GENDER DEPENDENT SURVIVAL OF ALLOGENEIC TROPHOBLAST STEM CELLS

A Dissertation

by

JESSICA A. EPPLE-FARMER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Physiology of Reproduction

GENDER DEPENDENT SURVIVAL OF ALLOGENEIC TROPHOBLAST STEM CELLS

A Dissertation

by

JESSICA A. EPPLE-FARMER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Duane Kraemer
Committee Members,	Ian Tizard
	Katrin Hinrichs
	Yanin Tian
Intercollegiate Faculty Chair,	Gary Acuff

August 2008

Major Subject: Physiology of Reproduction

ABSTRACT

Gender Dependent Survival of Allogeneic Trophoblast Stem Cells. (August 2008)
Jessica A. Epple-Farmer, B.A., Lincoln University;
M.S., Texas A&M University
Chair of Advisory Committee: Dr. Duane Kraemer

Pregnancy succeeds because the fetal allograft survives in the presence of a fully functional maternal immune system. The placenta, especially its trophoblast, provides the initial barrier between the maternal and fetal environment and, due to their location, trophoblast cells could be expected to be immune-privileged. Yet in the ectopic sites tested thus far, trophoblast stem cell transplants have failed to show noticeable immune privilege and appear to lack physiological support. However in this study, portal vein injected green fluorescent protein-labeled trophoblast stem cells were able to survive for several months in the livers of allogeneic female (14/14), but not male (0/4), mice. Gonadectomy experiments revealed that this gender-dependent survival does not require the presence of ovarian hormones (4/4) but the absence of testicular factors (5/5). In contrast, similarly labeled allogeneic embryonic stem cells were reliably rejected (11/11); these same embryonic stem cells survived when mixed with unlabeled trophoblast stem cells (13/13). The protective effect offered by the trophoblast stem cells did not require any immunological similarity with the co-injected embryonic stem cells. Neither the trophoblast stem cells nor the co-injected embryonic stem cells gave rise to tumors during the study period. Thus, this study demonstrates that, provided a suitable location and hormonal context, ectopic trophoblast stem cells may exhibit and confer immune privilege. These findings suggest applications in cell and gene therapy as well as provide a new model for studying trophoblast physiology and immunology.

DEDICATION

John Donne wrote that “No man is an island”. As I begin to assess all that has brought me to this juncture in my life I am humbled by the truth of those words. I am at this point because those who have come before me have paved the way, those who stand with me offer their support, and those who will follow deserve my assistance.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Duane Kraemer, and my committee members, Dr. Tizard, Dr. Tian, and Dr. Hinrichs, for their guidance and support throughout the course of this research. Special thanks goes to Dr. Binas for providing this project and introducing me to the wonders of stem cell research.

Thanks also go to my friends, colleagues, and the veterinary pathobiology department faculty and staff for making my time at Texas A&M University a memorable experience. I also want to extend my gratitude to the staff of the Comparative Medicine Program, which provided surgical guidance and animal care advice.

Finally, thanks to all my parents and grandparents, especially mom and dad, for your encouragement and instilling the belief that education is important. Thanks and appreciation also goes to my husband Chris for his faith, love, and support of my dreams. He refused to let me quit when I desperately wanted to and for that I will be eternally grateful.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION: THE IMPORTANCE OF RESEARCH

The isolation of mouse trophoblast stem (TS) cells (1) and the ability to promote differentiation of human embryonic stem (ES) cells into trophoblast cells in vitro (2) has increased the motivation of researchers to apply the phenomenon of trophoblast immune privilege (3-5) to allogeneic transplantation and cell therapy (6). Unfortunately, attempts to transplant TS cells or their in vitro-differentiated derivatives have been largely unsuccessful. When syngeneic TS cells were injected subcutaneously into non-pregnant females, they were reported to differentiate into giant cells within 5-10 days and to degenerate rapidly (7). When allogeneic TS cells and their differentiated derivatives were injected into the tail veins of non-pregnant females, they were removed from host lungs by natural killer cells within 24 hours (7). TS cells injected subcutaneously in high numbers into male nude mice formed giant trophoblast and spongiotrophoblast cells that were resorbed within 2 weeks (8). Thus, in the ectopic sites tested so far, all of the trophoblast cell transplants have failed to survive longer than a few weeks.

Research in sheep has illuminated the important role of the uterus in pregnancy by revealing that the uterus becomes moderately immune-privileged under the influence of progesterone (9). It is possible therefore that the failed trophoblast engraftments were due to injection at a poor location and so missing the synergistic interaction between the immune mechanisms of the trophoblast and the uterus. Based on this, it is possible that an injection into a partially immune-privileged location, other than the uterus, might permit allografted TS cells to survive. Furthermore if the allografted TS cells were able to survive in an ectopic location then they may also be able to provide protection for other nearby cells in a manner similar to the placenta protecting the fetus. This

---

This dissertation follows the style of *Proceedings of the National Academy of Science*.

protection, if successful, would enable the co-injected cells to survive in locations where they normally could not. These hypotheses were tested in the present experiments. As a final component of the project, the role played by sex hormones in the survival of ectopic TS cells was analyzed.

The results of these investigations demonstrate that (i) the liver provides a physiological and immunological environment permissive of prolonged TS cell survival. This TS cell survival (ii) displayed a lack of dependence on the systemically provided ovarian hormones and a susceptibility to testicular factors. This TS cell survival (iii) appeared to provide an immuno-protective effect on co-injected ES cells. Together, these results provide a framework for using transplanted TS cells in cell therapy, gene therapy, and as a model to study trophoblast physiology and immunology.

BACKGROUND

Previous Investigations

The use of TS cells in this project was based on studies that described the strain-dependent isolation, in vivo survival, lymphoid differentiation, and tolerogenicity of "rat embryonic stem cell-like cells" (RESC) (10). In that project, cell lines were generated from three different rat strains: Wistar-Kyoto (WKY), BDIX, and Sprague-Dawley (SD). These cells had characteristics similar to mouse ES cells in that they exhibited a compact morphology and expressed the enzyme alkaline phosphatase and the cell surface marker Stage-Specific Embryonic Antigen-1 (SSEA-1) (10). When the RESC line from the WKY background was injected into allogeneic rats, it was found that the cells: (i) survived and migrated to the liver, spleen and thymus, (ii) differentiated into B cells and macrophages that were detectable for 25+ weeks, (iii) failed to generate tumors, and (iv) conferred background cell specific tolerance to abdominal heart transplants. It was subsequently suggested however that the identity of this RESC line was more TS like then ES-like. This belief was based on the findings that the cells lacked the transcription factor Octamer-4 (Oct4), a crucial ES cell marker, and that the cells were able to grow in the absence of Leukemia Inhibitory Factor (LIF), an essential cytokine for the growth of mouse ES cells.

Early Embryonic Development

After fertilization, the ensuing cell divisions produce a mass of cells with an unlimited spectrum of differentiation possibilities that persist until the eight cell stage (11). At the eight cell stage, blastomeres begin increasing their cell-to-cell contact so that in future divisions they produce a compacted group of cells called the morula. As the cells within the early morula divide they can either take up a position on the inside where they are enclosed by other cells or remain on the outside in contact with the external environment. Late in the morula stage a cavity (the blastocoel) begins to form and marks the transition to the blastocyst stage. From the original totipotent cell type the

first restriction in potential occurs with the differentiation of trophoblast stem cells from what will become the inner cell mass (12). Trophectoderm cells can be distinguished by their expression of the transcription factor Cdx2 (13) while the cells of the inner cell mass express Oct4 (14). In the current developmental model, mutual antagonism between Oct4 and Cdx2 reinforce this segregation (15). Lineage-tracing experiments have demonstrated that the trophectoderm mainly originates from outer cells with the inner cells becoming the inner cell mass (16) as a result of cell polarity after cleavage (12). Later in development the trophectoderm will further differentiate into other placental cell types. The second restriction step occurs within 24 hours after blastocyst formation with the differentiation of the inner cell mass cells into either epiblast or primitive endoderm. These cell fates are further reinforced by the transcription factors Oct4 and Nanog (17) in the epiblast and Gata4/6 (18) in the primitive endoderm. In the current model for this developmental step the inner cell mass is a heterogeneous population of developmentally-committed cells that later migrate to the locations of the epiblast and primitive endoderm (15). The primitive endoderm will go on to form the parietal endoderm that covers the blastocoel surface of the blastocyst and the visceral endoderm of the yolk sac. The epiblast will continue to form the fetal cell populations as well as the mesodermal component of the yolk sac.

Stem Cell Types Analyzed in This Dissertation

Mouse stem cells have been isolated from each of the first three embryonic developmental lineages: the ES cells representing the epiblast, the TS cells representing the trophectoderm, and the extraembryonic endoderm (XEN) cells representing the primitive endoderm. This study has focused on the use of ES cells and TS cells in allogeneic cell transplantation.

ES Cells

In 1981 the first embryonic stem cell lines were derived from mouse blastocysts by two independent research groups (19, 20). The definition of ES cells is that they are

self-replicating stem cells that are derived from an embryo and can differentiate into all cells of the body. Investigators have however generated a list of more specific characteristics required of any ES cell line. These characteristics include that they originate from the inner cell mass of the blastocyst. They are capable of long-term self-renewal. They maintain a stable diploid karyotype. They can be differentiated into all three primary germ layers of the embryo (endoderm, mesoderm, and ectoderm). They are capable of integrating into all fetal tissues of a chimeric animal. They are germ line competent and can be cloned. They express the transcription factor Oct-4, and do not undergo X inactivation (21, 22).

There are several published protocols for the derivation and maintence of mouse ES cell lines. ES cells tend to grow in tightly packed colonies in which individual cells can not be identified. If these colonies have a rough appearance in which individual cells can be identified, this is evidence that the culture has started to differentiate. In order to prevent differentiation, the culture should be passaged frequently and medium replaced daily (23). When the cells are passaged it is also important to disaggregate the colonies down to a single cell level in order to prevent further differentiation.

Derivation of mouse ES cells begins with the collection of day 3.5 post coitus blastocysts. These blastocysts are allowed to expand and hatch in individual 4-well dishes where they will adhere and flatten to the provided feeder layer. At this stage the hatched blastocyst has a “fried egg” appearance and the inner cell mass is ready to be collected and disaggregated. From this point forward the ES-like colonies may be picked, disaggregated, and moved to new culture wells containing feeder cells until they reach a constant density that can then be trypsinized and further passaged.

TS Cells

The first trophoblast stem cell line derivation was reported in 1998 (1). This line could form all of the derivatives of the trophectoderm. The trophectoderm is the first cell type to differentiate from the developing embryo. It is involved in implantation and forms the fetal components of the placenta. Trophoblast stem cells, while not as highly

characterized as ES cells, have specific characteristics. These characteristics include their blastocyst derivation source, specific culture conditions (FGF_4, heparin, and contact with an embryo fibroblast feeder layer) (1) and their growth morphology, as tight relatively flat epithelial colonies with some spontaneous differentiation at the edge. TS cells are capable of long term self-renewal but can be differentiated by the removal of FGF_4 and feeder cells. They retain a stable diploid karyotype in their undifferentiated form and display primarily paternal X inactivation (24). TS cells characteristically express the genes Cdx-2 (13) and Eomesodermin (25) when undifferentiated.

There are several protocols for the derivation and maintence of TS cell lines each based upon the specific mouse strain being used. Maintaining a TS cell line on feeders requires medium changes every other day as opposed to the daily requirement of ES cells. Once the cells reach about 80% confluence they should be passaged every 4 days although some strains can grow longer without differentiating or separating from the substrate. Derivation of TS cell lines begins with the same blastocyst stage as ES cells. The culture medium is different (containing FGF_4 and Heparin instead of LIF) as is the timing of the first disaggregation (one day earlier in size of primary outgrowth). One derivation protocol calls for the first disaggregation and subsequent culture to take place within the same well as the original attachment with the medium supplemented with 70% feeder conditioned medium until the TS cells become an established cell line (23).

XEN Cells

Kunath et al (2005) (26) derived extraembryonic endoderm (XEN) stem cell lines from the primitive endoderm that forms on the blastocoel exposed surface of the inner cell mass (Figure 1). Within the developing conceptus, primitive endoderm cells form the parietal yolk sac (parietal endoderm) and the definitive yolk sac (visceral endoderm). Like the TS cells and ES cells, the XEN stem cell line also has its own unique characteristics (26). XEN cells maintain little cell-to-cell contact when grown at low density unlike ES and TS cell lines. The XEN cell lines require only feeders or feeder-conditioned medium as a supplement. They display mixed characteristics of both

parietal and visceral endoderm and in chimeras can form both parietal and visceral endoderm lineages. They also exhibit paternal X inactivation. The protocol for the derivation of XEN cells is the same as that for trophoblast derivation except that the blastocyst outgrowths are not promptly disaggregated. In fact, no disaggregation is performed until the primitive endoderm begins to take over the culture several days later.

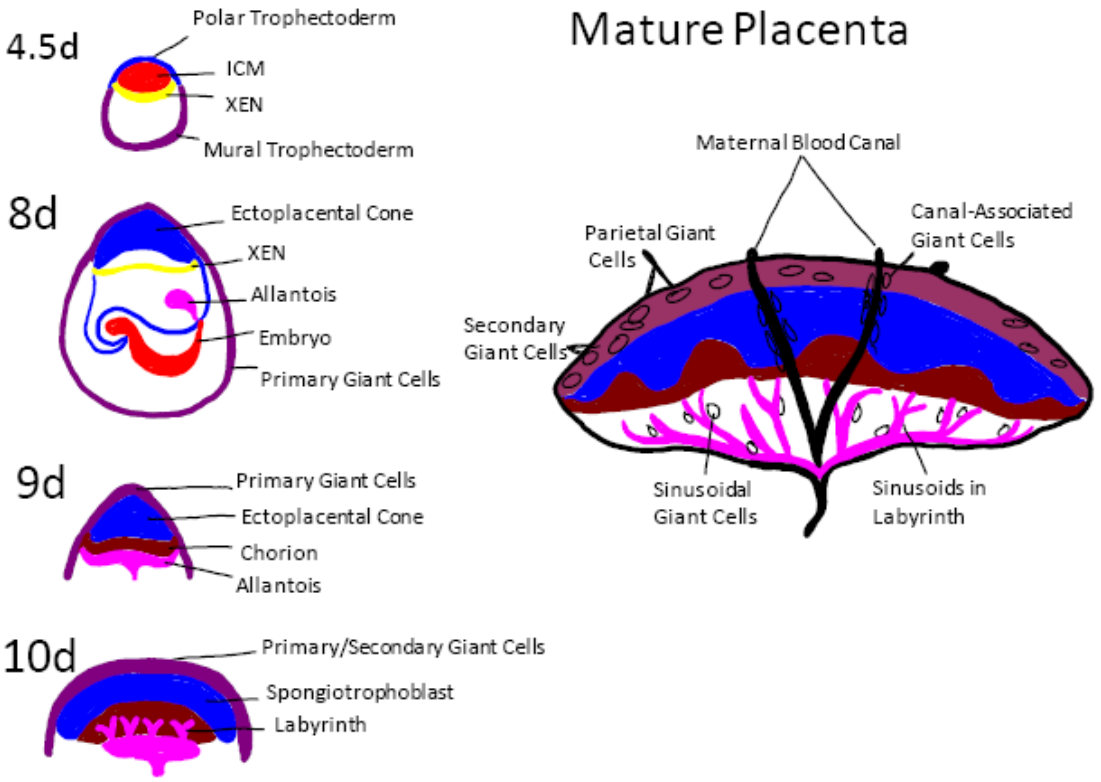

Figure 1. Mouse placental development highlighting the four subtypes of trophoblast giant cells.

Placental Structure and Function

Because TS cells are the precursors to a major component of the placenta, it is important to understand the roles they play in that organ. Figure 1 illustrates the different placental structures formed in the mouse as well as the type of trophoblast in

each location. Placental development begins as early as the late morula to early blastocyst stage with the expression of Cdx2 by the early trophoblast cells (13). In the mouse, mural trophoblast cells that line the blastocoel cavity differentiate into "primary" trophoblast giant cells and form part of the parietal yolk sac. The polar trophoblast cells that overlie the inner cell mass are different from their mural counterparts in that they continue to divide after implantation and form the structures of the chorionic ectoderm, the ectoplacental cone and secondary trophoblast giant cells (27). These polar trophoblast cells are also the source of TS cells. The secondary trophoblast giant cells that form after implantation at the edge of the ectoplacental cone are responsible for the connection of the fetus to the maternal blood supply. They do this by invading into the uterus and targeting maternal spiral arteries. These giant cells are polyploid, through endoreduplication, and can restructure their maternal environment through the production of angiogenic (28), vasodilatory (29), and anticoagulative products (30, 31). This results in the formation of pockets of maternal blood surrounding the conceptus. From these pockets of blood, nutrients and oxygen can pass directly through to the yolk sac and on to the embryo. In the spiral arteries the giant cells displace the endothelial cell lining and create entirely trophoblast lined blood spaces used to funnel maternal blood flow towards the site of implantation (32).

In addition to the trophoblast, another important cell lineage in the placenta is the primitive endoderm. Like the trophoblast, the primitive endoderm contributes exclusively to extraembryonic tissues and forms the parietal and visceral endoderm layers of the yolk sac. A third source of cells for the placenta comes from the extraembryonic mesoderm. At gastrulation, the extraembryonic mesoderm separates from the developing fetus and forms the allantois and the mesodermal components of the visceral yolk sac, amnion, and chorion. For the placenta to be fully functional the chorionic ectoderm layer must interdigitate with the allantois. This supplies the extraembryonic mesoderm cells from the allantios that are able to invade trophoblast cells and create the vascular network of the labyrinth (33). The fetal vessels will

eventually interdigitate in a counter-current orientation with the trophoblast-lined maternal blood vessels to ensure adequate transport of nutrients (Figure 2).

While the interdigitation is taking place, the trophoblast cells in the rodent differentiate into three distinct layers between the maternal blood and fetal capillaries. The outer-most layer consists of mononuclear trophoblast giant cells that are no longer dividing and in the rodent have endocrine functions (33). Below the mononuclear layer of giant cells are the structural layers of the spongiotrophoblast and the lower villous layer of the labyrinth. The labyrinth is thought to form from the chorion and innermost layers of the trophoblast; the outer trophoblast layer develops into the ectoplacental cone and later differentiates into the spongiotrophoblast as well as the mononuclear layer of giant cells (33, 34).

Recently, four distinct types of trophoblast giant cells have been identified through their gene expression patterns, time of formation, and location within the placenta (Figure 1). They include the primary giant cells, these are the original mural trophoblast cells formed in the peri-implantation period that express placental lactogen I (35) and function to line the implantation site and form part of the parietal yolk sac (36). Secondary giant cells form after implantation around the edges of the ectoplacental cone and express placental lactogen II (36). The spiral artery-associated giant cells are extremely invasive and migrate up to 300μm into the uterine wall where they make contact with the maternal spiral arteries (37). The canal-associated giant cells line the canals that funnel maternal blood into the labyrinth of the placenta and these produce placental isoferritin (Plf). Later, a fourth group of sinusoidal giant cells form within the cytotrophoblast layer of the labyrinth. These giant cells are associated with the sinusoids that carry maternal blood in the placental labyrinth layer, and express placental lactogen II (38).

Below the outer layer of mononuclear trophoblast giant cells is the spongiotrophoblast layer. Little is known about the spongiotrophoblast except that it expresses trophoblast specific protein alpha (Tpbpa) and that it is assumed to fulfill a structural role based on its location within the placenta. Below the spongiotrophoblast

layer is the placental labyrinth. The labyrinth contains two syncytial layers that can be differentiated by their expression of transcription factor glial cells missing 1 (Gcm1) and their distinct structural morphologies.

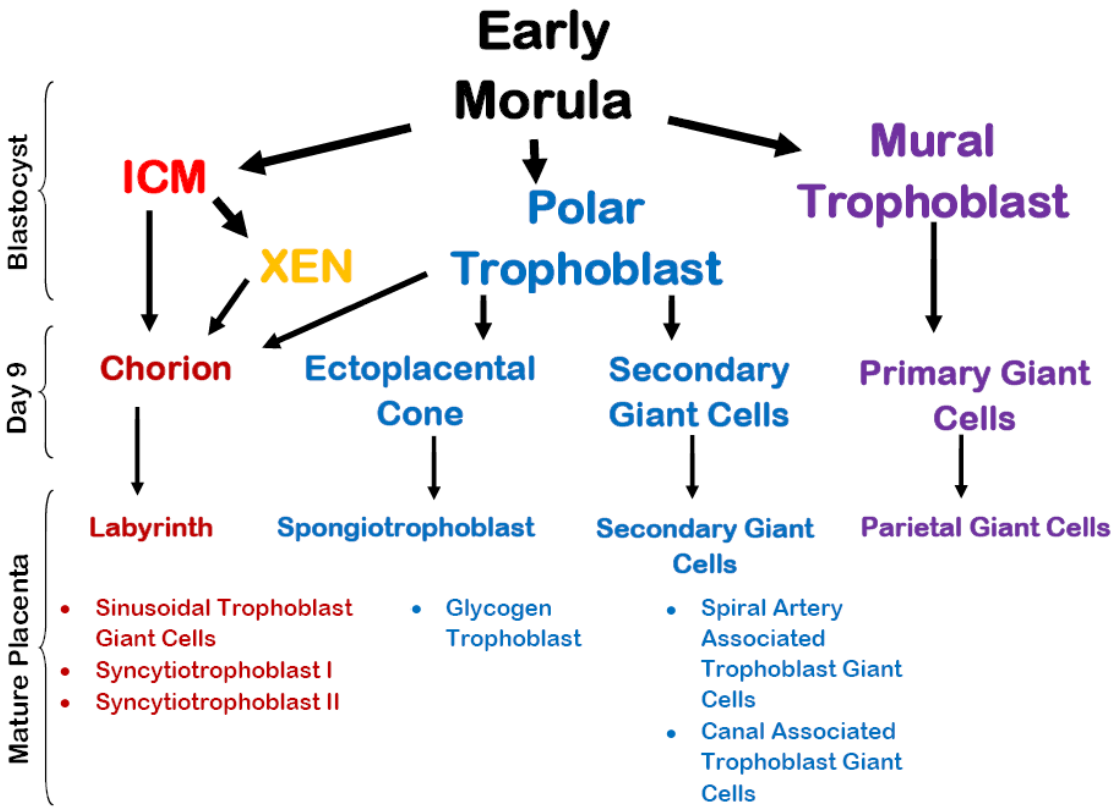

Figure 2. Flow chart illustrating the origin of each cell type in the mature placenta. The coloring reflects the placement of cell types in Figure 1.

Liver Structure and Function

In this study the liver is utilized as an experimental site for trophoblast engraftment. The liver is an organ of many functions including glycogen storage, bile production, decomposition of red blood cells, and detoxification. When nutrient rich blood leaves the stomach and small intestines, it travels to the liver for filtration prior to bodily dissemination. This blood enters the liver through the portal vein and then

infiltrates the lobes of the liver where the hepatocytes reside separated by vascular channels. The hepatocyte is the functional unit of the liver. When damaged, the liver has remarkable regenerative capacity both by hepatocytes, that are able to divide forming more hepatocytes, and by ovalocytes, that are able to form more hepatocytes and the epithelial cells that line the bile ducts. Like trophoblast cells, liver hepatocytes have also been known to fuse although much less is known about the process. The incidence of hepatocyte fusion is found to increase when the liver is under the strain of rapid regeneration (39). Fusion is also documented between hepatocytes and injected bone marrow stem cells when the latter are used for cell therapy (40, 41). Current research indicates that, in cell therapy, the specific cells fusing with the hepatocytes are bone marrow macrophages or cells that are their precursors (42).

How the Immune System Recognizes Self/Invaders

Every nucleated cell in the body expresses major histocompatibility complex (MHC) class I molecules that the immune system recognizes as self. Under normal circumstances the body's defense system does not attack its own cells and immune cells are subject to self-tolerance. The innate immune system is the generic part of immunity that keeps the body alive until the acquired immune system can kick in. The innate immune system contains a variety of cells including neutrophils, macrophages, mast cells, dendritic cells, and natural killer (NK) cells. They recognize foreign invaders based upon their toll like receptors (TLRs) and neutrophils, macrophages, mast cells, and dendritic cells are able to clear the invading microbes through phagocytosis. Before invaders are phagocytosed they are first contained in a process called inflammation; inflammation also serves as a beacon to call in more immune cells. In addition to phagocytosis, macrophages and dendritic cells are able to act as antigen-presenting cells for the acquired immune system by processing and presenting antigens on MHC class II receptors. The acquired immune system contains cells such as B and T cells that have customized receptors that allow them to recognize and respond to specific antigens. When B and T cells are created they undergo a negative selection process where they are

theoretically exposed to all the normal molecules in the body and are destroyed if they respond to them. This selection strongly encourages self-tolerance and in general, when B and T cells survive selection they contain receptors that only recognize foreign material.

B cells recognize antigen, i.e. foreign material, in its native state. The B cell receptor is a sample of the antibody it is prepared to manufacture in defense of the body. Before the B cell can become activated and start antibody production it has to have permission by a specific type of T cell. This acts as an additional check on the system to prevent an auto-immune response. T cells recognize only antigen that has been processed by other cells and is presented in an appropriate fashion along with a MHC self marker. If a cell is missing the MHC self marker or is displaying the wrong MHC it is targeted for destruction by NK cells of the innate immune system.

Because T cells control all acquired immune responses, a case can be made for calling them the master cells of the immune system. There are three general types of T cells and thus three different responses an activated T cell can generate. Helper T (Th) cells activate other immune cells, such as B cells and other T cells, and can facilitate the complex workings of the immune system. Th cells influence the microenvironment of an immune response through the cytokines that they produce. Th-1 cells secrete interlukin-2 and interferon-γ; these cytokines are responsible for creating a proinflammatory microenvironment that includes the presence of cytotoxic T (Tc) cells. Th-2 cells secrete interleukin-4 and interleukin-10; these cytokines aid in antibody production from B cells. In addition to influencing the microenvironment for other immune cells, the cytokines produced by Th-1 and Th-2 cells also have an inhibitory function on one another ensuring only one type of response occurs from a stimulus. Tc cells systematically destroy, via apoptosis, abnormal body cells that are viral-infested or have been transformed by cancer; Regulatory T (Treg) cells function to suppress the immune system through the production of IL-10 (43) and TGF-β (44). Both cytokines, IL-10 and TGF-β, play a predominant role in pregnancy immunology (see Natural Mechanisms to Prevent Fetal Rejection, below).

Methods to Prevent Immune Rejection

In standard organ transplantation, the MHC receptors and blood group antigens of the donor and recipient are closely matched. The recipient is treated with immunosuppressive medications such as cyclosporine and monitored for signs of rejection. The main drawbacks to this procedure are the prolonged exposure to the immunosuppressive medication and the limited supply of donor organs. Alternatives to this method of organ transplantation may include: tissue engineering, xenotransplantation, and the artificial generation of immune tolerance (45). While this study has mainly focused on the generation of immune tolerance using a pregnancy specific mechanism, all three therapies are briefly discussed here.

Tissue Engineering could substitute for whole organ transplantation through the creation of devices that act as a barrier against the host's immune system. This technique would combine allogeneic living secretory cells that would normally be rejected with an inert encapsulating material to create a barrier between the foreign cells and the immune system of the host. One successful example of this mechanism being tested in humans involves the allograft of Sertoli cells combined with islet cells to treat diabetes (46). Sertoli cells were utilized in this study for their secretion of TGF-β that provided a protective environment for the transplanted islets. Another form of tissue engineering is the use of syngeneic stem cells generated by cloning to engineer cells, tissues, or organs for transplantation. This technique has the advantage of creating syngeneic organs that the host immune system would have no reason to attack.

Xenotransplantation, animals as a transplantation source of cells/ organs for humans, is a further extension of tissue engineering. Pig heart valves have been used in humans since the 1975 and now pig islets are being investigated for the treatment of diabetes (46). In 2003, pigs were genetically engineered to remove the α 1-3 galactose epitope that triggers immune rejection in humans. This may eventually permit the use of non-human organ sources, but does not solve the need for persistent and aggressive immunosuppression.

Tolerance is achieved when foreign cells, tissues, or organs survive in a recipient's body without requiring immunosuppressive therapy and/or damaging the host immune system. Tolerance can be generated through lymphocyte anergy, clonal deletion of reactive lymphocytes, or manipulation of regulatory T cell function. Anergy is created when the lymphocytes that react specifically to the transplant antigens are turned off or killed before having a chance to mature and circulate. Many of the existing immunosuppressive drugs work by interfering with the lymphocytes ability to respond to such alloantigens. Clonal deletion is the natural process by which the body eliminates self-reactive B and T cells in the bone marrow and thymus. This process can be mimicked by killing the host immune cells through radiation and drugs, then performing a bone marrow transplant, with donor marrow cells. The donor marrow cells serve as a source of stem cells that then regenerate the host immune system resulting in acceptance of grafts from the same donor background. Regulatory T cells are a distant subset of T cells that function to suppress both the innate and acquired branches of the immune system. Their ability to suppress immune response gives regulatory T cells the ability to reduce allograft rejection.

Natural Mechanisms to Prevent Fetal Rejection

The survival of the fetus and its surrounding placenta is an enigma because of its success as an allograft/semiallograft within the fully functional maternal immune system. The fetus is typically considered a semiallograft because it contains genetic material from both the mother and the father, and as such should be rejected by the maternal immune system. This rejection should take place because the fetus displays not only the maternal but also the paternal pattern of MHC expression. With the advent of embryo transfer the maternal MHC of the fetus can also be different from the surrogate mother and still result in successful (allograft) pregnancies. Many different pregnancy-related tolerance mechanisms have been identified in both the rodent and human system. They originate from both the maternal and fetal side (Figure 3) and their relative importance is unclear. The maternal immune system tolerates the fetus through a

Maternal Contribution

- Hormonal Environment
  - Th1->Th2 shift
- Regulatory T Cells
  - TGFB
  - IL-10
- Regulatory Macrophages
  - TGFB
  - IL-10
- Uterine Natural Killer Cells
- Decidual Dendritic cells

Paternal Contribution

- Ejaculate
  - Causes changes in the female WBC population
    - Treg
    - reg Macrophages
    - uNK cells
  - Contains TGFB

Fetal Contribution

- Expresses non classical MHC class 1
  - HLA-C
  - HLA-E
  - HLA-G
- Fas-Ligand (CD95)
- IDO Synthesis
- IL-10 production
- Crry production
- Expression B7/CD28 Costimulation

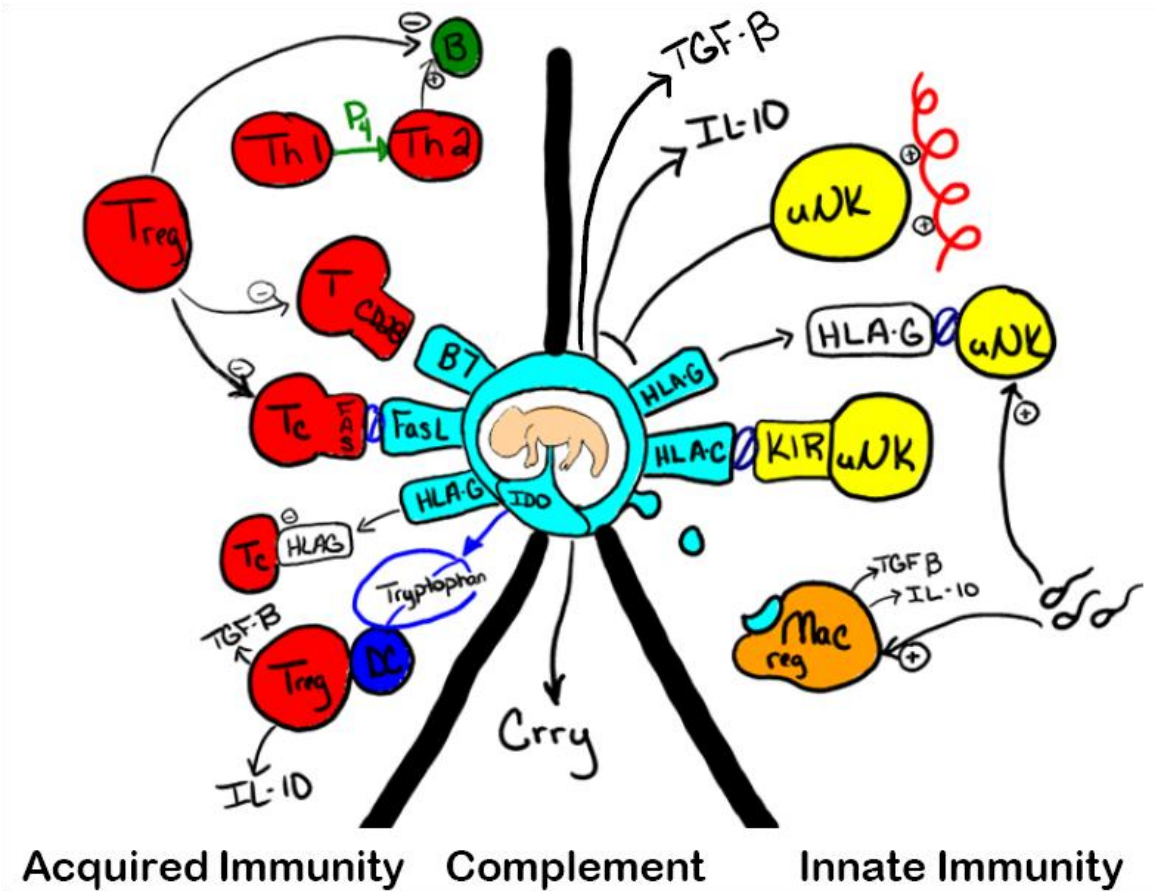

Figure 3. Diagram of mechanisms involved with pregnancy immunology separated by their role in the mother's immune system.

progesterone-mediated shift in the Th-1/Th-2 balance (47) and the production of Treg cells and regulatory macrophages. Changes in the Th-1/Th-2 balance influence the two possible responses the immune system can have towards a stimulus: the activation of cell-mediated immunity or the production of antibodies. To minimize inflammation within the uterus, the maternal immune system apparently shifts from a Th-1 to a Th-2 dominated process (47). Regulatory T cells prevent allograft rejection by generating peripheral tolerance (48). This regulation is facilitated by the production of IL-10 and TGF-β which suppresses both Th-1 triggered inflammation and Th-2 antibody formation. Macrophages serve a regulatory function by producing immunosuppressive (TGF-β) and anti-inflammatory (IL-10) molecules stimulated by the ingestion of apoptotic cells in low amounts (49). Macrophages accumulate in large numbers surrounding trophoblast cells at the site of implantation and remove apoptotic trophoblast cells. By removing the apoptotic trophoblast cells, the regulatory macrophages may prevent the release of cell contents that act as foreign antigens (50)

The trophoblast cells of the placenta also have a major role to play in maternal immunoregulation. The first mechanism utilized by the trophoblast cells is the expression of non-classical MHC class I molecules (HLA-C, HLA-E and HLA-G in humans and Qa-1 and Qa-2 in the mouse). MHC class I molecules are located on all nucleated cells and present endogenously produced proteins to cytotoxic T cells and natural killer cells. Foreign cells or virus-infected cells produce different proteins than normal cells and can be detected and destroyed based on the cell products bound to their MHC class I receptors. HLA-C, in humans, prevents destruction of the placenta by natural killer cells through binding to their killer inhibitory receptors (51). A parallel counterpart to HLA-C has not been identified in the mouse. HLA-E prevents NK cell cytotoxicity toward trophoblast cells and corresponds to Qa-1 in the mouse. HLA-G can be found in a number of forms including a soluble form that triggers apoptosis in activated cytotoxic T cells and inhibits the cytotoxicity and activation of natural killer cells (52). Qa-2 is the mouse equivalent to HLA-G. The second mechanism that the trophoblast cells employ is the induction of apoptosis through their expression of FAS

ligand (CD95L) (53). An activated T cell expressing FAS will be killed upon contact with trophoblast cells bearing the FAS receptor. The third mechanism utilized by trophoblast cells is the synthesis and secretion of indoleamine 2,3-dioxygenase (IDO). IDO aids in generating tolerance by locally depleting tryptophan thus inhibiting the proliferation of activated T cells (54) A fourth mechanism for trophoblast evasion of the maternal immune system involves the expression of the complement regulatory protein Crry, which inhibits the activation of the complement system (55). A fifth mechanism employed by trophoblast is the production of anti-inflammatory IL-10 (56) and immunosuppressive TBF-β (57). While these mechanisms have been shown to be involved with the peaceful coexistence between the mother and the placenta no governing order of importance has been established for the existing mechanisms. Their relative importance is unclear and may vary according to the stage of pregnancy and among species.

The father may play a small role in pregnancy immunology through the production of TGF-β in the seminal fluid as well as by providing paternal antigen. Seminal fluid promotes the recruitment and activation of macrophages, Treg cells and NK cells to the uterine environment (58). Thus the ejaculate may act as a priming event preparing the uterus for pregnancy in addition to providing the semen.

Testosterone and the Immune System

In the current study, testicular products appeared to play a key role in the rejection of allografted TS cells. Testosterone may directly affect TS cell survival or it may act on the immune system to create an environment that is antagonistic to trophoblast survival. Several reports indicate that androgens influence the immune response through their interaction with T cells, B cells, macrophages, and NK cells. Testosterone has two main effects on T cells; it both strengthens the Tc cell response and increases the numbers of Treg cells (59). While testosterone appears to have no effect on the ratio of (CD8+) Tc cells to (CD4+) Th cells, it increases the production of IFN-γ, and IL-1, and IL-6 (60) while reducing the production of IL-4 and IL-5 (61) by T cells.

This shift in cytokine production promotes inflammation which is presumably offset by the increased regulatory T cell presence. High testosterone is associated with a decrease in B cell numbers (62) most likely due to the downstream effects of diminished IL-4 production by T cells. High testosterone also causes a reduction in numbers of both macrophages and natural killer cells (59). The growth of macrophages is inhibited (63) and their apoptotic death is stimulated through the FAS/FASL pathway (64). Testosterone is also naturally found in the placental environment and appears to be utilized by the placenta to generate estrogen (65). Its sources include the male fetus, the fetal adrenal glands, and the trophoblast cells themselves. In placental explant studies testosterone had no effect on trophoblast survival at physiological levels and only caused cytotoxicity in super-physiological levels (66). The levels of testosterone needed to be cytotoxic to the trophoblast were not physiologically possible in either female or male mice. This however was not the case with macrophages, in that they were killed *in vitro* by physiologic normal male testosterone levels (63). Endogenous testosterone may therefore act through the immune system rather than directly on the trophoblast cells.

Liver Immunology- Oral Tolerance

The liver is an organ with tolerogeneic properties that has been compared to the placenta (67). Due to the livers downstream location in relation to the small and large intestine, it is constantly exposed to high levels of endotoxin from commensal bacteria as well as antigens from the food we eat. Most of the antigenic compounds that enter the liver pose no threat to the organism. Evidence of the liver's special tolerogeneic capacity can be seen in the long-term survival of liver allografts and subsequent systemic donor-specific tolerance to other organ transplants (68). Possible mechanisms involved in the generation of liver tolerance include the presence of high levels of IDO generated by antigen presenting cells (69), regulatory dendritic cells that produce IL-10, other less professional antigen presenting cells that cause anergy and apoptosis in T cells, and the presence of regulatory T cells (67). The comparison of the liver with the placenta is an appropriate one, since they share immunosuppressive properties.

Stem Cell Transplantation

Fetal Cell Transfer

In 1893 Georg Schmorl wrote the first paper documenting fetal-maternal cell trafficking in women who died of preeclampsia (70). In this paper he described the presence of "trophoblast sprouts" in the lungs as well as necrotic sites within the liver. At the time, this trafficking of fetal cells within the mother was considered to be the cause of disease; the connection may not be that strait forward.

In 2005 Bianchi et al described pregnancy-associated progenitor cells (PAPCs) (71) which are fetal stem cells found in maternal tissue usually through the detection of male antigen on their surface. These fetal cells circulate during all pregnancies and increase in frequency with gestational age (72). By the second trimester 1-6 fetal cells can be found per ml of maternal blood. After delivery the fraction of fetal cells within the circulation decreases, but these cells are suspected to persist in a maternal stem cell niche (72). Using PCR, fetal cells were found in the blood of 30-50% of normal mothers from a month to decades after the pregnancy (72, 73). Other studies have found microchimerism in 90% of women (74). The PAPCs express CD34 a marker of hematopoietic stem cells and some also express CD38 a marker of activated lymphocytes (74).

Time and the circumstances of parturition influence the transfer of fetal cells into the mother. Thus having a miscarriage or abortion increases, by 2.4 times, the chance that fetal cells will be found within the mother (75). Time after parturition also seems to play a role in the detection of fetal cells, as such cells were undetectable in several of the mothers of young sons (76).

While the persistence of fetal cells within the mother is apparently normal, studies suggest that there may be some association between high levels of microchimerism and certain autoimmune diseases such as systemic sclerosis. This is a disease in which there is excessive deposition of cartilage in the body and polymorphic eruption of pregnancy, in which a chronic hives-like rash appears on the abdomen of some pregnant women (73). Fetal cells may also have a beneficial effect on the mother.

In 2002 Johnson et al reported on 29 mothers with thyroid disease that had experienced some tissue repair with male cells (77). This group also documented a woman with hepatitis whose liver had evidence of repair by male cells from a fetus aborted 17 to 19 years earlier (77).

In contrast to the documented long-lasting microchimerism in humans, in 1995 Tafuri et al (Figure 4) showed that allogeneic tumors of male mice were only accepted in female mice who were pregnant by males of the same background as the tumor and their acceptance only lasted as long as the pregnancy (78). In this experiment, female mice with an H-2k background, were grafted with H-2b tumors then promptly rejected them. If the female, H-2k, mouse was mated with a male, H-2b, she would then accept the H-2b tumor, but this acceptance lasted only as long as the female was pregnant. In this experiment the tumor acceptance was paternal antigen specific; if the female was impregnated by a male that was not H-2b the H-2b tumor was rejected. This suggests that T cells may be specifically tolerized to paternal antigens during pregnancy.

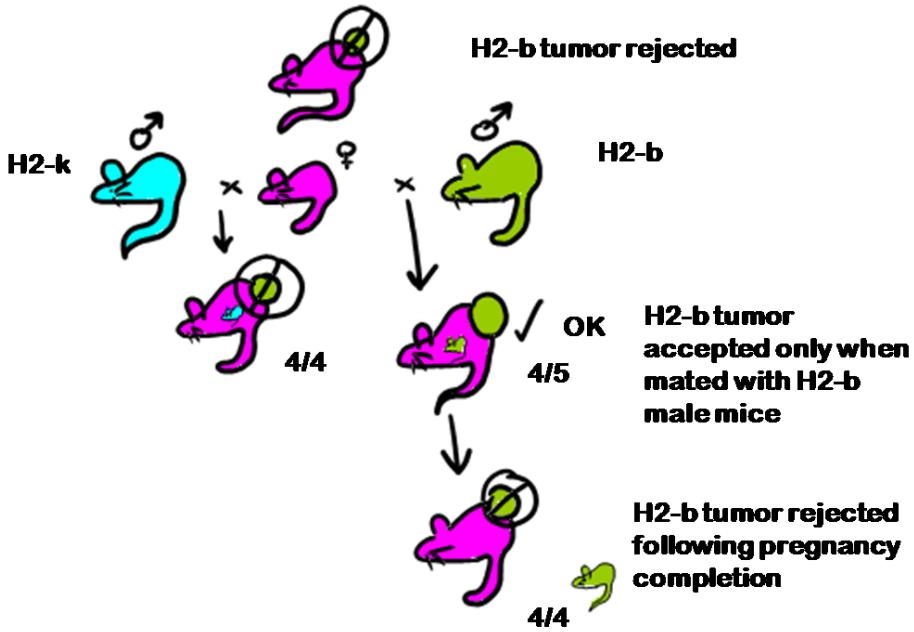

Figure 4. Diagram of experiment by Tafuri et al 1995 (78).

ES Cell Transfer

In addition to natural fetal microchimerism and pregnancy specific acceptance of tumor transplantation, a few studies have examined the fate of transplanted allogeneic ES cells (Table 1). In the paper that serves as a basis for this project, Fandrich et al reported the injection of ES-like rat cells into the portal vein of allogeneic rats (10). They found blood chimerism in some of these animals and those with chimerism went on to accept a heart allograft that was specific to the strain of ES cells injected. As noted earlier, it is possible that these injected cells were TS-like rather that ES-like. In 2005 Fabricius and Zavazava investigated the consequences of injecting 2 million ES cells into the supra orbital vein of mice (79). They reported greater than 5% white blood cell chimerism in wild-type mice with 80% of the mice retaining chimerism at 14 days and 75% retaining chimerism at 28 days. This experiment was repeated in FAS-deficient mice with 50% chimerism at 14 days and 0% chimerism at 28 days. These results led the researchers to question if FAS/FASL engagement was required for ES cell survival in allogeneic hosts. The results of this experiment were inconsistent and the number of animals used in each group was unclear. When a portion of this experiment was repeated on wild type mice as a control by the same lab in 2006, the results were the same as the earlier experimental FAS-deficient group. When wild type animals were injected with ES cells, they demonstrated chimerism in 30-40% of the mice at day 7, but no chimerism at one month (80). Thus the repeatability of the studies was questionable.

In 2005 Fair et al reported on the injection of 1 million mouse ES cells labeled with GFP (ESGFP) into the parenchyma of the mouse liver (81). Twenty days later they detected no GFP-labeled cells. However, if they differentiated the ES cells prior to injection, they found that approximately half (10/19) of the animals retained the ES cells in the liver. The mechanism of this differentiated ES cell survival remains unclear.

In 2006 Baertschiger et al reported that mouse ESGFP cells survived when injected into the liver of rats (4 of 12). This survival was detected through FACs sorting one month later and was followed in three months by a mouse skin allograft from the same donor haplotype that was promptly rejected (82).

Table 1. Allogeneic transplantation experiments with ES cells. In parentheses is the lab where the research was conducted to help identify multiple works by the same labs or individuals.

ALLOGENEIC ES CELL TRANSFER EXPERIMENTS				
Year & Group	**Cells Injected**	**Injection Location**	**Additional Sx**	**Results**
2002 Fandrich et al (10) (Binas & Zavazava)	1 million RESC	Portal Vein of rats	-	7d- Blood and liver chimerism
	1 million RESC	Portal Vein of rats	7d-(Abd) heart transplant	Heart transplants survived +100d when of the same background as the injected stem cells
2005 Fabricius et al (Zavazava) (79)	1 million ES cells	Orbital Vein	-	< 3% blood chimerism
	2 million ES cells	Orbital Vein	-	> 5% blood chimerism
	2 million ES cells	Orbital Vein w/t mouse	-	7d - 80% of the animals had blood chimerism 28d – 75% of the animals had blood chimerism
		Orbital Vein Fas deficient mouse	-	7d – 50% of the animals had blood chimerism 28d – 0% of animals had blood chimerism
2005 Fair et al (81) (Smithies)	1 million ES-GFP	Liver Paranchyma	-	20d- 0 chimerism in cryosectioned liver
	1 million differentiated ES-GFP	Liver Paranchyma	-	20d- 10/19 animals had chimerism in cryosectioned liver
2005 Swijnenburg et al (83)	1 million ES	Heart muscle	-	28d – strong immune invasion 28-56d complete rejection
	1 million invivo differentiated ES	Heart muscle	14d- heart transplant to syngeneic animal	28d – faster and stronger immune rejection
2006 Bonde and Zavazava (80)	2 million ES-YFP	Orbital Vein	-	14d – 35% of the animals had blood chimerism (2-5%)
	2 million ES-YFP	Orbital Vein irradiated mice	-	14d – 75% of the animals had blood chimerism 14d – liver cryosections contained labeled cells 28d- 0% of animals had blood chimerism
2006 Baertschiger et al (82)	1 million mouse ES-GFP	Portal Vein Rat	3mon- skin transplant	28d- 4/12 of animals had 4-16% blood chimerism 3mon-skin rejection
	1 million mouse ES-GFP	Portal Vein Immune-suppressed Rat	3mon- skin transplant	28d- 5/12 of animals had 6-36% blood chimerism 3mon-skin rejection
2006 Magliocca et al (84)	1 million ES-YFP	Portal Vein	7d- heart transplant same as cells	14d – transplant rejection; 0% blood chimerism; liver tumors
2007 Nussbaum et al (85)	½ million ES-GFP	Heart muscle	-	21d - cardiac teratomas but not directed toward cardiomeyocyte fate 35d -rejected all injected cells
2008 Koch et al (86)	1 million ES	Subcutaneously	-	0/10 tumors
	5 million ES	Subcutaneously	-	3/10 tumors
	20 million ES	Subcutaneously	-	9/10 Cause tumors

In 2006 Magliocca et al injected yellow-fluorescent mouse ES cells (ESYFP) into the portal vein of mice followed by a heart allograft a week later (84). The heart allografts were rejected in all animals, excluding the positive controls. When the livers were cryosectioned at two weeks, tumors were detected within the liver.

In 2005 Kofidis (83) and in 2007 Nussbaum (85) investigated the effects of injecting ES cells into the heart of allogeneic animals. Both groups reported tumor formation within the heart tissue and the Kofidis group demonstrated the presence of a strong lymphocyte infiltration. In 2008 Koch et al demonstrated injection of $2x10^6$ ES cells subcutaneously resulted in the formation of tumors. Koch et al also showed that the tumors were able to avoid rejection through the production of TGFβ (86). Overall, studies on ES cell allogeneic injection have resulted in either complete rejection of the cells or in tumor formation. The research by Fair et al may offer the most insight into ES cell tumor formation by having linked it with ES cell differentiation (81).

TS Cell Transfer

Early studies in trophoblast transplantation began prior to the creation and publication of trophoblast stem cells by Tanaka et al in 1998 (1) and are summarized in Table 2. Verstuyf (1989) utilized choriocarcinoma cells, a placenta tumor cell line, and demonstrated that tumor pieces inserted subcutaneously into mice formed masses after 10-14 days (87). The same pieces when inserted under the kidney capsule formed masses in only 5 days. These results were difficult to interpret because they utilized both male and female animals, made no mention of how long the masses persisted, if they caused the recipients death, or if they were rejected.

The usefulness of the placenta in transplantation gained new significance with the work of Tafuri in 1995 (Figure 4). Here allogeneic tumors of male origin were only accepted in females who were pregnant by males of the same background. The tumor acceptance in this experiment only lasted as long as the pregnancy and rejection followed parturition (78).

In 2002 Suzuki et al co-injected placental pieces with islets under the kidney capsule of allogeneic diabetic male mice (6). This coinjection allowed insulin to reach normal levels by day 3 although all transplanted tissue was rejected and cleared by day 14. Based on the result of the present study, the outcome of this experiment was likely influenced by the male-sex of the recipient animals.

In 2002 Erlebacher et al transplanted TS cells into an immunocompetent allogeneic environment (7). Here 200,000 TSGFP cells were injected into the tail vein of female mice. These cells were cleared by the lungs in 24 hours through the actions of NK cells. When the TSGFP cells were injected subcutaneously into female mice they differentiated into giant cells and were undetectable by 10 days. Thus these investigators demonstrated no successful engraftment of TS cells in either the lungs or skin of allogeneic female mice.

In 2006 Takahashi et al injected 10-15 TSGFP cells into SOCS3 knockout blastocysts in order to rescue the embryos (88). SOCS3 knockout embryos failed to survive due to placental defects that triggered excess trophoblast giant cell formation. When wild type TS cells were injected and the blastocysts transferred to recipients, 5 pups were born. This study demonstrated the first use of TS cells for rescuing a placental defect.

In early 2008 horse chorionic girdle explants were transferred to the vulval surface of virgin mares to detect migration and survival (89). This was a limited study that reported successful allogeneic engraftment, but failed to follow the grafts beyond one month. While the placental cell type (chorionic girdle) utilized in this study is specific to the horse, it does suggest the broad implication that the allogeneic trophoblast survival examined in this research is applicable to many species.

The results of the studies on rats by Fandrich et al may be included in the discussion of allogeneicly injected cell types due to the speculation, noted above, that cells they injected were possibly TS-like in origin. Overall, none of the reports of injected allogeneic TS cells showed that they were detected at times longer than one month after engraftment.

Table 2. Transplantation experiments involving placental cell types.

ALLOGENEIC TS CELL TRANSFER EXPERIMENTS				
Year & Group	**Cells Injected**	**Injection Location**	**Additional Sx**	**Results**
1989 Verstuyf (87)	Choriocarcinoma	Subcutaneous	-	14d- tumors
		Subcapsular	-	5d - tumors
2002 Suzuki (6)	Islets and placental tissue	Kidney capsule of male mice	-	3d- normal 14d- reabsorbed
2002 Erlebacher (7)	200,000 TS	Tail vein	-	24h – cleared from lungs
		Subcutaneous	-	No tumors
2002 Fandrich et al (10) (Binas & Zavazava)	1 million RESC	Portal Vein of rats ?sex	-	7d- Blood and liver chimerism
			7d-(Abd) heart transplant	Heart transplants survived +100d when of the same background as the injected stem cells
2006 Takahashi et al (88)	Mouse TSGFP	SOCS3-/- blastocyst	Uterine transfer	5pups/50 blast TS-injected survived 0pups/50 blast -/- neg control survived 8 pup/ 50blast Tetraploid embryo pos control
2008 de Mestre (89)	Horse chorionic girdle placental explants	vulvar mucosa of virgin mares		7d – 4/5 14 d– 4/4 21d – 2/2 28d – 0/1

MATERIALS AND METHODS

Experimental Design

The objective of this study was to replicate the RESC line results of Fandrich et al (10) with cells from better characterized mouse stem cell lines. The initial part of the study involved deriving mouse stem cell lines from an inbred genetic background, characterizing them in regard to the traditional stem cell markers, and comparing the results to the markers of the RESC line. Then the mouse cell lines (ES cells, TS cells and XEN cells) were each injected into a minimum of 5 allogeneic mice. The transplants were monitored for survival with the expectation that the placental stem cells would survive in the allogeneic environment. Finally, when one mouse stem cell line stood out with the ability survive in an allogeneic environment, then that cell line was co-injected with a different cell line that failed to survive. The hypothesis being that trophoblast cells are not only able to survive in an allogeneic environment but are also able to extend that protection to other cell types as seen in pregnancy semi-allografts.

Mouse Strain

All animals utilized in this study were purchased from Jackson Laboratories and housed in a controlled environment with a 12 hour day light schedule. The mice were fed a standard rodent 4% fat diet ad libitum with the additional calories of peanut butter added when the animals were being used for breeding or recovering from surgery. The TS cell line was generated from a C57/Bl6 X Balb/c F1 hybrid embryo with a haplotype of H-2b&d. The stem cell transplant recipient animals were C57/Bl6 (H-2d), Balb/c (H-2b), C57/Bl6 X Balb/c F1 (H2-b&d), and CBA (H-2k) mice. All procedures were approved by the Animal Care and Use Committee at Texas A&M University.

Derivation, Culture, and Treatment of Feeder Fibroblast Cells

In order to successfully culture any early stem cell line, a primary culture of mouse embryo fetal fibroblasts must be optimally maintained to support the stem cell

growth and prevent differentiation. It is important to note that the feeder fibroblasts are a primary cell line and only useful for a maximum of four passages. The primary culture has the ability to grow for a significantly longer time, but is no longer conducive for the support of stem cell growth. In this project, feeder cells derived from day 12 embryos generated the best results for both derivation and culture. To generate the feeder fibroblast cell lines, mated female mice were sacrificed 12 days following the appearance of the post coital plug. The uterus was excised and placed in sterile PBS for transport to a sterile working surface under a laminar flow hood. There the uterus was washed three more times and the embryonic vesicles exposed. Each early fetus had its head and liver removed to prevent unwanted cell types from appearing in the culture. The remaining bodies were pooled and disaggregated with the assistance of a tissue sieve and glass rod in the presence of DMEM with the addition of penicillin/streptomycin at 50µg/ml and 10% serum. The disaggregated pieces of tissue were then redistributed into 100mm dishes, allowed to reach confluence before being trypsinized (.25%), concentrated through centrifugation, resuspended then aliquoted in serum containing 10% DMSO, and finial stored in liquid nitrogen. When needed, the feeder cells were thawed and cultured then passaged in a ration of 1 to 3 two times a week. Confluent dishes utilized for stem cell culture were treated with mitomycin C overnight at 1µg/ml then trypsinized and redistributed generally at a ratio of 1 to 3 for ES cell and 1 to 6 for TS cell culture. Mitomycin C halted the replication of the feeder cell layer while permitting their continued metabolic function.

Derivation and Culture of Stem Cells

TS Cells

The TS cells utilized in this study were derived using a modification of the procedure described by Tanaka et al (1). Natural matings were set up between female Balb/c and male GFP labeled C57/Bl6 mice with intention of deriving a GFP labeled TS cell line. Feeder cells were prepared as previously described. The treated feeder cells were then disaggregated and replated in 4 well dishes (500 µl of 1x 10^5 cells/ml per

well) with TS medium the day before blastocyst collection. The morning before embryo recovery, the TS stock medium was replaced (DMEM, 20% ES qualified FBS, BME, P/S/F) with TS + F4Hβ medium (TS stock + 2µg/ml FGF4, 1µg/ml Heparin, 2ng/ml TGFβ) using 500 µl per well. That afternoon the lower oviduct and uterus of the 3.5dpc mice was flushed to collect blastocysts. Using sterile conditions, each blastocyst was placed in its own well and cultured at 37^{o}C with an atmosphere of 5% CO_2. The first day of culture was designated day 1. The blastocysts that were viable hatched within 24-48 hours on day 2 or 3. Twelve hours after hatching the blastocysts began to attach to the wells. By day 4, a small outgrowth would form from each embryo and the culture was fed an additional 500µl of 70% feeder conditioned medium supplemented with F4Hβ (70% FCM+F4Hβ). The next day (day 5) the outgrowth was disaggregated; to accomplish the first disaggregation the adherent embryo was gently washed with calcium and magnesium free PBS then treated with .1% Trypsin with EDTA (100µl) for about 5 min. After the first 2min., the embryo was observed through a stereo microscope located under the hood to maintain sterility. At 5min., 500µl of 70% FCM+F4Hβ was added back to the well and its contents pipetted repeatedly until the blastocyst were disaggregated into 3-4 pieces and the culture returned to the incubator. Care was taken not to completely disaggregate the TS cell aggregates, as this would lead to a differentiated culture. The timing for the first disaggregation was critical because if too much time passed and the outgrowth was too large the resulting TS cell line would be contaminated with endoderm. The morning following the first disaggregation, the medium was changed with 70% FCM+F4Hβ (day 6). The medium continued to be changed every two days until the next disaggregation step. The TS cell colonies began to appear in 5-6 days and were allowed to grow until they covered 50% of the culture well. Once the colonies reached the desired density, they were passaged again and moved to a new 4 well dish containing feeder cells of the appropriate density. If the TS culture continued to grow, on the following passage they were transferred into 2 wells of a 4-well dish before being transferred into a 35mm dish. Since TS cells grow best at high densities, care was taken not to passage the cells up to a large dish too fast or the

culture would be lost. When the culture was successfully growing in a 35 mm dish, that passage was counted as number 1 and a cell line was officially created. Once the line was established, it required passaging at a ratio of 1 to 3 twice a week and feeding every other day with TS F4Hβ medium (Figure 5).

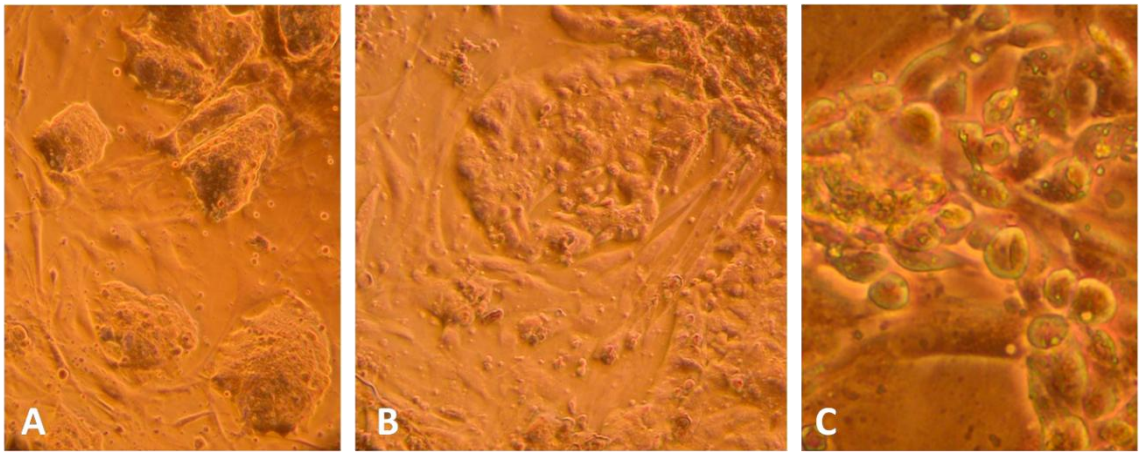

Figure 5. Cultured stem cells. A. ES cells (200x), B. TS cells (100x), C. XEN cells (400x). ES cells exhibit the classic morphology of small cells grown in a three dimensional cluster where the individual cell borders are indistinguishable. TS cell growth is typically two dimensional with a cobble stone appearance. XEN cells, when subconfluent, are larger and round with a distinct cell membrane.

ES Cells

The ES cell C57/Bl6 line was purchased from ATCC (SCRC-1002). These cells were reported to test positive for SRY, male specific antigen, and were cultured in the standard ES cell fashion, passaging at a ratio of 1 to 5 or 1 to 10 twice a week using ES cell culture medium (DMEM, 15% ES qualified FBS, P/S/F, BME, nonessential amino acids, 1,000 µ/ml LIF) (Figure 5). Later in the project a second ES cell line (CBA) was required and kindly given to the project by Dr. J. McWhir (Roslin Institute Edinburgh). Both ES cell lines were cultured under the same culture conditions.

XEN Cells

Extraembryonic Endoderm Stem cells (XEN) are the precursor to both the parietal and visceral endoderm (yolk sac). To derive XEN cells, feeder cells were prepared to the same density as the ES cell culture. One blastocyst was placed per well in a 4 well dish and cultured with DMEM supplemented with 20% ES qualified FBS, BME, and P/S/F. The medium was changed every few days and passage when the XEN cells covered 75% of the well (Figure 5).

Rat Embryonic Stem Cell–like Cells "RESC"

RESC line can grow on gelatin coated plates (Figure 6), mouse 12d feeder cells, or on immortalized rat feeder fibroblasts. RESC have the same medium requirements as ES cells and were cultured in the standard ES cell fashion, passaging 1 to 5 or 1 to 10 twice a week using ES cell culture medium (DMEM, 15% ES qualified FBS, P/S/F, BME, nonessential amino acids, 1,000 μ/ml LIF). The cells utilized for RNA comparisons were cultured on rat feeder fibroblast cells and provided by Dr. Binas (10).

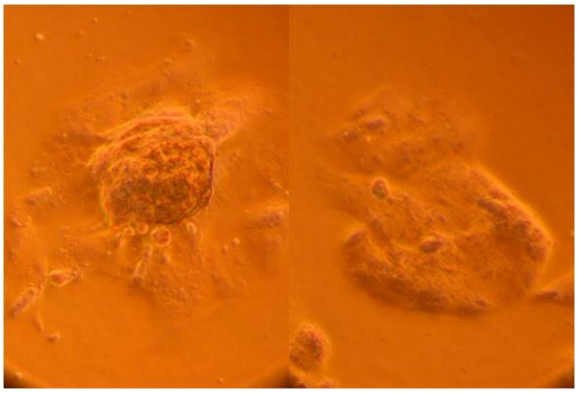

Figure 6. Picture of rat "RESC" line grown on gelatin coated plates. Note that the cell line displayed both the characteristic morphology of mouse ES cells (left) and TS cells (right).

Real Time PCR

In order to verify the identity of the newly derived TS cells, cultured cells were tested by RT PCR using the primers: Oct4 (gagggatggcatactgtggac, ggtgtaccccaaggtgatcc; 272); Nanog (tatcccagcatccattgcag, gtcctccccgaagttatggag; 252); Gata6 (gccgggagcaccagtaca, gtgacagttggcacaggacag; 419); Eomesodermin (cggcaaagcggacaataac, gttgtcccggaagcctttg; 361); Placental lactogen (ctgcttccatccatactccaga, gacaactcggcacctcaaga; 410); Errbeta (ctccagcatctccaggaagag, cacttggggaccagatgagc; 464); Hprt (cagtcccagcgtcgtgattag, atccagcaggtcagcaaagaac; 229); Cdx2 (ctctcggagagcccaagtgtg, gcagtccctaggaagccaagtga;162); and FAS ligand (aaccccagtacaccctctgaaa, ggttccatatgtgtcttcccattc, 108). Oct4 and Nanog are classic ES cell markers. Cdx2, Eomesodermin, Placental lactogen, Errbeta, and FAS ligand are all found in TS cells with Cdx2 and Errbeta diminishing in the cultures of differentiated TS cells. Gata6 is a marker for XEN and Hprt is a housekeeping gene used as an internal positive control. These studies were kindly performed by Dr. B. Debeb of Texas A&M.

Labeling of Cells

One newly derived hybrid TS cell line was established (see results); when this was examined under UV light, it had a 50% chance of generating fluorescence but did not fluoresce. As a result a decision was made to utilize pFUGW vector (90) from Dr. Baltimore (Caltech) to create fluorescently labeled TS cell and ES cell lines. Initially the lentiviral vector was engineered with DSRed2 but that proved ineffective as a marker with stem cells. Later, Dr. B. Debeb of Texas A&M was kind enough to retransform the cell line using a GFP labeled lentiviral vector (pFUGW). Both ES and TS cells were seeded at a moderate density in 60mm dishes and incubated overnight. Then 2 hours before transduction, the medium was changed and the cells transduced for 24 hours at a multiplicity of infection of ~1 in the presence of 8µg/ml Polybrene (Sigma). The transduced cells were then passaged at low density. After 7-14 days the fluorescent colonies of stem cells were picked, pooled, and further expanded.

Preparation of Cells for Injection

Cultured stem cells were trypsinized with 0.25% Trypsin and pipetted vigorously to ensure a near single-cell suspension. The cell suspension was then centrifuged, counted and aliquoted into tubes of one million cells each. The cells were resuspended in 200-250 µl of 0.1% PBS/BSA to prevent the stem cells from adhering to one another and stored on ice until injected (within the next 5 hours). When the TS and ES cells were co-injected, both cell types were trypsinized and counted independently then ½ million of each cell type was combined in each tube for injection.

Surgical Procedures

Portal Vein Injection

Mice were anesthetized with isoflurane to effect and injected with 0.008-0.029 mg/kg of buprenorphine to reduce discomfort. The animals were prepared for surgery, then a ventral midline skin and abdominal muscle wall incision (1cm) was made into the abdominal cavity. Sterile gauze was used as a draping material and was placed around the incision site. The cecum was exteriorized and enfolded in wet sterile gauze to prevent drying. The portal vein was visualized running parallel to the rostrocaudal axis of the mouse into the liver. The cell suspension was aspirated into a 1 ml syringe attached to a 25 gauge needle and injected into the lumen of the portal vein. Pressure was then applied to the injection site with wetted cotton-tipped applicators to reduce blood leakage. The viscera were then repositioned and the abdominal wall closed with absorbable sutures. The abdominal skin incision was then closed with 4-7 wound clips. In the cases of multiple surgeries such as ovarectomy or castration, the second surgery was performed at least 2 weeks after the gonadectomy to allow the animal time to recover and clear hormones from the system.

Ovarectomy

Using aseptic technique on fully anesthetized mice (as described above), a small incision was made on the dorsal aspect of the female mice over the ovarian fat pads.

The ovarian fat pad was grasped and exposed from the mouse with the oviduct and uterus attached. A loop of 2.00 absorbable suture was then tied between the oviduct and the ovary to ligate the ovarian artery and vein before the ovary was removed distal to the suture. The uterine horn was replaced in the body cavity and closed with absorbable suture. The skin incision was secured with 2 wound clips. Using this technique, both ovaries were removed from each mouse.

Castration

Using aseptic technique on fully anesthetized mice (as described above), the testes were pushed down into the scrotal sac by gently applying pressure to the abdomen. A 1cm incision was made through the skin along the midline of the scrotal sac. The midline wall appeared as a whitish line between the testes, in the sacs, under the covering membranes. A 5mm incision was made in the membrane on the left side of the midline wall. The testes were exposed using blunt forceps to grasp the testicular fat pad. The vas deferens and prominent blood vessel was ligated using 2.00 absorbable suture prior to removal of the testicle. This procedure was repeated on the right side, then the remaining fat pad was pushed back into the scrotal sac and the skin closed with an absorbable suture.

Blood Collection

Blood was collected when the animal was terminated in order to determine if the injected stem cells were differentiating into lymphocytes. Mouse blood (50µl) was collected in heprinized hematocrit tubes via tail tip bleeding. The tubes were then centrifuged for 5 min. and broken between the red and white blood cell layer to permit the extraction of the buffy coat. The white blood cells were resuspended in HEPES buffered medium containing Hoechst nuclear stain and examined for green fluorescence. If hematopoietic chimerism was present, then some of the white blood cells would have been co-labeled with green fluorescence from the GFP in addition to blue fluorescence from the Hoechst dye (Figure 7).

Histology and Microscopy

In order to view GFP labeled cells within tissue, mice were killed via CO_2 overdose and the liver removed. The liver tissue was first fixed in 4% paraformaldehyde at 4˚ C for 24 hours then incubated an additional 48- 72 hours in 30% sucrose at 4˚ C. Paraformaldehyde preserves the GFP in the cells while minimizing the auto fluorescence of the remaining tissue. Upon removal from sucrose the liver tissue pieces were placed in 4 cryomolds with OTC and frozen at -180˚C for a minimum of 24 hours before cryosectioning. Serial cryosections of 7-10 µm were cut from calculated locations within each liver piece using a Tissue Tek II cryotome (Miles, Inc.). The sections were viewed with an Olympus IX 71 microscope fitted with a wide GFP fluorescence filter (U-N31054). In order to prevent the cryosectioned tissue from separating from the slide, a thin coating of 1% celloidin solution was applied to the slides for 5 minutes then allowed to dry for 30 minutes prior to staining with the standard hematoxylin & eosin (H&E) protocol.

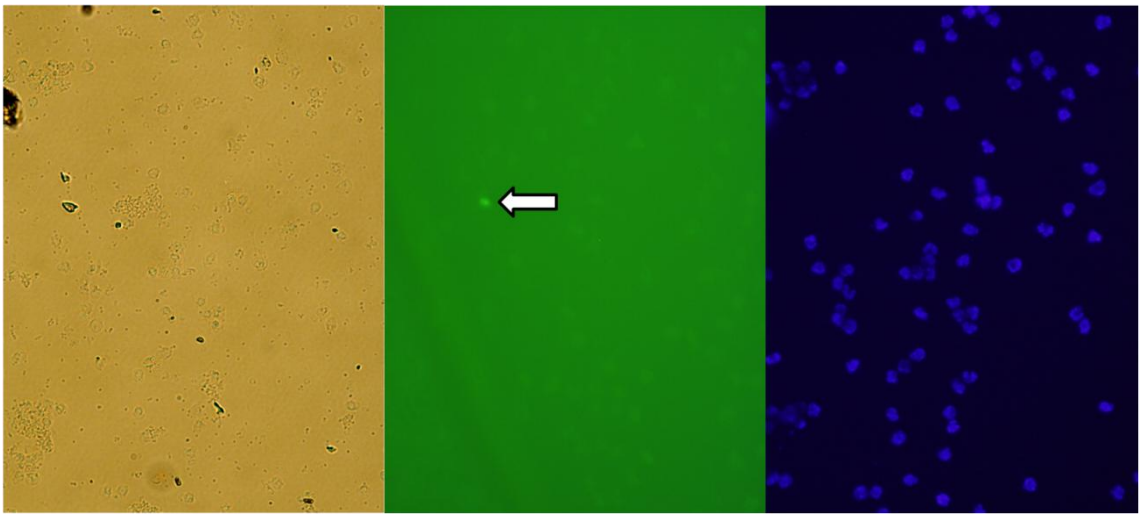

Figure 7. White blood smear panel showing parallel bright field image (left), GFP (center), and Hoechst stain (right). Note that the single positive GFP location does not correspond to any of the nuclei stained by Hoechst and thus is not a white blood cell.

Allograft Survival and Survival Index

The liver of each animal was cut into 8 pieces and placed into 4 cryomolds. Initially one quarter of the total liver was cryosectioned, resulting in about 50 equally spaced sections. If one of the cryosectioned slides contained four or more intact fluorescent cells, the allograft was considered to have survived and the remaining quarters not studied. If, however, the first quarter did not reveal allograft survival, an additional quarter was analyzed. As a semi-quantitative assessment of the survival, a "survival index" was constructed using the percentage of slides containing at least five or more distinct fluorescent cells compared to the total number of slides analyzed per animal and all results from each grouping of animals were pooled.

RESULTS

Derivation and Characterization of a New TS Cell Line

A new F1 cell line was derived from the mating of a C57Bl/6 GFP male mouse with a Balb/c female. Following embryo collection (1d), representative pictures of the initial growth and development up to the first disaggregation (6d) are documented in Figure 8A. Eventually the culture resulted in the TS cell line depicted in Figure 8B

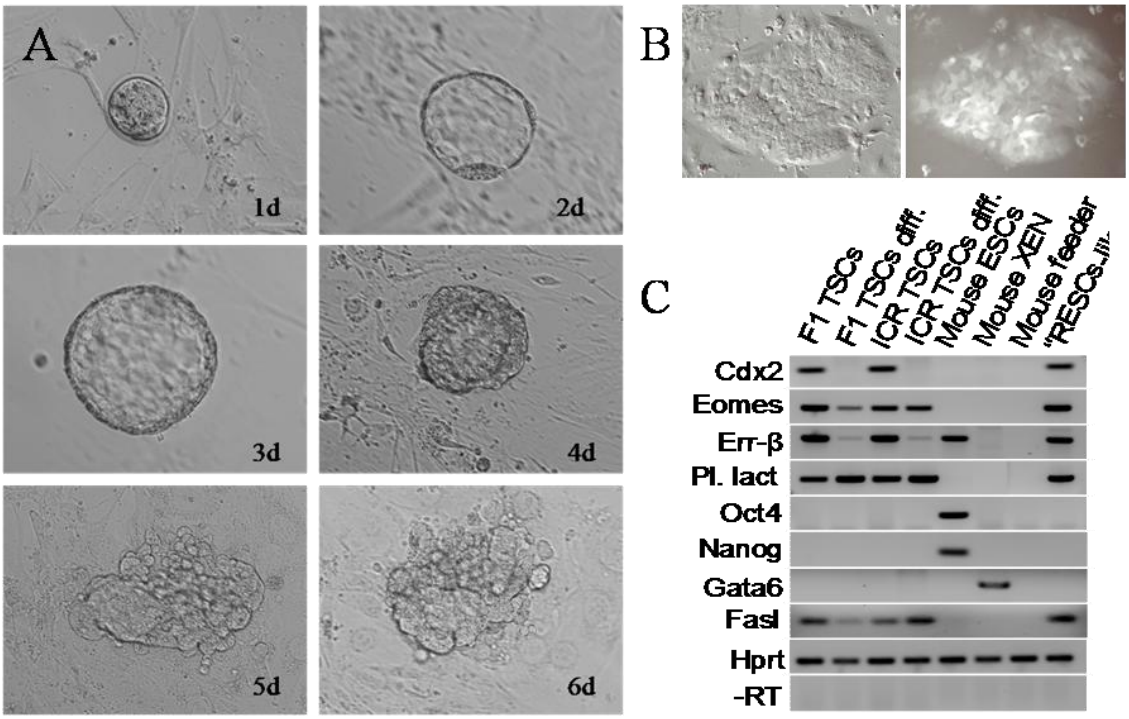

Figure 8. Derivation and characterization of new F1 TS cell line. A. Early pictures of the developing TS cell line from mouse embryo collection (day 1) to the first disaggregation (day 6). B.TS cell line transformed to express GFP. C. RT PCR results characterizing the newly derived trophoblast line (F1 TSCs) by comparing gene expression with, a differentiated culture of the new line (F1 TSCs diff.), the original trophoblast line (ICR TSCs), a differentiated culture of the original trophoblast cell line (ICR TSCs diff.), mouse ES cells (ESCs), mouse XEN cells (XEN), mouse feeder cells, and to the original rat stem cell line (RESCs). Note that the new trophoblast line displays the same characteristic gene expression as the original trophoblast line and the rat "RESC" line.

(left). While the matings to generate the trophoblast cell line involved a GFP heterozygous male mouse, the resulting cell line did not express the GFP protein. GFP is lethal to homozygous embryos so use of a heterozygous male was the only option but provided only a 50% possibility of creating a GFP positive cell line.

Due to the unlabeled nature of the newly derived TS cell line, a stably transfected cell line was needed to track the survival of the injected cells. The cell line was transformed using the FUGW lentiviral vector with GFP insert as can be seen in Figure 8B (right). The newly derived F1 TS cell line displayed the characteristic two dimensional growth patterns of other TS cell lines. RNA from the cell line was further characterized through RT PCR comparisons with the original TS cell line (1) and the RESC line (10) (Figure 8C). Following comparisons utilizing RT PCR, the F1 TS cell line expressed all of the traditional TS cell line markers (Cdx2, Eomesodermin, Err-β, Placental Lactogen, and FAS Ligand) and was negative for the ES (Oct4 and Nanog) and the XEN cell marker (Gata6). The RESC line, upon which this research was based, displayed an expression pattern more characteristic of TS cells then ES cells (Cdx2, Eomesodermin, Err-β, Placental Lactogen, and FAS Ligand), supporting the hypothesis that mouse TS cells are comparable to the RESC line.

TS Cells Survive Portal Vein Injection

Prior studies indicated that TS cells survived at least a month within the mouse liver (Table 2 Group 1) (91). This experiment was repeated using our own TS cell line (Figure 8) and confirmed that TS cells survive at least 1 month when injected into either Balb/c (Figure 9) or CBA female mice (Table 3 Group 2 & 5). In this study a total of 8 Balb/c female mice were injected with allogeneic TS cells; 5 animals survived the surgery and all 5 livers contained GFP labeled cells. The newly derived TS cell line (F1/ C57Bl/6[H2k-b] x Balb/c[H2k-d]) was considered allogeneic because cells from that cross should express both H2k-b and H2k-d haplotypes and thus should be rejected by both the Balb/c (H2k-d) and CBA (H2k-k) strains of mice. A total of 6 CBA mice were injected with allogeneic TS cells; 5 died during the injection procedure resulting in only

1 surviving animal. The results from this single female recipient suggested that MHC similarity was not required for TS cell allograft survival. When the livers were removed at one month, blood was collected for detection of hematopoietic chimerism and none was detected.

As a positive control, the TS cells were injected into animals with the same F1 Balb/c x B6 background. This F1 injection of TS cells would also detect potential tumor problems, similar to what ES cells exhibit when injected into syngeneic environments. A total of 6 F1 Balb/c x B6 female mice and 4 male mice were injected with allogeneic TS cells (Table 3 Group 3 & 4). Three of the 6 female animals survived the surgery and one month later all 3 livers contained GFP labeled cells. In the F1 males, 3 out of 4 survived the injection of TS cells but only 1 liver contained GFP positive cells one month later.

Table 3. Results of trophoblast injections into both allogeneic and syngeneic males and females after 1 month.

Trophoblast Portal Vein Injection Following 1 Month Engraftment				
	Trophoblast Background [Haplotype]	Recipient Animal [Haplotype]	Recipient Sex	Survival
1	TS – ICR [?]	Balb/c [H2k-d]	F	3/3
2	TS – B6xBalb/c [H2k-b&d]	Balb/c [H2k-d]	F	5/5
3	TS – B6xBalb/c [H2k-b&d]	B6xBalb/c [H2k-b&d]	F	3/3
4	TS – B6xBalb/c [H2k-b&d]	B6xBalb/c [H2k-b&d]	M	1/3
5	TS – B6xBalb/c [H2k-b&d]	CBA [H2k-k]	F	1/1

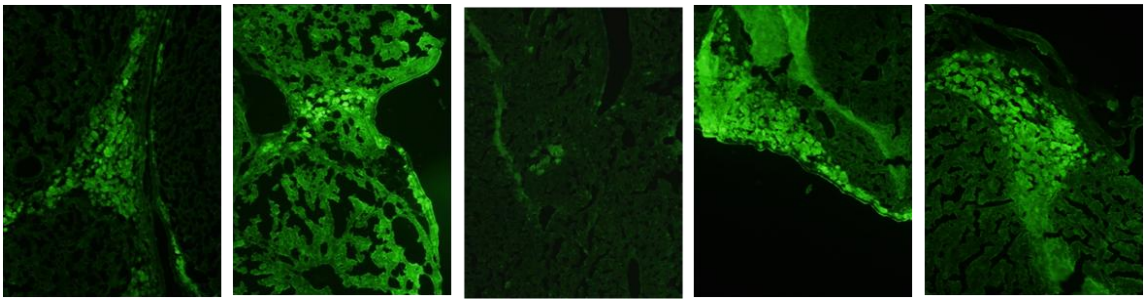

Figure 9. The presence of GFP labeled TS cells within livers one month after intra-portal injection (100x). Note the very high tissue autofluorescence in some samples as well as the brighter regions generated by tissue folds. The presence of TS cells is readily apparent despite the areas of high autofluorescence.

When the cryosectioned liver samples were examined under UV microscopy, they were compared to similarly prepared liver sections from both uninjected animals and GFP-labeled animals purchased from Jackson Laboratory (Figure 10). Liver samples from female recipients of syngeneic and allogeneic injections were completely indistinguishable from one another in regard to survival and location of the GFP-labeled cells. Following the unusual results of the syngeneic male injections (1/3), it was suggested that a sex difference in survival might exist. This was examined in greater

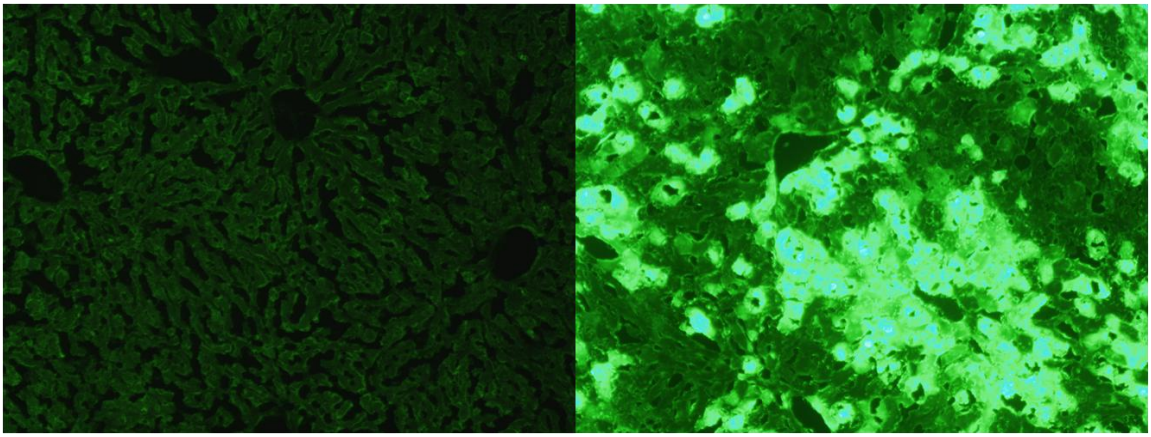

Figure 10. Representative liver slide from negative control animal (left) and positive control GFP labeled animal (right). 100x magnification.

detail later in the study. Syngeneicly injected TS cells failed to form the tumors (0/3) observed by others in the subcutaneous environment (7). This survival, but lack of growth and tumor formation, suggests that the TS cells may undergo differentiation following their allograftment.

Duration of TS Cell Survival

The initial experiments, highlighted in Table 3, demonstrated that TS cells could survive for at least one month without any detectable proliferation due to suspected differentiation. In order to determine the duration of this survival and to detect any slow proliferation, female recipient mice were examined at 2, 3, and 4 months after engraftment. The degree of survival was estimated by comparing the ratio of positive slides with the total number examined. The results demonstrated that there was no significant change in GFP-labeled cell numbers until 4 months when they were undetectable in 4/5 animals (Figures 11 & 12). This survival for at least 3 months without any detectable increase in cell numbers supports the concept that the TS cells differentiate when injected into the mouse.

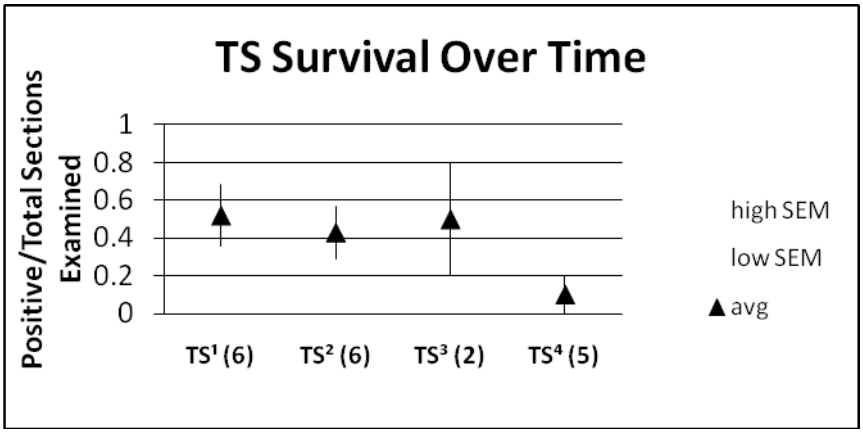

Figure 11. Allogeneic TS cell survival in liver at 1, 2, 3, and 4 months. This graph uses the standard error of the mean to display the ratio of slides with positive cell engraftment locations to the total number examined per animal. The sample number is given in parenthesis and the time of engraftment analysis is in superscript.

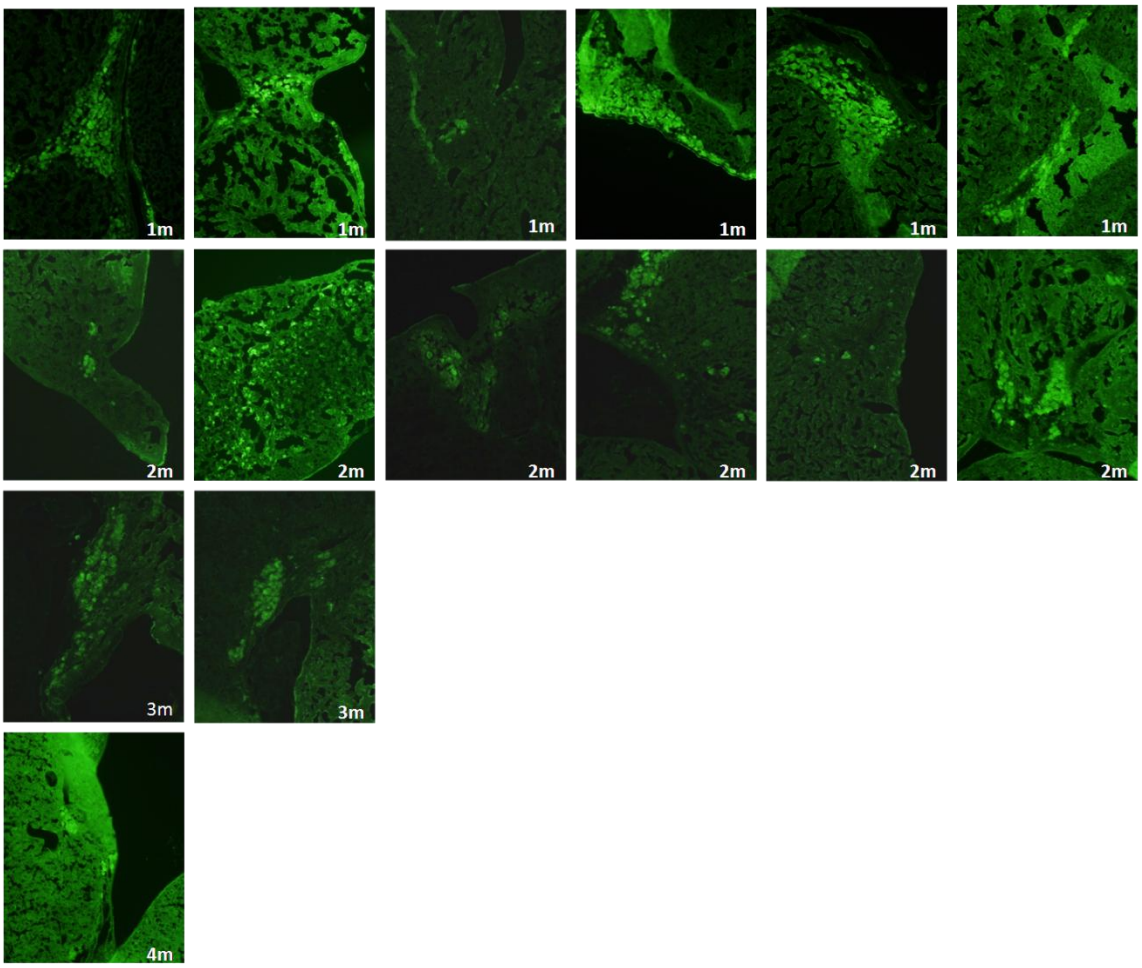

Figure 12. Examples of positive allogeneic engraftment locations from each of the GFP labeled TS cell injections. Top row are injections that were analyzed at 1 month; middle row are injections that were analyzed at 2 months; bottom left are the livers examined at 3 months; bottom right is the single location of cells found in 1/5 animals at 4 months.

ES Cell Allografts Are Rejected Following Portal Vein Injection

When allogeneic GFP-labeled ES cells from three different genetic backgrounds (129, B6, & CBA) were injected into female Balb/c mice, none of thirteen transplants were detectable at week 4 (Table 4 Group 1, 2, & 3). The preliminary experiment using 129 ES cells failed to survive 1 month within the mouse liver (0/2) (Table 4 Group 1) (91). This experiment was repeated with the inbred mouse strains B6 (0/6) and CBA (0/5) with the same negative results. One animal from the B6 group was excluded from

Table 4. ES cell survival in allogeneic and syngeneic animals after 1 month. Note that the control injection into syngeneic animals survived in 3/3 and generated tumors in 2/3.

ES Cell Survival 1 Month Following Portal Vein Injections				
	ES Cell Background [Haplotype]	**Recipient Strain [Haplotype]**	**Recipient Sex**	**Transplants Surviving**
1	ES – ICR	Balb/c [H2k-d]	F	0/2
2	ES – B6 [H2k-b]	Balb/c [H2k-d]	F	0/6
3	ES – CBA [H2k-k]	Balb/c [H2k-d]	F	0/5
4	ES – B6 [H2k-b]	B6 [H2k-b]	F	3/3 - tumors

the study due to visible tumor formations on the liver at the time of collection. This sporadic allogeneic ES cell tumor formation has been reported previously (10, 84, 86). In contrast to the failed ES cells allografts, isograft injections survived (3/3) and formed tumors (2/3) in the B6 background (Table 4 Group 4 & Figure 13). As expected, these tumors exhibited tissues from all three germ layers, attesting to the health and pluripotency of the ES cell lines used in this study. While the entire group of allogeneic

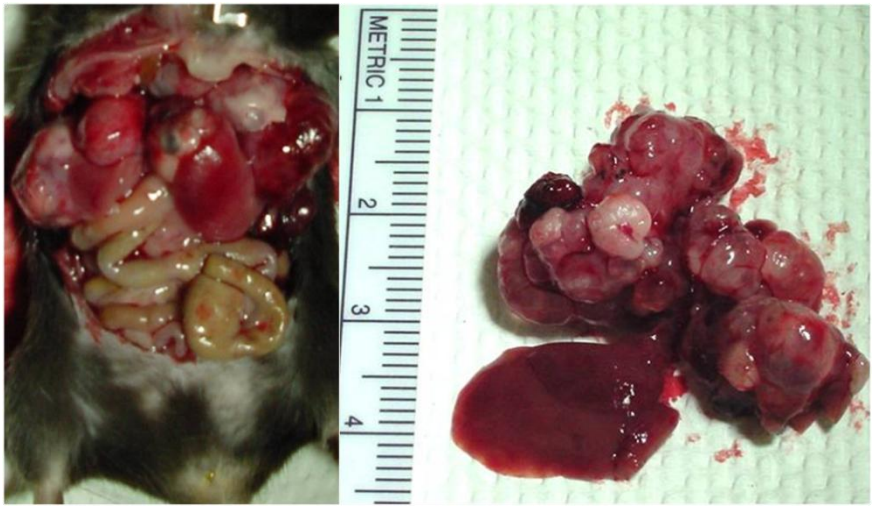

Figure 13. Representative pictures of tumors generated after control injections of ES cell into syngeneic animals.

ES cell injections failed to survive one month, at 2 weeks labeled cells were still present in an animal that was terminated early. The two week survival of ES cells has been demonstrated by other researchers at different injection locations (80, 86). The slow rejection of mouse ES cells has been attributed to their low immunogenicity and their production of TGFβ (86). In summary, GFP-labeled allogeneic ES cells failed to survive longer than two weeks within the livers of female mice.

XEN Injection into the Portal Vein

XEN cell intraportal injections were uniformly lethal (21/21) for unknown reasons. This difference in survival following injection is suspected to be due to the larger size of the XEN cells compared to the much smaller TS and ES cells.

Mouse TS Cells Injected into the Rat Portal Vein

Due to the survival of TS cell injections into mice, a xenograft was attempted with the same TS cell line. A single female rat was injected with 5 million TS cells. One month later GFP positive cells were observed at a single trace location (results not shown). A repeat of this experiment using 10 million cells gave negative results. Thus the results of this study were inconclusive.

TS Cells Rescue Co-injected ES Cells

Having successfully demonstrated that allografted TS cells but not ES cells survive allogeneic portal vein injection, the cells were mixed in order to determine if the TS cells could influence the survival of ES cells. The first series of injections used the newly derived unlabeled TS cells (F1 C57Bl/6xBalb/c) together with a commercially available B6 ES cell line that was transformed to express GFP (Table 5 Group 5). One month following the co-injection the livers displayed large clusters of labeled cells (Figure 14), suggesting that the TS cells had permitted the survival of the ES cells. Since the TS cells were F1 B6xBalb/c and the ES cells were B6, there was the possibility

Table 5. ES cell survival when co-injected with TS cells and examined at both one and two months. Note that the ES and TS cell haplotypes can differ and the ES cells still survive.

Co-Injection Survival Following Portal Vein Injection				
	Cell Type Injected	**Recipient Background**	**Engraftment Time**	**Transplants Surviving**
1	TS	Balb/c	1 month	5/5
2	TS	Balb/c	2 month	6/6
3	Sham	Balb/c	2 month	0/4
4	ES-B6	Balb/c	1 month	0/6
5	TS + ES-B6	Balb/c	1 month	5/5
6	TS + ES-B6	Balb/c	2 months	4/4
7	ES-CBA	Balb/c	1 month	0/5
8	TS + ES-CBA	Balb/c	1 month	4/4

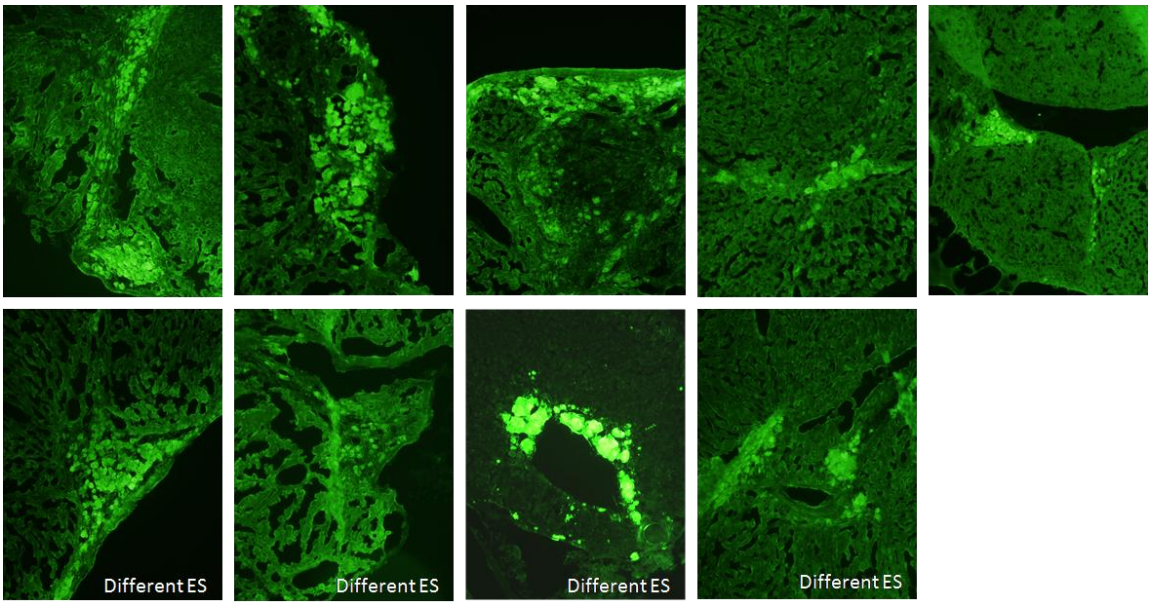

Figure 14. Examples of positive allogeneic engraftment by GFP labeled ES cells that were co-injected with unlabeled TS cells and examined at 1 month. The top row demonstrates C57Bl/6 ES cell engraftment and the bottom row demonstrates CBA ES cell engraftment when co-injected with TS cells into the portal vein of the liver.

that a haplotype overlap was required for successful ES cell engraftment. Subsequent co-injection experiments used CBA ES cells (H2k-k) from a completely different haplotype (Table 5 Group 7). This experiment suggested that the CBA ES cells survived to the same extent as the previously injected B6 ES cells (Figure 15). All of the allogeneic ES cell allografts survived at least one month when co-injected with TS cells. When co-injections using TS cells mixed with B6 ES cells were examined after two months, the percentage of GFP positive areas appeared to have almost tripled, suggesting that the ES cells had begun to proliferate (Table 5 Group 6 & Figure 15.); they did not, however, form tumors, suggesting that differentiation may have occurred.

In similar experiments, other investigators reported hematopoietic chimerism after (non-hepatic) injection of allogeneic mouse ES cells (80). This study also examined if GFP-expressing ES cells were present in the blood of injected or co-injected mice. Blood was collected at the time of liver examination and no hematopoietic chimerism was ever detected.

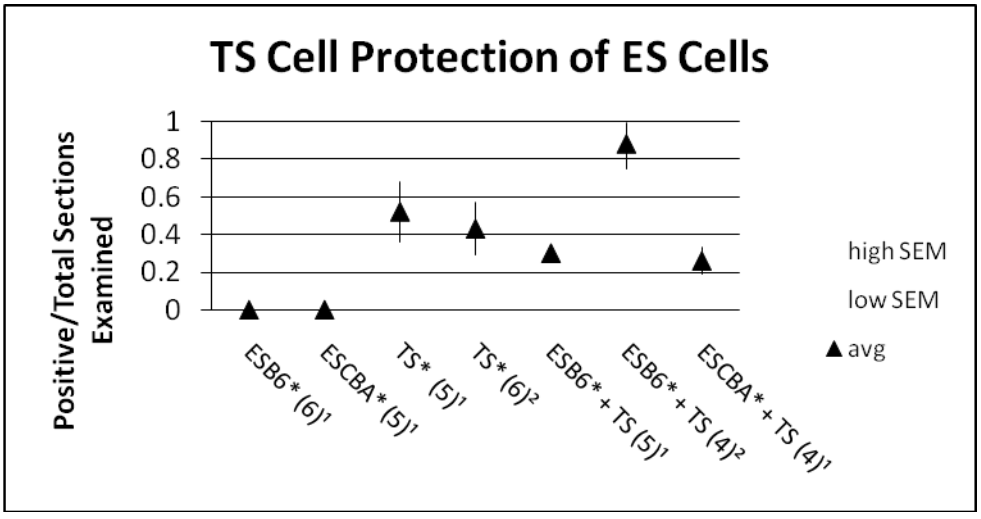

Figure 15. ES cell survival at 1 month and 2 months when co-injected with TS cells. Note that the number of slides with positive ES cell engraftment locations has increased by almost three fold. This graph uses the standard error of the mean to display the ratio of slides with positive cell engraftment locations to the total number examined per animal. The sample number is given in parentheses and the time of engraftment analysis is in superscript.

Table 6. Gender differences in TS cell survival. TS cells survive in intact females without regard to their background, ovarectomized females, and castrated males. TS cells fail to survive when transplanted into allogeneic males containing a single trace location in one animal when injected into syngeneic males.

Sex Effect on Trophoblast Survival 1 Month Following Injection				
Cell Type Injected		**Recipient Background**	**Recipient Sex / Gonadectomy**	**Transplants Surviving**
1	TS	syngeneic	F	3/3
2	TS	allogeneic	F	6/6
3	TS	allogeneic	F / ovarectomized	4/4
4	TS	allogeneic	M / castrated	5/5
5	TS	allogeneic	M	0/4
6	TS	syngeneic	M	1/3

TS Cell Survival Is Sensitive to Male Hormonal Influence

Initially all TS cell injections were performed on female animals. Other investigators have utilized male recipients, albeit unsuccessfully, or utilized both sexes and failed to distinguish the results by sex (6, 7, 87). The suitability of male mice as hosts was questioned in this study when they failed to demonstrate significant syngeneic TS cell survival (0/4) (Table 6 Group 5). It was speculated that the male mouse's inability to support TS cells resulted from either a lack of female hormones or as a result of adverse effects by male hormones. In order to distinguish between these possibilities, studies were conducted using both gonadectomized female (Table 6 Group 3) and male recipients (Table 6 Group 4). These experiments showed that castration completely abolished the gender difference while ovarectomy had no effect on TS cell survival (Figure 16). Together these results suggest it is the absence of testicular hormones instead of the presence of ovarian hormones that allows the survival of ectopic TS cells.

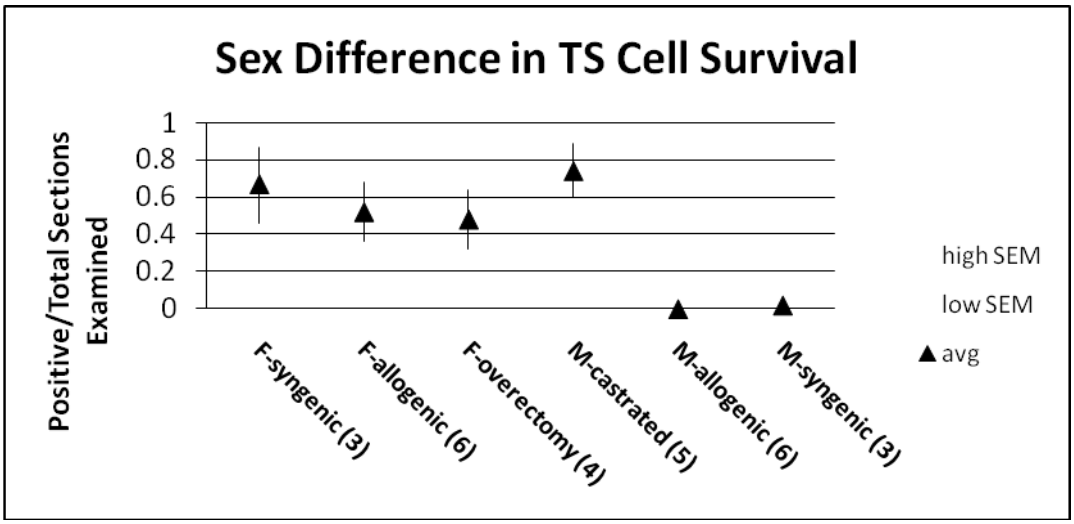

Figure 16. Gender differences in TS cell survival. TS cells survive in intact females without regard to their background, ovarectomized females, and castrated males. TS cells fail to survive when transplanted into allogeneic males and contain a single trace location in one animal when injected into syngeneic males. This graph uses the standard error of the mean to displays the ratio of slides with positive cell engraftment locations to the total number examined per animal. The sample number is given in parentheses.

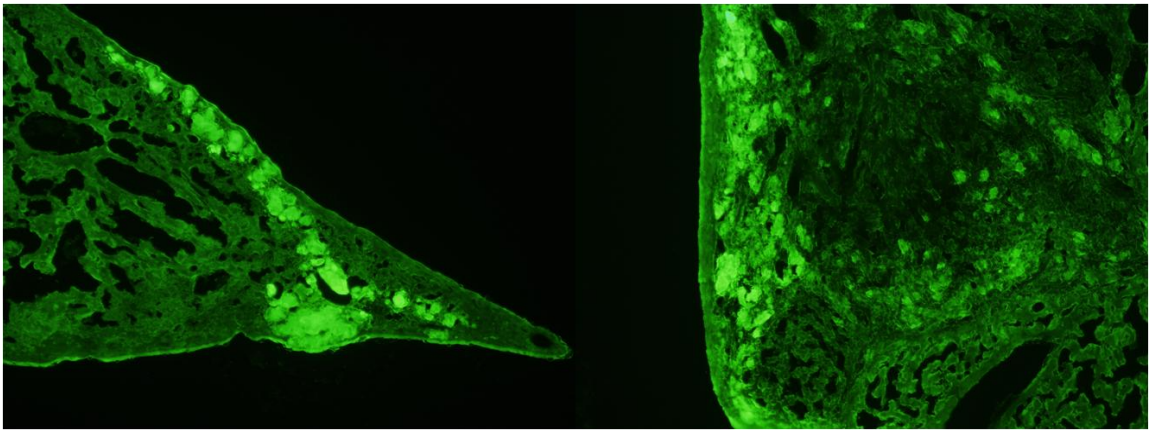

Figure 17. Examples of successful cell incorporation in both subcapsular and intramedullary regions of the liver. Both TS cells and ES cells co-injected with TS cells are found in each region of the liver.

Location of Successful Engraftment and Immune Cell Infiltration

Colony engraftment locations were consistent between all cell types injected and were focused in both the subcapsular and intramedullary regions of the liver (Figure 17). Serial sections from GFP positive locations were stained with H&E and examined. Similarities were evident in both the numbers and type of invading immune cells present within the tissue. These same sections contained peculiar brown cells at the engraftment site. Initially, when TS cells were injected into female mice and examined after a month there were low/moderate levels of lymphocytes and neutrophils present within the tissue

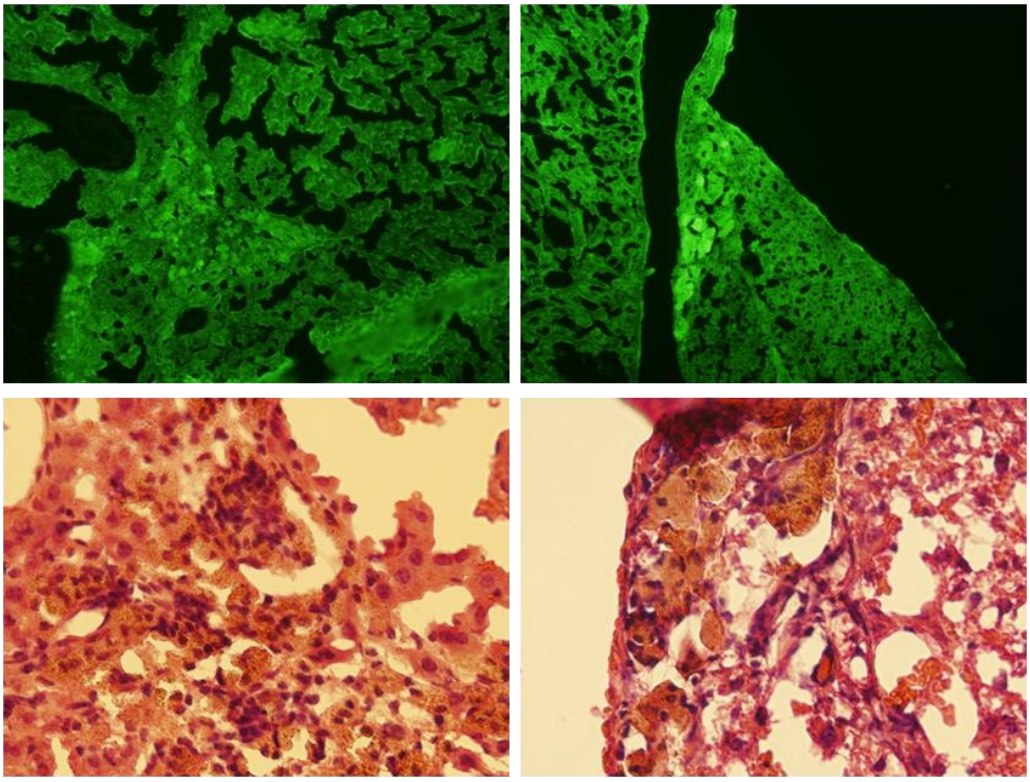

Figure 18. H&E stain of TS cell allograft locations within the liver at 1 (left) and 2 (right) months. Each location is magnified at 100x and 400x. Under 400x light microscopy, a moderate infiltration of neutrophils and round mononuclear cells (most probably lymphocytes) can be seen at 1 month and then at low levels at 2 months.

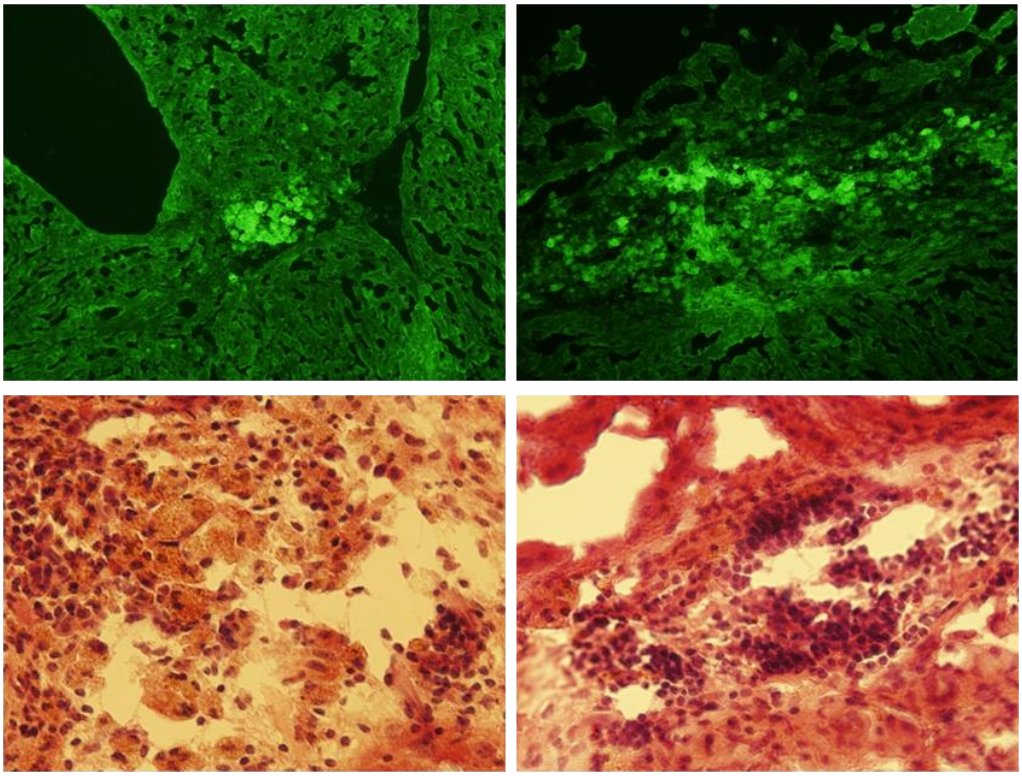

Figure 19. H&E stain of ES allografts at 2 ½ weeks. Each location is magnified at 100x and 400x. Under 400x light microscopy a high level of macrophages and round cells can be seen with plasma cells and neutrophils also in attendance.

(Figure 18). The numbers of invading cells dropped off to very low levels by two months and continued through month four with macrophages making up the majority of the infiltrating cell population at months three and four. The lymphocytes present within the cryosectioned tissue were determined through histological examination of cell morphology. ES cells injected alone were completely rejected by one month, but when one animal was examined at two and a half weeks with labeled cells still evident, large numbers of immune cells were present with macrophages and large lymphocytes predominating (Figure 19). Animals co-injected with both TS and ES cells displayed the same moderate lymphocyte population at the first month with levels dropping by the second (Figure 20). In general all animals with successfully engrafted cells displayed a similar moderate cell infiltration at one month that rapidly diminished by two months.

While high levels of lymphocytes typically indicates rejection that might not be the case in this model. Successful pregnancies are known to have a significant uterine lymphocyte infiltration. All examined trophoblast engraftments contained some lymphocytes, but numbers were fewer than the ES cell location at 2 ½ weeks.

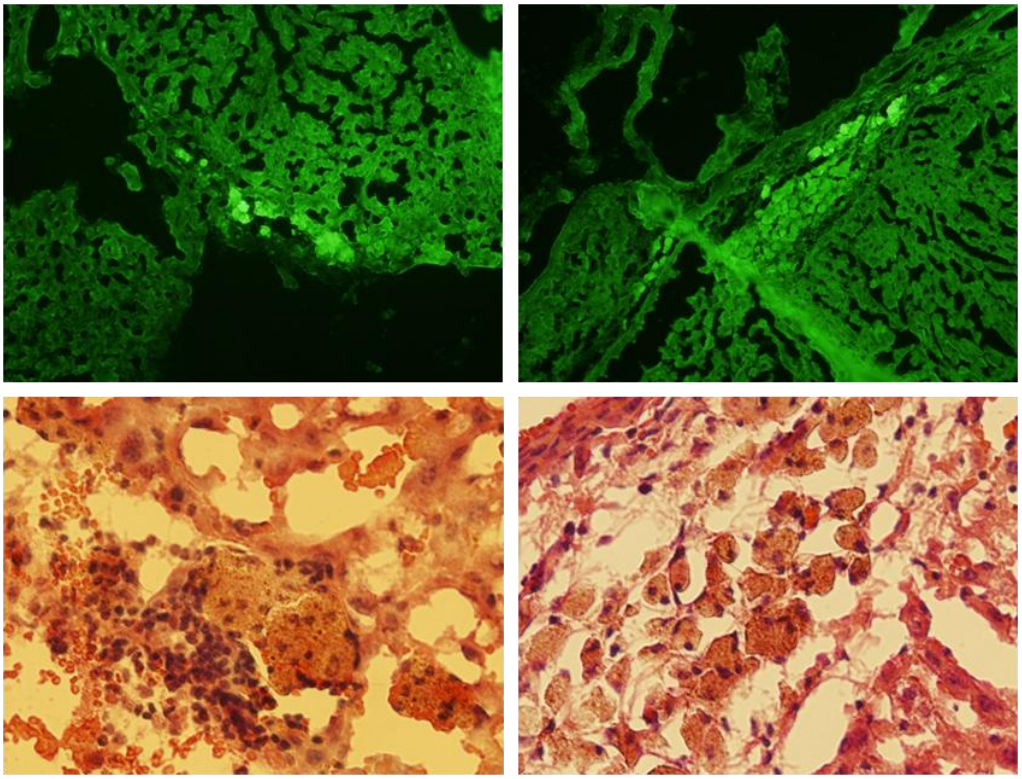

Figure 20. H&E stain of co-injection TS and ES cell allografts at 1 (left) and 2 (right) months. Each location is magnified at 100x and 400x. Under 400x light microscopy, a moderate level of round cells can be seen at 1 month and low levels at 2 months.

SUMMARY

There are three important findings reported in this study. First, allogeneic mouse TS cells appear to survive in the liver without rejection for over 3 months. This is significant because allogeneic TS cells have failed to survive for longer than 3 weeks in any of the other ectopic locations tested so far. It is possible that in this model, the liver plays a uterus-like role both physiologically and immunologically by supplying a nutrient rich environment that also contains a certain degree of immune privilege (92).

While the TS cells appear to survive in the liver for up to 4 months, the gestation length of mice is only 3 weeks. It is unclear whether this prolonged survival is due to a maximum 4 month lifespan of each trophoblast cell or if the TS cells replicate at a low level. The latter could result in a persistent population of TS cells but would mean that the trigger for their rejection would need to originate from outside the system of trophoblast growth. The survival of allogeneic TS cells, regardless of their background, suggests that the mechanisms involved in their survival are fundamental to the trophoblast identity. While the engraftment location, as discussed earlier, seems to be important, and possibly critical, the immune privileged characteristics of trophoblast cells (4, 5, 93) also influence their survival. This was clearly demonstrated when the GFP-labeled TS cells survived where the ES cells could not (81). Note that while the ES cells were reliably rejected in this model, this has not been a universal observation (84). Factors influencing ES cell survival include cell dosage, recipient age/strain/health, time point studied, and the immunogenicity of GFP (86, 94-98). Except when the environment is appropriate for tumor formation, allogeneic ES cells are generally rejected by 3 weeks (81, 83). This rejection is thought to be triggered following the differentiation of the stem cells into cell types with surface antigens that the recipient immune system can access and recognize as foreign. Tumor formation can be consistently triggered by using immunocompromised recipients or by injecting massive numbers of stem cells at a single location (86, 94-98). The general failure of allogeneic ES cells to survive makes using them in cell therapy problematic. The use of syngeneic

ES cells through therapeutic cloning would remove any rejection issue but would add the risk of tumor formation.

The second significant result of this study involves the role of recipient gender in transplanted TS cell survival. TS cell allografts were unable to survive in male recipients. This seemed to suggest that the female hormonal environment was necessary for trophoblast survival. When tested in gonadectomized mice, TS cells survived in both ovarectomized females and castrated males, indicating that survival does not require the presence of ovarian hormones, but the absence of testicular factors presumably male hormones. There are no reports in the literature that testicular factors have a detrimental influence on *in vivo* trophoblast survival. It has, however, been reported that super physiological doses of testosterone can be toxic to freshly isolated human trophoblast cells (66). When placental explants were exposed to normal physiological doses of testosterone they responded with increased estrogen production (65). Other research indicates that male hormones elicit a pro-inflammatory shift (99) in the immune system and that a proinflammatory environment is linked to spontaneous abortion (100). This proinflammatory influence in the immune system could explain the *in vivo* sex specific engraftment failure of TS cell transplants in the male while allowing the survival of their *in vitro* placental explant counterparts.

The third significant result of this study involves the apparent protection of ES cells by the presence of co-injected TS cells. When GFP-labeled ES cells were injected alone into allogeneic female animals they failed to survive in the liver and were undetectable within one month. The single animal examined at 2 ½ weeks still contained surviving ES cells. This finding is supported by the literature in which ES cells, injected into mice, survived for about 3 weeks (81, 83). Viability of the GFP-labeled ES cells was demonstrated when they were injected into control syngeneic female recipients; the ES cells survived in 3/3 mice and tumors were generated in 2 out of 3 animals. Examination of liver sections indicated the presence of all three developmental layers within locations of ES cell growth. The presence of all three layers suggests that the ES cell line was undifferentiated and healthy.

Once the extent of GFP-labeled ES cell allograft survival was established on its own, the ability of unlabeled TS cells to influence this survival could be assessed. In all cases, coinjection of TS cells with GFP-labeled ES cells permitted the survival of the ES cells for at least 1 month. This suggests that the ES cells benefited in some way from the change in local environment generated by their trophoblast cell neighbors (4, 5, 93). Given the tendency of trophoblast cells to fuse with each other in the placental environment (101), it cannot be excluded that the two cell types fused. This fusion might maintain the immune privilege of trophoblast cells and permit hybrid cell survival. However, the high efficiency of ES cell rescue and the increasing numbers of labeled cells in the second month argue against cell fusion being the cause of GFP-labeled cell survival. The increase in the numbers of ES cell positive locations from 1 to 2 months suggests that the ES cells were replicating. It is generally believed that fused trophoblast lose the ability to replicate there by providing a second argument against cell fusion. An important facet of coinjection survival is that TS cells appeared to provide the same protection to the ES cells of different lineages (CBA) as to the ES cells of similar lineage (B6). This nonspecific protection may be related to some of the non-specific mechanisms of local trophoblast immune suppression such as IDO, TGFβ, and IL-10 production.

The validity of the stem cell survival results is complicated by the amount of autofluorescence found within the liver. It is true that the liver contains a background level of autofluorescence that is readily observed, but that background is consistent and doesn't prohibit the identification of individual labeled cells. Pictured in Figure 10 are representative slides from a negative control uninjected animal and from a positive control GFP-labeled animal purchased from Jackson Laboratory. The identification of false positives was ruled out by the requirement of four or more positive cells in a given location to declare a section positive.

Due to the presence of the Kupffer cells within the liver, there is the possibility that the fluorescent cells, seen on the slides, are macrophages that have concentrated the debris of GFP-labeled stem cells. If this were the case, the label would have been

restricted to phagosomes as seen in Figure 21 (left). The difference in appearance of GFP ingested by phagocytic cells and GFP-labeled intact cells is readily apparent (50).

This is not to imply that immune cells are absent from the site of engraftment. Every cluster of labeled cells within the liver that was examined using H&E stain contained at least a few immune cells, but there were differences in the immune cell type and quantity if the stem cells were destined to survive. Both the surviving TS cells and co-injected ES cells with TS cells had the same moderate round cell infiltration at 1 month which dropped to low levels at 2 months. In contrast, the ES cells seen in the one animal examined at 2 ½ weeks were surrounded by numerous macrophages, round cells, neutrophils, and plasma cells. This cell presence surrounding the diminishing ES cells may suggest that the immune system is playing a role in the failure of allogeneic ES cells to survive.

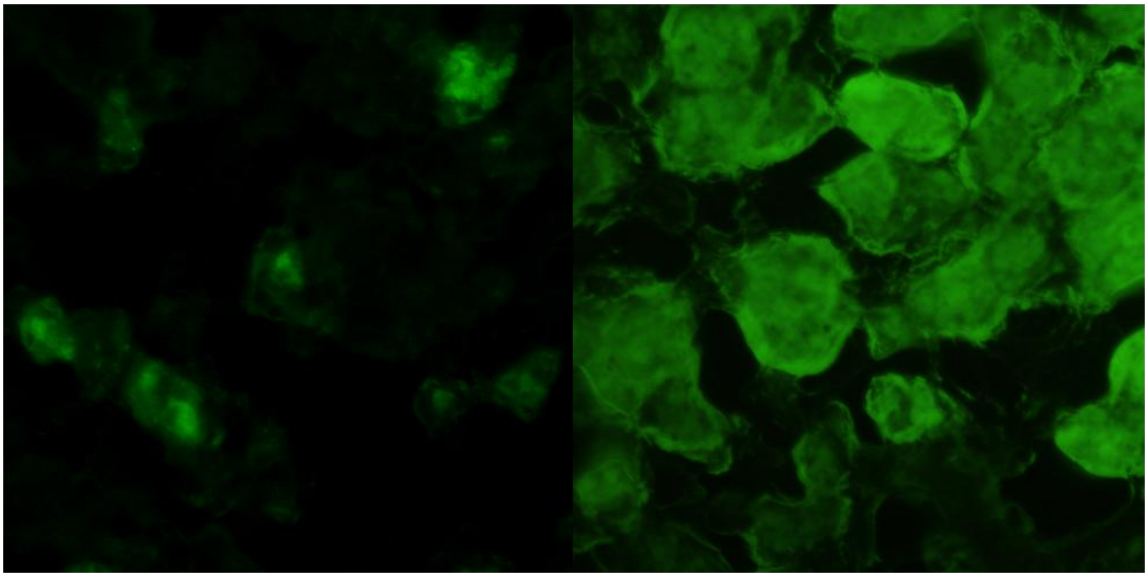

Figure 21. Comparison of phagocytic cells containing ingested GFP labeled cells (left) and large successfully engrafted GFP labeled stem cells (right). Pictures are of the same magnification. (400x)

The rat research, upon which this study was based, caused confusion with the assumption that the cell line used was ES-like in origin (10). The results of the current project confirm the suspicion of Buehr et al (102) that the RESC line was not wholly ES like in origin. Upon closer inspection the RESC line contained a gene expression profile more closely linked to mouse trophoblast cells, but displayed the unusual characteristics when injected making the line not completely TS-like. The RESC line, unlike the typical trophoblast lineage, expressed the ES marker Oct4, was able to generate blood chimerism in allogeneic injected animals (10), and could form tumors in syngeneic animals (91), all characteristics reported of ES cells. The RESC line displayed characteristics of both mouse ES and TS cell lines and appeared to share the immune privilege mechanisms displayed by the mouse trophoblast cells used in this project.

Like most research, this project has generated more questions than it could answer. The results of this study suggest that portal vein-injected TS cells or their coinjection with ES cells may prove a useful tool for cell therapy. Future research should determine the limitations associated with this therapy. Such details include determination if trophoblasts are able to protect cell types other than embryonic stem cells. How long the protection lasts; if it extends beyond the four month life span inherent to the trophoblasts in this study. Do both cell types have to be injected at the same time and at the same location? (It stands to reason that if cells from different backgrounds are injected; the protected area under influence by the trophoblast cells would probably be small, limited only to the organ or area of injection.) If the trophoblast were exerting a systemic effect, that could leave the animal open to microbial invasion. Finally, and most importantly, this model of trophoblast survival and protection should be tested in other species to determine if this important finding is limited to small rodents.

REFERENCES

1. Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, & Rossant J (1998) *Science* **282,** 2072-2075.

2. Xu RH, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, & Thomson JA (2002) *Nat Biotech* **20,** 1261-1264.

3. Margaret GP (2005) *Journal of Reproductive Immunology* **68,** 1-13.

4. Trowsdale J & Betz AG (2006) *Nat Immunol* **7,** 241-246.

5. Hunt JS (2006) *Immunological Reviews* **213,** 36-47.

6. Suzuki K, Ueda H, Yokono K, & Taniguchi H (2002) *Cell Transplant.* **11,** 455-457.

7. Erlebacher A, Lukens AK, & Glimcher LH (2002) *PNAS* **99,** 16940-16945.

8. Kibschull M, Nassiry M, Dunk C, Gellhaus A, Quinn JA, Rossant J, Lye SJ, & Winterhager E (2004) *Developmental Biology* **273,** 63-75.

9. Hansen PJ (1998) *Journal of Reproductive Immunology* **40,** 63-79.

10. Fandrich F, Lin X, Chai GX, Schulze M, Ganten D, Bader M, Holle J, Huang DS, Parwaresch R, Zavazava N, *et al.* (2002) *Nat Med* **8,** 171-178.

11. Johnson MH & McConnell JML (2004) *Seminars in Cell & Developmental Biology* **15,** 583-597.

12. Johnson MH & Ziomek CA (1981) *Cell* **24,** 71-80.

13. Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, & Rossant J (2005) *Development* **132,** 2093-2102.

14. Palmieri SL, Peter W, Hess H, & Scholer HR (1994) *Developmental Biology* **166,** 259-267.

15. Yamanaka Y, Ralston A, Stephenson R, & Rossant J (2006) *Developmental Dynamics* **235,** 2301-2314.

16. Fleming TP (1987) *Developmental Biology* **119,** 520-531.

17. Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, & Yamanaka S (2003) *Cell* **113,** 631-642.

18. Koutsourakis M, Langeveld A, Patient R, Beddington R, & Grosveld F (1999) *Development* **126,** 723-732.

19. Evans MJ & Kaufman MH (1981) *Nature* **292,** 154-156.

20. Martin GR (1981) *PNAS* **78,** 7634-7638.

21. Marshak DR, Gottlieb D, Kiger AA, Fuller MT, Kunath T, Hogan B, Gardner RL, Smith A, Klar AJS, Henrique D, *et al.* (2001) *Stem Cell Biology* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

22. Smith AG (2001) *Annual Review of Cell & Developmental Biology* **17,** 435-462.

23. Nagy A, Gertsenstein, M., Vintersten, K., and Behringer, R. (2003) *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

24. Patrick PL, Tam EA, & Williams SST (1994) *Developmental Genetics* **15,** 491-503.

25. Russ AP, Wattler S, Colledge WH, Aparicio SAJR, Carlton MBL, Pearce JJ, Barton SC, Surani MA, Ryan K, Nehls MC, *et al.* (2000) *Nature* **404,** 95-99.

26. Kunath T, Arnaud D, Uy GD, Okamoto I, Chureau C, Yamanaka Y, Heard E, Gardner RL, Avner P, & Rossant J (2005) *Development* **132,** 1649-1661.

27. Rossant J & Cross JC (2001) *Nat Rev Genet* **2,** 538-548.

28. Carney EW, Prideaux V, Lye SJ, & Rossant J (1993) *Molecular Reproduction and Development* **34,** 357-368.

29. Yotsumoto S, Shimada T, Cui CY, Nakashima H, Fujiwara H, & Ko MSH (1998) *Developmental Biology* **203,** 264-275.

30. Hemberger M, Nozaki T, Winterhager E, Yamamoto H, Nakagama H, Kamada N, Suzuki H, Ohta T, Ohki M, Masutani M, *et al.* (2003) *Developmental Dynamics* **227,** 185-191.

31. Weiler-Guettler H, Aird WC, Rayburn H, Husain M, & Rosenberg RD (1996) *Development* **122,** 2271-2281.

32. Hemberger M (2007) *Cellular and Molecular Life Sciences (CMLS)* **64,** 2422-2436.

33. Cross JC, Nakano H, Natale DRC, Simmons DG, & Watson ED (2006) *Differentiation* **74,** 393-401.

34. Hernandez-Verdun D (1974) *Cell and Tissue Research* **148,** 381-396.

35. Colosi P, Talamantes F, & Linzer DI (1987) *Mol Endocrinol* **1,** 767-776.

36. Hemberger M (2008) *Placenta* **29,** 4-9.

37. Hemberger M, Nozaki T, Masutani M, & Cross JC (2003) *Developmental Dynamics* **227,** 185-191.

38. Simmons DG, Fortier AL, & Cross JC (2007) *Developmental Biology* **304,** 567-578.

39. Böhm N & Noltemeyer N (1981) *Histochemistry and Cell Biology* **72,** 55-61.

40. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, & Grompe M (2003) *Nature* **422,** 897-901.

41. Vassilopoulos G, Wang PR, & Russell DW (2003) *Nature* **422,** 901-904.

42. Willenbring H, Bailey AS, Foster M, Akkari Y, Dorrell C, Olson S, Finegold M, Fleming WH, & Grompe M (2004) *Nat Med* **10,** 744-748.

43. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, & Wood KJ (2001) *J Immunol* **166,** 3789-3796.

44. Josien R, Douillard P, Guillot C, Muschen M, Anegon I, Chetritt J, Menoret S, Vignes C, Soulillou JP, & Cuturi M-C (1998) *J. Clin. Invest.* **102,** 1920-1926.

45. Israeli E, Fong, V., Mitchell, S., Harrison, J., and Schomer, A. (2004).

46. Valdes-Gonzalez RA, Dorantes LM, Garibay GN, Bracho-Blanchet E, Mendez AJ, Davila-Perez R, Elliott RB, Teran L, & White DJG (2005) *Eur J Endocrinol* **153,** 419-427.

47. Wegmann TG, Lin H, Guilbert L, & Mosmann TR (1993) *Immunology Today* **14,** 353-356.

48. Aluvihare VR, Kallikourdis M, & Betz AG (2004) *Nat Immunol* **5,** 266-271.

49. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, & Girkontaite I (1997) *Nature* **390,** 350-351.

50. Mor G, Romero R, & Abrahahams VM (2006) *Immunology of Pregnancy* (Landes Bioscience, Georgetown, TX).

51. Vilches C & Parham P (2002) *Annual Review of Immunology* **20,** 217-251.

52. Marchal-Bras-Goncalves R, Rouas-Freiss N, Connan F, Choppin J, Dausset J, Carosella ED, Kirszenbaum M, & Guillet JG (2001) *Transplantation Proceedings* **33,** 2355-2359.

53. Makrigiannakis A, Zoumakis E, Kalantaridou S, Coutifaris C, Margioris AN, Coukos G, Rice KC, Gravanis A, & Chrousos GP (2001) *Nat Immunol* **2,** 1018-1024.

54. Mellor AL, Sivakumar J, Chandler P, Smith K, Molina H, Mao D, & Munn DH (2001) *Nat Immunol* **2,** 64-68.

55. Xu C, Mao D, Holers VM, Palanca B, Cheng AM, & Molina H (2000) *Science* **287,** 498-501.

56. Lars S, Marie A, Mary-Ann S, Rikard H, & Ragnar M (2001) *Journal of Reproductive Immunology* **51,** 3-7.

57. Matsumoto J, Kawana K, Nagamatsu T, Schust DJ, Fujii T, Sato H, Hyodo H, Yasugi T, Kozuma S, & Taketani Y (2008) *Biochemical and Biophysical Research Communications* 236-241.

58. Gopichandran N, Ekbote UV, Walker JJ, Brooke D, & Orsi NM (2006) *Reproduction* **131,** 613-621.

59. Page ST, Plymate SR, Bremner WJ, Matsumoto AM, Hess DL, Lin DW, Amory JK, Nelson PS, & Wu JD (2006) *Am J Physiol Endocrinol Metab* **290,** E856-E863.

60. Nitsch SM, Wittmann F, Angele P, Wichmann MW, Hatz R, Hernandez-Richter T, Chaudry IH, Jauch KW, & Angele MK (2004) *Arch Surg* **139,** 157-163.

61. Araneo BA, Dowell T, Diegel M, & Daynes RA (1991) *Blood* **78,** 688-699.

62. Ellis TM, Moser MT, Le PT, Flanigan RC, & Kwon ED (2001) *Int. Immunol.* **13,** 553-558.

63. Cutolo M, Capellino S, Montagna P, Ghiorzo P, Sulli A, & Villaggio B (2005) *Arthritis Research & Therapy* **7,** R1124 - R1132.

64. Jin L, Ai X, Liu L, Wang Z, Cheng Y, Qiao Z. (2006) *Methods Find Exp Clin Pharmacol.* **28,** 283-293.

65. Ling W (1983) *Journal of Clinical Endocrinology & Metabolism* **57,** 439-441

66. Zhu XD, Bonet B, & Knopp RH (1997) *American Journal of Obstetrics and Gynecology* **177,** 196-209.

67. Crispe IN, Giannandrea M, Klein I, John B, Sampson B, & Wuensch S (2006) *Immunological Reviews* **213,** 101-118.

68. Sumpter T, Abe M, Tokita D, & Thomson AW (2007) *Hepatology* **46,** 2021-2031.

69. Lin YC, Chen CL, Nakano T, Goto S, Kao YH, Hsu LW, Lai CY, Jawan B, Cheng YF, Tateno C, *et al. Journal of Gastroenterology and Hepatology* **23** 243 - 2500.

70. Lapaire O, Holzgreve W, Oosterwijk JC, Brinkhaus R, & Bianchi DW (2007) *Placenta* **28,** 1-5.

71. Khosrotehrani K & Bianchi DW (2005) *J Cell Sci* **118,** 1559-1563.

72. Ariga H, Ohto H, Busch MP, Imamura S, Watson R, Reed W, & Lee T-H (2001) *Transfusion* **41,** 1524-1530.

73. Lambert NC, Lo YMD, Erickson TD, Tylee TS, Guthrie KA, Furst DE, & Nelson JL (2002) *Blood* **100,** 2845-2851.

74. Evans PC, Lambert N, Maloney S, Furst DE, Moore JM, & Nelson JL (1999) *Blood* **93,** 2033-2037.

75. Bianchi DW, Farina A, Weber W, Delli-Bovi LC, DeRiso M, Williams JM, & Klinger KW (2001) *American Journal of Obstetrics and Gynecology* **184,** 703-706.

76. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, & DeMaria MA (1996) *PNAS* **93,** 705-708.

77. Johnson KL, Samura O, Nelson JL, McDonnell WM, & Bianchi DW (2002) *Hepatology* **36,** 1295-1297.

78. Tafuri A, Alferink J, Möller P, Hämmerling GJ, & Arnold B (1995) *Science* **270,** 630-633.

79. Fabricius D, Bonde S, & Zavazava N. (2005) *Tranplantation* **79,** 1040-1044.

80. Bonde S & Zavazava N (2006) *Stem Cells* **24,** 2192-2201.

81. Fair JH, Cairns BA, LaPaglia MA, Caballero M, Pleasant WA, Hatada S, Kim HS, Gui T, Pevny L, Meyer AA, *et al.* (2005) *PNAS* **102,** 2958-2963.

82. Baertschiger RM, Mai G, Morel P, Armanet M, Sgroi A, Bosco D, Serre-Beinier V, Toso C, Hauwel M, Jaconi M, *et al.* (2006) in *World Transplantation Congress* (Boston, MA), p. 303.

83. Swijnenburg RJ, Tanaka M, Vogel H, Baker J, Kofidis T, Gunawan F, Lebl DR, Caffarelli AD, de Bruin JL, Fedoseyeva EV, *et al.* (2005) *Circulation* **112,** 166-172.

84. Magliocca JF, Held IKA, & Odorico JS (2006) *Stem Cells and Development* **15,** 707-717.

85. Nussbaum J, Minami E, Laflamme MA, Virag JAI, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H, & Murry CE (2007) *FASEB J.* **21,** 1345-1357.

86. Koch CA, Geraldes P, & Platt JL (2008) *Stem Cells* **26,** 89-98.

87. Verstuyf A, Sobis H, & Vandeputte M (1989) *International Journal of Cancer* **44,** 879-884.

88. Takahashi Y, Dominici M, Swift J, Nagy C, & Ihle JN (2006) *Journal of Biological Chemistry* **281,** 11444-11445.

89. deMestre A, Bacon S, Costa C, Leadbeater J, Noronha L, Stewart F, & Antczak D (2008) *Placenta* **29,** 158-169.

90. Lois C, Hong EJ, Pease S, Brown EJ, & Baltimore D (2002) *Science* **295,** 868-872.

91. Binas B (2002) Personal Communication, Texas A&M University, College Station, Texas.

92. Bowen DG, McCaughan GW, & Bertolino P (2005) *Trends in Immunology* **26,** 512-517.

93. Petroff MG (2005) *Journal of Reproductive Immunology* **68,** 1-13.

94. Stripecke R, del Carmen Villacres M, Skelton DC, Satake N, Halene S, & Kohn DB (1999) *Gene Therapy* **6,** 1305-1312.

95. Gambotto A, Dworacki G, Cicinnati V, Kenniston T, Steitz J, Tuting T, Robbins PD, & DeLeo AB (2000) *Gene Therapy* **7,** 2036.-2040

96. Skelton D, Satake N, & Kohn DB (2001) *Gene Therapy* **8,** 1813-1814.

97. Andersson G, Denaro M, Johnson K, Morgan P, Sullivan A, Houser S, Patience C, White-Scharf ME, & Down JD (2003) *Mol Ther* **8,** 385-391.

98. Bubnic SJ, Nagy A, & Keating A (2005) *Hematology* **10,** 289-295.

99. Fimmel S & Zouboulis CC (2005) *The Aging Male* **8,** 166 - 174.

100. Christiansen OB, Nielsen HS, & Kolte AM (2006) *Seminars in Fetal and Neonatal Medicine* **11,** 302-308.

101. Malassine A, Frendo JL, & Evain-Brion D (2003) *Hum Reprod Update* **9,** 531-539.

102. Buehr M, Nichols J, Stenhouse F, Mountford P, Greenhalgh CJ, Kantachuvesiri S, Brooker G, Mullins J, & Smith AG (2003) *Biology of Reproduction* **68,** 222-229.

VITA

Name: Jessica A. Epple-Farmer

Address: Room 124 VMR Building
College of Veterinary Medicine
Texas A&M University
College Station, TX 77843

Email Address: jepple@cvm.tamu.edu

Education: B.A., Animal Science, Lincoln University, 1998
M.S., Physiology of Reproduction, Texas A&M University, 2001
Ph.D., Physiology of Reproduction, Texas A&M University, 2008