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間葉系幹細胞は発生の初期においてまず神経上皮に由来する

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Mesenchymal stem cells, ES, neural crest, neuroepithelium, Sox1, ectoderm,
mesoderm,

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Summary

Mesenchymal stem cells (MSCs) are defined as cells that undergo sustained *in vitro* growth and are able to give rise to multiple mesenchymal lineages. Although MSCs are already used in regenerative medicine little is known about their *in vivo* behaviors such as developmental derivation. Here we show that the earliest wave of MSC in the embryonic trunk is generated from Sox1⁺ neuroepithelium but not from mesoderm. Using lineage marking by direct *gfp* knock-in and *Cre*-recombinase mediated lineage tracing, we show that Sox1⁺ neuroepithelium give rise to MSCs through neural crest. This pathway can be distinguished from the pathway through which Sox1⁺ cells give rise to oligodendrocytes by expression of PDGFR β and A2B5. MSC recruitment from this pathway, however, is transient and is replaced by MSCs from still unknown sources. We conclude that MSC can be defined as a definite *in vivo* entity recruited from multiple developmental origins.

Introduction

Mesenchymal stem cells (MSCs) have been defined by two features: the ability to undergo sustained proliferation *in vitro*, and the potential to give

rise to multiple mesenchymal cell lineages including osteocytes, chondrocytes, and adipocytes (Pittenger et al., 1999; Prockop, 1997). In recent years, MSCs have attracted attention as a source of cells for use in therapeutic applications for a number of reasons. Firstly, MSC lines have been isolated from human and animal tissues, both adult and embryonic (Deans and Moseley, 2000; Javazon et al., 2004; Pittenger et al., 1999; Prockop, 1997). Secondly, the use of MSCs is already a reality in regenerative medicine (Bianco et. al., 2001), a striking example of which is the recent success in correcting osteogenesis imperfecta (Horwitz et al., 2002). Finally, recent reports showing the existence of MSC-like cells (MAPCs) with unexpectedly high potential to give rise to cells of all three germ layers are expected to open new avenues into the understanding of the biology of MSCs (Jiang et al., 2002) .

Despite these increased expectations, the most significant problem in studying MSCs is the lack of knowledge concerning their *in vivo* characteristics, such as their development, exact tissue localization, and physiological role. Because of a difficulty in defining MSCs other than by the operational definition of *in vitro* self-renewal and differentiation potential, our knowledge of MSCs is solely based on the characterization

of cultured cells (Pittenger et al., 1999). Development of the CFU-F, a functional assay to measure fresh MSCs in tissue (Bianco et al., 2001; Friedenstein et al., 1970) improved the situation by defining markers that is exploited for purifying MSCs prospectively (Gronthos et al., 1994). But yet little is known about the behavior of MSCs *in vivo*.

The aim of this study is to identify MSC progenitors and track them prospectively to define MSC differentiation pathway. For instance, if MSCs are derived from mesoderm as have been widely believed (Dennis and Charbord, 2002), it should be possible to define their development prospectively by investigating whether purified mesoderm cells can give rise to MSCs. Once the earliest progenitor is identified, there are various ways to identify intermediate stages in the MSC differentiation pathway. To this end, we exploited ES cell cultures that support differentiation of various mesenchymal cell lineages (Kawaguchi et al., 2005; Sakurai et al., 2006; Wobus et al., 2002), as it is easier to dissect the differentiation process *in vitro* than in the living embryo. Thus, we began this study by attempting to define MSC progenitors in the *in vitro* differentiation of ES cells to adipocytes. Here, we present evidence that Sox1⁺ neuroepithelial cells supply the earliest wave of MSC differentiation, which occurs during

embryogenesis but is later replaced by MSCs from other origins in postnatal development.

Results

MSCs generated in ES cell culture are derived from neuroepithelium but not from mesoderm.

In order to specify the culture condition for ES cell differentiation to MSC, we compared two methods that have been used for inducing mesenchymal cell lineages from ES cells (Fig. 1A). The first condition (Condition A) is culture on collagen IV-coated dish under serum-containing medium, which supports generation of mesodermal cells that give rise to osteocytes, chondrocytes and myocytes (Sakurai et al., 2006). The other method involves the treatment of ES cells with pulse exposures to retinoic acid (RA) from day 2 to day 5 of culture (Condition B) followed by incubation under the adipocyte-inducing condition from day 11 (Dani et al., 1997) .

Cells expressing PDGFR α that is expressed in mesenchymal cells including adipocytes (Drozdoiff and Pledger, 1991) were generated under both culture condition, but they reached a peak on day 4 under Condition A and on day 9 under Condition B (Fig. 1B). Under both conditions,

adipogenic activity was detected only in the PDGFR α ⁺ population (Fig. 1C). With respect to the efficiency of adipogenesis as assessed by the TG content of the cultures, Condition B showed 10-fold higher activity than Condition A (Fig. 1C).

We then assayed the ability of MSCs to undergo sustained proliferation *in vitro*. Under the culture condition for maintaining MSC lines established from bone marrow and adipose tissue (MSC condition) (Pittenger et al., 1999), the PDGFR α ⁺ population induced under Condition B, but not A, exhibited sustained growth (Fig. 1D). Moreover, they underwent clonogenic growth and developed into fibroblast-like cell clones (a in Fig. 1F) that expressed molecular markers of mesenchymal cell lineage such as OB-CAD and PDGFR β ; they did not, however, express markers of mesoderm or neural crest (Fig. 1E). Of 10 clonal lines established, three lines gave rise to adipocytes, chondrocytes, and osteocytes (b-d in Fig. 1F). These results suggest that only PDGFR α ⁺ population induced under Condition B contains MSC progenitors.

Considering our previous study that Condition A is the optimum for mesoderm differentiation, this result suggests that MSCs generated in the ES cell culture are not derived from mesoderm. Indeed, no paraxial

mesoderm markers (Fig. 2A, Brachyury, Mesp2, and Mesogenin) were expressed in the PDGFR α ⁺ population induced by Condition B, whereas they were detected in the PDGFR α ⁺ population induced by Condition A. Though clearly distinctive each other, the two populations express common mesenchymal markers such as OB-CAD and PDGFR β (Fig. 2A). Taken together, the two PDGFR α ⁺ populations represent mesenchymal lineage but derived from distinct pathways.

To gain an insight into their origins, we assessed the expression of molecular markers for mesoderm, endoderm, and neural lineages of cells cultured under the two conditions. Mesoderm and endoderm markers were expressed under Condition A, but not B (Fig. 2B). In contrast, expression of neural markers was detected only under Condition B. Thus, the next issue is whether or not PDGFR α ⁺ MSC induced under Condition B are actually derived from neural lineage. In order to define neural lineage, we used Sox1 as a molecular marker, as it was shown to be the most specific marker for neuroepithelial cells (Aubert et al., 2003; Pevny et al., 1998). To visualize the Sox1⁺ neuroepithelial cells, we used an ES cell line to which the *gfp* gene has been inserted in the *sox1* allele (ES-Sox1^{gfp/+}) (Aubert et al., 2003). As expected, Condition B supports

preferential differentiation to Sox1⁺ cells (Fig. 2C and D). We then investigate whether or not Sox1⁺ neuroepithelium can give rise to PDGFR α ⁺ MSC. GFP⁺ but not GFP⁻ population in day 4 culture of ES-Sox1^{tgfp/+} cells under Condition B could give rise to adipocytes (Supplemental Data 1A). Neither GFP⁺ nor GFP⁻ cells expressed PDGFR α at day 4, but on culturing the sorted cells for 6 more days, the PDGFR α ⁺ population reached 30-40% in the culture of GFP⁺ cells (n=3), whereas only a low level of PDGFR α ⁺ was induced from the GFP⁻ cells (Fig. 2E). These Sox1⁺-derived PDGFR α ⁺ cells have already lost neural and neural crest markers such as Sox1, Sox10 and Wnt1 (Fig. 2F). Adipocytes were generated only from the PDGFR α ⁺ population in the culture of Sox1⁺ cells (Fig. 2G). Moreover, this PDGFR α ⁺ population had the potential to undergo sustained *in vitro* growth (data not shown) and to give rise to MSC lines that can give rise to the three lineages of adipocytes, chondrocytes, and osteocytes (Supplemental Data 1B). Taken together, Sox1⁺PDGFR α ⁻ and subsequent Sox1⁺PDGFR α ⁺ populations are successive intermediates in a MSC differentiation pathway.

Sox1⁺ MSC progenitors in early embryos

We next attempted to confirm this differentiation pathway in the embryo. ES-Sox1^{gfp/+} were aggregated with tetraploid embryos (Nagy et al., 1993), incubated *in utero* and harvested at E9.5. In this embryo, GFP expression was detected almost exclusively in neuroepithelial cells (Fig. 3A), whereas somitic cells are present in PDGFR α ⁺ population (Takakura et al., 1997). We removed the cranial and tail regions upon preparing single cell suspension of embryonic cells, as cranial neuroepithelial cells normally give rise to mesenchymal cell lineages through the neural crest (Le Douarin et al., 2004). Cells dissociated from the trunks of E9.5 embryos were divided into three populations, Sox1⁻PDGFR α ⁺, Sox1⁺PDGFR α ⁻, and Sox1⁻PDGFR α ⁻; no double-positive cells were detected (right panel in Fig.3A). Expression of molecular markers confirmed that Sox1⁺PDGFR α ⁻ cells from the trunk correspond to neuroepithelium and Sox1⁻PDGFR α ⁺ cells contain mesodermal but not neural cells (Supplemental Data 1C). Consistent with our *in vitro* experiments, adipocytes differentiated from both Sox1⁺ and PDGFR α ⁺ populations (Fig. 3B), but only Sox1⁺ cells underwent sustained growth under MSC condition (Fig. 3C). In separate experiments, we confirmed that cells expressing endodermal makers were present in the double negative fraction and failed to give rise to MSCs nor

adipocytes (Supplemental Data 1C).

In all attempts (n=3), multipotent MSC lines could be established directly from Sox1⁺ cells. Moreover, Sox1⁻PDGFR α ⁺ cells were generated by incubating Sox1⁺PDGFR α ⁻ cells in culture (Fig. 3D). Thus, in complete agreement with our *in vitro* results, only Sox1⁺ neuroepithelial cells in the trunk of E9.5 embryo contain MSC progenitors. While it was difficult to establish MSC clones directly from Sox1⁺ cells in the embryo, a fraction (7/576) of PDGFR α ⁺ cells that were induced by 6-day incubation of FACS-purified Sox1⁺ cells could undergo sustained clonogenic growth. Among them, 3 clones maintained the potential to give rise to adipocytes, chondrocytes, and osteocytes under conditions appropriate to those lineages (Fig. 3E).

Persistent labeling of Sox1⁺ neuroepithelial cells and P0⁺ neural crest cells

To determine the correlation between Sox1⁺ MSC progenitors in E9.5 embryos and MSCs in later life, we carried out a persistent labeling experiment of Sox1⁺ cells (Soriano, 1999). For this experiment, we exploited the Cre-recombinase-mediated labeling method by inserting *Cre* gene in the *Sox1* allele (see Experimental Procedures and Supplemental

Data 2A), and the resulting mice were crossed to the ROSA26R-EYFP strain, whose YFP expression was switched on by Cre-recombinase and constitutively maintained under ROSA promoter (Srinivas et al., 2001). Considering the possibility that Sox1⁺ neuroepithelial cells give rise to MSCs via the neural crest stage, we also used P0-Cre/YFP embryos. As compared with Wnt1 that is also used for neural crest labeling but expressed in the dorsal region of the neural tube (Parr et al., 1993; Pietri et al., 2003), the P0 promoter expresses genes only after the differentiation of neural crest cells (Yamauchi et al., 1999).

In the Sox1-Cre/YFP embryos, YFP expression was detected in the neural fold of E8.5 embryos and in the neural tube of E9.5 embryos (Fig. 4 A, B, D and E). Proportion of YFP⁺ cells (more than 80%) was maintained at the same level in the central nervous system up to adult stage (Supplemental Data 4E). Sox1 expression was segregated completely from the somites and restricted to the neuroepithelium in the trunk (Fig. 4G). While YFP expression in 1st branchial arch is low (Fig. 4E), nearly all neural tubes and dorsal root ganglia (DRG) of E11.5 embryos were YFP⁺ (Fig. 4H), suggesting that labeling efficiency per se is high. In contrast, in P0-Cre/YFP embryos, YFP expression was mainly observed in branchial

arches and cranial mesenchymal tissues (Fig. 4C and F) but not in neural tube. At E11.5, YFP expression was not detected in the neural tube, but was in the dorsal root ganglia (Fig. 4I).

MSCs in embryos are recruited from multiple sources

Using the Sox1-Cre/YFP mice, we first explored the presence of PDGFR α ⁺ cells derived from Sox1⁺ cells in E14.5 embryos. Again, we prepared cell suspensions only from the trunk region. YFP⁺PDGFR α ⁺ cells were detected in the trunk region of the two mouse strains (Fig. 5A). Thus, a PDGFR α ⁺ population derived from Sox1⁺ neuroepithelial cells as well as from P0⁺ neural crest cells are present in the trunk of the embryos. PCR analysis showed that YFP⁺ cells from the trunk of E14.5 embryo no longer express endogenous Sox1 as well as Cre, suggesting that they are the progeny of Sox1⁺ neuroepithelial cells generated before E14.5 (Upper panel in Supplemental Data 2B). In contrast, Cre expression is still observed in YFP⁺ cells from E14.5 P0-Cre/YFP embryos (lower panel in Supplemental Data 2B), suggesting sustained expression of P0 in some neural crest lineages. Section analysis showed that almost all YFP expression is detected in neural crest-derived tissues such as dorsal root

ganglia (Fig. 4I), and YFP+PDGFR α ⁻ cells expressed Sox10, a marker of trunk neural crest (Lower panel in Supplemental Data 2B).

Interestingly, the proportion of YFP+PDGFR α ⁺ cells in the cell suspension from the trunk of Sox1-Cre/YFP embryos was 8-fold less than that from P0-Cre/YFP embryos (Fig. 5A). This suggests that a considerable proportion of neural crest cells that differentiate to PDGFR α ⁺ cells do not originate from Sox1⁺ neuroepithelial cells. The same may be also the case for cranial region, as YFP⁺ cells in the mesenchymal population of the first branchial arch was far lower in Sox1-Cre/YFP embryos than in P0-Cre/YFP embryos (Fig. 4E and F).

Nonetheless, we found PDGFR α ⁺ cells derived both from neuroepithelial cells and from neural crest cells in the trunk of E14.5 embryo. The YFP+PDGFR α ⁺ populations from the Sox1-Cre/YFP and P0-Cre/YFP embryos were sorted and assayed for the potential to give rise to MSCs and CFU-F (Fig. 5B). Both populations expressed mesenchymal markers, such as OB-CAD and PDGFR β (Supplemental Data 2B). CFU-F was detected at the same frequency in the YFP⁻PDGFR α ⁺ and YFP+PDGFR α ⁺ populations in both stains of embryo, but almost no CFU-F was detected in the PDGFR α ⁻ populations, irrespective of YFP

expression. Moreover, YFP⁺PDGFR α ⁺ and YFP⁻PDGFR α ⁺ cells of both mouse strains exhibited sustained growth *in vitro* (Fig. 5C). These results strongly suggest that the MSC progenitors in the trunk of E14.5 embryos are PDGFR α ⁺ but derived not only from neural crest but also from other, as yet unidentified, sources. We also confirmed the multipotentiality of MSC established from YFP⁺PDGFR α ⁺ population of Sox1-Cre/YFP mice (Fig. 5D).

MSC progenitors are distinguished from oligodendrocyte precursors by expression of PDGFR β and A2B5.

YFP⁺PDGFR α ⁺ cells in the trunk of E14.5 Sox1-Cre/YFP embryos are likely to contain oligodendrocyte (OG) precursors (OGPs) generated in the neural tube (Stolt et al., 2006). We thus investigated whether or not MSCs in the PDGFR α ⁺ population derived from Sox1⁺ cells in E14.5 embryos are different from OGPs that express A2B5 (Kondo and Raff, 2000). YFP⁺ PDGFR α ⁺ cells in E14.5 Sox1-Cre/YFP mice contain both A2B5⁺ and PDGFR β ⁺ populations. Interestingly, the two populations are segregated almost completely (Fig. 6A). By RT-PCR analysis, markers for OGs are detected only in A2B5⁺ population (Fig. 6B). Sorted A2B5⁺

population could form neurosphere upon culturing under the condition described in previous reports (Morrison et al., 1999) (Supplemental Data 3A), whereas could not undergo sustained growth under MSC condition nor give rise to adipocytes (data not shown). As those neurosphere could give rise to OG, A2B5⁺ cells derived from Sox1⁺ neuroepithelium are OGP with self-renewing capacity (Supplemental Data 3B). The PDGFR β ⁺ population expresses none of OG markers used in this study and failed to form the neurospheres (Fig. 6B and Supplemental Data 3A). CFU-Fs are enriched in the PDGFR β ⁺ population (Fig. 6C). In addition, this population undergoes sustained growth and gives rise to adipocytes (Fig. 6D and E). Cells isolated from individual CFU-Fs gave rise to adipocytes but not OG (data not shown). The frequency of CFU-F in YFP⁺PDGFR α ⁺PDGFR β ⁺ population was 2%, and 50% of the colonies could develop to adipogenic cell lines. Thus, OGPs and MSCs, which are derived from neuroepithelium and express PDGFR α are fully segregated, respectively, to A2B5⁺ and PDGFR β ⁺ fractions at E14.5.

Transient wave of MSC generation from neuroepithelial and neural crest lineages

As bone and bone marrow has been the major sources of MSC (Deans and Moseley, 2000; Javazon et al., 2004), we focused the analysis of postnatal mice on cells dissociated from femoral and tibial bones by treatment with collagenase and dispase. YFP⁺PDGFR α ⁺ cells were detected in the bone cell preparation of neonates, 4-week and 12-week old mice of Sox1-Cre/YFP and P0-Cre/YFP (Fig. 7A and B, Supplemental Data 4A). However, the proportion of YFP⁺PDGFR α ⁺ cells in the bone cell preparation of neonates was far lower than in the whole embryonic trunk, and the population significantly decreased with age in both Sox1-Cre/YFP and P0-Cre/YFP mice (Fig. 7C). This is consistent with the result showing low expression of Ki67, suggesting MSCs derived from Sox1⁺ neuroepithelium do not proliferate after birth (Supplemental Data 4B, C). Similarly, YFP⁻PDGFR α ⁺ in the bone cell preparation of both mice decreased along with neonatal development (Fig. 7A and B, Supplemental Data 4A). Notably, CFU-F activity was found at the same level both in YFP⁺PDGFR α ⁺ and YFP⁻PDGFR α ⁺ populations representing, respectively, neural crest and non-neural crest pathways (Fig. 7D). The ability of YFP⁺PDGFR α ⁺ cells to give rise to the multipotent MSC was also confirmed, as before (Fig. 7E). Thus, PDGFR α ⁺ population in the bone

preparation of postnatal mouse contains MSCs derived from neuroepithelium and neural crest, but their contribution is not high. Moreover, they rapidly decrease during neonatal development (Fig. 7A-C, Supplemental Data 4A). As the YFP expression in central nervous system is maintained at the same level during postnatal development and YFP expression of MSC does not fade during culture, it is unlikely that decrease of YFP⁺ population during postnatal development is due to silencing of the YFP gene (Supplemental Data 4 D and E). Taken together, these results suggest that MSCs are differentiated through multiple routes with distinct cellular origins, among which the wave originating from neuroepithelium including the neural crest, although it is transient, is the earliest.

Discussion

The aim of this study was to define the developmental pathway of MSCs, which is largely unstudied *in vivo*. While we demonstrated two pathways for adipocyte differentiation, one from Sox1⁻PDGFR α ⁺ paraxial mesoderm and the other from Sox1⁺ neuroepithelium; only the latter could give rise to MSCs. This observation in ES cell culture was confirmed in E9.5

embryos. In the embryo, it is well established that cranial neural crest cells give rise to mesenchymal cells (LaBonne and Bronner-Fraser, 1999; Le Douarin et al., 2004). To avoid unnecessary confusion, this study focused only on the trunk region. We obtained PDGFR α ⁺ somitic cells and Sox1⁺ neuroepithelium from the trunk, which are segregated each other in FACS analysis (Fig. 3A). Consistent with the *in vitro* result, Sox1⁺ neuroepithelium in the trunk did give rise to MSCs. While somitic cells have been believed as progenitors of MSCs, none of the PDGFR α ⁺ cells in the E9.5 embryos could differentiate to MSCs, although they could give rise to adipocytes. Thus, the first MSC progenitors in the embryo, which are identifiable by the method herein, are derived from neuroepithelial cells. This observation, however, does not rule out a possibility that paraxial mesoderm has potential to give rise to MSCs under other appropriate conditions.

In the ES cell culture system, Sox1⁻PDGFR α ⁺ MSC progenitors induced from Sox1⁺ cells are negative for neural crest markers such as Sox10 and Wnt1 (Fig. 2F). We are however convinced that at least a part of MSCs pass through the neural crest stage during differentiation from Sox1⁺ cells to PDGFR α ⁺ MSCs, as Sox10 is induced during the culture of

Sox1⁺ cells (data not shown). We also investigated the expression p75, another neural crest marker (Morrison et al., 1999; Wilson et al., 2004), in cells derived from Sox1⁺ neuroepithelial cells. Consistent with the previous study (Wilson et al., 2004), p75 expression coincides with neural crest cells at E9.5 (Supplemental Data 5 A and B). Though PDGFR α has not been expressed in Sox1⁺ cells at E9.5 (Fig. 3A and Supplemental Data 5 C), we could detect p75⁺PDGFR α ⁺ population derived from Sox1⁺ cells at E14.5 and this population contained CFU-F (Supplemental Data 5 C and D). This observation also supports presence of neural crest pathway for MSCs differentiation, though p75⁺PDGFR α ⁺ cells also contain CFU-F.

The presence of neural crest-derived MSCs was also confirmed by Cre-recombinase-mediated genetic labeling method (Kilby et al., 1993). In this study, we compared Sox1-Cre/YFP embryos with P0-Cre/YFP embryos in which neural crest derived cells are labeled. In both strains, YFP⁺ cells that have an ability to form CFU-F were detected in the PDGFR α ⁺ population. Thus, in the embryo, at least a part of the earliest wave of MSCs are generated through Sox1⁺ neuroepithelium, P0⁺ neural crest and Sox1⁻PDGFR α ⁺ progenitor.

We have shown that Sox1⁺ cells in the trunk region can give rise to

PDGFR α ⁺ mesenchymal cell lineages, which contrasts to the widely accepted view that trunk neural crest cells do not contribute to mesenchymal cell lineages in normal development (Le Douarin et al., 2004). It has been suggested that trunk neural tube has the ability to give rise to mesenchymal cells (Shah et al., 1996). Moreover, a recent fate analysis of neural crest cells by persistent labeling demonstrated that trunk neural crest cells differentiate to myofibroblasts in the endoneural region (Joseph et al., 2004). These endoneural myofibroblast lineages may be identical to the neural crest-derived MSCs in the embryonic trunk. However, as cranial neural crest cells are also distributed in the trunk (Matsuoka et al., 2005), it has been difficult to determine the origin of neural crest cells in the trunk. In this respect, this study would be the clearest demonstration that trunk neural crest cells do give rise to mesenchymal lineages, as the contamination of cranial neural crest in Sox1⁺ trunk neuroepithelium is unlikely.

Another unexpected observation from our persistent labeling experiment is that PDGFR α ⁺ cells labeled by Sox1-Cre were 8-fold fewer than those labeled by P0-Cre. According to one current model, neural crest cells are derived mostly from the dorsal part of the neural tube (LaBonne

and Bronner-Fraser, 1999). If this is the case, Sox1-Cre should label all neural crest cells. It could be that the lower frequency of mesenchymal cells marked by Sox1-Cre may simply reflect low recombinase activity. However, Sox1-Cre was efficient in labeling other neuroepithelium-derived lineages, as nearly all neuroepithelial cells at E9.5, dorsal root ganglia cells and melanocytes (Supplemental Data 6) are YFP⁺ in Sox1-Cre/YFP mice. These results may suggest that Sox1⁺ neuroepithelial cells are not the sole source of neural crest cells. Recently, we proposed a hypothesis that ectodermal cells in close proximity to neuroepithelial cells are the actual source of cranial mesenchymal cells (Weston et al., 2004). The present study partly supports this hypothesis, though it shows at the same time that Sox1⁺ neuroepithelial cells do contribute to ectomesenchymal cells.

We were able to specify MSCs derived both from Sox1⁺ neuroepithelial cells and neural crest cells. In the *in vitro* CFU-F assay, about 1/40 of the YFP⁺PDGFR α ⁺ cells in the embryo and 1/4 of those in the neonatal bone preparation formed colonies, whereas almost no YFP⁺PDGFR α ⁻ cells did so. Thus, MSC progenitors are highly enriched in the PDGFR α ⁺ population of neonatal bone marrow. YFP⁺PDGFR α ⁺ cells from

Sox1-Cre/YFP mice also exhibited sustained growth on transfer to MSC condition, and multipotent MSC lines were established from them. This MSC progenitor is explicitly distinguished from oligodendrocyte progenitor by expression of PDGFR β and A2B5.

Though in E9.5 embryo Sox1⁺ cells are the only population that can give rise to PDGFR α ⁺ MSCs, MSCs derived from neuroepithelium appeared to be a transient population that is eventually replaced by those derived from unidentified pathways. Indeed, already in the E14.5 embryonic trunk, the proportions of YFP⁻PDGFR α ⁺ cells were 21% and 13% for Sox1-Cre/YFP and P0-Cre/YFP embryos, respectively, which are higher than those of YFP⁺PDGFR α ⁺ cells (1.3% and 9.4%, respectively). As the same levels of CFU-F are present in YFP⁺ and YFP⁻ populations, and MSCs could be established from both populations, the YFP⁻ population does contain *bona fide* MSCs. Taken together, these results suggest that a significant proportion of MSCs in E14.5 embryos are already derived from a non-neural crest pathway.

In this study, we showed that neuroepithelium-derived and neural crest-derived MSCs are present also in the cell preparation from femoral and tibial bones of postnatal mice. However, the proportions of neural

crest-derived as well as neuroepithelium-derived MSCs decrease progressively over the course of development, and the contributions of YFP⁺ cells to PDGFR α ⁺ cells in the bone cell preparation were 0.032% and 2.39% for Sox1-Cre/YFP and P0-Cre/YFP neonates, respectively. Moreover, the frequency of CFU-F in the PDGFR α ⁺ population was nearly the same in YFP⁺ and YFP⁻ cells. Thus, in the neonatal bone preparation, MSCs were enriched in PDGFR α ⁺ but most of them were derived from a non-neural crest pathway. As mice age, YFP⁺PDGFR α ⁺ cells practically disappeared from the bone preparation of both Sox1-Cre/YFP and P0-Cre/YFP strains. Taken together, these results suggest that the MSC differentiation pathway defined in this study represents the earliest, but transient, wave of MSC supply.

The concept of MSCs originating in multiple waves derived from distinct sources is reminiscent of various other stem cell systems. Hematopoiesis is divided into primitive and definitive hematopoiesis, which originate from distinct regions (Zon., 1995). As for mesenchymal cell lineages, a recent study showed that skeletal muscle cells are recruited through multiple waves derived from distinct pathways (Bailey et al., 2001). We have shown here that the earliest wave of MSC generation

originates in the neuroepithelial and neural crest cells. However, the derivation of the later waves remains for future investigation. While we speculate that mesoderm cells remain one possible source, a recent study suggests that hematopoietic cells may constitute the progenitors of MSCs in adult bone marrow (Ogawa et al., 2006).

To our knowledge, no experimental evidence is available for the origin of MSCs. Recently, however, neural crest cells have emerged as another potential source. Firstly, it is well established that neural crest cells are multipotent progenitors with the potential to give rise to a wide variety of tissue cells (LaBonne and Bronner-Fraser, 1999). Secondly, a neural crest origin may better account for the recent reports suggesting the existence of MSCs with neurogenic potential (Dezawa et al., 2001; Kopen et al., 1999). More recently, Fernandes *et al.* suggested the presence of multipotent stem cells in the hair papilla, which derive from neural crest (Fernandes et al., 2004). The question of which of those MSCs is of neural crest origin is an important issue for future research. In this respect, our data suggest that, while some MSC lines established from embryos may be derived from neural crest cells, it is unlikely that MSCs established from adult bone marrow are of neural crest origin. Moreover, we showed

that PDGFR β can be exploited as another marker of MSC that are restricted to mesenchymal cell lineage.

In conclusion, in this study we have defined the first wave of MSC differentiation by identifying the origin and intermediate stages of its differentiation, thereby demonstrating that MSCs are an actual tissue component rather than an *in vitro* artifact. Although the origin of the later waves remains for future study, this study will open a new avenue in the understanding of the behavior and role of MSCs.

Experimental Procedures

Cell culture

A variety of culture methods were required for this study such as ES cell differentiation, MSC culture, cultures for MSC differentiation to mature mesenchymal cells, CFU-F assay, neurosphere culture and oligodendrocyte differentiation culture. All details including ingredients in media are described separately in the Supplemental Experimental Procedure as well as in our previous papers cited in the reference. We exploited only monolayer culture of ES cells for inducing differentiation. Thus, while the original method of ES cell differentiation to adipocytes

exploited the embryoid body method (Dani et al., 1997), it was modified into the monolayer culture. ES cell lines used in this study are CCE, TT2 and ES-Sox1^{gfp/+} lines.

Injection of tetraploid embryos

Tetraploid embryo complementation was performed as described (Nagy et al., 1993), using C57BL/6 zygotes. Briefly, two-cell stage embryos were electro-fused and developed *in vitro* to the 4N blastocyst stage.

ES-Sox1^{gfp/+} cells were injected into blastocysts and transferred to pseudo-pregnant ICR females. At E9.5, the embryos were collected and those expressing GFP were selected for further use.

Mouse stains used for cell tracing

The Sox1-Cre knock-in strain was produced by homologous recombination by the targeting vector (Supplemental Data2A and Supplemental Experimental Procedure) into TT2 ES cells. Transient transfection with a Flp-expressing recombinant adenovirus was performed to remove the PGK-Neo cassette (Kondo et al., 2006). The absence of PGK-Neo cassette was confirmed by Southern blot analysis. P0-Cre mice and

ROSA26R-EYFP mice were kindly provided by Ken-Ichi Yamamura and Frank Costantini, respectively. PCR genotyping of mice was performed by using primers listed in Supplemental Data 7.

Preparation of single cell suspension.

Culture cells were dissociated by tryposin-EDTA or dissociation buffer (GIBCO). To prepare cells from embryo, the trunk region indicated in Fig.3 were pooled, minced, and incubated first with 2.4U dispase II in PBS(GIBCO) and subsequently dissociation buffer (GIBCO). To obtain bone cell suspension, femurs and tibias were removed, cut into small pieces, and treated with 0.2% collabonase (SIGMA) and 2.4U dispase II (GIBCO) at 37°C for 1hr with gentle agitation. Neonate and adult brain were minced and the fragments were treated with 0.05% Trypsin/EDTA (GIBCO) for 15 min at 37°C. Cells were stained with fluorescence labeled antibodies and sorted or analyzed by FACS Aria (Becton&Dickinson) and FACS Calibur (Becton&Dickinson) was used.

Immunohistochemistry

For histological analysis, embryos were fixed in 4% paraformaldehyde

overnight and embedded in cryomold for sectioning. The following antibodies were used as primary antibodies: APA5, 1:1000 or anti-p75 Ab (Chemicon), 1: 200; anti-GFP(NACALAI), 1:500 . TO-PRO3 iodide (Invitrogen) was used for nuclear staining. Samples were stained by appropriate fluorescence-tagged secondary antibodies and examined using a Radiance 2100 (BioRad) confocal imaging system. For Ki67 staining, the cytopinned cells were first incubated with anti-Ki67 (ABCAM) at 1/500 dilution. For statistical analysis, 250 cells were examined in each experiment.

RT-PCR assay

RT-PCR was performed as described previously (Tada et al., 2005). We used β -actin expression as a control. Primers used for RT-PCR assay is listed in Supplemental Data 7.

Acknowledgments

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Figure Legends

Figure 1. Two distinct pathways of ES cell differentiation to adipocytes

A. Two protocols for inducing adipocytes from ES cells. Condition A:

Culture on a collagen IV-coated dish with serum containing medium.

Condition B: Incubation with 10^{-7} M Retinoic Acid (RA) from day 2 to day

5 under serum containing medium. The medium was replaced by a

RA-free secondary culture on day 5. Under both conditions, adipogenesis

was induced by a cocktail of adipogenic reagents (see Supplemental

Experimental Procedures) added from day 11. B. Generation of

PDGFR α ⁺ cells during ES cell differentiation under the two conditions.

The proportion of PDGFR α ⁺ cells peaked on day 4 and day 9 under Condition A and B, respectively. C. Analysis of adipogenic activity. PDGFR α ⁺ and PDGFR α ⁻ cells (data not shown) were purified from day 4 (Condition A) or day 9 (Condition B) cultures, and 5×10⁵ cells were re-cultured under adipogenic conditions. Oil red O staining of at day 18 (Condition B) and day 25 (Condition A). Only PDGFR α ⁺ cells are adipogenic. Condition B is more efficient than Condition A in adipogenic activity as indicated by the triglyceride content in the inset of each panel (mean of triplicate cultures and standard deviation). D. PDGFR α ⁺ cells induced under Condition B but not Condition A underwent sustained growth *in vitro*. In vitro growth of PDGFR α ⁺ cells from cultures under Condition A and under Condition B were compared. E. RT-PCR analysis of lineage markers in MSC lines established from PDGFR α ⁺ cells induced under Condition B. OB-CAD, Vimentin and PDGFR β for mesenchymal lineage, Brachyury, Mesp2 and Mesogenin for mesoderm, and Sox10 and Wnt1 for neural crest (Matsuoka et al., 2005; Sakurai et al., 2006; Tada et al., 2005). F. Multipotency of MSC lines derived from PDGFR α ⁺ cells in Condition B culture. (a) Phase-contrast view. (b-d) Multipotency of MSC clones. Clones were established from bulk culture of MSCs, and their

potential to give rise to adipocytes (b, Oil red O staining on day 10), osteocytes (c, Alizarin red staining on day 28), and chondrocytes (d, Alcian blue staining on day 21) was analyzed. One-third of clones gave rise to all three lineages.

Figure 2. Sox1⁺ cells in ES cell culture are definite progenitors of MSCs

A. Expression of mesoderm and mesenchyme markers in PDGFR α ⁺ cells induced under Conditions A and B. PDGFR α ⁺ cells induced under each condition were purified and expression of molecular markers was evaluated by RT-PCR. Brachyury, Mesp2, and Mesogenin are markers for mesoderm and OB-CAD, Vimentin, and PDGFR α are common markers for mesenchymal cells. **B.** Expression of molecular markers for mesoderm, endoderm (GATA4, Foxa2), and neural lineages during ES cell differentiation under Conditions A and B. Cells were harvested every day from day 2 to day 5 and marker expression was examined by RT-PCR. Note that Condition A supports mesoderm and endoderm differentiation whereas Condition B does neural differentiation assessed by neural cell markers described previously (Li, 2002; McCormick et al., 1996). **C.** The kinetics of differentiation of Sox1⁺ and PDGFR α ⁺ cells under Condition B.

GFP⁺ cells peaked on day 4 but no PDGFR α ⁺ cells were detected in the culture up to day 4. **D.** Expression of neural and mesoderm markers in Sox1-GFP⁺ cells by RT-PCR analysis. No mesoderm markers are expressed. **E.** Generation of PDGFR α ⁺ cells from purified Sox1⁺ cells. The Sox1⁺ and Sox1⁻ populations purified from day 4 cultures under Condition B are re-cultured for 6 more days. PDGFR α ⁺ cells were generated only from Sox1⁺ population. **F.** Expression of lineage markers in Sox1⁺-derived PDGFR α ⁺ cells by RT-PCR. Note that mesenchymal⁻ but no neural⁻ and mesodermal-markers are detected. **G.** Adipogenic activity of cell fractions harvested from day 10 culture. Sox1⁺ and Sox1⁻ cells were purified from day 4 culture under Condition B, re-cultured for 6 more days, and separated into PDGFR α ⁺ and PDGFR α ⁻ fractions. Each fraction was re-cultured with adipocyte induction cocktail for 8 days. Adipocytes visualized by Oil red O staining were detected only in the PDGFR α ⁺ fraction derived from Sox1⁺ cells.

Figure 3. Sox1⁺ neuroepithelial cells are sole population of E9.5 embryos that can give rise to MSCs in vitro. **A.** GFP expression of a tetraploid chimera from ES-Sox1^{gfp/+} cells. Whole mount view under fluorescent

microscope (left panel) and section staining with anti-GFP (center panel).

Approximately 20% of blastocysts injected with ES-Sox1^{gfp/+} cells

developed to E9.5 embryos. Note that GFP expression is restricted in the

neural tube. Trunks (indicated by white lines in the left panel) of embryos

were pooled, dissociated, and analyzed for GFP and PDGFR α expressions

by flow cytometer. Expression of Sox1-GFP and PDGFR α were completely

segregated (right panel). **B.** Adipogenic activity of Sox1⁺PDGFR α ⁻ and

Sox1⁻PDGFR α ⁺ cells purified from E9.5 embryos. Adipogenesis was

assessed by Oil red O staining. Sox1⁺PDGFR α ⁻ cells generated more

adipocytes than Sox1⁻PDGFR α ⁺ cells. **C.** Growth activity of Sox1⁺ and

PDGFR α ⁺ populations from E9.5 tetraploid embryos. Only

Sox1⁺PDGFR α ⁻ cells underwent sustained growth under MSC condition

(mean value of triplicate culture). **D.** Differentiation of PDGFR α ⁺ cells

from Sox1⁺PDGFR α ⁻ cells in E9.5 embryos. Sox1⁺PDGFR α ⁻ differentiate

to Sox1⁻PDGFR α ⁺, Sox1⁺PDGFR α ⁻ and double-negative cells. **E.**

Multi-lineage differentiation from MSC clones derived from

Sox1⁻PDGFR α ⁺ cells generated from Sox1⁺ cells of E9.5 embryos. Cloned

cell lines were fibroblast-like in morphology (a) and could give rise to

adipocytes (b: Oil red O staining on day 14), osteocytes (c: Alizarin red

staining on day 35), and chondrocytes (d: Alcian blue staining on day 28).

Figure 4. Tracing of cells derived from Sox1⁺ neuroepithelial cells and P0⁺ neural crest cells in embryos. In Sox1-Cre/YFP embryos, YFP expression was detected in the neuroepithelium at E8.5 (A,D) , E9.5 (B,E,G) and E11.5 (H). PDGFR α expression (red color in D,G) is detected in the somite. In E9.5 and E11.5 embryos, YFP⁺ cells become detectable outside the neural tube such as branchial arch (arrow in E) and dorsal root ganglia (arrow in H). In contrast, no YFP expression is detected in the neural tube of P0-Cre/YFP embryo at E9.5(C, F) and E11.5 (I). On the other hand, YFP is expressed in neural crest of branchial arches (arrow in F) and neural crest-derived tissues such as cranial mesenchyme (C), otic vesicle (C,F) and dorsal root ganglia (arrow in I).

Figure 5. Presence of MSCs derived from Sox1⁺ neuroepithelium and P0⁺ neural crest in embryos. A. FACS analysis of YFP and PDGFR α expression in E14.5 Sox1-Cre/YFP (left) and P0-Cre/YFP (right) embryos. The trunk region of Sox1-Cre/YFP and P0-Cre/YFP E14.5 embryos were pooled and dissociated. YFP⁺PDGFR α ⁺ cells were detected in both strains.

B. PDGFR α ⁺ expression of CFU-F. Each fraction indicated in the figure was purified from Sox1-Cre/YFP (left) or P0-Cre/YFP (right) embryos at E14.5, and CFU-F assay was performed. Upper pictures show colonies developed in each dish. The number of colonies is shown in the lower graphs. Each bar represents the arithmetic mean and SD of triplicate assays. CFU-F activity was enriched in PDGFR α ⁺ populations irrespective of YFP expression. **C.** In vitro proliferative activity of PDGFR α ⁺ populations in E14.5 embryo. Irrespective of YFP expression, PDGFR α ⁺ cells can undergo sustained proliferation in the MSC culture condition. **D.** Multipotency of MSC clones. MSC clones were established from YFP⁺PDGFR α ⁺ cells in E14.5 Sox1-Cre/YFP embryos and examined their ability to give rise to adipocytes (left, Oil red O staining on day 14), osteocytes (center, Alizarin red staining on day 42), and chondrocytes (right, Alcian blue staining on day 35).

Figure 6. Distinction between oligodendrocyte and MSC in PDGFR α ⁺ population derived from Sox1⁺ neuroepithelial cells **A.** Expression of PDGFR β and A2B5 in YFP⁺PDGFR α ⁺ cells in E14.5 Sox1-Cre/YFP embryos. The single cell suspension was prepared only from the trunk

region. YFP+PDGFR α ⁺ cells are segregated to two distinct populations, A2B5⁻PDGFR β ^{+/weak} and A2B5⁺PDGFR β ⁻. B. Expression of neural markers in the PDGFR β ⁺ and A2B5⁺ populations. YFP+PDGFR α ⁺ cells in E14.5 Sox1-Cre/YFP embryos were further divided into A2B5⁺ and PDGFR β ⁺ populations and expression of neural cell markers in each fraction was evaluated by RT-PCR. Sox10, Olig1 and 2, MAP2 and Sox2, were used for markers for neural lineage including oligodendrocytes. Neural markers are not detected in PDGFR β ⁺ nor A2B5⁻ population, whereas detected in PDGFR β ⁻ and A2B5⁺ cells. C. CFU-F activity in PDGFR β ⁺ cells. PDGFR β ⁺ cells but not PDGFR β ⁻ cells in YFP+PDGFR α ⁺ cells have a potential to form CFU-F. (mean and SD of triplicate cultures). D. *In vitro* proliferative activity of PDGFR β ⁺ population. PDGFR β ⁺ and PDGFR β ⁻ populations were cultured under MSC condition. Only PDGFR β ⁺ cells underwent sustained growth. E. Adipogenic activity of PDGFR β ⁺ cells in YFP+PDGFR α ⁺ populations. Adipogenesis was assessed by Oil red O staining (left panel) or expression of adipocyte specific genes, PPAR γ and adiponectin (right panels).

Figure 7. Presence of neuroepithelium-derived and neural crest-derived

MSCs in postnatal bone preparation. Femoral and tibial bones were removed from neonatal and 4 week old mice, minced, and incubated with a 0.2% collagenase+2.4U dispase cocktail to prepare single cell suspension.

A. Expression of YFP and PDGFR α in the neonates of Sox1-Cre/YFP (left panel) and P0-Cre/YFP (right panel) mice. The proportion of YFP⁺PDGFR α ⁺ population is much lower than that of YFP⁺PDGFR α ⁺ population in both stains. **B.** The same assay of 4-week old Sox1-Cre/YFP (left panel) and P0-Cre/YFP(right panel) mice. Only a few PDGFR α ⁺ cells are detectable at this stage, irrespective of YFP expression. **C.** The proportions of neuroepithelium⁻ and neural crest-derived cells in neonatal and 4-week old mice as assessed by YFP expression. Note that the postnatal decrease is more conspicuous in PDGFR α ⁺ population than PDGFR α ⁻ one. Each value represents the arithmetic mean and SD of three mice (* p <0.05, ** p <0.01). **D.** Enrichment of CFU-F activity in PDGFR α ⁺ populations of neonates. Each fraction indicated in the figure was purified from the femoral and tibial bones of Sox1-Cre/YFP (left) or P0-Cre/YFP (right) neonates and frequency of CFU-F was examined. Upper panels show the colonies in representative dishes. The number of colonies in each fraction is shown in the lower graphs. Each bar represents the arithmetic

mean and SD of triplicate assays. Like fetal tissues, CFU-F activities were enriched in PDGFR α ⁺ populations irrespective of YFP expression. E.

Multipotency of MSC clones derived from bones of Sox1-Cre/YFP neonates.

MSC clones established from YFP⁺PDGFR α ⁺ fraction could give rise to adipocytes (left, Oil red O staining on day 14), osteocytes (center, Alizarin red staining on day 42), and chondrocytes (right, Alcian blue staining on day 35).

Figure 1 Takashima et al.

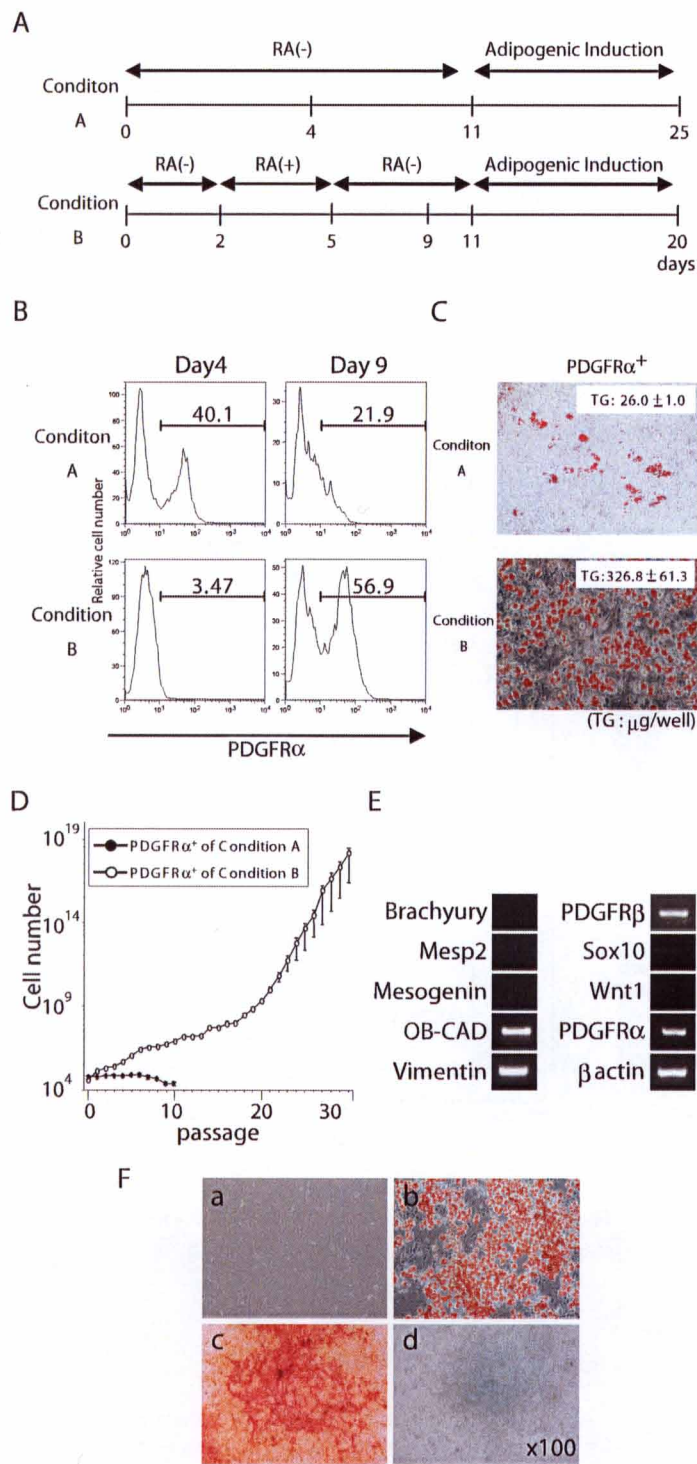


Figure 2 Takashima et al.

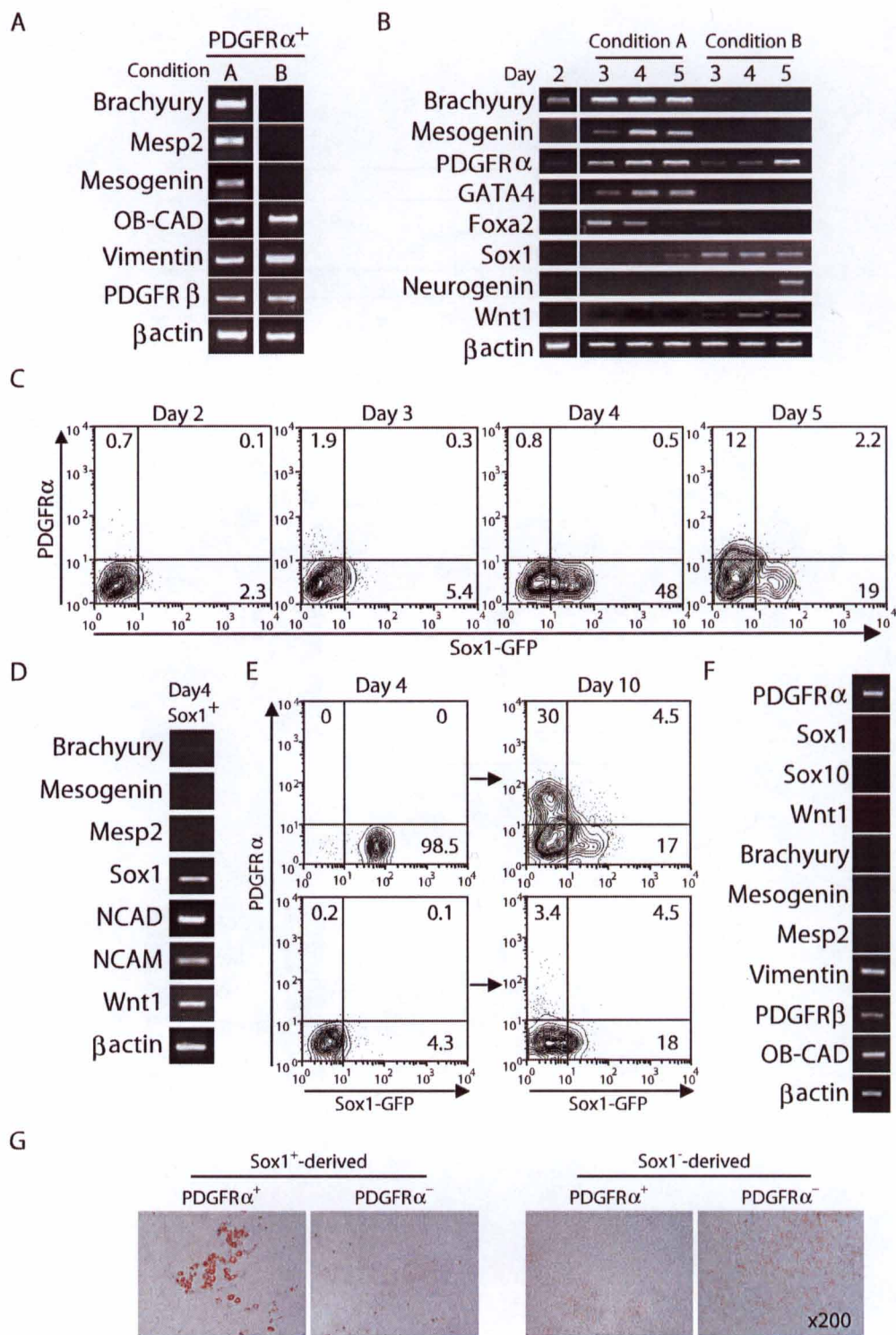


Figure 3 Takashima et al.

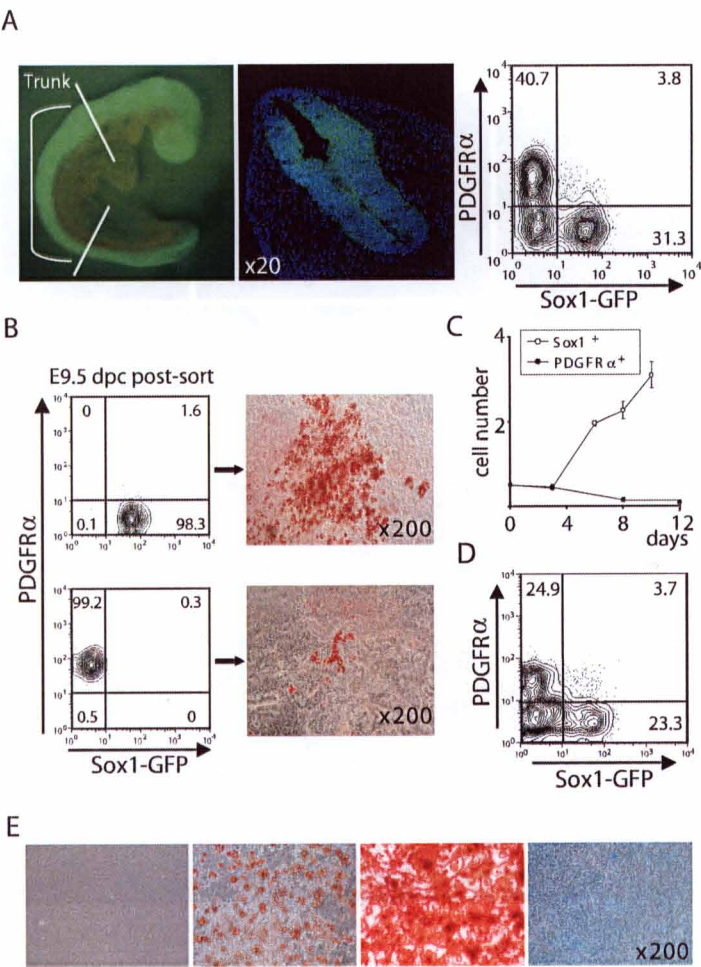


Figure 4 Takashima et al.

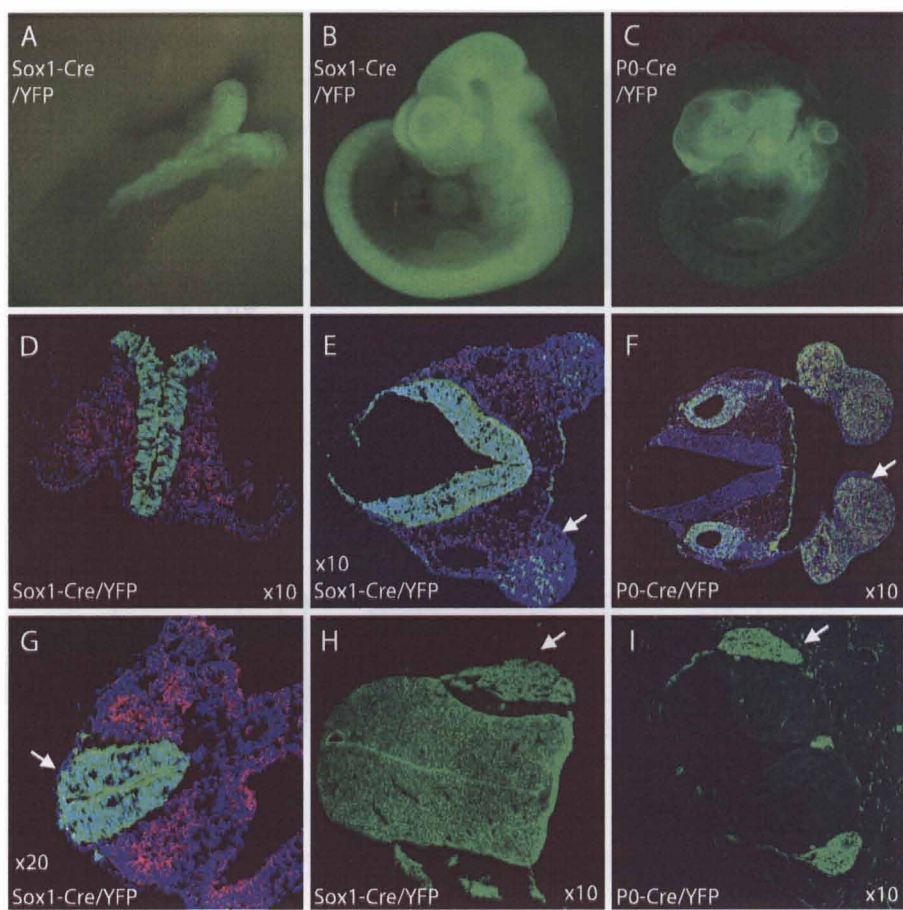


Figure 5 Takashima et al.

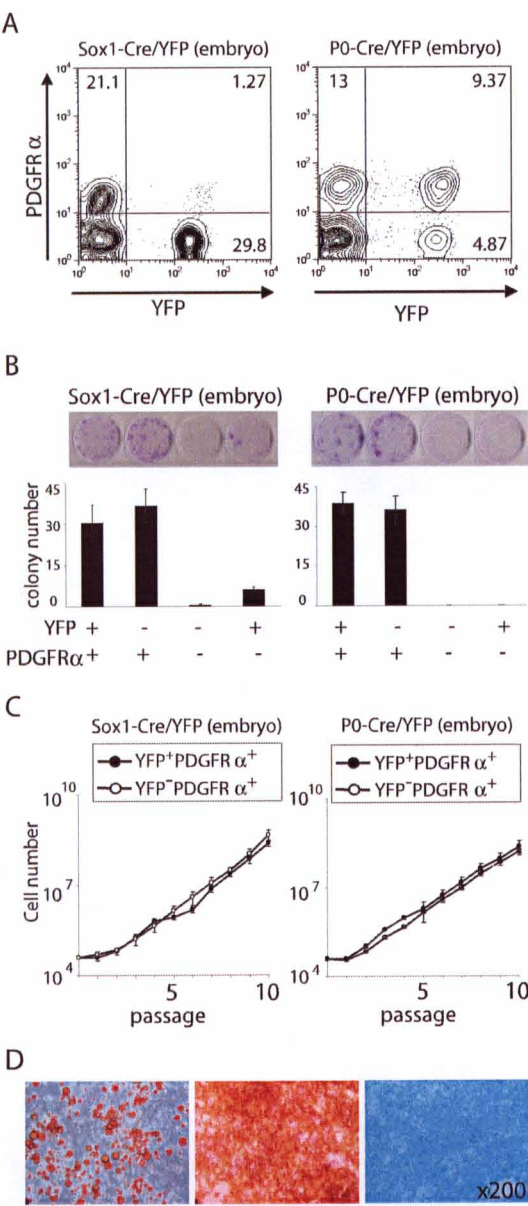


Figure 6 Takashima et al.

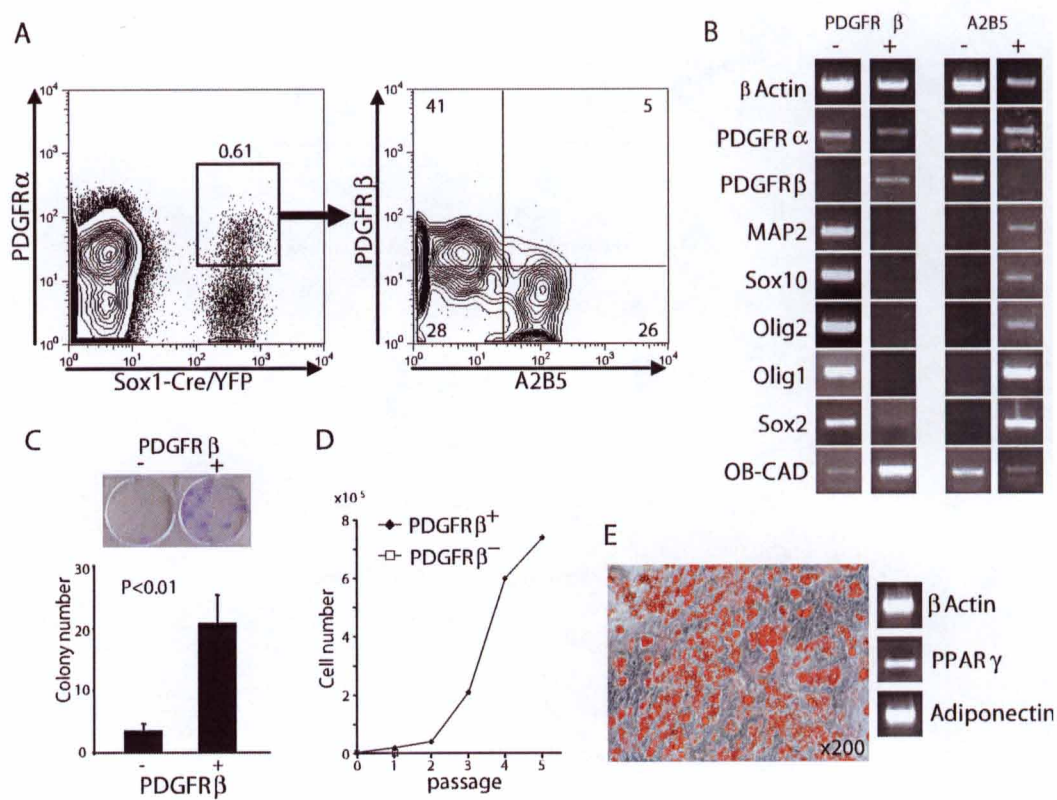
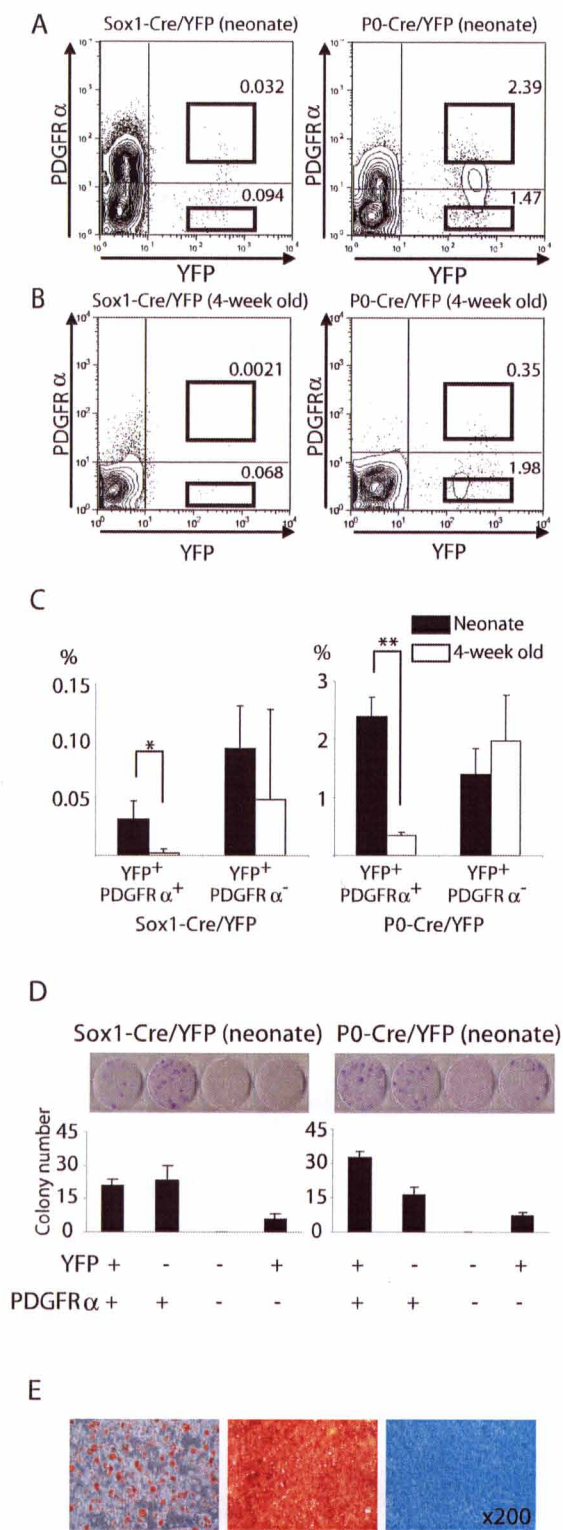


Figure 7 Takashima et al.



Supplemental Data 1 Takashima et al

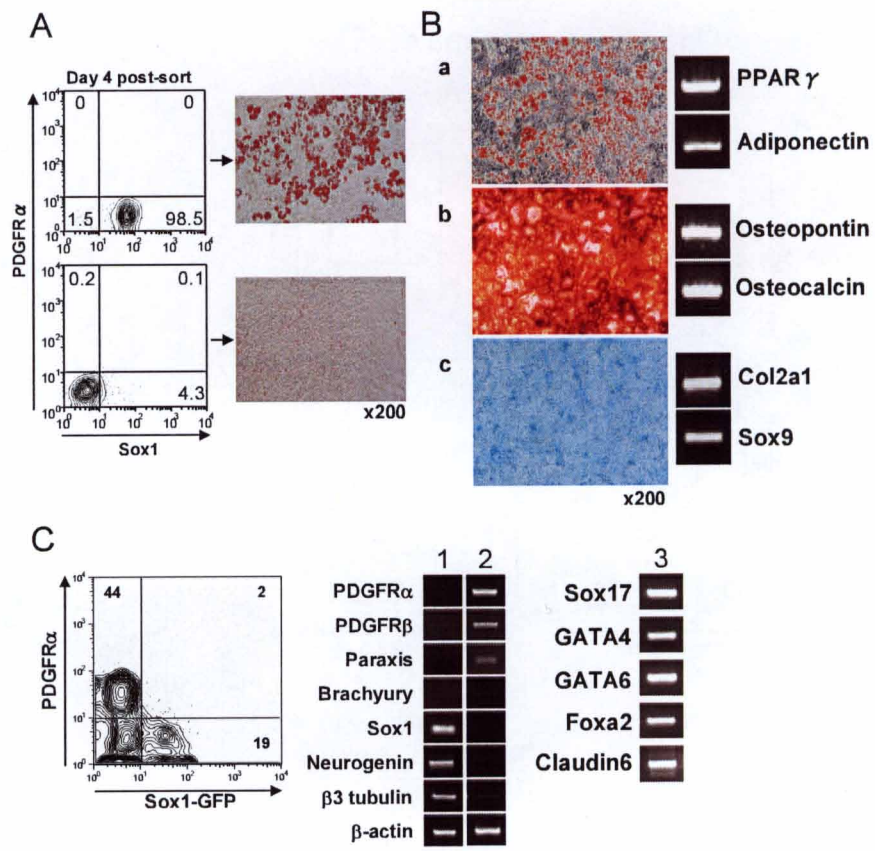


Figure legend for Supplemental Data 1

Stepwise ES cell differentiation to mature mesenchymal lineages through Sox1⁺ neuroepithelium and PDGFR α ⁺ progenitor cells.

A. Only Sox1⁺ cells derived from ES cells can give rise to adipocytes. The purity of sorted populations from day 4 culture of ES-Sox1^{gfp/+} cells under Condition B (left panels). When re-cultured with the adipocyte induction cocktail, adipocytes detected by Oil red Oil staining are generated only in the culture of Sox1⁺ cells (right panels). B. Sox1⁺ cells give rise to multipotent

MSC cell lines. As described in the text, Sox1⁺ cells differentiate to PDGFR α ⁺ cells that undergo sustained growth upon culturing under MSC condition.

(a-c) Cell lines have a potential to give rise to adipocytes (a, Oil red O staining on day 10), osteocytes (b, Alizarin red staining on day 42), and chondrocytes (c, Alcian blue staining on day 35). Generation of these lineages was also confirmed by RT-PCR assay of expression of molecular markers, PPAR γ and adiponectin for adipocytes, osteopontin and osteocalcin for osteocytes, and collagen 2a and Sox9 for chondrocytes (right panels).

C. Cells dissociated from E9.5 embryo derived from ES-Sox1^{gfp/+} consists of three populations in terms of expression of PDGFR α and Sox1. Sox1 single positive cells (Lane 1) express a series of neural cell markers, whereas PDGFR α single positive cells (Lane 2) express mesoderm markers such as paraxis. Double negative cells (Lane 3) express a series of endoderm markers.

Supplemental Data 2 Takashima et al.

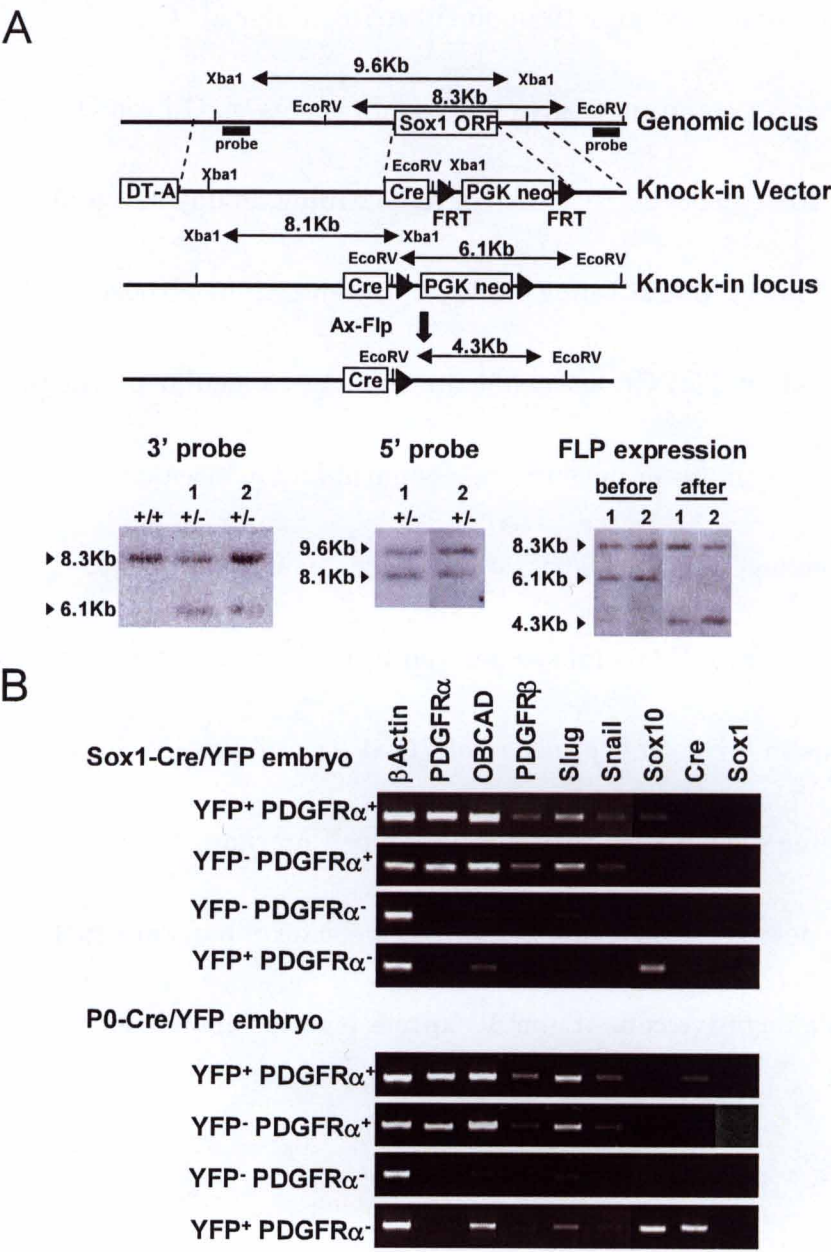


Figure legend for Supplemental Data 2

Generation of Sox1-Cre mouse strain and lineage tracing of cells derived from neuroepithelium and neural crest.

A. Strategy for inserting the *Cre* gene into the *Sox1* allele and generating Sox1-Cre mice. Cre recombinase cDNA was inserted into the ATG codon start site of Sox1 by in-frame (Upper panel). Finally, PGK-NEO cassette was removed by Flp recombinase expression. Correct recombination was confirmed by Southern blot analysis using 5' or 3' probes of Sox1 genomic DNA (Lower panels).

B. Gene expressions of FACS-purified populations derived from neuroepithelium or neural crest marked by Cre-recombinase mediated persistent labeling. In this experiment, the marked cells express YFP. YFP⁺PDGFR α ⁺, YFP⁻PDGFR α ⁺, YFP⁺PDGFR α ⁻ and YFP⁻PDGFR α ⁻ populations were purified by FACS from the trunks of both E14.5 Sox1-Cre/YFP (Upper panel) and P0-Cre/YFP (Lower panel) embryos. Gene expression was examined by RT-PCR. In both strains of mouse, PDGFR α ⁺ population strongly express mesenchymal markers such as OB-CAD, PDGFR β , Slug and Snail, irrespective of YFP expression, suggesting that these population contained mesenchymal lineage cells. It should be noted that the YFP⁺PDGFR α ⁺ fraction of P0-Cre/YFP embryo did not express Sox10, one of the most well-known markers for neural crest. YFP⁺ cells from the trunk of E14.5 Sox1-Cre/YFP embryo no longer expressed endogenous Sox1 nor Cre transgene. In contrast, Cre expression was still observed in

YFP⁺ cells from E14.5 P0-Cre/YFP mouse.

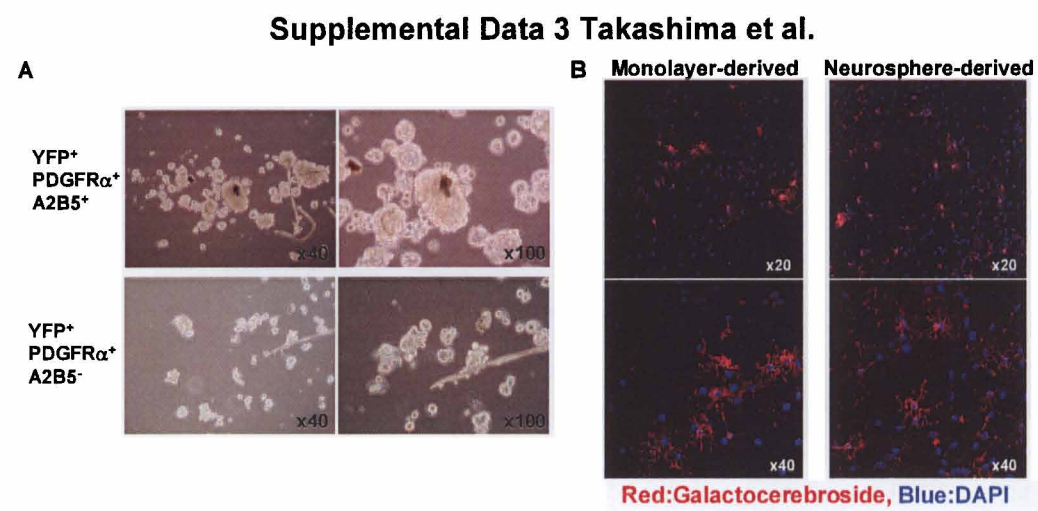


Figure Legend for Supplemental Data 3

Neurosphere formation of A2B5⁺ and A2B5⁻ cells in YFP⁺PDGFR α ⁺ population of E14.5 Sox1-Cre/YFP embryos.

A. Generation of neurospheres from A2B5⁺ cells in YFP⁺PDGFR α ⁺ population of E14.5 Sox1-Cre/YFP embryos. A2B5⁺ (upper panels) and A2B5⁻ (lower panels) cells in YFP⁺PDGFR α ⁺ population were purified from E14.5 Sox1-Cre/YFP embryos and re-cultured under a condition that allows

neurosphere formation as described previously (Bondurand et al., 2003). A large neurospheres were formed only from the A2B5⁺ population.

B. Potential of A2B5⁺ cells for Oligodendrocytic differentiation. The FACS-purified A2B5⁺ and A2B5⁻ cells were cultured either in neurosphere culture or in a monolayer culture as described previously (Monolayer culture, Morrison et al., 1999). Oligodendrocytic differentiation was induced as described previously (Kondo et al., 2000). A2B5⁺ cells maintained under monolayer (left) or neurosphere (right) conditions were able to generate oligodendrocytes. In contrast, A2B5⁻ cells failed to generate oligodendrocytes under these conditions (data not shown). The oligodendrocytic differentiation was confirmed by the galactocerebroside expression (red color in the Fig), a marker of oligodendrocytes, with the immunostaining (Kondo et al., 2000). The brain neural stem cells were used as a positive control (data not shown).

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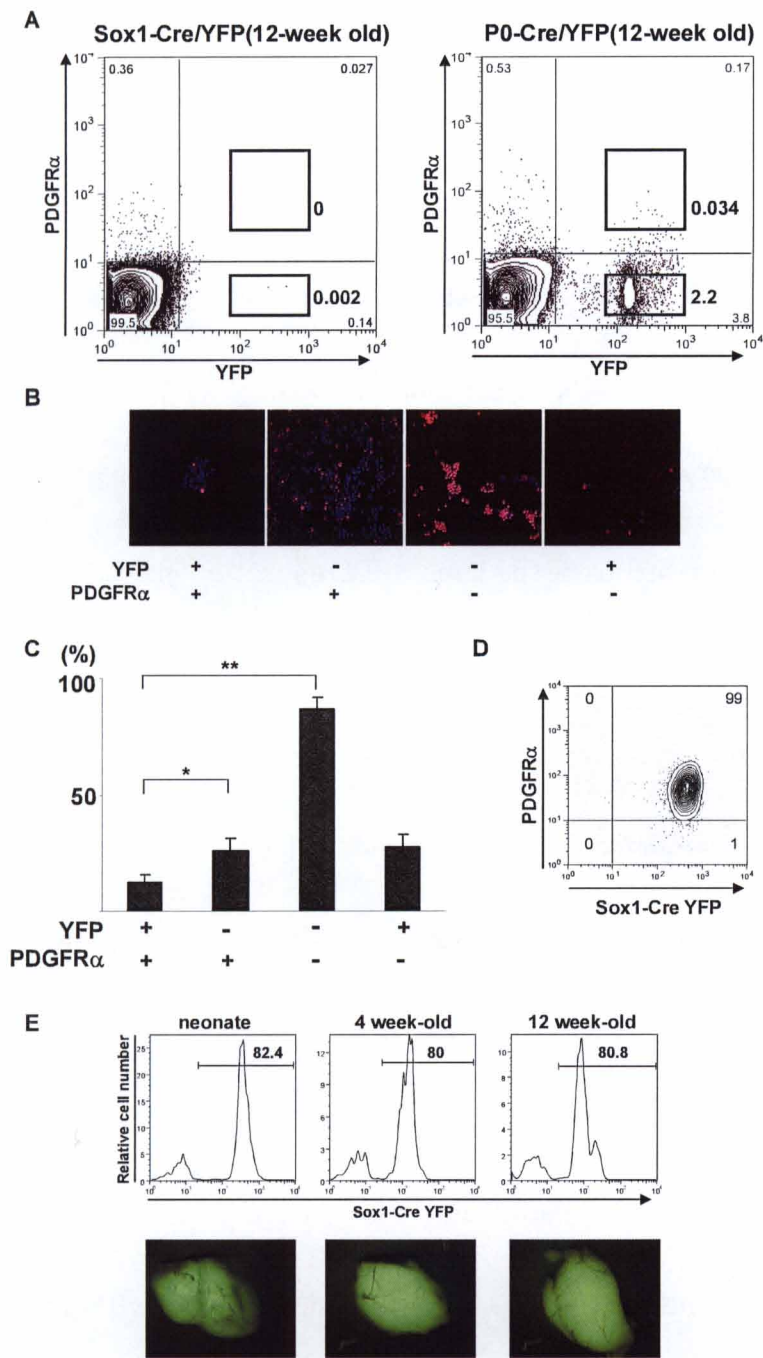


Figure Legend for Supplemental Data 4

Long term tracing of neuroepithelium- and neural crest-derived progenies.

A. Expression of YFP and PDGFR α in the bone cell preparation of 12-week old Sox1-Cre/YFP (left) and P0-Cre/YFP (right) mice. The cells dissociated from femoral and tibial bones of 12-week old Sox1-Cre/YFP and P0-Cre/YFP mice were pooled and dissociated to single cell suspensions. Almost no YFP⁺PDGFR α ⁺ and YFP⁻PDGFR α ⁺ cells were detected in both mice.

B. Ki 67 expression in YFP⁺PDGFR α ⁺ population of Sox1-Cre/YFP neonates. YFP⁺PDGFR α ⁺, YFP⁻PDGFR α ⁺, YFP⁻PDGFR α ⁻ and YFP⁺PDGFR α ⁻ populations were purified from the bone cell preparation of Sox1-Cre/YFP neonates and Ki 67 expression was examined by immunostaining with anti-Ki 67 antibody. Abundant Ki 67⁺ cells were detected in YFP⁻PDGFR α ⁻ population, whereas a few cells were positive for Ki 67⁺ expression in YFP⁺PDGFR α ⁺ population. C. Proportion of Ki67⁺ cells was enumerated.

The proportion of Ki 67⁺ cells in both PDGFR α ⁺ populations is much lower than that of double negative population. Interestingly, the proportion of Ki 67⁺ cells in YFP⁺PDGFR α ⁺ is significantly lower than that in YFP⁻PDGFR α ⁺ population. Each value represents the arithmetic mean and SD of four independent experiments (* p <0.05, ** p <0.01). D. Persistent expression of YFP in a MSC cell line established from YFP⁺PDGFR α ⁺ cells in E14.5 Sox1-Cre/YFP embryos. After multiple passages, YFP expression is

maintained at constant level. **E.** Proportion of YFP⁺ cells in the developing brains. Brains from neonates, 4 week-old and 8 week-old Sox1-Cre/YFP mice were removed and dissociated to single cell suspensions in order to examine the expression pattern of YFP by FACS. YFP is expressed over the entire brain (lower panel) and the proportion of YFP is maintained at constant level over many months (Upper panel).

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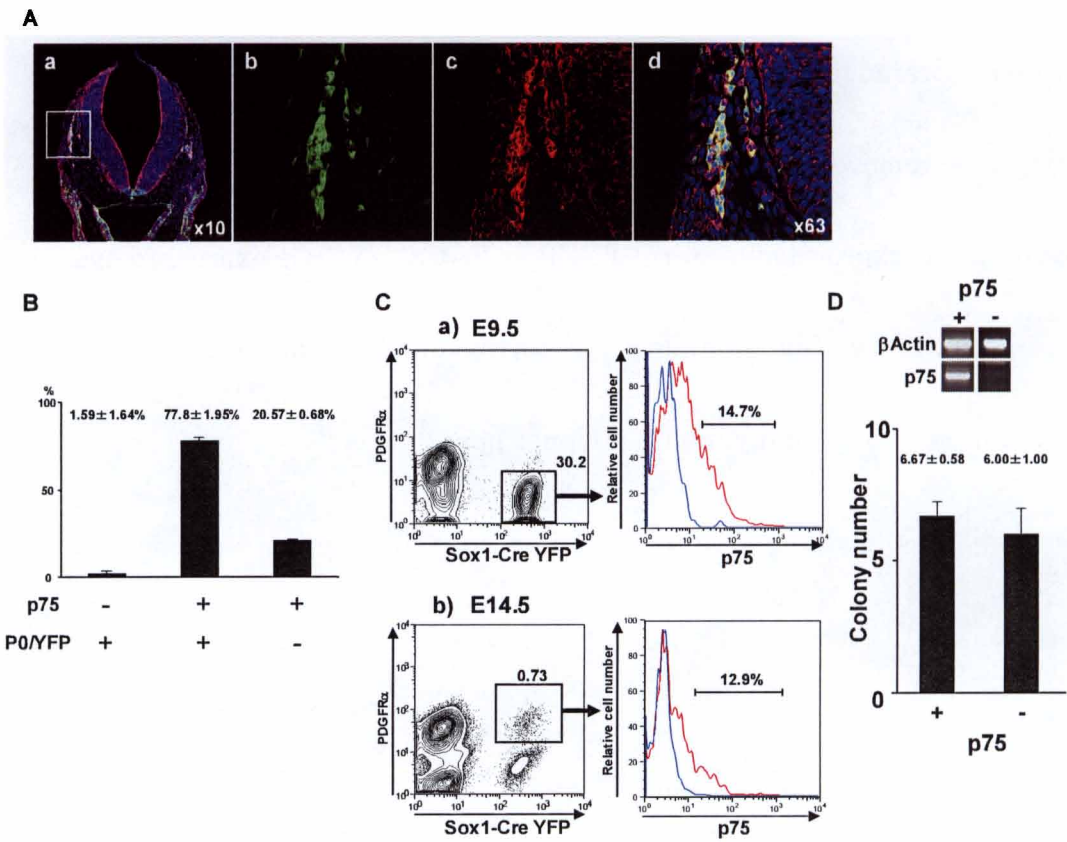


Figure Legend for Supplemental Data 5

Expression of p75NTR in YFP+PDGFRα⁺ cells of Sox1-Cre/YFP and P0-Cre/YFP embryos.

A. Immunohistochemical analysis of YFP and p75NTR expression in the trunk of E9.5 P0-Cre/YFP embryos. For staining we used anti-GFP (b) and anti-p75 (c) antibodies. The picture with merged images is also presented (d). Magnified views of the area indicated by the square in panel (a) are shown in

panels (b-d). Almost all of the YFP⁺ neural crest cells appear to express p75^{NTR} (b-c). The green, red and blue colors represent YFP, p75, and TO-PRO3 staining, respectively. **B.** Enumeration of YFP single positive, p75 single positive and YFP/p75 double positive cells in the trunk of E9.5 P0-Cre/YFP embryos. Note, most P0-Cre/YFP⁺ neural crest cells expressed p75^{NTR} (left and center bar in this graph). **C.** p75^{NTR} expression in the trunk of Sox1-Cre/YFP embryos. Trunk regions of Sox1-Cre/YFP embryos at E9.5 or E14.5 were pooled and dissociated to single cell suspensions for staining with p75 NTR, a neural crest cell marker (Morrison et al., 1999; Wilson et al., 2004). Staining with (red line) or without (blue line) the anti-p75^{NTR} antibody. (a) p75^{NTR} expression in YFP⁺PDGFR α ⁺ populations in E9.5 Sox1-Cre/YFP embryos. At this stage, PDGFR α is expressed in neither neuroepithelial cells nor neural crest cells that are marked by Sox 1-Cre/YFP. (b) On the other hand, in E14.5, a significant number of YFP⁺PDGFR α ⁺ cells appeared that consists of both p75⁺ and p75⁻ cells, though the proportion of p75⁺ cells is only 12.9%. **D.** CFU-F activity in p75⁺ and p75⁻ populations in YFP⁺PDGFR α ⁺ cells in the trunk of E14.5 Sox1-Cre/YFP embryos. The purity of sorted cells was assessed by RT-PCR analysis of p75 (upper panel). 250 cells purified by FACS were seeded for

CFU-F assay according to Supplement Experimental Procedures. Similar levels of CFU-F activity were detected in p75⁺ and p75⁻ populations. (mean value and SD of triplicate cultures).

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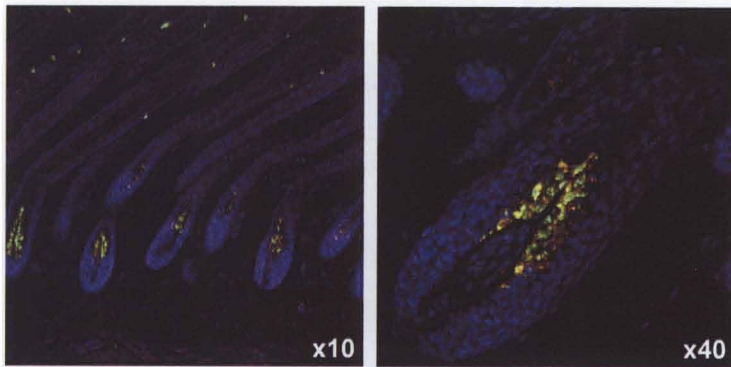


Figure Legend for Supplemental Data 6

Melanocytes in the skin of 1-week old Sox1-Cre/YFP mice. TRP2⁺

Melanocytes express YFP. Green, red and blue colors indicate YFP, TRP2

that is one of melanocyte markers, and TO-PRO3 (Nuclear staining).

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GENE	Forward	Reverse
Brachyury	5'-AACTTTCCTCCATGTGCTGAGAC-3'	5'-TGACTTCCCAACACAAAAGCT-3'
Mesogenin	5'-AGACCTATGCATAGGCGTAGGA-3'	5'-CCCAACACCAAATGGTAGG-3'
PDGFR α	5'-AATCCTGCAGACGAGAGCAC-3'	5'-GCCACCAAGGGAAAAGATT-3'
GATA4	5'-CGAGATGGGACGGGACACT-3'	5'-CTCACCCTCGGCCATTACGA-3'
Sox1	5'-CAATCTTGCATCCCGTTC-3'	5'-ACCCAGGTCTTATCCCATCC-3'
NCAM	5'-GCAGGTAGATATTGTTCCCA-3'	5'-CCCTTCCTTAAACTCCTGTGG-3'
Wnt1	5'-AGTCTGCACCTGCGACTAC-3'	5'-CAGCTCCTCCCTGGTGTAAA-3'
Sox10	5'-CAAGAGCAAGCCGACGTCAAGAG-3'	5'-AGCCTTCTTCTGGGTGCCGGTGGT-3'
β actin	5'-CCTAAGGCCAACCCTGAAAAG-3'	5'-TCTTCATGGTGCTAGGAGCCA-3'
Mesp2	5'-CCCAGAGCCTAGGAACAAGACT-3'	5'-AATGTTTATGTCCCCAGACAC-3'
PDGFR β	5'-GTCTGGTCTTTTGGGATCCT-3'	5'-AAGGCTGGTTACAGTTTGGC-3'
OB-CAD	5'-TCAGGGAACATTATGCCAC-3'	5'-TCTATGCCGTCTCCATCAAC-3'
Vimentin	5'-CCCTTAAAGGCACTAACGAGTC-3'	5'-CTTCTGGTACTGCACTGT-3'
Foxa2	5'-TGGCTGCAGACACTTCTACT-3'	5'-CAACATCAGTACAACCCCTGGT-3'
NCAD	5'-TATCATCATCCTGCTGATCCTTG-3'	5'-TGTCGCCAGTTTCTTGAAG-3'
Neurogenin	5'-ACCGACGAGGAGGACTGT-3'	5'-GGGGTCAGAGAGTGGTGATG-3'
Snail	5'-CTCCACAAGCACCAAGAGTCTG-3'	5'-TCCAGTAACCAACCCTGCTGAG-3'
Slug	5'-GCAGTGAGGGCAAGAGAAAG-3'	5'-CCTCCAAGAAGCCCAACTACAG-3'
Cre	5'-AGGCGTTTTCTGAGCATACC-3'	5'-TCGGATCATCAGCTACACCA-3'
β 3tubulin	5'-CTCAGTCTAGATGTGCTGCG-3'	5'-GCGGAAGCAGATGTCTAGA-3'
Adiponectin	5'-CAAAAGGGCTCAGGATGCTAC-3'	5'-CGTAGGTGAAGAGAACGGCCT-3'
PPAR γ	5'-GGAGATTCTCCTGTTGACCCA-3'	5'-GTGAGACATCCCCACAGCA-3'
Osteopontin	5'-GCTGTGTCCTCTGAAGAAAAGG-3'	5'-AGACTTGGTTCATCCAGCTGAC-3'
Osteocalcin	5'-AGCAGGAGGGCAATAAGGTAGT-3'	5'-GCTGCTGTGACATCCACTTG-3'
Collagen II α 1	5'-TCCTGCTGGAAAAGATGGTC-3'	5'-CTCTGTCTCCTTGTGGCCAGT-3'
Sox9	5'-GTGCAGCACAGAAGAACCA-3'	5'-GGGTGGCAAGTATTGGTCAA-3'
Paraxis	5'-TAGGCCTGGATGGCTAGATG-3'	5'-GCTCCATCTGCACCTTCTGT-3'
Sox17	5'-TTTGTGTATAAGCCCAGATGG-3'	5'-AAGATTGAGAAAACACGCATGAC-3'
GATA6	5'-AGACATAACATTCTTCGATGCG-3'	5'-TTCCAAGTGACCTCAGATCAGC-3'
Claudin6	5'-ACAAAGCTGACCGAGCACT-3'	5'-AGCAGCAAAAGGCCTGAG-3'
MAP2	5'-AAG GCCAAGAACACACGATTG-3'	5'-ACCAAGCCCTAAGCTTCGACTAA -3'
Olig1	5'-GCAGCCACCTATCTCCTCAT-3'	5'-GTGGCAATCTTGGAGAGCTT-3'
Olig2	5'-GTGTCTAGTCGCCATCGTC-3'	5'-CGATGTTGAGGTGTCGCAT-3'
Sox2	5'-GGAGTGGAACTTTTGTCCGA-3'	5'-TTCATGTAGGTCTGCGAGCTG-3'
Cre(genome)	5'-5'-GGACATGTTTCAGGGATCGCCAGGCG-3'	5'-GCATAACCAGTGAAACAGCATTGCTG-3'
ROSA26R(genome WT)	5'-GGAGCGGGAGAAATGGATATG-3'	5'-GCGAAGAGTTTGTCTCAACC-3'
ROSA26R(genome MT)	5'-AAA GTCGCTCTGAGTTGTTAT-3'	5'-GCGAAGAGTTTGTCTCAACC-3'

Figure Legends for Supplemental Data 7

Primer list used in this study.

Supplemental Experimental Procedures

Cell culture

CCE, TT2 and ES-Sox1^{gfp/+} cell lines were maintained as described previously (Era and Witte, 2000; Sakurai et al., 2006). Adipocytes were induced from ES cells according to the protocol described by Dani *et al* (Dani et al., 1997). While the original method used embryoid body culture, we induced the differentiation of ES cells on collagen IV-coated dishes with α MEM (GIBCO) containing 10% FBS and 5×10^{-5} M 2ME (SIGMA). The adipocyte induction cocktail contained 0.2 μ M insulin (SIGMA), 0.5 mM IBMX (3-isobutyl 1-methylxanthine) (SIGMA), 0.25 μ M dexamethasone (SIGMA), and 1 μ M troglitasone (Sankyo) and was added on day 11. At the end of the culture, the cells were fixed and stained with 0.5% Oil red O (SIGMA). The triglyceride content was measured using a triglyceride E-test Wako (Wako). This adipocyte induction cocktail was also used for FACS-purified PDGFR α ⁺ or Sox1⁺ cells to be re-cultured.

To evaluate the proliferative activity *in vitro*, cells were cultured with α MEM plus 10% FBS with passage every 3 days. Cell growth was assessed by determining the number of live cells. In some experiments, osteogenesis or chondrogenesis was induced as described previously (Sakurai et al., 2006).

The presence of osteocytes and chondrocytes was detected by Alizarin red and Alcian blue staining (SIGMA), respectively. RT-PCR analysis for adiponectin, osteopontin, and collagen IIa1 was also performed to confirm the staining results (Supplemental data 1B). The cloning of MSC lines derived from ES cell cultures was carried out by the limit cell dilution method, in which 30 cells were seeded in 96-well plates. The seeding efficiency was approximately 4%. Clones were established also from Sox1⁺ cells isolated from the trunk of embryos at E9.5. After FACS-purified Sox1⁺ cells had been grown *in vitro* for 2 weeks, Sox1⁻PDGFR α ⁺ cells were sorted by FACS and re-cultured again. One week later, the cells grown *in vitro* were harvested and seeded into a 96-well plate at 0.3/well density. The plating efficiency was 1.2% (seven positive wells in a total of 576 wells.). After clonal expansion, differentiation into adipocytes, osteocytes, and chondrocytes was induced separately as described above.

Each YFP⁺PDGFR α ⁺ or YFP⁻PDGFR α ⁺ cells was purified by FACS from the embryos and neonates of Sox1-Cre/YFP or P0-Cre/YFP mice. To evaluate the proliferative activity of these cells, cells were cultured as described in E9.5 embryo. In some experiments, clonal cell lines were established by single cell sorting by using FACS Aria with single cell deposition apparatus

(Becton & Dickinson). The plating efficiency was 1.0% in the trunk of Sox1-Cre/YFP embryo at E14.5 (4 positive wells in a total of 384 wells) and 0.5% in bones and bone marrows of Sox1-Cre/YFP neonates (2 positive wells in a total of 384 wells).

CFU-F assay

CFU-F assay was performed as described previously (Clarke, 2005). Briefly, 1500 or 1000 cells purified from E14.5 embryos or 300 cells purified from neonates were seeded on a 6 cm dish with α MEM containing 20% FBS. After 2 weeks, the cells were fixed by methanol and stained with Giemsa solution. Adherent colonies containing more than 50 cells were counted as a colony.

In order to examine the differentiation potentials, we picked up the colonies using cloning rings and Vacuum grease silicone (Beckman), and re-cultured under the MSC condition. The adipocytic differentiation of the expanded cells derived from each colony was induced as described above.

Oligodendrocytic differentiation

The purified cells, A2B5⁺ or A2B5⁻ cells in YFP⁺PDGFR α ⁺ population of

E14.5 Sox1-Cre/YFP embryos, were maintained as described previously (Morrison et al., 1999). For the induction into mature oligodendrocytes, the cells were cultured with neurobasal medium (GIBCO) supplemented by 0.5% FCS, 1x B27 (GIBCO), 1x Glutamax (GIBCO), 30 ng/ml of Thyroid hormone(T3, SIGMA), 5 mg/ml Heparin (SIGMA), 2ng/ml bFGF (Peprotec) and 10 ng/ml PDGF-AA (Peprotec) (Kondo et al., 2004). To confirm the oligodendrocyte generation, the expression of galactocerebroside (GC) were examined by immunostaining as described previously (Kondo et al., 2000).

In some experiments, the neurosphere formation was performed as described previously (Bondurand et al., 2003). Briefly, the FACS-purified cells were cultured on the plates coated by Pluronic F127 (SIGMA) with DMEM-low glucose medium (GIBCO) containing 15% chick embryo extract, 20 ng/ml recombinant human bFGF (R&D systems), 1% N2 supplement (GIBCO), 2% B27 supplement (GIBCO), 50 mM 2-ME, 35 mg/ml (110 nM) retinoic acid (SIGMA) and penicillin/streptomycin. We also generated the neurospheres from adult mouse brain as a positive control for the differentiation experiment.

Antibodies

Biotin-conjugated APA5(anti-PDGFR α) and Allophycocyanin (APC)-conjugated APB5(anti-PDGFR β) rat MoAbs were prepared as described previously (Nishikawa et al., 1998; Sano et al., 2001). Anti-galactocerebroside MoAb was a kind gift of Dr. Kondo (Eisenbarth et al., 1979). Anti-A2B5 MoAbs (Chemicon), rabbit anti-mouse p75NTR polyclonal Ab (Chemicon) (White et al., 2006), goat anti-TRP2 Ab (SantaCruz) and rat anti-GFP MoAb (for YFP staining, NACALAI) were used in FACS analysis and immunostaining. The Alexa Flour 633-conjugated and the PE-conjugated Goat anti-mouse IgMs (for A2B5) were purchased from Molecular Probe and Pharmingen, respectively. The Alexa Flour 633-conjugated Goat anti-rabbit (for p75) and the Alexa Flour 488-conjugated Goat anti-rat (for GFP) IgGs were purchased from Molecular Probe. These antibodies were used as secondary antibodies. PE-conjugated streptavidin (Pharmingen), PE-Cy7-conjugated streptavidin (Pharmingen) and APC-conjugated streptavidin (Molecular Probe) were used for detecting biotinylated-antibodies.

Generation of the Sox1-Cre Knock-in vector

To construct Sox1-Cre Knock-in vector, we took advantage of the gene

recombination methods as described previously (Ikeya et al., 2005). First, 5' and 3' Entry vectors were generated by PCR, using BamHI-digested pDONR-P4P1R and BamHI-digested pDONR-P2RP3, respectively, as templates. Each PCR primer was designed to include both recombination sequences recognized by the ET recombinase and 50–54 nucleotides of homologous regions to Sox1 gene. Next, the PCR products were introduced into *E. coli* carrying a BAC clone containing murine Sox1 genomic DNA. Through this transformation, 5' and 3' arms consisting of Sox1 genome were automatically inserted into the PCR products in *E. coli* by means of Red/ET recombination (Gene Bridges GmbH, Germany). The BAC clone was purchased from BACPAC Resources Center (Children's Hospital of Oakland Research Institute, URL <http://bacpac.chori.org/>). Proper recombination was confirmed by sequence.

We also generated a DNA fragment containing the *Cre-FRT-PGKneo-FRT* gene flanked with *att* sites by PCR (primers att-cre-F:
ggggacaagtttgtacaaaaaagcaggctctatggccaatttactgaccgtacac and att-cre-R:
ggggaccactttgtacaagaaagctgggtagcgccgcttttagaactagtggat) and cloned it into a pDONR 221 vector using BP clonase (Invitrogen).

Finally, to completely generate the Sox1-Cre knock-in vector, three vectors

were transfected into E. coli with the Destination vector bearing both DTA and recombination sites recognized by GATEWAY recombinase. Through this transfection, the 5' arm, 3' arm and *Cre-FRT-PGKneo-FRT* gene were automatically inserted into the Destination vector by means of GATEWAY recombination system.

< 5' Entry vector >

Template: pDONR-P4P1R (linearized with BamHI)

primerA:5'-ttttctggaagacagactggacacagctcatcccatctccctctggattccaacttttctatac
aaagttggcattat-3'

primerB:

5'-ctctctttgcggtaccggtgaacccgctagccgccagatgtacagcatggcaagttgtacaaaaaagtt
gaacgag-3'

< 3' Entry vector >

Template: pDONR-P2RP3 (linearized with BamHI)

primerC:

5'-aacatgcatataaaattagcacgtttcgatttcacagggaaacgggctttccactttgtacaagaaagtt
gaacgag-3'

primerD:

5'-gacctgggggtcagcctcaagccaaacccttttacaatcagtcgggaaacccaactttattatacaaagttg

gcattat-3'

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