The role of Sulfatase1 and Sulfatase2 in the development of oligodendrocyte in the mouse spinal cord

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**Introduction**

In the developing ventral spinal cord, Shh acts as a morphogen providing positional information to regulate cell fate decisions and structural organization in the ventral midline. Shh is secreted from the notochord and floor plate, and spreads to form a gradient along the dorsoventral axis. Depending on the concentration and duration of Shh exposure, Shh signaling regulates the activity of Gli transcriptional factors in the responding cells to control the expression of numerous target genes, such as patched1 and other transcription factors in the ventral progenitor cells, to establish five distinct domains: p0, p1, p2, pMN and p3 (listed from dorsal to ventral). After domain formation is complete, each progenitor domain generates distinct neuronal subtypes: V0, V1, V2, motor neurons (MN) and V3 neurons, respectively. The two most ventral neural progenitor domains, p3 and pMN, are defined by the expression of the transcription factors Nkx2.2 and Olig2, respectively [8]. In the mouse, pMN progenitors produce MNs during early embryonic stages (from E9-10.5) when neurogenin2 (Ngn2) expression overlaps with Olig2. After MN production is complete, Olig2 progenitors undergo a change in cell fate to become OPCs by E12.5 in the mouse. At this stage, the Nkx2.2-expressing region expands into the pMN domain following the downregulation of Ngn2 expression in response to increased Shh signaling, and Olig2+Nkx2.2+ cells differentiate into OPCs. Thus, precise regulation of Shh signaling is required not only for domain formation but also for the MN-to-OPC neuroglia switch in the developing spinal cord. However, the mechanism that regulates the spatiotemporal gradient of Shh remains unclear.

Many studies have demonstrated that HSPGs play critical roles in
regulating morphogen gradients and signaling activity. HSPGs are extracellular matrix macromolecules that consist of a core protein (e.g. glypican, syndecan, perlecan) and heparan sulfate (HS) glycosaminoglycan (GAG) chains, which attach to the core protein. HS consists of uronic acid-(1→4)-d-glucosamine repeating disaccharide subunits and undergoes sulfate modifications at the 2-O position on uronic acid and the N, 3-O and 6-O positions on the glucosamine HS units. These modifications are important for the regulation of numerous morphogen gradients and signaling pathways, including Shh signaling. For the 6-O-sulfate modification, two secretory proteins, Sulf1 and Sulf2, are important enzymes which specifically remove 6-O-sulfate groups from HS. In Drosophila and Xenopus, Sulf1 influences the Shh gradient by releasing Shh from the cell surface. Moreover, recent studies have shown that during development Sulf1 acts as a regulator of Shh signaling input into the pMN domain to regulate OPC differentiation in chick, zebrafish and mouse. Another sulfatase, Sulf2, which has similar enzymatic activity and specificity as Sulf1, is also expressed in the developing ventral spinal cord, but its function in this context is completely unknown.

In this study, we analyzed various Sulf KO mice, including Sulf1 KO, Sulf2 KO, Sulf double KO (DKO) mice and Sulf1/2 double heterozygous (Sulf1+/−Sulf2+/−) mice, and found that in the ventral spinal cord, both Sulf1 and Sulf2 activity is necessary for the precise MN-to-OPC fate switch.
Result

In this study, I found a novel function of Sulf2 as an endo-sulfatase involved in the control of MN to OPC fate change by regulating Shh signaling in the ventral spinal cord of mouse.

First I analyzed the expression pattern of Sulfs (Sulf1 and Sulf2), Shh and Shh signaling indicator Patched1 during mouse embryonic spinal cord development (E10.5-E12.5). In the mouse, Sulf mRNAs colocalized with Shh mRNA and gradually expanded dorsally from E10.5 to E12.5, which always followed strong Patched1 signal. These expression patterns gave me an hypothesis that in the mouse strong Shh signaling inputting was promoted by Shh released by Sulfs, and strong Shh signaling in turn induced Shh and Sulfs dorsal expansion. Consistent with this hypothesis, I found that in the Sulf1 KO or Sulf2 KO mouse ventral spinal cord, Shh expression was restricted to the floor plate at E11.5 and E12.5, and did not expand into p3 domain as in the WT mouse. Moreover, in Sulf1 KO or Sulf2 KO mouse, Patched1 expression was weaker than that in WT mouse at E11.5 and remained as E11.5 expression pattern even at E12.5.

Shh is required for the ventral spinal cord domain formation and the MN to OPC fate change in the pMN domain. Thus I checked the domain formation and progenitor differentiation in the pMN domain. I found that the pMN and the p3 domains position shifted ventrally, MN neuron generation prolonged and OPC generation was delayed at E12.5 in Sulf1 KO or Sulf2 KO mouse. These results showed that Sulf2 also plays an important role in MN to OPC fate change by regulating Shh signaling in the
ventral spinal cord like Sulf1 and thus has overlapping function as Sulf1.

However, Sulf1 or Sulf2 cannot compensate the loss of Sulf1 or Sulf2 respectively, in the developing mouse spinal cord. In vitro studies showed no evidence for Sulf1 and Sulf2 interaction and sulfatase activity of Sulf1 and Sulf2 was independent. Furthermore, similar phenotypes with Sulf1/Sulf2 single KO mouse were also observed in the Sulf1&2 DKO and Sulf1+/−/Sulf2+/− mice. Severe phenotypes were not found in the Sulf1&2 DKO mouse. These results suggest that a sulfatase threshold in the pMN domain required for precise MN to OPC fate switch change exists and indicates that MN to OPC fate change is not linear against Shh dose. Only when Shh concentration reaches a threshold, precise MN to OPC fate change can occurred.

Taken together, my work reports a new role for Sulf2 as a positive Shh signaling regulator to control MN to OPC fate change in the ventral spinal cord.


**Discussion**

In the embryonic ventral spinal cord, Olig2 progenitors change their fate from the generation of MN to that of OPC. Shh is indispensable for this fate change. My work demonstrates that a novel function for Sulf2 as a HS 6-O-sulfatase is involved in the control of MN to OPC fate change in the mouse ventral spinal cord, which is similar to that of Sulf1 KO mouse. My study provides evidence that in the mouse ventral spinal cord, sulfatases (both Sulf1 and Sulf2) dorsal expansion are induced by strong Shh signaling, and sulfatases regulate MN to OCP fate switch by promoting strong Shh signaling input in the pMN domain.

Shh organizes the pattern of cellular differentiation in the ventral spinal cord via multiple mechanisms, such as transcriptional upregulation of Patched1, transcriptional downregulation of Gli and differential stability of active and inactive Gli isoform. In addition, Shh signaling is essential to generate OPCs at E12.5. Therefore, in the early phase Shh (mainly released from the notochord) is required for cell fate specification (domain formation) of the ventral spinal cord, and later is required for MN to OPC fate change.

Recent works in chick and mouse have pointed to a function for Sulf1 in regulating the Shh signaling in Olig2 progenitors to control MN to OPC fate change in the pMN domain. Moreover, in the zebrafish ventral spinal cord, Sulf1, follows Shh expression, promotes strong Shh signaling input into the neighboring dorsal cells, and subsequently, this strong Shh signaling further induces Shh and Sulf1 upregulation in the dorsal cells. In the mouse, expression of sulfatases (Sulf1 and
strong part of Sulf2) overlapped with Shh mRNA expression, and always follows the strong Patched1 expression (Shh signaling) from E10.5 to E12.5. These expression patterns gave an insight that similar to zebrafish, in the mouse strong Shh signaling input was promoted by Shh released by 6-O-sulfatase activities of Sulf1 and Sulf2, then strong Shh signaling in turn induces dorsal expression of Shh and sulfatases. My work showed that in both Sulf1 KO and Sulf2 KO mice, Shh expression was not successful in expanding into the p3 domain, and was restricted to the floor plate even at E11.5 or E12.5. Not only Shh expression, but also Shh signaling indicator Patched1 expression was changed in Sulf1 KO and Sulf2 KO mice. At E11.5, Patched1 expression was weaker than that in WT which is consistent with phenotype in Sulf1 knockdown zebrafish. At E12.5, Patched1 expression remained as E11.5 expression pattern; a strong signal accumulating in the pMN domain. These phenotypes support the hypothesis that a similar mechanism operating in the zebrafish is operating in the mouse. Sulf1 and Sulf2 are well known to specifically remove glucosamine 6-O-sulfate group on HS chains of HSPGs. In addition loss of Sulf1 and/or Sulf2 in mice significantly increases the amount of trisulfated disaccharides in the central nervous system and other organs. Importantly, HSPGs regulate Shh processing and release, and HS acts as a scaffold or activator for Shh ligands. Moreover SPR analysis showed that Shh preferentially binds to highly sulfated HS. Works in Drosophila and Xenopus showed that Sulf1 influences the Hh/Shh gradient by releasing Hh/Shh from cell surface. Therefore, it is reasonable to consider that Sulf1 and Sulf2 lower Shh/HSPGs interaction by removing 6-O-sulfate group on HS chains to release Shh.
Subsequently, released Shh diffuses dorsally and inputs strong Shh signaling into the dorsal neighboring cells. This is why strong Shh signaling input always precedes the expression of sulfatases (Sulf1 and Sulf2 strong part) from E10.5 to E12.5. Therefore, in the Sulf1 KO or Sulf2 KO mouse, inefficient cleavage of 6-O-sulfate of heparan sulfate results in insufficient supply of Shh to the dorsal region, which leads to weak Shh signaling input, particularly in the pMN domain at E11.5. It is well known that strong Shh activity in the pMN domain is required for MN to OPC fate switch and for the Nkx2.2 expression expands into the pMN domain in response to a rise in Shh signaling and Olig2, Nkx2.2 double positive cells then differentiate into OPC. Because of the failure in inducing Shh to express in the p3 domain, the accumulation of Shh signaling in the pMN domain was not sufficient in the E11.5 Sulf KO mice, so the accumulation of Shh signaling in the pMN domain continued till E12.5. These might have caused ventral shift of p3 and pMN domain and delay in the MN to OPC fate change. MN generation, which should be over by E12.5, was still on going, and OPC generation was markedly reduced at E12.5.

Although the dorsal expansion of Shh, Sulf1 and Sulf2 appeared to be induced by strong Shh signaling, Sulf2 or Sulf1 expression was normal in Sulf1 KO mouse or Sulf2 KO mouse, respectively, at both protein and mRNA level. It suggests that Shh concentration required in sulfatase expansion is lower than that in MN to OPC generation.

Sulf2 or Sulf1 expressed normally in Sulf1 KO mouse or Sulf2 KO mouse, which raises a question why residual Sulf2 or Sulf1 activity cannot compensate the loss of
Sulf1 or Sulf2 in the developing mouse spinal cord. There may be two possibilities. One is that there is interaction between Sulf1 and Sulf2 for their sulfatase activities. But this possibility was ruled out by sulfatase activity assay. Another one is that for precise MN to OPC fate switch, Shh concentration in the pMN domain is required to reach a threshold. Shh released by sulf1 or Sulf2 alone is not sufficient for precise MN to OPC fate switch. This hypothesis was confirmed by the analysis of Sulf1&2 DKO and Sulf1+/-Sulf2+/-.

All 4 types of Sulf mutant mice (Sulf1 KO, Sulf2 KO, Sulf1&2 DKO and Sulf1+/-Sulf2+/-) appeared to show similar phenotypes and severer phenotype was not observed in Sulf1&2 DKO mouse. It suggests that MN to OPC fate change is not linear against Shh dose dependent. Only when Shh concentration reaches a threshold, precise MN to OPC fate change can occur.

Shh activity has generally been regarded to function in a concentration-dependent manner, but the duration of Shh signaling can also affect cellular responses. In the vertebrate central nervous system, the pattern of cellular differentiation is regulated by both the concentration and the duration time of Shh exposure. Dessaud et al. showed that changing the concentration or duration of SHH has an equivalent effect on intracellular signaling, and cells remain sensitive to change in Shh signaling for an extended time, reverting to antecedent identities if signaling levels fall below a threshold. My study showed that lack of Sulf activity result in insufficient Shh signaling input in the pMN domain and delays MN to OPC fate change but not inhibiting OPC generation completely. This is consistent with Dessaud et al. studies. This also supports that the longer duration of Shh signaling can compensate for the
low level of Shh and to control cell differentiation.

My present work characterized new function of Sulf2 as a positive regulator of Shh dependent MN to OPC fate switch. Compared to Sulf1, Sulf2 distribution was more widely spread in the ventral spinal cord, which can be divide into 2 parts: strong part and weak part. In this study, I characterized the function of strong part which was overlapping with Sulf1 expression. However, I could not clarify the function of weakly expressing part during embryonic spinal cord development. One posssibility involves FGF (fibroblast growth factor) signaling. Dynamic changes in HS 6-O sulfation correlate with modulated susceptibility to FGF signal transduction during neural development and Sulf1 and/or Sulf2 null mice showed increased signaling in response to FGF-2. In addition, FGFs also act as OPC inducer in the dorsal spinal cord. It is possible that weak part of Sulf2-expressing region is associated with signaling regulated by FGFs.

Although further research is needed to tease out the Sulf2 function, my study characterized a new role of Sulf2 as a positive Shh signaling regulator to control MN to OPC fate change in the ventral spinal cord. My study also indicated that there is a threshold in sulfatase activity (which probably reflects in the Shh dosage) to induce MN to OPC fate switch.