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Spatially distinct functions of PAX6 and NKX2.2 during gliogenesis in the ventral spinal cord

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ABSTRACT

During ventral spinal cord (vSC) development, the p3 and pMN progenitor domain boundary is thought to be maintained by cross-repressive interactions between NKX2.2 and PAX6. Using loss-of-function analysis during the neuron-glial fate switch we show that the identity of the p3 domain is not maintained by the repressive function of NKX2.2 on Pax6 expression, even in the absence of NKX2.9. We further show that NKX2.2 is necessary to induce the expression of Slit1 and Sulfatase 1 (Sulf1) in the vSC in a PAX6-independent manner. Conversely, we show that PAX6 regulates Sulf1/Slit1 expression in the vSC in an NKX2.2/NKX6.1-independent manner. Consequently, deregulation of Sulf1 expression in Pax6-mutant embryos has stage-specific implications on neural patterning, associated with enhancement of Sonic Hedgehog activity. On the other hand, deregulation of Slit1 expression in gliogenic neural progenitors leads to changes in Astrocyte subtype identity. These data provide important insights into specific functions of PAX6 and NKX2.2 during glial cell specification that have so far remained largely unexplored.

Introduction

The ventral spinal cord (vSC) in mice is organized into five progenitor domains that generate different subtypes of neurons and glia [1]. The two most ventral progenitor domains, known as p3 and pMN, are defined by the specific expression of Nkx2.2 and Olig2 during neurogenesis [2]. The p3/pMN progenitor boundary is believed to be maintained by cross-repression between PAX6 and NKX2.2 homedomain (HD) factors [3,4]. At the onset of the N/G switch, the pMN domain switches from generating motor-neurons to generate mostly Oligodendrocytes (OLs) and some Astrocytes (ASs) [5–8]. This process is preceded by a dorsal expansion of the p3 domain and a ventral retraction of the pMN domain [8–10]. In the chick embryo this boundary shift is characterized by a sharp increase of Sonic Hedgehog (SHH) protein in neuroepithelial cells located in the p3 domain [11,12]. The enzyme Sulfatase 1 (SULF1) has been proposed to mediate this process since Sulf1 expression expands from the Floor Plate (FP) to the p3 domain [12]. The functional significance of this phenomenon remains unknown, despite the suggestion that it may regulate the specification of p3-derived OLs in the chick embryo [12]. The genetic regulation of Sulf1 expression is also unknown.

More recently the domain-specific organization of the vSC has been proposed to govern the positional specification of three different subtypes of ventral astrocytes (VA), named VA1–3 ASs [13], that express Reelin and Slit1. White matter (WM) VA1 ASs express only Reelin and are specified in Pax6$^+$ progenitors located dorsal to the p2 domain. VA2 ASs express both Reelin and Slit1 and are specified in Nkx6.1/Pax6$^+$ progenitors (pMN–p2) and VA3 ASs are specified in Nkx2.2/Nkx6.1$^+$ (p3) progenitors and express only Slit1. It has been proposed that the combinatorial expression of PAX6 and NKX6.1 in ventricular zone (VZ) progenitors regulate the expression of both Reelin and Slit1 in the VZ and by doing so govern the subtype identity of the respective ASs [13]. Thus PAX6 induces Reelin and represses Slit1, while NKX6.1 (and NKX2.2) induce Slit1 and repress Reelin.

In this study, we considered NKX2.2 and PAX6 as primary candidates for regulating both Slit1 and Sulf1 in the vSC. We first show that under physiological *in vivo* conditions the proposed repressive function of NKX2.2 on Pax6 expression in the p3 domain is not supported by loss-of-function data even when NKX2.9 is switched off. This observation allowed us to establish a specific genetic requirement for NKX2.2 to initiate both Slit1 and Sulf1 expression in the vSC in a PAX6-independent manner. Conversely, we show that PAX6 has an NKX2.2/NKX6.1-independent function in regulating Sulf1/Slit1 expression in the pMN–p0 domains. Finally, we demonstrate that deregulation of Sulf1 and Slit1 expression has functional

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implications on the response of progenitors to SHH signaling and on AS subtype identity, respectively.

Materials and methods

Transgenic mice. The mouse mutant lines used were: $Pax6^{Sey/Sey}$ [14] and $Nkx2.2^{-KO}$ [15]. Genotyping was performed by PCR for Nkx2.2 and by morphological examination of the telencephalon and absence of eye development for $Pax6^{Sey/Sey}$ embryos. All animal procedures were performed in accordance with a license issued by the Chief of Veterinary Services of the Republic of Cyprus, as stipulated by National Law.

In situ hybridization and immunohistochemistry. Embryos were fixed overnight in 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), cryo-protected overnight in 20% (w/v) sucrose in PB and sectioned on a cryostat (14 μm). In situ hybridization was performed as described [16,17]. The probes used were: Nkx2.2 and Olig2 (Gift from Bill Richardson), Dbx1, Dbx2 (gift from Tom Jessell), Slit1 (gift from Marc Tessier-Lavigne), Pax6 (Gift from Peter Gruss), Nkx2.9 (gift from Johan Ericson). The rest of the probes were generated by PCR amplification with cDNA-specific primers for the gene of interest, ligated in pGEM-T Easy (Promega) and amplified by directional PCR to generate the template for antisense probe synthesis (T7 end). More information on the sequence is available on request. All images were captured using an Olympus microscope (Olympus SZX12) and digital camera (Olympus DP70). For domain-specific comparisons of gene expression, in situ hybridisation was carried out on consecutive sections and images captured were photo-converted using Adope Photoshop and aligned. Immunohistochemistry was performed using MEMFA-fixed embryos. The length of fixation was 20–40 min, depending on the stage and antibodies used. Sections were cut at cryostat (10–12 μm). Immunohistochemistry was performed according to standard procedures using the following primary antibodies: Shh, Nkx2.2, Nkx6.1, Pax3 (Hybridoma), Olig2 (Santa Cruz), Pax6 (Millipore). Images were captured on a TCSL confocal microscope (Leica, Germany).

Results

Temporal gene expression changes in the p3 and pMN domains

The neuron-glial (N/G) fate switch in the vSC in mice takes place between embryonic day 11.0 (e11.0) and e12.0 [18,19]. In agreement with previous data [8], we confirmed that between e10.5 and e12.5 the expression domain of Nkx2.2 expanded dorsally while the ventral limit of Olig2 and Pax6 retracted ventrally (not shown). Concomitant to this boundary shift, the expression of Sulf1 and Slit1 expanded from the FP in the vSC. Slit1 expression expanded beyond the pMN to reach the p2 domain as reported [13] while Sulf1 expanded to overlap partly with the ventral pMN (vpMN) domain (Supplementary Fig. 1A–D). At the same time Sulf2 expression, coding for an enzyme related to SULF1 [20], expanded to overlap with the p0 domain ($Dbx1^{+}$) while Nkx2.9 expression was switched off from the p3 domain (Supplementary Fig. 1E–H). Consistent with a role of SULF1 in regulating the local concentration of SHH in the p3 domain in the chick [11,12], we detected enhanced accumulation of SHH protein in the p3 domain at e11.5 that was not evident at e9.5 (Supplementary Fig. 1I–J). At the same developmental window the expression of Patched 1 (Ptch1), providing readout for Shh signaling [21], expanded from the p3 domain to reach more than two thirds of the VZ, suggesting that neuroepithelial progenitors became more exposed to SHH at e11.5 than at e9.5 (Supplementary Fig. 1K and L). These data suggest that during the N/G fate switch the boundary shifts observed in the p3/pMN domains affecting Nkx2.2/Pax6/Olig2 expression, are temporally associated with changes in the expression of Slit1, Sulf1 and Sulf2 and correlate with changes in the response of VZ progenitors to SHH activity.

Stage- and region-specific requirement for PAX6 and NKX2.2 to regulate the expression of Slit1 and Sulf1

We then asked how the expression of Sulf1/Sulf2 and Slit1 is genetically regulated. To test this we analyzed their expression in $Pax6^{Sey/Sey}$ and $Nkx2.2^{-/-}$ embryos. In both $Nkx2.2^{-/-}$ and $Pax6^{Sey/Sey}$ embryos analyzed at e10.5, Sulf1/2 and Slit1 were expressed in the FP like control embryos (not shown), suggesting that neither PAX6 nor NKX2.2 had any influence on their FP expression. However, at e12.5 in $Pax6^{Sey/Sey}$ embryos the expression of both Slit1 and Sulf1 was up-regulated in the VZ and the dorsal limit of their expression reached the ventral limit of Pax3 expression (Fig. 1A–D). In $Nkx2.2^{-/-}$ embryos both Slit1 and Sulf1 expression failed to expand in the VZ and were only expressed in the FP (Fig. 1E and F). One likely reason for this is the predicted ventral expansion of PAX6. However, Pax6 expression did not expand in the prospective p3 domain (Fig. 1G and H) despite the extinction of Nkx2.9 expression by this stage (Supplementary Fig. 1H). The expression of Sulf2 was not affected in any genetic background (not shown).

These data suggest that under physiological conditions in a stage- and region-specific manner, NKX2.2 controls the induction of Slit1 and Sulf1 expression in the vSC in a PAX6-independent manner. Conversely, loss of PAX6 led to co-extensive up-regulation of both Silt1/Sulf1 in the entire vSC despite the initial differences in their expression limits (Supplementary Fig. 2). They also suggest that, under physiological *in vivo* conditions, the proposed repressive function of NKX2.2 on Pax6 expression could not be corroborated by loss-of-function analysis at e12.5.

Stage-specific enhancement of Shh signaling in the absence of PAX6

Since SULF1 has been shown to promote accumulation of SHH in ectopic sites of expression [12], we wondered if the deregulation of Sulf1 expression in $Nkx2.2^{-/-}$ and $Pax6^{Sey/Sey}$ embryos had implications on Shh signaling and/or neural patterning. In $Nkx2.2^{-/-}$ embryos neural patterning was normal except from the ventral expansion of Olig2 as predicted by the repressive effects of NKX2.2 on Olig2 expression (not shown) [2,22]. In $Pax6^{Sey/Sey}$ embryos where both Sulf1 and Sulf2 were expressed in the p3–p0 region we found that at e10.5 Ptch1 expression did not change (Fig. 2A and B). However, at e12.5 we observed strong up-regulation of Ptch1 expression in the VZ of $Pax6^{Sey/Sey}$ embryos (Fig. 2C and D). To examine the consequences on neural patterning we compared the expression pattern of four Class I and three Class II genes at e10.5 and e12.5. As expected, Nkx2.2 expression expanded dorsally at both stages in $Pax6^{Sey/Sey}$ (Fig. 2E–H). The expansion of Nkx2.2 caused repression of Olig2 expression [2] (Fig. 2E–H) while Nkx6.1 expression did not change at any developmental stage (Fig. 2I–L). The analysis of the expression of Class I genes, which are directly repressed by SHH, was more informative. We first noted a dorsal shift of the ventral limit of Pax6 expression at e12.5. Importantly, this shift was not apparent at e10.5 despite the expansion of Nkx2.2 at both stages (Fig. 2M–P). Furthermore, Irx3 expression was reduced only at e12.5 despite the fact that Olig2 expression was almost abolished at both stages (Fig. 2Q–T) and loss of Olig2 should lead to ventral expansion of Irx3 in the pMN domain [8]. Finally, the expression of Dbx1 and Dbx2 was only reduced at e12.5, with Dbx2 being more affected than Dbx1 (Fig. 2U–Z2).

These data suggest that the stage-specific expression changes of several Class I genes in $Pax6^{Sey/Sey}$ embryos are likely to be due to the ventralization of p3–p0 progenitors brought about by expan-