Mini Review

Guiding ES cell differentiation into the definitive endoderm lineages

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The generation of specific lineages of the definitive endoderm from embryonic stem (ES) cells is an important issue in developmental biology, as well as in regenerative medicine. We have established a procedure, in which M15 cells, derived from a mesonephros, are used as supporting cell line for ES cell differentiation. Under selective culture conditions, by addition of Activin and bFGF, definitive endoderm are generated from the ES cells at a high efficiency. Then, under the presence of Activin and bFGF, the differentiation into pancreatic fates is further promoted, upon the expense of the hepatic fates. On the other hand, removal of Activin, following by the addition of HGF and Dexamethasone potentiated the differentiation of ES cells into hepatic lineages. Further maturation of the pancreatic precursor could be achieved by *in vivo* differentiation of the differentiated ES cells. Engraftment of the cells under kidney capsule further permit the differentiation of ES cells into mature cell types containing the three pancreatic cell lineages.

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Introduction

The ES cells are pluripotent cell lines derived from mouse blastocysts. In mouse, ES cells are proven to contribute to all adults tissues including germ cells, when introduced into host blastocyst[20]. Studies of the differentiation of ES cells to neural, hematopoietic and cardiac tissue revealed that ES cells in many aspects recapitulate that was known *in vivo* during normal development processes. Mimicking the signaling events in the mouse embryo, such as addition of growth factors in culture or over expression of transcription factors known to be involved in specific lineage decisions during normal development, can be used to direct ES cells to a certain lineage *in vitro*[30,31,33].

Studies in other animal models demonstrate that the definitive endoderm and mesoderm arise from a common bipotent progenitor, the mesendoderm. The differentiation of the definitive endoderm involves a fate selection process at which the divergence of an endoderm or a mesoderm fate occurs. Information on the genes that influence the formation of definitive endoderm

in the mouse comes largely from the analysis of gene-manipulated embryos[18]. However, the destruction of genes that function in the formation of mesoderm or endoderm often results in defective gastrulation and failure in the formation of endoderm and mesoderm[5]. Use of ES cells should allow *in vitro* dissection of these processes.

Previously, several reports claimed the generation of insulin-producing cells from ES cells via the differentiation of progenitors that express nestin[10,19]. Other researchers reported that this insulin staining resulted from an insulin uptake from the medium after using the same procedure to generate nestin expressing cells[22]. This highlights the importance of generating pancreatic cells from ES cells by following normal developmental processes. Several groups reported *in vitro* differentiation of mouse ES cells into mesendoderm[27], definitive endoderm[8,17,32] or foregut endoderm[9]. Other groups claimed the generation of definitive endoderm[6], Pdx1-positive foregut endoderm[13] or Insulin-expressing cells[7,12] from human ES cells. Although these above reports have shown the *in vitro* generation of definitive endoderm cells and insulin-producing cells from mouse and human ES cells, the molecular mechanisms for the inductive process remains largely unknown.

Differentiation of ES cells into Definitive Endoderm and Pancreatic cells

Pdx1 (*Pancreatic and duodenal homeobox gene 1*) is a regional endoderm marker whose expression marks the dorsal and ventral pancreatic buds, as well as a part of the stomach and duodenal endoderm[21]. Using an ES cell line with the lacZ reporter gene knocked into the *Pdx1* locus[21], we have found that ES cells are induced to a *Pdx1* expressing gut endodermal fate when co-cultivated with pancreatic rudiments or pancreatic mesenchyme[24]. However, the requirement for embryonic materials allows a limited production and analysis of the ES cell-derived differentiated cells. Given that the embryonic endoderm requires signals from the adjacent germ layers for subsequent regionalization into specific endoderm organs[29], a cultured cell line derived from the mesoderm could substitute for the embryonic materials and support the differentiation of the ES cells into pancreatic tissue.

Using an ES cell line bearing the *Pdx1* promoter driving GFP reporter gene, we found that M15, a mesonephros derived cell line, turned out to be an excellent endoderm inductive source. Culturing ES cells on a monolayer of M15 cells supports the ES cell differentiation into the definitive endoderm. It seems that when M15 are used as an inductive source, ES cells are induced into endoderm cells, which not only be able to differentiate into the pancreatic but also other definitive endoderm lineages, including the lung, stomach, liver and intestine.

The differentiation of ES cells to *Pdx1*/GFP-expressing cells is a multistep process. ES cells are first differentiated into mesendoderm, or into ectoderm (early phase). Then, the bipotential mesendoderm differentiate into mesoderm or definitive endoderm (middle phase). The definitive endoderm then turned into regional specific tissue of the endoderm (late phase). On day 8 of differentiation on M15, ES cells with GFP-expressing cells are observed to give rise in the edge of the colonies. The molecular bases of the signaling event involved in each step of the processes are summarized in Figure 1. Stimulation of undifferentiated ES cells by fibroblast growth factor (FGF) (a part of the ERK signaling cascade) triggers a transition from self renewal to lineage commitment. Activin and/or p38 MAPK then induce divergence into the mesendodermal lineage, and the inhibition of these signals directs differentiation into the neuroectodermal lineages. The differentiation of mesendoderm into definitive endodermal or mesodermal lineages, are promoted by the addition of Activin or bone morphogenetic protein (BMP), respectively. Then, Activin and bFGF, which are known as notochordal signals, maintain *Pdx1*/GFP expression in the endoderm, which in turn potentiate pancreatic differentiation. Each individual process is potentiated by either Activin or FGF, or both. Retinoic acid acts as an ectoderm inducer in early phase, but as a pancreatic inducer at late phase.

The growth factors mediated differentiation into the definitive endoderm turned out to be active without close contacting of the ES cells with M15 cells. However, the late phase, namely the regional differentiation into cells of various digestive organs, required a close contact of the ES cells with M15[26]. This was shown by a trans-filter assay. When ES cells and M15 were grown separately in filters, separately in upper or lower chambers, ES cells differentiated into definitive endoderm but not further not into *Pdx1*/GFP cells. These results suggest that differentiation of ES cells into definitive endoderm is mediated solely by soluble molecules. However, factors secreted by M15 are not sufficient to direct ES cells to differentiate into gut regional-specific endoderm lineages. Short range signals or intercellular interactions with supporting cells are required for the induction of *Pdx1* expression.

Since Activin and bFGF promote ES cell differentiation at all phases of induction, Activin and/or bFGF were added throughout the entire processes of ES differentiation in our M15 systems, namely days 0-8, to obtain a maximum yield of Pdx1-expressing cells. The simultaneous treatment of Activin and bFGF

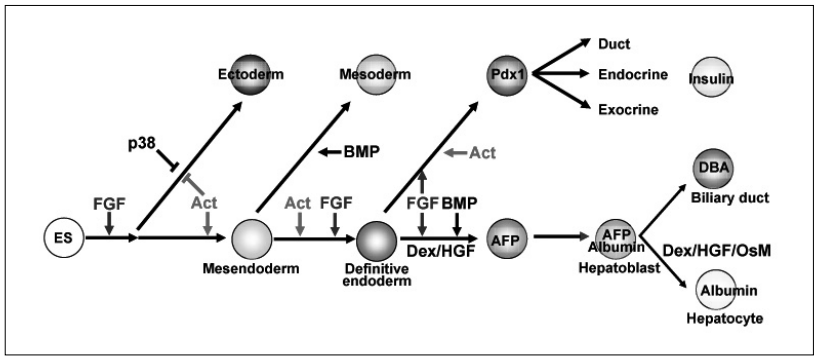

Fig.1 A schematic drawing of various lineages derived from ES cells upon differentiation into cells of the three germ layers and further into pancreatic and hepatic lineages. Growth factors or signal pathways, which potentiate or inhibit differentiation are shown.

resulted in an increase in both the number and extent of expanded ES colonies and 16-fold increase in the proportion of *Pdx1*/GFP+ cells within the total differentiated ES cells was observed (2% to 31%). When M15 cells fixed with paraformaldehyde were used for differentiation, a weaker but significant activity in supporting definitive endoderm differentiation and *Pdx1*/GFP+ cell formation from ES cells was observed. These results demonstrated that either cell-cell or cell-matrix interaction is important for pancreatic differentiation and that the effect of Activin and bFGF on ES cells is direct, rather than via indirect effects on the M15.

To test the differentiation potential, ES cells were grafted under the kidney capsule of *scid* (severe combined immunodeficiency) mice. Two weeks after transplantation, *Pdx1*/GFP+ grafts showed strong GFP fluorescent signals and teratomas were not observed. RT-PCR and immunocytochemistry analysis with the grafts of *Pdx1*/GFP+ cells revealed that the endocrine lineage markers, such as *Neurogenin3*, *Insulin1*, *Glucagon*, and *Pancreatic polypeptide*, as well as the exocrine marker *Amylase*, duct marker *Cytokeratin* 19, along with expression of other β cell markers, such as *Islet amyloid polypeptid*, *Kir6.2*, and *Glut2*, increased after transplantation. Immunohistochemical analysis revealed that C-peptide was detected in the *Pdx1*/GFP-positive cell after transplantation. Taken together, ES cell-derived *Pdx1*/GFP+ cells obtained under our present *in vitro* differentiation system have the potential to differentiate into all pancreatic lineages, namely endocrine, exocrine, and duct cells.

Hepatic differentiation of ES cells

The above procedure, using M15 cells supports differentiation into definitive endoderm, and upon addition of Activin and bFGF, the pancreatic differentiation was further potentiated. However, this pancreatic differentiation was at the expense of the hepatic differentiation, the withdrawal of activin and bFGF induced *Afp* expression[25]. To find an optimal condition in which ES cells dominantly adopt a hepatic fate instead of a pancreatic fate, the effects of Hepatocyte growth factor (HGF), oncostatin M (OsM) or dexamethasone (Dex) were studied, and it was revealed that the effects of promoting AFP expression are in an order of Dex > HGF > OsM. Addition of Dex and HGF was enough to result in a maximum potentiation of ES cell differentiation into AFP-expressing cells. OsM potentiates the accumulation of Albumin protein at later stages.

Since it is reported that BMP and FGF signaling are involved in hepatic differentiation[14,23], their effects on definitive endoderm cells were tested on the M15 procedure. Addition of bFGF increased AFP-positive cells, whereas addition of SU5402, a specific inhibitor of FGF receptor I, decreased AFP-positive cells. Noggin addition abolished most of the AFP-expressing cells, which was recovered by bFGF addition. These results show that BMP signaling is required in the induction of hepatocyte differentiation, whereas FGF signaling potentiates BMP signal.

The time course of the appearance of hepatic lineages were at the following orders: AFP single positive cells, immature hepatic precursors (AFP+/Albumin-), first appeared, then hepatoblast (AFP+/Albumin+) appeared. The hepatoblasts are bipotential progenitors capable to differentiate into hepatocyte and bile duct lineages. ICC analysis of the d30 differentiated ES cells, which followed the above M15 procedure, showed that cells of the hepatocyte lineage and bile duct lineage segregated and formed distinct colonies of Albumin+ or *Dolichos biflorus agglutinin (DBA)* +[25].

Functional characterization of the ES cell-derived hepatic cells revealed that in ES cell-derived day 30 differentiated cells, *Afp* transcripts decreased, and transcripts of mature hepatocyte markers, such as *Alb1*, *Keratin 7 (Cytokeratin 7)*, *Keratin 19 (Cytokeratin 19)*, or mature markers of cytochrome P450 enzyme *Cyp7a1*, *Cyp2b10*, *Cyp3a11*, *Cyp3a13*, *hydroxysteroid sulfotransferase (Sult2a1)*, *UDPglucuronosyltransferase (Ugt1a1)*,

organic anion transporting polypeptides (Slco1a4) or *bile salt export pump (Abcb11)*[4,28], increased to a substantial level. Most of the expression levels of the markers were similar to those of the fetal liver. *Ugt1a1*, *Cyp7a1*, *Cyp2b10*, *Cyp3a13* and *Slco1a4* expressions were higher than those of fetal liver, but lower than those of adult liver. Cyp3A4 is known to be involved in the metabolism of more than half of all currently used drugs. Several well-documented cases exhibiting clinically important drug-drug interactions and toxicities are implicated to be related to Cyp3A4 inhibition. In the case of the treatment of both drug-A which is substrate of Cyp3A4 and drug-B which has potential to inhibit Cyp3A4 activity, the blood concentration of drug-A is increased and sometimes cause toxicity. *Cyp3a11* and *Cyp3a13*, two important Cyp3A isoforms identified in the mouse, are expressed in differentiated ES cells. Functional assays for Cytochrome P450 3A activity of ES cell-derived hepatic cells at day 60 of differentiation, exhibited metabolic activities approximately one-fifth of that of adult liver.

KhES-1 human ES cells when grown on M15 cells exhibited similar behaviors with those of the mouse ES cells. Endoderm or hepatic markers, such *Sox17*, *Afp*, *Alb*, *Cytokeratin 7 (CK7)*, *Cytokeratin 18 (CK18)*, *Cytokeratin 19 (CK19)* and *Cyp7a1* were detected in differentiated ES cells on day 18. The mature hepatocyte markers such as *Albumin (Alb)*, *Cyp3a4*, liverspecific *organic anion transporting polypeptides 1B1 (Oatp1b1)*[11,15] were detected on day 18 and increased to a substantial level on day 50. Most cells on day 24 and day 50 were PAS-positive, demonstrating glycogen deposition. Ultra-structures evaluated by electron microscopy of the differentiated human ES cells at day 50 exhibited features of typical normal hepatocyte ultra-structure with active protein synthesis, some of the cells formed biliary canaliculi with microvilli, rich in glycogen granules and showed characteristic epithelial like structure with polarization.

Conclusion & Future perspectives

In conclusion, the use of M15 as supporting cells for the differentiation of ES cells are a powerful tool for regulating ES cell differentiation, especially into endodermal fate, such as pancreas or hepatic lineages. The differentiation process recapitulates normal development, thus helps understanding the molecular mechanism of the processes of induced differentiation of cells of the endoderm lineages. The applicability of this method to human ES cells would yield the potential tools for drug discovery or regenerative medicine. In future, following three points were keys to facilitate the stem cell research.

1) *In vitro* differentiation of lineage specific progenitors

Recently Melton's groups reported the large scale screening of small molecules, which promote ES cell differentiation into definitive endoderm[2] or Pdx1 positive pancreatic progenitor cells[3]. Such small molecules are useful for their potential application for the regenerative medicine or *in vitro* differentiation studies, or findings of reagents, which regulate gut regionalization into lung, liver, pancreas or intestine, and promote further differentiation to express functional mature pancreatic genes, insulin, glucagon or amylase.

2) *In vivo* differentiation

In previous studies, human ES cells generated *in vitro* showed insulin secretion in response to various regents but not to glucose *in vitro*[7]. When these cells were grafted under mouse fat pad, the insulin-expressing cells generated after three months engraftment exhibit many properties of functional beta-cells[16]. From these results, *in vivo* differentiation is anticipated to provide suitable environment for further maturation of ES cells derived cells.

3) Prospective isolation

To proceed *in vivo* differentiation studies and application for regenerative medicine, purification of ES cell-derived differentiated cells is necessary. Recently, it was reported that human ES cell derived hepatocyte-like cells can be enriched and recovered based on their asialoglycoprotein-receptor expression[1]. Many groups have attempted the identification of novel cell surface molecules for their potential use to prospectively isolate hepatic or pancreatic precursor cells using flow cytometry.

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