学位論文
Doctoral Thesis

Pancreatic differentiation of human iPS cells using a defined and completely xeno-free culture system
（ゼノフリー培養系を用いた ヒト iPS 細胞から インスリン産生細胞への分化誘導法の確立）

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Abstract

Background and Purpose:

Human induced pluripotent stem (hiPS) cells are considered a potential source for the generation of insulin-producing pancreatic β-cells because of their differentiation capacity. Many of the current differentiation protocols utilize a variety of undefined animal-derived products that may have unknown effects on cell characteristics and differentiation. The potential consequences of transplanting human cells exposed to animal-derived products into patients include an increased risk of graft rejection, immunoreactions, and microbial infections, prions, and yet unidentified zoonoses. Therefore, the establishment of a defined and completely xeno-free culture system with which functional and terminally differentiated endocrine cell types can be generated from hiPS cells is needed for future research and clinical applications.

Methods:

To address these issues, I established for the first time a defined and completely xeno-free culture system to derive INS-expressing β-like cells from hiPS cells using a synthetic scaffold (Synthemax II-SC Substrate, functionalized with short peptide sequences derived from the vitronectin protein, which is covalently linked to the synthetic acrylate polymer, Corning) and serum-free media containing humanized and/or recombinant supplements and growth factors.

Results:

In this study, I have developed a five-step xeno-free culture system to efficiently differentiate hiPS cells into insulin-producing cells in vitro. I found that a high NOGGIN concentration is crucial for specifically inducing the differentiation of cells first into pancreatic and duodenal homeobox-1 (PDX1)-positive pancreatic progenitors and then into neurogenin 3 (NGN3)-expressing pancreatic endocrine progenitors, while suppressing the differentiation into hepatic or intestinal cells. I also found that a combination of 3-isobutyl-1-methylxanthine (IBMX), exendin-4, and nicotinamide was important for the differentiation into insulin single-positive cells that express various pancreatic β-cell markers. Most notably, the differentiated cells contained

endogenous C-peptide pools that were released in response to various insulin-secretagogues and high levels of glucose.

Conclusions:

Our results demonstrate the feasibility of generating hiPS-derived pancreatic β-cells under xeno-free conditions and highlight their potential to treat patients with type 1 diabetes.

Key words: diabetes, pancreas, cell therapy, hiPS cells, xeno-free differentiation, β-cells

List of publications

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Abbreviations

hES	Human Embryonic Stem
hiPS	Human induced pluripotent stem
ELISA	Enzyme linked immunosorbent assay
MEFs	Mouse embryonic fibroblasts
Na-bu	Na-butyrate
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenetic protein 4
DAPT	A γ-secretase inhibitor
EGF	Epidermal growth factor
FGF10	Fibroblast growth factor 10
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor-1
IGF-2	Insulin-like growth factor 2
KGF	Keratinocyte growth factor
FGF7	Fibroblast growth factor 7
RA	All-trans retinoic acid
CYC	KAAD-cyclopamine, a sonic hedgehog signaling inhibitor
SANT-1	a sonic hedgehog signaling inhibitor
Ex-4	Exendin-4

FRKL	Forskolin
IBMX	3-isobutyl-1-methylxanthine
DM	Dorsomorphin
Nog	Noggin
ILV	(-)-indolactam V
NA	Nicotinamide
Dex	Dexamethasone
ALK5i	a TGFβ type 1 receptor kinase inhibitor II
TBI	a TGFβ type 1 receptor kinase inhibitor IV
SB	SB431542, a TGFβ type 1 receptor kinase inhibitor VI
TT	TTNPB
TBP	a PKC activator
LDN	a BMP inhibitor
Neu5Gc	*N*-glycolylneuraminic acid
OCT4	Octamer-4
SOX2	SRY box-2
TRA1–81	Tumor rejection antigen 1-81
SSEA-4	Stage-specific embryonic antigen-4
SSEA-1	Stage-specific embryonic antigen-1
SOX17	SRY box-17

FOXA2	Forkhead box protein A2
HNF4a	Hepatocyte nuclear factor 4a
PP	Pancreatic progenitor
PDX1	Pancreatic and duodenal homeobox-1
AFP	Alpha-fetoprotein
CDX2	Caudal-related homeobox 2
NGN3	Neurogenin 3
HNF6	Hepatocyte nuclear factor-6
NKX6.1	NK-related homeobox 6.1
HLXB9	Homeobox HB9
DAPI	4’,6-diamidino-2-phenylindole
EP	Endocrine progenitor
NEUROD1	Neurogenic differentiation 1
PAX4	Paired box 4
CP	C-peptide
DMSO	Dimethyl sulfoxide
ISL-1	Islet-1
GCK	Glucokinase
UCN3	Urocortin-3
IAPP	Islet amyloid polypeptide

GLP-1	Glucagon like peptide-1
DMEM	Dulbico’ modified eagle medium
RPMI	Roswell Park Memorial Institute (medium)
DMEM/F12	Dulbico’ modified eagle medium/F12
KSR	Knockout serum replacement
PS	Penicillin-streptomycin
L-Gln	L-glutamine
NEAA	Nonessential amino acids
2-ME	2-mercaptoethanol
CTS	Cell Therapy System
DE	Definitive endoderm
PG	Primitive gut tube
EC	Hormone-expressing endocrine cells
TGF-β	Transforming growth factor-β
PBS	Phosphate-buffered saline
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
INS	Insulin
GCG	Glucagon
SST	Somatostatin
PPY	Pancreatic polypeptide

GHRL	Ghrelin
AMY	Amylase
n.a	Not applicable
n.t	Not tested
n.d	Not determined

1. INTRODUCTION

1.1. The Pancreas

The pancreas is an abdominal gland connected to the duodenum and plays an important role in maintaining the nutritional homeostasis through the secretion of enzymes and hormones. This organ is composed of three principal cell types: acinar cells, duct cells, and endocrine cells (Fig. 1). Acinar cells produce and secrete digestive enzymes, such as lipases, proteases and nucleases, which are routed to the intestine by a branched ductal network. Acinar and duct cells compose the exocrine compartment of the pancreas and represent approximately 98% of the total organ mass. Endocrine cells, involved in regulating nutrient metabolism and glucose homeostasis, represent less than 2% of the pancreatic tissue and are organized into small cell clusters termed "islets of Langerhans. These islets, scattered throughout the exocrine tissue, are composed of five different endocrine cell subtypes: α β, g, ε and PP cells (Collombat et al., 2006). The central core of each islet is made up of β cells which are surrounded by other endocrine cells. The pancreatic β-cells produce insulin as a pro-hormone that is cleaved into mature hormone and C-peptide inside storage granules prior to secretion, which is regulated by extracellular stimuli, especially glucose. The pancreatic α, δ, ε, and PP cells also produce particular hormones such as, glucagon, somatostatin, ghrelin, and pancreatic polypeptide, respectively. Cellular uptake of glucose from the blood is regulated principally by the hormone insulin. Insulin and glucagon act coordinately to maintain glycemic homeostasis by regulating the storage, metabolism, and neogenesis of glucose. Insulin is secreted in response to an increase in blood glucose levels and acts in order to reduce glycemic levels by promoting the storage and/or metabolism of glucose in the liver, muscle and/or adipose tissues. Conversely, glucagon displays the opposite function by promoting glucose release and/or neogenesis in the case of hypoglycemia. Somatostatin and PP have been implicated in the regulation of other hormones and exocrine enzyme secretion (Adrian et al., 1978; Roncoroni et al., 1983; Csaba and Dournaud, 2001; Bonal and Herrera, 2008), but the function of ghrelin remains unclear (Wierup et al., 2002; Vignjevic et al., 2012).

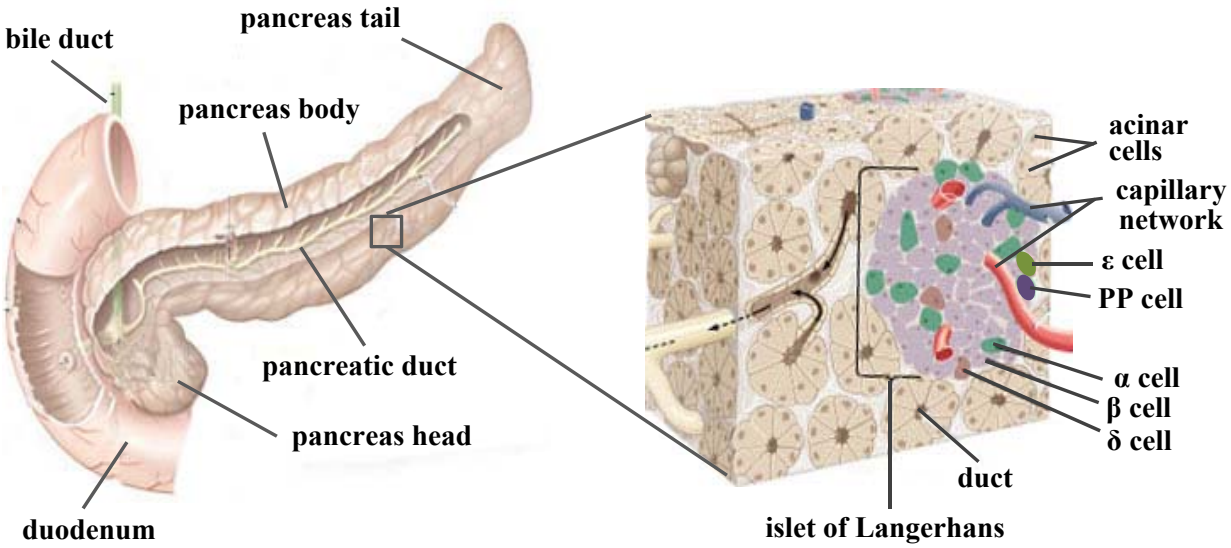

Fig. 1. Pancreas, it's composition and the enlarged view of the islet of Langerhans (Adapted from Encyclopaedia Britannica, Inc.).

1.2. Diabetes mellitus

Diabetes mellitus is a life-long disease characterized by chronic hyperglycemia. Type 1 diabetes results from a complete loss of insulin due to T-cell-mediated autoimmune destruction of β-cells in the pancreas, and type 2 results from relative insulin deficiency due to inadequate insulin secretion or end-organ insulin resistance (Donath and Halban, 2004; Rhodes, 2005). The classical symptoms of diabetes are polyuria (frequent urination), polydipsia (increased thirst), polyphagia (increased hunger), and weight loss. Long-term untreated diabetes is ultimately fatal; in the short term, it can lead to diabetic ketoacidosis and in the long term to chronic complications, such as cardiovascular disease, stroke, and microvascular diseases including retinopathy, nephropathy, and neuropathy (Nathan, 1993). Some type 2 diabetic patients can achieve normal blood glucose levels by a combination of lifestyle modifications and oral insulin secretagogues and sensitizers. In contrast, current type 1 diabetes treatment is solely dependent on the

administration of exogenous insulin, usually via injection. But, the difficulties in adjusting insulin requirement to the rapidly fluctuating demands cause the complications in diabetic patients.

Many research groups are developing cellular therapy to replace the destroyed β cells of type 1 diabetic patients. Currently, cell replacement is performed either by transplantation of whole pancreas or infusion of purified islets from cadaveric donors into the portal vein. Although promising; however, due to difficulties such as the scarcity of cadaveric donors compared to the large number of diabetic patients, the low yield of transplantable islets from cadaveric pancreas, and the necessity of chronic immunosuppression for preventing rejection of the allograft (Shapiro, 2011; Shapiro et al., 2006), an alternative surrogate cell source is required. Moreover, the number of functional β-cells that can be extracted from a single cadaveric pancreas is often not enough to restore euglycemia in one diabetic patient (White et al., 2009). This, again, illustrates the need for alternative sources of β-cells to treat the increasing number of diabetic patients. Therefore, in a search for alternative sources of β-cells, most current efforts aim at generating functional insulin-producing cells from pluripotent stem cells, e.g., embryonic stem (ES) cells or induced pluripotent stem (iPS) cells.

1.3. ES/iPS cells

Embryonic stem (ES) cells possess unlimited replicative properties (Takahashi et al., 2007; Thomson et al., 1998). Recent researches have shown that ES cells could be manipulated to generate a diverse cell types *in vitro* derived from all three germ layers. Induced pluripotent stem (iPS) cells established from somatic cells in the mouse and human (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007) also have the expansion and differentiation capacity of ES cells. Although there are many similarities between ES and iPS cells, the genetic modification makes iPS cells unsuitable for clinical applications; however, this problem could be solved by modifying the methods for their derivation (Kaji et al., 2009; Yu et al., 2009; Zhou et al., 2009; Kim et al., 2009). Therefore, both ES/iPS cells have the great potential for use in cell therapy and drug discovery.

1.4. Derivation and maintenance of human ES/iPS cells

The ES cells are derived from the inner cell mass (ICM) of blastocyst. They have unlimited self-renewal capability when they are grown with mitotically inactive feeder cells in the serum-containing medium, and also possess the potential to differentiate into any adult cell type (Thomson et al., 1998; Reubinoff et al., 2000; Cowan et al., 2004). Recently, several procedures have been reported for derivation and maintenance of hESCs on human feeder cells and on feeder-free matrices in various growth factor-supplemented media (Rao and Zandstra, 2005; Klimanskaya et al., 2005; Ludwig et al., 2005). Moreover, hESCs grown on feeders can be transferred to maintain under feeder-free conditions and vice versa without disturbing their pluripotency, although protein expression profile is slightly affected (Van Hoof et al., 2008). The generation of clinical grade cell lines could be possible by removing both feeder cells and nonhuman components.

The iPS cells are derived from various somatic cells by introducing a combination of several transcription factors, such as Oct3/4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Most iPS cells are made by retrovirus vectors, which integrate the reprogramming factors into host genomes. Retrovirus vectors can spontaneously infect various cell types and insert their coding genes into the host genome by reverse transcriptase, which allows continuous transgene expression during reprogramming. Expression of retroviral transgenes continues until the cells become iPS cells, and then the retroviral promoter is inactivated, possibly because of epigenetic modifications, such as histone methylation (Matsui et al., 2010). This guided reprogramming and automatic silencing mechanism is considered to be very important for iPS cell induction from somatic cells.

Generation of iPS cells from patient's somatic cells would be useful source for disease modeling, drug discovery, toxicology, and cell transplantation therapies (Okita and Yamanaka, 2010). So far, most patient-specific iPS cells have been established with retrovirus vectors. However, these iPS cells have numerous transgene integrations in the genome, and the

integrations may cause leaky expression which could interrupt the endogenous transcription factor network and lead to failure of differentiation. Another important problem of transgene integration is the tumorigenic risk after transplantation. In particular, c-Myc, one of the reprogramming factors, is a well-known oncogene, and its reactivation could give rise to transgene derived-tumor formation in chimeric mice (Okita et al., 2007). For making safe iPS cells, one important approach could be avoiding c-Myc transgene from reprogramming cocktail. Human and mouse iPS cells can be established from fibroblasts with only Oct3/4, Sox2, and Klf4, but the both the efficiency of iPS generation and their quality are significantly reduced (Nakagawa et al., 2008). The chimeric mice produced with c-Myc-free iPS cells did not show enhanced tumor formation in comparison to control mice. However, the retroviral insertion to the genome itself may disturb endogenous gene structure and increase the risk of tumors (Hacein-Bey-Abina et al., 2003). To generate safe iPS cells, various integration-free techniques have been reported, such as transient expression of the reprogramming factors using adenovirus (Zhou and Freed, 2009) or Sendai virus (Fusaki et al., 2009) vectors, the piggyBac system (Woltjen et al., 2009), episomal vectors (Yu et al., 2009; Okita et al., 2011), minicircle vector (Jia et al., 2010), and direct delivery of protein (Kim et al., 2009) or synthetic RNA (Warren et al., 2010). However, their efficiencies of iPS cell induction are lower than that with retrovirus vectors, possibly because of low transduction efficiency, and unstable expression (Okita and Yamanaka, 2010).

1.5. ES vs. iPS cells – similarities and differences

Like ES cells, iPS cells also have characteristic morphology, and unlimited proliferation capability in vitro, while maintaining their pluripotency. However, recent studies have revealed some differences between iPS cells and ES cells in terms of gene expression, epigenetic modification, and differentiation potentials.

DNA microarray analyses confirmed that gene expression profile of iPS cells does not completely resemble to that of ES cells (Okita et al., 2007). The iPS cells show epigenetic modification similar to those of ES cells in terms of DNA methylation and histone modification, but recent studies revealed that there are differences in DNA methylation of iPS cells to that of ES

cells (Doi et al., 2009; Okita et al., 2007). The iPS cells have some "memory" of their somatic origin, and are not identical to ES cells. There is still no sufficient evidence to determine whether the memory of human iPS cells is fatal for cell therapy. Recently, it has been reported that mouse iPS cells established from fetal and adult fibroblasts vary in their potential to differentiate into a neuronal lineage (Miura et al., 2009). Although both iPS cells can contribute to chimeric mice when transplanted into an early embryo, they show clear differences in their in vitro differentiation. Almost all iPS cells established from mouse embryonic fibroblast (MEF) cells became neuronal cells and only small portion of cells remained in an undifferentiated state like ES cells in an *in vitro* neuronal differentiation procedure. On the other hand, iPS cells established from adult tail fibroblasts tend to maintain their undifferentiated state. These undifferentiated cells could form tumors after transplantation into the mouse brain. Thus, the memory of iPS cells may have some influence on their safety.

In addition to the differences in gene expression and epigenetic modification, iPS cells also show difference in their differentiation potential compared to ES cells. Human iPS cells are able to make functional neuronal cells, blood cells, hepatocytes, and retinal cells. But, there are some reports that human iPS cells show attenuated differentiation potential into neuronal or hematopoietic lineages in comparison to ES cells (Hu et al., 2010; Feng et al., 2010). These results indicate limited application of human iPS cells, and suggest the need for improvement of reprogramming quality.

1.6. Variation in the safety and quality of iPS cell lines

The iPS cells have been derived from various tissues, including embryonic fibroblasts (Okita et al, 2007), adult tail-tip fibroblasts (TTFs) (Nakagawa et al., 2008), hepatocytes (Aoi et al., 2008), gastric epithelial cells (Aoi et al., 2008), pancreatic cells (Stadtfeld et al., 2008), neural stem cells (Silva et al., 2008; Kim et al., 2008; Eminli et al., 2008) and B lymphocytes (Hanna et al., 2008) in the mouse; and skin fibroblasts (Park et al., 2008; Yu et al., 2007; Takahashi et al., 2007), keratinocytes (Aasen et al., 2008), immature dental pulp stem cells (Beltrao-Braga et al., 2011), peripheral blood cells (Loh et al., 2009), and T lymphocytes (Brown et al., 2010) in the

human. Tissue origin of iPS cells appears to influence the differentiation potential and also the teratoma forming propensity after differentiation (Miura et al., 2009). In a study, secondary neurospheres (SNS) were generated from various mouse iPS cell clones whose origin were mouse embryonic fibroblast (MEF), TTF, hepatocyte (Hep) or gastric epithelial cell (Stm), and from ES cells, and examined their neural differentiation capacity and teratoma-forming propensity after transplantation into the brains of non-obese/severe combined immunodeficient (NOD/SCID) mice. Both iPS cell- and ES cell-derived SNS effectively differentiated into tri-lineage neural cells, that is, neurons, astrocytes and oligodendrocytes, *in vitro* or *in vivo,* but with different efficiency (Miura et al., 2009). SNS from TTF-iPS cells showed the highest teratoma-forming propensity, whereas those from MEF-iPS cells and Stm-iPS cells showed the lowest, being comparable to those from ES cells. SNS from Hep-iPS cells showed an intermediate propensity. This teratoma-forming propensity of SNS was not affected by the presence or absence of *c-Myc* retrovirus. Furthermore, reactivation of *c-Myc* or other transgenes in SNS or teratomas was not detected. However, it was observed that tumorigenicity depends on the number of undifferentiated cells in NS, and the number of undifferentiated cells in neuronal differentiation varies depending on tissue origin of iPS cells. The exact mechanisms underlying the different teratoma-forming propensities of iPS cells remain to be determined. Thus, the tissue origin of iPS cells, methods used for reprogramming and differentiation, and other factors that affect the safety and quality of iPS cells and their differentiation efficiency must be rigorously evaluated before their use in cell therapies (Miura et al., 2009).

1.7. Selection of good ES/iPS cell line

Human ES/iPS cells are potential tools for regenerative medicine. However, many issues have to be addressed before their clinical application. For example, differentiation propensities are reported to be different among ES/iPS cell lines (Osafune et al., 2008). Besides, depending on the origin or procedure of derivation, some iPS cell lines demonstrated resistance for differentiation or are tumorigenic (Miura et al., 2009; Tateishi et al., 2008). Abnormalities of karyotype and variations in the techniques used to obtain or maintain ES/iPS cell lines and epigenetic differences

among them have also been considered the vital factors to address. Epigenetic variations are more pronounced in iPS cells than ES cells. Thus, selection of good iPS cell lines, comparable to ES cells, is very important to employ them for differentiation into target lineages prior to use in specific cell therapy (Osafune et al., 2008). In addition, differentiated cells prepared from patient-specific iPS cells could increase the success rate of cell therapy in future.

1.8. ES/iPS cells – the surrogate cell sources for regenerative medicine

ES/iPS cells can be differentiated to a certain lineage *in vitro* by mimicking the major signaling pathways of embryonic development, such as addition of growth factors in culture or regulation of the transcription factors those are involved in specific lineage decisions (Yamashita et al., 2000; Wichterle et al., 2002; Ying et al., 2003). Generation of neural (Kobayashi et al., 2012; Nori et al., 2011), hepatic (Takayama et al., 2013; Yamazoe et al., 2013; Kawabata et al., 2012; Nakamura et al., 2012), cardiomyocyte (Arshi et al., 2013), adipocytes (Nishio and Saeki, 2014), and intestinal (Ogaki et al., 2013; Spence et al., 2011) cells/tissues from hES/iPS cells has been reported. Many researchers also proposed the differentiation procedures to pancreatic cell lineages from hES/iPS cells (Rezania et al., 2013; Kunisada et al., 2012; Rezania et al., 2012; Chen et al., 2009; Zhang et al., 2009; Kroon et al., 2008, Tateishi et al., 2008; Jiang et al., 2007a; Jiang W et al., 2007b; D'Amour et al., 2006). Therefore, ES/iPS cells are considered as the potential surrogate cell sources for regenerative medicine. The iPS cells have fewer ethical concerns than ES cells, which are derived from living embryos. However, further extensive researches are required to clearly understand the nature and the therapeutic usefulness of iPS cells.

1.9. Differentiation of human ES/iPS cells into pancreatic endocrine cells

The human ES cells (hESCs) were derived (Thomson et al., 1998) ~17 years after that of the mouse ES cells (mESCs) first derived successfully (Evans and Kaufman, 1981; Martin, 1981). Therefore, the differentiation protocols for insulin-producing cells were first established for mESCs. These protocols were dependent on either transfection with an expression construct harboring an antibiotic resistance gene under control of the insulin promoter (Soria et al., 2000),

or embryoid body formation (Shiroi et al., 2002). Insulin-producing cells with pancreatic β-cell characteristics were also successfully derived from hESCs (Assady et al., 2001). Later, numerous methods for differentiation of insulin-producing cells from human ES/iPS cells in monolayer culture have been reported (Table 1, Adapted from Van Hoof et al., 2009 and Hosoya, 2012). Formation of embryoid bodies, which mimic germ-layer specification during early embryogenesis, has also been applied in some methods for pancreatic differentiation of hESCs (Phillips et al., 2007; Shim et al., 2007; Mao et al., 2009; Schulz et al., 2012) (Table 1). Based on information from embryonic pancreatic development, each differentiation protocol has been designed where various cytokines or their signaling modulators were sequentially used.

Table 1. Various multistep procedures for pancreatic β-cell differentiation from human ES/iPS cells

References	Cell lines	Differentiation Conditions	Schematic of differentiation procedures						Transplant recipient	Transplantation site
			Step 1	Step 2	Step 3	Step 4	Step 5	Step 6		
D'Amour et al., 2006	CyT203	On low density MEFs	Activin A		CYC, FGF10		±Ex-4		n.a	n.a
			Wnt3A			RA	±DAPT	IGF-1, HGF		
Jiang et al., 2007a	H1,H7,H9	On Matrigel	Activin A Na-bu	EGF, Nog		NA, IGF-2			n.a	n.a
				bFGF						
Jiang et al., 2007b	H1, H9	On Matrigel	No factors	Activin A	RA	bFGF	bFGF, NA	bFGF, NA (Suspension)	Male BALB/c nude mice	Kidney capsule
Phillips et al., 2007	HES-1, HES-2, HES-3, HES-4, HUES-7, HUES-9	Suspension (EB formation), Matrigel	Activin A, BMP4	HGF, Ex-4, β-cellulin					Male SCID mice	Intraperitoneal injection
Shim et al., 2007	Miz-hES4, Miz-hES6	Suspension (EB formation)	No factors	Activin A	RA	Dissociation and plating, Fibronectin			Male BALB/c nude mice	Kidney capsule
Eshpeter et al ., 2008	H1	On Matrigel	Activin A Na-bu	EGF, Nog		NA, IGF-2	NA		Diabetic C57BL6 Rag -1/-1 mice	Kidney capsule
				bFGF						
Kroon et al., 2008	CyT49	On low density MEFs		Activin A	KGF	Noggin	No factors	Transplantation (In vivo maturation)	Male SCID-Beige mice	Epididymal fat pad
			Wnt3A			RA, CYC				
Cho et al., 2008	SNUhES3	On low density MEFs		Activin A	CYC, FGF10		DAPT, Ex-4 NA, β-cellulin	IGF-1, Ex-4, HGF, NA, β-cellulin	n.a	n.a
			Wnt3A			RA				

Continued…….

References	Cell lines	Differentiation Conditions	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	Transplant recipient	Transplantation site
Mao et al., 2009	PKU1.1	Suspension (EB formation), Gelatin	Fibronectin	bFGF	Ex-4	PLGA scaffold, Ex4				Male SCID mice	Kidney capsule and subcutaneous
Chen et al., 2009	HUES-2 HUES-4 HUES-8 HUES-9	On low density MEFs	Activin A		CYC		ILV		HGF	CD1 nude mice	Kidney capsule
			Wnt3A		FGF10			DAPT	IGF-1		
						RA	Ex-4				
Zhang et al., 2009	H1, H9	On Matrigel	Activin A Wortmannin	RA, Nog, FGF7	EGF	bFGF, NA, Ex-4, BMP4				n.a	n.a
Kunisada et al., 2012	253G1	On low density MEFs	Activin A		RA, DM SB431542	NA, FRKL Dex, Alk5i				n.a	n.a
			CHIR99021								
Rezania et al., 2012	H1 ESI-49	On Matrigel	Activin A			Nog		Suspension and rotating	Transplan-tation (In vivo maturation)	Male SCID-beige and STZ-injected diabetic mice	Kidney capsule
			Wnt3A			RA	TBP	Nog			
					FGF7	SANT-1	Alk5i	Alk5i			
Schulz et al., 2012	CyT49	Suspension (Aggregates)	Activin A		KGF	TT, CYC, Nog	KGF, EGF, Nog	Transplan-tation (In vivo maturation)		Male SCID-Beige mice	Epididymal fat pad
			Wnt3A		TBI						
			Spin at 95 rpm		105 rpm		95 rpm				
Rezania et al., 2013	H1	On Matrigel	Activin A			Nog		Suspension and rotating	Transplan-tation (In vivo maturation)	Male SCID-beige and STZ-injected diabetic mice	Subcutaneous (using Theracyte macro-encapsulation device)
			$NaHCO_3$		$NaHCO_3$	RA	±TBP				
			Wnt3A		FGF7	SANT-1		LDN			
						±Alk5i		Alk5i			

Growth factors applied in each step are summarized. MEFs, mouse embryonic fibroblasts; Na-bu, Na-butyrate; bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein 4; DAPT, γ-secretase inhibitor; EGF, epidermal growth factor; FGF10, fibroblast growth factor 10; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor 2; KGF, keratinocyte growth factor; FGF7, fibroblast growth factor 7; RA, all-trans retinoic acid; CYC, KAAD-cyclopamine; SANT-1, a sonic hedgehog signaling inhibitor; Ex-4, exendin-4; FRKL, forskolin; DM, dorsomorphin; Nog, noggin; ILV, (-)-indolactam V; NA, nicotinamide; Dex, dexamethasone; ALK5i, a TGFβ type 1 receptor kinase inhibitor II; TBI, a TGFβ type 1 receptor kinase inhibitor IV; TT, TTNPB; TBP, a PKC activator; LDN, a BMP inhibitor; SB431542, a TGFβ type 1 receptor kinase inhibitor VI; n.a, not applicable.

All of these protocols shown can induce insulin-positive cells as well as glucagon- and somatostatin-positive cells (Table 2). Pancreatic polypeptide- and ghrelin-positive cells were also induced in some protocols. The percentage of insulin-positive cells in culture varied among protocols.

Table 2. Pancreatic endocrine cell types induced and the percent of the insulin-positive cells generated by various procedures

References	Cell types induced	% of insulin-positive cells	Glucose-stimulated C-peptide /insulin secretion	Amelioration of hyperglycemia
D'Amour et al. 2006	INS+, GCG+, SST+, PPY+, GHRL+	7.3% (3-12%)	No	n.d
Jiang et al., 2007a	INS+, GCG+, SST+	4.1%	In vitro (moderate)	n.d
Jiang et al., 2007b	INS+, GCG+, SST+ [n.t: PPY+, GHRL+]	>15%	In vitro (moderate)	Yes
Phillips et al., 2007	INS+, GCG+, SST+	n.d	In vitro (marginal)	No
Shim et al., 2007	INS+, GCG+, SST+	n.d	n.d	Yes
Eshpeter et al ., 2008	INS+, GCG+, SST+	5.3%	In vivo (moderate)	No
Kroon et al., 2008	INS+, GCG+, SST+, PPY+, GHRL+	n.d	In vivo (moderate)	Yes
Cho et al., 2008	INS+, GCG+	n.d	n.d	n.d
Mao et al., 2009	INS+, GCG+, SST+, PPY+	n.d	n.d	No
Chen et al., 2009	INS+, GCG+, SST+, [n.t: PPY+, GHRL+]	0.8±0.4%	In vitro (marginal)	n.d
Zhang et al., 2009	INS+, SST+	25%	In vitro (moderate)	n.d
Kunisada et al., 2012	INS+, GCG+, SST+, GHRL+	11.8% (8.0-16.9%)	No	n.d
Rezania et al., 2012	INS+, GCG+, SST+, PPY+	~10% (Pre-transplantation)	In vivo (moderate)	Yes
Schulz et al., 2012	INS+, GCG+, SST+, [n.t: PPY+, GHRL+]	n.d	In vivo (moderate)	Yes
Rezania et al., 2013	INS+, GCG+, SST+, PPY+, GHRL+	~55-60% (Post transplantation)	In vivo (moderate)	Yes

INS, insulin; GCG, glucagon; SST, somatostatin; PPY, pancreatic polypeptide; GHRL, ghrelin; n.t, not tested (indicated by red color); n.d, not determined

In each differentiation protocol, human ES/iPS cells were directed *in vitro* through the key stages of embryonic pancreatic development: definitive endoderm (DE), primitive gut tube (PG), pancreatic progenitor (PP), endocrine progenitor (EP), and hormone-expressing endocrine (EC) cells.

Human ES cell could be differentiated into definitive endoderm cells by culturing under low-serum condition with activin A and mouse embryonic fibroblast (MEF) (D'Amour et al., 2005). The differentiation efficiency into definitive endoderm were further improved using Wnt3a in addition of activin A and in the complete absence of serum on the first day, followed by two days treatment with activin A and 0.2% serum (D'Amour et al., 2006). Definitive endoderm has also been induced from human ES cells grown on human feeder cells (Zhou et al., 2008), or cultured under feeder-free conditions in the absence of xeno factors (Zhou et al., 2008; King et al., 2008). It has also been shown that suppression of PI3K activity and insulin signaling are critical for efficient differentiation into definitive endoderm (McLean et al., 2007). Inhibition of PI3K signaling by wortmannin showed enhanced efficiency of definitive endoderm induction in another study (Zhang et al., 2009). The addition of activin A and Na-butyrate (a histone deacetylase inhibitor) at the initial stage also increases the expression levels of the definitive endoderm markers (Jiang et al., 2007a). Very recently, it has been shown that addition of CHIR99021 (an activator of Wnt signaling through inhibition of GSK3) along with activin A treatment resulted in enhanced efficiency of definitive endoderm differentiation *in vitro*, possibly by mimicking the regulated expression and action of both activin/Nodal and Wnt signaling pathways. In this study, Kunisada et al. have also compared Wnt3a and CHIR99021 effect under the same condition, and shown that CHIR99021 was more efficient than Wnt3a in inducing Sox17/Foxa2-positive definitive endoderm cells from various human iPS cells (Kunisada et al., 2012).

The definitive endoderm cells start to form primitive gut tube cells and adopt the primitive gut tube-like characteristics just after the removal of activin A. Fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling are known to regulate pancreatic lineage

differentiation during early embryonic development. Therefore, basic FGF (bFGF; also known as FGF2), keratinocyte growth factor (KGF; also known as FGF7), FGF10 and/or Noggin (an endogenous protein that inhibits BMP) are used in some methods to induce definitive endoderm and/or primitive gut tube cells into pancreatic progenitor cells (D'Amour et al., 2006; Jiang et al., 2007a; Kroon et al., 2008; Eshpeter et al., 2008; Zhang et al., 2009; Chen et al., 2009; Schulz et al., 2012; Rezania et al., 2012, 2013). Hedgehog expression is suppressed in the pancreatic primordium compared with that in surrounding organs, thus hedgehog signaling inhibitors (KADD-cyclopamine) or SANT-1 are used in many methods (D'Amour et al., 2006; Kroon et al., 2008; Cho et al., 2008; Chen et al., 2009; Schulz et al., 2012; Rezania et al., 2012, 2013). Notch signaling is known to control multiple steps of pancreatic differentiation. Notch signal activation has been implicated in the self-renewal of Pdx1-expressing pancreatic progenitors, whereas, Ngn3 expression in the pancreas occurs as a result of decreased Notch signaling. Continuous expression of FGF10 in embryonic pancreas activates Notch signaling and blocks endocrine differentiation, so the sequential use of FGF10 and DAPT, a gamma-secretase inhibitor that blocks Notch signaling, were included in some protocols (D'Amour et al., 2006; Cho et al., 2008; Chen et al., 2009). EGF treatment can increase the number of Pdx1-positive pancreatic progenitor cells *in vitro,* thus might be beneficial for generating large numbers of endocrine cells (Zhang et al., 2009). Furthermore, Chen et al. (2009) reported that (-)-indolactam V, a protein kinase C activator, efficiently induces the differentiation of human ESCs into Pdx1-expressing cells. In the method reported by Kunisada et al. (2012) Pdx1 expression is induced when Sox17-positive endoderm cells are exposed to retinoic acid and dorsomorphin (a BMP type I receptor inhibitor), while differentiation proceeds towards Ngn3-positive pancreatic endocrine precursor cells when SB431542, a transforming growth factor β (TGFβ) type I receptor kinase inhibitor VI, is added simultaneously. However, the derived progenitor cells expressed both Ngn3 and Pdx1 genes at the same stage of differentiation (Kunisada et al., 2012).

There are many other growth factors, such as insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF) and glucagon-like peptide-1 (GLP-1) or exendin-4 (a peptide analog of GLP-

1), have also been used to facilitate differentiation of all five pancreatic hormones-expressing endocrine cells. Although consistent well with organogenetic process of pancreas, however, D'Amour et al. (2006) have observed minor differences in the differentiation when DAPT, as well as exendin-4, IGF-1 and HGF, were omitted. Various classes of small molecules have also been reported to be effective for differentiating human ES/iPS cells into insulin-producing cells. Nicotinamide, a poly(ADP-ribose) synthetase inhibitor, has been shown to induce endocrine pancreatic differentiation and maturation (Otonkoski et al., 1993), and it is used in some protocols to improve the yield of pancreatic endocrine cells (Jiang et al., 2007a; Jiang et al., 2007b; Eshpeter et al., 2008; Cho et al., 2008; Zhang et al., 2009; Kunisada et al., 2012). Use of TGFβ type I receptor kinase inhibitor II (activin receptor-like kinase 5; ALK5i) in pancreatic-α and β-cell differentiation systems has also been reported (Kunisada et al., 2012; Rezania et al., 2012; 2013). Forskolin (an activator of adenylyl cyclase) and dexamethasone (a synthetic adrenocortical steroid) have also been shown to enhance cellular maturation; these agents could be combined with other small molecules to obtain synergistic effects (Kunisada et al., 2012).

The functionality and maturity of pancreatic β-like cells obtained by differentiation from human ES/iPS cells in vitro remain controversial. Detailed analysis of pancreatic β-like cells derived from human ES cells has revealed the expression of genes related to β-cell functions, such as glucose sensing, exocytosis and transcriptional factors involved in the pancreatic endocrine system (Basford et al., 2012). However, to date, pancreatic β-like cells generated from hES/iPS cells in vitro are largely polyhormonal (e.g., insulin/glucagon/somatostatin-triple positive) and exhibit limited capacity of glucose-stimulated insulin secretion (GSIS), a characteristic of functionally mature β-cells (Bruin et al., 2014; Chen et al., 2009; D'Amour et al., 2006; Kunisada et al., 2012; Zhang et al., 2009). These cells possibly resemble the immature β-cells that arise during primary transition (D'Amour et al., 2006). Although several of the above studies describe the detection of glucose stimulated C-peptide secretion from human ES/iPS cells-derived pancreatic endocrine cells *in vitro*, none of the above reported cells are capable of efficiently restoring euglycemia in diabetic animal model *in vivo*. Kroon et al. (2008) and Rezania et al. (2012; 2013) described a new strategy to generate glucose-responsive insulin-producing cells.

After pancreatic endoderm differentiation from human ES cells in vitro, these cells were transplanted into the immune compromised normoglycemic and/or diabetic model mice to allow further differentiation and maturation *in vivo*. Few months after transplantation, these grafts became fully responsive to glucose, and most of the endocrine cells became mature mono-hormonal cells.

There are still many points to address and problems to overcome, including transplantation site and the optimum number of transplantable cells, the efficacy of treatment and duration of action, immunogenicity, and safety issues including tumorigenicity before clinical application of ES/iPS derived cells in diabetic patients. Indeed, a simple, reproducible, safe, and scalable protocol for the differentiation of pancreatic β-cells from human ES/iPS cells is needed in the future.

1.10. Objectives of the study

So far, most studies reported the generation of pancreatic endocrine cells (ECs) in vitro from hES/iPS cells in feeder-cell culture systems (Chen et al., 2009; D'Amour et al., 2006; Kroon et al., 2008; Kunisada et al., 2012) or feeder-free culture systems (Jiang et al., 2007a, 2007b; Rezania et al., 2012; Zhang et al., 2009). Moreover, many of the current differentiation protocols utilize a variety of undefined animal-derived products that may have unknown effects on cell characteristics and differentiation. The potential consequences of transplanting human cells exposed to animal-derived products into patients include an increased risk of graft rejection, immunoreactions, and microbial infections, prions, and yet unidentified zoonoses (Martin et al., 2005; Cobo et al., 2005; Skottman and Hovatta, 2006). Some reports describe protocols that involved the use of xeno-free components to generate pancreatic ECs from human pluripotent stem cells (Micallef et al., 2012; Schulz et al., 2012). Micallef et al. (2012) used xeno-free media; however, they also used mouse embryonic fibroblasts for passaging. Schulz et al. (2012) expanded hES cells in xeno-free media without feeder cells but they used fetal bovine serum during differentiation. Therefore, the establishment of a defined and completely xeno-free culture

system with which functional and terminally differentiated endocrine cell types can be generated from hiPS cells is needed for future research and clinical applications.

To address these issues, I established for the first time a defined and completely xeno-free culture system to derive INS-expressing β-like cells from hiPS cells using a synthetic scaffold and serum-free media containing humanized and/or recombinant supplements and growth factors. I demonstrated that combined use of NOGGIN and 3-isobutyl-1-methylxanthine (IBMX) enhanced and directed hiPS-derived cells to differentiate into INS-expressing β-like cells. The differentiated cells secreted C-peptide in vitro in response to various insulin-secretagogues and high glucose levels and expressed several pancreatic β-cell markers.

2. MATERIALS AND METHODS

2.1 Cell lines

Toe, a hiPS cell line, was established from human embryonic lung cells by infection with retroviruses carrying *OCT4, SOX2, KLF4* and *C-MYC* genes by Toyoda M., Kiyokawa N., Okita H., Miyagawa Y., Akutsu H., and Umezawa A. at the National Institute for Child Health and Development, Tokyo, Japan (Yamazoe et al., 2013). I obtained the Toe cell line from the cell bank of the National Institute of Biomedical Innovation, Japan. The cells were initially proliferated and maintained in an undifferentiated state under xenogeneic conditions up to P26 (passage 26) as described previously (Shiraki et al., 2008) and were freeze-stored at -150°C. I also used KhES3, a hES cell line, obtained from Drs. Norio Nakatsuji and Hirofumi Suemori of Kyoto University; and 201B7, a hiPS cell line, obtained from Dr. Shinya Yamanaka of Kyoto University, Japan. Undifferentiated KhES3 and 201B7 were also maintained as described (Shiraki et al., 2008). Human ES cell study was approved by Kumamoto University's Institutional Review Board, following the hES cell guidelines of the Japanese government.

2.2. Xenogeneic and xeno-free undifferentiated hES/iPS cell culture

Freeze-stored hES/iPS cells were thawed and cultured on CellBIND cell culture dishes (Corning) coated with a xeno-free synthetic scaffold (Synthemax II-SC Substrate, functionalized with short peptide sequences derived from the vitronectin protein, which is covalently linked to the synthetic acrylate polymer, Corning) under feeder-free conditions with hES/iPS cell maintenance medium.

For xenogeneic culture, the maintenance medium was composed of Knockout DMEM/F12 (Life Technologies) supplemented with penicillin-streptomycin (50 units/ml penicillin, 50 μg/ml streptomycin [PS]; Nacalai Tesque), 2 mM L-glutamine (L-Gln; Nacalai Tesque), 1% nonessential amino acids (NEAA; Life Technologies), 0.1 mM 2-mercaptoethanol (2-ME; Sigma-Aldrich), 20% (v/v) Knockout serum replacement (KSR; Life Technologies), and 5 ng/ml recombinant human FGF2 (rhFGF2; PeproTech) reconstituted with 0.1% bovine serum albumin (Sigma-Aldrich). Freeze-stored hiPS cells were grown with this maintenance medium for 3–4 days and used for xenogeneic differentiation (Figure 2A).

For xeno-free culture, KSR and rhFGF2 used in the xenogeneic maintenance medium were replaced with the KSR xeno-free Cell Therapy System (CTS) (Life Technologies) and rhFGF2 reconstituted with 0.1% recombinant human albumin (Sigma-Aldrich), respectively. The medium was also supplemented with 1% KSR growth factor cocktail CTS (Life Technologies). Xeno-free maintenance medium is, therefore, free from animal-derived factors; rather, it contains all humanized and/or recombinant supplements and defined growth factors. Freeze-stored hES/iPS cells were first grown with xenogeneic maintenance medium for 3–4 days, and then sequentially passaged with xeno-free maintenance medium at a ratio of 1:3 every 3–4 days by dissociating cell colonies with a cell dissociation buffer (Life Technologies) before they were used for xeno-free differentiation (Figure 2A).

2.3. In vitro differentiation of undifferentiated hES/iPS cells

For pancreatic differentiation, undifferentiated hES/iPS cells were dissociated with TrypLE Select CTS (Life Technologies) after three consecutive passages under xeno-free conditions, seeded at a density of 1×10^5 cells/well on 96-well CellBIND cell culture plates coated with Synthemax II-SC Substrate. They were cultured for 1 day using xeno-free maintenance medium containing 10 μM ROCK inhibitor (Y-27632; Wako), followed by another 1–2 days of culture without ROCK inhibitor to 80–90% confluence. Cells were directed through the following key stages of pancreatic development: definitive endoderm (DE; stage 1), primitive gut tube (PG; stage 2), pancreatic progenitor (PP cells; stage 3), endocrine progenitor (EP; stage 4), and hormone-expressing endocrine cells (EC; stage 5) (Figure 4A).

At stage 1, cells were cultured for 2 days in DMEM-high glucose (Life Technologies) supplemented with PS, 2 mM L-Gln, 1% NEAA, 0.1 mM 2-ME, 2% (v/v) B27 supplement xeno-free CTS (Life Technologies), 100 ng/ml recombinant human activin A (Act, a member of the TGF-β superfamily, HumanZyme), and 3 μM CHIR99021 (a GSK3β-specific inhibitor, TOCRIS Bioscience), and subsequently for another 3 days cultured without CHIR99021.

At stage 2, cells were cultured for 2 days in RPMI 1640 (Life Technologies) supplemented with PS, L-Gln, NEAA, 2-ME, B27 supplement xeno-free CTS, 0.25 μM KAAD-cyclopamine

(Cyc, a sonic hedgehog signaling inhibitor, Stemgent), and 50 ng/ml recombinant human FGF10 (PeproTech).

At stage 3, cells were cultured for 6 days in DMEM-high glucose supplemented with PS, L-Gln, NEAA, 2-ME, B27 supplement xeno-free CTS, 2 μM all-trans RA (Stemgent), 0.25 μM Cyc, 10 μM SB431542 (SB, a TGF-β type I receptor kinase inhibitor VI; CALBIOCHEM), and 200 ng/ml recombinant human NOGGIN (Nog, a BMP inhibitor; R&D Systems), with the media changed every 2 days.

At stage 4, cells were cultured for 2 days in DMEM-high glucose supplemented with PS, L-Gln, NEAA, 2-ME, B27 supplement xeno-free CTS, 5 μM Alk5i (a TGF-β type I receptor kinase inhibitor II; CALBIOCHEM), 300 nM (-) indolactam V (ILV; a protein kinase C activator, R&D Systems), and 200 ng/ml Nog.

At stage 5, cells were cultured for 8 days in DMEM/F12 (Cell Science & Technology Inst.) supplemented with PS, L-Gln, NEAA, 2-ME, and B27 supplement xeno-free CTS. To this medium were added 50 ng/ml exendin-4 (Ex-4; a peptide agonist of the GLP-1 receptor, Cell Sciences), 10 mM nicotinamide (NA; Sigma-Aldrich), and/or 100 μM 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor; Wako), and/or 10 μM forskolin (FRKL, an adenylate cyclase activator; Wako). The media were changed every 2 days.

All factors were reconstituted using 0.1% recombinant human albumin, phosphate-buffered saline (PBS), or DMSO for xeno-free differentiation. A similar xeno-free technique for maintenance and differentiation of hiPS cells was used with the two other xeno-free scaffolds, CELLstart (Life Technologies) and rhVTN (Life Technologies), to compare the differentiation efficiency with that achieved with the Synthemax II SC substrate.

2.4. Flow cytometry

Flow cytometry was performed for the xenoantigenic factor Neu5Gc, as described previously (Martin et al., 2005). Briefly, 1.0×10^6 cells in a total volume of 50 μl were incubated at 4°C for 60 min with a chicken anti-Neu5Gc IgY antibody (Ab) (1:200 dilution, Sialix), chicken IgY negative control (1:200 dilution, Sialix), or without a primary antibody. After three washes, cells

were incubated at 4°C for 60 min with an Alexa Fluor 488-conjugated donkey anti-chicken antibody (1:500, Molecular Probes). Flow cytometry was performed on a BD FACSCanto Flow Cytometer (BD Biosciences) and analyzed using the FlowJo software (Tree Star, Inc.).

2.5. Quantitative RT-PCR analysis

Total RNA was extracted from cells using TRIzol and genomic DNA contamination was removed by digestion with deoxyribonuclease I (Sigma-Aldrich). Total adult human pancreas RNA was purchased from Clontech. cDNA was prepared from 2.0 μg of RNA with oligo-dT primers and the ReverTraAce RT-reagent kit (TOYOBO). The primer sequences used for quantitative RT-PCR are summarized in Table 3. Quantitative RT-PCR was carried out on a 7500 FAST Real-Time PCR System (Applied Biosystems) using the Thunderbird Sybr qPCR Mix (TOYOBO). The expression of each target gene was normalized to the expression level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.6. Alkaline phosphatase staining

The cultured cells were fixed with 4% (w/v) paraformaldehyde (Nacalai Tesque), washed with PBS and then incubated with alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$, and 0.1% Tween-20) for 30 min at room temperature. The staining reaction was carried out with 4-nitroblue tetrazolium chloride (35 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (17.5 μg/ml) (Roche Diagnostics) in the dark for 30 min at room temperature. Cells were then washed with 1 mM EDTA/PBS, fixed with 4% paraformaldehyde, and images were captured.

2.7. Immunocytochemistry

Immunostaining was performed as described previously (Yamazoe et al., 2013). Details on the primary antibodies are provided in Table 4. The secondary antibodies used were Alexa Fluor 488-, 568-, or 633-conjugated donkey or goat anti-mouse, anti-goat, anti-rabbit, anti-sheep, or anti-guinea pig IgG (1:1000 dilutions; Molecular Probes). Nuclei were counterstained with 4′,6-

diamidino-2-phenylindole (DAPI, Roche Applied Science). Images were captured using an ImageXpress Micro scanning system (Molecular Devices, Japan), and quantitative analysis was performed using the MetaXpress cellular image analysis software (Molecular Devices, Japan).

Table 3: Real-time PCR primers

Genes	Primer sequences		Product (bp)
	Forward	Reverse	
GAPDH	CGAGATCCCTCCAAAATCAA	CATGAGTCCTTCCACGATACCAA	288
OCT3/4	GTATTCAGCCAAACGACCATC	CTGGTTCGCTTTCTCTTTCG	176
SOX17	GCTTTCATGGTGTGGGCTAAG	CAGCGCCTTCCACGACTT	108
FOXA2	CTGAGCGAGATCTACCAGTGGA	CAGTCGTTGAAGGAGAGCGAGT	104
HNF1β	ATAGCTCCAACCAGACTCACA	AGGCTGTGGATATTCGTCAA	313
HNF4α	CCAAGAGATCCATGGTGTTCAA	TTGATGTAGTCCTCCAAGCTCA	274
PDX1	CTTGGAAACCAACAACTATTCAC	ATTAAGCATTTCCCACAAACA	218
HNF6	AAATCACCATTTCCCAGCAG	AGCTTTTCCACCGAGGTTTT	192
NKX6.1	CCAAGAAGAAGCAGGACTCG	TCAACAGCTGCGTGATTTTC	126
SOX9	AAAGGCAACTCGTACCCAAATTT	AGTGGGTAATGCGCTTGGAT	63
PROX1	AAAGCAAAGCTCATGTTTTTTTATACC	GTAAAACTCACGGAAATTGCTAAACC	135
HLXB9	GCACCAGTTCAAGCTCAACA	GCCTTTTTGCTGCGTTTCCATTTC	135
CDX2	CTCCTCCCCAGCTCTTCTCT	TCTTAGCTGCCTTTGGCTTC	195
AFP	TGCCAACTCAGTGAGGACAA	TCCAACAGGCCTGAGAAATC	356
NGN3	TCGAGAGAGAGCGTGACAGA	CTACCGGCGCAAAAGAATAG	175
PAX4	CAGACTGTGGCTCCTTCCTC	GGGTGCTCATAGGGAAAACA	224
NEUROD1	CTCCTTCGTTCAGACGCTTT	GTGGAAGACATGGGAGCTGT	226
INS	CATCAGAAGAGGCCATCAAG	TCTTGGGTGTGTAGAAGAAGC	200
GCG	CAGAAGAGGTCGCCATTGTT	TGGCTAGCAGGTGATGTTGT	192
SST	CCAACCAGACGGAGAATGAT	AGGGAAGAGAGATGGGGTGT	241
PPY	TGCCCATTTACTCTGGACTC	ATCTGCTCTGGTGTGGCATT	160
AMY	ATTCGCAAGTGGAATGGAGA	GCCCAACCCAATCATTAACA	283
ISL1	ATTTCCCTATGTGTTGGTTGCG	CGTTCTTGCTGAAGCCGATG	229
MAF-A	TTCAGCAAGGAGGAGGTCAT	CGCCAGCTTCTCGTATTTCT	216
GCK	GGAGAGAAAGCGCTGAGGAC	CTGGTTTGGGGTTTGAGGTT	160
UCN3	GAGGGAAGTCCACTCTCGGG	TGTTGAGGCAGCTGAAGATGG	137
IAPP	AGGCAGATCACAAGGTCAGG	GTGCAATCTCGGCTCACTG	186
SLC30A8	TGTCCCAGAGAGAGACCAGA	CCACGACCTCTGCAATCATG	163

2.8. C-peptide release and content assay

The C-peptide release and content assay was performed as described previously (Sakano et al., 2014) with minor modifications. Briefly, differentiated cells at the end of stage 5 were pre-incubated at 37°C for 30 min with DMEM (Life Technologies) containing minimal essential medium, 1% B27 supplement, and 2.5 mM glucose. Cells were washed twice with PBS and then incubated at 37°C for 1 h with DMEM containing 2.5 mM glucose at 100 µl per well. The culture medium was collected, and the same cells were further incubated with DMEM containing 20 mM glucose or DMEM containing 2.5 mM glucose supplemented with various stimulants, i.e., 2 µM (−)-Bay K8644 (Sigma-Aldrich), 100 µM tolbutamide (Wako), 250 µM carbachol (Sigma-Aldrich), 0.5 mM IBMX, or 30 mM potassium chloride (KCl) (Wako), at 37°C for another 1 h. The culture media were collected and stored at -20°C until analysis. Next, the cells were lysed with lysis buffer (0.1% Triton X-100 in PBS) supplemented with 1% protease inhibitor cocktail (Nacalai Tesque). C-peptide secretion into the culture media and C-peptide content of the cell lysates were measured using the human C-peptide ELISA Kit (ALPCO Diagnostics). The amount of C-peptide was normalized to the amount of total protein in the corresponding cell lysate.

2.9. Statistical analyses

Data are expressed as mean ± SEM (standard error of the mean). For comparisons of discrete data sets, unpaired Student's *t*-tests were performed. Significance levels or *P* values are indicated in the figure legends.

Table 4: Primary antibodies for immunofluorescent staining

Antibody	Source	Product code	Dilution
Mouse anti-OCT3/4	Santa Cruz Biotechnology, Inc.	sc-5279	1:100
Rabbit anti-NANOG	ReproCELL	RCAB003P	1:100
Mouse anti-SSEA4	R&D Systems, Inc.	BAM1435	1:100
Mouse anti-TRA 1-81	Millipore	MAB4381	1:100
Rabbit anti-SOX2	Millipore	AB5603	1:100
Mouse anti-SSEA1	BioLegend	125603	1:100
Goat anti-SOX17	R&D Systems, Inc.	AF1924	1:100
Rabbit anti-HNF3β/FOXA2	Millipore	# 07-633	1:300
Goat anti-HNF4α	Santa Cruz Biotechnology, Inc.	sc-6556	1:100
Goat anti-PDX1	R&D Systems, Inc.	AF2419	1:100
Rabbit anti-HNF6	Santa Cruz Biotechnology, Inc.	sc-13050	1:100
Rabbit anti-SOX9	Millipore	AB5535	1:200
Mouse anti-NKX6.1	Developmental Studies Hybridoma Bank	F64A6B4	1:100
Mouse anti-CDX2	BioGenex	MU392-UC	1:500
Mouse anti-AFP	MONO*SAN*	MON 4035	1:200
Sheep anti-NGN3	R&D Systems, Inc.	AF3444	1:200
Rabbit anti-PAX4	Abcam	ab42450	1:200
Goat anti-NEUROD1	R&D Systems, Inc.	AF2746	1:100
Guinea Pig anti-INSULIN	Dako	A0564	1:500
Rabbit anti-C-PEPTIDE	Cell Signaling Technology, Inc.	#4593	1:200
Mouse anti-GCG	Sigma-Aldrich	G2654	1:300
Goat anti-SST	Santa Cruz Biotechnology, Inc.	sc-7819	1:500
Rabbit anti-SST	Dako	A0566	1:500
Rabbit anti-Pancreatic polypeptide	Dako	A0619	1:300
Mouse anti-Amylase	Santa Cruz Biotechnology, Inc.	sc-46657	1:100
Rabbit anti-UCN3	Phoenix Pharmaceuticals, Inc.	G-019-28	1:500
Goat anti-ISL1	R&D Systems, Inc.	AF1837	1:100
Rabbit anti-IAPP	Abcam	ab15125	1:200

3. RESULTS

3.1. Self-renewal and maintenance of undifferentiated hiPS cells under xeno-free conditions

I found that the levels of *N*-glycolylneuraminic acid (Neu5Gc), an indicator of xenogeneic contamination in human pluripotent stem cell cultures (Martin et al., 2005), markedly decreased to an undetectable level in hiPS cells grown under xeno-free conditions after passage 2 (P2) (Figure 2B). In addition, hiPS cells grown under xeno-free conditions (P3) maintained their self-renewal capacity and pluripotency, as confirmed by positive alkaline phosphatase staining and the expression levels of octamer-4 (OCT4), NANOG, SRY box-2 (SOX2), tumor rejection antigen 1-81 (TRA1–81), and stage-specific embryonic antigen-4 (SSEA-4), which were similar to those of hiPS cells grown under xenogeneic conditions (Figure 3A). There was no detectable expression of stage-specific embryonic antigen-1 (SSEA-1), a marker associated with hES cell differentiation, suggesting that hiPS cells maintained the undifferentiated state under xeno-free conditions. HiPS cells grown under xeno-free conditions also exhibited a distinctive morphology of sharp-edged, flat, and tightly packed colony structures (Figure 3B), characteristic of pluripotent stem cells. Therefore, our xeno-free system is effective for keeping hiPS cells free of contamination from non-human derived factors, while maintaining their pluripotency.

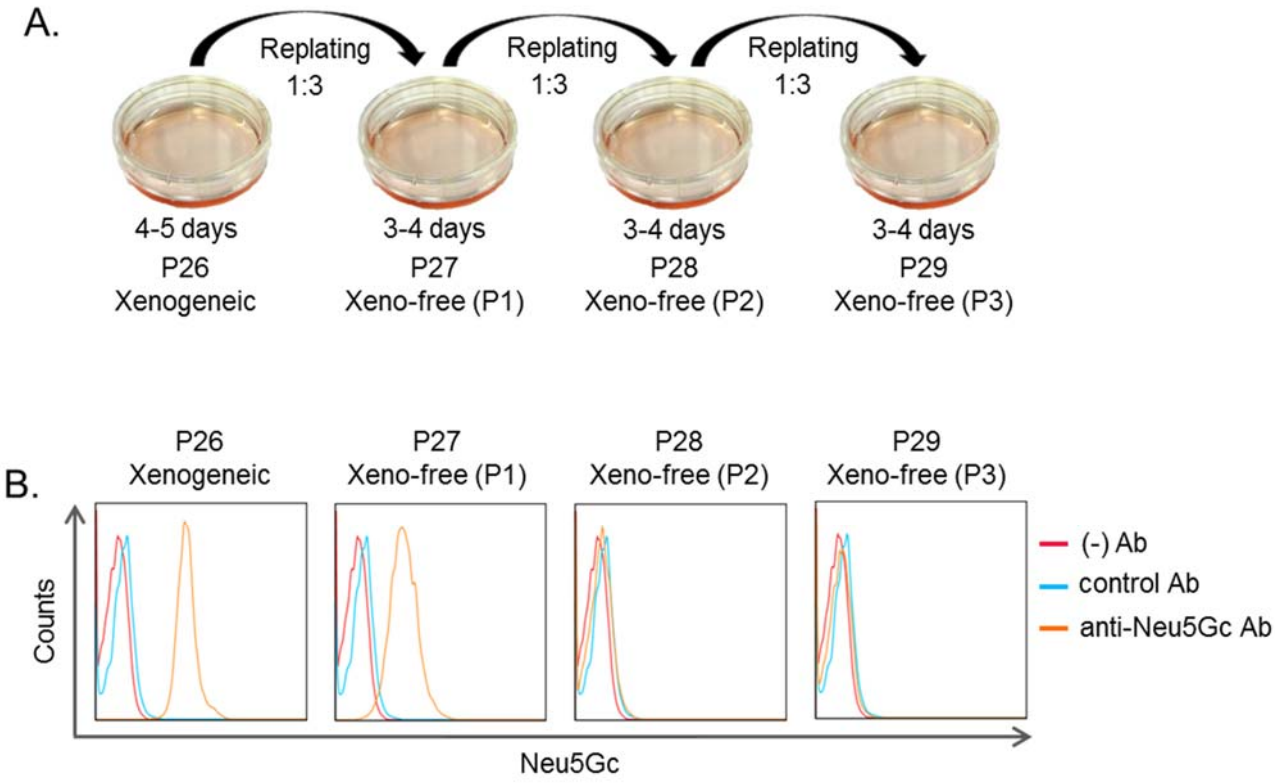

Fig. 2. Maintenance of undifferentiated hiPS cells under xeno-free conditions

HiPS cells grown under xeno-free conditions showed a decreased expression of Neu5Gc after two passages. A) Schematic drawing of the xeno-free culture system for proliferation and re-plating of undifferentiated hiPS cells. B) The expression of Neu5Gc, a marker of xenoantigenic contamination, in undifferentiated hiPS cells grown under xenogeneic or xeno-free conditions by flow cytometry. Cells were exposed to an anti-Neu5Gc antibody (orange), a control antibody (blue), or incubated without a primary antibody (red), and then stained with a secondary antibody for analysis. P, passage; Ab, antibody.

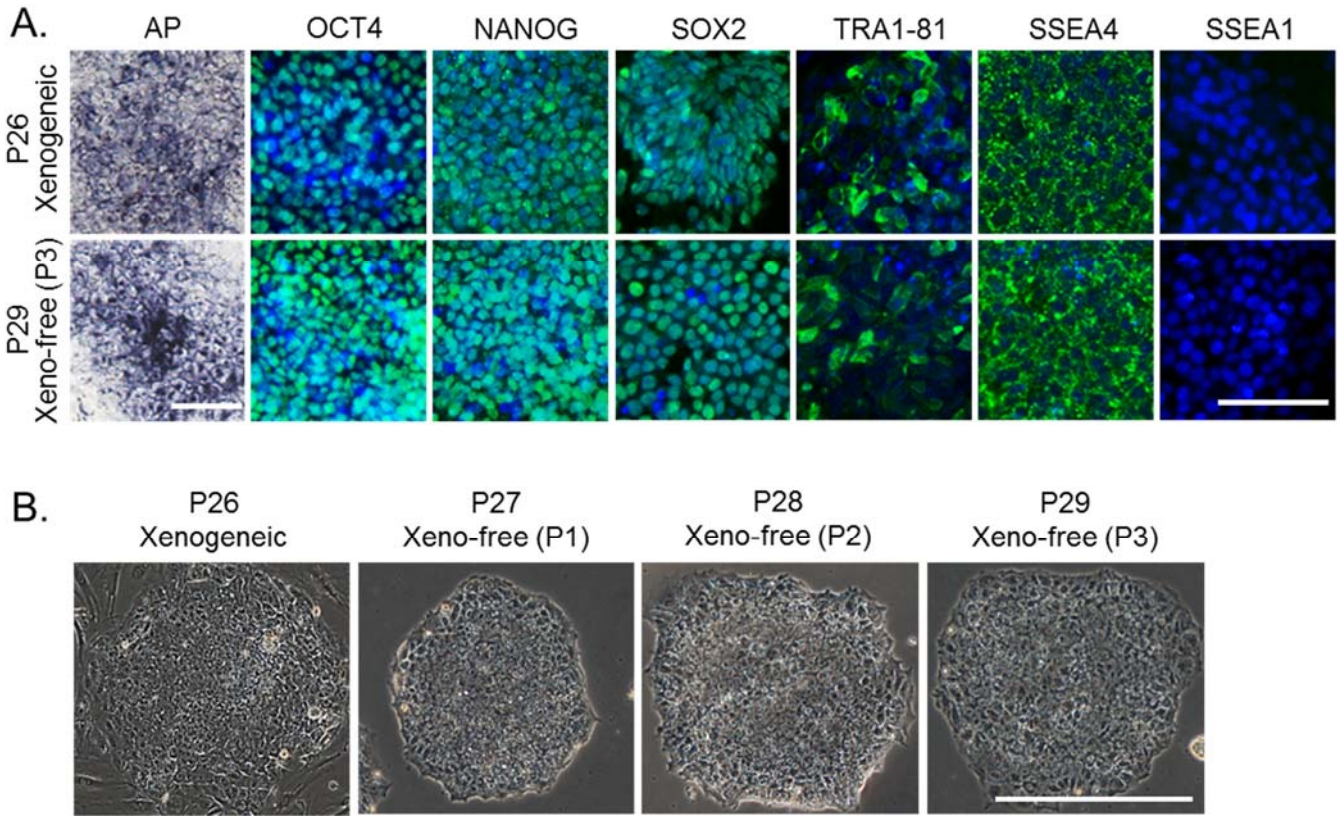

Fig. 3. Self-renewal and pluripotency of undifferentiated hiPS cells grown under xeno-free conditions

HiPS cells grown under xeno-free conditions showed pluripotent characteristics. A) Undifferentiated hiPS cells grown under xenogeneic and xeno-free conditions were subjected to alkaline phosphatase (AP) staining and immunostaining to compare the expression levels of pluripotency-associated markers. Under both xenogeneic and xeno-free conditions, hiPS cells exhibited a similar expression pattern of pluripotency markers, including OCT4, NANOG, SOX2, TRA-1-81, and SSEA4, confirming adaptation and maintenance of pluripotency under xeno-free conditions. SSEA1-positive cells were not observed among the colonies confirming the undifferentiated state of the cells. B) Bright-field images showing the morphology of hiPS cell colonies grown under xeno-free conditions. Cell nuclei were counterstained with DAPI. P, passage; Scale bars are 100 μm (A) or 1 mm (B).

3.2. Differentiation into pancreatic progenitor cells at high NOGGIN concentrations

I developed a five-step protocol for the differentiation of hiPS cells into pancreatic hormone-expressing cells under xeno-free conditions (Fig. 4A) by optimizing the protocol in a stepwise fashion.

At stage 1, hiPS cells were cultured in the presence of activin A (a member of the TGF-β superfamily) and CHIR99021 (a GSK3β-specific inhibitor) for two days, followed by an additional three days of culture in the presence of activin A alone to induce differentiation into definitive endoderm cells. At the end of stage 1, most cells differentiated into SRY box-17 (SOX17)/forkhead box protein A2 (FOXA2)-double positive cells (71.7 ± 2.8% of total cells) and expressed the transcript of the definitive endoderm marker gene *SOX17*, whereas the expression level of the marker gene of undifferentiated hiPS cells, *OCT4*, was markedly decreased (Fig. 4B).

At stage 2, fibroblast growth factor 10 (FGF10) and a hedgehog-signaling inhibitor, KAAD-cyclopamine, were added to allow the transition into primitive gut tube cells. I detected a large proportion of hepatocyte nuclear factor 4a (HNF4a)/FOXA2-double positive cells (77.7 ± 2.3% of total cells) and up-regulation of gut-tube marker genes *FOXA2*, *HNF1b*, and *HNF4a* at the end of stage 2 (Fig. 4C).

At stage 3, combined treatment with retinoic acid (RA), KAAD-cyclopamine, SB431542 (SB, a TGF-b type I receptor kinase inhibitor VI), and NOGGIN (Nog, a BMP signaling inhibitor) induced differentiation into pancreatic and duodenal homeobox-1 (PDX1)-positive pancreatic progenitor (PP) cells. A considerable proportion of alpha-fetoprotein (AFP)-positive hepatic, and caudal-related homeobox 2 (CDX2)-positive (mainly PDX1/CDX2-double positive) intestinal progenitors appeared when cells were treated with 100 ng/ml NOGGIN; their number was significantly reduced when cells were treated with 200–300 ng/ml NOGGIN (Fig. 4D, upper panels). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis also revealed that the expression of *CDX2* and *AFP* was significantly reduced at higher concentrations of NOGGIN. However, the expression of the posterior foregut genes *PDX1*, *hepatocyte nuclear factor-6 (HNF6)*, and *NK-related homeobox 6.1* (*NKX6.1*) (Habener et al., 2005; Jacquemin et al.,

2003) and that of the pancreatic dorsal bud gene *homeobox HB9 (HLXB9)* (Li et al., 1999) was significantly up-regulated (Fig. 4D, lower panels). These results suggest that BMP signaling is inhibitory for the differentiation into pancreatic lineages and high NOGGIN concentrations resulted in the differentiation into a high proportion of PP cells. Approximately 75% of the cells were PDX1-positive, 62% were PDX1/HNF6-double positive, 63% were PDX1/NKX6.1-double positive, and 59% were PDX1/SOX9-double positive, whereas only 8% of the cells were CDX2-positive and 4% cells were AFP-positive among all 4',6-diamidino-2-phenylindole (DAPI)-positive cells when 200 ng/ml NOGGIN was added to stage-3 media (Fig. 4D, upper panels and E), suggesting that high NOGGIN concentrations efficiently directed the differentiation into PP cells.

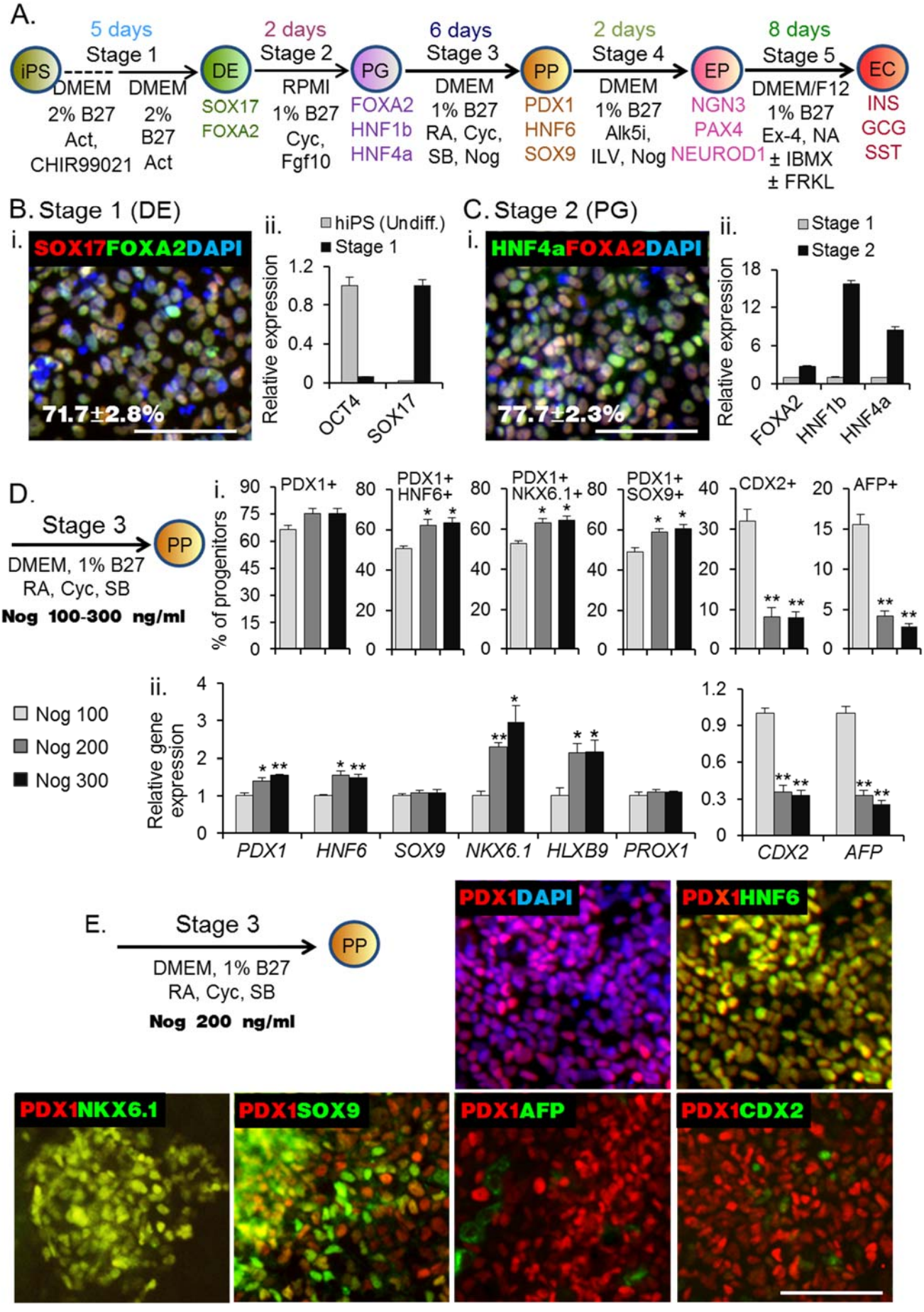
A.
5 days
Stage 1
2 days
Stage 2
6 days
Stage 3
2 days
Stage 4
8 days
Stage 5
iPS
DE
PG
PP
EP
EC
DMEM 2% B27 Act, CHIR99021
DMEM 2% B27 Act
SOX17 FOXA2
RPMI 1% B27 Cyc, Fgf10
FOXA2 HNF1b HNF4a
DMEM 1% B27 RA, Cyc, SB, Nog
PDX1 HNF6 SOX9
DMEM 1% B27 Alk5i, ILV, Nog
NGN3 PAX4 NEUROD1
DMEM/F12 1% B27 Ex-4, NA ± IBMX ± FRKL
INS GCG SST
B. Stage 1 (DE)
i.
SOX17FOXA2DAPI
71.7±2.8%
ii.
hiPS (Undiff.)
Stage 1
Relative expression
OCT4
SOX17
C. Stage 2 (PG)
i.
HNF4aFOXA2DAPI
77.7±2.3%
ii.
Stage 1
Stage 2
Relative expression
FOXA2
HNF1b
HNF4a
D.
Stage 3
PP
DMEM, 1% B27
RA, Cyc, SB
Nog 100-300 ng/ml
i.
% of progenitors
PDX1+
PDX1+ HNF6+
PDX1+ NKX6.1+
PDX1+ SOX9+
CDX2+
AFP+
Nog 100
Nog 200
Nog 300
ii.
Relative gene expression
PDX1
HNF6
SOX9
NKX6.1
HLXB9
PROX1
CDX2
AFP
E.
Stage 3
PP
DMEM, 1% B27
RA, Cyc, SB
Nog 200 ng/ml
PDX1DAPI
PDX1HNF6
PDX1NKX6.1
PDX1SOX9
PDX1AFP
PDX1CDX2

Fig. 4. In vitro xeno-free differentiation of hiPS cells into definitive endoderm (DE), primitive gut tube (PG), and pancreatic progenitor (PP) cells

The protocol was optimized to ensure hiPS cell differentiation into DE, PG, and PP cells. High concentrations of NOGGIN directed the differentiation into pancreatic lineages while suppressing differentiation into other lineages. A) Schematic of the differentiation procedure into definitive endoderm (DE; stage 1), primitive gut tube (PG; stage 2), pancreatic progenitor cells (PP; stage 3), endocrine progenitor cells (EP; stage 4), and endocrine cells (EC; stage 5). Act, activin A; CHIR99021, a GSK3β-specific inhibitor; FGF10, fibroblast growth factor-10; cyc, KAAD-cyclopamine; Nog, NOGGIN; RA, retinoic acid; SB (SB431542, a TGF-β type I receptor kinase inhibitor VI); Alk5i, a TGF-β type I receptor kinase inhibitor II; ILV, (-) indolactam V; Ex-4, exendin-4; NA, nicotinamide; IBMX, 3-isobutyl-1-methylxanthine; and FRKL, forskolin. B-(i) SOX17/FOXA2-positive cells, and (ii) relative mRNA expression of DE markers at the end of stage 1. mRNA expression was compared with that of undifferentiated hiPS cells. C-(i) HNF4a/FOXA2-positive cells and (ii) relative mRNA expression of PG markers at the end of stage 2. mRNA expression was compared with that of the stage-1 cells. D-(i) Percentages of PDX1-, PDX1/HNF6-, PDX1/NKX6.1-, PDX1/SOX9-, CDX2-, and AFP-positive cells generated with NOGGIN 100, 200, and 300 ng/ml (Nog 100, Nog 200, and Nog 300), and (ii) relative mRNA expressions of pancreatic, intestinal, and hepatic progenitor markers at the end of stage 3. E) Immunocytochemistry showing the expression patterns of PDX1- (red, pancreatic), HNF6- (green, pancreatic), NKX6.1- (green, pancreatic), SOX9- (green, pancreatic), CDX2- (green, intestinal), and AFP (green, hepatic)-positive cells generated with NOGGIN 200 ng/ml at stage 3. Cells were counterstained with DAPI (blue). The *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) transcript was used as internal RNA control. Results of both immunocytochemistry and quantitative RT-PCR are presented as mean ± standard error of mean (SEM) of three independent experiments (n = 3). Student's *t*-tests were performed against the values of Nog 100 or between two discrete data sets, $^{*}p < 0.05$, $^{**}p < 0.01$. Scale bar = 100 μm.

3.3. A high proportion of cells differentiated into neurogenin 3-positive pancreatic endocrine progenitor cells

Next, I optimized stage-4 medium by exploring the effect of indolactam V (ILV, a protein kinase C activator), Alk5i (a TGF-β type I receptor kinase inhibitor II), and different doses of NOGGIN on the induction of neurogenin 3 (NGN3)-positive endocrine progenitor (EP) cells in our xeno-free system. Results of quantitative RT-PCR showed that the expression of the *NGN3* transcript was significantly up-regulated, whereas those of *AFP* and *CDX2* did not change compared to stage-3 cells, when 300 nM of ILV, 5 μM of Alk5i, and 200 ng/ml of NOGGIN were added to stage-4 medium (Fig. 5A). Immunostaining results also showed that increasing the dose of NOGGIN up to 200 ng/ml resulted in an increase in NGN3-positive cells (up to 77% of total cells) and suppression of the reappearance of AFP- and CDX2-positive cells (Fig. 5A). Therefore, NOGGIN at a higher dose (200 ng/ml) is indispensable to direct the differentiation of PP cells into EP cells.

At stage 4, the expression levels of other EP genes such as *neurogenic differentiation 1 (NEUROD1)* and *paired box 4 (PAX4)* were also significantly increased (Fig. 5B). Immunostaining showed that most of the NGN3-positive cells co-expressed NEUROD1 and PAX4 when treated with ILV, Alk5i, and 200 ng/ml NOGGIN (Fig. 5B), reflecting the commitment to differentiate into EP lineages.

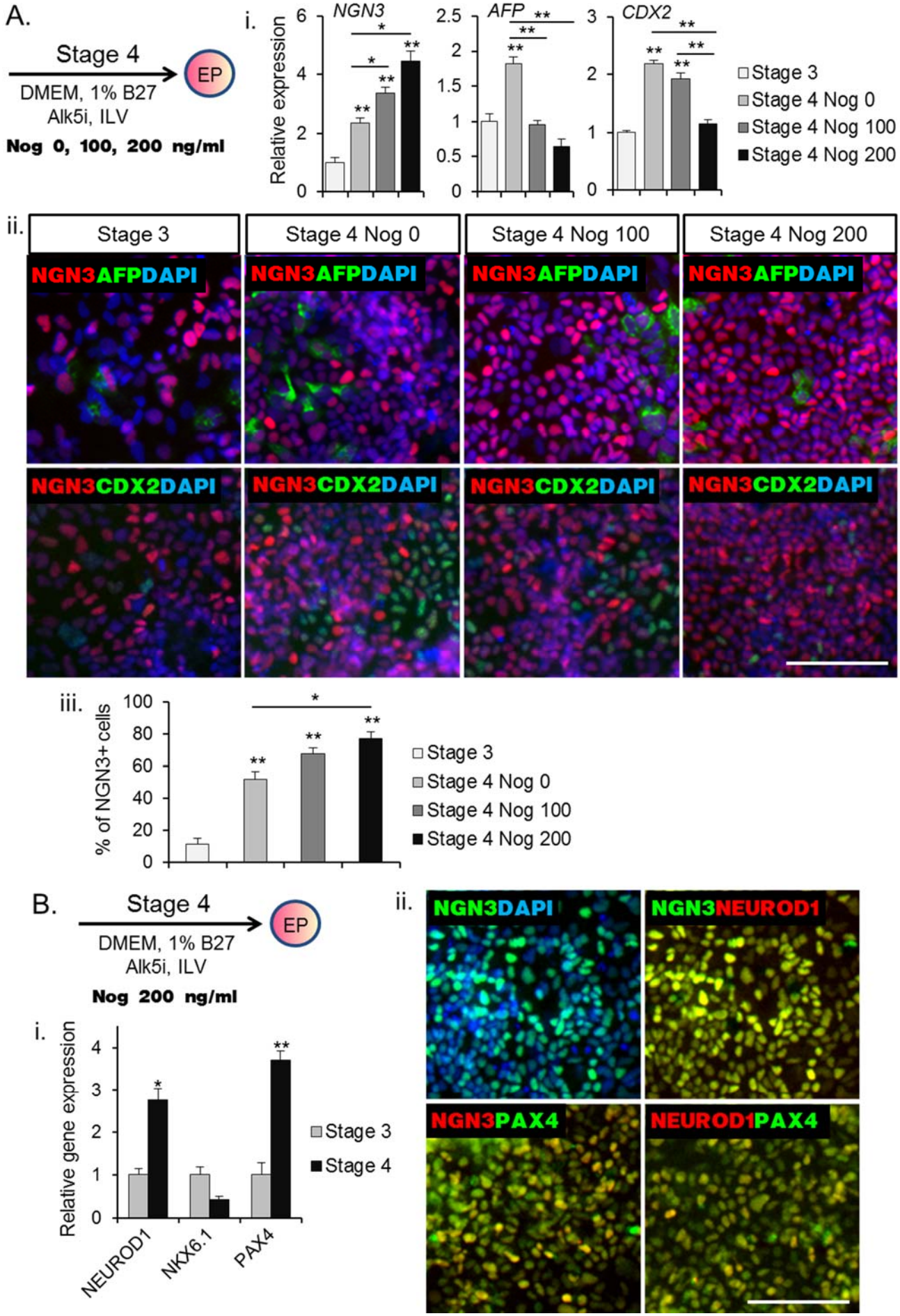
A.
Stage 4
EP
DMEM, 1% B27
Alk5i, ILV
Nog 0, 100, 200 ng/ml
i.
NGN3
AFP
CDX2
Relative expression
Stage 3
Stage 4 Nog 0
Stage 4 Nog 100
Stage 4 Nog 200
ii.
Stage 3
Stage 4 Nog 0
Stage 4 Nog 100
Stage 4 Nog 200
NGN3AFPDAPI
NGN3CDX2DAPI
iii.
% of NGN3+ cells
B.
Stage 4
EP
DMEM, 1% B27
Alk5i, ILV
Nog 200 ng/ml
i.
Relative gene expression
NEUROD1
NKX6.1
PAX4
Stage 3
Stage 4
ii.
NGN3DAPI
NGN3NEUROD1
NGN3PAX4
NEUROD1PAX4

Fig. 5. Differentiation from pancreatic progenitor cells (PPs) into endocrine progenitor cells (EPs)

NOGGIN at high concentrations is crucial for an efficient induction of NGN3-positive EPs and the suppression of differentiation into other lineages. A-(i) Relative mRNA expression levels of *NGN3*, *AFP*, and *CDX2* at stage-4 cells generated in media containing NOGGIN 0, 100, 200 ng/ml (Nog 0, Nog 100, Nog 200) in addition to 5 μM Alk5i and 300 nM ILV were analyzed by quantitative RT-PCR and compared with those of stage-3 cells. A-(ii) Immunocytochemistry showing expression patterns of NGN3-positive EPs (red) cells with AFP-positive hepatic progenitor cells (green) and CDX2-positive intestinal progenitor cells (green), and (iii) percentages of NGN3-positive cells among the total population generated with Nog 0, Nog 100, and Nog 200 at stage 4. B-(i) Relative mRNA expression of other EP cell markers generated with Nog 200 at stage 4. mRNA expression was compared with that of stage-3 cells. B-(ii) Immunocytochemistry showing co-expression of NGN3-positive cells (green or red) with NEUROD1 (red) and PAX4 (green) generated with Nog 200 at stage 4. Cells were counterstained with DAPI (blue). The *GAPDH* transcript was used as internal RNA control. Results are presented as mean ± SEM of three independent experiments (n = 3). Student's *t*-tests were performed against the values of stage-3 cells or between two discrete data sets, $^{*}p < 0.05$, $^{**}p < 0.01$. Scale bar = 100 μm.

3.4. IBMX induced endocrine progenitor (EP) cells to differentiate into INS-positive cells

I next examined the differentiation of EP cells into INS-expressing cells. First, I checked the effect of exendin-4 (a peptide agonist of the glucagon-like peptide 1 (GLP-1) receptor), and nicotinamide at stage 5; and observed that exendin-4 and nicotinamide together induced differentiation efficiency of EP cells into INS-expressing cells rather than they added separately (data not shown). Therefore, I added both exendin-4 and nicotinamide to the stage-5 medium for the subsequent experiments. Then, I tested the effect of IBMX (a phosphodiesterase inhibitor), and forskolin (FRKL, an adenylate cyclase activator) at stage 5 (Fig. 6A). Quantitative RT-PCR showed that *INS* expression was significantly up-regulated in the differentiated cells when IBMX

or FRKL or both were added to the culture media containing exendin-4 and nicotinamide (Fig. 6B). Under all conditions *glucagon* (*GCG)* transcript level was very low in the differentiated cells compared to that of *INS*. *Somatostatin* (*SST*) transcript was also significantly up-regulated when FRKL or IBMX plus FRKL were added to the culture media, whereas addition of IBMX did not significantly increase *SST* expression in the differentiated cells. Immunostaining showed that approximately 6–9% of cells of the total population were hormone-positive and 5–8% cells were C-peptide (CP)-positive, demonstrating the *de novo* synthesis of insulin in differentiated cells (Hansson et al., 2004; Rajagopal et al., 2003; Sipione et al., 2004) (Fig. 6C and D). The proportions of CP single-positive cells among the cells treated with IBMX, FRKL, or both were significantly higher than that treated with control (DMSO) (Fig. 6C and D). The proportion of GCG-positive (mostly CP/GCG-double positive) cells was very low under all conditions, but relatively lower in IBMX-treated cells and significantly lower in FRKL- and IBMX plus FRKL-treated cells than in DMSO-treated cells (Fig. 6C and D). In contrast, the proportion of SST-positive (CP/SST double- and SST single-positive) cells in FRKL- and IBMX plus FRKL-treated cells was significantly higher than in DMSO-treated cells (Fig. 6C and D). Under all applied culture conditions, only very few cells were positive for pancreatic polypeptide and amylase, which was confirmed by the very low expression levels of their transcripts in each experiment (data not shown).

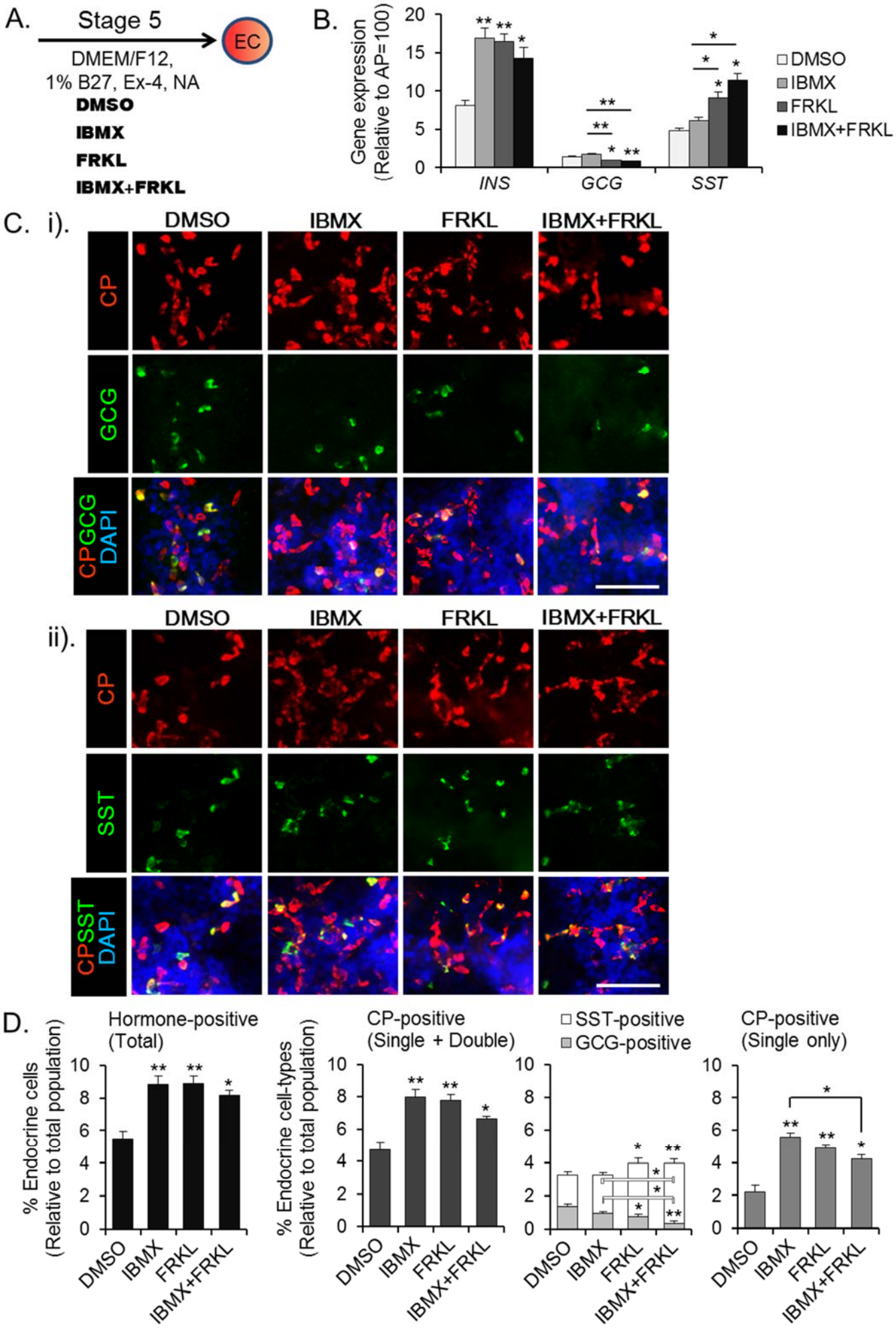
A.
Stage 5
EC
DMEM/F12,
1% B27, Ex-4, NA
DMSO
IBMX
FRKL
IBMX+FRKL
B.
Gene expression
(Relative to AP=100)
INS
GCG
SST
DMSO
IBMX
FRKL
IBMX+FRKL
C. i).
DMSO
IBMX
FRKL
IBMX+FRKL
CP
GCG
CPGCG
DAPI
ii).
CP
SST
CPSST
DAPI
D.
Hormone-positive
(Total)
% Endocrine cells
(Relative to total population)
CP-positive
(Single + Double)
% Endocrine cell-types
(Relative to total population)
SST-positive
GCG-positive
CP-positive
(Single only)

Fig. 6. Differentiation from endocrine progenitor cells (EPs) into pancreatic endocrine cells (ECs)

IBMX is crucial to increase the efficiency to induce INS single-positive cells. A) Factors added to stage-5 basal medium (DMEM/F12, 1% B27, Ex-4, and NA) to promote differentiation into pancreatic endocrine cells (ECs) were evaluated for the following parameters. B) Relative mRNA expression levels of the endocrine hormones insulin (*INS*), glucagon (*GCG*), and somatostatin (*SST*) in cells generated at the end of stage 5 were determined by quantitative RT-PCR. C-(i and ii) Immunocytochemistry showing hormone-positive cells generated at the end of stage 5. C-peptide- (CP, red), GCG- (green), and SST (green)-positive cells are shown. Cells were counterstained with DAPI (blue). D) Percentages of hormone-, CP-, GCG-, and SST-positive cells among the total cell population at the end of stage 5. The *GAPDH* transcript was used as internal RNA control. Expression levels of human adult pancreas genes were calculated and defined as 100. Results of both immunocytochemistry and quantitative RT-PCR are presented as mean ± SEM of three independent experiments (n = 3). Student's *t*-tests were performed against the values of DMSO treatment unless specifically indicated, or between two discrete data sets, $^{*}p < 0.05$, $^{**}p < 0.01$. AP, adult pancreas. Scale bar = 100 μm.

Next, I evaluated the mRNA expression levels of the pancreatic β-cell markers *NKX6.1*, *MAF-A*, *islet-1 (ISL-1)*, *glucokinase (GCK), urocortin-3 (UCN3)*, *islet amyloid polypeptide* (*IAPP*), and *SLC30A8 (ZnT8)*, in cells cultured under all four conditions by quantitative RT-PCR. The results showed that the expression levels of these pancreatic β-cell genes were significantly higher in IBMX-induced differentiated cells than in DMSO-induced cells (Fig. 7A). Then, C-peptide secretion in response to glucose was assessed in differentiated cells. Only marginal amounts of C-peptide were detected at an extracellular glucose level of 2.5 mM (Fig. 7B). In contrast, C-peptide secretion was significantly increased under all culture conditions in response to 20 mM glucose (approximately 2.06-, 2.74-, 2.46-, and 2.13-fold over basal level in DMSO-, IBMX-, FRKL-, and IBMX plus FRKL-induced cells, respectively). I also detected endogenous

C-peptide in cells under all four conditions; the levels of endogenous C-peptide were significantly higher in IBMX-induced and FRKL-induced cells than in DMSO-induced cells (Fig. 7C), confirming the presence of C-peptide pools in differentiated cells. I also added NOGGIN to stage-5 culture media and examined whether addition of NOGGIN could further enhance the differentiation efficiency of EP cells into INS-expressing cells (Fig. 8A). Both quantitative RT-PCR and immunostaining demonstrated that addition of NOGGIN to stage-5 culture media decreased the expression of *INS*, *GCG* and *SST* transcripts (Fig. 8B), and also the differentiation efficiency into pancreatic endocrine-positive (CP-, GCG- or SST-positive) cells (Fig. 8C), respectively. Moreover, C-peptide secretion was not significantly increased in response to 20 mM glucose over basal level under these conditions (Fig. 8D). Therefore, I excluded NOGGIN from stage 5 culture media.

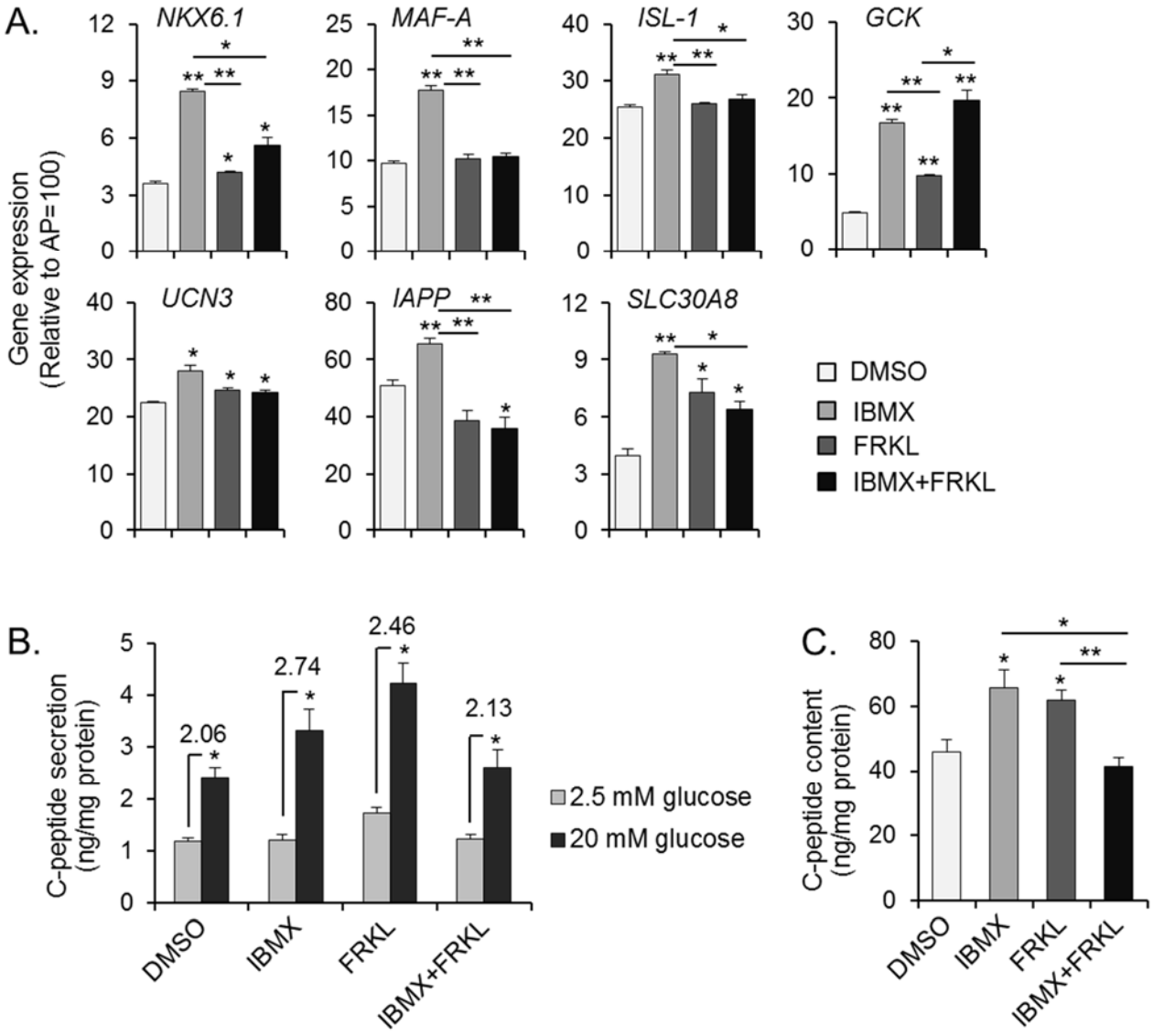

Fig. 7. Gene expression profile and glucose-stimulated C-peptide secretion

Expression levels of pancreatic β-cell markers and glucose-stimulated C-peptide secretion were determined in the derived endocrine cells (ECs). A) Relative expression levels of β-cell marker genes in differentiated cells generated at the end of stage 5 were determined by quantitative RT-PCR. B) In vitro glucose-stimulated C-peptide secretion of differentiated cells at the end of stage 5 was determined by ELISA. C-peptide secretion levels under stimulation with 20 mM glucose were compared with those detected under treatment with 2.5 mM glucose. Fold increases are shown on the top of each pair of bars. C) C-peptide contents in differentiated cells at the end of stage 5. The *GAPDH* transcript was used as internal RNA control. Expression levels of human

adult pancreas genes were calculated and defined as 100. Results of both quantitative RT-PCR and ELISA are presented as mean ± SEM of three independent experiments (n = 3). Student's *t*-tests were performed against the values of DMSO unless specifically indicated, or between two discrete data sets for both gene expression and C-peptide level analyses, *$p < 0.05$, **$p < 0.01$. For glucose-stimulated C-peptide secretion, *t*-tests were performed against the values of 2.5 mM glucose treatment, $^{*}p < 0.05$, $^{**}p < 0.01$. AP, adult pancreas.

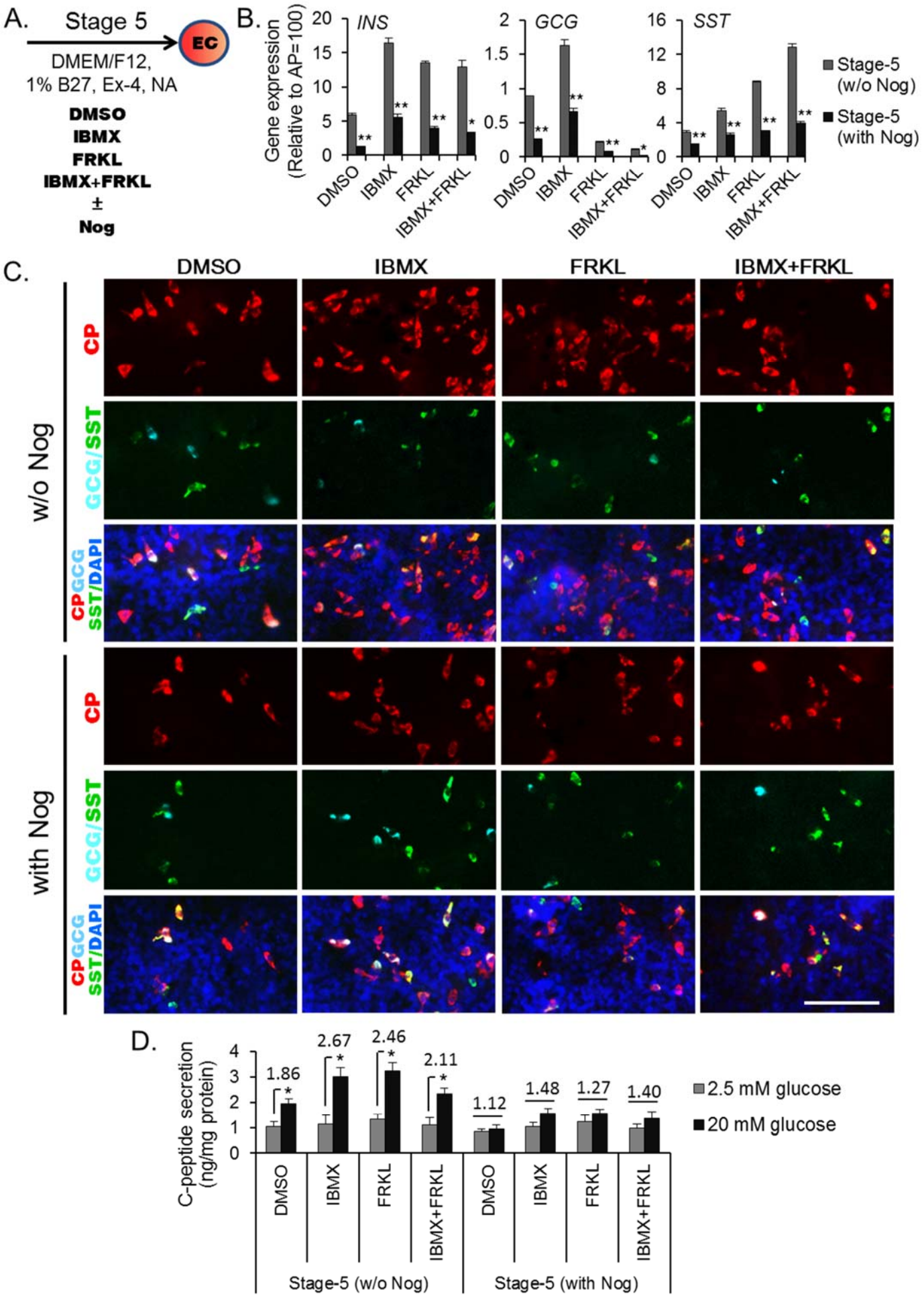
A.
Stage 5
EC
DMEM/F12,
1% B27, Ex-4, NA
DMSO
IBMX
FRKL
IBMX+FRKL
±
Nog
B.
Gene expression
(Relative to AP=100)
INS
GCG
SST
DMSO
IBMX
FRKL
IBMX+FRKL
Stage-5
(w/o Nog)
Stage-5
(with Nog)
C.
DMSO
IBMX
FRKL
IBMX+FRKL
w/o Nog
with Nog
CP
GCG/SST
CPGCG
SST/DAPI
D.
C-peptide secretion
(ng/mg protein)
1.86
2.67
2.46
2.11
1.12
1.48
1.27
1.40
2.5 mM glucose
20 mM glucose
DMSO
IBMX
FRKL
IBMX+FRKL
Stage-5 (w/o Nog)
Stage-5 (with Nog)

Fig. 8. NOGGIN at stage-5 culture media reduces the differentiation efficiency into pancreatic endocrine cells (ECs)

Addition of NOGGIN (Nog) to stage-5 culture media reduced the differentiation efficiency into pancreatic β-like cells. A) Nog added to stage-5 culture media to induce differentiation into pancreatic endocrine cells (ECs) was evaluated for the following parameters. B) Relative mRNA expression levels of the endocrine hormones *INS*, *GCG*, and *SST* in cells generated at the end of stage 5 were determined by quantitative RT-PCR. C) Immunocytochemistry showing hormone-positive cells generated at the end of stage 5 in absence or presence of Nog. C-peptide (CP, red)-, GCG (cyan)-, and SST (green)-positive cells are shown. Cells were counterstained with DAPI (blue). D) In vitro glucose-stimulated C-peptide secretion of differentiated cells at the end of stage 5 was determined by ELISA. C-peptide secretion levels under stimulation with 20 mM glucose were compared with those detected under treatment with 2.5 mM glucose. Fold increases are shown on the top of each pair of bars. The *GAPDH* transcript was used as internal RNA control. Expression levels of human adult pancreas genes were calculated and defined as 100. Results of both quantitative RT-PCR and ELISA are presented as mean ± SEM (n = 3). Student's *t*-tests were performed against the values of cells-derived in absence of Nog at stage-5 unless specifically indicated for gene expression, *$p < 0.05$, **$p < 0.01$. For glucose-stimulated C-peptide secretion, *t*-tests were performed against the values of 2.5 mM glucose treatment, $^{*}p < 0.05$, $^{**}p < 0.01$. AP, adult pancreas. Scale bar = 100 μm.

3.5. In vitro-generated INS-expressing cells exhibited pancreatic β-cell characteristics

To further confirm the pancreatic β-cell characteristics of the derived INS-expressing cells, I assessed the C-peptide secretion of differentiated cells (Fig. 9A) in response to various insulin secretagogues (Fig. 9B). Direct depolarization of the cells by addition of potassium chloride (KCl) to the medium or treatment of the cells with a K_{ATP} channel blocker, tolbutamide, or with an L-type voltage-dependent Ca^{2+} channel agonist, (–) BAY K8644, resulted in significant stimulation of C-peptide release, suggesting the presence of functional K_{ATP} channels (Sturgess et al., 1985)

and L-type voltage-dependent Ca^{2+} channels (Misler et al., 1992). The results also showed that increasing the cAMP level by using IBMX resulted in significantly increased C-peptide secretion, indicating the cells' responsiveness to cAMP, which influences insulin secretion (Prentki and Matschinsky, 1987; Pyne and Furman, 2003). Moreover, treatment of the differentiated cells with carbachol (a muscarinic agonist) also significantly increased the secretion of C-peptide. Immunocytochemical analysis also demonstrated that approximately 50% of all INS- or CP-positive cells co-expressed PDX1, UCN3, IAPP and ISL1 (Fig. 9C). Taken together, these results suggest that a fraction of INS-expressing cells derived from this culture system possesses pancreatic β-cell like characteristics.

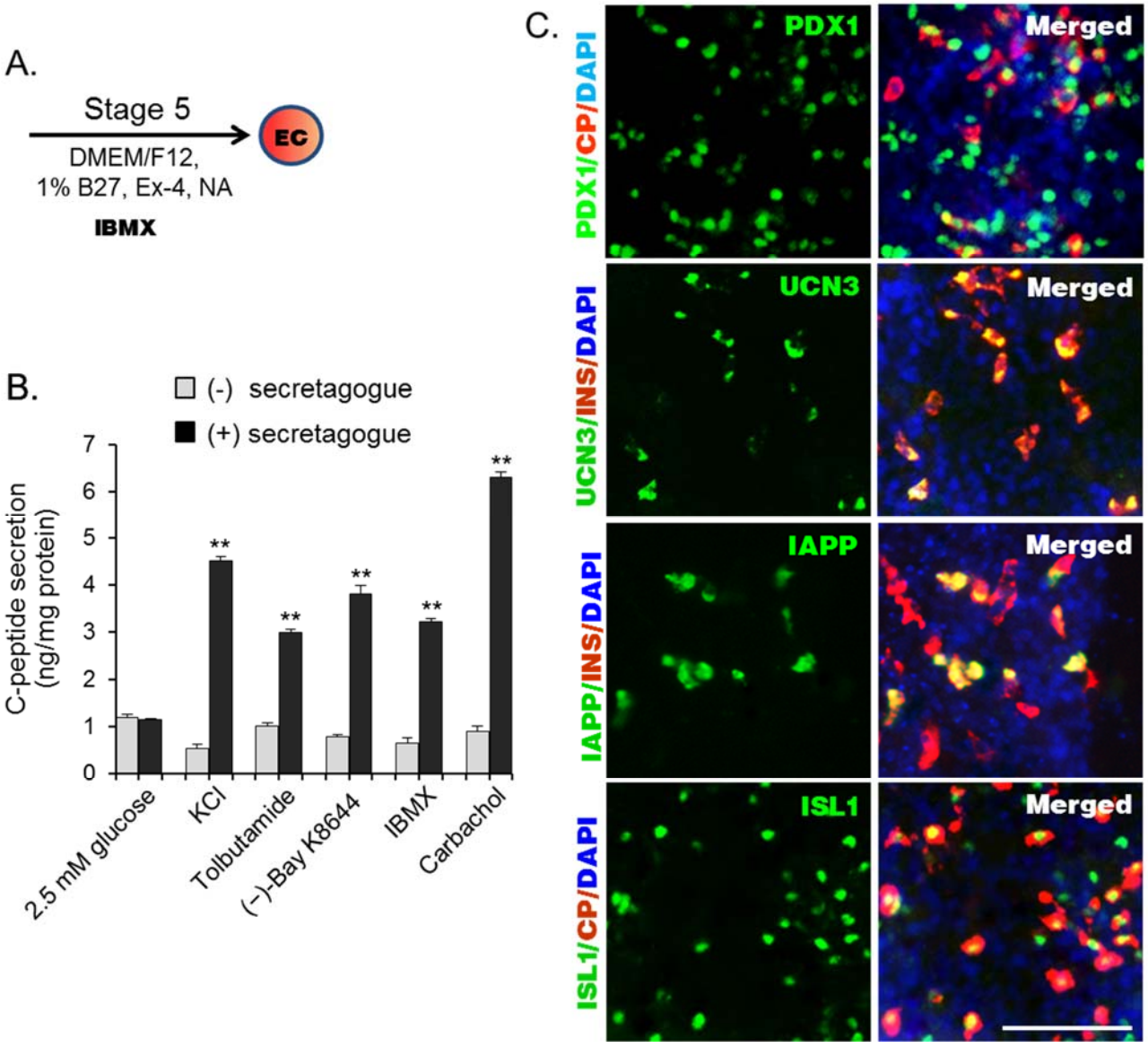

Fig. 9. Characterization of hiPS cell-derived INS-positive cells

HiPS cell-derived INS-positive cells secreted C-peptide in response to secretagogues and expressed markers of pancreatic β-cells. **A**) The IBMX-based condition was evaluated for the following parameters. B) In vitro C-peptide secretion levels of differentiated cells in response to various insulin secretagogues at the end of stage 5. C-peptide secretion levels under treatment with secretagogues were compared with those detected under treatment with 2.5 mM glucose without secretagogues. C) Co-expression of pancreatic β-cell markers in INS- or CP-positive cells. Approximately 50% of INS- or CP-positive cells (red) co-expressed PDX1 (green), UCN3 (green), IAPP (green), and ISL-1 (green). Cells were counterstained with DAPI (blue). Left panel, PDX1,

UCN3, IAPP or ISL1 staining (green); right panel, merged images are shown. Results of the C-peptide secretion levels are presented as mean ± SEM of three independent experiments (n = 3). Student's *t*-tests were performed against the values of 2.5 mM glucose without secretagogues treatment, $^{**}p < 0.01$. Scale bar = 100 μm.

3.6. NOGGIN and IBMX acted on hiPS-derived cells to enhance differentiation into INS single-positive cells

I next examined the key factors that promoted and properly directed hiPS-derived cells to differentiate into INS single-positive cells. I found that the *INS* expression (Fig. 10A and B) and proportion of INS-expressing cells (Fig. 10C and D) were significantly up-regulated only when I added 200 ng/ml of NOGGIN (Nog 200) at stages 3 and 4 and IBMX at stage 5. Importantly, adding both 200 ng/ml of NOGGIN at stages 3 and 4 and IBMX at stage 5 increased the proportion of INS single-positive cells, while it decreased the proportion of poly-hormonal cells. Interestingly, no hormone-positive cells were detected in the absence of NOGGIN, regardless of whether IBMX was used or not. C-peptide content (Fig. 10E) and glucose-stimulated C-peptide secretion (Fig. 10F) were also significantly increased when IBMX and 200 ng/ml of NOGGIN were used in the differentiation process. The above results support the hypothesis that NOGGIN (at high concentrations) at stages 3 and 4 and IBMX at stage 5 act to enhance and control the differentiation of hiPS-derived cells into INS single-positive β-like cells.

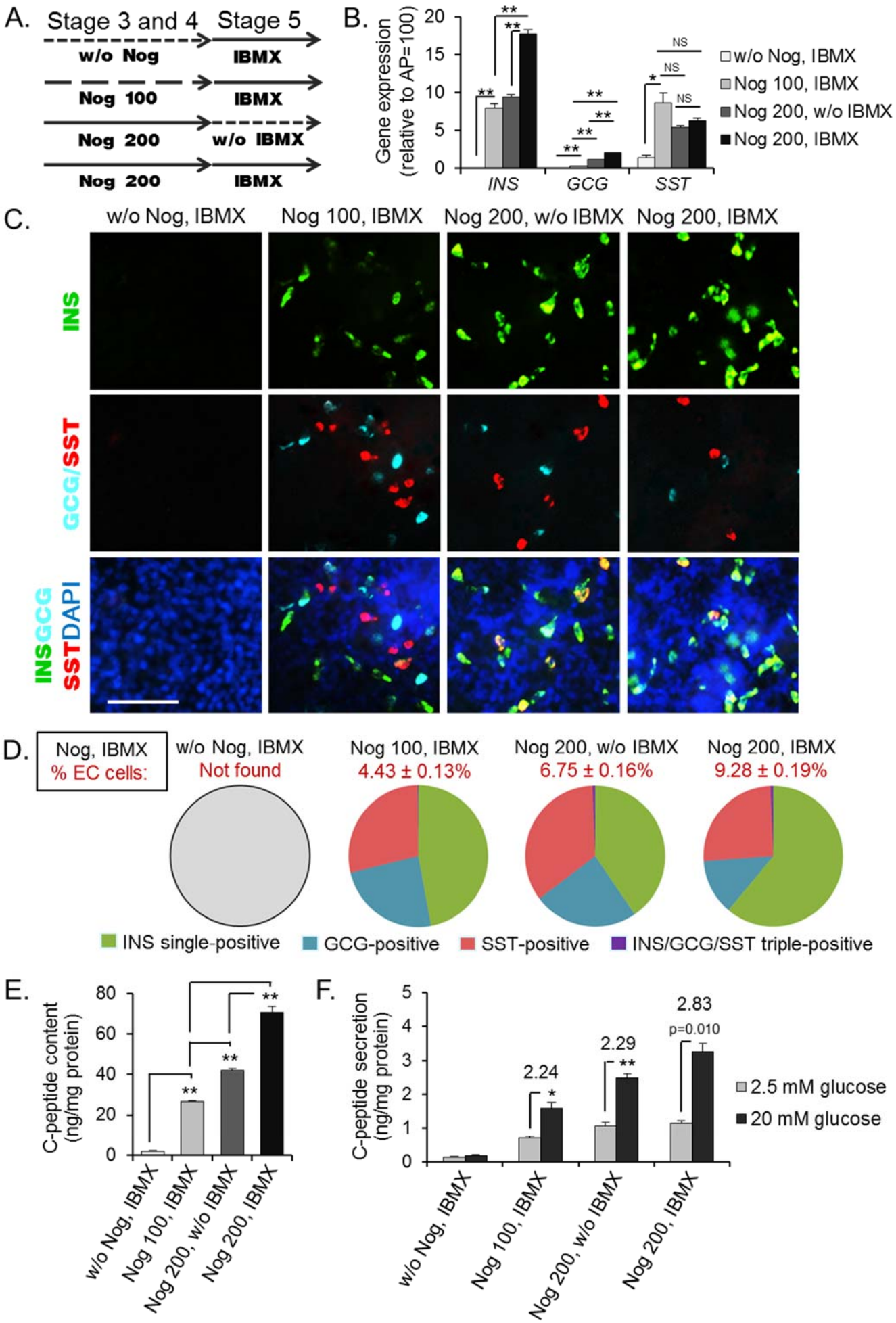
A.
Stage 3 and 4
Stage 5
w/o Nog
IBMX
Nog 100
IBMX
Nog 200
w/o IBMX
Nog 200
IBMX
B.
Gene expression (relative to AP=100)
INS
GCG
SST
w/o Nog, IBMX
Nog 100, IBMX
Nog 200, w/o IBMX
Nog 200, IBMX
C.
w/o Nog, IBMX
Nog 100, IBMX
Nog 200, w/o IBMX
Nog 200, IBMX
INS
GCG/SST
INSGCG
SSTDAPI
D.
Nog, IBMX
% EC cells:
w/o Nog, IBMX
Not found
Nog 100, IBMX
4.43 ± 0.13%
Nog 200, w/o IBMX
6.75 ± 0.16%
Nog 200, IBMX
9.28 ± 0.19%
INS single-positive
GCG-positive
SST-positive
INS/GCG/SST triple-positive
E.
C-peptide content (ng/mg protein)
w/o Nog, IBMX
Nog 100, IBMX
Nog 200, w/o IBMX
Nog 200, IBMX
F.
C-peptide secretion (ng/mg protein)
2.24
2.29
2.83
p=0.010
2.5 mM glucose
20 mM glucose

Fig. 10. Effect of NOGGIN and IBMX on the generation of INS single-positive β-like cells

INS single-positive cells were derived from pancreatic progenitor cells by treatment with Nog 200 and IBMX. A) Schematic drawing of the pancreatic differentiation procedure using various combinations of NOGGIN (w/o Nog, Nog 100, and 200 ng/ml with other components at stages 3 and 4) and IBMX (basal medium with or without IBMX at stage 5). B) Relative mRNA expression levels of *INS*, *GCG*, and *SST* in stage-5 cells were determined by quantitative RT-PCR and compared. C) Expression patterns of INS (green), GCG (cyan), and SST (red) in differentiated cells. Cells were counterstained with DAPI (blue). D) Numbers indicate the percentage of pancreatic endocrine cells among all DAPI-positive cells; within which the relative percentages of INS-, GCG-, and SST-positive sub-populations are shown in the pie charts. E) Endogenous C-peptide content in differentiated cells generated at the end of stage 5. F) In vitro glucose-stimulated C-peptide secretion of differentiated cells. C-peptide secretion levels under stimulation with 20 mM glucose were compared with those detected under treatment with 2.5 mM glucose. Fold increases are shown on the top of each pair of bars. The *GAPDH* transcript was used as internal RNA control. Expression levels of human adult pancreas genes were calculated and defined as 100. Results are presented as mean ± SEM (n = 3). Student's *t*-tests were performed between two discrete data sets for both gene expression and C-peptide level analysis, $^{*}p < 0.05$, $^{**}p < 0.01$. For glucose-stimulated C-peptide secretion, *t*-tests were performed against the values of 2.5 mM glucose treatment, $^{*}p < 0.05$, $^{**}p < 0.01$. AP, adult pancreas. Scale bar = 100 μm.

3.7. In vitro-generated INS-expressing cells were free of xenogeneic contamination

Next, I evaluated the degree of non-human-derived contamination by measuring the expression of Neu5Gc. Flow cytometry showed that the expression of Neu5Gc was below the detection limit at stage 4 and at the end of stage 5 when xeno-free culture condition was adopted (Fig. 11A-C), indicating that our entire differentiation system is xeno-free. I also examined the pancreatic differentiation of hiPS cells on other commercially available xeno-free scaffolds, e.g., CELLstart (composed of fibronectin) and rhVTN (recombinant human vitronectin), in our xeno-free system. I observed that the cells detached from CELLstart- and rhVTN-coated surfaces and

formed large clumps (data not shown). Therefore, CELLstart- and rhVTN-coated surfaces were less suitable than Synthemax-coated surfaces for long-term differentiation culture. Moreover, the differentiation efficiency into pancreatic lineages was better on Synthemax-coated surfaces than on CELLstart- or rhVTN-coated surfaces, as indicated by the significantly higher expression levels of pancreatic marker genes, i.e., *PDX1*, *HNF6*, *NKX6.1*, *HLXB9*, and *INS*, and significantly lower expression levels of hepatic (*AFP*) and intestinal (*CDX2*) marker genes (Fig. 11D).

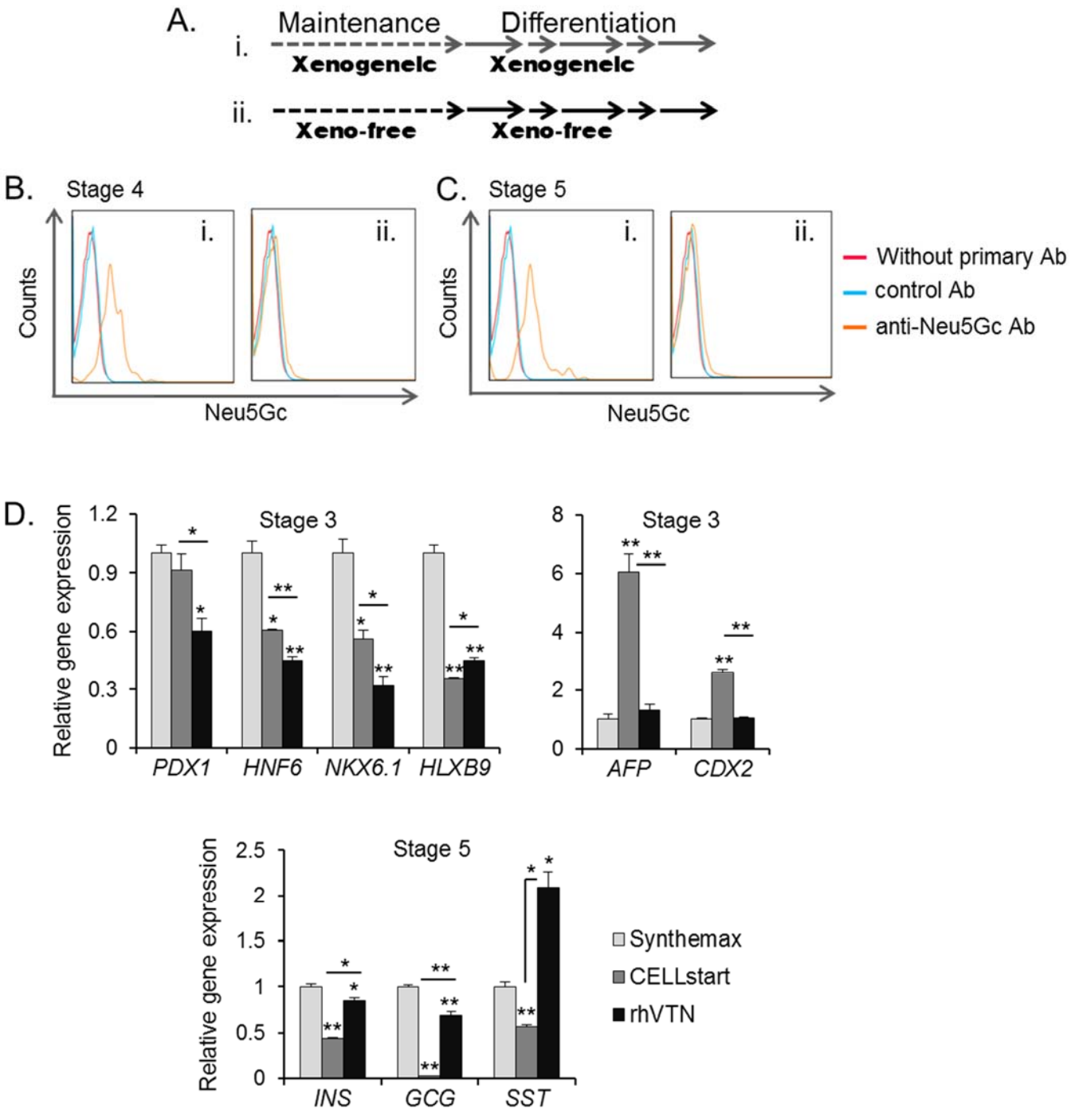

Fig. 11. Assay of xenoantigenic contamination in differentiated cell cultures

The entire differentiation system is free of xenogeneic contamination. A) Schematic drawing of the procedures. B, C) Detection of Neu5Gc expression in differentiated cells at the end of stage 4 (B) and stage 5 (C), cultured under xenogeneic (gray bars) or xeno-free (black bars) conditions. Cells were exposed to an anti-Neu5Gc antibody (orange), a negative control antibody (blue), or incubated without primary antibody (red), and then stained with a secondary antibody for analysis. D) Expression levels of pancreatic, hepatic (*AFP*), and intestinal (*CDX2*) marker genes at the end of stages 3 and 5 in cells differentiated under xeno-free conditions using xeno-free scaffolds (Synthemax, CELLstart, and rhVTN). The *GAPDH* transcript was amplified as internal RNA control. Results are presented as mean ± SEM (n = 3). Student's *t*-tests were performed against the values of Synthemax or between two discrete data sets. Significant differences were $^{*}p < 0.05$, $^{**}p < 0.01$.

3.8. Differentiation of other human ES/iPS cell lines into INS-positive cells

Finally, I examined whether our xeno-free differentiation system could be applied to other human ES/iPS cell lines. I used KhES3, a human ES cell line, and 201B7, a human iPS cell line, and compared their differentiation efficiency into INS-expressing cells to that of Toe (Fig. 12A). Quantitative RT-PCR showed that both KhES3- and 201B7-derived cells expressed *INS*, *GCG* and *SST* transcripts; the extents of which were similar to those of Toe-derived cells (Fig. 12B). Immunostaining results showed that both KhES3 and 201B7 cells differentiated into β-like cells (Fig. 12C), most of which were INS single-positive like that of Toe-derived cells. I also detected endogenous C-peptide contents in both KhES3- and 201B7-derived cells; the levels of which were similar to that of Toe-derived cells (Fig. 12D). In addition, I assessed KhES3- and 201B7-derived cells for their C-peptide secretion in response to high glucose concentration (Fig. 12E). Results indicated that C-peptide secretion was significantly increased in both KhES3- and 201B7-derived cells in response to 20 mM glucose (approximately 2.12-, and 2.70-fold over basal level, respectively), which were similar to that of the Toe-derived cells (2.55-fold over basal level).

These results suggest that our in vitro xeno-free system is suitable for pancreatic β-cell differentiation, not only from Toe but also from other human ES/iPS cell lines.

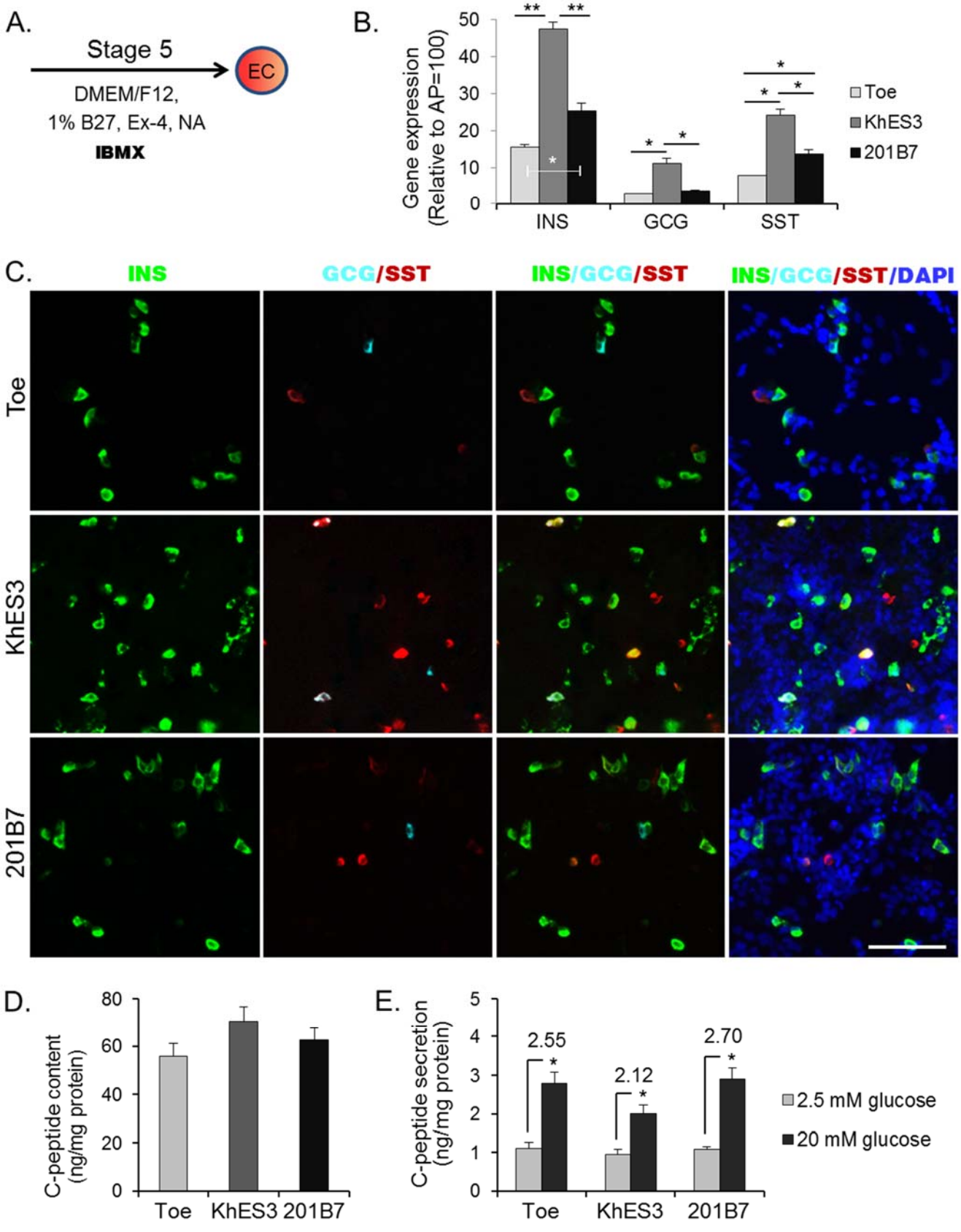

Fig. 12. Differentiation of other cell lines into pancreatic endocrine cells (ECs)

Xeno-free culture system is also suitable for other cell lines for the differentiation into pancreatic β-like cells. Two other cell lines, KhES3 (a hES cell line) and 201B7 (a hiPS cell line) efficiency differentiated into INS single-positive cells, which are similar to that of Toe. A) Factors added to stage-5 basal medium (DMEM/F12, 1% B27, Ex-4, and NA) to promote differentiation into pancreatic endocrine cells (ECs) were evaluated for the following parameters. B) Relative mRNA expression levels of the endocrine hormones *INS*, *GCG*, and *SST* in cells generated at the end of stage 5 were determined by quantitative RT-PCR. C) Immunocytochemistry showing hormone-positive cells generated at the end of stage 5. INS (green)-, GCG (cyan)-, and SST (red)-positive cells are shown. Cells were counterstained with DAPI (blue). D) C-peptide contents in differentiated cells at the end of stage 5. E) In vitro glucose-stimulated C-peptide secretion of differentiated cells at the end of stage 5 was determined by ELISA. C-peptide secretion levels under stimulation with 20 mM glucose were compared with those detected under treatment with 2.5 mM glucose. Fold increases are shown on the top of each pair of bars. The *GAPDH* transcript was used as internal RNA control. Expression levels of human adult pancreas genes were calculated and defined as 100. Results of both quantitative RT-PCR and ELISA are presented as mean ± SEM (n = 3). Student's *t*-tests were performed against the values of Toe unless specifically indicated, or between two discrete data sets for both gene expression and C-peptide content analyses, *$p < 0.05$, **$p < 0.01$. For glucose-stimulated C-peptide secretion, *t*-tests were performed against the values of 2.5 mM glucose treatment, $^{*}p < 0.05$, $^{**}p < 0.01$. AP, adult pancreas. Scale bar = 100 μm.

4. DISCUSSION

Diabetes mellitus is life-threatening disease, the prevalence of which is increasing worldwide. The available treatment can neither cure nor completely control the complications of this disorder. This may result in a substantial number of life losses in almost all countries in the world. Furthermore, current life-long treatment strategies also impose huge social and economic burden on a family. From the ancient time to the recent date human beings have been trying to unveil an effective and successful treatment strategy that can fully control this disorder and consequently save lives. Despite of many tremendous efforts, human beings are far away to be successful to find out an effective treatment strategy for diabetes mellitus. From the last decades to till date many researchers have been focusing on ES/iPS cell-based therapy for diabetes mellitus, as these cells are thought to be directed to pancreatic endocrine cells like many other somatic cells. So far, several research groups successfully generated pancreatic endocrine cells from ES/iPS cells, although these cells are not fully functional and therefore are not suitable for using them to treat diabetic patients. Being a part of the researchers in this field and as a continuous effort like other research groups, I are also paying attention to generate ES/iPS-derived pancreatic endocrine cells that could be of use as cell replacement therapy for diabetes mellitus. In this study, I established a five-step protocol and successfully generated INS-producing β-like cells from ES/hiPS cells in vitro that possess endogenous insulin pools and secrete C-peptide in a glucose-sensitive manner. Importantly, I found that optimization of the growth factors is important for directing the differentiation into INS single-positive β-like cells. I applied humanized and/or recombinant factors, chemically-defined supplements, and synthetic scaffolds in our in vitro culture system. Our protocol is simple, completely xeno-free, and does not include embryoid body formation, cell sorting, or reseeding on other plates.

I found that combined treatment with RA, KAAD-cyclopamine, SB431542, and NOGGIN was effective to induce pancreatic progenitor (PP) cells at stage 3. Hedgehog signaling has been reported to antagonize RA-mediated specification of pancreatic ECs during zebrafish and mouse embryonic development (Martín et al., 2005; Micallef et al., 2005; Stafford et al., 2006; Tehrani

and Lin, 2011). Thus, KAAD-cyclopamine and RA were used at this stage is because of their known importance. In our differentiation system, the number of AFP-positive cells was lesser at high NOGGIN concentrations (200–300 ng/ml), which is in agreement with previous reports that showed that BMP is required for hepatic differentiation but inhibitory for pancreatic differentiation (Mfopou et al., 2010; Cai et al., 2010). BMP signaling has also been reported to increase CDX2 expression through SMAD4 (Barros et al., 2008), which might explain the down-regulation of CDX2 at high NOGGIN concentrations.

At stage 4, I applied a combination of Alk5i, ILV, and NOGGIN and noted the efficient induction of endocrine progenitor (EP) cells from PP cells, which was consistent with recent reports (Rezania et al., 2012; Rezania et al., 2013). I successfully induced a high proportion of NGN3-expressing EP cells (Gu et al., 2002; Schwitzgebel et al., 2000), most of which co-expressed NEUROD1 and PAX4. Moreover, the *NGN3* transcript was highly expressed at this stage and gradually disappeared within one or two more days, which is consistent with the transient expression of this gene in vivo (Schwitzgebel et al., 2000). *NKX6.1*, an important regulator of the differentiation of pancreatic ECs, in particular, of β-cells (Habener et al., 2005), was expressed in both stage-3 and stage-4 cells, indicating that the progenitor cells derived in our culture system possess the potential to differentiate into pancreatic β-cells.

Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells (Otonkoski et al. 1993). In contrast, GLP-1 was reported to increase pancreatic β-cell mass (Buteau et al., 2003). Therefore, I added nicotinamide and exendin-4 (a peptide analog of the GLP-1 receptor) to the final differentiation medium. As expected, CP-expressing β-like cells appeared in the cell culture, however, to a lesser extent. Both IBMX and FRKL in addition to exendin-4 and nicotinamide further promoted differentiation into CP-expressing cells. IBMX and FRKL are known to increase the intracellular cAMP level, suggesting that the cellular cAMP level is one of the key factors that enhance the differentiation of INS-positive cells. Although IBMX and FRKL, in addition to exendin-4 and nicotinamide, similarly promoted the differentiation into

CP-positive cells, I considered that the combination of exendin-4, nicotinamide, and IBMX provided a better environment for the induction of EPs to differentiate into INS-expressing cells. This assumption was based on the following observations. First, the number of SST-positive cells was relatively higher in FRKL-induced cells than in IBMX-induced cells, indicating that although both IBMX and FRKL increased the intracellular cAMP level, FRKL might promote SST-positive cells by acting on other pathways. Second, the expression levels of β-cell specific genes were relatively higher in IBMX-induced cells than in FRKL-induced cells.

The differentiated cells obtained in our culture system secreted C-peptide in a glucose-dependent manner; the amount of secreted C-peptide was approximately 2.8 times higher than the basal level. Previous reports suggested that in vitro hES/iPS-derived INS-positive cells have a limited capacity to secrete C-peptide in a glucose-dependent manner (Bruin et al., 2014; Cheng et al., 2012; Kunisada et al., 2012; Chen et al., 2009; Zhang et al., 2009; Jiang et al., 2007a; Jiang et al., 2007b; D'Amour et al., 2006). Each report described varying degrees of in vitro glucose-stimulated C-peptide secretion, including an approximately 3-fold increase reported by Cheng et al. (2012), a 1.7-fold increase observed by Chen et al. (2009), a 2-fold reported by Zhang et al. (2009) and Jiang et al. (2007b), a 3.2-fold noted by Jiang et al. (2007a), and apparently no glucose-stimulated C-peptide secretion was reported by D'Amour et al. (2006), Kunisada et al. (2012), and Bruin et al. (2014). These variations and low levels of secreted C-peptide could be due to the generation of varying numbers of polyhormonal cells in the culture. The existence of polyhormonal cells has been reported during the primary transition stage of early fetal development in humans (De Krijger et al., 1992; Polak et al., 2000). The role and fate of polyhormonal cells during human fetal development are poorly understood; however, immunohistochemical characterization indicated that these cells possess α-cell transcription factor profile (Riedel, 2012). Previous studies reported that after transplantation, polyhormonal cells differentiated in vivo into GCG-expressing cells, and dynamic chromatin remodeling was reported to occur during this transition into matured cell types (Kelly et al., 2011; Xie et al., 2013, Basford et al., 2012; Rezania et al., 2011). Recently, it has been shown that hES cell-derived pancreatic

endoderm cells that were transplanted into immunodeficient mice underwent further differentiation and maturation into glucose-responsive INS-secreting cells (Kroon et al., 2008; Rezania et al., 2012; Rezania et al., 2013), suggesting that the pancreatic precursors obtained in vitro could mature in vivo. Here, in our differentiation culture, addition of IBMX to the culture medium effectively reduced the number of polyhormonal cells and increased the number of INS single-positive cells, which could explain the glucose-stimulated C-peptide secretion in vitro. Thus, our results revealed that it is possible to derive INS single-positive β-like cells that have the potential to secrete C-peptide in response to glucose by activating the relevant signaling pathways at the different developmental stages in vitro.

Both NOGGIN and IBMX play vital roles in the generation of INS single-positive cells from hiPS-derived cells. I confirmed that the addition of NOGGIN at stages 3 and 4 is indispensable to generate INS-positive cells, regardless of the presence or absence of IBMX, whereas addition of IBMX at stage 5 combined with high doses of NOGGIN (200 ng/ml) at stages 3 and 4 enhanced and regulated the generation of INS single-positive cells. The combined use of high NOGGIN concentrations and IBMX also increased the amount of endogenous C-peptide and glucose-stimulated C-peptide secretion, further supporting the hypothesis that the combined effect of NOGGIN and IBMX is crucial for the generation of INS single-positive β-like cells.

Although a fraction of our hiPS cells-derived pancreatic β-like cells mono-hormonal and are capable to secrete C-peptide in vitro in response to high glucose treatment, the extent of their glucose responsiveness is still not up to the mark. In contrast, hES-derived pancreatic progenitor cells when allowed to differentiate in vivo, they successfully differentiated into mature pancreatic β cells (Rezania et al., 2013, 2012, Kroon et al., 2008). This indicates that some in vivo factors are still missing in our in vitro growth factor cocktails. Therefore, various growth factors and signaling molecules involved in pancreas development process need to be more extensively screened for their potential effect on hES/iPS cells to differentiate in vitro into fully-functional pancreatic β cells, which could open the new avenue of success in the future. In this study, due to

the lack of suitable transplantation technique of hiPS-derived cells into the experimental animal model I could not examine the glucose controlling effect of our iPS-derived pancreatic cells in vivo. I failed to recover well-demarcated graft after transplantation of our hiPS-derived pancreatic cells into the kidney capsule of SCID mice, possibly due to the immunorejection of the host animal model. Therefore, I need to establish a suitable transplantation technique to examine the glucose-responsiveness of the iPS-derived pancreatic cells in vivo, which could indicate their extent of functionality and their actual potential to use them in the cell replacement therapy in the future.

In has been reported that once the hES/iPS-derived cells become poly-hormonal in vitro, they would never be differentiated into functionally-mature pancreatic β cells both in vitro and in vivo (Basford et al., 2012). Available reports suggest that a large proportion of the differentiated endocrine cells are poly-hormonal and an insignificant number of cells are mono-hormonal (Bruin et al., 2014; Kunisada et al., 2012; Zhang et al., 2009; Jiang et al., 2007a, 2007b, D'Amour et al., 2006). In our study, I also observed that a considerable fraction of differentiated cells are poly-hormonal. Therefore, it is necessary to know in detail the characteristics of these poly-hormonal cells and sought out the strategies for induction of these cell types into functionally-mature pancreatic β cells.

Batch-to batch variation in the differentiation efficiency of the same freeze-stored hES/iPS cell line, variation in the characteristics of various clone of one hES/iPS cell line, and vast differences in the differentiation efficiency among various ES/iPS cell lines are the major problems to establish a common method for generating pancreatic endocrine cells. During our study, I sometimes observed very limited differentiation propensity of some clones of the same hES/iPS cell lines. Therefore, in the future studies these issues should be addressed first in order to establish a suitable differentiation protocol, which could be reproducible and generate large numbers of pancreatic β cells.

Animal-derived products are undesirable for clinical use. Therefore, in future clinical applications, hiPS cells must be generated, maintained, and differentiated in xeno-free culture systems to minimize the risk. I used xeno-free scaffolds, supplements, and growth factors without any feeder cells, for both maintenance and differentiation. However, to increase the safety of hiPS-based cell therapies, it would be extremely necessary to generate hiPS cells without integrating vectors and continuous c-MYC expression. The generation of hiPS cells with transient expression from non-integrating vectors (Stadtfeld et al., 2008; Yu et al., 2009) may address these concerns.

To our knowledge, this is the first report of a complete xeno-free culture system in which hiPS cells have been differentiated into INS-positive cells. Indeed, our strategy provides evidence towards the possibility of differentiation of hiPS cells into pancreatic β-like cells without contamination by any non-human-derived factors. I believe that our differentiation strategy could be beneficial for the development of future cell therapies. Nevertheless, it also facilitates future in vitro studies on the mechanism of human pancreatic specialization and maturation. Although a number of limitations exist for the use of hES/iPS-derived cells in the cell replacement therapies, priority should be given on the following issues: 1) Safety of hES/iPS cell lines, 2) Establishment of good quality hES/iPS cell lines which could exhibit very high differentiation efficiency and less batch-to batch variation, 3) Establishment, maintenance and differentiation of hES/iPS cells should be carried out in defined and completely xeno-free culture system to avoid xenogeneic contamination and unknown effects, 4) Emphasis should be paid to understand the characteristics of the poly-hormonal cells and their induction into functionally-mature pancreatic β cells, and 5) Extensive screening of various growth factors and signaling molecules involved in the pancreatic development process that could induce hES/iPS cells in vitro into functionally-mature pancreatic β cells. Use of hES/iPS-derived pancreatic β cells in the cell replacement therapy of the diabetic patients could be possible in the near future only if attention should be given and successfully addressed the above issues.

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