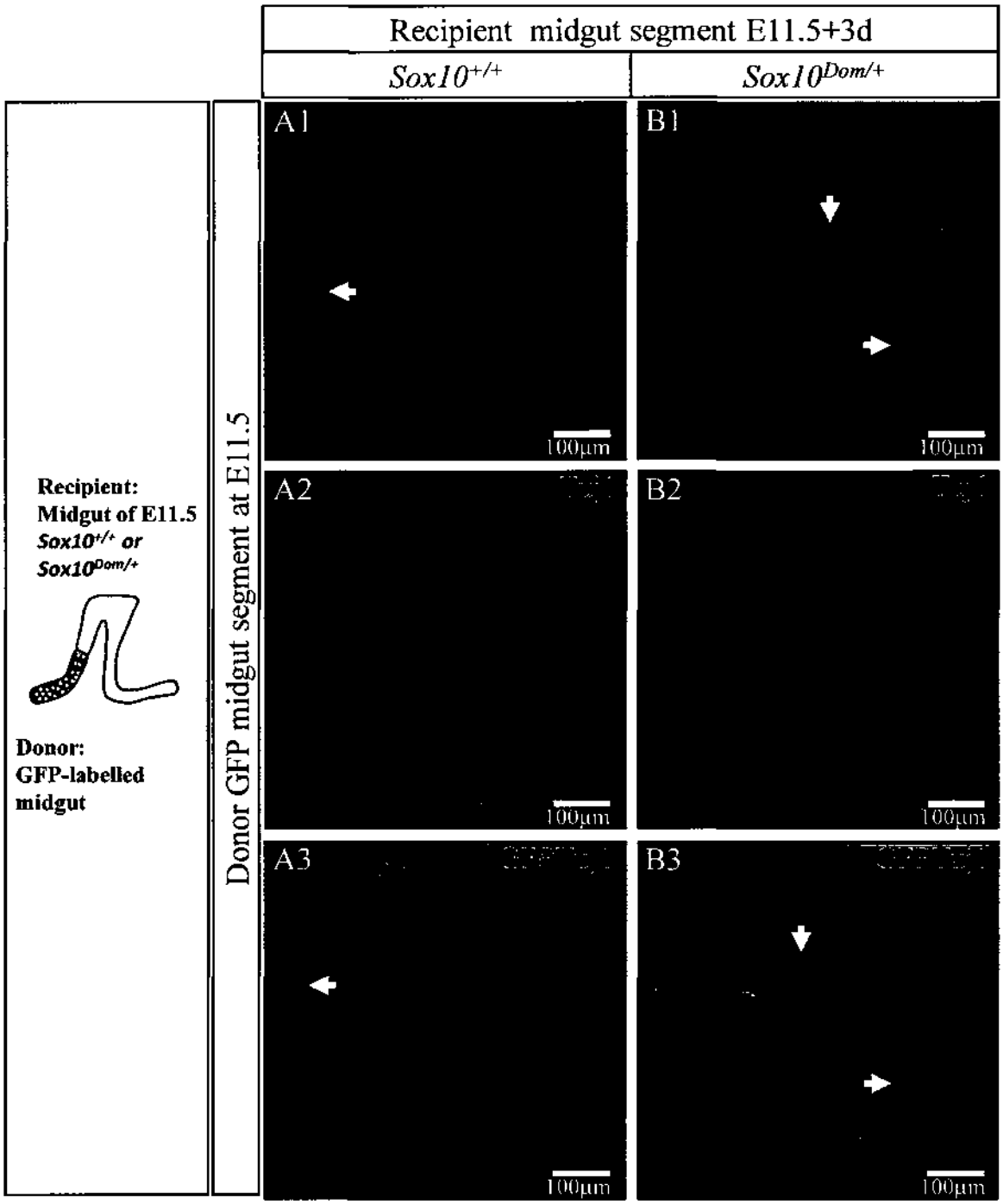

Recipient Dom midgut segment E11.5+3d
Sox10+/+
Sox10Dom/+
Sox10Dom/+
Sox10Dom/Dom
Recipient: Midgut of E11.5 Dom
Donor: GFP-labelled midgut
Donor GFP-labelled midgut segment at E11.5
A1
B1
C1
D1
A2
B2
C2
D2
A3
B3
C3
D3

Fig 3.13 Processes of donor enteric neural crest-derived cells extend into the ganglionic recipient segment

After three-day recombination organotypic culture, GFP-labelled processes (arrow in A1) from the donor midgut segment of the E11.5 EGFP mouse embryo are observed to extend into the ganglionic midgut segment of the E11.5+3d $Sox10^{+/+}$ embryo (A2) with a group of cells aggregated near the junction (A1 and A3). Note that the ganglionic recipient segments are packed with Tuj1 immunoreactive endogenous enteric neural crest-derived cells (A2 and A3).

Similarly, in the recipient midguts of the E11.5+3d $Sox10^{Dom/+}$ embryo (B1-B3), only very few donor GFP-labelled enteric neural crest-derived cells from the donor midgut of the E11.5 EGFP embryo are found in the recipient close to the recombination junction, and donor GFP-labelled processes (arrows in B1) are also found in the E11.5+3d $Sox10^{Dom/+}$ recipient midgut of the embryo with endogenous enteric neural crest-derived cells, which are Tuj1 immunoreactive but GFP negative.


Recipient midgut segment E11.5+3d
Sox10+/+
Sox10Dom/+
Recipient:
Midgut of E11.5
Sox10+/+ or
Sox10Dom/+
Donor:
GFP-labelled
midgut
Donor GFP midgut segment at E11.5
A1
B1
A2
B2
A3
B3
100µm

Chapter four

Colonization of embryonic and postnatal guts by enteric neural crest stem cells

4.1 Introduction

Enteric neuropathies comprise a vast and disparate array of congenital and acquired disorders of the enteric nervous system (Bassotti and Villanacci, 2006; De Giorgio and Camilleri, 2004; De Giorgio *et al.*, 2004; Grundy and Schemann, 2006; Milla *et al.*, 2002; Milla, 1988; Milla, 1998; Milla, 1999). These disorders range from a complete absence of enteric nerves in part of the bowel such as Hirschsprung's disease (HSCR) to more subtle abnormalities of the enteric nervous system structure or function (grouped within intestinal pseudoobstruction) (Thapar, 2007). More detailed information of Hirschsprung's disease was reviewed in Section 1.3.

4.1.1 Progress on treatments of enteric neuropathies

At present, therapeutic strategies for enteric neuropathies including Hirschsprung's disease are limited to surgery and the provision of artificial nutrition (Thapar, 2009). With the development of developmental biology and stem cell biology, the application of stem cells for treating diseases of tissue malformation, degeneration, trauma, and genetic deficiency gains momentum from scientific insights into new therapies (Daley, 2010).

Despite remaining issues, the last few years, exciting progress has linked developmental biology from the laboratory bench to the clinic. To date, central

nervous system derived neural stem cells were introduced into the pylorus of adult mice. They differentiated into neurons, expressed neuronal nitric oxide synthase (nNOS) and survived at least 8 weeks (Micci *et al.*, 2001). Embryonic stem cells derived Sox10-GFP-positive cells were also introduced into the cultured embryonic gut at the level of either intestine or cecum and generated neurons (Kawaguchi *et al.*, 2010). Human and mouse neurospheres derived from the enteric nervous system were transplanted into embryonic aganglionic mouse hindgut and shown to migrate appropriately, proliferate, differentiate into cells expressing S100 and PGP9.5 phenotypic markers which were specific to enteric glia and neurons respectively, and also form cells expressing gut neuropeptides such as vasoactive intestinal peptide (VIP) (Almond *et al.*, 2007). The enteric neural crest stem cells generated from the postnatal gut mucosa of mice and human (Metzger *et al.*, 2009) provide a new source and a potential on autologous transplantation for HSCR.

After the progenitor cells generated from embryonic mouse and neonatal human enteric nervous system had been transplanted into embryonic mouse hindgut explants and cultured for 8-12 days, functional assessment of the transplanted bowel confirmed that there was no significant difference between the contraction frequencies of ganglionic distal colon and transplanted distal colon, suggesting that transplanted neurons were functional (Lindley *et al.*, 2008). Synapses examined with transmission electron microscopy and calcium imaging of the transplanted neurospheres revealed the functional aspect of neurosphere-derived neurons (Lindley *et al.*, 2008). In the more mature gut environment, inflation stimulated contraction and electrical field stimulation-induced response were observed in grafted rat denervated colon with rat embryonic neuroepithelial stem cells while not observed in denervated colon (Liu *et al.*, 2007).

4.1.2 Transplantation approach *in vivo*

For the HSCR patients with absence of enteric ganglia in certain lengths of the gut, cell based therapy to supply the lost enteric neurons may be given at many locations by injections at multiple sites using fine micropipettes (Hotta *et al.*, 2009a; Kawaguchi *et al.*, 2010). However, the cells just injected into the abdomen by simple intra-peritoneal injection could colonize the ganglionic or aganglionic gut under the action of some unknown chemotactic agents (Martucciello *et al.*, 2007).

In utero transplantation (IUT) offers a potential treatment for a large number of diseases by transplantation of healthy cells into a fetus with a birth defect and is being pursued as a treatment for hematological and other kinds of birth defects in humans (Muench, 2005). With diagnosis of the genetic disease during the pregnancy, IUT has advantages such as: the cells can be delivered prior to the full development of the fetal immune system without the need for matching of major histocompatibility (MHC) antigens; the small size of the fetus obviates the need for a large graft and finding suitable donor tissues for IUT is less difficult than for postnatal transplantation (Muench, 2005). Using an ultrasound backscatter bio-microscopy, the enteric neural progenitor cells could be grafted into mouse embryonic gut by IUT (Sandgren and Pachinis, 2009).

4.1.3 Homing mechanism --- hematopoietic stem cell as an example

The initial event in the engraftment process, homing, is defined as the specific recruitment of circulating hematopoietic stem cell (HSC) to the bone marrow and involves the selective recognition of hematopoietic stem cells by the microvascular endothelium of the marrow and transendothelial cell migration into the extravascular

hematopoietic space. Current data suggests that homing involves the combined action of cell adhesion molecules (CAMs), cytokines, and chemotactic factors (Kerry *et al.*, 2004a).

Cell adhesion molecules play a critical role in mediating interactions between hematopoietic cells and the various components of the marrow stroma, as well as their associated biosynthetic products. Based on their structures and functions, these cell adhesion molecules can be divided into six main groups: the immunoglobulin (Ig) super-family (IgSF), integrin, cadherin, selectin, CD44, and mucin-like families (Kerry *et al.*, 2004a). Current data suggests key roles for the sialomucin receptor for P-selectin, PSGL-1 (P-selectin glycoprotein ligand-1) (Frenette *et al.*, 1998; Ma *et al.*, 1998), β1 integrin VLA4 (Very Late Antigen-4 or CD49d/CD29) (Papayannopoulou *et al.*, 2001), and the receptor for SDF-1(Stromal cell-derived factor-1), CXCR4 (a CXC chemokine Receptor, an alpha-chemokine receptor specific for SDF-1) (Lapidot, 2001), in the homing of hematopoietic stem cells to the bone marrow.

Cytokines act both locally by cell-to-cell contact or alternatively by binding to the extracellular matrix at sites where stem and progenitor cells adhere (Nathan and Sporn, 1991; Nugent and Newman, 1989). For example, macrophage colony stimulating factor (M-CSF) and stem cell factor (SCF) can exist in both a soluble and/or membrane-bound form, allowing them to mediate the adhesion of cells processing the relevant receptors (Flanagan *et al.*, 1991; Kerry *et al.*, 2004a; Long *et al.*, 1992; Massague, 1990; Rathjen *et al.*, 1990; Rettenmier *et al.*, 1987). Furthermore, cytokines including stem cell factor (SCF), thrombopoietin (TPO), Flt3 (FMS-like tyrosine kinase 3) -ligand, interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been shown to mediate interactions between hematopoietic cells and their microenvironment as a

consequence of their ability to modulated the affinity state of adhesion receptors on cytokine-responsive cells (Kinashi and Springer, 1994).

In contrast to the homing process, hematopoietic stem cell lodgement encompasses events following extravasation and is defined as the selective migration of cells to suitable hematopoietic microenvironment "niches" in the extravascular compartment of the bone marrow (Kinashi and Springer, 1994). Very little is known about the molecules that influence the site of the hematopoietic stem cell lodgement following homing to the bone marrow.

4.1.4 Gut motility --- function of the gastrointestinal tract

Several complex motor patterns, involving coordinated contractions and relaxations of the external muscle layers, have distinct roles in mature gut motility including segmentation, peristalsis and inter-digestive motility cycles (Hasler, 1999; Young, 2008). In adults, these motor patterns depend on the enteric nervous system. Gut motility can also be mediated or influenced by interstitial cells of Cajal (ICC) (Sanders, 1996).

In embryonic stage, propagating contractions, present in the small intestine of E14.5–E16.5 mice, are not mediated by interstitial cells of Cajal (ICC) or neurons for the following reasons. In the mouse duodenum, organized spontaneous contractions that propagate both orally and anally are observed in E14.5 mice, 6 days before birth; while these organized, propagating contractions, which are prominent at E16.5, are not neural mediated as they are unaffected by tetrodotoxin (TTX) which blocks action potential generation, and are present in the duodenum of mutant embryonic mice lacking enteric neurons (Burns *et al.*, 2009). Morphological ICC cannot be detected in the mouse duodenum of E14.5 to E16.5 mice (Burns *et al.*, 2009). Neural

mediated motility patterns, including mixing and migrating complexes, and slow wave activity (presumably mediated by ICC) are present in preparations of duodenum from E18.5 mice (2 days before birth) and in newborn mice (Burns *et al.*, 2009). Thus, the neural control of motility in the duodenum commences just before feeding. In the mouse jejunum, morphological ICC cannot be detected before E18, and slow waves cannot be recorded before E19 (Torihashi *et al.*, 1997; Ward *et al.*, 1997).

In the mouse embryonic colon, there are spontaneous contractions but enteric neurons are not required for colonic contractile activity or the propulsion of gut contents. Segments of colon removed from E11.5 mice before the arrival of enteric neuron precursors, and grown in culture, had shown rhythmic spontaneous contractions after 4 days culture *ex vivo* (Lindley *et al.*, 2008). These colonic explants at E11.5 lack enteric neurons, so the rhythmic contractions are not neural mediated. Shallow spontaneous propagating contractions have also been reported in the colon of E18.5 wild type mice and in mutant mice lacking colonic neurons, confirming that the contractile activity present in the colon of fetal mice is not neuronal mediated (Roberts *et al.*, 2007). Results from mutant late fetal mice showed that the movement of meconium (the contents of the gut lumen before birth consisting of epithelial cell debris, amniotic fluid, bile, and mucus) along the intestine is the same in fetal mice lacking enteric neurons as it is in wild type fetus (Anderson *et al.*, 2004). Other evidence is that colonic migrating motor complexes (MMCs) depending on neural activity cannot be detected in the colon of newborn or postnatal day 4 mice (Roberts *et al.*, 2007). While the rhythmic contractions in cultured aganglionic fetal colon were abolished by Kit functional blocking antibodies, ICC appeared to be required for the generation of rhythmic contractions in the fetal

mouse colon (Lindley *et al.*, 2008). During the development of embryonic intestine, the propagating contractions, first motor pattern is myogenic and depended on the entry of extracellular Ca^{2+} through L-type Ca^{2+} channels (Roberts *et al.*, 2010).

Neural activity was also reported that contractions in the distal colon of newborn mice occurred at a higher frequency and were more irregular in the presence of tetrodotoxin (TTX), suggesting that, although neurons were not required for their generation, the contractile activity was modulated by neural activity (Lindley *et al.*, 2008).

The intact and functional enteric nervous system plays important role in controlling the gastrointestinal motility in mature animal, but the first motility patterns in the duodenum and colon of fetal mice seems not requiring enteric neurons. Neural control of the small intestine commences shortly before birth, which is consistent with the fact that the mutant mice lacking enteric neurons from all or a majority of the gastrointestinal tract, died within 24 hours before birth (Pattyn *et al.*, 1999; Schuchardt *et al.*, 1994; Southard-Smith *et al.*, 1998).

Profound changes in intestinal motility occur during the postnatal period but the involvement of the enteric nervous system, a key regulator of gastrointestinal motility, in these modifications remains largely unknown (Abalo *et al.*, 2009; Rathna *et al.*, 1989). The nutritional, behavioral and environmental changes associated with weaning may be essential to attain full maturation of the myenteric plexus and gastrointestinal motility (Abalo *et al.*, 2009). Randomly contractions appear starting from postnatal 5 days (P5) and rhythmic phasic contractions occur starting P14, whose frequency and amplitude increase over time *ex vivo*. *In vivo*, bead latency is significantly reduced between P14 and P21. The increased proportion of ChAT (cholineacetyltransferase)-immunoreactive neurons is time dependent starting from

P14. The proportion of nNOS immunoreactive neurons increases as early as P5 and do not change afterwards (De vries *et al.*, 2010).

Well prior to the presence of milk in the gastrointestinal tract, intestinal contents (meconium) are propagated anally (McLain, 1963). Little is known about the mechanisms controlling the propagation of meconium along the intestine of the human fetus. However, some infants with long-segment Hirschsprung's disease present with the features of meconium ileus (Stringer *et al.*, 1994), strongly suggesting that enteric neurons are required for the propulsion of meconium in fetal humans. The discrepancy between these human data and the data showing that enteric neurons are not required for the propulsion of meconium before birth in mice might be due to the fact that humans are born at a developmentally later stage than mice (Burns *et al.*, 2009).

4.1.5 Challenges

Except these promising data, before providing a safe route to bring these advances into routine clinical practice, clinicians using cell based therapy for enteric neuropathies need to consider more questions prior to clinical application. These questions are listed as follows: what is the mechanism of certain stem cells to home in correct sites of the recipient? By what approach should the stem cells be transplanted? How much contribution will the transplanted stem cells have in the recipient? What are the criteria for measuring the therapeutic effect of the recipient gut with transplanted stem cells? How long will the therapeutic effect keep? Is there any risk of neoplastic growth of the transplanted cells?

4.1.6 Objective of this chapter

To explore the therapeutic potential of enteric neural crest stem cells, GFP-labelled enteric neural crest stem cells were isolated from embryonic gut and then cultured as neurospheres with methods described in Section 2.2.1 of Chapter 2. They were then transplanted into the prenatal and postnatal guts and the following will be carried out:

a. To compare the colonization of the embryonic hindgut of $Sox10^{Dom/Dom}$, $Sox10^{Dom/+}$ and $Sox10^{+/+}$ mice *ex vivo* by enteric neural crest stem cells migrated from the GFP-labelled neurosphere;
b. To compare the colonization of the embryonic cecum of $Sox10^{Dom/Dom}$, $Sox10^{Dom/+}$ and $Sox10^{+/+}$ mice *ex vivo* by enteric neural crest stem cells migrated from the GFP-labelled neurosphere;
c. To compare the colonization of the embryonic midgut of $Sox10^{Dom/Dom}$, $Sox10^{Dom/+}$ and $Sox10^{+/+}$ mice *ex vivo* by enteric neural crest stem cells migrated from the GFP-labelled neurosphere;
d. To compare the colonization of the postnatal colon in $Sox10^{Dom/+}$ and $Sox10^{+/+}$ mice *in vivo* by enteric neural crest stem cells which were transplanted on the external wall of the descending colon.

By using immunohistochemical staining on whole mount preparations and serial cross sections of the gut with transplanted GFP-labelled cells and comparing the colonization of the GFP-labelled cells in different recipients, information of the therapeutic potential of the enteric neural crest stem cells was derived and analyzed both *ex vivo* and *in vivo*.

4.2 Materials and Methods

4.2.1 Transplantation of GFP-labelled neural crest stem cells into E11.5 *Dom* embryonic gut *ex vivo*

GFP-labelled enteric neural crest stem cells were cultured as neurospheres according to the Section 2.2.1 of Chapter 2. Grafting of cells into embryonic gut was performed according to the method described by Natarajan (Natarajan *et al.*, 1999). The whole length of the gut from the stomach to the distal end of the hindgut was dissected out according to the method described in Section 2.2.1B of Chapter 2 from E11.5 *Dom* embryos. During transplantation, the gut was held by tungsten needles to an agarose block. A GFP-labelled neurosphere was grafted into the wall of the hindgut, cecum or midgut using a mouth pipette under a dissecting microscope. The successful transplantation of the neurosphere was checked under the inverted fluorescent microscope (Nikon). Then the gut with a transplanted GFP-labelled neurosphere was cultured in an organotypic culture for 5 to 7 days following the method described in Section 3.2.2.1 of Chapter 3.

4.2.2 Transplantation of GFP-labelled neurospheres into postnatal *Dom* mice by laparotomy

In order to prevent pain or unnecessary suffering, postnatal day 7 *Dom* mice were anaesthetized by an intraperitoneal injection of 2 µl/g of anesthesia solution (see Appendix II). Several minutes later, the postnatal mice were anesthetized. The surgical instruments were sterilized by heating at 160℃ for 4 hours and the operating board was disinfected by 70% alcohol before the surgery. By location the descending

colon after the abdominal cavity was cut open, the neurospheres containing approximately $6x10^5$ GFP-labelled enteric neural crest stem cells in 100 μl mouse self renewal medium were transplanted onto the external wall of the descending colon. The abdomen was then sutured. The postnatal mouse was lying on its back until recovered from anesthesia in a 37℃ wet warm chamber. Prepared quinine hydrochloride (see Appendix II) was administered to the surgical wound to prevent biting of the wound by the mouse's mother or other littermates, after surgery. 7-14 days later, the mice after surgery were sacrificed and the genotypes of the mice were confirmed by PCR by using tail tissues with the method described in Section 3.2.1 of Chapter 3. All the organs within the abdomen cavity were checked under a fluoresce microscope. The colon with GFP positive cells was fixed and stained following the methods described in Section 2.2.9 of Chapter 2 or embedded and sectioned serially before immunohistochemical staining following the methods described in Section 4.2.3 of Chapter 4.

4.2.3 Serial cryo-sectioning of the recipient postnatal colon and immunohistochemical staining

7 to 14 days after the surgery, the postnatal colon segments with GFP-labelled cells were fixed in 4% paraformaldehyde overnight and dehydrated by a graded series of 10%, 20% and 30% sucrose solution at least 8 hours. The segments were embedded with Optimal Cutting Temperature compound (OCT, Tissue-Tek) and stored at -20℃.

The cryosections of 10 μm were cut serially using a cryostat (Thermo) and mounted onto histological glass slides and stored at -20℃ after being dried overnight in darkness. The serial sections were immunochemically stained as

described in Section 2.2.9.2 of Chapter 2.

4.3 Results

4.3.1 Colonization of the *Dom* aganglionic hindgut segment in the organotypic gut culture by cells from the neurosphere

At E11.5, the colonization pattern of enteric neural crest-derived cells in *Dom* embryonic gut had been described in Section 3.3.1. Briefly, the enteric neural crest-derived cells expressing p75 and (or) Sox10 had colonized the midgut and part of the proximal hindgut just posterior to the cecum in $Sox10^{+/+}$ embryos, while in $Sox10^{Dom/+}$ embryos, the enteric neural crest-derived cells colonized the midgut anterior to the cecum only without any of them in the hindgut, and no enteric neural crest-derived cells were found either in the midgut or hindgut in the $Sox10^{Dom/Dom}$ embryos.

A GFP-labelled neurosphere was transplanted into each of the distal hindguts isolated from E11.5 *Dom* embryos (Fig. 4.1 A-C) and cultured for 5 to 7 days *ex vivo* in an organotypic gut culture before the hindguts were fixed and immunohistochemically stained with the Tuj1 antibody.

In the hindgut isolated from $Sox10^{Dom/Dom}$ (n=12), no endogenous enteric neural crest-derived cells were found during the period from the time of transplantation to the time of fixation. GFP-labelled cells migrated from the GFP-labelled neurosphere to the $Sox10^{Dom/Dom}$ recipient hindgut segment and colonized the adjacent regions of the transplanted site in both rostral and caudal directions (Fig. 4.1D1-D3). Some of the GFP-labelled cells then differentiated into Tuj1 immunoreactive neurons (Fig. 4.2A1-A3).

When the GFP-labelled neurosphere was transplanted into the *Sox10*$^{Dom/+}$ hindgut (n=18), no endogenous enteric neural crest-derived cells were found in the hindgut at the time of transplantation. After 5 to 7 days culture *ex vivo*, the hindgut was colonized by endogenous enteric neural crest-derived cells migrated from the rostral part of the gut in some *Sox10*$^{Dom/+}$ embryos (n=16). In the *Sox10*$^{Dom/+}$ hindgut segments with endogenous neural crest-derived cells after culture, GFP-labelled cells from the GFP-labelled neurosphere migrated in the rostral or caudal direction in the recipient hindgut segment and integrated into the neural network formed by endogenous enteric neural crest-derived cells (Fig. 4.1E1-E3). High magnification images showed that some of these GFP-labelled cells were Tuj1 immunoreactive neurons (Fig. 4.2B1-B3) and they interconnected with the Tuj1 positive endogenous neurons (GFP$^-$/Tuj1$^+$) to form a neural network. In the region without endogenous enteric neural crest-derived cells, GFP-labelled cells and fibers from the GFP-labelled neurospheres formed neural network (n=2, Fig. 4.1G1-G3 and Fig. 4.2D1-D3).

When the GFP-labelled neurosphere was transplanted into the aganglionic distal hindgut of wild *Sox10*$^{+/+}$ embryos (n=14) and cultured for 5 to 7 days *ex vivo*, the hindgut was completed colonized by the endogenous enteric neural crest-derived cells. GFP-labelled cells also could migrate from the GFP-labelled neurosphere and interconnected with the endogenous neurons migrated from the rostral end of the gut (Fig. 4.1F1-F3 and Fig. 4.2C1-C3).

The GFP-labelled cells could form a neural network in the hindgut of different genotypes. At the stage when the endogenous enteric neural crest-derived cells reached the distal end of the hindgut, the GFP-labelled cells were able to interconnect with them in *Sox10*$^{Dom/+}$ and *Sox10*$^{+/+}$ hindguts.

4.3.2 Colonization of the *Dom* cecum in the organotypic gut culture by cells from the neurosphere

At E11.5, the cecum has already been colonized by endogenous enteric neural crest-derived cells in the $Sox10^{+/+}$ embryonic gut. In the $Sox10^{Dom/+}$ embryos, some enteric neural crest-derived cells have colonized the cecum, but there are no enteric neural crest-derived cells in the cecum of the $Sox10^{Dom/Dom}$ embryo.

When the GFP-labelled neurosphere was transplanted into the cecum of the E11.5 $Sox10^{Dom/Dom}$ embryo (n=8) (Fig. 4.3A), GFP-labelled cells migrated from the neurosphere in the cecum (Fig. 4.4D1-D3). At high magnification, it was found that GFP-labelled cells formed a neural network in the cecum (Fig. 4.5A1-A3). When the neurosphere was transplanted into the cecum of the E11.5 $Sox10^{Dom/+}$ embryo (n=16) (Fig. 4.3B), GFP-labelled cells also migrated from the neurosphere (Fig. 4.3E1-E3) but there were fewer migrating cells than in the $Sox10^{Dom/Dom}$ cecum. In the $Sox10^{+/+}$ embryonic cecum (n=7) (Fig. 4.3C), GFP-labelled cells which migrated from the GFP-labelled neurosphere were even less as compared with those in the $Sox10^{Dom/Dom}$ and $Sox10^{Dom/+}$ cecum (Fig. 4.3F1-F3 as compared with Fig. 4.3D1-D3 and E1-E3). Some of the GFP-labelled cells migrated from the neurospheres were interconnected with endogenous enteric neural crest-derived cells in the $Sox10^{Dom/+}$ and $Sox10^{+/+}$ embryonic cecum (Fig. 4.4B1-B3 and C1-C3). The GFP-labelled fibers from the GFP-labelled neurosphere extended more extensively in the $Sox10^{Dom/+}$ and $Sox10^{+/+}$ embryonic cecum which had already been colonized by endogenous enteric neural crest-derived cells than in the $Sox10^{Dom/Dom}$ cecum without endogenous enteric neural crest-derived cells (Compare Fig. 4.3E1-E3 and F1-F3 with Fig. 4.3D1-D3).

4.3.3 Colonization of the *Dom* midgut in the organotypic gut culture by cells from the neurosphere

At E11.5, the midgut of the $Sox10^{Dom/+}$ and $Sox10^{+/+}$ embryos has been colonized by endogenous enteric neural crest-derived cells while there are no enteric neural crest-derived cells in the $Sox10^{Dom/Dom}$ midgut.

When a GFP-labelled neurospheres was transplanted to the $Sox10^{Dom/Dom}$ embryonic midgut (n=9) (Fig. 4.5A), GFP-labelled cells migrated away from the GFP-labelled neurosphere towards all directions including the rostral and caudal directions (Fig. 4.5D1-D3). High magnification of the GFP-labelled cells showed that some of them were Tuj1 immunoreactive (Fig. 4.6A1-A3). The GFP-labelled cells tend to form more and longer fibers in the midgut than in the hindgut and cecum of $Sox10^{Dom/Dom}$ (Fig. 4.5D as compared with Fig.4.1D and Fig.4.3D). In the midgut of $Sox10^{Dom/+}$ embryos (n=22), GFP-labelled cells migrated from the GFP-labelled neurospheres (Fig. 4.5E1-E3 and Fig. 4.6B1-B3) were fewer than those in the midgut of $Sox10^{Dom/Dom}$ embryos. In the midgut of $Sox10^{+/+}$ embryos (n=16), the GFP-labelled fibers also extended further away from the neurosphere than the GFP-labelled cells migrated from the GFP-labelled neurosphere (Fig. 4.5 F1-F3 and Fig. 4.6C1-C3).

4.3.4 Colonization of the postnatal colon of $Sox10^{Dom/+}$ and $Sox10^{+/+}$ neonates by GFP-labelled neurospheres *in vivo*

During the laparotomy, the GFP-labelled neurospheres were transplanted onto the descending colon of $Sox10^{Dom/+}$ and $Sox10^{-/-}$ neonates at postnatal 7 days. After 7 to 14 days of the transplantation, the mice were sacrificed and the genotypes of the

mice were confirmed by PCR. Abdominal and pelvic tissues and organs were checked under a fluoresce microscope (see the Table 6 below).

Table 6 Number of cases found with GFP-labelled cells in different tissues and organs

	Sox10$^{Dom/+}$ mice n=13	*Sox10*$^{+/+}$ mice n=10
Abdomen wall	12	10
Testis and appendix	9	4
Bladder	1	2
Renal	1	5
Spleen	2	2
Pancreas	2	4
Mesentery	11	7
Colon	13	9

In the *Sox10*$^{Dom/+}$ postnatal mice examined (n=13), GFP-labelled cells were found in all descending colon which had been transplanted with neurospheres. Some GFP-labelled cells were also found in the inner surface of the abdomen wall where stitches were made. In the *Sox10*$^{+/+}$ cases (n=10) mice which had undergone transplantation, GFP-labelled cells were found in the descending colon of 9 cases and in abdominal stitches of all cases. Occasionally, GFP-labelled cells were also found in other tissues and organs examined and their influences on the recipient mice and organs were not explored in the present study.

When GFP-labelled neurospheres were transplanted to the distal descending colon of *Sox10*$^{Dom/+}$ neonates at postnatal day 7 (Fig. 4.7A), which were then sacrificed 7 to 14 days later, GFP-labelled cells were found to scatter on the intestinal wall and some of them extended fibers on the wall (Fig. 4.7B and B'). Some of these

GFP-labelled cells and fibers were Tuj1 immunoreactive (Fig. 4.7C1-C3). Serial cross sections of the segments with GFP-labelled cells showed that in all cross sections without $GFP^-/Tuj1^+$ endogenous ganglion cells, the GFP-labelled cells and fibers were located in the serosa, longitudinal muscle layer and circular muscle layer (Fig. 4.7D1-D3 and E1-E3) and some of them were Tuj1 immunoreactive. These GFP-labelled and Tuj1 immunoreactive ($GFP^+/Tuj1^+$) were mostly found between the longitudinal muscle and circular muscle layer (Fig. 4.7F1-F3). However, in all cross sections with $GFP^-/Tuj1^+$ endogenous ganglion cells, the GFP-labelled cells located only in the serosa layer of the colon (Fig. 4.9B1-B3) and no GFP-labelled cells or fibers were found in the muscular layers.

Similarly, when GFP-labelled neurospheres were transplanted to the wall of descending colon of the $Sox10^{+/+}$ neonates at postnatal day 7 (Fig. 4.8A), GFP-labelled cells could be found in the wall of the descending colon (Fig. 4.8B). They seemed to be scattered into the neural networks formed by the endogenous enteric neural crest-derived cells (Fig. 4.8C1-C3). However, when the descending colon was examined with a confocal microscope, GFP-labelled cells were not located in the myenteric ganglia layer (Fig. 4.8D1-D3). Many of the GFP-labelled cells were still Tuj1 immunoreactive (Fig. 4.8E1-E3). In serial cross sections, GFP-labelled cells located mainly in the serosa of the colon (Fig. 4.9A1-A3) in which normal myenteric ganglion cells were found.

4.4 Discussion

It has already been known that the migration of enteric neural crest-derived cells in the embryonic *Sox10*$^{Dom/+}$ gut was delayed (Kapur *et al.*, 1996; Wang, 2006) and the delay may be cell autonomous described in Section 3.3.1 of Chapter 3 with some minor influences from the microenvironment of the gut of different genotypes. In this Chapter, GFP-labelled enteric neural crest stem cells were isolated and transplanted into different segments (hindgut, cecum and midgut) of the *Sox10*$^{Dom/Dom}$, *Sox10*$^{Dom/+}$ and *Sox10*$^{+/+}$ gut at E11.5. The *Sox10*$^{Dom/Dom}$ and parts of *Sox10*$^{Dom/+}$ segments were aganglionic at the time of transplantation. The enteric neural crest stem cells migrated from the neurosphere and formed neural networks in the region adjacent to the transplanted neurospheres after 5 to 7 days culture *ex vivo*. In the aganglionic gut segments, enteric neural crest stem cells were able to colonize the aganglionic segment, an observation consistent with previous studies (Almond *et al.*, 2007; Kawaguchi *et al.*, 2010; Lindley *et al.*, 2008; Metzger *et al.*, 2009; Natarajan *et al.*, 1999). These transplanted cells can organize into ganglia as well as differentiation into mature neuronal subtypes such as vasoactive intestinal polypeptide and nitric oxide synthase-containing neurons (Metzger *et al.*, 2009).

In the organotypic culture of the *Sox10*$^{Dom/+}$ and *Sox10*$^{+/+}$ gut segments transplanted with neurospheres, the transplanted cells could integrate with the endogenous enteric neural crest-derived cells which colonized the hindgut after transplantation. This evidence indicates that the transplanted enteric neural crest stem cells are able to interact with migrating endogenous enteric neural crest-derived cells to form a normal network of neuronal cells.

4.4.1 Factors influencing the colonization of exogenous enteric neural crest stem cells

In this Chapter, the transplanted enteric neural crest stem cells colonized and formed a cellular network in the *Sox10*$^{Dom/Dom}$ cecum, while in the *Sox10*$^{Dom/+}$ and *Sox10*$^{+/+}$ cecum in which endogenous enteric neural crest-derived cells were found, the migration and colonization of enteric neural crest stem cells into the gut segments were affected and neural fibers were observed to extend from the neurosphere to the gut segment. This observation is in line with the findings made in Chapter 3 (section 3.3.3.3), in which the presence of endogenous enteric neural crest cell within the recipient gut affected the colonization by exogenous enteric neural crest-derived cells. Similar, more enteric neural crest stem cells were found to migrate from the neurosphere into the aganglionic midgut of *Sox10*$^{Dom/Dom}$ embryos as compared with in the ganglionic midgut of the *Sox10*$^{Dom/+}$ and *Sox10*$^{+/+}$ embryos.

Apart from the presence of endogenous enteric neural crest-derived cells (Hotta *et al.*, 2009a; Meijers *et al.*, 1992), the extent of the maturation of the recipient gut segment may also affect the colonization of exogenous neural crest stem cells (Hotta *et al.*, 2009a). Presumably, graft cells may be easier to colonize less differentiated tissues. Hence, this may explain the decreased colonization ability in the *Sox10*$^{Dom/Dom}$ midgut by the enteric neural crest stem cells comparing to in the *Sox10*$^{Dom/Dom}$ cecum and hindgut, which are less mature than the midgut. The muscle layer, whose differentiation follows an anterior-to-posterior sequence, may affect the colonization of exogenous enteric neural crest-derived cells as implicated from the observations made in Chapter 3 (sections 3.3.3.2 and 3.4.2). Therefore it is also possible that the differentiation of the muscle layer may also be one of the contributing factors that affect the colonization of the neural crest stem cells in the

midgut region.

Stem cells derived from different sources may have different contributions to the recipient. Stem cells in different regions of the nervous system, such as sciatic nerve of peripheral nervous system and enteric nervous system, give rise to different types of mature cells (Bixby *et al.*, 2002). Cranial neural crest cells, as compared to trunk neural crest cells, have a position-determined higher proliferation rate (Zhang *et al.*, 2010). Sox10 expressing cells generated from embryonic stem cells exhibit molecular markers and differentiation phenotypes consistent with neural crest identity and they show the potential for therapeutic applications on Hirschsprung's disease (Kawaguchi *et al.*, 2010). Neurospheres containing enteric neural crest stem cells were derived from postnatal human gut mucosal tissue and injected into the center of the cultured aganglionic gut tissue of HSCR patients *ex vivo*. The cells from neurospheres were found to integrate within the recipient tissue and neural processes were found extending from the neurospheres (Metzger *et al.*, 2009). Neural stem cells or enteric neural crest stem cells when transplanted i*n vivo* were able to colonize in the pylorus and aganglionic colon of adult mice (Liu *et al.*, 2007; Martucciello *et al.*, 2007; Micci *et al.*, 2001). Here in this chapter, the results from the transplantation of GFP-labelled enteric neural crest stem cells to the wall of the colon by laparotomy showed that the enteric neural crest stem cells from E14.5 embryonic gut migrated into and colonized in the myenteric layers of aganglionic $Sox10^{Dom/+}$colon but they just distributed on the serosa of the ganglionic $Sox10^{Dom/+}$ and $Sox10^{+/+}$ colon.

During the development of the gut, enteric neural crest-derived cells invade the foregut as guided by GDNF (glial cell line derived neurotrophic factor), which is produced by the stomach mesenchyme and detected by the Ret receptor. When cells

are colonizing the midgut, GDNF expression is up-regulated in the more posterior cecum anlage (Natarajan *et al.*, 2002).

Signalling by the chemokine stromal cell-derived factor-1 (SDF-1, also known as CXCL12) via its receptor CXC chemokine receptor 4 (CXCR4) has been shown to be key components in the regulation of cell migration during the development of a variety of tissues including the nervous system (Killian *et al.*, 2009; Tran and Miller, 2003). CXCR4 receptors are expressed by dividing neural progenitor cells located in the subgranular zone (SGZ) (Bhattacharyya *et al.*, 2008), migrating mouse embryonic neural crest cells (Belmadani *et al.*, 2005), migrating zebrafish cranial neural crest cells (Chong *et al.*, 2001), skeletal muscles, heart and endothelium (Kucia *et al.*, 2005). SDF-1 is known to be expressed by stromal and endothelial cells, including bone marrow, heart, skeletal muscle, liver and brain (Kucia *et al.*, 2005) and within the migratory path of the neural crest cells to signal through CXCR4 to mediate positioning of dorsal root ganglia (Belmadani *et al.*, 2005). SDF-1/CXCR4 is an important signalling pathway in the hematopoietic stem cell homing and treatment of the heart and central nervous system injury (Madri, 2009; Schoenhard and Hatzopoulos, 2010; Zaruba and Franz, 2010). However the mechanism of gradient detection and polarization of these cells remains unknown (Dormann and Weijer, 2003).

Co-culture of smooth muscle cells from the aganglionic region with neurons form the neonatal mouse superior cervical ganglia *ex vivo* indicates that smooth muscle cells of the aganglionic colon is less favorable for neuronal development than the normally innervated region smooth muscle cells (Langer *et al.*, 1994). But in this study, the aganglionic segment is more permissive for the entry of enteric neural crest stem cells than the normal segment. Later some reports show the cytoskeletal

proteins are absent or markedly reduced in the aganglionic bowel of HSCR which may be responsible for the motility dysfunction in the aganglionic segment (Nemeth *et al.*, 2002) and the distribution of sarcoglycan subcomplex in colonic smooth muscle cells of aganglionic bowel is abnormal (Arena *et al.*, 2010). It is just a hypothesis that the impaired the smooth muscle in the aganglionic bowel may give certain cues for the homing of enteric neural crest stem cells not differentiated neurons. The ganglionic colon unlikely permitting the entry of enteric neural crest stem cells in this study may be due to the present of enteric ganglions and no effective chemotactic factors in the normal gut microenvironment.

There is less information about SDF-1/CXCR4 signalling in the enteric nervous system while it has been studied extensively on some other tissues such as central nervous system, heart, craniofacial development *et al* (Belmadani *et al.*, 2005; Galvez *et al.*, 2006; Killian *et al.*, 2009; Melchionna *et al.*, 2010; Zaruba and Franz, 2010). After laparotomy and transplantation, GFP-labelled cells were always found on the inner side of the abdomen wall. It may be explained by the factor that some homing agents released by the injured tissue of the abdomen may induced homing of the cells with stem cell properties. SDF-1 is highly expressed in regenerating skeletal tissue, where it plays a role in stem cell recruitment and vessel formation (Galvez *et al.*, 2006; Melchionna *et al.*, 2010). Neural stem cells isolated from rat and human brains express CXCR4 *ex vivo* (Pujol *et al.*, 2005; Tran *et al.*, 2004). *In vivo*, after traumatic brain injury, SDF-1 leaked from the injured area and diffused into the cortex, then the levels of *CXCR4* mRNA expression and CXCR4 protein synthesis increased significantly later and CXCR4-positive neural stem cells as well as elongated nerve fibers were attracted to the damaged area (Itoh *et al.*, 2009). So in the enteric nervous system, it is hypothesized that SDF-1/CXCR4 might play a role

in the neural crest cell migration and colonization. This hypothesis however awaits further investigation.

Only low percentages of transplanted cells survive in the transplantation site such as heart and hematopoietic organs for a long time (Muller-Ehmsen *et al.*, 2002; Pagani *et al.*, 2003). It has been proposed that there is some molecular signalling to benefit the stem cell therapy and chimeras formation (Flake *et al.*, 1986; Schoenhard and Hatzopoulos, 2010). Strategies to improve homing of the stem cells include enhancing adhesion protein and cytokine interaction between donor cells and host tissues (Ryzhov *et al.*, 2008; Vajkoczy *et al.*, 2003) and exploring the potential homing agents such as integrin-linked kinase (ILK) and hypoxia inducible factor-1α (HIF-1α), SDF-1, monocyte chemoattractant protein-3 (MCP-3), and VEGF (Schoenhard and Hatzopoulos, 2010).

4.4.2 Assessment of the therapeutic effect

In this Chapter, the functional recovery was not examined in the prenatal and postnatal gut with the transplanted GFP-labelled enteric neural crest stem cells. It has been reported that the contraction frequency was restored in embryonic hindgut after transplantation *ex vivo* (Lindley *et al.*, 2008). The inflation stimulated contraction and electrical field stimulation-induced response were observed in transplanted adult colon (Liu *et al.*, 2007). Some data however shows that at embryonic stage, propagating contractions, present in the small intestine of E14.5–E16.5 mice, are not mediated by interstitial cells of Cajal (ICC) or neurons. Neural control of motility in the duodenum commences just before feeding (Burns *et al.*, 2009; Torihashi *et al.*, 1997; Ward *et al.*, 1997). Morphological ICC cannot be detected in the mouse duodenum of E14.5 to E16.5 mice (Burns *et al.*, 2009), and in the mouse jejunum,

morphological ICC cannot be detected before E18. In addition, slow waves cannot be recorded before E19 (Torihashi *et al.*, 1997; Ward *et al.*, 1997). However, other reports show that ICC appears to be required for the generation of rhythmic contractions in the fetal mouse colon due to the rhythmic contractions in cultured aganglionic fetal colon are abolished by Kit functional blocking antibodies (Lindley *et al.*, 2008). The colonic migrating motor complexes (MMCs) depending on neural activity cannot be detected in the colon of newborn or postnatal day 4 mice (Roberts *et al.*, 2007). On the motility development of gut, there are so many controversial data that the contribution of the transplanted enteric neural crest stem cells in the embryonic and postnatal gut should be examined for a long time to assess the feasibility of using transplantation as a means of cell-based therapy.

4.5 Summary

In this Chapter, it was shown that GFP-labelled enteric neural crest stem cells were able to colonize the $Sox10^{Dom/Dom}$, $Sox10^{Dom/+}$ and $Sox10^{+/+}$ guts at E11.5. The enteric neural crest stem cells migrated from the neurosphere and in the recipient $Sox10^{Dom/Dom}$ gut they formed a neural network in the adjacent region of the transplanted neurospheres while in the $Sox10^{Dom/+}$ and $Sox10^{+/+}$ gut, they also integrated with endogenous enteric neural crest-derived cells which colonized the entire gut before or after transplantation. These results indicate that the exogenous enteric neural stem cells have the potential to compensate the loss of enteric neural crest-derived cells in the *Dom* embryos *ex vivo*.

Transplantation of GFP-labelled enteric neural crest stem cells to the wall of the colon by laparotomy showed that the enteric neural crest stem cells migrated into the myenteric layers and colonized the aganglionic segment of the $Sox10^{Dom/+}$ colon while in ganglionic segments of $Sox10^{Dom/+}$ and $Sox10^{+/+}$ colon, they just distributed on the serosa of the colon. These observations indicate that these GFP-labelled enteric neural crest stem cells may have the therapeutic application potential to compensate the deficiency of the enteric neural crest-derived cells in the postnatal period. The mechanism of underlying homing of the enteric neural crest stem cells to the region of enteric plexuses, the optimal procedure for transplantation and the functional contribution of enteric neural crest stem cells to the enteric nervous system need further clarifications.

4.6 Figures and Legends

Fig 4.1 Colonization of the aganglionic hindgut segment by cells from the neurosphere in the organotypic gut culture

(A) Diagram shows that at E11.5 there are no enteric neural crest-derived cells (as represented by black dots in B and C) in the midgut and hindgut in the *Sox10*$^{Dom/Dom}$ embryos. A GFP-labelled neurosphere derived from EGFP embryonic mouse gut is transplanted into the distal end of the hindgut of the *Sox10*$^{Dom/Dom}$ embryo (n=12).

(B) Diagram shows that the enteric neural crest-derived cells (black dots) have colonized the midgut just anterior to the cecum at E11.5 and the GFP-labelled neurosphere derived from EGFP embryonic mouse gut is transplanted into the distal end of the hindgut of the *Sox10*$^{Dom/+}$ embryo (n=18).

(C) Diagram shows that enteric neural crest-derived cells (black dots) have colonized the midgut and the proximal hindgut just posterior to the cecum in *Sox10*$^{+/+}$ embryos (n=14) at E11.5 and the GFP-labelled neurosphere is transplanted into the distal end of the hindgut of the *Sox10*$^{+/+}$ embryos.

The gut tube with GFP-labelled neurosphere was cultured *ex vivo* for 5-7 days and whole mount preparations of the gut segment was stained for Tuj1 expression. The preparations were examined under a confocal microscope.

(D1) to (D3) In the hindgut of the *Sox10*$^{Dom/Dom}$ embryo (n=12), there are no endogenous enteric neural crest-derived cells at the time of transplantation and harvest for fixation. GFP-labelled cells are observed in adjacent regions of the transplanted site and migrate along both towards rostral and caudal directions in the hindgut (D1). Most of GFP-labelled cells have differentiated into Tuj1 immunoreactive neurons (D2). (D3) is the merged image of (D1) and (D2).

(E1) to (E3) In the hindgut of the *Sox10*$^{Dom/+}$ embryo (n=18), the hindgut is colonized by endogenous enteric neural crest cells (GFP$^+$/Tuj1$^+$ cells in E3) migrated from the rostral hindgut in *Sox10*$^{Dom/+}$ embryos in many cases (n=16) (E2). GFP-labelled cells are able to migrate from the GFP-labelled neurosphere towards rostral and caudal (E1) and integrated into the neural network formed by endogenous enteric neural crest-derived cells (E3). (E3) is the merged image of (E1) and (E2).

In other *Sox10*$^{Dom/+}$ embryonic hindgut regions without endogenous enteric neural crest-derived cells (n=2), GFP-labelled cells and fibers (G1) are found to form a neural network (G2 and G3). Thin arrows in (G2) and (G3) show the migratory wave front of endogenous enteric neural crest-derived cells from the rostral hindgut. (G3) is the merged image of (G1) and (G2).

(F1) to (F3) In the hindgut of the *Sox10*$^{+/+}$ embryo (n=14), the hindgut is completely colonized by endogenous enteric neural crest-derived cells after cultured for 5 to 7 days *ex vivo* (F2). GFP-labelled cells are also observed to be able to migrate from the neurosphere (F1) and integrate into the endogenous neural network (F3). (F3) is the merged image of (F1) and (F2).

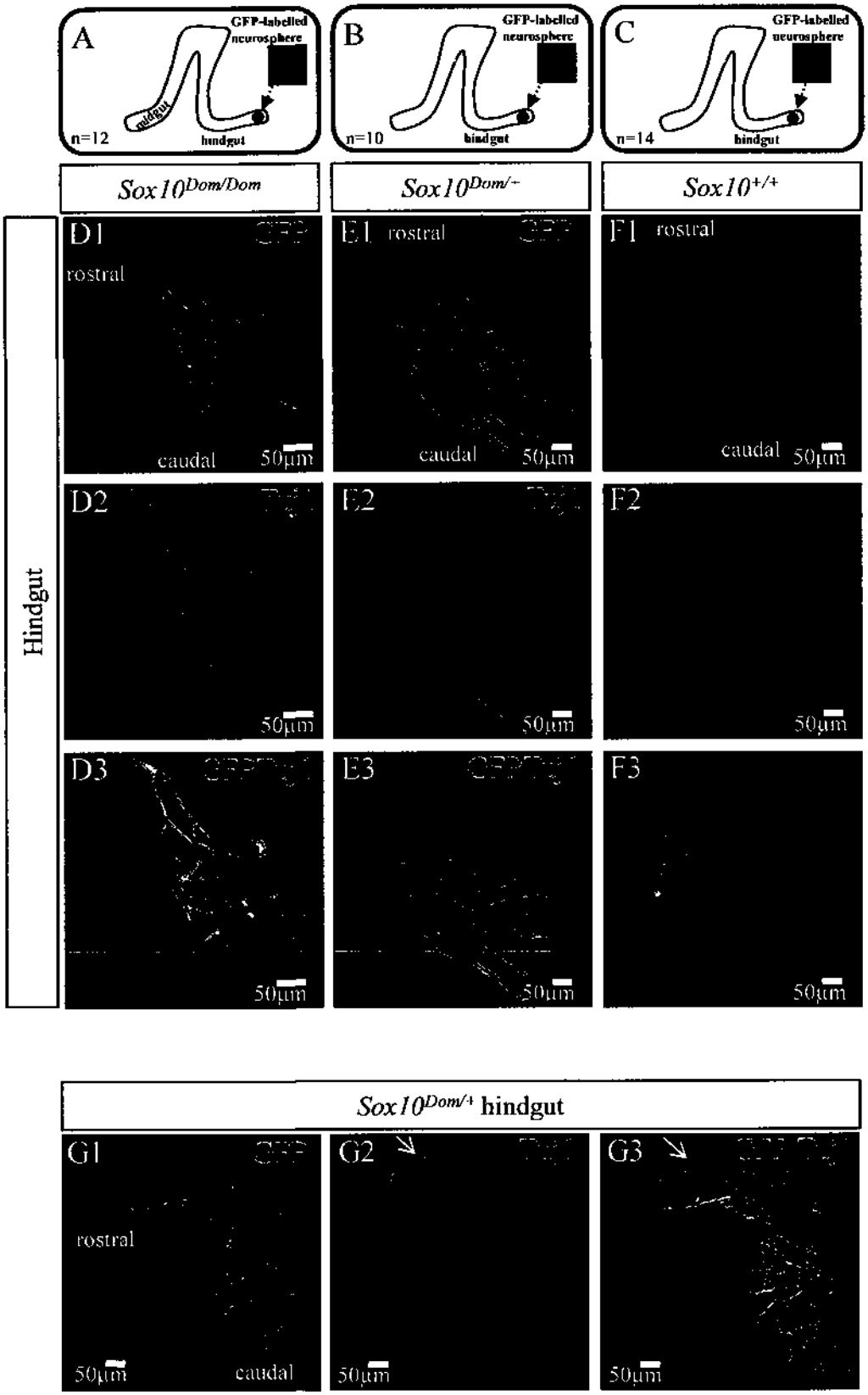

A
GFP-labelled neurosphere
midgut
hindgut
n=12
B
GFP-labelled neurosphere
hindgut
n=10
C
GFP-labelled neurosphere
hindgut
n=14
Sox10Dom/Dom
Sox10Dom/+
Sox10+/+
Hindgut
D1
rostral
caudal
50µm
E1
rostral
caudal
50µm
F1
rostral
caudal
50µm
D2
50µm
E2
50µm
F2
50µm
D3
50µm
E3
50µm
F3
50µm
Sox10Dom/+ hindgut
G1
rostral
50µm
caudal
G2
50µm
G3
50µm

Fig 4.2 Photomicrographs at high magnification on the colonization of the hindgut of different genotypes by cells from the neurosphere

(A1) to (A3) High magnification images show that in the *Sox10*$^{Dom/Dom}$ embryonic hindgut, some GFP-labelled cells (thick arrows in A1) have differentiated into Tuj1 immunoreactive neurons (thick arrows in A2). (A3) is the merged image of (A1) and (A2).

(B1) to (B3) High magnification images show in the *Sox10*$^{Dom/+}$ embryonic hindgut, GFP-labelled cells (thick arrows in B1), which are derived from the GFP-labelled neurosphere, are also Tuj1 immunoreactive neurons (thick arrows in B2). (B3) is the merged image of (B1) and (B2) and shows that GFP-labelled cells are interconnected with endogenous GFP$^-$/Tuj1$^+$ neurons.

(C1) to (C3) High magnification images show that in the *Sox10*$^{+/+}$ embryonic hindgut, most of the GFP-labelled cells, which are derived from the GFP-labelled neurosphere (C1), are not Tuj1 immunoreactive neurons (C2). (C3) is the merged image of (C1) and (C2) and shows that GFP-labelled cells are interconnected with the endogenous GFP$^-$/Tuj1$^+$ neurons.

(D1) to (D3) High magnification images show that in the region of *Sox10*$^{Dom/+}$ embryonic hindgut without endogenous enteric neural crest-derived cells, the GFP-labelled cells (thick arrows in D1) are Tuj1 immunoreactive neurons (thick arrows in D2), and form neural network together with GFP-labelled fibers (D2 and D3). (D3) is the merged image of (D1) and (D2).

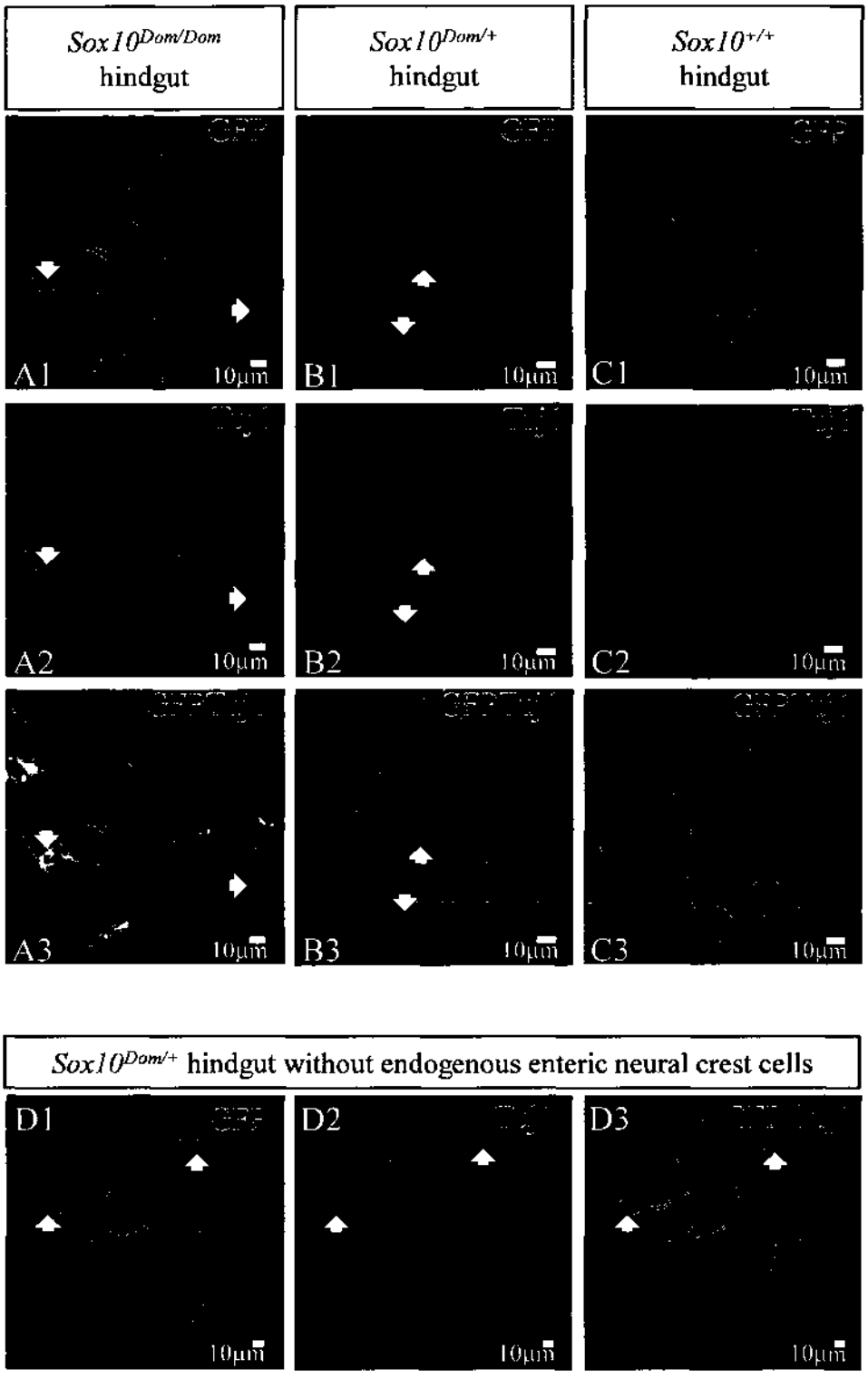

Sox10Dom/Dom hindgut
Sox10Dom/+ hindgut
Sox10+/+ hindgut
A1
B1
C1
A2
B2
C2
A3
B3
C3
10μm
Sox10Dom/+ hindgut without endogenous enteric neural crest cells
D1
D2
D3

Fig 4.3 Colonization of the cecum at E11.5 by cells from the neurosphere in the organotypic culture

(A) Diagram shows that at E11.5, a GFP-labelled neurosphere is transplanted into the cecum of the *Sox10*$^{Dom/Dom}$ embryo (n=8). No endogenous enteric neural crest-derived cells are observed within the cecum.

(B) Diagram shows that at E11.5, a GFP-labelled neurosphere is transplanted into the cecum of the *Sox10*$^{Dom/+}$ embryo (n=16). At this stage, endogenous enteric neural crest-derived cells (block dots) have colonized part of the cecum.

(C) Diagram shows that also at E11.5, a GFP-labelled neurosphere is transplanted into the cecum of the *Sox10*$^{+/+}$ embryos (n=7), which has already been colonized by endogenous enteric neural crest-derived cells (block dots).

The gut tube with the GFP-labelled neurosphere was cultured *ex vivo* for 5 to 7 days and whole mount preparations of the gut culture were stained for Tuj1 expression. The preparations were examined under a confocal microscope.

(D1) to (D3) When a GFP-labelled neurosphere is transplanted to the cecum of the E11.5 *Sox10*$^{Dom/Dom}$ embryo (n=8) and cultured for 5 to 7 days, GFP-labelled cells are observed in the cecum and regions of midgut and hindgut (D1). Tuj1 immunohistochemical staining (D2) shows the neural network formed by these exogenous GFP-labelled cells. (D3) is the merged image of (D1) and (D2).

(E1) to (E3) A GFP-labelled neurosphere is transplanted into the cecum of the *Sox10*$^{Dom/+}$ embryos. GFP-labelled cells from the neurosphere are observed in the cecum (E1) and many of them are Tuj1 positive cells (E2). GFP-labelled cells are found to interconnect with the non-GFP-labelled endogenous enteric neural crest-derived cells (E3). (E3) is the merged image of (E1) and (E2).

(F1) to (F3) Fewer GFP-labelled cells from the GFP-labelled neurosphere are observed as compared with the *Sox10*$^{Dom/Dom}$ and *Sox10*$^{Dom/+}$ recipients after 5 to 7 days in the organotypic culture *ex vivo* (F1). The colonization of GFP-labelled neurospheres in the cecum is organized in a pattern slightly different from that of endogenous enteric neural crest-derived cells (F2) although some GFP-labelled cells still form connections with endogenous enteric neural crest-derived cells (F3). (F3) is the merged image of (F1) and (F2).

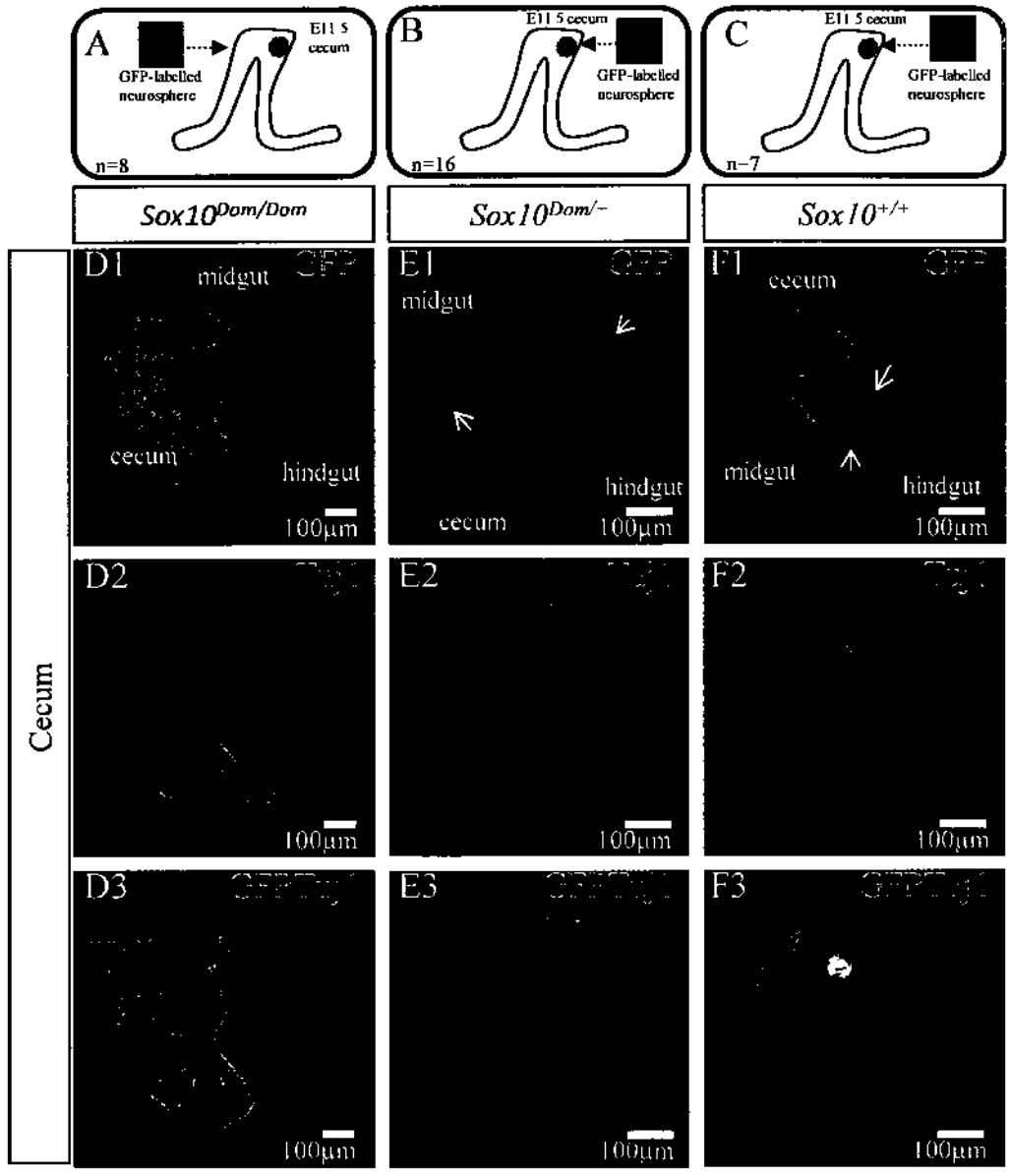

A
E11.5 cecum
GFP-labelled neurosphere
n=8
B
E11.5 cecum
GFP-labelled neurosphere
n=16
C
E11.5 cecum
GFP-labelled neurosphere
n=7
Sox10Dom/Dom
Sox10Dom/–
Sox10+/+
Cecum
D1
midgut
cecum
hindgut
100µm
E1
midgut
cecum
hindgut
100µm
F1
cecum
midgut
hindgut
100µm
D2
100µm
E2
100µm
F2
100µm
D3
100µm
E3
100µm
F3
100µm

Fig 4.4 Photomicrographs at high magnification on the colonization of the cecum of different genotypes by cells from the neurosphere

(A1) to (A3) High magnification images show that in the cecum of *Sox10*$^{Dom/Dom}$ embryo, GFP-labelled cells form a network of cells (A1) which have differentiated into Tuj1 immunoreactive neurons (A2). (A3) is the merged image of (A1) and (A2).

(B1) to (B3) High magnification images show GFP-labelled cells migrated from the GFP-labelled neurosphere (B1) in the *Sox10*$^{Dom/+}$ cecum. (B3) is the merged image of (B1) and (B2), which shows that GFP-labelled cells and fibers from the GFP-labelled neurosphere are interconnected with endogenous GFP$^-$/Tuj1$^+$ neurons and fibers.

(C1) to (C3) High magnification images show that fewer GFP-labelled cells migrated from the GFP-labelled neurosphere (C1) in the *Sox10*$^{+/+}$ embryonic cecum and some are Tuj1 immunoreactive (arrow in C2). (C3), which is merged image of (C1) and (C2), shows that GFP-labelled cells and fibers are interconnected with endogenous GFP$^-$/Tuj1$^+$ neurons and fibers.

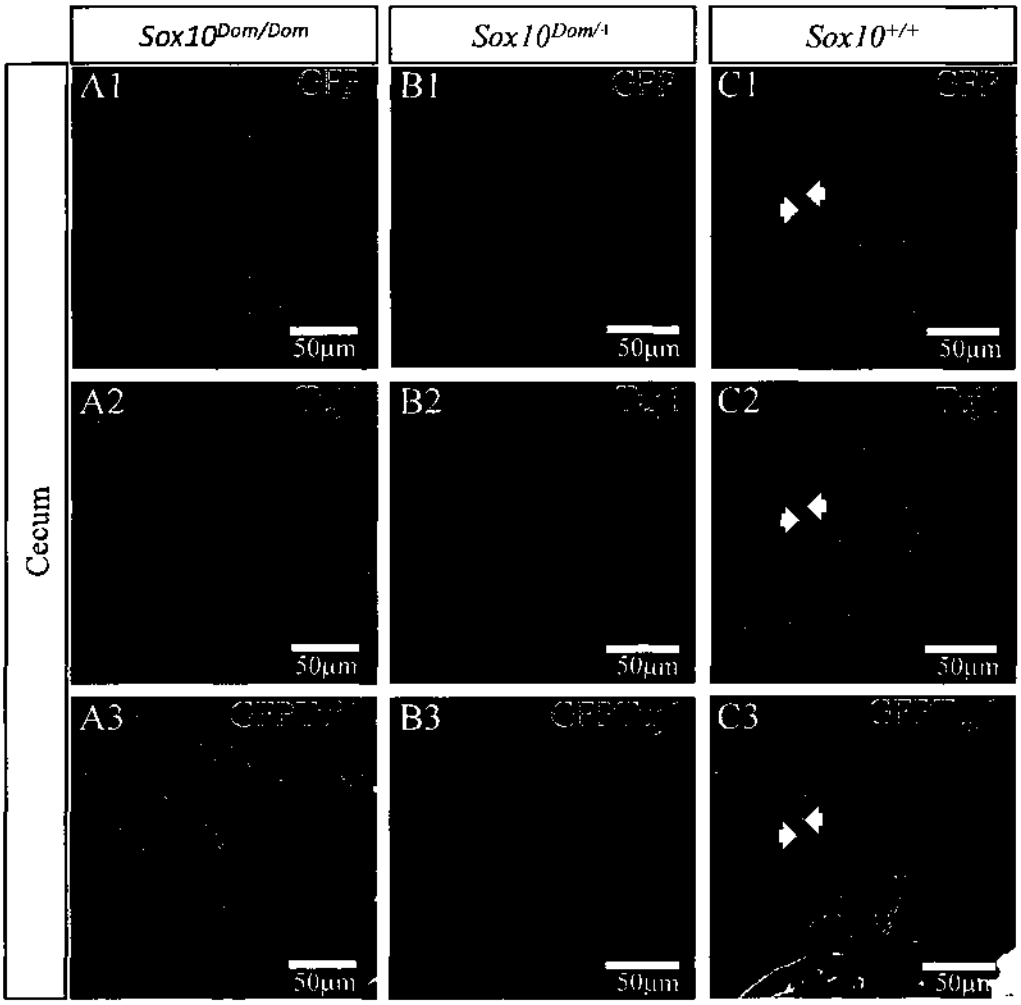

Sox10Dom/Dom
Sox10Dom/+
Sox10+/+
Cecum
A1
B1
C1
A2
B2
C2
A3
B3
C3
50μm

Fig 4.5 Colonization of the midgut at E11.5 by cells from the neurosphere in the organotypic gut culture

(A) Diagram shows the graft site of the GFP-labelled neurosphere in the aganglionic midgut of the *Sox10*$^{Dom/Dom}$ embryo (n=9).

(B) Diagram shows the graft site of the GFP-labelled neurosphere in the midgut of the *Sox10*$^{Dom/+}$ embryo, which has already been colonized by endogenous enteric neural crest-derived cells (black dots) (n=22).

(C) Diagram shows the graft site of the GFP-labelled neurosphere in the midgut of the *Sox10*$^{+/+}$ embryos, which has already been colonized by endogenous enteric neural crest-derived cells (black dots) (n=16).

The gut tube with GFP-labelled neurosphere was cultured *ex vivo* for 5 to 7 days and whole mount preparations of the gut were stained for Tuj1 expression. The preparations were examined under a confocal microscope.

(D1) to (D3) After 5 to 7 days culture *ex vivo*, in the midgut of *Sox10*$^{Dom/Dom}$ embryo (n=9) with transplanted GFP-labelled neurosphere, GFP-labelled cells with round and strong positive cell body (thick arrows in D1 and D3) and fibers (thin arrow in D1 and D3) from the GFP-labelled neurosphere are observed. Tuj1 immunohistochemical staining (D2) shows a sparsely neural network formed by these exogenous GFP-labelled cells and fibers. (D3) is the merged image of (D1) and (D2).

(E1) to (E3) In the midgut of the *Sox10*$^{Dom/+}$ embryos, few GFP-labelled cells migrated from GFP-labelled neurosphere (thick arrows in E1) are observed as compared with those in the *Sox10*$^{Dom/Dom}$ midgut recipient. In this *Sox10*$^{Dom/+}$ midgut recipient, endogenous enteric neurons and fibers (both are GFP$^-$/Tuj1$^+$) and exogenous GFP-labelled neurons (thick arrows) and fibers (thin arrows) are interconnected (E3). (E3) is the merged image of (E1) and (E2).

(F1) to (F3): In the *Sox10*$^{+/+}$ midgut, several GFP-labelled cells (thick arrows in F1 and F3) migrated from the GFP-labelled neurosphere are found. The midgut has already been fully colonized by endogenous enteric neural crest-derived cells (GFP$^-$/Tuj1$^+$) (F2). The GFP-labelled cells (thick arrows) and fibers (thin arrows in F1) (GFP$^+$/Tuj1$^+$) are integrated into the endogenous neural network (GFP$^-$/Tuj1$^+$) (F3) seen in the high magnification images Fig. 4.6 (C1)-(C3). (F3) is the merged image of (F1) and (F2).

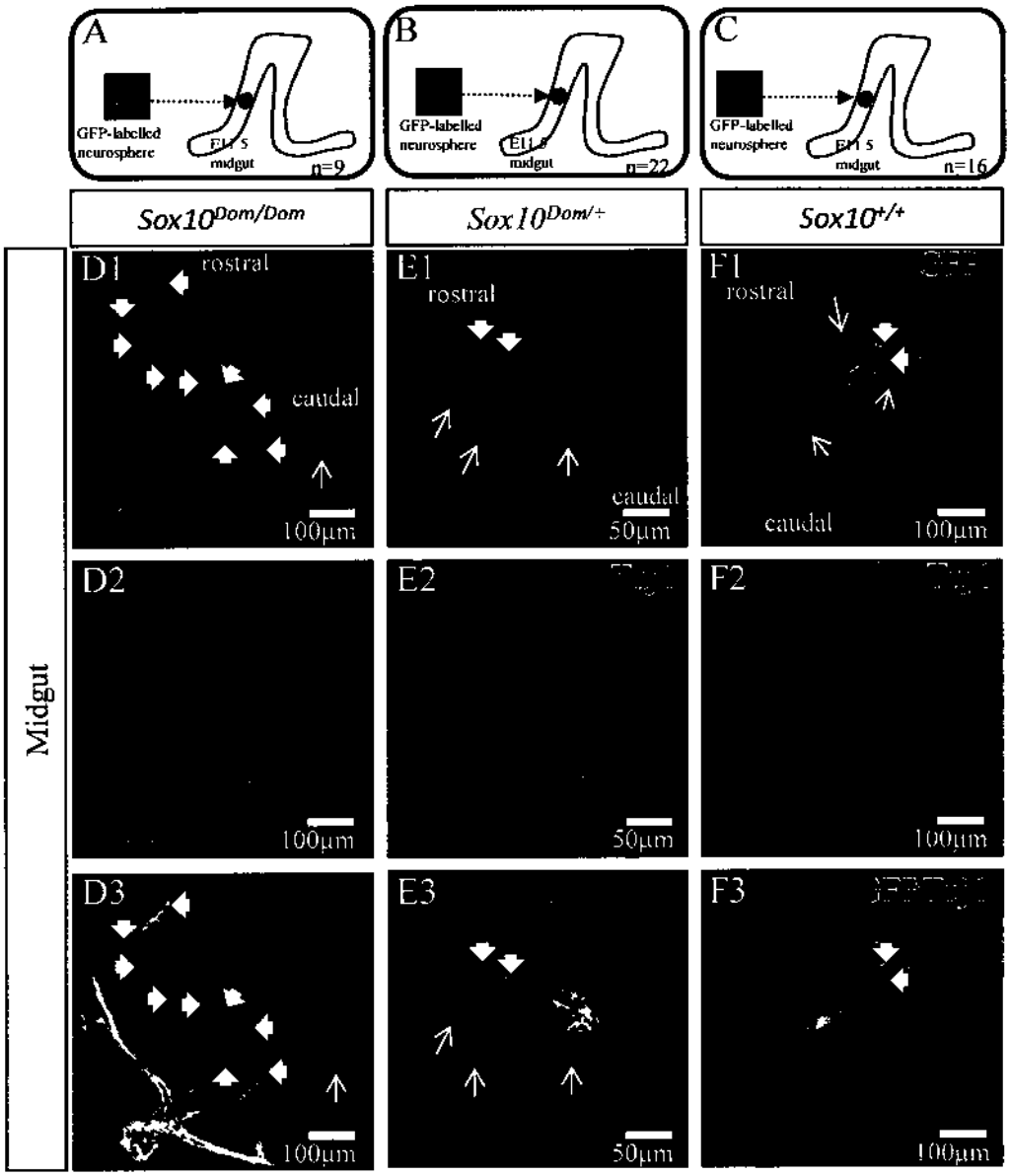

A
GFP-labelled neurosphere
midgut
n=9
B
GFP-labelled neurosphere
midgut
n=22
C
GFP-labelled neurosphere
midgut
n=16
Sox10Dom/Dom
Sox10Dom/+
Sox10+/+
Midgut
D1
rostral
caudal
100µm
E1
rostral
caudal
50µm
F1
rostral
caudal
100µm
D2
100µm
E2
50µm
F2
100µm
D3
100µm
E3
50µm
F3
100µm

Fig 4.6 Photomicrographs at high magnification on the colonization of the midgut of different genotypes by cells from the neurosphere

(A1) to (A3) High magnification images show there are at least two types of GFP-labelled cells migrated from the neurosphere in *Sox10*$^{Dom/Dom}$ midgut recipient at least. Some GFP-labelled cells are Tuj1 immunoreactive neurons (white arrows) while some (green arrows) are not Tuj1 immunoreactive cells. (A3) is the merged image of (A1) and (A2).

(B1) to (B3) High magnification images show in the *Sox10*$^{Dom/+}$ embryonic midgut recipient, some GFP-labelled cells (thick arrows in B1) are Tuj1 positive neurons (thick arrows in B2) and some thin fibers are Tuj1 positive (thin arrows in B2). (B3) is the merged image of (B1) and (B2) which shows that GFP-labelled cells and fibers are integrated with endogenous GFP$^-$/Tuj1$^+$ neurons and fibers.

(C1) to (C3): High magnification images show that in the *Sox10*$^{+/+}$ embryonic midgut recipient, few GFP-labelled cells migrated from the neurosphere are observed (thick arrows) while more GFP-labelled fibers (thin arrows in C1) extend from the GFP-labelled neurosphere. (C3) shows that GFP-labelled cells and fibers are integrated into the neural network of endogenous enteric neural crest-derived cells (GFP$^-$/TUJ1$^+$).

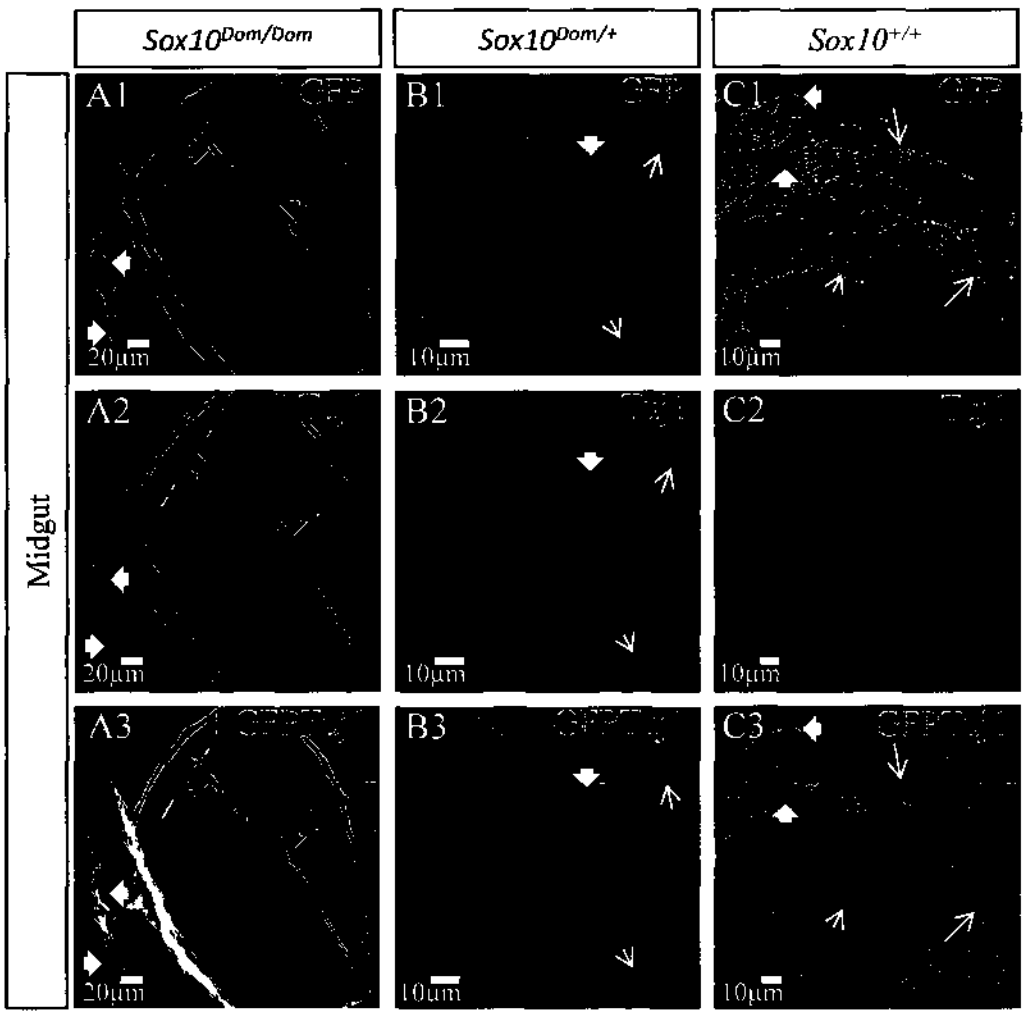

Sox10Dom/Dom
Sox10Dom/+
Sox10+/+
Midgut
A1
B1
C1
A2
B2
C2
A3
B3
C3
20μm
10μm

Fig 4.7 Colonization of the *Sox10$^{Dom/+}$* postnatal colon by cells from the neurosphere *in vivo*

(A) Diagram shows GFP-labelled neurospheres containing approximately 6x10^5 cells in 100 µl mouse self renewal medium are transplanted to the wall of the descending colon in the *Sox10$^{Dom/+}$* neonate at postnatal day 7 (n=13) following laparotomy.

(B) The operated postnatal mouse was sacrificed 7 to 14 days after transplantation of neurospheres. In a whole mount preparation, an interconnected network is formed by GFP-labelled cells and fibers on the gut wall of the descending colon. (B') shows the higher magnification of the boxed area in (B).

(C1) to (C3) Immunohistochemical staining the whole-mount preparations with Tuj1 antibody shows that GFP-labelled cells and fibers (C1) from neurospheres are Tuj1 immunoreactive (C2 and C3). (C3) is the merged image of (C1) and (C2).

(D1) to (D3) On serial cross sections of the descending colon with GFP-labelled neurospheres, GFP-labelled cells (D1) from the neurospheres are located in different layers of the gut wall including the serosa, the outer longitudinal muscle (LM) and inner circular muscle (CM). Endogenous myenteric ganglia (GFP$^-$/Tuj1$^+$) are not found on these sections.

(E1) to (E3) High magnification of the cross section after Tuj1 immunohistochemical staining shows that the GFP-labelled cells and fibers (E1) in the gut wall are Tuj1 immunoreactive (E2 and E3). (E3) is the merged image of (E1) and (E2).

(F1) to (F3) Even higher magnification image shows that a GFP-labelled cell (arrows in F1-F3) between the outer longitudinal muscle (LM) and inner circulation muscle (CM) is Tuj1 immunoreactive (F2 and F3). Sub M: Submucosa; Muc: Mucosa. (F3) is the merged image of (F1) and (F2).

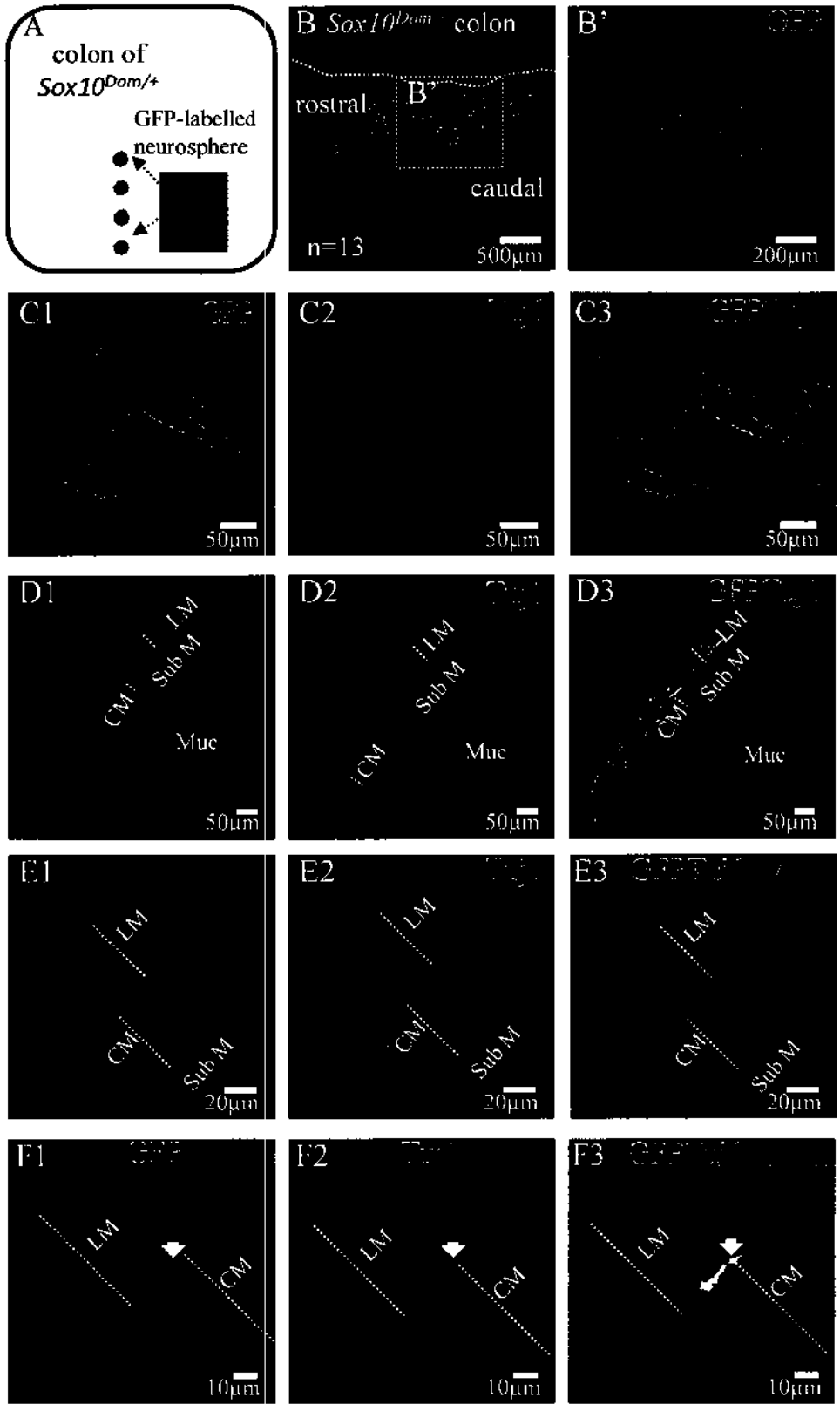

A
colon of
Sox10Dom/+
GFP-labelled
neurosphere
B Sox10Dom/+ colon
rostral
B'
caudal
n=13
500μm
B'
200μm
C1
50μm
C2
50μm
C3
50μm
D1
LM
Sub M
CM
Muc
50μm
D2
LM
Sub M
CM
Muc
50μm
D3
LM
Sub M
CM
Muc
50μm
E1
LM
CM
Sub M
20μm
E2
LM
CM
Sub M
20μm
E3
LM
CM
Sub M
20μm
F1
LM
CM
10μm
F2
LM
CM
10μm
F3
LM
CM
10μm

Fig 4.8 Colonization of *Sox10*$^{+/+}$ postnatal colon by cells from neurospheres *in vivo*

(A) Diagram shows GFP-labelled neurospheres are transplanted to the wall of the *Sox10*$^{+/+}$ descending colon at postnatal day 7 (n=9) following laparotomy.

(B) Whole preparation of the descending colon transplanted with GFP-labelled neurospheres shows that GFP-labelled cells are distributed on the wall of descending colon of the *Sox10*$^{+/+}$ postnatal mouse. White dotted lines show the outline of the colon.

(C1) to (C3) Confocal photomicrographs show GFP-labelled cells on the wall of *Sox10*$^{+/+}$ descending colon (C1) in a whole mount preparation. The gut segment contains GFP^{-}/Tuj1^{+} endogenous enteric ganglia (thick arrows in C2 and C3) by whole mount observation. (C3) is the merged image of (C1) and (C2).

(D1) to (D3) Confocal laser microscopic scanning on one single cellular layer shows that GFP-labelled cells are not the same layer as Tuj1 positive enteric ganglia (D2 and D3). (D3) is the merged image of (D1) and (D2), and the high magnification of the boxed area in D1 is shown in (E1) to (E3).

(E1) to (E3) High magnification of the boxed are in (D1) shows some GFP-labelled cells (thick arrows in E1) and GFP-labelled fibers (thin arrows in E1) are Tuj1 immunoreactive (thick arrows and thin arrows in E2 and E3). (E3) is the merged image of (E1) and (E2).

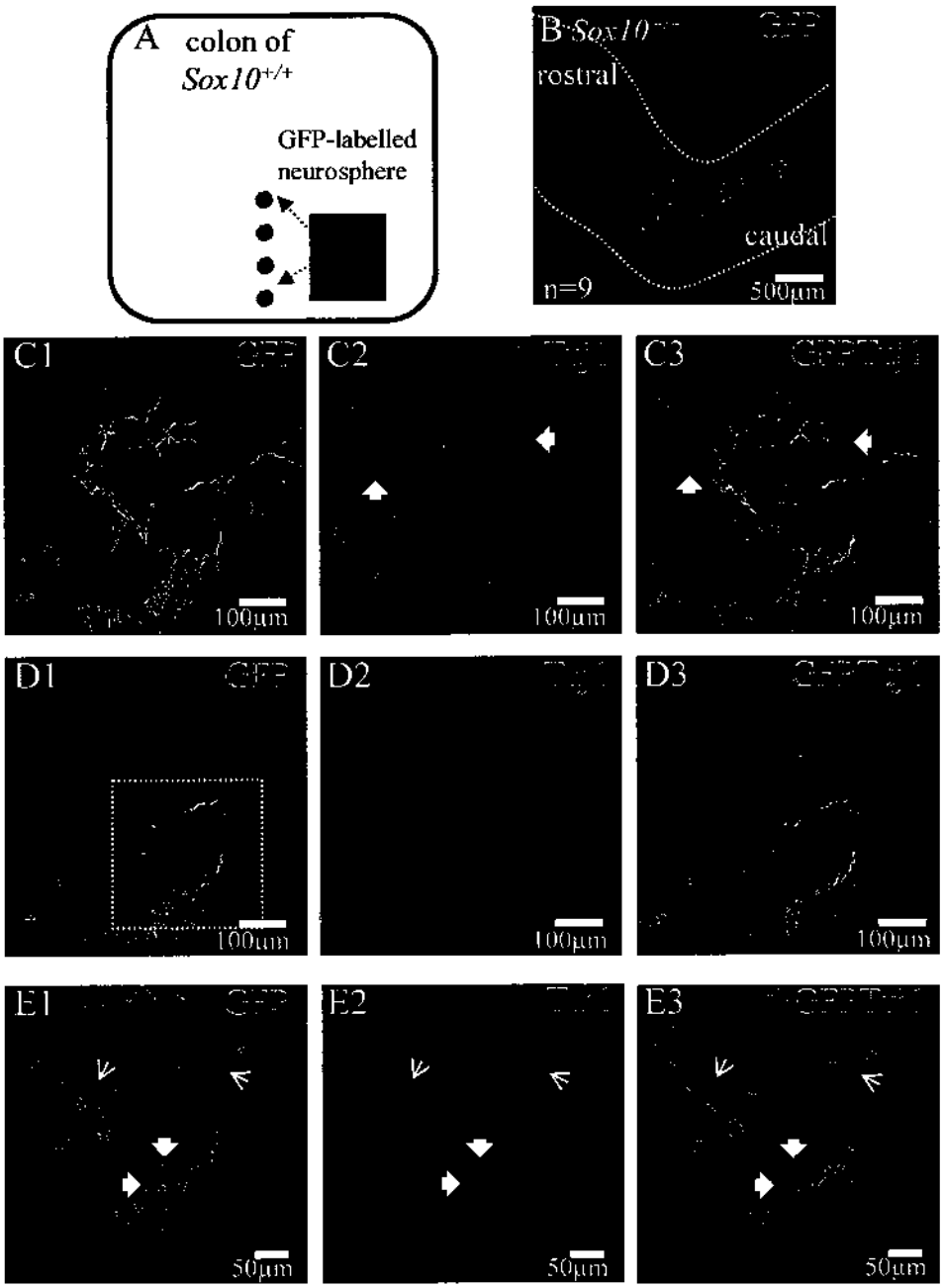
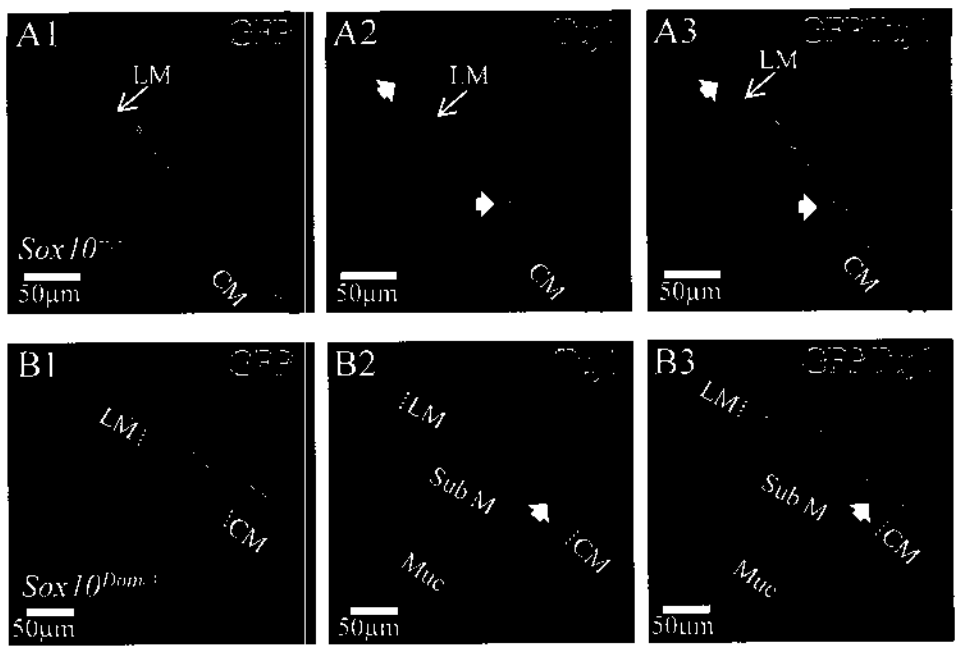

A
colon of
Sox10+/+
GFP-labelled
neurosphere
B Sox10-/-
GFP
rostral
caudal
n=9
500μm
C1
GFP
100μm
C2
Tuj1
100μm
C3
GFP Tuj1
100μm
D1
GFP
100μm
D2
Tuj1
100μm
D3
GFP Tuj1
100μm
E1
GFP
50μm
E2
Tuj1
50μm
E3
GFP Tuj1
50μm

Fig 4.9 Cross sections of the *Sox10*$^{+/+}$ and *Sox10*$^{Dom/+}$ postnatal descending colon with endogenous enteric ganglia and transplanted with GFP-labelled neurospheres

(A1) to (A3) Serial cross section of the *Sox10*$^{+/+}$ descending colon which has been transplanted with GFP-labelled neurospheres, shows that GFP-labelled cells in the serosa of the gut wall (A1) also express Tuj1 (A2). Tuj1 immunoreactive cells (GFP$^-$, derived from endogenous neural crest-derived cells) are also found in the myenteric layer (thick arrow in A2). (A3) is the merged image of (A1) and (A2).

(B1) to (B3) Serial cross section of the *Sox10*$^{Dom/+}$ descending colon which has also been transplanted with GFP-labelled neurospheres, shows GFP-labelled cells in the serosa of the descending colon (B1) while in the myenteric layer (thick arrow in B2 and B3), Tuj1 immunoreactive only cells (GFP$^-$, derived from endogenous neural crest cells) are found. (B3) is the merged image of (B1) and (B2).

Blue dotted lines outline the longitudinal muscular layer and circular muscular layer. CM, circular muscular layer; LM, longitudinal muscular layer; Sub M, submucosal; Muc, mucosa.


A1
LM
50µm
A2
LM
50µm
A3
LM
50µm
B1
LM
CM
50µm
B2
LM
Sub M
CM
Muc
50µm
B3
LM
Sub M
CM
Muc
50µm

Chapter five

Conclusions and discussion

There are still various diseases and syndromes such as congenital tissue malformations, tissue degeneration, trauma, and genetic deficiency which have no perfect and effective therapy at present (Daley, 2010). The promise of stem cell therapy has been realized in stem cell application with the advancement in the developmental biology and stem cell biology and with the pre-clinical experiments. Therapeutic strategies for enteric neuropathies including Hirschsprung's disease are limited to surgery and the provision of artificial nutrition (Thapar, 2009). With the aims to rescue the function of the gastrointestinal tract, many kinds of stem or progenitor cells have been used to explore their application potentials (Almond *et al.*, 2007; Kawaguchi *et al.*, 2010; Lindley *et al.*, 2008; Metzger *et al.*, 2009; Micci *et al.*, 2001). However, numbers of issues are unresolved about the basic biology of the enteric nervous system, the characteristics of the stem cells isolated from the enteric nervous system and the biological significance of these cells in the prenatal and postnatal periods.

In this thesis, enteric neural crest stem cells were first isolated as neurospheres from the embryonic gut at E14.5, which showed properties of stem cells with the demonstrated capacity of proliferation, self-renewal and differentiation. Then, the migratory ability of enteric neural crest-derived cells was shown to be related with the genotypes of enteric neural crest-derived cells, the developmental stage of the microenvironment through which enteric neural crest-derived cells migrate and the presence of existing endogenous enteric neural crest-derived cells. Lastly, after all

these migratory properties were revealed, experiments were proceeded to explore the therapeutic potential of enteric neural crest stem cells by transplantation to the prenatal and postnatal gut.

5.1 Cells used in cell-based therapy

Neural stem cells derived from the central nervous system (Micci *et al.*, 2001), Sox10-GFP-positive cells derived from embryonic stem cells (Kawaguchi *et al.*, 2010), and human and mouse neurospheres derived from the enteric nervous system (Almond *et al.*, 2007) have been used in the transplantation studies on animal *in vivo* and *ex vivo*. Enteric neural crest stem cells also have been reported to be isolated from the postnatal gut mucosa of mice and humans (Metzger *et al.*, 2009) which provide a new tissue source for autologous transplantation.

Different kinds of sources of stem cells are found with the advancement of techniques and continuous exploration, and new sources such as neural crest-like cells induced from human embryonic stem cells using small molecule Y27632 (Hotta *et al.*, 2009b) have been discovered. Neural crest stem cells are also found in the skin which are named epidermal neural crest stem cells (EPI-NCSCs) (Sieber-Blum *et al.*, 2004; Toma *et al.*, 2001; Wong *et al.*, 2006), in a cardiac side population (SP) (Tomita *et al.*, 2005), in the mouse cornea (Yoshida *et al.*, 2006; Yoshida *et al.*, 2005) and in the teeth (Miura *et al.*, 2003). Adult neural crest stem cells have been isolated from rat dorsal root ganglia (Li *et al.*, 2007). Most presently, stem cells with neural crest characteristics can be isolated from the bulge area of cultured human hair follicles (HFs) and this presence of stem cells with neural crest characteristics in HFs may offer new opportunities for the use of these cells in regenerative medicine (Biernaskie, 2010; Yu *et al.*, 2010). Mesenchymal stem cells (MSCs), a heterogeneous

subset of stromal stem cells in adult bone marrow, have been reported to be originated from at least two developmental origins, one of which is the neural crest (Morikawa *et al.*, 2009). The identification of neural crest stem cells in accessible adult tissues provides new potential sources for autologous cell therapy after nerve injury or disease (Nagoshi *et al.*, 2008).

Stem cells generated from different sources could have different contributions to the recipient. Stem cells in different regions of the nervous system such as those from the sciatic nerve of the peripheral nervous system and those from the enteric nervous system give rise to different types of mature cells (Bixby *et al.*, 2002). The cranial neural crest cells relative to the trunk neural crest cells, has a position-determined proliferation advantage favoring the formation of the enteric nervous system (Zhang *et al.*, 2010). More work should be done further to clarify the application value of neural crest stem cells from other sources in the enteric nervous system.

In this project, enteric neural crest stem cells were isolated from the embryonic gut tube and cultured as neurospheres for many passages *ex vivo* with the demonstrated capacity of proliferation, self-renewal and differentiation. Hence they clearly exhibited properties of stem cells. These studies will give some comparable information for the neural crest stem cells derived from other sources, although the exact kinds of cells that are most appropriate in the clinical application of stem cell therapy still need more experiments to be found out. Therefore, in this project, potential applications of these enteric neural crest stem cells isolated from the mouse embryonic gut tube were further explored in the transplantation experiments.

5.2 Effectors influencing migration

Though a number of stem cell sources have been found for the use in regenerative medicine through transplantation as described above, the cellular mechanism whereby the migratory ability of cells is affected through their interactions with their microenvironment should be considered. In the enteric nervous system, a number of genes and effectors involved in the development of the enteric nervous system have been summarized in the Section 3.1.2.

This study showed that the genotype of donor enteric neural crest-derived cells affected their migration within the recipient environment while the genotype of the recipient did not showed influences on the enteric neural crest-derived cells migration in the recombination organotypic culture. These observations are consistent with our previous findings (Wang, 2006) and support the idea that the defect of enteric colonization by the enteric neural crest-derived cells was not solely related to the microenvironment provided by the *Sox10* (*Dom*) mutant gut wall (Kapur, 1999). The migration of endogenous enteric neural crest-derived cells in the $Sox10^{Dom/+}$ (i.e. heterozygote of *Dom* in the present study) hindgut was delayed as compared with the migration in normal $Sox10^{+/+}$ (i.e. wild type in the present study) embryos as shown by Kapur (Kapur *et al.*, 1996) and by Wang (Wang, 2006). Time-lapse imaging study also showed that the migration of *Ednrb* mutant enteric neural crest-derived cells was delayed by ~24 hours behind those in same age-matched control gut (Druckenbrod and Epstein, 2009).

In addition to the cell-autonomous effect, it was also shown in the present study that the microenvironment through which enteric neural crest-derived cells migrated at different developmental stages and the presence of endogenous enteric neural

crest-derived cells in the environment along the migratory pathway also have effects on the colonization of donor enteric neural crest-derived cells.

In *Ret* and *Ednrb* mutant mice, older recipient colon is less permissive for enteric neural crest-derived cells migration than younger colon (Druckenbrod and Epstein, 2009; Hotta *et al.*, 2009a). In this study, in *Dom* mice, the recipient guts older than E13.5 were shown to be less and incompletely colonized than in younger gut tubes. The formation of a cellular network of enteric neural crest-derived cells with more spaces between chains of cells from E13.5 onwards coincided with the time at which the muscle layers of the gut tube started to differentiate. However, the impacts of the gradual maturation of the surrounding tissues on the migrating enteric neural crest-derived cells, especially the influences that newly arriving enteric neural crest-derived cells may encounter when they are placed in a more mature environment due to their delayed migration, are still poorly understood now.

The presence of endogenous enteric neural crest-derived cells along in the migratory pathway of donor enteric neural crest-derived cells or stem cells was shown in the present study to have a negative effect on their colonization as evidenced by the fact that donor enteric neural crest-derived cells were less able to colonized the ganglionic segments of the $Sox10^{+/+}$ and $Sox10^{Dom/+}$ midgut segments than the aganglionic $Sox10^{Dom/+}$ and $Sox10^{Dom/Dom}$ midgut segments at E11.5. Similar observations have also been made in the study where enteric neural crest-derived cells failed to enter most of the $Ret^{-/+}$ hindgut recipients at E14.5 to E16.5 where the hindgut has already been completely colonized by endogenous neural crest-derived cells (Hotta *et al.*, 2009a), and in quail-chick chimeric study, quail enteric neural crest-derived cells did not colonize segments of embryonic chick gut containing pre-existing enteric neural crest-derived cells (Meijers *et al.*, 1992). GFP-labelled

processes were found in the ganglionic *Sox10*$^{Dom/+}$ and *Sox10*$^{+/+}$ midgut segments extending from the donor gut segments. This indicates that the intimate interactions between the donor tissues and the recipient environment had taken place.

The impediment that was induced by the presence of existing enteric neural crest-derived cells may be explained by the contact inhibition of locomotion. Neural crest cells cultured *ex vivo* move away from each other and disperse quickly (Davis and Trinkaus, 1981). During this inhibition process, Wnt planar cell polarity (PCP, or non-canonical) pathway plays an important role and RhoA (One member of the Rho family of GTPases) as downstream molecule affects the PCP (Carmona-Fontaine *et al.*, 2008; De Calisto *et al.*, 2005; Matthews *et al.*, 2008). RhoA has a role in controlling the temporal dynamics of neural crest cell–cell interactions and cell movements to ensure accurate neural crest cell navigation and collective migration (Rupp and Kulesa, 2007).

Though there are some known effectors affecting the migration and colonization of donor enteric neural crest-derived cells or stem cells in the recipient environment, the results in this study showed that in the postnatal gut *in vivo*, the donor cells could migrate into the myenteric layers crossing the serosa. This may take a promise for the application of enteric neural crest stem cells using less damage methods to the gut unlike microinjection in multiple regions in the gut. The cells just injected through a simple intra-peritoneal injection to the regions near the gut wall could colonize the ganglionic or aganglionic gut segments, and similar finding have also been reported by Martucciello (Martucciello *et al.*, 2007). However, the mechanism of this kind of homing in the gut is unknown. SDF-1/CXCR4 signalling may play a role in chemoattraction because that SDF-1 has been shown to be a key component in the regulation of cell migration during the development of a variety of tissues including

nervous system (Killian *et al.*, 2009; Tran and Miller, 2003). It is also expressed within the migratory pathway of neural crest cells to dorsal root ganglia (Belmadani *et al.*, 2005). It is known that SDF-1/CXCR4 are important signalling molecules in the hematopoietic stem cell homing and the treatment of the heart and central nervous system injury (Madri, 2009; Schoenhard and Hatzopoulos, 2010; Zaruba and Franz, 2010). However the mechanism of gradient detection and polarization of these cells remains unknown (Dormann and Weijer, 2003). In the enteric neural nervous system which is the derivative of the neural crest, the existence and the role of this signalling pathway would be worth investigation.

5.3 Potential of cell-based therapy

Apart from the expression of genes of enteric neural crest-derived cells and donor stem cells affecting their own developmental functions, the microenvironment of the recipient also profoundly alters therapeutic effects. The key of a successful transplantation strategy is to rescue the lost function of the abnormal tissues or organs. Though there are more and more data showing that an increasing number of stem cell types possess the potential applications in the pre-clinical experimental study, there is still a long way from the laboratory bench to the clinic before stem cells can become truly applicable to clinical trials. One successful example up to date which has gone through this long way has been the development of hematopoietic stem cell transplantation in the clinic (Kerry *et al.*, 2004b).

The success in rescuing the defective enteric nervous system has been limited by the differentiated cell types that the donor cells can form in the recipient and the recovery of the contraction ability of the recipient gut segment. Central nervous system derived neural stem cells were found to be able to differentiate into neurons

and express neuronal nitric oxide synthase (nNOS) in the pylorus of adult mice (Micci *et al.*, 2001). Human and mouse neurospheres derived from the enteric nervous system differentiate to form cells expressing S100 and PGP9.5 phenotypic markers which are specific to enteric glia and neurons respectively, and expressing gut neuropeptides such as vasoactive intestinal peptide (VIP) in the aganglionic mouse embryonic hindgut (Almond *et al.*, 2007). Contraction frequency was examined in embryonic hindgut after transplantation *ex vivo* (Lindley *et al.*, 2008), and the inflation stimulated contraction and electrical field stimulation-induced response were observed in transplanted adult colon (Liu *et al.*, 2007).

In this study, using whole mount preparations and serial cross sections immunohistochemical staining on the gut with transplanted GFP-labelled cells and comparing the colonization of the GFP-labelled cells in different recipients *ex vivo* and *in vivo,* the enteric neural crest stem cells used were found that the colonization in the aganglionic segment of the prenatal and postnatal gut segment were better than that in the ganglionic segment *ex vivo* and *in vivo.*

The role of the enteric nervous system in the motility of the gastrointestinal tract during development is controversial. The interstitial cells of Cajal (ICC) or myogenic contraction may contribute to the motility in the development of the embryonic and newborn intestines (Burns *et al.*, 2009; Lindley *et al.*, 2008; Roberts *et al.*, 2010; Sanders, 1996). Hence the functional recovery of the rescued enteric nervous system by transplantation of stem cells needs to be assessed on a long term basis until the enteric nervous system and its microenvironment become mature and fully functional.

Appendix I

Preparations of solutions

Preparation of DNase I (Sigma, D4527):

1. Add 1.56 ml 0.9% NaCl to the 7.8 mg DNase I (5 mg/ml; 10X)
2. Aliquot the solution into eppendorf tubes (100 µl per tube) and stored at -20℃ for future use (10X)

Preparation of 1M HEPES pH7.4 (Sigma, H9136)

1. Add 5 ml double distilled water (DDW) to 2.383 g HEPES
2. Adjust pH to 7.4 by 1N NaOH
3. Add more DDW water to 10 ml
4. Aliquot the solution to eppendorf tubes (1.5 ml per tube) and store the solution at 4℃

Preparation of fluorescence-activated cell sorting (FACS) buffer/staining medium (150 ml)

L15 medium without phenol red (Gibico, 21083-27)	132 ml
1% pen/strep (Sigma, 10,000 U/ml stock)	1.5 ml
1mg/ml BSA (Sigma, A-9418, tissue culture grade)	150 mg
10 mM HEPES pH 7.4 (Sigma, H9136, 1M stock)	1.5 ml
10% DDW water	15 ml

Preparation of retinoic acid (Sigma: R2625; Final concentration: 3.5 mg/ml)

1. Add 14.3 ml 100% ethanol to the 50 mg Retinoic acid (3.5 mg/ml) (prepare in the dark because retinoic acid is sensitive to the light)
2. Aliquot the solution to eppendorf tubes (200 µl per tube)
3. Wrap the tubes with an aluminum paper and stored it at -20℃ for future use

Preparation of bFGF (Sigma, F3395; Final concentration: 25 µg/ml)

1. Prepare 5 mM Tris pH 7.6 by adding 995 µl DDW to 5 µl 1M Tris-HCl pH7.5

(Invitrogen, 15567-027)

2. Add 1 ml 5 mM Tris to 25 μg bFGF
3. Aliquot the solution to eppendorf tube (100μl per tube) and store the solution at -20℃ for future use

Preparation of human IGF-1 (R&D, 291-G1-050; Final concentration 2 μg/100 ml medium)

1. Add 1ml sterile PBS to the 50 μg IGF-1 (50 μg/ml)
2. Aliquot the solution to eppendorf tubes (2 μg/ 40 μl per tube) and store them at -20℃ for future use
3. When preparing the self-renewal medium, for neurosphere culture, add 40 μl (50 μg/ml) into 100 ml medium, so the final concentration of IGF-1 in the medium will be to 2 μg/100 ml

Preparation of collagenase IV (Worthington Biochemicals, LS004186; 100mg; final concentration: 10mg/ml)

1. Reconstitute to 100mg/ml by adding 1 ml HBSS with calcium and magnesium (Gibico, 24020) to 100 mg Collagenase IV powder
2. Aliquot to 10 eppendorf tubes (100 μl per tube) and stored the tubes at 20℃ for future use

Preparation of the dissociation medium for guts at E14.5

For 1ml dissociation medium:

Hank's balanced salt solution without calcium and magnesium (Sigma, H2387)	940 μl
Trypsin (0.5%; 0.025 g Trypsin in 5 ml DDW, Sigma, T9935)	50 μl
Collagenase IV (100 mg/ml in HBSS with calcium and magnesium)	10 μl

Preparation of mouse self-renewal medium

To make 100 ml of medium:

DMEM-low glucose (Gibico, 31600-034)	50 ml
Neurobasal medium (Gibico, 21103-049)	30ml
Chick Embryo Extract (CEE; US Biological, C3999)	15 ml

Penn/Strep (P/S) (Invitrogen, 15140-122)	1 ml
N2 (Gibico, 17502-048)	1 ml
B27 (Gibco, 17504-044)	2 ml
Retinoic Acid (RA; 117 μM stock = 35μg/ml; Sigma, R2625),	100 μl
2-mercaptoethanol (2ME; 50 mM stock; Gibico, 1985-023),	100 μl
bFGF (25μg/ml stock; Sigma, F3395)	80 μl
IGF-1 (20 ng/ml of medium; R&D, 291-G1-050)	40 μl
DDW	689 μl

When preparing the medium, put 25 ml of the DMEM-low in the 0.22 μm filter unit, and then add the rest of the ingredients. After drawing the medium through the filtration unit, put the rest 25 ml of the DMEM-low through the filter. Add the 2-mercaptoethanol first and pipette up and down. It is a strong reducing agent. Add the RA secondly and pipette up and down. Before dilution, both 2ME and RA can denature proteins in the medium. Make sure the CEE, N2, B27, and P/S stocks are made sure to be resuspended well before using. Do not freeze/thaw the ingredients more than twice except 2ME and RA which can be frozen/thawed as many times as needed.

Preparation of poly-D-lysine (PDL; Sigma, P7886: 10mg) (final concentration: 150 μg/ml):

1. Add 0.66 ml sterile distilled water to 10 mg of PDL (15 mg/ml)
2. Aliquot the solution into eppendorf tube (150 μl) and stored at -20℃ for future use (100X)
3. Coat each cover slip in the Petri dish with enough PDL, then transfer the PDL to another Petri dish
4. Do this until all the cover slips have been coated with PDL
5. Allow the cover slip to dry in a biological safety cabinet
6. Keep the PDL to reuse again and again

Preparation of the cover slip

1. Rinse the 13mm round cover slip on the grid with 2N HCl for 10 minutes
2. Wash the cover slip with distilled water for 10 minutes twice

3. Rinse the cover skip with acetone for 10 minutes
4. Wash the cover slip with distilled water for 10 minutes twice
5. Dry the cover slip in oven
6. Dry heat the cover slip in glass Petri dish wrapped in aluminum paper
7. Store the cover slip for future use

Coating the cover slips

1. Coat each cover slips with PDL
2. Allow the slips to dry in a biological safety cabinet
3. After the slips are dry, rinse with DDW
4. Allow the slips to dry in a biological safety cabinet
5. Store the plates in the incubator until the cells are ready to be plated

Appendix II

Preparation of the anesthesia solution with ketamine and xylazine

10% ketamine: 2% xylazine : saline=3:2:3

Postnatal mouse was anesthetized with an intraperitoneal injection of the anesthesia solution at 2 μl/g. Effects last 45 minutes to 1 hour.

Table 7 Preparation of 0.2 ml anesthesia solution

10% ketamine (Alfasan-Holland, 017018-4)	90 μl
2% xylazine (Alfasan Woerden-Holland, 040133)	60 μl
saline	90 μl
total	240 μl

Quinine hydrochloride

Table 8 Prepare in hood for surgery

Ether	2 ml
Collodion (BDH, K23097663628)	10 ml

1. Mix to glue like
2. Add a small plastic spoon of Quinine hydrochloride (BDH, 30008)
3. Mix until the mixture becomes transparent glue like texture and the mixture is stored at room temperature after sealed the container
4. In hood, paste on surgical wound to prevent bits on the wound by mice after surgery

References

Abalo, R., Vera, G., Rivera, A. J., Moro-Rodriguez, E. and Martin-Fontelles, M. I. (2009) Postnatal maturation of the gastrointestinal tract A functional and immunohistochemical study in the guinea-pig ileum at weaning *Neuroscience Letters* **467**, 105-110

Ahmed, S. (2009) The culture of neural stem cells *Journal of Cellular Biochemistry* **106**, 1-6

Akamatsu, W., Okano, H. J., Osumi, N., Inoue, T., Nakamura, S., Sakakibara, S. I., Miura, M., Matsuo, N., Darnell, R. B. and Okano, H. (1999) Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems *Proceedings of the National Academy of Sciences of the United States of America* **96**, 9885-9890

Almond, S., Lindley, R. M., Kenny, S. E., Connell, M. G. and Edgar, D. H. (2007) Characterisation and transplantation of enteric nervous system progenitor cells *Gut* **56**, 489-96

Amiel, J. and Lyonnet, S. (2001) Hirschsprung disease, associated syndromes, and genetics a review *Journal of medical genetics* **38**, 729-39

Amiel, J., Sproat-Emison, E., Garcia-Barcelo, M., Lantieri, F., Burzynski, G., Borrego, S., Pelet, A., Arnold, S., Miao, X., Griseri, P. et al. (2008) Hirschsprung disease, associated syndromes and genetics a review *Journal of medical genetics* **45**, 1-14

Anderson, R. B., Enomoto, H., Bornstein, J. C. and Young, H. M. (2004) The enteric nervous system is not essential for the propulsion of gut contents in fetal mice *Gut* **53**, 1546-7

Anderson, R. B., Stewart, A. L. and Young, H. M. (2006) Phenotypes of neural-crest-derived cells in vagal and sacral pathways *Cell and Tissue Research* **323**, 11-25

Angrist, M., Bolk, S., Thiel, B., Puffenberger, E. G., Hofstra, R. M., Buys, C. H., Cass, D. T. and Chakravarti, A. (1995) Mutation analysis of the RET receptor tyrosine kinase in Hirschsprung disease *Human Molecular Genetics* **4**, 821-30

Arena, S., Cutroneo, G., Favaloro, A., Sinatra, M. T., Trimarchi, F., Scarvaglieri, S., Mallamace, A., Arena, F., Anastasi, G. and Di Benedetto, V. (2010) Abnormal distribution of sarcoglycan subcomplex in colonic smooth muscle cells of aganglionic bowel *International journal of molecular medicine* **25**, 353-9

Ayme, S. and Philip, N. (1995) Possible homozygous Waardenburg syndrome in a fetus with exencephaly *American Journal of Medical Genetics* **59**, 263-5

Badner, J. A., Sieber, W. K., Garver, K. L. and Chakravarti, A. (1990) A genetic study of Hirschsprung disease *American Journal of Medical Genetics* **46**, 568-80

Barlow, A., de Graaff, E. and Pachnis, V. (2003). Enteric nervous system progenitors are coordinately controlled by the G protein-coupled receptor EDNRB and the receptor tyrosine kinase RET *Neuron* **40**, 905-16

Bassotti, G. and Villanacci, V. (2006) Slow transit constipation a functional disorder becomes an enteric neuropathy *World journal of gastroenterology* **12**, 4609-13

Baynash, A. G., Hosoda, K., Giaid, A., Richardson, J. A., Emoto, N., Hammer, R. E. and Yanagisawa, M. (1994) Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons *Cell* **79**, 1277-85

Belmadani, A., Tran, P. B., Ren, D. J., Assimacopoulos, S., Grove, E. A. and Miller, R. J. (2005) The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors *Journal of Neuroscience* **25**, 3995-4003

Benarroch, E. E. (2007) Enteric nervous system functional organization and neurologic implications *Neurology* **69**, 1953-7

Bez, A., Corsini, E., Curti, D., Biggiogera, M., Colombo, A., Nicosia, R. F., Pagano, S. F. and Parati, E. A. (2003) Neurosphere and neurosphere-forming cells morphological and ultrastructural characterization *Brain Research* **993**, 18-29

Bhattacharyya, B. J., Banisadr, G., Jung, H., Ren, D., Cronshaw, D. G., Zou, Y. and Miller, R. J. (2008) The chemokine stromal cell-derived factor-1 regulates GABAergic inputs to neural progenitors in the postnatal dentate gyrus *The Journal of neuroscience* **28**, 6720-30

Biernaskie, J. (2010) Human hair follicles "bulging" with neural crest-like stem cells *The Journal of investigative dermatology* **130**, 1202-4

Bitgood, M. J. and McMahon, A. P. (1995) Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo *Developmental Biology* **172**, 126-38

Bixby, S., Kruger, G. M., Mosher, J. T., Joseph, N. M. and Morrison, S. J. (2002) Cell-intrinsic differences between stem cells from different regions of the peripheral nervous system regulate the generation of neural diversity *Neuron* **35**, 643-656

Bolande, R. P. (1974) The neurocristopathies, a unifying concept of disease arising in neural crest maldevelopment *Human pathology* **5**, 409-20

Bondurand, N., Natarajan, D., Barlow, A., Thapar, N. and Pachnis, V. (2006) Maintenance of mammalian enteric nervous system progenitors by SOX10 and endothelin 3 signalling *Development* **133**, 2075-86

Bondurand, N., Natarajan, D., Thapar, N., Atkins, C. and Pachnis, V. (2003) Neuron and glia generating progenitors of the mammalian enteric nervous system isolated from foetal and postnatal gut cultures *Development* **130**, 6387-400

Bondurand, N., Pingault, V., Goerich, D. E., Lemort, N., Sock, E., Le Caignec, C., Wegner, M. and Goossens, M. (2000) Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome *Human Molecular Genetics* **9**, 1907-17

Bordeaux, M. C., Forcet, C., Granger, L., Corset, V., Bidaud, C., Billaud, M., Bredesen, D. E., Edery, P. and Mehlen, P. (2000) The RET proto-oncogene induces apoptosis a novel mechanism for Hirschsprung disease *The EMBO journal* **19**, 4056-63

Borghini, S., Bachetti, T., Fava, M., Di Duca, M., Cargnin, F., Fornasar, D., Ravazzolo, R. and Ceccherini, I. (2006) The TLX2 homeobox gene is a transcriptional target of PHOXB in neural-crest-derived cells *Biochemical Journal* **395**, 355-361

Brewer, G. J. (1999) Regeneration and proliferation of embryonic and adult rat hippocampal neurons in culture *Experimental Neurology* **159**, 237-47

Britsch, S., Goerich, D. E., Riethmacher, D., Peirano, R. I., Rossner, M., Nave, K. A., Birchmeier, C. and Wegner, M. (2001) The transcription factor Sox10 is a key regulator of peripheral glial development *Genes & development* **15**, 66-78

Brizzolara, A., Torre, M., Favre, A., Pini Prato, A., Bocciardi, R. and Martucciello, G. (2004) Histochemical study of Dom mouse A model for Waardenburg-Hirschsprung's phenotype *Journal of Pediatric Surgery* **39**, 1098-103

Brooks, A. S., Oostra, B. A. and Hofstra, R. M. (2005) Studying the genetics of Hirschsprung's disease unraveling an oligogenic disorder *Clinical genetics* **67**, 6-14

Brunet, J. F. and Pattyn, A. (2002) Phox2 genes - from patterning to connectivity *Current Opinion in Genetics & Development* **12**, 435-40

Burns, A. J., Champeval, D. and Le Douarin, N. M. (2000) Sacral neural crest cells colonise aganglionic hindgut in vivo but fail to compensate for lack of enteric ganglia *Developmental Biology* **219**, 30-43

Burns, A. J. and Douarin, N. M. (1998) The sacral neural crest contributes neurons and glia to the post-umbilical gut spatiotemporal analysis of the development of the enteric nervous system *Development* **125**, 4335-47

Burns, A. J. and Le Douarin, N. M. (1998) The sacral neural crest contributes neurons and glia to the post-umbilical gut spatiotemporal analysis of the development of the enteric nervous system *Development* **125**, 4335-4347

Burns, A. J. and Le Douarin, N. M. (2001) Enteric nervous system development analysis of the selective developmental potentialities of vagal and sacral neural crest cells using quail-chick chimeras *The Anatomical record* **262**, 16-28

Burns, A. J., Roberts, R. R., Bornstein, J. C. and Young, H. M. (2009) Development of the enteric nervous system and its role in intestinal motility during fetal and early postnatal stages *Seminars in pediatric surgery* **18**, 196-205

Burns, A. J. and Thapar, N. (2006) Advances in ontogeny of the enteric nervous system *Neurogastroenterology and Motility* **18**, 876-87

Campos, L. S. (2004) Neurospheres insights into neural stem cell biology *Journal of Neuroscience Research* **78**, 761-9

Campos, L. S., Leone, D. P., Relvas, J. B., Brakebusch, C., Fassler, R., Suter, U. and ffrench-Constant, C. (2004) Beta1 integrins activate a MAPK signalling pathway in neural stem cells that contributes to their maintenance *Development* **131**, 3433-44

Carlson, M. E., Hsu, M. and Conboy, I. M. (2008) Imbalance between pSmad3 and Notch induces CDK inhibitors in old muscle stem cells *Nature* **454**, 528-U9

Carmona-Fontaine, C., Matthews, H. K., Kuriyama, S., Moreno, M., Dunn, G. A., Parsons, M., Stern, C. D. and Mayor, R. (2008) Contact inhibition of locomotion in vivo controls neural crest directional migration *Nature* **456**, 957-61

Ceccherini, I., Bocciardi, R., Luo, Y., Pasini, B., Hofstra, R., Takahashi, M. and Romeo, G. (1993) Exon structure and flanking intronic sequences of the human RET proto-oncogene *Biochemical and Biophysical Research Communications* **196**, 1288-95

Chalazonitis, A., Rothman, T. P., Chen, J. and Gershon, M. D. (1998) Age-dependent differences in the effects of GDNF and NT-3 on the development of neurons and glia from neural crest-derived precursors immunoselected from the fetal rat gut expression of GFRalpha-1 in vitro and in vivo *Developmental Biology* **204**, 385-406

Chong, S. W., Emelyanov, A., Gong, Z. Y. and Korzh, V. (2001) Expression pattern of two zebrafish genes, cxcr4a and cxcr4b *Mechanisms of Development* **109**, 347-354

Conboy, I. M., Conboy, M. J., Wagers, A. J., Girma, E. R., Weissman, I. L. and Rando, T. A. (2005) Rejuvenation of aged progenitor cells by exposure to a young systemic environment *Nature* **433**, 760-764

Cowan, C. A., Atienza, J., Melton, D. A. and Eggan, K. (2005) Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells *Science* **309**, 1369-1373

Crane, J. F. and Trainor, P. A. (2006) Neural crest stem and progenitor cells *Annual Review of Cell and Developmental Biology* **22**, 267-286

Daley, G. Q. (2010) Stem cells roadmap to the clinic *Journal of Clinical Investigation* **120**, 8-10

Dalmau, J., Furneaux, H. M., Cordoncardo, C. and Posner, J. B. (1992) The Expression of the Hu (Paraneoplastic Encephalomyelitis Sensory Neuronopathy) Antigen in Human Normal and Tumor-Tissues *American Journal of Pathology* **141**, 881-886

Darby, I., Skalli, O. and Gabbiani, G. (1990) Alpha-Smooth Muscle Actin Is Transiently Expressed by Myofibroblasts during Experimental Wound-Healing *Laboratory Investigation* **63**, 21-29

Davis, E. M. and Trinkaus, J. P. (1981) Significance of cell-to cell contacts for the directional movement of neural crest cells within a hydrated collagen lattice *Journal of embryology and experimental morphology* **63**, 29-51

De Calisto, J., Araya, C., Marchant, L., Riaz, C. F. and Mayor, R. (2005) Essential role of non-canonical Wnt signalling in neural crest migration *Development* **132**, 2587-2597

De Giorgio, R. and Camilleri, M. (2004) Human enteric neuropathies morphology and molecular pathology *Neurogastroenterology and Motility* **16**, 515-31

De Giorgio, R., Guerrini, S., Barbara, G., Cremon, C., Stanghellini, V. and Corinaldesi, R. (2004) New insights into human enteric neuropathies *Neurogastroenterology and Motility* **16 Suppl** 1, 143-7

De vries, P., Soret, R., Suply, E., Heloury, Y. and Neunlist, M. (2010) Postnatal development of myenteric neurochemical phenotype and impact upon neuromuscular transmission in the rat colon *American journal of physiology Gastrointestinal and liver physiology*

Delalande, J. M., Barlow, A. J., Thomas, A. J., Wallace, A. S., Thapar, N., Erickson, C. A. and Burns, A. J. (2008) The receptor tyrosine kinase RET regulates hindgut colonization by sacral neural crest cells *Developmental Biology* 313, 279-92

Deleyrolle, L., Marchal-Victorion, S., Dromard, C., Fritz, V., Saunier, M., Sabourin, J. C., Tran Van Ba, C., Privat, A. and Hugnot, J. P. (2006) Exogenous and fibroblast growth factor 2/epidermal growth factor-regulated endogenous cytokines regulate neural precursor cell growth and differentiation *Stem Cells* **24**, 748-62

Discher, D. E., Mooney, D. J. and Zandstra, P. W. (2009) Growth factors, matrices, and forces combine and control stem cells *Science* **324**, 1673-7

Doetsch, F., Petreanu, L., Caille, I., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (2002) EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells *Neuron* **36**, 1021-1034

Dormann, D. and Weijer, C. J. (2003) Chemotactic cell movement during development *Current Opinion in Genetics & Development* **13**, 358-364

Draberova, E., Del Valle, L., Gordon, J., Markova, V., Smejkalova, B., Bertrand, L., de Chadarevian, J. P., Agamanolis, D. P., Legido, A., Khalili, K. et al. (2008) Class III beta-tubulin is constitutively coexpressed with glial fibrillary acidic protein and nestin in midgestational human fetal astrocytes Implications for phenotypic identity *Journal of Neuropathology and Experimental Neurology* **67**, 341-354

Drerup, C. M., Wiora, H. M., Topczewski, J. and Morris, J. A. (2009) Disc1 regulates foxd3 and

sox10 expression, affecting neural crest migration and differentiation *Development* **136**, 2623-32

Druckenbrod, N. R. and Epstein, M. L. (2009) Age-dependent changes in the gut environment restrict the invasion of the hindgut by enteric neural progenitors *Development* **136**, 3195-203

Durbec, P., Marcos-Gutierrez, C. V., Kilkenny, C., Grigoriou, M., Wartiowaara, K., Suvanto, P., Smith, D., Ponder, B., Costantini, F., Saarma, M. et al. (1996a) GDNF signalling through the Ret receptor tyrosine kinase *Nature* **381**, 789-93

Durbec, P. L., Larsson-Blomberg, L. B., Schuchardt, A., Costantini, F. and Pachnis, V. (1996b) Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts *Development* **122**, 349-58

Dutton, K. A., Pauliny, A., Lopes, S. S., Elworthy, S., Carney, T. J., Rauch, J., Geisler, R., Haffter, P. and Kelsh, R. N. (2001) Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates *Development* **128**, 4113-25

Ebert, A. D., Yu, J., Rose, F. F., Jr., Mattis, V. B., Lorson, C. L., Thomson, J. A. and Svendsen, C. N. (2009) Induced pluripotent stem cells from a spinal muscular atrophy patient *Nature* **457**, 277-80

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity *Cell* **75**, 1417-30

Edery, P., Lyonnet, S., Mulligan, L. M., Pelet, A., Dow, E., Abel, L., Holder, S., Nihoul-Fekete, C., Ponder, B. A. and Munnich, A. (1994) Mutations of the RET proto-oncogene in Hirschsprung's disease *Nature* **367**, 378-80

Egli, D., Rosains, J., Birkhoff, G. and Eggan, K. (2007) Developmental reprogramming after chromosome transfer into mitotic mouse zygotes *Nature* **447**, 679-U8

Ehrmann, J., Kolar, Z. and Mokry, J. (2005) Nestin as a diagnostic and prognostic marker immunohistochemical analysis of its expression in different tumours *Journal of Clinical Pathology* **58**, 222-223

Ellis, P., Fagan, B. M., Magness, S. T., Hutton, S., Taranova, O., Hayashi, S., McMahon, A., Rao, M. and Pevny, L. (2004) SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult *Developmental neuroscience* **26**, 148-65

Eng, L. F. (1985) Glial Fibrillary Acidic Protein (Gfap) - the Major Protein of Glial Intermediate Filaments in Differentiated Astrocytes *Journal of Neuroimmunology* **8**, 203-214

Episkopou, V. (2005) SOX2 functions in adult neural stem cells *Trends Neurosci* **28**, 219-21

Epstein, M. L., Mikawa, T., Brown, A. M. and McFarlin, D. R. (1994) Mapping the origin of the avian enteric nervous system with a retrovirial marker *Developmental Dynamics* **201**, 236-44

Flake, A. W., Harrison, M. R., Adzick, N. S. and Zanjani, E. D. (1986) Transplantation of fetal hematopoietic stem cells in utero the creation of hematopoietic chimeras *Science* **233**, 776-8

Flanagan, J. G., Chan, D. C. and Leder, P. (1991) Transmembrane Form of the Kit Ligand Growth-Factor Is Determined by Alternative Splicing and Is Missing in the Si(D) Mutant *Cell* **64**, 1025-1035

Frederiksen, K. and McKay, R. D. (1988) Proliferation and differentiation of rat neuroepithelial precursor cells in vivo *The Journal of neuroscience* **8**, 1144-51

Frenette, P. S., Subbarao, S., Mazo, I. B., von Andrian, U. H. and Wagner, D. D. (1998) Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow *Proceedings of the National Academy of Sciences of the United States of America* **95**, 14423-14428

Friedman, W. J. and Greene, L. A. (1999) Neurotrophin signaling via Trks and p75 *Experimental Cell Research* **253**, 131-42

Fu, M., Lui, V. C., Sham, M. H., Pachnis, V. and Tam, P. K. (2004a) Sonic hedgehog regulates the proliferation, differentiation, and migration of enteric neural crest cells in gut *The Journal of cell biology* **166**, 673-84.

Fu, M., Tam, P. K., Sham, M. H. and Lui, V. C. (2004b) Embryonic development of the ganglion plexuses and the concentric layer structure of human gut a topographical study *Anatomy and embryology* **208**, 33-41

Funahashi, H., Okada, Y., Sawai, H., Takahashi, H., Matsuo, Y., Takeyama, H. and Manabe, T. (2005) The role of glial cell line-derived neurotrophic factor (GDNF) and integrins for invasion and metastasis in human pancreatic cancer cells *Journal of surgical oncology* **91**, 77-83

Furness, J. B., Clere, N., Vogalis, F. and Stebbing, M. J. (2003) The enteric nervous system and its extrinsic connections *Yamada T, Alpers DH (eds) Textbook of gastroenterology Lippincott Williams & Wilkins, Philadelphia,*, pp 13-34

Galvez, B. G., Sampaolesi, M., Brunelli, S., Covarello, D., Gavina, M., Rossi, B., Costantin, G., Torrente, Y. and Cossu, G. (2006) Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability *Journal of Cell Biology* **174**, 231-243

Garzon-Muvdi, T. and Quinones-Hinojosa, A. (2010) Neural Stem Cell Niches and Homing Recruitment and Integration into Functional Tissues *Ilar Journal* **51**, 3-23

Gershon, M. D. (1999) The enteric nervous system a second brain *Hospital practice (Minneap)* **34**, 31-42

Gershon, M. D. and Erde, S. M. (1981) The nervous system of the gut *Gastroenterology* **80**, 1571-94

Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R. and Gruss, P. (1991) Pax-3, a novel murine DNA binding protein expressed during early neurogenesis *The EMBO journal* **10**, 1135-47

Graham, V., Khudyakov, J., Ellis, P. and Pevny, L. (2003) SOX2 functions to maintain neural progenitor identity *Neuron* **39**, 749-65

Gratwohl, A., Baldomero, H., Aljurf, M., Pasquini, M. C., Bouzas, L. F., Yoshimi, A., Szer, J., Lipton, J., Schwendener, A., Gratwohl, M. et al. (2010) Hematopoietic stem cell transplantation a global perspective *the journal of the American Medical Association* **303**, 1617-24

Gritti, A., Frolichsthal-Schoeller, P., Galli, R., Parati, E. A., Cova, L., Pagano, S. F., Bjornson, C. R. and Vescovi, A. L. (1999) Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain *Journal of Neuroscience* **19**, 3287-3297

Grundy, D. and Schemann, M. (2006) Enteric nervous system *Current opinion in gastroenterology* **22**, 102-10

Guillemot, F. and Joyner, A. L. (1993) Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system *Mechanisms of Development* **42**, 171-85

Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J. and Joyner, A. L. (1993) Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons *Cell* **75**, 463-76

Hackam, D. J., Filler, R. M. and Pearl, R. H. (1998) Enterocolitis after the surgical treatment of Hirschsprung's disease risk factors and financial impact *Journal of Pediatric Surgery* **33**, 830-3

Hao, M. M., Moore, R. E., Roberts, R. R., Nguyen, T., Furness, J. B., Anderson, R. B. and Young, H. M. (2010) The role of neural activity in the migration and differentiation of enteric neuron precursors *Neurogastroenterology and Motility*

Hari, L., Brault, V., Kleber, M., Lee, H. Y., Ille, F., Leimeroth, R., Paratore, C., Suter, U., Kemler, R. and Sommer, L. (2002) Lineage-specific requirements of beta-catenin in neural crest development *Journal of Cell Biology* **159**, 867-880

Hasler, W. L. (1999) Motility of the small intestine and colon In Yamada, T ed Textbook of Gastroenterology Philadelphia: Lippincott Williams & Wilkins 188-215

Hatano, M., Aoki, T., Dezawa, M., Yusa, S., Iitsuka, Y., Koseki, H., Taniguchi, M. and Tokuhisa, T. (1997) A novel pathogenesis of megacolon in Ncx/Hox11L 1 deficient mice *The Journal of clinical investigation* **100**, 795-801

Heanue, T. A. and Pachnis, V. (2006) Expression profiling the developing mammalian enteric nervous system identifies marker and candidate Hirschsprung disease genes *Proceedings of the National Academy of Sciences of the United States of America* **103**, 6919-24

Heanue, T. A. and Pachnis, V. (2007) Enteric nervous system development and Hirschsprung's disease advances in genetic and stem cell studies *Nature reviews Neuroscience* **8**, 466-79

Hearn, C. J., Murphy, M. and Newgreen, D. (1998) GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro *Developmental Biology* **197**, 93-105

Hendzel, M. J., Wei, Y., Mancini, M. A., VanHooser, A., Ranalli, T., Brinkley, B. R., BazettJones, D. P. and Allis, C. D. (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation *Chromosoma* **106**, 348-360

Herbarth, B., Pingault, V., Bondurand, N., Kuhlbrodt, K., Hermans-Borgmeyer, I., Puliti, A., Lemort, N., Goossens, M. and Wegner, M. (1998) Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease *Proceedings of the National Academy of Sciences of the United States of America* **95**, 5161-5165

Hirsch, M. R., Tiveron, M. C., Guillemot, F., Brunet, J. F. and Goridis, C. (1998) Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system *Development* **125**, 599-608

Hirschsprung, H. (1888) Stuhltra gheit neugeborener infolge von dilatation und hypertrophie des colons *Jornal brasileiro de doenças toracicas* **27**, 1

Hitoshi, S., Alexson, T., Tropepe, V., Donoviel, D., Elia, A. J., Nye, J. S., Conlon, R. A., Mak, T. W., Bernstein, A. and van der Kooy, D. (2002) Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells *Genes & development* **16**, 846-58

Holschneider, A. M. and Puri, P. (2008) **Hirschsprung´s Disease and Allied Disorders.**Third Edition

Hosoda, K., Hammer, R. E., Richardson, J. A., Baynash, A. G., Cheung, J. C., Giaid, A. and

Yanagisawa, M. (1994) Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice *Cell* **79**, 1267-76

Hotta, R., Anderson, R. B., Kobayashi, K., Newgreen, D. F. and Young, H. M. (2009a) Effects of tissue age, presence of neurones and endothelin-3 on the ability of enteric neurone precursors to colonize recipient gut: implications for cell-based therapies *Neurogastroenterology and Motility* **22**, 331-e86

Hotta, R., Pepdjonovic, L., Anderson, R. B., Zhang, D., Bergner, A. J., Leung, J., Pebay, A., Dottori, M., Young, H. M. and Newgreen, D. F. (2009b) Small molecule induction of neural crest stem cells from human embryonic stem cell-derived neural progenitor cells *Neurogastroenterology and Motility* **21**, Xvi-Xvi

Hunt, D. P., Morris, P. N., Sterling, J., Anderson, J. A., Joannides, A., Jahoda, C., Compston, A. and Chandran, S. (2008) A highly enriched niche of precursor cells with neuronal and glial potential within the hair follicle dermal papilla of adult skin *Stem Cells* **26**, 163-72

Iitsuka, Y., Shimizu, H., Kang, M. M., Sasagawa, K., Sekiya, S., Tokuhisa, T. and Hatano, M. (1999) An enhancer element for expression of the Ncx (Enx, Hox11L1) gene in neural crest-derived cells *The Journal of biological chemistry* **274**, 24401-7

Ishii, Y., Rex, M., Scotting, P. J. and Yasugi, S. (1998) Region-specific expression of chicken Sox2 in the developing gut and lung epithelium: Regulation by epithelial-mesenchymal interactions *Developmental Dynamics* **213**, 464-475

Itoh, T., Satou, T., Ishida, H., Nishida, S., Tsubaki, M., Hashimoto, S. and Ito, H. (2009) The relationship between SDF-1 alpha/CXCR4 and neural stem cells appearing in damaged area after traumatic brain injury in rats *Neurological Research* **31**, 90-102

Iwashita, T., Kruger, G. M., Pardal, R., Kiel, M. J. and Morrison, S. J. (2003) Hirschsprung disease is linked to defects in neural crest stem cell function *Science* **301**, 972-6

Jacobscohen, R. J., Payette, R. F., Gershon, M. D. and Rothman, T. P. (1987) Inability of Neural Crest Cells to Colonize the Presumptive Aganglionic Bowel of Ls Ls Mutant Mice - Requirement for a Permissive Microenvironment *Journal of Comparative Neurology* **255**, 425-438

Jaenisch, R. (2004) Human cloning - the science and ethics of nuclear transplantation *The New England journal of medicine* **351**, 2787-91

Jing, S., Wen, D., Yu, Y., Holst, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R. et al. (1996) GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF *Cell* **85**, 1113-24

Jing, S., Yu, Y., Fang, M., Hu, Z., Holst, P. L., Boone, T., Delaney, J., Schultz, H., Zhou, R. and Fox, G. M. (1997) GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family *The Journal of biological chemistry* **272**, 33111-7

Johnson, J. E., Birren, S. J. and Anderson, D. J. (1990) Two rat homologues of Drosophila achaete-scute specifically expressed in neuronal precursors *Nature* **346**, 858-61

Johnston, L. A. (2009) Competitive Interactions Between Cells: Death, Growth, and Geography *Science* **324**, 1679-1682

Julien, J. P. (1999) Neurofilament functions in health and disease *Current opinion in neurobiology* **9**, 554-60

Kamachi, Y., Sockanathan, S., Liu, Q., Breitman, M., Lovell-Badge, R. and Kondoh, H. (1995) Involvement of SOX proteins in lens-specific activation of crystallin genes *The EMBO journal* **14**, 3510-9

Kamachi, Y., Uchikawa, M. and Kondoh, H. (2000) Pairing SOX off with partners in the regulation of embryonic development *Trends in Genetics* **16**, 182-187

Kapur, R. P. (1999) Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10(Dom)/Sox10(Dom) mouse embryos *Pediatric and Developmental Pathology* **2**, 559-569

Kapur, R. P. (2000) Colonization of the murine hindgut by sacral crest-derived neural precursors experimental support for an evolutionarily conserved model *Developmental Biology* **227**, 146-55

Kapur, R. P., Livingston, R., Doggett, B., Sweetser, D. A., Siebert, J. R. and Palmiter, R. D. (1996) Abnormal microenvironmental signals underlie intestinal aganglionosis in Dominant megacolon mutant mice *Developmental Biology* **174**, 360-9

Kapur, R. P., Yost, C. and Palmiter, R. D. (1992) A transgenic model for studying development of the enteric nervous system in normal and aganglionic mice *Development* **116**, 167-75

Katayama, Y., Battista, M., Kao, W. M., Hidalgo, A., Peired, A. J., Thomas, S. A. and Frenette, P. S. (2006) Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow *Cell* **124**, 407-421

Kawaguchi, J., Nichols, J., Gierl, M. S., Faial, T. and Smith, A. (2010) Isolation and propagation of enteric neural crest progenitor cells from mouse embryonic stem cells and embryos *Development* **137**, 693-704

Kelly, E. B. (2007) Stem cells **Greenwood Press Westport, Connecticut, London.**, P3-4

Kelsh, R. N. (2006) Sorting out Sox10 functions in neural crest development *Bioessays* **28**, 788-98

Kerry, A., C, R., Jersome, R., Willem, E. F., Pwe, L. and Malcom, K. B. (2004a) Clinical bone marrow and bolld stem cell transplantation 2-3

Kerry, A., C, R., Jersome, R., Willem, E. F., Pwe, L. and Malcom, K. B. (2004b) Clinical bone marrow and bolld stem cell transplantation 59

Killian, E. C. O., Birkholz, D. A. and Artinger, K. B. (2009) A role for chemokine signaling in neural crest cell migration and craniofacial development *Developmental Biology* **333**, 161-172

Kim, D., Kim, C. H., Moon, J. I., Chung, Y. G., Chang, M. Y., Han, B. S., Ko, S., Yang, E., Cha, K. Y., Lanza, R. et al. (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins *Cell Stem Cell* **4**, 472-6

Kim, J., Lo, L., Dormand, E. and Anderson, D. J. (2003) SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells *Neuron* **38**, 17-31

Kinashi, T. and Springer, T. A. (1994) Steel Factor and C-Kit Regulate Cell-Matrix Adhesion *Blood* **83**, 1033-1038

Kiskinis, E. and Eggan, K. (2010) Progress toward the clinical application of patient-specific pluripotent stem cells *The Journal of clinical investigation* **120**, 51-9

Kondo, T. and Raff, M. (2000) Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells *Science* **289**, 1754-7

Kruger, G. M., Mosher, J. T., Bixby, S., Joseph, N., Iwashita, T. and Morrison, S. J. (2002) Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness *Neuron* **35**, 657-669

Kruger, G. M., Mosher, J. T., Tsai, Y. H., Yeager, K. J., Iwashita, T., Gariepy, C. E. and Morrison,

S. J. (2003) Temporally distinct requirements for endothelin receptor B in the generation and migration of gut neural crest stem cells *Neuron* **40**, 917-29

Kucia, M., Reca, R., Miekus, K., Wanzeck, J., Wojakowski, W., Janowska-Wieczorek, A., Ratajczak, J. and Ratajczak, M. Z. (2005) Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms Pivotal role of the SDF-1-CXCR4 axis *Stem Cells* **23**, 879-894

Kurer, M. H., Lawson, J. O. and Pambakian, H. (1986) Suction biopsy in Hirschsprung's disease *Archives of disease in childhood* **61**, 83-4

Kusafuka, T. and Puri, P. (1997a) Altered RET gene mRNA expression in Hirschsprung's disease *Journal of Pediatric Surgery* **32**, 600-4

Kusafuka, T. and Puri, P. (1997b) The RET proto-oncogene a challenge to our understanding of disease pathogenesis *Pediatric surgery international* **12**, 11-8

Lane, P. W. and Liu, H. M. (1984) Association of megacolon with a new dominant spotting gene (Dom) in the mouse *The Journal of heredity* **75**, 435-9

Lang, D., Chen, F., Milewski, R., Li, J., Lu, M. M. and Epstein, J. A. (2000) Pax3 is required for enteric ganglia formation and functions with Sox10 to modulate expression of c-ret *The Journal of clinical investigation* **106**, 963-71

Langer, J. C., Betti, P. A. and Blennerhassett, M. G. (1994) Smooth muscle from aganglionic bowel in Hirschsprung's disease impairs neuronal development in vitro *Cell and Tissue Research* **276**, 181-6

Lapidot, T. (2001) Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice - The role of SDF-1/CXCR4 interactions *Hematopoietic Stem Cells 2000 Basic and Clinical Sciences* **938**, 83-95

Le Douarin, N. and Kalcheim, C. (1999) The neural crest 2nd ed *Cambridge Cambridge University Press*

Le Douarin, N. M. and Teillet, M. A. (1973) The migration of neural crest cells to the wall of the digestive tract in avian embryo *Journal of embryology and experimental morphology* **30**, 31-48

Le Douarin, N. M. and Teillet, M. A. (1974) Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique *Developmental Biology* **41**, 162-84

Lee, H. O., Levorse, J. M. and Shin, M. K. (2003) The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors *Developmental Biology* **259**, 162-75

Leibl, M. A., Ota, T., Woodward, M. N., Kenny, S. E., Lloyd, D. A., Vaillant, C. R. and Edgar, D. H. (1999) Expression of endothelin 3 by mesenchymal cells of embryonic mouse caecum *Gut* **44**, 246-52

Lendahl, U., Zimmerman, L. B. and McKay, R. D. (1990) CNS stem cells express a new class of intermediate filament protein *Cell* **60**, 585-95

Li, H. Y., Say, E. H. and Zhou, X. F. (2007) Isolation and characterization of neural crest progenitors from adult dorsal root ganglia *Stem Cells* **25**, 2053-65

Lindley, R. M., Hawcutt, D. B., Connell, M. G., Almond, S. N., Vannucchi, M. G., Faussone-Pellegrini, M. S., Edgar, D. H. and Kenny, S. E. (2008) Human and mouse enteric

nervous system neurosphere transplants regulate the function of aganglionic embryonic distal colon *Gastroenterology* **135**, 205-216 e6

Lindvall, O. and Kokaia, Z. (2010) Stem cells in human neurodegenerative disorders--time for clinical translation? *The Journal of clinical investigation* **120**, 29-40

Liu, W., Wu, R. D., Dong, Y. L. and Gao, Y. M. (2007) Neuroepithelial stem cells differentiate into neuronal phenotypes and improve intestinal motility recovery after transplantation in the aganglionic colon of the rat *Neurogastroenterology and Motility* **19**, 1001-1009

Lo, L. and Anderson, D. J. (1995) Postmigratory neural crest cells expressing c-RET display restricted developmental and proliferative capacities *Neuron* **15**, 527-39

Lo, L., Tiveron, M. C. and Anderson, D. J. (1998) MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity *Development* **125**, 609-20

Lo, L. C., Johnson, J. E., Wuenschell, C. W., Saito, T. and Anderson, D. J. (1991) Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells *Genes & development* **5**, 1524-37

Long, M. W., Briddell, R., Walter, A. W., Bruno, E. and Hoffman, R. (1992) Human Hematopoietic Stem-Cell Adherence to Cytokines and Matrix Molecules *Journal of Clinical Investigation* **90**, 251-255

Iou, Y., Ceccherini, I., Pasini, B., Matera, I., Bicocchi, M. P., Barone, V., Bocciardi, R., Kaàriainen, H., Weber, D. and Devoto, M. (1993) Close linkage with the RET protooncogene and boundaries of deletion mutations in autosomal dominant Hirschsprung disease *Human Molecular Genetics* **2**, 1803-8

Louis, S. A., Rietze, R. L., Deleyrolle, L., Wagey, R. E., Thomas, T. E., Eaves, A. C. and Reynolds, B. A. (2008) Enumeration of neural stem and progenitor cells in the neural colony-forming cell assay *Stem Cells* **26**, 988-96

Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T., Bronson, R. T. and Springer, T. A. (1998) Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice *Proceedings of the National Academy of Sciences of the United States of America* **95**, 9448-9453

Madri, J. A. (2009) Modeling the neurovascular niche implications for recovery from CNS injury *Journal of physiology and pharmacology* **60 Suppl 4**, 95-104

Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996) Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development *Developmental Biology* **180**, 273-83

Martucciello, G., Brizzolara, A., Favre, A., Lombardi, L., Bocciardi, R., Sanguineti, M., Prato, A. P. and Jasonni, V. (2007) Neural crest neuroblasts can colonise aganglionic and ganglionic gut in vivo *European Journal of Pediatric Surgery* **17**, 34-40

Martucciello, G., Ceccherini, I., Lerone, M. and Jasonni, V. (2000) Pathogenesis of Hirschsprung's disease *Journal of Pediatric Surgery* **35**, 1017-25

Massague, J. (1990) Transforming Growth Factor-Alpha - a Model for Membrane-Anchored Growth-Factors *Journal of Biological Chemistry* **265**, 21393-21396

Massague, J. (1998) TGF-beta signal transduction *Annual review of biochemistry* **67**, 753-91

Mathe, G. (1968) Bone marrow transplantation In transplantation, ed F T Rappaport & J Dausset

New York Grune & Strantton, 284-303

Matthews, H. K., Marchant, L., Carmona-Fontaine, C., Kuriyama, S., Larrain, J., Holt, M. R., Parsons, M. and Mayor, R. (2008) Directional migration of neural crest cells in vivo is regulated by Syndecan-4/Rac1 and non-canonical Wnt signaling/RhoA *Development* **135**, 1771-1780

McBeath, R., Pirone, D. M., Nelson, C. M., Bhadriraju, K. and Chen, C. S. (2004) Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment *Developmental cell* **6**, 483-95

McCallion, A. S., Emison, E. S., Kashuk, C. S., Bush, R. T., Kenton, M., Carrasquillo, M. M., Jones, K. W., Kennedy, G. C., Portnoy, M. E., Green, E. D. et al. (2003) Genomic variation in multigenic traits Hirschsprung disease *Cold Spring Harbor Symposia on Quantitative Biology* **68**, 373-81

McKeown, S. J., Chow, C. W. and Young, H. M. (2001) Development of the submucous plexus in the large intestine of the mouse *Cell and Tissue Research* **303**, 301-5

McLain, C. R., Jr. (1963) Amniography Studies of the Gastrointestinal Motility of the Human Fetus *American Journal of Obstetrics and Gynecology* **86**, 1079-87

Meier-Ruge, W., Lutterbeck, P. M., Herzog, B., Morger, R., Moser, R. and Scharli, A. (1972) Acetylcholinesterase activity in suction biopsies of the rectum in the diagnosis of Hirschsprung's disease *Journal of Pediatric Surgery* **7**, 11-7

Meijers, J. H., van der Sanden, M. P., Tibboel, D., van der Kamp, A. W., Luider, T. M. and Molenaar, J. C. (1992) Colonization characteristics of enteric neural crest cells embryological aspects of Hirschsprung's disease *Journal of Pediatric Surgery* **27**, 811-4

Melchionna, R., Di Carlo, A., De Mori, R., Cappuzzello, C., Barberi, L., Musarò, A., Cencioni, C., Fujii, N., Tamamura, H., Crescenzi, M. et al. (2010) Induction of myogenic differentiation by SDF-1 via CXCR4 and CXCR7 receptors *Muscle Nerve* **41**, 828-35

Metzger, M., Caldwell, C., Barlow, A. J., Burns, A. J. and Thapar, N. (2009) Enteric nervous system stem cells derived from human gut mucosa for the treatment of aganglionic gut disorders *Gastroenterology* **136**, 2214-25 e1-3

Micci, M. A., Learish, R. D., Li, H., Abraham, B. P. and Pasricha, P. J. (2001) Neural stem cells express RET, produce nitric oxide, and survive transplantation in the gastrointestinal tract *Gastroenterology* **121**, 757-66

Milla, P., Cucchiara, S., DiLorenzo, C., Rivera, N. M., Rudolph, C. and Tomomasa, T. (2002) Motility disorders in childhood Working Group Report of the First World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition *Journal of Pediatric Gastroenterology and Nutrition* **35 Suppl 2**, S187-95

Milla, P. J. (1988) Gastrointestinal motility disorders in children *Pediatric clinics of North America* **35**, 311-30

Milla, P. J. (1998) Motility disorders in childhood *Baillière's clinical gastroenterology* **12**, 775-97

Milla, P. J. (1999) Acquired motility disorders in childhood *Canadian journal of gastroenterology* **13 Suppl A**, 76A-84A

Miura, M., Gronthos, S., Zhao, M. R., Lu, B., Fisher, L. W., Robey, P. G. and Shi, S. T. (2003) SHED Stem cells from human exfoliated deciduous teeth *Proceedings of the National Academy of Sciences of the United States of America* **100**, 5807-5812

Mollaaghababa, R. and Pavan, W. J. (2003) The importance of having your SOX on: role of SOX10 in the development of neural crest-derived melanocytes and glia *Oncogene* **22**, 3024-34

Molofsky, A. V., Pardal, R., Iwashita, T., Park, I. K., Clarke, M. F. and Morrison, S. J. (2003) Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation *Nature* **425**, 962-7

Montgomery, R. K., Mulberg, A. E. and Grand, R. J. (1999) Development of the human gastrointestinal tract: twenty years of progress *Gastroenterology* **116**, 702-31

Moore, S. W., Albertyn, R. and Cywes, S. (1996) Clinical outcome and long-term quality of life after surgical correction of Hirschsprung's disease *Journal of Pediatric Surgery* **31**, 1496-502

Morikawa, S., Mabuchi, Y., Niibe, K., Suzuki, S., Nagoshi, N., Sunabori, T., Shimmura, S., Nagai, Y., Nakagawa, T., Okano, H. et al. (2009) Development of mesenchymal stem cells partially originate from the neural crest *Biochemical and Biophysical Research Communications* **379**, 1114-9

Morin, X., Cremer, H., Hirsch, M. R., Kapur, R. P., Goridis, C. and Brunet, J. F. (1997) Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene Phox2a *Neuron* **18**, 411-23

Morrison, S. J., White, P. M., Zock, C. and Anderson, D. J. (1999) Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells *Cell* **96**, 737-49

Morshead, C. M. and van der Kooy, D. (2004) Disguising adult neural stem cells *Current opinion in neurobiology* **14**, 125-31

Muench, M. O. (2005) In utero transplantation: baby steps towards an effective therapy *Bone Marrow Transplant* **35**, 537-47

Muller-Ehmsen, J., Whittaker, P., Kloner, R. A., Dow, J. S., Sakoda, T., Long, T. I., Laird, P. W. and Kedes, L. (2002) Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium *Journal of Molecular and Cellular Cardiology* **34**, 107-116

Murakami, H., Iwashita, T., Asai, N., Iwata, Y., Narumiya, S. and Takahashi, M. (1999) Rho-dependent and -independent tyrosine phosphorylation of focal adhesion kinase, paxillin and p130Cas mediated by Ret kinase *Oncogene* **18**, 1975-82

Nagoshi, N., Shibata, S., Kubota, Y., Nakamura, M., Nagai, Y., Satoh, E., Morikawa, S., Okada, Y., Mabuchi, Y., Katoh, H. et al. (2008) Ontogeny and multipotency of neural crest-derived stem cells in mouse bone marrow, dorsal root ganglia, and whisker pad *Cell Stem Cell* **2**, 392-403

Nagy, N. and Goldstein, A. M. (2006) Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system *Developmental Biology* **293**, 203-17

Nagy, N., Mwizerwa, O., Yaniv, K., Carmel, L., Pieretti-Vanmarcke, R., Weinstein, B. M. and Goldstein, A. M. (2009) Endothelial cells promote migration and proliferation of enteric neural crest cells via beta1 integrin signaling *Developmental Biology* **330**, 263-72

Nagy, R. D., Tsai, B. M., Wang, M. J., Markel, T. A., Brown, J. W. and Meldrum, D. R. (2005) Stem cell transplantation as a therapeutic approach to organ failure *Journal of Surgical Research* **129**, 152-160

Narita, J., Miyaji, C., Watanabe, H., Honda, S., Koya, T., Umezu, H., Ushiki, T., Sugahara, S., Kawamura, T., Arakawa, M. et al. (1998) Differentiation of forbidden T cell clones and granulocytes in the parenchymal space of the liver in mice treated with estrogen *Cellular*

immunology **185**, 1-13

Natarajan, D., Grigoriou, M., Marcos-Gutierrez, C. V., Atkins, C. and Pachnis, V. (1999). Multipotential progenitors of the mammalian enteric nervous system capable of colonising aganglionic bowel in organ culture *Development* **126**, 157-168

Natarajan, D., Marcos-Gutierrez, C., Pachnis, V. and de Graaff, E. (2002) Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis *Development* **129**, 5151-5160

Nathan, C. and Sporn, M. (1991) Cytokines in Context *Journal of Cell Biology* **113**, 981-986

Nemeth, L., Rolle, U. and Puri, P. (2002) Altered cytoskeleton in smooth muscle of aganglionic bowel *Archives of pathology & laboratory medicine* **126**, 692-6

Newgreen, D. and Young, H. M. (2002a) Enteric nervous system development and developmental disturbances--part 1 *Pediatric and Developmental Pathology* **5**, 224-47

Newgreen, D. and Young, H. M. (2002b) Enteric nervous system development and developmental disturbances--part 2 *Pediatric and Developmental Pathology* **5**, 329-49

Nishimoto, M., Fukushima, A., Okuda, A. and Muramatsu, M. (1999) The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2 *Molecular and Cellular Biology* **19**, 5453-5465

Nugent, M. A. and Newman, M. J. (1989) Inhibition of Normal Rat-Kidney Cell-Growth by Transforming Growth Factor-Beta Is Mediated by Collagen *Journal of Biological Chemistry* **264**, 18060-18067

Nunez-Torres, R., Fernandez, R. M., Lopez-Alonso, M., Antinolo, G. and Borrego, S. (2009) A novel study of Copy Number Variations in Hirschsprung disease using the Multiple Ligation-dependent Probe Amplification (MLPA) technique *Bmc Medical Genetics* **10**

Okita, K., Nakagawa, M., Hong, H. J., Ichisaka, T. and Yamanaka, S. (2008) Generation of Mouse Induced Pluripotent Stem Cells Without Viral Vectors *Science* **322**, 949-953

Olden, T., Akhtar, T., Beckman, S. A. and Wallace, K. N. (2008) Differentiation of the zebrafish enteric nervous system and intestinal smooth muscle *Genesis* **46**, 484-98

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M. et al. (2001) Bone marrow cells regenerate infarcted myocardium *Nature* **410**, 701-5

Owen, R. D. (1945) Immunogenetic consequences of vascular anastomoses between bovine twin *Science* **102**, 400-401

Ozcelik, T., Lafreniere, R. G., Archer, B. T., 3rd, Johnston, P. A., Willard, H. F., Francke, U. and Sudhof, T. C. (1990) Synaptophysin structure of the human gene and assignment to the X chromosome in man and mouse *American Journal of Human Genetics* **47**, 551-61

Pagani, F. D., DerSimonian, H., Zawadzka, A., Wetzel, K., Edge, A. S. B., Jacoby, D. B., Dinsmore, J. H., Wright, S., Aretz, T. H., Eisen, H. J. et al. (2003) Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans - Histological analysis of cell survival and differentiation *Journal of the American College of Cardiology* **41**, 879-888

Page, R. L., Ambady, S., Holmes, W. F., Vilner, L., Kole, D., Kashpur, O., Huntress, V., Vojtic, I., Whitton, H. and Dominko, T. (2009) Induction of Stem Cell Gene Expression in Adult Human Fibroblasts without Transgenes *Cloning and Stem Cells* **11**, 417-426

Papayannopoulou, T., Priestley, G. V., Nakamoto, B., Zafiropoulos, V. and Scott, L. M. (2001) Molecular pathways in bone marrow homing dominant role of alpha 4 beta 1 over beta 2-integrins and selectins *Blood* **98**, 2403-2411

Paratore, C., Eichenberger, C., Suter, U. and Sommer, L. (2002) Sox10 haploinsufficiency affects maintenance of progenitor cells in a mouse model of Hirschsprung disease *Human Molecular Genetics* **11**, 3075-85

Paratore, C., Goerich, D. E., Suter, U., Wegner, M. and Sommer, L. (2001) Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling *Development* **128**, 3949-61

Parc, R., Berrod, J. L., Tussiot, J. and Loygue, J. (1984) Megacolon in adults Apropos of 76 cases *Annales de gastroenterologie et d'hepatologie (Paris)* **20**, 133-41

Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J. F. (1997) Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis *Development* **124**, 4065-75

Pattyn, A., Morin, X., Cremer, H., Goridis, C. and Brunet, J. F. (1999) The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives *Nature* **399**, 366-70

Peerani, R. and Zandstra, P. W. (2010) Enabling stem cell therapies through synthetic stem cell-niche engineering *Journal of Clinical Investigation* **120**, 60-70

Peirano, R. I. and Wegner, M. (2000) The glial transcription factor Sox10 binds to DNA both as monomer and dimer with different functional consequences *Nucleic Acids Research* **28**, 3047-55

Peters-van der Sanden, M. J., Kirby, M. L., Gittenberger-de Groot, A., Tibboel, D., Mulder, M. P. and Meijers, C. (1993) Ablation of various regions within the avian vagal neural crest has differential effects on ganglion formation in the fore-, mid- and hindgut *Developmental Dynamics* **196**, 183-94

Pevny, L. and Rao, M. S. (2003) The stem-cell menagerie *Trends in neurosciences* **26**, 351-9

Pevny, L. H. and LovellBadge, R. (1997) Sox genes find their feet *Current Opinion in Genetics & Development* **7**, 338-344

Pham, T. D., Gershon, M. D. and Rothman, T. P. (1991) Time of origin of neurons in the murine enteric nervous system sequence in relation to phenotype *The Journal of comparative neurology* **314**, 789-98

Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J. et al. (1996) Defects in enteric innervation and kidney development in mice lacking GDNF *Nature* **382**, 73-6

Pomeranz, H. D. and Gershon, M. D. (1990) Colonization of the Avian Hindgut by Cells Derived from the Sacral Neural Crest *Developmental Biology* **137**, 378-394

Pomeranz, H. D., Sherman, D. L., Smalheiser, N. R., Tennyson, V. M. and Gershon, M. D. (1991) Expression of a Neurally Related Laminin Binding-Protein by Neural Crest-Derived Cells That Colonize the Gut - Relationship to the Formation of Enteric Ganglia *Journal of Comparative Neurology* **313**, 625-642

Potten, C. S. and Loeffler, M. (1990) Stem-Cells - Attributes, Cycles, Spirals, Pitfalls and Uncertainties - Lessons for and from the Crypt *Development* **110**, 1001-1020

Potterf, S. B., Furumura, M., Dunn, K. J., Arnheiter, H. and Pavan, W. J. (2000) Transcription factor hierarchy in Waardenburg syndrome regulation of MITF expression by SOX10 and PAX3 *Human genetics* **107**, 1-6

Prior, H. M. and Walter, M. A. (1996) SOX genes Architects of development *Molecular Medicine* **2**, 405-412

Pujol, F., Kitabgi, P. and Boudin, H. (2005) The chemokine SDF-1 differentially regulates axonal elongation and branching in hippocampal neurons *Journal of Cell Science* **118**, 1071-1080

Raff, M. C., Miller, R. H. and Noble, M. (1983) A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium *Nature* **303**, 390-6

Rathjen, P. D., Toth, S., Willis, A., Heath, J. K. and Smith, A. G. (1990) Differentiation Inhibiting Activity Is Produced in Matrix-Associated and Diffusible Forms That Are Generated by Alternate Promoter Usage *Cell* **62**, 1105-1114

Rathna, P. A., Carol Lynn, B., Juan-R, M., Jean, P., Thomas, L. A. and Alan, D. H. (1989) **Postnatal Maturation of Small Intestinal Motility in Preterm and Term Infants** *Neurogastroenterology & Motility* **1**, 138 - 143

Rauch, U., Hansgen, A., Hagl, C., Holland-Cunz, S. and Schafer, K. H. (2006) Isolation and cultivation of neuronal precursor cells from the developing human enteric nervous system as a tool for cell therapy in dysganglionosis *International Journal of Colorectal Disease* **21**, 554-9

Rettenmier, C. W., Roussel, M. F., Ashmun, R. A., Ralph, P., Price, K. and Sherr, C. J. (1987) Synthesis of Membrane-Bound Colony-Stimulating Factor-1 (Csf-1) and down-Modulation of Csf-1 Receptors in Nih 3t3 Cells Transformed by Cotransfection of the Human Csf-1 and C-Fms (Csf-1 Receptor) Genes *Molecular and Cellular Biology* **7**, 2378-2387

Reynolds, B. A. and Rietze, R. L. (2005) Neural stem cells and neurospheres - re-evaluating the relationship *Nature Methods* **2**, 333-336

Reynolds, B. A., Tetzlaff, W. and Weiss, S. (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes *The Journal of neuroscience* **12**, 4565-74

Reynolds, B. A. and Weiss, S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system *Science* **255**, 1707-10

Reynolds, B. A. and Weiss, S. (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell *Developmental Biology* **175**, 1-13

Ro, S., Hwang, S. J., Muto, M., Jewett, W. K. and Spencer, N. J. (2006) Anatomic modifications in the enteric nervous system of piebald mice and physiological consequences to colonic motor activity *American journal of physiology Gastrointestinal and liver physiology* **290**, G710-8

Roberts, C. W., Sonder, A. M., Lumsden, A. and Korsmeyer, S. J. (1995) Development expression of Hox11 and specification of splenic cell fate *The American journal of pathology* **146**, 1089-101

Roberts, D. J. (2000) Molecular mechanisms of development of the gastrointestinal tract *Developmental Dynamics* **219**, 109-20

Roberts, D. J., Smith, D. M., Goff, D. J. and Tabin, C. J. (1998) Epithelial-mesenchymal signaling during the regionalization of the chick gut *Development* **125**, 2791-801

Roberts, R. R., Ellis, M., Gwynne, R. M., Bergner, A. J., Lewis, M. D., Beckett, E. A., Bornstein, J. C. and Young, H. M. (2010) The first intestinal motility patterns in fetal mice are not

mediated by neurons or interstitial cells of Cajal *Journal of Physiology-London* **588**, 1153-1169

Roberts, R. R., Murphy, J. F., Young, H. M. and Bornstein, J. C. (2007) Development of colonic motility in the neonatal mouse-studies using spatiotemporal maps *American journal of physiology Gastrointestinal and liver physiology* **292**, G930-8

Romeo, G., Ronchetto, P., Luo, Y., Barone, V., Seri, M., Ceccherini, I., Pasini, B., Bocciardi, R., Lerone, M., Kaariainen, H. et al. (1994) Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease *Nature* **367**, 377-8

Rupp, P. A. and Kulesa, P. M. (2007) A role for RhoA in the two-phase migratory pattern of post-otic neural crest cells *Developmental Biology* **311**, 159-71

Ryzhov, S., Solenkova, N. V., Goldstein, A. E., Lamparter, M., Fleenor, T., Young, P. P., Greelish, J. P., Byrne, J. G., Vaughan, D. E., Biaggioni, I. et al. (2008) Adenosine receptor-mediated adhesion of endothelial progenitors to cardiac microvascular endothelial cells *Circulation Research* **102**, 356-363

Sanchez, M. P., Silos-Santiago, I., Frisen, J., He, B., Lira, S. A. and Barbacid, M. (1996) Renal agenesis and the absence of enteric neurons in mice lacking GDNF *Nature* **382**, 70-3

Sanders, K. M. (1996) A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract *Gastroenterology* **111**, 492-515

Sandgren, K. and Pachnis, V. (2009) Ultrasound-Guided Grafting of ENS Progenitor Cells into Murine Embryonic Gut in vivo *Development of the enteric nervous system cells, signals and genes*, 65

Schafer, K. H., Hagl, C. I. and Rauch, U. (2003) Differentiation of neurospheres from the enteric nervous system *Pediatric surgery international* **19**, 340-4

Schoenhard, J. A. and Hatzopoulos, A. K. (2010) Stem Cell Therapy Pieces of the Puzzle *Journal of cardiovascular translational research* **3**, 49-60

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F. and Pachnis, V. (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret *Nature* **367**, 380-3

Schwartz, R. E., Reyes, M., Koodie, L., Jiang, Y. H., Blackstad, M., Lund, T., Lenvik, T., Johnson, S., Hu, W. S. and Verfaillie, C. M. (2002) Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells *Journal of Clinical Investigation* **109**, 1291-1302

Seaberg, R. M. and van der Kooy, D. (2003) Stem and progenitor cells the premature desertion of rigorous definitions *Trends in neurosciences* **26**, 125-31

Serbedzija, G. N., Burgan, S., Fraser, S. E. and Bronner-Fraser, M. (1991) Vital dye labelling demonstrates a sacral neural crest contribution to the enteric nervous system of chick and mouse embryos *Development* **111**, 857-66

Shamblott, M. J., Axelman, J., Wang, S., Bugg, E. M., Littlefield, J. W., Donovan, P. J., Blumenthal, P. D., Huggins, G. R. and Gearhart, J. D. (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells *Proceedings of the National Academy of Sciences of the United States of America* **95**, 13726-31

Shepherd, I. T., Beattie, C. E. and Raible, D. W. (2001) Functional analysis of zebrafish GDNF *Developmental Biology* **231**, 420-35

Shin, M. K., Levorse, J. M., Ingram, R. S. and Tilghman, S. M. (1999) The temporal requirement

for endothelin receptor-B signalling during neural crest development *Nature* **402**, 496-501

Shirasawa, S., Yunker, A. M., Roth, K. A., Brown, G. A., Horning, S. and Korsmeyer, S. J. (1997) Enx (Hox11L1)-deficient mice develop myenteric neuronal hyperplasia and megacolon *Nature medicine* **3**, 646-50

Sieber-Blum, M., Grim, M., Hu, Y. F. and Szeder, V. (2004) Pluripotent neural crest stem cells in the adult hair follicle *Developmental Dynamics* **231**, 258-69

Silva, A. T., Wardhaugh, T., Dolatshad, N. F., Jones, S. and Saffrey, M. J. (2008) Neural progenitors from isolated postnatal rat myenteric ganglia Expansion as neurospheres and differentiation in vitro *Brain Research* **1218**, 47-53

Skalli, O., Ropraz, P., Trzeciak, A., Benzonana, G., Gillessen, D. and Gabbiani, G. (1986) A monoclonal antibody against alpha-smooth muscle actin a new probe for smooth muscle differentiation *The Journal of cell biology* **103**, 2787-96

Sommer, L., Shah, N., Rao, M. and Anderson, D. J. (1995) The cellular function of MASH1 in autonomic neurogenesis *Neuron* **15**, 1245-58

Southard-Smith, E. M., Kos, L. and Pavan, W. J. (1998) Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model *Nature genetics* **18**, 60-4

Spiegel, A., Kalinkovich, A., Shivtiel, S., Kollet, O. and Lapidot, T. (2008) Stem Cell Regulation via Dynamic Interactions of the Nervous and Immune Systems with the Microenvironment *Cell Stem Cell* **3**, 484-492

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G. and Hochedlinger, K. (2008) Induced Pluripotent Stem Cells Generated Without Viral Integration *Science* **322**, 945-949

Stanchina, L., Van de Putte, T., Goossens, M., Huylebroeck, D. and Bondurand, N. (2010) Genetic interaction between Sox10 and Zfhx1b during enteric nervous system development *Developmental Biology* **341**, 416-28

Stemple, D. L. and Anderson, D. J. (1992) Isolation of a stem cell for neurons and glia from the mammalian neural crest *Cell* **71**, 973-85

Stringer, M. D., Brereton, R. J., Drake, D. P., Kiely, E. M., Agrawal, M., Mouriquand, P. D. and Tam, P. K. (1994) Meconium ileus due to extensive intestinal aganglionosis *Journal of Pediatric Surgery* **29**, 501-3

Swenson, O. (1996) Early history of the therapy of Hirschsprung's disease facts and personal observations over 50 years *Journal of Pediatric Surgery* **31**, 1003-8

Tada, M., Takahama, Y., Abe, K., Nakatsuji, N. and Tada, T. (2001) Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells *Current Biology* **11**, 1553-1558

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors *Cell* **131**, 861-72

Takahashi, K. and Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors *Cell* **126**, 663-76

Taraviras, S. and Pachnis, V. (1999) Development of the mammalian enteric nervous system *urrent opinion in genetics & development* **9**, 321-7

Thapar, N. (2007) Future horizons in the treatment of enteric neuropathies *Journal of Pediatric Gastroenterology and Nutrition* **45 Suppl 2**, S110-4

Thapar, N. (2009) New frontiers in the treatment of Hirschsprung disease *Journal of Pediatric Gastroenterology and Nutrition* **48**, S92-4

Thomas, S., Thomas, M., Wincker, P., Babarit, C., Xu, P., Speer, M. C., Munnich, A., Lyonnet, S., Vekemans, M. and Etchevers, H. C. (2008) Human neural crest cells display molecular and phenotypic hallmarks of stem cells *Human Molecular Genetics* **17**, 3411-25

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. and Jones, J. M. (1998) Embryonic stem cell lines derived from human blastocysts *Science* **282**, 1145-7

Tobin, J. L., Di Franco, M., Eichers, E., May-Simera, H., Garcia, M., Yan, J., Quinlan, R., Justice, M. J., Hennekam, R. C., Briscoe, J. et al. (2008) Inhibition of neural crest migration underlies craniofacial dysmorphology and Hirschsprung's disease in Bardet-Biedl syndrome *Proceedings of the National Academy of Sciences of the United States of America* **105**, 6714-9

Toma, J. G., Akhavan, M., Fernandes, K. J., Barnabe-Heider, F., Sadikot, A., Kaplan, D. R. and Miller, F. D. (2001) Isolation of multipotent adult stem cells from the dermis of mammalian skin *Nature Cell Biology* **3**, 778-84

Tomita, Y., Matsumura, K., Wakamatsu, Y., Matsuzaki, Y., Shibuya, I., Kawaguchi, H., Ieda, M., Kanakubo, S., Shimazaki, T., Ogawa, S. et al. (2005) Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart *The Journal of cell biology* **170**, 1135-46

Torfs, C. P. (1998) An epidemiological study of Hirschsprung disease in a multiracial California population *The Third International Meetings Hirschsprung disease and related neurocristopathies Evian, France*

Torihashi, S., Ward, S. M. and Sanders, K. M. (1997) Development of c-Kit-positive cells and the onset of electrical rhythmicity in murine small intestine *Gastroenterology* **112**, 144-55

Tran, P. B. and Miller, R. J. (2003) Chemokine receptors Signposts to brain development and disease *Nature Reviews Neuroscience* **4**, 444-455

Tran, P. B., Ren, D. J., Veldhouse, T. J. and Miller, R. J. (2004) Chemokine receptors are expressed widely by embryonic and adult neural progenitor cells *Journal of Neuroscience Research* **76**, 20-34

Tropepe, V., Sibilia, M., Ciruna, B. G., Rossant, J., Wagner, E. F. and van der Kooy, D. (1999) Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon *Developmental Biology* **208**, 166-88

Vajkoczy, P., Blum, S., Lamparter, M., Mailhammer, R., Erber, R., Engelhardt, B., Vestweber, D. and Hatzopoulos, A. K. (2003) Multistep nature of microvascular recruitment of Ex vivo-expanded embryonic endothelial progenitor cells during tumor angiogenesis *Journal of Experimental Medicine* **197**, 1755-1765

Vanderwinden, J. M., Gillard, K., De Laet, M. H., Messam, C. A. and Schiffmann, S. N. (2002) Distribution of the intermediate filament nestin in the muscularis propria of the human gastrointestinal tract *Cell and Tissue Research* **309**, 261-8

Verschueren, K., Remacle, J. E., Collart, C., Kraft, H., Baker, B. S., Tylzanowski, P., Nelles, L., Wuytens, G., Su, M. T., Bodmer, R. et al. (1999) SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5'-CACCT sequences in candidate target genes *The Journal of biological chemistry* **274**, 20489-98

Vescovi, A. L., Parati, E. A., Gritti, A., Poulin, P., Ferrario, M., Wanke, E., Frolichsthal-Schoeller, P., Cova, L., Arcellana-Panlilio, M., Colombo, A. et al. (1999) Isolation and cloning of

multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Experimental Neurology* **156**, 71-83.

Viegas, J. (2003). Stem cell research, Jennifer Viegas.-1st ed. Published The Rosen Publishing Group,Inc. New York.p41. *1st ed Published The Rosen Publishing Group,Inc New York.*, p41.

Vohra, B. P., Tsuji, K., Nagashimada, M., Uesaka, T., Wind, D., Fu, M., Armon, J., Enomoto, H. and Heuckeroth, R. O. (2006). Differential gene expression and functional analysis implicate novel mechanisms in enteric nervous system precursor migration and neuritogenesis. *Developmental Biology* **298**, 259-71.

Wakayama, T., Tabar, V., Rodriguez, I., Perry, A. C. F., Studer, L. and Mombaerts, P. (2001). Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. *Science* **292**, 740-743.

Wallace, A. S. and Burns, A. J. (2005). Development of the enteric nervous system, smooth muscle and interstitial cells of Cajal in the human gastrointestinal tract. *Cell and Tissue Research* **319**, 367-82.

Wang, L. (2006). Abnormal migration of vagal neural crest cells in Dominant megacolon mouse embryos.

Ward, S. M., Harney, S. C., Bayguinov, J. R., McLaren, G. J. and Sanders, K. M. (1997). Development of electrical rhythmicity in the murine gastrointestinal tract is specifically encoded in the tunica muscularis. *The Journal of physiology* **505 (Pt 1)**, 241-58.

Wegner, M. (1999). From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Research* **27**, 1409-1420.

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A. C. and Reynolds, B. A. (1996) Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *The Journal of neuroscience* **16**, 7599-609.

Wenger, M. and Markwalder, T. M. (1999). Surgically controlled, transpedicular methyl methacrylate vertebroplasty with fluoroscopic guidance. *Acta Neurochirurgica* **141**, 625-631.

Whitehouse, F. and Kernohan, J. (1948). Myenteric plexuses in congenital megacolon; study of 11 cases. *Archives of internal medicine* **82**, 75.

Widera, D., Zander, C., Heidbreder, M., Kasperek, Y., Noll, T., Seitz, O., Saldamli, B., Sudhoff, H., Sader, R., Kaltschmidt, C. et al. (2009). Adult palatum as a novel source of neural crest-related stem cells. *Stem Cells* **27**, 1899-910

Wilson, Y. M., Richards, K. L., Ford-Perriss, M. L., Panthier, J. J. and Murphy, M. (2004). Neural crest cell lineage segregation in the mouse neural tube. *Development* **131**, 6153-62.

Wong, C. E., Paratore, C., Dours-Zimmermann, M. T., Rochat, A., Pietri, T., Suter, U., Zimmermann, D. R., Dufour, S., Thiery, J. P., Meijer, D. et al. (2006). Neural crest-derived cells with stem cell features can be traced back to multiple lineages in the adult skin. *The Journal of cell biology* **175**, 1005-15.

Wu, J. J., Chen, J. X., Rothman, T. P. and Gershon, M. D. (1999). Inhibition of in vitro enteric neuronal development by endothelin-3: mediation by endothelin B receptors. *Development* **126**, 1161-73.

Wu, J. J., Rothman, T. P. and Gershon, M. D. (2000). Development of the interstitial cell of Cajal: origin, kit dependence and neuronal and nonneuronal sources of kit ligand. *Journal of Neuroscience Research* **59**, 384-401.

Yanagisawa, H., Yanagisawa, M., Kapur, R. P., Richardson, J. A., Williams, S. C., Clouthier, D. E., de Wit, D., Emoto, N. and Hammer, R. E. (1998) Dual genetic pathways of endothelin-mediated intercellular signaling revealed by targeted disruption of endothelin converting enzyme-1 gene *Development* **125**, 825-36

Yanchar, N. L. and Soucy, P. (1999) Long-term outcome after Hirschsprung's disease patients' perspectives *Journal of Pediatric Surgery* **34**, 1152-60

Yntema, C. L. and Hammond, W. S. (1954) The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo *The Journal of comparative neurology* **101**, 515-541

Yoshida, S., Shimmura, S., Nagoshi, N., Fukuda, K., Matsuzaki, Y., Okano, H. and Tsubota, K. (2006) Isolation of multipotent neural crest-derived stem cells from the adult mouse cornea *Stem Cells* **24**, 2714-2722

Yoshida, S., Shimmura, S., Shimazaki, J., Shinozaki, N. and Tsubota, K. (2005) Serum-free spheroid culture of mouse corneal keratocytes *Investigative Ophthalmology & Visual Science* **46**, 1653-1658

Young, H. M. (2008) Functional development of the enteric nervous system--from migration to motility *Neurogastroenterology and motility* **20 Suppl** 1, 20-31

Young, H. M., Bergner, A. J. and Muller, T. (2003) Acquisition of neuronal and glial markers by neural crest-derived cells in the mouse intestine *The Journal of comparative neurology* **456**, 1-11

Young, H. M., Hearn, C. J., Ciampoli, D., Southwell, B. R., Brunet, J. F. and Newgreen, D. F. (1998) A single rostrocaudal colonization of the rodent intestine by enteric neuron precursors is revealed by the expression of Phox2b, Ret, and p75 and by explants grown under the kidney capsule or in organ culture *Developmental Biology* **202**, 67-84

Young, H. M., Turner, K. N. and Bergner, A. J. (2005) The location and phenotype of proliferating neural-crest-derived cells in the developing mouse gut *Cell and Tissue Research* **320**, 1-9

Yu, H., Kumar, S. M., Kossenkov, A. V., Showe, L. and Xu, X. (2010) Stem cells with neural crest characteristics derived from the bulge region of cultured human hair follicles *The Journal of investigative dermatology* **130**, 1227-36

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R. et al. (2007) Induced pluripotent stem cell lines derived from human somatic cells *Science* **318**, 1917-20

Yuan, H., Corbi, N., Basilico, C. and Dailey, L. (1995) Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3 *Genes & development* **9**, 2635-45

Zaruba, M. M. and Franz, W. M. (2010) Role of the SDF-1-CXCR4 axis in stem cell-based therapies for ischemic cardiomyopathy *Expert Opinion on Biological Therapy* **10**, 321-335

Zhang, D. C., Brinas, I. M., Binder, B. J., Landman, K. A. and Newgreen, D. F. (2010) Neural crest regionalisation for enteric nervous system formation Implications for Hirschsprung's disease and stem cell therapy *Developmental Biology* **339**, 280-294

Zhou, H. Y., Wu, S. L., Joo, J. Y., Zhu, S. Y., Han, D. W., Lin, T. X., Trauger, S., Bien, G., Yao, S., Zhu, Y. et al. (2009) Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins *Cell Stem Cell* **4**, 381-384