Characterization of ALDH Positive Cells in the Human Fetal Pancreas

Amanda Oakie
*The University of Western Ontario*

**Supervisor**
Dr. Rennian Wang
*The University of Western Ontario*

**Graduate Program in Physiology and Pharmacology**

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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CHARACTERIZATION OF ALDH POSITIVE CELLS IN THE HUMAN FETAL PANCREAS

(Thesis format: Monograph)

by

Amanda Oakie

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Aldehyde dehydrogenase (ALDH) is an enzyme that regulates differentiation during pancreatic development, but determining the role of ALDH during endocrine differentiation is not well defined. The purpose of this study was to characterize transcription factors (TFs) and putative stem cell surface markers in ALDH^{hi} or ALDH^{lo} populations of the human fetal pancreas (18-22 weeks of age), and examine their expansion and differentiation in vitro and in vivo. Cell surface marker analysis demonstrated high co-localization of the stem cell marker CD133 with ALDH^{hi} cells. ALDH^{hi} cells also contained higher expression of islet-associated TFs and insulin. Expansion of sorted ALDH^{hi} cells resulted in down-regulation of endocrine TF genes, yet differentiated ALDH^{hi} islet-like clusters partially restored TF mRNA, but not protein, levels. Sorted cells transplanted into nude mice showed increased vascularization in ALDH^{lo} fibrin gels. In summary, this study demonstrated that ALDH^{hi} cells represent an endocrine progenitor pool important for β-cell differentiation.

Key Words: Aldehyde dehydrogenase, CD133, β-cell development, FACS, human fetal pancreas
Co-Authorship Statement

The methods described in Chapter 2 of this thesis were conducted by Amanda Oakie with contributions from other lab members. Dr. Rennian Wang and Dr. Fraser Fellows provided sample collection and preparation of pancreatic tissue. Dissociation of pancreatic sample to single cells for FACS, design of real-time RT-PCR primers, and technical support within the lab, including cDNA preparation and guidance for extended cell culture, were conducted by Jinming Li. FACS was performed by Dr. Kristin Chadwick of the London Regional Flow Cytometry Facility. Dr. Matthew Riopel performed surgery for the transplantation of fibrin matrix gels into nude mice. Editing of this thesis was conducted by Dr. Rennian Wang and Dr. David Hess.
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A special thank you to Dr. Kristin Chadwick for her advice and assistance during the cell sorting portion of this project. I would also like to thank the Hess lab for access to their lab space and equipment during cell sorting, and thanks to Gillian Bell for initial training and assistance regarding preparation for cell sorting.

I would like to thank my advisory committee, Dr. Jane Rylett, Dr. Dean Betts, and Dr. David Hess, for their suggestions and guidance over the years. Special thanks to Dr. Hess for volunteering to review my thesis and providing me with advice on my project.

I would like to thank and dedicate this thesis to my family. To my father, who encouraged me to pursue my passions, and to my mother, who is always there to talk with when I need advice. Thank you to my sister Jessica, whose wit and maturity will always surpass my own. A big thanks to my grandmothers, aunts, uncles, and cousins, who all encouraged me to continue down my academic path, but none more than my cousin Tina. Thanks to two of the best friends I could have asked for, Ana and AY. Finally, a big thanks to my canine companions Riley and Molly.

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</tr>
</thead>
<tbody>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>BAAA</td>
<td>BODIPY- aminoacetaldehyde</td>
</tr>
<tr>
<td>BAA</td>
<td>BODIPY- aminoacetate</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CK19</td>
<td>Cytokeratin 19</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle Threshold</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEAB</td>
<td>Diethylaminobenzaldehyde</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence- activated cell sorting</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter plot</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorophore Minus One</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cell</td>
</tr>
<tr>
<td>ISL1</td>
<td>Islet 1</td>
</tr>
<tr>
<td>mESC</td>
<td>Murine embryonic stem cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NGN3</td>
<td>Neurogenin 3</td>
</tr>
<tr>
<td>NKX2.2</td>
<td>NK2 homeobox 2</td>
</tr>
<tr>
<td>NKX6.1</td>
<td>NK6 homeobox 1</td>
</tr>
<tr>
<td>PAX4</td>
<td>Paired box gene 4</td>
</tr>
<tr>
<td>PAX6</td>
<td>Paired box gene 6</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDX-1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PSC</td>
<td>Pancreatic stellate cell</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RALDH</td>
<td>Retinaldehyde dehydrogenase</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SOX9</td>
<td>(Sex determining region Y)- box 9</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter plot</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethyl rhodamine isothiocyanate</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7- Aminoactinomycin D</td>
</tr>
<tr>
<td>9-cis RA</td>
<td>9-cis retinoic acid</td>
</tr>
</tbody>
</table>
Chapter 1 – Introduction

1.1 Significance of Study

The transplantation of functioning and glucose-sensitive β-cells into type I diabetic patients has been extensively examined for the restoration of endogenous insulin secretion and function. Although limited success is seen with transplanting existing donor islets (Ryan et al. 2002), recent interest has been invested in directed differentiation of uncommitted stem and progenitor cells towards functioning endocrine cells (D’Amour et al. 2006). Successfully producing cells that secrete insulin in response to blood glucose fluctuations requires the detailed examination of factors that give rise to β-cells in vivo. Once these developmental endocrine precursors have been identified and isolated, they can be expanded and matured in vitro for further analysis regarding their suitability for transplantation.

The work examined in this thesis provides insight into a putative stem cell marker, ALDH, that has been previously characterized in the developing human pancreas, and shown to influence endocrine cell development during the 1st and 2nd trimesters of pregnancy (Li et al. 2014). Expression of endocrine-committing transcription factors during development are also necessary, so the overlap of transcription factors within cells expressing putative stem markers needs to be determined in order to establish their suitability for further research addressing β-cell generation. This project contributes to the field of β-cell development by implementing fluorescence-activated cell sorting (FACS) for the separation of distinct putative stem cell populations within the human fetal pancreas. Furthermore, sorted cells are characterized and analyzed both in vitro and in vivo to determine the functions of the isolated populations.

1.2 Overview of the pancreas and diabetes mellitus

The pancreas is a secretory organ of the digestive system that contains two distinctive functioning units: the exocrine tissue (~98% of the total pancreas) and endocrine tissue (~2% of the pancreas). The exocrine portion is composed of acinar cells, which produce
and release digestive enzymes, and ductal cells, responsible for transporting these enzymes to the duodenum for the breakdown of the bolus. The endocrine cells are located within clusters known as the islets of Langerhans, and are heavily vascularized to allow for the release of glucose-regulating hormones into the blood stream. Islets found within the pancreas are composed of the following cell types and corresponding secreted hormones: α-cells (glucagon), β-cells (insulin), δ-cells (somatostatin), γ or PP cells (pancreatic polypeptide, or PP), and ε-cells (ghrelin). Human and murine islets differ in the morphology and composition of the islets; while murine islets have a higher percentage of β-cells that cluster in the center of the islet, human islets have higher contact between α-cells and β-cells (Brissova et al. 2005, Cabrera et al. 2006). Insulin production and secretion is critical for lowering glucose in circulation via a biphasic secretion response from the β-cells. Problems encountered with the reuptake of glucose, regulated by the glucose transporter class (GLUT) (Wood and Trayhurn. 2003), and production or secretion of insulin is categorized as diabetes mellitus. This disease manifests itself as two common diagnoses, type I or type II, based on the pathology of the disease. Type I diabetes is caused by autoimmune destruction of up to 90% of β-cells in the pancreas, and patients require an exogenous source of insulin to meet metabolic demands and avoid ketoacidosis (Shapiro et al. 2000). Type II diabetes is generally associated with insulin resistance across tissues accompanied by insulin dysfunction in β-cells (Masini et al. 2012), resulting in the inability to clear glucose from the circulating blood stream. These patients are typically able to improve glucose levels by exercise and diet changes.

In order to restore endogenous insulin production and secretion in type I diabetic patients, the Edmonton Protocol has been designed (Shapiro et al. 2000). This involves the transplantation of isolated islets from donor pancreata into the hepatic portal vein of immunosuppressed patients. Although these patients experience exogenous insulin dependence for the first year after these procedures, a low supply of cadaveric donors and transplantation complications due to aged islets from donors (Street et al. 2004) limit the use of this technique. Because of this, there is interest in investigating the formation of fully differentiated islets from programmed undifferentiated cells for the treatment of type I diabetic patients. By generating fully differentiated islets from a source that can
yield a large number of cells, it can be possible to correct the complications seen using cadaveric islets. In order to understand the developmental pathway of endocrine cells in the pancreas, the architecture of islets *in vivo* and specific factors that give rise to hormone-producing islet cells must be determined.

### 1.3 Development of the human fetal pancreas

The architecture of the human pancreas changes dramatically during the course of development. Fetal age, which is assigned two weeks later than the gestational age (marked at the beginning of the previous menstrual cycle), will be used throughout this section to describe pancreatic development. The pancreas first develops between 3 to 4 weeks of fetal age as two separate buds off of the duodenum, labeled the dorsal pancreatic bud and the ventral pancreatic bud (Piper et al. 2004, Jennings et al. 2013). The dorsal and ventral pancreatic buds fuse at approximately 7 to 8 weeks of fetal age to form the single immature pancreas (Piper et al. 2004), and the dorsal duct is normally lost in favour of the ventral pancreatic duct (Kamisawa. 2004). The pancreatic composition at 7 to 8 weeks of fetal age contains a high percentage of mesenchymal cells, with simple ductal structures and interspersed epithelial clusters (containing emerging endocrine cells) scattered throughout the mesenchyme (Lyttle et al. 2008, Sarkar et al. 2008). The ductal cell marker cytokeratin 19 (CK19) is also expressed throughout the epithelial cell clusters early in development before expression is restricted to duct-like structures (Sarkar et al. 2008). Around this time, insulin$^+$ cells are detected in the pancreas, and this is followed with glucagon$^+$ cells emerging at 7 to 8.5 weeks of fetal age (Piper et al. 2004, Jeon et al. 2009). Somatostatin$^+$ cells are seen as early as 8 to 8.5 weeks of fetal age, while PP$^+$ cells do not emerge until 10 weeks of fetal age (Piper et al. 2004, Jeon et al. 2009). Ghrelin$^+$ single cells are present in the pancreas at 11 weeks of fetal age (Andralojc et al. 2009). The grouping of insulin$^+$ and glucagon$^+$ cells close to one another is first reported at 10 weeks of fetal age, but prominent endocrine clustering of insulin$^+$ and glucagon$^+$ cells with somatostatin$^+$ and PP$^+$ cells is not observed until 15 weeks of fetal age (Jeon et al. 2009). Ghrelin$^+$ cells are not observed in the islets until 19 weeks of fetal age (Andralojc et al. 2009). These developing islets are found close to the ductal structures, and CK19 expression decreases in islets as they mature; CK19 staining is
weak in islets from 16 to 22 weeks of fetal age, and islets are completely negative for CK19 from 22 to 39 weeks of fetal age (Bouwens et al. 1997). Beginning at 8 weeks of fetal age, staining for vasculature could be seen in proximity to the forming endocrine cell clusters (Sarkar et al. 2008). These vessels invade islet clusters around 12 weeks of fetal age (Piper et al. 2004), which is necessary for hormone transport in mature islets. CEL, an indicator of early acinar cells, first appears in the epithelium at 9 weeks of fetal age (Sarkar et al. 2008). Lobular cell structures form during 12 to 18 weeks of fetal age (Adda et al. 1984), yet staining for amylase (an acinar secretory enzyme) emerges later on in the 2nd trimester of pregnancy (Sarkar et al. 2008). The weight of the human fetal pancreas increases to approximately 337 mg at 21 weeks of fetal age (compared to 9 mg at 8 weeks of fetal age), and the masses of islet cells, composed primarily of α-cells and β-cells, express transcription factors indicative of maturing islets (low NGN3 and high co-localization of PDX-1 and Insulin) (Lyttle et al. 2008).

1.4 TFs associated with endocrine cell differentiation

Successful commitment to islet and β-cell fate requires sequential expression of transcription factors during development of the human pancreas (Figure 1.1) (Lyttle et al. 2008). For endocrine cell commitment (and specifically β-cell fate), endoderm cells first up-regulate transcription factors necessary for pancreas development. PTF1α and PDX-1 are early pancreatic progenitor transcription factors expressed in the developing pancreatic buds and are required for pancreatic development from the duodenum (Offield et al. 1996, Kawaguchi et al. 2002, Burlison et al. 2008). SOX9, another pancreatic progenitor factor found during early development, maintains the progenitor cell pool during development and plays a prominent role during cell lineage determination via Notch activity (Lynn et al. 2007, McDonald et al. 2009, Shih et al. 2012). The cell type that develops from the pancreatic epithelium relies on activation of the Notch receptor, which increases the precursor transcription factor HES-1 (Apelqvist et al. 1999, Kopinke et al. 2011). HES-1 inhibits NGN3, the endocrine precursor transcription factor, during early stages of development (Jensen et al. 2000a and 2000b, Gu et al. 2002, Fujikura et al. 2006), but decreased Notch activation later on in development leads to expression of NGN3 (Shih et al. 2012). Once cells are committed to endocrine fate, there is further
regulation of transcription factor downstream of NGN3 required for islet cell commitment and function, including PAX6 (Sander et al. 1997), ISL1 (Du et al. 2009), and NKX6.1 (Schaffer et al. 2013). A detailed description of important transcription factors examined throughout this project is provided below.

1.4.1 SOX9

SRY (sex determining region Y)-box 9 (SOX9) is part of the high-mobility binding group domain family, and is recognized as a pancreatic progenitor transcription factor, as well as a marker of small intestine progenitors (Formeister et al. 2009, Belo et al. 2013). It is found upstream of key transcription factors responsible for commitment to pancreatic cell fate, and contributes to the progenitor pool during pancreatic development (Seymour et al. 2007 and 2012). In the developing human pancreas, strong SOX9 staining is first present in the immature pancreatic buds 32 days after conception (between 4 to 5 weeks of fetal age); however SOX9 decreases with age, with a noticeable drop at 17 to 18 weeks of fetal age (McDonald et al. 2012). Although inactivating Sox9 expression in the pancreas reduces Pdx-1 expression levels, pancreatic tissue still develops (Seymour et al. 2007, Dubois et al. 2011), indicating that multiple TFs are responsible for pancreas organogenesis. Co-localization of SOX9 with NGN3+, insulin+, and glucagon+ cells is seen at 8 to 11 weeks of fetal age, but a dramatic loss of SOX9 in these endocrine cells is observed at 20 to 21 weeks of fetal age (McDonald et al. 2012), indicating that SOX9 contributes to endocrine cell formation during the early stages of fetal development. At later stages of pancreatic development (20 to 21 weeks of fetal age in human), SOX9+ cells are primarily found in the pancreatic ducts (Shih et al. 2012, McDonald et al. 2012). This observation correlated with examined adult murine pancreata 8 weeks after birth, where Sox9 is retained in ductal cells (Furuyama et al. 2011). Sox9 also contributes to the exocrine compartment, as seen in the developing murine pancreas (Kopp et al. 2011), indicating that this TF is not restricted to a specific pancreatic cell lineage during development. Campomelic dysplasia (CD) patients, diagnosed with SOX9
Figure 1.1 Pathway of transcription factor activation for β-cell induction. The diagram above is a simplified pathway of β-cell differentiation that focuses on key TF expression mentioned in this thesis. The information in this figure is modified from results found in Lyttle et al. (2008), McDonald et al. (2012), and Riedel et al. (2012).
haploinsufficiency, demonstrate reduced islet cell size and dysfunction, with no observable effect on the surrounding exocrine tissue (Piper et al. 2002). Therefore, dose dependent studies have been conducted to focus on this observed effect. Developing murine pancreata with a ~50% decrease in Sox9 expression supported what is seen in CD patients, with normal pancreatic organogenesis but reduced islet size and number of endocrine cell precursors (Seymour et al. 2008). This is further supported by the siRNA knockdown of SOX9 gene in human fetal islet epithelial cell clusters (~50% reduction in SOX9), where a significant reduction of NGN3 and Insulin mRNA expression was observed (McDonald et al. 2012). Overall, these studies indicate that SOX9 is essential for maintaining the pancreatic progenitor pool and important for regulating the early development of endocrine islet cells.

1.4.2 PDX-1

Pancreatic and duodenal homeobox 1 (PDX-1) is a transcription factor that is part of the homeodomain-containing family (Kaneto et al. 2008) and regulates the cell fate of pancreatic and duodenal cells (Burlison et al. 2008). Although the exocrine transcription factor Ptf1a is important for endoderm commitment to the pancreatic fate, PDX-1 has been identified as an important transcription factor for endocrine cell development (Burlison et al. 2008, Lyttle et al. 2008). It first appears in the developing human pancreas at approximately 4 weeks of fetal age along the foregut area that is reported as the precursor to the mature duodenum and pancreas (Jennings et al. 2013). PDX-1 is also found after the dorsal and ventral buds fuse in the developing human pancreas at 7 weeks of fetal age (Lyttle et al., 2008, Sarkar et al. 2008). The endocrine, acinar, and ductal cells of the pancreas all arise from Pdx-1+ cells (Gu et al. 2002), emphasizing the important role that Pdx-1 plays during normal murine pancreas development. PDX-1 expression is also necessary in human embryonic stem cells in order to successfully produce endocrine cells (Johannesson et al. 2009). Furthermore, Pdx-1 expression is seen earlier than Ngn3 expression (Villasenor et al. 2008), indicating its upstream effects on endocrine cell differentiation. However, PDX-1 and NGN3 co-localization decreases with age in the developing fetal pancreas (Lyttle et al. 2008), which could represent a progenitor pool becoming committed to the endocrine cell fate. In addition, PDX-1 co-localization
decreases in CK19\(^+\) cells, increases in insulin\(^+\) and somatostatin\(^+\) cells, and is not present in committed glucagon\(^+\) cells during human fetal development (Lyttle et al. 2008), demonstrating specificity of this transcription factor towards distinct endocrine cell fate in later stages of development.

### 1.4.3 NGN3

Neurogenin 3 (NGN3) is a basic helix-loop-helix transcription factor (Jensen et al. 2000b) that commits pancreatic cells to the endocrine islet precursor cell fate (Gu et al. 2002, Gradwohl et al. 2000). It is downstream of the Notch receptor, which under normal circumstance activates the exocrine-promoting transcription factor HES-1 and inhibits NGN3 expression (Dror et al. 2007). In early stages of human fetal pancreatic development (8 to 11 weeks of fetal age), NGN3\(^+\) cells co-localize frequently with PDX-1\(^+\), insulin\(^+\), and glucagon\(^+\) cells; this co-expression decreases in pancreata at later stages of development (20 to 21 weeks of fetal age) (Lyttle et al. 2008), suggesting an important role for this factor during early initial endocrine commitment. However, recent information from human patients with NGN3 biallelic deletions reported that patients still produced insulin (Rubio-Cabezas et al. 2014). This indicates that there is an alternative differentiation pathway for insulin-secreting cells that does not require NGN3 expression early on in human development. Ngn3\(^+\) cells in the developing murine pancreas have been shown to co-localize with Sox9\(^+\) ductal cells, demonstrating an endocrine precursor rich area during development (Lynn et al. 2007). In addition, murine development studies have identified that Ngn3 is downstream of Pdx-1 (Oliver-Krasinski et al. 2009), indicating that endocrine cells emerge after initial pancreatic commitment. Further experiments in murine pancreata confirm a lack of Ngn3 expression in acinar cells during development, and that Ngn3 was absent from the pancreas of adult mice (Gradwohl et al. 2000).

### 1.4.4 NKX2.2 and NKX6.1

NK2 homeobox 2 (NKX2.2) is a transcription factor that contributes to endocrine cell fate. Nkx2.2 is seen throughout the dorsal pancreatic bud in early stage murine embryos,
but becomes restricted to the endocrine cells in later stage embryos (Sussel et al. 1998). Murine pancreata analyzed right before birth show that there is high co-localization of Nkx2.2 with insulin\(^+\) and glucagon\(^+\) cells, but not somatostatin\(^+\) cells (Sussel et al. 1998), suggesting that Nkx2.2 activity is only necessary for specific endocrine cell development. Unlike Ngn3, Nkx2.2 is still found within the endocrine cells of the islets in adult murine pancreata (Sussel et al. 1998), indicating that this transcription factor could be used to isolate certain mature islet cells. In addition, Nkx2.2 affects β-cell functionality and insulin secretion in adult murine pancreata (Doyle and Sussel 2007).

NK6 homeobox 1 (NKX6.1) is another transcription factor of the same homeobox gene group that is specifically associated with β-cell commitment (Sander et al. 2000). Research in the embryonic murine pancreas shows that early on in development (e10.5), Nkx6.1 is found in multipotent cells, and co-localizes with the acinar-committing TF Ptf1α (Schaffer et al. 2010), before becoming confined to the endocrine-producing area of the pancreas along the ductal cells after birth (Schaffer et al. 2010, Schaffer et al. 2013). Murine studies have demonstrated that Nkx6.1 is necessary for ensuring β-cell fate during development, but not other endocrine cell fates, and is not solely sufficient for β-cell development (Schaffer et al. 2013). Similar to Nkx2.2, Nkx6.1 is present throughout the developing pancreas in early stage murine embryos, but becomes restricted to insulin\(^+\) and ductal cells in later stages (Sander et al. 2000), before finally becoming β-cell restricted (Schaffer et al. 2013). Parallel studies in human fetal pancreata found that NKX6.1 co-localizes with insulin\(^+\) cells as early as 10 weeks of fetal age, with adult pancreata only displaying NKX6.1 co-localization in β-cells (Lyttle et al. 2008, Riedel et al. 2012).

1.4.5 ISL1

Islet 1 (ISL1) is a transcription factor in the LIM homeodomain family (Ahlgren et al. 1997) that has been linked to both mesenchymal and progenitor cells of the pancreas (Eberhardt et al. 2006). Isl1 cell expression affects all endocrine cells in the pancreas (Eberhardt et al. 2006, Andralojc et al. 2009). Embryonic mice with a mutation of Isl1 did not develop mesenchyme in the dorsal portion of the pancreas, and the isolated tissue
could not produce any of the islet endocrine cells from the epithelial buds (Ahlgren et al. 1997). Additionally, Isl1 is present in islet cells of the adult pancreas and have been shown to increase proliferation and maintain glucose tolerance in β-cells (Guo et al. 2011, Ediger et al. 2014), revealing that Isl1 modulates islet cells in the adult pancreas and is not restricted to pancreatic development. Mutations in Isl1 expression reduces Pax6+ cells in the postnatal murine pancreas, and is responsible for regulating the transcription of MafA, another transcription factor needed to establish proper glucose sensing in β-cells (Du et al. 2009).

### 1.4.6 PAX4 and PAX6

Paired box gene 4 (PAX4) is a transcription factor that, although not analyzed in this project, is heavily associated with β-cell development. It is directly antagonistic to the α-cell transcription factor Arx (Collombat et al. 2003 and 2005), and has been demonstrated to increase insulin+ cells during differentiation of mESCs programmed with vectors containing that specific gene (Błyszczuk et al. 2003). Additionally, PAX4 overexpression in adult rat islets resulted in increased β-cell proliferation (Brun et al. 2004).

Unlike PAX4, Paired box gene 6 (PAX6) is a transcription factor that is associated with insulin+ and glucagon+ cells during early endocrine cell development (Sander et al. 1997). However, studies of adult murine pancreata demonstrate that Pax6+ cells are found in all endocrine cell types and regulates via their hormone gene promoters (Sander et al. 1997). Pax6 mutant mice do not develop glucagon+ cells and display abnormal islets (St-Onge et al. 1997), suggesting that PAX6 is important for α-cell development and proper islet cell formation.

### 1.5 Pancreatic stem cells and endocrine cell differentiation

The production of new endocrine cells in the pancreas is a debated topic, with evidence of new adult β-cells arising from replication instead of stem cells as a possible source (Dor et al. 2004). Therefore, establishing stem cell sources within the pancreas is important for further understanding islet cell repopulation. Uncommitted progenitor cell pools are present during pancreatic development and organ maturation, while possible
pools of stem cells have been reported throughout the adult pancreas. This section will focus on the developing and adult human pancreas, and will discuss stem cell contributions to specific pancreatic cell fate.

1.5.1 Stem cell sources in the embryonic and fetal pancreas

The differentiation of immature cells in the fetal pancreas has been studied extensively to explain the relationship of precursor cells during endocrine cell commitment. Precursor cells are important for the overall development of the pancreas, as seen in the reduction of Pdx-1\(^+\) multipotent cells during murine development (Stanger et al. 2007). Since the maturation of the pancreatic tissue occurs throughout organogenesis, pancreatic cells readily express transcription factors that mark an uncommitted phenotype, including SOX9, PDX-1, and NKX6.1 (Lyttle et al. 2008, McDonald et al. 2012, Pan and Brissova. 2014); this indicates additional multipotency throughout the developing pancreas than what is seen in the adult structures. Nevertheless, examining the fetal pancreas for known stem cell markers (discussed in the following sections) assists with precursor identification.

Although in vitro stem cells sources do not capture in vivo pancreatic organogenesis, the use of embryonic stem cells, both human (hESCs) and murine (mESCs), are important for determining transcription factors and markers that link commitment of the developing pancreas to specific cell function (Kelly et al. 2011). Committing ESCs to definitive endoderm, characterized by the successful expression of Pdx-1, allows for accessible programming of this layer towards different cell fates in vitro (Micallef et al. 2005, D’Amour et al. 2006, Micallef et al. 2007). Further treatments of Pdx-1\(^+\) murine and human ESCs with stage-dependent protocols, designed to guide undifferentiated cells towards a committed pancreatic cell fate, have resulted in the production of endocrine cells in vitro (Jiang et al. 2007, Micallef et al. 2007). Additionally, hESCs geared towards β-cell fate and composed of a high population of NKX6.1\(^+\) cells are able to mature into insulin-secreting cells in vivo and alleviate diabetic symptoms when transplanted into STZ mice (Rezania et al. 2013), indicating the functional applications of this stem cell source.
1.5.2 Stem cell sources in the adult pancreas

The adult pancreas is a fully matured organ with functioning differentiated cells, though it has been noted that progenitor sites that contribute to endocrine cell development during fetal organogenesis lose their ability in the adult pancreas (Solar et al. 2009, Kopp et al. 2011). As a result, the isolation of cells with progenitor features from this source has been thoroughly examined to determine if there is an undifferentiated population that contributes towards the islet cell population. Indeed, adult murine islet and ductal cells were able to separately form colonies of pancreas-derived multipotent cell clusters (Seaberg et al. 2004), showing that there is potential for restoring undifferentiated cells in committed lineages. One identified bulk source is the epithelial compartment of the pancreas (Hao et al. 2006). Successful isolation of epithelial cells from islet and mesenchymal cells resulted in the ability to use this population in vitro as a stem cell source for endocrine cell production (Hao et al. 2006). In contrast, mesenchymal cells isolated from pancreatic islets expressed ISL1 and were able to form c-peptide-producing cell clusters under differentiation conditions, indicating that these cells are a pancreatic source capable of producing endocrine cells (Eberhardt et al. 2006). The ductal epithelium of the adult pancreas, identified as a progenitor pool in fetal pancreata, may also represent another source that contributes to new islets (Gao et al. 2003). Cells that exhibit stem cell traits have been previously isolated from isolated adult human pancreatic ducts and demonstrated endocrine cell lineage in vitro (Lin et al. 2006). Furthermore, adult patients suffering from nesidioblastosis are able to generate insulin$^+$ cells from pancreatic ductal cells (Gmyr et al. 2000), demonstrating lineage plasticity and the potential for β-cell production in vivo. Isolated adult murine ductal cells also display multipotency when cultured, and demonstrated endocrine transcription factors as well as guided islet differentiation (Ramiya et al. 2000). Finally, research focused on in vivo pancreatic duct ligation has been used to examine its effect on endocrine cell production during pancreatic injury, with supporting (Wang et al. 1995, Van de Casteele et al. 2014) and contrasting (Kopp et al. 2011, Rankin et al. 2013) evidence regarding the stimulation of β-cell neogenesis. Ligation drastically amplifies the presence of cells positive for the stem cell marker c-Kit (CD117) within the ducts (Peters et al. 2005), revealing that
progenitor cells can be stimulated within this tissue source. Furthermore, pancreatectomies in adult rats that had the pancreatic tail and most of the pancreatic head removed led to regeneration of the missing tissue via the ductal epithelium, again suggesting that the ducts contain progenitor cells capable of endocrine repair (Bonner-Weir et al. 1993). The acinar cells from the exocrine portion of the pancreas have also demonstrated precursor features, as seen during the transdifferentiation of acinar-to-ductal cells \textit{in vitro} and the subsequent appearance of PDX-1, necessary for the early stages of islet commitment (Rooman et al. 2000). An additional cell source that has been reported is the pancreatic stellate cell (PSC) (Kordes et al. 2012, Lonardo et al. 2012), which can be located throughout the pancreas around the ductal cells (Apte et al. 2012), and can also be isolated from pancreatic tumours (Lonardo et al. 2012).

Identifying multipotent cells within the developing pancreas is ideal for understanding the organogenesis of hormone-producing islet cells and can be utilized for reprogramming stem cells from multiple sources into \(\beta\)-cells. For the proper isolation of potential pancreatic islet progenitor cells that could be differentiate into islet cells, select surface stem cell markers must also be established.

1.6 Surface stem cell markers

Surface proteins located on the outer cell membrane are useful for isolating viable stem and progenitor cells by FACS for further analysis, since they can be tagged with antibodies without jeopardizing cell population. The clusters of differentiation (CD) markers are located on the outer membrane of cells and are commonly identified on hematopoietic stem and progenitor cells (Yin et al. 1997), and as a result this cell type is often studied for differentiation and regeneration properties. This project focused on the following three CD markers: CD133, CD34, and CD117 (\textbf{Figure 1.2}).
Figure 1.2 The presence of surface stem cell markers in the pancreas. CD133, CD34, and CD117 are not specific for one pancreatic cell lineage, and are found on multiple cell types throughout the development of pancreas. This figure is a summary of the studies described in Section 1.6, and encompasses human and murine pancreatic development and analysis studies.
1.6.1 CD133

CD133 (also known as Prominin-1) is a pentaspan membrane protein surface marker found on multiple cell types, including the tubular cells of the kidneys (Lindgren et al. 2011), the cells of the retina (Zacchigna et al. 2009), and on hematopoietic progenitor cells from different cell sources (Hess et al. 2006, Barraud et al. 2007, Snippert et al. 2009). It is also heavily associated with different forms of neoplastic cells and has been examined alongside ALDH to characterize and determine tumour aggressiveness (Alamgeer et al. 2013, Zhou et al. 2014). In addition, CD133 has recently been defined as an important proponent of epithelial-to-mesenchymal transition (EMT) and migration in neoplastic cells (Ding et al. 2012). CD133 levels in mESC are affected by the cell’s progression towards mature differentiation, where high levels were seen in cells that were committed but not yet differentiated (Kania et al. 2005). The expression of CD133 in the pancreas is strongly associated with ductal epithelial cells (Oshima et al. 2007, Lardon et al. 2008, Koblas et al. 2008, Rovira et al. 2010), and transdifferentiation of acinar cells to a ductal cell fate resulted in increased CD133 expression (Houbracken et al. 2011). The early stage human fetal pancreas has been reported to express CD133 on epithelial cells (Zhang et al. 2013), and this has also been reported in the developing murine pancreata (Hori et al. 2008). Additionally, pancreatic stellate cells (PSCs) and endothelial cells have been reported to express CD133 (Koblas et al. 2008, Kordes et al. 2012). Cells that express this marker on their surface have also been described as a precursor population for β-cells (Skurikhin et al. 2014). CD133 is present on the surface of Pdx-1+ epithelia in developing murine embryos (Hori et al. 2008), and ductal CD133+ cells in the human and murine fetal pancreas co-localize with NGN3 at 14 weeks of age (Sugiyama et al. 2007), demonstrating an endocrine precursor fate of this cell type. Expanded cells from human umbilical cord blood that are CD133+ also co-express PDX-1 and NGN3 (Pessina et al. 2010), showing the potential for non-pancreatic CD133+ cells to differentiate into islet-like cells. The contributions of CD133+ cells in giving rise to different cell fates in the pancreas may be age restricted, since there have been reports that adult murine pancreata have higher expression of acinar and endocrine cell in CD133- populations, and that ductal fate is propagated in CD133+ cells (Jin et al. 2013). In addition, adult human
pancreas cells that express CD133 do not co-localize with the stem cell markers OCT4 or SOX2 (Zhao et al. 2007), suggesting that CD133⁺ cells at this stage are associated more with mature ductal epithelium.

1.6.2 CD34

CD34 is a transmembrane protein (Simmons et al. 1992), and is most frequently associated with being a marker of hematopoietic stem cells (Ito et al. 2000, Hess et al. 2004). The multipotency of this stem cell marker has been extensively researched; CD34 cells have demonstrated the ability to reconstitute hematopoiesis and aid in the recovery of damaged tissue (Iwasaki et al. 2006). Although it is not commonly associated with the pancreas, the presence of CD34 has been previously examined throughout the tissue. Cells close to the ductal cells of the rat pancreas showed CD34 expression, and CD34 was seen dispersed throughout the islet (Joanette et al. 2004). CD34 has also been identified on fibrocytes surrounding the ductal and acinar cells of the adult human pancreas (Barth et al. 2002). It has also been identified as a marker of developing vasculature in the islets of the human fetal pancreas (Piper et al. 2004) and in the mesenchymal tissue early on in development (Castaing et al. 2005). In the bovine pancreas, CD34 is present on α- cells of the islets (Merkwitz et al. 2011). Although CD34 is recognized as a marker for hematopoietic stem cells, there was no co-localization seen in cells expressing the stem cell markers SOX2 or OCT4 in the adult pancreas (Zhao et al. 2007). However, CD34⁺ hESCs transplanted into sheep were able to provide higher levels of circulating human c-peptide than populations with heterogeneous cell expression (Goodrich et al. 2010), demonstrating that CD34⁺ hESCs have the potential to give rise to insulin-producing cell-like fate.

1.6.3 CD117

CD117 is a receptor tyrosine kinase found in progenitor cells from multiple sources, including bone marrow-derived hematopoietic cells (Wodja et al. 2003), bone marrow cells (Hess et al. 2003), and in tumour cell populations (Pietsch et al. 1998). Furthermore, CD117 is associated with endoderm cell commitment in precursor hESCs (Cheng et al.
The ligand of CD117 is stem cell factor (SCF), and SCF plays an important role for increasing endocrine expression in the human fetal pancreas (Li et al. 2006). In the rat pancreas, CD117 has been isolated in cells close to the ducts and within the islets (Joanette et al. 2004, Wang et al. 2004). Further examination of the islet populations showed that rat epithelial monolayers expanded from isolated islets on collagen I gels, contained an increased number of proliferating CD117-expressing cells with constant expression of Pdx-1, Ngn3, Pax4, and multiple undifferentiated cell markers, including Oct4 and α-fetoprotein (Wang et al. 2004). Islet-like clusters with increased insulin$^+$ and glucagon$^+$ cells were formed from the monolayer when cultured on laminin-rich matrigel, and the cells secreted insulin in response to glucose (Wang et al. 2004). In a pancreatic duct ligation model, the ligated portion showed an increase of CD117$^+$ cells in ductal and islet cells with increased Pdx-1 and CD117 co-localization (Peters et al. 2005), indicating possible pancreatic regeneration from a progenitor cell source. In the human fetal pancreas, CD117 expression in the ductal cells decreased with age, but co-localized with insulin$^+$ and glucagon$^+$ cells and remained consistent in islet cells (Li et al. 2007). Similar to human pancreata, the expression of CD117 drops in the islets, acinar, and ductal cells of postnatal rats after birth, with the majority of CD117 cells surrounding the islet (Yashpal et al. 2004). Murine pancreata during later stages of embryonic development showed high co-localization of CD117 with Ngn3 and insulin (Ma et al. 2012). In parallel to murine development, exogenous activation of CD117 in the human fetal pancreas leads to increased PDX-1 and insulin protein expression (Li et al. 2007). Further examination of CD117 revealed that reduction of CD117 in PANC-1 cells (a human pancreatic epithelial cancer cell line) led to decreased PDX-1 and insulin expression (Wu et al. 2010). Similar to CD34, mesenchymal cells isolated from human placenta express CD117; these cells were able to differentiate into islet-like clusters in vivo and demonstrated successful insulin production (Kadam et al. 2010). The transplantation of murine bone marrow-isolated CD117$^+$ cells into STZ mice reduced circulating blood glucose, and even outperformed larger cell transplantation populations of CD117$^+$ cells (Hess et al. 2003). Overall, previous works examining CD117 suggest that it is an important marker for β-cell differentiation and regeneration.
1.6.4 Additional surface stem cell markers

Alongside the surface markers listed above, additional cell surface markers have recently been studied and identified as indicators for pancreatic cell fate. CD24 cells highly co-express PDX-1 in definitive endoderm hESCs, and have the capacity to differentiate into insulin-producing cells compared to cells that do not express this surface marker (Jiang et al. 2011). It is important to note that the exclusivity of the CD24 marker to PDX-1 cells has been challenged in repeated hESC experiments, where CD24 was also present on cells that did not stain for PDX-1 (Naujok and Lenzen. 2012). Stage-specific embryonic antigen 4 (SSEA4) is another novel progenitor marker that can be found in cells surrounding the endocrine cells of the pancreas (Afrikanova et al. 2012). It co-localizes with CD133 in the adult human pancreas and NGN3 in the fetal human pancreas (Afrikanova et al. 2012). c-Met, like CD117, is another receptor tyrosine kinase (Bladt et al. 1995) that is found in small percentages on the acinar and ductal cells of the developing murine pancreas (Suzuki et al. 2004).

1.7 Aldehyde Dehydrogenase

Although cell surface markers are frequently used to identify stem cells, intracellular markers have also been examined. Aldehyde dehydrogenase (ALDH) is an NAD+ dependent enzyme (Hammen et al. 2002) found in the cytosol and mitochondria of cells in many different vertebrae (Jackson et al. 2011). It has been detected in various tissue types, including the endoderm-derived liver (Kiefer et al. 2012), lung (Hind et al. 2002), and stomach (Yin et al. 1988). ALDH is also currently regarded as a useful marker for identifying stem cells in normal and malignant tissue populations. As a result, recent research regarding ALDH has focused on its potential as a stem cell marker.

1.7.1 The ALDH family and Retinoic Acid

The 19 isoforms of ALDH are grouped within the families ALDH1 (1A1, 1A2, 1A3, 1B1, 1L1, 1L2), ALDH2, ALDH3 (3A1, 3A2, 3B1, 3B2), ALDH4 (4A1), ALDH5 (5A1), ALDH6 (6A1), ALDH7 (7A1), ALDH8 (8A1), ALDH9 (9A1), ALDH16 (16A1), and ALDH18 (18A1) (Jackson et al. 2011). Cytosolic ALDH isoforms found within the
ALDH1 class are responsible for retinoic acid conversion from retinaldehyde (Marchitti et al. 2008), and are subsequently known as the retinaldehyde dehydrogenase (RALDH) class (Niederreither et al. 2002) (Figure 1.3). They can affect the production of retinoic acid in the developing system, as seen in mice with ALDH1a1 deletions and stunted retinoic acid conversion (Molotkov and Duester. 2003).

Retinoic acid (RA), the product of the retinaldehyde dehydrogenase class, can induce differentiation towards specific cell fate (Andrews. 1984, Wobus et al. 1997, Cai et al. 2010), and can induce multipotent stem cells towards pancreatic cell fate (Micallef et al. 2005). Retinoic acid regulates two different types of receptors, the retinoic acid receptors (RAR) and the retinoid X receptors (RXR). Retinoic acid stimulates RAR-RXR dimer formation and binding of the dimer to DNA (Rastinejad et al. 2000), affecting the expression of retinoic acid response elements (Glass et al. 1991) and regulating gene transcription (Li et al. 2014). All-trans retinoic acid (ATRA) is one isoform of retinoic acid found to affect embryonic development (Klug et al. 1989). It binds to the RAR group, but has low affinity for RXR binding (Heyman et al. 1992). 9-cis retinoic acid (9-cis RA) is another retinoic acid isomer similar to ATRA, but has the ability to bind to RAR and RXR sites (Heyman et al. 1992). Consistent treatment of ALDH1-expressing cells with ATRA and 9-cis RA demonstrated dose-dependent down-regulation of ALDH (Moreb et al. 2005), indicating that sufficient production of RA can lead to down-regulation of its producing enzyme. Diethylaminobenzaldehyde (DEAB) is the major reversible inhibitor associated with halting ALDH activity (Russo et al. 1995) (Figure 1.3), and its actions have been demonstrated across multiple isoforms of ALDH (Moreb et al. 2012).

1.7.2 ALDH and its use as a stem cell marker

Primary cells with ALDH activity display characteristics associated with stem cells, including the ability to promote self-renew via proliferative capacity and differentiation towards to other cell types (Ginestier et al. 2007, Burger et al. 2009). As such, ALDH
Figure 1.3 Retinoic acid in cell differentiation and proliferation. Inhibition of retinoic acid production with DEAB treatment prevents differentiation of cells, but is reversed with exogenous retinoic acid treatment. The dimerization of RAR-RXR regulates TF expression, and stimulates cell proliferation and differentiation. Modified from Li et al. (2014).
has extensively been examined in different tissues and *in vitro* stem cell sources to further elucidate its role in multipotent cell fate (Corti et al. 2006). ALDH-expressing cells isolated from tissue demonstrated the ability to form replicating spheres *in vitro*, which retained the stem cell property of self-renewal (Ginestier et al. 2007, Lindgren et al. 2011, Li et al. 2010). In contrast, the inhibition of ALDH via DEAB has been linked to halting differentiation, illustrating the regulatory role of ALDH in multipotent populations. This was seen in hematopoietic stem cells that had been exposed to DEAB, where it resulted in an extension to their immature progenitor state (Muramoto et al. 2010). ATRA treatment has been found to rescue DEAB-inhibited cell populations and lead to differentiation (Figure 1.3) (Chute et al. 2006, Muramoto et al. 2010, Li et al. 2014), suggesting that while ALDH can serve as an identifying factor of stem cells, it is important for driving cell differentiation via regulation of retinoic acid. The retinoic acid-producing isoform Aldh1a1 is highly expressed in murine hematopoietic stem cells, but its deficiency in mice does not affect the function of this stem cell population (Levi et al. 2009). This suggests that although ALDH is an important factor in stem cells, it is not the sole regulator of stem and progenitor cells.

The relationship between ALDH and other markers of stem cell fate has also been demonstrated in different types of stem cells, aiding in the isolation of multipotent cells by using stem cell-associated markers to further characterize ALDH cell populations (Hess et al. 2004, Burger et al. 2009). Cells positive for the SSEA4 marker express higher levels of the ALDH1A1 isoform than SSEA4 negative cells in the developing human pancreas (Afrikanova et al. 2012), and in the adult murine pancreas, isolated ALDH+ cells that co-localize highly with the CD133 surface marker showed the ability to differentiate into multiple pancreatic cell types (Rovira et al. 2010). This establishes that by pairing the expression of stem cell markers with high ALDH activity in tissue, it may be possible to precisely isolate cells that display regenerative capabilities. However, previous research has shown variations, with hematopoietic stem cell studies demonstrating the presence (Storms et al. 2005) and absence (Armstrong et al. 2004) of the CD34 marker on ALDH+ populations, emphasizing the complexity of using ALDH co-localization studies for stem cell identification.
Although the presence of ALDH can indicate a normal stem cell population, stem cells found in cancerous tissue also express ALDH and have subsequently led to multiple papers examining this enzyme. It is important to note that ALDH does not serve as a guaranteed marker for cancerous cells; cells with low ALDH expression have also demonstrated neoplastic properties \textit{in vivo} (Prasmickaite et al. 2010), agreeing with developmental studies that show multiple markers that indicate stem cell multipotency. ALDH1 is high in cancers affecting many endoderm-derived tissues, including the pancreas, and has been linked to poor patient survival rates (Deng et al. 2010). In addition to this, the activity of ALDH in tumour tissue has been linked to chemotherapy drug resistance (Croker and Allan. 2012).

### 1.8 The Role of ALDH and RA in Pancreatic Development and Function

Current research examining pancreatic development has found that ALDH and its associated receptors can alter the cell composition of an organ. The ALDH1A1 isoform has been identified as the form that makes up a large majority of ALDH\textsuperscript{hi} pancreatic cells in both fetal (Li et al. 2014) and adult human pancreata (Chiang et al. 2009). Both RAR and RXR have been detected in the pancreas during embryonic murine formation, with isoform expression varying in different regions of the pancreas (Kadison et al. 2001, Kobayashi et al. 2002), indicating that there is ATRA and 9-cisRA activity during development (Kane et al. 2010). A summary can be found in Figure 1.4.

#### 1.8.1 Endocrine Fate and the Formation of Islet Cells

The ALDH1 family affects endocrine cells within the developing pancreas. Analysis of ALDH1 activity in the developing human fetal pancreas was extensively examined in our laboratory (Li et al. 2014), and the findings will be covered in brief below. Although overall ALDH activity decreased in the developing human pancreas late in the 2nd trimester of pregnancy, the retinaldehyde dehydrogenase isoform ALDH1A1 level increased as pancreas development progressed (Li et al. 2014), indicating an important developmental role for retinoic acid during late development. Along with these findings,
Figure 1.4 Generation of pancreatic cell lineages with retinoic acid treatment. Differentiation studies reveal that the application of retinoic acid promotes the proliferation of specific pancreatic cell lineages while repressing others. Summarized from the references in Section 1.8.
ALDH1 cells highly co-localized (~90%) with SOX9, PDX-1, and NGN3 (Li et al. 2014), and transcription factors downstream of NGN3 (NKX6.1, NKX2.2, and PAX6). Furthermore, isolated islet-epithelial clusters treated with ATRA led to significantly increased NGN3 and insulin in these clusters, indicating that endocrine as well as β-cell commitment is regulated by retinoic acid (Li et al. 2014). In tandem with the ATRA treatment, this study found that both the reduction of ALDH1A1 expression and inhibition of ALDH1A1 activity led to a reduction of insulin+ cells in these islet clusters (Li et al. 2014). In summary, the results from this study established the necessary role of ALDH1 and retinoic acid in the development of endocrine cells throughout pancreatic development.

Additionally studies have also focused on the contributions of ALDH and retinoic acid activity towards endocrine cell commitment by examining in vivo activity. Examination of the developing human pancreas found similar results seen in Li et al. (2014), where ALDH1A1 gene expression occurred parallel to detected Insulin expression and was particularly high during later developmental stages (Ostrom et al. 2008). Furthermore, insulin+ cell development from the endoderm is controlled by retinoic acid in zebrafish models (Stafford et al. 2006), suggesting that the role of retinoic acid in endocrine cell development is conserved throughout animal models. The mesenchymal cells of the pancreas are necessary for establishing its architecture, and have also been shown to play a role in promoting transcription factors needed for endocrine cell fate (Landsman et al. 2011). Examination of the developing murine pancreas shows high levels of Aldh1a2 in dorsal bud mesenchymal cells that subsequently affects Pdx-1 expression and endocrine cell formation, revealing that retinoic acid production from other cell types in the pancreas can affect islet development (Tulachan et al. 2003, Martin et al. 2005).

Embryonic stem cell studies conducted using hESCs have provided an important in vitro source for the examination of pancreatic fate commitment and subsequent islet development. Retinoic acid treatment of ESCs has repeatedly resulted in endoderm differentiation and expression of PDX-1 (Micallef et al 2005, Shim et al. 2007, Serafimidis et al. 2008, Johannesson et al. 2009, Nostro et al. 2011), and the continued differentiation of retinoic acid-treated hESCs can lead to the production of cells positive
for Insulin as well as inhibition of hepatic precursors from the definitive endoderm population (Cai et al. 2010), supporting the results seen during in vivo research.

1.8.2 Exocrine Fate

The exocrine portion of the pancreas is responsible for the release of digestive enzymes into the duodenum and arises from cells of the duodenum that retain expression of the pancreatic transcription factor Ptf1a (Kawaguchi et al. 2002). Current information on ALDH activity in regard to exocrine tissue development is limited, but studies have been conducted on the effects of retinoic acid on exocrine tissue. Treatment of murine embryonic pancreata with 9-cis RA reduces the percentage of amylase-expressing cells (Kobayashi et al. 2002), and similar results were seen when using ATRA on murine embryonic pancreata of a similar age (Shen et al. 2007). These results show that retinoic acid production in the pancreas largely inhibits the development of the acinar tissue, and suggests that ALDH activity is antagonistic of exocrine development. However, an increase in amylase tissue has been reported in developing murine pancreata treated with ATRA (Kadison et al. 2001), indicating that the role of retinoic acid in acinar development may be more complex than what is reported.

Ductal structures within the pancreas are closely tied to the exocrine portion due to their role of transporting digestive enzymes secreted from acinar cells, but are also associated with giving rise to islet precursors during pancreatic development (Bouwens et al. 1997). ALDH expression has been observed in adult murine ductal cells that make close contact with acini (Rovira et al. 2010) and is highly associated with ductal cells in the developing human fetal pancreas (Li et al. 2014), suggesting a possible progenitor pool in ductal cells. Although the presence of ALDH has been demonstrated in ductal cells, there is also reported information regarding the effects of retinoic acid on ductal cell development. 9-cis RA gives rise to increased ductal cells in murine embryonic pancreata (Kadison et al. 2001). Further analysis demonstrated that mesenchymal tissue is needed for 9-cis RA-induced ductal formations in the pancreas, due to the expression of RARs in this tissue type (Kadison et al. 2001, Kobayashi et al. 2002). However, there are conflicting results regarding the use of ATRA to promote ductal differentiation in murine embryonic
pancreata, with results showing promotion (Tulachan et al. 2003) and suppression (Shen et al. 2007) of ductal cell development. Overall, current available research examining retinoic acid treatment (notably 9-cis RA) indicates that it stimulates ductal cell formation.

Pancreatic stellate cells (PSCs) are characterized to be in either an unactivated quiescent stage or an activated stage that can produce extracellular matrices for repair (Apte et al. 2012). PSC quiescence is stimulated with retinoic acid treatment (Froeling et al. 2011), and proliferation of stellate cells and production of the extracellular component collagen from these cells are inhibited with ATRA and 9-cis RA treatments, implying that ALDH activity restricts the activated cell type (Jaster et al. 2003, McCarroll et al 2006).

1.9 Rationale, Objective, and Hypothesis

Although ALDH activity has been extensively studied in neoplastic stem cells and in stem cells from other sources (ex: hematopoietic stem cells), limited research has been conducted on ALDH and its role in β-cell differentiation during human fetal pancreas development. Our lab recently looked at ALDH co-localization with TF- and hormone-expressing cells in the whole human fetal pancreas during early 1st trimester (10 to 11 weeks of fetal age) and late 2nd trimester (20 to 21 weeks of fetal age) of pregnancy. The results demonstrated high co-localization of endocrine cell TFs and hormones with ALDH+ cells during development, leading to the conclusion that ALDH+ cells are important for endocrine cells throughout early and late human pancreatic development (Li et al. 2014). However, this previous study did not examine prospective sorted ALDH populations of the human fetal pancreas and characterize their markers and TFs in vitro and in vivo. Therefore, this project set out to separate ALDH populations from the developing human pancreas and assess their abilities to produce β-cells in vitro and in vivo. This thesis examined high (ALDHhi) or low (ALDHlo) ALDH populations in the developing human pancreas at 18 to 22 weeks of fetal age, and characterized the expression of putative stem cell markers and TFs in the sorted cells. Culture allowed for in vitro analysis of TF expression during expansion, while transplantation of sorted cells provided in vivo analysis of differentiation and maturation.
Objective

To characterize the transcription factors (TFs) and putative stem cell markers in purified cell populations with high ALDH activity (ALDH\textsuperscript{hi}) or low ALDH activity (ALDH\textsuperscript{lo}) sorted cell populations in the developing human fetal pancreas, and to examine their phenotype \textit{in vitro} and \textit{in vivo}.

Hypotheses

1. ALDH\textsuperscript{hi} cells denote a progenitor pool in the developing human pancreas.

2. ALDH\textsuperscript{hi} cells express factors indicative of commitment to the endocrine cell fate, and can give rise to endocrine cell populations \textit{in vitro} and \textit{in vivo}.

Specific Questions

1. Do CD133, CD34, or CD117 co-localize exclusively with the ALDH\textsuperscript{hi} populations in dissociated human fetal pancreatic cells?

2. Does the ALDH\textsuperscript{hi} sorted population contain TFs necessary for endocrine cell development?

3. Could ALDH\textsuperscript{hi} cells maintain their phenotypes during culture \textit{in vitro}?

4. Could cultured ALDH\textsuperscript{hi} cells give rise to islet-like cell clusters?

5. Does the subcutaneous transplantation of ALDH\textsuperscript{hi} sorted cells, wrapped in fibrin gel, enhance differentiation towards the endocrine cell type in nude mice?
Chapter 2 – Materials and Methods

2.1 Fluorescence-activated cell sorting

2.1.1 Pancreas Tissue Dissociation

Human fetal pancreata (18 to 22 weeks of fetal age) were obtained based on the approved protocol from the Health Human Sciences Research Ethics Board at Western University (Appendix A), in accordance with the Canadian Council on Health Sciences Research Involving Human Subjects guidelines. Fetal age is assigned two weeks later than the gestational age (based on the menstrual cycle), to account for the two weeks of pre-ovulation that precede conception. Pancreata were carefully dissected by removing all surrounding non-pancreatic tissue, and then digested with a dissociation buffer (1x Hank’s Balanced Salt Solution (HBSS) [Sigma, Oakville, ON, Canada]; 25 mM HEPES [Sigma]; DNase I [Roche, Mississauga, ON, Canada]) containing collagenase V (1 mg/ml) (Sigma) in a water bath at 37°C for 30 minutes (Al-Masri et al. 2010). To inhibit the active enzyme, dissociated samples were washed three times with cold wash buffer (1x HBSS (Sigma); NaHCO₃; Soybean Trypsin Inhibitor (STI); Bovine Serum Albumin (BSA); Pen/Strep; Fungizone). To prepare pancreatic single cell suspensions, the samples were further treated with 0.25x Trypsin-EDTA (Mediatech Inc., Manassas, VA) and incubated again at 37°C for 5 minutes. Confirmation of single cells was achieved by examining the sample using an inverted light microscope (Leica DM IL; Leica Microsystems, Bannockburn, IL, U.S.A.). Due to time constraints, cells were kept at 4°C overnight in CMRL-1066 media (Mediatech Inc., Manassas, VA) with 10% fetal bovine serum (FBS) (Life Technologies Inc., Burlington, ON, CA) for cell sorting the following day at the London Regional Flow Cytometry Facility, Robarts Research Institute.

2.1.2 Aldefluor and fluorophore-conjugated antibody staining

Pancreatic single cells were centrifuged to remove CMRL-1066 media and then suspended in Aldefluor buffer (Stem Cell Technologies Inc., Vancouver, BC, CA). Cell viability was examined using 0.2% trypan blue (Gibco, Life Technologies) staining with
the automated Cellometer T4 cell counter (Nexcelom Bioscience, Lawrence, MA, U.S.A.). Viability ranged between 80% to 90% and the total number of live cells was between $6 \times 10^6$ and $1 \times 10^7$ cells per pancreatic single cell preparation. Fluorophore Minus One (FMO) control tubes were set up as negative controls for all fluorophores used, and were prepared with approximately 100,000 viable cells from the bulk population. Aldefluor reagent (StemCell Technologies) was applied to detect the intracellular activity of ALDH. The cell population was incubated at 37°C for 30 minutes in order for ALDH to convert the fluorescent BODIPY-aminoacetaldehyde (BAAA) in the Aldefluor reagent to BODIPY-aminoacetate (BAA), a negatively charged anion trapped within the cell (Storms et al. 1999, Balicki. 2007). The Aldefluor FMO control tube was treated with DEAB to inhibit ALDH activity and BAAA conversion.

Fluorophore- conjugated antibodies listed in Table 2.1 were also used to examine surface stem cell marker populations (Hess et al. 2004). CD235a antibodies were used to remove erythrocytes from the sorted populations and prevent inaccurate cell population representation. Cells were incubated at 4°C for 30 minutes after antibody application. The viability marker 7-AAD (BD BioSciences, Mississauga, ON, CA) was added to each tube before sorting the cells in order to mark live versus dead cells within the final populations. Cells were stored on ice to retain Aldefluor fluorescence until the time of sort.

2.1.3 Fluorescence-activated cell sorting procedure

Prepared cells were sorted using the FACSAria III Cell Sorter (BD Biosciences, San Jose, CA, U.S.A.), and analyzed using FACSDiva software (BD Biosciences). FMO control tubes were used to properly gate the cell populations marked with each fluorophore. The sample tube was sorted into two of the following populations: 1) ALDH$^{hi}$ and ALDH$^{lo}$ populations; or 2) cell surface marker$^{hi}$/ALDH$^{hi}$ or ALDH$^{lo}$, and cell surface marker$^{lo}$/ALDH$^{hi}$ or ALDH$^{lo}$ populations.
**Table 2.1:** List of antibodies used for fluorescence-activated cell sorting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-human CD34 BV-conjugated</td>
<td>2 μl/ 1 x10⁷</td>
<td>BioLegend Inc., San Diego, CA, US</td>
</tr>
<tr>
<td>Mouse anti-human CD117 PE-conjugated</td>
<td>2 μl/ 1 x10⁷</td>
<td>BioLegend Inc., San Diego, CA, US</td>
</tr>
<tr>
<td>Mouse anti-human CD133 APC-conjugated</td>
<td>4 μl/ 1 x10⁷</td>
<td>Miltenyi Biotec Inc., Auburn, CA, US</td>
</tr>
<tr>
<td>Mouse anti-human CD133 PE-conjugated</td>
<td>2 μl/ 1 x10⁷</td>
<td>Miltenyi Biotec Inc., Auburn, CA, US</td>
</tr>
<tr>
<td>Mouse anti-human CD235a PerCP-Vio700-conjugated</td>
<td>8 μl/ 1 x10⁷</td>
<td>Miltenyi Biotec Inc., Auburn, CA, US</td>
</tr>
<tr>
<td>Mouse anti-human CD235ab APC-conjugated</td>
<td>2 μl/ 1 x10⁷</td>
<td>BioLegend Inc., San Diego, CA, US</td>
</tr>
</tbody>
</table>
2.2 Histological Analysis of Cell Populations

2.2.1 Paraffin Block Preparation of Sorted Cell Samples

Sorted cells were washed in PBS and fixed in 4% paraformaldehyde (PFA) (Fischer Scientific Company, Ottawa, ON, Canada) at 4°C overnight. The cell pellet was embedded in 2% agarose gel and then placed in cassettes for paraffin embedding as previously described (Lyttle et al. 2008). The processing procedure involved the following steps using an automated tissue processing machine (Shandon Citadel™ Tissue Processor, Thermo Electron Corporation; Waltham, Massachusetts, USA): 1) dehydration baths with increasing ethanol concentration (70% to 100%); 2) toluene baths; and 3) paraffin wax baths. The processed samples were embedded in paraffin-filled molds and fixed on a cold plate using an embedding machine (Thermo Shandon Histocentre 2, Thermo Scientific, Pittsburg, PA, U.S.A) to make paraffin blocks.

2.2.2 Immunofluorescence Staining

Paraffin blocks were sectioned using a microtome (Leica RM2245, Leica Biosystems) at 4µm, and set aside to warm overnight. Removal of all paraffin from slides was done in xylene baths, and cells were rehydrated in baths of decreasing ethanol concentration (from 100% to 70%). For the staining of transcription factors, slides were pretreated in either a citrate antigen retrieval solution (pH 6.0) or a trypsin-EDTA antigen retrieval solution (pH 8.9) for heat-induced antigen retrieval of fixed epitopes (Cattoretti et al. 1993). Blocking solution with 10% goat serum was applied in order to block the non-specific adhesion of antibodies. A list of primary antibodies used in this study is shown in Table 2.2, and slides were incubated overnight at 4°C after primary antibody application. Fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (JRL; West Grove, PA, U.S.A.) reactive to the primary antibody species were diluted (1:50 dilution) and incubated at room temperature for 1 hour. Cell nuclei were counter-stained by 4′,6-diamidino-2-phenylindole (DAPI) (10 mg/mL) (Sigma), and the slides were mounted with a cover slip using mounting medium (Dako, Burlington, ON, CA). Fluorescent staining signals were examined using a
fluorescent microscope (Leica DMIRE2; Leica Microsystems) and analyzed using the Image ProPlus software (Media Cybernetics Inc., Rockville, MD, U.S.A.). The cell counter program in the Image ProPlus software (Media Cybernetics Inc.) was used to quantify the total number of cells positive for the examined antigen, and this was normalized to the total number of cells per section and expressed as percentage. Secondary antibody was excluded from staining for negative controls.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-ALDH</td>
<td>1:100</td>
<td>BD Biosciences, Mississauga, ON, CA</td>
</tr>
<tr>
<td>Rabbit anti-CD31</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology Inc., CA, US</td>
</tr>
<tr>
<td>Rabbit anti-CD34</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology Inc., CA, US</td>
</tr>
<tr>
<td>Rabbit anti-CD117</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology Inc., CA, US</td>
</tr>
<tr>
<td>Rabbit anti-CD133</td>
<td>1:100</td>
<td>Biorbyt Ltd., Cambridge, U.K.</td>
</tr>
<tr>
<td>Rabbit anti-CK19</td>
<td>1:100</td>
<td>Abcam Inc., MA, USA</td>
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<tr>
<td>Mouse anti-CK19</td>
<td>1:50</td>
<td>Dako Canada Inc., Burlington, ON, CA</td>
</tr>
<tr>
<td>Rabbit anti-E-cadherin</td>
<td>1:50</td>
<td>Abcam Inc., MA, USA</td>
</tr>
<tr>
<td>Mouse anti-Glucagon</td>
<td>1:800</td>
<td>Sigma-Aldrich, Oakville ON, CA</td>
</tr>
<tr>
<td>Rabbit anti-Glucagon</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology Inc., CA, US</td>
</tr>
<tr>
<td>Mouse anti-HMS</td>
<td>1:100</td>
<td>Millipore Canada Ltd., Etobicoke, ON, CA</td>
</tr>
<tr>
<td>Mouse anti-Insulin</td>
<td>1:800</td>
<td>Sigma-Aldrich, Oakville ON, CA</td>
</tr>
<tr>
<td>Rabbit anti-Insulin</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology Inc., CA, US</td>
</tr>
<tr>
<td>Mouse anti-ISL1</td>
<td>1:100</td>
<td>DSHB, Iowa City, IA, US</td>
</tr>
<tr>
<td>Mouse anti-Ki67</td>
<td>1:100</td>
<td>BD Biosciences, Mississauga, ON, CA</td>
</tr>
<tr>
<td>Mouse anti-NGN3</td>
<td>1:100</td>
<td>DSHB, Iowa City, IA, US</td>
</tr>
<tr>
<td>Mouse anti-NKX2.2</td>
<td>1:100</td>
<td>DSHB, Iowa City, IA, US</td>
</tr>
<tr>
<td>Mouse anti-NKX6.1</td>
<td>1:100</td>
<td>DSHB, Iowa City, IA, US</td>
</tr>
<tr>
<td>Rabbit anti-PAX6</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology Inc., CA, US</td>
</tr>
<tr>
<td>Guinea Pig anti-PDX-1</td>
<td>1:800</td>
<td>Dr. Wright, University of Vanderbilt, US</td>
</tr>
<tr>
<td>Rabbit anti-PDX-1</td>
<td>1:800</td>
<td>Dr. Wright, University of Vanderbilt, US</td>
</tr>
<tr>
<td>Mouse anti-SOX9</td>
<td>1:100</td>
<td>Abcam Inc., MA, USA</td>
</tr>
<tr>
<td>Rabbit anti-SOX9</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology Inc., CA, US</td>
</tr>
<tr>
<td>Mouse anti-Vimentin</td>
<td>1:100</td>
<td>DSHB, Iowa City, IA, US</td>
</tr>
<tr>
<td>Rabbit anti-β-catenin</td>
<td>1:500</td>
<td>Millipore Canada Ltd., Etobicoke, ON, CA</td>
</tr>
</tbody>
</table>
2.3  Quantitative Real-time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

2.3.1  RNA Isolation

ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} cells gathered immediately after sort and expanded culture were washed with sterile PBS. The cell pellets were lysed in cell lysis buffer using the RNAqueous-4PCR kit (Life Technologies) according to the manufacturer’s instructions. In brief, the cell lysate was vortexed intermittently to break up cells and then treated with 64% ethanol. RNA was collected using a collection filter and washed with wash buffer (Life Technologies), and heated elution solution (Life Technologies) was used to collect the RNA from the filter. DNAse buffer (Life Technologies) and DNAse (Life Technologies) were added to the elution solution sample to eliminate DNA contamination, and samples were incubated at 37°C for 30 minutes. The isolated RNA solution was stored at -80°C prior to reverse transcription. RNA concentrations were measured using 250 µL of DEPC H\textsubscript{2}O and 1 µL of RNA sample, then analyzed using a Multiskan Spectrometer (Thermo Scientific). RNA samples with a concentration higher than 0.1 µg/µL were used for reverse transcription. The quality of the RNA was checked by running samples on a 1% agarose gel with ethidium bromide, and confirmed with the visual observation of 28S and 18S bands.

2.3.2  Reverse Transcription and cDNA Synthesis

RNA samples were heated with the addition of random hexamers (3000 ng/mL), Oligo dT, and DEPC H\textsubscript{2}O at 60°C for 5 minutes and 4°C for 5 minutes using the GeneAmp PCR System 2400 (Applied Biosystems Inc., Foster City, CA, U.S.A.). The master mix (4 µL of 5x buffer, 2 µL of DTT, 2 µL of 10 mM dNTPs, and 0.5 µL RNAsin) was added to each RNA sample and then centrifuged for 30 seconds. The samples were then heated on the GeneAmp PCR System (Applied Biosystems) at the following temperatures: 42°C for 90 minutes, 94°C for 5 minutes, and 4°C for 60 minutes. SuperScript® II Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) was added to each sample while in the PCR System at 42°C. A final concentration of 6 µg/ 80 µL for each sample was
achieved by diluting with sterile H₂O. The cDNA samples were then stored at -20°C prior to qRT-PCR reactions. Negative control samples were generated by excluding RNA during the reaction.

2.3.3 qRT-PCR Measurements

Each well for the real-time reaction was prepared using the following: 3 µL of sterile H₂O, 0.5 µL forward primer, 0.5 µL reverse primer, 1 µL cDNA sample, and 5 µL SYBR Green Master mix. The list of primers used and the annealing temperatures are listed in Table 2.3. Negative template controls were used to verify primer specific amplification, and prepared by substituting the primer volume with sterile H₂O. After adding the appropriate volumes, the reaction wells were mixed and loaded on to the Bio-Rad CFX Connect™ real-time PCR detection system (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, CA) and analyzed using the CFX Manager software (Bio-Rad). The melting curves were used to indicate if the qRT-PCR assays had amplified target sequences, and relative gene expression was analyzed using the comparative CT method ($2^{-\Delta\Delta CT}$) (Schmittgen et al. 2008) and the housekeeping gene 18S.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession Definition</th>
<th>Sequence 5’- 3’ <em>(Sense/Antisense)</em></th>
<th>Fragment Size (bp)</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INSULIN</td>
<td>NM_000207.2</td>
<td>TCA CAC CTG GTG GAA GCT CTC TA ACA ATG CCA CGC TTC TGC AGG GAC</td>
<td>179</td>
<td>56.9</td>
</tr>
<tr>
<td>NGN3</td>
<td>NM_020999.3</td>
<td>AGC CGG CCT AAG AGC GAG TT TTG GTG AGC TTC GCG TCG TC</td>
<td>158</td>
<td>59.3</td>
</tr>
<tr>
<td>NKX6.1</td>
<td>NM_006168.2</td>
<td>AGA CCC ACT TTT TCC GGA CA CCG CTG CTG GAC TTG TGC TT</td>
<td>335</td>
<td>56.6</td>
</tr>
<tr>
<td>PDX-1</td>
<td>NM_000209.3</td>
<td>CTC CTA CAG CAC TCC ACC TTG CCG AGT AAG AAT GGC TTT ATG</td>
<td>153</td>
<td>56.0</td>
</tr>
<tr>
<td>SOX9</td>
<td>NM_000346.3</td>
<td>ACA AGA AGG ACC ACC CGG ATT A AGC TCG CCG ATG TCC ACG TCG C</td>
<td>340</td>
<td>57.2</td>
</tr>
<tr>
<td>18S</td>
<td>NR_003286.2</td>
<td>GTA ACC CGT TGA ACC CCA TTC CCA TCC AAT CGG TAG TAG CG</td>
<td>151</td>
<td>55.8</td>
</tr>
</tbody>
</table>
2.4 Cell Culture Experiments

2.4.1 Examination of Extracellular Matrices on Sorted Cells

In order to examine which extracellular matrix preparation best promoted the viability of ALDH$^{hi}$ cells, a modified protocol used in Krishnamurthy et al. (2008) was implemented. Tissue culture plates (96 well, Falcon BD Beckson Dickson) were coated with 5 µg/mL of the following matrices at room temperature for 1 hour: control (1% BSA solution), Fibronectin, Laminin, Collagen I, and Collagen IV (BD Biosciences). ALDH$^{hi}$ cells were plated on top of wells coated with matrices at a density of 1 x10$^4$ cells/ well in 100 µL of DMEM/ F12 + 10% FBS (Life Technologies) media. Cell viability was measured at 24 hours and 5 days by treating the well with 100 µL of fresh DMEM/ F12+ 10% FBS media and 10 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) reagent (5 mg/mL), then incubating the samples for two hours at 37°C in the tissue culture incubator. The MTT positive viable cells (per well) were imaged using an inverted light microscope (Leica DM IL; Leica Microsystems) and analyzed with Image ProPlus software (Media Cybernetics Inc). Quantification of the number of viable cells was conducted by selecting five consistent fields in each well, and counting the number of MTT positive cells normalized to the total number of adhered cells per experimental group. Data were expressed as a percentage of viable cells with 3 experimental replications/group treatment.

2.4.2 Expansion Culture of ALDH$^{hi}$ and ALDH$^{lo}$ Cells

Based on the optimal viable cell results from ECM culture tests, the expansion culture was performed on the collagen I matrix coated culture plate. Sorted ALDH$^{hi}$ and ALDH$^{lo}$ cells were expanded on collagen I coated 12-well tissue culture plates with 1 mL DMEM/ F12 + 10% FBS (Life Technologies). Media was changed every 48 hours and cells were passaged once wells were 90 to 95% confluent. To examine expanded cell phenotype, cells were harvested for RNA extraction and histological analysis during expansion at different passages.
2.4.3 Differentiation Culture of ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} Cells

Passaged cells from ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} sorted cell expansion were plated in 12-well plates and cultured with 1mL of serum-free differentiation media (Gershengorn et al. 2004, Wu et al. 2010) in order to examine whether these cells could differentiate into endocrine cells. The cell cluster formation was monitored at 0 hours, 36 hours, and 7 days using an inverted light microscope (Leica DM IL; Leica Microsystems). Clusters were then collected for RNA extraction and histological analysis at day 7 of the culture.

2.5 Transplantation of Sorted Cells in Nude Mice

The transplantation of sorted cells was conducted to examine the effects that an \textit{in vivo} environment had on the differentiation of ALDH\textsuperscript{hi} sorted populations to endocrine lineage. Immediately after cell sorting, ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} cells were embedded in 100 µL of fibrin in a 96-well plate to form a 3D gel (Riopel et al. 2013) and cultured with 100 µL of Epicult-C plus 10% FBS media (StemCell Technologies, BC, Canada). After 24 hours of culture, ALDH\textsuperscript{hi} or ALDH\textsuperscript{lo} cells in fibrin gels were fixed in 4% PFA, embedded in 2% agarose gel, and processed in paraffin blocks for histological analysis. Additionally, ALDH\textsuperscript{hi} or ALDH\textsuperscript{lo} cells cultured overnight in fibrin gels were subcutaneously transplanted to nude mice, and an empty fibrin gel served as a negative control group. In brief, 8 week-old male nude mice were anesthetized with isoflurane and either empty gels or gels containing 24 hours cultured ALDH\textsuperscript{hi} or ALDH\textsuperscript{lo} cells were implanted subcutaneously. Mice were sacrificed at 1 week post-transplantation, where grafts were harvested for histological analyses. Animal work was performed using protocols approved by the Animal Use Subcommittee at the University of Western Ontario in accordance with the guidelines of the Canadian Council of Animal Care (\textit{Appendix D}).

2.6 Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis between ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} groups for all FACS and qRT-PCR experiments were done using a two-tailed paired
Student t-test using GraphPad Prism program (Version 5.0c, GraphPad Software Inc., La Jolla, CA, USA). Immunofluorescence analysis was performed using two-tailed unpaired Student t-tests to account for the use of unpaired sorted groups in these experiments. Statistical significance of analyzed data was achieved at p<0.05.
Chapter 3 - Results

3.1 FACS analysis for ALDH$^{hi}$ and ALDH$^{lo}$ groups

To isolate the ALDH$^{hi}$ cell population in the developing human fetal pancreas, fluorescence-activated cell sorting (FACS) analysis by the FACS Aria III Cell Sorter (BD Bioscience) was performed. Before cell sorting, the cell viability and cell diameter was examined using the automated Cellometer T4 cell counter. The bulk dissociated cells demonstrated high viability ($82.42 \pm 2.11\%$), determined by trypan blue staining, and consistent cell diameter size ($9.0 \pm 0.1 \mu m$). The average total live cell population was approximately $8.27 \times 10^6$ cells per dissociated human fetal pancreas. Viable dissociated human fetal pancreatic cells were first gated (isolation of populations of interest) using the fluorescent viability dye 7-aminoactinomycin D (7-AAD) (Figure 3.1A), and dead cells (7-AAD$^+$) were discarded during cell sorting. Erythrocyte removal from the sorted cell tubes was completed by gating out cells that stained positive for the erythrocyte surface marker CD235a (Glycophorin A) (Figure 3.1B). Sorted cell populations were verified based on cell size (Forward Scatter Plot, FSC) and cell internal complexity (Side Scatter Plot, SSC) (Figures 3.1C). Separating Aldefluor-expressing cells from the viable bulk cell population was done by using the DEAB negative control to represent the ALDH$^{lo}$ population during Aldefluor fluorescence gating (Figure 3.1D, E). The purity of the ALDH$^{hi}$ ($94.63 \pm 0.60\%$) and ALDH$^{lo}$ ($99.67 \pm 0.15\%$) sorted populations was confirmed analyzing their Aldefluor expression. Viable cells after sorting largely belonged to the ALDH$^{hi}$ sorted population ($65.87 \pm 3.54\%$), compared to the ALDH$^{lo}$ population ($28.37 \pm 3.50\%$) (p<0.001; Figure 3.1F).
Figure 3.1 Isolation of viable cell populations using fluorescence-activated cell sorting

(A) Removal of non-viable (7-AAD high) cells using gating during cell sorting. (B) Erythrocyte removal from the final sort population labeled by the cell surface marker CD235a antibody. (C) Single cell population based on cell size (Forward Scatter plot, FSC) and cell internal complexity (Side Scatter plot, SSC). (D) Separation of the sorted ALDH$^{hi}$ and ALDH$^{lo}$ pancreatic populations, using Aldefluor fluorescence gating to isolate ALDH activity. (E) ALDH inhibitor diethylaminobenzaldehyde (DEAB) was used as a negative control in conjunction with Aldefluor to set the population separation between ALDH$^{hi}$ and ALDH$^{lo}$ cells. (F) Quantification of ALDH$^{hi}$ and ALDH$^{lo}$ viability in the final sorted population. ALDH$^{hi}$ cells, green; ALDH$^{lo}$ cells, blue; non-viable and excluded cells, grey population. Data are expressed as mean ± SEM and were analyzed using paired t-tests (n=15 sorted pancreata/group). *** p<0.001 vs. ALDH$^{lo}$ sorted population.
3.2 Characterization of Stem Cell Surface Markers in Sorted ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} populations

The putative stem cell surface markers CD133, CD34, and CD117 expression have previously been demonstrated on a variety of stem cell lineages. Therefore, these cell surface markers were analyzed during FACS to determine their co-localization frequencies in the sorted populations. CD133\textsuperscript{+} cells were present in both ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} sorted populations, yet CD133 had significantly higher expression in ALDH\textsuperscript{hi} cells (65.82 ± 4.58\%) when compared to ALDH\textsuperscript{lo} sorted cells (8.15 ± 3.28\%) (p<0.05, Figure 3.2A,D). In comparison, the CD34\textsuperscript{+} marker was significantly higher in the ALDH\textsuperscript{lo} group (41.56 ± 2.78\%) than the ALDH\textsuperscript{hi} population (24.32 ± 2.86\%) (p<0.05, Figure 3.2B,D). No significant difference was detected for CD117\textsuperscript{+} expression between the ALDH\textsuperscript{hi} (12.64 ± 8.45\%) and ALDH\textsuperscript{lo} (10.56 ± 4.42\%) populations (Figure 3.2C,D).

To examine the ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} sorted cells for overlapping co-localization of surface stem cell markers, 3-colour multiparametric cell sorting analyses were performed. Overall, equivalent but low co-localization was seen in the ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} populations with CD117\textsuperscript{+}/CD34\textsuperscript{+} surface markers (Figure 3.3A). Lower co-localization of CD133\textsuperscript{+}/CD34\textsuperscript{+} surface markers (Figure 3.3B) and CD117\textsuperscript{+}/CD133\textsuperscript{+} surface markers (Figure 3.3C) were also observed in both ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} sorted group. These results show that the CD133\textsuperscript{+} population in ALDH\textsuperscript{hi} cells and CD34\textsuperscript{+} population in the ALDH\textsuperscript{lo} cells do co-localize with other stem cell surface markers, but are largely independent of the chosen surface markers examined in this study.
Figure 3.2 Expression of surface stem cell markers within ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} populations using FACS.

(A) Individual sorting plots for markers CD133, CD34, and CD117 (y-axis), and the co-staining of Aldefluor (x-axis) for the detection of co-localizing populations. Boxed areas on scatter plots represent separated populations based on Aldefluor and fluorophore-labeled antibody fluorescence intensity. (B) Quantification of the co-localized surface stem cell markers with ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} in sorted fetal pancreatic cells. White bar, ALDH\textsuperscript{hi} group; black bar, ALDH\textsuperscript{lo} group. Data are expressed as mean ± SEM and analyzed using paired t-tests (n=4-8 sorted pancreata/group). *** p<0.001 vs. ALDH\textsuperscript{lo} sorted population.
Figure 3.3 Co-localization between surface stem cell markers in the ALDH$^{hi}$ and ALDH$^{lo}$ populations.

Co-expression of sorted ALDH$^{hi}$ (left) and ALDH$^{lo}$ (right) populations, and quantitative analysis (far right) for CD117$^+$/CD34$^+$ (A), CD34$^+$/CD133$^+$ (B), and CD133$^+$/CD117$^+$ (C) in the sorted ALDH$^{hi}$ (left) and ALDH$^{lo}$ (middle) gated populations. Red lines indicate co-localization of stem cell surface markers with ALDH populations. White bar, ALDH$^{hi}$ group; black bar, ALDH$^{lo}$ group. Data are expressed as mean ± SEM and analyzed using paired t-tests (n=4 sorted pancreata/group)
3.3 Analysis of TFs in sorted ALDH$^{hi}$ and ALDH$^{lo}$ populations

Commitment to endocrine, and specifically β-cell, fate in the pancreas requires a sequence of transcription factor activation. Initial transcription factors responsible for pancreatic progenitor fate (SOX9), pancreatic cell fate (PDX-1), and endocrine cell commitment (NGN3) precede factors associated with islet cells (ISL1, NKX2.2, NKX6.1, PAX6). Therefore, the analysis of transcription factor expression in ALDH$^{hi}$ sorted cells is necessary to determine the commitment of these cells towards the endocrine cell fate. qRT-PCR results, used to examine the mRNA expression of the transcription factors, demonstrated that there was relatively higher mRNA levels of SOX9, PDX-1, NGN3, and NKX6.1 expression in the ALDH$^{hi}$ cells, but statistical significance was not reached due to a high variability between the sample groups (Figure 3.4). Using immunofluorescence staining to analyze the nuclear expression of these transcription factors, it was found that the SOX9$^+$ cells had higher expression in the ALDH$^{hi}$ sorted cells compared to ALDH$^{lo}$ group (55 ± 3% vs. 14 ± 1%, p<0.001, Figure 3.5). The PDX-1$^+$ cells also had higher percentages in ALDH$^{hi}$ group the ALDH$^{lo}$ population (20 ± 2% vs. 2.0 ± 0.3%, p<0.001, Figure 3.5). This data, along with the surface stem cell marker results from the previous section, could indicate that the ALDH$^{hi}$ sorted population may represent a pancreatic precursor pool, but other cell fate possibilities must be examined before concluding this. The major transcription factor for endocrine cell fate is NGN3, and although there was increased NGN3$^+$ labeling in the ALDH$^{hi}$ group (14 ± 5%) compared to ALDH$^{lo}$ group (3 ± 1%), there was no significant difference between the two cell populations (Figure 3.6). A similar pattern was observed in NKX2.2 staining where it was insignificantly higher in ALDH$^{hi}$ (4 ± 1%) sorted cells, compared to the ALDH$^{lo}$ (0.9 ± 0.2%) population (Figure 3.6). ISL1, an endocrine cell marker important for insulin secretion in β-cells (Ediger et al. 2014), did not make up a large population in the ALDH$^{hi}$ sorted group (3 ± 1%) but was significantly higher than the ALDH$^{lo}$ (0.6 ± 0.2%) sorted group (Figure 3.6). The transcription factors PAX6 (6 ± 2% vs. 0.8 ± 0.3% in ALDH$^{lo}$ group, p<0.05) and NKX6.1 (12 ± 2% vs. 2 ± 1% in ALDH$^{lo}$ group, p<0.01) are present in the ALDH$^{hi}$ sorted cells (Figure 3.7). Taken together, higher percentages of endocrine transcription factors are expressed in the
ALDH^hi sorted cells, suggesting that the ALDH^hi population could represent an early progenitor population with low levels of endocrine specification.
Figure 3.4 qRT-PCR analysis of ALDH$^{hi}$ and ALDH$^{lo}$ sorted cells.

mRNA expression of pancreatic transcription factors SOX9, PDX-1, NGN3, and NKX6.1 was normalized to the housekeeping gene 18S and analyzed using the comparative Ct method ($2^{-\Delta\Delta C_t}$). White bars, ALDH$^{hi}$ group; black bars, ALDH$^{lo}$ group. Data are expressed as mean ± SEM and analyzed using paired t-tests (n=4 sorted pancreata/group)
Figure 3.5 Pancreatic progenitor and pancreatic cell fate transcription factor analysis in the sorted populations.

(A) Representative immunofluorescence images of SOX9 and PDX-1 (green) nuclei staining in the sorted ALDH<sup>hi</sup> and ALDH<sup>lo</sup> populations. DAPI (blue) was used for nuclear counter staining. White arrows indicate cells positive for TF. Scale bar: 15 µm.

(B) Quantification of SOX9<sup>+</sup> and PDX-1<sup>+</sup> cells in the total sorted population. White bars, ALDH<sup>hi</sup> group; black bars, ALDH<sup>lo</sup> group. Data are expressed as mean ± SEM and analyzed using unpaired t-tests (n=3-5 sorted pancreata/group), *** p<0.001 vs. ALDH<sup>lo</sup> sorted population.
Figure 3.6 Analysis of transcription factors for commitment to endocrine cell differentiation.

(A) Representative images of NGN3, ISL1, and NKX2.2 (green) staining in the ALDH$_{\text{hi}}$ and ALDH$_{\text{lo}}$ sorted cell nuclei, labeled by DAPI (blue). White arrows indicate cells positive for TF. Scale bar: 15 µm. (B) Quantification of NGN3$^+$, ISL$^+$, and NKX2.2$^+$ cells in the total sorted population. White bars, ALDH$_{\text{hi}}$ group; black bars, ALDH$_{\text{lo}}$ group. Data are expressed as mean ± SEM and analyzed using unpaired t-tests (n=2-4 sorted pancreata/group) * p<0.05 vs. ALDH$_{\text{lo}}$ sorted population.
Figure 3.7 Analysis of transcription factors associated with islet endocrine cells.

(A) Representative images of NKX6.1 and PAX6 (green) staining in the ALDH$^{hi}$ and ALDH$^{lo}$ sorted cell nuclei, labeled by DAPI (blue). White arrows indicate cells positive for TF. Scale bar: 15 µm. (B) Quantification of NKX6.1$^+$ and PAX6$^+$ cells in the total sorted population. White bars, ALDH$^{hi}$ group; black bars, ALDH$^{lo}$ group. Data are expressed as mean ± SEM and analyzed using unpaired t-tests (n=3 sorted pancreata/group) * p<0.05, ** p<0.01 vs. ALDH$^{lo}$ sorted population.
3.4 Characterization of endocrine and ductal cells, and cell proliferation markers in sorted ALDH$^{hi}$ and ALDH$^{lo}$ populations

Immunofluorescence staining was used in order to characterize the pancreatic cell composition of the sorted ALDH$^{hi}$ and ALDH$^{lo}$ groups, and to determine if one pancreatic cell type is favoured in the ALDH$^{hi}$ sorted group. Insulin was found to be the most common of the endocrine hormone markers, with significantly higher insulin$^{+}$ cells in the ALDH$^{hi}$ population (5 ± 1%) and nearly undetectable levels in the ALDH$^{lo}$ population (0.8 ± 0.2%) ($p<0.05$; Figure 3.8). Somatostatin$^{+}$ cells were also increased in the ALDH$^{hi}$ sorted cells (3 ± 0.5%), compared to the ALDH$^{lo}$ sorted cells (0.7 ± 0.4%) ($p<0.05$; Figure 3.8). However, there was no significant difference in glucagon$^{+}$ cells between the sorted ALDH$^{hi}$ and ALDH$^{lo}$ groups (Figure 3.8). The epithelial cell markers, CK19 and E-cadherin, were expressed in relatively low levels in both the ALDH$^{hi}$ and ALDH$^{lo}$ populations (Figure 3.9). The expression of β-catenin, another epithelial-linked marker, was also examined and showed relatively similar expression levels in both ALDH$^{hi}$ and ALDH$^{lo}$ populations (Figure 3.10). Furthermore, the mesenchymal marker vimentin was present in the ALDH$^{hi}$ and ALDH$^{lo}$ populations, with a higher presence demonstrated in the ALDH$^{lo}$ group (Figure 3.10), indicating that the ALDH$^{hi}$ population is mostly devoid of mesenchymal cells. The active cell cycle marker Ki67 marks proliferating cells, and was used to examine the proliferation of the sorted cell populations. Interestingly, the ALDH$^{lo}$ population had a higher percentage of Ki67$^{+}$ cells (17 ± 1%) at the time of sort compared to the ALDH$^{hi}$ population (10 ± 1%) ($p<0.05$; Figure 3.11). This data suggests that cells in the ALDH$^{hi}$ population are not active in the cell cycle at the time of sort, and that the proliferating portion of the sorted pancreata is largely found in the ALDH$^{lo}$ cells.
Figure 3.8 Analysis of islet-specific hormone expressing cells in the sorted populations.

(A) Representative images for insulin (β-cell), glucagon (α-cell), and somatostatin (δ-cell) (green) staining in the ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} sorted cell nuclei, labeled by DAPI (blue). White arrows indicate positive cell staining. Scale bar: 15 µm. (B) Quantitative analysis of hormone\textsuperscript{+} co-localization in the sorted population. White bars, ALDH\textsuperscript{hi} group; black bars, ALDH\textsuperscript{lo} group. Data are expressed as mean ± SEM and analyzed using unpaired t-tests (n=3-4 sorted pancreata/group) * p<0.05 vs. ALDH\textsuperscript{lo} sorted population.
Figure 3.9 Characterization of sorted ALDH$^{\text{hi}}$ and ALDH$^{\text{lo}}$ populations for epithelial markers.

(A) Representative images for CK19 and E-cadherin, markers of epithelial staining (green), stained against the total ALDH$^{\text{hi}}$ and ALDH$^{\text{lo}}$ sorted cell nuclei, labeled by DAPI (blue). White arrows indicate positive cell staining. Scale bar: 15 µm. (B) Quantification of CK19$^+$ and E-cadherin$^+$ cells in the sorted cell population. White bars, ALDH$^{\text{hi}}$ group; black bar, ALDH$^{\text{lo}}$ group. Data are expressed as mean ± SEM and analyzed using unpaired t-tests (n=2-3 sorted pancreata/group).
Figure 3.10 Characterization of sorted ALDH$^{hi}$ and ALDH$^{lo}$ populations for epithelial and mesenchymal markers.

Representative images for epithelial marker $\beta$-catenin (A), and mesenchymal cell marker vimentin (B) (green), stained against the total ALDH$^{hi}$ and ALDH$^{lo}$ sorted cell nuclei, labeled by DAPI (blue). White arrows indicate positive cell staining. Scale bar: 15 µm. (C) Quantification of $\beta$-catenin$^+$ and vimentin$^+$ cells in the sorted cell population. White bars, ALDH$^{hi}$ group; black bar, ALDH$^{lo}$ group. Data are expressed as mean ± SEM and analyzed using unpaired t-tests (n=2-3 sorted pancreata/group)
Figure 3.11 Proliferative capacity of the ALDH$^{\text{hi}}$ and ALDH$^{\text{lo}}$ sorted cell populations using the cell cycle marker Ki67.

(A) Representative images for Ki67 proliferation in the sorted cell populations, labeled by DAPI (blue). White arrows indicate K67$^+$ cell staining. Scale bar: 15 µm. (B) Quantitative analysis of Ki67$^+$ cells in the total sorted cell populations. Data are expressed as mean ± SEM and analyzed using unpaired t-tests (n=3 sorted pancreata/group). * p<0.05 vs. ALDH$^{\text{lo}}$ sorted populations.
3.5 Examination of sorted ALDH$^{hi}$ cells cultured on extracellular matrix proteins

The selected extracellular matrices (ECM) coatings were tested to compare their suitability for promoting viability of ALDH$^{hi}$ sorted cells during short-term (24 hours) and long-term (5 days) culture conditions (Krishnamurthy et al. 2008). MTT staining was used to examine viable adhered cells at 24 hours (Figure 3.12A) and 5 days (Figure 3.12B) after initial seeding of 1 $\times$ 10$^4$ ALDH$^{hi}$ sorted cells/well. After 24 hours, viable adhered cells were scattered throughout wells in the control and ECM cultured groups (Figure 3.12A). Adhered cells cultured for a period of 5 days on control and select ECM coatings began to demonstrate cell-cell contact, forming viable monolayers; this was noticeable on the laminin, collagen I, and collagen IV coatings (Figure 3.12B). Fold change for the number of viable adhered cells on each ECM coating at 5 days was normalized to the 24 hour groups, and it was found that laminin and collagen I had a significantly higher fold change after 5 days of culture (Figure 3.12C). These results indicate that the laminin and collagen I coatings are ideal for the long-term promotion of viability and expansion in sorted pancreatic cells.
Figure 3.12 Effect of extracellular matrices on sorted ALDH\textsuperscript{hi} cell viability after 24 hours and 5 days of culture.

Phase-contrast images of ALDH\textsuperscript{hi} sorted cells on control- and select extracellular matrices- coated wells at 24 hours (A) and 5 days (B) after initial seeding of wells. MTT assays were used to confirm viability of adhered cells (dark blue). Scale bar: 100 µm. (C) Fold change of viable adhered cells on control and select extracellular matrices at 5 days. Data are expressed as mean ± SEM with an unpaired t-test analysis (n= 3 samples/ECM test). *p<0.05, **p<0.01 vs. viable adhered cells at 24 hours.
3.6 Characterization of sorted ALDH$^{\text{hi}}$ and ALDH$^{\text{lo}}$ cells expanded in vitro

Sorted ALDH$^{\text{hi}}$ and ALDH$^{\text{lo}}$ cell populations cultured for an extended period were analyzed to determine if the phenotype seen initially in the ALDH$^{\text{hi}}$ sorted cell population was maintained during long-term culture and expansion in vitro. During extended culture on collagen I–coated wells, the sorted ALDH$^{\text{hi}}$ and ALDH$^{\text{lo}}$ cells lost the epithelial-like cell monolayer and began to demonstrate spindle-shaped morphology (Figure 3.13A). Interestingly, both ALDH$^{\text{hi}}$ and ALDH$^{\text{lo}}$ cultured cells, which had demonstrated CK19 staining immediately after cell sorting, no longer expressed the CK19 marker in cultured cells (Figure 3.13B). The epithelial marker E-cadherin was retained in ALDH$^{\text{hi}}$ and ALDH$^{\text{lo}}$ cultured populations during expanding and passaged cultures, and the mesenchymal cell-associated marker vimentin was present in both populations in the expanding culture (Figure 3.13B). The presence of E-cadherin and vimentin after culture in both populations could imply that these cell types adhered and expanded in an in vitro environment, or that these cells had entered epithelial-to-mesenchymal transition (EMT), similar to previously reports in islet cell cultures (Gershengorn et al. 2004). qRT-PCR analysis of transcription factor expression in expansion culture groups showed relative decreases of SOX9, PDX-1, and NKX6.1 in the ALDH$^{\text{hi}}$ cultured cells, but maintained levels of NGN3 (Figure 3.14). The expression of INS mRNA showed similar levels between the ALDH$^{\text{hi}}$ and ALDH$^{\text{lo}}$ cultured groups, indicating that the difference seen in insulin expression immediately after cell sort was lost during the expanding culture period (Figure 3.14). Immunofluorescence staining was used to confirm these results as well as examine the change in pancreatic cell lineages of cultured cells. The cultured ALDH$^{\text{hi}}$ and ALDH$^{\text{lo}}$ groups retained SOX9 transcription factor immunofluorescence expression, but PDX-1, NGN3, and insulin staining were lost after expansion of the sorted cell populations (Figure 3.15), suggesting that these cells could be heading away from endocrine cell differentiation or that remaining cells belong to a non-endocrine pancreatic fate.
Figure 3.13 Morphological analysis of sorted ALDH$^{hi}$ and ALDH$^{lo}$ cell populations after extended culture.

(A) Representative phase-contrast image of spindle-shaped cultured cells immediately (0 days, left) and 3 days (right) after passaging onto collagen I coated wells. Scale bar: 200 µm. (B) Representative immunofluorescence image for E-cadherin, vimentin, and CK19 staining on cultured ALDH$^{hi}$ and ALDH$^{lo}$ cell populations. White arrows indicate positive cells in cultured population. Scale bar: 25 µm. (n=3-4 culture passages/group)
Figure 3.14 Expression of pancreatic transcription factors and hormones after expansion of ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} cells.

qRT-PCR analysis of SOX9, PDX-1, NGN3, NKKX6.1, and INS mRNA expression in cultured ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} cell populations. Data are normalized to the housekeeping gene 18S and analyzed using the comparative Ct method. White bars, ALDH\textsuperscript{hi} group; black bars, ALDH\textsuperscript{lo} group. Data are expressed as mean ± SEM and analyzed using unpaired t-tests (n=4 cultured passages/group)
Figure 3.15 Expression of transcription factors and the insulin hormone after expansion of ALDH$^{hi}$ and ALDH$^{lo}$ cells.

Representative immunofluorescence images for SOX9, PDX-1, NGN3, and insulin staining in cultured ALDH$^{hi}$ and ALDH$^{lo}$ cell populations. White arrows indicate marked nuclei SOX9$^+$ cells. Scale bar: 25 µm. (n=3-4 culture passages/group)
3.7 Differentiation of ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} cultured cells

To examine if the expanded sorted cells could form islet-like clusters and propagate towards β-cell fate, both ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} expanded cells were treated with differentiation medium and cultured for 7 days. Phase-contrast images under the inverted microscope demonstrated that islet-like clusters formed as early as 36 hours after differentiation medium was added, and large, dense clusters were seen more in the ALDH\textsuperscript{hi} group after 7 days of differentiation versus the ALDH\textsuperscript{lo} group (Figure 3.16). To determine if there were any changes in transcription factors and insulin gene expression after differentiation from cultured ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} cells, real-time RT-PCR analysis was conducted. The expression of PDX-1, NGN3 and INS mRNA was relatively elevated in the ALDH\textsuperscript{hi} cell clusters, compared to the ALDH\textsuperscript{lo} clusters (Figure 3.17), but no difference was seen when statistically analyzed. Similar expression levels of SOX9 and NXY6.1 were observed in those differentiated cell clusters (Figure 3.17).

Furthermore, immunofluorescence staining was conducted on differentiated ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} cell clusters to examine phenotypic changes that resulted from cluster formation. The weak expression of CK19, which was lost during the expansion of both cultured groups, was noted in both ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} clusters (Figure 3.18). Vimentin and E-cadherin were co-stained and still present in clusters after differentiation culture (Figure 3.18), implying that the mesenchymal-epithelial cell switch occurs during the differentiation culture in both ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} clusters. Weak SOX9\textsuperscript{+} staining could also be seen in the clusters and single cells from the ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} differentiated groups (Figure 3.18).
Figure 3.16 Islet-like cluster formation during differentiation of cultured ALDH$^{hi}$ and ALDH$^{lo}$ cells.

Representative phase-contrast images of single cells to cluster formation in cultured ALDH$^{hi}$ and ALDH$^{lo}$ cells at 0 hours, 36 hours, and 7 days following differentiation medium exposure. Scale bar: 200 µm.
Figure 3.17 Expression of pancreatic transcription factors and hormone in islet-like clusters.

qRT-PCR analysis of SOX9, PDX-1, NGN3, NKX6.1, and INS mRNA expression in ALDH$^{hi}$ and ALDH$^{lo}$ clustered cells after a 7-day period in differentiation media. Data are normalized to the housekeeping gene 18S and analyzed using the comparative Ct method. White bars, ALDH$^{hi}$ group; black bars, ALDH$^{lo}$ group. Data are expressed as mean ± SEM and analyzed using unpaired t-tests (mean ± SEM; n=3-5 cultured passages /group)
Figure 3.18 Morphological analysis of ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} clustered cells cultured in differentiating media for 7 days.

Representative immunofluorescence staining for CK19, E-cadherin, and SOX9 (green), and vimentin (red). Nuclei are stained by DAPI (blue). White arrows indicate CK19\textsuperscript{+}, E-cadherin\textsuperscript{+}, and SOX9\textsuperscript{+} cells, while yellow arrows indicate vimentin\textsuperscript{+} cells. Scale bar: 15 µm. (n=3 culture passages/group)
3.8 Examination and analysis of transplanted ALDH^{hi} and ALDH^{lo} sorted populations

Transplantation of fibrin-wrapped sorted populations into nude mice hosts was done to determine the effect that an \textit{in vivo} environment would have on the phenotype of the ALDH^{hi} population and differentiation towards the \(\beta\)-cell fate. ALDH^{hi} and ALDH^{lo} sorted cells wrapped in fibrin gel were analyzed at 24 hours of culture and 1 week after subcutaneous transplantation. It is noted that CD31\(^+\) cells were highly expressed in the ALDH^{lo} sorted cells compared to ALDH^{hi} cell population (\textbf{Figure 3.19A}). CD31\(^+\) cells were observed after 24h culture in fibrin gel and 1 week after subcutaneous transplantation of the ALDH^{lo} graft, and co-localization of the human-specific mitochondria marker HMS with CD31 was also seen occasionally in these grafts (\textbf{Figure 3.19B}), suggesting that vascularization is promoted in the ALDH^{lo} sorted population. Both fibrin-wrapped ALDH^{hi} and ALDH^{lo} cell grafts were clearly observed under the skin of the nude mice (\textbf{Figure 3.19C}). However, abundant vasculature formed around the fibrin-wrapped ALDH^{lo} cell graft and was clearly visible (\textbf{Figure 3.19C, right}) when compared to the fibrin-wrapped ALDH^{hi} cell graft (\textbf{Figure 3.19C, left}). Double immunofluorescence staining for Insulin and HMS (not shown) showed no noticeable change in co-localization or phenotype when compared to the sorted cells analyzed immediately after sort (0 hrs), and examination of the transplanted gels for these markers resulted in no bulk cell expression in either sorted cell group, demonstrating loss of insulin\(^+\) cells in the gel.
Figure 3.19 Analysis of an in vivo environment on vascularization in sorted and transplanted cells. (A) Representative immunofluorescence images for CD31 staining in sorted ALDH$^\text{hi}$ and ALDH$^\text{lo}$ populations (0 hours). White arrows, CD31$^+$ staining in population. Scale bar: 15 µm. (n=2-3 sorted populations/group). (B) Representative immunofluorescence images of CD31 (green) and HMS (red) staining in fibrin-gel wrapped sorted cells after 24 hours in culture and 1 week after subcutaneous transplantation. White arrow, positive staining in population. Yellow arrow, double positive staining. Dashed lines indicate co-localization of CD31 and HMS. Scale bar: 15 µm. (C) Representative graph images of vascularization in fibrin-wrapped ALDH$^\text{hi}$ and ALDH$^\text{lo}$ sorted cells after 1 week of subcutaneous transplantation in nude mice, graphs outlined by dashed lines (n=5 sorted populations/group).
Chapter 4 - Discussion

The purpose of this project was to examine the distribution of surface stem cell markers and endocrine-specific transcription factors in the ALDH$^{hi}$ sorted cell populations of the human fetal pancreas. This thesis hypothesized that ALDH$^{hi}$ cells represent a progenitor cell pool in the developing human pancreas that co-localizes with other established stem cell surface markers, expresses factors specific for endocrine cell differentiation, and propagates an endocrine cell population in vitro and in vivo. Using FACS and immunofluorescence staining to analyze the sorted populations, this thesis demonstrated that ALDH$^{hi}$ cells co-localized with stem cell markers (CD133), transcription factors (SOX9, PDX-1, NKX6.1), and endocrine hormones (insulin and somatostatin). Extended culture of ALDH$^{hi}$ sorted cells resulted in changes to the pancreatic cell population’s phenotype characterized immediately after sort, but differentiation induced cluster formation and restoration of some transcription factor expression in the expanded ALDH$^{hi}$ group. In summary, these results indicate that the ALDH$^{hi}$ cells of the human fetal pancreas could represent a potential endocrine precursor cell pool during pancreatic development. The following discussion section will address the five specific questions originally introduced at the beginning of this thesis.

4.1 Do CD133, CD34, or CD117 co-localize exclusively with the ALDH$^{hi}$ populations in dissociated human fetal pancreatic cells?

Analysis of the co-localization of established surface stem cell markers with ALDH$^{hi}$ human fetal pancreatic cells supports the categorization of this cell type as a precursor cell population during development. The co-staining of Aldefluor-treated pancreatic sorted cells against surface stem cell markers CD133, CD34, and CD117 resulted in distinctive expression of all three surface markers on ALDH$^{hi}$ sorted cells. However, this study only captures the later stages of human pancreatic development from 18 to 22 weeks of fetal age. The morphology of the pancreas in earlier stages (8 to 11 weeks of
fetal age) is vastly different from the more mature later stage organ, as examined previously (Lyttle et al. 2008), and the higher expression of ALDH in earlier developmental stages (Li et al. 2014) could affect co-localization percentages with these stem cell markers. For an accurate representation of ALDH and stem cell marker expression in the human fetal pancreas, the pancreas should be assessed during different developmental stages.

The highest percentage of co-localization was seen in CD133<sup>+</sup> cells within the ALDH<sup>hi</sup> sorted population, which correlated with our previous report in the human fetal pancreas (Li et al. 2014). CD133<sup>+</sup> cells in mESCs have been attributed as being not fully differentiated (Kania et al. 2005), which supports the work that the ALDH<sup>hi</sup> population with high CD133<sup>+</sup> signals in the developing pancreas could represent a pool of developmentally immature precursor cells. Due to the advanced morphology of the pancreas at this fetal age, the CD133<sup>+</sup> cell population could also be composed of the established ductal structures previously reported in human fetal pancreata (Sugiyama et al. 2007). This indicates that the ALDH<sup>hi</sup>/CD133<sup>+</sup> sorted cell population represents a heterogeneous pancreatic precursor pool. However, NGN3<sup>+</sup> cells have been found in the ductal cells that express CD133 (Sugiyama et al. 2007), implying that endocrine cells can develop from this population. Furthermore, the percent of CD133<sup>+</sup> cells that do not co-localize with ALDH<sup>hi</sup> cells is small, indicating that CD133<sup>+</sup> cells are almost always found on cells with high ALDH activity during later stages of pancreatic development. Thus, the ALDH<sup>hi</sup>/CD133<sup>+</sup> sorted population could denote one population for isolating a pancreatic precursor pool that can differentiate towards endocrine cell fate, due to both markers’ associations with islet cell production and the co-localization exclusivity seen during late stage development.

It is noted that only ~25% of ALDH<sup>hi</sup> cells co-localized with CD34; however, a significantly higher number of CD34<sup>+</sup> cells are found in the ALDH<sup>lo</sup> cell population. The association of CD34 with pancreatic progenitor cells is complex, since CD34<sup>+</sup> cells isolated from the murine pancreas can give rise to endothelial cells in culture (Oshima et al. 2007). Our previous study also found that endothelial cells (identified by CD31<sup>+</sup> cells) did not co-localize with ALDH in the developing human pancreas (Li et al. 2014),
suggesting that ALDH\textsuperscript{lo}/CD34\textsuperscript{+} population may represent a progenitor pool for endothelial development. It is possible that the smaller ALDH\textsuperscript{hi}/CD34\textsuperscript{+} population could be specifically related to endocrine cell differentiation, but there are not enough published results to support this statement. Thus, further studies are needed to analyze the composition of this population immediately after sort and examine its overlap with known endothelial cell markers.

Unlike CD133 and CD34, the marker CD117 is relatively unchanged between the sorted ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} populations. The percentage of CD117\textsuperscript{+} cells in both populations also varied heavily between sorts. This data does not agree with our previous study where ALDH\textsuperscript{hi} cells had significantly high co-localization with CD117\textsuperscript{+} cells compared to the ALDH\textsuperscript{lo} population (Li et al. 2014). One possible explanation for this difference is that sampled pancreata were acquired from different age groups; the previous study used pancreata from 13 to 22 weeks of fetal age, while this study used pancreata from 18 to 22 weeks. It is also important to note that there is a significant age-dependent decrease of CD117 expression in certain parts of the pancreas as age increases (Li et al. 2007). This could indicate that the later stage pancreata examined during this study no longer contain a sortable CD117\textsuperscript{+} population, and that the ALDH\textsuperscript{hi}/CD117\textsuperscript{+} sorted population, though it could contain endocrine precursor characteristics, does not represent a significant population of pancreatic cells at this fetal age. The ALDH\textsuperscript{hi}/CD34\textsuperscript{+} and ALDH\textsuperscript{hi}/CD117\textsuperscript{+} cells could be analyzed for endocrine precursor potential, but the goal of this study was to identify surface stem cell markers that co-localized highly with ALDH\textsuperscript{hi} cells during development at this fetal age. Therefore, future studies should primarily focus on characterizing ALDH\textsuperscript{hi}/CD133\textsuperscript{+} sorted cells.

The co-localization of CD133, CD34, and CD117 on ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} sorted cells was essential for examining if they exclusively co-localized with the ALDH populations, or if there was significant overlap between the surface cell markers. When analyzing the sorted populations, it was seen that CD117\textsuperscript{+}/CD34\textsuperscript{+} cells had the highest co-localization in both the ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} groups, while CD133\textsuperscript{+}/CD117\textsuperscript{+} and CD34\textsuperscript{+}/CD133\textsuperscript{+} cells co-localized in both groups at a lower frequency, demonstrating that none of the selected surface stem cell markers are independent from one another in the sorted
populations. Co-localization of these markers with the ALDH$^{hi}$ group has not been previously examined in the developing pancreas, but ALDH$^{hi}$/CD133$^+$/CD34$^+$/CD117$^+$ umbilical cord blood hematopoietic stem cells have been shown to improve diabetic conditions when transplanted into murine pancreata (Bell et al. 2012). This suggests that the overlapping populations of surface stem cell markers in the sorted ALDH$^{hi}$ cells aid in improving islet regeneration in damaged islets, and that ALDH$^{hi}$ populations with more than one stem cell marker attributed to them could be an important cell pool for regeneration and repair therapies in diabetics.

4.2 Does the ALDH$^{hi}$ sorted population contain TFs necessary for endocrine cell development?

An established set of transcription factors necessary for endocrine cell development has been reported in whole sections of the human fetal pancreas (Lyttle et al. 2008) and has also been briefly examined in co-localized ALDH cells of whole fetal pancreatic tissue (Li et al. 2014), but has not been characterized in sorted cells with ALDH$^{hi}$ activity. The results from qRT-PCR analysis demonstrated relatively increased gene expression of TFs important for endocrine cell differentiation in the ALDH$^{hi}$ sorted cells, which correlates with data gathered from immunofluorescence staining; however, none of them showed statistically significant differences between ALDH$^{hi}$ and ALDH$^{lo}$ populations at the mRNA level. The possible reasons associated with this result may be explained due to the following: (1) the extracted RNA concentrations from sorted single cells were lower (0.1-0.2 µg/µl) than those gathered from whole pancreas samples; (2) the fetal ages of gathered samples demonstrated extreme variability due to being separated by up to four weeks in developmental age; and (3) low cell viability after cell sorting.

Immunofluorescence staining was used to confirm the mRNA expression results and to detect transcription factor activity in the nuclei of sorted populations. There are a number of transcription factors that were highly expressed in the ALDH$^{hi}$ sorted population, including SOX9, PDX-1, NGN3, NKX6.1 and PAX6. SOX9 was the transcription factor with the highest nuclear presence in ALDH$^{hi}$ cells. The SOX transcription factor class has been heavily associated with propagating pancreatic development (McDonald et al.)
SOX9, which is up-regulated during early fetal pancreatic development and is reduced significantly with age (McDonald et al. 2012), has been previously linked to ALDH activity within a breast cancer-derived cell line, where treating cells with ATRA resulted in an increase of SOX9 mRNA (Muller et al. 2010). This correlates with our ALDH study, where DEAB treatment of isolated fetal islet-epithelial clusters reduced the number of SOX9+ cells (Li et al. 2014). The results from previous research combined with the results from this study indicate that SOX9 can be regulated by retinoic acid, and that ALDH activity encourages SOX9 expression during development. It is also important to note that the ALDHlo sorted cells also contain a small SOX9+ cell population. The expression of SOX9 in the pancreas can be induced by factors other than retinoic acid, notably through Fgf10-Fgfr2b signaling (Seymour et al. 2012, Belo et al. 2013). Thus, there could be a complex signaling network driving SOX9 expression, where SOX9 activity can be dependent or independent of retinoic acid production.

Since SOX9 is a transcription factor associated with pancreatic progenitors, its high percentage in the ALDHhi sorted population indicates that this group contains a significant portion of precursor cells. One progenitor fate that SOX9 populations could represent is the endocrine cell-producing ductal epithelium, due to the high number of CD133+ cells in the ALDHhi population and the localization of SOX9 in ductal cells during development (Sugiyama et al. 2007, Lardon et al. 2008, Kopp et al. 2011). SOX9 expression is also associated with NGN3+ and insulin+ cells as determined previously by over-expression and knockdown of SOX9 in islet-like cell clusters, indicating its role in β-cell development (McDonald et al. 2012). However, a higher number of PDX-1+, NGN3+, NKX6.1+, and insulin+ cells were seen in the ALDHhi group, all previously associated with promoting insulin+ cell-like fate (Lyttle et al. 2008). Previous research reported an increase of the transcription factors PDX-1 and NGN3 during the differentiation of isolated islet-like clusters from human fetal pancreata (Zhang et al. 2013), reflecting their up-regulation during the commitment towards islet cell fate in the pancreas. This information, combined with this thesis’s results, indicates that ALDHi sorted cells have a higher population of cells currently differentiating towards insulin-secreting cell fate. Endocrine precursor cells tend to lose their SOX9 expression once NGN3 activity is up-regulated (Shih et al. 2012, Belo et al. 2013), therefore the NGN3+
population in the ALDH\textsuperscript{hi} sorted group could be derived from the SOX9\textsuperscript{+} cells. Although SOX9 expression decreases in NGN3\textsuperscript{+} and insulin\textsuperscript{+} cells in later stages of development (McDonald et al. 2012), it is still seen in the sorted late stage cells, suggesting that SOX9\textsuperscript{+} cells in the ALDH\textsuperscript{hi} sorted group continue to contribute to the precursor cell pool. Indeed, our previous study found that the number of insulin\textsuperscript{+} cells in isolated islet-epithelial clusters was up-regulated by increasing ALDH activity and the presence of retinoic acid (Li et al. 2014); these results parallel the SOX9 research done on the same cell source, where increases of Insulin expression were observed with SOX9 overexpression (McDonald et al. 2012). Therefore, the higher association of insulin cells in the ALDH\textsuperscript{hi} sorted population could be promoted due to the high ALDH\textsuperscript{hi}/SOX9\textsuperscript{+} co-localization in the sorted population.

4.3 Could ALDH\textsuperscript{hi} cells maintain their phenotypes during culture \textit{in vitro}?

Extracellular matrix (ECM) proteins are present during structural development of the pancreas (Hisao et al. 1993) and have previously been used to increase cell survival in \textit{ex vivo} culture conditions (Pinkse et al. 2006). By modifying the protocol from Krishnamurthy et al. (2008), this study found that viability and expansion of the ALDH\textsuperscript{hi} sorted population significantly improved when cultured on laminin and collagen I coated wells. Collagen I has been shown to improve viability, proliferation, and function of INS-1 cells to maintain their cell phenotype (Krishnamurthy et al. 2008). Therefore, this study used collagen I matrix coating for ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} sorted cell culture and expansion. Interestingly, the expanded ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} sorted cells resulted in the formation of spindle-like monolayers in wells. Similar monolayers were also observed in other human islet pancreas studies (Gershengorn et al. 2004, Joglekar et al. 2009). Both ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} expanded cell populations showed vimentin and E-cadherin as well as the loss of CK19, indicating epithelial cell and mesenchymal phenotypes during culture. This could indicate that both cultured cell populations, which demonstrated similar cell lineage phenotypes of E-cadherin and \(\beta\)-catenin immediately after sort, enter epithelial-to-mesenchymal transition (EMT) for the purpose of expanding the population. There is
established evidence demonstrating that insulin-expressing cells from plated islets can enter EMT (Gershengorn et al. 2004). Additional research also showed correlations between the mesenchymal marker vimentin and the presence of insulin (Davani et al 2007, Cole et al. 2009). This could also account for the loss of CK19 from the cultured populations. However, a few vimentin$^+$ cells were present in the ALDH$^{hi}$ sorted population, and these cells could have adhered and successfully expanded from the initial seeded population, and thus the presence of vimentin may not necessarily indicate EMT. Previous research demonstrated that β-cells from isolated islets do not enter EMT and thus do not form mesenchymal cells (Atouf et al 2007). At least two possibilities could account for the presence of mesenchymal markers in the cultured cell population: initial mesenchymal cells from the original population that have expanded alongside e-cadherin$^+$ cells, or EMT as verified in differentiation experiments, where CK19$^+$ cells were restored in expanded cells that formed cell clusters.

Examining the mRNA expression of transcription factors in cells after extended culture resulted in maintained $NGN3$ expression in the ALDH$^{hi}$ group. One possibility for this could be due to the reported relationship between SOX9 and $NGN3$ expression in cells, since SOX9 is still present as determined by immunofluorescence staining of the expanded populations. Sox9 in developing murine pancreata is important for $Ngn3$ production, and increased $Ngn3$ led to down-regulated Sox9 expression (Shih et al. 2012). This could indicate that cultured cells from the ALDH$^{hi}$ population may continue with commitment to the endocrine cell fate, and once again implies that the SOX9$^+$ cells in the ALDH$^{hi}$ population are primed precursors for endocrine cell differentiation. However, the decrease in $NKX6.1$ and the low expression of $Insulin$ do not indicate β-cell specificity. Therefore, the retained presence of SOX9 after cell culture expansion could indicate an early endocrine precursor fate that can reach islet cell specificity when treated with differentiation factors, and this will be examined in the next section.
4.4 Do ALDH$^{hi}$ cultured cells give rise to islet-like cell clusters?

The formation of clusters was seen in ALDH$^{hi}$ wells when treated with media previously applied to initiate islet-like cluster differentiation (Gershengorn et al. 2004, Wu et al. 2010), though the ALDH$^{lo}$ population also had cluster formation. In vitro studies in different neoplastic populations have found increased sphere formation in ALDH$^{hi}$ neoplastic cultures when compared to the ALDH$^{lo}$ colonies (Li et al. 2010, Soehngen et al. 2013). The mRNA expression of transcription factors PDX-1 and NGN3, and the hormone Insulin, in the ALDH$^{hi}$ cluster group suggests that the ALDH$^{hi}$ group is primed towards endocrine cell differentiation after cluster formation. These changes detected at the level of gene expression reflect the NGN3 increase in ALDH$^{hi}$ expanded cells, and further reflect the importance of NGN3 as an endocrine precursor factor.

Strong immunofluorescence staining for E-cadherin and vimentin demonstrated that the mesenchymal and epithelial cells seen during undifferentiated culture and expansion were still present in ALDH$^{hi}$ and ALDH$^{lo}$ clusters, which corresponds to previous cluster differentiation retaining Vimentin expression (Gershengorn et al. 2004). CK19 and SOX9 expression were seen in differentiated clusters, and the relationship between the two during development should also be considered for these clustered cells, since SOX9$^+$ cells in the duct have been reported as a pancreatic cell precursor pool that can contribute to β-cell production during development (Kopp et al. 2011), and have a debated role regarding β-cell production in injured adult pancreata (Kopp et al. 2011, Van de Casteele et al. 2014). One interesting point to note is that SOX9 is retained at the protein level, but there is no change between ALDH$^{hi}$ and ALDH$^{lo}$ cluster SOX9 mRNA. It can be concluded from the expanded and differentiated data that SOX9 activity retains a pancreatic precursor fate that has the ability to form endocrine precursor cells. This study also demonstrated that specific culture conditions are needed to begin cell differentiation towards endocrine fate, and that SOX9 and NGN3 expression are important, but not sufficient, for cell commitment. Additional differentiation factors that have demonstrated insulin$^+$ cell production, like nicotinamide (Ye et al. 2006) and taurine (Gopurappilly et
al. 2013), could be implemented to further stimulate these clusters towards a β-cell–like fate.

4.5 Does the subcutaneous transplantation of ALDH$^\text{hi}$ sorted cells, wrapped in fibrin gel, enhance differentiation towards the endocrine cell type in nude mice?

Positive results have been reported in experiments combining a three-dimensional matrix system to support isolated islets in vitro and in vivo (Daoud et al. 2011, Hosseini-Tabatabaei et al. 2013, Riopel et al. 2013), so fibrin gels were implemented in the sorted populations to examine their differentiation potential in vivo. However, insulin staining was not seen in the cells after transplantation. There are many possible reasons as to why this was seen, including: 1) the low amount of cells transplanted into each graft; 2) the transplantation time interval in the host mouse could affect the differentiation potential of the sorted cells; and 3) the nude mice were in a normal glucose metabolic status, and thus the drive towards insulin$^+$ cells was not required. NKX6.1$^\text{hi}$ hESCs transplanted into mice for a 5-month time period were able to develop into endocrine cells that restored glucose tolerance in diabetic mice (Rezania et al. 2013), suggesting that cells may need a specific incubation time and a diabetic situation to develop in vivo.

The most significant change observed between the sorted cell groups was the vascularization seen in ALDH$^\text{lo}$ fibrin-wrapped sorted cells. The lack of CD31 in the ALDH$^\text{hi}$ population has been reported in our previous study (Li et al. 2014), and this result correlates to the current study and shows that ALDH$^\text{hi}$ cells do not promote vascularization. CD34 is heavily associated with developing vasculature in the human fetal pancreas, and has been shown to associate closely with developing islets (Piper et al. 2004, Sarkar et al. 2008). This is one possible reason as to why there is higher co-localization of CD34 in the ALDH$^\text{lo}$ sorted group. Therefore, further analysis should be conducted in the ALDH$^\text{lo}$ group to determine the co-localizaton of CD34 with CD31.
4.6 Proposed mechanism of ALDH\textsuperscript{hi} cell activity in the human fetal pancreas

By characterizing the ALDH\textsuperscript{hi} sorted population and analyzing the changes seen in culture, in clusters, and when transplanted, a mechanism for ALDH activity in the developing human fetal pancreata is proposed below (\textbf{Figure 4.1}). In the developing human fetal pancreata (18-22 weeks of fetal age), ALDH\textsuperscript{hi} sorted cells represent a CD133\textsuperscript{+} pancreatic population, with an enriched pool of endocrine precursor cells. In contrast, the ALDH\textsuperscript{lo} sorted group primarily co-localizes with the CD34 marker and contains a higher population of cells expressing mesenchymal and endothelial cell fate markers. Both populations contain epithelial cells, which may house the remaining ductal and acinar tissue in the sorted cells. This information demonstrates that separation of ALDH\textsuperscript{hi} cells from the total pancreatic population isolates a unique precursor source of cells geared towards endocrine cell differentiation.
Figure 4.1 Proposed fate commitment of cells with varying ALDH activity during human fetal pancreatic development. The ALDH$^{hi}$ developing population contains CD133-enriched cells and a pool of endocrine precursor cells. Additionally, the ALDH$^{lo}$ group expresses high levels of CD34 and represents a significant mesenchymal cell pool.
4.7 Limitations encountered in this thesis

Although FACS has previously been performed in ALDH<sup>hi</sup> cells from different cell types, extensive studies that solely utilize sorted ALDH<sup>hi</sup> cells from the human fetal pancreas had not been attempted. Established protocols had to be optimized since the sorted cells used were from a primary developing organ source. One limitation mentioned earlier was the unavoidable prolonged incubation of dissociated fetal cells. Maintaining these cells in this traumatized state may not have only affected the cell viability, but could have skewed the cellular composition of the final sorted population. Previous cell sorts demonstrated that almost half of the ALDH<sup>hi</sup> sorted group were E-cadherin<sup>+</sup> cells (Li et al. 2014), unlike the results of this thesis that found less than 10% of E-cadherin<sup>+</sup> cells were in the ALDH<sup>hi</sup> group. Furthermore, exocrine markers that have been previously found in the human fetal pancreas were not visible in the ALDH<sup>hi</sup> or ALDH<sup>lo</sup> sorted groups when using immunofluorescence labeling, although they were visible in ALDH<sup>hi</sup> and ALDH<sup>lo</sup> cells of whole fetal pancreas sections (Li et al. 2014). This is another critical limitation of this study due to the unknown percentage of ALDH<sup>hi</sup> sorted cells that expressed exocrine markers, and challenges using ALDH<sup>hi</sup> cells as precursor markers for endocrine fate since this population could also give rise to a significant number of exocrine cells as well. It is possible that the cells expressing exocrine markers were excluded from the sorts due to poor viability of this cell type. One solution for examining the whole cell composition of the sorted pancreas is to fluorescently label specific markers of each cell type (ex: using E-cadherin for epithelial cells) and analyze their expression during FACS. This will be able to tell us if the populations are at least present in the whole tissue sample, even if they are not viable sorted cells.

Due to the precise nature of cell sorting and the strict removal of all cells that do not fall within the gated populations of interest, a large amount of cells are excluded from the sorted population, which results in a low final cell number in both the ALDH<sup>hi</sup> and ALDH<sup>lo</sup> sorted populations. This resulted in sample limitations for all following experiments, including low RNA concentrations for qRT-PCR, smaller sample sizes for immunofluorescence analysis, and a limited number of cells for transplanted fibrin-wrapped sorted gels. Although the solutions for tackling these limitations are restricted, it
is possible to increase the sorted number without including cells outside of the sorted gates (and thus introducing cell populations that could contaminate the purity of the marker being examined) by merging fetal pancreatic samples of similar developmental ages. Proceeding with this introduces an additional limitation to this study: obtaining human fetal pancreata is difficult, so merging samples reduces the sample size number examined. It is also important to consider the unique development of each individual pancreas and the possibility of skewing results due to this. Genetic and maternal differences will be different between the samples, and these changes have the possibility of strongly affecting fetal development.

Finally, challenges associated with culturing the sorted populations for expansion and differentiation were encountered. Expanding the sorted ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} populations did not maintain their phenotypes immediately seen after sort, and resulted in a loss of ALDH activity. In addition, the transcription factor expression differences observed between ALDH\textsuperscript{hi} and ALDH\textsuperscript{lo} populations was also lost, meaning that further studies examining a larger population of these cells was not feasible without exogenous stimulation towards differentiation. While clustering of these expanded cells allowed for limited differentiation towards the endocrine cell fate, maintenance of these clusters was difficult to continue. Extended exposure to the serum-free medium not only reduced cell proliferation in previous experiments, but also increased cellular death at day 7 of differentiation (Wu et al. 2010). Using three-dimensional matrices to house these clusters after initial differentiation could be one method to extend cell viability and prolong cell proliferation in vitro, since this method has demonstrated some success in past studies (Riopel et al. 2013).

4.8 Future studies and concluding remarks

This thesis characterizes the surface stem cell markers, transcription factors, and hormones associated with ALDH\textsuperscript{hi} sorted cells of the human fetal pancreas, and examines the maintenance of their phenotype in vitro and in vivo. The high co-localization of ALDH\textsuperscript{hi} sorted cells with cell markers, notably CD133, and their high expression of SOX9 supports their role as a precursor for pancreatic cell fate. Analyzing endocrine-
linked markers and concluding that the $\text{ALDH}^{hi}$ sorted population had a high percentage of islet-specific transcription factors and hormone cells indicates the suitability of using ALDH activity as a marker for isolating endocrine precursors. In addition, prolonged culture of $\text{ALDH}^{hi}$ cells decreases pancreatic transcription factor expression and can encourage EMT conditions, but stimulation of cluster formation restores endocrine progenitor fate. Further studies should be conducted that focus on examining $\text{ALDH}^{hi}/\text{CD133}^+$ cells as ideal candidates for the generation of islet cells. By characterizing the $\text{ALDH}^{hi}/\text{CD133}^+$ sorted cell populations, it will be possible to compare the percentage of islet cell transcription factors and hormone cells to the $\text{ALDH}^{hi}$ sorted cells and see if the $\text{ALDH}^{hi}/\text{CD133}^+$ population has a specific commitment towards endocrine cell fate. Furthermore, the $\text{ALDH}^{hi}/\text{CD133}^+$ cells should be examined in an in vitro and in vivo environment to see if they display the phenotype that retains the expression of the pancreatic progenitor marker SOX9 as well as endocrine cell markers, allowing for the generation of islet cells. As a final point, transplantation of the differentiated $\text{ALDH}^{hi}$ and $\text{ALDH}^{lo}$ clusters wrapped in fibrin gel would be interesting to examine in order to see if an in vivo environment would encourage differentiation towards insulin-producing $\beta$-like cell clusters.
Chapter 5 - References


Moreb JS, Ucar D, Han S, Amory JK, Goldstein AS, Ostmark B, Chang LJ. 2012. The enzymatic activity of human aldehyde dehydrogenases 1A2 and 2 (ALDH1A2 and ALDH2) is detected by aldefluor, inhibited by diethylaminobenzaldehyde and has significant effects on cell proliferation and drug resistance. Chem Biol Interact 195(1):52-60.


Appendices
Appendix A: Use of Human Participants- Ethics Approval Form

Use of Human Participants - Ethics Approval Notice

Principal Investigator: Dr. Rennie Wang
Review Number: 10000
Review Level: Delegated
Approved Local Adult Participants: 100
Approved Local Minor Participants: 0
Protocol Title: Development of Human Fetal Pancreas
Department & Institution: Physiology, London Health Sciences Centre
Sponsor: Natural Sciences and Engineering Research Council

Ethics Approval Date: December 22, 2011
Expiry Date: April 30, 2016

Documents Reviewed & Approved & Documents Received for Information:

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This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines, and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB’s periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The UWO HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB0000004A.

Signature

Ethics Officer to Contact for Further Information

This is an official document. Please retain the original in your files.

The University of Western Ontario
Office of Research Ethics
Support Services Building Room 5150 • London, Ontario • CANADA – N6G 1G9
PH: 519-661-3036 • F: 519-850-2466 • ethics@uwo.ca • www.uwo.ca/research/ethics
# Appendix B: Classification II Laboratory Approval Form

## University of Western Ontario Permit Summary

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### Organism
- Human (primary), fetal pancreas and duodenum, Rodent (primary), mouse pancreatic islets, human (established), PANC-1, HEK293, Rodent (established), INS-1, AR42J

### Human
- Organs and tissues (unpreserved), organs and tissues (preserved)

### Gene Therapy
- Other OFF CAMPUS

### GMO
- B6 mice

### Toxin
- Streptozotocin, Tamoxifen

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University of Western Ontario
Permit Summary

Permit Holder: Wang, Rennian
Permit #: BIO-LHR-0046
Classification: 2
Department: Physiology
Phone: Ext. 
Email: 
Approval Date: Apr 25, 2014
Expiration Date: Apr 24, 2017

Permit Conditions
1. INTERNAL PERMIT HOLDER RESPONSIBILITIES
   Comply with UWO BioSafety Safety Policies and Standard Operating Procedures. Ensure that the Health Canada Biosafety Guidelines, relevant regulations and safe laboratory practices are followed.
   1.1 Receive adequate biosafety training from the institution. Permit Holders are responsible for the provision of specific training and instruction in biohazard agent handling that is necessary for the safe use of this material in their own laboratories. Supervisors must ensure that workers understand the health and safety hazards of the work or task (due diligence).
   1.2 Ensure that the UWO Biosafety Manual is available to all lab personnel under the permit.
   1.3 Report incidents of loss or theft of any biohazardous material immediately to the Biosafety Coordinator;

2. WORKER RESPONSIBILITIES
   Be familiar with the UWO Biosafety Manual, attend all required safety training sessions and obey all safety regulations required by the UWO Biosafety Committee.
   2.1 Report to the Permit Holder any incident involving known or suspected exposure, personal contamination or a spill involving a biohazardous agent.

I accept the above responsibilities as an Internal Permit Holder and I am accountable for following UWO BioSafety Guidelines and Procedures Manual for Containment Level 1 and 2 Laboratories.

Permit Holder Name ____________________________ Signed ____________________________ Date ____________________________

April-28-14
Appendix C: BioSafety Approval Form

April 28, 2014

Dear Dr. Wang:

Please note your biosafety approval number listed above. This number is very useful to you as a researcher working with biohazards. It is a requirement for your research grants, purchasing of biohazardous materials and Level 2 inspections.

Research Grants:
- This number is required information for any research grants involving biohazards. Please provide this number to Research Services when requested.

Purchasing Materials:
- This number must be included on purchase orders for Level 1 or Level 2 biohazards. When you order biohazardous material, use the on-line purchase ordering system (www.uwo.ca/finance/people/). In the “Comments to Purchasing” tab, include your name as the Researcher and your biosafety approval number.

Annual Inspections:
- If you have a Level 2 laboratory on campus, you are inspected every year. This is your permit number to allow you to work with Level 2 biohazards.

To maintain your Biosafety Approval, you need to:

- Ensure that you update your Biohazardous Agents Registry Form at least every three years, or when there are changes to the biohazards you are working with.
- Ensure that the people working in your laboratory are trained in Biosafety.
- Ensure that your laboratory follows the University of Western Ontario Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories.
- For more information, please see: www.uwo.ca/hr/safety/biosafety/.

Please let me know if you have questions or comments.

Regards,

Tony Hammoud
Biosafety Coordinator for Western

Researcher: Dr. Rennian Wang
Biosafety Approval Number: BIO-LHRI-0046
Expiry Date: April 24, 2017
Appendix D: Animal Use Protocol

AUP Number: 2008-038-04
AUP Title: Pancreatic Beta Cell Development: The Role of the c-Kit and Integrin Receptors
Yearly Renewal Date: 11/01/2014

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2008-038-04 has been approved, and will be approved for one year following the above review date.

This AUP number must be indicated when ordering animals for this project.
Animals for other projects may not be ordered under this AUP number.
Purchases of animals other than through this system must be cleared through the ACVS office.
Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee
Appendix E: Immunofluorescence staining for positive and negative antibody controls. ALDH<sup>hi</sup> sorted populations were stained for: Rabbit anti-SOX9 antibody and FITC-conjugated anti-rabbit IgG (+ve control); and FITC-conjugated anti-rabbit IgG only (2°–ve control). Nuclei populations are stained in blue (DAPI). Scale bar, 15 µm. Magnification, 40x
Appendix F: Melting curves (a) and derivative melting curves (b) of qRT-PCR products. Curves for 18S (left) and SOX9 (right) demonstrate the amplification of target sequences in qRT-PCR reactions.
Curriculum Vitae

AMANDA OAKIE

EDUCATION

**MASTER OF SCIENCE**

*Physiology and Pharmacology* - Western University  
September 2012 to Present  
London, Ontario

**BACHELOR OF SCIENCE**

*Biomedical Sciences* - University of Guelph  
September 2008 to June 2012  
Guelph, Ontario

PRESENTED ABSTRACTS

**Oakie A**, Li J, and Wang R. Examination of aldehyde dehydrogenase (ALDH) and stem cell marker-expressing cells for endocrine cell indicators in the human fetal pancreas

Accepted for presentation at:
- Lawson Health Research Day, Western University, March 2014
- Physiology and Pharmacology Research Day, Western University, November 2013
- Diabetes Research Day, Western University, November 2013

RELATED WORK EXPERIENCE

**Graduate Teaching Assistant**

Course: Physiology 3130y  
Western University  
September 2013 to April 2014

PUBLICATIONS

**Manuscripts Published**


| AWARDS |
|------------------|------------------|
| **Western Graduate Research Scholarship**  | September 2012- Present |
| Faculty of Graduate Studies               |                  |
| Western University                        |                  |
| **Poster Presentation, First Place**      | November 2013    |
| Diabetes Research Day                     |                  |
| Western University                        |                  |