

Investigation of the Disposition of a Marker Chromosome in a Mouse Model

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrechts-Universität
zu Kiel
Vorgelegt von

Bengi Acar-Perk

Kiel
2009

Referent/in: Prof. Dr. N. Arnold
Korreferent/in: Prof. Dr. T. Bosch

Tag der mündlichen Prüfung: 14.07.2009
Zum Druck genehmigt: Kiel

Prof. Dr. rer. nat. Lutz Kipp
Der Dekan

BU TEZİ, BANA HERZAMAN DESTEK OLAN, BENİ HİCBİR ZAMAN
YANLIZ VE CÖZÜMSÜZ BIRAKMAYAN AILEM NECMETTİN, HİLAL VE
ERGI ACAR'A ITAF EDİYORUM. İLK TEBRİK VE TESEKKÜR
ONLARINDIR.

İYİ Kİ VARSINIZ ve BENİM AILEMSİNİZ...

VE TABİİ Kİ YENİ HAYATIM, ESİM DENİZ PERK'e

ÖMÜR BOYU MUTLU BERABERLİĞİMİZE...

TABLE OF CONTENTS

1. INTRODUCTION

| | | |
|----------|--|----|
| 1. 1. | The mechanism of aneuploidy | 9 |
| 1. 2. | Mouse as a model | 12 |
| 1. 2.1. | Sex differentiation of the mice | 12 |
| 1. 2. 2. | History of Mouse Researches | 13 |
| 1. 2. 3. | General Information about Laboratory Mouse | 14 |
| 1. 2. 4. | Reproduction of Laboratory Mouse | 14 |
| 1. 2. 5. | Genetics of Inbred Laboratory Mouse | 18 |
| 1. 3. | CBA/CaH-T(14;15)6Ca/J translocated mouse strain | 21 |
| 1. 3. 1. | General information about CBA/CaH-T(14;15)6Ca/J strain | 21 |
| 1. 3. 2. | Estimated breaking point localisation by (14;15)6Ca/J strain | 21 |
| 1. 4. | M-FICTION | 22 |
| 1. 5. | Stem cell relevant markers | 23 |
| 1. 5. 1. | Oct4 | 23 |
| 1. 5. 2. | Cdx2 | 24 |
| 1. 6. | Aim of this study | 25 |

2. MATERIAL AND METHODS

| | | |
|-------------|--|----|
| 2. 1. | MATERIAL | 25 |
| 2. 1. 1. | Devices | 25 |
| 2. 1. 2. | Software | 26 |
| 2. 1. 3. | Hormones | 27 |
| 2. 1. 4. | Reagent and Chemicals | 27 |
| 2. 1. 5. | Culture dishes, pipets and slides | 30 |
| 2. 2. | METHODS | 31 |
| 2. 2. 1. | Suitable animal husbandry of experimental mice | 31 |
| 2. 2. 2. | Handling techniques of mouse | 32 |
| 2. 2. 2. 1. | Intraperitoneal injection | 32 |

| | | |
|----------------|--|----|
| 2. 2. 2. 2. | Cervical dislocation | 33 |
| 2. 2. 2. 3. | Opening the abdominal cavity and dissection of uterus | 34 |
| 2. 2. 3. | Inducing superovulation in female mouse | 34 |
| 2. 2. 3. 1. | Influence of mouse strain and age | 35 |
| 2. 2. 3. 2. | Doses of gonadotropins | 35 |
| 2. 2. 3. 3. | Time plan of gonadotropin injections | 36 |
| 2. 2. 4. | Gaining of mouse oocyte and early stage embryos | 38 |
| 2. 2. 4. 1. | Preparing the cultures for in- vitro cultivation and embryo handling transfer pipettes | 39 |
| 2. 2. 4. 2. | Collecting oocytes / zygotes and hyaluronidase treatment | 40 |
| 2. 2. 4. 3. | Collecting 4, 6, morula and blastocyst-stage embryos | 42 |
| 2. 2. 5. | Mechanical differentiation techniques to isolate ICM (Inner cell mass/embryoblast) and TE (Trophoectoderm/trophoblast) | 43 |
| 2. 2. 5. 1. | Trophectoderm biopsy technique | 43 |
| 2. 2. 5. 1. 1. | Embryo micromanipulation and zona slitting | 44 |
| 2. 2. 5. 1. 2. | Biopsy procedure | 46 |
| 2. 2. 5. 2. | Immunosurgery | 48 |
| 2. 2. 5. 2. 1. | Setting the reagents and equipments | 49 |
| 2. 2. 5. 2. 2. | Removing zona pellucida and Immunosurgery procedure | 49 |
| 2. 2. 5. 2. 3. | Fluorescence microscopy | 52 |
| 2. 2. 5. 3. | Stem cell culture | 52 |
| 2. 2. 5. 3. 1. | Preparing the culture mediums and solutions | 53 |
| 2. 2. 5. 3. 2. | Removing zona pellucida and culturing the embryos | 54 |
| 2. 2. 5. 3. 3. | Trypsin treatment | 55 |
| 2. 2. 5. 3. 4. | Different fixation techniques of ICM and TE slides | 56 |
| 2. 2. 6. | Immunofluorescence (IF) cell staining | 57 |
| 2. 2. 6. 1. | Preparation of required antibodies and slides | 58 |
| 2. 2. 6. 2. | Immunofluorescence protocol | 58 |
| 2. 2. 7. | Fibroblast cell culture from mouse skin | 60 |
| 2. 2. 7. 1. | Preparation of required solutions and mouse skin | 61 |
| 2. 2. 7. 2. | Mouse skin-fibroblast cell cultivation and preparation | 61 |

| | | |
|--------------|--|----|
| 2. 2. 7. 3. | Metaphase-chromosome preparation from mouse skin-fibroblast cell | 63 |
| 2. 2. 8. | Fluorescence in situ hybridization (FISH) protocol | 65 |
| 2. 2. 8. 1. | Preparation of required solutions | 65 |
| 2. 2. 8. 2. | Slide preparation | 66 |
| 2. 2. 8. 3. | Target and probe DNA denaturation / hybridization | 66 |
| 2. 2. 8. 4. | Washing protocols and counterstaining with 4'-6-diamidino-2 phenylindole (DAPI) | 67 |
| 2. 2. 8. 5. | Detection by fluorescence microscope | 68 |
| 2. 2. 8. 6. | Scoring criterias | 69 |
| 2. 2. 9. | Generation of FISH probe using Bacterial Artificial Chromosome (BAC) clones | 70 |
| 2. 2. 9. 1. | Preparing Lennox Bouillon (LB) solution and culturing the BACs | 71 |
| 2. 2. 9. 2. | BAC DNA purification | 72 |
| 2. 2. 9. 3. | Amplification DNA with Degenerate Oligonucleotid Primer-Polymerase Chain Reaction (DOP-PCR) | 73 |
| 2. 2. 9. 4. | Reamplification of the original banks | 75 |
| 2. 2. 9. 5. | Direct labelling of the BAC DNAs DOP-PCR products | 76 |
| 2. 2. 9. 6. | Precipitation protocol with addition of Mouse CotDNA and Hybmix | 79 |
| 2. 2. 10. | FICTION Technique (Fluorescence Immunophenotyping and Interphase Cytogenetics as a Tool for Investigation of Neoplasm) | 80 |
| 2. 2. 11. | ADAMTS gene expression | 81 |
| 2. 2. 11. 1. | RNA isolation from mouse tissue | 81 |
| 2. 2. 11. 2. | cDNA synthesis from rRNA for use in RT-PCR | 82 |
| 2. 2. 11. 3. | RT-PCR protocol | 83 |
| 3. | <u>RESULTS</u> | |
| 3.1. | General results of experimental mice usage | 84 |

| | | |
|-------------|--|-----|
| 3. 1. 1. | Establishing the superovulation protocol | 86 |
| 3. 1. 2. | FISH and Fibroblast Cell Culture from Mouse Skin | 89 |
| 3. 1. 3. | Embryo gaining | 92 |
| 3. 1. 4. | Mating | 96 |
| 3. 1. 4. 1. | Trophoblast Biopsy | 96 |
| 3. 1. 4. 2. | Immunosurgery | 101 |
| 3. 1. 4. 3. | Embryonic stem cell culture | 105 |
| 3. 2. | Oct4 and Cdx Immunofluorescence (IF) cell staining results | 110 |
| 3. 3. | Multi colour FISH probe | 115 |
| 3. 3. 1. | Generation of specific multi colour FISH | 115 |
| 3. 3. 2. | Break point localization | 118 |
| 3. 3. 3. | Expression of ADAMTS12 | 121 |
| 3. 4. | FICTION (Fluorescence Immunophenotyping and Interphase Cytogenetics as a Tool for Investigation of Neoplasms) results on mice TE and ICM cells | 122 |
| 4. | <u>DISCUSSION</u> | |
| 4.1. | Discussion of methods | 129 |
| 4. 1. 1. | Superovulation protocol | 129 |
| 4. 1. 2. | TE biopsy | 132 |
| 4. 1. 3. | Immunosurgery | 134 |
| 4. 1. 4. | Stem cell culture | 135 |
| 4. 2. | Discussion of results | 137 |
| 4. 2. 1. | Substrains for originating CBA/CaH-T(14;15)6Ca/J translocated mouse strain | 137 |
| 4. 2. 2. | Origin of CBA/CaH-T(14;15)6Ca/J translocated mouse strain | 137 |
| 4. 2. 3. | ADAMTS12 Expression | 140 |
| 4. 2. 4. | Oct4 Expression | 142 |
| 4. 2. 5. | Cdx2 Expression | 144 |
| 4. 2. 6. | FICTION results | 146 |
| 4. 3. | Conclusion | 151 |

| | | |
|-------|---------------------------|-----|
| 5. | <u>REFERENCES</u> | 153 |
| 6. | <u>SUMMARY</u> | 167 |
| 7. | <u>ZUSAMMENFASSUNG</u> | 169 |
| 8. | <u>APPENDICES</u> | 171 |
| 8. 1. | Abbreviations | 171 |
| 8. 2. | Curriculum Vitae | 173 |
| 8. 3. | Statements of Originality | 176 |
| 8. 4. | Acknowledgments | 177 |

1. INTRODUCTION

1. 1. The mechanism of aneuploidy

Understanding the nature and incidence of chromosome instability during early embryogenesis provides insight into the causes of human infertility and into the mechanisms underlying constitutional chromosomal rearrangements. Thirty percent of human conceptions result in a live birth (Macklon et al., 2002) more than 50% of spontaneous abortions have chromosomal imbalances (Benkhalifa et al., 2005; Fritz, 2001). Terminal deletions, duplications and isochromosomes, as well as mosaics are frequently observed in live births (Schinzel, 2001).

Chromosome instability is characterized by an elevated rate of gains or losses of complete chromosomes or segments of chromosomes per cell cycle resulting in cell-to-cell variability (Vanneste et al., 2009; Geigl et al., 2008).

Meiosis is the type of cell division that gives rise to eggs and sperm. Errors in the execution of this process can result in the generation of aneuploid gametes, which are associated with birth defects and infertility in humans.

It is well established that errors in chromosome segregation during human meiosis result in birth defects and infertility, but little is known about the molecular mechanisms used to segregate chromosomes and its leading such conditions.

The high rates of abnormal chromosome segregation (nondisjunction) occur in human female meiosis, leading to chromosomal aneuploidy and early pregnancy loss or offspring with developmental disorders (Geigl et al., 2008). Such embryos also have high rates of mosaic aneuploidy. It means that, in in-vitro environment some early blastomeres have abnormal chromosome number that indicates the mitotic nondisjunction events (Vanneste et al., 2009; Daphnis et al., 2008; Geigl et al., 2008; Baart et al., 2006; Li et al., 2005; Munne et al., 2005).

From clinical view, the majority of the studies are performed on embryos obtained from couples at risk for embryonic aneuploidy with advanced maternal age, recurrent implantation failure, recurrent miscarriages or severe male factor infertility. These factors are the main indications for preimplantation genetic

aneuploidy screening (Harper et al., 2008). Little is known about chromosomal imbalances in embryos from normal fertile women (Vanneste et al., 2009).

Techniques used for obtaining preimplantation cell samples involve; the removal of cells from the preimplantation embryo, accomplished by the removal of polar bodies and blastomeres at the early cleavage stage or extraembryonic trophoblast cells at the blastocyst stage. When the polar body or a single blastomere is analysed, only one attempt can be made at the analysis, severely limiting the diagnosis.

Genetic diseases of an embryo can be diagnosed prenatally either by chorionic villus sampling (CVS) or amniocentesis; however, other techniques have also been developed which allow diagnosis at the preimplantation stage of development.

New array-based techniques also allowed screening in single cells. These techniques revealed, not only mosaicism of aneuploidies and uniparental disomies in most cleavage-stage embryos but also frequent segmental deletions, duplications and amplifications that were reciprocal in sister blastomeres (Vanneste et al., 2009).

The frequency of missegregation in human gametes is remarkably high, particularly in the female. The nondisjunction by female occurs in about 10% of the meiosis in human oocytes. The presence of aneuploid cells in the cytotrophoblast layer or mesenchymal stroma may interrupt normal placental development or placental function during gestation but not the fetus. Therefore, chromosomal mosaicism seems to be the reason for different karyotypes within the placenta and unaffected fetus. It shows that the mosaic is generated in the embryo and that the trophoblast can function normally in the presence of trisomy or other aneuploidies (Astner et al., 1998; Tantravahi et al., 1996; Wolstenholme et al., 1995). Studies of early human embryos after in vitro fertilization (IVF) have shown even higher rates of aneuploidy than those found in early pregnancy or at birth. The discrepancy that may be accounted for by pregnancies lost before their detection. Now, microarray-based technologies reveal that structural chromosome abnormalities also occur at a shockingly high rate in early embryos

(Ledbetter, 2008). The low human fecundity can be explained, post-zygotic chromosome instability can be identified as a leading cause of constitutional chromosomal disorders (Vanneste et al., 2009).

The possibility of chromosomal false segregation next to somatic aneuploidies arises in early embryogenesis appears to be because of the aneuploidy induction in oogenesis arising during the first and second meiotic division. Numerous studies reporting mosaic whole-chromosome imbalances (Delhanty, 2005) and the sporadic reports on rare segmental aneuploidies (Daphnis et al., 2008; Voullaire et al., 2000; Wells & Delhanty, 2000) in human in vitro fertilized (IVF) embryos insinuate that chromosome instability might occur during human embryogenesis as well. According to the experiments on human beings, in 3% of chorionic villus sampling (CVS), the chromosomal abnormalities are being mosaics and do not necessarily affect the fetus (Astner et al., 1998).

To determine baseline rates of meiotic aneuploidy and structural imbalances before mitotic cell division, authors examined fertilized oocytes with low level of aneuploidy (Ledbetter, 2008; Munne et al., 2005). Vanneste et al. (2009) showed that, only 9% of IVF embryos have a normal karyotype in all blastomeres. The great majority show abnormalities of chromosome number or structure, such as large-scale duplications or deletions, or uniparental disomy, in which a chromosome pair is derived entirely from one parent. These abnormalities are often in mosaic form (Ledbetter, 2008; Munne et al., 2005). The normal diploid blastomeres, have to battle for survival to allow normal embryonic development and birth.

There is a unique challenge to understand how genes segregate and control the growth and differentiation of the mammalian embryo. Few genetic causes of infertility have been identified in humans. Nevertheless, genetic aetiologies are thought to underlie many cases of idiopathic infertility. The roots how genes control mammalian development, can be tracked back to experiments carried out in the early 1900s on the inheritance of coat colours of domestic animals. Mouse models with reproductive defects as a major phenotype are being rapidly created and discovered now with total over 200. These models are helping to define

mechanisms of reproductive function, to identify potential new contraceptive targets and genes involved in the pathophysiology of reproductive disorders (Matzuk et al., 2002).

1. 2. Mouse as a model

The achievements of the human and mouse genome projects provide increasing numbers of genes and phenotype-associated mutations (DeBry et al., 1996). Mouse genome project with molecular cytogenetic techniques generate BAC (bacterial artificial chromosomes) clones, with a subset linked to centromeric and telomeric genetic markers, to serve identifying mouse chromosomes 1-19 and X, Y and defining multiple band landmarks on each chromosome with unique BACs (Julie et al., 1999). The stability of BAC clones, the ease of BAC DNA purification, and the strong Fluorescence In-Situ Hybridization (FISH) signals resulting from the large insert size, make them superior molecular cytogenetic reagents (Korenberg et al., 1995)

Mouse is the smallest of the laboratory animal that belongs to one of numerous species of rodents. Therefore, it has the greatest demand as an experimental animal. Up to 80% of all animals used in laboratories, primarily in biology and psychology, are mice. The advantages for usage are their small size, rapid reproduction, inexpensiveness and easily handling. They are also sophisticated genetic tools, significant genetic resources, and relatively high position on the evolutionary scale. Since then, the mouse has become firmly established as the primary experimental mammal, and more information has accumulated on its genetics than on that of any other vertebrate, including humans (Nagy et al., 2003).

1. 2.1. Sex differentiation of the mice

By comparing the distance between the anal and genital orifices, sex of the mice can be decided quickly and briefly. Distance is always greater in the male than female but the testes are often retracted. Anus is also larger in male at all

postnatal stages. In some strains, the nipples of females are typically visible whereas the males are not.

1. 2. 2. History of Mouse Researches

The Chinese and Japanese are believed to have been the first to domesticate mice (Gregory et al., 2002; Astner et al., 1998) and the first recorded use of mice as research animals occurred 1664 when Robert Hooke studied the reactions of mice in experiments on air. Dr. Charles Little developed the first inbred strain, DBA (dilute, brown, non-agouti) in 1909. The first albino strain was developed by Halsey Bagg in 1915 for behavioural experiments; he named his strain Balb/c (Bagg albino). Nude mice were discovered in Glasgow, UK, in 1962; these resulted from a natural mutation and were immune deficient. Recombinant DNA research began in the 1970s, with the first successful transgenic mice produced in 1976. Knockout mice were first created in 1987 and in the mid-1990's, mapping of the mouse genome began, with sequencing to be completed soon thereafter.

The first report of attempts to culture mouse embryos in vitro to the blastocyst stage was by John Hammond. He succeeded in culturing eight-cell morula and some four-cell-stage embryos to blastocysts, but embryos removed at the two-cell stage soon died. With establishment in mediums for embryo culture and micro-drop culture technique, it is now possible and easier to culture embryos from oocyte to embryo stage. With the help of animal experiments, especially in in-vitro fertilization (IVF) field developed so much that this technique is now used with other assisted reproductive techniques routinely for patients (Tavmergen et al., 1991)

Every year millions of mice are used and there are hundreds of established inbred, outbred, and transgenic strains. Frequently used strains include the BALB/c and C57/Bl6 (which is a black mouse). Nude mice are strains developed from natural mutations. Hundreds (or even thousands) of transgenic and knockout strains of mice have been created, and more are being created today.

1. 2. 3. General Information about Laboratory Mouse

A laboratory mouse has a life span between 1.5 - 3 years. Approximate weight at birth is 1g, adult size changes between 15g (female) - 40g (male). Its pulse is 325 - 780 BPM, respiration is 60 - 220 RPM, genome size is about 2.6×10^9 bp with a normal diploid set of 40 chromosomes, predicts 30,000 genes. Mice age of sexual maturity is 5 - 8 weeks, useful breeding life of females 6 - 8 months and males 18 - 24 months.

A female mouse has an estrous cycle between 4 - 5 days, gestation continues for 19 - 21 days, weaning 8 - 12g for approximately 3 weeks at an age about 19-21 days, average litter size 6 - 8 pups.

1. 2. 4. Reproduction of Laboratory Mouse

Normally female mice have a reproductive cycle called estrous and this spontaneous cycle is divided into four phases:

- Diestrus – There are only small follicles present with large corpora lutea from the previous ovulation. Follicle development starts for the next ovulation.
- Proestrus - Development of some ovarian follicles accelerate.
- Estrus – Ovulation takes place, has a length of 4 to 6 days.
- Metestrus – Many corpora forms but they secrete for a short time.

These phases can be controlled by checking the histology of ovaries, uterus and vagina or by making smears. By controlling these phases, natural mating may occur with the copulation of males and females in estrus phase at about midpoint of a dark period.

Once animals have been together for more than a few days, mating will occur and particular components of the male ejaculate will coagulate to form a hard copulatory plug that blocks the vagina for 8 to 10 hours after mating which is considered as successful copulation.

Fertilization takes place in the upper reaches of the oviduct (a region referred to as the ampulla) and causes an immediate activation of the egg that induces the completion of the second meiotic division leading to the formation of the second polar body within two hours. In the 5 days following fertilization, fertilized oocyte respectively divide mitotically (each 2 daughter cells identical to the parent cell) and cleaved to 2 - 4 - 6 - 8 - 16 cell stage embryos, morula stage embryo blastocyst stage embryo and hatched embryo (Figure1 - 2).

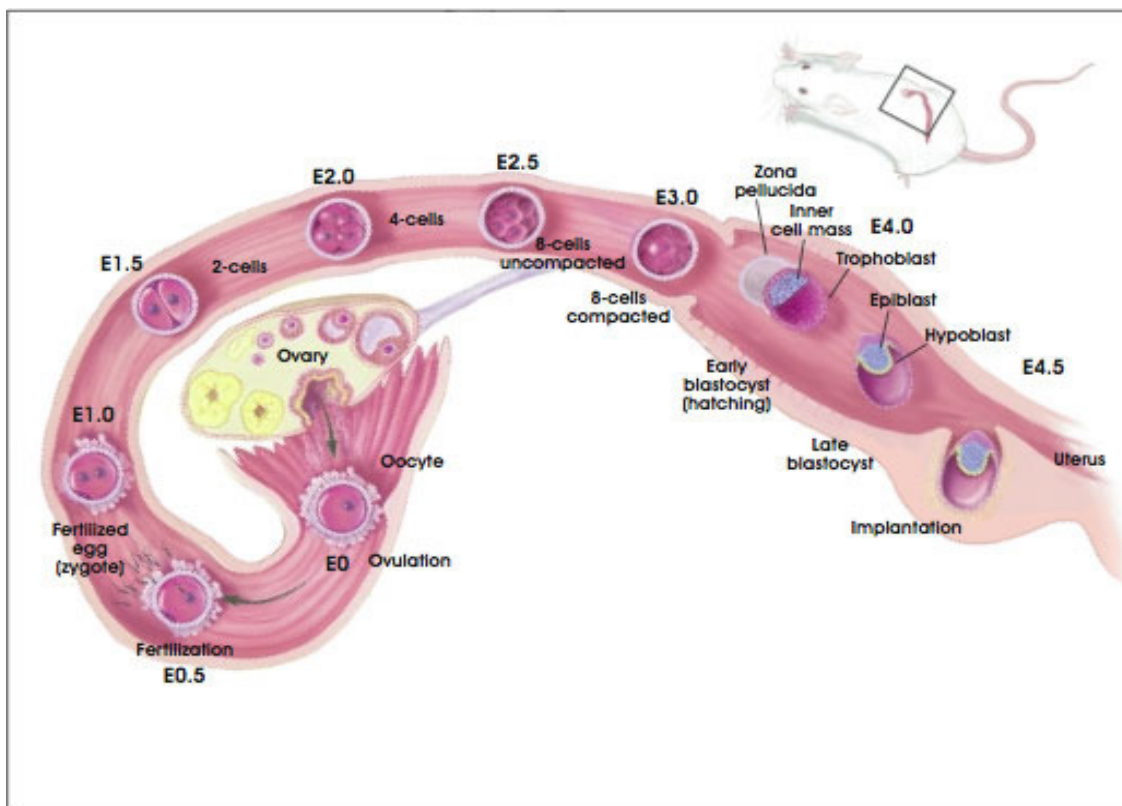


Figure 1: Development of the Preimplantation Blastocyst in Mice from Embryonic Day 0 (E0) Through Day 5 (E5.0) (Terese Winslow, 2001)

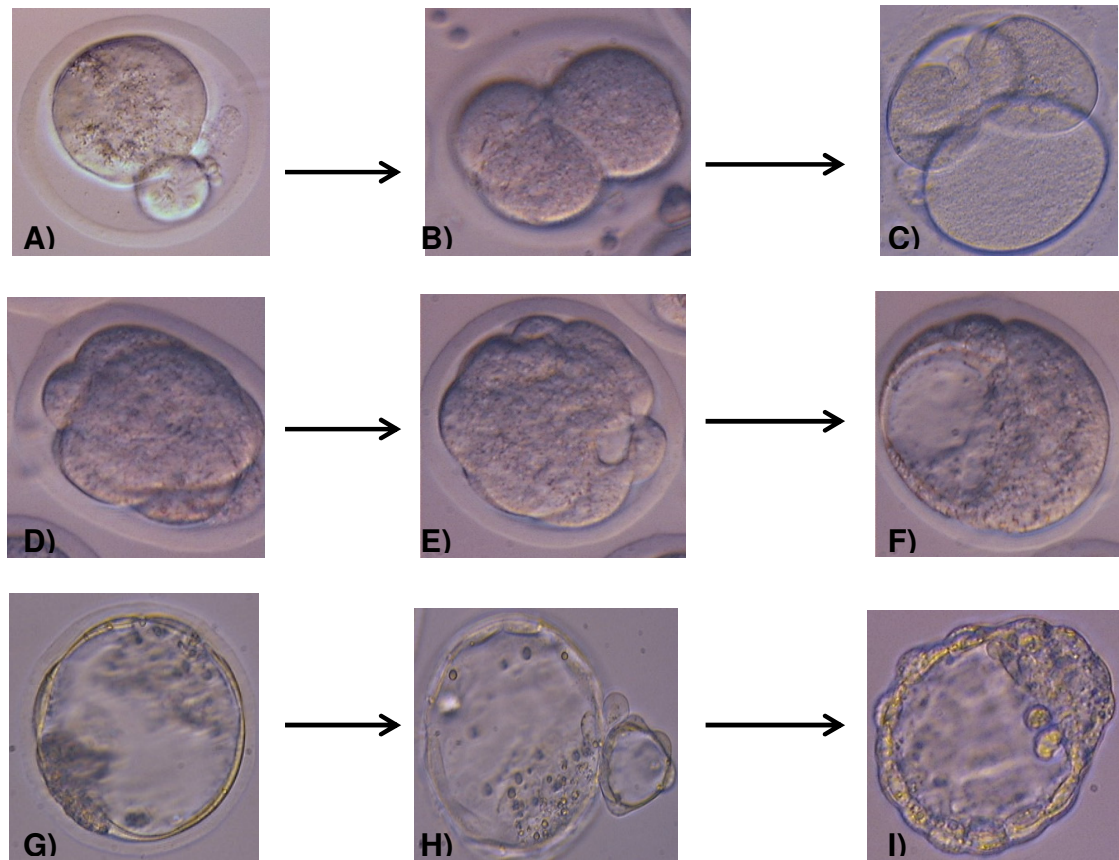


Figure 2: Morphology of mouse development: A) Mature oocyte with its first polar body. B), C), D) 3 - 4 – 8 compact cell stage embryos after fertilization. E) Morula stage embryo from cleaved compacted 8 cell stage embryos. F), G) Early and late blastocyst stage embryos. H) Hatching blastocyst from its zona pellucida. I) Hatched blastocyst without zona pellucida (magnification 400x).

Blastocysts were morphologically divided into three parts and embryologically graded into four categories: (respectively) (Figure 1 and 2)

1- On the embryonic pole, a group of cells which gives rise to an embryo – Inner cell mass (Embryoblasts) 2- A thin, outer cell layer which gives rise to the embryonic part of the placenta – Outer cell mass (Trophoblasts) 3- A fluid-filled space – Blastocoel and the blastocyst covered by a layer called the Zona Pellucida (Figure 3).

A - Early blastocyst - The blastocoelic cavity was less than one-quarter the blastocyst diameter. B- Blastocysts - The cavity was one-quarter to three-quarters the blastocyst diameter. C- Late blastocysts - The cavity was more than

three-quarters the blastocyst diameter. D - Hatched blastocysts – The cavity had the biggest size and blastocyst hatched from zona pellucida. These stages of development occurred between day 4 and day 7 in vitro with variation between the embryos and mice (Figure 3).

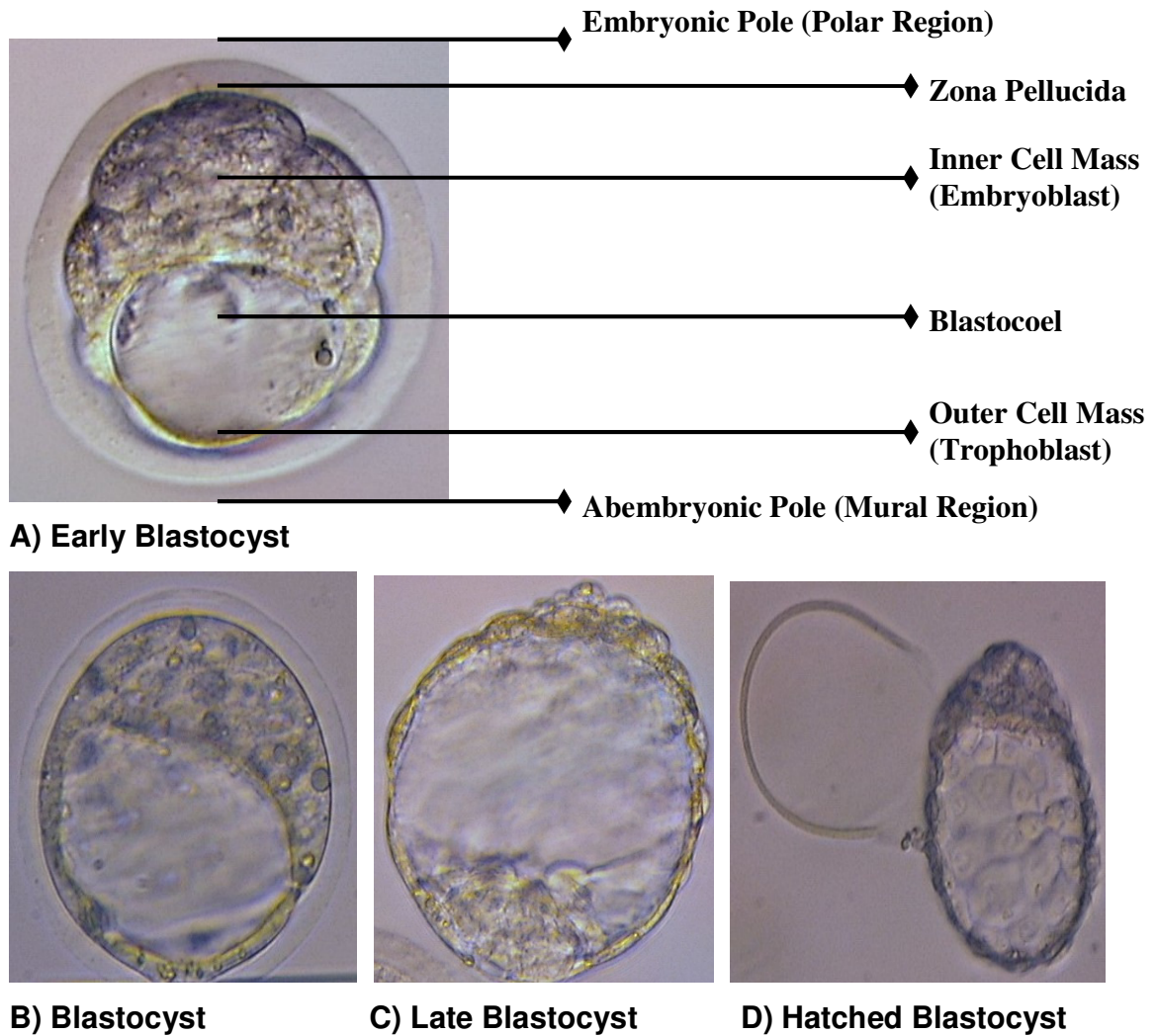


Figure 3: Morphological and embryological parts of blastocyst. A) Early blastocyst with inner cell mass (Embryoblasts), outer cell mass (Trophoblast), Blastocoel and the Zona Pellucida, B) Blastocysts, C) Late blastocysts, D) Hatched blastocysts with the zona pellucida on the left side (magnification 400x).

Trophoblast cells are found all around the outside of the blastocyst that are responsible for implantation into the uterine wall and will eventually differentiate

further into all the extra-embryonic tissues, such as placenta, amnion and chorion. The inner cell mass will eventually become the embryo and subsequent fetus (Figure 3).

There are two options for obtaining these preimplantation stage embryos from mice; natural mating with or without inducing the superovulation of female mice. In natural mating without hormonal treatment of female mice, the timings of ovulation and fertilization are controlled by environmental conditions. By inducing the superovulation of females, ovulation can be controlled by the injected hormones and a fertilization check is possible in a certain timeframe for further developments. Its utility is not only the establishment of timed pregnancies that would proceed to term, but also the production of large number of oocytes.

The protocol for superovulation of female mice is well established and many factors that can influence the outcome are known (Tarin et al., 2002; Ozgunen et al., 2001; Hogan et al., 1994). Superovulation of mice has been used for many years and exogenous gonadotropins are commonly used for superovulation in humans and animals to increase the number of oocytes for use in many fields of biology. New hormonal stimulation protocols are being introduced continuously to improve the quality of ovulated eggs, the chance of successful fertilization and pregnancy outcome.

1. 2. 5. Genetics of Inbred Laboratory Mouse

Prior to fertilization the mouse oocyte, which is suspended at meiosis I, divides by meiosis in its ovarian follicle until it reaches the metaphase II stage. Ovulated mature oocyte and its polar body have haploid chromosome sets that contain half the normal number of chromosomes. By fertilization, a haploid mature sperm cell binds to haploid mature oocyte and their pronuclear fuse to form a diploid zygote. After a few days, fused maternal and parental gene products in the zygote divides to form two identical cells and then cleaved asynchronously to 4, 6, 8, 16 cells stages and so on to form an embryo. During initial cleavages, the resulting daughter cells do not increase in size, rather the amount of cytoplasm of each

discernible targets has spurred the fantasy, and the goal to color-karyotype all mammalian chromosomes has been a long perceived one (Thomas et al., 1998) (Figure 5).

With only a few dozen gene differences, in principle metacentric chromosomes of humans and acrocentric chromosomes of mice are genetically very similar with ~30,000 genes and 3 picograms haploid genome. Ninety percent of the mouse genome could be lined up with a region on the human genome because the gene order in the two genomes is often preserved over large stretches, called conserved synteny (Korenberg et al., 1995). As you can see in the karyotyping schema metaphase chromosomes (Figure 5), mice have 20 haploid chromosomes including 19 autosomal pairs and the X and Y sex chromosomes.

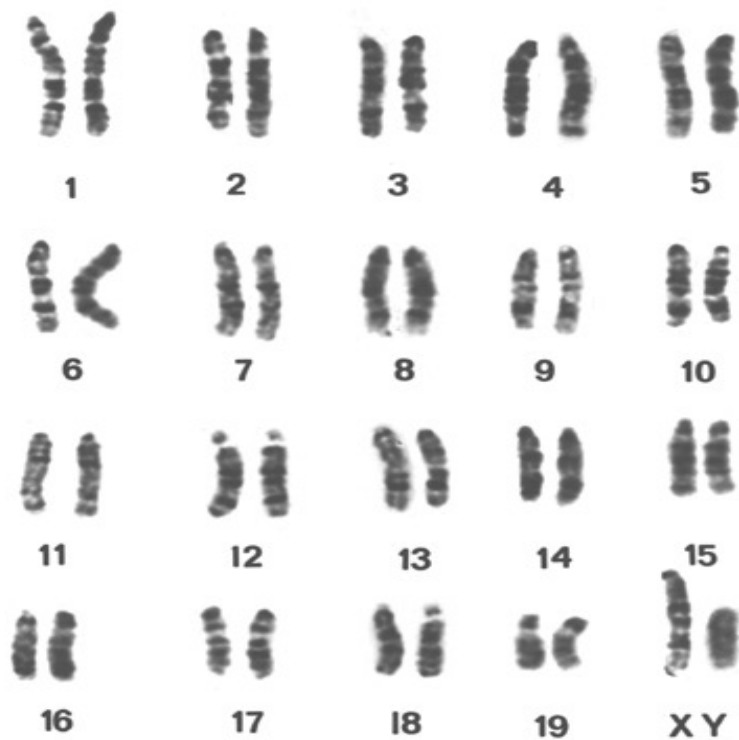


Figure 5: A complete diploid set of metaphase chromosomes from the laboratory mouse including 19 autosomal pairs and the X / Y sex chromosomes (This karyotype provided by Dr. Muriel Davisson Jackson Laboratory, Bar Harbor and www.informatics.jax.org)

1. 3. CBA/CaH-T(14;15)6Ca/J translocated mouse strain

CBA/CaH-T(14;15)6Ca/J strain is a primary repository strain and has stocks at the Jackson Laboratories (Stock Number: 000655) which carries spontaneous genetic mutations.

Jackson Laboratories described in research applications that this mouse could be used to support research in cancer researches like tumour incidence (lymphomas, mammary gland tumours, hepatomas) and genetic (as tissue / cell markers) researches. However, there is no confirming publication or study about tumour incidence. Using only substrain CBA/CaJ (Stock Number: 000654) the majority of studies found a higher frequency of spontaneous hepatomas in males than in females (Rithidech et al., 1999, Figueroa et al., 1994)

1. 3. 1. General information about CBA/CaH-T(14;15)6Ca/J strain

CBA/CaH-T(14;15)6Ca/J is an inbred strain maintained through sibling crosses. Breeders are maintained together for a period of 6 months. During this time, an average of 3-4 litters is produced. Average litter size is 5-6 pups. They are described as good breeders with no special husbandry requirements. First litters are generally born 4 weeks after breeders are set up (at 4 weeks of age). In spite of the female infertility problem and reduced male fertility that we encountered during our trials, Jackson Laboratories had no particular problems with non-productive animals that will be discussed later. CBA/CaH-T(14;15)6Ca/J has got a balanced reciprocal translocation between mouse chromosomes 14 and 15 which results in nondisjunction at a rate of 4.4% in males and 22.2% in females. T(14;15)6Ca reciprocal translocation is on Chr.14 and 15, Carter 6.

1. 3. 2. Estimated breaking point localisation by (14;15)6Ca/J strain

The international database resource for the laboratory mouse MGI defined the known break point regions in cells of mouse strain CBA/CaH-T(14;15)6Ca/J on

chromosome 15 in the cytoband A2 and on chromosome 14 in cytoband E3 (<http://jaxmice.jax.org/strain/000655.html>).

1. 4. FICTION

Phenotypic and genotypic analyses of cells are increasingly essential for understanding several mechanisms. In common cytogenetic techniques, the genetic analysis of single cells simultaneously with their morphological and immunophenotypic characterization are not possible.

A technique combining FISH with fluorescence immunophenotyping referred to as FICTION (fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms) was established in 1992 by Weber-Matthiesen. By applying this method, single cells could be analyzed with their morphological, immunophenotypic, and genetic features.

Fluorescence in situ hybridization (FISH) refers to the use of labelled nucleic acid sequence probes for the visualization of specific DNA or RNA sequences on mitotic chromosome preparations or in interphase cells. With the recent sequencing of genomes from numerous organisms, including human and mouse, the specific map location and availability of BAC clones has greatly facilitated the acquisition of locus-specific FISH probes and the assembly of systematic probe collections (Dorritie et al., 2004).

By combining FISH with immunofluorescence (IF), FISH technique applied with fluorescent probes that hybridize to their cellular counterparts at the place where the cellular target sequence is located can be rapidly detected and quantified all types of chromosomal aberrations, e.g., deletions, amplifications, or translocations, (Multiple chromosomal aberrations - multicolour FICTION (M-FICTION) by relevant probe design (Chano et al., 2002).

To overcome the limitations of conventional FICTION and to enhance the possibilities of combined morphologic, immunofluorescence (IF) and genetic analysis of single cells, more than two chromosomal loci could be studied simultaneously. The recent availability of many new fluorescent dyes, along with

improvements in digital imaging has led to the development of multicolor FISH techniques for karyotyping such as spectral karyotyping (SKY) and multicolour-FISH (MFISH).

Future multicolour-FISH applications include the detection of preneoplastic lesions, early stage and minimal residual diseases, or micrometastases.

1. 5. Stem cell relevant markers

The transcription factors believed to have a positive association with trophoblast specification have been inferred primarily in two ways: by their expression patterns in embryos, ES and TE cells and by the consequences of gene disruption on embryonic development. Many of these transcription factors also control the expression of genes characteristically expressed in trophoblast but not in the epiblast, primitive endoderm and their derivatives.

Commitment to ICM lineage is attributed to the function of the two transcription factors, Oct4 (encoded by Pou5f1) and Cdx (Strumpf et al., 2005).

1. 5. 1. Oct4

Oct4 is the best-studied gene essential for the maintenance of a pluripotent state in early embryonic and ES cells (Pesce and Schöler, 2001). Oct4 encodes a POU-domain transcription factor and expresses in pluripotent cells of the early embryo and in the embryonic and adult germ cells.

As the transcription factor Oct-4 is thought to be an essential factor in controlling early embryonic development and pluripotency, expression in the mouse and rhesus monkey is low in early cleavage-stage embryos. It appeared at the 16-cell stage and expressed only in the inner cell mass at the blastocyst stage. Recent studies in porcine and bovine embryos have shown significant differences in Oct-4 expression compared to that of the mouse and monkey (Obermiller et al., 2005). Immature oocytes expressed Oct-4 in the cytoplasm and germinal vesicle. Early embryos also showed cytoplasmic and nuclear staining.

In mice, Oct4 expression begins at the 4- to 8-cell stage and becomes restricted to inner cell mass (ICM) cells of the blastocyst and then to the epiblast, founder cells of the embryo proper (Palmieri et al., 1994). After gastrulation, Oct4 expression is restricted to the germ cell lineage (Pesce et al., 1998; Yeom et al., 1996). Although mouse embryos homozygous for a targeted deletion of Oct4 can develop into structures resembling blastocysts (Nichols et al., 1998), they do not form a pluripotent ICM and die shortly after implantation from an inability to differentiate into embryonic lineages. In vitro, variations in the level of Oct4 expression, as little as 30% above or below the normal level, regulate the differentiation of embryonic stem cells into putative endoderm or trophectoderm (TE), respectively (Niwa et al., 2000). Thus, subtle changes in Oct4 expression have predictable consequences for the early postimplantation embryo. Oct4 expression becomes restricted to the inner cell mass and epiblast.

1. 5. 2. Cdx2

Cdx genes (Cdx1, Cdx2, and Cdx4) encode a family of caudal-related transcription factors that mediate anterior–posterior patterning during embryogenesis through Hox gene regulation (Wang et al., 2008)

Cdx2, a caudal-type homeodomain transcription factor, has been reported to be specifically expressed in TE at blastocyst stage, and expression is maintained within proliferation (Beck et al., 1995). Heterozygous Cdx2 mutants show homeotic transformation defects and homozygous mutants die at the peri-implantation stage (Tamai et al., 1999; Chawengsaksophak et al., 1997).

Blastocoel formation initiates in Cdx2 mutants but epithelial integrity is not maintained and embryos fail to implant. Loss of Cdx2 results in failure to downregulate Oct4 in outer cells of the blastocyst and subsequent death of those cells. Thus, Cdx2 is essential for segregation of the ICM and TE lineages at the blastocyst stage by ensuring repression of Oct4 in the TE (Strumpf et al., 2005).

Oct4 is expressed in the ICM both species. Mouse trophectoderm is only specified when Oct4 becomes down regulated. Cdx2 is required early in the

mouse trophectoderm development and regulate trophectoderm differentiation prior to blastocyst formation

1. 6. Aim of the study

The aim of this study was to investigate and find out the distribution and heritage of somatic aberrations in early mouse embryogenesis and the heritage of aneuploidy in blastocyst stage mouse embryos especially in mouse trophoblast and embryoblast cells. With the help of chromosome preparation, segregation of translocations are observed and analyzed if the next generation 100% carry this translocation.

To address the question of which cell stayed in its stage and could be analysed as ICM or TE in late mouse development at the molecular level, we analyzed Oct4 and Cdx2 protein distribution in late blastocyst stage. Based on our identification of characteristic events involved in establishing molecular differences among individual cells, we propose a model for patterning in the late mouse embryo that may include stochastic processes.

2. MATERIAL AND METHODS:

2. 1. MATERIAL

2. 1. 1. Devices

- Incubator CO2 Begasung Heraeus, Germany
- Incubator CO2 Thermo Scientific, Germany
- Fluorescence-Microscop inclusive: Axioplan, Carl Zeiss, Jena
 - a) Fluorescence capable CCD-Camera, S30, IMAC
 - b) HBO-100/2-Quecksilberdampf Kurzbogenlamp, Osram
- Filter:
Fluorochrom: Anregung [nm] / Emission [nm]
DAPI: 360 / 461 AHF, Tübingen, Germany

FITC: 480 / 518 AHF, Tübingen, Germany
 DEAC: 436 / 480 AHF, Tübingen, Germany
 Spectrum Orange: 564 / 580
 Spectum Gold: 530 / 555 Vysis, Abbott, Wiesbaden, Germany
 Spectrum Blue: 400 / 450 Vysis, Abbott, Wiesbaden, Germany
 Texas Red: 592 / 612 AHF, Tübingen, Germany
 Cy5: 649 / 670 AHF, Tübingen, Germany

- Hybaid Omni Gene, MWG Biotech, Ebersberg, Germany
- HYBrite, Abbott, Wiesbaden, Germany
- Inversemicroscope, Olympus, Hamburg, Germany
- Refrigerated Centrifuge, Hettich, Tuttlingen, Germany
- Laser Octax, eyeware MWG Biotech, Ebersberg, Germany
- Manipulators CellTram vario/air Narishige, England, Eppendorf, Germany
- Micro-Manipulator, Luigs Neumann GmbH, Ratingen, Germany
- Microscope DMIRB, Leica Solms, Germany
- Slide-Booster Advalytix, Brunthal, Germany
- Stereomicroscop SZH10, SZX12, Olympus, Hamburg, Germany
- Stereomicroscop Axiovert 25, Axioplan, Carl Zeiss, Germany
- Sterile Werkbank with UV-Lamp, Clean Cab Herolab Wiesloch, Germany
- Thermocycler MJ Research PTC-150 und PTC 200, Biozym, Hessisch-Oldendorf, Germany
- Speed Vac Plus, Savant GMI, Minnesota, USA
- Waterbath, Haake/ Fisons, Karlsruhe, Germany
- Electronic heating cabinet, Memmert, Germany
- Laminar Flow Hood, window style, Nuaire Biological
- Laminar Flow Hood, K-System, Wisag
- Hot plate, 261450, MTG, Germany

2. 1. 2. Software

- Octax eyeware; MWG Biotech, Ebersberg, Germany

- ISIS; MetaSystems GmbH, Altlussheim, Germany

2. 1. 3. Hormones

- PMSG (pregnant mare serum gonadotropin), 1000 IU, Sigma
- hCG (human chorionic gonadotropin), 5000 IU, Organon or Pregnyl

2. 1. 4. Reagent and Chemicals

- D-MEM/F-12, 21331-020, Gibco BRL
- L-Glutamine, 25030-024, Gibco BRL
- Nonessential aminoacid solution (NEAA;100X), 11140-035, Gibco-BRL
- Penicilin/Streptomycin (100X), 15070-063, Gibco-BRL
- 2% Gelatin Solution (from bovine skin), G-1393, Sigma
- B-Mercaptoethanol (B-ME), M-7572, Sigma
- Pronase (Protease), P-8811, Sigma
- FBS, SH30070.03, Hyclone
- L-Glutamine 200mM (100X) liquid, 25030-024, Invitrogen
- MEM Non Essential Amino Acids (100X) liquid, 11140-035, Invitrogen
- Penicillin/Streptomycin (100X) liquid, 15070-063, Invitrogen
- ESGRO (LIF) (Mouse), ESG1106, Millipore/Chemicon
- PBS, L 1825, Biochrom
- PBS (1x without Ca²⁺ und Mg²⁺), Biochrom KG, Berlin, Germany
- Trypsin/EDTA, L 2143, Biochrom
- FCS (for skin culture, Fetal Calf Serum), S0113, Biochrom
- BSA (Bovin Serumalbumin), 11925, Serva
- Demecolcine solution, D1925 10 µg/mL in HBSS, AFC Qualified, Sigma
- Amethopterin hydrate, A7019, ≥95% powder, Sigma
- Thymidine, cell culture tested, T1895, Sigma
- M2-Medium, M 7167, Sigma
- M6-Medium, M7292, Sigma
- Ethanol, 1.00983.2500, Merck

- Methanol, 1.06009.2500, Merck
- Acetic Acid, 1.00063.2500, Merck
- Goat Serum (Normal), 10 ml, 0907, Dako
- Rabbit-Anti-Oct4-Antibody, unconjugated, AB 3209, Millipore/ Chemicon
- Rabbit-Anti-Cdx2-Antibody, unconjugated, AB 4123, Millipore/ Chemicon
- Antibody diluent with background reducing components, S 3022, Dako
- Polyclonal Goat-Anti-Rabbit Immunoglobulins, Biotinylated, E 0432, Dako
- Cy5-conjugated Streptavidin, PA45001, GE Healthcare, Germany
- AMCA-conjugated Streptavidin, 016-150-084, Jackson Immuno Research Laboratories
- Streptavidin/ FITC, F 0422, Dako
- Agarose, Biozym Hessisch-Oldendorf, Germany
- Antifade (inclusive 0,5µg/ml DAPI), Vector Laboratories, Peterborough, England
- DAPI, Boehringer, Ingelheim, Germany
- Acetic acid, Merck, Darmstadt, Germany
- Ethanol (absolut), Merck Darmstadt, Germany
- Fixogum, Marabu, Tamm, Germany
- Streptavidin labelled Cy5 Amersham, Little Chalfont, United Kingdom
- Formamid, Merck, Darmstadt, Germany
- Glycerol, Merck, Darmstadt, Germany
- HAM's F-10 Medium 1x10mM, Hepes Biochrom, Germany
- Hyaluronidase (80IU/ml), Linaris, Germany
- Menezotte Universal Kulturmedium, Medicult, Denmark
- Methanol, Merck, Darmstadt, Germany
- Mineral oil (embryo tested), Sigma-Aldrich Chemie, München, Germany
- NaCitrat, Merck, Darmstadt, Germany
- NaCl (500g), Sigma-Aldrich Chemical, München, Germany
- Poly-venyl-pyrrolidon (PVP) 10%, Medicult, Jyllinge, Denmark
- Spectrum Green-dUTP, Abbott- Vysis, Wiesbaden, Germany

- Spectrum Orange-dUTP, Abbott- Vysis, Wiesbaden, Germany
- Diethylamino Coumarin-5-dUTP, Perkin Elmer, Boston, USA
- Texas Red-dUTP, Chroma Tide/ MoBiTech,
- Digoxigenin-11-dUTP, Roche Diagnostics, USA
- Biotin-16-dUTP, Roche Diagnostics, USA
- Tween 20, Sigma-Aldrich Chemie, München, Germany
- Vecta Shield (without DAPI) Vector Laboratories, Peterborough, England
- Coupling liquid (Advason), Advalytix, Brunthal, Germany.
- BACMAX™ DNA purification kit, Epicentre Biotechnologies, Germany.
- Mouse COT-1 DNA, Invitrogen, Karlsruhe, Germany.
- HCl, Merck, Darmstadt, Germany
- LB Solution, 240230, Difco, Becton Dickinson Microbiosystems, USA
- Sequenase Ver 2.0, Amersham, Little Chalfont; United Kingdom
- Taq Polymerase, Amersham, Little Chalfont; United Kingdom
- MgCl₂, Merck, Darmstadt, Germany
- Replitherm-Reaktion-Buffer (20x, 1,5ml; 200mM Tris-HCl (pH 8,3), 1M KCl, 0,1%Tween 20 und 0,1% NP 40), Epicentre Tech. Biozym; Hamburg, Germany
- Sequenase dilution buffer, Amersham, Little Chalfont; United Kingdom
- Sequenase-DNA-Polymerase (Version 2.0, 200 Units) Amersham, Little Chalfont; United Kingdom
- Sequenase-Puffer (5x) Amersham, Little Chalfont; United Kingdom
- AmpliTaq-DNA-Polymerase (5 Units/µl), Applied Biosystems, Roche
- Ampuva-Water (5ml), Fresenius Bad Homburg, Germany
- dATP, 100mM Fermentas St. Leon-Rot, Germany
- dGTP, 100mM Fermentas St. Leon-Rot, Germany
- dCTP, 100mM Fermentas St. Leon-Rot, Germany
- dTTP, 100mM Fermentas St. Leon-Rot, Germany
- DOP-Primer
- Bisbenzimidazole, Hoechst 33258

- Aquadest
- Propidium iodide

2. 1. 5. Culture dishes, pipets and slides

- Center-Well-Organ-Culture-Dish, 60 X 15 mm Style, 353037, FALCON
- 4-well-slides, 4 Chamber Polystyrene Vessel, 354104, FALCON
- 4-well-culture dish, Nunclon DeltaSi, 176740, Nunc
- Serologische Pipettes: Sarstedt
 - 5ml: 86.1253.001
 - 10ml: 86.1254.001
 - 25ml: 86.1685.001
- Cellscraper, 83.1830, Sarstedt
- Tissue culture flask, 25cm², 83.1810.302, Sarstedt
- Multidish 4 wells, 176740, Nunc
- Cytospin slides, ca. 76X26X1 mm, 59910056/M, Thermo Electron GmbH
- 4-chamber-slides, 4 Chamber Polystyrene Vessel, 354104, Falcon
- Coverslides, 24x60mm, 18x18mm, 22x22mm, Menzel, Braunschweig, Germany
- Slides, SuperFrost Plus, Menzel, Braunschweig, Germany
- Biopsy pipette (18mm), Eppendorf, Hamburg, Germany
- Holding pipette, Microtech
- Hatching pipette, Microtech
- Pasteurpipettes, 230mm long, Assistent, Sondheim, Germany
- Petidish, 60x15mm, 50x9mm, Sarstedt, Nümbrecht, Germany
- Round tubes, 5ml, 14ml Sarstedt, Nümbrecht, Germany
- Serologic pipette, 10ml Sarstedt, Nümbrecht, Germany
- Sterile syringe 10ml, 1ml, Nümbrecht, Germany
- Sterile filter- pipette tips, 10, 100 Sarstedt, Nümbrecht, Germany

2. 2. METHODS

According to federal law, we obtained and had a permission to perform the animal experimentation presented in this study from “Ministerium für Landwirtschaft, Umwelt u. ländliche Räume des Landes”. The permission consisted of organ gaining, in-vitro or in-vivo grown embryos and oocytes derived from female mice that were cultured in vitro up to the hatched blastocyst stage and further manipulations on dissected materials for genetic investigations.

2. 2. 1. Suitable animal husbandry of experimental mice

In an ideal situation, caging of experimental mice should be maintained in a pathogen free environment. Because of this necessity, our animal facility recently constructed pathogen-free facility and was behind barriers. Our suppliers whose stocks were believed to be free of pathogens imported the animals without quarantine and our embryo donors and recipients were bred within the barrier.

Mice were kept in rooms with the temperature set at about 22°C and humidity was maintained at 55-60%. Lighting was not overly bright because too much light hurts the eyes of mice. Mice were housed with 12 hours of light and 12 hours of darkness each day and for our superovulation / mating experiments, they had a convenient lighting cycle 6 a.m. to 6 p.m. light, 6 p.m. to 6 a.m. dark. Regulation of the light cycle was strict because it has a great effect on breeding performance. Mice were normally fed a pelleted diet and water was available ad libitum at all times. Their bedding cages changed 3 times a week to prevent the build up of urea.

The transportation from vendors, environment, food, and light-cycle changes stress animals. They need time to adjust to new conditions before being used. 5 females were housed to a cage, and mixed with unfamiliar females without problems. Special attention was paid to the housing of males because of their propensity for fighting. Males generally do not fight if they are housed together from before sexual maturity through to old age.

2. 2. 2. Handling techniques of mouse

To immobilize a mouse, for several manipulations, we grasped its tail with our right hand and placed the mouse on top of the cage bars. Then gently grasped a pinch of loose skin over the shoulder area between the thumb and forefinger then scruffed the mouse with our left hand (Figure 1A). For any injection or sex checking, the head - body of the mouse needed to be immobilized smoothly. The body of the mouse was stretched by fixing the tail by crooking in the last two or three fingers (Figure 1B). Also all loose skin around the back of the neck was scruffed and gripped to avoid being bitten.

If the mice were excited and demonstrated high activity in the cage, thus making it difficult to grasp the base of the tail, the animal was first cupped at the top with a hand. The tail was then gently grasped at the base with the thumb and forefinger. We also noted that it was easier to work on the mice in the morning to early afternoon because they were more active just before the lights go out and therefore more difficult to handle later in the day.

Mice were handled and euthanized in sterilized places in the animal housing area. The mice were not taken out of the animal house before euthanizing, only uteruses at the horns junction were dissected and carried to the special laboratories in the woman clinic Kiel, for embryo manipulations in equilibrant M2 medium.

2. 2. 2. 1. Intraperitoneal injection

Intraperitoneal injection (IP) in mice is a common method of administering drugs, such as hormones and anaesthetics.

To give intra-peritoneal injections, first we restrained the animal as we described above and then disinfected the surface of injection area with 75% alcohol. Restraining the animal with the head lower than the hind legs allows the abdominal organs to fall away from the injection site.

The injection site was in the lower quadrant of the abdomen because vital organs were absent from this area and the tip of the 1 ml syringe needle placed slightly, pushed with a sharp jerk. We did not go further than 2-3 mm into the abdomen to prevent injection into the intestine and waited before withdrawing the needle to avoid exuding of the hormones (Figure 1B).



Figure 1: A) Handling mouse by grasping its tail and placing on top of the cage bars B) Intraperitoneal injection by immobilizing the mouse and stretching the body of the mouse with fixing the tail.

2. 2. 2. Cervical dislocation

Cervical dislocation is a common method for sacrificing animals with minimum pain and in the quickest manner. To sacrifice the mouse with cervical dislocation technique, we used a large scissors with a small end to immobilize the mouse at its neck.

To start with, we grasped mouse tail and placed it on top of the cage bars that it could grip with its paws. Then we applied a firm pressure with our scissor towards its neck to break it, at the same time gripped and pulled the tail to the other direction. By this way in few seconds mouse was dead but we waited until it stopped its movements that was not more than 50 seconds.

2. 2. 2. 3. Opening the abdominal cavity and dissection of uterus

To reduce the risk of contamination, firstly we disinfected the hairy mouse abdominal area with 70% ethanol and for further manipulations autoclaved surgical forceps, scissors were used.

After sacrificing the mouse with cervical dislocation technique, we laid the mouse on a clean paper used for surgical manipulations under a strong, focused operation light. Then we made a small cut at the midline, close to the bladder with our scissors. Below this small cut, we pinched the skin with our fingers toward the heart until the abdominal cavity was exposed (Figure 2A). After hairy skin was discarded and the peritoneum was exposed, it was cut with our fine scissors. Thus, all the organs, depending to the age and the strain, with fatty pads could be seen.

Because our aim was to dissect uterus with ovaries, we pushed away the coils of gut and cut the remaining fat pads out of the way. Two horn uterus with its ovaries lie like a “V” at the inferior part of abdominal cavity that could be afterwards easily seen. We grasped the ovaries, which lie at the ends of uterine horns, gently with fine forceps and tried to pull each side away from the body cavity. With this motion, the mesometrium was revealed which anatomically carries blood vessels and binds uterus and ovaries to the abdominal wall (Figure 2B). Mesometrium was trimmed away from the uterine cervix until the ovaries were fully exposed. Then the uterus was removed with ovaries by making cuts from the uterus horn junction (cervix) to the end of the ovaries. Lastly, the dissected two horn uterus with its ovaries was transferred to the tubes containing equilibrant M2 medium.

2. 2. 3. Inducing superovulation in female mouse

There were several factors in inducing the superovulation, which must be taken into consideration for success. Among these factors, the most important factors were age, strain of females, doses of gonadotropins and time of injection intervals.

2. 2. 3. 1. Influence of mouse strain and age

In order to optimize our superovulation protocol, we used different strains of mice with various ages.

Normally male mice plug superovulated female mice almost every time when they are present. Because the mating process is not really affected by the male mice strain, we only worked with C3H/HEN and C57BL/6 male strain.

Female sexual maturity, 3 -6 weeks of age varying from strain to strain, is an important factor, affecting the number and the quality of oocytes that are superovulated. This sexual maturity age is usually not available from commercial suppliers nor is the response to the hormonal superovulation in females change in each strain. Therefore, we examined four strains: B6D2F1, C3H/HEN, C57BL/6 and BALB/c.

The age interval that we analyzed for females was between 4 – 16 weeks and for the males was 6 - 32 weeks. We attempted to derive F₂ hybrid embryos from different combination of F₁ hybrid crossings. These were; B6D2F1xC57BL/6, B6D2F1xC3H/HEN, C3H/HENxC3H/HEN, C3H/HENxC57BL/6, C57BL/6xC3H/HEN, C57BL/6xC57BL/6, BALB/c xC3H/HEN, BALB/c x C57BL/6 B6D2F1 mouse origin was the crossing between female C57BL/6NCrI X male DBA/2NCrI and was obtained from Charles River (strain code 099), kept in our animal facility centre before use.

2. 2. 3. 2. Doses of gonadotropins

Superovulation regimen required a follicle-stimulating hormone (e.g. pregnant mare serum gonadotropin, PMSG) and a luteinizing hormone (e.g. human chorionic gonadotropin, hCG). PMSG simulates like an endogenous follicle-stimulating hormone (FSH) and oocyte – follicles were then induced then normally, depending on the dose given. Human chorionic gonadotropin hormone (hCG) was the second gonadotropin used to mimic the ovulation induction effect of luteinizing hormone (LH).

PMSG and hCG supplied 1000-IU and 5000-IU ampoules respectively as lyophilized powder. Both of them diluted in 0.9% NaCl solution to give the required concentration. Aliquots were stored at -20°C and thawed only once prior to use.

A dose response analysis was employed to examine the effects of different doses of PMSG and hCG on the incidence of mating, recovered total oocyte and embryo number, effect on in-vitro preimplantation development. As the effects of increasing doses of PMSG and hCG were seen, the best doses were determined for the protocol.

To optimize the PMSG dose, an arithmetic series of PMSG doses was prepared, ranging from 5 to 20 IU in 0.1 ml NaCl solution and for hCG dose ranging from 5 to 15 IU in 0.1 ml NaCl solution. Each mouse injected with PMSG subcutaneously followed by hCG.

2. 2. 3. 3. Time plan of gonadotropin injections

The setting of normal reproductive cycle takes time and is not always successful; therefore superovulation protocol was applied, independent of estrous phase, with a strict time plan of gonadotropin injection. The gonadotropin injections were performed with our end doses in different time intervals and designed a protocol for each cases like; obtaining oocyte, zygote, 2-6 cell stage embryo, morula-early blastocyst stage embryo.

Several experiments were performed to have the end time plan of gonadotropin injections, which were:

Day -2 - Pregnant mare serum gonadotropin (PMSG) treatment given in the afternoon (11.30 - 14.45) or in the evening (19.00 - 21.00)

Day 0 - Human chorionic gonadotropin (HCG) injected with keeping care the minimum time interval between the administration of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG), determined as 46 - 50 hours, which were then respectively in the afternoon (11.00 - 15.45) or in the evening (18.00 - 22.00). If our aim was to gain zygotes and embryos, each

female was placed with a male of proven fertility. To ensure high mating rates, the males were caged with females not more than once per week.

Day 1 – Between 08.00 -12.00h or 14.00 – 19.00 females were euthanized by cervical dislocation to gain oocytes or zygotes (in mating groups). Successful mating was defined as the presence of fertilized oocytes or embryos and also when females were checked for the presence of copulation plug in vagina (vaginal plugs). Normally plugs can easily be seen in most strains but must be checked early in the morning because they fall out or are no longer detectable ~ 12 hours after mating or earlier and usually without checking 60% or more females will mate depending on the strain. In addition, the presence of a vaginal plug indicates that the mating occurred, but does not mean that a pregnancy will result.

Day 2 – Whole day in different time intervals females were euthanized by cervical dislocation to gain 2 – 6 cell stage embryos.

Day 3 - Females were euthanized by cervical dislocation if it was possible to obtain 8 cell - morula stage embryos.

Day 4 - As a last step, we wished to have compacted morula – early stage blastocyst, and euthanized the females in different time intervals.

With variable times and days in the week, we optimize our protocol for different aims like; obtaining oocytes, 4 to 6 cell stage embryos, 8 to morula stage embryos and early to late blastocyst stage embryo.

After formulating the injection protocol to gain embryos and oocytes, every week 2 female were injected and afterwards caged with 2 males, which means we had two groups with separate pairings. To obtain morphologically good and large numbers of blastocysts, days -2, 0 and 4 played an important role. Euthanizing on the other days were generally undertaken to improve our results in terms of several factors such as; best timing for scarification and injection, the best stage of embryo for in-vitro cultivation, having the morphologically best looking, and most number of embryos.

2. 2. 4. Gaining of mouse oocyte and early stage embryos

After regular injections of PMSG and hCG, the mice were euthanized with cervical dislocation technique and the two horn uterus with ovaries were dissected as we described above.

From this step on dissected uteruses -ovaries were carried in tubes with equilibrated M2 medium to the embryology laboratories that were in woman clinic Kiel for embryo manipulations. Special care was given not to affect the oocytes and especially the embryos, which had not been gained from the dissected organs, from the sudden temperature differences.

All embryo manipulations were performed in a laminar flow hood, under stereomicroscope with a heating plate.

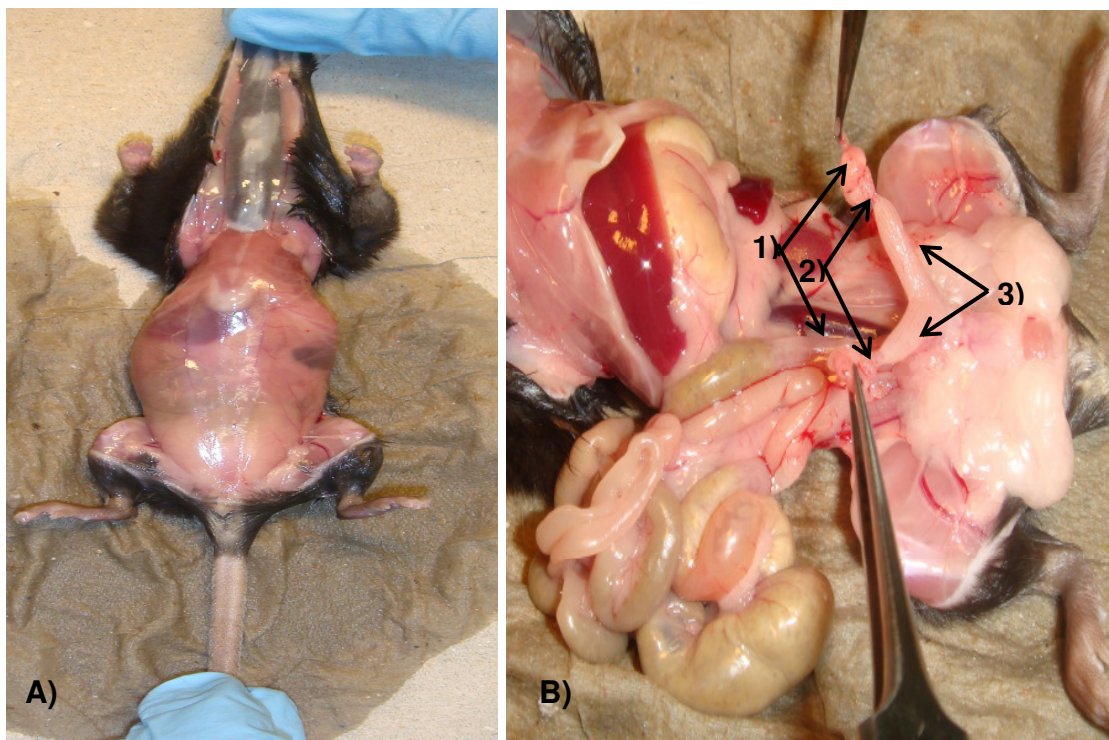


Figure 2: A) Opening the abdominal cavity of euthanized mouse. B) Sacrificing the mouse oviducts with uterus and arrows indicates: 1- ovaries, 2- fallopian tubes, 3-uterus

2. 2. 4. 1. Preparing the cultures for in- vitro cultivation and embryo handling transfer pipettes

It was important always to prepare the manipulation and culture mediums with tubes / dishes at least 2 hours before the dissection, because the mediums and the culture dishes had to be equilibrated and warmed up under a humidified 5%CO₂ atmosphere at 37°C in an incubator.

Care was taken that all the mediums and dishes prepared were kept in sterile to avoid fungi problems in our in-vitro settings. Therefore, we initially filtered our M2, M16 and Menazotte mediums with filters on the tubes.

The culture dishes that we prepared before hand to equilibrate were:

- 35-mm sterile plastic culture dishes with M2 medium for obtaining embryos, zygotes or oocytes from uterus - fallop tubes.
- Center well dishes with M16 or menazotte medium for overnight culturing or collecting all embryos, zygotes or oocytes directly after the recovery.
- Microdrop culture dishes, which were set up with the 30µl drops of M16 or HEPES-buffered Ham's F10 medium placed on the bottom of 35-mm sterile plastic culture dishes and covered with 5ml embryo tested mineral oil, for embryo manipulations or viewing the development.

Microdrop culture dishes were used and prepared with HEPES-buffered medium if we planned to make a further manipulation on the embryos like biopsies, while M16 was used if we wanted to view the development of zygotes – embryos, which was easier to observe through our microscope when they were transferred into drops. With HEPES-buffered, the pH value was stable during the manipulations and the embryos were not degenerated by the pH changes while the medium was out of the incubator. However, it was also not optimal to keep the embryos for a long time in this medium because of HEPES.

Oocytes and embryos were retrieved in M2 medium, which was used for all handling of oviducts, cumulus masses, ova and uteruses, and then placed in culture in M16 or Menazotte medium under a humidified 5%CO₂ atmosphere at 37°C.

Embryo handling mouth pipette consist of glass pasteur pipette, latex tubing with an aspirator mouthpiece and used to transfer the embryos/oocytes from dish to dish. The glass piece, which contact directly with the embryos/oocytes, was prepared in our laboratory from sterile glass pasteur pipettes with microburner. For that, we softened the pasteur pipettes in a flame by holding it from the both ends until the part on the fire was nearly going to bend. Then quickly withdrew the pipette from the heat, pulled both ends to produce a tube and two halves broke apart. Internal diameter of this tube was set depending on the manipulation type with the range ~30 - 300 μm . We tried to make a neat break to the half, which was for mouth-controlled holder, with the other half by snapping and afterwards we controlled the broken tip under the stereomicroscope if it was straight enough to pipette the embryos/oocytes without harming.

2. 2. 4. 2. Collecting oocytes / zygotes and hyaluronidase treatment

To collect the oocytes in nonmated groups and zygotes in mated groups, we euthanized the mice on day 1 that was respectively about 12 hours and 16 hours after hCG injection. However, to gain oocytes – zygotes, we needed only to isolate and control the oviducts. To search for the oocytes and zygotes, we transferred the euthanized oviducts from transfer tubes to 35-mm petri dishes containing equilibrated M2 medium at room temperature.

Oviduct anatomically, lies between the ovary and the uterine horn, consisting of 3 regions which are infundibulum, ampulla and isthmus. Ovulated oocytes and fertilized zygotes were recovered from the ampulla of the oviducts that could be distinguishable under 600x magnification of stereomicroscope by a swollen ampulla containing cumulus oocyte complexes (COC) and, in mated groups, cumulus zygote complexes (CZC). Sometimes we came across with the cumulus complexes also in infundibulum.

The cumulus masses of granulose cells containing eggs or zygotes were flushed from the ampulla with using a blunt needle, which was used to tear the swollen area with complexes. By teasing out into a straight segment on ampulla, the

cumulus masses flew out by themselves, if not we squeezed the oviduct gently with forceps and pushed them out. Occasionally, a few ova after flushed from the oviduct were found free from the cumulus cells or in a small, loosely packed cumulus mass. This was a typical sign of degeneration that we did not count these kinds of ova since they were most likely ovulated during the previous estrous cycle.

After releasing the clutch of ovulated eggs by teasing apart the oviduct, it was followed by treatment with hyaluronidase (80 IU/ml) to disperse the cumulus mass. Zygotes and oocytes were denuded by repeated pipetting in hyaluronidase solution for up to 2 minutes in room temperature, followed by several washes in M2 medium. We didn't leave them in hyaluronidase solution more than 2 minutes because it was harmful and could degenerate the zygotes and oocytes. Some oocytes could and were mechanically damaged with the fine pipettes during denudation. They were finally incubated in 30µl droplets of M16 or Menazotte culture medium under mineral oil.

Pre-embryos were subdivided into three groups:

1. 1 - cell (non-fertilized and fertilized oocytes),
2. 2 – cell (Figure 3),
3. >2- cell.

Any exhibiting poor morphology (e.g. absence of two polar bodies or zona pellucida, granular cytoplasm, fragmentation or other degenerative changes) were recorded and then discarded. The rest were used for in-vitro fertilization (IVF), for polar body biopsy that was done to improve our fluorescence in situ hybridization (FISH) technique, and for checking the stimulation protocol's efficiency.

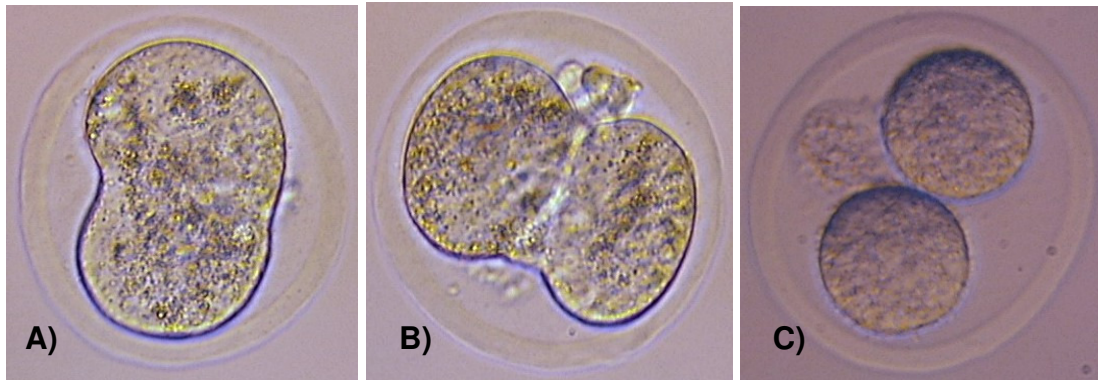


Figure 3: Dividing procedure of a zygote to form a two-cell stage embryo. A) Dividing of the zygote, B) Bridge between dividing the embryo, C) 2-cell stage embryo (magnification 400x).

2. 2. 4. 3. Collecting 4, 6, morula and blastocyst-stage embryos

We euthanized the female mice on day 3, 4 and 5 to collect respectively ~ 3 – 5 cell, ~ 6 - 8 cells and compact morula - early blastocyst stage embryos. Therefore we needed to isolate and control the oviduct and uterine. Either the oviduct or the uterus was flushed depending on the developmental stage. On day 4 and 5 we collected the embryos as we had for oocyte/zygote collection. It was also possible use another technique of flushing the oviduct but we did not favor it because of thin oviducts and sensitive embryos. For compact morula - early blastocyst stage embryos collection from each uterine horn was checked by the flushing technique that we would describe here.

To start with, like the other groups we transferred the euthanized uterus at room temperature from transfer tubes to 35-mm petri dishes containing equilibrated M2 medium. Then we cut each horn with scalpel close to the cervix, inserted 29-gauge needle from the part of the uterus, which would be close to utero-tubal junction. By flushing each uterine horn with 2 ml of M2 medium using a 1 ml syringe, we collected morula - early blastocyst stage embryos and then pooled them under a stereo dissecting microscope in another culture dish. To be sure that we collected all the embryos, we cut the uterus with scalpel close to the utero-tubal junction and flushed it from the cervix end.

Depending on the protocol that was going to be used, the embryos were cultured for one day and the number of blastocysts, < blastocysts and degenerated post-culture pre-embryos were recorded. These embryos were subdivided into three groups:

1. 4 - 6 cell stage,
2. 6 - 8 cell stage,
3. morula stage,
4. arrested morula stage,
5. blastocysts stage ,
6. hatched blastocysts stage
7. degenerated.

Poor morphologies (e.g. absence of zona pellucida, granular cytoplasm, fragmentation, arrested, degenerated pre-embryos or other degenerative changes) were reported and then these embryos were discarded.

First three groups were for to check the stimulation protocol and used embryo culturing medium efficiency with embryos development rate. Blastocyst stage embryos, as determined by the presence of a blastocoel and distinct inner cell mass with trophoectoderm, were used in trophoectoderm biopsy, immunosurgery, and stem cell culture which were performed to isolate inner cell mass (ICM – embryoblast) and trophoectoderm (TE – trophoblast).

2. 2. 5. Mechanical differentiation techniques to isolate ICM (inner cell mass / embryoblast) and TE (trophoectoderm / trophoblast)

Blastocysts were flushed from the uterine horns on the fourth day of pregnancy and incubated in M16 medium at 37⁰C and 5% CO₂.

2. 2. 5. 1. Trophoectoderm biopsy technique

Trophoectoderm biopsy has been described in several publications (Gomez et al, 1990; Muggleton-Harris, 1990). The method consisted of making an opening in

the zona pellucida opposite to the inner cell mass and then waiting 18-24 hours for herniation of the trophectoderm (Dokras et al., 1990, 1991) or aspirate trophectoderm prior to herniation (Muggleton-Harris and Findlay 1991). Aspirated section of the trophectoderm is then fixed to a glass slide for genetic investigations.

Technique can be performed in different ways like: (Muggleton-Harris et al., 1993, 1995; Dokras et al., 1990, 1991).

Laser – consisting of making a hole on the zona with low ms pulse of laser and afterwards letting trophectoderm to hatch then aspirate, Incision – involves removing the cells with the help of micro razor blade. Slit/excision – implies making a mechanical slit on the zona opposite to the inner cell mass with a finetipped (spear) pipette and excising the hatched trophectoderm cells. Hatch/excision – contains excising directly the hatching trophectoderm with a spear pipette.

We tried to obtain slit and laser versions but mainly focused on mechanical (slit) biopsy which was performed with the help of micropipettes and manipulator. The size of the slit and the stage of embryonic development at which slitting was performed were important for successful herniation to occur and biopsy to perform.

2. 2. 5. 1. 1. Embryo micromanipulation and zona slitting

As female mice superovulated and mated with males, they were then euthanized on the 3.day of pregnancy (70 - 72 h post-hCG administration and post-mating) by cervical dislocation. 8 cell - morula stage embryos flushed from the uterine horns with M2 medium and washed in medium M16. Embryos were cultured individually overnight in separate drops of M16 medium under oil in 37⁰C and 5% CO₂ incubator. The culture medium and oil were also equilibrated overnight and kept at 37°C prior to manipulation.

One hour before the manipulations, zona slitting, biopsy and culture dishes were prepared and left for incubation for equilibration. Manipulation dish contained on the top PVP drop and under it lying 3 lines, total 12 microdrops of HEPES-

buffered M2 medium (Figure 5A). PVP drop was for the holding pipette to control the pipette's inner pressure. The embryos were transferred individually to separate drops of HEPES-buffered M2 medium to stabilize the pH of the medium during micromanipulation.

Zona slitting procedure was started with the early blastocyst stage and blastocyst stage embryos, which would be suitable for slitting.

Blastocyst biopsy was performed using a pair of micromanipulation systems in conjunction with a phase contrast microscope and two manipulators that were applied to hold the blastocyst with holding-pipette and to slit the zona pellucida with closed-tip needle fitted with a heated stage. Different outer diameter of (120-150 μ m, 150-180 μ m, 95-120 μ m) holding-pipettes were used with inner diameter of 25 μ m and an angle of 45° and sharp. All the glass instruments and pipettes were siliconized to reduce adherence of cells to the glass.

This first part of the procedure simply consisted in making a slit in the zona between the two points pierced by the microneedle opposite to the inner cell mass, which should theoretically have taken 1 - 2 min.

Each blastocyst was held with the holding-pipette and immobilized close to the embryonic pole (Figure 4B). The trophoctoderm cells were withdrawn at the abembryonic pole, and a slit was started at the zona pellucida directly opposite the inner cell mass with siliconized closed-tip needle by inserting through the zona pellucida (Figure 4A). Special care was taken not to damage and pierce the trophoctoderm cell layer. After the siliconized closed-tip needle was inserted through the zona pellucida into the perivitelline space, the needle was then pierced back out through the zona and the holding pipette sucked the blastocyst out which was releasing the blastocyst suspended on the closed-tip needle. The lower end of the holding pipette zona pellucida was rubbed along and against the closed-tip needle on the area of the zona trapped to form a slit on this layer (Figure 4D). The size of the slit was standardized to one-quarter of the circumference of the blastocyst. After zona slitting, the zona-slit blastocysts were transferred immediately to the incubator and were incubated until a definite herniation of the trophoctoderm cells was visualized (Figure 4E).

There was another improved possibility to make a small opening in the zona pellucida which could be performed by laser. With using a 10-ms pulse from a infrared diode laser we thinned the zona away and made a hole.

2. 2. 5. 1. 2. Biopsy procedure

On day 4-5 (after 18 - 24 h after zona slitting), embryos were examined for expanding trophoctoderm. Definite herniation of the trophoctoderm layer searched that protruding through the zona slit (Figure 4A). If suitable cases were found, the embryo was marked for biopsy and trophoctoderm biopsies were carried out. If not, the embryo was cultured for another 8 - 12 hours and reassessed. We conducted biopsies under oil in falcon petri dishes and the herniating blastocysts were placed in drops of standard blastocyst medium. The embryos were oriented to ensure that the biopsies were performed on herniated trophoctoderm. Using an inverted microscope equipped with manipulators, the blastocyst was secured with a thick-walled and blunt glass holding pipette. Then the protruding trophoctoderm was at the 3 o'clock position and these herniating cells were biopsied when either the size of the herniation was approximately the same as the blastocyst or when it was half the diameter of the blastocyst (Figure 4D). As the blastocyst herniated through the slit on zona pellucida, the selected cells were gently pulled away from the blastocyst using a siliconized, closed-tip glass needle. The embryo was stabilized by the holding pipette and then excised, by gently rubbing the end of the needle across the waist of the herniation and against the bottom of the dish with a glass needle (Figure 4D). Biopsied TE cells were removed and placed to the side. The biopsied blastocyst would occasionally collapse during the manipulative procedure but would normally reform a blastocoelic cavity within the same hours in a culture.

After each biopsy was performed and as soon as a clump of trophoctoderm cells was removed from the blastocyst, the manipulated blastocyst was transferred to an individual drop of M16 and cultured overnight in incubators to assess the morphological changes like disaggregation or embryo degeneration the next day.

The biopsied trophoctoderm cells were placed on a glass slide under a stereomicroscope at a magnification of 400x, marked with circles by a diamond writer and given numbers of the corresponding embryo. We also marked between the circles using lines to find our way more easily with the fluorescence microscope (Figure 5B). The biopsied cells on the slide were fixed with 2 x 10 μ l of 3:1 Methanol / Acetic acid solution, then left to air dry (Tarkowski A. K., 1966) and finally left in methanol coplin jar for 5 minutes for possible FISH analysis.

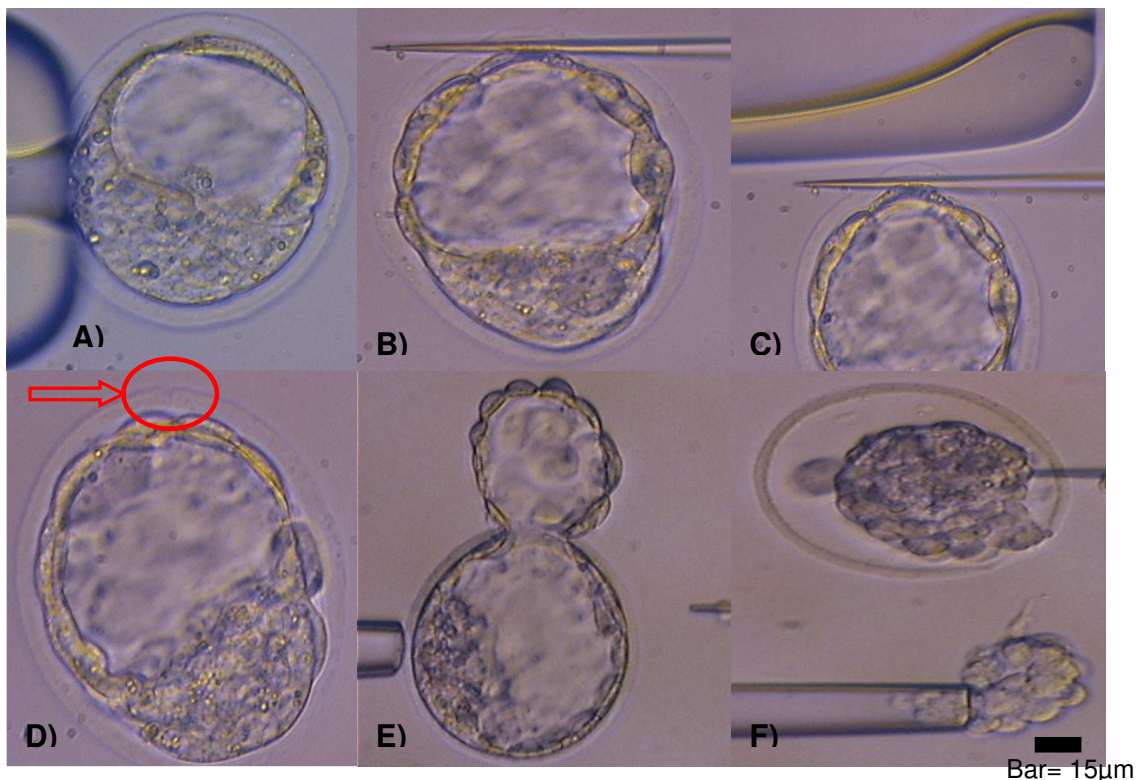


Figure 4: Stages of trophoctoderm biopsy in mouse blastocyst. (A) The early stage blastocyst held at the abembryonic by the holding pipette. (B) The siliconized closed-tip needle inserted through the zona into the perivitelline space without piercing the trophoctoderm layer, and the holding pipette is removed, herewith the blastocyst remains suspended by the needle. (C) The lower edge of the holding pipette is then rubbed against the trapped zona until the blastocyst falls free and a distinct slit is seen in the zona as indicated by the arrow. (D) Trophoctoderm cells herniating through the slit in the zona pellucida and had a large herniation. (E) Herniated cells separate from the blastocyst with the closed-tip needle, the holding pipette sucks away a number of cells away from the trophoctoderm cell layer and an operated blastocyst could be seen together with its clump of trophoctoderm cells, the rest embryo (magnification 400x; bar =15 μ m).

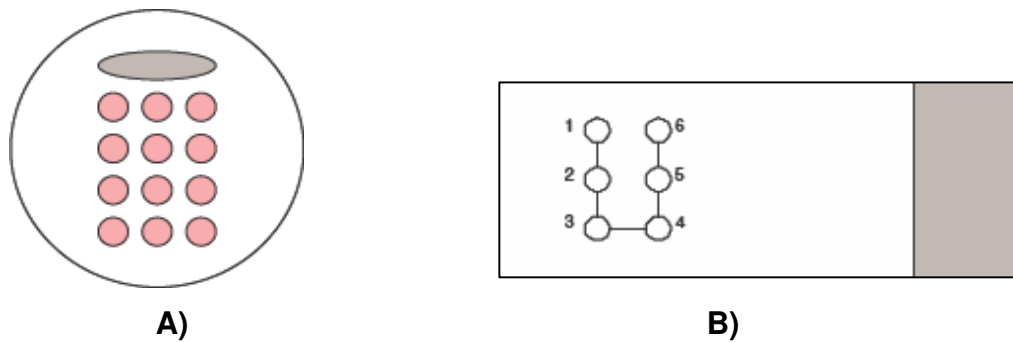


Figure 5: A) Biopsy dish for the biopsy and zona slitting. Grey big drop refers to PVP (Poly vinyl pyrrolidone) for holding pipet and pink small drops refer to HEPES-buffered M2 medium for the embryos. B) Represents a glass depression slide that contains fixed biopsied trophoctoderm cell in each circle. Between the circles marked with lines by diamond writer.

2. 2. 5. 2. Immunosurgery

Mouse blastocysts are susceptible to complement-dependent antibody cytotoxicity. Exposure of blastocysts to rabbit anti-mouse serum together with complement results in the death of all cells; however, when blastocysts are exposed to antiserum alone and then transferred to guinea pig complement, only the trophoblastic cells are killed. These results suggest that the mouse blastocyst is not permeable for certain antibodies. In need, inner cell masses can be easily separated from the remnants of trophoblastic cells.

Principle of this procedure was, destroying more or less the cells of trophoctoderm by brief exposure to interspecific rabbit sera antibodies directed against the mouse trophoctoderm antigens with mediating complement-reactive lysis that could be also described as an isolating method of blastocyst ICM by selectively killing of the outer trophoctoderm (TE). Immunosurgery can be used to obtain large quantities of pure inner cell masses in a short time period. The trophoctoderm lytic response was varied with antibody source.

2. 2. 5. 2. 1. Setting the reagents and equipments

Prior to the procedure, we prepared some reagents and equipments.

1. Special antibodies like guinea pig complement, anti-mouse serum or Rabbit anti-mouse antibody were divided to aliquots and stored at 4°C.
2. HEPES buffered M2-Medium mixed with 4mg/ml (BSA) bovine serum albumin (M2 + BSA), filtered through a 0,22 µm filter and incubated at least 1 hour in 37°C, 5% CO₂ incubator.
3. Defrosted anti-mouse serum (or Rabbit anti-mouse antibody) was heated at 56°C for 30 min to inactivate the complement. Then anti-mouse serum (or Rabbit anti-mouse antibody) diluted 1:5, 1:10, 1:25 with M2+BSA.
4. Guinea pig complement: Ready guinea pig complement was diluted 1:5 with M2+BSA and filtered through a 0,22 µm filter. Filtered complement divided to 100µl portions and stored at -70°C.
5. Plates were prepared for removal of the zona pellucida and immunosurgery (Figure 6) by setting up drops of M2 and Acid Tyrode solution, a single row per embryo intended for derivation, in tissue culture dishes. The drops were overlaid with mineral oil to prevent evaporation.
6. All plates were equilibrated at 37°C in a tissue culture incubator before use.
7. Pasteur pipettes were pulled for use in transferring embryos, the ends were cut and used with mouth pipette tubing.
8. Kaiser's glycerol gelatine was warmed at 37°C for fixing the cover slips.

2. 2. 5. 2. 2. Removing zona pellucida and Immunosurgery procedure

Superovulated females were mated overnight with males and after checking the presence of a vaginal plug next morning, females were killed by cervical dislocation on the 3.day of pregnancy, and 8 cell - morula stage embryos were flushed from the uteri with M2 medium. Gained embryos washed in medium M16 and 8 cell embryos were cultured overnight in separate drops 50-70 µl of M16

medium under oil at 37°C and 5%CO₂ incubator in which the culture medium and oil were equilibrated overnight and kept at 37°C prior to cultivation.

Next day blastocyst stage embryos were collected into a culture dish of M2 medium, then to begin with the procedure we transferred 10 – 20 blastocysts from culture dish to M2 drop of Acid Tyrode (AT) - M2 medium drop dish. AT plastic petri dish contained 3 drops of M2 medium and 3 drops of Acid Tyrode solution (pH 2.5) (Figure 6).

Blastocysts were transferred from M2 drop through series of AT drops and briefly held until all had been AT-processed. Zona pellucidas were removed by up and down pipetting with thin tipped pasteur pipette. Dissolution of zona pellucidas was observed and after zona dissolved, embryos were moved immediately into several fresh drops of M2 medium, washed through to neutralize AT reaction. The procedure repeated for the remaining embryos and same 200 µl drops were used. Before exposure to antiserum, the embryos were incubated at 37°C and 5% CO₂ incubator for 1-2 h.

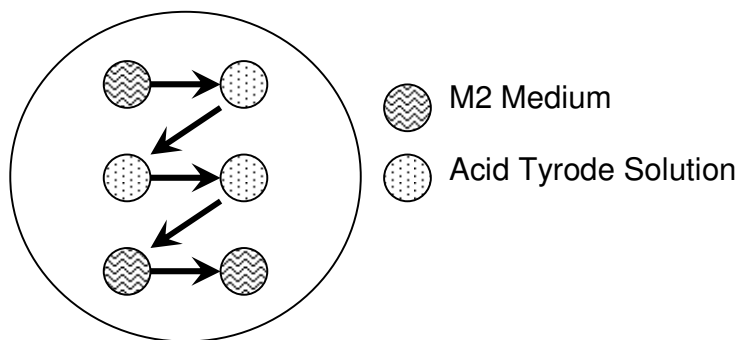


Figure 6: Figure represented the dish for zona pellucida removal, with Acid Tyrode's solution and M2 medium drops.

After the zona removal and incubation, we started with the immunosurgery procedure:

1. The ZP-free blastocysts were incubated in anti-mouse serum (or rabbit anti-mouse antibody) for 10 min at room temperature that was heat-inactivated and diluted 1:5, 1:10, 1:25, 1:50 and 1:100 with M2 + BSA.

2. Then the blastocysts were briefly washed 6 times with Phosphate Buffered Saline (PBS). They were not allowed waiting for a long time in PBS because of degeneration but also care was taken to ensure the anti-mouse serum was properly washed away.
3. It was followed by a maximal 5 - 6 min incubation in 100 µl guinea pig complement diluted 1:5 in M2+BSA with fluorochrome 10 µg/ml Propidium iodide (PI) at 37°C in the dark. Damaged trophoblastic layer cells were detached by smooth pipetting. The sequential incubations in antiserum and complement caused partial lyses of the exposed TE cells allowing the PI, which was excluded by some viable TE cells, access to their nucleus.
4. Embryos washed briefly in PBS to dissolve the rest of the PI, complement and brought to 4-well-glass object slides with chambers. These chambers were filled before with 100 µl ethanol to fix the embryos and each embryo pipetted to a single chamber. When fixation was finished, we took the ethanol away and marked the fixed embryos with a circle by diamond writer.
5. After fixation in ethanol, the second fluorochrome, bisbenzimidazole labelled the unlabelled inner cell mass nucleus. Absolute ethanol mixed with 20 µg/ml Bisbenzimidazole. 100 µl of this mixture was pipetted to each chamber with fixed embryos, then lids of the chambers closed and taped with surgical tapes to avoid the evaporation of the mixture. Slides were incubated for 1.30 hour at 4°C in dark.
6. After 1.30 hour incubation absolute Ethanol + Bisbenzimidazole mixture pipetted away and 1ml absolute Ethanol was pipetted to each chamber. Like the previous step, lids of the chambers closed and taped again to avoid the evaporation. Ethanol covered slides were incubated for 1 hour at 4°C in dark.
7. As a last step, we removed the Ethanol, pulled chambers away and fixed cover slips on the slides with glycerol gelatine.

After completion of differential labelling fluorescence micrographs of blastocysts were; the outer TE nuclei appear red due to labelling with propidium iodide, ICM nuclei labelled with bisbenzimidazole appear blue.

All manipulations of embryos were carried out using a stereo microscope and heated stage.

2. 2. 5. 2. 3. Fluorescence microscopy

Trophectoderm (TE) and inner cell mass (ICM) of mouse blastocysts nuclei distinguished by differentially labelling their nuclei observed with fluorescence microscopy. The two fluorochromes polynucleotide-specific fluorochromes, propidium iodide and bisbenzimidazole have widely different fluorescent spectra which can be separated using appropriate filter combinations.

Zeiss Axioplan 2 microscope equipped with CCD-camera was used for fluorescence microscopy. Digital Images were captured and further processed using the ISIS3 software.

Standard filter set was used: PI labelled nuclei appear red or pink with 617 nm TR (Texas Red)/Cy 3,5 filter and bisbenzimidazole labelled nuclei appear blue with 460 nm DEAC filter.

2. 2. 5. 3. Stem cell culture

Stem cell cultures provide another method of isolation embryoblasts and trophoblasts. Stem cells are the cells that serve as a normal reservoir for new cells needed to replace damaged or dying cells. The routine methods of embryonic stem cells culture contain passaging the colonies of ICM several times until reaching to the target tissue. Because our aim was to isolate pure, single embryoblasts and trophoblasts, we did not passage the ICM clumps rather then cultured with stem cell culture principles for 3 days and then isolated.

2. 2. 5. 3. 1. Preparing the culture mediums and solutions

- 0.1% Gelatine Coating for Culture Plates: 0.1% gelatine was prepared by diluting the 2% (20X stock) gelatine solution (after warming) 1:20 with cell culture grade water. The diluted 0.1% gelatine solution was transferred into 4-well-glass object slides with chambers. Each chamber was filled with 500 µl 0.1% gelatine solution and slides left for at least 20 minutes at 37°C incubation.
- Preparation of Pronase (TM) for Zona Removal: 500 IU/ml Pronase (TM) dissolved in DMEM, filtered, aliquoted as 22 µl portions and stored as a stock culture in -20°C. Working solution was prepared by diluting the stock as 10 IU/ml in a desired embryo culture media.
- Working solution of β-Mercaptoethanol: 70 µl stock β-ME was diluted in 10 ml D-MEM/F-12, aliquoted as 1,5 ml portions and stored at -20°C. Once it was warmed, it was stored at +4°C.
- Stock Culture Media Preparation: The media used for ES cells (mESC) are shown below:

| | | |
|--------------------------------|---------------------|-------|
| D-MEM/F-12 | 415ml | |
| FBS (Hyclone) | 75ml | |
| Penicillin/streptomycin (100X) | 5ml | |
| NEAA (100X) | 5ml | |
| β-ME working solution | | 500µl |
| Total volume | <u>500ml</u> | |

mESC medium prepared as a stock was aliquoted in 50ml tubes, stored at +4°C, and had to be used or discarded within 6 weeks. Before using the mESC medium on blastocysts, 1/100 L-glutamine (200mM) and 1000 U/ml LIF was added and the medium filtered through a 0,22 µm filter. After the incubation and removing of 0.1% gelatine solution, mESC was filled into 4-well-glass object slides with chambers.

The leukaemia inhibitory factor (LIF) controls the self-renewing capacity and undifferentiated state of ESCs in culture. ESCs can be maintained indefinitely in culture with pluripotential capacity in the presence of LIF. LIF is normally expressed in the TE and secreted into the ICM.

Ready for use Trypsin / EDTA was frozen in portions of 5 ml at -20°C and was always thawed before using.

2. 2. 5. 3. 2. Removing zona pellucida and culturing the embryos

Before the process 1ml embryo culture media is added to each well of the 4-well organ culture plate.

1. 20 µl Pronase (TM) was added into the first well of the plate with 1 ml culture medium (Final concentration: 10 IU/ml) approximately 30 minutes before zona removal.
2. Mouse blastocysts were firstly transferred to first well containing Pronase (TM) and incubated for 2-3 minutes.
3. Blastocysts that had a vanished zona pellucida were aspirated gently with a glass pipette and transferred into the well 2, 3 and 4, by gently washing in each. For gentle pipetting and removal of zona, we prepared glass pipettes with different ends to have the best pipetting without damaging the embryo.
4. Washed zona-free blastocysts were then transferred to the previously prepared culture plates. Zona-free blastocysts were not allowed to wait in Pronase (TM) because of the degeneration risk.

After zona removal, each zona-free blastocyst was transferred to 4-well-glass object slides with chambers and 2-3 day incubated at 37⁰C and 5% CO₂ incubator with mESC culture medium. We observed the blastocysts stem cell development every day and differentiated the cells easily under stereomicroscope (Figure 7).

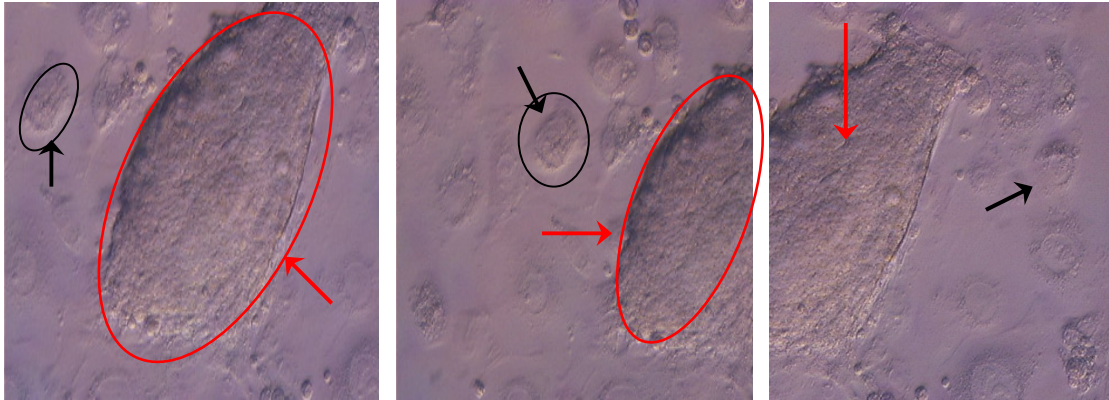


Figure 7: Red arrows and circles: Inner cell mass (ICM) clumps. Black arrows and circles: epithelioid peripheral trophoblasts outgrowths 2 days after incubation (magnification 200 x).

2. 2. 5. 3. 3. Trypsin treatment

The colonies were allowed to grow and to spread until they were large enough and were pretty piled up after 2 - 3 days incubation which means, the blastocysts were left undisturbed over the next 24 - 48 h to allow for the ICM lumps to attach to the monolayer.

Trypsin procedure had not needed to perform because of further passaging rather than isolating individual, single embryoblasts and trophoblasts from each embryo to investigate their chromosomal status through several tests.

Ready for use trypsin was thawed in portions of 5 - 7 ml before using and added 0,5 ml to each well of 4-well dishes.

Separation of the ICM lump was carried out using the blunt heat-sealed end of a finely drawn-out Pasteur pipette and stereomicroscope at high magnification. The ICM lump (Figure 7) was split very carefully before being picked up with a minimal volume of medium without removing the trophoblasts from the feeder layer. The aspirated lumps were then transferred to 1ml trypsin under the stereomicroscope and incubated for 20 - 30 min, for partial cell disaggregation at 37°C in a 5% CO₂ incubator. Longer application of trypsin was not allowed because of the degeneration risk.

The action of trypsin was completed after gentle breaking up the ICM cells by pipetting up and down 1 - 2 times until they dissociated into single cells and no clumps of cells remained. The trypsin / cell suspension was then carefully aspirated being transferred to sterile 1,5 ml micro tubes and filled with 500µl 1:10 diluted and sterile filtered FCS (fetal calf serum) in M2 medium pro micro tube. The trypsin reaction is inhibited by FCS. After neutralization, caps were centrifuged at 2000 rpm for 10 minutes, the supernatant was removed and the 50-100 µl cell pellets were washed twice with 1 ml PBS by centrifuging at 2000 rpm for 10 minutes. Finally, the supernatant was aspirated off and washed 50-100 µl embryoblast pellet were transferred dropwise to clean cytopsin slides circles which prevented the drop of embryoblast pellet from flying away. The slides were left to air dry for further fixation.

Without disturbing the rest trophoblasts (Figure 7), which were spread out from the peripheral regions on the 4-well-glass object slide chambers, mESC culture medium was carefully aspirated off and each chamber was washed and brandished once or twice with 1 ml PBS. Washing procedure with PBS was needed to reduce the background problems during our modified FICTION (Fluorescence Immunophenotyping and Interphase Cytogenetics as a Tool for the Investigation of Neoplasms) protocol but was kept short to avoid the degeneration of the cells. To better evaluate the area of adhered trophoblasts during the fluorescence microscope procedure, we marked the position of trophoblasts with a diamond tipped pen on the slide, removed the chambers and left the slides to air dry for further fixation.

2. 2. 5. 3. 4. Different fixation techniques of ICM and TE slides:

We tried to find optimal fixation methods for further applications that were immunofluorescence (IF) cell staining combined with fluorescence in situ hybridization (FISH).

Fixation methods included 4 different fixatives which were:

- 1:3 Methanol / Acetic Acid

- 1:1 Methanol / Acetone
- Pure Acetone
- 4% Paraformaldehyde (PFA) (4 g PFA resolved in 100 ml PBS, pH:7)

In principle, Methanol / Acetic Acid, Methanol / Acetone and pure Acetone fixation protocols were the same but just the first step as fixatives differs. 4% Paraformaldehyde fixation protocol differed greatly and is described below.

Methanol / Acetic Acid - Methanol / Acetone - pure Acetone Fixation:

1. Air-dried slides fixed with 2 X 10 µl drops of, pre-cooled at -20°C, 1:3 Methanol / Acetic Acid or 1:1 Methanol / Acetone or pure Acetone.
2. Slides left for air dry.
3. Slides transferred to, pre-cooled at -20°C, 100% methanol filled glass coplin jars and left for 5 minutes.
4. Slides washed 2 times for 5 minutes in PBS filled glass coplin jars.

4% Paraformaldehyde Fixation:

1. Air-dried slides fixed in fresh prepared 4% PFA filled glass coplin jars for 30 minutes.
2. Slides left for air dry.
3. Slides washed 2 X PBS filled glass coplin jar for 5 minutes.
4. For permeabilization in immunofluorescence (IF) cell staining, we applied 1% triton in PBS for 10 min.

After all the fixation protocols, the slides were not let to dry and continued on with the blocking step of immunofluorescence (IF) cell staining which is described below.

2. 2. 6. Immunofluorescence (IF) cell staining

Immunofluorescence principle is based on 3 steps with 3 set of antibodies which were; antibody against the wanted antigen, biotinylated antibody to identify the primary antibody and fluorescence conjugated streptavidin that binds to biotin.

This technique was used to visualize and distinguish the inner cell mass (ICM) and trophoectoderm (TE) cells. The expression of POU-domain transcription factor of Oct-4 with rabbit anti-oct4 polyclonal antibody and the expression of caudal-like transcription factor Cdx with rabbit anti-cdx polyclonal antibody were analyzed with fluorescence markers like; AMCA, FITC and Cy5. Direct labelling with AMCA, FITC and indirect labelling with Cy5 fluorescence used to find out the best merged images with the combination of multi colour FISH probe.

2. 2. 6. 1. Preparation of required antibodies and slides:

- Rabbit Anti-Oct4 affinity purified polyclonal antibody: To reconstitute the antibody, centrifuge the antibody vial was centrifuged at moderate speed (5,000 rpm) for 5 minutes to pellet the precipitated antibody product. The ammonium sulphate/PBS buffer solution was removed and discarded. 10 µl of residual ammonium sulphate solution does not affect the resuspension of the antibody. The protein pellet was not allowed to dry and the antibody pellet was resuspended in PBS. A 100µl buffer was needed to achieve a 1 mg/ml concentration with 100µg of precipitated antibody. The precipitated antibody was rehydrated for 1 hour at 4 – 25°C prior to use by gentle stirring without vortexing.
- Rabbit Anti-Cdx2 affinity purified polyclonal antibody: Prepared in the same way as Anti-Oct4. A 50µL buffer was needed to achieve a 1 mg/mL concentration with 50 µg of precipitated antibody. Precipitated antibody was rehydrated for 1 hour at 4 – 25°C prior to use without vortexing.
- Clean slides: stored in 1% HCl/ethanol at room temperature and dried prior to use.

2. 2. 6. 2. Immunofluorescence protocol

Corresponding ICM and TE slides were taken directly after fixation. The slides were not allowed to dry before proceeding with the immunofluorescence protocol steps. Steps differed by the fixative used for the slides.

1. Blocking:
 - a. Methanol/Acetic Acid - Methanol/ Acetone - pure Acetone fixated slides: The fixated cells were blocked with rabbit serum which was the species of the secondary antibody and left for 20 minutes at room temperature in a humidified chamber. Per sample 100 μ l rabbit serum diluted 1:10 with PBS was used.
 - b. 4% Paraformaldehyde fixated slides: We incubated the sections for 15 minutes in a humidified chamber with blocking solution containing 1% triton+2% NRS (normal rabbit serum) in PBS for 15 min.
2. The First Antibody: Excess blockings blotted from the slides and without washing the slides, directly first antibody rabbit-anti-mouse-Oct4 or rabbit Anti-Cdx2 antibody was applied. The first antibody diluted 1:200 with antibody diluent with background reducing components. Per section 100 μ l was pipetted and the slides were incubated in a humidified chamber overnight at 4°C. For 4% Paraformaldehyde fixated slides, blocking solution was used (1% triton + 2% NRS) to dilute the antibody. Before applying, it was strongly prohibited to vortex the rabbit-anti-mouse-Oct4 antibody, to ensure that the antibody structure was not annihilated.
- *** Negative Control: To control unspecific reactions, in few cases no primary antibody was used. Overnight in 4°C paraformaldehyde fixated slides were incubated only with blocking solution (1% triton + 2% NRS) and methanol / acetic acid - methanol / acetone - pure acetone fixated slides only with antibody diluent with background reducing components.
3. Washing: The next morning the primary antibody was drained off the sections and the slides were washed 3 x 10 minutes in PBS filled coplin jars.
4. The Secondary Antibody: Slides were incubated in a humidified chamber with vortexed, biotinylated secondary antibody solution for 1 hour at room temperature (per section 100 μ l goat-anti-rabbit antibody was diluted 1:200

- in antibody diluent with background reducing components, for 4°C paraformaldehyde fixated slides in blocking solution).
5. Washing: After one hour, the biotinylated secondary antibody was drained off and slides were washed 3 x 10 minutes in PBS filled coplin jars.
 6. The Immunofluorescence conjugated antibody: The FITC-conjugated Streptavidin, which gives a green colour to marked section, was diluted 1:200 in antibody diluent with background reducing components (for 4°C paraformaldehyde fixated slides in blocking solution), per section added 100 µl and incubated 1 hour at room temperature in a humid chamber. This step and preparations were applied in dark. AMCA -conjugated Streptavidin was diluted 1:400 and cy5-conjugated Streptavidin was diluted 1:1000 with antibody diluent with background reducing components. Per section the same amount of antibody used as FITC and AMCA gave direct blue colour whereas cy5 observed indirectly as green (Table 1).
 7. Washing: One hour later flouorchrom-conjugated Streptavidin sections were drained off and slides were washed 3 x 10 minutes in PBS filled coplin jars in dark.
 8. Air dry / DAPI /FISH: Slides were left to air dry in dark. To look for the immunofluorescence results the slides were mounted with mounting medium including DAPI, the cover slips were put on the slides or the FISH protocol was done. For the slides that AMCA fluorescence applied, mounting medium including DAPI was not used because of the same colour.

2. 2. 7. Fibroblast cell culture from mouse skin

We established a technique which mouse fibroblast cells could be achieved from mouse abdominal skin. The technique is not complicated and is based on tissue-cell culture.

2. 2. 7. 1. Preparation of required solutions and mouse skin

- Culture medium for MEF (embryonic mouse fibroblast):

| | |
|--------------------|-------|
| DMEM | 435ml |
| FCS | 50ml |
| L-Glutamin | 5ml |
| NEAA (100x) | 5ml |
| Pen / Strep (100x) | 5ml |

500ml

All ingredients were added to a 500ml, 0.22µm filter flask and filtered. The medium was kept always sterile and stored at 4 °C for up to 3 weeks.

- Ameothopterin solution: 25 mg Ameothopterin was pre-solved in 100µl 0,1N NaOH and vortexed in 1,5 ml eppendorf tubes. Then the solution was taken up in 2 ml HBSS and transferred into a 10 ml falcon tube. The vessel was rinsed several times with HBSS that was also transferred to the Falcon tubes until a volume of 10 ml. These 10 ml were then transferred to 490 ml HBSS and sterile filtered. Solution was aliquoted as 55 µl portions and stored at -20°C.
- Thymidin solution: 25 mg Thymidin was solved in 100 ml HBSS and sterile filtered. Aliquoted as 4ml portions and stored at -20°C.

2. 2. 7. 2. Mouse skin-fibroblast cell cultivation and preparation:

Mice were euthanized with cervical dislocation technique and the abdomen was saturated with ethanol. By using sterilized instruments, block of abdominal skins were cut, rinsed with MEF and placed into sterile petri dishes where the skins were cut to ~1mm² small square pieces. T75 flask rinsed with FCS and into each flask were placed 9-10 skin pieces. Important point was to fix the skin without hair parts (peritoneum face) to the surface of the flasks and placed apart (Figure 8) from the other skin pieces. Skin pieces remained in FCS for around 10 minutes and during the incubation while another mouse preparation was started.

After 10 minutes, FCS was aspirated off to give 800µl culture medium for MEF and skin tissues were cultivated in incubator for 3 days. Every 4.day the medium was refreshed by aspirating the spent media and replacing it with about 800-900µl fresh MEF Derivation Culture Media. We incubated the flasks, containing the skin tissue pieces, in a 37°C, 5%CO₂ tissue culture incubator. Contamination problem was solved later on by adding to our MEF medium Amphotericin B (2,5µg/ml).

After around 15 days, we started to view the cultures under an inverted microscope and if fibroblasts started to generate we removed the skin pieces to have enough area to allow fibroblasts to disperse. We inspected the cell layer covering the flask surface and if the flask was at least 90% confluent, the culture was ready for harvesting, if not we continued to culture the cells for another day.

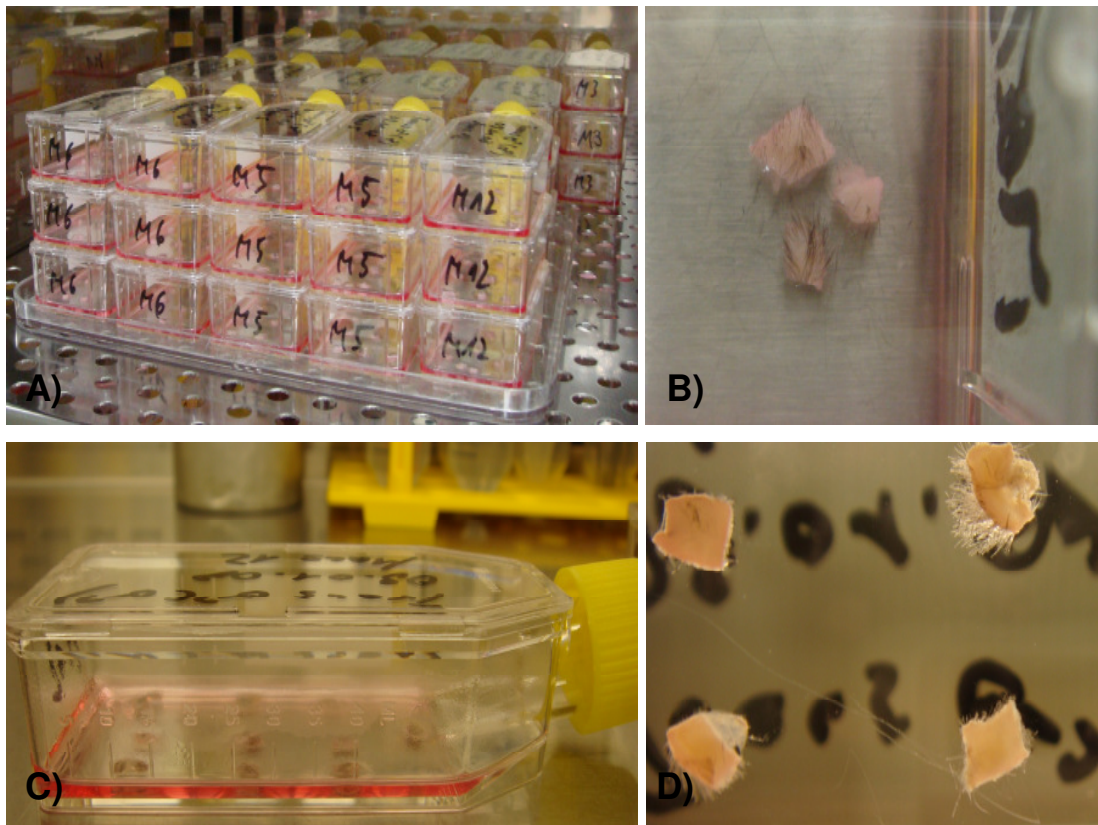


Figure 8: A) - C) T75 flasks, which contain small, square, skin pieces with MEF in a 37°C tissue culture incubator. C) Per flask, 9-10 skin pieces placed into it. B) - D) View of

the skins parts through the Flasks. D) Fixed skin without hair parts (peritoneum face) to the surface of the flasks and placed distanced from other skin pieces.

2. 2. 7. 3. Metaphase-chromosome preparation from mouse skin-fibroblast cell:

We prepared chromosome preparation from mouse skin-fibroblast cell to determine the exact breakpoint of our mice genome and to generate a suitable FISH probe for further analysis.

For this aim:

1. Growing embryonic mouse fibroblasts synchronized with a final concentration of 55ng Amethopterin/ml medium overnight in incubator and synchronized for longer high-resolution prophase or prometaphase preparations.
2. Next morning Amethopterin medium aspirated off, flasks washed twice with PBS (without Ca/Mg) to block the reaction and added Thymidin medium with a final concentration of 2 μ g /ml. Under this medium, the cells remain 7 hours in the incubator.
3. 7 hours later cells washed again with PBS (without Ca/Mg) and 10 μ g/ml Coleman - stock solution was given to the flasks for overnight incubation. Colcemid is a spindle inhibitor and helps to collect the cells at metaphase stage.
4. Medium with Colcemid in flasks aspirated off and slowly the cells in T75-culture flasks were washed once with PBS (without Ca/Mg).
5. Per flask, 3-5 ml trypsin was given to detach the cells from the flasks and incubated at room temperature for 10 minutes. We checked in an inverted microscope if cells were detached. After 10 minutes if there were still cells attached, we dislodged the cells from the flask surface by slapping the side of the flask with the heel of your hand 3-5 times and continued until the cell layer was dislodged.
6. When all the cells were detached, trypsin reaction was neutralized by the addition of 3-10 ml cell culture medium, which contained at least 10%

- FCS, to the T75 flasks and mixed it well with Trypsin solution. (Per flask trypsin: medium 1:1 or 1:2).
7. Each mouse detached cell suspension transferred to 50ml Falcon tubes and centrifuged at 1350 rpm for 10 minutes.
 8. After the centrifugation, supernatant was removed and approximately 2-3 ml 75mmol KCl solution (incubated in 37°C before using) was slowly given to the cell pellet. We pipetted the pellet up and down with plastic pasteur-pipette until there appeared to be a fine suspension of cells and no pellet remained.
 9. Rest of the tubes filled with end volume 7ml KCl solution, mixed properly with the cell suspension/KCl solution and incubated in water bath for 20-30min at 37°C. Incubation in a dilute KCl solution for 20–30 min generally achieves good spreading.
 10. 20-30 min later, a small drop was dripped on a slide to check the success of the hypotonic. We had to check it because over-treatment with hypotonic solution results in scattering of chromosomes, or rupture of the nuclei and loss of the chromosomes. Afterwards the tubes centrifuged at 1350 rpm for 10 minutes.
 11. Supernatant was removed and like KCl treatment 2-3ml Carnoy's fixation solution (fresh prepared and kept before at -20°C), a mixture of 3:1 methanol: glacial acetic acid, was slowly given to the cell pellet, then the rest of the tubes filled with the end volume 7ml fixation and mixed properly with the cell suspension. This suspension was incubated on ice for 30 minutes and cared to suspend the cells in the cell pellet to avoid clumping of cells and poor spreading. With fixation, hypotonic swelling was arrested and was maintained the cells in a stable state.
 12. 2 rounds of suspension in fresh Carnoy's and centrifugation of cells into a pellet by 1350 rpm for 10 minutes were employed, and in the end 2-3 ml fixative added to pellet, suspended to prepare cells for dropping onto slides.

13. One drop of the cell suspension was placed onto a clean slide that was stored in 1% HCl/ethanol at room temperature and was checked in an inverted microscope for the presence and quality of the metaphase. Then each mouse chromosome preparation was transferred to small eppendorf tubes and kept at -20°C.

2. 2. 8. Fluorescence in situ hybridization (FISH) protocol

Fluorescence in situ hybridisation (FISH) based on the denaturation of target DNA and probe DNA, which was labelled with fluorescent markers, to become single strand and then they were brought together that the probe DNA could hybridize to the complementary sequence of target DNA. FISH was used to analyse the signal intensity of the BAC clones labelled by DOP-PCR and the fluorophores labelling was performed by random prime labelling of purified BAC products.

Before using our self-generated FISH probe, the best fixation, denaturation, and hybridization protocols for the slides and the probe were tested, modified and determined. Therefore biopsied mice polar bodies and normal, translocated chromosome preparation from mouse skin-fibroblast cells were used and finally were examined for mouse TE and ICM cells.

All preparations and fixations prior to fluorescence microscope step performed under an inverted microscope and equipped with phase contrast optics using x100 magnification.

2. 2. 8. 1. Preparation of required solutions:

- Fixatives: Ice cold methanol and 3:1 methanol : acetic acid solution (-20°C)
- 20 X SSC solution: 175,32g NaCl (3M NaCl) + 88,23g Natriumcitrat (0,3M Nacitrat) + 1000ml aquadest (pH :7.1).

- 0.5 X SSC solution: 25ml 20 x SSC + 975ml aquadest (pH :7.1).
- SSCT solution: (4 x SSC / Tween) 200ml 20 x SSC + 500µl Tween + 800ml aquadest (pH: 7.1).
- Denaturisation solution: 70ml Formamid + 10ml 20XSSC + 20ml aquadest.

2. 2. 8. 2. Slide preparation

Before starting with FISH, cells were transferred to clean slides that were stored in 1% HCl / ethanol at room temperature, encircled the area with diamond writer and fixated. Fixation was carried out by dropping 2 x 10µl 3:1 methanol: acetic acid onto the area with cells and leaving for air dry. Subsequently the slides incubated in methanol for 5 minutes at room temperature and again left to air dry while continuing with the procedure.

By normal and translocated chromosome preparation from mouse skin-fibroblast cells, we did not need to perform fixation step, because they were already fixed during chromosome preparation procedure. Only a 10µl drop chromosome preparation was dripped onto the clean slide and let to dry.

2. 2. 8. 3. Target and probe DNA denaturation / hybridization

Probe DNA and slides (target DNA) had to be denaturized before hybridization to become single strand. Afterwards they brought together and hybridized that the probe DNA completes the sequence of target DNA to observe the signals on the chromosomes under fluorescence microscope.

For the denaturation of the slides, water-bath switched on 1 hour before with denaturation solution filled coplin jar to warm up the solution.

Target DNA denaturation protocol was;

1. washing 2 minutes with PBS,

2. dehydration in 70% , 90% and 100% ethanol for 2 minutes each at room temperature,
3. incubation of the slides for 2-3 hours at 60°C to fix the cells on glass surface for drastic denaturation procedure,
4. denaturation at 72°C with denaturation solution in water-bath for 3 minutes,
5. dehydration by incubating in 70% (ice-cold) , 90% and 100% ethanol for 3 minutes each, in room temperature to prevent annealing.
6. left the slides for air-dry.

Probe DNA, which was generated from BACs, was denaturated for ~35 minutes by simple PCR protocol as follows;

- Step 1: 75°C for 4:20 minutes
- Step 2: 75°C for 0:40 minutes
- Step 3: 4°C for 0:01 minutes, cooling down immediately to avoid the annealing of DNA. Instead of this step, probes can put immediately on ice.
- Step 4: 37°C forever, warming up again which we left the probes for 30 minutes before pipetting.

After denaturation, preparations were ready for hybridization that ~1µl probe (depending on the marked area of cells) added on the slides for hybridization and covered with the coverslips, then the coverslip sealed with rubber cement fixogum. This step was obtained quickly on a hot plate (37°C) without disturbing the probe. We placed the slides in a moist chamber at the appropriate hybridization temperature 37°C for overnight.

2. 2. 8. 4. Washing protocols and counterstaining with 4'-6-diamidino-2-phenylindole (DAPI)

The water-bath was switched on to 72°C with 0.5 x SSC solution filled coplin jar to warm up the solution 2 hours before the washing procedures.

FISH requires a certain number of washing steps after hybridization. After overnight hybridization, the rubber cement was removed with the coverslips and the slides were washed in 0.5 x SSC at 72°C in waterbath for 5 minutes. Then the slides were washed twice in SSCT for 2 minutes, followed by 1 minute in PBS and briefly in aquadest at room temperature to remove unspecific binding fluorescent signals. Excess water removed from the slide by wiping with soft paper around the chromosome preparation and cells and dried completely. The cells were counterstained with DAPI (4'-6-Diamidino-2-phenylindole) and covered with cover slides. DAPI is a fluorescence dye specific binding to the AT regions of DNA and counterstained the cell nucleuses or metaphase chromosomes. The signals were then viewed using the fluorescence microscope with the appropriate set of filters.

2. 2. 8. 5. Detection by fluorescence microscope

Fluorescence signals, concerning immunofluorescence, FICTION and FISH signals, were observed with fluorescence microscope equipped with a dual pass filter block and four single band pass blocks containing DAPI, FITC, Texas red, Spectrum Orange, DEAC and Cy5 under x600 magnification using fluorescence immersion oil. Each filter was responsible for the detection of one fluorescent signal (Table 1).

Images were recorded using a CCD (Charge Coupled Device) camera and analysed by ISIS system that was chromosome analysis software.

| Filter | Flouochrome | Detected Fluorescent signal | Excitation | Emission |
|------------------------|---------------------------|------------------------------------|-------------------|-----------------|
| DEAC | DEAC-dUTP | Spectrum Aqua | 426 | 480 |
| FITC | FITC-dUTP | Spectrum Green | 500 | 525 |
| Spectrum orange | Spektrum orange-dUTP | Spectrum Gold | 550 | 580 |
| Texas Red | Texas Red-12-dUTP | Spectrum Red | 595 | 615 |
| Cy5 | Cy5 labelled streptavidin | Indirect Spectrum Green | 649 | 670 |

Table 1: Fluorescence microscope filters equipped with a dual pass filter block, detected fluorochromes and fluorescent signals. Excitations and emissions of fluorescent signals also represents in table.

2. 2. 8. 6. Scoring criterias:

Slides were analyzed using fluorescence microscope equipped with the appropriate filter sets. Mercury vapour lamps and xenon lamps both emit light that excites fluorochromes.

In order to differentiate FISH errors from true mosaicism, scoring criteria were applied that included signal size and shape, distance between two hybridization signals and signal-nucleus size ratio (Munne et al., 1998). Minor hybridization spots with lower fluorescent intensity were not scored, as they are considered cross-hybridization signals to non-target chromosomes (Hopman et al., 1987). Spots found in close vicinity to one another, interconnected, or in paired arrangements counted as one signal.

Images were sequentially acquired in four black and white planes, pseudo-coloured appropriately, and then merged to form a composite colour image. In the merged image, the green plane represents the FITC signal, the light blue plane represents the DEAC signal, and the orange plane represents the Spectrum Orange and Texas red signal. The DAPI counterstained plane was pseudo-coloured dark blue. With these specific filters (Table 1), signals were observed on two chromatids of each chromosome.

After the precipitation of labelled BACs to form a multicolour FISH probe which will be explained below, we calculated to see signal as represents in Figure 9.

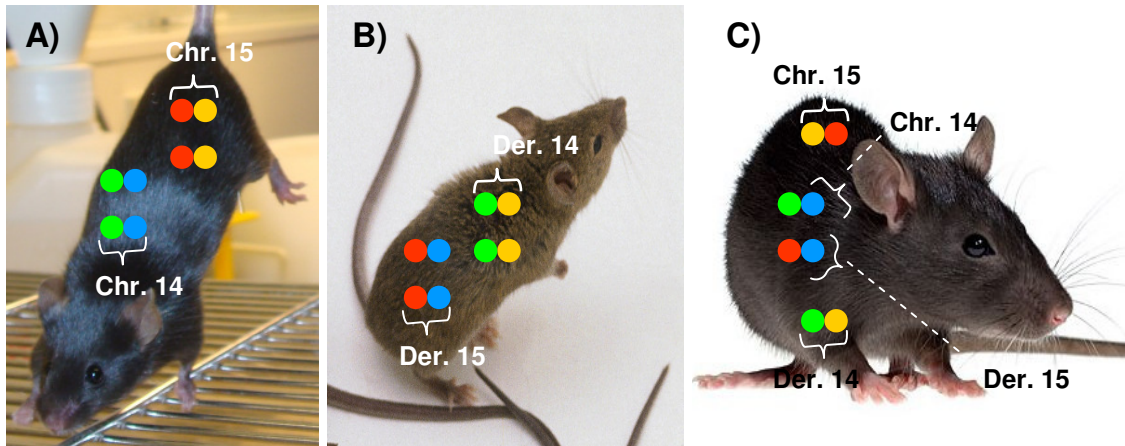


Figure 9: A) Homozygote normal mouse B) Homozygote translocated mouse C) Heterozygote translocated mouse.

2. 2. 9. Generation of FISH probe using Bacterial Artificial Chromosome (BAC) clones:

To form multicolour FISH, we mixed the labelled BACs in a certain ration which identified specific mouse chromosome bands or band orders, from RZPD (Deutsche Ressourcenzentrum für Genomforschung GmbH) and searched them from <http://genome.ucsc.edu> and <http://www.ensembl.org> web pages. Ordered some BACs listed with their position in table 1 and table 5 (15 BACs from chr.15 and 13 BACs from chr.14), briefly, they were:

- For chromosome 15: RP23-89K11, RP23-379J16, RP23-397B8, RP23-264J5, RP24-393C16, RP23-314D15, RP23-153I16, RP23-233J16, RP23-203G21, RP23-220O14, RP23-98B21, RP23-462M9, RP23-441F15, RP23-462K15, RP23-68N6.
- For chromosome 14: RP23-389I7, RP23-50C12, RP23-200H6, RP23-228P12, RP23-122C17, RP23-240O18, RP23-340M21, RP23-399K3, RP23-51F3, RP23-8P22, RP23-301G14, RP23-436G2, RP23-39J5.

Efficient culture of BAC and amplification, labelling of probes are crucial for successful sequence detection by fluorescent in situ hybridization (FISH).

To form multicolour FISH, labelled BACs were mixed the in a certain ration.

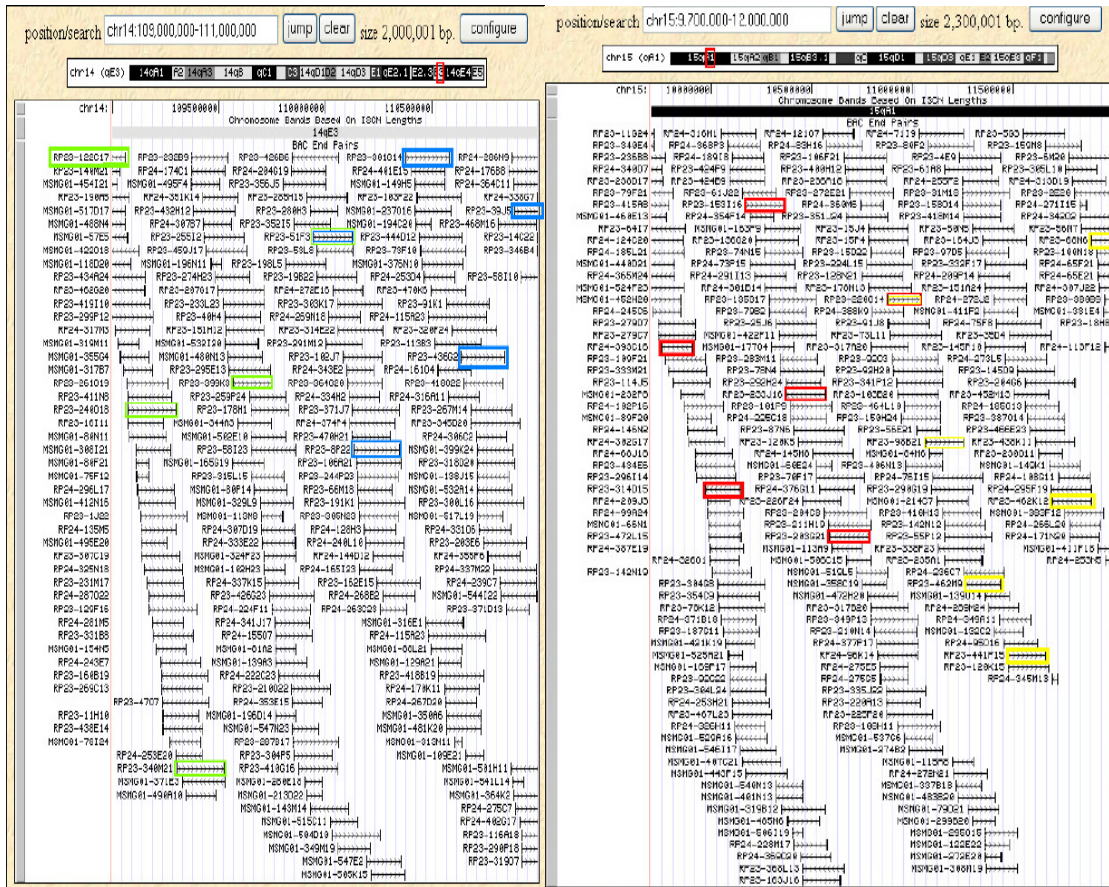


Figure 10: Listing of 20 BACs from chromosome 14 and 15, to generate m-FISH (<http://genome.ucsc.edu>).

2. 2. 9. 1. Preparing Lennox Bouillon (LB) solution and culturing the BACs

LB solution was prepared by the dissolution of 20gr LB powder in 1L distilled water that was needed to culture with BACs. When the powder was dissolved in water, it was autoclaved at 121°C for 2 hours and kept in 4°C for 8 weeks.

To start with, the BAC DNA purification protocol, the ordered BACs had to be grown in culture. Therefore, 4ml of LB medium was prepared containing 2µl chloramphenicol antibiotic in a 14 ml snap cap culture tube and inoculated with an isolated colony from a freshly streaked plate. The procedure repeated for each BAC and then the tubes were shaken (~247 rpm) at 37°C overnight.

2. 2. 9. 2. BAC DNA purification

Prior to starting BACMAX solutions 1, 3 and 4 placed on ice and 500µl of overnight cultured BACs transferred to 1,5 ml eppendorf tubes with 500µl glycerol for storage at -20°C. This storage had done to each BACs which could be thawed and used when needed.

The protocol performed to each BACs and continued as follows:

1. 1.5ml of the overnight cultured BAC was transferred to 2ml eppendorf tube. The cells were pelleted by centrifuging at 15,000xg for 1-3 minutes and the supernatant was discarded.
2. 200µl of BACMAX solutions 1 were added to the pellet and vortexed vigorously to resuspend the pellet completely. For lyses, 400µl of BACMAX solution 2 was added and mixed by inverting the tube 2-3 times very gently. To avoid shearing the BAC DNA the lysate was not vortexed, shaken or pipetted.
3. To lysate 300µl of BACMAX solutions 3 added and mixed by inverting the tube 2-3 times very gently. A white precipitate formed in tube, which should not be vortexed, incubated on ice for 15 minutes and then centrifuged at 15,000xg for 15 minutes at 4°C to pellet cellular debris.
4. The supernatant transferred to a 1ml eppendorf tube to minimize the shearing and 540µl isopropanol was added and mixed thoroughly by inverting the tube 4-6 times.
5. The nucleic acids precipitate by centrifugation 2 times at 15,000xg for 15 minutes at 4°C and isopropanol was totally decanted. When excess isopropanol pipetted off, the pellet was air-dried at room temperature for 3-5 minutes.
6. The pellet was resuspended in 250µl of TE Buffer by tapping, swirling the tube and 1µl of RiboShredder RNase Blend was added to the tube. Mixture was incubated for 30 minutes.
7. After 30 minutes 250µl of BACMAX solution 4 was added, mixed by tapping and incubated for 15 minutes on ice. It was then centrifuged at

- 15,000xg for 15 minutes at 4°C and the supernatant to another 1ml eppendorf tube.
8. To the recovered supernatant 1 ml absolute ethanol was added, mixed thoroughly by inverting the tube 4-6 times and centrifuged 2 times at 15,000xg for 15 minutes at 4°C to precipitate the nucleic acids.
 9. The pellet left for air-dry at room temperature for 3-5 minutes and afterwards 25µl aqua dest was added to it. The pellet was resuspended by tapping the tube and left for 10 minutes at room temperature.
 10. The yield of BAC DNA was quantitated by fluorimetry using a DNA specific dye Hoechst 33258. After measuring the DNA, it was kept at 4°C.

2. 2. 9. 3. Amplification DNA with Degenerate Oligonucleotid Primer-Polymerase Chain Reaction (DOP-PCR)

To start with the procedure we prepared the required PCR – solutions AB, C, D and E (Table 2).

The DOP-PCR procedure principles based on Telenius et al. (1992) and carried out in a Thermocycler. All the steps applied to each BAC DNA probe individually. The first program step had begun with 5µl AB solution with 1µl BAC DNA probe at 92°C for 5 minutes. Afterwards to each probe 0.24 µl C solution was pipetted and since the used Sequenase Ver 2.0 was not thermal stable, this step had to be repeated for eight cycles.

After eight cycles of the first phase, which could be controlled also from temperature profile, 45 µl of solution D was added. After cooling down to 56°C, 5µl solution E was added and 32 cycles were carried out without other additions. The program continued for 5 hours. The amplified DNA used for labelling and stored at -20°C for up to application several years.

Summary of DOP-PCR programme:

- Step 1: 92°C for 5 minutes
- Adding solution C
- Step 2: 25°C for 2:20 minutes
- Step 3: 34°C for 2:00 minutes

- Step 4: 90°C for 1:00 minute
- Step 5: Repeating from step 2 to 7, for additional 7 cycles.
- Adding solution D
- Step 6: 30°C for 2:20 minutes
- Step 7: 92°C for 1:00 minute
- Adding solution E
- Step 8: 56°C for 2:20 minutes
- Step 9: ramp at 0,1 °C/sec to 74 °C
- Step 10: 70°C for 2:00 minutes
- Step 11: 92°C for 1:00 minute
- Step 12: 56°C for 1:00 minute
- Step 13: ramp at 0,1 °C/sec to 74 °C
- Step 14: 70°C for 2:00 minutes
- Step 15: Repeating from step 11 to 31, for additional 31 cycles
- Step 16: 70°C for 10:00 minutes
- Step 17: 4°C for ever

| <u>Solution AB</u> | | <i>Stock solution</i> | <i>Final concentration</i> | <i>Amount of one approach (Vol 10µl)</i> |
|---------------------------|--------------------------------|------------------------------|-----------------------------------|--|
| | H ₂ O | | | 6,8 µ |
| | Sequenase Puffer | 5x | 0,6x | 1,2 µ |
| | Primer (DOP) | 50 µM | 5 µM | 1 µ |
| | dNTP | 2 mM | 0,2 mM | 1 µ |
| <u>Solution C</u> | | <i>Stock solution</i> | <i>Final concentration</i> | <i>Amount of one approach (Vol 4µl)</i> |
| | Sequenase Dilution Buffer | | | 3,5 µ |
| | Sequenase Ver.2.0 | 13 U/µl | 1,62 Uµl | 0,5 µ |
| <u>Solution D</u> | | <i>Stock solution</i> | <i>Final concentration</i> | <i>Amount of one approach (Vol 55µl)</i> |
| | H ₂ O | | | 36,7 µ |
| | Stoffel Frag. Puffer | 10x | 1x | 5,5 µ |
| | dNTPs | 2 mM | 0,22 mM | 6,05 µ |
| | Primer DOP | 50 µM | 1,1 µM | 1,21 µ |
| | MgCl ₂ | 25 mM | 2,5 mM | 5,5 µ |
| <u>Solution E</u> | | <i>Stock solution</i> | <i>Final concentration</i> | <i>Amount of one approach (Vol 7,5µl)</i> |
| | H ₂ O | | | 5,25 µ |
| | Stoffel Frag. Puffer | 10x | 1x | 0,75 µ |
| | MgCl ₂ | 25 mM | 2,5 mM | 0,75 µ |
| | Taq Polymerase (Stoffel Frag.) | 10 U/µl | 1 U/µl | 0,75 µ |

Table 2: PCR with degenerated oligonucleotid primes (DOP-PCR) – Mastermix (solutions AB, C, D and E) are shown in the table.

2. 2. 9. 4. Reamplification of the original banks

Reamplification in principle increases the amount of DNA and secondary DOP-PCR product can be used as a template for labelling DNA. The composition of secondary and subsequent amplification reaction is the same as that for primary amplification, but cycling parameters differ.

To have many trials FISH experiments with complex DNA probes caused to us by requiring large amounts of probe DNA which means, FISH probes needed to be labelled from DOP-PCR products frequently and that required too much template DNA with primary DOP-PCR products. With this reason, we regenerated the primary DOP-PCR products by reamplification protocol that continues for 3 hours. We performed several trials of FISH using metaphases and

only after the fourth round of reamplification were the probe quality considerably reduced.

The reamplification procedure based PCR, carried out in a Thermocycler and steps applied to each BAC DNA probe individually. The procedure started with 49µl master mix (Table 3) with 1µl BAC DNA probe 92°C for 4 minutes.

❖ Summary of Reamplification programme:

- Step 1: 92°C for 4 minutes
- Step 2: 92°C for 1 minutes
- Step 3: 56°C for 2:20 minutes
- Step 4: 70°C for 2 minutes
- Step 5: Repeating from step 2 to 5, for additional 25 cycles.
- Step 6: 70°C for 10 minutes
- Step 7: 4°C for ever

| <i>Master mix</i> | | <i>Stock solution</i> | <i>Final concentration</i> | <i>Amount of one approach (Vol 10µl)</i> |
|--------------------------|----------------------|------------------------------|-----------------------------------|---|
| | H ₂ O | | | 35,20 µl |
| | Replitherm. Puffer | 20x | 1x | 3 µl |
| | dNTPs | 2 mM | 0,2 mM | 5 µl |
| | Primer (DOP) | 50 | 2 | 2,00 µl |
| | MgCl ₂ | 25 mM | 2,5 µM | 5 µl |
| | Ampli Taq Polymerase | 5 U/µl | 0,03 U/µl | 0,3 µl |

Table 3: Required solution needed for reamplification procedure.

2. 2. 9. 5. Direct labelling of the BAC DNAs DOP-PCR products

For the simultaneous labelling of DOP-PCR products from BACs we prepared marking master mixes depending on the fluorochromes which were marked with UTPs (Table 4). As fluorochromes we used, Diethylaminocoumarin-5-dUTP (DEAC), Fluorescein-12-dUTP / Spectrum Green-dUTP, Spectrum Orange-dUTP, Texas Red-dUTP. 2 µl of template DNA added to fresh prepared 18µl

mastermix marked with the specific fluorochrome. The marked master mix contained 1x Replithempuffer, 2 µM DOP-Primer, 2.5 µM MgCl₂, 0.03 U/µl Ampli-Taq Polymerase. dNTP, dTTP and template concentration varied according to the fluorochrome. dTTP/ fluorochrome ratio by Texas Red-dUTP and Spectrum Orange-dUTP was 9:1 and by Spectrum Green was 2:1. Detailed pipetting table is below.

Procedure carried out in a Thermocycler, took around 4 hours and applied to each BAC DNA probe individually .The labelled DNA was used in probe preparation or stored at –20°C.

❖ The DOP amplified BAC DNA was labelled in a thermocycler as follows:

- Step 1: 92°C for 3 minutes to denaturate the DNA double strands
- Step 2: 92°C for 1 minutes to denaturate the DNA double strands
- Step 3: 56°C for 1 minutes to hybridize the DOP primer
- Step 4: Temperature increase of 0.1°C/second to 74°C
- Step 5: 70°C for 2 minutes to chain prolongation
- Step 6: Repeating from step 2 to 5, for additional 30 cycles.
- Step 7: 72°C for 5 minutes for final chain prolongation
- Step 8: 4°C for ever for cooling down for further processing

| | <i>Stock solution</i> | <i>Final concentration</i> | <i>Amount of one approach (Vol 20µl)</i> |
|-----------------------------|-----------------------|----------------------------|--|
| H ₂ O | | | 11,28 µl |
| Replithempuffer | 20x | 1x | 1 µl |
| Fluoro-lable-dNTP | 10x | 1x | 2 µl |
| dTTP | 1 mM | 0 mM | 0 µl |
| Spectrum orange-dUTP | 0,5 mM | 0,02 mM | 0,8 µl |
| DOP-Primer | 50 µM | 2µM | 0,8 µl |
| MgCl ₂ | 25 mM | 2,5 mM | 2 µl |
| Template | | | 2 µl |
| AmpliTaq Polymerase | 5 U/µl | 0,03 U/µl | 0,12 µl |

A) Marking the DOP-PCR products of BACs by Spectrum orange

| | Stock solution | Final concentration | Amount of one approach (Vol 20µl) |
|---|-----------------------|----------------------------|--|
| H ₂ O | | | 11,65 µl |
| Replithempuffer | 20x | 1x | 1 µl |
| Fluoro-lable-dNTP | 10x | 1x | 2 µl |
| dTTP | 1 mM | 0mM | 0 µl |
| Diethylaminocoumarin-5-dUTP/ DEAC-dUTP | 1 mM | 0,02 mM | 0,4 µl |
| DOP-Primer | 48 µM | 2µM | 0,833 µl |
| MgCl ₂ | 25 mM | 2,5 mM | 2 µl |
| Template | | | 2 µl |
| AmpliAq Polymerase | 5 U/µl | 0,03 U/µl | 0,12 µl |

B) Marking the DOP-PCR products of BACs by DEAC

| | Stock solution | Final concentration | Amount of one approach (Vol 20µl) |
|---|-----------------------|----------------------------|--|
| H ₂ O | | | 11,08 µl |
| Replithempuffer | 20x | 1x | 1 µl |
| Lable-dNTP | 10x | 0,5x | 1 µl |
| Fluorescein-12-dUTP / Spectrum Green-dUTP/FITC | 0,5 mM | 0,05 mM | 2 µl |
| dTTP | 1 mM | 0 mM | 0 µl |
| DOP-Primer | 50 µM | 2µM | 0,8 µl |
| MgCl ₂ | 25 mM | 2,5 mM | 2 µl |
| Template | | | 2 µl |
| AmpliAq Polymerase | 5 U/µl | 0,03 U/µl | 0,12 µl |

C) Marking the DOP-PCR products of BACs by Spectrum green

| | Stock solution | Final concentration | Amount of one approach (Vol 20µl) |
|--------------------------|-----------------------|----------------------------|--|
| H ₂ O | | | 11,25 µl |
| Replithempuffer | 20x | 1x | 1 µl |
| Fluoro-lable-dNTP | 10x | 1x | 2 µl |
| Texas Red-12-dUTP | 0,5 mM | 0,02 mM | 0,8 µl |
| dTTP | 1 mM | 0 mM | 0 µl |
| DOP-Primer | 48 µM | 2µM | 0,83 µl |
| MgCl ₂ | 25 mM | 2,5 mM | 2 µl |
| Template | | | 2 µl |
| AmpliAq Polymerase | 5 U/µl | 0,03 U/µl | 1,2 µl |

D) Marking the DOP-PCR products of BACs by Texas Red

Table 4: Table represented the required marking master mixes for each fluorochrome to mark the DOP-PCR products of BACs.

2. 2. 9. 6. Precipitation protocol with addition of Mouse CotDNA and Hybmix

After labelling, FISH probe with fluorochrome was not still ready to use. To reduce the background during evaluations, we added mouse CotDNA to each labelled probe in dark. The procedure applied to each BAC probe as follows:

- 20µl BAC probe mixed with 30µl Tris EDTA (TE). From this mixture, 10µl was taken and added to 10µl mouse CotDNA with 47µl 100% ice-cold ethanol. Labelled probe with mouse CotDNA vortexed properly and left for overnight at -20°C or 2 hours at -80°C.

After overnight or 2 hours incubation, the probe was centrifuged at 15,000xg for 30 minutes at 4°C and the supernatant was discarded. To be sure that of the rest alcohol was vanished, supernatant was dried in a speedvac for 10 minutes in dark. FISH probe was ready to use after adding 10µl Hybmix (2XSSC, 50% Formamid, 10% Dextranulfat, 1% Tween 20, pH 7,0), vortexing properly and leaving for 1 hour in room temperature to solve.

There by, combinatorial labelling involves the construction of a multiple-coloured probe set through mixing, in equal proportions, of differentially labelled chromosome.

On the basis of extensive control studies on touch preparation of mouse tissues from normal and translocated mice donors, the cut-off limit for detecting T(14;15)6Ca was set at 20%. The signals above the highest cut-off level calculated as the mean of false-positive cells.

2. 2. 10. FICTION Technique (Fluorescence Immunophenotyping and Interphase Cytogenetics as a Tool for Investigation Of Neoplasm)

Immunofluorescent detection of the indirect labelling of Cy5-Streptavidin with the 4-colour M-FISH paint set was undertaken in a single layer.

Therefore after IF application, ICM and TE slides were washed 3 times for 2 minutes with PBS and then briefly washed in distillate water. Before starting with denaturation procedure, slides were blowed to dry and M-FISH probe. Probe, which was generated from BACs, was denaturated for ~35 minutes by simple PCR protocol as described above.

Slides were denaturated at 72°C with denaturation solution in water-bath for 3 minutes and afterwards dehydrated by incubating in 70% (ice-cold), 90% and 100% ethanol for 3 minutes each, in room temperature to prevent annealing and left the slides for air-dry.

After denaturation protocols, for hybridization that ~1µl probe (depending on the marked area of cells) added on the slides for hybridization and covered with the coverslips, then the coverslip sealed with rubber cement fixogum and slides left in a moist chamber at hybridization temperature 37°C for overnight.

The next day the same washing protocol applied as applied to the other FISH marked slides. The rubber cements removed with the coverslips and the slides were washed in 0.5 x SSC at 72°C in waterbath for 5 minutes. Then the slides were washed twice in SSCT for 2 minutes, followed by 1 minute in PBS and briefly in aqua dest at room temperature.

Excess water removed from the slide by wiping with soft paper around the chromosome preparation and cells and dried completely. The cells were counterstained with DAPI (4'-6-Diamidino-2-phenylindole) and covered with coverslides.

The signals were then visualised by fluorescence microscope with the appropriate set of filters. The Cy5 signal was pseudo coloured red, directly fluorescent; FITC signal observed as green, DEAC signal observed as light blue, Spectrum Orange and Texas red signals observed as red.

2. 2. 11. ADAMTS gene expression

To perform RT-PCR (Real-Time PCR) for examining the ADAMTS gene expression, rRNA was isolated from mouse tissue and converted RNA to cDNA. Therefore, one translocated and one healthy mouse euthanized, their several organs excised; like lunge, heart, and kidney, used for the procedure. Best results were applied from lungs.

2. 2. 11. 1. RNA isolation from mouse tissue

For RNA isolation RNeasy mini kit was used and the required procedures were followed. Before starting with the protocol, 10 μ l β -Mercaptoethanol (β -ME) was added in 1ml Buffer RTL. That Buffer RTL was ready to use and to obtain working solution 4 volumes of 100% ethanol added to Buffer RPE.

The protocol continues as follows:

1. Only a portion of the tissue used, weighed and placed it into a suitably sized vessel for disruption and homogenization. Weighing tissue is the most accurate way to determine the amount and the tissue portions were not more than 30mg. 600 μ l Buffer RLT added to the vessel prior to disruption and homogenization.
2. The tissue was disrupted and homogenized with using a conventional rotor-stator homogenizer for 20-40 seconds to have uniformly homogeneous lysate.
3. Gained lysate centrifuged for 3 minutes at 14000 rpm at room temperature, the supernatant was removed by pipetting and transferred it to a new microcentrifuge tube.
4. 1 volume of 70% ethanol added to the cleared the lysate and mixed immediately by pipetting.
5. 700 μ l of the sample, including any precipitate that may have formed, transferred to a RNeasy spin column placed in a 2ml collection tube. The lid was gently closed, centrifuged for 15 seconds at 10000 rpm and the flow-through was discarded.

6. 350µl Buffer RW1 was added to RNeasy spin column, centrifuged for 15seconds at 10000rpm and the flow-through was discarded.
7. 10µl DNase stock solution was added to 70µl Buffer RDD and mixed by inverting the tube. This DNase incubation mix (80µl) was added to the RNeasy spin column membrane and incubated at room temperature for 15-30 minutes.
8. After incubation, step 6 was repeated and then 700µl Buffer RW1 was added to RNeasy spin column, centrifuged for 15seconds at 10000rpm to wash the spin column and the flow-through was discarded.
9. 500µl Buffer RPE was added to RNeasy spin column, centrifuged for 15seconds at 10000rpm to wash the spin column and the flow-through was discarded. This step repeated again but centrifuged for 2 minutes at 10000rpm.
10. The RNeasy spin column was placed into a new 2 ml collection tube and centrifuged for 1 minute at 14000rpm. The old collection tube with the flow-through was discarded.
11. The RNeasy spin column placed in a new 1,5 ml collection tube and 30µl RNase-free water was added directly to the membrane. It was centrifuged for 1 minute at 10000rpm to elute the RNA.
12. The RNA concentrations were measured, to know if the expected RNA yield was reached and stored at -20°C until the next application.

2. 2. 11. 2. cDNA synthesis from rRNA for use in RT-PCR

To convert RNA to cDNA, the QuantiTect Reverse Transcription Kit was used by following the required procedures. The protocol was optimized for use with 500ng of RNA. All the reactions set up on ice to minimize the risk of RNA degradation.

The protocol continues as follows:

1. The Template RNA was thawed on ice. gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-free water were also thawed but at room temperature (15-25°C).

2. DNA elimination reaction was prepared on ice according to components. These components were for translocated and normal mouse lunge, heart, an kidney with; 0,4µl template RNA + 2 µl Wipeout Buffer + 11,6 µl RNase-free water with a total volume of 14µl and for normal mouse lunge; 1,25 µl template RNA + 2 µl Wipeout Buffer + 10,75 µl RNase-free water with a total volume of each tissue 14µl.
3. They were incubated for 2 minutes at 42°C and then placed immediately on ice.
4. Reverse-transcription master mix prepared on ice by; 1µl Quantiscript Reverse Transcriptase, 4µl Quantiscript RT Buffer, 1µl RT Primer Mix and finally as template RNA, 14µl entire genomic DNA elimination reaction from step 2 was added.
5. Master Mix with the template RNA was incubated for 15 minutes at 42°C and afterwards incubated for 3 minutes at 95°C to inactivate Quantiscript Reverse Transcriptase.

2. 2. 11. 3. RT-PCR protocol

To know if the ADAMTS gene is expressed by normal and translocated mouse, we performed RT-PCR (Real-Time PCR), therefore isolated rRNA from mouse lunge, heart, kidney and converted rRNA to cDNA. Prior to RT-PCR procedure, master mixes prepared for each tissue which contained 12,5µl iQ SYBR green supermix + 2,5µl Mm-ADAMTS12-1-SG QuantiTect Primer Assay + 6µl MgCl₂ + 2,0µl H₂O and 2µl cDNA.

PCR protocol was as follows:

Cycle 1(1x): Step 1: 95.0°C for 03:00 minutes

Cycle 2 (40x): Step 1: 95.0°C for 00:30 minutes

Step 2: 55.0°C for 00:30 minutes

Step 3: 72.0°C for 00:30 minutes

Cycle 3 (1x) Step 1: 95.0°C for 00:30 minutes

Cycle 4 (1x): Step 1: 55.0°C for 01:00 minutes

Cycle 5 (80x): Step 1: 55.0°C for 00:10 minutes

Afterwards the products from RT-PCR brought to 1% agarose gel and checked for the bands.

3. RESULTS:

3.1. General results of experimental mice usage

For this project, in total 241 female mice were euthanized and 5 type of strains were examined which were B6D2F1, C3H/HEN, C57BL/6, BALB/c and CBA/CaHT(14;15)6Ca/J. These euthanized mice were used for several experiments to optimize the method and to have final results (Table 1).

| Type | Superovulation | FISH | Fibroblast cell culture | In vivo embryo gaining | Mating | | |
|----------------------|----------------|------|-------------------------|------------------------|-----------|---------------|-------------------|
| | | | | | TE biopsy | Immunosurgery | Stem cell culture |
| C3H/HEN | 12 | 4 | 0 | 5 | 3 | 0 | 0 |
| BALB/c | 15 | 10 | 0 | 8 | 3 | 0 | 0 |
| C57BL/6 | 31 | 11 | 2 | 10 | 4 | 0 | 0 |
| B6D2F1 | 0 | 0 | 0 | 7 | 25 | 38 | 42 |
| CBA/CaHT(14;15)6Ca/J | 0 | 0 | 5 | 1 | 0 | 2 | 8 |

Table 1: Distribution of euthanized mice due to the experiments performed which were applied to have the end protocols in the method and to have final results.

To establish the superovulation protocol and to gain morphologically good oocytes, 12 C3H/HEN, 15 BALB/c and 31 C57BL/6 in total 58 female mice were euthanized. 25 female mice, which were 4 C3H/HEN, 10 BALB/c, 11 C57BL/6, were used to improve the fixation, hybridization and denaturation parts of M-FISH with oocyte polar bodies. For testing and improving the M-FISH probe fibroblast cell cultures of 2 male C57BL/6, 3 male and 2 female CBA/CaHT(14;15)6Ca/J mice were used. After a steady superovulation protocol, we tried to find out the morphologically best embryo source, optimal mating and immunofluorescence protocols with 5 C3H/HEN, 8 BALB/c, 10 C57BL/6, 7 B6D2F1, 1

CBA/CaHT(14;15)6Ca/J, in total 31, female mice. To analyse embryoblasts and trophoblasts of an embryo, three techniques were tested to examine the best method for isolation of embryoblasts and trophoblasts separately. With the end method, we optimized our combined method FICTION and had the final results. The techniques with the number of used mice from each strain were respectively; TE biopsy with 3 C3H/HEN, 3 BALB/c, 4 C57BL/6, 25 B6D2F1 female mice with a total of 35, immunosurgery with 38 B6D2F1, 2 CBA/CaHT(14;15)6Ca/J female mice with a total of 40 and stem cell culture with 42 B6D2F1, 8 CBA/CaHT(14;15)6Ca/J female mice with a total of 50.

Our end protocol for which was obtained after the scarification of 89 female mice was:

- **Day -2** - Every tuesday between 11.30-13.30, intraperitoneal 7.5IU pregnant mare serum gonadotropin (PMSG) injection to each female mice obtained. The injected volume was always 0.15 ml and each week 1 – 3 female mice were injected.
- **Day 0** - Around 49 hours later, which is every thursday between 12.30-14.30, 5IU human chorionic gonadotropin (hCG) injected to each female mice. With this, we expected that ovulation occur between 11 and 14h after administration of hCG. The injected volume was always 0.10 ml. When our aim was to gain zygotes and embryos, females were caged singly with F1 hybrid stud males until euthanizing.
- **Day 1** - Every friday between 08.00 -12.00, we euthanized the females were by cervical dislocation to gain oocytes or zygotes in nonmated and mated groups respectively. In mated groups, plug control was also done on this day to check the fertilization.
- **Day 2** - When we wanted to observe 2 to 6 cell stage embryos from our mated groups, we separated the females from males and euthanized the females by cervical dislocation.
- **Day 3** - 8 cell to morula stage embryo were obtained on day 4 from mated groups. According to our plan mondays between 08.30 – 09.30, we euthanized the females by cervical dislocation and gained the embryos for further applications.

- **Day 4** - To gain blastocyst stage embryos, tuesdays between 08.30 – 09.30, we euthanized the females by cervical dislocation and males left a week rest. Late blastocyst stage embryos could be obtained afterwards, by killing the females in the afternoon (~12.00).

3. 1. 1. Establishing the superovulation protocol

To optimize the superovulation protocol 18 experiments performed with a total of 58 mice by which 12, 39 and 1 of them received 5 IU, 7.5 IU and 10 IU doses of PMSG, respectively, with a mean value of 6,76 IU, between 19:00 and 22:00 h on Day -2. Observed optimal PMSG dose was 7,5 IU and the best interval between PMSG and HCG injections necessary to obtain for consistent superovulation was approximately 48 hours with a mean value of 49,32 hours. Intervals of less or more than 48 hours, also resulted with a superovulation and not great differences observed. The other important factors like; age, strain of females, the dose and time of gonadotropin injections were playing a role. HCG gonadotropins were injected generally as 5 IU (mean value: 5,08 IU) on Day 0 and resulted with successfully induced superovulations (Table 2).

Three mouse strains were examined with an age interval of 5 -24 weeks (mean average of 13,46 weeks) and were euthanized on Day 1 with total number of 12 C3H/HEN, 15 BALB/c and 31 C57BL/6 mice respectively. The best interval between HCG injection and euthanizing of the mice were approximately 12 hours with a mean value of 12,15 hours.

From euthanized mice, in total 543 oocytes were gained and 302 of them had an ideal morphology with a regular round shape, clear cytoplasm and perivitellin space, normal zona pellucida thickness which was thinner than human oocyte zona pellucida and non-fragmented, big 1.polar body. Morphologically degenerated oocytes, total of 246, were excluded from the evaluations. (Figure1).

When we observed single mouse in each experiment, 5 – 8 weeks old C57BL/6 female mouse strain respond higher and better with oocyte morphology than

C3H/HEN and BALB/c, it was injected with 7.5 IU PMSG and 5 IU HCG gonadotropins with a time interval of 48h. The poorest response was obtained from C3H/HEN female strain when it got older even in the optimal conditions. Both gonadotropins worked best and had more, good quality oocytes-embryos at the following concentrations: 7.5 IU and 5 IU for PMSG and hCG respectively.

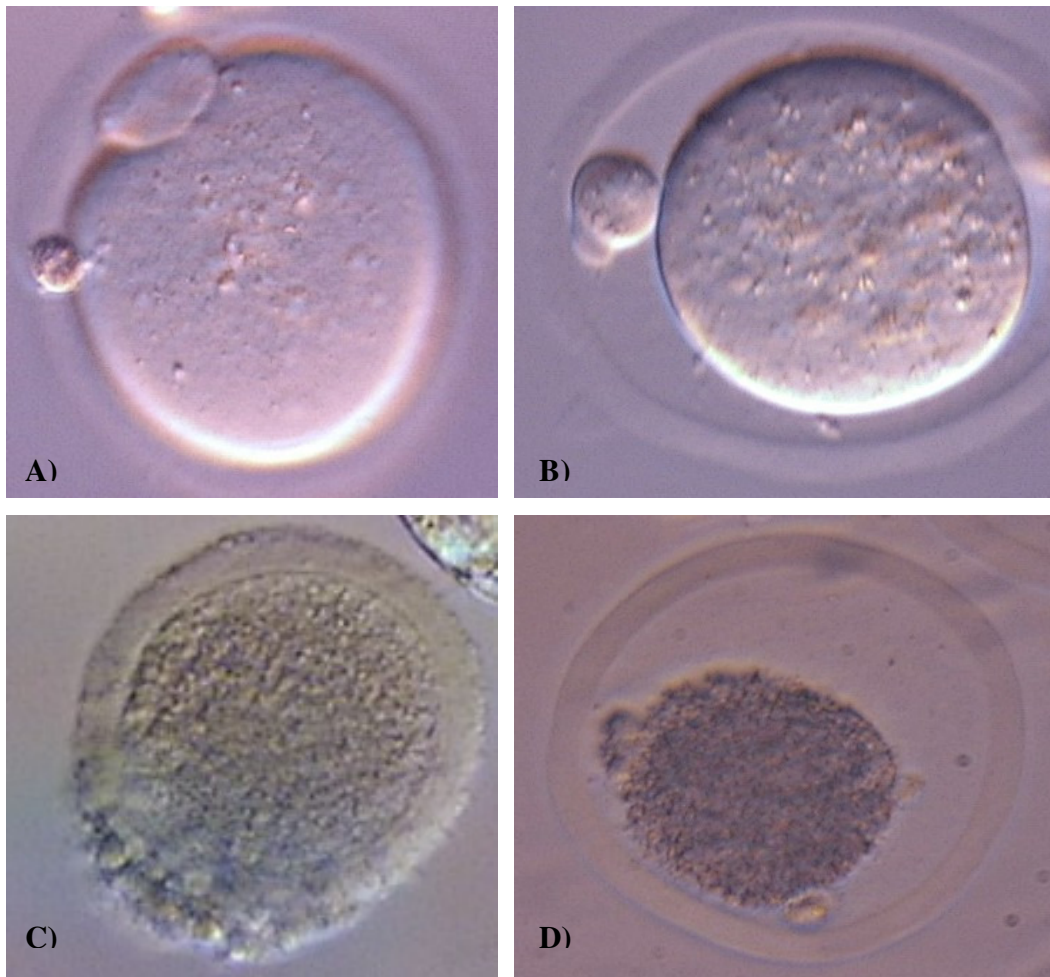


Figure 1: A) and B) Morphologically good mouse oocyte; regular round shape, clear cytoplasm and perivitellin space, non-fragmented, big 1.polar body. C) and D) Morphologically degenerated mouse oocytes; dark, fragmented cytoplasm, irregular perivitellin space, fragmented, very small or not existing 1.polar body (magnification 400x).

| Exp. | Σ of mice | Strain | Age of mouse (weeks) | Dose of PMSG (IU) | Time of PMSG injection | Time interval between PMSG and HCG injections | Dose of hCG (IU) | Time of hCG injection | Time interval between hCG injection and euthanizing | Time of euthanizing | Σ of gained oocyte | Σ regular oocyte | Σ degenerate oocyte |
|------|------------------|--------|----------------------|-------------------|------------------------|---|------------------|-----------------------|---|---------------------|---------------------------|-------------------------|----------------------------|
| 1 | 3 | e | 8 | 10- 5-7,5 | 20:00 | 48 | 5 | 20:00 | 13 | 09:00 | 31 | 26 | 0 |
| 2 | 3 | d | 9 | 5 - 7,5 | 20:00 | 48 | 5 | 20:00 | 13 | 09:00 | 20 | 20 | 0 |
| 3 | 3 | c | 12 | 5 - 7,5 | 19:00 | 48 | 5 | 19:00 | 13 | 08:00 | 28 | 18 | 10 |
| 4 | 3 | e | 12 | 5 - 7,5 | 19:00 | 48 | 5 | 19:00 | 13 | 08:00 | 52 | 40 | 12 |
| 5 | 3 | d | 13 | 5 - 7,5 | 19:00 | 69 | 5 | 15:45 | 16.30 | 09:00 | 17 | 17 | 0 |
| 6 | 3 | c | 14 | 5 - 7,5 | 21:30 | 48 | 5 | 21:30 | 15.30 | 13:00 | 0 | 0 | 0 |
| 7 | 4 | c | 12 | 5 - 7,5 | 21:00 | 48 | 5 | 21:00 | 12 | 09:00 | 45 | 45 | 0 |
| 8 | 4 | e | 14 | 5 - 7,5 | 19:15 | 48 | 5 | 19:15 | 12 | 08:00 | 47 | 44 | 3 |
| 9 | 2 | d | 16 | 7,5 | 20:00 | 48 | 5 | 20:00 | 12 | 08:00 | 27 | 10 | 17 |
| 10 | 3 | e | 6 | 5 - 7,5 | 19:00 | 48 | 5 | 19:00 | 13 | 08:00 | 43 | 18 | 25 |
| 11 | 1 | d | 5 | 5 | 20:00 | 48 | 5 | 20:00 | 12 | 08:00 | 43 | 6 | 37 |
| 11 | 3 | c | 5 | 7,5 | 20:00 | 48 | 5 | 20:00 | 12 | 08:00 | 0 | 0 | 0 |
| 11 | 1 | e | 7 | 5 | 20:00 | 48 | 5 | 20:00 | 13 | 08:00 | 0 | 0 | 0 |
| 12 | 2 | c | 7 | 7,5 | 20:00 | 48 | 5 | 20:00 | 12 | 08:00 | 8 | 0 | 8 |
| 13 | 2 | d | 12 | 7,5 | 20:00 | 48 | 7,5 | 20:00 | 12 | 08:00 | 23 | 5 | 18 |
| 14 | 1 | d | 13 | 7,5 | 21:00 | 47.30 | 5 | 20:30 | 12 | 08:00 | 2 | 1 | 1 |
| 15 | 2 | e | 9 | 7,5 | 19:00 | 48.30 | 5 | 19:30 | 11 | 08:00 | 24 | 14 | 10 |
| 16 | 5 | e | 20 | 5 - 7,5 | 21:20 | 49 | 5 | 22:00 | 12 | 10:00 | 59 | 15 | 44 |
| 17 | 5 | e | 21 | 5 - 7,5 | 20:00 | 50 | 5 | 22:00 | 11 | 09:00 | 43 | 4 | 39 |
| 18 | 5 | e | 24 | 7,5 | 22:00 | 47 | 5 | 21:00 | 12 | 09:00 | 31 | 19 | 12 |

Table 2: Table represents the applied experiments to optimize superovulation protocol. Female strains refers to; c: Balb/c, d: C3H/HEN and e: C57BL/6. Table represents; experiment number with number of euthanized female mice, strain and age (in weeks), time and doses of PMSG/HCG injections, time interval between PMSG-HCG injections and HCG injection- euthanizing in hours, time of euthanizing, number of total gained oocytes, number of regular /degenerated oocytes on euthanizing day.

3. 1. 2. FISH and Fibroblast Cell Culture from Mouse Skin

We developed a specific FISH probe for CBA/CaHT(14;15)6Ca/J translocated mice by assigning the mouse BACs to mouse banding landmarks, tested the FISH method on control and then applied on experimental groups. Therefore, mouse skin cells (fibroblasts) were cultured to test and to improve the specific FISH probe. Mouse oocytes were used to improve the FISH method.

To gain fibroblasts, common techniques were established generally from mouse blood. But for our aim, to make chromosome preparation for finding out our mice breaking point and generating special FISH probe, gained mouse blood was not enough. Because a mature mouse has in total 1.5ml blood in its body. Without scarification, from mouse cardiac, orbital sinus, tail vein or facial vein we could only gain 0.3ml blood and if the mouse was euthanized we could have 1 ml blood which was also not enough. With this reason, we established a technique which mouse fibroblast cells could be achieved from mouse abdominal skin and performed chromosome preparation.

The technique was not complicated and based on tissue-cell culture but had difficulties with culture environment because of hairy skin. We had to delete some of our groups concerning contamination but solved this problem later on by adding to our MEF medium Amphotericin B (2,5µg/ml).

4 C3H/HEN, 10 BALB/c, 11 C57BL/6 female mice were euthanized, a total of 25 mice, and gained 362 oocyte. From 362 oocytes 263 were degenerated, 355 oocytes had a good morphology, 277 oocyte left for overnight culture and polar body biopsy performed to 59 oocytes by carrying out a biopsy of mouse oocyte's 1.polar body. Results of 43 successful polar body biopsies were used to improve the fixation, hybridization and denaturation of the FISH method. The average age of euthanized female mice were 16 weeks and they received PMSG on Day -2 with a mean value of 6,76 IU. The time interval between PMSG – HCG injection and HCG - euthanizing averages were respectively 35 and 48 hours with mean value of 5 IU HCG.

For testing the generated FISH probe, cultures of homozygote normal, homozygote translocated and heterozygote control groups were used. Therefore

respectively, 2 C57BL/6, 5 CBA/CaHT(14;15)6Ca/J, 2 litters of CBA/CaHT(14;15)6Ca/J × C57BL/6 cross and 10 litters of B6D2F1 × CBA/CaHT(14;15)6Ca/J crosses were euthanized. Their skins were cultured to gain the fibroblasts for mouse chromosome preparations and regular metaphase band landmarks helped to test the generated FISH probe with clear results (Figure 2). Selecting after the contaminations of the cell cultures, high number of fibroblasts gained from 2 C57BL/6, 5 CBA/CaHT(14;15)6Ca/J, 2 litters of CBA/CaHT(14;15)6Ca/J × C57BL/6 cross and 10 litters of B6D2F1 × CBA/CaHT(14;15)6Ca/J crosses.

Multiple located signals observed by some BACs from 28 BACs on chromosomes 14 and 15; therefore, genetic markers from these regions were used to test again and generated FISH according to the results.

Using these metaphase spreads and generated FISH probe, signals were observed on two chromatids of each chromosome in an average of >98% chromosomes, with 50 metaphase landmarks.

Different fluorophores were also compared in the DOP amplified BAC clones including Spectrum Green, Spectrum Gold, Texas Red, DEAC, dioxygenin and biotin. FISH signals were obtained with all of them, but the BAC clones labelled with Spectrum Green, Spectrum Gold, Texas Red, DEAC provided the best hybridisation signals (Figure 2). The BAC clones labelled with dioxygenin and biotin had lower intensity of FISH signals compared to the other.

Four differently labelled BACs were hybridized and detected simultaneously on mouse metaphase spreads for chromosomes 14 and 15. By normal (standard) mouse fibroblast metaphase spreads, chromosomes (blue signal + green signal) 14 and (red signal + gold signal) 15 observed without any derivative products. Derivative products (red signal + blue signal = derivate chr.15 and green signal + gold signal= derivative chr.14) were seen alone by the chromosome preparation of homozygote translocated mouse fibroblasts. Metaphase spreads of heterozygote translocated mouse fibroblasts (translocated male mouse × normal female mouse) showed red signal + gold signal = chr.15, red signal + blue signal

= derivative chr.15, blue signal + green signal = chr.14, green signal + gold signal= derivative chr.14 (Figure 2).

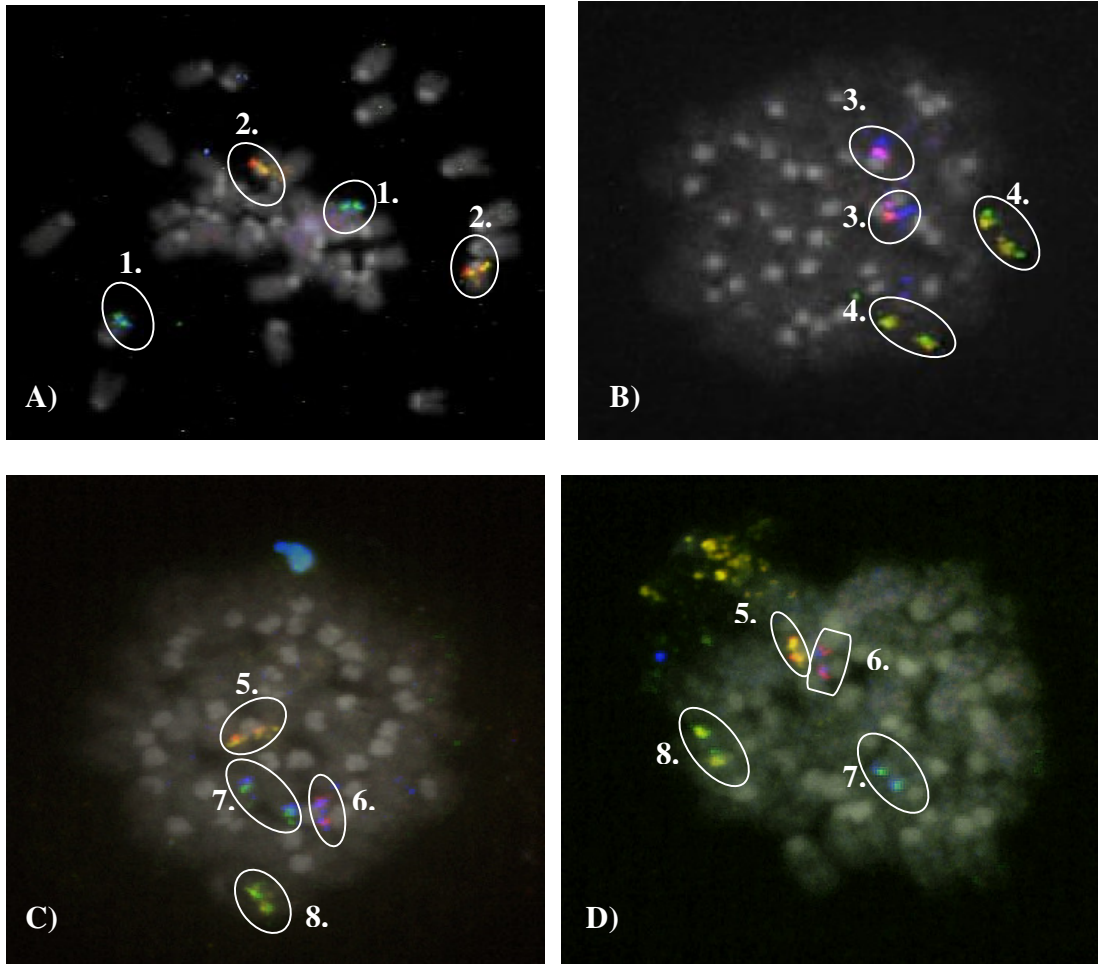


Figure 2: Dual-colour FISH mapping of BACs on chromosome preparations of mouse fibroblasts. Four differently labelled BACs were hybridized and detected simultaneously on mouse chromosomes 14 and 15. **A)** Normal (standard) mouse fibroblast metaphase spread, 1. Blue signal + green signal = chr.14, 2. Red signal + gold signal = chr.15 **B)** Translocated mouse fibroblast chromosome preparation, 3. Red signal + blue signal = derivative chr.15, 4. Green signal + gold signal= derivative chr.14. **C)** Fibroblast chromosome preparation of translocated female mouse x normal male mouse crossing kid - **D)** Metaphase spread of translocated male mouse x normal female mouse crossing kid, 5. Red signal + gold signal = chr.15, 6. Red signal + blue signal = derivative chr.15, 7. Blue signal + green signal = chr.14, 8. Green signal + gold signal= derivative chr.14 (magnification 600x).

3. 1. 3. Embryo gaining

To obtain morphologically best embryos and to optimize in vivo embryo gaining protocol, 31 superovulated females caged with males and performed 11 experiments. Euthanized female mice stem distribution was; 5 C3H/HEN, 8 BALB/c, 10 C57BL/6, 7 B6D2F1, 1 CBA/CaHT(14;15)6Ca/J. C57BL/6 males were used to breed with each female. Mean value of euthanized female and handled C57BL/6 male ages were respectively 9,6 and 15,18 weeks with an age interval between 4 – 23 weeks (Table 3). Embryos were collected from homozygous crosses and heterozygous CBA/CaHT(14;15)6Ca/J ×C57BL/6 cross and were for the imaging of further experiments.

We followed the same superovulation protocol that we optimized to gain oocytes, with an average PMSG dose of 7,5 IU and HCG dose of 5 IU. Until the 8.experiment, PMSG and HCG injections were performed generally in the late evenings between 21:00-22:00 with an interval mean value of 49,5 hours, respectively on Day -2 and 0. Through 8 – 11 experiments, PMSG and HCG injections were performed in the afternoons between 13:00-19:30 with the same interval value. In order to collect early morula and blastocyst stage embryos, mice were slaughtered at day 3 or 4, after HCG injection (day 0), with an average time interval of 2,9 days.

In total 174 embryos/oocytes gained with 62 oocytes, 46 degenerated embryos, 54 morula stages, 1 early blastocyst, 2 blastocyst stage embryos and 8 implanted embryos from euthanized mice. Degenerated and normal oocytes were excluded from the evaluations and 57 of the embryos could incubate overnight. From those overnight cultured embryos, 19 were degenerated, 5 were in morula stage and 33 were in blastocyst stage (Table 3). The percentage of embryos that reached the blastocyst stage after ~18 h in-vitro culture was 58% (33/57). 42% (22/57) of the embryos died, or developed with an abnormal growth pattern.

| Exp | Σ of mice | Strain | Age of female (weeks) | Age of male (weeks) | Time of PMSG inj. | Time of HCG inj. | Time interval between HCG inj. and euthanizing (day) | Time of euthanizing | Σ of gained oocytes 1.day | Σ of oocytes | Σ deg embryos | Morula | Early blastocysts | Blastocysts | Σ of cultured embryo (2.day) | Σ of deg embryos | Morula | Blastocysts |
|-----|------------------------|--------|-----------------------|---------------------|-------------------|------------------|--|---------------------|----------------------------------|---------------------|----------------------|--------|-------------------|-------------|-------------------------------------|-------------------------|--------|-------------|
| 1 | 5 | c | 4 | 4 | 22:00 | 21:00 | 2 | 09:00 | *x (8) | | | | | | 0 | | | |
| 2 | 5 | d | 4 | 4 | 21:30 | 21:00 | 2 | 09:10 | 14 | 9 | 5 | | | | 0 | | | |
| 3 | 1 | a | 4 | 4 | 22:00 | 21:00 | 3 | 08:30 | 3 | 1 | | | 1 | 1 | 2 | | | 2 |
| 3 | 2 | e | 17 | 19 | 22:00 | 21:00 | 3 | 08:40 | 3 | 1 | 2 | | | | 0 | | | |
| 4 | 1 | e | 5 | 20 | 22:00 | 21:00 | 3 | 09:20 | 7 | 2 | 3 | 2 | | | 2 | | | 2 |
| 4 | 2 | e | 5 | 20 | 22:00 | 21:00 | 4 | 09:30 | 11 | 4 | 5 | | | 1 | 1 | | | 1 |
| 5 | 2 | e | 21 | 21 | 22:20 | 21:10 | 3 | 09:30 | 4 | | 4 | | | | 0 | | | |
| 5 | 3 | c | 20 | 6 | 22:30 | 21:20 | 3 | 09:45 | 25 | 12 | 13 | | | | 0 | | | |
| 6 | 4 | a | 7 | 23 | 21:00 | 21:00 | 2 | 12:00 | 21 | 6 | 10 | 5 | | | 5 | | | 5 |
| 7 | 1 | x | 20 | 10 | 21:00 | 21:30 | 2 | 13:30 | 0 | | | | | | 0 | | | |
| 8 | 2 | e | 10 | 23 | 16:00 | 19:30 | 3 | 09:40 | (*z) 23 | 23 | | | | | 0 | | | |
| 9 | 1 | a | 4 | 12 | 13:00 | 14:00 | 3 | 13:00 | 20 | 1 | 2 | 17 | | | 17 | 9 | | 8 |
| 10 | 1 | e | 10 | 23 | 16:00 | 19:30 | 4 | 09:30 | 0 | | | | | | 0 | | | |
| 11 | 1 | a | 4 | 15 | 13:00 | 14:00 | 4 | 13:00 | 35 | 3 | 2 | 30 | | | 30 | 10 | | 20 |

Table 3: Applied experiments to optimize in vivo mating protocol with the numbers and morphology of embryos obtained from each breed at each age. Female strains refers to; a: B6D2F1, c: Balb/c, d: C3H/HEN, e: C57BL/6 and x: CBA/CaHT(14;15)6Ca/J. Table represents; the experiment number with number of euthanized female mice and strain, age of male and females in weeks, time of PMSG and HCG injections, time interval between HCG injection and euthanizing in days, time of euthanizing, number of gained embryos/oocytes on scarification day (1.day), number of oocytes on 1.day, number of degenerated embryos on 1.day, number of morula/early blastocyst/ blastocyst stage embryos on day 1 , number of overnight cultured/degenerated/morula/blastocyst stage embryos on day 2. *x: already implanted to the uterus. *z: Represents gained oocytes which were used to test immunofluorescence protocol for Oct4.

All superovulated females bred and ovulated normally. As the results are summarised in Table 3, by the first experiment a false negative vaginal plug was observed, which resulted in unexpected 8 implanted embryos. Vaginal plugs were controlled on the following experiments but were not taken as a fertilization criterion. 80% (25/31) of the mice were mated and the proportion of embryos from mated mice was not influenced by the doses of exogenous gonadotropins, rather then influenced by the different injection times. The percentage of embryos in morula stage and reaching the blastocyst stage was significantly higher in the

last 2 groups (85% (20/17), 86% (35/30) which were injected in the afternoons. The late evening PMSG and HCG injections had an overall negative influence on mating procedure and were not a preferable choice to gain morphologically optimal embryos as functioned before for the oocytes. We concluded by the last experiments that PMSG and HCG injections should be performed in the afternoons on the further experiments in mating. The main reason for injecting 2 females every week and having two groups with separate pairings was that every mouse did not always respond as we wished and had none or fewer embryos, oocytes then normal.

With the general observations of gained embryos, optimal mouse blastocyst consists of an inner cell mass (ICM) enclosed by the trophoctoderm (Figure 3). The origin of these two cell populations lies in the segregation of inner and outer cells in the early morula which means in mouse embryos compaction occurred in the 8-cell, in the transition from 4 to 8 cells stage. It is known that blastomeres decompact temporarily during mitosis in the mouse (Skrzecz and Karasiewicz, 1987). Eightyfive percent (47/55) of embryos appeared morphologically normal at the time of recovery and there was a clear progression by the overnight cultures when we stimulated the B6D2F1 female strain in the afternoons. Detailed observations revealed slight differences in the distribution of embryo gaining by the other strains, but morphologically optimal and higher number of embryos recovered from B6D2F1 female strain.

There were few important points that we have noticed with their mating performance. Sexual maturity that lies between 6 – 8 weeks of age is depending to the strain, individual caging before mating and a sufficient rest between plugging must be adjust.

The size of the ICM was the most important criterion of blastocyst quality. When the ICM extended to at least half the diameter of the embryo, the blastocyst was graded the highest quality that were generally obtained from B6D2F1 female strain.

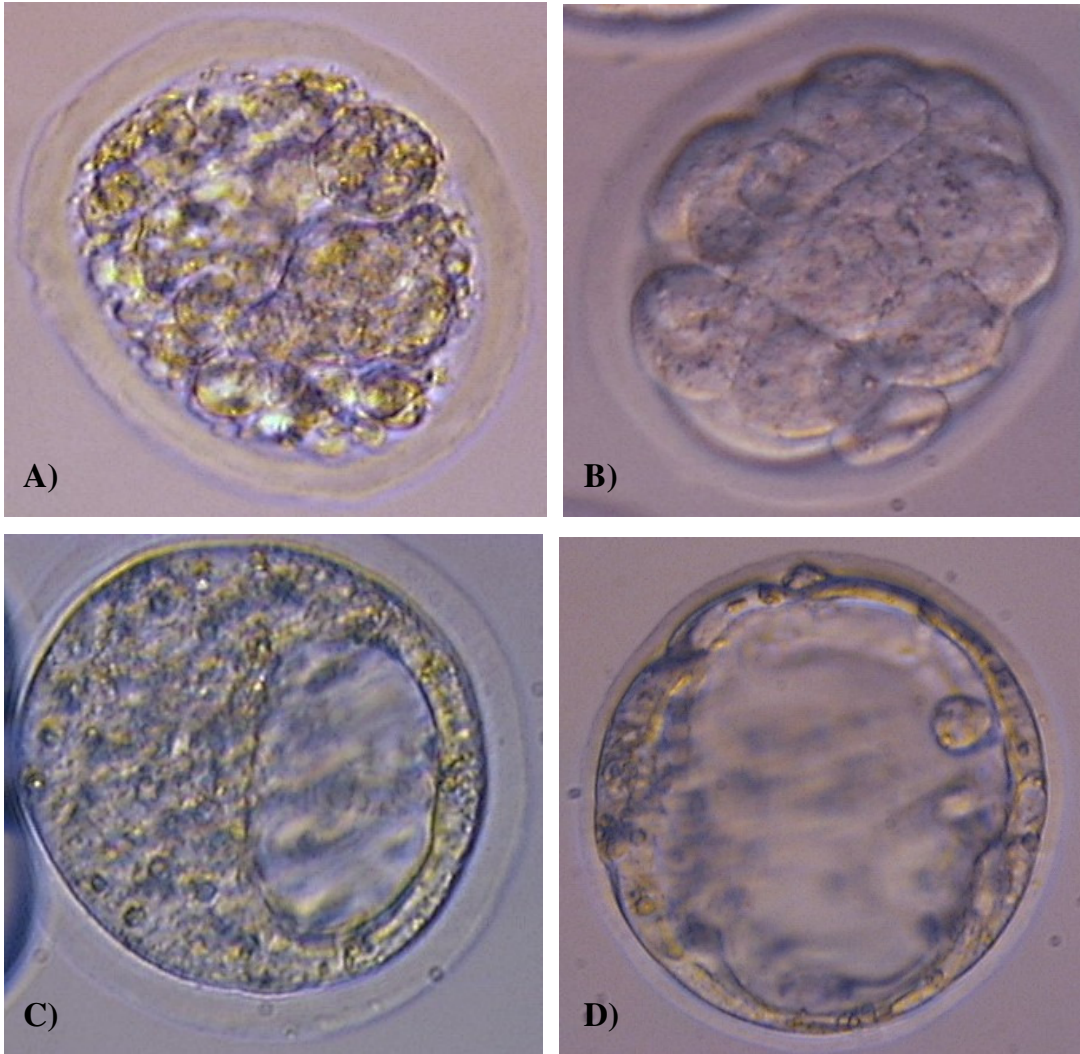


Figure 3: Morphological and embryological classifications in mating process. A - Degenerated and discarded embryo, B – Morula stage embryo, C - Early blastocyst stage embryo, D – Blastocyst (magnification 400x).

Also to find out if culture medium (M2, Menezotte and M6) was one of the primary factors in the overnight developments, embryos in each experiment group were cultured with different culture media and no difference was observed. Encountered retarded embryos, which were not included in the analysis, appeared in only one case (6. experiment) with 5 morula stage embryos.

As Jackson laboratories informed us with low fertility rate of CBA/CaHT(14;15)6Ca/J female mice, we had even no oocytes from this type which can be because of its high age. 23 gained oocytes in 8.experiment were

used by optimizing and testing immunofluorescence protocol with Oct4 / Cdx antibody.

3. 1. 4. Mating

Different methods for isolating individual trophoblasts (TE) and embryoblasts (ICM) from mouse blastocysts were performed and evaluated. As we described before, these methods were; trophoectoderm biopsy technique, immunosurgery and isolation by the help of stem cell culture. Our aim was to have our final results, with best method for the removal of greater numbers of cells which can also allow for repeat analysis, storage of samples for later reanalysis and for clear FISH results.

Therefore we performed 72 experiments, euthanized 125 superovulated female mice, fixed our mating protocol with 7.5 IU PMSG and 5 IU HCG gonadotropins injections between 12:00 – 17:00 which had a time interval of 48,5 hr.

All isolation procedures had a negative impact on the growth patterns of the isolated ICM and TE cells. Different abnormalities could be observed at the blastocyst stage during genetic analysis, including embryos lacking visible abnormalities.

For TE biopsy, immunosurgery and embryonic stem cell (ESC) culture respectively 35, 40, 50 superovulated female mice were euthanized and 14 experiments for TE biopsy, 25 for immunosurgery and 26 for embryonic stem cell experiments performed.

3. 1. 4. 1. Trophoblast Biopsy

In this part 14 experiments were performed with the aim of testing trophoectoderm microbiopsy technique for removal of TE from mouse blastocyst and the possibility of testing genetically biopsied TE with remaining ICM.

25 B6D2F1, 3 BalbC, 3 C3HHEN and 4 C57BL6 with a total of 35 female mice, which had an average of 6 weeks age, superovulated and mated with 22 C57BL/6, 9 BalbCXC3H/HEN males (median age 21 weeks). Females were then

ethanized on 4, 5 and 6. day of pregnancy and total of 353 embryos flushed from the uterine horns. To sum up 353 flushed embryos; 17, 20, 47, 159, 1 and 16 of them were respectively in 2 cell stage, 3 - 6 cell stage, 8 cell stage, morula, early blastocyst and blastocyst stage. 69 non-fertilized oocytes and 24 degenerated embryos were discarded that 258 (73%) embryos could be cultured overnight in the incubator. By the next day, 151 (59%) of the embryos were available for zona slitting. 29 were degenerated, 43 morula and 20 3 – 6 cell stage embryos were discarded (Table 4).

| Embryos | Morphological stages | Σ | % |
|-----------------------------|-----------------------------|----------|------------|
| Total gained embryos | | 353 | 100 |
| | Oocyte | 69 | 19% |
| | Degenerated | 24 | 7% |
| | 2 cell stage | 17 | 5% |
| | 3 - 6 cell stage | 20 | 6% |
| | 8 cell stage | 47 | 13% |
| | Morula | 159 | 45% |
| | Early blastocyt | 1 | ~1% |
| | Blastocyt | 16 | 4% |
| Overnight cultivated | | 258 | 73% |
| | Degenerated | 29 | 11% |
| | 3-6 cell stage | 20 | 8% |
| | Morula | 43 | 17% |
| | Early blastocyt | 29 | 11% |
| | Blastocyt | 103 | 40% |
| | Hatched blastocyt | 34 | 13% |
| | Available for biopsy | 151 | 59% |

Table 4: Table represents the numbers and percentages of the flushed embryos with overnight cultured embryos for TE biopsy procedure.

As described before, embryos that had formed on day 4, 5 and 6 had different stages. Nevertheless, each day form of the embryos was tested and the best stage for TE biopsy was selected. The stage of development at which slitting was performed was important for successful herniation to occur with minimal injury to the trophoectoderm. Mice, which were euthanized on day 3 of pregnancy (72 - 74 h post-hCG administration), had 80% morula and 8 cell stage embryos which

were optimal for zona slitting after overnight in vitro incubation. So the next day blastocyst or early stage blastocyst with distinct inner cell mass and trophoectoderm lining the cavity could be selected for manipulation.

Biopsy technique was performed by slit/excision and laser methods, but mainly focused on mechanical (slit) biopsy because of the better results. The size of the slit and the stage of embryonic development at which slitting was performed were important for successful herniation to occur and biopsy to perform.

Therefore, in the first set of experiments the zona pellucida of 59 embryos formed on day 4, 21 embryos were slitted and 38 embryos were destroyed by laser. Not in all cases, biopsy of the herniating cells was performed and slitting was possible. Making slit that should theoretically, written in different publications, took less than a minute for us at least 4 -8 minutes proceeded. One fact for that was, having the right inner diameter of the holding pipette, otherwise procedure took longer, embryo revolves during slitting and it can harm the inner cell mass or trophoectoderm cells. Therefore, the right holding-pipettes were determined to have an outside diameter of 120-150 μ m and inner diameter of 25 μ m with 35⁰ angled. With the further experiments, there were no problems with embryo revolving during slitting and biopsy but it did not significantly increase the biopsy results in the second set of experiments.

6,5 – 5,1 ms pulse laser was used to removed the zona away and made a hole, but pulse could not be optimized. The size of the holes, which was an important fact, made in the zona pellucida with laser was big that the blastocysts were hatched totally before the biopsy or was really small that no herniation was observed. With these trails, we had a 2% success (1/38) laser technique, technique was totally skipped.

In the second set of experiments the zona of 92 blastocysts at day 4 were slit and the next day (after 18 - 24 h after zona slitting) biopsy of the herniating trophoectoderm cells was attempted. Thirityfive embryo (38%) showed normal herniation of trophoectoderm through the slit and 14 (15%) blastocysts were successfully biopsied (Figure 4). Approximately clumps of 3 - 8 trophoectoderm cells were removed and the amount of trophoectoderm biopsied could be varied

depending on the time interval between slitting the zona and biopsying the herniation. Yielded cells were available for genetic analysis after fixing them on the slide.

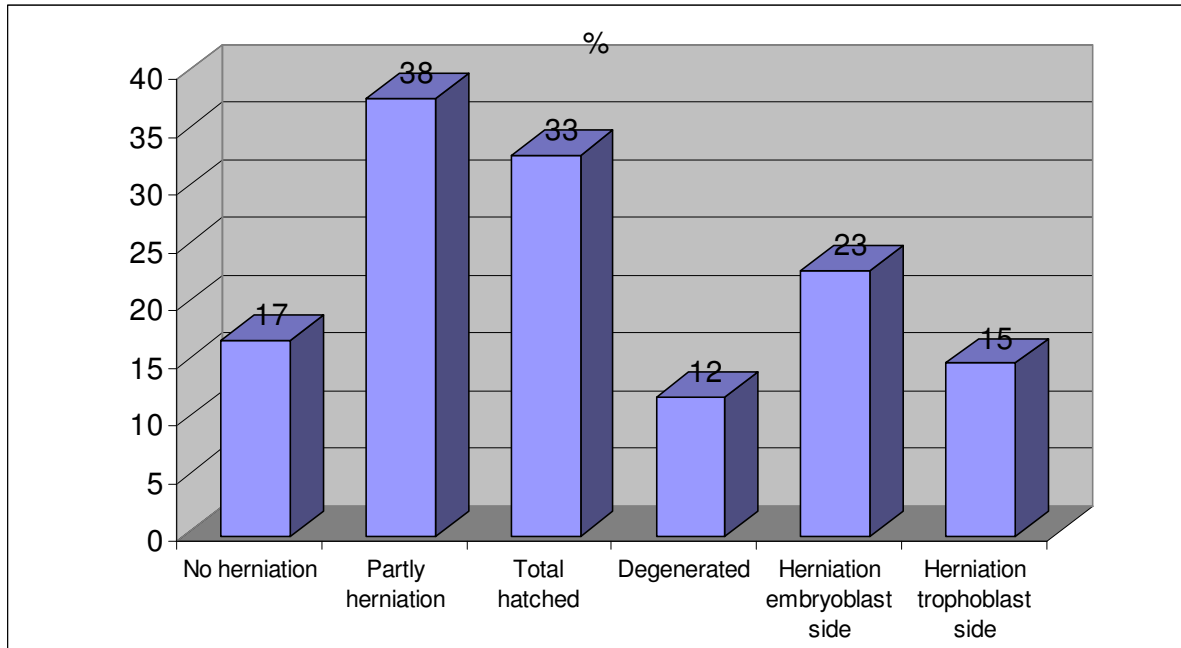


Figure 4: Overnight cultured embryos phases after zona slitting with their percentages.

Ratio of the biopsy was low because of several factors: Operative cellular injury and postoperative embryonic viability was assessed by blastocoele reexpansion, hatching inner cell mass development, trophoectoderm plating. After zona slitting in 19 blastocysts (21%) blastocoele cavity collapsed (Figure 4) and the next day 11 (12%) blastocysts were degenerated, failed to re-expand on further incubation due to the technical errors after zona slitting and during biopsy procedure. In 16 (17%) blastocyst no herniation or a very small herniation occurred, 30 (33%) hatched out of the zona completely before the biopsy was performed (Figure 4 - 5). From 21 (60%) of the herniated blastocysts a part of the inner cell mass herniated together with the trophoectoderm or just a part of inner cell mass herniated (Figure 5). Normally the location of the slit opposite to the inner cell mass allowed the controlled herniation of only trophoectoderm cells as the blastocyst developed in vitro which were in our cases only 14 blastocysts (40%).

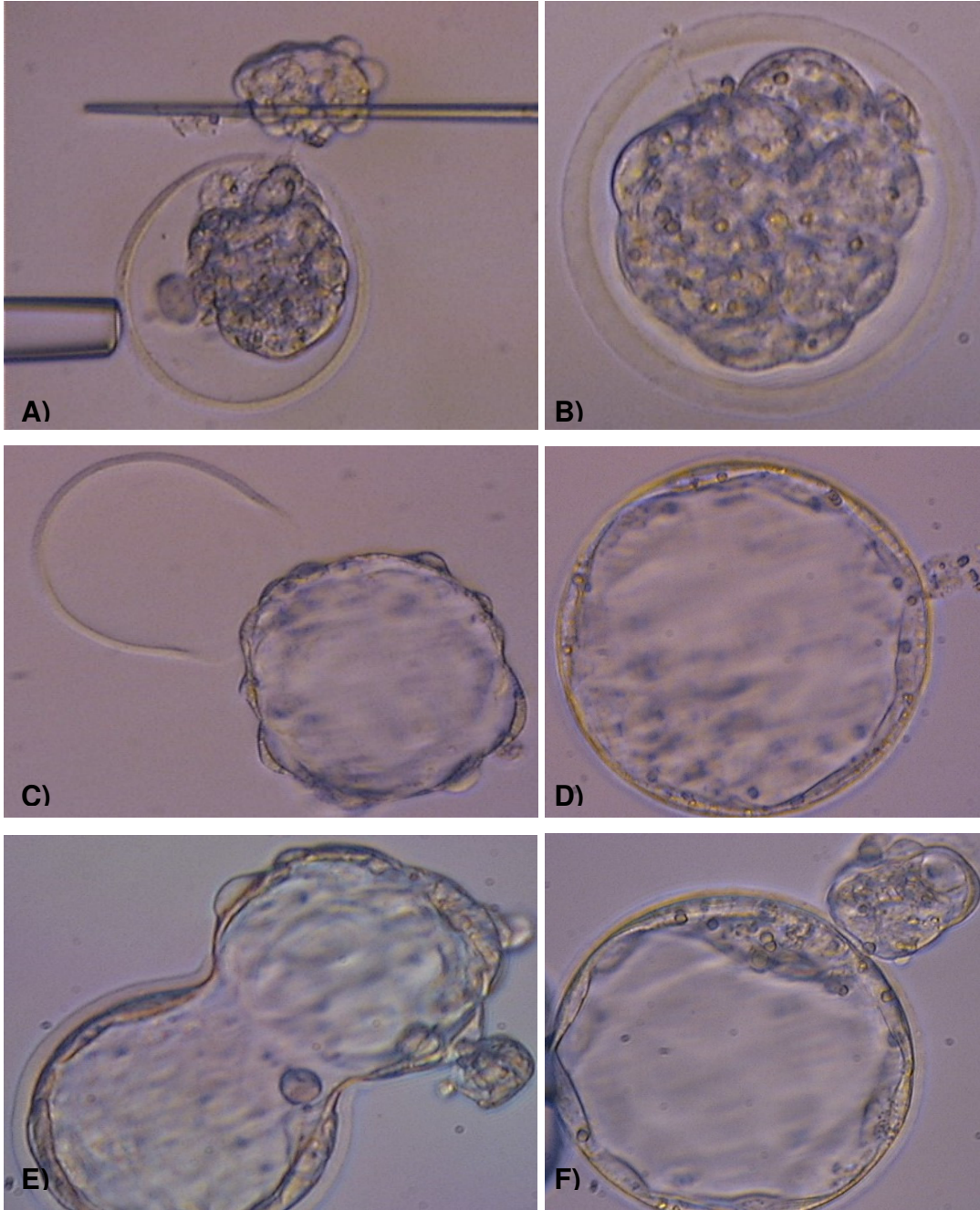


Figure 5: TE biopsy procedure failures: **A) – B)** After biopsy collapsed blastocoelic cavity and degenerated ICM, **C) – D)** Hatched out blastocyst before the biopsy was performed and a very small herniation. **E) – F)** The inner cell mass herniated together with the part of trophoblast and just the inner cell mass herniated (magnification 400x).

To conclude, TE biopsy was not an optimal technique for isolating high numbers of TE and ICM cells as also discussed in discussion.

3. 1. 4. 2. Immunosurgery

To obtain higher numbers of ICM and trophoctoderm cells to make their genetic analysis immunosurgery procedure was also performed. For this, nuclei of inner and outer cells were labelled different with two fluorochromes after partial complement-mediated lysis of the outer cells. Procedure principle was to destroy more or less the cells of trophoctoderm by brief exposure to antibodies directed against the mouse trophoctoderm antigens with mediating complement lysis and thereby isolating ICM.

In total 25 experiments were performed and 2 CBA/CaHT(14;15)6Ca/JXCBA/CaHT(14;15)6Ca/J, 35 B6D2F1, 5 B6D2F1XC57BLC3H/HEN, 6 CBA/CaHT(14;15)6Ca/J superovulated female (total:48) caged with 18 C57BL/6, 17 CBA/CaHT(14;15)6Ca/J, 2 BalbC X C3H/HEN males. Mean age of euthanized female and handled male ages were respectively 10 and 18 weeks with an age interval between 5 – 35 weeks. Females were euthanized on day 3 or 4 of pregnancy and 535 embryos were gained.

When we had a blink at the flushed embryos; 10 were 2 cell stage, 3 were 3 - 6 cell stage, 8 were 8 cell stage, 268 were morula, 40 were early blastocyst and 53 blastocyst stage embryos. 40 non fertilized oocytes and 113 degenerated embryos were excluded so that 361 (67,5%) embryos were cultured overnight. From overnight cultured embryos, 50 were degenerated, 3 were 3 - 6 cell stage and 106 were morula stages which were discarded. The remaining embryos that reached to early blastocyst, blastocyst and hatched blastocyst stage were in total 198 (55%), respectively 8, 185, and 9 embryos (Table 5).

| Embryos | Morphological stages | Σ | % |
|-----------------------------|-----------------------------|------------|--------------|
| Total gained embryo | | 535 | 100 |
| | Oocyte | 40 | 7,5% |
| | Degenerated | 113 | 21% |
| | 2 cell stage | 10 | 2% |
| | 3 - 6 cell stage | 3 | 0,56% |
| | 8 cell stage | 8 | 1,49% |
| | Morula | 268 | 50% |
| | Early blastocyt | 40 | 7,5% |
| | Blastocyt | 53 | 10% |
| Overnight cultivated | | 361 | 67,5% |
| | Degenerated | 50 | 14% |
| | 3-6 cell stage | 3 | 1% |
| | Morula | 106 | 29% |
| | Early blastocyt | 8 | 2% |
| | Blastocyt | 185 | 51% |
| | Hatched blastocyt | 9 | 3% |
| | Immunosurgery performed | 198 | 55% |

Table 5: Numbers and percentages of the flushed embryos with overnight cultured embryos for immunosurgery procedure showed in the table.

At the end of cultivation each group from expanded and hatched blastocysts were randomly selected, and 198 blastocysts treated with AT for ZP removal. To avoid damage during the achievement of complete removal of the zona pellucida to the ICM and the underlying trophectodermal cell layer, exposure to the AT (pH of 2.5–3) was carefully optimized. It was optimized by incubation of increasing times of trophectoderm and ICM during monitoring the morphology of the zona pellucida by visual microscopical inspection. The optimal incubation time was found to be in the range of 5 - 10 minutes. The ZP was completely digested in 189 blastocysts, and immunosurgery was performed on 198 blastocysts. It was more difficult to remove ZP in translocated mouse embryos, so AT treatment was resulted better for 20 - 25 minutes. Already ZP-free (hatched) or had the ZP removed entirely prior to exposure to antibody had no recognizable difference by their embryo morphology. Twenty intact blastocysts served as controls and immunosurgery was successful in all cases (Figure 6-1).

Embryos exposed simultaneously to antiserum for 10 minutes and complement for 5 minutes, displayed different distributions of dead cells depending on the dilution of antiserum. For differential staining of blastocysts; 14, 43, 102, 22, 17 embryos were respectively exposed to antiserum dilutions of 1:5, 1:10, 1:25, 1:50 and 1:100. The 1:25 (52% of embryos) dilution of antiserum was found to be optimal that the cells of the inner cell mass were intact and had clearly more TE cells in blastocysts to examine. When the embryos were exposed to antiserum, washed and subsequently were exposed to complement, all of the trophoblastic cells were destroyed in dilutions of antiserum up to 1:100. It was not possible to observe ICM cells because of too much TE cells in 1:5 dilution of antiserum.

To further check the permeability of mouse blastocysts for antiserum, several groups of blastocysts were exposed to antiserum at 1:25 dilution for 5, 10, 15, and 30 minutes and then were transferred them to complement for 5 min. Trophoblastic cells, in all cases, were there and the inner cell masses also maintained intact. However, the best time interval was for 10 minutes antiserum exposition with the highest number of TE.

Nevertheless, optimizing dilution and time interval of antiserum exposition did not always gave the expected results. In several experiments, even in the same groups we had different numbers of the ICM / TE cells by observing different labelling (Figure 6-12) and 52% of immunosurgery performed embryos used to solve this problem. In addition, inner cell masses looked like small homogeneous cell clumps, which did not let us to analyse the genetic stages of nucleuses clearly.

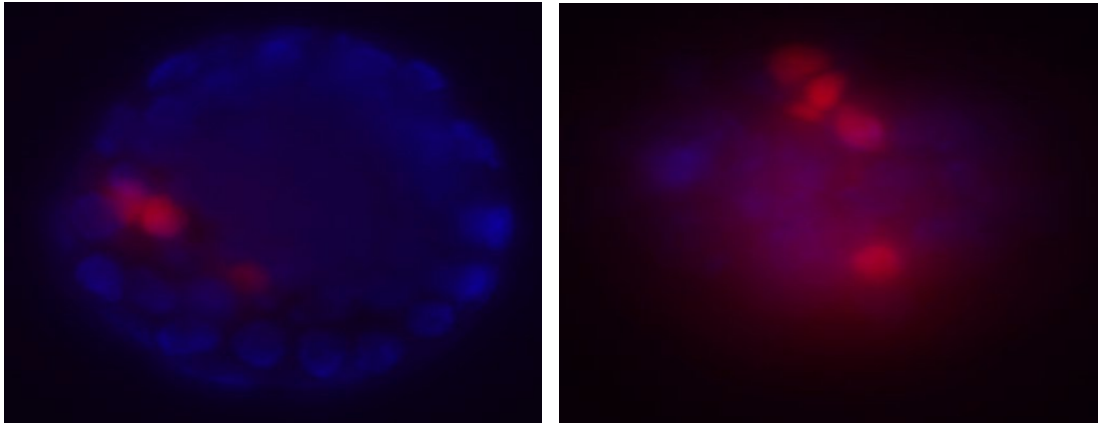


Figure 6 -1: Fluorescence micrographs of blastocysts after completion of differential labelling: Blastocysts undergoing partial lysis of the outer TE nuclei appear red due to labelling with propidium iodide, ICM nuclei labelled with bisbenzimidazole appear blue A) 3 TE cells nuclei observed in red colour, ICM nuclei labelled blue. B) 5 TE cells nuclei in red, 2 ICM nuclei labelled blue (magnification 600x).

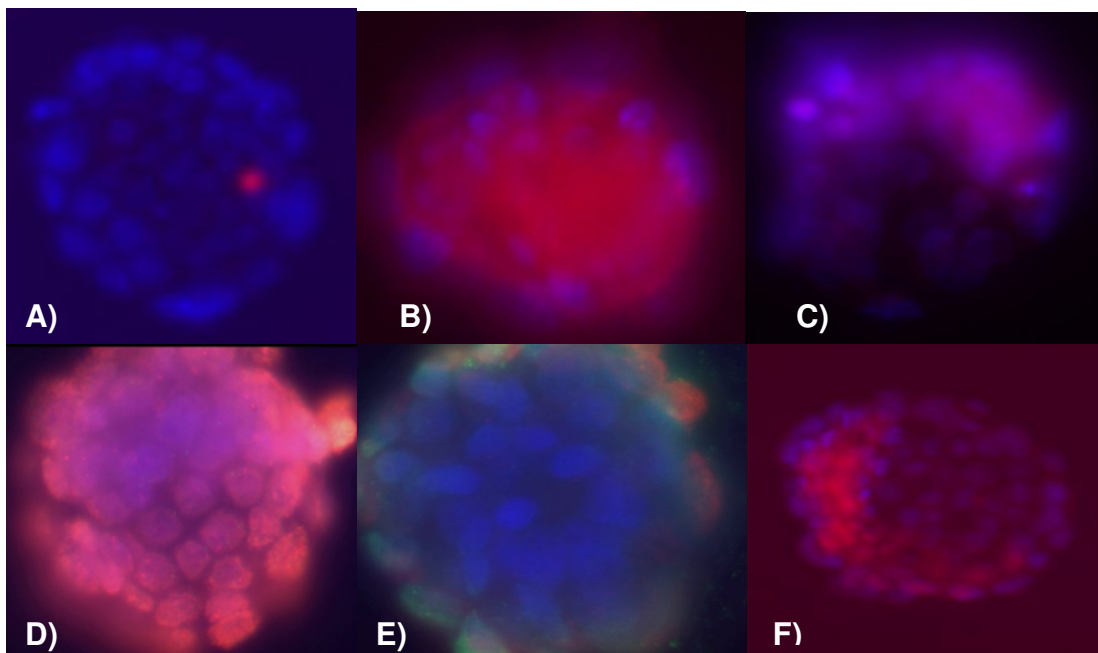


Figure 6 -2: Unspecific differential ICM / trophoctoderm staining examples; inner cells can be recognized by the blue fluorescence of the Hoechst staining and outer cells by a red to pink fluorescence due to accumulated propidium iodide. **A)** 1TE cell nuclei observed in red colour which means the TE cells were destroyed, other all of the nucleuses ICM. **B)** TE cells nucleus in red covered ICM nucleus in blue, both nucleuses were not clear to distinguish the borders. **C) – E)** ICM nucleus were in blue, no clear TE nucleus red to observe. **D)** TE nucleus were in red, no clear ICM nuclei to observe. **F)** Unclear number of TE nucleus were in red with ICM nucleuses (magnification 600x).

As a conclusion, immunosurgery also was not an optimal technique for individual analysis of TE and ICM cells that was discussed later.

3. 1. 4. 3. Embryonic stem cell culture

Animals carry stem cells throughout their entire life, from embryogenesis to senescence. Embryonic stem cells provide a model to study early human embryology, an investigational tool for discovery of novel growth factors and medicines, and a potential source of cells for use in transplantation therapy. Our experimental group was developed by the embryonic stem cell culture and 26 experiments were performed which contained different parts.

5 mouse strains were used with a total of 55 mice. 35 B6D2F1, 3 B6D2F1xCBA/CaHT(14;15)6Ca/J , 1 CBA/CaHT(14;15)6Ca/JxC57BL6, 6 CBA/CaHT(14;15)6Ca/J and 10 B6D2F1xC57BL6 superovulated females (7,5 IU PMSG and 5 IU HCG) had an age interval of 6 -23 weeks with an average of 12 weeks. They were mated with CBA/CaHT(14;15)6Ca/J and C57BL6 male strains (median age 19 weeks). On the 4th day of pregnancy, females were euthanized and 705 embryos were flushed from the uterine horns. Six hundred sixty-seven (95%) embryos were cultured overnight and 33 degenerated embryos were discarded.

When the stages of embryos were summarized on the day of scarification; 7 were in 2 cell stage, 28 were in 3 - 6 cell stage, 221 were morula stage, 136 were early blastocyst, 263 were blastocyst and 17 were already hatched (Table 5). After the overnight culture of 667 embryos, 48 were degenerated, 6 were in 3 – 6 cell stage and 75 were in morula stage, which means that 129 (19%) of the embryos were discarded on the 2nd day. For embryonic stem cell culture 519 (78%) embryos were available from 5 early blastocysts, 432 blastocysts and 101 hatched blastocysts (Table 5).

| | <i>Flushed embryos</i> | | <i>Overnight cultivated embryos</i> | |
|---------------------------|------------------------|-----|-------------------------------------|-----|
| | Σ | % | Σ | % |
| Total | 705 | 100 | 667 | 100 |
| Degenerated | 33 | 5 | 48 | 7 |
| 2 cell stage | 7 | 1 | 0 | 0 |
| 3-6 cell stage | 28 | 4 | 6 | 1 |
| Morula | 221 | 31 | 75 | 11 |
| Early Blastocyst | 136 | 19 | 5 | 1 |
| Blastocyst | 263 | 37 | 432 | 65 |
| Hatched Blastocyst | 17 | 3 | 101 | 15 |

Table 5: Number and percentage of the flushed embryos with overnight cultured embryos for embryonic stem cell culture procedure showed in the table.

Fourhundred sixtytwo (89%) embryonic cell cultures arising from 519 embryos were developed in culture and produced healthy ICM lumps which could be successfully separated within 2 - 3 days. Eleven percent of the blastocysts were lost before transferring to embryonic stem cell culture because of Pronase (TM) treatment that was needed for vanishing the zona pellucida. Blastocysts had a high degeneration risk to wait in Pronase (TM) solution even in few seconds more when they were zona-free already. Blastocysts were observed by stereomicroscope if their zona pellucida was dissolved during the procedure, which was sometimes not clearly to observe (Figure 7).

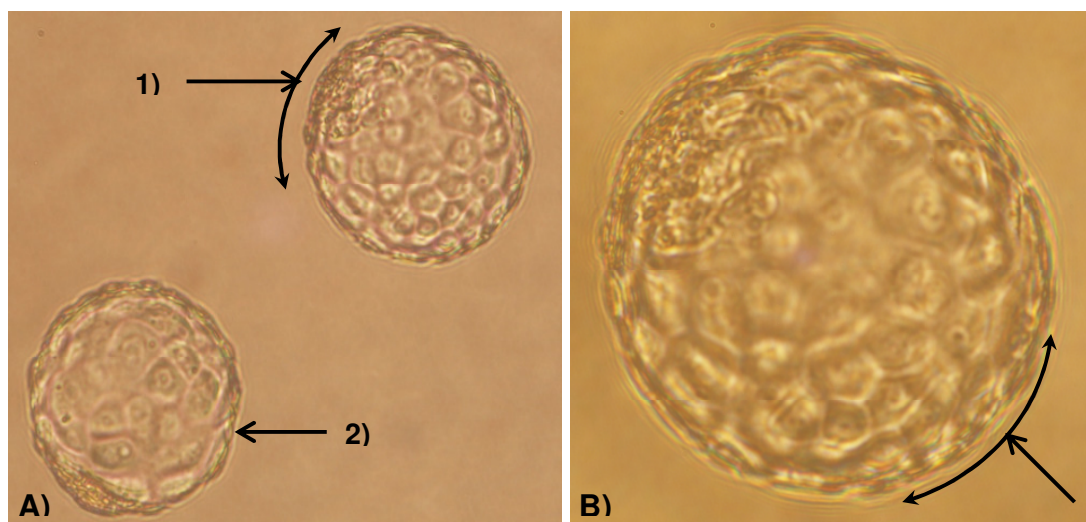
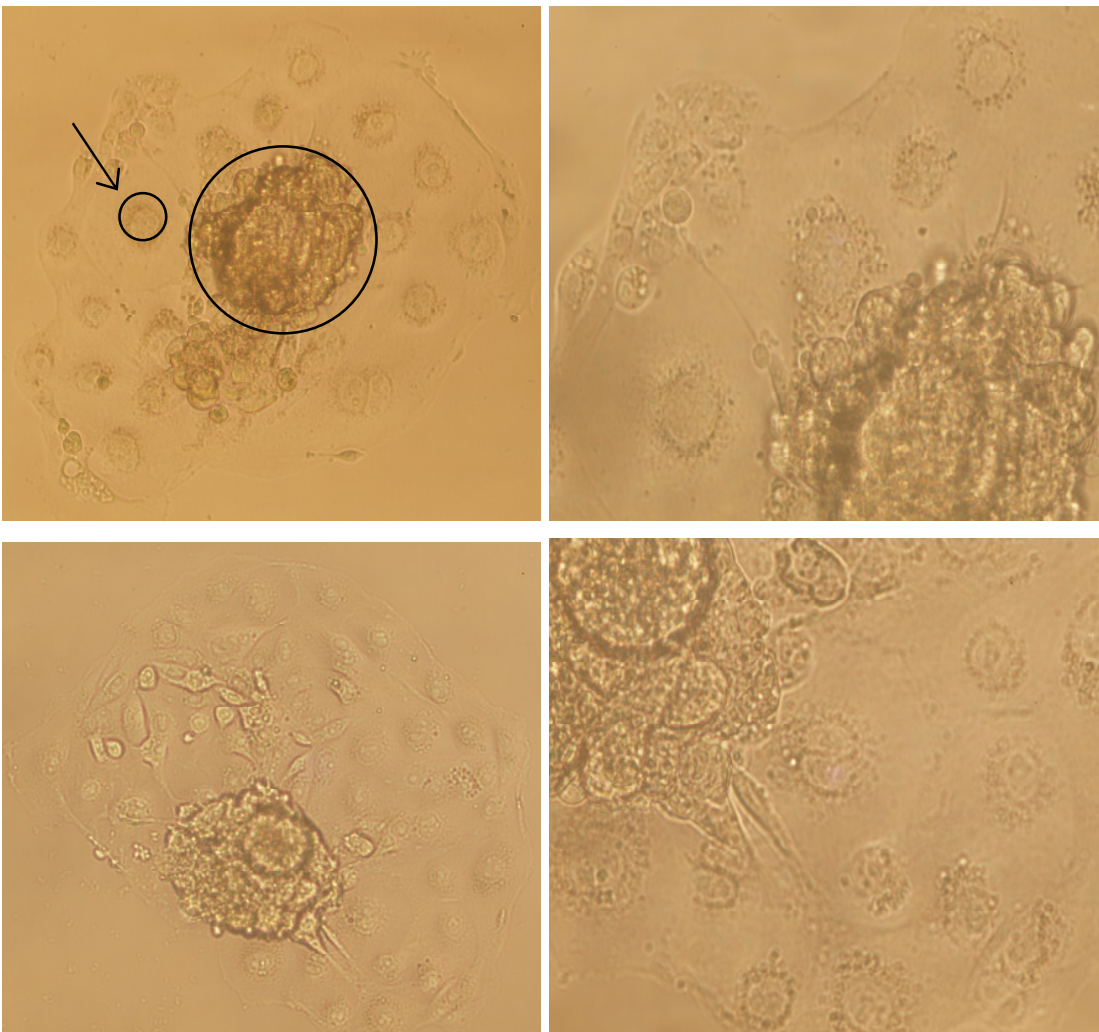


Figure 7: View blastocyst during Pronase (TM) treatment. A) 1 and B) - is the blastocyst with zona pellucida. Zona pellucida can be seen as a thin layer in bigger magnification. A) 2 represent zona-free blastocyst (magnification 200x, 400x).

A typical ICM cell colony and surrounding TE monolayer are shown in Figure 8. The cells showed the typical stem cell-like morphology reported in the literatures the ICM cells each cell was small, epithelioid morphology with large nuclear to cytoplasmic ratios that means a large nucleus and minimal cytoplasm. We also concluded that the trophoblasts began to spread out from the peripheral regions, became larger as a single-cell layer and attached to the gelatinized chamber slides during the embryoblasts piled up vertically as lumps (Figure 7). The efficiency of attachment (90%) and the growth rate of the ICMs were independent of the age of the mouse and maintained in cell culture through without differentiation.

As in material method was described to isolate ICM cells from stem cell culture slides, ICM lumps were aspirated and trypsin treatment was applied to each lump to have individual cells. The TE cells, which were left on the slide, were available for further treatment because of its monolayer and left for air dry. If ICM were not separated afterwards from the trophoblasts and feeder layer, they underwent degeneration in 4 - 5 days and appeared dark and spiky. Inner cells were present in all cultures and the proportion of inner cells did not significantly change between groups of embryos.

The ICM cells were actively mitotic and tightly packed in small nests or colonies in which it was difficult to recognize individual ICM cell without performing trypsin. Therefore, ICMs were subsequently disaggregated into small cell clusters using trypsin-EDTA before fixation and examination by fluorescence microscopy. Isolation, trypsin treatment, and fixation were harmful procedures for the cells that 26% (119/462) of ICM cells and 19% (89/462) of TE cells were lost during the procedures.



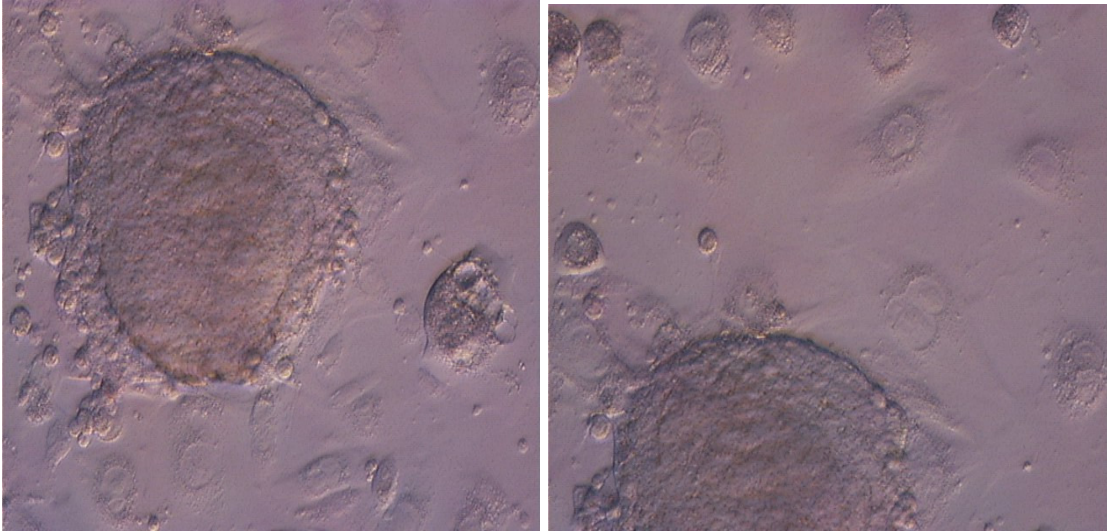


Figure 8: Developed embryonic stem cell culture on day 2 with inner cell mass lumps and epithelioid peripheral trophoblasts (magnification 400x).

The isolated ICMs were mechanically disaggregated, fixed to slide for further procedures and TE cells were fixed without disaggregation and isolation. We examined four fixation methods which were 1:3 Methanol/Acetic Acid, 1:1 Methanol/Acetone, Pure Acetone and 4% Paraformaldehyde (PEA). With Pure acetone fixation no FISH signals could be observed by 10 blastocysts and with 1:1 Methanol/Acetone fixation, background signals after immunofluorescence procedure were too intensive which did not let us to evaluate FISH signals by 12 blastocysts. PFA (Paraformaldehyde) and Methanol/Acetic Acid fixations performed respectively on 32 and 35 embryos with a success of 69% (22/32) and 86% (30/35). These results showed that FISH with immunofluorescence signals could be observed better and clearly, when ICM-TE cells fixed with Methanol/Acetic Acid after trypsin treatment. PFA fixation functioned better without trypsin treatment and in those cases by Methanol/Acetic Acid fixed slides, immunofluorescence signals were observed only on cell membrane that should be in nucleus.

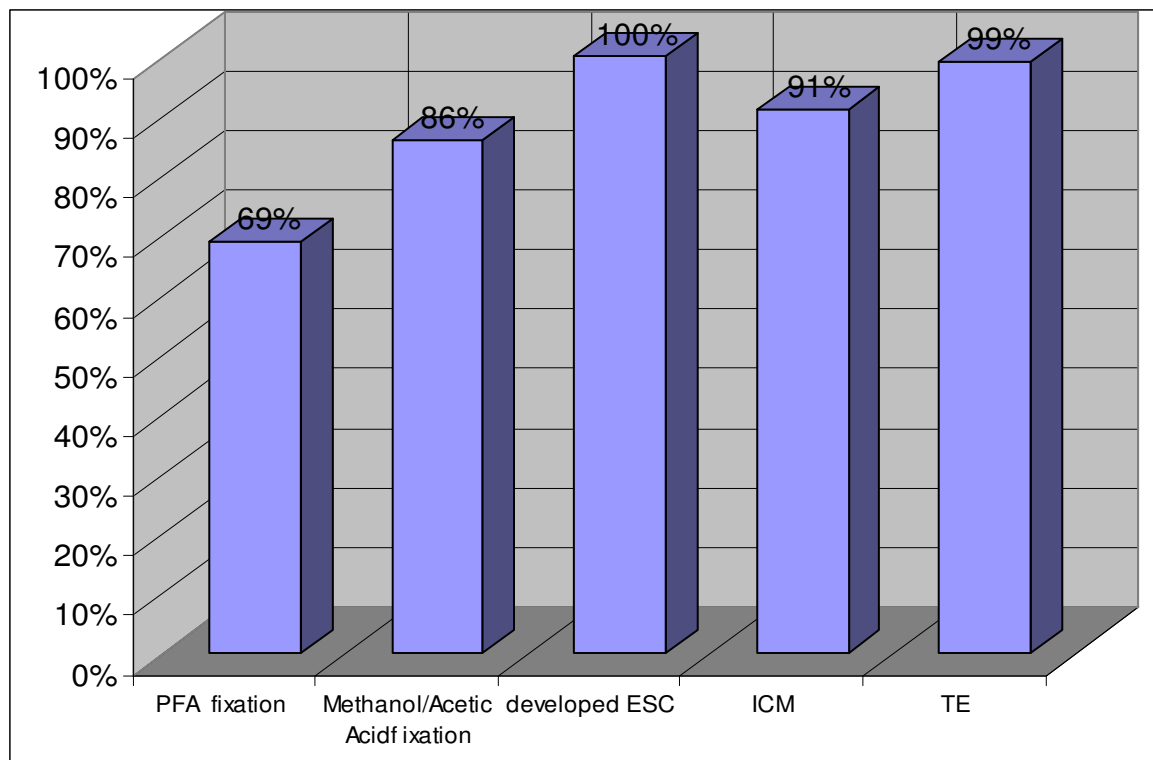


Figure 9: After the best fixation technique, isolated and fixed ICM and TE of blastocyst from develop embryonic stem cell culture.

In the end of the procedure, from 462 developed embryonic stem cell cultures ICM cells of 418 (91%) and TE cells of 455 (99%) fixed embryos were available for optimizing the immunofluorescence, FICTION (Fluorescence Immunophenotyping and Interphase Cytogenetics as a Tool for Investigation of Neoplasm's) procedures and for final results of FICTION.

418 embryo inner cells and 455 embryo TE cells were in ESC examined and the proportion of inner cells and TE did not significantly change between groups of embryos with different total cell numbers. The proportion of inner cells and TE were determined in each embryo with cell numbers ranging from 3 to 25.

3. 2. Oct4 and Cdx Immunofluorescence (IF) cell staining results

As described above, mouse pre-implantation development gives rise to the blastocyst, which is made up of at least three distinct cell types: the

trophectoderm (TE) that surrounds a cavity, and an inner cell mass (ICM) comprising the primitive endoderm (PE) and epiblast (EPI).

From 519 zona-free blastocysts, 462 blastocysts (89%) developed into ICM clumps with expanded TE cells in ESC. 419 (91%) ICM clumps (embryonic bodies) were successfully removed from adjacent trophectodermal, by mechanical dissection with hypodermic needles and enzymatically disaggregated the clumps with trypsin, fixed on the slides which were suitable for IF, analyzable for further FICTON. 455 (98%) TE slides were available for fixation and for further procedures (Table 6). With the help of fixed ICM and TE preparations, it was possible to observe cells on each preparation and the proportion of cells was determined in embryos with numbers ranging from 3 to 25.

By analyzing the distribution of the transcription factors Oct4 (Pou5f1) and Cdx2 at precisely defined stages in pre-implantation development, we were able to identify the cells of TE and ICM. Because the transcription factors Oct4 and Cdx2 believed to have a positive association with trophectoderm and embryoblast specification by their expressions in ICM cells and TE cells, these were used to differentiate the cells with IF.

The POU-domain transcription factor Oct-4 is essential for the pluripotent character of the mouse inner cell mass (ICM) and derivative embryonic stem (ES) cells. We analyzed the expression of Oct-4 with rabbit anti-Oct4 polyclonal antibody during ES culture of mouse embryos and found that Oct4 was down-regulated in TE cells, was present and maintained in the ICM cells. So only ICM cells showed intense Oct4 staining and in the trophectoderm signals were not visible by IF (Figure 10).

The caudal-like transcription factor Cdx2 expression with rabbit anti-cdx polyclonal antibody in embryos was also demonstrable by IF with distinct nuclear staining. Cdx2 expression was quite clearly confined to the nuclei of trophectodermal cells (Fig. 11) and was absent from the inner cell mass.

In total 4 ESC embryos were obtained as controls groups in parallel to Oct4 and Cdx2 groups to check the specificity of the antibodies. Control groups gave negative signals that supported our results.

Several fluorescence markers like AMCA, FITC and Cy5 used to find out the best-merged images with the combination of multi colour FISH probe. For this aim 60 ICM and 67 TE preparations, 60 – 68 ICM and TE preparations, 261 ICM and 279 TE preparations marked respectively with AMCA, FITC and Cy5 fluorescence (Table 6). From all of the groups, signals were observed but the best merged images obtained from indirect labelling with Cy5 fluorescence. Nuclear areas were not possible to observe with AMCA staining because of the same nuclear marker colour was blue with DAPI (Figure 10). FITC staining were clear and DAPI could be used in the same time (Figure 10) but FITC labelling was existing also used in our multi colour FISH probe which did not let us to use FITC also as a fluorescence in IF procedure. So the experiments obtained with AMCA and FITC, helped us to optimize the IF protocol and to see if was antibody working regularly. As shown in figure 10, Oct-4 and DAPI (blue) signals had been merged and the presence of DAPI nuclear staining (blue) were in the centre of cells, whereas all nuclei of the ICM were strongly Oct-4 positive, TE were low-level and apparently negative Oct-4 staining.

| | ESC | dev ESC | ICM preparations | TE preparations | Fluorescence |
|-------------|------------|----------------|-------------------------|------------------------|---------------------|
| Cdx | 49 | 43 | 40 | 49 | Cy5 |
| Oct4 | 31 | 31 | 29 | 31 | Cy5 |
| | 7 | 7 | 6 | 7 | Cy5 |
| | 18 | 18 | 16 | 18 | Cy5 |
| | 215 | 183 | 170 | 174 | Cy5 |
| | 8 | 8 | 6 | 7 | FITC |
| | 19 | 19 | 15 | 19 | FITC |
| | 46 | 42 | 39 | 42 | FITC |
| | 47 | 43 | 39 | 42 | AMCA |
| | 36 | 26 | 21 | 25 | AMCA |

Table 6: The POU-domain transcription factor Oct-4 and the caudal-like transcription factor Cdx immunofluorescence applications with fluorescence markers to ICM and TE of embryos from planned and developed embryonic stem cell culture procedure.

Cdx2 staining was obtained and observed on 40 ICM - 49 TE preparations. All TE cells on TE preparations showed nuclear Cdx2 positive staining, whereas ICM preparations showed low-level or negative Cdx2 staining.

The intensity of Oct4 fluorescence was never higher in the ICM than in the TE but the intensity of the fluorescent signals varied widely in the samples, ranging from negligible over clear to strong signals (Figure 10). We came across patterns of abnormal Oct4 expression in embryos included expression in both TE and ICM cells of the 2% embryos as well as absence of expression, but only clear signals were counted as positive. Because every cell in an isolated ICM lump should express Oct4 which suggests that Oct-4 expression in cells of the ICM can undergo down regulation in culture.

In 4% cases, it was occasionally difficult to distinguish between cytoplasmic and nuclear staining. It was unclear whether the cytoplasmic staining represents specific Cdx2 / Oct4 signal or background (Strumpf et al., 2005). Only the cases in which nuclear staining was clearly distinguishable from cytoplasmic staining was considered positive in this study and it was acceptable in some cases TE preparations had green/blue background.

In total, Cdx2 observed on 42 TE cells as positive and 4 TE (9%) cells weak positive. 36 ICM cells were Cdx2 negative and 6 ICM cells were Cdx2 weak positive (18%). When we have a look to the Oct 4 expression; 19 TE (4%) cells were weak positive and 445 TE cells were negative with 456 positive ICM cells were negative and 14 ICM (3%) cells were weak positive.

As described above, in most of the Cdx2 examined embryos (~30 embryos) Cdx2 signals were clearly weaker in the TE than Oct4 examined embryos with Oct4 signals in ICM cells. Moreover, with continues applications for FICTION, comparison of normalized mean fluorescence intensities of Oct4 and Cdx2 were clearly stronger by fluorescence of Oct4 expression. Because our aim was just to distinguish ICM and TE cells for examining their genetic statuses, mostly Oct4 was used to differentiate the cells.

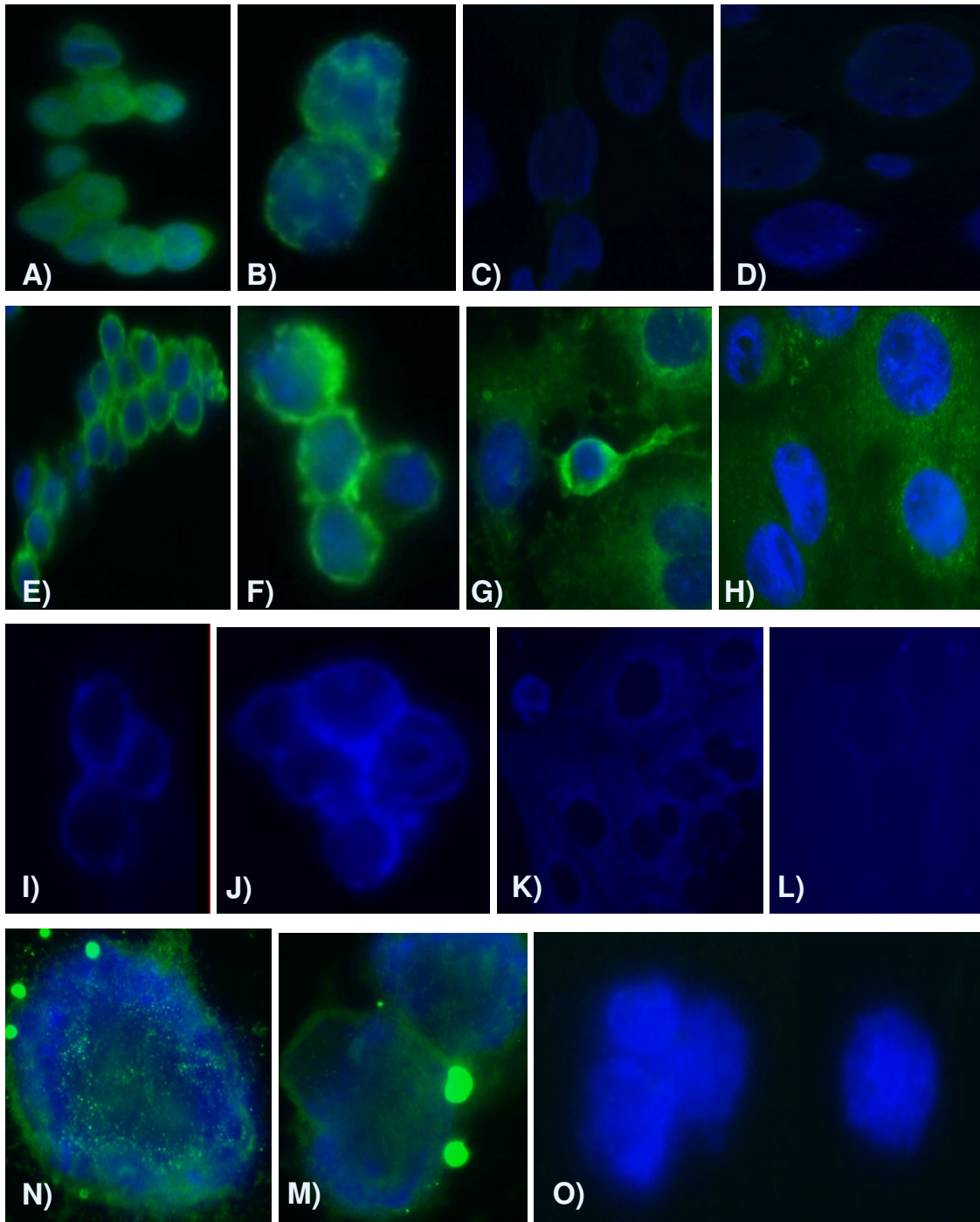


Figure 10: Photos from A, B, C, D, E, F, G, H represents; strong Oct-4 nuclear immunofluorescence is detected in the ICM cells with FITC and photos I, J, K, L with AMCA, while weaker or no signal observed over TE cells. Photos from N, M, and O represents; strong Cdx2 nuclear immunofluorescence is detected in the TE cells and while no signal observed over ICM cells (magnification 400x, 600x). Except the photos with AMCA (I, J, K, L), other nucleus were stained with DAPI in blue.

3. 3. Multi colour FISH probe

CBA/CaHT(14;15)6Ca/J mouse strain had a translocation with a breaking point between chromosomes 14 and 15. To find out the exact position of the breaking point and to generate multi colour FISH probe, we ordered 28 Bacterial Artificial Chromosomes (BACs) (Figure 11), isolated these BAC DNAs and tested on our normal, translocated mouse chromosomes preparations as described in material method.

3. 3. 1. Generation of specific multi colour FISH assay:

The positions of 28 BACs were enlarged in distance of 8.8 Mb on chromosome 15 in cytoband A2 and in a distance of 7.23 Mb on chromosome 14 in cytoband E3. All of the BACs gave detectable signals on mouse metaphase spreads, but by some BACs multiple locations of signals observed. Therefore, as a result after obtaining 75 experiments, 17 BACs were selected from chromosome 15 in a distance 1,70Mb and from chromosome 14 in distance of 2.26 Mb. As showed on figure 11, multiple located signals were observed by 11 BACS and were excluded. Four BACs located close to the breaking point, which was allowing us to identify the position and type of translocation.

Finally we decided to generate the dual colour FISH probe by using RP23-122C17, RP23-240O18, RP23-340M21, RP23-399K3 BACs in green and RP23-51F3, RP23-8P22, RP23-301G14, RP23-436G2, RP23-39J5 BACs in blue for chromosome 14. For chromosome 15, we generated a dual colour probe by using RP23-233J16, RP23-203 G 21, RP23-220O14 BACs in orange, and RP23-98B21, RP23-462M9, RP23-441F15, RP23-462K15, RP23-68N6 BACs in Texas red. The colocalisation of orange and blue, as well as red and green indicates to us the translocation products of chromosome 8 and 12. Figure 11 displays the chromosomal location of BACs including their positions in kb.

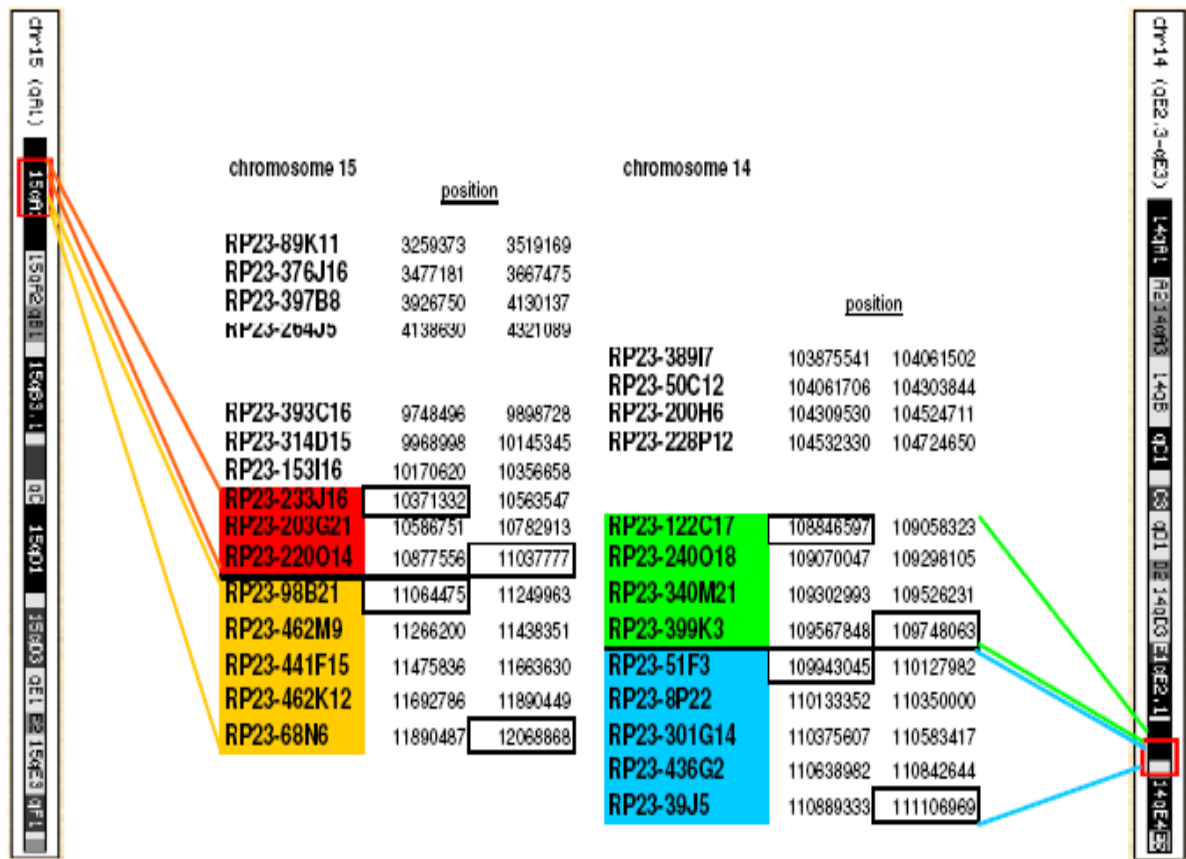


Figure 11: The distribution of 28 BACs on chromosome 15 in cyto band A2 and on chromosome 14 in cyto band E3. Differently labelled 17 BACs which were generating mFISH probe for CBA/CaHT(14;15)6Ca/J mouse strain.

The suspicion of a balanced translocation between chromosomes 14 and 15 was verified by mFISH and FISH was employed to assign mouse BACs to mouse banding landmarks. Our results confirmed that CBA/CaHT(14;15)6Ca/J mouse strain had a balanced translocation which maintained through sibling crosses.

The use of the mFISH ISIS Ver.3 software package from MetaSystems (Altussheim, Germany) and a Z-axis motorized microscope (Zeiss, Göttingen, Germany) allowed several focus planes to reconstruct into a single image. A final multicolour picture containing all of the hybridization signals was achieved by merging the information derived from each colour channel. With using our self-generated FISH probe, signals were observed on two chromatids of each

chromosome in an average of >98% of cells with a minimum of 1020 cells (TE+ICM) examined.

As shown in Figure 12, by regular heterozygote embryos chromosome 14, derivative 14, chromosome 15 and derivative 15 of ICM and TE cells respectively observed as Deac + Green, Green + Gold, Red + Gold and Red + Deac. In some cells also extra, unique signals detected which were interpreted as artefacts and ignored.

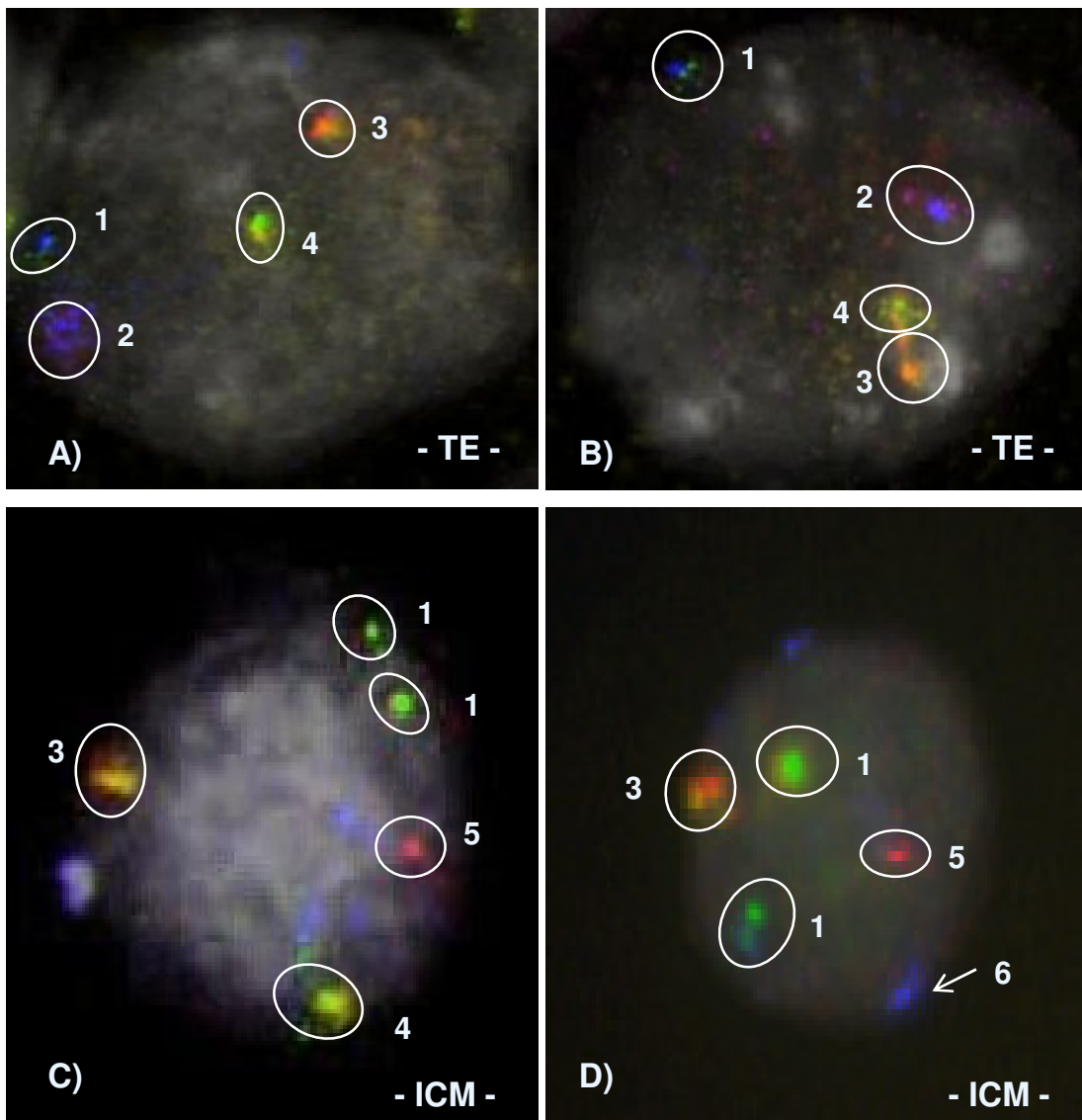


Figure 12: Merged FISH pictures of ICM and TE cells. Picture A and B represent TE cell, Picture C and D represent ICM cell. 1 - Deac + Green = Chr.14, 2 - Red + Deac = Der. 15, 3 - Red + Gold = Chr.15, 4 - Green + Gold = Der.14, 5 - Red signal = Artefact, 6 - Blue signal = Artefact (magnification 600x).

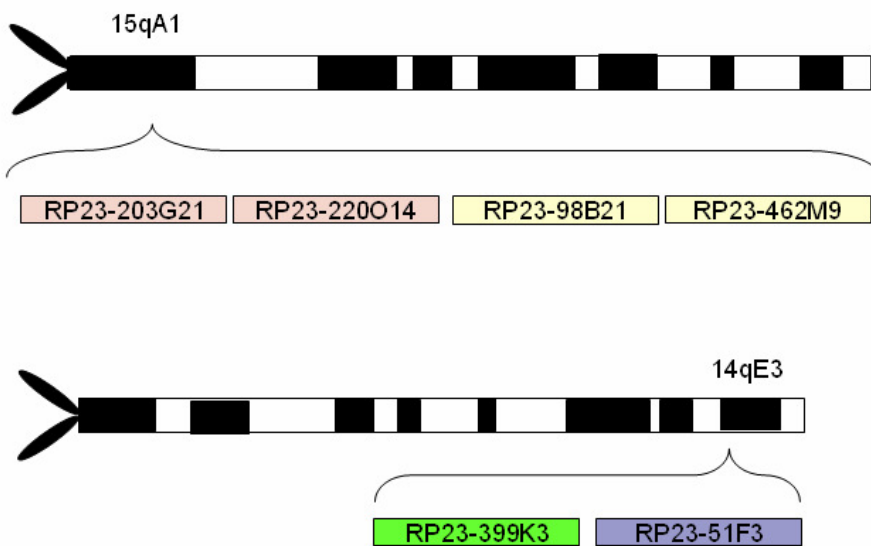
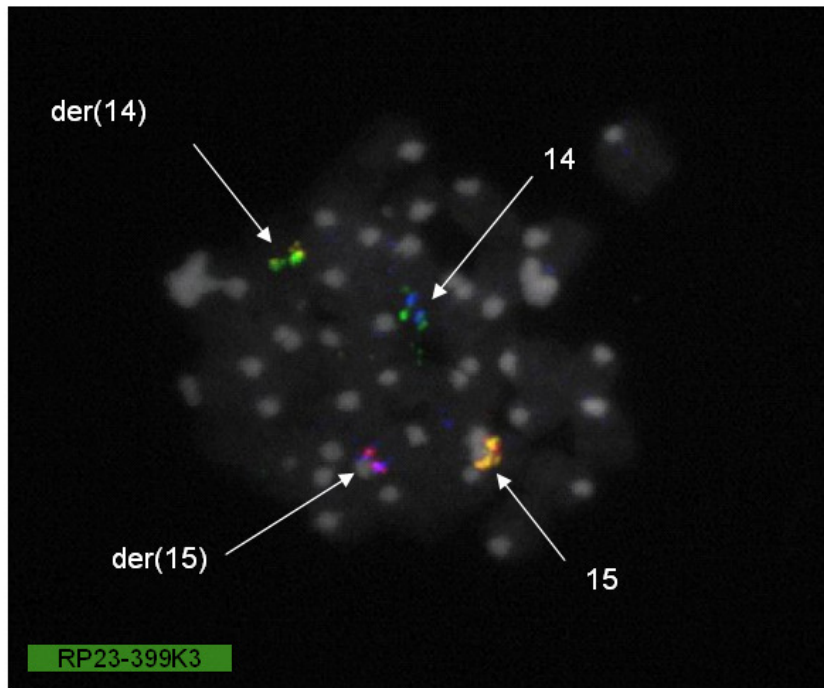
Of 1020 analyzed cells, 162 homozygote cells were served as a control group, 848 heterozygote cells were disposed as an experimental group which male strain carried translocation and only 10 cells were supposed to serve as another experimental group which female strain carried translocation.

After analyzing homozygote 85 ICM and 77 TE cells as a control group, cut off value of 20% also verified which was described in material method.

3. 3. 2. Break point localization

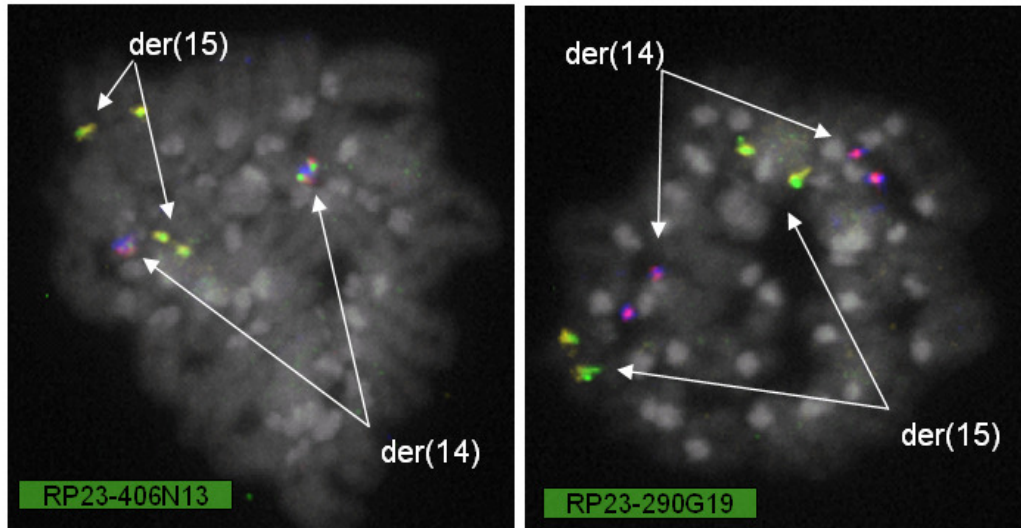
CBA/CaHT(14;15)6Ca/J mouse strain was used in different scientific projects but the breakpoint of this translocation has never has been examined in detail. The international database resource for the laboratory mouse MGI defined the known break point regions in cells of mouse strain CBA/CaHT(14;15)6Ca/J on chromosome 15 in the cytoband A2 and on chromosome 14 in cytoband E3 (http://www.informatics.jax.org/searches/marker_report_by_reference.cgi?5280). Both break point regions were delimited by covering 8 MB ranges with FISH probes generated from BACs. The neighbour BAC clones RP23-220O14 (red) and RP2398B21 (yellow) co localize on Chromosome 15 as well as RP23-399k3 (green) and RP23-51F3 (blue) on chromosome 14 on normal mouse chromosomes of heterozygote mice. On translocated chromosomes they recombine (Figure 13). A second multiplex hybridization on homologue CBA/CaH-T(14;15)6Ca/J strains was designed in the different use of a green sample. Instead of RP 23-399 k3 on chromosome 14 an other BAC clone (RP23-406N13) was labelled in green, which is overlapping the putative break point on chromosome 15 (Figure 14). Hereby, the green signal splits up on all translocation products (Acar-Perk et al., 2009)

The BAC clone RP23-290G19 localized a little more distal does not split. Thus, the breakpoint on chromosome 15qA1 is definitely anywhere between localization of RP23-98B21 and RP23-220O14, in a distance of 26699 bp in the second intron of ADAMTS12 (Acar-Perk et al., 2009).



On translocated chromosomes, RP23-220O14 (red) on chromosome 15 and RP23-51F3 (blue) on chromosome 14 as well as RP2398B21 (yellow) on chromosome 15 and RP23-399k3 (green) on chromosome 14 recombine.

Figure 13: Identification of the break point position by self generated FISH probes in heterozygote B6D2F1 x CBA/CaH-T(14;15)6Ca/J mouse strain (magnification 600x).

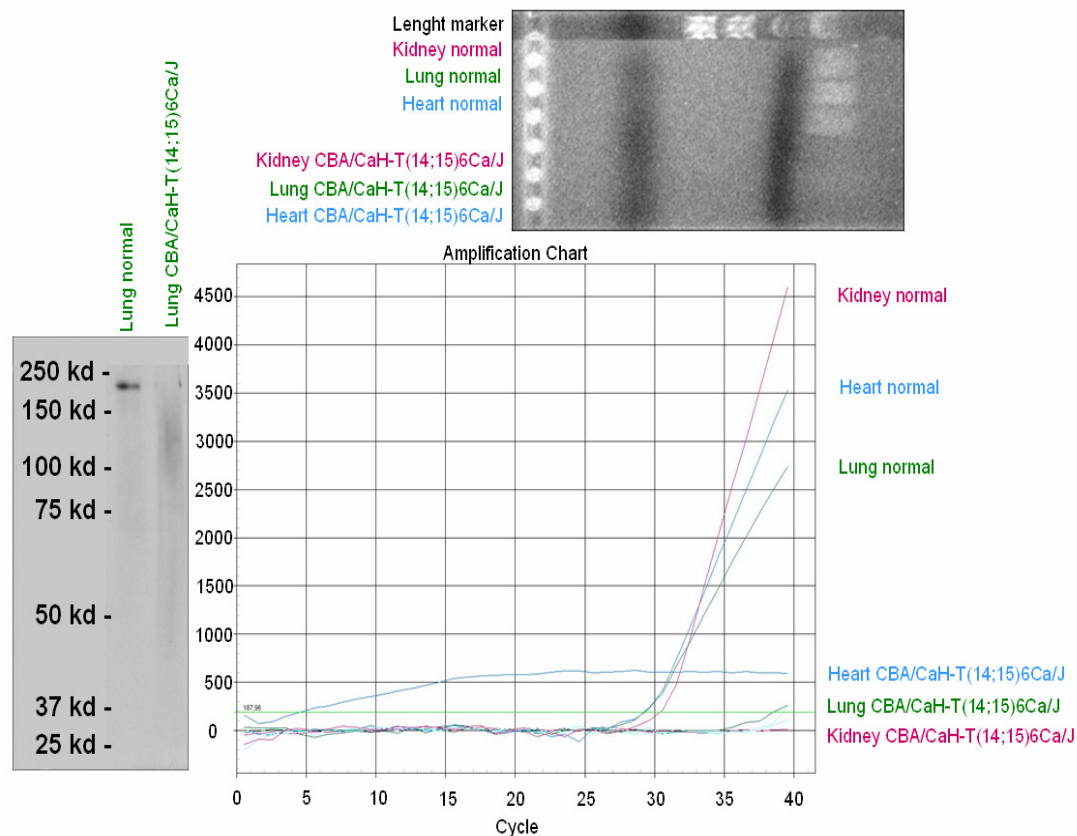


Upon left: The RP23-406N13 BAC clone was labelled in green and was overlapped the putative break point on chromosome 15 by splitting up green signals on all translocation products. **Upon right:** The BAC clone RP23-290 G19 localized more distal and did not split. **Below:** Map of BAC position.

Figure 14: Splitting of the ADAMTS12 signal in homozygote CBA/CaH-T(14;15)6Ca/J mouse strain (magnification 600x).

3. 3. 3. Expression of ADAMTS12

As a template for RT-PCR, we used cDNA produced out of the lungs, the hearts and the kidney from translocated mice as well as from normal mice, for the proof of an ADAMTS12 mRNA transcript. A transcription product was traceable in tissues of normal mice on an electrophoresis gel and with an I-Cycler (Figure 16) but not in mice with homozygote translocation chromosomes t(14;15)(qE3;qA1). A 178 kd pre peptide of the ADAMTS12 could be proved in the lungs of normal mice but not in homozygote CBA/CaH-T(14;15)6Ca/J mouse strains by western blotting experiment (Figure 15) (Acar-Perk et al., 2009).



Right: ADAMTS12 mRNA transcript product was traceable in tissues of normal mice on an electrophoresis gel and with an I-Cycler but not in mice with homozygote translocation chromosomes t(14;15)(qE3;qA1). **Left:** By western blot a 178kd transcript is detectable in lung of normal mice but not in homozygote translocation mice t(14;15)(qE3;qA1).

Figure 15: Expression of ADAMTS12 in CBA/CaH-T(14;15)6Ca/J and normal mouse organs.

3. 4. FICTION (Fluorescence Immunophenotyping and Interphase Cytogenetics as a Tool for Investigation Of Neoplasms) results on mice TE and ICM cells:

Combined immunophenotyping and fluorescence in situ hybridization (FISH) technique according to the “fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasms” (FICTION) technique had been successfully applied in 510 ICM and 510 TE cells of 73 mice embryos. Thus, we were able to visualize the Oct4 or Cdx2 antigen expression of cells with T(14;15)6Ca chromosomal translocation region directly.

The reliability of the multicolour FISH probe set to detect T(14;15)6Ca reciprocal translocation was verified by FICTION experiments with the homozygote control groups from B6D2F1 X C57BL/6 crossing embryos. 85 ICM cells and 77 TE cells were examined with 12 embryos (Figure 16). Twelve percentage of the cells were given irregular signals which were under the cut-off limit of 20% for detecting T(14;15)6Ca reciprocal translocation and was ignored. All ICM cells were Oct4 positive whereas TE cells were Oct4 negative.

As analyzed before by the chromosome preparations from fibroblast cell culture, observed signals from the homozygote control groups of 85 ICM and 77 TE cells were; blue signal + green signal for chromosome 14 and red signal + gold signal for chromosome 15, without any derivative products (Figure 16).

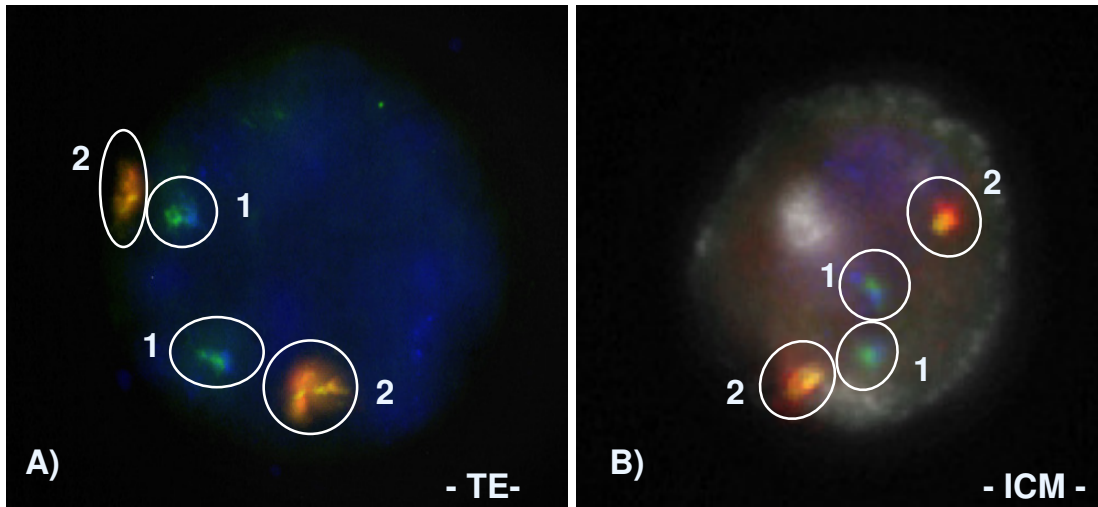


Figure 16: Merged FISH pictures represent the control group of homozygote ICM and TE cells with Oct4. Picture A represents TE cell by which is Oct4 negative and Picture B represents ICM cell with Oct4 positive signal (white background signal) No1: Deac + Green = Chr.14, No2: Red + Gold = Chr.15 (magnification 600x).

The translocation between chromosomes 14 and 15 on ICM and TE cells was verified by the FICTION experiments of 858 cells. As shown in Figure 17 with the hybridization of ICM and TE cells from the male confirmed T(14;15)6Ca reciprocal translocated embryos, 420 ICM and 428 TE cells were observed with a total of 60 embryos. Only 1 detectable embryo was gained from female confirmed T(14;15)6Ca reciprocal translocated embryos with 5 cells (Table 7). Immunofluorescent detection signals of Oct4 were clearly positive by 99% ICM cells and were Oct4 negative by TE cells.

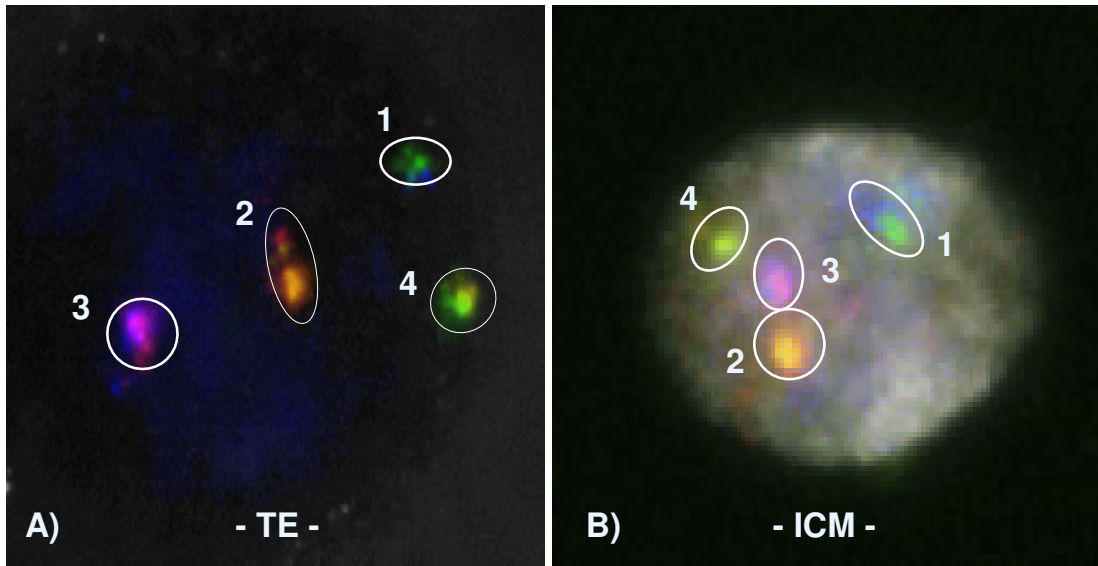


Figure 17: Merged FISH pictures of heterozygote ICM and TE cells with Oct4. Picture A represent TE cell by which Oct4 is negative, Picture B represent ICM cell which Oct4 is positive (white background signal). No1: Deac + Green = Chr.14, No2: Red + Gold = Chr.15, No3: Red + Deac = Der. 15, No4: Green + Gold = Der.14 (magnification 600x).

FICTION method, which contained 4-colour M-FISH with indirect labelling of Cy5-Streptavidin for Oct4 and Cdx2 transcription factors, performed on 8 embryos containing 40 ICM and 43 TE cells by labelling, detecting Cdx2 transcription factor. Fourhunderedseventy ICM and 467 TE cells in 65 embryos labelled and detected with Oct4 transcription factor during the procedure (Table 7). Eighty percent of TE cells gave positive signals with Cdx labelling and 99% ICM cells gave positive signals with Oct4 labelling (Figure 18).

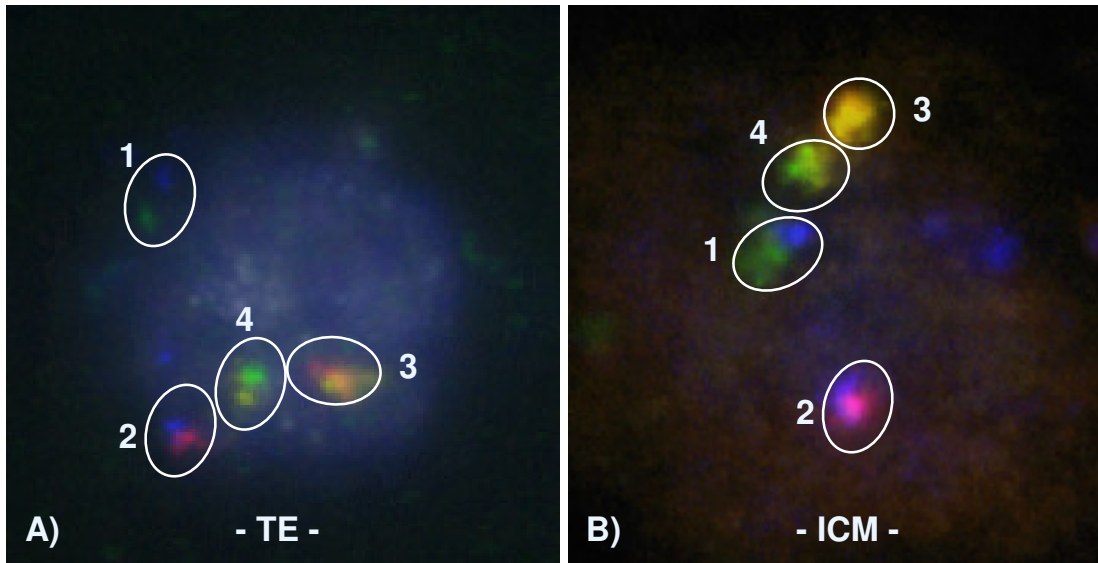
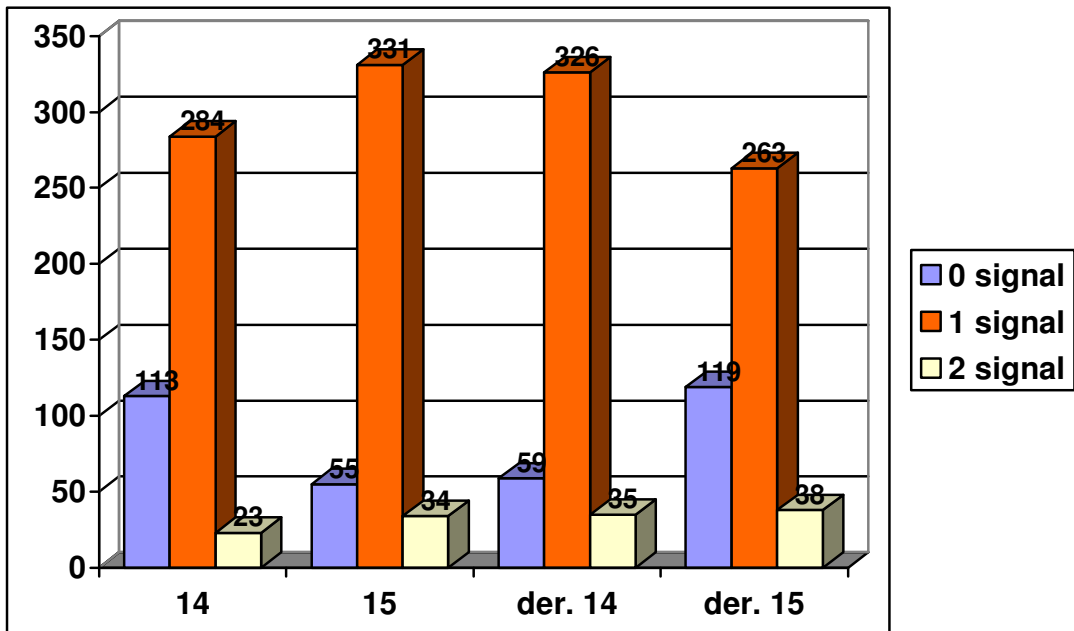


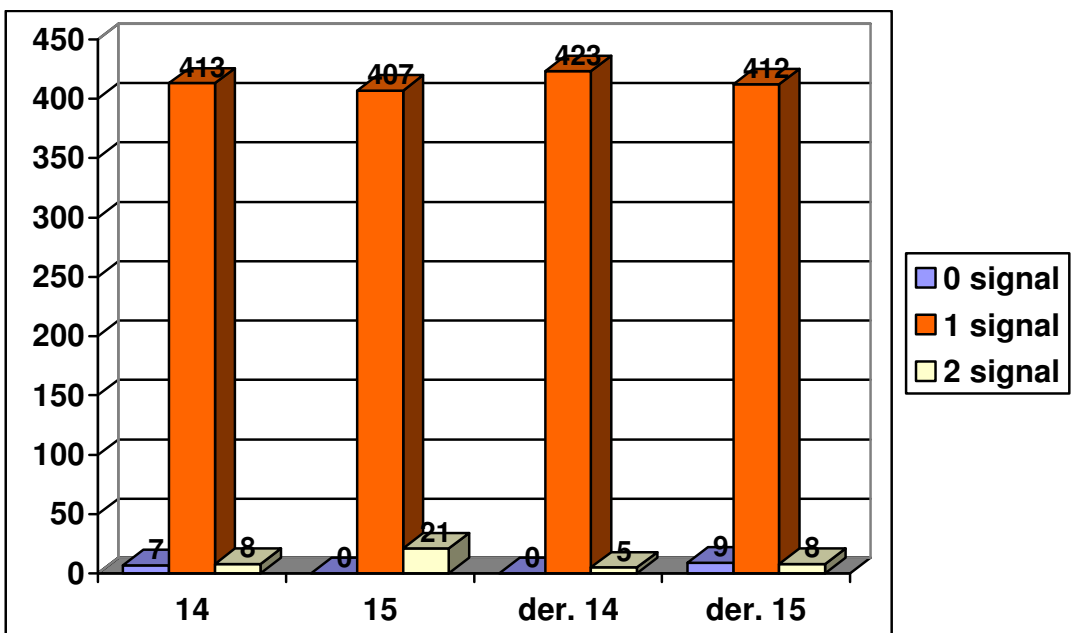
Figure 18: Merged FISH pictures of heterozygote ICM and TE cells with Oct4. Picture A represent TE cell by which Cdx is positive (white background signal), Picture B represent ICM cell which Cdx is negative. No1: Deac + Green = Chr.14, No2: Red + Deac = Der. 15, No3: Red + Gold = Chr.15, No4: Green + Gold = Der.14, (magnification 600x).

As shown in Figure 19A; chromosome 14, derivative 14, chromosome 15 and derivative 15 observed on heterozygote ICM cells respectively as 2 signals on 23, 34, 35 and 38 cells. No signal detected on heterozygote ICM cells for each chromosome and translocation product in 346 cells. Regular one signal counted for chromosome 14 on 284, for derivative 14 on 326, chromosome 15 on 331 and derivative 15 on 263 heterozygote ICM cells.

Heterozygote TE cells had 1 signal on chromosome 14 in 413 cells, by derivative 14 on 423 cells, by chromosome 15 on 407 cells and by derivative 15 on 412 cells. In contrast to the heterozygote ICM cells groups, just on 15 cells no signal observed and 2 signals for each chromosome and translocation product in total of 42 TE cells (Figure 19B).



A) Graphic represents observed signals on 420 heterozygote ICM cells which male strain carried balanced translocation.



B) Graphic represents observed signals on 428 heterozygote TE cells which male strain carried balanced translocation.

Figure 19: Revealed signals on 420 heterozygote ICM and 428 heterozygote TE cells which male strain carried balanced translocation in each chromatid.

Translocation products of chromosome 14 and 15 were observed among several aneuploid chromosomes in ICM cells by heterozygote-translocated embryos (Table 6). When the results from FICTION summarized, all the cells (ICM+TE) of heterozygote translocated embryos carried T(14;15)6Ca reciprocal translocations in a regular way but by ICM cells to this translocation also high numbers of aneuploidy observed (Figure 20).

| Crossing ♀ x ♂ | Σ ICM | Σ TE | Σ Embryo | Normal ICM | | Abnormal ICM | |
|-------------------|-------|------|----------|------------|----------|--------------|----------|
| | | | | Σ cell | Σ embryo | Σ cell | Σ embryo |
| T♀xN | 5 | 5 | 1 | 1 | 0 | 4 | 1 |
| NxN | 79 | 70 | 10 | 143 | 9 | 10 | 1 |
| | 6 | 7 | 2 | 4 | 1 | 5 | 1 |
| NxT♂ | 187 | 151 | 14 | 59 | 0 | 128 | 14 |
| | 30 | 13 | 2 | 5 | 0 | 4 | 2 |
| | 9 | 10 | 4 | 13 | 0 | 17 | 4 |
| | 25 | 13 | 5 | 8 | 0 | 17 | 5 |
| | 73 | 106 | 18 | 21 | 0 | 52 | 18 |
| | 25 | 25 | 5 | 12 | 0 | 13 | 5 |
| | 11 | 20 | 2 | 5 | 0 | 6 | 2 |
| | 60 | 90 | 10 | 25 | 0 | 35 | 10 |

Table 6: Detected ICM/TE cells with total number embryos and normal /abnormal ICM cells with the belonging embryo numbers were described. Normal ICM indicates translocation-carrying mice without aneuploid ICM cells. Abnormal ICM represents translocation products with aneuploidy in ICM cells. T♀xN: Translocated female mated with normal male mouse, NxN: Normal female mated with normal male mouse, NxT♂: Normal female mated with translocated male mouse.

These aneuploidies might occur by nondisjunction, because we observed 2 signals or no signal translocation products from chromosome 14 and 15 (Figure 20). There were no embryos without aneuploidy by ICM cells, which was not the case for TE (Table 6).

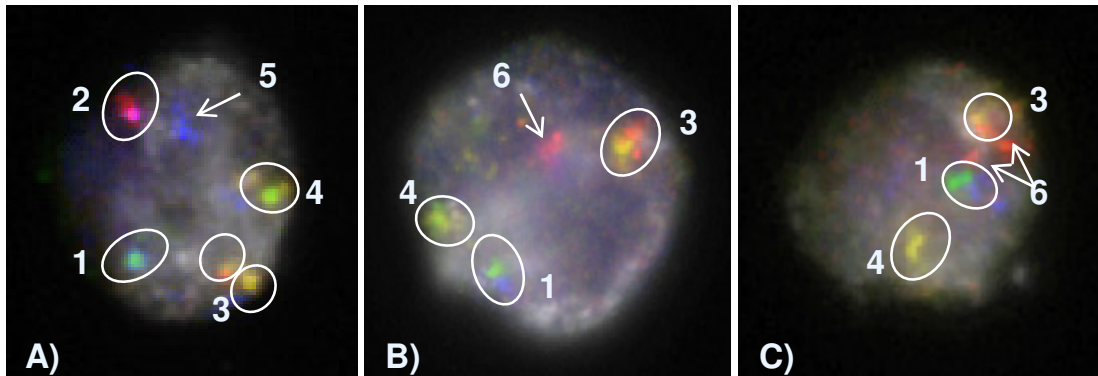


Figure 20: Merged FISH pictures of heterozygote ICM cells with Oct4 (white background signal). No1: Deac + Green = Chr.14, No2: Red + Deac = Der. 15, No3: Red + Gold = Chr.15, No4: Green + Gold = Der.14, No5: Blue = Artefact, No6: Red = Artefact (magnification 600x).

As mentioned before, only 1 detectable embryo was obtained from female confirmed T(14;15)6Ca reciprocal translocated embryos with 5 cells which we did not afterwards taken into consider because of females old age and less number of obtained cells.

4. DISCUSSION:

Discussion consists of two parts, the applied methods for optimization the technique and obtained results according to our aim.

First, we start the intensive discussion of methods and published protocols, which are suboptimal in our hands. Thus, optimization of the protocol is one of the important facts in this study.

Our results are new outcomes and different to published data before. Thus, we can improve new hypotheses of aneuploidies arose in mouse embryos.

4. 1 Discussion of methods

Different protocols for isolating individual ICM and TE cells from morula-blastocyst stage mouse embryos were evaluated. The benefits of the trophectoderm biopsy, embryonic stem cell lines and immunosurgery procedures are that the removal of greater numbers of cells can be achieved initially, so allowing for repeat analysis and / or storage of samples for later reanalysis. However, published protocols for immunosurgery and trophectoderm biopsy were not optimal for our aim. The best method for isolation and cultivation of high numbers of TE and ICM cells is the production of embryonic stem cell lines.

Normally isolation procedures had a negative impact, for example on the growth patterns of the isolated blastomeres. Different abnormalities could be observed at the blastocyst stage including embryos lacking visible compaction features, embryos with double blastocoelic cavities and embryos with no inner cell mass (trophoblastic vesicles) (Nys et al., 1987). A possible explanation for the formation of these abnormalities could be the incorrect division patterns caused by the lack of the zona pellucida: flattened groups or strands of cells are observed at the four- and eight-cell stage.

4. 1. 1 Superovulation protocol

Superovulation of female mice with exogenous gonadotrophins is routinely used for increasing the number of eggs ovulated by each female in reproductive and

developmental studies (Dagmar et al., 2004). These days superovulation is also a major procedure to generate transgenic rodents (Brooke et al., 2007).

To investigate the mechanisms involved superovulation Miller et al.,(1981) was administered a superovulatory dose of pregnant mare serum gonadotropin (PMSG, 40 IU) to seventy-six 30-day-old rats. Sixty-eight control rats received 4 IU, a dose that induces normal numbers of ovulations followed by normal pregnancy. Young W. et al. (1989) examined the effects of superovulatory treatment (20 IU PMSG) on follicular steroid contents and oocyte maturation. Immature female rats aged 28-30 days were injected with 4 or 20 IU PMSG and euthanized at 24, 48, 60, and 72 hours. Nuclear maturation of the majority of control oocytes recovered from oviducts at 72 hr and synchronized at metaphase II. However, superovulated oocytes displayed different stages varying from prophase I to metaphase II at 24, 48, and 72 hours. Legge M. et al. (1994) represented the optimum gonadotropin doses and chronology for the induction of superovulation in sexually mature hybrid mice. A regime of 12 IU PMSG, followed 48 hr later by 20 IU hCG administered 1 hr before the midpoint of the light cycle (1200), gave the maximum ovulatory response.

In our experimental settings, 5 – 8 weeks old C57BL/6 female from the mice respond higher and better with oocyte morphology than C3H/HEN and BALB/c, when it was injected with 7.5 IU PMSG and 5 IU HCG gonadotropins with a time interval of 48h. The poorest response was obtained from female of the C3H/HEN strain when it got older, even in the optimal conditions. We had better and good results when we preferred females of B6D2F1 mouse strain for mating process and males of the mouse C57BL/6 strain which should be at least 6 weeks old. Injection with 7.5 IU PMSG and 5 IU hCG worked best and had more, good quality oocytes-embryos. Previous publications (Hetherington et al., 1987; Hogan et al., 1986; Gates et al., 1969) have indicated that, between 1 and 5 IU of PMSG followed by 2.5 IU hCG after 45 to 48 hr will induce superovulation in mice. But Legge et al. (1994) mentioned that, although such low-dose protocols might induce ovulation in mature mice, they would be ineffectual for the production of large numbers of oocytes.

By embryo gaining, 80% (25/31) of our mice were mated and the proportion of embryos from mated mice was not influenced by the doses of exogenous gonadotropins, rather than influenced by the different injection times. The percentage of embryos in morula stage and reaching the blastocyst stage was significantly higher when mice were injected in the afternoons. Therefore, we concluded that PMSG and HCG injections should be performed in the afternoons and 85% (47/55) of embryos appeared morphologically normal at the time of recovery with a clear progression by the overnight cultures with B6D2F1 females. According to our results, the minimum interval between PMSG and HCG necessary to obtain consistent superovulation was approximately 44 hr. Intervals of less than 44 hr resulted in superovulation not all treated females. We have also noticed with that sexual maturity which lies between 6 – 8 weeks of age depending to the strain. PMSG treatment given in the morning or the afternoon on any day of the four-day estrous cycle with HCG given at 1700 hr two days later successfully induced superovulation. In general, PMSG treatment in the morning resulted in more consistent and abundant superovulation than when treatment was begun in the afternoon. However, Van der Auwera I. (1999) and Elmazar et al. (1989) have suggested that, effects of the hormone treatment on the maternal oviductal and uterine environment predominantly induce abnormal embryonic development after superovulation with gonadotrophins. No adverse effects of superovulation have been reported on fertilization or cleavage during the early phases of mouse preimplantation development. In rats, fertilization and implantation failure are common consequences of superovulation with PMSG (Walton & Armstrong 1983; Miller & Armstrong 1981).

Culture medium (M2, M16 and Menezotte) was also one of the primary factors in the overnight developments. Therefore, embryos in each experiment group were cultured with different culture media and no difference was observed. Encountered retarded embryos, which were not included in the analysis, appeared in only one case (6. experiment) with five morula stage embryos.

4. 1. 2. TE biopsy

The biopsy technique described with providing more cells and hence an increased amount of DNA for genetic analysis, providing for the loss of material during preparation and allowing duplicate analysis (Dokras et al., 1990). The technical approach to pre-implantation diagnosis reported suitable for defects where the gene product is expressed sufficient quantity for biochemical assay before implantation. Routinely TE biopsy has been performed in humans using mechanical means (Muggleton-harris et al., 1995 and 1993; Dokras et al., 1991, 1990) or using laser (Veiga et al., 1997). According to our results, it was not an optimal technique for isolating high numbers of TE and ICM cells. The technique was not as simple and harmless as described. In our experimental setting, the procedure was very traumatic to the embryos that most of them died shortly after or did not continue to develop. The method also was hard to perform on the material, independent from technical familiarity.

Carson et al. (1993) compared of four trophectoderm biopsy techniques in murine blastocysts, which were aspiration, incision, slit / excision, and hatch / excision. They concluded that; slit / excision technique, and hatch / excision techniques exhibited lower operative injury and the higher postoperative viability then aspiration or incision and appear to be the least damaging of the four methods. Cui et al. (1993) also agreed that the hatching rate appeared to be a simple, sensitive, and reliable method to evaluate the TE biopsy technique. Steven et al. (2005) and De Vos et al. (2001) compared near-infrared lasers with the use of partial zona lysis with acid Tyrode's solution. They found out that, the use of the laser shortens the time embryos were outside of incubators and had removed the risk of directly exposing embryos to potentially toxic acidic conditions.

In our cases laser pulse was not possible to optimize. The size of the holes was big that the blastocysts were hatched totally before the biopsy or were small that no herniation was observed. For having only a 2% biopsy success (1/38) rate with the laser, the technique was skipped.

Making slit which should theoretically, also written in different publications, took less than a minute, for us it took at least 4 -8 minutes. Considerable practice was required to perfect biopsy to avoid embryo damage, including aspirating more than one cell, breakage of the aspirated cell, and lysis of the remaining cells of the embryo, which we already had in our routine laboratory works like polar body biopsy. But our familiarity to the protocol, did not make the procedure easy to biopsy the trophectoderm

Dokras et al. (1991, 1990) recommend performing a hole or slitting in the area of the zona opposite to the inner cell mass (mural trophectoderm). In human embryos trophectoderm biopsy does not impair hatching and the subsequent outgrowth of the biopsied blastocyst in vitro (Dokras et al. (1991, 1990); Muggleton-Harris et al., 1993). However, in many instances the inner cell mass area protruded through the zona pellucida. It is possible to induce an appropriate area of the trophectoderm cells to herniate. Based on our observations, it appears reasonable to conclude that there is no particular site for shedding of the zona pellucida. All the shedding areas, however, differ distinctly in size (Yamazaki et al., 1989). From 21 (60%) of the herniated blastocysts a part of the inner cell mass herniated together with the trophoectoderm or a part of inner cell mass herniated. This showed that, there is no particular site that the zona pellucida of an embryo is shed prior to implantation. Normal controlled herniation of only trophoectoderm cells as the blastocyst developed in vitro, were in our cases only 14 blastocysts (40%).

Success of trophectoderm herniation and subsequent biopsy depended on the stage at which the blastocyst was slit. In the early blastocysts there was less injury to the trophectoderm herniation and in the late blastocysts, due to the expanded cavity, there was a greater chance of injury to the trophectoderm (Dokras et al., 1990). McArthur et al. (2005) represented that all blastocysts survived the biopsy procedure by reconstituting their blastocele and most continued to grow and develop. Ninety percent of blastocysts were able to cryostore and thaw successfully after the biopsy. In our set of experiments, zona pellucida of 92 blastocysts were slitted and afterwards in 19 blastocysts (21%)

blastocoelic cavity collapsed, 11 (12%) blastocysts degenerated or failed to re-expand the next day, 16 (17%) blastocyst had no herniation or a very small herniation occurred and 30 (33%) hatched out of the zona completely before the biopsy was performed.

Dokras et al. (1990) represented that, the number of trophectoderm cells obtained from human blastocysts depends on the time interval between slitting the zona and biopsy of the herniation. However, we removed approximately clumps of 3 - 8 trophectoderm cells were in each trial without time depending.

4. 1. 3. Immunosurgery

Immunosurgery also was not an optimal technique for individual analysis of TE and ICM because false positives and negatives which were not reliable and could not be solved by optimizing the protocol. There was a risk that not all of the TE cells may be removed during mechanical isolation and these may subsequently overgrow and inhibit the growth of ICM cells. On the other hand, strong background with cell clumps was not good choice for our multi colour FISH probe analysis.

Manipulation of the blastocyst with immunosurgery appeared to improve ICM plating efficiency, although in the immunosurgery group there was an enhanced growth of the plated ICMs, number and quality of the ESC colonies did not differ among the groups (Tanaka et al., 2005). The isolation rate of ICM was 100% when C57BL/6 blastocysts treated with antiserum and complement showed lysed trophoblasts and intact ICMs (Zheng et al., 2005). However, in some publications it was mentioned that immunosurgery of blastocyst-stage embryos is an effective procedure for isolating the TE and ICM cells of individual blastocysts, which were also counted by differentially labelling the nuclei (Verlinsky et al., 1998; Handyside and Hunter et al., 1986, 1984).

Nevertheless, optimizing 1:25 dilution of antiserum for 10 minutes, 1:5 dilution of complement for 5 min and 2 hours time interval of antiserum exposition did not always gave the expected results. We had even in the same groups we had different numbers of the ICM / TE cells by observing different labelling and 52%

of immunosurgery performed embryos used to solve these problems. In addition, inner cell masses that were isolated looked like small homogeneous cell clumps that did not let us to analyse the genetic stages of nucleuses clearly.

4. 1. 4. Stem cell culture

Embryonic stem (ES) cells are derived from the pluripotent inner cell mass (ICM) cells of the preimplantation blastocysts (Smith A. G., 2001).

Human embryonic stem cell (hESC) biology is expected to revolutionize the future of medicine by the provision of cell-based therapies for the treatment of a variety of deliberatig diseases. The very first study that describes successful separation of human ICM cells and their continued culture for at least two passages in vitro was published by Bongso et al. (1994). The storage of panels of HLA typed hESC lines containing information with respect to growth characteristics, phenotype, genotype, culture conditions, gender, and stability during prolonged passage, will be very useful for research and application (Bongso et al., 2005). Published data suggest that, the success rate in deriving hES cell lines is highly dependent on the quality of recovered blastocysts, isolation conditions and experience of the group (Mitalipova et al., 2003; Pera et al., 2000). Factors for successful hESC derivation include the availability of adequate quantities of good quality embryos and having an efficient culture procedure to support blastocyst development (Bongso et al., 2005). Therefore on the 4.day we discarded 33 (5%) degenerated embryos and on day 5 129 (19%) of the embryos were discarded which were not available for ESC. 5 early blastocysts, 432 blastocysts, 101 hatched blastocysts, total of 519 (78%)embryos, were brought to ESC and 462 (89%) embryos were developed in culture and produced healthy ICM lumps.

By ESC, blastocysts developed into ICM clumps and removed from adjacent trophectodermal and feeder cells, by mechanical dissection with hypodermic needles. ICM clumps then enzymatically disaggregated with trypsin before seeding. Actually, it is still difficult to determine how successful different researchers have been with the protocols. Reported the successful derivation

were, Cowan et al. (2004.) 17 hESC lines from 97 ICMs, Pickering et al. (2003) 3 lines from 58 embryos and Reubinoff et al. (2000) 2 lines from 4 blastocysts were isolated from early embryos. We cultured embryos only 2 – 3 days and did not passage or develop hESC lines for further developments because of our aim. We also described that isolation, trypsin treatment and fixation were harmful procedures for the cells that 26% (119/462) of ICM cells and 19% (89/462) of TE cells were lost during the procedures. In the end of our procedure, from 462 developed embryonic stem cell cultures ICM cells of 418 (91%) and TE cells of 455 (%99) fixed embryos were available for FICTION.

Although hESCs have a stable developmental capacity, some authors observed that prolonged cultivation in vitro can result in karyotypic changes. Draper et al. (2004) reported chromosomal anomalies in two hESC lines, that were cultured for extended periods of several months (22–60 passages). He suggested that culture methods that favor the growth of undifferentiated hESCs might weed out mutations that cause spontaneous differentiation and cell death. The cells may also have adapted, under the conditions or manipulations during culture in vitro, and selected for genes that will help them thrive (Pera et al., 2004). In contrast, Buzzard et al. (2004) did not observe chromosomal abnormalities in five other lines, which were cultured for the same number of passages and were propagated by the mechanical-dissection method. He suggested that the mechanical-dissection method of passaging, rather than enzymatic dissociation, may have contributed to the cytogenetic stability. Bongso et al. (1984) also showed that hESC were karyotypically normal and subsequently differentiated after the third passage. To conclude hES cells demonstrate high genomic instability and non-predictable differentiation after long-term growth.

These discussions and the outcome does not really concern our results because embryos cultured only 2 - 3days without passaging in ESC culture, which is a short time period for occurring chromosomal abnormalities and can not be reason for observed aneuploidies by ICM cells. Also it has been demonstrated that even aneuploid zygotes can be used as a source for derivation of hES cells (Suss-Toby et al., 2004) that could be used for research purposes.

4.2 Discussion of results

4. 2. 1. Substrains for originating CBA/CaH-T(14;15)6Ca/J strain

- **CBA**

It is an inbred mouse strain. Strain is developed by Strong in 1920 from a cross of a Bagg albino female and a DBA male. CBA was selected for a low mammary tumour incidence and C3H for a high incidence. Now widely distributed, and used as a general-purpose strain.

- **CBA/Ca or CBA/H**

Strain is developed by Haldane and Gruneberg in 1932 and examined by Carter 1947 and Harwell 1954. This substrain used in most British research.

- **CBA/H-T6**

T6 translocation backcrossed to CBA/H by Dr Lyon et al. (1969). Now homozygous for the marker translocation T(14;15) 6Ca, but otherwise congenic with CBA/H.

4. 2. 2. Origin of CBA/CaH-T(14;15)6Ca/J mouse strain

The CBA/CaH-T(14;15)6Ca/J cells carries balanced, translocated chromosomes with breakpoints in 15qA1 and 14qE3 (40,XX,t(14;15)(qE3;qA1) or 40,XY,t(14;15)(qE3;qA1)) in homozygotes. Jackson Laboratories do not have much more information about the CBA/CaH-T(14;15)6Ca/J origin but the strain has this translocation with spontaneous mutation which was discovered coincidentally. Non-disjunctions were detected in frequency of 4.4% in males and 22.2% in females (Jackson Laboratories). Benet et al. (2005) represented similar data that female translocation carriers (65.7%) are twice more frequent than male translocation carriers (34.3%) among the reciprocal translocation carriers.

Jackson Laboratories described that this mouse could be used to support research in cancer researches like tumour incidence and genetic researches. However, there is no confirming publication or study about tumour incidence. Only by the substrain CBA/CaJ, there are publications that high frequency of spontaneous hepatomas observed in males (Rithidech et al., 1999, Figueroa et al., 1994).

Spontaneous mutations normally occur very rarely and may alter the normal phenotype of the strain that did not happen to this strain. CBA/CaH-T(14;15)6Ca/J strain do not suffer and it is obviously that this strain has got chromosomal abnormality. Mice with spontaneous mutations are useful for elucidating basic biological processes, studying relationships between gene mutations and disease phenotypes, and modelling human disease. Investigators are strongly encouraged to research a recommended mouse model to be sure it is appropriate for their research.

According to the references, the mice were sent by M.F. Lyon, who had backcrossed the translocation to CBA/Ca for 13 generations and subsequently made homozygous for the translocation and maintained by brother x sister matings (inbreeding). The mice that arrived at the Jackson Laboratories were homozygous for the translocation and have been maintained as homozygous through at least 160 generations of brother x sister matings. As information obtained from Jackson Laboratories these mice supposed to have 100% translocation but they could not show a proof or test results. However, a simple recrossing will guarantee a homozygous balanced situation in a quarter of next generation (see Figure 1). The FISH-Test we developed proves a well usual method for testing chromosomal situation in our mice.

Homozygous inbred CBA/Ca mouse

Heterozygous CBA/H T(14;15)6Ca/J mouse

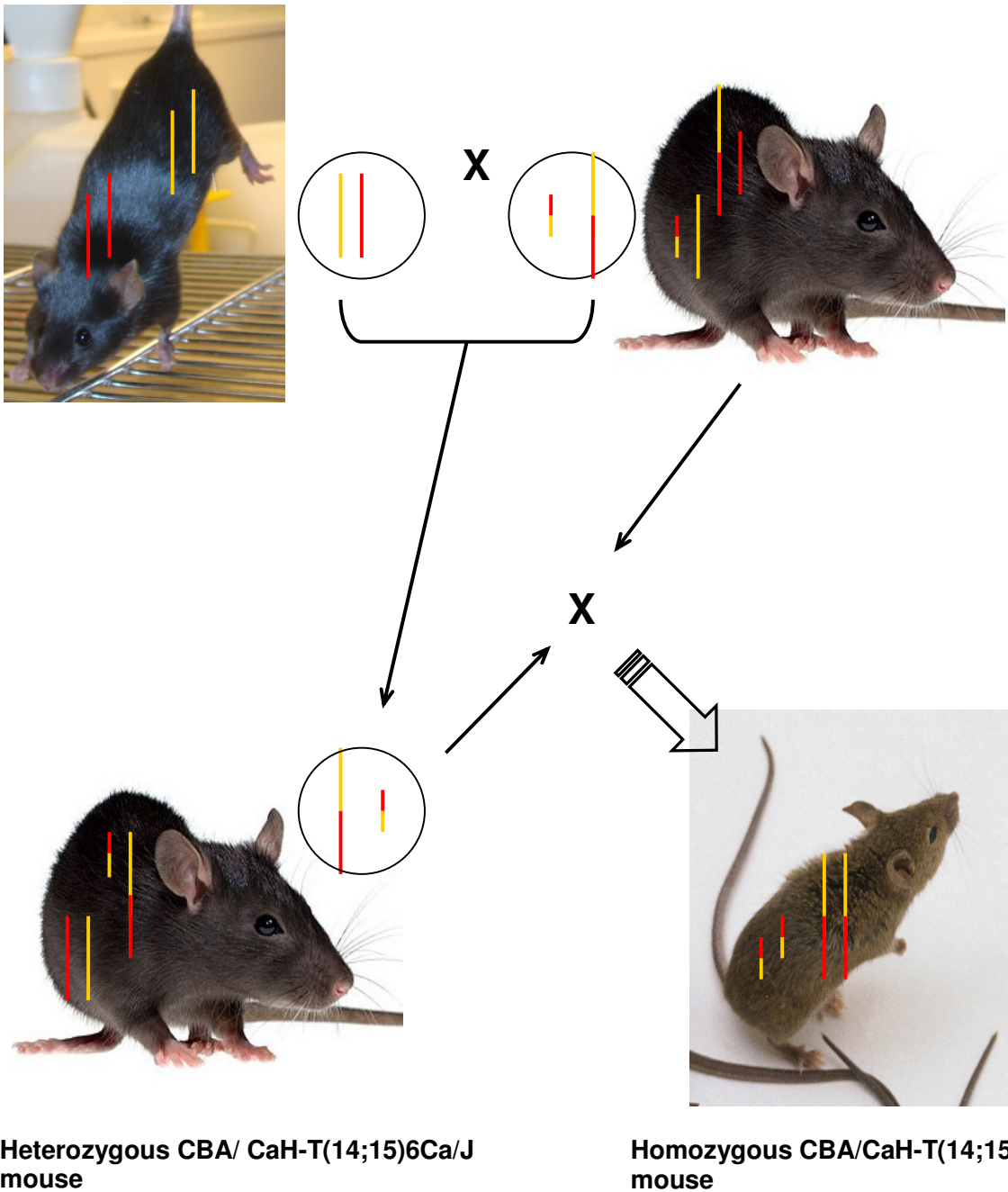


Figure 1: The reciprocal translocation, T(14;15)6Ca was introduced into this substrain by M.F. Lyon followed by 13 generations of backcrossing to CBA/Ca and subsequent inbreeding. CBA/CaH-T(14;15)6Ca/J mouse strain is homozygous for the reciprocal translocation, T(14;15)6Ca and is maintained by inbreeding.

4. 2. 3. ADAMTS12 Expression

As characteristic physical impairments approximately the halves of the gametes produced by heterozygotes produce zygotes, which die in uterus. Due to the translocated chromosomes, it is highly probable that abortions are arranged by unbalanced conditions in the zygote. One of the genes, which is located in the break point region, and has also the potential to cause this effect, could be ADAMTS12 in 15A1 of the CBA/CaH-T(14;15)6Ca/J strain. ADAMTS12 is synthesized as a precursor molecule that is first activated by cleavage of the 178 kd prodomain in a furin-mediated process and subsequently processed into two fragments of different size. The 120 kd N-terminal element involve the metalloproteinase and disintegrin domain, and a 83 kd C-terminal domain containing mostly thrombospondin TS-1 repeats (Cal et al., 2001)

Our data show cDNA products of ADAMTS12 in Lung-, Heart-, and Kidney-tissue of normal mice, whereas in homozygote CBA/CaH-T(14;15)6Ca/J mice it does not (Acar-Perk et al., 2009). A 178 kd pre protein product is only detectable in lung tissues of normal mice, but never in any tissue of homozygote CBA/CaH-T(14;15)6Ca/J strain by western blot. In vivo a human ADAMTS12 protein is traceable in foetal lung tissue only (Cal et al., 2001). In consequence, the expression of the ADAMTS12 on protein stage must be controlled after the transcription, but before the translation, because in heart and kidney RNA is proofable and protein not.

Luan et al., shows that ADAMTS12 is involved in the arthritic process. It is published that ADAMTS12 plays not a small rather an important role in the cartilage oligomeric matrix protein (COMP) degradation in the initiation and progression of arthritis as a COMP -interacting and -degrading (Liu et al., 2006). The C-terminal domain of metalloproteinase was found to be important for binding substrates and determining enzyme selectivity (Martel-Pelletier et al., 2001) ; as C-terminal TSP repeats of ADAMTS12 are required and sufficient for binding to COMP also supports this concept (Liu et al., 2006). Our results show that a loss of this domain does not have an effect mandatorily pathogenously,

because mice with ADAMTS12 knocked out are healthy. With corresponding questions, such the TS-domain is completely knocked or losses can be compensated with transfection vectors and comparisons lead to new insights (Acar-Perk et al., 2009).

While the function of ADAMTS12 is not clear in arthritis (Kevorkian et al., 2004) and asthma (Kurz et al., 2006) deficits of this gene contribute to faulty vascularisation and could not predict cancer proliferation (Cal et al., 2001). Human endothelia cells with additional copies of ADAMTS12 loose their ability to form tubules or spindle-shaped extensions under influence of vascular endothelial growth factor (Liamazares et al., 2007). The thrombospondin motif domain is required for this effect. These cells have diminished levels of active phosphorylated ERK, necessary for vascular development in tumours or under epithelial-mesenchymal transition (EMT) (Liamazares et al., 2007; O'Brien et al., 2004)

These are clear indications that ADAMTS12 influences the RAS MAPK-pathway. Furthermore, sustained activation of ERK seems to be crucial for cell scattering (Liang et al., 2001). Cells of Non-small-cell lung cancer cell line A549 with an additional ADAMTS12 expression produce smaller subcutaneous tumours in immune deficient SCID mice then those without. Therefore, ADAMTS12 unites aspects that correspond to function of tumour suppression.

Illnesses which often develop only in the forward life are particularly arthritis but also asthma. Thus, it is not wondering that the homozygote mice CBA/CaH-T(14;15)6Ca/J do not suffer arthritis or asthma, in general. In our hands, the mice only seldom reached an age that went out more then 25 to 30 weeks. However, there is no study of tumour development with these strains available. The failure of this gene probably therefore seems not to be highly pathogen.

In contrast with its restricted expression in normal tissues, ADAMSTS12 is highly expressed in gastric carcinoma and in other tumours. Furthermore, in tumour cell lines the expression can be induced by fibroblasts, producing grow factor β (Cal et al., 2001). In addition, anti-tumorigenic effects of ADAMTS12 in vivo were evaluated and observations suggested that ADAMTS12 expression might delay

tumour growth upon cells that produce this proteolytic enzyme (Liamazares et al., 2007). In the future, the regulatory roles of ADAMTS12 in angiogenesis or metastasis can be evaluated with help of this mouse strain, perhaps.

ADAMTS12 is only a "small wheel" in the interplay of many genes of the pathways. Its expression in Madin-Darby canine kidney (MDCK) cells prevents the tumorigenic effects of hepatocyte growth factor (HGF) by blocking the activation of the Ras-MAPK signalling pathway with the thrombospondin domains of the metalloproteinase (Liamazares et al., 2007). An ADAMTS12 protein lacking the thrombospondin domains, as we observed in homozygote mice CBA/CaH-T(14;15)6Ca/J, should be unable to inhibit the effects induced by HGF and cause cancer but these mice were totally healthy which means lacking the thrombospondin domains can be compensated and involved by other genes of these multi-function gene family.

4. 2. 4. Oct4 Expression

The transcription factor Oct-4 is thought to be an essential factor in controlling early embryonic development and pluripotency. Oct-4 expression in the mouse is low in early cleavage-stage embryos and appears at the 16-cell stage. It is expressed only in the inner cell mass at the blastocyst stage (Obermiller et al., 2005). A key first step in this process of specification is the down-regulation of Oct4, a transcription factor that acts as a negative regulator of trophoblast specification and genes normally up regulated as the trophectoderm first forms. It is believed to have a positive association with trophoblast specification (Nichols et al., 1998; Palmieri et al., 1994). Oct4 can also affect directly or indirectly many essential processes, such as chromatin remodelling, epigenetic regulation, apoptosis, cell cycle regulation, and signalling during early development. In fact, Oct4 may serve not only as a link between the germ cell and embryonic developmental programs, but also as a switch from a posttranscriptionally-regulated program to one that depends on the transcriptional network (Cauffman et al., 2005).

Normally, Oct4 is expressed in the nuclei of all cells of the cleavage stage embryo, but when the blastocyst forms, its expression is restricted to the ICM. When the Oct4 gene is knocked out, however, no ICM forms and all cells of the embryo default to trophectoderm (Nichols et al., 1998). Accordingly, trophectoderm is only specified in the mouse when Oct4 becomes down regulated. This generality does not apply across all species, Oct4 can be detected in trophectoderm until day 10, two to three days after the blastocyst first forms (Kirchhof et al., 2000; Van Eijk et al., 1999), although its expression there is clearly lower than in the ICM. Indeed, it may be that the mouse is the exception, since Oct4 is also expressed in early human trophectoderm (Hansis et al., 2000). These observations indicate that Oct4 is not a binary off-on switch but that its dosage is critical (Rossant et al., 2003). Dietrich et al. (2007) found that, Oct4 is present in all cells until late blastocyst, gradually disappearing from the TE thereafter. By Obermiller et al. (2005) Oct-4 staining was present in both the inner cell mass and in the trophoblast but staining appeared to be more vivid in the inner cell mass. In some cases, some trophoblast nuclei also did not stain for Oct-4. Obermiller et al. (2005) also represented that, in blastocysts Oct-4 transcripts, proteins were present in the ICM and the TE and added that as Oct-4 transcripts were also found in various differentiated cells, the presence of Oct-4 transcripts or proteins may not be sufficient for identifying undifferentiated cell lines in humans.

Oct4 levels do not affect Cdx2 accumulation and maintenance, suggesting that even if reciprocal inhibition between Oct4 and Cdx2 functions during in vivo patterning, additional regulatory mechanisms must be involved in its regulation (Tolkunova et al., 2006). Thus, Oct4 protein is present in all blastomeres long after Cdx2 is restricted, and it is not until then that Oct4 expression is low in Cdx2⁺ cells. Nevertheless, the proposed reciprocal inhibition pathway could function in the downregulation of Oct4 in Cdx2⁺ TE after the actual patterning process (Dietrich et al., 2007).

To sum up our results, we needed to detect Oct4 expression to distinguish the ICM and TE cells with FICTION and therefore in the same time checked the

expression during ESC culture in ICM and TE cells. Oct4 expression was clearly stronger positive in ICM cells when compared with the positive Cdx signals in the TE and therefore mostly Oct4 was used to differentiate the cells.

Our immunofluorescent detection signals of Oct4 were clearly positive in 99% ICM cells and were Oct4 negative in TE cells, by labelling 470 ICM and 467 TE cells in a total of 65 embryos. However, we also recognised patterns of abnormal Oct4 expression in both TE and ICM or complete absence in 2% embryos. Only clear and strong signals were counted as positive. Only the cases in which nuclear staining was clearly distinguishable from cytoplasmic staining was considered positive.

To conclude our results, Oct4 was a good marker for ICM. This finding does not support results from (Obermiller et al., 2005). Our results therefore confirm the statement of Dietrich et al. (2007) opposite to Obermiller et al. (2005).

4. 2. 5. Cdx Expression

The formation of trophectoderm is not simply a default pathway initiated by the down-regulation of the caudal-like transcription factor Cdx but probably requires specific transcription factors. (Rossant et al., 2003) like the homeodomain protein Cdx2. Cdx2 showed a reciprocal pattern of expression to Oct4 at the blastocyst stage (Hancock et al., 1999; Beck et al., 1995) and Cdx2 is absent from the ICM but expressed in trophectoderm by late blastocysts (Chawengsaksophak et al., 1997). Cdx knockout embryos fail to implant, although they develop to the early blastocyst stage but generally advance no further, and do not form trophoblast outgrowths when cultured (Roberts et al., 2004). Their embryos also cannot be coaxed to produce trophectoderm stem cells (Rossant, 2001).

Dietrich et al. (2007) represented that, the expression patterns of Cdx2 exhibits two specific phases, culminating in its restriction to TE. In the first phase, starting after compaction, blastomeres show highly variable Cdx2 protein levels and Cdx2 variability may originate from asymmetric cell divisions at the 8-cell stage in a non-stereotypic way. Furthermore, there is initially no reciprocal relationship

between Cdx2 and Oct4 protein levels. In the second phase, a definite pattern is established by a sorting process that accommodates intrinsic and extrinsic cues. Data from isolated blastomeres further suggest that differences in Cdx2 levels among blastomeres at morula stage may be generated by asymmetric cell divisions of the 8- to 16-cell stage transition (Dietrich et al., 2007).

Our FICTION results confirm the results from Dietrich et al. (2007) and Chawengsaksophak et al. (1997) that, 80% TE cells gave positive signals with Cdx labelling when detecting Cdx transcription factor detected on 8 embryos containing 40 ICM and 43 TE cells. We also applied simple Cdx IF staining and observed on 40 ICM - 49 TE preparations by all TE cells on TE preparations showed nuclear Cdx positive staining, whereas ICM preparations showed low-level or negative Cdx staining.

Cdx proteins act also as regulators of Hox expression that dose of Cdx protein normally plays a key role in the positioning of Hox gene expressions. Differential dose of Cdx protein along the axis is provided by the additive effects of the three Cdx proteins, as each has a different anterior boundary of expression. In addition, differential dose in gradients is provided by the decay of Cdx proteins in cells left behind by the regressing tail bud (Gaunt et al., 2008). Also increased Cdx protein dose effects upon axial patterning in transgenic lines of mice (Gaunt et al., 2008) and Cdx gene deficiency compromises embryonic hematopoiesis in the mouse (Wang et al., 2008).

According to the publications when we conclude our results, most of the Cdx examined embryos (~30 embryos) signals were clearly weaker in the TE than Oct4 examined embryos with Oct4 signals in ICM cells. In total, 9% TE cells weak positive with 18% Cdx weak positive ICM cells as compared to Oct- 4 weak positive signals in 4% TE cells with 3% ICM cells. The Oct4 signal was more stable than the CDX2 signal in the FICTION procedure. Because our aim was just to distinguish ICM and TE cells for examining their genetic statuses, as described above, mostly Oct4 was used to differentiate the cells that seems to be stronger marker for long procedures.

4. 2. 6. FICTION results

M-FICTION technique provides a new tool for the simultaneous and comprehensive characterization of morphological, immunophenotypic, and genetic features of single cells (Martin-Subero et al., 2002). It can be applied to basic researches by experimental cell biology. It can help to explain the mechanisms of recombination or to study interactions between DNA and proteins between chromosomes and centrosomes. (Roy et al., 2001). Moreover, contaminating tumor cells in stem cell transplants or minimal residual disease after treatment could be detected by M-FICTION. Martin-Subero et al. (2002) tested the performance of the technique by developing assays for the diagnosis of lymphoid malignancies. He designed M-FICTION assays for B-NHL and ALCL combine the detection of specific anti- genes with probes for the most characteristic chromosomal aberrations in these neoplasms. Martin-Subero et al. (2002) concluded that, M-FICTION was restricted to the investigation of a single antigen in combination with up to five FISH probes, which were sufficient to detect the diagnostically most important chromosomal aberrations in the tumors investigated. M-FICTION assays could be used for the early detection of cancer by which the early detection of bladder cancer cells in urine was improved by an M-FICTION assay combining commercially available assays relying on immunohistochemistry and FISH (Sokolova et al., 2000; Thomas et al., 1999). It can also visualize aberrations at gene and protein level simultaneously, which might prove clinically important in some cases of breast or pancreatic cancer with the conflicting results between immunohistochemistry and FISH (Jaffe, 1999). In principle technique is also applicable to paraffin-embedded sections, including tissue CHiPs. (Lindblom et al., 2000)

We applied M-FICTION technique for the first time to distinguish ICM and TE cells, which were isolated individually from ESC, to check the segregation of translocation and aneuploidies. Technique was successfully applied to 510 ICM and 510 TE cells of 73 mice embryos, and we were able to visualize the Oct4 or Cdx antigen expression of cells with T(14;15)6Ca chromosomal translocation region directly. The reliability of the multicolour FISH probe set to detect

T(14;15)6Ca reciprocal translocation was verified by FICTION experiments with the homozygote control groups of examining 85 ICM cells and 77 TE cells with 12 embryos. 12% of the cells gave irregular signals which was above the cut-off limit of 20% for detecting T(14;15)6Ca reciprocal translocation and could be ignored.

Different abnormalities could be observed at the blastocyst stage including embryos lacking visible compaction features, embryos with double blastocoelic cavities and embryos with no inner cell mass (trophoblastic vesicles) or genetic disorders.

Chromosomal mosaicism within the placenta makes cytogenetic interpretation difficult by some authors (Johnson et al., 1993; Fryburg et al., 1992; Kalousek, 1990), with euploidy or aneuploidy as well as with mosaicism in the fetus, which was not the case in our experimental settings by which TE cells (roots of placenta) and ICM cells (roots of fetus) could be isolated.

Chromosomal mosaicism is thought to be causative for different karyotypes within the placenta and a possible unaffected fetus itself, indicating that the mosaic is generated in the embryo and that the trophoblast can function normally in the presence of trisomy (Tantravahi et al., 1996; Wolstenholme, 1996; Fryburg et al., 1992; Williams et al., 1992; Gilbertson et al., 1990). More detailed analysis of the percentage of aneuploid cells present, and distribute between the cytotrophoblast and extra-embryonic mesoderm cell lineages, shows a highly specific pattern for each chromosome (Wolstenholme et al., 1996). The presence of aneuploid cells in the cytotrophoblast layer or mesenchymal stroma may also interrupt normal placental development or placental function during gestation. Association between confined placental mosaicism (CPM) and poor perinatal outcome has been reported (Henderson et al., 1996; Wolstenholme 1996; Williams et al., 1992; Kalousek et al., 1993; Kalousek et al., 1991). By placental mosaicism, placental function is impaired in an increased rate of spontaneous abortions and associated pregnancy complications (Sanchez et al., 1997; Henderson et al., 1996; Kalousek and Barrett, 1994; Pletcher et al., 1994; Johnson et al., 1993; Brady et al., 1992; Fryburg et al., 1992; Kalousek et al.,

1991; Kennerknecht and Terinde, 1990). Also there are reports of other cases showing that no severe developmental retardation in the newborn occurs if only the placenta carries the chromosomally abnormal cell line; therefore, the effect of confined placental mosaicism could be minimal or nonexistent (Wolstenholme et al., 1996; Schwinger et al., 1989).

These can be typical mosaicism cases, which are also frequently seen in human being. It was observed generally by TE rather than ICMs. Therefore, our findings do not confirm most of the studies. According to our results, translocation products of chromosome 14 and 15 were observed among several aneuploid chromosomes in ICM cells by heterozygote-translocated embryos. There were no embryos without aneuploidy by ICM cells, which was not the case for TE. With the results these mice supposed to have an illness by which we supposed to have abnormal embryos or abortions should be observed by their breeding. However, all the mice we received were healthy, had a normal breeding capacity and their embryos were also seem to be healthy. Astner et al. (1998) is also supporting our results with a case report in which, a trisomy 16 (47,XX,+16) mosaicism analysed in all placental cells by chorionic villus sampling (CVS) in the second trimester with a normal fetal karyotype in amniotic fluid cells and in fetal blood lymphocytes. After birth a detailed cytogenetic investigation was done, but only 46,XX karyotypes could be seen in neonatal blood lymphocytes and skin fibroblasts. In the term placenta, no trisomy 16 could be detected in numerous placental biopsies after birth (Astner A. et al., 1998).

In addition, Wolstenholme (1995) reported that maternal uniparental disomy (UPD) for chromosome 16 is associated with miscarriage or severe intra-uterine growth retardation can be compatible with a viable pregnancy. So UPD can be the reason in our cases by which both t(14.15) represent highly probably an UPD (Figure 1). Identical signal pattern on both derivative chromosomes make this very probable.

Analysis of reports of by confined placental mosaicism (CPM) without associated UPD indicates that the presence of high levels of trisomic cells in the placenta alone consistently produces a more variable inhibition of fetal growth, which may

also be associated with late pregnancy loss. The trisomic cell lines may well survive in placental tissues, but may have a proliferation disadvantage and therefore not detectable in term placentas after birth (Astner et al., 1998).

There are reports of chromosome 16 mosaicism as an example, found by CVS, which was very rarely confirmed in amniotic fluid cells or even in fetal blood lymphocytes and skin fibroblasts after birth (Henderson et al., 1996; Kalousek et al., 1993; Fryberg et al., 1992; Kennerknecht and Terinde 1990). It is possible that embryos with trisomy revert to disomy during the course of pregnancy, allowing for longer survival with various abnormalities in growth and morphogenesis. This indicates that however, we have to consider that aneuploid cells were obtained by direct preparation and are of embryoblastic origin rather than trophoblastic, the effect of aneuploidy could be minimal or nonexistent with the birth.

Pierce et al. (1989) represented, that blastocele fluid contains an activity toxic for cells with trophectodermal potential. Furthermore, he believed that other dead cells origin from embryonal carcinoma cells or ICM cells failed to differentiate normally.

This theory also explains why dead cells appear in midstage blastocysts, because dead cells have been reported in normal mid- to late stage blastocysts (Wilson, Smith, 1970; Potts et al., 1967). This mechanism could also explain the purity of cell type of most tissues and the absence of trophoblast or placental tissues in newborn mice. So it is an another theory from Pierce et al. (1989), programmed cell death is designed to eliminate redundant ICM cells with trophectodermal potential, and its mechanism of action is mediated by epigenetic factors in blastocele fluid.

When we analyzed the results from our FICTION, with the hybridization of ICM and TE cells from the male confirmed T(14;15)6Ca reciprocal translocated embryos and all the cells (ICM+TE) of heterozygote translocated embryos carried T(14;15)6Ca reciprocal translocations in a regular way. However, by ICM cells to this translocation also high numbers of aneuploidy observed.

Our results mainly seem to suit to Verlinsky et al. (1998), by which he searched for mosaicism in the inner cell mass in human blastocyst and found high aneuploidy rate by ICMs. However, according to Verlinsky et al. (1998), compared with the early cleavage-stage embryos, human blastocysts have significantly lower degree of mosaicism, which indicates that there is a selection against aneuploid cells and if the number of abnormal cells at the morula stage reaches some threshold level, this leads to whole-embryo degeneration.

We observed aneuploidies by nondisjunction and unbalanced predivision with 2 signals or no signal translocation products and chromosome 14 and 15. These aneuploidies generally observed in ICM cells as showed in results (346 cells no signal and 130 cells had extra 2 signals). In contrast to the heterozygote ICM cells groups, by heterozygote TE cells just on 15 cells no signal observed and on 42 cells 2 signals observed for each chromosome and translocation product. Munne et al. (1998) mentioned that, polyploid and haploid mosaic embryos were usually created at the first division. On the other hand, monospermic diploid mosaics were generated in 97% of cases at the second or later meiosis divisions. Unlike Verlinsky et al. (1998) and Dyce et al. (1987) our results do not show any mosaics between TE and ICM. All of our detected aneuploidies are located in ICM. Dyce et al. (1989) lifts up the hypothesis, that mitotic aneuploidies, which arise during the embryonic development after the onset of fluid accumulation, follow the development biological specification of the cell and will pass on. Due to our results, we would like to form a further hypothesis. We regard it as possible that a biological process recognizes aneuploide cells in an earlier phase of morula and the development specifically steers to the embryo. Also Verlinsky et al. (1998) suppose a selective system against aneuploidies in transition from morula to transition. However, he describes a selection of aneuploidie resulting in 10% of mosaicism in ICM, different to our results.

However, if the abnormal cells do not participate in inner cell mass formation, mosaicism may be a minor problem for preimplantation genetic diagnosis. Cozzi et al. (1999) reported a case with the existence of a trisomic germ cell line in a chromosomally normal woman with recurrent free trisomy 21 in descendants.

This can be explained, that the extra chromosome 21 could divide equal at first meiotic division rather than segregate as an entire univalent, resulting in an increased proportion of aneuploid meiosis II oocytes. There is a balanced condition at the T(14;15)6Ca mice differently than at Cozzi et al. (1999) has described for aneuploidies of human chromosome 21.

While Astner et al. (1998) is describing heaped aneuploidies in placenta instead of in the embryo, Pierce et al. (1989) explains this by a preconnected selection of disturbed cells in the embryo. Because we exclusively prove aneuploidies in ICM of heterozygots at a relatively early time, it is obvious that a corresponding repair mechanism is post-connected for this situation. The repair effect in Cozzi et al. (1999) probably is not effective in the early development of the embryo but during the Oogenese. However, this nevertheless is not excluded.

As described, the female descendants of T(14;15)6Ca mice show more non disjunctions than the male descendants. The balanced condition of the parents suggests that a reason for the accumulation of female aneuploidies lies in the defect of the ADAMTS12, with unknown correlation to their sex.

4.3 Conclusion

We have demonstrated for the first time the feasibility of simultaneous fluorescence immunophenotyping and FISH to check the segregation of translocation and aneuploidies by isolating ICM and TE cells, which were grown individually from ESC.

It was published, that culture and stimulation conditions may influence the aneuploidy frequency in embryos (Weghofer et al., 2008; Munne et al., 1997) and mouse embryos in general make a poor model for humans in genetic area, particularly given that mice have lower rates of aneuploidy compared to humans (Ledbetter, 2008). The high aneuploidy rate reported in our results, does not allow an interpretation of increased risk of aneuploidy for the humans who carry balanced translocation.

By M-FICTION, the diagnostic and prognostic genetic aberrations and the discriminating proteins identified in these and upcoming expression studies could

be detected sensitively and simultaneously at the single cell level. This technique can also be a new efficient method for many other fields of medical research, experimental cell biology and with the importance of M-FICTION for the diagnosis and research of embryology, it can be used in PGD.

Finding out mice lacking of ADAMTS12, will provide an essential tool to understand better the relevance of this enzyme in tumour-associated processes, asthma, arthritis, organogenesis, angiogenesis and reproduction.

For the future, the possibility that autonomus cytoplasmic activity found in mouse can be more investigated (Evsikov et al., 1994; Waksmundzka et al., 1984) and by lower species because of the possibility that it can affect the whole process.

The level of mosaicism in the trophectoderm is lower then that seen in the inner cell mass of the blastocyst would pioneer the further investigations of cell repair or elimination.

Although it was shown that poor quality embryos are often aneuploid, in our trials good quality embryos also contain chromosomal anomalies which can be shown that genetic status is independent from morphology and needed for further investigations in human, which will also help to have new view in PGD.

5. REFERENCES

- Acar-Perk B. , Bräutigam K., Grunewald R., Schmutzler A., Schem C., Arnold N., Jonat W., Weimer J. (2009) Mouse strain CBA/CAH-T(14;15)6CA/J carrying a homozygote reciprocal translocation T(14,15) with break in ADAMTS12 gene. Manuscript submitted in Chromosome Research.
- Astner A., Schwinger E., Caliebe A., Jonat W., Gembruch U. (1998) Sonographically Detected Fetal and Placental Abnormalities Associated with trisomy 16 Confined to the Placenta, A case report and review of the literature. *Prenatal Diagnosis* ,18: 1308–1315.
- Baart, E.B. , Martini E., van den Berg I., Macklon N.S., Galjaard R.J., Fauser B.C., Van Opstal D. (2006) Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF. *Hum Reprod*, 21: 223–233.
- Beck F., Erler T., Russell A., James R. (1995) Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. *Dev Dyn*, 204:219-227.
- Benet J., Oliver-Bonet M., Cifuentes P., Templado C., Navarro J. (2005) Segregation of chromosomes in sperm of reciprocal translocation carriers: a review. *Cytogenet Genome Res*, 111:281-90.
- Benkhalifa M., (2005) Array comparative genomic hybridization profiling of firsttrimester spontaneous abortions that fail to grow in vitro. *Prenat Diagn*, 25: 894–900.
- Bongso A., Fong C. Y., Ng S. C., Ratnam S. (1984) Human blastocyst culture and derivation of embryonic stem cell lines. *Theriogenology*, 41:161.
- Bongso A., Fong C. Y., Ng S. C., Ratnam S. (1994) Human embryonic behaviour in a sequential human oviductendometrial coculture system. *Fertil Steril*, 61: 976-978.
- Bongso A., Tan S. (2005) Human Blastocyst Culture and Derivation of Embryonic Stem Cell Lines. *Stem Cell Reviews Humana Press Inc.*, ISSN 1550-8943.

- Brady K., Polzin W.J., Kopelman J.N., Read J.A. (1992) Risk of chromosomal abnormalities in patients with idiopathic polyhydramnios. *Obstet Gynecol*, 79: 234–238.
- Brooke D., Orsi N., Ainscough J., Holwell S., Markham A., Coletta P. (2007) Human menopausal and pregnant mare serum gonadotrophins in murine superovulation regimens for transgenic applications. *Theriogenology*, 67:1409-1413.
- Buzzard J.J., Gough N.M., Crook J.M., Colman A. (2004) Karyotype of human ES cells during extended culture. *Nat Biotechnol*, 4:381-2.
- Cal S., Arguelles J.M., Fernandez P.L., López-Otín C. (2001) Identification, characterization, and intracellular processing of ADAM-TS12, a novel human disintegrin with a complex structural organization involving multiple thrombospondin-1 repeats. *J Biol Chem*, 276:17932-40.
- Chano T., Saeki Y., Serra S., Matsumoto K., Okabe H. (2002) Short Communication Preferential Expression of RB1-Inducible Coiled-Coil 1 in Terminal Differentiated Musculoskeletal Cells *American Journal of Pathology*, 161:359-64.
- Carson S. A., Gentry W. L., Smith A. L., Buster J. E. (1993) Animal Investigations, Trophectoderm Microbiopsy in Murine Blastocysts: Comparison of Four Methods. *Journal of Assisted Reproduction and Genetics*, 10:427-33.
- Cauffman G., Van de Velde H., Liebaers I., Van Steirteghem A. (2005) Oct-4 mRNA and protein expression during human preimplantation development. *Mol Hum Reprod*, 11:173-81.
- Chawengsaksophak K., James R., Hammond V.E., Kontgen F., Beck F. (1997) Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature*, 386:84-87.
- Cowan C.A., Klimanskaya I., McMahon J., et al. (2004) Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med*, 350:1353-1356.
- Cozzi J., Conn C.M., Harper J., Winston R.M., Rindl M., Farndon P.A., Delhanty J.D. (1999) A trisomic germ cell line and precocious chromatid segregation leads to recurrent trisomy 21 conception. *Hum Genet* 104:23-28.

- Cui K.-H., Verma P. J., Matthews C. D. (1993) Hatching rate: an Optimal Discriminator for the Assessment of Single-Blastomere Biopsy. *Journal of Assisted Reproduction and Genetics*, 10:157-162.
- Dagmar Z., Andrew J. W., Jack B. and Francesco M. (2004) Impaired fertility in T-stock female mice after superovulation. *Reproduction*, 128:573-58.
- Daphnis D.D., Fragouli E., Economou K., Jerkovic S., Craft I.L., Delhanty J.D., Harper J.C. (2008) Analysis of the evolution of chromosome abnormalities in human embryos from Day 3 to 5 using CGH and FISH. *Mol Hum Reprod*, 14: 117–125.
- DeBry R.W, M.F. Seldin. (1996) Human/mouse homology relationships. *Genomics* 33:337-351.
- Delhanty, J.D. (2005) Mechanisms of aneuploidy induction in human oogenesis and early embryogenesis. *Cytogenet Genome Res*, 111: 237–244.
- De Vos A., Van Steirteghem A. (2001) Aspects of biopsy procedures prior to preimplantation genetic diagnosis. *Prenatal Diagnosis*, 21:767– 780.
- Dietrich J.-E., Hiiragi T. (2007), Stochastic patterning in the mouse pre-implantation embryo. *Development*, 134:4219-4231.
- Dokras A., Sargent I. L., Ross C., Gardner R. L., Barlow D. H. (1990) Trophectoderm biopsy in human blastocysts. *Human Reprod* 5:821–825.
- Dokras A., Sargent I.L., Gardner R.L., Barlow D.H. (1991) Human trophoctoderm biopsy and secretion of chorionic Gonadotrophin. *Human Reprod*, 6:1453-1459.
- Dorritie K., Montagna C., Difilippantonio M.J., Ried T. (2004) Advanced Molecular Cytogenetics in Mouse and Human (Fish, Sky and CGH) *Expert Rev Mol Diagn* 4:663-676.
- Draper J.S., Smith K., Gokhale P. (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat Biotech*, 22:53–54.
- Dyce J., George M., Goodall H., Flemig T. P. (1987) Do trophoctoderm and inner cell mass cells in the mouse blastocyst maintain discrete lineage? *Development*, 100:685–698.

- Elmazar M. M., Vogel R., Spielmann H. (1989) Maternal factors influencing development of embryos from mice superovulated with gonadotropins. *Reprod Toxicol*, 3:135–138.
- Evsikov SV, Evsikov AV. (1994) Preimplantation development of manipulated mouse zygotes fused with the second polar bodies: a cytogenetic study. *Int J Dev Biol*, 38:725-30.
- Figuroa C.D., Taberner P.V. (1994) Pancreatic islet hypertrophy in spontaneous maturity onset obese-diabetic CBA/Ca mice. *Int J Biochem*, 26:1.
- Fritz B. (2001) Cytogenetic analyses of culture failures by comparative genomic hybridisation (CGH) re-evaluation of chromosome aberration rates in early spontaneous abortions. *Eur J Hum Genet*, 9:539–547.
- Fryburg J.S., Dimaio M.S., Mahoney M.J. (1992) Postnatal placental confirmation of trisomy 2 and trisomy 16 detected at chorionic villus sampling: a possible association with intrauterine growth retardation and elevated maternal serum alpha-fetoprotein. *Prenat Diagn*, 12:157–162.
- Gates A. H. (1969) Time relationship between neural stimulus for LH-releasing factor and ovulation in the PMS treated mouse. *Am Zool*, 9:1080.
- Gaunt S.J., Drage D., Trubshaw R.C. (2008) Increased Cdx protein dose effects upon axial patterning in transgenic lines of mice. *Development*, 135:2511-2520.
- Geigl J.B., Obenauf A.C., Schwarzbraun T., Speicher M.R. (2008) Defining 'chromosomal instability'. *Trends Genet*, 24:64-69.
- Gregory et al. (2002) A physical map of the mouse genome. *Nature*, 418:743-750.
- Gilbertson N.J., Taylor J.W., Kovar I.Z. (1990). Mosaic trisomy 16 in a live newborn infant. *Arch Dis Child*, 65:388–389.
- Gomez C.M., Muggleton-Harrist A.L. , Whittingham D.G. , Hoodt L.E. , Readhead C. (1990) Rapid preimplantation detection of mutant (shiverer) and normal alleles of the mouse myelin basic protein gene allowing selective implantation and birth of live young prenatal diagnosis/trophectoderm biopsy/polymerase chain reaction). *Proc Nati Acad Sci*, 87: 4481-4484.

- Hancock S.N., Agulnik S.I., Silver L.M., Papaioannou V.E. (1999) Mapping and expression analysis of the mouse ortholog of *Xenopus* Eomesodermin. *Mech Dev*, 81:205-208.
- Handyside A.H. and Hunter S. (1984) A rapid procedure for visualising the inner cell mass and trophectoderm nuclei of mouse blastocysts in situ using polynucleotide specific fluorochromes. *J Exp Zool*, 231:429-434.
- Handyside A.H., Hunter S. (1986) Cell division and death in the mouse blastocyst before implantation. *Roux's Arch Dev Biol*, 195:519–526.
- Hansis C., Grifo J.A., Krey L.C. (2000) Oct-4 expression in inner cell mass and trophectoderm of human blastocysts. *Mol Hum Reprod*, 6:999-1004.
- Henderson K.G., Shaw T.E., Barrett I.J., Telenius A.H.P., Wilson R.D., Kalousek D.K. (1996). Distribution of mosaicism in human placentae. *Hum Genet*, 97:650–654.
- Harper J., Sermon K., Geraedts J., Vesela K., Harton G., Thornhill A., Pehlivan T., Fiorentino F., SenGupta S., de Die-Smulders C., Magli C., Moutou C., Wilton L. (2008) What next for preimplantation genetic screening? *Hum Reprod*, 23:478-80.
- Hetherington C. M. (1987) Mouse husbandry. In *Mammalian Development: A Practical Approach*. Oxford IRL Press, 1-12.
- Hogan B., Constantini F., Lacy E. (1986) *Manipulating the Mouse Embryo*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory.
- Hogan B., Beddington R., Constantini F., Lacy E. (1994) *Manipulation of the Mouse Embryo*. Cold Spring Harbor, NY: Cold Spring Harbor Press.
- Hopman A.H.N., Wiegant J., van Duijn P. (1987) Mercurated nucleic acid probes, a new principle for non-radioactive in situ hybridization. *Exp Cell Res*, 169:357-368.
- Jaffe E.S. (1999) Hematopathology: integration of morphologic features and biologic markers for diagnosis. *Mod Pathol*, 12:109–115.
- Johnson M.P., Childs M.D., Robichaux A.G. III, Isada N.B., Pryde P.G., Koppitch F.C. III, Evans M.I. (1993). Viable pregnancies after diagnosis of trisomy 16

- by CVS: lethal aneuploidy compartmentalized to the trophoblast, *Fetal Diagn Ther*, 8:102–108.
- Julie R.K, Xiao-Ning C., Keri L.D., David N., Mary L.O., and Bruce W.B. (1999) Mouse molecular cytogenetic resource: 157 BACs link the chromosomal and genetic maps. *Genome Res*, 9:514-523.
- Kevorkian L., Young D. A., Darrah C., Donell S.T., Shepstone L, Porter S., Brockbank S.M.V., Edwards D.R., Parker A.E., Clark I.M. (2004) Expression profiling of metalloproteinases and their inhibitors in cartilage. *Arthritis Rheumatism* 50:131-141.
- Kirchhof N., Carnwath J.W., Lemme E., Anastassiadis K., Scholer H., Niemann H. (2000) Expression pattern of Oct-4 in preimplantation embryos of different species. *Biol Reprod*, 63:1698-1705.
- Kalousek D.K. (1990). Confined placental mosaicism. *Pediatr Pathol*, 10:69–77.
- Kalousek, D.K., Howard-Peebles, P.N., Olson, S.B., Barrett, I.J., Dorfmann, A., Black, S.H., Schulmann, J.D., Wilson, R.D. (1991) Confirmation of CVS mosaicism in term placentae and high frequency of intrauterine growth retardation association with confined placental mosaicism, *Prenat Diagn*, 11: 743–750.
- Kalousek D.K., Barrett I.J. (1994) Confined placental mosaicism and stillbirth. *Pediatr Pathol*, 14:151–159.
- Kalousek D.K., Langlois S., Barrett I., Wilson D.R., Howard-Peebles P.N., Johnson M.P., Giorgiutti E. (1993) Uniparental disomy for chromosome 16 in humans. *Am J Hum Genet*, 52:8–16.
- Kennerknecht I., Terinde R. (1990) Intrauterine growth retardation associated with chromosomal aneuploidy confined to the placenta. Three observations: triple trisomy 6, 21, 22; trisomy 16; and trisomy 18. *Prenat Diagn*, 10:539–544.
- Korenberg J.R., Chen X-N. (1995) Human cDNA mapping using a high resolution R-banding technique and fluorescence in situ hybridization. *Cytogenet & Cell Gent* 69:196-200.

- Kurz T., Hoffjan S., Hayes M. G., Schneider D., Nicolae R., Heinzmann A., Jerkic S. P., Parry R., Cox N. J., Deichmann K. A., Ober C. (2006) Fine mapping and positional candidate studies on chromosome 5p13 identify multiple asthma susceptibility loci. *J Allergy Clin Immunol*, 118:396-402.
- Ledbetter D.H. (2008) Cytogenetic technology-genotype and phenotype. *N Engl J Med*, 359:1728-30.
- Ledbetter D.H. (2008) Chaos in the embryo. An analysis of embryos derived from in vitro fertilization reveals high rates of structural abnormalities *nature medicine*. 14:577–583.
- Legge M. and Sellens M.H. (1994) Optimization of superovulation in the reproductively mature mouse. *Journal of Assisted Reproduction and Genetics*, 11:312–318.
- Liang, C.C., Chen H.C. (2001) Sustained activation of extracellular signal-regulated kinase stimulated by hepatocyte growth factor leads to integrin alpha 2 expression that is involved in cell scattering. *J Biol Chem* 276:21146-21152.
- Li M., DeUgarte C.M., Surrey M., Danzer H., DeCherney A., Hill D.L. (2005) Fluorescence in situ hybridization reanalysis of day-6 human blastocysts diagnosed with aneuploidy on day 3. *Fertil Steril*, 84:1395–1400.
- Lindblom A., Liljegren A. (2000) Regular review: tumour markers in malignancies. *BMJ*, 320:424-427. Erratum in: *BMJ* 320:867.
- Liu C.J., Kong W., Xu K., Luan Y., Ilalov K., Sehgal B., Yu S., Howell R.D., Di Cesare P.E. (2006) ADAMTS-12 associates with and degrades cartilage oligomeric matrix protein. *J Biol Chem*, 281:15800-15808.
- Llamazares M., Obaya A.J., Moncada-Pazos A., Heljasvaara R., Espada J., López-Otín C., Cal S. (2007) The ADAMTS12 metalloproteinase exhibits anti-tumorigenic properties through modulation of the Ras-dependent ERK signalling pathway. *J Cell Scien*, 120:3544-3552.
- Lyon M.F. and Hawkes S. (1969). *Mouse News Letter*, 41: 28.

- Macklon N.S., Geraedts J.P., Fauser B.C. (2002) Conception to ongoing pregnancy: the 'black box' of early pregnancy loss. *Hum Reprod Update*, 8: 333–343.
- Martel-Pelletier J., Welsch D.J., Pelletier J.P. (2001) Metalloproteases and inhibitors in arthritic diseases. *Best Pract Res Clin Rheumatol*, 15:805-829.
- Martin-Subero J.I., Chudoba I., Harder L., Gesk S., Grote W., Javier Novo F., Calasanz M.J., Siebert R., (2002) Multicolor-FICTION: Expanding the Possibilities of Combined Morphologic, Immunophenotypic, and Genetic Single Cell Analyses. *American Journal of Pathology*, 161:413-420.
- Matzuk M.M., Lamb D.J., (2002) Genetic dissection of mammalian fertility pathways. *Nat Cell Biol*, 4:s41-49.
- McArthur S.J., Leigh D., Marshall J.T., de Boer K.A. and Jansen R.P.S., (2005) Pregnancies and live births after trophoctoderm biopsy and preimplantation genetic testing of human blastocysts. *Fertility and Sterility*, 84:1628-1636.
- Miller B.G., Armstrong D.T. (1981) Superovulatory doses of pregnant mare serum gonadotropin cause delayed implantation and infertility in immature rats. *Biology of Reproduction*, 25:253–260.
- Miller B.G., Armstrong D.T. (1981) Effects of superovulatory dose of pregnant mare serum gonadotropin on ovarian function, serum estradiol and progesterone levels and early embryo development in immature rats. *Biol Reprod*, 25:261-271.
- Mitalipova M., Calhoun J., Shin S., Winger D., Schulz T., Noggle S., Venable A., Lyons I., Robins A., Stice S. (2003) Human embryonic stem cell lines derived from discarded embryos. *Stem Cells*, 21: 521–526.
- Muggleton-Harris A.L., Findlay I. (1991) In-vitro studies on 'spare' human preimplantation embryos in culture. *Human Reproduction*, 6:85-92.
- Muggleton-Harris A.L., Glazier A.M., Pickering S. (1993) Biopsy of the human blastocyst and polymerase chain reaction (PCR) amplification of the b-globin gene and a dinucleotide repeat motif from 2-6 trophoctoderm cells. *Human Reproduction*, 8: 2197–2205.

- Muggleton-Harris A.L., Glazier A.M., Pickering S., Wall M. (1995) Genetic diagnosis using polymerase chain reaction and fluorescent in-situ hybridization analysis of biopsied cells from both the cleavage and blastocyst stages of individual cultured human preimplantation embryos. *Human Reproduction*, 10:183–192.
- Munne S., Weier H.U.G., Grifo J., Cohen J. (1994) Chromosome Mosaicism in Human Embryos. *Biology of Reproduktion*, 51:373-379.
- Munne S., Magli C., Adler A., Wright G., de Boer K., Mortimer D., Tucker M., Cohen J., Gianaroli L. (1997) Treatment-related chromosome abnormalities in human embryos. *Hum Reprod*, 12:780-784.
- Munné S., Cohen J. (1998) Chromosome abnormalities in human embryos. *Hum Reprod Update*, 4:842-855.
- Munné S., Velilla E., Colls P., Garcia Bermudez M., Vemuri M.C., Steuerwald N., Garrisi J., Cohen J. (2005) Self-correction of chromosomally abnormal embryos in culture and implications for stem cell production. *Fertil Steril* 84:1328-1334.
- Nagy A., Gertsenstein M., Vintersten K., Behringer R. (2003) *Manipulating the Mouse Embryo, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Nichols J., Zevnik B., Anastasiadis K., Niwa H., Klewe-Nebenius D., Chambers I., Schöler H., Smith A. (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95:379–391.
- Niwa H., Miyazaki J.-i., Smith A.G. (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet*, 24:372–376.
- Nys M., Van Steirteghem A.C., (1987) Assessment of different isolation procedures for blastomeres from two-cell mouse embryos. *Human Reproduction*, 2:421-424.
- Obermiller A., Choi Y-H., Hinrichs K. (2005) Oct-4 Expression in the Equine Embryo. Summer Veterinary Student Research Fellows Program: Abstracts.

- O'Brien L. E., Tang K., Kats E. S., Schutz-Geschwender A., Lipschutz J. H., Mostov K. E. (2004). ERK and MMPs sequentially regulate distinct stages of epithelial tubule development. *Dev Cell*, 7:21-32.
- Ozgunen K.T., Erdogan S., Mazmanoglu N., Pamuk I., Logoglu G., Ozgunen T. (2001) Effect of gonadotrophin dose on oocyte retrieval in superovulated BALB/c mice. *Theriogenology* 56:435–445.
- Palmieri S.L., Peter W., Hess H., Schöler H.R. (1994) Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev Biol*, 166:259–267.
- Pera M.F., Reubinoff B., Trounson A. (2000) Human embryonic stem cells. *Journal of Cell Science*, 113:5–10.
- Pera M.F. (2004) Human embryonic stem cells: prospects for development. *Development*, 131:5515-5525.
- Pesce M., Schöler H.R. (2001) Oct-4: gatekeeper in the beginnings of mammalian development. *Stem Cells*, 19:271-278.
- Pickering S.J., Braude P.R., Patel M., et al. (2003) Preimplantation genetic diagnosis as a novel source of embryos for stem cell research. *RBM Online*, 7: 353-364.
- Pierce G.B., Lewellyn A.L., Parchment R.E. (1989) Mechanism of programmed cell death in the blastocyst. *Proc Natl Acad Sci* 86:3654-3658.
- Potts D.M., Wilson I.B. (1967) The preimplantation conceptus of the mouse at 90 hours post coitum. *J Anat*, 102:1-11.
- Reubinoff B.E., Pera M.F., Fong C.Y., et al. (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol*, 18:399-404.
- Ried T., Schröck E., Ning Y., Wienberg J. (1998) Chromosome painting: a useful art. *Human Mol Genet*, 7:1619-1626.
- Rithidech K., Dunn J.J., Bond V.P., Gordon C.R., Cronkite E.P. (1999) Characterization of genetic instability in radiation- and benzene-induced murine acute leukemia. *Mutat Res*, 428:33-39.

- Roberts R.M., Ezashi T., Das P. (2004) Trophoblast gene expression: Transcription factors in the specification of early trophoblast. *Reproductive Biology and Endocrinology*, 2:47.
- Rossant J. (2001) Stem Cells from the mammalian blastocyst. *Stem Cells*, 19:477-482.
- Rossant J., Chazaud C., Yamanaka Y. (2003) Lineage allocation and asymmetries in the early mouse embryo , *Philos Trans R Soc Lond B Biol Sci*, 358:1341-134.
- Roy L., Coullin P., Vitrat N., Hellio R., Debili N., Weinstein J., Bernheim A., Vainchenker W. (2001) Asymmetrical segregation of chromosomes with a normal metaphase/anaphase checkpoint in polyploid megakaryocytes. *Blood*, 97:2238–2247.
- Sanchez J.M., De Diaz S.L., Panal M.J., Moya G., Kenny A., Iglesias D., Wolstenholme J. (1997). Severe fetal malformations associated with trisomy 16 confined to the placenta. *Prenat Diagn*, 17:777–779.
- Schinzel A. (2001) *Catalogue of Unbalanced Chromosome Aberrations in Man*. Walter de Gruyter, New York.
- Schwinger E., Seidl E., Klink F., Rehder H. (1989) Chromosome mosaicism of the placenta: a cause of developmental failure of the fetus? *Prenat Diagn*, 9:639–647.
- Smith A.G. (2001) Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol*, 17:435–462.
- Sokolova I.A., Halling K.C., Jenkins R.B., Burkhardt H.M., Meyer R.G., Seelig S.A., King W. (2000) The development of a multitarget, multicolour fluorescence in situ hybridization assay for the detection of urothelial carcinoma in urine. *J Mol Diagn*, 2:116–123.
- Strumpf D., Mao C-A., Yamanaka Y., Ralston A., Chawengsaksophak K., Beck F., Rossant J., (2005) Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development*, 132: 2093-2102.

- Suss-Toby E., Gerecht-Nir S., Amit M., Manor D., Itskovitz-Eldor J. (2004) Derivation of a diploid human embryonic stem cell line from a mononuclear zygote. *Hum Reprod*, 19:670-675.
- Tamai Y., Nakajima R., Ishikawa T., Takaku K., Seldin M.F., Taketo M.M. (1999) Colonic hamartoma development by anomalous duplication in Cdx2 knockout mice. *Cancer Res*, 59:2965-2970.
- Tanaka N., Takeuchi T., Neri Q. V., Rosenwaks Z., Palermo G. D.. (2008) Cell Harvesting, Cdx gene deficiency compromises embryonic hematopoiesis in the mouse Effect of Immunosurgery on ICM Attachment and Embryonic Stem. *PNAS*, 105:7756-7761.
- Tantravahi U., Matsumoto C., Delach J., Craffey A., Smeltzer J., Benn P. (1996). Trisomy 16 mosaicism in amniotic fluid cell cultures. *Prenat Diagn*, 16:749-754.
- Tarin J.J., Perez-Albala S., Cano A. (2002) Stage of the estrous cycle at the time of pregnant mare's serum gonadotropin injection affects the quality of ovulated oocytes in the mouse. *Molecular Reproduction and Development*, 61:398–405.
- Tavmergen E., Tavmergen E. N., Capanoglu R. (1991) The experimental mouse model in building an in vitro fertilization center. *Zentralbl Gynakol*, 113:317-22.
- Telenius, H., Carter N.P., Bebb C.E., Nordenskjöld M., Ponder B.A., Tunnacliffe A. (1992) Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. *Genomics*, 13:718-25.
- Thomas L., Leyh H., Marberger M., Bombardieri E., Bassi P., Pagano F., Pansadoro V., Sternberg C.N., Boccon-Gibod L., Ravery V., Le Guludec D., Meulemans A., Conort P., Ishak L. (1999) Multicenter trial of the quantitative TRAK assay in the detection of bladder cancer. *Clin Chem*, 45:472–477.
- Tolkunova E., Cavaleri F., Eckardt S., Reinbold R., Christenson L.K., Schöler H.R., Tomilin A. (2006) The caudal-related protein cdx2 promotes trophoblast differentiation of mouse embryonic stem cells. *Stem Cells*, 24:139-44.
- Vanneste E., Voet T., Le Caignec C., Ampe M., Konings P., Melotte C., Debrock S., Amyere M., Vikkula M., Schuit F., Fryns J.P., Verbeke G., D'Hooghe T.,

- Moreau Y., Vermeesch J.R. (2009) Chromosome instability is common in human cleavage-stage embryos. *Nat Med*, 15:577-583.
- Van der Auwera I., D'Hooghe T. (1999) The influence of in vitro culture versus stimulated and untreated oviductal environment on mouse embryo development and implantation. *Human Reproduction*, 14:2570-2574.
- Van Eijk M.J., Rooijen M.A., Modina S., Scesi L., Folkers G., Tol H.T., Bevers M.M., Fisher S.R., Lewin H.A., Rakacolli D., Galli C., Vaureix C., Trounson AO, Mummery CL, Gandolfi F. (1999) Molecular cloning, genetic mapping, and developmental expression of bovine POU5F1. *Biol Reprod*, 60:1093-1103.
- Veiga A., Sandalinas M., Benkhalifa M., et al. (1997) Laser blastocyst biopsy for preimplantation diagnosis in the human. *Zygote*, 5: 351–354.
- Verlinsky Y., Evsikov S. (1998) Mosaicism in the inner cell mass of human blastocysts. *Human Reprod*, 13:3151-3155.
- Voullaire L., Slater H., Williamson R., Wilton L. (2000) Chromosome analysis of blastomeres from human embryos by using comparative genomic hybridization. *Hum Genet*, 106:210-7.
- Waksmundzka M., Krysiak E., Karasiewicz J., Czołowska R., Tarkowski AK. (1984) Autonomous cortical activity in mouse eggs controlled by a cytoplasmic clock. *J Embryol Exp Morphol*, 79:77-96.
- Walton E. A., Armstrong D. T. (1983) Oocyte normality after superovulation in immature rats. *Journal of Reproduction and Fertility*, 67:309–314.
- Wang Y., Yabuuchi A., McKinney-Freeman S., Ducharme D.M., Ray M.K., Chawengsaksophak K., Archer T.K., Daley G.Q.. (2008) Cdx gene deficiency compromises embryonic hematopoiesis in the mouse. *Proc Natl Acad Sci*, 105:7756-7761.
- Weimer J., Koehler M. R., Wiedemann U., Attermeyer P., Jacobsen A., Karow D., Kiechle M., Jonat W., Arnold N. (2001) Highly comprehensive karyotype analysis by a combination of spectral karyotyping (SKY), microdissection, and reverse painting (SKY-MD). *Chrom Res*, 9:395-402.

- Wells, D., Delhanty J.D. (2000) Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. *Mol Hum Reprod*, 6:1055–1062.
- Weghofer A., Munné S., Brannath W., Chen S., Tomkin G., Cekleniak N., Garrisi M., Barad D., Cohen J., Gleicher N. (2008) The impact of LH-containing gonadotropins on diploidy rates in preimplantation embryos: long protocol stimulation. *Hum Reprod*, 23:499-503.
- Williams J. III, Wang B.B., Rubin C.H., Clark R.D., Mohandas T.K. (1992) Apparent non-mosaic trisomy 16 in chorionic villi: diagnostic dilemma or clinically significant finding? *Prenat Diagn*, 12:163–168.
- Wilson I.B., Smith M.S. (1970) Primary trophoblastic invasion at the time of nidation. In: Hubinont PO, Leroy F, Robyn C, Leleux P (eds) *Ovo-implantation*, Karger, Basel.
- Winslow T (2001) *Medical and scientific illustration*.
<http://teresewinslow.com/news.asp>
- Wolstenholme J. (1995) An audit of trisomy 16 in man. *Prenat Diagn* 15:109-121.
- Wolstenholme J., (1996) Confined Placental Mosaicism for Trisomies 2, 3, 7, 8, 9, 16, 22 , Their Incidence, Likely Origins and Mechanisms for Cell Lineage Compartmentalization. *Prenatal Diagnosis*, 16:511-524.
- Yamazaki K., Kato Y. (1989) Sites of Zona Pellucida shedding by mouse embryo other than muran trophectoderm. *The journal of experimental zoology*, 249: 347-349.
- Yeom Y.I., Fuhrmann G., Ovitt C.E., Brehm A., Ohbo K., Gross M., Hübner K., Schöler H.R. (1996) Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. *Development*, 122:881–894.
- Yun Y.W., Yu F.Y., Yuen B.H., Moon Y.S. (1989) Effects of a superovulatory dose of pregnant mare serum gonadotropin on follicular steroid contents and oocyte maturation in rats. *Gamete Research*, 23:289 – 298.
- Zheng Y. L., Jiang M. X., OuYang Y. C., Sun Q. Y. and Chen D. Y. (2005) Production of mouse by inter-strain inner cell mass replacement. *Zygote*, 13:73–77.

6. SUMMARY

Errors in the execution of meiosis can result in the generation of aneuploid gametes, which are associated with birth defects and infertility. According to published results; in 3% of chorionic villus sampling (CVS), the chromosomal abnormalities are being mosaics and do not mandatory affect the fetus (Astner et al., 1998) in human beings. So far, little is known about the molecular mechanisms of chromosome segregation and its leading to conditions. Therefore, we wanted to find out the distribution of aneuploidy (and translocation) in early embryogenesis in mouse trophoblast (TE) and inner cell mass (ICM) cells. Our main interest is, if aneuploidy are rather provable in the TE side and cause abortions, or in ICM side that results with defected babies or development in the early embryogenesis.

In total, 241 female mice were sacrificed and 5 types of strains were examined in our experimental setting, which were B6D2F1, C3H/HEN, C57BL/6, BALB/c and CBA/CaHT(14;15)6Ca/J. The experimental group based on CBA/CaHT(14;15)6Ca/J mouse strain, which has a balanced reciprocal translocation between chromosomes 14 and 15. Although there are no single chromosome 14 and 15, except the translocation chromosome, these mice were totally healthy and had breeding capacity. Due to the homozygosity, it is possible to observe the segregation of chromosomes.

Trophectoderm (TE) biopsy, embryonic stem culture (ESC) and immunosurgery (IMS) protocols were evaluated for isolating individual ICM and TE cells. IMS and TE biopsy did not lead to our aim, because of their technical difficulties and false negative, positive results. Therefore, ESC technique was applied on 519 embryos and 462 (89%) embryos were developed healthy in culture.

Specific FISH probe was developed for CBA/CaHT(14;15)6Ca/J translocated mice with four differently labelled BACs. We have demonstrated the feasibility of simultaneous fluorescence immunophenotyping and FISH to check the segregation of translocation and aneuploidy for the first time by isolating ICM and TE cells. FICTION (fluorescence immunophenotyping and interphase

cytogenetics as a tool for investigation of neoplasms) technique was successfully applied in 510 ICM and 510 TE cells of 73 mice embryos. Thus, we were able to visualize the antigen expression of Oct4 as ICM marker or Cdx as TE marker together with the multicolour FISH probe. Eighty % TE cells gave positive signals with Cdx labelling and 99% ICM cells gave positive signals with Oct4 labelling.

The results from FICTION reveal that, all the cells (ICM+TE) of heterozygote translocated embryos carried the T(14;15)6Ca reciprocal translocation in a regular way. In heterozygote ICM cells we observed high numbers of aneuploidy than in heterozygote TE cells. Aneuploidies were observed by nondisjunction and unbalanced predivision. The level of mosaicism in the trophectoderm is lower than that seen in the inner cell mass of the blastocyst. This finding would pioneer the further investigations of cell repair or elimination. These results confirm those of Verlinsky et al. (2001). This, however, raises discussions about possible repair mechanisms once more.

In investigating the marker chromosome in more detail, we could identify the break point in 14qE3 on a limited extension of 200 kb. Comparisons with available gene bank data reveal that up to now within this area no genes were annotated. In 15qA1 we could reduce the extension of breakpoint region on about 27 kb in the proteolytic enzyme gene ADAMTS12 (a disintegrin and metalloproteinase with thrombospondin motifs) playing a crucial role in progression of vascularising as well as in cancer progression in human beings. With our results, this mouse strain lacking of ADAMTS12, will provide an essential tool to understand better the relevance of this enzyme in tumour-associated processes, asthma, arthritis, organogenesis, angiogenesis and reproduction.

7. ZUSAMMENFASSUNG

Fehler in der Ausführung der Meiose können zur Herstellung aneuploider Gameten führen, die mit Geburtsdefekten und Infertilität assoziieren. Laut veröffentlichter Ergebnisse, liegen in 3% der Proben von Chorionzotten Biopsien (CVS) chromosomale Abnormalitäten als Mosaik vor und beeinflussen nicht den menschlichen Fetus. Es ist bekannt, dass Fehler in der Chromosomenverteilung der Meiose zu Fehlbildungen und Infertilität führen, obwohl nur wenig über die molekularen Mechanismen der Chromosomensegregation und ihrer steuernden Bedingungen bekannt ist. Daher untersuchten wir die Verteilung von Aneuploidien (und Translokationen) in der frühen Mäuse-Embriogenese in Trophoblasten (TE) und der inneren Zellmasse (ICM). Unser Hauptinteresse galt der Frage, ob Aneuploidien eher im TE nachweisbar sind und Aborte verursachen oder eher in der ICM vorkommen und zu Fehlbildungen der Embryos führen.

Insgesamt wurden 241 weibliche Mäuse der fünf unterschiedlichen Stämme B6D2F1, C3H/HEN, C57BL/6, BALB/c and CBA/CaHT(14;15)6Ca/J für diese Untersuchung verwandt. Die experimentelle Gruppe basiert auf dem CBA/CaHT(14;15)6Ca/J Maus-Stamm, welcher eine homozygote, balancierte Translokation der Chromosomen 14 und 15 trägt. Die Mäuse sind völlig gesund und vermehren sich. Ihre Zellen tragen balancierte, reziproke Translokationschromosomen, die die Beobachtung ihrer Segregation ermöglichen.

Biopsie Trophectoderm (TE), embryonische Stammzellkultur (ESC) und Immunosurgery (IMS) Protokolle wurden bezüglich ihrer Fähigkeit individuelle ICM und TE Zellen zu isolieren beurteilt.

IMS und TE Biopsie führten wegen ihrer technischen Schwierigkeiten und falsch negativer, sowie falsch positiver Ergebnisse nicht zu unserem Ziel. Deshalb wurde die ESC-Technik bei 519 Embryos angewandt und 462 (89 %) der Embryos entwickelten sich gesund in Kultur.

Bestimmte FISH Untersuchungen mit vier unterschiedlich bezeichneten BACs wurden für CBA/CaHT(14;15)6Ca/J translozierte Mäuse entwickelt. Um zum ersten Mal die Segregation von Aneuploidien und Translokationen nach Isolation von ICM und TE Zellen zu überprüfen, haben wir die Machbarkeit einer gleichzeitigen Fluoreszenz-Immunophenotypisierung und FISH (FICTION) demonstriert. Die FICTION Technik wurde an 510 ICM und 510 TE Zellen von 73 Mausembryos erfolgreich angewandt. Auf diese Art waren wir in der Lage, OCT4 als Marker für ICM oder Cdx als TE Marker zusammen, mit der multicolor FISH-Probe darzustellen. Achtzig % TE Zellen ergaben positive Signale mit der Cdx Markierung, und 99% ICM Zellen ergaben mit der OCT4 Markierung positive Signale.

Die Literatur Ergebnisse zeigen, dass alle Zellen (ICM+TE) von heterozygot translozierten T(14;15)6Ca Embryos reziproke Translokationen auf reguläre Weise tragen. In ICM Zellen beobachteten wir eine höhere Anzahl von Aneuploidien, als in heterozygoten TE Zellen. Aneuploidien wurden in Form von 'Non-Disjunktionen' und 'unbalanced Predivisions' beobachtet. Der Grad an Mosaiken im Trophoektoderm ist nicht höher als jener, der in der ICM der Blastozyste beobachtet wurde. Diese Entdeckung könnte als Initiator für weitere Untersuchungen der Zellreparatur oder des selektiven Zelltodes sein. Diese Ergebnisse bestätigen jene von Verlinsky et al. (2001). Auch unsere Ergebnisse in Zusammenhang mit denen von Verlinsky et al. (2001), können die Diskussion über einen möglichen Reparaturmechanismus erneut anregen.

Durch eine detaillierte Untersuchung des Markerchromosoms konnten wir den Bruchpunkt in 14qE3 auf eine Ausdehnung von 200kb eingrenzen. Vergleiche mit verfügbaren Genomkartierungen konnten sicherstellen, dass sich nach heutigem Kenntnisstand in der Region keine Gene befinden. Die Ausdehnung des Bruchpunkts in 15qA1 konnte auf eine 27kb große Region begrenzt werden, in der sich das Gen ADAMTS12 (a disintegrin and metalloproteinase with thrombospondin motifs) befindet, welches eine wichtige Rolle bei der Vaskularisierung als auch bei der Krebsentwicklung im Menschen spielt. Nach unseren Ergebnissen, kann dieser Mausstamm, der ein trunkiertes Genprodukt

von ADAMTS12 trägt, ein wichtiges Werkzeug sein, welches zu einem besseren Verständnis für Prozesse der Tumorentstehung, Astma, Arthritis, Organogenese, Angiogenese und der Reproduktionsbiologie führen kann.

8. APPENDICES

8. 1. Abbreviations

- AMCA - Amino Methyl Coumarin Acetate
- BAC - Bacterial Artificial Chromosome
- cDNA - Complementary Deoxyribonucleic acid
- CVS - Chorionic Villus Sampling
- CZC - Cumulus Zygote Complex
- DAPI - 4,6-Diamidino-2-phenylindole
- DEAC - Diethyl Aminomethyl Coumarin
- dNTP - Desoxynukleotidtriphosphat
- DOP - Degenerate oligonucleotide priming
- DOP-PCR - Degenerate Oligonucleotid Primer-Polymerase Chain Reaction
- dTTP - Desoxythymintriphosphat
- FCS - Fetal Calf Serum
- FISH - Fluorescence in situ hybridization
- FITC - Fluorescence Isothiocyanat
- FSH - Follicle Stimulating Hormone
- hCG - Human Chorionic Gonadotropin
- hESC - Human Embryonic Stem Cell
- ICM - Inner Cell Mass / embryoblast
- IF - Immunofluorescence
- IMS - Immunosurgery
- IVF - In Vitro Fertilization
- MEF - Embryonic Mouse Fibroblast
- mESC - Embryonic Stem Cell medium

- M-FICTION - Multicolour Fluorescence Immunophenotyping and Interphase Cytogenetics as a Tool for Investigation of Neoplasm
- Mouse CotDNA - repeated mouse DNA sequences
- PB - Polar Body
- PBS - Phosphate Buffered Saline
- PCR - Polymerase chain reaction
- PGD - Preimplantation Genetic Diagnosis
- PMSG - Pregnant Mare Serum Gonadotropin
- PVP - Polyvenyl pyrrolidone
- RNA - Ribonucleic Acid
- TE - Trophoectoderm / trophoblast

8. 2. Curriculum Vitae

NAME: Bengi

SURNAME: Acar-Perk

BIRTH DATE: 19.08.1979

MARTIAL STATUS: Married

ADRESS: Falckstraße 15

24103 Kiel / Deutschland.

TELEPHONE: 0049 (0) 176 20322466

0049 (0) 4311281452

E-MAIL: bengiacar@hotmail.com

bengiacar@gmail.com

EDUCATION:

- Kiel University, International Doctorate Programme (Phd.) by the Faculty of Natural Sciences, Biology, since 2005.
- Istanbul University, Institute of Health Sciences, Cerrahpasa Medical Faculty Histology and Embryologie Master Degree Program (2002 – 2004).
- Istanbul University Cerrahpasa Medical Faculty Medical Biological Sciences Department (1998 – 2002).
- Private Kültür High – Middle - Elementary School (1987 – 1997).
- Halil Vedat Fıratlı Elementary School (1986 - 1987).

PUBLICATION:

Acar-Perk B. , Bräutigam K., Grunewald R., Schmutzler A., Schem C., Arnold N., Jonat W., Weimer J. (2009) Mouse strain CBA/CAH-T(14;15)6CA/J carrying a homozygote reciprocal translocation T(14,15) with break in ADAMTS12 gene. Manuscript submitted in Chromosome Research.

PRESENTATIONS:

- B. Acar – Perk

Title: Polar body diagnosis and FISH

Training course in Reproductive Medicine, Department of Gynaecological Endocrinology and Reproductive Medicine; Kiel University, Kiel, Germany, May-2009.

- B. Acar - Perk

Title: Animal experiments and practical demonstrations in mice

Training course in Reproductive Medicine, Kiel University, Department of Gynaecological Endocrinology and Reproductive Medicine; Kiel, Germany, May- 2009.

- B. Acar

Title: Polar body diagnosis and FISH

Training course in Reproductive Medicine, Kiel University, Department of Gynaecological Endocrinology and Reproductive Medicine; Kiel, Germany, March - October 2005 – 2006 - 2007.

- B. Acar

Title: Animal experiments and practical demonstrations in mice

Training course in Reproductive Medicine, Kiel University, Department of Gynaecological Endocrinology and Reproductive Medicine; Kiel, Germany, March - October 2005 - 2006 - 2007.

- B. Acar, J. Weimer, A.Salmassi, L. Mettler, W. Jonat, N. Arnold, A. Schmutzler

Title: Hohe Aneuploidieraten bei menschlichen Eizellen gefunden bei breiter Indikation zum Präimplantationscreening (PGS).

Congress: Deutsche Gesellschaft für Humangenetik (GFH); Bonn, Germany, 7. - 10. March 2007.

ABSTRACTS:

- Schmutzler, B. Acar, J. Weimer, A. Salmassi, N. Arnold, L. Mettler, W. Jonat

Title: High aneuploidy rate in human oocytes.

Congress: 2. Dachverband Reproduktionsbiologie und -medizin (DVR) Kongress; Bonn, Germany, 28. November – 1. December 2007.

- Schmutzler, B. Acar, J. Weimer, A. Salmassi, N. Arnold, L. Mettler, W. Jonat

Title: Bei breiter Indikation zum Präimplantationscreening (PGS) werden große Aneuploidieraten bei menschlichen Eizellen gefunden.

Congress: 123. Tagung der Norddeutschen Gesellschaft für Gynäkologie und Geburtshilfe; Göttingen, Germany, 7.- 9. September 2007.

- Schmutzler, B. Acar, J. Weimer, A. Salmassi, N. Arnold, L. Mettler

Title: High aneuploidy rates of human oocytes are found with broad indications for Preimplantation Genetic Screening (PGS).

Congress: Preimplantation Genetic Diagnosis International Society Conference (PGDIS); Melbourne, Australia, 13. - 16. June 2007

8. 3. Statements of Originality

Statement of Originality

I declare that this thesis has not been submitted in any form for another degree or diploma at any university. Results discussed in this thesis are my own work, and information derived from the literature or unpublished work of others has been acknowledged in the text and a list of references provided.

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel von mir eingesetzt worden sind. Des weiteren versichere ich, dass die vorliegende Dissertation weder ganz noch zum Teil bei einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegen hat.

Kiel, 25.05.2009

8. 4. Acknowledgments

I firstly want to thank to my doctoral adviser, Prof. Dr. Norbert Arnold, that he accepted me as a doctoral student to Molecular Oncology Department and gave me the possibility to do my Phd degree in Kiel. He was always helpful, polite and tolerant to me that under his guides I could bring my researcher to end in a foreign country. I could not wish a better doctoral adviser and prof. than him.

I further want to thank to Dr. Jörg Weimer who I called as David Copperfield because of his incredible talent to solve problems in expeditious ways. His never-ending patience in explaining the problems was always graceful. Without his help throughout the 4 years, this thesis might have needed more time to conclude.

I also want to thank faithfully to Prof. Dr. Mettler und Dr. Andreas Schmutzler for their trust in my scientific skills that I had the possibility to work in a field that I would like during completing my academic carrier. I first came to Kiel with the support of Prof. Dr. Mettler which I would never forget. Although Dr. Schmutzler had always stress, he had the time to listen and helped me even with my private problems.

I want to thank to Dr. Ali Salmassi in Turkish and happy to have always the home feeling with him. Çok teşekkür ederim hocam, iyi ki varsınız ve iyi ki bebaber çalışabileceğim imkanına sahip oldum. Ailem beni gözleri arkadan kalmadan size emanet ettiler ve hep bu sorunun olduğunda rahat oldular çünkü siz ordaydiniz.

Kerstin Koch I want to thank to you that from the beginning on you are not only my colleague also one of my best. Simply thank you for everything!

Regina Grunewald and Doris Karow I want to thank to both of you for a friendly atmosphere that we always had together. Regina, I learned genetic techniques from you with your never-ending patience. I am sorry again for the left open refrigerators, but we always laugh a lot afterwards that I will never forget. In addition, Doris you had always time for me to listen and to help. Thank you for the tips that you are given to use in new my married status.

Frank Rösel thanks a lot for the support in my animal experiments and helps. I also want to thank for your friendship.

Sybille Buck and Siegrid Harmann I want to thank to both you a lot, for the helps and motivation during my experiments.

Vielen lieben Dank auch an meine Familie, meine Freunde und meinen Mann

Last but not least: Meinen Eltern