A NOVEL TRANSGENIC RAT MODEL FOR THE STUDY OF
GERM CELL BIOLOGY

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Dedicated

to my husband Chris for his love

and encouragement and

to my best friend Linda for her

never ending support and

friendship

# A NOVEL TRANSGENIC RAT MODEL FOR THE STUDY OF GERM CELL BIOLOGY

by

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With over one million publications in scientific journals, the rat is a very important biological model in science. Unfortunately, since the introduction of genetic manipulation technology in the mouse, extension of this technology to the rat has proven to be very difficult. In an attempt to generate a transgenic line of rats expressing GFP in all cells of the body, a serendipitous integration of a ROSA-EGFP transgene resulted in exclusive expression of EGFP in the germ

cells of both sexes. EGFP expression was uniform and robust in cleavage stage embryos beginning at the late 2-cell stage and continuing through blastocyst development where expression became restricted to cells of the inner cell mass. Subsequent analysis showed high EGFP expression exclusively in primordial, embryonic, and adult germ cells. This unique expression pattern makes this EGFP marked locus the first molecular marker of the germline lineage in both sexes in mammals. FISH was used to localize the transgene insertion to chromosome 11q11-q12, proximal to *Grik1* and in close proximity to *Ncam2*. Analysis of the region did not identify known germ cell-specific genes but did identify 19 ESTs or transcribed loci present in testes, ovary, or pre-implantation libraries from mice or rats.

The unique germ cell specific expression of EGFP in these transgenic rats makes them an excellent novel tool to study germ cell origin, development, and differentiation. To evaluate the utility of the transgenic line for germ cell transplantation studies, non-selected, freshly isolated seminiferous tubule cells were transferred to the testis of recipient males. The donor cell population colonized the testis at a surprisingly high efficiency within 30 days following transfer. Since EGFP is a vital marker, the colonization process can be followed *in vivo* and the extent of colonization quantified. This assay was then used to define when developing germ cells first acquire apparent stem cell activity, and to assess the plasticity of adult SP bone marrow cells to enter the germ lineage.

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# CHAPTER ONE

## A Historical Perspective in Cloning and Germ Cell Research

### Cloning in Animals

*Introduction to cloning*

The year 2002 marked the 50th anniversary on nuclear transplantation research. In 1953, Briggs and King first reported that tadpoles could be derived from transferring the nucleus of a blastula cell to an enucleated egg (Briggs, 1952). This showed for the first time that nuclei from an embryonic cell retained the ability to develop into an animal and that the cytoplasm of the enucleated egg contained the factors needed to reprogram the nuclei. It then took over 40 years to show that a nucleus from a fully differentiated cell could be used to clone the first mammal, Dolly (Campbell et al., 1996). This section will review the history, achievements, impediments, and current focus in the exciting field of nuclear transplantation.

Nuclear cloning refers to the process that occurs when a nucleus from a donor cell is introduced into an enucleated oocyte to generate a cloned organism. Nuclear cloning can be further separated into two major events; the first is nuclear transfer that refers to the actual technique and the second is nuclear reprogramming that includes all of the events that occur after the donor nucleus is

transferred into a recipient oocyte. Nuclear reprogramming is defined as the ability of the cytoplasm of an unfertilized egg whose nucleus has been removed to return a fully differentiated nucleus to a totipotent state, having the potential to develop to a full term organism. The success of somatic cell nuclear transfer in mammals has allowed the scientific community to postulate and focus on the potential uses of cloning in medicine.

There are currently two definitions and ultimate purposes of cloning, reproductive and therapeutic. In reproductive cloning, adult animals would be produced for the potential of preserving an endangered species, or the generation of a genetically identical animal for commercial biotechnology (Gurdon and Byrne, 2003). In contrast, therapeutic cloning has the purpose of producing clonal populations of genetically identical cell types for the potential use in regenerative medicine (Jouneau and Renard, 2003) (Fig. 1.1).

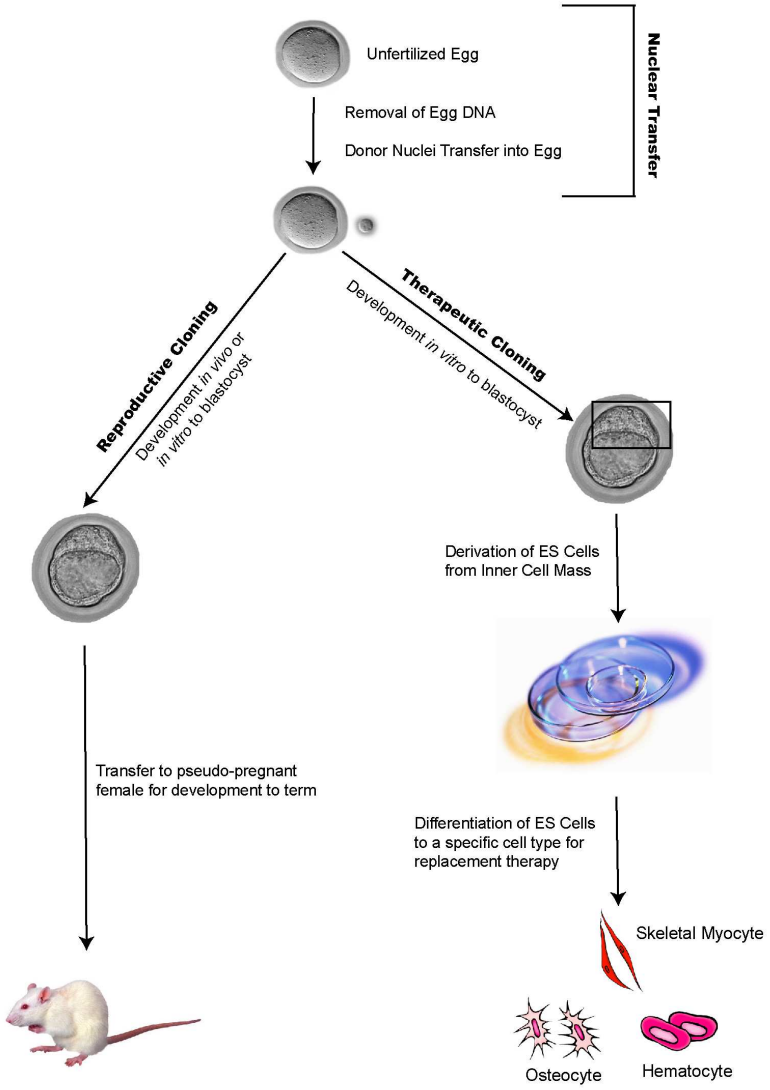


**Figure 1.1. Reproductive vs. Therapeutic cloning**. During reproductive cloning the cloned blastocyst is transferred into a surrogate mother, for in vitro development to term. During reproductive cloning the ICM of the cloned blastocyst is expanded and embryonic stem cell lines are derived that can potentially differentiate into different cell types.

Reproductive animal cloning has the potential to greatly impact the agricultural, biotechnical, and pharmaceutical industries. In the agricultural industry, reproductive cloning could improve the quality of livestock. For example, a cow with excellent milk production or with very lean muscle mass could be cloned. Only a few fertile cloned animals with a desired trait would be needed to generate a colony using selective breeding. Disease free animals could also help the agricultural industry. Bovine spongiform encephalopathy (mad cow disease) in cattle, scrapie in sheep, and Creutzfeld-Jacob disease in humans has been linked to the Prion Protein (PrP) (Paterson et al., 2003). Mad cow disease has almost shut down the beef export industry in England. If the PrP gene in the donor cells could be inactivated through knockout technology, the resulting clones would be PrP free and resistant to scrapie or mad cow disease. Cloning can also help the agricultural industry by preserving endangered species. For example, granulosa cells from the lone survivor of the Enderby Island cattle breed were used to clone Elise who is healthy and fertile. Elise will be bred using frozen Enderby sperm to expand this breed and hopefully keep this breed from extinction (Wells et al., 1998).

Finally, mammalian cloning has led to the possibility of producing large amounts of human proteins for use in treating disease that would benefit the pharmaceutical industry. Human coagulation factor IX is deficient in patients with hemophilia; currently this protein is isolated from blood plasma. Isolation of

the protein using this method, can possibly infect the recipient with infectious diseases carried in the blood (Paterson et al., 2003). One way to avoid this potential risk is to produce the protein outside of blood. The first example of this idea was the generation of a cloned transgenic sheep which expressed the human coagulation factor IX gene in its milk (Schnieke et al., 1997). A few years later, a goat that produced human anti-thrombin III that was secreted through her milk was cloned. This goat could yield 6 grams of protein per liter of milk demonstrating not only success in this new protein production technology but also demonstrating the high protein yield potential by using larger animals (Baguisi et al., 1999). These examples show the benefits reproductive cloning has in the pharmaceutical industry; transgenic animals can now be specifically engineered to produce medically needed proteins. Large domestic animals with identical genetic backgrounds could also aid the pharmaceutical industry during the initial phases of testing the effects of new drugs. Presumably, larger animals such as pigs may have responses to drugs that would be more applicable to humans due to their size and physiology which is closer to humans than mice and rats that are currently used in the early stages of clinical drug trials (Di Berardino, 2001).

Currently, there are 87401 people waiting for organ transplants and from January to November 2004, there were 24810 organ transplants from 2944 donors (Based on data form the Organ Procurement and Transplantation Network as of Feb. 7, 2005). From these data, it is clear that many people who need life saving

organ transplants will not receive one. In the biotechnological industry, xenotransplantation is an area of ongoing research. The main impediment to this technology has been histocompatibility issues. Cloning could help advance the xenotransplantation field by generating animals with specific genetic modifications in histocompatibility loci so that their organs would not be rejected by humans and could then be used for transplantation. Pig organs are ideal for xenotransplantation because the size of their organs closely matches the size of human organs and the physiology of the pig is compatible to humans (Lai et al., 2002). However, humans contain high levels of antibodies against the galactosyl$\alpha$1, 3-galactose found on the cell surface glycoproteins in pigs. The antibodies are the result of human's loss of galactosyltransferase activity during evolution (Kolber-Simonds et al., 2004). These antibodies cause a hyperacute rejection of porcine organs that occurs within minutes after the transplantation (Lai et al., 2002). In an effort to determine if histocompatibility issues can be overcome, mouse studies were done in which the $\alpha$1, 3-galactosyltransferase gene was knocked out. These mice had normal organs and when they were exposed to human serum, only a weak immune response was observed (Bondioli et al., 2001). Recently, the $\alpha$1,3-galactosyltransferase gene was deleted in pigs and sheep by nuclear transfer and studies are currently being conducted to determine the immune response to these tissues when transplanted (Denning et al., 2001; Kolber-Simonds et al., 2004; Lanza et al., 2002; Phelps et al., 2003). An

alternative avenue to pursue in xenotransplantation is to transplant tissues that possess human cell membrane proteins in the hope that the transplanted tissue will be seen as self and escape rejection. Towards this effort, transgenic pigs were produced, by cloning, to express the human CD59 (human complement inhibitory protein). When neurons from these cloned transgenic pigs were transplanted into immunosuppressed rats, aggressive tissue rejection was avoided (Imaizumi et al., 2000). This finding suggested that xenotransplantation is a possibility if specific proteins that the transplant recipient recognizes as self can be expressed in transgenic animals. However, xenografts, besides being rejected, may contain viruses which are hidden or unidentified because they do not cause infection in the pig but may manifest in humans (Paterson et al., 2003). Additionally, basic questions regarding the functionality of the transplanted pig organs in humans have yet to be revealed.

Therapeutic cloning is categorized as the production of cells using nuclear transfer procedures to generate clonal populations of genetically identical cell types for the potential use in replacement therapy in humans (Gurdon and Byrne, 2003) or the generation of stem cells for regenerative medicine (Jouneau and Renard, 2003). The goal of therapeutic cloning is to use human embryonic stem cells for directed differentiation *in vitro* into particular cell types. The differentiated cells would then be transplanted back into the original nuclei donor or potentially a histocompatible donor for therapeutic reasons. This type of

therapy would bypass some of the worries of tissue rejection that arise with xenotransplantation in that the tissue would be seen as self in the recipient. However, a major concern that must be addressed is that the cloned ES cell lines would still contain the egg donor's mitochondria and potentially some proteins could be made and this could cause the transplanted cells to be rejected. In a recent study in cows cloned cardiac, skeletal muscle, and renal cells were implanted back into the original nuclei donor. These implants were not rejected and became functional even though the mitochondria of the cells came from the recipient oocyte (Lanza et al., 2002). These results suggested that tissues from nuclear transplantation might not be subjected to rejection from an immune response.

Human therapeutic cloning is a topic under considerable ethical debate. While there is no intention to generate a full term baby, donor nuclei must be transplanted into a human unfertilized egg and allowed to develop to the blastocyst stage. The controversy arises in the fact that although the cloned blastocyst does not have the potential to develop full term *in vitro*, it has the potential to develop to term if placed into a uterus of a surrogate. Therefore, many people believe that any form of cloning is unethical and it should be banned. Many countries have taken steps to ban reproductive cloning but allow therapeutic cloning. For example, the Rosalind Institute in the UK just announced that they received a patent and governmental permission to clone human embryos

for therapeutic purposes. While some countries, such as the USA, have banned any type of human cloning, other countries have tried to circumvent the use of human oocytes to derive embryonic stem cells by using an oocyte from a different species such as the rabbit and cow. A report in 2003 described the derivation of human ES cell lines from rabbit oocytes (Chen et al., 2003), however to date no other laboratory has published data confirming their findings. In addition, there has also not been any other report on the differentiation capabilities of the stem cell lines that were generated. Therefore, the true potential for therapeutic cloning in medicine remains to be seen since it is only in the very early stages of development.

*History of Cloning*

It has been over 50 years since cloning by nuclear transfer was first reported by Briggs and King in 1952. During this time, Brigs and King were studying the developmental potential of embryonic nuclei in the frog (Gurdon and Byrne, 2003). Efforts to understand the developmental potential of nuclei began in 1888, by Wilhelm Roux and August Weismann who independently proposed the germ plasm theory. They believed that only the germ cells of the embryo carry all the nuclear determinants but each somatic cell contains only portions of the nuclear determinants required for that cell type (Di Berardino and McKinnell, 2004). Roux, in 1888, proved his germ plasma theory by taking a 2-cell frog

embryo and destroying one of the cells and this resulted in the development of only half a frog embryo (Di Berardino, 2004).

However, in 1901, Hans Spemann split a 2-cell newt embryo into two parts and each part developed into larvae that contradicted the germ plasm theory. In 1902, Spemann conducted the same type of experiment with a salamander embryo and each part developed into an adult. The latter experiment showed that each cell contained sufficient genetic information to direct development to an adult and again disproved the idea that the genetic material was divided between each cell. Then in 1938, Spemann demonstrated that salamanders retained totipotent nuclei up to the 16-cell stage (Spemann, 1938). Several researchers then extended nuclear transfer to amphibians and in 1952 Briggs and King published that they had successfully produced an adult frog (Rana pipens) by nuclear transplantation with embryonic nuclei (Briggs, 1952). In 1962, Gurdon using endoderm nuclei and intestinal epithelial nuclei was able, through nuclear transplantation, to obtain development to a zygote (Gurdon, 1962a; Gurdon, 1962b). In later studies he demonstrated that when nuclei from cultured adult *Xenopus laevis* skin cells were used as the donor, development to the tadpole stage was achieved (Gurdon et al., 1975). These studies suggested that if donor nuclei came from adult or later embryonic developmental stages, it was much harder to obtain adult frogs.

Following the success of amphibian cloning, researchers began to focus on extending the methodology to mammals. However, mammalian studies proved to be more difficult to carry out because of the small size of the mammalian embryo (< 0.1% the volume of an amphibian egg) (Gurdon and Byrne, 2003) and due to the fact that the mammalian embryo is very sensitive to the micromanipulation techniques especially when the plasma membrane is disrupted. Finally, in 1981 Illmensee and Hoope reported the birth of three mice generated by nuclear transfer (Illmensee and Hoppe, 1981). Several other groups were excited by these results and tried to reproduce this work, however, by 1984 four independent labs (McGrath and Solter, Surani, Modlinski, and Lovell-Badge) were unable to reproduce their findings. The field of mammalian cloning was at a stand still and in a now classic paper published in Science, they concluded that because the embryonic genome in the mouse was activated by the 2-cell stage there was an inadequate amount of time for the donor somatic nuclei to be reprogrammed. They concluded the paper by stating that "Cloning of mammals by simple nuclear transfer is biologically impossible" (McGrath and Solter, 1983). The cloning field in mammals then shifted its emphasis to larger domestic species in which the embryonic genome is activated at the 8 to 16-cell stage. Although amphibian cloning studies had employed unfertilized eggs as recipients, the belief at the time was that an egg whose zygotic genome had already been activated would be a better host for nuclear transplantation because the activated cytoplasm of the egg

would support reprogramming and development. This belief in the utility of the enucleated fertilized egg as the optimal recipient would be the sole reason why cloning attempts in mice failed. This oversight would not be recognized until a sheep and then a cow were cloned from embryonic nuclei from 8 to 16-cell embryos (Prather et al., 1987; Willadsen, 1986). In these experiments, the key to success was the use of an enucleated unfertilized egg as the recipient. Not long after the successful cloning of a mammal using embryonic nuclei; the first cloned animal, Dolly, from a fully differentiated adult cell type was announced (Campbell et al., 1996). Successful cloning has also been shown in a variety of other animals (Table 1.1).

**Table 1.1. Animals cloned from adult cells.**

| Animal | Donor Cell | Year | Ref |
|---|---|---|---|
| Sheep [a] | Mammary Gland | 1997 | Wilmut I et al. |
| | Granulosa | 2001 | Loi et al. |
| Mouse [b] | Cumulus | 1998 | Wakayama et al. |
| | Fibroblast | 1999 | Wakayama and Yangimachi |
| | Sertoli | 2000 | Ogura A et al. |
| | Neurons | 1998 | Wakayama et al. |
| | Mature Lymphocytes | 2002 | Hochedlinger K, and Jaenisch R |
| Cow [c] | Cummulus | 1998 | Kato Y et al. |
| | Oviduct | 1998 | Kato Y et al. |
| | Granulosa | 1999 | Wells DN, et al. |
| | Mammary Gland | 1999 | Zakhartchenko V et al. |
| | Muscle | 1999 | Shiga K et al. |
| | Fibroblast | 1999 | Zakhartchenko V et al. |
| | Uterine | 2000 | Kato Y et al. |
| Pig [d] | Granulosa | 2000 | Polejaeva IA, et al |
| | Fibroblast | 2001 | Bondioli et al. |
| Goat [e] | Granulosa | 2000 | Keefer et al. |
| | Cumulus | 2001 | Zou et al. |
| Cat [f] | Fibroblast | 2002 | Shin et al. |
| Rabbit [g] | Cummulus | 2002 | Chesné et al. |
| Rat [h] | Fibroblast | 2003 | Zhou Q, et al. |

**References for table**:

a - (Loi et al., 2001; Wilmut et al., 1997)
b - (Hochedlinger and Jaenisch, 2002; Ogura et al., 2000b; Wakayama et al., 1998; Wakayama and Yanagimachi, 1999)
c - (Kato et al., 1998; Kato et al., 2000; Shiga et al., 1999; Wells et al., 1999; Zakhartchenko et al., 1999)
d - (Bondioli et al., 2001; Polejaeva et al., 2000)
e - (Keefer et al., 2002; Zou et al., 2001)
f - (Shin et al., 2002)
g - (Chesne et al., 2002)
h - (Zhou et al., 2003)

With the recent successes of animal cloning, the questions of what are the requirements and what are the mechanisms that occur during reprogramming arise. Although, reprogramming was first defined over fifty years ago, very little is understood about the underlying mechanisms. Recent publications have elucidated some of the molecular mechanisms that are required for nuclear

reprogramming to occur. It is known that in normal development, the oocyte and sperm are transcriptionally silent until fertilization when their chromatin is remodeled to allow basic transcription to begin (Solter, 2000). In contrast, the donor nucleus is transcriptionally active and therefore it must first be silenced and then initiate an expression pattern that would be required for a zygote. This process is termed dedifferentiation. One study showed that when the nucleus of a differentiated frog cell line was placed into a frog oocyte, nuclear proteins were redistributed and taken up by the oocyte cytoplasm. They were also able to identify biochemically a chromatin remodeling nucleosomal adenosine triphosphatase (ISWI) that is necessary for reprogramming to commence (Kikyo et al., 2000). This study is the first example of the identification of a protein that plays an important role in reprogramming and may lead to an understanding of one of the molecular mechanisms that is involved in reprogramming. The donor nuclei's chromatin structure is also very different from that of a sperm or oocyte. If the donor nucleus is from a terminally differentiated cell, its DNA has undergone extensive reorganization which includes regional heterochromatization which can impede nuclear factors that are needed to remodel the DNA so that reprogramming can begin (Piedrahita et al., 2004). It is also interesting to note that mouse studies using embryonic stem cells as the donor cells have a looser chromatin structure than differentiated nuclei and appear easier to reprogram (Wakayama et al., 2001). This may be due to the ability of nuclear factors to

access the chromatin and silence the ES cell gene transcriptional activity. Finally, in normal development, once the basic transcription is established and the male and female pronuclei fuse to yield a 2N zygote, the embryonic genome must be activated. Depending on the species, the embryonic genome is activated between 2-4 days after fertilization. For cloning to be successful, the donor nucleus must be reprogrammed in the oocyte's cytoplasm. The donor nucleus must therefore, discontinue its own gene expression profile and adopt the expression pattern of a zygote. Consequently, it is believed that the reprogramming of the donor nucleus must occur before the embryonic genome is activated (Solter, 2000). Based on normal development studies, transcriptional silencing, chromatin remodeling, and embryonic genome activation are required. Therefore, it can be hypothesized that these same events must also occur in the development of cloned embryos. However, the key players, molecular pathways, and timing requirements for these processes to occur so that reprogramming is successful remain largely unknown.

*Cloning efficiency*

In order for reprogramming to occur, the donor nucleus and egg cytoplasm need to undergo very complex processes in a very short and limited period. These two factors taken together may be the reason that although the offspring of cloned animals do appear normal, "there are almost no normal clones" says Whitehead Institute for Biomedical Research MIT professor Rudolf Jaenisch M.D. The

following section will summarize the problems in the cloning field at this time and provide the potential reasons why there are so few "normal" clones. One of the most revealing reasons is the inefficiency of cloning. An accumulation of all the published cloning data indicates that only 0-10% of live births result from cloning depending on species and when the donor nucleus is derived from adult cells, the percentage of success drops below 1%. Table 1.2 summarizes the cloning efficiencies reported in different species and differing donor cell age.

**Table 1.2. Cloning efficency**

| Animal | Donor Cell Age | NT oocytes No. | Live Births No. | Cloning efficiency % |
|---|---|---|---|---|
| Sheep [a] | Adult | 1273 | 8 | 0.63 |
| | Fetal | 1087 | 14 | 1.29 |
| Mouse [b] | Adult | 14033 | 154 | 1.10 |
| | Newborn | 1778 | 16 | 0.90 |
| | Fetal | 3287 | 23 | 0.70 |
| Cow [c] | Adult | 6738 | 66 | 0.98 |
| | Newborn | 338 | 4 | 1.18 |
| | Fetal | 6142 | 39 | 0.63 |
| Pig [d] | Adult | 5222 | 7 | 0.13 |
| | Newborn | 458 | 4 | 0.87 |
| | Fetal | 3300 | 8 | 0.24 |
| Goat [e] | Adult | 519 | 10 | 1.93 |
| | Fetal | 1487 | 21 | 1.41 |
| Cat [f] | Adult | ? | 6 | ? |
| Rabbit [g] | Adult | 2000 | 6 | 0.30 |
| Rat [h] | Adult | 231 | 5 | 2.16 |

**References for table:**


a - (Denning et al., 2001; Loi et al., 2002; Loi et al., 2001; McCreath et al., 2000; Schnieke et al., 1997; Wilmut et al., 1997)

b - (Ogura et al., 2000a; Ogura et al., 2000b; Ono et al., 2001; Tsuda et al., 2003; Wakayama et al., 1998; Wakayama et al., 2001; Wakayama and Yanagimachi, 1999; Wakayama and Yanagimachi, 2001b; Yamazaki et al., 2001)

c - (Gibbons et al., 2002; Kato et al., 1998; Kato et al., 2000; Kishi et al., 2000; Shiga et al., 1999; Wells et al., 1999; Zakhartchenko et al., 1999)

d - (Bondioli et al., 2001; Polejaeva et al., 2000)

e - (Keefer et al., 2002; Zou et al., 2001)

f - (Shin et al., 2002)

g - (Chesne et al., 2002)

h - (Zhou et al., 2003)

Table 1.2 shows that donor nuclei from the fetal tissues and the newborn have a higher percentage of live births compared to nuclei from adult tissues. It has been theorized and supported by these published reports that during development and cell differentiation, the cell loses its nuclear potency. Although clones have been produced from adult populations of cells it is not known if the donor cell is a terminally differentiated cell. It remains a possibility that the clone derived from an adult cell came from an adult stem cell that resided in tissues from which the cells were collected (Mullins et al., 2003). Attempts to generate mouse clones from lymphocytes, a terminally differentiated cell, were unsuccessful (Wakayama and Yanagimachi, 2001b). However, it has been shown that terminally differentiated mature B and T-cells were able to produce cloned mice but only by using an indirect cloning method that involves two steps (Hochedlinger and Jaenisch, 2002). In the two-step cloning method, ES cells are first derived from a cloned blastocyst. The derived ES cells are then used in tetraploid embryo complementation (Nagy et al., 1993). Tetraploid embryo complementation involves taking diploid ES cells and injecting them into a tetraploid blastocyst. A tetraploid blastocyst is only able to form a placenta but not an embryo; the resulting mouse will therefore be entirely derived from the injected diploid ES cells (Hochedlinger and Jaenisch, 2003).

To make matters even worse, the number of cloned animals that survive past birth is very low. Based on published reports, when a somatic cell is used as

the donor, the cloning efficiency is below 1% in almost every species except the goat and rat (Table 1.3).

**Table 1.3. Survival of nuclear transfer clones.**

| Animal | Donor Cell Age | Live Births No. | Survived No. | Survival Efficiency % | Cloning Efficiency % |
|---|---|---|---|---|---|
| Sheep [a] | Adult | 8 | 3 | 37.50 | 0.24 |
| | Fetal | 14 | 10 | 71.43 | 0.92 |
| Mouse [b] | Adult | 154 | 134 | 87.01 | 0.95 |
| | Newborn | 16 | 11 | 68.75 | 0.62 |
| | Fetal | 23 | 18 | 78.26 | 0.55 |
| Cow [c] | Adult | 66 | 48 | 72.73 | 0.71 |
| | Newborn | 4 | 3 | 75.00 | 0.89 |
| | Fetal | 39 | 19 | 48.72 | 0.31 |
| Pig [d] | Adult | 7 | 7 | 100.00 | 0.13 |
| | Newborn | 4 | 2 | 50.00 | 0.44 |
| | Fetal | 8 | 7 | 87.50 | 0.21 |
| Goat [e] | Adult | 10 | 7 | 70.00 | 1.35 |
| | Fetal | 21 | 18 | 85.71 | 1.21 |
| Cat [f] | Adult | 1 | 1 | 100 | ? |
| Rabbit [g] | Adult | 6 | 4 | 66.67 | 0.20 |
| Rat [h] | Adult | 5 | 4 | 80.00 | 1.73 |

**References for table:**

a - (Loi et al., 2001; Wilmut et al., 1997)
b - (Hochedlinger and Jaenisch, 2002; Ogura et al., 2000b; Wakayama et al., 1998; Wakayama and Yanagimachi, 1999)
c - (Kato et al., 1998; Kato et al., 2000; Shiga et al., 1999; Wells et al., 1999; Zakhartchenko et al., 1999)
d - (Bondioli et al., 2001; Polejaeva et al., 2000)
e - (Keefer et al., 2002; Zou et al., 2001)
f - (Shin et al., 2002)
g - (Chesne et al., 2002)
h - (Zhou et al., 2003)

It is interesting to note that cloned species seem to share some common abnormalities once they develop to full term. The most common problem is large offspring syndrome that has been documented in cattle, sheep, mice, and goats (Piedrahita et al., 2004). This large offspring phenotype appears to be a side effect of cloning since the phenotype is not transmitted to offspring generated by natural mating (Tamashiro et al., 2002). Respiratory distress is a common cause of death in newborn cattle, sheep, mice, and goats. It is also common to see

defects in the extra embryonic tissues, especially the placenta that controls the growth and survival of the fetus (Jouneau and Renard, 2003). In mice, the spongiotrophoblast layer of the placenta is very large and this results in the disruption of the placental structure and inhibits the placenta from functioning properly (Tanaka et al., 2001). It has also been reported that in 40% of pregnancies there is a large accumulation of fluid in the hydroallantois (Mullins et al., 2003). The defects found in the extra embryonic tissues are one reason so few clones make it to term. Studies have shown that one third of all cloned pregnancies in large animals are aborted during gestation (Paterson, 2002). There are also specific species abnormalities that are seen. In cattle, clones commonly have pulmonary hypertension, dilated cardiomyopathy, diabetes, joint defects, prolonged gestation and enlarged organs (Cibelli et al., 2002). In pigs, abnormal number of teats and malformed limbs have been observed (Archer et al., 2003). In sheep, kidney, liver, and brain defects have been noted (Schnieke et al., 1997). A recent study that focused on health issues reported that 64% of cattle, 40% of sheep, and 93% of mice that are generated by cloning exhibit some form of abnormality (Cibelli et al., 2002). Unfortunately, it can be concluded from the reports on surviving clones that even when cloning is successful in terms of a live birth, it is obvious that the reprogramming of the donor nuclei is not complete or it is prone to many errors.

*Factors contributing to cloning efficiency*

The fact that there are any surviving clones should be considered a remarkable accomplishment considering that the donor nuclei must undergo a complete transformation. This donor nuclei must first be transcriptionally silenced, then a new gene expression pattern must be activated, a process that would include chromatin reorganization and a change in methylation patterns. With so many different and complex processes that are involved in reprogramming, it is not known where the errors are occurring that give rise to the abnormal phenotypes of surviving clones. The following section will review some of the processes where reprogramming errors could lead to abnormal clones. Epigenetic signals include histone acetylation and DNA methylation of chromatin. These signals do not change the sequence of the DNA but allow for the regulation of genes so that different types of cells are able to develop (Santos and Dean, 2004). Therefore, epigenetic reprogramming is the process that the donor nuclei must undergo for the correct development of clones to occur. The donor nucleus already contains epigenetic modifications of the particular cell type. These modifications become more complex as the cell develops along defined lineages. These epigenetic modifications must first be erased and then new modifications must occur for development to progress. Studies have shown that there are vast differences between the methylation patterns of the sperm and egg versus the donor nuclei. Further studies have shown that the methylation

patterns of a cloned preimplantation embryo have many errors when compared to that of a normal same stage preimplantation embryo (Hochedlinger and Jaenisch, 2003). The lack of expression of embryonic genes can give some insight into the inefficiency of cloning. The expression of Oct4, a marker of pluripotency, was evaluated and found not to be expressed in 60% of the cloned preimplantation embryos (Bortvin et al., 2003). This finding suggests that failure to reactivate the Oct4 gene may contribute to embryonic lethality in somatic-cell clones. However, there has not yet been enough work done to know specifically what subset of embryonic genes must be activated for reprogramming to be successful.

Another process to consider in clones is the reestablishment of telomere length. All vertebrate DNA has long tandem arrays of hexameric sequence (TTAGGG) at the end of the chromosomes. The telomeres control the lifespan of a cell by stabilizing the chromosome from errors during the replication process of the cell cycle (Mullins et al., 2003). Telomere length becomes shorter every time the cell replicates and once the telomere reaches a minimal point, the cell becomes senescent so that DNA damage will not occur. Therefore, the older the cell donor age, the shorter the telomeres should be, and if the telomere length were not reset during cloning, the clone would theoretically have a shorter lifespan. Cloning groups have looked into this possibility and the results are mixed. For instance, Dolly's telomeres were shorter than age-matched control sheep, yet another group found that cloned calves telomeres were longer than age-

matched controls (Shiels et al., 1999; Tian et al., 2000). To confuse matters even more, when senescent cells were used as donors, the clones had an increase in telomere length compared to age-matched controls and serial cloning of mice for six generations did not display any significant difference in telomere length (Lanza et al., 2000; Wakayama et al., 2000). These studies, although differing, seem to suggest that reprogramming of telomere length either has a beneficial response in that the telomeres are longer than even age-matched controls or does not seem to cause the cloned animal to have a shorter lifespan.

*Factors to improve cloning efficiency*

Somatic nuclear transfer in mammals has the potential to be extremely beneficial to biomedical science. However, since the birth of Dolly, mammalian cloning has proved to be very inefficient and the mechanisms and requirements of reprogramming remain poorly understood. To understand nuclear reprogramming, the mechanisms that preserve the genome during differentiation must be elucidated and the factors in the egg cytoplasm that allow and initiate reprogramming must be identified. First, the efficiency of nuclear reprogramming must be increased and markers of reprogramming must be found in order to study and understand the basic underlying principals of reprogramming. The current focus and strategies of the cloning field's efforts to achieve these goals will be discussed in further detail. It is not known if there is an ideal donor cell to use in

cloning. Varieties of somatic cells have been used as nuclear donors; these include cumulus cells, Sertoli cells, oviductal cells, mammary epithelial cells, and fibroblasts. However, cloned animals have been generated from all of these different cell types with relatively the same efficiency making it unclear at this time to name a superior donor cell type. For example, in cattle and mice, cumulus cells gave the highest cloning efficiency, in pigs fibroblast cells gave the highest cloning efficiency, while in goats and sheep granulosa cells gave the highest cloning efficiency (Bondioli et al., 2001; Keefer et al., 2002; Loi et al., 2002; Tian et al., 2003; Wakayama and Yanagimachi, 2001b). Many different cell types have proven to be suitable nuclear donors and only a few cells, such as Sertoli cells and brain cells from the mouse, are unsuitable for cloning.

The reason why one cell type is suitable and another is not is not clear at this time and understanding this is of interest. One aspect of the donor cell under consideration is to determine which stage of the cell cycle is the most conducive to nuclear reprogramming. The synchrony between the donor cells and the recipient oocyte is believed to be a very important factor for nuclear reprogramming to occur. Studies with sheep have indicated that donor nuclei that are quiescent or arrested in the G0 phase by serum starvation make the best donors (Campbell et al., 1996; Wilmut et al., 1997). In other studies using donor cells that are in either G1 in the cow or mouse; or G2 in the mouse have also been successful as long as the egg was kept in the 2N. G2 cells have a 4N content of

DNA therefore by allowing the extrusion of a polar body following activation of the egg  the reconstructed egg is 2N (Cibelli et al., 1998; Yanagimachi, 2002). Yet in even other studies, comparing serum starved vs. no starvation of donor cells, viable clones were obtained from both groups (Tian et al., 2003). These studies indicate that quiescence is not necessary but whether donor cells that are in G0 or G1 give a higher cloning efficiency are still unknown.

The age of the donor cell is another factor to consider when evaluating cloning efficiency. Somatic fibroblasts from either embryonic, newborn, or adult stages of development have been used successfully in cloning (Table 1.2). Generally, cells from newborns or embryos gave a slightly higher cloning efficiency. Adult cells, regardless of the type of cell, did not seem to change the cloning efficiency. They did however have a higher rate of late term abortions and more abnormalities compared to newborn and fetal cells (Tian et al., 2003). The passage number of the donor cell has also been evaluated to determine if there was a correlation with the efficiency of cloning. Successful cloning has been reported using somatic cells with late passages up to sixteen (Kubota et al., 2000). Another study showed development to the blastocyst stage from an embryonic fibroblast at passage 36 (Roh et al., 2000). These studies show that developmental potential to blastocyst stage can occur with later cell passage number and at an efficiency rate that does not significantly differ from early passage efficiency rate. This is a very important finding since generating cloned

knockout animals would require donor cells to be selected *in vitro* for the correct modifications. This screening and selection process usually takes several passages to achieve.

As previously discussed, huge epigenetic changes must occur to the donor nucleus for reprogramming to be successful. Depending on the donor cell used, differing amounts of modifications are needed. The published data clearly supports the correlation between the development potential of a donor cell and the degree of differentiation it has undergone in a specific cell lineage (Dean, 2003). These complex modifications in differentiated cells may affect how efficient the donor cell can be reprogrammed. Therefore, it has been theorized that if donor cells can be treated with reagents that erase their epigenetic modifications then they may make better nuclear donors and increase cloning efficiency. Two different pharmacological reagents trichostatin-A (TSA) that increases histone acetylation and 5-aza-deoxy-cytidine (5-aza-dC) that decreases DNA methylation have been studied to examine their effects on donor cell efficiency (Tian et al., 2003). When donor cells were first treated with 5-aza-dC, development to the blastocyst was reduced in cows (Enright et al., 2003; Jones et al., 2001). However, when TSA was used to treat donor cells development to blastocyst was significantly increased in cows but decreased in mice (Enright et al., 2003; Tian et al., 2003). These studies suggest that if modifications can be done to the donor cell before nuclear transfer it may help the development potential and cloning

efficiency. Although two of the three studies had negative effects on development potential, these drugs cannot be totally dismissed because the dosage and timing of treatment varied between studies and the conditions used may not be the optimal ones for specific species and donor cell type.

A final area that is being studied to increase cloning efficiency is oocyte activation after the donor nucleus has been transferred into the recipient. During normal fertilization the oocyte becomes activated when the sperm binds to the membrane of the egg and initiates calcium oscillations and prevents any other sperm from entering the egg (Solter, 2000). For cloning purposes the oocyte must be artificially activated either by chemical treatment with strontium chloride or by electrical pulse stimulation. These methods can induce calcium oscillations that are necessary for egg activation. However, this artificial activation differs from fertilization. For example, inositol-1,4,5-triphosphate receptor is known to be down regulated after fertilization, but in activated oocytes this down regulation has not been observed (Jellerette et al., 2000). Additionally, when the donor nuclei are transferred to a mouse oocyte, the chromosomes immediately begin to condense and soon after activation resemble a metaphase plate. The chromosomes are then segregated into two structures termed pseudo-pronuclei that do not exist in normal fertilization (Wakayama et al., 1998). When donor cells are fused to an oocyte, depending on the parameters of the electrical pulses used, the oocyte may become activated at the same time as the cell fusion.

Therefore, finding the optimal conditions for activation and the best time to initiate activation needs to be studied. One study in mice reported that the highest development potential was when the egg was activated 3-6 hours after nuclei injection (Wakayama et al., 1998). Another study in mice reported that the addition of DMSO to the activation medium significantly increased the developmental potential to blastocyst (Wakayama and Yanagimachi, 2001a). Studies using these activation parameters have not yet been tested in other species probably because nuclear transfer in larger species is done by cell fusion instead of nucleus injection and the oocytes are activated by electrical stimulation instead of chemical treatment. In the future, alternative ways to activate the egg may better recapitulate fertilization and may even increase the cloning efficiency. A recent report showed that nitric oxide when injected into an egg mimicked normal fertilization (Kuo et al., 2000). This has yet to be tested in cloning but may prove to be beneficial in increasing the cloning efficiency.

In summary, the field of animal cloning is in its very early stages. The potential uses and impact it may have in science is tremendously exciting. However, increasing the development potential and overall cloning efficiency is necessary. Then global questions about the nature of nuclear reprogramming and the exact mechanisms of reprogramming can be elucidated.

## Germ Cells

### *Origin of germ cells in invertebrates and mammals*

The union of a sperm and egg commences the production of a new organism in sexual reproduction. These unique cells, also known as gametes, transmit all the genetic information from one generation to the next, therefore, allowing the survival of a species. The precursors of gametes are germ cells. Many of the molecular mechanisms involved in the establishment and development of the germ cell lineage remain a mystery. The following will give a brief review of the published reports by many different laboratories working towards understanding the origin and differentiation of germ cells.

Primordial germ cells (PGCs) the precursors to germ cells first arise as a small cluster of cells in the invertebrate embryo. Many studies have shown that invertebrates have a germ plasm in the embryo. The germ plasm is a portion of the cytoplasm that directs cells located in this region to enter the germ line (Wylie, 2000). In *Drosophila*, the germ plasm is located in the posterior portion of the embryo after the blastula stage. A small group of cells termed pole cells are found in this region and will become germ cells. Experiments have shown that any nuclei placed in this germ plasm will also become a germ cell (McLaren, 2001; Wylie, 1999). Germ cells in *C. elegans* are first set aside in the 16-24 cell stage of development. The germ plasm is located in the posterior portion of the embryo at this stage. The P4 cell of the *C. elegans* is found in the germ plasm and

it is believed that P granules found in the germ plasm determine the P4 cell fate to the germ lineage (Wylie, 1999). The *Xenopus laevis* also has a germ plasm that contains germinal granules that are similar to the P granules found in *C. elegans*. The plasm is located in the endoderm tissue of the egg and cells located in the germ plasm will become germ cells (Wylie, 1999). Finally, in the chicken, the germ cell lineage is not determined until later cleavage stages. At the epiblast stage of development there is a region that specifically expresses the *chicken vasa homologue* (*cvh*); cells found in this region become germ cells (McLaren, 2001). It is not known if this region of cvh expression is a germ plasm. For invertebrates, it seems that the germ cell lineage is determined by the location of cells in a specific area often referred to as the germ plasm of the embryo.

In order to understand the mechanisms involved in germ cell fate in invertebrate embryos, the germ plasm has been studied to determine what proteins make up this plasm and to determine if early markers for germ cell specification exist. This highly specialized region in the embryo contains proteins, fibrils rich in RNA, and electron dense masses known as polar granules in *Drosophila*, P granules in *C. elegans*, and germinal granules in *Xenopus* (Wylie, 2000). A number of orthologous genes have been found in the germ plasm; for example, RNA binding protein Nanos originally found in *Drosophila* functions to inhibit a number of genes from being expressed so that germ cell migration can occur (Kobayashi et al., 1996). Homologues of this gene have been found in *Xenopus*

(*Xcat2*) and *C. elegans* (*Nos1* and *Nos2*); they are localized to the germ plasm and are required for germ cell differentiation (Kloc et al., 2001; Subramaniam and Seydoux, 1999). These genes appears to be required to either establish and or maintain the silencing of genes during germ cell development (Wylie, 2000). Another example is Vasa, a RNA binding protein of the DEAD box family found in *Drosophila* and a homologue Xcat3 in *Xenopus* is localized to the germ plasm (McLaren, 1999). Other important genes that have been identified seem to be specific for the species since no orthologs have been found. In *Xenopus, Xklp,1* a kinesin-like protein is responsible for the movement of granules in the germ plasm in the egg and has no apparent orthologs (Vernos et al., 1995). Another example of a protein found only in the *Xenopus* is *Xpat*, which localizes to the germ plasm when PGC move into the mesentery and it functions to target other proteins in granules to the germinal particles (Hubbard, 2003; Hudson and Woodland, 1998). Finally, *PIE1* is a transcriptional repressor in *C. elegans* that will not allow the cells in the germ plasm to enter any other cell lineage (Wylie, 2000). These genes are just a small glimpse into the molecular mechanisms that control the fate of a germ cell in invertebrates.

The germ plasm is crucial in establishing germ cells in invertebrates however; germ plasm does not exist in all species suggesting that the mechanisms for germ cell determination are also different. Studies in mammals have proven that germ cell determination is not a universal process. In mouse development,

following implantation, the blastocyst becomes organized into an epiblast from which all embryonic and extra-embryonic tissues are derived (Gardner, 1988). All embryonic tissues are derived from the inner cell mass of the blastocyst while the extra embryonic tissues are derived from the trophectoderm of the blastocyst. The germ cells presumably would originate from the ICM, yet germ cells are not defined until the gastrulation stage of development. During gastrulation, the epiblast divides into three primary germ layers (endoderm, ectoderm, and mesoderm) and the extra-embryonic tissues. The germ layers are the precursors to all fetal tissues and the germ cells differentiate from the mesoderm layer (Loebel, 2003). Germ cells are first seen in the mouse embryo as a cluster of cells in the extra embryonic mesoderm at E7.5 due to their high alkaline phosphatase activity (Ginsburg et al., 1990). Very elegant transplantation studies were performed to determine if germ cells could be identified before E7.5 in the mouse. These studies proved that marked single cells taken from different portions of the epiblast and placed in the proximal region of the epiblast next to the extra embryonic ectoderm were able to become germ cells at E6.5 (Lawson and Hage, 1994). Therefore, the germ cell lineage is not yet defined at this embryonic stage. Recent studies have shown that the commitment to the germ cell lineage is dependent on exposure to *BMP4* released from the extra embryonic ectoderm that is adjacent to the extra-embryonic mesoderm (Fig. 1.2).

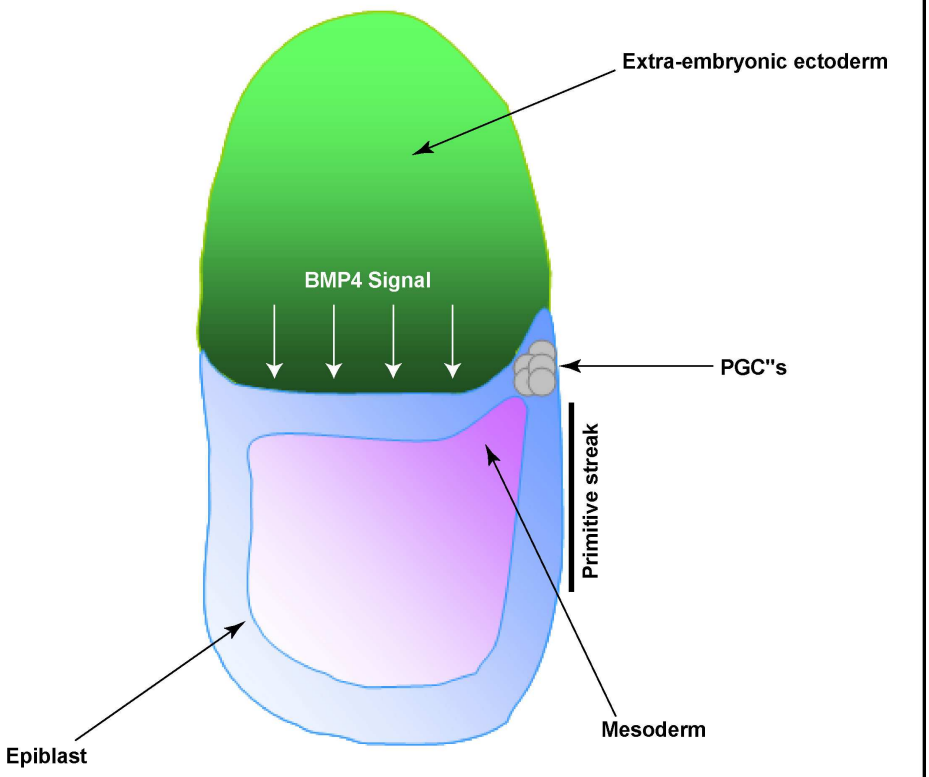


**Figure 1.2. Location of primordial germ cells in the mouse on E7.5.** Schematic of mouse epiblast when PGC's are first identified by high alkaline phosphatase activity. The PGCs are located in the mesoderm in the proximal region of the epiblast. A gradient of BMP4 from the extra-embryonic tissue is one signal that determines a cells entry into the germ cell lineage.

However, the *BMP4* signal is not sufficient to determine germ cell fate because cells taken from the proximal epiblast at a time when BMP4 is present do not become germ cells in culture (Lawson et al., 1999). It has also been shown that BMP2 is present in the epiblast and cooperates with BMP4 to initiate a SMAD signaling pathway (Kierszenbaum and Tres, 2001). It is not known if BMP4 and/or BMP2 act directly on germ cells or the identity of downstream genes that

are activated or repressed upon SMAD activation by BMPs. Discovery of the BMP signaling pathway's involvement in germ cell fate is just a small piece of this complex process; many questions remain concerning the processes that must take place to form a germ cell.

One hindrance to understanding the mechanism of germ cell determination in mammals is that the expression pattern of early germ cell markers found in other species does not correlate with expression patterns found in mice. In addition, there are no known specific germ cell markers in the epiblast stage of development in the mouse when germ cells are first defined. An example of this is the *Drosophila vasa* gene that marks the emergence of germ cells in the germ plasm of this species. In the mouse the *vasa* homologue, *mvh,* is not expressed until after germ cells have migrated to the genital ridges (Raz, 2000). Another gene, *Gustavus (Gus)* is required for the localization of vasa in *Drosophila* and is also found in mammals but the function in mammals remains unknown (Hubbard, 2003). Mouse germ cells can be identified at E7.5 due to their high alkaline phosphatase activity (Chiquoine, 1954). Alkaline phosphatase activity is present at equal levels in most embryonic cells before E7.5, making it impossible to identify germ cells before E7.5 (McLaren, 2003). However, alkaline phosphatase activity is the earliest known marker for mouse germ cells. Oct4, a marker for pluripotency, also marks germ cells during development but like alkaline phosphatase activity, it is not specific for germ cells in the early stages of

embryonic development (Pesce and Scholer, 2000). E-cadherin, another protein found in early germ cells, is necessary to facilitate cell-to-cell interactions to activate intracellular signaling molecules like MAPK and PI3K. This protein like Oct4 and alkaline phosphatase, is found not only in germ cells but also in all pluripotent cells in the developing mouse embryo (Pece et al., 1999). Fortunately, a few germ cell specific markers do exist. These include, fragilis, which is an interferon inducible transmembrane protein whose expression is dependent on BMP4 and possibly involved in cell-cell interactions required for PGC determination and Stella, which is first expressed in nacesent PGCs (Matsui and Okamura, 2005). Unfortunately, these genes are not highly expressed in any specific cells until the embryo is at developmental stage E7.5 (Lange et al., 2003). Their expression pattern mimics the alkaline phosphatase activity suggesting that germ cells may not be determined until mouse E7.5. It is unclear exactly how or when mammalian germ cells enter this lineage. Future studies focused on identifying the molecular players in the mouse germ cell specification will lead to understanding this complex process.

*Germ cell migration to the genital ridge*

Once a cell has entered the germ cell lineage, it must migrate from the site of origin to the genital ridge. During migration, the number of PGCs increase through mitotic divisions and undergo random X inactivation in females

(McMahon et al., 1981). As discussed earlier, most of the molecular pathways and mechanisms involved in germ cell development are unknown; and this is true for the migration process of PGCs. A few markers have been discovered in both invertebrates and mammals. In *Drosophila*, genetic screens have revealed a number of important genes for the migration process. *Columbus,* a lipid metabolizing enzyme called HMG CoA reductase, functions to send a signal to attract PGCs to the gonadal mesoderm (Van Doren et al., 1998). Another gene *Wunen,* encoding a phosphatidic acid phosphatase involved in lipid metabolism, functions to repulse PGCs from the gut endoderm (Zhang et al., 1997). Finally Nanos, a RNA binding protein, inhibits a set of genes from being expressed so that germ cell migration can occur (Kobayashi et al., 1996). The elucidation of the function of these genes has shed some light on the regulation of germ cell migration in invertebrates. It seems likely that germ cell migration involves controlled expression of specific genes and chemoattractants or repellants that direct the germ cells through the developing embryo.

In mammals, the migration of PGCs differs from invertebrates. In the mouse, 24 hours after the establishment of the germ line, the visceral endoderm moves to form the hindgut and the cluster of germ cells in the endoderm is distributed along the hindgut between E7.5-E9.0 (McLaren, 2000). The PGCs leave the hindgut and migrate to the neighboring mesenchyme between E9.5-E10.0. All the PGCs have migrated to the gonad primordia by E11.5 (Wylie,

2002). It is not known if the migration of PGCs in the mouse is an active or passive process. There may be signals from somatic cells that drive the PGCs to be distributed along the hindgut, or the distribution of the PGCs could be the result of the endoderm's expansion to form a hindgut (Freeman, 2003). Although the mechanisms that drive the germ cells to the genital ridge in the mouse is not fully understood due to the movement of PGCs among somatic cells, there must be germ cell and extracellular matrix protein interactions. Laminin, type IV collagen, is expressed and restricted to the basal lamina under the epithelium of the gonad primordial. Migrating PGCs colonize the gonad on the laminin positive regions through interaction with an integrin (Wylie, 2002). Unfortunately, there are no markers that identify PGCs migration from the proximal epiblast to the genital ridge. Functional homologues to proteins that mark invertebrate PGC migration have not proven to be important in mammals.

*Development of the embryonic gonads from the genital ridge*

Once the PGCs have migrated to and colonized the genital ridge, they can begin their next step in differentiation. Division continues but at a much slower rate and motility is now completely lost. PGCs are often called gonocytes at this stage of development. Germ cell mouse markers have been identified in PGCs that have reached the genital ridge. These include SSEA-1 that is first expressed on E9.5 when the first PGCs reach the ridge, mouse vasa homolog (mvh) that is

also first expressed when PGCs reach the genital ridge, and germ cell nuclear antigen (GCNA1) that is first expressed on E12.5 when sex determination begins. However, only GCNA1 and mvh are germ cell specific markers. It is also known that germ cells associate with somatic cells presumably via membrane bound Steel factor and its receptor c-kit found on germ cells (De Felici et al., 1996).

By E12.5 PGCs that have colonized the genital ridge, have been exposed to hormones and cellular interactions that will help to influence whether they will become an egg or sperm. At this stage, the male and female genital ridge can be distinguished from each other. The male genital ridge has a distinct testicular cording pattern. The male germ cells become mitotically arrested, differentiate into prospermatogonia, and will not enter meiosis until about 1 week after birth (McLaren, 1992). The female ridge has a molted or stippled pattern and the inactive X chromosome in the germ cells is reactivated. Female germ cells cease mitosis at E13.5 and enter the first meiotic division which will be arrested at the diplotene stage at birth (Nakatsuji and Chuma, 2001). Following sex determination, structures known as embryonic gonads are formed. The embryonic gonads remain inactive until the birth of the mouse. Germ cells then enter the final stage of differentiation, oogenesis in the female, or spermatogenesis in the male. This complex process of germ cell development is summarized in Fig. 1.3.

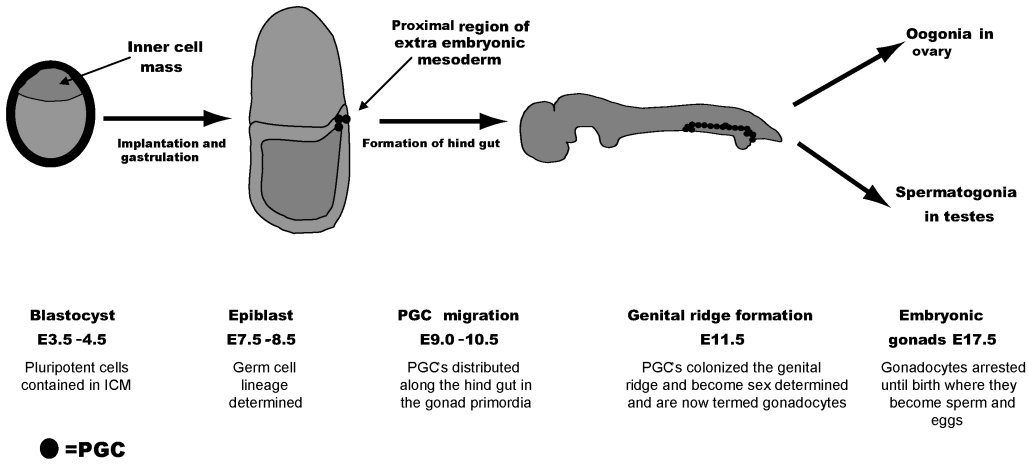


**Figure 1.3. Germ cell development in the mouse.** Schematic of germ cell development in the mouse illustrating the location of the germ cells at during different time-points in development. All embryonic cells are derived from the inner cell mass of a blastocyst. During gastrulation, primordial germ cells are set aside the proximal region of the epiblast in the extra-embryonic mesoderm. The PGC will then migrate to the genital ridge were the embryonic ovary and testis will develop.

Although we have developed a better understanding of germ cell specification and differentiation in the last ten years, many of the mechanisms and players involved in these processes remain elusive. Even more frustrating is the fact that these processes are not universal so new findings in one organism may not have any relevance to another. Future studies focusing on identifying key players that control entry into the germ lineage and the differentiation of germ cells to the sperm and egg will lead to constructing the molecular pathways of germ cell development.

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# CHAPTER TWO

## Cloning a Mouse and Rat

### Introduction

The first cloned mammal from an adult somatic cell was a sheep named Dolly and this feat immediately claimed international fame (Wilmut et al., 1997). The following year the first cloned mouse (Wakayama et al., 1998a) and cow (Kato et al., 1998) were reported and this success confirmed that adult somatic cells could be reprogrammed when placed into an enucleated oocyte. With the success of cloned mammals, new opportunities using cloning to investigate complex mechanisms such as genomic reprogramming and imprinting became possible. Using the mouse model to examine these processes has many advantages. Their short generation and lifespan allow studies to be completed in a few years as compared to decades if larger domestic animals are used (Tamashiro et al., 2003). Additionally, many mouse genes have been well characterized, the mouse genome has been completely sequenced, and these genes have been mapped to specific chromosomes. Therefore, the mouse ideally could be used to find genes required for reprogramming. For example, reconstructed oocytes could be collected at specific time points, mRNA isolated, and molecular techniques such as microarray analysis could be performed so that genes that are actually transcribed during reprogramming could be elucidated. However, the

past eight years have shown that cloning is a very inefficient process and less than five percent of reconstructed oocytes will make it to term (Yanagimachi, 2002). Therefore, the focus on cloning has been on trying to improve the development of reconstructed oocytes, and to identify early markers of reprogramming so that a basic understanding of the molecular mechanisms that regulate the reprogramming process can be developed.

The rat is one of the most extensively studied animals in science. There are over one million published papers using the rat as the biological model. The rat is a good animal model due to its small size that allows for easy housing and maintenance, short gestation period that allows for rapid expansion of a colony, and the ease of performing surgical techniques without significant complications (Waynforth, 1992). The rat was the first species to be bred for research in 1850, and became the model of choice by physiologists (Lindsey, 1979). Rat strains were generally developed in individual laboratories based on a particular trait they displayed. There are currently over 234 different inbred strains that exhibit certain phenotypes that relate to human disorders or diseases (Jacob and Kwitek, 2002). The most common rat models found today are used to study immunogenetics, transplantation, cancer-risk assessment, cardiovascular disease, and behavior (Gill et al., 1989). Unfortunately, the rat has taken a back seat to mouse models in recent years due to the difficulty in genetically modifying this species. Transgenic rats produced by pronuclei injection were first reported in

1990 by two different groups (Hammer et al., 1990; Mullins et al., 1990). However, due to the difficulty in manipulating rat oocytes, this technology has not reached the same level of use as in the mouse. Gene targeting is also done with relative ease in the mouse since the derivation of embryonic stem cells in 1981 (Evans and Kaufman, 1981). These embryonic stem (ES) cells can be maintained in culture and genes can be targeted through homologous recombination. Although many groups have attempted to derive rat embryonic stem cells, to date no pluripotent lines have been established. Therefore, specific genetic deletions or insertions are not yet possible in the rat. Other methods must be explored to extend this technology to this species. For example, the development of culture systems for spermatogonial stem cell propagation is an alternative strategy that may allow specific gene targeting within germ cells (Hamra et al., 2002).

The main goals I wanted to accomplish were to first reproduce the mouse cloning experiments at UTSW-Cecil and Ida Green Center for Reproductive Biological Sciences to demonstrate that I had mastered all the required skills needed to clone a mouse to full term; secondly, to extend the cloning technology to the rat. The rat, in the year 2001, had not yet been cloned and if a rat could be cloned then this would open the door to specific gene targeting by genetically modifying the rat with homologous recombination. Knock-out and knock-in technologies are only available in the mouse because genetic modifications using homologous recombination are most commonly done using embryonic stem cells

that can be incorporated into the blastocyst of the mouse and this allows the genetic modifications to enter the germline. Unfortunately, embryonic stem cell lines do not exist in the rat. However, if rats can be cloned, then somatic cells such as fibroblasts could be genetically modified and selected for the correct modifications. These modified cells could then be used as the donor nucleus in nuclear transfer experiments. Rats generated through cloning would contain desired modifications and the need for rat ES cells would not be essential. This new technology has been used in sheep to delete the *PrP* gene (Denning et al., 2001), demonstrating that it is possible to expand the gene targeting technology if embryonic stem cells are unattainable. The transfer of this technology to the rat will allow for the establishment of genetically defined rat models of human disease.

**Materials and Methods:**

*Collection of metaphase II mouse oocytes*

Female C57Bl6/SJL F1 mice were induced to superovulate by an intraperitoneal (ip) injection of 7 units of Gestyl (Organon Pharmaceuticals, West Orange, NJ) followed 36 hours later by another ip injection of 7 units of hCG (Sigma Aldrich, St. Louis, MO). Twelve hours after hCG stimulation, cumulus enclosed oocyte masses were collected from the oviducts. Oocytes were isolated from the cumulus masses by placing the masses in Brinster's modified oocyte medium

(mBMOC-3) (Hammer, 1998) supplemented with 0.1% hyaluronidase IV (Sigma-Aldrich, St. Louis, MO). Following enzymatic treatment, the oocytes were washed three times in mBMOC-3 medium to remove any traces of hyaluronidase and placed in at 37.5°C, under 5% $CO_2$ atmosphere.

*Enucleation of metaphase plates in the oocyte*

Isolated oocytes were placed in a microdrop overlaid with mineral oil on a glass slide attached to a specifically constructed aluminum base. The microdrop consisted of mBMOC-3-HEPES supplemented with 5μg/ml of cytochalasin B (Sigma-Aldrich, St Louis, MO). After five min incubation in the microdrop, the glass slide with the oocytes was then placed on the microscope stage. Each oocyte was then picked up by the holding pipette and rolled around until the metaphase plate, which could be visually identified with differential interference contrast (DIC) microscopy, was positioned in the 12-3 o'clock position (Fig. 2.1.A, Fig. 2.2.A-B). The metaphase plate was removed by first drilling a hole in the zona pellucida with the assistance of a Piezo Micromanipulator Controller PMM150 (Prime Tech Ltd., Ibaraki, Japan) piezo drill, that was attached to the injection pipette. The injection pipette was then placed adjacent to the metaphase plate and it was then aspirated into the injection pipette taking only a minimal amount of oocyte cytoplasm (Fig. 2.1.B-D, Fig. 2.2.B-D). Following enucleation, oocytes were washed in mBMOC-3 medium to remove any traces of cytochalasin

B, and held in mBMOC-3 at 37.5°C, under 5% $CO_2$ until required for the injection of the donor nuclei.

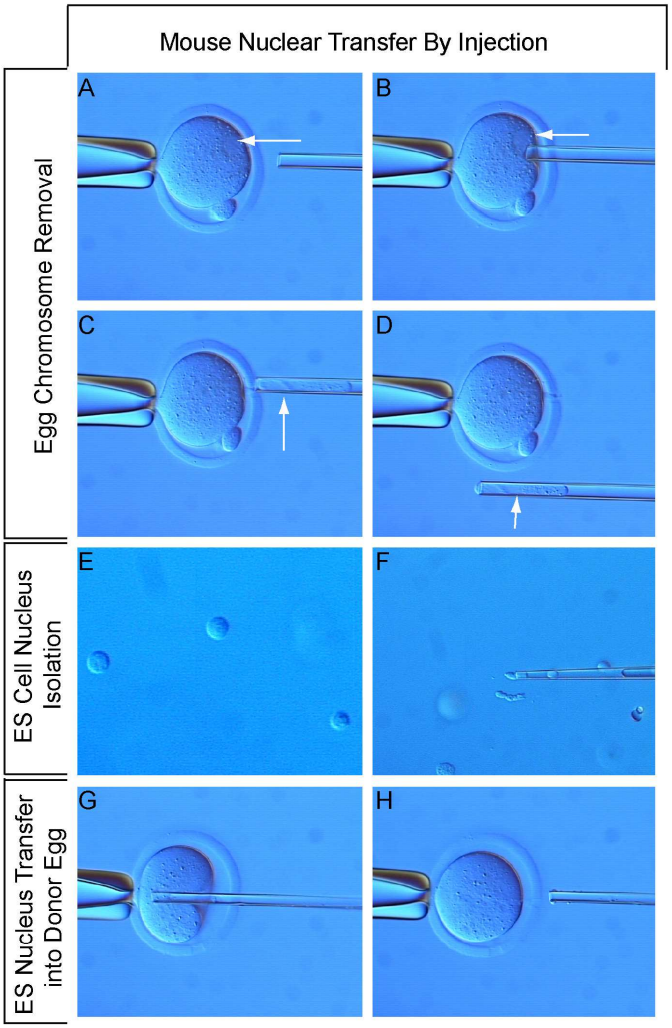



**Figure 2.1. Mouse nuclear transfer by injection technique.** Bright field DIC photomicrographs (A-D) Removal of recipient egg's DNA that is found arrested in the metaphase II stage of meiosis. A white arrow indicates the metaphase plate. (E-F) Isolation of donor nucleus, the donor cell membrane is disrupted and the nucleus is isolated from the cytoplasm by drawing the cell in and out of the pipette. (G-H) The donor nucleus is injected into the recipient egg with the aid of a Piezo drill attached to the injection pipette.

*ES cell line and culture conditions*

An ES cell line called Rosa-LacZ obtained from Richard Behringer was used in this study. Donor ES cells were used between passage 11-21 and were grown in stem cell isolation media (SCIM) (Robertson, 1987). Briefly, SCIM consists of DMEM-lo (Invitrogen/GibcoBRL, Carlsbad, CA) supplemented with; 20% FBS-ES cell qualified (Hyclone, South Logan, UT), 1000units/ml of ESGRO (Chemicon International, Temecula, CA), 1% antibiotic stock (Invitrogen/GibcoBRL, Carlsbad, CA), 1% beta-mercaptoethanol (Invitrogen/GibcoBRL, Carlsbad, CA), 1% non-essential amino acids (Invitrogen/GibcoBRL, Carlsbad, CA), 1% nucleoside (Sigma-Aldrich, St. Louis, MO),and 1% L-glutamine (Invitrogen/GibcoBRL, Carlsbad, CA). Cells were maintained on an irradiated STO feeder layer. Cells were split 1:4 every 24 hours.

*ES cell nuclei isolation and injection*

A 35mm culture dish of Rosa-LacZ ES cells, 75-100% confluent, was treated for 15 min with 0.25% trypsin (Invitrogen/GibcoBRL, Carlsbad, CA), at 37.5°C, under 5% $CO_2$. The trypsin solution was inhibited by the addition of SCIM that contained 20% FBS. The cells were moved to a 50ml conical tube and pipetted up and down 5-10 times to produce single cells. The cells were then spun down at 100 X g for 3 min and the pellet was resuspended with 3 ml of SCIM. The cell

suspension was moved to a 35 mm culture dish treated with 0.1% gelatin and incubated for 30 min at 37.5°C under 5% $CO_2$ to allow the STO feeder cells to adhere to the culture dish. The supernatant fluid containing mostly ES cells was collected and centrifuged at 100 X g for 3 min to collect all the cells. ES cells used for injection were resuspended in 1ml SCIM and then a small portion of the resuspended cells were added to a microdrop of SCIM supplemented with 10% PVP to inhibit the cells from sticking to each other and the glass injection pipette (Fig. 2.1.E). Cells were then placed in a microdrop overlaid with mineral oil on a glass slide attached to a specifically constructed aluminum base and placed on the microscope stage. Single cells were drawn in and out of a glass injection pipette from Humagen (Charlottesville, VA) with an 8-10 micron opening until the plasma membrane was disrupted. The nuclei were then slowly aspirated in and out of the injection pipette until most of the cytoplasmic material surrounding the nucleus was gone (Fig. 2.1.F). Nuclei were injected into the oocyte with the assistance of the piezo drill within 10 min of isolation. The tip of the injection pipette touched the oocyte plasma membrane at the 3 o'clock position. The pipette then was advanced almost to the opposite side of the oocyte to stretch out the oocyte membrane and one or two piezo-pulses were applied to disrupt the membrane (Fig. 2.1.G). The injection pipette contained a small amount of mercury or Flurionert (Sigma-Aldrich, St. Louis, MO) because these compounds have a higher density and are able to transmit the piezo pulses efficiently to the

oocyte. The nucleus was slowly dispelled into the oocyte cytoplasm and the injection pipette was slowly removed leaving the nuclei in the oocyte (Fig. 2.1.H). Injected oocytes were then placed in mBMOC-3 medium at 37.5°C, under 5% $CO_2$ for 1-3 hours.

*ES cell fusion to the oocyte*

ES cells used for fusion were then resuspended in 1ml of SCIM and placed in a microdrop overlaid with mineral oil on a glass slide attached to a specifically constructed aluminum base and placed on the microscope stage. Single intact cells were picked up by an injection pipette and placed in the perivitelline space of the enucleated oocyte (Fig. 2.2.E). Enucleated oocytes whose polar body and been removed and an ES cell placed adjacent to the oocyte in the perivitelline space were placed in a fusion chamber containing fusion medium that consisted of 300mM mannitol supplemented with 0.1mM $MgSO_4$, 0.1mg/ml polyvinyl alcohol, and 3mg/ml of BSA. Oocytes were first equilibrated in the fusion medium for 1-2 min and fusion was induced using an ECM2001 (BTX, San Diego, CA) a DC pulse of 1800V/cm to align the oocyte –ES cell perpendicular to the wires of the fusion chamber, once aligned a pulse of 100V/cm at 1.5MHz for 9μs was manually given to induce fusion. The oocytes were then washed and placed back into mBMOC-3 medium and cultured for 1 hour at 37.5°C, under 5%

$CO_2$. After one hour, fusion was accessed under a stereoscope and oocytes that had fused were activated (Fig. 2.2.)

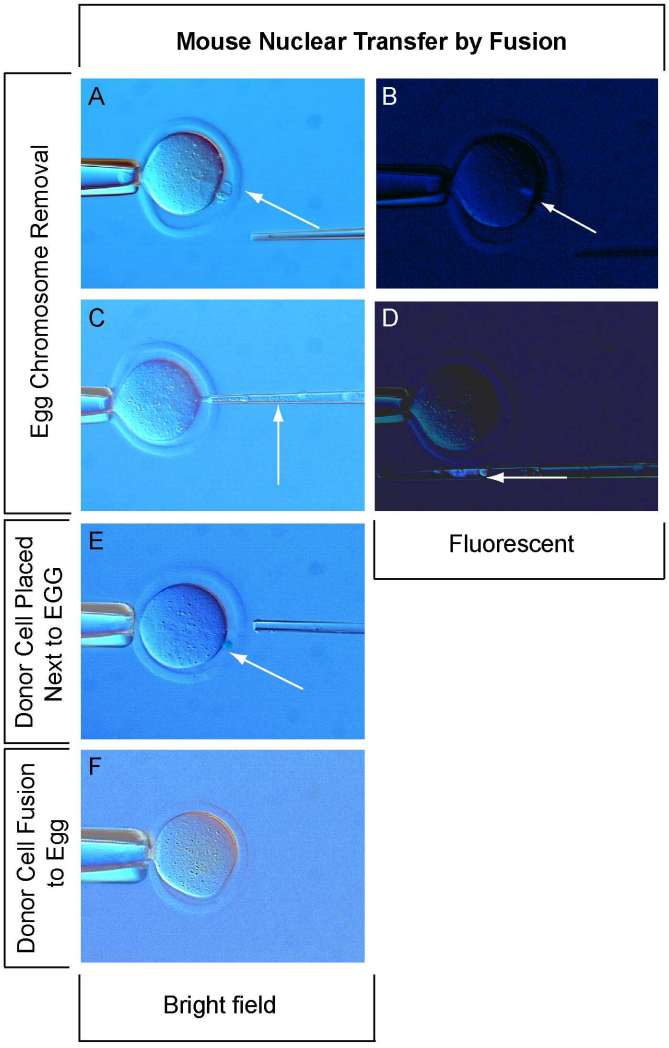



**Figure 2.2. Mouse nuclear transfer by fusion technique.** Bright field DIC photomicrographs of the different stages in mouse cloning using fusion. (A and C) Removal of the donor egg's metaphase plate white arrows indicate the DNA. (E) Donor cell which is placed next to the recipient egg prior to fusion; white arrow indicates donor cell. (F) Recipient egg immediately after fusion. (B and D) Fluorescent photomicrographs of (A and C) white arrows indicate the DNA.

*Oocyte activation*

Following donor nuclei injection or donor cell fusion, reconstructed oocytes were placed in 10mM of $Ca^{2+}$-free mBMOC-3 containing 10mM $SrCl_2$ and 5μg/ml of cytochalasin B and incubated for 6 hours at 37.5°C under 5% $CO_2$. Following activation, the oocytes were washed 3 times in mBMOC-3 and then cultured in mBMOC-3 at 37.5°C under 5% $CO_2$.

*LacZ staining in Preimplantation Embryos*

Oocytes were removed from MMBMOC-3-3 medium and washed in Phosphate Buffer Saline (PBS) without Ca or Mg. Oocytes were fixed in 1% paraformaldehyde and 0.2% glutaraldehyde for 5 min and then washed 3 times in PBS plus 10mg/ml of BSA (PBA). Oocytes were stained in LacZ staining solution (PBS plus 1mg/ml of X-gal, 5mM potassium ferricyanide, and 5mM potassium ferrocyanide) for 10min to 1 hour at 37°C. After staining, oocytes were washed one time in PBA, fixed in 10% neutral buffered formalin for 10 min, washed in PBA, and stored at 37°C in PBA.

*Embryo Transfer*

Reconstructed oocytes at the 2-cell stage were transferred to the oviducts of B6CBAF1 on day 0.5pc pseudo-pregnant females that had been mated to B6SJLF1 vasectomized males.

*Collection of superovulated rat oocytes*

Female Sprague Dawley rats (75-100g) were induced to superovulate by an intraperitoneal (ip) injection of 20 units of Gestyl (Organon Pharmaceuticals, West Orange, NJ) followed 48 hours later by an ip injection of 20 units of hCG (Sigma Aldrich, St. Louis, MO). Twelve hours after hCG stimulation, cumulus-oocyte masses were collected from the oviducts. Oocytes were isolated from the masses by placing the cumulus masses in R1ECM medium (Specialty Media, Phillipsburg, NJ) supplemented with 0.5% hyaluronidase. Following treatment, the oocytes were washed three times to remove any traces of hyaluronidase and placed in R1ECM medium at 37.5°C under 5% $CO_2$

*Visualization of DNA in ovulated rat oocytes*

Isolated oocytes were incubated for 5 min in a microdrop of RIECM-HEPES supplemented with 2μg/ml of Hoechst 33342 (Sigma-Aldrich, St. Louis, MO). The oocytes were then placed on a glass depression slide, visualized with a Leica inverted equipped with a UV light source and a fluorescent cube.

## Results

*Establishment of nuclear transfer protocol in the mouse*

The first part of my project focused on learning and becoming proficient at micromanipulation techniques using the mouse as the model organism. The second part was to establish mouse cloning at UTSW Medical Center. My initial cloning attempts followed the published protocols for mouse cloning (Wakayama et al., 1998a; Wakayama et al., 1999; Wakayama and Yanagimachi, 1999a; Wakayama and Yanagimachi, 2001a). However, in the early mouse cloning reports, the specific timing and conditions were not fully worked out so detailed protocols were not available. Therefore, after many attempts and systematic variations in the timing and the order of steps, a protocol that routinely resulted in some *in vitro* development of reconstructed oocytes was obtained. Figure 2.3 illustrates the mouse cloning protocol that I followed in my studies.

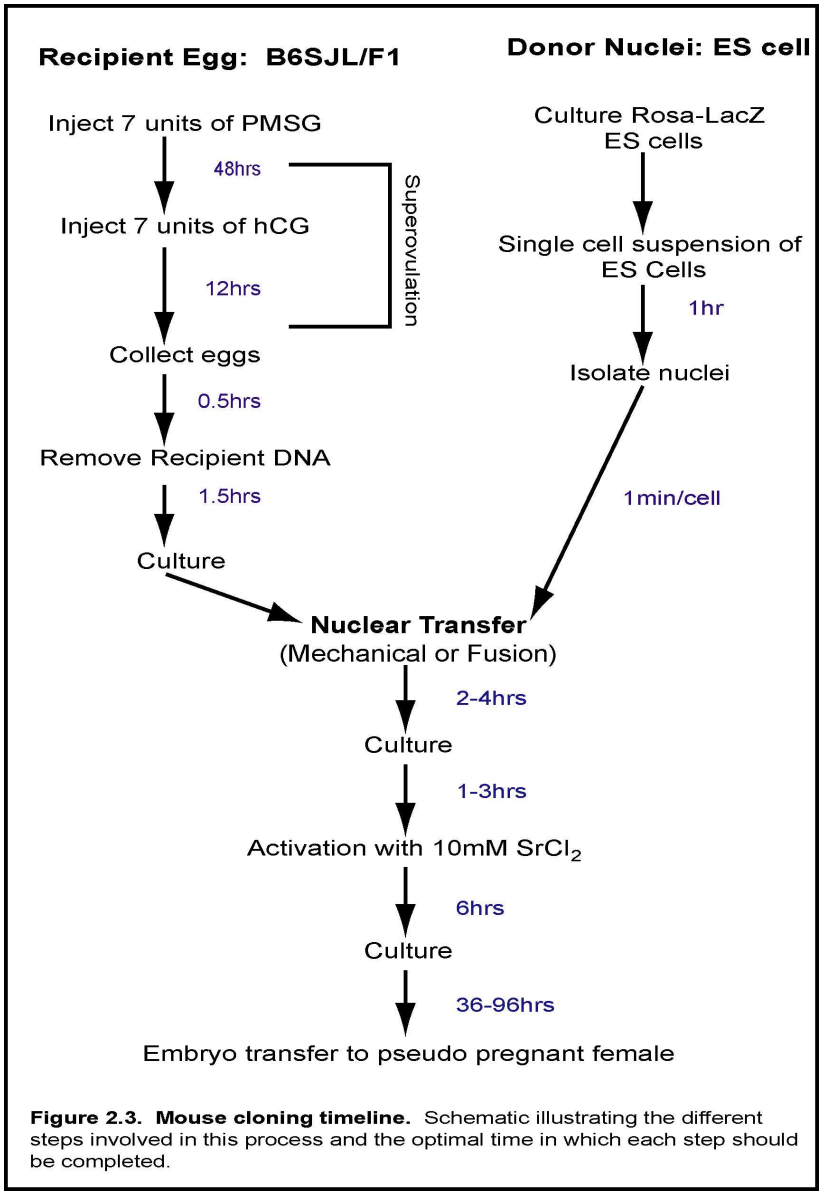



**Figure 2.3. Mouse cloning timeline.** Schematic illustrating the different steps involved in this process and the optimal time in which each step should be completed.

*Development of reconstructed oocytes*

Previous studies have shown that when embryonic stem (ES) cells are used as the donor nuclei, the reconstructed eggs have a higher rate of development than those derived from adult somatic cell donors. Therefore, ROSA-LacZ mouse

embryonic stem (ES) cells were chosen as the donor cell because of the higher expected developmental potential, and as important, the development of cloned oocytes could be followed since the LacZ was expressed in all cells (Kisseberth et al., 1999). Unfortunately, LacZ is not a vital marker; therefore, once the reconstructed oocyte was stained for expression further development of a cloned oocyte was impossible. However**,** certain morphological changes occur following the injection of a donor nucleus into an enucleated oocyte that can be considered indicators of re-initiation of development. After chemical activation of a cloned mouse oocyte, the ES cell nucleus forms a structure that is similar to pronuclei formed during normal fertilization. This nuclear structure in reconstructed, activated oocytes is called a pseudo-pronucleus (Wakayama et al., 1998b). Oocytes that have this structure are considered to have under gone activation successfully (Fig. 2.4.A). Another morphological indicator of successful initiation of development is the formation of two blastomeres. When the 1-cell divides forming 2-cells with equal amounts of cytoplasm (Fig. 2.4.B) then further development is possible; conversely, when the 1-cell divides forming two-asymmetrical cells (Fig. 2.4.C) further development was rarely, if ever observed. An interesting observation I noted is that if an enucleated oocyte has been injected with a donor nucleus but does not initiate development it fragments (Fig. 2.4. D) and dies several days later.

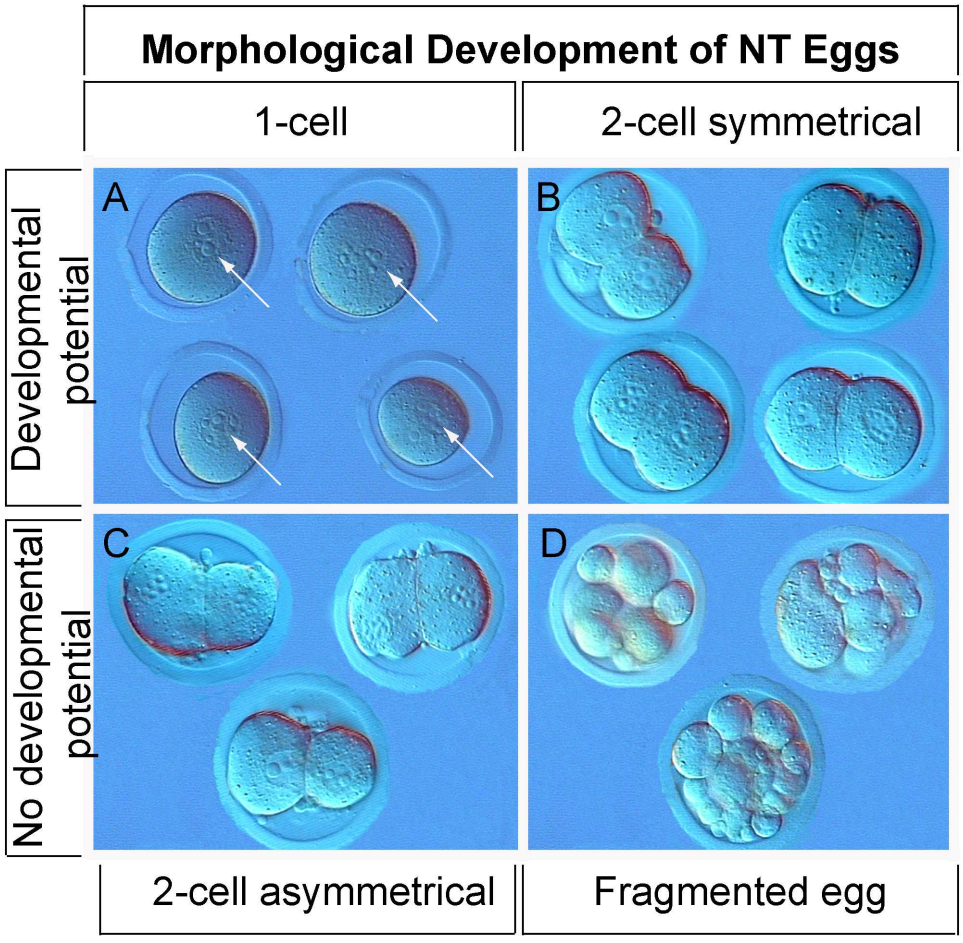



**Figure 2.4. Morphological development of nuclear transfer reconstructed eggs.** Bright field DIC photomicrographs (A and B) Eggs which have *in vitro* developmental potential, (A) 10-14 hours after egg activation those eggs that have been properly activated, form pseudo-pronuclei indicated by white arrows. (B) 24-36 hours after activation one cell eggs divide into 2 symmetrical blastomere cells. (C-D) Eggs which have no developmental potential, (C) 24-36 hours after activation, one cell eggs divide into 2 asymmetrical cells are unable or do not divide further. (D) Eggs which have either not been properly activated or are unable to divide will begin to fragment in to many small membrane bound balls of egg cytoplasm.

Because the donor ES cells harbored a ROSA-LacZ transgene, we could study the development of cloned oocytes by staining the oocytes at each stage of development from post activation until the blastocyst stage (Fig. 2.5).

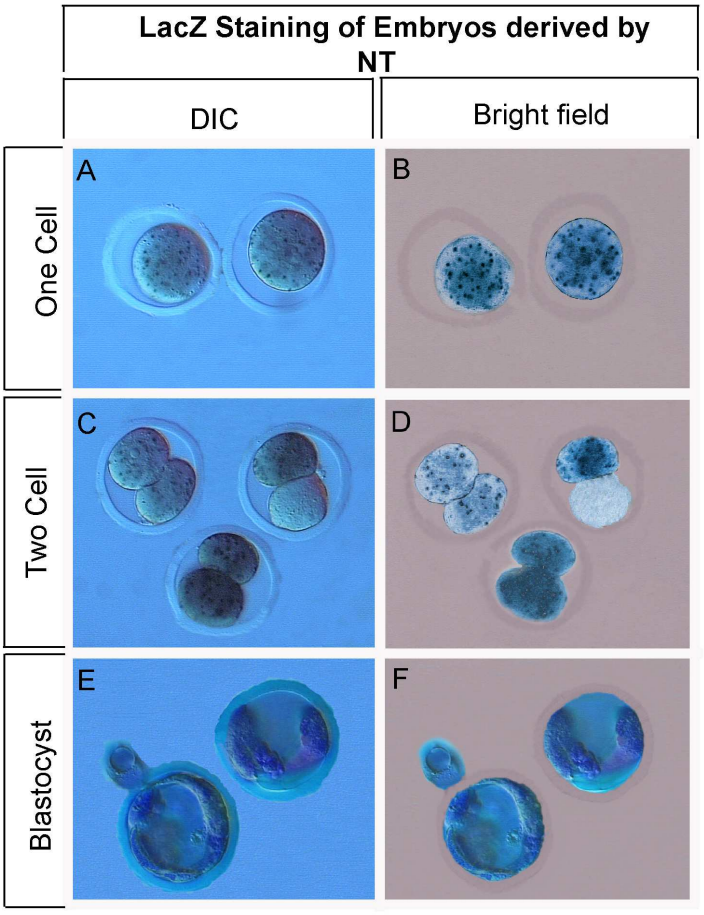
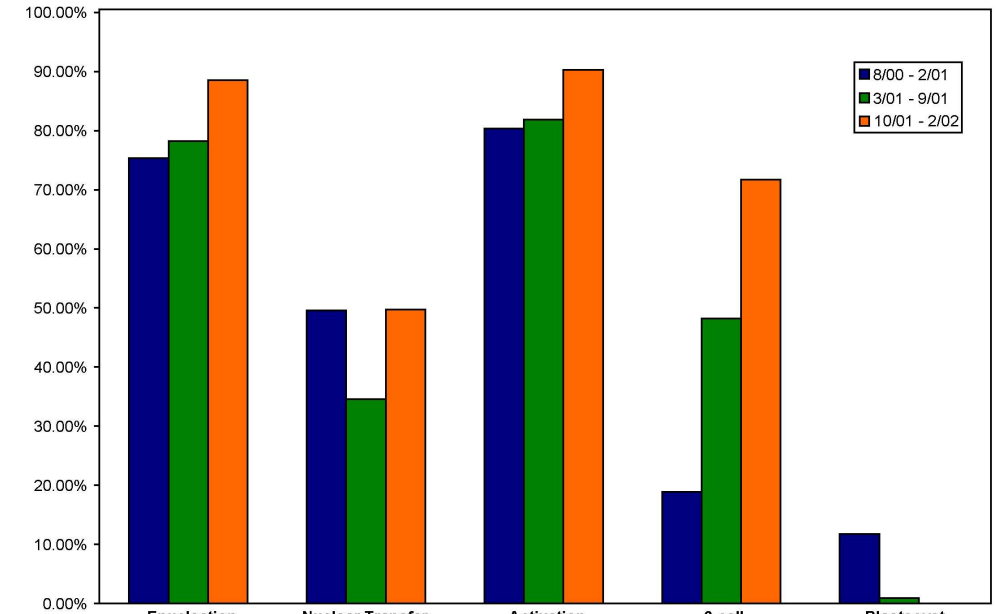



**Figure 2.5. Development of mouse eggs constructed by nuclear transfer using donor nuclei marked with LacZ.** (A, C, and E) Brightfield DIC photomicrographs of different stages egg development in vitro. (B, D, and E) Brightfield photomicrographs of same eggs as in (A,C, and E) to illustrate the staining pattern. (A and B) One-cell eggs expressing the LacZ marker. (C and D) Two-cell eggs showing different levels of LacZ expression. (E and F) Reconstructed eggs at the blastocyst stage expressing LacZ in all cells.

Another advantage of using marked donor cells was that it served to verify that the reconstructed oocyte was derived from the donor nucleus. A punctuate pattern of LacZ expression was observed as early as 8 hours after chemical

activation of the reconstructed oocyte (Fig. 2.5.A-B). The expression pattern at the 2-cell stage differed from oocyte to oocyte (Fig. 2.5.C-D). The relative amount of LacZ expression from each experimental group ranged from very weak and punctuate to intense and homozygous. It was also observed that not only was the expression different between oocytes but in some cases, the expression differed between the two blastomeres of an oocyte (Fig. 2.5.D). Unfortunately, the differing levels and patterns could not be correlated with developmental potential since this was not a vital marker. Only a small number of reconstructed oocytes made it to the blastocyst stage *in vitro* and LacZ expression at this stage was evenly distributed in both the inner cell mass and trophectoderm (Fig. 2.5.E-F). Table 2.1 summarizes the experiments in which the ROSA-LacZ ES cell nuclei were injected into enucleated mouse oocytes.

**Table 2.1. Summary of mechanical nuclear transfer experiments.**




| | Enucleation | Nuclear Transfer | Activation | 2-cell | Blastocyst |
|---|---|---|---|---|---|
| 8/00 - 2/01 | 75.33% | 49.56% | 80.36% | 18.89% | 11.76% |
| 3/01 - 9/01 | 78.22% | 34.58% | 81.88% | 48.23% | 0.92% |
| 10/01 - 2/02 | 88.55% | 49.75% | 90.29% | 71.71% | 0.00% |

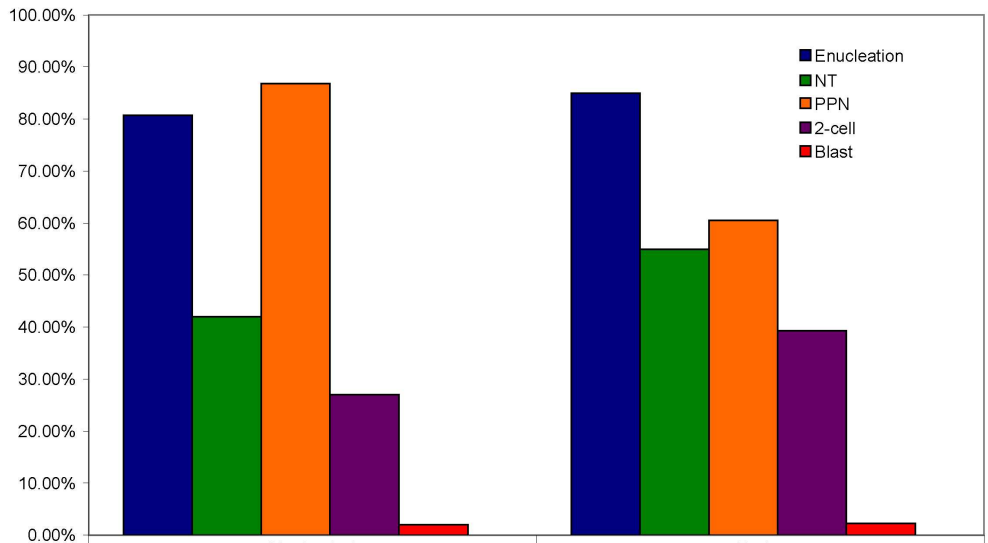
| Enucleation | | | NT | | | Activation | | 2-cell | | Blastocyst | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| attempted | survived | % | attempted | survived | % | PPN # | % | # | % | # | % |
| 2405 | 1942 | 80.75 | 1686 | 708 | 41.99 | 578 | 86.79 | 153 | 26.98 | 2 | 1.96 |
| 604 | 513 | 84.93 | 337 | 185 | 54.90 | 112 | 60.54 | 44 | 39.29 | 1 | 2.27 |

This table highlights the progress I made in mastering the diverse steps involved in the nuclear transfer technique. The percentage of oocytes surviving the enucleation step increased from 75% to 88%; however, the percentage of oocytes surviving the injection of the donor nuclei did not significantly increase. The percentage of oocytes successfully activated (assessed by pseudo-pronuclei formation) increased by 10% during the course of my experiments. This was

most likely due to the standardizing the protocol so that the oocytes were cultured at least one hour after injection of the donor nuclei and then allowing the activation was allowed to occur for exactly 6 hours. The percentage of two cells increased more than 50% and this was most likely due to conforming to the optimal time allowed for each step in this process (Fig. 2.3). Finally, the number of reconstructed oocytes that developed to the blastocyst stage did not increase over the experimental period. However, this is not a true representative number because, for a large portion of experiments, oocytes that had developed to the 2-cell stage *in vitro* were transferred to the oviducts of a pseudo-pregnant female to allow development *in vivo*. By doing this, the blastocyst stage was not assessed.

The most common method used in nuclear transfer experiments in species other than the mouse is membrane fusion. In the fusion method, an intact donor cell is placed next to an enucleated oocyte and a series of short electrical pulses induce the fusion of the donor cell and the oocyte membranes. I used this method in a small number of experiments in order to determine if the fusion method would lead to higher development efficiency than the injection method. Table 2.2 compares the injection or mechanical method to the fusion method.

**Table 2.2. Efficiency of cloning steps: Fusion vs. mechanical nuclear transfer.**




| | Mechanical | Fusion |
|---|---|---|
| Enucleation | 80.75% | 84.93% |
| NT | 41.99% | 54.90% |
| PPN | 86.79% | 60.54% |
| 2-cell | 26.98% | 39.29% |
| Blast | 1.98% | 2.27% |

| | Enucleation | | | NT | | | PPN | | 2cell | | Blast | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | attempted | survived | % | attempted | survived | % | # | % | # | % | # | % |
| Mechanical | 2405 | 1942 | 80.75 | 1686 | 708 | 41.2 | 578 | 86.79 | 153 | 26.98 | 3 | 1.9 |
| Fusion | 604 | 513 | 84.93 | 337 | 185 | 54.90 | 112 | 60.54 | 44 | 39.29 | 1 | 2.3 |

The results indicated that although a higher percentage of oocytes survived the electrical fusion step as compared to the injection of the nucleus, there was not a significant difference in the percentage of oocytes that developed to the 2-cell stage or to the blastocyst stage. Since nucleus injection was used more often in mouse cloning, most of my experiments were done using this method.

*Comparison of nuclear transfer efficiencies in the mouse field*

The initial goal for the mouse cloning experiments I conducted was to reproduce the cloning of a mouse in our laboratory as a proof of principal that I could master the techniques required for successful cloning. In order to assess my progress I compared the developmental efficiencies at different stages to the efficiencies obtained by others in the field. Table 2.2 summarizes the data from all published mouse cloning experiments (Hochedlinger and Jaenisch, 2002; Munsie et al., 2000; Ogura et al., 2000a; Ogura et al., 2000b; Ono et al., 2001; Wakayama et al., 1998b; Wakayama and Yanagimachi, 1999a; Wakayama and Yanagimachi, 2001a; Wakayama and Yanagimachi, 2001b; Zhou et al., 2001).

The leader in this field is Teruhiko Wakayama, a former member of the Yanagimachi laboratory. He published the first report of cloned mice, while he

was a member of the Yanagimachi laboratory, and has subsequently published many papers demonstrating the successful development of reconstructed mouse oocytes. He has reconstructed over 10,000 oocytes to date and no one working in this field has come close to achieving these numbers. Therefore, his efficiencies are considered the gold standard. It is clear from Table 2.3 that a major problem in the mouse-cloning field is the lack of development of reconstructed oocytes to the blastocyst stage. The Yanagimachi laboratory routinely obtains over 40% of reconstructed oocytes developing to the blastocyst stage, however the rest of the field is only able to obtain less than 10% of reconstructed oocytes developing to blastocysts. Table 2.3 indicates that 65-88% of reconstructed oocytes that survive the injection procedure become activated. This suggests that it is not likely that micromanipulation of the oocytes is the problem. More likely, other candidate factors must be considered such as the strain of mouse used, type of donor cell, and experimental conditions such as the reagents must be taken into consideration. These factors will be reflected on in detail in the discussion.

*Spontaneous activation of rat oocytes*

A second goal was to extend cloning technology to the rat since at the time I initiated my research efforts it had not been accomplished. Rat oocytes were collected from superovulated WT Sprague Dawley females to determine if the metaphase plate could be identified using Normanski optics in lieu of polarized

light or fluorescent staining. The DNA could be identified very easily using Normanski however; the oocyte was not arrested in metaphase II. The oocytes were stained with Hoechst dye to clearly visualize the chromosomes and determine which stage of meiosis they were in (Fig. 2.6.A-F).

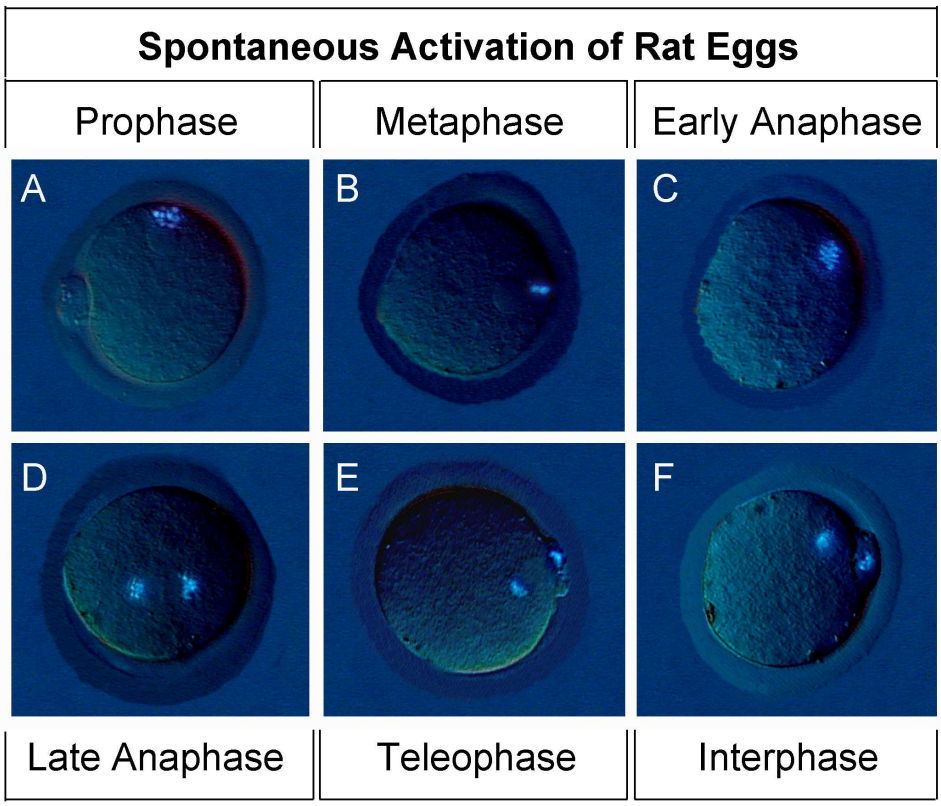



**Figure 2.6. Superovulated rat eggs are not arrested in metaphase II but are spontaneously activated.** Flourescent photomicrographs of superovulated rat eggs collected 12 hours post hCG stimulation. (A-D) Ovulated rat eggs whose DNA represents all stages of meiosis.

Superovulated oocytes represented all stages of meiosis confirming previous reports of the spontaneous activation of rat oocytes (Zernicka-Goetz, 1991b).

Spontaneous activation of the oocyte would make cloning rats very difficult since the oocytes must be arrested at metaphase for development of a reconstructed oocyte to occur. This is due to events that must occur to the donor nuclei upon injection into the oocyte. For example, a metaphase II arrested oocyte contains maturation promoting factor that induces nuclear envelope breakdown and premature chromosome condensation (Wakayama and Yanagimachi, 2001a). Nuclear envelope breakdown and chromosome condensation does not occur in activated oocytes, since maturation promoting factor is no longer present in the cytoplasm of the oocyte (Wakayama et al., 1998a). To see if the superovulation protocol could be optimized so that the majority of the oocytes were properly arrested in metaphase, the time of oocyte collection after hCG treatment was varied. It was determined that oocytes were released from the ovary between 8-10 hours after injection of hCG. Collection of oocytes beginning from 8 -13 hours post hCG did not change the percentage of oocytes arrested in metaphase (data not shown). Therefore, the oocytes must be spontaneously activated immediately upon release from the oviducts. In order for the cloning technology to be extended to the rat, reagents must be found that can inhibit the oocyte from resuming meiosis until the reconstructed oocyte is reactivated. Advances made towards solving this problem will be further detailed in the discussion section.

**Discussion**

To date, mice have been cloned from adult, embryonic, male, and female somatic cells. However, the efficiency of live births is below 2% and even those that develop to term usually display many abnormalities (Yanagimachi, 2002). Wakayama's work demonstrated that a high percentage of reconstructed oocytes could develop to the blastocyst; other groups have tried to repeat his experiments but have not been able to attain the same success rates. Unfortunately, I was not able to attain development to full term and *in vitro* development to the blastocyst stage occurred in less than 3% of reconstructed embryos. The same experimental procedures used by the Yanagimachi laboratory were implemented in the studies I employed. This included, using the Piezo drill for nuclei injection, the BTX ECM2001 to induce cell fusion, and adhering to the same timing between nuclear transfer and activation. There were some modifications: the culture medium used was mBMOC-3 instead of CZB or KSOM-AA; the donor mouse oocytes were derived from C57Bl/6 X SJL F1 females instead of C57Bl/6 X DBA/2 F1; and injection pipettes were manufactured from Humagen Co. instead of making them in the laboratory. A factor that contributed to the lack of reconstructed oocyte development was the micromanipulation technique; only 42% of manipulated oocytes survived the injection of the nuclei compared to over 90% survival of oocytes manipulated by Wakayama et al. Other groups reported that the percentage of oocytes surviving the injection procedures ranged between 40-50%

(Table 2.3), suggesting that although the technique in theory is straightforward; it is very difficult and requires some sort of artistic touch to achieve high survival rates. However, the micromanipulation technique is not the only factor, since 80% of the reconstructed oocytes that survived injection of a nucleus were successfully activated. Therefore, other undefined factors may also be important for success in cloning.

It is hard to make definitive conclusions about the requirements for successful cloning in the mouse because there are only a small number of groups who have been successful. Upon closer analysis, there are many differences between the various groups' protocols making it almost impossible to determine what is required in a cloning protocol to guarantee success. For example, the specific strain of mouse varies between groups. No groups used an inbred strain; however, they do use different F1 strains. The most commonly used F1 donor oocytes are from B6D2F1 mice (Gao et al., 2003; Munsie et al., 2002; Wakayama and Yanagimachi, 1999b; Zhou Q., 2000); however, the work presented here used B6SJLF1 mice. It cannot be concluded if the strain of mouse used here would lead to a lower cloning efficiency, but data shown by T. Wakayama at the Stem Cells Keystone Symposium in January 2004, indicated that the efficiency rate differed when the only variable was strain of donor oocytes. His data showed that inbred strains and some F1 strains never produced a viable clone. The B6SJLF1

strain was unfortunately not tested in his study so it only remains a possibility that this F1 strain is incapable of supporting reconstructed oocyte development.

Another variable is the medium the oocytes are cultured in, the most common medium is CZB. The data here represents eggs cultured in mBMOC-3 medium, which was shown in 1972 to support *in vitro* development of mouse eggs (Hammer, 1998). This medium became the basis for many of the media now commonly used for egg culture. The main difference between CZB and mBMOC-3 is the absence of glucose which is replaced with glutamine and increased amount of pyruvate in CZB (Biggers, 1998). However, one group reported that a modified version of CZB termed CZBG led to a higher development rate (Gao et al., 2003). The CZBG was CZB supplemented with glucose therefore making its components very similar to those found in mBMOC-3. Although, both CZB and mBMOC-3 can be used to culture fertilized eggs to blastocyst it is unclear if one medium or a specific component of the medium is better for *in vitro* development of reconstructed oocytes.

Another area of variability is the activation of the reconstructed oocyte. It was originally believed that the activation of the embryonic genome by the late 2-cell stage was not enough time to reprogram a differentiated nuclei to pluripotency (McGrath and Solter, 1983). However, since this thought has been proven incorrect, reprogramming is thought to begin as soon as the donor nucleus is exposed to the oocyte cytoplasmic contents. In order, to optimize the

reprogramming of the donor nucleus, the timing and conditions of activating the metaphase arrested donor oocyte have been extensively studied. Initial experiments suggested that when the reconstructed oocyte was cultured for one to three hours before chemical activation with $SrCl_2$ then *in vitro* development increased (Wakayama et al., 1998a). Therefore, the protocol followed in this report used these parameters. Later experiments showed that the addition of DMSO to the activation medium lead to a higher developmental efficiency in mouse regardless of the amount of time in culture after nuclei injection (Wakayama and Yanagimachi, 2001a). However, regardless of when activation was initiated, the percentage of full term births remained very low in the mouse. In the experiments where the fusion method was used, chemical activation with $SrCl_2$ was also used, but the possibility of the eggs becoming activated during the fusion process cannot be ruled out. Reconstructed oocytes that were immediately activated following nuclei injection showed a 30-40% decrease in their ability to develop to blastocysts *in vitro* (Wakayama and Yanagimachi, 2001a). Therefore, if the oocyte was activated during the fusion process this may be one reason in this study that the *in vitro* development to the 2-cell stage was decreased by over 13% (Table 2.2) when compared to 2-cell development when nuclei were transferred using the injection technique.

Unfortunately, despite manipulating over 3000 eggs, a reconstructed oocyte never developed to term. The *in vitro* development was also very

disappointing, fewer than 3% of the reconstructed oocytes developed to the blastocyst stage and most reconstructed oocytes were blocked at the 2-cell stage. Variability among the different protocols being used in the field has been discussed; but a specific area that caused such low development cannot be pinpointed at this time. Although, most cells were blocked at the 2-cell stage, it is not necessarily the cloning technique per se because 46% of the reconstructed oocytes survive the micromanipulations and of these surviving eggs, over 86% become activated based on the appearance of pseudo-pronuclei. Therefore, development past the 2-cell stage may be inhibited by the strain of donor oocyte or the components of the culture medium. In order for this development problem to be solved, further experiments must be done using the same protocol and varying each condition independently.

In an effort to extend the cloning technology to rats, the spontaneous activation of superovulated oocytes was examined. Regardless of the time at which superovulated rat oocytes were collected, very few were found arrested in the metaphase stage. Most oocytes were found to be activated and in all stages of the meiosis. Previous studies had reported that rat oocytes are spontaneously activated upon release from the oviduct (Zernicka-Goetz, 1991b). Additionally, *in vitro* parthenogenetic development of spontaneously activated oocytes was successful only to the 4-cell stage (Zernicka-Goetz, 1991a). Therefore, transferring this technology would be impossible unless oocyte activation could

be inhibited. Since I was unable to achieve full term development in the reconstructed mouse, further studies involving the rat were not done. However, in 2003 the first report of a fertile cloned rat was achieved, (Zhou et al., 2003). The spontaneous activation of the oocyte was overcome by inhibiting the action of maturation promoting factor (MPF). They used MG132 a protease inhibitor that blocks the meiotic metaphase to anaphase transition and stabilizes the metaphase II stage (Zhou et al., 2003). The use of this inhibitor has opened the doorway to extending gene targeting to the rat. However, until the cloning efficiency for live births increases above 1-3%, the generation of many genetic models of human disease in rats will not be seen in the near future.

Another approach to inhibit rat eggs from spontaneous activation is to mimic *in vitro* the same the conditions that ovulated oocytes are found in upon release from the ovary. Previous work has shown that second messenger cAMP plays an important role in sustaining the meiotic arrest of ovulated oocytes (Downs et al., 1989). When the intracellular levels of cAMP are elevated, the oocyte is prevented from resuming meiosis and the decrease of intracellular cAMP will initiate resumption of meiosis. Hypoxanthine, a naturally occurring purine derivative, is present at high levels in the follicular fluid. It has been suggested that hypoxanthine maintains the meiotic arrest in oocytes by suppressing phosphodiesterase (PDE) activity (Downs et al., 1985). When rat ovulated oocytes were treated with PDE3 inhibitors or derivatives of cAMP, the

oocytes did not resume meiosis *in vitro* (Wiersma et al., 1998). Therefore, if a combination of PDE and protease inhibitors can be used to keep rat oocytes arrested long enough for the nuclear transfer procedure to be completed, the generation of genetically modified rat models for development or disease models can be exploited.

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# CHAPTER THREE

## Generation and Characterization of a Novel Transgenic Rat with Germ Cell Specificity

### Introduction

With the emergence of transgenic technology in the 1980's, a fragment of DNA can be introduced and stably integrated into the genome of animal. This newly introduced DNA has been shown to express the protein that it encodes and is transmitted to its progeny (Young, 1999). Use of a vital marker, such as EGFP, in combination with transgenic techniques makes it possible for the first time to score and follow a population of cells *in vivo.* This technology is essential for studies that require one cell population to be distinguished from another *in vivo*; for example, development of cloned embryos could be followed if the donor nucleus is from a transgenic animal that ubiquitously expresses EGFP. Previous reports have described the use of an 800-bp fragment of the ROSA26 promoter that is able to direct ubiquitous expression of human placental alkaline phosphatase and enhanced green fluorescent protein (EGFP) during embryonic and postnatal development in mouse and rat (Kisseberth et al., 1999). Using the ROSA26 promoter to express EGFP, transgenic rats were generated to serve as the source of donor nuclei for rat cloning experiments described in Chapter 2.

However, one of the transgenic lines did not display a ubiquitous expression pattern. This expression appeared to be limited to the germ cells of both the male and female. Germ cells have the ability to transmit genetic information from one generation to the next. The precursors to these cells, primordial germ cells (PGCs), were first identified in the mouse and shown to contain high alkaline phosphatase (AP) activity. The PGCs form a small group of cells located in the extraembryonic region of the proximal epiblast of E7.25 mouse embryos (Chiquoine, 1954; Ginsburg et al., 1990; Lawson and Hage, 1994). The PGCs then migrate from the base of the allantois through the dorsal mesentery to the genital ridges; in the mouse almost all PGCs reach the genital ridge by E11.5 (Bendel-Stenzel et al., 1998). Within the genital ridge, the PGCs interact with somatic cells to form sex cords that serve as precursors to the seminiferous tubules of the testis and the ovarian follicles. The sex cords can be identified by E13.5 (McLaren, 1992; Wylie, 2002) and develop into the embryonic gonads and finally into the adult testis and ovary.

Although PGC's contain high alkaline phosphatase activity (Chiquoine, 1954; Ginsburg et al., 1990), other markers such as Oct3/4 (Okazawa et al., 1991; Yoshimizu et al., 1999), SSEA1 (stage specific embryonic antigen 1) (Fox et al., 1981), fragillis (Lange et al., 2003), nanog (Chambers et al., 2003), nanos (Tsuda et al., 2003), vasa (Tanaka et al., 2000), and GCNA (germ cell nuclear antigen)

(Enders and May, 1994) also have been suggested as reliable molecular markers for germ cells. However, AP, oct3/4, SSEA1, nanog, and fragillis are also expressed in undifferentiated cells of the early embryo (blastocyst to epiblast in the mouse), and therefore do no distinguish germ cells from other undifferentiated cells. Markers such as vasa and GCNA are germ cell specific, but are not expressed during all stages of germ cell development and GCNA is sex-specific. Thus, a single molecular marker of the germ cell lineage has not been reported. The following will describe the generation and characterization of a novel transgenic line of rats that expresses EGFP specifically in germ cells during virtually all stages of male and female germ cell development. This transgenic line is referred to as Germ Cell Specific-EGFP (GCS-EGFP).

**Materials and Methods**

*Construction of ROSA-EGFP transgene and production of transgenic rats*

A 0.8 kb SalI-BamHI ROSA26 fragment (Zambrowicz et al., 1997) was inserted between the SalI and BamHI restriction sites of the pEGFP-N1 plasmid (Clontech/BD Biosciences, Palo Alto, CA) to generate the ROSA-EGFP transgene (Kisseberth et al., 1999; Zambrowicz et al., 1997). The 1.8 kb SalI and AlfII ROSA-EGFP fragment was separated from vector DNA by gel electrophoresis and the fragment was isolated from the gel by perchlorate elution. Transgenic rats were produced by microinjection of the 1.8 kb ROSA-EGFP

fragment into the pronucleus of Sprague Dawley rat eggs as described (Young, 1999); 6 transgenic rats were produced. Founders were mated with Sprague Dawley wildtype (WT) rats and four independent lines were established. Genotyping of founders was by dot blot analysis and progeny by either dot blot (Fig. 3.1)

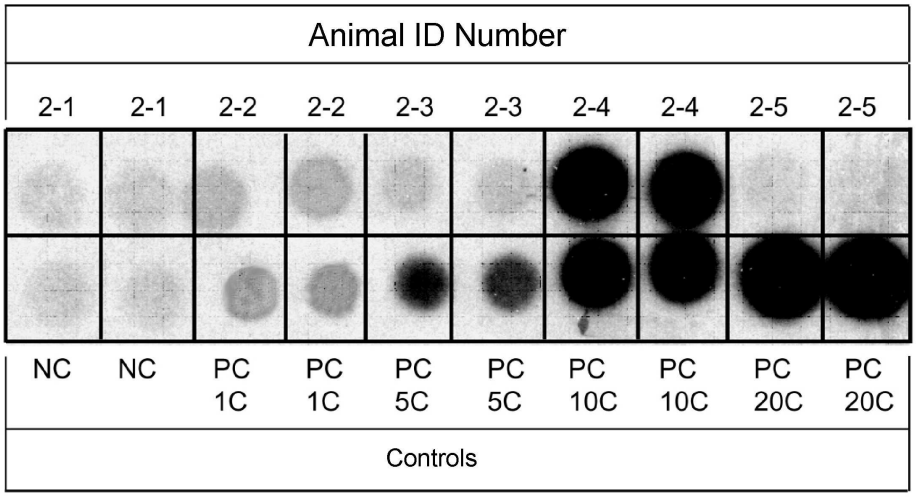


**Figure 3.1 Transgenic lines generated from the ROSA-EGFP construct.** DNA dot blot analysis of pups delivered from female number 2. Only pup 2-4 expressed the transgene in multiple copies. (NC) negative control, and (PC) positive control denoting approximate copies of the transgene. Pup 2-4 expresses between 5-10 copies of the transgene.

or PCR analysis of genomic DNA isolated from tail biopsies. PCR was performed using the forward primer EGFP5-1 (5' AACTTCAGGGTCAGCTTGC) and the reverse primer EGFP3-1 (5'GGTGTTCTGCTGGTAGTGGTC) corresponding to nucleotides 971 to 1492

of the ROSA-EGFP DNA fragment that amplified a DNA product of 521bp. Unless otherwise specified homozygous transgenic rats and WT Sprague Dawley rats were used for all described experiments. Animals were housed in SPF condition cages with a 12-hour light and 12-hour dark cycle and fed Teklad Mouse/Rat Diet (Harlan Teklad, Madison, WI*) ad libitum.*

*Southern Blot Analysis*

For Southern blot analysis of the ROSA-EGFP transgene integration sites, genomic DNA from the livers of adult Sprague Dawley WT and GCS-EGFP rats were digested with the restriction endonuclease BamHI, XbaI, StyI, BanII, DraI, MseI, and NcoI. 20μg of digested DNA was loaded per lane, separated on a 0.8% agarose gel, and transferred to overnight to a Hybond N+ nylon membrane (Amersham Pharmacia Biotech, Pisscataway, NJ). Isolated EGFP-SV40 DNA fragments were labeled with [ $-^{32}$P] dCTP and used as a probe. Each membrane was pre-hybridized for 1 hour, and hybridized with the 50ng of labeled probe for 2 hours with Clontech Express Hybridization solution (Clontech/BD Biosciences, Palo Alto, CA) at 65°C. Following hybridization, the membranes were washed at room temperature for 15 min in (2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS) and then washed at 50°C for 5 min in (0.1% SSC, 0.1% SDS), followed by exposure to autoradiography film.

*Genome Walker*

Amplification of unknown DNA region adjacent to a known DNA region can be done using the Universal Genome Walker kit (Clontech/BD Biosciences, Palo Alto, CA). Briefly, genomic DNA from GCS-EGFP adult rat liver was digested in five separate batches by five different enzymes that generate blunt ends: *DraI, EcoRV, PvuII, ScaI* and *StuI*. Each batch of digested genomic DNA was then ligated separately to the Genome Walker Adaptator. The resulting products were submitted to two successive PCR amplifications with two different pairs of primers. A pair of primers was composed of an adaptator primer and an EGFP or SV40 primer located on the 5' transgene associated with the unknown flanking DNA. The gene specific primers used for amplification are listed in Table 3.4. Adaptor primers, AP1, for the first round of PCR, and AP2, for the nested PCR, were provided with the kit. PCR was performed with 60 ng of DNA, 10 pmol of primers, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.5 U *Taq* Advantage Genomic Polymerase Mix (Clontech/BD Biosciences, Palo Alto, CA) in 50 µl of the 10X Tth PCR reaction buffer was performed with seven cycles (94 °C for 25 s, 72 °C for 3 min) followed by 32 cycles (94°C for 25 s, 67 °C for 3 min) with a final elongation step of 7 min at 72°C. Nested PCR was performed with 1µl of PCR product, in the same conditions as for the first PCR reaction mix. Amplification was performed with five cycles (94°C for 25 s, 72 °C for 3 min) followed by 20 cycles (94°C for 25 s, 67°C for 3 min) with a final elongation step of 17min at

72°C. PCR products were visualized on a 1.0% agarose gel, containing ethidium bromide.

*Inverse PCR*

Genomic DNA from GCS-EGFP adult rat liver was digested with restriction endonucleases *XbaI, BanII, MseI, NcoI, StyI,* and *DraI* then self-ligated with the addition of T4 polymerase. The first round of PCR on the circularized DNA was performed using primers listed in Table 3.4. PCR was performed with 100 ng of DNA, 10 pmol of primers, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.25 U Qiagen Taq Polymerase (Qiagen, Valencia, CA) in 50µl of the 10X Qiagen reaction buffer. Amplification was performed with a 3 min initial denaturizing step at 94°C for 3 min followed by 35 cycles (94°C for 30s, 68°C for 2 min) with a final elongation step of 10 min at 72°C. PCR products were visualized on a 1.5% agarose gel containing ethidium bromide.

*Cloning of the Inverse PCR and Universal Genome Walker Kit fragments*

Amplified PCR products were excised from the agarose gel and purified using the Qiaex II Kit (Qiagen, Valencia, Ca.). Purified PCR products were directly cloned into pPCR2.1 plasmid using the TA cloning kit (Invitrogen/GibcoBRL Carlsbad,

CA). Plasmid DNA was amplified and purified using Wizard mini prep kit (Promega, Madison, WI) and sent for automated sequencing.

*Lambda Screen*

A Lambda library constructed by Stratagene (La Jolla, CA) from genomic liver DNA of adult male GCS-EGFP rat. The lambda library was packaged, amplified, and 1 X $10^6$ plaques were screened. Briefly, 50,000 pfu/plate were grown up overnight on 150mm plates of agar. Plaques were transferred to nitrocellulose filters and were hybridized at 65°C overnight with a [$\alpha^{32}$P]dCTP probe corresponding to the entire EGFP-SV40 DNA fragment isolated from pEGFP-N1 (Clontech/BD Biosciences, Palo Alto, CA). Filters were washed in 2X SSPE (1X SSPE = 180 mM NaCl, 10 mM $NaH_2PO_4$, 1 mM EDTA, pH 7.4) + 1.0% SDS at room temperature for 10 min, 2X SSPE + 1.0% SDS at 65 °C for 10 min, and 0.2X SSPE + 0.1% SDS at 65 °C for 10 min. Positive plaques were picked, re-amplified, and re-screened until the all the plaques on a single plate gave positive signal to the EGFP-SV40 probe. Lambda DNA was isolated using Qiagen lambda Midi Kit (Qiagen, Valencia, Ca.) and digested with KpnI, and the resulting fragments were subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA) and sequenced by automated sequencing.

*FISH analysis*

Rat embryonic fibroblasts were isolated from E15.5 homozygous embryos by standard procedures (Hogan, 1994). Slides for cytogenetic analysis were prepared essentially as previously described (Islam and Levan, 1987). Briefly, cell cultures were treated with 0.2 mg/ml of 5′-bromo-2′-deoxyuridine (BrdU) for 17 hours. Subsequently, the cells were washed three times and cultured for 6 h in medium supplemented with 0.05 μg/ml thymidine. Mitotic figures were accumulated by adding 0.05 μg/ml Colcemid (Sigma Aldrich, St. Louis, MO) during the final 30 min, and metaphase cells were harvested by mitotic shake-off; a procedure which selects cells which have entered mitosis and have become morphologically rounded which allows them to become easily detached from the culture plate by gentle shaking. The cells were resuspended in 0.07 M KCl at room temperature for 10 min, washed, and fixed in three dilutions of methanol:acetic acid (9:1, 5:1, and 3:1). Dual-color FISH analysis was performed using a biotinylated (Nick Translation Systems, Gibco/BRL, Carlsbad, CA) BAC DNA probes for rat reference genes *Grik1/Ncam2/Kcjn6/Mx1* genes and a digoxigenine-11-dUTP labeled (DIG-NICK Translation Mix, Roche Diagnostics GmbH, Mannheim, Germany) DNA probe for EGFP reporter gene construct (Behboudi et al., 2002). Approximately 500ng of the co-precipitated probes along with about 15-fold excess of sonicated total rat genomic DNA were co-hybridized to each slide. Detection of the dual-color labeling was performed using a mixture of

Rhodamine-conjugated antidigoxigenin and FITC-conjugated avidin (Invitrogen/GibcoBRL Carlsbad, CA). The chromosome preparations were washed, counter-stained for chromosome identification, and the fluorescence signals were visualized as described previously (Helou et al., 1999).

*Imaging of EGFP fluorescence in embryos*

Prepubertal female rats were superovulated by a standard regimen (Young, 1999) and placed overnight with stud males. The presence of sperm in the vaginal lavage or a copulatory plug the following morning indicated mating had occurred and was scored as day E0.5. Pre-implantation embryos were collected on the specified day of development in R1ECM medium (Specialty Media, Phillipsburg, NJ), washed in R1ECM, and held in R1ECM drop cultures overlaid with oil until use. Blastocyst implantation was delayed as previously described (Evans, 1999). Briefly, WT females were mated with GCS-EGFP homozygous males and the morning of finding a copulatory plug was designated as E0.5. On E4.5, 50μg tamoxifen (Sigma-Aldrich, St. Louis, MO) in an aqueous solution of corn oil was injected intraperitoneally and 5mg Depo-Provera (Upjohn Co., Kalamazoo, MI) was injected subcutaneously. Delayed blastocysts were collected on E8.5 in R1ECM, washed in R1ECM, and held in drop cultures in R1ECM until use. Epiblasts were dissected on day E8.5 and held in PBS until use. The genital ridge was dissected from E12.5 and E15.5 embryos in PBS, and gonads were dissected

from E19.5 embryos in PBS. Embryos and epiblasts were visualized with a Nikon Eclipse TE2000-U inverted microscope using an EGFP filter. The genital ridges and embryonic gonads were visualized with a Nikon SMZ1500 stereoscope using an EGFP filter.

*Imaging of EGFP fluorescence in adult tissues*

Tissues were either directly visualized for EGFP expression using a Nikon SMZ1500 stereoscope or fixed in 4% paraformaldehyde overnight at 4$^{o}$C, washed in PBS, placed in 30% sucrose overnight at 4$^{o}$C to equilibrate, embedded in freezing medium Tissue Tek OCT (optimal cutting temperature) (Sakura Finetek U.S.A., Inc. Torrance, CA), and frozen in a biocooler Histobath 2 (Shandon Lipshaw, USA) containing isopentane at -55$^{o}$C. Frozen tissues were then sectioned at 10-12 μm on a Leitz cryostat and mounted on positively charged glass slides; Fisherbrand Superfrost Plus Slides (Fisher Scientific Co., Pittsburgh, PA), rehydrated with PBS, and immediately visualized for EGFP expression using an inverted Olympus IX70 microscope (Olympus Inc. Melville, NY).

*Imaging of EGFP fluorescence in mature sperm*

Mature spermatozoa were collected from the cauda epididymis of WT and homozygous GCS-EGFP male rats that had mated with a female within the previous 7-10 days. The cauda epididymis was placed into one ml of a

fertilization medium (Brinster medium for oocyte culture with 30mg/ml of BSA) under oil and the body of the cauda epididymis was cut and gently squeezed to express the sperm cells. The spermatozoa were allowed to disperse by swim out for one hour during incubation at 37°C in 5% $CO_2$ atmosphere. An aliquot of the sperm preparation was then placed directly on to a glass slide and viewed using Leica TCS SP2 AOBS-confocal microscope (Leica Microsystems, Wetzlar, Germany).

*Alkaline phosphatase staining*

Epiblasts from E8.5 and genital ridges from E12.5 embryos were isolated by microdissection and fixed in 4% paraformaldehyde for 2 h at 4ºC. The tissues were washed three times in PBS, incubated for one hour in 70% ethanol, and washed three times in distilled water. Tissues were stained with α-naphthyl phosphate/fast red TR (Sigma-Aldrich., St. Louis, MO) for 15 min at room temperature (Ginsburg et al., 1990), mounted on slides, overlaid with 70% glycerol and viewed on a Nikon Eclipse TE2000-U inverted microscope.

*Quantitative real-time PCR*

Total cellular RNA was isolated from multiple organs of WT rats, homozygous GCS-EGFP rats, and line 7-10 rats using RNA Stat-60 (Tel-Test, Friendswood TX). One microgram of total RNA was reverse transcribed using random primers and Superscript III reagents (Invitrogen, Carlsbad, CA). Samples were diluted

1:10 and 3 μl were used for the PCR reaction. The PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and EGFP primers: forward EGFP2-5(5' GGGCACAAGCTGGAGTACAAC) and reverse EGFP2-3(5' TCTGCTTGTCGGCCATGATA), which were designed in Primer Express Ver. 2.0. Real-time PCR analysis was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), and the PCR reaction was analyzed using the Sequence Detection System Ver. 2.1. For expression analysis, all samples were normalized to the ribosomal RNA18S signal and the expression of EGFP in transgenic tissues was compared to the background signal in WT testis.

**Results**

*Generation and characterization of ROSA-EGFP transgenic rat lines*

Four lines of ROSA-EGFP transgenic rats were generated, designated as lines Hsd:SDTgN(ROSA-EGFP) 2-4, 4-2, 7-9 and 7-10Reh; three of which contained the transgene on autosomes and one (7-9) that harbored the transgene on the Y chromosome. The murine ROSA promoter sequences used in the EGFP reporter transgene to target expression had been used in both transgenic mice and rats (Kisseberth et al., 1999; Zambrowicz et al., 1997) to drive nearly ubiquitous expression of reporter genes. We expected a similar expression pattern for our ROSA-EGFP transgenic rats. In two (line 4-2 and 7-9) of the four lines, one with

an autosomal integration and the other Y-linked, there was no apparent EGFP expression in any of the 15 tissues examined, either macroscopically (Fig. 3.2) or microscopically.

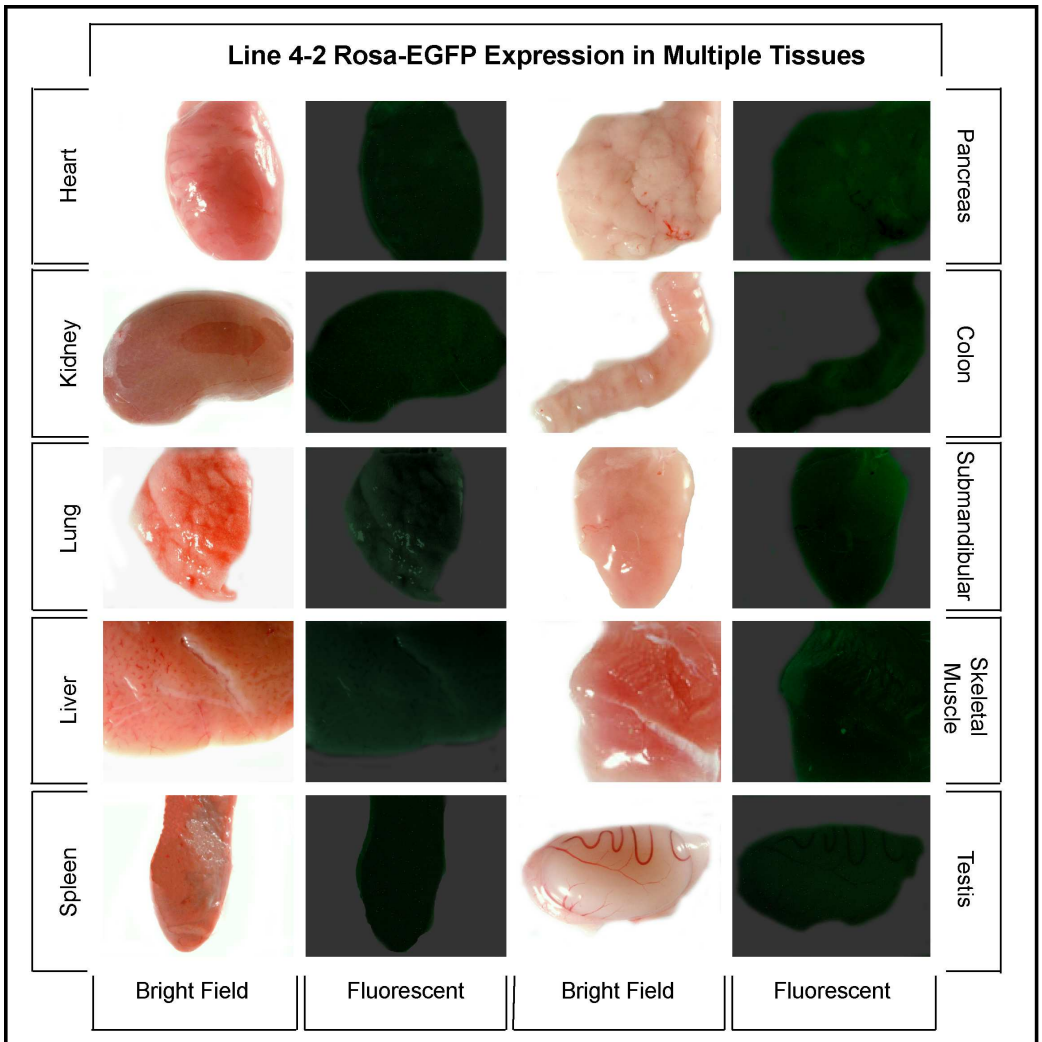


**Figure 3.2. EGFP expression in transgenic line ROSA-EGFP 4-2. Tissues (heart, pancreas, kidney, colon,** submandibular, liver, muscle, spleen, and testis) from adult rats were immediately removed, placed in ice cold PBS, and viewed using an EGFP fluorescent filter on a Nikon stereoscope. No EGFP expression could be found above background.

In the 7-10 line, EGFP was expressed in every organ examined, but the abundance of EGFP fluorescence varied between organs and often appeared cell-specific within an organ (Fig. 3.3).

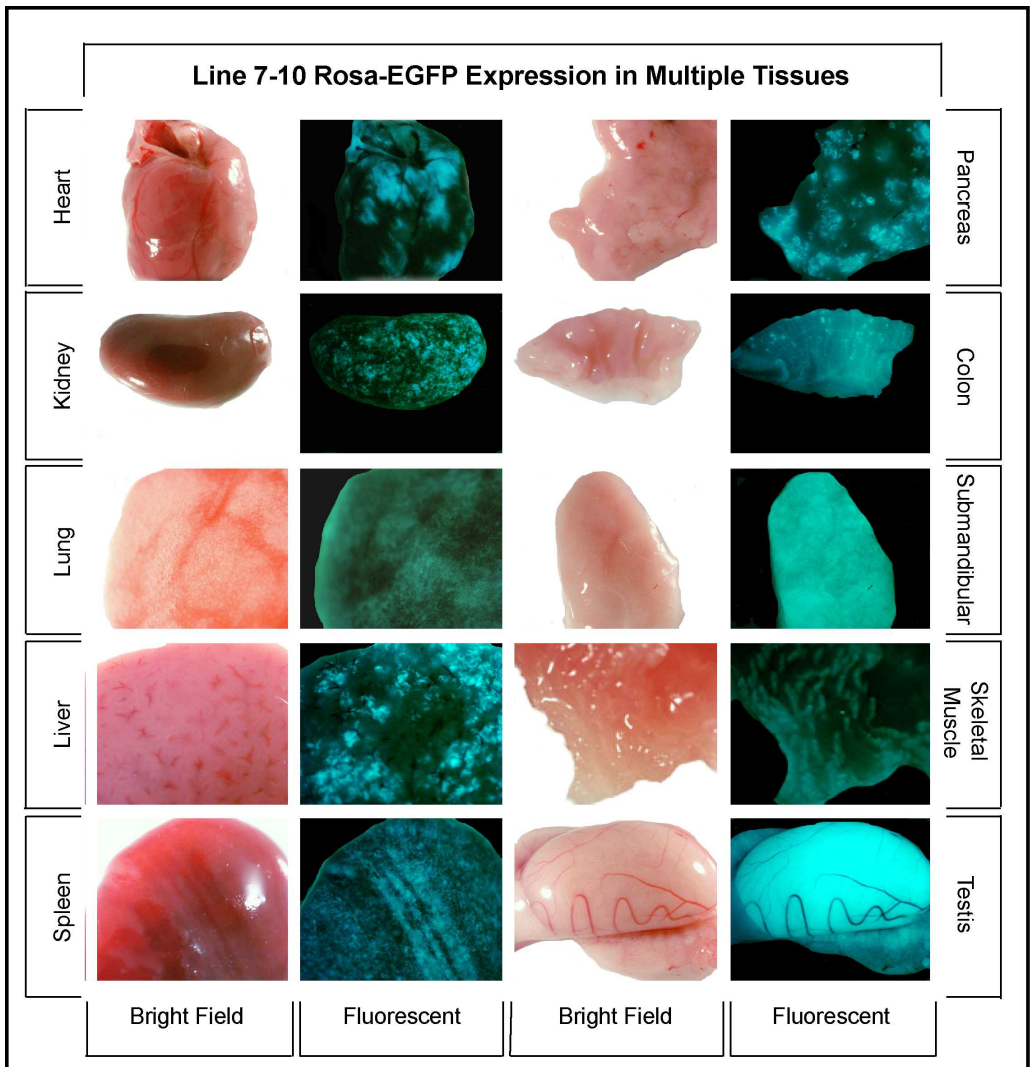


**Figure 3.3. EGFP expression in transgenic line ROSA-EGFP 7-10. Tissues (heart, pancreas, kidney, colon,** submandibular, liver, muscle, spleen, and testis) from adult rats were immediately removed, placed in ice cold PBS, and viewed using an EGFP fluorescent filter on a Nikon stereoscope. EGFP expression could be found in all tissues examined.

In the fourth line (line 2-4, designated GCS-EGFP), expression of EGFP appeared limited to the germ cells (Fig. 3.4). The two lines without obvious transgene expression were discarded and the other two were bred to transgene homozygosity. Progeny from both lines develop normally and do not display obvious abnormalities associated with transgene insertion or expression. A summary of founder lines is found listed in Table 3.1.

**Table 3.1. Summary of founder transgeniclines generated from the ROSA-EGFP construct.**

| Line | Transgene Location | EGFP Expression |
|---|---|---|
| 2-4 | Autosome | Germ Cell |
| 4-2 | Autosome | No expression |
| 7-9 | Y chromosome | No expression |
| 7-10 | Autosome | Ubiquitious |

We characterized the EGFP expression pattern in lines 7-10 and GCS-EGFP more extensively. In the GCS-EGFP rats, EGFP fluorescence was not detectable in the brain, heart, jejunum, kidney, liver, or skeletal muscle (Fig. 3.5). EGFP expression was robust in ovulated unfertilized eggs (Fig. 3.4.G and H) and in adult male germ cells including mature sperm cells (Fig. 3.4.E and F).

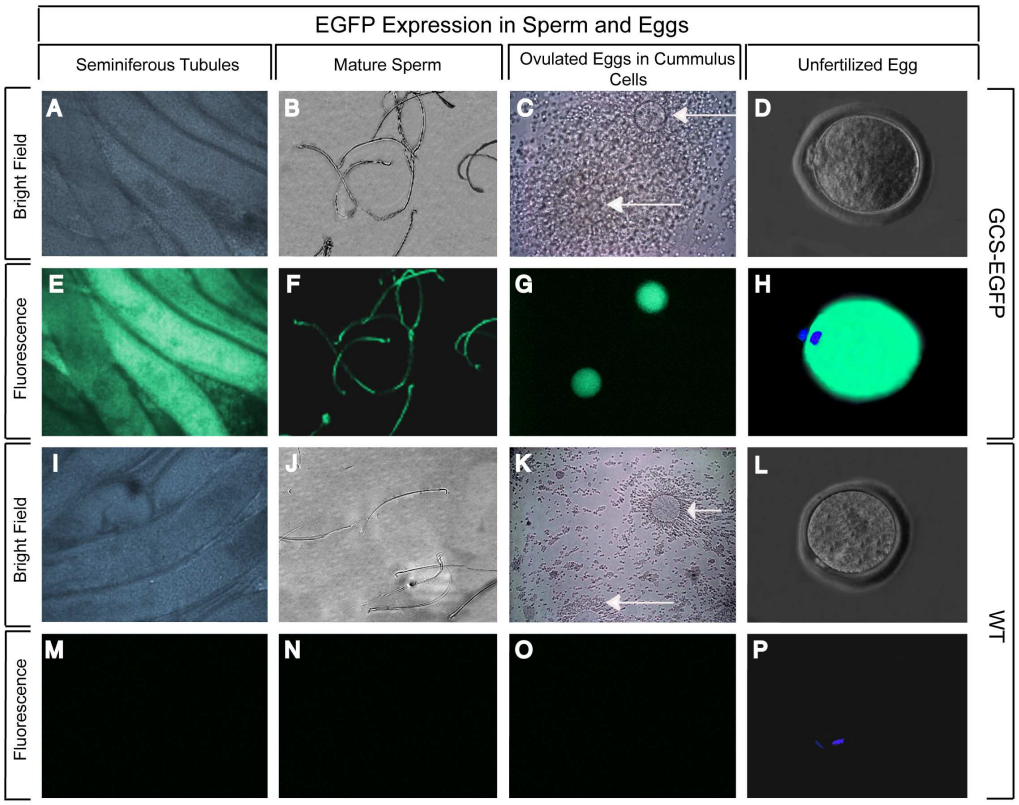


**Figure 3.4. EGFP is expressed in spermatozoa and eggs in GCS-EGFP rats.** (A-H) Germ cells from GCS-EGFP rats; (A-D) DIC photomicrographs (E-H) fluorescent photomicrographs of same specimens as in (A-D). (A, E) Isolated seminiferous tubules dissected from the testis, (B, F) cauda epididymal sperm cells, (C, G) unfertilized eggs in the associated cumulus masses, and (D, H) a denuded unfertilized, metaphase-II egg. (I-P) Germ cells from WT Sprague Dawley rats; (I-L) DIC photomicrographs (M-P) fluorescent photomicrograph of the same specimens in (I-L). (H and P) Eggs were counterstained with Hoechst 33442 to visualize DNA (blue).

Expression of EGFP mRNA, analyzed by quantitative PCR, demonstrated that expression of EGFP was confined to the testis and ovary (not present in muscle, fat, liver, small intestine, large intestine, spleen, kidney, heart, submandibular, brain, and stomach) (Table 3.2).

**Table 3.2. Relative EGFP expression in tissues of GCS-EGFP and 7-10 ROSA-EGFP rat lines**

| Tissue | Transgenic Lines | |
|---|---|---|
| | GCS-EGFP | Line 7-10 |
| Skeletal muscle (soleus) | Bkg | 2.7 |
| White Fat | Bkg | 5.6 |
| Liver | Bkg | 5.8 |
| Jejunum | Bkg | 5.7 |
| Colon (proximal) | Bkg | 5.6 |
| Spleen | Bkg | 8.6 |
| Kidney | Bkg | 7.1 |
| Heart (right ventricle) | Bkg | 6.9 |
| Submandibular | Bkg | 1.5 |
| Brain (frontal lobe) | Bkg | 5.2 |
| Stomach | Bkg | 6.9 |
| Ovary | 5.1* | 8.6 |
| Testis | 11.7 | 9.6 |
| Epididymis (cauda) | 6.1 | 9.7 |

Total RNA was subjected to real time quantitative PCR as described in Materials and Methods. Each value represents the ratio of EGFP mRNA relative to the background (Bkg) value in WT.

* 6 week old GCS-EGFP females were injected intraperitoneally. with 20 units of Gestyl (Organon Pharmaceuticals, West Orange, NJ) sacrificed and ovaries collected 72 hours after treatment.

In line 7-10, EGFP expression was observed in all tissues examined (Fig. 3.5); there was robust EGFP fluorescence in the testis, ovary, and kidney. Expression in the testis was evident in germ, Sertoli, and Leydig cells. In the ovary, EGFP fluorescence was substantial in both germ and cumulus cells. Quantitative PCR of RNA isolated from this subset of organs confirmed the expression of EGFP and identified the ovary, testis, epididymis, and spleen as the sites of highest EGFP expression (Table 3.2). Given the unique pattern of germ cell specific expression in the GCS-EGFP line, a more thorough examination of the EGFP

expression pattern was performed and the characterization of the site of transgene insertion was pursued.

*Characterization of the transgene insertion and assignment of the transgene chromosomal position*

Based on the nearly ubiquitous EGFP expression pattern in line 7-10 and on previously described ubiquitous expression patterns of ROSA-EGFP and ROSA-alkaline phosphatase in mice and rats (Kisseberth et al., 1999), we surmised that the unique germ cell-specific expression in line 2-4 was due to the chromosomal position of the transgene. A genomic dot blot analysis of the transgene insertion using an EGFP probe indicated at least five copies of the transgene were incorporated in the genome (Fig. 3.1).

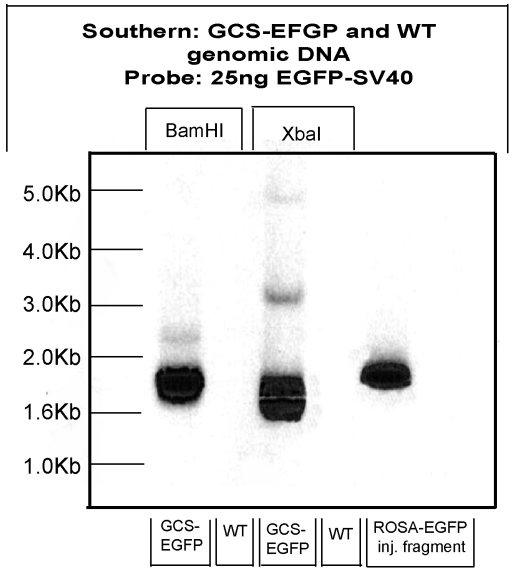


**Figure 3.6 Southern Blot Analysis for transgenic line GCS-EGFP.** Total liver DNA from GCS-EGFP and Sprague Dawley WT rats digested with BamHI or XbaI was probed with a radiolabeld EGFP-SV40 probe. Signal was observed in only GCS-EGFP rats the original ROSA-EGFP injected fragment was 1.8KB. GCS-EGFP junction fragments were at found at 2.4 KB when digested with BamHI and 3.2Kb when digested with XbaI. A smaller 1.6KB signal was found with the XbaI digestion indicating at least one copy of the transgene was inserted in a tail to tail fashion.

Southern analysis using probes to the EGFP and SV40 regions of the transgene indicated there where two copies oriented in a tail-tail pattern (Fig. 3.6). Further mapping of the transgene insertion site by southern analysis was done to identify the approximate size of junction fragments (Fig. 3.7).

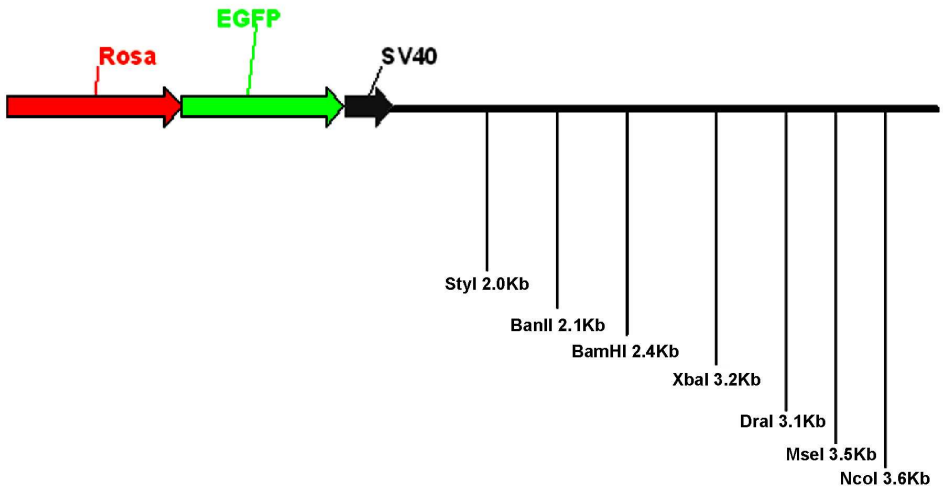


**Figure 3.7. GCS-EGFP transgene insertion junction fragments.** Schematic of southern analysis results to map the transgene insertion site. Enzymes that cut the transgene were used to find the approximate length of DNA flanking the the transgene

As a first approach to identify the DNA regulatory regions that target the germ cell lineage, and to ultimately identify the structural gene that marks this lineage, we used two PCR based methods, the Universal Genome Walker Kit (BD Biosciences, San Jose, CA) and inverse PCR on the 3'transgene sequence. Using

both methods a band of 3.2Kb was amplified which corresponded to the junction fragment obtained when XbaI was used to digest the genomic DNA (Fig. 3.8).

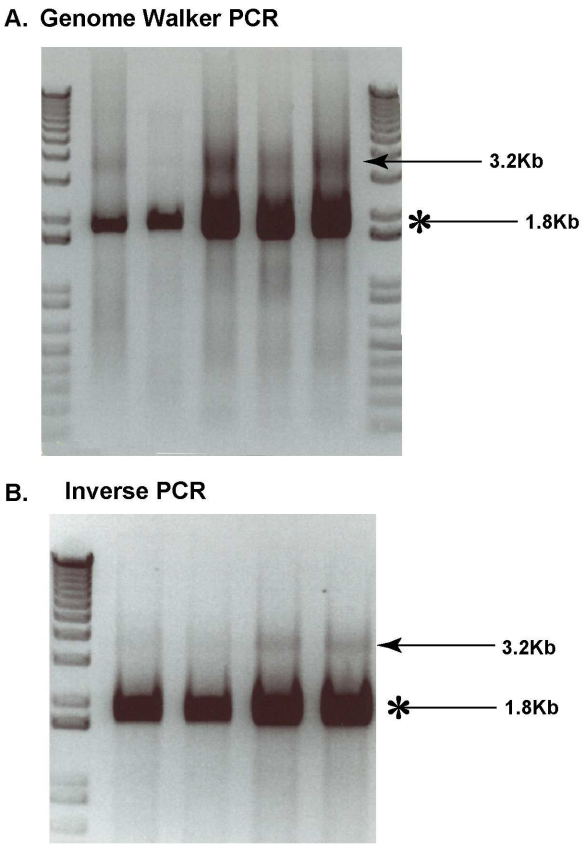


**Figure 3.8. Genome Walker PCR and Inverse PCR.** Two different PCR based strategies were used in order to identify DNA flanking the transgene. Results (A) using the genome walker PCR kit from Clontech and (B) using inverse PCR; a large 1.8Kb (black asterisk) representing the transgene was always amplified and a 3.2Kb (black arrowhead) junction fragment was amplified.

Unfortunately, from this amplified fragment, at most only 50bp of flanking sequence was obtained; this was insufficient to identify a unique sequence in the rat genomic database. With multiple copies of the transgene present, PCR based

methods to identify flanking DNA are often less effective due to multiple priming sites. A second method used to obtain flanking sequence was to screen a library. A lambda library was constructed (Stratagene Co., La Jolla, CA) from liver DNA from the GCS-EGFP adult rat and screened for plaques which containing the transgene plus some flanking sequence. The initial screening yielded 31 individual positive colonies, three subsequent rounds of enrichment screening yielded 5 positive clones (Fig. 3.9).

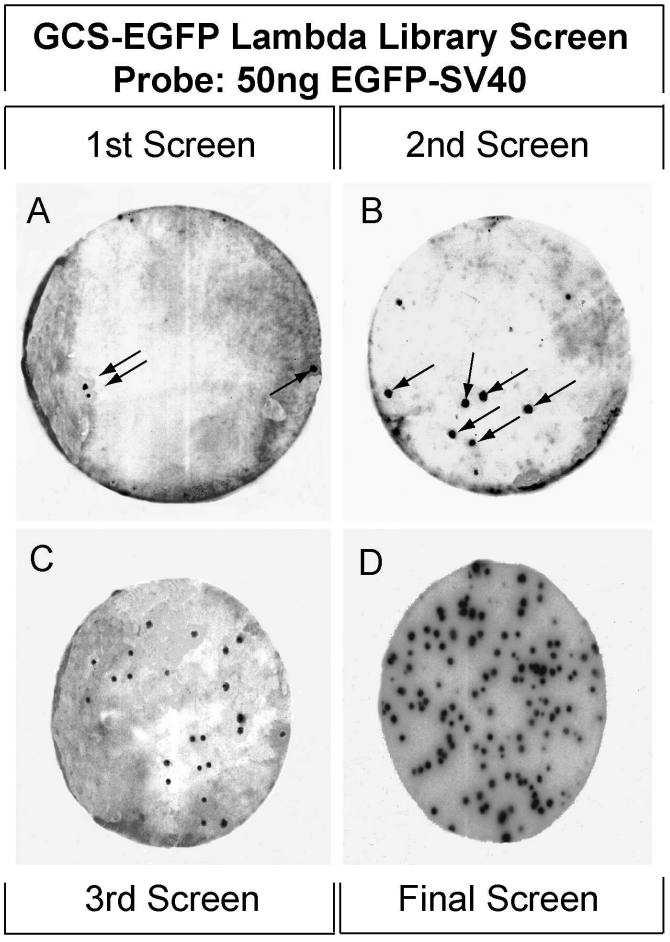


**Figure 3.9. Lambda library screen.** Total liver DNA from GCS-EGFP rat was partially digestedand ligated to a lambda plasmid to generate a library in order to identifytransgene flanking sequence. The Lambda library was screened using a radiolabeled EGFP-SV40 probe. (A-D) 4 consecutive rounds of screening were performed to identify individual clones which contained the transgene with a portion of flanking DNA sequence. Black arrows incidate the positive clonesthat were picked for further enrichment.

These clones were purified and verified by southern analysis and PCR to be positive for EGFP (data not shown). The positive clones were subcloned and sequenced. However, the transgene itself was never obtained through DNA sequencing and the five different clone sequences obtained were mapped to four

different chromosomes. These results indicated that the clones were either false positives or that the during the injection of the transgene into the pronucleus, damage occurred to the genomic DNA that may have caused small fragments of genomic DNA to break off and religate next to transgene upon it's insertion. Most transgenes generated by microinjection are present in multiple copies and may contain small stretches of endogenous DNA between copies of the transgene (Palmiter and Brinster, 1986). If this is true for the insertion site of the GCS-EGFP line then conventional methods used to identifying flanking DNA, such as screening a library or PCR based methods, become very difficult to obtain definitive results.

To determine the chromosomal location of the transgene, Caroloa Norlander in Dr. Levan's laboratory performed fluorescence *in situ* hybridization (FISH) analysis on GCS-EGFP homozygous colcemid-treated embryonic fibroblasts. The chromosome preparation was spread onto slides and an EGFP probe was used for hybridization (Fig. 3.10 A). The transgene was assigned to chromosome 11 in the q11-q12 region. To narrow the region, dual hybridization was performed using an EGFP probe and a BAC clone probe that contained a known gene on chromosome 11. Initial experiments showed that the EGFP signal was proximal to *Mx1* (at 11q21: 37.5 Mb: Rat Genome Project build 2.1; Fig. 3.10 B). Another BAC clone containing *Kcnj6* (at 35.5 Mb) was shown to be distal to the EGFP insertion at 11q12 (data not shown). Two other genes that were

proximal to *Mx1* and *Kcnj6* were chosen for further FISH analysis, namely *Ncam2 (at 20.7 Mb;* Fig 3.10 C) and *Grik1* (at 27.7 Mb; data not shown). Both of these BAC's gave signals at 11q11 and showed that the EGFP insertion site was proximal to *Grik1* and in close proximity to *Ncam2* (Fig. 3.10 D).

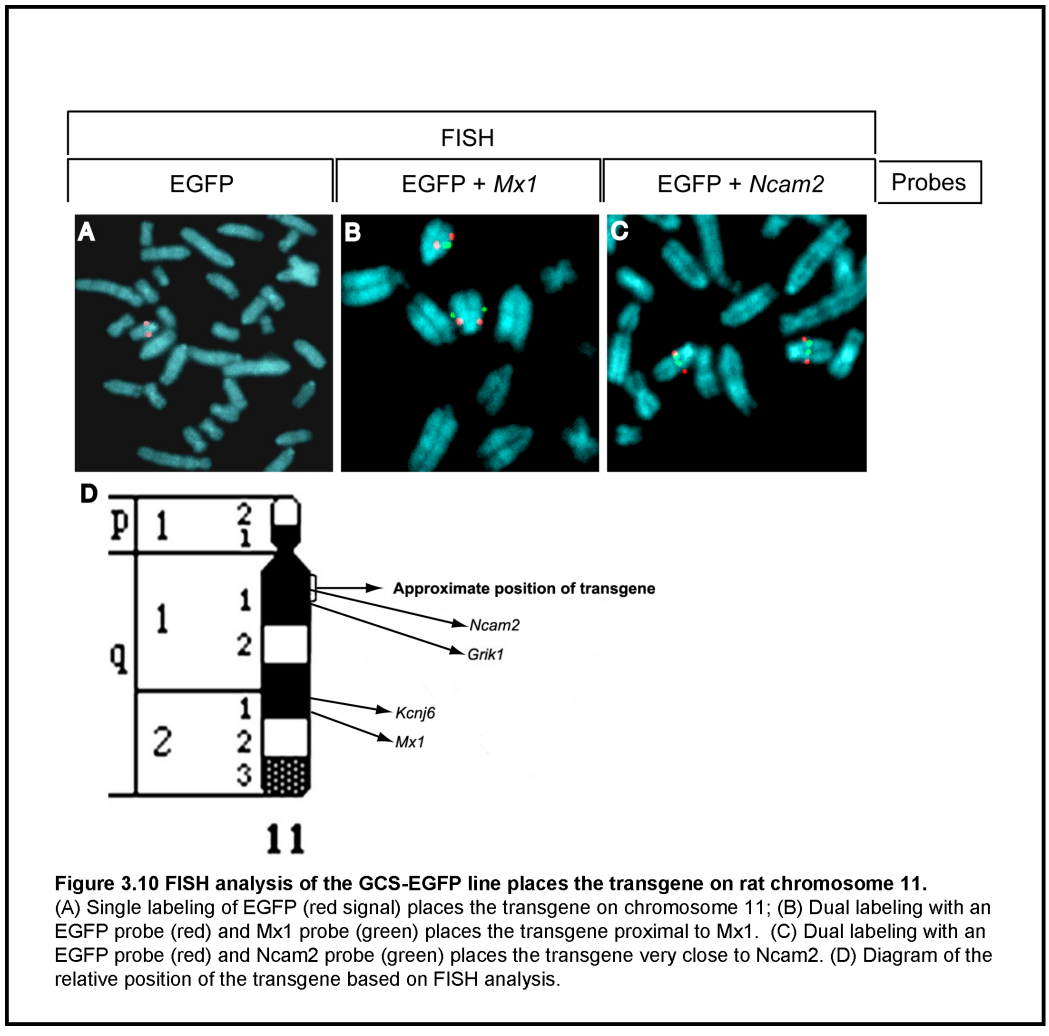


**Figure 3.10 FISH analysis of the GCS-EGFP line places the transgene on rat chromosome 11.** (A) Single labeling of EGFP (red signal) places the transgene on chromosome 11; (B) Dual labeling with an EGFP probe (red) and Mx1 probe (green) places the transgene proximal to Mx1. (C) Dual labeling with an EGFP probe (red) and Ncam2 probe (green) places the transgene very close to Ncam2. (D) Diagram of the relative position of the transgene based on FISH analysis.

*Transgene is expressed in the epiblast, genital ridge, embryonic gonads, and adult germ cells*

EGFP expression pattern was examined in post-implantation embryos (E8.5-E19.5) by fluorescence microscopy. EGFP fluorescence was first observed in the proximal epiblast with a small population of cells displaying strong fluorescence on E 8.5 (Fig. 3.11.A and I); these are conceivably primordial germ cells (PGC). In E13.5 embryos, the genital ridge (Fig. 3.11.B and J) showed strong fluorescence while the adjacent mesonephros was devoid of an EGFP signal. In E15.5 embryos, the male and female genital ridges can be distinguished from each other in that the male genital ridge (Fig. 3.11.D and L) exhibits a distinct cording pattern while the female ridge is mottled (Fig. 3.11.C and K). The genital ridges from both sexes of the GCS-EGFP rats were strongly fluorescent. The male and female embryonic gonads (Fig. 3.11.M and N) also showed strong EGFP fluorescence while the surrounding somatic support tissues were negative. EGFP fluorescence in the adult ovary of GCS-EGFP rats was confined to eggs in all stages of maturation (Fig. 3.11.G and O) while in the adult testis EGFP fluorescence was robust only within the seminiferous tubules (Fig. 3.11.H and P).

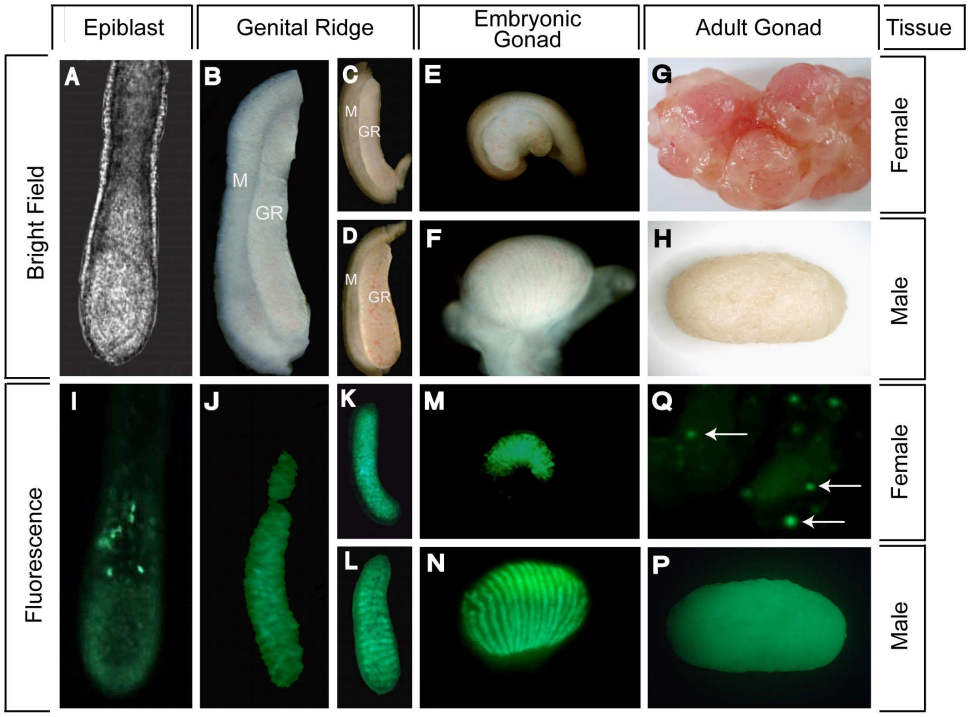


**Figure 3.11. EGFP expression during development is limited to germ cells.** (A-F) GCS-EGFP homozygous embryos at the following developmental stages - (A) epiblast E8.5, (B) early genital ridge E12.5, (C) female genital ridge E15.5, (D) male genital ridge E15.5, (E) female embryonic gonads E19.5 and (F) male embryonic gonads E19.5, GCS-EGFP homozygous adult (G) ovary and (H) testis. (I-P) Images of same specimens in (A-H) photographed using an EGFP fluorescent filter. Arrows in O indicate the location of fluorescent eggs in the cumulus masses.

As PGCs are identified principally by location, morphology, and high alkaline phosphatase activity, experiments were designed to determine if there was concordance between cells showing GFP expression and cells expressing high alkaline phosphatase activity. Cells in the proximal region of the epiblast and cells in the genital ridge had very high alkaline phosphatase activity (Fig. 3.12.B and D), and these same cells displayed abundant EGFP fluorescence (Fig. 3.12.A,

and C). Thus, early and late primordial germ cells in the GCS-EGFP rat appear to express EGFP.

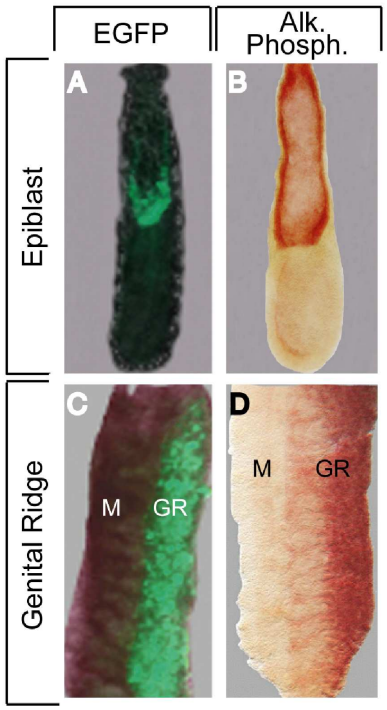


**Figure 3.12. EGFP expression colocalizes with the expression of alkaline phosphatase, a marker for germ cells.** (A, B) GCS-EGFP homozygous embryos at E8.5; (A) Phase- contrast and fluorescent overlay reveals EGFP expression is localized to the extra-embryonic portion of the epiblast with a population of the EGFP cells located in the proximal region. (B) Alkaline phosphatase activity is high in the same subpopulation of cells. (C, D) Genital ridges from E13.5 GCS-EGFP embryos; (C) Phase-contrast and fluorescent overlay reveals EGFP expression is localized to the genital ridge (GR) and not the adjacent mesonephros (M) tissue. (D) Alkaline phosphatase activity (red orange) is highest in the genital ridge.

*Transgene expression in pre-implantation embryos*

EGFP expression was characterized in pre-implantation embryos to determine the temporal and spatial patterns of expression during early germline

delineation. To accomplish this, homozygous females and males were mated to WT rats and embryos were collected at one-cell, 2-cell, 4-cell, morula, and blastocyst stages of development and examined for EGFP fluorescence. In fertilized eggs collected from transgenic females there was uniform robust EGFP expression from the one cell to the morula stages of development (Fig. 3.13.A, a-f and h-m). There was a significant, reproducible increase in the expression of EGFP at the late 2-cell stage onward, the known time of transition from maternal to zygotic based transcription (Zernicka-Goetz, 1994). The initiation of EGFP expression at the late 2-cell stage was confirmed in embryos collected from WT females mated with transgenic males. In eggs from this cross, there is no maternal EGFP message or protein and thus we could definitely establish the onset of transgene expression. There was no EGFP fluorescence in either the one or early 2-cell eggs (Fig. 3.13.B, a-b and h-i). In contrast, there was weak but discernible expression in both blastomeres of the late 2-cell stage eggs (Fig. 3.13.B, c and j) indicating that transgene expression is initiated during the earliest period of zygotic transcriptional activation.

Somewhat surprisingly, expression in the early to mid blastocyst stage of development was localized to both the inner cell mass and trophoblast (Fig. 3.13.A and 3.13.B, g and n). This non-ICM restricted pattern of expression may be due to active transcription of the transgene in these two compartments or more likely, may be a reflection of the extended half-life of EGFP (~20 hours). To discriminate between these two possibilities, delayed implantation was induced in WT females mated with transgenic males, collected blastocysts at E8.5, and examined them for the localization of EGFP expression. In all of the blastocysts examined (n=50), EGFP fluorescence was localized exclusively to the ICM with no discernible expression in trophectoderm (Fig. 3.13.D, a-c). This result strongly suggests that EGFP expression is limited to the ICM, the sole derivative of the germ cell lineage.

**Discussion**

This study describes and characterizes a line of transgenic rats in which a reporter transgene is fortuitously expressed exclusively in the germ cell lineage in both males and females. These data established that expression is initiated at the late 2-cell stage of embryogenesis and is localized to blastomeres of cleavage stage eggs, cells in the ICM of the blastocyst, proximal epiblast, and in primordial, embryonic, and adult germ cells. The transgene insertion site has been localized

to rat chromosome 11q11-q12, a region that interestingly harbors a number of testes-specific ESTs and transcribed loci. Based on these findings, the EGFP marked locus makes this strain of rats a powerful tool for the study of germ cell origin, development and migration, and potentially for the derivation of rat embryonic stem (ES) cells, germline stem (GS) cells (Zwaka and Thomson, 2005), and embryonic germ (EG) cells.

The generation of transgenic animals expressing a reporter gene cassette such as EGFP, LacZ, or growth hormone is a very common strategy for defining and characterizing the regulatory regions of a gene that direct tissue or cell specific expression (Hadjantonakis and Nagy, 2001; Sasaki and Hogan, 1996). In most instances the random integration of a transgene into the host genome does not alter expression of the neighboring genes, and therefore does not perturb the normal physiology of the transgenic animal (Tidhar et al., 2001). However, in approximately 10% of transgene insertions, the integration event seems to disrupt the expression of endogenous genes, leading to unexpected phenotypes most often discernible when the line is bred to homozygosity (Palmiter and Brinster, 1986). A second phenomenon common to transgene insertions generated by microinjection is improper expression of a transgene due to the influence of neighboring strong regulatory elements. This generally occurs when one uses "ubiquitous" or minimal promoters to drive transgene expression. The ROSA promoter sequences used in our transgene are known to drive the reporter genes,

EGFP and alkaline phosphatase, in a ubiquitous fashion (Kisseberth et al., 1999), and thus it can be assumed that the novel pattern of transgene expression in the GCS-EGFP line is due to positional effects.

Currently, there are no known markers for germ cells that are either expressed throughout germ cell development or that are exclusive to germ cells. Markers such as vasa, Oct4, and alkaline phosphatase identify germ cells, but are somewhat of limited value in that they either are not germ cell specific or they are not present throughout germ cell development (Chiquoine, 1954; Gertz, 1999; Tanaka et al., 2000). Identifying the regulatory sequences and ultimately the gene that drives germ cell-specific expression would provide a valuable tool to study germ cell delineation throughout development. The GCS-EGFP line is fully fertile in both males and females suggesting that the coding sequence of the putative germ cell-specific gene was likely not functionally disrupted by the insertion event. This type of positional effect on transgene expression is common and has been shown to occur in the following examples: the hsp68-LacZ transgene was expressed in developing neural tissue due to the influence of the *dystonia* enhancer (Kothary et al., 1988), the IE-LacZ transgene was expressed only in the apical ectodermal ridge (Gardner and Kappen, 2000) and HSVtk-LacZ transgene was expressed only in developing neurons (Allen et al., 1990). The identification of the hypothesized gene that targets germ cells may lead to an understanding of the mechanisms that determine and maintain germ cell fate.

Since I was unable to obtain sufficient flanking DNA sequence for further analysis, I used FISH to identify the chromosomal position and localized the transgene to rat chromosome 11q11-q12. This chromosomal region is syntenic to mouse chromosome 16 and human chromosome 21. Interestingly, this region encompasses the Down syndrome critical region genes 1-6. Based on dual FISH analysis we were able to narrow down this region (q11-q12) even further. There are 32 rat and 62 mouse loci mapped to this region and of these 18 rat and 24 mouse genes have been identified (NCBI mouse and rat genome database and Celera mouse database). I looked at the expression pattern of the assigned genes in this region in an attempt to identify candidate genes that had a similar pattern of expression, or that had germ cell expression at any stage of germ cell development. A gene was not found that was exclusively expressed in both the male and female germ cells; however, there were nine rat or mouse transcribed loci expressed in the testis, ovary, or pre-implantation embryo. Recently nine Riken clones from a testis-specific library were mapped to mouse chromosome 16 in the region that is syntenic to rat chromosome 11 in our region of interest (Table 3.3). In conclusion, the elucidation of the gene and or the regulatory regions that are driving the germ cell specific expression may help lead to an understanding of germ cell fate and differentiation. Additionally, the unique germ cell specific expression pattern of this transgene makes this rat line a very powerful tool to be used to address important questions in the germ cell field.

**Considerations and Future Directions**

The gene controlling the unique expression pattern must be identified. Many attempts were made initially to elucidate this gene or regulatory region by obtaining DNA sequence that flanked the transgene. However, no more than 50bp of flanking sequence was obtained. The 50bp segment of flanking sequence was not specific and received over 100 hits when blasted on the NCBI rat and mouse databases. Some limitations experienced when trying to identify flanking sequence included; inability to obtain 5' flanking sequence, multiple copies of the transgene, tail to tail integration of one of the transgenes, and difficulty in obtaining PCR products due to a high GC content. Since the transgene contained the mouse ROSA26 promoter that shares 94% homology with the rat ROSA 26 promoter, experiments were designed to look for flanking sequence at the 3' end only. This strategy was chosen so that DNA regions flanking the endogenous ROSA26 promoter were not obtained. With multiple copies and different orientations of the transgene, identification of the transgene through the use of PCR based methods are usually more difficult since there will be more than one priming site, resulting in the amplification of only fragments of the transgene. Regulatory regions are also very rich in GC content; a GC box is usually found in multiple copies in the promoter region usually near the TATA box (Hapgood et al., 2001). High GC content also makes the amplification of PCR products very difficult. Different modifications to the common PCR protocol have been shown

amplify GC rich promoter regions. These include the use of the reagents, DMSO, betaine, and 7-deaza-2'-deoxyguanosine, that lower the melting temperature during PCR (Chakrabarti and Schutt, 2002; Jung et al., 2002) and modifying the PCR cycling parameters to optimize the amplification of an unknown region (Choi et al., 1999; Hecker and Roux, 1996). The previously described inverse PCR and Genome Walker results were achieved only when a combination of DMSO and betaine were included in the PCR reaction mixture and a modified PCR cycling program known as Touchdown PCR was used. Touchdown PCR parameters consisted of one cycle (96 °C for 3 min, 25 cycles of 94 °C for 45sec., 72 °C for 1 min -1 °C/cycle), followed by 16 cycles (94 °C for 45sec., 58 °C for 1 min., 72 °C for 3 min), and a final elongation step (72 °C for 10 min). These particular conditions amplified a 3.2 kb junction fragment made by XbaI digestion. Based on the restriction mapping of this transgene insertion site, other junction fragments exist and amplification of these fragments may be successful by varying different components of the PCR reaction mixture or changing the PCR cycling parameters.

If the junction fragment cannot be amplified and sequenced, the precise regulatory region that drives the expression of EGFP to the germ cells in this transgenic strain should be defined. Since FISH analysis places the transgene on rat chromosome 11q12-q12, BAC's containing this region can be obtained. Regulatory regions have been studied by generating transgenic animals that are

co-injected with a BAC and a reporter gene. The transgenic animals generated by these co-injections can be then screened for founders whose reporter gene expression recapitulates the expression pattern of interest (DiLeone et al., 2000). The corresponding BAC that contains the regulatory region can be enzymatically digested and transgenics generated again by co-injection with an isolated BAC fragment and a reporter gene. These animals can be screened for the expression pattern of interest. This process can be repeated with the fragment of interest until the desired expression pattern is lost indicating a necessary region of the regulatory region has been removed. Although this method would not identify the gene, it would allow for the exact regulatory region that drives the EGFP expression to germ cells to be defined.

Finally, since the rat genome has been sequenced and FISH analysis has placed the transgene on chromosome 11, the genes mapped to this region can be studied. Table 3.3 lists the genes in the rat and mouse and their known mRNA expression. This table revealed a number of potential candidates that are testis or ovary specific. The next step will be to determine whether any of the genes or ESTs are also exclusively expressed in germ cells of the opposite sex. This can be done initially by quantitative real time PCR and further confirmed through *in situ* hybridization experiments. Candidates that are found in both oogonia and spermatogonia can then be further studied to determine if their expression extends

to different stages of germ cell development. These experiments may then lead to elucidating the gene that specifically marks germ cells.

**Table 3.3: Expression pattern of genes located on rat chromosome 11q11-q12 and the syntenic region on mouse chromosome 16(as of April 8, 2005)**

| Gene Symbol | Mouse Unigene | Mouse Expression | Rat Unigene | Rat Expression |
|---|---|---|---|---|
| 1700041M19Rik | Mm.372181 | Pre-implantation Embryo | Does not exist | No information at this time |
| Usp25 | Mm.40986 | Multiple tissues | Rn.23509 | Ovary, brain; dorsal root ganglion; |
| Rat transcribed locus | Does not exist | No information at this time | Rn.26727 | Omnibus Database |
| Rat transcribed locus | Does not exist | No information at this time | Rn.94354 | Cartilaginous tumor library |
| Rat transcribed locus | Does not exist | No information at this time | Rn.131271 | Cartilaginous tumor library |
| Rat transcribed locus | Does not exist | No information at this time | Rn.112932 | Testis and Brain |
| Rat transcribed locus | Does not exist | No information at this time | Rn.131270 | Heart |
| C130023A14Rik | Mm.220632 | Brain, Mammary Gland, Late-Gestation Embryo, Adult | Does not exist | No information at this time |
| 8030498J20Rik | Mm.218964 | Pancreas, Pituitary Gland, Testis, Embryo | Does not exist | No information at this time |
| 9530003O04Rik | Mm.184166 | Adult | Does not exist | No information at this time |
| Transcribed locus | Mm.202403 | Pancreas, Embryo | Does not exist | No information at this time |
| 9330154C14Rik | Mm.328655 | Male diencephalon | | |
| Locus similar to BTG3 | Mm.311644 | Multiple tissues | Does not exist | No information at this time |
| Cxadr | Mm.66222 | Multiple tissues | Rn.113837 | Intestine, fetal heart, liver |
| Rat transcribed locus | Does not exist | No information at this time | Rn.45690 | Multiple tissues |
| Btg3 | Mm.2823 | Multiple tissues | Rn.8897 | Multiple tissues |
| Rat transcribed locus | Does not exist | No information at this time | Rn.33175 | Ovary, lung |
| 4933417O19Rik | Mm.84968 | Eye, Testis Embryo | Does not exist | No information at this time |
| D16Ertd472e | Mm.37332 | Multiple tissues | Rn.27471 | Multiple tissues |
| Rat transcribed locus | Does not exist | No information at this time | Rn.18313 | Adult Tissues |
| Chodl | Mm.7789 | Multiple tissues | Does not | No information |

| | | | | |
|---|---|---|---|---|
| | 5 | | exist | at this time |
| Prss7 | Mm.5184 | Multiple tissues | Rn.83908 | No information at this time |
| **1700066C05Rik** | Mm.219782 | **Testis** | Does not exist | No information at this time |
| Ncam2 | Mm.258759 | Multiple tissues | Does not exist | No information at this time |
| Rat transcribed locus | Does not exist | No information at this time | Rn.45155 | Dorsal root ganglion |
| Transcribed locus | Mm.226395 | Brain, Embryo | Does not exist | No information at this time |
| Transcribed locus | Mm.372310 | Brain, Embryo | Does not exist | No information at this time |
| Transcribed locus | Mm.39609 | Brain, Adult | Does not exist | No information at this time |
| **Transcribed locus** | Mm.247738 | **Pre-implantation Embryo** | Does not exist | No information at this time |
| ENSMUST00000033585 | Mm.188 | Multiple tissues | Does not exist | No information at this time |
| Transcribed locus | Mm.245081 | Spleen | Does not exist | No information at this time |
| **4930553E22Rik** | Mm.261415 | **Testis mRNA library** | Does not exist | No information at this time |
| A730009L09Rik | Mm.127269 | Brain | Does not exist | No information at this time |
| **4930529L06Rik** | Mm.243853 | **Testis** | Does not exist | No information at this time |
| **4930551I23Rik** | Mm.84635 | **Testis mRNA library** | Does not exist | No information at this time |
| BIC noncoding mRNA | Mm.261808 | Multiple tissues | Does not exist | No information at this time |
| Mrpl39 | Mm.103655 | Multiple tissues | Does not exist | No information at this time |
| Rat transcribed locus | Does not exist | No information at this time | Rn.17615 | Brain, eye, pituitary gland, embryo |
| Jam2 | Mm.41758 | Multiple tissues | Rn.6473 | No information at this time |
| Transcribed locus | Mm.374465 | Retina | Does not exist | No information at this time |
| Gabpa | Mm.18974 | Multiple tissues | Rn.76236 | Intestine, fetal |
| Atp5j | Mm.353 | Multiple tissues | Rn.5790 | Multiple tissues |
| Gene model 311 | Mm.301399 | Thymus | Does not exist | No information at this time |
| Rat transcribed locus | Does not exist | No information at this time | Rn.45570 | Brain, colon, eye, lung, embryo |
| App | Mm.2775 | Multiple tissues | Rn.2104 | Multiple tissues |

| | | | | |
|---|---|---|---|---|
| | 85 | | | |
| Rat transcribed locus | Does not exist | No information at this time | Rn.131264 | Cartilaginous tumor library |
| **Rat transcribed locus** | Does not exist | No information at this time | Rn.1528 | **Ovary,** multiple tissues |
| LOC433045 | Mm.355446 | Brain, Mammary Gland, Skin, Adult | Does not exist | No information at this time |
| B830010I11Rik | Mm.170971 | 10 days neonate medulla oblongata cDNA | Does not exist | No information at this time |
| Cyyr1 | Mm.213203 | Multiple tissues | Does not exist | No information at this time |
| **Transcribed locus** | Mm.372998 | **Testis** | Does not exist | No information at this time |
| LOC433046 | Mm.310871 | Multiple tissues | Does not exist | No information at this time |
| Adamts1 | Mm.1421 | Multiple tissues | Rn.7897 | Multiple tissues |
| **4930556C24Rik** | Mm.108627 | **Testis** | Does not exist | No information at this time |
| Transcribed locus | Mm.139860 | Brain, Mid-Gestation Embryo | Does not exist | No information at this time |
| Transcribed locus | Mm.372689 | Muscle, Placenta, Testis, Embryo | Does not exist | No information at this time |
| **Rat transcribed locus** | Does not exist | No information at this time | Rn.16839 | **Ovary,** multiple tissues |
| Adamts5 | Mm.112933 | Multiple tissues | Rn.107051 | Multiple tissues |
| **Rat transcribed locus** | Does not exist | No information at this time | Rn.18479 | **Embryo** |
| Rat transcribed locus | Does not exist | No information at this time | Rn.131262 | Adult Tissues |
| N6-DNA-methyltransferase isoform 1 | Does not exist | No information at this time | Rn.107202 | Adult Tissues |
| Rat transcribed locus | Does not exist | No information at this time | Rn.113851 | Brain |
| **1700007H22Rik** | Mm.80901 | **Testis** | Does not exist | No information at this time |
| Transcribed locus | Mm.357673 | Other | Does not exist | No information at this time |
| D630011L08 | Mm.132956 | Kidney, Liver, Uterus, Neonate, Adult | Does not exist | No information at this time |
| **4933405A01 Rik** | Mm.50828 | **Testis** | Does not exist | No information at this time |
| **Transcribed locus** | Mm.328671 | **Testis** | Does not exist | No information at this time |
| **Transcribed locus** | Mm.233725 | **Testis** | Does not exist | No information at this time |
| 5830445C04Rik | Mm.2965 | Multiple tissues | Does not | No information |

| | | | | |
|---|---|---|---|---|
| | 32 | | exist | at this time |
| Zfp294 | Mm.249005 | Multiple tissues | Rn.44204 | Multiple tissues |
| ORF5 | Mm.162406 | Multiple tissues | Rn.98689 | Liver |
| Usp16 | Mm.152941 | Multiple tissues | Rn.55800 | Multiple tissues |
| Cct8 | Mm.328673 | Multiple tissues | Rn.98524 | Multiple tissues |
| B130034C11Rik | Mm.166361 | Brain, Eye, Liver, Thymus, Mid-Gestation Embryo, Adult | Does not exist | No information at this time |
| ORF63 | Mm.194466 | Multiple tissues | Rn.32787 | Kidney, muscle |
| Rat transcribed locus | Does not exist | No information at this time | Rn.92101 | Found only in library 1652: UI-R-C3 |
| Bach1 | Mm.5183 | Multiple tissues | Rn.29793 | Multiple tissues |
| Rat transcribed locus | Does not exist | No information at this time | Rn.13802 | Multiple tissues |
| 2810407A14Rik | Mm.123865 | Multiple tissues | Does not exist | No information at this time |
| Grik1 | Mm.5134 | Multiple tissues | Rn.10449 | Dorsal root ganglia, pituitary |
| 4930420G21Rik | Mm.296547 | Spleen, Testis, Adult | Does not exist | No information at this time |
| **4930590A17Rik** | Mm.160295 | **Testis, Pre-implantation Embryo, Brain** | Does not exist | No information at this time |
| Claudin 17 | Mm.126860 | E16-head only | Rn.19782 | Found only in library 1387: UI-R-Y0 |

**Does not exist** indicates that the sequence has not been given a unique Unigene identifier in that particular species

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# CHAPTER FOUR

## GCS-EGFP Rat as a Model to Study Stem Cells

### Introduction

There is currently a very intensive interest in embryonic and somatic stem cells due to their potential ability to differentiate to a variety of distinct cell types to replace cells and tissues damaged from disease, injury, or age. The most commonly used definition for a stem cell is a cell that can proliferate indefinitely, has the ability to self-renew, and is able to differentiate into multiple lineages (Weissman, 2000). Stem cells can be divided into three groups based on their potential. The earliest stem cells are totipotent, which means the cells have the ability to contribute to all cells in an organism. In mammals, only the fertilized egg and cells from the first few cleavages retain totipotency. During development, the zygote becomes a blastocyst that consists of two cell types, the inner cell mass (ICM) and the trophectoderm. Cells from the inner cell mass are pluripotent, which means the cells have the ability to develop to all cells of the embryo proper (Rossant, 2001; Wells et al., 2003).

In 1981, embryonic stem (ES) cells from mice were first derived by the *in vitro* culture of ICM in conditioned medium (Evans and Kaufman, 1981; Martin, 1981). It was later shown that a key factor in the conditioned medium that maintained the cells in an undifferentiated state was the cytokine leukemia inhibitory factor (LIF) (Williams et al., 1988). In 1987, site directed targeted

mutagenesis in ES cells through homologous recombination was successfully demonstrated in the mouse genome (Thomas and Capecchi, 1987). This strategy was used with ES cells to create mutant cell lines that were then injected into blastocysts to create chimeric mice that harbored silent mutations (Thomas and Capecchi, 1987). Since the introduction of this methodology, thousands of transgenic knock-out and knock-in mice strains have been generated for the investigation of gene expression, regulation, and to serve as mouse models of different human diseases.

Mouse ES cell lines are also capable of differentiating into many different somatic cell types when specific factors are added to stimulate differentiation (Czyz et al., 2003). A current focus in mouse ES cell biology is to find the exact factors and conditions needed to stimulate differentiation to a specific functional cell type. The only other pluripotent stem cells that can be cultured *in vitro* are embryonic carcinoma (EC) cells that are derived from teratocarcinomas and embryonic germ (EG) cells that are derived from primordial germ cells (Czyz et al., 2003). To date, human ES and EG cell lines have been generated and work is currently focused on the differentiation of these cells so that they can be used as a source for transplantation therapies in medicine.

Many adult tissues in the mammal can regenerate cells during normal homeostasis or following injury. These tissues contain adult stem cells that are restricted in their ability to differentiate and are therefore characterized as

multipotent. Adult stem cells have been isolated from skeletal muscle, retina, neurons, liver, pancreas, and bone marrow (Czyz et al., 2003; Garry et al., 2003). Recently, it has been shown that adult stem cells can be transplanted into ectopic sites and will transdifferentiate into distinct cell types (Alison et al., 2002; Filip et al., 2004). For example, hematopoietic stem cells, which are the most extensively studied AS cell to date, are able to transdifferentiate into myocytes and hepatocytes (Masson et al., 2004). These observations open the possibility that adult stem cells could be used in regenerative medicine. More importantly, if future studies prove that adult stem cells exhibit the same plasticity as ES cells then stem cell therapies would reduce or possibly avoid the use of stem cells derived from the human embryo. Therefore, a great deal of effort is being focused on discovering the properties and potentiality of adult stem cells.

In 1994, the Brinster laboratory reported the transplantation of male germ cells to the testis of sterile mice resulted in the colonization of the testis by the spermatogonial stem cells and the restoration of spermatogenesis (Brinster and Zimmermann, 1994). I wanted to determine the usefulness of the GCS-EGFP transgenic line. Therefore, I took advantage of the germ cell specific expression of the GCS-EGFP rats and used these transgenic rats to first assess the utility of the transgenic line for germ cell transplantation studies.

Germ cells originate from PGCs; however, it is not clear when stem cells first appear during embryonic germ cell development. Germ cells are sex-

determined after they have migrated to the genital ridges, and male germ cells can be observed in corded pattern by E15.5 in the rat (McLaren, 1998). Male germ cells enter mitotic arrest after the formation of the embryonic gonads and it is not until after birth when they will resume spermatogenesis (Wylie, 1999). Spermatogonial stem cells are responsible for maintaining spermatogenesis throughout a male's life. The GCS-EGFP ratline is an ideal animal to determine when male germline stem cells first appear in germ cell development. To accomplish this I isolated pure populations of germ cells at distinct developmental stages and then transferred them to recipient testes to assess their colonization potential.

During the last several years, the degree of plasticity of adult somatic stem cells has been an area of extreme controversy. A population of hematopoietic stem cells, termed the side population (SP), can be isolated from bone marrow by fluorescein-activated cell sorting (FACS) based on the ability of the cells to efflux Hoechst dye through an ABC type II transporter (Goodell et al., 1996). These cells are capable of contributing to diverse lineages *in vivo* which include; skeletal muscle, hepatocytes, and vascular endothelial cells (Ferrari et al., 1998; Petersen et al., 1999; Shi et al., 1998). I again used the transplantation assay and the GCS-EGFP rats to demonstrate another utility of this ratline and to determine the plasticity of an adult hematopoietic stem cell. I isolated the SP population of cells

from the bone marrow of male GCS-EGFP rats and transferred them to recipient testes to assess if they had the ability to transdifferentiate to male germ cells.

ES cells from diverse mouse strains are now routinely derived; however, in rats the derivation of an ES cell line has remained elusive. Many groups have attempted to establish a stable embryonic rat cell line that is able to contribute to the germline in the past 10 years (Iannaccone et al., 1994; Kawase et al., 2000; Ouhibi et al., 1995; Vassilieva et al., 2000). However, there is only one report on the establishment of a rat embryonic "stem-like" line (Vassilieva et al., 2000). However, this line of cells has not been shown to contribute to the germline through the generation of chimeric animals. If a rat ES cell line can be established its use in knockout experiments to manipulate the rat genome would create new animal disease models that may complement mouse models and aid in the research areas in which the rat is the preferred animal. For example, the pharmaceutical industry favors the rat model for the routine use of evaluating drug compounds (Charreau et al., 1996). Therefore, in an attempt to expand the ES cell technology to the rat, I used the GCS-EGFP rat blastocysts to derive a rat ES cell line.

**Material and Methods**

*Seminiferous tubule cell isolation from testis*

Seminiferous tubules were isolated from the testes of 23-day-old homozygous GCS-EGFP rats. The tubules were mechanically disaggregated and enzymatically digested with dispase (Invitrogen/GibcoBRL Carlsbad, CA), dissociated into a cellular suspension, and filtered through a 20μm nylon mesh. Cells were then counted and resuspended at a concentration of $1x10^7$ cells/ml (Hamra et al., 2004).

*PGC isolation from the genital ridge*

PGC's were isolated from homozygous GCS-EGFP E13.5 embryos. The genital ridge was isolated by microdissection in PBS as previously described (Hogan, 1994). Isolated genital ridges were enzymatically digested with 2.5% trypsin (Invitrogen/GibcoBRL Carlsbad, CA), mechanically disaggregated to single cells, and filtered through a 20μm nylon mesh. Cells were then counted and resuspended in DMEM.

*Germ cell isolation from embryonic gonads*

Germ cells were isolated from homozygous GCS-EGFP E19.5 male embryos. The tubules were mechanically disaggregated and enzymatically digested with 2.5% Trypsin (Invitrogen/GibcoBRL Carlsbad, CA), dissociated into a cellular

suspension, and filtered through a 20μm nylon mesh. Cells were then counted and resuspended in DMEM.

*Germ cell transplantation*

Twelve day-old WT Sprague Dawley male rats were injected intraperitoneally with 12.5 mg/kg of busulfan (4 mg/ml in 50%DMSO) and used as recipient males at 24 days of age. Donor cells were loaded into an injection needle fashioned from a 100 μl glass capillary microcaps (Cole-Parmer Instruments Co., Vernon Hills, IL) and cells were transplanted into the seminiferous tubules of an anesthetized recipient rat by retrograde injection through the *rete* testis (Hamra et al., 2002; Ogawa et al., 1997). Trypan blue was added to the cell suspension to visualize transfer into the tubules. Recipient rats were analyzed for donor cell colonization on day 30 or 60 after transfer by direct visualization of EGFP expression using a fluorescent Nikon SMZ1500 stereomicroscope. The seminiferous tubules were dissected from the testis and processed for the quantitation of soluble GFP as described (Hamra et al., 2004). The testis lysates were assayed for fluorescent intensity using recombinant EGFP with a carboxyl-terminal histidine tag as a standard. For the recipient testis receiving PGCs or embryonic germ cells, three months after transfer the recipient rats were sacrificed, the testes removed, the seminiferous tubules isolated and digested with proteinase K. DNA was isolated using standard procedures and PCR was

performed using 3ug of input DNA and the primers for PCR was performed using the forward primer EGFP-F (5' CTCGTGACCACCCTGACCTACGG) and the reverse primer EGFP-R (5' ATGCCCTTCAGCTCGATGCGGTT) corresponding to nucleotides 1004 to 1195 of the ROSA-EGFP DNA fragment that amplified a DNA product of 191bp; or forward primer EGFP5-1 (5' AACTTCAGGGTCAGCTTGC) and the reverse primer EGFP3-1 (5'GGTGTTCTGCTGGTAGTGGTC) corresponding to nucleotides 971 to 1492 of the ROSA-EGFP DNA fragment that amplified a DNA product of 521bp.

*Bone marrow SP cell isolation by flow cytometry*

Side population **(**SP) cell isolation from bone marrow was performed as previously described (Goodell et al., 1996), with some modifications. Briefly, bone marrow was isolated from 8 week old homozygous GCS-EGFP male rats by flushing the tibia and femur with Hanks Balanced Salt Solution supplemented with 2% Fetal Calf Serum and 10mM HEPES (HBSS+) (Invitrogen/Gibco, Carlsbad, CA) using an 18 gauge needle. The cells were pipetted up and down to obtain a single cell suspension, then counted, and resuspended at $1X10^6$cells/ml in HBSS+. Cells were stained with 5 μg/ml Hoechst 33342 (Sigma Aldrich, St. Louis, MO) at 37$^o$C for 30 min, washed once with PBS, and resuspended at a concentration of 1 X $10^7$ cells/ml in cold HBSS+. Cells were then sorted using a MoFlo flow cytometer (Cytomation, Inc., Fort Collins, CO) using a krypton laser

at 361 nm to excite the Hoechst dye. Fluorescence emission was collected with a 405/30 BP filter (Hoechst Blue) and a 670/40 BP filter (Hoechst Red) (Fig. 4.1 A).

The SP cells were collected and stored on ice and then sorted a second time by flow cytometry. The SP fraction appeared about 98% pure and showed no EGFP fluorescence (Fig. 4.1 B). To ensure that the cells isolated expressed an ABC

type II transporter, the bone marrow prior to sorting was incubated with Hoechst 33342 alone as a control (Fig. 4.1 C), with Hoechst 33342 and Verapamil, an ABC transport inhibitor (Fig. 4.1 D), or with furnitremorgin C (FTC), an ABC type II transport inhibitor (Fig. 4.1 E). The profile suggested that both drugs were able to block the efflux of the Hoechst 33342 dye, confirming that the population of cells represented the SP fraction.

*SP Cell Transplantation*

Twelve day-old WT Sprague Dawley male rats were injected intraperitoneally with 12.5 mg/kg of busulfan (4 mg/ml in 50%DMSO) and used as recipient males at 24 days of age. The busulfan will destroy the majority of the rats germ cells. Donor cells were loaded into an injection needle made from a 100 µl glass capillary and cells transplanted into the seminiferous tubules of an anesthetized recipient rat by retrograde injection through the *rete* testis as described by (Hamra et al., 2002; Ogawa et al., 1997). Trypan blue was added to the cell suspension to visualize transfer into the tubules. Recipient rats were analyzed for donor cell colonization 30 days after transfer by direct visualization of EGFP expression using the Nikon SMZ1500 stereoscope. Six months after transfer the recipient rats were sacrificed, the testes removed, the seminiferous tubules isolated and digested with proteinase K. DNA was isolated using

standard procedures and PCR was performed using 3ug of input DNA and the primers for EGFP as previously described.

*Histological Analysis*

Testis were removed and fixed overnight in 4% paraformaldehyde and then embedded in paraffin. The tissue was then cross-sectioned at 5μm and the slides were processed then stained with hematoxylin and eosin.

*Rat ES cell derivation*

The GCS-EGFP rat strain was used for the isolation and derivation of ES cells. Prepubertal female rats were superovulated by a standard regimen (Young, 1999) and placed overnight with stud males. The presence of sperm cells in the vaginal lavage or a copulatory plug the following morning indicated mating had occurred and was scored as day E0.5. Blastocyst implantation was delayed as previously described (Hunter and Evans, 1999). Briefly, WT females were mated with GCS-EGFP homozygous males and the morning of finding a copulatory plug was designated as E0.5. On E4.5, 50μg tamoxifen (Sigma-Aldrich, St. Louis, MO) in an aqueous solution of corn oil was injected intraperitoneally and 5mg Depo-Provera (Upjohn Co., Kalamazoo, MI) was injected subcutaneously. Delayed blastocysts were collected on E8.5 in R1ECM, washed in R1ECM, and placed in organ culture dishes onto a feeder layer. Embryonic fibroblasts were isolated from E15.5 rat embryos and served as the feeder layers. A stable line of BMP4

Cos cells that secreted BMP4 into the media were also used as a feeder layer. One day prior to ICM plating the feeder cells were irradiated and plated onto gelatinized organ culture dishes. Rat ICM's were grown in stem cell isolation medium (SCIM) (Robertson, 1987b). Briefly, SCIM consists of DMEM-lo (Invitrogen/GibcoBRL, Carlsbad, CA) supplemented with; 20% FBS-ES cell qualified (Hyclone, South Logan, UT), 1000units/ml of rat LIF (Chemicon International, Temecula, CA), 1% antibiotic stock (Invitrogen/GibcoBRL, Carlsbad, CA), 1% beta-mercaptoethanol (Invitrogen/GibcoBRL, Carlsbad, CA), 1% non-essential amino acids (Invitrogen/GibcoBRL, Carlsbad, CA), 1% nucleoside (Sigma-Aldrich, St. Louis, MO), and 1% L-glutamine (Invitrogen/GibcoBRL, Carlsbad, CA) and 5μg/ml FGF. ICM outgrowths were picked off the plate, treated for 2 min with 0.25% Trypsin (Invitrogen/GibcoBRL, Carlsbad, CA), and then mechanically disaggregated using a glass pipette. Aggregates of 10-50 cells were placed onto fresh feeder layers and cultured. Compacted homogenous colonies were picked and passaged every 2 days. Colonies were disaggregated by either mechanical disaggregation with a glass pipette or with treatment for 2 min with 0.25% Trypsin (Invitrogen/GibcoBRL, Carlsbad, CA) and then mechanical disaggregation.

*AP Staining*

Rat cells were washed three times in PBS and then fixed in 10% formalin for 2 min at room temperature. Alkaline phosphatase activity of the cells was assessed by histochemical staining using an alkaline phosphatase staining kit from Chemicon (Chemicon International, Temecula, CA); cells were kept in PBS, and viewed on a Nikon Eclipse TE2000-U inverted microscope.

**Results**

*Transfer of GCS-EGFP seminiferous tubule cells to recipient testes*

Unsorted seminiferous tubule cells from GCS-EGFP rats (Fig. 2A, 2B) were transferred to the testis of recipient males to establish the efficiency of this heterogeneous population of cells to take residence in a recipient testis. Colonization efficiency is the measure of the inherent stem cell activity of a given population of cells. Presumably the germline stem cells constitute a very small proportion of the total seminiferous tubule cell preparation of an adult testis (Brinster and Zimmermann, 1994; Dobrinski et al., 2000; Hamra et al., 2002; Nagano and Brinster, 1998). Recipient males were sacrificed at either 30 or 60 days following cell transfer, the testis examined by fluorescent microscopy and the entire tubular mass processed for quantification of the abundance of GFP. The testis from all recipient males that were sacrificed 30 days after receiving seminferous tubule cells from GCS-EGFP rats had abundant GFP expressing cells

distributed throughout the tubules (Fig.4. 2C and 2D), and had quantities of GFP/testis that ranged between 200-300ng. Extending the time of colonization from 30 to 60 days approximately doubled the abundance of GFP per testis (Fig. 4.2E).

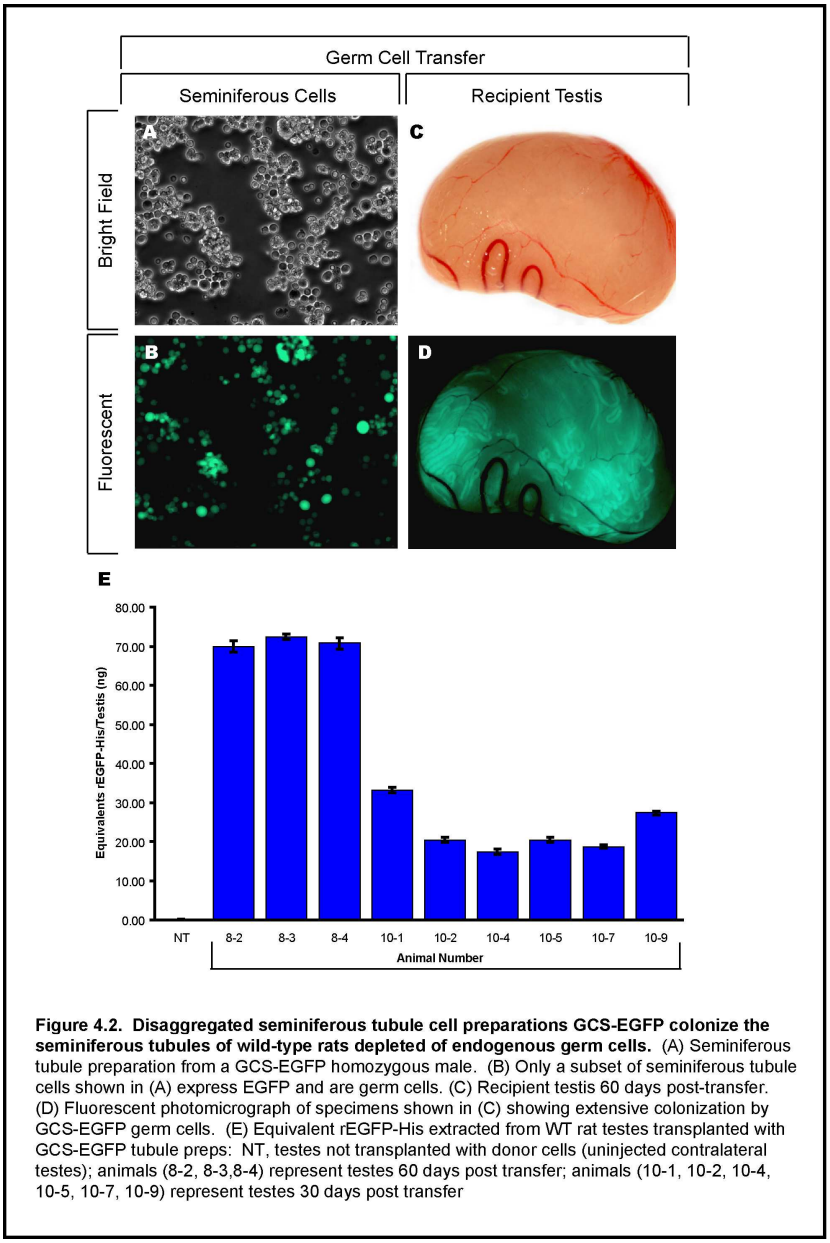


**Figure 4.2. Disaggregated seminiferous tubule cell preparations GCS-EGFP colonize the seminiferous tubules of wild-type rats depleted of endogenous germ cells.** (A) Seminiferous tubule preparation from a GCS-EGFP homozygous male. (B) Only a subset of seminiferous tubule cells shown in (A) express EGFP and are germ cells. (C) Recipient testis 60 days post-transfer. (D) Fluorescent photomicrograph of specimens shown in (C) showing extensive colonization by GCS-EGFP germ cells. (E) Equivalent rEGFP-His extracted from WT rat testes transplanted with GCS-EGFP tubule preps: NT, testes not transplanted with donor cells (uninjected contralateral testes); animals (8-2, 8-3,8-4) represent testes 60 days post transfer; animals (10-1, 10-2, 10-4, 10-5, 10-7, 10-9) represent testes 30 days post transfer

*Transfer of GCS-EGFP genital ridge cells to recipient testes*

PGCs were isolated, based on their location and EGFP positive expression, from the genital ridge of E13.5 GCS-EGFP homozygous embryo (Fig. 4.3 A and B)

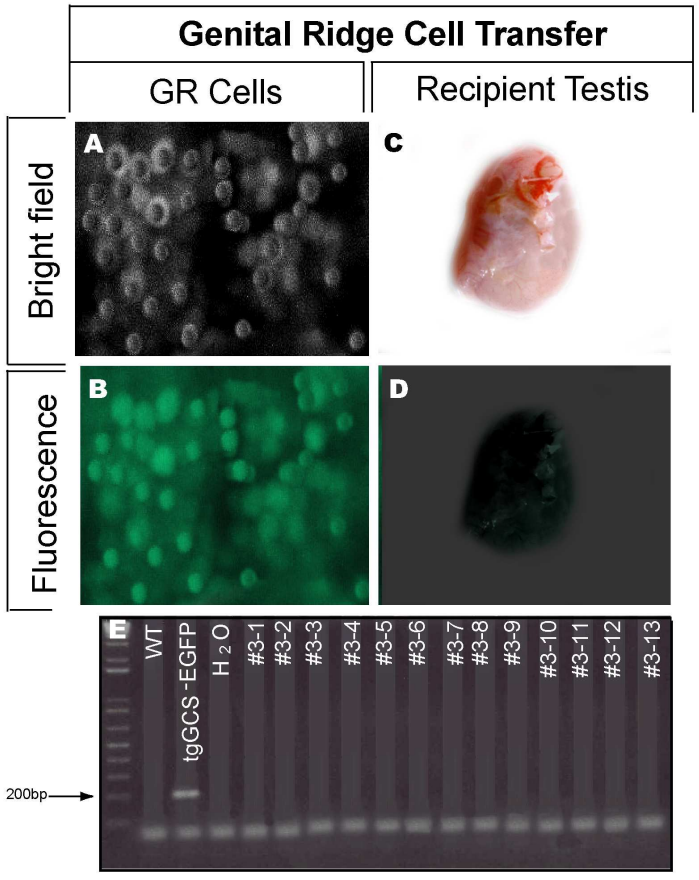


**Figure 4.3. PGCs isolated from the genital ridge of E13.5 GCS-EGFP homozygous embryos are not able to colonize the seminiferous tubules of WT rats depleted of endogenous germ cells.** (A) Isolated PGC cells from a homozygous GCS-EGFP E13.5 genital ridge. (B) Fluorescent photomicrograph of cells in (A), the PGCs express EGFP. (C) Recipient testis after 3 months. (D) Fluorescent photomicrograph of specimen in (C), showing no evidence of EGFP in any of the tubules. (I) PCR analysis of one PGC donor cell transfer experiment. Testes were collected from recipient animals, tubules isolated, digested, extracted, and PCR performed on DNA preparations with EGFP primers. The numbers 3-1 to 3-13 refers to recipient animal numbers, WT refers to wild-type testis

The cells were transferred to the seminiferous tubules of a WT recipient rat. The testes were examined for EGFP fluorescence at one and three months following transfer (Table 4.1). There was no EGFP expression observed in any of the recipient testis (Fig. 4.3 C and D). PCR analysis of the recipient testis also did not amplify EGFP (Fig. 4.3 E).

However, the recipient testis was observed to be much smaller compared to the contralateral testis that had not received any cells (Fig. 4.4 A) and upon further examination, the recipient testis contained a solid mass. The testes were removed, paraffin embedded, and processed for sectioning to determine the cell morphology in order to identify the mass. H&E staining revealed that the mass was a tumor containing a small homogenous population of undifferentiated cells (Fig. 4.4 B and C).

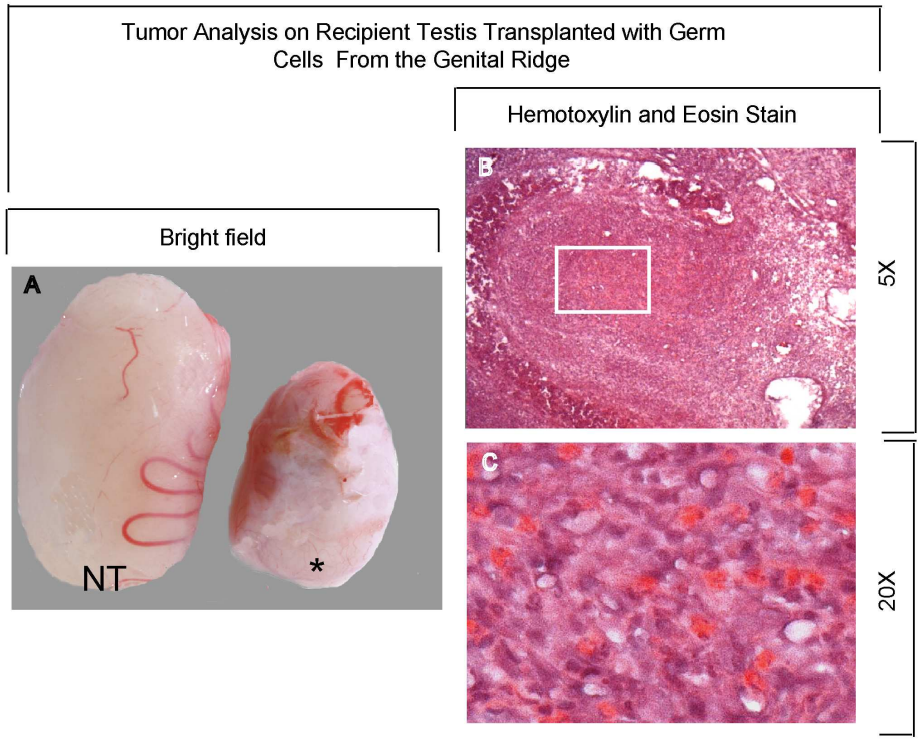


**Figure 4.4. PGCs from the genital ridge transferred to the testis of a wild-type rats depleted of endogenous germ cells form tumors.** (A) Bright field photomicrograph of recipient testis 90 days after PGC's from E13.5 were transferred. (*)Injected testis on the right side and (NT) testes not transplanted with donor cells (uninjected contralateral testes) on the left side. (B and C) Testis in (A*) was fixed in paraffin and sliced in to 5μm sections H&E staining was performed on the sections to determine morphology. (B) 5X magnification (C) 20X magnification of the boxed area in (B).

*Transfer of GCS-EGFP male embryonic gonadal cells to recipient testes*

Male embryonic germ cells were isolated from E19.5 GCS-EGFP homozygous embryos and expressed EGFP robustly (Fig. 4.5 A and B.) They were transferred to the seminiferous tubules of a WT recipient as previously described. Thirty days after the transfer EGFP positive tubules were observed in recipient testis (Fig 4.5 C and D). EGFP colonization was observed in 100% of the recipient testis indicating that the embryonic testis contains spermatogonial

stem cells. Experimental details are summarized in Table 4.2. One male from each experiment was mated to a WT female three months after the transplantation to determine if complete spermatogenesis had occurred by producing offspring that were positive for the EGFP transgene. Males not being tested for fertility, had their tubules enzymatically digested and genomic DNA extracted and analyzed for the presence of the transgene by PCR (Fig. 4.5 E). A 200bp band was present in tubules isolated from animals receiving embryonic germ cells.

However, evaluating three litters of pups produced by each male revealed that only WT rats, no EGFP positive offspring were obtained. Previous studies have

shown that 60 % of busulfan treated rats are able to regain their fertility by three months post treatment (Ryu et al., 2003). This suggests that no EGFP positive pups were obtained because not enough seminiferous tubules were initially colonized with donor cells.

**Table 4.2. E19.5 Embryonic Germ Cells Transplantations to Recipient Testis.**

| Donor Cells | Recipient Animals (No.) | Cells Transferred (No. X $10^4$) | EGFP Tubules (No.) |
|---|---|---|---|
| E19.5 | | | |
| Embryonic | 5 | 7.7 | 5+ ** |
| Gonads | 10 | 24.8 | 10+ ** |
| | 7 | 11 | 7+ ** |

** Represents number of colonies observed in the testis with the tunica in place. Since the tubules of some of the animals were not disaggregated since they were kept alive for fertility studies, this number may be an underestimate of the colonization efficiency.

*Transfer of GCS-EGFP SP bone marrow cells to recipient testes*

The colonization assay described was used to assess the plasticity of adult bone marrow SP stem cells to become germ cells. Purified populations of GCS-EGFP SP cells did not express EGFP (Fig. 4.6 A and B) and were transferred to 43 recipient males and the recipient testes were examined for EGFP fluorescence from between one to six months following transfer (Table 4.3).

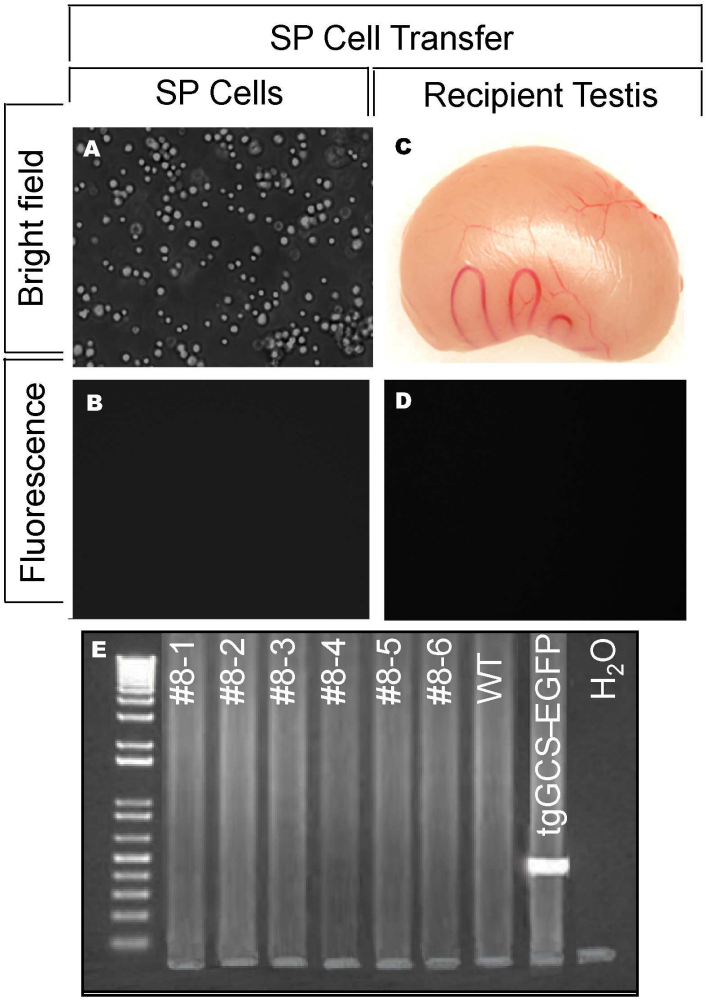


**Figure 4.6. SP bone marrow cells from GCS-EGFP rats fail to colonize the seminiferous tubules of wild-type rats depleted of endogenous germ cells.** (A) Isolated SP bone marrow cells from a homozygous 2 month old male GCS-EGFP rat. (B) Fluorescent photomicrograph of cells in (A), the SP cells do not express EGFP. (C) Recipient testis 60 days post-transfer. (D) Fluorescent photomicrograph of specimens shown in (C) showing no evidence of EGFP in any of the tubules. (E) PCR analysis of one SP donor cell transfer experiment. Testes were collected from recipient animals, tubules isolated, digested, extracted, and PCR performed on DNA preparations with EGFP primers. The numbers 8-1 to 8-6 refers to recipient animal numbers, WT refers to wild-type testis.

After six months, the testes were removed and the seminiferous tubules were dissociated and examined for EGFP fluorescence. There was no evidence of EGFP expression (Fig. 4.6 C and D). The tubules from these animals were then

enzymatically digested and genomic DNA extracted and analyzed for the presence of the transgene by PCR. A 500bp amplified band representing EGFP was present in the tubules isolated from animals receiving seminiferous tubule cells, but an EGFP signal was not evident in the tubule preparation from the 6 animals that received the SP cells (Fig. 4.6 E).

**Table 4.3. Seminiferous tubule cell and bone marrow stem cell transplantations into recipient testis.**

| Donor Cells | Recipient Animals (No.) | Cells Transferred (No. X $10^{3)}$ | EGFP Tubules (No.) |
|---|---|---|---|
| SP Cells | 2 | 22 | 0 |
| | 3 | 50 | 0 |
| | 4 | 25 | 0 |
| | 6 | 100 | 0 |
| | 3 | 50 | 0 |
| | 4 | 50 | 0 |
| | 7 | 50 | 0 |
| | 6 | 50 | 0 |
| | 8 | 50 | 0 |

*Derivation of Rat ES Cells*

The derivation of ES cells lines from rat blastocyst-stage embryos was attempted using SCIM medium on irradiated feeder layers (Fig. 4.7 A). Delayed embryos were used in some of these experiments because in the mouse it has been shown to improve the chances of successfully deriving ES cell lines (Hunter, 1999). In primary culture, the ICM was picked off the plate after 4 days and disaggregated into small clumps of 10-50 cells that were seeded onto fresh feeder layers. A population of robust EGFP positive cells was detected in the ICM and primary colonies (Fig 4.7 D and E). Cells were maintained in culture for up to 20

days and undergoing at least six passages. The colony's morphology that was picked for passaging was compact and usually flat (Fig. 4.7 A-C and G-J). Table 4.4 summarizes the expansion of the ICM outgrowths and Table 4.5 summarizes the variations and outcomes for each experiment.

**Table 4.4. Summary of rat ES cell derivation: Expansion of ICM outgrowths.**

| Expt. Name | No. Rats | No. Blastocysts | No. ICM Outgrowths | No. 1° Colonies | No. 2° Colonies |
|---|---|---|---|---|---|
| JC-3 | 1 | 7 | 7 | 36 | 53 |
| JC-4 | 1 | 9 | 7 | 40 | 60 |
| JC-5 | 1 | 14 | 13 | 62 | 36 |
| JC-6 | 1 | 9 | 7 | 30 | 0 |
| JC-7 | 3 | 13 | 12 | 22 | 0 |
| GCS-1 | 1 | 6 | 6 | 20 | 0 |
| GCS-2 | 1 | 11 | 11 | 21 | 0 |
| GCS-3 | 2 | 6 | 6 | 37 | 91 |

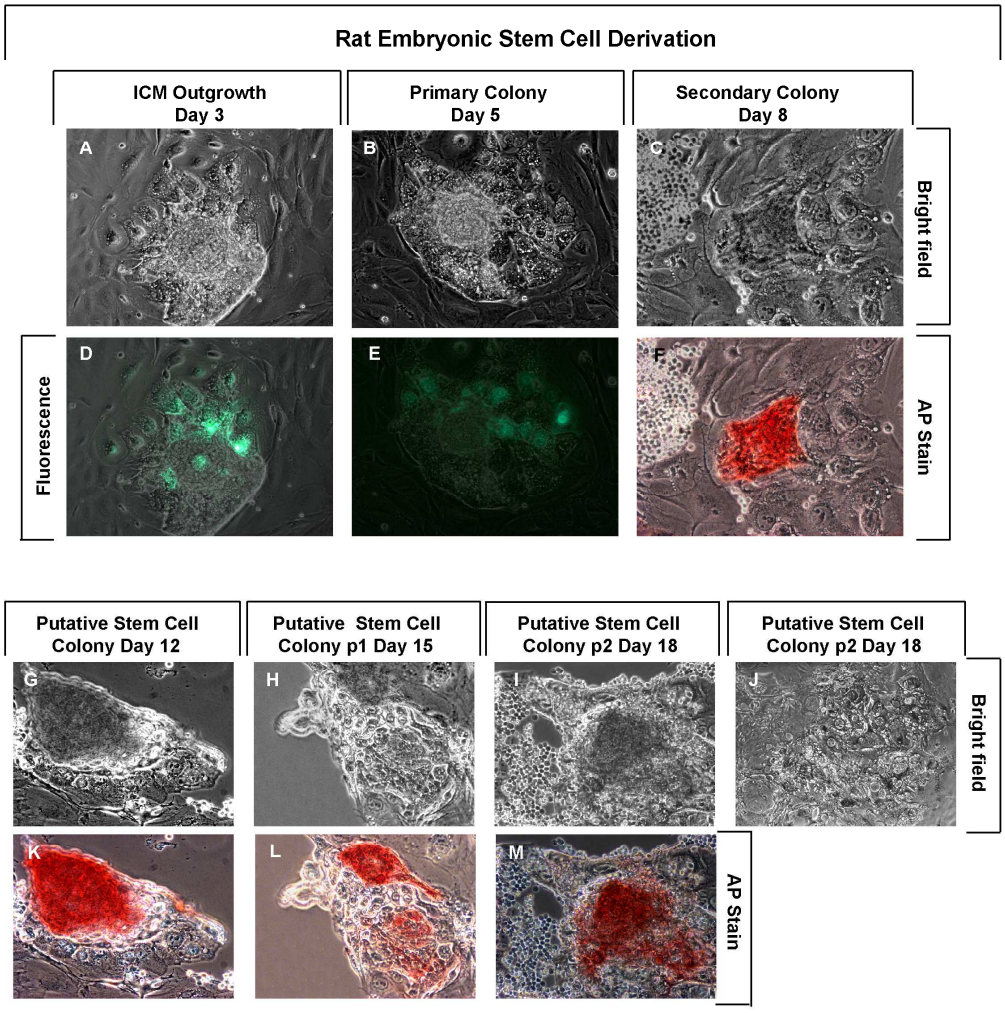


**Figure 4.7. GCS-EGFP rat ES cell derivation.** Outgrowths of inner cell mass from delayed blastocysts cultured onto primary rat embryonic fibroblasts. (A-C and G-J) Bright field DIC photomicrographs of ICM cultures depicting the morphological changes that occur in subsequent passages. (D-E) Fluorescent photomicrographs of (A and B) only a subset of cells are positive for EGFP. (F and K-L) Endogenous alkaline phosphatase activity of the colonies shown in the bright field photomicrographs indicating that the colonies retain some pluripotency.

**Table 4.5. Summary of rat ES cell derivation: Experimental variations and outcomes.**

| Expt. Name | Blastocyst Age* | Immuno-surgery | Feeder Layer** | Days in Culture | No. of Passages | Media Variations*** | Comments |
|---|---|---|---|---|---|---|---|
| JC-3 | Delayed E6.5 | Yes | REF | 12 | 5 | Human LIF + 15ng/ml bFGF | All colonies differentiate into trophectoderm (TE) |
| JC-4 | Delayed E7.5 | Yes | REF | 12 | 5 | 50% BRL-CM Human LIF | All colonies differentiate into giant cells |
| JC-5 | Delayed E.7.5 | No | BMP4 cos cells | 10 | 3 | 50% BRL-CM Human LIF | All colonies differentiate into giant cells or TE |
| JC-6 | Delayed E8.5 | No | BMP4 cos cells | 11 | 3 | 50% BRL-CM Human LIF | All cells differentiated into giant cells or TE |
| JC-7 | E4.5 | No | REF | 7 | 2 | 50% BRL-CM Human LIF | All cells differentiated into giant cells endoderm |
| GCS-1 | E4.5 | No | REF | 8 | 1 | Rat LIF | Colonies disaggregate to less than 10 cells per aggregate all colonies differentiated |
| GCS-2 | E4.5 | No | REF | 8 | 2 | Rat LIF | Large flat colonies surrounded by endoderm |
| GCS-3 | Delayed E8.5 | No | REF | 26 | 7 | Rat LIF | At passage 7 transferred aggregates to 35mm dish colonies differentiated into endoderm or did not grow |

* Blastocyst Age - embryos collected after E4.5 were treated with Tamoxifen and Depo-Provera to delay implantation

**Feeder Layer Cells - REF-Rat Embryonic Fibroblasts
BMP4 Cos Cells - Stable Cell Lines expressing BMP4 which excrete BMP4 into medium

***Media Variations - All Cultures were grown in SCIM (Stem Cell Isolation Medium) supplemented with Either 200units/ml of Human LIF or 1000units/ml of Rat LIF or conditioned media containing Rat LIF secreted from Buffalo Rat Liver cells.

Colonies showed a steady proliferation following disaggregation of the ICM outgrowths and 1° colonies however; differentiated cells always surrounded these colonies. AP staining for these compact colonies revealed high levels of AP activity (Fig. 4.7 E and K-M); suggesting that the colonies remained pluripotent. The expansion and proliferation of cells dramatically decreased following passage

of 2° colonies. Most colonies became very flat and differentiated. Unfortunately, no colonies of undifferentiated compact cells were present after in culture after 26 days.

**Discussion**

It has previously been shown that matrix-selected male germline stem cells isolated from the GCS-EGFP rats were capable of forming functional spermatozoa when transferred to WT recipient testes (Hamra et al., 2004; Hamra et al., 2002). Here, I demonstrate that a non-selected, crude preparation of adult seminiferous tubule cells contain a sufficient number of germline stem cells to efficiently colonize a recipient testis within 30 days following transfer and the extent of colonization doubles with an additional 30 days. The surprisingly efficient colonization of a recipient testis using only a crude preparation of cells suggests that adult male germline stem cells are remarkably effective at reconstituting the germ cell compartment. Given that all of the previous estimates of the colonization potential of a population of non-selected tubule cells have relied on the use of β-galactosidase marked donor cells, these studies suggest that the colonization potential was likely underestimated due to the necessity of performing histochemical staining on fixed tissue. The use of donor stem populations with robust expression of a vital marker, such as EGFP, will facilitate

the accurate assessment of colonization potential and allow ease of manipulation of such a population of cells while maintaining viability.

I used the colonization assay to determine when developing germ cells first give rise to spermatogonial stem cells since at the time these experiments were done it was unclear. Male germ cells are evident as PGCs located in the epiblast of the rat embryo on E8.5 and they proliferate and migrate to the genital ridges from E9.0-E13.5. By E15.5, the male germ cells become sex determined and enter a state of quiescence. The embryonic gonads have formed by E18.5 and resumption of the cell cycle or spermatogenesis begins shortly after birth (Wylie, 2002). From this study, I demonstrated that spermatogonial stem cells are present in the E19.5 embryonic gonad but not in the early genital ridge. At the time I was conducting these studies, a paper was published that examined this question in mice. The group found that germ cells from the early genital ridge were unable to colonize a recipient testis but germ cells isolated from the genital ridge after sex determination and from the embryonic gonads were able to colonize the recipient testis (Ohta et al., 2004). These observations are in direct support of my data in rats. PGC's upon arrival at the genital ridges are still undergoing proliferation and also demethylation to erase parental imprints (Hajkova et al., 2002), however, imprinting is reestablished after sex determination (Ueda et al., 2000). Suggesting that PGCs may not have the ability to differentiate into spermatogonial stem cells until imprinting has occurred.

Although embryonic gonadal cells were able to colonize the recipient testis, the formation of colonies was not as robust as when adult germ cells were used in the transplantation assay. When adult germ cells are transferred, 60% of the recipient males are able to produce progeny from the transplanted cells (Brinster and Zimmermann, 1994). However, no progeny were obtained from fetal germ cells, suggesting that the number of spermatogonial stem cells present in the embryo is relatively low. In the mouse study, progeny from the transplanted cells were also not obtained through natural mating. They were able to obtained progeny but only through *in vitro* fertilization using the intracytoplasmic sperm injection technique (Ohta et al., 2004).

One interesting observation in my studies was the formation of tumors in recipient testes transplanted with PGC's from the early genital ridge. Teratocarcinomas can be induced with a high frequency in mice by transplanting embryonic stem cells under the testicular capsule of an adult host (Stevens, 1970). The formation of tumors with the PGC's suggests that the cells retain some pluripotentency and have not yet been fully committed to the germ cell lineage. Although, analysis of these tumors did not indicate that any of the cells had differentiated into different cell types this may be due to the time at which the testes were analyzed. Recipient testes were analyzed at 1-3 months after transplantation and histological analysis was done when the tumor was first observed so the tumor cells may not have had enough time to differentiate.

Finally, I used the colonization assay to examine the plasticity of bone marrow SP to become germ cells. I transferred the SP cells directly into the seminiferous tubules and allowed the cells to colonize the testis for a period of 1-6 months. However, there was no evidence of EGFP expression in tubules isolated from all 43 recipients. Recent studies have shown that in most cases where SP cell differentiation has occurred, the SP cells were first transferred to the bone marrow and allowed to repopulate the hematopoietic lineage of the recipient suggesting that their fate is restricted (Bonnet, 2002; Goodell et al., 2001; Herzog et al., 2003). It has been suggested that an increased ability of SP cells to form a different lineage in tissues occurs when the end organ is first subjected to some degree of damage prior to transfer (Alvarez-Dolado et al., 2003; Camargo et al., 2003; Hirschi and Goodell, 2002; Moore and Quesenberry, 2003). In these experiments, tissues were only partially damaged, while, in my assay the recipient testis was extensively depleted of germ cells. I may have caused too much damage to the germ cell compartment and thus the SP stem cells are not able to colonize the testis without the presence of sufficient residual testis stem cells. Another possibility is that the SP cells are recruited to the damaged tissue and then fuse with an existing cell in that tissue. Upon fusion, the SP cell is reprogrammed to become a different cell type (Alvarez-Dolado et al., 2003; Moore and Quesenberry, 2003; Prockop, 2003; Rudnicki, 2003; Wang et al., 2003). This later theory could explain why no colonization was detected since the

recipient testis was treated with busulfan, very few endogenous germ stem cells would have survived, and thus the probability of the SP cells fusing with germ cells would be very rare. Finally, it has been shown by transplantation assay that donor cell colonization and stem cell expansion is dependent on the ability of the donor cells to migrate within the tubules to the basement membrane and form cellular associations with the recipient Sertoli cells that support the donor cells differentiation (Nagano and Brinster, 1998). It is possible that the SP cells we transferred are not responsive to signals in the seminiferous tubule environment that would promote their migration to the basement membrane and association with Sertoli cells. Therefore, differentiation and colonization of SP cells in the testis would be unlikely.

I used the GCS-EGFP transgenic line as a source of blastocysts to derive a rat ES cell line. Rat blastocyst have been shown to differentiate when cultured on specific feeder layers (Ouhibi et al., 1995). However, the blastocysts in these studies could not be maintained in culture in an undifferentiated state. There has only been one ES "like" ratline published. This line was shown to remain in culture for at least 16 passages and exhibited expression of pluripotent markers such as high alkaline phosphatase activity and SSEA-1(Vassilieva et al., 2000). However, this line has not been shown to produce chimeras, a crucial test for pluripotency, or shown to differentiate to a specific cell type. Therefore, I attempted to derive a line that would be able to produce chimeras and lead to

expanding gene targeting techniques to the rat. The initial derivation attempts were done following a protocol that is commonly used to derive mouse ES cells with variations in the disaggregation of the colonies and the supplements added to the medium (Robertson, 1987a). However, stable rat stem cell lines were not derived. In my studies, cultures of alkaline phosphatase positive colonies were maintained for a maximum of 26 days /seven passages before all colonies had differentiated or no longer were alkaline phosphatase positive. Mouse ES cell colonies are disaggregated to single cells for each passage (Downing and Battey, 2004), however, rat blastocysts cultures if disaggregated to single cells will no longer form compact colonies but form differentiated cell types such as endoderm. Several methods of disaggregation were tried; however, it became apparent regardless of the method, that the aggregates must be at least 10 cells for proliferation to occur. Recently, another group reported the derivation of undifferentiated colonies from rat blastocysts that were unable differentiate to any of the germ layer of cells due to a loss of Oct4 (Buehr et al., 2003). They found that although the colonies were positive for high alkaline phosphatase activity, the amount of Oct4 varied in the different lines and that as time passed in culture the Oct4 expression was lost. Oct4, a POU family, member is necessary for pluripotency in mouse ES cells. The loss of Oct4 leads to the rapid differentiation to trophectoderm (Niwa et al., 2000; Pesce and Scholer, 2000). Vassilleva et al. also reported varying levels of Oct4 between the different lines. The loss of Oct4

in rat ICM or blastocyst cultures will make the derivation of stable rat ES cell lines virtually impossible. Future studies to generate rat ES cell lines must focus on finding culture conditions that maintain Oct4 in culture. In conclusion, I have demonstrated that unique germ cell specific expression pattern of this transgene makes this rat line a very powerful tool that can be used to address important questions in the germ cell field.

**Future Directions**

The GCS-EGFP rat line can be used to identify the roles of novel genes and known genes in the delineation of the germline during development. Since EGFP is a vital marker, FACS can isolate germ cells at all stages of embryonic development and the RNA can be analyzed using microarrays to develop markers for distinct stages of germ cell development. Additionally, these genes could be studied to determine their function during germ cell development and may eventually lead to an understanding of the signaling pathways involved in this complex process. Currently PGCs in the mouse are first identified at E7.5 by high alkaline phosphatase activity; earlier identification is not possible because AP activity levels are high in many cells in the embryo at earlier time points (McLaren, 2000). The GCS-EGFP rat may allow the identification of markers of the germ lineage at the earliest embryonic stages.

Finally, there have not been any reports of the generation of teratocarcinomas from rat epiblasts or genital ridges. However, in the mouse, teratocarcinomas are routinely seen when these cells are transplanted under the tunica of the testis (Buehr et al., 2003). In my studies, transplantation of the genital ridge cells to the testis formed undifferentiated tumors. Therefore, it would be interesting to see if they are able to form teratocarcinomas. The formation of teratocarcinomas would be an indicator that the PGCs at the early genital ridge retain pluripotentency. The PGCs could then be cultured to form embryonic germ cell (EG) lines. EG lines have been derived from migrating PGCs and PGCs in the early genital ridge in the mouse. These lines are able to produce chimeras and differentiate to all three germ lines *in vitro* (Durcova-Hills et al., 2001; Durcova-Hills et al., 2003; Resnick et al., 1992; Richards et al., 1999). If stable EG lines in the rat are derived, the need for ES lines for genetic manipulation techniques may be eliminated since EG cells are pluripotent. Recently, pluripotent stem cells were derived from the neonatal mouse testis (Kanatsu-Shinohara et al., 2004). Derivation of embryonic germ cell lines from neonatal mouse testes were previously described by this group (Kanatsu-Shinohara et al., 2003); and they found that culturing these cells without bFGF and $Sl^4$-m220 gave rise to colonies that were indistinguishable from mouse ES cells. They were also able to show that these cells had the same properties as ES cells in that they formed teratocarcinomas and could produce chimeras. Long-

term culture of neonatal rat testis cells is routinely done in the Garbers laboratory (Hamra et al., 2004). Therefore, it would be of great interest to see if ES cells in the rat can be derived from neonatal testes cultures since the derivation of ES cell lines from the rat blastocyst have remained elusive.

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# VITAE

Jennifer Tran Croy Cronkhite was born in Vietnam, on October 28, 1974, and brought to the USA by her adoptive parents, Susan and Michael Croy, in April 1975. After many moves throughout Pennsylvania and Florida, she graduated in Florida, from Winter Park High School in 1993. She then went on to earn a Bachelor's of Science Degree in Molecular and Microbiology in 1997 from the University of Central Florida. She then moved to Dallas, Texas where she worked in Dr. Muallem's laboratory at the University of Texas Southwestern Medical Center in Dallas, as a research technician. Then in August 1998, she entered the Ph. D. program in the Division of Cellular and Molecular Biology at the University of Texas Southwestern Medical Center. In 1999, she joined the Integrative Biology program and began her doctoral research under the joint guidance of Robert Hammer Ph. D. and David Garbers Ph. D. In 2002, she married her long time love, Christopher Cronkhite.

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