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Reprogramming of human exocrine pancreas cells to beta cells

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1. Introduction

The ridges in Waddington’s illustrious epigenetic landscape that represent the process of cellular decision-making during development have flattened and morphed into an “epigenetic disc” [1]. Waddington proposed his conceptual landscape in 1957, but the “creodes” he coined as a metaphor for unidirectional lineage commitment were soon challenged by paradigm shifts [2]. Spemann originally conceived the fantastical experiment of somatic cell nuclear transfer (SCNT) to answer the question whether the nucleus of a differentiated cell retains the genomic capacity to direct all types of differentiation [3]. Nuclear transplantation was elaborated in experiments performed by Briggs and King, but the factual possibility of transition between epigenetic states in differentiated cells was first demonstrated in Gurdon’s seminal paper [4]. In addition to SCNT, stressful conditions that endanger cell integrity and longevity, whether due to isolation of cells from their in vivo restrictive niches or in response to in vivo tissue damage, have been shown to result in extreme fate changes (plasticity) such as dedifferentiation (return to stemness) and reprogramming (interconversion of differentiated cell types) in an attempt to escape from damage and contribute to regeneration of the damaged tissue as soon as the risk has subdued (reviewed in [5, 6]). A further blow to the concept of a hierarchical organization of cell differentiation was delivered with the demonstration of transcription factor (TF) induced reprogramming, typically exemplified by Myod1-mediated reprogramming of fibroblasts into myotubes [7]. The value of this groundbreaking concept (reviewed in [8]) has been demonstrated extensively, especially in the blood system ([9-13], reviewed in [14]). One of the most spectacular experiments in developmental biology in the 1990’s was arguably the ectopic expression of eyeless in various imaginal disc primordia of Drosophila, causing the formation of ectopic, morphologically normal and stimulus-sensitive eyes on the wings, legs, and antennae [15]. Since a single TF could induce morphogenesis of such a complex structure as the insect eye, great expectations were directed towards the general use of master control genes for reprogramming purposes in regenerative medicine. Although the underlying cellular and molecular mechanisms remain largely elusive, a two-way relationship between transcription factor binding and chromatin structure is essential during the reprogramming process ([16-18], reviewed in [19, 20]). Reprogramming approaches should not only envisage the overexpression of TFs imposing a particular target cell fate, but also TFs disturbing the differentiation status of the original cell and TFs antagonizing genes inappropriate for the desired cell type [21-24]. Diabetes has been one of the prime interests of regenerative medicine as it is a major and increasing public health problem which could be cured by the replacement of a single cell type, the pancreatic beta cell [25]. Moreover, with the notable exception of persistent hyperinsulinemic hypoglycemia of infancy [26], the existence of an inherent regenerative potential of the human (type 1) diabetic pancreas is probably one of the most controversial aspects of current diabetes research [27]. This review will focus on the current knowledge on reprogramming of human pancreatic exocrine cells towards beta cells.

2. Reprogramming exocrine cells of the adult human pancreas

For several reasons, pancreatic duct and acinar cells represent particularly useful targets for reprogramming into beta cells as a cell replacement therapy for diabetes. First, cellular reprogramming is considered more efficient when the cells of origin share a similar ontogeny as the envisioned final cell type. The epigenetic memory, defined as the retention of regulatory labels on DNA and DNA-interacting proteins in the origin cell, is considered a hurdle to reprogramming. Obviously, this hurdle is less difficult to cross between cells with similar epigenetic landscapes (reviewed in [28]). Indeed, duct and acinar cells follow a similar developmental trajectory as their pancreatic endocrine counterparts and, although the exact origin of adult human beta cell progenitors remains uncertain, a substantial amount of evidence has put forward the “trunk” cells lining the early ducts as plausible candidates (reviewed in [29, 30]). Second, both duct cells ([31], reviewed in [32]) and acinar cells (reviewed in [33]) show a high degree of cellular plasticity. Although this phenomenon has been mostly studied in rodents, recent studies have uncovered plasticity of
human exocrine cells as well ([34-37], reviewed in [38, 39]). A theoretical framework has been proposed for the therapeutic application of induced metaplasia, referring to reversal of harmful metaplasias on the one hand and generation of useful reprogrammed cells on the other [40]. Third, besides non-beta cells, pancreatic exocrine cells share the most similar microenvironment with beta cells as compared to any other cell type. Since the differentiated state of a mature cell is most stable when the cell resides in its native environment, in situ reprogramming of pancreatic exocrine cells to beta cells is likely favorable over reprogramming in an environment other than pancreas, in order to avoid an altered phenotype or function of the reprogrammed cells [41]. Sufficiency of the pancreatic microenvironment in promoting endocrine differentiation has been suggested as acinar to endocrine conversion was seen in zebrafish upon suppression of Ptf1a, a key regulator of acinar cell fate [21]. Fourth, acinar and duct cells remain abundantly present and unaffected in patients with diabetes, in theory permitting in vivo or ex vivo reprogramming and autotransplantation. In addition, acinar cells can be isolated in large quantities from healthy donor organs. Since acinar cells constitute about 80% of the pancreatic mass, successful in vivo reprogramming of only a small acinar fraction would theoretically be sufficient to adequately correct diabetes while simultaneously maintaining its digestive function. The manipulation of acinar cells, however, also warrants caution with regard to the occurrence of pancreatitis or ductal adenocarcinomas [38]. Furthermore, although reprogramming in the context of a chronic diabetic environment appears possible in rodents [42], endothelial (progenitor) cell dysfunction in diabetic patients [43] could potentially influence the reprogramming process given the importance of the vasculature in islet ontogeny and function (reviewed in [44]). Last, the ease of in vitro manipulation of duct cells and of transdifferentiated acinoductal cells [45] supports further attempts to explore their potential as a source for new beta cells [46]. However, the rapid acinoductal transdifferentiation and extensive cell death in response to cell isolation [34, 45, 47-49] needs to be overcome to allow long-term primary culture of human acinar cells.

Our current understanding of the transcriptional regulation of pancreas organogenesis has advanced significantly over the past years owing to genome-wide gene expression analysis of pancreatic progenitor cell subsets [50]. This knowledge has been used to induce experimental reprogramming, mainly through in vitro and in vivo gain-of-function studies.

2.1. Guides for in vitro reprogramming: Neurogenin 3 and Lgr5

The class B basic helix-loop-helix TF neurogenin 3 (NEUROG3) is a master control gene during embryogenesis of the endocrine pancreas. Loss-of-function mice lack expression of several essential regulators of pancreatic endocrine development, resulting in a complete absence of endocrine precursors and mature endocrine cells [51]. On the other hand, a number of seminal gain-of-function experiments have demonstrated its potential as a master gene for reprogramming. Ectopic expression of Neurog3 in the early embryonic mouse pancreas directed the development of pancreas precursor cells prematurely and exclusively into glucagon-producing cells [52, 53]. When introduced into early chicken endoderm, Neurog3 induced premature differentiation into glucagon- and somatostatin-producing cells [54]. Manipulation of the Delta-Notch pathway by ectopic, adenovirus-mediated expression of NEUROG3 drives adult human pancreatic duct cells towards a neuroendocrine phenotype expressing low amounts of insulin [46]. Genome-wide mRNA profiling to characterize the extent of this NEUROG3-mediated duct-to-endocrine cell reprogramming identified a gene set that needs to be activated to obtain full beta cell reprogramming. Almost half of NEUROG3-activated genes in adult human duct cells belong to this gene set, but overall, NEUROG3 failed to induce full reprogramming with only 6% of the estimated path completed. NEUROG3 activated only 9% of a beta cell-abundantly expressed gene set, underscoring incomplete reprogramming [55]. In their landmark study, Zhou et al. demonstrated that adenoviral overexpression of three TFs Neurog3, Mafa, and Pdx1, coined M3 factors, was sufficient to convert murine adult acinar cells in vivo into beta-like cells [18]. The original protocol was significantly improved through polycistronic expression of the M3 factors since the obtained beta-like cells
persisted long time \textit{in vivo}. This persistence seemed critically dependent on the induction of a sufficiently large number of insulin$^+$ cells to reach an apparent threshold for self-assembly into islet-like structures [56]. Perturbation of gene expression patterns using multiple TFs has been widely applied to the exocrine pancreas, however, assuming a linear relationship between cellular phenotype and TF expression is too simplistic and has proven inadequate [57]. Most likely, full endocrine reprogramming will have to include epigenetic modifications, such as a dynamic regulation of Polycomb repression, shaping the identity of differentiated beta cells [58]. Another route towards successful reprogramming might be through addressing the help of pioneer TF families which dynamically open chromatin in the absence of cell division, thereby creating a permissive environment for others TFs to bind [59]. In undifferentiated endoderm cells, FoxA and GATA are among the first to engage silent genes and enable subsequent access for other TFs (reviewed in [60]). FoxA1 and FoxA2 are essential TFs for Pdx1 expression and pancreas growth \textit{in vivo} [61] and GATA-4 was shown to be a marker of exocrine pancreas differentiation, whereas GATA-6 marks development of the endocrine pancreas (reviewed in [62]).

Wnt signaling plays a crucial role in the regulation of multiple adult stem and progenitor cell types [63]. The Wnt target gene Lgr5 (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) was recently discovered as a marker for intestinal epithelial stem cells. Lineage tracing studies subsequently identified Lgr5$^+$ cells in non-intestinal tissues with high cellular turnover such as stomach and skin, while devoid from organs with low cellular turnover such as liver and pancreas (reviewed in [64]). However, in analogy with the findings in liver [65], upon tissue injury by partial-duct ligation (PDL), expression of Lgr5 was induced in regenerating pancreatic ducts. When isolated from PDL pancreas or activated by Wnt signaling, Lgr5-expressing duct cells could be clonally expanded into budding cysts, called organoids and their potential to differentiate into endocrine cells upon transplantation, albeit at low efficiency, was demonstrated [66]. It remains to be determined whether Injury-induced facilitation of TF-mediated reprogramming of Lgr5$^+$ cells is possible in analogy with Sox2-mediated reprogramming in the injured cerebral cortex [67]. Nevertheless, this novel \textit{in vitro} model provides us with a tool to further optimize endocrine differentiation protocols.

2.2 \textit{In vivo} models of beta cell regeneration as toolboxes: partial duct ligation and EGF/CNTF

The existence of beta cell precursor cells in adult mice has been shown \textit{in vivo} in PDL [68]. An increase in the functional beta cell mass through precursor cell differentiation and proliferation, rather than by self-duplication of pre-existing beta cells only, was demonstrated [68]. However, these findings have proven controversial [69, 70], highlighting the inherent disadvantage of variability of the PDL technique, depending on the study population and operator. Nevertheless, the existence of adult Neurog3$^+$ facultative progenitor cells initiated further studies into the role of Neurog3 in beta cell proliferation in adult pancreas. Beta cell mass expansion induced by PDL was attributed for 86% to proliferation of pre-existing beta cells while, at sufficiently high Neurog3 expression levels, up to 14% originated from non-beta cells [71]. Conditional inactivation of Neurog3 in mature beta cells but not in non-beta cells or newly formed beta cells decreased beta cell proliferation and total insulin content of the pancreas demonstrating Neurog3’s essential role in these processes in PDL pancreas [71]. The lack of detecting a contribution of duct cells to beta cells after PDL has been used as evidence against neogenesis [72-74], however our observation of the doubling in number of small endocrine cell clusters in PDL pancreas, together with the increased overall volume of tissue represented by beta cells (without appreciable beta cell hypertrophy), strongly supports continuous neogenesis [75]. Sorted beta cells from PDL pancreas show an increase in Ki67 mRNA, as well as G2/M phase regulators CYCLIN B2, CKS2 and CDK1. A positive correlation between Ki67 and cyclin B2 mRNA expression in these cells suggests that the high level of cyclin B2 gene expression independently gauges beta cell proliferation beyond the G1-S phase [75]. An initiating factor in progenitor activation and/or beta cell proliferation may be the near complete disappearance of acinar tissue after PDL, in analogy to plasticity of isolated cells upon release from their restrictive \textit{in vivo} niche. Such a recapitulation of elements of embryonic development has also been observed in a
model of acute pancreatitis with early major loss of acinar tissue [76]. As an experimental model, PDL can thus be used to distillate factors important for beta cell regeneration. For example, activation of alpha cell proliferation in PDL pancreas has been shown to require interleukin (IL)-6 signaling [77], while immune cells appear to play only a limited role overall [78].

Subjecting adult rat exocrine pancreatic cells in a 3-day culture period to the combination of epidermal growth factor (EGF) and leukaemia inhibiting factor (LIF), a member of the IL-6 family in vitro resulted in the generation of insulin producing beta cells [79]. This combinational treatment induced a transient expression of both Neurog3 and its upstream activator hepatocyte nuclear factor 6. Inhibition of EGF and LIF signaling by pharmacological antagonists of the JAK2/STAT3 pathway, or by knockdown of Neurog3 by RNA interference prevented the generation of new insulin+ cells [80]. Extending the insights gained from the in vitro experiments, transient cytokine therapy with EGF and Ciliary Neurotrophic Factor (CNTF), a cytokine of the IL-6 family but with an improved in vivo toxicity profile compared to LIF, was elegantly shown to induce acinar cell reprogramming to beta-like cells, capable of restoring normoglycemia in mice with chronic hyperglycemia. The regenerative process was shown to depend on STAT3 signaling and to require a threshold number of Neurog3 expressing acinar cells. Epigenetic profiling of the newly formed beta-like cells showed increased accessibility to the beta cell-specific insulin promotor and methylation of the acinar cell-specific amylase promotor [42]. Overexpression of constitutively active mitogen-activated protein kinase (MAPK) and STAT3 by lentivirus transduction of isolated human exocrine cells allowed for partial endocrine reprogramming, providing proof-of-concept in human. The reprogramming process however is inefficient and the obtained beta-like cells are immature. Subsequent short-term (6 weeks) transplantation under the kidney capsule of immune-compromised mice allowed for significantly improved endocrine differentiation. In long term transplantation, a further significant rise in circulating human C-peptide could be observed from day 90 onwards, indicating further endocrine differentiation [81].

3. Future perspectives

In order to advance regenerative medicine in diabetes, it is crucial to address some of the major challenges in exocrine reprogramming. First, the efficiency of reprogramming (i.e. the extent of reprogramming) varies strongly but is generally low. This not only hampers reaching full function but may also result in reversion to the original cell type or even malignant transformation. Models of TF perturbation often require high expression levels of reprogramming factors [28]. The short half-life of NEUROG3 may explain the low efficiencies of exocrine-to-beta cell reprogramming by ectopic expression of NEUROG3 or activation of its endogenous gene. Indeed, NEUROG3 protein stability is regulated by the ubiquitination and proteasome-mediated degradation and rapid turnover of NEUROG3 appears regulated both by binding to its heterodimeric E protein partner and by the presence of cdk inhibitors [82]. NEUROG3 is a target for the SCF-type E3 ubiquitin ligase substrate recognition component FBW7. Loss of FBW7 increases NEUROG3 stability and induces duct-to-beta cell transdifferentiation in the adult pancreas [83]. Further studies are needed to unravel the regulation of NEUROG3 stability while methods to prolong its half-life may improve reprogramming efficiencies. In addition, low reprogramming efficiencies may be explained by our still incomplete understanding of master regulators and networks that drive pancreatic endocrine development [84]. A more complete knowledge of these regulators and networks as well as of the role of microRNAs and other non-coding RNAs may identify new tools to overcome barriers to reprogramming, especially of epigenetic nature ([85], reviewed in [86]). More efficient reprogramming of fibroblasts towards hematopoietic, neuronal or cardiac fates has been attained by the forced expression of pluripotency-inducing factors (especially Oct4) during the early stage of reprogramming to induce a transient state of plasticity (without reaching full pluripotency), followed by induction of directed differentiation with lineage-specific TFs or defined culture medium [87, 88]. However, the risk for persistence of pluripotency markers which may lead to tumorigenesis in the reprogrammed cells may
limit this approach. Unravelling the nature of rate-limiting barriers in cellular reprogramming will be required in order to advance from stochastic reprogramming towards a deterministic approach.

Second, the relation between TF expression and cell fate is not linear and relates to dynamic gene regulatory networks. Recently, a hierarchical mechanism in direct lineage reprogramming of fibroblasts to neuronal cells was described in which Ascl1 functioned as an “on target” pioneer factor by occupying most cognate genomic sites in fibroblasts only when these loci exhibited a particular trivalent histone modification signature [89]. The other reprogramming factors did not directly interact with the chromatin but recruited other regulators, thereby highlighting the importance of a precise match between pioneer factor(s) and the chromatin context at key target genes. A correlation between histone modifications and reprogramming potential has been observed in islet cells from human pancreas [90]. Mathematical modeling may outperform current empirical approaches to determine the optimal sequence of multiple TF perturbations as well as identify synergic and antagonistic effects. Simulations of perturbation experiments using *Mafa*, *Neurog3* and *Pdx1* have shown that optimal exocrine-to-beta cell reprogramming is only achieved when *Mafa* and *Neurog3* are perturbed first. Recently, an influence of the order of expression of reprogramming factors on the acquisition of a mature beta cell phenotype has been described for liver to pancreas transdifferentiation [91].

Third, the epigenetic memory of the cells of origin remains a major obstacle to efficient reprogramming. Indeed, despite induction of a transcriptional profile consistent with reprogramming to the desired cell type, often some residual gene expression specific to the cell type of origin persists, a problem which may prove challenging to resolve since epigenetic memory even persists in embryos, generated from somatic cell nuclei [92]. On the other hand, although exocrine cells are closely developmentally related to their endocrine counterparts, key endocrine promoters are silenced in the former. In order to obtain full reprogramming into functional beta-like cells, a long and as yet obscure process of *in vivo* maturation is often needed. This black box of maturation has long been deemed necessary for human embryonic stem cell (hESC) directed differentiation *in vitro*, however recent advances have identified suitable culture conditions supporting maturation into functional beta-like cells [93, 94]. In order to attain full maturation *in vitro* of beta cells and especially of highly organized islets diverse signals arising from their supportive *in vivo* microenvironment will need to be integrated. Further unravelling of the signaling between beta cells and extracellular matrix [95-97], vessels (reviewed in [98]), neurons [99-101], and the other pancreatic islet cells [102] will likely bring the goal of islet grafts with normal physiology closer. For now, the recently identified culture conditions for directed hESC differentiation are widely applied to reprogram differentiated exocrine cells *in vitro* in a similar manner as the conditions generated in PDL have allowed us to establish Lgr5⁺ pancreas organoids. Such cross-pollination of *in vitro* and *in vivo* efforts will further advance the field of regenerative medicine in diabetes. Ultimately, clinical translation will require replacement of current viral-based gene delivery methods by equally efficient but safer approaches such as non-viral gene delivery methods (reviewed in [103]) or by the use of small molecules [104] and/or modulation of intracellular signaling that mimic TF mediated reprogramming and epigenetic plasticity [42, 105]. Drug discovery in biomimetic platforms such as stem cell-based organoid models is expected to provide valuable knowledge [106]. Nevertheless, caution is warranted in the extrapolation of data obtained from rodent models to *in vitro* or *in vivo* reprogramming of human cells as exemplified by the failure of EGF and LIF to induce reprogramming of human exocrine cells [79].

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5. References


