The Role of Proinflammatory Cytokines on Pancreatic Cell Plasticity

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Ivan Achel Valdez

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The Role of Proinflammatory Cytokines on Pancreatic Cell Plasticity

Abstract

Diabetes mellitus is a complex and heterogeneous group of metabolic diseases characterized by a disruption in blood glucose homeostasis. This could result from an autoimmune destruction of insulin-producing pancreatic beta cells or insulin resistance with subsequent beta cell dysfunction. Thus, a major goal of diabetes research is developing strategies to replenish beta cells. One emerging and promising strategy is harnessing pancreatic plasticity—the ability of pancreatic cells to undergo cellular interconversions—and numerous studies implicate this phenomenon in diverse states of physiological stress or pancreatic injury. In this study, we investigated the effect of inflammatory cytokine stress on the differentiation potential of ductal cells in a human cell line in vitro; on mouse ductal cells by pancreatic intraductal injection in vivo; and throughout the progression of autoimmune diabetes in the non-obese diabetic (NOD) mouse model. We demonstrate that inflammatory cytokine insults stimulate epithelial-to-mesenchymal transition (EMT) as well as the endocrine program in human pancreatic ductal cells via STAT3-dependent NGN3 activation. Furthermore, we show that inflammatory cytokines activate ductal cell proliferation as well as ductal-to-endocrine cell reprogramming in vivo independently of hyperglycemic stress. Together, our findings provide novel evidence of inflammatory cytokines in directing ductal-to-endocrine cell differentiation and have significant implications for diabetes and cytokine-driven beta cell regeneration.
“No dejes para mañana lo que puedas hacer hoy”

“Cada esfuerzo tiene su recompensa”

-Emma Candelaria Valdez

For your guiding principles and unwavering support, mom, this thesis is dedicated to you.
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<td>ADA</td>
<td>American Diabetes Association</td>
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<tr>
<td>AP</td>
<td>Artificial Pancreas</td>
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<tr>
<td>CNTF</td>
<td>Ciliary Neurotrophic Factor</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>EMT</td>
<td>Epithelial-to-Mesenchymal Transition</td>
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<td>ESCs</td>
<td>Embryonic Stem Cells</td>
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<td>GMD</td>
<td>Gestational Diabetes Mellitus</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
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<tr>
<td>Hnf6</td>
<td>Hepatocyte Nuclear Factor 6</td>
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<tr>
<td>HPL</td>
<td>Human Placental Lactogen</td>
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<tr>
<td>IDF</td>
<td>International Diabetes Federation</td>
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<tr>
<td>IFNγ</td>
<td>Interferon Gamma</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin 1 Beta</td>
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
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<tr>
<td>iPSCs</td>
<td>Induced Pluripotent Stem Cells</td>
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<td>LADA</td>
<td>Latent Onset Diabetes of Adults</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
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<tr>
<td>LIRKO</td>
<td>Liver Insulin Receptor Knock Out</td>
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<tr>
<td>MafA</td>
<td>V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog A</td>
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<tr>
<td>MMF</td>
<td>Mycophenolate mofetil</td>
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<td>MODY</td>
<td>Maturity Onset Diabetes of the Young</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Ngn3</td>
<td>Neurogenin 3</td>
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<td>Nkx6.1</td>
<td>NK6 Homeobox 1</td>
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<tr>
<td>NOD</td>
<td>Non-Obese Diabetic</td>
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<tr>
<td>PDL</td>
<td>Pancreatic duct ligation</td>
</tr>
<tr>
<td>Pdx1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PPx</td>
<td>Partial Pancreatectomy</td>
</tr>
<tr>
<td>pStat3</td>
<td>Phosphorylated signal transducer and activator of transcription 3</td>
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<tr>
<td>T1D</td>
<td>Type 1 Diabetes</td>
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<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
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<tr>
<td>TNFa</td>
<td>Tumor Necrosis Factor alpha</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER ONE

INTRODUCTION
1.1 DIABETES MELLITUS OVERVIEW

1.1.1 History

Diabetes mellitus is one of the oldest diseases known to man. It was first recognized by the ancient Egyptians around 1500 B.C.E. as a rare condition that led to excessive urination and weight loss (Ahmed, 2002, Polonsky, 2012). Around the same time, Indian physicians identified the disease and classified it as “madhumeha” or honey urine noting that the urine would attract ants (Poretsky, 2010). It was not until the 2nd century C.E., however, that the Greek physician, Aretaeus of Cappadocia, coined the term "diabetes" (Greek for “to pass through”) to describe this disease (Laios et al., 2012). Later, in 1675, the word “mellitus” (Latin for “honey-sweet”) was added by Thomas Willis after rediscovering the sweet taste of patients’ urine (Ahmed, 2002, Laios et al., 2012). However, the actual measurement and detection of elevated glucose levels in the urine of these patients did not occur until 1776 in the work of English physician, Matthew Dobson (Ahmed, 2002, Polonsky, 2012).

Despite its long history, not very much was known about the mechanisms of diabetes until very recently. No effective treatment was available, and this disease led to certain death. Ironically, even though our understanding of the underlying causes, treatments and prevention of diabetes has tremendously increased during the last century, diabetes is now more prevalent than it has ever been. Thus, our quest to combat it, with a combination of scientific investigation and social effort, continues (Polonsky, 2012, Alberti and Zimmet, 2014).
1.1.2 Pathophysiology

Much of our understanding of the pathophysiology of diabetes arose from studying the pancreas and the discovery of insulin (Rosenfeld, 2002). In 1889, Joseph von Mering and Oscar Minkowski found that removing the pancreas from dogs resulted in fatal diabetes (Brogard et al., 1992). These experiments provided the first clue that the pancreas plays a role in regulating blood glucose levels. In 1910, Edward Albert Sharpey-Schafer hypothesized that the deficiency of a single factor, which he termed insulin (from the Latin word “insula” meaning “island”) and which he believed was derived from the pancreatic islets, was responsible for diabetes. But it was not until 1921 that Fredrick Banting and Charles Best actually discovered, purified and used insulin to successfully treat a human patient (Rosenfeld, 2002, Polonsky, 2012).

Today, we use the term diabetes mellitus to describe a complex and heterogeneous group of metabolic diseases characterized by a disruption in blood glucose homeostasis (Polonsky, 2012, Ahmed, 2002, Olokoba et al., 2012). There are several different types of diabetes, but the most common are type 1 diabetes (T1D) and type 2 diabetes (T2D). Although their underlying mechanisms are different, they are both characterized by high blood glucose levels, a condition known as hyperglycemia. The World Health Organization defines these levels as having fasting plasma glucose levels > 126mg/dl or 2hr plasma glucose levels > 200mg/dl following a 75g oral glucose load (World Health Organization, 2006). Proper glucose metabolism is vital, as glucose is a main source of energy for our body’s cells (Chang et al., 2004). Sustained, elevated blood glucose levels, however, can lead to severe diabetic complications, including ketoacidosis, retinopathy and associated blindness, kidney disease leading to renal failure, cardiovascular disease, stroke, and
neuropathy that may potentially result in amputations (Brownlee, 2005, Domingueti et al., 2015, van Belle et al., 2011, Bhatt et al., 2015). Thus, one of the main goals of diabetes research focuses on improving glycemic control, which could help prevent these severe, secondary complications and drastically improve patients’ health.

1.1.3 Diabetes Statistics: A Global Epidemic

It is becoming increasingly apparent that diabetes is a global epidemic, and the severity of this disorder is unequivocally highlighted by its statistics (Zimmet et al., 2001, Whiting et al., 2011). In 2015, 415 million people were estimated to have diabetes, and this number is expected to increase 54% to 642 million by 2040 (Figure 1-1) (International Diabetes Federation, 2015 Diabetes Atlas). In the United States alone, nearly 30 million people representing approximately 10% of the entire population, have diabetes today, and strikingly, 1 in 3 American adults are projected to have it by 2050 (Boyle et al., 2010).

The increased prevalence of diabetes has also imposed a huge financial burden on our health care system, which is only expected to increase in the future (1998, Zimmet et al., 2001). Diagnosed diabetes results in $320 billion in total (direct and indirect) costs annually (International Diabetes Federation, 2015 Diabetes Atlas). Even more alarming is the fact that >85 million people age 20 and older have pre-diabetes, a condition that can develop into T2D, in the United States, and 9 out of 10 people are not aware of it (American Diabetes Association, 2012). Without the proper lifestyle and/or medical interventions and lifestyle modifications, these people have a very high risk of developing full blown T2D. As it stands, diabetes is the 7th leading cause of death in the United State and the 8th leading cause in the world (World health Organization, 2014).
Figure 1-1. Global Predictions for the Diabetes Epidemic from 2015-2040. Despite increased awareness and new developments in the treatment of type 1 and 2 diabetes and prevention of type 2 diabetes, it is estimated that the prevalence of the disease in people ages 20-79 will increase 54% worldwide, from 415 million to 642 million, by 2040. Source: International Diabetes Federation (IDF) 2015 Diabetes Atlas.
1.2 CLASSIFICATION OF DIABETES

1.2.1 Type 1 Diabetes

Type 1 diabetes (T1D), sometimes referred to as Juvenile Diabetes, is one of the most common chronic disease in children and adolescents (Zimmet et al., 2001). It is characterized by a highly-specific autoimmune destruction of the insulin-producing beta cells within the pancreatic islets of Langerhans (Figure 1-2). This leads to an absolute deficiency of insulin and consequently elevated blood glucose levels, forcing patients to become dependent on an exogenous and life-long insulin therapy that must be stringently monitored (Mathis et al., 2001). Even though the exact cause of T1D remains unknown, it is understood that both genetic and environmental factors play a role in the etiology of the disease. To date, more than 40 genetic loci have been associated with T1D (Burren et al., 2011, Noble and Erlich, 2012). Out of these, the HLA (Human Leukocyte Antigen) region in chromosome 6p21 remains, by far, the greatest contributor of T1D genetic susceptibility (Rich et al., 2006, Noble and Erlich, 2012). Albeit speculative, some of the environmental factors that have been proposed to precipitate T1D, particularly in genetically susceptible individuals, include: viruses, cow’s milk, wheat proteins and toxic substances, such as nitrates (Knip et al., 2005, Placzkiewicz-Jankowska et al., 2007).

Since the 1950s, the incidence of T1D has been steadily increasing in developing countries, and some estimates indicate that it will double by the year 2020 (Patterson et al., 2009, Todd, 2010). In the United States, there are approximately 1.25 million cases of T1D, which accounts to approximately 5-10% of all diabetes cases (American Diabetes Association, 2012). However, some studies estimate that the number of T1D cases is largely
underestimated and 5-15% of people diagnosed with T2D may, in actuality, have T1D (Palmer et al., 2005, Atkinson, 2012).

1.2.2 Type 2 Diabetes

Type 2 diabetes (T2D) is by far the most prevalent type of diabetes accounting for approximately 90% of all diabetes cases. While T1D has traditionally been more common in children and T2D in adults, the prevalence of T2D in children and adolescents has been drastically increasing to the point that some estimates predict that the order may be reversed by 2020 (Fagot-Campagna et al., 2000). One of the primary reasons for this increased prevalence is the rise in globalization and industrialization in many societies that is conducive to a sedentary lifestyle and obesity, the predominant factors involved in the development of T2D (Fagot-Campagna et al., 2000, Zimmet et al., 2001, Olokoba et al., 2012).

In addition to environmental factors, there is a strong genetic foundation for T2D. Having relatives with T2D substantially increases the risks of developing this disease (Fagot-Campagna et al., 2000). The concordance among monozygotic twins is close to 100% and about 25% of them have a family history of T2D (Rother, 2007).

Unlike T1D, T2D does not begin by a decrease in insulin production but rather by a decrease in sensitivity of the body’s tissues, such as fat and muscle, to the effects of insulin, a condition known as insulin resistance (Olokoba et al., 2012). In fact, during the early stages of the disease, hyperinsulinemia (i.e. a relative increase in insulin levels in the bloodstream) may actually occur due to compensatory islet hyperplasia (Kulkarni et al., 1999) or beta cell work overload (Prentki and Nolan, 2006). As the disease progresses, beta cell dysfunction,
Figure 1-2. Beta cell dysfunction: The unifying theme of type 1 and type 2 diabetes.

Schematic representation and comparison of T1D and T2D disease progression. T1D is characterized by the autoimmune attack of pancreatic beta cells possibly triggered by environmental factors, such as viruses, which leads to beta cell dysfunction and eventually depletion. T2D has a strong genetic component, which, in combination with environmental factors such as obesity and lack of exercise, leads to insulin resistance, beta cell dysfunction and loss. (Adapted from the Kulkarni Laboratory Website, 2016).
de-differentiation or death may occur (Talchai et al., 2012) (Figure 1-2). The actual percentage of beta cell loss in T2D is unknown, but some studies estimate that this number is close to 50% (Butler et al., 2003), and the remaining cells may not be completely functional. Together, type 1 and 2 diabetes account for the vast majority of diabetes cases, but there are other types which will be briefly discussed.

1.2.3 Other Types of Diabetes

Gestational diabetes mellitus (GDM)

GDM is a condition in which a woman without prior history of diabetes temporarily exhibits high blood glucose levels during pregnancy, particularly during the third trimester (Hollander et al., 2007). Even though its exact cause is still not known, some studies have found that the changes in adipokines (e.g. leptin, adiponectin, TNFα, IL-6, resistin) and maternal hormones (e.g. human placental lactogen and the human placental growth hormone) can give rise to peripheral insulin resistance and hyperglycemia (Barbour et al., 2007). The prevalence of GDM has been reported to vary significantly, ranging from 1% to 14%, depending on which diagnostic criteria is used and which population is studied (Agarwal et al., 2005). Nevertheless, the highest prevalence is noted in Asian, African, Indian and Hispanic women (Carolan et al., 2012). If left untreated, some studies have reported that GDM can lead to adverse maternal and perinatal outcomes, including an increased risk of developing glucose intolerance, obesity, metabolic syndrome, and diabetes for both the mother and her offspring (2004).
Maturity Onset Diabetes of the Young (MODY)

MODY, also known as monogenic diabetes, refers to a group of autosomal dominant hereditary disorders (currently 11 known types) caused by a single mutation in a number of different genes that primarily affect pancreatic beta cell function (Gardner and Tai, 2012). It is estimated that MODY affects around 1-2% of all people with diabetes. Since some types of MODY exhibit similar symptoms as T1D and T2D, such as hyperglycemia and increased thirst and urination, it is often misdiagnosed (Gardner and Tai, 2012).

Latent Autoimmune Diabetes of Adults (LADA)

Latent autoimmune diabetes of adults (LADA), also known as late-onset diabetes of adults or diabetes type 1.5, is a subtype of autoimmune diabetes that shares characteristics of both T1D and T2D (Chatzianagnostou et al., 2015). It affects approximately 5-10% of people with diabetes (Brahmkshatriya et al., 2012). Since it is usually diagnosed in people over 30 years of age, and because the pancreatic beta cells of these patients initially still produce insulin, it is often misdiagnosed as T2D. However, since beta cell destruction inevitably occurs as the disease progresses, LADA patients will eventually need exogenous insulin administration (Chatzianagnostou et al., 2015, Brahmkshatriya et al., 2012).

1.3 CURRENT TREATMENTS AND RESEARCH STRATEGIES

1.3.1 Current Treatments

Since the 1920s, the gold standard treatment for autoimmune diabetes has been insulin replacement therapy. Under this treatment regimen, life-long monitoring of blood
glucose levels and multiple daily insulin injections are required. Although insulin injections are effective, the quality of life for some diabetes patients remains low as a result of life-endangering hypoglycemic episodes known as “insulin shock” and long-term diabetic complications (Polonsky, 2012). A study performed between 2004 and 2005 found that insulin was the most common drug implicated in adverse events treated in emergency rooms, accounting for 8% of total emergency room admissions, in the United States (Budnitz et al., 2006).

With increased technological advances, there are now newer and more effective treatments for normalizing blood glucose levels. One example is insulin pumps, which are small, computerized devices that can deliver steady (“basal”) or surge (“bolus”) doses of insulin (Polonsky, 2012). More recently, there has been some success with the use of artificial pancreases (AP) in clinical trials (Tauschmann et al., 2016), which use computer algorithms to measure and release insulin as needed.

When it comes to T2D, numerous studies have demonstrated that lifestyle and diet modifications, such as healthy eating and regular exercise, can significantly make a difference and reduce its incidence (Chen et al., 2012, Hu et al., 2001, Willi et al., 2007). In addition, there are many different kinds of oral or injected therapeutics available to T2D patients that act on beta cells or peripheral tissues (Bosi, 2009). The most common medication for T2D is metformin, which works by decreasing the amount of hepatic glucose production, improving insulin sensitivity, and thus exerting protective effects on pancreatic beta cells (Bosi, 2009). Lastly, insulin therapy may also be used in the later stages of T2D patients who exhibit beta cell dysfunction in combination with medication and lifestyle interventions.
1.3.2 Current Strategies for Beta Cell Regeneration

A. Suppressing Apoptosis / Enhancing Proliferation of Existing Beta Cells

Beta cell dysfunction and/or loss is a hallmark of diabetes, and significant effort is placed on finding ways to increase beta cell mass (Figure 1-3). One potential strategy is suppressing beta cell apoptosis, which occurs due to inflammatory cytokine insults in T1D, and to some extent in T2D (Cnop et al., 2005). Therefore, some groups focus on finding small-molecule inhibitors with protective effects against cytokine-induced beta cell death, such as inhibition of histone deacetylases (HDAC) with suberoylanilide hydroxamic acid (SAHA) or trichostatin A (TSA) (Chou et al., 2012).

In addition, one of the most commonly implemented strategies to enhance beta cell mass is increasing beta cell proliferation from existing beta cells. Beta cells are terminally differentiated cells and, under normal conditions, they exhibit extremely low proliferation rates (Teta et al., 2005). However, beta cell mass has also been shown to be dynamic in response to environmental cues and/or states of physiological stress, such as pregnancy (Rieck and Kaestner, 2010) and insulin resistance (Escribano et al., 2009). The compensatory islet hyperplasia that occurs in response to insulin resistance, as in the Liver Insulin-Receptor Knock Out (LIRKO) mouse model of insulin resistance, is a dramatic example that demonstrates the remarkable ability of endogenous beta cells to proliferate (Michael et al., 2000). Islet cell mass in the LIRKO mouse model has been found to increase up to 6-fold and, importantly, cell proliferation to be specific to pancreatic beta cells (El Ouamari et al., 2013). Due to such observations, many groups have focused on identifying factors that drive beta cell proliferation in states of insulin resistance (Flier et al., 2001, El Ouamari et al., 2013, So et al., 2015). Recently, there have been promising studies which identify
compounds such as SerpinB1 that enhance human, mouse and zebrafish beta cell proliferation (El Ouaamari et al., 2016). Other recent strategies for enhancing beta cell proliferation include the inhibition of TGF-beta signaling (Dhawan et al., 2016) and of dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) (Dirice et al., 2016) with small-molecule inhibitors. Despite their tremendous potential for beta cell regeneration, a possible downside of such approach for T1D patients lies in the fact that, by the time diabetes is diagnosed, more than 90% of pancreatic beta cells would have already been destroyed (van Belle et al., 2011). For that reason, it is possible that other strategies, such as advancements in stem cell directed differentiation methodologies, might help to overcome this issue.

B. Stem Cell Research

The ability of human pluripotent stem cells—embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs)—to differentiate into practically any cell of the human body makes them a particularly attractive and exciting strategy for pancreatic beta cell regeneration. In theory, the number of beta cells that could be generated with this approach is unlimited, and numerous groups have made significant progress in differentiating stem cells into beta-like cells in vitro that can be transplanted into mice and restore euglycemia (Kroon et al., 2008, Pagliuca et al., 2014, Rezania et al., 2014). However, to date, there has been limited success in efficiency rates and in reproducibly deriving mature, functional, glucose-responsive beta cells (Kushner et al., 2014).

Unlike ESCs, autologous stem cells such as iPSCs have been shown to avoid alloimmune, but not the autoimmune, responses. Thus, current efforts are being directed
towards finding ways to protect these cells via encapsulation techniques (Kroon et al., 2008, D'Amour et al., 2005), as has been done with stem cells transplanted into streptozotocin (STZ) treated mice and the non-obese diabetes (NOD) mouse model of autoimmune diabetes (Lee et al., 2009).

C. Islet Transplantations

Pancreas transplantation is the only accepted procedure that has demonstrated some level of success in normalizing blood glucose levels of insulin-dependent patients (Harlan et al., 2009). The Edmonton protocol, developed by scientist in the University of Alberta in Edmonton, Canada, is the most successful and widely used protocol for islet transplantations, and it continues to be refined. However, despite the advancements it has made, there are still several critical issues that make it a difficult and somewhat impractical strategy. Some examples include the relatively short duration of euglycemia achieved, which is currently 1 to 5 years after islet transplantation in about 10% of the patients (Ryan et al., 2005), and the extremely limited number of organ donors, as each recipient receives islets from one to as many as three donors (Bruni et al., 2014).

Another major disadvantage of this strategy is the simultaneous need for immunosuppressive drug therapy, especially since current immunosuppressive drugs have been shown to interfere with proper beta cell function (Nir et al., 2007, Robertson, 2004). For example, rapamycin (sirolimus), a commonly used immunosuppressant in islet transplantation procedures, has been shown to affect islet engraftment (Zhang et al., 2006), interfere with angiogenesis (Cantaluppi et al., 2006), induce insulin resistance (Fraenkel et al., 2008), and inhibit in vivo beta cell replication (Zahr et al., 2007). Other immuno-
Figure 1-3. Current Beta Cell Regeneration Strategies. Diagram of some of the current research strategies for beta cell regeneration which include: 1) identification of factors that induce replication of existing beta cells or prevent apoptosis; 2) pluripotent stem cell (ESC or iPSC) differentiation; 3) islet transplantations; 4) cellular transdifferentiation.
suppressive drugs, such as tacrolimus, corticosteroids, and MMF (mycophenolate mofetil), have been shown to decrease insulin transcription (Robertson, 2004) and inhibit beta cell neogenesis (Gao et al., 2007). Moreover, due to the need for life-long immunosuppressive drug therapy, this clinical procedure is not currently available for children or people with good glycemic control (Bruni et al., 2014).

D. Cellular Transdifferentiation

The idea of cellular transdifferentiation or cellular interconversions is not new. Evidence of transdifferentiation is well established and has been reported by numerous groups in numerous tissues (Shen et al., 2004). Our understanding of the mechanisms involved in this process has been evolving rapidly in recent years due to advancements in the discovery of transcription factors involved in the field of developmental biology. Today, we have exciting new evidence that demonstrates that functional, insulin-producing, beta-like cells can be derived from intestinal (Bouchi et al., 2014, Chen et al., 2014), stomach (Ariyachet et al., 2016) and even other pancreatic cells (Figure 1-3) (Baeyens et al., 2014b).

1.4 THE PLASTIC PANCREAS

1.4.1 Pancreatic Development and Anatomy

During gastrulation, the epiblast is partitioned into three germ layers: 1) ectoderm, which goes on to form skin and the central nervous system, 2) mesoderm, which will form blood, bone and muscle, and 3) endoderm, which will form the respiratory and digestive
Figure 1-4. Overview of pancreatic development. Pluripotent stem cells found in early developmental stages have the ability to adopt one of the three germ layers: ectoderm, mesoderm, and endoderm, from which pancreatic cells are derived. A subset of endoderm cells acquires PDX1 expression and becomes pancreatic endoderm, which has the ability to branch out into acinar, ductal and NGN3$^+$ endocrine progenitor cells. Pancreatic progenitor cells further differentiate into the five hormone-expressing cells of the pancreatic islet. (Source: Pagliuca and Melton, 2014).
tracts (Figure 1-4). Endoderm tissue then folds into a primitive gut tube that later branches off into different organs. A subset of these endoderm cells acquires the expression of key transcription factors such as Pancreatic and Duodenal Homeobox 1 (PDX1), and becomes pancreatic endoderm, which has the ability to branch out into acinar, ductal and Ngn3+ endocrine progenitor cells. Ngn3 is a critical basic helix-loop-helix transcription factor that is necessary for the specification of endocrine cells in the pancreatic islets, and serves as a marker of undifferentiated endocrine progenitor cells (Rukstalis and Habener, 2009). Pancreatic Ngn3+ progenitor cells further differentiate into five hormone-expressing cells that make up the pancreatic islets of Langerhans (Pagliuca and Melton, 2013) (Figure 1-4).

The adult pancreas is a highly diversified glandular organ that houses two major cell types which contribute to the endocrine and exocrine compartments (Reichert and Rustgi, 2011). Endocrine cells constitute ~2% of the pancreas and consists of five hormone-secreting cells—alpha cells that secrete glucagon, beta cells that secrete insulin, delta cells that secrete somatostatin, gamma or PP cells that secrete pancreatic polypeptide, and epsilon cells that secrete ghrelin. Together, they make up the pancreatic islets of Langerhans (Figure 1-5).

The remainder and vast majority of the pancreas is made up of the exocrine portion and contains acinar and ductal cells. Acinar cells are responsible for secreting digestive enzymes into the small intestine to help digest carbohydrates, lipids and proteins, and ductal cells secrete bicarbonate to help neutralize stomach acidity (Reichert and Rustgi, 2011).

1.4.2 Previous Studies on Pancreatic Plasticity

The existence of a “pancreatic stem cell” that is capable of replenishing lost beta cells has not been established and, thus, remains a controversial topic. However, evidence of
Figure 1-5. Anatomy of the Adult Pancreas. The majority of the pancreas consists of exocrine tissue, which is made up of acinar cells that secrete pancreatic enzymes delivered to the intestine to facilitate digestion of food, and ductal cells that secrete bicarbonate to neutralize stomach acidity. Scattered throughout the exocrine tissue are thousands of clusters (which account for only 5% of pancreatic mass) of endocrine cells known as islets of Langerhans. Within the islet, alpha (α) cells produce glucagon; beta (β) cells, insulin; delta (δ) cells, somatostatin; gamma (γ) cells, pancreatic polypeptide, and epsilon (ε, not shown), ghrelin – all of which are delivered to the blood. Source: (Trucco, 2005).
inherent pancreatic plasticity—the ability of pancreatic cells to undergo cell-to-cell interconversions within the pancreas—has gained considerable attention. Over the last several years, a wave of literature uncovering the potential of both endocrine and exocrine (ductal and acinar) cells to transdifferentiate and regenerate pancreatic beta cells, in vivo and in vitro, has emerged (Valdez et al., 2015). The work carried out by these groups is critical, as it highlights the intrinsic capability of the adult pancreas to regenerate specifically in times of need, such as injury and cellular stress (Figure 1-6).

Endocrine Cell Plasticity

One example of endocrine cell plasticity is alpha-to-beta cell transdifferentiation. Using non-physiological models, some studies have shown that diphtheria toxin-induced beta cell depletion can promote alpha-to-beta cell conversion (Thorel et al., 2010). In addition, Chung et al. combined pancreatic duct ligation (PDL) with high-dose alloxan (a chemical that selectively destroys beta cells) in mice to demonstrate that, in adult animals, beta cells are primarily derived from alpha cells (Chung et al., 2010). More recently, the same group showed that alpha-to-beta-to-delta cells could be observed in mice in vivo following caerulein (a chemical that induces acute pancreatitis) treatment and in human type 1 diabetic pancreatic sections ex vivo (Piran et al., 2014).

Another example of endocrine cell plasticity is beta cell de-differentiation. Previous studies have reported that physiological stress in the islets of several type 2 diabetes mouse models might lead rodent beta cells to regress to a less mature NGN3-expressing progenitor stage (Talchai et al., 2012). Interestingly, upon relief from the hyperglycemic stress via insulin therapy, another group was able to demonstrate that these NGN3+ de-differentiated
cells are capable of re-differentiating into mature insulin$^+$ cells (Wang et al., 2014). Finally, stressors encountered during human islet isolation have been shown to stimulate inflammatory signaling and beta cell de-differentiation (Negi et al., 2012).

*Exocrine Cell Plasticity*

Due to its abundance, exocrine tissue is a strong endogenous candidate for beta cell neogenesis. Previous studies have shown that inflammation can induce exocrine acinar cells to give rise to progenitor cells in cases of autoimmune or chronic pancreatitis in humans (Ko et al., 2013). More recently, Baeyens et al. (2014) used diabetic mice in an elegant study to demonstrate that acinar cells are capable of restoring a glucose-responsive, functional beta cell mass upon a transient cytokine (EGF and CNTF) exposure through the activation of STAT3 (signal transducer and activator of transcription 3) and NGN3 (Baeyens et al., 2014a).

The exocrine ductal cell is another highly studied pancreatic cell type that has been implicated in cellular plasticity. Using mice, various groups have reported that pancreatic ductal cells can serve as a source of NGN3$^+$ endocrine progenitors following stressors, such as pancreatic injury by duct ligation (Bonner-Weir et al., 1993), partial pancreatectomy (Walker et al., 1992), or targeted beta cell destruction via constitutive interferon gamma (IFN$\gamma$) expression (Gu et al., 1995a, Gu et al., 1995b, Gu and Sarvetnick, 1993). Using human primary ductal cells, it has been demonstrated that the transgenic expression of NGN3 along with other critical endocrine factors is important for their conversion into glucose-responsive, insulin-secreting endocrine cells (Lee et al., 2013). In addition, some studies have
Figure 1-6. Pancreatic Cell Plasticity. Schematic representation of pancreatic plasticity induced by various stressors. Stress induced by cytokines, nutrients, injury, and other physiological stressors has been implicated in the induction of intrapancreatic cell-to-cell conversions. The inherent plasticity of the pancreas is promising for regenerative medicine, as beta cell regeneration is a major goal for future diabetes therapies. (Source: Valdez et al., 2016).
shown that humans that undergo physiological stress in the form of insulin resistance exhibit an increase in insulin$^+$ ductal cells relative to control subjects (Mezza et al., 2014).

1.4.3 Relevance of Pancreatic Plasticity and Thesis Focus

Pancreatic cell plasticity has been reported in most pancreatic cells, both endocrine and exocrine, using a variety of models and experimental approaches (Figure 1-6), but the unifying theme of these studies is cellular stress. Despite the current treatments currently available, alternative therapeutic options are needed to address the many challenges of diabetes. Harnessing the inherent cellular plasticity of the pancreas as a source of new beta cells is a promising field of regenerative medicine.

The focus of this work is to explore the effects of inflammatory stress on the differentiation potential of exocrine ductal cells. Chapter 2 addresses the effects of inflammatory cytokines in a human ductal cell line in vitro. We demonstrate that a cocktail of three inflammatory cytokines, TNF$\alpha$, IL-1$\beta$ and IFN$\gamma$, can induce epithelial-to-mesenchymal transition (EMT) and induce the expression of NGN3 via STAT3 activation in human ductal cells. Chapter 3 explores the in vivo effects of an acute insult of the same inflammatory cytokines by pancreatic intraductal injection in mice and the chronic effect of pancreatic insulitis throughout the progression of autoimmune diabetes in the non-obese diabetic (NOD) mouse model. This work demonstrates that inflammatory cytokines can activate pancreatic ductal cell proliferation and the endocrine program in mouse ductal cells in vivo independently of hyperglycemic stress. Finally, Chapter 4 provides a summary and discussion of our findings in the context of existing literature and provides caveats and future perspectives for the field of pancreatic palasticity and regenerative medicine.
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CHAPTER TWO

IN VITRO

EFFECTS OF INFLAMMATORY CYTOKINE INSULTS ON HUMAN PANCREATIC DUCTAL CELL DIFFERENTIATION
Proinflammatory cytokines induce endocrine differentiation in pancreatic ductal cells via STAT3-dependent NGN3 activation

Ivan Achel Valdez1,2, Ercument Dirice1, Manoj K. Gupta1, Jun Shirakawa1, Adrian Kee Keong Teo1,3,* Rohit N. Kulkarni1,*

1Section of Islet Cell and Regenerative Biology, Joslin Diabetes Center, Department of Medicine, Brigham and Women’s Hospital, Harvard Stem Cell Institute, and Harvard Medical School, Boston, MA 02215, USA.

2Department of Cell Biology, Program in Biological and Biomedical Sciences, Graduate School of Arts and Sciences, Harvard University, Cambridge, MA 02138, USA.

3Discovery Research Division, Institute of Molecular and Cell Biology, Proteos, Singapore 138673, Singapore.

*Corresponding Author

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Writing 1st draft of manuscript and participating in subsequent revisions.
2.1 ABSTRACT

A major goal of diabetes research is developing strategies to replenish pancreatic insulin-producing beta cells. One emerging and promising strategy to accomplish this is harnessing pancreatic plasticity—the ability of pancreatic cells to undergo cellular interconversions—and many studies implicate this phenomenon in diverse states of physiological or cellular stress. In this chapter, we investigate the effects of inflammatory cytokine stress, specifically by TNFα, IL-1β and IFNγ stimulations, on the differentiation potential of ductal cells in a human cell line in vitro. We demonstrate that inflammatory cytokine insults stimulate epithelial-to-mesenchymal transition (EMT) as well as the endocrine program in human pancreatic ductal cells via STAT3-dependent NGN3 activation. Our findings provide evidence and mechanistic insights of inflammatory cytokine-driven ductal-to-endocrine cell transdifferentiation, an area that has significant implications for beta cell regeneration.

2.2 INTRODUCTION

The ability of the pancreas to host an array of intrapancreatic cellular conversions offers promise to the fields of regenerative biology and diabetes research. A growing and compelling line of evidence in rodents and humans suggests that pancreatic cell plasticity allows the generation of insulin-producing cells from non-beta cell sources, particularly during times of need (e.g. beta cell loss, pancreatic injury or metabolic stress) (Valdez et al., 2015). Various groups have argued for or against this phenomenon in an ongoing debate about the origin of the newly formed beta cells (Dor et al., 2004, Nir et al., 2007, Kopp et al., 2011, Xiao et al., 2013, Solar et al., 2009, Kulkarni et al., 2004). Therefore, and given its
potential therapeutic applications, it is timely to address these questions to obtain a better understanding of the mechanisms that control pancreatic cell plasticity.

For many decades, countless insights have been gained about normal and pathological processes by studying human cells in vitro (Park et al., 2008), and numerous groups have conducted studies in vitro that demonstrate pancreatic cell plasticity. For example, ductal-to-beta cell transdifferentiation has been achieved by the forced expression of various transcription factors (Ngn3, MafA, Pdx1 and Pax6) by adenoviral transduction (Lee et al., 2013, Swales et al., 2012), and growth factor stimulations (Activin A and Exendin 4) (Kim et al., 2013). Acinar-to-beta cell transdifferentiation has also been observed following adenoviral transduction of Pdx1, Ngn3 and MafA (Minami et al., 2011, Akinci et al., 2012) and with stimulation with EGF + LIF (Baeyens et al., 2005). In all of these cases, the exocrine-derived insulin-producing cells generated have been shown to be able to respond to glucose stimulations (Lee et al., 2013, Minami et al., 2011) or restore euglycemia in diabetic mice (Kim et al., 2013, Akinci et al., 2012, Baeyens et al., 2005). Nevertheless, despite these promising observations, the cellular and molecular mechanisms that drive pancreatic cell interconversions are not well understood.

In this chapter, we conducted in vitro studies to investigate the effects of inflammatory cytokines, TNFα, IL-1β and IFNγ, on the differentiation potential of the human ductal cell line, PANC-1. Our work demonstrates that inflammatory cytokines lead to cellular stress and triggers characteristics of ductal-to-endocrine cell differentiation in PANC-1 cells. We show that PANC-1 cells undergo morphological changes, EMT, and STAT3-dependent upregulation of key endocrine progenitor marker, NGN3, upon inflammatory cytokine stimulations.
2.3 RESULTS

2.3.1 Inflammatory cytokines induce epithelial-to-mesenchymal transition in the human epithelial ductal cell line, PANC-1

The human ductal epithelial cell line, PANC-1, a commonly used cell line for in vitro differentiation studies (Wu et al., 2010, Lefebvre et al., 2010, Hardikar et al., 2003), was used for our in vitro cytokine stimulation analyses. These cells were treated either with a single cytokine or different combinations of the three proinflammatory cytokines, TNFα, IL-1β, and IFNγ at different time points. The optimal dose of cytokines (Cx) was determined by assessing the gene expression of cellular stress marker, iNOS, by qPCR analyses (Figure 2-1). As expected, the results demonstrate that a cocktail of the three cytokines has a stronger effect than individual or any of the different combinations of two cytokines as measured by iNOS mRNA expression upregulation (Figure 2-1A). In addition, decreasing the dose to [1/2X], [1/10X] and [1/100X] is not as effective as [1X] in inducing iNOS mRNA expression, while increasing the dose to [2X] or [10X] does not make a significant difference relative to the [1X] condition (Figure 2-1B and 2-1C). Therefore, based on these optimization studies, we finalized the optimal dose for cytokine stimulations, which is referred to as [1X] and consists of the following doses: TNFα [50ng/mL]; IL-1β [25ng/mL]; IFNγ [100ng/mL]. All experiments henceforth are based on a [1X] dose unless specified otherwise.

Having determined the optimal dose of cytokines, the mRNA levels of cellular stress markers, iNOS and CHOP, were measured by qPCR analysis in untreated or cytokine-treated PANC-1 cells at different time points: 24hrs, 48hrs and 72hrs (Figure 2-2A). These analyses demonstrated an increase in iNOS and CHOP gene expression at all time points relative to
Figure 2-1. Inflammatory cytokine dose-optimizations based on cellular stress marker, iNOS, mRNA expression.

(A-C) Dose optimization of iNOS gene expression by qPCR analyses upon cytokine stimulations: (A) 72hr treatment with either single or combination of [1X] cytokines, (B) 72hr treatment with [1/2X], [1/10X] or [1/100X] dose of cytokines, and (C) 72hr treatment with [1X], [2X] or [10X] dose of cytokines relative to untreated controls.

Data represent mean ± SEM. *p≤0.05 (n=3 for each group). qPCR data was normalized to beta actin. All experiments were repeated at least on two independent occasions.
Figure 2-2. Inflammatory cytokines induce cellular stress and hinder cell growth in the human ductal cell line, PANC-1.

(A) qPCR analysis of iNOS and CHOP in untreated (Ctrl) or cytokine-treated [1X] Cx concentrations: TNFα [50ng/mL]; IL-1β [25ng/mL]; IFNγ [100ng/mL]) PANC-1 cells.

(B) Representative immunofluorescence images of untreated or cytokine-treated cells for Ki67 and cleaved caspase-3 staining at 72hrs (top panels) and quantification of Ki67 or cleaved caspase-3 / DAPI (bottom panels). Scale bar = 100µM.

(C) Growth curve of PANC-1 cells at 24, 48 and 72hrs after [1X] cytokine stimulations.

Data represent mean ± SEM. *p≤0.05 relative to untreated controls. (n=3 for each group).

qPCR data was normalized to beta actin. All experiments were conducted on at least two independent occasions.
untreated controls (Figure 2-2A). As another marker of cellular stress, cleaved caspase-3 expression was assessed by immunofluorescence analyses. These analyses revealed a significant increase in the apoptosis marker, cleaved caspase-3, following cytokine stimulations relative to untreated controls (Figure 2-2B; left, upper and lower panels).

An important decision progenitor cells must make is to proliferate or differentiate, as many cells, including endocrine progenitor cells throughout beta cell development (Kim et al., 2015), must temporarily defer their proliferative abilities while undergoing differentiation (Zhu and Skoultchi, 2001, Gingold et al., 2014). To assess proliferation, we performed Ki67 immunostaining (Figure 2-2B; right, upper and lower panels) and cell growth curve analyses (Figure 2-2C). Cytokine-treatment of PANC-1 cells led to a significant decrease in proliferation marker, Ki67, at 72hrs (Figure 2-2B; right, upper and lower panels) and reduced cell number at all time-points relative to untreated controls (Figure 2-2C).

Another important indicator of cellular differentiation is morphological change (Gallo et al., 2007). Thus, we observed cells by brightfield microscopy in untreated and cytokine-treated conditions and found that untreated cells exhibited a normal epithelial-like morphology (Lieber et al., 1975), while cytokine-treated cells appeared more mesenchymal-like at all 3 time-points (Figure 2-3; representative data shown for 72hrs) (Attali et al., 2007).

Epithelial-to-mesenchymal transition (EMT) has been associated with the differentiation of epithelial cells toward a pancreatic endocrine progenitor state (Rukstalis and Habener, 2007). Therefore, we assessed the levels of the ductal cell marker, carbonic anhydrase II (CA-II), the epithelial marker, e-cadherin (CDH1), and the mesenchymal marker, SNAIL2, by qPCR. These analyses demonstrated a significant decrease in mRNA levels of ductal cell marker, CA-II, and epithelial marker, CDH1, at all time points, as well
Figure 2-3. Morphological change in PANC-1 cells upon inflammatory cytokine stimulations. Representative brightfield images of PANC-1 cells under untreated (Ctrl) or [1X] cytokine-treated conditions at 72hrs. Scale = 100µM.
as a significant increase in mesenchymal marker, SNAIL2 (Rukstalis and Habener, 2007) at 72hrs post-stimulation (Figures 2-4A-C). To confirm the expression of these genes at the protein level, we performed protein immunoblot analyses. These experiments also revealed a decrease in CDH1 and an increase in SNAIL2 in PANC-1 cells treated with cytokines at 72hrs relative to untreated controls (Figures 2-4D and 2-4E).

Together, these data suggest that proinflammatory cytokine-induced stress triggers the loss of ductal cell identity in the human epithelial ductal cell line, PANC-1, and stimulates their differentiation from an epithelial cell type towards a mesenchymal-like cell.

2.3.2 Proinflammatory cytokines upregulate NGN3 in human ductal cells via STAT3 signaling

To further address the hypothesis that cytokine-induced stress induces the differentiation of the human ductal cell line, PANC-1, towards the endocrine cell lineage, we assessed the expression of the endocrine progenitor marker, NGN3. Following [1X] cytokine stimulation, qPCR analyses revealed an upregulation of NGN3 expression at all time points in cytokine-treated conditions relative to untreated controls (Figure 2-5A). These results were corroborated at the protein level by immunofluorescence analyses (Figures 2-5B and 2-5C).

In addition to NGN3, gene expression of other endocrine markers, NKX6.1 (Figure 2-6A) and HNF6 (Figure 2-6B), were also assessed in PANC-1 cells by qPCR analyses. We found that the mRNA expression of both of these endocrine genes was upregulated upon cytokine stimulation (Figures 2-6A and 2-6B).

Next, we wanted to assess the specificity of cytokine-induced endocrine progenitor upregulation. To accomplish this, we used another cell line of similar (endodermal)
Figure 2-4. Inflammatory cytokines induce Epithelial-to-Mesenchymal Transition (EMT) in cytokine-treated human ductal cells.

(A-C) qPCR analysis of (A) ductal marker, CA-II; (B) epithelial marker, CDH1; and (C) mesenchymal marker, SNAIL2, in untreated or cytokine-treated cells at 24, 48 and 72hrs.

(D, E) Protein immunoblot analysis (D) and quantification (E) of CDH1 (E-cadherin) and SNAIL2 after 1X Cx treatment at 72hrs.

Data represent mean ± SEM. *p<0.05 and **p<0.005 relative to untreated controls. (n=3 for each group). qPCR data was normalized to beta actin. All experiments were conducted on at least two independent occasions.
developmental origin—the human liver cell line, HepG2. Stimulation with the same cocktail of inflammatory cytokines did not affect the mRNA expression levels of NGN3, NKKX6.1 or HNF6 relative to untreated controls at any time-point (Figures 2-6C-E).

We then wanted to investigate the gene expression levels of NGN3 in PANC-1 cells after cytokine removal. To do this, we divided the experiment into three groups:

1) 96hrs untreated control (Ctrl)
2) 48hrs without cytokines + 48hrs with cytokines, (Cx-|Cx+)
3) 48hrs with cytokines + 48hrs without cytokines (Cx+|Cx-)

As expected, there was a down-regulation in the expression of the stress marker, iNOS gene, after cytokine removal (Cx+|Cx-), but, interestingly, NGN3 mRNA and NGN3 protein levels, as well as NKKX6.1 and HNF6 mRNA levels remained elevated even after cytokine removal (Figure 2-7A-E). qPCR analyses revealed an autocrine upregulation of TNFα and IL-1β gene expression as has been previously reported (Brewington et al., 2001) but not that of IFNγ (Figure 2-7F). These observations could, in part, explain the persistent and elevated expression of endocrine genes even upon cytokine removal (Cx+|Cx-) (Figure 2-7B-F).

To test this, we treated PANC-1 cells with a cocktail of specific receptor antagonists (TNFαR [25µM]; IL-1R [50µM]; IFNγR [25µM]) after cytokine removal and examined NGN3 protein expression by immunoblot analysis. We found that NGN3 expression is markedly decreased in the presence of cytokine receptor antagonists (Cx+|Ag) relative to the cytokine-treated (Cx-|Cx+) and cytokine-depleted (Cx+|Cx-) conditions (Figure 2-7E), suggesting that inflammatory signaling is necessary for NGN3 expression upregulation in PANC-1 cells.
Figure 2-5. NGN3 is upregulated in human ductal cells upon inflammatory cytokine stimulation.

(A) qPCR analysis of NGN3 in untreated or [1X] cytokine-treated (TNFα, IL-1β and IFNγ) PANC-1 cells at 24hrs, 48hrs and 72hrs.

(B, C) Representative immunofluorescence images (B) and quantification (C) of untreated or cytokine-treated cells for NGN3 (red) and DAPI (blue) at 72hrs.

Data represent mean ± SEM. *p≤0.05 (n=3 for each group). qPCR data was normalized to beta actin. Experiments were conducted on at least two independent occasions.
Figure 2-6. Upregulation of endocrine markers is specific to PANC-1 cells.

(A, B) qPCR analyses of endocrine progenitor markers, (A) *N*K*6*.1 and (B) *H*NF6, in untreated (Ctrl) and [1X] cytokine-treated (Cx) PANC-1 cells at 24, 48 and 72hrs.

(C-E) qPCR analyses of endocrine progenitor markers, (C) *N*G*N*3, (D) *N*K*6*.1, and (E) *H*NF6, in untreated (Ctrl) and [1X] cytokine-treated (Cx) human liver cell line, HepG2, at 24, 48 and 72hrs. Data represent mean ± SEM. *p*≤0.05 (n=3 for each group). qPCR data was normalized to beta actin. Experiments were conducted on at least two independent occasions.
Finally, since NGN3 is a downstream effector of STAT3 (Kaucher et al., 2012, Baeyens et al., 2006, Lemper et al., 2015), and STAT3 has been shown to be activated by inflammatory signaling (Yu et al., 2009, Ivashkiv and Donlin, 2014), we assessed whether NGN3 upregulation in our proinflammatory cytokine-treated PANC-1 cell model is mediated through STAT3 activation. To this end, we used a phosphorylated STAT3 (pSTAT3) small-molecule inhibitor, Stattic, (Selleckchem) and assessed the levels of pSTAT3 by immunoblot analyses (Figure 2-8). Our results demonstrate that [1X] cytokine stimulation at 72hrs induces an increase in pSTAT3 levels relative to untreated and DMSO-treated controls, which is abrogated upon the addition of Stattic [5µM] and decreased to control levels upon Cx+Stattic [5µM] treatment (Figure 2-8). Importantly, NGN3 protein expression is upregulated only upon [1X] cytokine stimulation relative to untreated and DMSO controls, but not when cells are treated with Stattic [5µM] or Cx+Stattic [5µM] (Figure 2-8).

Together, the results from these experiments demonstrate that proinflammatory cytokines, TNFα, IL-1β and IFNγ, upregulate NGN3 expression specifically in the human ductal cell line, PANC-1. Furthermore, we show that NGN3 mRNA and NGN3 protein expression is sustained even upon cytokine removal due an autocrine upregulation of inflammatory cytokines, which can be abrogated by the addition of specific cytokine receptor antagonists. Finally, we demonstrate that cytokine-induced NGN3 expression upregulation requires the activation of pSTAT3.
Figure 2-7. NGN3 upregulation in human ductal cells is sustained through an autocrine inflammatory cytokine release.

(A-D) qPCR analysis of (A) iNOS, (B) NGN3, (C) NKX6.1 and (D) HNF6 after 1) 96hrs untreated conditions (ctrl), 2) 48hr without cytokines + 48hr with cytokines (Cx−|Cx+), or 3) 48hrs with cytokines + 48hrs of cytokine removal (Cx+|Cx−).

(E) Protein immunoblot analysis of NGN3 after 1) 96hrs untreated conditions, 2) 48hr without cytokines + 48hr with cytokines (Cx−|Cx+), 3) 48hrs with cytokines + 48hrs of cytokine removal (Cx+|Cx−), 4) 48hrs with cytokines + 48hrs of cytokine removal with specific cytokine receptor antagonists (TNFαR [25µM]; IL-1R [50µM]; IFNγR [25µM]) (Cx+|Ag). Beta actin was used as a loading control.

(F) qPCR analysis of TNFα, IL-1β, and IFNγ in untreated and [1X] cytokine-treated PANC-1 cells at 48hrs. Data represent mean ± SEM. *p≤0.05 (n=3 for each group). qPCR data was normalized to beta actin. Experiments were conducted on at least two independent occasions.
Figure 2-8. Cytokine-induced NGN3 upregulation in human ductal cell line, PANC-1, is mediated through phosphorylated STAT3.

Protein immunoblot analysis of pSTAT3, total STAT3, and NGN3, in untreated control, DMSO-treated control, 1X cytokine-treated (Cx), Stattic [5µM], or [1X] Cytokine + Stattic [5µM] treatment after 48hrs. Beta actin was used as a loading control.
2.4 DISCUSSION

Pancreatic cell plasticity has been demonstrated in numerous studies, and a common theme within the field is focused on the emergence of NGN3, a key transcription factor and master regulator of pancreatic endocrine cell fate. While there are some links between inflammatory stress and exocrine ductal cell transdifferentiation, detailed cell and molecular mechanisms have not been fully established. Hence, in this chapter, we sought to investigate the effects of cytokines, specifically TNFα, IL-1β, and IFNγ—critical cytokines implicated in the pathogenesis of both type 1 and type 2 diabetes—on the differentiation potential of human ductal cells.

Our work demonstrates that inflammatory signaling induces morphological changes, epithelial-to-mesenchymal transition (EMT) and the upregulation of endocrine progenitor marker, NGN3, via STAT3 activation in the human ductal epithelial cell line PANC-1. We also show that NGN3 upregulation is abrogated upon STAT3 and inflammatory cytokine receptor inhibition. It has been previously demonstrated that NGN3 expression is mediated through STAT3 activation in vitro (Kaucher et al., 2012, Baeyens et al., 2006, Lemper et al., 2015) and in vivo (Baeyens et al., 2014) using rodent acinar cells. Our study now highlights the relevance of STAT3 and NGN3 in the context of a human ductal cell line in vitro.

As previously demonstrated, the transgenic expression of key endocrine transcription factor, NGN3, via adenovirus administration is critical for the generation and expansion of insulin-secreting beta cells from human primary ductal cells (Lee et al., 2013). Here, we demonstrate and highlight the relevance of inflammatory cytokine stimulation in inducing the expression of NGN3, providing a mechanistic insight into cytokine-induced ductal-to-endocrine differentiation.
2.5 MATERIALS AND METHODS

PANC-1 Cell Line
Two cell lines were used for these in vitro experiments. The human pancreatic ductal epithelial cell line, PANC-1, derived from the pancreatic adenocarcinoma of a 56-year old male, was used for these experiments. Cells were maintained in DMEM medium supplemented with 10% FBS, and stimulations were carried out using this medium. For all experiments, PANC-1 cells were plated at 1x10^6 cells/well of a 12-well plate, and each condition was carried out in triplicate. All experiments were performed a minimum of two times.

HepG2 Cell Line
The human hepatocyte cell line, HepG2, was used as a non-endocrine cell line control. Cells were maintained in DMEM medium supplemented with 10% FBS, and stimulations were carried out using this medium. For stimulation experiments, HepG2 cells were plated at 1x10^6 cells/well of a 12-well plate, and each condition was carried out in triplicate. All experiments were performed two times.

Inflammatory Cytokine Stimulations
Cells were exposed to individual or combinations of TNFα, IL-1β, and IFNγ in dose-response and time-course experiments. TNFα [50ng/mL]; IL-1β [25ng/mL]; IFNγ [100ng/mL] corresponds to [1X] concentration. All other stimulation doses (2X, 10X, 1/2X, 1/10X, 1/100X) are relative to [1X].
Cytokine Receptor Antagonist Stimulations

Human recombinant interleukin-1 receptor antagonist (Creative Biomart®, Catalog No. IL1RN-05H) concentration [50µm]; Human recombinant IFNγ receptor antagonist (Sigma-Aldrich®, Catalog No. 15152) concentration [25µM]; Human TNFα receptor antagonist (Santa Cruz®, Catalog No. R-7050) concentration [25µM]. Cells were treated with a cocktail of these cytokine receptor antagonists for 48hrs.

pSTAT3 Inhibitor (STATTIC) Stimulations

pSTAT3 inhibitor, STATTIC (Selleck Chemicals LLC, Catalog No. S7024), was used at a concentration of [5µM] for 48hrs before extracting RNA or protein from cells.

Immunofluorescence studies

Cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature, washed twice, blocked with 5% donkey serum in PBS triton X (PBS-T 0.1%) for 1hr at room temperature, and stained with appropriate primary antibodies overnight at 4 degree Celsius and appropriate secondary antibodies for 1hr at room temperature. Nuclei were stained with DAPI for 5 minutes at room temperature. At least 1,000 cells were counted per well for quantification analyses.

qPCR Analyses

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer’s recommendations followed by cDNA synthesis using standard RT-PCR protocols. Approximately 25ng of cDNA template was mixed with 2x Sybr Green PCR Master Mix
(BioRad) and diluted to 1x with 100nM primers and dH₂O.

**Immunoblot analyses**

Total cellular proteins were harvested using RIPA buffer and immunoblotting was performed using standard immunoblotting techniques. Membranes were developed using chemiluminescent substrate, low sensitivity (Pico reagent) or high sensitivity (West Femto maximum sensitivity reagent) from Thermo Scientific Inc., (Waltham, MA).

**Statistical analyses**

Data are expressed as mean ± SEM and a two-tailed Student t test or ANOVA was used to confirm significance at p≤0.05

**Primary Antibodies**

<table>
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<td>BD</td>
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</tr>
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<td>Ki67</td>
<td>M 7187</td>
<td>DAKO</td>
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<td>F25A1B3</td>
<td>DSHB</td>
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<td>Rabbit</td>
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**Secondary Antibodies**

Alexa Fluor® 488 Donkey anti-goat IgG (A-11055, Life Technologies) 1:400
Alexa Fluor® 594 Donkey anti-rabbit IgG (711-586-152, Jackson ImmunoResearch) 1:400
Alexa Fluor® 647 Donkey anti-guinea pig IgG (706-605-148, Jackson ImmunoResearch) 1:400
Alexa Fluor® 594 Donkey anti-mouse IgG (715-585-150, Jackson ImmunoResearch) 1:400
**Primers for qPCR**

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**2.6 CONTRIBUTIONS AND ACKNOWLEDGEMENTS**

This work was a collaboration between Ivan A. Valdez, Manoj Gupta, Adrian K.K. Teo and Rohit N. Kulkarni. I.A.V. was responsible for the overall direction of the project, cell culture, immunostaining and qPCR analyses (Specifically, Figures 2-1; 2-2; 2-3; 2-4A-C; 2-4E; 2-5; 2-6; 2-7A-D; 2-7F) and writing the 1st draft of the manuscript. M.G. performed immunoblot analyses (Figures 2-4D and 2-8). A.K.K.T. was involved in overseeing the project, providing technical assistance and guidance. R.N.K provided guidance, mentorship and supervision of the work. All four authors participated in subsequent revisions of the manuscript.
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CHAPTER THREE

IN VIVO

EFFECTS OF INFLAMMATORY CYTOKINES ON DUCTAL CELLS BY PANCREATIC INTRADUCTAL INJECTION AND THROUGHOUT DIABETES PROGRESSION IN THE NOD MOUSE MODEL
Proinflammatory cytokines induce endocrine differentiation in pancreatic ductal cells via STAT3-dependent NGN3 activation

Ivan Achel Valdez$^{1,2}$, Ercument Dirice$^1$, Manoj K. Gupta$^1$, Jun Shirakawa$^1$, Adrian Kee Keong Teo$^{1,3,*}$, Rohit N. Kulkarni$^1,*$

$^1$Section of Islet Cell and Regenerative Biology, Joslin Diabetes Center, Department of Medicine, Brigham and Women’s Hospital, Harvard Stem Cell Institute, and Harvard Medical School, Boston, MA 02215, USA.

$^2$Department of Cell Biology, Program in Biological and Biomedical Sciences, Graduate School of Arts and Sciences, Harvard University, Cambridge, MA 02138, USA.

$^3$Discovery Research Division, Institute of Molecular and Cell Biology, Proteos, Singapore 138673, Singapore.

*Corresponding Author

Adapted from the in vivo studies of the following manuscript, which is published in the journal of Cell Reports:


Personal Contributions:

Experimental design and overall direction of project.

Figures: 3-1C; 3-3; 3-4; 3-5; 3-6; 3-7; 3-8

Writing 1st draft of manuscript and participating in subsequent revisions.
3.1 ABSTRACT

The existence of a pancreatic stem cell remains elusive, but pancreatic plasticity—the transdifferentiation from one pancreatic cell type to another—such as evidence of insulin-producing beta-like cells within exocrine tissue (acinar and ductal cells) has gained considerable attention in recent years. Interestingly, many studies implicate this phenomenon in diverse states of physiological/pathophysiological, metabolic, and cellular stress or pancreatic injury. Here, we investigated the effects of inflammatory stress on the differentiation potential of ductal cells by 1) performing a pancreatic intraductal injection of a cocktail of cytokines in vivo and 2) throughout the progression of pancreatic insulitis in the non-obese diabetic (NOD) mouse model of autoimmune diabetes. We show that inflammatory cytokines activate ductal cell proliferation and ductal-to-endocrine cell reprogramming in vivo independently of hyperglycemic stress. Our findings provide novel evidence of inflammatory cytokines in directing ductal-to-endocrine cell differentiation, with implications for beta cell regeneration.

3.2 INTRODUCTION

The concept of intrapancreatic cell-to-cell conversion, particularly under states of injury or cellular stress, is not new. Research on pancreatic injury models, such as pancreatic duct ligation (PDL) and partial pancreatectomy (PPx), has been around since the 1600s, but evidence of acinar and ductal cell differentiation toward islet cells following these pancreatic injury models began to emerge in the first decade of the 1900s (Granger and Kushner, 2009). More recently, lineage-tracing experiments demonstrated that carbonic anhydrase II (CAII)-
expressing ductal epithelial cells within the pancreas give rise to new acini and islets after birth and PDL (Inada et al., 2008). In addition, Harry Heimberg’s group demonstrated that in response to PDL, cells in the ductal lining of the mouse pancreas can be converted into endocrine progenitors guided by the expression of NGN3, a key transcription factor for endocrine pancreas specification (Xu et al., 2008). These NGN3+ progenitors can subsequently contribute to beta cell neogenesis and proliferation, leading to the doubling of beta cell volume. Importantly, a selective knock out (KO) of NGN3 in pre-existing beta cells does not ablate this effect, suggesting that beta cell neogenesis occurs from non-beta cells (Van de Casteele et al., 2013).

In addition to pancreatic injury models, multiple lines of evidence also suggest that cytokine- and nutrient-induced physiological stressors can promote exocrine-to-beta cell transdifferentiation. Glucose challenge experiments performed by Lipsett and Finegood in rats indirectly suggested that acinar cells transdifferentiate into beta cells (Lipsett and Finegood, 2002). In zebrafish, high nutrient concentrations can also promote ductal-to-beta cell transdifferentiation (Ninov et al., 2013). The ability of cytokines to promote exocrine-to-beta cell transdifferentiation as well as beta cell neogenesis has also been reported. In 1993, Gu and Sarvetnick first demonstrated that transgenic mice overexpressing IFNγ in pancreatic beta cells displayed proliferation and differentiation of pancreatic ductal cell into endocrine cells (Gu and Sarvetnick, 1993). After a gap of two decades, Susan Bonner-Weir’s group demonstrated that another cytokine, TNF-like weak inducer of apoptosis (TWEAK), stimulates ductal cell proliferation and transient expression of NGN3, ultimately giving rise to new pancreatic beta cells (Wu et al., 2013). More recently, Baeyens et al. interestingly demonstrated that transient epidermal growth factor (EGF) and ciliary neurotrophic factor
(CNTF) treatment stimulate NGN3 expression in acinar cells. These acinar cells subsequently transdifferentiated into beta cells and restored euglycemia in diabetic mice (Baeyens et al., 2014).

Based on all of these studies and our in vitro findings presented in Chapter 2, in this chapter we explored the in vivo effects of an acute insult of inflammatory cytokines, TNFα, IL-1β and IFNγ, by pancreatic intraductal injection in mice and the chronic effect of pancreatic insulitis throughout the progression of autoimmune diabetes in the non-obese diabetic (NOD) mouse model. We demonstrate that inflammatory cytokines can activate pancreatic ductal cell proliferation and the endocrine program in mouse ductal cells in vivo independently of hyperglycemia. Taken together, these experiments, as well as those of many other groups discussed above, highlight the plastic nature of the pancreas favoring beta cell regeneration in states of stress.
3.3 RESULTS

3.3.1 Pancreatic intraductal injection of inflammatory cytokines does not affect blood glucose levels.

To explore the effects of proinflammatory cytokine stimulation in vivo, we performed a single injection of the same cocktail of cytokines that was used during our in vitro studies—TNFα, IL-1β, and IFNγ—directly into the pancreatic duct of 8-10 week old C57BL/6 mice. We performed these injections at a [1X], [2X], or [10X] dose, and compared them to saline-injected controls. Following the intraductal injections, we measured blood glucose levels weekly for 3 weeks and injected BrdU intraperitoneally in all mice at 3 days and at 5 hours prior to sacrifice at a concentration of [100mg/kg BW] (Figures 3-1A and 3-1B). We did not observe significant differences in blood glucose levels among the cytokine- or saline-injected groups, ruling out possible effects induced by hyperglycemic stress (Figure 3-1C).

3.3.2 Pancreatic intraductal injection of inflammatory cytokines stimulates ductal cell proliferation and an increase in insulin+ ductal cells in mice.

Three weeks after injection, we harvested the pancreata of mice and performed immunohistochemistry analysis to assess ductal cell proliferation. To do this, we performed co-immunostaining for BrdU and the ductal cell marker, DBA (dolichos biflorus agglutinin). These analyses revealed increased levels of ductal cell proliferation in the [2X] and [10X] cytokine-injected mice relative to saline-injected controls (Figures 3-2A and 3-2B). No significant difference was observed between the [2X] and [10X] concentrations.
Figure 3-1. Pancreatic intraductal injections of inflammatory cytokines does not affect blood glucose levels.

(A) Pancreatic intraductal injection experimental design. Intrapancreatic injections into the pancreatic duct of C57BL/6 (B6) mice aged 8-10 weeks were performed using saline or a single injection of the cocktail of inflammatory cytokines (TNFα, IL-1β, IFNγ) at [1X] [2X] and [10X] doses. (B) Representative image of mouse undergoing pancreatic intraductal injection surgery. Evans blue dye shows inflated pancreas. (C) Blood glucose levels of saline, [1X], [2X] or [10X] cytokine-injected C57BL/6 mice. (n=4-6 per group).
Figure 3-2. Pancreatic intraductal injection of cytokines stimulates ductal cell proliferation in the pancreatic ducts of mice.

(A, B) Immunofluorescence images (A) and quantification (B) of saline injected, [1X], [2X] or [10X] cytokine injected mice for BrdU+ (red) and DBA (green) at 3 weeks post-injection. Nuclei were stained with DAPI (Blue). Scale bar = 100μM.

Data represent mean ± SEM. # and *p≤0.05 relative to saline injected controls. (n=4-6 for each group).
3.3.3 Pancreatic intraductal injection of cytokines stimulates insulin-like beta cells in the pancreatic ducts of mice.

To assess beta cell neogenesis in the pancreatic ducts of these mice, we performed co-immunostaining of insulin and DBA. Strikingly, immunostaining analyses revealed an increase in the number of insulin$^+$ ductal cells in a dose-dependent manner in cytokine-injected mice relative to saline-injected controls (Figures 3-3A and 3-3B).

Moreover, to further characterize these cells and investigate whether these insulin$^+$ ductal cells displayed features of true beta cells, we co-immunostained insulin with other markers of beta cell identity—secretory granule protein, Chromogranin A, (Lukinius et al., 2003), and the insulin precursor, proinsulin (de la Rosa and de Pablo, 2011). Our results demonstrated that the number of Chromogranin A/DBA (Figures 3-3C and 3-3D) and Proinsulin/DBA (Figures 3-3E and 3-3F) double$^+$ cells significantly increased in all cytokine injection groups relative to saline-injected controls. In addition, we found that the majority of insulin$^+$ ductal cells also expressed proinsulin (Figures 3-3E and 3-3F), whereas the number of Chromogranin A$^+$ ductal cells was higher than that of insulin$^+$ ductal cells in all groups (Figures 3-3C and 3-3D). Since Chromogranin A is also expressed in the secretory granules of other endocrine cells (Lukinius et al., 2003), our results suggest that some ductal cells may perhaps also adopt a non-beta, endocrine cell fate.

Importantly, we did not observe NGN3$^+$ ductal cells in these sections (data not shown), in contrast to our findings in vitro. These findings suggest that the acute effects of a single proinflammatory cytokine injection is unlikely to be sufficient to maintain endocrine cells in an undifferentiated state for a prolonged period, perhaps due to a transient expression of NGN3, in pancreatic ducts.
Figure 3-3. Pancreatic intraductal injection of cytokines stimulates insulin-like beta cells in the pancreatic ducts of mice.

(A-F) Representative immunofluorescence images and quantification of saline injected, [1X], [2X] or [10X] cytokine injected mice for (A, B) Insulin, DBA and DAPI (C, D) Chromogranin A and DBA, and (E, F) Proinsulin and BDA at 3 weeks post-injection. Scale bar = 100µM. Data represent mean ± SEM. # and *p≤0.05, ## and **p≤0.005, ***p≤0.0005 relative to respective saline injected controls. (n=4-6) for each group.
3.3.4 Characterization of diabetes progression in NOD mouse model.

Previous studies using the non-obese diabetic (NOD) mouse model have reported an increase in pancreatic ductal cell proliferation and in the number of insulin$^+$ and glucagon$^+$ ductal cells in diabetic conditions (O'Reilly et al., 1997). Furthermore, it has been reported that cells harvested from the ductal epithelium of NOD mice contain progenitor cells capable of clustering and forming functional islets in vitro that can be re-implanted into NOD mice to restore normoglycemia (Ramiya et al., 2000).

Based on these reports, we sought to investigate whether chronic pancreatic immune cell infiltration could generate NGN3$^+$ endocrine progenitor cells in the pancreatic ducts of NOD mice at various stages of autoimmune diabetes progression. We harvested the pancreata of mice at three pre-diabetic (3, 8 and 12 weeks of age) and two diabetic stages (new onset diabetes at 1-2 weeks post-diabetes onset and established diabetes at 5-6 weeks post-diabetes onset) (Figure 3-4A). Blood glucose levels were measured weekly, and mice with glucose levels greater than 200mg/dl on two consecutive days were considered diabetic (Figure 3-4B). To further characterize diabetes progression in these mice, serum levels of insulin, C-peptide and glucagon were also assessed (Figures 3-4C-E).

3.3.5 Increase in serum cytokine levels and insulitis commence prior to the onset of diabetes correlating with immune cell infiltration into the pancreas.

To assess serum levels of TNFα, IL-1β and IFNγ, we performed a high-sensitivity cytokine multiplex assay and observed that the levels of all three cytokines peaked prior to the onset of diabetes and decreased at the established diabetes stage (Figure 3-5A-C). Levels of eosinophil chemoattractant, Eotaxin (Garcia-Zepeda et al., 1996) and cytokine, IL-6, were
also highest before the onset of diabetes (Figures 3-5D-E). Even though the serum levels of IFNγ have previously been reported to gradually increase before diabetes onset (Schloot et al., 2002), to our knowledge, no study has observed a decrease in serum cytokine levels after diabetes is established (5-6 weeks post diabetes) (Figure 3-5).

3.3.6 Ductal cell proliferation and pancreatic insulitis peaks at new onset diabetes in NOD mice

In addition to assessing serum cytokine levels, we confirmed immune cell infiltration in the pancreas by performing haematoxylin and eosin (H&E) staining and quantifying insulitis scores. These results demonstrate that pancreatic insulitis commences prior to the onset of hyperglycemia and decreases at the established diabetes stage (Figures 3-6A and 3-6A’).

We then assessed ductal cell proliferation at all stages of diabetes progression by immunofluorescence analyses. Our study demonstrates, for the first time to our knowledge, that ductal cell proliferation (BrdU/DBA double+ cells) peaks at diabetes onset and significantly decreases when diabetes is established (Figures 3-6B and 3-6B’), correlating with the increase in pancreatic insulitis and serum cytokine levels in the pre-diabetes stages.

To control for potential effects of aging in a non-NOD mouse model, we used C57BL/6 mice at 4, 12 and 20 weeks of age. As expected, H&E staining did not reveal pancreatic insulitis (Figures 3-7A and 3-7B), and immunofluorescence analyses did not reveal a significant increase in ductal cell proliferation (Figures 3-7C and 3-7D). On the contrary, we observed a significant decrease in BrdU/DBA double+ cells, suggesting an attenuation in ductal cell proliferation with age (Figures 3-7C and 3-7D).
Figure 3-4. Experimental design and diabetes progression in the NOD mouse model.

(A) Experimental design using female NOD mouse model of autoimmune diabetes.

(B) Blood glucose levels of NOD mice throughout diabetes progression.

(C-E) Serum levels of metabolic hormones (C) Insulin, (D) C-Peptide and (E) Glucagon and cytokines measured by multiplex Luminex assay.

Data represent mean ± SEM. *p≤0.05 (n=4-9 for each group).
Figure 3-5. Serum cytokine levels increase prior to new onset of diabetes and decreases at the established stage.

(A-C) Serum levels of inflammatory cytokines, TNFα, IL-1β and IFNγ, measured by Luminex multiplex assay.

(D-E) Levels of eosinophil chemoattractant, Eotaxin, and cytokine, IL-6, measured by Luminex multiplex assay.

Data represent mean ± SEM. *p≤0.05 (n=4-9 for each group).
Figure 3-6. Pancreatic ductal cell proliferation and insulitis peaks at new onset diabetes in NOD mice.

(A, A’) Representative brightfield images (A) and quantification (A’) of islet insulitis at different time points of diabetes progression. Scale bar = 100µM.

(B, B’) Representative immunofluorescence images (E) and quantification (E’) of BrdU+ (red) ductal (DBA+ green) cells in NOD mice throughout diabetes progression. Nuclei were stained with DAPI (Blue). Scale bar = 100µM.

Data represent mean ± SEM. *p≤0.05 **p≤0.005 (n=4-9 for each group).
3.3.7 Ductal cell proliferation and emergence of NGN3\(^+\) and pSTAT\(^+\) ductal cells correlate with immune cell infiltration, and not hyperglycemia, in NOD mice

To assess ductal-to-endocrine cell transdifferentiation throughout the progression of autoimmune diabetes in the NOD mouse model, we conducted immunofluorescence analyses of NGN3 and DBA. These analyses revealed the emergence of NGN3/DBA double+ cells before the onset of diabetes (Figures 3-8A and 3-8A’). Although some insulin/DBA double+ were detected in the pre-diabetic and new-onset diabetes stages, no insulin+ ductal cells were found in the established diabetes stage despite the presence of NGN3 (data not shown). These observations suggest that the prolonged effects of inflammatory stress in the established diabetes stage may maintain cells in an undifferentiated, NGN3\(^+\), state and limit the formation of insulin\(^+\) ductal cells. Importantly, immunofluorescence analyses also revealed the presence of pSTAT3/DBA double+ cells in NOD mice (Figures 3-8B and 3-8B’). These results correlate with the emergence of NGN3 expression in ductal cells (Figures 3-8A and 3-8A’), increased serum proinflammatory cytokine levels (Figure 3-5) and the development of pancreatic immune cell infiltration observed at pre-diabetic stages (Figures 3-6A and 3-6A’). Finally, and as expected, immunofluorescence analyses did not reveal the presence of NGN3/DBA or pSTAT3/DBA double+ cells at any of the time points studied in the aging control using C57BL/6 mice (data not shown). Therefore, our data suggest that ductal cell proliferation and the emergence of NGN3\(^+\) or pSTAT3\(^+\) ductal cells are driven by insulitis and elevated cytokine levels and not hyperglycemia or aging.
Figure 3-7. Pancreatic ductal cell proliferation decreases with age in C57BL/6 mice.

(A, B) Representative brightfield images (A) and quantification (B) of islet insulitis at different time points in C57BL/6 mice. Scale bar = 100µM.

(C, D) Representative immunofluorescence images (C) and quantification (D) of BrdU (red) ductal (DBA⁺; green) cells in C57BL/6 (B6) mice at 4 weeks, 12 weeks, and 20 weeks of age. Nuclei were stained with DAPI (Blue). Scale bar = 100µM.

Data represent mean ± SEM. ***p≤0.0005 (n=3 for each group).
Figure 3-8. The emergence of NGN3+ and pSTAT3+ ductal cells correlate with immune cell infiltration, and not hyperglycemia, in NOD mice.

(A, A’) Representative immunofluorescence images (A) and quantification (A’) of NGN3+ (red) ductal (DBA; green) cells in NOD mice throughout diabetes progression. Scale = 20µM.

(B, B’) Representative immunofluorescence images (B) and quantification (B’) of pSTAT3+ (red) ductal (DBA; green) cells in NOD mice throughout diabetes progression. Scale = 20µM. (n=4-9 for each group).
3.4 DISCUSSION

In this chapter, we investigated whether stress induced by inflammatory cytokine insults could induce ductal-to-endocrine cell differentiation. While previous studies have reported the effect of chronic inflammatory cytokine stimulation on mouse ductal cells in vivo (Gu et al., 1997; Gu et al., 1995a; Gu et al., 1995b), our study is unique for several reasons. First, we report that acute inflammatory cytokine (TNFα, IL-1β and IFNγ) stimulation via pancreatic intraductal injection is able to increase ductal cell proliferation in vivo as measured by BrdU incorporation. The decrease, rather than increase, in ductal cell proliferation observed in vitro could be potentially due to the direct and sustained exposure of the target cells to cytokines that may not be observed in vivo. Second, the significant increase in insulin+ ductal cells in our intraductal injection model suggests that an acute insult of proinflammatory cytokines is sufficient to activate ductal-to-endocrine cell reprogramming. Third, the co-expression of insulin with proinsulin and chromogranin A in ductal cells indicates that they possess characteristics of true beta cells. In addition, by following the progression of diabetes in a chronic model of autoimmune diabetes, the NOD mouse, we observed that ductal cell proliferation and the emergence of NGN3+ and pSTAT3+ ductal cells correlate with the presence of serum cytokine levels and pancreatic insulitis prior to the onset of diabetes.

Together, our intraductal injection and NOD mouse diabetes progression studies highlight the importance and specificity of inflammatory cytokines in pancreatic cell plasticity and, importantly, rule out potential contributions of hyperglycemic stress. Although the detrimental effects of inflammatory cytokines on beta cells and the pathogenesis of diabetes have been widely documented (Kikodze et al., 2013; Rabinovitch and Suarez-
Pinzon, 2003), they have also been reported to stimulate beta cell proliferation in mice (Dirice et al., 2014; Dirice and Kulkarni, 2016). Moreover, the Joslin Medalist Study, which consists of patients who have had autoimmune diabetes for at least 50 years, has also reported the presence of scattered insulin$^+$ cells within the pancreatic exocrine tissue of all subjects after postmortem analyses (Keenan et al., 2010). These findings, in combination with our work, suggest that inflammatory signaling is able to activate beta cell neogenesis from both beta and non-beta cell sources in vivo—a strategy that holds high clinical and translational relevance since diabetic patients are typically exposed to inflammatory cytokines and prolonged low-grade inflammation.

### 3.5 MATERIALS AND METHODS

**BrdU administration**

Intraperitoneal injections of BrdU were performed in all mice 3 days and 5 hours prior to sacrifice at a concentration of [100mg/kg BW] (El Ouaamari et al., 2013).

**Pancreatic intraductal injections**

For this experiment, 8-10 week old male C57BL/6 mice were anesthetized with Avertin (240 mg/kg intraperitoneally). An incision of 3 cm was performed through the center of the abdomen. Then the duodenum was exposed and the common bile duct was clamped near the liver. The intraductal injection was performed using a 28G1/2 U-100 Insulin Syringe into the pancreatic duct from the duodenum through the ampulla of Vater. The needle was clamped and 150 µl of saline or a single dose of a cocktail of proinflammatory cytokines ([1X] = TNFα [50ng/mL]; IL-1β [25ng/mL]; IFNγ [100ng/mL], n=3), ([2X] = TNFα [100ng/mL];
IL-1β [50ng/mL]; IFNγ [200ng/mL], n=6), ([10X] = TNFα [500ng/mL]; IL-1β [250ng/mL]; IFNγ [1000ng/mL], n=4) or saline injections (n=5) were administered. After clamp removal, the wound on the ampulla was sealed with 3M Vetbond™ Tissue Adhesive (3M Animal Care). Abdominal muscle layer was closed with interrupted suture and the overlying skin was sutured using Needle Dafilon® Polyamide Monofilament (B. Braun). Blood glucose levels were measured once a week in the morning for 3 weeks. Pancreata were harvested 3 weeks post-injection.

**NOD mouse diabetes progression studies**

Female NOD mice (n=4-9 per group) were purchased from The Jackson Laboratory (Farmington, CT USA) and maintained in a pathogen–free animal facility at Joslin Diabetes Center. Diabetes was tracked by measuring their morning blood glucose levels using a Contour® Blood Glucose Meter (Bayer) once a week every week. Mice were considered diabetic after two consecutive days of blood glucose levels being greater than 200mg/dl (Dirice et al., 2014). A total of n=4-9 mice per group were used and divided into 5 groups: 3 weeks (n=5), 8 weeks (n=5) and 12 weeks of age (n=4), new onset diabetes (1-2 weeks after onset; n=9) and established diabetes (5-6 weeks after onset; n=8). The pancreases of these animals were harvested and sectioned for immunohistochemical analyses (Dirice et al., 2014).

**Insulitis scores**

Pancreatic sections of NOD mice were stained with H&E were ranked for insulitis using the following parameters: 0 = normal islet morphology with no insulitis, 1 = peri-insulitis, 2 =
insulitis, and 3 = islet remnant as reported previously (Dirice et al., 2014).

*Serum Cytokine Measurements using Multiplex Assay*

We collected blood samples from heart puncture of all NOD mice and obtained serum. Samples were stored at -70°C until analysis. Serum cytokine levels were measured by Luminex bead-based multiplex assay using a dual-laser Luminex-100 instrument (Millipore) by the Joslin Diabetes Center Assay Core.

*Immunohistochemistry*

Pancreases were harvested, fixed with 4% paraformaldehyde, and embedded in paraffin. Sections were blocked with 5% FBS in PBS Triton X 0.1% and stained using primary antibodies and appropriate secondary antibodies [1:400]. Nuclei were stained with DAPI. At least 1,000 cells were counted per animal.

*Statistical analyses*

Data are expressed as mean ± SEM and a two-tailed Student t test or ANOVA was used to confirm significance at $p \leq 0.05$.

*Study approval*

All animal experiments were conducted after approval by the Institutional Animal Care and Use Committee (IACUC) of the Joslin Diabetes Center in accordance with National Institutes of Health (NIH) guidelines.
3.6 CONTRIBUTIONS AND ACKNOWLEDGEMENTS

This work was a collaboration between Ivan A. Valdez, Adrian K.K. Teo, Ercument Dirice and Rohit N. Kulkarni. I.A.V. was responsible for the overall direction of the project, immunostaining analyses, measuring weekly blood glucose levels, analyzing serum cytokine level data obtained after submitting serum samples to the Joslin Assay Core (Specifically, Figures 3-1C; 3-3; 3-4; 3-5; 3-6; 3-7; 3-8) and writing the 1st draft of the manuscript. A.K.K.T. was involved in overseeing the project and providing experimental design guidance. E.D. performed intraductal injection surgeries, harvested pancreata and participated in leading the direction of the project. R.N.K provided guidance, mentorship and supervision of the work. All four authors participated in subsequent revisions of the manuscript. We would also like to thank Dr. Susan Bonner-Weir and Brooke Sullivan for providing NGN3 antibody and technical assistance with the immunostaining protocol.
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CHAPTER FOUR

DISCUSSION
4.1 OVERVIEW

Diabetes Mellitus, a group of metabolic disorders and a global epidemic that currently affects over 415 million people worldwide, occurs because of an autoimmune destruction of pancreatic beta cells or beta cell dysfunction coupled with a poor response to insulin by muscle, fat, and liver cells termed insulin resistance. Although insulin therapy and diabetes medications are adequate to regulate glucose homeostasis, the quality of life for some diabetes patients remains low as a result of life-endangering hypoglycemic episodes (insulin shock) and multi-organ complications (e.g., eyes, vascular system, kidneys, and nervous system). Therefore, current translational efforts in the regenerative medicine arena focus on novel cell replacement therapies for the replenishment of insulin-producing beta cells. Some of these strategies include: enhancing beta cell mass by increasing existing beta cell proliferation or suppressing beta cell apoptosis, directed differentiation of human pluripotent stem cells (ESCs or iPSCs), and transdifferentiation or neogenesis from endogenous endodermal derivatives such as hepatocytes and gastrointestinal, pancreatic ductal, acinar, or other endocrine cells (discussed in Chapter 1).

Within the field of cellular transdifferentiation, pancreatic cell plasticity, the interconversion of one pancreatic cell type to another within the pancreas, is a dynamic and timely area of investigation that has been the focus of intense examination in recent years (Valdez et al., 2015). A growing and compelling line of evidence in rodents and humans suggests that pancreatic cell plasticity allows the generation of insulin-producing cells from non-beta cell sources, particularly during times of need (e.g. beta cell loss, pancreatic injury or metabolic stress) or pancreatic cellular stress.

The focus of this thesis was to investigate the effect(s) of proinflammatory cytokine-
induced stress specifically caused by TNFα, IL-1β, and IFNγ, three of the most critical proinflammatory cytokines implicated in the pathogenesis of type 1 and 2 diabetes (Wang et al., 2010, Al-Shukaili et al., 2013, Spranger et al., 2003), on the differentiation potential of human ductal cells in vitro (Chapter 2) and mouse ductal cells in vivo (Chapter 3). In this chapter, we conclude our journey by stepping back and looking at the bigger picture. We begin by discussing our findings within the context of the current advances in pancreatic plasticity, both in vivo and in vitro, we discuss current controversies, potential challenges and provide a future perspective for the field.

4.2 ADVANCES IN PANCREATIC CELL PLASTICITY

4.2.1 In Vivo Plasticity

The concept of intrapancreatic cell-to-cell conversions, particularly under states of injury or cellular stress, is not new as evidence of exocrine cell differentiation toward islet cell phenotypes began to emerge in the first decade of the 1900s. More detailed and recent evidence of this phenomenon has been reported in rodents in 1) PDL and PPx pancreatic injury models, 2) beta cell depletion by diphtheria toxin, alloxan and caerulein treatment, and 3) IFNy, EGF + CNTF, and TWEAK cytokine stimulation, as well as in humans in states of insulin resistance, and alcoholism/autoimmune pancreatitis (Ko et al., 2013) (Summarized in Table 4-1; top section).

Our work on the effects of specific inflammatory cytokine insults by pancreatic intraductal injection and of insulitis in the NOD mouse expands our knowledge of the role cytokines regarding ductal-to-endocrine differentiation and contribute several unique observations to the field. First, our pancreatic intraductal injection model demonstrates that
an acute insult of inflammatory cytokines is sufficient to stimulate ductal cell proliferation and a dose-dependent generation of insulin$^+$, but not NGN3$^+$, cells within the pancreatic ducts. Second, by co-immunostaining insulin with pro-insulin and Chromogranin A, we show that insulin$^+$ ductal cells share characteristics of true beta cells. Finally, our NOD autoimmune diabetes progression model gives us a valuable window of opportunity, during which pancreatic insulitis and serum inflammatory cytokine levels increase prior to the onset of hyperglycemia, to explore the role inflammatory stress plays on pancreatic ductal cells. We demonstrate, for the first time to our knowledge, that pancreatic immune cell infiltration can give rise to the emergence of NGN3/DBA and pSTAT3/DBA double$^+$ cells independently of hyperglycemic stress.

4.2.2 In Vitro Plasticity

For many decades, countless insights have been gained about normal and pathological processes by studying human cells in vitro (Park et al., 2008), and numerous groups have conducted studies in vitro that demonstrate the plasticity of pancreatic cells (Summarized in Table 4-1; bottom section). For example, ductal-to-beta cell transdifferentiation has been achieved by forced expression of endocrine transcription factors, Ngn3, MafA, Pdx1 and Pax6, via adenoviral transduction (Lee et al., 2013, Swales et al., 2012). Treatment of human ductal cells with Activin A and Exendin 4 in vitro has also been reported to induce their conversion into insulin$^+$ cells which, after transplantation into STZ-induced diabetic mice, can restore normoglycemia (Kim et al., 2013). Acinar-to-beta cell transdifferentiation has also been demonstrated by spontaneous transition in culture (De Waele et al., 2014), adenoviral transduction of transcription factors such as Pdx1, Ngn3 and MafA (Minami et al.,
2011) (Akinci et al., 2012), stimulation with EGF + LIF (Baeyens et al., 2005), and with cytokines, TNFα and RANTES Liou et al., 2013). Finally, endocrine cell plasticity has been reported primarily in the form of beta cell de-differentiation. For example, stress encountered during human islet isolation has been shown to stimulate inflammatory signaling and induce beta cell de-differentiation via the emergence of progenitor-specific transcription factors (Negi et al., 2012).

Despite these observations, the mechanisms that drive pancreatic cell interconversions, particularly ductal-to-endocrine cell transdifferentiation in vivo or in vitro, have remained largely elusive. In rodent acinar cells, NGN3 expression has been shown to be mediated through STAT3 activation in vitro (Kaucher et al., 2012, Baeyens et al., 2006, Lemper et al., 2015) and in vivo (Baeyens et al., 2014) after cytokine (EGF + CNTF) treatment. However, our in vitro studies now highlight the relevance of inflammatory signaling, STAT3 and NGN3 in the context of a human ductal cell line. We demonstrate that inflammatory cytokines (TNFα, IL-1β and IFNγ) lead to cellular stress and trigger characteristics of ductal-to-endocrine cell differentiation in PANC-1 cells. Upon inflammatory cytokine treatment, PANC-1 cells display morphological changes, epithelial-to-mesenchymal transition (EMT), and pSTAT3-dependent upregulation of key endocrine progenitor marker, NGN3. Together, our study provides novel mechanistic insights into a naturally occurring phenomenon that may potentially open the door to novel beta cell regeneration strategies for diabetic patients.
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<th>Table 4-1. Summary of literature on pancreatic plasticity.</th>
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<tr>
<td>(Lipsett and Finegood, 2002)</td>
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<td>(Gu and Sarvetnick, 1993)</td>
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<td>(Wu et al., 2013)</td>
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<td>(Pan et al., 2013)</td>
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<td>(Van de Casteele et al., 2013)</td>
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<td>(Thorel et al., 2010)</td>
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<td>(Chung et al., 2010)</td>
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<td>(Piran et al., 2014)</td>
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<p>| <strong>In Vitro</strong>                                              |</p>
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<th><strong>Ref</strong></th>
<th><strong>Species</strong></th>
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<th><strong>Type of Cellular Plasticity</strong></th>
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<tr>
<td>(Baeysens et al., 2005)</td>
<td>Rat</td>
<td>EGF + LIF</td>
<td>Exocrine-to-Beta</td>
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<td>(Zhan et al., 2009)</td>
<td>Rat</td>
<td>HGF and BTCd4</td>
<td>Ductal-to-Beta</td>
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<td>(Minami et al., 2005)</td>
<td>Mouse</td>
<td>EGF + Nicotinamide</td>
<td>Acinar-to-Beta</td>
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<td>(Akkinci et al., 2012)</td>
<td>Mouse</td>
<td>Adenoviral transduction of PDX1</td>
<td>Acinar-to-Beta</td>
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<td>(Liou et al., 2013)</td>
<td>Mouse</td>
<td>Caerulein (cytokines TNFα &amp; RANTES)</td>
<td>Acinar-to-Ductal</td>
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<td>(Hourbracken et al., 2011)</td>
<td>Human</td>
<td>Spontaneous transition in culture</td>
<td>Acinar-to-Ductal</td>
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<td>(Inman et al., 2011)</td>
<td>Human</td>
<td>EGF</td>
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<td>(Swales et al., 2012)</td>
<td>Human</td>
<td>Adenovirus - Ngn3</td>
<td>Ductal-to-Beta</td>
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<tr>
<td>(Lee et al., 2013)</td>
<td>Human</td>
<td>Adenovirus - Ngn3, MafA, Pdx1 and Pax6</td>
<td>Ductal-to-Beta</td>
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<td>(Kim et al., 2013)</td>
<td>Human</td>
<td>Activin A + Extendin 4</td>
<td>Ductal-to-Beta</td>
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<tr>
<td>(De Waele et al., 2014)</td>
<td>Human</td>
<td>Spontaneous transition in culture</td>
<td>Acinar-to-Ductal</td>
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<td>(Bouckenooghe et al., 2005)</td>
<td>Human</td>
<td>Culture and expansion</td>
<td>Beta Cell Dedifferentiation</td>
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<td>(Negi et al., 2012)</td>
<td>Human</td>
<td>Islet isolation and culture</td>
<td>Beta Cell Dedifferentiation</td>
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<tr>
<td>(Piran et al., 2014)</td>
<td>Human</td>
<td>Alloxan + Caerulein</td>
<td>Alpha-to-Beta-to-Delta</td>
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4.3 PLASTICITY: CONTROVERSIES IN THE FIELD

Although the evidence highlighted throughout this work suggests the presence of beta cell progenitors within adult exocrine cells, there are also numerous opposing studies indicating that this is not the mechanism by which adult beta cells regenerate, at least in rodents. In pancreatic injury models such as partial pancreatectomy (PPx) (Dor et al., 2004) and diphtheria-toxin-based beta cell depletion (Nir et al., 2007), it was found that new beta cells predominantly arise from proliferation of pre-existing beta cells during adult life. Subsequent studies proposed that pancreatic ductal cells give rise to beta cells only during pancreatic embryogenesis but not after birth following experimentally-induced injury models such as PDL (Kopp et al., 2011) and alloxan-induced beta cell ablation (Solar et al., 2009). Using lineage-tracing studies in various models of beta cell loss, Xiao et al. did not find evidence of beta cell neogenesis in adult mice despite an upregulation of NGN3 in proliferating duct cells and in pre-existing beta cells (Xiao et al., 2013). Rankin et al. performed pancreatic duct ligation (PDL) and comprehensive whole pancreas beta cell quantification to conclude that no progenitors contribute to beta cell mass regeneration. Accordingly, PDL stimulates excessive pancreatic injury, thereby affecting the way by which beta cell content can be accurately measured (Rankin et al., 2013).

Despite this intensive debate on the presence/absence of rodent beta cell progenitors which can regenerate the adult pancreas, it is now generally accepted that transdifferentiation of cells from one type to another is indeed a possibility. In a recent Perspective, Van de Casteele et al. summarized and discussed the work by various laboratories in an attempt to explain the discrepancies (e.g. experimental design or quantification analyses) that exist in the field of injury(PDL)-induced beta cell regeneration (Van de Casteele et al., 2014).
4.4 IMPORTANCE OF ENDOCRINE TRANSCRIPTION FACTOR, NGN3

A common theme within the field of pancreatic cell plasticity and trans- or de-differentiation mechanisms is focused on the re-emergence of NGN3 expression in terminally differentiated adult cells. NGN3 is a transcription factor and a master regulator that determines pancreatic endocrine cell fate during embryogenesis (Rukstalis and Habener, 2009), but its importance in beta cell neogenesis is currently debated. Some studies indicate that injury by PDL does not result in NGN3 upregulation; others suggest that its activation does occur without resulting in beta cell neogenesis (Van de Casteele et al., 2014). Conversely, there are also studies overexpressing NGN3 in pancreatic duct cells which demonstrate that it is sufficient to induce endocrine cell fate (Boretti and Gooch, 2007). Work by Johansson et al. has shown that the temporal increment of NGN3 expression promotes pancreatic progenitors to differentiate into distinct endocrine cell types in mice (Johansson et al., 2007), but it should be noted that the expression pattern of NGN3 in the mouse is distinctly different from that in humans (Jennings et al., 2013). Importantly, NGN3 is also purportedly expressed in the adult pancreas whereby it is essential for the expression of beta cell genes NeuroD1, Pax4, MafA and insulin (Wang et al., 2009). Further investigations are warranted to determine the significance of re-emergence of NGN3 expression in these transdifferentiation observations. Speculatively, should the NGN3 expression result in increased alpha cell formation, as suggested by Talchai et al. (Talchai et al., 2012), the increased glucagon and GLP-1 could possibly promote beta cell formation (Rukstalis and Habener, 2009). Furthermore, it may be worthwhile to ectopically stimulate NGN3 expression to further investigate the transdifferentiation of human exocrine or alpha cells to beta cells.
4.5 CHALLENGES, FUTURE PROSPECTS AND IMPLICATIONS

Despite its controversies, there is ample evidence indicating that the pancreas possesses the plasticity that allows it to regenerate under states of stress, either by physiological means, such as inflammation and insulin resistance, or non-physiological means, such as injury or cytotoxic insults. However, several key challenges remain to be addressed. First, it is possible that misappropriated plasticity may be a threat as inflammatory stress can in fact lead to pancreatitis, a major risk factor for developing pancreatic adenocarcinoma (Puri and Hebrok, 2010). It is important to recognize that embryonic pathways, such as NOTCH signaling that plays a role in acinar regeneration during pancreatic development, have also been linked to neoplastic transformation (Puri and Hebrok, 2010). The second challenge relates to understanding and controlling how stress mechanisms can be used to induce the desired cell type. For example, during states of metabolic stress, Domenico Accili’s group demonstrated that de-differentiated beta cells are able to re-differentiate, but adopt an alpha cell fate instead (Talchai et al., 2012). Furthermore, the finding that NGN3^ progenitors observed in diabetic mice can re-differentiate into insulin^+ cells upon insulin therapy is critical, as it suggests that the presence of cellular stress may also play a role in hindering the re-differentiation of these NGN3^ progenitors into mature beta cells (Wang et al., 2014). Notably, these observations support our findings in which the effects of established diabetes in NOD mice maintain the expression of NGN3 while at the same time seem to hinder the formation of insulin^+ cells in pancreatic ducts.

In conclusion, stress-induced pancreatic cell plasticity is a controversial area of research that is still not fully understood. Nevertheless, there is great promise that unraveling
its intricate mechanisms will allow us to translate these findings into therapies that contribute to diabetes treatments. These findings warrant further investigation into the field of cytokine-driven exocrine ductal cell reprogramming, an area that holds translational relevance for the fields of diabetes and pancreatic regenerative medicine.
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APPENDIX 1

Published Perspective
Cellular Stress Drives Pancreatic Plasticity

Ivan A. Valdez$^{1,2,†}$, Adrian K. K. Teo$^{1,†,*}$ and Rohit N. Kulkarni$^{1,*}$

$^1$Section of Islet Cell and Regenerative Biology, Joslin Diabetes Center, Department of Medicine, Brigham and Women’s Hospital, and Harvard Medical School, Boston, Massachusetts 02215, USA.

$^2$Department of Cell Biology, Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, Massachusetts 02215, USA.

†Co-first authors

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Contributions:

Writing manuscript’s 1st draft
Figure1 and Table 1
Participation in subsequent revisions
BETA CELL REGENERATION

Cellular stress drives pancreatic plasticity
Ivan A. Valdez,1,2§ Adrian K. K. Teo,3,* Rohit N. Kulkarni1

Controversy has long surrounded research on pancreatic beta cell regeneration. Some groups have used nonphysiological experimental methodologies to build support for the existence of pancreatic progenitor cells within the adult pancreas that constantly replenish the beta cell pool; others argue strongly against this mode of regeneration. Recent research has reinvigorated enthusiasm for the harnessing of pancreatic plasticity for therapeutic application—for example, the transdifferentiation of human pancreatic exocrine cells into insulin-secreting beta-like cells in vitro; the conversion of mouse pancreatic acinar cells to beta-like cells in vivo via cytokine treatment; and the potential redifferentiation of dedifferentiated mouse beta cells in vivo. Here, we highlight key findings in this provocative field and provide a perspective on possible exploitation of human pancreatic plasticity for therapeutic beta cell regeneration.

A metabolic disorder, diabetes occurs because of inadequate insulin production by the pancreatic beta cells coupled with a poor response to insulin by muscle, fat, and liver cells (termed insulin resistance). Although insulin therapy and diabetes medications are adequate to regulate glucose homeostasis, the quality of life for some diabetes patients remains low as a result of life-endangering hypoglycemic episodes and multi-organ complications (e.g., eyes, vascular system, kidneys, and nervous system). Therefore, current translational efforts in the regenerative medicine arena focus on cell replacement therapies for the replenishment of insulin-producing beta cells. Some of these strategies involve the generation of beta cells from pluripotent stem cells and endogenous mesenchymal derivatives such as hepatocytes and gastrointestinal, pancreatic ductal, acinar, or other endocrine cells (1).

The existence of a “pancreatic stem cell” remains elusive, but pancreatic plasticity—that is, transdifferentiation from one pancreatic cell type to another—such as evidence of insulin-producing beta-like cells within exocrine tissue (acinar and ductal cells) has gained considerable attention. Early experiments carried out by Bonner-Weir and Heimberg suggest some form of pancreatic plasticity and an intrinsic ability of the adult pancreas to regenerate (see Table 1). These studies demonstrate that beta cells can be restored in times of need, such as during injury and cellular stress. More recently, a wave of literature uncovering the potential of exocrine and endocrine cells to transdifferentiate and regenerate pancreatic beta cells, both in vitro and in vivo, has emerged. Furthermore, work by several groups has brought to light the phenomenon of beta cell redifferentiation under physiological and pathophysiological states.

From a clinical point of view, these findings underscore the inherent plasticity of the pancreas and suggest the possibility of stimulating endogenous beta cell neogenesis or regeneration in vivo without the need for invasive methodologies. Here, we briefly summarize the literature and present a perspective on how plasticity associated with human pancreatic exocrine and endocrine cells, despite existing controversies, might potentially be harnessed for the regeneration of the beta cells in diabetic patients.

EXOCRINE CELL PLASTICITY

Nonphysiological stresses. The concept of intrapancreatic cell-to-cell conversion, particularly under states of injury or cellular stress, is not new. Research on pancreatic duct ligation (PDL) and partial pancreatectomy (PPx) has been carried out since the 1960s, but evidence of acinar and ductal cell differentiation toward islet cell phenotypes in these pancreatic injury models began to emerge in the first decade of the 1990s (2). More recently, cell lineage-tracing experiments demonstrated that, after birth and after PDL, injury, carbonic anhydrase II (CAII)—expressing ductal epithelial cells in the pancreas give rise to new acinar tissue (clusters of exocrine pancreas cells that synthesize, store, and secrete digestive enzymes) and islets of Langerhans (which contains, alpha, beta, delta, PP, and epsilon cells) (3). In addition, Heimberg and colleagues demonstrated that in response to PDL, cells in the ductal lining of the mouse exocrine pancreas can be converted into endocrine progenitors guided by the expression of neurogenin 3 (Ngn3), a key transcription factor for endocrine pancreas specification (4). These Ngn3+ progenitor cells can subsequently contribute to beta cell neogenesis and proliferation, leading to the doubling of beta cell volume. Importantly, a selective knock out (KO) of Ngn3 in preexisting beta cells does not ablate this effect, suggesting that beta cell neogenesis occurs from non-beta cells (5).

Lineage tracing of exocrine acinar—-endocrine cell transdifferentiation after PDL in rodents was first reported by Pan et al. (6). After PDL and elimination of pre-existing beta cells via treatment with streptozotocin (STZ, a chemical of microbial origin that is toxic to beta cells and used to create rodent diabetes models), the authors lineage-traced pancreas-specific transcription factor 1a (Ptf1a)—expressing acinar cells and showed that these cells give rise to endocrine beta cells via Ck19+/Hnf1β+/Sox9+ ductal and Ngn3+ endocrine pancreas progenitor intermediates in vivo. Acinar cell redifferentiation into a progenitor-stage pancreatic cell (Pdx1+/Ecadherin-Beta-catenin+) after chemically (caerulein, an oligopeptide that causes pancreatitis)–induced pancreatic injury in vivo has also been reported (7).

Collectively, these nonphysiological models demonstrate that exocrine cells in rodents, upon severe injury, prompt beta cell regeneration via transdifferentiation mechanisms (Fig. 1). Human pancreatic exocrine cells (ductal cells) were successfully converted into beta-like cells recently, presenting a potential translational opportunity for human beta cell replacement (8, 9).

Physiological stresses: Cytokines and nutrients. Multiple lines of evidence suggest that cytokine– and nutrient-induced physiological stresses also can promote exocrine cell-to–beta cell transdifferentiation (Table 1). Glucose challenge experiments performed by Lipsett and Finegood in rats indirectly suggested that acinar cells transdifferentiate into beta cells (10). In zebrafish,
high nutrient concentrations can also promote ductal cell–to–beta cell transdifferentiation (11). The ability of cytokines to promote exocrine cell–to–beta cell transdifferentiation as well as beta cell neogenesis has also been reported. In 1993, Gu and Sarvetnick first demonstrated that transgenic mice overexpressing interferon γ (IFN-γ) in pancreatic beta cells displayed proliferation and differentiation of pancreatic ductal cells into endocrine cells (12). After a hiatus of two decades, another cytokine, tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK), was shown to stimulate ductal cell proliferation and transient expression of Ngn3, ultimately giving rise to new pancreatic beta cells (13). More recently, Baeyens et al. demonstrated that transient epidermal growth factor (EGF) and ciliary neurotrophic factor (CNTF) treatment stimulates Ngn3 expression in acinar cells, which subsequently transdifferentiated into beta cells and restored euglycemia in diabetic mice (14).

Taken together, these experiments in model organisms highlight the plastic nature of the pancreas and suggest that it favors beta cell regeneration in states of stress. Notably, the fact that transient exposure to cytokines promotes acinar cell–to–beta cell transdifferentiation in mice (14) means that it might be possible to use such an intervention to regenerate human pancreatic beta cells in vivo (Fig. 1). This is of high clinical and translational relevance because diabetic patients are typically exposed to inflammatory cytokines and prolonged low-grade inflammation. This means that the appropriate use of the correct cytokines may eventually serve to restore pancreatic beta cell function (15).

PLASTICITY: ADULT CONTROVERSIES

Although the evidence highlighted above suggests the presence of beta cell progenitors within adult exocrine cells, there are also numerous opposing studies indicating that this is not the mechanism by which adult beta cells regenerate (at least in rodents). In pancreatic injury models such as PPx (16) and diphtheria toxin–based beta cell depletion (17), it was found that new beta cells arise predominantly from proliferation of preexisting beta cells during adult life. Subsequent studies proposed that pancreatic ductal cells give rise to beta cells during pancreatic embryogenesis but not after birth following experimentally induced injury models such as PDL (18) and alloxan-induced beta cell ablation (19). Using lineage-tracing studies in various models of beta cell loss, Xiao et al. did not find evidence of beta cell neogenesis in adult mice despite an up-regulation of Ngn3 in proliferating duct cells and in preexisting beta cells (20). Rankin et al. performed PDL and comprehensive whole pancreas beta cell quantification to conclude that no progenitors contribute to beta cell mass regeneration. Accordingly, PDL stimulates excessive pancreatic injury, thereby affecting the way by which beta cell content can be accurately measured (21).

Despite this intensive debate on the presence or absence of rodent beta cell progenitors that can regenerate the adult pancreas, transdifferentiation of cells from one type to another is now generally accepted as a possibility. In a recent Perspective, Van de Casteele et al. discussed the work by various laboratories in an attempt to explain the discrepancies that exist in the field of injury (PDL)-induced beta cell regeneration (22).
community plays a role in islet-cell interconversion, which has enormous implications for the treatment of human type 1 diabetes (25).

Physiological stress. Physiological stresses such as insulin resistance, chronic hyperglycemia, multiparity, aging, and oxidative stress also have been implicated in epigenetic actions that alter islet cell mass and interconversions (26). The comparison of insulin-resistant versus insulin-sensitive human pancreatic samples by Mezza et al. suggests that insulin resistance is linked to alpha (and ducal) cell–to-beta cell transdifferentiation in human type 2 diabetes patients (27). In addition, beta cell plasticity in states of physiological stress has also been reported. In 1999, Jonas et al. demonstrated that PPx-induced hyperglycemia is marked by beta cell dysfunction and a loss of mature beta cell gene expression (28). More recently, Talchai et al. reinvigorated the concept of beta cell plasticity by demonstrating that physiological stresses such as chronic hyperglycemia, multiparity, and aging induce beta cell dysfunction and transdifferentiation (29). The dedifferentiated beta cells reexpress Ngn3, and some of the cells revert to alpha cells. These results were subsequently confirmed in other mouse models of metabolic stress (29). Remarkably, dedifferentiated Ngn3+Ins− cells can be redifferentiated into mature Ngn3+Ins+ beta cells after insulin therapy, raising hopes that the loss of beta cell function in humans might be reversible (30). Another recent study indicates that, in islets from human type 2 diabetes patients, oxidative stress–associated beta cell dysfunction induces cytoplasmic translocation and inactivation of MAPA, NKX6.1, and PDX1, all of which are mature beta cell transcription factors (31). Collectively, these studies indicate that beta cell "dysfunction" may be a reflection of stress-induced beta cell dedifferentiation.

From a translational medicine standpoint, the ability to (i) halt the dedifferentiation process of beta cells, (ii) redifferentiate these stress-derived beta cell "progenitors," and (iii) facilitate alpha cell–to-beta cell conversion would allow potential repopulation of the functional beta cell pool in diabetic patients.

**NGN3, A CENTRAL PLAYER**

A common theme within the field of pancreatic plasticity—mechanisms of trans- or dedifferentiation—is focused on the reemergence of Ngn3 expression in terminally differentiated adult cells. Ngn3 is a transcription factor and master regulator that determines pancreatic endocrine cell

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**Table 1. Pancreatic plasticity: Summary of literature.**

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**Abbreviations:** PDL, pancreatic duct ligation; PPx, partial pancreatectomy; EGF, epidermal growth factor; CNF, ciliary neurotrophic factor; LIF, leukemia inhibitory factor; HGF, hepatocyte growth factor; BTCd4, Betacellulin–delta 4; H, human; M, mouse; R, rat; Z, zebrafish.
fate during embryogenesis, but researchers are currently debating its importance in beta cell neogenesis. Some studies indicate that injury by PDI does not result in Ngn3 up-regulation, whereas others suggest that activation of Ngn3 does occur but without resulting in beta cell neogenesis (20, 22). Conversely, there are also studies that show that overexpression of Ngn3 in mouse and rat pancreatic duct epithelial cells is sufficient to induce endocrine cell fate (32). Work by Johansson et al. has shown that the temporal increment of Ngn3 expression promotes pancreatic progenitors to differentiate into distinct endocrine cell types in mice (33), but the expression pattern of Ngn3 in the mouse is distinctly different from that in humans (34).

Importantly, Ngn3 is also purportedly expressed in the adult pancreas, whereby it is essential for the expression of beta cell genes that encode NeuroD1, Pdx4, Mafa (pancreatic developmental transcription factors), and insulin (35). Further investigations are warranted to determine the significance of reemergence of Ngn3 expression in these transdifferentiation observations. Speculatively, should the Ngn3 expression result in increased alpha cell formation, as suggested by Talchai et al. (29), the increased glucagon and glucagon-like peptide 1 (GLP-1) secreted by the new alpha cells might promote beta cell formation (36). Furthermore, it may be worthwhile to ectopically stimulate Ngn3 expression should it eventually be determined to be critical for the transdifferentiation of human exocrine or alpha cells to beta cells.

CHALLENGES AND PROSPECTS

Ample evidence indicates that the pancreas possesses the plasticity that allows it to regenerate under various states of stress. However, several challenges remain. First, misappropriated plasticity might be a threat, as inflammatory stress can lead to pancreatic-atitis—a major risk factor for developing pancreatic adenocarcinoma (37). It is important to recognize that embryonic pathways, such as NOTCH signaling, that play a role in acinar regeneration during pancreatic development have also been linked to neoplastic transformation (37). The second challenge relates to understanding and controlling how stress mechanisms can be induced to induce the desired cell type. For example, during states of metabolic stress, Talchai et al. demonstrated that dedifferentiated beta cells are able to redifferentiate, albeit adopting an alpha cell fate instead (29). Further, the finding that Ngn3 progenitors observed in diabetic mice can redifferentiate into insulin cells upon insulin therapy is noncritical, because it suggests that the presence of cellular stress might also hinder the redifferentiation of these progenitors into mature beta cells (30). In conclusion, stress-induced pancreatic plasticity is a controversial area of research on a process that still is not fully understood. Nevertheless, there is great promise that unraveling its intricate mechanisms will allow us to translate these findings into therapies that contribute to pancreatic regenerative medicine.

REFERENCES AND NOTES

25. Z. Wang, N. W. Wink, C. G. Nicholas, M. S. Remedi, Pancreatic β-cell dedifferentiation in diabetes and dedifferenta-


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APPENDIX 2-1

Contributions to Additional Co-Authored Publications
Comparable Generation of Activin-Induced Definitive Endoderm via Additive Wnt or BMP Signaling in Absence of Serum

Adrian Kee Keong Teo¹, Ivan Achel Valdez¹, Ercument Dirice¹ and Rohit N. Kulkarni¹

¹Section of Islet Cell and Regenerative Biology, Joslin Diabetes Center, Department of Medicine, Brigham and Women’s Hospital, and Harvard Medical School, Boston, Massachusetts 02215, USA.

Article published in the journal of Stem Cell Reports:


Contributions:

Immunofluorescence and FACS analyses

Figures: 1D-E; 3C; 4B-C; S3B-C; S3E

Editing manuscript
Comparable Generation of Activin-Induced Definitive Endoderm via Additive Wnt or BMP Signaling in Absence of Serum

Adrian Kee Keong Teo,1,*, Ivan Achel Valdez,1 Er cement Dirice,1 and Rohit N. Kulkarni1,*
1Section of Islet Cell and Regenerative Biology, Joslin Diabetes Center; Department of Medicine, Brigham and Women’s Hospital; and Harvard Medical School, Boston, MA 02215, USA
*Correspondence: rohit.kulkarni@joslin.harvard.edu (R.N.K.), drainteo@gmail.com (A.K.K.T.)
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SUMMARY

There is considerable interest in differentiating human pluripotent stem cells (hPSCs) into definitive endoderm (DE) and pancreatic cells for in vitro disease modeling and cell replacement therapy. Numerous protocols use fetal bovine serum, which contains poorly defined factors to induce DE formation. Here, we compared Wnt and BMP in their ability to cooperate with Activin signaling to promote DE formation in a chemically defined medium. Varying concentrations of WNT3A, glycogen synthase kinase (GSK)-3 inhibitors CHIR99021 and 6-bromoindirubin-3'-oxime (BIO), and BMP4 could independently co-operate with Activin to effectively induce DE formation even in the absence of serum. Overall, CHIR99021 is favored due to its cost effectiveness. Surprisingly, WNT3A was ineffective in suppressing E-CADHERIN/CDH1 and pluripotency factor gene expression unlike GSK-3 inhibitors or BMP4. Our findings indicate that both Wnt and BMP effectively synergize with Activin signaling to generate DE from hPSCs, although WNT3A requires additional factors to suppress the pluripotency program inherent in hPSCs.

INTRODUCTION

Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), collectively known as human pluripotent stem cells (hPSCs), can potentially be differentiated into clinically useful cell types for in vitro disease modeling, drug screening, and cell replacement therapy. Given the explosion in diabetes and its complications worldwide, the directed differentiation of hPSCs into definitive endoderm (DE) and subsequently pancreatic cells is of immense interest (Teo et al., 2013a). In 2005, Novocell (now ViaCyte) reported the ability to derive >80% of DE from hESCs with the use of 100 ng/ml Activin A (hereafter referred to as “Activin”) in the presence of 0.2%–2% fetal bovine serum (FBS; D’Amour et al., 2005). To complement Activin/Nodal signaling in inducing DE formation, Wnt and BMP signaling activators were then introduced (Table S1 available online). Developmentally, this mimics the complex Nodal, Wnt (Wnt3, Wnt3a and Wnt5a) and Bmp (Bmp4) signaling, which operate during gastrulation, primitive streak, and early DE formation in the mouse embryo (Arnold and Robertson, 2009; Lawson et al., 1999; Liu et al., 1999; Ohinata et al., 2009; Teo et al., 2011; Yamaguchi et al., 1999).

The coactivation of Wnt and Activin/Nodal signaling (albeit in the presence of FBS) is commonly used as described by D’Amour et al. (2006). They reported that Activin and WNT3A (specifically 100 ng/ml Activin, 25 ng/ml WNT3A, and 0.2% FBS) can induce more than 80% of DE cells (D’Amour et al., 2006). Alternatively, activation of Wnt signaling via the inhibition of glycogen synthase kinase (GSK)-3 (specifically 100 ng/ml Activin or 500 nM IDE1, 3 μM CHIR99021 [GSK-3 inhibitor], and 2% FBS) has also been recently reported to induce 70%-80% of DE cells (illing et al., 2013; Kunisada et al., 2012). In contrast, the work of Teo, Dunn (Phillips et al., 2007; Teo et al., 2012), and Vallier (Vallier et al., 2009) independently demonstrated that 10–50 ng/ml of BMP4 can synergistically act with Activin (in a defined medium without FBS) to induce more than 80% of DE cells. However, it is still uncertain how Wnt compares with BMP in cooperating with Activin/Nodal signaling in a chemically defined medium to induce DE formation.

We sought to clarify and define the Activin-Wnt-BMP signaling relationship using hiPSCs that we derived (Teo et al., 2013b). Here, we report a head-to-head comparison of Wnt versus BMP in combination with Activin signaling in a chemically defined medium without FBS to induce DE formation. Unlike previous reports (25 ng/ml WNT3A plus FBS; Table S1), we observed that a high dose of WNT3A (100 ng/ml, without FBS) is required to maximally induce DE in the presence of 100 ng/ml Activin. The activation of Wnt signaling with the use of two independent GSK-3 inhibitors (CHIR99021 or 6-bromoindirubin-3'-oxime [BIO]; Sato et al., 2004) or BMP signaling with BMP4 can also maximally induce DE cells. Thus, both Wnt and BMP signaling can effectively cooperate with Activin signaling to induce DE formation.

Effective differentiation of hPSCs into DE is coupled with the suppression of E-CADHERIN/CDH1 and pluripotency. Whereas BMP signaling has been reported to suppress E-CADHERIN/CDH1 and pluripotency (Teo et al., 2011; 2012), we observed that WNT3A is not as effective as
Comparable DE formation via Wnt or BMP Signaling

A Low dose WNT3A

B High dose WNT3A

C

D

E Percentage of SOX17+ cells at Day 3

(legend on next page)
GSK-3 inhibitors in suppressing both E-CADHERIN/CDH1 and the pluripotency program. This could partly explain the continued requirement for FBS when deriving DE with WNT3A. Therefore, our study identifies the unprecedented need for an additional compound to suppress pluripotency when combining Activin and WNT3A to generate DE cells without serum.

RESULTS

High Dose of WNT3A Is Sufficient to Cooperate with Activin to Induce DE without Serum

Many previous studies have reported reliance upon Activin, WNT3A, and FBS to differentiate hPSCs into DE (Table S1). To determine whether WNT3A can cooperate with Activin in a chemically defined medium in the absence of serum (RPMI 2% B27) to induce DE from hPSCs, we used 10–30 ng/ml WNT3A with 0–100 ng/ml Activin in optimization experiments. Dismally, 30 ng/ml WNT3A and 100 ng/ml Activin only marginally increase the expression of DE markers SOX17 and FOXA2 (Figures 1A and 1C–E). Unlike previous reports, a low dose of WNT3A is insufficient to effectively promote DE formation in serum-free conditions. We subsequently increased the dose of WNT3A and determined that 100 ng/ml WNT3A can effectively result in maximal DE marker gene expression (Figures 1B–1E) without a dose-responsive increase in mesodermal markers BRACHYURY and TBX6, or extraembryonic marker SOX7 (Figure S1A). Morphologically, cells induced with 100 ng/ml Activin and 30 or 100 ng/ml WNT3A looked indistinguishable but different from no growth factor condition (Figure 1C). However, immunostaining and quantitative analyses for SOX17 DE marker clearly demonstrated that 100 ng/ml Activin + 100 ng/ml WNT3A gave rise to DE cells with a comparable efficiency as that of 100 ng/ml Activin + 30 ng/ml WNT3A + 0.5% FBS, as opposed to 100 ng/ml Activin + 30 ng/ml WNT3A (Figures 1D and 1E). Together, these findings suggest that FBS is necessary for synergistic activity with 100 ng/ml Activin and 25 ng/ml WNT3A to efficiently induce DE formation. Thus, FBS + 25 ng/ml WNT3A can be replaced with a high dose of WNT3A (100 ng/ml) to induce DE.

GSK-3 Inhibitors, CHIR99021 and BIO, Cooperate with Activin to Induce DE without Serum

To further define the synergism between Wnt and Activin signaling in inducing DE without serum, we adopted complementary approaches to activate Wnt signaling by inhibiting the downstream signaling molecule GSK-3, using two independent GSK-3 inhibitors, CHIR99021 or BIO, which block the degradation of β-catenin, thereby allowing its nuclear translocation and activation of downstream target genes. Kunisada et al. (2012) and Illing et al. (2013) used a GSK-3 inhibitor to demonstrate that 3 μM CHIR99021 with 100 ng/ml Activin or 500 nM IDE1 and 2% FBS induces 70%–80% of DE cells.

Initially, we used a range of doses of CHIR99021 (0.5, 3, or 9 μM) together with Activin to prompt DE differentiation (Figure 2A). Interestingly, 9 μM CHIR99021 induces maximal CXCR4 gene expression independent of the requirement for Activin. However, cardinal DE markers EOMES, SOX17, and FOXA2 are suppressed at such a dose, suggesting that excessive Wnt signaling activation is refractory to DE formation (Figure 2A). Thus, we lowered the doses of CHIR99021 to 1, 3, and 5 μM. Morphologically, cells induced with 100 ng/ml Activin and 1 or 3 μM CHIR99021 were indistinguishable but different from no growth factor condition. We finally confirmed that 3 μM CHIR99021 (together with 100 ng/ml Activin) maximally induces DE differentiation in our chemically defined medium (Figures 2B and 2C) without a dose-responsive increase in mesodermal markers BRACHYURY and TBX6, or extraembryonic marker SOX7 (Figure S1B). The increasing dose of CHIR99021 increases mesodermal marker gene expression independent of the dose of Activin (Figure S1B), indicating a fine balance in its use for DE (1–3 μM) versus mesoderm (>3 μM) formation.

To complement the use of CHIR99021 in deriving DE cells, we used another GSK-3 inhibitor, BIO (0.5, 2, or 5 μM), to...
activate Wnt signaling and observed that ≥ 2 μM resulted in excessive cell death (data not shown). Subsequently we determined that 1.5 μM BIO together with 100 ng/ml Activin is optimal for generating DE cells from hPSCs without serum (Figure 2D), again without a dose-responsive increase in mesodermal markers BRACHYURY and TBX6, or extraembryonic marker SOX7 (Figure S1C).

**Wnt and BMP Signaling Can Enhance Activin-Induced DE with Comparable Efficiencies without Serum Supplementation**

Next, to confirm that BMP4 and Activin can induce DE from hPSCs without serum, we performed similar dose-response experiments. These experiments ascertained that 100 ng/ml Activin and 25–50 ng/ml BMP4 can elicit maximal expression of DE markers CXCR4, SOX17, and FOXA2 (Figure 3A), without a dose-responsive increase in mesodermal markers BRACHYURY and TBX6, or extraembryonic marker SOX7 (Figure S1D). Because both Wnt and BMP can cooperate with Activin signaling to generate DE cells in our chemically defined differentiation condition, we then compared the various optimal DE-inducing conditions in the same experiment. Time course analyses indicated that Wnt and BMP signaling-based conditions induced a peak expression of mesendodermal marker EOMES between days 2 and 3 and DE markers CXCR4 and SOX17 on day 3 in one hPSC and one hESC line (Figures S2A–S2C). Thus, we compared these DE-inducing conditions on day 3 of differentiation.

Interestingly, the combination of 100 ng/ml Activin and 3 μM CHIR99021 resulted in the highest expression of mesendoderm (EOMES, MIXL1) and DE (CXCR4, SOX17, FOXA2) marker gene expression (Figure 3B), although the percentage of SOX17+ DE cells (as determined by cell count) is comparable with the other DE-inducing conditions (Figures S3B and S3C). Fluorescence-activated cell sorting (FACS) analyses performed on two different hPSC lines further confirmed the observation that the various optimal DE-inducing conditions generated comparable percentages of CXCR4+SOX17+ DE cells (Figures 3C and S3E). Reassuringly, cells differentiated in the optimal DE-inducing conditions morphologically resembled each other but were different from no growth factor condition (Figure S3D). Among the optimal DE-inducing conditions, 3 μM CHIR99021 induced mesendodermal marker BRACHYURY and a marginal increase in mesodermal marker TBX6 but none of them induced extraembryonic tissue marker SOX7 dramatically (Figure S3A).

To further ascertain that the various optimal DE-inducing conditions were comparable with published methods, we undertook a head-to-head comparison with protocols outlined in Kroon et al. (2008) and Touboul et al. (2010), using the same differentiation medium (RPMI 2% B27). Because Touboul et al. (2010) utilized 20 ng/ml FGF2 (F20) and 10 μM LY294002 (LY10) in addition to Activin and BMP4, we applied the same amount of FGF2 and LY294002 to all our DE-inducing conditions because the aim was to determine if the various Wnt and BMP signaling-induced DE (in the presence of 100 ng/ml Activin) were comparable with published methods. In our comparison performed on two different hPSC lines, we found that our various optimal DE-inducing conditions were comparable with Touboul et al. (2010) and at times superior to Kroon et al. (2008) in terms of the induction of DE (CXCR4, SOX17, FOXA2) marker gene expression (Figure S4A).

Next, we investigated whether our DE-inducing conditions generated DE derivatives comparably. To this end, we differentiated these DE cells into pancreatic progenitors using a published protocol (Teo et al., 2012) with some modifications (A.K.K.T. et al., unpublished data). Our DE-inducing conditions gave rise to pancreatic progenitor markers SOX9 and PDX1 comparably with Kroon et al. (2008) in two different hPSC lines (Figure S4B), demonstrating equivalence in terms of their differentiation potential.

**Wnt Signaling via WNT3A Is Not as Effective as BMP4 or the GSK-3 Inhibitors, CHIR99021 and BIO, in Suppressing E-CADHERIN and Pluripotency of hPSCs during DE Induction**

During DE differentiation, hPSCs undergo epithelial to mesenchymal transition evidenced by morphological changes (Figures 1C, 2C, and 3D) and a decrease in epithelial marker E-CADHERIN/CDH1 (D’Amour et al., 2005). In addition, pluripotency program is suppressed, with rapid plummeting of pluripotency factor SOX2 expression.
Comparable DE formation via Wnt or BMP Signaling
followed by NANOG and lastly OCT4 (Teo et al., 2011, 2012). To ascertain that both Wnt and BMP signaling-induced DE cells are committed toward DE cell fate, we investigated the impact of varying doses of Wnt and BMP signaling activators on epithelial marker E-CADHERIN/CDH1 and pluripotency factor gene expression. Despite a high dose of WNT3A being able to significantly induce DE marker gene expression (with 100 ng/ml Activin), the expression level of E-CADHERIN/CDH1 did not decrease with increasing doses of WNT3A (Figure 4A). Surprisingly, pluripotency factors SOX2 and NANOG were only marginally affected by increasing doses of WNT3A with a background of 100 ng/ml Activin (Figures 4A–4C). In contrast, increasing doses of BMP4 and GSK-3 inhibitors, CHIR99021 and BIO, resulted in significant suppression of E-CADHERIN/CDH1 and pluripotency factors SOX2 and NANOG (Figures 4A–4C). Collectively, these data suggest that additional factors are required to work with WNT3A to suppress E-CADHERIN/CDH1 and pluripotency factors during differentiation toward DE. Therefore, CHIR99021, BMP4, and BIO may be preferred for DE differentiation because they also aid in the suppression of pluripotency (Figures 4A–4C).

**DISCUSSION**

We report the use of a chemically defined differentiation medium to compare DE-inducing effects by Wnt or BMP signaling in the presence of Activin signaling. This direct comparison demonstrates that both Wnt and BMP can independently synergize with Activin signaling to comparably and effectively induce DE cells from hPSCs in the absence of FBS. Numerous reports have established that 25 ng/ml WNT3A is optimal for inducing DE in the presence of Activin and FBS (Table S1). However, 10–30 ng/ml WNT3A is insufficient to synergize with 100 ng/ml Activin to effectively generate DE cells in our serum-free system. The need for 100 ng/ml WNT3A for efficient DE formation in a chemically defined system suggests that FBS (which exhibits batch-to-batch variability) introduces additional disturbances that normally assist in the induction of DE cells. Although Kunisada et al. (2012) and Illing et al. (2013) used CHIR99021 to induce DE differentiation from hPSCs, they also included FBS in their protocol. We now provide several alternative methods for effectively inducing DE cells, all via the activation of Wnt signaling pathway (in addition to the indispensable Activin signaling pathway). These include 100 ng/ml WNT3A, 3 μM CHIR99021, and 1.5 μM BIO, all of which exhibit similar effects in DE induction. This three-pronged approach confirms the ability of Wnt to synergize with Activin signaling to induce DE in a chemically defined medium. Our comparison with Koon et al. (2008) (WNT3A-based) and Touboul et al. (2010) (BMP4-based) further demonstrates that these three Wnt signaling-based optimized conditions induce DE comparably and efficiently, and give rise to DE derivatives with equivalent potential. However, it is surprising to note that WNT3A is not as effective in downregulating E-CADHERIN/CDH1 and pluripotency factors as compared to GSK-3 inhibitors (or even BMP4) in a serum-free system. It is known from early mammalian development that the expression of Oct4, and in particular Sox2 and Nanog (Avalion et al., 2003; Hart et al., 2004) coincides with the in situ expression of Wnt3, Wnt5a and Wnt5a but not that of Bmp4 (Arnold and Robertson, 2009; Liu et al., 1999; Tam and Loebel, 2007; Teo et al., 2011; Yamaguchi et al., 1999). Thus, given the overlapping expression of Wnt and pluripotency genes but not that of Bmp, it is conceivable that Wnt does not play a major role in suppressing pluripotency in contrast to BMP. In this study, we reveal fundamental mechanistic differences between the two developmental signaling pathways: (1) BMP signaling suppresses pluripotency and simultaneously promotes DE formation, whereas (2) Wnt signaling mostly serves to synergistically promote DE formation and may require additional factors to suppress pluripotency. FBS, which is commonly added in presence of WNT3A, could be promoting both differentiation and suppressing pluripotency. Although the use of GSK-3 inhibitors results in a downregulation of E-CADHERIN/CDH1 and pluripotency factors SOX2 and NANOG, and GSK-3 inhibition activates downstream Wnt signaling, the inhibition of GSK-3 could potentially affect other signaling pathways in which GSK-3 is active, such as cellular proliferation and migration (Doble et al., 2011; Illing et al., 2013).
### A

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### B

- A100
- A100 B25
- A100 W100
- A100 C3
- A100 Bio1.5
- A100 W30+0.5%FBS

### C

**Percentage of SOX2+ cells at Day 3**

![SOX2+ cells at Day 3](image)

**Percentage of NANOG+ cells at Day 3**

![NANOG+ cells at Day 3](image)

### D

**Choice of DE induction methodology based on cost-effectiveness**

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<td>5</td>
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*Legend on next page*
and Woodgett, 2003). This is in contrast to the specific WNT3A-induced Wnt signaling. In our experiments, we also observed that WNT3A (up to 200 ng/ml) was not as effective as the GSK-3 inhibitors in elevating β-catenin transcript expression during DE differentiation (data not shown). This could imply that WNT3A-induced Wnt signaling events occurred solely at the protein level. Further, this suggests that GSK-3 inhibitors potentiate the increased presence of β-catenin, thereby strengthening Wnt signaling (besides inhibiting the GSK-3-containing destruction complex). Therefore, the inhibition of multifunctional GSK-3 coupled with the increased β-catenin expression could partly account for the differences observed between WNT3A-induced and GSK-3 inhibition-induced Wnt signaling. Specific to CHIR99021 (a potent and highly selective GSK-3 inhibitor as compared to BIO), the strong inductive effects on mesendodermal (EOMES, MIXL1, BRACHYURY) marker gene expression (Kubo et al., 2004; Tada et al., 2005) and a marginal increase in mesodermal marker TRX6 indicate that excessive Wnt signaling via CHIR99021 (>3 μM) is likely to promote mesoderm while suppressing DE formation.

Taken together, CHIR99021 appears to be the preferred choice in combination with Activin (rank 1, Figure 4D) in generating DE cells based on its superior ability to induce mesendoderm as well as its cost effectiveness (~2 cents/ml of media). BMP4 would be favored if not for its ~20-fold greater cost (rank 2, Figure 4D), whereas BIO, although less expensive, exhibits cytotoxic effects (rank 3, Figure 4D) and WNT3A is both expensive and requires a high dose to be effective (rank 4, Figure 4D).

In summary, we demonstrate that both Wnt and BMP can cooperate with Activin signaling to induce DE with comparable efficiencies in a chemically defined serum-free medium. DE generated from both Wnt-Activin and BMP-Activin signaling can give rise to derivatives such as pancreatic progenitor cells (Kunisada et al., 2012; Teo et al., 2012). Thus, DE made via these two differing signaling pathways is conceptually consistent and physiologically relevant. Future genome-wide comparisons could reveal interesting mechanistic insights specific to Wnt and BMP signaling that are relevant for DE specification.

EXPERIMENTAL PROCEDURES

Cell Culture
hiPSCs derived from AG16102 skin fibroblasts (Coriell Institute) or hESCs (CHB8) were cultured in conditions as described previously (Teo et al., 2012). To initiate DE differentiation, hiPSCs were treated with Collagenase IV and Dispase at 1:1 ratio for 3–5 min before being manually passaged into small clumps and placed in a 70 μm cell strainer to deplete the feeders (Teo et al., 2012). hiPSCs were then differentiated 2 days later in RPMI 2% B27 (no vitamin A; serum-free chemically defined medium; Teo et al., 2012) supplemented with the appropriate growth factors and chemical compounds. Cells were harvested after 3 days of differentiation. To generate pancreatic progenitors, DE was further differentiated with 50 ng/ml Activin for 2 days, 50 ng/ml fibroblast growth factor-2 (FGF2), 3 μM all-trans retinoic acid (RA), and 10 mM nicotinamide (NIC) for 5 days; 50 ng/ml FGF2, 3 μM RA, 10 mM NIC, and 20 μM DAPT for 4 days; and 50 ng/ml FGF2, 10 mM NIC, and 20 μM DAPT for 3 days.

qRT-PCR, Immunostaining, Western Blot, and FACS Analyses
Methods for qRT-PCR, immunostaining, western blot, and FACS analyses have been described previously (Teo et al., 2012, 2013b). The percentage of DE and pluripotent cells was obtained by dividing the number of SOX17+, SOX2+, and NANOG+ cells over the total number of DAPI+ cells. The average of three separate images was used for cell counting analyses. A p value <0.05 indicates statistical significance by Student’s t test. All error bars represent SD of three biological replicates in at least two independent experiments. Primers and antibodies are provided in Table S2.

SUPPLEMENTAL INFORMATION
Supplemental information includes four figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.05.007.
ACKNOWLEDGMENTS

The hiPSC line used was derived in the Joslin DRC iPS Core Facility (NIH 5 P30 DK036836-27). FACS analysis was supported by the HSCI/DRC Flow Core (NIH P30DK036836). We thank G. Daley, M.D., Ph.D. for CHB8 hESCs. A.K.K.T. is supported by a Juvenile Diabetes Research Foundation postdoctoral fellowship. I.A.V. is supported by a HSCI Stemllicht Director’s Fund Award and an NIH F31DK098931 award. R.N.K. is supported by the HSCI, NIH grants RO1 DK 67536 and RO1 DK 055523, and a grant from AstraZeneca.

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REFERENCES

Comparable Generation of Activin-Induced Definitive Endoderm via Additive Wnt or BMP Signaling in Absence of Serum

Adrian Kee Keong Teo, Ivan Achel Valdez, Ercument Dirice, and Rohit N. Kulkarni
Supplemental Information

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1. Lack of synergism between Wnt, BMP and Activin signaling in inducing mesoderm or extra-embryonic tissue formation. (“Related to Figures 1, 2 and 3”) Expression of mesoderm (BRACHYURY, TBX6) and extra-embryonic tissue (SOX7) markers in hiPSCs differentiated for three days in the presence of increasing doses of Activin (0, 25, 50 or 100 ng/ml) and (A) high dose (50, 100 or 200 ng/ml) of WNT3A ligand or (B) narrow range (1, 3 or 5 μM) of CHIR99021 or (C) BIO (0.5, 1 or 1.5 μM) or (D) BMP4 (0, 25 or 50 ng/ml). Asterisk (*) indicates $p < 0.05$ compared to no growth factor condition. All error bars indicate standard deviation of three biological replicates.

Supplemental Figure S2. Definitive endoderm markers EOMES, CXCR4 and SOX17 exhibit peak expression on Day 3 in a detailed time course analysis. (“Related to Figure 3”) Expression of definitive endoderm markers (A) EOMES, (B) CXCR4 and (C) SOX17 in hiPSCs and hESCs (CHB8) differentiated for three days in the presence of A100 B25, A100 W100, A100 C3, A100 Bio1.5, A100 W30 + 0.5% FBS or as per Kroon et al. (2008). Asterisk (*) indicates $p < 0.05$ compared to Day 1 condition. All error bars indicate standard deviation of three biological replicates.

Supplemental Figure S3. Wnt and BMP signaling can enhance Activin-induced definitive endoderm with comparable efficiencies and negligible mesoderm or extra-embryonic tissue formation. (“Related to Figure 3”) Expression of mesoderm...
(BRACHYURY, TBX6) and extra-embryonic tissue (SOX7) markers in hiPSCs differentiated for three days in the presence of (A) no growth factor (A0), 100 ng/ml Activin alone (A100), A100 plus either 25 ng/ml BMP4 (B25), 100 ng/ml WNT3A (W100), 3 μM CHIR99021 (C3) or 1.5 μM BIO (Bio1.5). Asterisk (*) indicates $p < 0.05$ compared to no growth factor (A0) condition. All error bars indicate standard deviation of three biological replicates. (B) Immunostaining for the SOX17 definitive endoderm marker on hiPSCs differentiated for three days in the presence of A100, A100 B25, A100 W100, A100 C3, A100 Bio1.5 or A100 W30 + 0.5% FBS. Scale bar: 50 μm. (C) The percentage of SOX17+ cells determined by cell count after differentiation of hiPSCs for three days in the presence of A100, A100 B25, A100 W100, A100 C3, A100 Bio1.5 or A100 W30 + 0.5% FBS. Asterisk (*) indicates $p < 0.05$ compared to A100 condition. (D) Morphology of hiPSCs differentiated for three days in the presence of no growth factors (A0), A100, A100 B25, A100 W100, A100 C3 or A100 Bio1.5. Scale bar: 200 μm. (E) The percentage of CXCR4+SOX17+ definitive endoderm cells determined by FACS analyses after differentiation of hESCs (CHB8) for three days in the presence of A100 B25, A100 W100, A100 C3, A100 Bio1.5 or A100 W30 + 0.5% FBS.

**Supplemental Figure S4.** The various optimal DE-inducing conditions perform comparably with published protocols and generate pancreatic progenitors comparably. ("Related to Figure 3") (A) Expression of definitive endoderm (CXCR4, SOX17 and FOXA2) markers in hiPSCs and hESCs (CHB8) differentiated for three days in the presence of 20 ng/ml FGF2 (F20) and 10 μM LY294002 (LY10) + A100 B25, A100 W100, A100 C3, A100 Bio1.5 or as per Kroon et al. (2008) or Touboul et al. (2010). (B) Expression of pancreatic progenitor (SOX9, PDX1) markers in hiPSCs and
hESCs (CHB8) differentiated for 17 days with varying conditions for the first three days (A100 B25, A100 W100, A100 C3, A100 Bio1.5 or as per Kroon et al. (2008)). Asterisk (*) indicates $p < 0.05$ compared to Kroon et al. (2008) condition. All error bars indicate standard deviation of three biological replicates.

SUPPLEMENTAL TABLE LEGEND

Supplemental Table S1. Published studies reporting protocols for differentiation into definitive endoderm involving serum, Wnt and BMP. (“Related to Figure 3”)

Supplemental Table S2. Primers and antibodies used in this study. (“Related to Figures 1, 2, 3 and 4”)
Supplemental Figure S1: Teo et al.,

A  High dose WNT3A

B  GSK3 inhibitor CHIR99021 narrow range

C  GSK3 inhibitor BIO

D  Low dose BMP4
Supplemental Figure S2: Teo et al.,

A  

**EOMES**

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B  

**CXCR4**

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C  

**SOX17**

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Supplemental Figure S3: Teo et al.,

A. BMP vs Wnt Signaling

Relative expression (fold over growth factors)

- BRACHYURY
- TBX6
- SOX7

B. A100 B25 A100 W100 A100 C3 A100 Bio1.5 A100 W30 + 0.5 % FBS

C. Percentage of SOX17+ cells at Day 3

D. Unstained control A100 B25 A100 W100 A100 C3 A100 Bio1.5 A100 W30 + 0.5 % FBS

E. hESC (CHB8)
Supplemental Figure S4: Teo et al.,
### SUPPLEMENTAL TABLE

**Supplemental Table S1. Published studies reporting protocols for differentiation into definitive endoderm involving serum, Wnt and BMP.**

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<td>King et al., Regen Med, 2008</td>
<td>Activin FBS only</td>
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<td>Agarwal et al., Stem Cells, 2008</td>
<td>100 ng/ml Activin A + 0.5% FBS</td>
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<td>Sumi et al., Development, 2008</td>
<td>100 ng/ml Activin A + 2% FBS</td>
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<td>Shim et al., Diabetes, 2007</td>
<td>100 ng/ml Activin A + 20% FBS</td>
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<td>D’Amour et al., Nat Biotechnol, 2006</td>
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<td>Kroon et al., Nat Biotechnol, 2008</td>
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<td>500 nM IDE1 + 3 μM CHIR99021 + 10 ng/ml BMP4 + 5 μM LY294002 + 2% FBS</td>
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<td>Kunisada et al., Stem Cell Res, 2011</td>
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<td>Illing et al., Stem Cell International, 2013</td>
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<td>Hay et al., PNAS, 2008</td>
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<td>Van Hoof et al., Stem Cell Res, 2011</td>
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<td>Phillips et al., Stem Cells Dev, 2007</td>
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APPENDIX 2-2

Contributions to Additional Co-Aauthored Publications
Early Developmental Perturbations in a Human Stem Cell Model of MODY5/ HNF1B Pancreatic Hypoplasia

Adrian Kee Keong Teo1,*, H. H. Lau, Ivan Achel Valdez1, Ercument Dirice1, Erling Tjora2, Helge Raeder2 and Rohit N. Kulkarni1,*

1Section of Islet Cell and Regenerative Biology, Joslin Diabetes Center, Department of Medicine, Brigham and Women’s Hospital, and Harvard Medical School, Boston, Massachusetts 02215, USA.

2Department of Pediatrics, Haukeland University Hospital, and the KG Jebsen Center for Diabetes Research, Department of Medicine 2, University of Bergen, 5021 Bergen, Norway

Article published in the journal of Stem Cell Reports:


Contributions:
Immunofluorescence and FACS analyses

Figures: 3B-C; 4A; 5A; S1C-D

Editing manuscript
Early Developmental Perturbations in a Human Stem Cell Model of MODY5/HNF1B Pancreatic Hypoplasia

Adrian Kee Keong Teo,1,2,3,4,5,* Hwee Hui Lau,2 Ivan Achel Valdez,1 ERCument Dirice,1 Erling Tjora,6,7 Helge Raeder,5,7 and Rohit N. Kulkarni1,4
1Section of Islet Cell and Regenerative Biology, Joslin Diabetes Center, Department of Medicine, Brigham and Women’s Hospital, Harvard Stem Cell Institute, Harvard Medical School, One Joslin Place, Boston, MA 02215, USA
2Discovery Research Division, Institute of Molecular and Cell Biology, 61 Biopolis Drive, Proteos #06-07, Singapore 138673, Singapore
3School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore
4Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117596, Singapore
5Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 308232, Singapore
6Department of Pediatrics, Haukeland University Hospital, 5021 Bergen, Norway
7Department of Clinical Science, KG Jebsen Center for Diabetes Research, University of Bergen, 5021 Bergen, Norway
*Correspondence: akeo@imcb.a-star.edu.sg (A.K.K.T.), rohit.kulkarni@joslin.harvard.edu (R.N.K.)
http://dx.doi.org/10.1016/j.stemcr.2016.01.007
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SUMMARY

Patients with an HNF1B<sup>STABLE</sup> mutation (MODY5) typically exhibit pancreatic hypoplasia. However, the molecular mechanisms are unknown due to inaccessibility of patient material and because mouse models do not fully recapitulate MODY5. Here, we differentiated MODY5 human-induced pluripotent stem cells (hiPSCs) into pancreatic progenitors, and show that the HNF1B<sup>S148L/+</sup> mutation causes a compensatory increase in several pancreatic transcription factors, and surprisingly, a decrease in DHNF1A protein. Thus, patients typically develop MODY5 but not neonatal diabetes despite exhibiting pancreatic hypoplasia.

INTRODUCTION

Maturity-onset diabetes of the young (MODY) is a type of autosomal dominant monogenic diabetes classically characterized by non-ketotic, non-insulin-dependent diabetes occurring before the age of 25 years (Teo et al., 2013a). Many MODY genes are pancreatic developmental transcription factors, with the notable exception of GCK, CEL, and INS. Although MODY was discovered more than two decades ago, the molecular mechanisms underlying transcription factor MODY in humans is largely unclear because mouse models do not fully recapitulate this disease (Maestro et al., 2007) and the lack of a suitable human model.

Patients with MODY5 commonly exhibit pancreatic hypoplasia due to an autosomal dominant mutation in the HNF1B gene (Edghill et al., 2006). HNF1B is a member of the complex pancreatic transcription factor network which includes HNF1A and HNF4A. HNF1B<sup>+</sup> cells in the pancreatic trunk epithelium are multipotent pancreatic progenitors which play a role in endocrine and exocrine development (Haumaitre et al., 2005). Although MODY5 was discovered in 1997, to date the effects of an autosomal dominant mutation in HNF1B on human pancreas development and the molecular mechanisms underlying pancreatic hypoplasia remain not fully understood. MODY5 phenotype in humans cannot be phenocopied by Hnf1b<sup>−/−</sup> mice since they do not develop diabetes (Haumaitre et al., 2005), highlighting the need for a suitable human model to study the perturbations in pancreas development (Teo et al., 2013a).

Human-induced pluripotent stem cell (hiPSC)-derived pancreatic cells now provide an excellent opportunity to study this monogenic diabetes phenotype. In this study, we established a well-controlled MODY5-hiPSC pancreatic differentiation model to elucidate the molecular mechanisms underlying MODY5 pancreatic hypoplasia. We differentiated four control and six mutant hiPSC lines, and observed that mutant HNF1B<sup>S148L/+</sup> elicits a compensatory increase in definitive endoderm (DE) and pancreatic transcription factor gene expression. Mutant HNF1B<sup>S148L</sup> directly accounted for an increased PDX1 gene expression. These pancreatic transcription factor network perturbations could possibly explain the occurrence of maturity-onset diabetes rather than neonatal diabetes despite pancreatic hypoplasia. Importantly, pancreatic PAX6 gene expression, known to be important for pancreatic β-cell function, was distinctly down-regulated in MODY5 pancreatic progenitors, which in part explains the early-onset diabetes and pancreatic hypoplasia in MODY5 patients.
RESULTS

Establishment of a Human Stem Cell Model for MODY5

We recently reported the derivation of several hiPSC lines from a MODY5 family (Haldorsen et al., 2008) for in vitro disease modeling of monogenic diabetes (Teo et al., 2013b). Our experimental design included a “node” comprising a healthy family member (N805-6) and two members of the family with an autosomal dominant (S148L) mutation in the HNF1B gene, of which one of them has developed diabetes (N805-2) whereas the other has not (N805-1) (Figure 1A). Three independent hiPSC lines from each subject were established (iN805-6A/B/C, iN805-1A/B/C, and iN805-2A/B/C) and verified for the absence or presence of the S148L (C443T in exon 2) mutation in the HNF1B gene (Figures 1B and S1A).

We then set out to establish a human pancreatic differentiation protocol for disease modeling of MODY5 in vitro (Figure 1C) based on a chemically defined medium (no serum) (Teo et al., 2014), which is a modified version of our recently reported protocol (Teo et al., 2012, 2015). Careful time-course analyses of differentiated control hiPSCs (derived from AG16102) (Teo et al., 2013b) indicated that pluripotency factors OCT4 and NANOG plummet by day 3, and that hiPSCs transit through EOMES+ MIXL1+ mesoderm before differentiating into DE marked by CXCR4, SOX17, GATA4, and PAX6 (Figure 1D).

Further differentiation toward foregut endoderm and pancreatic progenitors by day 17 revealed an up-regulation of numerous key pancreatic progenitor markers such as ISL1, HLXB9, HNF1B, PAX6, SOX9, PDX1, PTF1A, RXF6, NEUROD1, HNF6, DLK1, SOX4, and MAFB, indicating that our pancreatic differentiation protocol is suitable for studying the impact of HNF1B S148L/+ mutation on early pancreatic development and its transcriptional network (Figures 1E and S1B). Immunostaining on day 12 and fluorescence-activated cell sorting (FACS) analyses on day 17 further confirmed the protein expression of key pancreatic developmental genes (Figures S1C and S1D). Since HNF1B transcripts exhibit peak expression between days 7 and 10, we performed genome-wide microarray analyses on day-10 differentiated control hiPSCs, and confirmed the up-regulation of pancreas-related genes such as PDX1, HNF1B, ISL1, RXF6, PAX6, GATA4, GATA6, SOX9, and PTF1A, and a reciprocal down-regulation of numerous pluripotency-related genes (Figures 1F and Table S1). Gene ontology (GO) analyses on up- and down-regulated genes (fold change >2) indicated general changes in developmental processes and possibly a down-regulation of vascular development (Figure S2A).

MODY5-hiPSC-Derived Pancreatic Progenitors Exhibit a Compensatory Increase in DE and Pancreatic Markers but Down-Regulation of PAX6 Gene Expression

We first differentiated the MODY5-hiPSCs into DE (day 3), gut endoderm (days 5–7), and early foregut progenitors (day 10) (Figure 2). Hnf1β is expressed as early as embryonic day 8.75 (E8.75) in the mouse primitive gut/foregut (Ott et al., 1991), corresponding to ~days 3–7 of the hiPSC in vitro differentiation. Interestingly, we observed an early compensatory increase in DE markers CXCR4, SOX17, FOXA2, GATA4, and GATA6 on day 5 and beyond (Figure 2) in the mutant hiPSCs, just when HNF1B gene is beginning to be expressed (Figure 1E; days 3–5). This suggests that the early (low) expression of mutant HNF1B S148L/+ during gut endoderm development is already causing an early compensatory increase in DE and gut endoderm markers.

Given the phenotypic similarities in pancreatic agenesis/hypoplasia caused by HNF1B, PDX1, PTF1A, GATA4, and GATA6 mutations, we hypothesized that the dorsal pancreatic agenesis in MODY5 (HNF1B S148L/+; Haldorsen et al., 2008) could be directly linked to the down-regulation of downstream pancreatic genes GATA4, GATA6, PDX1, and PTF1A. Surprisingly, time-course transcriptional analyses of MODY5-hiPSC-derived pancreatic progenitors indicate higher gene expression of HNF1B, PDX1, GATA4, and GATA6 in mutant hiPSCs (iN805-1A/B/C and iN805-2A/B/C; three independent lines; each in biological triplicate) compared with two control hiPSCs (non-family-related control iAG16102 and family control iN805-6A/B; two independent lines; each in biological triplicate) (Figure 3A). These findings were also substantiated by immunostaining of HNF1B and PDX1 on day 12 (Figure 3B; representative of two to three hiPSC lines) and FACS analyses of SOX9 and PDX1 on day 17 (Figure 3C; two to three hiPSC lines per subject were pooled together) in MODY5-hiPSC-derived pancreatic progenitors.

Subsequently, to address the hypothesis that mutant HNF1B S148L/+ results in its decreased expression and thus leads to pancreatic hypoplasia and MODY, we analyzed the transcriptional profile to potentially identify a gene(s) which is down-regulated in the mutant hiPSC-derived pancreatic progenitors. Contrary to expectations, we found a compensatory increase in FOXA2, ISL1, HLXB9, and RXF6 (Figures S2B and S2C), suggesting that this set of genes (including HNF1B, PDX1, GATA4, and GATA6) play an important role in alleviating the negative effects of mutant HNF1B S148L/+ so as to delay the overall impact of pancreatic hypoplasia and/or mutant HNF1B S148L/+ on diabetes onset. Surprisingly, PAX6 was the singular gene among the many examined to exhibit down-regulation...
in mutant hiPSC-derived pancreatic progenitors (Figure 3A). Following this candidate gene approach, we performed microarray analyses for cells obtained from days 0, 12, and 17 (Figure S3A and Table S2). Reassuringly, our genome-wide analyses indicated that our candidate gene approach has captured all the key changes in pancreatic genes: increase in PDX1, ISL1, HNF1B, TCF2, RFX6, FOXA2, GATA4, GATA6, and HLXB9 (MNX1) expression in the mutant hiPSC-derived pancreatic progenitors (Figure S3A). In addition, PAX6 was also the most relevant pancreatic gene (expressed in our differentiation) to be down-regulated in the mutant hiPSC-derived pancreatic progenitors (Figure S3A). Further GO analyses on the up- and down-regulated genes (fold change >2) on days 12 and 17 mostly reflected changes in developmental processes (Figures S3B and S3C). Uniquely, neural development appeared to be more down-regulated in MODY5 pancreatic progenitors on day 17 (Figure S3C).

We then performed chromatin immunoprecipitation (ChIP) analyses and confirmed that HNF1B binds onto the genomic loci of HLXB9 and HNF1B (Figure S3D), suggesting that these two genes could be the earliest mediators of pancreatic transcription factor network control. To evaluate whether the HNF1B mutation affected pancreatic progenitor cell growth, we performed a cell counting assay and, interestingly, observed that there is a huge retardation of cell growth from day 12 onward, when the mutant HNF1B protein exhibits peak expression (Figure S3E). This strongly suggests that the mutant HNF1B protein affects pancreatic cell growth, thereby accounting for the pancreatic hypoplasia phenotype in MODYS.
Wild-Type HNF1B Suppresses, While Mutant HNF1B Increases, PDX1 Gene Expression

The compensatory increase in both HNF1B and PDX1 gene expression in the mutant hiPSC-derived pancreatic progenitors prompted us to further investigate this molecular relationship. FACS analyses performed on day-12 differentiated control hiPSCs confirmed that 36.2% are PDX1+ (Figure 4A) whereas 91.4% are HNF1B+ (Figure 5A), corroborating their transcriptional profile (Figure 1E). We first performed luciferase assays to study the transcriptional regulation of cardinal pancreatic gene PDX1 (Stoffers et al., 1997) by HNF1B. Overexpression of HNF1B from days 10–12 in differentiated control hiPSCs surprisingly suppressed PDX1 transcriptional activity, in both the ~7 kb full-length PDX1 promoter construct and a construct containing areas I–III of PDX1 promoter (~2.4 kb) known to be the principal control region of PDX1 gene expression (Gerrish et al., 2000) (Figure 4B). This indicates a repressive pattern of HNF1B whereby HNF1B gene expression is decreasing when PDX1 is beginning to be expressed from days 10–12 (Figure 1E).

HNF1B protein functions either as a homodimer or a heterodimer with the structurally related HNF1A (Mendel et al., 1991; Rey-Campos et al., 1991). We thus overexpressed both HNF1B and HNF1A from days 10–12 in differentiated control hiPSCs, only to discover that HNF1A does not have any transcriptional regulatory effect on PDX1, at least during this early pancreatic progenitor stage when PDX1 is beginning to be expressed (Figure 4C). This is consistent with the observation that HNF1A is expressed very late during pancreatic differentiation (Figure S1D). Thus, the suppressive effect of HNF1B on PDX1 transcriptional activity is likely due to homodimers in action.

Very little is known about the target genes of HNF1B in a developing pancreas. HNF4A is one potential candidate (Thomas et al., 2001). HNF1 binding sites have been found to be present in pancreas-/islet-specific P2 (Thomas et al., 2001) and P1 promoters (Taraviras et al., 1994) of the Hnf4a gene (Eeckhoute et al., 2003). Thus, we cloned both P1 and P2 promoters of HNF4A, and performed similar luciferase assays. Dismally, HNF1B overexpression did not have any effect on P1 promoter and only a very marginal effect on P2 promoter activity in our stem cell model on day 12 (Figure S4A), suggesting that the regulation of HNF4A by HNF1B (or HNF1A) is likely apparent only at later stages of pancreatic development.

To delineate whether the wild-type HNF1B or mutant HNF1B<sup>S148L</sup> allele is responsible for the compensatory increase in pancreatic gene expression (Figure 3A), we next overexpressed wild-type HNF1B or mutant HNF1B<sup>S148L</sup> from days 7–10 in differentiated control hiPSCs. Interestingly, overexpression of mutant HNF1B<sup>S148L</sup> resulted in an increase in PDX1 gene expression but was insufficient to up-regulate other pancreatic transcription factors such as GATA4, GATA6, or SOX9 (Figures 4D and S4B). On the contrary, overexpression of wild-type HNF1B from days 7–10 did not alter the expression level of PDX1 or other genes we investigated, including GATA4, GATA6, and SOX9 (Figures 4E and S4C). ChIP analyses performed on HNF1B also did not reveal binding on PDX1 genomic loci (Cebola et al., 2002).
and data not shown). Last but not least, qPCR and western blot analyses confirmed that the HNF1B and HNF1A genes/proteins were overexpressed successfully (Figures S4D and S4E). Together, these data indicate that mutant HNF1B<sup>S148L</sup> (and not HNF1B or HNF1A) gene expression is responsible for the compensatory increase in PDX1 gene expression in mutant hiPSC-derived pancreatic progenitors. The molecular mediator(s) of increased GATA4 and GATA6 gene expression in mutant hiPSC-derived pancreatic progenitors is unclear and requires further investigation.

**HNF1B Is Not Directly Involved in Gene Regulation of PAX6**

Pax6 is known to be expressed during early pancreatic development (E9–9.5) in cells committed exclusively to the endocrine cell fate (Ashery-Padan et al., 2004; Sander et al., 1997). Among the transcription factors we analyzed, only PAX6 transcripts were clearly down-regulated (Figure 3A). Since PAX6 exhibits a similar (but right-shifted) gene-expression profile to that of HNF1B, we sought to investigate whether HNF1B or HNF1B<sup>S148L</sup> is involved in the regulation of PAX6 gene expression. FACS analyses on day-12 differentiated control hiPSCs (PAX6 peak transcript expression) (Figure 1E), indicated that 27.2% are PAX6<sup>+</sup>, of which 22.0% are also HNF1B<sup>+</sup> (Figure 5A). This percentage of PAX6<sup>+</sup> cells is consistent with the report that PAX6 protein is detected only in a small subset of cells in the pancreatic endoderm at E9–9.5 (Sander et al., 1997). 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only resulted in a marginal suppression of PAX6 promoter activity (Figure 5C). Overexpression of wild-type HNF1B from days 7–10 in differentiated control hiPSCs (Figure 4E) did not alter PAX6 gene-expression levels (Figure 5D). Overexpression of mutant HNF1B S148L alone (Figure 4D) was not sufficient to suppress PAX6 gene-expression levels (Figure 5E). Furthermore, HNF1B was not found to bind onto the PAX6 promoter region (Cebola et al., 2015 and data not shown). This indicates that the decrease in PAX6 gene expression is an indirect effect of the mutant HNF1B S148L/+, and is possibly a result of the altered pancreatic transcription factor network.

The loss of Pax6 expression early on during pancreatic development reduces pancreatic insulin content (Ashery-Padan et al., 2004; Sander et al., 1997) and affects postnatal pancreatic β-cell function, which results in early-onset diabetes (Ashery-Padan et al., 2004). In this study, we found that PAX6 gene expression is decreased in mutant (HNF1B S148L/+) hiPSC-derived pancreatic progenitors. To translate our findings to the MODY5 patients, we tracked the clinical phenotype of the N805 patients over time. N805-2 (HNF1B S148L/) exhibited high fasting glucose levels (16.3 mmol/l) and required insulin (58–68 units/24 hr), whereas N805-1 (HNF1B S148L/), who was not diabetic, presented with elevated fasting glucose levels (normal range: 4.0–6.0 mmol/l) and elevated 2-hr glucose levels after a standard oral glucose challenge (75 g) (impaired glucose tolerance range: 7.8–11.1 mmol/l) after the age of 8 years (Figure 5F). N805-2 presented low fasting insulin (2.4 mIE/l) whereas N805-1 exhibited a transition...
from normal to subnormal levels of fasting insulin, at 5.4 mIE/l (normal range: 6.0–27.0 mIE/l) (Figure 5F). As diabetes develops, plasma glucagon levels usually rise due to impaired α-cell glucose-sensing function and lack of appropriate suppression (Dunning et al., 2005). This scenario fits with the substantially elevated plasma glucagon levels (72.2 pmol/l) in the diabetic individual N805-2 and the moderately elevated plasma glucagon levels in the prediabetic individual N805-1 (46.9 pmol/l; normal range: 14.3–43 pmol/l) (Figure 5F).

**DISCUSSION**

We have successfully established a human stem cell model to study the molecular mechanisms underlying MODY5. Our experimental design, which includes a non-family control hiPSC line and three independent hiPSC lines from each of the three subjects in a MODY5 family, is a well-controlled experimental “node” which minimizes potential genetic background influence and line-to-line variation in hiPSC differentiation. While we observe HNF1B gene expression in the family control data to resemble that in the MODY5-hiPSC-derived pancreatic progenitors on rare occasions (Figure 2), suggesting initial genetic background effects (difference between control lines becomes attenuated later), the use of non-family control samples provides confidence in the overall distinct phenotypes we observed in our in vitro stem cell model. In addition, more than 90% of day-12 differentiated cells are HNF1B+, making our pancreatic differentiation protocol well suited for studying MODY5.
HNF1B has been reported to be a transcriptional activator (Rey-Campos et al., 1991), but any positive regulation of PDX1 gene expression by HNF1A and/or HNF1B could be specific to pancreatic β cells (Ben-Shlushan et al., 2001) and not pancreatic progenitors, implying that HNF1B and/or HNF1A switch their binding partners during pancreatic development to regulate specificity in gene regulation.

We observed that mutant HNF1B5148L (and not HNF1B or HNF1A) gene expression is responsible for the compensatory increase in PDX1 gene expression in mutant hiPSC-derived pancreatic progenitors. This could, in part, be acting to counter the increased transcriptional repression by wild-type HNF1B (Figures 3B and 3C), although increasing HNF1B gene expression for 3 days from days 7 to 10 appears to be insufficient to significantly suppress PDX1 gene expression (Figure 4E). This gene regulation is likely to be indirect, since we did not find HNF1B protein to be bound onto the genomic loci of PDX1 (Cebola et al., 2015). It is interesting to note that β-cell-specific knockout of Hnf1β similarly results in an increase in Pdx1 gene expression (Wang et al., 2004), indicating that perturbations in HNF1B gene expression, either early during pancreatic development or in mature β cells, both prompt compensatory PDX1 gene expression.

The presence of a wild-type HNF1B allele and the combinatorial effects of wild-type-mutant HNF1B dimers in MODY5 (HNF1B5148L/+ ) patients partly explains the compensatory up-regulation of numerous pancreatic transcription factors. The lack of down-regulation of critical pancreatic genes PDX1, PTF1A, GATA4, and GATA6 implies that the mechanism underlying dorsal pancreatic agenesis in MODY5 is independent of these genes, which are also known to result in pancreatic agenesis/hypoplasia when mutated (D’Amato et al., 2010; Lango Allen et al., 2012; Sellick et al., 2004; Stoffers et al., 1997). This compensation in pancreatic transcriptional network due to an HNF1B autosomal dominant mutation may also account for the diabetes onset relatively later in life seen in MODY5 compared with the early onset observed in genetic forms of neonatal diabetes (Teo et al., 2013a) (Figure 5G). In this context, it is interesting that the MODY5 subjects with dorsal pancreatic agenesis also exhibit compensatory increase in physiological acinar function with hypersecretion from the remaining ventral portions of the pancreas (Tjora et al., 2013).

Hnf1β−/− mice with pancreatic agenesis exhibit loss of numerous pancreatic genes, including Pax6 expression (Haumaitre et al., 2005). Pax6-deficient pancreatic progenitors that are unable to mature and reach terminal differentiation later in life cannot be rescued by postnatal neogenesis (Ashery-Padan et al., 2004). Thus, the reduction in pancreatic endocrine cell number in Pax6−/− embryos (Sander et al., 1997) and altered acinar structure (Hart et al., 2013) could partly account for the pancreatic hypoplasia phenotype in MODY5 patients. Given that PAX6 directly regulates genes that regulate β-cell function (Ashery-Padan et al., 2004; Wen et al., 2009), we speculate that the loss of PAX6 gene expression early on in the MODY5 patients also partly accounts for the β and α islet cell dysfunction, and contributes to their diabetic phenotype (Figure 5G). This concept is concordant with the observations that patients with PAX6 heterozygous mutations also develop glucose intolerance (Wen et al., 2009; Yasuda et al., 2002). It is interesting to note that administration of exendin-4, a glucagon-like peptide 1 receptor (GLP-1R) agonist, to Pax6−/− mice can rescue the metabolic abnormalities observed, either via increased insulin secretion or β-cell regeneration (Ding et al., 2009), suggesting a potential for treatment for patients lacking pancreatic PAX6 gene expression, and the MODY5 patients in this study.

Collectively, we have evaluated the impact of a MODY5-causing point mutation (S148L) in the HNF1B gene on human pancreas development using a unique human stem cell model. We report molecular phenotypes (up- and down-regulation of pancreatic genes) when HNF1B is increasingly expressed from days 7 to 10, and observed that these phenotypes extend beyond the expression window of HNF1B. These data provide insights into the long-term impact of the MODY5 mutation on the pancreatic transcriptional network and the subsequent development of human pancreatic progenitors into mature functional endocrine cells. This is in agreement with Haumaitre et al. (2006), who indicated that MODY5 is certainly due to defective morphogenesis of the pancreas. Future efforts to further differentiate these hiPSCs into pancreatic β cells will reveal the impact of transcriptional perturbations on β-cell formation and function.

EXPERIMENTAL PROCEDURES

Cell Culture
Informed consent was obtained from MODY5 patients. This study was reviewed and approved by the Institutional Review Boards at Haukeland University Hospital and Joslin Diabetes Center, and in accordance with the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. hiPSCs used were tested mycoplasma negative and were cultured as described previously (Teo et al., 2013b, 2014). hiPSCs were differentiated into pancreatic progenitors as described previously (Teo et al., 2014). In general, 2–3 independent hiPSC lines per subject were used, and biological triplicates were used for each hiPSC line.

Sequencing of MODY5-hiPSCs
The method for sequencing has been described previously (Teo et al., 2013b).
qRT-PCR, Immunostaining, FACS, Western Blot, and ChIP Analyses

Methods for qRT-PCR, immunostaining, FACS, western blot (Teo et al., 2014), and ChIP analyses (Cebolla et al., 2015; Teo et al., 2011, 2015) have been described previously. p < 0.05 indicates statistical significance by Student’s t test (two-sided; equal variance). In general, qRT-PCR and immunostaining data of MODY5-hiPSC-derived cells are representative of one non-family control hiPSC line (IAG16102), two family control hiPSC lines (iN805-6A/C), and three lines each for the two MODY5 patients (iN805-1A/B/C and iN805-2A/B/C). All error bars indicate SD of three biological replicates. Two to three hiPSC lines per subject were pooled together before being set up in triplicate for FACS analyses. Primers and antibodies used are provided in Table S3.

Microarray

Microarray was performed by the Molecular Genetics Core Facility at Children’s Hospital, Boston. Two biological replicates for undifferentiated and day-10 differentiated control hiPSCs were analyzed. One sample from IAG16102, iN805-6, iN805-1, and iN805-2 each was used for day-0, -12 and -17 analyses.

Gene Ontology Overrepresentation Analysis

The overrepresentation of GO biological process categories was assessed using DAVID.

Overexpression Studies

HNF1B gene was amplified from cDNA obtained from differentiated hiPSCs using PfuUltra II Fusion HS DNA Polymerase with a melting temperature (Tm) of 50°C and extension at 72°C for 2 min. HNF1B cDNA was then subcloned into pCDH-FlagV5 vector using EcoRI and XhoI RE sites to obtain pCDH-hHNF1B. HNF1B cDNA was also subcloned into PCR-Blunt II-TOPO vector, and hHNF1B/C443TF and hHNF1B/C443TF primers were used to amplify hHNF1B^S148L using PfuUltra II Fusion HS DNA Polymerase with an extension at 72°C for 5 min. HNF1B^S148L cDNA was then subcloned into pCDH-FlagV5 vector using EcoRI and XhoI RE sites to obtain pCDH-hHNF1B^S148L. pcDNA3.1-hHNF1A was a gift from Y.-I. Chi. Primers used are provided in Table S3. Day-6 differentiated control hiPSCs were split and replated onto 12-well plates. These cells were transfected with hPAX6 promoter in the absence and presence of pCDH-Flag, pCDH-HNF1B, pCDH-HNF1B^S148L, and/or pcDNA3.1-hHNF1A on day 7 and harvested on day 10. The method for luciferase assay has been described previously (Teo et al., 2011).

Hormone Assays

Glucagon was assessed with a radioimmunoassay from Millipore. This assay reports a precision (coefficient of variation) for high levels (72.4 pmol/l) of 10.1%, for intermediate levels (52.2 pmol/l) of 12.6%, and for low levels (28.8 pmol/l) of 9.2%. Prior to analysis, we collected blood samples in plasma tubes containing 250 kIU Trasvlo1 (Aprotinin) per ml of whole blood. This resulted in a final concentration of approximately 500 kIU Trasvlo1 per ml of serum or plasma, and the aliquot was frozen at –70°C.

ACCESSION NUMBERS

Microarray data have been uploaded to GEO with accession number GEO: GSE74885.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.01.007.

AUTHOR CONTRIBUTIONS

A.K.K.T. designed the study, performed most of the experiments, collected and analyzed data, and wrote the paper; H.H.L. performed experiments for manuscript revision; I.A.V. performed FACS analyses; E.D. contributed to discussions; E.T. collected patient data; H.R. contributed to the design of the study and to data interpretation; R.N.K. contributed to conceptual discussions, supervised the studies, and edited and approved the paper. All authors discussed the results and commented on the manuscript.

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REFERENCES


References


Supplemental Information

Early Developmental Perturbations in a Human Stem Cell Model of MODY5/HNF1B Pancreatic Hypoplasia

Adrian Kee Keong Teo, Hwee Hui Lau, Ivan Achel Valdez, Ercument Dirice, Erling Tjora, Helge Raeder, and Rohit N. Kulkarni
SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURE LEGENDS

“Figure S1. Establishment of a stem cell model for MODY5, Related to Figure 1”

(A) S148L (C443T in exon 2) mutation (or lack thereof) in HNF1B has been verified in iN805 hiPSCs at least thrice. (B) Expression of pancreas-related (NEUROD1, HNF6, DLK1, SOX4 and MAFB) markers in hiPSCs differentiated for 17 days. All error bars indicate standard deviation of three biological replicates in an independent experiment. (C) Immunostaining for HNF1B, PAX6, ISL1, SOX9 and PDX1 on hiPSCs differentiated for 12 days. Scale bar: 200 μm. (D) The percentage of PDX1⁺, SOX9⁺, HNF1B⁺, HNF1A⁺ and PTF1A⁺ cells after differentiation of hiPSCs for 17 days.

“Figure S2. Transcriptional perturbations in MODY5-hiPSC-derived pancreatic progenitors, Related to Figures 1, 2 and 3” (A) Gene ontology (GO) analyses of differentially up- and down-regulated genes (fold change >2) in control hiPSCs differentiated for 10 days. (B) Gene expression of SOX17, FOXA2, ISL1, HLXB9, SOX9 and RFX6 in non-family control and iN805 hiPSCs differentiated for 0, 12, 14 and 17 days. Data are representative of at least 2 independent hiPSC lines per subject. The experiment was replicated at least twice. All error bars indicate standard deviation of three biological replicates in an independent experiment. (C) Immunostaining for HNF1B, SOX9 and ISL1 on iN805 hiPSCs differentiated for 12 days. Data are
representative of at least 2 independent hiPSC lines per subject. The experiment was replicated at least twice. Scale bar: 200 μm.

“Figure S3. Further characterization of pancreatic differentiation model for MODY5-hiPSCs, Related to Figures 2 and 3” (A) Gene expression heat map of representative pancreas-related genes in undifferentiated (first four columns), Day 12 differentiated (middle four columns) and Day 17 differentiated (last four columns) control and iN805 hiPSCs (up-regulation in red, down-regulation in blue). The colors in the heat map depict gene expression in units of standard deviation from the mean across all samples. GO analyses of differentially up- and down-regulated genes (fold change >2) in iN805 versus control cells on (B) Day 12 or (C) Day 17. (D) ChIP demonstrates that HNF1B is bound onto genomic loci of HLXB9 and HNF1B on Day 12. (E) Cell count was performed on control and iN805 hiPSCs differentiated for 17 days. All error bars indicate standard deviation of three biological replicates in an independent experiment.

“Figure S4. HNF1B has no impact on HNF4A or SOX9 transcription, Related to Figure 4” (A) Luciferase assay showing the effect of HNF1B overexpression on HNF4A transcriptional activity in hiPSCs differentiated for 12 days. Expression of SOX9 in hiPSCs differentiated for 10 days and overexpressing increasing amounts of (B) mutant HNF1B<sup>S148L</sup> or (C) HNF1B from Day 7-10. Control experiments demonstrating that the overexpression of (D) HNF1B or (E) HNF1A is successful. Experiments were replicated
at least twice. All error bars indicate standard deviation of three biological replicates in an independent experiment.
SUPPLEMENTAL TABLE LEGENDS

“Table S1. Microarray performed on undifferentiated and Day 10 differentiated control-hiPSCs, Related to Figure 1”

“Table S2. Microarray performed on undifferentiated, Day 12 and Day 17 differentiated control and iN805 hiPSCs, Related to Figures 2 and 3”

“Table S3. Primers and antibodies used, Related to Figures 1, 2, 3, 4 and 5”
Figure S1: Teo et al.,

A

**MODY5 (HNF1B) (S148L)**

- iN805-6B
  - CAG TCG CAC CAG TGG CAC
  - TCG to TTG

- iN805-1B
  - CAG TCG CAC CAG TGG CAC
  - TCG to TTG

- iN805-6C
  - CAG TCG CAC CAG TGG CAC
  - TCG to TTG

- iN805-1C
  - CAG TCG CAC CAG TGG CAC
  - TCG to TTG

- iN805-2B
  - CAG TCG CAC CAG TGG CAC
  - TCG to TTG

- iN805-2C
  - CAG TCG CAC CAG TGG CAC
  - TCG to TTG

B

- **NEUROD1**
- **HNF6**
- **DLK1**
- **SOX4**
- **MAFB**

C

Day 12

- HNF1B
- PAX6
- ISL1
- SOX9
- PDX1

- DAPI
- DAPI
- DAPI
- DAPI
- DAPI

D

Day 17

- Rabbit 2° Control
- Rabbit PDX1
- Rabbit SOX9
- Guinea Pig 2° Control
- Guinea Pig PDX1

- Goat 2° Control
- Goat HNF1B
- Goat HNF1A
- Rabbit 2° Control
- Rabbit PTF1A
Figure S2: Teo et al.,

A

Up-regulated on Day 10 (FC >2)

- Cellular developmental process
- Regionalization
- Pattern specification process
- Chordate embryonic development
- Embryonic development ending
- Multicellular organismal process
- Organ morphogenesis
- Organ development
- Anatomical structure
- System development
- Developmental process
- Multicellular organismal process
- Anatomical structure morphogenesis

Down-regulated on Day 10 (FC >2)

- Negative regulation of cellular process
- Blood vessel development
- Response to organic substance
- Regulation of signal transduction
- Vasculature development
- Negative regulation of biological process
- Anatomical structure formation
- Regulation of biological quality
- Anatomical structure development
- System development
- Response to chemical stimulus
- Multicellular organismal process
- Developmental process

B

- SOX17
- ISL1
- SOX9
- FOXA2
- HLXB9
- RFX6

C

Day 12

iN805-6 Control
HNF1B
SOX9
MERGE
DAPI

iN805-1 Mut+ Dia-
HNF1B
SOX9
MERGE
DAPI

iN805-2 Mut+ Dia+
HNF1B
SOX9
MERGE
DAPI
Figure S3: Teo et al., 1E-15 to 1E-09

A

B

Up-regulated in MODY5 on Day 12 (FC > 2)

- Cell development
- Generation of neurons
- Positive regulation of biological process
- Nervous system development
- Regulation of cell proliferation
- Positive regulation of cellular process
- Tissue development
- Heart development
- Organ morphogenesis
- Developmental process
- Multicellular organismal process
- Anatomical structure morphogenesis
- Multicellular organismal process
- System development
- Anatomical structure development
- System development

Down-regulated in MODY5 on Day 12 (FC > 2)

- Chordate embryonic development
- Neurogenesis
- Tissue development
- Generation of neurons
- Organ morphogenesis
- Developmental process
- Cellular developmental process
- Cell differentiation
- Nervous system development
- Multicellular organismal process
- Anatomical structure morphogenesis
- System development
- Developmental process
- Anatomical structure development
- Multicellular organismal process

C

Up-regulated in MODY5 on Day 17 (FC > 2)

- Nervous system development
- Cell development
- Gland development
- Regulation of multicellular organismal process
- Heart morphogenesis
- Organ morphogenesis
- Tissue development
- Multicellular organismal process
- Heart development
- Organ development
- Developmental process
- System development
- Developmental process
- Multicellular organismal process
- Anatomical structure development

Down-regulated in MODY5 on Day 17 (FC > 2)

- Regulation of neurogenesis
- Regulation of nervous system development
- Cell differentiation
- Organ morphogenesis
- Embryonic morphogenesis
- Neurogenesis
- Generation of neurons
- Regulation of neuron differentiation
- Anatomical structure morphogenesis
- Developmental process
- Anatomical structure development
- Multicellular organismal process
- Nervous system development
- Multicellular organismal process
- System development

D

HLXB9 genomic locus

HNF1B genomic locus

Relative expression

Control

Mut+ Dia-

Mut+ Dia+

E

Cell count during differentiation

Fold change relative to Day 0

Control

MODY5

P = 0.058

Day 0

Day 5

Day 12

Day 17

1E-08

1E-07

1E-06

1E-05

1E-04

1E-03

1E-02

1E-01

1E-00
Figure S4: Teo et al.,

A

Day 12

HNF1B has no impact on HNF4A transcriptional activity

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<th>HNF4A P1 promoter</th>
<th>HNF4A P2 promoter</th>
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<td>OE HNF1B</td>
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B

Day 10

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<tr>
<td>1x mut HNF1B</td>
</tr>
<tr>
<td>2x mut HNF1B</td>
</tr>
<tr>
<td>3x mut HNF1B</td>
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C

Day 10

<table>
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<th>Relative expression (fold over control vector)</th>
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<tr>
<td>GFP</td>
</tr>
<tr>
<td>1x HNF1B</td>
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<tr>
<td>2x HNF1B</td>
</tr>
<tr>
<td>3x HNF1B</td>
</tr>
</tbody>
</table>

D

HNF1B

ACTIN

PCDH-GFP
PCDH- HNF1B
PCDH-HNF1B

E

HNF1A

ACTIN

PCDNA3.1
PCDNA3.1-HNF1A
PCDNA3.1-HNF1A

HNF1B

Relative expression

PCDH-GFP
PCDH-HNF1B

HNF1A

Relative expression

PCDNA3.1
PCDNA3.1-HNF1A