Dbx1 Is a Direct Target of SOX3 in the Spinal Cord

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Abstract

SoxB1 sub-family of transcriptional regulators are expressed in progenitor (NP) cells throughout the neuroaxis and are generally downregulated during neuronal differentiation. Gain- and loss-of-function studies indicate that Sox1, Sox2 and Sox3 are key regulators of NP differentiation and that their roles in CNS development are largely redundant. Nevertheless, mutation of each SoxB1 individually results in a different array of CNS defects, raising the possibility that SoxB1 proteins have subtly different functions in NP cells. To explore the mechanism of SOXB1 functional redundancy, and to identify genes that are most sensitive to loss of the Sox3 gene, we performed genome wide expression profiling of Sox3 null NP cells. Nineteen genes with abnormal expression were identified, including the homeobox gene Dbx1. Analysis of Sox3 null embryos revealed that Dbx1 was significantly reduced in the neural tube and developing brain and that SOX3 bound directly to conserved elements associated with this gene in cultured NP cells and in vivo. These data define Dbx1 as a direct Sox3 target gene whose expression, intriguingly, is not fully rescued by other SOXB1 transcription factors, suggesting that there are inherent differences in SoxB1 protein activity.

Introduction

SOX3 is a member of the SOX (Sry-related HMG box) family of transcription factors, of which 20 members have been identified in mammals. SOX genes generally have developmentally-regulated expression and play important roles in cell specification, self-renewal and differentiation in a broad range of embryonic contexts [1]. Within the developing central nervous system (CNS), Sox3, and the closely related genes Sox1 and Sox2 (which together make up the SoxB1 subgroup), are expressed in neuroprogenitor cells throughout the neuroaxis and are downregulated upon differentiation [2,3]. Overexpression studies in chick embryonic spinal cords and cultured murine neuroprogenitor (NP) cells indicate that SOXB1 proteins function as inhibitors of neurodifferentiation and that they have subtle differences in their activities. However, while this is an attractive hypothesis, NP cells expressing only one SOXB1 gene have not been definitively identified. Furthermore, contrary to this possibility, structures that are normally formed in Sox3 null mice exhibit specific CNS defects within the hippocampus, corpus callosum and hypothalamus despite the widespread Sox3 expression in NP cells throughout the developing brain [5,6]. In addition, humans with polyalanine tract expansion mutations in SOX3 have a relatively mild phenotype that includes infundibulum hypoplasia, hypothalamic-pituitary axis dysfunction and incompletely penetrance of intellectual disability [7,8]. CNS-specific deletion of Sox2 or Sox1 in mice also results in regionally-restricted defects as opposed to a general NP phenotype [9,10,11,12,13].

While these studies provide strong support for SOXB1 functional redundancy, the existence of congenital CNS defects in Sox3, Sox2 and Sox1 single gene mutant mice also indicates that there is a specific requirement for each SOXB1 factor in a (relatively small) subpopulation of NP cells. This phenomenon can be explained by at least three possibilities. Firstly, given that subtle differences in Sox1, Sox2 and Sox3 gene expression have been identified in the developing CNS [3,14,15], it is possible that some NP cells express only one SOXB1 gene. Deletion of the single expressing SoxB1 gene in these cells would likely cause a specific developmental defect due to the complete absence of SOXB1 activity. However, while this is an attractive hypothesis, NP cells expressing only one SOXB1 gene have not been definitively identified. Furthermore, contrary to this possibility, structures that are normally formed in Sox3 null mice exhibit specific CNS defects within the hippocampus, corpus callosum and hypothalamus despite the widespread Sox3 expression in NP cells throughout the developing brain [5,6]. In addition, humans with polyalanine tract expansion mutations in SOX3 have a relatively mild phenotype that includes infundibulum hypoplasia, hypothalamic-pituitary axis dysfunction and incompletely penetrance of intellectual disability [7,8]. CNS-specific deletion of Sox2 or Sox1 in mice also results in regionally-restricted defects as opposed to a general NP phenotype [9,10,11,12,13].

To explore the mechanism of SOXB1 functional redundancy, and to identify genes that are most sensitive to loss of the Sox3 gene, we performed genome wide expression profiling of Sox3 null NP cells. Nineteen genes with abnormal/delayed expression were identified that included the homeobox gene Dbx1. In vivo analysis of Sox3 null embryos revealed that Dbx1 was significantly reduced in the neural tube and developing brain and that SOX3 bound
directly to conserved elements associated with this gene in cultured NP cells and in vivo. These data define Dbx1 as a direct SOX3 target gene whose expression, intriguingly, is not fully rescued by other SOXB1 transcription factors, suggesting that there are inherent differences in SOXB1 protein activity.

**Methods**

**ES Cell Generation**

Sox3 null embryonic stem cells were previously targeted as described in (Hughes et al 2013). Sox3 null mice have been published previously [5].

**ES Cell Culture and Neurodifferentiation**

R1 ES cells were maintained on irradiated MEFs in standard conditions and neurodifferentiated as monolayers using N2B27 as previously described [16].

**Microarray and qRT-PCR**

RNA from differentiated cells was extracted using a RNeasy mini kit (Qiagen) and cDNA generated using a High Capacity RNA-to-cDNA kit (ABI). Expression profiling was performed on four wild type and four Sox3 null Day 4 samples using Affymetrix GeneChip Mouse Gene 1.0 ST Arrays (full dataset available at Gene Expression Omnibus: GSE53760). 2-way-ANOVA, using batch as a factor, was used to identify the significantly regulated genes. qRT-PCR validation was performed previously described [17].

**Immunohistochemistry**

IHC was performed as described previously [3].

**Chromatin Immunoprecipitation (ChIP)**

In vitro ChIP was performed as per [4] using 5 ug of SOX3 or IgG antibodies, with Dynabeads blocked over night with 200 ug BSA, 200 ug yeast tRNA and 200 ug glycogen. In vivo ChIP was performed with wild type 10.5 dpc embryonic heads, dissociated with scalpel blades and fixed for 10 minutes using the above protocol. ChIP samples were analysed by qPCR (StepOne Plus, Applied Biosystems) using Fast SYBR (Life Technologies). Signals were considered positive when $2^{-}\Delta\Delta Ct_{\text{sample (non-enriched region)} - Ct_{\text{sample (peak region)}}} > 2$ following normalisation to $\Delta \text{CtIgG}$ and $\Delta \text{CtInput}$ of the corresponding qPCR run.

**Ethics Statement**

Animal experiments were approved by the University of Adelaide Animal Ethics Committee (project number: S-2012-242). All studies were conducted in accordance with the principles...
of animal replacement and reduction and experimental refinement.

Results

Sox3 Expression during In vitro Neural Progenitor Differentiation

To begin to identify SOX3 target genes in NP cells, we utilised the N2B27 culture system, which has previously been shown to generate NP cells from ESC with high efficiency [16]. To characterise \(\text{Sox3}\) expression during N2B27 neuroinduction, we performed qRT-PCR (Fig. 1A) and immunohistochemistry analysis (Fig. 1B;[3]). At the mRNA level, \(\text{Sox3}\) expression was virtually undetectable in ESC (Day 0). \(\text{Sox3}\) expression was upregulated by Day 2, and increased further at Day 4 and Day 6. Consistent with these data, weak expression of SOX3 protein was evident in Day 2 cultures. By Day 4, robust expression of SOX3 was detected in most cells. At Day 6, robust expression of SOX3 could still be detected in most cells, although some cells (particularly those at the periphery of colonies) had begun to downregulate SOX3.

To investigate the impact of \(\text{Sox3}\) deletion on NP differentiation, we performed N2B27 neuroinduction using our previously-generated \(\text{Sox3}\) null ES cells that contain a GFP reporter knock-in allele [17]. FACS analysis of Day 4 NP cultures revealed that the majority (over 60%) of cells were GFP\(^+\), which is comparable to the proportion of cells that are SOX3\(^+\) in the WT NP cultures (Figure 1B,C). To determine whether the loss of \(\text{Sox3}\) has a major impact on induction of neural progenitors, expression of neural progenitor markers (including other \(\text{SoxB1}\) genes) was analysed by qRT-PCR. No significant difference in \(\text{Sox1}, \text{Sox2}\) or \(\text{Pax6}\) expression was detected in samples utilized for microarray analysis, at Day 4 (Fig. 1D). No change in expression of the other \(\text{SoxB1}\) subgroup members was also observed on Day 6 (data not shown). As expected, \(\text{Sox3}\) expression was not detected in the mutant samples (Fig. 1E). Together these data indicate that \(\text{Sox3}\) is rapidly upregulated during N2B27 neurodifferentiation and that loss of \(\text{Sox3}\) does not overtly affect NP induction across this timeframe, probably due to functional redundancy with other \(\text{SOXB1}\) proteins.

Identification of SOX3 Target Genes

To identify putative SOX3 target genes, we compared the global gene expression profile of Day 4 NP cell cultures derived from \(\text{Sox3}\) null cells and a control cell line containing a conditional \(\text{Sox3}\) allele [17]. Microarray analysis was performed in quadruplicate using 4 biological replicates from two independent experiments. A total of 19 genes had significantly (\(p \leq 0.05\)) altered expression with a fold change of 1.4 or more, as determined by batch corrected two-way ANOVA analysis (Table 1). Fifteen and 4 genes were upregulated and downregulated, respectively. \(\text{Sox3}\) provided an internal control for this experiment and, as expected, this gene was significantly downregulated in the \(\text{Sox3}\) null samples. Further validation of a subset of the misregulated genes was conducted using qRT-PCR on independent samples. \(\text{Dbx1}\) (\(p = 0.049\)) and \(\text{Fezf2}\) (\(p = 0.0228\)) showed significant downregulation in mutant cells (Fig. 2A). Significantly elevated expression of \(\text{Efnb3}\) (\(p = 0.0229\)), \(\text{Cspg5}\) (\(p = 0.0251\)), \(\text{Fam174b}\) (\(p = 0.0065\)), \(\text{Ddr2}\) (\(p = 0.0386\)) and \(\text{Slit1}\) (\(p = 0.0456\)) was also confirmed in the independent samples. These data identifies a number of potential SOX3 targets in cultured NP cells.

Table 1. Table of significantly regulated genes with a fold change equal to or greater than 1.4 in Day 4 N2B27 differentiated ES cells.

| Gene Symbol | RefSeq   | stepup p-value | Fold-Change | Fold-Change (Description) | Independently Validated |
|-------------|----------|----------------|-------------|---------------------------|-------------------------|
| Sox3        | NM_009237 | 0.01           | -4.10       | KO down vs WT             | Yes                     |
| Cspg5       | NM_001166273 | 0.02      | 1.49        | KO up vs WT               | Yes                     |
| Ddr2        | NM_022563 | 0.02           | 1.83        | KO up vs WT               | Yes                     |
| Slt1        | NM_015748 | 0.04           | 1.87        | KO up vs WT               | Yes                     |
| Tagln3      | NM_019754 | 0.04           | 1.54        | KO up vs WT               |                         |
| 1200009022Rik | NM_025817 | 0.04           | 1.41        | KO up vs WT               |                         |
| Tmem163     | NM_028135 | 0.04           | -1.45       | KO down vs WT             |                         |
| Slc44a5     | NM_01081263 | 0.05      | 1.44        | KO up vs WT               |                         |
| Fzd2        | NM_080433 | 0.05           | -1.61       | KO down vs WT             |                         |
| Fam174b     | NM_001162532 | 0.05      | 1.64        | KO up vs WT               |                         |
| Fgf3        | NM_008010 | 0.05           | 1.65        | KO up vs WT               |                         |
| Fli2        | NM_201518 | 0.05           | 1.46        | KO up vs WT               |                         |
| Dbx1        | NM_001005232 | 0.05      | -2.35       | KO down vs WT             |                         |
| Gpr56       | NM_018882 | 0.05           | 1.56        | KO up vs WT               |                         |
| Efnb3       | NM_007911 | 0.05           | 1.59        | KO up vs WT               |                         |
| Nexit       | NM_199465 | 0.05           | 1.72        | KO up vs WT               |                         |
| Ctgf        | NM_010217 | 0.05           | 1.45        | KO up vs WT               |                         |
| Cp          | NM_001042611 | 0.05     | 2.04        | KO up vs WT               |                         |
| Ednrh       | NM_007904 | 0.05           | 1.53        | KO up vs WT               |                         |

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Figure 2. Validation of putative Sox3 targets. A: qRT-PCR validation of gene expression levels of down regulated genes, Dbx1 and Fezf2 (n = 3). B: qRT-PCR validation of gene expression levels of up regulated genes Ddr2, Slit1, Cspg5, Efnb3 and Fam174b (n = 3). C: Percentage of significantly regulated genes with SOX3 binding sites, as closest gene. RQ: relative quantification normalised to β-actin.

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Figure 3. Validation of putative Sox3 targets in vivo. A: Dbx1 gene expression within whole 9.5 dpc embryos. B: Dbx1 gene expression within 10.5 dpc dissected heads. C: qRT-PCR analysis of mRNA levels of Sox1, Sox2, Slit1 and Fezf2 showing no significant difference. RQ: relative quantification normalised to β-actin.

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Correlation between SOX3 Binding Sites and Sox3 Targets

Differentially expressed genes in Sox3 null NPs potentially represent genes that are direct SOX3 targets. To investigate this possibility, we examined the differentially expressed genes for SOX3 binding sites using a previously published SOX3 ChIP-seq data set generated using a comparable NP differentiation system [4]. A significant enrichment of SOX3 binding sites was present in genes with altered expression in Sox3 null NP cells. Over 83% of the differentially expressed genes (Table 1) have at proximal (closest gene) SOX3 binding site (Fig. 2C). In contrast, SOX3 binding sites were detected in only 25% of 20 randomly selected genes that do not display altered expression in Sox3 null NPs. The enrichment of SOX3 binding sites near genes with significantly altered expression genes suggests that many of these genes are direct targets of SOX3.

In vivo Validation of SOX3 Targets

Next, we investigated whether the differentially-expressed NP culture genes were affected by the loss of Sox3 in vivo. This analysis was performed using 9.5 dpc embryos (somite stages 12–15) as embryos at this stage of development have an abundance of NP cells and a paucity of differentiating neurons. In addition, like Day 4 NP cell cultures, Sox1 and Sox2 expression in not significantly altered in Sox3 null embryos at this stage (Fig. 3C). qRT-PCR analysis of Slit1 and Foxf2 showed no significant difference in expression between WT and Sox3 null embryos at this developmental time point (Fig. 3C). In contrast, Dbx1 expression was significantly lower in Sox3 null embryos at 9.5 dpc (Fig. 3A), consistent with its downregulation in Sox3 null NP cells. A significant reduction in Dbx1 expression was also evident in 10.5 dpc embryonic heads (Fig. 3).

To determine the impact of Sox3 loss-of-function on DBX1 expression at the cellular level, we compared the number of DBX1+ cells in Day 4 NP cultures using immunohistochemistry (Figure 4 A). DBX1+ cells were readily identified in control cultures and were located in a subpopulation of SOX3+ cells located at the periphery of cell clusters. In contrast, the number of DBX1+ cells was significantly and dramatically reduced in SOX3 KO cultures (Figure 4B). To determine whether there is a similar reduction in DBX1+ cells in Sox3 mutant embryos, we compared DBX1 expression in 9.5 dpc transverse sections through the trunk region. Again, a clear reduction in the number of DBX1+ cells within the spinal cord of Sox3 null mice was evident (Fig. 4C). Together these data suggest that Dbx1 is regulated by SOX3 in vivo and that other SoxB1 factors cannot fully compensate for the loss of SOX3.

SOX3 Binds to the Dbx1 Locus In vivo

To investigate whether Dbx1 is directly targeted by SOX3 in Day 4 NP cells, we performed ChIP-PCR on five SOX3 binding sites located in or near the Dbx1 locus that were previously
identified by ChIP-seq analysis ([4]; Fig. 5A). Significant (p ≤ 0.05) enrichment of all five sites was detected by SOX3 ChIP-PCR, when compared to the IgG isotype control (Fig. 5B). To determine if SOX3 bound these sites in vivo, we analysed SOX3 binding to one of the most enriched sites (Intronic site 2) using chromatin extracted from 10.5 dpc embryonic heads. Significant enrichment (p ≤ 0.001) of this SOX3 binding this site was observed in comparison to the IgG negative control (Fig. 5C). Together, these data indicate that Dbx1 is a direct target of SOX3 in vivo.

Discussion

Despite the widespread expression of Sox3 in progenitor cells throughout the neuroaxis, the CNS phenotypes of mice and humans with Sox3 mutations are relatively mild. It is generally believed that other SOXB1 proteins (SOX1 and SOX2) can function redundantly with SOX3 to “rescue” the SOX3 deficiency. However, the lack of a complete phenotypic rescue suggests that, at least in some NP cells, the level of SOX1 and SOX2 expression may be important for neurodevelopment. Alternatively, each of the SOX1 and SOX2 factors could bind preferentially to a unique subset of SOX1 targets. As a result, target gene expression resulting from the loss of a single SOX1 gene would only be partially rescued by the other two SOX1 factors. To explore this issue in more detail, we attempted to identify genes that were sensitive to the loss of Sox3 in NP cells. To do this we used a previously targeted Sox3 null ES cell line in which the single Sox3 exon was replaced with GFP [17]. N2B27-induced generation of conditionally targeted Sox3 null NP cells resulted in rapid upregulation of Sox3/GFP at the mRNA and protein level. The NP markers Sox1 and Pax6 were also rapidly induced and showed comparable kinetics in the control and mutant cultures. In addition, expression of Sox2 was comparable between the two populations. Notably, no overt defects in the morphology or global gene expression (see below) were identified in Sox3 null NP cells. This is likely due to the expression of Sox1 and Sox2 in N2B27-induced NP cells and is consistent with the mild CNS phenotype of Sox3 null embryos.

To maximise the likelihood of identifying genes that are directly regulated by SOX3, we performed gene expression profiling at Day 4, the earliest time point at which high levels of Sox3 were expressed in the majority of NP cells. Of over 24,000 genes assayed by microarray, only 19 had significantly different expression levels at Day 4. Expression differences (when tested by qRT-PCR) were validated on independent samples for a majority of the targets, thereby confirming the authenticity of the microarray data. However, not all differentially expressed genes validated on independent samples (Tmem163, Ednrb, and Slc44a5, data not shown). The relatively small number of differentially expressed genes is interesting to consider in the context of a recently-published ChIP-seq analysis of a similar NP cell type, which showed that SOX3 binds at thousands of genomic sites, many of which are likely to have regulatory functions [4]. Preliminary ChIP-seq analysis from our laboratory also indicates that SOX3 binds many of these sites in Day 4 N2B27-induced NP cells (D.M. and P.T. unpublished data). The most parsimonious explanation for the small number of differentially expressed genes is that Sox1 and/or Sox2 (both of which are expressed by Day 4 NP cells) can bind and functionally compensate for loss of
SOX3. Consistent with this hypothesis, almost all SOX2 binding sites in NPs also bind SOX3 [4]. However, while SOX3B1 redundancy provides an explanation for the unaltered expression of most genes associated with SOX3 binding sites, it also suggests that differentially expressed genes cannot be fully compensated by SOX1 and SOX2. Given the HMG domain of SOX3B1 proteins is not identical, it is possible that they have subtly different preferences for sequence-specific binding in vivo, as has been suggested by in vitro DNA binding experiments [18]. In addition, amino acid differences within the HMG box (or outside of it) may influence interactions with DNA binding partners such as the POU-class transcription factors [19,20]. Regardless of the mechanism, it is important to note that the differentially expressed genes are highly enriched for SOX3 binding sites (over randomly selected genes without significantly different expression levels), supporting the idea that they represent direct SOX3 targets. As a number of these putative direct target genes are upregulated, it appears that SOX3 could function as a repressor in NP cells [21] in addition to its more established role as an activator [2].

Interestingly, many of the genes identified by the microarray analysis have established roles in neurodevelopment. For example, Cpg53 has been associated with intellectual disability disorders [22], and ectopic Slit1 expression has been published in mice with abnormal corpus callosum development [23]. Intellectual disability is present in a subset of individuals with SOX3 mutations [7], while Sox3 null mice have abnormal corpus callosum development [5], thereby providing a link between these putative targets and processes with a functional requirement for SOX3.

Another putative SOX3 target was the downregulated transcription factor gene Fezf2. Fezf2 has been shown to be important for the early development of the posterior hypothalamus/thalamus in zebrafish and mouse [24,25]. Fezf2 expression has been previously published to be downstream of the SOXC group genes Sox4 and Sox11, double deletion of which leads to a loss of Fezf2 expression. Regulation of Fezf2 by SOX4/11 gene is mediated by a cis acting enhancer binding site which, interestingly, was also identified as a SOX3 binding site in NP cells by ChIP-seq and independently within our lab (data not shown; [4,26]). These data suggest that, in addition to acting as a pioneer factor [4], Sox3 may directly regulate Fezf2 expression in NP cells.

**Dbx1 is a Direct SOX3 Target In vivo**

To investigate whether NP targets were dysregulated in vivo, we compared their expression by qRT-PCR using whole 9.5 dpc embryos. Of the seven putative targets analysed (Fig. 3C, data not shown), only one (Dbx1) had significantly different expression that matched the genome-wide expression profiling analysis. The lack of in vivo validation for other targets may reflect a phenotypic or stage-specific difference in the NP cells induced by N2B27 differentiation compared with their 9.5 dpc embryonic counterparts. Consistent with this idea, expression analysis of Sox3 targets in Day 6 NP cells revealed that many genes no longer had significantly different expression levels or had greatly reduced expression fold changes (Fig. S1). Dbx1 expression, however, was reduced in whole 9.5 dpc embryos lacking Sox3. In addition, DBX1+ cells were not detected in the spinal cord of Sox3 null embryos at 9.5 dpc and were massively reduced in Day 4 null NP cells. Further, SOX3 bound to evolutionarily conserved elements at the Dbx1 locus in cultured NP cell and embryos. Together these data indicate that Dbx1 is directly activated by SOX3 in NP cells.

**Supporting Information**

**Figure S1** Expression of putative Sox3 targets at day 6 of neural progenitor differentiation. A: Putative targets Dlx1 and Slit1 relative gene expression levels by qRT-PCR at day 6 of N2B27 differentiation, (n = 3 normalised to day 4). B: Putative targets Cp, Fezf2 and Efnb3 showing reduced and/or insignificant fold change at day 6 of N2B27 differentiation (n = 3, normalised to day 4). RQ: relative quantification normalised to β-actin. (TIF)

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**Author Contributions**

Conceived and designed the experiments: NR DM PT. Performed the experiments: NR DM. Analyzed the data: NR DM PT. Contributed reagents/materials/analysis tools: NR DM PT. Wrote the paper: NR PT.
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