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Reduced serum concentration is permissive for increased *in vitro* endocrine differentiation from murine embryonic stem cells

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ABSTRACT

Embryonic stem cells (ESCs) have been shown to be capable of differentiating into pancreatic progenitors and insulin-producing cells *in vitro*. However, before ESC derivatives can be used in clinical settings, efficient selective differentiation needs to be achieved. Essential to improving ESC differentiation to islet endocrine cells is an understanding of the influences of extrinsic signals and transcription factors on cell specification. Herein, we investigate the influence of serum-supplemented growth conditions on the differentiation of murine ESCs to endocrine lineages in the context of over-expression of two pancreatic transcription factors, Pdx1 and Ngn3.

To study the effect of different serum formulations and concentrations on the ability of murine ESCs to differentiate into endocrine cells *in vitro,* cells were grown into embryoid bodies and then differentiated in various serum replacement (SR), fetal calf serum (FCS) and serum-free conditions. Using immunohistochemistry and quantitative real-time RT-PCR (QPCR), we found that, of the conditions tested, 1% SR differentiation medium resulted in the highest levels of insulin-1 mRNA and significantly increased the total number of insulin-expressing cells. Applying this knowledge to cell lines in which *Pdx1* or *Ngn3* transgene expression could be induced by exposure to doxycycline we differentiated TetPDX1 and TetNgn3 ESCs under conditions of either 10% FCS or 1% SR medium. In the presence of 10% serum, induced expression of either *Pdx1* or *Ngn3* in differentiating ESCs resulted in modest increases in hormone transcripts and cell counts. However, changing the serum formulation from 10% FCS to 1% SR significantly enhanced the number of insulin+/C-peptide+ cells in parallel with increased insulin-1 transcript levels in both inducible cell lines. In summary, these data demonstrate that induced expression of key pancreatic transcription factors in combination with low serum/SR concentrations increases endocrine cell differentiation from murine ESCs.

1. Introduction

Embryonic stem cells (ESCs) are envisaged as a source for replacement β cells for patients with diabetes. Characterized by the absolute or relative deficiency of insulin due to the destruction of β cells, diabetes is a stereotypical disease that would be amenable to potential stem cell-based therapies. Currently, most patients are treated with insulin injections, but it is well-documented that exogenous insulin therapy is not entirely effective in either correcting hyperglycemia, halting the myriad secondary metabolic and organ-specific complications, or preventing dangerous hypoglycemic episodes. Successful human islet transplantation from deceased donors now offers a strong proof-of-principle that a cell-based therapy can be effective in reversing abnormal glucose regulation (Shapiro et al., 2000). However, despite successful pancreas and islet transplantation it is clear that the available supply of deceased donor tissue is not adequate to meet the increasing needs of millions of affected patients. Furthermore, the quality of tissues isolated from deceased donors is in many cases suboptimal, which may impact the overall long-term function of the transplant. As a result, new sources of β cells are needed; ESCs which have the capacity for self-renewal and multi-lineage differentiation may be one source.

In order to design differentiation protocols that promote endocrine specification from ESCs, it is useful to explore normal developmental cues, including regulation by transcription factors and soluble signals. During pancreas development, endocrine cells normally differentiate from a subset of cells within the pancreatic

Abbreviations: FCS, fetal calf serum; SR, serum replacement; ESC, embryonic stem cell

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epithelium and migrate into the surrounding mesenchyme where they coalesce into multicellular aggregates forming the islets of Langerhans, comprising α, β, δ, PP and ε cells expressing glucagon, insulin, somatostatin, PP and ghrelin, respectively. Pancreatic duodenal homeobox 1 (Pdx1 or Ipf1) and neurogenin 3 (Ngn3, or Neurog3) are two transcription factors of central importance in regulating which foregut cells are specified to endocrine fate. Pdx1 is one of the first homeodomain proteins to be expressed in the posterior foregut region and is required for pancreas development (Offield et al., 1996; Jonsson et al., 1994). In mid-gestation in the mouse (e9.5–15.5), a subset of pancreatic epithelial cells begins to express Ngn3, a bHLH transcription factor that is required for endocrine lineage specification (Gradwohl et al., 2000). It is generally accepted that Pdx1+ pancreatic epithelial cells that also express Ngn3 represent endocrine progenitors *in vivo,* as lineage-tracing experiments have shown that Ngn3-expressing cells give rise to all endocrine cell types (Schwitzgebel et al., 2000; Gu et al., 2002; Gradwohl et al., 2000). Normally, transient Ngn3 expression then leads to target gene expression (NeuroD1, Nkx2.2, Pax4, etc.) in these endocrine progenitors as they initiate commitment to specific endocrine lineages. Ngn3 expression is ultimately extinguished in endocrine cells by the end of gestation and is not normally expressed post-natally. During or after commitment, the developing endocrine progenitors migrate from the pancreatic epithelium into the surrounding mesenchyme and coalesce into islets through largely unknown mechanisms.

Numerous recent studies have demonstrated that both murine and human ESCs are indeed capable of differentiating into pancreatic lineage cells including insulin-producing β-like cells *in vitro*, though the percentage of cells produced is quite low (Soria et al., 2000; Kahan et al., 2003a; Skoudy et al., 2004; Assady et al., 2001; Brolen et al., 2005; Segev et al., 2004; Xu et al., 2006; Ku et al., 2007). Many of these differentiation protocols used non-specific culture conditions containing high concentrations of serum, which may contain factors that inhibit pancreatic progenitor specification and/or impair endocrine differentiation. Indeed, in serum-based culture conditions, numerous Pdx1+ cells can be detected, yet insulin+ cells are relatively rare (Kahan et al., 2003a; Odorico et al., 2004; Xu et al., 2006). Some success has been achieved in generating partially enriched cultures of β cells through manipulation of developmentally relevant growth factors (i.e. Activin A and others) in low serum-containing, or in serum-free medium in order to direct differentiation to definitive endoderm (Shi et al., 2005; D'Amour et al., 2006; Shim et al., 2007; Jiang et al., 2007a, 2007b; Phillips et al., 2007; Serafimidis et al., 2008), yet the efficiency of endocrine differentiation remains low, despite achieving highly enriched definitive endoderm.

Others studies have focused instead on guiding endocrine cell differentiation from ESCs by over-expressing key pancreatic transcription factors, including Pdx1, Pax4, Ngn3 and Nkx2.2 (Miyazaki et al., 2004; Blyszczuk et al., 2003; Blyszczuk et al., 2004; Lin et al., 2007; Treff et al., 2006; Serafimidis et al., 2008; Boretti and Gooch, 2007; Shiroi et al., 2005). The outcomes achieved in some of these studies may have been significantly affected by experimental factors such as the constitutive expression of the transgene (Blyszczuk et al., 2003; Lin et al., 2007; Shiroi et al., 2005) or inappropriate timing of induced expression (Miyazaki et al., 2004; Treff et al., 2006). In addition, the presence of serum, which may contain undefined, potentially inhibitory factors, may also affect results (Treff et al., 2006; Shiroi et al., 2005). Indeed, few studies have explored the effects of growth medium modifications or reduced serum-containing medium together with transcription factor over-expression on ESC-derived endocrine cell types.

During development, the expression of transcription factors in temporally and spatially restricted patterns sets up the endoderm for differentiation into the organs to which it will eventually give rise, and for specifying distinct cell types within those organs. Transgenic studies in mice have also shown that ectopic expression of transcription factors can have profound effects on development in many different organ systems, in some cases leading to ectopic organ formation, hypertrophied tissues, altered cell fates, or reconstituted organogenesis (Halder et al., 1995; Heller et al., 2001; Schwitzgebel et al., 2000; Kawaguchi et al., 2002; Johansson et al., 2007, among others). Furthermore, the outcomes of such experiments can depend greatly on the context in which the transcription factor is expressed (i.e. cell type or stage of development). For example, induced expression of Ngn3 in pancreatic epithelium at an early stage during pancreas development results in almost exclusive α-cell differentiation whereas later expression produces predominantly insulin or somatostatin cell types (Apelqvist et al., 1999; Schwitzgebel et al., 2000; Johansson et al., 2007). Thus, by analogy to *in vivo* pancreas development, it is essential to understand the proper temporal regulation of combined intrinsic and extrinsic developmental signals in order to generate more enriched populations of particular endocrine cells from ESCs *in vitro*.

Herein, we describe the extensive testing of different concentrations of serum, serum replacement (SR) and serum-free media on murine ESC differentiation to islet cell types. In so doing, we address the question whether serum inhibits pancreatic lineage commitment and/or endocrine differentiation from ESCs. Our observations in unmanipulated murine ESC lines prompted us to test whether serum affected the ability of induced Ngn3 or Pdx1 expression to enhance differentiation of ESCs into islet endocrine cells. These experiments showed that low serum concentrations allowed endocrine differentiation to proceed from unmanipulated ESC lines at a greater rate than if cells were allowed to differentiate in high serum-containing media, and that induced expression of Ngn3 or Pdx1 further enhanced endocrine differentiation, particularly in the context of low serum differentiation. In addition, we found that efficient endocrine differentiation depended on the timing of transgene expression, suggesting that the context of the environment or developmental state of cells is relevant in improving endocrine specification from ESCs.

2. Materials and methods

2.1. Cell lines

The Ainv15 ESC line (a gift from M. Kyba/G. Daley and available at ATCC) is the parent line for the tetracycline-inducible lines and is used in this study as a non-inducible control (Kyba et al., 2002, 2003). The generation and characterization of the TetNgn3 and TetPdx1 lines were described previously (Treff et al., 2006; Vincent et al., 2006). The R1 cell line (Nagy et al., 1993) was a gift from Andras Nagy.

2.2. Cell culture

Tissue culture reagents were purchased from Invitrogen unless otherwise noted. Undifferentiated TetNgn3, TetPdx1, Ainv15 and R1 ESCs were cultured in gelatinized (Sigma) 100 mm tissue culture plates (BD Biosciences) on a layer of γ-irradiated neomycin-resistant mouse embryonic fibroblasts (Specialty Media) in the presence of DMEM-High Glucose with 15% fetal calf serum (FCS, Hyclone), 100 U penicillin/streptomycin, 2 mM L-glutamine, 5.5×10^{-2} M β-mercaptoethanol, 0.11 mM nonessential