Chromatin remodeling and histone modification in the conversion of oligodendrocyte precursors to neural stem cells

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We showed previously that purified rat oligodendrocyte precursor cells (OPCs) can be induced by extracellular signals to convert to multipotent neural stem-like cells (NSLCs), which can then generate both neurons and glial cells. Because the conversion of precursor cells to stem-like cells is of both intellectual and practical interest, it is important to understand its molecular basis. We show here that the conversion of OPCs to NSLCs depends on the reactivation of the *sox2* gene, which in turn depends on the recruitment of the tumor suppressor protein Brca1 and the chromatin-remodeling protein Brahma (Brm) to an enhancer in the *sox2* promoter. Moreover, we show that the conversion is associated with the modification of Lys 4 and Lys 9 of histone H3 at the same enhancer. Our findings suggest that the conversion of OPCs to NSLCs depends on progressive chromatin remodeling, mediated in part by Brca1 and Brm.

Oligodendrocyte precursor cells (OPCs) are arguably the best-characterized precursor cells in the vertebrate central nervous system (CNS) (Barres and Raff 1994). They can differentiate in vitro into either oligodendrocytes or type-2 astrocytes (2As), depending on extracellular signals: thyroid hormone (TH), for example, promotes oligodendrocyte differentiation (Barres et al. 1994), whereas bone morphogenetic proteins (BMPs) promote 2A differentiation (Mabie et al. 1997). Furthermore, if purified OPCs are first induced by BMPs to develop into 2As and are then cultured in basic fibroblast growth factor (bFGF), they convert to multipotent neural-stem-like cells (NSLCs), which can self-renew and produce neurons and type-1 astrocytes, as well as oligodendrocytes and 2As (Kondo and Raff 2000a). The conversion to 2As seems to be a necessary first step in reprogramming OPCs to become NSLCs: if OPCs are freshly purified from the developing rat optic nerve and cultured directly in bFGF without an initial exposure to BMPs, they do not convert to NSLCs (Kondo and Raff 2000a). Since OPCs are multipotent (Kondo and Raff 2000a), present

throughout the adult CNS, and are stimulated to proliferate by CNS damage (Dawson et al. 2003), they are an attractive endogenous source of cells for CNS repair, especially if one could learn how to control their behavior in vivo.

In the present study, we examine changes in the expression of several candidate genes during the conversion of OPCs to 2As and NSLCs. We show that the treatment of OPCs with BMP2 rapidly induces the expression of several genes known to be expressed in at least some neural stem cells (NSCs), including *sox2*, which encodes a member of the SRY-related, HMG-box-containing, transcription factor family (Gubbay et al. 1990). Sox2 is one of the earliest known transcription factors expressed in the developing neural tube, and there is increasing evidence that it is essential for the maintenance of NSCs (Zappone et al. 2000; Bylund et al. 2003; Graham et al. 2003). We demonstrate that depletion of Sox2 by RNA interference (RNAi) blocks the proliferation of NSLCs and causes them to differentiate into neurons. We provide evidence that *sox2* expression in NSCs and NSLCs depends on both Brca1 and Brm, which is the catalytic subunit in a subset of SWI/SNF chromatin-remodeling complexes. Finally, we show that the conversion of OPCs to NSLCs is associated with the recruitment of Brca1 and Brm to an enhancer in the *sox2* promoter and

that histone H3 in the enhancer is modified during the conversion to an active form, which is methylated at Lys 4 (K4) and acetylated at Lys 9 (K9). These findings suggest that the conversion is associated with extensive chromatin remodeling, which is mediated in part by Brca1 and Brm.

Results

2As express several NSC markers

We first addressed whether the BMP-induced conversion of OPCs to 2As is associated with the expression of antigens that are expressed by at least some NSCs. We purified OPCs from postnatal day 6 (P6) rat optic nerve and expanded them for 4 wk in PDGF, in the absence of TH to avoid their differentiation into oligodendrocytes (Barres et al. 1994). We then recultured the cells for 2 d on PDL-coated slide flasks, either in PDGF alone or in PDGF plus BMP2, and then fixed and immunolabeled them with the A2B5 monoclonal antibody (Eisenbarth et al. 1979), which labels rat OPCs (Raff et al. 1983); anti-GFAP antibodies, which label astrocytes (Bignami et al. 1972) and some NSCs (Doetsch et al. 2002); and antibodies against Nestin, PSA-NCAM, LeX/SSEA1, or Sox2, all of which are expressed by at least some NSCs (Lendahl et al. 1990; Seki and Arai 1991; Li et al. 1998; Zappone et al. 2000; Capela and Temple 2002). Whereas >90% of both OPCs and 2As expressed A2B5 and Nestin, no OPCs expressed GFAP, PSA-NCAM, SSEA1, or Sox2; >90% of the BMP-induced 2As, however, expressed GFAP, 80% expressed SSEA1, 6% expressed PSA-NCAM, and 60% expressed Sox2 (Fig. 1A; data not shown). Thus, BMP2 rapidly induces many OPCs to express antigens that are characteristic of at least some NSCs.

NSLCs and NSCs express similar mRNAs

We next used RT–PCR to analyze several potentially relevant mRNAs in OPCs, 2As, NSLCs, and NSCs, including *sox2*, *nestin*, *gfap*, *brca1*, *olig2*, *musashi1*, *hes1*, *hes5*, *bmi1*, *id1-4*, and *bcrp1* mRNAs, all of which have been shown to be expressed by at least some NSCs or by more restricted neural precursors. *Olig2*, *hes1*, and *hes5* encode basic helix–loop–helix (bHLH) transcription factors (Akazawa et al. 1992; Sasai et al. 1992; Lu et al. 2000; Takebayashi et al. 2000; Zhou et al. 2000); *musashi1* encodes an RNA-binding protein (Nakamura et al. 1994); *bmi1* encodes a polycomb family transcription factor (van Lohuizen et al. 1991); *id1-4* encode HLH proteins that inhibit cell differentiation (Benezra et al. 1990; Christy et al. 1991; Sun et al. 1991; Biggs et al. 1992; Riechmann et al. 1994); and *bcrp1* encodes an ABC transporter expressed by various types of stem cells (Doyle et al. 1999).

OPCs were cultured in PDGF alone. We prepared 2As by culturing OPCs in PDGF plus BMP2 for 2 d, and we prepared NSLCs by culturing the 2As in bFGF alone for 1 wk. We prepared NSCs from embryonic day 14.5
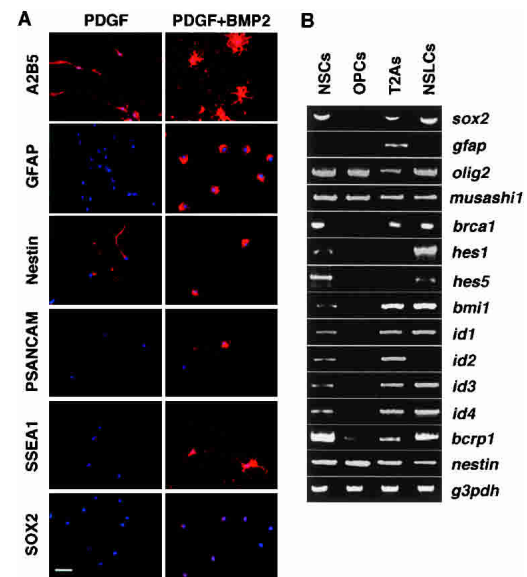

Figure 1. Expression of NSC-characteristic molecules in OPCs, 2As, NSCs, and NSLCs. (*A*) Purified P6 OPCs were expanded in PDGF without TH for 4 wk. They were then cultured in either PDGF (OPCs) or in PDGF plus BMP2 (2As) for 2 d and stained (in red) with the antibodies indicated. The cells were counterstained with Hoechst 33342 to visualize all nuclei (blue). Bar, 25 μm. (*B*) Purified OPCs were cultured as above for 4 wk and then in PDGF alone (OPCs), in PDGF plus BMP2 for 2 d (2As), or in PDGF plus BMP2 for 2 d and then in bFGF for 7 d (NSLCs). NSCs were prepared from E14.5 mouse brain by culturing the cells as floating neurospheres on uncoated dishes in bFGF for 2 wk. RNA was extracted and analyzed by RT–PCR, as described in Materials and Methods.

(E14.5) mouse telencephalon and expanded them in bFGF for 2 wk (Nakashima et al. 1999).

As shown in Figure 1B, NSCs expressed all of the examined mRNAs except *gfap*, whereas OPCs expressed only *olig2*, *musashi1*, and *nestin* mRNAs. (We showed previously that freshly isolated P0 rat OPCs express all four known mammalian *Id* mRNAs but that *id4* rapidly declines as OPCs proliferate in vitro and in vivo [Kondo and Raff 2000b]; the present results indicate that the other three *id* mRNAs also eventually decrease as OPCs proliferate in culture and are no longer detectable after 4 wk.) The 2As expressed all but two of the examined mRNAs (*hes1* and *hes5*, both of which encode effectors of Notch signaling), although *olig2* mRNA decreased significantly compared to its expression in OPCs. When the 2As were cultured in bFGF for a week (to become NSLCs), they re-expressed both *hes1* and *hes5* mRNAs but lost *gfap* mRNA, and *olig2* mRNA returned to the level seen in OPCs. Thus, a 2-d exposure to BMP2 induced a global change in gene expression in OPCs, and the patterns of expression in the mRNAs examined were similar in NSCs and NSLCs.

NSLC proliferation and maintenance depends on Sox2

As Sox2 is up-regulated when OPCs are treated with BMP2 (see Fig. 1), we investigated whether the depletion

of Sox2 by RNAi would influence the proliferation and/ or differentiation of NSLCs. We constructed expression vectors encoding either green fluorescence protein (GFP) alone (control) or GFP and a small interfering RNA specific for *sox2* (*sox2*-siRNA). We first examined whether *sox2*-siRNA depleted endogenous Sox2. We transfected NSLCs with either the control vector or *sox2*-siRNA, cultured them in bFGF for 3 d, and then fixed and immunolabeled them for both GFP and Sox2. Compared to the cells transfected with the control vector, the cells transfected with *sox2*-siRNA showed a greatly reduced expression of endogenous Sox2 (Fig. 2A).

We then transfected OPCs with either the control vector or *sox2*-siRNA, cultured them either in PDGF alone for 7 d (OPCs) or in PDGF plus BMP2 for 2 d and then bFGF for 5 d (NSLCs). We found that the NSLCs transfected with *sox2*-siRNA proliferated less than the NSLCs transfected with the control vector (data not shown), and we quantified this by adding bromodeoxy-uridine (BrdU) for 8 h and then staining the cells with anti-BrdU antibody; as shown in Figure 2B, >30% of the OPCs transfected with *sox2*-siRNA and 50% of the NSLCs transfected with the control vector incorporated BrdU, whereas none of the NSLCs transfected with *sox2*-siRNA incorporated BrdU. Thus, the proliferation of NSLCs apparently depends on Sox2.

To determine whether the knock-down of Sox2 by RNAi causes the spontaneous differentiation of NSLCs, we transfected OPCs, cultured them to produce NSLCs as above, and then fixed and immunolabeled the NSLCs for both GFP and a neural-specific antigen—either Nestin, GFAP, neurofilaments (NFs), which are specifically expressed by neurons (Julien 1999), or galactocerebroside

(GC), which is specifically expressed by oligodendro-cytes (Raff et al. 1978). Whereas over 70% of the cells transfected with the control vector expressed Nestin, only 6% of the cells transfected with *sox2*-siRNA did so (Fig. 2C), suggesting that Sox2 is required to maintain Nestin expression in NSLCs. Similarly, whereas 10% of the cells transfected with the control vector expressed GFAP, none of the cells transfected with *sox2*-siRNA did so (Fig. 2D), suggesting that Sox2 helps prolong the expression of GFAP as 2As convert to NSLCs in the presence of bFGF. In contrast, 40% of the cells transfected with *sox2*-siRNA expressed NFs, whereas none of the cells transfected with the control vector did so (Fig. 2E), suggesting that Sox2 normally inhibits the spontaneous differentiation of NSLCs into neurons. We could not detect any cells expressing GC among the cells transfected with either vector (data not shown), suggesting that Sox2 is not required to prevent the spontaneous differentiation of NSLCs into oligodendrocytes.

Sox2 expression in NSLCs and NSCs depends on Brca1

As Sox2 seems to play an important part in maintaining the proliferation and undifferentiated state of NSLCs, we investigated how *sox2* expression is controlled in NSLCs and NSCs. There were two reasons for thinking that Brca1 might be involved in this control, even though it was originally discovered as a tumor suppressor gene that predisposes women to breast and ovarian cancer (Miki et al. 1994). First, *brca1* is expressed in the ventricular zone of the developing rodent brain, as well as in cultured embryonic and adult NSCs (Marquis et al. 1995; Korhonen et al. 2003). Second, *brca1*$^{-/-}$ mice die as em-
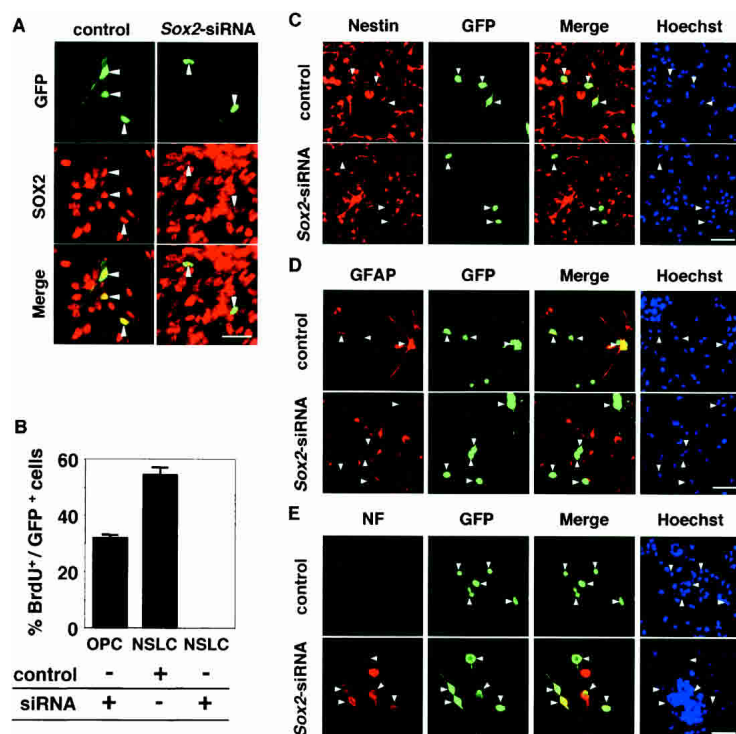

Figure 2. Effect of *sox2*-siRNA. (*A*) NSLCs were transfected with either a control vector encoding GFP alone or *sox2*-siRNA, cultured in bFGF for 3 d, and then stained for both GFP (green) and Sox2 (red). (*B*) OPCs were transfected as above and cultured either in PDGF alone (OPCs) or in PDGF plus BMP2 for 2 d and then bFGF for 5 d (NSLCs); BrdU was then added for 8 h, and the cells were then fixed and immunolabeled for BrdU. The proportion of GFP-expressing cells that were BrdU+ is shown as the mean ± SD of three cultures. (*C*–*E*) OPCs were transfected and cultured in PDGF plus BMP2 and then bFGF for 5 d. The cells were then stained for both GFP (green) and the neural markers Nestin (*C*), GFAP (*D*), or NFs (*E*)—all in red. The cell nuclei were stained with Hoechst 33342 (blue). The GFP-positive cells are indicated with arrows. Bars: *A*,*C*–*E*, 25 µm.

bryos, with CNS defects that are very similar to those seen in *sox2*⁻/⁻ mice (Hakem et al. 1996; Liu et al. 1996; Avilion et al. 2003). We therefore examined the expression of Brca1 protein in NSCs, OPCs, 2As, and NSLCs. As shown in Figure 3A, Brca1 was not detectably expressed in OPCs but was expressed by 2As, NSLCs, and NSCs, indicating that its expression is upregulated when OPCs are induced to become 2As and NSLCs.

To determine the relationship between Brca1 and Sox2 expression, we constructed an expression vector encoding both GFP and a siRNA specific for *brca1* (*brca1*-siRNA). To show that *brca1*-siRNA could deplete endogenous Brca1, we transfected NSLCs with either the control vector or *brca1*-siRNA, cultured them in bFGF for 3 d, and then fixed the cells and immunolabeled them for both GFP and Brca1. As shown in Figure 3B, transfection with *brca1*-siRNA significantly reduced the expression of endogenous Brca1. We then transfected NSLCs with either *brca1*-siRNA or *sox2*-siRNA, cultured them as just described, and immunolabeled them for either GFP and Brca1 or GFP and Sox2. Whereas all of the NSLCs transfected with *sox2*-siRNA still ex-

Figure 3. Role of Brca1 in *sox2* expression in NSLCs. (*A*) NSCs, OPCs, 2As, and NSLCs were immunostained for Brca1 (green) and for Nestin, A2B5, or GFAP (in red). (*B*) NSLCs were transfected either with a control vector encoding GFP alone or with *brca1*-siRNA, cultured in bFGF for 3 d, and then stained for GFP (green) and Brca1 (red). (*C,D*) NSLCs were transfected with the control vector, *sox2*-siRNA, or *brca1*-siRNA, cultured in bFGF for 3 d, and then stained for both GFP (green) and either Brca1 or Sox2 (in red). Arrows indicate the GFP-positive cells. Bar: *A–D*, 25 μm.

pressed Brca1 (Fig. 3C), >60% of the NSLCs transfected with *brca1*-siRNA did not express significant amounts of Sox2 (Fig. 3D). Transfection of cultured NSCs with *brca1*-siRNA resulted in a similar loss of Sox2 expression (data not shown). These findings suggest that Sox2 expression in both NSCs and NSLCs depends on Brca1.

To determine if Brca1 is sufficient to induce *sox2* expression in OPCs, we transfected OPCs with an expression vector encoding both mouse Brca1 and GFP, cultured them in bFGF for 7 d, and fixed and immunostained them for GFP and Sox2. None of the GFP-positive cells were labeled for Sox2 (data not shown), suggesting that overexpression of *brca1* on its own cannot induce Sox2 expression in OPCs; apparently, other factors are needed.

Both Brca1 and Brm associate with an *sox2* enhancer in cultured NSCs

To study the regulation of *sox2* expression in our NSCs, we constructed a series of *sox2*-enhancer–luciferase expression vectors. We inserted the 5′-promoter region of the mouse *sox2* gene, or various deleted forms (R1–R3) of the promoter, into the pGL3-promoter vector, which contains a minimal SV40 promoter upstream of a firefly luciferase cDNA (Fig. 4A). We transfected NSCs and 293 cells with these vectors, along with a vector (pEF-Rluc) encoding a sea pansy luciferase. After 24 h, we assayed the firefly luciferase activity, normalized against the sea pansy luciferase activity. As shown in Figure 4A, both the full *sox2* promoter and the R1-containing promoter in which R2 and R3 were deleted produced strong luciferase activity in NSCs, but both the R2-containing and R3-containing promoters in which R1 was deleted did not. These findings suggest that R1 contains an enhancer(s) for *sox2* expression in cultured NSCs. In contrast, even the full promoter did not produce luciferase activity in 293 cells (Fig. 4A).

We then used chromatin immunoprecipitation (ChIP) to determine whether Brca1 is associated with the R1 DNA sequence in NSCs. We fixed NSCs in formaldehyde, sonicated them in a cell lysis buffer, and harvested the extracts. We precipitated Brca1 and its associated DNA from the extracts with anti-Brca1 antibodies and Protein-A-Sepharose, purified the precipitated DNA fragments, and analyzed them by PCR. As shown in Figure 4B, we could detect the R1 but not the R2 or R3 DNA sequences, indicating that at least some Brca1 is associated with the R1 sequence in the *sox2* promoter in NSCs and may thus be involved in stimulating *sox2* expression in these cells.

Although Brca1 is unlikely to bind directly to undamaged DNA, it has been shown to associate with SWI/SNF chromatin-remodeling complexes, which contain several subunits, including Brg1 and Brm (Bochar et al. 2000). To determine if Brca1 is associated with SWI/SNF complexes in NSCs, we did a ChIP analysis as just described, but using antibodies against Brm or Brg1; we also used antibodies against Brca1 as a positive control
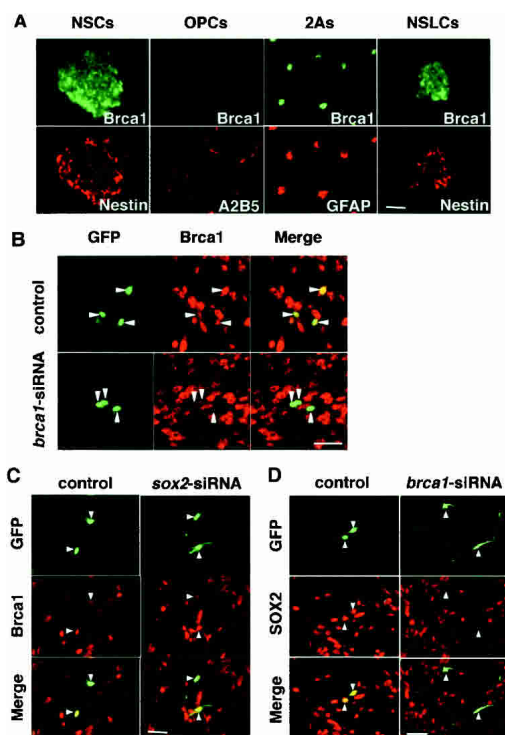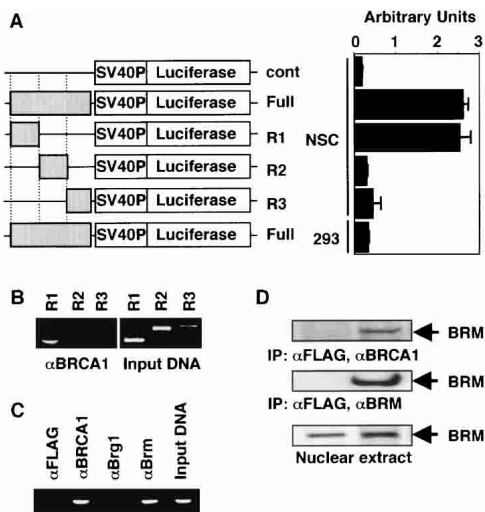
Figure 4. Association of Brca1 and Brm on the R1 *sox2* enhancer in NSCs. (*A*) NSCs or 293 cells were cotransfected with both pEF-Rluc, which encodes a sea pansy luciferase, and one of the *sox2*-enhancer–firefly-luciferase expression vectors containing the full-length or deleted versions of the *sox2* promoter (shown on *left*). The activity of firefly luciferase was analyzed after 24 h, normalized to the sea pansy luciferase activity. (*B*) ChIP analysis to determine if Brca1 is bound to the R1 DNA sequence in the mouse *sox2* promoter. Extracts of formaldehyde-fixed NSCs were immunoprecipitated with Brca1 antibodies and Protein-A-Sepharose, and genomic DNA fragments in the immunoprecipitate were analyzed by PCR with specific primer sets to amplify the R1, R2, or R3 sequences. The gel positions of the R1, R2, and R3 sequences are shown on the *right* (input DNA). (*C*) ChIP analysis to determine whether the R1 coprecipitates with Brm or Brg1 subunits of the SWI/SNF complex; anti-Brca1 antibodies and anti-Flag antibodies were used as positive and negative controls, respectively. The gel position of the R1 sequence is shown at the *right* (input DNA). (*D*) Coprecipitation of Brca1 and Brm proteins from extracts of NSCs. The extracts were immunoprecipitated with antibodies against Brca1, Brm, or Flag, and Protein-A-Sepharose, and the immunoprecipitates were analyzed by Western blotting using anti-Brm antibodies.

and against the Flag epitope as a negative control. As shown in Figure 4C, both the anti-Brca1 and anti-Brm antibodies precipitated the R1 sequence, whereas the anti-Brg1 and anti-Flag antibodies did not, suggesting that Brca1 is associated with Brm-containing SWI/SNF complexes in NSCs.

To confirm that Brca1 and Brm are in the same protein complexes, we prepared extracts of NSCs and treated them with antibodies against Brca1, Brm, or Flag, followed by Protein-A-Sepharose, and then analyzed the immunoprecipitates by Western blotting using anti-Brm antibodies. As shown in Figure 4D, both the anti-Brca1 and anti-Brm antibodies precipitated Brm, whereas the anti-Flag antibody did not, suggesting that at least some Brca1 and Brm are in the same complexes in NSCs. Taken together, these results suggest that both Brca1 and Brm associate with the R1 *sox2* enhancer and help activate the transcription of *sox2* in NSCs.

Brca1 and Brm are recruited to the R1 when OPCs convert to 2As and NSLCs

To investigate whether Brca1 and Brm are recruited to the R1 when OPCs are induced to convert to 2As and NSLCs, we fixed OPCs, 2As, and NSLCs in formaldehyde and used them for ChIP analysis as described above. As shown in Figure 5A, whereas neither anti-Brca1 nor anti-Brm antibodies precipitated the R1 sequence in OPCs, both of them did so in 2As and NSLCs (Fig. 5A). This suggests that both Brca1 and Brm are recruited to the R1 during the conversion of OPCs to 2As and NSLCs.

A dominant-negative Brm decreases Sox2 expression in NSLCs

To examine the relationship between Brm and *sox2* expression, we constructed expression vectors encoding either GFP and wild-type human Brm (wBrm) or GFP and a dominant-negative form of human Brm (mutBrm), which has an inactive ATPase domain (Muchardt and Yaniv 1993; Muchardt et al. 1998; Bourachot et al. 1999). We transfected NSLCs with the vectors and immunolabeled the cells after 5 d for GFP and Sox2. Whereas all of the NSLCs transfected with wBrm expressed Sox2
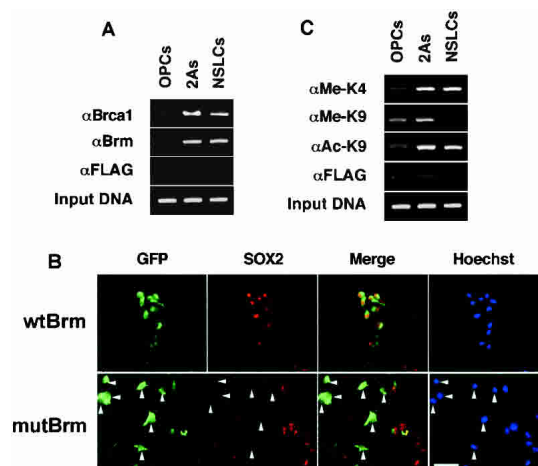

Figure 5. Recruitment of Brca1 and Brm and histone H3 modification on the *sox2* R1 enhancer during the conversion of OPCs to 2As and NSLCs. (*A*) ChIP analysis showing that R1 coprecipitates with Brca1 or Brm. Extracts of formaldehyde-fixed OPCs, 2As, and NSLCs were precipitated with either anti-Brca1 antibodies, anti-Brm antibodies, or anti-Flag antibody and Protein-A-Sepharose. Precipitated genomic DNA fragments were analyzed by PCR with the primer set for the R1 sequence. (*B*) NSLCs were transfected with the wBrm or mutBrm vector, cultured in bFGF for 3 d, and then immunostained for both GFP (green) and Sox2 (in red). The Sox2-negative cells are indicated with arrowheads. Bar, 25 µm. (*C*) ChIP analysis showing the modifications of histone H3 associated with the R1 enhancer during OPC conversion. The cell extracts were precipitated with anti-dimethyl-H3-K4, anti-dimethyl-H3-K9, or anti-acetyl-H3-K9 antibodies and Protein-A-Sepharose, and the precipitated genomic DNA fragments were analyzed as in *A*.

(Fig. 5B), <30% of the NSLCs transfected with mutBrm did so (Fig. 5B), suggesting that Brm and its ATPase-dependent chromatin-remodeling activity promotes the expression of *sox2* in NSLCs.

Histone H3 is modified when OPCs convert to 2As and NSLCs

There is increasing evidence that histone modification plays an important part in the control of gene expression during development (Heard et al. 2001; Litt et al. 2001; Lunyak et al. 2002; Song and Ghosh 2004). The acetylation (Ac) of histone H3 at K9 and its methylation (Me) at K4, for example, is associated with transcriptional activation, whereas the methylation of H3 at K9 is associated with transcriptional silencing. We examined histone H3 modification in the R1 enhancer of *sox2* during the conversion of OPCs to NSLCs, using ChIP analysis. As shown in Figure 5C, antibodies specific for methylated K9 in H3 (Me-K9), but not antibodies specific for Me-K4 or Ac-K9, precipitated the R1 sequence from OPC extracts, consistent with the lack of expression of *sox2* in these cells; in contrast, antibodies specific for either Me-K4 or Ac-K9 in H3 precipitated the R1 sequence from extracts of both 2As and NSLCs, consistent with the expression of *sox2* in both these cell types. Interestingly, antibodies specific for Me-K9 in H3 also precipitated the R1 sequence from extracts of 2As but not from extracts of NSLCs, consistent with the increased expression of *sox2* mRNA in NSLCs compared to 2As (see Fig. 1B). These findings suggest that histone H3 is progressively modified as OPCs convert to 2As and then NSLCs.

Discussion

In this study, we have examined some of the molecular changes that occur in OPCs when the cells are induced by extracellular signals to convert to 2As and then to NSLCs. One of the changes we find that is likely to be important is the induction of *sox2*. Although *sox2* is not expressed in OPCs, it is expressed in 2As and NSLCs, as well as in NSCs, which are thought to give rise to OPCs. We have focused our attention on the chromosomal mechanisms involved in the re-expression of *sox2* when OPCs convert to 2As and NSLCs. We provide evidence that the re-expression depends on the recruitment of chromatin-remodeling complexes to the R1 enhancer in the *sox2* promoter and is associated with modifications of H3 histones at this enhancer.

We show that OPCs rapidly acquire several molecular characteristics of NSCs when induced by BMPs to become 2As, which provides a plausible explanation for why this induction is apparently a required first step in the conversion of purified OPCs to NSLCs stimulated by extracellular signals (Kondo and Raff 2000a). Recently, it has been reported that newborn mouse (Belachew et al. 2003) and adult human (Nunes et al. 2003) OPCs can behave as NSCs in culture, even without purposeful 2A induction. In these studies, however, the OPCs were ex-

posed to FCS, which contains BMPs (Kondo and Raff 2004); since even a brief exposure to BMPs is enough to induce 2A development (Mabie et al. 1997), it is possible that BMP-induced 2A development occurred in these experiments.

We show here that Sox2, which is induced when OPCs convert to 2As and NSLCs, plays an important part in maintaining both the proliferation and undifferentiated state of NSLCs. When Sox2 is knocked down using RNAi, NSLCs stop proliferating, lose the expression of Nestin, and spontaneously differentiate into neurons. This finding is consistent with previous observations in vivo that overexpression of *sox2* in mouse NSCs blocks their differentiation and that inhibition of Sox2 in these cells causes their premature exit from the cell cycle and differentiation into neurons (Graham et al. 2003).

Sox2 expression in both NSCs and NSLCs apparently depends on the tumor suppressor gene *brca1*, which is induced along with *sox2* when OPCs convert to 2As and NSLCs. When Brca1 levels are knocked down in these cells using RNAi, Sox2 protein is substantially decreased. In contrast, when Sox2 levels are knocked down in these cells, Brca1 protein is not detectably decreased, suggesting that Brca1 expression does not depend on Sox2. Although Sox2 expression in NSLCs and NSCs seems to depend on Brca1, overexpression of Brca1 is not enough on its own to induce Sox2 expression in OPCs, suggesting that other factors are also required.

Another protein that seems to be involved in Sox2 expression in NSLCs is Brm, an ATPase subunit in certain SWI/SNF chromatin-remodeling complexes. When a dominant-negative form of Brm, containing an inactive ATPase domain, is expressed in NSLCs, Sox2 protein is substantially decreased, suggesting that the ATPase activity of Brm helps stimulate *sox2* expression in these cells. Most importantly, we show using ChIP analysis that both Brca1 and Brm are associated with the R1 enhancer sequence in the *sox2* promoter in NSCs, 2As, and NSLCs, but not in OPCs; we show using a luciferase assay that this enhancer sequence is required for *sox2*-promoter-driven gene expression in NSCs. In coprecipitation assays, we demonstrate that at least some Brca1 and Brm are in the same protein complexes in NSCs.

Taken together, these findings strongly suggest that SWI/SNF remodeling complexes containing Brm and Brca1 are recruited to the R1 enhancer in the *sox2* promoter during the conversion of OPCs to 2As and NSLCs, where they help activate *sox2* expression. It was shown previously that the expression of *sox2* in the developing CNS depends on a patchwork of separate enhancers, each controlling a distinct spatial and temporal pattern of expression (Uchikawa et al. 2003). The R1 site that we identify here is contained in the enhancer that was previously found to be essential for *sox2* expression in telencephalic NSCs in both mouse and chicken (Zappone et al. 2000; Uchikawa et al. 2003). Since both Brca1 and Brm are expressed in proliferating NSCs in vivo and in vitro (Marquis et al. 1995; Korhonen et al. 2003; this

study), it is likely that they both help activate *sox2* expression in NSCs in vivo.

Interestingly, Brm-containing SWI/SNF complexes are also recruited to the promoters of both *hes1* and *hes5* genes in C2C12 myoblasts (Kadam and Emerson 2003), raising the possibility that these complexes might be required for stem cell maintenance in some nonneural tissues. Moreover, another chromatin-remodeling factor ISWI has been shown to help reprogram a somatic nucleus when it is transplanted into an unfertilized *Xenopus* egg (Kikyo et al. 2000), suggesting that chromatin remodeling may be a general feature of reprogramming in which cells (or nuclei) revert to a state with increased developmental potential.

We have also used ChIP analysis to show that H3 histones associated with the R1 sequence in the *sox2* promoter undergo progressive modification when OPCs are induced to convert to 2As and then NSLCs. The changes are consistent with the progressive activation of *sox2* expression from OPCs-to-2As-to-NSLCs: in OPCs these H3 histones are in an inactive form, with K9 methylated and unacetylated and K4 unmethylated; in 2As they are in a more active form, with K4 methylated and K9 both methylated and acetylated, and in NSLCs they are in an even more active form, with K4 methylated and K9 acetylated but unmethylated. These changes are summarized in the schematic model shown in Figure 6.

In summary, we have shown that the conversion of OPCs to 2As and NSLCs is associated with extensive changes in gene expression, including the reactivation of *sox2* expression. We have provided evidence that the re-expression of *sox2* depends on chromatin remodeling by Brca1- and Brm-containing SWI/SNF complexes, which are recruited to the R1 enhancer in the *sox2* promoter, and is associated with progressive modifications in H3 histones at this enhancer. It will be a major challenge to identify all the other genes and gene regulatory proteins involved in these conversions.
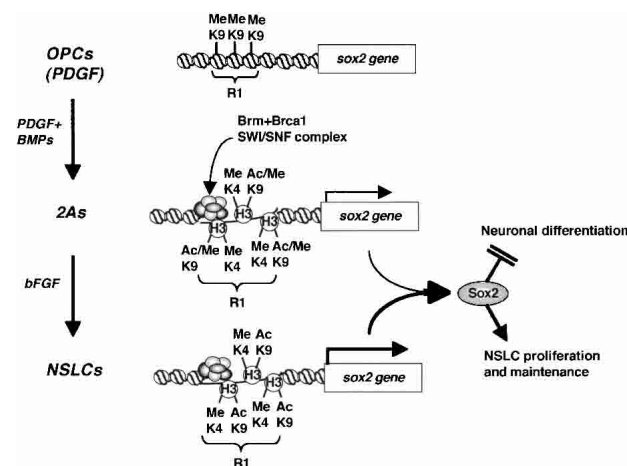

Figure 6. A model for how Brca1- and Brm-containing SWI/SNF chromatin-remodeling complexes and histone H3 modifications may help activate *sox2* transcription when OPCs are induced to convert to 2As and NSLCs.

Materials and methods

Animals and chemicals

Animals were obtained from the Animal Facilities at University College London and at University of Cambridge Centre for Brain Repair. Chemicals were purchased from Sigma, except where indicated. Recombinant human PDGF-AA, human BMP2, and human bFGF were purchased from Peprotech.

Cell culture

P6 rat optic nerve OPCs were purified to >98% purity by sequential immunopanning as described previously (Barres et al. 1992) and cultured in poly-D-lysine (PDL)-coated 100-mm culture dishes (Falcon) in serum-free Dulbecco's Modified Eagle's medium containing bovine insulin (10 μg/mL), human transferrin (100 μg/mL), BSA (100 μg/mL), progesterone (60 ng/mL), putrescine (16 μg/mL), sodium selenite (40 ng/mL), N-acetyl-cysteine (60 μg/mL), forskolin (5 μM), PDGF (10 ng/mL), penicillin, and streptomycin (GIBCO; culture medium). To induce 2A differentiation, OPCs were cultured in PDL-coated culture dishes or slide flasks (Nunc) in the presence of PDGF and BMP2 (10 ng/mL) for 2–3 d as described before (Kondo and Raff 2004). To induce the conversion to NSLCs, 2As were cultured in the presence of bFGF (10 ng/mL) for 5–10 d in uncoated dishes. If cultures were maintained for longer than 4 d, half of the medium was replaced every 2 d.

NSCs were prepared from E14.5 mouse telencephalon as described before (Nakashima et al. 1999) and expanded in bFGF as floating spheres. For immunostaining, the spheres were cultured on poly-L-ornithine (PLO, 15 μg/mL; SIGMA)- and fibronectin (1 μg/mL; Invitrogen)-coated eight-well chamber slides (Nunc) in the presence of bFGF for up to 5 d, as described before (Nakashima et al. 1999).

RT–PCR analysis

RT–PCR was carried out as described previously (Kondo and Raff 2000b). Dimethylsulfoxide (DMSO) was added to the reaction mixture: 5% DMSO for *olig2*, *brca1*, *musashi1*, and *nestin*, and 10% DMSO for *sox2* and *id1-4*. Cycle parameters for *sox2*, *olig2*, *musashi1*, *hes1*, *hes5*, *bmi1*, and *nestin* were 10 sec at 94°C, 20 sec at 58°C, and 90 sec at 72°C for 35 cycles. For *gfap*, *brca1*, *id1-4*, and *bcrp1*, they were 10 sec at 94°C, 20 sec at 58°C, and 45 sec at 72°C for 35 cycles. For *g3pdh*, they were 15 sec at 94°C, 30 sec at 53°C, and 90 sec at 72°C for 23 cycles.

The following oligonucleotide DNA primers were synthesized: For *sox2*, the 5′ primer was 5′-ATGTATAACATGATG GAGACGGAGC-3′, and the 3′ primer was 5′-TCACATGTGC GACAGGGGCAGTGT-3′. For *gfap*, the 5′ primer was 5′-GA GATGATGGAGCTCAATGACC-3′, and the 3′ primer was 5′-CTGGATCTCCTCCTCCAGCGA-3′. For *olig2*, the 5′ primer was 5′-ATGGACTCGGACGCCAGCCT-3′, and the 3′ primer was 5′-TCACTTGGCGTCGGAGGTGAG-3′. For *musashi1*, the 5′ primer was 5′-CCTGGTTACACCTACCAGTTC-3′, and the 3′ primer was 5′-TCAGTGGTACCCATTGGTGAAG-3′. For *brca1*, the 5′ primer was 5′-ATGGATTTATCTGCTGTTC GAATTC-3′, and the 3′ primer was 5′-AAACCATTTGCA CACTGCATTCC-3′. For *bmi1*, the 5′ primer was 5′-ATG CATCGAACAACCAGAAT-3′, and the 3′ primer was 5′-TCACTTTCCAGCTCTCCA-3′. For *nestin*, the 5′ primer was 5′-GCTACATACAGGACTCTGCTG-3′, and the 3′ primer was 5′-AAACTCTAGACTCACTGGATTCT-3′. The primers for *hes1*, *hes5*, *id1-4*, *bcrp1*, and *g3pdh* were described previously (Kondo and Raff 2000b,c; Kondo et al. 2004).

Vector construction

To deplete *sox2* or *brca1* mRNA and to mark the transfected cells, we constructed vectors based on the pMY vector (gift from T. Kitamura, University of Tokyo, Tokyo, Japan) (Misawa et al. 2000), which encodes *green fluorescence protein* (*gfp*) and U6-promoter-driven siRNA for either *sox2* (*sox2*-siRNA) or *brca1* (*brca1*-siRNA). The synthesized *sox2*-siRNA oligonucleotides were 5′-TCGACAACCAAGACGCTCATGAAGT CAAGAGCTTCATGAGCGTCTTGGTTTTTTA-3′ and 5′-AG CTTAAAAAAACCAAGACGCTCATGAAGCTCTTGACTT CATGAGCGTCTTGGTTG-3′. The synthesized *brca1* siRNA oligonucleotides were 5′-TCGACAGGGCCTTCACAATGTC CTTTCAAGAGAAGGACATTGTGAAGGCCCTTTTTTT-3′ and 5′-CTAGAAAAAAAGGGCCTTCACAATGTCCTTCTC TTGAAAGGACATTGTGAAGGCCCTG-3′.

To construct the series of *sox2*-enhancer–firefly luciferase expression vectors, mouse *sox2* 5′ genomic DNA (gift from R. Lovell-Badge, NIMR, London, UK) was amplified and cloned into a pGL3-promoter vector (Promega), producing sox2-full-pGL3-pro (full), sox2-R1-pGL3-pro (R1), sox2-R2-pGL3-pro (R2), or sox2-R3-pGL3-pro (R3). The following oligonucleotide DNA primers were synthesized: For *R1* (–3833 ~ –3444), the 5′ primer was 5′-GTCAAATAGGGCCCTTTTCAG-3′, and the 3′ primer was 5′-AAGCCAACTGACAATGTTGTGG-3′. For *R2* (–3465 ~ –2684), the 5′ primer was 5′-CCACAACATTGTC AGTTGGCTT-3′, and the 3′ primer was 5′-TAGTCTGAA CCTCTCCAATG-3′. For *R3* (–2704 ~ –1883), the 5′ primer was 5′-CATTGGAGAGGTTCAGACTA-3′, and the 3′ primer was 5′-CTGCCCCAGGTTCTCCTTAAG-3′. Full *sox2* 5′ genomic DNA (–3833 ~ –1883) was amplified with R1 5′ primer and R3 3′ primer. The transcription start site is considered to be position +1 (Wiebe et al. 2000).

To determine whether Brm has a role in *sox2* expression, we constructed vectors based on the pMY vector, which encodes GFP (to mark transfected cells) and either wild-type human Brm (wBrm) or mutant human Brm (mutBrm), which has an inactive ATPase domain (gift from C. Muchardt, Institut Pasteur, Paris, France).

Transfection

Transfection into OPCs and NSLCs was performed using the Nucleofector procedure according to the supplier's instructions (AMAXA). In brief, 2×10^6 cells were suspended in the Rat NSC Nucleofector Solution (100 µL) with the expression vector (10 µg), and the cells were transfected with the vector using the Nucleofector device. OPCs and NSLCs were then cultured in PDGF or bFGF, respectively.

To examine the effect of the transgenes, the transfected cells were harvested, and 5×10^3 cells per well were recultured in a PLO- and fibronectin-coated eight-well chamber slide (Nunc). To examine the effect on cell differentiation, the cells were cultured for 5 d in bFGF, fixed, and stained for neural markers (see below). To examine the effect on cell proliferation, BrdU (20 µM) was added after 5 d; 8 h later, the cells were fixed and stained for BrdU (see below), and the proportion of GFP$^+$ cells that were BrdU$^+$ was determined.

Immunocytochemistry

Immunostaining was carried out as described previously (Kondo and Raff 2000a). The following antibodies were used to detect intracellular antigens: monoclonal anti-Nestin (Pharmingen; diluted 1:200), rabbit anti-Sox2 (gift from R. Lovell-Badge; diluted 1:500), rabbit anti-Brca1 (Santa Cruz; diluted 1:200), mono-

clonal anti-GFAP (Sigma; 1:200), rabbit anti-GFAP (DACO; diluted 1:400), a mixture of rabbit anti-NF antibodies (Affiniti; diluted 1:100), monoclonal anti-GFP, and rabbit anti-GFP (Molecular Probe; diluted 1:200). To detect surface antigens, we used the A2B5 monoclonal antibody to detect OPCs (hybridoma supernatant; diluted 1:5) (Eisenbarth et al. 1979), monoclonal anti-PSA-NCAM (gift from T. Seki, University of Tokyo, Tokyo, Japan; diluted 1:1000), and monoclonal anti-SSEA1 (Santa Cruz; diluted 1:200). The antibodies were detected with Texas-Red-conjugated goat anti-mouse IgG or IgM (Jackson Immunoresearch; diluted 1:200), fluorescein-conjugated goat anti-rabbit IgG (Jackson Immunoresearch; diluted 1:200), and Alexa-488-coupled goat anti-mouse IgG and Alexa-594-coupled goat anti-rabbit IgG (Molecular Probe; diluted 1:200). Cells were stained for BrdU as previously described (Kondo and Raff 2000b), by using a monoclonal anti-BrdU antibody (hybridoma culture supernatant; diluted 1:5) (Magaud et al. 1988), followed by Texas-Red-conjugated goat anti-mouse IgG. To visualize all nuclei, the cells were counterstained with bisbenzimide (Hoechst 33342; 1 µg/mL).

Luciferase assay

NSCs were cultured on PLO- and fibronectin-coated six-well plates (Nunc). On the following day, cells were transfected with 2 µg of the full, R1, R2, or R3 vectors encoding firefly luciferase and 0.2 µg of the internal control vector pEF-Rluc (gift from S. Nagata and K. Shimozaki, Osaka University, Osaka, Japan) encoding sea pansy luciferase using the Superfect Transfection Reagent according to the supplier's instructions (QIAGEN). After 24 h, the activities of the two types of luciferase were measured using the Dual-Luciferase Reporter Assay System according to the supplier's instructions (Promega).

ChIP assay

ChIP assays were performed as described in Shang et al. (2000). In brief, we fixed 2×10^6 NSCs, OPCs, 2As, or NSLCs in 4% formaldehyde, suspended them in a cell lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl at pH 8.1, 5 mg/mL aprotinin, and 3 mM PMSF), sonicated them, and harvested cell extracts by centrifugation. The extracts were incubated with antibodies overnight and then immunoprecipitated with Protein-A-Sepharose (50 µL of 50% suspension). The following antibodies were used for the assay; anti-Brca1 antibodies (10 µg), anti-Brm antibodies (Santa Cruz; 2 µg), anti-Brg1 antibodies (Santa Cruz; 2 µg), anti-dimethyl-histone H3-K4 antibodies (UPSTATE; 1:100), anti-dimethyl-histone H3-K9 antibodies (UPSTATE; 1:100), and anti-acetyl-histone H3-K9 antibodies (UPSTATE; 1:100). Precipitates were heated to reverse the formaldehyde cross-linking. The DNA fragments in the precipitates were purified by phenol/chloroform extraction and EtOH precipitation and then used for PCR with primer sets for R1, R2, and R3. Cycle parameters were 10 sec at 94°C, 20 sec at 60°C, and 90 sec at 72°C for 30 cycles.

Immunoprecipitation

Immunoprecipitation assays were performed as described in Fukuda et al. (2004). In brief, 2×10^6 NSCs were harvested, suspended in NP-40 lysis buffer (10 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 5 mg/mL aprotinin, and 3 mM PMSF), and sonicated. After centrifugation, cell extracts were immunoprecipitated with anti-Flag M2, anti-Brca1, or anti-Brm antibodies and Protein-A-Sepharose. Immunoprecipitates were analyzed using anti-Brm antibodies.

Acknowledgments

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