

Embryonic stem cells as a source of hematopoietic and vascular endothelial cells in vitro

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The murine embryonic stem cell line is the only totipotent cell line currently available, although extensive attempts have been made to establish such lines from other species. The usefulness of this line has been proved over the last few decades, because many strains of mutant mice have been developed from embryonic stem cells in which the genes were altered by means of homologous recombination.

Because of its totipotency, the embryonic stem cell line should have the potential to be used for production of many types of mature cells that could be used in cell supplement treatments. However, such use has been hampered by a number of obstacles, the most important of which is difficulty controlling the differentiation of embryonic stem cells into a desired cell lineage. Although embryonic stem cell differentiation can be blocked by means of the addition of leukemia inhibitory factor (LIF) to cultures, removal of LIF induces uncontrolled differentiation into various cell lineages, and the culture becomes occupied with a mixture of mature cells and unidentified cells in intermediate stages of differentiation.

The problem can be solved in two ways. Probably the best method is to use a combination of cytokines that drive embryonic stem cell differentiation into a given pathway. To do this, however, we need to understand more about the molecules that control cell specification. The other method, which is easier to achieve, repeatedly selects the intermediate stages of a desired cell lineage from the unwanted ones until pure, fully differentiated cells are eventually produced in vitro. Herein, we present our attempts in the latter direction to produce vascular endothelial cells and blood cells from embryonic stem cells.

DIFFERENTIATION INTO HEMANGIOBLAST, PUTATIVE PRECURSORS OF HEMATOPOIETIC CELLS, AND VASCULAR ENDOTHELIAL CELLS

Among the many cell types in the mammalian body, vascular endothelial cells and erythrocytes are those that

Abbreviations used

LIF:	leukemia inhibitory factor
mAb:	monoclonal antibodies
PDGFR:	platelet-derived growth factor receptor
RTKP:	receptor tyrosine kinases of the PDGFR family

appear most rapidly during embryogenesis. In murine embryos, both vascular endothelial cells and erythrocytes appear in the yolk sac on day 7.5 of gestation, when most other cell types remain in the intermediate stages of differentiation. This suggests that the intermediate steps in differentiation from totipotent embryonic ectoderm to hematopoietic and angiogenic cells may be fewer than those required for differentiation of other cell types, although it also may be a matter of differentiation speed. In fact, only three distinct steps of this process have been defined (Fig. 1). From the fertilized egg to the embryonic ectoderm of the egg cylinder stage, each embryonic cell preserves multipotency. The first step of cell specification occurs at 6.5 days of gestation (dpc), when a structure called the *primitive streak* is formed on the egg cylinder. Mapping studies have indicated that mesodermal and endodermal cells are generated only by passing through this structure.¹

With respect to the hematopoietic and vascular endothelial cell lineages, the cells in the most proximal part of the primitive streak differentiate into the proximal lateral mesoderm, which then differentiates into extraembryonic mesoderm and lateral plate mesoderm. Both can give rise to vascular endothelial cells. Studies have strongly suggested that some progeny of the lateral plate mesoderm can give rise to hematopoietic cells.² Hence the two mesoderms are nearly the same with respect to differentiation potential, although cells that migrating to the extraembryonic region differentiate most rapidly into mature erythrocytes and vascular endothelial cells through a stage called the *hemangioblast*.³ Because our purpose was to obtain a culture system that generated only hematopoietic cells and vascular endothelial cells, the issue was whether intermediate cells in this differentiation pathway can be differentiated from other cell lineages and collected at high purity for further differentiation culture.

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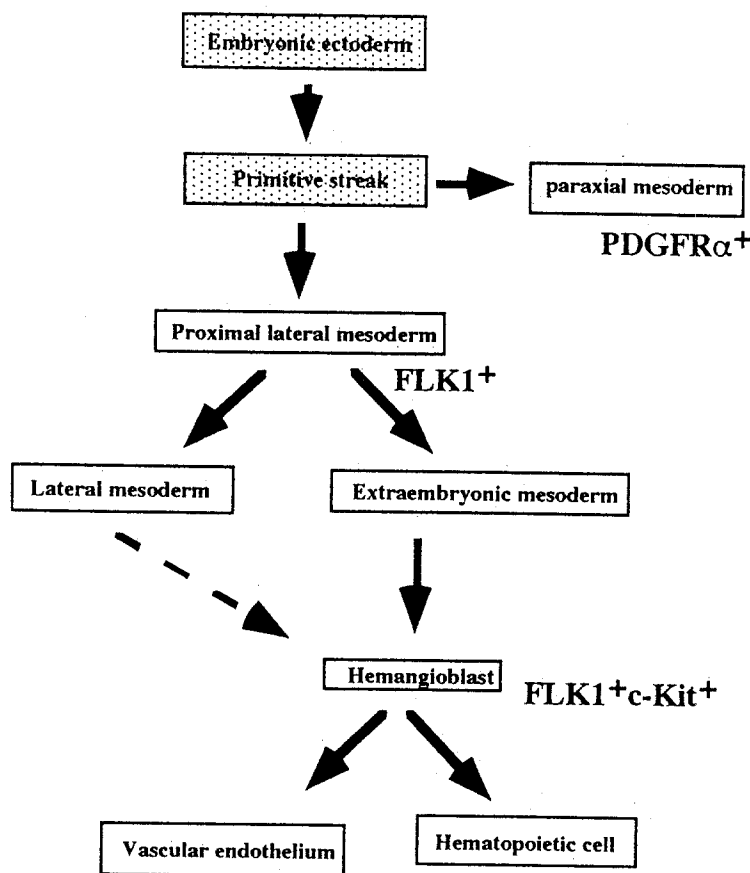


FIG. 1. Differentiation pathway of vascular and hematopoietic cell lineages from totipotent embryonic ectoderm. The totipotent stages are indicated by shaded boxes.

RECEPTOR TYROSINE KINASES OF THE PDGF RECEPTOR FAMILY ARE SUITABLE SURFACE MARKERS TO ISOLATE EARLY MESODERMAL CELLS

In hematopoietic and vascular endothelial cell differentiation, the proximal lateral mesoderm is the first intermediate stage after the embryonic ectoderm. Although various molecules have been shown to be specifically expressed in this mesoderm, most of them are intracellular molecules and therefore difficult to stain in living cells. When we searched for information on mesoderm-specific molecules that are potentially expressed on the cell surface, we found reports suggesting that receptor tyrosine kinases of the platelet-derived growth factor receptor (PDGFR) family (RTKP) are specifically expressed by mesodermal cells. To confirm the results of those studies, we established monoclonal antibodies (mAb) to PDGFR α , c-Kit, and Flk1 and tested whether these mAb can stain mesodermal cells specifically. Although c-Kit was expressed by various cells other than mesodermal cells, PDGFR α and Flk1 were specifically expressed by mesodermal cells of the gastrulating embryo. More interesting was that whole-mount immunohistochemical examination of embryos demonstrated that PDGFR α and Flk1 were expressed by distinct sets

of mesodermal cells that corresponded to the paraxial and proximal lateral mesoderm, respectively (Fig. 1). No RTKP-positive cells were present in the primitive streak of the embryos. This finding indicated that only mesodermal cells exfoliated from the primitive streak were specified by these mAb.

It is important to note that our mAb recognize the extracellular domain of RTKP, so they can be used for marking and sorting live cells that express these markers. Indeed, staining of live cells dissociated from the gastrulating embryo demonstrated that distinct cell populations expressed these RTK in varying combinations.

OTHER MOLECULAR MARKERS THAT DEFINE EARLIER OR LATER STAGES OF DIFFERENTIATION

Because the mesoderm is an early intermediate stage of differentiation toward fully specified cells, it is important to differentiate it from earlier and later stages with other surface markers. For this purpose, we used E-cadherin for the earlier stages, V-cadherin for vascular endothelial cells, and CD45 and Ter119 for hematopoietic cells. E-cadherin was first identified by Takeichi's group at Kyoto University⁴ and is expressed from the eight-cell embryo to the embryonic ectoderm but not in

the mesoderm. On the other hand, V-cadherin is expressed by mature vascular endothelial cells. CD45, the common leukocyte antigen, has been used as a specific marker of the hematopoietic cell lineage, except for erythroid cells. By staining gastrulating embryos, we confirmed that these surface markers can be used to define specific populations of vascular endothelial cells and hematopoietic cells.

CULTURE TO INDUCE MESODERMAL SUBSET FROM EMBRYONIC STEM CELLS

Two methods have been used to induce angiohematopoietic cell differentiation of embryonic stem cells in vitro—embryoid body culture and stromal cell-dependent culture.⁵⁻⁷ Removal of LIF is essential for inducing embryonic stem cell differentiation with both methods. In the first method, embryonic stem cells are allowed to form a spherical epithelial body called an *embryoid body*, in which many other cell lineages, including hematopoietic cells, are generated spontaneously. Because this process appears to follow the pattern of early embryogenesis, that is, formation of an egg cylinder and subsequent generation of mesoderm, this method has gained popularity. However, because all differentiation processes occur spontaneously inside the epithelial sphere, differentiation is difficult to observe or manipulate.

The other method is to use stromal cell lines to provide an appropriate microenvironment for differentiation of embryonic stem cells into hematopoietic cells. A study by Nakano et al. demonstrated that a stromal cell line, OP9, provides a microenvironment that induces various hematopoietic cell lineages.⁷ However, the stromal cell-based microenvironment is different from that in the embryo, so the culture itself is selective for certain cell lineages.

Nevertheless, any available culture conditions are selective, because we are unable to reproduce embryogenesis in vitro. Therefore, the extent to which these in vitro microenvironments, either embryoid body or stromal cell-dependent culture, can reproduce the actual processes within the embryo should be evaluated with a set of markers for each intermediate stage. We used the surface markers described in the preceding section to determine which of the intermediate stages can be detected during embryonic stem cell differentiation.

We also attempted to establish culture conditions in which embryonic stem cells differentiate into various mesodermal cells. After a number of preliminary experiments, we eventually used type IV collagen as the matrix. The other culture conditions were the same as those reported by Nakano et al.⁷ As depicted in Fig. 1, both PDGFR α ⁺ and Flk1⁺ cells representing nascent mesoderm were detected in this culture as early as day 3 after removal of LIF. These cell populations have never been detected in embryonic stem cell cultures with LIF. Almost no V-cadherin⁺, CD45⁺, or Ter119⁺ cells were detected under our culture conditions, even after incu-

bation for up to 6 days. This indicated that our culture conditions were permissive for mesodermal cells but not for more differentiated cells.

We sorted each subset of cells and recultured them on a stromal cell clone OP9 that has been shown to support embryonic stem cell differentiation into hematopoietic cells. As would be expected from the known processes of embryonic development, only Flk1⁺ cells were able to give rise to vascular endothelial cells and hematopoietic cells. Of note is that V-cadherin⁺ cells were generated as early as 1 day after the reculture of Flk1⁺V-cadherin cells. Likewise, a substantial number of CD45⁺ and Ter119⁺ cells were detectable on day 2 of reculture. Thus all of the necessary molecules for angiohematopoietic differentiation are expressed on OP9 cells. Identification of such molecules is an important issue for the future.

In addition to mature vascular endothelial cells and hematopoietic cells, a number of intermediate stages from the proximal-lateral mesoderm were identified. These included Flk1⁺c-Kit⁺V-cadherin⁻ CD45⁺ and Flk1⁺c-Kit⁻dull V-cadherin⁺ CD45⁻ cells that corresponded to cells committed to the hematopoietic and vascular endothelial cell lineages, respectively. Thus we were able to select the intermediate cells that give rise to angiohematopoietic cell lineages and to produce them specifically in vitro.

PROSPECTS

We describe an outline of our approach to regulating in vitro differentiation of hematopoietic and vascular endothelial cells. The markers described herein might not be sufficient for defining all the steps in the differentiation pathway, but the key steps are differentiated. Moreover, the culture system described is highly efficient, so much so that it is possible to expand clonogenic cells at various intermediate stages. With further progress, we may be able to control the differentiation of hematopoietic cells in such a way that all immature mesodermal cells are driven to select a given differentiation pathway such as that for erythrocytes.

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