Advances and complications of regenerative medicine in diabetes therapy

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Abstract

The rapid development of technologies in regenerative medicine indicates clearly that their common application is not a matter of if, but of when. However, the regeneration of beta-cells for diabetes patients remains a complex challenge due to the plurality of related problems. Indeed, the generation of beta-cell mass expressing marker genes is only a first step, with maintaining permanent insulin secretion, their protection from the immune system and avoiding pathological modifications in the genome being the necessary next developments.
The prospects of regenerative medicine in diabetes therapy were promoted by the emergence of promising results with embryonic stem cells (ESCs). Their pluripotency and proliferation in an undifferentiated state during culture have ensured the success of ESCs in regenerative medicine. The discovery of induced pluripotent stem cells (iPSCs) derived from the patients' own mesenchymal cells has provided further hope for diabetes treatment. Nonetheless, the use of stem cells has significant limitations related to the pluripotent stage, such as the risk of development of teratomas. Thus, the direct conversion of mature cells into beta-cells could address this issue. Recent studies have shown the possibility of such transdifferentiation and have set trends for regeneration medicine, directed at minimizing genome modifications and invasive procedures.
In this review, we will discuss the published results of beta-cell regeneration and the advantages and disadvantages illustrated by these experiments.

Introduction

Diabetes mellitus is a widespread and socially significant disease that leads to a deterioration in the quality of life and life expectancy of patients. Patients with type I diabetes (T1D) are

40 characterized by a deficiency of the pancreatic beta-cells mass, which can represent a loss of
41 between 70–100% (Lam & Cherney, 2018). Traditionally, T1D can be separated from type II
42 diabetes (T2D) by the factors triggering it, age of manifestation, and the useful strategies for
43 treatment. T2D is considered to develop due to insulin resistance rather than initial beta-cell loss.
44 However, to compensate for the imbalance, the beta-cells produce more insulin but then, after
45 years of hypersecretion, the pool of beta-cells is depleted by up to 65% in some cases. As a
46 consequence, this group of T2D patients also needs exogenous insulin administration (Butler et
47 al., 2003; Eckel et al., 2011).
48 However, exogenous control of insulin dosage cannot compensate for the sensitive adjustment
49 normally made by beta-cells. This often leads to hyper- or hypoglycemia with corresponding
50 complications increasing with the time. Hyperglycemia increases the development and
51 progression of microvascular complications such as retinopathy, nephropathy, and neuropathy
52 (Gibbons, 2020; Williams, Nawaz & Evans, 2020). Furthermore, the most severe complications
53 are those related to hypoglycemia in T1D patients, resulting in neurocognitive dysfunction (Kodl
54 & Seaquist, 2008). Thus, recovery of the pool of beta-cells is an attractive strategy for treating
55 patients with diabetes (Figure 1). Currently, only surgical islet transplantation provides such an
56 opportunity (Shapiro, Pokrywczynska & Ricordi, 2017; Matsumoto & Shimoda, 2020). Islet
57 transplantation has been developed mainly for T1D patients and involves both transfusion into
58 the portal vein and embolization within the liver, and allows the avoidance of complications
59 related to whole organ transplantation. However, such an approach is complicated by the limited
60 source of donor tissue available and by immune rejection, requiring immuno-suppression therapy
61 (Posselt et al., 2010). Furthermore, there are no approved methods in regenerative medicine that
62 can address the specific characteristics required of beta-cells.
63 Our current understanding of lesion mechanisms in beta-cells is based on rodent studies.
64 Although it appears that human pancreas does show a corresponding lack of regeneration
65 capacity under systemic disorders. Therefore the translation of these observation to human beta-
66 cells should be handled with caution (Zhou & Melton, 2018). Indeed, a comprehensive analysis
67 of a diverse array of animal models and human cell cultures might be fruitful in initiating new
68 advances in regenerative medicine.
69
70 ## Survey methodology
71 The purpose of this article is to present a comprehensive review of the literature regarding recent
72 methods of regenerative medicine in diabetes mellitus. To that aim, we performed
73 PubMed searches for such keywords and terms as "beta-cells", "pluripotent stem cells", "3D
74 culturing", "transdifferentiation", and "diabetes mellitus" among others. We then analyzed
75 articles from Q1 journal ranking quartiles within the subdiscipline, using the SJR citations
76 referenced. Although this is a comprehensive review, it is not exhaustive. It should be noted that
77 we focused particularly on the limitations of the current methods, as solutions to these problems
78 should help progress the development of regenerative medicine. However, it must be

79 remembered, that the question regarding the problems underlying beta-cell preservation remains
80 open and needs further investigation.
81
82 CHARACTERISTICS OF BETA-CELLS
83 Beta-cells are one of the five cell types that form the islets of Langerhans, the hormone-
84 producing structures of the pancreas. The islets are heterogeneously distributed in the pancreas
85 and, according to recent studies, have a variety of content and functions. Patients with removal of
86 the pancreatic tail showed increased glucose level indicators post-operatively than did patients
87 with pancreatic head ectomy (Menge et al., 2009). Menge et al. showed, that the number of islets
88 in the tail is greater than that in the head of the pancreas Such a difference can be explained by
89 the distinct embryogeneses of the pancreas head and tail: while the head originates from the
90 dorsal and ventral pancreatic bud, the body and tail are formed only from the ventral pancreatic
91 bud. (Pandol, 2010). That also influences the heterogeneity of the beta-cells, as has been
92 confirmed by single-cell RNA-seq technologies. Recent studies have revealed two
93 subpopulations of beta-cells with distinct molecular, physiological, and ultrastructural features
94 (Bader et al., 2016; Baron et al., 2016). However, further investigations of their roles in diabetes
95 are needed.
96 Beta-cell deficiency affects the ratios of other cell types in the islets of Langerhans. There are
97 complex cell-to-cell interactions in the islets, resulting in a unique cytoarchitectural structure.
98 Recent studies with beta-cell culturing in three-dimensional (3D) gels have shown the
99 importance of such interactions (Weber, Hayda & Anseth, 2008; Xu et al., 2020). In these
100 experiments the 3D gel contained extracellular matrix (ECM) proteins and directly influenced
101 cytoskeletal organization, promoting beta-cell maturation and insulin secretion. As is known,
102 diabetes patients exhibit dramatic changes not only in their hormone levels but also in the
103 structure and intrinsic cell communications within the pancreas. In this case, 3D hydrogel
104 cultures are more promising for diabetes treatment than 2D ones.
105 In T1D the deficiency of beta-cells is a result of immune attack by T- lymphocytes. Nakayama et
106 al. showed that in the NOD mouse model the insulin epitope is a prime target for effector T-
107 lymphocytes: B:9-23 peptide promotes the development of insulin autoantibodies (Nakayama et
108 al., 2007). But it is not only insulin production that is affected by this T-lymphocyte targeting;
109 due to the effect of epitope spreading, the islet-specific glucose-6-phosphatase catalytic subunit–
110 related protein (IGRP) is also targeted after insulin (Prasad et al., 2012)).IGRP is a
111 multicomponent integral membrane system that plays a crucial role in the terminal step of the
112 gluconeogenic and glycogenolytic pathways, catalyzing the hydrolysis of glucose-6-phosphate.
113 Krishnamurthy et al. demonstrated that the development of an autoimmune responses to multiple
114 autoantigens increases the progression of T1D (Krishnamurthy et al., 2006).
115 Herewith, histological analyzes show that the destruction of the beta-cells has a lobular
116 character, and some of the islets show insulin staining (Gianani et al., 2010) This can be
117 explained by the above-mentioned difference in the beta-cell populations.
118

119 BETA-CELL SOURCES *IN VIVO*
120 To date, the existence of a definite native source for beta-cell regeneration remains in dispute
121 (Teta et al., 2007). There are at least four suggested sources: beta-cells themselves, acinar cells,
122 ductal cells, and alpha-cells.
123 Adult pancreatic beta-cells are formed mainly by self-duplication, this evidence having been
124 demonstrated using a lineage tracing approach (Dor et al., 2004). Using a mouse model, Dor et
125 al. showed that at least 85% of new beta-cells were formed from pre-existing beta-cells. This
126 conclusion applied to both the normal adult pancreas and to pancreas subjected to partial ectomy.
127 However, special circumstances, e.g. pregnancy or diabetes, may activate other sources of beta-
128 cells. Acinar, ductal and endocrine cells possess Pdx1/Ptf1-positive multipotent pancreatic
129 progenitors during organogenesis. The endocrine specification of these progenitors is initiated by
130 the Neurogenin3 (NEUROG3) and neurogenic differentiation 1 (NEUROD1) transcription
131 factors (TFs), while the late stages of maturation of beta-cells is regulated by TFs including V-
132 maf musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA), V-maf
133 musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB), paired box gene 6 (PAX6),
134 and estrogen-related receptor gamma (ESRRG) (Zhu et al., 2017). Thus, alterations in the
135 ectopic expression of key transcription factors can change the fate of pancreatic cells (Table 1).
136 Embryonic ducts give rise to both differentiated endocrine and duct cells but once the pancreatic
137 duct epithelium acquires a differentiated phenotype it does not contribute significantly to new
138 beta-cells. Lineage-tracing studies by several research groups have shown that the ducts in adults
139 do not appear to be a source of beta-cells (Kopinke & Murtaugh, 2010; Kopp et al., 2011).
140 However, lineage-tracing analysis in studies by Inada et al. and Zhang et al. did demonstrate that
141 such a possibility exists (Inada et al., 2008; Zhang et al., 2016). Such discrepancies may be
142 explained by heterogeneity in the duct cell populations and of the different gene regions that
143 were used for tracing.
144 Dirice et al. showed a contribution of duct cells to the compensatory beta-cell pool by neogenesis
145 during pregnancy, with increased insulin demand (Dirice et al., 2019). Furthermore, using an
146 injury model of the pancreas in mice, Xu et al. also revealed the neogenesis of beta-cells from
147 duct cells (Xu et al., 2008). The injury involved partial duct ligation with the restriction of
148 digestive enzyme drainage from the exocrine pancreas into the duodenum. This ligation caused
149 degeneration of the acinar cells and the extensive proliferation of duct cells. Xu et al. found that
150 such ducts not only highly express NGN3, but also have the ability to form islets of Langerhans
151 in NGN3-deficient embryos. This confirmed NGN3 as a key regulator both in beta-cell
152 neogenesis and in their embryonic development.
153 Acinar cells are the most abundant type of exocrine pancreatic cells, producing enzymes for food
154 digestion. They could also be a source of beta-cells, but attempts to prove this suggestion by
155 lineage tracing methods in mouse models have been unsuccessful (Desai et al., 2007), indicating
156 that acinar cells do not normally transdifferentiate into beta-cells. Nonetheless, by exhibiting
157 significant transcriptional plasticity during culture, acinar cells remain an attractive source for *in*
158 *vitro* beta-cell generation. Thus, Pan et al. showed the ability of acinar-to-beta-cell conversion,

159   including an intermediate step with pancreatic multipotent progenitor cells (Pan et al., 2013).
160   Clayton et al. performed direct reprogramming of acinar cells on a transgenic mouse by the
161   virus-based induction of PDX1, MAFA, and NGN3 (Clayton et al., 2016) Another important
162   output from their study concerns the impact of inflammation on the reprogramming result.
163   Marked inflammation processes promote an acinar-to-ductal cell transition and inhibit the
164   number of beta-cells.
165   Despite the fact, that alpha and beta-cells play antagonistic roles, alpha-cells seem to be the most
166   promising source for beta-cell recovery, since they are the second-largest endocrine cell group in
167   the pancreas and have tight connections with beta-cells. Several studies have demonstrated the
168   possibility of alpha-to-beta transdifferentiation (Thorel et al., 2010; Bru-Tari et al., 2019). While
169   the experiments include different diabetes modeling methods in mice, their results are united by
170   the conclusion that the conversion can occur with immature alpha-cells. Only this limited
171   population has the necessary plasticity for transition in the lack of insulin. To force this transition
172   a Collombat group applied small molecules mimicking the effects of the ectopic expression of
173   Pax4 and inhibiting Arx in alpha-cells (Ben-Othman et al., 2017). Small molecules refer to
174   organic compounds with low molecular weight (<900 daltons), that may regulate biological
175   process. A Collombat group identified that gamma-aminobutyric acid (GABA) induces alpha-to-
176   beta transdifferentiation (Table 1). GABA is synthesized in beta-cells and has previously been
177   described as a regulator of hormone secretion and as a signaling molecule in islet cell
178   communication (Franklin & Wollheim, 2004). Notably, GABA-mediated transdifferentiation
179   was limited by the number of alpha-cells, which decreased in line with the concomitant increase
180   in numbers of beta-cells.
181   To sum up: pre-existing beta-cells appear to be the principal *in vivo* source of new beta-cells,
182   while the contribution of other sources is less significant. However, several groups have
183   demonstrated the feasibility of artificially inducing transdifferentiation of pancreatic non-beta-
184   cells *in vivo* (Zhou et al., 2008; Li et al., 2014). Meanwhile, as the induced beta-cells are located
185   in the native milieu, researchers have an opportunity for the direct comparison of endogenous
186   and induced beta-cells. Despite promising results, there are some points that need further
187   investigation. For instance, Zhou et al. observed a lack of organization of the induced beta-cells
188   that can impair their function. Such result may be explained by DNA methylation, which pre-
189   existed in the regulatory elements of the non-beta-cells. Detailed epigenetic maps allow this
190   problem to be overcome and a more targeted effect to be obtained (Gifford & Meissner, 2012).
191   There are also many problems with integrating viral delivery, and these can be even more
192   complicated *in vivo,* resulting in off-target effects.
193
194   BETA-CELL SOURCES *IN VITRO*
195   *Ex-vivo* generation of beta-cells remains an attractive strategy in regeneration medicine,
196   however, the differentiated cells normally have low proliferation activity. For these purposes,
197   different agonists have been tested: nutrients, growth factors, intracellular signaling molecules,
198   and small molecules (Huang & Chang, 2014). Currently, however, the proliferation of beta-cells

199   in tissue culture results in a loss of the beta-cell phenotype, making it difficult to use them for
200   diabetes therapy (Efrat, 2008). A proposed method of redifferentiation showed only low
201   efficiency (Kayali et al., 2007).
202   To date, the most promising approaches for beta-cell generation include the differentiation of
203   stem cells and the generation of beta-cells while bypassing pluripotency (Table 2).
204   1.   ESC differentiation
205   The differentiation of ESCs into beta-cells *in vitro* was developed in the early 2000s (Keller,
206   1995). The Baetge group developed the first directed differentiation protocol and identified the
207   main principles for stem cell differentiation into beta-cells (D'Amour et al., 2006). The first step
208   in the differentiation of ESCs is a very critical stage in the formation of the definitive endoderm
209   (DE) lineage (Baetge, 2008). This step is essential for the successful differentiation of the
210   pancreatic lineage. The second step involves foregut endoderm formation and requires the
211   addition of transforming growth factor-beta. Retinoic acid application is essential for the third
212   step of the pancreas specification. Retinoic acid contributes to the efficient transition to the
213   pancreatic lineage and prevents the differentiation of the pancreatic endoderm into endocrine
214   cells. During the fourth step, foregut endoderm cells are recruited to the pancreatic and endocrine
215   lineages. These cells have a high expression of PDX1 and transient expression of NGN3. During
216   the fifth step the range of different hormones normally produced by endocrine cells start to be
217   secreted. The ratio of beta-cells generated depends on the characteristics of the cell culture media
218   and the success of the previous stages.
219   Each step requires strict monitoring of the expression of marker genes by immunohistochemical
220   analysis, flow cytometry, and RT-PCR. Such an approach is essential for determining the
221   homogeneity of the phenotypes and the efficiency of the multiple differentiation steps. However,
222   even rigorous monitoring cannot guarantee the absence of teratoma formation from the
223   undifferentiated ESCs. There is still a need for developing the standards needed in ESC therapy
224   to provide safety (Hentze et al., 2009)
225   2.   iPSC differentiation
226   The limited sources of ESCs and allograft rejection of transplanted ESCs have forced a search
227   for alternative methods of beta-cell regeneration. The discovery of induced pluripotent cells
228   derived from human somatic cells has initiated a new chapter in regenerative medicine.
229   The use of iPSCs has allowed the generation of patient-specific beta-cells for autologous
230   transplantation, with adult fibroblasts being frequently used for this process, representing an
231   almost unlimited source for the generation of iPSCs.
232   Most of such iPSCs lines are made using retrovirus vectors that integrate the reprogramming
233   factors involved in stem cell pluripotency. Such an approach is associated with the risk of
234   tumorigenesis. Therefore the latest methods of iPSC generation involve non-integrating gene
235   delivery, such as the Sendai virus, plasmid transfection, the piggyback transposon system, and
236   minicircle vectors.
237   Recent studies have introduced a new strategy for the direct differentiation of iPSCs to
238   pancreatic beta-cells (Hogrebe et al., 2020). The principle is based on cytoskeleton

239 reorganization, whereby F-actin is depolymerized. It has been shown that latrunculin A and B
240 guide stem cell differentiation towards a beta-cell fate, shortening the time for endocrine
241 transcription factor expression and overcoming the requirement for 3D culture (Table 1).
242 These advances bring iPSC technology to the top of the most promising regeneration approaches.
243 However, the risks found with teratoma formation from ESCs, for instance, also exist for iPSCs.
244   3. Mature cell transdifferentiation
245 The direct conversion of adult cells without the establishment of a pluripotency step would allow
246 avoiding oncological complications. Early studies have revealed a capacity of pancreatic cells,
247 such as ductal, acinar and non-beta endocrine cells to transdifferentiate into beta-cells by using a
248 specific combination of multiple factors (PDX1, NGN3, and MAFA predominantly) (Baeyens et
249 al., 2005; Minami et al., 2005).
250 While the pancreatic cells have increased capacity for being reprogrammed into beta-cells, there
251 are still difficulties with culturing sufficient quantities of these cells. Besides, the pancreas is a
252 retroperitoneal organ and is very difficult to assess for biopsies.
253 Thus, there is a need to search for alternative sources for transdifferentiation: ones that are both
254 easy to culture and biopsy. In this case, human fibroblasts appear particulary promising. It has
255 previously been shown that fibroblasts can be directly reprogrammed into neurons, hepatocytes,
256 or cardiomyocytes (Zhu, Wang & Ding, 2015; Babos & Ichida, 2015; Chen et al., 2017). In these
257 studies, the researchers applied a cell-activation and signaling-directed (CASD) strategy. In the
258 first step of this approach, a key set of reprogramming factors (transcription factors or small
259 molecules) turn the fibroblasts into a plastic and unstable state. During this step, the cells have a
260 transient expression of pluripotency. However, several studies have shown, that such transient
261 expression of the pluripotency genes does not necessarily mean a transient establishment of the
262 pluripotency intrinsic for iPSCs (Maza et al., 2015; De Los Angeles et al., 2015). Margariti et al.,
263 in their article, termed these "partial-iPS (PiPS) cells" and showed, that PiPS cells in this state
264 did not form tumors *in vivo* (Margariti et al., 2012) To provide an appropriate level of safety for
265 the medical use of CASD technology, several researchers have suggested a CA step, using small
266 molecules instead of transgenic reprogramming factors (Table 1) (Hu et al., 2015; Li et al.,
267 2015). Furthermore, such non-integrating methods make the CA step faster and more effective
268 compared with manipulations of the genome. However, the low reproducibility of the protocols
269 involving small molecules limits their application (Kim, Jeong & Choi, 2020).
270   Subsequent treatment with sequential combinations of growth factors and soluble lineage-
271 specific signals proceed to the second step of the CASD approach. During the lineage-specific
272 SD step the unstable intermediate population of cells generates progenitors of the desired cell
273 line.
274 Currently, CASD technology has great potential in regenerative medicine and has raised hopes
275 for its clinical application. However, further investigations are needed for comprehensive
276 determination of the CA step and for obtaining homogeneity of cell conversion during the SD
277 step.
278   4. Adult stem cell differentiation

279 One other promising source for beta-cell generation is adult stem cells (ASCs), and this has
280 attracted close attention in recent years. ASCs, also called somatic stem cells, are located in
281 specialized niches and serve for maintaining tissue homeostasis. Such niches with ASCs have
282 been found in numerous tissues and organs, including the pancreas. However, these populations
283 are rare and cannot provide complete regeneration of the beta-cell mass in patients with systemic
284 endocrine disorder. Thus, various research groups have suggested proliferating ASCs *in vitro*
285 (Gurusamy et al., 2018). Mitutsova et al. have shown, that non-adherent muscle-derived adult
286 stem cells (MDSCs) have the potential to differentiate into beta-cells during *in vitro* culturing
287 (Mitutsova et al., 2017). In their experiment a multipotent stem cell population isolated from
288 adult skeletal muscle was able to form islet-like cell clusters. Furthermore, adult MDSCs injected
289 into hyperglycemic diabetic mice, successfully differentiated into beta-cells *in vivo* and
290 contributed to reducing the glucose levels. Despite these promising results, the application of
291 adult MDSCs is restricted by complications of the muscle biopsy, which requires several areas of
292 expertise and optimal cryoprocessing of the fresh specimens. In the light of this, bone-marrow
293 and adipose-derived stem cells (BMDSCs and ADSCs) are more readily available (Trivedi et al.,
294 2008; Phadnis et al., 2011). BMDSCs contain two distinct populations: hematopoietic stem cells
295 and mesenchymal stem cells, of which the mesenchymal stem cell population is more successful
296 in adopting a pancreatic fate (Carlsson et al., 2015). Zhao et al. have identified a novel cell
297 population of monocytes from adult human blood that can differentiate into efficient insulin-
298 producing cells *in vitro.* These peripheral blood mononuclear cells express ESC transcription
299 factors along with the hematopoietic markers (Zhao et al., 2007). The authors showed that, after
300 30 days of culturing, these mononuclear cells began to express the insulin gene transcription
301 factors MafA and Nkx6.1 and to produce insulin.
302 ADSCs have certain advantages compared with BMDSCs (Takahashi et al., 2019). First the
303 isolation of adipose-derived stem cells presents less harvesting difficulty(Wang et al., 2018).
304 Second, ADSCs have been shown to have a higher differentiative potential, while BMDSCs
305 tend toward osteogenesis and chondrogenesis (Dmitrieva et al., 2012). The third advantage
306 involves the immunosuppressive capacity of ADSCs. It has been shown that ADSCs do not
307 excite alloreactivity of incompatible lymphocytes, suppressing the mixed lymphocyte reaction by
308 inducing the expansion of Tregs (Puissant et al., 2005). Furthermore, ADSCs contribute to an
309 increase in anti-inflammatory cytokines (Kocan et al., 2017) However, the increased expression
310 of inflammatory cytokines and long-term fibrinolytic modifications in T2DM patients might
311 impair the efficiency of therapy by adipose-derived stem cells (Serena et al., 2016; Brovkina et
312 al., 2019).
313 Urine-derived stem cells (USCs) can also be applied as a potential stem cell source for diabetes
314 therapy (Zhao et al., 2018). The advantages of USC use consists in their noninvasive isolation
315 and expandable and simple methods of culturing. However, Zhao et al. noticed that USCs are
316 useful only in compensating for minor apoptosis of beta-cells, but not in cases of extensive
317 depletion as in T1D patients.

318 One of the major problem of the application of ASCs is their limited transdifferentiation
319 efficiency, which affects from 5 to 25% of the cells (Sharma & Rani, 2017). Another problem is
320 the low level of insulin secreted by differentiated ASCs and their limited lifespan without
321 stemness. Nonetheless, despite these current limitations the ASC approach has undeniable
322 advantages over using iPSCs since it presents a lower risk of generating teratomas and
323 autoimmune responses *in vivo*.
324 METHODS FOR BETA-CELL PRESERVATION
325 The protection of beta-cells is no less important than their generation. This is an especially
326 significant issue in the early phases of T1D or T2D when beta-cell mass is maximal. The
327 common strategy for T2D treatment is targeting insulin-resistance and beta-cell dysfunction.
328 Metformin and PPAR-gamma agonists are applied to decrease the insulin resistance, while
329 sulphonylureas and glinides enhance secretion by the beta-cells. Some of the drugs effective in
330 T2D treatment have also been considered for T1D patients. For example, verapamils, calcium
331 channel blockers, and glucagon-like peptide-1 receptor agonists decrease beta-cell injury and
332 exert beta cell-protective effects in experimental models of T1D (Chen et al., 2008; Lotfy et al.,
333 2014). However, due to the immunogenic development of T1D, immunotherapy for beta-cells is
334 more effective. Immunotherapy of T1D has two main approaches: the first is manipulation of the
335 autoimmune response independently of beta-cell specificity, while the second uses beta-cell
336 antigen-specific strategies. The limited specificity of the first approach can suppress acquired
337 immunity. In this case, antibody-based therapy is more promising. However, current studies of
338 anti-CD3 monoclonal antibodies show, that the protection of the residual beta-cell mass is short-
339 term and can result in cytokine release and unwanted inflammation (Chatenoud & Bluestone,
340 2007, p. 3).
341 In regenerative medicine, the protection of generated beta-cells is also a paramount aim.
342 However, first of all, we have to define what exactly should be protected – the beta-cell mass or
343 its function. Beta-cells have different populations and some of them survive during immune
344 attack (Keenan et al., 2010). Further investigation has revealed, that the surviving populations
345 are of lower granularity cells with stem-like features and increased rates of proliferation, but they
346 have a lower insulin content (Rui et al., 2017). Talchai et al. suggested these characteristics are
347 due to beta-cell dedifferentiation (Talchai et al., 2012). Thus, the reemergence of endocrine
348 progenitor-like cells results in beta-cell dysfunction. In such cases the beta-cells maintain the
349 expression of marker genes, like Pdx1 and MafA, but adopt a dedifferentiated fate and do not
350 release insulin. Recent work by Lee et al. confirms that dedifferentiated beta-cells exhibit
351 reduced levels of beta-cell autoantigens and increased expression of immune inhibitory markers
352 (Lee et al., 2020). Evidently, the preservation of beta-cell function and their mature populations
353 should be a priority.
354 There are two main approaches that enable the protection of transplanted cells from the immune
355 system without the need for systemic immunosuppression: islet encapsulation and "open" islet
356 transplantation(Korsgren, 2017; Peloso et al., 2018; Ernst et al., 2019). The encapsulation
357 strategy uses a biocompatible membrane that wraps the beta-cells. Such containers permit the

358 diffusion of oxygen and nutrients while protecting against larger molecules, including antibodies.
359 This strategy has several advantages. Since the mature beta-cells are more fragile, immature cells
360 are preferable for transplantation. Encapsulation both isolates immature cells from stress
361 conditions and protects them from the host immune system. A further advantage of encapsulation
362 is the possibility of immediate removal of specific containers if tumorigenesis is detected.
363 Despite some promising results, current encapsulation technology has serious limitations. One of
364 these is a difference in the kinetics of glucose and insulin diffusion compared with native islets
365 (Korsgren, 2017). Insulin secretion in encapsulated beta-cells is delayed, leading to a risk of
366 persistence of hypoglycemia. Another problem is of limited oxygen and nutrient delivery to the
367 encapsulated cells due to deficiencies in the vascularization processes (Rafael et al., 2000). A
368 third problem is the process of fibrosis that starts with recognition of nonspecific protein
369 adsorption and leads to the formation of a fibrotic capsule around the implant. Nanotechnology
370 provides opportunities to overcome or mitigate the aforementioned problems. A variety of
371 nanoscale chemical, physical, and biological properties of the interfacing polymers has been
372 shown to modulate the immune response and to enhance the vascularization processes. In this
373 case, the composition of the polymer plays a significant role in the biocompatibility. Hydrogels
374 and alginate matrix are commonly used in encapsulation technology. The modification of these
375 polymers with certain compounds and proteins both decreases the magnitude of fibrosis and
376 promotes vascularization. Thus, polyethylene glycol (PEG) with affinity peptides has been
377 shown to significantly reduce the level of chemokines, thereby contributing to a reduction of
378 inflammation (Lin et al., 2010). Hemoglobin-conjugated hydrogels improve the islet
379 oxygenation, while crosslinked antioxidant enzymes can protect the hemoglobin from hypoxic
380 and free radical stress (Nadithe, Mishra & Bae, 2012). Increasing vascularization is a challenging
381 task, because this process is accompanied by inflammation, and this negatively influences islet
382 survival. The current strategy for solving this problem includes modification of the surface
383 roughness and porosity of the polymers, combined with anchoring vascular endothelial growth
384 factor to their surfaces. Collagens, fibronectin, and laminin in the islet coat conduce the
385 vascularity of implanted islets by mimicking the natural islet matrix (Salvay et al., 2008). Vlahos
386 et al. engineered submillimeter collagen cylinders (modules) coated with endothelial cells, and
387 these promoted islet vascularization and resulted in a proangiogenic M2-like macrophage
388 response (Vlahos, Cober & Sefton, 2017). Also, the larger pores were able to act as effective
389 scaffolding for the vasculature. Brauker et al. demonstrated that a 0.8–8-μm pore size allowed
390 the penetration of host blood vessels into the device if the large-pore membrane was laminated to
391 a smaller-pore inner membrane (Brauker et al., 1995).
392 Finally, the layer-by-layer method allows the engineering of islet polymer films with versatile
393 functions and optimal composition, thickness, and mechanical properties (Wilson et al., 2011).
394 In contrast to the encapsulation strategy "open" islet transplantation permits host interaction with
395 the implanted material. The stem cell educator therapy proposed by Zhao et al. reverses
396 autoimmunity and allows the preservation of transplanted islets (Zhao et al., 2012; Zhao, 2012).
397 This therapy is based on cord blood-derived multipotent stem cells being co-cultured with the

398   patient's lymphocytes in a closed-loop system. After this, the educated lymphocytes are
399   separated and returned to the patient's circulation. The authors demonstrated that stem cell
400   educator therapy increases in the number of Tregs and restores the cytokine balance (Zhao et al.,
401   2012). Peripheral blood-derived insulin-producing cells are an alternative source that display the
402   characteristics of islet beta-cell progenitors and therefore can also provide a potential resource
403   for educator therapy (Zhao et al., 2007).
404   The application of stem cells may also play a part in the protection of the transplanted beta-cells.
405   Such an impact is due to the ability to induce the proliferation of Tregs and the promotion of
406   neovascularization (Caballero et al., 2007; Chen et al., 2020). Thus, a hybrid transplantation,
407   which combined islet transplantation with mesenchymal stem cells, was shown to improve graft
408   function (Figliuzzi et al., 2009). Furthermore, *in vitro* co-culturing of an islet graft with
409   mesenchymal stem cells contributed to oxygenation and nutrient supply, as well as decreasing
410   the levels of inflammatory cytokines (Arzouni et al., 2017). Therefore, co-culturing appears to
411   increase both the survival of the beta-cells and islet quality.
412   Summing up, the development and combining of new technologies in regenerative medicine
413   provides a means to overcome the very real complications of beta-cell recovery. However,
414   despite the promising results, the effectiveness and reproducibility of the suggested solutions
415   need further verification.
416
Conclusions
417

418   These days regenerative medicine is advancing rapidly, breaking new ground. iPSC-technology
419   is replacing ESCs, to provide an almost unlimited source for the generation of desired autologous
420   cell lines. Currently, stem cell technologies are taking the lead in the regeneration of beta-cells,
421   however, there are certain risks due to manipulations of the genome and the capacity of
422   undifferentiated cells for forming teratomas. To overcome these limitations, the transient
423   introduction of RNAs, recombinant proteins, or chemical mimics can substitute virus-mediated
424   introduction. Meanwhile, the direct conversion of mature cells or the differentiation of adult stem
425   cells can reduce the risk of teratoma development. Thus, the current trends of regenerative
426   medicine are aiming towards safety and immune-compatibility.
427   However, despite the substantial success of regenerative medicine overall, there are still many
428   whitespaces in diabetes treatment when using beta-cells. Without a primary determination of the
429   diversity and functions of different beta-cell populations, current technologies for beta-cell
430   reproduction represent a colossus with feet of clay and will continue to have limitations.
431   Epitranscriptome modifications also require further investigations, especially for
432   transdifferentiation approaches, since most of the studies suggest that the newly generated beta-
433   cells retain the epigenomic landscape of the initial cell line.
434   Evidently, beta-cell preservation remains a stumbling block in diabetes treatment. Finding a
435   solution to this problem represents the core task both for treating early stage patients and for
436   patients with a total loss of beta-cells.
437

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The key regulators of beta-cells

Designation	Function	References
Transcript factors		
Pdx1	Essential in early and mature stages of pancreatic cell development. In early-stage regulates the formation of the pancreas from foregut endoderm. In the late stage transactivates the insulin gene	(Kaneto et al., 2007; Zhu et al., 2017)
Hb9	Regulates development of the dorsal pancreas	(Arkhipova et al., 2012)
Ngn3	Critical regulator for pancreatic endocrine fates	(Akinci et al., 2012, p. 3; Zhu et al., 2017)
NeuroD	Regulates pancreatic endocrine cell differentiation and insulin gene transcription	(Zhu et al., 2017)
Mafa	Regulates glucose-responsive insulin secretion. This TF is expressed only in beta-cells	(Wang et al., 2007; Nishimura, Bonner-Weir & Sharma, 2009; Zhu et al., 2017)
Pax4	Regulates of beta-cell specification	(Napolitano et al., 2015)
Pax6	Increases the insulin expression	(Arkhipova et al., 2012)
Nkx6.1	Guides endodermal progenitors toward beta-cell fate, must be generated before certain endocrine genes turn on	(Taylor, Liu & Sander, 2013)
Nkx2.2	Critical regulator of pancreatic endocrine cell specification and differentiation	(Doyle & Sussel, 2007)
Small molecules		
GNF-9228	Selectively stimulates proliferation of beta-cells	(Pavathuparambil Abdul Manaph et al., 2019)
5'-azacytidine (5-AZA)	DNA methylation inhibitor; increases the reprogramming process 10-fold	(Hohmeier et al., 2020)
Valproic acid (VPA)	Increases the reprogramming process 100-fold	(Hohmeier et al., 2020)
Indolactam V (ILV)	Induces differentiation of hesc lines toward Pdx1-positive pancreatic progenitors	(Hohmeier et al., 2020)
BRD7389	Induces insulin protein expression in alpha-cells	(Hohmeier et al., 2020)
latrunculin A and B	Induce endocrine differentiation by the reorganization of the cytoskeleton	(Hogrebe et al., 2020)
GABA(gamma-aminobutyric acid)	Induces alpha-to-beta-cell neogenesis; participates in maintaining the beta-cell mass and in protecting beta-cells from apoptosis in vitro	(Ben-Othman et al., 2017)
MicroRNAs		

miR-15a, miR-15b, miR-16 and miR-195	Decrease the level of NGN3 gene expression	(Joglekar et al., 2007; Wong et al., 2018)
miR-7	Inhibition of proliferation in Langerhans islets	(Wang et al., 2013; Wong et al., 2018)
RNA-binding proteins		
polypyrimidine tract-binding protein 1 (PTBP1)	Regulates the stability of insulin mRNA	(Magro & Solimena, 2013)
human antigen D (HuD)	Binds to insulin mRNA and controls its translation	(Lee et al., 2012)
protein-disulfide isomerase (PDI)	Key regulator of glucose-stimulated insulin biosynthesis	(Kulkarni et al., 2011)

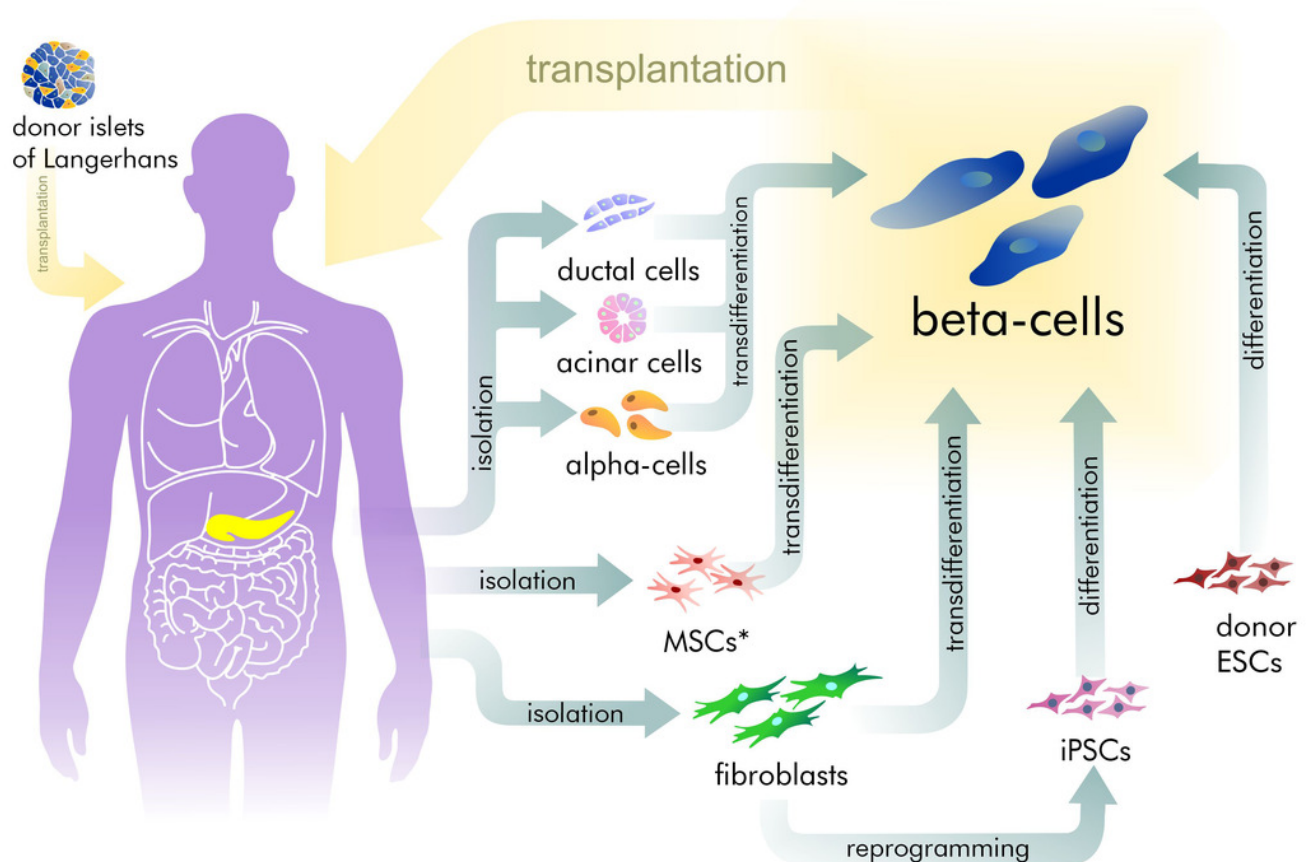
The limitations of approaches for the generation of beta-cells

1   Table 2

	Ex-vivo generation of beta-cells	ESCs differentiation *in vitro*	iPSCs differentiation *in vitro*	Non-beta pancreatic cells transdifferentiation *in vivo*	Non-beta pancreatic cells transdifferentiation *in vitro*	Fibroblasts transdifferentiation *in vitro*	References
Limited sources	Yes	Yes	No	Yes	Yes	No	(Huang & Chang, 2014)
Risk of teratoma development	No	Yes	Yes	No	No	No	(Hentze et al., 2009)
Allograft rejection	No	Yes	No	No	No	No	(Hentze et al., 2009)
Lack of organization into islets	No	Yes	Yes	Yes	Yes	Yes	(Zhou et al., 2008)
Lack of reproducibility of the protocols	No	No	No	Yes	Yes	Yes	(Kim, Jeong & Choi, 2020)
Off-target effects after manipulation with genome	No	No	Yes/No*	Yes	Yes/No*	Yes/No*	(Clayton et al., 2016)
The necessity of deep invasion for cell product preparation	Yes	No	No	No	Yes	No	(Trivedi et al., 2008; Matsumoto & Shimoda, 2020)
The necessity of deep invasion for transplantation of final cell product	Yes	Yes	Yes	No	Yes	Yes	(Shapiro, Pokrywczynska & Ricordi, 2017; Matsumoto & Shimoda, 2020)

3   *the presence of off-target effects will depend on the reprogramming methods (integrating or non-integrating)

Figure 1

The existing approaches for beta-cell recovery

*Muscle-derived MSCs, adipose-derived MSCs, bone-marrow-derived MSCs