Modelling Hirschsprung disease treatment with organ culture techniques

M.Sc. thesis
Molecular Genetics, Cell-and Developmental Biology specialization

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Abbreviations

1.	ENS	Enteric nervous system
2.	GI	Gastrointestinal
3.	HSCR	Hirschsprung disease
4.	ENSCs	Enteric neural stem/progenitor cells
5.	NCCs	Neural crest cells
6.	ENCCs	Enteric neural crest cells
7.	ENSDCs	Enteric nervous system-derived cells
8.	CNS	Central Nervous System
9.	10ss	10 somite-stage
10.	L1CAM	L1 cell adhesion molecule
11.	GDNF	Glial cell line-derived neurotrophic factor
12.	EDN3	Endothelin-3
13.	ECM	Extracellular matrix
14.	CSPGs	Chondroitin-sulphate proteoglycans
15.	LMMP	Longitudinal muscle/ myenteric plexus
16.	HSPGs	Heparan-sulphate proteoglycans
17.	PBS	Phosphate-buffered saline
18.	DPBS	Dulbecco’s Phosphate-Buffered Saline
19.	GFAP	Glial fibrillary acidic protein
20.	Tuj1	Neuron-specific class III beta-tubulin
21.	SMA	Smooth muscle actin
22.	DIV	Days *in vitro*
23.	RA	Retinoic acid
24.	WT	Wild type
25.	CS-56	Chondroitin sulphate 56
26.	VIP	Vasoactive intestinal peptide
27.	ChAT	Choline acetyltransferase
28.	S100	Ca^{2+} binding protein
29.	Wnt	Wingless-related integration site

Introduction

The enteric nervous system (ENS) is a complex neuronal-network composed of enteric neurons and glial cells within the wall of the gastrointestinal (GI) tract. The ENS plays an essential role in the development and normal function of the gastrointestinal tract. These normal roles include absorption, motility, secretion and regular immune function (Cheng et al., 2017). During embryogenesis, the enteric nervous system is formed by a neural tube derived highly migratory multipotent stem cells, the neural crest cells (NCCs). Abnormalities occurring during development of the ENS can cause several types of congenital diseases, including gastroparesis, oesophageal achalasia, anal achalasia, intestinal pseudo-obstruction, slow-transit constipation, and Hirschsprung disease (HSCR).

Hirschsprung disease is one of the most well-known clinically relevant disorders that can only be treated by colonic resection. HSCR affects mostly the distal bowel, and it is caused by the failure of neural crest-derived cells to complete migration in the gastrointestinal tract, leaving variable length of distal intestine "aganglionic," which deprives them of innervation thus making them paralyzed. Currently, our knowledge regarding the genetic background of HSCR is not yet complete. A variety of genes contribute in the neonatal development of HSCR including the cell line-derived neurotrophic factor (GDNF) and its receptor *RET*, and the endothelin-3 *(EDN3)* and its receptor the *EdnrB* (Mueller & Goldstein, 2022). Rapid development of the stem cell field could potentially mean that surgery may not be the only way to treat this disease. Previous studies reported successful isolation of human and mouse postnatal gut-derived enteric neural stem/progenitor cells (ENSCs) which can be cultured as floating neurospheres (NS) (Cheng et al., 2017). Implanted enteric neurospheres have shown promising gastrointestinal motility following *in vivo* transplantation in mouse colon or intestinal *ex vivo* in muscle layer explant (Cheng et al., 2017; Almond et al., 2007).

Despite the accumulating scientific literature describing the different aspects of the abnormal development of the gut, our knowledge is not yet complete regarding the mechanism involved in this abnormal development of the intestinal neuropathies. Stem cell and regenerative medicine appear to be very promising approaches to this problem (Pan et al., 2021). The aim of this study was to get a better insight about the extracellular matrix properties of the transplanted neurospheres and the host intestinal tissues.

1. Overview of the ENS

1.1 Anatomy of the enteric nervous system

The enteric nervous system (ENS) is the largest autonomous network in the peripheral nervous system, extending along the whole segment of the gastrointestinal tract. The GI tract is primarily composed of a complex network of enteric neurons and enteric glial cells. The ENS has multiple roles which are essential for the normal function of the GI tract including absorption and digestion of nutrients, secretion, immunity and motility. The ENS and the central nervous system (CNS) have the same neuroectodermal origin. The ENS is composed of approximately 100 million functional neurons which are composed of 18 subtypes (Brookes, 2001). Although the ENS receives central sympathetic and parasympathetic regulation, the ENS is able to function independently from the CNS (Nagy & Goldstein, 2017). The ENS can also sometimes be referred to as the "second brain," despite the ENS having extrinsic innervation from the CNS. The enteric neurons can be categorised into 4 main neuron types: intestinofugal neurons, primary afferent neurons, motoneurons, and interneurons. A recent study in our laboratory identified a new cell type which are present in the enteric ganglia: the intraganglionic macrophages (Dora et al., 2021; Dora et al., 2018). This myeloid cell type has a similar immunophenotype to that of the microglia in the CNS.

Histologically the ENS is organised into two major circular plexuses which are composed of the interconnected network of enteric neurons and glial cells forming a ganglia. (Sasselli et al., 2012). The two major plexuses are the myenteric (Auerbach's) plexus and the submucosal (Meissner's) plexus. Both of these plexuses are positioned on either side of the circular muscle layer (Fig. 1). While the myenteric plexus extends along the whole GI tract, the submucosal plexus is absent in the oesophagus (Timmermans et al., 2001). The enteric neural circuits are composed of enteric neurones arranged in networks of enteric ganglia connected by interganglionic strands. Most enteric neurons which are involved in motor functions are located in the myenteric plexus while some primary afferent neurones are located in the submucous plexus. The ENS comprises primary afferent neurons, which are sensitive to chemical and mechanical stimuli, interneurones and motorneurones that act on the different effector cells including smooth muscle, pacemaker cells, mucosal glands, blood vessels, and epithelia. This complex system of intestinal neurons also play an essential role in regulation of immune responses and endocrine and paracrine functions.

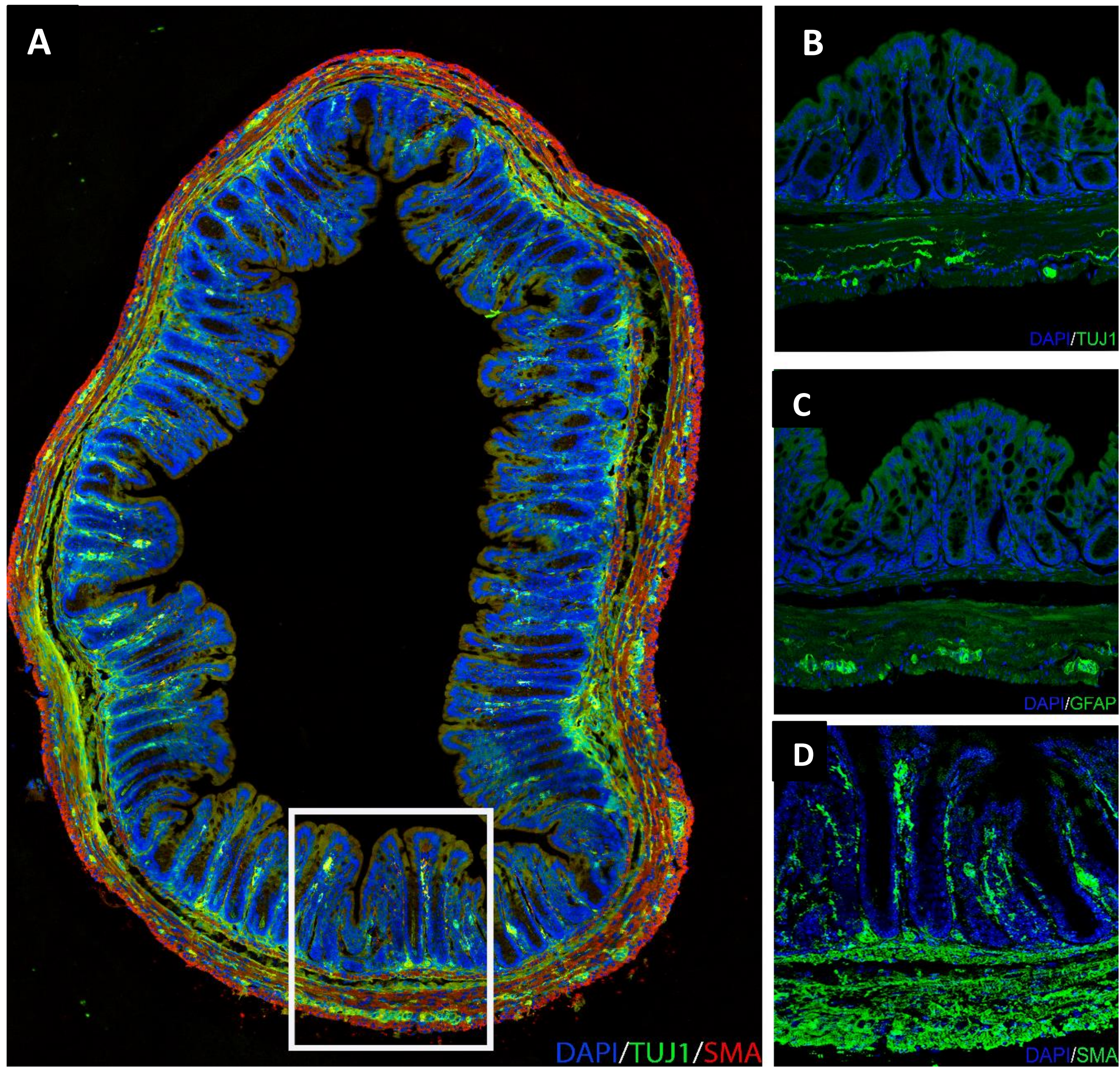

Figure 1: Histology of the mammalian ENS. (A) Double immunostaining of 8 weeks old mouse colon cross section, with the neuron-specific class III beta-tubulin (Tuj1; green), the smooth muscle actin marker (SMA; red), and DAPI (blue). Inset in (A) is magnified in (B-D). (B) Immunolabeling with Tuj1 antibody shows the neuronal network of the ENS. (C) GFAP stains the enteric glia in the myenteric plexus. (D) SMA immunofluorescence staining shows the muscle layer in the mouse colon.

1.2 Development of the enteric nervous system

During embryonic development a transient group of neuroectodermal cells in vertebrates, the neural crest cells (NCCs) colonise the gut. NCCs are multipotent stem cells that give rise to many different cell types including melanocytes, head connective tissue, bone, smooth muscle, neurons and glia (Martik & Bronner, 2021; Tang & Bronner, 2021). The first demonstration that the ENS originates from neural crest populations was demonstrated by Yntema and Hammond, when ablated postotic and anterior spinal levels of chicken neural crest cells resulted in the absence of enteric ganglia (Yntema & Hammond, 1954). These NCCs have a highly migratory mesenchymal-like cell phenotype, and their first movements include a delamination from the closing cranial neural folds as an epithelial-mesenchymal transition (EMT) and from the closed trunk neural tube (Bronner & LeDouarin, 2012). Separate groups of NCCs originate from specific axial levels of the neural tube (cardiac, vagal, sacral, and trunk). These features allow migration throughout the entire embryo, and colonise multiple organ primordia to differentiate into a variety of cell types, including endocrine cells, melanocytes, connective tissue of the head, and the glia and neurons of the peripheral nervous system - which include the enteric nervous system (Bronner & LeDouarin, 2012; Le Douarin & Teillet, 1973; Yntema & Hammond, 1954). After delaminating from the neural tube, vagal NCCs migrate along two separate pathways: dorsolaterally and ventrally. The former takes place at the 10-somite stage (10ss; embryonic day 8.5(E8.5) in mice), when at the level of the somites 1-3 the cells start to migrate dorsolaterally under the ectoderm which results in colonising the pharyngeal arches and the cardiac outflow tract. The latter takes place at the 13ss, when a second cell population arises from the same somite level and starts to form the sympathetic and dorsal root ganglia while other cells enter the foregut and differentiate to the ENS (Kuo & Erickson, 2010). Here a question arises: How could the NCCs determine if their cell fate should result in cardiac or ENS cell type? The answer to this question is that the two NCC populations have a different type of segregation cue which can separate them. While the cardial NCCs destined for the heart express *CXCR4*, the vagal NCCs colonising the foregut are *CXCR4* negative. Expression of *CXCR4* receptor results in the migration of the cells towards its ligand *CXCC12,* which is expressed in the pharyngeal mesoderm and the conotruncal mesenchyme of the developing heart (Escot et al., 2013). *CXCR4* negative NCCs from the 4-7 somites migrate only ventrally, and join the migration stream into the foregut. The trunk NCCs which are present posterior to the vagal region do not enter the foregut. The most likely possibility for this migration pattern is that the foregut expresses *Slit2* which results in the repulsiveness of the *Robo*-expressing trunk NCCs, but not in the Robo-negative vagal NC-

derived cells (Zuhdi et al., 2015; De Bellard et al., 2003). The entry of NCCs into the foregut occurs at E9.5 in mice and at week 4 of gestation in humans (Fig. 2).

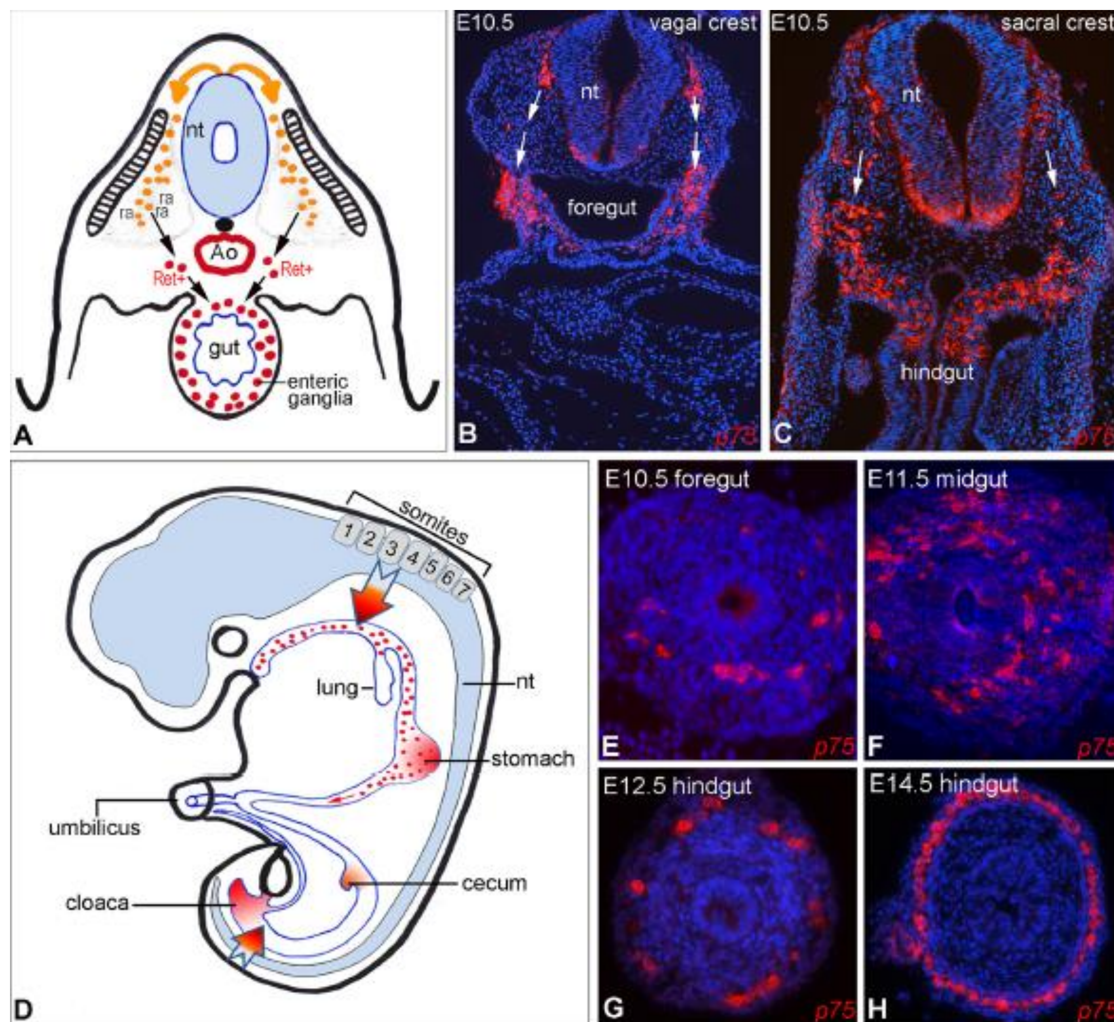

Figure 2: Development of enteric neural crest-derived cells (ENCCs) in the mouse embryo. (A) The neural crest cells (orange dots) originate from the neural tube. Developmental steps of neural crest cell formation: First, by epithelial-mesenchymal transition neural crest cells delaminate from the epithelial neural tube. Second, mesenchymal phenotype delaminated cells arrive throughout the retinoic acid (RA) rich sclerotomal compartment of the somites. Third, neural crest cells that express *RET* receptor colonize the foregut mesenchyme. (B) p75+ vagal neural crest cell migration from the neural tube into the foregut at E10.5. (C) p75+ sacral neural crest cells migrate into the hindgut mesenchyme at E10.5. (D) Vagal and sacral derived neural crest cells contribute to the formation of the ENS on a schematic drawing (Red arrows show the entry of ENCCs to the gut. Red dots represent the ENCCs in the foregut). (E-H) Mouse p75+ NCC colonisation in the gut from E10.5 to E14.5 (Nagy & Goldstein, 2017).

When the NCCs migrate towards the foregut, they first move through the anterior half of the somites, between the dermomyotome and sclerotome, then enter the gut mesenchyme - at which stage these cells are called enteric neural crest-derived cells (ENCCs) (Bronner-Frasser & Marianne, 1993). These cells express the *RET* receptor, travel, and colonise along the whole length of the gut tube in a cranio-caudal direction. In mice, the migratory ENCCs are randomly distributed in the outer mesenchyme of the midgut and the foregut, in which the smooth muscle layer has not been completely differentiated (Young & Newgreen, 2001; Burns & Le Douarin, 1998). In contrast to the midgut, the smooth muscle cells of the hindgut differentiate before the wavefront of the ENCCs reach the ceca and enter the proximal hindgut. As the circular smooth muscle cells differentiate, the ENCCs become restricted to the outermost layer of the gut wall - more precisely, they occur between the smooth muscle layer and serosa, where later the myenteric ganglia differentiate (Nagy & Goldstein, 2017). The submucosal plexus formation happens as the ENCCs from the myenteric plexus start to migrate radially towards the epithelium (Uesaka et al., 2013). In the case of mammalian development, the myenteric plexus develops before the submucosal plexus. Interestingly, both the myenteric and submucosal plexuses develop early during the avian and human embryonic development, whereas in mice and rats the colorectal submucosal plexus develops only postnatally (Wallace & Burns, 2005; McKeown et al., 2001).

Vagal-derived ENCCs are not the sole source of NCCs for the ENS, there is a second NC population as well, called sacral-derived ENCCs. Neural tube ablation has revealed that NC caudal to somite 28 contributes to the ENS in the colorectum (Nagy et al., 2012; Nicole et al., 1973; Yntema & Hammond, 1955). The avian sacral-derived NCs migrate through the tail bud mesenchyme and colonise both sides of the cloaca and the distal hindgut (Nagy et al., 2007). In mice, sacral NCC migration starts at E9.5 and arrives in the cloaca at E11.5 (Wang et al., 2011). First arriving ENCCs differentiate into the pelvic plexus, from where fibres extend from the pelvic ganglia. Sacral ENCCs colonise the distal hindgut, which ultimately results in the contribution of creating enteric neurons and glial cells (Wang et al., 2011; Nagy et al., 2007). The current literature only observed this phenomenon in mouse and chick embryos, but not in human embryos.

Vagal and sacral-derived ENCC's intrinsic properties are very different - the former being more invasive. Experiments including comparative microarray analysis in avian model systems have revealed higher expression of *RET* transcripts in vagal NC-derived ENCCs which could be an explanation of their migratory invasiveness. In relation to this hypothesis the

overexpression of *RET* signalling in sacral NCC was observed. This stimulation resulted in an increased colonisation potential which explains the fundamental differences in the contribution to the ENS between the sacral and vagal-derived ENCCs (Delalande et al., 2008).

1.3 Molecular regulation of the enteric nervous system development

Many aspects contribute to the normal development of the ENS, one of particular importance is the available ENCC progenitor cell pool. According to the experimental studies performed in avian embryos, reducing the size of the vagal neural crest results in different length of aganglionosis in the distal gut (Barlow et al., 2008; Burns et al., 2000). Another study revealed that inhibiting the normal apoptotic cell death of the vagal NCCs resulted in hyperganglionosis in the foregut (Wallace et al., 2009). To achieve normal gut colonisation, a certain density of the ENCCs must be present (Barlow et al., 2008). Current literature has also revealed, that the "chain migration" (a process in which cell migration is enhanced when ENCCs are in contact with each other) is characteristic for the migratory behaviour of the ENCCs (Druckenbrod & Epstein, 2007; Druckenbrod & Epstein, 2005). Another study has revealed that the isolated ENCCs did not migrate as directly and quickly, as chains of cells, which leads to the assumption that the cell-cell contact during ENS formation is essential (Landman et al., 2007). L1 cell adhesion molecule (L1CAM) is a protein, which is responsible for maintaining such cell-cell contacts. Mutation of the *L1CAM* gene results in lesser ENCCs contacts and HSCR (Anderson et al., 2006; Mm et al., 2004). A proposed explanation for this is that the ENCCs migrate very slowly, so by the time they reach their destination, they are unable to colonise properly the distal gut (Druckenbrod & Epstein, 2009). Therefore, cell proliferation which regulates the migratory speed of ENCCs is crucial in the normal development of the ENS (Landman et al., 2007; Simpson et al., 2007).

Our current knowledge about the role of the extracellular matrix (ECM) during the ENS formation is not complete. Early studies have revealed that abnormal expression of the ECM molecules resulted in HSCR phenotype in mouse models (Parikh et al., 1994). The ECM in the intestine is composed of glycoproteins, such as laminin, fibronectin, tenascins, and other molecules like collagens and proteoglycans which regulate ENCCs through their surface receptor expression. Therefore, the precise regulation of the ECM molecules in both time and space contributes to normal development of the ENS. In endothelin-3 *(EDN3)* null mice, an increased expression of laminin, collagen IV, perlecan and other types of proteoglycans were observed in the aganglionic segments of the gut. This finding can implement the fact that altered ECM composition might contribute to the formation of aganglionosis (Rothman et al., 1996;

Tennyson et al., 1990; Payette et al., 1988). Researchers discovered an increased laminin expression in aganglionic segments in human HSCR samples, consistent with the findings obtained from the mouse model (Parikh et al., 1992). Another study confirmed that ECM molecules form a dynamic and complex scaffold, which is responsible for providing specific signals and a physical surface for the ENCC migration which therefore regulate many aspects of the normal ENS development (Chevalier et al., 2016; Payette et al., 1988).

Recent *in vitro* studies revealed that the molecule laminin, fibronectin, vitronectin and collagen type I all supported ENCC migration (Akbareian et al., 2013; Breau et al., 2009; Nagy et al., 2009; Nagy & Goldstein, 2006). However, molecules such as collagen type VI inhibit ENCC migration (Soret et al., 2015). Moreover, chondroitin-sulphate proteoglycans (CSPGs), versican and collagen type IX, could have an inhibitory effect on the migrational success of ENCCs. Pro-migratory ECM molecules are mostly distributed along the whole length of the developing gut, even in the inner mesenchyme in which the ENCCs are absent. Taking into consideration this distribution, we could hypothesise that the ECM molecules that are non-permissive or inhibitory to the migration of ENCCs must control the formation of the concentric enteric plexuses (Nagy & Goldstein, 2017).

1.4 Hirschsprung disease and therapeutic possibilities

Hirschsprung disease, or megacolon congenitum, is a developmental disorder which occurs when the ENS is absent in the myenteric and submucosal plexuses, generally around the region of the distal intestine. It is well characterised by the absence of ganglion cells (aganglionosis), which can potentially extend along the whole intestine. As previously discussed, the ENS originates from the neural crest-derived cells which migrate from the proximal region to the distal intestine. In Hirschsprung disease, this migration is completely absent, or in some cases the survival or differentiation of the ganglion cells are not satisfactory in the distal colorectum (Mueller & Goldstein, 2022; Langer, 2011). Our current knowledge about the origins of this disease is very limited. Mutations in the proto-oncogene *RET* and the genes of the endothelin-receptor family can cause HSCR. The mechanism by which they cause the abnormal development of the ENS is currently unknown. HSCR can be diagnosed based on neonatal distal intestinal obstruction, chronic constipation, or enterocolitis. Radiologically, there is massive dilatation of the bowel segment proximal to the ileus. In the neonatal stage it is present in the form of distended abdomen and feeding intolerance, bile-stained aspirate or frank emesis (Langer, 2011). In very rare and unfortunate cases the disease can lead to cecal perforation. The passage of faeces in most cases are delayed or even obstructed, which can be

explained by the abnormal and unregulated gut movement. This can appear radiologically, as there is massive dilatation of the bowel segment proximal to the ileus. Currently, the only known solution for HSCR treatment is bowel resection immediately after birth. The surgery is mostly performed by a procedure called "pull-through procedure", which includes the removal of the diseased part of the colon, and then pulling the normal section through the colon from the inside and attaching it to the anus (Langer, 2011). This method is mostly performed by laparoscopic methods which are less invasive. Even though the surgery's success rate is approximately 93.8%, the postoperative care is still essential and it can be a lifelong challenge. In more severe cases, a stoma needs to be applied to the abdomen of the patient. After the surgery, there is a possibility for recurring constipation and severe enterocolitis.

As the regenerative medicine and stem cell biology field improves daily, there could be other solutions regarding the cure to this developmental disorder. One of these solutions could be stem cell therapies, namely, creating enteric neurospheres (NS). The enteric neurospheres are organoid-like 3D structures which consist mainly of enteric neuronal precursor, glial, and differentiated neuronal cells isolated from the postnatal intestinal wall (Cornelia & Hagl, 2003). The first neurospheres created originates from the central nervous system (CNS), and only later, the protocol was optimised for creating neurospheres from the ENS. The newer ENS protocols included the separation of the muscularis layer from the mucosal layer which resulted in the creation of longitudinal muscle/ myenteric plexus preparations (LMMPs). Our current knowledge about neurosphere transplantation and their integration into the gut wall is not yet complete. Recently, Cooper et al. generated neurospheres from postnatal (P2-P4) *Wnt1-cre;R26R-YFP/YFP* mice and transplanted them into *Ednrb*^{tm1Ywa} mice lacking endothelin receptor type B (a model for Hirschsprung Disease) and into wild type mouse (Cooper et al., 2016). They found that the selected YFP+ mouse ENCCs created well-defined enteric neurospheres of approximately 20 μm in diameter within 2 weeks *in vitro*, which increased their size to approximately 120 μm within a month. These neurospheres expressed neuron specific markers such as Tuj1 and the glial marker, GFAP. Undifferentiated precursor cells were positive for SOX10 and negative for GFAP. When transplanted into the wild type mouse, from the neurospheres neuron specific Tuj1 immunoreactive projections formed. These transplanted neurospheres have shown several types of ENS cell marker positivity including vasoactive intestinal peptide (VIP), choline acetyltransferase (ChAT), calcium binding protein (S100), GFAP and neuronal nitric oxide synthase (nNOS). According to this paper, neurospheres can be created from postnatal gut, and successfully integrate into host gut - increasing possibility

for the application of autologous ENCCs transplantation into euganglionic end aganglionic gut segments (Cooper et al., 2016).

The importance of the ECM regarding the migration of the neurospheres is essential. This hypothesis was first suggested by (Rauch & Schäfer, 2003) when they observed how ECM molecules such as fibronectin and laminin influence the migration of the NCCs and the development of the ENS. (Cheng et al., 2017) further supported their findings, when they observed the migrational aspects of the NSs in different gut environments. They found, that human-derived enteric neural stem cells, which were transplanted into the chick embryo and *Edrnb* $^{-/-}$ mice *ex vivo* and *in vivo* behaved as undifferentiated neural crest cells when they were placed along the normal migration pathways; the embryonic hindgut and neural crest were prominent to the postnatal-derived ENSCs; the postnatal aganglionic gut showed lesser migration capacity, but supported neuronal differentiation of the ENSCs; in the *ex vivo* aganglionic gut segment the ENSCs showed only limited intramural migration in the myenteric layer (Cheng et al., 2017). These findings raise the possibility that the ECM plays an essential role in the integration of the ENSCs and that the embryonic and postnatal, and even the HSCR ECM pattern is different. This hypothesis was further supported by a recent study by (Navoly & McCann, 2021) in which it was demonstrated that the collagen components within the ECM undergo a remodelling process as the donor cells migrate in the intestine. ENSC integration within the gut wall led to elevated expression of ECM related genes, such as Collagen 1a2 *(Col1a)* and Collagen 4a1 *(Col4a)*, whereas other genes like Fibronectin 1 *(Fn1)*, Elastin *(Eln)*, Laminin a1 *(Lama1)*, Laminin b1 *(Lamb1)* had the same expression level as in the *ex vivo* cultured tissues. Heparan-sulphate proteoglycans (HSPGs), also contribute in the ENS development and in the successful integration of NCs within the host gut environment (Nagy et al., 2018).

2. Aims

Enteric neurospheres established from postnatal enteric nervous system progenitor cells have been proven to be an appropriate source of cells for the treatment of Hirschsprung's disease. One limitation of the transplantation is the reduced cell emigration from the transplanted neurospheres. Despite the complete literature regarding the intestinal ECM, it is still unknown how its composition changes after cell transplantation. Therefore, identifying the changes in the ECM pattern could contribute to the successful cell implantation.

Specific aims of my diploma work were the following:

- Generation and characterization of mouse enteric neurospheres.
- Transplantation of enteric neurospheres onto LMMPs, and identification of specific ECM produced by the transplanted cells and host tissue environment.

3. Materials and methods

3.1. Animals

For the experiments postnatal day 3 (P3) and P28 old wild type mice (*Mus musculus*; C57Bl6/J) were used. Mice carrying the *Wnt1*$^{cre/+}$ knock-in allele (Pánczél et al., 2021) were obtained from Dr. Liam Ridge (UCL GOS Institute of Child Health, UK) and were maintained in heterozygous form. *ROSA26R-tdTomato (R26RtdT)* reporter (#007914) mice were obtained from Jackson Laboratories. *Wnt1-Cre* mice were crossed with *R26R-tdT* reporter mice to obtain *Wnt1-Cre;tdTomato (Wnt1;tdT)* mice in which all neural crest-derived cells are fluorescently labelled. The animals were kept at room temperature in the animal facility of the Institute of Anatomy, Histology and Embryology, Semmelweis University. Wild-type animals were the kind gift of Zoltán Jakus Dr. from Semmelweis University School of Medicine, Department of Physiology. Mouse experiments were approved by the Institutional Animal Care and Use Committees of Semmelweis University.

3.2. Histological analysis of the samples

3.2.1. Gelatine embedding for cryosectioning

Tissue embedding was performed according to a previously described protocol (Dora et al., 2018). Postnatal gut segments were isolated, LMMPs and enteric neurospheres were generated from wild type mice P28 and fixed with 4% paraformaldehyde (PFA; Reanal Labor; cat.no: #25550-0-13-38) for 2 hours. After washing with 1x PBS (3x5 minutes) the samples were incubated in 15% sucrose (Reanal puriss; cat.no: #07140-0-08-38) in 0,1 M phosphate-buffer (PBS, pH=7,2) at 4 °C overnight. The next day the sucrose was replaced with 7,5 % gelatine (Sigma; cat.no: #G2500) and tissues were further incubated at 37 °C for 2 hours. During incubation a gelatine base was created on a plastic mold surface. The samples were placed onto the gelatine base, and oriented correctly under a stereomicroscope and covered by a second gelatine layer. The proximal and distal ends of the gut were marked so later during cryosectioning the samples could be oriented correctly. Blocks were then cut out with a scissor, and placed onto a piece of cardboard with the help of Tissue-Tek cryomatrix (Sakura Europe; cat.no: #4583) and frozen in -50°C isopentane (2-methylbutane, Sigma, cat.no: #M32631) for 2 minutes. Until sectioning blocks were stored at -80 °C. Sectioning was performed with a Shandon cryotome at -28-30 °C. 12 μm thick sections were picked up on poly-L Lysine (Sigma; cat.no: #P8920) coated slides, and stored at -20 °C until immunostaining was performed.

3.3. Immunohistochemistry

Frozen sections were rehydrated in warm 1x PBS for 10 minutes. For detection of cytoplasmic and nuclear antigens rehydration was performed in 1% Triton-X PBS (Triton-X100: Sigma; cat.no: #9036-19-5). Sections were incubated with primary antibodies (Table 1) diluted in 1% PBS-BSA (Bovine Albumin, Sigma; cat.no: A9647) - (50-80 μl/section) for 60 minutes in a wet chamber at room temperature. This was followed by a washing step in 1x PBS (3x5 minutes). Alexa Fluor IgG (H+L) and IgM secondary antibodies were used in accordance with the primary antibody's isotype and host specificity (anti-mouse, anti-goat, anti-rabbit). The secondary antibodies were diluted 1:200 in 1x PBS. The incubation time for the secondary antibodies was 45 minutes at room temperature in dark. For double immunostaining, the previous procedure was repeated with a different primary antibody, and an isotype specific secondary antibody (IgG2a, IgG2b). Cell nuclei were stained with DAPI (4,6-diamino-2-phenylindole dihydrochloride; Invitrogen; cat.no: #D1306) Slides were washed in 1x PBS (3x5 minutes) then coverslips were mounted using a water based mounting solution (Poly-Aqua Polyscience Inc., Warrington PA, USA; cat.no: #18606) and stored at 4°C.

Table 1: Primary antibodies used during immunostaining

Antibody	Source	Specificity	Binding structures	Dilution	Source and catalogue number
Tuj1	mouse	IgG2a	neurons (type III β-tubulin)	1:100	Santa Cruz (sc-80016)
SOX-10	mouse	IgG1	neural crest cells and glial precursors	1:100	Santa Cruz (sc-365692)
SMA (1A4)	mouse	IgG2a	alpha-smooth muscle actin	1:200	DAKO (MO851)
GFAP	rabbit	IgG1	mature enteric glia	1:200	DAKO (ZO334)
HuC/HuD	mouse	IgG2b	neuron specific ELAV-like protein 3	1:50	Invitrogen (A21271)

Agrin	goat	IgG (H+L)	extracellular matrix	1:50	R&D Systems (AF550)
CS-56	mouse	IgM	anti-chondroitin sulfate	1:100	Sigma (C8035)
Collagen IV	rabbit	IgG (H+L)	native collagen epitopes	1:200	Santa Cruz (sc-29010)

Table 2: Secondary antibodies used during immunostaining

Secondary antibody	Emitting wavelength of the fluorochrome	Organism of origin	Supplier
anti-rabbit IgG (H+L)	488 nm	Donkey	Invitrogen (A21206)
anti-goat IgG (H+L)	488 nm	Donkey	Invitrogen (A11055)
anti-mouse IgG (H+L)	488 nm	Donkey	Invitrogen (A21202)
anti-mouse IgM	488 nm	Goat	Invitrogen (A21042)
anti-rabbit IgG (H+L)	594 nm	Goat	Invitrogen (A11012)
anti-mouse IgG (H+L)	594 nm	Donkey	Invitrogen (A21203)

3.4. Used solutions

1 litre of 10x PBS: 1,42 g $Na_2HPO_4 \cdot 2\ H_2O$,8 g NaCl, 0,2 g KH_2PO_4, 0,2 g KCl, distilled water

1x PBS: 1/10 of 10x PBS mixed with 9 parts of distilled water and stored at RT

15 m/V% sucrose: 15 g D(+) sucrose (Reanal puriss; cat.no: #07140-0-08-38) dissolved in 100 ml 1x PBS

7,5 m/V% Gelatine: 75 g D(+) sucrose (Reanal puriss; cat.no: #07140-0-08-38) and 37.5 g gelatine (Sigma, G2500) dissolved in 500 ml warm 1x PBS on a magnetic mixer.

4% Buffered Paraformaldehyde (PFA, pH= 7,4): 20 g of paraformaldehyde (Reanal Labor, cat.no: #25550-0-13-38) dissolved in 400 ml 80ºC distilled water on a magnetic mixer. 100 µl 1N NaOH was added to help dissolve any remaining PFA precipitate. The solution was filtered through a filter paper and 50 ml of 10x PBS was added, then completed with distilled water to the final volume of 500 ml.

1% PBS-BSA :1 g of BSA (Bovine Serum Albumin; Sigma; cat.no: #A9647) dissolved in 100 ml PBS. After filtration 0,1 % Na-azid was added (NaN_3).

Ethanol (75%): absolute 100% ethanol mixed with H_2O (3:1)

Penicillin/Streptomycin PBS: 5 ml Penicillin-Streptomycin (10,000 U/mL) (Gibco; cat.no: #15140122) + 495 ml 1x PBS

Modified NeuroCult Basal Medium: 490 ml of Basal medium (StemCell Technologies; cat.no: #0570) + 5 ml Penicillin/Streptomycin PBS or 5 ml Antibiotic-Antimycotic Solution (100X) (Gibco; Cat.no: #15240096)

Dissociation enzyme mix: 2 ml of dispase II (250 $\mu g/ml^{-1}$; StemCell Technologies, Vancouver, Canada; cat.no: #07913) + 2.5 mg of collagenase XI. (1 mg/ml^{-1}; Sigma Aldrich, St. Louis, MO; cat.no: #c7657) + 4 ml of Modified Neurocult Basal Medium.

Proliferation Medium: 1 ml of Neurocult Proliferation Supplement (StemCell Technologies; cat.no: #05701) + 9 ml of Modified NeuroCult Basal Medium.

Completed DMEM: 500 ml of DMEM (Sigma; cat.no: # D6429) + 50 ml Fetal Calf Serum (FCS; Sigma; cat.no: #F9665) + 5 ml Penicillin/Streptomycin (Gibco; cat.no: #15140122) + 5 ml MEM Non-Essential Amino Acid Solution (Sigma; cat.no: #M7145) + 5 ml MEM Vitamin Solution (Sigma; cat.no: #M6895)

3.5 Neurosphere techniques

3.5.1. Low-adherence plate

2 ml/well of Anti-Adherence Rinsing Solution (Stem Cell Technologies; cat.no: #07010) was added to a 6 well plate (Thermo Scientific; cat.no: #140675) and placed on a shaker (PSU-10i, Biosan) at 80 rpm at room temperature. After 1 hour of incubation the Anti-Adherence Rinsing Solution was replaced with Modified NeuroCult Basal Medium, and placed back on the shaker for 1 hour at room temperature. Before plating of the cells, the Modified Basal Medium was removed.

3.5.2. LMMP preparation

LMMPs (mouse longitudinal muscle-myenteric plexus) were isolated from P28 wild type mice colon. The isolated distal colon was transferred into Penicillin/Streptomycin PBS + 15% sucrose on ice and cleaned thoroughly with the help of sterile forceps and sterile blunt-ended syringe. The cleaned distal colon was pulled onto a glass rod, then a small incision was made along the mesenteric border with the forceps. After the incision the LMMPs were removed by vertical movement along the whole gut with the help of sterile q-tips or micro forceps, and placed into 10X Antibiotic-Antimycotic solution for 1 minute for the prevention of contamination. After 1 minute, the LMMPs were collected into a 50 ml centrifuge tube containing 2 ml of Modified NeuroCult Basal Medium.

3.5.3. Neurosphere culturing

LMMPs were dissociated in 15 ml of dissociation enzyme mix at 37 °C for 45 minutes in case of the small intestine and 60 minutes in case of the colon. During digestion gentle pipetting (trituration) and the usage of vortex is advised. The cell suspension was passed through a 40 µm cell strainer (Corning cell strainer; cat.no: #431750) into a 50 ml centrifuge tube (Eppendorf; cat.no: #0030122178) and then completed with Modified NeuroCult Basal Medium (50 ml). This was followed by 15 minutes of centrifugation on 500 g (1500 rpm). The pellet was resuspended, and pipetted into a 15 ml Falcon tube (Eppendorf; cat.no; #0030122151), which was completed with 15 ml Modified NeuroCult Basal Medium. Another 15 minutes of centrifugation and pellet resuspension was performed. During centrifugation, 1 ml of NeuroCult Proliferation Supplement (StemCell Technologies; cat.no: #05701) was added to 9 ml NeuroCult Basal Medium (this resulted in the creation of the "Proliferation Medium") and then further completed with cytokines: 20 µl epidermal growth factor (EGF; 20 ng/ml;

StemCell Technologies; cat.no: #78016) + 10 µl basic fibroblast growth factor (bFGF; 10 ng/ml; StemCell Technologies; cat.no: #78003) + 10 µl heparin (StemCell Technologies; 0.2%; cat.no: #07980). After resuspension the excess Basal Medium was removed, and replaced with NeuroCult Proliferation Medium with cytokines up to final 6 ml. 2 ml of cell suspension was pipetted into each well of the anti-adherence 6 well-plate and incubated at 37 °C in a humidified atmosphere of 5% CO_2.

3.5.4. Neurosphere dissociation and passage

Neurospheres cultured 4-7 days *in vitro* (DIV) were observed under phase-contrast microscope. Viable neurospheres are semi-transparent and phase-contrast bright. Ideally, these cultures should contain neurospheres measuring approximately 100 µm in diameter. To prevent cell loss a 1 ml pipette tip was washed with NeuroCult Proliferation medium. The culture medium containing the neurospheres was removed and transferred into a 15 ml sterile centrifuge tube. Cells were centrifuged at 100 g for 5 minutes. After centrifugation cells were resuspended in 5 ml Dulbecco's Phosphate-Buffered Saline (DPBS no calcium, no magnesium; Thermo Fisher Scientific; cat.no: #14190144) and centrifugation was repeated under the same conditions. After the second centrifugation DPBS was aspirated leaving the neurospheres at the bottom of the tube with approximately 100 µl of DPBS remaining. 1 ml of Accutase (Stem Cell Technologies; cat.no: #07920) was added and cells were incubated at room temperature for 10 minutes. During enzymatic digestion, the neurospheres were resuspended with a 1 ml pipette tip. Next, 4 ml of fresh NeuroCult Proliferation Medium without cytokines was added to the tube. Cells were centrifuged at 200 g for 5 minutes and the supernatant was gently aspirated. Finally, cells were resuspended in fresh Complete NeuroCult Proliferation Medium. The desired cell density is 100 000 cells/well in 6 well plate in 2 ml final volume. The resuspended spheres were then transferred to a new culture dish and incubated at 37 °C in a humidified atmosphere of 5% CO_2.

3.5.5. Neurosphere transplantation onto ECM protein coated petri-dishes

Wnt1;tdT-derived neurospheres (7 DIV) were transplanted onto culture plates coated with 20 µg/ml fibronectin (Sigma; cat.no: #F0556) and 2 µg/ml agrin recombinant protein (R&D Systems; cat.no: #6624-AG-050) and kept in culture for 48 hour in serum-free DMEM . After 48 hours cultures were fixed in 4% PFA and were further prepared for immunostaining.

3.5.6. Neurosphere transplantation onto LMMP

LMMPs were isolated as previously described and pinned down using micro pins onto a sterile silicone bottomed Petri dish filled up with completed Dulbecco's Modified Eagle Medium (DMEM; -high glucose, Sigma Aldrich; cat.no: #D5671-1L). *Wnt1;tdT*-derived neurospheres aged 7 days *in vitro* were transplanted onto the surface of wild type LMMPs using micro forceps under sterile conditions and incubated at 37 °C in a humidified atmosphere of 5% CO_2. At 14 DIV the samples were fixed in 4% PFA and embedded according to the previously described embedding protocol.

3.6. Evaluation of the samples

Confocal images were obtained by the Zeiss LSM 780 type confocal microscope with Zeiss ZEN microscope software. The evaluation of the images was performed with ImageJ and Adobe Photoshop CS 7.01 softwares. Neurosphere cultures were photographed during DIV 0 and DIV 21 under Zeiss Axiovert 135 microscope.

4.Results

4.1. Generation of enteric neurospheres.

To generate enteric neurospheres small and large intestine were collected. Postnatal day 28 (P28) *Wnt1;tdT* mice were euthanized by pouring water onto dry ice within a closed box. Sterile surgical scissors and forceps were used for dissection. Before enzymatic digestion, tissue staining was used to assess the distribution of *Wnt1* transgene within the gut wall. Similar to previous reports (Becker et al., 2022), *Wnt1* expressing cells were located in the mucosal, submucosal and myenteric plexuses of the ENS (Fig. 3).

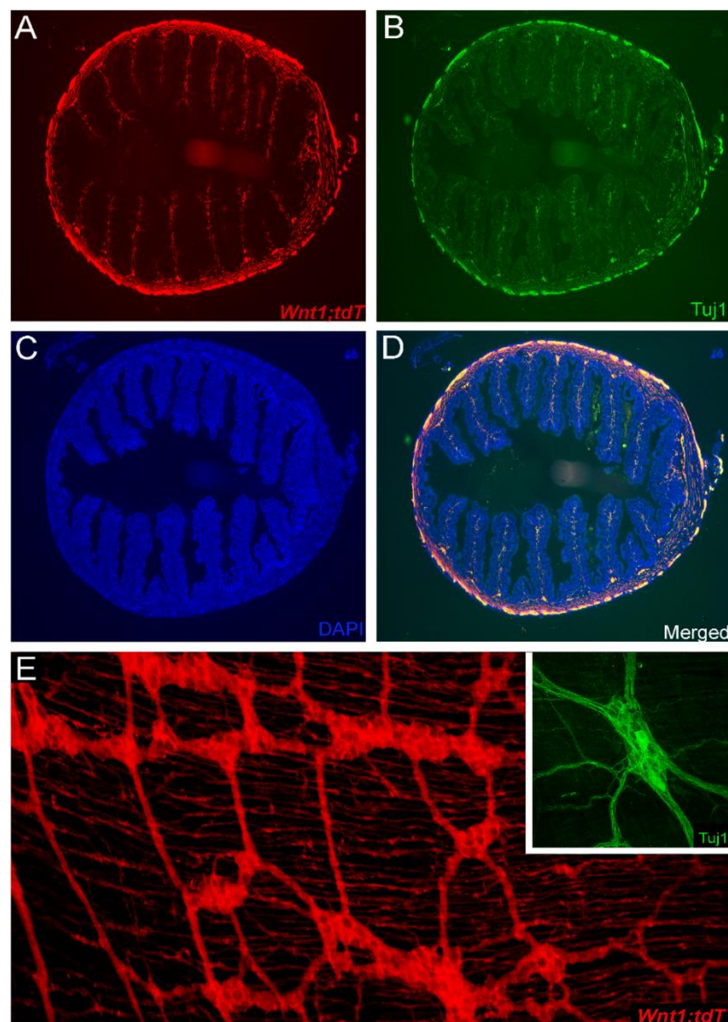

Figure 3: Histology of the adult *Wnt1;tdT* transgenic mouse colon. Cryosections from P28 mice colon. (A) *Wnt1* transgene expression shows the circular and longitudinal muscle layer within the mouse colon. (B) Immunostaining with Tuj1 monoclonal antibody marks enteric neurons in the mouse colon. (C) Cell-nuclei (DAPI) staining in the colon. (D) Merged images of *Wnt1;tdT* (red), Tuj1 (green) and DAPI (blue). (E) Whole-mount preparation of *Wnt1;tdT* mice showing all neural crest-derived (red) neuronal network within the muscle layer (inset shows Tuj1+ neuron).

From the proximal end of the oesophagus to the internal anal sphincter the ENS consists of a range of nerve cell types gathered in numerous ganglia that are interconnected by nerve fiber bundles. Whole mount immunofluorescence staining of *Wnt1;tdT* mouse colon shows specific agrin expression around *Wnt1* expressing ENS (Fig. 4).

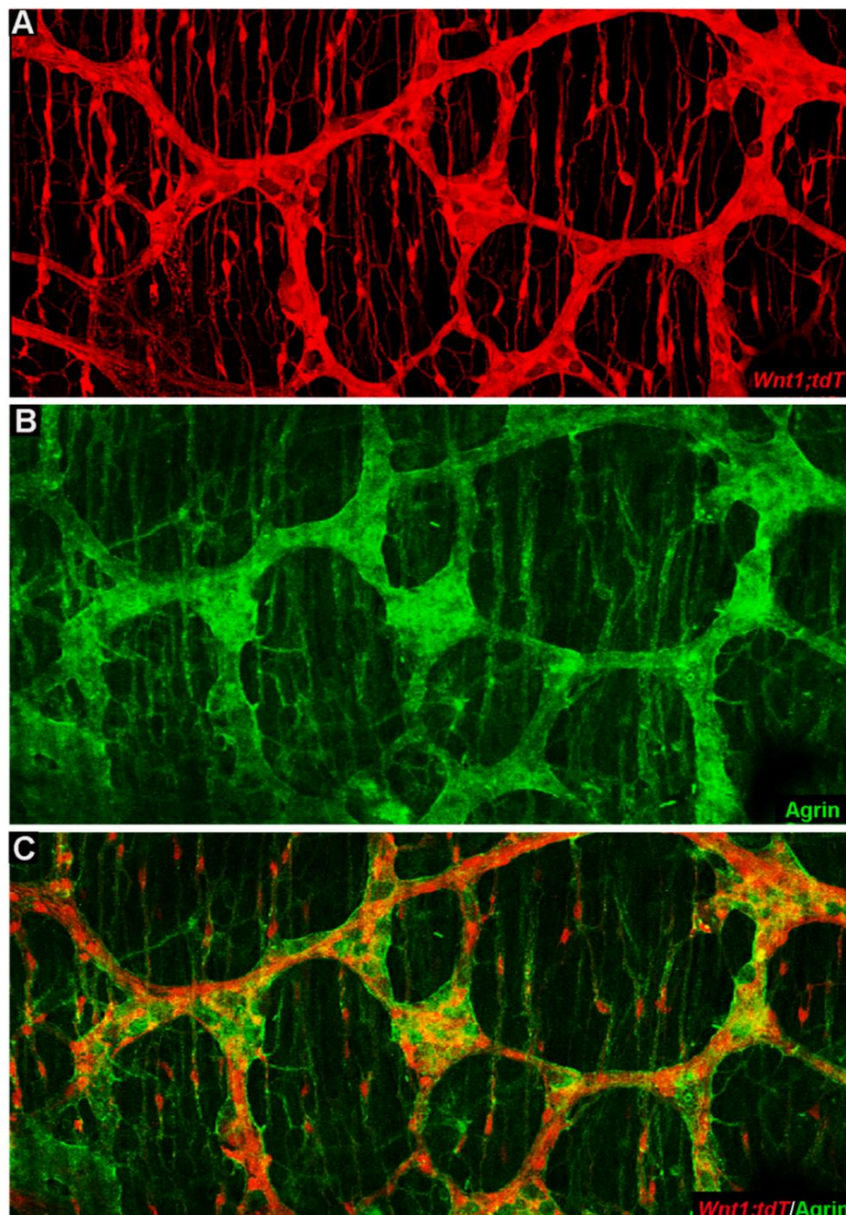

Figure 4: Immunohistochemistry of *Wnt1;tdT* transgenic mice LMMP. Representative confocal images of single and merged channels magnified from Fig. 3E. (A) Neural crest-derived cells are represented with red color within the *Wnt1;tdT* muscle layer. (B) Immunostaining with the extracellular matrix specific antibody agrin (green) on the wholemount section. (C) Merged color channels (Agrin; green, *Wnt1;tdT* ; red)- indicating, that agrin is specifically present within the muscle layer surrounding the neural crest-derived cells.

To isolate and culture the ENSDCs the gut segments were dissociated in mixture of dispase II and collagenase XI. The cell suspension was cultured at a density of 50,000 cells/ml in proliferation medium, consisting of NeuroCult NSC Basal Medium supplemented with epidermal growth factor and basic fibroblast growth factor for 7 days to promote formation of free-floating neurosphere-like bodies (Fig. 5A). Neurospheres were dissociated every 7 days and allowed to form new free-floating neurospheres in low attachment tissue culture dishes (Fig. 5 B,C).

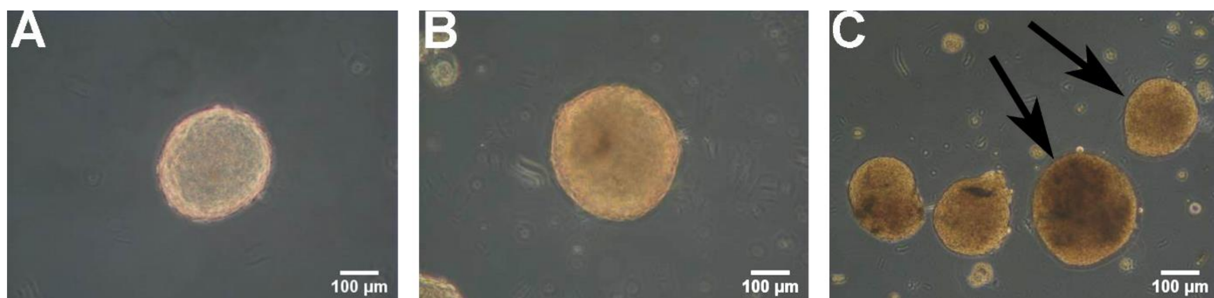

Figure 5: Mouse enteric neurospheres. Phase contrast images of mouse enteric neurospheres. (A) After 7 days of culture enteric neurosphere grow approximately 120 µm diameter in size. (B) 10 days old enteric mouse neurosphere. (C) 14 days old *in vitro* enteric neurospheres before the passage. Arrows point towards a neurospheres with different sizes and morphology. The measurements were calculated with Image J software.

During the three-weeks of *in vitro* culture, the neurospheres were regularly checked under a phase contrast microscope every other day to examine the spheroid-like structure formation. At the end of the culture period, the neurospheres were collected into a 50 ml sterile falcon tube and centrifuged at 500g for 5 minutes. The supernatant was removed and 4% PFA was poured into the falcon tube. After 20 minutes another centrifugation was performed at 500g for 5 minutes. After centrifugation the supernatant was removed and neurospheres were stained with Nile blue vital dye (0.1%) to visualize during the gelatine embedding and cryosectioning procedures (Fig. 6).

4% PFA fixation and the gelatine embedding were performed on 7 days and 21 days. Serial cryosections were immunostained with SOX10, Hu, Tuj1, GFAP, and SMA specific antibodies (Fig. 6).

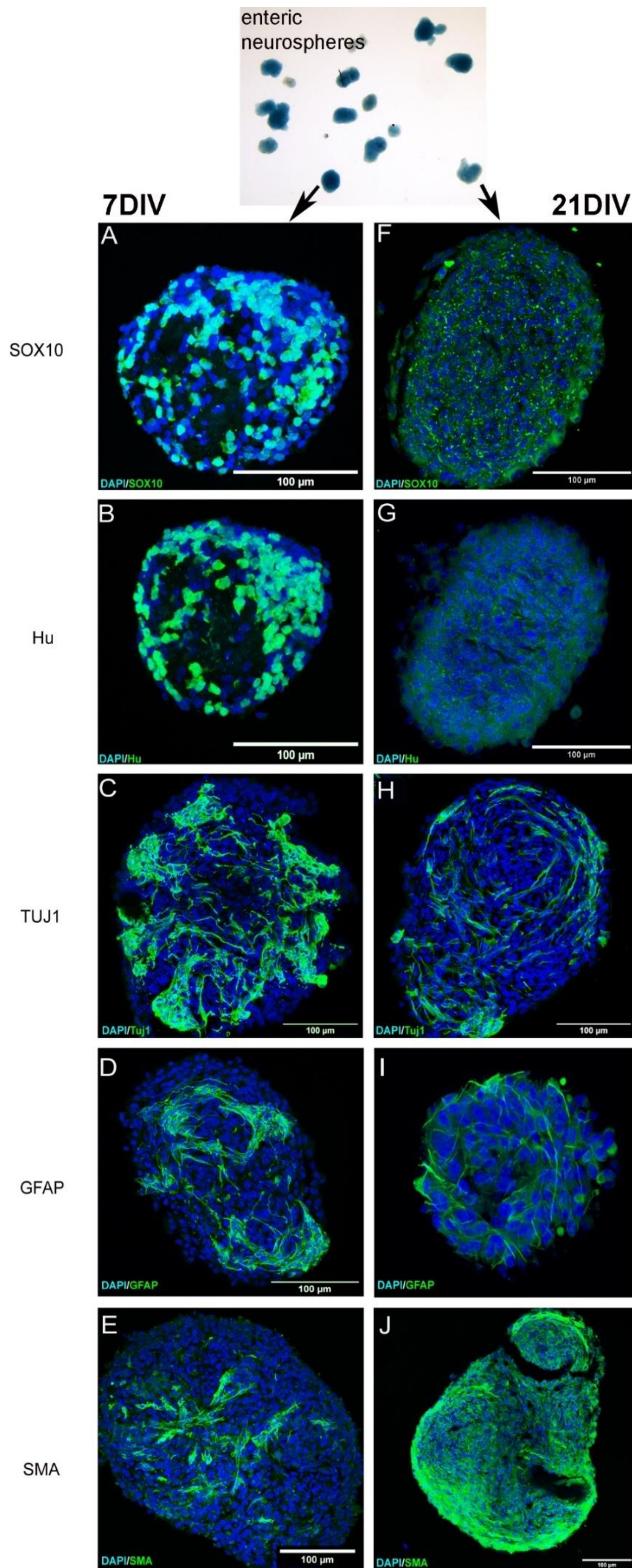

Figure 6: Characterization of mouse enteric neurospheres. (A) Bright-field view of representative 7- and 21-days old mouse enteric neurospheres stained with 0.1% Nile blue vital dye and further prepared for cryosectioning (arrowheads point towards magnified confocal images of immunostained enteric neurospheres). (A-E) Immunofluorescene on 7 days old enteric neurospheres (7DIV) confirmed that SOX10 (A), Hu (B), Tuj1 (C), GFAP (D), and SMA (E) are expressed in 7 days old neurospheres. (F-J) 21 days old enteric neurospheres (21DIV) show decreased expression of SOX10 (F), Hu (G), Tuj1 (H), GFAP (I), and increased SMA immunoreactivity (J).

Consistent with previous literature, all these markers were expressed in 7 days old enteric neurospheres (Schmitteckert et al., 2019; Binder et al., 2015; Almond et al., 2007). Majority of the cells were positive for SOX10, which suggests that these cells have stem cell-like features. Neuron specific Hu positivity supports the presence of neural crest-derived neuron. The smooth muscle actin specific antibody showed relatively low expression indicating the smooth muscle presence in the enteric neurospheres whereas the 21-day *in vitro* neurosphere contains more SMA+ cells. In contrast, the 21-day old enteric neurospheres showed decrease for SOX10 and Hu specific antibodies. There was also a reduction in the neuronal (Tuj1) and the glial specific antigen expression (GFAP). These results indicate that after the passages the old neurospheres reduce their neuronal and glial cell content. Furthermore, these observations confirm that enteric neurospheres derived from the mouse intestine can be propagated in culture up to 21 days and early enteric neurospheres are composed of neural crest-derived cells that are capable of differentiating into enteric neurons and/or enteric glia.

Consecutive sections of 7-day- old mouse enteric neurospheres were also stained with extracellular matrix specific antibodies including Agrin, CS-56, and Collagen IV (Fig. 7). The immunofluorescence showed intense agrin immunoreactivity. In contrast CS-56 and collagen IV are not expressed in the neurospheres.

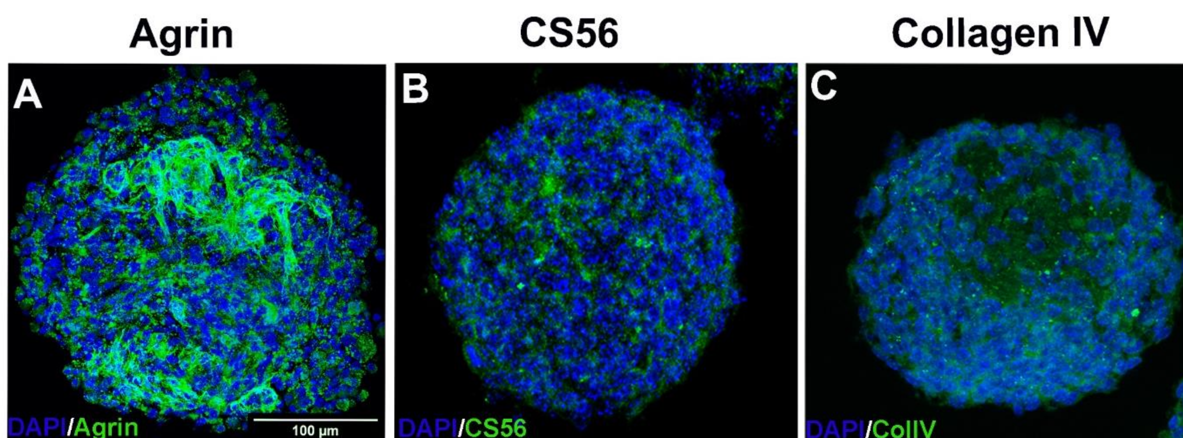

Figure 7: The extracellular matrix pattern of 7 days old mouse enteric neurospheres. Neurospheres immunostained with agrin (A), CS-56 (B) and collagen IV (C). Scale bar 100µm

A recent paper from our lab demonstrated that chicken neural crest cells migrate well on fibronectin-coated plates, and they are unable to migrate in the presence of agrin (Nagy et al., 2018). *In vitro* techniques were used to determine the effect of agrin on neurosphere cell migration. We confirmed the inhibitory role of agrin (2 µg/ml) by culturing neurospheres derived from *Wnt1;tdT* mouse gut explanted to agrin-coated surface. No enteric nervous

system-derived cell (ENSDC) migration was observed from these postnatal neurospheres (Fig. 8D-F), whereas robust migration occurred on the fibronectin-coated (20 μg/ml) surface (Fig. 8A-C), similar to the avian neural crest cells.

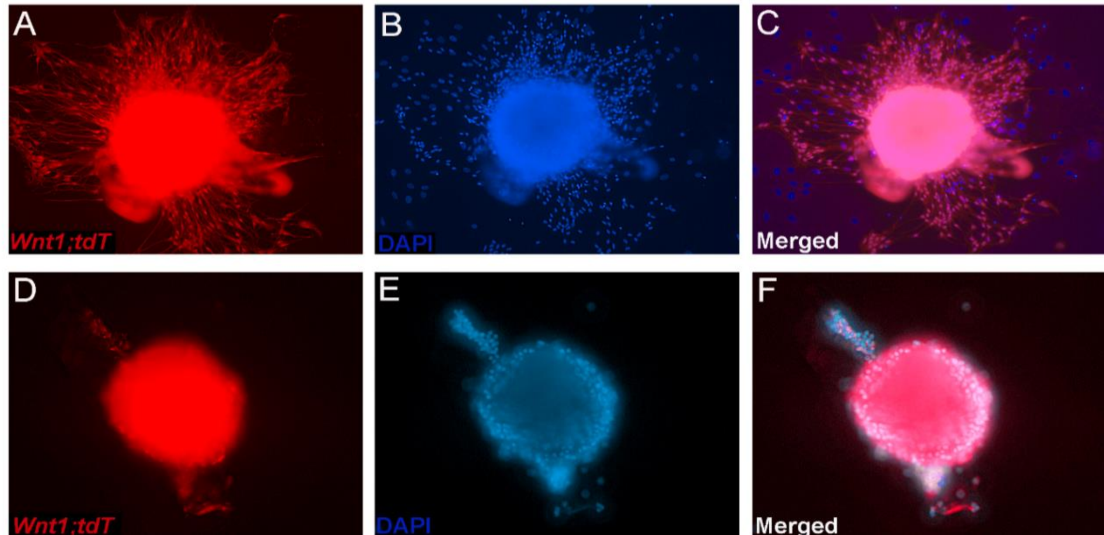

Figure 8: Agrin is inhibitory, whereas fibronectin is permissive for ENSDC migration. (A-C) Neurosphere derived ENSDC migrate well on the fibronectin coated plastic surface. (D-F) By contrast, the inhibitory role of agrin was confirmed by culturing *Wnt1;tdT* derived neurospheres on agrin-coated Petri-dish, which inhibits ENSDC migration. Nuclei were stained with DAPI (blue).

4.2. Transplantation of enteric neurospheres onto LMMPs, and identification of specific ECM produced by the transplanted cells and host tissue environment.

The longitudinal muscle/ myenteric plexus (LMMP) was isolated from both the small intestine and colon as described previously (Levin et al., 2020; Hotta et al., 2016). P28 old wild-type mice were euthanized the same way as previously described. After dissection the gut segments were washed thoroughly with Pen/Strep PBS + 15m/V% sucrose and then pulled onto a glass rod. Using fine forceps, the longitudinal muscle/ myenteric plexus was isolated and pinned down to a silicone coated Petri-dish (Fig. 9). After a 7-day *in vitro* culture, the tissue sample was processed for immunofluorescence staining. Immunostaining included the neuron specific antibody Tuj1 and the smooth-muscle actin specific antibody SMA. Both antibodies showed cell specific immunoreactivity in the LMMP indicating the neuronal and muscle survival in the absence of the gut mucosa. According to Levin et al., LMMP preparations maintain *ex vivo* peristalsis up to 2 weeks along with proliferation of smooth muscle, glia and neurons (Levin et al., 2020)

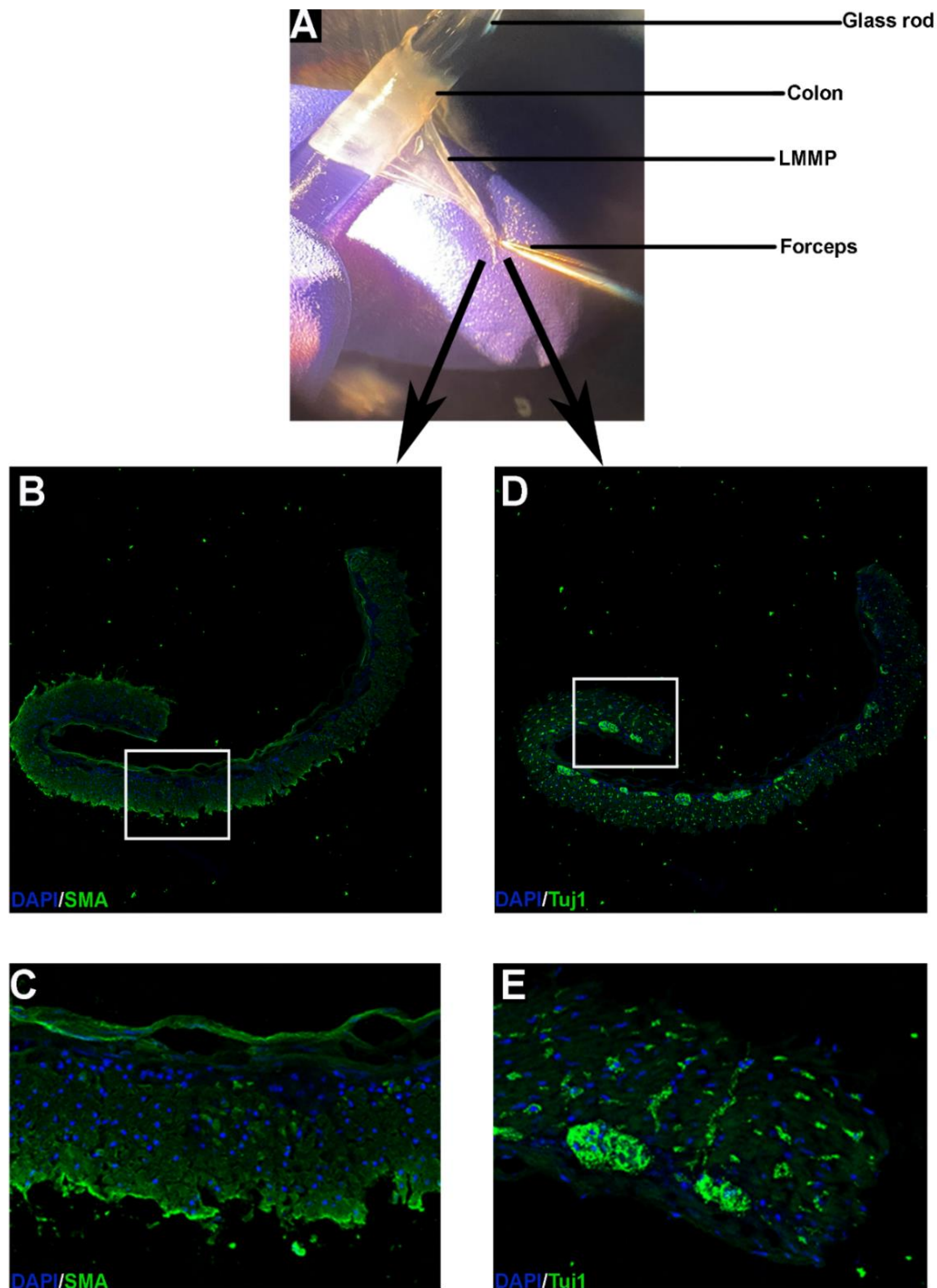

Figure 9: Isolation, culture and histology of the longitudinal muscle/ myenteric plexus preparation. (A) P28 old wild type mouse LMMP was isolated by pulling the approximately 3 cm long colon segment onto a sterile glass rod and removing the LMMP by peeling it off vertically. (B-E) Immunostaining of the LMMP preparations cultured for 7 days. Insets in (B) and (D) are magnified in (C) and (E). (B) Immunostaining with the smooth muscle actin specific antibody showing the muscle layer (green) and DAPI (blue). (D) Immunocytochemistry performed on the mouse LMMP with the neuron specific antibody Tuj1 (green) shows the remaining neuronal network in the LMMP and DAPI (blue). (C) Magnified inset from (B) representing the muscle layer (green) and DAPI (blue). (E) Magnified inset from (D) representing Tuj1+ neurons (green) and DAPI (blue).

Longitudinal muscle/myenteric plexus isolated from wild type mice was pinned down in a silicone coated Petri dish and filled with DMEM culture medium. 7 day old *Wnt1;tdT*-derived enteric neurospheres were transplanted into the LMMP using fine forceps under stereomicroscopic visualization and then further cultured for 7 days. After one week *in vitro* culture, the samples were removed and fixed in the Petri dish, embedded in gelatine, and further processed for cryosectioning and immunostaining to localize the *Wnt1;tdT* expressing cells and to characterize the ECM. Consecutive sections were immunostained with the ECM-specific antibody agrin and CS-56 (Fig. 10). The immunostaining confirmed the successful enteric neurosphere transplantation and integration into the muscle layer. The immunofluorescence staining pattern of agrin specific antibody suggests, that the enteric neurospheres are producing inhibitory extracellular matrix proteins to remodel their microenvironment (Nagy et al., 2018).

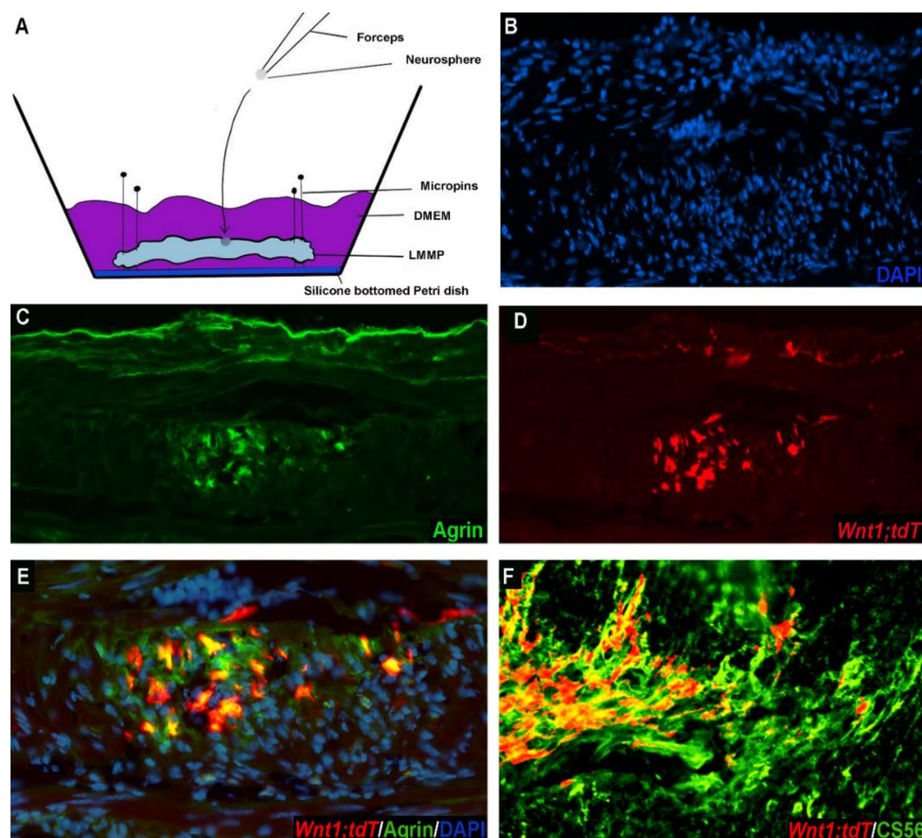

Figure 10: Enteric mouse neurosphere transplantation onto the muscle layer and the ECM pattern. (A) Neurospheres were microsurgically transplanted into the muscle wall of the mouse LMMP and 7 days later, *Wnt1:tdT* cell engraftment was observed in the gut wall. (B-F) Transplanted *Wnt1:tdT* cells are located between the muscle layers showing reduced migration of cells within the LMMP. (E) High-magnification confocal images confirm that *Wnt1:tdT* mouse-derived ENSDCs produce agrin. (F) Interestingly, accumulation of CSPG immunoreactivity can be observed around the transplanted neurosphere. Note that (E) is a magnified view of (C).

5. Discussion

The enteric nervous system is vulnerable to a wide range of congenital and acquired disorders that disrupts the function of its neurons or lead to their loss. The resulting neuropathies are some of the most challenging clinical conditions to manage. Transplantation of enteric neurospheres derived from neural stem cells isolated from postnatal gut offers the possibility of a novel cell-based cure given their potential ability to replace missing or dysfunctional neurons. From the perspective of harnessing such cells for therapy there has been a tremendous progress in the field, however, better characterization of potential target diseases is needed.

During my student research work one of the tasks was the generation and characterization of enteric neurospheres from postnatal mouse intestine. I have successfully generated enteric neurospheres from *Wnt1;tdT* transgenic mice small and large bowel, and confirmed their immunophenotype by immunohistochemistry. I found, that enteric neurospheres cultured for 7 days are much more suitable for transplantation and have a stem cell like immunophenotype in contrast to the 21 days old where a drastic decrease occurs in these features. My observations are in line with those studies, which show that early enteric neurospheres are composed of a heterogeneous cell population of stem and progenitor cells, neuronal, glial, and smooth muscle cells (Schmitteckert et al., 2019).

My other aim was to identify specific ECM proteins in the enteric neurospheres microenvironment and analyse if these ECM proteins have any effect on the enteric neurospheres migration capability. I have successfully confirmed, that the enteric neurospheres produce specific ECM molecules like agrin, and show no expression of other ECM proteins like CS-56 and Collagen IV. This finding is consistent with the previous literature, showing that the ENSDCs produce agrin type of heparan-sulphate proteoglycan ECM molecule (Nagy et al., 2018).

To study the function of agrin molecule, postnatal *Wnt1;tdT* enteric nervous system-derived cells were cultured on agrin and fibronectin coated Petri-dishes. The results obtained showed increased migration on the fibronectin coated surface in contrast to the inhibitory role of agrin. The literature is very detailed on fibronectin function being a well-known ECM protein which promotes ENSDC migration during ENS development (Yasui et al., 2022). The inhibitory role of agrin or ENSDCs is possibly mediated by the co-expression of its receptor the B-dystroglycan (Winder, 2001). A previous study demonstrated that after ENCCs complete the colonization of the embryonic GI tract start to express agrin and B-dystroglycan. Inhibition of agrin by using agrin-blocking antibody resulted in increased ENCDC migration (Nagy et al.,

2018). Our preliminary data shows that in contrast to highly migratory embryonic neurospheres, adult enteric neurospheres following transplantation strongly express agrin, which may contribute to the inhibitory microenvironment of the transplanted cells.

Next, I wanted to get a better insight regarding the migration capacity of the enteric neurospheres. Therefore, adult *Wnt1;tdT* derived enteric mouse neurospheres were transplanted into adult mouse LMMPs and cultured for 7 days *in vitro.* My detailed immunofluorescent staining studies show a limited ENSDC migration and elevated expression of specific ECM proteins at the transplantation site. Consistent with the literature, neurospheres cannot migrate and colonize the whole gut segment, they reside only at the transplantation site (Cheng et al., 2017; Hotta et al., 2010). Other studies also showed that the migration capacity of enteric neurospheres is highly dependent on the ECM composition of the host tissue. For example, it was reported that Collagen I, Collagen IV, Laminin and heparan-sulphate proteoglycan are present in the myenteric ganglia and are all permissive to the migration of enteric neurospheres (Raghavan & Bitar, 2014; Raghavan et al., 2013; Peter et al., 1986). Previous reports have shown that ENSCs transplanted into aganglionic colon successfully survive *in vivo*, giving rise to differentiated glial cells and neurons with processes extending into the smooth muscle layers (Cheng et al., 2017; Cooper et al., 2016; Hotta et al., 2016; Anitha et al., 2008). However, when ENSDCs are delivered into the intestinal wall, we find that these donor ENSCs do not migrate well and do not integrate extensively with the host ENS.

There are many possible factors contributing to the inefficient integration of ENSDCs into the postnatal intestinal wall, including lack of adequate neurotrophic support or the non-permissive extracellular microenvironment which results in limited anatomic and functional recovery after cell transplantation. It is possible that the postnatal environment inhibits ENSDC precursor migration and integration, preventing a satisfactory integration between transplant and host, and thereby blocking the potentially beneficial effect of cell transplantation on functional recovery. Our recent data showed that in addition to the ENSDC derived agrin the chondroitin sulphate proteoglycans (CSPGs) are expressed in the gut wall and they are inhibitory to ENCC migration during ENS development (Nagy et al., 2016). Similarly, recently it was shown that expression of CSPGs, like versican, is tightly associated with the formation of barrier tissues that constrain neural crest cell migration (Szabó et al., 2016).

Interestingly, we find that CS-56 is upregulated in the ECM around transplanted ENSDCs (Fig. 10F). This expression pattern contrasts with that seen for collagen IV, which we find mainly in the basement membranes (data not shown). Based on these observations, we hypothesized that CS-56 deposition diminishes the beneficial effects of ENSDC transplantation

by inhibiting the migration and integration of the transplanted ENSDCs into the host gut. Therefore, the digestion of CSPGs might provide a more favourable environment for the kinetics of transplanted progenitor cells, resulting in improved regeneration of the ENS.

In conclusion, migration, proliferation, and differentiation of enteric neural crest cells (ENCCs) are dependent on reciprocal interactions between NCCs and the gut microenvironment, including the mesenchyme, extracellular matrix (ECM), blood vessels and the gut endoderm. While the ECM influences ENS development, ENCCs also affect ECM composition. Given that ENS development depends on the coordinated interactions between ENCCs and their local extracellular environment, successful development of ENSDC transplantation requires a precise combination and regulation of both a cell population with high stemness and a permissive ECM.

Ongoing studies involve modification of neurospheres with lentiviral transduction and *in vivo* cell transplantation to determine the long-term effect in the postnatal gut environment. One of our future aims is the transduction with agrin silencing by short-hairpin RNA (shRNA) silencing and adenoviral vector (AAV) transfection which in theory will inhibit agrin and promote ENSDC migration.

Summary

The enteric nervous system (ENS) in one of the most complex parts of the peripheral nervous system in vertebrates. The complexity of the ENS is due to the vast number of neurons and glial cells.

Hirschsprung disease is a well-known congenital malformation of the ENS (~1/5000 live births), characterized by the congenital absence of the myenteric and submucosal plexuses of the colon, leading to intestinal obstruction and abnormal colonic distension in newborns. The surgical treatment of HSCR is imperfect, with long-term postoperative gastrointestinal complications like constipation and enterocolitis, therefore novel therapies are needed.

Neural crest-derived progenitor cells of the enteric nervous system (ENSDCs) are a potential source of neurons and glia that could be used in future transplant therapies to help restore normal gut function in patients with HSCR. Transplantation of these stem cells offers an innovative approach for treating HSCR and other enteric neuropathies. We and others have developed techniques to isolate ENS progenitor cells from embryonic and postnatal mouse. These cells can be maintained and amplified in culture as floating neurospheres, which are aggregates of ENS derived stem cells and their differentiating neuronal and glial cell progeny.

In this diploma work, ENS derived ENSDC progenitors were transplanted in a mouse model of a gut wall to analyze the cellular integration of the neurosphere derived cells and compare the ECM expression of the donor cells and host environment. It was found that enteric neurospheres express agrin. Furthermore, I found that *in vitro* treatment with agrin inhibited ENSDC migration. After 1-week the grafted ENSDCs were visualized by *Wnt1;tdT* transgenic red fluorescence combined with agrin and CS-56 specific immunolabelling. After co-staining with agrin specific antibody, I found that *Wnt1* mouse derived ENSDCs strongly produce agrin, while chondroitin sulphate specific CS-56 antigen was also upregulated around transplanted neurospheres.

My study adds agrin and CS-56 to the list of the inhibitory ECM which ENSDCs interact with during cell transplantation experiments. My findings suggest that agrin production prevents cell emigration from the transplanted neurospheres. Similarly, CS-56 antigen is highly secreted from the gut wall after transplantation, which further strengthens the inhibitory environment. These observations should be leveraged to develop novel cell transplantation protocols where inhibitory ECM molecules are degraded from the stem cell environment.

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Statement

STATEMENT

Name: Simon Balázs
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ELTE Faculty of Science: Biology MSc

Specialization: Molecular Genetics, Cell- and Developmental Biology

Title of diploma work: Modelling Hirschsprung disease treatment with organ culture techniques

As the author of the diploma work I declare, with disciplinary responsibility that my thesis is my own intellectual product and the result of my own work. Furthermore I declare that I have consistently applied the standard rules of references and citations.

I acknowledge that the following cases are considered plagiarism:
– using a literal quotation without quotation mark and adding citation;
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– representing another person's published thoughts as my own thoughts.

Furthermore, I declare that the printed and electronical versions of the submitted diploma work are textually and contextually identical.

Budapest, 2022.05.07.

Signature of Student