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Generation of polyhormonal and multipotent pancreatic progenitor lineages from human pluripotent stem cells

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ABSTRACT

Generation of pancreatic β-cells from human pluripotent stem cells (hPSCs) has enormous importance in type 1 diabetes (T1D), as it is fundamental to a treatment strategy based on cellular therapeutics. Being able to generate β-cells, as well as other mature pancreatic cells, from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) will also enable the development of platforms that can be used for disease modeling and drug testing for a variety of pancreas-associated diseases, including cystic fibrosis. For this to occur, it is crucial to develop differentiation strategies that are robust and reproducible across cell lines and laboratories. In this article we describe two serum-free differentiation protocols designed to generate specific pancreatic lineages from hPSCs. Our approach employs a variety of cytokines and small molecules to mimic developmental pathways active during pancreatic organogenesis and allows for the *in vitro* generation of distinct pancreatic populations. The first protocol is designed to give rise to polyhormonal cells that have the potential to differentiate into glucagon-producing cells. The second protocol is geared to generate multipotent pancreatic progenitor cells, which harbor the potential to generate all pancreatic lineages including: monohormonal endocrine cells, acinar, and ductal cells.

1. Introduction

According to worldwide statistics, the incidence of T1D continues to increase at alarming rates [1,2]. T1D results from the autoimmune attack and destruction of pancreatic β-cells and despite improvements in insulin administration, patients with T1D eventually develop complications severely impacting their quality of life [2]. As a potential treatment option, the use of *in vitro*-generated human β-cells has gained much traction in recent years, as this method can theoretically provide an infinite supply of physiologically suitable cells. Indeed, a clinical trial using hESC-derived pancreatic progenitor cells is ongoing in the USA (NCT02239354) to test the safety of this approach for the treatment of T1D patients [3]. The approach used to generate pancreatic cells from hPSCs aims at recapitulating embryonic steps leading to pancreatic development and it has been successfully utilized by several groups to generate cell populations that have the potential to ameliorate hyperglycemia in mouse models of T1D [4–7].

Pancreatic organogenesis can be divided into four fundamental stages: (1) the formation and (2) patterning of definitive endoderm, (3) specification of the pancreatic epithelium, and (4) lineage commitment of endocrine/exocrine cells [8]. Definitive endoderm formation occurs during gastrulation. This process begins when epiblast cells, guided by Wnt and Nodal signaling, undergo an epithelium-to-mesenchyme transition in order to form the primitive streak [9–11]. Subsequently, the most anterior region of the primitive streak is exposed to high concentrations of Nodal signaling, which drives definitive endoderm formation. Following gastrulation, the endoderm layer folds into a primitive gut tube that can be divided into three regions: the foregut, midgut, and hindgut, which are all patterned by growth factors secreted by nearby tissues. This patterning gives rise to the different organ-buds along the primitive gut tube. Two pancreatic buds, which are marked by the expression of homeobox gene Pdx1, arise in the posterior foregut. Pdx1-expressing cells form the two outgrowths of epithelium that evaginate dorsally and ventrally. At this stage, signaling molecules secreted by the dorsal aorta and the notochord influence dorsal bud development, while those secreted by the cardiac mesenchyme and the lateral plate mesoderm influence ventral bud development. Several loss-of-function animal models have shown that retinoid signaling is indispensable for dorsal pancreatic

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specification [12–14]. In addition, studies in the mouse embryo demonstrate that Bmp inhibition influences pancreatic over hepatic fate in the ventral bud, while studies in the chick embryo show that Shh inhibition provided by the notochord is necessary for pancreatic development [15,16]. The eventual rotation of the gut tube fuses both the dorsal and ventral buds together, forming the pancreatic primordium, which expands into the surrounding mesenchyme. The pancreatic mesenchyme creates a permissive environment for expansion and differentiation for the pancreatic epithelium by secreting factors such as Fgf10, responsible for maintaining Notch activation [17,18]. Subsequently, endocrine and exocrine lineage commitment occurs from a pool of multipotent progenitor cells (MPCs) expressing transcription factors such as Pdx1, Nkx6-1, and Ptf1a. Both Pdx1 and Nkx6-1 expression become exclusive to endocrine/ductal progenitors while Ptf1a expression characterizes the acinar progenitors [19,20]. Maturation of endocrine cells leads to the generation of α-, β-, γ-, δ-, and ε-cells that secrete glucagon, insulin, pancreatic polypeptide, somatostatin, and ghrelin, respectively [8].

Since most of our knowledge of pancreas organogenesis comes from the mouse, it is important to realize key differences exist when we attempt to translate this process to human development by using cytokines and small molecules to drive *in vitro* differentiation of hPSCs along the four stages of pancreatic development. For example, both species exhibit an embryonic polyhormonal cell population producing both insulin and glucagon, and lacking expression of Nkx6-1 [21,22]. In the mouse, the formation of these polyhormonal cells occurs between embryonic day 10.0 (E10.0) and E12.0 and is known as the first transition, while the formation of MPCs with the potential to give rise to adult endocrine cells is known as the second transition and occurs between E12.5 and E15.5 [8]. In humans, monohormonal cells arise at gestational week 7.5 (Gw7.5), prior to the polyhormonal population at Gw9.0, suggesting that the development of these populations in humans do not follow the same temporal pattern seen in the mouse [23,24]. To elucidate this and other differences that could distinguish humans from other model systems, we can now take advantage of the hPSC-directed differentiation system, as it provides the unique opportunity to study human development *in vitro*.

Here we report two serum-free differentiation protocols utilizing monolayer culture that can selectively generate either polyhormonal cells or MPCs from a variety of hESC and hiPSC lines. Our previous work has identified that inhibition of TGFβ signaling is important for the development of polyhormonal cells [25], while combining BMP inhibition with EGF and nicotinamide signaling results in efficient NKX6-1$^+$ endoderm induction, containing progenitors of adult pancreatic lineages [26]. Additionally, we demonstrated that the size of this NKX6-1$^+$ population is determined by the duration of treatment with retinoic acid, FGF10, and inhibitors of BMP and SHH pathways. In this article, we will refer to these two protocols as first and second transition differentiation protocols, respectively. When transplanted into NODscid gamma (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice, purified INSULIN$^+$ cells generated with the first transition differentiation protocol give rise to glucagon-producing cells, while the progenitors generated with the second transition differentiation protocol give rise to exocrine and endocrine cells, including monohormonal insulin-expressing cells [26,27].

2. Materials

2.1. Reagent lists

See Tables 1 and 2.

Table 1
Media and differentiation components.

Reagent	Company	Catalog #
1-Thioglycerol (MTG)	Sigma	M6145
2-Mercaptoethanol	Life Technologies	21985-023
Activin A	R&D	338-AC/CF
Ascorbic acid	Sigma	A4544
B-27 Supplement, minus vitamin A	Life Technologies	12587-010
bFGF	R&D	233-FB
BSA	Sigma	A9647
CHIR990210	Tocris	4423
DMEM/F-12	Life Technologies	11330
DMEM, high glucose	Life Technologies	12100
DNase I	VWR	80510-412
Dorsomorphin	Sigma	P5499
EGF	R&D	236-EG
FBS	HyClone	SH30071.03
FGF10	R&D	345-FG
Gelatin from porcine skin	Sigma	G1890
Glutamine	Life Technologies	25030
Ham's F12 Medium	cellgro	10-080
IMDM	Life Technologies	12440
Knockout serum replacement	Life Technologies	10828
L-685,458 (γ-Si)	Tocris	2627
L-Glutamine	HyClone	SH30034
Matrigel	BD	356230
MEM non-essential amino acids solution	Life Technologies	11140
N-2 Supplement	Life Technologies	17502-048
Nicotinamide	Sigma	N0636
Noggin	R&D	3344-NG
Paraformaldehyde 16% solution	EMS	15710
Penicillin/streptomycin	Life Technologies	15070-063
Retinoic acid	Sigma	R2625
ROCK inhibitor	Tocris	1254
RPMI	Life Technologies	11875
SANT-1	Tocris	1974
Saponin	Sigma	S4521
SB431542	Sigma	S4317
Sodium bicarbonate 7.5% solution	Life Technologies	25080-094
Sodium pyruvate solution	Sigma	S8636
TrypLE express	Life Technologies	12605

Table 2
Antibodies and stains.

Reagent (working dilution)	Company	Catalog #
C-Peptide (1:1000)	DSHB	GN-ID4
CD117 PE (1:100)	Life Technologies	CD11705
CXCR4 APC (1:50)	BD	551966
Cytokeratin 19 (1:800)	Abcam	ab15463
DAPI (1:2500)	Biotium	40043
Donkey Anti-Guinea pig IgG (H + L), Alexa Fluor 488 conjugate (1:800)	Jackson Immuno Research	706-545-148
Donkey Anti-Mouse IgG (H + L), DyLight 488 conjugate (1:800)	Thermo Scientific	SA5-10166
Donkey Anti-Mouse IgG (H + L), Alexa Fluor 647 conjugate (1:800)	Life Technologies	A-31571
Donkey Anti-Mouse IgG (H + L), DyLight 594 conjugate (1:200)	Thermo Scientific	SA5-10168
Donkey Anti-Rabbit IgG (H + L), DyLight 550 conjugate (1:200)	Thermo Scientific	SA5-10039
Donkey Anti-Rat IgG (H + L), Alexa Fluor 488 conjugate (1:800)	Life Technologies	A-21208
Hematoxylin and Eosin Staining Kit	Scytek	HAE-1
Insulin (1:1000)	Dako	A0564
Glucagon (1:500)	Sigma	G2654
Goat Anti-Rat Ig, PE conjugate (1:200)	BD	550767
NKX6-1 (1:2000)	DSHB	F55A10
Pancreatic Polypeptide (1:500)	Peninsula Laboratories	T-4088
Rabbit Anti-Sheep IgG (H + L), Alexa 647 conjugate (1:800)	Jackson Immuno Research	313-605-003