Commentary

Stem Cell INVESTIGATION

Editing genetics, stem cells are prophetic, what's the best way to model cells of diabetics?

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The pancreas is composed of many different cell types, including the endocrine beta cells that are lost or dysfunctional in those afflicted with diabetes. Beta cells normally sense the level of glucose in the blood and secrete an appropriate amount of insulin in response, which binds to receptors on peripheral tissues like muscle and fat to induce glucose uptake. Current treatments for diabetics hinge on constant injections of external insulin, leaving large fluctuations in blood glucose levels and devastating consequences such as kidney failure and retinopathy. If we can create a transcriptional map of the directions pancreatic cells take in route to their ultimate fate, then we can mimic these developmental routes *in vitro* to make new beta cells for diabetics and restore normoglycemia. Mouse models have provided a glimpse into the complexities of pancreatic development, with groundbreaking discoveries into the roles of many critical genes, including Pdx1, which is responsible for the formation of the pancreas and later beta cell maintenance (1-3), and Ngn3 the master regulator of endocrine specification (4,5). Elegant gene targeting experiments carried out by Johansson *et al.* (6) have shown that the timing in which Ngn3 is expressed during embryogenesis dictates the resulting fate of these endocrine progenitors. By deleting Ngn3 and re-expressing a Ngn3:CreER fusion protein that translocates to the nucleus to induce endocrine specification upon tamoxifen administration, they have shown overlapping temporal competence windows in which the fate of these induced endocrine progenitors are biased (6). However, it is unclear how many of the developmental mechanisms found in mice and other model organisms are conserved in human development. In addition, many discoveries from mice are ineffective when used in attempts to treat human diabetes due to discrete species distinctions (7), illustrating the necessity of determining conservation for therapeutic effectiveness, not only in the context of pancreatic development but in the context of all tissues.

But how can we study the route human cells take during development to ultimately become beta cells? Once we build a knowledge base regarding the *in vivo* developmental fate choices of cells from model organisms, we can take this information and apply it during human pluripotent stem cell (hPSC) differentiation. The study of model organisms is still of monumental importance; we must first have an understanding of the basic developmental mechanisms that occur in complex *in vivo* systems, where different cell types interact throughout a shifting milieu. But this practice—using what has been discovered in mice to model human development in a dish—is what has led to the substantial progress in making beta cells *in vitro* (8-16). These cells can be used for drug screening or to potentially replenish the lost or dysfunctional cells in diabetics, but they also provide a convenient system to study biological mechanisms that are otherwise inaccessible: the mechanisms of human embryonic development. While the differentiation of hPSCs into pancreatic cells has been a large focus due to the direct clinical potential for diabetics, this technology is only limited by our own knowledge of cellular developmental programs, and can be theoretically applied to study the development of any human tissue.

The advent of fast, reliable, programmable gene-editing technologies such as TALENs (transcription activator like effector nucleases) and CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-

associated) permit unprecedented interrogation of gene function in a wide array of systems (17-19). These gene-editing technologies were discovered in bacteria, again emphasizing the importance of model organisms, but can be implemented to efficiently modify the genome of many species, including human cells, with fewer off target events than previous methods (20-24). Genetic engineering can be used for gain-of-function or loss-of-function analysis, or even to introduce or correct disease-causing mutations (25).

In the June 2016 issue of *Cell Stem Cell*, Zhu *et al.* elegantly combined gene-editing technologies in hPSCs to probe the relevance of previously discovered murine genetic mechanisms to human pancreatic development and disease (26). To study the role of genes during human development, they created gain of function and loss of function hPSC lines. For gain of function analysis, the authors used TALENs to target the *AAVS1* safe harbor site in hPSCs for integration of a M2rtTA (reverse tetracycline-controlled transactivator) and a tetracycline-response element (TRE) to drive gene expression. This M2rtTA is downstream a strong promoter and thus constitutively produced; in normal context in the absence of doxycycline, the TRE promoter is not activated and the gene of interest is silenced. Upon treatment with doxycycline, the available M2rtTA binds to the TRE promoter to drive expression of the gene of interest. Zhu and colleagues [2016] not only show high efficiency of transgene insertion after electroporation (roughly half), but also designed donor vectors compatible with Gateway cloning, making the fluent generation of hPSC lines overexpressing any gene of interest accessible. Cells inducibly expressing GFP show uniform expression of GFP and normal expression of key markers at different stages of pancreatic differentiation, such as Oct4 (hPSC), Sox17 (definitive endoderm), and Pdx1 (pancreatic progenitor), showing that this system can be used to uniformly overexpress genes during pancreatic development. Therefore, they studied the role of two well-known factors involved in endocrine development in mice: Ngn3 and Notch. They find that the genetic networks outlined in *in vivo* murine studies are conserved in *in vitro* human pancreatic development, with differentiating hPSCs overexpressing Ngn3 at the multipotent pancreatic progenitor stage greatly increased in endocrine formation while those overexpressing NotchIC were completely blocked (5,27). It would be interesting to see the effect of inducible Hes1 expression at these stages, as this is the downstream transcriptional target of activated Notch that has been suggested as primarily responsible for the repression of pancreatic endocrine fate (28). The establishment of this platform enables these types of studies to be performed.

In a past study from the same lab, an hPSC line that inducibly expresses Cas9 upon doxycycline treatment allowed for gene knockout with transfection of guide RNAs (gRNAs) (29,30). This system was used by Zhu *et al.* [2016] to interrogate the function of eight pancreatic transcription factors, six of which (Pdx1, Rfx6, Ptf1a, Glis3, Mnx1, and Ngn3 but not Hes1 or Arx) are associated with permanent neonatal diabetes mellitus (31-35). A total of 62 hPSC mutant lines were created, with each gene targeted by two gRNAs, four mutant lines per gene, and two isogenic control lines per targeting experiment. The clonal lines were analyzed throughout differentiation, at the hPSC, definitive endoderm, pancreatic progenitor, and beta cell stages. Their findings corroborate with conclusions from mouse studies, with a requirement for Pdx1, Rfx6, and Ngn3 in proper endocrine differentiation, an increase in endocrine cell formation after loss of Hes1, and a loss of glucagon expressing cells and reduction in insulin expressing cells after deletion of Arx (5,28,36). However, they find new insights into a requirement of Rfx6 for pancreatic progenitor specification, not through a change in proliferation or cell death but through direct reduction of Pdx1. This system further allowed for dosage analysis of Pdx1, revealing a striking haploinsufficiency of Pdx1 in endocrine differentiation. Closer analysis of the Ngn3 mutant line revealed few glucagon expressing cells similar to what has been shown in mouse studies, but also that sparse insulin positive cells form in the absence of Ngn3 expression, contrary to what has even been seen in mouse studies (5). The persistence of insulin positive cells in the absence of Ngn3 was intriguing, as patients with Ngn3 mutations are seldom diagnosed with diabetes and even those who are produce some level of blood C-peptide (the inactive form of insulin). To test the possibility that these patients are still able to make beta cells in the absence of Ngn3 function, the authors made an hPSC line carrying a patient specific Ngn3 mutation, and show that this mutation indeed renders this gene inactive but a small percentage of C-peptide positive cells still form. The mutations in these Ngn3 lines were corrected, and cells were analyzed to show that the mutant phenotypes were a direct result of the mutation and not due to CRISPR/Cas9 off target effects. However, the few C-peptide positive cells that form were not identified as bona fide beta cells, and they may be beta-like cells that are impaired in function. To determine

this, the C-peptide positive cells would need to be purified and analyzed next to a similar number of control cells for glucose responsiveness, however with so few C-peptide positive cells (~0.05%), it would be difficult to isolate a high enough number for physiological assessment. Finally, the authors combine technologies, knocking out Ngn3 and inducibly re-expressing it to analyze the temporal competence states identified by Johansson *et al.* (6) in human development. Ngn3 was re-expressed at different steps after pancreatic progenitor differentiation, showing that more hormone producing cells form during early stages and revealing a competence window in which endocrine cells formation is most robust. Whether there are distinct windows of competence in which progenitors are biased towards different endocrine cell fates however is still not clear, and it would be fascinating to see if these biases exist. Thus, this system allowed the discernment of genetic mechanisms regulating pancreatic development that are specific to the human context.

Zhu *et al.* (26) devised a sophisticated platform enabling the analysis of a large number of genes during human development. Using these tools, a multitude of genes can be assessed, and the developmental steps that are perturbed can be teased out and further investigated for deep, mechanistic analysis. While the authors show the benefits of these hPSC lines in context of pancreatic development, these cells can be used to understand the genetics governing development of any human cell type. As we learn more from model organisms, we can apply this knowledge to the development and study of human cells in a clinically relevant context.

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Footnote

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