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COMMENTARY

Toward a better understanding of enteric gliogenesis

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ABSTRACT

Most of gastrointestinal functions are controlled by the enteric nervous system (ENS), which contains a vast diversity of neurons and glial cells. In accordance with its key role, defective ENS formation is the cause of several diseases that affect quality of life and can even be life-threatening. Treatment of these diseases would greatly benefit from a better understanding of the molecular mechanisms underlying ENS formation. In this regard, although several important discoveries have been made over the years, how the full spectrum of enteric neuronal and glial cell subtypes is generated from neural crest cells during development still remains enigmatic. Because they also have stem cell properties, such knowledge would be especially important for the enteric glial cell lineage. In a recent study, we identified the NR2F1 transcription factor as a new key regulator of enteric gliogenesis. Here we discuss our recent findings and briefly review what is already known about the mechanisms and signaling pathways involved in enteric gliogenesis, with an emphasis on Hedgehog and Notch signaling.

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Heterogeneity of both neurons and glia in the enteric nervous system

Buried within the wall of the whole gastrointestinal tract, the enteric nervous system (ENS) is the most complex division of the peripheral nervous system. Although it works in concert with the central nervous system (CNS) to control the numerous gastrointestinal functions, the ENS can function independently from the CNS and is therefore often described as the "second brain".[1,2] This extensive neural network intrinsic to the gastrointestinal tract contains a vast amount of enteric neurons and glial cells that are mainly grouped in ganglia distributed into 2 interconnected plexuses. One plexus–the myenteric or Auerbach's plexus–is located between the longitudinal and circular muscle layers of the gut wall where it provides innervation to both muscle layers to control peristalsis. Another plexus–the submucosal or Meissner's plexus–lies underneath the mucosal epithelium and is involved in several mucosal processes like sensing the environment within the lumen, regulating gastrointestinal blood flow, and controlling epithelial cell function.

Both the total number and diversity of enteric neurons also explain why the ENS is often referred to as the second brain. There are 3 broad classes of enteric neurons: sensory neurons (or intrinsic primary afferent neurons), interneurons and motor neurons.[1] Different types of receptor on sensory neurons in the mucosa and muscles respond to mechanical, thermal, osmotic and chemical stimuli. Interneurons integrate the sensory input from sensory neurons and send it to motor neurons. Motor neurons then interact with smooth muscle cells and others effectors cells to directly control gastrointestinal motility, blood flow and secretion. Enteric motor neurons is a heterogeneous population that can be further subdivided in 5 classes of neuronal subtypes: excitatory or inhibitory neurons to gut muscle, secretomotor/vasodilator neurons, non-vasodilatator secretomotor neurons and neurons innervating enteroendocrine cells.[3]

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Present in similar amount or even outnumbering enteric neurons depending of species, enteric glial cells were initially considered to be only passive support cells for enteric neurons. This traditional view has changed a lot over the last few years and enteric glial cells are now recognized as being essential for virtually all ENS-controlled gastrointestinal functions. The importance of glial cells in the ENS is notably highlighted by a multitude of digestive (e.g. ulcerative colitis, Crohn's disease, infectious enteritis and slow transit constipation) and even extradigestive (e.g., Parkinson disease and obesity) disorders that are associated with altered enteric glia.[4-6] Due to their shared neural crest origin, enteric glial cells were first considered to be the Schwann cells of the gut but detailed analysis of their morphology as well as their relationship to neurons later suggested that enteric glial cells are more similar to astrocytes.[7] Recent work further challenged this view and now suggests that enteric glia have a unique hybrid transcriptome profile overlapping (in order of importance) with the signature of Schwann cells, oligodendrocytes and astrocytes.[8] This apparent hybrid identity is most likely also reflective of the heterogeneity within the enteric glial cell population. For example, while most enteric glial cells co-express SOX10, PLP1 and S100β, only a subset of them also express GFAP.[8] Other work focusing on the diversity of enteric glial cells led to the identification of 4 specific types based on their morphology and their location along the serosa-to-lumen axis: star-shaped "protoplasmic gliocytes" within myenteric ganglia (type I), elongated "fibrous gliocytes" within fiber tracts (type II), mucosal and intramuscular gliocytes with 4 primary processes (type III_{mucosa} and Type $III_{MP/SMP}$, respectively) and bipolar intramuscular gliocytes (type IV).[9,10] Whether each of these subtypes has an associated physiologic role remains to be determined but differences in dye filling, calcium transient and receptor expression strongly suggest that enteric glial subtypes are functionally distinct.[2,9]

Another interesting feature of enteric glial cells is their remarkable plasticity. Indeed, they have been shown to possess a neurogenic potential *in vitro* and *in vivo* even though they are restricted to a glial fate in their native environment.[11,12] Furthermore, enteric glia are also capable of performing the functions of oligodendrocytes and astrocytes when transplanted into the CNS.[13] Harnessing the plastic capabilities of enteric glia thus holds great promise for the development of cell-based therapies for many diseases but the conditions and factors involved remain to be identified.[4]

Formation of enteric glial cells from neural crest cells

Enteric neurons and glia are both derived from multipotent neural crest cells (NCCs) originating from the dorsal tip of the developing neural tube.[14] This cell population is divided into several subpopulations depending of their origin along the anterior-posterior axis of the neural tube: cranial, cardiac, vagal, trunk and sacral. The vast majority of ENS progenitors (also refer to as enteric NCCs) has a vagal origin,[14] although minor contingents are also provided by the sacral region [15] and by NCC-derived Schwann cell precursors within the extrinsic nerves of the developing bowel.[16] In the mouse, enteric NCCs of vagal origin initially colonize the foregut mesenchyme around embryonic day (E) 9.5 and then migrate in the rostrocaudal direction to reach the end of the hindgut by E14.5. This stage also roughly corresponds to the arrival of enteric NCCs of sacral and Schwann cell origin. Differentiation of ENS progenitors is initiated soon after their entry into the developing bowel and, as in other parts of the nervous system, gliogenesis occurs after neurogenesis has begun.[17-19] In mice, neuronal precursors can be detected as early as E10-E10.5 just behind the migration front of enteric NCCs of vagal origin whereas glial precursors cannot be detected until E11.5-E12.[19]

A great deal is already known regarding markers that can be used to reliably distinguish between undifferentiated ENS progenitors from neuronal and glial precursors at different stages of differentiation (Fig. 1).[20,21] However, much less is known regarding the mechanisms underlying the associated cell fate decisions.[22] As seen in other developmental systems, cell fate decisions in the ENS are believed to be orchestrated by the combination of extrinsic factors from the gut mesenchyme and direct cell-cell communication between adjacent enteric NCCs. Pertaining to gliogenesis, such a combination is well exemplified by the functional interaction between Hedgehog and Notch signaling pathways.[23-26]

With both the ligand and the receptor being transmembrane proteins, the Notch signaling pathway allows direct cell-cell communication. Following

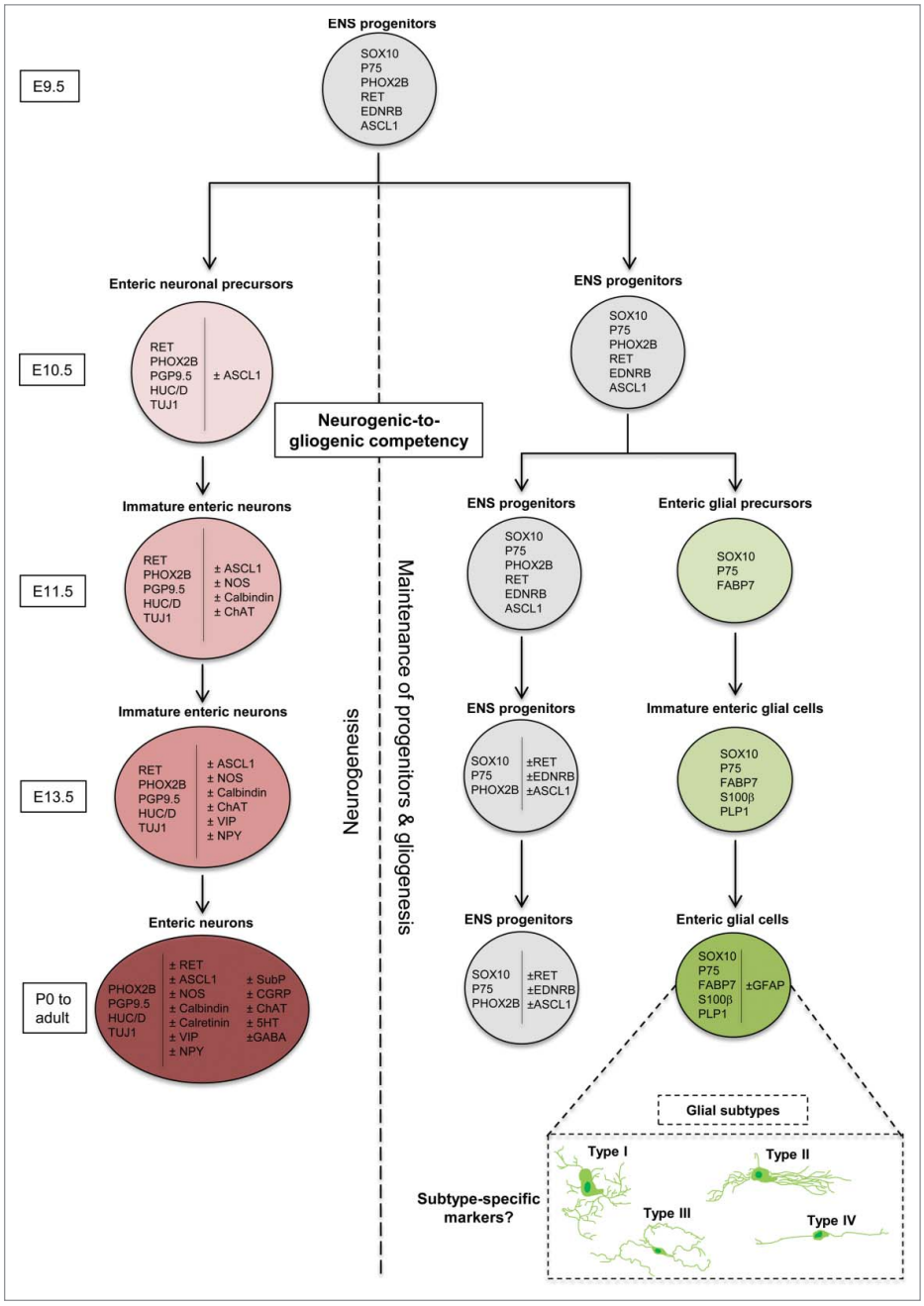

Figure 1. Key cell-specific markers during ENS formation. Enteric NCCs of vagal origin (ENS progenitors) enter the foregut around E9.5 and start to migrate rostrocaudally. Soon after their arrival, a subset of these ENS progenitors starts to differentiate into neurons while the majority is maintained in an undifferentiated and proliferative state. The competency of a subset of ENS progenitors to differentiate into enteric glial cells is only acquired around E11.5. From E15.5 onwards, colonization of the gut is completed but neuronal and glial differentiation continue until birth and during a short postnatal period. Adapted from refs. 20, 21.

interaction with its ligand, the Notch intracellular domain (NCID) is cleaved and thereby free to translocate to the nucleus where it associates with the DNA-binding protein RBPJ to activate transcription of target genes such as members of the *Hes* family.[27] In the developing ENS, many Notch pathway receptors and ligands (of both the DLL and JAG families) are present at the right place and the right time to influence glial differentiation (Table 1). As in other parts of the nervous system, Notch signaling seems critically required in enteric NCCs for the maintenance of undifferentiated progenitors as well as for the switch from neurogenesis to gliogenesis.[24-26,28,29] Analysis of mice with NCC-specific deletion of *Pofut1*–which encodes an o-fucosyltransferase that modifies the Notch receptors for optimal activity–suggests that such a dual role

Table 1. Extract of *Spot*$^{Tg/Tg}$ vs control RNAseq data from e12.5 enteric NCCs.[39]

		FPKM			
	Transcript isoform	***Spot*$^{Tg/Tg}$**	**Ctl**	Fold Change	Significant($P \leq 0.01$)
***Nr2f1* locus**	*A830082K12Rik-001*	9.10	2.07	+4.4	yes
	Nr2f1–001	94.92	7.07	+13.4	yes
Selected glial markers	*Cnp-001*	48.96	31.36	+1.6	yes
	Erbb3–001	81.34	46.32	+1.8	yes
	Fabp7–001	190.92	62.34	+3.1	yes
	Foxd3–001	55.92	33.54	+1.8	yes
	Mbp-003	3.47	5.24	−1.4	yes
	Mpz-001	3.56	0.95	+3.9	yes
	Nr2f2–001	7.31	6.25	+1.2	no
	Plp1–002	25.74	14.79	+1.8	yes
	Pmp22–001	4.24	4.66	−1.1	no
	Pou3f1–001	0.67	0.18	+3.7	yes
	S100b-001	3.20	0.56	+5.6	yes
	Sox2–001	27.91	21.66	+1.3	yes
	Sox8–001	17.27	23.69	−1.4	yes
	Sox10–201	64.41	36.05	+1.8	yes
	Zeb2–002	22.87	17.49	+1.3	no
Selected components of the Notch pathway	*Dll1–001*	7.36	10.44	−1.4	yes
	Dll3–001	7.62	7.49	–	no
	Dll4–001	0.59	1.33	−2.2	yes
	Hes1–001	14.89	25.72	−1.7	yes
	Hes5–001	0.11	0.81	−7.1	yes
	Hes6–001	29.32	36.63	−1.2	no
	Hey1–001	4.04	6.16	−1.5	yes
	Hey2–001	3,70	3,45	+1.1	no
	Jag1–001	5,67	3,08	+1.8	yes
	Jag2–201	1,23	2,86	−2.2	yes
	Notch1–001	15,49	10,63	+1.5	yes
	Notch2–001	13.95	17.42	−1.2	yes
	Notch3–001	6.24	5.16	+1.2	yes
	Notch4–001	0.28	0.41	+1.6	no
Selected components of the Hedgehog pathway	*Gas1–001*	8.03	10.19	−1.3	yes
	Gli1–201	1.08	2.31	−2.1	yes
	Gli2–001	1.23	1.70	−1.4	yes
	Gli3–001	7.86	5.93	+1.3	yes
	Ptch1–001	5.92	9.22	−1.5	yes
	Ptch2–001	0.47	0.90	−1.9	yes
	Smo-001	37.18	27.86	+1.4	yes
	Sufu-001	4.01	3.91	–	no

Note. FPKM, fragments per kilobase of transcript per million mapped reads.

could be due to the indirect downregulation of *Sox10* expression.[25] *Sox10* encodes a HMG-box transcription factor also known to be critically required in the developing ENS for both the maintenance of the progenitor pool and the acquisition of the glial fate.[30] Based on work in other systems, it was proposed that *Sox10* could normally be repressed by the pro-neuronal transcription factor ASCL1 (MASH1),[31] which would itself be negatively regulated at the transcriptional level by the HES1 transcription factor downstream of Notch signaling.[32] However, other work suggests a more complex mechanism that also involves Hedgehog signaling.[23,24]

Sonic Hedgehog (*Shh*) and *Indian Hedgehog* (*Ihh*) are both expressed in the gut endoderm from the earliest stages of gut tube closure onwards.[33-35] In accordance with the fact that Hedgehog ligands can act over long distances, their absence in mice revealed essential roles for the proper formation of all gut layers including the developing ENS.[35] Enteric NCCs express all the necessary machinery for Hedgehog signaling (Table 1) and disruption of the genes encoding either IHH or SHH secreted proteins was shown to notably results in partial intestinal aganglionosis or ectopic ganglia formation, respectively.[35] In the canonical pathway, binding of Hedgehog ligands to PTCH receptors relieves the inhibition of the SMO signal transducer, which role is to counteract SUFU to ultimately promote the formation of the activator form of the GLI transcription factors at the expense of their repressor form.[27] Constitutive activation of Hedgehog signaling in the NCC lineage via targeted disruption of *Ptch1* or

Sufu in mice highlighted an important role in the regulation of enteric gliogenesis.[23,24] Indeed, activation of the Hedgehog pathway was shown to trigger premature glial differentiation of enteric NCCs, an effect that was notably demonstrated to occur through activation of Notch signaling.[24] Moreover, recent work suggests that Hedgehog-induced gliogenesis could also involve direct activation of *Sox10* expression by GLI transcription factors.[23]

In brief, the current knowledge strongly suggests that both Hedgehog and Notch signaling pathways sit at the top of the gene regulatory network that controls enteric gliogenesis. Other progliogenic pathways like GGF2/ERBB3 and LGI4/ADAM22 appear to be required for subsequent phases of expansion and maturation of enteric glial cells.[36,37] However, it is also clear that more work is required to elucidate how the network is precisely wired downstream of all the involved signaling pathways. Importantly, this work might eventually reveal if the network is differentially wired as a function of glial subtypes.

NR2F1 is a newly identified potent regulator of enteric gliogenesis

Via an insertional mutagenesis screen focused on the identification of neurocristopathy-associated genes,[38] we recently generated a new mouse model of Waardenburg syndrome type 4 called *Spot*.[39] As observed in the human pathology, homozygous *Spot* mice ($Spot^{Tg/Tg}$) are depigmented and display spatial orientation defects as well as intestinal blockage, resulting respectively from a lack of NCC-derived melanocytes (in the skin and inner ear) and ENS (in the colon). Detailed analysis of the developing intestines revealed that the *Spot* mutation negatively impacts migration and proliferation of enteric NCCs due to their premature differentiation toward the glial lineage. This phenotype was found to be caused by transgene insertion-mediated perturbation of a silencer element that leads to NCC-specific upregulation of the orphan nuclear receptor gene *Nr2f1* and its overlapping antisense long non-coding RNA *A830082K12Rik*. Targeted overexpression of *Nr2f1* in NCCs under the control of the U3 *Sox10* enhancer further revealed that a gain of *Nr2f1* alone can cause aganglionosis independently of *A830082K12Rik*. Altogether, these data allowed us to conclude that the NR2F1 transcription factor is a novel key player in enteric gliogenesis.[39]

Although the mechanism of NR2F1 action during enteric gliogenesis is currently unknown, we can predict that its co-expressed paralogue NR2F2 is also normally involved (Table 1). Indeed, NR2F1 and NR2F2 (also known as COUP-TFI and COUP-TFII) have been previously reported to control in a redundant manner the neurogenic-to-gliogenic temporal change during the specification of neural stem/progenitor cells (NSPCs).[40] This work revealed that *Nr2f1/2* are transiently upregulated in NSPCs during the early neurogenic period to make these cells responsive to the gliogenic cytokines LIF and BMP2.[40] In support of a similar mechanism in the developing ENS, our RNAseq data from E12.5 $Spot^{Tg/Tg}$ enteric NCCs show that *Erbb3*–which encodes the receptor of the gliogenic growth factor GGF-2–is noticeably upregulated in comparison to control (Table 1).[39] A more direct role is also possible. Indeed, the early enteric glial marker *Fabp7* (Fig. 1) has been previously reported as a direct NR2F1 target gene in the brain and inner ear,[41] and is robustly upregulated in $Spot^{Tg/Tg}$ enteric NCCs (Table 1). Moreover, other recent studies have reported that some micro-RNAs are downstream effectors of NR2F1/2 in the inner ear and NSPCs.[42,43] Definitive identification of the downstream effectors of NR2F1/2 transcription factors in enteric NCCs will require further studies but all of the observations mentioned above suggest that NR2F1/2 could trigger the neurogenic-to-gliogenic transition by playing both instructive and permissive roles.

More work will also be required to determine how *Nr2f1/2* expression is normally regulated during ENS formation. Because of their presumed position at the top of the gene regulatory network that controls enteric gliogenesis, both Hedgehog and Notch signaling pathways should be seriously considered for such a role. This possibility is supported in part by prior reports of SHH-mediated regulation of *Nr2f2* expression in the neural tube and stomach.[44,45] Another not necessarily mutually exclusive possibility could involve epigenetic mechanisms. Indeed, epigenetic regulation is known to be important for multiple aspects of NCC development including glial differentiation.[46] For instance, HDAC1/2 activity has been shown to be essential for the differentiation of NCCs into Schwann cells and satellite glia, in part through direct activation of *Mpz* expression.[47] Furthermore, the ZEB2 transcription factor has been shown to be a critical regulator of Schwann cell maturation through

HDAC/NuRD-mediated repression of genes that inhibit maturation such as *Hey2, Sox2* and *Ednrb*.[48,49] When focusing on the regulation of *Nr2f1* only, a role for its antisense long non-coding RNA *A830082k12Rik* is also likely. Indeed, based on their co-regulation in *Spot*$^{Tg/Tg}$ enteric NCCs (Table 1),[39] *A830082k12Rik* is expected to activate *Nr2f1* transcription in *cis* as described previously for several similar cases. [50]

Conclusion

Determining the exact mechanism of action of NR2F1/2 in enteric gliogenesis and the different mechanisms and signaling pathways involved in the regulation of their expression will surely allow for a better understanding of the neurogenic-to-gliogenic competency of enteric NCCs. This exciting work will again greatly benefit from the *Spot* mouse line.

Disclosure of potential conflicts of interest

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