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Strategies for differentiating embryonic stem cells (ESC) into insulin-producing cells and development of non-invasive imaging techniques using bioluminescence

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Abstract Diabetes is a chronic autoimmune disease that affects 4–5% of the world's population. If the present trends continue, diabetes would soon become a major/leading health problem worldwide. Hence there is an urgent need to develop novel approaches for the treatment of diabetes. While transplantation of the pancreas or that of isolated pancreatic islets can lead to the cure of the disease in some patients, immunological complications and the chronic shortage of donors makes it impossible to adequately treat all patients. Interestingly, embryonic stem cells (ESC) have emerged as a possible source of pluripotent cells that can be coaxed into insulin-producing cells (IPCs) that can be used to treat diabetes. However, until appropriate protocols have been established, this new technology will be difficult to tap into. Our laboratory is interested in developing new strategies for harnessing the pluripotency of ESC and differentiating them into IPCs that are stable and will continue to produce insulin in vivo. A second aspect is the non-availability of non-invasive imaging protocols. We show here that transcriptionally targeted luciferase expression can be used successfully to non-invasively monitor the transplanted cells in vivo.

Keywords Embryonic stem cells · Diabetes · Insulin producing cells

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Introduction

Autoimmune destruction of pancreatic β cells leads to type 1 diabetes, a highly complex disease characterized by hyperglycemia, leading to an increased risk of cardiovascular disease [1]. Whole organ pancreas transplantation is the preferred treatment of diabetes that leads to sustained euglycemia and insulin independence in a vast majority of the patients [2]. Despite significant progress in the treatment of diabetes, pancreatic transplantation is associated with perioperative mortality and significant morbidity. More recently, treatment of diabetes has been significantly improved by the Edmonton protocol which combines several unique strategies and an improved immunosuppressive protocol [3]. Despite encouraging results, however, the major limitations of this innovative protocol include an inadequate supply of deceased donor-derived islets. Furthermore, since the donor islets are histoincompatible with the recipient, transplantation necessitates a life long immunosuppressive therapy with undesirable side effects including renal failure. These major limitations have led to the search for the development of innovative therapeutic modalities for the treatment of type 1 diabetes. One of the most promising approaches is based upon the generation of insulin-producing cells (IPCs) from embryonic stem cells (ESC). However, so far the results are still disappointing.

ESC which are derived from the inner cell mass of the early developing embryo are pluripotent and are able to undergo multi-lineage differentiation into highly specialized cells representing all three germinal layers [4–6]. A number of recent reports have claimed successful differentiation of ESC into IPCs [7–11]. A vast majority of these studies have derived the IPCs using the embryoid body formation route. However, the major limitations of these studies have been an inefficient differentiation and an inability to purify the ESC-derived IPCs. Further the insulin production by the ESC-derived IPCs has been suboptimal and consequently, transplantation studies using these ESC-derived IPCs in the diabetic mice have failed to correct hyperglycemia. Yet another potential drawback of these studies has been the lack of real-time non-invasive monitoring of the transplanted IPCs in vivo. Thus, it is necessary to develop novel protocols that closely mimic pancreatic β-cell development and their in vivo physiologic functioning, especially responses to glucose levels.

In order to maximize the differentiation potential our major research, efforts have been intensely focused upon understanding the molecular mechanisms underlying ESC differentiation into IPCs as well as to develop novel molecular imaging modalities. Toward achieving a greater understanding of how the ESC go through lineage commitment into IPCs, we have developed novel approaches for the differentiation of ESC into IPCs. Our first approach relies upon generation of the definitive endoderm from ESC which could then be further differentiated into (IPCs). Our second molecular approach is based upon differential transcriptional regulation of ESC-specific NANOG and pancreatic β-cell-specific rat insulin-promoters in ESC-derived cells undergoing differentiation into IPCs. Here, we provide a brief overview of the salient features, key research findings, and future research directions.

Developmental regulation of pancreatic β cells

During embryonic development, the pancreas develops from the ventral and dorsal parts of the foregut endoderm. The adult human pancreas is functionally composed of endocrine and exocrine cells. The endocrine functional unit, the islet of Langerhans, is composed of

five cell types: α-cells producing glucagons, β-cells secreting insulin, δ-cells producing somatostatin, ε-cells secreting ghrelin, and PP cells producing pancreatic polypeptide. Significant efforts have been directed toward understanding the transcriptional network regulating the development of the pancreatic islets [12, 13]. Initially, the transcription factors Hnf6, Hlxb9 and Foxa2 (Hnf3β) are expressed in the region of the foregut endoderm committed to the pancreatic fate [14–16]. During early pancreas development, signals from the notochord repress the expression of Sonic hedgehog, a negative regulator of pancreatic and duodenal homeobox gene 1 (Pdx-1) one of the earliest known and a key transcription factor in pancreas development [17–19]. Pdx-1 is known to play a central role in regulating the expression of several β-cell specific genes and is critically involved in pancreatic morphogenesis in mice and humans [20, 21]. Pdx-1 is expressed globally in the epithelium of early pancreatic buds and finally becomes restricted to β-cells, where it plays a pivotal role in insulin expression and glucose response [22]. Although, Pdx-1, expression may not be required for pancreatic determination of the endoderm, it is indeed crucial for the development of pancreatic endocrine and exocrine cells as it acts by stimulating proliferation, branching, and differentiation of the pancreatic epithelium [20, 22, 23]. Notch signaling has been shown to control multiple steps of pancreatic differentiation [24, 25]. Neurogenin3 (Ngn3) a member of the basic helix-loop-helix (bHLH) family of transcription factors is essential for further specification of the endocrine progenitors, since mice carrying a homozygous targeted disruption of Ngn3 gene lack any islet cells [26]. Ngn3 is required for the development of all endocrine lineages of the pancreas and is both necessary and sufficient to drive the differentiation of islet cells during pancreas development. Overexpression of Ngn3 in the developing pancreas results in accelerated differentiation of endocrine progenitor cells. An important regulator of the early cell-fate decisions in the islet is the homeodomain transcription factor Nkx2.2 which is expressed at the onset of pancreatic epithelium formation and later becomes restricted to mature α-, β- and PP cells [27]. Subsequently, an upregulation of Pdx1 and Nkx6.1 and activation of MafA directs the maturation of the β-cells [28–31]. The bHLH transcription factor Beta2/NeuroD is an important regulator of both insulin gene transcription in pancreatic β-cells. Mice homozygous for a targeted disruption of *beta2/neuroD* gene survive until birth but die within 3–5 days as a result of severe hyperglycemia [32, 33]. The members of the paired box homeoprotein family, Pax4 and Pax6, play an important role in the differentiation of the pancreatic endocrine cells [34, 35]. While Pax6 expression is detected throughout pancreas development and is present in all endocrine cells, Pax4 is required for the development of pancreatic β- and δ-cells. Mice lacking Pax4 fail to develop β-cells and are diabetic, in contrast to Pax6 mutant mice which display the lack of α-cells [34, 35].

Derivation of definitive endoderm from ESC

While significant strides have been made in differentiating ESC into neural, hematopoietic, and cardiac tissue, limited knowledge has precluded their differentiation into a definitive endodermal lineage. To be able to fully exploit the potential of ESC to generate IPCs, it is important to recapitulate the in vivo development of pancreas by the ESC in vitro.

The development of pancreas from the foregut follows three major steps: the formation of endoderm regulated by forkhead box transcription factors, pancreatic biogenesis regulated by homeobox transcription factors, and finally the differentiation of endocrine and exocrine cells regulated by basic helix-loop-helix transcription factors. During the course of embryonic development, the definitive endoderm is derived from the mesendoderm of

the anterior segment of the primitive streak which corresponds to the early and mid-gastrula organizer [36–38]. This process is initiated while cells from the specific regions of the epiblast ingress through the primitive streak and undergo an epithelial-to-mesenchymal transition to form the definitive endoderm and mesoderm [38–40]. Genetic analyses have also identified that the WNT and TGF-β signaling pathways are essential for the formation of the primitive streak and definitive endoderm during gastrulation [41–45]. Many studies recently demonstrated that a TGF-β-like molecule, Activin A, could act through the signaling pathway as another TGF-β member, Nodal, and efficiently induce endoderm formation from differentiating ESC [46–51]. Thus, to be able to efficiently produce the desired differentiated cell types from ESC, derivation of specific intermediate precursor cell types is the critical step.

Figure 1 depicts a summary of the steps involved during the differentiation of ESC into the definitive endoderm from either human or mouse ESC. There are two major approaches for differentiating ESC into the definitive endoderm. In the embryoid bodies (EBs) formation protocol, the ESC are grown as EBs either in the presence or absence of Activin A and, these EBs are plated on an adherent matrix-coated substrate in serum-free medium containing Activin [48, 49]. In the other protocol which bypasses the EB formation, the ESC are directly cultured in serum-free medium containing Activin A to form mesoendoderm and then further differentiated into the definitive endoderm and mesoderm [50, 52]. Recently, Baetge and colleagues have developed a protocol to differentiate human ESCs into definitive endoderm and IPCs using low-serum medium containing a high concentration of Activin [46, 47]. Although the functional cell population produced through these protocols remains very low, these studies have shown significant advancement in directing ESC toward specific lineages.

During differentiation of ESC, the definitive endodermal cells morphologically appear as an epithelial monolayer (Fig. 2A) and express Foxa2, Sox17, CXCR4, GSC, and CER along with other genes which have been defined as definitive endoderm markers. However, some of these genes are not only expressed in definitive endoderm but in other germ layers. These studies have utilized genetically marked ESC for monitoring and selecting the definitive endoderm. Although it is a feasible approach, it becomes impractical due to

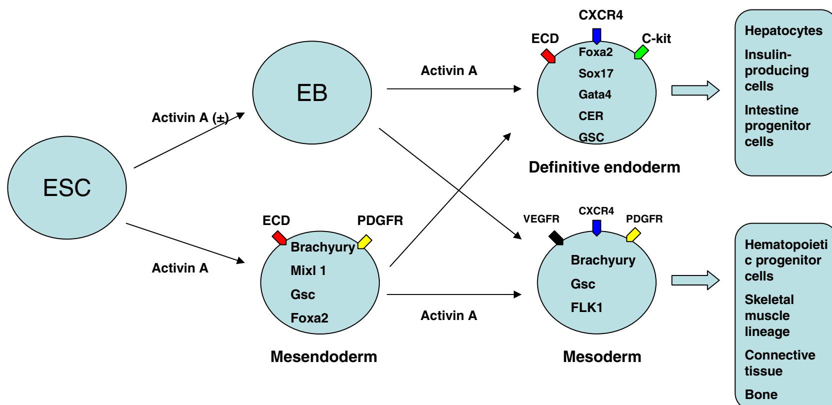

Fig. 1 Differentiation of ESC into definitive endoderm and expression of molecular markers at various stages. To achieve the definitive endoderm, embryoid body (EB) formation has been reported as an approach that can be done under either the presence or absence of Activin A

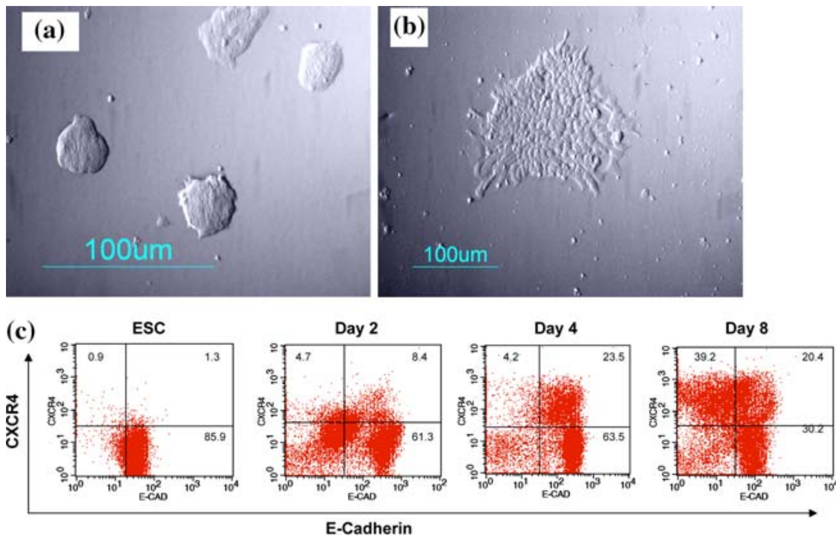

Fig. 2 The cell surface expression of CXCR4 and E-cadherin in Elm3 ESC undergoing differentiation. (**a**) Undifferentiated mouse ESCs displayed well-rounded clusters. However, during the cultivation with Activin A, the differentiated mouse ESCs displayed an epithelial monolayer appearance (**b**). Thus ESC can be utilized for the derivation of the definitive endoderm that can be applied to produce IPCs. (**c**) Flow cytometric analysis of CXCR4 and E-cadherin expression of the cells that were cultured under serum-free medium containing activin. All ESC are E-cadherin positive. However, during the differentiation procedure, a gradual increase in the expression of CXCR4 is noted culminating in >50% being CXCR4 positive by day 8

several selection steps that are required. Therefore, it is necessary to develop an applicable approach without using genetic manipulation.

Our studies indicate that the murine ESC differentiation into definitive endoderm can be successfully achieved using low-dose Activin A treatment in the presence of serum free medium for 8 days (Fig. 2B). Time course studies of ESC lines Elm-3 and R1 undergoing Activin A treatment reveal a gradual decrease in the expression of E-cadherin followed by a corresponding increase in the expression of CXCR4. CXCR4 has been identified as a cell-surface marker representing definitive endoderm under defined culture conditions [43, 48]. Although CXCR4 is not exclusively expressed in the definitive endoderm, it is a useful cell-surface marker available for purifying the definitive endoderm from a differentiated cell population. Keller and colleagues have recently reported that an endoderm progenitor cell population could be enriched from genetically unmanipulated mouse ESCs based on co-expression of c-kit and CXCR4. These endodermal progenitor cells could be further differentiated and led to efficient generation of hepatic progenitor cells under defined culture conditions [48].

Differentiation of ESC into IPCs by EB formation and their molecular imaging

One of the major limitations of the currently employed approaches for ESC differentiation into IPCs includes an inability to monitor the differentiation process in vitro and the fate of the transplanted cells in vivo post-transplantation. To overcome this limitation, we have

devised a unique approach by exploiting the tissue specificity of ESC-specific NANOG gene promoter and IPCs-specific RIP to monitor the ESC differentiation events in vitro as well as in vivo. NANOG, a homeodomain gene, has been shown to play a key role in maintaining the pluripotency of the ESC. We have generated eukaryotic expression vectors in which the luciferase gene has been placed under the transcriptional control of either the NANOG promoter or RIP. These expression vectors have been stably integrated into Elm3 and R1 ESC. In an undifferentiated state, both these cell lines express higher levels of NANOG as a result of which they exhibit higher luciferase activity (Fig. 3). In contrast, RIP which is active specifically in the IPCs is selectively turned off in these undifferentiated ESC lines thereby showing negligible luciferase expression. As the ESC undergo differentiation into IPCs, the NANOG promoter becomes transcriptionally inactive while RIP exhibits significant transcriptional activity leading to enhanced luciferase expression.

The islet transplantation and engraftment are critical to the success of diabetes treatment especially because a substantial number of islets fail to implant or are destroyed by immunological responses. Thus, it is extremely important to be able to follow the fate of the transplanted IPCs in vivo non-invasively. To study the dynamic fate of transplanted IPCs in the recipient mice, we have developed a real-time non-invasive bioluminescence imaging (BLI) modality. This approach is based upon the detection of RIP-driven luciferase expression and permits the detection of early events underlying graft survival and function in vivo. A comparison of NANOG promoter and RIP activity in undifferentiated ESC transplanted subcutaneously in mice followed by BLI is depicted in Fig. 4. The results clearly indicate a significant luciferase activity by the NANOG promoter in the undifferentiated ESC whereas the RIP activity is almost negligible. These findings highlight the fact that this approach can be successfully used to monitor the transcriptional activities of various tissue specific promoters non-invasively in vivo by real-time BLI. Furthermore,

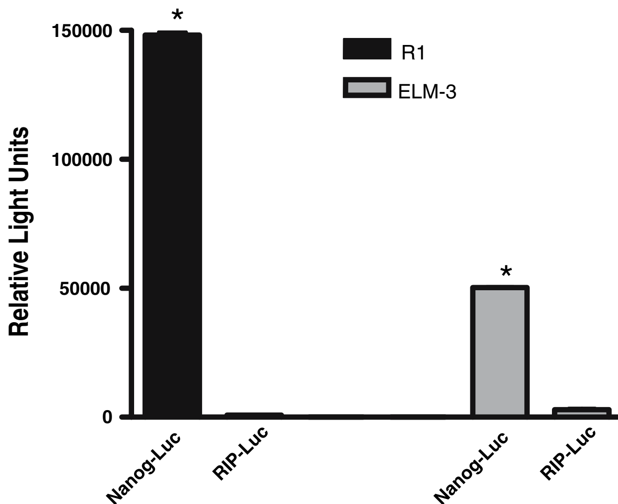

Fig. 3 Differential activation of NANOG and RIP in ESC: The undifferentiated ESC lines R1 and Elm3 were transfected with equal amounts of either NANOG promoter or RIP driven luciferase expression vectors NANOG-Luc or RIP-Luc. The luciferase activity was monitored 72 h post-transfection by luciferase reporter assay. NANOG promoter exhibits a significant luciferase activity in R1 and Elm3 ESC while RIP is transcriptionally silent

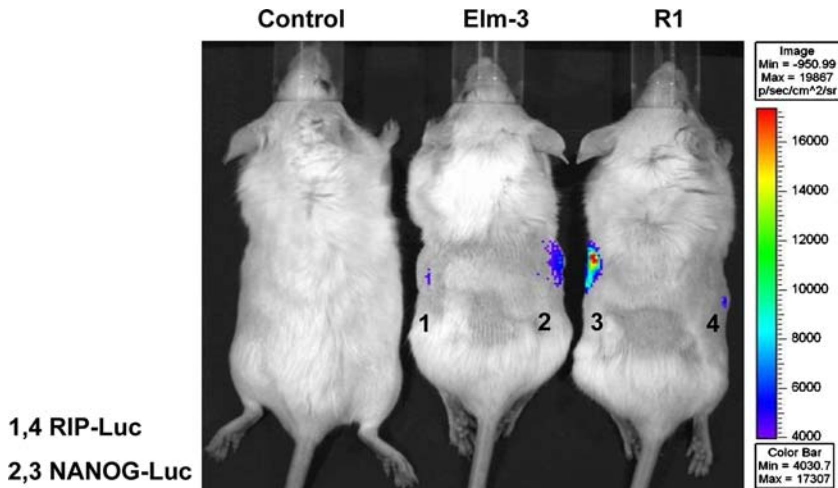

Fig. 4 Real-time in vivo BLI of the transplanted ESC: The ESC expressing luciferase under the transcriptional control of either NANOG promoter or the RIP promoter were subcutaneously transplanted in mice and subjected to BLI 72 h post-transplantation. The NANOG promoter displays a significant transcriptional activity as compared to RIP in both R1 as well as Elm3 ESC

BLI has the potential for the real-time non-invasive monitoring to follow the fate of the transplanted IPCs, graft survival and its functioning in vivo.

Future directions

The basic idea that stem cells represent a potential source of tissue for the replacement or the regeneration of the damaged tissue is very exciting. However, in order to harness the true potential of the multipotent stem cells a significant number of challenges need to be overcome before ESC could be used successfully for the treatment of type 1 diabetes. While embryonic stem cells possess enormous differentiation potential, they also exhibit several characteristics of tumor cells, such as unlimited proliferation capacity, clonal growth, and lack of contact inhibition. When the undifferentiated ESC are transplanted into syngeneic host they result in teratoma or teratocarcinoma formation [53]. The ongoing studies on ESC differentiation into IPCs have demonstrated that only a very small fraction of the total ESC undergo differentiation into IPCs. Furthermore, currently there is a lack of a suitable technology that can be used to physically separate the ESC-derived IPCs from the undifferentiated ESC. Consequently, transplantation of the mixed cell population not only fails to correct diabetes on a long-term basis but the remaining undifferentiated cell population could also lead to tumor formation thereby ultimately self-defeating the very purpose of using the ESC-derived IPCs for the treatment of diabetes. This major drawback merits serious consideration for developing innovative strategies for the active separation of tumorigenic cells from the ESC-derived IPCs. Addressing this very critical issue will lead to a significant advancement in the field as it will allow selective enrichment of the IPCs while eliminating not only tumorigenic cells but also of other non-pancreatic cells.

Successful islet transplantation depends upon and is directly influenced by a wide variety of factors including blood supply during early engraftment, non-specific

inflammation, immunosuppression, and rejection. A major limitation in the field of islet transplantation is the inherent inability to non-invasively monitor the transplantation efficiency including islet mass, graft survival, islet neovascularization, angiogenesis, islet blood flow as well as islet function in vivo. Critical to the success of islet transplantation is the development of novel non-invasive imaging modalities for the real-time in vivo monitoring of the transplanted IPCs. Thus far, direct in vivo imaging of either the native or the transplanted IPCs has met with limited success due to their relatively small size and low numbers distributed over a large area within the pancreas. Non-invasive BLI is emerging as a powerful tool for investigating a wide range of biological processes in real-time in vivo and has tremendous potential for studying the dynamics of islet transplantation to develop novel protocols to advance this exciting field.

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