Regeneration of Pancreatic Insulin-Producing Cells By *In Situ* Adaptive Cell Conversion

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Abstract

The impaired ability to produce or respond to insulin, a hormone synthesized by the pancreatic β -cells, leads to diabetes. There is an excruciating need of finding new approaches to protect or restore these cells once they are lost. Replacement and *ex vivo* directed reprogramming methods have an undeniable therapeutic potential, yet they exhibit crucial flaws. The *in vivo* conversion of adult cells to functional insulin-producing cells is a promising alternative for regenerative treatments in diabetes. The stunning natural transdifferentiation potential of the adult endocrine pancreas was recently uncovered. Modulating molecular targets involved in β -cell fate maintenance or in general differentiation mechanisms can further potentiate this intrinsic cell plasticity, which leads to insulin production reconstitution.

Introduction

Diabetes, i.e. the excessive production of urine (polyuria) associated with tenacious thirst (polydipsia), is a symptom common to many different diseases characterized by a debilitating persistent excess of glucose in circulating blood (hyperglycemia). The high blood sugar is typical of diabetes *mellitus* (distinct from diabetes *insipidus*, a “brain-kidney” disease). Diabetes mellitus results from the impaired ability of either (i) the endocrine pancreas to produce the hormone insulin, or (ii) the target tissues (brain, liver, muscle...) to respond to it, or both. The two prevailing forms of diabetes, named Type 1 and Type 2 (T1D and T2D), are characterized by the loss of insulin-producing islet cells: total or near total in T1D, due to an autoimmune destruction, or variable and partial in T2D^{1,2}. A cure for insulin-dependent diabetes requires the reconstitution of a functional β -cell mass, either through *in situ* regeneration or by cell-based replacement therapies, i.e. the transplantation of surrogate β -like cells obtained from stem cells^{3,4} (Figure 1).

In recent years, several observations have revealed an astonishing intrinsic plasticity in the pancreatic islets of Langerhans⁵. These findings allow envisioning new strategies for treating diabetes by exploiting the *in vivo transdifferentiation* potential of diverse pancreatic cell types (Figure 1). Due to space constraints, in this mini-review we will solely address the main advances towards this goal by focusing exclusively on the experimental settings in which reprogramming into insulin production (either natural or guided, of pancreatic or extra-pancreatic cells) satisfied the following criteria: i) was described *in vivo*, ii) occurred during postnatal life, and iii) reinstated, transiently or permanently, blood sugar levels (glycemia) in diabetic animals.

Choosing a β -cell reconstitution strategy: replacement vs. regeneration

The last 20 years have seen a rapid development of β -cell replacement strategies, the most common being the allotransplantation of healthy pancreatic islets from cadaveric donors⁶ (Figure 1). This method is limited by the scarcity of donors and the inherent graft decay, with only 44% of the recipients being insulin-independent 3 years after transplantation⁷. Consequently, finding a more accessible source of β -cells is mandatory for the development of regenerative therapies to treat diabetes.

An increasingly popular line of research focuses on generating mature insulin-producing cells *in vitro* starting from human induced pluripotent stem cells (hiPSC), derived from somatic cells of normal donors (such as fibroblasts), as an alternative to islet allotransplantation (Figure 1). Although this approach has the advantage of generating a potentially unlimited number of β -like cells, it still faces some controversy regarding graft rejection complications^{8,9}, thus requiring further research directed at designing optimal delivery

methods (encapsulation devices)¹⁰, or developing genetically-modified β -like cells from autologous patient-derived iPSC¹¹. Also, most current cell differentiation protocols have limiting flaws linked to heterogeneous yields and tumorigenesis^{3,12,13}.

An alternative approach to the *in vitro/ex vivo* differentiation of surrogate β -cells is the exploitation of the natural *in vivo* β -cell regenerative capacity of the pancreas, primarily by stimulating β -cell self-replication¹⁴⁻¹⁶ (Figure 1). Nevertheless, this approach is inadequate for treating patients with complete or near-complete absence of β -cells, as reported in many T1D cases, among other limitations.

Adaptive transdifferentiation is a conserved regeneration mechanism

The body has developed two main natural strategies to replenish lost cell populations, which are different depending upon the capacity of the cells to enter the cell cycle (summarized in Figure 2).

Cell transdifferentiation, conversion, reprogramming or fate change, is a stable switch in cell identity, where a terminally differentiated cell converts into a different mature cell-type, with or without experiencing a transitional proliferative stage (reviewed in¹⁷⁻²²). It occurs naturally in response to various stressors (reviewed in²³) and represents an ancient and widespread regenerative strategy among metazoans, being described from cnidarians to vertebrates (reviewed in^{19,24-26}; Table 1). However, the transdifferentiation nature of the regenerative process remains controversial in some cases, because it may occur alongside other regenerative mechanisms^{22,27,28}. Two examples are fin regeneration in fish and limb regeneration in amphibians, where cell lineage tracing experiments have revealed that most cell types are lineage-restricted²⁹: upon injury, differentiated cells in the proximity of the wound form the blastema, i.e. they de-differentiate before rebuilding the original tissue by giving rise to the original cell type without a change in fate³⁰⁻³². Interestingly though, fin regeneration still occurs after osteoblast ablation, thus proving that bone can regenerate from alternative sources³³.

Two different mechanisms of cell transdifferentiation were described already a century ago: (1) direct conversion, whereby cell type A trans-fates directly into cell type B, in absence of cell division and displaying a transient hybrid phenotype, and (2) indirect conversion, where cell type A de-differentiates, proliferates and re-differentiates acquiring the B cell type fate. The first process is characteristic for morphallaxis, while the second is a type of epimorphosis³⁴ (Figure 2).

In order to determine the occurrence of a cell fate conversion event in response to injury, the resulting differentiated cell type must be i) lineage-traced to its original progenitor, and ii) characterized at the morphological and molecular levels³⁵. These changes can be reflected by

wide differences in the transcriptional landscape, including, for instance, the expression of specific genes such as those encoding peptidic hormones and the enzymes involved in their conversion and secretion²¹.

Cells and their progeny can be irreversibly (genetically) tagged *in vivo*, in transgenic animals^{36,37} (reviewed in³⁸⁻⁴⁰). The first *in vivo* cell lineage tracing analysis during mouse embryonic development using the Cre/loxP system was performed by one of us in a study of pancreatic cell fate allocation⁴¹. A few examples of *in vivo* lineage-tracing studies in development and regeneration in other organs are indicated here^{5,42-47}. This cell labeling method eliminates any ambiguity regarding the identity of the precursors of converted cells⁴⁸⁻⁵⁰. Moreover, the same transgenic setup may be used for the simultaneous constitutive or inducible, transient or irreversible, modulation of key genes in the cell type of interest.

The lack of a cell tracing system is a major limitation in any study involving human diabetic patients⁵¹. It is also a limiting factor in analyses of experimental diabetes: for instance, in a murine model of severe injury in which pancreatic duct ligation was combined with alloxan-induced β -cell destruction, the authors claimed that α -cells convert into β -like cells based only on marker co-expression, which is never conclusive⁵².

Guided pancreatic cell transdifferentiation: the artificially induced cell plasticity

In homeostatic conditions, β -cells are located in endocrine units termed islets of Langerhans, intermingled between the acini and ducts of the exocrine pancreas. Besides the preponderant β -cells, pancreatic islets contain other endocrine cell types, each secreting a different hormone: glucagon (α -cells), somatostatin (δ -cells) and pancreatic polypeptide (PP-cells).

The regenerative potential of the pancreas was first described almost 20 years ago, yet without adequate lineage tracing tools, when several studies reported the emergence of new endocrine cells, including insulin-producing cells, following tissue injury (reviewed in²⁶). The authors suggested an exocrine cell transdifferentiation process, rather than the recruitment of “dormant” stem cells.

Recent years have witnessed attempts at generating insulin-producing cells *in vivo* by ectopically inducing β -cell-specific programs in extra-pancreatic and pancreatic cells, either by gene therapy or pharmacologically. The objective of any guided cell-fate switching strategy is to asymptotically reach a “perfect” conversion while minimally interfering with the original target cell population, so as to avoid paradoxical side effects. One example is the study of Melton and colleagues⁵³, who reprogrammed exocrine cells into insulin production in mice by injecting into the pancreas different combinations of adenoviruses encoding 9, 6 or 3 key β -cell transcription factors; the highest conversion rate inversely correlated with adenoviral cocktail complexity. Only 3 key transcription factors were sufficient for β -cell

conversion induction: Pdx1 (required during early pancreas development as well as for β -cell maintenance), Ngn3 (required for islet endocrine progenitor fate allocation) and MafA (required for β -cell maturation). In a long-term study, Zhou and colleagues reported the persistence of induced β -like cells for up to 13 months and, interestingly, their aggregation into islet-like structures⁵⁴. These studies and others⁵⁵ showed that these 3 factors are absolutely necessary for an efficient acinar-to- β -like transdifferentiation.

In another study⁵⁶, Heimberg and colleagues reported that the acinar cell transdifferentiation can be pharmacologically induced with cytokines (epidermal growth factor and ciliary neurotrophic factor treatment). The regenerated β -like cells were able, like in the Zhou study, to rescue diabetes and maintain euglycemia for up to 8 months in mice where diabetes was induced by streptozotocin (STZ) administration.

The endocrine pancreas is a natural choice as source of new β -like cells. Due to the difference in ratio between non- β - and β -cells in islets (β -cells represent almost 80% of the islet cell mass in rodents), one concern of the *in vivo* islet cell conversion towards the β -cell fate is the risk of decreasing or even losing another islet cell type. In this regard, Collombat et al. reported that the transgenic misexpression of the β -cell-specific transcription factor Pax4 in α -cells (Pax4OE) leads to a loss of 77% of them, together with a progressive increase in insulin+ cells⁵⁷. Indeed, most Pax4OE α -cells became β -like cells. The authors hypothesized that the constant α -to- β -cell conversion process depleted the α -cell compartment, i.e. the pancreatic glucagon content, hence triggering a compensatory α -cell neogenesis through the mobilization of ductal precursors. The newly formed α -cells adopted a β -cell fate, generating a positive feedback loop, which ultimately resulted in islet hyperplasia⁴⁹ (reviewed in⁵⁸). Nevertheless, the authors' interpretation is refuted by the lack of any compensatory α -cell neodifferentiation in a genetic model of almost complete α -cell ablation⁵⁹, thus raising the possibility that an alternative explanation accounts for the observed series of events. In fact, by using a genetic model allowing the specific and inducible ablation of 98% of adult α -cells, we observed that the remaining 2% α -cell mass maintains basal glucagon levels (glucagonemia)⁵⁹. This is incidentally encouraging, since an induced massive α -cell conversion could be envisioned in guided α -cell conversion protocols (see below).

The important concept, however, is that the ectopic expression of just one single β -cell-specific transcription factor in α -cells is sufficient to steadily transdifferentiate them towards a stable β -cell phenotype. Moreover, the Pax4OE-induced β -like converted α -cells were able to restore the glycemic control in mice made diabetic with STZ⁵⁷.

Extra-pancreatic tissues: a new source for guided β -cell generation

Likely, the number and type of factors required for acquiring a β -like cell phenotype vary depending on the targeted source cell type. Most transdifferentiation studies concern the

pancreas, but there is also interest in organs with a related developmental origin, such as the liver^{60,61} and gut⁶².

In one such study, Slack and colleagues were able to generate glucose sensing insulin-secreting cells in diabetic mice by converting Sox9⁺ cells, which are located in the small bile ducts of the liver⁶³. Interestingly, transdifferentiation was induced by a single polycistronic adenovirus encoding the same 3 factors Pdx1, Ngn3 and MafA, indicating that this trio is also highly efficient at inducing a β -like cell fate in extra-pancreatic tissues.

Overexpression of β -cell-specific factors is not the only alternative for making β -like cells. For instance, although the transcription factor FoxO1 is expressed in β -cells, it is not a β -cell-specific marker, and its inactivation does affect neither the formation nor the cell architecture of the endocrine pancreas. By inactivating FoxO1 in gut Ngn3⁺ enteroendocrine precursor cells of mice⁶⁴, Accili and co-workers directed their differentiation towards a β -cell fate. The resulting cells expressed β -cell maturity markers and were able to cure diabetes.

This group also showed that, intriguingly, the constitutive selective inactivation of FoxO1 in β -cells leads to their de-differentiation in situations of metabolic stress⁶⁵. This observation has led the authors to postulate that, instead of dying, perhaps some β -cells dedifferentiate in T2D.

Spontaneous reprogramming of pancreatic islet non- β -cells: the innate plasticity

To accurately determine if the adult pancreas can regenerate new β -cells once they are lost, like in T1D, and to characterize the cellular and molecular programs activated in the pancreas in response to β -cell loss, we used specific cell tracing tools in a transgenic model of inducible acute, rapid, selective and total β -cell removal^{48,50}.

We found that, unexpectedly, β -cell ablation triggers the natural and spontaneous reprogramming of a small fraction (~2%) of the adult glucagon-producing α -cells into insulin production. This α -to- β conversion includes an intermediary hybrid transitional stage of insulin+/glucagon+ bihormonal cells, which was confirmed by genetic lineage tracing analyses⁴⁸. Of note, this is one of the very few reported cases of naturally occurring direct transdifferentiation in the animal kingdom, as it occurs in the absence of cell proliferation. Probably as a consequence of the proliferation-independent character of the change of identity process, α -cells retain the capacity of engaging into insulin production in aged mice as well, demonstrating a persistence of plasticity throughout life, which could have clinically relevant implications⁵⁰.

Further studies on the influence of age on β -cell reconstitution following total β -cell ablation revealed that juvenile prepubescent mice always recover from diabetes after near-total β -cell ablation⁵⁰. Moreover, their recovery was faster, being able to regain a glycemic control by 5 months of regeneration, correlated with a rapid replenishment of the β -cell pool. Surprisingly,

genetic lineage-tracing studies revealed a completely novel regeneration mechanism, involving the massive recruitment of another hormonal cell type, δ -cells, which de-differentiate to a progenitor stage, reenter the cell cycle, and recapitulate embryonic development to become insulin producers. Interestingly, this early timeline of the juvenile regeneration mechanism resembles closely the one described for lens regeneration in urodeles (reviewed in^{5,21,25}). The δ -to- β reprogramming is confined to a very restrictive window at the beginning of regeneration and is required for generating a “minimal pool” of insulin producing β -like cells. These regenerated β -like cells secreted insulin and expressed typical β -cell-specific markers, yet they displayed striking differences in the expression of key cell cycle regulators. For this reason, these cells were able to undergo several rounds of proliferation and finally reconstituted up to 30-70% of the age-matched control β -cell mass. Notably, even after 2 years of regeneration the recovered β -cell mass never exceeded the one of age-matched controls suggesting a stringent regulation of the regeneration event, consistent with the observations in classical regenerative structures and systems.

The efficient juvenile regeneration mechanism raises the interest for developing “rejuvenation” strategies aimed at fostering or maintaining the juvenile regenerative mechanism later in life⁵⁰. In this respect, the initial, yet limited, comparative transcriptional analysis following total β -cell loss revealed that *FoxO1* and its downstream effectors display a divergent behaviour in juvenile and adult δ -cells upon injury. *FoxO1* is a transcription factor involved in cell cycle and senescence regulation. Its decrease in juvenile δ -cells and the detected low levels of its direct targets, such as senescence markers *Cdkn1a* (p21) and *Cdkn1b* (p15Ink4b), was consistent with an increased proliferative capacity. In contrast, following injury, *FoxO1* and cyclin-dependent kinase inhibitors were upregulated in adult δ -cells, thus potentially explaining their incapacity to engage into an efficient regenerative program during adulthood. Interestingly, the juvenile mechanism could be somewhat mimicked with a pharmacological inhibition of *FoxO1* after injury, which promoted the δ -to- β conversion in adulthood⁵⁰.

Prospective

The production of experimental models for selective cell ablation *in vivo* will continue to lead to discoveries about tissue homeostasis and adaptive cell plasticity during embryonic development and during regeneration in pathological conditions or after injury, as has been the case in the pancreas^{48,66}.

In vivo generation of surrogate β -cells from alternative non- β -cell sources represents a promising approach to treat diabetes, especially when β -cell loss is total. It appears that there

may be efficient regeneration of β -cells in children with T1D or having undergone subtotal pancreatectomy^{5,67,68}. Also, insulin+/glucagon+ bihormonal cells have been described in human diabetic patients⁶⁹⁻⁷¹ and upon *ex vivo* epigenetic manipulation⁷², suggesting that human α -cells might also display the plasticity allowing insulin production. Yet, whether these are reprogrammed α -cells remains mysterious; since it is not possible to lineage-trace them to their origin, these could on the contrary be de-differentiated β -cells that start expressing glucagon, as claimed by some⁵¹.

The natural regeneration potential of the pancreas is age-dependent, with two distinct reprogramming mechanisms being employed according to the age at which the regenerative stressor occurs. The efficient juvenile regeneration is proliferation-dependent and hence restricted to a narrow window during early post-natal life. Current evidence suggests that unknown factors promote the accumulation of senescence markers in adult pancreatic islet cells, thus triggering a steep proliferation potential decrease and blocking the efficient juvenile regenerative program. These factors could be linked to sexual maturation, ageing, dietary change or other. In contrast, the adult regeneration mechanism is proliferation-independent and consequently the cell senescence status does not affect the 1:1 direct transdifferentiation event (age-independent). A consequence of these observations is that, besides cell sources and target molecules, one should also consider the age of a tissue when designing replacement strategies.

Understanding the cellular and molecular basis of innate adaptive islet plasticity and pancreas development is a requirement for designing efficient guided transdifferentiation strategies. In recent years we have seen a diversification of methods for the generation of replacement β -cells from non- β -cells (Figure 3), including the up-regulation of β -cell-specific factors, as well as the modulation of general regulators of cell differentiation and the cell cycle, both in pancreatic and extra-pancreatic tissues. Given the functional complexity of β -cells, it is unlikely that the artificial modulation of one single factor will suffice to produce a bona-fide β -cell equivalent; rather, a design involving the combinatorial regulation of key β -cell factors and the niche influence will be required.

Future studies will help identifying the critical molecular targets and signals, which foster the plasticity leading to the generation of replacement β -like cells.

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Figure Legends

Figure 1. Synopsis of present-day and tentative approaches to treat diabetes. Today, physicians try to maintain and improve insulin secretion and β -cell survival / function; in extreme situations, the only solution is transplantation (of isolated islets or total pancreas). The two prospective broad strategies of the *regenerative medicine* approach are β -cell replacement and β -cell regeneration. The two largely rely on the exploitation of the recently discovered cell plasticity of the adult.

Developing an efficient protective immunomodulation against β -cell autoimmunity will be an additional requirement in T1D conditions.

Figure 2. The natural strategies to replenish lost cell populations *in vivo* rely upon *adaptive increased cell proliferation*, in tissues with high renewal rates, or on *adaptive changes of cell identity* (conversion), in tissues with low proliferation capacity.

At the tissular injury level, limb amputation does not imply the loss of a given specific cell type, since in the remaining member all cell types are present, in contrast with selective cell ablation situations; therefore, limb regeneration after partial amputation appears as “low tissular injury” condition. The examples listed are referenced in **Table 1**.

Figure 3. Integrative view of the innate and guided cell conversion approaches aimed at reconstituting lost β -cells, classified according to the cell type of origin and the nature of the reprogramming stimuli.

DT, diphtheria toxin-mediated β -cell ablation (as used in references #49 and #51); PDL, pancreatic duct ligation (surgical method to trigger pancreatitis in rodent models, and therefore study pancreatic tissue remodeling).

Organism	Organ / Tissue	Cell Conversion Type	References
Cnidarians (<i>Hydra vulgaris</i> AEP)	Hypostome (Granular mucous cells)	Direct	Siebert et al. 2008 ⁷³
Echinoderms (<i>Eupentacta fraudatrix</i>)	Digestive tract (anterior rudiment)	Direct	Mashanov et al. 2005 ⁷⁴
Echinoderms (<i>Holothuria forskali</i>)	Cuvierian tubules	Indirect	VandenSpiegel et al. 2000 ⁷⁵
Echinoderms (<i>Apostichopus japonicus</i>)	Respiratory trees	Direct & Indirect	Dolmatov and Ginanova 2009 ⁷⁶
Fish (Zebrafish) (<i>Danio rerio</i>)	Liver	Indirect	He et al. 2014 ⁷⁷
Fish (Zebrafish) (<i>Danio rerio</i>)	Heart	Indirect	Zhang et al. 2013 ⁷⁸
Teleosts (Zebrafish) (<i>Danio rerio</i>)	Fin rays (osteoblasts-depleted fins)	Indirect	Singh et al. 2012 ³³
Teleosts (Zebrafish) (<i>Danio rerio</i>)	Lateral line and inner ears (sensory hair cells)	Direct	Reviewed in: Monroe et al. 2015 ⁷⁹
Amphibians: Anures (Frog) (<i>Xenopus laevis</i>)	Lens regeneration & Neural retina regeneration	Indirect	Yoshii et al. 2007 ⁸⁰
Amphibians: Urodeles (Newt) (<i>Notophthalmus viridescens</i>) (<i>Triturus viridescens</i>) (<i>Cynops pyrrhogaster</i>)	Wolffian lens regeneration & Neural retina regeneration	Indirect	Reviewed in: Barbosa-Sabanero, et al. 2012 ⁸¹ ; Tsonis et al. 2004 ⁸²
Avians (Chick) (<i>Gallus gallus</i>)	Cochlea (sensory hair cells)	Direct & Indirect	Reviewed in: Stone and Cotanche 2007 ⁸³
Mammals (Mouse) (<i>Mus musculus</i>) JUVENILES	Pancreas (β -cells)	Indirect	Chera et al. 2014 ⁵⁰
Mammals (Mouse) (<i>Mus musculus</i>) ADULTS	Pancreas (β -cells)	Direct	Thorel, Népote et al. 2010 ⁴⁸
Mammals (Mouse) (<i>Mus musculus</i>)	Skin and heart	Direct	Davis, Burr et al. 2012 ⁸⁴ Reviewed in: Hinz 2007 ⁸⁵
Mammals (Mouse) (<i>Mus musculus</i>)	Peripheral nerves (Wallerian degeneration)	Indirect	Arthur-Farraj et al. 2012 ⁸⁶ Reviewed in: Jessen et al. 2015 ⁸⁷ Jessen et al. 2015 ²²
Mammals (Mouse) (<i>Mus musculus</i>) NEONATAL	Cochlea (sensory hair cells)	Direct & Indirect	Bramhall et al. 2014 ⁸⁸ Cox et al. 2014 ⁸⁹ Reviewed in: Richardson and Atkinson 2015 ⁹⁰
Mammals (Mouse) (<i>Mus musculus</i>)	Liver (biliary epithelial cells)	unknown	Yanger et al. 2013 ⁹¹

Table 1. Documented examples of regeneration through adaptive cell plasticity in kingdom Animalia.

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Using triazole–thiomorpholine dioxide alginate capsules, the authors were able to transplant glucose-responsive mature β -cells, which were derived from human embryonic stem cells. This is the first study showing glycemic correction in diabetic mice without any immunosuppression.
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Using skin biopsies from two MODY2 subjects, the authors generated induced glucose-responsive mature β -cells and showed that higher glucose levels are needed to stimulate insulin secretion in GCK mutant β -cells. This is the first study showing mutation-specific phenotype reversion upon gene sequence correction by homologous recombination.
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Figure 1
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Figure 2
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GUIDED TRANSDIFFERENTIATION **SPONTANEOUS TRANSDIFFERENTIATION**

Figure 3
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