Editorial

Stem Cell INVESTIGATION

Studying pancreas development and diabetes using human pluripotent stem cells

David W. Scoville, Anton M. Jetten

Cell Biology Group, Immunity, Inflammation, and Disease Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA

Correspondence to: Anton M. Jetten. Immunity, Inflammation, and Disease Laboratory, NIEHS, NIH, Research Triangle Park, NC 27709, USA. Email: jetten@niehs.nih.gov.

Received: 12 October 2016; Accepted: 21 October 2016; Published: 14 November 2016.

doi: 10.21037/sci.2016.11.03

View this article at: http://dx.doi.org/10.21037/sci.2016.11.03

Introduction

For over two decades now, studies into pancreatic development have relied largely on the mouse as a model organism. These studies have generated a wealth of information into the genetic regulatory networks involved in pancreas formation, identifying key transcription factors involved in early pancreatic differentiation, endocrine cell specification, and mature hormone-producing cell production [reviewed recently in (1)]. Many of these genes have been confirmed to play a role in β-cell development in humans as well, with some factors linked to monogenic forms of diabetes, while others through Genome-Wide Association Studies (GWAS) have been linked to increased risk of developing either type 1 or type 2 diabetes mellitus.

In order to examine human pancreatic β-cell development and differentiation, González and colleagues (2) previously developed a human embryonic stem cell (hESC) system containing a doxycycline-induced Cas9 expression, allowing for inducible gene editing. This same group then utilized these inducible Clustered regularly interspaced short palindromic repeats (iCRISPR) cells as a human model system of pancreatic β-cell differentiation (3). Using this system, they were able to examine the effects of both knocking out and overexpressing key genes identified in mouse studies, and examining their effects in human cells. We will discuss these findings here, as well as the broader implications of using human stem cell differentiation as a model for human development and disease.

Mouse models on pancreatic differentiation provide a wealth of information

Embryonic development of the mouse pancreas is generally broken up into two stages, referred to as the primary and secondary transition. During the primary transition, the pancreatic epithelium undergoes changes in morphology, while maintaining high levels of proliferation. It begins in the mouse embryo around embryonic day (e)9.5, with an outgrowth of dorsal and ventral pancreatic buds from the dorsal foregut endoderm. These cells express the transcription factor Pdx-1, one of the earliest markers of primitive pancreas, without which the pancreas is unable to form. This phenotype in mice is mimicked in humans, with homozygous loss of PDX-1 responsible for pancreatic agenesis (4,5). Subsequent expression of Ptf1a is necessary for further development of both the endocrine and exocrine pancreas in mice as well as humans (6,7). While Rfx6 is also expressed during these early time points in mice, it only appears to be required for endocrine pancreatic development, a phenotype shared in humans with mutations in RFX6 (8,9). Mnx1, formerly known as Hlbx9 or Hb9, is expressed early, but unlike the previously mentioned transcription factors, Mnx1-null mice fail to develop a dorsal pancreas, and the resulting pancreas has fewer and dysfunctional islets. Similar phenotypic results were observed in humans with mutations in MNX1 (10). Hes1 is a notch signaling pathway component, and its loss in mice disrupts normal development of the pancreas, yet still allows for formation of both exocrine and endocrine cells (11).

During the primary transition, the only hormone-

positive endocrine cells produced are a small number of glucagon+ cells. Whether these cells play a role in development and postnatal function (and what that role might be) is not currently understood. Starting at ~e13.5, the pancreas enters the secondary transition, where Ngn3 expression marks endocrine progenitor cells that will differentiate into the mature, hormone producing cells of the Islets of Langerhans. Ngn3 is only highly expressed prior to hormone expression, and loss of Ngn3 prevents the development of endocrine progenitor cells and consequently pancreatic islets. Arx is specific to the glucagon-producing α-cell lineage, and its loss prevents development of α-cells specifically, with a concomitant increase in β- and δ-cells (12). Glis3 was the most recent addition to this list, with loss of functional Glis3 leading to a severe reduction in both β- and pp-cell number postnatally, as well as formation of cystic pancreatic ducts (13). Ngn3, Arx, and Glis3 represent the relatively "late actors", as their expression is restricted to the establishment of defined endocrine progenitor cells [or, in Glis3's case, endocrine and ductal cells (14)].

Taken together, these genes play critical roles at multiple stages of pancreatic development, from epithelial rearrangement to lineage determination to maturation, and their loss leads to a variety of pancreatic phenotypes. Additionally, most of these genes have been associated with clinical manifestations of impaired pancreas development or diabetes in humans (i.e., PDX-1, RFX6, PTF1A, MNX1, NGN3, and GLIS3). It should be noted, however, that these genes represent only a fraction of those involved in mouse pancreatic development, as well as those involved in the development of Diabetes.

In vitro differentiated stem cells offer a possible treatment for type 1 diabetes

Understanding human β-cell differentiation took two large steps forward in 2005 and 2006, with the development of protocols to differentiate first definitive endoderm (15), and then insulin secreting β-cells from hESC (16). This initial protocol was developed from knowledge gleaned from mouse development, and attempted to recreate this step-like pathway in human cells. Further refinements in this protocol in 2014 (17) has led to efficient means to differentiate large numbers of hESC [or induced pluripotent stem cells (iPSC)] into functional β-cells capable of transplantation. However, comparison of these *in vitro* differentiated β-cells to normal human islet β-cells indicated significant differences in gene expression, and while they displayed glucose-responsive insulin secretion, levels of insulin secretion were still below that of endogenous islet β-cells. While Pagliuci *et al.* (17) suggest that these differences may represent artifacts of the isolation procedure used to obtain human islets from cadaveric donors, it also remains possible that these "β-like" cells may represent an immature or even dysfunctional status, and not a stable population capable of long-term treatment.

To distinguish between these possibilities, however, requires a far more in-depth understanding of human β-cell development than we currently possess. While studies in the mouse model organism continue to be the most promising avenue for discovery, validation and comparison to human β-cell development will be necessary in the future as differences between human and mouse have been reported. For example, heterozygous mutations in the GATA6 gene in humans can lead to pancreatic agenesis or severe pancreatic hypoplasia (18), while mutations or deletions of GATA4 have been linked to diabetes but the mechanism remains unclear (19). Yet pancreas-specific knockout of Gata4 or Gata6 in mice produced only a mild phenotype that was resolved after birth (20,21), suggesting functional redundancy between Gata4 and Gata6 in mice, but not in humans. This highlights the shortcomings of translating mouse studies to human disease: when looking at the mouse studies alone, one may have assumed that GATA4 or GATA6 mutations in humans would have little effect on pancreas development. It also highlights the need for a better model of human pancreatic development, in order to further test hypotheses generated from mouse models in human systems.

Utilizing CRISPR in a human model system

CRISPR has opened the door to extensive study of the genome (both mouse and human). CRISPR utilizes the Cas9 endonuclease and specific RNA targeting sequences to create double stranded DNA breaks at the desired genomic site, which are repaired via a mechanism of nonhomologous end joining, an error-prone method of DNA repair causing random insertions or deletions at the site of the DNA break. In addition, more than one targeting sequence can be used to create multiple DNA breaks, allowing for removal of a specific sequence from the genome, or subsequent replacement with a new DNA sequence of choice via homology directed repair. In this manner, CRISPR allows for the direct manipulation of the genome not only to disrupt genes by deletions, but also to rewrite the genomic

code itself. This is particularly advantageous for the study of the human genome, as specific point mutations or deletions associated with disease can be studied for their effects *in vitro*.

This technique is not without its own set of technical issues. The plasmids used to allow genetic rewriting must include the Cas9 endonuclease, possible selection markers for enrichment of affected cells, as well as the guide RNAs. The resulting ~10 kB plasmid is often difficult to transfect, leading to an overall reduction in the efficiency of the process, and increased amount of screening for mutations and deletions. In order to address these issues, González and colleagues developed an inducible CRISPR (iCRISPR) system (2). They used transcription activator-like effector nucleases (TALENs) to insert a doxycycline-inducible Cas9 sequence into the *AAVS1* locus, a locus allowing robust expression similar to the *Rosa26* locus in mice. Using this system, González and colleagues could focus on transfecting only the guide RNA (~100 bp) targeting a specific locus, which allowed for much higher transfection efficiency with less cellular toxicity. After transfection, the cells could be treated with doxycycline for inducible deletion of the targeted gene. In addition, they show that instead of inserting inducible Cas9 into the AAVS1 locus, they can insert a transgene as well, allowing for study of an inducible gene overexpression/misexpression. This allows for studies on human gene function following differentiation of hESC into different cell types.

In a study by Zhu *et al*. (3), the same investigators utilized their iCRISPR system to study the function of a number of transcription factors involved in pancreatic β-cell differentiation, including PDX-1, PTF1A, MNX1, RFX6, HES1, NGN3, ARX, and GLIS3. These genes were selected for their differing roles across pancreatic and β-cell differentiation (as described above), and for their association with human disease, specifically diabetes. In order to study their effects, they used a model of β-cell differentiation based on the protocols published previously (16,17). They then utilize iCRISPR to study overexpression of NGN3, deletion of each of the above-mentioned genes, and to further characterize NGN3 function by mimicking a point mutation found in a human patient. Not surprisingly, NGN3 is shown to be important for the development of glucose-responsive β-cells, similar to its role in mice. Yet in the absence of NGN3 a small number of endocrine cells are still able to form, including insulin-producing cells. This is in direct contrast to murine models, where insulin+ cells are never observed in Ngn3 knockouts (22,23). This raises interesting questions about the time course of mouse endocrine development, and how it relates to human development. In mice, only a small number of glucagon+ cells are produced prior to Ngn3 expression, and it remains unclear whether these cells are functional in the adult. Zhu and colleagues show that a small number of both insulin+ and glucagon+ cells are produced in in the absence of NGN3, suggesting an extended first wave of pancreatic differentiation in humans that may have functional importance. This demonstrates that while the developmental timing of stem cell systems is significantly different than normal human development, important information can still be discovered.

Additionally, Zhu and colleagues showed that PTF1A, MNX1, and GLIS3 knockout stem cells still produce insulin+ cells at the roughly the same rate as normal stem cells. This is in contrast to knockout mouse models of these factors, which show reduced numbers of β-cells. These differences may be due to the endpoint examined, as only ~7% of cells are differentiated into insulin+ cells, and the relative maturity of these cells was not extensively examined. Indeed, mouse studies of these factors have indicated defects in mouse β-cell differentiation may arise relatively late, and that early embryonic differentiation may only be minimally affected. However, in contrast to the GATA4/GATA6 example mentioned earlier, it remains possible that there is functional redundancy of these factors in the human pancreas that is absent in the mouse, highlighting the need for further research in this area. Future studies into the role of these factors in humans will undoubtedly be aided by the iCRISPR system.

The future of differentiated stem cells looks CRISPR

Looking to the future, CRISPR should allow for direct manipulation of the human genome for a variety of purposes. One such purpose, illustrated by Zhu and colleagues, is for the study of specific point mutations implicated in disease, in order to characterize their effect using an *in vitro* human model system. Another intriguing possibility is that, in the near future, a patient with a specific disease-causing mutation could have induced pluripotent stem cells made from their own tissue, the mutation corrected via CRISPR, and stem cells re-differentiated into the necessary tissue. Indeed, the differentiated β-cells from the 2014 protocol (17) are already being examined for use in possible clinical trials, using a newly developed

encapsulation device (24). Over the next few decades, we will likely see a dramatic increase in the use of differentiated stem cells for the treatment of a number of diseases.

Therefore, it has become imperative to determine whether the function of specific disease related genes in mice is preserved in humans. CRISPR provides a significant step forward, not only for the aforementioned treatment options, but also because it will allow researchers to determine the function of individual genes and mutations. Zhu *et al.* demonstrate that their iCRISPR system can be used for both gain of function and loss of function studies of individual genes or multiple genes simultaneously, as well as allowing for the inducible expression of genes during stem cell differentiation. This will allow researchers to confirm information obtained from mouse models in a therapeutically relevant model of β-cell development, and relate it back to diabetes in humans. Future studies should seek to further clarify apparent differences between mouse and human pancreatic development, such as the maintenance of MAFB expression in human β-cells (whereas it is largely silenced in mouse β-cells at 2–3 weeks), the apparent non-redundancy of GATA4 and GATA6, or to compare the differences between first-wave and second-wave endocrine cells in the two systems. Information gained from these experiments should prove critical in understanding the role of these factors in the development of diabetes and therapeutic strategies.

Acknowledgements

Funding: This research was supported by the Intramural Research Program of the National Institute of Environmental Health Sciences, the National Institutes of Health (Z01-ES-100485).

Footnote

Provenance: This is an invited Editorial commissioned by Editor-in-Chief Zhizhuang Joe Zhao (Pathology Graduate Program, University of Oklahoma Health Sciences Center, Oklahoma City, USA).
Conflicts of Interest: The authors have no conflicts of interest to declare.

Comment on: Zhu Z, Li QV, Lee K, *et al.* Genome Editing of Lineage Determinants in Human Pluripotent Stem Cells Reveals Mechanisms of Pancreatic Development and Diabetes. Cell Stem Cell 2016;18:755-68.

References

1. Romer AI, Sussel L. Pancreatic islet cell development and regeneration. Curr Opin Endocrinol Diabetes Obes 2015;22:255-64.
2. González F, Zhu Z, Shi ZD, et al. An iCRISPR platform for rapid, multiplexable, and inducible genome editing in human pluripotent stem cells. Cell Stem Cell 2014;15:215-26.
3. Zhu Z, Li QV, Lee K, et al. Genome Editing of Lineage Determinants in Human Pluripotent Stem Cells Reveals Mechanisms of Pancreatic Development and Diabetes. Cell Stem Cell 2016;18:755-68.
4. Stoffers DA, Zinkin NT, Stanojevic V, et al. Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. Nat Genet 1997;15:106-10.
5. Schwitzgebel VM, Mamin A, Brun T, et al. Agenesis of human pancreas due to decreased half-life of insulin promoter factor 1. J Clin Endocrinol Metab 2003;88:4398-406.
6. Sellick GS, Barker KT, Stolte-Dijkstra I, et al. Mutations in PTF1A cause pancreatic and cerebellar agenesis. Nat Genet 2004;36:1301-5.
7. Weedon MN, Cebola I, Patch AM, et al. Recessive mutations in a distal PTF1A enhancer cause isolated pancreatic agenesis. Nat Genet 2014;46:61-4.
8. Smith SB, Qu HQ, Taleb N, et al. Rfx6 directs islet formation and insulin production in mice and humans. Nature 2010;463:775-80.
9. Mitchell J, Punthakee Z, Lo B, et al. Neonatal diabetes, with hypoplastic pancreas, intestinal atresia and gall bladder hypoplasia: search for the aetiology of a new autosomal recessive syndrome. Diabetologia 2004;47:2160-7.
10. Bonnefond A, Vaillant E, Philippe J, et al. Transcription factor gene MNX1 is a novel cause of permanent neonatal diabetes in a consanguineous family. Diabetes Metab 2013;39:276-80.
11. Jensen J, Pedersen EE, Galante P, et al. Control of endodermal endocrine development by Hes-1. Nat Genet 2000;24:36-44.
12. Collombat P, Mansouri A, Hecksher-Sorensen J, et al. Opposing actions of Arx and Pax4 in endocrine pancreas development. Genes Dev 2003;17:2591-603.
13. Kang HS, Kim YS, ZeRuth G, et al. Transcription factor Glis3, a novel critical player in the regulation of pancreatic beta-cell development and insulin gene expression. Mol

Cell Biol 2009;29:6366-79.
14. Kang HS, Takeda Y, Jeon K, et al. The Spatiotemporal Pattern of Glis3 Expression Indicates a Regulatory Function in Bipotent and Endocrine Progenitors during Early Pancreatic Development and in Beta, PP and Ductal Cells. PLoS One 2016;11:e0157138.
15. D'Amour KA, Agulnick AD, Eliazer S, et al. Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 2005;23:1534-41.
16. D'Amour KA, Bang AG, Eliazer S, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006;24:1392-401.
17. Pagliuca FW, Millman JR, Gürtler M, et al. Generation of functional human pancreatic β cells in vitro. Cell 2014;159:428-39.
18. Lango Allen H, Flanagan SE, Shaw-Smith C, et al. GATA6 haploinsufficiency causes pancreatic agenesis in humans. Nat Genet 2011;44:20-2.
19. Shaw-Smith C, De Franco E, Lango Allen H, et al. GATA4 mutations are a cause of neonatal and childhood-onset diabetes. Diabetes 2014;63:2888-94.
20. Carrasco M, Delgado I, Soria B, et al. GATA4 and GATA6 control mouse pancreas organogenesis. J Clin Invest 2012;122:3504-15.
21. Xuan S, Borok MJ, Decker KJ, et al. Pancreas-specific deletion of mouse Gata4 and Gata6 causes pancreatic agenesis. J Clin Invest 2012;122:3516-28.
22. Gradwohl G, Dierich A, LeMeur M, et al. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A 2000;97:1607-11.
23. Wang S, Hecksher-Sorensen J, Xu Y, et al. Myt1 and Ngn3 form a feed-forward expression loop to promote endocrine islet cell differentiation. Dev Biol 2008;317:531-40.
24. Vegas AJ, Veiseh O, Gürtler M, et al. Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. Nat Med 2016;22:306-11.

doi: 10.21037/sci.2016.11.03
Cite this article as: Scoville DW, Jetten AM. Studying pancreas development and diabetes using human pluripotent stem cells. Stem Cell Investig 2016;3:80.

