9. Immuno-cytostaining is the easiest method for clarifying the presence of endothelial colonies in the culture. We routinely examine the expression of either VE-cadherin or PCAM-1(CD31) as the markers for endothelial cells (***16***, ***18***).

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Figure legends

Fig. 1 Differentiation pathway of mesoderm in *in vitro* ES cell culture.

The analyses of differentiated ES cells reveal the three types of mesoderm cells, PDGFRα^{+}VEGFR2^{+} population (PDGFRα and VEGFR2 double positive population, DP), PDGFRα^{+}VEGFR2^{-} population (PDGFRα single positive population, PSP) and PDGFRα^{-}VEGFR2^{+} population (VEGFR2 single positive population, VSP). The DP is the most immature and can give rise to both the PSP and the VSP. Both the VSP and the PSP exhibit the specific properties of paraxial and lateral mesoderm respectively,

Fig. 2 Fate of the ES cell-derived mesoderm cells

(A) Day4 ES cell differentiation. CCE ES cells are cultured on type IV collagen-coated dishes with the differentiation medium in the absence of LIF. Four days after the induction, differentiated ES cells are harvested and the expression of PDGFRα and VEGFR2 are examined by FACS. Four populations (PDGFRα^{+}VEGFR2^{+},DP; PDGFRα^{+}VEGFR2^{-}, PSP; PDGFRα^{-}VEGFR2^{+}, VSP; PDGFRα^{-}VEGFR2^{-}, DN) are observed in day 4 differentiated ES cells. (B) Marker expression by quantitative RT-PCR (qPCR). PSP expresses the markers specific for paraxial mesoderm such as Tbx6 and Mesp2. In contrast, VSP expresses the markers specific for lateral mesoderm such as GATA2 and Tal1, suggesting that it represents lateral mesoderm in actual embryo. Black and white squares indicate the expression in

PSP and VSP, respectively. (**C**) Marker expression of bone and cartilage cells. The ES cell-derived mesoderm populations are cultured under distinct conditions that allow the differentiation of osteocytes or chondrocytes. After differentiation, RNA is purified and the expression levels of individual specific markers are measured by qPCR. Culture cells derived from the PSP expresses osteogenesis-(Bglap1 and Bglap2, **left panel**) and chondrogenesis-(col2a1 and col10a1, **right panel**) related genes at higher level than that of the VSP. Black and white squares indicate the expression in PSP and VSP, respectively. (**D**) The expression level of βHI in the cultures of PSP and VSP. The expression level of βHI is measured by qPCR and normalized by GAPDH expression level. Culture of VSP exhibits the higher expression of βHI than that of PSP. This suggests that VSP generates hematopoietic cells more efficiently than PSP. (**E**) The number of endothelial colonies derived from different mesoderm populations. 500 sorted cells are cultured on confluent OP9 cell layer for three days. Endothelial colonies are visualized by VE-cadherin immunostaining. The number of VE-cadherin^{+} colonies is counted in each well of 24-well plates. (Error bars = SD). The frequency of endothelial progenitors in the PSP is a quarter of those of the VSP.

Table 1 Primers for quantitative RT-PCR

Gene		Sequence
GAPDH	sense	5'-GGAGCGAGACCCCACTAACA-3'
	antisense	5'-GCCTTCTCCATGGTGGTGAA-3'
Tbx6	sense	5'-CCCAACTATGCAGCCAACACT-3'
	antisense	5'-CTGTGTGATCCTAGGGTTCTGGTA-3'
Mesp2	sense	5'-CTGAAAACCTTGGGAACAGGAT-3'
	antisense	5'-GGCTCTTTCTAGGGACTGGTGTAA-3'
GATA2	sense	5'-CGGCCTCTTCTTCTGCAGG-3'
	antisense	5'-TGGTACTTGACGCCATCCTTG-3'
Tal1	sense	5'-CCCACCAGACAAGAAACTAAGCA-3'
	antisense	5'-GGCCAGGAAATTGATGTACTTCA-3'
Bglap1	sense	5'-GAGGACCATCTTTCTGCTCACTCT-3'
	antisense	5'-GACATGAAGGCTTTGTCAGACTCA-3'
Bglap2	sense	5'-GCGCTACCTTGGAGCTTCAG-3'
	antisense	5'-CATACTGGTTTGATAGCTCGTCACA-3'
Col2a1	sense	5'-CCTTGGACGCCATGAAAGTT-3'
	antisense	5'-CTTGCTGCTCCACCAGTTTTT-3'
Col10a1	sense	5'-CCTGGTTCATGGGATGTTTTATG-3'
	antisense	5'-TGGCGTATGGGATGAAGTATTG-3'
βH1	sense	5'-TGTTTACCCATGGACTCAGAGATTC-3'
	antisense	5'-CTTTCTTGCCATGGGCTCTAA-3'

Figure 1 Era

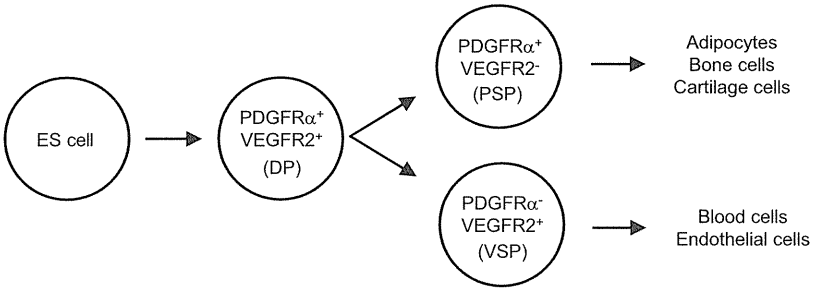
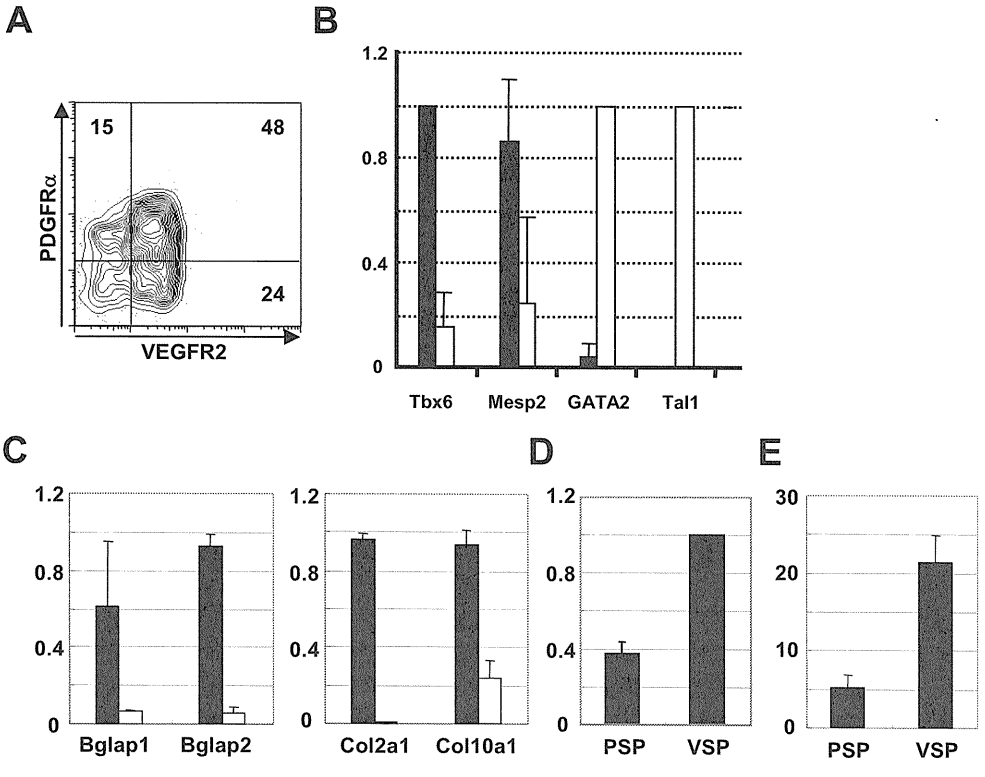

ES cell
PDGFRα+
VEGFR2+
(DP)
PDGFRα+
VEGFR2-
(PSP)
Adipocytes
Bone cells
Cartilage cells
PDGFRα-
VEGFR2+
(VSP)
Blood cells
Endothelial cells

Figure 2 Era


A
B
15
48
24
PDGFRα
VEGFR2
1.2
0.8
0.4
0
Tbx6
Mesp2
GATA2
Tal1
C
D
E
Bglap1
Bglap2
Col2a1
Col10a1
PSP
VSP
30
20
10

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Differentiation of mesodermal cells from pluripotent stem cells

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Abstract The pluripotency of embryonic stem cells has been well demonstrated by a vast variety of studies showing the induction of differentiation into desired cell types that have the potential to be used not only in basic studies but also in medical applications. The induction of mesodermal cells, especially blood cells, from embryonic stem cells is notable from the point of view of transplantation, and the methods for this induction have improved over the last few years, with more defined culture conditions in place. Concurrently, the generation of induced pluripotent stem cells from somatic cells opens the possibility of autologous transplantation. In fact, there are a growing number of reports demonstrating that several mesodermal cells can be differentiated from induced pluripotent stem cells using the same methods used for embryonic stem cells. This review summarizes recent advances in the differentiation of mesodermal cells from embryonic stem cells and induced pluripotent stem cells.

Keywords Mesoderm · Pluripotent stem cells · ES cells · iPS cells

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1 Introduction

Embryonic stem (ES) cells originate from the inner cell mass (ICM), the internal cell component of the blastocyst, and are pluripotent cell lines with the ability to self-renew, infinitely proliferate, and the potential to broadly differentiate into many cell types, including mesodermal cells and their descendants [1, 2]. Besides the sustained growth and pluripotency, the efficient homologous recombination in ES cells provides a useful system for genetic manipulation by creating gene-targeted mice. As ES cells have been shown to differentiate into various lineages under appropriate culture conditions in vitro, ES cells are also available as an experimental tool to elucidate the molecular mechanisms of embryonic development and differentiation. Moreover, the establishment of human ES cells has facilitated their utilization in research for regenerative therapies [3]. The advantages of using ES cells are as follows: (1) although the number of cells in the mouse ICM or adult tissue stem cells is too limited for direct analysis, ES cells are capable of proliferating and providing enough cells for this; (2) ES cells can also be manipulated genetically in vitro, and are available to generate chimeric mice, allowing the analysis of the function of a gene of interest in vivo. However, there are several disadvantages of using ES cells. Some canonical methods of differentiation tend to suffer from contamination by irrelevant cells because of the difficulties of separating the cells of interest, which complicates any subsequent analysis. As shown in transplantation experiments, ES cells produce teratomas in recipient mice and insufficient removal of undifferentiated ES cells may lead to tumorigenesis, one of the major obstacles for the application of the differentiated cells in regenerative medicine. To solve these problems, it is necessary to introduce some steps to concentrate the intermediate precursor/progenitor cells

using cell surface markers and to simplify the differentiation procedure using serum-free media or avoiding coculture with other cells if possible. For the last decade, researchers have been developing methods to differentiate ES cells into their preferred tissues, including mesoderm (summarized in Table 1). From the point of view of clinical demand for therapeutic transplantations, hematopoietic cells are the most notable descendants of mesodermal cells. Understanding the properties of the stem/progenitor cells and fine-tuning their culture conditions are indispensable for the precise induction of the descendant cells of interest. The establishment of secure and safe methods of manipulation of ES cells into terminally differentiated cells will lead to clinical applications, such as regenerative treatment. This review summarizes the recent advances in the manipulation of ES cells into mesodermal tissues.

2 Mesoderm development in vivo

On the basic of the biological mechanisms observed in normal embryogenesis, a variety of ES cell differentiation processes have been developed in vitro. To control the differentiation of ES cells into mesodermal tissues, it is important to precisely refer to the in vivo process of mesodermal tissue development. In mammalian embryogenesis, the ICM gives rise to the primitive endoderm and epiblast, which is the source of the three primary germ layers during gastrulation (Fig. 1). The formation of the germ layers, and their subsequent fates, are determined through a process dependent upon spatial and temporal regulatory control. The primitive streak, one of the first signs of gastrulation, is a key structural marker to discriminate mesodermal precursor cells [4]. The cells at the anterior region of the primitive streak form the anterior mesendoderm, a bipotential cell population that has the capacity to generate definitive endoderm or axial mesoderm. At the mid- to late-streak stage, the mesoderm emerging from the primitive streak enters between the endoderm and ectoderm to form the paraxial, intermediate, and lateral mesoderm in order of their proximity to the primitive streak. The mesodermal precursor populations give rise to the following: the prechordal plate and notochord (from axial mesoderm); somites, which develop into muscles, bones, and cartilage (from paraxial mesoderm); kidneys, gonads, and their respective duct systems (from intermediate mesoderm); and heart, blood vessels, and blood cells (from lateral mesoderm). In addition, the epiblast forms the extraembryonic mesoderm outside of the embryo, which gives rise to the endothelial and hematopoietic cells of the yolk sac vasculature. However, to reproduce this complicated biological process by simple experimental procedures is clearly difficult. It requires the dissection of the process into elementary steps using various biological markers and the compiling of each step in the correct order. Section 3 explains how to induce and analyze the differentiation of ES cells into cells corresponding to mesodermal cells or tissues of interest on culture dishes.

3 In vitro differentiation of mesodermal cells

3.1 General consideration

Commonly used protocols for ES cell differentiation are categorized into three types according to the method of culturing: embryoid-body (EB) formation [5], culture on feeder cells [6], and simple monolayer culture on extracellular-matrix-coated dishes (Fig. 2). EB formation is the most popular method whereby three-dimensional cell aggregates are formed and undergo a developmental process corresponding to the events of early embryogenesis, in which the cells lose their pluripotency and differentiate into ectoderm, endoderm, and mesoderm. However, the multiplicity of differentiated cell lineages complicates subsequent analysis, and the heterogeneous size of the cell aggregates tends to cause non-uniform differentiation processes due to differences in extracellular adhesion and intercellular signaling by the cells of interest. It is therefore difficult to control and direct ES cells into mesodermal cells using exogenous signals. To overcome these disadvantages, investigators have developed two-dimensional culture methods. Coculture with feeder cells allows the selective induction of the cell lineage of interest. The induction of the hematopoietic cell lineage from ES cells is achieved by coculturing with stromal cell lines [7]. The modification of the feeder cells enables the elimination of unnecessary cell lineages, such as the use of stromal cell line OP9 to avoid macrophage proliferation, for example [8]. Furthermore, ST2 stromal cells, derived from bone marrow cells, have the potential to support osteoclastogenesis from hematopoietic cells, thus a sequential coculture of mouse ES cells with OP9 cells followed by ST2 cells could efficiently introduce osteoclasts [9]. However, the feeder culture system is technically complicated because the condition of the feeder cells tends to affect the reproducibility of the differentiation. By using a simple monolayer culture, the inducible cell lineage is more selective than the feeder culture, and it is easier to control and observe the differentiation process, and to collect the differentiated cells. Another advantage of monolayer culture is the flexibility and selectivity of culture dish matrix components. Collagen IV is capable of directing ES cell differentiation into mesoderm lineages, including hematopoietic, endothelial, and smooth muscle cells [10]. Although each cell monolayer could be uniformly treated with the same culture conditions, lots of trials are needed to establish the defined culture conditions for the cell types of

Table 1 Summary of mesodermal cell differentiations from ES and iPS cells

Differentiated cell types	Cell types	Selection markers	Culture conditions	Supplement factors	References
Mesodermal cells	Mouse ES	GFP-Gsc, E-cadherin	Embryoid body	Activin A	[13]
	Mouse ES	PDGFRα, VEGFR2	Collagen IV-coated dish		[24]
	Mouse ES	GFP-Brachyury	Embryoid body	Activin A	[89]
Hematopoietic cells	Mouse ES		Coculture with OP9 stromal cells		[90]
	Mouse ES	VEGFR2, VE-cadherin	Collagen IV-coated dish	VEGF, SCF, IL-3, EPO, G-CSF	[10]
	Mouse ES		Embryoid body	BMP4, Activin A, bFGF, VEGF	[29]
	Mouse ES	GFP-Brachyury, VEGFR2	Embryoid body	BMP4, Activin A, Wnt3a	[15, 30]
	Mouse ES		Embryoid body	HoxB4 (retrovirus)	[32]
	Human iPS	CD43	Coculture with OP9 stromal cells		[75]
Erythrocytes	Human ES		Coculture with murine fetal liver-derived stromal cells		[34]
Megakaryocytes	Mouse ES	Coculture with OP9 stromal cells		TPO	[91]
	Human ES	Coculture with OP9 stromal cells		VEGF	[35]
Lymphoid cells	Mouse ES		Coculture with OP9 stromal cells	IL-7	[6]
	Mouse ES		Coculture with OP9 stromal cells	Dll1 (expressed on OP9 cells)	[92]
	Human ES	CD34	Coculture with OP9 stromal cells	Flt3-L, IL-3, IL-7, SCF	[36]
Dendritic cells	Mouse iPS		Coculture with OP9 stromal cells	GM-CSF, IL-4, TNFa, anti-CD40	[72]
Macrophage	Mouse iPS		Coculture with OP9 stromal cells	M-CSF, IL-4, TNFa, anti-CD40	[72]
Vascular endothelial cells	Mouse ES	VEGFR2, VE-cadherin	Collagen IV-coated dish	VEGF, SCF, IL-3, EPO, G-CSF	[10]
	Mouse ES		Embryoid body	VEGF, IGF-1, Epo, bFGF, IL-11	[41]
	Mouse ES	VEGFR2	Collagen IV-coated dish	VEGF	[23]
	Mouse iPS	VEGFR2	Collagen IV-coated dish	VEGF	[71]
	Human iPS	CD31, CD43	Coculture with OP9 stromal cells		[75]
Cardiac cells	Mouse ES	VEGFR2, CXCR4	Coculture with OP9 stromal cells	Dkk-1, Frizzled/Fc	[47]
	Mouse ES	VEGFR2, Isl1, Nkx.25	Embryoid body		[48]
	Mouse ES		Embryoid body	Noggin	[49]
	Mouse iPS	VEGFR2	Collagen IV-coated dish and coculture with OP9 stromal		[69]
	Human iPS		Embryoid body		[73]
Skeletal muscle cells	Mouse ES	PDGFRα	Collagen IV-coated dish		[57]
	Mouse ES	PDGFRα, VEGFR2	Embryoid body	Pax3 (Dox inducible)	[58]

Table 1 continued

Differentiated cell types	Cell types	Selection markers	Culture conditions	Supplement factors	References
Osteocyte	Mouse ES		Embryoid body	Ascorbic acid, β-glycerophosphate, vitamin D3	[54]
	Human ES		Without embryoid body	Ascorbic acid, β-glycerophosphate, dexamethasone	[53]
Chondrocyte	Mouse ES		Embryoid body	BMP-2 or BMP-4	[55]
Adipocyte	Mouse ES		Embryoid body	Retinoic acid	[59, 61]
	Human iPS		Embryoid body	Retinoic acid	[74]

interest. Another factor affecting the differentiation process of ES cells is the composition of the culture medium. Culture media containing fetal bovine serum are often difficult to reproduce because the combination of factors in serum varies among serum lots. It is well-known that the TGFβ, BMP, Wnt, and FGF families play important roles in mesoderm induction in all vertebrates [11]. To exclude the possibility that undefined factors in serum affect the differentiation, the use of serum-free conditions with supplements of chemically defined factors should be adopted. So far, there are several reports that serum-free culture conditions can induce the differentiation of mesodermal cells from ES cells using humoral factor supplements (summarized in Table 1). A serum-free medium containing activin can induce the development of mesendoderm from ES cells on collagen IV-coated dishes, which could potentially give rise to endoderm and mesoderm [13]. The addition of BMP4 to serum-free cultures leads to the induction of mesoderm from ES cells [12]. Culturing with defined media is indispensable from the viewpoint of clinical applications of pluripotent stem cells.

However, it is rather difficult to specify the differentiated cells in in vitro culture systems because of the lack of availability of positional information, unlike for cells in the embryo. Thus, effective selection markers are required to define and track the differentiation of a particular cell lineage in culture, where the differentiation of multiple lineages occurs simultaneously (Fig. 3). Several cell surface markers or knockin/transgenic reporter strategies have been utilized to provide information on the various intermediate stages that occur during ES cell differentiation, and to select the differentiated cells of interest. Tracing the differentiated cell lineage by marker proteins also provides new information about the differentiation pathways. In fact, the existence of mesendoderm, which can give rise to both endoderm and mesoderm, was demonstrated using the Goosecoid (Gsc) gene as a tracing marker [13]. Brachyury (T), another lineage tracing marker, is expressed throughout the anterior–posterior region of the primitive streak and the notochord, and is associated with the appearance of mesodermal precursor cells [14]. A 500 bp upstream promoter region of the T gene drives GFP expression in the middle portion of the primitive streak [15], and a combination of these markers, human CD4 targeted to the Foxa2 and T-gene-induced GFP, could distinguish the cell populations corresponding to the anterior and posterior regions of the primitive streak [16], respectively.

Subsequent cell lineages of mesodermal precursor cells can also be concentrated using the following markers. MIXL1, a homeobox gene involved in early hematopoietic specification, is another marker of the primitive streak and is suitable for the isolation of primitive hematopoietic precursors [17, 18]. Two cell surface markers, PDGFRα

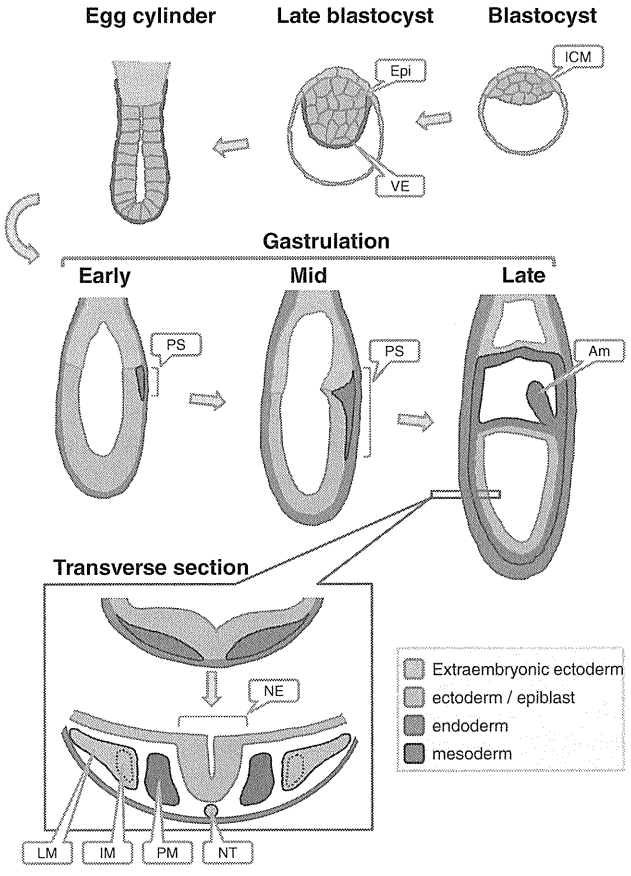

Fig. 1 An overview of mesodermal development in mouse embryos. The embryos are shown in mid-sagittal section, anterior to the left. The stages represent the blastocyst (around embryonic day 4.0 (E 4.0)), late blastocyst (E4.5), egg cylinder (E5.0), and early- (E5.5), mid- (E6.0), and late- (E6.5) stages of gastrulation. The *red square* speech balloon shows transverse sections of the embryo after gastrulation (upper section is E7.5, lower section is E8.0). *ICM* inner cell mass, *Epi* epiblast, *VE* visceral endoderm, *PS* primitive streak, *Am* amnion, *NE* neuroepithelium, *NT* notochord, *PM* paraxial mesoderm, *IM* intermediate mesoderm, *LM* lateral mesoderm

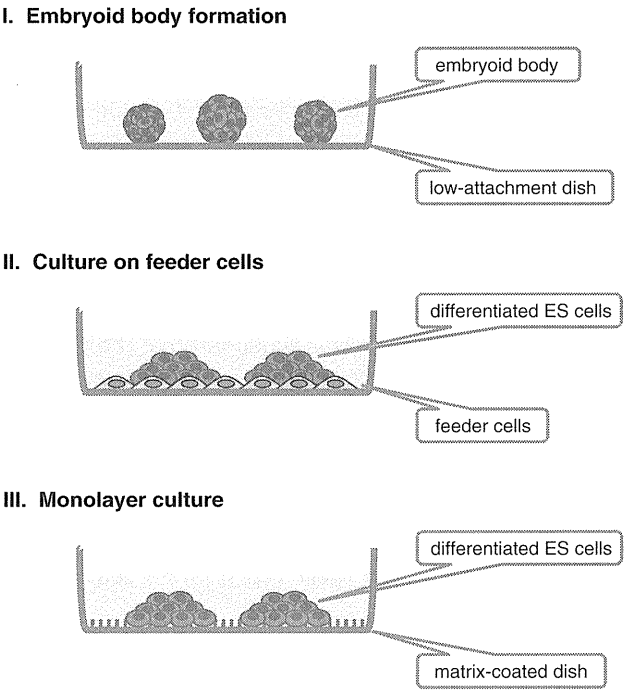

Fig. 2 Schematic diagram of the culturing methods used for ES cell differentiation. Culturing methods used for ES cell differentiation into mesodermal cell lineages are generally categorized into three types: *I* formation of embryo-like aggregates of ES cells by culturing on low-attachment dishes; *II* culture on feeder cells, such as OP9 stromal cells; and *III* culture on plates coated with a defined matrix, such as collagen IV

(platelet-derived growth factor receptor a) and VEGFR-2 (vascular endothelial growth factor receptor 2), can be used to dissect the early processes of divergence in mesodermal differentiation. PDGFRα is one of the receptors expressed in paraxial mesoderm during mouse embryogenesis and an antibody specific for it can recognize paraxial mesoderm

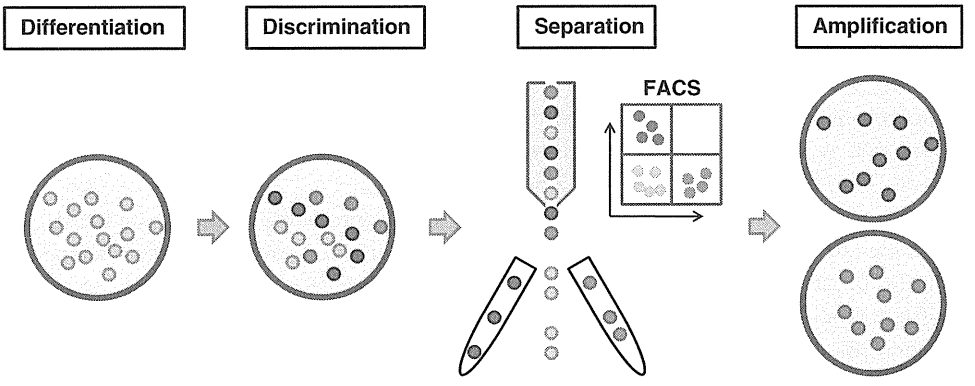

Fig. 3 Schematic diagram of the differentiation and concentration of mesodermal cells from ES cells. ES cells are differentiated by three types of culturing methods as shown in Fig. 2 (*Differentiation*). Differentiated cells are distinguished using mesodermal marker gene promoter-driven fluorescent proteins such as Brachyury, Goosecoid, and MIXL1, or fluorescently labeled antibodies against mesoderm surface markers, such as PDGFRα and VEGFR-2 (*Discrimination*), separated using flow cytometry (*Separation*), and cultured with differentiated mesodermal cell-specific media (*Amplification*)

cells at late primitive streak stages [19, 20]. VEGFR-2 is an indicator of the development of lateral mesoderm [21], and can be utilized to isolate lateral mesoderm cells with the potential to be hematopoietic precursors [22], or vascular progenitors of endothelial cells [23]. Mouse ES cells cultured on collagen-IV-coated dishes differentiate into PDGFRα+/VEGFR-2+ immature mesodermal precursors, which give rise to two populations: VEGFR-2+/PDGFRα− single-positive (VSP) population, which potentiates endothelial and hematopoietic differentiation, and a VEGFR-2−/PDGFRα+ single-positive (PSP) population, which dominantly expresses lineage markers for myocytes, osteocytes, and chondrocytes [24]. The combination of each culture method and mesodermal marker has the potential to minimize these disadvantages and improve differentiation efficiency (Fig. 3). In the following sections, we describe recent progress in the differentiation of each type of mesodermal tissue cell from ES cells.

3.2 Hematopoietic cells

The vast majority of clinical organ transplantations are blood cell transplants, including bone marrow transplantation. However, we have not yet succeeded in adequately maintaining and proliferating hematopoietic stem cells in vitro, while there are many difficulties in undertaking allogeneic transplantation. The induction of hematopoietic cells from ES cells represents the longest studied differentiation pathway. To date, a number of experimental trials have been conducted to evaluate the feasibility of producing hematopoietic lineage cells from mouse and human ES cells [25–27]. Most of them have required either embryoid body formation or coculture with stromal cells, with the addition of cytokines, such as interleukins, CSF, or both. On the other hand, ES cells cultured on a collagen IV coated two-dimensional plane could differentiate into hematopoietic lineage cells, demonstrating that neither the three-dimensional structure in the embryoid body nor feeder cells are required for this process [10].

Which population in ES-cell-derived mesoderm can give rise to hematopoietic lineage cells? The fact that the VEGFR2+ population that emerges after mouse ES cell differentiation generates hematopoietic lineage cells demonstrates that mouse ES cells can differentiate into hematopoietic cells through the lateral mesoderm-like cells [28]. Biomarkers can be useful to further elucidate the process of the differentiation of hematopoietic lineage cells from ES cells with a combination of other factors. For example, it was demonstrated in an ES cell culture system that the appropriate timing of the expression of SCL, an essential molecule for the early development of hematopoietic systems, is required for definitive hematopoietic precursor cell differentiation [28]. Recent reports show that the selective differentiation of mouse ES cells into hematopoietic progenitor cells is achieved by stepwise treatments with serum-free media containing Wnt/BMP/activin or Bmp4/activin/bFGF/VEGF, although stable and efficient hematopoietic engraftment of the cells has not been demonstrated [29, 30]. Moreover, the induction and identification of hematopoietic stem cells is still difficult without genetic manipulation. Several homeotic selector genes are expressed in definitive hematopoietic stem cells and have been studied as they are candidate genes for hematopoiesis regulation. One of these genes, HoxB4, enhances hematopoietic repopulation without interfering with hematopoietic differentiation when the gene is overexpressed in adult bone marrow [31]. Ectopic expression of HoxB4 in mouse differentiated ES cells enhances its contribution to multilineage hematopoietic cell engraftment, suggesting that HoxB4 expression confers "stemness" potential on ES-cell-derived hematopoietic cells [32]. However, a number of trials are yet to demonstrate the engraftment of multilineage hematopoietic cells from human ES cells, which may be due to immune-mediated rejection [33].

Several recent studies have focused on the enucleated terminally differentiated hematopoietic cells, such as erythrocytes and megakaryocytes, which are advantageous owing to the absence of tumorigenicity by X-ray irradiation. Enucleated and functionally matured erythrocytes are generated from human ES cells by coculturing with mouse fetal-liver-derived stromal cells [34]. Human ES cells cultured with VEGF on stromal cells, such as 10T1/2 or OP-9, formed sac-like structures containing hematopoietic progenitors, and gave rise to mature megakaryocytes, which have the ability to release platelets [35]. In addition, lymphoid lineage cells could be induced from mouse and human ES cells by coculturing with OP9 cells in medium containing lymphoid cytokines [6, 36]. These findings open the possibility for clinical applications of terminally differentiated, functional hematopoietic cells induced from human ES cells. More details of the differentiation of hematopoietic cells are provided in another article in this issue by Sakamoto.

3.3 Endothelial cells

It has been clearly demonstrated that hematopoietic cells are generated from vascular endothelial cells using in vitro ES cell differentiation culture systems [10]. In fact, according to recent reports, the generation of hematopoietic cells from aorta endothelial cells was confirmed in vivo [37, 38]. Moreover, ES cells have made it possible to demonstrate the derivation of both endothelial cells and hematopoietic cells from a single cell [39]. This enabled the identification of the aorta as an intraembryonic site for hematopoietic cell production [40]. Thus, it is important

that endothelial cells are correctly induced from ES cells for the differentiation of hematopoietic cells. As is the case for other tissue cells, EB formation had been utilized to differentiate ES cells into endothelial precursor cells and develop vascular-like structures, although it was difficult to trace the vascular differentiation process at the cellular level [41, 42]. Two-dimensional culture and the use of some cell surface markers could solve these difficulties and improve the efficiency and accuracy of the differentiation process. By detailed analysis of the process of embryonic vasculogenesis, the majority of vascular tissues and blood cells are considered to be derived from a common progenitor cell of the lateral mesoderm because the VEGFR2 knockout mouse, known as a marker of lateral mesoderm, failed to develop hematopoietic and endothelial cells [43]. Indeed, VEGFR2 is a cue for concentrating the vascular lineage cells from in vitro differentiated mouse ES cells without using EB formation. The VEGFR2+ cell population could be directed to differentiate into endothelial cells by VEGF and mural cells by PDGF-BB under serum-free culture conditions, and form vessel-like structures in a three-dimensional culture containing serum and VEGF [23]. However, from the point of view of regenerative medical applications, the differentiation stages of vascular lineage cells might be critical for the effective engraftment of the transplanted cells into the recipient, because differentiated vascular progenitor cells, a mixture of endothelial cells and mural cells, contribute to neovasculogenesis in adult tissues more effectively than VEGFR2+ E-cadherin mesoderm-like cells [44]. A recent report demonstrates that the mixture of endothelial cells and mural cells differentiated from human ES cells contributes to vascular regeneration in recipient mice, suggesting that the reconstituted vascular structures may have potential as a source of therapeutic vascular regeneration [45].

3.4 Cardiac cells

Clarifying the mechanisms of cardiac muscle differentiation improves the understanding of the pathogenesis of congenital heart diseases, such as cardiac anomaly. It is well-known that beating cardiomyocytes are easily developed during EB formation of mouse ES cells [5], but terminally differentiated cardiomyocytes stop proliferating and thus the EB has a very low proportion of the differentiated cardiomyocytes in the cell mass [46]. As previously described in Sects. 3.2 and 3.3, cardiomyocytes could also be enriched by the combination of a two-dimensional culture and cell surface marker selection. By differentiating mouse ES cells with collagen-IV-coated culture dishes and selection of the VEGFR2+ E-cadherin-population corresponding to lateral mesoderm, the proportion of differentiated cardiomyocytes could be increased 2- to 3-fold compared with EB formation [47]. Although the VEGFR2+ E-cadherin-population contains not only cardiomyocyte progenitors but also vasculogenic progenitors, additional selection by CXCR4 effectively concentrates the cardiomyocyte progenitors. As another strategy for amplification of cardiac progenitor cells from ES cells, it was also reported that the Isl1+/Nkx2.5+/VEGFR2+ population, differentiated by EB formation, could be a multipotent cardiovascular progenitor that can give rise to cardiac myocytes, smooth muscle cells, and endothelial cells [48]. Several humoral factors are also reported to modify the efficiency of cardiomyocyte differentiation. Treatment of the VEGFR2+ E-cadherin-population with a BMP inhibitor, noggin, suppressed the induction of cardiomyocyte progenitor cells [47]. On the other hand, another group reported that temporal- and phase-restricted treatment with noggin was an effective way of inducing cardiomyocyte differentiation of mouse ES cells [49]. Wnt proteins were also reported as biphasic factors that enhance or inhibit cardiomyocyte differentiation of ES cells, depending on the phase of the differentiation process [50]. According to a recent report, treatment with an Src family kinase inhibitor, PP2, or the expression of a dominant negative focal adhesion kinase increased ES cell cardiomyocyte differentiation, suggesting that adhesion signaling in ES cells may control cardiomyocyte differentiation [51]. Thus, it will be possible to increase the efficiency of the cardiomyocyte differentiation of ES cells using defined humoral factors and several cell signaling inhibitors at each stage of differentiation.

3.5 Osteocytes, chondrocytes, adipocytes, and myocytes

Osteocytes, chondrocytes, adipocytes, and myocytes are other mesodermal descendants, which organize the assembly of supportive tissues of somatic cells and are well characterized as derivatives of mesenchymal stem cells (MSCs). Regeneration of the supportive tissues, such as bone, cartilage, and skeletal muscle, is indispensable for the treatment of age-related and intractable tissue degeneration.

Differentiation of human ES cells into osteocytes has been developed into an efficient culturing system without using embryoid body formation [52, 53]. In vitro osteocyte differentiation of mouse ES cells has been developed to produce mineralized osteoblasts [54]. Chondrogenic differentiation of ES cells was also accomplished by supplementation with growth factors of the TGFβ family [55]. Although several methods have been developed to induce the differentiated cells of each supportive tissue from ES cells, it is difficult to reconstitute the supportive tissue structure with the terminally differentiated cells correctly

because the mechanism is a complex process accomplished by a combination of specialized cells, for example, osteoblast and osteoclast cell-coordinated formation of bones. However, this could be overcome by exploiting some selective markers that recognize the progenitor cells of interest and transplanting the pre-terminally differentiated cells. The PDGFRα+ population from ES-cell-derived mesodermal progenitors can differentiate not only into chondrocytes and osteocytes, but also into muscle satellite cells that are thought to be a candidate for contributing to adult skeletal muscle regeneration [56, 57]. Indeed, sorting of the PDGFRα-positive and VEGFR2-negative population, a combination of selection markers corresponding to the paraxial mesodermal cells, concentrated skeletal muscle progenitors efficiently from mouse ES cells and were functionally engrafted into muscular dystrophic mice [57, 58]. Adipocytes, similar to muscle and bone cells, are generally thought to be derived from MSCs. The efficient differentiation of ES cells into adipocytes was established by retinoic acid treatment during embryoid body formation [59]. However, it was reported that retinoic acid-treated mouse ES cells caused a reduction in the level of several mesodermal markers [60]. It is still unclear whether adipocytes only originate from mesoderm because Sox1-positive neuroepithelial cells from mouse embryos can give rise to MSCs and adipocytes [61]. As another factor triggering adipogenesis, mechanical stimulus on cell membranes might also influence adipose differentiation because the adipocyte–osteocyte fate of MSCs is found to be regulated via RhoA-ROCK signaling [62]. In addition, one of the Rho GTPase regulators, p190-B RhoGAP, is known to be able to switch the adipocyte–myocyte fate of mouse embryonic fibroblasts [63]. It might be possible that a common progenitor derived from ES cells can switch the cell fate from adipocyte to osteocyte or myocyte, although it will be necessary to clarify the precise origin of adipocytes and carry out lineage-tracing analysis to confirm this.

4 Generation of mesodermal cells from iPS cells

Exploitation of induced pluripotent stem (iPS) cells represents recent exciting progress in the direct reprogramming of adult somatic cells. Four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc, could transform terminally differentiated mouse fibroblasts into pluripotent cells, so-called iPS cells, that resemble ES cells in morphology, growth patterns, and gene expression [64]. Several reports confirmed the applicability of this innovative method to various cell sources and species, for example, mouse hepatocytes and gastric epithelial cells [65], human fibroblasts [66, 67], and human peripheral blood cells [68]. Reports concerned with the induction of mesodermal tissue cells from iPS cells are being published in rapid succession: these include those from studies on cardiomyocytes [69, 70], vascular endothelial cells [71], and dendritic cells and macrophages [72] derived from mouse iPS cells; cardiomyocytes [73], adipocytes [74], and vascular endothelial and hematopoietic cells [75] derived from human iPS cells. The differentiation of mesoderm lineage cells from iPS cells seems to proceed in a similar way to that from ES cells. For example, cardiovascular cells and vascular endothelial cells are derived from the VEGFR2+ population in differentiated iPS cell cultures, and the time course and efficiency of the differentiation were found to be comparable to those of mouse ES cells [69, 71]. Some problems for the usage of iPS cells have been identified, such as a high frequency of tumor formation in mice [76], perhaps caused by the expression of *c*-Myc, and chromosomal damage introduced by random viral vector integration. Although it was reported that none of the mice transplanted with iPS-derived hematopoietic progenitor cells showed any evidence of tumor formation in a sickle cell anemia model [77], the possibility still remains that iPS-derived mesodermal cells may develop into malignant tumors, as was reported following transplantations of ES-derived mesodermal precursor cells [57] and hematopoietic precursor cells [78]. As mentioned above, the number of reports concerning the differentiation of mesoderm-derived descendants from human iPS cells is increasing. However, it is still unclear whether mesoderm cells can be induced from iPS cells. These problems might be resolved by improvements to the preparation procedures, such as omission of *c*-Myc or gene transfer with non-viral vectors [79–81]. By using adult mouse neural stem cells as the source, the exogenous introduction of Oct3/4 is sufficient to generate iPS cells [82]. Moreover, recent reports have raised the possibility of the preparation of human iPS cells suitable for medical applications. Supplementing culture media with some chemical compounds, inhibitors of chromatin-modifying enzymes, could increase the efficiency of iPS cell generation [83, 84]. In the future, it may be possible to replace all transcription factors with chemical compounds and to produce iPS cells without gene transfer, although the safety of the human iPS cells that are produced remains to be seen.

5 Future perspectives

Most mesodermal tissues, such as blood, bone, and cartilage, are structurally more uniform than ectoderm- or endoderm-derived tissues, that is, in vitro-reconstituted mesodermal tissues might be in the vanguard of clinical regenerative treatment using manipulated pluripotent stem cells. Analysis of the mesodermal differentiation process of

ES cells will provide advantages, not only for clinical applications, but also for the study of mesoderm development. If in vitro differentiation of ES cells precisely recapitulates in vivo embryogenesis, the molecular mechanisms observed in in vitro culture systems can be fed back to in vivo embryonic development.

Human ES cells have huge potential for regenerative medicine, but both ethical controversies and immunological rejection are major obstacles to the use of human ES cells as a source of clinical treatment [85]. iPS cells would avoid the problems of immunological identity as they would be developed from the autologous somatic cells of the recipient. The establishment of iPS cells from human terminally differentiated cells might lead to individualized therapy instead of the use of human ES cells. In fact, it was experimentally demonstrated that iPS cells are a suitable source for the treatment of sickle cell anemia [77] or hemophilia [86] in a mouse model. Nonetheless, as most of the methods of differentiation applied to iPS cells are based on the culture and differentiation procedures of ES cells, it is indispensable to clarify the differentiation process of ES cells in detail. The issue of tumorigenicity will remain regardless of which pluripotent cells are used as a source. It is important to introduce differentiation at a high efficiency and to completely exclude tumorigenic undifferentiated cells. To achieve this, more fundamental studies are required to dissect the intermediate processes producing the differentiated cells of interest and to explore unknown cell surface markers for concentration of target cells. Indeed, the importance of effective selection of a specific cell lineage was confirmed by the success of functional engraftment of skeletal muscle cells derived from mouse ES cells into muscular dystrophic mice [58]. Another improvement to be achieved is to optimize the differentiation culture conditions and chemically define the molecular components indispensable for differentiation. Sequential treatment with several small molecules can differentiate mouse and human ES cells into pancreatic progenitors at a higher efficiency than conventional procedures based on TGFβ family members [87, 88], although this was assessed under low-serum conditions. The ultimate goal of ES cell manipulation is to mimic the process of differentiated cell specification under chemically defined conditions and to generate a specific type of mature cell at high purity, safety, and reproducibility in vitro. Precise manipulation of mesodermal cell development from ES cells may lead to the identification of mesodermal stem cells and the development of methods for their maintenance. If mesodermal stem cells can be maintained in vitro, it will help our understanding of the molecular mechanisms of self-renewal and multipotency. On the basis of our findings, we might then be able to induce mesodermal stem cells from somatic cells. Thus, we can avoid the unnecessary risk of transformation by using pluripotent cells and take advantage of the induced "mesodermal" stem cells for clinical applications.

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Notch2 Activation in the Embryonic Kidney Depletes Nephron Progenitors

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ABSTRACT

Successive activation of Wnt4 and Notch2 generates nephrons from the metanephric mesenchyme. Mesenchymal-to-epithelial transition requires Wnt4, and normal development of the proximal nephron (epithelia of glomeruli and proximal tubules) requires Notch2. It is unknown, however, whether Notch2 dictates the fate of the proximal nephron directly. Here, we generated a mutant strain of mice with activated Notch2 in *Six2*-containing nephron progenitor cells of the metanephric mesenchyme. Notch2 activation did not skew the cell fate toward the proximal nephron but resulted in severe kidney dysgenesis and depletion of *Six2*-positive progenitors. We observed ectopic expression of *Wnt4* and premature tubule formation, similar to the phenotype of *Six2*-deficient mice. Activation of Notch2 in the progenitor cells suppressed *Pax2*, an upstream regulator of *Six2*, possibly through *Hesr* genes. Taken together, these data suggest that a positive feedback loop exists between Notch2 and Wnt4, and that Notch2 stabilizes, rather than dictates, nephron fate by shutting down the maintenance of undifferentiated progenitor cells, thereby depleting this population.

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The mammalian kidney, the metanephros, is formed by reciprocally inductive interactions between two precursor tissues; namely, the metanephric mesenchyme and the ureteric bud. Glial cell line-derived neurotrophic factor (Gdnf) is secreted by the mesenchyme and attracts the ureteric bud toward the mesenchyme.[1–3] The attracted ureteric bud in turn secretes Wnt9b and induces the mesenchyme.[4] Upon this induction by the ureteric bud, mesenchymal cells secrete Wnt4, which plays an essential role in the mesenchymal-to-epithelial transition.[5,6] The mesenchyme sequentially forms condensates, renal vesicles, C- and S-shaped bodies, and terminal epithelia of the glomeruli and renal tubules.

A previous report retrospectively suggested the presence of multipotent cells in embryonic kidneys, after demonstrating that cells in several portions of the nephron were derived from single stem cells by using *LacZ* gene transduction with a retrovirus into single mesenchymal cells.[7] We prospectively proved this concept by establishing a novel colony assay. When we plated dissociated mesenchymal cells at a low density onto feeder cells stably expressing *Wnt4*, single cells formed colonies consisting of several types of epithelial cells that are found in glomeruli and renal tubules.[8] We further found that only cells strongly expressing *Sall1*, a zinc finger nuclear factor expressed in the metanephric mesenchyme and essential for kidney development, formed colonies.[8,9] Therefore, multipotent progenitors of nephron epithelial cells do exist and reside in the *Sall1*-high population of the mesenchyme.[10]

Nephron progenitors have also been shown to express another transcription factor, *Six2*. Kobayashi *et*

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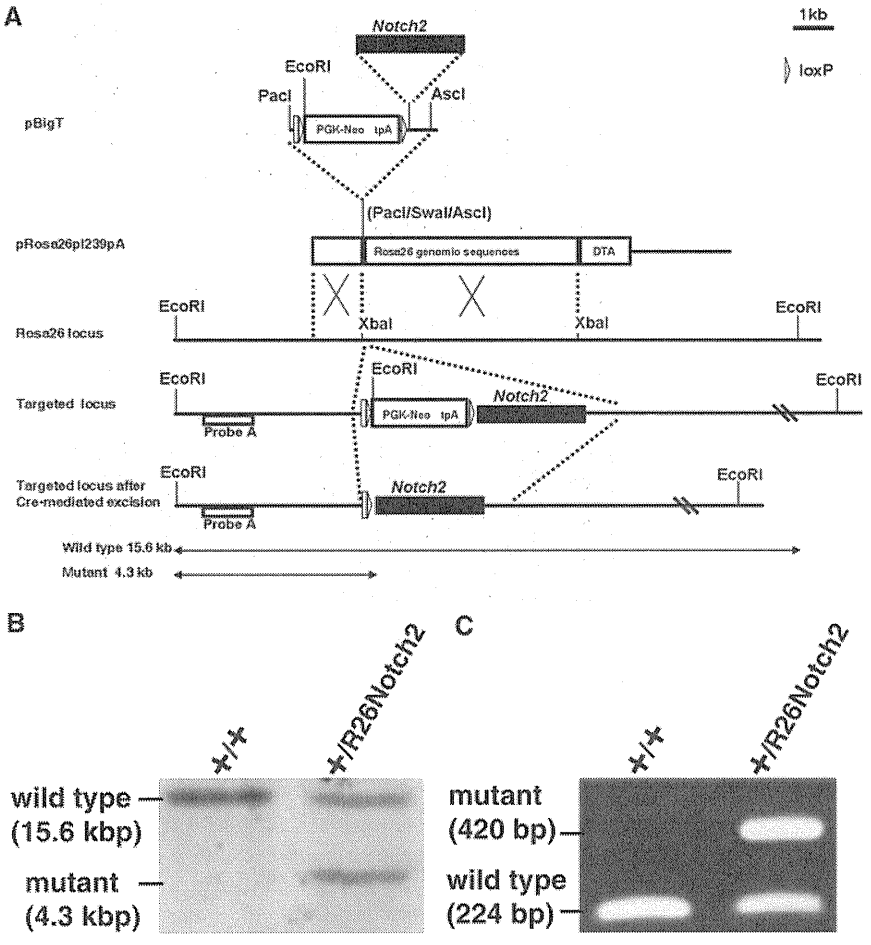

Figure 1. *R26Notch2* mice are generated. (A) Targeting strategy of *Flag-Notch2* in the *Rosa26* locus. A *Flag-Notch2* fragment is inserted into the *pBigT* vector containing an adenovirus splice acceptor sequence followed by a *PGK-neo* cassette and a tpA stop sequence flanked by two loxP sites. The resultant plasmid is inserted into *pRosa26pl239pA* (a modified *pRosa26-1* vector) and subsequently integrated into the *Rosa26* locus in ES cells by homologous recombination. (B) Targeted ES clones are confirmed by Southern blot analysis. Genomic DNA is digested with *Eco*RI and hybridized with probe A. (C) PCR amplification of mouse tail DNA.

al.[11] marked *Six2*-positive cells using *Six2Cre*, a mouse strain expressing Cre recombinase under the control of the *Six2* promoter, and they demonstrated that *Six2*-positive mesenchyme does indeed give rise to epithelia of the glomeruli and renal tubules of the nephron *in vivo*. Because *Six2* is expressed in *Sall1*-high mesenchymal cells, the *Sall1*-high and *Six2*-positive mesenchyme may represent a nephron progenitor population in the embryonic kidney.[12] *Six2* is expressed in the undifferentiated mesenchyme that caps the ureteric bud (dorsal side of the ureteric bud), and its expression pattern is reciprocal to that of *Wnt4*, which is expressed near the ureteric stalk (ventral side). *Wnt4* is upregulated in the dorsal portion of the mesenchyme in *Six2*-deficient mice, and the mice exhibit ectopic and premature tubulogenesis,[13] suggesting that *Six2* is required to maintain the undifferentiated progenitor population by opposing *Wnt4*-mediated epithelialization.

Six2 expression is regulated by the Pax2/Eya1/Hox11 complex, and the binding sites for this complex are present in the *Six2* proximal promoter, which is essential for *Six2* expression in the mesenchyme *in vivo*.[14] *Six2* expression is reduced in mice lacking each of the complex components, whereas crossing of these mutant mice revealed physiologic significance for the interactions among the complex components.[14,15] Therefore, the Pax2/Eya1/Hox11 complex appears to be a bona fide regulator of *Six2 in vivo*. This complex also regulates the expression of *Gdnf*, which is essential for ureteric bud attraction.[14]

Six2-positive nephron progenitors differentiate into epithelia upon *Wnt4* stimulation, but a fate decision is required for further differentiation toward glomerular podocytes or proximal and distal renal tubules. *Notch2* is required for the differentiation of proximal nephron structures (podocytes and proximal tubules), because mesenchyme-specific *Notch2* deletion in mice leads to impaired formation of these proximal structures.[16–18] Although proximodistal polarity is still initiated in the absence of *Notch2*, *Notch2* is essential for the final establishment of the proximal nephron fates. In humans, *Notch2* haploinsufficiency causes Alagille syndrome, which is associated with renal abnormalities.[19] Interestingly, endogenous *Notch1* cannot compensate for *Notch2* deficiency in mice, although Notch1 activity is present in the kidney, and increases in its activity enhance the formation of proximal tubules at the expense of podocytes and distal nephrons.[18]

To gain insights into Notch2 functions, we generated mice with activated Notch2 in *Six2*-positive nephron progenitors. Interestingly, *Notch2* activation did not specify the proximal nephron fate but instead caused progenitor depletion by shutting down the *Six2*-mediated program for progenitor maintenance.

RESULTS

Generation of a Mouse Strain Allowing Notch2 Activation in a Cre Recombinase-Dependent Manner

By using homologous recombination in embryonic stem (ES) cells, we generated *R26Notch2* mice containing a controllable *Notch2* cassette in the *Rosa26* locus that confers ubiquitous expression.[20] The Flag-tagged intracellular domain of *Notch2* was introduced into the *pBigT* vector,[21] which contains *PGK-neo* and three SV40 terminator sequences flanked by two loxP sites (Figure 1A). The *pBigT* vector containing *FlagNotch2* was