PLURIPOTENT STEM CELL DERIVED ENDODERM IN INTERSPECIES BLASTOCYST COMPLEMENTATION

A THESIS SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA

BY: NOAH C. DENMAN

IN PARTIAL FULFILLMENT
OF THE REQUIERMENTS
FOR THE DGREE OF
MASTER OF SCIENCE

Faculty Advisor:

James Dutton, PhD

Jan, 2020

ACKNOWLEDGMENTS

Dr. Susan Keirstead, PhD

Dr. James Dutton, PhD

Dr. Clifford Steer, PhD

Dr. Walter C. Low, PhD

Dr. Mercy Ruiz, PhD

Dr. Andrew Crane, PhD

Caitlin Hill, M.S.

ABSTRACT

Every year in the United States approximately 4.5 million people are living with liver disease. Of those, an estimated 45,000 will die due to complete liver failure. This accounts for the 12th leading cause of death in the United States. There is currently no way to prevent death once the liver has failed and the only therapeutic option is transplantation. While 8,250 transplants occur every year clearly there exists an organ shortage that needs to be addressed (faststats @cdc.org 2019 a,b) (Liver Disease Stories @ liverfoundation.org, 2019).

Several techniques are being used to try to address this organ shortage. Of these whole organ blastocyst complementation may hold significant promise. This technique allows for the generation of chimeric animals with target organs that are derived from a donor cell population. The therapeutic goal of whole organ complementation is to produce human organs in large animal surrogates. However, both a species and developmental timing barrier exist which may preclude human stem cells from efficiently contributing to the body plan of mammalian species such as the pig. There are also ethical concerns that must be considered when performing these techniques.

This study addresses the species and developmental timing barriers associated with interspecies blastocyst complementation as well as current ethical concerns. In this work donor cells engineered for increased cell survivability were prepared for injection and cells from the non-human primate *Callithrix jacchus* (marmoset) was used as non-human primate substitute. For this study pluripotent stem cells from three species were used, human induced pluripotent stem cells (hiPSCs), marmoset embryonic stem cells (ESCs), and mouse induced pluripotent stem cells (miPSCs). Definitive endoderm was differentiated from these three cell types. In addition, the hiPSCs and the marmoset ESCs were engineered to constitutively express the either the reporter protein GFP alone or the anti-apoptotic BCL2 protein and the reporter protein GFP. Human iPSC BCL2/GFP-derived endoderm, marmoset ESC and ESC BCL2/GFP-derived endoderm, miPSC and miPSC-derived endoderm were prepared for injection into mouse embryos with a homozygous *Hhex* KO background. This studied aimed to investigate if preventing cellular apoptosis

allows donor cells to overcome the species and developmental timing barriers and enables their progeny to complement a developmental niche and generate liver in a host embryo. Endoderm was successfully induced in all three species, with varying degrees of purity, and miPSC, human iPSC-derived endoderm, and marmoset ESC-derived endoderm were injected into mouse embryos with an *Hhex* KO background. Those embryos were then implanted into pseudo pregnant surrogate hosts. At e9.5 the remaining implanted embryos were harvested and analyzed. Only embryos injected with miPSCs displayed chimerism between the host and donor cells with no contribution was seen in embryos injected with either human iPSC or marmoset ESC-derived endoderm.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1:

INTRODUCTION

BLASTOCYST COMPLEMENTATION

A recent review by Angles et. al., 2018, summarizes interspecies blastocyst complementation well and reviews the technical and ethical barriers present in the research.

Interspecies chimeras, where embryos are created with cells from two separate species, have been generated successfully for decades. Large animal chimeras such as sheep-goats were being created as early as 1984. (Fehilly et. al. 1984) These were whole animal chimeras that produced live animals containing a mosaic of cells from each species and demonstrated it was possible to generate living animals containing cells from more than one species.

Whole organ production using interspecies blastocyst complementation is a technique that leverages the ability to generate an animal with cells from more than one species to produce a chimeric animal with a target organ comprised from the progeny of cells from the donor species introduced at the blastocyst stage. The long term therapeutic goal of whole organ blastocyst complementation is to produce human organs suitable for transplantation in humans, that are grown in large surrogate animals. In interspecies blastocyst complementation the blastocyst of the host animal has donor cells injected into the inner cell mass (ICM). to enable the progeny of these cells to incorporate into the host embryo and contribute to the to the host body plan.

DEVELOPMENTAL NICHE

To produce a whole organ using the technique of interspecies blastocyst complementation a developmental niche needs to be identified and generated in the host. This developmental niche can be created by identifying and ablating the expression of a host gene target that would be necessary for the early development of a specific organ's progenitor cells and without expression of this gene the organ will fail to develop (Angeles et al. 2018 b). The resulting developmental niche allows only progeny of the donor cells introduced at the blastocyst stage to contribute exclusively to the development of the organ or tissues as the host cells are unable to. After the host zygote has been successfully edited to contain a developmental niche and cultured to the blastocyst stage, donor cells are injected into the edited blastocyst. As development proceeds, progeny of the introduced donor cells which

are capable of producing the gene products that the host cannot, contribute to forming the organ or tissue that is the target of the developmental niche (Angeles et al. 2018 b). In previous successful studies of rat/mouse pancreas complementation the pancreatic developmental niche was produced by a homozygous knockout of the gene Pdx1. This knockout in mouse or rat blastocysts allowed for the complementation by either rat or mouse donor cells and the production of pancreases derived almost entirely from donor progeny of the donor cells (Kobayashi, et. al. 2010). The developmental defect in the host allows for missing organ tissue to be generated by donor cells where their expression of the gene product ablated in the host cells allows them to respond correctly to developmental and growth cues and signals. However, this does not allow for entirety of the organ tissue to be grown from cells that are directly descended from the progeny of the cells complementing the host gene defect. In most organs and tissues there is vasculature, extracellular matrix, cells of the nervous system, and possibly other cells types which are not derived from the pool of gene ablated cells in the host and so are still produced by host in the target organ. This means that all the cells in the organs produced by blastocyst complementation will not be 100% donor-derived unless further gene ablations are made eg. multi-complexing the organ specific niche production KO with gene ablation such as ETV2 knockout to reduce host vasculature contribution.

Interspecies studies have also been performed between rats and mice that have successfully complemented kidney development. The empty developmental niche was created by performing a homozygous silencing of the gene Spalt like transcription factor 1 (Sall1). While successful complementation was achieved, the kidney highlights one of the hurdles that still need to be overcome. The kidney has multiple cells of origin and while complementing the Sall1 KO allows for many of the cell types of the kidney tissues to be generated from donor cell progeny, structures such as collecting ducts arise from separate developmental origins not influenced by Sall1 expression (Usui, et. al. 2012). The need to silence additional genes to fully complement kidney tissue, illustrates again the need to be able complement blastocysts with multiplexed gene editing in order to produce organs entirely from donor cells.

LIVER BLASTOCYST COMPLEMENTATION

The gene of interest for creating the developmental niche for this study is the haematopoietically - expressed homeobox protein (HHEX). Expression of this gene has been detected in several primordium organs including the thyroid, lungs and liver. The homeobox containing domains of the second and third exon of HHEX are thought to be required for determining early liver progenitor cell fate. Homozygous knockout of the HHEX gene creates a phenotype where no liver is produced and this genotype is embryonic lethal in mice by embryonic day 10.5 (Ken, W. 2000); (Myint et. al. 1999).

BARRIERS AND HURDLES

There exist several hurdles that must be overcome in order to perform interspecies blastocyst complementation. There is a species barrier, which may preclude species that are more distantly related than rats are to mice from successful complementation. There is also a developmental timing barrier that may prevent donor cells that are not matched in developmental timing from surviving and successfully integrating into the host embryo (Angeles et al. 2018 b). In addition, the NIH currently has a moratorium on funding the use of human pluripotent stem cells in any interspecies blastocyst complementation studies.

Successful chimera generation studies to date suggest that it is most successful when performed between closely related species, such as the sheep and goat or rat and mouse (Angeles et al. 2018 b). To highlight this the first study to complement a targeted organ with nearly 100% donor cells was between rats and mice and the targeted organ was the pancreas (Angeles et al. 2018 b). The potential need for the donor cells to be closely related evolutionarily to the host organism is known as the species barrier. Why does this barrier exist? There is a large amount of evolutionary conservation in early mammalian development and early mammalian embryos from different species exhibit similar morphology and developmental landmarks. One possible explanation for the species barrier might be that amongst mammals the transcription factor networks and morphogen signaling active during development are very similar but exhibit species-specific differences. This could explain why the donor cells from more distantly related species have difficulty surviving long enough to incorporate into the host embryos. Another possible explanation for the species barrier is that different organisms have different

developmental speeds. This species barrier suggests that it may be necessary to find a closely related species to humans in order to successfully produce organs for transplant. As well, a host animal would need to be sufficiently large in order to accommodate the desired organ size. Animals such as pigs or sheep are attractive candidates as they are large animals with organs that are of comparable size to humans; they also have short gestation times and are relatively inexpensive to keep. Both pigs and goats however, may be subject to the species barrier when the donor cells are human. It might be that we need a non-human primate such as the baboon to serve as a host as these are more closely related to humans than either pigs or goats. However, there exist multiple draw backs to possible use of non-human primates. There are significant ethical considerations and more restrictions placed on the use of non-human primates in scientific studies. As well, non-human primates like baboons are far more expensive to keep than their non-primate counterparts (Angeles et al. 2018 a); (Angeles et al. 2018 b).

Another consideration while performing interspecies blastocyst complementation is the developmental stages of the host embryo and the donor cells. Previous studies suggest that both the embryo and the donor cells need to be in the same stage of development (Angeles et al. 2018 b). While mouse ESCs readily contribute to intraspecies chimera formation it is likely that the human ESCs will not. This is due to the fact that the mouse pluripotent cells represent a different stage of development than their human analog. The reason for this difference in developmental staging is that mouse ESCs are derived from the blastocyst stage while human ESCs are derived at the epiblast stage (EpiS) of development. While these developmental periods are related and both exist prior to a defined germ layer formation, they are distinct. Human ESCs have been described as being in a primed state., while mouse ESCs are considered to be in a naïve state and readily give rise to chimeric animals (Angeles et al. 2018 b). The success of rat/mouse complementation lends itself to the idea of matching development speed as rats and mice both have gestation times of approximately 20 days and it is these two animals that the most successful complementation has been demonstrated for both the pancreas and the kidneys (Kobayashi, et. al. 2010); (Usui, et. al. 2012).

There are also ethical concerns that must be addressed when attempting to perform interspecies blastocyst complementation. The NIH currently has a moratorium on funding the use of human pluripotent stem cells in any blastocyst complementation study. The reason for this is in successful interspecies complementation trials, including when there is an empty developmental niche, there may also be a random integration of donor cells into all tissues of the chimeric animal (Fehilly, et. al. 1984); (Usui, et. al. 2012); (Kobayashi, et. al. 2010). It is this random integration that causes concern. This is especially true for the possibility of using non-human primates as a host organism. There is a suggestion that the human donor cell contribution to the host animal's brain may fundamentally change the way those animals process information and a concern that they might be able to experience enhanced suffering or that they will meet as yet to be defined criteria of increased cognition that will grant them a unique status that will preclude further experiments as a direct result of human cell contribution to the central nervous system and neuronal tissue. (Angeles et al. 2018 a)

POSSIBLE SOLUTIONS TO THE BARRIERS AND HURDLES

If a species barrier exists, how might it be possible that we could produce human organs in pigs or sheep? It might be possible because the molecular cues that drive early differentiation are more similar than they are different. While it appears that the conditions needed to maintain pluripotency and cell viability are species dependent, the early signaling for germ layer differentiation appears to be highly conserved. For instance, induction of human endoderm phenotype in vitro is driven primarily by activin signaling and the activation or suppression of WNT (Loh et. al. 2013). Similarly, studies suggest that homologs of those same two molecules activin and WNT drive endoderm differentiation in mice ESCs (Zhong et. al. 2017). Marmoset endoderm also appears to be driven by activin as studies suggest that it appears to be the primary signaling molecule that drives this germ layer differentiation (Diekmann et. al. 2015). Thus, these three mammalian species all have highly conserved molecular pathways that drive their germ layer differentiation. It is therefore possible that after introduction of PSCs into a different species' blastocyst that those cells may survive and integrate contributing to the host body plan.

It might be possible to help overcome the developmental timing hurdle using methods that prevent cell death. There are studies that suggest that if the donor cells are engineered to express anti-apoptotic genes it might be possible to overcome the species barrier. One possible gene target is the gene polycomb group RING finger protein (BMI1) which has been shown to have anti-apoptotic effects. BMI1 expression is known to inhibit the Ink4A-ARF pathway, which is linked to p53 and pRB, both known tumor suppressors (Wang, et. al. 2017); (Ivanchuk, et. al. 2001). It has been shown that forced expression of BMI1 during the initial injection, subsequent embryo culture, and post surrogate implantation increases the integration rate of naïve human ESCs in mouse embryos to 16.7% with contribution to either the ICM, the trophoblast or both, an increase from 0% (Huang, et. al. 2018). Another possible gene target to prevent cell death is B-Cell Leukemia/Lymphoma 2 (BCL2). BCL2 acts as anti-apoptotic gene by delaying the advancement of the cell from quiescence to S phase and preventing the cell from entering into the cell cycle (Janumyan et. al. 2003). A recent study suggests that over expression of BCL2 increases the ability for cells to incorporate and contribute embryonic tissue. Contribution to the embryonic tissue from engineered donor cells was observed in Wang, et. al. 2018, while this contribution was not observed from donor cells that do not over express BCL2. Both of these studies were performed by expressing either BMI1 or BCL2 using tetracycline – inducible systems which may be necessary to prevent uncontrolled anti-apoptosis. The expression of anti-apoptosis genes may address the difficulties that arise from varied developmental timing by allowing the cells to survive long enough to integrate and contribute to the embryo. However, it remains to be seen if the donor cells will continue to contribute to post-embryonic tissues. In the BCL2 and BMI1 models, embryos were assessed for human cell integration at several time points and the further into development, the percentage of embryos that contained human cells decreased. Furthermore, it has been shown when human stem cells form teratomas in immunodeficient mice that they maintain a developmental speed congruent with other human cells and do not adopt the mouse developmental speed (Angeles et al. 2018 b). This difference in developmental speed could be an explanation for why there is a decline in the percentage of mouse blastocysts that contain human donor cells as development progresses. Even though the cells appear to incorporate they are still developing at a human speed which might not be compatible with

mouse gestation times. It is possible that mice are evolutionarily too distant from humans to successfully perform blastocyst complementation. This separation might preclude human cells from being able to contribute to any postembryonic tissue in this species. Humans are more closely related to pigs and sheep than mice and it might be possible that while the human gestation time is more than double that of a pigs the human donor cells will react more favorably to the porcine developmental molecular cues allowing for complementation.

ADDRESSING ETHICAL CONCERNS

This study tried to overcome ethical concerns by differentiating human iPSCs into definitive endoderm for injection into mouse *Hhex* KO embryos. This allowed for research using human cells while not using pluripotent human iPSCs in blastocyst complementation studies. hiPSCs that constituently over expressed the anti-apoptotic BCL2 gene, were differentiated into definitive foregut endoderm (Janumyan et. al. 2003); (Loh, et. al 2014). Without the ability to give rise to the ectoderm or mesoderm germ layers these human cells would be unable to contribute to the brain or germ line of the host animal. Marmoset ESCs were also used as non-human replacement to further address the ethical concerns. Endoderm derived from marmoset ESCs engineered to constituently over express BCL2 were also used for injections into mouse *Hhex* KO embryos. Since marmoset ESCs were being used as non-human primate substitute the use of marmoset ESC-derived endoderm was tested as well. It was possible that marmoset cells would overcome the developmental timing barrier more effectively as their gestation time is closer to the mouse's. Endoderm-derived from mouse iPSCs would have not have to overcome the species barrier and so it was planned to use these cells in mouse embryos that contained the *Hhex* KO background. Also, to ensure that the *Hhex* KO embryos were chimeric competent mouse iPSCs were injected as a control.

CHAPTER 2:

METHODS

hiPSC CULTURE

Human iPSCs that were to be differentiated into definitive endoderm were maintained in a pluripotent state prior to differentiation. Human iPSC lines were maintained in T25 flasks. Media was changed daily. Cells were maintained in Essential E8 medium (Thermo-Fisher A15169-01) or TeSR – E8 Kit for hESC/hIPSC maintenance (STEMCELL technologies 05990). Basal E8 media was switched from Thermo-Fisher to STEMCELL for reasons of availability. Cells were maintained for 3-4 days before passaging depending on cell confluency. Cultures that were approximately 70% or more confluent were passaged on day 3. If cultures appeared to be less than 70% confluent on day 3 they were allowed to grow until day 4 when they were passaged. Cells were passaged using a sodium citrate solution (4.4g of sodium citrate and 25g of potassium chloride in one liter of deionized sterile water). To passage cells, E8 was aspirated from the cells and 0.1 mL/cm^2 sodium citrate was introduced and allowed to incubate for 5-7 mins at 37^o C and 5% CO_2. Sodium citrate was then aspirated from the flask and 1 mL of E8 was added. The cells were then mechanically dislodged from the T25 flask and transferred to a conical 15 mL tube, diluted to 3 mL E8, and the cell colonies were mechanically separated using a 5mL serological pipette. They were then seeded at a 1:4 - 1:8 split ratio depending on pre-passage confluency and day of passage.

MARMOSET ESC CULTURE

Marmoset ESCs were maintained in T25 flasks. Media was changed daily. Cells were maintained in whole marmoset media which contains 497mL Essential E8 (Thermo-Fisher - A15169-01), 10mL E8 Supplement (Thermo-Fisher - A15171-01), 5 mL Glutamax (Life Technologies - 35050-061), 5mL lipid concentrate (Life Technologies - 11905-031), 100ng/mL Nodal (R&D - 3218-ND), and 1.94 µg/mL Glutathione (Sigma – G4251). The formulation of the whole marmoset media and the marmoset cj367 ESC line were gifts from Dr. Ted Golos at the Wisconsin National Primate Research Center. ESCs were passaged using the same methods as human iPSCs. They were then seeded at a 1:4 to 1:8 split ratio depending on pre-passage confluency and day of passage.

MOUSE iPSC CULTURE

Mouse iPSCs were cultured on irradiated mouse embryonic fibroblast cells (iMEFs) (R&D – PSC001). Cells were maintained in 6 well plates in whole mouse iPSC media. Whole mouse iPSC media contains 156mL KO DMEM (Thermo-Fisher – 10829018), 20 mL of knockout serum replacement (Thermo-Fisher – A3181501), 2 mL L-Glutamine (Invitrogen – CX30310), 2 mL MEM Non-Essential Amino Acid Solution (100x) (STEMCELL Technologies 11140050), then in a 50mL Aliquot: 5µL Mouse Recombinant LIF (STEMCELL Technologies – 78056), and 100µL β-mercaptoethonol (Santa Cruz Biotechonology – sc-202966). Mouse iPSCs were passaged using 0.1 mL/cm^2 StemPro Accutase Cell Dissociation Reagent (Thermo-Fisher – A1110501) for 5 mins. The Accutase/cell solution was then pipetted into a 50 mL conical tube and diluted with whole mouse iPSC media to 12mL. Cells were then centrifuged for 5 mins at 1200 RPM. Supernatant was then aspirated and the cells resuspended in 6 mL of whole mouse iPSC media. Cells were then seeded onto a new 6 well plate with mouse fibroblast feeder cells at a split ratio of 1:50 – 1:100 depending on prior confluency.

IMMUNOCYTOCHEMISTRY

Cells to be characterized by immunocytochemistry were first fixed and blocked using the standard Dutton lab protocol. Fixation was performed using a 10% formalin solution which the cells were exposed to for 10 mins. The formalin solution was then aspirated and the cells were washed with dPBS at 0.1 mL/cm^2 and stored in 0.2 mL/cm^2 dPBS at 4^{o}C. Cells were then permeabilized for 10 mins using permeabilization buffer containing 0.2% (v/v) Triton X-100 in dPBS, at 0.1 mL/cm^2. Permeabilization buffer was then aspirated and cells were blocked using blocking buffer containing 0.1% (v/v) Tween 20, and 1% (w/v) bovine serum albumin in dPBS, at 0.1 mL/cm^2 for 2 hours. Primary antibody/blocking buffer solutions were added to cells at 0.1 mL/cm^2 and incubated overnight at 4^{o}C. The following day primary antibodies were aspirated and cells were then washed three times with blocking buffer at 0.1 mL/cm^2 before secondary antibody/blocking buffer solutions were added at 0.1 mL/cm^2 and incubated at room temperature in the dark for 30 mins. Secondary antibodies were then aspirated and a DAPI counterstain at 1:1000 dilution ratio/blocking buffer solution was added at 0.1 mL/cm^2 and incubated at room in the dark for 10 mins.

Counterstain solution was then aspirated and the cells were washed in 0.1 mL/cm^2 dPBS. Cells were then stored in 0.2 mL/cm^2 dPBS wrapped in foil and at 4^{o}C.

MICROSCOPY

All microscopy was performed using a Leica DMI 6000 inverted microscope.

hiPSC ENDODERM DIFFERENTIATION

The goal of this research is to overcome the species and developmental timing barriers that may reduce the ability of human donor cells from integrating into mouse blastocysts and allow the donor cells to contribute to post embryonic tissues. To do this hiPSCs needed to differentiate into a definitive foregut endoderm to address the ethical concerns of using hiPSCs in blastocyst complementation. First the Loh protocol (Loh, et. al. 2014) was attempted to induce endoderm differentiation. To do this the 3A3 line of hiPSCs (Parr, et. al. 2016) were plated onto a 12 well plate coated with the truncated vitronectin, , protein (Fisherscientific – A31804) 1:100 in dPBS. The iPSCs were plated between 8% to 10% confluency. The cells were then cultured in Essential E8 (Thermo-Fisher 1517001) media for 24hrs to allow for the cells to attach to the plates and allow them to double the size of their colonies. Culturing the iPSCs for 24hrs prior to differentiation gives the cells more stability by permitting the colonies to become established which allows them to respond to the differentiation induction more readily. In accordance with the Loh protocol CDM2 basal differentiation media was prepared, which consisted of: 50% v/v IMDM (Thermo Fisher - PI88367), 50% v/v F12 (Thermo Fisher – 88424), which was then supplemented with 1 mg/mL polyvinyl alcohol (Thermo Fisher - 8.43871.1000), 1% v/v of chemically-defined lipid concentrate (Gibco – 11905-031), 450 µM monothioglycerol (Sigma-Aldrich - M6145), 0.7 µg/mL insulin (Sigma-Aldrich – I9278), and 15 µg/mL transferrin (Fisher Scientific – 501870561). CDM2 basal media was filtered through a 250 mL sterile filter and stored at 4^o C. To begin the differentiation into definitive endoderm after 24hrs of culture in E8, the media was aspirated, and the cells were exposed to CDM2 basal media supplemented with Activin A 100 ng/mL (Biolegend 592004), CHIR99021 2 µM (Stemgent) and PI-103 50 nM (Tocris – 2930) for 24 hours. Then the cells were treated for 48 hours with Activin A 100 ng/mL, LDN193189 250nM (R&D Systems – 399850) in CDM2 basal media. Activin was used because across mammalian species activin is a key

requirement in endoderm differentiation (Zhong, et. al. 2017); (Loh, et. al. 2014); (Diekmann, et. al. 2015). CHIR99021 is a Wnt agonist and WNT is well known pathway involved in the patterning of many embryonic tissues. Early WNT activation during germ line differentiation will pattern endoderm and excludes ectoderm differentiation. As well, inhibition of several protein kinases and the mTORC pathways by PI-103 further excludes the cells from entering an ectoderm lineage (Loh, et. al. 2014). The second step of this protocol exposes cells to activin and LDN193189 for 48 hours. Continued activin activation continues to drive endoderm lineage and LDN193189 acts as a BMP antagonist which prevents adoption of mesoderm lineage fates.

Characterization of cells differentiated by the Loh protocol was conducted using immunocytochemistry. Fixing, permeabilizing, and characterization using immunocytochemistry were performed using standing lab protocols. Co-expression of FOXA2, SOX17, and/or CXCR4 are utilized as indication of induction toward a definitive endoderm phenotype (Ang, et. al. 2018); (Cho, et. al. 2012); (Katsumoto, Kume, 2011); (Loh, et. al. 2014). SOX17 monoclonal mouse antibody (R&D MAB19241) at a dilution ratio of 1:1000 and FOXA2 monoclonal goat antibody (R&D AF2400) at a dilution ration of 1:250 were used to detect the presence of SOX17 and FOXA2 respectively. Secondary antibodies used were donkey-anti mouse - 488 fluorophore (Invitrogen - A21202) and donkey-anti goat – 555 fluorophore (Invitrogen - A21432) Despite several attempts at reproducing this protocol few cells were positive for either FOXA2 or SOX17 and even fewer co-expressed these two proteins. There were no positive controls used. Undifferentiated human iPSCs were tested for expression of endoderm proteins using the same primary and secondary antibodies as a negative control. Further negative controls using secondary antibody only was performed. Due to lack of reproducibility another protocol for endoderm differentiation was tested.

The commercially available PSC Definitive Endoderm Induction Kit (PSC Media) (Life Technologies cooperation - A3062601) was selected. This media was designed from the Loh protocol. The kit comes as two separate media, medium A for day one and medium B for day two. Media A contains both activin A and a WNT activator. Media B contains activin A, a WNT inhibitor, and a BMP inhibitor. 3A3 hiPSCs were seeded onto a 12 well

plate coated with truncated vitronectin 1:100 (Fisherscientific – A31804) at 8% to 10% confluency. The iPSCs were cultured in Essential E8 (Thermo-Fisher 1517001) for 24 hours to allow for cell attachment and colony expansion. Cells were then exposed to Definitive Endoderm Induction Kit medium A for 24hrs. After 24hrs medium A was aspirated and the cells were exposed to Definitive Endoderm Induction Kit medium B for 24hrs. After 48hrs the cells were then fixed and prepared for immunocytochemistry.

Immunocytochemistry of the 3A3 hiPSC derived definitive endoderm from the PSC Media was performed to characterize the cell state and verify the ability of the media to differentiate hiPSCs into endoderm. To do this the proteins SOX17 and FOXA2 were detected for using the human SOX17 monoclonal mouse antibody (R&D MAB19241) at a dilution ratio of 1:1000 and human FOXA2 monoclonal goat antibody (R&D AF2400) at a dilution ratio of 1:250. Secondary antibodies used were donkey-anti mouse - 488 fluorophore (Invitrogen - A21202) at a dilution ration of 1:1000 and donkey-anti goat – 555 fluorophore (Invitrogen - A21432) at a dilution ratio.

To determine that the PSC Media could reliably induce endoderm differentiation in multiple cell lines, endoderm induction in 6B4 hiPSCs (Geng, et. al 2018) and 9-1 hiPSCs (Zhang, et. al. 2013) was tested. Two biological replicates were seeded and treated to the same conditions as the 3A3 hiPSCs.

Immunohistochemistry of the biological replicate hiPSC-derived definitive endoderm from the PSC media was performed to characterize the induced phenotpye and immunochemistry used to verify the ability of the PSC Media to induce endoderm differentiation in multiple cell lines as described. To do this the proteins SOX17 and FOXA2 were detected using the same antibodies that were used against the 3A3 hiPSCs. However, in the 6B4 replicate the SOX17, 555 channel presented with what appeared to be indiscriminate binding in the negative control undifferentiated hiPSCs. To address this a different SOX17 primary antibody, the human SOX17 monoclonal goat antibody (R&D AF1924) at a 1:200 dilution ratio was used. Secondary antibody used for the SOX17 probe was donkey-anti goat 555 fluorophore (Invitrogen – A21432). However, the SOX17 antibody (R&D AF1924) probes for an epitope that does not exist in the marmoset homolog. To ensure that the cells were co-labeled for multiple definitive endoderm proteins

when probed for using immunocytochemistry in hiPSC-derived endoderm, miPSCs-derived endoderm and marmoset ESC-derived endoderm the monoclonal antibody against the C-terminal domain in the CXCR4 protein was selected; the CXCR4 monoclonal rabbit antibody (Abcam - AB219178) which is predicted to bind to non-human primate, mouse, and human CXCR4 protein was chosen. Immunocytochemistry was performed using this CXCR4 antibody at a dilution ratio of 1:250. Secondary antibody used was donkey-anti rabbit 488 fluorophore (Invitrogen – A21428).

To attempt to overcome the developmental timing barrier human iPSCs needed to be edited to over express BCL2 and be tagged with green fluorescent protein (GFP). 9-1 BCL2/GFP hiPSCs and 9-1 GFP hiPSCs were a gift from Recombinetics, Inc.

To determine that the changes in the cells genome of both 9-1 BCL2/GFP and 9-1 GFP hiPSCs would not adversely affect their ability to differentiate the cells were cultured using the same protocol described above. The cells were then exposed to the 48hr PSC Media protocol. After 48hrs the cells were fixed and prepared for immunocytochemistry as described above.

Immunohistochemistry of the 9-1 BCL2/GFP^{+} hiPSC-derived definitive endoderm from the PSC Media was performed to characterize the cell state and verify the ability of the media to differentiate hiPSCs into endoderm. Detection of SOX17, FOXA2 and CXCR4 was performed using the using the same antibodies as previous assays.

MARMOSET ENDODERM DIFFERENTIATION

To differentiate the marmoset cj367 ESCs into definitive endoderm, the PSC Media 48hr protocol which had been used to induce endoderm differentiation in hiPSCs was used and the cells were then fixed and prepared for immunohistochemistry as previously described. Cells were also exposed to each both media A and media B for an additional 24 hours to assess if prolonged exposure to the molecular cues that drive endoderm induction was required. After 96hrs media was aspirated and the cells were fixed and prepared for immunohistochemistry.

Immunocytochemistry of the marmoset cj367 ESC-derived definitive endoderm induced by the PSC Media 48hr protocol was performed to characterize the cell phenotype and to assess the ability of the media to differentiate marmoset ESCs into endoderm. The presence of FOXA2 and CXCR4 were assessed using the FOXA2 monoclonal goat antibody (R&D AF2400) and C-terminal monoclonal antibody (Abcam – AB219178). Secondary antibodies used were donkey-anti rabbit - 488 fluorophore (Invitrogen – A21428) and donkey-anti goat – 555 fluorophore (Invitrogen - A21432).

Marmoset cj367 ESCs that were designed to constitutively overexpress BCL2 and are GFP^+ were a gift from Recombinetics. To prepare these cells for injection into mouse blastocysts required two distinct cell populations. The first population of ESCs were maintained using the same marmoset ESC cell culture methods previously described. To differentiate the marmoset cj367 BCL2/GFP^+ and cj367 GFP^+ ESCs into endoderm, cells were seeded onto a 12 well plate coated with truncated vitronectin at approximately 8% to 10% confluency. The cells were then cultured in whole marmoset media for 24hrs to allow for cell attachment and colony expansion. The cells were then washed with dPBS at 0.1 mL/cm^2. The cells were then exposed to PSC Media A for 24hrs. After 24hrs medium A was aspirated and cells were exposed to PSC Meida B for 24hrs. After 48hrs the cells were then fixed and prepared for immunohistochemistry using standard lab protocols.

Immunocytochemistry of the marmoset cj367 BLC2/GFP^+ ESC-derived definitive endoderm that had been exposed to the PSC 48hr protocol to characterize the cell state and assess the ability of the media to differentiate marmoset ESCs into endoderm. The presence of FOXA2 and CXCR4 was detected using the human FOXA2 monoclonal goat antibody (R&D AF2400) and C-terminal Human monoclonal antibody (Abcam – AB219178). Secondary antibodies used were donkey-anti rabbit - 647 fluorophore (Invitrogen – AB219178) and donkey-anti goat – 555 fluorophore (Invitrogen - A21432).

MOUSE ENDODERM DIFFERENATION

Mouse iPSCs do not have to contend with species barrier in intraspecies complementation. It was therefore hypothesized that endoderm derived from miPSCs might be able to integrate into the host embryo. SOX17, FOXA2, and CXCR4 are present on both human

and mouse endoderm cells. Studies also show that Activin and WNT play crucial roles in mouse endoderm induction. (Zhong, et. al. 2017)

In order for miPSCs to remain pluripotent they are cultured on iMEFs, that needed to be depleted from the cell culture prior to differentiation. Depletion was performed after miPSC passaging, the remaining resuspended cells were diluted to 7.5 mL in whole mouse iPSC media and pipetted into an untreated T75 flask and allowed to incubate for 1 to 1.5 hrs. iMEFs adhere to the untreated flask leaving the miPSCs in suspension. The supernatant was then collected and the miPSCs were seeded onto 6 well plates coated with 10% gelatin at an 8% to 10% confluency. Cells were cultured for 24hrs in whole mouse iPSC media to allow for cell attachment and colony expansion. The cells were then washed with F12 for approximately 1hr. The cells were then exposed to PSC Media A for 24hrs. After 24hrs medium A was aspirated and cells were exposed to PSC Media B for 24hrs. After 48hrs the cells were then fixed and prepared for immunohistochemistry.

Immunohistochemistry of the 3F10G miPSC GFP^+ -derived definitive endoderm was performed to assess if endoderm induction in miPSCs using the PSC Media was possible.. The previously used monoclonal antibodies against the proteins FOXA2, SOX17 and CXCR4 were projected to bind to the mouse homologs of these proteins and the antibodies described previously were used.

CELL SUSPENSION

To prepare the 9-1 BCL2/GFP^+ hiPSC-derived endoderm cells for embryo injection they needed to be in a single cell suspension with approximately $1x10^6$ cells / ml. Cells in 5 wells of a 12 well plate that had been treated with the PSC media 48hr protocol were treated with citrate passaging solution for 6 mins and incubated at 37^o C and 5% CO_2. The cells were mechanically dislodged from the wells and the citrate/cell solution collected and diluted with PSC Media B to 10 mL and centrifuged for 5 min at 1200 RPM. The supernatant was then aspirated and the cells were washed in 5 mL dPBS. The solution was then centrifuged for 5 mins at 1200 RPM. The supernatant was then aspirated and the cells were resuspended and mechanically dissociated using a serological pipette in 2 mL media

B. 1.5 mL of the cells suspension was transferred to a 1.7 mL micro centrifuge tube and placed in ice for transport.

To prepare the 3F10G GFP^+ miPSC-derived endoderm cells for embryo injection the cells needed to be in a single cell suspension with approximately $1x10^6$ cells/ml. To do so the same methodology as hiPSC-derived endoderm as previously stated.

To prepare the 3F10G mouse iPSCs for embryo injection the cells were exposed to 0.1 mL/cm^2 StemPro Accutase Cell Dissociation Reagent (Thermo-Fisher – A1110501) for 5 mins. Accutase was then pipetted into a 50 mL conical and diluted with whole mouse iPSC media to 12 mL. Cells were then centrifuged for 5 mins at 1200 RPM. The supernatant was then aspirated and the cells were washed with 5 mL of dPBS. Cells were then feeder depleted in a T75 for 1.5 hrs. The remaining media was collected and centrifuged for 5 mins at 1200 RPM. The supernatant was then collected and the cells were resuspended in 2 mL of whole mouse media and mechanically dissociated using a serological pipette. 1.5 mL of cell suspension was transferred to a 1.7 mL micro centrifuge tube and placed in ice for transport.

To prepare the cj367 marmoset ESC-derived endoderm for embryo injection the cells needed to be in a single cell suspension with approximately $1x10^6$ cells per ml. The methodology previously described for hiPSC-derived endoderm was used.

To prepare the cj367 marmoset ESCs for embryo injection the same protocol was followed as the marmoset ESC derived endoderm except the cells were diluted and delivered in whole marmoset media instead of PSC Media B.

CRYOSECTIONING & IMMUNOHISTOCHEMISTY

Of the cells prepared for injection only the Marmoset BCL2/GFP ESC-derived endoderm, human BCL2/GFP iPSC-derived endoderm and mouse GFP iPSCs were used for injection. Embryos with the *Hhex* homozygous KO were approximately embryonic day 3.5 to 4.7 at time of injection and approximately 5 to 10 cells were introduced. Embryos were then implanted into pseudo pregnant host mice. 85 embryos were injected with marmoset cj367 BCL2/GFP ESC-derived endoderm, 51 embryos were injected with 3F10G mouse iPSCs,

and 51 embryos were injected with human BCL2/GFP iPSC-derived endoderm. Injections and embryo transfers were performed by Dr. Yun You at the University of Minnesota.

Embryos were harvested at approximately e9.5. Embryos #89 and #75 were fixed in 4% paraformaldehyde for one day and suspended in 30% sucrose 70% dPBS v/v for tissue protection during freezing. Embryos were suspended into O.C.T compound and placed dry ice until frozen. Embryos were cut into 6 µm sections using a Leica CM3050 S cryostat. Complementation was assessed by the presence of the reporter GFP.

The proteins FOXA2, HHex, and insulin were detected in embryo #75 using immunohistochemistry to assess if the complemented 3F10G miPSCs filled the empty developmental niche left by the homozygous HHex KO in the host embryo. Antibodies used were Insulin monoclonal guinea pig antibody (Cell Marque 2731-15), HHex monoclonal rabbit antibody (R&D MAB83771), and FOXA2 monoclonal goat antibody (R&D MAB1924).

The protein Insulin was probed for in embryo #89 using immunohistochemistry and the same insulin antibody that was used on embryo #75.

CHAPTER 3:

RESULTS

The purpose of this research was to test methods to help overcome the perceived developmental and timing barriers associated with whole organ interspecies blastocyst complementation. Furthermore, this research attempted to address ethical concerns by using hiPSC derived endoderm cells to prevent donor cell contribution into the host brain. Induction of endoderm differentiation in the hiPSC using a protocol by Loh et. al. 2014 was attempted. 3A3 iPSCs were seeded onto either vitronectin or Matrigel and the induction of endoderm was attempted. After the 72hr protocol cells had undergone what morphologically appeared to be an epithelial to mesenchymal transition (EMT) and expression of the pluripotency transcription factors OCT4 and NANOG was lost. During this EMT the cells cytoplasmic to nuclear ratio increased, the cells flattened and started to migrate away from the cell colony. However, expression of proteins SOX17 and FOXA2 indicative of definitive endoderm phenotype, was only detected a low levels by immunocytochemistry and the proteins were rarely co-expressed (data not shown).

HUMAN iPSC

Following the unsuccessful induction using the Loh protocol, another method was tested to induce endoderm differentiation using the proprietary media PSC Definitive Endoderm Induction Kit (Life Technologies A3062601). Following the previously described PSC 48hr differentiation protocol, the 3A3 iPSC-derived endoderm cells were characterized with immunocytochemistry for nuclear proteins SOX17 and FOXA2 which are both known to be co-expressed in early foregut endoderm in multiple mammalian species including human, mouse, and marmoset. (Loh et. al. 2014) (Zhong et. al. 2017) (Ang et. al. 2018) (Cho et. al. 2012) (Diekmann et. al. 2015) (Figure 1.) Immunocytochemistry for SOX17 and FOXA2 showed fluorescence concurrently with DAPI at 97.75% and 97.6%. (Figure 1. d, e) Immunocytochemistry also shows that of the cells that express SOX17 97.61% also express FOXA2. (Figure. 1. e, f) The induced endoderm lost expression of pluripotency transcription factors of NANOG and OCT4 (figure 1. a, b). Together these suggest that differentiation was successful and that definitive endoderm was induced. Additional biological replicates 6B4 hiPSCs and 9-1 hIPSCs were differentiated using the same protocol for additional verification. (data not shown)

Figure 1

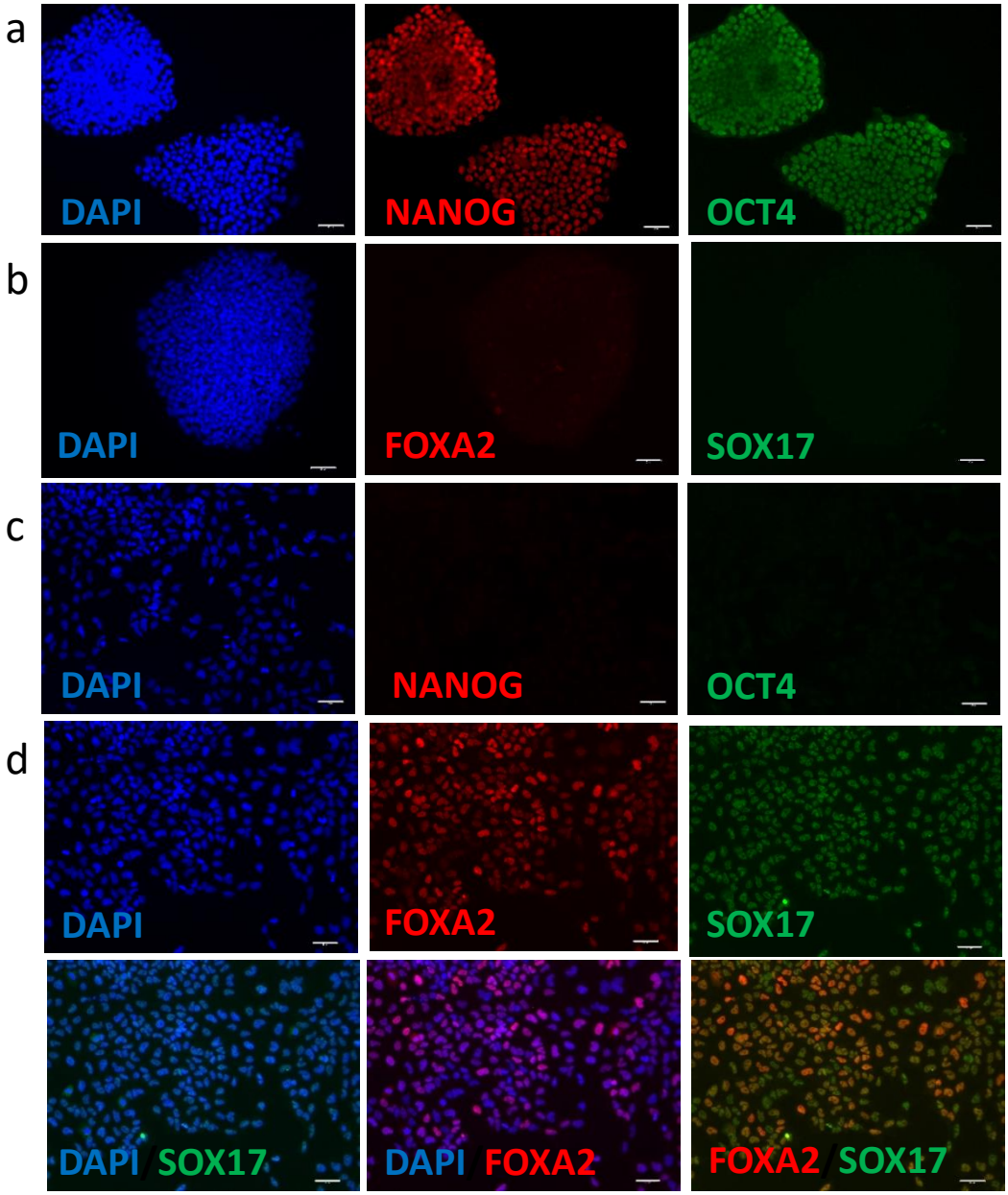


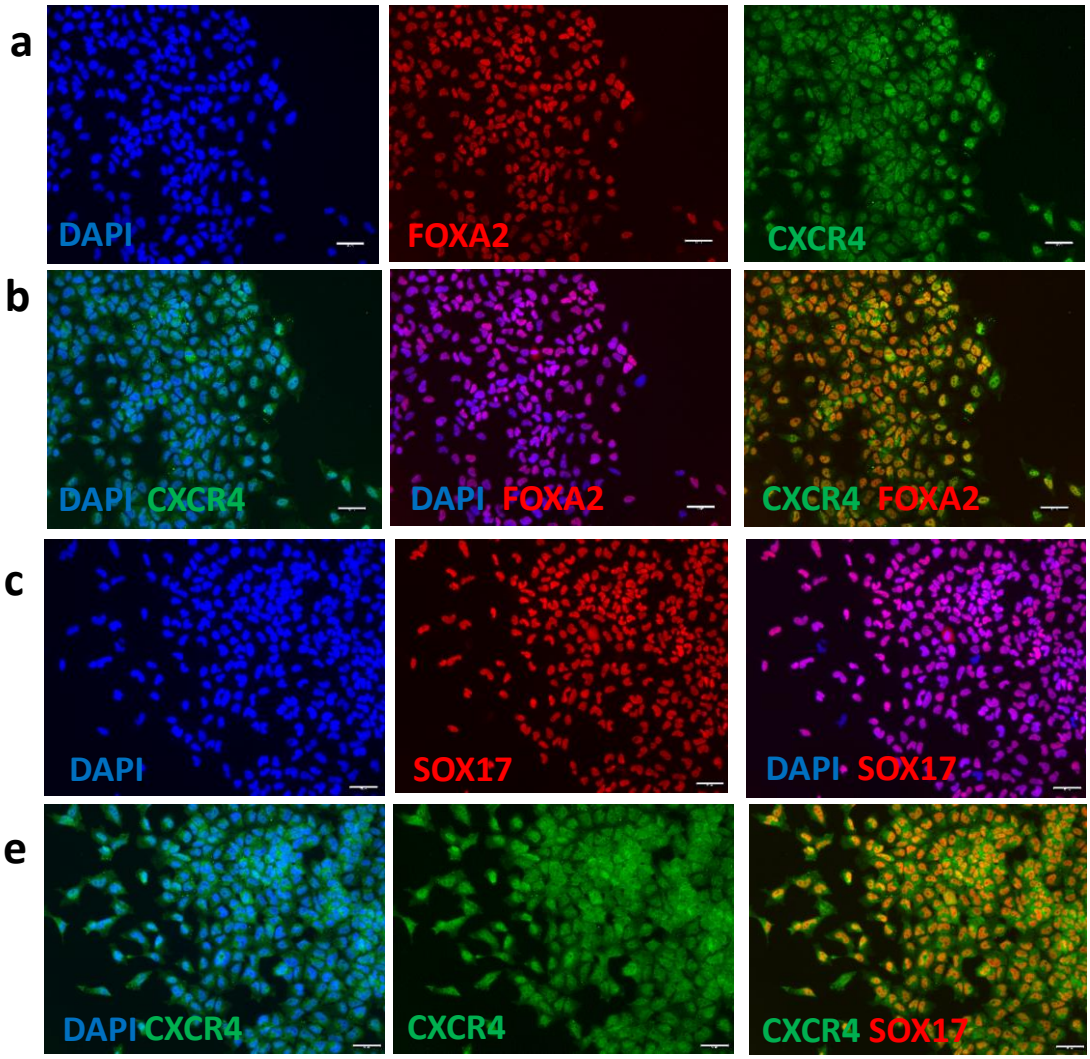
Figure 1. Immunocytochemistry of 3A3 hiPSCs and 3A3 hiPSC induced Endoderm **a**. 3A3 undifferentiated hiPSCs left to right DAPI, Red – NANOG, and Green – OCT4. **b**. 3A3 undifferentiated hiPSCs left to right DAPI, Red – FOXA2, Green – SOX17. **c**. 3A3 derived endoderm left to right DAPI, Red – NANOG, Green – OCT4. n = 3 **d. top row** 3A3 derived endoderm left to right DAPI, Red – FOXA2, Green – SOX17. n = 3 **bottom row** 3A3 derived endoderm left to right Merged DAPI/ SOX17, DAPI/FOXA2, FOXA2/SOX17 n =3. Images at 20x magnification, Scale bars 50 µM. **e**. Example of images used for cell count analysis of 3A3 definitive endoderm using DAPI counterstain, FOXA2, and

While SOX17 is known to be expressed in marmoset as well as human endoderm an antibody that was projected to bind to an epitome present on the marmoset SOX17 protein was not able to be obtained. Therefore, a protein which is present in both species that identifies endoderm was required. The cytoplasmic protein CXCR4 is also known to be present in early endoderm and an antibody that was projected to bind to not just human and marmoset but also mouse CXCR4 was used. (Diekmann et. al. 2015) (Katsumoto et. al. 2011) After the 48hr endoderm induction the CXCR4 antibody detected protein in the 9-1 hiPSC derived endoderm (Figure 2. c, d, e, f). Immunocytochemistry detected the CXCR4 epitome in 99.95% of the cells when compared to DAPI staining. (Figure 2. b, e, f) CXCR4 was also shown to be co-expressed in induced endoderm cells with SOX17 and FOXA2 at 98.27% and 97.47% respectively. (Figure 2. b, e, f, g) The presence of these proteins being expressed in the majority of the cells suggests that the PSC Definitive Endoderm Induction Kit is capable of inducing differentiation in human PSC to a definitive endoderm lineage.

GENE ENGINEERING

It has been suggested that apoptosis needs to be inhibited to overcome the species and developmental timing barriers and allow human cells to fill desired developmental niches (Huang et. al. 2018); (De Los Angeles, et al. 2018a); (Wang et. al. 2018). To address this, hiPSC 9-1s and marmoset ESC cj367s were engineered to constituently express either GFP and the antiapoptotic protein BCL2 or just GFP alone. GFP was inserted into both cells using a commercially designed plasmid designed to insert the GFP gene into the protein coding gene citrate lyase beta like protein (CLYBL); the marmoset ESCs was transfected with a linearized plasmid, and the human were transfected as a knock-in construct using Talens (Cerbini et. al. 2015). BCL2 constitutive expression was performed using an AAVS1 safe harbor locus in both human and marmoset. All gene editing, selection, characterization, and verification was done by Recombinetics, Inc.

Figure 2



e

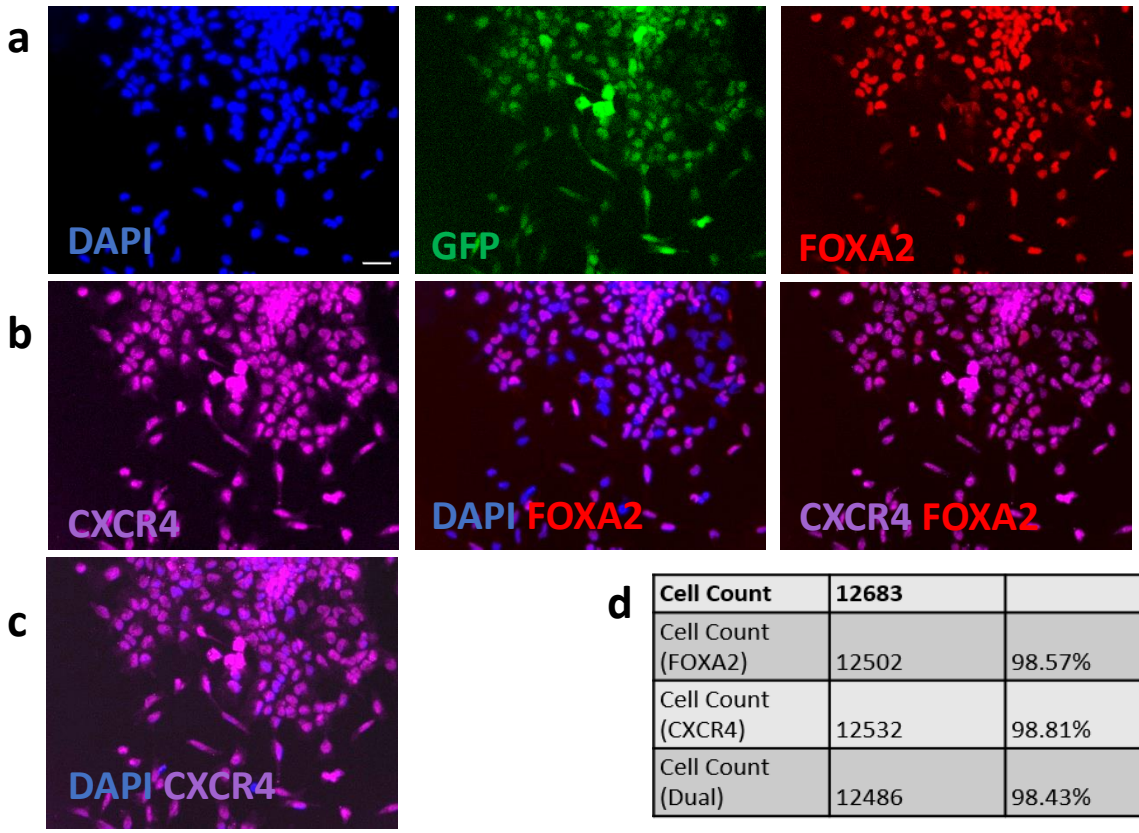
Cell Count	10432	
Cell Count (Sox17)	10208	97.85%
Cell Count (CXCR4)	10386	99.55%
Cell Count (Dual)	10252	98.27%

f

Cell Count	9832	
Cell Count (FOXA2+)	9666	98.31%
Cell Count (CXCR4+)	9706	98.72%
Cell Count (Dual+)	9584	97.47%%

Figure 2. Immunocytochemistry of Endoderm derived from hiPSCs **a**. 9-1 hiPSC derived endoderm left to right DAPI, Red – FOXA2, and Green – CXCR4. **b**. 9-1 hiPSC derived endoderm left to right merged DAPI/ CXCR4 n=3. **c. 9**-1 hiPSC derived endoderm left to right DAPI, Red – SOX17, Green – CXCR4. n = 3 **d.** 9-1 hiPSC derived endoderm left to right Merged DAPI/ CXCR4, DAPI/SOX17, CXCR4/SOX17 n =3. Images at 20x magnification, Scale bars 50 µM. **e**. Gen5 software cell count analysis of 9-1 derived endoderm using DAPI counterstain, SOX17 and CXCR4. **f.** Gen5 software cell count analysis of 9-1 derived endoderm using DAPI counterstain, FOXA2 and CXCR4.

Figure 3



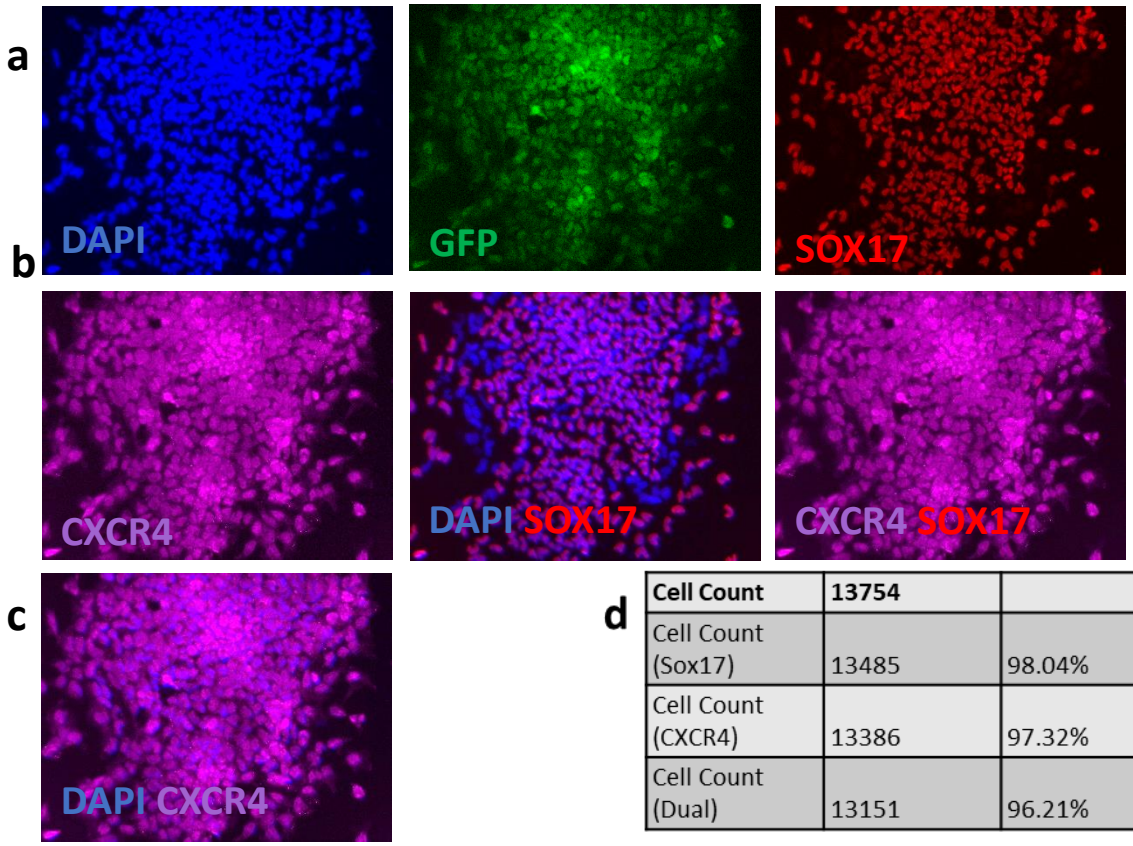
d

Cell Count	12683	
Cell Count (FOXA2)	12502	98.57%
Cell Count (CXCR4)	12532	98.81%
Cell Count (Dual)	12486	98.43%

Figure 3 Immunocytochemistry of 9-1 BCL2/GFP hiPSC induced Endoderm a. 9-1 BLC2/GFP hiPSC derived endoderm left to right DAPI, and Green – GFP, Red – FOXA2 n=3. **b**. 9-1 BLC2/GFP hiPSC derived endoderm left to right Far Red – CXCR4, merged image DAPI/FOXA2, merged image CXCR4/FOXA2 n=3. **c**. 9-1 BLC2/GFP hiPSC derived endoderm merged left to right DAPI/CXCR4, n = 3 Images at 20x magnification, Scale bars 50 µM **d.** Gen 5 software cell count analysis of 9-1 BCL2/GFP derived endoderm DAPI, FOXA2, and CXCR4.

To attempt to overcome the species barrier the PSC Media would need to induce endoderm differentiation in 9-1 GFP and GFP/BCL2 hiPSCs. 9-1 BCL2/GFP hiPSCs and 9-1 GFP hiPSCs were treated with the same PSC Kit 48hr induction protocol as previously described. 9-1 BCL2/GFP hiPSCs were treated with the same 48hr induction protocol as previously described. Immunocytochemistry shows presence of FOXA2, SOX17, and CXCR4 epitomes at 98.57%, 98.04%, and 98.04% respectively. (Figure 3. a, b, c, d - Figure 4. a, b, c, d) Immunocytochemistry of 9-1 GFP induced endoderm presented with the presence of FOXA2, SOX17, and CXCR4 at approximately the same rates as rates as previously shown (data not shown).

Figure 4



d

Cell Count	13754	
Cell Count (Sox17)	13485	98.04%
Cell Count (CXCR4)	13386	97.32%
Cell Count (Dual)	13151	96.21%

Figure 4. Immunocytochemistry of 9-1 BCL2/GFP hiPSC induced Endoderm a. 9-1 BLC2/GFP hiPSC derived endoderm left to right DAPI, and Green – GFP, Red – SOX17 n=3. **b**. 9-1 BLC2/GFP hiPSC derived endoderm left to right Far Red – CXCR4, merged image DAPI/SOX17, merged image CXCR4/SOX17 n=3. **c**. 9-1 BLC2/GFP hiPSC derived endoderm merged left to right DAPI/CXCR4, n = 3 Images at 20x magnification, Scale bars 50 µM **d.** Gen 5 software cell count analysis of 9-1 BCL2/GFP derived endoderm DAPI, SOX17, and CXCR4.

MARMOSET

Endoderm was induced in cj367 marmoset ESCs so that marmoset ESC derived endoderm could be injected into mouse blastocysts to attempt to overcome the developmental timing and species barriers. Co-expression of the proteins SOX17, FOXA2, and CXCR4 are used to identify marmoset endoderm as well as human endoderm, and the pathways that drive endoderm specification are highly conserved between these two species; (Loh et. al. 2014) (Ang et. al. 2018) (Cho et. al. 2012) (Diekmann et. al. 2015) It was hypothesized that it would be possible to use the same media that induces human endoderm to induce marmoset

endoderm. Marmoset cj367 ESCs were subjected to the same 48hr protocol as the hiPSCs using the PSC Media. Morphologically the cells appeared to have undergone EMT and when analyzed using immunocytochemistry the treated cells no longer expressed the pluripotency transcription factors NANOG and OCT4. (Figure 5. a) Immunocytochemistry of the treated cj367 marmoset cells found that they co-expressed FOXA2 and CXCR4 at 82.88% and 81.15% respectively with a co-expression of 80.03%. (Figure 5. b, c, d).

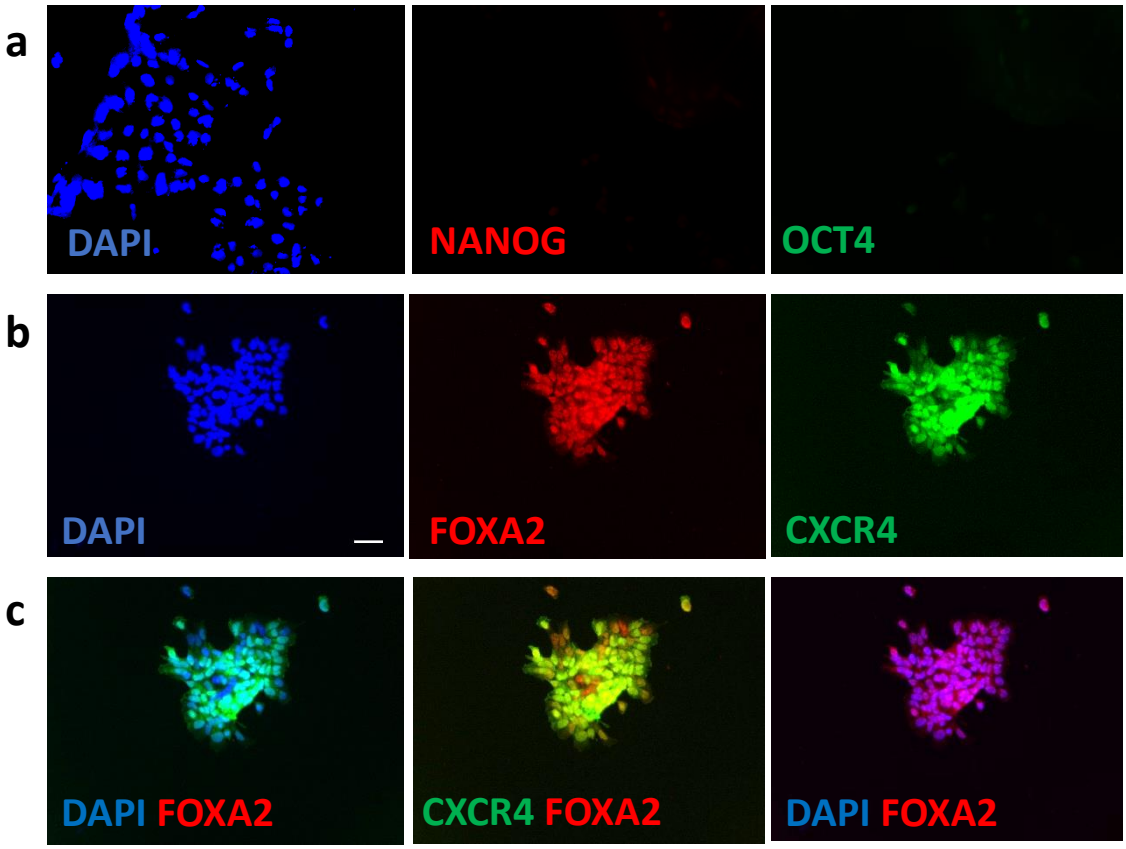
Marmoset cj367 BCL2/GFP ESC derived endoderm was to be used in the complementation studies to attempt to overcome the species and developmental barriers. For this the PSC Media's capability to induce endoderm in the cj367 BCL2/GFP and cj367 GFP ESCs was verified. The ESCs were exposed to the same 48hr differentiation protocol and analyzed via immunocytochemistry. Morphologically these cells appeared to have undergone EMT. Immunocytochemistry showed presence of FOXA2 and CXCR4 in the induced endoderm at rates of 82.45% and 81.97% respectively. (Figure 6. a, b, c)

MOUSE

Mouse iPSCs were chosen to be injected into *Hhex* KO embryos as a control as the mouse iPSCs do not need to overcome either the species or developmental timing barriers in this context. Mouse iPSC-derived endoderm was chosen to serve as a possible proof of concept. The GFP positive mouse line 3F10G was used in these experiments (Greder, et. al. 2012). The primary signaling pathway, the Activin-Nodal pathway, which drives differentiation of endoderm in mice is conserved in humans. (Loh, et. al. 2014) (Zhong, et. al. 2017).

First the Zhong protocol was attempted to induce endoderm differentiation in miPSCs. The miPSCs were exposed to this protocol for 5 to 7 days. There was significant cell death and the cells that did survive did not appear to have undergone EMT. Immunocytochemistry showed an absence of endoderm proteins SOX17, FOXA2, or CXCR4. (data not shown)

Figure 5



d

Cell Count	6875	
Cell Count (FOXA2)	5698	82.88%
Cell Count (CXCR4)	5579	81.15%
Cell Count (Dual)	5512	80.17%

Figure 5. Immunocytochemistry of cj367 marmoset ESC induced endoderm a. cj367 marmoset ESC derived endoderm left to right DAPI, Red – NANOG, and Green – OCT4 n=3. **b**. cj367 marmoset ESC derived endoderm left to right DAPI, Red – FOXA2, Green – CXCR4 n=3. **c**. cj367 marmoset ESC derived endoderm merged left to right DAPI/FOXA2, CXCR4/FOXA2, DAPI/FOXA2 n = 3. Images at 20x magnification, Scale bar 50µM **d**. Gen5 software cell count analysis of cj367 derived endoderm using DAPI counterstain, FOXA2 and CXCR4.

Figure 6

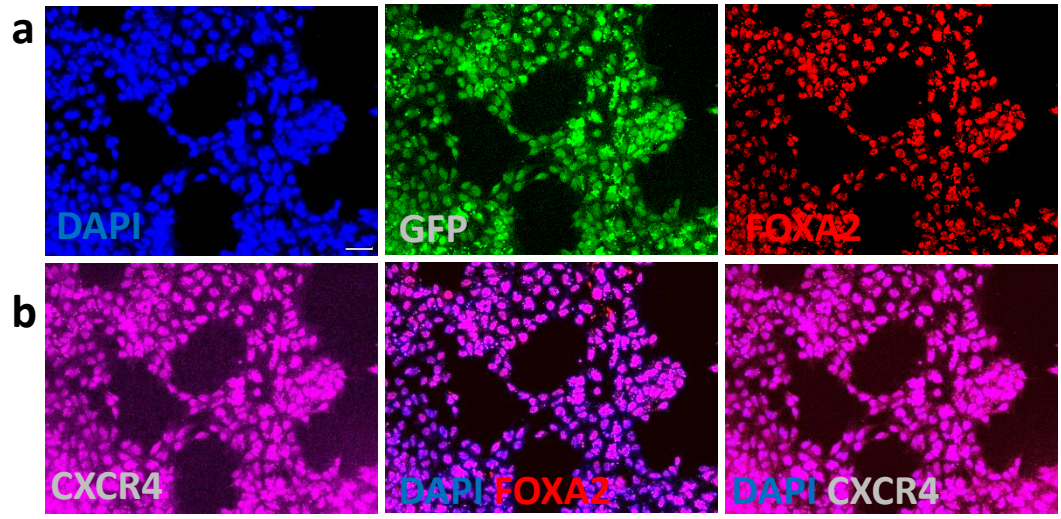


Figure 6. Immunocytochemistry of cj367 BCL2/GFP marmoset ESC derived endoderm a. cj367 BCL2/GFP marmoset ESC derived endoderm left to right DAPI, Green – GFP, Red – FOXA2 n=3. **b**. cj367 marmoset ESC derived endoderm left to right DAPI, Far Red – CXCR4, Red – FOXA2 n=3. **c**. cj367 BCL2/GFP marmoset ESC derived endoderm merged left to right 647 – CXCR4, merged image DAPI/FOXA2, CXCR4/, DAPI/CXCR4 n = 3. Images at 20x magnification, Scale bar 50µM

Since the main signaling pathway in endoderm induction is conserved between human and mouse the 3F10G miPSCs were exposed to the 48hr PSC Media protocol. Morphologically approximately 40-60% of the colonies underwent EMT. The remaining colonies did not. The colonies that did undergo EMT were analyzed using immunocytochemistry and those cells were positive for SOX17, FOXA2, and CXCR4. The percentage of cells in culture presented positive for co-expression of these proteins was 60.03%, 61.62%, 59.66% respectively. (Figure 7. a, b, c, d, e, f) After the 48hr protocol the 3F10G induced endoderm no longer expressed pluripotency markers NANOG and OCT4. (Figure 8. a, b)

BLASTOCYST COMPLEMENATION

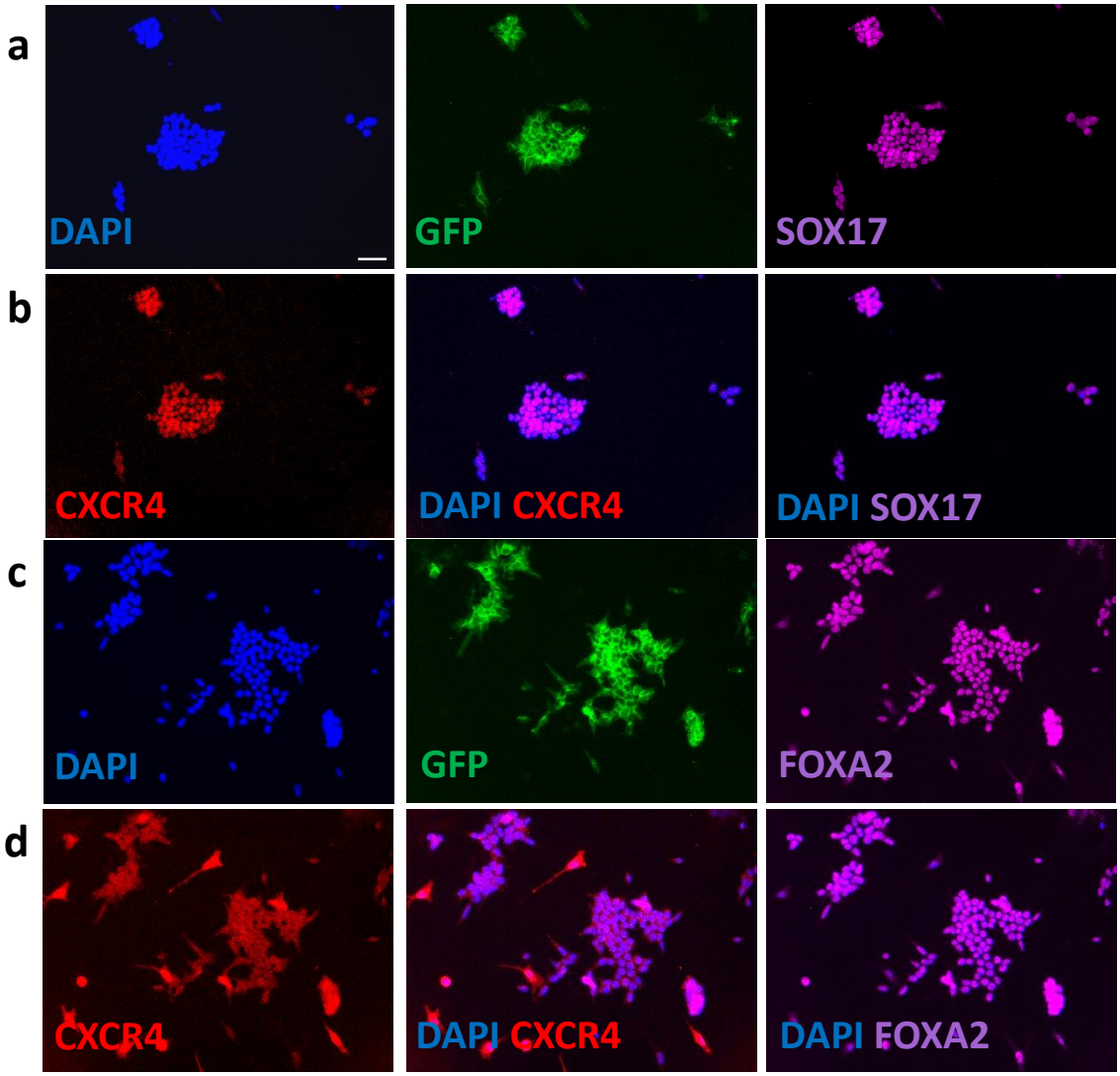
Homozygous knockouts in mouse zygotes of the *HHex* gene at the second exon using CRISPR-Cas9 was performed by Recombinectics, Inc. Cells to be injected included induced endoderm from 9-1 BCL2/GFP hiPSC, ESC derived endoderm from cj367

marmoset-ESCs and cj367 marmoset ESCs, 3F10G miPSCs and 3F10G miPSC derived endoderm. The cells were cultured and differentiated using the PSC Media, or kept pluripotent. Injections were to be performed over the course of two days. 3F10G miPSC-derived endoderm and cj367 marmoset ESCs however, were not injected due to lack of embryos that had matured to the appropriate developmental age. Of the cells injected only the 3F10G miPSCs injected embryos successfully complemented and contributed to the body plan, as assessed by GFP detected expression. (Figure 9.) There was no detectable GFP signal in embryos that had either 9-1 BCL2/GFP derived endoderm or cj367 marmoset ESC derived endoderm identified after whole embryo imaging. (Figure 9.) Of the embryos complemented with 3F10G miPSCs all of them contained high levels of GFP expression when the whole embryo was imaged using a dissecting microscope. (Figure 9. a, b, c)

EMBRYO HISTOLGOY

3F10G miPSC complemented embryo #75 and 9-1 BCL2/GFP hiPSC derived endoderm embryo #89 were selected to be sectioned to assess the amount of contribution and the ability of the donor cells to fill the empty developmental niche created by the homozygous KO of *HHex*. Detection of proteins FOXA2, insulin, and HHex in miPSC complemented embryo #75 was performed using immunohistochemistry. 54 sections were analyzed using immunohistochemistry, which confirmed the presence of FOXA2 in some sections of embryo #75 however, no detectable signal was present for the proteins HHex and insulin. (Figure 10. a,b,c,d) After sectioning there was no visible GFP present in 9-1 BCL2/GFP hiPSC-derived endoderm complemented embryo #89 in all 210 sections. (Figure 10. e) Immunohistochemistry of embryo #89 did not detect Insulin protein in 48 sections. Figure (10. c)

Figure 7



e

Cell Count	3684	
Cell Count (FOXA2)	2270	61.62%
Cell Count (CXCR4)	2198	59.66%
Cell Count (Dual)	2139	58.06%

f

Cell Count	4058	
Cell Count (SOX17)	2436	60.03%
Cell Count (CXCR4)	2402	59.19%
Cell Count (Dual)	2349	57.89%

Figure 7. Immunocytochemistry of 3F10G miPSC derived endoderm a. 3F10G miPSC derived endoderm left to right DAPI, Green – GFP, Far Red – SOX17 n=3. **b**. 3F10G miPSC derived endoderm left to right Red – CXCR4, merged images DAPI/CXCR4, DAPI/SOX17 n=3. **c**. 3F10G miPSC derived endoderm merged left to right DAPI, Green – GFP, Far Red – FOXA2 n = 3. **d.** Left to Right Red – CXCR4, merged images DAPI/CXCR4, DAPI/FOXA2 n=3. Images at 20x magnification, Scale bar 50µM. **e**. Gen 5 software cell count analysis of 9-1 BCL2/GFP derived endoderm DAPI, FOXA2, and CXCR4. **f.** Gen 5 software cell count analysis of 9-1 BCL2/GFP derived endoderm DAPI, SOX17, and CXCR4.

Figure 8

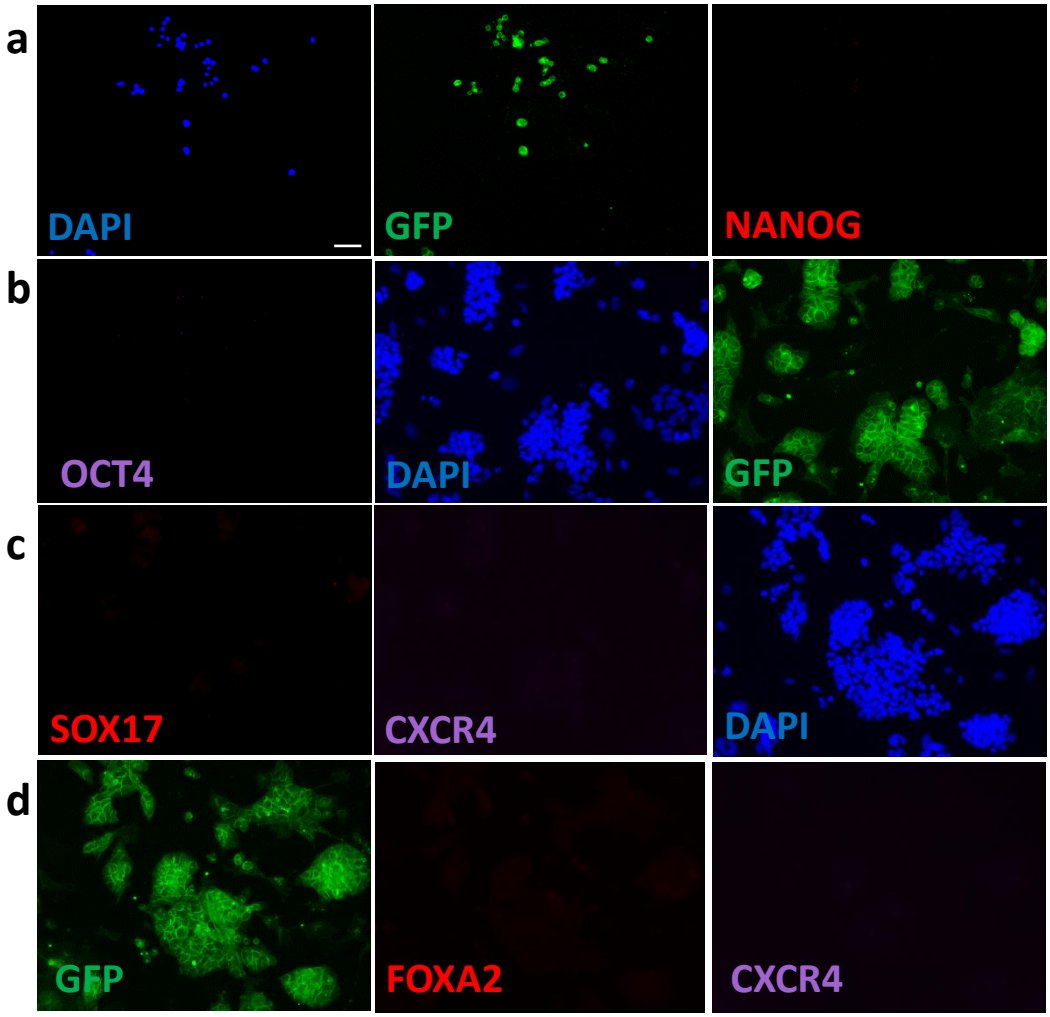


Figure 8. Immunocytochemistry of 3F10G miPSC derived endoderm a. 3F10G miPSC derived endoderm left to right DAPI, Green – GFP, Red – NANOG n=3. **b**. 3F10G miPSC derived endoderm left to right Far Right – OCT4, 3F10G miPSC DAPI, Green – GFP n=3. **c**. 3F10G miPSC left to right Red – SOX17, Far Red – CXCR4, DAPI n = 3. **d.** Left to Right Green – GFP, Red – FOXA2, Far Red – CXCR4 n=3. Images at 20x magnification, Scale bar 50µM.

Figure 9

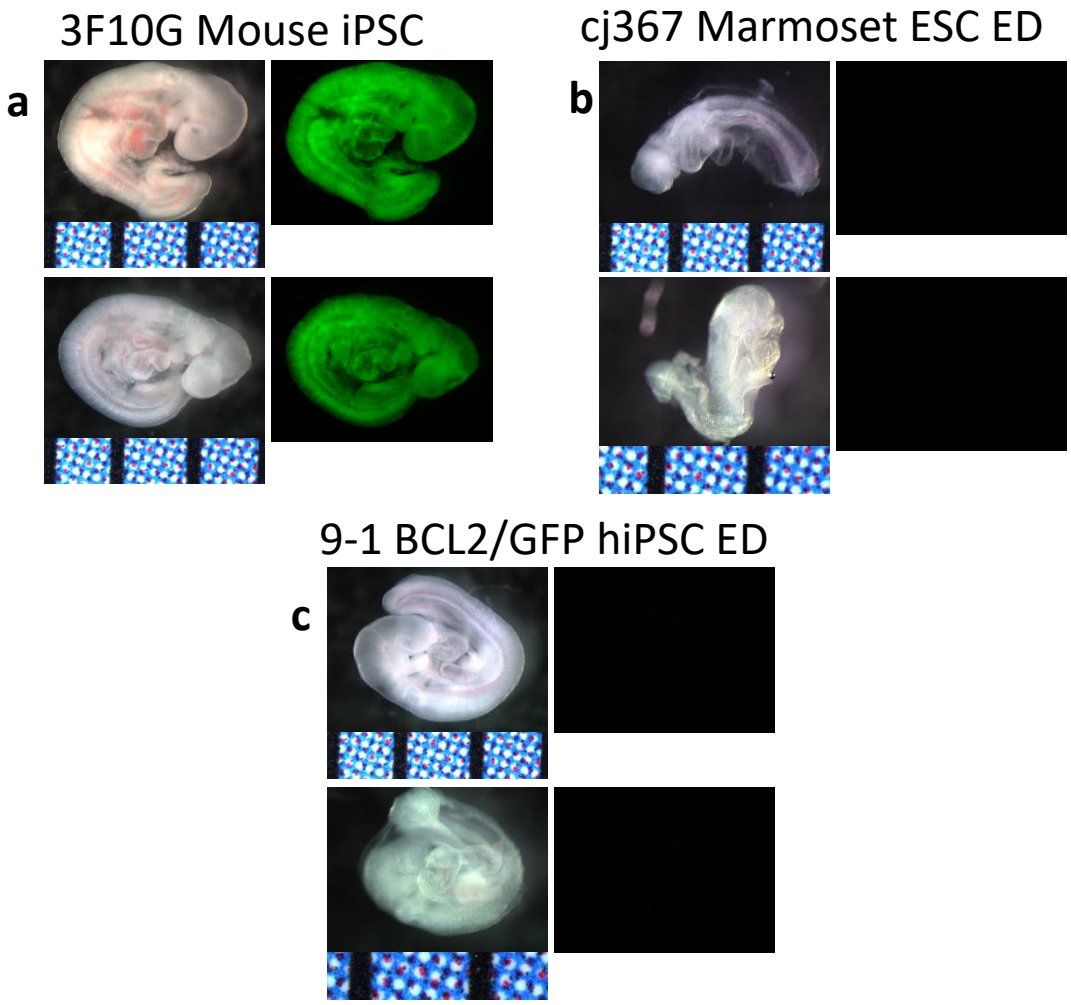


Figure 9. HHex KO mouse e9.5 embryos a. e10.5 HHex KO embryo complemented with 3F10G miPSCs at the blastocyst stage, with representative GFP images n=13. **b**. e10.5 HHex KO embryo injected with cj367 marmoset ESC derived endoderm at the blastocyst stage, with representative GFP images n=9. **c**. e10.5 HHex KO embryo injected with 9-1 BCL2/GFP hiPSC derived endoderm at the blastocyst stage, with representative GFP images n=3. Images are taken with a dissection microscope, scale bars are 1 mm each.

Figure 10

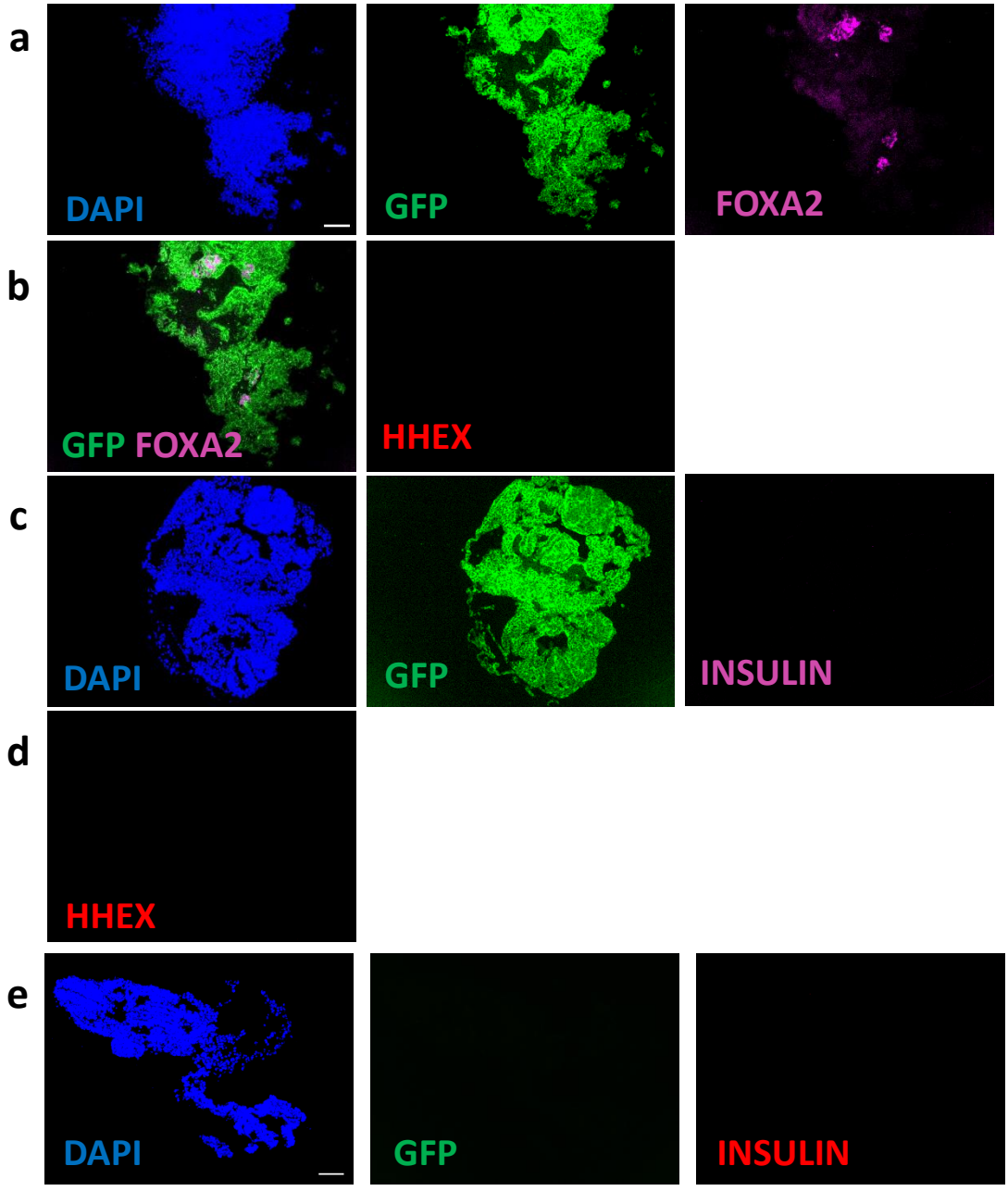


Figure 10. Immunohistochemistry 6 µm embryo sections a. Immunohistochemistry of 3F10G miPSC complemented HHex KO B6 mice embryos left to right DAPI, Green – GFP, Far Red – FOXA2. **B.** Immunohistochemistry of 3F10G miPSC complemented HHex KO mice embryos left to right merged image GFP/FOXA2, Red – HHex. **c**. Immunohistochemistry 3F10G miPSC complemented HHex KO mice embryos left to right DAPI, Green – GFP, Far Red – insulin. **d.** Immunohistochemistry of 3F10G miPSC complemented B6 mice embryo 555- HHex **e.** Immunohistochemistry 9-1 BCL2/GFP hiPSC induced endoderm injected into HHex KO mouse embryo left to right DAPI, Green – GFP, Red- insulin. All images at 10x magnification, Scale bar 100 µM.

CHAPTER 4:

DISCUSSION

This study showed that *intraspecies* blastocyst complementation in an *Hhex* KO background can allow for the donor cells to contribute to nearly 100% of the total body plan. In interspecies blastocyst complementation it was unable to show that derived endoderm cells are chimeric competent as these cells were not detected as contributing to any embryonic tissue in an *Hhex* KO background.

There is research that claims that it may be possible to overcome the developmental timing barrier by constitutively expressing an anti-apoptotic gene such as BCL2 or BMI1. (Wang, et. al. 2018) (Huang, et. al. 2018) However, the previous work sought to permit primed hiPSCs to integrate into host embryos. While they report success, it appeared that the longer the embryos were developed the fewer cells survived. This could mean that the ability of the donor cells to survive long enough for both the embryo and the donor cells to be at the same developmental age might not be enough to see interspecies complementation in evolutionarily distant species such as human and mouse. However, those studies were not seeking to fill an empty developmental niche. If that niche existed it is hypothetically possible that donor cells from more distantly related species might be able to compete with the native cells because they would fulfill a necessary developmental function that the host lacks. Therefore, it is reasonable to ask the question if hiPSCs that overexpress an anti-apoptotic gene would be able to fulfill an empty developmental niche in a mouse model. However, currently there is a moratorium on federal funding for the use of hiPSCs in blastocyst complementation studies. This requires that nonhuman-primate analogs be used in this research. However, due to having fewer embryos for injections than was originally planned this study was unable to assess if the cj367 marmoset BCL2/GFP ESCs were capable of filling the empty developmental niche left by the homozygous *HHex* KO. Work currently being done at Recombinetics, Inc. has found that these ESCs are capable of surviving and proliferating in pig embryos with this homozygous *HHex* KO, however it did not appear as if they contributed to any tissues but rather formed their own unstructured colony. It is hypothetically possible that given more trials these cells will in fact fill the empty developmental niche given that embryonic viability would indeed depend on that niche being filled.

This study sought to complement the germ layer of endoderm specifically. This would require several things to happen. The cells or their progeny would first need to survive long enough to be present when the host embryo forms the germ layers that will later give rise the host body plan. As well, the donor cells would then need to be in the appropriate physical location to associate with the host cells that are fated to the same lineage. The donor cells would then need to respond to host's molecular and mechanical cues in order to integrate and fill the engineered empty developmental niche.

While it might be possible that donor cells with the appropriate germ layer phenotype could contribute to a host embryo, the species barrier might be too large for human cells to complement a mouse embryo. The previous studies of BMI1 and BCL2 suggest that it is possible for the donor iPSCs to survive implantation, but it is unclear if they would contribute to any tissue in the final body plan. The molecular cues in mouse and human, while similar, are not identical and protein homologs often interact differently with cells across species. Indeed, this can be seen by looking at the effect of the PSC Media on the three different species in this study. The media which was designed to work in hiPSC and ESC lines effectively induced endoderm differentiation at nearly 100% efficiency. While the marmoset ESCs reacted to the molecular queues of the media they were less efficient at responding to them as can be seen by the approximately 80% induction of endoderm. Then when the miPSCs were exposed to the same protocol there was only approximately a 60% induction of endoderm. It may be that as the species became more distantly related the molecular cues that determine their development become more species specific. However, a population of miPSC derived endoderm cells would be most likely to successfully integrate and complement a mouse host embryo as an intraspecies complementation. Therefore the immediate future studies to investigate if it is indeed possible to compliment an embryo with a multipotent germ layer should be done using intraspecies complementation as a proof of concept. This study was not able to perform the number of injections needed to develop any statistical relevance investigating if any integration of donor derived cells had occurred. Further studies will need to be performed at a larger scale.

BIBLOGRAPHY

Ang, L. T., Tan, A. K. Y., Autio, M. I., Goh, S. H., Choo, S. H., Lee, K. L., … Lim, B. (2018). A Roadmap for Human Liver Differentiation from Pluripotent Stem Cells. *Cell Reports*, *22*(8), 2094–2106. https://doi.org/10.1016/j.celrep.2018.01.087

Boroviak, T., Loos, R., Lombard, P., Nichols, J., Smith, A., Bertone, P., … Okahara, J. (2015). Lineage-Specific Profiling Delineates the Emergence and Progression of Naive Pluripotency in Mammalian Embryogenesis Resource Lineage-Specific Profiling Delineates the Emergence and Progression of Naive Pluripotency in Mammalian Embryogenesis, 366–382. https://doi.org/10.1016/j.devcel.2015.10.011

Cerbini. et. al. (2015, Jan). Transcription activator-like effector nuclease (TALEN)-mediated CLYBL targeting enables enhanced transgene expression and one-step generation of dual reporter human induced pluripotent stem cell (iPSC) and neural stem cells (NSC) lines. PLOS ONE. 10(1). https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0116032

Cho, C. H. H., Hannan, N. R. F., Docherty, F. M., Docherty, H. M., Lima, M. J., Trotter, M. W. B., … Vallier, L. (2012). Inhibition of activin/nodal signalling is necessary for pancreatic differentiation of human pluripotent stem cells. *Diabetologia*, *55*(12), 3284–3295. https://doi.org/10.1007/s00125-012-2687-x

De Los Angeles, A., Hyun, I., Latham, S., Elsworth, J., & Redmond, E. (2018)a. Human-Monkey Chimeras For Modeling Human Disease: Opportunities and Challenges. *Stem Cells and Development*, *27*(23), scd.2018.0162. https://doi.org/10.1089/scd.2018.0162

De Los Angeles, A., Pho, N., & Redmond, D. E. (2018)b. Generating human organs via interspecies chimera formation: Advances and barriers. *Yale Journal of Biology and Medicine*, *91*(3), 333–342. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/30258320

Diekmann, U., Naujok, O., Blasczyk, R., & Müller, T. (2015). Embryonic stem cells of the non-human primate *Callithrix jacchus* can be differentiated into definitive endoderm

by Activin-A but not IDE-1/2. *Journal of Tissue Engineering and Regenerative Medicine*, *9*(4), 473–479. https://doi.org/10.1002/term.1709

FastStats – Chronic Liver Disease or Cirrhosis. (n.d.). Retrieved May 5, 2019a from https://www.cdc.gov/nchs/fastats/liver-disease.htm

FastStats – Leading causes of death. (n.d.). Retrieved May 5, 2019b from https://www.cdc.gov/nchs/fastats/leading-causes-of-death.htm

Liver Disease Communities. Liver Disease Stories – ALF. (2018) Retrieved May 5, 2019 from https://liverfoundation.org/

Transplant trends – 2018 transplants by organ type – (2018) Retrieved Sept. 17, 2019 from https://unos.org/data/transplant-trends/

Fehilly, C. B., Willadsen, S. M., & Tucker, E. M. (1984). Interspecific chimerism between sheep and goat. *Nature*, *307*(5952), 634–636. https://doi.org/10.1038/307634a0

Geng, Z., Walsh, P. J., Truong, V., Hill, C., Ebeling, M., Kapphahn, R. J., … Dutton, J. R. (2017). Generation of retinal pigmented epithelium from iPSCs derived from the conjunctiva of donors with and without age related macular degeneration. *PLoS ONE*, *12*(3). https://doi.org/10.1371/journal.pone.0173575

Huang, K., Zhu, Y., Ma, Y., Zhao, B., Fan, N., Li, Y., … Pan, G. (2018). BMI1 enables interspecies chimerism with human pluripotent stem cells. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-07098-w

Ivanchuk, S. M., Mondal, S., Dirks, P. B., & Rutka, J. T. (2001). The INK4A/ARF Locus: Role in Cell Cycle Control and Apoptosis and Implications for Glioma Growth. *Journal of Neuro-Oncology*, *51*(3), 219–229. https://doi.org/10.1023/A:1010632309113

Janumyan, Y. M., Sansam, C. G., Chattopadhyay, A., Cheng, N., Soucie, E. L., Penn, L. Z., … Yang, E. (2003). Bcl-xL/Bcl-2 coordinately regulates apoptosis, cell cycle

arrest and cell cycle entry. *The EMBO Journal*, *22*(20), 5459–5470. https://doi.org/10.1093/emboj/cdg533

Katsumoto, K., & Kume, S. (2011). Endoderm and mesoderm reciprocal signaling mediated by CXCL12 and CXCR4 regulates the migration of angioblasts and establishes the pancreatic fate. *Development*, *138*(10), 1947–1955. https://doi.org/10.1242/dev.058719

Ken, W. (2000). Homeobox Gene Hex Is Essential for Onset of Mouse Embryonic Liver Development and Differentiation of the Monocyte Lineage. *Science Direct*, *1161*(3), 1155–1161. https://doi.org/10.1006/bbr

Kobayashi, T., Yamaguchi, T., Hamanaka, S., Kato-Itoh, M., Yamazaki, Y., Ibata, M., … Nakauchi, H. (2010). Generation of Rat Pancreas in Mouse by Interspecific Blastocyst Injection of Pluripotent Stem Cells. *Cell*, *142*(5), 787–799. https://doi.org/10.1016/J.CELL.2010.07.039

Loh, K. M., Ang, L. T., Zhang, J., Kumar, V., Ang, J., Auyeong, J. Q., … Lim, B. (2014). Efficient endoderm induction from human pluripotent stem cells by logically directing signals controlling lineage bifurcations. *Cell Stem Cell*, *14*(2), 237–252. https://doi.org/10.1016/j.stem.2013.12.007

Myint, Z., Inazu, T., Tanaka, T., Yamada, K., Keng, V. W., Inoue, Y., … Noguchi, T. (1999). Genomic organization and promoter analysis of a mouse homeobox gene, Hex. Zhong, W., Lai, Y., Xia, Z., Yuan, Y., Ouyang, H., … Chen, Q. (n.d.). O OR RI IG GI IN NA Wnt and Nodal signaling simultaneously induces definitive endoderm differentiation of mouse embryonic stem cells. *Rom J Morphol Embryol*, *2017*(2), 527–535. Retrieved from http://www.rjme.ro/

National Institute of Health: Office of Science Policy - Next Steps on Research Using Animal Embryos Containing Human Cells (n.d.). Retrieved October 3, 2019. https://osp.od.nih.gov/2016/08/04/next-steps-on-research-using-animal-embryos-containing-human-cells/

Parr, A. M., Walsh, P. J., Truong, V., & Dutton, J. R. (2016). cGMP-compliant expansion of human iPSC cultures as adherent monolayers. In *Methods in Molecular Biology* (Vol. 1357, pp. 221–229). Humana Press Inc. https://doi.org/10.1007/7651_2015_243

Terzic, D., Maxon, J. R., Krevitt, L., Dibartolomeo, C., Goyal, T., Low, W. C., … Parr, A. M. (2016). Directed Differentiation of Oligodendrocyte Progenitor Cells from Mouse Induced Pluripotent Stem Cells. *Cell Transplantation*, *25*(2), 411–424. https://doi.org/10.3727/096368915X688137

Usui, J., & Kobayashi, T. (2012). Generation of Kidney from Pluripotent Stem Cells via Blastocyst Complementation. *AJPA*, *180*(6), 2417–2426. https://doi.org/10.1016/j.ajpath.2012.03.007

Wang, W., Zhu, Y., Huang, K., Shan, Y., Du, J., Dong, X., … Liu, J. (2017). Suppressing P16Ink4a and P14ARF pathways overcomes apoptosis in individualized human embryonic stem cells. *FASEB Journal*, *31*(3), 1130–1140. https://doi.org/10.1096/fj.201600782R

Wang, X., Li, T., Cui, T., Yu, D., Liu, C., Jiang, L., … Hu, B. (2018). Human embryonic stem cells contribute to embryonic and extraembryonic lineages in mouse embryos upon inhibition of apoptosis. *Cell Research*. Nature Publishing Group. https://doi.org/10.1038/cr.2017.138

Ye, L., Zhang, S., Greder, L., Dutton, J., Keirstead, S. A., Lepley, M., … Zhang, J. (2013). Effective Cardiac Myocyte Differentiation of Human Induced Pluripotent Stem Cells Requires VEGF. *PLoS ONE*, *8*(1). https://doi.org/10.1371/journal.pone.0053764

Zhong, W., Lai, Y., Yu, T., Xia, Z. S., Yuan, Y. H., Ouyang, H., … Chen, Q. K. (2017). Wnt and Nodal signaling simultaneously induces definitive endoderm differentiation of mouse embryonic stem cells. *Romanian Journal of Morphology and Embryology*, *58*(2), 527–535. Retrieved from http://www.rjme.ro/