The role of epithelial-mesenchymal interactions and extracellular matrix in the development of the enteric nervous system

Doctoral thesis

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1. INTRODUCTION

The enteric nervous system is a network of neurons and glial cells within the wall of the gastrointestinal tract that is critically important in regulating motility and other fundamental aspects of gut function. Due to its autonomous function and morphological characteristics (the number of nerve cells in the intestinal tract exceeds the number of spinal cord neurons) often referred to as „the second brain". Our work focusing on Hirschsprung disease, a congenital neurocristopathy characterized by the absence of enteric ganglia (aganglionosis) from variable lengths of distal bowel due to failure of enteric neural crest-derived cells to complete their migration.

Extensive research over the past few decades has provided important insights into the development and function of the enteric nervous system, but little is known about the molecular mechanism of the congenital abnormalities affecting the enteric nervous system. The proper development of the enteric nervous system requires the coordinated regulation of enteric neural crest-derived cell migration, proliferation and differentiation and these processes rely on reciprocal interaction between enteric neural crest-derived cells and their microenvironment, including extracellular matrix (ECM) and soluble factors. Many of the molecular factors involved in the pathomechanism of the development and disorders of the enteric nervous system have been identified: mesenchymal factors such as GDNF, Endothelin-3 and BMP-4 play a critical role in the congenital malformation of the enteric nervous system, but little is known about the function of epithelial derived factors. Netrin promotes the radial migration of enteric neural crest-derived cells from the myenteric to submucosal region and also prevent premature apoptosis of enteric neural crest-derived cells, but the role of Hedgehog (Hh) proteins is less clear. According to Fu and colleagues, when Sonic hedgehog (Shh) is added to intestinal explants, Shh inhibits the migration and differentiation of enteric neural crest-derived cells. However, addition of Shh to mouse enteric neurospheres increased enteric neural crest-derived cell proliferation which contradicts the proliferation observed in the enteric nervous system of Shh mutant mice. Addition of cyclopamine (an inhibitor of Shh pathway) to zebrafish embryos inhibited enteric neural crest-derived cell proliferation and led to intestinal aganglionosis. The contradictory results of *in vivo* and *in vitro* experiments suggest a complex role for Hh activity during enteric nervous system development.

In order to clarify the role of the gut epithelium during enteric nervous system patterning, we used the avian and mouse embryos as model systems and applied a variety of techniques, including tissue recombination, embryonic chimeras, intestinal organ cultures, retrovirus-mediated gene misexpression, and *in situ* hybridization. Expression studies of Shh receptors (Ptc1 and Ptc2) in various model systems have yielded conflicting results, which made it necessary to determine the expression pattern of Ptc1. Given the lack of Ptc1 expression by enteric neural crest-derived cells, and the significant changes in ECM expression induced by Shh, our results suggest that Shh does not directly affect enteric neural crest-derived cell development, but rather disrupts the extracellular microenvironment and indirectly leads to aganglionosis.

However, the difference between the expression of ECM proteins in normal and aganglionic hindgut in Hirschsprung-disease was previously described, their exact expression pattern and role in regulating enteric nervous system development is largely unknown. In the second part of our work we attempted to characterize the interactions between enteric neural crest-derived cells and their extracellular microenvironment. Our results suggest that enteric neural crest-derived cells govern their own migration by actively remodeling their microenvironment through secretion of ECM proteins.

2. OBJECTIVES

The Stem Cell and Experimental Embryology Laboratory at Semmelweis University in collaboration with the Department of Pediatric Surgery at Harvard University, Boston, aims to characterize the embryonic development of the enteric nervous system in the normal bowel and in the hindgut of Hirschsprung disease. The investigation of enteric neural crest cells, which form the enteric nervous system is important not just to understand the pathogenesis of neurointestinal diseases but for the future applicability of stem cell therapies. The extracellular microenvironment which determines the development of stem cells is important not only in the propagation of therapeutic cells but also plays an important role in creating a beneficial tissue environment after implantation.

The aims of our study were:

1. To characterize the enteric neural crest-derived cell migration and proliferation during enteric nervous system ontogenesis.

2. To clarify the role of the gut epithelium-derived Shh during enteric nervous system patterning.

3. To characterize the ECM expression pattern in the microenvironment of the enteric neural crest-derived cells during normal and pathological enteric nervous system development.

3. METHODS

3.1. Animals

Fertilized White Leghorn chicken (Gallus gallus domesticus, White Leghorn SPF, Phylaxia Sanophi, Hungary) and mouse embryo were used. Embryos were staged according to the number of embryonic days (E). Eggs were obtained from commercial breeders and incubated in our laboratory, at 38°C. Ednrb$^{-/-}$ (Ednrb$^{tm1Ywa/J}$; #003295), Tau$^{GFP/+}$ [Tau (Mapt) KO, #004779], Col18$^{-/-}$, Plp1^{GFP} (PLP-EGFP), Wnt1-Cre;tdTomato (Wnt1;tdT) transgenic mice were kindly provided by Prof. Allan Goldstein (Harvard Medical School). The design conditions of the animal experiments were approved by the Animal Ethical Committee of Semmelweis University, Budapest, Hungary.

3.2. Histological processing of tissue samples

The tissue samples were fixed in 4% formaldehyde (PFA) in phosphate buffered saline (PBS) for 1 hour, rinsed with PBS, and infiltrated with 15% sucrose-PBS overnight at 4°C. Before embedding, the medium was changed to 7,5% gelatin containing 15% sucrose at 37°C for 1-2 hours, and the tissues rapidly frozen at -50°C in isopentane (2-methylbutane) and stored -80°C until the section were made. The gelatin-embedded blocks were cut with Shandon cryotom at -24°C working temperature. For light and immunofluorescent microscopy, 12 μm sections were prepared and collected on poly-L-lysine coated slides.

3.3. Immunohistochemistry and immunofluorescence

Frozen sections were incubated with primary antibodies for 45 minutes, followed by biotinylated anti-mouse IgG, anti-mouse IgM, anti-guinea pig IgG and anti-rabbit IgG. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 10 minutes. After washing, the ABC complex (avidin-biotinylated peroxidase complex) was applied and the sections were incubated for 30 minutes. The binding sites of the primary antibodies were visualized by 4-chloro-1-naphthol.

For immunofluorescence, sections were incubated with primary antibody for 45 minutes and secondary antibody (Alexa Fluor 594-, 350- and 488 conjugated anti-mouse IgG, Alexa Fluor 594- and 488 conjugated anti-mouse IgG1, Alexa Fluor 488 conjugated

anti-mouse IgM, Alexa Fluor 594- and 488 conjugated anti-goat, Alexa Fluor 350- and 647 conjugated anti-guinea pig, Alexa Fluor 594- and 488 conjugated anti-rabbit from Invitrogen) for 1 hour. For cell proliferation, 10 μM 5-ethynyl-20-deoxyuridine (EdU) was added to the media 3 hours prior to fixation. EdU incorporation was detected using the Click-iT EdU Imaging Kit (Invitrogen, Carlsbad, CA). Cell nuclei were counterstained with DAPI (4',6-diamidine-2-phenylindole-dihydrochloride). Sections were covered with aqueous Poly/Mount and examined by using a Zeiss Axiophot microscope or with a Zeiss LSM 710 laser-scanning confocal microscope. Images were compiled using ImageJ and Adobe Photoshop CS 7.01.

3.4. *In situ* hybridization

In situ hybridization was performed for chick Shh, Ptc1 and Ptc2 on paraffin sections using digoxigenin-labeled riboprobes (kindly provided by Cliff Tabin) according to standard protocols.

3.5. Intestinal organ culture assay

Gut was removed from E5 chick and embedded in type-I collagen-based matrix in a suitable culture dish. The collagen matrix (700 μl DMEM, 6 μl 1N NaOH and 294 μl collagen) was placed in the center of the culture dish, while penicillin-streptomycin-PBS solution was in the outer part of the dish. Embryos were embedded between two layers of the collagen matrix and then the organ culture was incubated in a CO_2 incubator at 37 °C for 48-72 hours. By adding to the culture, we examined the effect of recombinant mouse Shh protein (2 μg/ml) or cyclopamine (1-3 μM).

3.6. Chorioallantoic membrane (CAM) transplants

For CAM grafts E5 chick intestine was harvested and the cloaca removed. Ganglionic intestine, termed "+ceca," included the hindgut, ceca, and proximal midgut, which contains enteric neural crest-derived cells at this stage. Aganglionic intestine, termed "-ceca," included only the post-cecal intestine and therefore has no enteric neural crest-derived cells. To generate a hindgut containing only sacral enteric neural crest-derived cells E5 hindgut (preganglionic) was harvested without ceca, but with cloaca, and these intestines were grafted onto the CAM of an E9 host embryo. After 9 days of

incubation the graft, together with the surrounding CAM, was excised and processed for immunohistochemistry.

3.7. Microinjection of Shh overexpressing replication competent retrovirus (Shh-RCAS) and cyclopamine

Shh misexpression was achieved with the replication-competent retroviral vector RCAS. During this experiment we have used Narishige microinjector device with 100 µl Hamilton syringe. For infection 2-5 µl Shh-RCAS retrovirus or cyclopamine (3 µM) solution was injected into the E5 chicken hindgut wall, which was cultured on the CAM for 9 days.

3.8. Mouse-chick intestinal chimeras

Intestinal chimeras were constructed as we previously described. Hindgut was dissected from E11.5 mouse embryos. Ceca were removed, and these preganglionic hindguts, labeled with charcoal, were transplanted into the coelomic cavity of E3 chick embryos using a blunt-ended glass. During the experiment the vitelline membrane and amnion were carefully opened under the right wing bud. A longitudinal incision was made between the wing bud and heart by using a sterile tungsten needle. The eggs were closed with adhesive tape and allowed to develop at 37°C for 9 days. The survival rate for the chimeras (n=9) was 65-70%.

3.9. Neurosphere transplants to aneural chick hindgut

Spontaneously generated neurospheres from 3-week-old Wnt1;tdT mouse intestine were kindly provided by Dr. Ryo Hotta (Massachusetts General Hospital, Harvard Medical School Boston). One or two neurospheres were implanted into the proximal hindgut of E5 chick embryos. The hindgut was placed onto the CAM of E9 host chick embryo and, 7 days later, the gut was removed and processed for immunohistochemistry (n=25).

3.10. *In vitro* migration assay

Enteric neural crest-derived cell migration was assessed by stripe choice assay. During the experiment 6 cm plastic culture dishes were coated in alternate stripes of control proteins (10 µg/ml laminin or 20 µg/ml fibronectin) or ECM proteins (2 µg/ml

agrin or 50 μg/ml endostatin) using silicon matrices available from Prof. Martin Bastmeyer (Karlsruhe Institute of Technology, Germany). For each assay E7 chick midguts were placed perpendicular to the stripe pattern and covered with a small volume of serum-free DMEM culture medium containing penicillin and streptomycin. After initial incubation, DMEM containing 10 ng/ml GDNF was added to the culture. The distance of enteric neural crest-derived cell migration along each stripe was assessed at 24 h.

Enteric neural crest-derived cell migration was also analyzed by plating Wnt1;tdT-derived neurospheres onto culture plates coated with 20 μg/ml fibronectin and culturing for 48 h in either serum-free DMEM or medium supplemented with 20 μg/ml function-blocking agrin antibody as described Chakraborty and colleagues. Cell migration was measured using ImageJ by dividing the field of view into octants and measuring the distance from the neurosphere edge to the farthest Wnt1;tdT cell in each octant. Three to four neurospheres were analyzed per condition.

4. RESULTS

4.1. The role of epithelial-derived Shh growth factor during enteric nervous system development

4.1.1. Shh inhibits enteric nervous system development of the embryonic hindgut

In order to clarify the role of the Shh signaling during enteric nervous system development we used the avian and mouse embryos as model systems and applied a variety of techniques, including tissue recombination, embryonic chimeras, intestinal organ cultures, retrovirus-mediated gene misexpression, and *in situ* hybridization.

In the intestinal organ culture we find that Shh overexpression significantly decreases enteric neural crest-derived cell proliferation and promotes their premature differentiation into neurons, accounting for the aganglionic phenotype. Retrovirus-mediated overexpression of Shh *in vivo* confirms these results, leading to distal aganglionosis with severe hypoganglionosis in the proximal colon. Inhibition of Shh signaling, by addition of cyclopamine (organ culture, CAM transplant) resulted in large, ectopic ganglia adjacent to the epithelium. In cyclopamine-treated and untreated cultures, intestinal smooth muscle differentiation appears to be undisturbed, while Shh overexpression was affected the radial symmetry of the gut wall (mucosa, submucosa, tunica muscularis) and the smooth muscle development. Shh treatment increased the proliferation of mesenchymal but had no effect on the rate of enteric neural crest-derived cell proliferation. By contrast, cyclopamine dramatically decreased the proliferation of submucosal mesenchymal cells. To determine whether Shh induces enteric neural crest-derived cell apoptosis, we examined the expression of activated caspase 3, a marker of programmed cell death. There was no caspase 3 expression in any of the treatment groups. We find that Shh overexpression promotes enteric neural crest-derived cell premature differentiation into neurons, accounting for the aganglionic phenotype. Our observations from chicken embryos were also confirmed by experiments with mouse embryonic gut.

4.1.2. Shh receptors were not expressed by enteric neural crest-derived cells

In embryonic avian hindgut Ptc1 *in situ* hybridization combined with p75/HNK1 immunofluorescence to mark enteric neural crest-derived cells showed no overlap, and therefore no Ptc1 expression by enteric neural crest-derived cells. We found a similar expression pattern of Ptc1 in the mesenchyme, but not the enteric nervous system, in

embryonic mouse hindgut. Although Shh has a major influence on enteric nervous system development, the absence of Shh receptor expression on enteric neural crest-derived cells indicates that the effect is mediated indirectly via alterations in the microenvironment.

4.1.3. Shh signaling affects the expression of ECM proteins

The lack of Ptc1 expression by enteric neural crest-derived cells suggest that Shh does not directly affect enteric neural crest-derived cell development, but rather disrupts the microenvironment and indirectly leads to aganglionosis. We determined how modulating Shh signaling affects the expression of ECM proteins known to be permissive or inhibitory to neural crest cell migration. The most dramatic ECM effects, however, were observed with members of the CSPG family, which are known inhibitors of neural crest cell migration. We observed significant Shh-induced changes in collagen IX, CS-56 and versican isoforms. Collagen IX and versican are normally expressed strongly in the inner mesenchymal layer, just beneath the epithelium. After Shh treatment, both collagen IX and versican were dramatically upregulated and distributed throughout the mesenchyme, whereas cyclopamine reduced expression of these chondroitin sulfate proteoglycans, limiting them to a small ring of subepithelial mesenchyme.

4.2. The role of the ECM expression pattern in the microenvironment of the enteric neural crest-derived cells during normal and pathological enteric nervous system development

In addition to chondroitin sulfate proteoglycans we next characterized other ECM proteins expression before (E5), during (E8) and after (E14) enteric nervous system colonization of the chicken hindgut to identify ECM patterns that might suggest a role in enteric nervous system development. We found strong expression of HSPG proteins (collagen XVIII and agrin) around the submucosal and myenteric ganglia during enteric nervous system formation and consequently interrogated specific members of this ECM family. We show that collagen XVIII is expressed by enteric neural crest-derived cells at the wavefront of migration. By contrast, agrin is notably absent from the wavefront. Agrin is produced by enteric neural crest-derived cells only later during development, first appearing in the avian hindgut at E10, ~2 days after enteric nervous system colonization has completed. Given the proximity of collagen XVIII and agrin to enteric neural crest-derived cells, we examined whether enteric neural crest-derived cells are required for their expression using experimentally and genetically aganglionic models.

4.2.1. Enteric neural crest-derived cells are required for normal expression of collagen XVIII and agrin around enteric nervous system

Given the proximity of collagen XVIII and agrin to enteric neural crest-derived cells, we asked whether enteric neural crest-derived cells are required for their expression by using experimentally and genetically aganglionic models (3-week-old Ednrb$^{-/-}$ mice). The proximal, ganglionated colon of Ednrb$^{-/-}$ mice shows strong collagen XVIII expression around enteric ganglia and the interconnecting fibers. In contrast, the aganglionic distal colon lacks collagen XVIII expression in the mesenchyme. Peri-ganglionic expression of agrin is also absent in the aganglionic segment. These results suggest that enteric neural crest-derived cells are indeed required for the periganglionic expression of collagen XVIII and agrin.

4.2.2. Collagen XVIII and agrin are produced by enteric neural crest-derived cells

In vivo results from CAM cultures and transgenic embryos suggest that collagen XVIII and agrin are produced by enteric neural crest-derived cells. To answer this question, chick-mouse chimeras were generated. E11.5 mouse hindgut was isolated without the cecum or the cloaca. This preganglionated gut was transplanted into the coelomic cavity of an E3 chick host. Immunofluorescence imaging of serial sections of the grafted colon confirms the chick origin of the graft's enteric nervous system. The origin of extracellular matrix proteins was also confirmed by species-specific antibodies. Collagen XVIII and agrin are all labeled by chick-specific antibodies, confirming that these ECM proteins are produced by chick-derived cells. To confirm that enteric neural crest-derived cells are the source of collagen XVIII and agrin, we used spontaneously generated neurospheres. These neurospheres were generated from FACS-sorted 3-week-old Wnt1;tdT mouse intestine. One or two neurospheres were implanted into the proximal hindgut of E5 chick embryos. The hindgut was placed onto the CAM of E9 host chick embryo and, 7 days later, the gut was removed and processed for immunohistochemistry (n=25). As in chicken embryos, mouse-derived enteric neuronal stem cells also produce collagen XVIII and agrin.

To determine which enteric nervous system cell type produces these HSPG proteins in the postnatal intestine neurospheres were spontaneously generated from Plp1^{GFP} (all enteric glia show green positivity) and TauGFP$^{/+}$ (all enteric neuron show green positivity) intestine to generate enteric neural crest-derived cell cultures containing fluorescently labeled glia and neurons, respectively. PLP1+ enteric glial cells express

both collagen XVIII and agrin. By contrast, Tau+ enteric neurons express agrin, but not collagen XVIII.

4.2.3. The effect of agrin and collagen XVIII on enteric neural crest-derived cell migration

To explore the functional significance of these HSPG proteins, we examined the effects of collagen XVIII and agrin on enteric neural crest-derived cell migration. Using *in vitro* stripe-choice assays, we demonstrate that in contrast to collagen XVIII, agrin strongly inhibit the migration of multipotent enteric neural crest-derived cells on fibronectin by interfering with cell-substrate adhesion. During our experiment, using *in vitro* stripe-choice assays, we demonstrate that in contrast to collagen XVIII, agrin strongly inhibit the migration of multipotent enteric neural crest-derived cells, presumably through its interaction with its receptor, dystroglycan, which is expressed by enteric ganglia. Collagen XVIII, on the other hand, is permissive to, although not essential for, migration. The different function of the two heparan sulphate proteoglycans can explain the spatiotemporal differences in their expression patterns.

We confirmed the inhibitory role of agrin by culturing neurospheres derived from Wnt1;tdT mouse gut on an agrin-coated surface. No enteric neural crest-derived cell migration was observed from those postnatal neurospheres. We performed immunofluorescence on Wnt1;tdT-derived enteric neurospheres and identified the undifferentiated progenitors based on their co-expression of Sox10 and Phox2b. As expected, these cells do not express agrin. Agrin inhibition is thus enhancing the migration of a population of agrin-expressing cells in the neurosphere, that are not undifferentiated progenitors, but more likely committed precursors, that retain migratory potential. The ongoing migratory capacity of committed precursors in the enteric nervous system has been described previously.

5. CONCLUSIONS

Our results support an essential role for epithelial-mesenchymal interaction and ECM in the pathogenesis of the neurointestinal disorders such as Hirschsprung-disease.

1. Using *in situ* hybridization in combination with immunofluorescence techniques we revealed, that Hedgehog signaling receptors (Ptc1, Ptc2) are not expressed by neural crest stem cells.
2. With mouse and chicken intestinal organ cultures and retrovirus-mediated *in ovo* overexpression we demonstrated, that Shh inhibits neural crest stem cells proliferation and promotes neuronal differentiation. This effect is mediated indirectly via alterations in the microenvironment: Shh strongly induced the expression of versican and collagen type IX, which chondroitin sulfate proteoglycans are inhibitory to neural crest cell migration.
3. Using chick-mouse intestinal chimeras and enteric neurospheres, we have showed that vagal- and sacral-derived enteric neural crest cells from both species secrete collagen XVIII and agrin.
4. We demonstrated that while glia express collagen XVIII and agrin, enteric neurons only express the latter.
5. With *in vitro* migration assay, we demonstrated, that collagen XVIII is permissive whereas agrin is strongly inhibitory to enteric neural crest-derived cell migration.

Our results suggest, that enteric neural crest-derived cells not only respond to their local environment, but enteric neural crest-derived cells actively shape their local ECM environment in a cell-autonomous way and that these environmental changes in turn regulate enteric neural crest-derived cell development. Knowledge of the extracellular matrix production function of enteric neural crest-derived cells is essential for the successful regenerative therapy.

6. PUBLICATIONS OF THE AUTHOR

6.1. Publications related to the doctoral thesis

Nagy N, **Barad C,** Graham HK, Hotta R, Cheng LS, Fejszak N, Goldstein AM. (2016) Sonic hedgehog controls enteric nervous system development by patterning the extracellular matrix. Development. doi:10.1242/dev.128132.
IF: 5,843

Nagy N*, Barad C*, Hotta R, Bhave S, Arciero E, Dora D, Goldstein A.M. (2018) Collagen 18 and agrin are secreted by enteric neural crest cells to remodel their microenvironment and regulate their migration during ENS development. Development. doi:10.1242/dev.160317.
IF: 5,413

(*These authors contributed equally to this work)

6.2. Other publications

Dora D, Arciero E, Hotta R, **Barad C**, Bhave S, Kovacs T, Balic A, Goldstein AM, Nagy N. (2018) Intraganglionic macrophages: a new population of cells in the enteric ganglia. J Anat 1–10. doi: 10.1111/joa.12863.
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