Expert Opinion





# Gene transfer to induce insulin production for the treatment of diabetes mellitus

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*Background*: Gene transfer can induce insulin production from non-β-cells. Multiple gene transfer protocols have demonstrated efficacy correcting diabetes-associated hyperglycemia and growth abnormalities *in vivo*. *Objectives*: To review the literature reporting induction of insulin secretion from non-β-cells by gene transfer. *Methods*: Database search of literature in Ovid Medline. *Results/conclusions*: Gene transfer for the treatment of diabetes mellitus has advanced significantly, but remains premature for clinical translation. Approaches inducing metaplasia produce β-like-cells that normalize glycemia in diabetic rodents. Insulin gene transfer strategies provide somewhat inferior glycemic control, but avoid the overproduction of counter-regulatory hormones. Both approaches will require extensive investigations into their effects on host cells and tissues, and the efficacy of neither has been satisfactorily verified in a large animal model.

**Keywords:** diabetes mellitus, gene transfer, insulin, transdifferentiation

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## 1. Introduction

Obtaining glycemic control is a critical component of care for patients with diabetes mellitus [1-4]. Yet, available therapy is unable to normalize blood glucose in the majority of patients [5,6]. Treatment for all patients with Type 1 diabetes, and many with Type 2, includes multiple daily, or continuous, subcutaneous insulin injections, and repeated measurements of capillary blood glucose. The inability of patients to coordinate the timing and dose of insulin injections with meals and activity frequently results in hyperglycemia with the progression of complications, and acute hypoglycemia with neural impairment [1-4].

Limited efficacy and patients' desires to eliminate insulin injections have driven exploration of alternative therapies. The breakthrough 'Edmonton protocol' enhanced pancreatic islet cell transplantation, reducing adverse events, and eliminating glucocorticoids from the immunosuppressive regimen [7]. However, islet availability continues to limit widespread application [7]. Reports from experienced centers indicate the need for from one to four donor pancreata to treat a single patient with Type 1 diabetes mellitus, and even successful transplants lose efficacy over time [8,9]. Consequently, pressures persist to identify an alternate source of insulin producing cells.

Various gene transfer protocols have been used to induce insulin production from non-β-cells. However, most approaches fall loosely into one of two categories: i) transfer of genes to induce metaplasia; and ii) transfer of proinsulin expression sequences. In general, approaches inducing metaplasia use genes expressing transcription factors to direct cells toward a β-cell-like phenotype that includes insulin production and a regulated secretory pathway. In contrast, transfer of a proinsulin expression sequence is generally intended to enable the production and

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secretion of insulin, without altering other aspects of the host cell phenotype.

Although many gene transfer approaches have shown success *in vitro*, the following discussion is limited to those that have been successfully applied to the treatment of hyperglycemia *in vivo*. The limitations in achieving glycemic control and the extent of knowledge concerning broader effects of gene transfer are assessed. In addition, the choice of target tissue, the ability to secrete mature insulin in sufficient quantity, and the benefits of regulating insulin production or secretion are discussed. Finally, speculation is provided on further developments in the field.

## 2. Induced metaplasia to produce insulin

Metaplasia is the conversion of one cellular phenotype to another, and includes lineage shifts among stem cells, and phenotype switches occurring during embryogenesis [10]. Transdifferentiation is a specific form of metaplasia, referring to a phenotypic shift of one differentiated cell type to another [10], whose accurate determination requires cellular ancestor–descendent analyses [11]. Multiple investigators have termed their gene transfer of transcription factors to induce insulin production in non-islet tissues 'transdifferentiation'. However, the precise form of metaplasia is often undetermined.

The pancreas, including the Islets of Langerhans, shares common embryonic origins with the liver and gut endoderm [12]. Recent elucidation of the timing and hierarchy of trophic and transcription factors critical to the development of β-cells, in particular pancreas duodenum homeobox-1 (Pdx1), neurodifferentiation-1 (Neurod1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (avian) (Mafa, mouse; killer cell lectin-like receptor subfamily G, member 1 [Klrg1], rat), neurogenin 3 (Neurog3) and beta-cellulin, have established the theoretical basis to redirect cell development of some tissues toward an islet cell-like phenotype by expressing these factors [13-18]. The liver does not normally produce β-cell-specific transcription factors. However, work in amphibians suggests that their forced expression can direct liver cells toward a pancreatic phenotype [12]. Ferber *et al.* established this concept in mature mammals, demonstrating in mice that hepatic transduction with adenovirus constitutively expressing rat Pdx1 (Ad/rPdx1) stimulates expression of endogenous β-cell-specific proteins, including murine insulin-I, murine insulin-II and prohormone convertase (PC) 1/3 [15]. Livers of transduced mice produced mature, fully cleaved forms of insulin [15]. Importantly, Ad/rPdx1 administered systemically normalized blood glucose in mice made diabetic with streptozotocin (STZ) [15]. Ad/rPdx1 treatment appears to function, in part, by inducing sustained expression of the endogenous mouse homolog of Pdx1 [16]. Indeed, adenoviral expression of the rat protein declined to baseline values after 30 days, while murine Pdx1 was hepatically expressed for 180 days postinfection [16]. More significantly, transduced livers produced insulin for up to 8 months postinfection, considerably longer than expected from standard adenoviral transduction [16]. However, not all treated animals responded, and responses were inconsistent. Only 75% of rPdx1 expressing mice expressed mouse insulin-II, and only 10% expressed insulin-I, the form of insulin produced most copiously in normal mice [15]. Insulin-I expression could be enhanced by co-expression of transcription factors Mafa and Neurod1 with Pdx1 [19]. However, responses to intraperitoneal glucose tolerance tests waned within 14 days following expression of these three transcription factors, thus suggesting that this enhancement may have also limited the duration of insulin expression [19]. Most importantly, Ad/rPdx1 treatment induced hepatic expression of islet proteins other than insulin, including glucagon and somatostatin, which may negate some of the glucose lowering effects of insulin [16]. Multiple groups also reported that delivery of Pdx1 induced expression of pancreatic acinar cell markers and enzymes contributing to hepatic toxicity, fibrosis and death [16,20]. However, such adverse results may not be inherent to the transdifferentiation approach. Coupling the murine Pdx1 C-terminus with VP-16, an activation domain protein from herpes simplex virus, and administration by adenovirus *in vivo*, reduced the requisite viral infectious load, and avoided expression of pancreatic acinar cell proteins (amylase) in the liver [21]. As observed with full-length Pdx1, the combined expression of additional transcription factors (Neurod1 or Neurog3) further enhanced both insulin expression and glycemic responsiveness in diabetic mice [17]. Alternatively, hepatic transduction with a helper-dependent adenovirus expressing Neurod1, a transcription factor whose action lies downstream of Pdx1, in conjunction with expression of betacellulin, an islet tropic factor, also avoided severe liver dysfunction [20]. This model induced expression of insulin processing enzymes PC1/3 and PC2 in addition to insulin, and formation of intracellular inclusions staining positively for insulin by electron microscopy, reminiscent of true β-cell insulin granules [20].

It remains unclear precisely which cells in the liver produce insulin following transduction with islet transcription factors. In addition to forming clusters of insulin-producing cells in the vicinity of the portal triads, transduction with vectors expressing both Neurod1 and betacellulin induced them under the liver capsule [20]. Transduction of subcapsular areas is not commonly expected following adenoviral gene delivery [22]. The observation that insulin-producing hepatic oval cells *in vitro* also stained for glucagon, somatostatin and pancreatic polypeptide, similar to Pdx1-expressing rat livers, suggested that oval cell development could account for the results *in vivo* [20]. However, more recent reports suggested that mature hepatocytes may also be susceptible to transdifferentiation. For example, forced expression of Pdx1 in isolated human hepatocytes inhibited CEBPβ/liver activator protein activity, leading to dedifferentiation and possibly redirection toward islet cell phenotypes [23], and lentiviral-induced Pdx1 expression was reported to induce

insulin production in isolated rat hepatocytes [24]. Continued expression of transferrin and albumin in Ad/Pdx1 VP-16-transduced hepatocytes that subsequently produce insulin also suggests that the initial infection occurred in differentiated hepatocytes [21].

In addition, the gene transfer effects are not always clearly discernable from those exerted by extracellular factors. For example, hyperglycemia without gene transfer can induce insulin expression in bone marrow-derived cells [25,26]. Insulin-producing cells were shown to subsequently fuse with cells in diverse tissues, including liver [25,26]. Moreover, experimental outcomes using cells transdifferentiated *in vitro* are clearly different from those exposed to environments *in vivo* [27-29]. Cao *et al.*, in particular, documented an insulin inducing effect of high glucose levels on Pdx1 expressing hepatocytes, confirming a modifying role for soluble factors in transdifferentiation [29]. Thus, available data indicate that the precise combination of transcriptional and environmental factors required to skew metaplasia toward the production of insulin secretion cells is unknown.

Multiple groups have attempted to exploit the shared ontogeny of gut endothelium and pancreas cells with hepatocytes, which suggests they may also be susceptible to a redirection toward a β-cell phenotype [30-33]. Adenoviral delivery of Pdx1 into the common bile duct stimulated pancreatic ductal hyperplasia, and generated cells staining positive for insulin that were external to existing islets [30]. Combined transgenic expression of two β-cell transcription factors, Pdx1 and Isl1, induced the rat intestinal cell line (IEC-6) to produce insulin, as well as GLUT2, GK, Kir6.2, and SUR1, transcripts integral to glucose sensing in β-cells [31,32]. However, despite a favorable gene expression profile, intraperitoneal injection of these cells only modestly and transiently reduced blood sugar [31]. More recently, native basal expression of Pdx1 and Isl1 in some rat intestinal epithelial cells was supplemented by inducing expression of an additional β-cell transcription factor: Mafa. Orally administered adenovirus expressing Mafa to STZ diabetic rats induced insulin staining of gut endothelial cells [33]. However, insulin secretion from these cells was not glucose responsive, thus limiting the ultimate use of this approach [33].

Due to concerns of inducing immortal phenotypes *in vivo*, approaches to induce metaplasia have also been applied *ex vivo*. Such approaches might also address cell sourcing problems limiting islet transplantation. Using retroviral transduction to stably express rat Pdx1, Zalzman *et al.* demonstrated glucose-stimulated human insulin secretion from isolated fetal human liver epithelial progenitor cells [34]. The transduced human fetal hepatocytes expressed multiple genes consistent with a β-cell phenotype, including insulin, and ameliorated hyperglycemia when implanted in STZ diabetic NOD-SCID mice [34]. However, total insulin content on a per cell basis was estimated to be < 2% of human islets [28]. They also failed to express other genes important for β-cell glucose sensing (e.g., GLUT2, GK and SUR1) and expressed elastase, a marker of pancreatic acinar cells [34]. Interestingly, these deficiencies were addressed by additionally treating transduced fetal hepatocytes with Activin A *in vitro*, which significantly upregulated GLUT2, GK, SUR1 and PC 1/3, as well as insulin expression [28]. In addition, Activin A pretreatment diminished the expression of non-β-cell products (glucagon, pancreatic polypeptide and α1-anti-trypsin) [28]. Although insulin content remained < 15% of islets, responses to a glucose challenge were normalized in some treated animals [28]. Importantly, these data support the concept of beneficially modulating gene expression following metaplasic induction [28].

Due to controversies regarding the use of fetal tissues, studies *ex vivo* with differentiated liver cells are noteworthy. Treatment of adult human liver cells with a Pdx1-expressing adenovirus *in vitro* stimulated human insulin production, but only following exposure to nicotinamide and epidermal growth factor [18]. Transplantation of these insulin-producing liver cells under the renal capsule ameliorated STZ-induced hyperglycemia and produced detectable circulating levels of human C-peptide in NOD-SCID mice [18]. More recently, another group confirmed that lentiviral transduction to overexpress exogenous Pdx1 in cultured adult rat hepatocytes induces insulin expression without soluble factor treatment [24]. Again, renal capsular implantation in diabetic SCID mice reduced hyperglycemia [24]. Similar to previous approaches [16,20], transduction *ex vivo* resulted in expression of non-insulin genes, such as glucagon, somatostatin, elastase and amylase, that may worsen glycemia or produce hepatic fibrosis [18,24]. However, these results also provided support for contentions that terminally differentiated cells may retain some developmental plasticity.

Some groups attempt to direct stem cells toward an insulin-producing phenotype. Karnieli *et al.* transduced human, bone marrow-derived mesenchymal stem cells (BMSCs) expanded in primary culture with a Pdx1 expression vector, inducing cells from 9 of 14 donors to stain for insulin, glucagon and somatostatin [27]. Despite low expression of GLUT2 and GK and absent expression of Kir6.2, SUR1, NEUROD1 and PC2, insulin secretion was sensitive to increasing glucose concentrations in selected cultures [27]. While secreting relatively small quantities of insulin compared with human islets when assessed *in vitro*, insulin-positive cells transplanted under the renal capsule of STZ-SCID mice, effectively reduced blood sugars [27]. Evaluation of transplanted cells *in situ* revealed robust insulin production and profoundly diminished glucagon content compared with preimplantation, and gene expression of glucagon and somatostatin were diminished, while insulin expression was increased 19-fold [27]. In addition, expression of GLUT2 and GK were increased, and expression of NEUROD1, SUR1 and Kir6.2 were newly detected [27]. Thus, although it appears that *in vitro* experimentation is useful to guide exploration, environments *in vivo* may profoundly affect transduced cells.

## 3. Transfer of insulin genes into non-β-cells to induce insulin secretion

Rather than redirect multiple facets of cell phenotype, another approach simply transfers a proinsulin expression sequence into non-β-cells. Early studies improved hyperglycemia by implanting fibroblasts stably transfected with a proinsulin gene intra-peritoneally in diabetic mice [35]. Subsequent modifications of the endoprotease recognition sites bounding the C-peptide have facilitated proinsulin processing in non-β-cells that express the furin protease [36,37]. Investigators obtained transgenic insulin expression in a wide variety of tissues, including pituitary, pancreas, fat, enteroendocrine cells, bone marrow-derived stem cells and muscle [38-47].

Direct comparison of protocols using various vectors, promoters and tissue targets are lacking. However, several approaches to produce transgenic insulin from non-β-cells have demonstrated insulin action sufficient to limit metabolic derangements in diabetic animals. Assorted proinsulin expressing vectors, targeted to various tissues, improved survival, inhibited ketosis and reversed weight loss associated with β-cell depletion [39,41,42,47-49]. In addition, experience gained with various transgenes clarified some basic concepts of insulin gene therapy and demonstrated that not all insulin gene therapy approaches are equally effective. For example, approaches using either insufficiently active promoters, or expression sequences limiting synthesis to proinsulin, failed to fully control glycemia [50-52]. Moreover, early work revealed the necessity of regulating transgene production to avoid lethal hypoglyemia. Investigators using viral transduction and constitutive expression to induce hepatic insulin production, lowered blood glucose in STZ-treated rats [49,53], but viral doses sufficient to control glycemia during feeding produced hypoglycemia in fasted animals [49,54]. Importantly, these studies confirmed the existence of a therapeutic window for transgenic insulin production, and underscored the need to couple insulin production to systemic glucose levels.

Muscle constitutes a large, accessible, vascularized cell mass that is a major site of insulin-responsive glucose disposal. Transfer of an exogenous insulin gene into muscle cells *in vivo* by various methods produced sufficient insulin to control hyperglycemia in diabetic rodents. Successful transfer methods include direct injection of plasmids [55-58], plasmid injection followed by electroporation [59,60], and viral vectors [61,62]. During the initial 5 weeks, STZ mice treated by intramuscular injection of plasmids, expressing both furin and a cleavage site-modified mouse insulin II B10 mutant, increased serum insulin and controlled fed glycemia [55]. However, fasting these animals induced hypoglycemia [55]. Conversely, by 8 weeks, fasting glucose values were normal, but fed values had increased to levels comparable to untreated diabetic mice, suggesting a waning insulin effect [55]. In transgenic mice, insulin expression in skeletal muscle cells driven by a myosin light-chain 1 promoter/enhancer combination increased serum insulin levels, sustained body weight, and normalized fasting hyperglycemia in STZ-treated mice [47]. Insulin produced from muscle improved glucose disposal, and normalized serum triglycerides, NEFA and β-hydroxybutyrate levels, thus confirming effects beyond glycemia [47]. However, despite insulin production from the entire musclature, fed blood glucose values of STZ-treated mice, although attenuated, remained abnormally elevated [47]. These mice were subsequently crossed with another line to produce transgenic mice overexpressing both insulin and glucokinase (GK), a hexokinase restricted to liver and β-cells, in skeletal muscle [61]. Isolated overexpression of GK in muscle reduced glycemia in STZ mice [63], thus raising the expectation that combined expression with insulin would improve glycemic control. However, dual skeletal muscle expression of GK and insulin failed to normalize fed glycemia [61]. This may have been due to limited promoter activity, irrespective of tissue mass, as adeno-associated virus-mediated dual transduction of GK and insulin driven by a strong viral promoter in skeletal muscle of STZ mice did normalize serum insulin levels as well as fed glycemia [61].

Similar to muscle, livers present a large tissue mass accessible by direct injection [50,64] via the hepatic portal or arterial vasculature [22], or via the systemic venous circulation when using adenovirus vectors in rodents [65]. Hepatocytes possess prodigious synthetic capacity, and express the constitutively functional GLUT2 glucose transporter and the high $K_m$ hexokinase, GK, recapitulating critical molecular components of the glucose sensing system present in β-cells [66]. Livers exhibit significant regenerative capacity that theoretically reduces the risk of treatment-induced target organ insufficiency, and are initial targets for insulin action. Expectedly, some of the most successful insulin gene therapy approaches have targeted the liver [48,67-70].

One approach uses a promoter carrying stimulatory multimers derived from the rat liver pyruvate kinase (L-PK) glucose response element, upstream of an inhibitory insulin responsive element within the rat insulin-like growth factor binding protein-1 (rIGFBP-1) promoter. Glucose stimulated and insulin inhibited this promoter in primary cultured hepatocytes [71]. When coupled to a modified human proinsulin expression sequence, and packaged in a replication-defective adenovirus, the resultant vector induced near-normoglycemia in multiple rodent models of diabetes [48,68]. Moreover, as an indication of this transgene's capacity to respond to demand *in vivo*, hepatic insulin gene therapy-treated animals were shown to grow normally and tolerate a 24-h fast without lethal hypoglycemia [68,70]. The glucose-sensitive rat L-PK promoter, in conjunction with the SV40 enhancer, was also used by Lee *et al.* to induce normoglycemia in both STZ-treated rats and NOD mice following transduction with an adeno-associated virus expressing a single-chain insulin analog [67]. Chen *et al.* also exploited a combined stimulatory response to glucose and an inhibitory response to insulin intrinsic to the glucose-6-phosphatase promoter to

drive transgenic insulin expression in rats [69]. To increase a relatively weak transcriptional activity, dual copies of the aldolase B enhancer sequence were incorporated into the promoter, enhancing activity sufficient to produce normal serum insulin levels in STZ-treated rats that responded appropriately to feeding [69]. All three of these approaches confirmed the use of engineering hepatically active promoters that incorporate adequate transcriptional activity and metabolic responsiveness.

Experimental glucose-responsive hepatic insulin expression has been pursued most intensely in rodents. However, two recent reports describe application *ex vivo* in diabetic pigs. Okitsu *et al.* injected a reversibly immortalized human hepatocyte cell line engineered for glucose responsive insulin production into the hepatic portal system [72]. Chen *et al.* electroporated and reimplanted autologous hepatocytes with a glucose-responsive human insulin-expressing transgene [73]. Although neither study obtained complete normalization of glycemia, their results support the use of hepatic insulin production beyond rodent models of diabetes.

Regulation of transgenic insulin production in the liver has most commonly been achieved at the level of transcription. However, regulation by non-glucose signals and use of synthetic or non-mammalian promoters has also been explored. Auricchio *et al.* designed a rapamycin inducible promoter to drive regulated expression of transgenic insulin from mouse liver [74]. Graded doses of rapamycin reduced blood sugars in treated diabetic mice, thus suggesting the use of a premeal dosing schedule to control prandial glycemic excursions [74]. Although not targeted to the liver, a similar approach used synthetic nuclear hormone receptors consisting of multiple Gal4 DNA binding domains, an estrogen or progesterone ligand binding domain, and a serum response element binding protein-1a activation domain to stimulate insulin production from a transgene driven by a Gal4-sensitive promoter [75]. Subcutaneous implantation of cells expressing insulin from this promoter reduced glucose in diabetic mice within 2 h of exposure to either RU486 or 17β-estradiol [75].

Compared with β-cells, transcriptional regulation of transgenic insulin production is burdened by relatively slow secretion kinetics. In addition, a long message half-life may lead to sustained poststimulatory production of transgenic insulin and resultant hypoglycemia [48]. Shortening the half-life of the transgene message improved the fidelity between glucose stimulation and insulin production *in vitro*, and ameliorated poststimulation hypoglycemia *in vivo* [76] (Thulé PM, manuscript in preparation). However, minute to minute regulation of insulin secretion may ultimately require post-translational regulation. In an example of post-translational regulation, Rivera *et al.* engineered rapid secretion kinetics into (human fibrosarcoma) cells usually limited to constitutive secretion. They expressed a transgenic insulin that was linked via a furin cleavage site to multiple conditional adhesion domains (CAD) [77]. Without intervention, transgenic insulin aggregated via the CAD, and was retained within the endoplasmic reticulum [77]. However, following administration of a CAD ligand, transgenic insulin de-aggregated, the CAD were enzymatically removed, and insulin was secreted via the constitutive pathway [77]. Importantly, altering the number of CAD repeats attached to the transgenic insulin adjusted the basal ratio of retained to secreted transgene product with a rigorous dose–response relationship [77]. Implantation into diabetic nude mice reduced glycemia within 30 min following exposure to a CAD ligand, and glucose-lowering effects of ligand administration dissipated within 3 – 4 h [77]. Questions about the potential to elicit endoplasmic reticulum stress responses in such a model may limit ultimate use. However, the study provided proof of principle that secretory kinetics can be successfully modulated at a post-translational step.

Fibroblasts represent an accessible, large and expandable cell source, and were used for initial proof-of-principle demonstrations for insulin gene therapy [35,78]. Unrestrained growth, and continuously increasing hormone production, with resultant lethal hypoglycemia restricted expanded application of proinsulin-secreting fibroblasts [35,78]. Agarose encapsulation prior to intraperitoneal implantation in STZ diabetic mice limited growth of proinsulin-expressing fibroblasts, and avoided lethal hypoglycaemia, but significantly reduced glycemic efficacy [79]. More recently, Kim *et al.* reported an innovative system to modulate single-chain insulin analog production from NIH3T3 fibroblasts implanted under the skin. Transfecting both a synthetic steroid hormone receptor and a transgene promoter responsive to this synthetic receptor, they engineered transcriptional stimulation responsive to dermal application of estrogen or progesterone [75]. Although obtaining adequate glucose control using this approach alone would require modification of regulation dynamics, it could conceivably be combined with other methods.

Fat is a readily accessible tissue, of nearly unlimited quantity. Fat is also an important insulin target and secretes multiple metabolically active peptides [80]. Proinsulin expressed in 3T3L1 adipocytes co-localized to GLUT4-containing vesicles, suggesting that transgenic insulin could be released by stimuli that induce GLUT4 translocation, such as insulin itself [81]. When applied *in vivo*, adenoviral-mediated proinsulin expression from epididymal fat modestly reduced glycemia in a mouse model of Type 2 diabetes [42]. In contrast, subcutaneous implantation of retrovirus-infected primary adipocytes expressing modified human insulin effectively controlled 16-h fasted blood glucose in STZ diabetic mice for up to 10 weeks [82].

Liver, muscle, fat and fibroblasts all lack the prohormone convertases required to produce mature insulin from proinsulin. In contrast, pituitary cells express prohormone convertase 1/3 and 2, as well as carboxypeptidase H, and should thus be able to process proinsulin to mature insulin [83,84]. In addition, they possess a regulated secretory pathway similar to β-cells [39]. Intermediate lobe mouse

pituitary cells transduced to express proinsulin and transplanted into the renal capsules of hyperglycemic NOD mice successfully normalized blood sugars [39]. While transplanted pancreatic islets induced an immune reaction in immune-competent mice, insulin-producing pituitary cells did not, thus indicating that transgenic insulin-expressing non-β-cells may have an advantage over islet transplants by evading recurrent autoimmune destruction in Type 1 diabetes [39]. Although insulin-expressing pituitary cells were not glucose responsive, this limitation was addressed by co-expressing the glucagon-like protein receptor (GLP-1R) [40]. Pituitary cells expressing both GLP-1R and insulin demonstrated secretory responsiveness to glucose [40]. Oral glucose loading increased human insulin in the serum of NOD/SCID mice receiving transplants of these insulin producing pituitary cells, but effects on diabetic hyperglycemia have not been reported [40].

Entero-endocrine cells residing within the stomach and intestines also exhibit vesicular secretion and prohormone convertases capable of insulin processing. Expression of human proinsulin in gastric G-cells driven by the gastrin promoter generated mature human insulin in gastric antral extracts of transgenic mice [44]. Human insulin co-localized to gastrin-containing vesicles, and insulin was secreted after exposure to peptone, a stimulant of gastrin secretion [44]. However, although survival was dramatically improved in a genetic model of Type 1 diabetes, glycemic improvement was modest [44]. A more impressive effect was observed in transgenic mice expressing human preproinsulin from the glucose-dependent insulinotropic peptide (GIP) promoter. In these mice, insulin co-localized with GIP in duodenal cells, and STZ-treated transgenic mice had normal responses to an oral glucose tolerance test [45]. Although not using enteroendocrine cells, Barry *et al.* achieved a similar anatomic location in diabetic BB Wor rats by implanting insulin-secreting aortic smooth muscle cells in the gastric subserosa [85]. Although they reduced exogenous insulin requirements, the relatively smaller number of insulin producing cells failed to fully control glycemia [85].

Similar to fibroblasts, BMSCs exhibit a capacity for expansion *ex vivo*, and have been targeted for insulin gene transfer. Following isolation and transduction with a human proinsulin expressing retrovirus, BMSCs were expanded *in vitro*, and subsequently injected into the liver parenchyma [46]. Implantation increased serum levels of human insulin in STZ diabetic mice, and normalized 6-h fasting glucose values for up to 42 days [46]. Although these results are promising, the implanted cells, and thus their potential for transformation and expansion, remain incompletely characterized.

### 3.1 Non-glycemic effects of insulin production in non-β-cells

Effects of transgenic insulin expression in cells and tissues are evaluated infrequently. Although available data suggest that ectopic insulin expression impacts native gene expression, it remains less clear whether these impacts carry risk or fortuitous benefit. Human proinsulin expressed in primary rat hepatocyte cultures suppressed GLUT2 and GK expression [86]. Moreover, consistent with suppression of two proteins critical for translocating and trapping glucose in hepatocytes, proinsulin expression reduced glycogen deposition, despite high levels of glucose and insulin, a phenomenon also observed *in vivo* (Thulé PM, manuscript in preparation) [86]. Other investigators have demonstrated variable effects on liver glycogen with hepatic expression of proinsulin [49,51,87,88]. It remains unresolved whether the observed intrahepatic effects are attributable to proinsulin expression, or associated with the choice of promoter. In support of the former, constitutive expression of proinsulin in HUH7 cells, which naturally express GLUT2 and GK, induced expression of both Neurod1, and prohormone convertases 1/3 and 2 [89]. Electron microscopy revealed dense, insulin-staining secretory granules, and these cells secreted insulin within minutes following exposure to glucose [89]. In another study, HepG2 hepatoma cells, which do not normally express GLUT2, expressed Kir6.2, the inward rectifying potassium channel normally associated with β-cells, following co-expression of GLUT2 and proinsulin [90]. During patch clamp studies, membrane currents in these liver-derived cells were diminished after glucose or sulfonylurea challenges, and maximized by exposure to diazoxide [90,91]. Consistently, glucose and sulfonylureas stimulated, and diazoxide inhibited insulin secretion, replicating response patterns typical of β-cells [90,91]. Additional provocative findings of glucose-regulated insulin secretion from liver *in vivo* were observed with lentivirus delivery of an insulin transgene. Ren *et al.* reported glucose-responsive insulin secretion and normalization of glycemia in STZ diabetic rats for up to 500 days [92]. Interestingly, expression of modified human proinsulin induced expression of multiple other islet proteins, including transcription factors (Neurod11, Pdx1), and some hormones (somatostatin and glucagon), but not native insulin or PC2 [92]. Interestingly, these findings stand in contrast to metaplasia studies where vectors expressing these same transcription factors induced expression of endogenous insulin and PC 2 [15,20]. The reasons for these discrepancies are unclear and may reflect unrecognized consequences of expressing transgenic insulin in hepatocytes.

Expressing insulin from ectopic anatomic sites and in tissues other than β-cells may also produce indirect systemic effects. Elevated glucagon has been consistently observed in a model of regulated hepatic insulin gene transfer [48,70,93]. This is at least partially accounted for by circulating insulin levels insufficient to suppress endogenous α-cell secretion [48,94], but may suggest a dysregulated counter-regulatory hormone response, as well, as fasting glucagon levels were abnormally suppressed [93]. In addition, mildly increased free fatty acids, and moderately increased triglycerides, have often been

observed in diabetic animals expressing insulin from the liver [48,87,93], although not universally [70]. These elevations may again relate to circulating insulin levels, but may also be associated with altered fuel oxidation [93]. Consistent with altered fuel metabolism, STZ diabetic rats expressing insulin from the liver produced more heat and exhibited smaller intra-abdominal fat accumulations than normal control animals with similar glucose levels [93].

## 4. Conclusions

Gene transfer approaches designed to produce insulin from non-β-cells have achieved variable success in treating hyperglycemia. The transfer of islet-associated transcription factors can induce metaplasia of gut, liver and pancreatic acinar cells *in vivo*. Indeed, induced metaplasia has generated insulin-secreting cells with the critical molecular components of a glucose-sensing and a vesicular secretory system. Consequently, metaplastic insulin producing cells exhibit glucose-responsive insulin secretion kinetics similar to native β-cells and exquisite glycemic control in diabetic rodents. However, it remains difficult to selectively obtain insulin-producing cells while excluding other pancreatic cell types, such that most induced metaplasia approaches induce secretion of many different islet hormones. In addition, the precise cellular targets of these approaches remain unclear, as does the potential for malignant transformation [10]. In general, the effect of approaches inducing metaplasia on changes in gene expression, chromatin structure, or other host functions has not been evaluated in the sited literature. Indeed, it remains unclear why a certain transcription factor works in some cell types and not in others. Moreover, little is known about the need for regulating the level or duration of expression of transcription factors, or the benefits of expressing several transgenes for achieving advanced differentiation. However, understanding how environmental elements, both *ex vivo* and *in vivo*, modify the metaplastic process, while currently rudimentary, could potentially address these issues.

By transferring proinsulin expression sequences, investigators have induced insulin secretion from a variety of tissues, including muscle, liver, fibroblasts, entero-endocrine, fat and pituitary cells. Neuroendocrine cells, like entero-endocrine and pituitary cells, which possess a regulated secretion system, with or without the molecular components of a glucose-sensing system, tend to be inaccessible. Conversely, more clinically accessible cells, like liver and muscle, tend not to express a glucose-sensing and regulated secretion system. In these latter cells, inducing appropriately regulated insulin secretion remains a significant hurdle. None the less, ectopic insulin expression in non-β-cells can ameliorate hyperglycemia and reverse many short-term metabolic dysfunctions associated with diabetes mellitus. The most effective approaches have used hepatocytes to obtain some form of metabolic regulation of insulin production. This is most commonly achieved at the level of transcription. It remains unclear how transgenic insulin expression affects host tissues, as well as broader metabolic parameters. Importantly, there are scant data as to whether gene transfer is efficacious in a large animal model of diabetes [72,73].

## 5. Expert opinion

Inducing the production of insulin from endogenous non-β-cells in diabetic rodents effectively controls glycemia, modifies both carbohydrate and fat metabolism, and restores normal growth. Transdifferentiation approaches in particular offer the profound benefit of creating cells that accurately recapitulate β-cell-like glucose-responsive insulin secretion. However, there are considerable barriers to applying induced metaplasia in humans. The potential for malignant transformation and the stability of proliferation and secretion characteristics must be examined. In addition, to advance metaplastic approaches, the targeted cell types must be clearly identified, and a determination of a minimum set of metamorphizing transcription factors established that selectively produce desired phenotypes. This should restrict metaplasia to a select cell population and permit the exclusion of cells likely to secrete counter-regulatory hormones, that may undermine efficacy, or toxic products, that may damage host cells. Ideally, target cell identification would permit isolation, expansion and manipulation *ex vivo*. However, data suggest that although cellular responses to gene transfer *in vitro* are informative, they appear insufficient to predict behavior *in vivo*. Consequently, investigations of how the milieu *in vivo* affects metaplastic cells will be critical.

Initial data indicate that inducing insulin expression in non-β-cells is more straightforward. However, inherent differences between β-cells and non-β-cells represent a major challenge to obtaining sufficient insulin secretion with appropriate secretion dynamics. Targeted cell types have ranged from muscle cells and fibroblasts, which share no molecular components of the glucose-sensing system present in β-cells and lack regulated secretion mechanisms, to cells with metabolically responsive vesicular release of transgenic insulin, such as entero-endocrine and pituitary cells. Between these two poles, adipocytes exhibit vesicular secretion, but are not naturally glucose responsive with respect to transgene secretion, whereas hepatocytes share GLUT2 and GK expression with β-cells, but are limited to a constitutive secretion system. On one hand, fat, liver and muscle cells are numerous and easily accessible, while on the other, entero-endocrine and pituitary cells are inaccessible outside of animal models. As no single cell type is optimal, it seems rationale to consider combinations of cell types to produce transgenic insulin. One scenario would use fat, muscle or liver cells to provide basal insulin. Use of hepatocytes would provide metabolic responsiveness on a time scale compatible with the second phase of insulin release from β-cells, or similar to the basal insulin injections in current clinical use.

In contrast, a transduced entero-endocrine cell, K-cells for example, would provide rapidly responsive insulin release, similar to the first phase of insulin release from β-cells, analogous to the use of rapid acing insulin at mealtimes and for rapid corrections. That said, entero-endocrine cells are not currently accessible for manipulation in individual animals, much less humans. Until they are, it should be recognized that the limits of engineering secretion dynamics by combining interventions at multiple steps in the synthetic and secretory process have not been fully explored.

Even with a reasonable approach to addressing secretory dynamics, two barriers hinder clinical translation of insulin gene transfer. First, as with induced metaplasia, the inherent risks of insulin transgene expression have been inadequately explored. The work of Ren *et al.* in which transgenic insulin expression in the liver appears to stimulate metaplasia underscores the potential for insulin gene transfer to exert unexpected effects on host tissues [92]. These effects must be rigorously explored prior to entertaining clinical experiments. Second, experiments must be conducted in a large animal model of diabetes mellitus. With two exceptions [72,73], reports of efficacious insulin gene therapy *in vivo* are limited to rodent experiments. Although results have supported the premise that insulin gene transfer may be clinically effective, significant differences between rodent and human physiology do not permit direct extrapolation. Induced metaplasia or insulin gene transfer, or a combined approach, would advance rapidly if it were able to convincingly improve glycemia in a diabetic large animals or primates.

In conclusion, gene transfer for the treatment of diabetes mellitus has advanced significantly over the past decades, but remains premature for clinical translation. Approaches inducing metaplasia have proved capable of inducing the production of β-like-cells that normalize glycemia in diabetic rodents. Insulin gene transfer strategies provide somewhat inferior glycemic control, but are less burdened by the overproduction of counter-regulatory hormones. Both approaches will require extensive investigations into their effects on host cells and tissues, and the efficacy of neither has been broadly verified in a large animal model. Ultimately, comparison with current clinical therapies must demonstrate acceptable risks and significant benefits, including effects on diabetic complications. Only comparative clinical studies will define the reasonable application of insulin producing non-β-cells for the treatment of diabetes mellitus.

## Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.



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