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SOX9, acting downstream of Sonic hedgehog signalling, induces and maintains neural stem cells

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Neural stem cells (NSCs) are uncommitted cells of the central nervous system (CNS) defined by their multipotentiality and ability to self renew. We found that these cells are not present in substantial numbers in the CNS until after E10.5 in mouse and E5 in chick. This coincides with the induction of SOX9 in neural cells. Gain and loss-of-function studies indicate that SOX9 is essential for multipotent NSC formation. Moreover, Sonic Hedgehog is able to stimulate precocious generation of NSCs by inducing *Sox9* expression. SOX9 is also necessary for the maintenance of multipotent NSCs, as shown by *in vivo* fate mapping experiments in the adult subependymal zone (SEZ) and olfactory bulbs. In addition, loss of SOX9 leads ependymal cells to adopt a neuroblast identity. These data identify a functional link between extrinsic and intrinsic mechanisms of NSCs specification and maintenance, and establish a central role for SOX9 in the process.

Neural stem cells (NSCs) are self-renewing, multipotent cells that generate the main differentiated cell types of the nervous system, including neurons, astrocytes, oligodendrocytes^{1, 2} and ependymal cells that line the ventricles³. Understanding how NSC identity is established and maintained, as well as what regulates their proliferation and differentiation, is vital to realise their potential in regenerative medicine. The entire CNS is derived from cells of the neuroepithelium, which begins to form shortly after implantation of the embryo, coincident with gastrulation. At these early stages, the cells of the neuroepithelium undergo self-renewal and while they generate some neuronal subtypes, they produce few if any glial cells. The latter arise in earnest only from midgestation stages. In both the embryonic and adult brain, cells with radial glial or astroglial morphology, exhibit NSC-like characteristics and are responsible for generating many neuronal and glial cell types⁴. The prospective identification of these NSCs *in vivo* has been hindered, however, by the inability to identify molecular markers consistently and uniquely expressed in NSCs. Nevertheless, cells with NSC properties can be isolated from many regions of the embryonic and adult CNS and propagated *in vitro* as clonal populations of cells known as neurospheres⁵. These *in vitro* experiments can be complemented by *in vivo* cell fate assays, which allow retrospective identification of NSCs and tests of their properties following experimental manipulation.

Several extrinsic factors are thought to play critical roles in the maintenance and proliferation of NSCs. These include Wnts, FGFs, EGF, and Sonic Hedgehog (SHH)⁵⁻⁹. The latter can promote the proliferation and mobilization of NSCs both *in vitro* and *in vivo*¹⁰, in the adult⁸ and the embryo¹¹. However, the intrinsic factors regulated by SHH that control NSC properties, such as self-renewal and differentiation remain unclear. Several members of the SOX family of HMG transcription factors are expressed in NSCs, where they are thought to play important roles¹²⁻¹⁴. Notably, SOX2 is expressed in the epiblast (and by embryonic stem cells; ESCs), and subsequently in cells of the neuroepithelium and multipotent NSCs^{4, 12, 15} and has been linked to SHH signaling^{16, 17}. Nevertheless, although SOX2 appears necessary for NSC formation, its expression in the early embryo and in neuroepithelial cells prior to the emergence of NSCs (see below) indicates that it is not sufficient to explain the difference between neuroepithelial cells and multipotent NSCs.

We utilised the neurosphere assay⁵ to show that multipotent NSCs do not appear in substantial numbers until after E10.5 in mice and E5 in chick. These time points coincide with the onset of gliogenesis¹⁸. Two members of the SOXE family, SOX9 and SOX10, are expressed in radial glia, astrocytes and oligodendrocyte progenitors in the embryonic and adult CNS, and have been implicated in maintaining stem cells in other tissues^{12, 15, 18-22}. SOX9 is not detected in neuroblasts of the adult subependymal zone (SEZ), however it is expressed by transit amplifying cells and ependymal cells in the same region²³ (data not shown). Given the involvement of *Sox9* in the specification of glial cells^{18, 24} and data showing that certain types of glial cell can be NSCs⁴, we investigated the role of SOX9 in NSCs. Gain and loss of function experiments revealed that *Sox9* is both necessary and sufficient for neurosphere formation from neuroepithelial cells. We also found that SHH can induce precocious generation of multipotent NSCs, and appears to do so by inducing *Sox9* expression. In addition we found that *Sox9* is required to maintain multipotentiality of NSCs both *in vitro* and *in vivo* in the adult as well as embryonic CNS.

RESULTS

Induction of multipotent NSCs

The early neuroepithelium grows rapidly, largely by self-renewal of precursors, and while these precursors will eventually give rise to the entire adult CNS, this potential is not realised until later. This suggests that multipotent NSCs are not present at early stages. To define the timing of their appearance we used the neurosphere assay⁵. Consistent with previous studies, neurospheres were generated efficiently from E14.5 spinal cord and dorsal telencephalon (on average 215 and 308 neurospheres, respectively, from 5×10^5 cells) (**Fig. 1a**). Neurospheres could also be generated at E11.5 and were multipotent (**Supplementary Fig. 1c**), however, markedly fewer were obtained from E10.5 CNS tissue (**Fig. 1a**). Moreover, almost no neurospheres could be generated from E9.5 neural tissue (**Fig. 1a**), although isolated cells survived in culture. The few small aggregates that did form were not multipotent and were unable to generate secondary

neurospheres. A similar developmental profile of NSC formation was observed in a non-mammalian vertebrate: neurospheres were generated from E6 chick spinal cord and dorsal telencephalon (**Fig. 1b**). These neurospheres appeared to behave in the same way as mammalian neurospheres: passaging demonstrated self-renewal capacity and, upon differentiation, a mixture of neurons and glia were generated (**Fig. 1c** and **Supplementary Fig. 1a**). Prior to E6, however, significantly fewer neurospheres were produced from both anterior and posterior chick neural tissue (**Fig. 1b**). Thus, in both mouse and chick, neurosphere forming NSCs only appear in the CNS several days after neural induction.

Since SHH can promote the expansion of NSCs^{4, 11}, we asked whether SHH was sufficient to induce NSCs from neural tissue not normally capable of producing neurospheres. The addition of SHH to E9.5 mouse dorsal telencephalon and spinal cord tissue resulted in a ~25 fold increase in the induction and propagation of neurospheres (**Fig. 1d**). Passaging and differentiation assays confirmed that these contained cells with NSC properties (**Supplementary Fig. 1b**). Consistent with the role of Gli proteins as transcriptional mediators of SHH signalling, electroporation of an activated-*Gli* construct²⁵ was also sufficient to promote NSC properties in dorsal telencephalon and spinal cord tissue from E4 chick (**Fig. 1e**).

***Sox9* is expressed in NSCs**

Together these data predict that a key determinant of NSC formation must be activated in cells of the CNS after the formation of the neuroepithelium and it should be inducible by SHH signalling. Evaluation of the transcriptional changes induced in neural cells by SHH signalling focused our attention on *Sox9*, the expression of which was activated to a similar degree as other known target genes (**Fig. 1f**). We therefore investigated the expression of SOX9. Neurospheres derived from the dorsal telencephalon and spinal cord contained many cells expressing SOX9 and the related SOXE protein, SOX10. The expression of these proteins largely coincided with SOX2, which marks proliferating neural progenitors, moreover, SOX9 was expressed by radial glial-like NS5 cells¹³ (**Supplementary Fig. 2a, b**).

In vivo, the expression of SOX9 corresponds with sites containing neurosphere-forming NSCs and SOX9 was expressed in cells positive for GLAST, a marker of radial glia and astrocytes^{4, 18, 24} (**Fig.2a, b, Supplementary Fig.8, 11**). Prior to E10.5, SOX9 expression was low or undetectable in neural tissue. Expression commenced in a few SOX2-positive neuroepithelial cells at E10.5 (the spinal cord at E10.5 expressed higher levels of SOX9 in more ventral regions), was found in many by E11.5, and continued throughout embryogenesis. From E11.5, SOX9 was co-expressed with SOX2 in almost all cells of the developing CNS (**Fig. 2a, b, Supplementary Fig. 11a,b**). Moreover, co-expression of SOX9 with SOX2 was observed in the adult SEZ (Fig. 2c), which harbours NSCs and can generate neurospheres, and in the ependymal layer (**Fig. 2c**). To determine whether SOX9 was present in slowly dividing quiescent NSCs as well as more proliferative progenitors, adult animals were pulsed with bromodeoxyuridine (BrdU) and the distribution of BrdU and SOX9 assessed in the SEZ (**Fig. 2d**) between 24 h and 3 months later. Almost all BrdU positive cells expressed SOX9 at each time point, including those 3 months after administration (**Fig. 2d**). This suggests that SOX9 is expressed in cells with the low mitotic index characteristic of quiescent stem cell populations²⁶. Together these data indicate that SOX9 is expressed in regions of neural tissue that generate neurospheres, in cells with the attributes of NSCs.

***Sox9* is sufficient to induce NSC formation**

We next asked whether SOX9 was sufficient to induce neurosphere forming multipotent NSC in the CNS. We took advantage of mice carrying a conditional *Sox9* transgene (*Z/Sox9*) (**Fig. 3a, Supplementary Fig. 7**) in which Cre-mediated recombination results in the stable expression of SOX9. Few if any neurospheres were produced from E9.5 control neural tissue. In contrast, after transfection with a Cre-expression construct, neurospheres were generated at a frequency equivalent to those produced from E9.5 neural tissue exposed to SHH (**Fig. 3b**). Passaging of primary neurospheres generated in this way produced many secondary neurospheres, indicating that expression of *Sox9* was sufficient to confer self-renewal (**Fig. 3b**). Differentiation demonstrated that both primary and secondary neurospheres were multipotent (**Fig. 3c, d**).

Notably, the ratio of neurons to glia produced on differentiation of these neurospheres was comparable to that obtained from neurospheres derived from E14.5 and E11.5 wild-type dorsal telencephalon or spinal cord, rather than the predominant neuronal differentiation obtained from E9.5 tissue (**Fig. 3c, d, Supplementary Fig. 1c**). This further suggested that expression of *Sox9* in E9.5 neural cells was sufficient to induce NSCs with the properties of those found in the more mature CNS. Consistent with SOXE proteins having a conserved role in the formation of NSCs, similar data was obtained in the chick (**Supplementary Fig. 4d**). Together these results indicate that SOX9 is sufficient to promote NSC formation.

These data prompted us to determine whether SOXE proteins enhanced NSC formation in post-natal neural tissue. In agreement with previous studies, the SEZ of adult mice produced neurospheres⁴. Expression of *Sox9* by transfection of Cre into the SEZ of adult *Z/Sox9* mice resulted in a two-fold increase in the number of primary neurospheres (**Fig. 3e, f**). Moreover, the generation of neurospheres occurred more rapidly after induction of *Sox9* (**Fig. 3e**). Significantly, these produced secondary neurospheres at twice the rate of untransfected neurospheres (**Fig. 3f**). Differentiation of both primary and secondary neurospheres generated following Cre-mediated activation of *Z/Sox9* were multipotent (**Fig. 3g**), however their potential shifted towards gliogenesis, with 3 fold more astrocytes and half as many neurons differentiating compared to controls (**Fig. 3g**). These data suggest that misexpression of *Sox9* in adult neural cells was sufficient to enhance neurosphere generation *in vitro* and to increase the number of NSCs in these neurospheres.

Sox9 is required for NSC properties *in vivo* and *in vitro*

Germline deletion of *Sox9* results in severe defects in NSCs but also morphological defects in the CNS precluding a full assessment of NSC formation and behaviour (**Supplementary Fig. 3b, c**). We therefore generated embryos lacking *Sox9* specifically in the CNS using Nestin-Cre²⁷ induced recombination of a conditional allele (**Fig. 4b**)^{18, 24, 28}. Animals with this genotype die at or just after birth. We assessed the dorsal telencephalon at E14.5 (**Fig. 4a, c**) and the SEZ at E18.5 *in vivo* (**Fig. 5a,**

Supplementary Fig. 6a). At E14.5 a reduction in S100+ astrocytes, NG2+ oligodendrocyte precursors and TuJ1+ neurons was observed together with an increase in PSA-NCAM+ neuroblasts (**Fig. 4a, c**). At E18.5, no astrocytes, fewer neurons and more neuroblasts were observed in neural tissue lacking *Sox9* compared to wild type littermates (**Fig. 5a, Supplementary Fig. 6a**). In addition to this, we observed larger lateral ventricles in null embryos compared to wild type littermates (**Fig. 5a**). This is reminiscent of the dilated cerebral ventricles observed in individuals with Campomelic Dysplasia (caused by heterozygous mutations of *SOX9*)²⁹.

To assess NSCs, we assayed neurosphere formation at E14.5 (**Fig. 4d**) and E18.5 (**Fig. 5b**). There was a significant reduction in the numbers of neurospheres generated from the CNS of embryos lacking *Sox9* in these regions compared to wild type and heterozygous littermates (**Fig. 4d, 5b**). Moreover, the frequency of secondary neurosphere generation from *Sox9*-deficient primary neurospheres was also significantly reduced (**Fig. 4d, 5b**). These data provide evidence that SOX9 is required for the maintenance and production of NSCs. Nevertheless, some NSCs were present even in the absence of *Sox9*. One explanation for this could be functional substitution by SOX10. Consistent with this, the proportion of cells expressing SOX10 was markedly increased in neurospheres generated from *Sox9* deficient neural tissue (**Supplementary Fig. 4b**). This suggests that there is a selective generation of neurospheres from SOX10 expressing NSCs in the absence of SOX9 (**Supplementary Fig. 4a**). Differentiation of E14.5 *Sox9* deficient neurospheres resulted in the generation of neurons and astrocytes. However, astrocyte numbers were reduced (**Fig. 4e, f**). No oligodendrocytes were apparent in these cultures (**Fig. 4e, f**). Differentiation of *Sox9* deficient neurosphere cells at E18.5 revealed a similar pattern: there was a severe reduction in numbers of astrocytes and oligodendrocytes, but there was also a significant decrease in neurons (**Fig. 5c, Supplementary Fig. 6b**). Together these data suggest that SOXE proteins, and particularly SOX9, are crucial for the specification of multipotent NSCs within the developing and adult CNS.

***Sox9* acts downstream of SHH to induce and maintain NSCs**

We next turned our attention to the relationship between SHH signalling and *SoxE* genes. We first analysed whether SHH induced *Sox9* or *Sox10* expression. Consistent with the transcriptional profiling data, exposure of neuroepithelial cells to SHH induced *Sox9* and *Sox10*, with similar kinetics (**Fig. 6a**). We also found a modest increase in *Sox2* expression. We then asked whether blocking SOXE activity inhibited the NSC promoting activity of SHH signalling. For this experiment, E4 chick neural cells, which normally produce few if any NSCs, were transfected with Gli3A^{HIGH}, a dominant active version of a transcriptional mediator of SHH signalling²⁵. As expected, this resulted in the generation of significant numbers of neurospheres (**Fig. 6b**), similar to the numbers induced either by the addition of SHH itself, or by forced *Sox9* expression. In contrast, co-transfection of a dominant negative version of SOX9 together with Gli3A^{HIGH} inhibited the NSC-promoting activity of the latter (**Fig. 6b**).

We next asked if SOX9 activity is sufficient to induce NSC when SHH signalling is inhibited. The number of neurospheres formed from E14.5 mouse dorsal telencephalon was reduced significantly by blocking SHH signalling with cyclopamine³⁰ (**Fig. 6c**). This inhibitory effect of cyclopamine was, however, reversed by transfection of Cre into *Z/Sox9* cells (**Fig. 6c**). Together these data provide evidence that the effects of SHH signalling on NSC formation depend on induction of *Sox9* and possibly other *SoxE* genes.

Adult NSCs of the SEZ require *Sox9* for multipotentiality

Our *in vitro* and *in vivo* data showed that *Sox9* is necessary for multipotentiality of NSCs during development. We therefore asked whether *Sox9* is required for multipotentiality *in vivo* in the adult mouse SEZ. A Cre expressing adenovirus³¹ was injected into the lateral ventricle of mice carrying Flox-*Sox9* and/or R26R^{EYFP} alleles^{32, 33}. This targets the cells lining the ventricle, including NSCs which have been shown to extend processes through the ependymal cell layer to the ventricle⁴. Cre-mediated recombination was reported by the expression of EYFP, which also allowed the fate of the cells to be tracked *in vivo*. Analysis of mice containing only the R26R^{EYFP} allele revealed that the infected cells of the SEZ expressed SOX9, SOX2 and proliferate (Ki67

positive) (**Fig. 7b, e**). In addition, GFP labelled cells gave rise to astrocytes (as marked by GFAP), oligodendrocytes (NG2), and neurons (NeuN), showing that NSC's are indeed being targeted by the adenovirus (**Fig. 7a, c, d**). We used the neuroblast markers PSA-NCAM and DCX to reveal GFP positive neuroblasts (**Fig. 7a, d, Supplementary Fig. 9a**). Flox-*Sox9*^{het}; R26R^{EYFP} SEZ cells infected with Cre also expressed SOX9 and SOX2, proliferated (**Fig. 7b, e**), and were multipotent, although the relative proportion of glial cell types was reduced compared to the R26R^{EYFP} controls (Fig. 7a, c, d). By contrast, Flox-*Sox9*^{hom}; R26R^{EYFP} SEZ cells no longer expressed SOX9 after Cre-mediated recombination (**Fig. 7 b, e**). Ki67 expression was much reduced in these cells, while SOX2 expression appeared unaffected. We failed to detect GFP-positive cells expressing GFAP, NG2 or NeuN, suggesting that in the absence of SOX9, NSCs lose the ability to give rise to astrocytes, oligodendrocytes and neurons (**Fig. 7a, c**). Moreover, the expression of Arl13b and CD133 were also markedly decreased in targeted cells suggesting that ependymal cells were also lost (**Supplementary Fig.9b, Supplementary Fig. 10a, b**). By contrast many GFP positive cells expressing the neuroblast markers PSA-NCAM and DCX were observed in the region of the SEZ (**Fig. 7a, d, Supplementary Fig. 9a**) and the rostral migratory stream (RMS). Thus, loss of SOX9 appears to promote the differentiation of cells into PSA-NCAM/DCX-positive cells that resemble neuroblasts (**Fig. 7a, Supplementary Fig. 9c**). This might be a consequence of NSCs acquiring neuroblast identity and/or the ependymal cells differentiating into neuroblasts³⁴.

We then analysed neurogenesis in the olfactory bulbs of mice 6 weeks after the initial Cre virus injection. Compared to controls, there was a marked increase in the number of transfected cells in the olfactory bulbs of Flox-*Sox9*^{hom}; R26R^{EYFP} mice (**Fig. 8b**). Strikingly, these cells expressed the neuroblast marker PSA-NCAM, and almost none of the cells lacking *Sox9* expressed the mature neuron marker NeuN. No difference in cell death was observed. Moreover, a larger proportion of the transfected cells in the olfactory bulb of Flox-*Sox9*^{hom}; R26R^{EYFP} mice retained the BrdU label, compared to the control mice (**Fig. 8a, c**). Together these data suggest that SOX9, in the adult SEZ, is required for NSC self-renewal, multipotentiality and for the maintenance of ependymal

cells. The removal of *Sox9* results in these cells differentiating into neuroblasts and migrating into the olfactory bulb.

DISCUSSION

Our data provide evidence that SOX9 is a key determinant of multipotent NSCs in both the embryonic and adult CNS. We show that the NSC promoting activity of SHH signalling is mediated at least in part by induction of *Sox9*. Previously, SOX9 has been shown to be expressed by radial glia^{18, 22}, at least some of which possess NSC characteristics⁴ and had been implicated in the switch from neurogenesis to gliogenesis^{18, 24} in progenitors of the embryonic spinal cord. Here we show that SOX9 is expressed at times and in locations where NSCs reside in cells displaying the hallmarks of NSC identity. In addition, radial glial-like NS5 cells¹³ express SOX9. Consistent with the association between SOX9 expression and NSC activity, both loss and gain of function experiments indicate that SOX9 is required for the generation and maintenance of multipotent NSCs in the embryo and the generation of neurospheres *in vitro*.

The data reveal a link between SHH signalling and SOX9 expression within the CNS. SHH and *Gli*-activators are able to induce *Sox9* transcription and result in neuroepithelial cells precociously acquiring neurosphere forming ability. Moreover, the effect of ectopic SHH on cells within the adult SEZ⁸ is mimicked by ectopic SOX9. Whether this regulation of *Sox9* by SHH is direct is not known. However, *Sox9* expression also appears to require SHH signalling in hair follicles²⁰, where an enhancer element 1.1 Mb 5' to *Sox9* is reported to show direct activation by *Gli*^{20, 35}, raising the possibility of direct regulation also in neural tissue.

How SOX9 exerts its action remains to be determined. Like other members of the SOX family, its activity is context dependent and usually reliant on partner proteins. SOX2, which is associated with all multipotent cells of the CNS, represents a candidate for one such partner. The induction of SOX9 *in vivo* (and/or SOX10) correlates with, and appears to be required for, the transition of SOX2 expressing neuroectoderm progenitors to a multipotent stem cell identity and from E11.5

onwards SOX9 is coexpressed with SOX2 in almost all cells of the developing CNS. It is noteworthy that prior to the emergence of neurosphere forming NSCs in neural tissue, SOX9 is critical for the generation of neural crest from the dorsal neural tube, where its relationship to SOX2 is antagonistic rather than cooperative^{19,36}. Therefore, expression of SOX9 in the absence of SOX2 may characterise a neural crest progenitor whereas the later induction of SOX9 in SOX2 expressing neuroectoderm cells promotes NSC formation.

Misexpression of SOX9 is not sufficient to turn all SOX2 expressing neuroepithelial cells into neurosphere forming NSCs. Indeed, the frequency of neurosphere generation was never more than about 1%, whether from the telencephalon, spinal cord or primary neurospheres. It is likely that some of the remaining SOX9-positive cells are committed progenitors of astrocytes or oligodendrocytes. Moreover, this suggests that other mechanisms, such as lateral inhibition via Notch signalling, contribute to the acquisition of NSC properties³⁷. Recently it has been shown that Wnt signalling is required for single cell expansion and multipotentiality during development *in vitro*³⁸, while inhibition of BMP signalling in the adult hippocampus can expand NSCs *in vitro* and *in vivo*³⁹. Thus a number of signalling pathways must contribute to NSC character, perhaps with region specific variations.

Previously, SOX9 was shown to be essential for the ‘switch’ from neurogenesis to gliogenesis in caudal regions of the developing CNS¹⁸. In the absence of *Sox9*, astrocyte and oligodendrocyte progenitor numbers were reduced, and a transient increase in motor neurons was observed¹⁸. In agreement with these data, our findings indicate a decrease in astrocyte and oligodendrocyte differentiation, *in vitro* and *in vivo*, in anterior regions of the CNS in the absence of *Sox9*. Concomitant with this, the number of PSA-NCAM-positive neuroblasts was increased. Moreover, the pattern of neurons throughout the cortex was disrupted in *Sox9* mutant embryos. This was particularly evident at E18.5 where there also appeared to be a complete loss of astrocytes. This suggests that in the absence of *Sox9*, cells adopt a less mature state rather than progressing to a fully multipotent NSC.

The genetic cell fate mapping in adult mice revealed that *Sox9* is also required to maintain NSC multipotentiality within the SEZ of the lateral ventricles. Cells lacking *Sox9* fail to give rise to astrocytes, oligodendrocytes and neurons. Proliferation was also markedly attenuated. However, the number of neuroblasts differentiating from *Sox9* null cells was increased, both within the SEZ and olfactory bulbs. Consistent with the idea that SOX9 inhibits neuronal differentiation, knockdown of miR-124, which targets *Sox9*, has been shown to increase SOX9 expression and decrease neurogenesis²³. However, this study did not address the effect of the complete loss of SOX9 on neurogenesis, nor the role of SOX9 on NSC function. Our data indicate that neurogenesis in the olfactory bulb is decreased in the absence of *Sox9* and cells lacking *Sox9* remain as neuroblasts. The failure of these neuroblasts to progress to mature neurons (which does occur in *Sox9* heterozygotes), could indicate an additional intrinsic role for at least low levels of SOX9 or a requirement for the simultaneous production of new glial cells.

Strikingly, many of the GFP-expressing *Sox9* null cells that acquired neuroblast marker expression were located in the position normally occupied by ependymal cells, i.e. the cell layer lining the ventricles. These cells lacked cilia and no longer appeared epithelial (**Supplementary Fig. 9b**), suggesting that ependymal cells lacking SOX9 had differentiated into neuroblasts. This finding is in agreement with a recent study indicating that ependymal cells can give rise to neuroblasts³⁴. Furthermore, embryos lacking *Sox9* throughout the CNS do not form CD133 positive ependymal cells surrounding the lateral ventricle at E18.5 and P0 (**Supplementary Fig. 10a, b**). Since radial glia give rise to ependymal cells³, and glial-like stem cells are compromised in the absence of *Sox9*, the effect on ependymal cell generation would be expected. In addition, however, our data suggest that *Sox9* is required for the maintenance of differentiated ependymal cells.

It is noteworthy that there is no significant difference in the overall proliferation, or the numbers of mature neurons or neuroblasts between the olfactory bulbs of R26R^{EYFP} mice and Flox-*Sox9* homozygous; R26R^{EYFP} mice (**Supplementary Fig. 12**). This suggests that the differences observed for the recombined cells, in which *Sox9* is no longer expressed, is due to a lack of SOX9 and not to a hypomorphic effect of the Flox-*Sox9* allele.

Together, these data provide evidence that SOX9 is required for multipotentiality of NSCs, and that in its absence, cells adopt an early neuronal fate. This is consistent with a model in which SOX2 expressing progenitors in the early neuroectoderm gain multipotentiality by expressing SOX9. In this view, a multipotent neural stem cell would be required to express SOX2 in order to generate neurons, SOX9 to give rise to astrocytes and SOX10 in order to generate mature oligodendrocytes¹⁸. Since SHH expression appears to be regulated by *Sox2*¹⁶, at least in the hippocampus, it is possible that SOX2 expressing NE cells gain multipotentiality by inducing SHH expression, which in turn, either directly or indirectly, induces the expression of SOX9 (**Fig. 6**).

As well as CNS tissue, *Sox9* appears to function in several other tissue stem cells. *Sox9* plays an essential role in stem cells in hair follicles of adult skin⁴⁰. In addition, SOX9 is required in multipotent mouse retinal progenitor cells⁴¹ and associated with stem cells in the gut and lung^{42, 43}. Furthermore, the observation that SOX9 is highly up-regulated in several neural tumours^{44, 45} and basal cell carcinomas²⁰ raises the possibility that it confers stem-like properties to tumour cells. The manipulation of SOX9 expression or activity may therefore have several applications in addition to providing further understanding of the stem cell state.

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Author Contributions

CES, JB and RLB initiated the project. CES performed the *in ovo* electroporations, neurosphere culturing, immunohistochemistry, RT-PCR and quantification and data analysis of all the *in vivo* and *in vitro* experiments, except for the acquisition and analysis of the microarray data (CC). SLW, BG and KSEC generated the *Z/Sox9* mice. MC supplied many of the constructs used in this study. AS performed the adenovirus injections and SB perfused the adult mice. MVGG carried out the BrdU injections. CES, RLB and JB were involved in the study design and wrote the manuscript.

METHODS

Mouse lines.

Mice containing a *Sox9^{Flox}* allele³³ were crossed with the transgenic line nestin-Cre^{24, 27}. Generation of *Z/Sox9* mice: A 4.8 kb fragment of mouse genomic DNA (from 82bp upstream of the start of transcription of *Sox9* to 1119bp downstream of the polyadenylation sequences), including the *Sox9* coding region, its two introns and 1.1 kb of 3' flanking DNA, together with an IRES2-EGFP (Clontech) sequence inserted between the *Sox9* stop codon and the polyadenylation site (at + 3237 bp), was cloned downstream of the *loxP*-flanked β geo/3xpA of the pCall2 vector (gift of A. Nagy⁴⁶) to generate *pZ/Sox9* expression construct (Supplementary Fig. S5). *pZ/Sox9* was transfected into R1 embryonic stem (ES) cells⁴⁷ by electroporation and ES clones containing a single copy of the transgene were used to generate the mouse line *Z/Sox9*. All experiments on mice and chicks were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Expression Constructs and Chick *in ovo* electroporation

The *Sox9*, VP16-*Sox9*, dominant negative *Sox9*, Gli3A^{HIGH}, Ptc1^{Δloop2} and GFP constructs have been described previously^{19, 25, 36, 48}. The CMV:cre construct was a gift of S. O'Gorman, (Salk Institute, La Jolla, CA). *In ovo* electroporations of E3 chick embryos were performed as previously described¹⁹.

Neurosphere culture and differentiation

The neurosphere growth and differentiation assays with cells from the dorsal telencephalon (DT) and spinal cord (SC) of mouse and chick embryos between E9.5 – E14.5 and HH 18_29, respectively, were carried out as described^{5, 49}. SHH protein (60 µg ml⁻¹), control protein (CP) (60 µg ml⁻¹), or cyclopamine (5 µM) was added on the third day of mouse primary neurosphere culture, and replenished after each media change. Dissociated embryonic DT and SC from *Z/Sox9* mice were electroporated with a Cre expression construct. Adult mouse single cells from the SEZ were transfected using an Amaxa nucleofactor, according to the manufacturers instructions. Quantification of differentiated cell types: The percentages of DAPI positive cells co-stained for specific markers was calculated in 4 random fields of view in 3 separate wells (n = 12).

Immunohistochemistry and BrdU incorporation

BrdU injections were carried out as described in²⁶. Differentiated neurospheres and tissue sections were fixed, incubated in blocking solution (PB, 0.1% triton, 1% donkey serum) for 30 minutes, then immunohistochemistry performed with SOX9 (R&D Systems), SOX10³⁶, SOX2 (Millipore), TuJ1 (Covance), GFAP (Sigma), CNPase (Millipore), NG2 (Millipore), PSA-NCAM (Millipore), S100 (Dako), GLAST (Millipore), DCX (Santa Cruz), Ki67 (abcam), BrdU (abcam), CD133 (eBiosciences), cleaved PARP (abcam), Arl13b (gift from T. Caspary). Immunofluorescence images were obtained with a Leica TCS SP confocal using a UV 10x/0.4 or 40x/0.5 NA dry HC-PLAPO lens (Leica). Images were processed using the Adobe Photoshop CS v8.0 (Adobe Systems) software.

Adenovirus injection

An adenovirus expressing Cre³¹ was diluted to 4 x 10⁷ pfu ml⁻¹ in artificial cerebrospinal fluid and 5µl injected into the right lateral ventricle of the following adult (8- 12 weeks): R26R^{EYFP}, Flox-Sox9^{het}; R26R^{EYFP}, Flox-Sox9^{hom}; R26R^{EYFP}, 3 mice per group. In relation to the bregma the injection was anterior 0mm; lateral 1.4 – 1.3 mm; ventral 3.5 mm. 4 weeks after the injection, mice were perfused with 4% paraformaldehyde and the brains sectioned using a vibratome to a thickness of 70 µm. Sections were blocked using 10% donkey serum for 15 minutes and then immunohistochemistry carried out as described above. For analysis of neurogenesis in the olfactory bulbs, the Cre adenovirus was injected as described above, 3 weeks after this injection, BrdU injections were carried out as described in²⁶. The mice were perfused 6 weeks after the initial injection of the adenovirus expressing Cre. Quantification: The total number of recombined (EYFP / GFP positive) cells were counted in 4 SEZ or olfactory bulb sections from 3 different mice. The percentage of these GFP cells co-stained for specific markers was then calculated.

RT-PCR

RNA was extracted from neurospheres (Qiagen RNeasy kit) and reverse transcribed with Omniscript RT (Qiagen) kit. PCR primer sequences were as follows; *Sox2*, forward 5'-CACAACCTCGGAGATCAGCAA, reverse 5'-CTCCGGGAAGCGTGTACTTA; *Sox8*, forward 5'-AGGCGAAGGAAGAGTGTGAA, reverse 5'-CCCTTCCAGCCTTAGCTCTT; *Sox9*, forward 5'-AGCTCACCAGACCTGAGAA, reverse 5'-CTCCAGCAATCGTTACCTTC; *Sox10*, forward 5'-AGGCCTCACTGCTCCTGTTA, reverse 5'-TTGACCAGTTCACATTCA; *Actb*,

forward 5'-TCATGCCATCCTGCGTCTGGACCT, reverse 5'-
CCGGACTCATCGTACTCCTGCTTG. PCR profile: 94 °C, 15' (1 cycle), 95 °C, 30", 55 °C,
30", 72 °C, 60" (30 cycles) and 72 °C, 8' (1 cycle).

Acquisition and analysis of microarray data

HH 11 embryos were electroporated *in ovo* with Gli3A^{HIGH}, Ptc1^{Δloop2} or GFP, and embryos collected either 14h or 36h later. Transfected cells were collected by FACS and expression profiled using GeneChip Chicken Genome Array (Affymetrix). Hybridization to microarrays, sample preparation and array processing were carried out according to manufacturers instructions (Affymetrix). Analysis of microarray data was performed using GeneSpring 7.2 and Bioconductor⁵⁰. Protocols as described in Cruz C. et. al., submitted.

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Figure 1: Acquisition of neurosphere forming ability in mouse and chick CNS and the effect of SHH signalling.

(a), Number of neurospheres generated from 5×10^5 cells from mouse dorsal telencephalon (DT) and spinal cord (SC) between E9.5 and E14.5. Few if any neurospheres form prior to E10.5. (b), Few neurospheres are formed from chick DT until E4 and from SC until E6. (c), Assay of the differentiation potential of E14.5 mouse and E6 chick DT neurospheres indicated that similar ratios of neurons (TuJ1), oligodendrocytes (CNPase) and astrocytes (GFAP) were obtained from both species. Quantitation was performed on 100 cells in 4 independent fields of view. (d), In comparison to control protein (CP), exposure to SHH promoted neurosphere formation from E9.5 mouse DT cells and enhanced self-renewal of NSCs as shown by increased secondary neurosphere formation. (e), E4 Chick SC cells electroporated with GFP gave rise to few if any neurospheres. By contrast, expression of Gli3A^{HIGH} significantly increased neurosphere formation (n = 12). (f), Evaluation of transcriptional changes in HH11 chick neural cells indicated that *Sox9* expression was induced by SHH signalling. Columns represent the mean expression of three biological replicates for neural cells expressing each of the indicated constructs; a = Ptc1^{Δloop2}, b = GFP, c = Gli3A^{HIGH}. Rows represent the expression level of the indicated genes, increased expression in gold, decreased in blue.

Figure 2: The expression of SOX9 coincides with the formation of NSCs.

Immunohistochemistry on horizontal sections for SOX2, SOX9 and GLAST, indicated that SOX2 was expressed at all stages from E9.5-18.5 in the dorsal telencephalon (top is rostral, bottom is caudal) (a) and spinal cord (top is dorsal, bottom is ventral) (b) as well as in the adult (8 week) subependymal zone (SEZ) and ependymal cells (c). SOX9 was absent at E9.5 and expressed in only a small number of cells at E10.5, but in many progenitors from E11.5, including in the adult SEZ. From E10.5, many of the SOX2+ cells co-express SOX9 (a, b). (d), Sections of brains from adult mice in which BrdU was injected daily for 12 consecutive days, prior to assaying 1, 3, 7, 28 and 84 days after the last injection. Immunohistochemistry for BrdU and SOX9 indicated that more than 80% of BrdU positive cells were SOX9 positive at each assay point, and 100% were positive after 28 days. (e), Illustration of a coronal section showing the SEZ (boxed area). Scale bars = 50 μm.

Figure 3: Ectopic *Sox9* can induce precocious neurosphere formation from embryonic CNS, and increase neurosphere formation from the adult SEZ.

(a), Schematic of the *Z/Sox9* transgene; *Sox9* is expressed after cre-mediated removal of the loxP flanked LacZ gene. (b), In contrast to controls, primary neurospheres were obtained from the dorsal telencephalon and spinal cord of E9.5 *Z/Sox9* transgenic embryos transfected with Cre. These were able to generate many secondary neurospheres. There was a significant difference in the number of primary neurosphere between *Z/Sox9* (Tg+Cre) dorsal telencephalon and spinal cord and controls, (Tg-Cre and WT) (Student's t-test, P<0.002). (c, d), The proportion of cell types differentiating from E9.5 dorsal telencephalon (c) and spinal cord (d) after cre-mediated expression of *Sox9* (E9.5+S9)

were comparable to those from WT E14.5 DT (c) and E11.5 SC (d) cultures respectively. Cell type-specific markers: TuJ1, neurons; CNPase, oligodendrocytes; GFAP, astrocytes. (e), Cells from the adult subependymal zone (SEZ) from Z/Sox9 mice were electroporated with Cre. After 4 days in culture, neurospheres were already apparent in Cre electroporated cultures (Tg + Cre), whereas no neurospheres were visible at this stage in controls (Tg – Cre). Scale bars = 50µm. (f), Significantly more neurospheres were generated in Cre transfected samples compared to control cultures. ($P < 0.005$, Student's t-test ($n = 12$)). The number of secondary neurospheres was also increased. (g), Differentiation of primary neurospheres from the SEZ of Z/Sox9 mice after electroporation with Cre. The number of TuJ1 neurons, O4 oligodendrocytes and GFAP astrocytes were counted and plotted as fold change relative to cells differentiating from WT tissue. More astrocytes and less neurons formed from Tg + Cre compared to WT. Bars indicate standard error.

Figure 4: Sox9 is necessary for neurosphere formation.

Embryos lacking *Sox9* throughout the developing CNS were generated by nestin specific Cre-mediated recombination of the *Flox-Sox9* targeted allele (b). (a, c), *In vivo* analysis at E14.5 revealed that S100+, NG2+, TuJ1+ and BrdU+ cells were much reduced in the *Sox9* null DT. By contrast, PSA-NCAM+ cells were increased in *Sox9* null tissue. White lines demarcate the telencephalic vesicle. Scale bars = 50µm. (d), Spinal cord (SC) and dorsal telencephalon (DT) were dissected at E14.5. In contrast to wild type (WT) and heterozygous (het) controls, *Sox9* null tissue (null) from both regions gave rise to small numbers of neurospheres. In dorsal telencephalon cultures, 80% fewer neurospheres were generated from *Sox9* null cultures than WT ($P < 0.008$). Neurospheres from *Sox9* deleted CNS tissue also gave far fewer secondary neurospheres. In *Sox9* null spinal cord cultures there was a four-fold decrease in primary and secondary neurosphere generation versus WT ($P < 0.001$) ($n = 5$). Bars indicate standard error. (e), Neurospheres or cells generated from *Sox9* null dorsal telencephalon tissue were differentiated for 5 days and immunohistochemistry carried out. These cells did not express SOX9 and were not able to give rise to oligodendrocytes (CNPase). They did express SOX2 and were able to generate neurons (TuJ1) and astrocytes (GFAP). However, *Sox9* null astrocytes lacked long processes typical of astrocyte morphology. Scale bars = 20 µm. (f), The proportion of cells differentiating from *Sox9* null cultures compared to WT cultures. Fewer astrocytes and oligodendrocytes, but a similar number of neurons were observed. Error bars indicate standard error.

Figure 5: Sox9 is necessary for neurosphere formation at E18.5, and required for multipotentiality *in vitro* and *in vivo*.

(a), Coronal sections (12µm) of brains from E18.5 embryos deficient in *Sox9* (null) revealed a loss of GFAP (astrocytes) expression compared to wild type (WT) tissue. Neurons (TuJ1) and BrdU+ cells were observed in the mutants, but fewer than in controls. PSA-NCAM+ cells were reduced in null dorsal telencephalon tissue compared to WT. In addition, larger, miss-shapen lateral ventricles were observed in null tissue when compared to WT CNS. White lines indicate the lateral ventricles of the subependymal

zone. Scale bars = 50 μ m. (n = 9). **(b)**, *Flox-Sox9*; Nestin Cre mice were dissected at E18.5. *Sox9* null tissue from both dorsal telencephalon ($P < 0.001$) and spinal cord ($P < 0.001$) (n = 5, Student's t-test, bars indicate standard error) produced very few neurospheres compared to wild type (WT) and heterozygous (het) controls. **(c)**, Upon differentiation, E18.5 *Sox9* null dorsal telencephalon (null) produced fewer astrocytes (GFAP), oligodendrocytes and neurons (TuJ1) than WT. In addition, astrocytes from *Sox9* null embryos lacked long processes and only weakly expressed GFAP compared to WT astrocytes. Scale bars = 20 μ m.

Figure 6: SOX9 and SOX10 mediate the effects of SHH signalling.

(a), RT-PCR demonstrates the induction of *SoxE* genes in E9.5 DT and SC cells after the addition of SHH for 18 or 24 hours compared to control protein (CP). *Sox2* was expressed with or without addition of SHH. β -actin was used to assess input RNA levels. (The gels have been cropped, for full gel images see Supplementary Figure 13). **(b)**, Few neurospheres were generated from E3 chick neural tubes dissected 24 hours after electroporation with pCAGGS-IRES-nls-GFP (GFP). By contrast when Gli3A^{HIGH} (Gli), a constitutive activator form of Gli3 was introduced by electroporation, many neurospheres were obtained. Likewise, the transfection of WT *Sox9* (pCAGGS-*Sox9*-IRES-nls-GFP) induced substantial numbers of neurospheres. Co-electroporation of a dominant negative form of SOX9 (d/n S9), lacking the transactivation domain, together with Gli3A^{HIGH}, resulted in a reduction in neurosphere generation, compared to Gli3A^{HIGH} alone. **(c)**, Cells from *Z/Sox9* E14.5 mouse dorsal telencephalon were able to form many neurospheres after electroporation with GFP, whereas the addition of 5 μ M cyclopamine (cyc) reduced the number generated by half. However, the expression of *Sox9* by electroporation of Cre (*Z/Sox9*) restored the ability of cyclopamine treated cells to generate substantial numbers of neurospheres. Bars indicate standard error. (n = 12).

Figure 7: Sox9 is necessary for NSC function in vivo in the adult.

R26R^{EYFP}, *Flox-Sox9*^{het}; R26R^{EYFP} (het) and *Flox-Sox9*^{hom}; R26R^{EYFP} (hom) adult (8- 10 week) mice were injected in one lateral ventricle with an adenovirus expressing Cre. Analysis was carried out 4 weeks post injection. GFP identifies cells in which Cre-mediated recombination has taken place. **(a)**, The fate of the cells surrounding the lateral ventricles after recombination were analysed. GFP cells in the R26R^{EYFP} mice gave rise to GFAP⁺ astrocytes, NG2⁺ oligodendrocytes, NeuN⁺ neurons and neuroblasts expressing PSA-NCAM and DCX. GFP cells from hom animals generated few astrocytes, oligodendrocytes or neurons. By contrast, recombined cells expressed the neuroblast markers PSA-NCAM and DCX. **(b)**, GFP cells in the R26R^{EYFP} mice surrounding the lateral ventricles expressed SOX9 and SOX2, arrows indicate GFP cells co-expressing Ki67. GFP expressing cells in hom mice did not express SOX9, however SOX2 expression was unchanged. **(c)**, Fold change in the cell types expressing GFP from het and hom mice. A significant decrease in the number of GFAP⁺ astrocytes ($P < 0.001$), NG2⁺ oligodendrocytes ($P < 0.001$) and NeuN⁺ neurons ($P < 0.001$) was found in hom samples compared to control animals. Conversely, there was a significant increase in the co-expression of GFP and PSA-NCAM/DCX ($P < 0.0001$; $P < 0.004$ respectively) **(d)**. GFP

cells did not co-localise with SOX9 in hom samples ($P < 0.0001$) (e). There was no significant difference in the number of SOX2 positive cells. A decrease in proliferation (Ki67) was observed in GFP expressing cells in hom brains compared to controls ($P < 0.00003$). P values were calculated by Student's t-test, $n = 9$. Bars indicate standard error. Scale bars = 20 μm . f, illustration of a coronal section showing the analysed area, the SEZ (boxed area).

Figure 8: Sox9 is necessary for NSC function *in vivo* in the adult olfactory bulb.

R26R^{EYFP}, *Flox-Sox9*^{het}; R26R^{EYFP} (het) and *Flox-Sox9*^{hom}; R26R^{EYFP} (hom) adult (8- 10 week) mice were injected in one lateral ventricle with an adenovirus expressing Cre. BrdU was injected 3 weeks after the initial Cre virus injection. Analysis of the olfactory bulbs was carried out 6 weeks post injection. GFP identifies cells in which Cre-mediated recombination has taken place. (a), A greater number of GFP positive cells were observed in the olfactory bulbs of hom mice compared to R26R^{EYFP} mice. GFP cells in R26R^{EYFP} olfactory bulbs co-expressed the neuronal marker NeuN, GFP cells in het tissue expressed NeuN and some GFP cells expressed the neuroblast marker PSA-NCAM. *Sox9* negative, GFP positive cells in the olfactory bulbs of hom mice expressed PSA-NCAM. No GFP cells in R26R^{EYFP}, het or hom tissue were undergoing apoptosis (PARP +). Few GFP cells in sections of hom olfactory bulbs retained BrdU. (b), Quantification of the number of GFP positive cells in sections of the olfactory bulbs from R26R^{EYFP} mice and hom mice. A greater number of GFP positive cells were found in hom mice compared to R26R^{EYFP} ($P < 0.0006$). (c), Quantification of the percentage of GFP positive cells co-expressing NeuN, PSA-NCAM, PARP and BrdU. The differences between R26R^{EYFP} and hom mice was significant ($*P < 0.00003$; $**P < 0.00002$; $****P < 0.05$, $n = 9$, Student's t-test). There was no significant difference between GFP; PARP double positive cells ($***P < 1$). Bars indicate standard error. Scale bars = 20 μm . (d), illustration of a sagittal section showing the rostral migratory stream (RMS) from the lateral ventricle (LV) to the olfactory bulb. Coronal section through the olfactory bulb (vertical line in d) is represented in e.

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Article Title:	SOX9, acting downstream of Sonic hedgehog signalling, induces and maintains neural stem cells
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Supplementary Item & Number (add rows as necessary)	Title or Caption
Supplementary Figure 1	Neurospheres induced by SHH from E9.5 mouse CNS and generated from the chick CNS are multipotent.
Supplementary Figure 2	SOX9 is expressed in NS5 cells and neurospheres, whereas SOX10 is expressed in neurospheres but not in NS5 cells.
Supplementary Figure 3	<i>Sox9</i> is necessary for neurosphere formation.
Supplementary Figure 4	<i>Sox10</i> expression is upregulated in neurospheres generated from <i>Sox9</i> null CNS. <i>SoxE</i> genes are sufficient to induce precocious neurosphere generation.
Supplementary Figure 5	Proliferation is decreased in CNS tissue lacking <i>Sox9</i> .
Supplementary Figure 6	<i>Sox9</i> is required for multipotentiality <i>in vivo</i> and <i>in vitro</i> .
Supplementary Figure 7	The <i>pZ/Sox9</i> expression construct.
Supplementary Figure 8	The expression of <i>SoxE</i> mRNA coincides with the timing of neurosphere formation in the dorsal telencephalon.
Supplementary Figure 9	<i>Sox9</i> is necessary for the maintenance of ependymal cells <i>in vivo</i> in the adult.
Supplementary Figure 10	<i>Sox9</i> is necessary for ependymal cell formation.
Supplementary Figure 11	SOX9 is co-expressed with SOX2 after E10.5 in the developing CNS.
Supplementary Figure 12	There is no significant difference between the types of unrecombined cells in the R26R ^{EYFP} and Flox- <i>Sox9</i> ^{hom} ;

	R26R ^{EYFP} olfactory bulbs after Cre-mediated recombination.
Supplementary Figure 13	SHH signalling induces <i>SoxE</i> genes.













