DIRECTED DIFFERENTIATION OF ENDODERMAL CELLS FROM MOUSE EMBRYONIC STEM CELLS

by

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ABSTRACT

Pluripotent embryonic stem cells hold a great promise as an unlimited source of tissue for treatment of chronic diseases such as Type 1 diabetes and chronic liver disease. Various attempts have been made to produce cells that can serve as precursors for pancreas and liver. By using all-*trans*-retinoic acid, basic fibroblast growth factor, dibutyryl cAMP, and cyclopamine, an attempt has been made to produce definitive endoderm and subsequently cells that can serve as pancreatic and hepatocyte precursors from mouse embryonic stem cells. By using retinoic acid and basic-FGF, in the absence of embryoid body formation, mouse embryonic stem cells were differentiated at different culture periods. Four protocols of varying lengths of culture and reagents and their cells were analyzed by quantitative PCR, immunohistochemistry and static insulin release assay for markers of trilaminar embryo, pancreas and hepatocytes. Inclusion of DBcAMP and extension of culture time resulted in cells that display features of definitive endoderm by expression of Sox 17 and FOXA2 and minimal expression of primitive endoderm and other germ cell layers such as ectoderm and mesoderm. These cells produced insulin and C-peptide and secreted insulin in a glucose responsive manner. However, they seem to lack mature insulin secretion mechanism. There was a production of hepatocyte markers (AFP-2 and transthyretin) but there was insufficient data to assess for convincing production of hepatocytes. In summary, one of the protocols produced cells that displayed characteristics of definitive endoderm and they may serve as pancreatic endocrine precursors.

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ADP	Adenosine Diphosphate
AFP	Alfa fetoprotein
ATP	Adenosine Triphosphate
FBS	Fetal Bovine Serum
bFGF	Basic Fibroblast Growth Factor
bHLH	Basic Helix Loop Helix
BMP	Bone morphogenetic protein
DBcAMP	Di-butyryl Cyclic Monophosphate
DMEM	Dulbecco's Modfied Essential Medium
EBs	Embryoid Bodies
EGF	Epidermal Growth Factor
EPI	Epiblast
ES cells	Embryonic Stem
ExE	Extraembryonic Ectoderm
FGF	Fibroblast growth factor
GRIP cells	Glucose Responsive Insulin Producing Cells
HGF	Hepatocyte Growth Factor
Hlxb	Homeobox Transcription Factor
HNF	Hepatocyte Nuclear Factor
IL	Interleukin
LIF	Leukemia Inhibitory Factor
LPS	Lipopolysaccharide

Mixl	Mix-Like Gene
MTG	Thioglycerol
PCR	Polymerase Chain Reaction
PE	Primitive Endoderm
PEF	Embryonic Fibroblasts
Ptf	Pancreas Transcription Factor
SHH	Sonic Hedge Hog
SMO	Spemann-Mangold Organizer
Sox	Sry-related HMG-box transcription factor
TE	Trophectoderm
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TTR	Transthyretin
VE	Visceral Endoderm

CHAPTER 1: INTRODUCTION

1. Diabetes Mellitus

1.1. History

From the time the term diabetes mellitus was first coined by Aretaios in Cappadocia in the first century [1], diabetes has been recognized as a disease that has afflicted patients worldwide. The discovery of insulin by Frederick Banting and Charles Best in 1921 in Toronto [2] has revolutionized the management of diabetes, converting it from a fatal to chronic disease which continues to affect millions of people worldwide.

1.2. Clinical Problem

Type 1 diabetes and its complications are becoming increasingly common and they pose a growing burden on the health care system. Diabetes affects estimated 200 million people worldwide. It is the third most common disease and the fourth leading cause of death in North America [3]. It affects 16 million Americans and consumes 1 out of 8 health care dollars in United States [4]. Insulin therapy, although effective, does not prevent the progression of the devastating microvascular and macrovascular complications. These complications which include, nephropathy, retinopathy, neuropathy and cardiovascular disease, claim many lives every year.

1.3. Treatments

The mainstay of treatment established by the Diabetes Control and Complications Trial is tight control of glucose levels by frequent insulin injection or by a pump which hays been shown to be protective against microvascular complications [5]. However, insulin therapy did not prevent development of secondary complications. The risk of tight

glucose control was a significantly increased risk of hypoglycemic episodes resulting in seizures or coma [5].

In addition to rigorous medical therapy, whole pancreas transplantation has been proven to prolong survival, and alleviate complications of diabetes [6 7 8]. However, Type 1 diabetes patients with complications of diabetes may not be suitable candidates for whole pancreas transplantation with or without simultaneous kidney transplantation due to morbidity associated with these operations.

An alternate form of islet replacement therapy is islet transplantation which was first attempted in 1894 by Williams [9]. Islet transplantation had not seen any successes in humans until the 1980s when anecdotal reports of allogeneic islet transplants began to appear [10]. The high success rates reported by Shapiro et al. in 2000 [11] propelled a multinational study on human islet transplantation and further infused energy and excitement into research into this field [12]. Although, these results are encouraging, islets are harvested from multiple donors and some patients required multiple transplants resulting in shortage of islets [11].

1.4. Challenges of Islet Transplantation

The main problem in islet transplantation is the shortage of donors. In light of the shortage of donors, in order for the islet transplantation to be widely accepted in the treatment of Type I diabetes, an alternate source of glucose responsive, insulin producing (GRIP) cells need to be identified. This need has inspired researchers to find ways of producing islets from stem cells.

Intensive research in making these GRIP cells in both humans and animals have taken place. In humans, Bonner- Weir et al. achieved islet budding from ductal tissue [13].

In experimental animals, a few groups produced islet like cell clusters by selecting for nestin positive cells based on the hypothesis that the pancreas and the central nervous system share genetic and developmental pathways [14, 15]. However, the initial excitement has waned as two studies have shown that the insulin detected in these cells originated from uptake from insulin containing media rather than de novo insulin synthesis [16, 17].

1.5. Insulin

1.5.1.Chemistry of Insulin

Human insulin is a small protein with a molecular weight of 5808 daltons. It is composed of two amino acid chains connected to each other by two disulfide linkages. If these two amino acid chains are split apart, the funtional activity of insulin is lost.

Insulin is synthesized in beta cells of the pancreas to preprohormone form and subsequently cleaved in the endoplasmic reticulum to form a proinsulin. The proinsulin is further cleaved in the Golgi apparatus to form insulin before being packaged into secretory granules. Still, about one-sixth of the insulin is secreted in proinsulin form which has no biological activity.

The half life of insulin is about 6 minutes and it is present in the bloodstream within 10-15 minutes before being cleared by insulinase mainly in the liver, and to the lesser extent in the kidneys and the muscles[18].

1.5.2. Mouse Insulin

There are two nonallelic insulin genes (insulin I and II) expressed during mouse development. Fetal yolk sac and liver predominantly express insulin II but also insulin I at much lower levels[19, 20]. Neuronal cells express only insulin II[21], and the pancreas expresses both insulin I and II [22].

1.5.3. Mechanism of Release

Tight regulation of insulin is essential in maintaining glucose homeostasis. Glucose stimulated insulin secretion is biphasic both *in vitro* and *in vivo*. The mechanism underlying this biphasic nature seems to depend on how far the cytoplasmic secretory vesicles are located from the cell membrane. The first phase secretion seems to involve the pre-docked vesicles whereas the second phase involves the new vesicles that are recruited to the cell membrane [23].

The mobilization and the release of the insulin containing vesicles is orchestrated by glucose which is taken up into the beta cells by liver-type glucose transporter Glut2 which eventually leads to elevation of total intracellular ATP content (or an increase in ATP:ADP ratio)[24]. The ATP then binds to the ATP-sensitive K^+ channel (K_{ATP}) leading to its closure and depolarization of the plasma membrane from -70mV to 0mV[25, 26]. This depolarization in turn opens the voltage sensitive L-type Ca^{2+} channels leading to Ca^{2+} influx[27]. It is this Ca^{2+} influx that mobilizes the secretory vesicles and causes their fusion with the cell membrane[28, 29]. Furthermore, Ca^{2+} is taken up by the mitochondria which enhances mitochondrial ATP synthesis and continued glucose signaling[30].

1.5.4. Function of Insulin

Insulin has profound effects on metabolism and storage of excess energy substances. With respect to carbohydrate metabolism, insulin causes uptake, storage, and use of glucose by almost all tissues of the body including the skeletal muscles, liver and the adipose tissues. Insulin also plays a role in fat metabolism. It spares fat by causing the body to utilize glucose rather than fat. In addition, insulin inhibits the action of hormone sensitive lipase and promotes glucose transport into the fat cells, leading to increased fat

storage. It causes active transport of amino acids into the cells and inhibits catabolism of proteins.

2. End Stage Liver Disease

2.1. History of Liver Disease

The liver has been an organ of interest since early civilization. In Greek mythology, Prometheus was chained to the Caucasus mountain and he was tortured by an eagle that attacked and ate parts of his liver on a daily basis. His liver then grew back overnight, ready for another round of "hepatophagy" by the eagle [31]. In Mesopotamia and in ancient Egypt, it was thought to be the "seat of life" and the "seat of the soul" [32]. Galen held a view that the liver was the source of all veins and the factory that made all the blood and view was maintained for nearly 1500 years until the cardiocentric view of the body came into the picture. Aristotle believed that the heart was the source that the body depended on for soul, heat and force and his views were supported by William Harvey's discovery of the circulation which was driven by the heart [33]. Although the world no longer holds the hepatocentric view of life, the liver is an essential organ that affects patients in the clinical setting today.

2.2. Clinical Problem and Treatments

End stage liver disease is becoming an increasingly common clinical problem caused by various factors such as viral infections (hepatitis B and C), toxins (alcohol and drugs), and autoimmune diseases. The presentation of the end stage liver disease is characterized by hepatic encephalopathy, coagulopathy, ascites, malnutrition and complications of portal hypertension such as bleeding esophageal varices.

Orthotopic liver transplantation is the mainstay treatment for liver replacement therapy. However, due to the increasing shortage of donor organs for liver transplantation, a considerable number of patients die while on the waiting list [34]. Despite the efforts to increase the donors by using split livers, living related donors, and marginal livers, the demand still outweighs the supply. As of August 8, 2005, 17,495 patients were on the waiting list for a liver nation wide in the United States [34]. This shortage has raised an interest in alternative forms of liver replacement therapy such as hepatocyte transplantation and bioartificial liver devices.

There have been anecdotal reports of hepatocyte transplantation in clinical setting and the most successful reports have been for patients with metabolic diseases. Some of the diseases that have seen short term successes include Crigler-Najjar syndrome type 1 [35], glycogen storage disease type 1a [36], urea cycle deficiency [37] and peroxisomal biogenesis disease [38]. In patients with fulminant liver failure, hepatocyte transplantation has been used as a bridge to orthotopic liver transplantation [39]. Although the number of hepatocytes involved in hepatocyte transplantation is lower than what would be involved in an orthotopic transplant, there is still a need for donors. To address this issue, researchers have looked into stem cells: (1) liver specific stem cells, also known as the oval cells, and (2) embryonic stem cells.

2.3. Alternate Sources of Liver Tissue

Embryonic stem cells, due to their pluripotent nature, have received much interest as an unlimited source for transplantation. Some encouraging preliminary studies have shown that *in vitro and in vivo*, the embryonic stem cells are capable of developing into hepatocyte like cells [40-43]. These studies used embryoid bodies (EBs) from embryonic

stem cells as the starting point but did not focus on producing hepatocyte like cells through the endoderm intermediate.

During embryogenesis, distinct cell lineages are established from pluripotent cells. The trophectoderm and primitive endoderm develop into extraembryonic tissues while the primitive ectoderm develops into all three germ layers including ectoderm, mesoderm and the definitive endoderm. It is the definitive endoderm that gives rise to liver, pancreas, thyroid and the digestive tract [44]. If there are residual embryonic stem cells that are still pluripotent, transplantation of these cells result in development of teratomas [45].

2.4. Concept of Liver Regeneration

In order to achieve successful differentiation of embryonic stem cells to hepatocytes, in addition to endoderm development, the endodermal cells need to be exposed to the growth factors and cytokines that are involved in liver regeneration. The ability for liver to regenerate was demonstrated definitively by Higgins and Anderson in 1931 [46] and the mechanism of liver regeneration was recently reviewed by Taub [47]. Factors such as tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-6 are released by Kupffer cells and stellate cells in response to lipopolysaccharide (LPS). These cytokines, in cooperation with signals such as hepatocyte growth factor (HGF) from stellate cells, epidermal growth factor (EGF) from the duodenum and salivary gland, insulin from the pancreas, triodothreonine from the thyroid gland and norepinephrine from the adrenal gland, push the hepatocytes from Go to S phase of the cell cycle, thus resulting in hepatocyte proliferation [47].

3. Embryonic Development and Embryonic Stem cells

3.1. Human Embryonic Development

First Week

After fertilization, the zygote embarks on a series of regulated cell divisions termed cleavage. Cleavage is not accompanied by cell growth, resulting in many daughter cells called blastomeres without increasing the total size of the embryo. By 3 days post fertilization, the embryo consists of 6-12 cells, and by day 4, the embryo cosists of 16-32 cells and when it reaches 32 cells, it takes on an appearance of a mulberry and hence it is termed a morula (from Latin *morum*, mulberry).

The cells of the morula not only give rise to the embryo proper but also to the placenta and related structures. In keeping with this development down the different paths, the blastomeres group in different parts: some cells to the center of the morula and some cells to the outside. The centrally placed blastomeres are called the inner cell mass and the blastomeres in the periphery are called the outer cell mass. The inner cell mass gives rise of the embryo proper and it is called the embryoblast and the outer cell mass gives rise to the placenta and is called trophoblast.

On day 4 of development, the morula begins to absorb fluid and the collection of this fluid, results in a cavity called the blastocyst cavity. The inner cell mass is now compacted to one side of the cavity and the outer cell mass, trophoblast, arranges itself into a thin, single layered epithelium. The embryo is now termed the blastocyst[48] . The embryonic stem cell lines are derived by harvesting the inner cell mass.

Second Week

In the second week, the embryoblast splits into the epiblast (primary ectoderm) and the hypoblast (primary endoderm) which constitute the bilaminar germ disc lying between the blastocyst cavity and the amniotic cavity. The cells of this germ disc develop into the embryo proper. The first new cavity to form in the second week is the amniotic cavity which develops within the epiblast and eventually is lined by the amniotic membrane which is formed by amnioblasts and separates the amniotic cavity from the cytotrophoblasts that arise from the trophoblasts. The amniotic cavity expands continuously and eventually surrounds the entire embryo.

The cells of the hypoblast migrate out over the cytotrophoblast and line the blastocyst cavity. These cells form a thin membrane of extraembryonic endoderm also known as the exocoelomic membrane or Heuser's membrane. The former blastocyst cavity is now called the primary yolk sac. Between the primary yolk sac and the cytotrophoblast, a thick, loose reticular, acellular layer called the extraembryonic reticulum is formed. Within the extraembryonic reticulum forms the chorionic cavity.

Third Week

On about day 15 of development, the primitive streak is formed along the bilaminar embryo. The primitive streak is a longitudinal structure which is consisted of a longitudinal groove called the primitive groove, a depression at the caudal end of the groove called the primitive pit and the mound surrounding the groove called the primitive node. The primitive streak establishes the longitudinal axis and the bilateral symmetry of the embryo.

On day 16, the cells of the epiblast begin to migrate through the primitive streak into the space between the epiblast and the hypoblast. This process of involution and

ingress is called gastrulation in which pluripotent epiblast cells are allocated to three principal germ layers – endoderm, ectoderm and mesoderm. As the epiblast cells ingress through the primitive streak, they undergo an epithelial-to-mesenchymal transition (EMT) and become either mesoderm or definitive endoderm. It is the definitive endoderm or entoderm which eventually gives rise to the future gut and other intraabdominal organs. Once the definitive endoderm is formed, the epiblast cells continue to migrate and move into the space between the epiblast and the definitive endoderm to for the intraembryonic mesoderm. When the intraembryonic mesoderm and the definitive endoderm have been formed, the epiblast takes on a new name, ectoderm, completing the formation of trilaminar embryo, all formed from the epiblast.

In the fourth week, the flat embryo grows rapidly undergoing the process of cephalocaudal and lateral folding and the embryo takes on an elongated cylindrical appearance. The innermost part of this cylinder is the endodermal primary gut tube consisted of the foregut, midgut, and the hindgut. The foregut gives rise to the duodenal endoderm. Both liver and the pancreas arise from the duodenal endoderm. The hepatic diverticulum from the duodenal endoderm grows into the septum transversum and gives rise to the hepatic parenchyma and the bile ducts. The dorsal and the ventral pancreatic diverticula also form from the duodenal diverticulum and later fuse to form one pancreas.

3.2. Mouse Embryonic Development

Early Development

In mice, after fertilization, the zygote develops into a blastocyst. By day 4.5, mouse blastocyst contains three cell types: epiblast (EPI), trophectoderm (TE) and primitive endoderm (PE). Trophectoderm gives rise to the extraembryonic ectoderm

(ExE) and all the trophoblast lineages that eventually form the majority of the fetal part of the placenta[50]. As illustrated in Figure 1, the PE gives rise to the visceral (VE) and parietal endoderm. The EPI gives rise to the entire fetus, allantois and the extraembryonic mesoderm cells that line the visceral yolk sac. On day 6, the embryo is referred to be in the early blastula stage. The blastula is also known as the blastosphere and is a stage prior to the gastrula.

While the trophoblasts (outer cells of the preimplanted embryo) are invading the uterine wall, the inner cell mass develops an epithelial structure distally. This structure has been variously named, primitive endoderm (PE), extraembryonic, primary endoderm or hypoblast. The primitive endoderm gives rise to two structures: parietal endoderm and the visceral endoderm[49]. Parietal endoderm gives rise to the parietal yolk sac which is critical for absorption of nutrients during early development and it protects the embryo from mechanical damage. The visceral endoderm later in gestation, together with extraembryonic mesoderm, gives rise to the visceral yolk sac[49]. These extraembryonic tissues are precursors of the placenta and serve multiple functions: they are required for invasion of the uterine lining, they protect the embryo and facilitate the embryo-maternal interactions.

Gastrulation

Gastrulation in mice, as in humans, is a set of morphogentic processes that occur during development that transforms an unstructured embryo into a gastrula with three germ layers: endoderm, mesoderm and ectoderm. Coined by Haeckel, it is derived from the Greek word, 'gaster' which means a stomach or a gut[51]. The early and later gastrula stages are illustrated in Figure 2. In mice, the early gastrula looks like a cup and

undergoes four morphogenetic movements: internalization, epiboly, convergence, and extension[51]. Internalization brings the cells of the prospective mesoderm and endoderm beneath the prospective ectoderm via an opening in the blastula called the primitive streak also known as the blastopore. Epiboly movements spread and thin germ layers. Convergence and extension movements narrow the germ layers mediolaterally, and elongate the embryo from head to tail. These movements are preceded and accompanied by processes that are largely in part controlled by the Spemman-Mangold organizer (SMO) located in the dorsal or axial aspect of the blastopore[52].

After gastrulation, the ectoderm gives rise to epidermis and neural tissues; the mesoderm gives rise to the muscles, cardiovascular, urogenital and skeletal elements of the body; and the endoderm will give rise to the digestive tract and its associated organs including the pancreas and the liver.

3.3. Pancreatic Differentiation

In mice, the pancreas arises from two patches of epithelium that bud dorsally and ventrally in the gut epithelium between the stomach and the duodenum on day E9.5. The ventral and dorsal pancreatic buds fuse by E16-17. A local block in signaling to endoderm by Notch specifies the location of budding from the gut. Pancreatic mesenchyme collects around the gut epithelium and induces pancreatic bud formation and branching. Each bud forms branched structures and the acini are visible by day E14.5[53]. The endocrine cells appear early during branching and represent 10% of the pancreas by day E15.5. Pancreatic tissue arises from the cells of definitive endoderm where the transcription factor Pdx1 (pancreas-duodenum homeobox) is expressed and where the extracellular signaling molecule sonic hedge hog (Shh) is repressed. The

ventral and the dorsal portions of the pancreas develop separately (Figure 3(a)). The dorsal pancreas receives its signals from the notochord and the aorta. The signaling from the notochord most likely inhibits Shh in the endoderm that will become the pancreas[53]. The aortic endothelial signals also are crucial in dorsal pancreatic development[54]. The ventral pancreas is not in contact with the notochord or the aorta but receives the inductive signals from the lateral plate mesoderm. The ventral pancreas owes its specification to Ptf1a (pancreas transcription factor, a basic helix-loop-helix transcription factor, or bHLH) whilst the dorsal pancreas owes its specification to the homeobox transcription factor Hlxb9. The overview of the transcription factors involved in pancreas and beta cell development is illustrated in Figure 3(b) adapted from Soria[55].

3.4. Hepatic Differentiation

Liver parenchyma, gallbladder and the biliary system bud from the duodenal endoderm (Figure 3(c)). From day 7.5 to 8.5 of the embryonic period of the mouse, anterior and posterior invaginations develop in the embryonic foregut and hindgut pockets. By E8.5-9.5, in the anterior-ventral domain of the foregut endoderm, develops buds for liver, lung, thyroid, and the ventral portion of the pancreas[56, 57].

The key element in hepatic differentiation comes from its interactions with the cardiac mesoderm. The cardiac mesoderm is fashioned at approximately E7.5 in mouse and it secretes fibroblast growth factor (FGF) which is necessary to induce hepatic fate in the endoderm. In addition to this signal, bone morphogentic protein (BMP) signaling from the septum transversum mesenchyme[56, 57].

3.5. Embryonic Stem Cells

Stem cells are a group of cells that are: (1) clonogenic, capable of unlimited self-renewal by symmetric division; (2) able to divide asymmetrically, one daughter cell resembling the mother, the other daughter giving rise to multiple types of cells representing the three germ layers (ectoderm, mesoderm and endoderm); and (3) it must originate from an adult or an embryonic source [58].

The embryonic stem (ES) cells are produced by harvesting the inner cell mass of the blastocyst and testing them for their function *in vivo*. These cells can be tested by injecting them into immunodeficient mice to produce teratomas or the markers of embryonic stem cells can be traced to mouse pups and assess the contribution of these cells to different types of tissue[59, 60]. Another approach which is used commonly in tissue culture is formation of embryoid bodies *in vitro* containing all three germ layers [61-63].

The human embryonic stem cell lines are created from cleavage- and blastocyst-stage embryos, produced by *in vitro* fertilization. The blastocysts are treated with Tyrode's solution to remove the zona pellucida, and immunosurgery is used to remove the inner cell masses[64]. The mouse embryonic stem cells are produced by isolating the inner cell mass (ICM) of the blastocyst. ICM is a precursor to the epiblast and it is plated onto a feeder layer of embryonic fibroblasts. These mitotically inactivated embryonic fibroblasts were found to result in efficient establishment of ES cell cultures, and resulted in production of ES cells with high differentiation capacity[65, 66]. The apparent benefit is thought to be from possible nutrient or trophic factor support provided by the fibroblasts, hence the name 'feeder' cells. The embryonic stem cells are initially cultured on these embryonic fibroblats in a medium containing leukemia inhibitory factor (to keep them

from differentiating). The conventional tissue culture medium is supplanted with 2-mercaptoethanol, 10-20% fetal calf serum[67]. After several days of culture, they are subsequently passaged with mechanical dissociation initially and eventually undergo enzymatic passage with trypsin then replated onto fresh feeders. Various types of differentiated colonies arise as well as the undifferentiated colonies. The undifferentiated colonies are individually dissociated and replated. If secondary colonies of undifferentiated cells arise, these can generally be expanded further and ES cell lines can then be established.

The mouse ES cells have been differentiated into various cell types in vitro with moderate efficiency. These cell types include: yolk sac endoderm[68], yolk sac mesoderm[68], primitive and definitive hematopoietic cells[61, 68-70], lymphoid precursors[71], mast cells[72], dendritic cells[73], endothelial cells[74, 75], cardiomyocyte[68, 76], striated muscle[77], smooth muscle[75], adipocyte[78], osteoblast[79], chondrocyte[80], keratinocyte[75, 81], neurons[82, 83], astrocyte[84], and oligodendrocyte[85, 86].

Due to their pluripotency, they can also form teratomas but have relatively consistent with their epiblast origin. The tissues derived from the ES cell lines are of epiblast origin (Figure 4), but they can produce cells of hypoblast (parietal endoderm, visceral endoderm and yolk sac) *in vitro* but rarely *in vivo*[67]. They contribute poorly to the extraembryonic endoderm and rarely to trophoblast[87].

3.6. Embryoid Bodies

The embryonic stem cells are initially cultured in the presence of feeder cells (namely embryonic fibroblasts) and leukemia inhibitory factor[88] that inhibit differentiation. One of the ways to generate differentiated cell types is through formation

of embryoid bodies (EBs). The EBs are three-dimensional, multicellular aggregates of differentiated and undifferentiated cells that ES cell spontaneously develop into once they are taken out of the environment containing LIF and the feeder cells [89]. The EBs range from simple aggregates of cells to more complex structures resembling embryos. A number of studies published previously have attempted to use EBs to make cells that resemble pancreas or tissues that have pancreatic fate. The formation EBs have been a useful step in differentiation of the embryonic stem cells but the time it takes for them to form, adds extra time to the overall culture time. They are also not suitable for easy maintenance and re-passaging due to their bulky structure.

3.7. Production of Definitive Endoderm

During embryogenesis, the liver and the pancreas arise from definitive endoderm[90] which is produced through the process of gastrulation from the embryonic ectoderm of the epiblast [91]. The dearth of markers of definitive endoderm has made it difficult to identify culture conditions to guide the ES differentiation down the endodermal route. Recently, transcriptional factors such as Sry-related HMG-box transcription factor Sox 17[92], primitive streak marker mix-like gene Mixl1[93], and FOXA2 (previously known as the hepatocyte nuclear factor (HNF) 3β)[94-97] have been proposed as markers of definitive endoderm. However, these markers are also expressed in primitive/parietal/visceral endoderm. This calls for monitoring of development of these markers as well as decreased expression of markers that are limited to primitive, parietal and visceral endoderm (e.g Sox 7). Furthermore, to assess for enrichment of the culture population with cells of definitive endoderm, a relative decrease in the expression of markers of mesoderm (e.g. brachyury) and ectoderm (e.g. Sox 1, Pax 6) should be observed. Finding

a way to efficiently produce definitive endoderm from the embryonic stem cells would be the key to ultimately produce beta cells and hepatocytes.

Some factors in the literature have been proposed to promote endoderm differentiation. Activin A, a member of the transforming growth factor-beta (TGF-β) superfamily has been reported to promote endoderm formation at high concentrations [98, 99]. D'Amour et al. reported an efficient protocol to produce definitive endoderm in human embryonic stem cells using Activin A and low serum yield culture populations that are enriched in definitive endoderm [100]. This group of investigators also outlined criteria for determining the development of definitive endoderm using multiple markers that would mimic the patterns of expression in gastrulation and production of definitive endoderm. An increase in expression of markers of definitive endoderm (Sox 17, GSC, FOXA2 (HNF3β) and MIXL1) in combination with a concomitant decrease in expression of primitive endoderm marker (Sox 7), mesoderm markers (brachyury, MEOX1) and ectoderm markers (Sox 1 and ZIC1) was used in this study to signify the production of definitive endoderm.

All-*trans*-retinoic acid (RA) has been shown to stimulate endoderm differentiation in teratocarcinoma stem cells and this effect was potentiated by addition of dibutyryl cyclic-AMP (DBcAMP) in culture [101]. More recently, RA has been implicated in embryonic endoderm differentiation pattern, especially in the early pancreas formation [102, 103]. Cyclopamine, a steroid alkaloid teratogen and an inhibitor of Sonic hedge hog (SHH) signaling inhibitor, has been shown to promote pancreatic differentiation [104].

As the process of embryogenesis and development of definitive endoderm becomes more understood, efficient production of the definitive endoderm would form

the cornerstone of production of embryonic stem cell derived pancreatic and hepatic tissues.

4. Differentiation of Embryonic Stem Cells

4.1. All-trans-retinoic acid and Dibutyryl cAMP

Although initially Strickland and Mahdavi described a production of endoderm by their experiments on the teratocarcinoma cells[101] the endoderm produced were proved later to be of parietal endoderm in nature. A growing body of evidence suggests that the combination of retinoic acid and DBcAMP induces production of parietal endoderm (extraembryonic) rather than definitive endoderm. Futaki et al., to further elucidate the signals involved in parietal endoderm and basement membrane production during rodent development, silenced the expression of various genes by short RNA interfering vectors[105]. They treated the mouse F9 embryonal carcinoma cells with retinoic acid and DBcAMP and discovered that Sox 7 plays an important role in development of parietal endoderm and likely is required for production of GATA-4 and GATA-6[105]. The studies using F9 teratocarcinoma cells by Thompson and Gudas suggested that retinoic acid produced parietal endoderm with Rex-1 deletion[106]. Harris and Childs confirmed these findings by studying the global gene expression patterns during differentiation of F9 embryonal carcinoma cells in the presence of retinoic acid[107].

4.2.Retinoic Acid Mechanism of Action

Retinoic acid is one of the more biologically active derivatives of retinol (vitamin A). Retinoids are a group of natural and synthetic compounds that are structurally related to retinol. Retinoids function as an important regulatory signaling molecules for cell growth and differentiation during embryogenesis and malignant transformation[108].

The actions of the retinoids are mediated through retinoid receptor proteins that are members of the steroid/thyroid/retinoid family hormone receptor family. There are two broad types of retinoic acid receptors: (1) the RARs and RXRs and (2) the CRABPs. The RARs and RXRs act as ligand inducible transcription factors by binding to *cis*-acting retinoic acid response elements (RAREs) on DNA, resulting in increased transcription of the target genes. Generally, there are two groups of retinoid receptor proteins: RARs and RXRs. RARs α, β, and γ bind both all-*trans*-RA and 9-*cis*-RA, an isomer of all-*trans*-RA. RXRs, on the other hand, bind to 9-*cis*-RA but to some all-*trans*-RA.

The CRABPs (CRABP-1 and CRABP-2), although their functions are not completely understood, they affect the sensitivity of the cell to the external concentration of all-trans-RA. CRABP-1 reduces the amount of RA available to regulate the gene expression in the nucleus by converting RA to more polar, oxidized derivatives.

Retinoic acid regulates differentiation of different cell types by altering the expression of a wide variety of transcription factors. This effect is either primary (mediated directly by the receptor binding to the RAREs that control gene expression) or secondary (the effects occur without direct binding of the receptor to the RAREs). One of the more important transcription factors regulated by RA is homeobox gene. In vertebrate development, RA's ability to activate certain homeobox transcription factors plays a critical role.

4.3. Cyclopamine

Cyclopamine, an alkaloid from plants from the Veratum genre, is of a steroid structure related to cholesterol. It inhibits the cellular response to Sonic hedgehog (Shh) by blocking the membrane protein Smoothened. Shh binds to its receptor Patched and the

inhibition of this interaction is a key event for differentiation of pancreas during development. When Shh binds to Patched, Smoothened is activated and produces a cascade of signals resulting in the inhibition of pancreatic differentiation[109]. The signals from the notochord mediate this repression of Shh signaling. Other endoderm derived abdominal organs such as the liver, stomach and duodenum require the Shh signaling. Kim and Melton observed heterotopic formation of pancreatic cell types in stomach and intestinal anlagen after treatment with cyclopamine.

4.4. Production of Islet Like Clusters from Embryonic Stem Cells: Previous Studies

Discovering a way to culture cells *in vitro* that function like islets (i.e. produce insulin in response to high glucose to normalize hyperglycemia) is the Holy Grail of diabetes research. Lumelsky et al. in 2000 and Hori et al. in 2002 claimed to have produced insulin producing cells from mouse embryonic stem cells by using strategies used to induce production of neural cells. However, initial excitement stirred by these articles waned as studies refuting these findings showed that the insulin observed in these studies was not synthesized but taken up from the media [16 17]. Furthermore, the initial studies lacked evidence of C-peptide production which is considered a surrogate marker of *de novo* insulin synthesis.

D'amour et al. by using activin A, exendin-4, cyclopamine, and all-trans-retinoic acid, showed that human embryonic stem cells can differentiate into endocrine cells capable of synthesizing pancreatic hormones including insulin, glucagons, somatostatin, pancreatic polypeptide and ghrelin[110]. The same group of scientists went on to produce cells that produce insulin and C-peptide from human embryonic stem cells. These cells, when engrafted into immune deficient mice showed production of these two hormones

and were responsible for decreasing blood glucose levels in streptozocin treated diabetic mice[111].

4.5. Differentiation of Embryonic Stem Cells to Hepatocytes

Various articles have attempted to produce hepatic tissues from embryonic stem cells[112, 113]. They have listed a number of markers that can be used as surrogate markers of hepatic tissue production. These include: alpha-feto protein, albumin, transthyretin (TTR), tyrosine aminotransferase (TAT, marker of perinatal, and post natal hepatocyte specific differentiation), glucose-6-phosphatase (G6P), and SEK1. The expression of AFP-2 and TTR is not enough to support the production of hepatocytes or hepatocyte precursors.

Kuai et al. differentiated mouse embryonic stem cells in the presence of retinoic acid, hepatocyte growth factor (HGF) and β-nerve growth factor (β-NGF)[113]. These cells displayed immunohistochemistry evidence of α_1-antitrypsin and alpha fetoprotein. Also, they displayed expression of glucose-6-phosphatase, albumin, transthyretin, hepatic nuclear factor 4, and SAPK/ERK kinase-1 (SEK-1) on PCR assay.

Hamazaki et al. cultured the embryoid bodies in the setting of the growth factors, FGF, HGF, oncostatin (OSM), dexamethasone, and ITS (insulin, transferring, and selenium)[112]. They found that induction of TAT and G6P was observed regardless of expression of a functional SEK-1 gene. They concluded that these conditions produce cells that may serve as precursors for hepatocytes and SEK1 signaling pathway is not indispensable in later stage maturation of hepatocytes.

All the hepatocyte markers described above are not truly hepatocyte specific. Asahina et al. found the enzyme cytochrome 7a1 (Cyp7a1) to be truly hepatocyte specific

in that it is not expressed in yolk sac but in liver only[114]. Cyp7a1 is the rate-limiting enzyme in the conversion of cholesterol to bile acids in the liver[115]. They found that by adding dexamethasone to the culture, the mRNA expression of cyp7a1 was increased. Dexamthasone has been shown to increase Cyp7a1 mRNA expression in rat primary hepatocytes[116].

4.6. Markers of Hepatocyte Differentiation

Transthyretin (TTR)

Transthyretin (previously referred as prealbumin) is a transport protein produced in liver. TTR and retinol-binding protein (RBP) are the two proteins that are involved in transporting retinol in plasma from the liver to extrahepatic tissues. TTR is a 55.0k Da tetrameric protein which is composed of four identical subunits. TTR also has a high affinity sites for binding thyroid hormones. TTR is also produced in the choroids plexus of the brain and the yolk sac[118]. TTR is expressed throughout development, early, middle and late stages[118].

Alpha-1-Antitrypsin (AAT)

Alph-1antitrypsin is a glycoprotein primarily synthesized in the liver and secreted into the serum where its function is to prevent non-specific neutrophil protease-induced host tissue injury. It is expressed in the endoderm and in the liver throughout liver maturation[119]

Alpha Feto Protein (AFP)

Alpha feto protein is an early fetal hepatic marker as well as primitive endoderm. Its levels decrease as the liver develops into a mature stage. The expression of this marker supports but does not prove the production of hepatocyte production.

Albumin

Albumin is the most abundant protein produced in the mature hepatocytes. Its expression does start in the early hapacyte development stage and reaches its maximal level in mature hepatocytes. It is not completely hepatocyte specific for it is also expressed weakly in yolk sac[112, 120].

Tyrosine Aminotransferase (TAT)

Tyrosine aminotransferase is an enzymatic marker for post natal hepatocyte specific differentiation. This enzyme is not produced in significant quantity prior to birth but during early neonatal period, undergo rapid activation[121].

Glucose-6-Phosphatase

Glucose-6-Phosphatase is predominantly expressed in the liver during the late gestational or perinatal stage.

SEK 1

SEK1, also known as MKK4 and JNKK, is a member of the mitogen activated protein kinase activator family. It is a direct activator of stress activated protein kinases (SAPKs, Jun-N-terminal kinases, JNKs), and assumes this function in the setting of a various cell stresses including: change in osmolarity, toxins, DNA damage and heat shock or inflammatory cytokines. SEK1 plays a crucial role in early hepatogenesis. In SEK1 null mouse, the hepatocytes during development undergo massive apoptosis[122].

5. Summary

This report describes an attempt at differentiation of mouse embryonic stem cells to definitive endoderm that can serve as pancreatic and hepatic precursors without formation of the EBs. We hypothesized that mouse embryonic stem cells can be

differentiated by using various factors which include, all-*Trans* retinoic acid, basic fibroblast growth factor (bFGF) and dibutyryl-cyclic AMP, to produce cells that display features of definitive endoderm. Additional specific aims include: (1) evaluate the produced cells for characteristics of beta cells and hepatocytes; (2) evaluate the effects of lengthening the culture period on the production of definitive endoderm, beta cells, and hepatocytes.

We document the findings that are consistent with a production of mixture of definitive endoderm and extraembryonic endoderm. Extension of time in culture and inclusion of DBcAMP resulted in production of cells that preferentially differentiated into definitive endoderm and produced and secreted insulin in the glucose responsive manner. There was expression of hepatocyte markers in some cells but there was insufficient data to definitively conclude production of hepatocytes. Conceptually, the outline of the experiments performed is illustrated in Figure 5(a).

CHAPTER 2: METHODS

1. Experimental Design

The experimental design is outlined in Figure 5(b). The embryonic stem cells were cultured as described in the following sections. To address the question of the potential effects of length of time of culture, they were cultured in "standard conditions" and "extended conditions." The culture time in the extended condition was longer than the standard condition. The cells produced from each of these conditions were evaluated for production of definitive endoderm by quantitative PCR. The production of beta cells was evaluated by expression of beta cell markers on Q-PCR and production of insulin and C-Peptide on immunohistochemistry and radioimmunoassay for insulin and C-Peptide. Furthermore, different culture conditions were compared with respect to production of insulin to assess if a particular condition produced cells that expressed more insulin than other conditions. The cells were assessed for expression of hepatocyte markers on Q-PCR.

2. Differentiation of Embryonic Stem Cells

The C57/BL6 mouse embryonic stem cells were cultured *in vitro*. These were cultured in attempts to produce endodermal cells, cells that display features of islets and hepatocytes. First set of conditions were created to produce endodermal cells and subsequently insulin producing cells. This set is called "Standard Conditions." As the data will show, the Standard Conditions did not produce definitive endodermal cells. The question of insufficient time in culture was raised and therefore, a new set of conditions were created. The main feature of the second set is lengthening of the culture time. This set is called "Extended Conditions." Both of these conditions were analyzed with quantitative PCR,

immunohistochemistry and static insulin secretion assay as outlined in the following sections. The cells from the Standard Conditions were analyzed for hepatocyte markers.

2.1. Production of Endoderm and Islet Like Cells

2.1.1. Standard Conditions (Figure 6)

Step 1: Embryonic Stem Cell Maintenance

C57/BL6 mice embryonic stem (ES) cells were cultured on a feeder layer of gamma irradiated PEF (embryonic fibroblasts). These cells were incubated at 37°C with 95% O_2/5% CO_2 in a ES cell maintenance medium comprised of 15% FBS (Gemini Bio-Products, Woodland, CA), 1mM sodium pyruvate, 2mM glutamine, 0.1mM non-essential amino acids, 10ng/ml leukemia inhibitory factor [88], 100μM 1-thioglyercol (MTG) and high glucose DMEM (Stem Cell Technologies, Vancouver, Canada) for 2-3 days until they were 70-80% confluent.

Step 2

These ES cell colonies were dissociated with trypsin/EDTA solution and plated onto gelatinized tissue culture dishes and incubated at 37°C with 95% O_2/5% CO_2 with 15% FBS (Gemini Bio-Products, Woodland, CA), 0.1 mM non-essential amino acids, 1 mM MTG, 2 mM of L-glutamine, 10^{-7} M of all-*Trans*-retinoic acid (Sigma, St. Louis, MO) and 25 ng/ml of human basic fibroblast growth factor (bFGF) (Stem Cell Technologies, Vancouver, Canada), and high glucose DMEM (Stem Cell Technologies, Vancouver, Canada) for 3 days.

Step 3

These cells were trypsinized, harvested, plated and incubated at 37°C with 95% O_2/5% CO_2 with 15% FBS, 2 mM of L-glutamine, 0.1mM non-essential amino acids, 1 mM

MTG, $2x10^{-3}$ M of dibutyryl cAMP (Sigma, St. Louis, MO) and high glucose DMEM for 2 days.

Step 4

These cells were then trypsinized, harvested, plated onto petri dishes, and incubated in suspension and allowed to form cell clusters at 37°C with 95% O_2/5% CO_2 with ES-Cult basal medium-A (Stem Cell Technologies, Vancouver, Canada) which contains DMEM/Ham's Nutrient Mixture F-12, 1.55 g/L D-glucose, 0.5 mM glutamine, and 22.4 g/L Sodium Bicarbonate. 1x N2 supplement-A (Stem Cell Technologies, Vancouver, Canada) which contains 2.5 mg/ml rh Insulin, 10 mg/ml human transferrin (iron saturated), 0.52 μg/ml Sodium Selenite, 1.61 mg/ml Putrescine, 0.63 mg/ml Progesterone in phosphate buffered saline was added to the medium as well as 25 ng/ml bFGF, 1x B27 supplement (Stem Cell Technologies, Vancouver, Canada). The cells were cultured in this medium for 6 days or 3 days with the addition of 5 μM of cyclopamine (Biomol, Plymouth Meeting, PA).

Step 5

These cell clusters were harvested, transferred to petri dishes and incubated at 37°C with 95% O_2/5% CO_2 with ES-Cult basal medium-A, 1x N2 supplement, 10 mM nicotinamide (poly-ADP-ribose synthetase inhibitor known to differentiate and increase beta cell mass in cultured human fetal pancreatic cells and protect β-cells from desensitization induced by glucose toxicity), and $2x10^{-3}$ M of dibutyryl cAMP or 5 μM cyclopamine and incubated for 6 days.

2.1.2. Extended Conditions for Production of Endodermal Cells and Islet Like Cells (Figure 7)

Step1: Embryonic Stem Cell Maintenance

The embryonic stem cells were maintained in the same manner as the standard conditions.

Step 2

These ES cell colonies were dissociated with trypsin/EDTA solution and plated onto gelatinized tissue culture dishes and incubated at 37°C with 95% O_2/5% CO_2 with 15% FBS (Gemini Bio-Products, Woodland, CA), 0.1 mM non-essential amino acids, 1 mM MTG, 2 mM of L-glutamine, 10^{-7} M of all-*Trans*-retinoic acid (Sigma, St. Louis, MO) and 25 ng/ml of human basic fibroblast growth factor (bFGF) (Stem Cell Technologies, Vancouver, Canada), and high glucose DMEM (Stem Cell Technologies, Vancouver, Canada) for 3 days.

Step 2 Extended

The Step 2 cells from the standard protocol were dissociated with trypsin/EDTA solution and plated onto gelatinized tissue culture dishes and incubated at 37°C with 95% O_2/5% CO_2 with 15% FBS, 0.1 mM non-essential amino acids, 1 mM MTG, 2mM of L-glutamine and 25 ng/ml of bFGF and high glucose DMEM for 14 days.

Step 4 Extended

These cells were then trypsinized, harvested, plated onto petri dishes, and incubated in suspension and allowed to form cell clusters at 37°C with 95% O_2/5% CO_2 with ES-Cult basal medium-A with 1x N2 supplement-A, bFGF, 1x B27 supplement. The

cells were cultured in this medium for 3 days with the addition of 5 μM of cyclopamine or for 6 days without cyclopamine.

Step 5 Extended

The cell clusters cultured in Step 4 in the presence of cyclopamine were further cultured in 5 μM cyclopamine in a medium containing ES-Cult basal medium-A, 1x N2 supplement, 10 mM nicotinamide for 6 days (cyclopamine extended). The cell clusters cultured in Step 4 in the absence of cyclopamine were cultured in the presence of $2x10^{-3}$ M of dibutyryl cAMP for 6 days at 37°C with 95% O_2/5% CO_2 (DB cAMP extended).

2.1.3. Quantitative PCR

RNA was isolated from cells in each step using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. The isolated RNA was treated with DNAse (Sigma) and quantified with spectrophotometer, Ultrospec® 3000 (Pharmacia Biotech Ltd, Cambridge, UK). Primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and spanned introns where possible. Amplicons were between 80-120bp for efficient amplification. Primer efficiencies were determined by using an exponential dilution series of islet cDNA. Only primers pairs with an efficiency greater than 0.8 were used in subsequent analyses. A 7500 Real Time PCR System (Applied Biosystems) and SYBR Green PCR master mix (Applied Biosystems) were used for all reactions. cDNA's were obtained by reverse transcription (RT) of 1 μg of total RNA from newly isolated tissue for each RT. 10 ng of generated cDNA was used in each reaction with all reactions done in duplicate. Samples were normalized to β-actin, and the fold increase compared to step 1 or islets as calculated using $2^{-\Delta\Delta CT}$ [123] as appropriate unless otherwise noted. The markers of definitive endoderm used were: Sox

17, HNF3β (Foxa2), and MIXL1. Sox 7 was used as the marker of primitive endoderm. The marker of mesoderm used was brachyury. Neuroectoderm marker used was Sox 1. Pax 6 was used as a pre-beta cell endocrine cell marker [124, 125]. Beta cell markers include: Nkx6.1, Pdx-1 and Insulin I and II. The primer sequences are listed in Table 1. Prevalidated primers for Sox 7, Sox1, brachyury and MIXL1 were purchased from Applied Biosystems.

2.1.4.. Immunohistochemistry

The Step 5 cell clusters were fixed in 4% formalin and subsequently embedded in paraffin by coating the cells with agarose gel first. Immunohistochemistry was performed using standard protocols. The primary antibodies used were as follows: mouse anti-mouse monoclonal antibody for insulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:200 dilution, goat anti-rat antibody for C-peptide (Linco Research, St. Charles, Missouri) at 1:100 dilution, rabbit polyclonal antibody for glucagon at 1:100 dilution (DAKO, Mississauga, Ontario, Canada). The secondary antibodies used include: donkey anti-goat Alex Fluor 488 (Molecular Probes, Burlington, Ontario) at 1:200 dilution, donkey anti-mouse Alex Fluor 594 (Molecular Probes, Burlington, Ontario) at 1:200 dilution, donkey anti-rabbit Texas Red (Jackson ImmunoResearch Laboratories, West Grove, PA). A universal blocking reagent Power Block^{TM} (Biogenex, San Ramon, CA) was used. Zeiss inverted light microscope was used to visualize the flurochromes.

2.1.5. Insulin and C-Peptide Quantification

The Step 5 cell clusters from culture protocols were recovered in insulin free, low glucose recovery medium composed of F12 (Ham's)/DMEM (no glucose) (Gibco/Invitrogen, Burlington, Ontario) and 10 mM of nicotinamide overnight. The

clusters were then washed in Krebs-Ringer bicarbonate buffer (KRBB) with 0.1% BSA in 1.67 mM D-glucose. The cells were then lysed by 1M glacial acetic acid. In a separate experiment, the clusters were washed in KRBB with 0.1% BSA in 1.67 mM D-glucose and exposed to 1.67 mM or 16.7mM D-glucose. Insulin radioimmunoassay (RIA) was performed to determine the insulin level using a human insulin radioimmunoassay kit according to the manufacturer's instructions (MP Biomedicals, Irvine, CA). According to the manufacturer, human insulin radioimmunoassay detects both murine insulin-I and II. The C-peptide level was measured using a rat C-peptide radioimmunoassay kit according to the manufacturer's instructions (Linco Research, St. Charles, MO). This C-peptide RIA reliably detects C-peptide II and not C-peptide I [126]. The insulin and C-peptide content were calibrated per DNA content (μg) determined by spectrophotometer, Ultrospec® 3000 (Pharmacia Biotech Ltd, Cambridge, UK).

2.1.6. Transmission Electron Microscopy

Step 4d clusters were fixed in EM grade 4% formaldehyde and embedded in Lowicryl K4M resin Ultrathin sections were prepared on an RMC MT-6000-XL ultramicrotome (Sorvall, Microtomes, Wilmington, DE) and examined for the presence of secretory granules with Hitachi H7600 transmission electron microscope.

2.1.7. Transplantation

C57/BL6 mice (4-6 week old, body weight 20-25g) were treated by intraperitoneal injection of 185 mg/kg - 200mg/kg streptozotocin (Sigma) to induce diabetes. Following the injection, blood glucose levels were monitored by AccuCheck glucose detector through tail vein sampling. Within 5 days of injection, the mice became hyperglycemic. The blood glucose levels ranged from 22.2 to 31.4 mmole/L. Transplantation of the

clusters from the DBcAMP treated cells of the extended protocol (4d) were performed under general anesthesia into the spleen. Each mouse received approximately 3000 cell clusters (150 clusters per gram of mouse), (n=6) and the control group received 0.1 ml of PBS injection into the spleen as a sham surgery (n=7). Post operatively, the mice were fed *ad libitum*, and their blood glucose was monitored daily for 7 days.

2.2 Differentiation of Endodermal Cells to Hepatocytes

The embryonic stem cells from C57/B6 mice were cultured with the same protocol as outlined in Figure 6 (Standard Condition). The embryonic stem cells when maintained in Step 1, they were labeled with green fluorescence protein (GFP). The aim was to test whether the Standard Condition produced cells with any hepatocyte features. In order to assess for the presence of hepatocyte markers, the cells from the standard protocol were analyzed by quantitative PCR for hepatocyte markers, alfa fetoprotein (AFP)-2 and transthyretin (TTR), (primers listed in Table 1).

To take advantage of the liver's regenerative properties *in vivo*, the following experiment was designed and carried out. The Step 3 cells were injected into the liver that had received a chemical injury (see 2.2.1. Liver Injection for details). This was to introduce the pluripotent cells into the microenvironment of the liver that is undergoing injury and regeneration. The embryonic stem cells that were labeled with GFP were used for this part of the experiment in order to distinguish native liver tissues from the any step 3 cell derived tissues. As outlined in Figure 7, the cells from step 3 were harvested and used for liver injection experiments (2.2.1.)

2.2.1. Liver Injection for Production of Hepatocytes

C57/BL6 mice (4-6 week old, body weight 20-25g) were treated with 0.5mg/g of D-galactosamine by intraperitoneal injection to induce chemical liver injury. The mice were monitored post injection. The step 3 cells which were labeled with GFP were isolated and prepared for injection. Forty eight hours after the mice were treated with D-galatosamine, 5 million GFP positive Step 3 cells were injected into the left lobe of the liver. Total of 4 mice were injected with the Step 3 cells. The mice were fed *ad libitum*.

2.3 Statistics

Data are presented as mean $\pm$ standard deviation (S.D.). The Student's t-test was used to compare the means. The p value <0.05 was defined to be statistically significant.

CHAPTER 3: RESULTS

3.1. Differentiation of Embryonic Stem Cells to Endoderm

3.1.1. Endoderm Markers Detected by Quantitative PCR

Standard Protocol

As shown in Figure 9, the two markers of definitive endoderm, Sox 17 and FOXA2, were upregulated as the ES cells were differentiated to latter stages of the standard protocol. There was about 100 fold increase in the expression of these two markers as the cells were cultured in the last step (steps 5a and 5b, see Figure 6). In addition to the markers of definitive endoderm, Sox 7, a marker of primitive endoderm showed upregulation as well in the latter stages implying that the production of primitive endoderm contributed to the increase in expression of Sox 17 and FOXA2. Brachyury, a marker of mesoderm, and Sox 1 and Pax 6, markers of ectoderm were expressed at low levels and even decrease in expression compared to the ES cells throughout the steps. MIXL1, a primitive streak marker was also expressed at low levels. This standard protocol likely produces primitive, extraembryonic endoderm rather than definitive endoderm.

Extended Protocol

As illustrated in Figure 10, there was an upregulation of markers of definitive endoderm: Sox17 (10-12 fold) and FOXA2 (5-6 fold) in the latter steps of the protocol relative to their respective expression in ES cells (Step 1). However, expression of Mixl1 was not upregulated. Sox 7, a primitive endoderm marker did not show increased expression except a slight increase in step 5d. Also, the expression of Sox 7 with the culture was associated with high Ct values in the 30's. The Ct value is a cycle number at

which the increase in the cDNA becomes exponential. High Ct value suggests that a high number of cycles were required to result in the increase and therefore, there are not many copies of the gene of interest in the sample tested. These results imply that the increased expression of Sox 17 and FOXA2 may be due to production of definitive endoderm. The slight increase in the expression of Sox 7 implies that the endoderm population is a mixture of definitive endoderm and extraembryonic endoderm. Brachyury, a mesoderm marker, and Sox 1, a neuroectoderm marker did not show appreciable increase with the Ct values in the 30's which point towards enrichment of definitive endoderm tissues without significant production of other germ layers.

3.2. Differentiation of Embryonic Stem Cells to Insulin Producing Cells

3.2.1. Immunohistochemistry

Standard Protocol

The cells from the last step of each protocol were stained for insulin and C-Peptide. The mouse islets were first stained with the aforementioned antibodies to test them and also to serve as a reference (Figure 11). As illustrated in this figure, the mature mouse islets stained strongly for both insulin and C-Peptide. The cells cultured in the standard protocol did not have a significant number of cells that were positive for insulin or C-Peptide (Figure 12). Qualitatively, there did not appear to be difference between the cells that were cultured in the presence of cyclopamine and in the presence of DBcAMP.

Extended Protocol

The proportion of the cells stained positive for insulin and C-Peptide was very small in this protocol (Figure 13). There did not appear to be a difference between the cells that were cultured in the presence of cyclopamine and in the presence of DBcAMP in terms

of the proportion of cells that stained positive. In addition, the extended protocol did not significantly increase the proportion of cells that stained positive.

3.2.2. Quantitative PCR

The generation of cells of the pancreatic lineage was assessed by measuring expression levels of the following markers: insulin I and insulin II (beta cells), the transcriptional regulators Nkx 6.1 and Pdx-1(pancreas development) and an endocrine marker (Pax 6).

Standard Protocol

From the quantitative PCR data, the markers including Insulin-I, insulin-II, Nkx 6.1, Pdx-1 show progressively increased expression from Step 3 to Steps 4 and 5 (Figure 14). However, insulin-I which is more specific than insulin-II for beta cells, showed an increased expression in Step 5 to 10 fold at most in DBcAMP condition, whereas insulin-II was expressed throughout the steps at high levels. Insulin-II is also expressed in other tissues including fetal liver, neurons and yolk sac and these other tissues may have contributed to its expression throughout the steps. Pax 6 which is an endocrine marker showed a modest increase in the last step of each protocol.

Extended Protocol

The Q-PCR analyses revealed an approximately 100 fold increase in expression of insulin-I and insulin II in the last step of the protocol (Figure 15). Of note is the observation that insulin I progressively increased with each step. In contrast, insulin II, was expressed at a constant level throughout steps 2-4. Both Nkx6.1 and Pax 6, markers of endocrine differentiation increased throughout the differentiation process. In contrast, Pdx-1 levels were increased during steps 2 and 3, before falling during step 4.

3.2.3. Insulin and C-peptide Content and Secretion

The results of glucose stimulated insulin release assays are illustrated in Figure 16. As a surrogate for changing glucose concentrations *in vivo*, a static glucose responsive assay was performed. As mentioned previously, the cell clusters from the last step of each protocol were exposed to a low glucose environment (1.67 mM) and a separate cell clusters from the same culture dish were exposed to a high glucose environment (16.7 mM). The insulin secreted by each group of cell clusters were measured and calibrated to the amount of DNA present in the cell clusters. The cells from the standard protocol (5a and 5b) did not exhibit glucose responsive behavior. Insulin secretion by 5a cells in the low glucose environment was 0.5 $\pm$ 0.2 mcIU/ml/mcg of DNA vs. high glucose environment, 0.9 $\pm$ 0.3 mcIU/ml/mcg of DNA ($P < 0.3$). Insulin secretion by 5b cells in the low glucose environment was 0.7 $\pm$ 0.4 mcIU/ml/mcg of DNA vs. high glucose environment, 1.4 $\pm$ 0.9 mcIU/ml/mcg of DNA ($P < 0.2$). The 4c cells, which were produced from the extended protocol and were treated with cyclopamine in the last step showed a trend toward glucose responsive secretion of insulin (0.6 $\pm$ 0.2 mcIU/ml/mcg DNA vs. 1.0 $\pm$ 0.2 mcIU/ml/mcg DNA, $P < 0.05$). The 4d cells from the extended protocol that were treated with DBcAMP showed insulin secretion in the glucose responsive manner (3.5 $\pm$ 0.5 vs. 14.8 $\pm$ 2.7 mcIU/ml/mcg DNA, $P < 0.03$). None of the cells that were tested secreted C-Peptide in a glucose responsive manner (Figure 17).

These findings as well as the total insulin and C-Peptide content of each group of cells that were tested are listed in Table 2. As illustrated in Table 2, the DBcAMP extended protocol (4d) yielded cells with insulin content twice as high as all other

conditions. However, this apparent difference in insulin content did not translate into a difference in C-peptide content (Table 3) although, it did translate into a significant insulin release to exposure to glucose as illustrated in Figure 16 ($P < 0.03$). The apparent absence of difference in C-peptide content is likely due to the fact that the RIA used for this step reliably detects C-peptide II only[126]. These cells had a high basal insulin secretion as evidenced by 17.2 ± 3.1 % secretion at low glucose concentration and 29.3 ± 1.1 % secretion at high glucose concentration compared with cells from other conditions that demonstrated much lower insulin secretion

3.2.4. Transmission Electron Microscopy of Secretory Granules

Electron microscopy revealed the presence of secretory granules in the cytoplasm of 4d cells (Figure 18). This implies the presence of an endocrine hormone synthesis and packaging mechanism at the cellular level as a beta cell would have insulin containing secretory granules in the cytoplasm. However, it is not possible to tell whether these secretory granules contain insulin without having performed insulin staining on the electron microscopy slides.

3.2.5. Transplantation of DBcAMP Treated Cells from the Extended Protocol

Following the treatment of C57/B6 mice with streptozotocin, the difference in the pre-transplant mean blood glucose between the transplant group and the sham surgery group was not statistically significant ($p < 0.6$). As illustrated in Figure 19, on post transplant day 1, the blood glucose was significantly lower in the transplanted group ($P < 0.02$) but this was not sustained after this day. The mice in both groups remained severely diabetic after post transplant day 1.

3.3 Differentiation of Embryonic Stem Cells to Hepatocytes

3.3.1. Expression of Hepatocyte Markers

As illustrated in Figure 20, two hepatocyte markers were measured by Q-PCR. There was a significant increase in the expression of these markers (approximately 100-1000 fold) in the latter steps of the protocol. These findings do not prove that there are hepatocytes produced in the protocol since these markers are not hepatocyte specific but are produced by yolk sac as well.

3.3.2. Liver Injection

In the post-operative period, the mice started to gain weight with obvious abdominal distention. The mice were sacrificed and a laparotomy was performed which revealed a heterogeneous mass arising from the injection site on the liver (Figure 21). The mass was stained with hematoxylin and eosin (H&E) and examined with an experienced pathologist. As shown in Figure 22, the features of this mass were most consistent with a teratoma containing tissues such as smooth muscle, cartilage and occasional pancreatic tissues. These findings suggest that the cells initially injected into the liver are likely of all three germ layers capable of chaotic differentiation into teratomas.

CHAPTER 4: DISCUSSION

4.1. Differentiation of Embryonic Stem Cells to Definitive Endoderm

Due to their pluripotent nature, the embryonic stem cells have the potential to differentiate into the liver and the pancreas. Since both the liver and the pancreas arise from the epithelium of the primitive gut tube formed by the definitive endoderm, in order to differentiate ES cells to liver and pancreas, the definitive endoderm first needs to be produced. In addition to the challenges of optimizing the culture conditions for endoderm development, scarcity of the markers of definitive endoderm make it difficult to assess for the presence of definitive endoderm. In contrast to the well studied area of development fate and movement of the endodermal progenitors and their derivatives, the genetic determinants that regulate and stimulate production of definitive endoderm are just beginning to be understood. In mice, based on targeting experiments, Mixl1 and Sox17 have been proven to be involved with the formation of definitive endoderm [92, 93]. From the phenotypic analysis of the Sox17-null mutant embryo showed that Sox17 plays a crucial role in maintenance and differentiation of the definitive endoderm of the embryonic gut [92]. Of note, in this study, the authors detected Sox17 mRNA in the visceral endoderm as well.

Some researchers have taken FOXA2 (HNF3β) also as a marker of definitive endoderm [22, 95, 96, 127]. FOXA2 is essential for the development of foregut and midgut endoderm and the loss of FOXA activity results in the loss of the endoderm of the fore- and midgut along with embryonic structures of the notochord [95, 97, 128]. Other important transcription markers include GATA-4 and 6. GATA-4 and GATA-6, zinc finger transcription factors expressed in the yolk-sac endoderm, and mesoderm that regulate

differentiation, gene expression, and cell proliferation in various tissues. They have been implicated in folding of the embryo and yolk sac closure[129, 130]. Recently, GATA-4 has been implicated to be expressed in the exocrine pancreas and in the prehepatic endoderm whereas GATA-6, which regulates the expression of the nuclear receptor HNF4, is expressed in the endocrine pancreas and is essential for liver bud expansion [131, 132].

4.2. Endoderm Characteristics of the Cells in this Protocol

In this study, retinoic acid and DBcAMP in the standard protocol did not seem to produce appreciable amount of definitive endoderm or insulin producing cells. The increase in the expression of Sox 17 in this protocol likely contributed significantly by primitive endoderm production. This is supported by the growth of teratomas from the liver when step 3 cells from the standard protocol were injected into it. Culturing the cells in the presence of bFGF and serum for 14 days after having them exposed to all-*trans*-retinoic acid in the extended protocol produced definitive endoderm as evidenced by an increased expression of FOXA2 and Sox 17 in a stepwise fashion. There were minimal changes in relative levels of Sox 7 except for a slight increase in the production of Sox 7 in the last step but the high Ct values of this marker would suggest that the absolute level of expression of Sox7 in this protocol was low. These findings are most consistent with production of definitive endoderm production with minimal contribution of extraembryonic endoderm. Whether the cells were exposed to cyclopamine or DBcAMP did not seem to affect the relative amount of these markers that have been produced.

In addition, the cells from the extended protocol did not seem to produce significant levels of markers of neuroectoderm or mesoderm as evidenced by low levels of Sox 1 and brachyury. These findings suggest that the differentiation of these cells with

the extended protocol is more directed towards definitive endoderm rather than chaotic differentiation of all three germ layers.

4.3. Production of Islet Like Clusters from this Protocol

In general, the extended protocol produced cells that have more characteristics of islets. The extended protocol produced higher expression of insulin I and II than the standard protocol. The cells produced in the DCcAMP in the extended protocol (4d) produced the most amounts of insulin and secreted insulin in the glucose responsive manner. However, the key marker of pancreatic differentiation, Pdx-1 showed a decrease in the 4d cells which initially may seem inconsistent with production of cells that display islet characteristics. In Figure 4 (b), Pdx-1 shows an increase in the early pancreatic development, then decreases in the endocrine precursors stage before increasing its levels in the beta cell stage. It is plausible that these cells are in the endocrine precursor stage but not at the mature beta cell stage.

Although insulin and C-peptide was detected by immunohistochemistry and quantified in this study, only a small proportion of cells in the clusters seemed to express insulin and C-peptide. After quantifying the insulin and C-peptide content in these cells, the DBcAMP condition in the extended protocol (4d) proved to contain the most amount of insulin per a calibrated amount of DNA. This finding did not translate into the findings in C-peptide quantification. This is likely due to the fact that the human insulin assay used in this study detects both mouse insulin-I and -II whereas the C-peptide radioimmunoassay kit used in this study reliably detects C-peptide II only and is of a limited use in detecting C-peptide I [126]. The high percentage of insulin secretion (29.3 $\pm$ 1.1 %) observed in these cells imply that the mechanisms of glucose responsive insulin

secretion are not well developed and that these cells secrete insulin in a primitive manner. This is corroborated by the transplantation data where there was a transient decrease in the blood sugar in the transplanted mice on post transplant day 1 only but this decrease was not sustained. These findings further supports that the 4d cells produced in the extended protocols are pancreatic endocrine cell precursors rather than mature beta cells.

With regards to expression of genes of interest, increased expression of the endocrine marker Pax 6 seemed to support the production of endocrine tissues; however, the presence of insulin II observed throughout the steps imply that there is some contribution of non-endocrine tissues. During development in mice, fetal liver and yolk sac express insulin II [19], and neuronal cells express insulin II only [21]. Pancreas expresses both insulin I and II [22].

4.4. Production of Hepatocytes

The quantitative PCR data showed the production of two hepatocyte markers in a sequential fashion. Although, AFP-2 and transthyretin are well known hepatocyte markers but they can also by produced by yolk sac; therefore, these markers are not specific and exclusive to hepatocytes. The production of teratoma in the liver where the step 3 cells were injected supports the conclusion that the step 3 cells are under differentiated and still contain all three germ layers. Based on these results, there is insufficient data at this time to support a production of hepatocytes or their precursors. More experiments testing for other markers of hepatocytes (albumin, tyrosin aminotransferase, or SEK) are needed to adequately assess for the production of hepatocytes.

CHAPTER 5: CONCLUSIONS

In summary, the protocols described in this study yielded some cells that have characteristics of definitive endoderm. The 4d cells in the extended protocol produced definitive endoderm and cells that are can serve as pancreatic endocrine cells. Extension of the protocol with the inclusion of DBcAMP in the culture conditions produce cells with the highest insulin content which are glucose responsive but seem to lack a mature secretion mechanism. The insulin produced by these cells is likely of mixed origin (insulin I and insulin II) and further studies are underway to improve the production of definitive endoderm along with the insulin content and secretion mechanism in these clusters. There is insufficient evidence to suggest that the hepatocytes were produced by any of the protocols.

CHAPTER 6: FUTURE DIRECTIONS

The future focus of the current work will be on improving the production of definitive endoderm and further enrichment of the cell population of definitive endoderm. This will be done by using a report system, green fluorescent protein (GFP) which is under the influence of the mouse insulin promoter to test the efficiency of different culture conditions using activin, exendin-4.

Table 1. Primers for developmental markers, β cell markers and hepatocyte markers.

Developmental Markers

Definitive Endoderm

L-Sox17	CTTTATGGTGTGGGCCAAAG
R-Sox17	GGTCAACGCCTTCCAAGACT
L-Hnf3β (Foxa2)	CATCCGACTGGAGCAGCTA
R-Hnf3β (Foxa2)	TGTGTTCATGCCATTCATCC

Mesoderm

Brachyury	Prevalidated primers purchased from Applied Biosystems

Neuroectoderm

Sox 1	Prevalidated primers purchased from Applied Biosystems

Primitive Endoderm

Sox 7	Prevalidated primers purchased from Applied Biosystems

β cell markers

L-Insulin I	CTTCAGACCTTGGCGTTGGA
R-Insulin I	ATGCTGGTGCAGCACTGATC
L-insulin II	GGCTTCTTCTACACACCCATGT
R-insulin II	GGTCTGAAGGTCACCTGCTC
L-Nkx6.1	CCCGGAGTGATGCAGAGT
R-Nkx6.1	TTCTCTTCCCATGTTTGTCCA
L-Pax6	TCAGCAGCAGCTTCAGTACC
R-Pax6	CCCAACATGGAACCTGATGT
Pdx-1	Prevalidated primers purchased from Applied Biosystems.

Hepatocyte markers

L-AFP-2	AAGGATCTGTGCCAAGCTCA
R-AFP-2	GTTCAGGCTTTTGCTTCACC
L-Transthyretin	CTGGACACCAAATCGTACTGG
R-Transthyretin	CAGAGTCGTTGGCTGTGAAA

Background (β-actin)

L-β-actin	GCTCTTTTCCAGCCTTCCTT
R-β-actin	CGGATGTCAACGTCACACTT

Table 2. Total insulin content and secretion in response to exposure to low (1.67mM) and high (16.7mM) glucose concentration. Student's t-test was used to determine the differences in percent secretion.

Culture Conditions	Total Insulin Content	% secretion* Glucose concentrations		P value
	(μIU/ml/μg DNA)	low	high	
Standard Protocol				
DBcAMP	21.3 ± 7.0	2.7 ± 1.2	6.9 ± 3.3	0.06
Cyclopamine	25.1 ± 5.2	2.2 ± 0.9	2.6 ± 0.9	0.5
Extended Protocol				
DBcAMP	50.4 ± 7.1	17.2 ± 3.1	29.3 ± 1.1	0.03
Cyclopamine	20.3 ± 4.6	3.7 ± 1.4	4.9 ± 1.4	0.3

* per cent secretion is calculated by insulin secretion divided by the total insulin content multiplied by 100%. This was calculated to evaluate the proportion of insulin secreted by cells from each culture condition when exposed to low (1.67 mM) and to high (16.7 mM) glucose concentrations.

Table 3. Total C-peptide content and secretion in response to exposure to low (1.67mM) and high (16.7mM) glucose concentration. Student's t-test was used to determine the differences in percent secretion.

Culture Conditions	Total C-Peptide Content	% secretion* Glucose concentrations		P value
	(pM/μg DNA)	low	high	
Standard Protocol				
DBcAMP	11.9 ± 3.6	4.0 ± 1.2	4.2 ± 0.9	0.8
Cyclopamine	20.3 ± 6.8	3.4 ± 1.1	3.4 ± 0.6	0.9
Extended Protocol				
DBcAMP	20.6 ± 3.2	4.7 ± 0.2	4.4 ± 1.0	0.9
Cyclopamine	23.1 ± 5.3	2.6 ± 0.9	3.9 ± 1.4	0.1

* per cent secretion is calculated by C-peptide secretion divided by the total C-peptide content multiplied by 100%. This was calculated to evaluate the proportion of C-peptide secreted by cells from each culture condition when exposed to low (1.67 mM) and to high (16.7 mM) glucose concentrations.

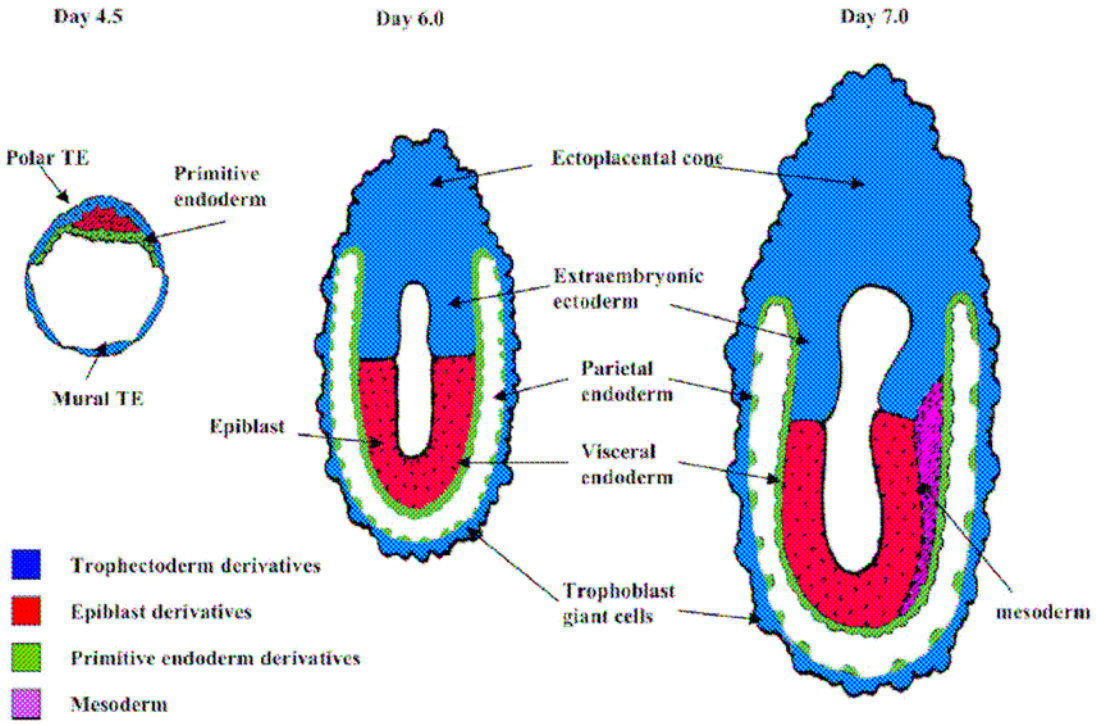


Figure 1. Development of mouse embryo. In the blastocyst stage (day 4.5), the embryo is composed on the trophectoderm (TE), primitive endoderm (PE), and epiblast (EPI). The TE give rise to the extraembryonic ectoderm (ExE) and the fetal part of the placenta. The PE gives rise to the parietal and visceral endoderm. The epiblast gives rise to the fetus itself. Adapted from Rossant. Sem Cell and Devel Biol 2004;15:573-581.

Figure 2. Early embryonic development in mice.The early gastrula stage in the mouse (left) is composed of the neuroectoderm (light blue), mesoderm (red), definitive endoderm (yellow), epidermis from nonneural ectoderm (dark blue), extraembryonic tissues (green), and the Spemann-Mangold organizer region (SMO). The late gastrula (right) is composed of the endoderm derived structures in yellow (ge: gut endoderm), mesoderm derived structures in red (som: somites, psm: presomitic mesoderm, pm: prechorrdal mesendoderm), and the neuroectoderm derived structures in light blue (fb: forebrain, mb: midbrain, hb: hindbrain). The epidermis (epi) in dark blue derived from the nonneural ectoderm is posterior to the neural ectoderm derived tissues. Adapted from Solnica-Krezel. Curr Biol 2005;15:R213-R228.

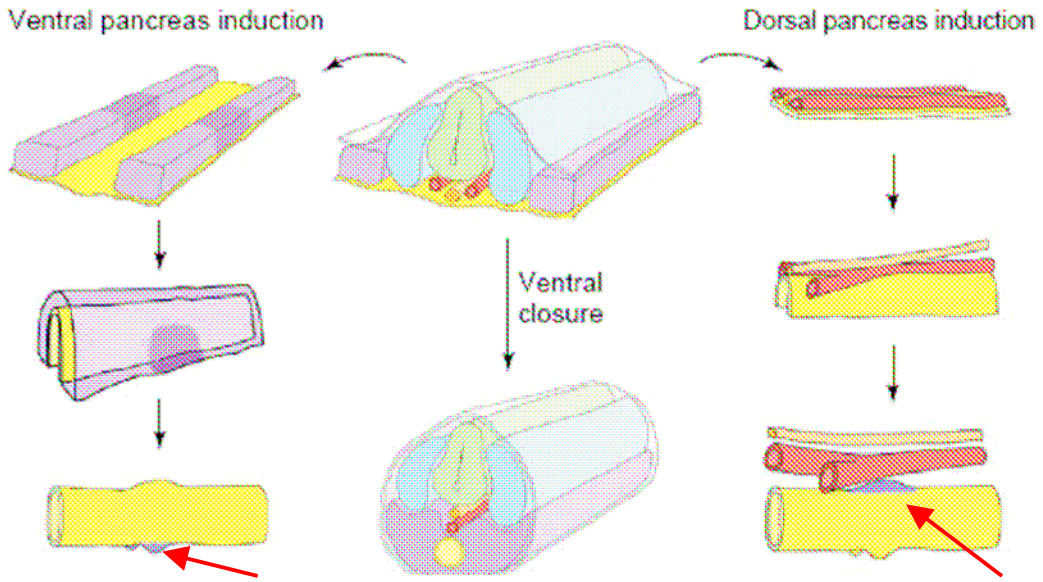


Figure 3 (a). Schematic illustration of early pancreatic differentiation. The signals from lateral plate mesoderm (purple) induced ventral pancreas differentiation(blue with a red arrow) from the endoderm (yellow). The signals from the notochold (orange) and the aorta (red) are required for dorsal pancreas induction (blue and with red arrow). Adapted from Kumar and Melton. Curr Opin Genet Devel 2004; 13:401-407.

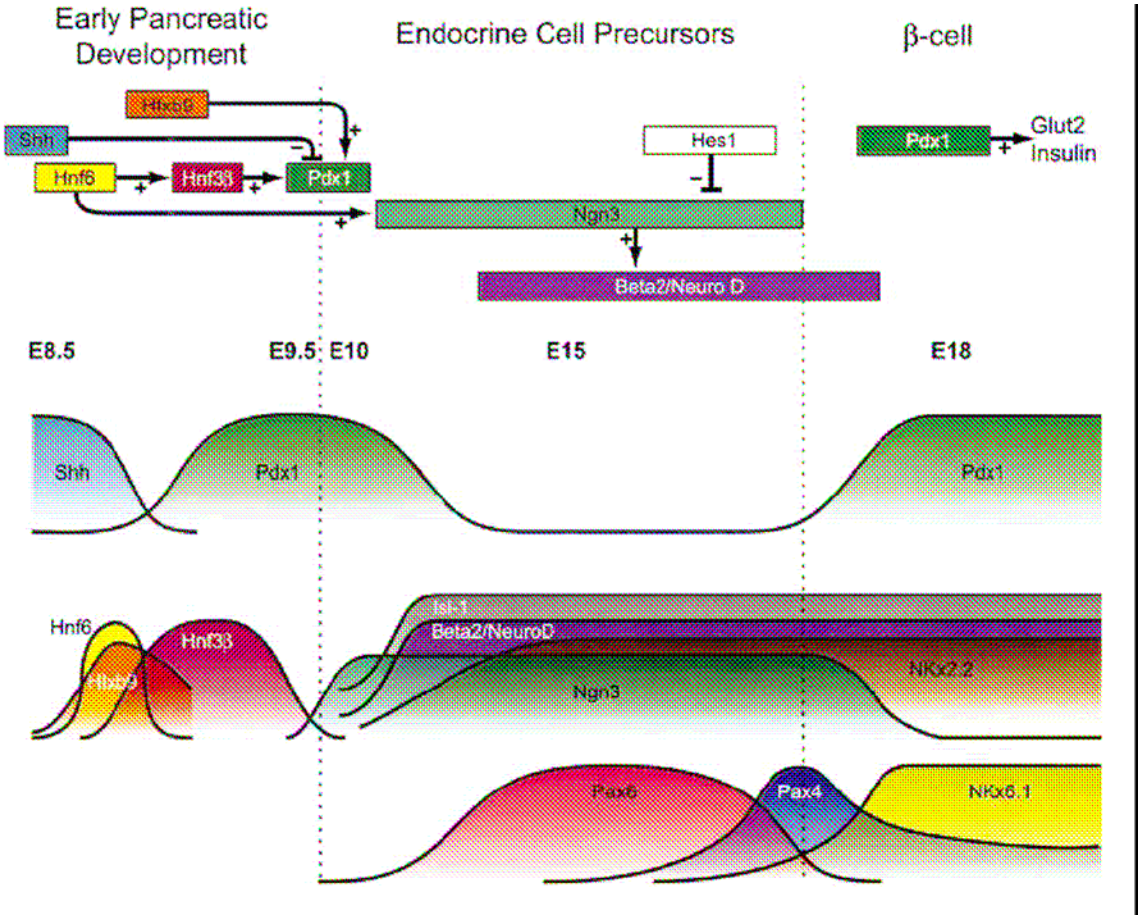


Figure 3 (b). Sequential expression of transcription factors in mouse pancreas and beta cell development. Adapted from Soria. Differentiation 2001; 68:205-219.

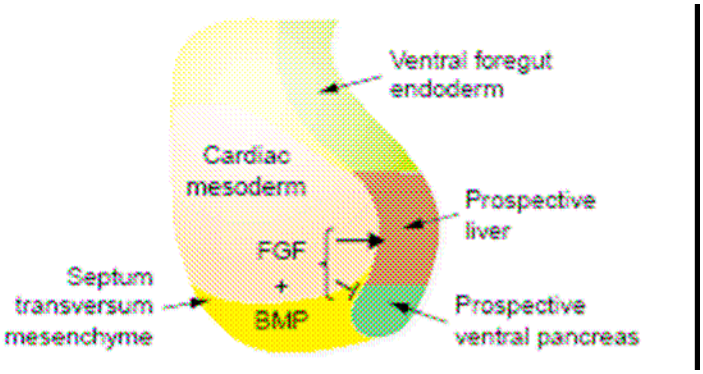


Figure 3 (c). Liver development in mouse. The prospective liver tissue arises from the endodermal cells of the duodenum. The FGF signaling from the cardiac mesoderm, and BMP signaling from septum transversum mesenchyme induces the endoderm to liver fate (Adapted from Zaret, Curr Opin Genet Devel 2001;11:568-574).

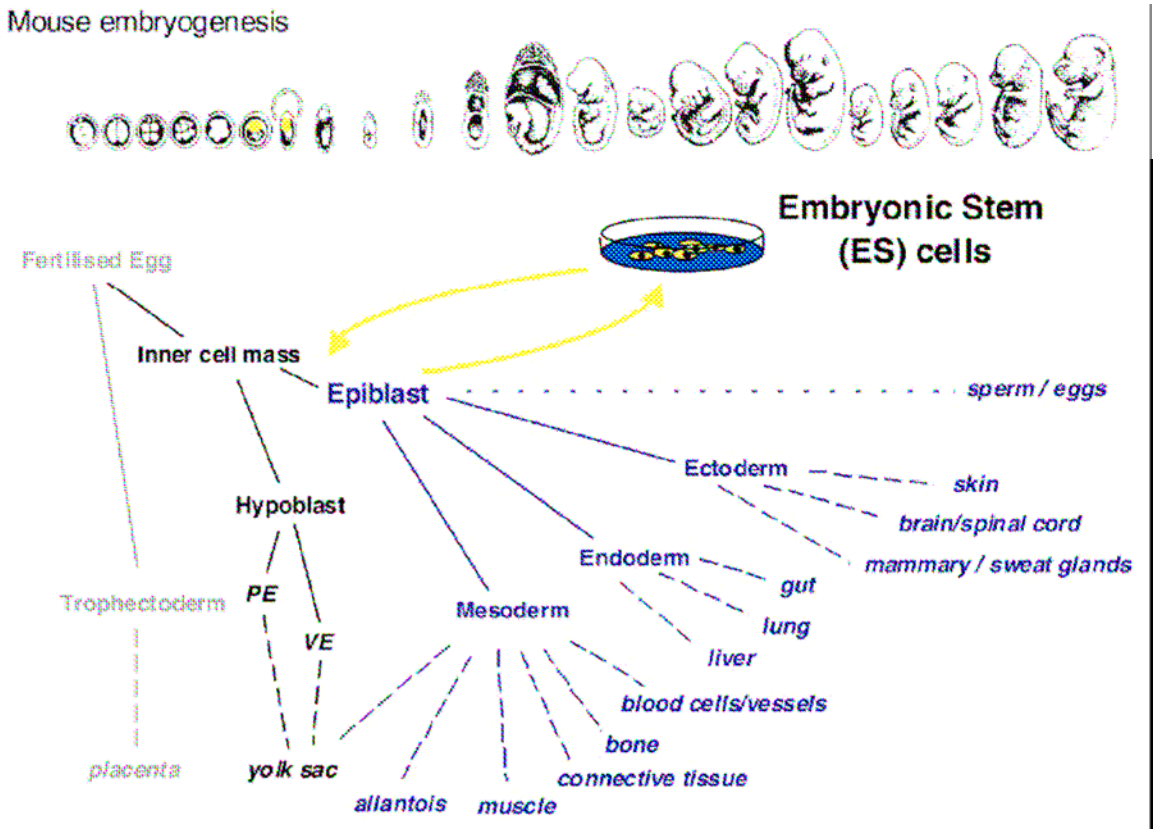


Figure 4. Embryonic stem cells in the context of mouse development. The upper panel shows the mouse embryogenesis and the inner cell mass (yellow) from which the ES cells are harvested. The hypoblasts lineages that can be produced by ES cells are outlined in blue. The ES cells can produce the derivatives of hypoblasts *in vitro* but rarely *in vivo*. Adapted from Smith. Annu Rev Cell Dev Biol 2001;17:435-62.

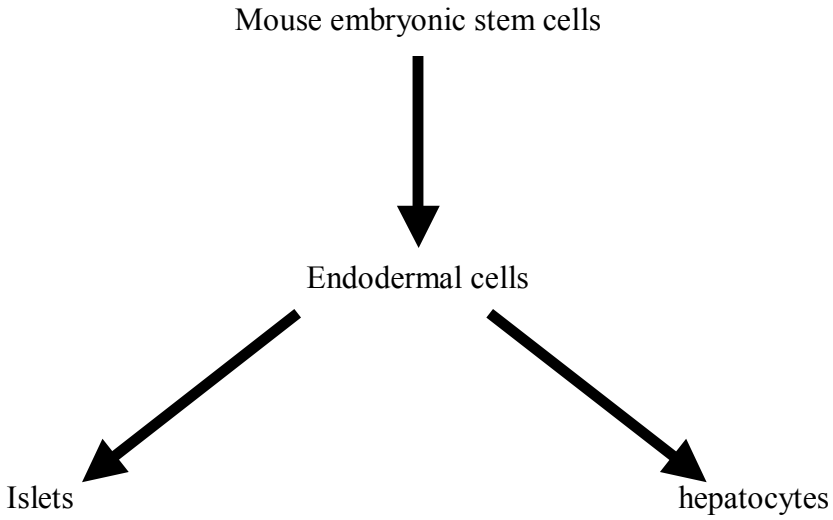
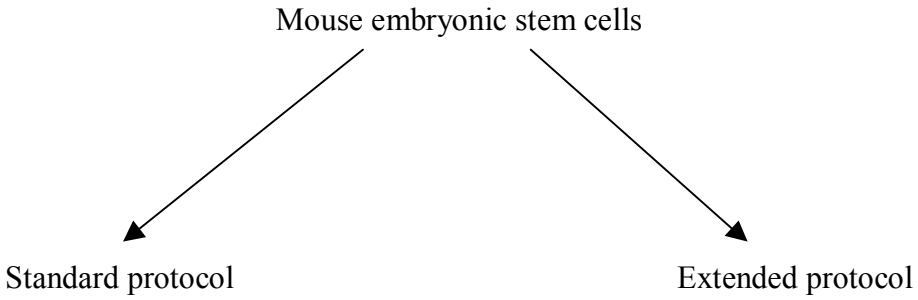


Figure 5(a). A conceptual overview of differentiation pathway from mouse embryonic stem cells to endodermal cells *in vitro*. An attempt was made to produce endodermal cells followed by further differentiation into islets and hepatocytes.



1. Production of definitive endoderm

(a) markers of definitive endoderm by Q-PCR

2. Production of beta cells

(a) markers of beta cells by Q-PCR
(b) insulin and C-peptide production by immunohistochemistry, radioimmunoassay
(c) insulin content and secretion abilities of the cells: static insulin assay

3. Production of hepatocytes

(a) markers of hepatocytes by Q-PCR

Figure 5(b). An overview of the experimental design. To evaluate the effects of different culture time period on production of definitive endoderm, two different culture conditions with different time periods (standard and extended) were tested. The production of definitive endoderm was assessed by Q-PCR. The production of beta cells was assessed by Q-PCR, immunohistochemistry, and radioimmunoassay. The secretion abilities of the cells were tested by static insulin assay. The production of hepatocytes was assessed by Q-PCR.

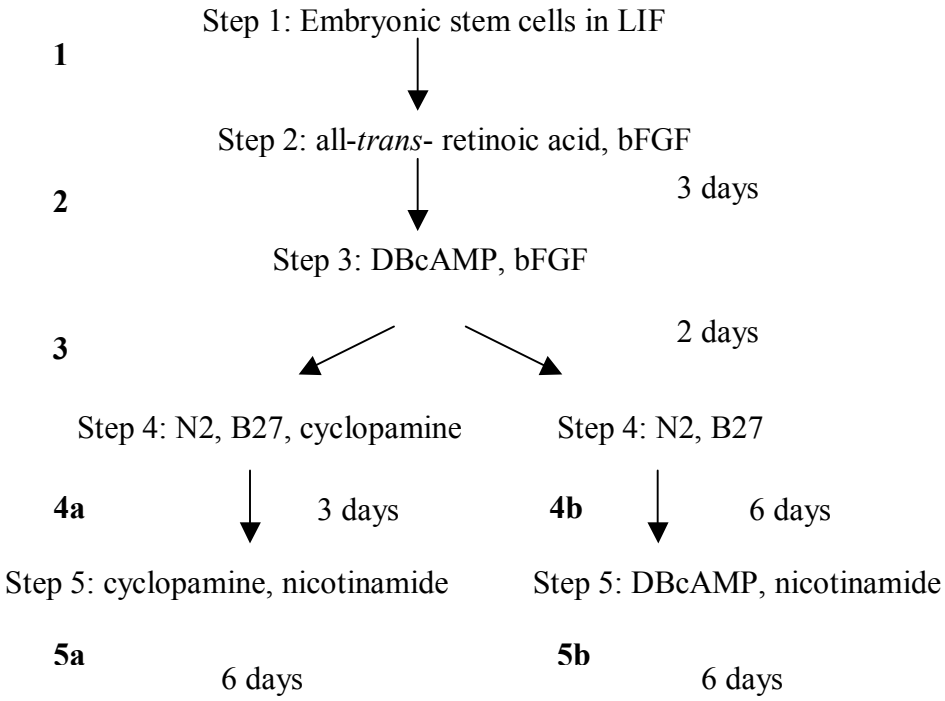


Figure 6. Differentiation pathway of mouse embryonic stem cells in the standard protocol. The ES cells are maintained in leukemia inhibitory factor (LIF) (1) before undergoing differentiation in Step 2 for 3 days (2), Step 3 for 2 days (3). The cells are then cultured in Step 4 for 3 days in N2 and B27 and cyclopamine (4a) or with N2 and B27 only (4b). The 4a cells were then cultured in cyclopamine and nicotinamide for 6 days (5a) and in DBcAMP and nicotinamide for 6 days (5b).

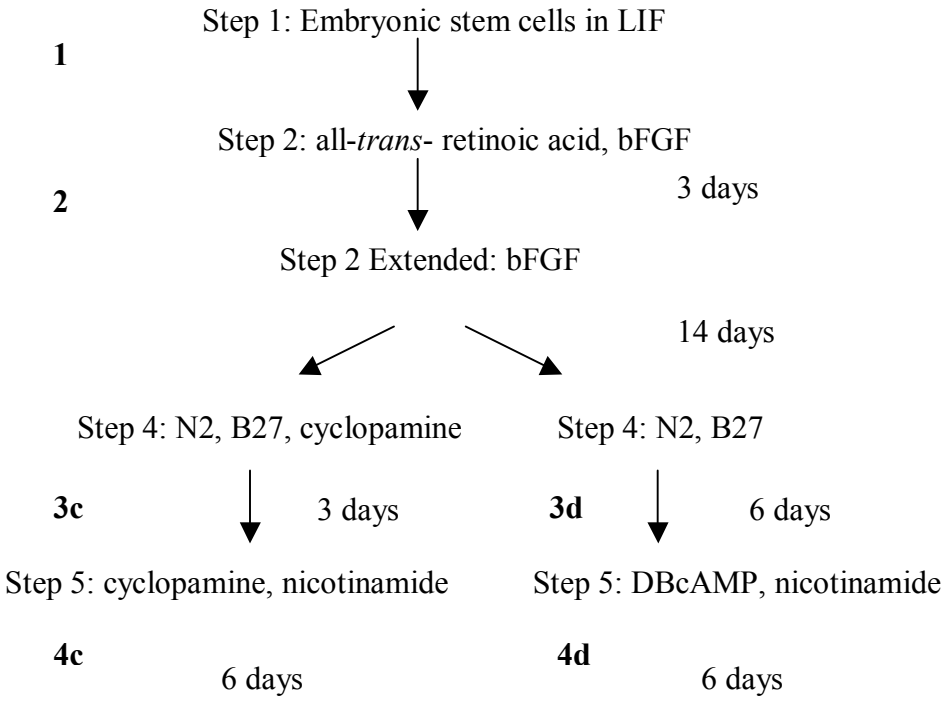


Figure 7. Differentiation pathway of mouse embryonic stem cells in the extended protocol. The ES cells were maintained in leukemia inhibitory factor (LIF) (denoted with 1) before undergoing differentiation in Step 2 for 3 days (denoted with 2). After this step, the retinoic acid was removed from the culture, and the cells were cultured for 14 days. The cells were then cultured in Step 4 for 3 days in N2 and B27 and cyclopamine (3c) or with N2 and B27 only (3d). The 3a cells were then cultured in cyclopamine and nicotinamide for 6 days (4c) and in DBcAMP and nicotinamide for 6 days (4d).

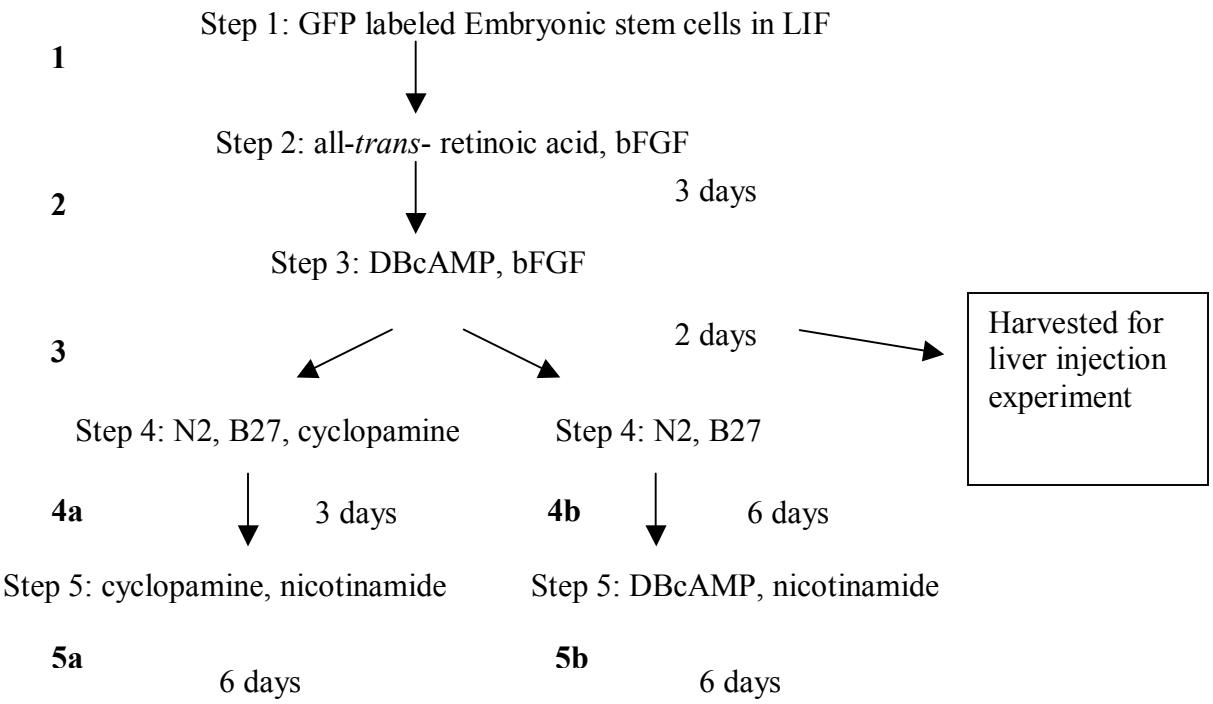
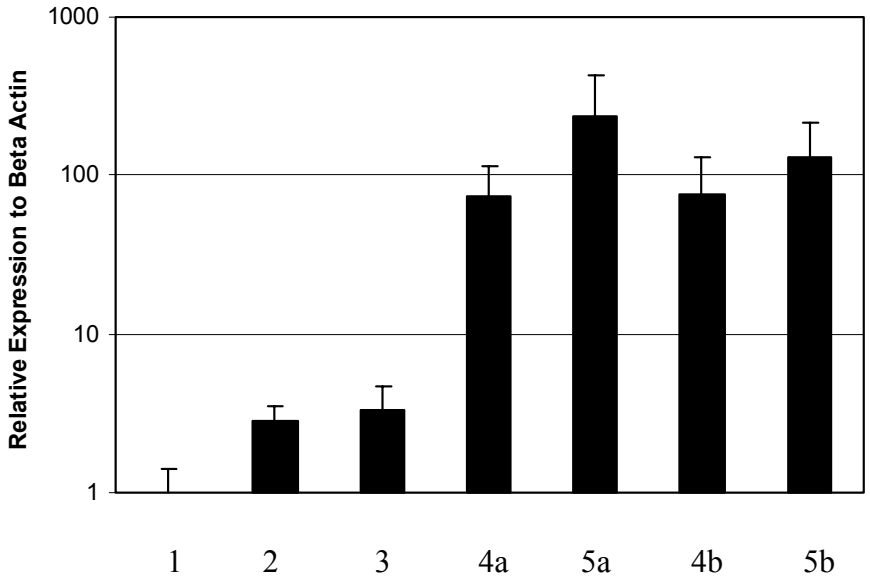
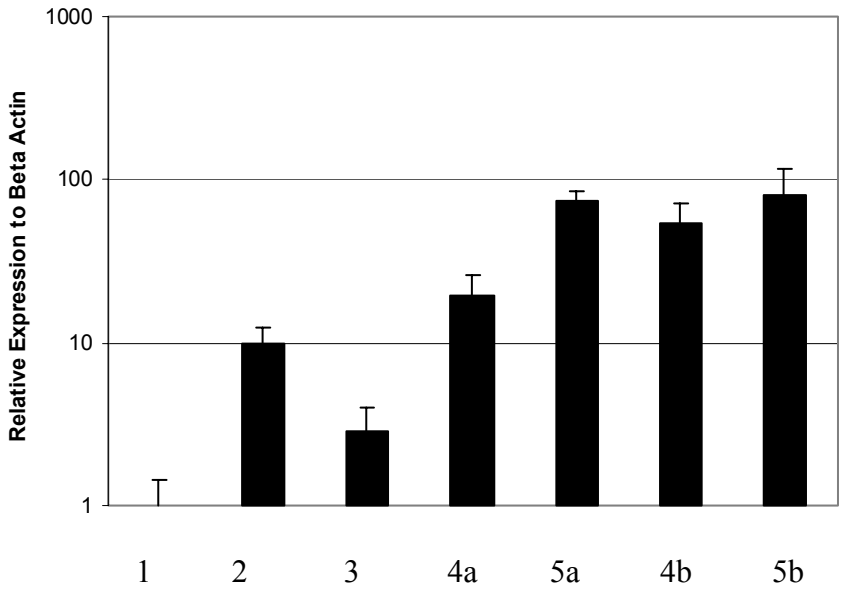


Figure 8. Differentiation pathway of mouse embryonic stem cells. The ES cells are labeled with green fluorescence protein (GFP) and maintained in leukemia inhibitory factor (LIF) (1) before undergoing differentiation in Step 2 for 3 days (2), Step 3 for 2 days (3). The cells are then cultured in Step 4 for 3 days in N2 and B27 and cyclopamine (4a) or with N2 and B27 only (4b). The 4a cells were then cultured in cyclopamine and nicotinamide for 6 days (5a) and in DBcAMP and nicotinamide for 6 days (5b).

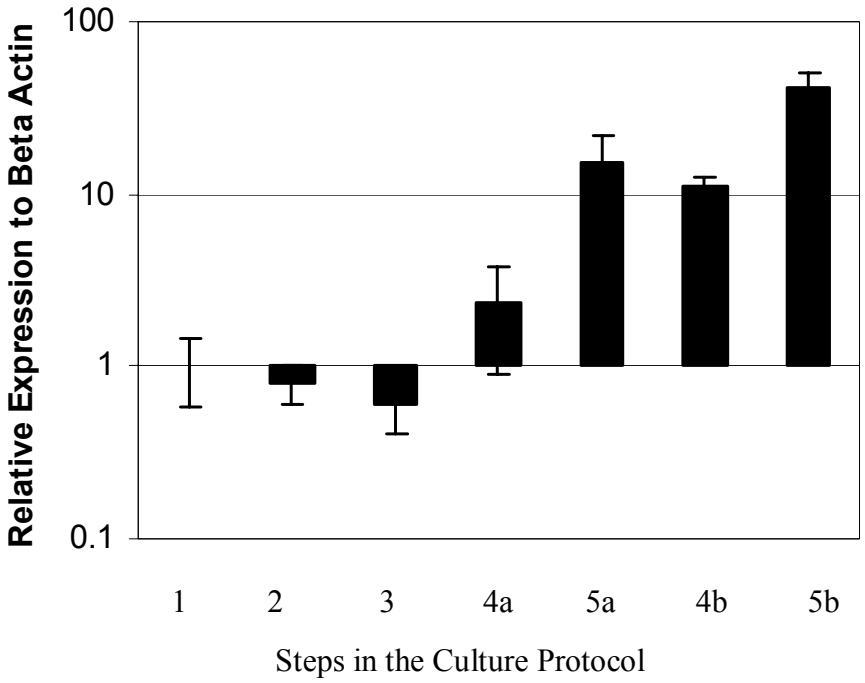
(a)


Sox 17
1000
100
10
1
Relative Expression to Beta Actin
1
2
3
4a
5a
4b
5b
Steps in the Culture Protocol

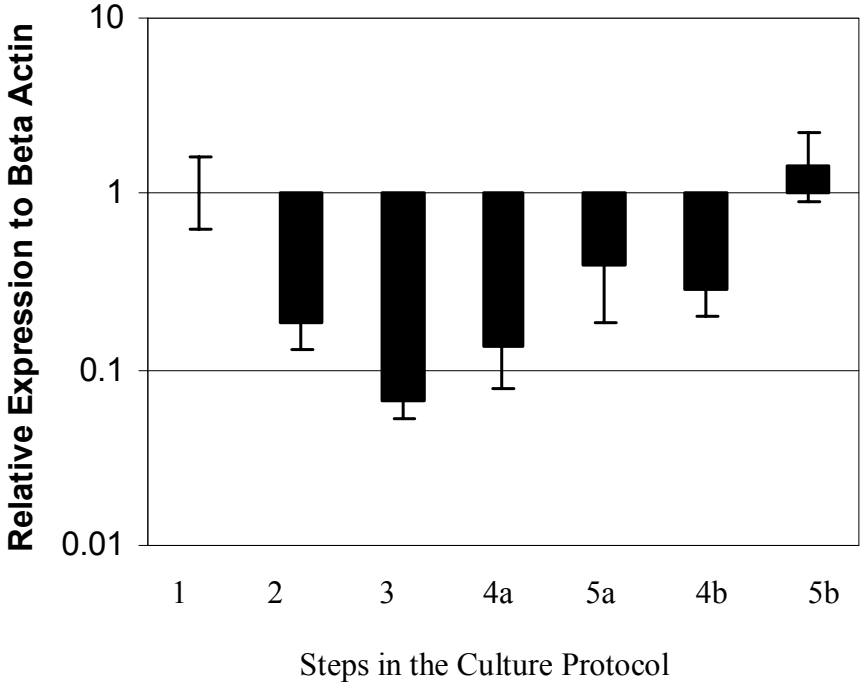
(b)


FOXA2
1000
100
10
1
Relative Expression to Beta Actin
1
2
3
4a
5a
4b
5b
Steps in the Culture Protocol

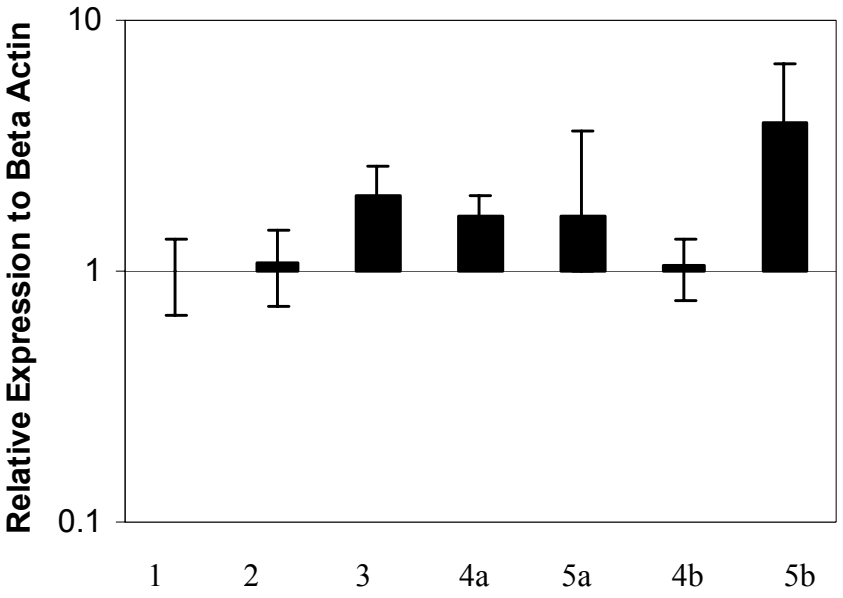
(c)


Sox7
Relative Expression to Beta Actin
100
10
1
0.1
1
2
3
4a
5a
4b
5b
Steps in the Culture Protocol

(d)


Brachyury
Relative Expression to Beta Actin
10
1
0.1
0.01
1
2
3
4a
5a
4b
5b
Steps in the Culture Protocol

(e)



(f)

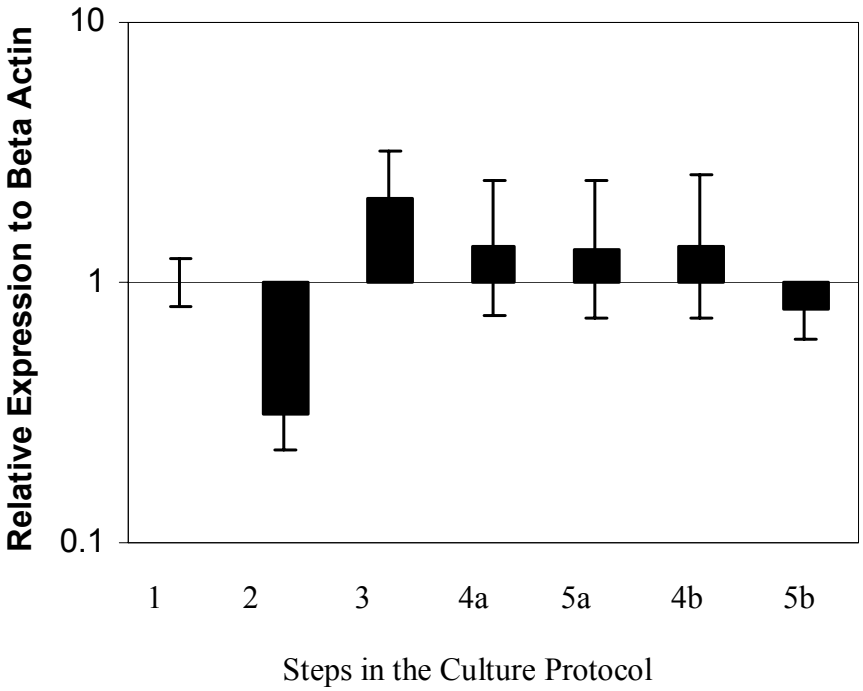
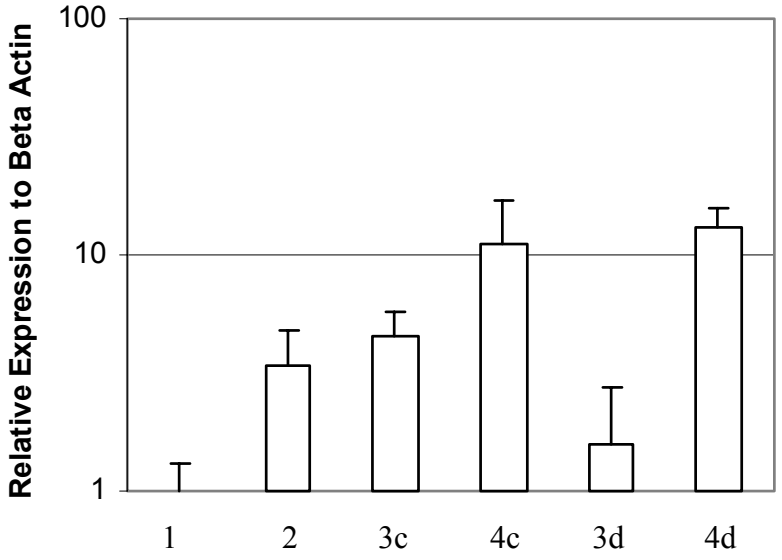


Figure 9. Expression of endodermal markers (Sox 17, FOXA2 and MIXL1), primitive endoderm marker (Sox 7), neuroectoderm marker (Sox 1), and mesoderm marker (brachyury), measured as relative expression to beta actin in embryonic stem cells (step 1). The embryonic stem cells were cultured in all-*trans*-retinoic acid and bFGF in Step 2, and DBcAMP and bFGF in step 3. Subsequently, they were cultured in suspension in the presence of cyclopamine, N2, B27 in step 4a and cyclopamine and nicotinamide in Step 5a. The step 3 cells were also cultured in N2 and B27 only in Step 4b followed by DBcAMP and nicotinamide in step 5b.

Sox 17

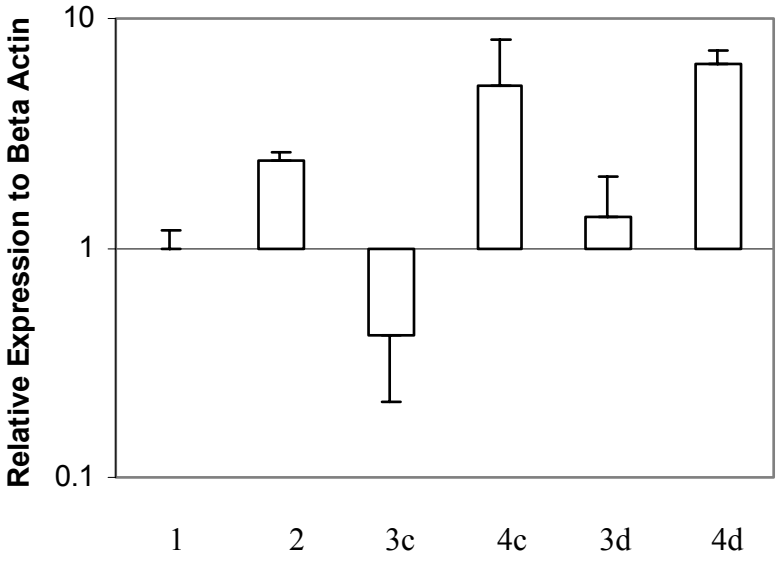
(a)



Steps in the Culture Protocol

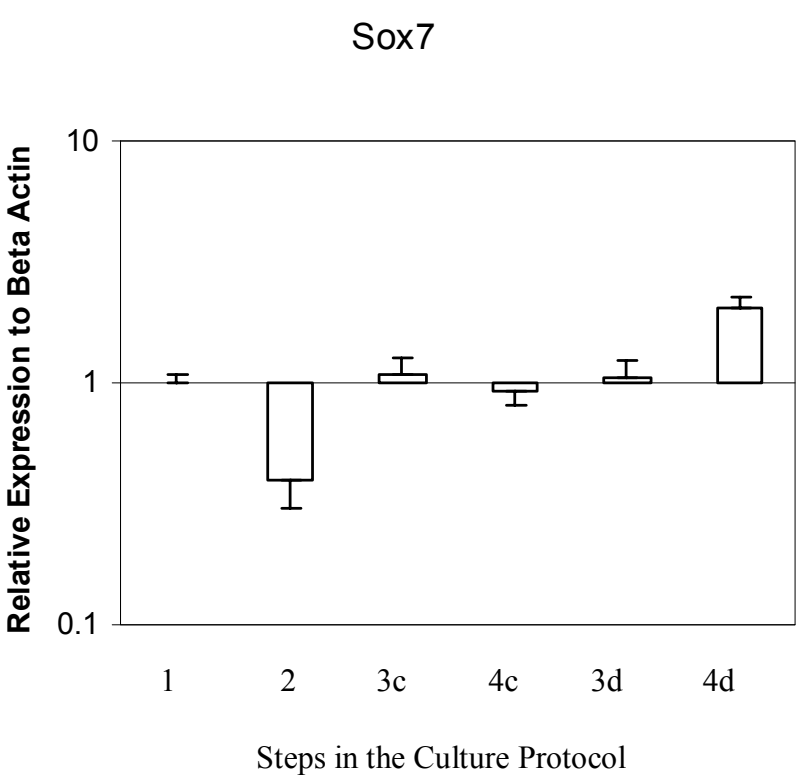
FOXA2

(b)

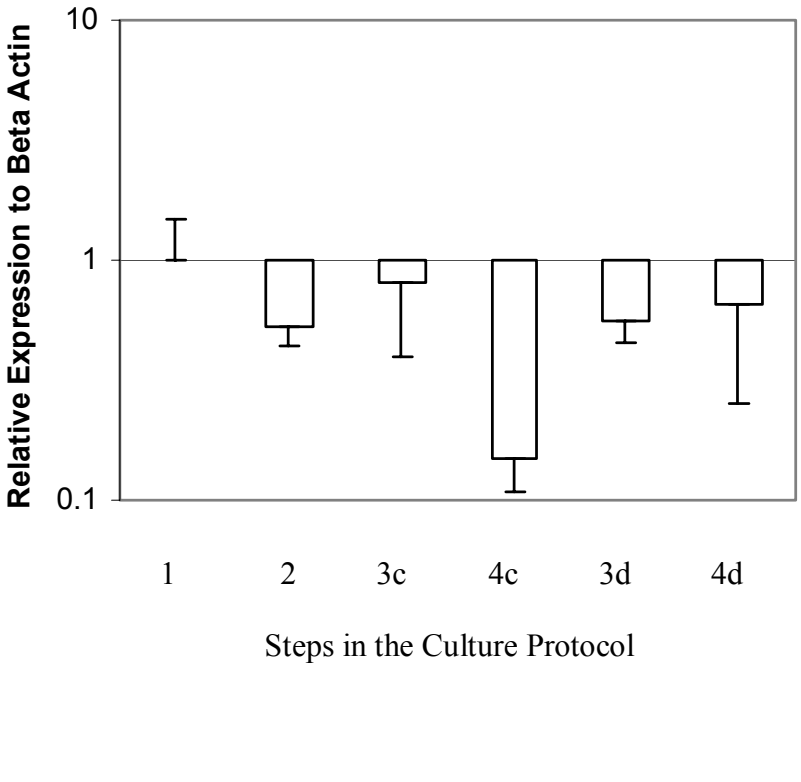


Steps in the Culture Protocol

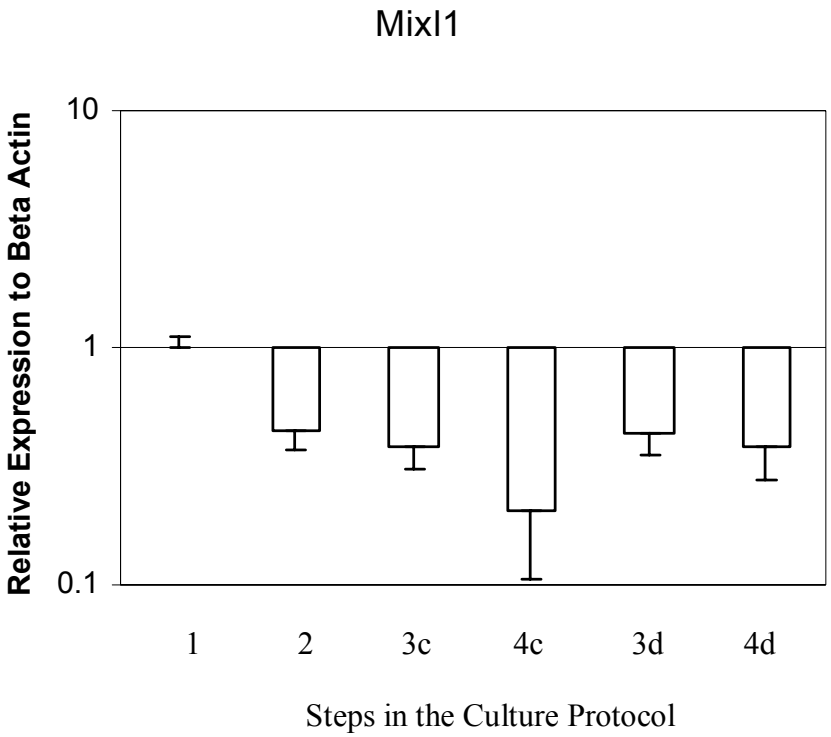
(c)


Sox7
Relative Expression to Beta Actin
10
1
0.1
1
2
3c
4c
3d
4d
Steps in the Culture Protocol

(d)


Brachyury
Relative Expression to Beta Actin
10
1
0.1
1
2
3c
4c
3d
4d
Steps in the Culture Protocol

(e)



(f)

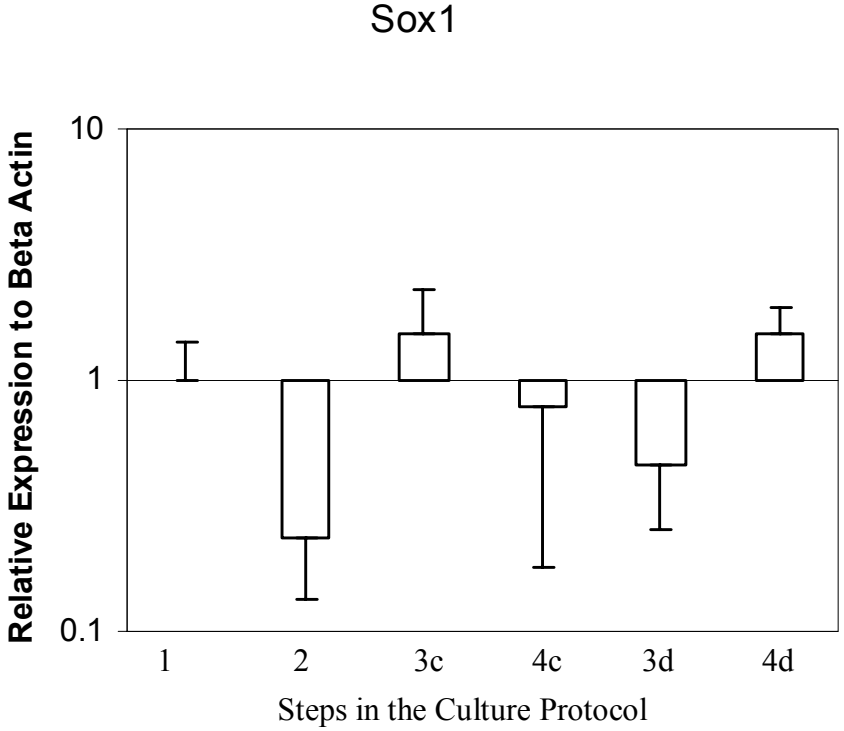


Figure 10. Expression of endodermal markers (Sox 17, FOXA2 and MIXL1), primitive endoderm marker (Sox 7), neuroectoderm marker (Sox 1), and mesoderm marker (brachyury), measured as relative expression to beta actin in embryonic stem cells (step 1). The expression of these markers were measured in each of the extended culture protocol. The embryonic stem cells were cultured in the presence of all-trans-retinoic acid and bFGF (step 2) followed by 14 days in the absence of all-trans-retinoic acid. The step 2 cells were then cultured in N2, B27, cyclopamine (step 3c) followed by cyclopamine and nicotinamide (step 4c). The step 2 cells were also cultured in N2 and B27 (step 3d) followed by DBcAMP and nicotinamide (step 4d).

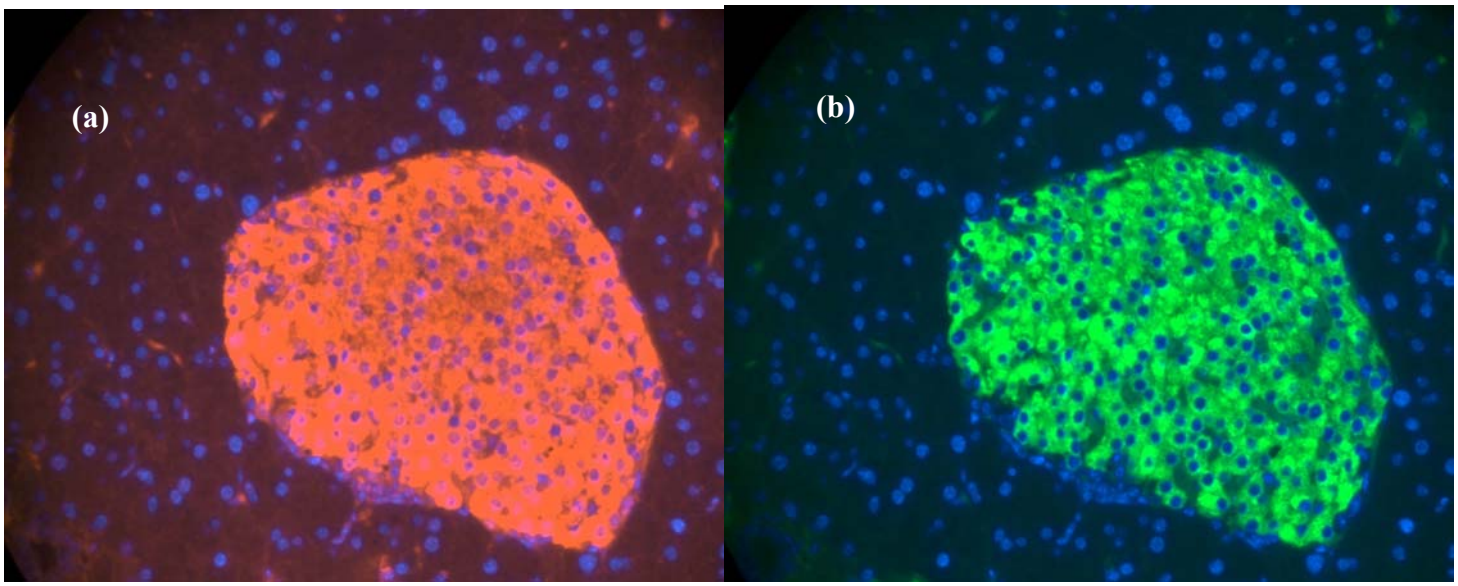


Figure 11. Immunohistochemistry of (a) insulin stain (200x), (b) C-peptide (200x) of adult mouse islets for reference purposes. Insulin stain was done with mouse anti-mouse monoclonal primary antibody at 1:200 dilution and donkey anti-mouse Alex Fluor 594 secondary antibody at 1:200 dilution. C-peptide stain was done with goat anti-rat primary antibody at 1:100 dilution and donkey anti-goat Alex Fluor 488 secondary antibody at 1:200 dilution. DAPI (nuclear stain) is in blue.

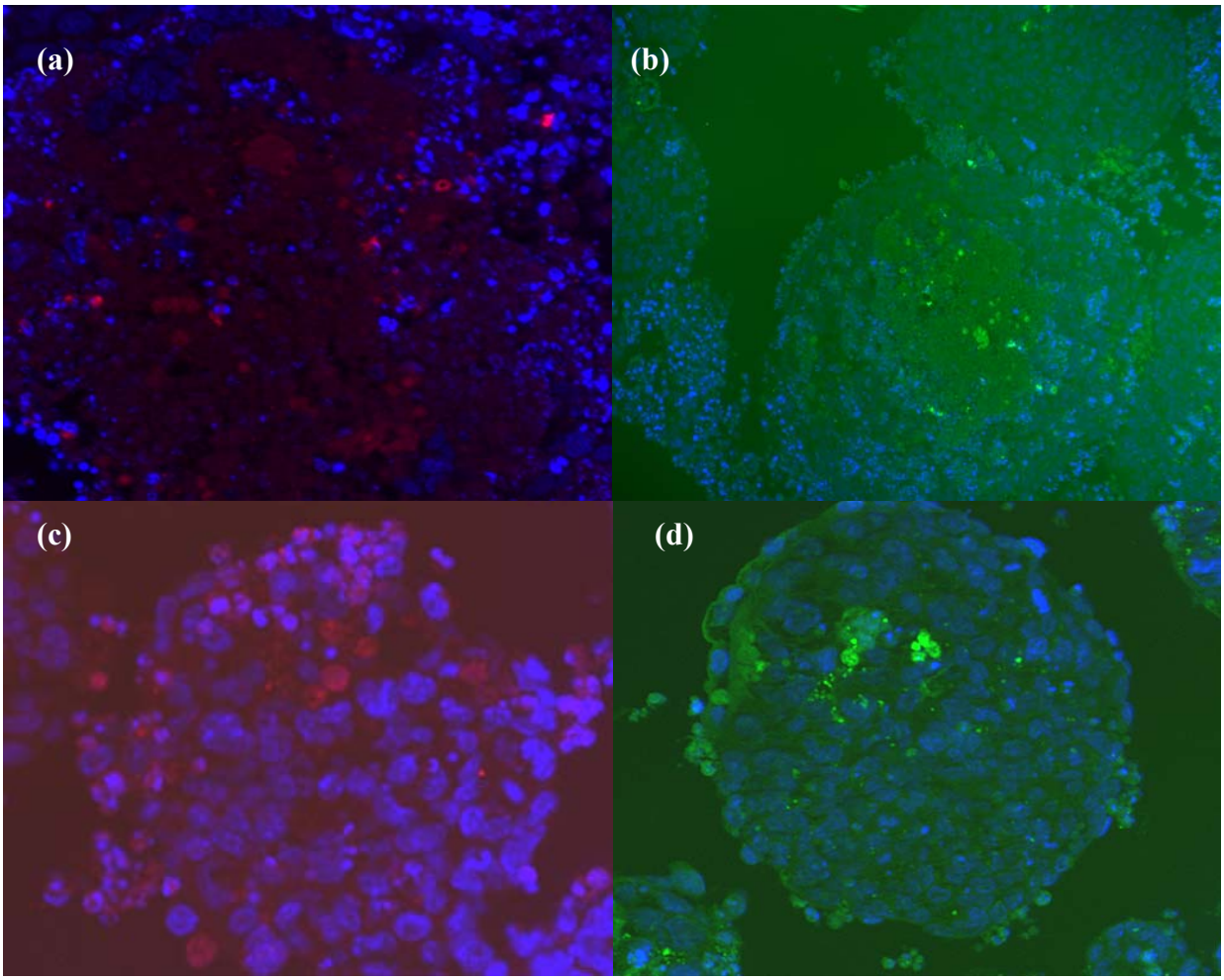


Figure 12. Immunohistochemistry staining for insulin (a and c), (200x), C-peptide (b and d), (200x) of cells clusters from step 5 of the regular protocol: (a) insulin stain of 5a, (b) C-peptide stain of 5a, (c) insulin stain of 5b, and (d) C-peptide stain of Db. Insulin stain was performed with mouse anti-mouse monoclonal primary antibody at 1:200 dilution and donkey anti-mouse Alex Fluor 594 secondary antibody at 1:200 dilution. C-peptide stain was done with goat anti-rat primary antibody at 1:100 dilution and donkey anti-goat Alex Fluor 488 secondary antibody at 1:200 dilution. DAPI (nuclear stain) is in blue.

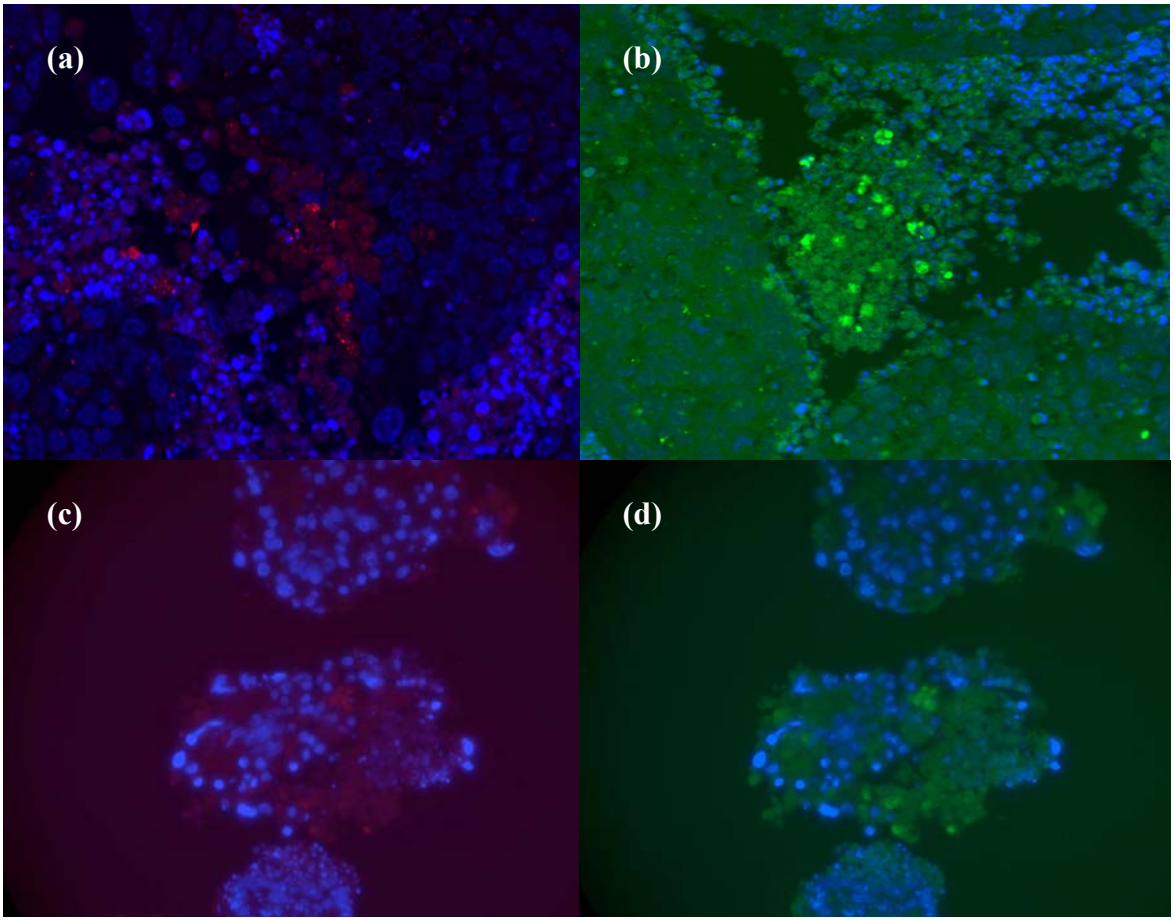
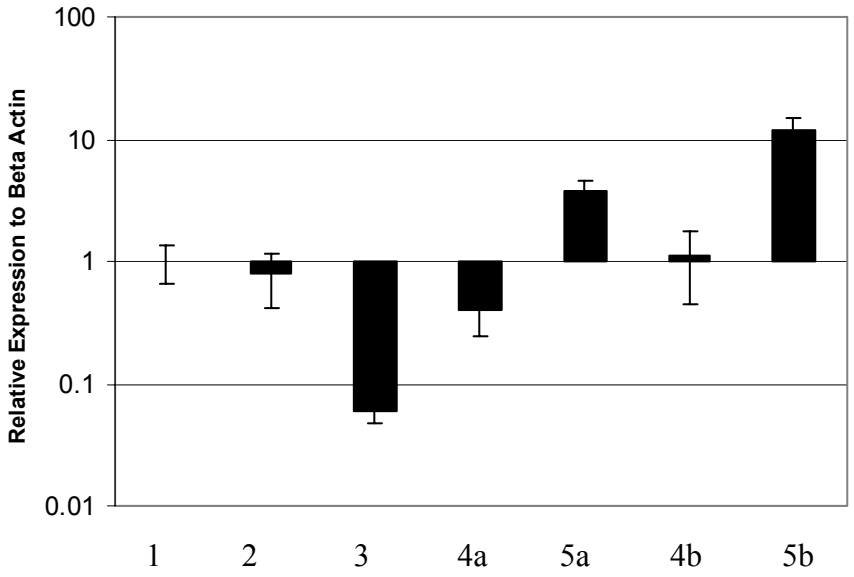
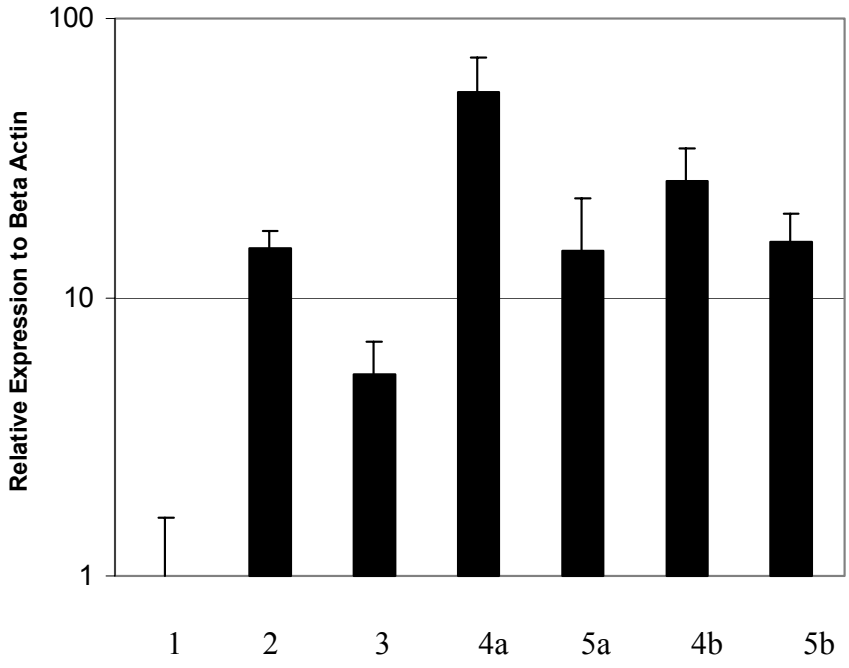


Figure 13. Immunohistochemistry staining for of insulin (a and c), (200x), C-peptide (b and d), (200x) of cells clusters from step 5 of the extended protocol: (a) insulin stain of 4c, (b) C-peptide stain of 4c, (c) insulin stain of 4d, and (d) C-peptide stain of 4d. Insulin stain was performed with mouse anti-mouse monoclonal primary antibody at 1:200 dilution and donkey anti-mouse Alex Fluor 594 secondary antibody at 1:200 dilution. C-peptide stain was done with goat anti-rat primary antibody at 1:100 dilution and donkey anti-goat Alex Fluor 488 secondary antibody at 1:200 dilution. DAPI (nuclear stain) is in blue.

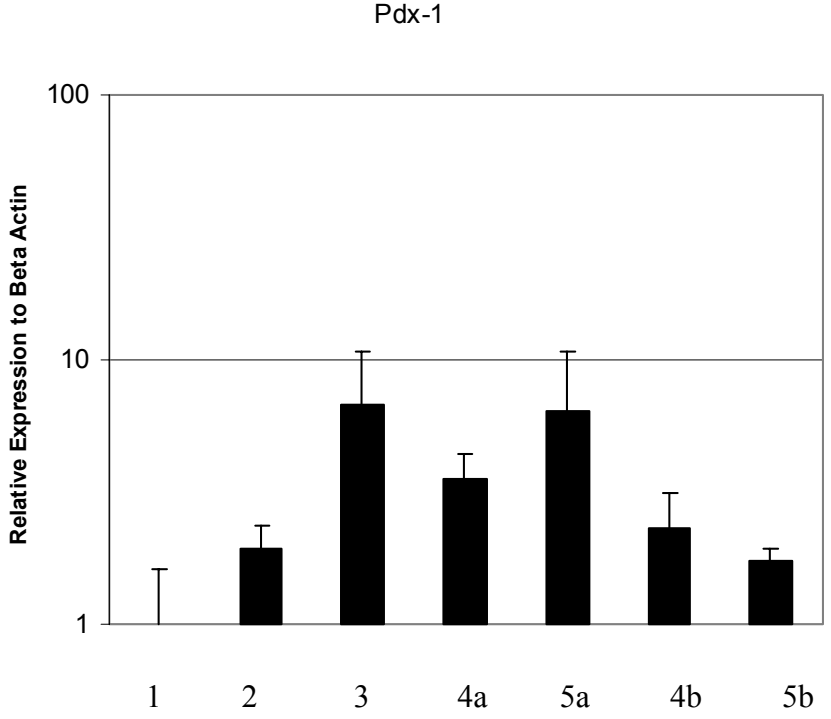
(a)


Insulin-I
100
10
1
0.1
0.01
Relative Expression to Beta Actin
1
2
3
4a
5a
4b
5b
Steps in the Culture Protocol

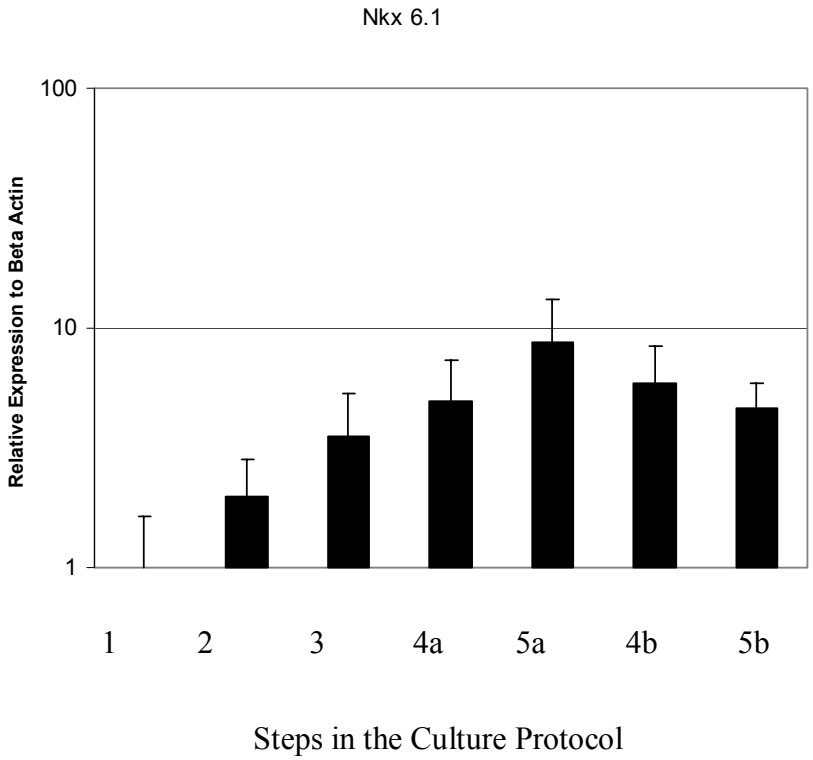
(b)


Insulin-II
100
10
1
Relative Expression to Beta Actin
1
2
3
4a
5a
4b
5b
Steps in the Culture Protocol

(c)


Pdx-1
100
10
1
Relative Expression to Beta Actin
1
2
3
4a
5a
4b
5b
Steps in the Culture Protocol

(d)


Nkx 6.1
100
10
1
Relative Expression to Beta Actin
1
2
3
4a
5a
4b
5b
Steps in the Culture Protocol

(e)

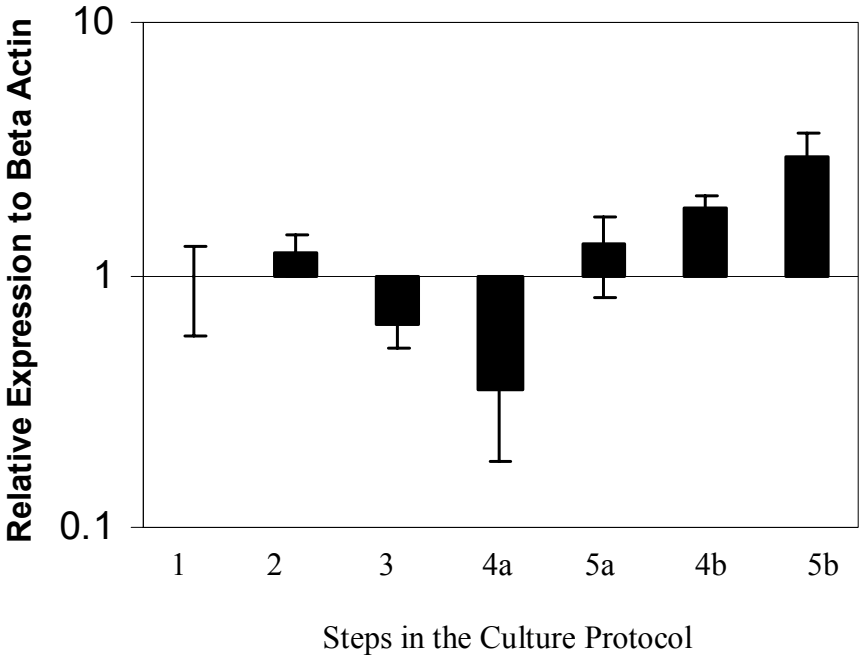
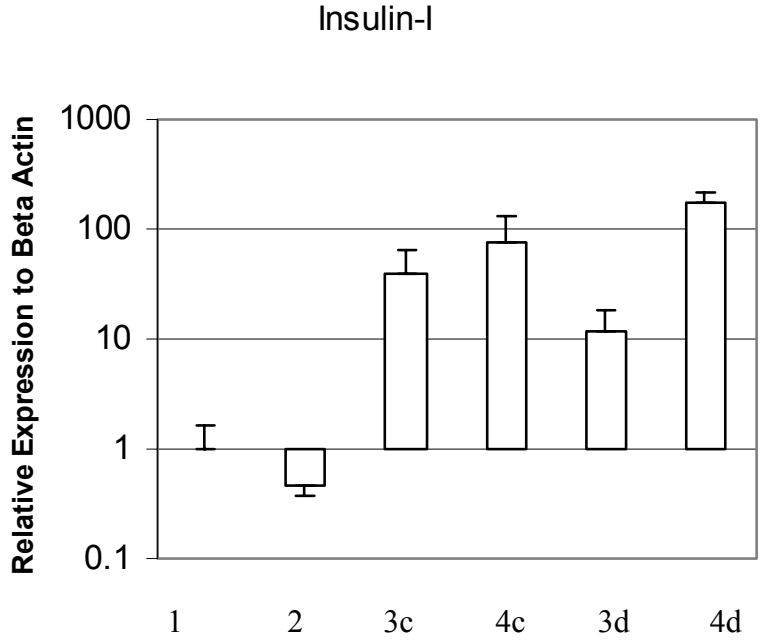
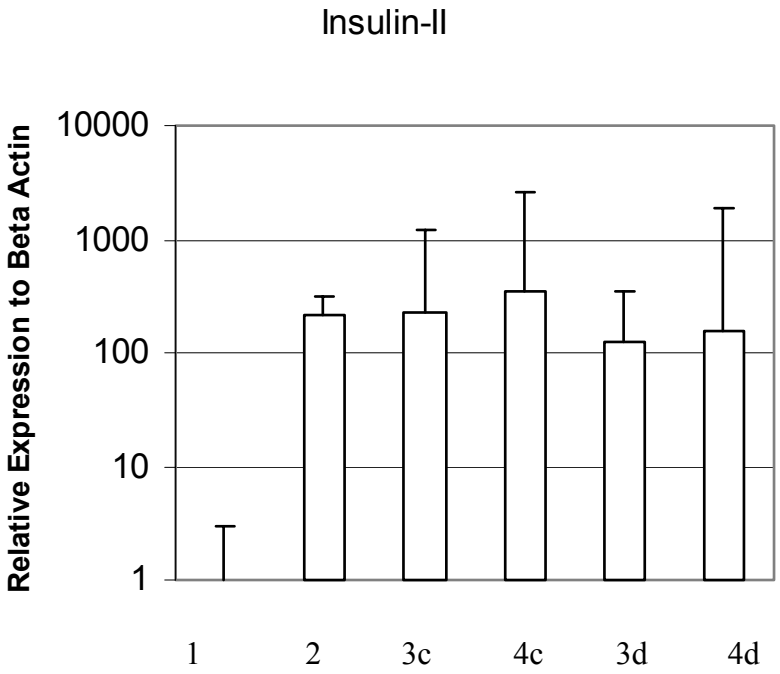


Figure 14. Expression of beta cell markers in cells produced using the regular protocol measured by quantitative PCR using SYBR green. Marker expression was normalized to the level of beta actin in the embryonic stem cells (Step 1). The embryonic stem cells were cultured in all-*trans*-retinoic acid and bFGF in Step 2, and DBcAMP and bFGF in step 3. Subsequently, they were cultured in suspension in the presence of cyclopamine, N2, B27 in step 4a and cyclopamine and nicotinamide in Step 5a. The step 3 cells were also cultured in N2 and B27 only in Step 4b followed by DBcAMP and nicotinamide in step 5b.

(a)

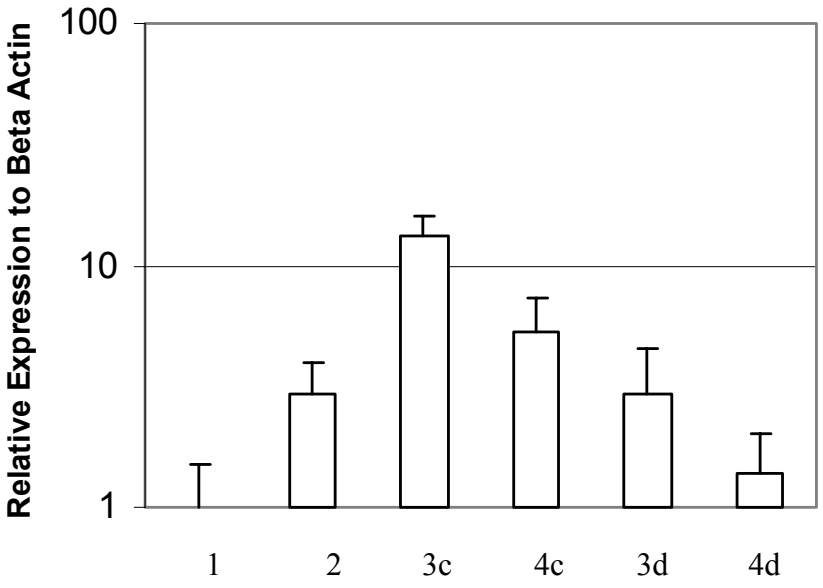

Insulin-I
Relative Expression to Beta Actin
1000
100
10
1
0.1
1
2
3c
4c
3d
4d

(b)


Insulin-II
Relative Expression to Beta Actin
10000
1000
100
10
1
1
2
3c
4c
3d
4d

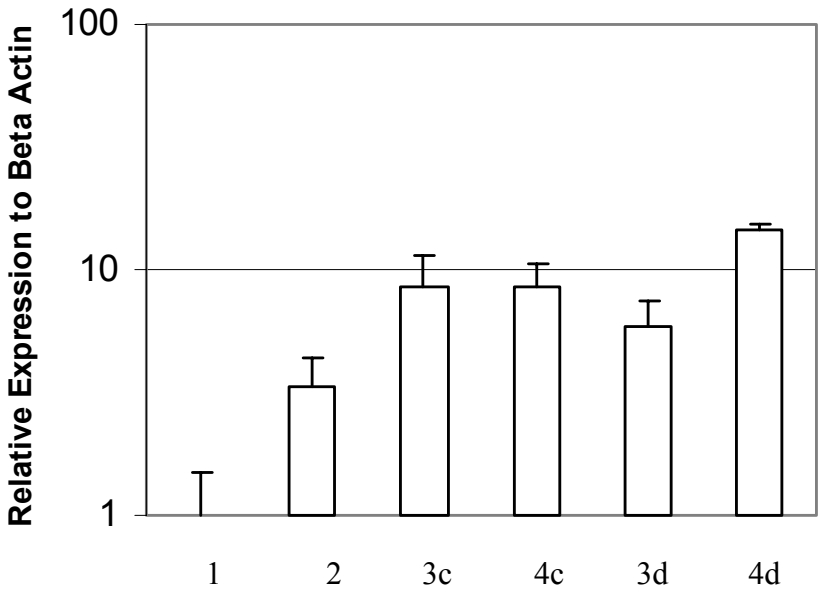
Pdx-1

(c)



Nkx 6.1

(d)



(e)

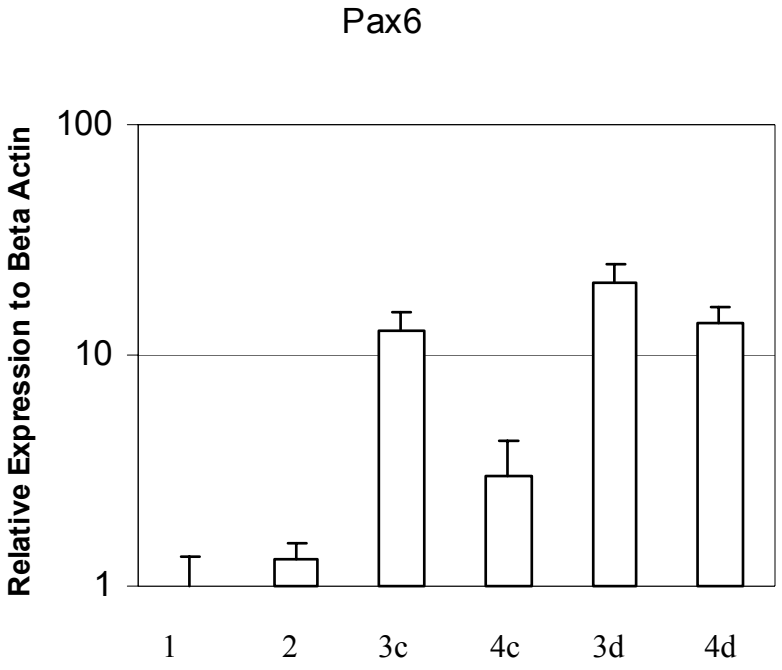


Figure 15. Expression of beta cell markers in cells produced from the extended protocol. Marker expression was normalized to the level of beta actin in the embryonic stem cells (Step 1). The expression of these markers were measured in each of the extended culture protocol. The embryonic stem cells were cultured in the presence of all-trans-retinoic acid and bFGF (step 2) followed by 14 days in the absence of all-trans-retinoic acid. The step 2 cells were then cultured in N2, B27, cyclopamine (step 3c) followed by cyclopamine and nicotinamide (step 4c). The step 2 cells were also cultured in N2 and B27 (step 3d) followed by DBcAMP and nicotinamide (step 4d).

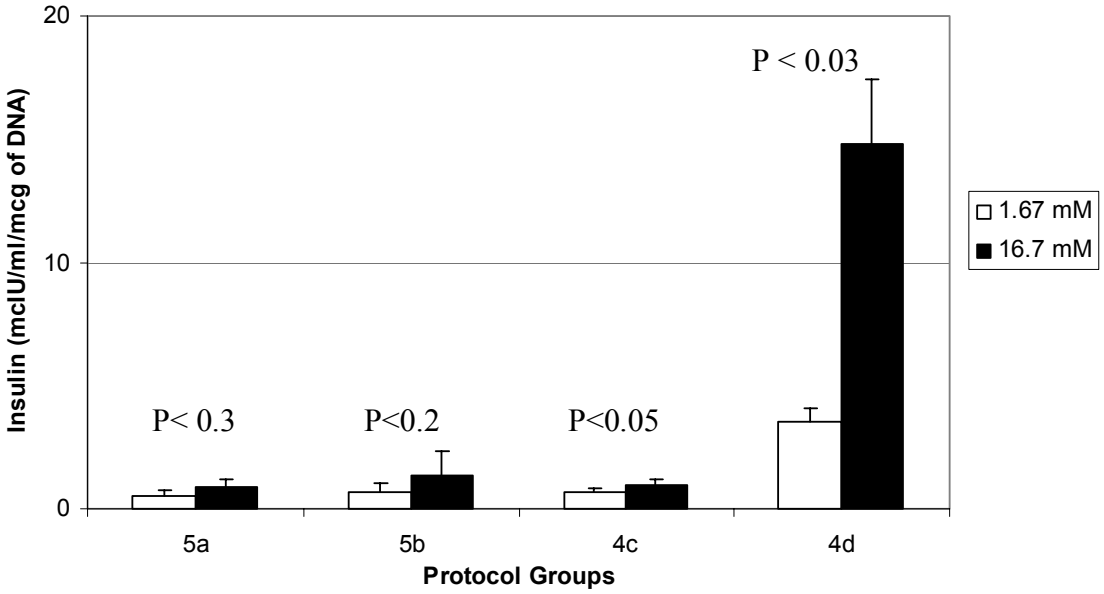


Figure 16. Static glucose responsive insulin release assay for cell clusters from the last step of each protocol. The cell clusters were incubated either in 1.67 mM of D-glucose or 16.7mM of D-glucose. The amount of secreted insulin was measured for each cell cluster by insulin radioimmunoassay and expressed as mcIU of insulin per ml per mcg of DNA. There was no statistically significant secretion of insulin by 5a cells (0.5 ± 0.2 vs. 0.9 ± 0.3, $P < 0.3$) and 5b cells (0.7 ± 0.4 vs. 1.4 ± 0.9, $P < 0.2$). There was a trend towards glucose responsive secretion by 4c cells (0.6 ± 0.2 vs. 1.0 ± 0.2, $P<0.05$). There was a statistically significant secretion of insulin by 4d cells in responsive to a high glucose environment (3.5 ± 0.5 vs. 14.8 ± 2.7, $P < 0.03$).

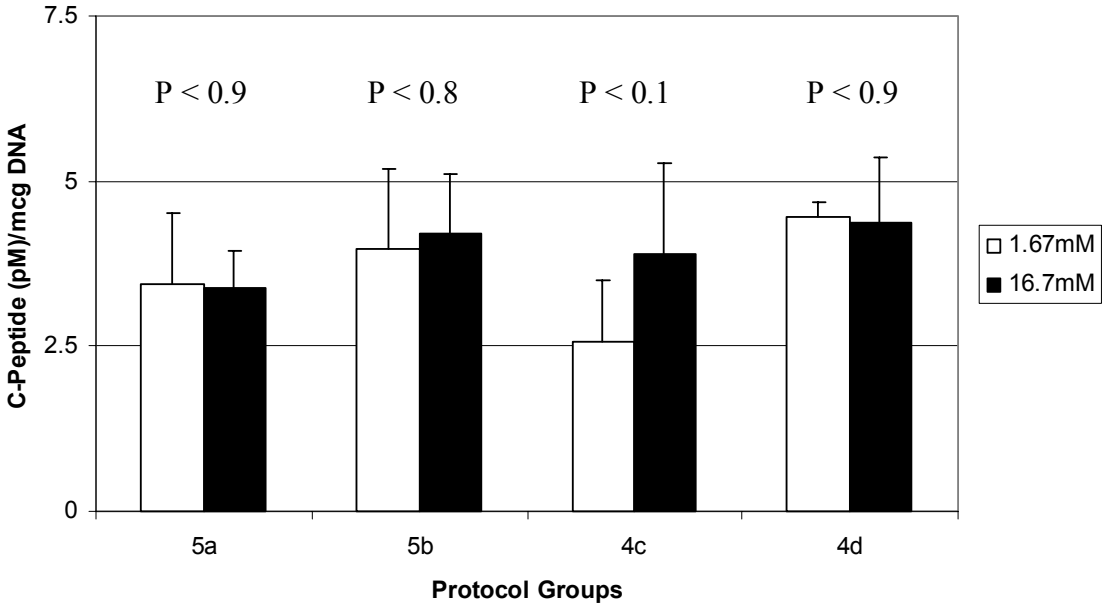


Figure 17. Static glucose responsive C-Peptide release assay for cell clusters from the last step of each protocol. The cell clusters were incubated either in 1.67 mM of D-glucose or 16.7mM of D-glucose. The amount of secreted C-Peptide was measured for each cell cluster by a radioimmunoassay. None of the conditions produced cells that secreted C-peptide in a glucose responsive manner: (a) 5a cells, 3.4 $\pm$ 1.1 vs. 3.4 $\pm$ 0.6 pM/mcg DNA, $P < 0.9$; (b) 5b cells, 4.0 $\pm$ 1.2 vs. 4.2 $\pm$ 0.9 pM/mcg DNA, $P < 0.8$; (c) 4c cells, 2.6 $\pm$ 0.9 vs. 4.0 $\pm$ 1.4 pM/mcg DNA, $P < 0.1$; and (d) 4d cells, 4.5 $\pm$ 0.2 vs. 4.4 $\pm$ 1.0 pM/mcg DNA, $P < 0.9$).

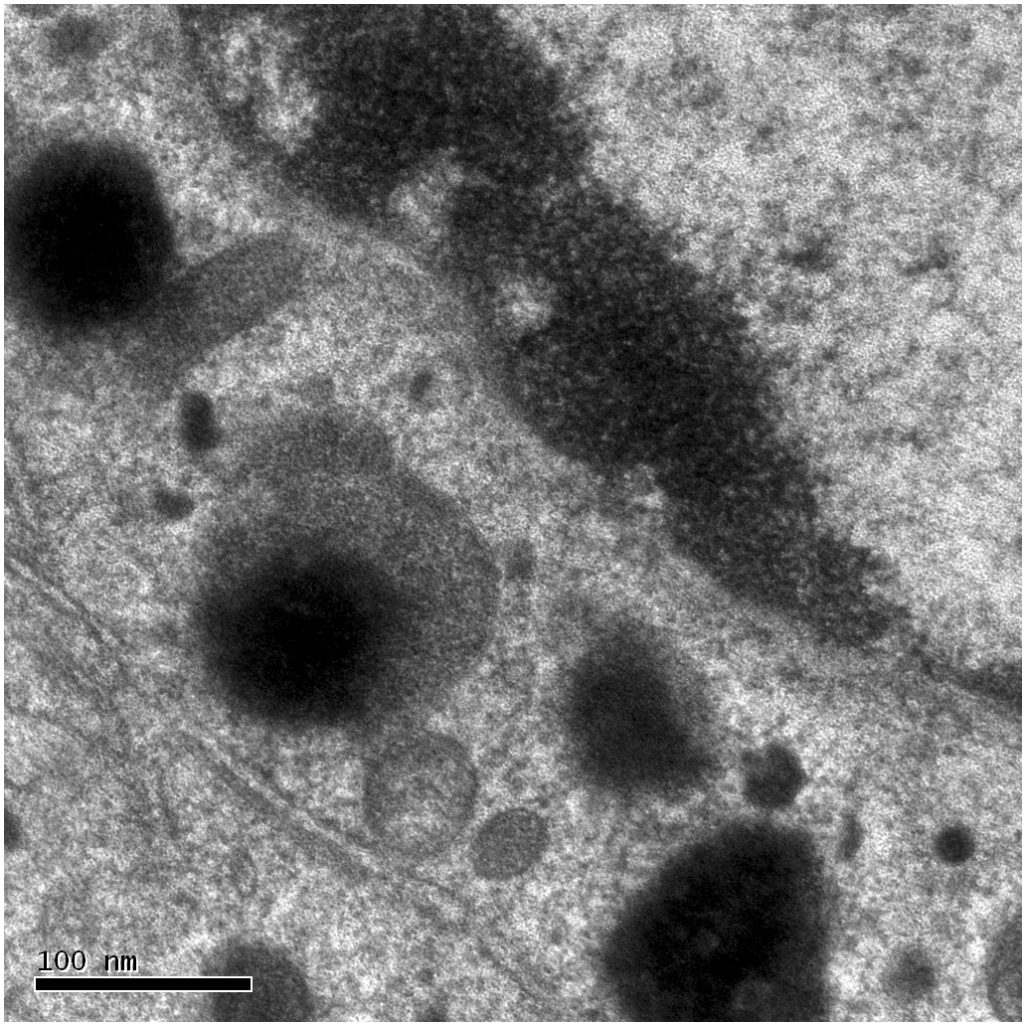


Figure 18. Transmission electron micrograph of the cytoplasm of the 4d cell clusters from the extended protocol demonstrating secretory granules.

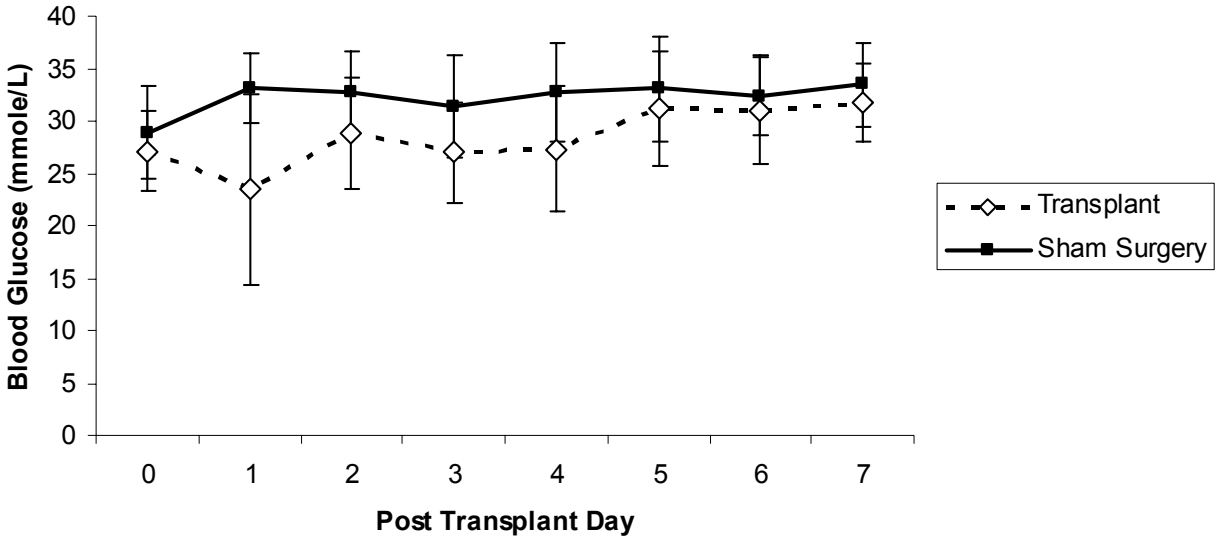


Figure 19. Results of splenic transplantation of DBcAMP treated cell clusters (4d) from the extended protocol. Approximately, 3,000 cell clusters (150 per gram of mouse) were transplanted into the spleen of streptozotocin treated C57/Black6 mice. The control group received an injection of PBS into the spleen after treatment of streptozotocin.

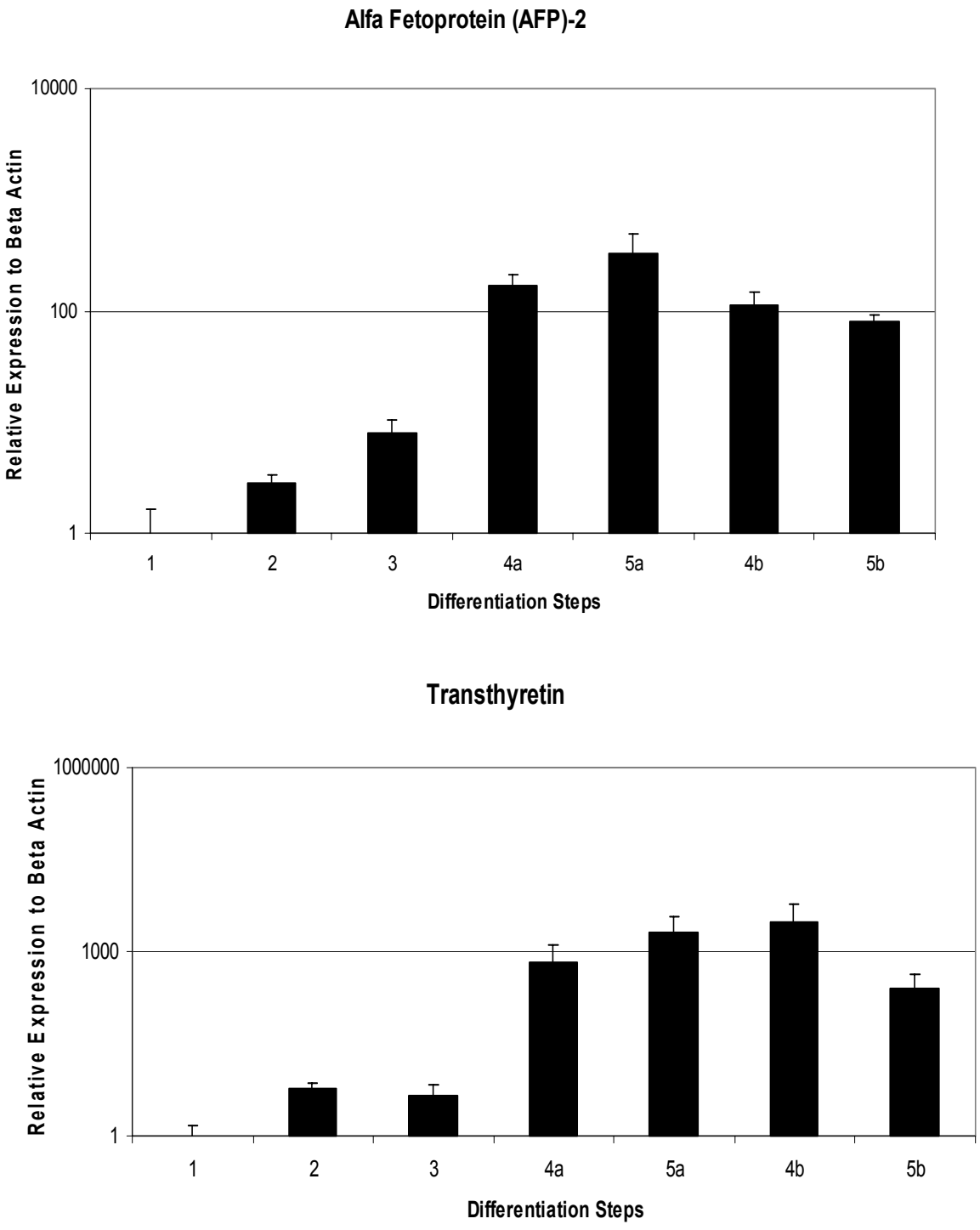


Figure 20. Expression of hepatocyte markers (AFP-2 and transthyretin) in the differentiation steps of the regular protocol outlined as relative to their expression levels in Step 1 (embryonic stem cells).

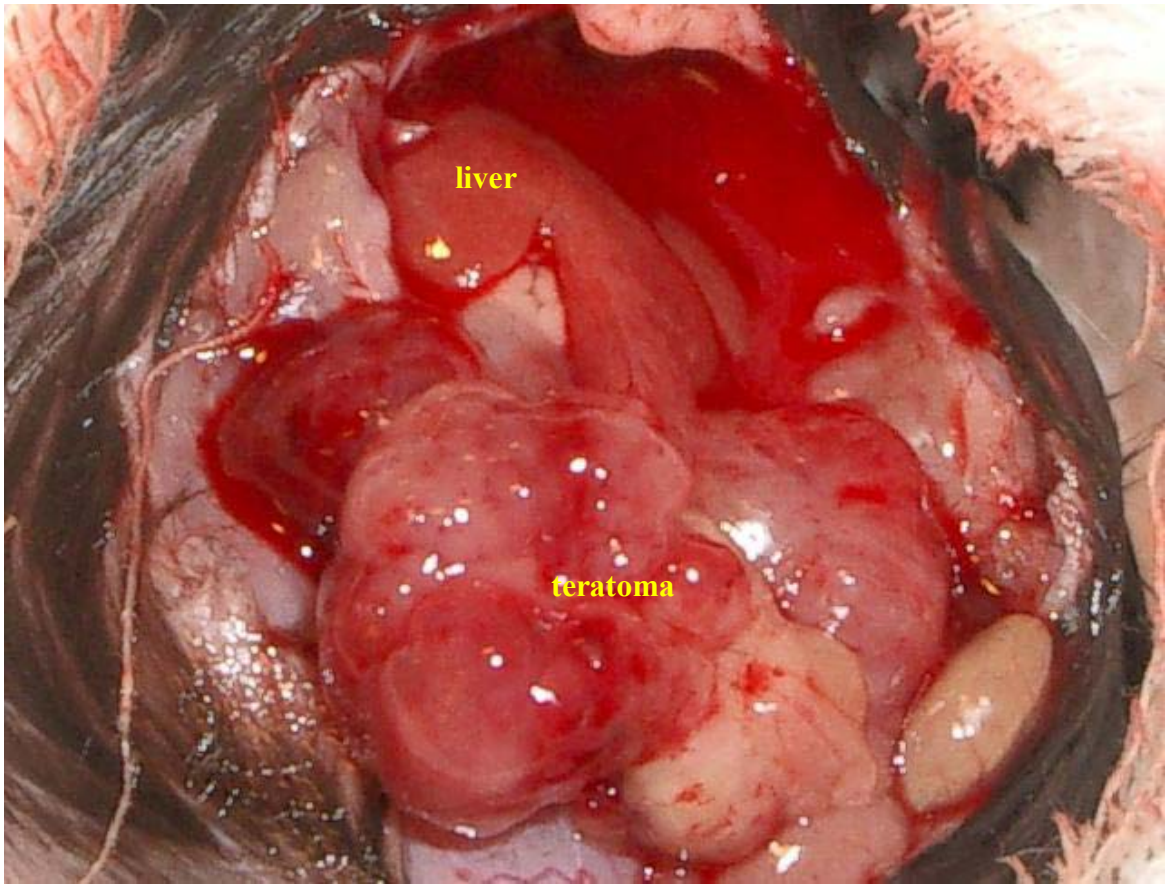


Figure 21. A photograph of a teratoma arising from the liver after an injection of the Step 3 cells from the regular protocol. The abdomen of the mouse is open and the teratoma fills the abdominal cavity, protruding from the edge of the liver where the step 3 cells were injected.

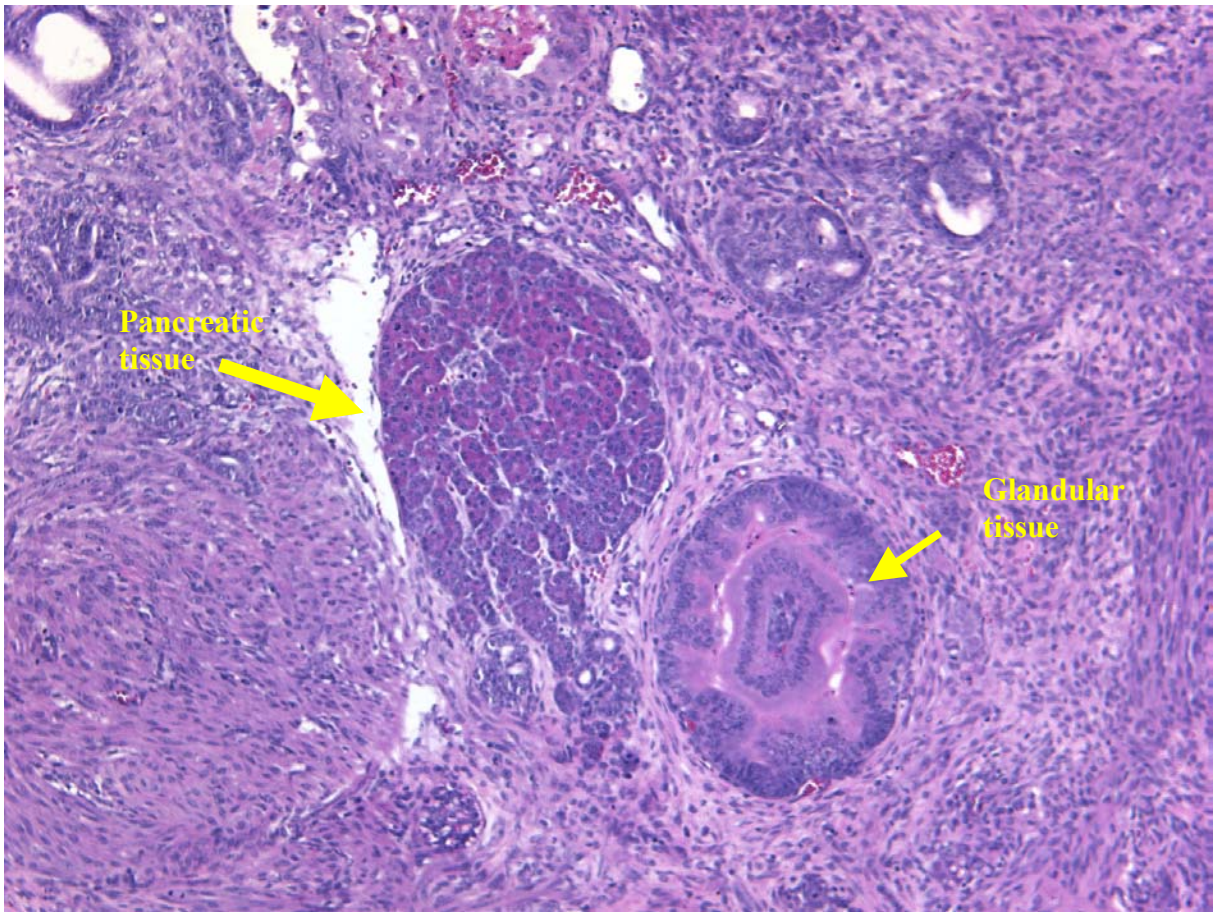


Figure 22. Hematoxylin and eosin stain of the teratoma arising from the injection site of step 3 cells from the regular protocol (200x). There is a presence of tissues from all three germ cell layers including the pancreatic tissues, glandular tissues, smooth muscles and cartilage

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