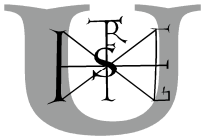

Szent István Egyetem
Állattenyésztés-tudományi Doktori Iskola

Elaboration of chimera techniques for cloning, masculinization and genetic manipulations

Kiméra-technikák kidolgozása klónozás, ivarátfordítás és genetikai manipulációk céljára

PhD értekezés

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Szent István Egyetem, Állattenyésztés-tudományi Doktori Iskola

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Az iskolavezető jóváhagyása A témavezetők jóváhagyása

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2. ABBREVIATIONS

2n - diploid
4n - tetraploid
BAC- bacterial artificial chromosome
Cre recombinase - an enzyme that catalyzes a type of site-specific homologous recombination
d.p.c. - days post coitum
dsRNA - double-stranded ribonucleic acid
EG cells - embryonic germ cells
EGFP - enhanced green fluorescent protein
ES cells - embryonic stem cells
ESGRO medium – type of ES cell culture medium from GIBCO company
EYFP – enhanced yellow fluorescent protein
FCS - fetal calf serum
FISH - fluorescence in situ hybridization
Flp - restriction fragment length polymorphism
GFP - green fluorescent protein
GnRH - gonadotropin-releasing hormone
hCG - human chorionic gonadotropin
hpg - hours post gestation
i.m. -intramuscular
i.p. - intraperitoneal
ICM - inner cell mass
ISH - in situ hybridization
LIF - leukemia inhibitory factor
LIFR - leukemia inhibitory factor receptor
MEF -mouse embryonic fibroblast
MGFP - modified green fluorescent protein
MQ water– molecular quantified water
p.c. - post coitum
PCR - polymerase chain reaction
PMSG – pregnant mare serum gonadotropin
r.p.m. – rotation per minute
RNAi - ribonucleic acid interference
R.T. – room temperature
RT PCR – reverse transcription polymerase chain reaction
TS cells - trophoblast stem cells
UV - ultraviolet light
XEN - stem cell lines from the extraembryonic endoderm

3. SUMMARY

The main task during my PhD work was to develop efficient chimera producing methods using mouse and rabbit embryos. Applying these enhanced methods I wanted to get more information about the process of sex determination and cell commitment during embryonic development.

First I examined the factors influencing the chimera forming ability of ES cells. I produced chimeras by aggregating ES cell clumps with 8-cell stage host embryos. My task was to determine the reason of difference in germ cell forming ability of different ES cell lines. We examined the chimera forming capability of R1 and R1/E mouse ES cell lines. By increasing the passage number, we could get less chimera animals, and only the R1/E ES cell line derived cells could contribute to the germ cells. We found, that the number of aneuploid cells, in R1 ES cell line, dramatically increased after 10 passages. We made X- and Y-chromosome FISH analyses. We found, that the aneuploid R1 and R1/E ES cells contained the Y-chromosome, so not the loss of the Y-chromosome caused the problem at the germ cell formation. The karyotype analysis demonstrated that in the case of R1 ES cell line, the 41 and 42 chromosome containing cells had autosomal trisomies. The proportion of trisomic cells increased proportionally with the passage number. The aneuploid ES cells can contribute to the different tissues of chimera animals, but cannot form viable germ cells.

In the second part of my work I produced chimeras using single blastomeres derived from EGFP expressing eight-cell stage embryos and diploid or tetraploid host embryos. First I followed the fate of EGFP expressing diploid blastomere derived cells in 3.5 and 4.5-day-old chimera embryos in vitro. My finding suggest that both diploid and tetraploid cells could participate in the ICM of chimeras, and selection against tetraploid cells begins before the cavitation. The diploid blastomere derived cells have significantly higher chance to contribute to the ICM, so in this way the percentage of diploid blastomere derived cells participating to the ICM of chimeric embryos could be increased.

After that I composed chimeras using single blastomeres, derived from sexed eight-cell stage embryos and tetraploid and diploid host embryos. I wanted to know wether a single blastomere could participate in the tissues of developing chimeras and is it possible to revert the sex of female (XX) host embryos using single blastomeres derived from XY embryos. I could demonstrate that a single male blastomere was able to reverse the 8-cell stage female embryo's gender. In our experiments, to generate mice with clonal origin, single blastomeres obtained from the same sexed EGFP labeled 8-cell stage embryos were complemented with unsexed tetraploid carriers. I could produce EGFP labeled twins and triplets with pre-planned gender. The clonal origin of the members of twins and the triplet was confirmed by micro-satellite analysis.

As a first step toward the ES cell derived transgenic rabbits, we had to develop a method for sex chimera rabbit production. My task was to develop a method for chromosome analysis of chimera rabbits and using FISH method for identification of the chromosomal localization of some newly sequenced genes in rabbit.

Chromosome analysis was performed from peripheral blood lymphocyte cultures. Complete mitoses (2n=44) were analyzed for the number of the smallest acrocentric chromosomes. Metaphases containing nine of the smallest chromosomes (pairs of 18, 19, 20, 21 and Y) were evaluated as male, those containing eight as female cells. All chimeric rabbits including the XX/XY hypogonadic buck were fertile and did not show growth abnormalities.

FISH technique was performed using four different rabbit BAC clones. The LIFR gene is located in man on chromosome 5p13.1, which based on comparative rabbit-human mapping data corresponds to rabbit chromosome 11. Since, we have mapped the LIFR gene in rabbit on OCU11p11.1 our result agrees with the human – rabbit comparative map and refines it.

4. ÖSSZEFOGLALÁS

Feladatom hatékony egér és nyúl kiméra előállító módszerek kidolgozása volt és az, hogy az így létrehozott kiméra embriók fejlődését vizsgálva újabb információkat szerezzek az ivari determináció, illetve a sejtek elköteleződése során zajló folyamatokról.

Munkám első részében az egér embrionális őssejtek (ES sejtek) kiméra alkotó képességét befolyásoló tényezőket vizsgáltam. Azt figyeltük meg, hogy az eltérő eredetű ES sejtvonalakból származó sejtek eltérő mértékben képesek részt venni a kiméra egerek szöveteinek kialakításában és ivarsejtek létrehozásában. Én az R1 és R1/E sejtvonalakat hasonlítottam össze. Az ES sejtek passzázs számának növekedésével egyre kevesebb kiméra állatot lehet kapni, és csak az R1/E ES sejtek tudtak ivarsejteket is létrehozni. Megállapítottam, hogy az R1 sejtek esetében már kezdetben is magas volt az aneuploid sejtek aránya. X és Y kromoszóma FISH analízist végezve azt találtam, hogy az aneuploid R1 és R1/E sejtek is tartalmaznak Y kromoszómát, így nem az Y kromoszóma elvesztése okozza az ivarsejt képzésben megfigyelt hiányosságokat. A kariotípus analízis azt mutatta, hogy a 41 és 42 kromoszómát tartalmazó aneuploid R1 ES sejtek autószomális triszómiát tartalmaznak. A triszómiát tartalmazó sejtek aránya a passzázs szám növekedésével nőtt. Az aneuploid sejtek ugyan korlátozott mértékben, de részt tudtak venni kimérák szöveteinek kialakításában, de már nem voltak képesek életképes ivarsejteket képezni.

A munkám második részében olyan kimérákat állítottam elő, melyekben egyetlen, EGFP-t expresszálló nyolcsejtes embrióból származó blasztoméra sejtet aggregáltattam nyolc sejtes diploid, vagy tetraploid gazda embrióval. Az EGFP-t expresszálló blasztomerából származó sejtek sorsát a 3.5-4.5 napos embriókban nyomon követve, megállapítottam, hogy mind a diploid, mind a tetraploid gazda embrió ICM-jébe be tudnak épülni ezek a sejtek, de mivel a tetraploid sejtek elleni szelekció még a hólyagcsíra kialakulását megelőzően megkezdődik, a tetraploid gazda embrióban a diploid blasztomerából származó sejtek nagyobb arányban tudtak beépülni az ICM-be, mint diploid gazda embriók esetében.

Később szexált embrióból származó EGFP-t expresszálló blasztomérákat aggregáltattam diploid gazda embriókkal. Arra voltam kíváncsi, vajon egyetlen XY genotípusú embrióból származó blasztoméra képes-e részt venni az állat különböző szöveteinek felépítésében, illetve képes-e átfordítani egy XY genotípusú blasztoméra az XX genotípusú gazda embrióval aggregáltatva létrehozott kiméra állat ivarát. Bebizonyítottam, hogy egyetlen XY genotípusú blasztomer is képes az XX genotípusú embrió ivarát megváltoztatni. Kísérletünk további részében egy-egy olyan blasztomert aggregáltattam tetraploid gazda embriókkal, amely ugyan abból az EGFP-t expresszáló, szekált embrióból származtak. Ezzel a módszerrel sikerült ikerpárokat és hármas iker egereket is előállítanom. Ezzel a tetraploid komplemntációs módszerrel lehetségessé vált előre meghatározott nemmel rendelkező, identikus ikrek előállítása. Az ikrek klonális eredetét a mikroszatellit analízis is igazolta.

ES kiméra nyulak létrehozásához egy ivarsejt kiméra utódok létrehozására is alkalmas kiméra előállítási módszert kellett kifejleszteni. Ehhez kapcsolódva olya kromoszóma vizsgálati rendszert dolgoztam ki, mely alkalmas annak megállapítására, hogy az adott kiméra sejtjei milyen gonoszómákat hordoznak. FISH módszerrel, a csoportunkban újonnan szekvenált néhány nyúl gén kromoszómális lokalizációját is sikerült meghatározni. A kromoszóma vizsgálatokat limfocita tenyészetekből származó premarátumokon végeztem. A komplett mitózisokat (2n=44) analizálva meghatároztam a legkisebb akrocentrikus kromoszómák számát. Kilenc kis akrocentrikus kromoszóma (egy-egy pár 18, 19, 20, 20, 21 és Y) esetében a sejt XY-, nyolc ilyen kromoszóma esetében pedig XX genotípusú. A vizsgált kiméra nyulak mindegyike, még a hipogonádiás XX/XY bak is fertilis volt.

A FISH analízis négy különböző BAC klón felhasználásával történt. Az eredmények igazolták, hogy a nyúl LIF receptor gén a 11-es nyúl kromoszómán, az OCU11p11.1 régióban található.

5. INTRODUCTION AND THE AIM OF MY WORK

Recently the interest in chimeras increased again when embryonic stem cells became available (until now mostly in the mouse) and when subtle genetic manipulations at the level of single genes enabled one to modify and produce genetically transformed ES cells. This represents an alternative way of modifying the genome more precisely and predictably than injection of genes into pronuclei of zygotes, but requires transferring the transformed cells into 'carrier' embryos in order to introduce them (and their descendants) into the germ line to obtain gametes. Although mouse ES cells have supported discoveries in different research fields, their value was established as a tool to enable targeted mutagenesis. Several years earlier it was demonstrated that, when injected into mouse blastocysts, genetically altered ES cells could generate transgenic offspring. The application of these techniques with homologous recombination technology provided scientists with a controlled process to generate an unlimited variety of transgenic mice with engineered, predetermined genomes.

The main task was to develop efficient chimera producing methods using mouse and rabbit embryos. Applying these enhanced methods I wanted to get more information about the process of sex determination and cell determination during embryonic development.

First I examined the factors influencing the chimera forming ability of ES cells. I produced chimeras by aggregating ES cell clumps with 8-cell stage host embryos. I analyzed the ratio of chimera new-born and germ cell chimera animals. My task was to determine the reason of difference in germ cell forming ability of different ES cell lines.

In the second part of my work I produced chimeras using one blastomere derived from EGFP expressing eight-cell stage embryos and diploid or tetraploid host embryos. First I followed the fate of EGFP expressing diploid blastomere derived cells in 3.5 and 4.5-day-old chimera embryos in vitro. I examined the contribution pattern of diploid blastomere derived cells in chimera embryos. After that I have to compose chimeras using single blastomeres, derived from sexed eight-cell stage embryos and tetraploid host embryos. I wanted to know whether a single blastomere could participate in the tissues of developing chimeras and is it possible to revert the sex of female (XX) host embryos using single blastomere derived from XY

embryo.

As a first step toward the ES cell derived transgenic rabbits, we have to develop a method for sex chimera rabbit production. My task was to develop method for chromosome analysis of chimera rabbits, and using FISH method for identification of the chromosomal localization of newly sequenced rabbit genes.

6. OVERVIEW OF LITERATURE

6.1. *DEFINITION OF CHIMERAS*

The term “chimera” is used to describe composite animals containing genetically different cell populations originating from more than one embryo. Mythological chimeras demonstrate the basic principles of this type of creatures composed from parts of several individuals, like the Etruscan chimera from Arrezzo in 5th century B.C. **(*Figure 1.*)**. Natural chimeras may also develop by polocyte fertilization of twin oocytes in a single zona pellucida, joint implantation, or *via* placental circulation between dizygotic twins, like cattle freemartins. According to this definition, blood, bone marrow and organ transplant recipients are also chimeras.

The chimeric animals are carriers of an alien genotype and therefore are useless if they are only somatic and not germ line chimeras. The embryonic chimera has proven to be very useful for elucidating gene function in the mouse beyond the simple characterization of mutant phenotypes. Its application is not limited to the embryological study of early development but also to the analysis of organogenesis, postnatal maturation and function. When used in combination with molecular tools that can modify genetic activity in a time- and lineage-specific manner in the cell population under scrutiny, the chimera offers practically unlimited options for precise and large-scale analyses of gene function.

Genetic activity can be modified conditionally by gene-driven and inducible Cre recombinase activity (NOVAK, 2000), or up- and down-regulate gene function by applying molecular reagents such as morpholine, anti-sense oligonucleotides and RNAi.

A unique feature of embryonic chimera is that foreign cells introduced to the embryonic environment may be provided with all the possible lineage options available normally to the cells during development. As a result, the cells are subject to a test of the full range of lineage potency, which may reveal the true extent of pluripotency (CLARKE, 2000; JIANG, 2002). Chimera analysis is expected to become an essential tool for the most comprehensive and stringent *in vivo* assessment of the characteristics of embryonic and adult tissue stem cells of mouse and other mammalian species. Chimeras are more than just a tool for making mouse mutants; they are crucial for analyzing the biological effects of genetic changes.

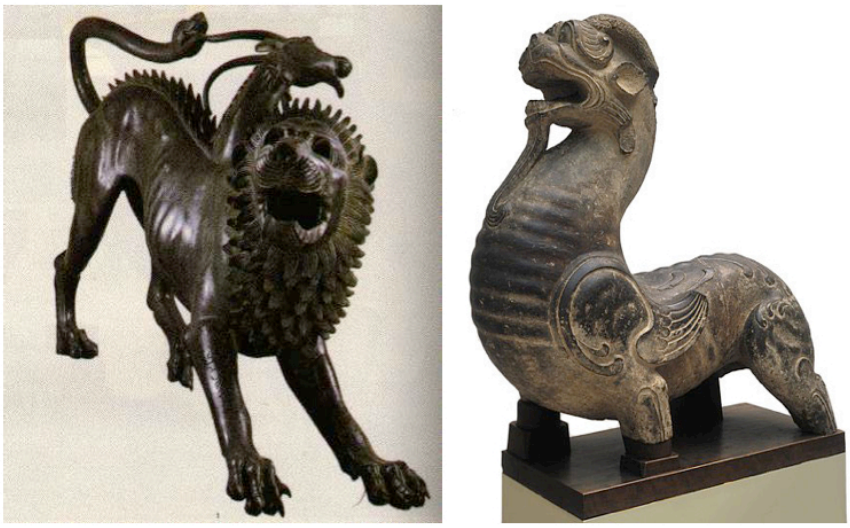


Figure 1: *Chimeras in mythology*

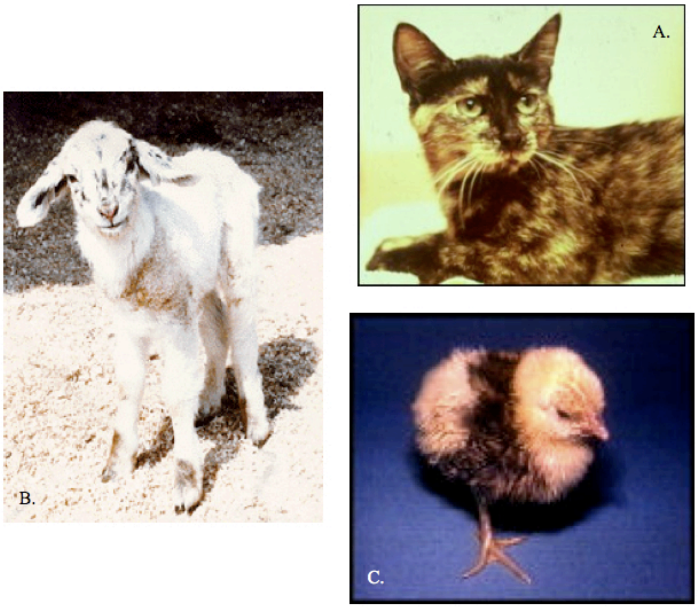


Figure 2: *Mosaics and chimeras*

6.2. *MOUSE CHIMERAS*

6.2.1. TYPES OF MOUSE CHIMERAS

The first mouse embryonic chimeras were produced in 1961 by A. K. Tarkowski and repeated by Beatrice Mintz, in 1964, by aggregating two eight-cell stage embryos (TARKOWSKI, 1961; MINTZ, 1964). The result was one normal-sized mouse, whose tissues were a mixture of cells derived from the two embryos. Tarkowski removed the zona pellucida of two genetically distinct eight-cell stage embryos, put them in contact and after the aggregation they formed one single conglomerate composed from two eight-cell stage embryos. The aggregates resulted in viable live chimeras after transferring them into a pseudopregnant female. This was the first laboratory experiment of chimera embryo production; in this case he used a method called: aggregation method.
Gardner in 1968 developed a new technique for animal chimera production (GARDNER, 1968). Injection of embryonic cells into blastocysts was first used for transferring dissociated inner cell mass (ICM) cells. The original technique required usage of five instruments and permitted the introduction of a whole ICM into the blastocoel, but not that of single cells. But later he developed the method of single cell injection (GARDNER, 1971).
The making of chimeras by injecting cells into the blastocysts, devised by Richard Gardner, opened up new possibilities for introducing foreign cells into the embryo. From the 1960s to the 1980s, chimerism has become one of the most important tools for investigating basic aspects of early embryonic development, differentiation and sex determination (MCLAREN, 1984; HUNTER, 1995) and for producing transgenic animals when embryonic germ cells (EG cells) or embryonic stem cells (ES cells) are injected into blastocysts or aggregated with blastomeres.
Chimeras can be made by combining two whole (eight-cell) embryos or by combining subsets of blastomeres of two or more cleavage (two- to eight-cell) stage embryos. Because the early embryonic cells are not yet restricted in their lineage at these stages, they are equally capable of contributing to both extra-embryonic and embryonic lineage. When two diploid eight-cell embryos or blastomeres of two diploid embryos are aggregated, chimerism can occur in the epiblast, the primitive endoderm and trophectoderm. By contrast, when the inner cell mass (ICM) cells of a diploid blastocyst are used to make the chimera, whether injected micro-surgically into morula or blastocyst, or aggregated with eight-cell stage diploid embryos, they can

contribute only to the epiblast and to the primitive endoderm, and not to the trophectoderm, because of the restricted lineage potency of the ICM cells.
ES cells in the same situations behave more like epiblast cells. They contribute only to germ layers that give rise to all the embryonic tissues and some extra embryonic tissues (including the amnion, the mesoderm of the yolk sac, the allantois and the embryo-derived blood vessels in the placenta (BEDDINGTON and ROBERTSON, 1989).
After the derivations of the first ES cell lines, chimeras gained additional importance and represented the vehicles for transmitting the ES cell genome *in vivo*. ES cells can be introduced in two ways into the pre-implantation stage embryos: injected micro-surgically into morula or blastocyst (GARDNER, 1968), or aggregated with eight-cell stages diploid embryos (WAGNER, 1985; NAGY, 1990; WOOD, 1993). Both techniques can result efficient germ line transmission.
András Nagy and his coworkers aggregated ES cells with tetraploid mouse embryos for first time in 1990 (NAGY, 1990). In diploid ES cell/tetraploid embryo chimeras, the ES cells contribute primarily to the epiblast-derived tissues, whereas cells of the tetraploid embryo mainly give rise to the extra embryonic primitive endoderm and trophectoderm. The almost complete segregation of descendants of the ES and tetraploid cells provides powerful means for revealing the effect of the mutation in the embryonic versus the extra-embryonic tissues. In addition, as exclusively the ES cells constitute the fetus properties at this type of chimeras, ES cell derived embryos and adult mice of the same genotype as the ES cells can be produced immediately for phenotypic studies (NAGY, 1990; 1993). Trophoblast stem (TS) cells, which are permanent cell lines derived either from the trophectoderm of the blastocyst or from early post-implantation trophoblasts (TANAKA, 1998), their derivatives in the chimeras following injection into the blastocyst contribute only to trophectoderm. At presently, chimera can be produced with diploid embryos, tetraploid embryos, ES cells and TS cells, all this combinations represent unique developmental potential and restriction determining the allocation of their derivatives in the resulting chimeras.

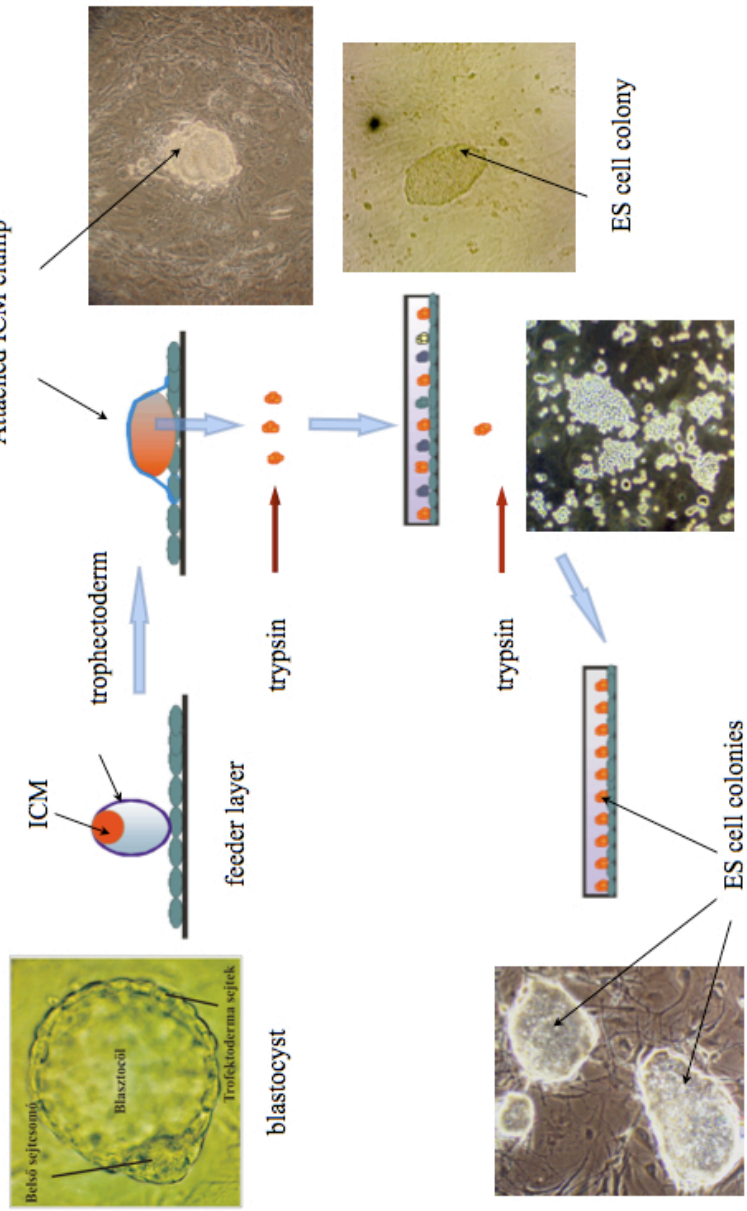


***Figure 3:** Mouse ES cell line establishment (Photos: E. Gócza)*

Using this technique, the injection of embryonic cells directly into the blastocoel cavity genetic alteration performed in ES cells can be maintained in the offspring and later propagated via sexual reproduction by producing germ line-transmitting chimeras (BRADLEY, 1984). Chimeras can be produced as well by aggregation of embryonic cells with morula stage embryos (NAGY, 1993; WOOD, 1993). These two methods for chimera production: aggregation and injection have some similarities, but they also have major differences, advantages and disadvantages. Injection requires expensive equipment (inverted microscope with phase contrast optics, micro-manipulators), it is a slow process (you can inject maximum 50-100 blastocysts / day), but you can select individually the ES cells to be injected and the embryos are not kept *in vitro* for a long time. The aggregation method can be performed simply using a good stereo dissecting microscope and 200, or more aggregates/day can be produced. Since in the aggregation methods the zona pellucida has to be removed, the embryos became very sensitive to the in vitro condition and the work with them becomes more difficult, because they will be very sticky. Anyway when ES cells quality is high, both techniques generally work equally efficiently in terms of producing chimeric embryos.
Chimeras can be produced also by the help of the nuclear transfer technique. This smart technique differs from the other two techniques (i.e., aggregation and injection) in that it doesn't consist of incorporating two types of cells into one embryo, but in replacing a nucleus in one blastomere at the 2-cell stage with a genetically different nucleus from another 2-cell (or 8-cell) embryo (KONO and TSUNODA, 1989). The manipulated blastomere is a nucleo-cytoplasmic hybrid and carries the mitochondrial DNA of the recipient embryo.

6.3. *CHIMERAS IN ANIMAL KINGDOM*

Although the mouse is the unquestionable leader and hero among experimental mammalian chimeras, chimeric animals were produced also in the rat (MAYER and FRITZ, 1974), rabbit (GARDNER and MUNRO, 1974; MOUSTAFA and DIXON, 1974), sheep (TUCKER, 1974; FEHILLY, 1984a) and cattle (BREM, 1984; SUMMERS, 1984). Studies on chimerism in farm animals, so far carried out on a limited scale, will explode as soon as embryonic stem (ES) cells become available in these species.

6.3.1. OBSERVATIONS ON THE SEX OF XX/XY CHIMERAS

Mosaics and chimeras are animals that have more than one genetically-distinct population of cells. The distinction between these two forms is quite clearly defined, although at times ignored or misused. In mosaics, the genetically different cell types all arise by somatic mutation among some cells from a single zygote, whereas chimeras originate from more than one zygote. Mosaics are not uncommon; in fact, roughly half of the mammals on earth are a type of mosaic. A chimera, on the other hand, is not something you're likely to come across, unless you are an experimental embryologist or cattle breeder.

6.3.1.1. *Cytogenetic mosaics*

The term mosaic is usually applied to an animal that has more than one cytogenetically-distinct population of cells. For example, in a human mosaic, some of the cells might be 46, XX and some 47, XXX. The fraction of cells having each genotype is quite variable, reflecting how early during embryo-genesis the mosaicism originated. In most but not all cases, the mosaicism can be detected in cells from all tissues.
What is the clinical significance of mosaicism? If the proportion of cytogenetically abnormal cells in a mosaic is sufficiently large, that individual will manifest disease. Conversely, if the abnormal cells are proportionally small in comparison to cytogenetically normal cells, the normal cells may be sufficient to prevent disease or reduce its severity. For example, majority of humans having Turner's syndrome (X-chromosome monosomy) are infertile. Many of the fertile Turner's individuals are found to be mosaics with a substantial fraction of normal cells (46,XX/45,XO mosaics).

6.3.1.2. *X-chromosome mosaicism*

Early in embryo-genesis in mammals, all but one X-chromosome is functionally inactivated through a process called X-chromosome inactivation. Because in placental mammals this inactivation occurs randomly, all normal females have roughly equal populations of two genetically different cell types and are therefore a type of functional mosaics. In roughly half of their cells, the paternal X-chromosome has been inactivated, and in the other half the maternal X-chromosome is inactive.

This has a number of important biological and medical implications, particularly with regard to X-linked genetic diseases.
Tortoiseshell or calico cats provide a unique opportunity to observe X-chromosome inactivation and help visualization how it affects all females **(*Figure 2.A*.)**.

6.3.2. CHIMERAS IN OTHER SPECIES

Chimeric cattle are not at all rare. When a cow has twins, it is almost inevitable that anastomoses develop between the fetal circulatory systems early in gestation. This leads to exchange of blood between the two fetuses. Fetal blood contains hematopoietic stem cells, and each fetus is permanently "seeded" with stem cells from its twin. The result is that both animals are hematopoietic chimeras. A variable fraction of all their cells that are derived from hematopoietic stem cells (peripheral blood cells, Kupffer cells in the liver, lymphocytes and macrophages in lymph nodes and spleen, etc) are from the twin.
Major clinical significance is seen when one fetus is a female and the other one is a male. In such cases, the female fetus is exposed to hormones from the male and is masculinized. Such female cattle are called free-martins. The external genital tract of a free-martin looks like a female, although usually infantile. The degree to which the internal genital tract is masculinized varies, but typically, the vagina is very short and uterine horns are rudimentary. Pretty obviously, these animals are sterile. Freemartins are seen occasionally in other species, although much less commonly than in cattle, and in some species like horse and marmosets the XX/XY females are regularly fertile.
There are reports of naturally-occurring chimerism in a variety of species. Such individuals undoubtedly do occur, although they are quite rare. The most likely pathogenesis in such cases is the spontaneous fusion of two early embryos into one (KOVÁCS, 1977). This is suspected because chimeras are also produced experimentally, and have been a valuable research tool in several biomedical disciplines. The basic technique is to combine two very early embryos such that their cells intermix and the resulting conceptus has cells from both original embryos. This technique has been widely applied with mice and has also been applied to ruminants.
The chimeric animal shown below is a baby "geep", made by combining a goat and sheep (FEHILLY, 1984b). Notice the chimerism evident in the skin - big patches of skin on front and rear legs are covered with wool, representing

the sheep contribution of the animal, while a majority of the remainder of the body is covered with hair, being derived from goat cells **(*Figure 2.B.*)**.
Inter-specific avian chimeras are very useful for proliferating endangered avian species and studying immune rejection of donor cells in the chimeras. Quail–chicken inter-specific chimeras have been produced by the transfer of stage X blastodermal cells (NAITO, 1991). The injected quail blastodermal cells differentiate into various tissues and organs including gonads in the quail–chicken chimeras (WATANABE, 1992), but production of viable offspring derived from the donor blastodermal cells has not been successful.
Chimeric mice and sheep/goat chimeras have been most useful in answering fundamental questions about developmental biology and pathology. There is also some potential that this technique can be applied to problems such as rescue of endangered species. It is possible, for example to construct a goat/ sheep chimera such that a goat fetus is "encased" in a sheep placenta. This enables a sheep to carry a goat to term, which will not occur if you simply transfer goat embryos into sheep (the sheep will immunologically reject the goat placenta and fetus). It may be possible to extend this procedure to allow embryos from severely endangered species to be carried by recipient mothers from another species.

6.4. *REGULATION OF SEXUAL DIMORPHISM IN MAMMALS*

Sexual dimorphism has been the subject of interest for centuries. In 355 BC, Aristotle postulated that sexual dimorphism arose from differences in the heat of semen at the time of copulation. In his scheme, hot semen generated males, whereas cold semen made females. In medieval times, there was great controversy about the existence of a female pope, who may have in fact had an inter-sex phenotype (NEW and KITZINGER, 1993). Recent years have seen a resurgence of interest in mechanisms controlling sexual differentiation in mammals. Sex differentiation relies on establishment of chromosomal sex at fertilization, followed by the differentiation of gonads, and ultimately the establishment of phenotypic sex in its final form at puberty. Each event in sex determination depends on the preceding event, and normally, chromosomal, gonadal, and somatic sex all agree. There are, however, instances where chromosomal, gonadal, or somatic sex do not agree, and sexual differentiation is ambiguous, with male and female characteristics combined in a single individual. Well-characterized individuals of 40, XY mouse females who have the syndrome of pure gonadal dysgenesis, and a subset of true hermaphrodites are phenotypic males with a 40, XX

karyotype. Analysis of such individuals has permitted identification of some of the molecules involved in sex determination, including *SRY* (sex-determining region on the Y-chromosome) fulfilling the genetic and conceptual requirements of a testis-determining factor. Mammalian sexual differentiation is a complex process that begins with the establishment of genetic sex (XX or XY) at the time of fertilization. In mice, the bipotential gonads arise from the coelomic epithelium of the urogenital ridges and initially are indistinguishable in males and females. Between 10.5 and 12.5 days post coitum (dpc), a gene on the Y-chromosome, designated *Sry*, initiates the male developmental pathway, in the absence of *Sry*, ovaries develop (KOOPMAN, 1990). Although *Sry* unambiguously initiates the male developmental pathway, most of the mechanisms that mediate testes development remain to be defined.

6.4.1. PRODUCTION OF SEX CHIMERAS

Chimeric mice provided an experimental system in which the correlation of chromosomal sex with phenotypic sex and the proportions of chimeric components in the gonadal tissues could be evaluated. Tarkowski and Mintz made the first mouse embryonic chimeras in the 1960s, by aggregating two eight-cell embryos (TARKOWSKI, 1961; MINTZ, 1964). Sex chimeras (XX/XY) developed mostly into fertile males, and true hermaphroditism was a rare event (TARKOWSKI, 1964; BRADBURY, 1987).

Some XX/XY males (and probably the majority of them) pass during the fetal life through the hermaphroditic state with their gonads being ovotestes; the ovarian parts must disappear later and the normal male phenotype develops (BRADBURY, 1987; JANKOWSKA, 1992). Genetically female germ cells are unable to undergo spermatogenesis (MINTZ, 1968; MYSTKOWSKA and TARKOWSKi, 1968) – this is a speciality of mammals, as in non-mammalian vertebrates sex-reversal permits the germ cells to undergo gametogenesis characteristic for the acquired sex. XX/XY chimeras develop occasionally into fertile females. Genetically male germ cells can undergo oogenesis and form functional oocytes (FORD, 1975; FORD and EVANS 1977). Although this is a very rare event, such a possibility has been confirmed on several occasions in chimeras produced by injection of genetically male ES cells into genetically female blastocysts (BRONSON, 1995).

6.4.1.1. *Cloning using chimeras*

The pioneering studies of Tarkowski (TARKOWSKI and WROBLEWSKA, 1967) revealed that a single blastomere isolated from a 2-cell stage embryo could develop into an adult mouse. In cases where both blastomeres were let to develop separately, identical twin animals were produced (MULLEN, 1970). Later, similar studies were performed with blastomeres isolated from 4- and 8-cell stage embryos, which can implant, but only sporadically form small egg cylinders (ROSSANT, 1976). In other mammalian species, like rabbit and sheep, single blastomeres of the 4-cell and 8-cell embryo can develop into adults (MOORE, 1968; WILLADSEN, 1981).
By aggregating single blastomeres from 4-cell embryos with carrier embryos and transferring them into surrogate females, a few live-born mice appeared to originate exclusively from single 4-cell blastomeres (KELLY, 1977).
In chimeric mice, produced from the combination of diploid (2n) and tetraploid (4n) cells, tetraploid contribution to the embryo proper does occur at low degree, but the 4n cells are principally restricted to the extra-embryonic tissues. MacKay (MACKAY, 2005) found that tetraploid cells were not initially excluded from the epiblast in 4n/2n chimeric blastocysts, but preferentially lost from the epiblast lineage following the embryo implantation. In the light of this contribution pattern of tetraploid cells, chimeras created using single blastomeres of the 4-cell and 8-cell embryos supported by tetraploid carrier embryos were composed. This complementation resulted healthy mice (TARKOWSKI, 2001). Moreover, taking advantage that the carrier tetraploid embryos do not contribute to the embryonic tissues, but they are abundant in extraembryonic tissues, identical triplets or twins were produced from single 1/8 blastomeres and pairs of 1/16 blastomeres (TARKOWSKI, 2005).

6.5. *ES CELL CHIMERAS*

6.5.1. PRODUCTION AND CHARACTERIZATION OF ES CELL LINES

Embryonic stem cells (ES cells) are stem cells derived from the inner cell mass of an early stage embryo known as a blastocyst. Mouse embryos reach the blastocyst stage 4, 5 days post fertilization, at which time they consist of 50-150 cells. ES cells are pluripotent. This means they are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. These include each of the more than 220 cell types in the adult body. Pluripotency distinguishes ES cells from multipotent progenitor cells found in the adult; these only form a limited number of cell types. When given no stimuli for differentiation, (i.e. when grown in vitro), ES cells maintain pluripotency through multiple cell divisions. The presence of pluripotent adult stem cells remains a subject of scientific debate.
Comparing with pluripotent cell, the cell able to differentiate into only one type of tissue/cell type it is a unipotent cell. The most common of these in humans are skin cells. This cells has a unique property: self-renewal. This property distinguishes it form most other terminally differentiated non-stem cells. A totipotent cell has the ability of a single cell to divide and produce all the differentiated cells in an organism, including extraembryonic tissues. Totipotent cells formed during sexual and asexual reproduction include spores and zygotes.
Mouse embryonic stem (mES) cells remain undifferentiated in the presence of leukemia inhibitory factor (LIF), and activation of signal transducer and activator of transcription 3 (STAT3) via LIF receptor (LIFR) signaling appears sufficient for maintenance of mES cell pluripotency. LIF has been shown to play an important role in the development and implantation of blastocysts in mice, and rabbits. In rabbit, the expression levels of LIF and LIFR in uterine epithelium were gradually increased during the pre-implantation period and reached their highest levels on days 6.5 of pregnancy, just before blastocyst implantation.
The isolation and genetic manipulation of embryonic stem (ES) cells represents one of the most important and far-reaching achievements in mammalian developmental biology. Following a significant foundation laid down by research on embryonic carcinoma (ES) cells, ES cells were first derived from blastocysts in culture by Evans and Kaufman, and Gail Martin (EVANS and KAUFMAN, 1981; MARTIN, 1981)(***Figure 3.***).
Soon thereafter, Bradley et al. (BRADLEY, 1984) showed that ES cells were

capable of contributing in many different tissues of chimeras generated by blastocyst injection, including the germ line. ES cells are typically used as vehicles for modifying the mouse genome. However, ES cells can also be used for chimera studies. Several ES cell lines (NAGY, 1990; EGGAN, 2001) are capable to produce completely ES-cell-derived animals very efficiently. This approach can be used directly to generate and analyze heterozygous and potentially homozygous mutants (EGGAN, 2002). ES cells can also be manipulated in vitro to generate differentiated cell types that may be used in the future for cell-based therapies. Recently, an additional type of stem cell, trophoblast stem (TS) cell (TANAKA, 1998), has been isolated from blastocyst. These cells hold promise for providing new insights into trophoblast differentiations and placental biology Permanent stem cell lines from the extra embryonic endoderm (XEN) have been established recently (KUNATH, 2005). When XEN cells are injected into the blastocyst they display a bias toward parietal endoderm differentiation. ES, TS and XEN cell lines complete the effort of establishing permanent cell lines from all the three developmental distinct lineage of the blastocyst (***Figure 4.***)

For demonstrating the pluripotency of ES cells, germ-line transmission of ES cells genome can be tested (SCHOONJANS, 2003). The ability of the newly established ES cell lines to colonize the germ-line of a host embryo can be tested by chimeras injection of these ES cell lines after one or more passages into host blastocyst, or by the aggregation with eight cell stage diploid embryos, or four cell tetraploid embryos, and implantation of this chimeric embryos into pseudo-pregnant foster mothers using standard procedures. Results of germ line transmission using the injection method show that all ES lines tested resulted in chimeric offspring. These chimeras had the capability to pass the ES cell genome to the next generation, as it is judged from offspring by the coat color of the ES cell strain after mating with relevant recipient females (***Figure 5.***)

With this experiment the pluripotency of ES cells and their capacity to colonize the host embryo and added new character in the resulting organism was demonstrated.

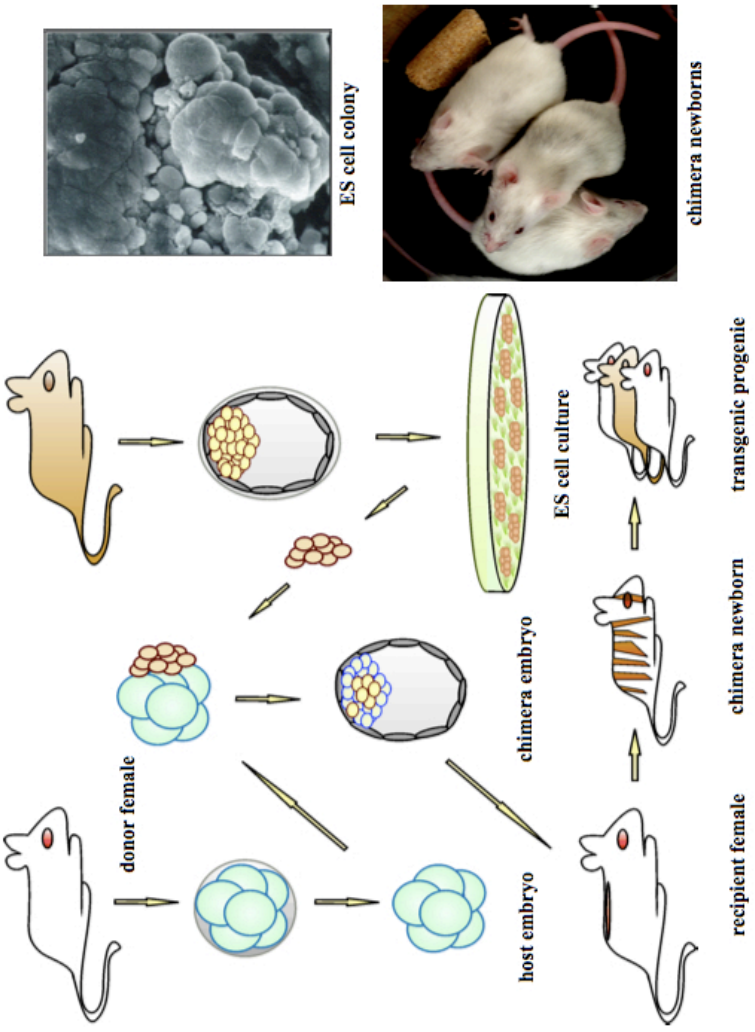


***Figure 5:** Production of mouse ES cell chimeras (Photos: G.Takács, E. Gócza)*

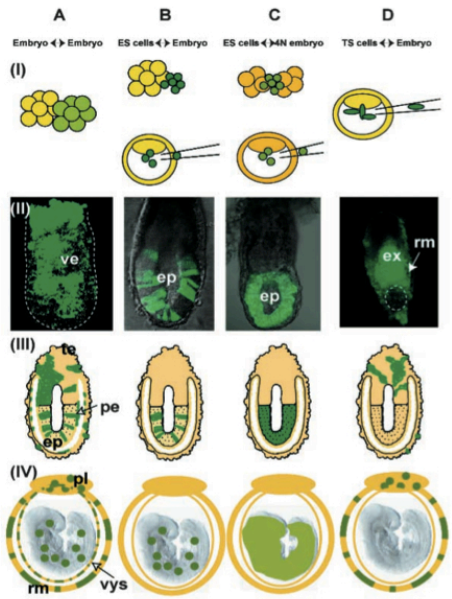


Figure 4: *Types of mouse chimeras (Figure: J. Rossant, 2003)*

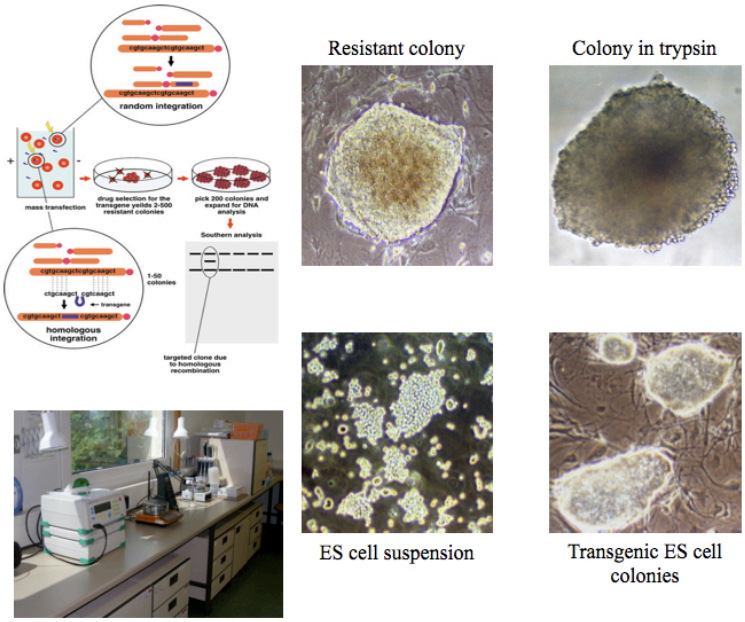


Figure 6: *Production of transgenic ES cell lines (Photos: E. Gócza)*

6.5.2. PRODUCTION OF TRANSGENIC ES CELLS

Techniques for producing transgenic mice developed in the last few decades. Random transgene integration has helped to elucidate the role of a number of genes. During development, the endogenous promoter associated with the gene controls the expression of many genes in a tissue-specific manner, both temporally and spatially. By introducing a stretch of DNA into a cell, which artificially associates the coding region of the gene under investigation with a constitutive promoter, for instance those derived from CMV, SV40 or the ubiquitin gene, it is possible to drive expression of the gene at levels and in tissues beyond that typically found in development. Such over-expression studies have been successful in revealing genes which can act as master fate regulators, controlling the lineage into which cells will develop (PEVNY, 1998; FUJIKURA, 2002; CHAMBERS, 2003; NIWA, 2005). Targeted mutation or knockout of genes has become the tool of choice for investigating the phenotypic consequences associated with gene ablation (HASTY, 2001). Gene targeting requires the construction of a targeting cassette containing at least three main elements: DNA sequence for insertion or replacement of the target loci, a selectable marker for reclamation of the targeting event from the background of non-targeted cells and flanking arms that are homologous to the endogenous regions surrounding the target locus. Introduction of the targeting cassette to the cell will occasionally lead to the substitution of the endogenous loci with the incoming targeting cassette, which can be distinguished, from wild-type cells by Southern blot or PCR analysis. Whilst gene targeting was perfected in cell lines other than murine ES cells (SMITHIES, 1985), it soon became clear that ES cells were ideally suited for this task (DOETSCHMAN, 1987; THOMAS and CAPECCHI, 1987). ES cells exhibit several properties which are particularly important in facilitating their functionality in transgenic research: firstly, the rapidity with which these cells proliferate (cell cycle time of <12 h)(STEAD, 2002) and consequently the number of cells, for instance $>1\times10^7$ cells per 10-cm-diameter plate, that can routinely be obtained. These extremely large numbers allow the relatively frequent occurrence of very rare recombination or integration events in a single experiment. Secondly, clonal sub-lines can be created from a single cell with high efficiency (>10%)***(Figure 6.)***. This permits these rare recombination or integration events to be retrieved, typically via drug resistance selection, from the background of wild-type ES cells that have not undergone the desired modification. Finally, after

transgenic manipulation, ES cells can retain pluripotency and, most importantly, germ line competence, enabling the heritable transmission of the genetic alterations. Initial experiments indicated that these traits could be exploited in order to generate transgenic ES cells, random integration of cassettes including drug resistance genes was demonstrated, along with subsequent germline transmission from chimeric animals (LOVELL-BADGE, 1985; GOSSLER, 1986; ROBERTSON, 1986). Not long after the initial gene targeting experiments in mouse ES cells, the first mice were generated from ES cells that had undergone targeted mutation of a specific gene (KOLLER, 1989). The number of genes which have been targeted in a similar way now exceeds 7,000 (CAPECCHI, 2005); a number which highlights the importance of mouse transgenesis in our understanding of gene function. Notwithstanding, the generation of targeted gene knockouts is a laborious process, taking many months to construct the targeting vector, target the allele in ES cells and generate transgenic animals from the ES cells. The discovery that an endogenous cellular mechanism could be subverted to silence gene expression has lead to a faster way of reducing target gene expression. RNA interference (RNAi) capitalizes upon a cellular response to the presence of double-stranded RNA in which endogenous mRNAs are inhibited or degraded if they share sequence homology to the double-stranded RNA (dsRNA) (reviewed in MITTAL, 2004). This technology has been utilized to analyse gene function in vitro and in vivo, enabling recapitulation of traditional gene knockout phenotypes (HENKEMEYER, 1995; NIWA, 2000; KUNATH, 2003(a,b); VELKEY and O'SHEA, 2003). Once protocols had been established for altering the DNA of ES cells and the subsequent generation of live animals carrying these changes, more elaborate methods for analyzing gene function became possible. Most notable amongst these technologies was the application of site-specific recombinases. Site-specific recombinases utilize short DNA sequences as both recognition sites for the recombinase and substrates for the recombination event. The length of recombinase recognition sites is typically on the order of several dozen bp, making the sequence sufficiently unique to occur very infrequently in most mammalian genomes. The lack of such pseudo-recombination sites in the target genome is an important aspect of high fidelity, efficient recombination (NAGY, 2000). Most notable in the family of recombinases are Cre and Flp. Several observations have established that enzymes, which originate in yeast, bacteria or bacteriophages, could work in mammalian cells (SAUER and HENDERSON 1988, 1989, 1990). Subsequently, Cre has become the dominant recombinase used in mammalian transgenics.

6.5.3. PRODUCTION OF ES CELL LINES, CARRYING GENE BASED REPORTER CONSTRUCTS

Genetically manipulated mice, often incorporating gene based reporters, are frequently being used to model and understand mammalian development and disease process. Fluorescent protein reporter currently represent a superior alternative to other gene-based reporters such as the bacterial lacZ or human placental alkaline phosphatase in that their visualization is non invasive and as such does not require cromogenic substrates. Fluorescence can be monitored in real time in vivo and in vitro, and has the added advantage that it can be quantified.
The prototype fluorescent protein reporter is the green fluorescent protein; derived from the bioluminescent jellyfish Aequorea victoria (HADJANTONAKIS, 2002). Green fluorescent variants of wild types GFP with improved thermostability and fluorescence emission, including enhanced green fluorescent protein (EGFP) and MGFP, have gained popularity for use in mice. Recently, several additional mutants of wild type GFP with altered excitation and emission spectral profiles (fluorescent in colors other than green) as well as improved thermostability and fluorescence have been described (CORMACK, 1996). It was demonstrated earlier that EGFP and EYFP represented developmentally neutral reporters. The GFP variant reporters are unique in allowing non-invasive multi spectral visualization in live samples ***(Figure 7.)***. The EGFP and EYFP-expressing transgenic ES cell provided sources of cells and tissues for combinatorial, double-target recombination experiments, chimeras or transplantations (HADJANTONAKIS, 2002).

6.5.4. PRODUCTION OF MOUSE ES CELL CHIMERAS AND TRANSGENIC MICE

Transgenic animals are a powerful tool for studying gene function and testing drugs. Many human genetic diseases can be modeled by introducing the same mutation into a mouse or other animal. Although similar genetic manipulations can be performed in tissue culture, the interaction of transgenes with proteins, hormones, neurotransmitters, and other components of an intact organism provide a much more complete and physiologically relevant picture of the transgene's function. With the development of transgenic livestock and plants, new uses for this technology have become apparent, some of potentially great economic and medical value. These include the ability to produce medically important recombinant

proteins and antibodies on an industrial scale, as well as disease-resistant creatures.
The use of transgenic mice in biochemical research and study of embryonic development is now indispensable. Several general reviews have been published, including those by PALMITER and BRINSTER (1986), JAENISCH (1988) and HANAHAN (1989). The most common use of transgenic mice is for studies of tissue-specific and developmental stage specific gene regulation, and for experiments of the phenotypic effects of transgene expression.

6.5.5. GERM LINE CHIMERA MICE

Mouse pluripotent embryonic stem (ES) cells, once reintroduced into a mouse blastocyst, can contribute to the formation of all tissues, including the germ-line ***(Figure 7.)***. However, the reason why this contribution often appears erratic is poorly understood (LONGO, 1997) tested the notion that the chromosome make-up may be important in contributing both to somatic cell chimerism and to germ line transmission. They found that the percentage of chimerism of ES cell-embryo chimeras, the absolute number of chimeras and the ratio of chimeras to total pups born all correlated closely with the percentage of euploid metaphashes in the ES cell clones injected into the murine blastocyst. The ES cells containing 50 up to 100% euploid methaphases, did transmit to the germline, in contrast none of the ES cell clones with more than 50% of chromosomally abnormal metaphashes were transmitted to the germ-line. After 20 passages ES cells in vitro rapidly become severally aneuploid (ROBERTSON, 1986), and again they correlated closely with the percentage of chimerism and with the number of ES cell embryo chimeras obtained per number of blastocysts injected. In the same time, the ability of these clones to contribute to the germline was lost when the proportion of euploid cells dropped below 50%. In conclusion aneuploidy, rather than 'loss of totipotency', in ES cells, is the major cause of failure in obtaining contributions to all tissues of the adult chimera, including the germ-line. Because euploidy is predictive of germ-line transmission, karyotype analysis is crucial in any gene-targeting experiment.

6.5.6. TRANSGENIC FARM ANIMALS USING EMBRYONIC STEM CELLS

Embryonic stem cells with pluripotent characteristics have the ability to participate in organ and germ cell development after injection into

blastocysts or by aggregation with morulae. True ES cells (that is, those lambs carrying the prion protein knockout locus died shortly after birth (DENNING, 2001). Cloned cattle with a knockout for the prion locus have also been generated CYRANOSKI (2003). Transgenic animals with modified prion genes will be an appropriate model for studying the epidemiology of spongiform encephalopathies in humans, and are crucial for developing cells able to contribute to the germ line, currently only available from inbred mouse strains (KUES, 2005). In mouse genetics, ES cells have become an important tool for generating gene knockouts, gene knockins and large chromosomal rearrangements. Embryonic stem-like cells and primordial germ cell cultures have been reported for several farm animal species, and chimeric animals without germ line contribution have been reported in swine (SHIM, 1997) and cattle (CIBELLI, 1998). Recent data indicate that somatic stem cells may have a much greater potency than previously assumed (NIEMAN, 2005). Whether these cells will improve the efficiency of chimera generation or somatic nuclear transfer in farm animals has yet to be shown.
In swine species RUI et al. (2003) showed embryonic germ (EG) cells share common features with porcine embryonic stem (ES) cells, including morphology, alkaline phosphatase activity and capacity for in vitro differentiation. Porcine EG cells are also capable of in vivo development by producing chimeras after blastocysts injection; however, the proportion of injected embryos that yield a chimera and the proportion of cells contributed by the cultured cells in each chimera are too low for practical use in genetic manipulation. Moreover, somatic, but not germ-line chimerism, has been reported from blastocyst injection porcine ES or EG cells.

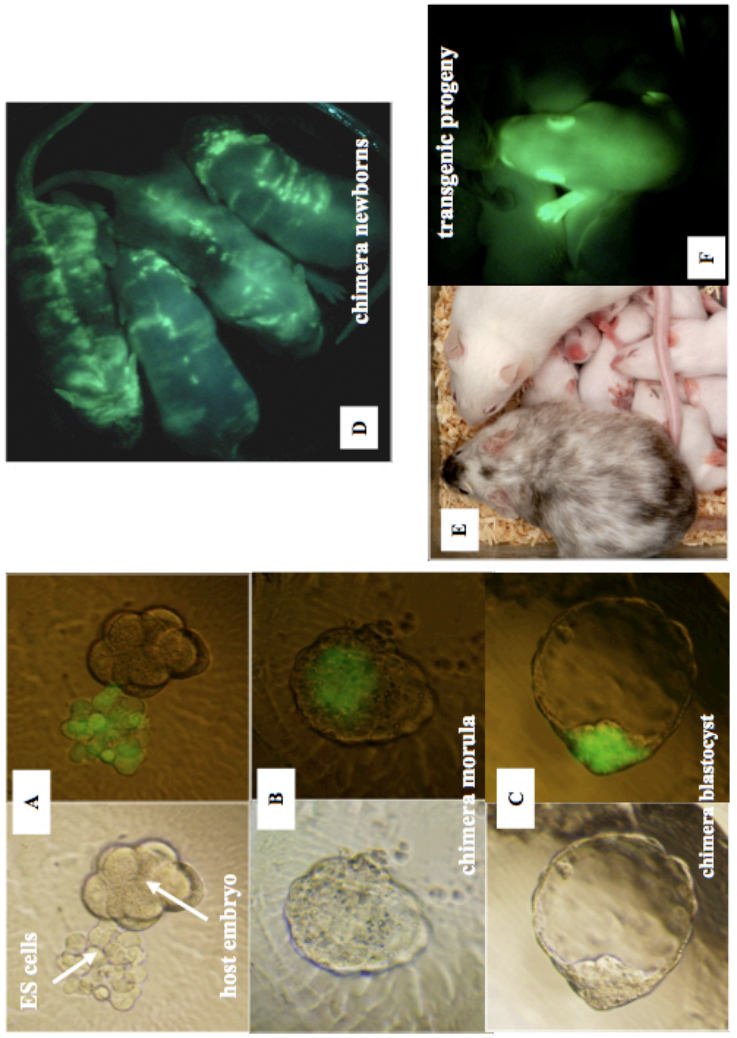


Figure 7: *Production of transgenic mouse ES cell chimeras (Photos: V.B. Carstea and G. Takács)*

6.6. *CHROMOSOME INVESTIGATIONS IN CHIMERA ANALYSES*

The in situ hybridization is a powerful and unique technique that correlates molecular information of a DNA sequence with its physical location along chromosomes and genomes. It thus provides valuable information about physical map position of sequences and often is the only means to determine abundance and distribution of repetitive sequences.
WALDEYER first prefaced the term "chromosome", in 1888. It means body (soma) that takes up color (chromo). Eukaryotic chromosomes all follow similar mitotic and usually meiotic cycles of DNA replication, condensation, division, and de-condensation. The morphology of metaphase chromosomes is relatively conserved and the size, centromere (primary constriction) position, and presence and location of the nucleolar organizers (secondary constriction) are characteristic for individual chromosome types. From the 1930s, and well before the controversy over whether DNA, proteins, or even other molecules were important for heredity was resolved, chromosome numbers and morphology were studied, often using carmine- and eosin-based stains or Feulgen-reaction. The chemical nature of DNA was of small direct importance to chromosome analysis over the decades following the elucidation of DNA structure, although the knowledge that DNA encoded the genes and was responsible for inheritance made their study relevant and important. A number of improvements in chromosome analysis came through C-banding, differential Giemsa staining of heterochromatin and recognition of gene-rich and gene-poor regions, early and late replicating regions, G- and R-bands (SCHWARZACHER, 1974) and such analyses are still used today for many human prenatal and cancer analyses. In fluorescent chromosome banding (SCHWEIZER, 1981), the fluorescent dyes bind directly to DNA either uniformly or base pair specifically and certain fractions of chromosomes, different types of heterochromatin, or the R- and G- bands of mammalian chromosomes can be differentiated and some information about the DNA sequence behind the chromosome elucidated.
The ultimate correlation of DNA sequence composition and molecular data to the structure and organization of chromosomes and nuclei is achieved through DNA : DNA in situ hybridization (ISH). The technique was first described by PARDUE and GALL (1969) and JOHN (1969) and their insight came directly from the thought that chromosomal DNA could be made single stranded (denatured) and allowed to hybridize (form double helix molecules) in situ with labeled probe DNA molecules before the sites of hybridization

were detected. Their work was carried out with radioactively labeled satellite DNA and both groups showed that the sequences with particular base pair compositions (leading to differential buoyant density) were concentrated in particular regions of chromosomes, including the nucleolar organizing sites.
In situ hybridization is now widely used for mapping DNA sequences to their physical location within the genome, for correlating linkage groups to specific chromosomes, and for understanding genome organization and the three-dimensional spatial distribution of DNA sequences in inter-phase and meiosis. In situ hybridization enables identification and characterization of chromosomes and chromosome segments, providing markers for recent or evolutionary chromosome rearrangements and for changes in sequence abundance during evolution and disease. Many of the answers obtained from chromosomal ISH are difficult to discover using any other method: repeated sequences show multiple bands in gel electrophoresis that are difficult to separate, interpret, and assign to loci, and large-clone counting and sequencing projects are not able to access long and relatively homogeneous stretches of repetitive sequences, whereas linkage mapping gives limited data about where recombination is occurring in the genome. With pure molecular methods, genomic organization, dynamics, and evolution are very hard to interpret when a sequence is present in even dozens of copies, whereas FISH can study sequences that represent half a genome and are present in thousands of copies.

6.7. *RABBIT CHIMERAS*

6.7.1. PRODUCTION OF RABBIT CHIMERAS

The rabbit has several advantages over other laboratory animals and transgenic rabbits are used both as large animal models of genetic and acquired diseases and as bioreactors for producing recombinant proteins (BŐSZE, 2003). Present utilization of transgenic rabbits is limited by the low efficiency of micro-injection and the absence of proven embryonic stem cell (ES) lines. Cloning of genetically modified donor cells would be an attractive alternative to targeted gene modification. It is promising that the first cloned rabbits from adult somatic cells did not show any obvious morphological abnormalities in the offspring (CHESNE, 2002). Nevertheless somatic cell cloning is still a very demanding method with a number of unpredictable perturbations in gene expression (WILMUT, 2002). Therefore a rabbit ES cell line, which would allow precise genetic modification and

could colonize the germ line, would be very useful.
It was a decade ago when pluripotency of cultured rabbit inner cell mass (ICM) cells as demonstrated by analyzing chimeric fetuses (GILES, 1993) but despite years of effort, reliable rabbit ES cell lines have not been obtained (HOUDEBINE, 2002). ES-like cells have been isolated and used to create chimeric rabbits (GRAVES and MOREADITH, 1993; SCHOONJANS, 1996) but germ-line chimerism or transmission of transgenes from these animals was not reported.
The characteristics of rabbit ES like cells, such as morphology, LIF independence, and trophoblast cell differentiation capability, were similar to those of the primate ES like cells but unlike those, it would be difficult to establish rabbit ESC lines because there was no report on the existence of long-term surviving of rabbit ES like cell lines in vitro until 2007, when Wang and his group have established one stable rabbit ES cell lines (WANG, 2007), derived from blastocysts fertilized in vivo and cultured in vitro and other three ES cell lines derived from parthenogenetic blastocysts sharing the characteristics of ES like cells without any differences among them. These rabbit ES like cells shared similar morphologic characteristics with primate ES like cells and expressed cell surface markers and the genes of pathways related to the self-renewal of ES like cells. As large animals with many characteristics similar to humans, rabbits have advantages over mice in size and physiology, which suggests that rabbit ES like cells may be a good model for some specific human diseases in preclinical trials.
To evaluate the degree of chimerism and the extent of germline transmission we took advantage of a recently developed transgenic rabbit line (HIRIPI, 2003), homozygous for the presence of the human blood coagulation factor VIII (hFVIII). Towards that aim an indispensable first step have to be to develop an efficient method to create rabbit chimeras from pre-implantation stage embryos.

6.7.2. A TRUE HERMAPHRODITE RABBIT CHIMERA

The developmental potential of rabbit embryonic cells was studied by Shi Yan SHENG and Wu Xue BAO in 1990 through making chimeras by separate introduction of inner cell mass from 96-h-old p. c., 120-h-old p. c., and 144-h-old p. c. of grey rabbits into 96-h-old p. c. blastocysts of New-Zealand white rabbits. A total of five overt coat color chimeras were obtained including two fertile males, two fertile females and one sterile male, from the ICM cells of 96-h-old and 120-h-old embryos but none was obtained from

144-h-old cells. Histological examination of the gonads showed that the sterile chimera derived from 120-h-old ICM cells had ovotestes on both sides.
Follicles and seminiferous tubules developed in the cortex and medulla of the gonad, respectively. Neither of them developed into functional germ cells.
Analysis of karyotypes of peripheral blood lymphocytes showed that both XX and XY cells coexisted. These results indicated that the hermaphrodite sterile chimera was an XX/XY sex chimera derived from ICM cells of a donor and a recipient with different sexes. From the results mentioned above we may conclude that the ICM cells at 120-h-old p. c. are still pluripotent, they can not only participate in development into somatic components but also develop into germ cells.
A novel technique of chimeric somatic cell cloning was developed by SKRZYSZOWSKA (2006a,b), to produce a transgenic rabbit (NT20). Karyoplasts of transgenic adult skin fibroblasts with Tg(Wap-GH1) gene construct as a marker were micro-surgically transferred into one, previously enucleated, blastomere of a 2-cell non-transgenic embryo, while the second one remained intact. The reconstructed embryos either were cultured in vitro up to the blastocyst stage or were transferred into recipient females immediately after the cloning procedure. Embryos developed to the blastocyst stage mostly from the single non-operated blastomeres, while the reconstructed blastomeres were damaged and degenerated. Some embryos did not exceed the 3- to 4-cell stages and some were inhibited at the initial 2-cell stage. By molecular analysis, the transgene was detected in the cells of 4 blastocysts. When embryos where transferred into 9 pseudo-pregnant recipient-rabbits (an average of 18 embryos per recipient) four recipient-females became pregnant and delivered a total of 24 (14.7%) pups. Molecular analysis confirmed that two pups (1.2%), one live and one stillborn, showed a positive transgene signal. Live transgenic rabbit NT20 appeared healthy and anatomically as well as physiologically normal. The results of their experiments showed that transgenic adult skin fibroblast cell nuclei, which have been introduced into the cytoplasmic micro-environment of single enucleated blastomeres from 2-cell stage rabbit embryos, are able to direct the development of chimeric embryos not only to the blastocyst stage but also up to term.

7. MATERIALS AND METHODS

7.1. *MOUSE CHIMERAS*

7.1.1. MOUSE EMBRYO PRODUCTION

CD1 mouse were provided by Charles River Laboratories, Hungary. Production of diploid and tetraploid embryos and the chimera aggregation method were carried out according to published protocols (NAGY, 2003).
The EGFP expressing transgenic B5/EGFP mouse line B5 (HADJANTONAKIS, 1998) was kindly provided by Andras Nagy (Mount Sinai Hospital, Toronto, Canada). These mice were produced by random integration of a transgene containing Green Fluorescent Protein driven by chicken beta-actin promoter and CMV intermediate early enhancer through electroporation into R1 ES cells (NAGY, 1993). This B5/EGFP mouse cell line has been maintained on CD1 background in our laboratory.
Females were super-ovulated by 7 IU PMSG intraperitoneally (i.p.) at 13 p.m. and two days later by 7 IU hCG at 13 p.m., i.p., and set up for mating with males. We checked the plug on the next day morning. We recovered the 2-cell stages embryos from super-ovulated or naturally mated females on day 1.5 (day 0.5 dpc is the day of the plug). After one day long cultivation, we could get 8-cell stages embryos (2.5 dpc).
Under dissecting microscope we flushed the embryos from the oviduct using 0.30 needle attached to a one ml syringe, filled with M2, and continued the procedure with the second oviduct.
We collected the embryos using a hand pipette attached to a glass capillary, then washed them through several drops of M2 and at last KSOM media ***(Appendix)***. We transferred the embryos into a tissue culture dish containing pre-equilibrated KSOM covered with mineral oil (SIGMA). The dish was placed into the incubator (at 37°C, 5% CO_2 in air).

7.1.2. TETRAPLOID EMBRYO PRODUCTION

The two-cells of 2-cell stage outbred CD1 embryos were electrofused using a CF-150B pulse generator (Biochemical Laboratory Services Ltd., Budapest, Hungary) optimized for blastomere fusion of pre-implantation stage embryos (NAGY, 1990) at 39 hpg. The embryos were placed into a drop of 0.3M mannitol (Sigma), supplemented with 0.3% bovine serum albumin (BSA, Sigma) and fused on a GSS-250 electrode (BLS Ltd.,

Budapest, Hungary). This was accomplished by first orienting the embryos with a weak (0.7 V) AC field followed by 2 repetitions of a 30 V, 40μs pulse fusogenic stimulus.
Fused embryos were cultured until 4-cell stage for 24 hrs in separate drops of KSOM medium covered with mineral oil (Sigma) at 37°C in an atmosphere of 5% CO_2 in air. Sterile mineral oil used in embryo culture dishes to cover the culture medium prevents in this way the evaporation of medium and maintains the temperature when removing the dishes from the thermostat for checking the development.

7.1.3. ISOLATION OF DIPLOID BLASTOMERES

The zona pellucida of 8-cell stage embryos was removed using Acidic Tyrode's solution (Sigma) at 58 hpg. The zona-free embryos were then placed in Ca/Mg free PBS (supplemented with 1% BSA (Sigma) for 10 minutes, and gentle pipetting isolated individual blastomeres. One blastomere, from each embryo, was taken for sexing and the remaining ones were kept in KSOM medium until their sex was revealed.

7.1.4. SEXING BLASTOMERES BY SINGLE CELL PCR ANALYSIS

The multiplex PCR is based on the method published by CHONG (1993). The Zfx and Zfy specific primer pairs were planned to amplify differently sized PCR products. The required time was minimalized to three hours including the embryo biopsy and PCR reaction, to avoid any reduction in the viability of embryos.
The removed single blastomeres were sexed with a complex X- and Y-specific polymerase chain reaction (CARSTEA, 2005). Blastomeres are transferred individually into 0.2 ml Eppendorf tubes containing 2 μl sterile water and incubated with proteinase K (0.5mg/ml, Fluka) at 56°C for 45 min. Following digestion, the proteinase K was denatured at 98°C for 10 min. Two pairs of sex specific primers, Zfx and Zfy were added to a PCR mix with a total volume of 20 μl. The reaction conditions were the following: 95°C for 5 min, followed by 35 cycles of 40 sec at 95°C, 1 min at 61°C and 1 min at 72°C. The expected sizes of the PCR products were 104 bp (Zfx) and 299 bp (Zfy) respectively.

The sequences of the female and male specific primer pairs were the followings:

Zfx_L: 5- aacatcctgaacaccttgcc - 3 (nt 1637-1656, Genbank ac. no. NM_011768)
Zfx_R: 5- tagcttgtggctctccaggt - 3 (nt 1740-1721, Genbank ac. no. NM_011768)
Zfy_L: 5- ccatcagcactcaaaaagca - 3 (nt 1767-1686, Genbank ac. no. X14382)
Zfy_R: 5- gcctttgtgtgaacggaaat - 3 (nt 2065-2046, Genbank ac. no. X14382)

7.1.5. AGGREGATION OF SEXED DIPLOID BLASTOMERES WITH TETRAPLOID OR DIPLOID HOST EMBRYOS

For the aggregation process, drops of KSOM medium in tissue culture dishes were floated with mineral oil, and small depressions were created in the plastic by the use of an aggregation needle (DN-10, BLS Ltd., Budapest, Hungary) as described by NAGY, (2003). Aggregates of a single sexed EGFP expressing blastomere and one sexed diploid ***(Figure 8)*** or unsexed 4-cell-stage tetraploid embryo ***(Figure 9)*** were placed in the aggregation wells at 63 hpg, and the cultures were incubated at 37°C in 5% CO_2 for 24 h.

7.1.6. TRANSFER OF CHIMERIC BLASTOCYSTS

Following 24 h of culture (at 87 hpg), blastocysts were transferred to the uterus of the 2.5 dpc pseudopregnant recipient females. Newborn mice were recovered by Cesarean section at 19 dpc and fostered by surrogate females.

7.1.7. MICROSATELLITE ANALYSIS

Out of the 28 microsatellites (Research Genetics) selected for the analysis, 17 were polymorphic (D1Mit262, D2Mit113, D3Mit352, D4Mit166, D5Mit1, D5Mit155, D6Mit105, D6Mit200, D7Mit22, D8Mit85, D9Mit163, D10Mit16, D11Mit2, D12Mit63, D13Mit26, D15Mit13, D16Mit136). The 17 polymorphic microsatellite markers represented 15 chromosomes across the mouse genome. The PCR-markers, allele separation, and silver staining were performed as described by VARGA (1997).

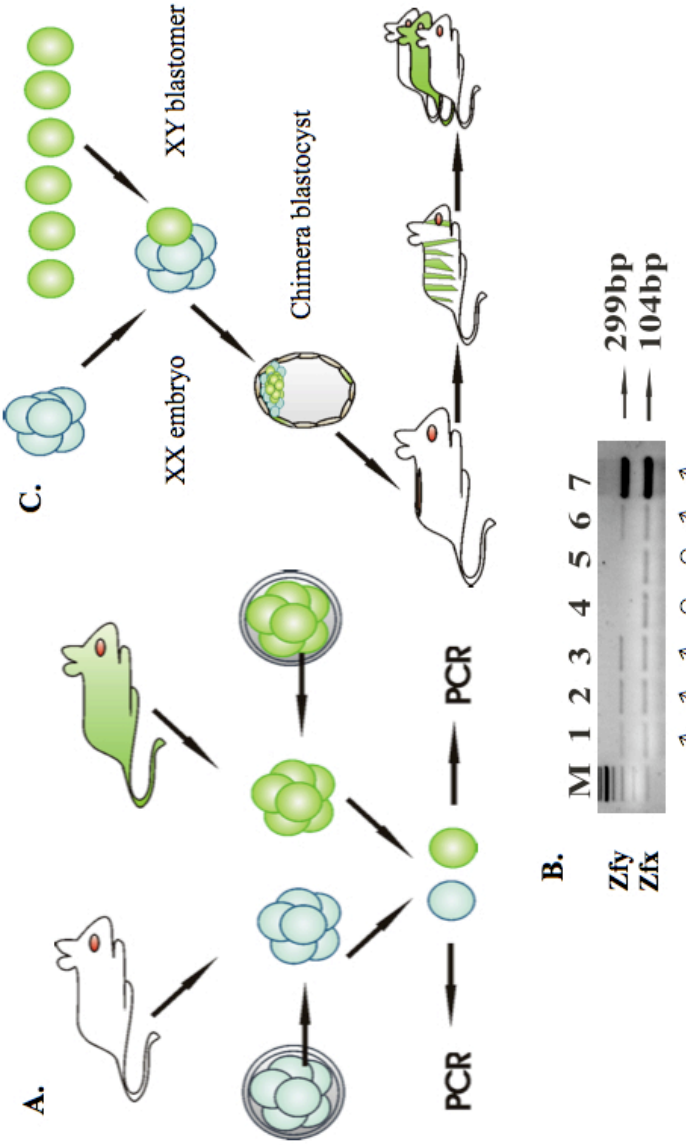


***Figure 8:** Production of cloned sexed triplets (Figures: V.B. Carstea, 2007)*

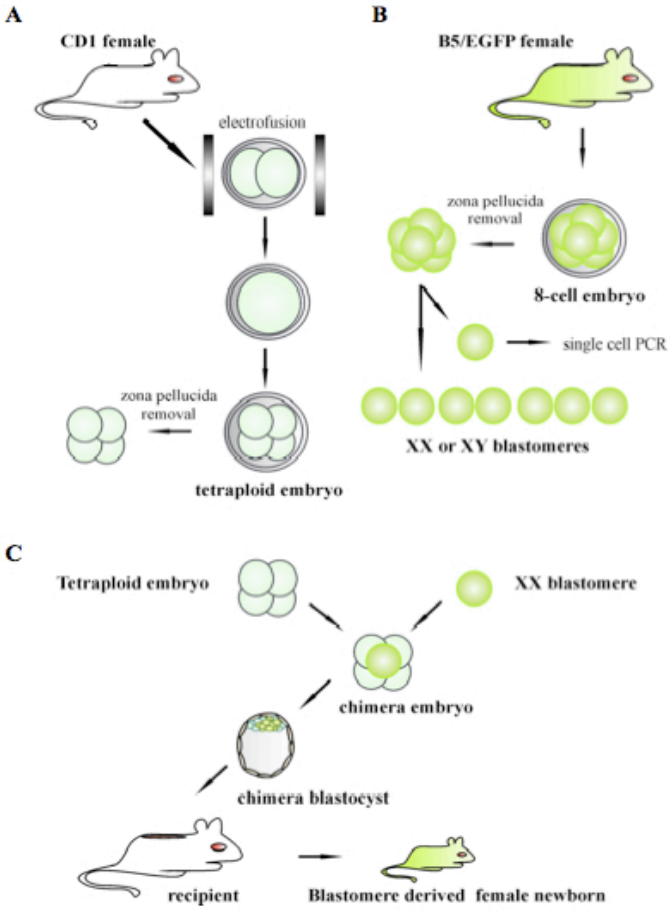


Figure 9: *Production of cloned sexed triplets (Figure: V.B. Carstea, 2007)*

7.2. *MOUSE ES CELL LINES*

7.2.1. MOUSE ES CELL CULTIVATION

The ES cell line R1 was established from a (129/Sv x 129/Sv-CP)F1 3.5-day blastocyst (*NAGY, 1993*). R1 ES cell lines were kept on primary embryonic mouse fibroblast feeder layer, in Dulbecco's modified Eagle's medium (KO-DMEM medium)(GIBCO) supplemented with glutamax (Gibco, 100x), 50 µg/ml streptomycin (SIGMA), 50U/ml penicillin (SIGMA), 50mM β-mercaptoethanol (SIGMA), 0.1mM non-essential amino acids (GIBCO), 1000 units/ml of leukemia inhibitory factor (ESGRO) and 20% fetal calf serum (FCS, HyClone)(***Appendix***).

7.2.2. ES CELL PREPARATION FOR CHIMERA PRODUCTION

It is important to maintain optimal ES cell culture conditions all the time, but particularly for ES cell clones to be used for generation of chimeric animals. Three or four days prior to aggregation, we thawed a line of ES cells on a plate with MEF, and changed the medium next day. One or two days prior to aggregation, we passaged sub-confluent ES cells to the gelatin-coated plates. On the day of the experiment, after preparation of the embryos, we removed the medium, and washed the cells with PBS. We added a minimal amount of trypsin to just cover the cells (e.g. 0.5 ml per 60 mm plate), we placed it in to the incubator for 1-2 minutes or left at room temperature. After that we added ES cell medium to the plate. With very gentle pipetting we could create clumps of the correct size (10-15 cells/clump).

7.2.3. ES CELL CHIMERA PRODUCTION

The zona pellucida of the eight-cell stage embryos was removed by acidic Tyrode's solution. The zona free embryos were placed individually into depressions of the aggregation plate. Clumps of 10-15 ES cells were picked up from the medium and transferred aside the zona free embryo. After 24 hours most aggregates were transferred into the uterus of pseudo-pregnant females.

7.2.4. KARYOTYPING OF MOUSE ES CELLS

Early passages of R1 cells have normal karyotype, but it has been reported that chromosome instability can be observed with long-term passage of ES cells (DRAPER, 2004), so it is therefore necessary to test periodically the karyotype of ES cells.
The following method was developed to test the karyotype of ES and fibroblast cells in our laboratory.
Karyotyping was done from ES cells grown with high density on mitomycin-C treated feeder layer. The cells were passed to the gelatin-coated plate one day before the procedure. The next day, 10 µl colcemid solutions were added to the plate to make a final concentration of 0.02 µg/ml. The incubation time was 2.5 hours at 37°C, 5% CO_2. After 2.5 hours the colcemid containing medium was removed, we washed the plate with PBS, and than added 1 ml, 0.05% trypsin-EDTA solution to it. After 10 minutes adding 3ml FM medium stopped the reaction. We used P1000 tips for resuspending the ES cells. The cell suspensions were transferred to a 15 ml tube and centrifuged at 1000 rpm (200g) for 7 mins, at room temperature. The supernatant was removed, and the pellet was resuspended in 4 ml of warm (37°C) hypotonic solution (0.56% KCl) added drop by drop and the suspension was kept on room temperature, for 10 minutes. At the end, 0.5 ml of freshly prepared fixative (methanol: acetic acid 3:1, 4°C), was added drop by drop. The cells were centrifuged again at 1000 rpm (200g) for 7 min, (on RT), the supernatant was carefully removed and after we added 4 ml fixative to the pellet drop by drop. We repeated the same method twice. The cellular suspension was stored on 4°C (or -20°C). The slides were treated in chrome sulfuric acid for degreasing them (1 hour) than we washed the slides three times, and kept in MQ water on 4°C until usage. Before chromosome preparation, the cells were centrifuged again with 1000 rpm (200g) for 7 min, (on RT). The supernatant was carefully removed and 1 ml fresh fixative was added. We dropped 2-3 drops of suspension to a cold and wet slide. The slides were dried on RT and than stained with 7.5% Giemsa solution (50 ml MQ water and 4 ml Giemsa Stock Solution (SIGMA GS-500)), for 10 minutes. The slides were washed under flowing tap water for 1 minute, dried and covered. Slides used for FISH were not stained.

7.2.5. **FISH** TECHNIQUES IN MOUSE

For FISH techniques the chromosome preparations were kept from three days up to one week at room temperature.
Materials used for FISH: 2xSSC/0.5% NP40 (0.5 % Tween 20), distilled

water, protease 10%, 10xPBS, 1xPBS, specific probes for hybridization for the X-and Y-chromosome, thermostat at 37 ˚C.
The entire reaction needed two days. On the first day we washed the slides in 2xSSC/0.5% NP40 (0.5% Tween 20) for 10 min (5-30 min). This step is used to clean the slides and remove the impurity. During the time of incubation in water bath at 37˚C we prepared the protease solution. We put the slides in the protease solution for 10 minutes. The protease is used for digesting the cytoplasm around the chromosomes in metaphase, in this case the probe hybridization on to the chromosome was easier. After protease digestion we washed the slides in 1xPBS for 5 min to stop the protease action. The next step was dehydration washing the slides in ethanol 70-90 -100% successively for 2 min each after we air dried the slides. We added the probe at the selected area (containing cells in metaphases), the probe was kept at -20˚C until use. Next step was denaturation, the DNA is double helix and for hybridization we had to separate the filaments strands of the double helix in two single ones and in this case the probe could attach to the complementary DNA. For denaturation step we kept the slides together with the probe covered with a glass cover-slip at 72˚C for two minutes. After removing the slides from the hot plate we kept them at room temperature for 5 min. Hybridization was done by keeping the slides after denaturation in the incubator at 37˚C for 16-24 hours. To prevent the evaporation of the probe we kept the slides in a humid chamber using 2xSSC.
On the second day we removed the glass cover-slip in 2xSSC at 37˚C. Removing the probe from the nonspecific DNA segments was carried out by a washing step for 10 min at 37˚C using 50% formamide/2xSSC and after it for 5 min in 1xPBS. The chromosomes were stained by DAPI-VECTA staining (10-15 μl/ 22x22 mm cover slip). We kept the slides at 4˚C until analysis. Slides may be kept for up to one month at 4˚C, to prevent drying the coverslip was fixed to the slide by surrounding it with nail polish. Filters, UV-excitation and 100x oil immersion objective were used in the fluorescent microscope for visualization.

7.3. *RABBIT CHIMERAS*

All animals used were sexually mature New-Zealand White rabbits. Super-ovulation and embryo recovery from the donor does oviducts was performed as published by HIRIPI et al. (2003). Eight-cell embryos were obtained 44 hours after insemination.

7.3.1. RABBIT CHIMERA PRODUCTION

From the flushed pre-compacted transgenic embryos the mucin coat and zona pellucida were removed by incubation in 0.5% pronase (Sigma P8811) solution for 10 minutes. The blastomeres were separated individually by pipetting the zona free embryos up and down in a glass capillary tube several times. The recipient 16-cell stage embryos were obtained from wild type fertilized does. The holding pipettes for the recipient embryos during the cell injection were prepared from borosilicate glass capillaries by a micro-capillary puller (Backhoffer, Type 462) and a micro-forge (Narishige, MF-9). The tip of the pulled capillary was broken at 80-100μm inner diameter. Contrary to the traditional method of making holding capillaries the mouth of the pipette was not fire polished, in order to keep the rabbit embryo covered by mucin coat in a stable position during micro-manipulation. For the injection pipette the tip of the pulled glass capillary was broken with the glass bulb of the micro-forge at 40 μm inner diameter. After that the capillary was grounded at 45° with a microgrinder (Narishige, PB-7) and a small spike was melted to the tip of the pipette. Only one blastomere was injected into the recipient embryo by a micro-controller regulated syringe.
Following micro-manipulation, the chimeric construct embryos were cultured *in vitro* until compaction in RDH medium (JIN, 2000) in a CO_2 incubator at 38.5° C, 5% CO_2 and 98% relative humidity in air. The RDH medium was prepared by mixing Ham's F10, RPMI and DMEM media (1:1:1), supplemented with 5mM taurine (Sigma, T8691) and 0.3% BSA (Sigma, A3311).
Recipient does received 84 μg GnRH analog i.m. (Receptal, Intervet International B.V. Boxmeer, Holland) in 11-12 hours asynchrony with the donor rabbits. 4-12 compacted morula stage embryos were transferred to each oviduct of the recipient does by laparoscopy (BESENFELDER, 1998).

7.3.2. ANALYSIS OF RABBIT CHROMOSOMES

7.3.2.1. *Chromosome analysis from peripheral blood lymphocytes*

Chromosome analysis was performed from peripheral blood lymphocyte cultures (MOORHEAD, 1960). Complete mitoses (2N=44) were analyzed for the number of the smallest acrocentric chromosomes. Metaphases containing nine of the smallest chromosomes (pairs of 18, 19, 20, 21 and Y) were

evaluated as male, those containing eight as female cells (FRANKENHUIS, 1990).

7.3.2.2. *FISH technique used for rabbit chromosome analysis*

FISH technique was performed using four different rabbit BAC clones: two BAC clones containing the LIFR gene (102H02 and 206F02) and two other BAC clones (304A7 and 779H10) screened for the POU5F1(Oct4) gene. For these two latter clones, since POU5F1 is located on human chromosome HSA6p21.3 in the region containing the major histocompatibility complex, they were expected to belong to a contig of BAC clones covering this region in the rabbit genome on rabbit chromosome 12.

DNA preparation

We extracted DNA from the BAC clones grown on LB medium (6 ml) using standard protocols and purified it with the SNAP kit K 1900 from Invitrogen. DNA was quantified by agarose (1%) gel electrophoresis using lambda DNA digested with HindIII as control DNA. DNA quantities varied from 40 to 600 ng depending on the BAC clones. Approximately 200 ng of purified DNA was then labeled by nick-translation with biotin-14-dATP using the BioNick labeling kit 18247-015 from Invitrogen at 15°C for one hour. The labeled DNA was purified on Sephadex G50 columns to eliminate free nucleotides from the preparation. The labeled DNA was supplemented with 100-fold excess of unlabeled and sonicated total rabbit DNA and herring sperm DNA, precipitated and slightly dried before resuspension in the hybridization mixture (50% [vol/vol] deionized formamide, 10% [wt/vol] dextran sulphate, 2xSSC, 40 mM sodium phosphate and 10xDenhardt's solution, pH 7) at a concentration of about 8-10 ng/µl.

R-banded chromosome preparations

Chromosome spreads was already available in the laboratory (INRA France) and time was too short to carry out such preparations. Chromosome spreads were prepared from rabbit fibroblast cell cultures from normal New-Zealand White female embryos as described by HAYES et al. (HAYES, 1991). To obtain R-banded chromosomes, cell cultures were synchronized with an excess of thymidine and treated with 5-bromodeoxyuridine (BrdU) during the second half of the S phase. Fluorescence in situ hybridization (FISH) was performed essentially as described by HAYES et al. (HAYES 1992).

RNase treatment of slides

Chromosome preparations were treated with ribonuclease A, to remove endogenous RNA which can be a source of background. Slides were incubated in 2xSSC buffer pH7 containing 100 µg/ml of ribonuclease A (10 mg/ml stock solution diluted 100 fold) for 1 hour at 37°C, rinsed once in 2xSSC buffer for 10 minutes at room temperature, dehydrated by three successive 10 minute washes at room temperature in 50%, 75% and 100% ethanol and finally air dried.

Chromosomal DNA denaturation

Slides were incubated for 2 minutes at 70°C in preheated 2xSSC buffer pH7 containing 70% (vol./vol.) deionised formamide. The slides were treated one by one, allowing an interval of several minutes between each treatment in order to maintain the temperature. They were then rinsed for 2 minutes in each of three successive baths of 2xSSC, 2xSSC and 0.1xSSC buffer pH7 kept on ice and were dehydrated by four successive 2 minute washes in ice cold 50%, 75%, 100% and 100% ethanol. They were stored in the last 100% ethanol bath and dried quickly just before addition of the probe.

Probe DNA denaturation

Probes were denatured at 100°C for 10 minutes and then placed on ice for 10 minutes, before being deposited on the slides.

Hybridization

10 µl of hybridization medium containing about 100 ng of the denatured probe was placed on the cell spread, which has been localized in advance, and was covered with a piece of plastic film of the type sold for domestic cooking and freezing which adheres well to the slide and does not damage the chromosome preparation. The slides were incubated at 37°C for about 20 hours in a medium saturated by 2xSSC buffer pH7 containing 50% form-amide.

Post-hybridization washes

After hybridization, the cover-slips were carefully removed and the slides were washed for 3 minutes in each of the four following baths at 45°C to eliminate unhybridised or non-specifically hybridized DNA, two baths in 2xSSC buffer pH7 containing 50% form-amide and two baths in 2xSSC buffer pH7.

Detection of hybridized probes

Immunodetection of the hybridization signals on chromosomes was performed in four steps: saturation of nonspecific antibody binding sites, reaction with the first anti-biotin antibody, reaction with a second fluorescein labeled antibody directed against the first antibody, chromosome counter-staining with propidium iodide.

Slides were incubated for 10 minutes in each of successive baths of PBT solution at room temperature. 50 to 100 µl of anti-biotin antibody solution was then dropped on the slides, cover slips added, and the slides were incubated for 1 hour at 37°C in a humid chamber protected from light. After removal of cover-slips and two washes in PBT solution, 50 to 100 µl of fluorescein labeled anti-goat IgG antibody was dropped onto the slides, which were again covered and incubated as described above. A few minutes before the end of the incubation with the second antibody, 100 µl of a solution of propidium iodide at a final concentration of 1 ng/µl was placed on the slides and incubation was continued for further 6 minutes at room temperature in darkness, after which the slides were rinsed in PBS, mounted in the same solution with a cover-slip, and stored at 4°C until examination.

Microscopy and image capture

Chromosome preparations were observed under a fluorescence microscope in the presence of PPD11 used to mount the slides and reveal the R-banding pattern. The microscope was equipped with a 100 W mercury lamp for fluorescence excitation and with a filter set (transmission interval 450-490 nm) permitting observation of the light emitted by the fluorescent dyes, yellow-green fluorescence emitted by fluorescein and red-orange emitted by propidium iodide. Thus, the hybridization signals (yellowish green) and the RBP chromosome bands (red and dark) could be observed simultaneously, and hybridised DNA fragments could be mapped precisely on chromosomes (Figures III-1a and III-1b). The preparations were observed under a Zeiss Axioplan 2 epifluorescence microscope and the Applied Imaging Cytovision software was used to capture and analyze the images.

8. RESULTS

8.1. *ES CHIMERAS*

8.1.1. THE INFLUENCE OF PASSAGE NUMBER ON CAPACITY OF ES CELLS TO FORM GERM LINE CHIMERAS

The aim of this study was to examine the factors, influencing the chimera forming ability of mouse embryonic stem cells (ES cells). By aggregating ES cell clumps with 8-cell stage host embryos we were able to produce chimera embryos. By transferring the chimera embryos to the uterus of recipient females, chimeric animals were obtained. The differentiated cells, derived from ES cells can contribute to chimeric animals tissues. The real pluripotent ES cells can differentiate into germ cells of chimera animals, too. Using transgenic ES cells, we can produce transgenic mice carrying targeted gene modification via germ cell chimeras.

At the moment no farm animal derived real pluripotent ES cell lines are available, so it is not possible to generate transgenic farm animals using ES cell chimera embryos. Wider view about the factors influencing chimera production could also help in the production of transgenic farm animals using ES cell chimeras.

In our work, we examined the chimera forming capability of R1 and R1/E mouse ES cell lines. We considered that the passage number affects the chimera-forming capability of the ES cells. By increasing the passage number, the rate of viable chimera animals decreased and only the R1/E ES cell line derived cells could contribute to the germ cells.

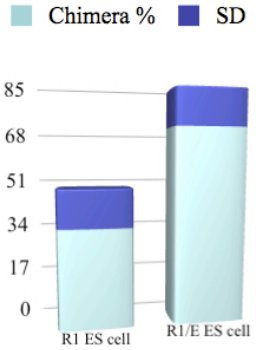
In our experiments the host embryo was CD1 and the coat color specific for CD1 is white, for R1 and R1/E ES cells line the coat color expressed into the chimera newborns is dark brown. In this way we could determinate the ratio of chimerism in the newborn pups. The capacity of chimera production in experiments when we used R1/E ES cells for aggregation is higher (21.2%) than when the aggregation was made between CD1 host embryos and R1 ES cells (6.8%). We could get germ line chimeras only when CD1 embryos were aggregated with R1/E ES cells ***(Figure 10., Table 1***).

1.table

ES cell line	Newborn/ transferred *% (±SD)*	Chimera/ transferred *% (±SD)*	Chimera/ newborn *% (±SD)*	ES% *(±SD)*	Germ cell chimera
R1	19.8±11.0	6.8±3.3	35.2±13.6	28.3±16.1	-
R1/E	32.2±11.4	21.2±6.7	70.2±13.6	62.5±16.3	+

Using R1/E ES cells for chimera production, the ratio of chimera embryo production (chimera/transferred embryo%) was higher (21.2%) than using R1 ES cells (6.8%). We could get germ line chimeras only using R1/E ES cells.

Figure 10: *Examination of R1/E and R1 ES cell line chimera forming ability (Photo: G. Takács)*



R1/E ES germ cell chimera male and his transgenic progenies

8.1.2. KARYOTYPING AND FISH ANALYSIS

We analyzed the pluripotency of ES cell lines by immunostaining and RT PCR analysis, but the experiments didn't show significant differences in the expression of pluripotent markers (Oct4, SSEA-1, Nanog) we couldn't explain why only in the case of R1/E ES cells we got germ line chimeras.
The results of chromosome analysis showed that the number of aneuploid cells in R1 ES cell line dramatically increased after eleven passages. The euploid cells in mouse contain 40 chromosomes. During the passages, the number of cells with 38, 42 or more chromosomes increased ***(Figure 11)***. In R1/E cells, the number of euploid chromosome contain cells were higher, and the increase in the number of aneuploid chromosomes was not so drastic ***(Figure 12)***. First we thought that during cell division Y-chromosomes could not arrange correctly between the two newly derived progeny cells, and after cell division it could became one 39 X0 cell. To prove our conception we made X- and Y-chromosome FISH analyses ***(Figure 13)(Table 2)***. We found, that the aneuploid R1 and R1/E ES cells contain only one X- and one Y-hromosome, so not the loss of Y-chromosome caused the problem at germ cell formation.

2.table

ES cell lines	**Number of chromosomes** *examined karyotype/karyotypes containing one Y*		
	39	**40**	**41**
R1/E p15	-	5/5	2/2
R1/E p21	-	2/2	1/1
R1 p17	3	4/4	-
R1 p23	2/2	4/4	3/3

We analyzed the karyotype of R1/E and R1 ES cells at different passage numbers (p15, p21, p17, p23). All of the euploid (40 chromosomes) and aneuploid (39, 41 chromosomes) ES cells contained a single Y-chromosome.

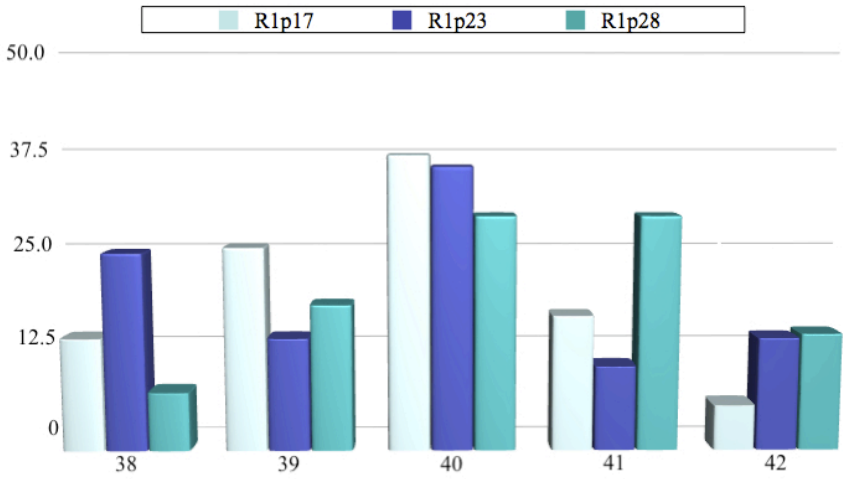


Figure 11: *Chromosome number of R1 ES cell line at different passage numbers (p17, p23, p28) and chimeras*

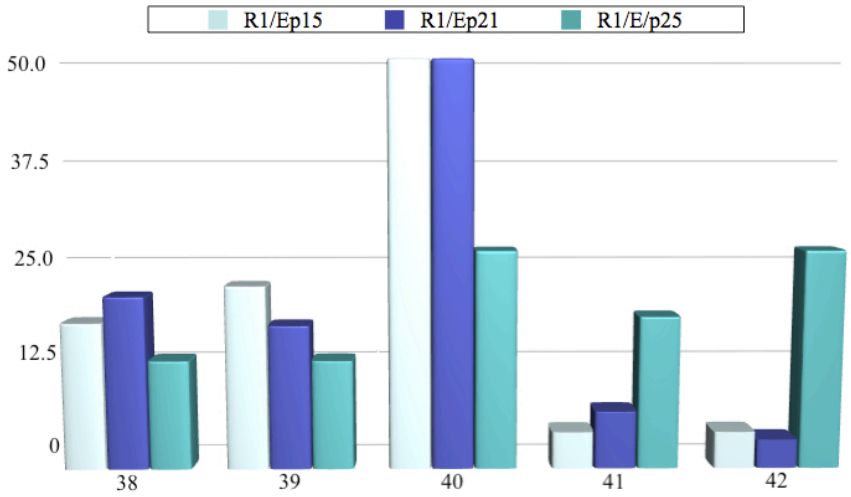


Figure 12: *Chromosome number of R1/E ES cell line at different passage numbers (p15, p21, p25)*

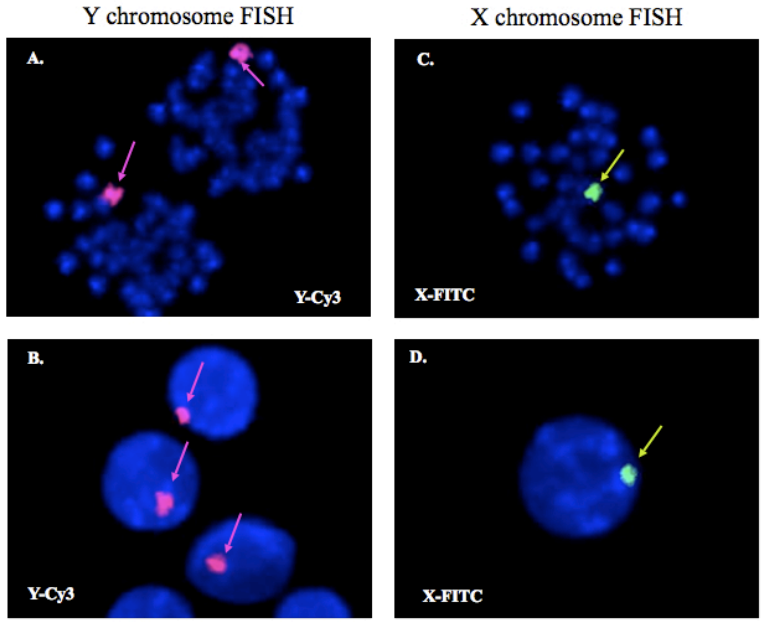


Figure 13: *FISH analysis of R1/E and R1 ES cells (Photo: V.B. Carstea, A. Kozma)*

Finally, we made karyotype analyses of R1 and R1/E ES cells at different passages. It was demonstrated by G- banding that in the case of aneuploid R1 ES cells the 41 and 42 chromosome containing cells hold autosomal trisomies ***(Figure 14, Table 3)***. The proportion of trisomic cells increased proportionally with passage number. The aneuploid ES cells can contribute to the different tissues of chimeric animals but cannot form viable germ cells.

3.table

Number of chromosomes	Number of Trisomies
Ch2	1
Ch3	3
Ch5	1
Ch7	1
Ch3 and Ch5	1

We analyzed the number of Ch2, Ch3, Ch5, Ch7 and Ch3-Ch5 chromosome trisomy containing cells in R1 ES cells at p23 passages.

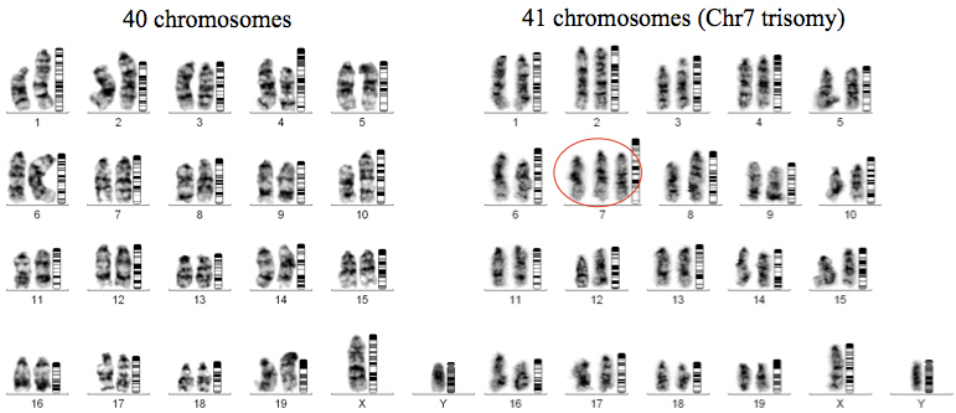


Figure 14: *G-banded karyotypes of R1 ES cells (p23)*
(Photo: R. Katona)

8.2. *MASCULINIZATION PHENOMENON*

8.2.1. POST-IMPLANTATION DEVELOPMENT OF SEX DETERMINED DIPLOID/ DIPLOID CHIMERIC BLASTOCYST

The capacity of a single male blastomere to induce masculinization upon aggregating it with a diploid female 8-cell stage host embryo was investigated. Single blastomeres from 8-cell embryos were isolated and aggregated with diploid host embryos.

In the first series of experiments, single blastomeres derived from sexed, EGFP expressing, diploid 8-cell stage male embryos [(2n)(1-cell)], were aggregated with sexed diploid 8-cell stage female embryos [(2n)(7-cells)], as one cell was removed for sexing. The aggregates were cultured in vitro and transferred at the early blastocyst stage to the uterus of pseudo-pregnant females ***(Figure 8)***. 84 embryos were transferred and out of those 39 formed only deciduas. 12 of the 22 newborns were chimeras visualized by the presence of EGFP positive cells in their skins. 11 of the 12 sex chimeras were males and one was female ***(Table 4.)***. This female was an XX/XY chimera since we could identify both XX- and XY-cells in its tail tissue. Although this mouse displayed a normal fertile female phenotype, none of its 31 offspring were found to inherit the EGFP marker. After sacrificing the animal, we could detect in average 10% EGFP expressing cells by histological analysis in tissues of its different organs (skin, kidney, oviduct, uterus and ovary). All the other eleven chimeras developed to fertile, healthy males, and their offspring of both sexes inherited the EGFP marker protein ***(Table 5.).*** This finding demonstrates that a single male blastomere was able to reverse the 8-cell stage female embryo's gender.

4.table

Type of chimeras	[XY(2n)(1-cell)]/ [XX(2n)(7-cells)]
Number of transferred embryos	84
Ratio of resorption (Resorption/transferred %)	39 (46.4)
Ratio of newborns (Alive and stillborn mice /transferred %)	22 (26.2)
Ratio of mouse born alive (Alive mice /transferred %)	14 (16.7)
No of chimeras	12 (85.7)
No of chimeras born alive	12
Ratio of male chimeras (Males/Chimeras %)	11 (91.7)
Ratio of female chimeras (Females/Chimera %)	1 (8.3)(A)

Single blastomeres derived from sexed, EGFP expressing, diploid 8-cell stage male embryos [XY(2n)(1-cell)] were aggregated with sexed diploid 8-cell stage female embryos [XX(2n)(7-cells)]. 84 embryos were transferred, 39 formed only deciduas, 12 of the 22 newborns were chimeras. 11 of the 12 sex chimeras were males and one of them (A) was female.

5.table

Chimeras	Sex	The percentage of EGFP positive cells in the skin of newborns (%)	Number of progenies	EGFP expressing progenies %		EGFP non-expressing progenies %	
				male	female	male	female
A.	female	10	31	*0.0*	*0.0*	*32.3*	*67.7*
B.	male	10	14	*28.6*	*14.3*	*28.6*	*28.6*
C.	male	30	13	*46.2*	*7.7*	*15.4*	*30.8*

Chimera (A) was normal fertile female with XX/XY genotype, but none of its 31 offspring expressed the EGFP marker. Chimera (B) and (C) were males with XX/XY genotype and could inherit the EGFP marker to the offspring.

8.2.2. DISTRIBUTION OF EGFP POSITIVE CELLS DERIVED FROM A SINGLE EGFP EXPRESSING BLASTOMERE BETWEEN THE EMBRYONIC LAYERS OF CHIMERIC BLASTOCYSTS

At first we analyzed the developmental potential of a single sexed EGFP expressing blastomere, derived from diploid eight-cell stage embryo, in 2n (1-cell), XY / 2n (7-cells), XX and in 2n (1-cell) / 4n (4-cells) chimera embryos ***(Figure 9)***. The distribution of EGFP expressing blastomere-derived cells in different parts of chimera embryos was evaluated at 82 hpg (3.5 dpc) and 106 hpg (4.5 dpc). Since we used a single EGFP expressing blastomere, the contribution of EGFP derived cells could be evaluated more accurately, compared to traditional 2n/4n chimera embryos. Significant differences were found between the diploid and diploid/tetraploid chimeras in the contribution of EGFP expressing blastomere-derived cells to the inner cell mass (ICM) and trophectoderm (TE) ***(Table 6)***. 106 diploid XX/XY and 101 diploid/tetraploid chimera embryos were analyzed at 3.5 dpc along with 50 diploid XX/XY and 30 diploid/tetraploid 4.5 dpc chimera embryos. 41.5% of the 3.5 dpc diploid/tetraploid chimeras contained EGFP expressing cells only in the ICM compared to the diploid/diploid chimeras in which the ratio of those embryos was 20%. At 4.5 dpc the ratio of the diploid/tetraploid chimeras that contained EGFP expressing cells only in ICM was somewhat reduced (36.7%), but was still significantly higher as compared to 24.4% in the diploid/diploid chimeras.

On the contrary, the percentage of chimeras containing EGFP expressing cells only in TE was 23.6% and 26.7% in the diploid/tetraploid and 46.4% and 41.7% in the diploid group at 3.5 and 4.5 dpc, respectively.

6.table

Distribution of EGFP expressing cells between the embryonic layers		**2n(1-cell)/4n(4-cells) ±SD (No. of embryos)**	**2n(1-cell)/2n(7-cells) ±SD (No. of embryos)**
3.5 dpc embryos	ICM only	41.5±5.3*a* (n=41)	20.0±7.4*b* (n=19)
	ICM and TE	34.9±9.2 (n=38)	33.6±4.8 (n=36)
	TE only	23.6±5.1*c* (n=22)	46.4±5.7*d* (n=51)
4.5 dpc embryos	ICM only	36.7±3.3*e* (n=11)	24.4±3.0*f* (n=12)
	ICM and TE	36.7±3.3 (n=11)	33.9±0.7 (n=17)
	TE only	26.7±0.0*g* (n=8)	41.7±2.2*h* (n=21)

The distribution of the EGFP expressing single blastomere derived cells between the embryonic layers was analyzed in (2n)(1-cell)/(4n)(4-cells) and (2n)(1-cell)/(2n)(7-cells) 3.5 and 4.5 dpc chimera blastocysts. We represented here the percentage of the EGFP expressing cell containing embryos: only in the inner cell mass, or in the inner cell mass and trophectoderm and only in the trophectoderm layer. Results labeled with a,b letters were significantly different at 0.002 level; c,d at 0.01 level; e,f at 0.02; g,h at 0.001 level using Student's t-probe.
ICM – inner cell mass, TE – trophectoderm, n - number of analyzed chimeras

8.2.3. A SINGLETON, TWINS AND A TRIPLET OF PREDICTED GENDER

To generate mice with clonal origin, single blastomeres obtained from the same sexed EGFP labeled 8-cell stage embryos were complemented with unsexed tetraploid carriers ***(Figure 9)***. We developed a quick and reliable multiplex PCR strategy for sex-diagnosis at the single cell level by simultaneous amplification of the homologous, but non-allelic Zfx and Zfy genes present on the X- and Y-chromosomes. From 12 transferred 2n/4n aggregates, where single EGFP expressing blastomere was combined with a 4-cell stage tetraploid embryo 2n (1-cell) / 4n (4-cells), we got two (16.7%) live newborns, one male (M) and one female (F). From 36 transferred 2n, XY (1-cell)/4n (4-cells) aggregates, where a single XY, EGFP expressing blastomere was combined with a 4-cell stage tetraploid embryo, we got five (13.9%) live male newborns: two pairs of twins (A1, A2 and B1, B2) and a singleton (D1). From the transfer of four 2n, XX (1-cell)/4n (4-cells) chimera embryos a set of live female triplet was born (C1, C2, C3) (Table 6). Five of the males and all of the triplet females did reach the adulthood. We tested the fertility of one twin male (B1) and the triplet females (C1, C2, C3). We could get healthy progeny in all cases ***(Table7)***.

8.2.4. CLONAL ORIGIN OF THE TWINS AND TRIPLET BORN FROM 2N/4N BLASTOCYSTS

The clonal origin of the members of twins and the triplet respectively was proven by microsatellite analysis ***(Table 8)***. The 17 polymorphic microsatellite markers represented 15 chromosomes across the mouse genome. The analysis distinguished four groups (G1-G2-G3-G4) of mice: A1 and A2 males; B1 and B2 males; the single D1 male; C1, C2, C3 females ***(Figure 15)***. Animals within each group had uniform genotype for all microsatellite markers, but the groups differed from each other with respect of the different markers.

7.table

Type of chimeras	**2n, (1-cell) / 4n, (4-cells)**	**2n, XY(1-cell) / 4n,(4-cells)**	**2n, XX(1-cell) / 4n, (4-cells)**
Number of transferred embryos	12	36	4
Ratio of resorptions *(Resorptions / Transferred %)*	8 (66.7)	11 (30.6)	1 (25.0)
Ratio of newborns *(Newborns / Transferred %)*	2 (16.7)	11 (30.6)	3 (75.0)
Ratio of live born pups *(Live pups / transferred %)*	2 (16.7)	5 (13.9)	3 (75.0)
Ratio of males *(Males / Chimeras %)*	1 (50.0)	5 (100.0)	0 (0.0)
	M	(A1, A2, B1, B2, D1)	-
Ratio of females *(Females / Chimeras %)*	1 (50.0)	0 (0.0)	3 (100.0)
	F	-	(C1,C2,C3)
Number of progenies of tested adult mice *(Male / Progenies %)*	-	B1: 25 (48.0)	C1: 18 (66.7)
			C2: 18 (50.0)
			C3: 23 (43.5)

Chimeras were created by aggregating single sexed, EGFP expressing blastomeres, isolated from 8-cell embryos with non-sexed tetraploid carrier embryos 2n (1-cell) / 4n (4-cells), or 2n, XY(1-cell) / 4n, (4-cells), or 2n, XX(1-cell) / 4n, (4-cells). The aggregates were cultured in vitro, and transferred as blastocysts, to the uterus of pseudo pregnant females. From the transferred 2n (1-cell) / 4n (4-cells) blastocysts one male (M) and one female (F) newborn, from the single XY diploid cells 5 living male newborns were born: 2 pairs of monozygotic twins (A1, A2 and B1, B2) and one singleton (D1). From the single XX diploid cells a set of live monozygotic female triplet (C1, C2, C3) was born. We checked the fertility of B1 male and C1, C2, C3 females. We got progenies from each examined adults.

8.table

DMit markers	A1	A2	B1	B2	D1	C1	C2	C3
	G1		G2		G3	G4		
D1Mit262	b	b	ab	ab	ab	ab	ab	ab
D2Mit113	c	c	bc	bc	ac	bc	bc	bc
D3Mit352	b	b	b	b	b	ab	ab	ab
D4Mit166	b	b	b	b	ab	ab	ab	ab
D5Mit1	ab	ab	a	a	ab	ab	ab	ab
D5Mit155	ab	ab	ab	ab	a	a	a	a
D6Mit105	ab	ab	b	b	ab	bc	bc	bc
D6Mit200	b	b	b	b	b	ab	ab	ab
D7Mit22	a	a	ab	ab	ab	ab	ab	ab
D8Mit85	b	b	ac	ac	ac	ab	ab	ab
D9Mit163	b	b	ab	ab	a	a	a	a
D10Mit16	ab	ab	a	a	a	a	a	a
D11Mit2	b	b	ab	ab	ab	b	b	b
D12Mit63	ab	ab	a	a	a	ab	ab	ab
D13Mit26	a	a	a	a	a	b	b	b
D15Mit13	b	b	ab	ab	b	ab	ab	ab
D16Mit136	a	a	ab	ab	b	ab	ab	ab

DMit genotypes of the eight mice developed from XX/XY sex-chimera embryos belonging to four groups (G1-G4): A1 and A2 males (G1); B1 and B2 males (G2); the single D1 male (G3); C1, C2 and C3 females (G4).
a,b,c – allele forms

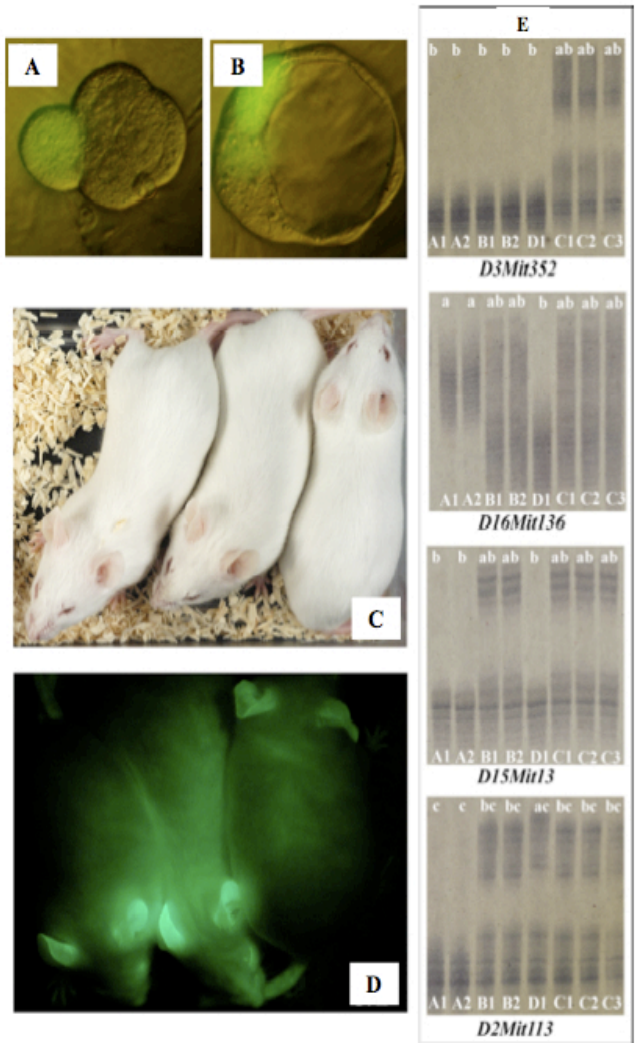


Figure 15: *Microsatellite analysis of cloned triplets (Photo: V.B. Carstea, G. Takács, L. Varga)*

8.3. *RABBIT CHIMERAS*

8.3.1. CHROMOSOME ANALYSIS FROM PERIPHERAL BLOOD LYMPHOCYTES

The normal diploid number of chromosomes (2N = 44) for the domestic rabbit was first established by Painter in 1926 using amnion cells from 14-day embryos (CHAN, 1977). Identification of unbanded X- and Y-chromosomes is problematic in this species as none of them are surely distinguishable from autosomes (Frankenhuis et al., 1990). Sexing by counting the smallest chromosomes in complete mitoses was successfully carried out in all four animals ***(Figure 16)***.
The two chimeric does were XX/XX, one of the bucks XY/XY. The hypogonadic young male proved to be an XX/XY chimera with 54 XX and 14 XY cells but later developed to a normal fertile male ***(Table 9)***.

9.table

Chimeric rabbit #	**Phenotypic sex**	**Ratio of male metaphases** *(%)*	**Ratio of female metaphases** *(%)*
16	doe	0	100
374	doe	0	100
334	buck	100	0
375	hypo-gonadic buck	14 (20.5)	54 (79.5)

The two chimeric does were 44, XX; one of the bucks 44, XY. The hypo-gonadic male proved to be an XX/XY chimera with 54 XX and 14 XY cells.

8.3.2. KARYOTYPING USING FISH TECHNIQUES

The DNA was extracted by Maxi prep Kit extraction (Qiagen) and labeled as described above. For these experiments, hybridized chromosome preparations were very clean, but no signal was detected in all cases. It is hypothesized that the purified DNA was not correctly labeled maybe due to the presence of an inhibitor of the labeling reaction.
Therefore, it was decided to prepare purified DNA extracted by Mini prep Kit for the second set of FISH experiments.
In this case, hybridization signals were observed in all cases with intensities variable according to slides. We could map the clones containing the LIFR

gene to rabbit chromosome OCU11p11.1 and the clones assumed to contain the POU5F1 gene to OCU1q21.1 as shown in the ***(Figure 17)***

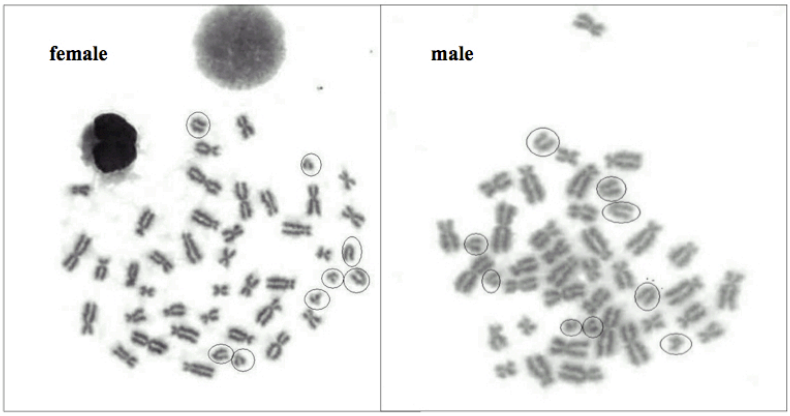


Figure 16: *Analysis of female and male rabbit chimera chromosomes from blood samples (Photo: V.B. Carstea, T. Révay)*

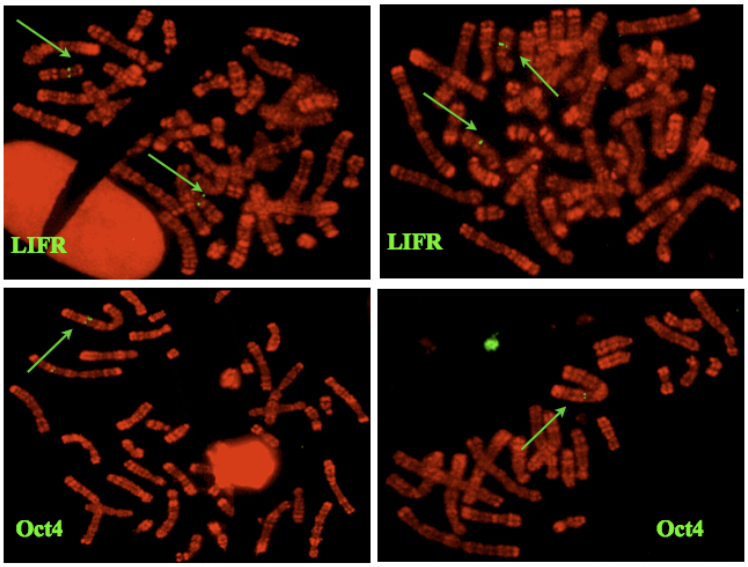


Figure 17: *Rabbit chromosome FISH from rabbit embryonic fibroblast cells (Photo: V.B. Carstea, H. Hayes)*

9. NEW SCIENTIFIC RESULTS

1. I could demonstrate using karyotype analysis and FISH examination that in R1 ES cell line, the 41 and 42 chromosome containing cells had autosomal trisomies, and the proportion of trisomic cells increased by the passage number. These aneuploid ES cells can contribute to the different tissuses of chimera animals, but cannot form viable germ cells.
2. I proved that both diploid and tetraploid cells could participate in the ICM of chimeras, and selection against tetraploid cells begins before the cavitation, so the diploid blastomere derived cells have significantly higher chance to contribute to the ICM upon using tetraploid carrier embryo.
3. I demonstrated that a single male blastomere was able to reverse the 8-cell stage female embryo's gender.
4. I produced EGFP labeled twins and triplets with pre-planned gender through an improved and efficient tetraploid embryo complementation method.
5. I mapped rabbit LIFR gene using FISH technique to the rabbit chromosome OCU11p11.1 region.

10. DISCUSSION

10.1. *ES CHIMERAS*

We analyzed the capacity of ES cells for chimera formation. By aggregation between ES cells and 8-cell embryos we obtained chimera embryos and after their transfer into the foster mother uterine horns we could obtain chimera newborns. ES cell can develop further in any chimera tissues and in these way, they can contribute to the formation of the germinal line. Using transgenic ES cells for chimera production, and by obtaining germ line chimeras we produced transgenic animals. In farm animals, the ES cell lines establishing and chimera production is more difficult. Understanding the phenomena in mouse will help to apply the technique on farm animals in the near future.

In our work, we examined the chimera producing ability of R1 and R1/E mouse ES cell lines. We found that the passage number affects chimera-forming capacity of the ES cells. By increasing the passage number, we could get less chimera animals, and only the R1/E ES cell line derived cells could contribute to the germ cells.

We also analyzed the ES cells using immunostaining and RT-PCR for pluripotency markers and we couldn't find significant differences between these two cell lines. Using chromosomal analyzes we found that the increasing of passage number affects the chromosome numbers of R1 and R1/E cell lines. In mice, the normal diploid chromosome number is 40, but after 15-20 passages of the cells lines, we found more or less, than 40 chromosomes in an increasing proportion of the cells.

At chromosome analysis, we found, that the number of aneuploid cells, in R1 ES cell line, dramatically increased after 10 passages. We thought that the reason is that during the cell division the Y-chromosome could not be correctly distributed between the two newly derived progeny cells. To prove our conception, we made X- and Y-chromosome FISH analyses. We found, that the aneuploid R1 and R1/E ES cells contained only one X- and one Y-chromosome, so not the loss of the Y-chromosome caused the problem at the germ cell formation. At last, we made the karyotype analysis of R1 and R1/E ES cells at different passages. The karyotype analysis demonstrated that in the case of R1 ES cell line, the 41 and 42 chromosome containing cells had autosomal trisomies. The proportion of trisomic cells increased proportionally by the passage number. The aneuploid ES cells can contribute to the different tissuses of chimera animals, but cannot form viable germ

cells.

10.2. *MASCULINIZATION PHENOMENON*

Using the advantage of aggregation chimeras made of single diploid EGFP expressing blastomeres, we were able to follow precisely the fate of the EGFP labeled cells, which was not possible in the traditional 4n/2n chimera embryos.

ISHIGURO (2005) suggested that distribution of 4n cells in 4n/2n chimeras was normal until day 3.5, but data of EVERETT and WEST, (1998), obtained in early blastocysts showed that 4n cells are preferentially allocated to the mural trophectoderm, and in the later-stage blastocysts, evidence was found for selection against 4n cells. In addition to this, EVERETT (2000) demonstrated that ploidy could influence the relative position of blastomeres in the preimplantation embryo. Our findings together with Everett's observation suggest that both diploid and tetraploid cells could participate in the ICM of chimeras, and selection against tetraploid cells begins before the cavitation. The diploid blastomere derived cells have significantly higher chance to contribute to the ICM upon using tetraploid carrier embryo, so in this way the percentage of diploid blastomere derived cells participating to the ICM of chimeric embryos could be increased. Our data underline the observation of MACKAY and WEST (2005) that tetraploid cells contributed to all four blastocyst tissues and were not initially excluded from the epiblast in 4n/2n chimeric blastocysts.

The novel ability to visualize marked cells in living embryos has implications for both cell migration and cell lineage analyses. When EGFP is expressed under tissue-specific promoters, it should be a useful analysis to delineate the fates of subpopulations of cells throughout embryogenesis and postnatal development. Chimeras composed of sexed, EGFP expressing blastomeres and diploid host embryos are valuable tools to gain insights into the development and phenotype of different sex chromosomal abnormalities. In future term these chimeric mice provide an experimental system in which the correlation of chromosomal sex with phenotypic sex and the proportions of chimeric components in the gonadal tissues could be evaluated.

The advent of molecular genetics has made twin studies more useful than ever because of the power of quantitative trait loci analyses (LUFT, 2001; MORLEY, 2005). Twin studies have been convincing in demonstrating the familial aspects of ischemic heart disease (REED, 1991), in showing the effect of genetic variance on cholesterol and its fractions (CHRISTIAN, 1987).

Serial nuclear transfer of embryo-derived blastomeres, as donor cells, could generate mouse clones, which develop to the blastocyst stage with high frequency. The transfer of these blastocysts to recipient mice resulted a set of live young, which included one set of identical sextuplet and two sets of identical quadruplet mice (KWON, 1996).

Since the pioneering experiments of WILMUT (1997) a number of different species including pigs, goats, horses, cats have been cloned with the somatic cell nuclear transfer technique, although the technology still has relatively low success rates and there seem to be substantial problems with the welfare of some of the cloned animals (VAJTA, 2006).

Recent data show that blastocysts, derived from embryonic stem (ES) cell cloning by nuclear transfer into an enucleated oocyte, developed at a high rate, correctly expressed the pluripotential marker gene Oct4 in ICM cells and displayed normal growth in vitro (JOUNEAU, 2006). The majority of them implanted in the uterus of recipient females, but most of them died before mid-gestation. This study indicated that in nuclear transfer experiments, the trophoblast cell lineage is the primary source of the defects, which results in high mortality during early embryo-genesis. Only chimeras formed by the aggregation of NT and tetraploid embryos reveal no growth abnormalities at gastrulation, because tetraploid embryo derived cells are able to contribute to the trophoblast cell lineage and help to form normal trophoblast layer (JOUNEAU, 2006).

In another point of view, unlike clones produced by nuclear transfer, which exhibit various degrees of mitochondrial heterogeneity (EVANS, 1999), chimeras produced with the tetraploid embryo complementation method are identical with respect to both nuclear as well as mitochondrial DNA. Mitochondrial function is normally controlled by a dual genome system with cooperation between nuclear- and mitochondrial- encoded genes (KHAN, 2007). In nuclear transfer procedures, varying quantities of donor cell mitochondria are transferred with nuclei into recipient oocytes, and mitochondrial heteroplasmy has been observed. Cytoplasm or purified mitochondria from somatic cells were transferred into oocytes. All injected oocytes with somatic cytoplasm or mitochondria showed delayed parthenogenetic development when compared to control oocytes injected with buffer (TAKEDA, 2005).

Here we report the production of EGFP labeled twins and triplets with pre-planned gender through an improved and efficient tetraploid embryo complementation method. Genetically identical twins or triplets created with this method would substantially reduce the numbers of animals required for

generating statistically valid data due to elimination of genetic variation, in particular for investigating environmental influences on criteria, which are mainly genetically conditioned.

10.3. *RABBIT CHIMERAS*

In rabbit species the high rate of chimeric construct embryos born alive could be partly explained by the adaptation of the asynchronous timing for hormonal treatments of the recipient does. The chimeric rabbits including the XX/XY hypo-gonadic male were fertile and did not show any other growth abnormalities. In the case of chimeric mice the relationship of chromosomal sex to functional germ cells has been long time analyzed in detail. It was first described by TARKOWSKI (1961) and confirmed by several authors that XX/XY chimeric mice constructed at an early embryonic stage develop as male (MULLEN, 1971; GEARHARDT and OSTER-GRANITE 1981; YOSHINO et al., 1994; TARKOWSKI 1998). Our data underline this observation in rabbits since the XX/XY animal developed as a buck, albeit the ratio of male cells in its blood was only 20%.

BABINET and BORDENAVE (1980) in their pioneering experiment reported germ line chimerism assessing it in the lymphoid organs with the help of allotypic immunoglobulin markers in the founders and their progeny. However, at that time no other markers were available to estimate the extent of chimerism. Since then a number of different laboratories have reported the formation of rabbit chimeras, but germ-line transmission in these animals was not confirmed (YANG and FOOTE, 1988; GILES, 1993; SCHOONJANS, 1996; MOENS, 1996).

Concerning the LIFR gene, it is located in man on chromosome 5p13.1, which based on comparative rabbit-human mapping data corresponds to rabbit chromosome 11. Since, we have mapped the LIFR gene in rabbit on OCU11p11.1. Our result agrees with the human – rabbit comparative map (KORSTANJE, 1999; CHANTRY-DARMON, 2003) and refines it.

Concerning the clones containing the POU5F1 gene, we have mapped them to rabbit chromosome OCU1q21.1 and not to OCU12q11.1 as expected. Therefore, our result provides additional data that these clones do not contain the POU1F5 gene, but most probably a pseudo-gene. The rabbit BAC library will be screened again for the POU5F1 gene using different primers.

11. APPENDIX

11.1. *REFERENCES*

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11.4. *MEDIA*

11.4.1. MEDIA FOR EMBRYO CULTURE

11.4.1.1. *M2, M16 medium*

STOCK A M2, M16	10x	(100ml/g)	(4C-on)
SIGMA WATER	W1503	30	ml
NaCl	S5886	5534	g
KCl	P5405	356	g
KH2PO4	P5655	162	g
MgSO4	M2543	143	g
Glucose	G7021	1	g
Penicillin G	P3032	6	g
Streptomycin	S9131	5	g
NaLactate (60% syrupe)	L7900	4349	g
SIGMA Embryo transfer water	to 100ml volume	70	ml
STOCK B M2, M16	10x	(100ml/g)	4C
SIGMA WATER	W1503	30	ml
NaHCO3	S5761	2101	g
SIGMA Embryo transfer water	to 100ml volume	70	ml
STOCK C M2, M16	100x	(10ml/g)	-20C
SIGMA WATER	W1503	10	ml
Na pyruvate	118-40-030	36	g
STOCK D M2, M16	100x	(10ml/g)	-20C
SIGMA WATER	W1503	10	ml
CaCl2x2H2O	C7902	252	g
STOCK E M2	100x	(100ml/g)	-20C
SIGMA WATER	W1503	100	ml
HEPES	H4034	5958	g
phenol red	P5530	1	g

11.4.1.2.*KSOM medium*

STOCK A KSOM	10x	(100ml/g)	4C
SIGMA WATER	W1503	30	ml
NaCl	S5886	555	g
KCl	P5405	186	g
KH2PO4	P5655	476	g
MgSO4	M2543	2408	g
Glucose	G7021	36	g
Penicillin G	P3032	6	g
Streptomycin	S9131	5	g
NaLactate (60% syrupe)	L7900	187	g
SIGMA Embryo transfer water	to 100ml volume	kb. 70	ml
STOCK B KSOM	10x	(100ml/g)	4C
SIGMA WATER	W1503	30	ml
NaHCO3	S5761	21	g
SIGMA Embryo transfer water	to 100ml volume	kb. 70	ml
STOCK C KSOM	100x	(10ml/g)	-20C
SIGMA WATER	W1503	10	ml
Na pyruvate	118-40-030	22	g
STOCK D KSOM	100x	(10ml/g)	-20C
SIGMA Embryo transfer water	W1503	10	ml
CaCl2x2H2O	C7902	25	g
STOCK F KSOM	100x	(10ml/g)	-20C
SIGMA Embryo transfer water	W1503	10	ml
EDTA	E6511	38	g
PHENOL RED solution	5%		4C
PHENOL RED	P5530	5	g
SIGMA Embryo transfer water	W1503	10	ml

M16	10 ml	20 ml	50 ml	
STOCK A M16, M2	1	2	5	g
STOCK B M16, M2	1	2	5	g
STOCK C M16, M2	1	2	5	g
STOCK D M16, M2	1	2	5	g
BSA (A3311)	4	8	2	g
H2O	78	156	39	ml

M2	10 ml	50 ml	100 ml	
STOCK A M16, M2	1	5	10	g
STOCK B M16, M2	16	8	16	g
STOCK C M16, M2	1	5	1	g
STOCK D M16, M2	1	5	1	g
STOCK E M16, M2	84	42	84	g
BSA (A3311)	4	2	4	g
H2O	78	39	78	ml

KSOM BASIC SOLUTION			
STOCK A KSOM		10	ml
STOCK B KSOM		10	ml
STOCK C KSOM		1000	ml
STOCK D KSOM		1000	ml
STOCK F KSOM		100	ml
MEM AAS	M5550	1000	ml
MEM NON ESSENTIAL AAS	M7145	500	ml
PHENOL RED SOLUTION		10	ml
GLUTAMAX	GIBCO 11140 (100x)	1000	ml
SIGMA Embryo transfer water	W1503	kb. 76	ml
KSOM BASIC SOLUTION		20	ml
BSA	A3311	2	g
	osmolarity	256	
	pH	74	
	2x10ml	*Filtered (0.2um filter)*	

11.4.2. MEDIA FOR EMBRYONIC STEM CELL CULTURE

GIBCO FM basic solution		*filtered (0.2um filter)*	
D-MEM (GIBCO 31966-047)	1x solution	490	ml
penicillin (SIGMA P3032)	60%	5	ml
streptomicin (SIGMA S9131)	1%	5	ml
	Sum:	**500**	**ml**

GIBCO Fibroblast medium (FM+10%FCS)			*filtered (0.2um filter)*
GIBCO FM basic solution		400	ml
st. FCS (EUROCLONE)(ECS0180L)		45	ml

KO-EM ES cell cultural medium (15% FCS)(for chimeras)			*filtered (0.2um filter)*		
KnockOut D-MEM (GIBCO 10829-018)	1x solution	80	ml	40	ml
penicillin	60%	1	ml	500	ml
streptomicin	1%	1	ml	500	ml
glutamax (GIBCO 35050)	100x	1	ml	500	ml
2-merkaptoethanol stock		1	ml	500	ml
mouse LIF (Chemikon ESG1106)		100	ul	50	ml
MEM NON ESSENTIAL AAS (GIBCO 11140)	100x	1	ml	500	ml
FCS (HYCLONE SH30070.03)		15	ml	75	ml
	Sum:	100	ml	50	ml

KO-EM ES medium for ES cell line establishment (20% FCS)			*filtered (0.2um filter)*		
KnockOut D-MEM (GIBCO 10829-018)	1x solution	75	ml	375	ml
penicillin	60%	1	ml	500	ml
streptomicin	1%	1	ml	500	ml
glutamax (GIBCO 35050)	100x	1	ml	500	ml
2-merkaptoethanol stock		1	ml	500	ml
mouse LIF (Chemikon ESG1106)		100	ul	50	ml
MEM NON ESSENTIAL AAS (GIBCO 11140)	100x	1	ml	500	ml
FCS (HYCLONE SH30070.03)		20	ml	10	ml
	Sum:	100	ml	**50**	ml

EM ES cell cultural medium (15% FCS) (*before differentiation*)			*filtered (0.2um filter)*		
D-MEM (GIBCO 31966-047)	1x solution	81	ml	405	ml
penicillin	60%	1	ml	500	ml
streptomicin	1%	1	ml	500	ml
2-merkaptoethanol stock		1	ml	500	ml
mouse LIF (SIGMA L5158)		40	ul	50	ml
MEM NON ESSENTIAL AAS (GIBCO 11140)	100x	1	ml	500	ml
FCS (EUROCLONE ECS0180L)		15	ml	75	ml
	Sum:	100	ml	**50**	ml
2x Freezing medium					
Fibroblast medium	15	ml	3	6	ml
FCS (EUROCLONE ECS0180L)	5	ml	1	2	ml
DMSO (st.)(SIGMA D2650)	5	ml	1	2	ml
Sum:	**20**	**ml**	**5**	**10**	**ml**

Stock solution	**%**	**sterilization**		**Contrentation**	**solvent**	**usage**	**stocks**
gelatin	10%	autokl.	*4C, 3month*	0,1g gelatin	100ml PBS	4ml/6cm/ 20min/RT, 10 min to dry	
mytomicin	1mg/ml	st.	*4C, 2weeks*	2mg mytomicin	2ml autokl. MilliQ water	60ul/6ml , 2.5 hour, 37C	20x60 ul stock -70C
2-merkapto-ethanol (SIGMA M7522)	7ul/l (0.7v%)	st.	*-20C*	14ul Mercapto-ethanol	20ml PBS	1ml merkaptoet. stock./ 100ml ES medium	20x1ml fagy.

11.4.3. STOCK SOLUTIONS FOR EMBRYONIC STEM CELL CULTURE MEDIA

PBS solution					
Matherials	*FW*			*autoclaved*	
Qviz		1000	ml	2000	ml
KCl	7455	20	g	4	g
KH2PO4	13610	20	g	4	g
NaCl	5840	800	g	160	g
Na2HPO4	14200	115	g	23	g
EDTA solution					
Matherials	*FW*			*autoclaved*	
PBS oldat		1000	ml	500	ml
EDTA(Na4)	38020	2	g	1	g
Trypsin solution					
Matherials	*FW*	*4x50 ml, -20C*		*filtered (0.2um filter)*	
Q viz		250	ml		4C
KCl	7455	100	g		RT
NaCl	5840	2000	g		RT
Na2HPO4	14200	25	g		RT
D-glucose	18020	250	g		RT
Trizma base	1211	750	g		RT
phenol red	3764	3	g		RT
penicillin G	3564	15	g		4C
streptomicin	1457	25	g		4C
trypsin (2.5 g/l)	1-250	625	g		4C
Trypsin-EDTA solution					
Matherials	*FW*	4C, 2 weeks		*filtered (0.2um filter)*	
Trypsin oldat	50	ml			
EDTA	200	ml			

Name of solution	Concentration	sterilization		Matherials		st. MQ water	
penicillin	60%	filt.	4C	**60**	**g**	**100**	**ml**
streptomicin	**1%**	filt.	4C	**100**	g	100	ml
NaHCO3	**750%**	filt	4C	**1875**	g	250	ml
pyruvic acid	**110%**	filt.	4C	**110**	g	100	ml
NaOH	**1N**	filt.	4C	**400**	g	100	ml
NaH2PO4	**109%**	filt.	4C	**109**	g	100	ml
tripán blue	**20%**	filt.	4C	**1**	g	50	ml
NaCl	**425%**	filt.	4C	**425**	g	100	ml

Cell counting staining solution *(4C)*		(0.16% stock)	
tripán blue	**20%**	40	ml
NaCl	**425%**	10	ml

Cell counting sol. *fressly prepared*		
tripan staining solution	80	ml
cell suspension	20	ml

Materials	Cat. Numb. (SIGMA)	
mytomicin-C	M0503	*4C*
mineral oil	M6145	*RT*
pronase	P8811	*-20C*
MTG	M6145	*4C*
GIEMSA	GS-500	4C

11.4.4. STOCK SOLUTION FOR FISH

20xSSC	Cat. Number	Fw:	
MQ			900 ml
NaCl	S5886	58,44g	175,3 g
Na-citrate	S4641	2941	88,4 g
MQ	to		1000ml

2xSSC	Cat. Number	Fw:	*pH: 7*
MQ			180 ml
20xSSC	S6639		20 ml

10xPBS	Cat. Number	Fw:	*4C-on*
MQ			900 ml
NaCl (SIGMA)	S5886	584	76 g
Na2HPO4x2H2O	K12550380	17799	12,46 g
NaH2PO4x1H2O	A420746	13799	4,68 g
MQ			1000 ml-ig

1xPBS	Cat. Number	Fw:	*pH: 7*
MQ			180 ml
10xPBS			20 ml

Formamide/SSC 50%	Cat. Number	Fw:	*pH: 7*
Formamide (Fluka)	47671		50 ml
2xSSC			50 ml

0,005% pepszin	Cat. Number	Fw:	
MQ			99 ml
1N HCl (SIGMA)	H9892		1 ml
10% pepsin stock(before use)			50 ul

0,5% NP40/2xSSC	Cat. Number	Fw:	*pH: 7*
2xSSC			100 ml
NP-40 (Fluka)	74385		500 ul

10 % pepsin stock	Cat. Number	Fw:	*50ul/tube on -20C*
MQ			10 ml
pepsin	Sigma 6887		1g

For hybridization:	Cat. Number	
DAPI-VECTA SHIELD	H1200	BioMarker
Total Mouse Chr. Y Red	CP6121-R	Qbiogene, BioMarker
Total Mouse Chr. X Green	CP6120-G	Qbiogene, BioMarker
Whole Mouse Chr. Y Red ready to use	1200-YMCY3	CAMBIO