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PURSUING CELL REPLACEMENT THERAPY FOR THE TREATMENT OF DIABETES MELLITUS: ADULT STEM CELL-BASED APPROACH, ENDOGENOUS β-CELL REGENERATION, OR BOTH?

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LA TERAPIA SOSTITUTIVA CELLULARE PER LA CURA DEL DIABETE MELLITO: UTILIZZO DI CELLULE STAMINALI ADULTE, RIGENERAZIONE β-CELLULARE ENDOGENA, O ENTRAMBE?

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Riassunto

Il diabete mellito è causato da un assoluto (tipo 1) o relativo (tipo 2) difetto delle β-cellule nelle isole pancreatiche di Langerhans. La somministrazione di insulina esogena, tuttavia, non rappresenta una cura per diabete nella misura in cui non è in grado di prevenire le complicanze croniche della malattia. Il trapianto di isole pancreatiche isolate da organi di donatori ha dimostrato come la terapia sostitutiva cellulare possa essere una realistica opzione terapeutica. Tale approccio è però limitato dalla scarsità di donatori e dalla necessità di terapia immunosppressiva a vita. Un'attraente strategia alternativa è rappresentata dal ripristino della massa β-cellulare attraverso 1) trapianto di cellule surrogate glucoso-responsive ed insulino-secernenti oppure 2) stimolazione della rigenerazione β-cellulare endogena.

L'utilizzo di cellule staminali adulte nella terapia sostitutiva β-cellulare è recentemente diventato oggetto di inteso studio. La β-cellula, tuttavia, è notevolmente sofisticata, e molte delle sue caratteristiche sono difficili da ottenere nelle cellule surrogate. Pertanto, nonostante il progresso fatto nell'ultima decade, una sua applicazione clinica è ad oggi incerta.

Un approccio differente è rappresentato dalla stimolazione della rigenerazione endocrina endogena. Vi sono evidenze che la massa funzionale β-cellulare sia capace di espandersi in risposta ai cambiamenti della richiesta metabolica così come di quelli fisiopatologici. Inoltre numerosi studi nel modello animale hanno dimostrato che il pancreas è capace di rigenerare in risposta a diversi tipi di danno. Tuttavia, le cellule responsabili e la reale magnitudine di tale processo rigenerativo sono ancora soggette a controversie.

In questo progetto di Dottorato sono stati investigati entrambi gli approcci, ossia **1)** l'abilità di cellule staminali adulte, nello specifico le cellule limbari simil-fibroblastiche, di differenziarsi *in vitro* in cellule producenti insulina, e **2)** la capacità delle cellule duttali pancreatiche di rigenerare *in vivo* cellule endocrine ed esocrine in seguito a danno in un modello murino transgenico.

Summary

Diabetes is caused either by an absolute (type 1) or relative (type 2) defect of insulin-producing β-cells in the pancreatic islets of Langerhans. Nevertheless, exogenous insulin is not a cure for diabetes, as it cannot prevent the chronic and devastating complications of the disease. Transplantation of islets isolated from the pancreas of organ donors provided strong support to cell-based therapy as a realistic treatment option. However, this approach is severely limited by a shortage of human organ donors and the need of lifelong immunosuppressive therapy. An attractive alternative strategy would be to restore functional β-cell mass by either 1) transplantation of surrogate glucose-responsive, insulin-secreting cells or 2) activation of endogenous β-cell regeneration.

The use of adult stem cells for β-cell replacement therapy recently became an area of intense study. The β-cell is, however, remarkably sophisticated, and many of the features of this highly differentiated secretory cell fail to be faithfully mimicked in surrogate cells. Therefore, in spite of the progress that has been made over the last decade, widespread clinical application is still uncertain.

A different approach might be fostering pancreatic endogenous regeneration. There is accumulating evidence that functional pancreatic β-cell mass expands in response to changes in metabolic demand as well as pathophysiological changes. In addition, several studies on animal models demonstrated that the injured adult pancreas is able to regenerate. However, the cell of origin in pancreatic regeneration is still subject of controversial discussion.

With my PhD research project I focused on both strategies, by exploring **1)** the ability of fibroblast-like limbal stem cells to differentiate *in vitro* into insulin-producing cells, and **2)** the potential of pancreatic ductal cells to contribute *in vivo* to regeneration of endocrine and exocrine cells following injury in a transgenic mouse model.

Rationale and overview of the two PhD projects

Type 1 diabetes (T1D) is caused by the autoimmune destruction of pancreatic β-cells, which leads to their virtually complete eradication. By the time of clinical diagnosis, patients have lost sufficient functional β-cell mass for glucose homeostasis and are dependent on exogenous insulin to survive. Type 2 diabetes (T2D) has a complex pathogenesis characterized by both insulin-resistance and β-cell failure, and a third of the patients eventually require insulin treatment to prevent death from hyperglycemia resulting from the loss of pancreatic islets. The discovery of insulin has prevented death from acute diabetes. However, even tight glucose control does not prevent the systemic complications, such as renal failure, proliferative retinopathy leading to blindness, peripheral neuropathy and vascular disease.

Transplantation of whole pancreas or purified insulin-producing islets is the preferred approach to achieve glucose homeostasis, especially for a specific population of T1D patients who do not respond to conventional therapy. Even though promising results including reduction or freedom from insulin, better glucose stability and less risk of complications have been achieved, the major obstacle to widespread use of pancreatic or islet transplantation is the shortage of suitable donor tissues and the inferior long-term results, including graft rejection and nephrotoxicity from the immunosuppressive agents.

A stem cell-based approach to β-cell replacement therapy has been recently proposed as a solution. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPS), which are able to self-renew and virtually differentiate into any phenotype, might provide an unlimited supply of surrogate β-cells. However, their clinical application is affected by ethical and technical challenges. Hence, adult multipotent stem cells are now being widely evaluated, although the scarcity of the source, the invasive procedures often required to isolate this cells, and their restricted differentiation potential have limited their use in translational medicine.

An alternative strategy could be exploiting the intrinsic capacity of the adult pancreas to produce new β-cells endogenously. During pancreatic organogenesis, stem cells within the duct pancreatic epithelium give rise to both the endocrine and acinar cells. Therefore it seems reasonable to think that the regeneration process could start within the ductal compartment, recapitulating embryonic and fetal development. In addition, there are similarities between islet regeneration and embryonic pancreas development at the gene expression level.

Given the relevance of for the treatment of diabetes, during this three-year PhD research I attempted to investigate both approaches to β-cell regeneration, i.e. the ability of adult stem cells to

differentiate *in vitro* into insulin-producing cells (**PROJECT 1**), and the potential of pancreatic endogenous regeneration in a transgenic mouse model (**PROJECT 2**).

PROJECT 1 was carried out a the University of Palermo, Italy, and involved the use of fibroblast-like limbal stem cells (f-LSCs) of the eye as a source of adult stem cells to be used for β-cell replacement therapy. The limbus is a highly specialized region of the eye located in the transitional zone between the cornea and the conjunctiva. It is well known that the limbal niche hosts a population of epithelial stem cells (LESCs) with restricted fate potential, and which are responsible of the continuous renewal of the corneal surface. However, it has been recently suggested that the limbus also contains stromal fibroblast-like stem cells (f-LSCs), with apparent broader plasticity. The lack of agreement on specific molecular hallmarks for the identification of the pluripotent limbal stromal cells has so far limited the investigation of their differentiation potential. In our study, we showed that the human limbus contains uncommitted cells, which could be potentially harnessed for the treatment of diabetes. First we described a subpopulation of f-LSCs characterized by robust proliferative capacity, stable expression of pluripotent stem cell markers and self-renewal ability. We then demonstrated that f-LSCs are able to generate pancreatic endocrine cells when subjected to a four-step differentiation protocol which directs f-LSCs through a series of intermediates similar to those occurring during pancreatic organogenesis, efficiently leading to *in vitro* production of functional hormone-expressing cells.

PROJECT 2 was carried out at the University of Pittsburgh, USA, and focused on assessing the ability of endogenous pancreas to regenerate after injury in a transgenic mouse model. In the past years there have been conflicting results on a cell of origin in pancreatic regeneration, predominantly due to lack of specific markers for the pancreatic precursors/stem cells, as well as differences in the targeted cells and severity of tissue injury in the experimental models proposed. In the attempt to overcome these discrepancies, we took advantage of the diphtheria toxin receptor (DTR)-mediated conditional targeted cell ablation model to ablate specific cell populations, control the extent of injury, and avoid induction of the inflammatory response. Specifically, we crossed $R26^{DTR}$ or the reporter $R26^{DTR/lacZ}$ mice with mice expressing Cre recombinase under control of the Pdx1 (global pancreatic) or Elastase (acinar-specific) promoters. In the global model, exposure of $PdxCre;R26^{DTR}$ mice to diphtheria toxin leads to extensive ablation of all pancreatic epithelial cells with the exception of ductal cells, which are serendipitously spared. Surviving cells within the ductal compartment contribute to substantial regeneration of both endocrine and acinar cells via recapitulation of the embryonic pancreatic developmental program. By contrast, following selective ablation of acinar tissue in the $ElaCre\text{-}ERT2;R26^{DTR}$ mice, regeneration occurs by direct conversion of ductal cells to acinar lineage.

Background and review of the literature

1. Can the endocrine pancreas regenerate?

The pancreas is composed by two distinctly different tissues: the exocrine component, i.e. pancreatic acinar cells that produce digestive enzymes and ductal cells that responsible of their excretion; and the endocrine component, i.e. the islets of Langerhans, constituted by hormone secreting cells. In the islet, the α cells produce glucagon; the β cells, insulin; the δ cells, somatostatin; γ cells, pancreatic polypeptide (**Figure 1**).

Diabetes is caused either by an absolute (type 1 or T1D) or relative (type 2 or T2D) defect of insulin-producing β-cells in the pancreas (Atkinson et al., 2001). Therefore, regardless of the different pathogenesis, diabetes is the perfect candidate for cell replacement therapy. Currently the two available alternatives for β-cell replacement therapy are whole pancreas or isolated islet transplantation (Shapiro et al., 2000). However, these approaches are severely limited by a shortage of human organ donors and the need of lifelong immunosuppressive therapy. In the absence of other clearly suitable and renewable sources of surrogate β-cells, an alternative strategy to exogenous cell replacement therapy might be fostering endogenous β-cell regeneration. Therefore, knowledge of the mechanisms regulating β-cell plasticity in both embryonic and adult life, as well as in pathological conditions, is of particular interest.

During pancreatic development, β-cells derive from a population of endocrine precursors arising from the pancreatic epithelium (Gittes, 2009). Activation of cell-specific transcription factors guides the initially multipotent progenitors and determines their differentiation into mature β-cells. The final size of the endocrine pancreas is limited by the size of the progenitor cell pool in the developing pancreatic bud (Stanger et al., 2007). After birth, most β-cells are considered quiescent, however, it has been shown that the β-cell mass can adaptively expand under some physiologic or pathologic circumstances, such as pregnancy and obesity, both in mammals and rodents (Bernard-Kargar & Ktorza, 2001).

In mouse models there is also evidence that pancreas preserves the ability to regenerate the β-cell mass in response to several non-physiological injuries, such as selective chemical destruction or surgical excision (Trucco, 2005; Thorel et al., 2010). Furthermore, in non-obese diabetic mice (NOD) it has been shown that recovery of sufficient endogenous insulin production is possible via combination of strategies involving reversal of the autoimmune attack (Zorina et al., 2003; Kodama et al., 2003; Suri et al., 2006; Chong et al., 2006; Nishio et al., 2006).

In humans it is still debated whether this recovery is possible and, if so, to what extent it is feasible. Spontaneous recovery of β-cell function has been reported in patients with recent onset of

T1D, suggesting the possibility of β-cell regeneration despite the underlying autoimmunity (Karges et al., 2004, 2006; Meier et al., 2006a; Butler et al., 2007). Additionally, the observation that people with long-standing T1D still possess β-cells despite their destruction by the enduring autoimmunity and glucotoxicity, suggests that new β-cell formation must be occurring throughout life (Meier et al., 2005). However, it remains largely unclear through which molecular and cellular mechanisms it occurs.

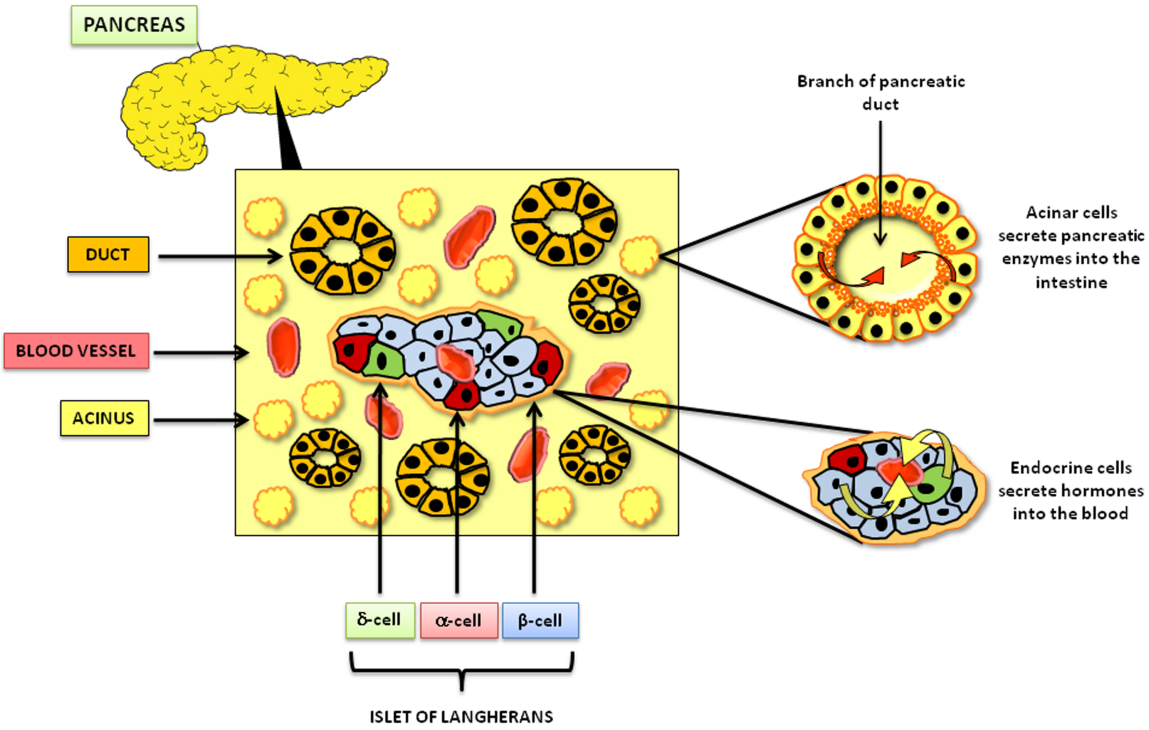


Figure 1. *Cells of the pancreas.*

2. Models of artificially induced diabetes

To address the question whether the pancreas possesses the ability to regenerate, several models have been used to artificially reduce the β-cells mass in order to stimulate a pancreatic response. Most of these models allow exploring the plasticity of the pancreas in the absence of concurrent autoimmunity, which might prevent or block any attempt of β-cell restoration.

Partial pancreatectomy

Partial pancreatectomy was shown to induce limited re-growth of the remnant organ in rats, although near total (>90%) removal is necessary (Pearson et al., 1977). In comparison to liver regeneration second to partial hepatectomy, subtotal pancreatectomy is followed only by a limited regenerative growth that is proportional to the size of the excision. In addition, regeneration is mostly related to the exocrine tissue, while the endocrine part was shown to rescue, at its best, only

approximately 30% of its initial mass (De Leon et al., 2003). The extent of the surgical intervention seems to be important, and could explain discrepancies in some reports describing absent (Dor et al., 2004) or vigorous pancreatic regeneration (Bonner-Weir et al., 1993) after 70% and 90% organ resection, respectively. In addition, hyperglycemia, which is present only in the latter case, could act as co-stimulator. Using 70–80% pancreatectomy in C57BL/6 mice, Guo and colleagues found the same regeneration pattern as in 90% pancreatectomy (Guo et al., 2010).

Pancreatic duct ligation

Pancreatic duct ligation (PDL) was also used to determine obstruction and consequently local inflammation and stimulation of pancreatic regeneration. During the first week post-ligation an increase in the β-cell number and presence of intermediate ductal/endocrine (Wang et al., 1995) or acinar/endocrine phenotypes (Bertelli & Bendayan, 1997; Inada et al., 2008) has been observed. Additional stimulation in the expansion of the β-cell mass following PDL can be achieved by gastrin infusion (Rooman et al., 2002).

Wrapping of the pancreas with cellophane

Wrapping the pancreas with cellophane has been also used to induce islet neogenesis from ducts, and it has been reported to reverse streptozotocin-induced diabetes in hamsters (Rosenberg et al., 1996).

Selective β-cell destruction

Selective β-cell destruction can be obtained by chemical ablation with streptozotocin or alloxan, alone and in combination with pancreatectomy (Finegood et al., 1999; Wang et al., 1996). Streptozotocin (STZ) is a drug that leads to cell death by DNA alkylation, while alloxan is a generator of oxygen free radicals causing extensive DNA damage. Adult mice rendered diabetic with a high dose of STZ or alloxan are unable to recover endogenous β-cell function (Szkudelski, 2001). Interestingly, β-cell neogenesis can be stimulated in STZ-diabetic newborn rats by administration of the hormone glucagon-like peptide-1 (GLP-1), resulting in improved glucose homeostasis persisting at adult age (Tourrel et al., 2001). In another murine experimental model of alloxan-induced beta-cell destruction, treatment with gastrin and epidermal growth factor (EGF) was found to restore glycemic control and 30–40% of the normal β-cell mass within 7 days (Rooman & Bouwens, 2004). Combination of the same growth factors proved to be effective also in facilitating islet β-cell neogeneisis in NOD mice with autoimmune diabetes (Suarez-Pinzon et al., 2005). In addition, rescue of endogenous islet function was shown in STZ-diabetic mice after removal of kidney bearing syngeneic islets, which temporarily maintained mice normoglicemic (Yin et al., 2006), thus indicating that glucose control might be relevant to facilitate the regenerative process. On the other hand, the

possibility that recovery of the endogenous β-cell function may occur independently of effects on glucose control exists, i.e. by the mediation of cytokines, which may activate residual β-cell proliferation or progenitor cell differentiation. To note, in the study by Yin et al., a facilitating role was exerted by the presence of the spleen, which probably plays an indirect role as modulator of the inflammatory process in the pancreas, thereby stimulating recovery of the STZ-damaged islets. STZ seems to trigger a pancreatic regenerative response also in non-human primate models, although it does not lead to a substantial endogenous β-cell recovery in absence of additional stimuli, like the failure of exogenous islet transplantation in the liver (Bottino et al., 2009).

Method	Target Cells	Potential Mechanism for Regeneration
Pancreatectomy	Endocrine Exocrine	Replication of pre-existing β-cells Reactivation of embryonic program
PDL	Endocrine Exocrine	Regeneration through CA-II, Sox9+ and Ngn3+ progenitors Regeneration through CA-II progenitors
Streptozotocin	β-cell	β-cell neogenesis from ductal cells
Alloxan	β-cell	β-cell neogenesis from ductal cells

Table 1. *Summary of the models used to investigate β-cell regeneration. PDL: Pancreatic duct ligation CA-II: carbonic anhydrase II.*

3. Lineage Tracing Techniques

Lineage tracing techniques have been widely used to investigate both the ontogeny of pancreatic cell fates during mouse embryogenesis as well as the identification of progenitor cells *in vivo* during regeneration (see **Figure 2** and **3** for further explanations). In lineage analysis, specific cells are labeled or marked so that their progeny can be identified later during development. In the pancreas, lineage analysis has been used to recognize not only the progenitor cells giving rise to mature endocrine and exocrine cells, but also the stage at which each set of progenitors is restricted to a particular cell fate. Lineage tracing is also useful to label and isolate marked cells in order to study their gene expression profile and in vitro differentiation.

In pancreatic lineage analysis, cells can be labeled using distinct approaches. A physical label - such as dye or a replication-incompetent retrovirus - can be directly injected into embryos to label cells within a tissue. The tissue is allowed to mature *in vivo* or in culture, and the cell types that become labeled reveal the lineage of the starting cells. However, since this method marks cells indiscriminately, in most tissues it cannot be used to label specific sub-populations. A more reliable

approach is to genetically mark progenitor cells using endogenous gene expression patterns. This method selectively labels cells that express a particular gene, thus revealing the fate of their progeny. In most cases, a tissue specific promoter (for example Pdx-1) driving Cre recombinase is used to irreversibly tag cells. Other options include the use of a transgene driven by a specific promoter within different cell types, and lineage ablation, either using gene-inactivation mutants (knockout) or transgenic expression of cellular toxins. All these approaches have been used to follow pancreatic cell lineages (reviewed in Gu et al. 2003).

Cre/LoxP system

Cells can be irreversibly marked using the Cre/LoxP system, thus permitting detection of progeny cells that no longer express the gene of interest. This system uses two transgenic mouse lines, the "reporter" and the "deletor" (**Figure 2**). The first transgenic mouse uses a promoter (promoter 1), which can be tissue specific or ubiquitous, to drive the expression of a reporter gene, such as LacZ or green fluorescent protein (GFP). The second mouse carries a transgene that uses a different tissue specific promoter (promoter 2) to drive the expression of Cre recombinase. In the absence of the Cre deletor transgene, the expression of the reporter protein is prevented by a STOP cassette (multiple repeats of a poly-adenylation signal) upstream of the reporter coding sequence. However, in the presence of Cre recombinase, two LoxP sites flanking the blocking sequence permit this block to be removed. Thus, in double transgenic animals, the reporter gene will be expressed in cells following the excision event, thereby labeling all progeny derived from those precursors that express the deletor transgene.

A fine-tuning of the Cre/LoxP system can be achieved with the Cre-ERT recombinase, which is a fusion between the catalytic domain of the Cre recombinase and the ligand-binding domain of a modified estrogen receptor. The Cre-ERT protein requires an artificial ligand, tamoxifen, to catalyze LoxP mediated recombination. Because tamoxifen is active within mouse embryos for less than 48 h, cells expressing Cre-ERT at a specific developmental stage can be selectively labeled by administration of tamoxifen during that stage. After tamoxifen treatment, the conventional Cre recombinase activates the reporter transgene expression as soon as Cre protein is generated, and labeled cells accumulate in any lineage where Cre has been expressed. This type of recombinase can be used to follow selectively the progeny of cells born at defined developmental stages, including postnatal growth and during regeneration.

Figure 2. *Design plan for direct cell lineage analysis. The reporter line uses promoter 1 (Pro.1, black line) to drive reporter gene expression (green rectangle). Upstream of the reporter gene coding region is a STOP cassette made of three repeat of a polyA signal (red rectangle). Flanking the blocking signal are two LoxP sites (blue arrows). Promoter 1 can be tissue specific or ubiquitous. In the deletor line, another tissue specific promoter (Pro. 2, black line) is used to drive the expression of Cre recombinase (yellow rectangle). When the two mouse lines are crossed, Cre is expressed in the cells in which promoter 2 is active, thus deleting the blocking signal. This results in the expression of the reporter gene in cells that also express promoter 1.*

Lineage analysis based on simple transgenes

A simpler transgenic approach drives expression of a reporter gene, such as LacZ or green fluorescent protein (GFP), under the promoter of interest. The drawback of this approach is that any progeny of these cells, which cease expression of the chosen protein, cannot be followed. In addition, since this method marks cells from the first time the promoter is activated, and as these cells accumulate during development it becomes impossible to distinguish new members of the population. Thus, one cannot distinguish the progeny of cells born during embryogenesis from those born in adults.

Lineage analysis based on cell ablation

Another method to investigate lineage relationship is cell ablation. This can be accomplished by specific gene inactivation mutations (knockout), such as in the *Pdx1* knockout mouse, which has no mature pancreatic cells. Alternatively, a tissue specific promoter can be used to drive the expression of a cellular toxin, such as the Diphtheria Toxin A (DTA) subunit. In these transgenic animals, the DTA will kill those cells whose progenitors express that specific transgene. A similar approach is represented by the Diphtheria Toxin Receptor (DTR)-mediated conditional cell ablation model. DTR is a membrane-anchored form of the heparin-binding EGF-like growth factor (HB-EGF precursor). The human and simian HB-EGF precursors bind DT and function as toxin receptors, whereas HB-EGF from mice and rats do not bind the toxin and therefore remain insensitive to DT. Thus, transgenic expression of the simian or human DTR in mice can render murine cells DT-sensitive. Recently, a mouse strain was generated (iDTR), in which the gene encoding DTR has been introduced into the

ROSA26 locus ($R26^{DTR}$), but its expression is dependent on the Cre-mediated removal of a transcriptional STOP cassette. Therefore, only Cre-expressing cells and their progeny will undergo Cre-recombinase activity and subsequently will transcribe DTR. Although viable and normally functioning, these cells are rapidly killed upon DT administration (**Figure 3**) (Buch T et al. 2003).

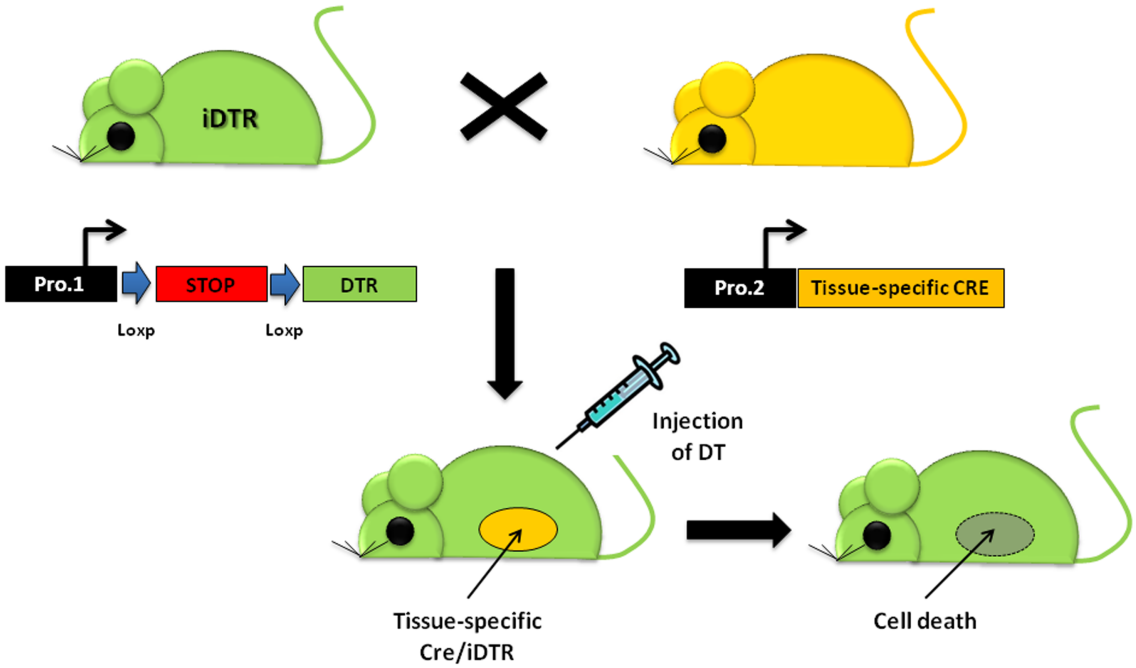


Figure 3. *Design plan of the inducible DTR mouse strain (iDTR). The STOP cassette, which prohibits DTR expression, is removed by crossing the iDTR strain to a tissue-specific Cre-expressing mouse strain. Consecutive expression of the DTR renders the respective tissues sensitive to cell death induced by injection of diphtheria toxin.*

4. Evidence of pancreatic progenitors/stem cells in the pancreas

Several cells in the pancreas have been described as potential sources of β-cell renewal.

Pancreatic ductal progenitor cells

During pancreatic organogenesis, stem cells within the duct pancreatic epithelium give rise to both the endocrine and acinar cells (Gittes, 2009). Therefore it seems reasonable to think that the regeneration process could start within the ductal compartment, recapitulating embryonic and fetal development (**Figure 4**). In addition, there are similarities between islet regeneration and embryonic pancreas development at the gene expression level.

Evidence of adult duct cells harboring stem cells capable of differentiating into β-cells was reported both *in vivo* and *in vitro* (Dudek et al., 1991; Ramiya et al., 2000; Bonner-Weir et al., 2000, 2008; Gao et al., 2003). In 2000 Ramiya et al. claimed that long-term cultivation of pancreatic ductal epithelial cells isolated from pre-diabetic, adult, non-obese diabetic mice contained nestin-positive

stem cells able to differentiate into islets of Langerhans (Ramiya et al., 2000). These "surrogate" islets responded *in vitro* to glucose challenge, and reversed insulin-dependent diabetes after being implanted into diabetic NOD mice. Similar observations were reported using more defined culture conditions in which isolated human pancreatic duct preparations led to formation and propagation of human islet-like structures (Bonner-Weir et al., 2000, 2008 Gao et al., 2003). Ogata et al. also derived a similar subset of islet-like insulin secreting cells from pancreatic ducts of neonatal rats (Ogata et al., 2004). After incubation with activin A and betacellulin, cells showed tolbutamide- and glucose-responsive insulin secretion. Transplantation of these pseudo-islets in STZ-diabetic NOD mice improved blood glucose levels. Hao et al. confirmed the existence of endocrine stem or progenitor cells within the epithelial compartment of the adult human pancreas, by isolating stem cells from the non-endocrine fraction after islet separation of adult human pancreas digests (Hao et al., 2006). Following elimination of the contaminating mesenchymal cells, the highly purified population of non-endocrine pancreatic epithelial cells (NEPECs) was transplanted under the kidney capsule of immunodeficient (SCID) mice. Although NEPECs produced only low amounts of insulin, when co-transplanted with fetal pancreatic cells, they were capable of endocrine differentiation. No evidence of β-cell replication or cell fusion was observed. To directly test whether ductal cells serve as pancreatic progenitors after birth and give rise to new islets, a transgenic mouse expressing human carbonic anhydrase II (CAII) promoter was generated. This study showed that CAII-expressing cells within the pancreas act as progenitors that give rise to both new islets and acini normally after birth and after injury (PDL) (Inada et al., 2008).

Additional evidence of the existence of endocrine precursor cells within the ductal compartment come from the detection of the nuclear transcription factor Neurogenin-3 (Ngn3) in the ducts during regeneration after STZ. Ngn3 is a basic helix–loop–helix transcription factor, which is able to commit pancreatic cells to an endocrine cell fate (Schwitzgebel et al., 2000). Lack of Ngn3 leads to an absence of islets (Gradwhol et al., 2000); its ectopic expression determines premature over-commission towards the endocrine lineage (Apelqvist et al., 1999). Presence of Ngn3 is very convincing evidence that pancreatic regeneration starts from pancreatic progenitors and mimics the same pathway followed during normal development (**Figure 4**). By using an inducible Cre-ERT-LoxP system to mark the progeny of cells expressing either Ngn3 or Pdx1 at different stages of development, Gu et al. showed that endocrine/exocrine and ductal lineages are separated before E12.5 (Gu et al., 2002). Authors demonstrate that while cells expressing Pdx1 give rise to all three types of pancreatic tissue (exocrine, endocrine and duct), only the subset $Pdx1^{+}/Ngn3^{+}$ cells are islet progenitors. The duct cells that do not contain progeny of $Ngn3^{+}$ cells presumably give rise to the adult duct system and account for the heterogeneity in developmental potential among 'duct-like structures'. Kodama et al. also suggested that in STZ-treated mice regeneration occurs mainly from intra-islet $Ngn3^{+}$ progenitor cells rather than from ductal precursors (Kodama et al., 2005).

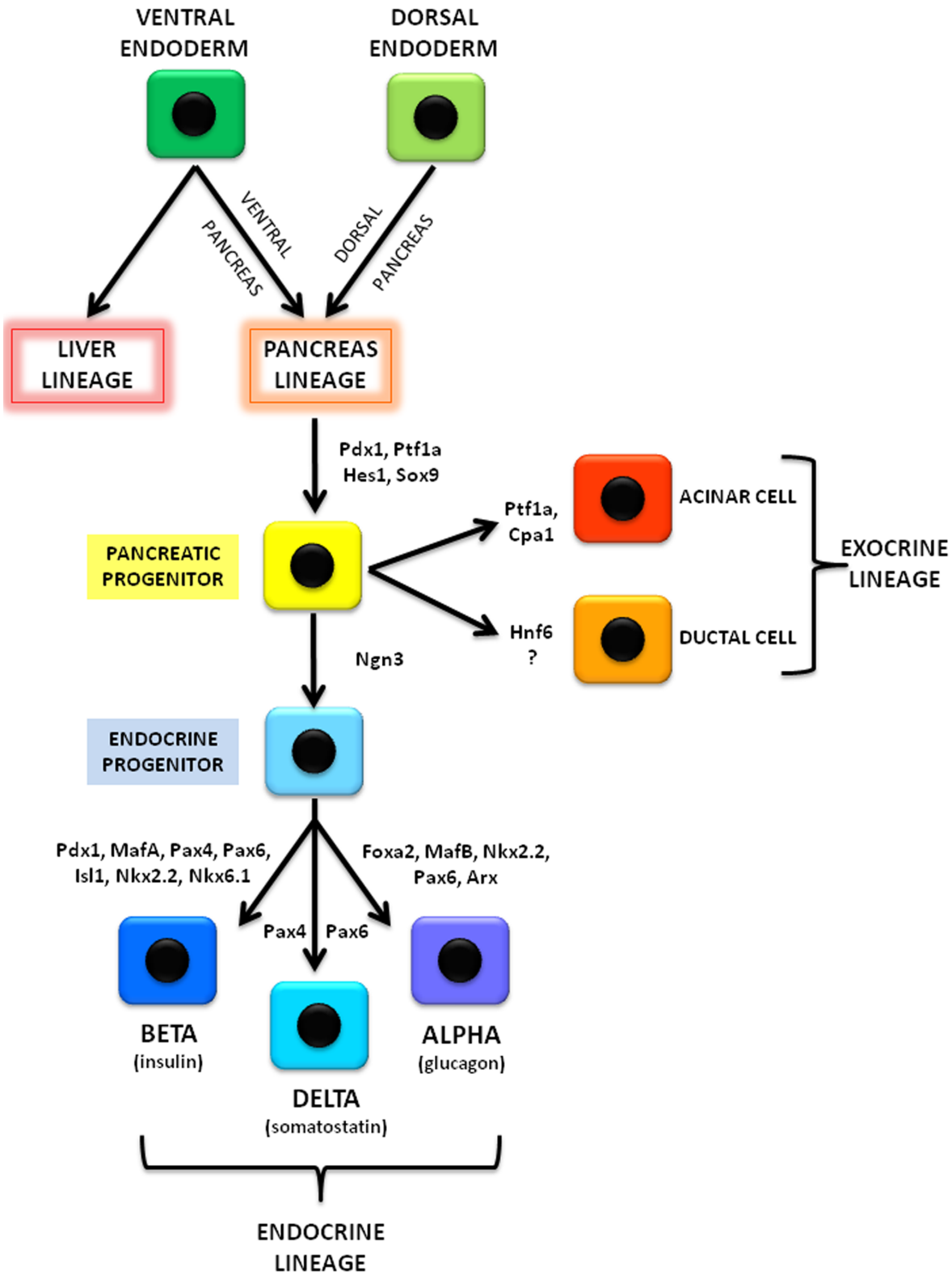


Figure 4. *Regulatory nuclear transcription factors controlling cell type lineages during embryonic pancreas development.*

Recently, Xu et al. showed that PDL in the pancreatic tail resulted in some β-cell proliferation and, more strikingly, to a large upregulation of Ngn3 gene expression (Xu et al., 2008). Ngn3 positive cells were found to be closely associated with ducts, and possibly cell of the ductal lineage themselves. After isolation, these cells could give rise to all islet cell types, including glucose responsive β-cells, both *in situ* and when cultured in $Ngn3^{-/-}$ embryonic pancreas explants. In addition, White et al. utilized a system based on Ngn3–enhanced green fluorescent protein knock-in mouse model to isolate endocrine progenitor cells from embryonic pancreata to generate an ample gene expression profile of these progenitors and their immediate descendants (White et al., 2008). On the other hand, a recent publication reported low level expression of Ngn3 in adult endocrine cells, raising concerns about using Ngn3 expression as a marker of endocrine progenitors and neogenesis in adult pancreas (Wang et al., 2009). Furthermore, another study indicate that although duct ligation leads to Ngn3 expression in a sub-population of cells within the ducts, it does not induce appropriate cues to allow for completion of entire β-cell neogenesis program (Kopp et al., 2011).

The potential for the pancreatic organ to recover the endocrine function after injury has been also investigated in non-human primates, where a ductal involvement was observed in STZ-diabetic monkeys who recovered endogenous β-cell function following pig islet transplantation in the liver (Bottino et al., 2009). Further evidence that hormone-positive cells arise from the ducts comes from the comparison of 16 donor pancreata and biopsied pancreata from 8 simultaneous pancreas/kidney transplantations (Martin-Pagoda et al., 2008). While in the donor pancreata the frequency of $insulin^{+}$ duct cells was low (0.45%), in five pancreatic transplants with recurrent autoimmunity, 57.5% of the duct cells expressed insulin protein. If new islets were generated from pre-existing ductal tissue, transient co-expression of hormones and residual duct markers could be expected. Indeed, this has been demonstrated in grafts of purified human duct cells (Yatoh et al., 2007).

Pancreatic non-ductal progenitor cells

Besides ductal progenitors, other groups proposed that pancreatic stem cells might also reside within the islets or in the acinar compartment.

Acinar cells

Acinar cells, which represent the main portion of pancreatic tissue, have been shown to transdifferentiate into islet cells *in vivo* and *in vitro*, through the generation of duct cells as an intermediate step (Rooman et al., 2000; Lardon et al., 2004; Baeyens et al., 2005; Lipsett et al., 2007). Lineage tracing has been used *in vitro* to further strengthen the conclusion that endocrine cells can be generated from exocrine cells via transdifferentiation (Minami et al., 2005). Earlier *in vivo* lineage tracing experiments in mice showed that acinar cells scarcely contribute to generate

new islet β-cells and duct cells (Desai et al., 2007). However, more recent studies by Collombat et al. demonstrated that upon expression of Pax4, adult α-cells can transdifferentiate to β-cells (Collombat et al., 2009). The ectopic expression of Pax4 forces endocrine precursor cells as well as mature α-cells, to adopt a β-cell fate. In addition, since α-cells were constantly recruited and converted to β-cells, the resulting glucagon deficiency provoked a compensatory and continuous glucagon^{+} cell neogenesis through Ngn3^{+} precursors. On the other hand, Arx mis-expression in β-cells, using either an Ins^{Cre} or in adult β-cells using an inducible Pdx1^{CreERT} reduced insulin-expressing cells and increased α- and PP-cells (Collombat et al., 2007). This α-to-β-cell transdifferentiation was also recently showed in a transgenic model of diphtheria-toxin-induced acute selective near-total β-cell ablation (Thorel et al., 2010). Lineage-tracing to label the glucagon-producing α-cells before β-cell ablation tracked large fractions of regenerated β-cells as deriving from α-cells, revealing a previously unknown flexibility in the functioning of the pancreas in relation to hormone secretion, with the potential for exploiting it to cure diabetes. Generation of β-cells from α-cells has also been shown with a unique model that combines PDL with alloxan-mediated β-cells destruction (Chung et al., 2010). In this model, large numbers of β-cells were generated primarily from α-cells by two mechanisms: the first involved extensive α-cell proliferation, which provided a large pool of precursors that, in turn, became β-cells via asymmetric division; the second demonstrated that β-cells could form directly from α-cells via transdifferentiation. This latter mechanism was suggested by the finding of intermediate cells co-expressing α- and β-cell-specific markers, which were detectable in the first week after injury, declining gradually in number by the second week when double-positive cells converted into mature β-cells, as shown by loss of glucagon and expression of MafA.

Nestin-positive cells

Nestin-positive cells have been identified within adult rat islets as being able of differentiating into insulin-positive cells *in vitro* (Zulewski et al., 2001). Nestin is an intermediate filament protein expressed by the neural lineage which was also found in the pancreas (Edlund, 2002), although not all groups agree that nestin is expressed during development of the human pancreatic epithelium (Piper et al., 2002). Lineage-tracing experiments in mice imply that nestin^{+} cells contribute to the vasculature as well as acinar lineage but not the endocrine lineage (Treutelaar et al., 2003; Esni et al., 2004; Delacour et al., 2004).

Proliferative human islet precursor cells (hIPCs)

Proliferative human islet precursor cells (hIPCs) were obtained *in vitro* from preparations of adult human islets after extensive *in vitro* proliferation (Gershengorn et al., 2004). Authors believed that these cells, showing a mesenchymal phenotype, derived from insulin-expressing cells undergoing

epithelial-to-mesenchymal transition (EMT). hIPCs could be re-differentiated into insulin-expressing islet-like cell aggregates (ICAs) and secreted insulin when transplanted under the kidney capsule of immunodeficient mice**.** However, many criticisms were advanced from other groups, claiming that, at least in mouse pancreatic cultures, islet-derived fibroblast-like cells are not derived via EMT from pancreatic β-cells (Chase et al., 2007; Atouf et al., 2007). Later, Gershengorn et al. further confirmed the basic differences between human and mouse cultures, and claimed that hIPCs are special kind of pancreatic mesenchymal stromal cells (Morton et al., 2007). More recently, using a lineage-tracing *in vitro* technique, Russ et al. found evidence for massive proliferation of cells derived from human β-cells. Nevertheless, it appears that induction of significant replication *in vitro* results in dedifferentiation. (Russ et al, 2008).

5. Evidence of pancreatic progenitors/stem cells outside the pancreas

Stem cells, which can be isolated from embryonic, fetal, and adult tissues, might represent an unlimited source of β-cells because of their ability to self-renew by asymmetric division while retaining the potential of differentiating under the proper conditions into the desired phenotype. Despite the enormous scientific potential of human stem cell research, its clinical application is still limited by many challenges. **Embryonic Stem Cells (ESCs)** possess the widest differentiation potential and should, therefore, be the best candidates for β-cell replacement therapy. Several studies have reported the differentiation of both mouse and human ESCs into insulin-producing cells. However, their use is associated with ethical concerns; they may give rise to tumors when implanted outside their physiologic niche, and are, by definition, only allogeneic. **Fetal stem cells** (placental, amniotic, trophoblastic, cord blood) do not give rise to ethical issues. However, they are considered only multipotent, i.e. less plastic than ESCs; in addition, their transdifferentiation potential towards the β-cell phenotype has been poorly investigated. Induced pluripotent stem cells, created by reprogramming differentiated post-mitotic human somatic cells (e.g., adult human dermal fibroblasts) with transduction of defined transcription factors, are currently far from clinical application because of the problems associated with the use of retroviruses and oncogenes for reprogramming. **Adult stem cells (ASCs)**, which possess the advantage of being potentially autologous and ethically acceptable to most people, apparently lack the pluripotency of ESCs. This has limited so far their use in β-cell replacement therapy. Yet it has been reported that some ASCs, such as bone marrow stem cells, can give rise to cell types other than their default ones, including β-cells. However, a mechanism of cell fusion has been proposed, as apparently these cells do not become insulin-producing cells *per se*, rather they take part in islet vascularization eventually promoting β-cell regeneration.

Liver stem cells

Pancreas and liver share the same origin from the embryonic endoderm (Zaret, 2000). It has been reported that transdifferentiation of pancreas into liver occurs both *in vitro* and *in vivo* in animal models after a number of experimental treatments (Rao et al., 1986, 1995; Dabeva et al., 1997; Kralowski et al., 1999; Shen et al., 2000). The opposite conversion of liver into pancreas is also possible (Horb et al., 2003). Zalzman et al. were able to immortalize a population of human fetal liver epithelial progenitor cells that once transfected with the Pdx-1 gene, generated a stable population of insulin-producing cells (Zalzman et al., 2003). Intraperitoneal transplantation of these cells into immunodeficient mice led to reversal of diabetes for 80 days. However, Yang et al. showed that expression of Pdx-1 in hepatocytes does not result in the formation of functional endocrine pancreas in Pdx-1 deficient mice, thus suggesting that Pdx-1 is necessary but not sufficient to induce differentiation of pancreatic tissue (Yang et al., 2002).

Splenic stem cells

The hypothesis that the spleen may harbor stem cells capable of differentiating into β-cells has also been investigated. Faustman and colleagues initially showed that splenocytes contributed to the reversal of autoimmunity in the NOD mouse model when injected with Freund's complete adjuvant (Ryu et al., 2001). Later they also suggested that splenocytes might directly contribute to islet regeneration by differentiation into β-cells (Kodama et al., 2003). However, these findings proved to be controversial and several other groups, although confirming to some extent the recovery from the autoimmune attack following splenocytes injections, failed to display evidence of a direct contribution of donor cells to β-cell regeneration (Suri et al., 2006; Chong et al., 2006; Nishio et al., 2006). Nonetheless, Yin and colleagues also supported a facilitating role of the spleen in regeneration of endogenous β-cell mass (Yin et al., 2006).

Bone marrowstem cells

There are numerous reports suggesting that the bone marrow not only harbors haemopoietic stem cells, which are committed to differentiate into blood cells, but also mesenchymal stem cells (MSCs), capable of differentiation into β-cells (Oh et al., 2004; Moriscot et al., 2005). MSCs were reported to differentiate *in vivo* into glucose-competent pancreatic endocrine cells when transplanted in NOD mice (Ianus et al., 2003). However, following studies resulted in controversial outcomes suggesting that MSCs do not become *per se* insulin producing cells, rather they take part in islet vascularization, eventually promoting β-cell regeneration (Hess et al., 2003 Chamson-Reig et al., 2010). Transplantation of human MSCs was also shown to induce repair of pancreatic islets and renal glomeruli in immunodeficient mice (NOD/SCID) suffering from STZ-induced diabetes (Lee et al., 2006). The role exerted by bone marrow cells by quenching autoimmunity, allowing therefore

functional recovery of residual β-cell mass, has been proven by Zorina et al. (Zorina et al., 2003). In the autoimmune diabetes model of NOD, restoration of endogenous β-cell function to physiologically sufficient levels was achievable after allogeneic bone marrow transplantation. Abrogation of autoimmunity and consequent β-cell mass recovery interestingly occurred even when allogeneic bone marrow cell tranplantation was performed after the clinical onset of diabetes. A recent study has suggested that bone marrow cells might have a role in permitting survival of endogenous β-cells also in humans (Voltarelli et al., 2007). Authors reported insulin independence for up to a year in more than half the cases of a small number of patients with recent-onset T1D. Patients were administered high-dose immunosuppressive therapy to kill autoreactive T cell clones followed by autologous non-myeloablative stem cell transplantation. Nonetheless, because autologous bone marrow cell transplantation could not change indefinitely the genetic susceptibility to develop autoimmune diabetes, autoimmunity recurred soon after full immunocompetence was re-established. Therefore different approaches should be used to obtain durable abrogation of β-cell specific autoimmunity and allow recovery of insulin production.

Collectively these series of reports suggest that bone marrow cells - although do not give rise directly to new insulin producing cells - can indirectly facilitate regeneration of the endocrine pancreas, perhaps by secreting appropriate regenerative factors that still need to be characterized.

Adipose stem cells

Other mesenchymal stem cells have been taken into consideration as a potential source of β-cells. Human and rat multipotent adipose tissue-derived stem cells (ADSCs) have been reported to generate insulin-producing cells after transduction with Pdx1 gene. The surrogate β-cells obtained improved glucose sensitivity when transplanted under the renal capsule of STZ-induced diabetic rats (Lin et al., 2009). In addition, intraportal infusion of human ADSCs together with bone marrow stem cells could increase endogenous insulin levels by reducing exogenous insulin requirements in patients affected by T1D (Trivedi et al., 2008).

Limbal stem cells

The limbus is a highly specialized region of the eye hosting a well-recognized population of epithelial stem cells (LESCs), which continuously renew the corneal surface (Davanger et al., 1971). The limbal niche is characterized by stromal invaginations which provide anatomical and functional dimensions to maintain “stemness”, protect stem cells from traumatic and environmental insults, allow epithelial-mesenchymal interactions, and supply access to chemical signals that diffuse from the rich underlying vascular network (Dua et al., 2005). LESCs are thought to generate a large number of functional differentiated progeny via transient amplifying cells (TACs), which eventually migrate centripetally towards the centre of the cornea. LESC deficiency results in severe corneal damage and

its treatment by transplantation of limbal explants is currently performed for ocular surface reconstruction. Limbal grafts are taken with a minimally invasive procedure from the uninjured eye in case of unilateral defects, and from either living or cadaver donors in bilateral disorders, as they do not express class II histocompatibility molecules (Dua et al., 2000; Kheirkhah et al. 2008; Shortt et al., 2008).

LESCs have been widely characterized (Chen et al., 2004; De Paiva et al., 2005; Di Iorio et al., 2005; Du et al., 2000; Dua et al., 2005), and investigated for their differentiation potential, which seems to be restricted to the corneal fate.

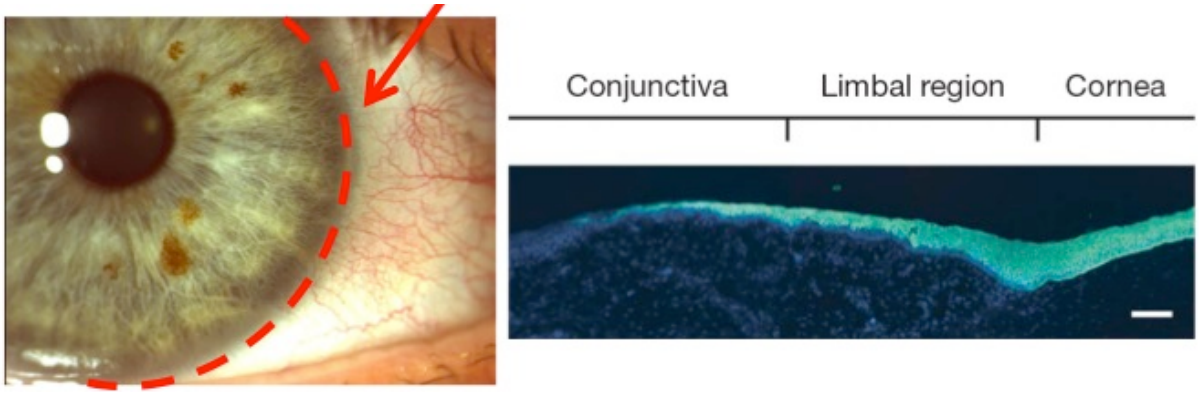


Figure 5. *The limbus is located in the transitional zone between cornea and conjunctiva (red arrow).*

There is recent evidence that the limbal niche hosts also stromal fibroblast-like stem cells (f-LSCs), with apparent multilineage transdifferentiation potential (Dravida et al., 2005; Du et al., 2005; Polisetty et al., 2008). Until recently, the identification of f-LSCs has been mainly based on general stem cell properties, such as slow cell cycle during homeostasis *in vivo*, high capacity to maintain a normal and stable genome during replication, lack of differentiation during proliferation and ability for asymmetric division. Several putative markers have been proposed for f-LSCs, however they are also expressed by the majority of corneal epithelial basal cells, TACs and occasionally also by conjunctiva cells (Chen et al., 2004; De Paiva et al., 2005; Di Iorio et al., 2005). This lack of agreement on specific molecular hallmarks for the identification of the pluripotent subpopulation among the limbal stromal cells has so far limited the investigation of their differentiation potential to a few studies (Dravida et al., 2005; Polisetty et al., 2008).

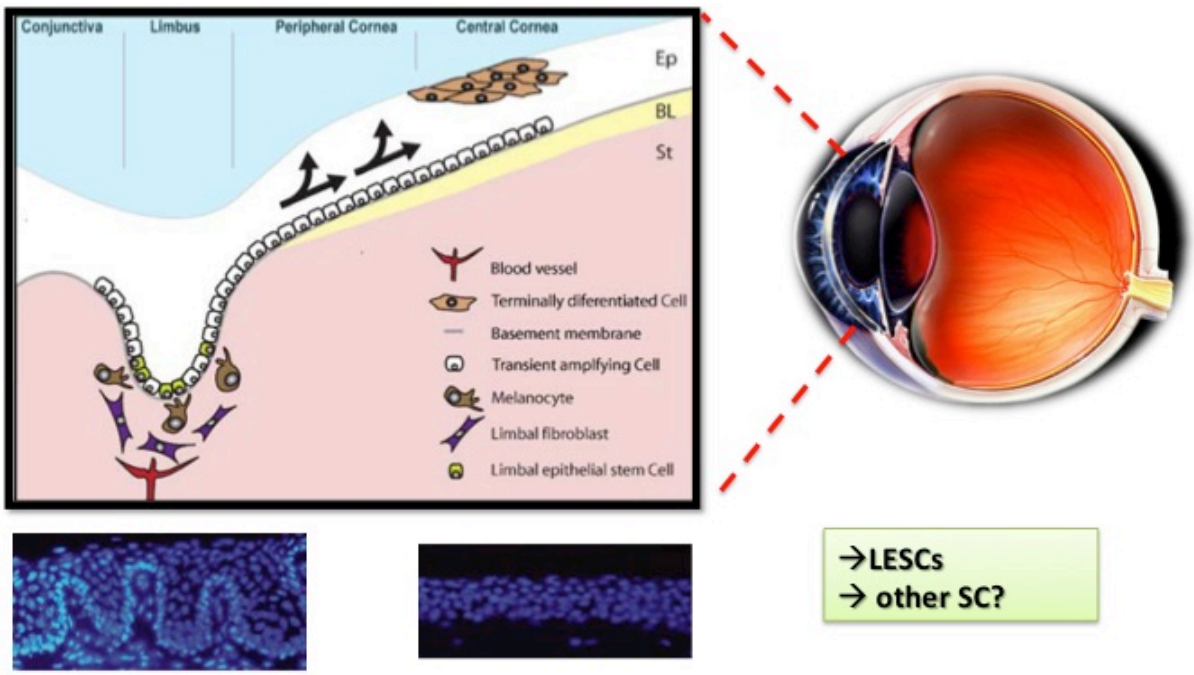


Figure 6. *Schematic representation of the limbal niche. The limbus is characterized by stromal invaginations (palisades of Vogt) which protect stem cells from traumatic and environmental insults, allow epithelial-mesenchymal interactions, and supply access to chemical signals that diffuse from the rich underlying vascular network.*

5. Evidence of β-cell regeneration via proliferation of pre-existing β-cells

During the fetal stage, differentiation is the major mechanism for forming new β-cells, while replication is enhanced during the perinatal and neonatal period. Lineage-tracing experiments in rodents provided convincing proof for the theory that the adult β-cells predominantly arise from other β-cells without significant contributions from underlying stem or progenitor cell population (Cano et al., 2008). Several studies conducted by the group of Melton and colleagues showed that under normal conditions, after pancreatic resection (Dor et al., 2004), or in diabetic status induced by transgenic expression of diphtheria toxin (Nir et al., 2007), mouse β-cells possess significant capacity for spontaneous regeneration, sufficient to recover from overt diabetes. Authors claim that failure of β-cell regeneration in both autoimmune and pharmacological models of diabetes is due to confounding factors disguising the innate regenerative response, such as the persistence of autoreactive T cells. To further sustain this hypothesis, it has been pointed out how protocols for blocking autoimmunity in NOD mice (Chatenoud et al., 1994) and in humans with T1D (Herold et al., 2002, 2005, Bresson et al., 2006) resulted in partial remission from the disease, although it is not clear whether this is due to a true regeneration process or a recovery of dysfunctional β-cells. However, the irreversibility of chemically induced diabetes, where the β-cell population is selectively destroyed leaving no opportunity for regeneration, seems to support this hypothesis. Recently, Brennand et al. also demonstrated that in adult mice all β-cells, and not a subpopulation, equally

contribute to islet growth and maintenance (Brennand et al., 2007). Two approaches were performed to address this issue. First, evaluation of the replicative potential of the entire β-cell mass was performed by monitoring the disappearance of a fluorescent marker accompanying cell division. Second, clonal analysis of dividing β-cells was completed. Because a uniform loss of label across the entire cell population was observed and all clones were of comparable size, authors conclude that the β-cell pool homogeneously possess replication ability.

In humans, increased β-cell replication has been reported adjacent to intrapancreatic gastrinomas, suggesting that adult human β-cells can be driven again into the cell cycle (Meier et al., 2006b). Support to this remark was also given by another study which assessed on a population of young non-diabetic individuals that β-cell replication is the primary mechanism responsible for the postnatal expansion of the β-cell mass (Meier et al., 2008). In particular, it was appraised that β-cell mass is able to (1) expand by several folds from birth to adulthood, (2) this is accomplished by an increase in number of β-cells per islet with a concomitant expansion in islet size (3) the relative rate of β-cell growth is higher in infancy and gradually declines thereafter to adulthood with no secondary accelerated growth phase during adolescence, (4) β-cell mass (and presumably growth) is highly variable between individuals, (5) a high rate of β-cell replication is coincident with the major postnatal expansion of beta-cell mass. A summary of the theories on β-cell origin is presented in **Figure 7**.

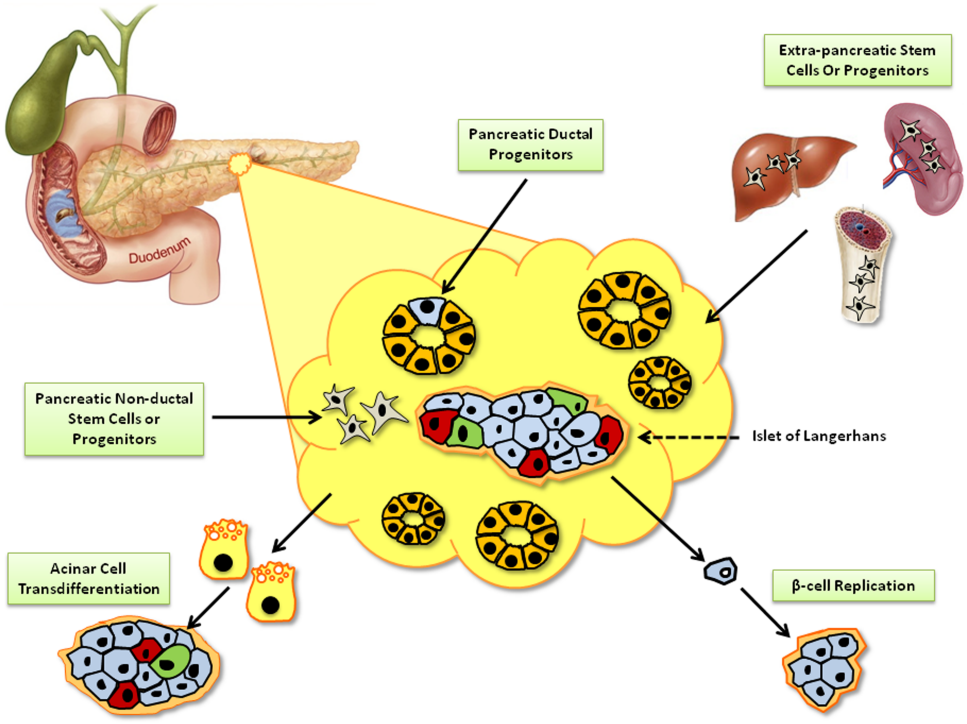


Figure 7. *Schematic illustration of potential cell sources for postnatal* β-cell *regeneration*

PROJECT 1:

IN VITRO GENERATION OF PANCREATIC ENDOCRINE CELLS FROM HUMAN ADULT FIBROBLAST-LIKE LIMBAL STEM CELLS

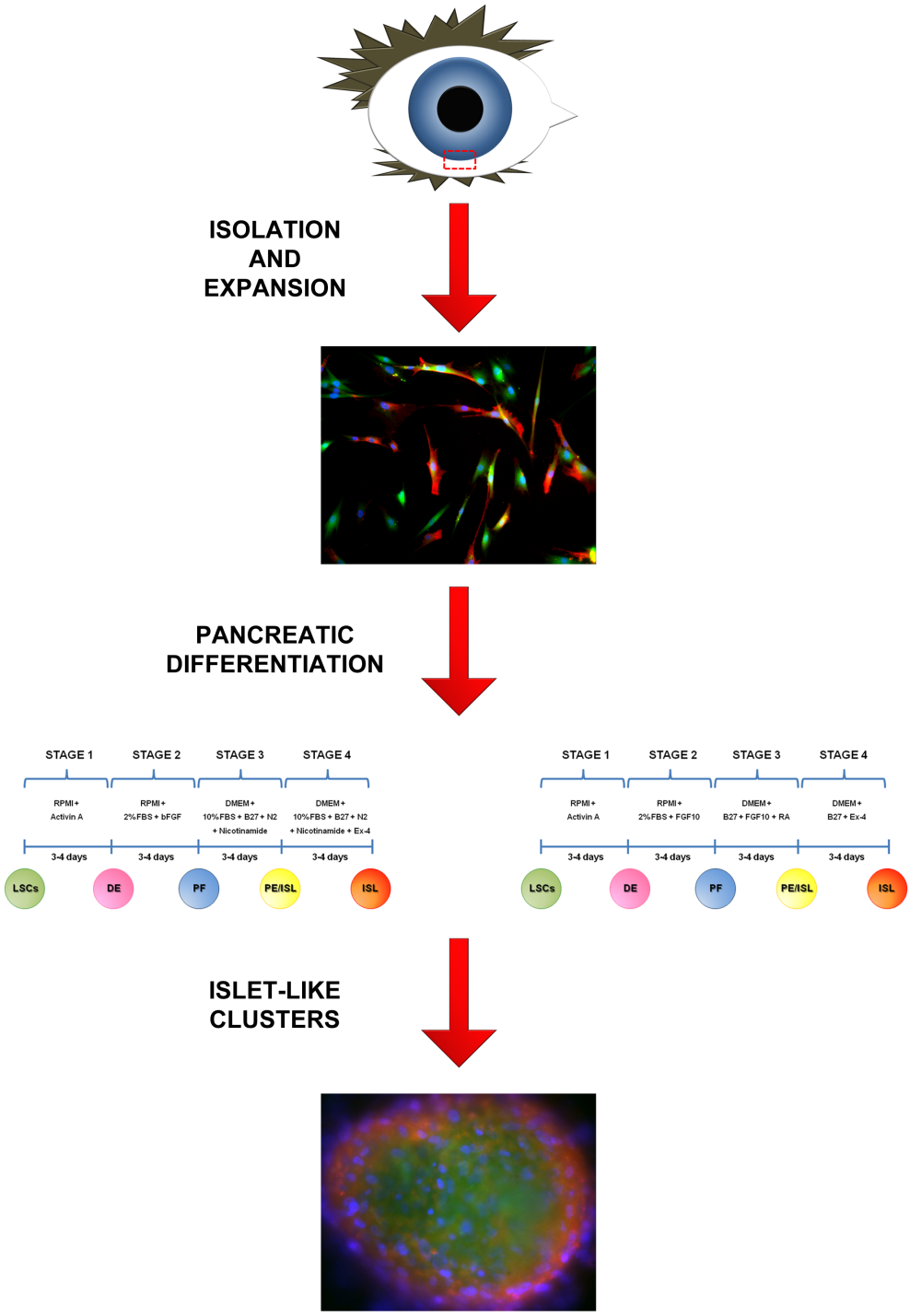
BACKGROUND AND AIMS

A variety of stem cells may be potentially harnessed for the treatment of type 1 diabetes. Embryonic stem cells (ESCs), which are able to self-renew and virtually differentiate into any phenotype, might provide an unlimited supply of surrogate β-cells. However, their clinical application is affected by ethical and technical challenges. Hence, adult multipotent stem cells are now being widely evaluated. Nevertheless the scarcity of the source, the invasive procedures often required to isolate this cells, and their restricted differentiation potential have limited their use in translational medicine (Aguayo-Mazzucato et al., 2010).

The purpose of our study was to identify a novel population of uncommitted cells that could offer advantages over the countless stem cell sources proposed so far for β-cell replacement therapy. The limbus is a highly specialized region of the eye hosting a well-recognized population of epithelial stem cells (LESCs), which continuously renew the corneal surface (Davanger et al, 1971). The limbal niche is characterized by stromal invaginations which provide anatomical and functional dimensions to maintain "stemness", protect stem cells from traumatic and environmental insults, allow epithelial-mesenchymal interactions, and supply access to chemical signals that diffuse from the rich underlying vascular network (Dua et al., 2005). A critical advantage of limbal cells is that they are easily accessible with a well-established and minimally invasive procedure (Dua et al., 2000; Kheirkhah et al., 2008; Shortt et al., 2008). LESCs have been widely characterized (Chen et al., 2004; De Paiva et al., 2005; Di Iorio et al., 2005; Du et al., 2005; Dua et al., 2005), and investigated for their differentiation potential, which seems to be restricted to the corneal fate. However, human ocular stem cell research has been mainly focused on the tissue-specific differentiation that may be of clinical significance in the context of eye diseases, as demonstrated by their clinical use in ocular surface reconstruction (Dua et al., 2000; Kheirkhah et al., 2008; Shortt et al., 2008). There is recent evidence that the limbal niche hosts also stromal fibroblast-like stem cells (f-LSCs), with apparent multilineage transdifferentiation potential (Dravida et al., 2005; Du et al., 2005; Polisetty et al., 2008). Phenotype of f-LSCs is reportedly characterized by variable expression of several stem cells markers, which are distinct from those described for LESCs (4,9,10). However, the lack of agreement on specific molecular hallmarks for the identification of the pluripotent subpopulation among the limbal stromal cells has so far limited the investigation of their differentiation potential to a few studies (Dravida et al., 2005; Polisetty et al., 2008).

Here we describe a subpopulation of f-LSCs characterized by robust proliferative capacity, stable expression of several pluripotent stem cell markers and self-renewal ability. We then demonstrate that f-LSCs are able to generate pancreatic endocrine cells. To this end, we have developed a four-step differentiation protocol aimed at directing f-LSCs through a series of intermediates similar to those occurring during pancreatic organogenesis, efficiently leading to

production of functional hormone-expressing cells. With our stage-specific approach we obtained up to 77% of insulin-producing cells. More importantly, differentiated f-LSCs possessed the ability to secrete C-peptide in response to glucose and other stimuli, similar to mature β-cells.



Graphical abstract of Project 1.

MATERIAL AND METHODS

Establishment of limbal cell cultures and karyotype analysis

Two-three mm^2 limbal biopsies were obtained from 14 patients undergoing surgery for ocular diseases not involving the conjunctiva or corneal surface. Patients gave written informed consent and the IRB of the University of Palermo approved the study. Briefly, explants were plated in cell culture-treated flasks following fine dissection with a sterile blade. After 24-48 hours, adherent colonies of fibroblast-like cells (f-LSCs) and small cuboidal cells (epithelial) were obtained. At the same time, floating spherical cell clusters or 'limbospheres' started forming. Limbospheres progressively increased in number and size and attached to the plastic surface, eventually generating highly proliferating fibroblast-like outgrowths. To better select f-LSCs from epithelial cells, forming limbospheres were transferred into new flasks. Limbal cells were cultured in F12/DMEM medium supplemented with 10% embryonic stem cell-tested FBS (PAA), 1X ITS (PAA) and 4 ng/ml bFGF (Sigma-Aldrich) (Expansion Medium). Cultures were maintained in 5% CO_2 in a humidified incubator at 37°C. Population doublings were calculated as [$\log_{10}$ (final cell number/starting cell number)/$\log_{10}2$]. Karyotype analysis was performed by cytogenetic standard protocol on all primary cell cultures.

Flow cytometry

Cells were treated with FcR blocking reagent (Miltenyi Biotec) and incubated with primary antibody at 4°C for 20 minutes. Cells were washed twice with PBS and incubated with secondary antibodies at 4°C for 20 minutes in the dark. Intracellular staining was performed using Cytofix/Cytoperm and Perm/Wash buffer kits (BD Pharmingen), according to the manufacturer's instructions. The sources of antibodies and dilutions used are summarized in **Table 1**. Freshly isolated PBMCs and HeLa cells were used as positive controls for haematopoietic markers and p63(ΔN), respectively. Data were acquired on a FACSCalibur and analyzed using CELLQuest Pro software (Becton Dickinson).

Assessment of self-renewal ability

Prior to assay, cells were sorted for SSEA4 to ensure a purified f-LSC population. Single-cell suspension of undifferentiated f-LSCs was labeled with mouse anti-human SSEA4 antibody (Santa Cruz Biotechnology, sc-21704). Magnetic isolation was carried out by MACS® technology using goat anti-mouse IgG microbeads (Miltenyi Biotec), according to the manufacturer's instructions. After sorting, cell purity was evaluated by flow cytometry and immunofluorescence. Sorted $SSEA4^+$ f-LSCs were then labeled with CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes, Invitrogen), according to the manufacturer's instructions. Cells were then cultured either in Expansion Medium or in RPMI 1640 supplemented with 10% FBS (Basal Medium), fixed with 2% paraformaldehyde at

24, 48 and 72 hrs and stained for SSEA4. Images were acquired with DM IRB inverted microscope equipped with DC300F digital camera system. Alternatively, self-renewal assay was performed over a 15-day period as previously described (Zito et al., 2008). Briefly, sorted SSEA4$^+$ cells were cultured as described above and SSEA4 expression was detected by flow cytometry on days 0, 4, 8, 12 and 15. At the same time points cell proliferation was assessed by Cell Proliferation Elisa, BrdU (colorimetric) kit (Roche), according to the manufacturer's instructions. Apoptosis was assessed by Annexin V-FITC Apoptosis Detection kit (BD Pharmingen) following the manufacturer's instructions. Data are representative of at least five independent experiments.

ANTIBODY	HOST	CODE NUMBER	DILUTION
SSEA4	Mouse	Santa Cruz Biotechnology, sc-21704	1:20
OCT3/4	Mouse	Santa Cruz Biotechnology, sc-5279	1:20
c-Kit	Mouse	Chemicon, MAB1164	1:50
Thy-1	Mouse	Chemicon, CBL415	1:50
HLA-DR	Mouse	Santa Cruz Biotechnology, sc-18875	1:20
TRA 1-60	Mouse	Biolegend, 330602	1:100
TRA 1-81	Mouse	Biolegend, 330702	1:100
Sox2	Rabbit	BioLegend, 630801	1:100
p63(ΔN)	Rabbit	BioLegend, 619002	1:100
ABCG2	Mouse	BioLegend, 332002	1:50
CD105	Mouse	BioLegend, 323202	1:100
CD133/2	Mouse	Miltenyi Biotec, 130-090-853	1:50
C-peptide/Proins	Mouse	Millipore, CBL94	1:20
Insulin	Guinea pig	Dako, A0564	1:100
Glucagon	Rabbit Mouse	Santa Cruz Biotechnology, sc-13091 Sigma-Aldrich, G2654	1:20 1:250
Somatostatin	Rabbit	Dako, A0566	1:250
Synaptophysin	Mouse	Novocastra, CL-SYNAP-299	1:100

Table 1. *Sources of antibodies and dilutions used for flow cytometry.*

Differentiation towards the pancreatic phenotype

Differentiation with Protocol A was performed in 9 f-LSC cultures, and each cell culture was differentiated three or more times. Protocol B was tested in parallel experiments in 3 out of 9 f-LSC cultures used for Protocol A. Protocol A: In **stage 1**, cells were cultured for 2-3 days in RPMI 1640 supplemented with 100 ng/ml Activin A (Peprotech); in **stage 2**, cells were treated for 3-4 days with RPMI 1640 supplemented with 2% embryonic stem cell-tested FBS (PAA) and 50 ng/ml bFGF (Sigma-Aldrich); in **stage 3**, cells were cultured for 3-4 days with advanced DMEM supplemented with 10% embryonic stem cell-tested FBS, 1% B27 (PAA), 2% N2 (PAA) and 1 mM nicotinamide (Sigma-Aldrich); in **stage 4**, cells were cultured for 3-4 days in advanced DMEM supplemented with 10% embryonic stem cell-tested FBS, 1% B27, 2% N2, 1 mM nicotinamide and 50 ng/ml Exendin-4 (Sigma-Aldrich).

Protocol B: in **stage 1** cells were cultured as described in Protocol A; in **stage 2** cells were treated with 50 ng/ml FGF10 (Sigma-Aldrich); **stage 3** was carried out in advanced DMEM supplemented with 1% B27, 50 ng/ml FGF10 and 2 µM RA (Sigma-Aldrich); in **stage 4** cells were cultured in advanced DMEM supplemented with 1% B27 and 50 ng/ml Exendin-4. All data represented were generated with Protocol A using the same primary cell culture, except where differently indicated.

Immunofluorescence

Cells were differentiated in culture-slides (BD Biosciences), fixed for 15 min at room temperature (RT) in 2% (wt/vol) paraformaldehyde, permeabilized with 0.1% PBS/Triton X-100, washed in PBS and blocked for 30 min in 3% PBS/BSA. Primary antibodies were incubated over night at 4°C, while secondary antibodies were incubated for one hour at RT. The sources of antibodies and dilutions used are summarized in **Table 2**. Mouse anti-Nkx6.1 and mouse anti-Ngn3 antibodies were gifts from O. Madsen. Images were acquired with DM IRB inverted microscope equipped with DC300F digital camera system or TCS SP5 confocal microscope (all by Leica Microsystems). All fileds are representative of at least five independent experiments.

ANTIBODY	HOST	CODE NUMBER	DILUTION
Sox17	Rabbit	Santa Cruz Biotechnology, sc-20099	1:50
FOXA2/HNF3β	Goat	Santa Cruz Biotechnology, sc-6554	1:50
MafA	Rabbit	Sigma-Aldrich, SAB2101414	1:250
PDX1	Rabbit	Millipore, AB3503	1:100
C-peptide/Proins	Mouse	Millipore, CBL94	1:50
Insulin	Guinea pig	Dako, A0564	1:200
Glucagon	Mouse	Sigma-Aldrich, G2654	1:500
Somatostatin	Rabbit	Dako, A0566	1:500
GLUT2	Rabbit	Santa Cruz Biotechnology, sc-9117	1:50
Synaptophysin	Mouse	Novocastra, CL-SYNAP-299	1:200

Table 2. *Sources of antibodies and dilutions used for immunofluorescence analysis.*

Western Blot analysis

Cells were harvested and resuspended in 200 µl of 1X ice-cold NP40 lysis buffer supplemented with a cocktail of protease inhibitors (Complete™, Roche). Proteins of human islets were obtained as previously described (Ricordi et al., 1988). Electrophoresis of 3 µg (for human islets) or 15 µg (for differentiated f-LSCs) lysates was performed on 4–20% polyacrylamide gels (Invitrogen). The sources of antibodies and dilutions used are summarized in **Table 3**. Horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) and chemiluminescent substrate (SuperSignal West Pico, Pierce) were used for detection with a Bio-Rad Chemidoc XRS Imager. Data are representative of at least five independent experiments.

ANTIBODY	HOST	CODE NUMBER	DILUTION
PDX1	Rabbit	Millipore, AB3503	1:2000
C-peptide	Mouse	Millipore, CBL94	1:500
GLUT2	Rabbit	Santa Cruz Biotechnology, sc-9117	1:500
Glucokinase	Rabbit	Santa Cruz Biotechnology, sc-7908	1:500
β-actin	Mouse	Sigma-Aldrich, A5441	1:500

Table 3. *Sources of antibodies and dilutions used for western blot analysis.*

Real-time quantitative PCR (qRT-PCR)

mRNA isolation and subsequent cDNA synthesis were performed using μMACS® One-step cDNA Kit (Miltenyi Biotec) according to the manufacturer's instructions. PCR primers were purchased from Qiagen (QuantiTect® Primer Assays, Qiagen) and are listed in Table 4. All reactions were performed with Quantitect Sybr Green PCR Kit (Qiagen) using a LightCycler 1.5 Instrument (Roche). Reactions were performed at least in triplicates. Specificity of the amplified products was determined by melting peak analysis. Quantification for each gene of interest was performed in relation to a standard curve represented by the appropriate cDNA plasmid. Quantified values were normalized against the housekeeping gene β-actin. cDNA of human islets was obtained from adult cadaveric donors as previously described (Ricordi et al., 1988).

GENE	QIAGEN QUANTITECT PRIMER ASSAY	CATALOG NUMBER
SOX17	Hs_SOX17_1_SG	QT00204099
FOXA2	Hs_FOXA2_1_SG	QT00212786
PDX1	Hs_PDX1_1_SG	QT00201859
NGN3	Hs_NEUROG3_2_SG	QT01019823
ISL1	Hs_ISL1_1_SG	QT00000294
NKX6.1	Hs_NKX6-1_1_SG	QT00092379
PAX4	Hs_PAX4_1_SG	QT00022358
NeuroD	Hs_NEUROD1_1_SG	QT00203189
MAFA	Hs_MAFA_2_SG	QT01035398
INS	Hs_INS_2_SG	QT01531040
GCG	Hs_GCG_1_SG	QT00091756
SST	Hs_SST_1_SG	QT00004277
PPY	Hs_PPY_2_SG	QT01671012
GHRL	Hs_GHRL_1_SG	QT00041377
GCK	Hs_GCK_1_SG	QT00000812
GLUT2	Hs_SLC2A2_2_SG	QT01008399

Table 4. *List of primers used for qRT-PCR.*

Transmission Electron Microscopy

Pellets were fixed for 20 minutes in 2% paraformaldehyde; post-fixed for 15 minutes with 1% osmium tetroxide; dehydrated through graded ethanol concentrations and embedded in epoxy resin (Durcupan ACM). 80 nm thick sections were cut with an Ultracut-Reichert Microtome, mounted on nickel grids and stained with 1% uranyl acetate and lead citrate. Analysis was performed on a Zeiss EM 109 electron microscope.

Insulin content and C-peptide release assays (static incubation)

After discarding differentiation media, cells (both monolayer and islet-like clusters) were washed several times and then incubated for 1 hour in Krebs-Ringer solution with bicarbonate and HEPES (KRBH), followed by 1 h incubation in KRBH containing 2 mM D-glucose (basal condition). Cells were then incubated for another 1 hour in stimulating conditions with 20 mM D-glucose, 100 µM Tolbutamide or 30 mM KCl, respectively (all from Sigma-Aldrich). Plates were incubated at 37°C on a rotating shaker and the supernatant was sampled at basal conditions and at the time points 1', 2', 3' 5', 7', 10', 15', 30' and 60'. C-peptide content and release were assessed with C-peptide ELISA Kit (Mercodia) on cell lysates or supernatants, respectively. Proinsulin content was measured on cell lysates with Proinsulin Elisa Kit (Mercodia). The fold increase was calculated for each culture condition by dividing the C-peptide concentration in the stimulation supernatant at 60' by the C-peptide concentration in the basal supernatant. Total insulin content was calculated as the sum of C-peptide (pM) and proinsulin (pM) per µg of total proteins. 500,000 differentiated f-LSCs and an equal mass of islet equivalents (IE) (~250 islets with an estimate of 2,000 cells per IE) were used. Data are representative of five independent experiments.

RESULTS

f-LSCs express pluripotent markers and exhibit self-renewal ability

Limbal explants were finely dissected with a sterile blade and cells were plated in cell culture-treated flasks. After 24-48 hrs, a few colonies of small cuboidal (epithelial) cells and several colonies of fibroblast-like cells (f-LSCs) were observed. At the same time, the single cell suspension gave rise to floating spherical cell clusters, which we termed 'limbospheres'. Limbospheres progressively increased in number and size and after few days attached to the plastic surface, eventually giving rise to highly proliferating fibroblast-like outgrowths (**Figure 1 and Figure 2A)**.

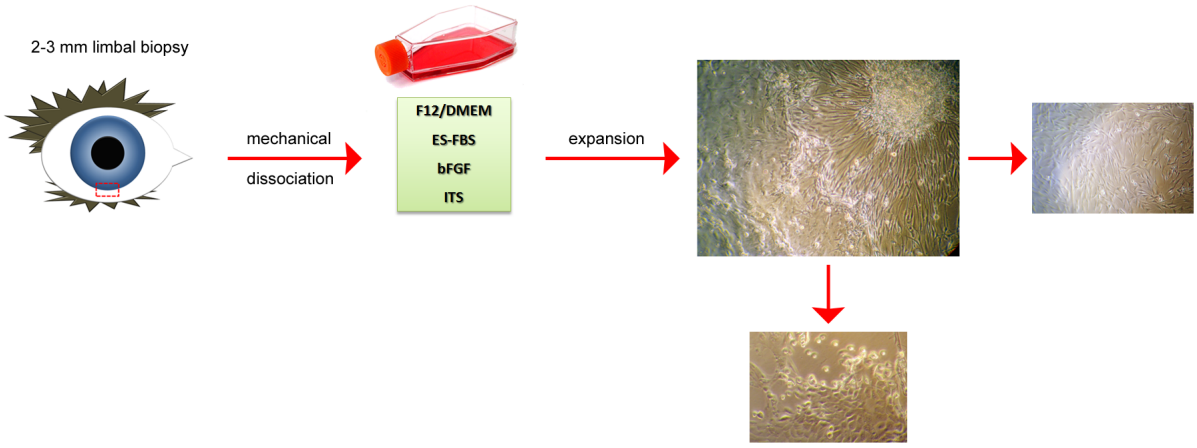


Fig.1. *Sketch illustrating isolation of f-LSCs from limbal explants.*

f-LSCs soon prevailed after one week of culture, however to better select f-LSCs from epithelial cells, forming limbospheres were transferred in new flasks. Upon replating, limbospheres exclusively generated f-LSCs. Cell morphology and growth rate of f-LSCs are shown in **Figures 2A and 2B**; Primary f-LSC cultures were obtained from all 14 limbal biopsies and maintained normal karyotype (46XX or 46XY) during long-term culture. **Figure 2C** shows a representative karyotype at P30, corresponding to ~60 population doublings.

Immunophenotype of f-LSCs was assessed by flow cytometry (**Figure 2D**). Freshly digested limbal specimens (which included also epithelial cells) showed significant expression of the pluripotent stem cell marker SSEA4 (mean ± SD: 65.2 ± 7.6%; data not shown), which had been previously proposed as a reliable marker for f-LSCs (Dravida et al., 2005). After 10-12 population doublings in stem cell Expansion Medium - or at earlier passages in primary cultures obtained by sub-culturing limbospheres – the highly proliferating f-LSCs showed increased positivity for SSEA4 (90.8 ± 7.6%). $SSEA4^+$ f-LSCs co-expressed the stem cell markers OCT4 (double positive (DP): 92.7 ±

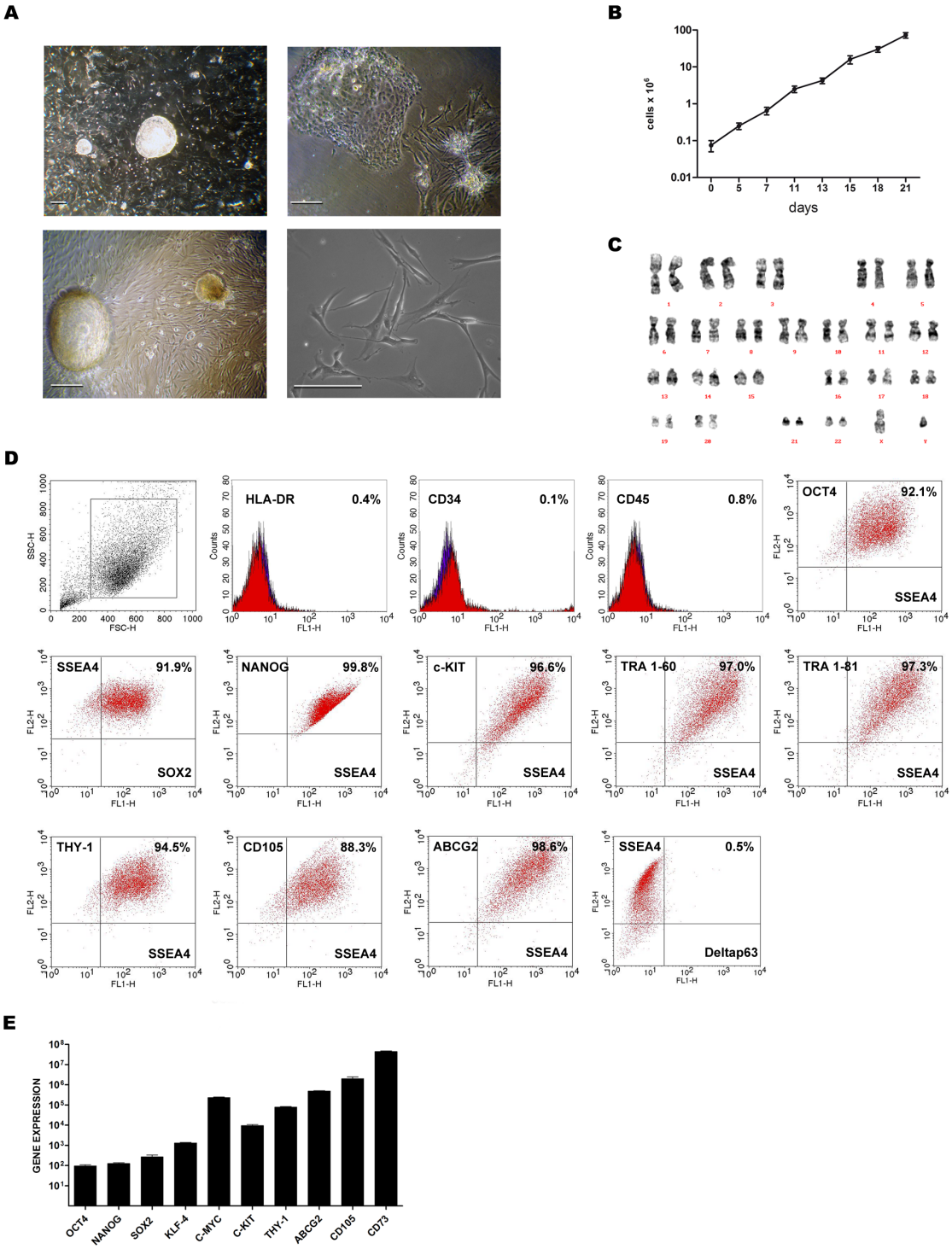


Figure 2. f-LSCs express several pluripotent stem cell markers and exhibit self-renewal ability. (A) *Morphology of cultured f-LSCs. After 24-48 hrs in culture, the single cell suspension gives rise to floating spherical cell clusters or 'limbospheres' which progressively increase in number and size and attach to the plastic surface (upper left). Colonies of small cuboidal adherent cells (epithelial) are also observed (upper right); however f-LSCs soon prevail after one week of culture. Upon replating, limbospheres are able to generate only highly proliferating fibroblast-like outgrowths (lower left and lower right). Primary cultures were obtained from all 14 limbal biopsies as indicated in the Method section. Scale bars 400 µ.* ***(B)*** *Kinetics of f-LSCs in expansion medium. Cells were counted using trypan blue exclusion dye at each passage.* ***(C)*** *f-LSCs maintain a normal karyotype in long-term cultures. Figure shows a representative karyotype at P30, corresponding to ~60 population doublings.* ***(D)*** *FACS analysis shows f-LSCs are positive for several nuclear and surface stem cell markers. Data are representative of at least five independent experiments.* ***(E)*** *qRT–PCR confirms expression of pluripotent stem cell markers. Each bar represents mean ± SE (log scale) of the gene of interest in all 14 primary cell cultures. Quantification for each gene of interest was performed in relation to its own standard curve (arbitrary units) and expression was normalized for the housekeeping gene β-actin.*

4.3%), SOX2 (DP: 91.9 ± 5.6%), NANOG (DP: 98.6 ± 1.3%), c-KIT (DP: 93.6 ± 3.3%), TRA 1-60 (DP: 95.3 ± 4.1%), TRA 1-81 (DP: 95.0 ± 3.1%), THY-1 (DP: 94.5 ± 3.6%), CD105 (DP: 88.3 ± 2.3%), and the limbal marker ABCG2 (DP: 94.9 ± 4.8%) (4,9). CD133 was weakly expressed or absent (DP: 5.2% ± 2.9 only in 4 out of 14 primary cultures; data not shown). f-LSCS were also negative for CD34, CD45, HLA-DR and the LESC marker ΔNp63 (Di Iorio et al., 2005). To exclude that the absence of these markers in f-LSCs was due to experimental artifacts, freshly isolated PBMCs and HeLa cells were used as positive controls (data not shown). The expression of all stem cell markers was unaffected by long-term culture and was assessed up to 92 population doublings (data not shown). The "stem-like" profile of f-LSCs was confirmed by qRT–PCR (**Figure 2E**), which also showed expression of *KLF-4*, *c-MYC* and *CD73*. **Table 4** summarizes the markers that identify f-LSCs.

MARKER	LOCALIZATION	EXPRESSION	MARKER	LOCALIZATION	EXPRESSION
SSEA4	surface	+	**CD34**	surface	-
c-KIT	surface	+	**CD45**	surface	-
TRA 1-60	surface	+	**HLA-DR**	surface	-
TRA 1-81	surface	+	**ΔNp63**	nuclear	-
ABCG2	surface	+	**OCT4**	nuclear	+
THY-1	surface	+	**SOX2**	nuclear	+
CD105	surface	+	**KLF-4**	nuclear	+
CD73	surface	+	**NANOG**	nuclear	+
CD133[§]	surface	-	**c-MYC**	nuclear	+

[§]Found weakly expressed only in 4/14 f-LSCs

Table S2. *Expression of stem cell antigens in f-LSCs*

f-LSCs were also evaluated for the ability to divide asymmetrically, a key stem cell feature (**Figure 3**). Prior to assay, cells were sorted for SSEA4 to ensure a purified $SSEA4^+$ f-LSC population (**Figure 3A**). Cells were then labeled with fluorescent dye CFSE in order to track cell division (**Figure 3B**). Cells were either cultured in Expansion Medium (supporting maintenance of the pluripotent phenotype) or in a Basal Medium (and thus allowing asymmetric division). Immunofluorescence analysis performed at 24, 48 and 72 hrs in Basal Medium showed increasing number of $CFSE^+/SSEA4^-$ cells, while several mitotic cells showed polarized SSEA4 distribution (**Figure 3C and 3D arrows**). In addition, flow cytometry analysis after 72 hrs showed that the number of $SSEA4^+$ cells remained unchanged while the total cell population increased, thus suggesting asymmetric division. By contrast, at 72 hrs cells cultured in Expansion Medium maintained SSEA4 expression as their number progressively increased, indicating cells divided symmetrically during proliferation (data not shown). Asymmetrical division of $SSEA4^+$ cells was further evaluated indirectly over a 15-day period by culturing f-LSCs in the same conditions described above (**Figure 3E**). SSEA4 expression detected by flow cytometry gradually decreased from 98% on day 0 (after sorting) to 58% on day 8, and

maintained a steady state up to the last day of the assay. Instead, cell proliferation (detected by BrdU) was observed during the entire culture period. Assessment of apoptosis by Annexin V-FITC at the same time points demonstrated that the decrease in SSEA4 expression overtime was not caused by cell death (<2.7%, data not shown).

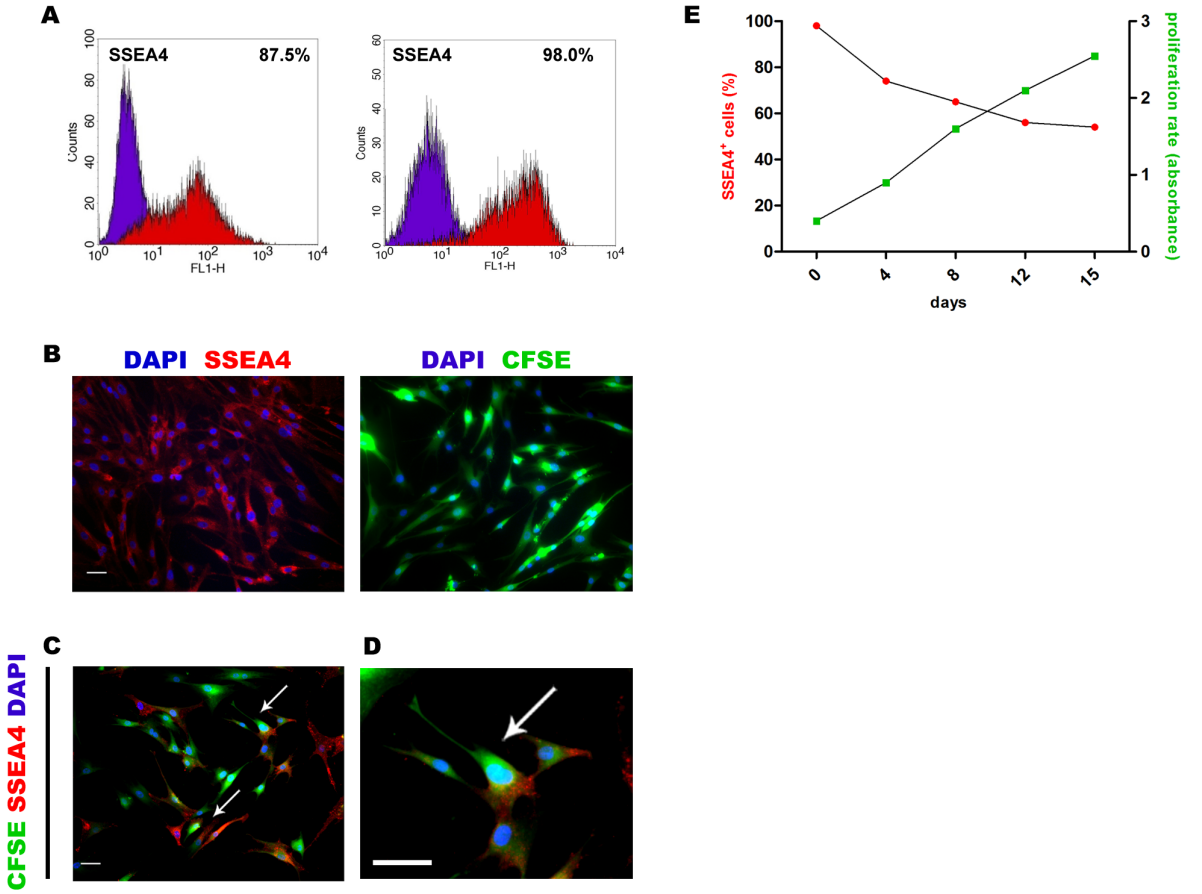


Figure 3. Self renewal ability of f-LSCs. (A) *f-LSCs are sorted for SSEA4 prior to assay, in order to ensure a purified population. Left: before sorting; Right: after sorting. Purple peak: negative control. Red peak: SSEA4.* ***(B)*** *SSEA4$^+$ cells (red) are then labeled with fluorescent dye CFSE (green) in order to track cell division. The dye–protein adducts that form in labeled cells are retained by daughter cells after either cell division or cell fusion, and CFSE is not transferred to adjacent cells. Note that all cells are positive for SSEA4 and CFSE after sorting.* ***(C)*** *Cells are cultured in a basic medium not containing bFGF. Immunofluorescence analyses performed at 24, 48 and 72 hrs show increasing number of CFSE$^+$/SSEA4$^-$ cells, suggesting they divide asymmetrically. Arrows indicate polarized SSEA4 expression in pre-mitotic or mitotic cells. Scale bar 100 µ.* ***(D)*** *Higher magnification of (C). Scale bar 100 µ.* ***(E)*** *Asymmetrical division of SSEA4$^+$ cells evaluated indirectly over a 15-day period. SSEA4 expression detected by flow cytometry decreases from 98% on day 0 (after sorting) to 58% on day 8, and maintains a steady state up to the last day of the assay. Instead, cell proliferation detected by BrdU continues for the entire culture period, thus suggesting that SSEA4$^+$ cells divide asymmetrically. Data are representative of five independent experiments.*

Directed differentiation of f-LSCs cells towards the pancreatic endocrine phenotype

We developed a four-step protocol for differentiation of f-LSCs into pancreatic endocrine cells through a series of intermediates mimicking *in vivo* pancreatic organogenesis. Stepwise, we added factors and supplements known to direct/support pancreatic differentiation of embryonic stem cells,

such as Activin A, bFGF, B27, N2, nicotinamide and exendin-4 (Protocol A, **Figure 4A**) (Lumelski et al., 2001; D'Amour et al., 2005; Shim et al., 2007; Iwamuro et al., 2010). The substitution or addition of other molecules - such as FGF10 and retinoic acid (RA) - was also investigated based on previous evidence reporting improvement in endoderm formation and increase in insulin content of ESC-derived surrogate beta cells (Protocol B, **Figure 4B**) (D'Amour et al., 2006; Jiang et al., 2007).

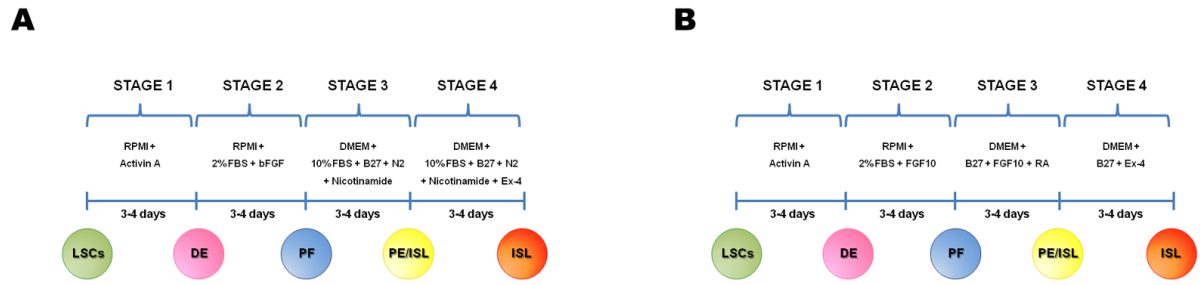


Figure 4. Four-step protocols for differentiation of f-LSCs into pancreatic hormone–expressing cells. *Media, growth factors, supplements and range of duration for each stage are indicated.* ***(A)*** *Protocol A.* ***(B)*** *Protocol B. Stage 1 is the same in the two protocols, and leads to formation of definitive endoderm, which is a crucial step for the subsequent differentiation towards the pancreatic phenotype. DE, definitive endoderm; PF, posterior foregut; PE, pancreatic endoderm; ISL, hormone-expressing islet cells.*

The differentiation process was followed by immunofluorescent and qRT-PCR analyses. At **stage 1 and 2** (days 1-7), we focused on generating definitive endoderm (DE) and posterior foregut (PF). During stage 1 we used high concentrations of Activin A in serum-free media, while in stage 2 Activin A was removed, and either bFGF (Protocol A) or FGF10 (Protocol B) were added in the context of low FBS supplementation. Soon upon Activin A removal, a consistent percentage of f-LSCs transitioned to DE as indicated by the remarkable upregulation of *SOX17* and *FOXA2* mRNAs (**Figure 5**) and the subsequent protein detection by immunofluorescence (**Figs. 6A and 6B**). Of note, differentiating cells progressively aggregated into spherical cell clusters resembling human islets. At the beginning of stage 2 we also observed significant upregulation of *PDX1*, which is indicative of PF formation (**Figure 5**) (Murtaugh, 2007). $PDX1^+$ cells could be detected in both the forming islet-like clusters and in the surrounding monolayer (89.3 ± 4.1% at the end of stage 2). $PDX1^+$ cells co-expressed NKX6.1, which is characteristic of pancreatic epithelium (**Figure 6C)** (Murtaugh, 2007). *NGN3* expression, as expected, was transient and peaked at the end of stage 2, along with the increase of *ISL1*, *PAX4*, *NKX6.1* and *NEUROD1*, all transcription factors controlling endocrine cell differentiation (**Figure 5**) (Gradwohl et al., 2000; Gu et al., 2002). Indeed, low levels of these pancreatic genes were already detected at the end of stage 1, thus suggesting that a high dose of Activin A alone was able to initiate transcription of DE and PF markers. Nevertheless, if Activin A was not removed and either

bFGF or FGF10 were added, generation of hormone-producing cells in the subsequent stages did not take place or was significantly reduced (data not shown).

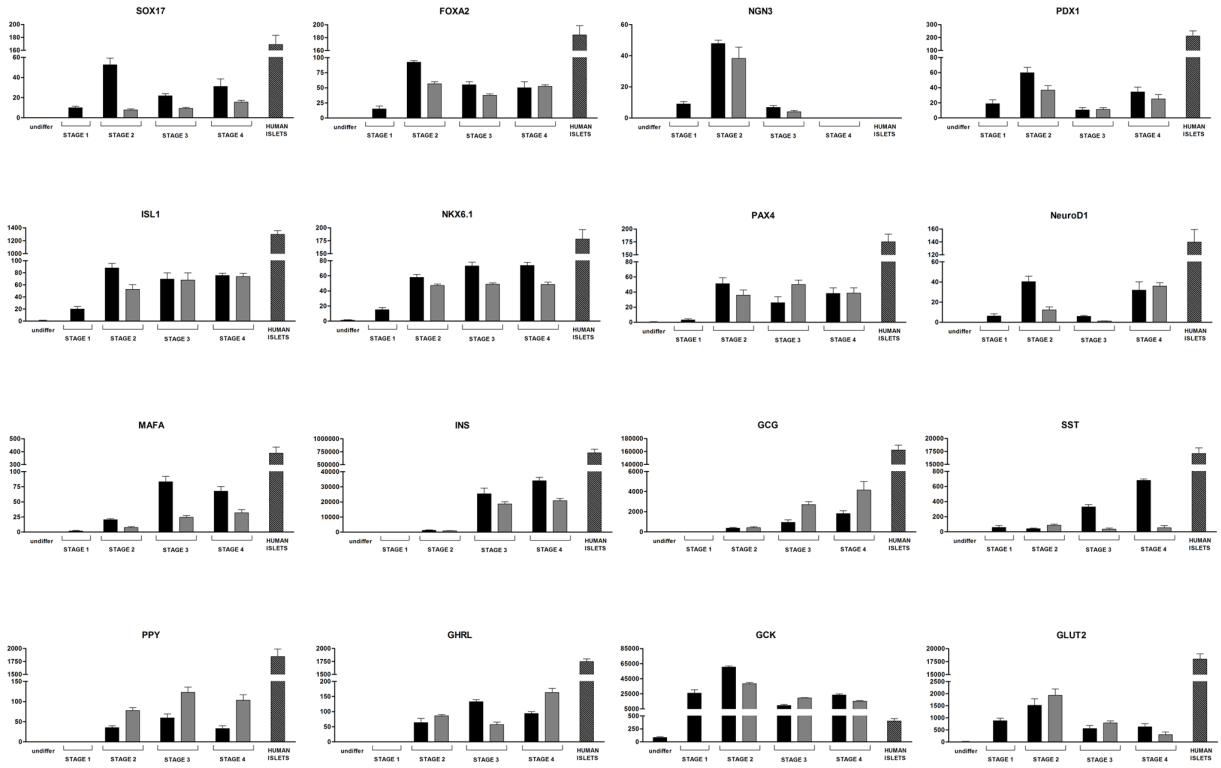


Figure 5. Expression of pancreatic/β-cell markers during differentiation. *f-LSCs express pancreatic, islet and β-cell markers in temporal succession, according to stages of differentiation. From stage 2 black bars indicate Protocol A, grey bars indicate Protocol B and striped bars indicate expression in ~500 handpicked human islets. Bars represent gene expression at the end of each stage. Quantification for each gene of interest is performed in relation to a standard curve (arbitrary units) and gene expression is normalized for the housekeeping gene β-actin. Values are showed as mean ± SE of 9 experiments for Protocol A and 3 experiments for Protocol B. See also Figure S1B for decrease of stem cell markers during differentiation.*

By the end of stage 2, NGN3 showed a nuclear localization in a substantial number of PDX1^{+} cells, consistent with endocrine determination (**Figure 6D**). At the end of stage 2, we also noted upregulation of *GLUT2* and *glucokinase* (*GCK*), along with the appearance of *insulin* (*INS*), *glucagon* (*GCG*), *somatostatin* (*SST*), *pancreatic polypeptide* (*PPY*) and *ghrelin* (*GHRL*) transcripts (**Figure 5**). However, no hormone-producing cells were found by immunofluorescence at this stage (**Figure 6E**). In addition, by day 8 the expression of stem cell markers was already negligible (**Figure 7**).

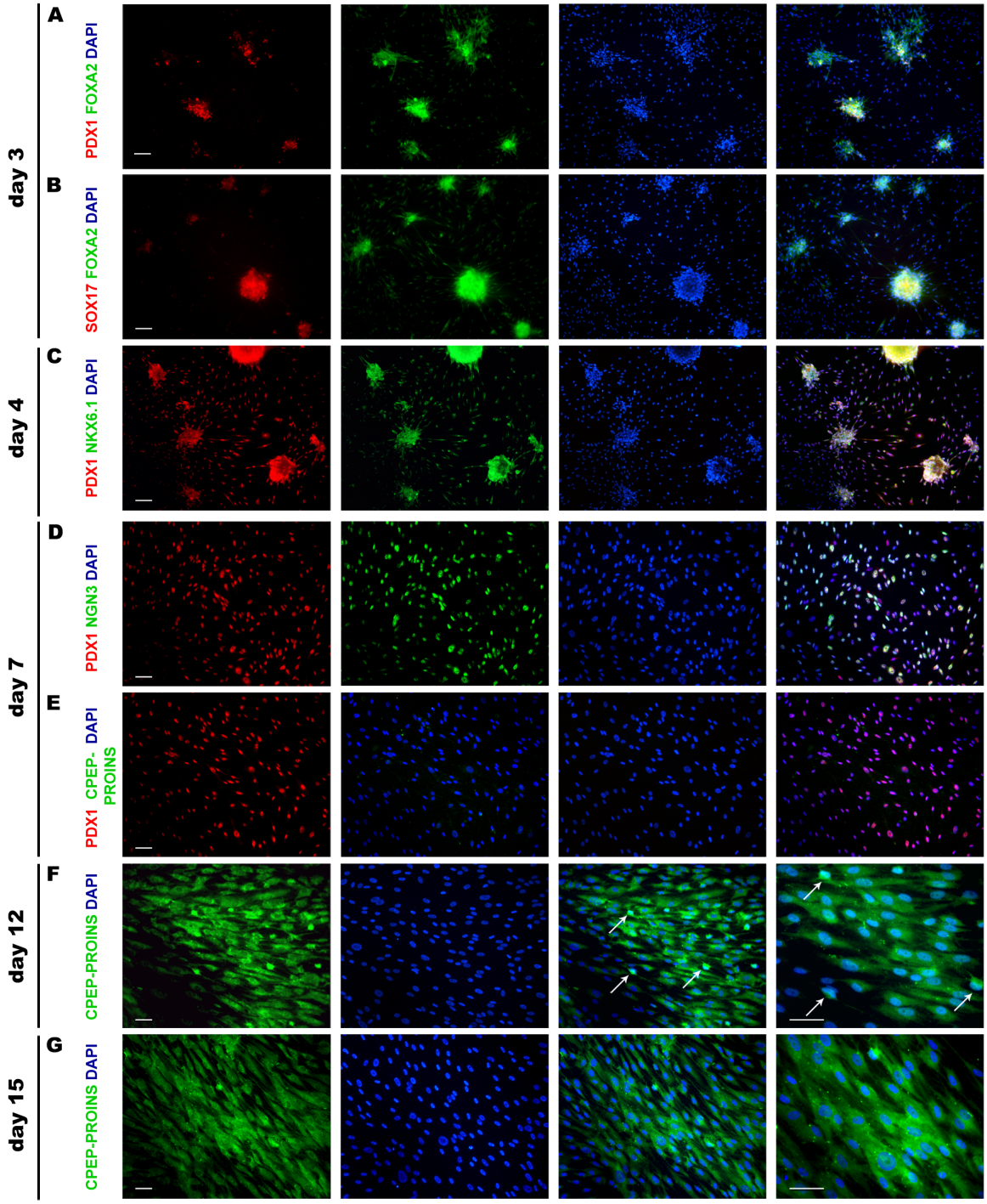


Figure 6. *(**A-C**)* ***Immunofluorescence analysis of transition from definite endoderm (DE), to posterior foregut (PF) and pancreatic epithelium.*** *Soon upon Activin A removal, f-LSCs transition to DE as indicated by the staining for SOX17* ***(A)*** *and FOXA2* ***(B)****. Notably, at this stage PDX1 staining is already detectable in the islet-like clusters but not in the monolayer. (**C**) At day 4 PDX1^{+} cells co-express NKX6.1, which is characteristic of pancreatic epithelium. PDX1 can be detected in both the forming islet-like clusters and in the surrounding monolayer, suggesting cells transitioned to PF (PDX1^{+} cells: 89.3 ± 4.1%).* ***(D-G) Immunofluorescence analysis of transition from pancreatic endoderm (PE) to hormone-expressing cells. (D)*** *By day 7 PDX1 colocalizes with NGN3, consistently with endocrine determination. (**E**) No hormone-producing cells are found by immunofluorescence at this stage.* ***(F)*** *Differentiating cells express C-PEP at day 12. Arrows indicate polarized C-PEP/PROINS distribution, as in immature hormone^{+} cells.* ***(G)*** *In later-stage cells the hormone fills and delineates the cytoplasm, consistently with a more mature phenotype. Scale bars 200 µ in (A-C) and 100 µ in (D-G). All fields are representative of at least five independent experiments.*

At **stage 3** (days 8-12), bFGF was removed and B27, N2 and nicotinamide were added to further improve the yield and maturation of pancreatic endocrine precursors (Protocol A). Alternatively, FGF10 was maintained for 3-4 more days, in presence of B27 and RA (Protocol B). At the end of this stage, by about 12 days of differentiation, we observed consistent upregulation of *INS* and *MAFA* mRNAs. Transcripts for *GCG*, *SST*, *GHRL* and *PPY* were also detectable (**Figure 5**). Differentiating cells showed a perinuclear or polarized localization of C-PEP/PROINS, consistent with immature phenotype (**Figure 6F**). By contrast, in later-stage cells the hormone filled and delineated the cytoplasm (**Figure 6G**). The absence of NGN3 staining at this stage (data not shown), along with the very low levels of *NGN3* mRNA, indicated that the majority of cells already underwent endocrine determination.

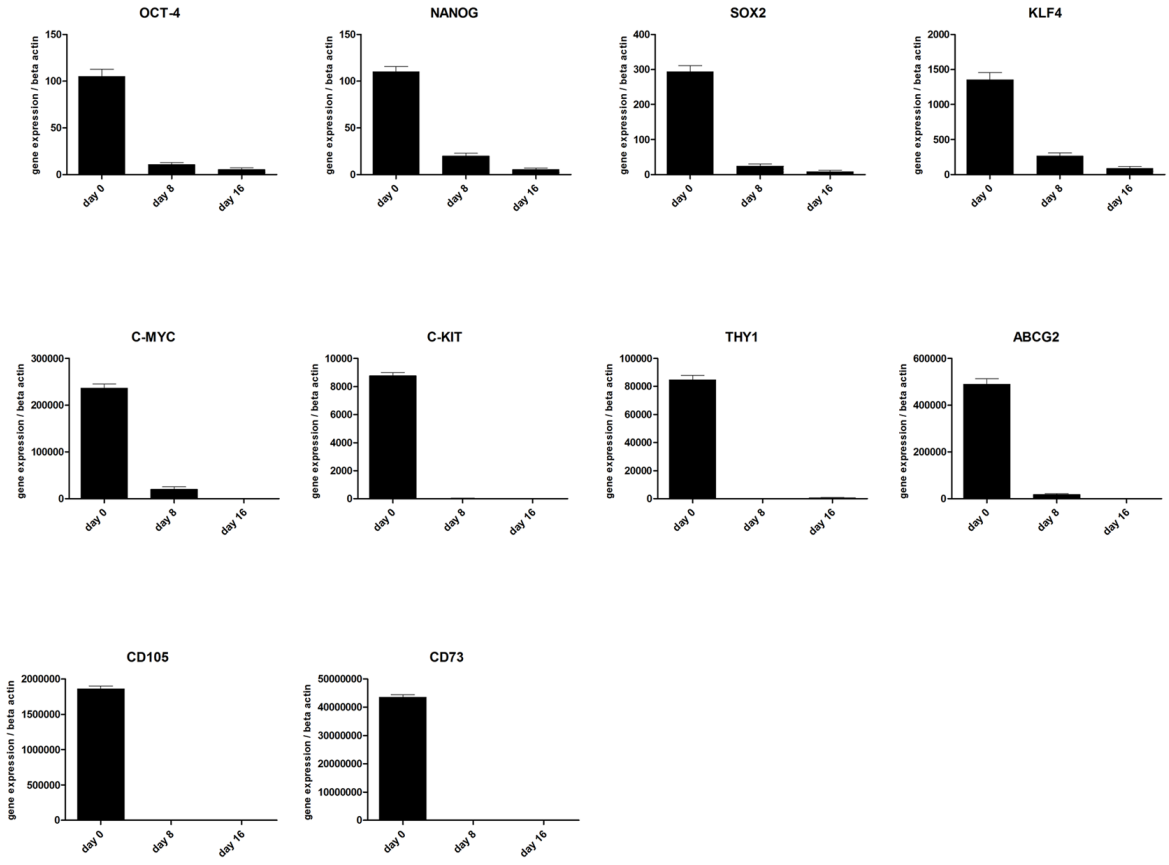


Figure 7. Decrease of stem cell markers during pancreatic differentiation. *The expression of stem cell markers is almost negligible already at day 8.*

At **stage 4** (days 13-15), the islet-like clusters increased in number and size (**Figure 8A**). In addition, cells double stained for C-PEP/PROINS and INS, suggesting further maturation. Interestingly, in bigger islet-like clusters staining for proinsulin was more marked in the cells of the inner core, while outer cells stained strongly for mature insulin (**Figure 8B**). The glucose transporter GLUT2, which is part of the "glucose-sensor" along with GCK, showed a similar localization (**Figure 8C**). By contrast, smaller islet-like cluster had a more homogeneous C-PEP/PROINS and INS

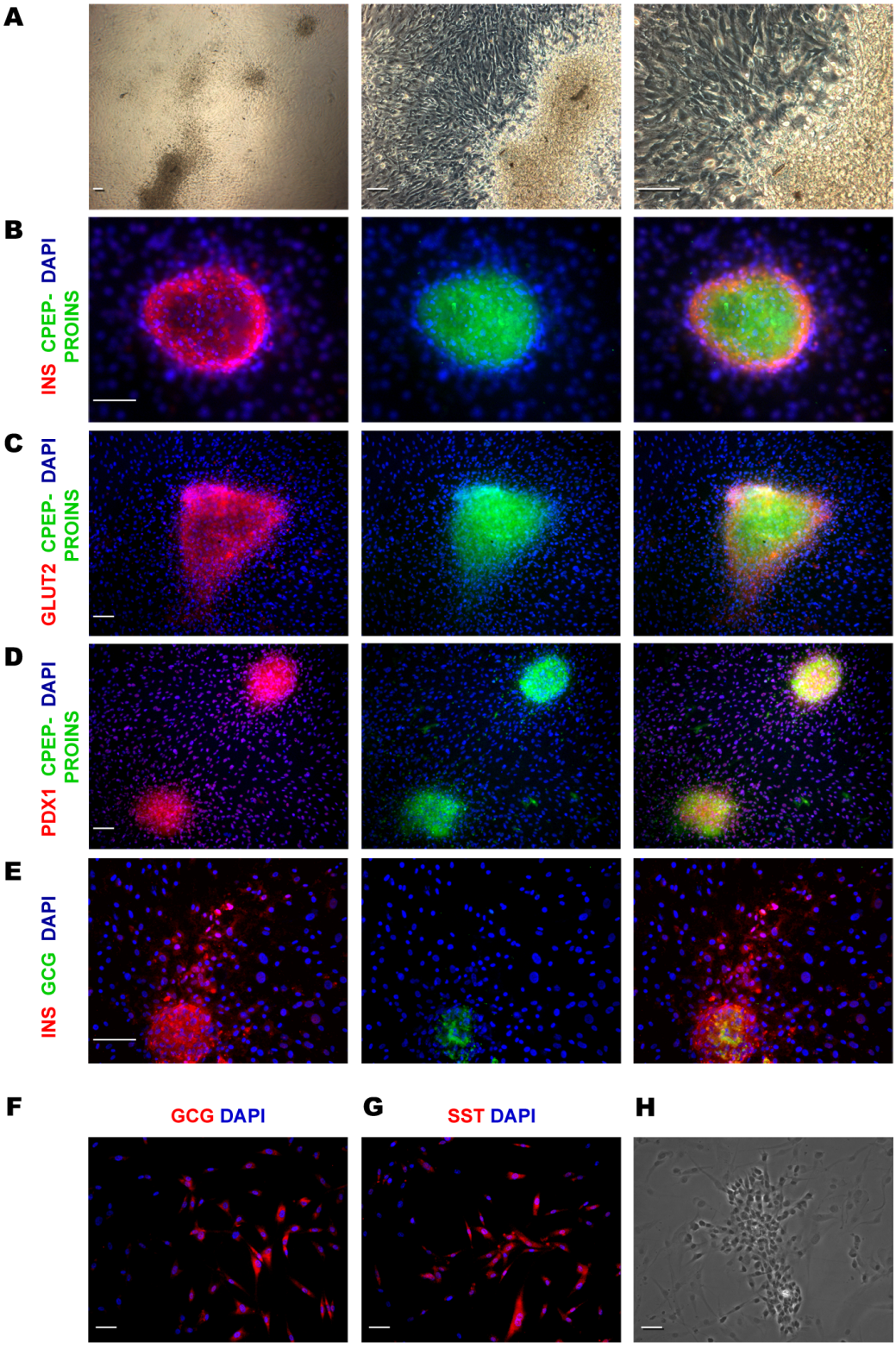


Figure 8. Differentiation into fully competent hormone-producing cells. (A) *Morphological appearance of end-stage islet-like clusters.* ***(B)*** *Double staining for C-PEP/PROINS and INS indicates that the hormone is efficiently processed. In these bigger islet-like clusters C-PEP/PROINS is preferentially located in the inner core, while outer cells stain strongly for mature INS. The increased of the aggregates size may hinder or impair uniform exposure of inner cells to medium and factors. (**C**) The glucose transporter GLUT2 shows a pattern similar to INS staining. (**D**) PDX1 is expressed in both the monolayer and the islet-like clusters, where colocalizes with C-PEP/PROINS. (**E**) An islet-like structure with a few cells of the inner core staining for GCG. Note that small islet-like cluster shown in (D) and (E) display homogeneous hormone distribution. (**F**) Rare single GCG^{+} and SST^{+}* ***(G)*** *cells were randomly found in the monolayer.* ***(H)*** *Phase contrast micrograph of a 2-D aggregate of cells. Scale bars 200 µ in (A)-(E) and 100 µm in (F-H). All fields are representative of at least five independent experiments.*

distribution (**Figures 8D and 8E**). In some islet-like clusters a few cells of the inner core also expressed GCG (Figure 8E). Rare single SST$^+$ and GCG$^+$ cells were randomly found in the monolayer (**Figures 8F and 8G**). Confocal image of an islet-like cluster is shown in **Figure 9A**. At this stage, almost all C-PEP/PROINS$^+$ cells also expressed MAFA and PDX1 (**Figure 9B**). Western blot analysis expression confirmed protein expression of GLUT2, PDX1 and GCK (**Figure 9C**).

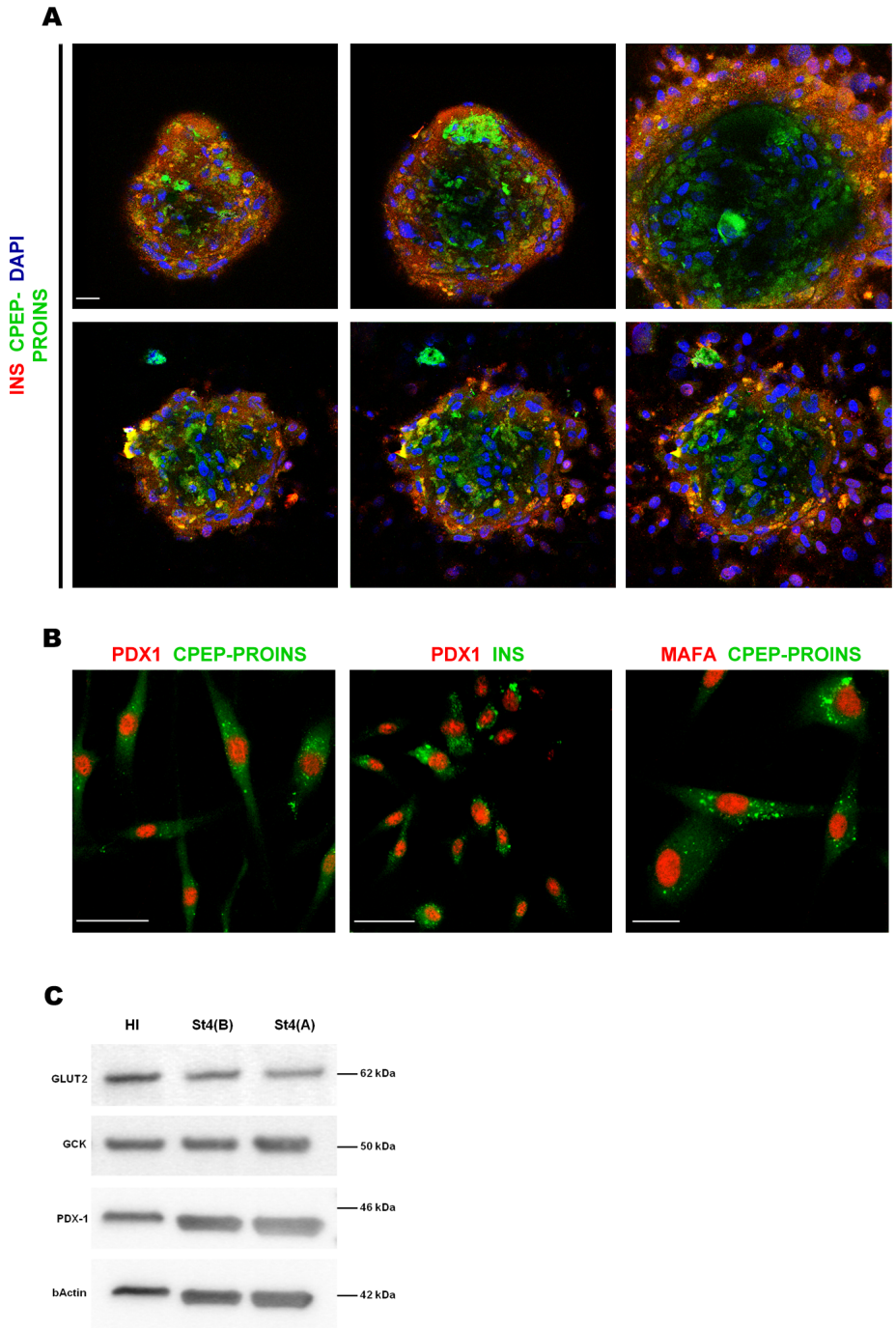


Figure 9. Additional characterization of differentiated f-LSCs. (A) *three micrographs taken from Z-stacks of an islet-like cluster stained for C-PEP/PROINS and INS. (**B**) At stage 4 almost all C-PEP/PROINS$^+$ or INS$^+$ cells co-express MAFA and PDX1 (confocal micrographs). C-PEP and INS stainings show a granulated pattern, consistently with presence of secretory granules. Scale bars 50 µ in left and middle micrographs; 25 µ in right micrograph. (**C**) Western blot analysis for islet markers GLUT2, GCK and PDX1 at stage 4 (St4). (St4(A)) indicates differentiation with Protocol A and (St4(B)) indicates Protocol B. 3 µg or 15 µg of protein lysates were loaded for human islets (HI) and differentiated f-LSCs, respectively.*

No substantial differences were observed between protocol A and Protocol B in inducing the expression of the majority of islet and β-cell specific transcription factors. However, concerning hormone production, protocol A was more efficient in inducing formation of islet-like clusters. Minor differences were observed in the differentiation outcome among the primary cell cultures used.

Quantification of differentiated f-LSCs

To quantify hormone-expressing cells, we performed flow cytometry after completing differentiation at stage 4 (**Figure 11A**). Analysis showed an average of 72.1 ± 5.3% positive cells for C-PEP/PROINS, with higher rates obtained with Protocol A. GCG^+ and SST^+ cells were 10.6 ± 2.4% and 8.2 ± 2.6%, respectively. qRT-PCR analysis showed higher *INS* expression in the islet-like clusters in comparison to monolayer cells (data not shown).

Differentiated f-LSCs possess secretory granules

To confirm the degree of maturation of hormone-expressing cells, we investigated whether they formed secretory granules. Confocal microscopy of C-PEP and INS stained cells showed a granulated pattern of the cytoplasm, consistent with the existence of secretory granules (**Figure 11B**) (D'Amour et al., 2005). Several INS^+ cells co-expressed the vesicle protein synaptophysin (SYP), and flow cytometry analysis indicated an average percentage of co-expression of 69.0 ± 3.9% (**Figure 11B**). Electron microscopy also revealed secretory granules in the cytoplasm and next to the cell membrane. Some of them showed a clear halo surrounding a less dense core, a morphology that is characteristic of insulin-containing vesicles (**Figure 11C**).

Insulin content and insulin release from differentiated f-LSCs

To confirm *de novo* synthesis and release of insulin, we investigated the ability to secrete C-peptide in response to secretory stimuli (**Figure 11**) (Rajagopal et al., 2003). Stimulation with 20 mM glucose, 100 μM of the secretagogue tolbutamide or direct depolarization with 30 mM potassium chloride (KCl) resulted in a robust C-peptide secretion in the culture medium during 2-h static incubation. All of the three stimuli showed rapid kinetics with a peak of secretion within 5 minutes. A biphasic profile was observed after glucose stimulation with a second phase of secretion around 10 minutes from the beginning of incubation. Fold stimulation of C-peptide release over the respective basal condition during 1-hr static incubation resulted in 4- to 6-fold increase after glucose stimulation, 5- to 11-fold increase after tolbutamide, and 8- to 12-fold after KCl (**Figure 11E**). C-peptide secretion after 1-hr static incubation was 15 to 28-fold less than that observed for adult human islets incubated in the same conditions (**Figure 11F**). To accurately evaluate the ability to process insulin, we measured both C-peptide and proinsulin by ELISA on cell lysates. Total insulin content in differentiated f-LSCs was about 160-fold lower than in adult human islets. However, the average

proportion of total insulin content attributable to C-peptide in differentiated f-LSCs was comparable to that of human islets (~98%; range 97-99.5%) (**Figure 11G**). Morphology of an islet-like cluster after 1-hr static incubation with 20 mM glucose is shown in Figure **11H**.

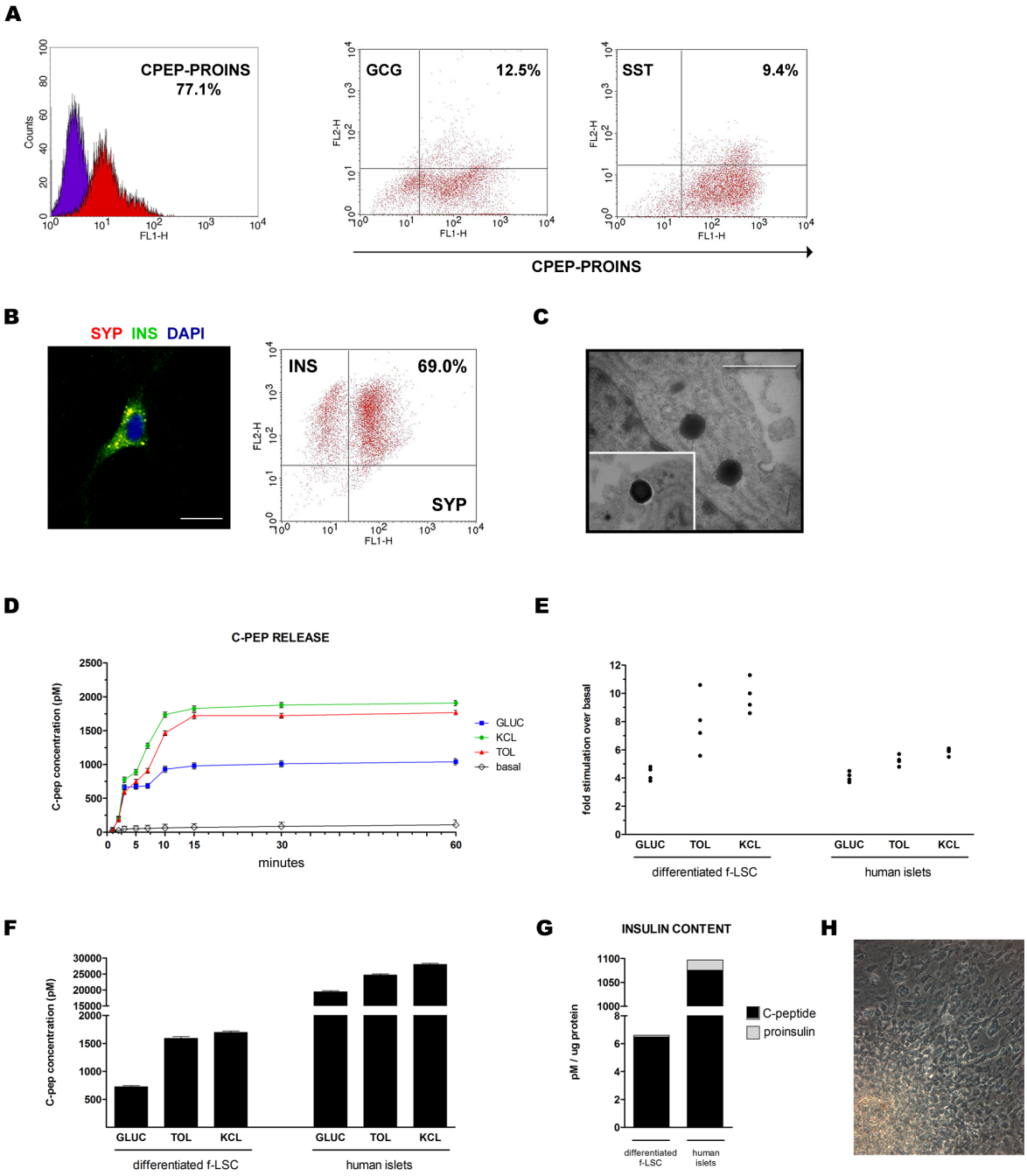


Figure 11. (A-C) Quantification of differentiated f-LSCs at the end of stage 4. (A) *Flow cytometry shows up to 77% cells expressing C-PEP/PROINS. GCG and SST co-expressed C-PEP/PROINS, and at a much lower levels.* ***(B)*** *Left: confocal micrograph of one INS$^+$ cell co-staining with synaptophysin (SYP), indicating the hormone is stored in granules. Right: Flow cytometry shows that ~70% of cells co-express SYP and INS.* ***(C)*** *Transmission Electron Microscopy shows presence of secretory granules. Insert shows a granule with a clear halo surrounding a less dense core, a morphology that is characteristic of insulin-containing vesicles. Scale bar, 1 µ.* ***(D-E) Functional assessment of differentiated f-LSCs at the end of stage 4. (D)*** *Stimulation with 20 mM glucose (GLUC), 100 µM of the secretagogue tolbutamide (TOL) or direct depolarization with 30 mM potassium chloride (KCl) during 2-h static incubation of both monolayer and islet-like clusters. All of the three stimuli show rapid kinetics with a peak of secretion within 5 minutes. A biphasic profile can be observed after glucose stimulation with a second phase around 10 minutes from the beginning of incubation. Basal secretion is C-peptide release during 1 h incubation in KRBH containing 2 mM D-glucose.* ***(E)*** *Fold stimulation of C-peptide*

*release over the respective basal condition during 1-hr static incubation. Values were calculated for each culture condition by dividing the C-peptide concentration in the stimulation supernatant at 60' by the C-peptide concentration in the basal supernatant. (**F**) C-peptide secretion after 1-hr static incubation compared to isolated adult human islets. (**G**) Total insulin content (sum of C-peptide + proinsulin) in differentiated f-LSCs and isolated adult human islets/µg total protein. Total insulin content in differentiated f-LSCs is about 160-fold lower than in adult human islets. However, the average proportion of total insulin content attributable to C-peptide in differentiated f-LSCs is ~98%, comparable to that of human islets. 500,000 differentiated f-LSCs and an equal mass of islet equivalents (IE) (~250 islets with an estimate of 2,000 cells per IE) were used for the experiments shown in (A-G). (**H**) Morphology of an islet-like cluster after 1-hr static stimulation with 20 mM glucose. D-E: data represents mean ± SE of 5 experiments.*

Positive controls for antibodies used throughout differention were tested on β-TC3 cell line and are shown in **Figure 10**.

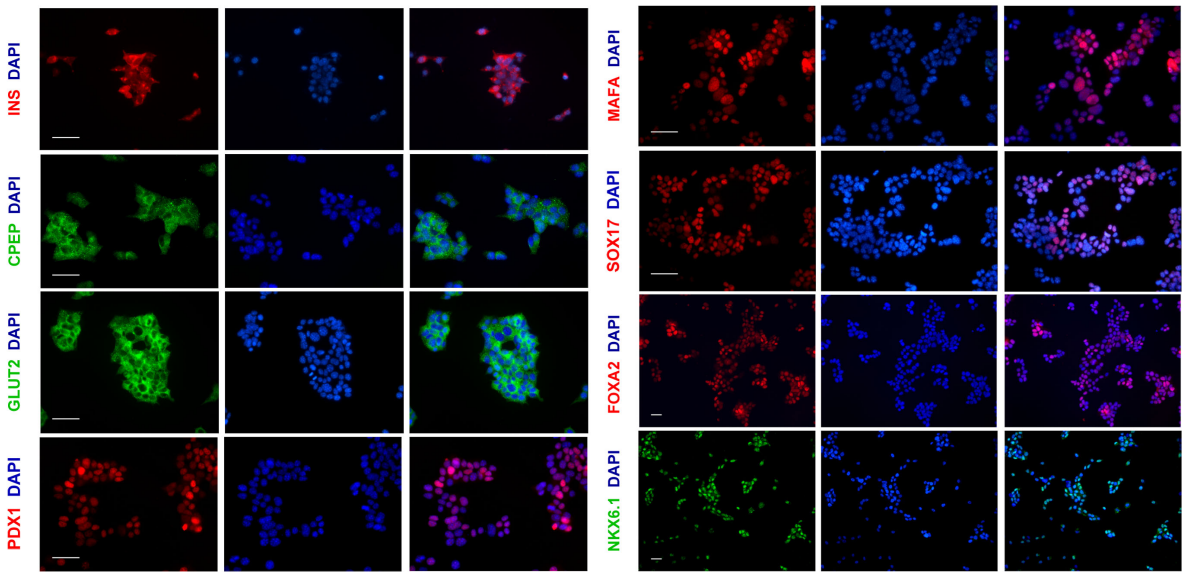


Figure 10. *Positive controls of antibodies on β-TC3 cell line. Scale bars 100 µm.*

DISCUSSION

Cultured human limbal epithelial stem cells have been successfully used for corneal reconstruction (Dua et al., 2000; Kheirkhah et al., 2008; Shortt et al., 2008). However, human ocular stem cell research has been mainly focused on the tissue-specific differentiation that may be of clinical significance in the context of eye diseases. Here we show how the limbus hosts a fibroblast-like stem cell population that could be harvested for clinical use in type 1 diabetes. The phenotypic characterization of f-LSCs has been variably described (Dravida et al., 2005; Du et al., 2005; Polisetty et al., 2007). We have identified a core set of attributes that uniquely characterizes f-LSCs, such as the expression of the well-established stem cell surface antigens SSEA4, TRA 1-60, TRA 1-81, THY1, c-KIT, CD105, CD73 and the limbal stem cell marker ABCG2. f-LSCs also expressed several nuclear transcription factors, such as OCT4, NANOG, SOX2, KLF-4 and c-MYC, which are involved in self-renewal and maintenance of pluripotency of both embryonic and adult stem cells (Takahashi et al., 2007; Yu et al., 2007). The LESC marker ΔNp63 was negative, confirming the non-epithelial nature of f-LSCs, while the absence of CD34 and CD45 argues against a haematopoietic origin. f-LSCs did not express HLA-DR histocompatibility molecules on their surface. This feature could be advantageous in the transplantation setting as it might confer reduced immunogenicity. In addition, the ability to maintain an undifferentiated status and normal karyotype during long-term *in vitro* culture is another critical advantage because expansion of the stem cell pool before differentiation is required to eventually obtain a large number of transplantable surrogate β-cells. We also found that cultured f-LSCs are able to form non-adherent spherical clusters, or limbospheres. The generation of spheres has been first observed for progenitors/stem cells belonging to the central nervous system and neural crest-derived tissue (Reynolds et al., 1992), and culture conditions developed for their formation are known to enrich stem-like cells with self-renewal ability and differentiation potential. The self-renewal assays provided the proof of principle of the stem properties of f-LSCs.

Differentiation of f-LSCs into a surrogate β-cell type was achieved using factors and supplements, which have been described to direct and/or sustain differentiation of other stem cells towards the pancreatic phenotype (Lumelski et al., 2001; D'Amour et al., 2005; D'Amour et al., 2006; Jiang et al., 2007; Shim et al., 2007; Iwamuro et al., 2010). Different concentrations, times of administration and lengths of application were tested in order to find the optimal conditions to induce β-cell differentiation through progressive stages of commitment that mimics normal pancreatic development. The expression of specific endodermal, pancreatic, islet and β-cell markers, as well as functional properties of f-LSC-derived insulin-producing cells, were assessed during differentiation.

With our four-step approach, we first focused on obtaining DE and PF (stages 1-2). The serum-free conditions, combined with the addition of Activin A and its subsequent removal, were critical in blocking f-LSC self-renewal and in inducing mesendoderm differentiation (D'Amour et al., 2005; D'Amour et al., 2006; Chng et al., 2010). Remarkably, cells started gathering in islet-like clusters, which progressively increased in size during differentiation. At **stage 1**, transition to DE was demonstrated by the appearance of SOX17 (Séguin et al., 2008; Spence et al., 2009) and FOXA2 (Lee et al., 2005). Of note, both transcription factors were persistently expressed up to stage 4, albeit at lower levels. This finding is obvious for *FOXA2*, being also a well-recognized pancreatic and β-cell nuclear transcription factor. SOX17 is expressed during human fetal pancreas development, when its transcription is significantly higher than in adult human islets (Suarez-Pinzon et al., 2005). The persistence of *SOX17* mRNA expression thus may be explained by the arrest of a certain number of cells at DE/PF stages or may mirror its physiological post-natal expression. Notably, at stage 1 PDX1 staining was already detectable. PDX1 is a master regulator of pancreas development and specification. During embryogenesis, PDX1 marks the pre-pancreatic endoderm before it has visibly thickened (Murtaugh, 2007). Experiments of lineage tracing showed that at this stage $PDX1^+$ cells represent progenitors of all the mature pancreatic cell types, including duct, islet and acinar cells (Gu et al., 2002). *PDX1* is subsequently silenced to eventually appear in the mature β-cell as a regulator of insulin gene transcription. The expression of *PDX1* mRNA we observed during differentiation seems to mirror this biphasic pattern.

At **stage 2**, we further induced transition towards posterior foregut and pancreatic endoderm. A considerable upregulation of PDX1 at both mRNA and protein levels was observed, therefore confirming that the majority of f-LSCs (both in the monolayer and in the forming clusters) differentiated into pancreatic precursors. In particular, many $PDX1^+$ cells also co-expressed NKX6.1, which suggests transition towards the pancreatic epithelium. Simultaneously, NGN3 became also detectable, indicating initial commitment to the endocrine lineage. NGN3 is transiently expressed during endocrine specification and is responsible of the induction of a battery of transcription factors that constitute a 'core program' of endocrine development (Gradwohl et al., 2000). They include *ISL1*, *NEUROD1*, *NKX6.1* and *PAX4* genes, which are also known to act relatively late in beta cell specification (Murtaugh). These factors were indeed detected already at the end of stage 2.

At the end of stage 2, we also observed upregulation of *GLUT2* and *GCK*, two genes which play a crucial role in maintaining blood glucose homeostasis, forming the so-called "β-cell glucose sensor" (Im et al., 2006). The early upregulation of GLUT2, in absence of insulin detection, recalls the embryonic development phases when GLUT2 is expressed temporarily in pancreatic non-β-cells, likely acting as a signal for further development (Herrera, 2000). The same finding has been described in GCG^+ cells during attempt of regeneration in STZ-diabetic non-human primates (Bottino et al., 2009).

During **stage 3**, maturation of pancreatic progenitors into hormone-producing cells and in particular β-cells, was allowed. Consistent with this observation, we observed remarkable up-regulation of *MAFA*. This finding is extremely indicative, as no other characterized islet-enriched transcriptional factor is expressed exclusively in β-cells, where it functions as a potent activator of insulin gene transcription together with PDX1 and NEUROD1 (Matsuoka et al., 2004; Wang et al., 2007). NGN3 mRNA and protein were undetectable in stage 3, suggesting that its expression is extinguished before the final differentiation of the hormone-producing cells. At this early stage of hormone maturation, however C-peptide did not fill the cytoplasm, being stored in the apical pole of the cells.

Differentiation into fully competent hormone-producing cells was completed by the end of **stage 4**, when the GLP-1 analog Exendin-4 was added. Exendin-4 has been shown to increase β-cell replication, neogenesis and expansion of the β-cell mass both *in vitro* and *in vivo* (Xu et al., 1999; Suarez-Pinzon et al., 2005; Suarez-Pinzon et al., 2008a and 2008b). At this stage, C-PEP/PROINS and INS co-localized in immunofluorescence, indicating acquisition of a more mature phenotype. Indeed, the efficient conversion of proinsulin to insulin requires cleavages at both junctions of the connecting segment linking the B and A chains to release insulin and C-peptide. These products normally are stored within the mature secretory granules. As the C-PEP/PROINS antibody used reacts specifically with C-peptide and does not cross-react with insulin or other peptide hormones, the double staining for both C-peptide and insulin demonstrates that the hormone was efficiently processed. In addition, sustained protein synthesis was suggested by the observation that the hormone extensively filled and delineated the cytoplasm. At this stage the islet-like clusters increased consistently in number and size. Notably, in bigger islet-like clusters C-PEP/PROINS was more marked in the cells of the inner core, while INS and GLUT2 staining was stronger in outer cells. By contrast, smaller islet-like clusters showed a homogeneous insulin distribution. We thus believe that the increased size of the aggregates may hinder or impair uniform exposure of inner cells to medium and factors, leading to accumulation of proinsulin due to decreased cleavage of the pro-hormone. The granulated pattern observed by confocal microscopy suggested granules were docked in the cytoplasm of hormone-producing cells. In addition, INS/SYP co-staining suggested the hormone was incorporated, at least transiently, in secretory granules. This was also confirmed by electron microscopy, which revealed granules in the cytoplasm and next to the cell membrane. Co-staining of C-PEP with INS, MAFA, PDX1 and GLUT2 at day 15 confirmed maturation of cells.

Assessment by flow cytometry at the end of differentiation showed that ~70% of the cells (and as many as 77%) eventually differentiated into cells expressing insulin (assessed as C-peptide). These differentiation rates are about 10-fold higher than earlier *in vitro* studies (D'Amour et al., 2006; Jiang et al., 2007). However, insulin gene transcription was about 20-fold lower compared to isolated human islets, suggesting that hormone production *per cell* is lower than in adult human

beta cells. This observation is further supported by the finding that insulin content/μg total protein was about 160-fold lower than what observed in adult islets. Interestingly, differentiated cells processed ~98% of their proinsulin content, which is comparable to what observed in mature β-cells. In addition, analysis of the secretory function in response to glucose, tolbutamide and KCl, confirmed that differentiated f-LSCs are able to respond to different stimuli in a regulated manner by secreting mature insulin (assessed by C-peptide production). The observed biphasic kinetics of stimulated C-peptide secretion suggests that f-LSC-derived INS^+ cells possess two different pools of granules. In particular, the rapid kinetics of the first phase (peaking within 5 minutes in all conditions) indicates the existence of pre-formed vesicles that are promptly secreted, while the sustained secretion in the second phase suggests presence of a slowly releasable pool. Notably, this biphasic time course can be observed in perifused islets (Henquin et al., 2002). By contrast, the rapid increase observed after incubation with KCl and tolbutamide was probably due to the continuous and strong membrane depolarization induced by these two stimuli. These findings collectively suggest that insulin positive cells have developed the secretory machinery that is typical of β-cells. Flow cytometry also showed that a few insulin-positive cells co-expressed glucagon (~10%) and somatostatin (~8%), a feature of newly forming endocrine cells. Indeed few insulin-positive islet-like clusters showed glucagon staining in the inner core. Polyhormonal cells have been described in both rodents and humans during the primary transition stage of early fetal development (Teitelman et al., 1993; Polak et al., 2000). Thus hormone co-expression in a subpopulation of our cells may indicate the persistence of immature cells. Alternatively, polyhormonal cells may result from partial failure of our differentiation protocol to maintain the appropriate transcription factor code.

CONCLUSIONS ON PROJECT 1

The purpose of our study was to identify a novel population of uncommitted cells, which could offer advantages over the countless stem cell sources proposed so far for β-cell replacement therapy. In our study we have identified a core set of attributes that uniquely characterizes f-LSCs, such as the expression of well-established stem cell surface antigens and self-renewal ability. Interestingly, f-LSCs do not express HLA-DR histocompatibility molecules on their surface, a feature that could be advantageous in the transplantation setting. The ability to maintain an undifferentiated status and normal karyotype during long-term *in vitro* culture is another critical advantage as a large number of cells is required for transplantation. *In vitro* differentiation of f-LSCs into fully competent hormone-producing cells was successfully achieved with a four-step protocol, suggesting that f-LSCs could be used a valuable source of adult stem cells for β-cell replacement therapy. In particular, with our stage-specific approach, up to 77% of f-LSCs eventually differentiated into cells expressing insulin (also assessed as C-peptide) and exhibited phenotypic features of mature beta cells, such as expression of critical transcription factors and presence of secretory granules. Although insulin content was about 160-fold lower than what observed in adult islets, differentiated cells processed ~98% of their proinsulin content, similar to mature beta cells. Moreover, they responded *in vitro* in a regulated manner to multiple secretory stimuli, including glucose. Future studies are needed to improve the amount of functional surrogate β-cells and ultimately assess their therapeutic potential after transplantation for the reversal of diabetes.

PROJECT 2:

DUCT CELLS CONTRIBUTE TO REGENERATION OF ENDOCRINE AND ACINAR CELLS FOLLOWING PANCREATIC DAMAGE IN ADULT MICE

BACKGROUND AND AIMS

Unraveling the mechanisms involved in the maintenance of the pancreatic cell mass in both physiologic and pathologic conditions has important clinical repercussions. Different models have been used to address the question of whether the pancreas possesses the ability to regenerate after injury, and several "cells of origin" have been proposed to account for this process (Puri et al., 2010; Bonner-Weir S, et al., 2010). Variation between different studies is likely due to the disparities among the animal species used, as well as the type and extent of injury, which may affect pancreatic cells in different ways. A solution could be cell type–specific ablation, which may be a better method for analyzing the in vivo function of cells during regeneration. This type of injury can be achieved by using streptozotocin (for β-cells) or cerulein (for acinar cells). However, the massive inflammatory response, often accompanying some of the injury models, may act as a confounding factor.

Several studies have suggested that, under physiologic conditions, adult β-cells predominantly arise from already existing β-cells via replication, implying that regeneration might not involve specialized progenitors (Dor et al., 2004; Teta et al., 2007; Brennand et al., 2007). However, during pancreatic organogenesis, progenitors within the pancreatic ductal epithelium give rise to both endocrine and acinar cells (Gittes, 2009). Therefore, it seems reasonable to assume that the regeneration process in the adult pancreas following injury would involve ductal cells (Inada et al., 2008; Puri et al., 2010; Bonner-Weir S, et al., 2010). In addition, a recent report has argued for the existence of a rare multipotent subpopulation of insulin-expressing cells within the adult human and mouse islets with the capacity to give rise to neuronal and pancreatic cell type lineages (Smukler et al., 2011). These results strongly suggest that while maintenance of the β-cell mass during adult life may primarily involve cell replication, following insult the adult mouse pancreas is capable of robust regeneration by the recruitment of different cell types, including non-β-cells. In particular, the mechanisms involved in β-cell regeneration seem to be determined by the nature and the extent of tissue damage, which apparently dictates whether new β-cells may be generated by replication of existing β-cells, differentiation of progenitor cells residing within or in proximity to ducts (Inada et al., 2008; Xu et al., 2008; Collombat et al., 2009) or, as recently reported, from transdifferentiation of α-cells (Nir et al., 2007; Collombat et al., 2009).

The regeneration of exocrine pancreas has also been reported following several injury models, including partial pancreatectomy and cerulein treatment. Lineage tracing studies indicated that the recovery of acinar mass in these models occurs not via differentiation of new acini from ductal or other progenitors, but by expansion of those acinar cells that survived injury (Jensen et al., 2005; Desai et al., 2007; Fendrich et al., 2008; Morris et al., 2010).

In an effort to determine a potential link between the extent and type of tissue damage and the nature of the regenerative response, we have studied pancreatic regeneration following ablation of different pancreatic cell lineages using the diphtheria toxin receptor (DTR)-mediated conditional targeted cell ablation model. The DTR is a membrane-anchored form of the heparin-binding epidermal growth factor–like precursor (HBEGF precursor) (Naglich et al., 1992). While the human and simian HBEGF precursors function as receptors for diphtheria toxin (DT), the HB-EGF from mice and rats does not bind the toxin and therefore remains insensitive to DT (Mitamura et al., 1995). Thus, transgenic expression of simian or human DTR in mice renders DT sensitive the otherwise naturally DT-resistant mouse cells (Saito et al., 2001; Jung et al., 2002; Cha et al., 2003). Recently, a mouse strain was generated ($R26^{DTR}$) in which a loxP-flanked STOP cassette and the open reading frame of simian DTR has been introduced into the ROSA26 locus (Buch et al., 2005). In the $R26^{DTR}$ strain, the gene encoding DTR is under the control of the Rosa promoter, but its expression is dependent on Cre-recombinase removal of the STOP cassette; hence, only Cre-expressing cells and their progeny will transcribe DTR. Although viable and normally functioning, the DTR-expressing cells are rapidly killed up on DT administration, allowing control over the extent of injury based on the targeted cells.

In our PdxCre;$R26^{DTR}$ transgenic line, all pancreatic epithelial cells are killed via DT administration with the exception of ductal cells, which are serendipitously spared. This represents the most severe (but still survivable) pancreatic injury model of regeneration yet reported. We show that following extensive ablation of both acinar and endocrine tissue, surviving cells within the ductal compartment contribute to regeneration of new endocrine and acinar populations through a process that seems to recapitulate the embryonic pancreatic developmental program. By contrast, following selective ablation of only acinar cells (thus in a less severe type of damage) in the ElacreERT2;$R26^{DTR}$ transgenic line, regeneration occurs through direct differentiation of ductal cells to acinar lineage.

MATERIALS AND METHODS

Mice

Mice used in this study were maintained according to protocols approved by the University of Pittsburgh IACUC. The Rosa26^{DTR} (Buch et al., 2005) and ElaCreERT2 (Ji et al., 2008) strains were generated in the laboratories of Dr. Ari Waisman (Johannes-Gutenberg-University, Mainz, Germany) and Craig Logsdon (University of Texas MD Anderson Cancer Center), respectively, whereas the PdxCre mice (Wells et al., 2007) were obtained from the Mouse Models of Human Cancer Consortium. The Rosa26^{lacZ} mice were purchased from The Jackson Laboratories.

Diphtheria Toxin (DT) treatment

To induce the human Diphteria Toxin Receptor (DTR) expression globally in the pancreas, we crossed R26^{DTR} or R26$^{DTR/lacZ}$ with PdxCre mice. Eight week-old mice were injected i.p. daily for 5 days with 0.5 ng/g body weight of DT (Sigma), and sacrificed at different time points, up to 45 days after DT-administration **(Figure 1A)**. Throughout this report, days 1-7, 8-25 and 26-45 after last DT-injections are referred to as early-, mid-, and late-stages respectively, because of variable progression of the phenotype after ablation. To allow DTR expression selectively in acinar cells, we crossed R26^{DTR} or R26$^{DTR/lacZ}$ with ElaCreERT2 mice. Eight-week-old mice were first injected i.p. with tamoxifen for 5 days to activate Cre. After a 7-day wait to allow for Cre-recombinase action, 0.5 ng/g body wt DT injections were performed for 5 consecutive days. Mice were killed 1, 3, 5, 7, or 8 days after DT administration (**Figure 1B**).

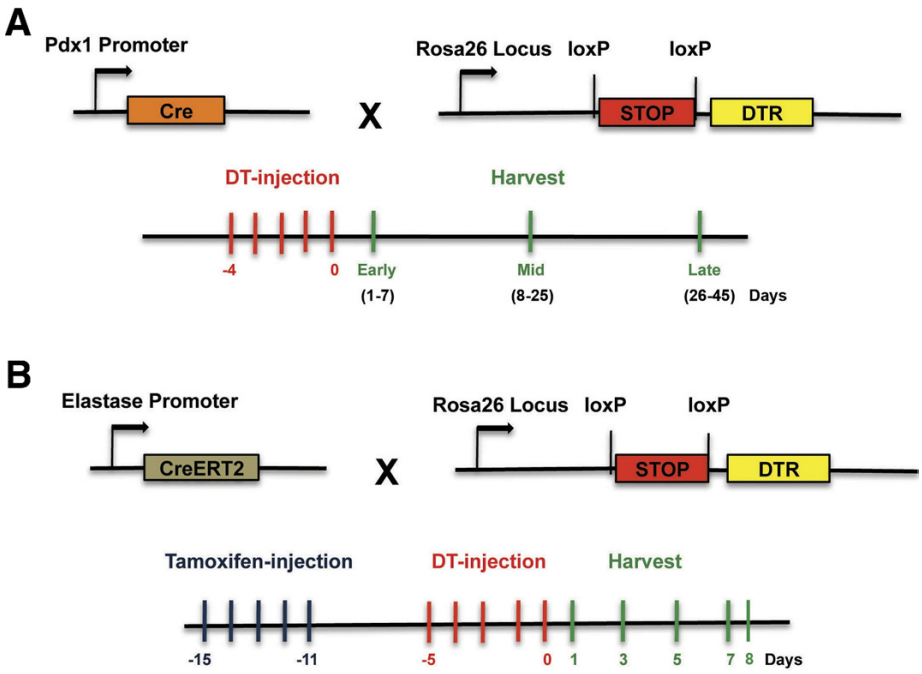


Figure 1. *Experimental design of the (**A**) PdxCre;R26^{DTR} and (**B**) ElaCreERT;R26^{DTR} transgenic mouse model.*

Tamoxifen Treatment

Tamoxifen (Sigma-Aldrich) was dissolved in 100% ethanol at 100 mg/mL and subsequently resuspended in corn oil (Sigma-Aldrich) at a concentration of 10 mg/mL. Six-week-old mice were injected intraperitoneally with 2 mg tamoxifen for 5 consecutive days, for a total of 10 mg.

Immunofluorescence and Quantification Analysis

For immunolabeling on cryopreserved sections, harvested pancreata were fixed overnight at 4°C in 4% paraformaldehyde, incubated overnight at 4°C in 30% sucrose solution and subsequently embedded with OCT compound. Cryosections (5-6 µm) were collected serially so that each slide would contain semi-adjacent sections across the entire tissue. Sections were permeabilized with 0.1% PBS/Triton X-100, washed in PBS and blocked for 30 minutes in 10% normal donkey serum in 0.1% PBS/Tween. For BrdU staining, slides were pre-treated with 2M HCl for 30 minutes at room temperature prior to permeabilization and blocking. For CK19 staining, tissues were snap-frozen in OCT and sections were subsequently fixed in 4% paraformaldehyde at room temperature for 10 minutes prior to permeabilization and blocking. Primary antibodies were incubated overnight at 4°C, while secondary antibodies were incubated for one hour at room temperature. Images were acquired on a Zeiss Imager Z1 microscope with a Zeiss AxioCam driven by Zeiss AxioVision Rel.4.7 software. For quantification analysis, marker-positive cells were counted using ImageJ software. Primary antibodies are listed in **Table 1.**

ANTIGEN	SPECIES	COMPANY	CAT. NO.	DILUTION
E-cadherin	Goat	R&D	AF748	1:200
E-cadherin	Rat	Invitrogen	13–1900	1:200
panCK	Rabbit	DAKO	Z0622	1:100
CK19	Rat	DSHB	TROMA III	1:100
CD31	Rat	BD	BD550274	1:50
Insulin	Guinea pig	Linco/Millipore	4011–01	1:1000
Insulin	Mouse	Abcam	Ab8305	1:500
Glucagon	Guinea pig	Linco/Millipore	4031–01F	1:1000
Glucagon	Rabbit	Linco/Millipore	4030–01F	1:2000
Pdx-1	Rabbit	Abcam	AB47267	1:2000
Pdx-1	Goat	Abcam	AB47383	1:10,000
Sox9	Rabbit	Millipore	AB5535	1:1000
Ngn3	Guinea pig	gift	M. Sander	1:500
Amylase	Rabbit	Sigma	A8273	1:300
Amylase	Goat	Santa Cruz	Sc-12821	1:250
DBA	FITC-conj	Vector Lab	FL1031-2	1:100
BrdU	Rat	Abcam	AB6326	1:100
β-gal	Chicken	Abcam	9361	1:1000
HB-EGF	Goat	R&D	AF-259-NA	1:500

Table 1. *Sources of antibodies and dilutions used for immunofluorescence analysis.*

Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories: biotin-conjugated anti-rabbit, anti-rat, anti-guinea pig (all 1:500); biotin-conjugated anti-goat (1:250); Cy2-conjugated and Cy3-conjugated streptavidin (1:500); Cy5-conjugated streptavidin (1:100); and Cy2- and Cy3-conjugated donkey anti-guinea pig, anti-rabbit, anti-rat, anti-mouse, anti-goat (all 1:300).

Hematoxilin/Eosin staining

For H&E staining, tissues were fixed in 4% PFA, dehydrated in ethanol, and paraffin-embedded. Hematoxylin-Eosin staining was performed on 5 µn-thick sections according to standard protocol.

X-gal staining

For β-galactosidase staining (X-gal staining), tissues were fixed at room temperature for two hours in 2% paraformaldehyde, washed in PBS and Rinse Buffer (2 mM MgCl2, 0.01% Na-deoxycholate, 0.02% NP-40 in PBS), and then incubated overnight at 37°C with X-gal solution [1mg/ml X-gal (Research Products International Corp.), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ in Rinse Buffer]. Following washes in PBS, tissues were post-fixed in 4% paraformaldehyde at room temperature for 10 minutes, incubated in 30% sucrose solution overnight and subsequently embedded with OCT compound. Cryosections were prepared as described for immunolabeling and counterstained with hematoxylin.

BrdU-labeling (pulse-chase) and TUNEL Assay

BrdU-labeling was performed by injecting i.p. 1mg BrdU (Sigma) for two consecutive days prior to sacrifice. Alternatively, BrdU (0.8 g/L) was provided in drinking water for two days, with water changed daily. Apoptotic cells were recognized by TUNEL (terminal dUTP nick-end labeling) with a TUNEL Apoptosis Detection Kit (Millipore), following the manufacturer's instructions.

Real-time quantitative PCR (qRT-PCR)

After harvesting, tissues were preserved in RNAlater (Ambion) and stored until usage. mRNA isolation and subsequent cDNA synthesis were performed using µMACS® One-step cDNA Kit (Miltenyi Biotec) according to the manufacturer's instructions. PCR primers were purchased from Qiagen (QuantiTect® Primer Assays, Qiagen). Reactions were performed at least in triplicates with Quantitect Sybr Green PCR Kit (Qiagen) using a LightCycler 1.5 Instrument. Specificity of the amplified products was determined by melting peak analysis. Quantification for each gene of interest was performed with the $2^{-(delta)(delta)Ct}$ method. Quantified values were normalized against the housekeeping gene GAPDH, which proved to be stable across the samples.

GENE	QIAGEN QuantiTect PRIMER ASSAY	CAT. NUMBER
Notch1	Mm_Notch1_1_SG	QT00156982
Hes1	Mm_Hes1_1_SG	QT00313537
Mist1	Mm_Bhlha15_1_SG	QT00315182
Ptf1a	Mm_Ptf1a_1_SG	QT00124187
PDX1	Mm_Pdx1_1_SG	QT00102235
NGN3	Mm_Neurog3_1_SG	QT00262850
NeuroD	Mm_Neurod2_1_SG	QT00248892
MAFA	Mm_Mafa_2_SG	QT01037638
INS2	Mm_Ins2_1_SG	QT00114289
GCK	Mm_Gck_1_SG	QT00140007
GLUT2	Mm_Slc2a2_1_SG	QT00103537

Table 2. *List of Primers Used for qRT-PCR*

RESULTS

Cell ablation and regeneration of adult pancreatic endocrine and acinar cells in PdxCre;R26^{DTR} mice

Eight-week-old PdxCre;R26^{DTR} mice were injected with DT and sacrificed at different time points (**Figure 2A**). Within one week after the last DT injection (early stage), the pancreas had sustained a massive loss of the epithelial cell population, quantifiable in a 97% reduction of acinar tissue and a 96% reduction of insulin- or glucagon-expressing cells. Interestingly, ductal epithelial cells were serendipitously spared (**Figure 2A and B**). Immunostaining for insulin, glucagon, and amylase (**Figure 2C**) documented the initial loss and the subsequent recovery of the endocrine and acinar cells.

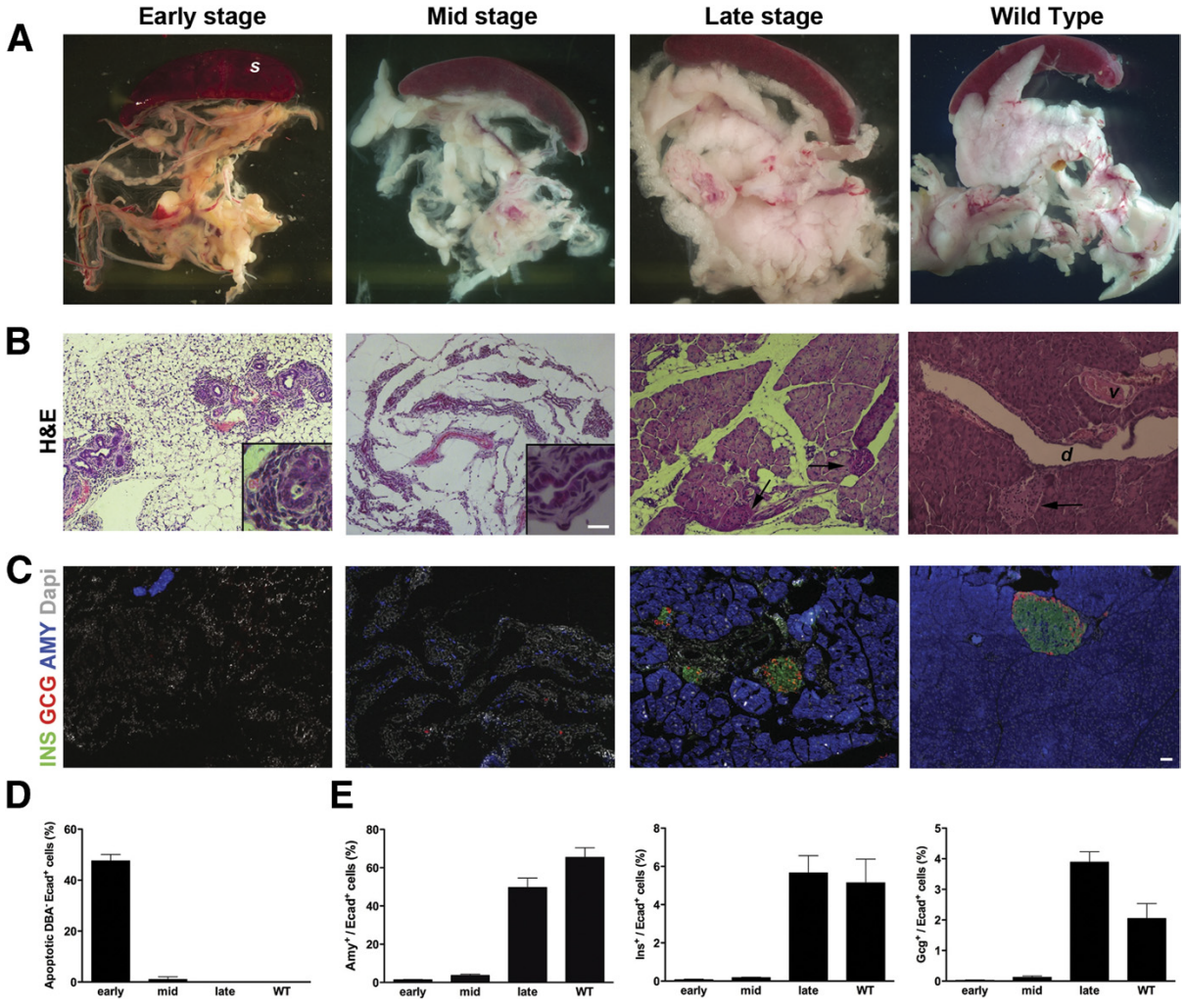


Figure 2. *Loss and regeneration of pancreatic mass in DT-treated PdxCre;R26DTR mice. After DT treatment, mice were killed at early, mid, or late stage.* ***(A)*** *Macroscopic appearance shows progressive rescue of pancreatic mass during regeneration.* ***(B)*** *Representative H&E staining of pancreata at the same time points. Notably, upon DT treatment, all epithelial cells were killed but ducts (insets). Arrows show islets.* ***(C)*** *Immunostaining for amylase, insulin, and glucagon confirmed the initial loss and subsequent regeneration of both exocrine and endocrine tissues. Wild type (WT) is an age-matched adult control pancreas.* ***(D)*** *Quantification of apoptotic cells (percent) among the nonductal epithelial (DBA$^-$/Ecad$^+$) population shows the massive cell loss after DT treatment.* ***(E)*** *Quantification (percent) of amylase$^+$, insulin$^+$, and glucagon$^+$ cells normalized by number of E-cadherin$^+$ cells. S, spleen; d, duct; v, vessel. Scale bars = 20 μm.*

TUNEL assay analysis showed that the majority of β-cells and the nonductal DBA$^-$/E-cadherin$^+$ epithelial cells surrounding the DBA$^+$ structures were apoptotic one day after the injections (**Figure 3A**). The apoptotic rate among the non-ductal epithelial cells was almost 50% in the early stage, significantly declined during the following days, and by day 10 it was less than 1% (**Figure 2D** and **Figure 3B**). The pancreas regained 60% of its original acinar mass and regenerated the endocrine compartment in 3 to 4 weeks (late stage; **Figure 2E**). Mice exhibited hyperglycemia during the regeneration process for up to 3 weeks (non-fasting blood glucose levels >600 mg/dL), after which blood glucose levels eventually declined to approximately basal values (~200 mg/dL) in 63% of mice. Notably, all mice killed at late time points showed recovered acinar tissue, and 80% showed islets characterized by MafA and Glut2 staining, suggesting the presence of functional β-cells. By contrast, 20% of late-stage pancreata exhibited only scattered small hormone$^+$ cell clusters with no β-cells expressing MafA or Glut2 (data not shown).

Survival of the Pancreatic Duct Cells in DT-Treated PdxCre;R26^{DTR} Mice

Based on the widespread positivity (85%–90% of total pancreatic cells) for X-gal staining in the PdxCreR26lacZ pancreas (**Figure 3C**), the apparent resistance of duct cells to DT was a surprising result. To ensure that survival of the duct cells was not due to lack of DTR expression, we stained wild-type and mid stage (post-DT treatment) PdxCre;R26^{DTR} pancreata with antibodies specifically recognizing DTR (simian HB-EGF) (**Figure 3D–F**). As shown in **Figure 3E**, DTR is clearly expressed in surviving DBA$^+$ duct-like structures, but for unknown reasons duct cells remain insensitive to DT.

Lineage-tracing analysis in the regenerating pancreas of PdxCre;R26$^{DTR/lacZ}$ mice

To study more rigorously the contribution of cells within the ductal compartment to the regeneration process, 8-week-old PdxCre;R26$^{DTR/lacZ}$ mice were treated with DT and then killed during early, mid or late stages after DT injection. Shortly after DT treatment, the only X-gal$^+$ structures detectable in the pancreas were the surviving duct cells (**Figure 4**), whereas during mid and late stages staining revealed extensive contribution of X-gal$^+$ cells to parenchymal restoration, including acinar and endocrine cells (**Figure 4A** and **4B**). In addition, no cells co-expressing β-galactosidase (β-gal) and insulin, or β-gal and amylase, could be found during early stage (**Figure 4C**), while β-gal$^+$ cells expressing insulin (**Figure 4D**) or amylase (**Figure 4E**) were detected during mid stage. Notably, our analyses also revealed that some segments within the ductal network did not express the Cre recombinase, and as a result ductal branches originating from those areas contained X-gal$^-$ cells (inset in **Figure 4A**).

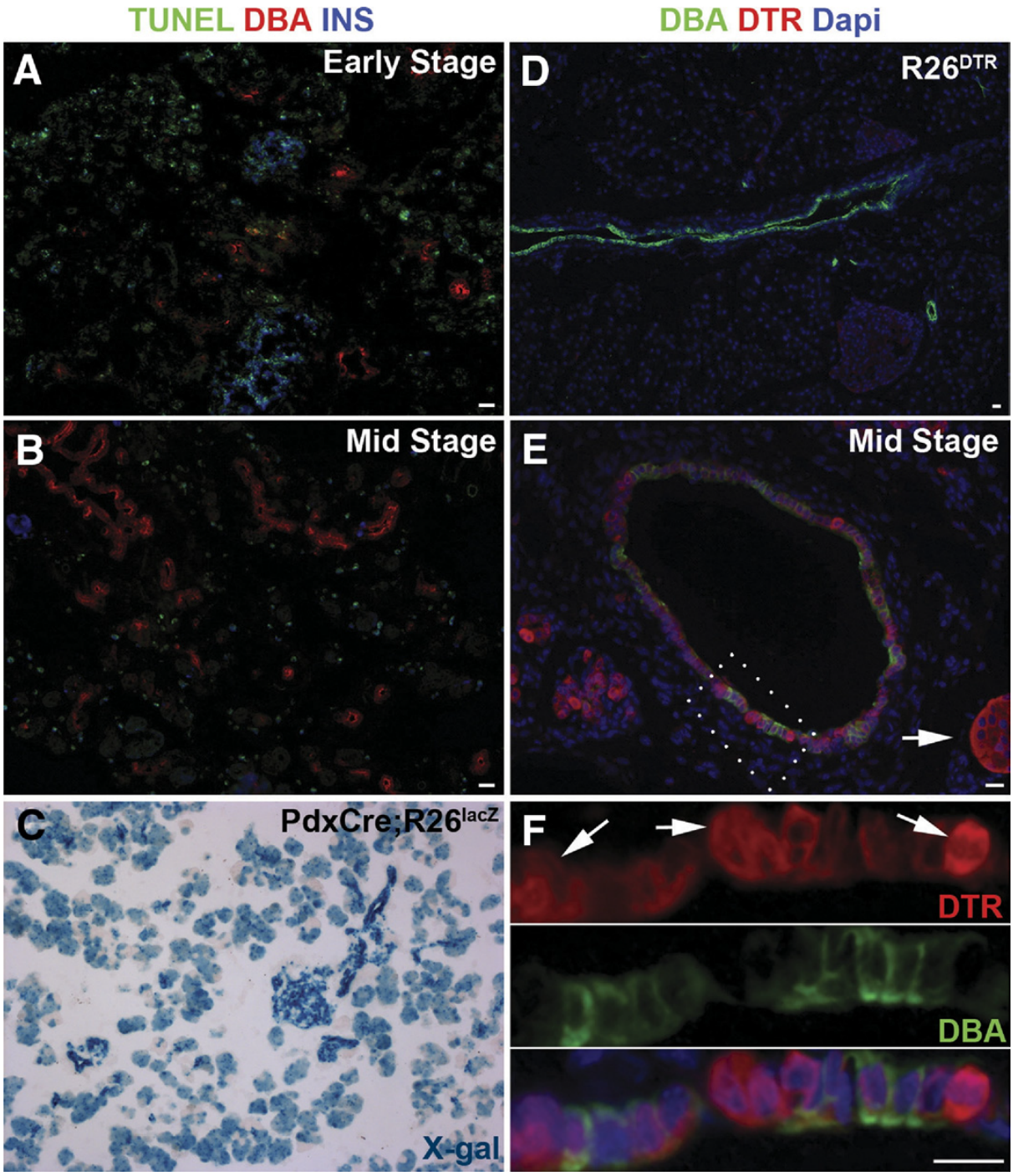


Figure 3. (A) *TUNEL assay in PdxCre;R26^{DTR} mice. DBA/TUNEL/insulin triple staining showed a high level of apoptosis among non-DBA^{+} epithelial cells (A) early after insult but* ***(B)*** *not during mid stage regeneration.* ***(C)*** *X-gal staining of PdxCre;R26^{lacZ} pancreas revealed high penetrance of Cre-transgene in all pancreatic epithelial cell lineages in the PdxCre strain. Immunofluorescent analyses of sections obtained from* ***(D)*** *R26^{DTR} control and* ***(E,F)*** *mid stage regenerating PdxCre;R26^{DTR} pancreas for detection of DBA and DTR revealed expression of DTR in DBA^{+} cells.* ***(F)*** *Higher magnification of E. Arrow in E highlights a regenerated acinus. Arrows in F mark cytoplasmic and membrane localization of DTR in DBA^{-} cells within the duct-like structures. Scale bars = 20 μm.*

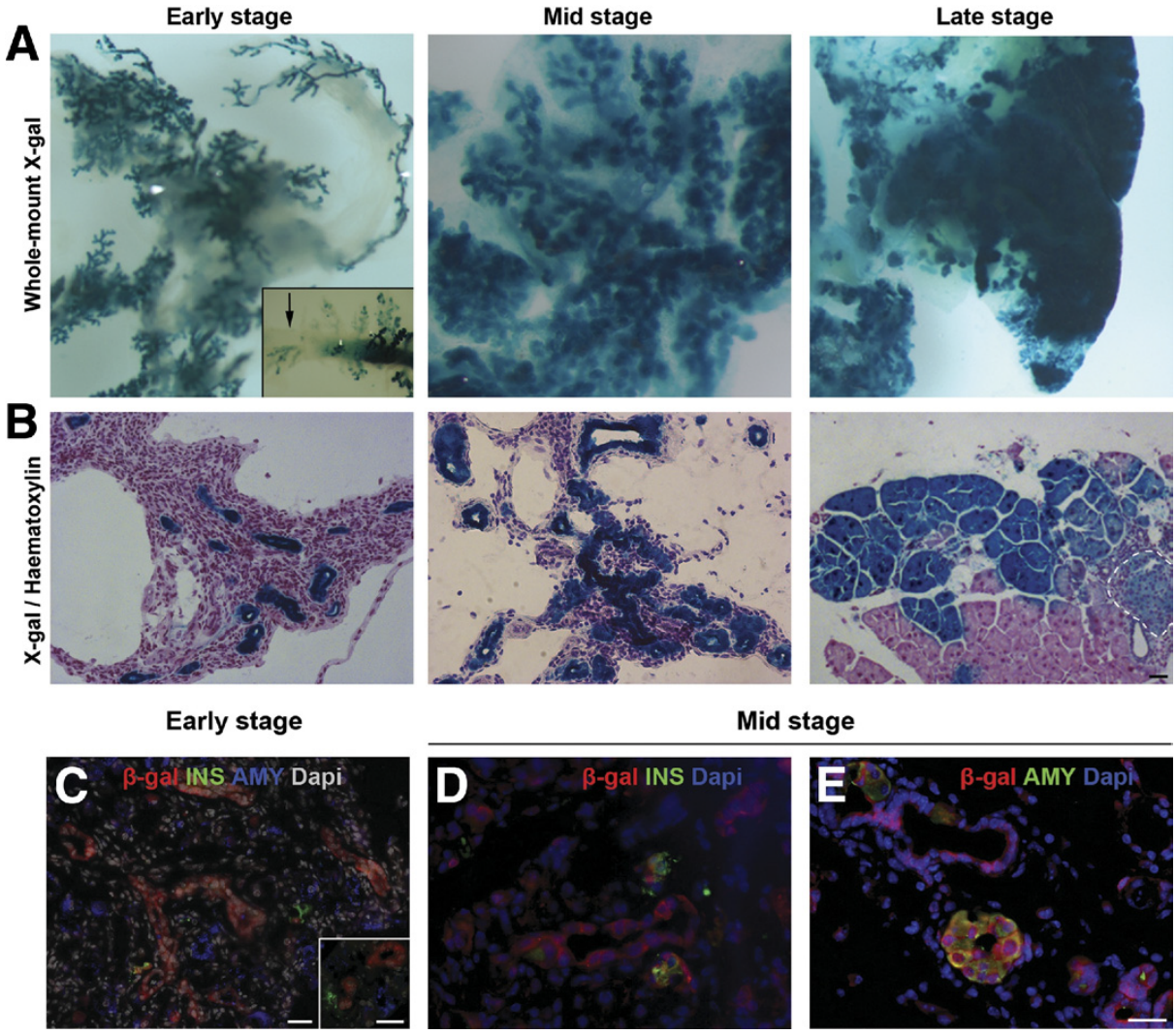


Figure 4. *Lineage tracing analyses in the regenerating PdxCre;R26$^{DTR/lacZ}$ model.* ***(A)*** *Whole-mount X-gal staining of early-, mid-, and late-stage regenerating pancreata. Shortly after DT treatment, the only X-gal^{+} structures detectable were the surviving ducts, whereas mid- and late-stage staining revealed extensive contribution of X-gal^{+} cells to parenchymal restoration. (Inset) X-gal^{-} segment within the ductal network (arrow).* ***(B)*** *X-gal stained pancreata, counterstained with hematoxylin. Dashed line highlights an islet.* ***(C)*** *β-gal/insulin/amylase immunostaining of PdxCre;R26$^{DTR/lacZ}$ pancreas early after injury showed no β-gal expression in insulin^{+} or amylase^{+} cells. Inset shows higher magnification.* ***(D)*** *β-gal/insulin and* ***(E)*** *β-gal/amylase staining of mid stage PdxCre;R26$^{DTR/lacZ}$ pancreata showed double^{+} cells. Scale* ***bars =*** *20 μm.*

Cells within the ductal compartment are highly proliferative during regeneration

To identify the proliferating cells within each compartment that may give rise to the regenerated endocrine and acinar cells, representative tissues from different time points were stained for the proliferation marker phospho-histone-H3 (PHH3). Cells within the ductal compartment, as well as surviving acinar cells, were found to be the main proliferating cells during early and mid stages. In particular, the peak of proliferation in acinar tissue was reached during mid stage, whereas ductal cells kept proliferating consistently throughout late stage. PHH3^{+}/endocrine^{+} cells were detected only from the end of mid stage onward and peaked in late stage (**Figure 5A**). To better analyze the

fate of proliferating cells, we performed a pulse-chase approach. Bromodeoxyuridine (BrdU) was administrated via the drinking water on the final day of DT injections and on the following day; subsequently, pancreata were harvested later on day 1 (**Figure 5B**), day 10 (**Figure 5C** and **5D**), or day 30 after last DT injection (**Figure 6**). On day 1 (pulse), the duct-like structures were the main epithelial cells incorporating BrdU (**Figure 5B**). On day 10 (chase), the presence of insulin$^+$/BrdU$^+$ cells (**Figure 5C**) and amylase$^+$/BrdU$^+$ cells (**Figure 5D**) suggested that these cells or their precursors must have been proliferating immediately after injury. Interestingly, at this time point, abundant amylase$^+$/BrdU$^+$ cells were found within both the small and large duct-like structures (inset in **Figure 5E**), further supporting the hypothesis that the main source of regenerating acinar cells, which peak during mid stage, resides in the ducts rather than in proliferating residual Cre$^-$ acinar cells. On day 30, some β-cells, acinar cells, and cells within the larger ducts (but not the smaller ducts) still retained the BrdU labeling (**Figure 6**).

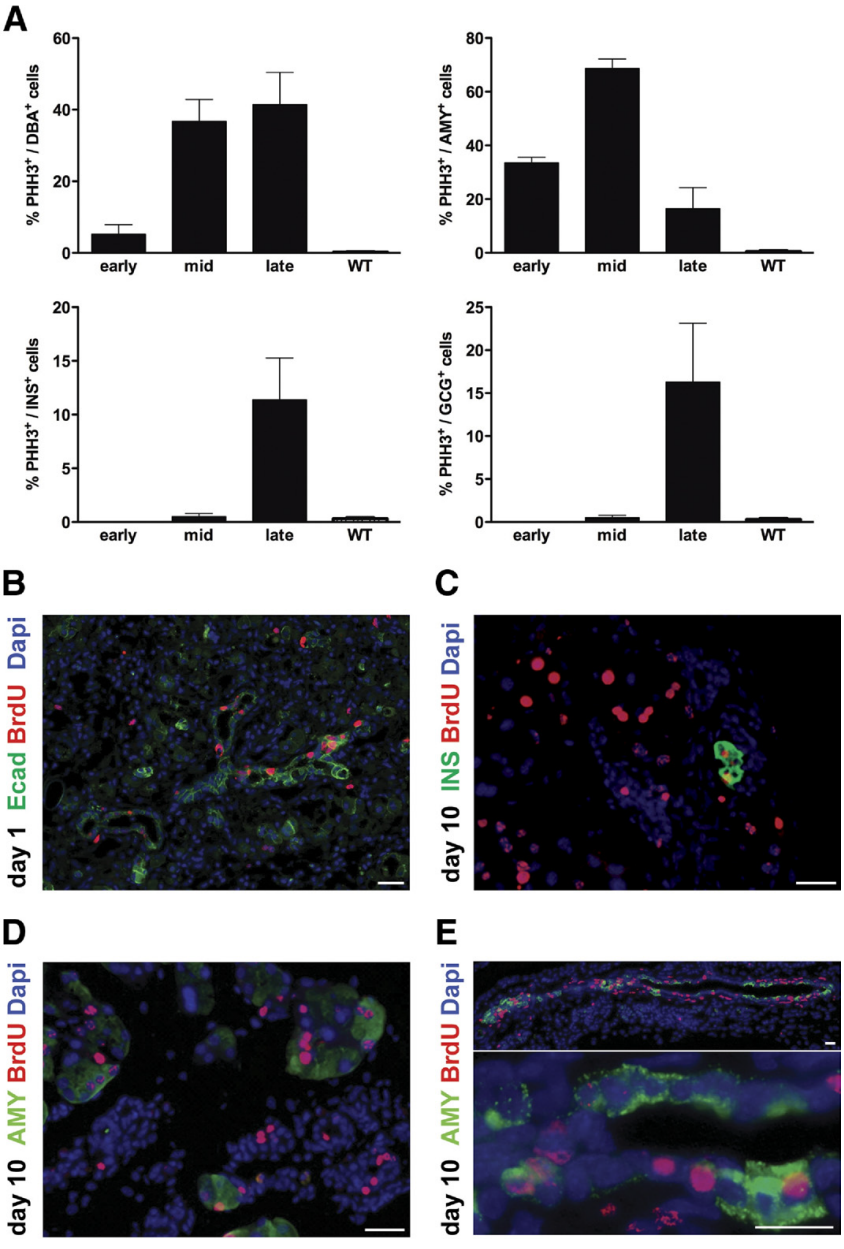


Figure 5. Cells within the ductal compartment are highly proliferative in regenerating PdxCre;R26^{DTR} pancreata. **(A)** Quantification (percent) of proliferating DBA$^+$, amylase$^+$, or insulin$^+$ cells. PHH3, phospho-histone H3. Wild type (WT) is an age-matched adult control pancreas. **(B)** Pulse-chase BrdU experiments. On day 1 (pulse), the duct-like structures were the main epithelial cells incorporating BrdU. On day 10 (chase), presence of **(C)** insulin$^+$/BrdU$^+$ cells and **(D)** amylase$^+$/BrdU$^+$ cells suggested that these cells or their precursors must have been proliferating immediately after injury. **(E)** Several amylase$^+$/ BrdU$^+$ cells were found within both the small and large duct-like structures, further supporting the hypothesis that the main source of regenerating acinar cells resides in the ducts rather than in proliferating residual Cre$^-$ acinar cells. Scale bars = 20 μm.

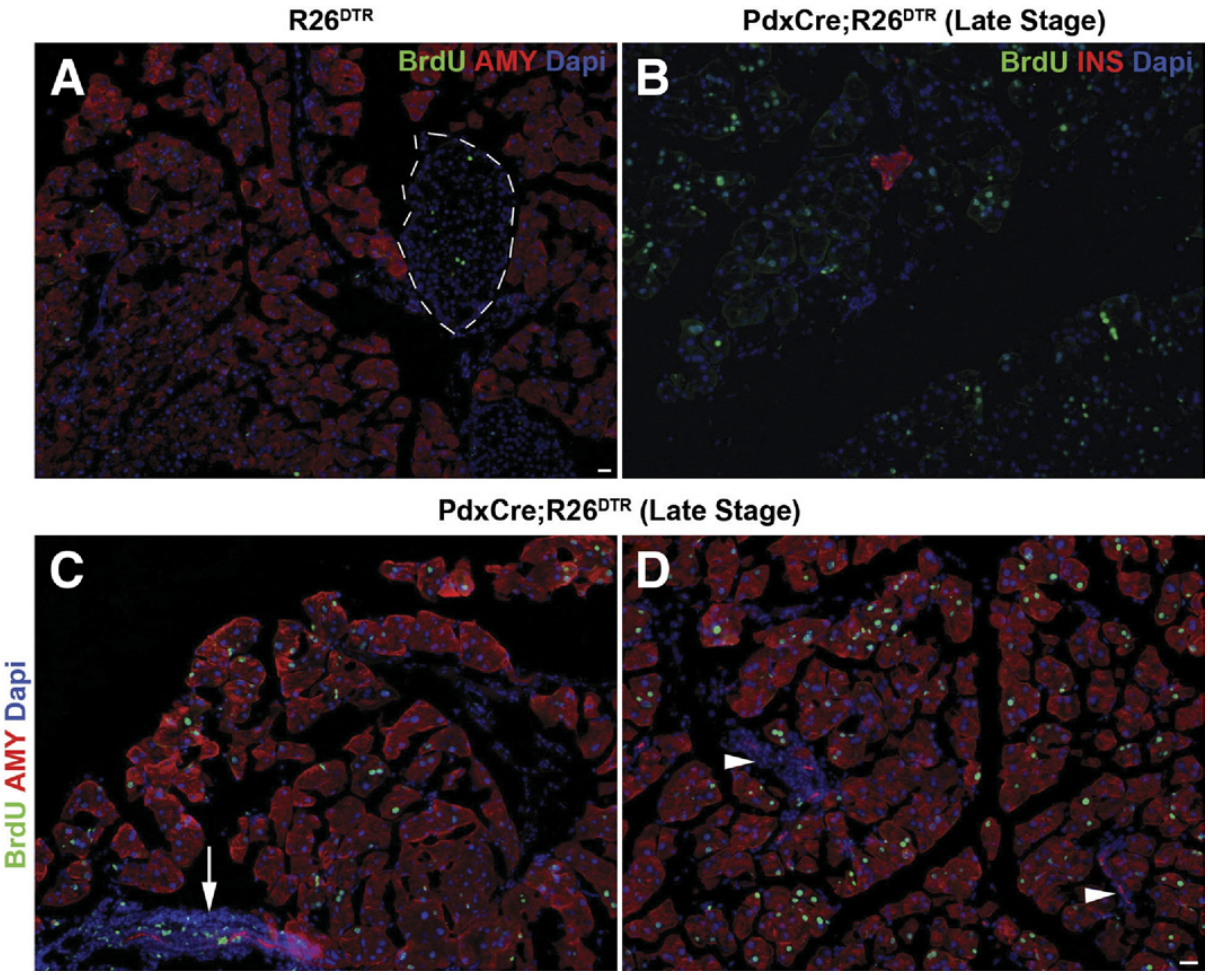


Figure 6. *Pulse-chase BrdU experiments. Immunofluorescent analyses of (**A**) R26^{DTR} control or (**B–D**) day 30 after DT treatment regenerating PdxCre;R26^{DTR} pancreas using antibodies against (A, C, and D) BrdU/amylase or (B) BrdU/insulin. BrdU was administrated for 2 days on the day of last DT injection and the following day via the drinking water, and the pancreas was harvested on day 30 after BrdU withdrawal. Arrow in C marks a large duct with retained BrdU. Arrowheads in D highlight smaller ducts with no retained BrdU. Dotted lines highlight an islet. Scale bars = 20 μm.*

Regeneration process recapitulates the pancreatic developmental program in PdxCre;R26^{DTR} mice

To further characterize the cells within the ductal compartment during regeneration, pancreata from DT-treated PdxCre;R26^{DTR} mice were stained for markers of committed endocrine progenitors and differentiated acinar, endocrine, or duct cells. Mature duct cells are generally identified as DBA^{+}/SOX9^{+}/PDX1^{-} (Kobayashi et al., 2002; Greenwood et al., 2007; Seymour et al., 2007). Interestingly, after DT injection, the surviving ductal cells re-expressed PDX1 (**Figure 7A** and **B**). Notably, once regeneration was completed, Pdx1 expression was once again restricted to β-cells (**Figure 7C**). These regenerating ductal structures retained Sox9 expression, resembling the DBA^{+}/SOX9^{+}/PDX1^{+} undifferentiated epithelial cells in the developing pancreas **(Figure 7D–F)**.

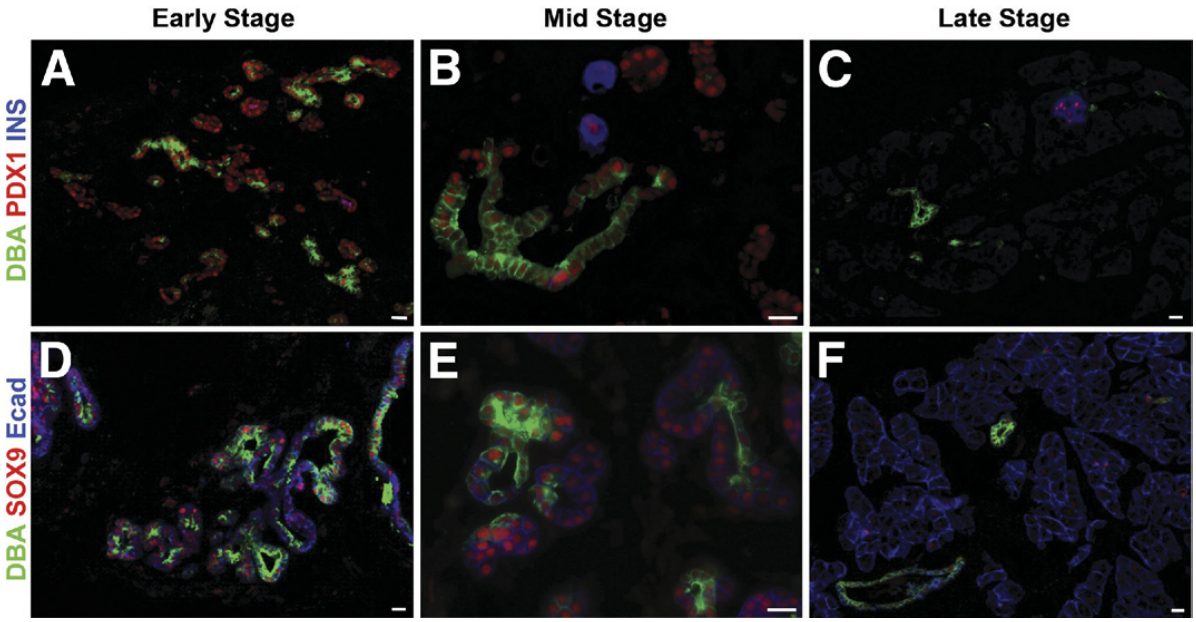


Figure 7. *Immunofluorescent analyses of sections obtained from* ***(A and D)*** *early-stage,* ***(B and E)*** *mid stage, or* ***(C and F)*** *late-stage regenerating DT-treated PdxCre;R26^{DTR} pancreas for detection of (A–C) DBA/PDX1/insulin or (D–F) DBA/SOX9/E-cadherin. (A and B) In DT-treated PdxCre;R26^{DTR} pancreata, surviving ductal cells re-expressed PDX1 in early and mid stages. (C) Notably, once regeneration was completed, Pdx1 expression was once again restricted to β-cells. Scale bars = 20 μm.*

The presence of NGN3^{+}, insulin^{+}, amylase^{+} or glucagon^{+} cells within the duct-like structures (**Figure 8A-D**) further supports that in this severe injury model, cells within the ductal network contribute to formation of both endocrine and acinar cells by recapitulation of the pancreatic developmental program. Surprisingly, a subpopulation of PDX1^{+}/glucagon^{+} cells was also detected in the ducts of regenerating pancreas during mid stage (**Figure 8D**, arrows), perhaps suggesting α-to-β cell transdifferentiation.

Although duct cells in the control pancreas were all DBA^{+}/cytokeratin^{+} (**Figure 8E**), in the regenerating PdxCre;R26^{DTR} pancreas a significant number of DBA^{+} cells had lost cytokeratin expression (**Figure 8F**, arrows). This phenomenon was more commonly found in the epithelial buds arising from bigger ducts, thus suggesting these cells were undergoing dedifferentiation and/or transdifferentiation into other cytotypes. During late stage of regeneration, all DBA^{+} ducts once again expressed cytokeratin (data not shown).

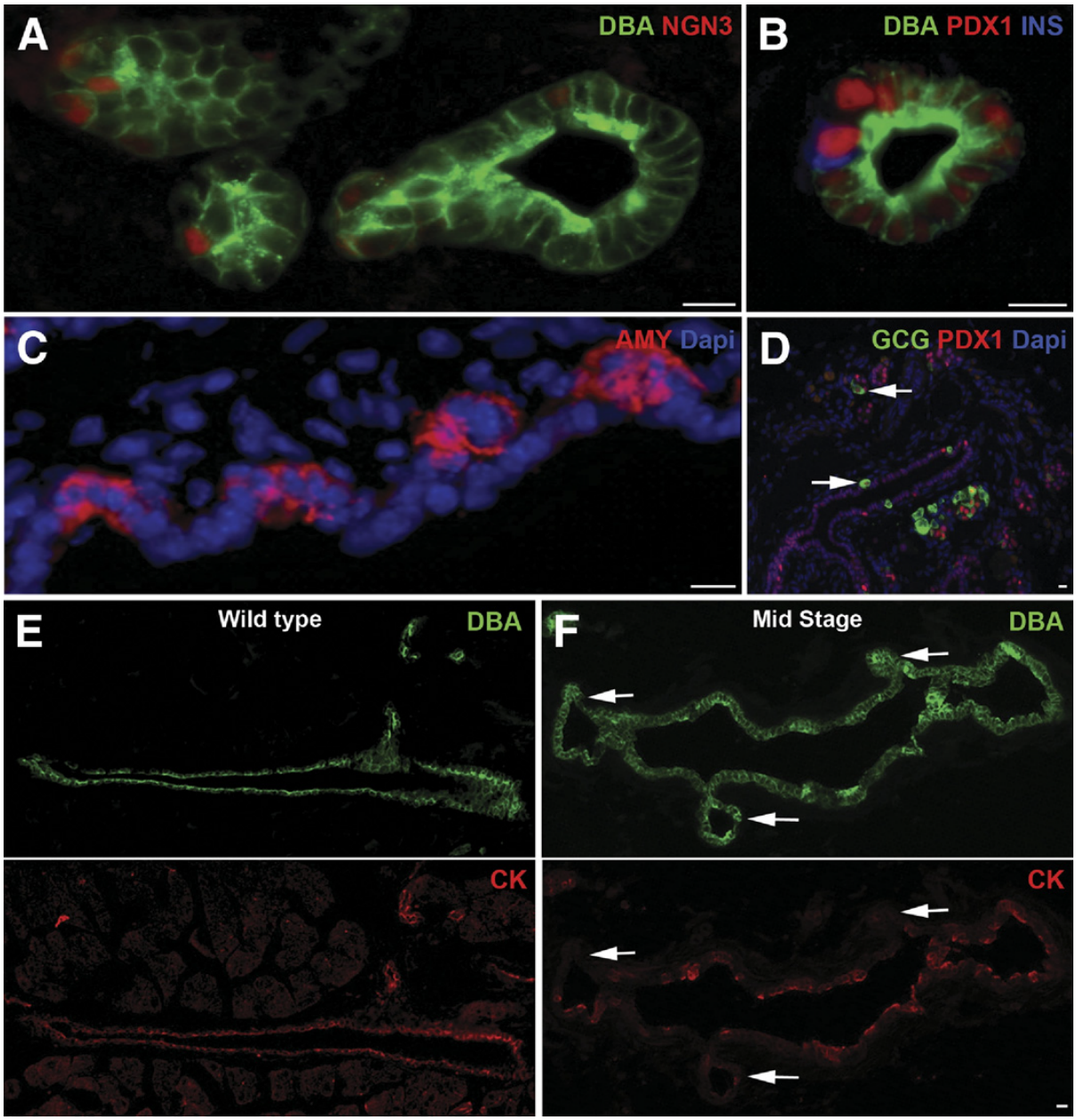


Figure 8. *Recapitulation of pancreatic developmental program in regenerating PdxCre;R26^{DTR} pancreata. During mid stage, ductal structures showed presence of endocrine progenitor marker* ***(A)*** *Ngn3^{+},* ***(B)*** *PDX1^{+}/insulin^{+},* ***(C)*** *amylase^{+}, or* ***(D)*** *glucagon^{+} cells. A subpopulation of PDX1^{+}/glucagon^{+} cells was also detected during mid stage (arrows).* ***(E)*** *Duct cells in wild-type pancreas were all DBA^{+}/cytokeratin^{+} (CK).* ***(F)*** *In the regenerating pancreas, a significant number of DBA^{+} cells had lost cytokeratin expression (arrows). Wild type is an age-matched adult control pancreas. Scale bars = 20 μm.*

The hypothesis that the regeneration process mirrors the pancreatic developmental program was further supported by qRT-PCR analysis of tissues from early-, mid-, and late-stage PdxCre;R26^{DTR} pancreata (**Figure 9**). Throughout early and mid stages, we found expression of genes that are normally transcribed during embryonic pancreatic organogenesis, such as Notch1 and Hes1, and genes that are expressed in endocrine progenitors, such as Ngn3. Parallel to their decrease in the late stage, from mid stage onward we found upregulation of both genes associated with exocrine acinar tissue (Mist1, Ptf1a) and mature endocrine cells (Ins2, Gcg, Gck, Beta2NeuroD, Glut2). Pdx1,

which is normally expressed in both early pancreatic precursors and mature β-cells, was indeed found to be expressed already during early stage and then consistently upregulated in mid and late stages.

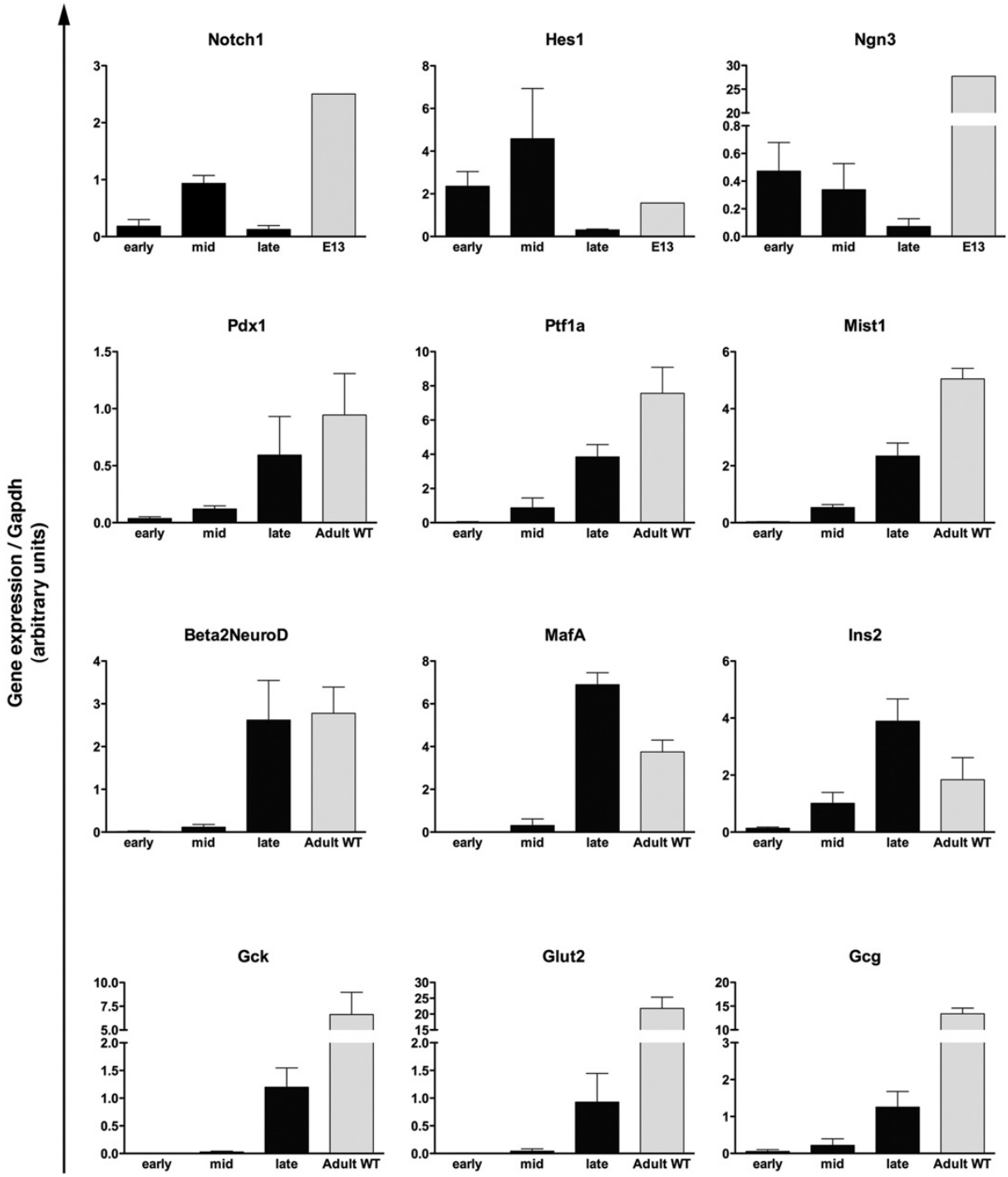


Figure 9. *Expression (qRT-PCR) of embryonic and adult pancreatic markers in the regenerating PdxCre;R26^{DTR} pancreata. Bars represent gene expression (mean ± SE) of mice killed at early, mid, or late stage (n = 5 for each time point). Wild type (WT) is age-matched adult control pancreata (n = 5); E13 is pooled pancreata from WT litters (n = 8 embryos).*

Acinar-Specific Cell Ablation and Regeneration in the ElaCreERT2;R26^{DTR} Mouse Model

DT treatment in PdxCre;R26^{DTR} mice results in ablation of all pancreatic cells derived from Pdx1^{+} progenitor cells (cells that had expressed the Cre recombinase and thereby activated the DTR gene). To evaluate whether the mechanisms involved in regeneration are different when the extent of cell ablation is more limited, we selectively targeted acinar cells by using mice expressing a tamoxifen-sensitive Cre under the acinar-specific elastase promoter (ElaCreERT2), in combination with the Rosa26^{DTR} construct. In this transgenic line (ElaCreERT2;R26^{DTR}), expression of the DT receptor is restricted to acinar cells, and only after tamoxifen treatment. Mice were then killed 1, 3, 5, 7, and 8 days after DT injection (**Figure 1B**). The peak of injury, identifiable by massive acinar destruction, was reached on day 1 after DT treatment, when sections showed the pancreas consisted mainly of islets, ducts, blood vessels, and adipose tissue (**Figure 10A**).

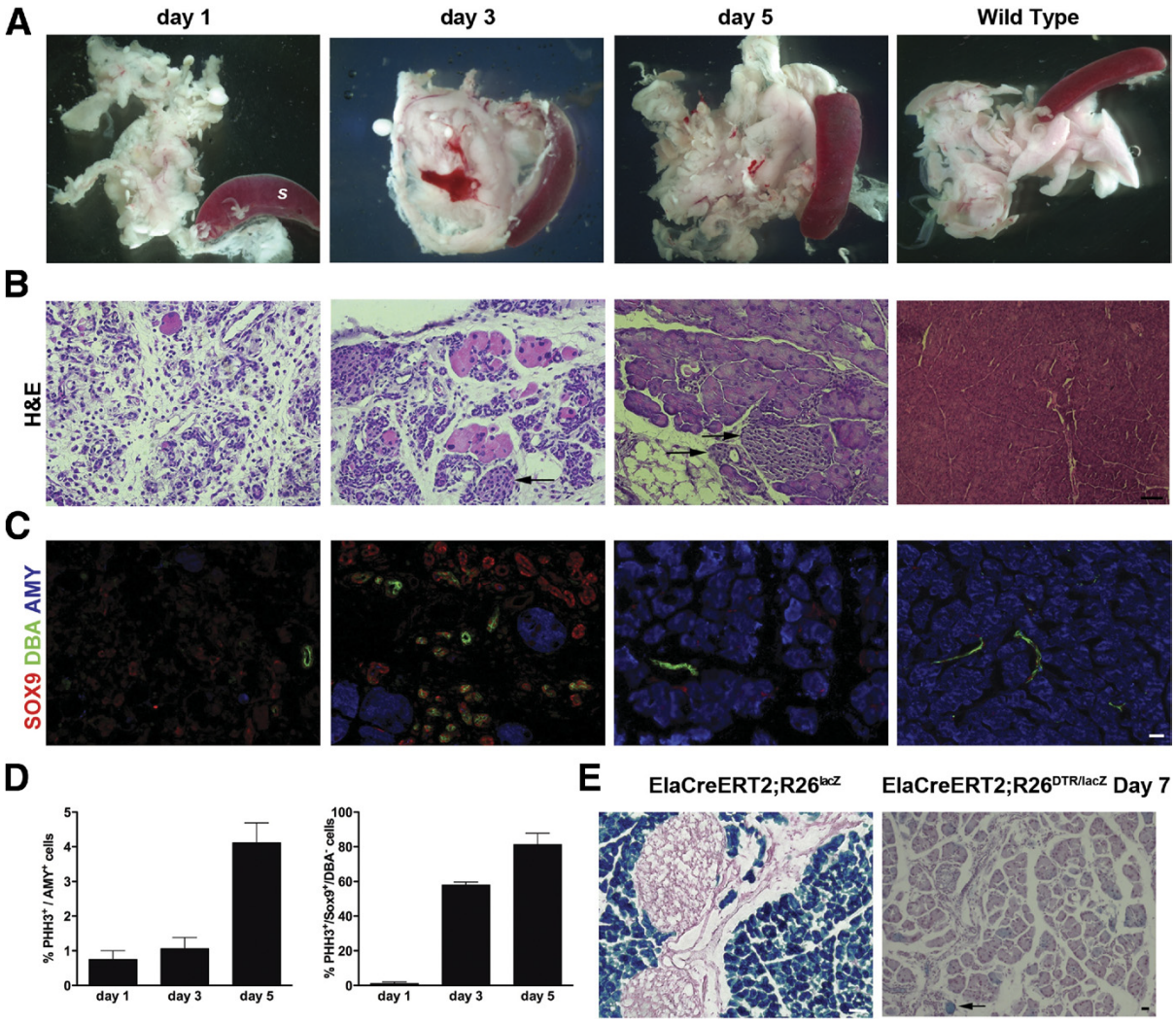


Figure 10. *Acinar-specific cell ablation and regeneration in DT-treated ElaCreERT2;R26^{DTR} pancreata.* ***(A)*** *Massive acinar cell loss was observed on day 1 after DT treatment, peaked on day 3, and was nearly complete on day 5. S, spleen.* ***(B)*** *Representative H&E staining of pancreata at the same time points. Arrows highlight islets.* ***(C)*** *Immunostaining for DBA/SOX9/amylase in the regenerating ElaCreERT2;R26^{DTR} pancreas showed several SOX9^{+}/DBA^{-} duct-like structures during peak of regeneration. By contrast, ducts in wild type are DBA^{+}/SOX9^{+}.* ***(D)*** *X-gal staining showed minimal contribution of preexisting acinar cells to acinar regeneration.* ***(E)*** *Quantification (percent) of proliferating amylase^{+} and SOX9^{+}/DBA^{-} cells. Arrows highlight X-gal^{+} acinar cells. Scale bars = 20 μm.*

Remarkably, and presumably due to the apoptotic nature of acinar cell ablation, pancreas did not exhibit significant local inflammation. By day 3, acinar cells were significantly regenerating (**Figure 10B**), and from days 5 (**Figure 10C**) to 8 (data not shown) the regeneration process was almost complete. In addition to intact islets (**Figure 11**), the ElaCreERT2;R26^{DTR} mice also displayed normal blood glucose levels throughout the regeneration process (data not shown), further confirming the sparing of endocrine cells. Interestingly, by day 3, we could detect the presence of numerous SOX9^{+}/DBA^{-} duct-like structures (**Figure 10C**), which were found to be highly proliferating (60%) (**Figure 10D**). The proliferation rate among these duct-like structures increased to 80% on day 5; however, due to the concomitant decrease in their total number, the main proliferative compartment on day 5 was represented by acinar cells.

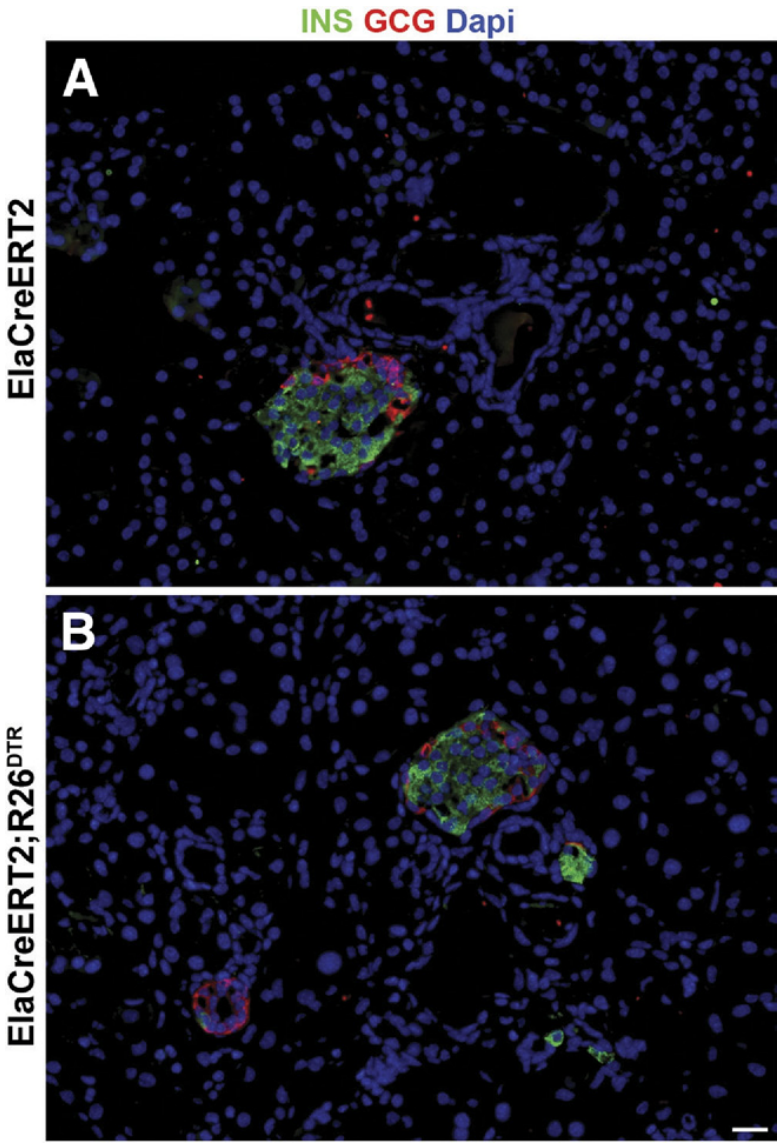


Figure 11. *Acinar-specific cell ablation in tamoxifen-induced DT-treated ElaCreERT2;R26^{DTR} pancreas. Double immunostaining of tissues obtained from* ***(A)*** *control ElaCreERT2 or* ***(B)*** *regenerating ElaCreERT2;R26^{DTR} pancreas using antibodies recognizing insulin or glucagon showed the survival of non-acinar cells. Scale bar = 20 μm.*

It has been shown that following cerulein treatment, preexisting acinar cells in the adult pancreas are the main source for regeneration of new acinar cells (Fendrich et al., 2008; Morris et al., 2010). To determine whether preexisting acinar cells were the origin of newly regenerated acinar cells in DT-treated ElaCreERT2;R26^{DTR} pancreas, we used the ElaCreERT2;R26$^{lacZ/DTR}$ transgenic line. Following tamoxifen and then DT treatment, the pancreata were harvested on day 7 and analyzed by X-gal staining. As shown in **Figure 10E**, the vast majority of regenerated acinar tissue was X-gal negative. qRT-PCR analysis on ElaCreERT2;R26^{DTR} pancreata harvested at different time points revealed the expression of embryonic pancreatic markers, such as Notch1 and Hes1, in the first phase of regeneration (peak at day 3) and the up-regulation of acinar-related genes, such as Mist1 and Ptf1a, in the late phase of regeneration (peak at day 8) (**Figure 12A**). Collectively, these data further support the hypothesis that a mechanism other than replication of preexisting acinar cells is involved in acinar regeneration.

Regeneration Occurs Through Different Mechanisms in PdxCre;R26^{DTR} and ElaCreERT2;R26^{DTR} Models

Comparison of the regenerative processes in the PdxCre;R26^{DTR} and ElaCreERT2;R26DTR pancreata revealed important differences between the two models. Perhaps the most impressive difference was the absence of PDX1 in the ductal structures of the regenerating ElaCre-ERT2;R26^{DTR} pancreas compared with the DBA^{+}/PDX1^{+}/SOX9^{+} duct-like structures seen in the PdxCre;R26^{DTR} pancreas (**Figure 12B**). Interestingly, during early stages of regeneration in the PdxCre;R26^{DTR} pancreas, not all SOX9^{+} cells expressed Pdx1, whereas during mid stage almost all SOX9^{+} cells also expressed PDX1 (**Figure 12A**). In addition, the duct-like structures in the regenerating PdxCre;R26^{DTR} pancreata exhibited morphologic resemblance to embryonic pancreatic epithelium (**Figure 12C**).

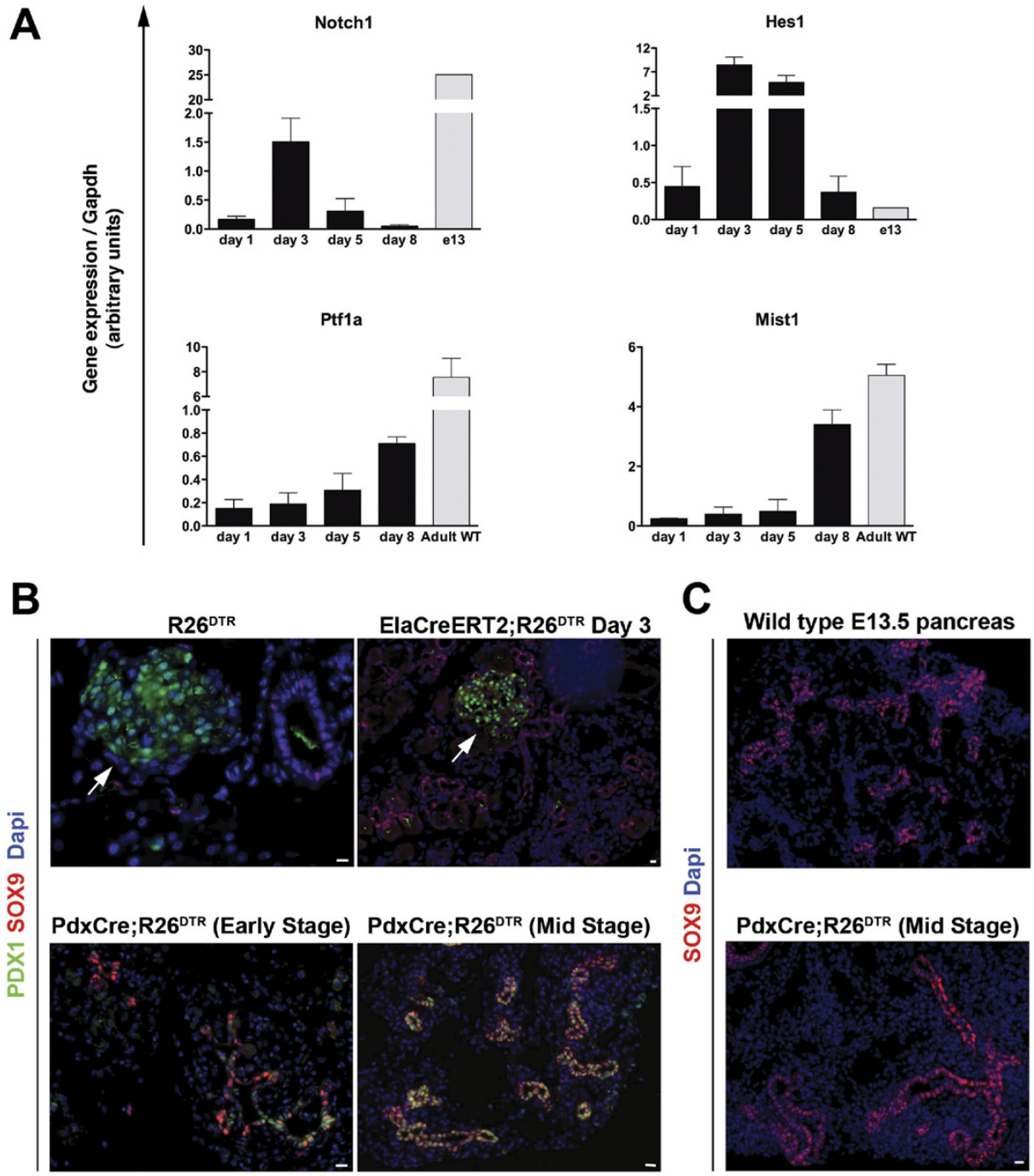


Figure 12. (A) *Expression (qRT-PCR) of embryonic and adult exocrine markers in the regenerating ElaCreERT2;R26^{DTR} pancreata. Bars represent gene expression (mean ± SE) of mice killed at different time points (n = 5 for each group). Wild type (WT) is age-matched adult control pancreata (n = 5); E13 is pooled pancreata from WT litters (n = 8 embryos). (B and C) The regeneration mechanism is dictated by the severity of injury.* ***(B)*** *Immunostaining analysis of SOX9 and PDX1 on R26^{DTR} control pancreas, day 3 ElaCreERT2;R26^{DTR}, and early-stage or mid-stage regenerating PdxCre;R26^{DTR} pancreata showed coexpression of these markers only in DT-treated PdxCre;R26^{DTR} pancreas. Arrows highlight islets.* ***(C)*** *SOX9 expression in the regenerating PdxCre;R26^{DTR} pancreas displayed striking similarities to an E13.5 embryonic pancreas. Scale bar = 20 μm.*

DISCUSSION

A number of different animal models have been used to investigate pancreatic plasticity, including duct ligation, partial resection, and chemical ablation. However, there is no firm conclusion as to the origin of the cells destined to become acinar, ductal, or endocrine. In an attempt to overcome the discrepancies among the different studies, we used a DTR-mediated conditional and targeted cell ablation model, which enabled us to evaluate the extent of injury (ie, the cell type that is damaged) on the regenerative response. In our PdxCre;$R26^{DTR}$ transgenic line, which represents the most severe pancreatic injury model of regeneration yet reported, all pancreatic cells are killed via DT administration, with the exception of ductal cells, which are serendipitously spared. Notably, in this model, cell death is quickly achieved after DT administration via massive apoptosis. Because no relevant local inflammation response is generated during this process, the numerous confounding variables incurred with other models are ruled out.

Lineage tracing studies have shown that all pancreatic epithelial cells originate from a pool of $PDX1^+$ progenitors (Gu et al., 2002). Thus, our initial assumption was that in PdxCre;$R26^{DTR}$ double-transgenic mice all descendents (endocrine, acinar, and ductal) of $Cre^+/PDX1^+$ progenitors would express DTR and therefore should be killed by DT. However, the surprising persistence of numerous DBA^+ ductal structures seen after DT injection indicated that cells within the ductal compartment somehow survived the insult. In our rigorous search for the cell(s) of origin, we used PdxCre;$R26^{DTR/lacZ}$ mice in which cells with Cre activity will become DTR^+ and $lacZ^+$ double positive. The regenerated PdxCre;$R26^{DTR/lacZ}$ pancreata consisted of mixed populations of X-gal^+ and X-gal^- cells, indicating a contribution from both preexisting differentiated cells (X-gal^-) and ductal cells (X-gal^+ or X-gal^- as shown in Figure 4D). Our analysis showed a 30% proliferation rate among acinar cells at early stage. However, given the 97% loss of acinar tissue and the actual low number of $amylase^+$ cells immediately after injury, these cells most likely did not contribute significantly to overall acinar regeneration. Taken together, these data strongly suggest that the ductal compartment is a potential source of new cells. However, it cannot distinguish whether mature duct cells or putative progenitors residing within ducts were the source of regeneration. Furthermore, it is possible that in some cells, due to low Cre-recombinase activity, only one of the two floxed genes (the STOP cassette flanking either the DTR or lacZ genes) would be excised. Because it would seem unlikely that any non-ductal pancreatic epithelial cell with DTR on its surface would survive the toxin treatment, the main concern would be that some endocrine or acinar cells might express lacZ but not DTR. However, because we could not detect any differentiated X-gal^+ cells immediately after injury, it is unlikely that their contribution to regeneration is significant. In a recent report, Solar et al. showed that postnatal duct cells do not contribute to new acinar or endocrine cells during normal growth or

after injury, based on HNF1b expression as a ductal marker (Solar et al., 2009). Nevertheless, the injury models used in that report were pancreatic duct ligation or alloxan treatment for β-cell ablation, neither of which is as severe as the injury induced in the PdxCre;$R26^{DTR}$ pancreas following DT treatment.

Further evidence of ductal involvement during regeneration comes from the BrdU pulse-chase experiments. Because our analysis showed that DBA^+ duct-like structures were the main epithelial cells that were proliferating after injury, the presence of several $BrdU^+$/$insulin^+$ or $BrdU^+$/$amylase^+$ cells during chase phase in the mid stage of regeneration further suggested a progenitor-progeny relationship between the proliferating DBA^+ duct-like structures immediately after injury, and the regenerated endocrine and acinar cells in subsequent stages. Notably, a few $BrdU^+$ cells could be found within the larger ducts after 30 days from pulse phase. We speculate that this retention of BrdU may reflect their "stemness." One of the main features of adult stem cells is indeed their ability to divide asymmetrically, ie, to give rise to one daughter stem cell and another daughter cell that undergoes differentiation. Thus, we believe these $BrdU^+$ ductal cells represent the result of perhaps one or two rounds of asymmetric division, whereas the differentiating cells that underwent extensive proliferation to give rise to regenerated pancreas eventually became $BrdU^-$.

So far, our data indicate that in this severe ablation model the regeneration process involves proliferation and differentiation of ductal progenitors, mirroring the pancreatic developmental program. This hypothesis is further supported by the finding that, shortly after DT treatment, the surviving ductal cells re-expressed PDX1, resembling the DBA^+/$SOX9^+$/$PDX1^+$ undifferentiated epithelial cells in the developing pancreas, and the endocrine progenitor marker Ngn3. In addition, in the epithelial buds arising from bigger ducts during mid stage, a subset of DBA^+ cells had lost cytokeratin expression, implying that these cells may be differentiating and/or transdifferentiating into other cytotypes. This would also explain the presence of a subpopulation of $PDX1^+$/$glucagon^+$ cells not only within the ducts, but also in the forming endocrine clusters. Because α-cells typically do not express PDX1, it is tempting to speculate that these $PDX1^+$/$glucagon^+$ cells may represent α-cells converting to β-cells, as recently reported (Thorel et al., 2010; Chung et al., 2010). Notably, during late stage of regeneration, all DBA^+ ducts once again expressed cytokeratin, and PDX1 expression was restricted to β-cells. In addition, qRT-PCR analysis of tissues from early-, mid-, and late-stage PdxCre;$R26^{DTR}$ pancreata confirmed expression of genes that are normally transcribed during embryonic pancreatic organogenesis.

In the PdxCre;$R26^{DTR}$ mice, we saw a consistent level of cell ablation and subsequent regeneration for all pancreatic cell types in the early stage and mid stage pancreata, respectively. The acinar cell recovery then consistently continued through late stage to form normal-appearing acinar tissue. By contrast, only 80% of mice regenerated enough endocrine tissue by the late stage to reverse the diabetic state caused by DT treatment. In light of recent studies reporting that glucose

metabolism influences beta cell replication (Porat et al., 2011), it would be interesting to determine whether in our model maintaining normoglycemia or moderate hyperglycemia affects regeneration.

In response to injury, the exocrine pancreas also activates regenerative processes to maintain tissue homeostasis. We thus investigated whether regeneration would occur through the same mechanisms following selective ablation of acinar tissue. It has been shown that in cerulein-induced acinar cell death, surviving acinar cells can serve as the main source for acinar regeneration (Fendrich et al., 2008; Morris et al., 2010). Nevertheless, in our DT-treated ElacreERT2;R26^{DTR} pancreas, where virtually all acinar cells were killed, the acinar compartment still regenerated rapidly. In particular, regeneration coincided with the expansion of DBA^{-}/SOX9^{+} cells, while proliferation of amylase^{+} cells was detected only in a subsequent stage. A recent lineage-tracing study has reported that, under normal conditions, SOX9^{+} cells within the ductal compartment contribute to the maintenance of acinar mass in the adult pancreas (Furuyama et al., 2011). Therefore, we speculate that in the absence of a significant number of surviving acinar cells, the regenerative mechanism taps into these SOX9^{+} cells, as in an accelerated version of a physiological process.

The identity of the cells of origin in our acinar regeneration model is still to be determined as they may be mature duct cells, unidentified progenitors, terminal duct cells, or centroacinar cells (CACs). In the adult pancreas, SOX9 is expressed in duct cells (which are also DBA^{+}) and CACs. To our knowledge, it has not been established whether or not CACs are DBA^{+}. If they do, then the DBA^{-}/SOX9^{+} cells observed in DT-treated ElaCreERT2;R26^{DTR} pancreas likely do not derive from CACs and may be either dedifferentiated duct cells that lost their ability to bind DBA or another unidentified cell type residing within the ducts. By contrast, if CACs are DBA^{+}, it seems likely that the DBA^{-}/SOX9^{+} cells derive from CACs. Furthermore, in the adult pancreas, Notch-Hes1 signaling appears particularly high in terminal duct and/or CACs, which can give rise to acinar cells following isolation and culture (Miyamoto et al., 2003; Rovira et al., 2010; Kopinke et al., 2011). Therefore, reactivation of Notch and Hes1 during acinar regeneration in ElaCreERT2;R26^{DTR} pancreas would argue for terminal duct cells and/or CACs to be the source for this process. Although these data do not provide irrefutable proof of the cell of origin, the lineage tracing studies in ElaCreERT2;R26$^{DTR/lacZ}$ model strongly suggest that preexisting acinar cells are not the major contributor to acinar regeneration in this model.

CONCLUSIONS ON PROJECT 2

Our findings show that following extensive ablation of acinar and endocrine cells in the PdxCre;R26^{DTR} mice, or acinar-specific ablation in the ElaCreERT2;R26^{DTR} mice, epithelial cells within the ductal network are capable of contributing to both endocrine and acinar regeneration, although through different mechanisms and perhaps through different cell types. However, duct cells might not be the "preferred" regenerative source in physiologic conditions but could be hierarchically recruited based on the severity of injury. During embryonic pancreatic organogenesis, stem cells within the duct pancreatic epithelium give rise to both the endocrine and acinar cells. Therefore, it seems reasonable to assume that the regeneration process in our extremely severe PdxCre;R26^{DTR} injury model would recapitulate the embryonic program. By contrast, in a less severe injury model (exclusive acinar ablation in ElaCreERT2;R26^{DTR} pancreas), reprogramming of ductal cells to acinar lineage is sufficient. This could explain why acinar cell recovery is completed within a week in the ElaCreERT2;R26^{DTR} model, whereas it requires from 3 to 4 weeks to complete in PdxCre;R26^{DTR} model. Identifying signals that may initiate these different responses may provide a key understanding of how new pancreatic cells in general, and β-cells in particular, can be generated.

FINAL COMMENTS

There have been extraordinary recent advances in the understanding of diabetes because of its priority as a major health problem and the remarkable development of scientific methods in genomics, genetics, cell biology and other fields. The potential of stem cell approaches for diabetes is particularly attractive because the development of both forms of diabetes is dependent upon deficiency of pancreatic β-cells, and the diabetic state can be reversed using β-cell replacement therapy. The major challenges facing this approach are finding an adequate supply of islet cells and preventing transplanted or regenerated cells from being killed by immune destruction from autoimmunity and/or transplant rejection. There have been hopes that it might be possible to replace the β-cell deficit that occurs in diabetes by regenerating new β-cells from adult tissues. The pancreas has received the most attention, in particular regarding the potential for replication of pre-existing β-cells or neogenesis. While there could be stem cells in the pancreas itself, observations to date point to the pancreatic duct epithelium as the most likely potential source for new islet formation. However, caution is required in interpreting these studies. The extrapolation of information from animal studies can be misleading and not necessarily indicative for regenerative therapy in diabetic patients because mechanisms of regeneration/maintenance of the β-cell mass might be different across species. However, even if the mechanisms regulating the maintenance of the β-cell mass in physiologic conditions are the same in humans, monkeys and rodents, we cannot exclude that during pathological conditions different pathways - species-specific and injury-specific for type and magnitude - might be activated. Understanding the potential contribution of each mechanism will be crucial to find better curative approaches for diabetes.

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Project 1. In vitro generation of pancreatic endocrine cells from human adult fibroblast-like limbal stem cells

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Project 2. Duct cells contribute to regeneration of endocrine and acinar cells following pancreatic damage in adult mice

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PUBLICATIONS AND AWARDS

The work presented in this PhD thesis has been object of publications and oral communications at national and international meetings. They are listed here for reference:

Peer-reviewed Publications:

- **Criscimanna A**, Zito G, Taddeo A, Richiusa P, Pitrone M, Morreale D, Lodato G, Pizzolanti G, Citarrella R, Galluzzo A, Giordano G. *In vitro* generation of pancreatic endocrine cells from human adult fibroblast-like limbal stem cells. Cell Transpl. 2011 June 9 (Epub ahead of print).
- **Criscimanna A**, Speicher JA, Houshmand G, Shiota C, Prasadan K, Ji B, Logsdon CD, Gittes GK, Esni F. Duct Cells contribute to Regeneration of Endocrine and Acinar Cells Following Pancreatic Damage in Adult Mice. Gastroenterology. 2011 Oct;141(4):1451-1462.e6. Epub 2011 Jul 18.

Book Chapters:

- **Criscimanna A**, Bertera S, Esni F, Trucco M, Bottino R. The Enigma of β-Cell Regeneration in the Adult Pancreas: Self-Renewal Versus Neogenesis. Type 1 Diabetes Complications, David Wagner (Ed.), ISBN: 978-953-307-788-8, InTech. Available at: *http://www.intechopen.com/articles/show/title/the-enigma-of-946-cell-regeneration-in-the-adult-pancreas-self-renewal-versus-neogenesis*

Oral Comunications at National and International Meetings:

- **Criscimanna A**, Zito G, Richiusa P, Pizzolanti G, Coppola A, Bommarito A, Carissimi E, Pitrone M, Giordano C. Limbal stem cells as a potential source of pancreatic beta cells. 10th International Congress of the Immunology of Diabetes Society, May 17-20 2009, Malmo, Sweden. OC158, p.90
- **Criscimanna A**, Zito G, Taddeo A, Richiusa P, Pitrone M, Morreale D, Lodato G, Citarrella R, Pizzolanti G, Galluzzo A, Giordano C. Le cellule staminali limbari fibroblastiche (f-LSCs) come fonte di cellule pancreatiche endocrine in vitro. XXIII National Congress of the Italian Society of Diabetology (SID). 9-12 June 2010, Padova, Italy. OC17, p.24
- Giordano C, **Criscimanna A**, Zito G, Taddeo A, Richiusa P, Pitrone M, Morreale D, Lodato G, Pizzolanti G, Citarrella R, Galluzzo A. Generation of pancreatic endocrine cells from human adult fibroblast-like limbal stem cells. 46th EASD Meeting. 20-24 September 2010, Stockholm, Sweden. OP 22

- **Criscimanna A.** "Dynamics of Endocrine Regeneration in Adult Mouse Pancreas". Annual Rangos Research Symposium. June 23, 2011. Pittsburgh, USA

Honors and Awards

The project relative to Limbal Stem Cells has been awarded the "IDS-10 Award from the Immunology of Diabetes Society" during the 10th International Congress of the Immunology of Diabetes Society, held in Malmo, Sweden, 17-20 May, 2009 (recipent: Angela Criscimanna); and the "Best Young Research Scientist Award for year 2009" from the University of Palermo, Italy (recipent: Angela Criscimanna). In addition, a European Patent Request has been submitted on behalf of Carla Giordano, Aldo Galluzzo, Angela Criscimanna and Giovanni Zito.

The project relative to Pancreatic Regeneration has been awarded the 2010 Cochrane-Webber Grant from the University of Pittsburgh, USA (Funding: 30,000$; Title: Dynamics of Endocrine Regeneration in Adult Mouse Pancreas; PI: Angela Criscimanna); and the first prize for Outstanding Poster at the Annual Rangos Research Symposium, held in Pittsburgh, USA.