In vitro pancreatic islet cluster expansion facilitated by hormones and chemicals

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Abstract

Tissue regeneration, such as pancreatic islet tissue propagation in vitro, could serve as a

promising strategy for diabetes therapy and personalized drug testing. However, such a protocol has not been realized yet. Propagation could be divided by expansion in vitro and passaging. Even the in vitro expansion of the islet has not been achieved to date. Here, we describe a method to enable the expansion of islet clusters isolated from pregnant mice or wild-type rats by employing a combination of specific regeneration factors and chemical compounds in vitro. The expanded islet clusters expressed insulin, glucagon and somatostatin, which are markers corresponding to pancreatic β cells, α cells and δ cells, respectively. These different types of cells grouped together, were spatially organized and functioned similarly to primary islets. Further mechanistic analysis revealed that forskolin in our recipe contributed to renewal and regeneration, whereas exendin4 was essential for preserving islet cell identity. Our results provide a novel method for the in vitro expansion of islet clusters, which is an important step forward in developing future protocols and medium used for islet tissue propagation in vitro. Such method is important for future regenerative diabetes therapies and personalized medicine using large amounts of pancreatic islets derived from the same person.

Introduction

Diabetes is characterized by insulin resistance and dysfunctional insulin secretion. Recent progress in the generation of functional insulin-producing pancreatic β cells from stem cells differentiation or transdifferentiation in vitro has shed light on cell-replacement therapies for the treatment of diabetes[1-4]. However, compared to tissue/organ replacement therapy derived from somatic islets of the same person for curing diabetes[5,6], these methods still have the following deficits: (1) The function of a tissue, such as pancreatic islets, cannot be fully substituted by a single β cell type. Islets contain at least three main cell types, and together, they contribute the functional integrity adaptations to responses to different physiological changes[7-10]. In fact, recent reports indicate the essential roles of the islet circuit in maintaining glucose metabolism homeostasis[7,10]. (2) The identity of the β cells generated from transdifferentiation or stem cells is not defined, and their functions are not guaranteed. These cells normally have different gene expression patterns or certain mutations compared to somatic β cells. (3) Some of the β-like cells generated from stem cells may still have pluripotency potential, which may correlate to a risk of tumour development. (4) Finally, these cells may elicit unwanted immune responses.

Ideally, propagating islets isolated from the same patient to the desired number and then transplanting them into the patient is a promising regenerative strategy for diabetes therapy with

benefits that stem cell or transdifferentiation based cell-replacement treatment cannot match. This method will minimize the immune response, decrease the identity difference and result in a better ability to adapt to different growth niche after transplantation. In addition, the organoids generated from pancreatic islets propagated from patients can be used for drug testing because they mostly modelling composition, architecture and function of primary tissue. Recently, significant progress has been made in which somatic intestinal, gastric, colonal, hepatic and pancreatic ducts were cultured and propagated to organoids in vitro[11-19], which lays the foundation for future regenerative therapy. However, these organoids mostly exhibit epithelial properties and are difficult to be induced into endocrine cells. Thus, the in vitro propagation of functional endocrine organoids especially islets, is still required to be established.

Propagation of islets could be divided by two processes: 1) expansion of the islet cells in vitro. 2) Propagating the expanded islet cells by multiple passages. Here, we reported a method for 3D culture with a novel special medium recipe that enabled the in vitro expansion of pancreatic islet clusters isolated from pregnant mice or wild-type rats. Further mechanistic analysis suggested that forskolin (FSK) in the recipe is required for maintaining cell renewal and regeneration, whereas exendin4 is essential for preserving islet identity. Therefore, our results identified a method allowed an in vitro expansion of the pancreatic islet clusters, which could be a key step for propagating patient-specific pancreatic islets for future regenerative diabetes therapy.

Results

Recipe for the in vitro pancreatic islet expansion medium (PIEM)

To identify the optimal conditions for pancreatic islet expansion in vitro, we reviewed the chemicals, proliferation and regeneration factors used in the culture of other organoids, such as those in pancreatic ducts, cholangiocytes and hepatocytes [14,16,20,21], and pancreatic islet organoids derived from stem cells or fibroblasts (Figure 1) [1-4]. According to their reported functions, these chemicals and factors are classified into the following groups: islet identity, cell division and proliferation, and cell renewal and regeneration (Figure 1). By screening different combinations of compounds and factors, a medium composed of 12 chemicals and hormones, as well as the general nutrient nicotinamide, B27 and GlutaMAX, was created and was found to robustly support the in vitro expansion of dispersed islet clusters isolated from pregnant mice or wild-type rats. We named this in vitro pancreatic islet expansion medium

103 PIEM.

104

105 **Expansion of mouse islet clusters in vitro**

106 Previous studies have shown that pregnant mouse islets maintain expansion ability in vivo,
107 whereas wild-type mouse islets undergo little expansion[22,23]. We therefore used islets from both
108 pregnant and wild-type mice, as pregnant mice could serve as an easier starting point for
109 investigating the regeneration process. We isolated pancreatic islets from both wild-type and
110 pregnant C57BL/6 mice by collagenase P perfusion, sedimentation and handpicking. These
111 pancreatic islets were separated into single cells or cell clusters by dispase II digestion and
112 mechanical dissociation. The single cells or cell clusters were cultured in 3-dimensional (3D)
113 Matrigel. Only dispersed islet clusters isolated from pregnant mice showed significant
114 expansion during nine-day culture (Figure 2A). Approximately 5~15% of the pancreatic islet
115 clusters isolated from pregnant mice were able to expand from a surface area of 6000-9000 μm^2
116 to a surface area of 20000-30000 μm^2 (Figure 2A and 2C). In contrast, the dispersed single islet
117 cells from pregnant or wild-type mice or islet clusters from wild-type mice showed no
118 significant expansion (Figure 2B and 2D).

119 We next used qRT-PCR to examine the expression of key islet markers in the expanded islet
120 clusters. The expanded islet clusters maintain expression of the crucial pancreatic β cell markers
121 *insulin-1 (Ins1), insulin-2 (Ins2)* and *Mafa*; the α cell markers *glucagon (Gcg)* and *Mafb*; and
122 the δ cell marker *somatostatin (Sst)* (Figure 2E).

123 To confirm that specific cell types coexisted in the in vitro expanded islet clusters, which is
124 a hallmark of expanded islet clusters, we performed immunostaining for insulin, glucagon and
125 somatostatin, which are markers for pancreatic α cells, β cells and δ cells, respectively. The
126 expanded islet clusters in Matrigel were too tiny, which caused great difficulties in slice
127 preparation for immunostaining. We therefore immunostained the whole clusters as described
128 in the material and methods section. The result unambiguously identified that glucagon, insulin,
129 and somatostatin staining was present in different cells grouped together in the same expanded
130 islet clusters (Figure 3A and 3B).

131 We next examined the functional integrity of the expanded islet clusters. In response to
132 combined stimulation with 20 mM glucose and 100 nM GLP-1, the expanded islet clusters
133 demonstrated a significant increase in insulin secretion (Figure 3C). In response to high glucose
134 stimulation, both other labs and our lab have shown that UCN3 serves an endogenous paracrine
135 factor secreted by pancreatic β cells to stimulate endogenous somatostatin secretion from
136 pancreatic δ cells[7,10,24]. We therefore stimulated the expanded islet clusters with both glucose
137 and UCN3 to minimize the amount required for islet cluster usage. The in vitro expanded islet
138 clusters displayed significantly more somatostatin release in response to glucose and UCN3
139 stimulation (Figure 3D). Moreover, the expanded islet clusters secreted more glucagon in

140 response to 25mM Arginine stimulation, hallmarking the normal function of pancreatic islet α
141 cells (Figure 3E). These results confirmed the functional integrity of the in vitro expanded islet
142 clusters isolated from pregnant mice.
143 Although the expanded pancreatic islet clusters showed similar SST and Glucagon secretion
144 compared to the primary isolated islets in response to specific physiological stimulations, it is
145 worth to note that their insulin secretion in response to the combined stimulation of glucose and
146 GLP-1 is significantly weakened. Some extent degeneration during the islet expansion may
147 occur during current in vitro expansion condition, which awaits for further investigation and
148 methods optimization in our future work.
149
150 **Expansion of rat islet clusters in vitro**
151 We then examined the expansion of the dispersed rat islet single cells and clusters in PIEM.
152 Similar to those from pregnant mice, approximately 5~15% of the rat pancreatic islet clusters
153 were able to expand from a surface area of 5000-8000 μm^2 to a surface area of 20000-25000
154 μm^2, whereas dispersed rat islet single cells showed no such expansion ability (Figure 4A and
155 4B). Furthermore, qRT-PCR revealed that the expanded rat islet clusters maintained the same
156 expression of the α cell markers *Gcg* and *Mafb* as the primary rat islets but showed significantly
157 higher expression of the β cell markers *Ins-1, Ins-2* and *Mafa* and the δ cell marker *Sst* (Figure
158 4C).
159
160 **Increased gene expression related to dedifferentiation, pluripotency and proliferation in**
161 **expanded islet clusters**
162 We next examined whether the expanded islet clusters increased the expression level of genes
163 functionally associated with cell proliferation, renewal and regeneration, which are important
164 factors for in vitro regeneration. Importantly, significantly higher expression of *Ki67*, *Ccnd1*
165 and *Pcna* was found in expanded islet clusters than in islets derived from pregnant mice or
166 wild-type rats, highlighting the increased proliferation ability of the expanded islet clusters
167 (Figure 5A and 5D). Moreover, significantly higher *Nanog* and *Sox9* expression were found in
168 expanded islet clusters derived from both pregnant mice and rats (Figure 5B and 5E). These
169 results provide putative explanations accounting for the better proliferation and renewal
170 abilities of isolated islet clusters than integral islets.
171 We suspected that these islets gained proliferation and pluripotency due to dedifferentiation.
172 Thus, we examined dedifferentiation markers in expanded islet clusters and compared them
173 with those in isolated pancreatic islets. Interestingly, the expanded islet clusters showed
174 significantly higher expression of *Ngn3* and *Hlxb9*, markers that characterize pancreatic islet
175 progenitor cells (Figure 5C and 5F). Although the expanded islet clusters derived from pregnant
176 mice showed higher expression of *Gata6*, they showed decreased expression of *Gata4*, both of

which are pancreatic progenitor markers (Figure 5C and 5F). Similarly, the expanded islet clusters derived from rats did not display significant pancreatic progenitor characteristics, as they showed decreased expression of *Gata6* (Figure 5C and 5F). The data suggested that these expanded islet clusters underwent one-step dedifferentiation towards islet progenitor cells, but they did not ultimately reach the pancreatic progenitor cell stage. Taken together, this one-step dedifferentiation of isolated islet clusters cultured in PIEM towards pancreatic islet progenitor cells contributed to the gain of cell proliferation, renewal and regeneration functions of expanded islet clusters.

Essential role of FSK and exendin4 in PIEM

During our formula component screening and recipe formation for PIEM, we identified that FSK and exendin4 are both required for robust islet cluster expansion in vitro. It is known that exendin4 induced cAMP accumulation after it activates GLP-1R and downstream Gs proteins[25]. FSK is known as a cAMP agonist through direct binding to adenyl cyclase[26]. Moreover, recent reports indicate that FSK is required for proliferation of liver organoids in addition to A83-01[27]. Interestingly, removing FSK or exendin4 from PIEM has different effects on the gene expression profiles of islet identity, proliferation, renewal and regeneration markers. Removing FSK from PIEM had partial effects on islet identity and cell proliferation markers, including decreased expression of *Mafa*, *Sst* and *Ccnb1* (Figure 6A and 6B). In particular, FSK was required for the expression of cell renewal and regeneration markers, including *Nanog* and *Sox17* (Figure 6C). In contrast, exendin4 was essential for islet identity and proliferation because it maintained the expression of the pancreatic β cell markers *Ins-1*, *Pdx1* and *Mafa* and the proliferation markers *Ki67*, *Ccnb1* and *Cdk4* (Figure 6A and 6B). In particular, exendin4 was required for the expression of *Ngn3* and *Hxlb9*, two islet progenitor markers (Figure 6D). These results indicated that FSK in PIEM renders cell renewal and regeneration ability to isolated islet clusters, whereas exendin4 is essential for maintaining islet β cell identity, proliferation and dedifferentiation.

Discussion

The in vitro expansion of primary pancreatic β cells or pancreatic islet tissues has not been reported before. Here, we developed a medium recipe, which we named PIEM, that enabled the proliferation of dispersed islet clusters isolated from pregnant mice or wild-type rats in vitro (Figure 7). Isolated islet clusters normally grow from an initial surface area of 5000-9000 μm^2 to a surface area of 20000-30000 μm^2, with an estimated approximately 5-fold increase in cell number. Compared to primary pancreatic islets, expanded cell clusters have similar or higher mRNA expression levels of insulin, glucagon and somatostatin, which are markers of

pancreatic β cells, α cells and δ cells, respectively. Expanded islet clusters demonstrated normal insulin and somatostatin secretion in response to physiological stimulation and normal glucagon secretion in response to amino acids, suggesting that the clusters recapitulate specific functions of pancreatic islets. Immunostaining further confirmed that the cells expressing insulin, glucagon and somatostatin were grouped together and spatially organized in expanded islet clusters similar to primary islets. These data indicated that the expanded islet clusters behaves similar to an in vitro organoid, thus serving as an initial step for future islet propagation methods developing in vitro. The ultimate goal of developing efficient methods for propagation islets in vitro is to fulfil the demand for regenerative diabetes therapy and personalized drug tests.

It is worth noting that we observed the expansion of islet clusters isolated from only pregnant mice and wild-type rats in PIEM, not those isolated from wild-type mice. Pregnant mice are well known for their increased pancreatic β cell proliferation[28-31], which provides a useful starting point for testing medium recipes. Although we did not observe the proliferation of islet clusters isolated from wild-type mice, we did observe the expansion of islet clusters isolated from wild-type rats, which share more properties identical to human islets than mouse islets. An urgent need is to test whether PIEM enables the expansion of primary islet clusters isolated from human patients.

Another notable observation is that only dispersed islet clusters were able to proliferate in vitro, whereas dispersed single primary islet cells could not. Two important factors may contribute to this discrepancy. First, the islet clusters contain multiple cell types, which are not only the origins of the different cell types in the finally expanded islet clusters but also may form certain cell circuits and gradient hormone concentrations to support in vitro expansion. Second, primary single cells must undergo harsher digestion than islet clusters, which means that the extracellular parts of the membrane or matrix proteins, including receptors, ion channels and transporters, may be digested and their function impaired. These membrane or matrix proteins may be key for in vitro tissue expansion. Therefore, future analysis of the different expression patterns and functions of membrane proteins and signalling circuit differences between isolated islet clusters and single cells may provide more clues for unravelling the secrets of the in vitro expansion of islets and provide guidance for a better strategy for in vitro islet cluster expansion.

We noticed that expanded islet clusters have higher expression of *Ngn3* and *Hlxb9*, which are markers of pancreatic islet progenitor cells. This observation indicated that in vitro expanded islets underwent certain dedifferentiation. However, we doubt that the islet clusters gained proliferation ability through this dedifferentiation. Further experiments with withdrawal of key chemicals in the medium will test this hypothesis. For clinical usage, the in vitro expansion of 100 patient islets to 1 million islets without significant gene mutations, while

preserving pancreatic islet function, and transferring them back to the patient to recover glucose homeostasis is ideal. However, the current approach of PIEM allows only approximately 5-fold growth of approximately 5%-15% of isolated islet clusters. The expansion of the islet clusters was only observed in the first passage. Therefore, it is unlikely to cause significant genomic instability. However, the current acquired in vitro expanded islet cluster cells do not contain sufficient amounts for RNA-seq to verify the gene expression profile or transplantation to confirm their in vivo functions. More important, the final usage of in vitro propagated islet clusters for regenerative therapy to treat diabetes in general require millions of pancreatic islets. Therefore, a future revision of the current PIEM composition and method for in vitro islet propagation is in an urgent demand.

One of the future directions for the optimization of in vitro islet propagation methods could be as follows: (1) initial expansion and dedifferentiation of islet clusters to islet progenitor-like cell clusters, (2) rapid proliferation of islet progenitor-like cell clusters to a sufficient amount, (3) differentiation of progenitor-like cell clusters to functional integral islets, (4) transplantation or (5) exploitation of these expanded islet tissues for drug tests. Therefore, manifesting the markers for each stage and searching chemicals that are useful for each procedure in the above protocol is our next goal to fulfil expanded islet clusters regeneration in vitro in the future.

Taken together, we have developed a recipe for PIEM, and our 3D culture method enabled the initial generation of expanded islet clusters via the in vitro expansion of isolated islet clusters from pregnant mice or wild-type rats in the first passage. Our study is one important step forward for the realization of a method to fulfil in vitro propagation of pancreatic islet in vitro, which may facilitate new therapies development to treat diabetes.

Materials and methods

Key resources table

Reagent	Source	Identifier
Antibodies		
Anti-Insulin	Santa	sc-9168
Anti-Glucagon	Sigma	G2654
Anti-Somatostatin	Abcom	ab30788
Donkey anti-Mouse IgG (H+L) (secondary antibody)	Invitrogen	A10037
Donkey anti- Rabbit IgG (H+L) (secondary antibody)	Invitrogen	A21206

Donkey anti-Rat IgG(H + L) (secondary antibody)	Abcam	ab150154
Chemicals, Peptides, and Recombinant Proteins		
TRIzol	Thermo	15596-026
Cell Recovery Solution	Corning	354253
Matrigel	Corning	356231
GlutaMax	Thermo	35050-061
Penicillin-Streptomycin	Biological Industries	03-031-1B
recombinant human EGF	Peprotech	AF-100-15
recombinant human FGF10	Peprotech	100-26
CHIR-99021	BioGems	2520691-1MG
N-Acetylcysteine	Sigma-Aldrich	A9165-25G
Nicotinamide	Sigma-Aldrich	N0636
B27 Supplement (minus Vitamin A)	Thermo	12587-010
gastrin-1, human	MedChemExpress	HY-P1097
A83-01	Adooq Bioscience	A12358
Y-27632	Adooq Bioscience	A11001
Forskolin	TargetMol	T2939
Exendin4	ChinaPeptides	
5-Iodotubercidin	Adooq Bioscience	A13948
L-Ascorbic acid	Sigma-Aldrich	A4544
Triton X-100	Solarbio	T8200
Donkey Serum	Solarbio	SL050
DAPI	Beyotime	C1002
Critical Commercial Assays		
Glucagon ELISA Kit	Cloud-Clone Corp	CEB266Mu
Insulin ELISA Kit	merck-millipore	EZRMI-13K
Somatostatin EIA Kit	Phoenix Pharmaceuticals	EK-060-03
Software and Algorithms		

Prism Graphpad		https://www.graphpad.com
Adobe illustator	Adobe	http://www.adobe.com/products/illustrator.html
ImageJ		https://imagej.nih.gov/ij/
Experimental Models: Animals/Cell Lines		
C57B6	Shandong University animals care facility	
Wistar	Shandong University animals care facility	
Oligonucleotides		
Primer: Gcg Forward for mouse cDNA: GGCACATTCACCAGCGACTA	This paper	N/A
Primer: Gcg Reverse for mouse cDNA: GAGAAGGAGCCATCAGCGTG	This paper	N/A
Primer: Ins1 Forward for mouse cDNA: TGGACTATAAAGCTGGTGGGC	This paper	N/A
Primer: Ins1 Reverse for mouse cDNA: TGTGTAGAAGAAGCCACGCT	This paper	N/A
Primer: Ins2 Forward for mouse cDNA: GCTATCCTCAACCCAGCCTAT	This paper	N/A
Primer: Ins2 Reverse for mouse cDNA: CTCCAGTGCCAAGGTCTGAA	This paper	N/A
Primer: Mafa Forward for mouse cDNA: GGCACATTCTGGAGAGCGAG	This paper	N/A
Primer: Mafa Reverse for mouse cDNA: TCACAGAAAGAAGTCGGGTGC	This paper	N/A
Primer: Mafb Forward for mouse cDNA: TACCAGCAGATGAACCCCGA	This paper	N/A
Primer: Mafb Reverse for mouse cDNA: GCTAGTGGGTAGCTGTTGCG	This paper	N/A
Primer: Sst Forward for mouse cDNA: CCACCGGGAAACAGGAACTG	This paper	N/A
Primer: Sst Reverse for mouse cDNA: TTGCTGGGTTCGAGTTGGC	This paper	N/A
Primer: Ngn3 Forward for mouse cDNA: ACGCAATTTACTCCAGGCGA	This paper	N/A
Primer: Ngn3 Reverse for mouse cDNA: GAGGCGCCATCCTAGTTCTC	This paper	N/A
Primer: Hlxb9 Forward for mouse cDNA: GAACCTCTTGGGGAAGTGCC	This paper	N/A
Primer: Hlxb9 Reverse for mouse cDNA:	This paper	N/A

TCTTTGGCCTTTTTGCTGCG		
Primer: Gata4 Forward for mouse cDNA: AGATCGCGCCGGTTTTCTG	This paper	N/A
Primer: Gata4 Reverse for mouse cDNA: GATCACCCACCGGCTAAAGA	This paper	N/A
Primer: Gata6 Forward for mouse cDNA: TTTCCGGCAGAGCAGTAAGAG	This paper	N/A
Primer: Gata6 Reverse for mouse cDNA: GAAACGCTTTGGCAGGCAC	This paper	N/A
Primer: Sox9 Forward for mouse cDNA: CGGAACAGACTCACATCTCTCC	This paper	N/A
Primer: Sox9 Reverse for mouse cDNA: GCTTGCACGTCGGTTTTGG	This paper	N/A
Primer: Ki67 Forward for mouse cDNA: CAAGGCGAGCCTCAAGAGATA	This paper	N/A
Primer: Ki67 Reverse for mouse cDNA: TGTGCTGTTCTACATGCCCTG	This paper	N/A
Primer: Nanog Forward for mouse cDNA: AGGACAGGTTTCAGAAGCAGA	This paper	N/A
Primer: Nanog Reverse for mouse cDNA: CCATTGCTAGTCTTCAACCACTG	This paper	N/A
Primer: Ccnb1 Forward for mouse cDNA: GCGTGTGCCTGTGACAGTTA	This paper	N/A
Primer: Ccnb1 Reverse for mouse cDNA: CCTAGCGTTTTTGCTTCCCTT	This paper	N/A
Primer: Ccnd1 Forward for mouse cDNA: TGACTGCCGAGAAGTTGTGC	This paper	N/A
Primer: Ccnd1 Reverse for mouse cDNA: CTCATCCGCCTCTGGCATT	This paper	N/A
Primer: Pcna Forward for mouse cDNA: TTGCACGTATATGCCGAGACC	This paper	N/A
Primer: Pcna Reverse for mouse cDNA: GGTGAACAGGCTCATTCATCTCT	This paper	N/A
Primer: Pdx1 Forward for mouse cDNA: CCTTTCCCGAATGGAACCGA	This paper	N/A
Primer: Pdx1 Reverse for mouse cDNA: GGGCCGGGAGATGTATTTGT	This paper	N/A
Primer: Sox17 Forward for mouse cDNA: CCAAAGCGGAGTCTCGCAT	This paper	N/A
Primer: Sox17 Reverse for mouse cDNA: GCCTAGCATCTTGCTTAGCTC	This paper	N/A
Primer: Cdk4 Forward for mouse cDNA: GCCACTCGATATGAACCCGT	This paper	N/A

Primer: Cdk4 Reverse for mouse cDNA: CACAGACATCCATCAGCCGT	This paper	N/A
Primer: Gcg Forward for rat cDNA: TTCACAGGGCACATTCACCA	This paper	N/A
Primer: Gcg Reverse for rat cDNA: CTATGGCGACTTCTTCCGGG	This paper	N/A
Primer: Insulin1 Forward for rat cDNA: ACCCTAAGTGACCAGCTACAATC	This paper	N/A
Primer: Insulin1 Reverse for rat cDNA: CGGGTCCTCCACTTCACGAC	This paper	N/A
Primer: Insulin2 Forward for rat cDNA: ACCTTTGTGGTTCTCACTTGGT	This paper	N/A
Primer: Insulin2 Reverse for rat cDNA: CAGTGCCAAGGTCTGAAGGTCA	This paper	N/A
Primer: Mafa Forward for rat cDNA: GACAAGTTTGCGCAGGCCG	This paper	N/A
Primer: Mafa Reverse for rat cDNA: GTATTCACCGTTCTCGGGGC	This paper	N/A
Primer: Mafb Forward for rat cDNA: GCAACGGTAGTGTGGAGGAC	This paper	N/A
Primer: Mafb Reverse for rat cDNA: GAGCTGCGTCTTCTCGTTCT	This paper	N/A
Primer: Sst Forward for rat cDNA: GCTACTGGAGTCGTCTCTGC	This paper	N/A
Primer: Sst Reverse for rat cDNA: GGCATCGTTCTCTGTCTGGT	This paper	N/A
Primer: Ngn3 Forward for rat cDNA: GTTCCAATTCCACCCCACCT	This paper	N/A
Primer: Ngn3 Reverse for rat cDNA: CGCAGGGTCTCGATCTTTGT	This paper	N/A
Primer: Hlxb9 Forward for rat cDNA: CGGCGCTTTCCTACTCGTAT	This paper	N/A
Primer: Hlxb9 Reverse for rat cDNA: TCCCCAAGAGGTTCGATTGC	This paper	N/A
Primer: Gata4 Forward for rat cDNA: TGAATGGTATCAACCGGCCC	This paper	N/A
Primer: Gata4 Reverse for rat cDNA: TTTGAATCCCCTCCTTCCGC	This paper	N/A
Primer: Gata6 Forward for rat cDNA: TCATCACCACCCGACCTACT	This paper	N/A
Primer: Gata6 Reverse for rat cDNA: GCATGCGTTGCACAGGTAAT	This paper	N/A
Primer: Sox9 Forward for rat cDNA: CACAAGAAAGACCACCCCGA	This paper	N/A

Primer: Sox9 Reverse for rat cDNA: TGCACGTCTGTTTTGGGAGT	This paper	N/A
Primer: Ki67 Forward for rat cDNA: ACAGGGCTTAGGAAACAGTCC	This paper	N/A
Primer: Ki67 Reverse for rat cDNA: GGTTCTAACTGGTCTTCCTGGT	This paper	N/A
Primer: Nanog Forward for rat cDNA: AAGTCCCTTCCCTTGCCGT	This paper	N/A
Primer: Nanog Reverse for rat cDNA: CTCGGGACCAGACAGCTTTAG	This paper	N/A
Primer: Ccnb1 Forward for rat cDNA: GGGTGTCTTCTCAGATCGGC	This paper	N/A
Primer: Ccnb1 Reverse for rat cDNA: TCCACAGGTTTTGGTAGGGC	This paper	N/A
Primer: Ccnd1 Forward for rat cDNA: TCAAGTGTGACCCGGACTG	This paper	N/A
Primer: Ccnd1 Reverse for rat cDNA: CTACTTGGTGACTCCCGCCT	This paper	N/A
Primer: Pcna Forward for rat cDNA: GACGGGGTGAAGTTTTCTGC	This paper	N/A
Primer: Pcna Reverse for rat cDNA: GACAGTGGAGTGGCTTTTGTG	This paper	N/A

Animals

C57BL/6 mice and rats of Wistar strain were housed and bred under specific-pathogen-free conditions at Shandong University animals care facility. Female animals were used. The numbers of animals are indicated in the figure legends within each experiment. All animal care and experiments were reviewed and approved by the Animal Use Committee of Shandong University School of Medicine.

Pancreatic islet isolation and digestion into single cells or cell clusters.

We isolated pancreatic islets from C57BL/6 mice, pregnant C57BL/6 mice and Wistar rat. Briefly, mice and rat were killed by cervical dislocation and then pancreas were isolated from them individually. Adult pancreas were digested by Collagenase P (Roche, 11213873001) at 37°C for 18-25 minutes. Digestion was stopped by cold Hank's balanced salt solution (136.9 mM NaCl; 5.4 mM KCl; 1.3 mM $CaCl_2$; 0.8 mM $MgSO_4$; 0.44 mM KH_2PO_4; 0.34 mM Na_2HPO_4; 5.55 mM D-glucose; 4.4 mM $NaHCO_3$, pH=7.4) followed by sedimentation for three

293 or five times at 4℃. The islets were collected by hand picking using a stereomicroscope and
294 were cultured overnight in islet complete media containing 5.6 mM glucose (Biological
295 Industries), 10% FBS (Gibco),0.1% penicillin/streptomycin.Next we obtained single cells and
296 cell clusters of different diameters (20-150μm)under the microscope by controlling enzyme
297 concentration, digestion and settling time. Islets briefly after naturally settling for 1 minute in
298 KRBB buffer (135 mM NaCl; 4.7 mM KCl; 1.2 mM KH_2PO_4; 10 mM Hepes; 3 mM D-glucose;
299 5 mM $NaHCO_3$; 0.1% BSA; 1% penicillin/streptomycin, pH=7.4), preheated EDTA-KRBB
300 solution was added, gently piped for 2 min, then placed in a 33℃ water bath for 10 min; after
301 clearing the supernatant, digested with dispase II(0.1-0.5 U/ml, Roche, 04942078001) in a 33℃
302 water bath for 3~8 min to obtain single cells or islet cell clusters. Mechanical fragmentation
303 was achieved by gentle pipetting (5–10 times) with a glass dropper in a 15ml glass centrifuge
304 tube. Using DMEM/F-12 (Biological Industries) medium with 10% FBS (Gibco) and 1%
305 penicillin/streptomycinfor termination of digestion.Collecting single cells or cell clusters by
306 centrifugation (300×g, 5 min, Room temperature).
307
308 **Production of Rspo1-conditioned medium**
309 The RSPO1 conditioned medium is home-made and will be described in other papers.
310
311 **Expanded islet clusters culture**
312 Isolated islet cell clusters were washed twice with DMEM/F-12 (Biological Industries),
313 counted and mixed with Matrigel in glass centrifuge tubes. 30,000–50,000 cells or 10-20 cell
314 clusters were used per well of a pre-warmed 24-well plate (the volume of 50 μl). After Matrigel
315 was solidified for 30min at 37℃, pancreatic islet expansion medium (PIEM) was added. PIEM
316 consists of DMEM/F-12 (1% GlutaMax,1% Penicillin-Streptomycin) plus 15% RSPO1
317 conditioned medium (home-made), 3 μM CHIR-99021 (BioGems), 1 μM A83-01 (Adooq
318 Bioscience), 10 nM gastrin-1 (MedChemExpress), 1.25 mM N-acetylcysteine (Sigma), 10 μM
319 Y-27632 (Adooq Bioscience), 10 μM forskolin (TargetMol), 50 ng/ml Exendin-4
320 (ChinaPeptides), 50 μg/ml L-Ascorbic acid (Sigma), 250 nM 5-Iodotubercidin (Adooq
321 Bioscience), 50 ng/ml FGF10 (PeproTech), 50 ng/ml EGF (PeproTech), 10 mM Nicotinamide
322 (Sigma), B27 Supplement (minus Vitamin A) (Thermo). Cultures were kept at 37℃, 5% CO_2
323 in a humidified incubator. During culturing, medium was refreshed every three days.
324
325 **RNA extraction and qRT-PCR.**
326 RNA from islets and islet single cells as well as islet cell clusters derived from C57BL/6
327 mice, pregnant C57BL/6 mice and Wistar rat, Including the corresponding islet clusters were

extracted with TRIzol reagent (Thermo). We used the tip to pick up the expanded islet clusters from the Matrigel using a stereomicroscope. Cell Recovery Solution(Corning) is used to recover cells from Matrigel. Add 0.2-0.5 ml per 24-well plate cold Cell Recovery Solution. Scrape the gel layer into an cold 1.5 ml Microcentrifuge Tube and leave it on ice for 0.5-1 hour until the gel complete dissolved. Collecting cells by centrifugation (200×g, 5 min, 4℃). And we used TURBO DNA-free TMKit (Invitrogen) to remove DNA contamination from the extracted RNA. cDNA synthesis were used the qRT-PCR Kit (Toyobo, FSQ-101). We conducted quantitative reverse-transcriptase PCR (qRT-PCR) in the LightCyclerqPCR apparatus (Bio-Rad) with the FastStart SYBR Green Master (Roche). All primers used for qRT-PCR assay are listed in Key Resources Table.

Immunofluorescence staining

Expanded islet clusters in martrigel were fixed in 4% paraformaldehyde for 30 min at room temperature, followed with blocking and permeabilizing in PBS with 0.5% Triton X-100 (Solarbio, T8200) and 5% donkey serum (Solarbio, SL050) for 30min at room temperature. Then, samples were incubated with primary antibody at 4℃ overnight, followed by incubation with secondary antibody for 2h at room temperature. DAPI (Beyotime, C1002) was used to stain the nucleus and find islets. The following antibodies were used for immunofluorescence: anti-INSULIN (1:200, sc-9168; Santa), anti-SOMATOSTATIN (1:600, ab30788; Abcam), anti-GLUCAGON (1:200, G2654; Sigma). Expanded islet clusters imaging was performed on Zeiss LSM 780 and processed using ImageJ or Adobe illustator software.

Insulin secretion measurement

The insulin secretions were preformed similar to previously described in our group[7,9,24]. Fifty day 7 expanded islet clusters (cell number is comparable to 1/10 of primarily isolated islets, 1000~2000 cells) or ten islets for each group were starved in 2.8 mM glucose MKRBB buffer (5 mM KCl, 120 mM NaCl, 15 mM Hepes, 24 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH=7.4) for 1 hour at 37℃, and then treated with 20 mM glucose and 100 nM GLP1, whereas the control groups were treated with 2.8 mM glucose for 30 min. The supernatant fractions were collected to measure the secreted insulin. The insulin levels were measured with the Millipore

358 Rat / Mouse Insulin (Cat. # EZRMI-13K), as what indicated by the manufacturer's instructions.

359

360 **Somatostatin measurement**

361 The somatostatin secretions were preformed similar to previously described in our group[7,9,24].
362 Fifty day 7 expanded islet clusters or ten primary islets for each group were starved in 1 mM
363 glucose MKRBB buffer for 1 hour at 37℃, then treated with 20 mM glucose and 100 nM UCN3,
364 whereas the control groups were treated with 1 mM glucose for 1 hour. The supernatant
365 fractions were collected for somatostatin measurement using the Phoenix Pharmaceuticals
366 ELISA kit (EK-060-03), as what indicated by the manufacturer's instructions.

367

368 **Glucagon measurement**

369 The glucagon secretions were preformed similar to previously described in our group[7,9,24].
370 Fifty day 7 expanded islet clusters or ten primary islets for each group were starved in 12 mM
371 glucose MKRBB buffer for 1 hour at 37℃, and then treated with 1 mM glucose and 25 mM
372 Arginine, whereas the control groups were treated with 5 mM Arginine for 1 hour. The
373 supernatant fractions were collected to measure the secreted glucagon. The glucagon levels
374 were measured with the Cloud-Clone Corp Mouse Glucagon (Cat. CEB266Mu), as what
375 indicated by the manufacturer's instructions.

376

377 **Statistical analysis**

378 All the qRT-PCR data were performed independently for at least three times. Statistical
379 analyses were performed using GraphPad Prism 7 software. Experimental data were performed
380 using unpaired two-tailed Student's t-test. Results are presented as mean ± SEM; $P < 0.05$ was
381 considered statistically significant.

382

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Competing interests

The authors declare no competing interests.

Author contributions

X.Y. started the idea for in vitro expansion of islet tissues since 2010. X.Y. J.-P.S supervised the overall project design and execution. H.-L.H provided key experience and protocols for in vitro 3D tissue culture and initial components advices. X.Y. J.-P.S. and H.-L.H. initiated the project. J.P.-S organized the screening idea. X.Y. J.-P.S and J.-Y.L designed the screening details for chemicals and other factors. X.Y., J.-P.S., J.-Y.L. and J.C. designed the cell culture and all other experiments. J.-Y.L., J.C., Y.-Q.D., W.P., X.Y. and J.-P.S. participated in data analysis and interpretation. Z.Z., Y.-Q.D. and R.-J.C. provided the new material preparation methods. J.W., Z.Y., F.Y. and J.A. provided insightful idea. Y.-F.X., H.L., W.-T.A. and J.W. participated in molecular biology and animal experiments. X.Y. and J.-P.S wrote the manuscript. H.-L.H, R.-J.C., J.-Y.L., J.C., Y.-Q.D. and W.P. revised manuscript and provided key insights for discussion.

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Professor Xiao Yu. (yuxiao@sdu.edu.cn), Jin-Peng Sun (sunjinpeng@bjmu.edu.cn) or Hui-Li Hu. (huhuili@sdu.edu.cn).

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Figure legends

Figure 1. Flowchart for the development of the medium recipe used for pancreatic islet expansion.

The chemicals and hormones used in previous studies for stem cell expansion, differentiation and pancreatic islet cell conversion (upper panel) are classified by their reported function related to cell division and proliferation, islet identity, and cell renewal and regeneration (middle panel). The common factors are summarized and combined for the formulation of the medium recipe used for in vitro pancreatic islet cluster expansion.

Figure 2. In vitro expansion of pregnant mouse islet clusters.

(A) Representative images of single islet cells or islet clusters (approximately 6000-9000 μm^2 original surface area) derived from pregnant mice after 3D culture in pancreatic islet cluster expansion medium (PIEM) at the indicated time points. Experiments were performed on pregnant mice. The scale bar in the single-cell image represents 50 μm, and one in the cluster image represents 100 μm.

(B) Representative images of dispersed wild-type mouse islet single cells or clusters at various times after 3D culture with PIEM. The scale bar in the single-cell image represents 50 μm, and one in the cluster image represents 100 μm.

(C) Numbers of expanded clusters per 100 islet cell clusters. The experiments were performed in triplicate and with pregnant mice. The data are represented as the mean ± SEM.

(D) Numbers of expanded clusters per 100 islet cell clusters. The experiments were performed in triplicate on wild-type mice. The data are represented as the mean ± SEM.

(E) Comparison of islet cell identity marker expression in pregnant mouse pancreatic islet clusters vs isolated primary mouse islets. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; clusters were compared with primary mouse islets. The data are shown as the mean ± SEM of at least three independent experiments. The data statistics were analysed using an unpaired two-tailed Student's t-test.

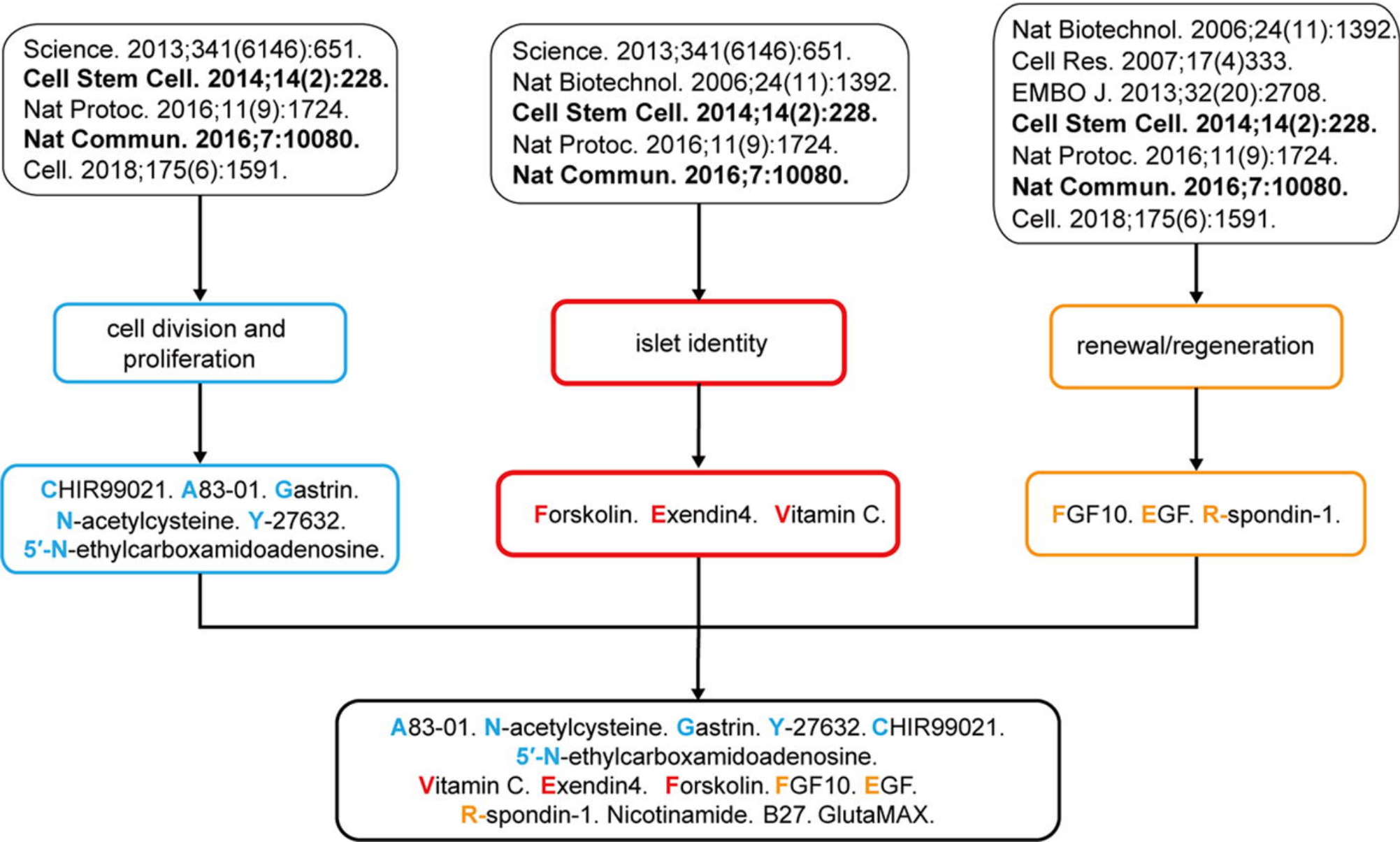
Figure 3. Immunostaining for pancreatic islet cell markers and functional examination of in vitro expanded pancreatic islet clusters.

(A) Immunofluorescence staining for insulin and glucagon in expanded pancreatic islet clusters derived from pregnant mice; the scale bar represents 100 μm.

512 (B) Immunofluorescence staining for insulin and somatostatin in expanded pancreatic islet
513 clusters derived from pregnant mice; the scale bar represents 100 μm.
514 (C) Insulin secretion of primary islets or expanded islet clusters treated with 20 mM glucose
515 and 100 nM glucagon peptide 1 (GLP-1) were measured for 30 minutes. The control groups
516 were treated with 2.8 mM glucose. ***, $p < 0.001$, stimulation groups were compared with
517 control groups. ###, $p < 0.001$, expanded islet clusters were compared with primary islets.
518 (D) Somatostatin secretion of primary islets and expanded islet clusters treated with 20 mM
519 glucose and 100 nM UCN3 were measured for 1 hour. The control groups were treated with 1
520 mM glucose. **, $p < 0.01$, stimulation groups were compared with control groups. NS, no
521 significance, expanded islet clusters were compared with primary islets.
522 (E) Glucagon secretion of primary islets and expanded islet clusters treated with 25 mM
523 Arginine were measured for 1 hour. The control groups were treated with 5mM Arginine. ***,
524 $p < 0.001$, stimulation groups were compared with control groups. NS, no significance,
525 expanded islet clusters were compared with primary islets.
526 (C-E) The data are shown as the mean ± SEM of at least three independent experiments. The
527 data statistics were analysed using an unpaired two-tailed Student's t-test.
528
529 **Figure 4. In vitro expansion of rat islet clusters.**
530 (A) Representative images of rat islet single cells or clusters (approximately 5000-8000 μm^2
531 original surface area) at various times after 3D culture with PIEM. The scale bar in the single-
532 cell image represents 50 μm, and one in the cluster image represents 82.5 μm.
533 (B) Numbers of expanded clusters per 100 islet cell clusters. The experiments were performed
534 on rats in triplicate. The data are represented as the mean ± SEM.
535 (C) Comparison of the expression of islet cell identity markers in rat expanded clusters
536 compared with primary islets. *, $p < 0.05$; **, $p < 0.01$; clusters were compared with primary
537 rat islets. The data are shown as the mean ± SEM of at least three independent experiments.
538 The data statistics were analysed using an unpaired two-tailed Student's t-test.
539
540 **Figure 5. qRT-PCR analysis of gene expression in** expanded clusters **compared to primary**
541 **islets.**
542 (A) Comparison of the expression of proliferation and cell cycle markers in pregnant mouse
543 expanded clusters vs. isolated primary islets.
544 (B) Comparison of the expression of pluripotency and regeneration markers in pregnant mouse
545 expanded clusters vs. isolated primary islets.
546 (C) Comparison of the expression of islet and pancreatic progenitor markers in pregnant mouse
547 expanded clusters vs. isolated primary islets.
548 (D) Comparison of the expression of proliferation and cell cycle markers in rat expanded

549 clusters vs. isolated primary islets.
550 (E) Comparison of the expression of pluripotency and regeneration markers in rat expanded
551 clusters vs. isolated primary islets.
552 (F) Comparison of the expression of islet and pancreatic progenitor markers in rat expanded
553 clusters vs. isolated primary islets.
554 (A-F) *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; clusters were compared with primary islets. The
555 data are shown as the mean ± SEM of at least three independent experiments. The data statistics
556 were analysed using an unpaired two-tailed Student's t-test.
557
558 **Figure 6. Essential roles of FSK and exendin4 in in vitro pancreatic islet expansion**.
559 (A-D) qRT-PCR analysis of the expression of islet cell identity markers (A), proliferation and
560 cell cycle markers (B), pluripotency and regeneration markers (C) and islet and pancreatic
561 progenitor markers (D) in pregnant mouse clusters after 3 days of culture with PIEM or PIEM
562 without forskolin or exendin4.
563 (A-D) *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; cell clusters cultured in PIEM without FSK or
564 exendin4 were compared with those cultured in complete PIEM. The data are shown as the
565 mean ± SEM of at least three independent experiments. The data statistics were analysed using
566 an unpaired two-tailed Student's t-test.
567
568 **Figure 7. Scheme of the current approach and future direction.**
569 Schematic depicting the isolation, seeding and the expansion of primary islet cell clusters. After
570 the islets were isolated from pregnant mice or wild-type rats, the islets were digested into
571 appropriate cell clusters by incubation with the dispase II. The digestion time were optimized
572 and the mechanical blow force was used for clusters generation. These clusters were cultured
573 3D in PIEM to achieve the in vitro expansion of the islet cell clusters.
574
575
576

Figure 1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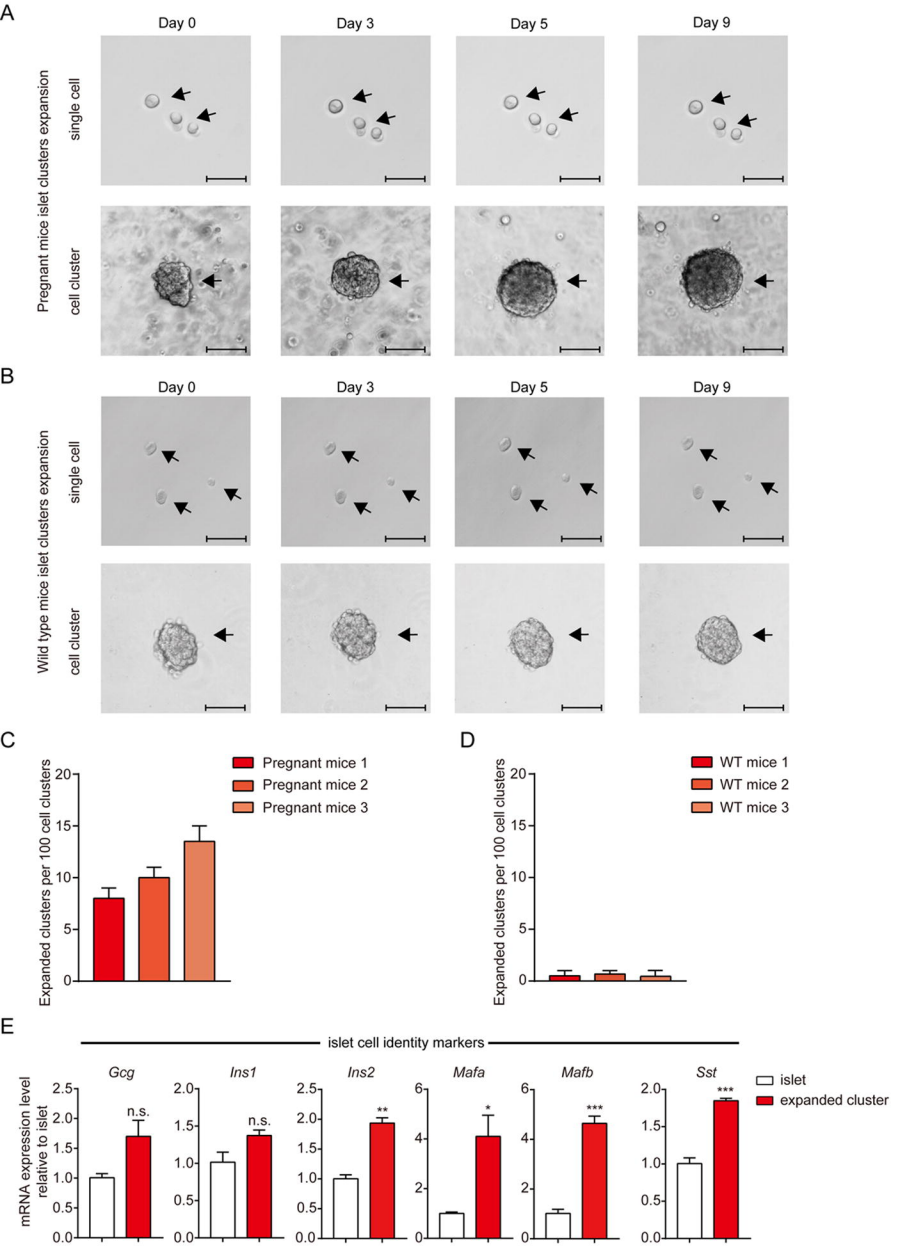
cell division and proliferation
islet identity
renewal/regeneration
CHIR99021. A83-01. Gastrin. N-acetylcysteine. Y-27632. 5′-N-ethylcarboxamidoadenosine.
Forskolin. Exendin4. Vitamin C.
FGF10. EGF. R-spondin-1.
A83-01. N-acetylcysteine. Gastrin. Y-27632. CHIR99021. 5′-N-ethylcarboxamidoadenosine. Vitamin C. Exendin4. Forskolin. FGF10. EGF. R-spondin-1. Nicotinamide. B27. GlutaMAX.

Figure 2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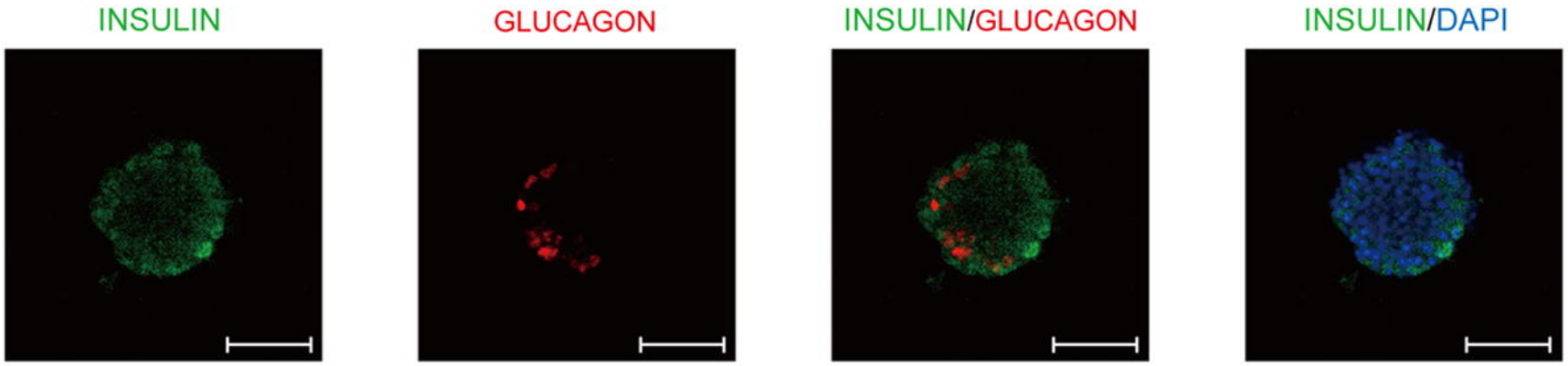
Day 0
Day 3
Day 5
Day 9
Pregnant mice islet clusters expansion
single cell
cell cluster
B
Day 0
Day 3
Day 5
Day 9
Wild type mice islet clusters expansion
single cell
cell cluster
C
Expanded clusters per 100 cell clusters
Pregnant mice 1
Pregnant mice 2
Pregnant mice 3
D
Expanded clusters per 100 cell clusters
WT mice 1
WT mice 2
WT mice 3
E
islet cell identity markers
mRNA expression level relative to islet
Gcg
Ins1
Ins2
Mafa
Mafb
Sst
islet
expanded cluster
n.s.
n.s.
**
*
***
***

Figure 3

A



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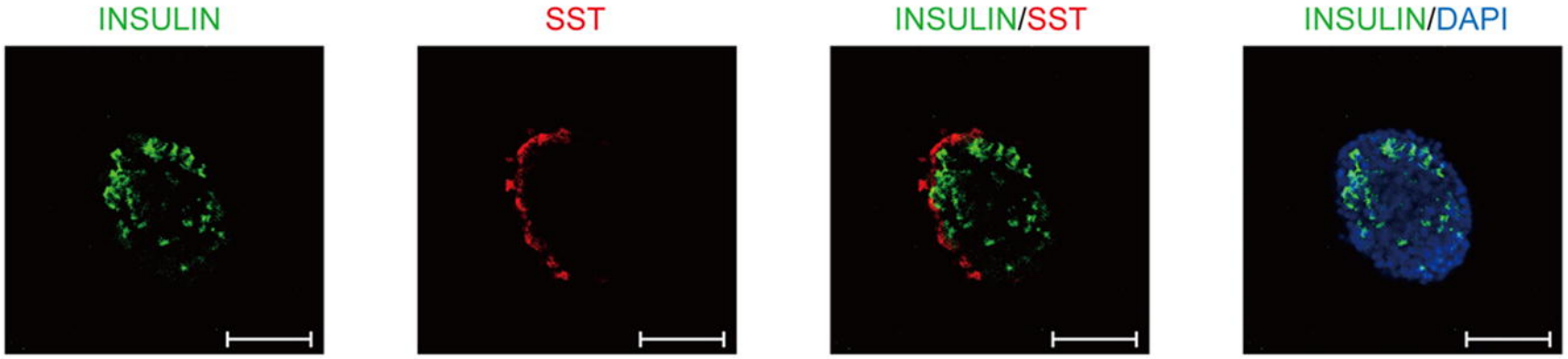

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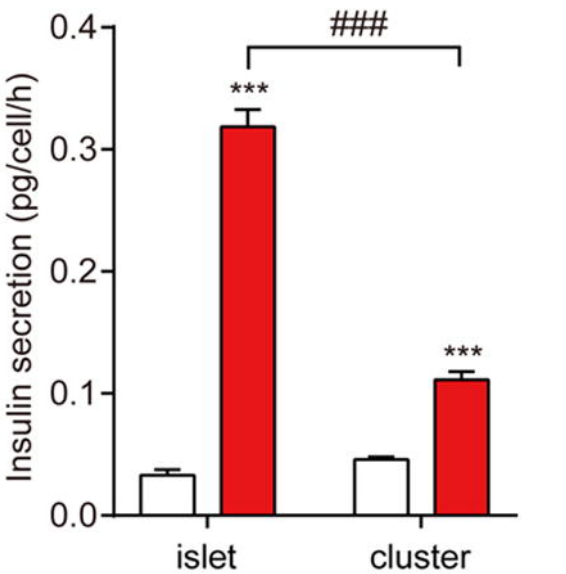

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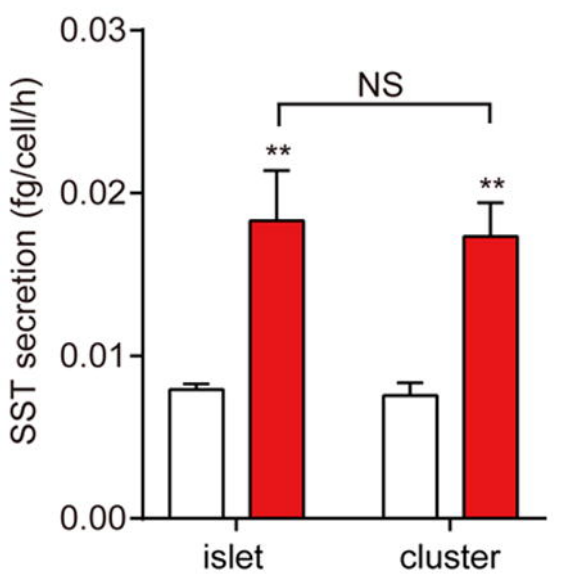

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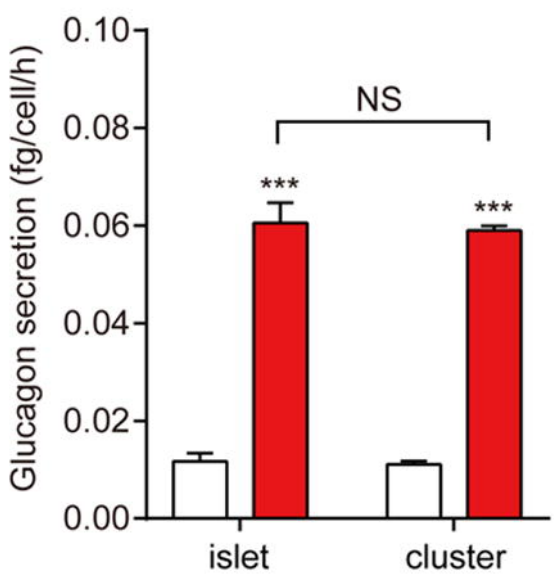


Figure 4

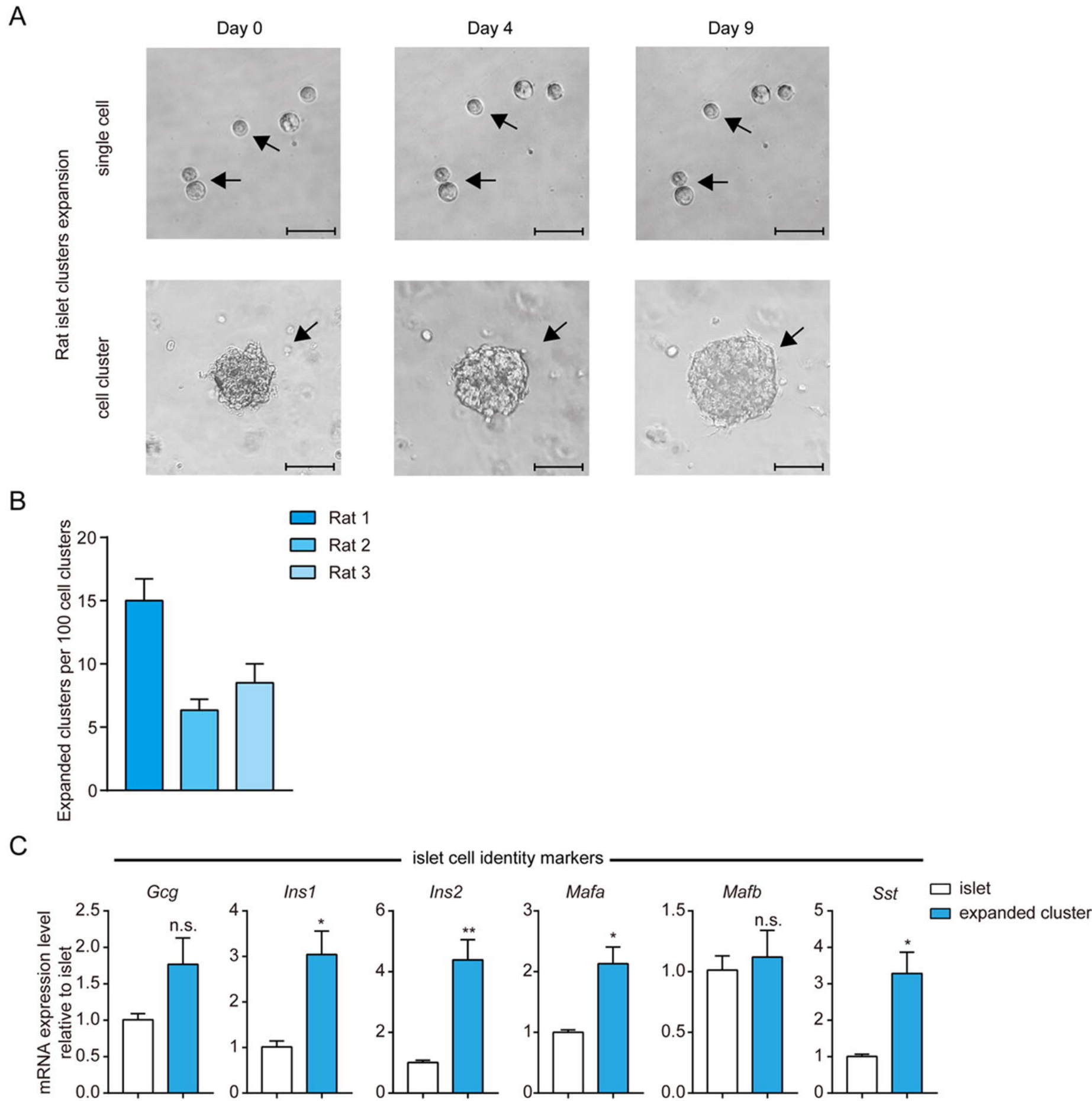
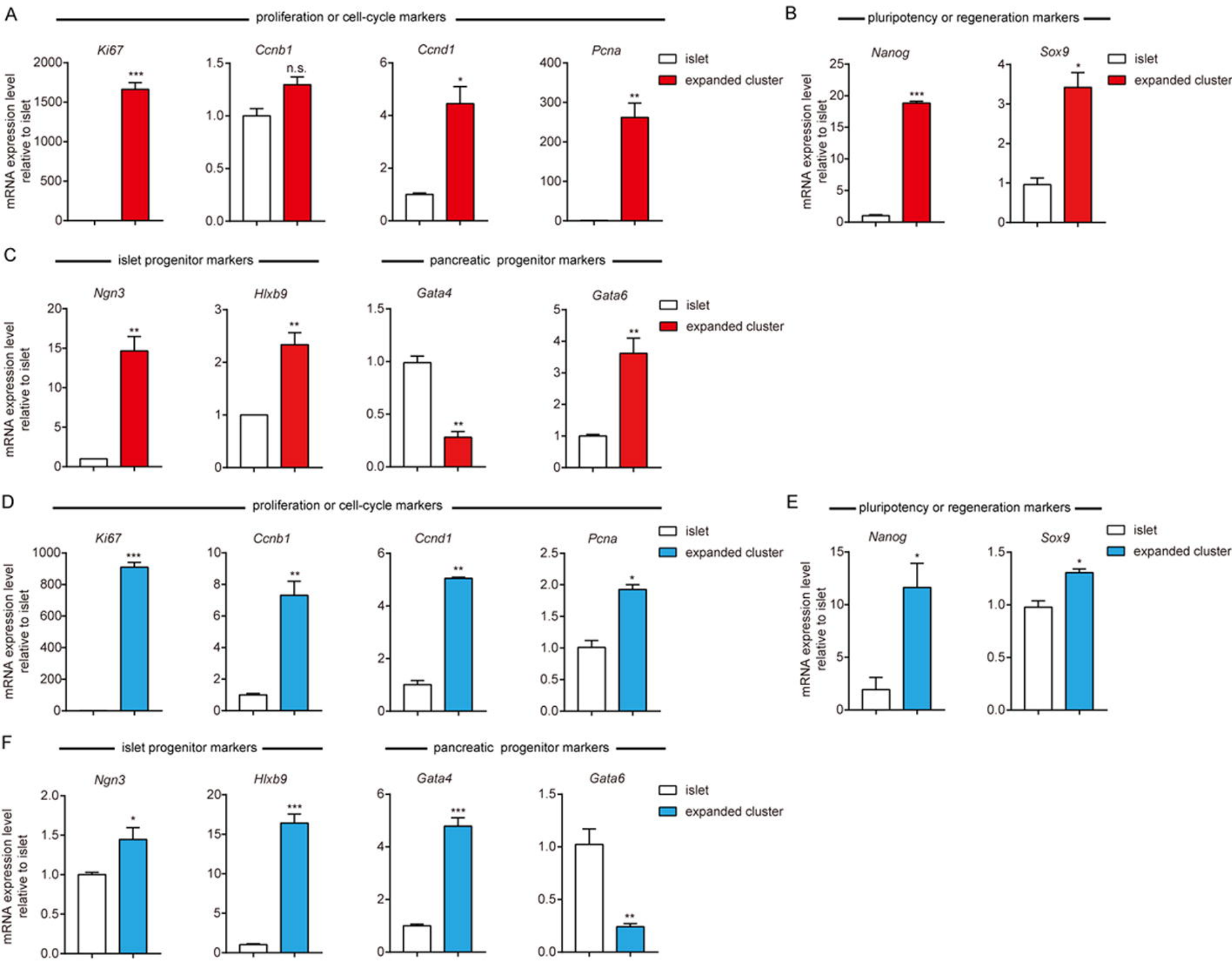

A
Day 0
Day 4
Day 9
Rat islet clusters expansion
single cell
cell cluster
B
Rat 1
Rat 2
Rat 3
Expanded clusters per 100 cell clusters
0
5
10
15
20
C
islet cell identity markers
islet
expanded cluster
mRNA expression level relative to islet
Gcg
Ins1
Ins2
Mafa
Mafb
Sst
n.s.
*
**
*
n.s.
*

Figure 5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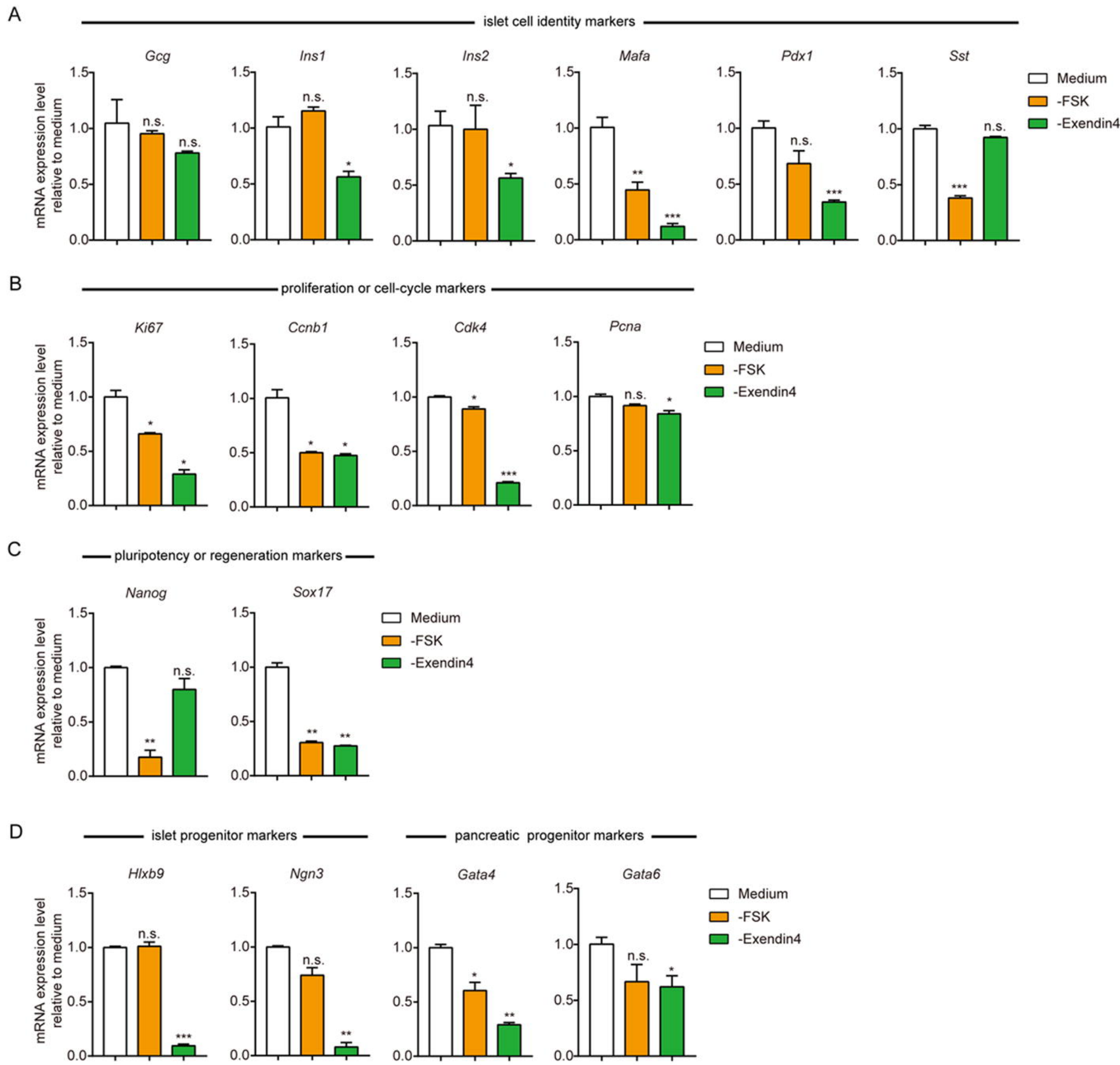
proliferation or cell-cycle markers
Ki67
Ccnb1
Ccnd1
Pcna
mRNA expression level relative to islet
islet
expanded cluster
n.s.
B
pluripotency or regeneration markers
Nanog
Sox9
C
islet progenitor markers
pancreatic progenitor markers
Ngn3
Hlxb9
Gata4
Gata6
D
E
F

Figure 6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islet cell identity markers
Gcg
Ins1
Ins2
Mafa
Pdx1
Sst
mRNA expression level relative to medium
Medium
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-Exendin4
B
proliferation or cell-cycle markers
Ki67
Ccnb1
Cdk4
Pcna
C
pluripotency or regeneration markers
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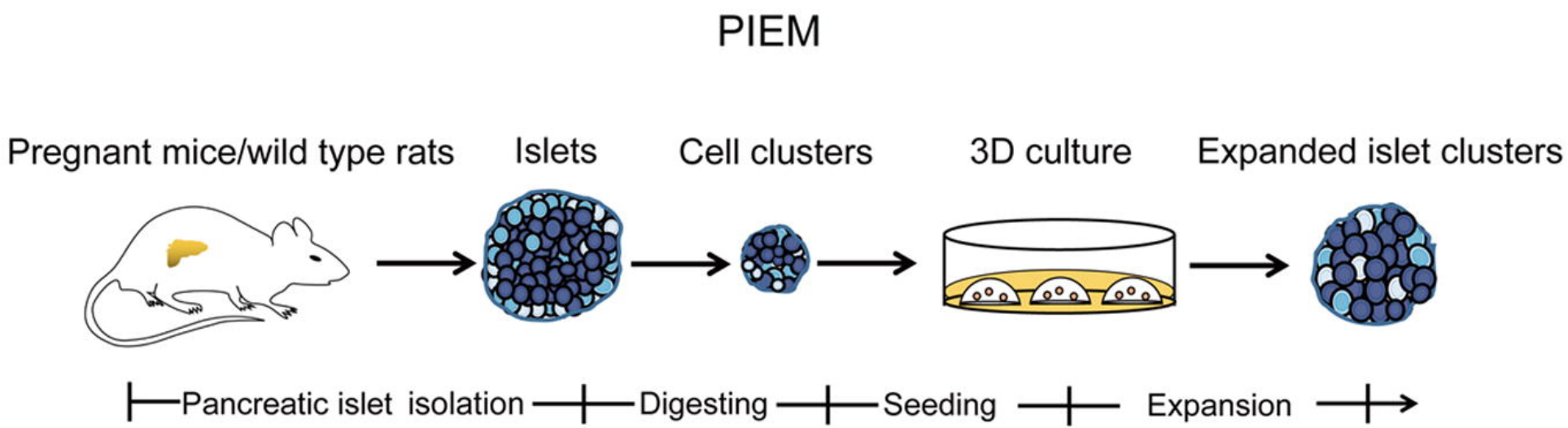
Sox17
D
islet progenitor markers
pancreatic progenitor markers
Hlxb9
Ngn3
Gata4
Gata6

Figure 7


PIEM
Pregnant mice/wild type rats
Islets
Cell clusters
3D culture
Expanded islet clusters
Pancreatic islet isolation
Digesting
Seeding
Expansion