THE FEASIBILITY OF INTERSPECIFIC RESCUE OF ENDOTHELIAL/HEMATOPOIETIC LINEAGE DEFICIENCY

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

The feasibility of interspecific rescue of endothelial/hematopoietic lineage deficiency

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ABSTRACT

THE FEASIBILITY OF INTERSPECIFIC RESCUE OF ENDOTHELIAL/HEMATOPOIETIC LINEAGE DEFICIENCY

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The ultimate goal of this project is to develop animal models with specific organs derived from human. For example, we could produce chimeras in which a human vascular endothelium and a human hematopoietic system were present in a mouse, rat or other species. Large animal models (*e.g*. human-swine chimeras) with these modifications could ultimately be used as a source of cells, tissue and organs for xenotransplantation (Cooper, 2003) and in pre-clinic trials. Small animal models (*e.g*. human-mouse) would be extremely valuable in studies of: (a) leukemia and lymphoma; (b) cardiovascular disease; (c) blood-borne infectious pathogens; (d) vaccine development; and (e) hematopoietic system pathology. Here we propose to develop a mouse model which will establish "proof of principle" for future development of large animal (*e.g.* pig) models which could be used as actual human solid organ and blood donors as well as in pre-clinic trials. As a proof of concept, we intended to develop interspecific chimeras between mouse and rat to test the possibility that embryonic stem cells (ESCs)

from one species are able to survive and develop in a blastocyst from a difference species. Then we intended to make a rat-mouse chimera with a vascular endothelium and a hematopoietic system from a rat genetic background and the other tissues and organs from a mouse genetic background. The first series of experiments of this thesis work were designed to test the feasibility for the interspecific chimeras (mouse-rat and rat-mouse) that can be made by ESC-blastocyst injection method. The second series of experiments of this thesis work were designed to test the feasibility of making such a chimera by using rat ESCs to rescue endothelial/hematopoietic system deficient mouse embryos that will otherwise die.

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CHAPTER 1: INTRODUCTION

Drug discovery and development are very expensive. The costs of bringing a successful drug to market are estimated to be between $0.8–1.7B, or put another way $1-3M per day (U.S. EPA, June 2011; FDA's "Critical Path" Initiative Janet Woodcock, 2004). Of all compounds developed for use in humans only a small fraction are eventually approved by the Food and Drug Administration (FDA). Each year, currently only ~0.001% of all Investigational New Drugs (INDs) reach the market with a high percentage of failure late in clinical development and only about 20 truly novel drugs (new chemical entities; NCE) are approved for marketing (U.S. EPA, June 2011; FDA's "Critical Path" Initiative Janet Woodcock, 2004). This approval comes only after heavy investment in pre-clinical development and clinical trials, as well as a commitment to ongoing safety monitoring. Because of the extremely high cost and the low success rate of developing new drugs, better tools, such as better animal models to select those drug candidates that are predicted to have a high probability of safety and effectiveness early in the process are critically needed. Accordingly, the sooner drug candidates are identified as likely to be non-effective *vs.* likely to be effective, the sooner companies can "kill" a given product's development and save millions of dollars.

Pre-clinical development is a stage in the development of a new drug that begins before clinical trials (testing in humans) can begin, and during which important

safety and pharmacology data is collected. The main goals of pre-clinical studies (also called preclinical studies and nonclinical studies) are to determine a drug's pharmacodynamics (PD), pharmacokinetics (PK) and toxicity through animal testing. These data allow researchers to estimate a safe starting dose of the drug for clinical trials in humans. Typically, preclinical trials involve the use of two animal species, one small animal species (usually mice or rats) (Lapidot *et al.,* 1992) and one large animal species (usually pigs or monkeys) (Truty and Smoot, 2008). New genetically modified and/or humanized animal models are often much more predictive of human responses to drugs and treatments for diseases than traditional animal models (Brolen *et al.,* 2005). Humanized animal models can provide insights into *in vivo* human biology that would otherwise not be possible due to ethical, logistical and technical constrains (Alajati *et al.,* 2008; Bissig *et al.,* 2007). However, so far, so-called humanized animal models are defined as immunodeficient rodents engrafted with human cells or tissues, or transgenic animals that express human genes. These models result in animals that are partially “humanized” at the level of tissues, organs, or cells. The approach proposed in this thesis will likely produce a new method to make humanized mice and later pigs to be used as animal models in testing drugs and biologics for safety and efficacy. These new animal models made with this method will provide greater sensitivity in identifying drugs that will not make it through human clinical trials so that using these new animal models will eventually save companies tremendous time and money.

Besides contributing to preclinical trials, enhanced animal models, specifically humanized animal models, may also provide the benefit of more rapid discovery of effective prevention and treatment of cancer, cardiovascular diseases, diabetes and other catastrophic human diseases. In the last few years, the FDA has launched a “Critical Path” program in response to the recent slowdown in innovative medical therapies reaching patients (http://www.fda.gov/oc/initiatives/criticalp ath/). The FDA Critical Path program is directly related to the new NIH Roadmap Clinical and Translational Science Award (CTSA) program (http://www.ncrr.nih.gov/clinical_research resources/clinical_and_and_translational science awards) directed towards integrating basic, translational and clinical research (Fig. 1.1). In addition, the human and mouse genome sequences are now available and the complete pig sequence will be available soon. Together, this new availability of technology creates an unprecedented opportunity to establish new, genetically engineered animal models to expedite the development of new treatment modalities to address and relieve human pain and suffering due to diseases.

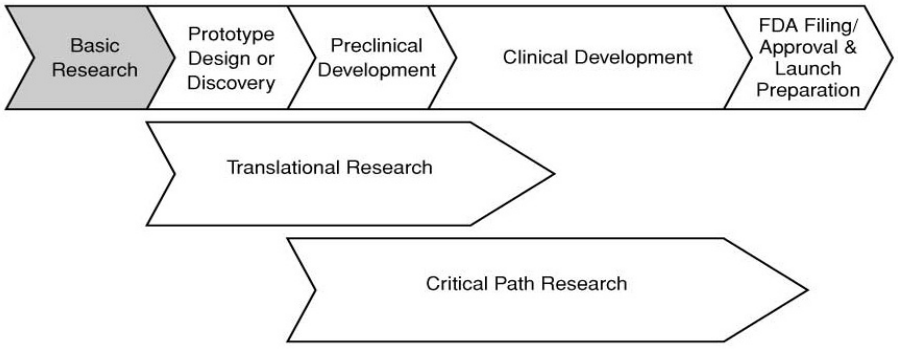


FIG. 1.1: The relationship between the FDA Critical Path initiative and the NIH CTSA initiative. (From: FDA's "Critical Path" Initiative Janet Woodcock, 2004).

Humanized animal models also will benefit organ transplantation. Transplantation of organs is a well-known and accepted life-saving procedure for many diseases such as end-stage kidney, liver, heart and lung diseases. From a medical point of view, organ transplantation is often preferable to an alternative form of therapy to end-stage human diseases. However, the number of donated cadaver organs does not meet the current clinic needs. The low availability of donor organs limits the application of this technique and leads to unnecessary loss of life. Experimental techniques such as xenotransplantation are extremely important to determine new methods of creating organ availability. Although the efforts in xenotransplantation continue to improve the prospect of using animals as organ donors, the transplant will always be non-human. Production of organ donors that harbor organs composed of human-origin cells, also called interspecies chimeras, may be an alternative, non-human source of human tissues. The new humanized animal models made with the method proposed in this thesis may

serve this purpose as alternative organ donors. In addition to serving as a possible source of therapeutic tissues or organs, humanized animal models can serve as models to study disease progression or therapeutic interventions. New genetically modified and/or humanized animal models are often much more predictive of human responses to drugs and treatments for diseases than traditional non-chimeric animal models. For example, one humanized mouse model made especially for human diabetes study permitted the study of human islets or their precursor in the presence of a robust human immune system which cannot be provided by non-humanized animal models (Ron, 2002). These humanized mouse models provide an important preclinical bridge between *in vitro* studies and rodent models and the translation of discoveries in these model systems to the clinic.

The work described in this dissertation evaluates the feasibility of making interspecific chimeras by embryonic stem cell (ESC)-blastocyst injection and of rescuing the endothelial/hematopoietic lineages in a vascular insufficiency mouse model through the use of rat ESCs. The technologies tested in this thesis could potentially be utilized to produce a new method to make humanized mice or pigs (later stage). Such animal models could be used in testing drugs and biologics for safety and efficacy which will provide a new paradigm for drug discovery/development with greater sensitivity in identifying drugs. Eventually these humanized animal models also can be used as organ donors in xenotransplantation.

Chimeras

The first experimental intra-species chimeras (between two mouse strains) were created more than 50 years ago by removal of the zona pellucida from two genetically distinct eight-cell-stage embryos which were then placed in physical contact with each other during *in vitro* culture(Tarkowski, 1961). These aggregates resulted in viable live chimeras after transfer to surrogate mothers. Since then, many important biological questions about embryonic and organ development have been addressed using chimeras as a research tool. Especially with the advent of ESCs, chimera experiments gained additional importance. In mice, chimeras have become the vehicles for transmitting the ESC genome *in vivo*. There are two predominant methods to produce chimeras with ESCs: injection chimeras(Gardner, 1968) and aggregation chimeras (Nagy *et al.,* 1990; Wood *et al.,* 1993). Injection chimeras are made by injecting ESCs into blastocysts and is the most common method for introducing genetic alterations performed in ESCs into mouse (Bradley *et al.,* 1984). Also, chimeras can be generated by aggregation of ESCs with precompaction morula-stage embryos.

Besides mouse chimeras, chimeric cattle have been produced between parthenogenotes and normal embryos as well as between genetically modified cells and normal embryos (Boediono *et al.,* 1999; Wells and Powell, 2000). Chimeric sheep have been produced by blastocyst injection with pluripotent cells

(Butler *et al.,* 1987). In pigs, blastocyst injection is an established, robust technology and chimeric piglets have been produced from blastocyst injection by multiple labs over the last 15 years (Kashiwazaki *et al.,* 1992; Nagashima *et al.*, 2004; Notarianni *et al.,* 1997; Rui *et al.,* 2004; Wheeler, 1994). Additionally, chimeric swine embryos have been constructed with tetraploid and normal embryos (Prather *et al.,* 1996).

Interspecies chimera production in livestock has a ~30 year history (Fehilly *et al.*, 1984a, b). The first interspecific chimera was produced from aggregation of an unequal number of sheep and goat eight-cell embryos and transfer to a sheep or a goat recipient female (Fehilly *et al.,* 1985; Fehilly *et al.,* 1984a). In rodents, chimeric blastocysts produced by combining embryonic cells of two different species have been used in investigations of cell lineages and interaction during development (Frels *et al.,* 1980; Rossant *et al.,* 1983). In most of these studies, live offspring were not produced successfully(Gardner and Johnson, 1973). Recently however, a Japanese group successfully produced interspecific live chimeras between mouse and rat (Kobayashi et al.). One possible reason for non-successful aggregation chimeras could be a contribution of cells from the heterologous species (relative to the recipient female) in the placenta which could prohibit implantation of the embryos. An accidental formation of mouse-rat chimera showed that this potential problem can be circumvented based on the theory that ESCs are destined to contribute to the embryo proper but not to trophoblast cells (Beddington and Robertson, 1989; Brenin *et al.,* 1997).

Therefore, by ESC-blastocyst injection, Kobayashi *et al.,* (2010) showed that interspecies chimeras between mouse and rat can be made and are able to develop to term. More importantly, their data demonstrated that mouse blastocysts injected with rat ESCs could be rescued and result in a live chimera with a rat endocrine pancreas. One year later, a similar report demonstrated that rat ESCs can contribute to various organs in mouse-rat interspecies chimeras (Isotani *et al.,* 2011). Similarly, they also showed a formation of thymus from rat ESCs by making an interspecies chimera with *nu/nu* mice. In contrast to previous work, their data suggested the contribution of rat ESCs into germs cells (Isotani *et al.,* 2011).

Researchers also investigated the possibilities of making humanized chimeras in rodents (James *et al.,* 2005; Yokoo *et al.,* 2005). Previous reports have demonstrated that making mouse-human chimeras is possible and that human pluripotent (embryonic stem cells; hESC), as well as multipotent (mesenchymal stem cells; hMSC; and immature dental pulp stem cells; hIDPSC) stem cells are able to respond to the spatial and temporal program of the mouse embryonic environment during development (James *et al.,* 2005; Siqueira da Fonseca *et al.,* 2009; Yokoo *et al.*, 2005). Yokoo *et al* also demonstrated that hMSCs can commit to the fate of kidney if they are placed within a developing kidney in whole-embryo culture (Yokoo *et al.,* 2005). Other developmental fates and/or organ structures can arise from hMSC in heterologous systems, depending on the embryonic environment. Siqueira de Fonseca *et al.* (2009) showed that

hIDPSCs were capable of engrafting and proliferating in mouse morula and blastocysts and capable of participating in development in preterm (E18) chimeras. One possible reason may be that the mouse embryonic environment influences the epigenome of human cell so that the human cells express genes controlling their differentiation and development in such a way as to be synchronous with their surrounding environment (Jaenisch and Bird, 2003; Siqueira da Fonseca *et al.,* 2009).

Pluripotent Cell Lines

The first ESC line was established from mouse embryos in 1981, following a method virtually identical to rabbit models used about 16 years earlier (Cole *et al.,* 1965; Evans and Kaufman, 1981). The knowledge of pluripotency and differentiation of ESCs has revolutionized science by providing a platform for generating knockout/knockin mice. Martin Evans has been honored with the Nobel Prize for Physiology and Medicine (2007) for his contribution towards development of animal models of disease through ESC mediated gene targeting. Almost two decades after the establishment of mouse ESCs, human ESCs were first derived in 1998 from the inner cell mass (ICM) of blastocyst stage embryos that were discarded after *in vitro* fertilization (IVF) and after embryo donations (Thomson *et al.,* 1998).

"Self-renewal" *i.e.,* the ability to undergo indefinite self-renewing and symmetric cell divisions to maintain the population, and "pluripotency" which indicates their ability to differentiate into any of the different known cell types derived from ectoderm, endoderm and mesoderm lineages are the two hallmarks of these cells. To date, the differentiation program of embryonic stem and progenitor stem cells is one of the central questions in biology. In the new era of regenerative medicine, one of the central experimental plans has been predicated on driving the differentiation of ESCs along specific lineages, expansion and purification of the cell type of interest, and *in vivo* repopulation of damaged or degenerating organs by ESC-derived differentiated cells. For example, an exciting new study showed that mouse ESCs can rescue otherwise lethal cardiac defects in mouse embryos (Fraidenraich *et al.,* 2004).

The use of human embryonic stem cells (hESCs) in cell replacement therapies has been limited due to ethical and technical issues (Chung *et al.,* 2006). The ethical issues have driven strong interest in alternative stem cells that do not require the destruction of a human embryo. Adult stem cells may be another hope, however, there has been an extensive debate about the benefits and drawbacks of adult stem cells *vs.* hESCs use in therapies (Raff, 2003; Gerecht-Nir S, Itskovitz-Eldor, 2004). Unlocking the therapeutic potential of human ESCs has remained an essential but elusive goal. To circumvent this problem, one alternative would be using ESC-like cells, such as embryonic germ (EG) cells (Raff, 2003). Besides ethical issues associated with the use of human embryos,

these are a very limited cell source that deters large-scale therapeutic application. Also, human ESCs will not be immune-compatible with a heterologous patient which thus restricts their medical potential as well. Therefore, investigators are trying to generate patient-matching pluripotent cells. Recently, adult somatic cells have been genetically modified and reprogrammed to undergo a process of dedifferentiation by inducing the expression of pluripotency-related genes. These cells are called induced pluripotent stem cells (iPSCs) and offer potential as a promising alternative for hESCs. These iPSCs exhibit the morphology of ESCs and express ESC markers (Takahashi and Yamanaka, 2006). Soon after the first report that Takahashi and Yamanaka (2006) induced mouse somatic cells into pluripotent stem cells by introducing four factors *Oct-4*, *Sox-2*, *c-Myc* and *Klf-4*, other groups described iPSCs derived from multiple murine and human somatic cell types (Li *et al.,* 2010; Zhou *et al.,* 2010). Now it is possible to generate iPSCs from patients with specific diseases (Park *et al.,* 2008; Soldner *et al.,* 2009) and these cell lines will help to produce animal models with specific diseases as well as discovery of new drugs. The iPSC field is developing with a tremendous speed. To date, iPSC have been successfully established from several species such as rhesus monkey, rat, dog, pig, marmoset, rabbit and horse (Honda *et al.,* 2010; Liao *et al.,* 2009; Liu *et al.,* 2008; Nagy *et al.,* 2011; Roberts *et al.,* 2009; Shimada *et al.,* 2010). Similarly, iPSCs have been successfully derived from other somatic cell populations, for example, neural cells, stomach and liver cells and pancreatic β cells (Aoi *et al.,* 2008; Eminli et al., 2008; Stadtfeld *et al.,* 2008).

Cell Tracking Marker and *Rosa26* locus

Green fluorescent protein (GFP) gene was isolated from jellyfish, *Aequorea Victoria* that naturally transduce the blue chemiluminescence of the protein aequorin into green fluorescent light by energy transfer (Chalfie *et al.,* 1994). GFP has become a useful tool in many fields of life science research by allowing the observation of cell behavior, gene expression and metabolic process in live cells. This reporter gene is unique compared to other reporters because its detection does not require fixation, and it is possible to visualize the protein in a live specimen by illumination with a specific excitation wavelength coupled with an emission filter and photon detection system. GFP is particularly useful in ESC-based gene and genome manipulations in the rodents. Now, investigators are able to utilize different fluorescently tagged proteins to follow gene expression and cellular behavior. For example, in mouse, enhanced green (EGFP), yellow (EYFP), and cyan (ECFP) fluorescent protein have been used for many years (Hadjantonakis and Nagy, 2000; Srinivas *et al.,* 2001). Another naturally fluorescent protein, DsRed, started to be widely used in other model systems, such as yeast, *E. coli*, *C. elegans*, and zebrafish. Later, DsRed was investigated in mouse ESCs and in living animals and a transgenic ESC line/mouse line with widespread DsRed expression was reported (Vintersten *et al.,* 2004).

Gene targeting technology has been widely used for making animal models through the use of ESCs. These marker genes can be inserted into the gene *Rosa26* which has proven to be a very reliable insertion site to ensure marker gene expression. The *Rosaβgeo 26* (Gtrosa26) (A DNA construct) was initially derived from pools of ESCs infected with the retroviral gene trap vector Gen-*Rosaβgeo* at low multiplicity of infection (Friedrich and Soriano, 1991). Then ESCs harboring a single proviral copy were injected into blastocysts and mice containing the *Rosa26* insertion were isolated. These *Rosa26* mice express *lacZ* in all tissues of the embryos including the hematopoietic lineages (Zambrowicz *et al.,* 1997). *Rosa26* insertion method is thus very useful for a number of applications, including chimera analysis. The characterization of this locus has led to two methods for generating transgenic mice that express a transgene in a generalized fashion. First, it is possible to target genes to the *Rosa26* locus. For example, this site has been used to produce a variety of knock-in lines which include reporter lines for site specific recombinases. For the second method, the promoter of the *Rosa26* locus has been characterized in ESCs (Zambrowicz et al., 1997) and has been used for broad expression of transgenes in mice (Kisseberth *et al.,* 1999). In addition, when uniform expression is desired, targeting to the locus by introducing the desired gene into the first intron of the locus is a better way to conveniently achieve this. For instance, Nagy's lab reported a novel stem cell line named *Rosa26-rtTA-IRES-EGFP* which targeted the *rtTA* transgene into the *ROSA26* locus to ensure reliable *rtTA* expression in a broad range of cell types (Belteki *et al.,* 2005). This system allowed for the spatial

and temporal regulation of transgene expression *in vivo* by combining with *rtTA-tet-On* system.

In past years, many ESC lines have been established and their differentiation potential has been tested both *in vivo* and *in vitro*. However, none of these studies addressed the *in vivo* physiological fate of such stem cells or progenitor cells as a part of normal development. To address this, a novel cell-mapping system was developed that was based on expressing Cre or Flp recombinase in a stem cell or progenitor cell population (Dymecki and Tomasiewicz, 1998; Gu *et al.,* 2002; Zinyk *et al.,* 1998). Cre recombinase of the bacteriophage P1 is the most widely used recombinase in combination with transgenesis and gene targeting in the mouse (Nagy, 2000). This prokaryotic enzyme was first shown to work in the mouse by Lakso (Lakso et al., 1992). The alternative recombinase that is also well known is Flp recombinase from yeast (*Saccharomyces cerevisiae*). Its mechanism of action is similar to c*re/loxP* and a newer developed Flp has improved the efficiency of this recombinase (Buchholz *et al.,* 1998; Rodriguez *et al.,* 2000).

Cre-mediated excision of the “floxed” sequences (*i.e., loxP*-flanked STOP sequences) or Flp-mediated excision of the *Frt*-flanked STOP sequences in the reporter constructs results in the permanent expression of the reporter in all the descendant cells. This strategy permits analysis of the fate of precursor cells throughout life in complex organ systems *in vivo*, when *cre* or *flp* driven by

specific promoters and/or enhancer elements in mice are introduced in these precursors. For example, fate-mapping of *Flk1*$^{+}$ cells in mice showed that *Flk1*$^{+}$ cells also exhibit a differentiation potential for the other mesodermal lineages besides endothelial cells (Motoike *et al.,* 2003). So far, over a hundred *cre* mouse lines have been developed with well characterized spatial and temporal Cre expression patterns (Nagy *et al.,* 2009). In order to better track this recombination event *in vivo*, it is very important to label cells after Cre-mediated recombination and non-recombined cells. For example, one double fluorescent Cre recombinase reporter mouse expresses membrane-targeted tdTomato (a DsRed variant) prior to Cre excision and membrane-targeted EGFP following Cre excision, thereby allowing live visualization and distinction of recombined and non-recombined cells (Muzumdar *et al.,* 2007).

Since Cre-mediated recombination is permanent, it will be more feasible and convenient if the recombination can be timed *in vivo* so that the cell fates can be tracked more precisely. To achieve this goal, a mutant ligand binding domain of the human estrogen receptor (ER^{T}) has been fused to the Cre recombinase (Metzger and Chambon, 2001). This mutation prevents binding of its natural ligand (17β-estradiol) at normal physiological concentrations, but renders the ER^{T} domain responsive to 4-hydroxy (OH)-TM (tamoxifen) (Danielian *et al.,* 1993; Littlewood *et al.,* 1995). Fusion of *cre* with ER^{T} leads to the ER^{T}-dependent cytoplasmic sequestration of Cre by Hsp90 (Mattioni et al., 1994), thereby preventing Cre-mediated recombination which is a nuclear event. However,

binding of 4OH-TM leads to a disruption of the interaction with Hsp90, permitting access of Cre-ER^T to the nucleus and initiation of recombination. Transgenic mouse lines produced with this modification are tamoxifen dependent, therefore, inducible. The advent of conditional null alleles combined with the availability of *cre*-ER^T has led to a large number of studies using these methods to remove gene function in a cell autonomous manner. By taking advantage of this inducible Cre-ER^T system, many transgenic mouse lines have been produced to generate cell/organ specific spatiotemporally controlled somatic mutation. The system has also been used in enriching for desired cell types in mouse stem cell differentiation studies *in vivo*. In these studies, the administration of tamoxifen was needed to induce Cre-mediated recombination during embryogenesis. Some reports have shown tamoxifen treatment during pregnancy can interfere with normal delivery (Danielian *et al.,* 1998; Gothert *et al.,* 2005). Hayashi and McMahon demonstrated that a single, intraperitoneal injection of tamoxifen into a pregnant mouse at 8.5 days post coitum (dpc) led to detectable recombination in the developing embryo within 6 hours of injection and efficient recombination of a reporter gene in derivatives of all three germ layers within 24 hours of injection(Hayashi and McMahon, 2002). In addition, by varying the dose of tamoxifen injected, the percentage of cells undergoing a recombination event in the embryo can be controlled. One report showed that cell markers can be tracked during embryogenesis into adulthood by intraperitoneal injections of 5-10 mg to the pregnant female mice at as early as 6.5 dpc (Samokhvalov *et al.,* 2007).

Cell Ablation

Cell ablation (also known as genetic amputation and tissue ablation) can be used as a tool to produce transgenic animal models lacking a cell type. Cell ablation experiments are also important for answering questions regarding lineage and developmental or physiological functions of particular cell types *in vivo* (O'Kane and Moffat, 1992). Physical ablation techniques, such as laser ablation or microsurgery, can be applied to accessible cells in many organisms. However, in mice, methods using “toxigenes” such as herpes virus thymidine kinase (TK) and diphtheria toxin (DT) have been more popular and widely used (Evans, 1991). For example, cell-specific ablation has been achieved in transgenic animals through the expression of toxin genes under the control of cell-specific regulatory elements such as promoters or enhancers.

The principle of cell ablation through TK is that TK catalyzes the formation of thymidylate by the phosphorylation of thymidine. However, this enzyme can also phosphorylate non-toxic thymidine analogs to produce a toxic product. For example, ganciclovir (GCV) acts as one pro-drug, which in itself is not toxic, but is phosphorylated by TK to yield GCV-triphosphate which causes chain termination and single-strand breaks upon incorporation into DNA (Aghi *et al.,* 2000). In mice that carry the wild type TK gene as a transgene, reporter expression of viral TK was shown at a high level in the thyroid gland and

ectopically in the testis resulting in male sterility (al-Shawi *et al.,* 1991; Braun *et al.,* 1990). This is because the presence of a cryptic testis-specific promoter within the TK reporter gene causes transcription initiation at sites within the coding sequence (Bernier *et al.*, 1994). Later, one report showed that modified TK were less likely expressed at high levels in non-targeted tissues (Ellison and Bishop, 1998). With these new modified transgenes, researchers were able to easily generate and maintain lines of mice because males were often fertile and transmitted the transgenes to their offspring. In addition, the toxic effect in TK-GCV system is reversible and dependent upon drug administration.

Another suicide gene that is popularly used in cell ablation experiments is the diphtheria toxin (DT) from *Corynebacterium diphtheria*, which is so toxic to cells that a single molecule may be enough to kill (Honjo *et al.,* 1971). It catalyzes the ADP-ribosylation of eukaryotic elongation factor-2 (eEF2) by ADP-ribosylating the unusual amino acid diphthamide to inactivate this protein. Like TK, DT has been frequently used for negative selection in gene targeting and for conditional transgenesis in ablation studies. DT is normally composed of two subunits, A and B. Subunit B is responsible for the internalization of the toxin upon binding to its cell surface receptor. Once inside the cells, subunit A will induce apoptosis in the target cells (Collier, 2001; Honjo *et al.,* 1971). Only the gene for subunit A is used for cell ablation studies. Since the transgene product is produced within a cell, membrane transport is not required. Additionally, since DT can be released from

expressing cells upon death, it is important to prevent the transfer of transgene-expressed DT into neighboring cells

Development of the Vascular Endothelium and Hematopoietic System and Introduction of Stem Cell Leukemia*Scl* (*Tal1*)

During the rapid growth of the early embryos, a mechanism where simple diffusion provides the necessary nutrients and removes metabolic byproducts for the ever-increasing cell number is converted to a mechanism of circulated transport which is provided by the system of blood and vasculature(McGrath *et al.,* 2003). The first blood cells are found in the yolk sac at embryonic day (E) 7.0-7.5 at which point they appear as islands of blood cells (Palis *et al.,* 1999, Ji *et al.,* 2003). Later on, blood cells are surrounded by the endothelial cells that will form blood vessels. The yolk sac supports primitive blood formation, with these early blood cells being mainly nucleated red blood cells carrying the embryonic and fetal-type hemoglobins that are presumably optimal for the developing embryo and then the fetus. Around 1.5 days after these blood islands appear, blood formation in the embryo is observed at or near the dorsal aorta. Blood-forming activity attributed to hematopoietic stem cells (HSCs) is evident in this region at around E10.5, though some evidence for simultaneous emergence of lymphomyeloid hematopoiesis in the yolk sac, embryo, and placenta (Rhodes *et al.,* 2008). The site of blood formation then migrates to the fetal liver, thymus and spleen, and eventually to the bone marrow. Data showed that many factors play

an important role in HSC migration during embryonic development and are essential for the emergence of intraembryonic hematopoiesis (Ghiaur *et al.,* 2008).

Vasculogenesis occurs in parallel with hematopoiesis in mammals. It is first seen in the embryonic yolk and then later during development in the embryo proper. In the yolk sac, the visceral endoderm elicits soluble signals which target the underlying mesoderm to induce the formation of primitive endothelial and hematopoietic cells. Primitive endothelial and hematopoietic cells combine to form blood islands that then fuse to form a primitive network of tubules known as a capillary or vascular plexus. The close proximity of the two precursor cells and the developmental relationship between the formation of blood and blood vessels suggest a shared parent cell from which both are derived: the hemangioblast (Lancrin *et al.,* 2009). Also, mutations of certain transcription factors in mice and zebrafish will delete both blood cells and blood vessels. Furthermore, the earliest blood cells and the earliest blood vessel cells share many of the same rare proteins on their surface (Choi *et al.,* 1998; Wood *et al.,* 1997).

There are two models used to describe how embryonic blood is developed into adult blood (Ueno and Weissman, 2007). They are called the separate model and the common model, respectively. In the first model it is hypothesized that embryonic blood-forming cells are generated in the primitive streak, proliferated in the yolk sac and migrate to the embryo proper, but are eventually lost. The

adult blood-forming cells are generated in the aorta–gonad–mesonephros (AGM) region followed by seeding of the adult bone marrow later (Samokhvalov *et al.,* 2007). In the second model it is proposed that adult blood-forming cells in the AGM regions at least partly originate from *Runx1*-positive progenitors in the yolk sac (Samokhvalov et al., 2007). The yolk-sac blood islands are formed by multiple progenitors that are mainly already lineage-committed to a blood or an endothelial fate. Some data showed that at least some of these yolk-sac blood cells could be derived from local progenitors with both blood and endothelial fates, which means that there is probably a heterogeneous population of hematopoietic and endothelial progenitors at this stage. However, their relationship to later HSCs is still unknown. To reveal the relative contribution of yolk sac-derived cells to the definitive hematopoietic progenitor cell pool that seeds the fetal liver, Lux *et al.* (Lux *et al.,* 2008) found that the primitive erythroblasts, which circulate in the conceptus and the definitive hematopoietic progenitor cells which seed the liver are all derived from the yolk sac.

In eukaryotes, the generation of diverse cell types depends on mechanisms that establish and maintain specific programs of gene expression. Transcription factors play a central role in this process and act in a combinatorial manner to activate/repress specific sets of target genes in the cellular environment. In the hematopoietic system, differentiation and leukemogenesis are controlled by the combined interactions of transcription factors from diverse families (Enver and Greaves, 1998; Shivdasani and Orkin, 1996; Sieweke and Graf, 1998).

Hematopoiesis is characterized by the production of many distinct cell types through the regulated differentiation of pluripotent HSCs and oligo-potent progenitors (Morrison *et al.,* 1995). Hematopoiesis occurs in successive waves during embryo development and the hematopoietic system represents a powerful model to study the mechanisms through which transcription factors control differentiation and lineage commitment decisions (Orkin, 2000). Drake and Fleming (Drake and Fleming, 2000) examined the temporal and spatial expressions of an array of proteins associated with the hematopoietic and endothelial lineages in the developing mouse embryo, such as *Scl* (*Tal1*), *Flk*, platelet/endothelial cell adhesion molecule (PECAM), CD34, VE-cadherin, and *Tie2*. Their work indicated that Scl^{+}/Flk^{+} cells were specified to form precursors of endothelial cells and Scl^{+}/Flk^{-} cells were specified to form extraembryonic hematopoietic cells. Their data also indicated that *Scl* was expressed earlier than the others such as *Flk, Tie2*. Besides *in vivo* work, Era *et al.,* (Era *et al.,* 2008) have developed novel mouse ESC lines and defined culture conditions that permit the induction of distinct types of mesoderm cells in order to study the divergence of vascular endothelial cell and hematopoietic cell fates.

Gene-targeting studies in mice have identified a series of regulators that function at various stages of hematopoietic cell differentiation and in specific blood cell lineages as well as endothelial lineages. To identify the candidate factors regulating embryonic HSC and endothelial lineage development, many mouse models in which specific genes have been deleted (knock-out models) have been

made. These models have shown that a number of transcription factors are essential for specification of the hematopoietic program and for normal hematopoietic development during embryogenesis. Of note, the *Scl* knockout embryos showed a complete failure of both primitive and definitive hematopoiesis resulting in embryonic death as early as 8.5 dpc (Robb *et al.,* 1995).

The *Scl* gene, also known as *Tal1*, encodes a transcription factor with a basic helix-loop-helix (bHLH) motif. Basic helix-loop-helix transcription factors play a vital role in the regulation of cell-type identities during embryonic development. For example, the MyoD family drives skeletal myogenesis, similar to the regulation of neurogenesis by NeuroD proteins and lymphocyte development by E proteins (Lee, 1997; Quong *et al.,* 2002; Tapscott, 2005). Expression of *Scl* is observed predominantly within the hematopoietic system (Visvader *et al.,* 1991) and is also detected in spinal cord from E9.5 in the developing skeletal system. In addition, its expression is observed in intra- and extra-embryonic as well as adult endothelium (Hwang *et al.,* 1993; Kallianpur *et al.,* 1994). During mouse embryonic development, *Scl* is expressed in intra- and extra-embryonic mesoderm at E7.5, in the yolk sac blood islands at E8.5, and later in adult hematopoietic tissues. The gain-of-function experiments demonstrate that *Scl* can direct early mesodermal cells to form hematopoietic and endothelial progenitors at the expense of other non-axial mesodermal cell fates (Gering *et al.,* 1998). Ectopic expression of *Scl* mRNA in zebrafish embryos showed overproduction of common hematopoietic and endothelial precursors,

perturbation of vasculogenesis as well as concomitant loss of pronephric duct and somitic tissue. However, notochord and neural tube formation were unaffected (Gering *et al.,* 1998). These results also suggested that *Scl* specified formation of hemangioblasts, the proposed common precursor of blood and endothelial lineages.

Besides the important function during embryonic development, *Scl* is also required for adult hematopoietic stem cells and endothelial cell development as $Scl^{-/-}$ ESCs failed to contribute to definitive hematopoiesis in chimeric mice generated with wild-type embryos and failed to contribute to remodeling of the primary vascular plexus in the yolk sac (Visvader *et al.,* 1998). Furthermore, examination of $Scl^{-/-}$ embryoid bodies demonstrated a complete failure of expression of hematopoietic genes (Elefanty *et al.,* 1997). In summary, *Scl* specifies hematopoietic and endothelial lineages development from early mesoderm and acts as a critical regulator of hematopoiesis with a crucial function in the formation of hematopoietic stem cells (HSCs) and in the specification of endothelial precursors (Bloor *et al.,* 2002; Liao *et al.,* 1998).

To further define the function of *Scl* in normal hematopoiesis, some (Robb *et al.,* 1995; Shivdasani *et al.,* 1995) have generated mice with a null mutation of the *Scl* gene. They found that the blood islands and major vitelline vessels were not seen in the yolk sac of $Scl^{-/-}$ embryos by E8.75-E9.0 and $Scl^{-/-}$embryos died around E8.5-9.5. To rescue the hematopoietic and vascular defects due to the

lethal *Scl*$^{-/-}$ mutation, Huss *et al.,* (Huss et al., 2005) injected GFP marked *Scl*$^{+/+}$ adult stem cells into *Scl*$^{-/-}$ blastocysts, but there was no indication of phenotypic rescue of the mutant blastocysts. Sanchez *et al.,* (Sanchez *et al.,* 2001) expressed *Scl* under the control of the *Scl* 3' enhancer and found that expression of exogenous *Scl* rescued the formation of early hematopoietic progenitors and also resulted in normal yolk sac angiogenesis. In contrast, erythropoiesis remained markedly deficient in rescued embryos. This observation is consistent with the inactivity of the stem cell enhancer in erythroid cells and reveals an essential role for *Scl* during erythroid differentiation *in vivo*. Sinclair *et al.* used a yeast artificial chromosome containing the human *SCL* locus and completely rescued the embryonic lethal phenotype of *Scl*$^{-/-}$ mice.

Further research has indicated there are two distinct elements within the *Scl* locus. One is the *Scl* 5' enhancer that directs expression to the endothelium and some embryonic hematopoietic progenitors (Gothert *et al.,* 2004; Sinclair *et al.,* 1999). The other is the *Scl* 3' enhancer which is responsible for *Scl* expression in early hematopoietic progenitors and embryonic endothelium (Sanchez *et al.,* 2001). To address the *in vivo* function of these two elements, two different mouse models were made. An endothelial-*Scl*-*Cre*-ER^{T} mouse model was generated in which endothelial-specific expression of the tamoxifen-inducible Cre recombinase was driven by the 5' endothelial enhancer (Gothert *et al.,* 2004). This transgenic line provides a useful tool for the analysis of gene function in quiescent adult endothelium and during pathologic angiogenesis. Data showed

that Cre-mediated recombination happened in almost all the endothelial cells of the developing vasculature by breeding this mouse line with Cre reporter mice. The same group generated another transgenic mice line, which expressed tamoxifen inducible Cre recombinase under the control of *Scl* 3' enhancer (HSC-*Scl-Cre*-ER^T). Similarly, by breeding with Cre reporter mice, this transgenic mouse line was characterized by high-level specificity within hematopoiesis for HSCs although some activity in endothelial cells was observed as well.

ESCs *in vitro* differentiation assay

ESCs are derived from the inner cell mass of 3-4 day old blastocysts. ESCs are pluripotent and retain the capacity to contribute to all three germ layers when reimplanted back into a blastocyst. This potential, combined with genetic manipulation, has revolutionized many fields by facilitating the ability to generate transgenic, chimeric and knockout mice for gene function studies *in vivo*. In addition to *in vivo* differentiation capability, ESCs can differentiate *in vitro* into complex structures called embryoid bodies (EBs) which contain a number of different cell types. Assay systems have been devised for the detection of a variety of cell types including endothelial, muscle and hematopoietic progenitors. Among these assays, the *in vitro* hematopoietic and endothelial differentiation of mouse ESCs has been extensively examined at both the cellular and molecular levels.

First, mouse ESCs are removed from their feeder layer, and placed in suspension culture in the absence of the growth factor LIF, which promotes maintenance of the pluripotential state (Smith *et al.,* 1988). In 2-4 days, ESCs form aggregates which consist of an outer layer of hypoblast-like cells (extraembryonic visceral endoderm) surrounding an epiblast-like core (primitive ectoderm) (Maye *et al.,* 2004; Rathjen *et al.*, 2002). At this stage, the EB resembles the anterior prestreak stage embryo which is able to generate derivatives of all three primary germ layers: definitive endoderm, mesoderm, and ectoderm (Keller, 2005). The embryoid body core continues to express the ESC marker *Oct4* and begins expressing the primitive ectoderm marker *FGF5 (*Hebert *et al.,* 1991; Rathjen *et al.,* 2002). Between days 6 and 8, the core undergoes cavitation and forms an inner epithelial layer (Coucouvanis and Martin, 1995). Cells within this layer can be committed to definitive ectoderm, characterized by *Sox2* and *Otx2* expression (Maye *et al.,* 2004; Rathjen *et al.,* 2002).

The formation of EBs is the principal step and has been utilized widely as a trigger of *in vitro* differentiation of both mouse and human ESCs. Three basic methods, namely, liquid suspension culture in bacterial-grade dishes (Doetschman *et al.,* 1985) culture in methylcellulose semisolid media (Wiles and Keller, 1991), and culture in hanging drops (Hopfl *et al.,* 2004) are usually used for the formation of EBs to induce the formation of a variety of cell types from ESCs. In addition to the three basic methods, the method using a round-bottomed 96-well plate (Ng *et al.,* 2005) is applied to form EBs from a

predetermined numbers of ESCs.

The method used in this thesis to test the *in vitro* differentiation capability into hematopoietic and endothelial lineages of ESCs is named the “two-step differentiation method” (TEMCELL Technologies). In the first step, ESCs are suspended as single cells in methylcellulose-based medium which promotes their primary differentiation into EBs. This permits determination of the frequency with which differentiating ESCs form EBs and allows quantitation of EBs at various times throughout the primary differentiation. In the second step, EBs are disrupted into single cells and replated in methylcellulose-based medium containing a cocktail of hematopoietic or endothelial cytokines. The various types of hematopoietic progenitors or endothelial cells present in the EBs then grow out into discrete hematopoietic colonies or endothelial cells that are easily identified in the methylcellulose cultures. One alternative is to differentiate ESCs into hemangioblasts which is the common progenitor of HSCs and endothelial progenitor cells according the protocol established in 1998 (Choi *et al.,* 1998).

In Summary

In this thesis, as proof-of-concept, we first tested the possibility of making interspecific chimeras between mouse and rat by the ESC-blastocyst injection method. Data showed that it is possible to produce viable interspecific chimeras

between mouse and rat. Secondly, we tried to rescue the phenotype of endothelial/hematopoietic lineages deficient embryos by injecting rat ESCs into mouse *Scl* null blastocysts followed by embryo transfer. Our data showed no phenotypic rescue of *Scl* null blastocyst. In the meanwhile, rat ESCs were detected in variant organs in 16.5dpc embryos.

CHAPTER 2: Production of Interspecific Chimeras between Mouse and Rat by ESC-blastocyst Injection and a Gall Bladder Formation in a Mouse➔rat chimera

Summary: Since the first experimental mouse-mouse intraspecific chimera was created more than 40 years ago, interspecific chimeric blastocysts produced by combining embryonic cells of two different species have proven useful in investigating embryonic development in rodents. We have injected mouse/rat embryonic stem cells (ESCs) into rat/mouse blastocysts, respectively; followed by embryo transfer. Our data showed that ESCs contributed to xenogenic blastocysts to different degrees at 11.5 dpc, 16.5 dpc and in live interspecific chimeras. ESC-derived cells were found throughout the body and yolk sac with the varying degrees of ESC contribution but never in placenta. Interestingly, the mouse ESCs were able to complement the rat hooded gene mutation. One mouse➔rat chimera showed the formation of a gall bladder suggesting complementation of the unknown mechanism that results in the absence of a gall bladder in rats. Together, these data demonstrate that it is possible to generate donor ESC derived-organs using a xenogenic environment. However, although adult interspecific chimeras could be produced, poor postnatal developments such as shorter life-span as well as kidney and limb deformation in live chimeras were observed.

INTRODUCTION

Chimeric animals were initially produced experimentally by aggregation of two independent embryos of the same species (Mintz, 1962; Tarkowski, 1961). In rodents, several species combinations have been used to make interspecific chimeric blastocysts from aggregated blastomeres (*Mus musculus-Rattus norvegicus, M. musculus-Clethrionomys glareolus, M. musculus-Mus caroli*) (Frels *et al.,* 1980; Rossant *et al.,* 1982; Rossant *et al.,* 1983). There appears to be few species barriers for embryo aggregation since *O. aries-C. hircus* (Fehilly *et al.,* 1984a), *B. indicus-B. taurus* (Williams *et al.,* 1990), and *B. taurus-M.musculus* (Wells, unpublished) blastocysts are readily formed after aggregation of precompaction blastomeres. Although similar efforts resulted in blastocysts *in vitro*, live chimeric animals were not observed from simple blastomere aggregations after embryo transfer to recipient females (Rossant, 1976; Tachi and Tachi, 1980). The lack of development *in vivo* after blastocyst formation may be explained by physiological incompatibilities or by immunological rejection of cells that are heterologous to the recipient female. When chimeras are produced that do not have contribution to trophoblastic lineages, implantation can occur. For example, Rossant *et al*, (1976) showed that interspecific aggregation of ICMs with morula could form apparently normal 5.5 dpc concepti of which a high proportion were inter-specific chimeras. There is

one reported live interspecific chimera produced from injected embryos (Fehilly *et al.,* 1984a), generally referenced as a “geep”. This animal was produced by injection of goat (*Capra hircus*) ICM into a sheep (*Ovis aries*) blastoceole cavity (Fehilly *et al.,* 1984b). Previous observations have shown that foreign cells that contribute to extra-embryonic lineages like trophectoderm or primitive endoderm may inhibit uterine implantation and/or further development (Fehilly *et al.,* 1984a; Rossant *et al.,* 1982; Tarkowski, 1962). Since ESCs can be used to produce chimeras, we hypothesized that ESC injection could be used to replace ICM injection in interspecific chimera production. Recently, Kobayashi *et al.,* (2010) and Isotani *et al.,* (2011) reported viable chimeras between rat and mouse by the use of ESC injection into blastocysts. In addition, Kobayoshi *et al.,* demonstrated rat complementation of a mouse pancreas deficiency. Similarly, Isotani *et al.,* (2011) demonstrated rat complementation of a mouse thymic deficiency. These reports clearly demonstrate that ESCs can be used to produce interspecific chimeras and illustrate the opportunity to study cross-species cell-cell interactions in viable animals.

By injection of mouse/rat ESCs into rat/mouse blastocysts, respectively, we have produced interspecific chimeras from ESCs of both species. Our data confirm that mouse/rat ESCs could survive and proliferate when injected into heterologous blastocysts and were able to produce viable adult interspecific chimeras. Our data showed that ESC-derived cells generally responded in concordance to the host embryo developmental program. However, some

developmental events were influenced by the ESC species-specific developmental program. Although viable interspecific chimeras were produced and some of them survived to adulthood, neonatal deaths and abnormal development after birth were observed. Also our data showed that ESCs may be able to contribute to the organs in the niche formed in a different species. For example, a gall bladder was observed in a mouse➔rat chimera while normally the gall bladders are not formed in rats.

RESULTS

Production of mouse➔rat and rat➔mouse chimeras

The contribution and impact of heterologous ESCs during development was unknown when this project was initiated. Prior to any attempts to obtain viable chimeric offspring, it seemed prudent to evaluate these parameters prior to full-term gestation. In order to investigate ESC contribution in the context of a heterologous host embryo during embryonic development, potential mouse➔rat and rat➔mouse chimeras were collected at 11.5 dpc and 16.5 dpc (Fig. 2.1). ESC contribution was observed in multiple organs and tissues (Fig. 2.2). In addition to contribution to the fetus proper, ESC-derived cells were also observed in the yolk sac but were never observed in placenta. An inability of ESC to contribute to the placenta is consistent with previous observations (Fig. 2.2) (Kobayashi *et al.,* 2010).

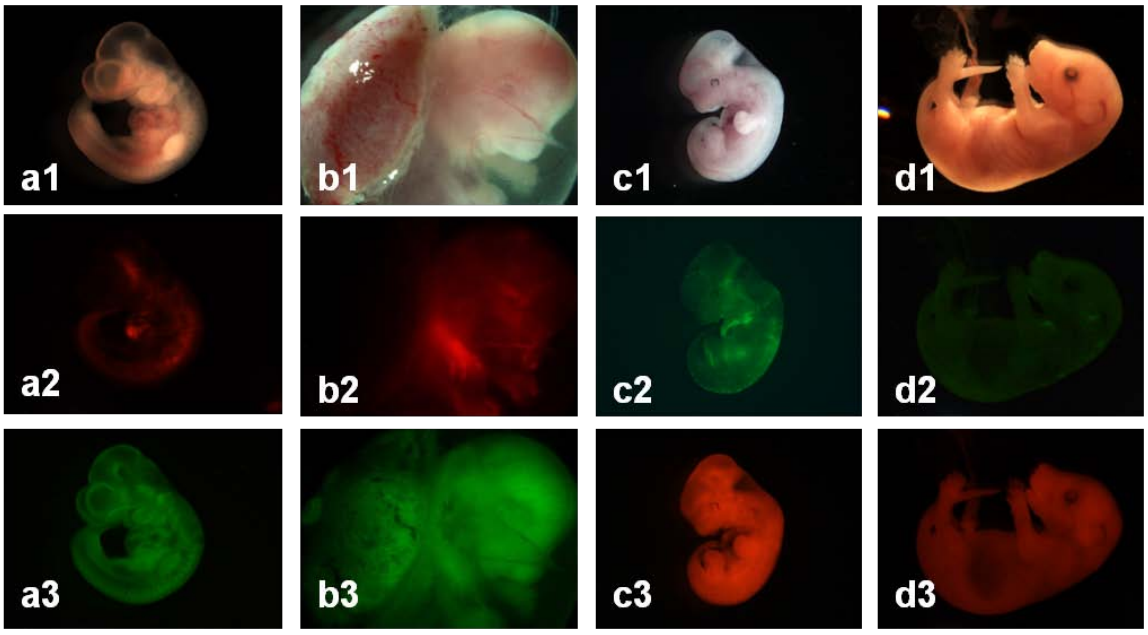


FIG. 2.1. Florescence images for the interspecific fetuses. (a) and (b): mouse➔rat chimeric fetuses, respectively at 11.5 dpc (0.75X) and 16.5 dpc (0.75X). (c) and (d): rat➔mouse chimeric fetuses, respectively at 11.5 dpc (0.75X) and 16.5 dpc (0.375X). As seen in Panels a and b, mouse ESCs (red) were observed all over the body and yolk sac but not in placenta (b). As shown in Panels c and d, rat ESC (green) contribution was observed obviously at 11.5 dpc, however, it was not obvious at 16.5 dpc.

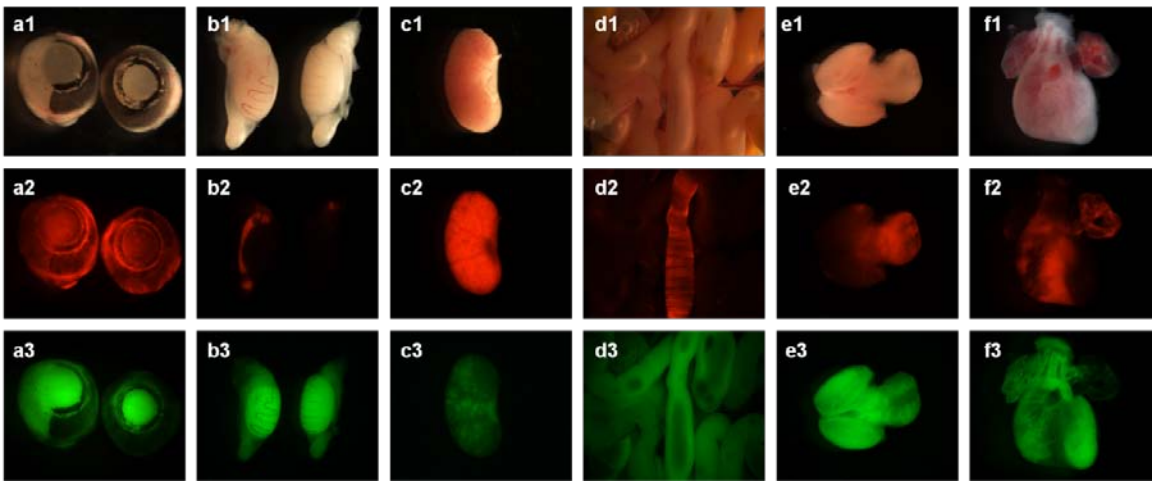


FIG. 2.2. In mouse➔rat chimeras, mouse ESCs (red) contributed to various organs. Panels a, b, c, d: 3-days-old chimera. Panels e and f: 16.5 dpc embryo. First row: Organ amples visualized under brightfield; Second row: Same samples as in first row visualize under TexRed filter. Red color indicates cells of mouse origin. Third row: same samples as in first row visualized under GFP filter. Green color indicates cells of rat origin. (a) Eyes. a1 showed the contribution of mouse ESCs because of observation of black spots which came from the mouse ESCs; (b) Epididymis and testis; (c) Kidney; (d) Intestine; (e) Brain; (f) Heart.

Since no obvious detrimental impact was noted in either type of chimera, an attempt was made to produce chimeric offspring (Supplemental data: Video 1 showed one viable mouse➔rat chimera). Both mouse➔rat and rat➔mouse chimeric offspring were successfully produced based initially on the coat color and confirmed later by fluorescence and PCR. Table 2.1 summarizes the

chimerism rate in mouse➔rat chimeras and i n rat➔mouse chimeras, respectively at 11.5 dpc, 16.5 dpc and live offspring (>12 weeks old).

Table 2.1a: Chimerism in mouse➔ rat chimeras

	Total number of embryos/animals examined	Red Positive: indicating the mouse origin	Red Negative	% of embryos/animals examined that were chimeric
11.5 dpc	21	8	13	38%
16.5 dpc	20	11	9	55%
Live offspring (>12 weeks old)	33	17	16	52%

Table 2.1b: Chimerism in rat➔mouse chimeras

Date	Total number of embryos/animals examined	Green Positive: indicating the rat origin	Green Negative	% of embryos/animals examined that were chimeric
11.5 dpc	24	11	13	46%
16.5 dpc	20	6	14	30%
Live offspring (>12 weeks old)	68	7	61	10%

Our data showed that mouse ESCs contributed widely throughout the body in mouse➔rat chimeras, including heart, vasculature, blood, kidneys and pancreas. However, rat ESCs contributed only to a few organs in rat➔mouse chimeras, for example in brain, lungs and heart. To further confirm interspecific chimerism, genomic DNA of various tissues/organs from adult mouse➔rat chimeras was collected and genotyped by PCR with the primers specific for DsRed (mouse) and EGFP (rat). The genetic contribution of the ESCs in a variety of tissues was evident by the ability to detect the presence of the DsRed or EGFP gene by PCR

(Fig. 2.3). Although expected based on fluorescence, these data independently confirm chimerism.

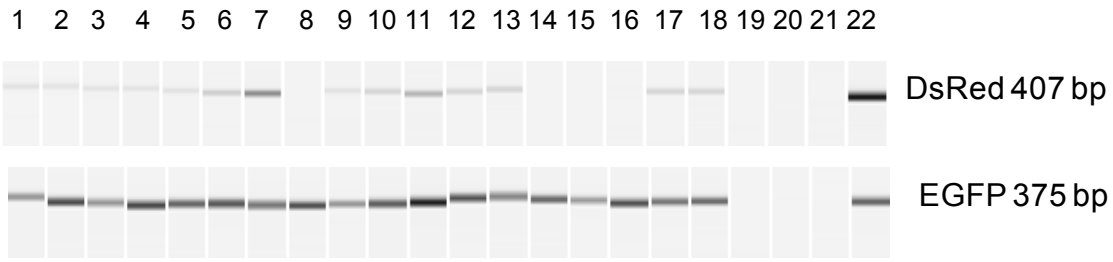


FIG. 2.3. PCR results confirm the mouse ESC contribution (DsRed) in mouse➔rat chimeras.

Line 1: Spleen; Line 2: Pancreas; Line 3: Bladder; Line 4: Liver; Line 5: Adrenal; Line 6: Kidney (right); Line 7: Kidney (left); Line 8: Eye; Line 9: Thymus; Line 10: Stomach; Line 11: Intestine; Line 12: Uterus; Line 13: Ovary; Line 14: Brain; Line 15: Tongue; Line 16: Heart; Line 17: Lung; Line 18: Thyroid; Line 19: DI water; Line 20: Blank; Line 21: Blank; Line 22: Positive control.

As an additional measure of chimerism, the contribution of ESCs in the peripheral blood was quantified. The peripheral blood was collected and analyzed by FACS. The data showed that there was a range of 0.02% to 73.6% of mouse ESC-derived cells (DsRed positive) in the peripheral blood samples

obtained from mouse➔rat chimeras (Fig. 2.4). A video of blood flow under the red fluorescence added more evidence (Supplemental data: Video 2). The percentage of mouse ESC contribution in the peripheral blood had a positive correlation with the coat color contribution (Fig. 2.4). In contrast to mouse ESCs, no rat ESC-derived cells (GFP positive) were observed from peripheral blood samples obtained from rat➔mouse chimeras (data not shown). Similarly, low levels of coat-color chimerism were observed in rat➔mouse chimeras.

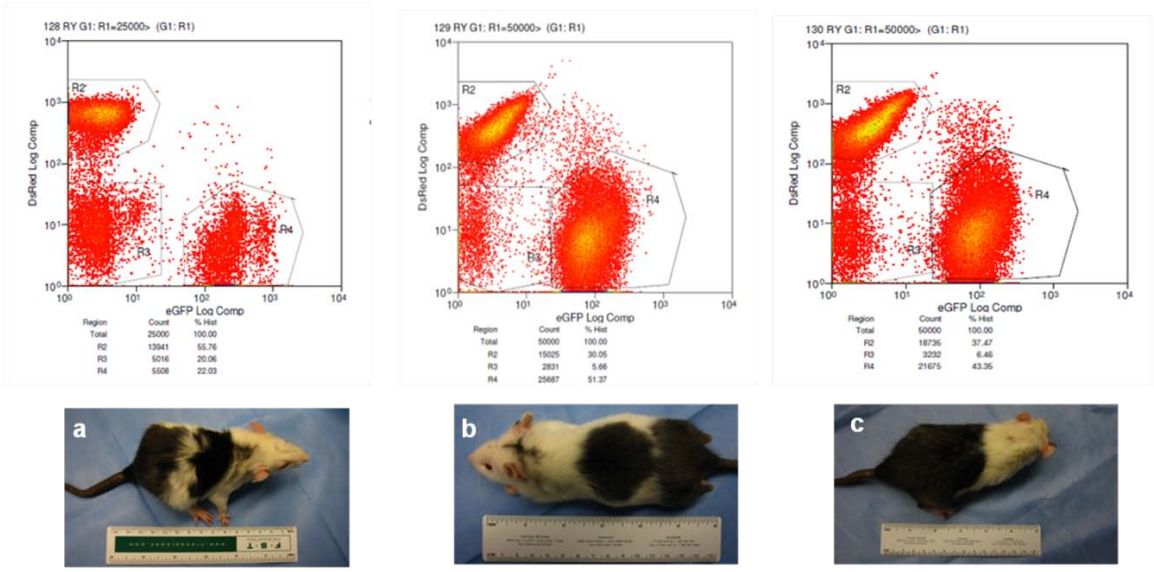


FIG. 2.4. FACS results showed the contribution of mouse ESCs in the peripheral blood samples from mouse➔rat chimeras. The percentage of mouse ESC contribution in the peripheral blood had a positive correlation with the coat color contribution. R2: DsRed positive; R3: dead cells or non-labeled cells; R4: EGFP positive. (a, b, c) showed the pictures of individual chimeric animals corresponding the FACS data. Of note, the chimera in (c) had a gall bladder.

Typically, adult rats are ten times larger than adult mice. In theory and also in congruence with our observation, the size of newborns was determined by the species of the host blastocyst/recipient female. However, the size of adult chimeras varied. Our data showed that the size of mouse➔rat chimeras, in general, was smaller than rats, while the size of rat➔mouse chimeras was similar to that of mouse. A correlation between body weight and ESC contribution was estimated (Fig. 2.5). Particularly, in mouse➔rat chimeras, the more black hair (mouse ESC-derived cells) observed in the coat color, the smaller the chimera. In other words, chimeras with high mouse contribution to coat color looked more like a mouse. However, one mouse➔rat chimera which had over 50% black hair on the back showed similar size to a control rat (Fig. 2.5c).

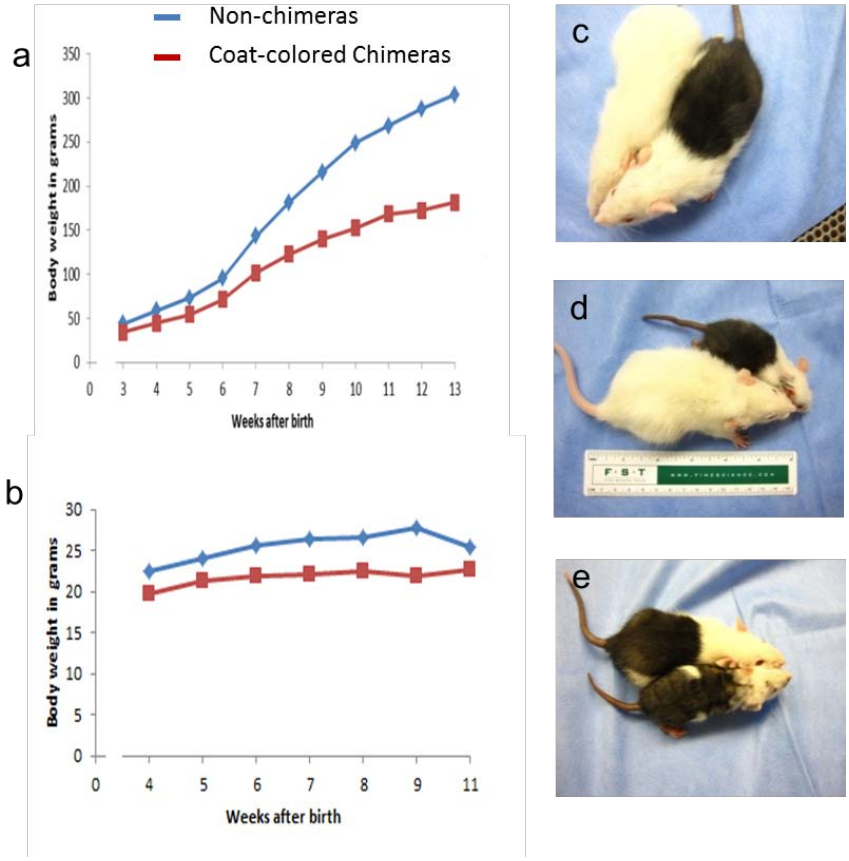


FIG. 2.5. Comparison of Average body weights at different ages among mouse➔rat chimeras. (a) Average body weight of one litter of mouse➔rat chimeras. (b) Average body weight of one litter of rat➔mouse chimeras. (c, d, e) pictures of some mouse➔rat chimeras which showed the black hair (from mouse ESCs) were located in the rear half of the body and also showed that contribution of mouse ESCs contribute to the size of the chimeras as well. (c and d) two mouse➔rat chimeras showed similar percentage of mouse ESC contribution

based on the coat color change, while one had a similar size as a rat (c) while another one is much smaller than a rat (d).

Complementation of hooded mutation in mouse➔rat chimeras

For all experiments, the ESCs and the host embryos harbored genetics for different coat colors. The rat blastocysts used for the mouse➔rat chimeras carry the hooded mutation and a tyrosinase mutation which give rise to rats with an albino coat. The mouse ESCs in these experiments have a 129S6xB6 genetic background which gives rise to mice with a black coat. As expected, mixed coat-color of black patches and white patches in the mouse➔rat offspring was observed (Fig 2.4 and 2.5). Interesting, almost all mouse➔rat chimeras showed a color pattern that was opposite of the hooded phenotype (Fig 2.4 and 2.5). In hooded rats, the hooded allele delays the migration of melanocytes from the neural crest (Yamamura and Markert, 1981). Consequently, the areas furthest from the dorsal midline are not populated by melanocytes and those areas produce unpigmented, white hair. In mouse➔rat chimeras, it seems that mouse ESC-derived melanocytes migrated only or preferentially to the areas where the rat melanocytes did not migrate, such as the rear, back, and the belly. Areas such as the face and shoulders, rarely displayed black hair. Our hypothesis is that, the endogenous, albino rat melanocytes out-competed the ESC-derived mouse melanocytes in the compartment that would normally be filled by rat

melanocytes in a hooded rat. The remainder of the melanocyte niche was then filled with mouse melanocytes.

Interestingly, all of the rat➔mouse chimeras showed a small patch of white hair (from rat ESCs) on the forehead only: no obvious white hair was observed elsewhere on the body. Since the rat ESC line used in these studies has a SD genetic background it is not known as to whether this line carries a hooded mutation. Albino hooded rat ESCs would be expected to affect coat color primarily over the head and shoulder regions.

Gall bladder formation in a mouse➔rat chimera

One of the most noticeable differences in organ morphogenesis between the mouse and the rat is that the mouse has a gall bladder while the rat does not. Kobayashi's group (2010) argued that the host blastocysts seem to impose additional morphogenetic regulations besides controlling the size and growth of the chimera. They offer this possibility as a mechanism to explain why the adult mouse➔rat chimeras produced did not have gall bladders despite a significant contribution from mouse ESCs. Their hypothesis is that the host ICM cells have a "morphogenetic" capacity that controls the behavior of injected stem cells at all developmental stage.

However, one mouse➔rat chimera produced here did have a gall bladder of mouse ESC origin (Fig. 2.6) suggesting that mouse cells are able to fill the gall bladder developmental niche during embryonic organogenesis. Several factors were considered to conclude that the mouse derived structure (it displayed DsRed fluorescence which indicated mouse ESC origin) was a bona fide gall bladder 1) it was found in the correct location, 2) a bile-like liquid inside the structure was observed and, 3) histological examination showed that the architecture of the gall bladder wall was composed of a serosal layer of dense collagen, two circumferential bands of muscle, and an inner collagenous layer (Fig. 2.6). The architecture of the mouse-in-rat gall bladder had many similarities to a mouse gall bladder, including the thin muscular smooth muscle wall and simple columnar epithelial mucosa. Given all the evidence in addition to the fact that rats do not produce gall bladders, this gall bladder was suggested to be of mouse origin. Of particular note was the pancreatic chimerism in the same mouse➔rat chimera which had a gall bladder, in that more than 95% (based on visual appraisal, no quantitative measuring method was used) of this pancreas showed red fluorescence indicating that mouse ESCs gave rise to almost the whole pancreas (Fig. 2.6).

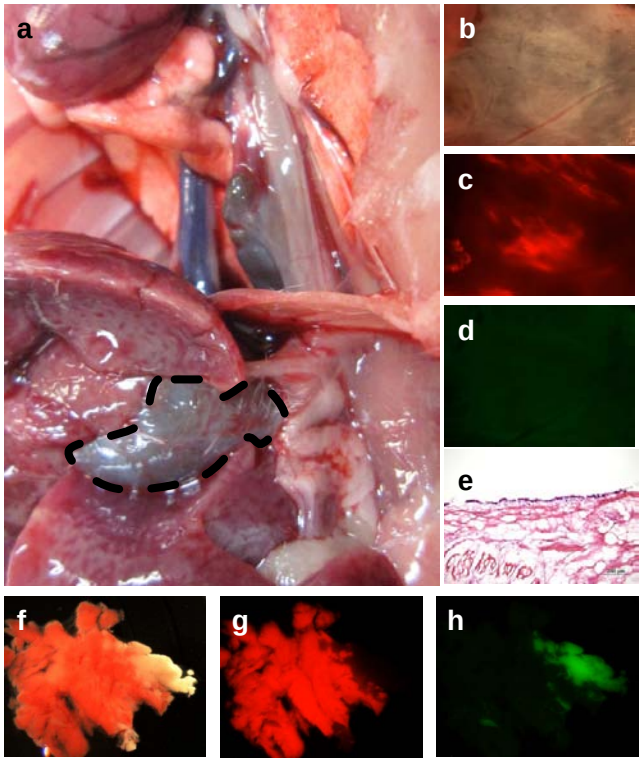


FIG. 2.6. A Gall bladder was observed in one mouse➔rat chimera (a, in the black frame). (b) the gall bladder under the normal light. (c) the gall bladder showed red florescence. (d) the gall bladder did not show green florescence. (e) Histology exam showed similar structure as that of a mouse gall bladder. (f, g, h) the same mouse➔rat chimera had a pancreas in which majority of it was derived from mouse cells (red positive).

To analyze the contribution of ESCs in the mouse➔rat chimeras, various organs (brain, heart, lung, kidney and testis) were frozen and examined by a confocal imaging system. The confocal images (Fig. 2.7) showed that red fluorescent cells (mouse ESC-derived cells) were present in many organs in mouse➔rat chimeras and the individual cells were either red (mouse origin) or green (rat origin) but

never both. Even though different degrees of contribution from mouse ESCs were observed, germ cells in testicle or ovary never showed any red fluorescent cells indicative of mouse cells (Fig. 2.7). As the injected mouse ESCs were confirmed as germ-line-competent stem cells in intraspecific settings, these results suggest an incompatibility in the signals/response during germ cell induction, migration, or development between these two species.

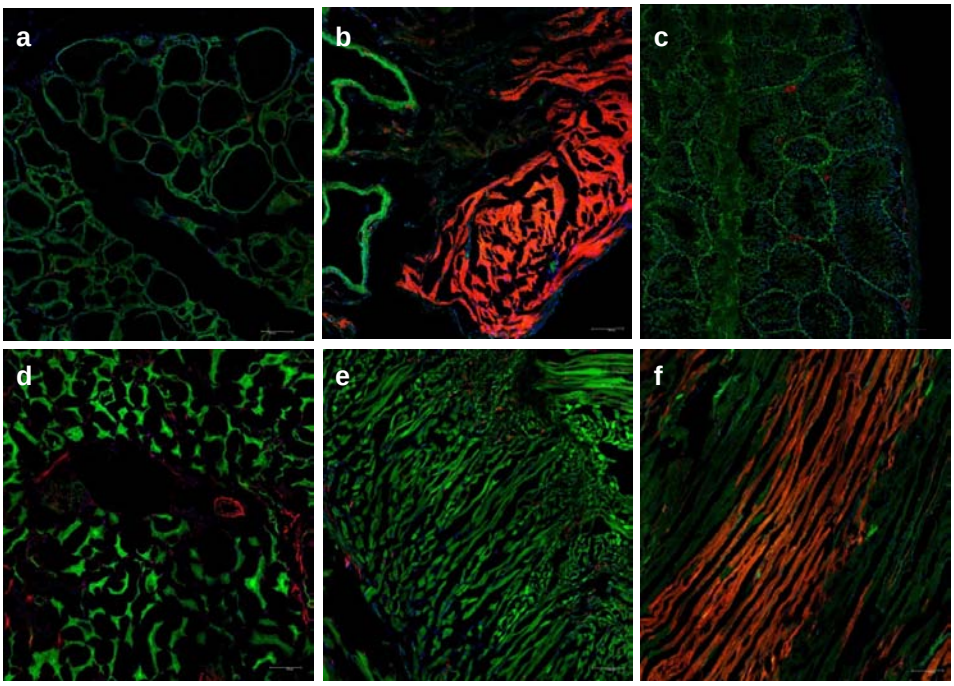


FIG. 2.7. Confocal pictures showed the cells from mouse origin (red) and cells from rat origin (green) in different organs collected from one mouse➔rat chimera in which half of coat color was black. This chimera was healthy and behaved normal. Cells in these pictures are either red or green but never both. (a): thyroid (b): bladder (c): testis (d): kidney (e): tongue (f): heart. All images are taken at 200X magnification.

Abnormality of interspecific chimeras

Although adult interspecific chimeras were produced, poor postnatal development, such as slow growth, slow weight gaining was frequently observed, especially in mouse➔rat chimeras. With greater coat color contribution, more abnormalities were observed. In mouse➔rat chimeras, because of the poor postnatal development, four chimeras did not survive to the weaning date (3 weeks old), three chimeras were euthanized at eight weeks old due to the poor development, and only three of seventeen total chimeras (18%) survived to over 14 weeks old and appeared healthy which was consistent with previous report (Kobayashi *et al.,* 2010). However, all rat➔mouse chimeras grew to adulthood (over 8 weeks old).

In addition to the short life-span, mouse➔rat chimeras commonly displayed kidney abnormalities. One mouse➔rat chimera had an absence of kidneys at birth. Some offspring showed abnormal kidney formation (Fig. 2.8). For example, severe hydronephrosis was seen frequently (12 out of 17). Histology showed interstitial fibrosis, lymphoid infiltrates, tubular regeneration and occasional tubules containing degenerated cells and proteinaceous fluid in the areas of the cortex to the renal papilla were noted (Fig. 2.8).

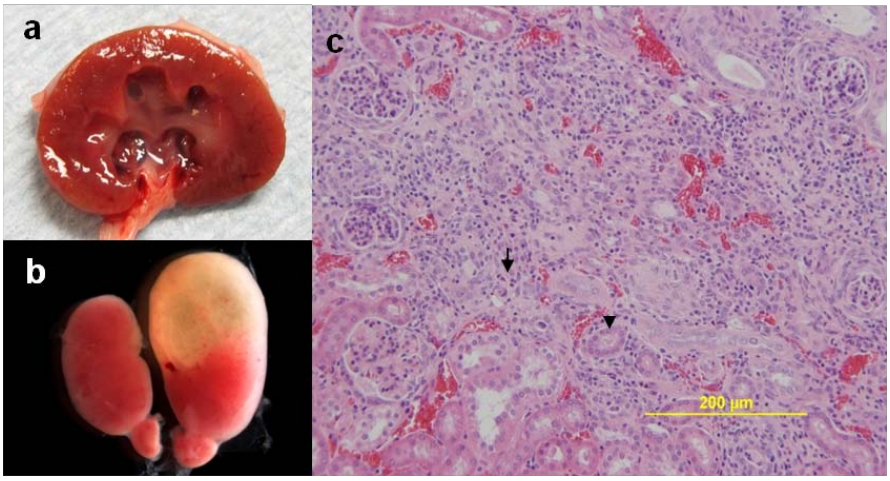


FIG. 2.8. Kidney abnormality observed in 12 out of total 17 mouse➔rat chimeras. (a): mild hydronephrosis. (b): one of two kidneys in one mouse➔rat chimera showed severe hydronephrosis. (c): histology for the abnormal kidney. The arrow head indicates the regenerating tubules with close epithelial cells. The arrow indicates the cells in between the tubules which are lymphocytes and fibroblasts making collagen (fibrosis). The scale bar in (c) represents 200 μm.

Abnormal limb formation was also observed. The percentage of this abnormality among chimeras was 14% (1 out of 7 rat➔mouse chimeras) and 88% (15 out of 17 mouse➔rat chimeras) (Fig. 2.9) respectively. Mouse➔rat chimeras presented more severe abnormalities in organ development than rat➔mouse chimeras. This observation is likely due to the degree of ESC contribution in

mouse➔rat chimera. For example, every mouse➔rat chimeras with limb abnormalities showed over 50% coat color contribution (based on visual appraisal, no quantitative measuring method was used). Given the fact that mouse ESCs did not affect rat embryonic development in the early stage at 11.5 dpc and 16.5 dpc, this limb-deformation might be the result of aberrant interspecies cell interactions during later embryonic or postnatal development. This deformation may be indirectly responsible for the slow weight gain observed in mouse➔rat chimeras before and after weaning since it may have negatively impacted the animal's ability to feed properly.

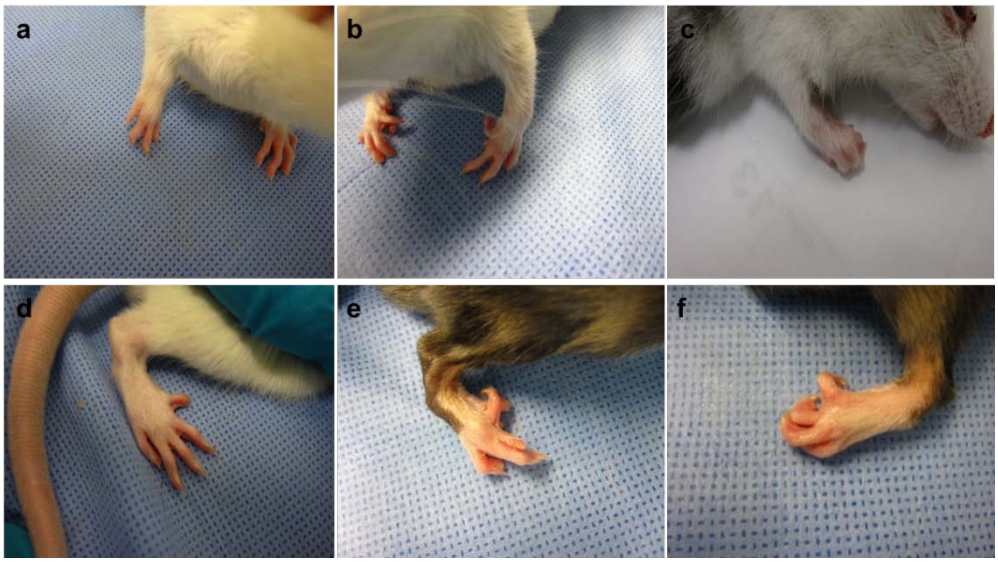


FIG. 2.9: Skeletal abnormalities observed in mouse➔rat chimeras. The percentage of this abnormality among chimeras was 88% (15 out of 17 mouse➔rat chimeras). (a) normal front legs/toes (b, c) abnormal front legs/toes. (d) normal back legs/toes. (e, f) abnormal back legs/toes. (b, c, d, e) all these

four animals showed over 50% black coat color which was contributed by mouse cells (based on visual appraisal, no quantitative measuring method was used).

One mouse➔rat chimera of which the coat color change was over 60% (subjective observation) showed many irregular bumps in abdominal organs. Histology analysis showed that heterogenous basophilic cells such as neoplastic basophilic cells, plasma cells and plasmacytoid cells infiltrated almost all the sections examined from abdominal organs, such as ileum, kidney, liver, pancreas, thymus, lymph nodes and pancreas (Fig. 2.10). For example, the architecture of the lymph nodes is replaced with noncohesive basophilic rounds cells with multifocal clusters of large eosinophilic to amphophilic epithelioid cells. The noncohesive basophilic cells infiltrated the subcapsular sinus and the capsule as well as the mesentery adjacent to the lymph nodes. The cellular infiltrates observed had a tissue distribution pattern and morphologic appearance similar to immunocytomas which is described as a tumor primarily of the Louvain (LOU) rat strain and is also called plasmacytomas in mice and people.

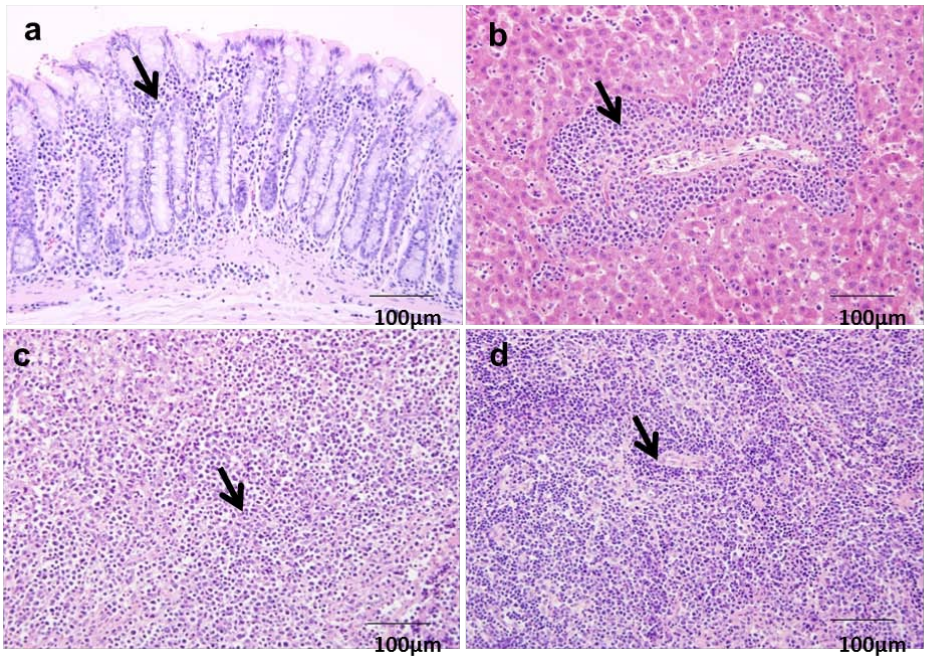


FIG. 2.10. Only one mouse➔rat chimera showed plasmacytomas. Histology analysis showed that heterogenous basophilic cells (see arrows) such as neoplastic basophilic cells, plasma cells and plasmacytoid cells infiltrated almost all the sections examined from abdominal organs, such as intestine (a), liver (b), lymph nodes (c) and spleen (d). The scale bars in (a, b, c, d) represent 100 μm.

The degree of chimerism had a positive correlation with the organ abnormality and a negative correlation with the body weight based on the observation in mouse➔rat chimeras. The rat➔mouse chimeras do not show these correlations. As described earlier, it is likely that the difference is due to the extent of chimerism since a high degree of rat ESC contribution to chimera was not observed.

DISCUSSION

Although the first interspecific chimera between sheep and goat, “geep”, was produced almost 30 years ago, production of live chimeras between mouse and rat was only recently reported (Isotani *et al.,* 2011; Kobayashi *et al.,* 2010). As previous work indicated that aggregation of early embryos would lead to trophectoderm formation of xenogenic cells that are harmful to embryonic development during the uterine implantation, the use of ESC circumvented this problem (Beddington and Robertson, 1989). Recently, one Japanese group reported the first interspecific chimera between mouse and rat by injecting mouse/rat ESCs into the rat/mouse host blastocysts. Here, by injection of ESCs into blastocysts from the different species, both mouse➔rat and rat➔mouse interspecific chimeras were produced. Our data showed that mouse/rat ESCs can survive, proliferate and differentiate in developing interspecific blastocysts. It has been demonstrated that these interspecific chimeras are viable and can survive until adulthood albeit with some abnormalities. As observed by others, in mouse➔rat chimeras, mouse ESC-derived cells were distributed throughout the body, such as brain, liver, heart, kidney and intestine *etc.* (Fig. 2.2) during fetal and adult development after being injected into rat blastocysts, while these mouse ESC-derived cells were never observed in placenta (Fig. 2.1). In rat➔mouse chimeras, rat ESC-derived cells were distributed to several organs including the brain, pancreas and adrenal but were not observed in other organs or placenta.

In summary, our data showed that ESCs can differentiate into pancreas (endoderm), blood (mesoderm) and hair (ectoderm) after being injected into a blasctocyst from different species. Our data suggest that this approach may be used as a tool to test the pluripotency of new ESC or iPSC lines by injection of cells into mouse or rat blastocysts followed by embryo transfer. It is also possible to inject human ESCs or iPSCs into a mouse (James *et al.,* 2006) or rat or even pig blastocysts and to allow early embryonic development of human-animal chimeras that could potentially be used to test the differentiation capacity of human pluripotent cells in addition to conventional teratoma formation experiments. Human-animal chimeras may prove useful in pre-clinical applications such as drug testing *in vivo*. However, extensive consideration of the ethical and social issues is warranted prior to initiation of such experiments. Some of these issues may be addressed with stem cells that have genetic modifications that severely limit their developmental potential to restriction their contribution to predetermined tissue compartments.

One most noticeable difference between a rat and a mouse is the size. Previous data suggested that the size of chimeras was somehow determined by the species of the host blastocysts, which is consistent with our observation that newborn chimeras were the size expected for the species of the host blastocyst and surrogate. However, our data also showed that the percentage of coat color chimerism correlated with the size of adult chimeras. For example, the

rat➔mouse chimeras have similar size as of mouse and little contribution of rat ESCs based on the coat color change was observed accordingly. The mouse➔rat chimeras are much smaller than a rat but larger than a mouse. In this case, the greater the contribution of mouse ESCs throughout the body, the closer the chimeras were to the size of a mouse. In mouse➔rat chimeras, there is a negative correlation between contribution of ESCs and body weight (Fig. 2.5). There were two mouse➔rat chimeras that were rat-sized when they had small percentage of contribution of mouse ESCs (a tiny patch of black hair on the junction of hips and tail). Even though it is suggested that the contribution of donor cells somehow was involved in determination of the extent of chimera growth, it remains to be elucidated whether it is the relative contribution of the embryo itself, the uterine environment, or the donor cells in size determination. Since immunogical compatibiltiy is required between the placenta and the surrogate, transfer of chimeric embryos to surrogates of the ESC species is not a possibility. Previous research showed that such experiments have failed because of the need for compatibility between the placenta and the uterus (Rossant *et al.,* 1982). Further insight into the role of the uterine environment may be gained by injection of mouse ESCs into rat tetraploid embryos since tetraploid cells are not able to contribute to the embryo itself, but instead create the primitive endoderm derivatives and the trophectoderm (Tarkowski *et al.,* 1977). Due to cell complementation, complete segregation of descendants of ESCs and tetraploid cells is achieved, resulting in fetuses and viable offspring that are completely derived from mouse ESCs (Nagy *et al.,* 1990; Nagy *et al.,* 1993). However, a

fetus resulting from such an experiment is unlikely to be chimeric. Functionally, this experiment would place a mouse fetus in a rat placenta that is gestated by a rat uterus.

Our data also suggested that the hooded gene mutation in rats was complemented by mouse ESCs. In hooded rats melanocytes are restricted to the head, shoulders, and along the central, dorsal line of the body. The rat blastocysts used to produce the mouse➔rat chimeras carried the hooded mutation and a tyrosinase mutation giving rise to rats with an albino coat, while the counterpart mouse ESCs with 129S6XB6 background give rise to mice with a dark coat. Most mouse➔rat chimeras produced had black hair (from mouse ESCs) in the rear back, feet or the belly where hooded rats do not have pigmented cells. Thus, our hypothesis is that the empty melanocyte niche produced from the rat hooded mutation was complemented by the mouse ESCs. Similarly, gall bladder formation was also observed in one mouse➔rat chimera. Normally, the postimplantation development of rat and mouse embryos is very similar, but there are differences in organ-morphogenesis. One of the noticeable differences is the presence of a gall bladder in mice and its absence in rats. Kobayashi's mouse➔rat chimeras were ''rat-like'' in that the gall bladder was absent even with a significant contribution from mouse cells to abdominal organs. They speculated that cells of the ICM possess a ''morphogenetic'' capacity that controls the behavior of injected stem cells at all developmental stages. In contrast to Kobayashi's group, formation of a gall bladder in a mouse➔rat

interspecific chimera was observed here even though it was an uncommon event (1 out of 17) (Fig. 2.6). The presence of a gall bladder suggests that it is possible for mouse ESCs to supplement the host development program. The mechanism that omits a gall bladder from the normal development program in rats is not known. It is possible that a morphogenic signal is absent and gall bladder formation is not initiated. It is also possible that a development signal is present to initiate gall bladder formation, but rat cells cannot respond. Since a single mouse➔rat chimera was observed in this study that harbored a gall bladder, these two possible mechanisms cannot be settled. However, since rat cells were not observed in the gall bladder in this case, our observations are consistent with a mechanism that involves the ability of rat cells to respond to developmental quest that initiate gall bladder formation.

In conclusion, the interspecific chimeras between mouse and rat can be produced and can grow to adulthood. These chimeras could potentially be used to solve many fundamental biological questions as well as have utility in the clinical arena in areas such as xenotransplantation. All in all, the approach discussed here could be an initial step toward future regenerative medicine.

MATERIALS AND METHODS

Animals

F344-Tg (EGFP) F455 rats (RRRC 00307) were obtained from the Rat Resource and Research Center (RRRC) at University of Missouri. Wild type F344 females were purchased from Charles River. CD recipient rats were purchased from Charles River. SD females were purchased from Harlan. Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J (Stock #007576) were purchased from the Jackson Laboratory. FVB mice were purchased from Taconic and r ecipient mice (CD1) were purchased from Charles River. All animal procedures were performed with an approved University of Missouri Animal Care and Use (ACUC) protocol.

Mouse and rat ESC Culture and Maintenance

Rat ESC line SD-Tg.EC8/Rrrc was used. This ESC line was derived from stock SD-Tg(GFP)2BalRrrc (Lois *et al.,* 2002) obtained from the Rat Resource and Research Center (RRRC#0065). SD-Tg.EC8/Rrrc has stable GFP expression, a normal male karyotype, expresses the stem cell markers *Oct 4*, *Nanog*, and *Sox2* and was able to generate chimeras when injected into SD X DA blastocyst (Men *et al.,* submitted). The ESC line was cultured in N2B27+2i rat ESC media described previously (Buehr *et al.,* 2008).

Mouse DsRed ESC line (DsRed transgene) was obtained from MMRRC (stock #011988-MU, University of Missouri-Columbia, Columbia, MO). Undifferentiated

mouse ESCs were grown on mouse embryonic fibroblasts cells in Dulbecco's minimal essential medium (DMEM) (Invitrogen, Carlsbad, CA), supplemented with 15% ES-qualified fetal bovine serum (Hyclone, Logan, UT), 0.1 mM nonessential amino acids (Gibco BRL), 2.0 mM glutamine (Gibco BRL), 1mM sodium pyruvate (Gibco BRL), 100μg／ml penicillin／streptomycin solution (Gibco BRL), 0.1 mM 2-β-mercaptoethanol (Sigma), and 1000 U/ml LIF (Chemicon, Temecula, CA). Cells were passaged every two days and maintained at 37°C and 5% CO_2. This cell line has been characterized previously (Vintersten *et al.,* 2004)

Production of chimeras using ESC-blastocyst injection

Rat blastocysts were collected at 4.5 dpc from wild type F344 females naturally mated with homozygous F344-Tg (EGFP) F455/Rrrc male with rat 2-cell embryo culture medium (Oh *et al.,* 1998). Female mice were superovulated following a standard protocol (Legge and Sellens 1994). Female mice were administered 5 units PMSG (pregnant mouse serum gonadotrophin) through I/P injection followed by 5 units hCG (human chorionic gonadotrophin) 48 hours later. Blastocysts were collected at 3.5 dpc from FVB females mated with homozygous Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J male with FHM media.

Micromanipulation dishes will be prepared using HEPES buffered rat (2-cell media) or mouse flushing medium (FHM) under mineral oil. Embryos were graded and sorted based on formation and expansion of the blastocoel cavity. Groups of 15-20 embryos were handled and each embryo received 8-15 ESCs.

ESC-blastocyst injection was done using a standard protocol (Bradley *et al.,* 1984).

On the injection day, ESCs were prepared in 60mm cell culture dishes. ESCs were trypsinized into single cell suspension and resuspended into ESC injection media (added 200 µl of 1 M HEPES to 10 ml of ES medium) and stored on ice for 30 minutes. To remove the dead cells, ¾ of the medium were depleted and the cells were resuspended in new cold ESC injection medium. These cells were maintained for injection on ice for 3-4 hours.

Blastocysts transfer was accomplished by standard surgical embryo transfer procedures into pseudopregnant recipients at 2.5 dpc (mouse, naturally mated) or 3.5 dpc (rat, naturally mated), respectively. Uterine embryo transfer was performed immediately after the completion of a replicate of blastocyst injections. The surrogates were dissected at E11.5 and E16.5 or allowed to go to term.

Genotyping

To detect the GFP transgene, primers GFP F: 5'- CGC ACC ATC TTC TTC AAG GAC GAC-3' and GFP R: 5'-AAC TCC AGC AGG ACC ATG TGA TCG-3' were used. PCR conditions were 94 ^{o}C for 3 minutes, then 94 ^{o}C for 30 seconds, 60 ^{o}C for 30 seconds and 72 ^{o}C for 1 minute for 35 cycles followed by 72 ^{o}C for 10 min in a 20 µl mixture containing 1 µl (~25ng) DNA (extracted from organs), 0.2µl (1 unit) of FastStart *Taq* DNA Polymerase (Roche), 0.2µl (1µM) of each primer, 3.6

µl (4.5 mM) dNTP, 2µl of 10x reaction buffer. Amplicons of 375 bp were detected on the Qiaxcel (Qiagen) with the Qiaxcel DNA Screening Kit (Cat# 929004). Alignment Marker (QX Alignment Marker 15bp/1kb, Cat# 929521, Qiagen) and size Marker (QX DNA Size Marker 50bp-800bp, Cat# 929556, Qiagen) were used.

To detect the DsRed transgene, primers DsRed F: 5'- TGT CCC CCC AGT TCC AGT AC-3' and DsRed R: 5'-GTCCACGTAGTAGTAGCCGG-3' were used. PCR conditions were 94 ^{o}C for 3 minutes, then 94 ^{o}C for 30 seconds, 58.8 ^{o}C for 30 seconds and 72 ^{o}C for 1 minute for 35 cycles followed by 72 ^{o}C for 10 min in a 20 µl mixture containing 1 µl (~25ng) DNA (extracted from organs), 0.2µl (1 unit) of FastStart *Taq* DNA Polymerase (Roche), 0.2µl (1µM) of each primer, 3.6 µl (4.5 mM) dNTP, 2µl of 10x reaction buffer. Amplicons of 407 bp was detected on the Qiaxcel (Qiagen) with the Qiaxcel DNA Screening Kit (Cat# 929004). Alignment Marker (QX Alignment Marker 15bp/1kb, Cat# 929521, Qiagen) and size Marker (QX DNA Size Marker 50bp-800bp, Cat# 929556, Qiagen) were used.

Flow cytometry

Whole peripheral blood samples were collected immediately after euthanizing the animals. For every 200 µl of a whole blood sample, 2 ml lysing buffer (BD Biosciences Cat. No. 349202) was added followed by 15 minute incubation at room temperature. The samples were then centrifuged at 200*g* for 5 minutes

and then washed with PBS once. The cell pellets were resuspended in cell dissociation buffer (GIBCO) and were analyzed by Beckman Coulter CyAn ADP High-Performance Flow Cytometer at the CIC Center at the University of Missouri-Columbia.

Embedding and sectioning of samples

All samples were dissected in PBS and fixed overnight at 4°C in 4% paraformaldehyde. Embedding in O.C.T was done 24 hours later on dry ice. Confocal pictures were taken using a Leica SPE II microscope equipped with EGFP and Texas Red filter sets.

CHAPTER 3: Generation of *Scl* null Embryos

Summary: The first blastocyst complementation experiment was reported in 1993 in which mouse ESCs complemented the deficiency of T and B lymphocyte lineages in *Rag2* knockout mouse embryos (Chen *et al.,* 1993). Recently, another report showed the generation of rat pancreas in *Pdx* knockout mice that are pancreas deficient (Kobayashi *et al.,* 2010). It has been suggested that organ deficient embryos may provide a developmental niche for ESCs from the same or different species to complement the deficiency (Kobayashi *et al.,* 2010). Our work has proven that interspecific chimeras (mouse➔rat or rat➔mouse) can be made by the ESC-blastocyst injection method and mouse/rat ESCs contribute to different organs and tissues in the interspecific chimeras. Based on the principle of blastocyst complementation and the successful production of interspecific chimeras, we hypothesized that rat ESCs could complement the deficiency of vascular and hematopoietic lineages in *Scl* knockout mouse embryos which otherwise die at embryonic day 8. This chapter describes the method we used to produce *Scl* knockout embryos.

INTRODUCTION

Basic helix-loop-helix (bHLH) transcription factors are crucial regulators, also referred to as 'master regulators' of cell-type identities. The stem cell leukemia gene, *Scl* (also called *Tal-1*), encodes a basic helix-loop-helix transcription factor which plays a pivotal role in both hematopoiesis and endothelial development. Its function is similar to the role of the *MyoD* family during skeletal myogenesis and the regulation of neurogenesis by NeuroD proteins and lymphocyte development by E protein (Lee, 1997; Quong *et al.,* 2002; Tapscott, 2005). *Scl* expression is observed predominantly within the hematopoietic system (Green *et al.,* 1992; Visvader *et al.,* 1991) both during embryonic development and adult development. In the meantime, it also is observed in intra- and extra-embryonic and adult endothelium (Green *et al.,* 1992; Hwang *et al.,* 1993; Kallianpur *et al.,* 1994). During mouse embryonic development, *Scl* is critical for the generation of all hematopoietic lineages and normal yolk sac angiogenesis. For example, *Scl* is expressed in intra- and extra-embryonic mesoderm as early as E7.5, in the yolk sac blood islands at E 8.5, and thereafter in adult hematopoietic tissues (Green *et al.,* 1992). The gain-of-function experiments demonstrate that *Scl* can program

early mesodermal cells to form hematopoietic and endothelial progenitors at the expense of other non-axial mesodermal cell fates (Gering *et al.,* 1998). Ectopic expression of *Scl* mRNA in zebrafish embryos leads to overproduction of common hematopoietic and endothelial precursors (Gering *et al.,* 1998).

With further investigation, two distinct elements within the *Scl* locus were discoved. One is the *Scl* 5' enhancer which directs expression to the endothelium and some embryonic hematopoietic progenitors (Gottgens *et al.,* 2004; Sinclair *et al.,* 1999); the other is the *Scl* 3' enhancer which is responsible for *Scl* expression in early hematopoietic progenitors and embryonic endothelium (Gottgens *et al.,* 2002; Sanchez *et al.,* 2001). Since $Scl^{-/-}$ ESCs failed to contribute to definitive hematopoiesis in chimeric mice generated with wild-type embryos, it is proved that *Scl* is also required for adult hematopoietic stem cell development besides playing an important role during embryonic development (Visvader *et al.,* 1998). This same report showed that *Scl* is also required for endothelial cell development, as $Scl^{-/-}$ ESCs failed to contribute to remodeling of the primary vascular plexus in the yolk sac. Furthermore, examination of $Scl^{-/-}$ embryoid bodies demonstrated a complete failure of expression of hematopoietic genes (Elefanty *et al.,* 1997). Later report also showed that *Scl* also plays an important role in the specification of endothelial precursors (Liao *et al.,* 1998). Together, these data indicate that *Scl* is capable of specifying hemangioblast development from early mesoderm.

To further define the function of *Scl* in normal hematopoiesis *in vivo*, some (Elefanty *et al.,* 1999; Elefanty *et al.,* 1998) generated mice with a null mutation of the *Scl* gene by disrupting *Scl* with a *lacZ* gene insertion. It was found that $Scl^{-/-}$ embryos died around embryonic day 8.5 and the blood islands and major vitelline vessels were not seen in the yolk sac of $Scl^{-/-}$ embryos. To rescue the hematopoietic and vascular defects associated with the lethal $Scl^{-/-}$ mutation, Huss *et al*.(2005), injected GFP-marked $Scl^{+/+}$ cells into *Scl* mutant blastocysts, but there was no indication of phenotypic rescue of the mutant blastocysts. Sánchez *et al.* (2001) found that *Scl* exogenous expression driven by a *Scl* 3' enhancer in $Scl^{-/-}$ embryos rescued the formation of early hematopoietic progenitors and also resulted in normal yolk sac angiogenesis. By contrast, erythropoiesis remained markedly deficient in rescued embryos. This observation is consistent with the inactivity of the stem cell enhancer in erythroid cells and reveals an essential role for *Scl* during erythroid differentiation *in vivo*.

Gene targeting studies have shown that the transcription factor *Scl* is critically important for embryonic hematopoiesis, but the early lethality of *Scl* null mice has precluded the genetic analysis of its function in the adult. To address this issue, a conditional knockout of *Scl* was created using Cre/*loxP* technology (Hall *et al.,* 2003). It was found that deletion of *Scl* in adult mice perturbed megakaryopoiesis and erythropoiesis with the loss of early progenitor cells in both lineages. *Scl* is critical for megakaryopoiesis and erythropoiesis, but is dispensable for production of myeloid cells during adult hematopoiesis (Hall *et al.,* 2003).

Cre mouse lines

The site-specific DNA recombinase Cre has been widely used to develop tools for controlling gene expression in mice (Kilby *et al.,* 1993). Cre mediates the recombination of two directly repeated target sites (*loxP*), with excision of the DNA segment flanked by the *loxP* sites. Segments of DNA flanked by *loxP* sites are said to be “floxed”. Such recombination can function to activate a gene by excising a floxed DNA segment that blocks expression because it either separates the regulatory and coding sequences of the gene or interrupts the gene's open reading frame (Lakso *et al.,* 1992). Similarly, DNA excision can inactivate a gene if an essential fragment of the gene is floxed (Gu *et al.,* 1994). Gene activation or inactivation *in vivo* can be achieved by mating two different animals, one carrying a ‘target gene’ with appropriately placed *loxP* sites and one carrying a *cre* transgene. In most cases, the specificity of the system is dependent upon stringent regulation of *cre* expression.

In order to obtain *Scl* knockout embryos for this project, we took advantage of two mouse Cre lines. The first line is called C57BL/6-Tg(Zp3-cre)93Knw/J (for ease, the altered allele and this strain will be designated $Zp3^{cre}$ in this thesis). Lewandoski *et al.,* reported a mouse line in which c*re* expression is controlled by regulatory sequences from the mouse zona pellucida 3 (*Zp3*) gene (Lewandoski *et al.,* 1997), which is normally expressed exclusively in the growing oocyte prior

to the completion of the first meiotic division (Kinloch *et al.,* 1993). Data show that in $Zp3^{cre}/Zp3^{cre}$ mice, Cre-mediated recombination of the target gene occurs in 100% of oocytes. More importantly, Cre activity is not detected in the somatic tissues of most $Zp3^{cre}/Zp3^{cre}$ mice. Another transgenic mice line we used, FVB-Tg (Ddx4-cre)1Dcas/J (designated $Vasa^{cre}$ in this thesis) in which Cre is efficiently and specifically expressed in germ cells, was reported in 2007 by Gallardo *et al*. *Vasa* expression is confined to the germ cell lineages in species including *Drosophila*, zebrafish, mouse, and human (Castrillon *et al.,* 2000; Lasko and Ashburner, 1988). In mouse, *Vasa* expression begins at the time of gonad colonization by primordial germ cells and is detected by E12.5 in both sexes (Tanaka *et al.,* 2000). *Vasa* promoter driven Cre recombination was observed beginning at E15 and was greater than 95% efficient in male and female germ cells by birth. The authors claimed that there was no ectopic activity in adult mice, although some animals showed more widespread *lacZ* expression. These two lines are useful for genetic analysis of diverse aspects of germ cell function and gametogenesis.

Our hypothesis is that the pluripotent cells from the same species (mouse) or different species (rat) will replace the vascular endothelium and hematopoietic system in recipient blastocysts from a genetically modified mouse line where homozygotes fail to develop either vascular endothelium or a hematopoietic system. The purpose of this chapter is to describe how to obtain *Scl* null embryos by breeding different available mouse strains. These embryos were used for the

experiments described in Chapter 4.

RESULTS:

Strategy 1: Production of *Scl* null embryos using B6.129S1-Tal^{tm2Wehi}

The B6.129S1-*Tal*^{tm2Wehi} strain was generated by insertion of the *lacZ* reporter gene into the 5' untranslated region of the *Scl* gene. This mutation also deletes all *Scl* coding sequences creating a null allele (Elefanty *et al.,* 1998). For convenience, this allele will be designated as *Scl*^{lacZ}*.*

Homozygous *Scl* null embryos were obtained by mating *Scl*$^{+}$/*Scl*^{lacZ} heterozygous males with *Scl*$^{+}$/*Scl*^{lacZ} heterozygous females. This mating resulted in embryos with the following genotypes: 25% *Scl* null embryos (*Scl*^{lacZ}/*Scl*^{lacZ}), 25% wild type embryos (*Scl*$^{+}$/*Scl*$^{+}$) and 50% heterozygous embryos (*Scl*$^{+}$/*Scl*^{lacZ}) (Fig. 3.1 and 3.2).

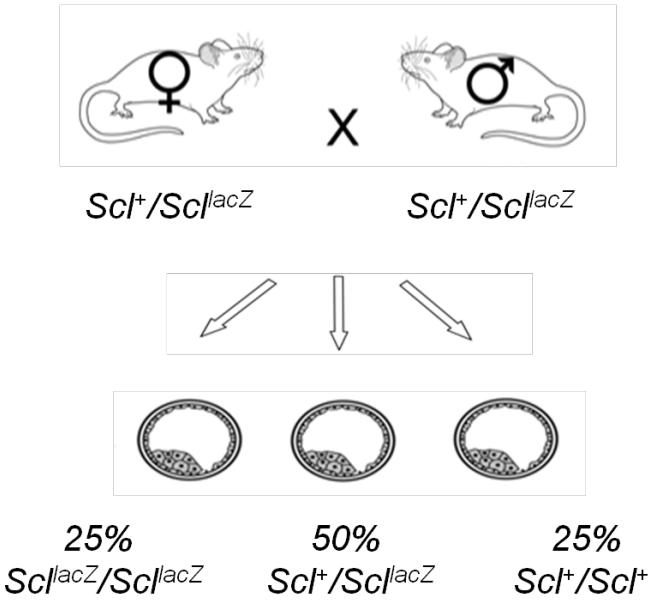


FIG. 3.1: Breeding scheme to obtain *Scl* null embryos. By this breeding method, 25% of total embryos collected were homozygous for the *Scl* null allele (*Scl*^{lacZ}/*Scl*^{lacZ}).

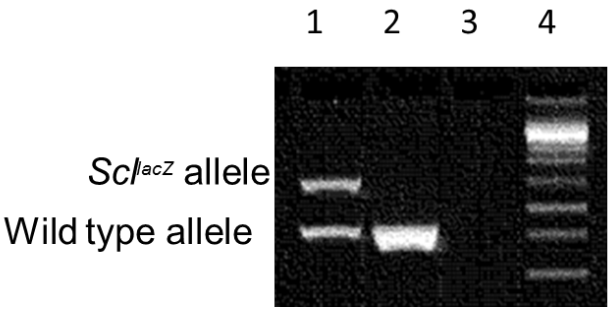


FIG. 3.2: PCR results of genotyping assay for detection of *Scl* allele. The 600bp band represents the *Scl*^{lacZ} allele and the 424bp band represents the wild type.

Lane 1: *Scl*$^{+}$/*Scl*^{lacZ} offspring; Lane 2: wild type offspring; Lane 3: No DNA control; Lane 4: 1kb+ ladder.

An alternative breeding scheme for producing *Scl* null embryos was also used for the experiments described in Chapter 4. This scheme involved the use of a strain carrying a floxed allele of *Scl* that has full wild type function (Hall *et al.,* 2003). In this strain, a floxed neomycin resistance cassette was inserted into intron 6 of the *Scl* gene and a single *loxP* site was introduced into the non-coding region of exon 6 via homologous recombination. The floxed *neo* cassette was removed by mating mutant animals to Cre deleter strain TgN(EIIa-cre)C5379Lmgd, leaving behind a single *loxP* site in intron 6 and a single *loxP* site in exon 6 (Hall *et al.,* 2003). For convenience, this allele will be designated as *Scl*^{loxP}. In the breeding scheme used here, *Scl*$^{+}$/*Scl*^{lacZ} females were mated to *Scl*^{loxP}/*Scl*^{loxP} males resulting in offspring where 50% were *Scl*^{lacZ}/*Scl*^{loxP}. By intercrossing animals with this genotype, it is possible to generate 25% *Scl* null (*Scl*^{lacZ}/*Scl*^{lacZ}), 25% *Scl*^{loxP}/*Scl*^{loxP} and 50% *Scl*^{lacZ}/*Scl*^{loxP} embryos (Fig. 3.3). A PCR-based genotyping assay allows the three different alleles (*Scl*$^{+}$, *Scl*^{lacZ} and *Scl*^{loxP}) to be distinguished from one another (Fig. 3.4).

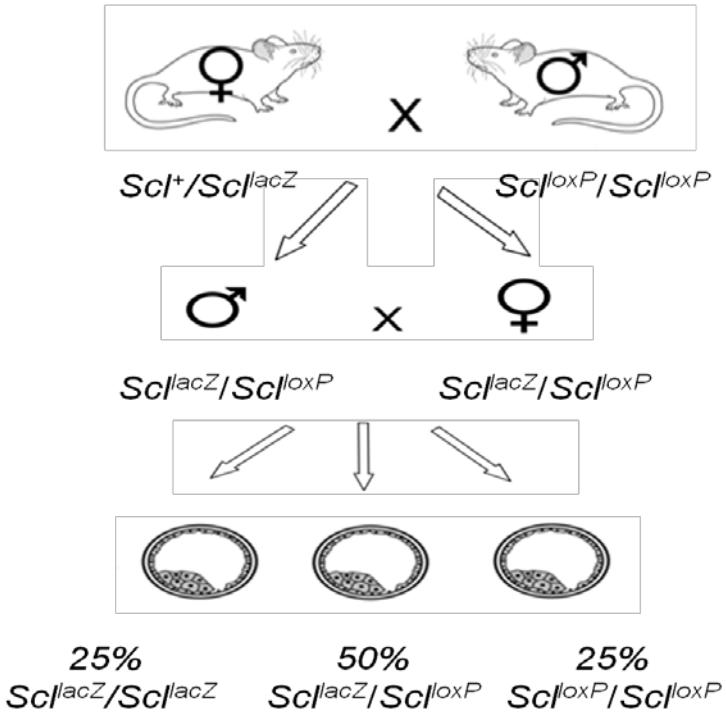


FIG. 3.3: Schematic of the breeding process. By this breeding method, 25% of total embryos collected are homozygous for the *Scl* null allele (Scl^{lacZ}/Scl^{lacZ}).

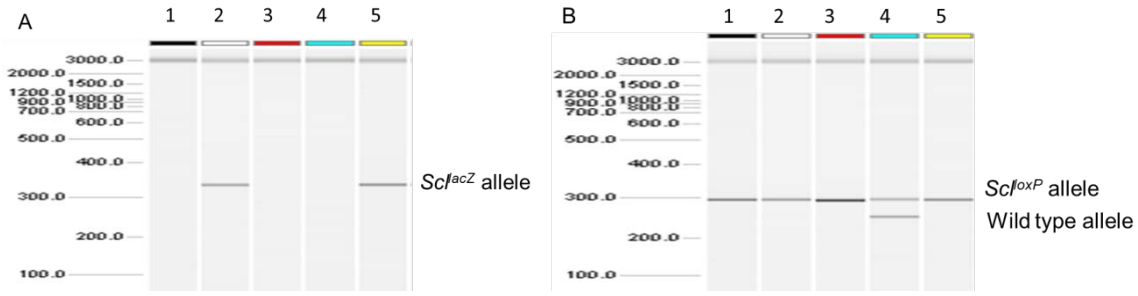


FIG. 3.4: PCR results of genotyping assay for the Scl^{lacZ}, Scl^{loxP} and wild type alleles. Panel A and B showed two different PCR assays for the same samples. Lane 1 and 3: Scl^{loxP}/Scl^{loxP} offspring; Lane 2 and 5: Scl^{lacZ}/Scl^{loxP} offspring; Lane

4: *Scl*$^{+}$/*Scl*^{loxP} offspring. Alignment Marker (QX Alignment Marker 15bp/1kb, Qiagen) and size Marker (QX DNA Size Marker 50bp-800bp, Qiagen) were used.

Strategy 2: Production of *Scl* knockout embryos

While the breeding strategy described above is straightforward, it results in only 25% of the embryos having the genotype of interest. It is possible to use an alternative approach which allows 100% of resulting embryos to be homozygous for *Scl* knockout alleles (Fig. 3.5). In order to achieve this goal, mouse line C57BL/6-Tg(Zp3-cre)93Knw/J (designated *Zp3*^{cre}) was used to generate female breeders and mouse line FVB-Tg(Ddx4-cre)1Dcas/J (designated *Vasa*^{cre}) was used to generate male breeders in which the *Scl* gene was deleted specifically in the germ cells.

First, *Zp3*^{cre}/*Zp3*^{cre} homozygous female mice were bred to the *Scl*^{loxP}/*Scl*^{loxP} homozygous male mice in order to get *Zp3*$^{+}$/*Zp3*^{cre} *Scl*$^{+}$/*Scl*^{loxp} (Fig. 3.5, 3.6) female mice. Hypothetically, *Zp3*-driven Cre mediated recombination of the floxed *Scl* would occur in 100% of oocytes in female mice with this genotype (Lewandoski *et al.,* 1997). When a *Zp3*$^{+}$/*Zp3*^{cre} *Scl*$^{+}$/*Scl*^{loxp} female mouse was bred to a homozygous *Scl*^{loxP}/*Scl*^{loxP} male, Cre recombinase present in the oocytes is expected to mediated knockout of the floxed *Scl* allele contributed by the female. The floxed *Scl* allele contributed by the male will stay floxed. The resulting offspring have a genotype of *Zp3*$^{+}$/*Zp3*^{cre} *Scl*^{loxP}/ *Scl*$^{\Delta}$ (Δ: deleted allele

that results after Cre recombination) (Fig. 3.5, 3.7). Female mice with the *Zp3*$^{+}$/*Zp3*^{cre} *Scl*^{loxP}/*Scl*$^{\Delta}$ genotype were used as breeders for the final mating step to produce 100% *Scl* knockout embryos.

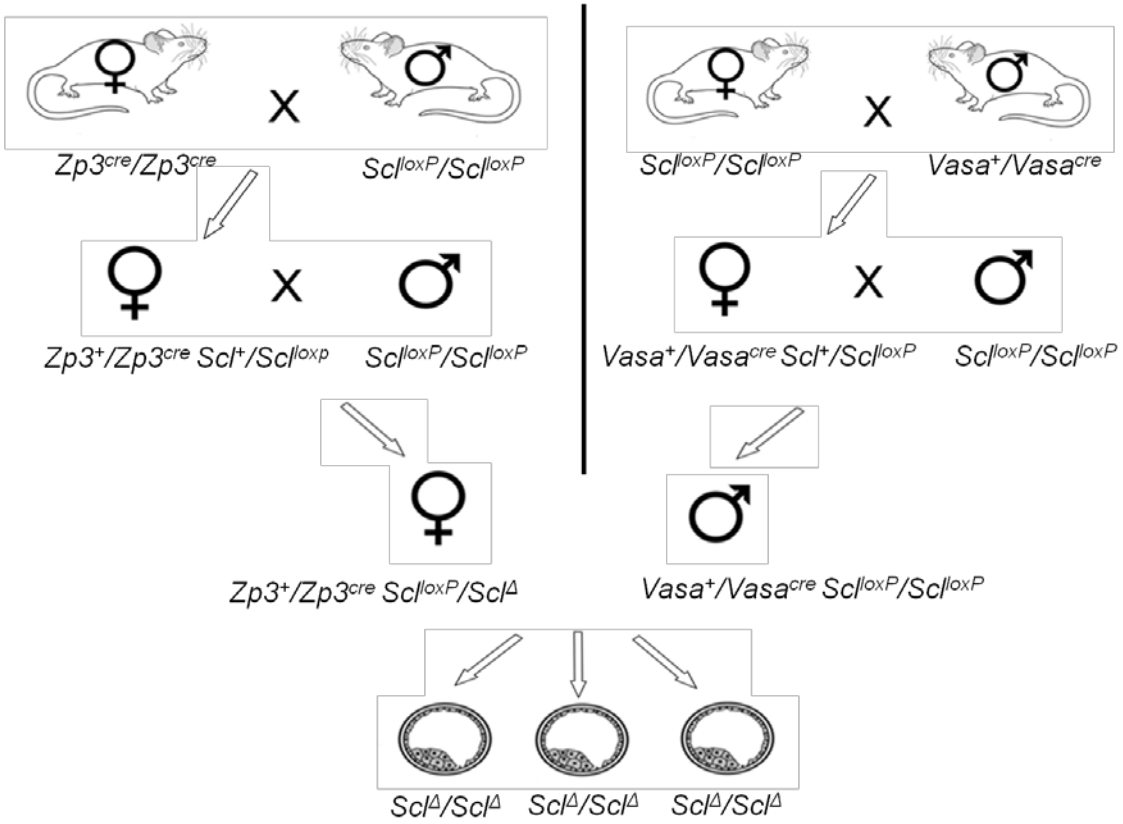


FIG. 3.5: Schematic of the breeding process to produce 100% *Scl* knockout embryos. (Δ: deleted allele after Cre recombination at the *loxP* sites).

Next, a similar strategy was used for the production of the male breeders needed for the final mating to produce 100% *Scl* knockout embryos. *Vasa*$^{+}$/*Vasa*^{cre} male

mice were bred to *Scl*^{loxP}/*Scl*^{loxP} homozygous female mice in order to produce *Vasa*$^{+}$/ *Vasa*^{cre} *Scl*$^{+}$/*Scl*^{loxP}mice (Fig. 3.5, 3.8). Hypothetically, in mice with this genotype, Cre was specifically and efficiently expressed in germ cells (Gallardo *et al.,* 2007). This resulted in gametes in which the floxed *Scl* allele has been subjected to recombination resulting in a knocked out (Δ) *Scl* allele. Female mice with the *Vasa*$^{+}$/*Vasa*^{cre} *Scl*$^{+}$/*Scl*^{loxP} genotype were bred to *Scl*^{loxP}/*Scl*^{loxP} homozygous males. Offspring of this mating have a genotype of *Vasa*$^{+}$/*Vasa*^{cre} *Scl*^{loxP}/*Scl*^{loxP} in somatic cells, while all germ cells have a *Vasa*$^{+}$/*Vasa*^{cre} *Scl*^{loxP}/*Scl*$^{\Delta}$ genotype (Fig. 3.5).

Lastly, females with the *Zp3*$^{+}$/*Zp3*^{cre} *Scl*^{loxP}/*Scl*$^{\Delta}$ genotype were bred to males with the *Vasa*$^{+}$/*Vasa*^{cre} *Scl*^{loxP}/*Scl*^{loxP} genotype in order to obtain 100% *Scl*$^{\Delta}$ /*Scl*$^{\Delta}$ knockout embryos (Fig. 3.5).

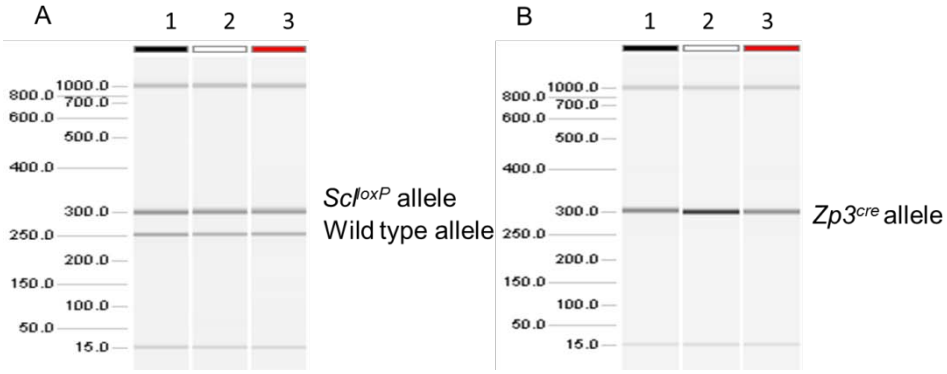


FIG. 3.6: PCR assay for *Zp3*$^{+}$/*Zp3*^{cre} *Scl*$^{+}$/*Scl*^{loxp} genotype resulting from the breeding between *Zp3*^{cre}/*Zp3*^{cre} homozygous female mice and the *Scl*^{loxP}/*Scl*^{loxP} homozygous male mice. Panel A: assay to detect *Scl*^{loxP} allele and wild type

allele. Panel B: assay to detect *Zp3*^{cre} allele. Lane 1, 2, 3: *Zp3*$^{+}$/*Zp3*^{cre} *Scl*$^{+}$/*Scl*^{loxp} offspring. Alignment Marker (QX Alignment Marker 15bp/1kb, Qiagen) and size Marker (QX DNA Size Marker 50bp-800bp, Qiagen) were used.

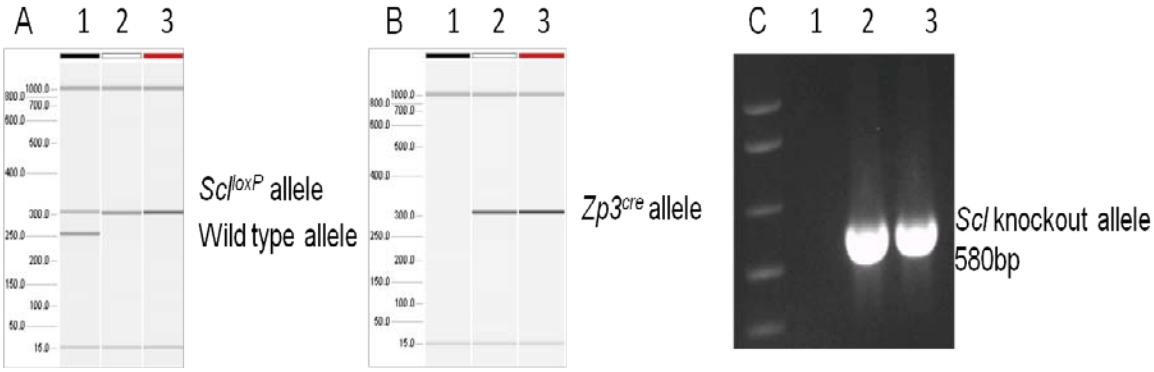


FIG. 3.7: Genotype of offspring from the breeding between female *Zp3*$^{+}$/*Zp3*^{cre} *Scl*$^{+}$/*Scl*^{loxp} and male *Scl*^{loxP}/*Scl*^{loxP}. Panel A: assay to detect *Scl*^{loxP} allele and wild type allele. Panel B: assay to detect *Zp3*^{cre} allele. Panel C: assay to detect the *Scl* knockout allele. Lane 1: *Zp3*$^{+}$/*Zp3*$^{+}$ *Scl*$^{+}$/*Scl*^{loxP} offspring. Lane 2 and 3: *Zp3*$^{+}$/*Zp3*^{cre} *Scl*^{loxP}/*Scl*$^{\Delta}$ offspring that were used for final breeding. Alignment Marker (QX Alignment Marker 15bp/1kb, Qiagen) and size Marker (QX DNA Size Marker 50bp-800bp, Qiagen) and 1kb+ ladder (Panel C) were used.

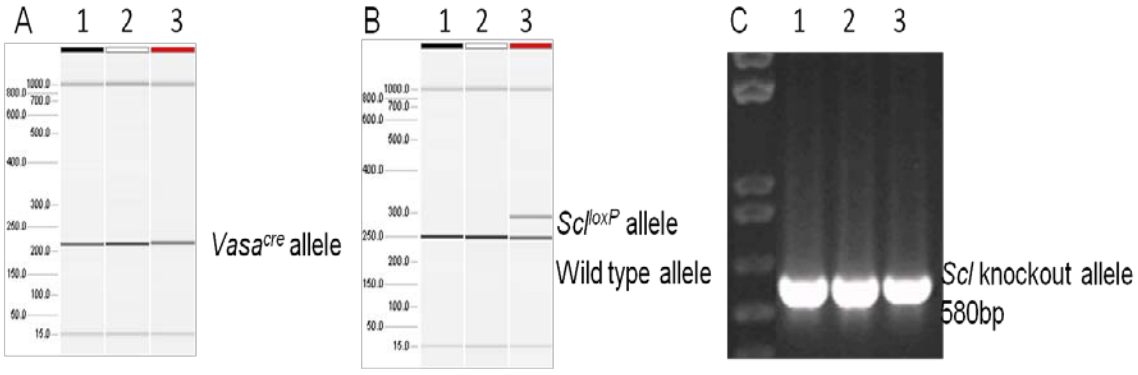
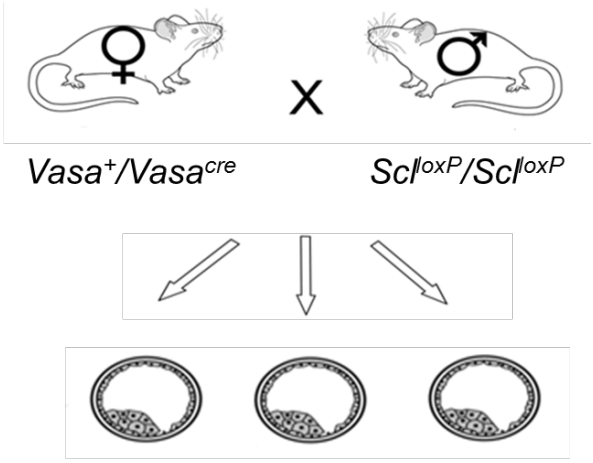


FIG. 3.8: Genotype of offspring from the breeding between female *Vasa*$^{+}$/*Vasa*^{cre} and male *Scl*^{loxP}/*Scl*^{loxP}. Panel A: assay to detect *Vasa*^{cre} allele. Panel B: assay to detect *Scl*^{loxP} and wild type allele. Panel C: assay to detect the *Scl* knockout allele. Lane 1 and 2: *Vasa*$^{+}$/ *Vasa*^{cre} *Scl*$^{\Delta}$/*Scl*$^{+}$ offspring. Lane 3: *Vasa*$^{+}$/*Vasa*^{cre} *Scl*$^{+}$/*Scl*^{loxP}/*Scl*$^{\Delta}$offspring. Alignment Marker (QX Alignment Marker 15bp/1kb, Qiagen) and size Marker (QX DNA Size Marker 50bp-800bp, Qiagen) and 1kb+ ladder (Panel C) were used.

High ectopic Cre activity in the mouse line of *Vasa*$^{+}$/*Vasa*^{cre}

In order to produce 100% of resulting embryos to be homozygous for *Scl* knockout alleles, we took advantage of the mouse lines that caused specific deletion of *Scl* in the germs cells. For example, *Vasa*$^{+}$/*Vasa*^{cre} crossed with the mouse line with floxed *Scl* (Fig. 3.5) results in the specific deletion of *Scl* in male germ cells. Deletion of *Scl* in several somatic cell types or global *Scl* deletion was not desirable since it causes embryonic lethality if both *Scl* alleles are deleted. A

previous report showed potent parent-of-origin effects within this mouse line (Gallardo *et al.,* 2007). For example, in our hands, when *Vasa*$^{+}$/*Vasa*^{cre} females were bred to *Scl*^{loxP}/*Scl*^{loxP} homozygous males, it was observed that all the progeny underwent global Cre-mediated recombination even though they did not inherit Cre. This was consistent with results reported by Gallardo *et al.* (2007) (Table 3.1). This observation can be explained by the persistence of active Cre protein in the egg.



A: *Vasa*$^{+}$/*Vasa*^{cre} *Scl*$^{+}$/*Scl*^{loxP}

B: *Vasa*$^{+}$/*Vasa*^{cre} *Scl*$^{+}$/*Scl*$^{\Delta}$

C*: Vasa*$^{+}$/*Vasa*$^{+}$ *Scl*$^{+}$/*Scl*^{loxP}

FIG. 3.9: Breeding scheme between *Vasa*$^{+}$/*Vasa*^{cre} females and *Scl*^{loxP}/*Scl*^{loxP} males as well as possible genotypes for the offspring.

Table 3.1

Possible genotypes for the offspring from crossing

Vasa^{+}/*Vasa*^{cre} females and *Scl*^{loxP}/*Scl*^{loxP} males

Genotype	Number of offspring	Percentage of animals with observed genotype
Vasa^{+}/*Vasa*^{cre} *Scl*$^{+}$/*Scl*^{loxP}	0	0
Vasa^{+}/*Vasa*^{cre} *Scl*$^{+}$/*Scl*$^{\Delta}$	29	74%
Vasa^{+}/*Vasa*$^{+}$ *Scl*$^{+}$/*Scl*$^{\Delta}$	10	26%
Total	39	100%

While in the male *Vasa*$^{+}$/*Vasa*^{cre} mice, global Cre-mediated recombination should not be a concern. Gallardo *et al.,* tested the function and specificity of male *Vasa*$^{+}$/*Vasa*^{cre} line *in vi*vo by crossing *Vasa*$^{+}$/*Vasa*^{cre} male with female *Gt(ROSA)*^{26sor}*lacZ* reporter mice (Soriano, 1999). They claimed that strong *lacZ* expression, which indicated Cre-mediated recombination, was found in the gonads of all the progeny but not in most animals, skin, and all other tissues. They stated that less than 20% of animals showed more global expression.

In this experiment, when *Vasa*$^{+}$/*Vasa*^{cre} males were used to cross with *Scl*^{loxP}/*Scl*^{loxP} homozygous females (Fig. 3.9), we expected to observe a minority of animals with the genotyping that showed the Cre-mediated deletion in somatic cells. Surprisingly, our data showed 63% of total offspring showing the deletion of *Scl* in somatic cells in addition to the 3% of total offspring showing the partial deletion of *Scl* in somatic cells (Table 3.2) while the previous report showed less than 20% of animals showed Cre-mediated recombination.

Table 3.2

Genotyping results of offspring from

Vasa^{+}/*Vasa*^{cre} males X *Scl*^{loxP}/*Scl*^{loxP} females cross.

Genotype	Number of offspring	Percentage of animals with observed genotype
Vasa^{+}/*Vasa*^{cre} *Scl*$^{+}$/*Scl*^{loxP}	17	13%
Vasa^{+}/*Vasa*$^{+}$ *Scl*$^{+}$/*Scl*^{loxP}	27	21%
Vasa^{+}/*Vasa*^{cre} *Scl*$^{+}$/*Scl*$^{\Delta}$	82	63%
Vasa^{+}/*Vasa*^{cre} *Scl*$^{+}$/*Scl*$^{\Delta}$/*Scl*^{lox}	4	3%
Total	130	100%

DISCUSSION

This chapter describes different breeding schemes used to produce *Scl* null embryos. There are two strategies to achieve this goal. One is to breed heterozygous Scl^{+}/Scl^{lacZ} mice or heterozygous Scl^{lacZ}/Scl^{loxP} mice in order to produce 25% *Scl* null embryos. Another one is to take advantage of germ line specific deletion technology to knockout the *Scl* allele in the gametes so that 100% null embryos can be produced. Ideally, this should be the best strategy for the rescue experiment in Chapter 4. However, this strategy involves three mouse lines and is very time-consuming because of three generations of breeding before the final breeding for the embryo collection. Thus, breeding heterozygous Scl^{lacZ}/Scl^{loxP} mice was chosen and used for the experiments described in Chapter 4.

High ectopic Cre activity in $Vasa^{+}/Vasa^{cre}$ males was observed when bred to Scl^{loxP}/Scl^{loxP} females. As high as 66% of total offspring showed Cre-mediated *Scl* deletion in somatic cells which is not consistent with previous reports (Gallardo *et al.,* 2007). This high ectopic Cre activity in $Vasa^{+}/Vasa^{cre}$ males would result in more global *Scl* deletion which would cause embryo lethality when both *Scl* alleles are deleted globally. The reason for this observation is still unclear. More experiments, such as crossing $Vasa^{+}/Vasa^{cre}$ with different mouse lines with *loxP*-floxed genes, need to be done to further explain this observation.

MATERIALS AND METHODS

Acquisition of mouse lines and animal care

Frozen embryos of *Scl*$^{+}$/*Scl*^{lacZ} and *Scl*^{loxP}/*Scl*^{loxP} were generously provided by Dr. Lorraine Robb, The Walter and Eliza Hall Institute of Medical Research, Victoria Australia. Embryo transfer was performed and the genotypes of all animals were confirmed by PCR genotyping assays. *Vasa*$^{+}$/*Vasa*^{cre} (FVB-Tg(Ddx4-cre)1Dcas/J. Stock number: 006954) and *Zp3*^{cre}/*Zp3*^{cre} (C57BL/6-TgN(Zp3-Cre)93Knw. Stock number: 003651) were purchased from the Jackson Laboratory (Bar Harbor, Maine). This study was approved by the Institution Animal Care and Use Committee of University of Missouri-Columbia. Animals housed at the University of Missouri Veterinary School were maintained in microisolators and veterinary medical oversight was provided by the director of the MU animal facility.

DNA preparation

Tail snips were collected either from 16.5 dpc embryos or three week old offspring. DNA extraction was performed with DNeasy® Blood & Tissue Kits from Qiagen (Valencia, CA). For each tail (0.4-0.6 cm), 180 µl ATL lysing buffer and 20 µl proteinase K were added followed by incubation at 56°C overnight. After vortexing for 15 s, 200 µl Buffer AL and 200 µl ethanol (96–100%) were added to the sample. The mixture was transferred into the DNeasy Mini spin column and

centrifuged at 6000 X g for 1 minute. The columns were then washed with 500 µl buffer AW1 and 500 µl AW2. Lastly, to elute DNA, 150 µl AE buffer was added directly onto the DNeasy membrane followed by incubation at room temperature for 1 minute followed by centrifugation for 1 minute at 6000 X g.

Genotyping

For the breeding of *Scl*$^{+}$/*Scl*^{lacZ} heterozygous males with *Scl*$^{+}$/*Scl*^{lacZ} heterozygous females in Strategy 1:

To detect the *LacZ* allele, primers *LacZ* F: 5'-GAA TCT CTA TCG TGC GGT GGT TGA-3' and *LacZ* R: 5'-GCC GTG GGT TTC AAT ATT GGC TTC-3' were used. PCR conditions were 94 ^{o}C for 5 minutes, then 94 ^{o}C for 30 seconds, 72 ^{o}C for 30 seconds for 35 cycles followed by 72 ^{o}C for 7 min in a PCR tube (Sigma) with a 20 µl mixture containing 1 µl (~25ng) DNA (extracted from tail snips), 0.2µl (1 unit) of FastStart *Taq* DNA Polymerase (Roche), 0.2µl (1µM) of each primer, 3.6 µl (4.5 mM) dNTP, 2µl of 10x reaction buffer. Amplicons of 341bp (*Scl LacZ* targeted allele) were detected by gel electrophoresis on 3% agarose gel in 1xTBE buffer for 30 minutes at 100 V.

For all the other breedings:

To detect three different *Scl* alleles (*Scl*$^{+}$, *Scl*^{lacZ} and *Scl*^{loxP}), primers MH97-F 5'-TCC CAA GCC CAA AGA TTT CCC CAA TG-3' and MH98-R 5'-GCA AGC TGG ATG GAT CAA CAT GGA CCT-3' were used to identify wild type allele, *loxP*

floxed allele and deleted allele respectively. PCR conditions were 95 ^{o}C for 3 minutes, 94 ^{o}C for 30 seconds, 60 ^{o}C for 30 seconds and 72 ^{o}C for 30 seconds for 35 cycles followed by 72 ^{o}C for 10 minutes in a PCR tube (Sigma) with a 20 µl mixture containing 1 µl (~25ng) DNA (extracted from tail snips), 0.2µl (1 unit) of FastStart *Taq* DNA Polymerase (Roche), 0.2µl (1µM) of each primer, 3.6 µl (4.5 mM) dNTP, 2µl of 10x reaction buffer. Amplicons of 250 bp (wild type allele), 300 bp (floxed *Scl* allele) and 580 bp (*Scl* knockout allele) were detected by gel eltrophoresis on 3% agarose gel in 1xTBE buffer for 30minutes at 100V.

To detect the *cre* allele, primers cre-F 5'-CGA GAT TGA GGG AAG CAG AG-3' and cre-R 5'- GCA AAC GGA CAG AAG CAT TT-3' were used. PCR conditions were 95 ^{o}C for 3 minutes, then 94 ^{o}C for 30 seconds, 68 ^{o}C for 30 seconds, 72 ^{o}C for 30 seconds for 35 cycles followed by 72 ^{o}C for 10 min in a PCR tube (Sigma) with a 20 µl mixture containing 1 µl (~25ng) DNA (extracted from tail snips), 0.2µl (1 unit) of FastStart *Taq* DNA Polymerase (Roche), 0.2µl (1µM) of each primer, 3.6 µl (4.5 mM) dNTP, 2µl of 10x reaction buffer. Amplicons of 320bp (*cre* allele) were detected by gel electrophoresis on 3% agarose gel in 1xTBE buffer for 30 minutes at 100 V.

PCR conditions were 94 ^{o}C for 3 minutes , then 94 ^{o}C for 30 seconds, 51.7 ^{o}C for 1 minute and 72 ^{o}C for 1 minute for 35 cycles followed by 72 ^{o}C for 2 minutes in in a PCR tube (Sigma) with a 50 µl mixture containing 1 µl (~30ng) DNA (extracted from tail snips), 1 µl (2 units) of FastStart *Taq* DNA Polymerase

(Roche), 2µl (1µM) of each primer, 8 µl (0.2 mM) dNTP and 34 µl of 1x reaction buffer. This resulted in 300bp *Cre* transgene allele after running on Qiaxcel using Method AM320, with 15bp-1kb alignment marker and 5—800bp DNA size marker.

PCR products (in second part of Strategy 1 and in Stragegy 2) smaller than 1KB were analysed on the Qiaxcel (Qiagen) with the Qiaxcel DNA Screening Kit (Cat# 929004). Alignment Marker (QX Alignment Marker 15bp/1kb, Cat# 929521, Qiagen) and size Marker (QX DNA Size Marker 50bp-800bp, Cat# 929556, Qiagen) were used. All the analysis was performed by the Genetic Testing Services lab at University of Missouri-Columbia.

CHAPTER 4: Complementing *Scl* Deficient Embryos with Rat ESCs

Summary: The stem cell leukemia gene (*Scl*) encodes a basic helix-loop-helix transcription factor which plays an essential role in both endothelial and hematopoietic development. *Scl* is first expressed in extra-embryonic mesoderm and is required for the generation of hematopoietic lineages and angiogenesis. The absence of *Scl* gene results in embryonic lethality as early as embryonic day (E) 8.5. Rat embryonic stem cells (ESCs) have the capability to differentiate into the three germ layers both *in vivo* and *in vitro*. Here, we injected rat ESCs into mouse *Scl* deficient blastocysts at 3.5 days post coitum (dpc) and investigated their capability to reverse genetic defects in *Scl* deficient blastocysts. Data showed that rat ESCs did not persist during mouse embryonic development. Only two expecting genotypes (Scl^{lacZ}/Scl^{loxP} and Scl^{loxP}/Scl^{loxP}) were detected from Scl^{lacZ}/Scl^{loxP} intercrosses. There was no detection of the third genotype of Scl^{lacZ}/Scl^{lacZ} (*Scl* deficient) at 16.5 dpc which indicated that there was no phenotypic rescue of the *Scl* deficient blastocysts. Even though rat ESCs were not capable of rescuing the *Scl* deficiency during embryonic development, they were found to contribute to different organs/tissues in an allogeneic blastocyst environment.

INTRODUCTION

Embryonic stem cell (ESC) research is an area of investigation with enormous therapeutic potential. ESCs are able to differentiate into cells from the three germ layers both *in vivo* and *in vitro* so that ESCs are extremely important for studies in regenerative medicine. In modern regenerative medicine, many scientists have tried to repopulate damaged organs *in vivo* using ESC-derived, lineage-specific, *in vitro* differentiated cells. Other investigators have provided direct evidence that mouse ESCs can rescue otherwise lethal organ defects in mouse embryos by directly injecting ESCs into blastocysts. For example, by injecting as few as 15 wild type mouse ESCs into blastocysts with lethal cardiac defects, Fraidenraich *et al.,* successfully rescued a subset of the cardiac defects and prevented embryo death (Fraidenraich *et al.,* 2004). Using the same method, investigators rescued the mouse embryos with Duchenne muscular dystrophy and central nervous system demyelination disease (Schneider *et al.,* 2009).

By targeting *Scl* with a *lacZ* transgene, mice with a null mutation of the *Scl* gene were generated (Robb *et al.,* 1995; Shivdasani *et al.,* 1995). *Scl* deficient embryos were found dying around E 8.5 and the blood islands and major vitelline vessels were not seen in these embryos. To rescue the hematopoietic and vascular defects associated with the lethal *Scl* mutation, Huss *et al.,* injected GFP marked adult stem cells from peripheral blood into *Scl* deficient blastocysts,

but there was no indication of phenotypic rescue of the blastocysts (Huss *et al.,* 2005). Another group found that expression of exogenous *Scl* driven by the stem cell enhancer (3' enhancer: active in hematopoietic progenitors and vascular endothelium during development) rescued the formation of early hematopoietic progenitors and also resulted in normal yolk sac angiogenesis (Sanchez *et al.,* 2001). By contrast, erythropoiesis remained markedly deficient in these rescued embryos. This observation is consistent with the inactivity of the stem cell enhancer in erythroid cells and reveals an essential role for *Scl* during erythroid differentiation *in vivo*. A 130-kilobase yeast artificial chromosome containing the human *SCL* locus completely rescued the embryonic lethal phenotype of *Scl* deficient embryos (Sinclair *et al.,* 2002). However, all these reports did not show any live rescued pups.

In experiments described in Chapter 2 it was shown that rat ESCs can proliferate and develop when injected into mouse blastocysts to produce viable rat➔mouse chimeras. In Chapter 3, different methods to produce *Scl* deficient mouse embryos were described. In this Chapter, work to investigate if *Scl* deficient mouse embryos can be rescued by injection of rat ESCs is described.

RESULTS

To generate *Scl* deficient blastocysts, Scl^{lacZ}/Scl^{loxP} males were mated to Scl^{lacZ}/Scl^{loxP} females (see Chapter 3). A total number of 338 blastocysts were collected and these blastocyst were injected with 10-15 rat ESCs. The rat ESC-injected mouse blastocysts were transferred into foster mothers, and 91 embryos were recoverd at embryonic stage 16.5 dpc. The embryos were genotyped at the *Scl* locus.

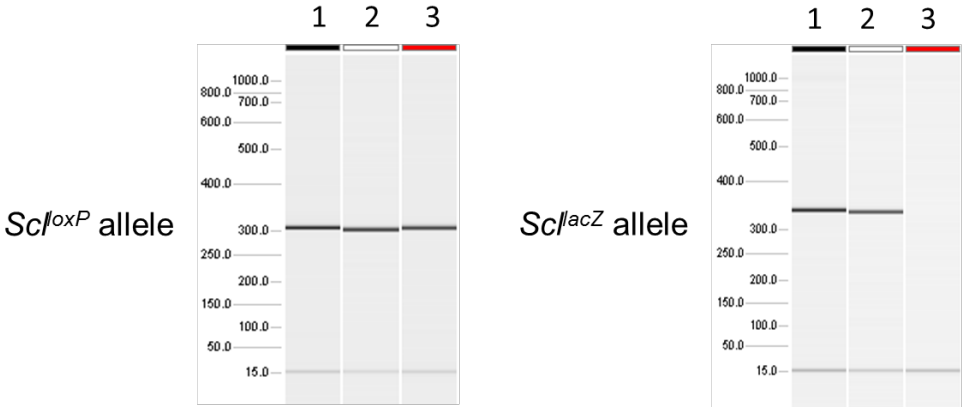


FIG. 4.1: Genotyping results for the presence of Scl^{loxP} and Scl^{lacZ} alleles in DNA samples obtained from the collected embryos. The same samples were analyzed using two different assays respectively for the allele of Scl^{loxP} and of Scl^{lacZ}. The Scl^{loxP} allele produces an amplicon of 300 bp and the Scl^{lacZ} allele produces an amplicon of 341 bp. Lane 1 and 2: Scl^{lacZ}/Scl^{loxP} embryo; Lane 3: Scl^{loxP}/Scl^{loxP} embryo. QX Alignment Marker 15bp/1kb (Qiagen) was used.

Of the 91 embryos recovered and genotyped, 60 embryos (66%) had the *Scl*^{lacZ}/*Scl*^{loxP} genotype. Thirty-one embryos (34%) had the *Scl*^{loxP}/*Scl*^{loxP} genotype (Table 4.1). Theoretically, in a *Scl*^{lacZ}/*Scl*^{loxP} X *Scl*^{lacZ}/*Scl*^{loxP} cross, the possible genotypes of the embryos are 25% *Scl*^{loxP}/*Scl*^{loxP}, 25% *Scl*^{lacZ}/*Scl*^{lacZ}, and 50% *Scl*^{lacZ}/*Scl*^{loxP}. Because embryos with the *Scl*^{lacZ}/*Scl*^{lacZ} genotype die prior to 16.5 dpc, we would not expect to recover embryos with this phenotype unless rescue by the rat ESCs occurred.

Our data are consistent with the expected percentage of embryos with genotypes of *Scl*^{lacZ}/*Scl*^{loxP} and *Scl*^{loxP}/*Scl*^{loxP} (50% and 25%). Embryos with the *Scl*^{lacZ}/*Scl*^{lacZ} genotype were not identified by PCR. This supports the hypothesis that the rat ESCs failed to rescue the phenotype of the *Scl* deficient embryos and therefore by E16.5, no homozygous *Scl*^{lacZ} embryos survived.

Table 4.1

Genotyping results for the rat ESC-injected embryos collected at 16.5 dpc (mouse blastocysts from *Scl*^{lacZ}/*Scl*^{loxP} male X *Scl*^{lacZ}/*Scl*^{loxP} female injected with GFP labeled rat ESCs).

	Embryos numbers	Percentage of embryos with observed genotype
Total embryos injected and transferred	338	N/A
Total embryos collected at 16.5 dpc	91	N/A
Scl^{lacZ}/*Scl*^{loxP}	60	65%
Scl^{loxP}/*Scl*^{loxP}	31	34%
Scl^{lacZ}/*Scl*^{lacZ}	0	0

Of the 91 embryos recovered on E16.5, 29 embryos were GFP positive which was 32% of total injected embryos (Table 4.2). Among these 29 embryos, 8 embryos showed delayed development (Fig. 4.2). Specifically, these embryos were much smaller than the embryos from the same foster mother. Limbs were not well-formed and abdominal organs could not be identified or separated when dissected. Among these 8 embryos, 5 embryos were *Scl*^{lacZ}/*Scl*^{loxP} and 3 embryos were *Scl*^{loxP}/*Scl*^{loxP} (Table 4.2).

Table 4.2

Genotypes of EGFP positive embryos collected

Genotype	Number of embryos with observed genotype		Percentage of embryos with observed genotype
Scl^{lacZ}/*Scl*^{loxP}	14	5D*	48%
		9N**	
Scl^{loxP}/*Scl*^{loxP}	15	3D*	52%
		12N**	
Total EGFP^{+} embryos (rat ESCs origin)	29		100%

*D: embryos exhibiting delayed development

**N: embryos exhibiting normal development

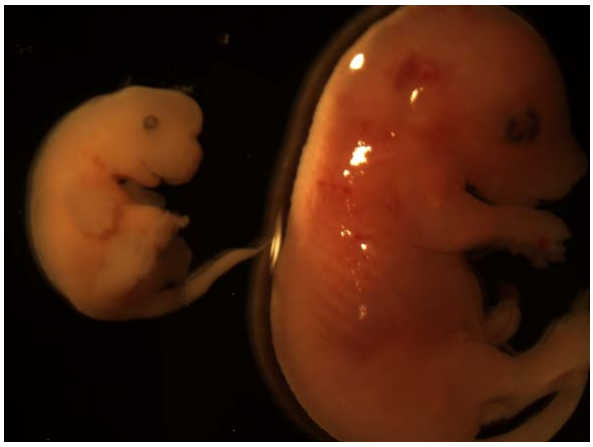


FIG. 4.2: Two embryos collected at 16.5dpc from the same recipient. The embryo on the left showed delayed development and the embryo on the right showed normal development.

DISCUSSION:

These studies were performed to investigate if rat ESCs are capable of rescuing *Scl* deficient mouse embryos. To achieve this goal, we injected 10-15 rat ESCs directly into *Scl* deficient mouse blastocysts followed by embryo transfer.

Based on Mendelian principles, the intercross between Scl^{lacZ}/Scl^{loxP} males and Scl^{lacZ}/Scl^{loxP} females generates 50% of total embryos with the Scl^{lacZ}/Scl^{loxP}, 25% of total embryos with the Scl^{loxP}/Scl^{loxP} genotype and 25% of total embryos with the Scl^{lacZ}/Scl^{lacZ} (*Scl* deficient) genotype. A total of 91 embryos were obtained at 16.5dpc out of 338 rat ESC-injected blastocysts. Among these 91 embryos, 29 (32%) embryos were EGFP positive which indicated the persistence of rat ESCs and 31 of the embryos showed the Scl^{loxP}/Scl^{loxP} genotype (Table 4.1). In theory, 10 embryos with the Scl^{lacZ}/Scl^{lacZ} genotype should have been observed if they were rescued by rat ESCs. However, our data did not show any embryos with this genotype. It strongly suggests that *Scl* deficient embryos were not rescued by rat ESCs, specifically, rat ESC line SD-Tg.EC8/Rrrc. After our studies were performed, it was shown that this particular rat cell line, while capable of forming chimeras when injected into rat SD X DA blastocyst, was not germline competent (Men *et al.,* submitted). The inability of this rat ESC line to rescue the Scl deficiency could be due to characteristics of this particular cell line. For example, this cell line might not be robust enough to compete with the mouse origin cells in an exogenetic environment. As discussed in Chapter 2, the same cell line was

used to make rat➔mouse chimeras. It is true that we did observe the production of rat➔mouse chimeras when injecting those rat ESCs into mouse blastocysts. However, the contribution of this rat ESC line in rat➔mouse chimeras was minimal. First of all, rat ESC (green) contribution was observed obviously at 11.5 dpc, however, it was not obvious at 16.5dpc. Secondly, in rat➔mouse adult chimeras, rat ESCs contributed only to a few organs, for example, brain, lungs and heart. Similarly, low levels of coat-color chimerism were observed in rat➔mouse chimeras. Lastly and the most importantly, no rat ESC-derived cells (GFP positive) were observed from peripheral blood samples obtained from rat➔mouse chimeras. These data make our observation that no *Scl* deficient embryos were rescued by this rat ESC line less unexpected. In theory, a different rat ESC line, proven to contribute to the endothelial/hematopoetic lineages both *in vitro* and *in vivo*, could work.

In summary, there was no sign of phenotypic rescue detected in rat ESC-injected mouse *Scl* deficient blastocysts even though rat ESC contribution was observed in several organs dissected from 16.5 dpc embryos. In the meanwhile, our data showed quite a few embryos with delayed development in Scl^{loxP}/Scl^{loxP} and Scl^{lacZ}/Scl^{loxP} embryos at 16.5 dpc (Table 4.2). The reason for this is unclear. Since these delayed embryos were not *Scl* deficient embryos, one possible reason for delayed development might be incompatible interactions between rat and mouse cells during early embryonic development which was observed as well during production of rat➔mouse chimeras.

MATERIALS AND METHODS

Maintenance of rat ESCs:

The rat embryonic stem cell line SD-Tg.EC8/Rrrc was used for these studies. This line was derived from RRRC# 65 stock SD-Tg(GFP)2BalRrrc (Lois *et al.,* 2002) an outbred Sprague Dawley (SD) stock carrying a ubiquitously expressed EGFP transgeneSD-Tg.EC8/Rrrc has a normal male karyotype, stableGFP expression and expresses the stem cell markers Oct 4, Nanog, Sox2. Chimeras were formed when injected into rat blastocysts (Men *et al,* submitted).

Rat blastocysts were gently flushed out from the uteruses of E4.5 timed-pregnant rats with N2B27+2i medium. After the removal of the zona with acid tyrodes solution (Sigma), whole blastocysts were transferred into 4-well plates and cultured in N2B27+2i medium. After five to seven days, the outgrowths of blastocysts were disaggregated and replated in N2B27+2i media. Emerging ESC colonies were then trypsinized and expanded. Undifferentiated rESCs were maintained on mitomycin-c treated mouse embryonic fibroblasts cells in N2B27+2i medium (Ying *et al.,* 2003) containing 1 mM MEK inhibitor PD0325901 (Axon Groeningen, The Netherlands), 3 mM GSK3 inhibitor CHIR99021 (Axon), FGF receptor inhibitor SU5402 (Calbiochem, La Jolla, CA, USA), and 1000 U/ml of rat LIF (Millipore). Medium was changed every day, and cells were split with 0.05% trypsin every three days.

Acquisition of mouse lines and animal care

Frozen embryos of *Scl*$^{+}$/ *Scl*^{lacZ}and *Scl*^{loxP}/*Scl*^{loxP} were generously provided by Dr. Lorraine Robb, The Walter and Eliza Hall Institute of Medical Research, Victoria Australia. Embryo transfer was performed and produced heterozygous pups which have been confirmed the genotype by PCR. This study was approved by the Institution Animal Care and Use Committee of University of Missouri-Columbia. Animals housed at the University of Missouri Veterinary School were maintained in microisolators and veterinary medical oversight provided by the director of the MU animal facility.

Genotyping:

To detect three different *Scl* alleles (*Scl*$^{+}$, *Scl*^{lacZ} and *Scl*^{loxP}), primers MH97-F 5'-TCC CAA GCC CAA AGA TTT CCC CAA TG-3' and MH98-R 5'-GCA AGC TGG ATG GAT CAA CAT GGA CCT-3' were used to identify wild type allele, *loxP* floxed allele and deleted allele respectively. PCR conditions were 95 ^{o}C for 3 minutes, 94 ^{o}C for 30 seconds, 60 ^{o}C for 30 seconds and 72 ^{o}C for 30 seconds for 35 cycles followed by 72 ^{o}C for 10 minutes in a PCR tube (Sigma) with a 20 µl mixture containing 1 µl (~25ng) DNA (extracted from tail snips), 0.2µl (1 unit) of FastStart *Taq* DNA Polymerase (Roche), 0.2µl (1µM) of each primer, 3.6 µl (4.5 mM) dNTP, 2µl of 10x reaction buffer. Amplicons of 250 bp (wild type allele), 300 bp (floxed *Scl* allele) were detected on the Qiaxcel (Qiagen) with the Qiaxcel DNA Screening Kit (Cat# 929004). Alignment Marker (QX Alignment Marker 15bp/1kb, Cat# 929521, Qiagen) and size Marker (QX DNA Size Marker 50bp-

800bp, Cat# 929556, Qiagen) were used. All the analysis was performed by the Genetic Testing Services lab at University of Missouri-Columbia.

CHAPTER 5: General Discussion and Future Directions

The final goal of this project is to develop chimeric animal (mouse or rat) models with specific cells and tissues having genotypes and phenotypes from different species. Ultimately and specifically, we intended to make an animal model with a vascular endothelium and a hematopoietic system derived from human pluripotent cells while all other tissues and organs are from a different species. For example, we are able to make interspecific chimeras between human and mouse in which the vascular endothelium and hematopoietic system are human, while all the other organs are from mouse. Establishment of this enabling technology will provide the basis for improved animal models of all types of human disease, reduce the time and cost of getting new drug candidates through the approval process, as well as create new opportunities for xenotransplantation. This new technology will immediately impact the development of improved diagnostic and treatment approaches in the areas of cancer, cardiovascular disease, infectious disease, including emerging pathogens, and vaccine development, including vaccines against select agents.

The overall goal of the research presented in this dissertation was to conduct a proof-of-concept in which we would make a mouse model with a vascular endothelium and a hematopoietic system from one genetic background while all other tissues and organs would be derived from a different genetic background. To achieve this goal, the original plan was to use genetically modified mouse

ESCs that can only differentiate into the endothelium and hematopoietic system to rescue endothelium/hematopoietic lineages deficient mouse embryos. As preliminary steps to achieving this goal, the feasibility of generating rat/mouse interspecies chimeras was tested.

Early investigations focused on whether we could make interspecific chimeras between mouse and rat by ESC-blastocyst injection. Before we initiated the interspecific chimeras study, no such experiments had been successfully reported even though the first interspecific chimera between sheep and goat was reported almost 30 years ago. One possible reason for the failure of these kinds of experiments could be a contribution of cells from different species in the placenta. ESCs can circumvent this potential problem since ESCs only contribute to the embryo proper, never placenta. Based on the principle of ESC-blastocyst complementation, we injected mouse or rat ESCs into blastocysts from the other species followed by embryo transfer to the recipients that are the same species as the blastocysts. Our data showed that generation of interspecific chimeras between mouse and rat (mouse➔rat and rat➔mouse chimeras) is possible and injected ESCs are able to distribute to various organs throughout the body, such as brain, lung, heart, GI track, kidney as well as ovary and testis. Viable interspecific chimeras were successfully produced and some survived to adulthood without obvious abnormalities. For all experiments, the ESCs and the host embryos harbored genetics for different coat colors. As expected, mixed coat-color of black patches and white patches in the mouse➔rat and rat➔mouse

offspring was observed. Mixed coat-color was very obvious in mouse➔rat chimeras while it was not so obvious in rat➔mouse chimeras which only show small patches of white hair (rat cell origin) limited to the head region. In mouse➔rat chimeras, the pattern of coat-color change indicated that mouse ESCs might have rescued the hooded gene mutation in rats. It is suggested that ESCs are able to occupy the niche of deficiency in an allogeneic environment. Similarly, a gall bladder was observed in one mouse➔rat chimera which also supported this suggestion.

Although viable interspecific chimeras can be produced, high embryonic lethality and poor postnatal development are observed quite frequently. It seems that the chimeras show more abnormalities with the higher degree of chimerism, especially in mouse➔rat chimeras. Frequently, abnormal kidney and limb formation in live offspring were observed. Histology data also showed severe hydronephrosis, mostly in the kidneys and some in the heart. Another obvious abnormality observed was the abnormal limbs in mouse➔rat chimeras. One explanation could be some complementation between mouse- and rat-derived cells may not work across species which might result in retarded postnatal development as well as causing the abnormal formation of different organs, especially in kidney and limb structure.

Together, our observations in Chapter 2 showed that xenogenic host embryo microenvironments regulate the development of donor ESCs in a temporal-

spatial manner so that the interspecific chimeras can be produced and some of them survived to the adulthood without severe abnormalities. The developmental program imprinted in blastocyst-derived and ESC-derived cells may compete to form chimeric organs during morphogenesis and organogenesis. The balance between host and donor cells thus might be very critical at least at certain points during embryonic and/or postnatal development. Future experiments can be designed to generate chimeras between species evolutionarily more distant, such as chimeras between mouse and pig, in order to better answer developmental questions related to xenogenic chimeras described in this dissertation.

Along with our experiments, there were two reports published regarding interspecific chimeras respectively in 2010 and 2011. Kobayashi's group demonstrated that mouse *Pdx* $^{-/-}$ blastocysts injected with rat ESCs result in a live chimera with a rat endocrine pancreas (Kobayashi *et al.,* 2010). Similarly, another report showed a formation of thymus from rat ESCs by injecting rat ESCs into *nu*/*nu* mouse blastocysts (Isotani *et al.,* 2011). These data showed that it is possible to produce an entire organ of one species in a different species. So, once we successfully produced interspecies chimeras in our lab and we had an EGFP-labeled rat ESC line available to us, the possibility of generating a vascular and hematopoietic system from rat in a vascular and hematopoietic system deficient mouse embryo was tested. To achieve this goal, we used *Scl* deficient embryos (described in Chapter 3) which die at E8.5 due to the absence

of vascular and hematopoietic systems. By taking advantage of Cre-*loxP* technology, female and male mice with a germline *Scl* deletion were generated and they were used to produce embryos with a *Scl* deficient genotype (see Chapter 3). There is also another method to produce 25% of embryos with a *Scl* deficient genotype (see Chapter 3) and this method was used for the rescue experiments discussed in Chapter 4.

Our hypothesis is that these *Scl* deficient embryos will provide a vascular and hematopoietic developmental niche that can be filled by interspecies rat ESCs. Therefore, injection of rat ESCs into *Scl* deficient mouse embryos should result in the generation of vascular and hematopoietic systems entirely derived from injected rat ESCs. *Scl* deficient embryos were injected with 10-15 rat ESCs followed by embryo transfer. At 16.5dpc, these foster females were dissected and examined. Unfortunately, our data did not show any evidence of phenotypic rescue of *Scl* deficient embryos. That is, PCR results never confirmed the *Scl* deficient genotype (Scl^{lacZ}/Scl^{lacZ}) from the rat ESC-injected fetus. However, our data showed that rat ESC-derived cells are found in various organs and tissues, such as lung and GI track as well as brain and the testes at 16.5dpc. One possible reason could be the rat ESC line used doesn't have the capability to differentiate into endothelial/hematopoietic lineages specifically in *Scl* deficient blastocysts. To address this possibility, additional rat ESC lines could be tested. For example, the rat ESC line used in this experiment does not go germ line when injected into rat blastocysts (Men *et al,* submitted). Other cell lines may be

more robust than this one in terms of differentiation capability both *in vitro* and *in vivo*. Another possible reason is that the small number of injected rat ESCs are not adequate to replace the whole endothelial/hematopoietic system since only 10-15 ESCs are injected into blastocysts. One way to circumvent this potential issue would be to genetically modify the ESCs so that these ESCs can only differentiate into endothelial/hematopoietic lineages. So all these injected rat ESCs will only differentiate into the lineages which are deficient in the mouse blastocysts in order to adequately replace the whole endothelial/hematopoietic lineages.

Altogether, our data confirmed that interspecific chimeras between mice and rats can be produced by the ESC-blastocyst injection method and viable chimeras could reach adulthood. However, *Scl* deficient blastocysts were not rescued by the rat ESCs used in this project when injected into the blastocysts. So what did we learn here and what is the next? As discussed before, it is possible to inject human pluripotent cells into a mouse blastocyst and allow limited development of human-mouse chimeras in order to test the differentiation potential of human pluripotent cells in addition to the traditional teratoma test. Furthermore, given the fact that mouse-rat chimeras can be taken as a first step, could production of human-pig chimeras be possible and an alternative approach for the xenotransplantation? It is true that the availability of human pluripotent cells such as ESCs and iPSCs, show more possible opportunities for tissue and cell replacement therapies in clinics, while the replacement of whole organs, such as

lung, kidney and liver, is more complicated with huge biological and technical challenges. Plus, the evolutionary distance between human and non-human animals can add extra difficulties in the production of human-nonhuman chimeras. Another concern is that, in mouse➔rat chimeras, mouse ESC-derived cells were found in various organs including brain and gonads. One alternative to this is to use to genetically modify human pluripotent cells in order to appropriately control the differentiation capability so that they can only differentiate into certain tissues or organs *in vivo*. For example, two constructs (p*CO3.3-CAG-loxP-TK-loxP-tdTomato* and *Scl*-Cre*ER*^{T}) can be designed specifically for the rat (human) and then can be electroporated into rat (human) ESCs so that these ESCs can only differentiate into endothelial/hematopoietic lineages under the induction of drugs (Tamoxifen and GCV). These cells conditionally express a suicide gene Herpes Simplex Virus-Thymidine Kinase (HSV-TK) that kills cells at a disadvantage once it is expressed and its killing effect is under control of the tamoxifen inducible system. Simply, the ESCs will be injected into embryos which are vasculature and hematopoietic system deficient, *i.e. Scl*$^{-/-}$ blastocysts. With induction of GCV and tamoxifen, Cre recombinase will be expressed at the same time as of the endothelial/hematopoietic cell-specific enhancers' expression. Then Cre will recognize the *loxP* site to cut *TK* out. All the endothelial/hematopoietic progenitor cells derived from ESCs will survive because of the depletion of TK while the other lineages will die contrarily. The embryo will be a host for the injected cells, establishing all the organs and tissues except endothelial and hematopoietic system. Altogether, injecting these genetically modified ESCs into mouse

endothelial/hematopoietic deficient blastocysts will result in the live interspecific mice with endothelial/hematopoietic lineages derived from rat (human). Later on, the same principle can be applied to make humanized animal models in which specific organs are derived from human in a non-human environment.

All in all, such experiments with human pluripotent cells will be complicated, time consuming, difficult to interpret and may not become part of the standard protocols regulating the medical use of human pluripotent cells. The work present here could be potential protocol for researchers seeking to better understand the biology of stem cells and mammalian development. The approach described here will be of use not only for better understanding of the mechanism of organogenesis but also as an initial step toward the ultimate regenerative medicine of the future.

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VITA

I was born in Ningling, Henan province in China to my parents, Guangchao Zhao and Yiling Wang. I have two lovely younger sisters: Chongcan and Xiaozi. After finishing high school located in my hometown in 1998, I studied in Zhengzhou University (orginal Henan Medicine Univeristy) located in Zhengzhou, capital city of Henan province for my M.D. My mom is a medical doctor and she always wanted me to become a doctor. During these five years, I found my interests in search, which helps me understand diseases on molecular level. That is why I went for my master degree in Beijing Military Medicine Institute afterwards and that is where I met John in an international conference held in Beijing in 2004. It was fate that helped me meet with John and after discussion with him about my research, I was offered a PhD position in his lab at the University of Missouri.

It is a quick review of my previous experiences. So after I complete the PhD study here, I plan to pursue a career in the biotech or pharma industry where hopefully I can make the most use of my knowledge in science and learn more about business management in the future.