Cloning, Stem Cells and Epigenetic Reprogramming After Nuclear Transfer

by

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B.S., Microbiology
University of Illinois at Urbana-Champaign, 1996

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Biology

at the
Massachusetts Institute of Technology

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Abstract

The process by which a single totipotent cell becomes a complex organism is a unidirectional program, with each mitotic division generating new cells that gradually differentiate towards more specified fates and specialized functions. Nuclear transfer (NT) experiments have demonstrated the epigenetic nature of development and showed, that although differentiated cells have a very limited developmental potential, the nuclei of these cells retain the potency to direct embryogenesis after reintroduction into the unfertilized oocyte. Herein, we have used the mouse as a model system for understanding both the nature of epigenetic reprogramming that occurs after NT as well as the ramifications it has for the development of cloned animals. Specifically, we investigated how epigenetic states are reprogrammed after NT and demonstrated that the inactive X chromosome is reactivated in NT embryos, resulting in normal X inactivation in female clones. Additionally, investigations into the factors that influence the survival of cloned animals, indicate that there are considerable genetic influences on the cloning process. These genetic factors modify the survival of mice cloned from ES cells by influencing the developmental potential of the donor ES cells rather then the reprogramming process itself. This realization has subsequently led to the development of novel methods for the expedited production of complex mutant mice, which are also described. Finally, we have created cloned embryos by NT from both cortical and mature olfactory sensory neurons to address question of nuclear equivalence in the brain and to investigate whether generation of synaptic diversity or odorant receptor choice, are mediated by genetic as well as epigenetic events.

Thesis Supervisor: Rudolf Jaenisch
Title: Professor of Biology
Dedication

I would like to dedicate this thesis to Phillip Morrison and to the memory of Phylis Morrison. Of all of the people that I have known while in graduate school, they perhaps taught me the most about growing, thinking, learning and living.
Acknowledgements

First and foremost, I would like to thank Rudolf Jaenisch for welcoming me into his laboratory and allowing me to take on this rather risky thesis project. Rudolf provided a great deal of patient support over many long months when it was very unclear as to whether these experiments would be a success. Perhaps most importantly, I would like to thank Rudolf for being a true mentor both scientifically and professionally. My time in Rudolf’s lab will be an experience I will never forget and always treasure.

These experiments simply would not have been possible without the help of everyone in the Jaenisch lab, where I have made a number of good friends. I would like to thank Britt Rideout and Laurie Jackson-Grusby who taught me most of what I now know about mouse genetics and epigenetics. Britt and Laurie and were also always around to listen to my latest crazy idea for an experiment. David Humphereys and Konrad Hchedlinger made up, with Britt, the core of the cloning group while I was in Rudolf’s lab and were always helpful with ideas and collaborations. Maribel Rios, Caroline Beard, Sandra Luikenhaus, Joost Gribau, Sarah Cherry and all the other members of the Jaenisch lab past and present made the lab a fantastic place to work, live and grow.

The fourth floor of the Whitehead Institute has been one of the most exciting and scintillating places I can imagine working. I would especially like to acknowledge Alex Bortvin, Sasha Ebrahimi and Michael Tackett for being great collaborators. Also, Elizabeth Wiellette and Vince Troppe who helped make the floor a great place to work. Also special thanks to Elizabeth for proofreading the unpublished portions of this thesis.

I would like to thank my thesis committee: Tyler Jacks, David Page and Bob Weinberg for their support and earnest help in guiding these experiments and my career. Tyler, David and Bob were often the Ying to Rudolf’s Yang.

Lastly, I would like to thank my family and friends for endless patience and support. Without them, I never would have made it.
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Chapter 1: Introduction

Nuclear Reprogramming: Biological and Technological Constraints

The realization of mammalian cloning by somatic cell nuclear transfer has raised considerable interest in the mechanisms by which the mammalian oocyte returns the gene expression profile of a somatic cell to one that is appropriate and necessary for the development of a single cell zygote. Investigations into the importance, nature and efficiency of this process, dubbed “reprogramming”, as well as its influence on the development of cloned animals, are in their earliest stages. In this chapter, I summarize our current understanding of the reprogramming process, as it occurs after nuclear transfer. Furthermore, I will attempt to build a framework for understanding how both the technological parameters of the cloning process as well as the biological limitation of nuclear reprogramming, might relate to the inefficiencies and phenotypes observed during the development of cloned animals.

Nuclear transfer technology and its influence on the development of cloned mammals.

Viable sheep (Campbell et al., 1996), cows (Cibelli et al., 1998), pigs (Onishi et al., 2000)(Polejaeva et al., 2000), goats (Baguisi et al., 1999) and mice (Wakayama et al., 1998) have all been produced through the direct introduction of somatic nuclei into enucleated oocytes (Figure 1.1). Unfortunately, the universally low efficiency of nuclear transfer experiments has made it difficult to dissociate technical difficulties from true biological phenomena in cloning research. However, as nuclear transfer technology becomes more standardized and as research focus shifts from whether cloning is possible to which parameters influence its success, a glimpse of the variables that determine a successful cloning outcome has begun to emerge. In this chapter
Figure 1.1: Mammalian cloning by nuclear transfer

**Normal development**
- sperm (1n)
- fertilization
- zygote (2n)
- blastocyst
- adult mouse

**Cloning**
- oocyte (1n)
- adult cell (2n)
- enucleated oocyte
- NT and artificial oocyte activation
- reconstructed embryo (2n)
- blastocyst
- cloned mouse

**Figure 1.1.** The mouse as a model for mammalian cloning by nuclear transfer. During normal development, the two haploid genomes contained within the gametes unite at fertilization giving rise to a diploid zygote. After fertilization in the oviduct, the developing cleavage stage embryo travels to the uterus where it implants as a blastocyst. Just more then two weeks after implantation the newborn mouse is born and then develops to adulthood. In cloning, a somatic cell is removed from the adult mouse and its nucleus is transplanted into an oocyte which has had its genetic material removed (enucleated). After NT, the oocyte is artificially activated and the cloned embryo begins to develop. After in vitro culture to the blastocyst stage, the embryo is transferred to the oviduct or uterus of a suitable recipient mother, where it will occasionally develop to term.
I begin by focusing in on four key parameters of nuclear transfer technology that influence the development of cloned embryos; *(i)* the state and nature of the recipient cytoplasm, *(ii)* the cell cycle status of the donor cell, *(iii)* the identity, differentiation state and developmental potency of the donor cell and finally, *(iv)* genetic influences on the successful development of cloned embryos. After discussing these technological constraints, I will summarize some aspects of epigenetic reprogramming after nuclear transfer.

**The recipient oocyte and its cytoplasm.** A robust, reproducible method for nuclear transfer was first pioneered in the mouse embryo (McGrath and Solter, 1983). With this technique, a zygotic pronucleus or embryonic nucleus was transferred as a karyoplast via Sendai virus-mediated membrane fusion into another enucleated zygote. Zygotes reconstituted in this way, with the pronucleus of another zygote, were competent to develop to the blastocyst stage with high efficiency and on to birth after embryo transfer (McGrath and Solter, 1983). However, when the same nuclear transfer methodology was used to introduce nuclei from either cleavage stage blastomeres or the inner cell mass (ICM) into zygotes, the cloned embryo rarely completed more than a few cell divisions (Table 1.1) (McGrath and Solter, 1984).

Conceptually similar methods utilizing electrically mediated membrane fusion have been used to introduce either embryonic or adult ovine cells, into unactivated, Metaphase II oocytes. In these experiments, the preimplantation embryos developed to the blastocyst stage with a reasonable efficiency and cloned sheep were produced (Wells et al., 1997)(Wilmut et al., 1997). Nuclear transfer of both embryonic and adult cells into MII oocytes has also been used to produce cloned cattle (Table 1.1) (Sims and First, 1994)(Wells et al., 1999)(Kato et al., 1998). The positive outcome of these efforts relative to earlier attempts in the mouse seems to lie in the nature of the recipient cytoplasm, suggesting that some property of the MII oocyte is critical for cloning success.

The most thorough analysis of the nature of the recipient cytoplasm and the development
Table 1.1

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Species</th>
<th>Efficiency of <em>in vitro</em> development to morulae/blastocyst (%)</th>
<th>Developmental potency after embryo transfer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zygote</td>
<td>Mouse</td>
<td>0</td>
<td>None</td>
<td>(McGrath and Solter, 1984) (Wakayama et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Cow</td>
<td>0</td>
<td>None</td>
<td>(Tani et al., 2001)</td>
</tr>
<tr>
<td>MII oocyte then serial transfer to zygote</td>
<td>Mouse</td>
<td>29%</td>
<td>Adult</td>
<td>(Ono et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>N/A</td>
<td>Adult</td>
<td>(Polejaeva et al., 2000)</td>
</tr>
<tr>
<td>Oocyte MII</td>
<td>Mouse</td>
<td>70%</td>
<td>Adult</td>
<td>(Wakayama et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Cow</td>
<td>65%</td>
<td>Adult</td>
<td>(Wells et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>20-40%</td>
<td>Adult</td>
<td>(Wilmut et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>30%</td>
<td>Adult</td>
<td>(Onishi et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td>N/A</td>
<td>Adult</td>
<td>(Baguisi et al., 1999)</td>
</tr>
<tr>
<td>Activated Oocyte, (Anaphase or Telophase)</td>
<td>Mouse</td>
<td>6%</td>
<td>None</td>
<td>(Wakayama et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>12%</td>
<td>Adult</td>
<td>(Campbell et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Goat</td>
<td>N/A</td>
<td>Adult</td>
<td>(Baguisi et al., 1999)</td>
</tr>
</tbody>
</table>
of cloned embryos examined the effects of cumulus cell NT into murine MII oocytes, activated telophase oocytes and zygotes (Wakayama et al., 2000). These experiments, utilizing a direct injection method of nuclear transfer (Wakayama et al., 1998), demonstrated that the efficiency of in vitro embryonic development dropped off markedly as the oocyte cytoplasm transitioned from meiosis to G1 of the first cell cycle (Table 1.1). Nuclear transfer into MII oocytes followed by either immediate or delayed activation resulted in efficient preimplantation development and full term development. Preactivated oocytes, however, developed in vitro with a very low efficiency after NT and cloned mice could not be produced. As observed previously (McGrath and Solter, 1984), nuclear transfer into zygotes resulted in very early cleavage failure of the cloned embryo. When the chromosomes of the embryos generated by NT into enucleated zygotes were analyzed, all contained gross karyotypic abnormalities. In contrast, 70% of embryos produced by NT into enucleated MII oocytes possessed an intact diploid complement of chromosomes. These experiments suggest the metaphase environment is somehow critical for preparing the somatic chromosomes for preimplantation development after NT.

In the MII oocyte, MPF (Metaphase/Maturation Promoting Factor) levels are high (reviewed by Fulka et al., 1996). After fertilization or artificial oocyte activation, MPF levels decrease allowing the embryo to complete meiosis and commence development. High MPF levels in the oocyte cytoplasm lead to somatic cell nuclear envelope breakdown and premature chromosome condensation (Wakayama et al., 1998). These events do not occur after NT into enucleated zygotes (Wakayama et al., 2000). It therefore seems likely that either the physical condensation of the chromosomes, induced cell cycle synchronization of the donor nucleus and oocyte cytoplasm, or some other consequence of high MPF levels must be responsible for protecting the somatic DNA from damage after NT. Whether cytoplasm rich in MPF has intrinsic properties, necessary for developmental reprogramming, other than those bestowing protection from DNA damage, has yet to be determined. Interestingly, cloned goats (Baguisi et al., 1999) and sheep (Campbell et al., 1996) have been generated by introducing somatic nuclei into
activated oocytes (Table 1.1), indicating that exposing the donor nucleus to cytoplasm rich in MPF may not be strictly required for the successful development of cloned animals. Further investigation will be required to determine whether this discrepancy reflects interesting species specific differences in nuclear remodeling after NT or simply variation in MPF half-life following oocyte activation.

**Cell cycle status of the donor cell.** Comparative analysis of cloning experiments with a variety of murine cells has begun to demonstrate a clear correlation between the efficiency of NT embryo development to the blastocyst stage and the proportion of donor cells in the G1 phase of the cell cycle (Table 1.2). When sertoli (Ogura et al., 2000), cumulus (Wakayama et al., 1998), and serum starved fibroblast cells (Wakayama and Yanagimachi, 1999), all primarily in a G1 (2N) state, were used for NT, a majority of activated embryos developed to the blastocyst stage. In contrast, when rapidly cycling embryonic stem (ES) cells were used as nuclear donors (Wakayama et al., 1999)(Rideout et al., 2000)(Eggan et al., 2001), only a small percentage of NT embryos completed cleavage development. As most ES cells in a given population are in S phase (unpublished observation), this may be the cause of their poor *in vitro* development after nuclear transfer. Consistent with this interpretation, methods that force a higher proportion of ES cells into the G1 phase of the cell cycle, such as partial serum withdrawal, also increase their *in vitro* developmental potential after NT (Wakayama et al., 1999) (Table 1.2).

Presumably, the importance of the donor nucleus cell cycle state is directly linked to its compatibility with the oocyte cytoplasm. As mentioned above, high MPF levels in the oocyte cause donor nuclear envelope breakdown and premature chromosome condensation. Chromatin structure in the S- phase of the cell cycle may be incompatible with this condensation leading to DNA damage and zygotic arrest. In contrast, G1 and G2/M phase chromatin seem to be compatible with this condensation.

In sheep (Wilmut et al., 1997) and cattle (Cibelli et al., 1998), nuclear transfer procedures
Table 1.2

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell Cycle Stage</th>
<th>Reconstructed embryos developing to blastocyst stage(%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulus</td>
<td>G1,G0</td>
<td>70%</td>
<td>(Wakayama et al., 1998)</td>
</tr>
<tr>
<td>Sertoli</td>
<td></td>
<td>65%</td>
<td>(Ogura et al., 2000)</td>
</tr>
<tr>
<td>Fibroblast</td>
<td></td>
<td>58%</td>
<td>(Wakayama and Yanagimachi, 1999)</td>
</tr>
<tr>
<td>ES cells</td>
<td>(Log) High serum</td>
<td>10-20%</td>
<td>(Wakayama et al., 1999)(Rideout et al., 2000)(Eggan et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Small(G1?) low serum</td>
<td>55%</td>
<td>(Wakayama et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Large cell-nocodozol (G2-M) high serum</td>
<td>43%</td>
<td>(Wakayama et al., 1999)</td>
</tr>
</tbody>
</table>

16
often utilize simultaneous fusion and activation, preventing extrusion of somatic chromosomes into a polar body. In the mouse (Wakayama et al., 1998), delayed activation combined with chemicals preventing polar body extrusion ensure that the entire chromosomal content of the G1 (2N) donor cell remains in the oocyte. Further evidence that the numeral chromosome content of the donor cell is critical, rather than a particular cell cycle state per se, also comes from experiments using ES cells as nuclear donors (Wakayama et al., 1999). In these studies, ES cell nuclei were arrested in mitosis by nocodazole treatment and transferred into MII oocytes. Several hours after NT, a functional metaphase spindle was observed in a subset of the reconstructed embryos. However, complete retention of all DNA from a G2/M phase would create a tetraploid (4N) embryo, a chromosome content incompatible with full-term development (Kaufman and Webb, 1990). To prevent a tetraploid state, oocyte activation was performed in the absence of cytoskeleton depolymerizing agents. After activation, part of the, presumably 4N donor cell chromosomal complement was shown to be extruded into the second polar body. Similarly reconstructed and activated embryos developed to the blastocyst stage and to term with a high efficiency.

In conclusion, a G0 cell cycle state is clearly not necessary for the successful development of cloned embryos as once suggested (Wilmut et al., 1997). However, the somatic nucleus must be in a conformation compatible with nuclear envelope break down and chromosome condensation. In addition, careful consideration of the donor cell cycle state, combined with methods for ensuring correct numeral chromosome content after activation, such as inclusion or exclusion of drugs preventing polar body extrusion, are critical.

The inherent developmental potential of the donor cell. Comparing the efficiency of nuclear transfer experiments using both somatic and embryonic cells lends support to the hypothesis that intrinsic developmental potency of the donor cell plays a role in the cloning outcome (Table 1.3). When mouse cumulus (Wakayama et al., 1998) and tail-tip cells (Wakayama
<table>
<thead>
<tr>
<th>Species</th>
<th>Cell type</th>
<th>Morulae/ Blastocysts developing to term (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Cumulus</td>
<td>2-3%</td>
<td>(Wakayama et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Sertoli</td>
<td>3.5%</td>
<td>(Ogura et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Fibroblast</td>
<td>1%</td>
<td>(Wakayama and Yanagimachi, 1999)</td>
</tr>
<tr>
<td></td>
<td>ES cells</td>
<td>5-21%</td>
<td>(Wakayama et al., 1999)(Rideout et al., 2000)(Egkan et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Blastomere</td>
<td>25%</td>
<td>(Tsunoda and Kato, 1997)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Mammary</td>
<td>3.5%</td>
<td>(Wilmut et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Fetal</td>
<td>7.5%</td>
<td>(Wilmut et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Fibroblast</td>
<td></td>
<td>(McCreade et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Embryo cells</td>
<td>4.5%</td>
<td>(Wilmut et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>ICM ES like</td>
<td>4%</td>
<td>(Wells et al., 1997)</td>
</tr>
<tr>
<td>Cow</td>
<td>Cumulus</td>
<td>10%</td>
<td>(Wells et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Oviductal</td>
<td>15%</td>
<td>(Kato et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Fetal</td>
<td>14%</td>
<td>(Cibelli et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Fibroblast</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ICM ES like</td>
<td>12%</td>
<td>(Sims and First, 1994)</td>
</tr>
<tr>
<td>Pig</td>
<td>Granulosa</td>
<td>7%</td>
<td>(Polejaeva et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>(serial NT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fetal fibroblast</td>
<td>1%</td>
<td>(Onishi et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>(Single NT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat</td>
<td>Fetal fibroblast</td>
<td>3.5%</td>
<td>(Baguisi et al., 1999)</td>
</tr>
</tbody>
</table>
and Yanagimachi, 1999) were used as nuclear donors, only 1-3% and 0.5% respectively, of embryos transferred to surrogate mothers developed to term. In contrast, 5-25% of blastocysts generated by nuclear transfer with ES cell (Wakayama et al., 1999)(Rideout et al., 2000)(Eggan et al., 2001) or blastomere (Tsunoda and Kato, 1997) nuclei survived until birth.

This increased developmental efficiency suggests that the ES cell and blastomere genome must be either more amenable to, or require less, epigenetic reprogramming than that of somatic cells. For instance, it would be expected that an ES cell nucleus would already express genes critical for early development such as Oct4 (Nichols et al., 1998). In cumulus cell or tail-tip nuclei, these genes are not expressed and must be reactivated after NT. Furthermore, cumulus cells and tail-tip fibroblasts have chromatin configurations and DNA methylation levels appropriate for expression of genes necessary for maintenance of their differentiated state. Presumably the chromatin and methylation configurations that drive expression of these genes must be reset to an embryonic state in order to ensure that they are not inappropriately expressed either during cleavage or later in development. It may be that maternal stores of embryonic proteins and RNAs present in the oocyte support development of NT embryos derived from differentiated cells through cleavage stages of preimplantation development. Inability to appropriately reprogram gene expression, followed by depletion of these stores, could lead to the frequent developmental failure observed shortly thereafter (Wakayama and Yanagimachi, 2001).

In farm animals such as cattle and sheep, there is no apparent difference in cloning efficiency when using embryonic cells (Wells et al., 1997)(Sims and First, 1994) rather than somatic cells (Wells et al., 1999)(Wilmut et al., 1997) as nuclear donors. However, this observation could reflect that the embryonic cells used for these studies, derived from both cattle and sheep embryos, had decreased intrinsic developmental potential relative murine ES cells. Therefore, the inconsistency between experiments in mouse and cattle/sheep may again result from differences in experimental design rather than reflecting species-specific differences in the reprogramming process.
It remains a subject of debate whether all nuclei have the potency to direct embryogenesis after NT and whether surviving clones are derived from rare somatic stem cells, with increased developmental potential, present at a low frequency in the donor cell population (Liu, 2001). Thus the great inefficiency of cloning might merely reflect the rare nature of those donor cells with the developmental capacity to direct development after NT. However, experiments using differentiated, mature B and T lymphocytes as donors have now demonstrated that even highly differentiated cells can give rise to ES cell lines and then cloned mice after nuclear transfer, albeit at an extremely low efficiency (Hochdelinger and Jaenisch, 2002). At the other extreme, it may be that all somatic nuclei are competent to direct development of cloned offspring after NT. If this is the case, inherent inefficiencies and errors in reprogramming must cause erratic and/or stochastic expression of genes critical for embryogenesis, resulting in developmental failure. Consistent with erratic reprogramming, it has been shown that some bovine NT blastocysts failed to express appropriate levels of FGF4, FGFr2 and IL 6 (Daniels et al., 2000). Furthermore, at certain reptetive sequences, some bovine NT blastocysts were shown to have DNA methylation levels more similar to the donor cell population than to control blastocysts (Kang et al., 2001).

Recently, it has been proposed that tissue specific stem cells in the adult may have greater plasticity then previously believed and that it is possible to isolate these cells for directed experimentation (Clarke, et al., 2000). Although the nature of these cells is still controversial, comparing the efficiency of cloning using adult stem cells and more differentiated cells from the same tissue may shed light on which aspects of cloning are limited by the developmental potential of the donor cell.

**Genetic influences on the cloning process.** The outbred nature of livestock precludes genetic analysis of cloning in cattle, goats, pigs or sheep. In contrast, the mouse is an ideal model for the genetic dissection of nuclear reprogramming. When ES cells from various strains were used as nuclear donors, inbred 129, C57/B6 and F1ES cell lines all gave rise to newborn clones
(Wakayama et al., 1999)(Rideout et al., 2000)(Eggan et al., 2001). Interestingly however, clones from four independent inbred ES cell lines died shortly after birth due to respiratory failure (Wakayama et al., 1999)(Rideout et al., 2000)(Eggan et al., 2001) while most clones derived from five different F1 ES cell lines survived to adulthood (F1, I.E. ES cell lines derived from embryos resulting from the intercross of parents with two independent inbred genetic backgrounds.) (Table 1.4)(Rideout et al., 2000)(Eggan et al., 2001).

Neonatal lethality has also been reported in mice entirely derived from inbred ES cells injected into tetraploid blastocysts (ES cell-tetraploid) (Figure 1.2)(Nagy et al., 1990)(Nagy et al., 1993). Like inbred clones, ES cell-tetraploid pups derived from inbred ES cell lines died shortly after delivery with signs of respiratory distress (Nagy et al., 1990)(Eggan et al., 2001). In contrast, most ES cell-tetraploid neonates derived from six F1 ES cell lines, developed into fertile adults (Eggan et al., 2001)(Table 1.4). These results suggest that the death of inbred ES cell clones is not a direct result of the nuclear transfer procedure itself but instead is due to the limited developmental potential of inbred ES cells. It is possible the decreased developmental potential and respiratory failure observed in inbred ES NT embryos could be due to delayed developmental timing relative to their F1 counterparts. However, experiments demonstrating that NT embryos derived from both F1 (Wakayama et al., 1998) and inbred 129 cumulus cells (Wakayama and Yanagimachi, 2001) survive from birth to adulthood at a high frequency seem to argue against this conclusion. It is therefore reasonable to speculate that inbred ES cells may suffer some ill effect due to long term cell culture, which ultimately leads to the demise of ES cell derived offspring. Experiments suggesting that prolonged in vitro passage of ES cells can further aggravate these phenotypes in ES cell derived offspring lend further support to this hypothesis (Nagy et al., 1993). Interestingly, an F1 genetic background seems to be protective against these detrimental effects as offspring from all F1 genotypes tested survived to adulthood, even at high passage (Eggan et al., 2001)(Eggan et al., 2002). Cloned animals of other species, including sheep, also often display signs of respiratory distress at birth, especially following prolonged
### Table 1.4

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Genetic Background</th>
<th>Survival to Term (%)/</th>
<th>Survival from Term to Adult (%)</th>
<th>References</th>
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<tbody>
<tr>
<td>Cumulus</td>
<td>Various F1</td>
<td>1-2%</td>
<td>85%</td>
<td>(Wakayama et al., 1998) (Wakayama and Yanagimachi, 2001)</td>
</tr>
<tr>
<td></td>
<td>Inbred C/57</td>
<td>0</td>
<td>N/A</td>
<td>(Wakayama and Yanagimachi, 2001)</td>
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<tr>
<td></td>
<td>Inbred 129</td>
<td>1.5%</td>
<td>95%</td>
<td>(Wakayama and Yanagimachi, 2001)</td>
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<tr>
<td>ES cells</td>
<td>Various F1</td>
<td>8-17%</td>
<td>50-85%</td>
<td>(Wakayama et al., 1999)(Rideout et al., 2000)(Eggen et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Inbred C/57</td>
<td>17%</td>
<td>0</td>
<td>(Eggen et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Inbred 129</td>
<td>1-18%</td>
<td>0</td>
<td>(Eggen et al., 2001)</td>
</tr>
<tr>
<td></td>
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<td>1.6%</td>
<td>20% (n=1)</td>
<td>(Wakayama et al., 1999)</td>
</tr>
<tr>
<td>ES cells</td>
<td>Various F1</td>
<td>18%</td>
<td>85%</td>
<td>(Eggen et al., 2001)</td>
</tr>
<tr>
<td>into 4N</td>
<td>Inbred C/57</td>
<td>4%</td>
<td>0</td>
<td>(Eggen et al., 2001)</td>
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<tr>
<td>Blasts</td>
<td>Inbred 129</td>
<td>10%</td>
<td>0</td>
<td>(Eggen et al., 2001)</td>
</tr>
</tbody>
</table>
Figure 1.2: Production of mice from ES cells by tetraploid embryo complementation

| In vitro culture isolated zygotes | Electrofusion Produces one-cell Tetraploid embryo | In vitro culture to blastocyst | Embryonic stem cell microinjection | Embryo transfer | ES cell-derived mice |

Figure 1.2. Production of completely ES cell derived (ES cell-tetraploid) mice by tetraploid embryo complementation (Nagy, et al., 1990). Fertilized zygotes can obtained from the oviduct post coitum and cultured in vitro over night to the two-cell stage. The two diploid blastomeres in the two cell embryo can be induced to fuse back into a single cell with an electrical pulse. This one cell tetraploid embryo will then develop in vitro to the blastocyst stage. If transferred in utero, such blastocysts can not develop beyond early postimplantation stages. However, if these blastocysts are injected with diploid ES cells, they can give rise, after embryo transfer, to a conceptus in which the embryonic lineages are derived completely from the injected ES cells while the extraembryonic lineages are derived from the host tetraploid blastocyst.
donor cell _in vitro_ culture (McCleath et al., 2000). It is therefore possible that these unknown effects of long-term donor cell _in vitro_ culture, observed in mice cloned from ES cells, may also be relevant to the survival of other cloned animals.

**Epigenetic Reprogramming after Nuclear Transfer**

The most interesting issue in cloning by nuclear transfer is the problem of epigenetic reprogramming (Gurdon and Colman, 1999). For clones to complete development, genes normally expressed during embryogenesis but silent in the somatic donor cell must be reactivated. To date, the efficiency of deriving live cloned animals has been low and independent of the source of the cell type used as nuclear donor, with two notable exceptions. Nuclei isolated from ES cells and embryonic blastomeres generated viable cloned animals with a significantly higher efficiency than that of any somatic donor cell-type (Table 1.3). As stated above, this observation is consistent with the notion that the genome of pluripotent embryonic cells is easier to reprogram than that of a somatic cell. If this is the case, what is the nature of the reprogramming that must occur for a somatic cell clone to survive?

**Germ line transmission relieves epigenetic damage accumulated during the cloning process.** Remarkably, it has proven possible to clone mice from cumulus cell nuclei for up to six generations (Wakayama et al., 2000). However, with each round of reproductive cloning, the generation of offspring became increasingly more difficult (Wakayama et al., 2000). Although these mice displayed few overt developmental defects, they seem to have accumulated genetic or epigenetic damage that prevented their further propagation by nuclear transfer. The observation that the sexual offspring of cloned animals are normal and fertile suggests that the abnormalities are epigenetic rather than genetic in nature (Wakayama et al., 1998). In a similar experiment, bovine embryos were created by nuclear transfer, cultured to the blastocyst stage, disaggregated and subjected to repeated rounds of cloning (Peura et al., 2001). The results showed that repeated
cloning led to a gradual decrease in the production of blastocysts (Peura et al., 2001). Together, these serial cloning experiments suggest that bypassing the normal removal and reestablishment of epigenetic information, which normally occurs during gametogenesis, can aggravate the developmental inefficiency observed when cloning animals.

Even more insidious than evidence that incomplete epigenetic reprogramming leads to developmental failure (Daniels et al., 2000)(Kang et al., 2001), are signs that considerable epigenetic damage may be tolerated by mammalian development and only manifested in cloned animals late in life or after multiple rounds of cloning (Jaenisch and Wilmunt, 2001)(Humpherys et al., 2001)(Wakayama et al., 2000). Thus epigenetic abnormalities are present in clones and may cause the many abnormal phenotypes characteristic of cloned animals (Rideout et al., 2001).

In normal development, the reprogramming of the genome occurs during gametogenesis, a complex process that assures that the genome of the two gametes when combined at fertilization can faithfully activate early embryonic genes (Hilscher, 1999)(Barton et al., 1984)(Kafri et al., 1992). In cloning, reprogramming has to occur in the short interval between transfer of the donor nucleus into the egg and the onset of blastomere cleavage, a cellular context dramatically different from normal fertilization. The challenge now lies in identifying the epigenetic errors, arising either as a result of faulty reprogramming after NT or incurred by the donor cell nucleus during in vitro cultivation and aging that are responsible for cloning phenotypes. For the following discussion it is useful to distinguish epigenetic information coded before formation of the zygote from that which is established in the developing embryo after fertilization. For example, parent-specific changes in DNA methylation and chromatin structure that lead to monoallelic expression of imprinted genes are programmed during gametogenesis and maintained after fertilization (Tilghman, 1999)(Bartolomei and Tilghman, 1997). In contrast, X chromosome inactivation (Lyon, 1999) and telomere length adjustment (DePinho, 2000) are processes occurring after fertilization.

I will first discuss advances in our understanding of how reprogramming of zygotically
controlled epigenetic processes, such X inactivation and telomere length adjustment, occur after nuclear transfer. In addition, I will comment on the identity of candidate genes and processes, such as maintenance of imprinted gene expression, whose regulation is controlled by information established during gametogenesis and whose improper or incomplete reprogramming may lead to the phenotypes observed in cloned animals.

**Reprogramming X chromosome inactivation.** Dosage compensation in mammals is achieved by extinguishing gene expression from one X chromosome in female somatic cells, a process known as X inactivation (Lyon, 1999). The inactive X chromosome (X\textsubscript{i}) differs from both the active chromosomes X (X\textsubscript{A}) and the autosomes in its heterochromatic and transcriptionally silent state. The characteristics of a heterochromatic state exemplified by the X\textsubscript{i} include increased concentration of macrohistone H2a (Csankovszki et al., 1999), histone H4 hypoacetylation (Jeppesen and Turner, 1993), and increased DNA methylation at promoter sequences (Norris et al., 1991).

Dosage compensation in female embryos by X inactivation is a developmentally regulated process, commencing during preimplantation cleavage with both X chromosomes transcriptionally active, occurring in a stereotypical series of molecular events (Figure 1.3) (Panning et al., 1997). First, an unidentified parental epigenetic mark, or imprint, causes preferential inactivation of the paternal X in the extraembryonic trophectoderm (TE), a tissue that contributes to the placenta and is necessary for implantation of the embryo (Marahrens et al., 1997) (Takagi and Sasaki, 1975) (Lyon, 1999). After TE allocation and extraembryonic X-inactivation, the paternal imprint is subsequently lost in those cells giving rise to the embryonic (epiblast) lineage where X chromosome choice is random (Epstein et al., 1978) (Krätscher and Gartler, 1978). Once chromosomal choice and transcriptional silencing have occurred, the heterochromatic state imposed on the X\textsubscript{i} is stable, with the X\textsubscript{i} state being faithfully passed on to all of the mitotic daughters of the respective chromosome chosen for inactivation (Lyon, 1999).
Figure 1.3: X inactivation during normal development

**X-Inactivation During Normal Development**

- **Zygote**
  - Inner cell mass
  - Embryo
  - Trophectoderm (TE)
  - Extraembryonic Tissues (Placenta)

**Figure 1.3.** X chromosome inactivation is a developmentally regulated process occurring during female embryogenesis. During the maturation of the gametes an imprint of unknown molecular nature is installed on one of the two parental X chromosomes. This imprint is arbitrarily represented as a circle around the maternal (pink) X chromosome. After fertilization both the paternal (blue) and maternal X chromosomes are expressed. However, the imprint is retained through cleavage stages of development and leads to non-random, imprinted inactivation of the paternal X chromosome in the trophectoderm. The transcriptionally silent state of the inactive X is represented by shading over the inactive chromosome. In contrast, during proliferation of the inner cell mass, the gametic imprint is lost, leading to random X inactivation in the epiblast. Once the choice of which X to inactivate has been made and X inactivation has been completed, this inactive state is stably inherited by all of its mitotic daughters.
The existence of cloned female animals indicated that dosage compensation must occur following nuclear transfer of a female donor nucleus. However, it was unclear whether X inactivation would be random, as in normal animals, or non-random as in the TE lineage (Gurdon and Colman, 1999). The issue was whether the heterochromatic state of the X_{i} in the donor nucleus would determine the state of this chromosome in all cells of the clone resulting in non-random inactivation. Alternatively, the epigenetic mark imposed on the X chromosomes during embryonic development of the donor animal could have been erased after nuclear transfer and randomly reestablished on either of the two chromosomes at the time of inactivation.

Using genetically marked X-chromosomes it was demonstrated that X inactivation occurs randomly in the epiblast lineage of cloned mice (Eggnan et al., 2000). These data indicate that the epigenetic marks which distinguish X_{a} and X_{i} in somatic cells are removed and reestablished on either X during the cloning process, resulting in random X inactivation in the cloned animal. In contrast, epigenetic marks on X_{a} and X_{i} in the somatic donor cell are not removed in the TE lineage of the clone and predispose the X_{a} of the donor cell to be active and the donor X_{i} to be inactive. Thus, TE X inactivation occurs in cloned animals largely as it does in normal development. The gametic imprint-like mark, present in the somatic cell, is removed only after allocation of the TE lineage, but before differentiation of the epiblast lineage, leading to imprinted extraembryonic X inactivation (Lyon, 1999).

In contrast to somatic cells, both X-chromosomes are active in female ES cells, suggesting the lack of an imprint like epigenetic mark on either chromosome (Panning et al., 1997)(Wutz and Jaenisch, 2000). When ES cells were used as nuclear donors, random X inactivation was observed in the epiblast as well as the TE of the cloned embryo. This observation is consistent with the existence of a mechanism that ensures random X inactivation in the TE lineage in the absence of an epigenetic mark on the donor chromosomes (Eggnan et al., 2000).

These observations indicate that the different epigenetic information present on the somatic donor X-chromosomes is not immediately removed following nuclear transfer, but is interpreted
in the TE lineage resulting in non-random inactivation. After TE allocation, the epigenetic marks distinguishing the two donor X-chromosomes are removed and randomly reestablished in the epiblast lineage allowing for random inactivation in the somatic cells of the cloned female. In summary, the process of X inactivation is faithfully recapitulated in cloned female embryos and resembles that in normal females (Figure 1.4) (Eggan et al., 2000).

**Telomere length adjustment in cloned embryos.** A characteristic of most somatic cells dividing *in vivo* or *in vitro* is the progressive shortening of their telomeres (DePinho, 2000). Once telomeres shorten below a critical length, apoptotic pathways are activated and the cell loses its ability to divide (DePinho, 2000). It is known that telomerase, the enzyme that maintains telomere length, is expressed during gametogenesis and in embryonic cells consistent with maximal telomere length in the early embryo (DePinho, 2000). Importantly, it has been shown that expression of telomerase in aging cells can restore telomere length and rescue a cell from mitotic senescence (Counter et al., 1998).

The observation that Dolly and other cloned sheep had shortened telomeres (Shiels et al., 1999) raised the question of whether the gradual erosion of telomere length which occurs during both the *in vivo* and *in vitro* aging of the donor cell nucleus might lead to premature aging of cloned animals. However, in contrast to the shortened telomeres observed in cloned sheep, cloned cattle have telomere lengths either similar to or longer than their age-matched controls. When near-senescent embryonic bovine cells were used as nuclear donor, telomere length and cellular proliferative life span was completely restored or even enhanced by the cloning process (Lanza et al., 2000). Further studies investigating telomere length in ear biopsies taken directly from a large numbers of cloned and control offspring, concluded that telomere length in cloned neonatal cattle was nearly identical to that of newborn controls (Tian et al., 2000)(Kato et al., 2000). Notably, it was determined that telomere length was similar in fibroblasts derived from both clones that died shortly after delivery and clones that survived to adulthood suggesting that neither telomere
Figure 1.4: X inactivation in cloned mouse embryos

Figure 1.4. X inactivation in cloned mouse embryos. In the somatic female donor cell, one X chromosome is transcriptionally active (blue) while the other X chromosome is transcriptionally repressed (red)(shaded). After successful nuclear transfer, both X chromosomes become transcriptionally active, however, some epigenetic difference remains between the two X chromosomes (circle) which acts as a "somatic imprint". This somatic imprint leads to non random inactivation of the X chromosome which had been transcriptionally repressed in the donor nucleus. Thus this somatic imprint is functionally equivalent to the gametic imprint which leads to preferential inactivation of the paternal X during normal extraembryonic development. In contrast, and again similar to normal development, this somatic imprint is lost during proliferation of the ICM leading to random X inactivation in the epiblast of the clone.
length maintenance nor premature cellular aging play a role in the neonatal mortality of cloned cows (Tian et al., 2000). It has also been demonstrated that telomerase activity is reactivated in cloned preimplantation bovine embryos with slightly delayed yet similar kinetics to those in normal fertilized controls (Betts et al., 2001).

Species specific differences in the mechanisms of either telomere length maintenance or reconstruction between cows and sheep are possible explanations for the differences observed. However, the observation that telomere length in cloned mice is also normal (Wakayama et al., 2000), suggests that exposing the telomeres to telomerase early in embryogenesis (Betts et al., 2001), similar to telomerase over-expression in senescent cells (Counter et al., 1998), is sufficient to restore normal telomere length and extend cellular life span.

**Loss of normal imprinted gene expression as a potential cause of the phenotypes observed in cloned animals.** Unlike X-inactivation and telomere length maintenance, epigenetic information controlling imprinted gene expression is primarily established during gametogenesis with allele-specific marks being only maintained postzygotically (Figure 1.5) (Bartolomei and Tilghman, 1997)(Latham et al., 1994)(Tremblay et al., 1995). It therefore seems likely that any alterations in epigenetic information that controls imprinted gene expression, and incurred by the donor cell nucleus, would be present in any resulting cloned embryo. It has recently been realized that in vitro maintenance of both mammalian embryos and cell lines can lead to changes in DNA methylation that disrupt normal regulation of imprinted genes (Figure 1.6)(Dean et al., 1998)(Doherty et al., 2000). Since the most common phenotypes observed in cloned animals are neonatal death and fetal growth abnormalities, this loss of epigenetic information is of particular relevance in the preparation of donor cells used for nuclear transfer (Eggan et al., 2001)(Wakayama and Yanagimachi, 1999)(McCreath et al., 2000)(Hill et al., 1999). Similar phenotypes have been observed in both human patients and in mice as a consequence of both naturally occurring and targeted mutations disrupting imprinted gene expression (Tilghman,
Figure 1.5. Establishment and interpretation of gametic imprints. Imprinted genes are genes expressed in a monoallelic parent of origin specific manner. For instance, \( H19 \) is expressed exclusively from the maternal (pink) chromosome while the linked \( Igf2 \) locus is expressed only from the paternal (blue) chromosome. In the gametes the two parental chromosomes carry different epigenetic marks, namely a differentially methylated region at \( H19 \) is methylated (\( *** \)) on the paternal chromosome but demethylated on the maternal chromosome. It is thought that this methylation marks codes for monoallelic expression that is then read in the somatic tissues leading to paternal \( Igf2 \) expression (arrow) and maternal \( H19 \) expression. However, in the primordial germ cells, there is a dominant demethylating activity that erases this gametic imprints so they can then be reprogrammed in a sex specific manner in maturing gametes, completing the circle.
Figure 1.6: Environmental and tissue specific effects lead to loss of imprinting

Figure 1.6. Environmental and tissue specific effects can lead to loss of imprinting (LOI). Although in most tissues in vivo, H19 and Igf2 are normally reciprocally expressed, disruptions in imprinted gene expression can occur. This locus is particularly sensitive to environmental effects such as in vitro cell or embryo culture, which can lead to biallelic methylation at the H19 DMR, causing biallelic expression of Igf2 and silencing of the active H19 locus. LOI and biallelic expression of Igf2 with accompanying changes in DNA methylation are also observed in some tumors. In addition there can also be tissue specific effects on imprinting. For instance, imprinting at this locus is tightly maintained in the liver and other endodermal organs. However, in some derivatives of the neuroectoderm, biallelic expression of Igf2 can be observed.
1999) (Jaenisch, 1997). These apparent similarities suggest that aberrant imprinting might be one of the causal molecular mechanisms for the abnormal phenotypes observed in cloned animals.

Recent evidence suggests that abnormal imprinted gene expression might indeed be the source of some phenotypes observed in cloned animals. Analysis of imprinted gene expression in neonatal mice derived by nuclear transfer of ES cells revealed improper expression of several imprinted genes (Figure 1.7) (Humpherys et al., 2001). Most strikingly, H19 expression was extinguished and Igf2 levels were increased in both the placentas and organs of many clones. Other clones expressed intermediate or normal levels of these imprinted genes. In all cases the methylation status at the H19 differentially methylated region correlated well with that genes expression. Stochastic losses in expression of other imprinted genes, such as Meg1/Grb10 and Pegl/Mest were also observed. In contrast, some imprinted genes seemed to retain their normal expression (Snrpn and Peg3) and methylation (Igf2r DMR2) levels. Strikingly, all of the epigenetic abnormalities found within the cloned animals could also be found in the donor ES cell populations from which they were derived. Furthermore, when imprinted gene expression was analyzed in mice generated from the same donor cells by tetraploid embryo complementation, similar abnormalities in gene expression were observed. As a whole, these results suggest that for the subset of imprinted genes analyzed, epigenetic abnormalities were present in the ES cells prior to nuclear transfer and were not likely to be a direct result of the cloning procedure per se (Humpherys et al., 2001).

It therefore was critical to understand whether imprinted gene expression is also disturbed in animals cloned from primary cells. Loss of information encoding imprinted gene expression in vivo seems rare but appears to be aggravated by in vitro culture (Dean et al., 1998) (Doherty et al., 2000). It could have been that animals cloned from non-cultured cumulus cells possessed imprinted gene expression patterns more like those of normal off-spring, while clones derived from cultured cells, such as tail-tip or embryonic fibroblasts showed disruptions in imprinted gene expression similar those observed in ES cell-derived NT animals. However, both candidate
**Figure 1.7:** *H19* and *Igf2* expression in the placentas of cloned animals

**Placental Northern**

*In vitro* manipulated controls

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mice cloned from embryonic stem cells</th>
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</thead>
<tbody>
<tr>
<td><em>H19</em></td>
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</tr>
<tr>
<td><em>Igf2</em></td>
<td>![Image]</td>
</tr>
<tr>
<td><em>Gpdh</em></td>
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</tr>
</tbody>
</table>

**Norm**

Avg = 0.1g

Norm Avg = 0.8g

**Neonatal Survival**

![Image]

**Figure 1.7.** *H19* and *Igf2* expression in the placentas of mice cloned from embryonic stem cells (Humpherys et al., 2001). Expression of *H19* and *Igf2* were determined by Northern blot analysis. Each lane contains RNA from an individual control or cloned placenta. The phenotypic data, including placental weight, birth weight and neonatal survival, for each cloned animals appears below the gene expression data. For neonatal survival, a green bar represents successful respiration and long term survival while a red bar indicates that the animal died shortly after cesarean section. Note the often reciprocal expression of *H19* and *Igf2* expression which is consistent with methylation of the normally demethylated allele of *H19* leading to biallelic expression of *Igf2*. Additionally, note the lack of correlation between abnormalities in the expression of these imprinted genes and the phenotypic parameters recorded.
based approaches and array based expression analysis (Inoue et al., 2002)(Humpherys et al., In press) indicate that even animals cloned from primary cumulus and sertoli cell nuclei display abnormal levels of imprinted gene expression. Consistent with the notion that multiple pathways may lead to loss of imprinting, mice cloned from these cells showed a different spectrum of gene expression abnormalities than that in ES cell derived clones.

Thus far, no clear correlation can be made between disruption in expression of any one imprinted gene with the phenotypes observed in clones (Humpherys et al., 2001). However, the apparently stochastic nature of epigenetic insults, such as those incurred during ES cell culture, combined with the reciprocal functions of many imprinted genes, may complicate this analysis (Humpherys et al., 2001)(Dean et al., 1998). Consistent with this hypothesis, mounting evidence in both sheep and mice suggests that prolonged in vitro culture of donor cell populations prior to nuclear transfer may increase the likelihood of neonatal complications in cloned offspring (Eggen et al., 2001) (McCreath et al., 2000). It will therefore be important to determine whether long-term culture of somatic cells can cause disruptions in imprinted gene expression similar to those seen in embryonic stem cells and if phenotypes in clones can be linked directly to any of these disruptions.

**What molecular mechanisms control reprogramming?** It is now clear that even nuclei from highly differentiated cells, such as lymphocytes, can be reprogrammed after nuclear transfer to a pluripotent state which is competent to direct embryonic development (Hochedlinger and Jaenisch, 2002). Furthermore, although several epigenetic characteristics of a differentiated cell are returned to an embryonic ground state by nuclear transfer (Lanza, et al 2000)(Eggen, et al 2000), the mechanisms by which this reprogramming is accomplished remain largely unknown. In fact, it remains unclear to what extent reprogramming is a passive process, involving dilution of factors required for maintenance of a differentiated state into the oocyte cytoplasm, or an active process requiring specific factors within the oocyte that actively remodel the differentiated
chromatin to reestablish pluripotency.

Some combination of these two mechanisms seems likely. For instance, restoration of telomere length must be an active processes, recruiting telomerase and other factors required for telomere polymerization and extension. However, silencing of tissue-specific genes and X chromosome reactivation after nuclear transfer may be accomplished primarily by dilution of tissue specific transcription and heterochromatin factors, which are not found in the oocyte. Meanwhile, other epigenetic information established during gametogenesis, such as allele specific DNA methylation encoding imprinted gene expression may be completely refractory to reprogramming. If reprogramming indeed requires enzymatic work by molecular machines within the mammalian oocyte, the identification of these enzymes as well as the chromatin components or genes that they target are obviously of considerable interest.

Because of cloning's inherent inefficiencies, nuclear transfer experiments using lymphocytes and other terminally differentiated cells as nuclear donors must be carefully designed and carried out if we are to understand the mechanisms by which epigenetic reprogramming is accomplished. In addition, as suggested by recent NT studies with the mouse, increased standardization of nuclear transfer technology from laboratory to laboratory, and if possible from organism to organism, will substantially improve our ability to synthesize experimental data into coherent hypothesis regarding why cloning by nuclear transfer succeeds or fails.

Acknowledgements

I would like to thank R. Janeisch, K. Hochedlinger, W. Rideout and C. Beard for kindly reading this chapter and offering their opinions. Figure 1.1 was a kind gift from K. Hochedlinger.
Chapter 2:

X Chromosome Inactivation in
Cloned Mouse Embryos

Abstract

To study whether cloning resets the epigenetic differences between the two X chromosomes of a somatic female nucleus, we monitored X inactivation in cloned mouse embryos. Both X chromosomes were active during cleavage of cloned embryos, followed by random X inactivation in the embryo proper. In the trophectoderm (TE), X inactivation was non-random with the inactivated X of the somatic donor being chosen for inactivation. When female embryonic stem cells with two active X chromosomes were used as donors, random X inactivation was seen in the TE and embryo. These results demonstrate that epigenetic marks can be removed and reestablished on either X chromosome during cloning. Our results also suggest that the epigenetic marks imposed on the X chromosomes during gametogenesis, responsible for normal imprinted X inactivation in the TE, are functionally equivalent to the marks imposed on the chromosomes during somatic X inactivation.

Notes

These studies would not have been possible without the patient help of H. Akutsu who took many days to help ensure that nuclear transfer technology was successfully ported from the University of Hawaii to MIT. H. Akutsu performed an equal share of the NT experiments in this study. K. Hochdelinger prepared first strand cDNA from cloned RNAs. W. Rideout found the Grpr SSLP. J. Dausman and R. Flannery provided expert mouse support. I would like acknowledge L. Jackson-Grusby and W Rideout for many helpful discussions. This work was supported by the Victoria and Bradley Geist Foundation, the Kosasa Family Foundation, and the Harold Castle Foundation to R. Yanagimachi and by NIH grants 5-R35-CA44339 and
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Introduction

Dosage compensation in mammals is achieved by the silencing of one X chromosome in female somatic cells, a process known as X inactivation (See Figure 1.3)(Lyon, 1961). Prior to implantation, both X chromosomes in female embryos are transcriptionally active (Epstein et al., 1978)(Krater and Gartler, 1978)(Monk and Harper, 1979). Upon differentiation, one X chromosome is chosen for inactivation and silenced (Epstein et al., 1978)(Krater and Gartler, 1978)(Monk and Harper, 1979). An untranslated RNA, encoded by the Xist gene is both necessary and sufficient for the initiation of X chromosome inactivation (Wutz and Jaenisch, 2000)(Marahrens et al., 1997)(Penny et al., 1996)(Lee and Jaenisch, 1997). The Xist RNA is stably transcribed from the center of inactivation (Xic) on the inactive X (X\(_i\)) and associates with the inactive chromosome over its entire length (Borsani et al., 1991)(Brockdorff et al., 1991)(Clemson et al., 1996). Xist expression is involved in both the initiation of inactivation and the choice of which chromosome will be inactivated (Marahrens et al., 1997)(Penny et al., 1996)(Marahrens et al., 1998)(Clere and Avner, 1998). The (X\(_i\)) also differs from other chromosomes in histone content (Csankovski et al., 1999), histone H4 hypoacetylation (Jeppesen et al., 1993), replication timing (Preist et al., 1967), and increased DNA methylation at promoter sequences (Norris et al., 1991). The maintenance of the X\(_i\) and active X (X\(_a\)) chromosomes is dependent on epigenetic marks imposed upon the X chromosomes. For example, on the X\(_i\), Xist is unmethylated and expressed, whereas on X\(_a\) it is methylated and silent (Beard et al., 1995). Induced demethylation of Xist on X\(_a\) leads to inappropriate Xist expression and silencing of
X-linked genes (Panning and Jaenisch, 1996).

X inactivation is random in the embryonic (epiblast) lineage of the embryo (Epstein et al., 1978)(Kratzer and Gartler, 1978)(Monk and Harper, 1979). In contrast, an unidentified mark, called an imprint, causes selective inactivation of the paternal X in the extraembryonic trophoderm (TE), a tissue that contributes to the placenta and is necessary for implantation of the embryo (Marahrens et al., 1997)(Takagi and Sasaki, 1975)(West et al., 1982)(Kay et al., 1994). An unresolved question is whether the epigenetic marks on \( X_s \) and \( X_i \) in somatic cells are functionally the same as those imposed on the X chromosomes during gametogenesis.

It has been suggested that the cloning of mammals by nuclear transfer requires epigenetic reprogramming of the differentiated state of the donor cell to a totipotent, embryonic ground state (Gurdon and Coleman, 1999)(Kiko and Wolfe, 2000). However, there is no direct molecular evidence for reprogramming. For example, it is unclear whether the epigenetic modifications of \( X_s \) and \( X_i \) in a female somatic cell are reversible during cloning (Gurdon and Coleman, 1999). To address these questions, we used an X-linked reporter transgene (\( X^{GFP} \)), with a cytomegalovirus promoter driving expression of the Green Fluorescent Protein (GFP). This reporter is subject to silencing by X inactivation (Hadjantonakis et al., 1998) and allowed us to distinguish the state of X inactivation in different lineages of cloned embryos.

Results

X-linked gene expression in normal and cloned preimplantation mouse embryos.

Control zygotes were isolated from normal females mated with hemizygous transgenic males (\( X^{GFP}/Y \)). The control embryos were cultivated in vitro and assessed for fluorescence at successive cleavage stages (Table 2.1A). All embryos were dark at the one, two, four and early eight cell stages, but 50%, presumably \( X/X^{GFP} \), began to fluoresce after compaction. The other 50%, presumably \( X/Y \), never fluoresced. These observations suggest that the paternally inherited transgene, carried in female embryos, was active during cleavage, consistent with
Table 2.1: X linked GFP expression in normal and cloned preimplantation mouse embryos

<table>
<thead>
<tr>
<th>Parents or Donor Cell</th>
<th>Genotype</th>
<th>Stage of development</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 Cell</td>
</tr>
<tr>
<td>A Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XGFP/X</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>XGFP/γ</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>X/Y</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>X/GFP</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B Clones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tail Tip Cells</td>
<td>XGFP/X</td>
<td>−</td>
</tr>
<tr>
<td>Cumulus Cells Tail Tip Cells</td>
<td>X/XGFP</td>
<td>−</td>
</tr>
<tr>
<td>6TG Selected Tail Tip Cells</td>
<td>Hprt-XGFP</td>
<td>−</td>
</tr>
<tr>
<td>Parthenotes</td>
<td>X/X</td>
<td>−</td>
</tr>
</tbody>
</table>

I tested for reactivation of the inactive X chromosome in cloned embryos generated by transfer of nuclei from X<sup>GFP</sup> female tail tip or cumulus cells into enucleated oocytes (Table 2.1B)(Wakayma et al., 1998)(Wakayama et al., 1999). Because of random X chromosome inactivation in these somatic donor cells, 50% of the donor nuclei were expected to have an active X<sup>GFP</sup> (X<sub>i</sub><sup>GFP</sup>) and 50% an inactive X<sup>GFP</sup> (X<sub>i</sub><sup>GFP</sup>). Green fluorescence was not seen in one, two, or four cell nuclear transfer embryos but was observed in all cloned transgenic embryos by the morula and blastocyst stages (Table 2.1A, Figure 2.1B). The fluorescence in cloned embryos was similar to that in normal female embryos carrying a paternally inherited transgene, suggesting that the GFP transgene, which was expected to be derived from a somatically inactivated X in 50% of all clones, was reactivated after nuclear transfer. However, because the X inactivation status of the donor cells was not determined prior to nuclear transfer, it was possible that all morulae and blastocysts had been derived from transfer of nuclei with an X<sub>i</sub><sup>GFP</sup>.

To confirm that X chromosome reactivation occurred after nuclear transfer, I used a drug selection scheme to generate donor cells with a known X inactivation status (Figure 2.1A)(Hooper et al., 1987). X<sup>GFP</sup>/Y males were crossed with homozygous Hypoxanthine-Guanine Phosphoribosyltransferase (Hprt-) mutant females (X<sup>Hprt-/X<sup>Hprt-</sup></sup>) and tail tip fibroblasts were derived from daughters carrying the transgene (X<sup>Hprt-/X<sup>GFP</sup></sup>). The fibroblasts were grown in 6-thio-guanine (6TG) to select for cells with an X<sub>i</sub><sup>GFP</sup> and in hypoxanthine, aminopterin, thymidine (HAT) medium to select for cells with an X<sub>i</sub><sup>GFP</sup> (Fig 1A). 6TG selection yielded a population in which > 99% of cells had an X<sub>i</sub><sup>GFP</sup>, as assayed by fluorescence activated cell sorting (FACS) (Figure 2.2A). When 6TG selected cells were used as nuclear donors (Table 2.1B), 100% of nuclear transfer morula and blastocysts expressed GFP in every cell. These results demonstrate that genes on X<sub>i</sub> are reactivated after nuclear transfer (Table 2.1A, Fig 2.1B).
**Figure 2.1: X linked GFP expression in female clones**

(A) Drug selection scheme for obtaining populations of tail tip cells with a predetermined X inactivation state. (B) X linked GFP transgene is reactivated after nuclear transfer. Bright and dark field photomicrographs of control and nuclear transfer blastocysts (Table 2.1A). All transgenic control and nuclear transfer blastocysts clearly express GFP. P denotes parthenogenetic control blastocyst. Arrowhead points to a nuclear transfer embryo arrested at the two-cell stage; note lack of fluorescence. Arrow marks nuclear transfer embryo in which one blastomere of the two-cell embryo has arrested and does not express GFP, while the other blastomere continued to divide and all cells are GFP positive. Bar = 100 μm. (C) X linked GFP transgene was expressed in the placentas of female E12.5 control embryos (X^{Gfp}/X^{Hprt}) with the maternally inherited transgene but was not expressed if the transgene was paternally inherited (X^{Hprt}/X^{Gfp}). (D) X inactivation state in the TE and epiblast of cloned embryos. HAT selected (X^{Gfp}) or 6TG selected (X^{Gfp}) tail tip cells were used as donor cells for nuclear transfer and embryos and placentas were recovered at E12.5. Both embryos and placentas derived from the transfer of HAT selected (X^{Gfp}) donor were fluorescent. In contrast, 6TG selected (X^{Gfp}) nuclei gave rise to GFP expressing embryos with placentas that did not express GFP. Bars = 1 mm.
Verification of somatic donor cells with a known X inactivation status. HAT-selected and 6TG-selected cells were used as donors for nuclear transfer to investigate the X inactivation status in the TE and in the epiblast lineage. In contrast to 6TG selection of tail tip fibroblasts, which yielded a population in which > 99% of cells had an X\textsubscript{i}\textsuperscript{GFP}, HAT selection resulted in a population in which only 79% of cells expressed GFP (Figure 2.2A) instead of the expected 100%. This result suggested either incomplete HAT selection or complete HAT selection with non-specific silencing of the transgene in some tail tip donor cells even when present on the X\textsubscript{a}. Consistent with effective HAT selection for cells with an X\textsubscript{a}\textsuperscript{GFP}, 6TG counter-selection of the previously HAT selected cells resulted in death of all cells, indicating that every cell expressed the functional \textit{Hprt} gene located on the same chromosome as the GFP transgene. Therefore, it is likely that the transgene, even when carried on the X\textsubscript{a}, is subject to some non-specific silencing in the adult cells. Nuclei from HAT or 6TG selected tail tip cells were injected by myself and H. Akutsu into enucleated oocytes and the reconstructed embryos were transferred to recipient females (Table 2.1B). Embryos were recovered from recipient mothers at E12.5 and GFP expression was assessed.

\textbf{X inactivation in the embryonic lineages of cloned mice.} GFP expression in the epiblast lineage was analyzed by FACS of mouse embryonic fibroblasts (MEFs) from cloned embryos derived from 6TG or HAT selected donor cells. In control experiments 45% to 65% of MEFs isolated from individual, female embryos expressed GFP, consistent with random X inactivation (Figure 2.2C compare to Figure 2.1D). 35% of MEFs from cloned embryo 6TG 1, which was derived from a 6TG (X\textsubscript{i}\textsuperscript{GFP}) selected donor cell, expressed GFP (Figure 2.2B), indicating that the X\textsubscript{i}\textsuperscript{GFP} was reactivated in 35% of cells and inactive in 65% of cells. Similarly, two cloned embryos, HAT 1 and HAT 2, derived from HAT (X\textsubscript{a}\textsuperscript{GFP}) selected donor cells, expressed GFP in 35% and 78% of MEFs, respectively (Figure 2.2B). Fibroblasts for FACs could not be obtained from clone 6TG 2. However visual inspection revealed a mosaic pattern of GFP expression in
Figure 2.2: FACS analysis of X linked GFP expression in cultured fibroblasts

A \(X^{Hprt-}\times X^{GFP}\) Donor Tail Tip Cells

- Unselected: 22% GFP
- 6TG Selection: 0.3% GFP
- HAT Selection: 79% GFP

B \(X^{Hprt-}\times X^{GFP}\) Tail Tip Clone MEFs

- 6TG Clone 1: 35% GFP
- HAT Clone 1: 35% GFP
- HAT Clone 2: 78% GFP

C MEF Controls

- X\(^{GFP/}\)Y: 96-100% GFP
- X/X: 0-1% GFP
- \(X^{Hprt-}\times X^{Gfp}\): 45-65% GFP

Figure 2.2. FACS analysis of GFP expression. The Fl-1 "high" population contained GFP+ cells and is displayed in brown on the histogram while the FL-1 "low" population was GFP and is represented by the black line overlay. (A) GFP expression in unselected and selected tail tip donor cells prepared as in Figure 2.1A. (B) GFP expression in mouse embryonic fibroblasts (MEFs) derived from E12.5 cloned embryos (Table 2.2, Figure 2.1D). (C) GFP expression in MEFs from control E12.5 embryos. For controls %GFP+ is displayed as the range observed in the number of cells expressing GFP from N>10 individual embryos.
Table 2.2: Development of cloned embryos after nuclear transfer

<table>
<thead>
<tr>
<th>Female Donor Cells</th>
<th>Oocytes Reconstructed</th>
<th>Pronuclear Formation</th>
<th>2-Cell (% Pronuclear)</th>
<th>Morula Blastocyst (% Pronuclear)</th>
<th>Embryos Transferred (% Pronuclear)</th>
<th>Midgestation Embros Recovered (% Transferred)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulus Cells</td>
<td>76</td>
<td>56 (74)</td>
<td>38 (58)</td>
<td>17 (30)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tail Tip Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X(^{GP})/X</td>
<td>26</td>
<td>12 (46)</td>
<td>10 (83)</td>
<td>8 (67)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>X(^{GP})/X(^{GP})</td>
<td>131</td>
<td>63 (48)</td>
<td>56 (89)</td>
<td>31 (49)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tail Tip Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X(^{GP})/X(^{GP}) HAT</td>
<td>162</td>
<td>82 (51)</td>
<td>64 (78)</td>
<td>—</td>
<td>61 (74)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>6TG</td>
<td>233</td>
<td>187 (80)</td>
<td>154 (82)</td>
<td>30 (88)</td>
<td>135 (72)</td>
<td>3 (2.2)</td>
</tr>
<tr>
<td>6TG</td>
<td>81</td>
<td>34 (42)</td>
<td>30 (88)</td>
<td>9(30)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tail Tip Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57/M.cast</td>
<td>704</td>
<td>218 (31)</td>
<td>191 (88)</td>
<td>—</td>
<td>189 (87)</td>
<td>7 (3.7)</td>
</tr>
<tr>
<td>ES Cells 129/M.cast</td>
<td>142</td>
<td>36 (25)</td>
<td>35 (97)</td>
<td>—</td>
<td>34 (94)</td>
<td>2 (6)</td>
</tr>
</tbody>
</table>
the embryo proper, characteristic of random X inactivation (Figure 2.1D). These results indicate that either X chromosome can be chosen for inactivation, regardless of the X inactivation status of the donor nucleus, and strongly suggest that X inactivation in the somatic lineage of clones is random.

To confirm random X inactivation in the epiblast of clones, I generated *M. musculus* (*M. musc*)/ *M. castaneus* (*M. cast*) F1 females. These F1 females allowed the use of strain specific simple sequence length polymorphisms (SSLPs) in the X-linked genes *Xist* and *Grpr* to distinguish between expression from the two X chromosomes (Blake et al., 2000)(Hendrich et al., 1993). Some skewing of X inactivation was expected in these F1 animals because *M. cast* carries the strong *Xce* and *M. musc* the weak *Xce* alleles (Cattanach and Rasberry, 1994). This was evident in slightly higher expression of the *M. musc Xist* allele and lower expression of the *M. musc Grpr* allele in fibroblasts isolated from control F1 female embryos (Figure 2.3A).

Midgestation clones were derived by myself and H. Akutsu from tail tip fibroblasts of F1 females used as donors for nuclear transfer (Table 2.2). Similarly to controls, strain-specific RT-PCR showed that both *M. musc* and *M. cast Xist* and *Grpr* transcripts were expressed in the clones with the *M. cast X* being more active then the *M. musc X*. These results confirm that X inactivation is random in the somatic lineage of cloned female embryos (Figure 2.3A).

**X inactivation in the embryonic lineages of cloned mice.** To assess X inactivation in the TE lineage, I investigated GFP expression in the placentas of clones derived from HAT or 6TG selected donor tail tip cells. Control hemizygous female embryos inheriting the transgene from their mother (*X^{Gfp}/X^{Hprt}*) were expected to express GFP in all TE cells due to imprinted X inactivation, giving rise to fluorescent placentas (Figure 2.1C)(Hadjantonakis et al., 1998). Conversely, normal hemizygous female embryos inheriting the transgene from their fathers (*X^{Hprt}/X^{Gfp}*) did not express GFP in the TE and had dark placentas due to imprinted X inactivation (Figure 2.1C)(Hadjantonakis et al., 1998). Two clones recovered at E12.5 from HAT (*X^s_{GFP}*)
Figure 2.3. Strain specific RT-PCR analysis of Xist and Grpr expression. (A) Expression analysis of embryonic RNAs from four control and six cloned embryos. F denotes RNA from MEFs, E, RNA from whole embryonic tissue, A, amniotic membrane RNA. (B) Placental RNAs from four normal, seven tail tip cloned and two ES cell cloned embryos. (C) Embryonic RNAs from female ES cell clone 1 and the donor cells used to produce the tail tip clones. Ratio appearing beneath each lane represents quantification of the ratio of M.mus to M.cast Xist expression as measured by phospoimaging.
selected donor cells had fluorescent placentas and embryos (Table 2.2, Figure 2.1D). In contrast, two cloned embryos derived from 6TG (X<sup>GFP</sup>) selected donor cells had nonfluorescent placentas but showed fluorescence in the embryo proper (Table 2.2, Figure 2.1D). Two additional implants containing only placentas were also recovered (Table 2.2). One of these placentas, derived from a HAT selected donor, was fluorescent, the other from a 6TG selected donor was not (data not shown). These observations suggest that X inactivation in the TE lineage of cloned embryos is non-random and that the status of X inactivation in the donor nucleus determines which X chromosome is active or inactive.

I also analyzed allele-specific expression in placentas of cloned female embryos derived from F1 *M. musc* / *M. cast* tail tip cells. RT-PCR was performed on 7 placentas whose embryos were all cloned from cells derived from a single F1 female (Figure 2.3B). Three of the 7 clones expressed only the *M. musc Xist* allele (clones 1, 3 and 4) while three expressed only the *M. cast Xist* allele (clones 2, 5, and 7). As expected, all control placentas, (n=3) expressed only the paternal *M. cast Xist* allele. The placenta from one tail tip clone showed expression of both Xist alleles (clone 6). This embryo had the smallest placenta and was severely malformed suggesting that perhaps inappropriate reprogramming of the genome had occurred. These results confirm that X chromosome inactivation in the TE lineage of cloned embryos is non-random.

**X inactivation in mice cloned from female ES cells.** Early work has shown that both X chromosomes are active in female ES cells and that one X is chosen for inactivation only after induction of differentiation (Wutz and Jaenisch, 2000)(Panning et al., 1997)(Sheardown et al., 1997). It is therefore presumed that neither X chromosome in undifferentiated ES cells carries a distinguishable epigenetic mark. This hypothesis predicts that transfer of ES cell nuclei into enucleated oocytes might produce clones with random X inactivation in both the TE and epiblast lineage. To test this, I produced clones from a female F1 *M. musc* / *M. cast* ES cell line (Table 2.2). Two live implants were recovered at E 13.5, one containing a normal embryo with
placenta and the other containing a normal placenta with a growth-retarded fetus. Allele-specific RT-PCR on RNAs from these normal appearing placentas revealed expression of both Xist alleles suggesting that random X inactivation had occurred (Figure 2.3B). These results imply that clones derived from donor cells which have not yet undergone X inactivation do not carry an epigenetic mark on either X that would predispose one of them to be inactivated in the TE lineage. Expression analysis of both Xist and Grpr in the epiblast of the first female ES cell clone showed, as expected, that random X inactivation also occurred in the embryo proper (Figure 2.3C).

Discussion

In summary, we have demonstrated that X inactivation is random in the epiblast lineage of cloned mice. This random inactivation indicates that the epigenetic marks that distinguish Xa and Xb in somatic cells can be removed and reestablished on either X during the cloning process, resulting in random X inactivation in the cloned animal. In contrast, the epigenetic marks on Xa and Xb in the donor cell are not removed in the TE lineage of the clone and predispose the Xa of the donor cell to be active and the donor Xb to be inactive. However, this somatically acquired mark is ignored during early cleavage stages of development as both X chromosomes are expressed. This expression is consistent with that in normal cleavage development where both Xist alleles are expressed and both X chromosomes are active (Sheardown et al., 1997). Similarly, the gametic imprint in normal development is removed only after allocation of the TE lineage, but before differentiation of the epiblast lineage, leading to imprinted X inactivation in extraembryonic tissue and random inactivation in the embryo proper (See Figure 1.4)(Kay et al., 1994).

The nature of the marks responsible for imprinted X inactivation in the TE lineage is not known (McDonnell et al., 1998). However, our results imply that the epigenetic marks acquired during random X inactivation in the somatic lineage are functionally equivalent to the marks
acquired during gametogenesis, as they both can determine which X will be active and which will be inactive in the TE lineage. Finally, clones derived from ES cells which contain two active and as yet unmarked X chromosomes showed random X inactivation in both the TE and epiblast lineages. This observation indicates that in the trophectoderm random instead of imprinted X inactivation occurs in the absence of somatic, or gametic, epigenetic modification.

Materials and Methods

Generation of parthenogenetic control embryos. Parthenogenetic blastocysts were obtained by activating unmanipulated B6D2F1 recipient oocytes for 5.5 hours in Ca\(^{2+}\) free media containing 10 mM Sr\(^{2+}\) and 5 µg ml\(^{-1}\) Cytochalasin followed by 3 days of in vitro embryo culture.

Embryo transfer of cloned embryos. Two-cell embryos (n=10 to 15) were transferred to each oviduct of Swiss Webster recipients mated with vasectomized males the previous evening. After 11 to 12 days, recipient females were sacrificed and analyzed for the presence of living implants.

Preparation of tail-tip cells for NT. A tail tip biopsy was prepared from a 4 week old X\(^{Hprt/}\)/X\(^{GFP}\) female, freed of skin, minced into small pieces and placed in culture. Tail tip cells were cultured as previously described (Wakayama et al., 1998) or in the presence of HAT (Lexicon Genetics) or 2 µg ml\(^{-1}\) 6TG.

Isolation of midgestation transgenic control embryos. One cell zygotes were obtained from superovulated X\(^{GFP/}\)/X\(^{GFP}\) or X\(^{Hprt/}\)/X\(^{Hprt}\)-females mated with X\(^{Hprt/}\)/Y or X\(^{GFP/}\)/Y males and cultured overnight. Embryos were transferred to recipient females. In the case of paternal transmission of the transgene, all green embryos were assumed to be female and placental
fluorescence was observed. In the case of maternal transgene transmission, embryos were photographed and embryonic fibroblasts were prepared as described (Figure 2.2). FACS profile of GFP expression was then used to identify the sex of the recovered embryo.

Quantification of GFP expression by fluorescence activated cell sorting. Briefly, cells were trypsinized, washed once in Dulbecco’s Modified Eagle Medium (DMEM) with 15% Fetal Calf Serum (FCS) and resuspended in DMEM with 10 μg ml⁻¹ propidium iodide (PI). Live cells were gated on the basis of size and PI exclusion. When Fl-1 (GFP fluorescence) and Fl-2 (auto-fluorescence) were compared, two discrete populations of cells were observed and gated.

Allele specific analysis of X linked gene expression by RT-PCR. Strain-Specific RT PCR was performed with primers previously described (Blake et al, 2000)(Hendrich et al, 1993). Briefly, first strand cDNA was primed from DNase-treated RNA using random hexamer primers. We then carried out 27 rounds of amplification with end-labeled forward primer and cold reverse primer (linear range) for Xist and Grpr on both RT⁺ and RT⁻ samples. PCR products were never observed in RT⁻ controls. PCR-products were purified and either applied directly to a 6% sequencing gel (Grpr) or first digested with Taq I (Xist) and then applied.
Chapter 3:

Hybrid-Vigor, Fetal Overgrowth and Viability of Mice Derived by Nuclear Cloning and Tetraploid Embryo Complementation

Abstract

To assess whether heterozygosity of the donor cell genome was a general parameter, crucial for long-term survival of cloned animals, we tested the ability of ES cells with either an inbred or F1 genetic background to generate cloned mice by nuclear transfer. Most clones derived from five F1 ES cell lines survived to adulthood. In contrast, clones from three inbred ES cell lines invariably died shortly after birth due to respiratory failure. Neonatal lethality has also been reported in mice entirely derived from inbred ES cells that had been injected into tetraploid blastocysts (ES cell-tetraploids). Like inbred clones, ES cell-tetraploid pups derived from inbred ES cell lines died shortly after delivery with signs of respiratory distress. In contrast, most ES cell-tetraploid neonates, derived from six F1 ES cell lines, developed into fertile adults. Cloned pups obtained from both inbred and F1 ES cell nuclei frequently displayed increased placental and birth weights while ES cell-tetraploid pups were of more normal weight. The potency of F1 ES cells to generate live, fertile adults was not lost after either long term in vitro culture or serial gene targeting events. We conclude that genetic heterozygosity is a crucial parameter for postnatal survival of mice that are entirely derived from ES cells by either nuclear cloning or tetraploid embryo complementation. In addition, our results demonstrate that tetraploid embryo complementation using F1 ES cells represents a simple, efficient procedure for deriving animals with complex genetic alterations without the need for a chimeric intermediate.
Notes

H. Akutsu performed half of the nuclear transfer experiments in this study. Half of the cloned mice reported here were produced by H. Akutsu and myself at the University of Hawaii. The other half of the cloned mice were produced by myself at MIT. The previously unpublished ES cell lines reported in this study were produced by M. Klemm. The B6129 line was produced by W. Rideout and J. Loring. The assistance of J. Loring in culturing and maintaining the many ES cell lines used in this study was invaluable. Targeted V6.5 ES cell lines were kindly provided by L. Jackson-Grusby and R. Pasemoto. J. Dausman and R. Flannery provided technical mouse support. Tissue sectioning was performed by J. Reis. I thank L. Jackson-Grusby, W Rideout and A. Wutz for many helpful conversations. This work was supported by the Victoria and Bradley Geist Foundation, the Kosasa Family Foundation, and the Harold Castle Foundation to R. Yanagimachi and by NIH grants S-R35-CA44339 and RO1-CA84198 to R. Jaenisch.

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Introduction

Cloned mammals have been generated through the transfer of embryonic and somatic nuclei into enucleated oocytes. However, a serious impediment to the general utility of the procedure is the low survival rate of cloned animals as only 1-5% of reconstructed, nuclear transfer embryos develop into animals surviving to adulthood (Wilmut et al., 1997)(Young et al., 1998)(Cibelli et al., 1998)(Wells et al., 1999)(Baguisi et al., 1999)(Polejaeva et al., 2000)(Wakayama et al., 1998)(Wakayama et al., 1999)(Ogura et al., 2000). In several species,
including sheep (Wilmut et al., 1997)(Young et al., 1998), cows (Cibelli et al., 1998)(Wells et al., 1999), and mice (Wakayama et al., 1998)(Wakayama et al., 1999)(Ogura et al., 2000)(Rideout et al., 2000), placental abnormalities, fetal overgrowth and respiratory failure have been reported and are often associated with neonatal mortality. Factors that contribute to these abnormalities, also referred to as "large offspring syndrome" (Young et al., 1998), as well as the parameters that affect the long-term survival of clones remain undefined.

Vertebrate development involves programmed changes in gene expression that promote the differentiation of totipotent embryonic cells into somatic cells that build the body of the animal. It has been proposed that the pattern of gene expression inherent to a differentiated somatic cell nucleus must be returned to a totipotent embryonic state, or "reprogrammed", for nuclear clones to develop normally (Gurdon and Coleman, 1999)(Kikyo and Wolfe, 2000). In support of this hypothesis, we have demonstrated that the inactive X chromosome in female somatic cells is reprogrammed after nuclear transfer to a transcriptional and epigenetic state appropriate for embryonic development (This Thesis, Chapter 2; Eggan et al., 2000). It has been suggested however, that inefficient and faulty reprogramming may limit the long-term survival of clones or may be related to their abnormal phenotypes (Young et al., 1998)(Gurdon and Coleman, 1999)(Kikyo and Wolfe, 2000). For example, conflicting evidence has been reported concerning the deregulation of telomere length in clones derived from somatic cell nuclei, where shortened (Shiels et al., 1999), normal (Betts et al., 2000), and increased (Lanza et al., 2000) telomere lengths have all been observed.

We have previously found that a significantly higher proportion of clones derived from ES cell nuclei than clones derived from somatic nuclei developed to term (Rideout et al., 2000). This raised the question of whether the nucleus of an undifferentiated embryonic cell is easier to reprogram than that of a terminally differentiated somatic cell. In other words, is the potency of the ES cell nucleus to direct development of a clone intrinsically higher than that of a somatic nucleus? Alternatively, clone survival may be influenced by undefined parameters of the nuclear
transfer procedure itself that may differentially affect survival or development of clones derived from ES cell as opposed to somatic nuclei. For example, it has been proposed that the cell cycle stage of the donor cell and recipient oocyte are critical for normal clone development and survival but no clear evidence is available (Wilmut et al., 1997)(Cibelli et al., 1998)(Wakayama et al., 1999). Also, in the direct injection method used for cloning the mouse (Wakayama et al., 1998), the isolation of the donor nucleus involves extensive physical manipulations and it is possible that ES cell nuclei are inherently more resistant to mechanical damage then some somatic cells.

ES cells can be used as a model donor cell to determine whether the abnormal phenotypes of cloned animals are a consequence of the nuclear transfer technique itself or instead due to inherent properties of the donor cell nucleus. ES cells have the unique ability to autonomously direct embryonic development either after nuclear transfer (Rideout et al., 2000)(Wakayama et al., 1999) or after injection into tetraploid host blastocysts (Nagy et al., 1990)(Nagy et al., 1993)(Wang et al., 1997). Tetraploid mouse blastocysts are not capable of completing normal development independently (Kaufman et al., 1990), but when complemented by the introduction of diploid ES cells, develop into conceptuses where the embryo proper (epiblast) is derived entirely from the ES cells and the extraembryonic lineages arise from the tetraploid host cells (See Figure 1.2)(Nagy et al., 1990)(Nagy et al., 1993)(Wang et al., 1997). Thus, introduction of ES cells into tetraploid embryos is another method, independent of nuclear cloning, for generating mice that are completely derived from ES cells. Comparing mice derived by these two methods from the same donor ES cell lines should yield insight into the causes of abnormal phenotypes observed in cloned animals. Phenotypes observed in both ES cell clones and ES cell-tetraploid mice, are more likely due to the intrinsic developmental properties of the donor ES cell nucleus whereas phenotypes observed only in nuclear clones may be specific to the nuclear transfer procedure itself.

Previously, we have shown that mice cloned from 129/Sv inbred ES cells developed to
term but invariably died shortly after birth (Rideout et al., 2000). In contrast, when a 129/Sv x C57BL/6 F1 ES cell line was used as a source of nuclear donors, all newborns survived to adulthood (Rideout et al., 2000). These results raised the question of whether the difference in survival was due to unknown characteristics of the particular F1 and inbred ES lines used as donors or whether chromosomal heterozygosity, often referred to as “hybrid vigor”, was advantageous for long-term survival of clones.

In this study, we have compared the phenotypes of animals derived from F1 and inbred ES cells created by either tetraploid embryo complementation or nuclear cloning. We report that hybrid vigor is a general attribute beneficial for the survival of both types of ES cell derived animals. These results suggest that the death of inbred ES cell clones is not a direct result of the nuclear transfer procedure itself but instead is due to the limited developmental potential of inbred ES cells. In contrast, as fetal and placental overgrowth were primarily observed in cloned ES cell animals, our data indicate that the dramatic loss of neonatal growth control observed in cloned animals is either a direct result of the nuclear transfer procedure itself or is secondary to their abnormal placenta.

Results

Nuclear transfer with F1 and inbred ES cells. H. AKutsu and I compared donor nuclei derived from four different ES cell lines of three inbred backgrounds (129/Sv, C57BL/6 and BALB/c) with six different F1 lines (129/Sv x C57BL/6, C57BL/6 x 129/Sv, BALB/c x 129/Sv, 129/Sv x M. castaneus, C57BL/6 x BALB/c and 129/Sv x FVB) in nuclear cloning experiments. Cells from each of these ES cell lines can contribute efficiently to the germ-line after incorporation into chimeric animals (data not shown). We successfully reconstructed and activated 817 oocytes using inbred ES cell donor nuclei and 783 oocytes using F1 ES cell donor nuclei as judged by pronucleus (PN) formation. The efficiency of PN formation for all cell lines, inbred or F1, was approximately 70% (Figure 3.1). Activated oocytes with a visible PN derived
Figure 3.1. In vitro preimplantation development of ES cell clones. Values are displayed as the percentage of embryos reaching each developmental stage. For pseudo pronuclear (PN) formation rate, efficiency is expressed as the percent of total oocytes surviving reconstruction for each ES cell line. In the case of 2-cell and blastocyst stage development, efficiency is expressed as the percent of embryos out of all with PN. Data from two independent 129/Sv x 129/Sv ES cell lines was similar and therefore combined.
from either an inbred or F1 nucleus developed to the blastocyst stage with about 20% efficiency. The efficiency of cleavage-stage development was similar for clones derived from the four inbred and five F1 ES cell lines indicating that neither genetic background nor genetic heterozygosity influence in vitro pre-implantation development of ES cell clones (Figure 3.1).

To assess full-term development of inbred and F1 ES cell clones, blastocysts were transferred to pseudo-pregnant foster mothers. When delivered by caesarian section at E19 of gestation, 15 of 182 cloned inbred blastocysts (8%) and 28 of 169 cloned F1 blastocysts (17%) were found to have developed to term. However, all inbred clones died within a few minutes after delivery of apparent respiratory failure (Table 3.1A). In striking contrast, 78% of clones (22 of 28 pups) derived from the various F1 ES cell donors initiated breathing and developed into healthy adults (Table 3.1B). As all pups derived from inbred ES cells died at birth and the great majority of clones derived from F1 ES cell nuclei survived, these results confirm previous conclusions (Rideout et al., 2000) that heterozygosity of the donor cell genome is critical for the survival of ES cell clones. In addition, these results indicate that heterozygosity is of general, rather than anecdotal, importance in the survival of mice cloned from ES cells.

**Survival of ES cell animals derived by tetraploid embryo complementation.** To test whether heterozygosity of the donor ES cell genome was playing a role in the cloning process itself or instead influencing the developmental potency of the donor ES cell nucleus, I transferred cells from inbred and F1 ES lines into tetraploid blastocysts. Injection of ES cells into the blastocoel cavity of tetraploid blastocysts was aided by the use of a piezo-driven micromanipulator. After micromanipulation, the resulting composite embryos were transferred to recipient females. I injected 312 tetraploid blastocysts with four different inbred ES cell lines. After transfer to recipient females, these embryos gave rise to 20 pups (6%) that were alive and active at caesarian section. However, 17 of the 20 newborns died of respiratory failure within 30 minutes. Of the three remaining pups, two were unable to sustain respiration and died within
Table 3.1A

Survival of Inbred ES Cell Clones

<table>
<thead>
<tr>
<th>ES Cell Line</th>
<th>Genotype</th>
<th>Total Active PN</th>
<th>Blastocyst Stage-ET (%PN)</th>
<th>Pups Alive at Term (%ET)</th>
<th>Pups Surviving to Adult-hood (%Alive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J1</td>
<td>129/Sv</td>
<td>352</td>
<td>68(19)</td>
<td>6(9)</td>
<td>0</td>
</tr>
<tr>
<td>V18.6</td>
<td>129/Sv</td>
<td>178</td>
<td>40(22)</td>
<td>7(18)</td>
<td>0</td>
</tr>
<tr>
<td>V26.2</td>
<td>C57BL/6</td>
<td>164</td>
<td>40(24)</td>
<td>2(5)</td>
<td>0</td>
</tr>
<tr>
<td>V39.7</td>
<td>BALB/c</td>
<td>123</td>
<td>34(28)</td>
<td>0(0)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>817</td>
<td>182(22)</td>
<td>15(8)</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.1. Development of Inbred (A) and F1 (B) ES cell clones. Total active PN refers to the number and percent of reconstructed oocytes with observable pseudo-pronuclei. Blastocyst stage-E.T. refers to the number and percent of embryos with pseudo-pronuclei that developed to the blastocyst stage and were subsequently transferred to pseudo-pregnant recipient females. *Includes three independent subclones targeted at the Rosa26 locus.
### Table 3.1B

**Survival of F1 ES Cell Clones**

<table>
<thead>
<tr>
<th>ES Cell Line</th>
<th>Genotype</th>
<th>Total Active PN</th>
<th>Blastocyst Stage-ET (%PN)</th>
<th>Pups Alive at Term (%ET)</th>
<th>Pups Surviving to Adulthood (%Alive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V6.5*</td>
<td>C57B/6 x 129/Sv</td>
<td>381</td>
<td>79(21)</td>
<td>18(23)</td>
<td>15(80)</td>
</tr>
<tr>
<td>129B6</td>
<td>129/Sv x C57BL/6</td>
<td>66</td>
<td>18(27)</td>
<td>3(17)</td>
<td>2(100)</td>
</tr>
<tr>
<td>F1.2-3</td>
<td>129/Sv x M. cast</td>
<td>143</td>
<td>27(18)</td>
<td>3(11)</td>
<td>2(67)</td>
</tr>
<tr>
<td>V8.1</td>
<td>129/Sv x FVB</td>
<td>69</td>
<td>19(28)</td>
<td>2(11)</td>
<td>2(100)</td>
</tr>
<tr>
<td>V17.2</td>
<td>BALB/c x 129/Sv</td>
<td>99</td>
<td>21(21)</td>
<td>2(10)</td>
<td>1(50)</td>
</tr>
<tr>
<td>V30.30</td>
<td>C57BL/6 x BALB/c</td>
<td>25</td>
<td>5(20)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>-</td>
<td><strong>783</strong></td>
<td><strong>169(22)</strong></td>
<td><strong>28(17)</strong></td>
<td><strong>22(78)</strong></td>
</tr>
</tbody>
</table>
the next few hours (Table 3.2A). Only one inbred ES cell-tetraploid pup was able to sustain respiration and developed to adulthood. The manifestation of respiratory failure was remarkably similar to that observed in neonatal, inbred ES cell clones. In contrast, of 344 tetraploid blastocysts injected with 6 different F1 ES cell lines, 60 (18%) developed to birth, 51 of which (85%) survived to adulthood (Table 3.2B). Therefore, genetic heterogeneity of the donor ES cells has a significant effect on long-term survival of both nuclear clones and ES cell-tetraploid pups. In addition, these results suggest that the death of inbred ES cell clones is not a consequence of the nuclear transfer procedure but is rather due to the decreased developmental potency of inbred ES cells as compared to their F1 counterparts.

Inbred ES cell derived animals die of respiratory failure. Both ES cell-tetraploid pups and clones derived from inbred ES cells appeared to suffer from respiratory distress after delivery. Because of this similarity, I performed histological analysis of both F1 and inbred completely ES cell derived neonates. Examination of the lungs from inbred clones as well as from inbred ES cell-tetraploid pups revealed that the alveoli were not inflated. In contrast, the lungs of newborns derived from F1 ES cells were fully inflated and alveoli were expanded (Figure 3.2). In addition, interstitial bleeding within the body cavity and failed closure at the ventral mid-line were occasionally seen in inbred ES cell derived from mice of both types (data not shown). The extraembryonic tissues of the ES cell-tetraploid animals, such as the placenta, are largely derived from the tetraploid host blastocyst rather than the donor ES cells (Nagy et al., 1990), as in ES cell clones. Therefore, my data suggest that the underlying defect leading to the common cause of neonatal lethality observed in both types of inbred ES cell derived animals is likely localized to the embryonic lineages. Together, these observations suggest that the failure to initiate breathing and/or sustain normal circulation likely contributed to postnatal death of both inbred clones and inbred ES cell-tetraploid pups and that their death is not directly linked to placental malfunction.
Table 3.2A

Survival of Inbred ES Cell-Tetraploid Pups

<table>
<thead>
<tr>
<th>ES Cell Line</th>
<th>Genotype</th>
<th>4N Blasts Injected</th>
<th>Pups Alive at Term (%Inj)</th>
<th>Pups Respiring After C-Section (%Alive)</th>
<th>Pups Surviving to Adulthood (%Alive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J1</td>
<td>129/Sv</td>
<td>120</td>
<td>9(7.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V18.6</td>
<td>129/Sv</td>
<td>48</td>
<td>5(10)</td>
<td>1(20)</td>
<td>0</td>
</tr>
<tr>
<td>V26.2</td>
<td>C57BL/6</td>
<td>72</td>
<td>3(4)</td>
<td>1(33)</td>
<td>0</td>
</tr>
<tr>
<td>V39.7</td>
<td>BALB/c</td>
<td>72</td>
<td>3(4)</td>
<td>1(33)</td>
<td>1(33)</td>
</tr>
<tr>
<td>Total</td>
<td>Inbred</td>
<td>312</td>
<td>20(6)</td>
<td>3(15)</td>
<td>1(5)</td>
</tr>
</tbody>
</table>

Table 3.2. Development and survival of inbred (A) and F1 (B) ES-tetraploid mice. *Three ES cell subclones targeted at the Rosa26 locus. **ES cell subclone serially targeted, once at the Rosa26 locus and once with a random insertion.
<table>
<thead>
<tr>
<th>ES Cell Line</th>
<th>Genotype</th>
<th>4N Blasts Injected</th>
<th>Pups Alive at Term (% Inj)</th>
<th>Pups Respirating After C-Section (% Alive)</th>
<th>Pups Surviving to Adulthood (% Alive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V6.5</td>
<td>C57BL/6 x 129/Sv</td>
<td>72</td>
<td>18(25)</td>
<td>17(94)</td>
<td>16(89)</td>
</tr>
<tr>
<td>V6.5*</td>
<td>C57BL/6 x 129/Sv</td>
<td>60</td>
<td>11(18)</td>
<td>9(81)</td>
<td>9(81)</td>
</tr>
<tr>
<td>V6.5**</td>
<td>C57BL/6 x 129/Sv</td>
<td>20</td>
<td>1(15)</td>
<td>1(100)</td>
<td>1(100)</td>
</tr>
<tr>
<td>129B6</td>
<td>129/Sv x C57BL/6</td>
<td>48</td>
<td>2(4)</td>
<td>1(50)</td>
<td>1(50)</td>
</tr>
<tr>
<td>F1.2-3</td>
<td>129/Sv x M. Cast.</td>
<td>48</td>
<td>4(8)</td>
<td>3(75)</td>
<td>3(75)</td>
</tr>
<tr>
<td>V8.1</td>
<td>129/Sv x FVB</td>
<td>24</td>
<td>7(30)</td>
<td>7(100)</td>
<td>7(100)</td>
</tr>
<tr>
<td>V17.2</td>
<td>BALB/c x 129/Sv</td>
<td>48</td>
<td>13(27)</td>
<td>12(92)</td>
<td>11(85)</td>
</tr>
<tr>
<td>V30.11</td>
<td>C57BL/6 x BALB/c</td>
<td>24</td>
<td>4(30)</td>
<td>4(100)</td>
<td>3(75)</td>
</tr>
<tr>
<td>Total</td>
<td>F1</td>
<td>344</td>
<td>60(18)</td>
<td>54(90)</td>
<td>51(85)</td>
</tr>
</tbody>
</table>

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Figure 3.2: Lung histology of entirely ES cell derived mice

Figure 3.2. Hematoxylin and Eosin staining of lung sections from ES cell derived and control neonatal mice. Neonatal mice delivered by cesarean section were observed for 45 minutes for respiration. After observation, lungs were removed and placed over night in Bouin’s fixative. Following fixation, tissue was paraffin embedded, sectioned and stained.
Embryonic and placental overgrowth in ES cell derived mice. Embryonic and placental overgrowth and dysfunction have been suggested as potential causes of neonatal mortality in cloned livestock (Young et al., 1998)(Cibelli et al., 1998)(Wells et al., 1999) and mice (Wakayama et al., 1998)(Wakayama et al., 1999). This speculation led me to investigate the role of increased birth and placental weight in the survival of mice completely derived from ES cells. Neonatal mice cloned from ES cells were found to have a mean embryo weight of 2.1 grams (Figure 3.3A) and a mean placental weight of 0.32 grams (Figure 3.3B). These weights were significantly higher than those of ES cell-tetraploid pups, normal pups or pups derived from normal embryos in vitro cultured to the blastocyst stage (Figure 3.3). Neither birth nor placental weights of ES cell-tetraploid pups were significantly different from neonates derived from in vitro cultured, control embryos (Figure 3.3).

The increase in birth weight observed in ES cell clones was occasionally severe. A pup with extreme overgrowth is illustrated in Figure 3.4. This figure compares the largest recovered clone (birth weight 4.0 grams, placental weight 0.70 grams) with the largest ES cell-tetraploid pup derived from the same ES cell line (birth weight 1.8 grams, placental weight 0.15 grams).

Both extremely large and more normally sized embryos and placentas were observed in cloned conceptuses derived from both inbred and F1 ES cells. Significantly, while both large and more normal F1 ES cell clones survived postnatally, both large and more normal sized inbred ES cell clones died. It has been previously suggested that neonatal and placental overgrowth might be related to neonatal lethality in cloned animals (Young et al., 1998)(Cibelli et al., 1998)(Wells et al., 1999)(Wakayama et al., 1998)(Wakayama et al., 1999). But in our experiments no correlation between placental or embryonic overgrowth and neonatal survival was apparent.

Our data confirm other studies that suggest that either in vitro culture or transfer of embryos to pseudo-pregnant recipient mothers can cause increased placental and embryonic birth weight (Young et al., 1998). Because placental and embryonic weights of ES cell clones were
Figure 3.3. Birth (A) and placental (B) weights of ES cell derived and control neonatal mice. Birth and placental weights of clones were significantly higher than either ES cell-tetraploid mice (P<0.0001, P<0.0001) or in vitro cultured controls (P<0.0001, P<0.0001) in pair-wise comparisons. Neither birth nor placental weights of ES cell-tetraploid mice were significantly different from in vitro cultured controls (P>0.05, P>0.05). Birth and placenta weights of normal mice were significantly lower than cloned, ES cell-tetraploid or in vitro cultured pups (for all P<0.004) Pair wise comparisons were performed using the students T-test. Data from normal pups were recorded from litters with a size less than or equal to three. Cross-bars mark the mean weight for each data set. In vitro cultured, control animals, were generated by isolating two-cell stage embryos, culturing them to the blastocyst stage and then transferring them to recipient females.
Figure 3.4 Phenotype of animals derived from ES cells

Figure 3.4. ES cell clones display increased neonatal birth and placental weight. These two animals were derived from the same ES cell line, F1.2-3, one cloned by nuclear transfer, the other derived by tetraploid embryo complementation. Note the dramatic increase in both neonatal and placental size in the cloned pup. Bars = 1 cm.
significantly higher than those of ES cell-tetraploid mice or other control mice, our data indicate that the loss of neonatal growth control is either a direct consequence of the nuclear cloning procedure or secondary to some placental defect inherent to clones.

**Survival of mice after long term in vitro culture and gene targeting of ES cells.** It has been previously shown that prolonged passage of ES cells is detrimental to their developmental potency (Nagy et al., 1993)(Wang et al., 1997). Survival of F1 ES cell pups seemed to be due to the superior developmental potency of F1 ES cells rather than to the method by which they were produced. To investigate whether continuous *in vitro* culture would impair the survival of F1 ES cell-tetraploid pups, a 129Sv x C57BL/6 ES cell line (V6.5) was kept in culture for 25 passages. When these cells were injected into tetraploid blastocysts at either passage 15 or 25, live pups were generated at a frequency similar to that observed with low passage cells (data not shown). Similarly, ES cell-tetraploid mice were efficiently generated from ES cells that carried a targeted insertion at the Rosa26 locus and had been subjected to puromycin selection (Table 3.2B). In addition, F1 ES cells were subjected to two consecutive rounds of drug selection. An F1 ES cell subclone carrying the insertion at the Rosa 26 locus was transfected with a tet-inducible promoter driving expression of a hygromycin-thymidine kinase cassette and subclones expressing the construct were isolated by hygromycin selection. Injection of these double-selected cells into 20 tetraploid blastocysts resulted in one full-term pup, which survived to adulthood (Table 3.2B). My results indicate that live, adult mice, entirely derived from ES cells can be generated from F1 ES cells even after long-term passage of the cells in culture or after consecutive rounds of drug selection.

**Discussion**

In this study we compared development and long-term survival of mice derived by either nuclear cloning or tetraploid embryo complementation in an effort to understand some
of the parameters that lead to abnormal development of cloned animals. We were interested
to determine whether phenotypic abnormalities frequently seen in cloned mice such as loss of
neonatal growth control, respiratory failure and neonatal mortality are a consequence of the
nuclear transfer procedure itself or are due to the intrinsic developmental potency of the nucleus.
Our results indicate that respiratory competence and neonatal survival depend on the genetic
make-up of the donor cell nucleus whereas neonatal overgrowth is either a consequence of the
nuclear transfer procedure or caused by the abnormal placenta of cloned animals.

**Hybrid vigor and the survival of completely ES cell derived mice.** We have demon-
strated that genetic heterozygosity is a crucial parameter influencing postnatal survival of mice
derived from ES cells by nuclear cloning or by tetraploid embryo complementation. Pups derived
from inbred ES cells by either method die perinatally with a similar phenotype of respiratory
failure. In contrast, the great majority (80 to 85%) of pups derived from F1 ES cells by either
procedure survived to adulthood.

Adult ES cell mice have been previously generated at a low frequency from early passage
R1 ES cells by tetraploid embryo complementation (Nagy et al., 1993) and nuclear transfer
(Wakayama et al., 1999). The R1 ES cell line was derived from an intercross between two
different 129 substrains (Nagy et al., 1993), consistent with our observations that some level of
heterozygosity in the donor cell genome is important for postnatal survival of ES cell pups. The
common respiratory phenotype observed in both cloned and ES cell-tetraploid mice suggests that
the neonatal lethality observed in ES cell clones is not due to the nuclear transfer procedure *per
se* but more likely due to some inherent inability of inbred ES cells to maintain developmental
potency. Our observation that heterozygosity of the donor ES cell genome rescues the neonatal
lethality observed in both types of ES cell-mice, even after long-term in vitro culture, is
consistent with this conclusion.
**Neonatal overgrowth is specific to cloned animals.** Increased placental and embryonic weights have been reported in cloned farm animals and in mice (Young et al., 1998)(Wakayama et al., 1998)(Wakayama et al., 1999). In our experiments increased birth and placental weights were only observed in ES cell clones and not in ES cell-tetraploid pups suggesting that this phenotype is secondary to placental dysfunction. It is not clear why the different nuclear transfer procedures used for cloning of mice or farm animals all lead to neonatal overgrowth. It is widely assumed that the “reprogramming” of the genome from a state that is appropriate for the donor cell to one that is appropriate for an early embryonic state is crucial for successful nuclear cloning (Gurdon and Coleman, 1999)(Kikyo and Wolfe, 2000). Faulty gene expression during development may result from failed epigenetic reprogramming or epigenetic instability in the donor cell population and may contribute to the phenotypes observed in mammalian clones (Gurdon and Coleman, 1998)(Kikyo and Wolfe, 2000)(Humpherys et al., 2001)(Humpherys et al., In Press).

**Practical implications.** The possibility of deriving mice directly from ES cells without the production of a chimeric intermediate has great potential for facilitating the generation of animals with multiple genetic alterations. In conventional approaches, targeted ES cells are injected into diploid blastocysts to generate chimeric founders. The derivation of mice carrying the desired mutations or transgenes requires out-crossing of the chimeras with wild type mice. Thus, the generation of compound animals that combine multiple desired mutations or transgenes in their genome entails time-consuming and expensive cycles of crossing mice derived from different chimeric founders. In contrast, the ES cell-tetraploid technology in combination with F1 ES cells allows assembling multiple genetic alterations in the same ES cell line by consecutive gene targeting cycles in vitro prior to generating mutant animals.

For example, the generation of a mouse with a homozygous, targeted mutation, would normally require a single round of in vitro ES cell gene targeting, followed by production of a
chimeric mouse. The chimeric founder would then be bred to generate heterozygous offspring that are interbred to generate mice homozygous for the desired mutation. The whole process requires a minimum of three mouse generations (or nine months of breeding). If other mutations or transgenes are to be introduced into the mutant background, the targeted mice need to be crossed with the respective transgenic strain which causes segregation of the alleles necessitating additional breeding cycles. In contrast, multiple genetic alterations could be introduced into F1 ES cells by consecutive targeting, each step requiring 2 to 4 weeks of tissue culture, followed by injection of the multiply targeted cells into tetraploid blastocysts. Unlike nuclear cloning technology, which has proven both difficult to master and transfer from laboratory to laboratory, the ES cell-tetraploid technology can easily be adopted by any laboratory experienced in the production of chimeric mice by ES cell blastocyst injection.

At present, the mechanisms that permit long-term survival of clones and ES cell-tetraploid pups derived from F1 but not from inbred ES cells are unclear. Though it is generally assumed that “hybrid vigor” is an important parameter in animal survival under various selective conditions, it is not apparent whether wide-ranging chromosomal heterozygosity or heterozygosity at only a few crucial modifier loci is required. Examining the potency of ES cells that have been derived from embryos generated by backcrossing F1 mice and their parental inbred strains may clarify this question.

Materials and Methods

Production of ES cell clones. Nuclear transfer of ES cell nuclei into enucleated metaphase II oocytes was carried out as previously described (Wakayama et al., 1998)(Wakayama et al., 1999). 1-3 hours after nuclear transfer, oocytes were activated for 5 hours with 10mM Sr²⁺ in Ca²⁺ free media in the presence of 5 mg/ml of Cytochalasin B. Embryos were cultured in vitro to the blastocyst stage and transferred to recipient mothers.
**Embryo culture.** All embryo culture was carried out in microdrops on standard bacterial petri-dishes (Falcon) under mineral oil (Squibb). Modified CZB media (Chatot et al., 1990) was used for embryo culture unless otherwise noted. Hepes buffered CZB (Chatot et al., 1990) was used for room temperature operations while long term culture was carried out in bicarbonate buffered CZB at 37° with an atmosphere of 5% CO₂ in air.

**Preparation of two cell embryos for electrofusion.** B6D2F1 females were superovulated by IP injection of 7.5 IU PMS (Calbiochem) followed 46-50 hours later with 7.5 IU HCG (Calbiochem). After administration of HCG, females were mated with B6D2F1 males. Fertilized zygotes were isolated from the oviduct 24 hours later. Zygotes were left in Hepes buffered CZB with 0.1% bovine testicular hyaluronidase for several minutes at room temperature to remove any remaining cumulus cells. After washing, zygotes were transferred to a new culture dish containing drops of bicarbonate buffered CZB and placed at 37° overnight to obtain two-cell embryos.

**Preparation of tetraploid embryos by electrofusion.** 40 hours post HCG the blastomeres of two-cell embryos were electrofused to produce once cell tetraploid embryos. Electrofusion was carried out on an inverted microscope using the lid of a petri-dish as a micromanipulation chamber. Platinum wires were used as both electrodes and micromanipulators to align two cell embryos for fusion. A group of 15 two-cell embryos was placed on the stage in a 200 ml drop of M2 media (Sigma). Embryos were aligned with the interface between their two blastomeres perpendicular to the electrical field and a single electrical pulse of 100V with a duration of 100 ms was applied to each individually. Manipulation of a single group took less then five minutes. After electrofusion, embryos were returned to CZB media at 37°. Embryos that had not undergone membrane fusion within 1 hour were discarded.
Culture of ES cells. Derivation, culture and targeted mutagenesis of ES cells were carried out as previously described (Hogan et al., 1994) with ES cell lines derived from both inbred and F1 blastocysts. ES cells were cultured in DMEM with 15% FCS containing 1000 U/ml Leukocyte Inhibiting Factor on gamma-irradiated primary feeder fibroblasts. For blastocyst injection ES cells were trypsinized, resuspended in DMEM and first pre-plated on a standard 10 cm tissue culture dish for thirty minutes to remove feeder cells and debris.

Piezo micromanipulator injection of tetraploid blastocysts. For microinjection, 5-6 blastocysts were placed in a drop of DMEM with 15% FCS under mineral oil. A flat tip microinjection-pipette with an internal diameter of 12-15 mm was used for ES cell injection. 50 ES cells were picked up in the end of the injection pipette. The blastocyst to be injected was held in the vicinity of the inner-cell-mass with a standard holding pipette. The injection pipette, containing the ES cells was pressed against the zona opposite the inner-cell-mass. A brief pulse of the Piezo (Primetech Pmm, Ibaraki, Japan) was applied and the injection needle was simultaneously pushed through the zona and trophectoderm layer into the blastocoel cavity. About 10 ES cells were then expelled from the injection pipette and pushed against the inner cell mass of the blastocyst. After injection of the entire group, blastocysts were returned to CZB media and placed at 37° until transfer to recipient females.

Recipient females and caesarean sections. Ten injected blastocysts were transferred to each uterine horn of 2.5 days post coitum pseudopregnant Swiss females that had mated with vesectomized males (Hogan et al., 1994). Recipient mothers were sacrificed at E 19.5 and pups were quickly removed from the uterus. After cleaning fluid from their air passages, pups were placed under a warming light and respiration was observed. Surviving pups were fostered to lactating BALB/c albino mothers.
Chapter 4:

Nuclear equivalence in the nervous system: cloned embryos produced from the nuclei of cortical and mature olfactory sensory neurons

Abstract

In order to investigate whether somatic recombination events participate in either the development or postnatal function of the nervous system, I have produced cloned embryos by nuclear transfer (NT) using cortical and mature olfactory sensory neurons. I employed site specific activation of a GFP reporter gene by neural specific expression of Cre recombinase to allow for better characterization of donor cell populations, prospective identification of donor cells for NT and retrospective evidence that NT embryos were derived from the donor neurons of interest. NT embryos generated from marked populations or donor neurons developed to the blastocyst stage and gave to rise to embryonic stem cell lines with an efficiency similar to embryos generated from other somatic cells commonly used for NT. After tetraploid embryo complementation, these ES cell lines gave rise to full-term cloned mice, validating my approach.

Notes

OMP::Cre;Z/EG mice were generated by K. Baldwin. K. Baldwin performed the immunostaining controls on the olfactory epithelium from OMP::Cre;Z/EG mice. Dissociation of olfactory epithelium for NT was carried out by K. Baldwin or by M. Tackett. Q. Chaing kindly taught me to produce cortical cultures from CaMKII::Cre;Z/EG mice and produced some cultures for NT. K. Hochedlinger kindly assisted on occasion with ES cell culture. Thank you to M. Rios, W. Rideout, L. Jackson-Grusby, K. Hochedlinger and S. Akbarian for helpful discussions. This work was funded by NIH grants 5-R35-CA44339 and RO1-CA84198 to R. Jaenisch.
This thesis chapter has not yet been published

**Introduction**

Of critical importance for understanding the development and function of the mammalian nervous system is elucidation of the mechanisms that underlie the generation of an incredibly large number of neurons, each with its own purpose and identity. The idea that some generator of genetic diversity in the nervous system, analogous to that used to generate diversity in the immune system, might be used to achieve this level of complexity was first posed more then ten years (Dreyer et al., 1967) before the discovery of V(D)J recombination (Hozumi and Tonegawa, 1976) (reviewed by Chun and Schatz, 1999).

Further interest in the possibility that genetic rearrangements might occur during neuronal differentiation were raised by the discovery of the large gene family encoding odorant receptors. (Buck and Axel, 1981). Studies with *in situ* hybridization (Ressler et al., 1993) and later by RT-PCR (Malnic et al., 1999) demonstrated that each olfactory sensory neuron specifically expresses only one of the approximately 1,000 different odorant receptor genes. Further work showed that only one of the two alleles encoding a particular receptor is expressed, in a manner similar to the immunoglobulin loci (Chess et al., 1994). These observations prompted speculation that odorant receptor choice in olfactory sensory neurons might be dictated by some as of yet unidentified DNA rearrangement (Mombaerts et al., 1996). However, to this date, the mechanism by which olfactory receptor choice occurs remains obscure.

Additionally, the question of whether DNA recombination events might play a role in generating diversity among neurons in the developing CNS, particularly the cerebral cortex, has been reinvigorated by recent genetic studies. Mice carrying mutations in several different genes (XRCC5, DNA Ligase IV and KU), encoding factors required for repairing DNA double strand breaks (DSB's) by non-homologous end joining (NHEJ), displayed wide spread apoptosis of newly born postmitotic neurons in the developing cortex, leading to embryonic lethality. (Gao et
al., 1998) (Frank et al., 1998). In addition to the neuronal phenotype, these mice showed a discrete block in the maturation of B and T lymphocytes as well as an increased sensitivity to ionizing radiation. These phenotypes are consistent with the known function that these NHEJ gene products have in repairing DSBs created by DNA damage and by the recombination-activating gene (RAG) products during V(D)J recombination (Li et al., 1995).

The apoptotic death of neurons observed in NHEJ mutants is particularly intriguing as detection of RAG expression has been reported only in developing lymphocytes and the exact subset of newborn neurons dying in these mutant mice (Chun et al., 1991). This observation has raised the possibility that these neurons might be dying due to developmentally induced, unrepaiured DSBs. However, only RAG1 and not RAG2 expression could be detected in these neurons, and RAG2 mutant mice show no obvious neuronal phenotype (Chun et al., 1991) (Mombaerts et al., 1992). Partial rescue of apoptosis in p53, NHEJ double mutant mice strongly suggests that these cells are inducing programmed cell death due to the presence of unrepaiured DSBs, rather than some unknown secondary function of these gene products specific to the nervous system (Gao et al., 2000). However, it remains to be determined whether apoptosis in these mutant neurons is due to an unrepaiured, developmentally induced DSRB, or instead due to some increased sensitivity of these cells to DNA damage (Gao et al., 2000).

A satisfactory resolution of the involvement of recombination events in neuronal function or development has been slowed by the lack of reliable cell culture systems in which either olfactory receptor choice or neuronal differentiation can be studied. Additionally, the small numbers of neurons in the olfactory epithelium that express any given olfactory receptor, and the unknown nature of any putative rearrangement that might occur in developing cortical neurons make, this question refractory to standard molecular or genetic analyses.

Cloning neurons from the cortex and olfactory epithelium by NT provides an opportunity to circumvent the technical problems surrounding these questions of neuronal nuclear equivalence. Any loss or rearrangement in the genetic material occurring during neuronal differentia-
tion would be expected to be present in every cell of a cloned embryo derived from that neuron. Therefore, if olfactory receptor choice were mediated by an instructive recombination event, then it might be expected that a cloned mouse derived from an olfactory neuron could express only one olfactory receptor, namely that which was expressed in the donor cell. Also, if a recombination event is responsible for generating synaptic diversity in the cortex, then it might be expected that a mouse cloned from a cortical neuron would have a simplified repertoire of neuronal synapses, perhaps leading to some detectable phenotype. Furthermore, cloning offers an opportunity to greatly amplify the amount of material derived from a single neuron, allowing standard molecular genetic techniques to be applied to this problem.

Unfortunately, the extremely inefficient nature of cloning (Wilmut et al., 1997) (Wakayama et al., 1998), particularly from differentiated cells (Hochedlinger and Jaenisch, 2002), combined with the heterogeneous nature of donor cell populations generally used for NT, make it difficult to retrospectively identify the exact nature of any particular donor cell giving rise to a cloned animal. In fact, this lack of confidence has led to the proposal that the inefficient nature of cloning may be due to the rare frequency of adult stem cells in a particular donor cell population rather than the result of inadequate or incomplete reprogramming (See Chapter 1, differentiation state of the donor cell, for a full discussion). This uncertainty is of particular concern when cloning from an extremely heterogeneous population of cells such as the cerebral cortex, which is known to contain a discrete population of stem cells (Johansson et al., 1999); (Doetsch et al., 1999). Clearly, for these studies to be meaningful, we must be able to conclude absolutely that cloned mice were derived from terminally differentiated neurons, rather than neuronal progenitors which may or may not have undergone a developmentally induced genomic change.

In an attempt to retrospectively confirm that NT embryos were derived from bona fide neurons, we have used the Cre/LoxP site-specific recombination system (Lakso et al., 1992) to specifically mark post-mitotic neurons within our donor cell populations. In the donor animals, a transgenic Cre recombinase under control of a neuron-specific promoter is activated upon
neuronal differentiation. Cre expression leads to site-specific recombination at an independent transgenic reporter allele, thereby permanently activating expression of a GFP reporter gene in the donor nucleus (Figure 4.1). Thus, donor cells are marked, and can then be chosen for NT based on intrinsic changes in genes expression caused by developmental events or terminal differentiation, rather then by potentially arbitrary morphological criteria that are subject to human error. Importantly, this strategy allows more rigorous characterization of donor cell populations for NT, prospective picking of GFP-expressing donor neurons for NT, and retrospective confirmation that GFP-positive NT embryos were indeed derived from the donor cells of interest.

A further complication in using NT to search for phenotypic consequences of recombination in the brain are the many stochastic epigenetic phenotypes found in cloned embryos and animals (Young et al., 1998);(Wakayama et al., 1998);(This Thesis Chapter 3). In a standard nuclear transfer experiment, one nucleus is injected into a single oocyte, giving rise to a single cloned embryo (Figure 4.2A). Therefore, in such an experiment, it would be difficult to distinguish between phenotypes resulting from the stochastic epigenetic consequence of the cloning procedure and the effect of decreased nuclear potency caused by some developmentally induced somatic mutation in a donor neuron. To circumvent this complication, we have utilized a two-step cloning procedure, which has recently been used to produce cloned mice from B and T lymphocytes (Figure 4.2B) (Hochedlinger and Jaenisch, 2002). In the first step of this approach, ES cell lines are produced from NT embryos. After ES cell line production, a theoretically unlimited number of mice can be produced from a given line by tetraploid embryo complementation (Nagy et al., 1990). Thus, many cloned mice may be produced from a single donor neuron, supplying an opportunity to observe stereotypical defects in the animals which might be due to some genetic change that occurred in the donor cell.

Toward understanding the importance of hypothetical genetic process that may occur during neuronal development and function, I have produced embryos and ES cell lines by NT with genetically marked populations of donor cells from the cerebral cortex and olfactory
**Figure 4.1**: Strategy for genetic marking of specific populations of neurons for nuclear transfer studies

A

**Z/EG Cre-recombinase reporter**

Novak et al 2000

\[ \begin{align*}
\text{pCAGGS} & \rightarrow \text{LacZ} \quad 3\text{xpA} \quad \text{EGFP} \quad \text{pA} \\
\text{Cre mediated recombination} & \\
\text{pCAGGS} & \rightarrow \text{EGFP} \quad \text{pA}
\end{align*} \]

B

Cell-type specific Cre mediated recombination

OMP::Cre knock-in

CaMKII Cre 93 transgene

Should be specific to:

Mature olfactory Neurons

CNS postmitotic neurons

**Figure 4.1**. A strategy for marking populations of neural donor cells for prospective and retrospective identification in nuclear transfer studies. (A) Cre recombinase dependent GFP expression in Z/EG reporter mice. The engineered allele pictured is inserted in the genome as a random transgene. In the unrecombined state, the ubiquitous CAGGGS promoter drives expression of LacZ in all tissues. GFP is not expressed because a series of triple polyadenylation signals arrest transcription. After exposure to the Cre recombinase, the LacZ gene and transcriptional terminator are removed by site specific recombination, permanently activating GFP expression. Though only the cell type of interest is marked in the donor animal, as GFP expression after recombination is also driven by the CAGGS promoter, GFP is expressed in all cells of NT derived embryos and offspring. (B) Cre recombinase mouse strains used in this study. In order to mark populations of neural cells we have produced mice that contain both the Z/EG reporter and neural specific Cre recombinases. OMP::Cre is an allele in which the recombinase has been targeted to the endogenous Olfactory Marker Protein Locus a gene expressed exclusively in all mature olfactory neurons. In this engineered allele, Cre is introduced in such a way that both the intact OMP gene and the recombinase are expressed from the same transcript. An internal ribosome entry site allows both proteins to be expressed by this bicistronic message. This allele allows for Cre expression from the endogenous OMP locus and provides extremely tight cell type specific recombination. In contrast the CaMKII Cre 93 line is a random transgenic insertion of a portion of the Calcium Modulated Kinase II gene promoter transcriptionally fused to the Cre recombinase. This promoter is expressed in many subsets of postmitotic neurons throughout the postnatal brain leading to widespread recombination in the CNS.
Figure 4.2: A two step cloning procedure for producing many cloned mice from a single donor nucleus

A

Normal cloning procedure

Marked donor neuron

B

Two-step cloning procedure

Tetraploid blastocyst

Marked donor neuron

ES cell intermediate

Figure 4.2. A two step cloning procedure for producing multiple mice from a single donor cell. (A) In the classic nuclear transfer experiment, a single nucleus is introduced into the enucleated oocyte, which is then artificially activated. After in vitro development to the blastocyst stage, this embryo is transferred in utero where it might develop into a single cloned animal. (B) For these studies we have taken a two step approach. In this approach a single donor cell is injected into an enucleated oocyte and the cloned embryo is cultured to the blastocyst stage. Instead of being transferred in vivo, this embryo is explanted in vitro and an ES cell line is derived. After ES cell line derivation, multiple mice may be produced by tetraploid embryo complementation. As these mice are all completely derived from the ES cell line, they are also all clones descended from the original donor nucleus. This strategy is particularly beneficial for our studies as the production of many mice from a single donor neuron will help us discern between stochastic, epigenetic phenotypes caused by the cloning procedure and defects which might be caused by some genetic event which occurred in the donor nucleus.
epithelium. Furthermore, I have produced cloned mice from cortical ES cell lines, validating our approach.

Results

**Isolation and characterization of marked cortical neurons for NT.** To produce populations of genetically marked cortical neurons suitable for NT, Q. Chaing and I generated cortical cultures from postnatal day 1.5 CaMKII::Cre;Z/EG compound heterozygous mice. In these mice and cortical cultures, transgenic Cre expression is driven by a fragment of the Calcium Modulated Kinase II (CaMKII) gene promoter, randomly integrated into the genome as a transcriptional fusion with the Cre recombinase gene (Minichiello et al., 1999)(Rios et al., 2001). Thus Cre expression and therefore recombination and GFP activation at the Z/EG allele should occur only in postmitotic neurons where CaMKII is normally expressed (Figure 4.1). After preparation, transgenic cortical cultures were maintained for 72-144 hours in the presence of cell cycle inhibitors before NT.

To assess the neuronal specificity of Cre-mediated recombination in these cortical cultures, I performed double immunostaining studies with both antibodies specific to GFP and antibodies specific to proteins expressed exclusively in either neuronal or glial cells (Figure 4.3). Double staining experiments with antibodies specific to GFP and a neuronal form of tubulin demonstrated that approximately 98% of cells that underwent recombination had a neuronal identity (Figure 4.3A,B). However, staining experiments with antibodies specific to GFP and GFAP revealed that a small subset of the recombined cells were GFAP positive (about 2%), suggesting that some marked cells were glial rather then neuronal in identity (Figure 4.3CD). These results have clear ramifications for the interpretation of NT experiments using these donor cells, which are discussed at length below.
Figure 4.3: Characterization of Cre mediated recombination in cortical cultures generated from CaMKII::Cre; Z/EG double heterozygous mice

Figure 4.3. Characterization of Cre mediated recombination in cortical cultures derived from CamKII::Cre; Z/EG double heterozygous mice. Cortical cultures were produced from P1.5 animals and then cultured for 72 hours in vitro in the presence of the cell cycle inhibitor ara C. (A) Immunofluorescence with an anti-GFP antibody and a FITC conjugated secondary antibody (Green) reveals a large number of cells in the culture which have undergone site-specific recombination and activated GFP expression. (B) Same field, immunostained with antibodies specific to a neuronal specific form of tubulin, visualized with a Texas red conjugated secondary antibody, then with A, overlayed. Note that essentially all of the green cells in A directly overlay with the red staining cells in B. In preliminary cell counts 98% of GFP positive cells were also positive for this neuronal form of tubulin. (C) Double immunostaining with antibodies specific to GFP (Green) and GFAP (red). GFAP is specifically expressed in subsets of glial cells. The two GFAP, GFP double positive cells in this field indicate that recombination is occurring in a small number of glial cells. Preliminary counts indicate that approximately 2% of the GFP positive cells are also GFAP positive. (D) GFAP immunostaining overlayed with DAPI blue nuclear staining strongly suggests that GFP and GFAP are present in the same cell.
Isolation and characterization of marked olfactory sensory neurons for NT. To obtain populations of marked olfactory sensory neurons for NT, K. Baldwin generated OMP::Cre; Z/EG compound heterozygous mice. In these mice, the gene sequence encoding the Cre recombinase has been targeted to the 3’ untranslated region of the endogenous olfactory marker protein (OMP) locus. Thus unlike in CaMKII::Cre mice, in OMP::Cre mice, the recombinase is expressed from the endogenous OMP locus. An internal ribosome entry site allows both OMP and the Cre recombinase to be expressed from this bicistronic message. Extensive studies have demonstrated that OMP and therefore also Cre, are expressed only in mature olfactory sensory neurons after olfactory receptor choice and expression.

To confirm that Cre-mediated recombination occurred only in mature olfactory neurons, K. Baldwin performed immunostaining with both neuron and GFP-specific antibodies on sections from OMP::Cre; Z/EG compound heterozygous mice (Figure 4.4). GFP positive cells could only be detected in the olfactory epithelium in locations consistent with their identity as olfactory sensory neurons. Furthermore, all GFP-positive cells also expressed the neural cell specific-marker, Neural Cell Adhesion Molecule (NCAM)(Figure 4.4). Mature olfactory neurons transduce olfactory stimuli via axonal projections to discrete structures in the olfactory bulb called glomeruli. GFP immunostaining of sections from the olfactory bulb of these mice revealed the presence of GFP-positive glomeruli, indicating that GFP-positive cells in the olfactory epithelium were projecting to the bulb, further confirming their identity as mature olfactory neurons (data not shown). GFP-positive cells could not be detected in locations outside of either the olfactory epithelium or bulb.

Unlike the cortex, which contains a diverse array of cell types, the olfactory epithelium is an anatomically simple tissue containing only the olfactory sensory neurons, the basal stem cells that repopulate the epithelium with mature neurons, and sustentacular support cells which line the lumen of the nasal cavity (Caggiano et al., 1994). As their name suggests, basal cells are located in the cell layer of the epithelium most distal from the lumen. GFP-positive cells
Figure 4.4: Characterization of recombination in the olfactory sensory epithilium of OMP::Cre; Z/EG double heterozygous mice.

Figure 4.4. Characterization of Cre mediated recombination in the olfactory sensory epithilium of OMP::Cre; Z/EG double heterozygous mice. Immunostaining of a 20 micron section from the olfactory epithelium. (A) Overlay of immunostaining with anti-Neural Cell Adhesion Molecule (NCAM)(red), anti-GFP(green) antibody and a DAPI nuclear stain (blue) (B) Anti-GFP immunostaining alone. (C) anti-NCAM staining alone. Note the absence of GFP positive cell bodies in both the sustentacular cell layer proximal to the lumen of the olfactory cavity and the basal cell layer most distal to the lumen.
were never found in this basal cell layer and were never found less than 3 nuclei away from this layer. Sustentacular cells are a non-neuronal, NCAM-negative population. As all GFP-positive cells were also NCAM-positive, there was no recombination in sustentacular cells. Thus, Cre-mediated site-specific recombination in these mice is highly specific and only occurs in mature olfactory sensory neurons.

**In vitro development of cloned embryos generated by NT with cortical and olfactory sensory neurons.** To produce cloned embryos derived from neuronal cells, I performed NT with marked population of donor cells from Cre;Z/EG reporter compound heterozygous mice. After trypsinization of cortical cultures derived from CaMKII::Cre;Z/EG mice or dissociation of the olfactory epithelium from OMP::Cre;Z/EG mice, cell populations were obtained that contained GFP-positive cells in similar ratios to those seen in control experiments. These GFP-positive cells could be prospectively identified by epifluorescence microscopy and picked for NT. I reconstructed 315 NT embryos with the nuclei of GFP positive cells from CaMKII::Cre;Z/EG cortical cultures (Table 4.1). 211 (67%) of the reconstructed embryos formed pseudo-pronuclei demonstrating that NT has been successful and that oocyte activation had occurred. 77 (36% of those with pronuclei) developed in vitro to the blastocyst stage.

When GFP-positive cells picked from the freshly dissociated olfactory epithelium of OMP::Cre;Z/EG compound heterozygous mice were used for NT, 208 of 323 embryos reconstructed (64%) formed pronuclei (Table 4.1). 38 of the 208 embryos with pronuclei (18%), proceeded to develop to the blastocyst stage. The lower efficiency of in vitro development to the blastocyst stage of olfactory neuron-derived NT embryos, relative to those derived from cortical neurons, may be due to variation in the enzymatic dissociation of the olfactory epithelium, which occasionally led to extremely poor cellular viability and NT embryo development. In contrast, preparation of donor cell populations from the cultured cortical neurons was more reproducible.

Importantly, 100% of the blastocysts derived by NT from both populations of donor
Table 4.1: Results of nuclear transfer with marked populations of donor neurons

<table>
<thead>
<tr>
<th>Cre mouse strain</th>
<th>Oocytes surviving (%inj)</th>
<th>Total active PN (%surv)</th>
<th>Blastocysts (%PN)</th>
<th>ES cell lines derived (%PN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMKII</td>
<td>315 (78)</td>
<td>211 (67)</td>
<td>77 (36)</td>
<td>4 (2)</td>
</tr>
<tr>
<td>OMP</td>
<td>323 (85)</td>
<td>208 (64)</td>
<td>38 (18)</td>
<td>2 (1)</td>
</tr>
</tbody>
</table>

Table 4.1. In vitro development of NT embryos generated with marked cortical and olfactory sensory donor neurons.

%inj = % injected.
%surv = % surviving injection.
PN = active pronucleus indicating successful NT and artificial oocyte activation.
cells were GFP positive when observed by GFP epifluorescence microscopy, demonstrating their neuronal provenance (Figure 4.5A, C). To ensure that NT did not lead to precocious activation of the Cre recombinase or inappropriate expression of the GFP reporter, we examined GFP fluorescence in embryos created using both GFP negative cells from the neuronal populations and cumulus cells as nuclear donors. When GFP negative donor cells from either the CaMKII::Cre; Z/EG or OMP::Cre;Z/EG neural populations were chosen for NT, all of the embryos produced were GFP negative (data not shown). Similarly, when cumulus cells from CaMKII::Cre;Z/EG double heterozygous mice were used as nuclear donors, none of the embryos expressed GFP (data not shown).

**Derivation of ES cell lines from neuronal NT embryos.** I explanted 77 blastocysts derived by NT from marked cortical cells and 38 blastocysts derived from marked olfactory sensory neurons onto mouse embryo fibroblasts to derive ES cell lines. After dissociation of primary ICM outgrowths, 4 blastocysts derived from cortical cells (2% of those embryos which formed pronuclei) and 2 blastocysts derived from olfactory sensory neurons (1% of those that formed pronuclei) gave rise to cell lines with a clear ES cell like morphology (Table 4.1) (Figure 4.5B,D). These 6 independent ES cell lines were all GFP positive when observed by epifluorescence microscopy, retrospectively demonstrating that they were descended from marked members of their respective donor cell populations.

**Generation of cloned mice by tetraploid embryo complementation with neuronal derived ES cell lines.** To produce cloned mice from the neuronal derived ES cells, I injected these ES cell lines into tetraploid blastocysts (Table 4.2). I injected a total of 319 blastocysts with 3 of the cortical derived ES cell lines and then transferred the blastocysts to pseudopregnant recipient females. After cesarean section, a total of 9 full term pups (3%) were recovered. Of these 9 pups, 5 established a strong respiratory rhythm and 3 survived to adulthood. All 9
Figure 4.5: Blastocysts and ES cell lines produced by nuclear transfer with marked populations of donor neurons

Figure 4.5. Bright field images of and GFP fluorescence in blastocysts and ES cell lines derived from marked populations of donor neurons (See Table 4.1). (A) Blastocysts produced by nuclear transfer with CaMKII:Cre marked donor cells from cortical culture. "-" is a negative control blastocysts. (B) ES cell line CC1, produced from one of the blastocysts pictured in A. (C) Blastocysts produced by nuclear transfer using OMP::Cre marked donor neurons isolated directly from freshly dissociated olfactory epithelium. (D) ES cell line ON1, produced from one of the blastocysts in C. In all cases NT blastocysts and ES cell lines were GFP positive, retrospectively demonstrating they were derived from the marked subset of the donor cell populations.
Table 4.2: Production of cloned mice by tetraploid embryo complementation with ES cell lines derived from marked donor neurons

<table>
<thead>
<tr>
<th>ES cell line</th>
<th>Cell-type derived from</th>
<th>4N blastocysts injected</th>
<th>Pups at term (%Inj)</th>
<th>Pups respirating after C-section (%Alive)</th>
<th>Pups surviving (%Alive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1</td>
<td>Cortical</td>
<td>87</td>
<td>7 (8%)</td>
<td>5 (71%)</td>
<td>3 (43%)</td>
</tr>
<tr>
<td>CC2</td>
<td>Cortical</td>
<td>200</td>
<td>1 (0.5%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CC3</td>
<td>Cortical</td>
<td>pending</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC4</td>
<td>Cortical</td>
<td>132</td>
<td>2 (1.5%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ON1</td>
<td>Olfactory neuron</td>
<td>137</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ON2</td>
<td>Olfactory neuron</td>
<td>Pending</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2. Production of cloned mice by tetraploid embryo complementation.

%inj = % blastocysts injected.

%Alive = % Alive at term
pups were GFP positive in all tissues confirming that they were completely derived from the GFP positive cortical ES cells (Figure 4.6). The three surviving adults were still viable and fertile 4 months after birth, showing no obvious behavioral abnormalities. A gross histological examination of the brain from one these mice showed no obvious structural or anatomical abnormalities (data not shown). I have also injected 137 tetraploid blastocysts with one of the olfactory neuron derived ES cell lines but have not yet been able to produce full-term offspring.

Discussion

Fifty years ago, the first nuclear transfer experiments were carried out in amphibians to investigate whether cellular differentiation during development involved gradual restriction, or loss, of genetic material (Briggs and King, 1952). At that time it seemed reasonable to speculate that differences in cell fate and function might be mediated by losses or changes in the genetic material, thereby providing a genetic explanation for the lack of developmental plasticity inherent to terminally differentiated cells. More than twenty years of modern molecular biology have now revealed that the vertebrate genome remains largely intact in most differentiated cells, and that development is primarily achieved by changes in the epigenetic configuration of the genome, rather than by changes in its physical structure or sequence (Rideout et al., 2001). B and T lymphocytes are one notable exception to this rule, as execution of a developmentally induced genetic rearrangement is critical for both their survival and function in immunity (Mombaerts et al., 1992).

In this work, I have used cloning technology to create embryos by NT from populations of neuronal donor cells in order to investigate whether unknown genomic rearrangements, reminiscent of those occurring during V(D)J recombination, occur during the development of the nervous system. In particular, I was interested in determining whether olfactory receptor choice in olfactory sensory neurons is mediated by epigenetic or genetic mechanisms. Additionally, I sought to produce cloned mice from neurons of the cerebral cortex to explore whether
Figure 4.6: Neonatal mouse cloned from a marked cortical cell

(A) Normal new-born

(B) CC1 ES cell-tetraploid

Figure 4.6. Cloned mouse produced by tetraploid embryo complementation from ES cell line CC1. (A) Bright field and GFP expression in a normal wild-type newborn. (B) Bright field and GFP expression in a newborn generated from the cortical derived ES cell CC1. Note the obvious green fluorescence which demonstrates both the ES cell and cortical provenance of this animal.
recombination events play a role in generating neuronal diversity in the CNS.

I used the Cre/LoxP site-specific recombination as a cell lineage marker to genetically mark populations of donor neurons for nuclear transfer. This allowed me to not only prospectively pick specific populations of neurons for nuclear transfer and also allowed me to retrospectively confirm that NT embryos and ES cell lines were derived from the desired donor cells. Furthermore, by specifically marking populations of donor cells, I was able to more thoroughly characterize the constituency of our donor cell population then has been done previously. By immunofluorescence, I determined that 98% of the marked cells in cortical cultures derived from CaMK::Cre;Z/EG compound heterozygous mice were postmitotic neurons. However, 2% of the marked cells expressed an antigen (GFAP) that suggests they were of glial rather then neuronal identity. Therefore, I must remain skeptical as to the exact identity of any particular donor cell from our marked population of cortical cells that gave rise to any one particular cloned embryo. In contrast, careful analysis has revealed that only mature olfactory sensory neurons are marked in compound heterozygous OMP::Cre;Z/EG mice. Thus I can be confident that any NT embryo, ES cell line, or mouse created from this marked population of olfactory neurons is indeed derived from a mature olfactory neuron.

The percentage of NT embryos derived from either the olfactory epithelium or cortical culture donor cells that developed to the blastocyst stage (18%, 36% respectively) was substantially higher then the percentage of contaminating non-neuronal cells present in the marked donor cell population (0%, 2% respectively). Therefore, I can formally conclude that NT can reverse mitotic exit after terminal neuronal differentiation and that neural derived cloned embryos are competent to develop to the blastocyst stage.

The efficiency by which neuronal NT embryos developed to the blastocyst stage was similar to the efficiency of development of embryos derived from other non-dividing or slowly dividing cells, such as sertoli cells (65%) (Ogura et al., 2000), and serum starved fibroblasts (58%)(Wakayama and Yanagimachi, 1999) (See Table 1.2). However, the efficiency of generat-
ing blastocysts was higher then that observed when B and T cells were used as nuclear donors (<5%) (Hochedligner and Jaenisch, 2002). As neither resting lymphocytes in the lymph nodes nor post-mitotic neurons are actively dividing, this difference in efficiency cannot be trivially explained by differences in the relative proportion of donor cells in S-phase of the cell cycle, as it can between relatively quiescent sertoli cells and rapidly cycling ES cells. This difference could potentially be explained by technical differences between the two cell types, such as susceptibility to damage during NT, but might instead reflect differences in epigenetic reprogramming. As these two cell types have dramatically different chromatin configurations, with resting lymphocytes being substantially more transcriptionally repressed then postmitotic neurons, this is an attractive hypothesis.

I was able to generate ES cell lines from several neuronal NT embryos. The efficiency by which I was able to derive these lines (2% of successful cortical NTs, 1% of successful olfactory neuron NTs) was again comparable to the efficiency by which ES cell lines have been previously generated after somatic cell NT (1-3%) (Wakayama et al., 2001) (Rideout et al., 2002). Unfortunately, the percentage of successful NT experiments with marked cortical cells that gave rise to ES cell lines (2%) was not different from the percentage of non-neuronal contaminants in the marked donor cell population (2%). Therefore, I cannot be confident that these ES cell lines, or the mice produced from them, were descended from bona fide neurons. In contrast, I can be confident as to the provenance of the two ES cell lines we have generated from marked olfactory neurons as there were no detectable non-neuronal cellular contaminants in this population of donor cells.

Cloned mice were produced from cortical NT ES cell lines by tetraploid embryo complementation. After injection into tetraploid blastocysts, all three of the cortical derived ES cells lines used gave rise to living pups that were completely ES cell derived as judged by GFP expression. Several of these pups from one of the ES cell lines, survived to adulthood. Thus, using Cre-mediated recombination as a marker of cellular differentiation is compatible with the
production of cloned mice. Furthermore, any toxicity caused by Cre recombinase expression (Loonstra et al., 1999) in the donor cell did not abrogate its ability to produce cloned animals, validating our approach. Our results also confirm those of others (Yamazaki et al., 2001), indicating that there is a population of cells in the cerebral cortex that retains the capacity to redirect development after NT. However, as stated above, the efficiency at which we able to derive ES cell lines from cloned cortical cells was sufficiently low that the cellular ancestry of these clones is confounded by the presence of contaminating non-neuronal cells in the marked donor cell population. Therefore, if we are to achieve our goal of producing cloned animals from cortical neurons, we must consider either methods for eliminating contaminating glial cells from our current system, or generate new strains of mice in which Cre expression is more tightly restricted within cortical neurons. Until one of these aims is met, we remain unable to address the role that genetic events might play in the function of cortical neurons.

Additionally, we have produced 2 NT ES cell lines from marked donor cells that we can formally demonstrate were mature olfactory sensory neurons. Efforts to produce cloned mice by tetraploid embryo complementation from one of these lines have, as of yet, been unsuccessful. However, any mice that are eventually produced from either of these ES cell lines will provide an excellent substrate for addressing the question of whether olfactory receptor choice is mediated by epigenetic or genetic events. The hypothesis that olfactory receptor choice is mediated exclusively by epigenetic mechanisms would predict that mice produced from these cell lines would have a normal repertoire of olfactory receptor expression. In contrast, if olfactory receptor choice is mediated in part by a DNA rearrangement event, then it might be expected that only a subset or perhaps only one odorant receptor would be expressed, perhaps leading to gross anatomical changes in the organization of the olfactory bulb.

In summary, using the nervous system as a model, we have demonstrated that the Cre/LoxP site-specific recombination is an effective means of genetically marking specific populations of donor cells for NT and that cells marked by this method can give rise to cloned
animals. Furthermore, we have demonstrated that cell cycle exit occurring during neuronal
differentiation is reversible, as NT embryos derived from both cortical and olfactory sensory
neurons can commence cleavage and developed to the blastocyst stage. Finally, we have
produced ES cell lines from mature olfactory sensory neurons, which in further studies will
be used to address whether olfactory receptor choice is regulated by genetic or epigenetic
mechanisms.

Materials and Methods

**Preparation of donor neurons for NT.** Cartical cultures were generated from
CaMKII:Cre neonatal mice as previously described (Xia et al., 1996). Olfactory epithelium were
dissected in L15 medium (Gibco)at 4 degrees then chopped into tiny pieces and incubated with
Type IV collagenase at 1ug/ml at 37 degrees for 15 minutes with occasional vigorous shaking.
The Collagenase digestion was stopped by addition of DMEM + 10% FBS. Cells were spun
down and resuspended in trituration medium (PBS + 30% Glucose + 10% FBS + Pen/ strep)
and triturated with several widths of pipette tips to produce single cell suspensions. Cells were
pelleted resuspended in PBS 10% FBS (for FACS) or L15 + 10% FBS for picking. Clumps were
removed with mesh when necessary.

**Derivation of ES cell lines from NT embryos.** Production of ES cell lines from NT
embryos was exactly as described (Hochedlinger and Jaenisch., 2002).

**Production of mice by tetraploid embryo complementation.** Production of mice by
tetraploid embryo complementation was exactly as described (Eggen et al., 2001).

**Generation of cloned embryos by NT.** Production of cloned embryos by NT was
essentially as described (Wakayama et al., 1998)(Eggen et al., 2001) except that only GFP
positive cells from the two donor populations, as identified by epifluorescence, were picked and
used for NT.
Chapter 5:

Male and female mice derived from the same ES cell clone by tetraploid embryo complementation

Abstract

We have devised a general strategy for producing female mice from 39XO embryonic stem (ES) cells derived from male cell lines carrying a targeted mutation of interest. We show that the Y chromosome is lost in 2% of sub-clones from 40XY ES cell lines rendering the identification of targeted 39XO sub-clones a routine procedure. After gene targeting, male and female mice carrying the mutation can be generated by tetraploid embryo complementation from the 40XY ES cell line and its 39XO derivatives. A single intercross then produces homozygous mutant offspring. Because this strategy avoids outcrossing and therefore segregation of mutant alleles introduced into the ES cells, the time and expense required for production of experimental mutant animals from a targeted ES cell clone is substantially reduced. Our data also indicate that ES cells have inherently unstable karyotypes, however this instability does not interfere with production of adult ES cell-tetraploid mice.

Notes

Many experiments I carried out in this chapter were subsequently repeated in a confirmatory manner by employees of Artemis Pharmaceuticals GmbH who included, A. Rhode, C. Samuel, T. Hennek, H. Tintrup, B. Zevnik and R. Kuehn. The results from Artemis confirmed ours and added significant weight to our conclusions and they are therefore included in this chapter. All experiments carried out with Art4.12 ES cell lines and derivatives were performed by Artemis. Whereas all experiments with V6.5, R26FR and WT ES cell lines were carried out by myself. J. Loring and J. Erwin assisted with ES cell culture and molecular analysis. I. Jentsch

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performed all FISH analysis. Karyotyping of ES cell tetraploid mice and identification of XX and XO offspring by real-time quantitative PCR were carried out by Artemis. C. Beard provided the Cola1 targeted ES cell clones. I would like to thank L. Jackson-Grusby, D. Page, W.M. Rideout, C. Beard, K. Hochedlinger, A. Bortvin, M. Rios and D. Menke for helpful discussions. This work was supported in part by NIH grants 5-R35-CA44339 and RO1-CA84198 to R. Jaenisch. I. Jentsch received a stipend from TILL I.D., Gräfeling, Germany.

This thesis chapter has been published as:

Introduction

Conditional control of gene expression (Gu et al., 1994)(Rodriguez et al., 2000)(Lakso et al., 1992) has enabled genetic dissection of processes in the adult, such as learning, memory (Minichiello et al., 1999) and cancer progression (Shibata et al., 1997) for genes in which germ-line null mutations lead to embryonic or neonatal lethality. However, conditional mutagenesis requires the introduction of multiple experimentally engineered genes into a single animal. Unfortunately, the production of mice carrying multiple mutant alleles or transgenes necessitates time consuming and laborious breeding. Here we describe a strategy that simplifies and accelerates the production of complex mutant mice.

I demonstrated that mice derived by tetraploid embryo complementation (ES cell-tetraploid) from F1 ES cells survive at a higher frequency then those derived from inbred cell lines, indicating that hybrid vigor plays a significant role in their neonatal survival (This Thesis Chapter 3; Eggen et al., 2001). Importantly, this increase in efficiency allows the routine production of adult male F1 ES cell-tetraploid mice that carry one or more mutations (Eggen et al., 2001).
While generating ES cell-tetraploid mice we noted that female mice were occasionally produced from male ES cell lines. This observation was consistent with previous results that showed ES cells are karyotypically unstable after long-term culture (Nagy et al., 1993) and that XO female mice are viable and fertile (Cattanach, 1962). It appeared possible, therefore, that spontaneous Y chromosome loss would allow generation of male and female mice from a single targeted ES cell clone (Figure 5.1A).

Here, we demonstrate that the Y chromosome is lost from male ES cells at a high frequency and that female ES cell-tetraploid mice can be intentionally produced from targeted male ES cell lines that have undergone Y chromosome loss. These heterozygous XO females are fertile, when mated with ES cell-tetraploid males derived from 40XY ES cell lines carrying the same mutation, thereby expediting production of homozygous mutant offspring (Figure 5.1A).

Results

High frequency of Y chromosome loss in male ES cells. To determine the frequency of Y chromosome loss in ES cell sub-clones, I used a Y chromosome-specific probe for Southern hybridization analysis (Figure 5.2A) (Lamar et al., 1984). I screened 883 sub-clones from 3 independent ES cell lines (passage 6-8) and found that 17 had lost the Y chromosome (Table 5.1). The frequency of Y chromosome loss did not seem to vary significantly between the inbred J1 ES cell line (1.7%) and the F1 V6.5 cell line (1.8%), although it was slightly higher in the F1 cell line B6129 (2.7%) (Table 5.1). This suggested that spontaneous Y chromosome loss could be used to isolate 39X0 sub-clones from targeted 40 XY ES cell lines for the production of female mice (Figure 5.1A).

Two complementary approaches were used to identify targeted X0 sub-clones from XY cell lines. In the first approach, sub-clones from the male V6.5 ES cell line, already targeted once at the Rosa26 locus with a flp recombinase reporter (R26FR) (Passemoto et al., 2002), were isolated by limiting dilution and assayed for the Y chromosome by Southern hybridization. This
Figure 5.1: Two schemes for producing mice from targeted ES cells

A

Accelerated production of mutant mice

40XY targeted ES cell line
↓
Intrinsic karyotypic instability
↓
39X0 targeted ES cell line

4N blastocyst injection

B

Normal production of mutant mice

40XY targeted ES cell line

2N blastocyst injection

Figure 5.1. Standard and accelerated production of mutant mice from ES cells. (A) The accelerated production of homozygous mutant offspring can be achieved by isolating 39X0 sub-clones from targeted 40XY ES cell lines and producing ES cell derived males and females by tetraploid embryo complementation. These heterozygous mice can then be immediately intercrossed to produce homozygous mutant offspring, eliminating independent segregation of alleles and considerably shortening the time required to generate experimental animals. (B) Standard production of mutant mice from heterozygous ES cells requires generating chimeric founder animals by introducing targeted male ES cells into diploid blastocysts. Chimeric founders must then be out crossed to fix the mutation in the male and female germ-line. These male and female heterozygous offspring are finally intercrossed to produce homozygous mutant progeny.
Figure 5.2: Y chromosome loss in in male ES cells

**Figure 5.2.** Isolation of targeted 39XO sub-clones from 40XY ES cell lines. (A) Y chromosome specific Southern analysis shows the presence of Y chromosome repetitive sequences in males but not female DNA. (B) Ethidium bromide stained loading control for figure 1C. (C) Y chromosome repeat Southern analysis showing that most of these R26FR sub-clones still contained the Y chromosome (312-314) while one had lost the Y (315). (D) Southern analysis with a Colla 5' probe demonstrated lanes were equally loaded with DNA and that sub-clones 89,90,92 contain the knock in (k.i.) allele while sub-clone 91 was not targeted, showing only the wild type (w.t.) allele. (E) Filter from figure 1D, hybridized with the Y repeat specific probe, demonstrating that sub-clone 90 in addition to having been targeted had lost the Y chromosome. In contrast, sub-clones 89, 91 and 92 had retained the Y. (F) Metaphase FISH karyotype analysis of sub-clones Art4.12R9-H10 (Y chromosome repeat positive) and Art4.12R9-Y-C8 (Y chromosome repeat negative) identified by Y chromosome specific Southern analysis, demonstrating that most cells from these sub-clones had 40XY and 39XO karyotypes respectively.
Table 5.1

<table>
<thead>
<tr>
<th>ES cell line</th>
<th>Genetic background</th>
<th>Passage # or transfection</th>
<th>Total sub-clones screened</th>
<th>Y repeat negative sub-clones (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V6.5</td>
<td>F1</td>
<td>P8-9</td>
<td>448</td>
<td>8(1.8)</td>
</tr>
<tr>
<td>B6129</td>
<td>F1</td>
<td>P6</td>
<td>146</td>
<td>4(2.7)</td>
</tr>
<tr>
<td>J1</td>
<td>Inbred</td>
<td>P7</td>
<td>289</td>
<td>5(1.7)</td>
</tr>
<tr>
<td>R26FR</td>
<td>F1</td>
<td>limit dilution</td>
<td>210</td>
<td>3(1.4)</td>
</tr>
<tr>
<td>V6.5</td>
<td>F1</td>
<td>Cre-ER</td>
<td>384</td>
<td>6(1.6)</td>
</tr>
<tr>
<td>Art4.12</td>
<td>F1</td>
<td>Cre-ER</td>
<td>384</td>
<td>8(2)</td>
</tr>
<tr>
<td>V6.5</td>
<td>F1</td>
<td>Cola1</td>
<td>80</td>
<td>1(1.3)</td>
</tr>
<tr>
<td>total</td>
<td>-</td>
<td>-</td>
<td>1941</td>
<td>35(1.8)</td>
</tr>
</tbody>
</table>
effort yielded targeted ES cell sub-clones that had lost the Y chromosome (such as sub-clone 315) with a frequency (1.4%) similar to that observed in the parental wild-type cell line (Table 5.1, Figures 5.2B, 5.2C).

In a second approach, researchers at Artemis and myself endeavored to simultaneously identify newly targeted XY and XO sub-clones. At Artemis, the V6.5 and Art4.12 ES cell lines were transfected with a Rosa26 targeting vector carrying a CreER transgene and the sub-clones were screened for both gene targeting and Y chromosome loss (Table 5.1). These experiments identified targeted sub-clones that had either retained (sub-clone Art4.12R9-H10) or lost the Y chromosome (sub-clone V6R9-Y-F4). Artemis also found sub-clones which had lost the Y chromosome but which carried random transgene integrations (sub-clone 4.12R9-Y-C8). Similarly, when the V6.5 cell line was transfected with a Colla targeting vector and 80 transformants were analyzed, I identified targeted sub-clones that had either lost (Colla #90) or retained (Colla #89) the Y chromosome (Table 5.1, Figures 5.2D, 5.2E).

To confirm that ES cells lacking the repetitive sequences recognized by our Y-specific probes possessed a 39X0 karyotype, Artemis counted Giemsa-stained chromosomes in metaphases spreads from 9 sub-clones. For all lines analyzed, a majority of spreads were found to have 39 or fewer chromosomes (data not shown). Furthermore, multiplex metaphase fluorescence in situ hybridization (FISH)(Jentsch et al., 2001) analysis of one of the sub-clones, carried out by I. Jentsch, (4.12R9-Y-C8) confirmed that it consisted primarily of cells with a 39X0 karyotype (Figure 5.2F).

**Female mice generated from 39X0 cell lines.** To determine if targeted 39X0 ES cell lines could be used to generate female mice, two of the 39X0 Flp reporter subclones (R26FR #66, R26FR #315) and two of the 39X0 Cre-ER sub-clones (V6R9-Y-F4, Art4.12R9-Y-C8) were injected into tetraploid blastocysts by both myself and Artemis. After tetraploid embryo complementation, the 39X0 cell lines all gave rise to surviving ES cell-derived offspring with
an efficiency similar to that previously observed (Table 5.2)(Eggen et al, 2001). Invariably, the 17 surviving mice derived from these four 39XO sub-clones were female (Table 5.2, Figure 5.3A). All female mice had an agouti coat color establishing their ES cell provenance (Figure 5.3A). In contrast, all animals derived from the parental 40XY ES cell line (R26FR) carrying the original targeted mutation were male (Table 5.2, Figure 5.3A). Similarly, only male offspring were observed after tetraploid blastocyst injection of the targeted 40XY Cre-ER (Art4.12R9-H10) cell line identified in parallel with 39XO subclones (Table 5.2).

All female ES cell-tetraploid mice were fertile when crossed to ES cell-tetraploid males, with an average first litter size of 6 (total litters analyzed = 14; litter size range = 3-11). As it has been reported that XO female mice have shortened reproductive life spans and decreased litter sizes (Cattanach et al., 1962);(Brook, 1983), I continued breeding three of the ES cell-tetraploid females with ES cell-derived males for six months. During this time, the females gave birth to litters at regular intervals with an average litter size of 7 (total litters analyzed = 20; litter size range = 3-12), demonstrating that decreased fertility in XO mice was not significant enough to interfere with their use in efficiently producing homozygous mutant offspring.

To verify that animals homozygous for the targeted mutation could be produced, I used a PCR-based assay to genotype offspring for the presence of the flp reporter (Figure 5.3B). Of 34 offspring from crosses between females and males derived from the same targeted ES cell clone (Figure 5.3C), five were wild-type, 20 heterozygous and 9 were homozygous for the targeted Rosa26 allele (Figure 5.3D), as expected for Mendelian segregation of the mutant locus. Similar results were obtained from intercrossing Cre-ER ES cell-tetraploid males and females (data not shown).

Female offspring derived by mating 39XO females to 40XY males are expected to consist of 2/3 normal 40XX females and of 1/3 39X0 females (due to preferential loss of XO embryos (Brook, 1983)). Because XO females have been reported to be subfertile (Cattanach, 1962)(Brook, 1983), we wished to identify XX females for further breeding. Artemis therefore
### Table 5.2

<table>
<thead>
<tr>
<th>ES cell line</th>
<th>Y repeat genotype</th>
<th>4N blastocysts injected</th>
<th>ES cell derived mice</th>
<th>Sex of adult mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>R26FR</td>
<td>+</td>
<td>68</td>
<td>7(10.2)</td>
<td>3(4.4)</td>
</tr>
<tr>
<td>R26FR #66</td>
<td>-</td>
<td>63</td>
<td>7(11.1)</td>
<td>6(9.5)</td>
</tr>
<tr>
<td>R26FR #315</td>
<td>-</td>
<td>46</td>
<td>5(10.8)</td>
<td>5(10.8)</td>
</tr>
<tr>
<td>Art4.12R9-H10</td>
<td>+</td>
<td>72</td>
<td>8(11.1)</td>
<td>8(11.1)</td>
</tr>
<tr>
<td>Art4.12R9-Y-C8</td>
<td>-</td>
<td>80</td>
<td>7(8.75)</td>
<td>5(6.25)</td>
</tr>
<tr>
<td>V6R9-Y-F4</td>
<td>-</td>
<td>182</td>
<td>15(12)</td>
<td>1(0.8)</td>
</tr>
</tbody>
</table>
Figure 5.3: Fertile male and female mutant mice produced from the same targeted ES cell line

Figure 5.3. Mutant female mice produced from 39X0 ES cells by tetraploid embryo complementation when crossed to ES cell-tetraploid males produce homozygous mutant offspring. (A) Male and female ES cell-tetraploid mice produced from targeted 40XY R26FR and 39X0 R26FR #315 ES cell lines. (B) Rosa26 genotypes of control wild-type (w.t.), heterozygous, and homozygous animals, carrying the targeted Rosa26 insertion. (C) Rosa26 genotyping of ES cell-tetraploid mice derived from R26FR, R26FR #66, and R26FR #315 cell lines demonstrating these mice are heterozygous for the targeted allele. (D) Rosa26 genotypes of the first 23 offspring delivered by R26FR #315 ES cell-tetraploid females after mating with R26FR ES cell-tetraploid males. Wild-type offspring and offspring heterozygous or homozygous for the targeted Flp reporter allele were observed at approximately the expected ratios.
employed a quantitative PCR assay using a primer pair specific to the \textit{Hp}rt gene to determine X-chromosome copy number. PCR analysis on tail DNA from 14 female offspring, derived from ES cell-tetraploid intercrosses, identified 11 mice with 2 X-chromosomes and 3 mice with 1 X-chromosome (data not shown).

\textbf{Karyotypic instability in ES cells but not ES cell derived mice.} Since we were exploiting chromosomal instability to obtain females, we were concerned that increasing karyotypic abnormalities might limit the number of times a given cell line could be targeted before losing its potential to generate mice. To directly test the influence of chromosomal abnormalities in ES cells on the development of ES cell-tetraploid animals, I. Jentsch karyotyped 18 different targeted ES cell lines and assessed their ability to produce mutant mice by tetraploid embryo complementation. Chromosomal analysis was carried out by both Giemsa staining (not shown) and mouse-multiplex-FISH (Jentsch et al., 2001) which unambiguously identified each of the chromosomes in metaphase spreads, allowing us to monitor karyotypes for chromosome loss, duplications, translocations and large deletions. Her analysis revealed that the cell lines harbored a range of abnormalities (Table 5.3, see Table 5.4 for a complete description of the abnormalities observed). While some ES lines consisted primarily of cells with a normal karyotype, other lines possessed only a minority of normal 40XY cells.

Despite this variation in karyotypic stability, all 18 sub-clones gave rise to viable offspring by tetraploid embryo complementation (Table 5.3). Surprisingly, there was no significant correlation between either the percentage of cells with abnormal karyotypes or the type of chromosomal abnormality in a particular cell line and the efficiency by which that cell line produced mice (Regression analysis: $R^2=0.02$). This observation raised the question of whether the ES cell-tetraploid animals showed karyotypic variation similar to the donor ES cells, or whether the viable mice were derived from only those ES cells in the population with a diploid karyotype. Artemis therefore performed chromosomal analysis on bone marrow cells from 4
Table 5.3

<table>
<thead>
<tr>
<th>ES cell line</th>
<th>Times serially transfected</th>
<th>Metaphase FISH</th>
<th>ES cell derived mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% 40XY</td>
<td># 40XY</td>
</tr>
<tr>
<td>4.12R9H10flpE9</td>
<td>2x</td>
<td>86</td>
<td>13</td>
</tr>
<tr>
<td>4.12R9H10flpE6</td>
<td>2x</td>
<td>80</td>
<td>12</td>
</tr>
<tr>
<td>4.12R9A12flpA11</td>
<td>2x</td>
<td>80</td>
<td>12</td>
</tr>
<tr>
<td>4.12R9A12flpC2</td>
<td>2x</td>
<td>73</td>
<td>11</td>
</tr>
<tr>
<td>V6R9-C3-2</td>
<td>1x</td>
<td>73</td>
<td>11</td>
</tr>
<tr>
<td>4.12R9-G6a</td>
<td>1x</td>
<td>66</td>
<td>10</td>
</tr>
<tr>
<td>4.12R9-A12a</td>
<td>1x</td>
<td>60</td>
<td>9</td>
</tr>
<tr>
<td>V6R9-E12</td>
<td>1x</td>
<td>53</td>
<td>8</td>
</tr>
<tr>
<td>4.12R9H10flpE2</td>
<td>2x</td>
<td>53</td>
<td>8</td>
</tr>
<tr>
<td>4.12R9-Y-C8</td>
<td>1x</td>
<td>50a, b</td>
<td>8c</td>
</tr>
<tr>
<td>4.12R9B8flpF1</td>
<td>2X</td>
<td>47</td>
<td>7</td>
</tr>
<tr>
<td>V69-D7</td>
<td>1x</td>
<td>47</td>
<td>7</td>
</tr>
<tr>
<td>4.12R9H10</td>
<td>1x</td>
<td>46</td>
<td>7</td>
</tr>
<tr>
<td>4.12R9-B8</td>
<td>1x</td>
<td>43</td>
<td>6</td>
</tr>
<tr>
<td>4.12R9B8flpC11</td>
<td>2x</td>
<td>43</td>
<td>7</td>
</tr>
<tr>
<td>4.12R9-C9a</td>
<td>1x</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td>4.12R9A12flpD8</td>
<td>2x</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td>V6R9-G8a</td>
<td>1x</td>
<td>20</td>
<td>3</td>
</tr>
</tbody>
</table>

* Mice derived from these lines were used for somatic cell karyotyping.

a%39X0, #39X0, #<39>.
Table 5.4

<table>
<thead>
<tr>
<th>ES cell line</th>
<th>Times serially transfected</th>
<th>40XY %</th>
<th># 40XY</th>
<th># &lt;40&gt;</th>
<th>Metaphase FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.12R9H10flpE9</td>
<td>2x</td>
<td>86</td>
<td>13</td>
<td>2</td>
<td>13 40,XY&lt;br&gt;1 40,XY,+Y,-11&lt;br&gt;1 64&lt;n&gt;XY,-X,+Y,-1,+2,+3,-5,-6,+8,&lt;br&gt;+9,+10,+11,+12,+13,+14,+15,+17,+18,+19</td>
</tr>
<tr>
<td>4.12R9H10flpE6</td>
<td>2x</td>
<td>80</td>
<td>12</td>
<td>3</td>
<td>12 40,XY&lt;br&gt;1 41,XY,del(8),+del(8)</td>
</tr>
<tr>
<td>4.12R9A12flpA11</td>
<td>2x</td>
<td>80</td>
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112
control mice and 6 ES cell-tetraploid animals derived from 4 different targeted F1 ES cell lines which contained a range of chromosomal abnormalities (27-73% metaphases with 40 chromosomes) (Table 5.3). Counting chromosomes in metaphase spreads revealed that the ES cell-tetraploid mice, like control mice, exhibit a normal karyotype, as judged by the number of metaphases with 40 chromosomes (ES cell-tetraploid mouse: 114/120 metaphases (95%); Controls: 77/80 metaphases (96%)).

**Prolonged in vitro culture and maintenance of pluripotency.** Because successful production of male and female mice carrying multiple targeted mutations requires that ES cells remain pluripotent, Artemis assessed developmental potency after subjecting the cells to consecutive rounds of in vitro manipulation and selection. They found that neither one nor two rounds of in vitro selection reduced the total frequency at which F1 ES cells gave rise to viable offspring. After injection with the wild-type ES cell line Art4.12, 10.4% of tetraploid blastocysts gave rise to viable mice (N=77 viable mice). Similarly, tetraploid blastocysts injected with ES cells from Art4.12 sub-clones, that had undergone 1 or 2 rounds of manipulation, developed into viable off-spring with efficiencies of 13.6% (N=53 viable mice, 6 different sub-clones) and 11.5% (N=81 viable mice, 9 different sub-clones).

Finally, both myself and Artemis produced ES cell-tetraploid mice from Art4.12 sub-clones that have been subjected to 3 (viable mice from 9 different sub-clones) and 4 consecutive rounds of in vitro selection (viable mice from 4 different sub-clones). However, the number of injected blastocysts is too small to establish the relative efficiency of producing mice from these ES cell lines. Nevertheless, our results demonstrate that in principal, ES cells retain the potency to generate ES cell-tetraploid mice even after several consecutive rounds of in vitro genetic manipulation.
Discussion

The conventional production of genetically engineered mice is time consuming and involves (i) generation of mutant alleles in ES cells by homologous recombination, (ii) production and breeding of chimeric founder mice and (iii) crosses between the resulting mouse strains carrying the desired allele to produce homozygous mutant offspring (Figure 5.1B). The principal advantage of the tetraploid complementation technology described in this chapter is the demonstration that both heterozygous males and females may be produced directly from the same mutant ES cell line. This advance allows homozygous mutant mice to be produced in a single breeding cycle (Figure 5.1A), eliminating the need for a chimeric intermediate. We showed that 39X0 sub-clones can be readily identified from several independent male ES cell lines using established cell culture and molecular techniques, demonstrating the general utility of this procedure. As the frequency of Y chromosome loss is high, the additional work required for identification of XO sub-clones is compensated for by the shortening of breeding cycles needed to produce mice that are homozygous for a mutation and/or carry multiple transgenes.

The conventional derivation of mice carrying more than one mutant allele of interest involves the independent production of targeted ES cell lines and mutant mouse strains followed by two cycles of mating to yield compound heterozygous and finally compound homozygous mutant animals. With the strategy reported here, it should be possible to isolate 39X0 derivatives of cell lines sequentially targeted two or more times and then to produce both male and female compound heterozygous mutant mice. Knocking-out multiple genes in a single ES cell line would allow rapid production of mutant mice with all possible combinations of heterozygous and homozygous genotypes, exploring many potential phenotypes in a single cross. In addition to the expedited production of mutant mice, the tetraploid complementation approach will allow female germ-line transmission of targeted mutations or transgenes that either directly interfere with spermatogenesis (Al-Shawi et al., 1991) (Wang et al., 2001) or cause embryonic lethality after paternal inheritance (Marahrens et al., 1997).
A prerequisite for producing male and female ES cell tetraploid mice carrying multiple mutations is that ES cells retain their potency to generate mice by tetraploid embryo complementation, even after several rounds of in vitro drug selection. Our results indicate that F1 ES cell lines subjected to one or two rounds of selection produce ES cell-tetraploid mice as efficiently as the parental cell line. Furthermore, even 3 or 4 consecutive rounds of genetic manipulation did not abolish the potency of the F1 ES cells to generate ES cell-tetraploid mice, establishing the efficacy of our approach.

It must be noted, however, that the offspring of F1 ES cell-tetraploid mutant mice are a genetically heterogeneous F2 population. If mutant animals with an inbred genetic background were required, substantial backcrossing would be necessary, abrogating many benefits of our strategy. This is similar to conventional gene targeting experiments, where the production of homozygous mutant mice is routinely achieved by crossing heterozygous F1 mice generated by test crossing chimeric founders with a mouse strain of another coat color. However, with conventional methods it is possible to produce mutant mice on an inbred background by using inbred cell lines, mating chimeras to the appropriate strain and then using molecular methods to identify heterozygous offspring. Thus, unless efficient production of ES cell-tetraploid mice from inbred ES cell lines can eventually be achieved, it is important to consider whether expedited mutant production or an inbred genetic background is the priority when choosing between the strategy reported here and more conventional methods.

Finally, it has been previously reported that certain karyotypic abnormalities in ES cells may interfere with both production of ES cell-tetraploid mice (Nagy et al., 1993) and germ-line transmission of targeted mutations (Liu et al., 1997). We were therefore concerned that increasing karyotypic abnormality might limit the number of times we could target ES cells before mutant mouse production. Surprisingly we found that chromosomal abnormalities in most, if not all ES cell lines, did not interfere with their potency to generate adult mice by tetraploid embryo complementation. Interestingly, chromosomal analysis revealed that the abnormalities found in
the donor ES cells were not present in the animals. Therefore, it may be that only a small subset of the injected ES cells, those with a normal karyotype, contribute to embryo formation while the cells with karyotypic abnormalities are selected against during development. However, any chromosomal abnormality resulting in a selective growth advantage in vitro (Liu et al., 1997) would be expected to overgrow the population and eliminate its competence to generate mice. Therefore, if chromosomal analysis is performed to exclude cell lines dominated by cells with abnormal karyotypes, there may be no limit to the number of times an F1 ES cell can be genetically manipulated before mutant mouse production.

Materials and Methods

ES cell culture. Culture and targeted mutagenesis of ES cells were carried out using standard methods (Hogan et al., 1994).

Identification and isolation of targeted 40XY and 39X0 sub-clones. For isolation of 39X0 R26FR sub-clones, 5000 targeted ES cells were plated in a 10cm dish. Eight days later, ES colonies were picked and expanded for freezing. For Y chromosome Southern analysis of controls and R26FR ES cell sub-clones, DNA was digested with EcoRI, blotted and probed with a 720bp MboI fragment of pY2 (Lamar et al., 1984). For Y chromosome analysis of Colla subclones, DNA was digested with PstI and first analyzed for gene targeting with a 5’ Colla probe. After, hybridization with the Colla 5’ probe, the filter was reprobed with the pY2 MboI fragment. For Y chromosome analysis of V6.5 and Art4.12 clones transfected with the CreER vector, DNA was digested with EcoRV and analyzed for gene targeting with a 5’ Rosa26 probe then reprobed with the 1.5 kb EcoRI fragment of pY353 (Bishop et al., 1987).

Rosa26 genotyping of targeted ES cells. ES cell-tetraploid mice and their offspring. Genotyping of targeted ES cell clones was carried out by southern analysis with the Rosa26 5’
probe after EcoRI digestion (Soriano et al., 1999). Rosa26 genotyping of animals was carried out by PCR on tail DNA (Laird et al., 1991) as reported (Soriano et al., 1999).

**Production of male and female mice by tetraploid embryo complementation.** Production of mice by tetraploid embryo complementation was carried out exactly as described (Eggan et al., 2001).

**Chromosome analysis of ES cell sub-clones with metaphase FISH.** For the chromosome analysis of ES cell clones, 4 x 10^6 cells were cultured for 1 hour in the presence of 0.2 mg/ml Demicolchicin (Sigma, St. Louis, MO), trypsinised, resuspended in a hypotonic (0.56%) KCl solution and incubated for 8 minutes. The cells were sedimented, resuspended in freshly prepared fixative (3:1 ethanol:glacial acetic acid) and washed 2 times in fixative. Finally, cells were resuspended in 500ml fixative and dropped on precleaned glass slides. After air drying the metaphase spreads were analyzed by mouse multiplex-FISH as described previously (Jentsch et al., 2001). For chromosome analysis of ES cell-tetraploid mice, bone marrow cells sampled from femur were processed as described above, except that cells were not cultivated and fixation was performed at 4°C o/n. Slides were stained for 5 minutes in 2% Giemsa’s stain solution (Merck, Whitehouse Station, NJ), washed in water and air-dried. The chromosome numbers of 20 suitable metaphase spreads were counted at 1000x magnification under oil immersion.

**Determination of X-chromosome copy number.** X-chromosome copy number was determined by quantitative, real time PCR with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Genomic DNA was isolated from tail (Laird et al., 1991) and further purified by phenol/chloroform extraction. A primer pair and probe specific to the HPRT gene were used to quantify X-chromosome content, while another primer pair and probe specific for the Rosa26 locus served as an autosomal reference (HPRT primer 1: AGCGTTTCT-
GAGCCATTGCT; HPRT primer 2: TGCTACCGCTCCGGAAAG; HPRT probe (FAM labeled): CCCAACGCTCTCCCTCGCCG; Rosa26 primer 1: CTCTTCCCTCGTGATCTGCAA; Rosa26 primer 2: CCACACACCAGGTAGCCTTTAA; Rosa26 probe (VIC labeled): CCAGTCTTTCTAGAAGATGGGC GGAGTCT). 1ng of genomic DNA was tested in triplicate for amplification of HPRT or Rosa26 sequences using TaqMan Universal Master mix reagents (Applied Biosystems). The assay was standardized with DNA from normal male or female mice and the genotype of experimental offspring was determined by comparing HPRT and Rosa26 threshold cycles.
Chapter 6: Conclusion

The process by which a single totipotent cell becomes a complex organism is a unidirectional program, with each mitotic division generating new cells that gradually differentiate towards more specified fates and specialized functions. Nuclear transfer (NT) experiments have demonstrated the epigenetic nature of development and have shown that although a differentiated cell has a very limited developmental potential, the nuclei of cells retain the potency to direct embryogenesis after reintroduction into the unfertilized oocyte. Herein, we have used the mouse as a model system for understanding both the nature of epigenetic reprogramming that occurs after NT as well as its ramifications for the development of cloned animals. Specifically, we have investigated how archetypical epigenetic states are reprogrammed after NT and demonstrated that the inactive X chromosome is reactivated in NT embryos, resulting in normal X inactivation in female clones. Additionally, we have used animals derived by both tetraploid embryo complementation and nuclear transfer to investigate the factors that effect the survival of cloned animals, demonstrating that there are genetic influences on the cloning process. Interestingly, we showed these genetic factors modify the survival of mice cloned from ES cells by influencing the developmental potential of the donor ES cells rather than the reprogramming process itself. This realization has subsequently led to the development of novel methods for the expedited production of complex mutant mice. Finally, we have created cloned embryos by NT from both cortical and mature olfactory sensory neurons to address questions of nuclear equivalence in the brain and to investigate whether generation of synaptic diversity and odorant receptor choice are mediated by genetic or epigenetic events.

The phenotypes of mice completely derived from ES cells. A major question surrounding cloning by nuclear transfer is: What are the causes of abnormal phenotypes observed in cloned
animals? I reasoned that creating animals completely derived from embryonic stem cells by both NT and tetraploid (4N)-embryo complementation, and then comparing their phenotypes, might provide insight into factors that influence the cloning outcome. Injection of ES cells into 4N blastocysts, or 4N-embryo complementation, creates a conceptus in which the embryonic lineages are completely derived from the ES cells (Nagy et al., 1990). Thus from the perspective of the embryonic lineages, these animals can be considered “clones”. However, they are produced by a method independent of nuclear transfer, providing a control for NT-derived animals.

Comparing mice derived from ES cells by cloning and 4N-embryo complementation has helped determine which phenotypes (Eggan et al., 2001) (Chapter 3) and gene expression states (Humpherys et al., 2001) in cloned animals are due to intrinsic properties of the donor cell and not direct consequences of errors in reprogramming. As mice generated by tetraploid embryo complementation are produced by a method completely independent of nuclear transfer, any phenotype present in these animals cannot be a direct result of nuclear transfer. For example, neonatal respiratory failure was frequently observed in mice derived from ES cells by nuclear transfer (Wakayama et al., 1999) (Rideout et al., 2000). Animals produced by tetraploid embryo complementation proved to have an identical respiratory phenotype (Eggan et al., 2001), suggesting that this neonatal defect was caused by some problem intrinsic to the donor cell, rather than a direct result of faulty reprogramming. Further investigation has allowed us to conclude that neonatal respiratory failure in both cloned and ES cell-tetraploid animals was due to the reduced developmental potency of the inbred ES cell lines from which they were derived (Chapter 3) (Eggan et al., 2001). Importantly, these observations have lead directly to the development of strategies for the expedited production of mutant mice (Chapter 5)(Eggan et al., 2002).

Mice produced by 4N-embryo complementation have also played a pivotal role in our efforts to determine the mechanisms that lead to abnormal imprinted gene expression in cloned mice (Figure 1.7). We observed that several imprinted genes, including $H19$ and $Igf2$, were
abnormally expressed in the placentas and organs of mice cloned from ES cells (Humpherys et al., 2001). This observation raised the question of whether these changes in gene expression were directly caused by the cloning process or instead due to preexisting epigenetic abnormalities in the donor ES cells. Analysis of imprinted gene expression in mice produced from the same ES cell lines by 4N-embryo complementation revealed almost identical gene expression abnormalities. These abnormalities could then be traced back to epigenetic defects in the donor ES cells. Thus loss of normal imprinted gene expression in these cloned animals was caused by environmentally induced, epigenetic instability in the donor cell population rather then by the NT procedure per se.

More recently, mice produced by tetraploid embryo complementation have provided a control for cloned mice in transcriptional profiling experiments (Humpherys et al., in press). These experiments have allowed us to produce distinct lists of candidate genes whose disrupted expression is specifically caused by NT or alternatively is the result of intrinsic properties of the donor cell.

The nature of epigenetic information which can and cannot be reprogrammed after NT. The production of Dolly the sheep irrefutably demonstrated that some nuclei in the adult retained the capacity to redirect embryogenesis after transfer into an enucleated oocyte. However, it remained unclear whether cloning involved a process of active reprogramming, in which the epigenetic conformation of the genome was reprogrammed to an embryonic ground-state (Gurdon and Coleman, 1999).

To address whether or not developmental reprogramming was occurring at the molecular level, we investigated whether NT could remove the epigenetic marks that impose the silent transcriptional state of the inactive X chromosome and then reinstate them on either X, resulting in random X inactivation (Eggan et al., 2001)(Chapter 2). Our experiments using genetically-marked X chromosomes revealed that the inactive X chromosome was reactivated after NT, and
that X inactivation occurred in cloned females in a manner largely analogous to that in which it occurs in normal mice (Eggen et al., 2001)(Chapter 2). Our documentation of normal X inactivation in cloned mice, provided some of the first molecular evidence for active reprogramming of epigenetic information after NT.

Subsequent to our studies, abnormalities in X linked gene expression have been reported in cloned neonatal cattle (Xue et al., 2002). However, the foundation of this study was the quantification of X linked gene expression by RT-PCR in tissues derived from deceased neonatal clones, which was found to be lower then in surviving clones. From these results, the authors conclude that deceased clones possessed aberrant patterns of X inactivation that lead to neonatal mortality. However, cloned cattle derived from male and female cells die of neonatal complications with similar frequency (Lanza et al., 2000) (Wells et al., 1999), strongly arguing against a role for abnormal dosage compensation in these phenotypes. Furthermore, if cloning does cause chromosome-wide disruptions in dosage compensation as suggested (Xue et al., 2002), then development of these embryos would arrest during early embryogenesis, similar to mutant embryos in which normal X-inactivation is genetically disrupted (Marahrens et al., 1997). It seems a more likely explanation for the observed expression changes between surviving and deceased clones is the relative stability of RNA in vital and post-mortem tissue.

In contrast to epigenetic information affecting X inactivation, epigenetic information encoding mono-allelic expression of imprinted genes is not restored to a functional state in cloned mice (Humpherys et al., 2001). This observation is significant because many cloned animals display symptoms of “large offspring syndrome” including fetal and placental overgrowth, phenotypes that are reminiscent of several genetic diseases perturbing imprinted gene expression (Rideout et al., 2001). However, further investigation will be required to determine whether these abnormalities in gene expression can be directly linked to cloning phenotypes.

Information controlling imprinted gene expression is primarily established during gametogenesis, with allele specific marks being only maintained postzygotically (Figure 1.5) (Bartolo-
mei and Tilghman, 1997) (Latham et al., 1994) (Tremblay et al., 1995). Therefore it is perhaps not surprising that in the absence of gametogenesis, that normal imprinted gene expression cannot be restored in cloned embryos. In contrast, both X inactivation and telomere length restoration, processes normally occurring in the developing embryo, are robustly reprogrammed after NT. Unlike genomic imprinting, these processes are not directly dependent on epigenetic information established during gametogenesis. Thus, it seems reasonable to extrapolate that other epigenetic processes that are primarily controlled post-zygotically might be robustly reprogrammed after NT. In contrast, processes that are reliant on the highly specific epigenetic conformation of the haploid genomes within of the parental gametes are less likely to occur normally in clones.

This paradigm of pre-zygotic versus post-zygotic programs of gene expression could be particularly relevant to the placental abnormalities that are universal to all cloned mammals. During mammalian development, the extraembryonic lineages are the first to be allocated and to differentiate. Thus, it seems reasonable that these lineages would be most dependent on patterns of gene expression preprogrammed by the epigenetic confirmation of the gametes. The somatic nucleus, in contrast, is certainly not poised to activate genes required for extraembryonic differentiation. Therefore, it may be that failure to prepare the genome for extraembryonic differentiation, in the extremely short window between NT and trophectoderm allocation, is the cause of universal placental overgrowth and dysfunction in cloned animals.

To test this hypothesis, it will be crucial to derive mice from cells that have the endogenous developmental potential to generate the extraembryonic lineages. In particular, the embryonic blastomeres are an attractive donor cell-type for this analysis, as they retain the endogenous potency to direct both embryonic and extraembryonic development. Our model suggests that embryos created by NT with embryonic blastomeres should have patterns of gene expression most similar to normal embryos, since these cells would be prepared to execute extraembryonic development and would therefore lack aberrant placental phenotypes.
Epigenetic vs. genetic control of development. Cloning by nuclear transfer provides an opportunity to test whether certain developmental events are controlled by epigenetic or genetic events (Briggs and King, 1952). For instance, X inactivation is a developmentally regulated epigenetic event which can be completely reprogrammed after NT, leading to normal patterns of X inactivation in the cloned animal (Eggan et al., 2001). In contrast, generation of immunoglobulin gene diversity by V(D)J recombination is in part a genetic process that cannot be reprogrammed in NT. Therefore, cloned animals produced from mature B lymphocytes have a "pseudo monoclonal" character and express a much more limited repertoire of immunoglobulin gene products (Hochedlinger and Jaenisch, 2002).

We have used embryos cloned from both cortical and olfactory sensory neurons in a preliminary effort to investigate whether genomic rearrangements, reminiscent of those that occur during V(D)J recombination, mediate CNS synaptic diversity or odorant receptor choice. Because of the inefficient nature of cloning terminally differentiated cells by NT, we used a strategy that genetically marks donor neural nuclei for both prospective and retrospective identification during the cloning process. Animals carrying both a neuron specific Cre recombinase as well as a recombinase dependent GFP reporter gene were used as a source of donor cells. Theoretically, Cre-mediated recombination, and therefore fluorescence, should occur only in the neural cell populations, allowing these cells to be easily selected for NT.

To generate cloned mice derived from neurons, we have undertaken a two step cloning strategy that was previously used to generate monoclonal mice from differentiated B and T lymphocytes (Hochedlinger and Jaenisch, 2002). In this strategy, ES cell lines are derived from NT embryos, followed by production of ES-cell derived animals by tetraploid embryo complementation. This strategy allows many mice to be made from a single donor cell, permitting discrimination between stochastic epigenetic phenotypes caused by the cloning procedure and stereotypical genetic phenotypes, which might be caused by some unknown somatic genetic
rearrangement,

Unfortunately, in our donor cell populations derived from the cerebral cortex, glial cells appeared at a frequency which prevented us from concluding that the ES cell lines we generated, and therefore the mice we had produced, were derived from bona fide neurons. Secondly, although we have been able to derive neuronal NT ES cells from mature olfactory sensory neurons, we have not yet been able to generate mice from these ES cell lines. Therefore, although we have validated our approach for producing cloned mice from marked donor cells we cannot conclude that there were neurons. At the moment, we are not able to conclude whether genetic events play a role in the function of these two neuronal sub-types.

**Future applications of NT: Therapeutic and reproductive cloning.** In closing, it would seem irresponsible to study even the most basic aspects of cloning by nuclear transfer and not consider the ramifications one’s work might have on the nascent field of human cloning. Beyond the use of nuclear transfer for scientific investigations and the production of transgenic livestock, attempts at both human therapeutic and reproductive applications of cloning technology are underway. Human reproductive cloning would involve the transfer of a human somatic nucleus into an enucleated human oocyte, with the intent of creating an embryo, which could then be implanted *in utero* and possibly carried to term, developing into a viable new-born. Human therapeutic cloning would similarly involve the transfer of a human somatic cell into enucleated oocyte to create a cloned embryo. However, in this case, the intent would not be to create a newborn, but instead to use this embryo in the derivation of an ES cell line that might then be used for cell replacement therapy without fear of immune rejection (Gurdon and Coleman, 1999).

Rational scientific and medical discussions as to the relative feasibility, safety and utility of both therapeutic and reproductive cloning are critical. All existing scientific data suggests that any attempts at human reproductive cloning would likely be at best extremely inefficient
and at worst extremely dangerous for both the cloned child and the mother carrying the cloned fetus. The inefficiencies in development and phenotypes inherent to cloned animals have held remarkably constant across all species from which clones have bee produced (Wilmut et al., 1997) (Cibelli et al., 1998) (Wakayama et al., 1998) (Eggen et al., 2001), suggesting that there are conserved biological mechanisms that underlie these phenotypes. Therefore, barriers to human reproductive cloning are not merely technical, as they were for the production of the first children by in vitro fertilization, but biological in nature. Many of these barriers to normalcy, including restoration of normal patterns of imprinted gene expression, are now and will likely remain insurmountable for the foreseeable future. Furthermore, as we are still cataloging the variety of phenotypes and gene expression abnormalities present in cloned animals (Tamashiro et al., 2002) (Humpherys et al., in press), it seems premature to conclude that we can devise comprehensive methods for their prevention.

In contrast, prospects for the use of human cloning technology for therapeutic applications seem more promising. In all cases documented, (Wakayama et al., 2001) (Rideout et al., 2002) there have been no appreciable differences between NT and normal ES cell lines, in their ability to differentiate in vivo into various therapeutically relevant cells such as neurons (Wakayama et al., 2001) or hematopoietic stem cells (Rideout et al., 2002). Furthermore, in vitro production of donor cells allows for selection of functional cell types for transplantation, reducing concerns that epigenetic abnormalities resulting from NT would interfere with therapeutic function. However, careful considerations must be made of how the safety of these ES cell lines can be tested before transplantation.

Socio-ethical discussions concerning the potential benefits and pitfalls of the application of nuclear transfer technology to human subjects remains critical. Human reproductive and therapeutic cloning raise considerable ethical questions that must be openly discussed and vigorously debated in public forums before steps to proceed with or ban either of these technologies are made.
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Appendix 1:

Cloning Mice by Direct Injection Nuclear Transfer

Few experimental methodologies are as captivating or as remarkable as the generation of a viable embryo through the introduction of a somatic nucleus into an enucleated oocyte (Wilmut et al., 1997). The mouse in particular is an excellent model system for cloning by nuclear transfer as it allows the full power of mouse genetics as well as a wealth of embryological knowledge to be used to address the nature of epigenetic and developmental reprogramming (Wakayama et al., 1998). However, the mouse remains one of the more difficult mammals to clone, with transfer of NT technology from laboratory to laboratory occurring at a slow rate. With these difficulties in mind I have written this appendix, which summarizes the basic methodologies we use for the generation of cloned embryos by direct injection nuclear transfer (Wakayama et al., 1998). For general information on embryo culture, mouse husbandry and embryo surgery I recommend a brief review elsewhere (Hogan et al., 1998).

Micromanipulations. All micromanipulations during NT are carried out with standard hydraulic micromanipulators (Narishige, M188NE or equivalent) on an inverted microscope with Hofman modulation contrast (such as Nikon TE200). The lid of a petri dish is routinely used as a micromanipulation chamber (Figure A1.1). We use and only recommend the Primetech Piezo micromanipulator. To cope with the significant backpressure required for piezo microinjection, we recommend the IM6-2 microinjector (Narishige), which also allows for the fine control required for NT manipulations.
Figure A1.1: Micromanipulators and inverted microscope set for Piezo micromanipulation

Figure A1.1. Micromanipulators and inverted microscope set for Piezo micromanipulation. Inverted microscope with Hofman modulation contrast set for nuclear transfer using the lid of a petri dish as a micromanipulation chamber. Note the correct angle of inclination for the Piezo micromanipulator which is between 15-20 degrees.
Isolation of Metaphase II oocytes for NT. A complete description of superovulation and microdrop embryo culture can be found elsewhere (Hogan et al., 1994) Ovulated MII oocytes for NT are isolated from 8-10 week old superovulated B6D2F1 females. Superovulation begins with IP injection of 5 IU of pregnant mare’s serum (PMS) (Calbiochem). 46-48 hours after PMS, 5 IU of human chorionic gonadotropin (HCG) (Calbiochem) is injected. 14-15 hours after administration of HCG, females are sacrificed and oviducts are isolated as previously described (Hogan et al., 1994). After dissection, oviducts are placed directly into mineral oil in a petri dish covering 3, 200μl drop of Hapes buffered CZB media (HCZB), one of which contains .1% bovine testicular hyaluronidase (Sigma). Next, oocytes are isolated in small drops of oviductal fluid by tearing the walls of the oviductal ampullae. Tearing causes the oocytes in cumulus complexes to spill out into the mineral out. Once all cumulus complexes have been removed from the oviducts they can be more easily and simultaneously moved into the drop of HZB containing hyaluronidase. After several moment the hyaluronidase will begin to dissociate the cumulus complexes. After 10 minutes, the oocytes can be moved via mouth controlled aspirator assembly to one of the other drops of HCZB media. Washing the oocytes through the final drop of HCZB should eliminate any remaining cumulus cells. After washing, oocytes should be transferred into MCZB media, which has been preequilibrated at 37° under 5% CO2 in air. Alternatively, we now also use KSOM with amino acids for long term embryo culture (Specialty media.) Oocytes should be washed through several drops of medium to remove residual HZCB. Oocytes should be used for enucleation within 2 hours after isolation.

Enucleation of MII oocytes. After isolation, groups of 15-20 oocytes are placed on the microscope stage in HCZB containing 5μg ml-1 cytochalasin B (Sigma). The oocytes should rest in cytochalasin containing medium for 5 to 10 minutes to allow actin-cytoskeletal depolymerization to occur. After cytoskeletal depolymerization, nucleation is performed as
described at length in Figure A1.2. After enucleation, oocytes should be washed through several drops of KSOM or MZB and returned the incubator. This process can then be repeated until the desired number of enucleated oocytes has been obtained.

**Preparation of cumulus cells for NT.** Cumulus cells for NT are isolated from cumulus complexes in parallel with MII oocytes. After 5 minutes of hyaluronidase treatment, cumulus cells are aspirated into a transfer pipette with a minimal amount of media and deposited in a 500μl drop of HZB under mineral oil in a small dish. This dish is then placed on ice until cells are needed for NT.

**Preparation of tail-tip cells for NT.** For isolation of mouse tail-tip cells for nuclear donors, the mouse is appropriately anesthetized, and then 1/2 of the tail is amputated. After amputation the tail wound is cauterized and the biopsy is placed into wescodyne for 2 minutes. After wescodyne treatment, the tail is washed several times through Hepes buffered saline (HBS) and the outer layer of skin is removed. After several more washes through (HBS) and finally through Dulbecco’s modified eagle medium (DMEM) with 15% fetal calf serum (FCS), the tail is chopped into very small pieces with a scalpel on the lid of a petri dish. These pieces are then split equally among 2-3 wells of a 6 well dish. These wells are then filled with DMEM containing 15% FCS. After 3 days the medium is changed and fibroblasts should be observed growing on the bottom of the dish. These cultures are best used 1-2 weeks after derivation. 24-48 hours prior to NT, DMEM with 15% FCS is replaced with DMEM containing .05% FCS in order to force the donor cells to exit the cell cycle. Finally, for NT cells are trypsenized, washed twice in DMEM with 15% FCS and placed on ice until NT.
Figure A1.2: Piezo micromanipulator enucleation of metaphase II mouse oocytes
Figure A1.2. Piezo micromanipulator enucleation of metaphase II mouse oocyte. (A) Mouse MII oocyte immobilized with a standard holding pipette. The MII spindle has been circled. Note that the spindle has an optical nature different from the rest of the oocyte and can be seen as a more refractive portion of the cytoplasm. (B) Application of Piezo micromanipulator combined with gentle suction to the enucleation pipette leads to zona “drilling”, which allows access of the blunt needle to the periviteline space. (C) Before aspirating the spindle into the enucleation pipette it is worth while to touch the spindle with the end of the pipette. If the spindle moves, then one can be confident that the needle is in the correct location and that the spindle will be removed with a minimum of cytoplasm. (D) After the pipette is in the correct location, suction is applied to the enucleation pipette leading to aspiration of the spindle into the pipette. Not that the refractive portion of the cytoplasm is being drawn into the pipette. (E) Once the spindle is 2/3 to 3/4 inside the pipette, the pipette should be drawn away from the oocyte. If done properly the spindle will remain in the pipette, removing the minimum of cytoplasm. (F) To confirm that enucleation was complete, the spindle can be observed after expulsion from the enucleation pipette. Unlike the rest of the ooplasm, which will form into a sphere when discarded from the needle, the spindle has a rigid character and will remain in a bar shaped karyoplast. Often the karyoplast will have a distinct “dog-bone” appearance.
**Direct injection nuclear transfer.** For direct injection nuclear transfer, donor cells are diluted 1:10 into a drop of HCZB containing 11% W/V PVP. It is critical that that cells are mixed thoroughly with the drop to ensure that the cells are not damaged by the ionic gradient between the media with and without the volume excluding PVP. In general, a micro injection pipette just smaller than the diameter of the donor cell is used for nuclear transfer (cumulus cell 6 mm, tail-tip cell 8-10 mm). Donor nuclei are isolated by aspirating intact cells into the injection pipette and slowly working them back and forth in the needle, while applying gentle pulses with the piezo micromanipulator. 3-10 nuclei are generally isolated for NT at one time. Just before, donor nucleus isolation a group of 10-20 enucleated oocytes should be placed on the stage in HCZB containing 5 µg ml-1 cytochalasin B. After donor nucleus isolation, the instruments are moved to the drop of media containing the enucleated oocytes and direct injection NT can be performed as describe in Figure A1.3. After NT, reconstructed embryos are washed through several drops of KSOM or MCZB and returned to the incubator.

**Oocyte activation and subsequent culture of cloned embryos.** We generally activate reconstructed NT embryos 1-3 hours after NT in Ca++ free MZCB medium containing 10 mM Sr++ and 5µg ml-1 cytochalasin B. Oocytes are activated under these conditions for 5.5-6 hours. We generally culture a maximum of 5 NT embryos per 20 ml drop of activation media. Oocyte death during activation, which can lead to Ca++ precipitation and further oocyte death, is not uncommon. Keeping a minimal number of oocytes in each drop lowers this risk to any particular NT embryo. After activation, NT embryos are washed through several drops of KSOM or MCZB. After washing, embryos are cultured in KSOM or MCZB until embryo transfer.

**Embryo transfer of cloned embryos.** For production of cloned mice, we have had best success transferring NT embryos at the 2, 4 or 32 cell stage to the oviducts of 0.5 days post coitum (dpc) Swiss Webster or B6D2F1 pseudopregnant females. We have not found transfer
Figure A1.3: Piezo micromanipulator direct injection nuclear transfer
Figure A1. continued: Piezo micromanipulator direct injection nuclear transfer
Figure A1.3. Piezo micromanipulator direct injection nuclear transfer with hole removal method. (A) After cell pickup, the instruments are returned to the culture drop containing enucleated oocytes. The oocyte to be injected should be immobilized with the largest portion of the periviteline space facing the injection pipette. (B) The blunt injection pipette cannot enter the periviteline space without the use of the Piezo. (C) Once the Piezo element is applied, the zona can be drilled. Zona drilling will cause a plug shaped piece of zona to be aspirated into the needle. After drilling, this piece of zona should be expelled in the periviteline space. (D) Expelling media into the periviteline space brings the donor nucleus towards the oocyte. (E) Just before the donor nucleus reaches the end of the pipette, the pipette should be moved forward into the oocyte forming a channel of ooplasmic membrane around the injection pipette. (F) A brief pulse of the piezo breaks the oolema, creating a small hole, allowing the donor nucleus to be deposited inside the oocyte. Note nucleus just outside of injection pipette, proximal to the opening of the holding pipette. (G) Quickly after nucleus drop off, the needle should be withdrawn just to the opening of the channel created in the membrane. (H) Once at the opening of the channel, suction should be applied. Proper suction will aspirate the membrane into the injection pipette. If this is done correctly, the hole created by microinjection is aspirated into the needle. (I) If the needle is removed carefully and quickly, the fluid ooplasmic membrane will pinch off from the end of the needle, topologically removing the hole from the membrane! (J) After hole removal the small amount of cytoplasm should be expelled from the injection needle.
of NT morula/blastocyst stage embryos to the uteri of 2.5 dpc pseudopregnant as consistently successful as others (Wakayama et al., 1998) The oviduct transfer method we use is a variation of that described elsewhere (Hogan et al., 1994). For oviduct transfer mice are anesthetized with the barbiturate, Avertin. In the mean time, NT embryos are retrieved from the incubator and moved from microdrop culture under mineral oil to microdrop culture under air. This transfer prevents oil droplets clinging on the transfer pipette from entering the reproductive tract. To access the oviduct, a small incision is made one inch from the dorsal midline, at the clear depression in the back of the animal. After cutting through the skin, another incision is made in the body cavity, being careful to avoid blood vessels and nerves. The ovary and oviduct should be clearly visible (see Hogan et al., 1994 for a diagram of the ovary and oviduct) after the second incision is made, a pair of forceps can be used to reach in and careful draw the ovary and oviduct out of the body cavity. Next locate the end of the oviduct, emanating from the ovarian bursa. Once this location has been identified, immobilize the ovary with a hemostat. Now, pick up a small amount of embryo culture media in the transfer pipette then two small air bubbles and then approximately 15 NT embryos in a small amount of media. Now hold the transfer pipette and a syringe with a 30.5 gauge needle in the same hand; poke a small hole in the oviduct with the needle just where it leaves the ovarian bursa. Be careful to avoid blood vessels in the oviduct. After making the hole, drop the syringe, adjust your grip on the transfer pipette and place the pipette into the hole made by the syringe. Next blow the embryos and the two-three air bubbles into the oviduct. The embryos should be pushed around by the air bubbles and land in the ampule. After transfer, carefully replace the ovary back into the body cavity, close the hole in the body wall with a single suture and staple closed the hole with two or three autoclips. Repeat on other oviduct or proceed to another recipient. We find this transfer method less invasive and more reliable than the oviduct transfer method in which the bursa is ripped from the ovary and the embryos introduced via the infundibulum (Hogan et al., 1994).
Cesarean section and cross fostering of cloned animals. We routinely carry out cesarean section in the morning of recipient 19.5 dpc. After sacrifice of the recipient, full-term pups are quickly but carefully removed from the uterus, the umbilicus is carefully cut and pups and their placentas arrayed in a way such that their identities can be maintained. After removal from the uterus, neonates are quickly wiped off with a Q-tip or kim-wipe paying special attention to airways. Pups are kept under a heat lamp until cross-fostering. Generally we prefer to cross-foster to Balb/C foster mothers which have delivered the previous day. To cross foster, remove the Balb/C litter is removed from the cage with a little bedding. Pups to be fostered and bedding are thoroughly mixed until pups have taken on the smell of the bedding. We generally leave 6-8 pups per foster mother.
Appendix 2:

Production of Completely ES cell Derived Mice by Tetraploid Embryo Complementation

Production of embryonic stem (ES) cell-tetraploid embryo chimeras, or tetraploid embryo complementation, has proven to be a powerful method in mammalian developmental biology. Tetraploid embryo complementation involves the production of tetraploid embryos, generally by electrofusion at the two-cell stage, followed by injection of tetraploid blastocysts with ES cells and finally transfer of these composite embryos into a suitable surrogate mother. In chimeric embryos generated by these means, the extraembryonic trophoblast lineages are completely derived from the tetraploid host embryo, while the embryonic lineages are dominated by derivatives of the ES cells (Nagy et al., 1990). The use of tetraploid embryo complementation has become particularly widespread for establishing the relative importance of a gene's function in embryonic or extraembryonic development (Rossant and Cross, 2001). By aggregating mutant embryos with tetraploid embryos or by injecting mutant ES cells into tetraploid blastocysts, it can be tested whether a developmental phenotype observed in a mutant can be rescued by the normal trophoblast tissues derived from the tetraploid component of the conceptus. Additionally, as the contribution of tetraploid cells to the embryonic lineages is either non-existent or vanishingly small, tetraploid embryo complementation provides an important means for creating completely ES cell-derived embryos and mice (Nagy et al., 1990).

Mice completely derived from ES cells by tetraploid embryo complementation (ES cell-tetraploid) survive to birth at a high frequency, formally demonstrating that ES cells are sufficiently pluripotent to give rise to all of the embryonic lineages (Nagy et al., 1990). However, it was observed that neonatal ES cell-tetraploid mice died due to respiratory failure with an extremely high penetrance (Nagy et al., 1990). This respiratory distress prevented the produc-
tion of ES cell-derived adults and therefore greatly limited the utility of this procedure. A number of ES cell lines have subsequently been identified that allow the production of adult ES cell-tetraploid mice (Nagy et al., 1993; Wang et al., 1997). However, the properties of these ES cell lines that permitted neonatal survival remained unclear. Furthermore, the efficiency by which viable mice could be produced from these lines, particularly after long-term in vitro culture, was still limited. Thus, although normal and wild-type mutant mice could be produced by tetraploid embryo-complementation, the use of this method for the production of mice from targeted ES cell lines did not become widespread due to its inefficient and unpredictable nature.

Recently, we have demonstrated that hybrid vigor plays a critical role in the survival of ES cell-tetraploid animals (Eggn et al., 2001). Animals derived from ES cells with an inbred genetic background die of respiratory failure, as previously observed (Nagy et al., 1990; Eggn et al., 2001). However, mice generated from a number of ES cell lines with an F1 genetic background develop to term and survive to maturity with a high frequency (Eggn et al., 2001). Remarkably, several of these lines retain the potency to efficiently generate viable mice even after as many as 5 serial rounds of in vitro gene targeting (Eggn et al., 2002). Thus, tetraploid embryo complementation with F1 ES cell lines represents a reasonable, reliable and easily adopted methodology for the production of mice from genetically engineered ES cells.

This appendix briefly describes methods currently used in our laboratory for the production of viable ES cell-derived mice by tetraploid embryo complementation. My aim is to provide sufficient information concerning experimental methodologies and strategies such that the reader will be able to use information garnered here to accelerate the production of mutant mice in their laboratory. With this goal in mind, these protocols are written with the assumption that the reader has prior knowledge of methodologies required for the standard production of mutant and transgenic mice. These methods include targeted mutagenesis of ES cell by homologous recombination, injection of ES cells into diploid blastocysts for creation of chimeric offspring and creation of transgenic mice by pronuclear injection. For those not well versed in these arts
and as a companion to this chapter, we recommend the excellent text by Hogan et al. Additional reviews and information concerning tetraploid embryo complementation and the production of tetraploid embryos can also be found elsewhere.

**Overall Strategy**

The conventional production of genetically engineered mice is highly time consuming and involves the generation of mutant alleles in ES cells by homologous recombination, the production and breeding of chimeric founder mice and finally, crosses between the resulting mouse strains carrying the desired allele to produce homozygous mutant offspring (Figure A2.1A). The primary advantage in using tetraploid-embryo complementation in the production of mutant mice is that completely ES cell-derived mice can be produced directly from ES cells without a chimeric intermediate (Eggan et al., 2001). Thus for instance, experimental mice carrying multiple mutations or transgenes may be directly produced from engineered ES cells without breeding (Figure A2.1B).

The utility of ES cell-derived mouse production has been further increased by our recent observation that the Y chromosome is lost from male ES cells at a high frequency (2%)(Eggan et al., 2002). Harnessing Y chromosome loss allows 39X0 female ES cell-tetraploid mice to be produced from targeted male ES cell lines. These heterozygous XO females are fertile when mated with ES cell-tetraploid males derived from 40XY ES cell lines carrying the same mutation (Eggan et al., 2002; Cattanach, 1962). Thus, our strategy allows homozygous mutant mice to be produced in a single breeding cycle, thereby greatly expediting production of homozygous mutant offspring (Figure A2.1C).

Importantly, 39X0 subclones can be readily identified from male ES cell lines using established cell culture and molecular techniques, allowing our strategy to be utilized by any laboratory already carrying out gene-targeting experiments (Eggan et al., 2002). We find that the
Figure A2.1: Production of mutant mice by 4N embryo complementation

Figure 1. Standard and accelerated production of mutant mice from ES cells. (A) Standard production of mutant mice from heterozygous ES cells requires generating chimeric founder animals by introducing targeted male ES cells into diploid blastocysts. Chimeric founders must then be out crossed to fix the mutation in the male and female germ-line. These male and female heterozygous offspring are finally intercrossed to produce homozygous mutant progeny. (B) Animals carrying multiple transgenes can be rapidly generated using serial gene targeting followed by the production of mice by tetraploid embryo complementation. For instance, tetracycline inducible gene expression requires two transgenes, a transactivator and an inducible target gene. Both of these components can be targeted to the F1 ES cells. Mice with tetracycline inducible gene expression can then be generated by tetraploid embryo complementation. (C) The accelerated production of homozygous mutant offspring can be achieved by isolating 39X0 sub-clones from targeted 40XY ES cell lines and producing ES cell derived males and females by tetraploid embryo complementation. These heterozygous mice can then be immediately intercrossed to produce homozygous mutant offspring, considerably shortening the time required to generate experimental animals.
additional work required for identification of XO subclones is greatly compensated for by the shortening of breeding cycles needed to produce mice that are homozygous for a mutation or that carry multiple transgenes.

The conventional derivation of mice carrying more than one mutant allele of interest involves the independent production of targeted ES cell lines, then mutant mouse strains, followed by two cycles of mating to yield compound heterozygous and finally compound homozygous mutant animals. Using tetraploid embryo-complementation, it is possible to isolate 39X0 derivatives of cell lines sequentially targeted multiple times and then to produce both male and female compound heterozygous mutant mice. Mating these compound heterozygotes allow production of mutant mice with all possible combinations of heterozygous and homozygous genotypes in a single mouse cross. This approach could be extremely beneficial for the genetic analysis of entire gene families or genetic pathways, allowing the rapid exploration of many potential phenotypes.

In addition to the expedited production of mutant mice, the production of female mice from male ES cell lines allows female germ-line transmission of targeted mutations or transgenes that either directly inhibit spermatogenesis (Al-Shawi et al., 1991; Wang et al., 2001) or cause developmental failure after paternal inheritance (Marahrens et al., 1997).

An important requirement for the routine production of male and female ES cell tetraploid mice carrying multiple mutations is that ES cells retain their potency to generate mice by tetraploid embryo complementation after multiple rounds of gene targeting. We have previously demonstrated that F1 ES cell lines subjected to one or two rounds of selection produce ES cell-tetraploid mice as efficiently, or more efficiently then the parental cell line (Eggan et al., 2001; Eggan et al., 2002). Furthermore, even up to 5 consecutive rounds of genetic manipulation do not abolish the potency of the F1 ES cells to generate ES cell-tetraploid mice, establishing the efficacy of the strategy described here (Eggan et al., 2002).

However, before embarking on these methods it is important to consider that the offspring
of F1 ES cell-tetraploid mutant mice are a genetically heterogeneous population of F2 animals. If mutant animals with an inbred genetic background are required for quantitative or qualitative analysis of a particular phenotype, substantial backcrossing would be necessary, abrogating many benefits of this approach.

**ES Cell Culture and Genetic Manipulations**

**Derivation, culture and in vitro gene-targeting of F1 ES cells.** Isolation, culture and gene-targeting of F1 embryonic stem cells is carried out essentially as described (Hogan et al., 1994). ES cells are cultured in ES cell medium (DMEM with 15% fetal calf serum (Hyclone), 0.1mM non-essential amino acids (Gibco), 2mM L-glutamine, 50 IU Penicillin, 50 IU Streptomycin (Gibco) and 0.1 mM Beta-mercaptoethanol (Sigma), 1000 U/ml LIF) on gelatinized tissue culture ware (Falcon) pre-plated with a mono-layer of gamma-irradiated primary mouse embryo fibroblasts (MEFs).

**Subcloning of ES cells to identify 39X0 derivatives of targeted cell lines.** To isolate 39X0 sub-clones of a targeted ES cell line we generally expand the ES cell line, trypsenize, count the cells and plate 5000 ES cells in a 10cm dish preplated with irradiated MEFs. Approximately eight days after plating, well defined ES colonies can be seen. 200-300 colonies are picked and expanded on female MEFs for freezing and DNA isolation. It is essential to expand the ES cell sub-clones on female MEFs as contaminating Y chromosome DNA from male MEFs may confound Y chromosome genotyping of the ES cell subclones. Y chromosome genotyping of subclones can be robustly carried out either by Southern hybridization or by PCR on crudely prepared genomic DNA (Laird et al., 1991). After identification, frozen sub-clones can be expanded and female mutant mice can be produced by tetraploid embryo complementation.
Y chromosome genotyping by Southern hybridization. For Y chromosome Southern analysis of ES cell subclones, DNA is digested with EcoRI, blotted and probed with a 720bp MboI fragment of the plasmid pY2 (Lamar and Palmer, 1984). Alternatively, DNA can be digested with PstI, blotted and probed with the 1.5 kb EcoRI fragment of the plasmid pY353 (Bishop and Hatat, 1987). Both of these probes hybridize to highly repetitive sequences located primarily on the Y chromosome, giving a bright signal at a range of molecular weights. It is advisable to always run a female DNA control on each blot so that the male specific pattern of bands can be easily determined. 39XO, subclones can then be recognized by the lack of these male-specific hybridization signals but remaining cross-reactivity with a small number of autosomal repeats which are also observed in the female control. 39XO subclones should be found at approximately a 1.5-2% frequency (Eggen et al., 2002).

Identification of 39XO subclones by PCR. PCR genotyping for the Y chromosome can also rapidly be carried out using primers specific for the Y linked Zfy locus. Zfy primer 1: GAT AAG CTT ACA TAA TCA CAT GGA. Zfy primer 2: CCT ATG AAA TCC TTT GCT GC. Also, primers specific for the Y chromosome EST Tet35 have been successfully used to screen for loss of the Y chromosome. For TET35 PCR, primer 1: CTCATGTAGACCAAGATGACC, primer 2: GGAATGAATGTGTCCCATGTCG. For these primer sets we perform PCR for 30-35 cycles, annealing at 60 C for 10-15 seconds and extending for 30 seconds at 72 C. These PCR products are then run on a 1% agarose gel to assay for the presence of the Y chromosome.

Karyotyping of ES cell lines during serial gene-targeting. It has been observed that certain karyotypic abnormalities in ES cells may interfere with both production of ES cell-tetraploid mice and germ-line transmission of targeted mutations (Nagy et al., 1993; Wang et al., 1997; Liu et al., 1997). Surprisingly, we have found that chromosomal abnormalities present in most, if not all ES cell lines, did not interfere with their potency to generate adult mice by tetraploid
embryo complementation (Eggan et al., 2002). Our chromosomal analyses have revealed that the
abnormalities found in donor ES cell lines are not present in the animals derived from them.
It may be that only a small subset of the injected ES cells, those with a normal karyotype,
contribute to embryo formation while the cells with karyotypic abnormalities are selected against
during development. However, any chromosomal abnormality resulting in a growth advantage
in vitro can be expected to completely overgrow the population of karyotypically normal cells
and eliminate its competence to generate mice (Liu et al., 1997. Therefore, if multiple rounds
of gene-targeting are to be performed before mutant mouse production, it may be prudent to
perform chromosomal analysis at each round of targeting to exclude lines dominated by cells
with abnormal karyotypes. If these precautions are taken, it may be that there is no limit to
the number of times an F1 ES cell line can be genetically manipulated before mutant mouse
production.

Production of Tetraploid Embryos

Tetraploid mouse embryos can be produced from diploid embryos by a number of
methods including preventing cytokinesis with Cytochalasin B (Edwards, 1958) and by chemi-
cally inducing cell-cell fusion with PEG (Eglitis, 1980). However, embryos for tetraploid
embryo complementation are generally created by electrically fusing the two blastomeres of a
two-cell embryo. The popularity of this method stems from the simplicity by which a large
number of tetraploid embryos can be generated and the extremely high efficiency by which
these embryos complete preimplantation development. In short, diploid zygotes are isolated from
super-ovulated females, cultured to the two-cell stage, when electrofusion is performed and then
cultured to the blastocyst stage at which time ES cells are injected.

In our experience, one of the most critical determinants of successfully producing mice
by tetraploid embryo complementation is the proper in vitro culture and maintenance of the
preimplantation embryos. If special attention is not paid to proper in vitro culture technique, embryo health may be compromised rendering tetraploid blastocyst production inefficient and frustrating. In our hands, tetraploid embryos develop to the blastocysts stage with 90-95% efficiency. If this efficiency is not routinely achieved, post-implantation development may be less then optimal and efficient production of mice may be compromised. In order to routinely use tetraploid embryo complementation for the production of mutant mice, researchers should expect to produce and inject a minimum of 50 tetraploid blastocysts per experiment.

Isolation and in vitro culture of preimplantation embryos. Fertilized zygotes used for the production of tetraploid embryos are isolated from superovulated B6D2F1 (NCI) females that have been mated with B6D2F1 males. Eight to twelve week old females are generally maintained in our colony for at least two weeks after arrival from the vendor before superovulation. This delay ensures time for the females to undergo day-night cycle adjustment. To induce superovulation, we first inject 5 IU of pregnant mares serum (PMS)(Calbiochem) followed 48 hours later by 5 IU of human chorionic gonadotropin (HCG) (Calbiochem). These injections are generally performed in the early afternoon. After HCG injection, females are individually housed with a B6D2F1 male overnight. Approximately 20-24 hours after HCG injection, fertilized zygotes are isolated from the oviduct. Superovulation of 8-10 females is generally sufficient for a single experiment, allowing isolation of around 200 fertilized zygotes.

All preimplantation embryo culture is carried out in microdrops on standard bacterial petri dishes (Falcon) under mineral oil (Squib). A detailed description of microdrop embryo culture and zygote isolation can be found elsewhere (Hogan et al., 1994). Briefly, after sacrificing the superovulated females, the oviducts are dissected and placed in a petri dish flooded with mineral oil, containing several 30 ml drops of M2 medium (Sigma) and several drops of M2 medium containing 0.1% w/v bovine testicular hyaluronidase (Sigma). Under the dissecting microscope the ampula containing the fertilized zygotes should be easily observed. Using a sharp
pair of forceps, the ampula can be torn releasing the zygotes, still surrounded by their cumulus masses, into the mineral oil. These cumulus masses can then be pushed into the drops of M2 media containing hyaluronidase. We find that this method allows for rapid quantitative isolation of zygotes with minimal hypertonic shock to the embryos. After several minutes in M2 with hyaluronidase the cumulus masses surrounding the zygotes will dissolve allowing the zygotes to be isolated. After cumulus mass dissociation, the zygotes should be washed through several drops of M2 media to both eliminate the hyaluronidase and to remove excess cumulus cells. Zygotes are then immediately transferred to microdrops of KSOM media (Specialty Medium) and placed at 37 with an atmosphere of 5% CO2 in air for overnight culture.

**Electrofusion of two-cell embryos.** Application of a direct current (DC) electrical-pulse across the cellular membranes separating the two blastomeres of a two-cell embryo leads to the opening of small holes in the membrane. Because of the direct juxtaposition of the two cells, these openings often resolve into small cytoplasmic bridges. As a spherical shape is the lowest energy equilibrium-state for the membrane, these bridges continue to widen until the two cells have completely fused to form one continuous cytoplasm containing two nuclei (Figure A2.2A). Thus two diploid-cells become one tetraploid-cell. For most efficient fusion at the lowest current, the electrical field must be perpendicular to the membrane junction between the two cells of the embryo. The two-cell embryo can be aligned in this manner either manually via micromanipulation or electrically with the application of an alternating current (AC) in a non-electrolyte buffer. Devices capable of applying sufficient AC and DC electrical fields are available from several sources. We recommend the CF-150B pulse generator BLS Ltd. Hungary.

Under the two sets of conditions described below it will be apparent in several minutes whether membrane fusion has begun, although it may take as long as an hour for all embryos to complete fusion. After two hours, tetraploid embryos having undergone cell-fusion must be separated from diploid embryos that have not fused. Using these methods we routinely observe
Figure A2.2: Electrofusion of two-cell mouse embryos

Figure A2.2. Electrofusion of two-cell mouse embryos. (A) Two-cell embryos 10 minutes after DC fusion. Note that the embryos are at various stages of cell fusion. (B) A two-cell embryo in which one of the two blastomeres has died during the electrofusion procedure. These diploid embryos must be removed to ensure that they do not confound the results of the tetraploid embryo complementation experiment (See text).
fusion rates of 95% without embryo lysis. However, occasionally embryos in which one of the
two blastomeres has lysed due to the electrical-treatment are observed (Figure A2.2B). Lysis
of one of the blastomeres results in a one-cell diploid embryo that can be easily confused with
the tetraploid embryos. These diploid one-cell embryos often continue to develop into diploid
blastocysts and can confound interpretation of tetraploid embryo-complementation experiments
if not eliminated. Exact field strengths and conditions may vary between pulse generators and
should be experimentally determined. In general, if the rate of cell-fusion is low, then the
DC voltage or pulse width should be increased. In contrast, if excessive embryo lysis or poor
preimplantation development is observed, voltage or pulse width should be decreased.

**Manual alignment and DC fusion of two-cell embryos.** We perform manual alignment and DC
fusion on an inverted microscope using the lid of a petri-dish as a micromanipulation chamber.
Platinum wires (200-300 mm in diameter) are used as both electrodes and micromanipulators to
align two cell embryos for fusion. A group of 15-25 two-cell embryos are placed on the stage
in a 500 ml drop of M2 media (Sigma). Note that in this case, the M2 media is not covered in
mineral oil, as mineral oil tends to coat the electrodes, causing substantial variability in electrical
field strength and cell-fusion. As media not covered in mineral oil tends to evaporate quickly,
changing the ionic strength of the media, it is critical to work quickly. In order to prevent
 hypertonic shock, operation on a single group should not take more then 5-10 minutes. For
cell-fusion, embryos are aligned with the interface between their two blastomeres perpendicular
to the electrical field and a single electrical pulse (1.5 kV/cm for 90 ms) is individually applied
to each in turn. After electrofusion, embryos are washed through several drops of KSOM and
returned to KSOM media at 37°. Each subsequent round of electrofusion should be carried out
in a fresh drop of M2 medium.

**Electrofusion by AC alignment and DC pulse.** AC alignment of the embryos, followed by
DC fusion allows many embryos to be simultaneously aligned and fused. When acting on a two-cell embryo, the AC field generates a membrane potential, polarizing both cells within the embryo. This cellular polarization has two effects. First, it causes adjacent membranes to become attracted to each other forming a tighter junction between the cells, thus helping to mediate DC cell-fusion. Second, the polarization causes the embryo to begin to rotate until the axis of the embryo is parallel to the electrical field. Thus, the interface of the two blastomeres is electrically aligned perpendicular to the subsequent DC pulse. With this method as many as 50-100 two-cell embryos can be simultaneously fused.

In order to obtain an AC field in the medium a non-electrolyte salt (generally mannitol) is required. For AC alignment and DC fusion we use a fusion medium composed of 0.3M mannitol, 0.1mM MgSO4, 50 mm CaCl2 and 3% BSA at pH 7.4. The medium should be stored frozen and is generally good for several months. Before fusion, two-cell embryos must be equilibrated in fusion medium. Equilibration is accomplished by placing the embryos at the top of a large drop of fusion medium (1 ml) and allowing them to settle to the bottom. After equilibration, the embryos may be transferred into the fusion chamber.

For this fusion method we use a fusion chamber consisting of two parallel platinum wires (500 mm gap width) immobilized on a glass petri dish (such as GSS-500, BLS Ltd.). The chamber is flooded with electrofusion medium and the equilibrated embryos are placed midway between the two electrodes. The AC field should be initiated before the embryos settle to the bottom of the chamber in order to facilitate rotation of the embryos in the electrical field. We recommend trying an initial AC field strength of two AC volts, however, the field strength required to rotate the embryos may vary from chamber to chamber and is largely influenced by electrode gap distance. The minimal field strength required to rotate the embryos should be experimentally identified and used subsequently. Some embryos may not align in the field due to adherence to chamber or other embryos and can be nudged with the end of a capillary, allowing them to align. Other embryos may never align due to asymmetry between the two blastomeres.
and will not undergo cell fusion.

Once embryo alignment is deemed to be satisfactory, the DC pulse (1.5 kV for 90 ms) is applied to initiate cell-fusion. After the DC pulse the embryos should be removed from the chamber and washed through several large drops (200 ml) of KSOM before being returned to the incubator for long-term culture.

**Culture of tetraploid embryos to the blastocyst stage.** After electrofusion, the embryos are cultured to the blastocyst stage in KSOM. The tetraploid embryos should continue to develop with timing analogous to their diploid two-cell counterparts. About 24 hours after fusion, 4-cell tetraploid embryos will undergo compaction (with similar timing to compaction of diploid 8-cell embryos). 48-56 hours after fusion tetraploid embryos should start to inflate a blastocoel cavity and be ready for ES cell-injection. Substantial delays in either compaction or blastocyst development may indicate that cell-fusion parameters were not gentle enough or that the embryo culture medium has become too old. We generally find that new KSOM medium must be made every two-weeks and that medium quality is one of the most important parameters for successful, rapid preimplantation development of tetraploid embryos.

**Piezzo-Micromanipulator injection of tetraploid blastocysts with ES cells.** We perform blastocyst injections on an inverted microscope with Hoffman modulation contrast optics using the lid of a petri dish as micromanipulation chamber (Figure A2.3). For microinjection, 10-15 blastocysts are placed in a drop of ES cell media without LIF under mineral oil. A flat tip microinjection-pipette with an internal diameter of 12-15 mm is used for ES cell injection. It is critical that the internal diameter of the injection pipette is sufficiently large that the cells are not constricted inside the injection needle. If the pipette is too small the ES cells will be lysed during blastocyst injection. 50 ES cells are generally picked up in the end of the injection pipette so that four or five blastocysts may be injected at a time. The blastocyst to be injected is held with a
Figure A2.3: Piezo micromanipulator injection of ES cells into a blastocyst
Figure A2.3: Piezo micromanipulator injection of ES cells into a blastocyst. (A) ES cells are collected in a flat tipped microinjection pipette that is slightly larger than the ES cells. If the diameter of the needle is not larger then the ES cells, then the ES cells may be killed during microinjection. After ES cell pick-up, the instruments are moved to the drop of media containing several blastocysts. (B) The blastocysts to be injected should be held adjacent to the inner cell mass if present, which may often not be the case in tetraploid blastocysts. (C) The flat tipped injection pipette is pressed against the zona and (D) the piezo micromanipulator is applied allowing the pipette to enter the blastocoel cavity. After ES cell deposition (E), the needle is removed (F) and the next blastocyst can be injected.

standard holding pipette. The injection pipette, containing the ES cells is then pressed against the zona-pallucida. A brief pulse of the Piezo-element (Primetech Pmm, Ibaraki, Japan) is applied and the injection needle is pushed through the zona and trophoderm into the blastocoel cavity. About 10 ES cells are expelled from the injection pipette and pushed against the inner surface of the blastocyst. After injection of the entire group, blastocysts are returned to KSOM media and placed at 37° until transfer to recipient females.

Embryo transfer to recipient females. After blastocyst injection, 10-12 tetraploid-ES cell embryos are transferred to each uterine horn of 2.5 days post coitum (dpc) pseudopregnant Swiss Webster recipients exactly as described (Hogan et al., 1994).

C-section and cross fostering of ES cell-tetraploid mice. Because of the high incidence of respiratory distress in ES cell-derived neonates (Nagy et al., 1990; Eggan et al., 2001), we find it extremely beneficial to deliver pups by C-section on the morning of recipient dpc 19.5. To deliver neonates, the recipient mother is sacrificed, and the pups are rapidly removed from the uterus. Special care should be taken when cutting the umbilical cords of these neonates as the umbilicus is occasionally herniated, which can cause excessive bleeding. After removal from the uterus the pups should be quickly dried with a Q-tip, paying special attention to clear all fluid from the airways of the mice. Finally, the pups are placed under a heat lamp until they are cross-fostered.

We cross-foster ES cell-tetraploid neonates delivered by C-section to Balb/c mother
which have given birth either the same or previous day. To cross foster, we briefly mix the ES cell-tetraploid neonates with a small amount of bedding from the foster mother’s cage, remove the foster mother’s litter and quickly replace it with the ES cell-tetraploid neonates. If the number of ES cell-tetraploid neonates to foster is small, we will generally also return several of the foster mother’s pups. Using these methods, around 85% of ES cell-tetraploid neonates can be expected to survive to adulthood. Rates of term development and postnatal survival for ES cell-tetraploid mice derived from several different inbred and F1 ES cell lines appear in Tables 3.2A, 3.2B, 5.2, 5.3.

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