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Abstract

Hepatic and pancreatic differentiation from ES cells is of great interest for the impact that this knowledge could have on the treatment of hepatic and diabetic patients. The liver and pancreas initially develop by budding from the embryonic endoderm. Thus, the development of the endoderm represents an important step and has an integral common role in initiating the early stages of pancreatic and liver development. We know that the development of hepatocytes and insulin-producing pancreatic beta-cells from ES cells represents the culmination of a complex developmental program. However, there has been recent progress in directing ES cells to endoderm and early-stage hepatic and pancreatic progenitor cells. We here discuss the role of the microenvironment, transcriptional factors and cytokines, which have been recognized as important molecules during the major steps of the development of the liver and pancreas. We also present the most recent advances and efforts taken to produce definitive endoderm-committed ES cells for the further differentiation of hepatocyte-like and insulinproducing cells. Recent progress in the search for new sources of hepatocytes and beta-cells has opened up several possibilities for the future of new perspectives for future of new prophylactic and therapeutic possibilities for liver diseases and diabetes.

KEYWORDS: embryonic stem cells (ES cells), diff erentiation, hepatocyte like-cells, insulin-producing cells, defi nitive endoderm

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Review

Endoderm Induction for Hepatic and Pancreatic Differentiation of ES Cells

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Hepatic and pancreatic differentiation from ES cells is of great interest for the impact that this knowledge could have on the treatment of hepatic and diabetic patients. The liver and pancreas initially develop by budding from the embryonic endoderm. Thus, the development of the endoderm represents an important step and has an integral common role in initiating the early stages of pancreatic and liver development. We know that the development of hepatocytes and insulin-producing pancreatic β-cells from ES cells represents the culmination of a complex developmental program. However, there has been recent progress in directing ES cells to endoderm and early-stage hepatic and pancreatic progenitor cells. We here discuss the role of the microenvironment, transcriptional factors and cytokines, which have been recognized as important molecules during the major steps of the development of the liver and pancreas. We also present the most recent advances and efforts taken to produce definitive endoderm-committed ES cells for the further differentiation of hepatocyte-like and insulin-producing cells. Recent progress in the search for new sources of hepatocytes and β-cells has opened up several possibilities for the future of new perspectives for future of new prophylactic and therapeutic possibilities for liver diseases and diabetes.

Key words: embryonic stem cells (ES cells), differentiation, hepatocyte like-cells, insulin-producing cells, definitive endoderm

Certain kinds of liver failure can motivate a lethal condition requiring treatment by liver transplantation or alternatively hepatocyte transplantation [1]. The success of islet transplantation, in between the laboratory and the clinic, has proven that cell therapy can cure diabetes [2]. However, given the current global donor shortage and the need for several infusions, the use of hepatocyte and islet transplantation has been seriously restricted.

Facing an increasing worldwide population of hepatic and diabetic patients whose care requires extensive economic and health care resources, several candidate cell types are being explored as sources for

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generating unlimited amounts of hepatocytes and insulin-producing cells for transplantation. Among them, human embryonic stem cells are the most attractive, due to their pluripotent nature and their suitability for cell-replacement therapy [3]. Thus, an exact understanding of the developmental processes that lead to a specific cell fate might help us to recapitulate the events *in vitro* and engineer artificial cells and tissues to combat liver diseases and diabetes. Important progress has been reported in inducing ES cells to the endoderm stage, a common developmental stage for liver and pancreas cells. The definitive endoderm gives rise to the major cell types of the digestive tract and associated organs, including the liver and pancreas [4]. This short review focuses on the major steps of endoderm development, which may contribute to a better understanding of the main factors involved in the hepatic and β-cell differentiation process. Moreover, we discuss the role of the major transcriptional factors, driving the hepatic and pancreatic development. Finally, we discuss recent efforts to produce hepatocytes and β-cells suitable for transplantation.

Endoderm Formation and Induction

Heterotopic transplantation studies have demonstrated that by mid-to-late gastrulation, cells are determined to give rise to the endoderm [5]. Several early endodermal transcription factors, including orthodenticle homologue (Otx2), homeobox expressed in ES cells 1 (Hesx1), homeobox (Hex), and caudal-related homeobox 2 (Cdx2), are regionally expressed prior to the time that organ specific genes are activated [6]. Then, within the PS, the mesendoderm cells regulate the expression of several genes, such as goosecoid (GSC) forkhead box A2, (Foxa 2), chemokine C-X-C motif receptor 4 cxcr4, sex determining region-Y box 17 (Sox17a/b), Brachyury, E-cadherin, vascular endothelial growth factor receptor-2, (VEGFR2), VE-cadherin, platelet-derived growth factor receptor-a (PDGFRa), and GATA-binding protein 4, (GATA-4) for the cell-fate differentiation of the definitive endoderm and mesoderm progenitors (see Fig. 1) [4–6]. Extraembryonic endoderm cells share the expression of many genes with the definitive endoderm, including the often-analyzed transcription factors Sox17, FoxA1 and FoxA2 [7]. The common transcriptional machinery in the definitive and visceral endoderm implies a similarity in the mechanism of specification of the 2 tissues.

Thus, it is tempting to consider that common signaling events induce Sox17 and the FoxA genes [8]. However a recent work suggested that 2 conditions are required to induce approximately 70%–80% of definitive endoderm from human ES cells: signaling by Activin/Nodal family members and release from inhibitory signals generated by PI3K through insulin/IGF [9, 10].

From Hepatic Specification to the Mature Hepatic Phenotype

Growth factor signaling from the cardiac mesoderm and septum transversum mesenchyme specifies the underlying endoderm to adopt a hepatic fate [11]. The growth factors identified were fibroblast growth factos (FGFs) and bone morphogenic proteins (BMPs). Using a tissue explants assay, it was demonstrated that FGFs (acidic or basic) could be substituted for the cardiac mesoderm in inducing the ventral endoderm to elicit a hepatogenic response (see Fig. 2) [11, 12]. Cocultures of chick cardiac mesoderm

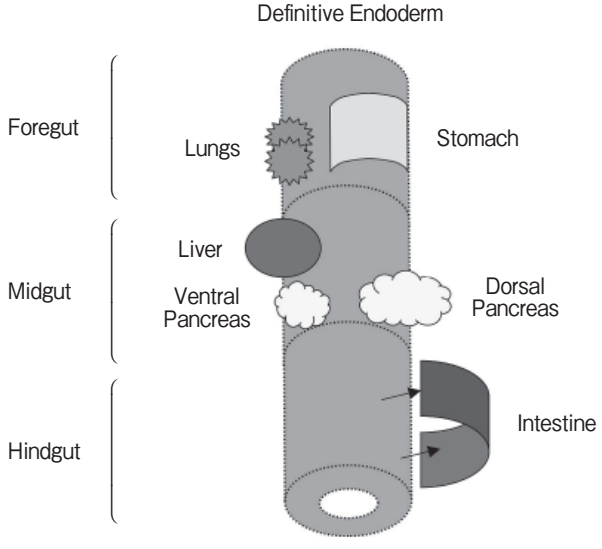

Fig. 1 Representation of definitive endoderm and its derivatives. The figure shows how the definitive endoderm is responsible for deriving the entire gastrointestinal tract and lungs; in particular, the portion in the midgut is capable of generating hepatic and pancreatic tissue.

were shown recently to induce hepatic differentiation in mouse ES cells. Recently, some reports have proved the importance of FGFs and BMPs in mouse ES cells differentiation toward a hepatic phenotype. Furthermore, interactions with endothelial cells, a mesodermal derivative in this inductive sequence, are crucial for this early budding phase in hepatic induction [13].

In the endoderm, the onset of Foxa gene expression precedes the induction of the hepatic program by FGF signals. Furthermore, Foxa proteins are able to displace nucleosomes present in the regulatory region of the albumin gene before the gene becomes activated, but other transcription factors that bind to this region are unable to do so [14]. Foxa2 binding can reverse chromatin-mediated repression of alpha-fetoprotein (Afp) gene transcription *in vitro* [14, 15].

Hepatocyte growth factor (HGF) is critical to the signaling pathway that controls the proliferation of fetal liver cells [16]. Genetic studies in mouse embryos showed that the proliferation and outgrowth of the liver bud cells require the interaction of HGF [16]. Hematopoiesis plays an important role in hepatic maturation. After the liver bud emerges from the gut tube, hematopoietic cells migrate there and propagate. The hematopoietic cells secrete oncostatin M (OSM), a growth factor belonging to the interleukin-6 (IL-6) family [17]. OSM stimulates the expression of hepatic differentiation markers and induces morphologic changes and multiple liver-specific functions such as ammonia clearance, lipid synthesis, glycogen synthesis, detoxification, and cell adhesion [18]. Also glucocorticoids have been shown to be involved in hepatic maturation and were found to modulate the proliferation and function of adult hepatocytes. In the fetal liver, physiological concentrations of dexamethasone (Dex), a synthetic glucocorticoid, suppress AFP production and DNA synthesis and up-regulate albumin production [19].

From the Primitive Pancreas to the Mature Endocrine Islet Phenotype

The endoderm can give rise to all pancreatic tissues, as demonstrated by tissue culture and *in vivo* transplantation [20]. To get to the mature hormone-producing endocrine phenotype, the primitive gut has to go through a few crucial steps: patterning of the

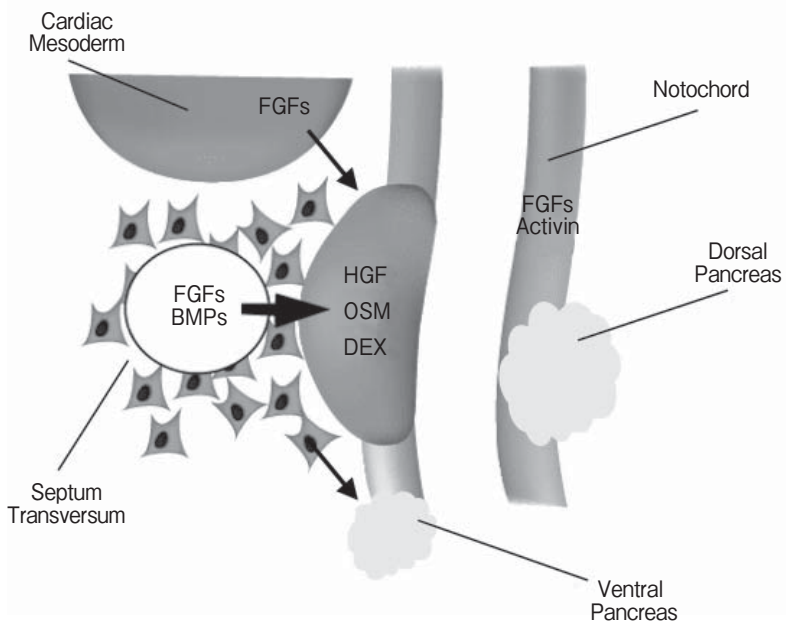

Fig. 2 Liver and pancreas specific derivation.
The figure shows extracellular signals from neighboring tissues, which regulate the tissue- and cell type-specific differentiation.

endoderm, inhibition of gut formation by hh suppression, mesenchyme conditioning and finally epithelial expression of key transcription factors [21]. The pancreas follows a profile of cytodifferentiation in three phases depending on the amount of enzymes and hormones secreted, with 2 main transitions: a primary regulatory transition, defined by conversion of pre-differentiated cells to a proto-differentiated state where pancreas specific proteins are present, and a secondary regulatory transition, with the conversion of proto-differentiated tissue to differentiated cells with full protein synthesis and no proliferative capacity [22].

With some differences, the development of the ventral and dorsal buds follows the same differentiation pathway to achieve the pancreatic phenotype: repression of the hh genes and expression of critical homeobox gene products such us pancreas-duodenum homeobox 1 (pdx-1) and the homeobox transcription factor hlxb9. In the dorsal pancreas, a series of notochord-derived factors, such as activin-βB and fibroblast growth factor (FGF) have been reported to participate in this process (see Fig. 2). On the other hand, development of the ventral pancreas seems to be a notochord-independent procedure, which endodermal transcription factors such us the *Hex* homeobox gene controls indirectly by maintaining the proliferation rate and consequently the positioning of ventral foregut endoderm cells relative to the mesoderm (see Fig. 2) [23].

As endocrine cells emerge from the epithelium and migrate into the mesenchyme, the lack of Notch signalling results in high levels of the bHLH transcription factor neurogenin3 (Ngn3), promoting the endocrine fate [24]. Further differentiation is achieved by a multipotent pancreatic progenitor coexpressing Pdx1, Hlxb9, Nkx6–1, Nkx2–2, Nkx6–2, and Sox9 [25–27], by the primary regulatory transition. The surrounding epithelium then gives rise to the committed cell types by the expression of several specific transcription factors, among others, Isl1, Pax4, Pax 6, and NeuroD/BETA2 [28–32] and others, depending on their specific endocrine lineage, namely α (glucagon), β (insulin), PP or δ (pancreatic polypeptide) and ε cells (ghrelin).

Current Status of Hepatocyte-like Cell Differentiation from Human ES Cells

Several approaches have been used to differentiate and to obtain enriched populations, and human hepatic-like cells have been isolated and characterized for their phenotypes. One study used gene manipulation to select the cells through an albumin promoter. However, the cells expressing a hepatic phenotype were isolated from EBs; thus few cells were produced, and the functionality of the cells was not tested [33]. In one of the few reports on human ES cells, combined treatment with insulin, DEX and collagen type I followed by sodium butyrate, led to increased numbers of mature hepatic gene-expressing cells (10–15%) [34]. The lack of success of these early attempts at differentiating human ES cells into functional hepatocytes has focused attention on the fundamentals of normal embryonic development, knowledge of which is essential to better understand the early stages of definitive endoderm formation. A recent important contribution is a protocol in which the use of activin A in combination with serum-free conditions, resulted in enrichment to definitive endoderm cells (up to 80%) by human ES cells [9]. Using a modification of this protocol and a combination of protocols previously reported using mouse ES cells, Cai *et al.* reported that the addition of FGF, BMP, and HGF can induce hepatic fate, and that the later addition of OSM and Dex to the cell culture induced even more differentiated hepatocyte-like cells in a total time of 18 days [35]. We recently combined the techniques of various efforts to generate functional hepatocytes from mouse ES cells. The differentiation protocol was simple, used defined reagents and yielded to date the most efficiently differentiated hepatocyte-like cells. Starting with a suspension culture system, where early endodermal development is initiated, ES cells were subsequently transferred to plates and cultured in the presence of fibroblast growth factor-2 and activin A. The predifferentiated cells were then further developed toward hepatocytes in a defined coculture together with human nonparenchymal liver cells (endothelial cell line, cholangiocyte cell line and stellate cell line) under the influence of the hepatocyte growth factor, dimethyl sulfoxide, and dexamethasone. An improvement of hepatic maturation was observed when a coculture with liver nonparenchymal

cell lines was applied. Several cytokines and growth factors important for liver regeneration and development were identified in the conditioned medium of the cell lines [36, 37].

Current Status of β-cell Differentiation from Human ES Cells

Another possibility for specifying stem/progenitor cells is to use the appropriate sequence and combination of a permeable peptide, the protein transduction domain from HIV-TAT fused with specific transcription factors. Transduction of PDX-1, BETA 2/NeuroD and TAT-Ngn3 has been able to enhance insulin gene transcription and facilitate differentiation toward the β-cell lineage [38]. With the help of transgenic mice expressing a tamoxifen-inducible form of Ngn3, Grapin-Botton and colleagues have shown that endocrine progenitors change competence over time within an epithelium-intrinsic mechanism, demonstrating that pancreas endocrine progenitors are committed to generate different endocrine cell types at different stages [39]. To date the exact role for the mesenchyme in coordinating progenitor cell proliferation and differentiation is incompletely understood. It has been previously shown that FGF10 is produced by embryonic pancreatic mesenchymal cells and is required for the proliferation of early pancreatic progenitors [40]. However, additional factors generated by the mesenchyme should be investigated, as FGF10 does not provide complete growth when compared with mesenchyme.

In diabetes mellitus, even if β-cells are the main cell type affected, there is a general endocrine islet dysfunction, which results in inefficient blood glucose homeostasis. The ultimate goal for cell therapy in diabetes would be to restore euglycemia. This raises the question whether insulin-producing stem cells would be sufficient. In an effort to mimic the normal pancreatic development, D'Amour *et al.* recently published a protocol to generate hormone-secreting islet-like clusters [41]. Although immature in respect to the clusters' secretory capacity, their approach is the first to succeed in generating a hormone-secreting cluster, however it must be further improved to produce therapeutic β-cells. Furthermore, another protocol generating the sametype of islet clusters has been recently reported, where insulin-producing cells secreted human c-peptide in a glucose- dependent manner [42].

Conclusions

Research to repopulate damaged livers and restore the β-cell deficiency of diabetes is being pursued aggressively. There is optimism about disparate strategies for generating supplies of hepatocytes and β-cells sufficient for transplantation in the near future. Exact understanding of the developmental processes that lead to a specific cell fate might help us to recapitulate the events *in vitro* and engineer artificial liver and β-cells to combat liver diseases and diabetes.

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