From the Department of Clinical Sciences, Division of Obstetrics and Gynecology, Huddinge University Hospital Karolinska Institute, Stockholm, Sweden

NEW MICROMANIPULATIVE TECHNIQUES IN REPRODUCTIVE BIOLOGY

José Inzunza

Stockholm 2003

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7349-568-9

To my family

ABSTRACT OF THE THESIS

New micro manipulative techniques have facilitated development to help patients who suffer from infertility and spontaneous abortion, as well as those who carry severe genetic diseases and have risks of delivering an affected child. Preimplantation genetic diagnosis (PGD), which is based on obtaining blastomere biopsy from an eight-cell embryo, has made it possible to diagnose severe defects caused by a genetic factor.
In the animal laboratory, ovarian transplantation is a new technique that provides a tool in research to save valuable mutants and transgenic animals that have an infertility not caused by germ cells. The infertility can be due to prepubertal lethality, and inability to mate or incapacity to complete a successful fertilization, gestation or parturition.
The creation of a technique for transfer of mouse embryos into germ free recipients to produce gnotobiotic mice will facilitate researchers to study the micro flora of transgenic and special mutants more specifically.
In this thesis we improved three micro manipulative techniques and the possibilities of applying them in research laboratories and in in-vitro fertilization units.
1) We created an improvement in the embryo biopsy micro technique that is used to allow the preimplantation genetic diagnosis (PGD) using a single-needle to perform the blastomere biopsy. 2) We used the ovarian transplantation micro-surgical technique to save valorous mutants and transgenic animals when the reason for infertility was other than non-functional germ cells. 3) We created a new micro technique to allow transfer of transgenic animals to a germ free state, through the embryo transfer into a germ- free or gnotobiotic recipient.

We tested and successfully applied a single-needle approach to obtain blastomere biopsies from human preimplantation embryos for preimplantation genetic diagnosis (PGD). The method was first evaluated in a mouse system and shown to be compatible with a high degree of in-vitro and in-vivo development of biopsied mouse embryos. Biopsied mouse embryos after transfer to recipient mice underwent implantation, normal development and delivery. Successful human preimplantation diagnosis, followed by pregnancies and birth of healthy babies, was established with two out of three couples carrying a risk to transmit chromosomal abnormalities leading to severe diseases. This is the first report of the use of a single-needle approach in human PGD.

We evaluated an ovary transplantation method by transplanting ovaries from females belonging to a non-reproductive BALB/cByJ mutant mouse strain. All transplanted mice, BALB/c.C57BL/6By, produced offspring and 94 % of the progeny originated from the transplanted ovaries. The mean litter size and the mating period needed for productive mating to occur were similar to what is observed for corresponding control mice.
Subsequently we also performed ovary transplantation of estrogen beta-receptor knockout (ERβ-/-) ovaries into wild type and normal C57Bl/6 recipient mice. The litters' sizes were similar after transplantation of ERβ-/- and WT ovaries to normal recipients, hence referring to an extra-ovarian cause of subfertility.

Lastly we demonstrated that it is possible to obtain germ-free animals using embryo transfer of conventional or transgenic mice to germ-free recipients inside a steel isolator. We have developed a unique embryo transfer technique that will facilitate researchers to use this procedure as a tool in changing the status of different mutants into germ-free mice.
All these three techniques are powerful tools in the field of reproductive medicine and biology.

LIST OF PUBLICATIONS

This thesis is based on the following papers which will be referred to in the text by their Roman numerals

I. **Inzunza J**, Iwarsson E, Fridström M, Rosenlund B, Sjöblom P, Hillensjö T, Blennow E, Jonse B, Nordenskjöld M, Ährlund-Richter L.
Application of single-needle blastomere biopsy in human preimplantation genetic diagnosis
Prenatal Diagnosis, **18**:1381-8,1998.

II. Iwarsson E, Ährlund-Richter L, **Inzunza J**, Rosenlund B, Fridström M, Hillensjö T, Sjöblom P, Nordenskjöld M, Blennow E.,
Preimplantation genetic diagnosis of a large pericentric inversion of Chromosome 5.
Molecular Human Reproduction.vol 4 no. 7 pp 719-723, 1998.

III. Lavebratt C, **Inzunza J**, Petersson S, Iwarsson K, Schalling M, Ährlund-Richter L.
Ovary transplantation method resulting in high reproductive performance in mice
Scand.J.Lab.Anim.Sci. No.2. 1998. Vol. 25

IV. **Inzunza J**, Cheng G, Warner M, Hreinsson J, Ährlund-Richter L, Gustafsson J-Å, Hovatta O.
Reproductive function after orthotopic transplantation of oestrogen receptor beta knockout mouse ovaries to wild type mice littermates and vice versa.
Manuscript

V. **Inzunza J**, Midtvedt T, Fartoo M, Rozell B, Norin E, Österlund E, Persson A-K, Ährlund-Richter L.
Safe and reproducible shift to gnotobiotic or germ-free status of mouse stocks via embryo transfer in a sealed isolator environment.
Manuscript submitted

CONTENTS

1 Abbreviations

ART	assisting reproduction technology
IVF	in vitro fertilization
ICSI	intracytoplasmic sperm injection
PGD	pre implantation genetic diagnosis
ICM	inner cells mass
TRPs	transcription-requiring proteins
DNA	deoxiribonuclei acid
CVS	chorionic villus sampling
CCS	Cri du Chat syndrome
DGS	DiGeorge syndrome
FISH	fluorescence in situ hybridization
PCR	polymerase chain reaction
ZD	zona drilling
FSH	follicle stimulating hormone
LH	luteinizing hormone
CL	corpora lutea
GF	germ-free
PMS	pregnant mare's serum gonadotrophin
hCG	human chorionic gonadotropin
PBS	phosphate-buffered saline
HHb	haemoglobin β-chain
ERß	estrogen receptor beta
WT	wild type
TGB	tryptose and glucose broth
RCM	reinforced clostridial medium
THM	thioglycollate medium
ACM	anaerobic clostridial medium
cAMP	cyclic adenosine monophosphate

2 Introduction and literature review

2.1 Historic remark:

The evolution of embryology and the modern reproductive biology started with Reinier De Graaf (1641-1673) and Antonie van Leeuwenhoek (1632-1723). Both created their own theories on the reproduction and inheritance mechanisms. One discovered and described the follicles in the ovaries, and the other discovered the sperm. De Graaf stated that the egg contained an entire human in miniature, and that the semen stimulated its growth. Leeuwenhoek on the other hand, stated that the sperm contained the pre-formed human being, the homunculus that contained all the genetic information from the father, which meant that the woman only served as an incubator for the homunculus. However, we know today from embryology that neither the egg nor the sperm contain a complete or developed embryo. Only in the course of the second half of the 19th century was it recognized that the eggs and sperms are equivalent.
Nevertheless they are both considered the founders of the modern reproduction biology.
The discovery of the ovum by Karl von Baer (1792-1869) and his observation of the stages of embryogenesis, contributed to a better understanding of the mechanisms in reproduction physiology. At the same time the study of the development of egg (ova) in rabbits, done by Edouard Van Baneden (1845-1910) determined a great knowledge concerning the egg development, which until then was solely on a descriptive and observational stage.
The middle of the 20th century can be considered as a crucial time for the active development of mammalian embryology, under which a great number of scientists were active in obtaining special means for the in-vitro culture of the embryo. Anne McLaren, W.White and John D Biggers were able to find means of cultivating the mouse eggs in a chemical "medium" and later transfer them into the uterus of a pseudo pregnant female mouse. In 1959, M.C Chang was the first to perform and succeed with the in vitro fertilization in rabbit, paving the way for artificial fertilization. This method was first used in humans by Robert Edwards and Patrick Steptoe in 1978, with the result of the birth of Louisa Brown.
In 1963 Ralph Brinster reported a method for cultivating eggs in micro drops of medium under oil (Brinster 1963), which has become universally used. Two years later, he identified pyruvate as the central and essential energy source for early development of egg (Brinster 1965). These two developments have revolutionized in-vitro studies of mammalian egg culture and egg manipulation. Researchers like Andrzej Tarkowski started putting forward theories on the embryonic differentiation, concepts such as ICM trophectoderm and the theory of cell determination during the development of the embryo. Other researchers were interested in the function and the role of the microparticles, which during the 60's made it clear that there existed gene expression in the first steps of the embryonic development (Alexandre 2001). Embryology is now concerned with understanding development from the viewpoint of the activation and transcription of DNA sequences, which will allow us to approach the first causes or underlying genetic and epigenetic mechanisms of development. As a result, embryology and genetics have fused into a wider biological sub discipline, developmental biology (Murillo *at al* 2001).
The solid foundation of understanding about the biology of early mammalian eggs was consolidated during the 60´s and 70´s, and subsequent studies have broadened this understanding. The impact of a reliable egg culture method has provided a possibility to perform complicated manipulative procedures on preimplantation stages of mammalian embryos. In no area has this been more important than in the development of transgenic animals (Palmiter *et al*., 1987; Peschon *et al.,* 1987). All methods for generating germ line genetic modifications rely on the ability to maintain and manipulate eggs and early

developmental stages in-vitro without loss of developmental competence. The importance of efficient egg culture for manipulation and transgensis is fundamental and enabling for the development of new embryo micro manipulative techniques. (Hammer, 1998).

2.2 Preimplantation embryo development

The preimplantation embryo development begins with the sperm-oocyte interaction of specific recognition of complementary receptors on the surfaces of the two gametes and terminates in syngamy, also with the union of male and female chromosomes; this complex mechanism is called fertilization (William, 2001)
Fertilization is a sequence of events that starts with contact between a spermatozoon and an ovum, leading to their fusion, which stimulates the completion of oocyte maturation with release of the second polar body. Male and female pronuclei then form and merge, synapsis follows, which restores the diploid number of chromosome and results in biparental inheritance and the determination of sex. The process of fertilization leads to the formation of a zygote and ends with the initiation of its cleavage. After fertilization the zygote divides by normal mitosis into smaller cells called blastomeres. During this stage the embryo does not grow, as a result the volume of pre-embryo is the same until the early blastocyst stage, day four in mouse and day five in human (Hogan *et al*., 1994; William, 2001). After this stage the embryo increases in size by active accumulation of fluid in the central blastocoelic cavity, this stage is called blastocyst expansion. In fertilization the sex cell from a male individual in the form of his sperm, containing a random selection of chromosomes from both his mother and father, combines with a female's sex cell, an ova containing a random selection of chromosomes from both her mother and father. This has been further randomized by recombination. Thus the individual that develops from this union will have a random selection of alleles from both of his, or her, grandparents, and from increasing numbers of ancestors from past generations.
With the complex process in which the sperm and egg chromosome recombine, mistakes do occur from time to time. As in mitosis, an error in copying a gene may occur, the difference being that it will be handed down to succeeding generations. Such a germ line mutation may be harmful, may be beneficial, or simply have no apparent effect at all.

2.2.1 Cleavage stage

Approximately 24 hours after fertilization the zygote starts a series of mitotic divisions, these divisions are also called cleavage and are not accompanied by cell growth, they divide the zygote into smaller cells called blastomeres. The first cleavage division divides the embryo along a plane at right angles to its equator and in line with polar bodies. After 40 hours from fertilization the second division takes place, which produces four equal blastomeres, here one of the two blastomeres divides meridionally and the other divides equatorially, this type of cleavage is called rotational cleavage (Gulyas, 1975). By three days the embryo consists of 6 to 12 cells, and by four days it consists of 16 to 32 cells. When the embryo reaches 32 cells it has the appearance of a small mulberry and is therefore, called a morula. During cleavage, in each division the resulting blastomeres are approximately half the size of the parent blastomere. Cleavage is also a period of intense DNA synthesis and replication, without growth. Differences arise between the blastomeres that may result from the unequal distribution of cytoplasmic components as already laid down in the oocyte during oogonesis, or from changes occurring in the blastomeres during development. Each blastomere nucleus will be subjected to a different cytoplasmic environment that in turn may differentially

influence the genome activity and subsequent potential for cell differentiation (Harper *et al.,* 2001)

2.2.2 Genome activation in early embryo

The oocyte immediately after ovulation already possesses the complete biochemical machinery for protein synthesis; during growth and maturation reserves of maternal mRNAs, proteins, organelles for protein synthesis accumulates. Activation of the new embryonic genome provides novel transcripts and reprograms the pattern of gene expression to direct further development. This critical transition takes place during the early cleavage stages of the embryo, at the four-to eight-cell in humans and up to the mid two-cell stage (27 hours postfertilzation) in mouse embryo (Piko and Clegg 1982), and maternal mRNA rapidly disappears while the zygote genome gradually increases its expression. A small amount of maternal message is needed almost until the blastocyst stage (Harper *et al*., 2001). Protein synthesis is also completely dependent on new RNAs read from the embryonic DNA for all stages after maternal zygotic transition. Transcription-requiring proteins (TRPs) are the first group of proteins produced. This group of proteins disappears after the 8-cell stage. In embryos in which DNA synthesis and cell division have been inhibited, TRPs still appear at the right time. This indicated that transcription is initiated by a clock that ticks, regardless of DNA synthesis. The fact that TRPs also appear in embryos, into which nuclei from a later embryonic stage have been transplanted, is a strong indication that the clock function is situated in the cytoplasm (Bras *et al.,* 1996). Previous failures at any stage of oocyte development, maturation and handling can affect development even after genome activation. (William, 2001; Harper *et al.,* 2001).

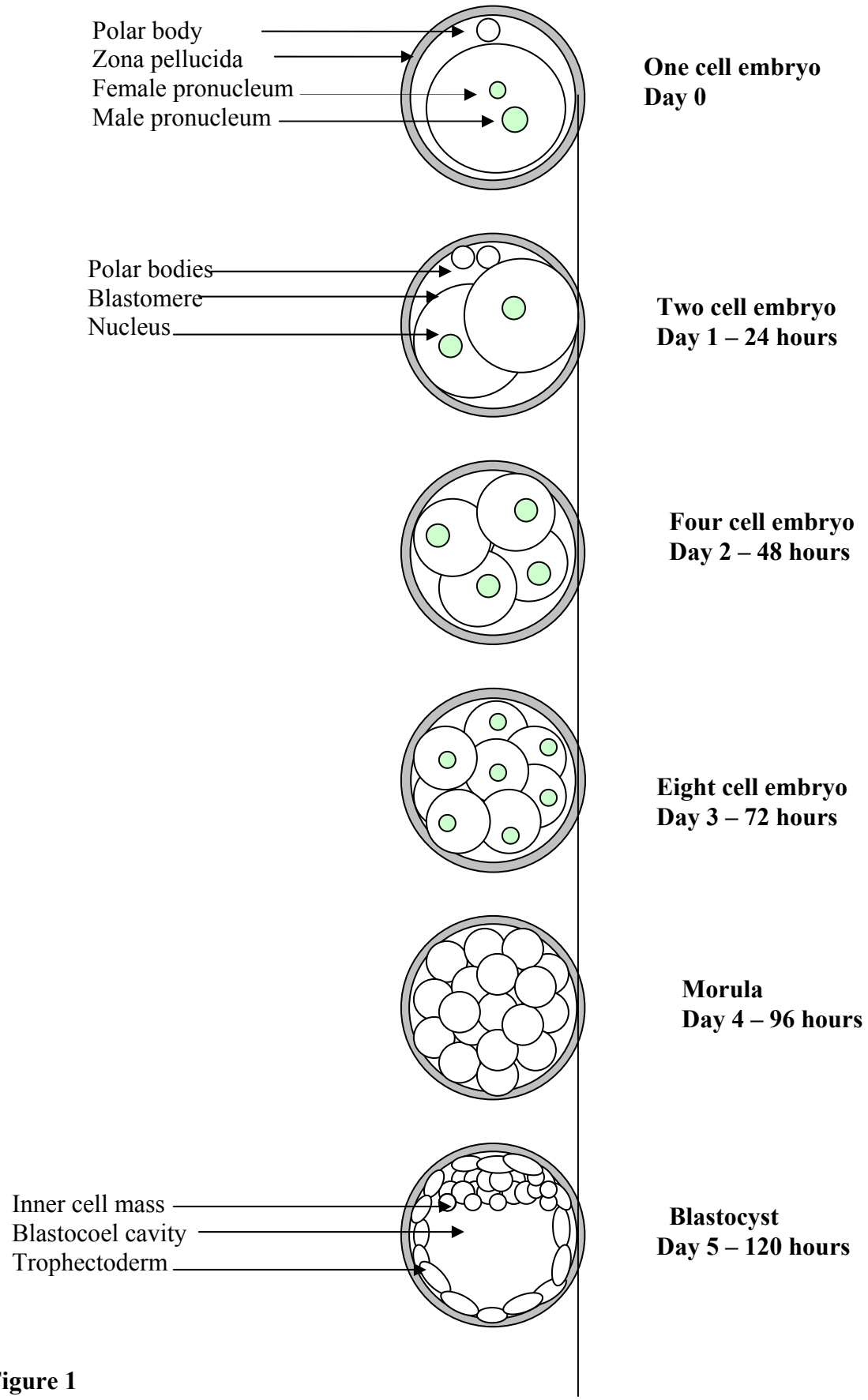


Figure 1
Pre implantation embryo development (different cleavage stages)

2.2.3 Cell polarization and compaction

Starting at the four-cell and eight-cell stage of development, the originally round and loosely adherent blastomeres begin to flatten, developing an inside-outside polarity that maximizes cell-to-cell contact among the blastomeres at the center of the mass (Nikas *et al*., 1996). As differential adhesion develops, the outer surfaces of the cells become covex and their inner surfaces become concave. This reorganization, called compaction, involves the activity of cytoskeletal elements and adhesion of the blastomeres (William, 2001). The cells of the compacted embryo become highly polarized, and are tightly associated and communicating. Surface polarity can be seen by the appearance of dense microvillar and amicrovillar regions, and cytoplasmic polarity can be seen in the distribution of endocytotic vesicles, actin filaments, and the location of the cell nucleus. Therefore, the four-cell embryo probably contains some of the proteins required for compaction. Although the factors which trigger the timing of its onset are not known, experimental evidence suggest that this may be regulated by post-translational modification of specific proteins such as E-cadherin (Haper *et al*.,2001). The protein E-cadherin (uvomorulin) is expressed in the oocyte, and during all stages of preimplantation development. It is uniformly distributed on the surface of blastomeres, and accumulates in the regions of intercellular contact during compaction. E-cadherin phosphorylation can be observed in the mouse eight-cell embryo. Culturing embryos in calcium-free medium prevents compaction, and this also inhibits E-cadherin phosphorylation. In human embryos, tight junctions begin to appear on day 3, at the 6-10-cell stage, heralding the onset of compaction. Scanning electron microscopy studies showed evidence of compaction on day 4, when the majority of embryos with 10 or more cells revealed dense microvilli with a polarized distribution over the free surface of the compacted blastomeres (Nikas *et al*., 1996). In the mouse, gap junctions are expressed at the eight-cell stage. In human, gap junctions are not apparently well developed until the early blastocyst stage, when intercellular communication is clearly seen between ICM cells (Dale *et al*., 1991; Harper *et al*., 2001).

2.2.4 Cavitation, blastocyst expansion and hatching

By 4 days of development between the 16-and 32-cell stage, a second morphological change occurs, known as cavitation. Activation of Na+, K+ ATP-ase systems result in an energy-dependent active transport of sodium pumped into the central area of the embryo (Harper *et al*., 2001), followed by osmotically driven passive movement of water to form a fluid-filled cavity (blastocoele). The movement of other ions such as chloride and bicarbonate also contributes to blastocoele formation (Harper *el al*., 2001). The stage of blastocoele formation and the expansion phenomena is important for the continued development and differentiation of the ICM. The ICM is bathed in a specific fluid medium which contains factors and proteins that influences the cell proliferation and differentiation. Another factor which can influence the differentiation of the outer cells into the primitive endormal cells is the position of the ICM cells in relation to the fluid cavity.

By day 4/5 the blastocyst initially does not shows any increase in size, but it eventually expands over the next one or two days (day 5/6) due to the active accumulation of fluid in the central blastocoelic cavity. At these early stages the embryo is still enclosed in the zona pellucida, which keeps the cells together before compaction and also prevents the fusing of two embryos.
In the transition from morula to blastocyst the embryo enters the uterus, where the

throphectoderm cells produce proteolytic enzymes which help the blastocyst to “hatch” free from the zona pellucida. Proteolytic enzymes are believed to exist in the uterine environment as well, however there is little knowledge concerning the molecular basis for hatching.
The uncovered cell layers of the hatched blastocyst start making firm contact with the uterus lumen and implantation starts.

2.3 Micromanipulative techniques

Micromanipulative techniques in reproduction biology are important tools especially in assisting reproductive technology (ART), and in laboratory animal research. The manipulative techniques were developed to overcome the limitation of classic methodology in the IVF clinics and in the embryo research investigation. The sheep Dolly (Wiltmut *et al.,* 1997) was the culmination of a series of prior manipulation techniques that allowed the development of the cloning technique. Cloning can be done with embryonic cells as well as with somatic cells. The latter is a difficult technique, for success numerous attempts are required.
With the discovery of the DNA recombinant technique, new possibilities were available which allowed the use of DNA sequences (genes) to introduce them into cells through the cellular transfection and expression of the newly introduced genes. This has permitted the production on an industrial level of synthetic proteins which no more than 30 years ago were impossible to even imagine obtaining The transgenesis technique developed, meaning the creation of series of transgenic animals, produced through the technique of microinjection of DNA fragments into an embryo in the pronuclear stage (Palmiter *et al.*, 1987; Hogan *et al* 1994). This technique allows the fragment of DNA to integrate into the genome of the animal. With this technique models of animals that carry a determined genetic disease have been created, whether it is with or without this specific gene. For all of this it has been necessary to create and improve new manipulation techniques, one of these techniques is the manipulation of the embryo, which during the last 20 years has resulted in finding and developing sophisticated methods like nuclear transfer.
In the IVF laboratories the advancements of the embryonic manipulation techniques has allowed the development of new technologies. The implementation of the technique called intracytoplasmic sperm injection (ICSI) (Palermo *et al*.,1992), has helped many couples with the problem of male infertility. At the same time the preimplantation genetic diagnosis (PGD) was coming forward (Handyside *et al*., 1989; 1990), this technique allows biopsy of one or two cells from the embryo and analysis of the known defect from these cells. Diagnosis of the possible genetic disease, which will cause severe damages and in many cases are lethal, thus helps to avoid spontaneous abortion or the birth of a child affected by a severe genetic disease. The development of the embryonic manipulation techniques in reproductive biology has been a great advance in understanding the embryo and its genetic characteristics. The advance and the applications have been fast and with a wide spectrum of interdisciplinary applications.
The cryoperservation techniques of the gametes and of the embryos allow us to conserve the biological material of reproduction for a long time and the use of them when it is most appropriate (Whittinghan *et a*l., 1974), (Mukaida *et al*., 1998), (Hsieh *et al*., 1999), (Poirot *et al*., 2002),

2.4 Preimplantation genetics diagnosis PGD

Preimplantation genetic diagnosis (PGD) is a technique that can be used during in-vitro fertilization (IVF) procedures to test embryos for genetic disorders prior to their transfer to the uterus. PGD makes it possible for couples or individuals with serious inherited disorders to decrease the risk of having a child who is affected by the same problem (Handyside *et al*., 1989; 1990), (Cassel *et al.,* 1997), (Verlinsky *et al*., 1996), (Munne *et a*l., 1995; 1998).
PGD is an alternative to traditional prenatal diagnosis such as chorionic villus sampling (CVS) and amniocentesis and is a result of close collaboration between clinical and molecular geneticist, fertility clinicians and clinical embryologist.
Fluorescent in situ hybridization (FISH)) allows the detection of chromosomal aberrations (structural or numerical) and gender determination in cases of sex-linked diseases (Coonen *et al.*, 1998), whereas polymerase chain reaction (PCR) at single-cell level allows the specific detection of single-gene defects (Findlay *et al.,* 1996)

PGD can be performed in couples who have been affected by repeated pregnancy loss due to genetic disorders. It is also appropriate in couples who already have one child with a genetic problem and are at high risk of having another. The conditions for which this technique has been successfully applied are presented in table I. The list is not complete as new applications appear all the time. There are over 200 disorders that could potentially be prevented by gender selection of embryos, but an exact diagnosis of the disorder is, of course, prioritized (ESHRE -PGD consortium, 1999; 2000)

PGD are performed on embryos in two ways: either by combining an egg and sperm in the laboratory (i.e. IVF), or by flushing out the uterus five to seven days after fertilization.
The last procedure is only applied in animal studies. One or two cells are removed from each embryo under microscopic guidance and analyzed for the presence of genetic disorders, and only the unaffected embryos are replaced in the female's uterus.

PGD was first performed in 1989 (Handyside *et al*., 1989). It has since been successfully applied to a wide variety of single gene disorders or chromosomal abnormalities. In single gene disorders where the gene structure is known, such as cystic fibrosis or Tay-Sachs, the actual genes of the sampled embryo can be examined for the presence of the condition.
Other genetic conditions, such as Duchenne muscular dystrophy or hemophilia, affect only males. In these cases, while the exact gene defect may not be known, the DNA of the biopsied cell can be examined to determine the sex of the embryo and thus making it possible to restore only female embryos. Finally, in cases of severe recurrent chromosomal diseases, such as Downs syndrome (which has an extra chromosome), the number and character of several chromosomes of the sampled embryo can be determined (Munné *et al*., 1998; Harper *et al*., 2001).

Table I, Diseases which have been diagnosed by blastomere biopsy.

- Achondroplasia
- Adenosine deaminase def.
- Alfha 1 antitrypsin deficiency
- Alzheimer disease
- Beta thalassemia
- Charcot-Marie-Tooth disease
- Cri du chat syndrome
- Cystic fibrosis
- DiGeorge syndrome
- Downs syndrome
- Duchenne muscular dystrophy
- Fragil X syndrome
- Gaucher disease
- Gender
- Hemophilia A and B
- Huntington disease
- Lesch-Nyhan syndrome
- Myotonic dystrophy
- Neurofibromatosis I
- p53 cancers
- Phenylketonuria
- Retinoblastoma
- Retinitis pigmentosa
- Sickle cell disease
- Spinalmuscular atrophy
- Tay-Sachs disease
- Turner syndrome

In Sweden, the use of PGD is only allowed when there is a risk for early death for which there is no cure or treatment available. The IVF unit at Huddinge University Hospital, in collaboration with the Department of Molecular Medicine at the Karolinska Hospital and the Unit for Embryology and Genetic Research, Novum, Karolinska Institute, Stockholm is one of the two units in Sweden offering PGD. From June 1996 to October 2000, 18 couples carrying structural chromosomal aberrations were treated in this unit (Fridström *et al*., 2001)

2.4.1 DiGeorge syndrome

DiGeorge syndrome (DGS) is a rare congenital disease whose symptoms vary greatly between individuals but commonly include a history of recurrent infections, heart defects, and characteristic facial features (Wilson *et al*., 1993).
DGS is caused by a large deletion from chromosome 22, produced by an error in recombination at meiosis. This deletion means that several genes from this region are not present in DGS patients. It appears that the variation in the symptoms of the disease is related to the amount of genetic material lost in the chromosomal deletion (Driscoll *et al*., 1993), (McLean *et al*., 1993),
Although it is well know that the DGS gene is required for the normal development of the thymus and related glands, counteracting the loss of DGS is difficult.
Some effects, for example the cardiac problems and some of the speech impairments, can be treated either surgically or therapeutically, but the diminished production of T-cells (produced by the thymus) is more challenging and requires further research on immune function.

2.4.2 Cri du chat syndrome

Cri du chat syndrome (CCS) is a rare chromosomal disorder, with an incidence of 1 in 20 000 to 1 in 50.000, that results from a partial deletion of the short arm of chromosome 5 (Niebuhr, 1978).
CCS is associated with mental retardation and a facial phenotyp. The syndrome is usually diagnosed postnatally. The presence of cat-like cry appear to be the only strong specific feature that can be linked to the loss of 5p material, other characteristic include a round face, microcephaly, micrognathia, hypertelorism, epicanthic folds, low-set ears, short stature and

short fingers with clinodactyly. Individuals later show severe psychomotor and developmental delay (Stefanou *et al*., 2002)

2.4.3 Robertsonian translocation of chromosome 14 and 15:

Robertsonian translocations usually involve two different chromosomes for example non-homologous (non-homologous translocation), and those involving the fusion of homologous chromosome (homologous translocation) these are very rare. In about 80 % of Robertsonian translocations the chromosomes are two of the group D (13-15).
There are three possible mechanisms of formation of Robertsonian translocation: fusion at the centromere (centric fusion), union following breakage in one short arm and one long arm (reciprocal translocation), and union following breakages in both short arms (Guichaoua *et al.,* 1986). The former two produce a translocation chromosome with one centromere (monocentric), and the latter results in a chromosome with two centromeres (dicentric). In some dicentric chromosomes, one centromere is suppressed, and the chromosomes appear monocentric.
Robertsonian translocation in which chromosomes 14 and 15 (t14:15) are involve belongs to the category of the non-homologous translocations. This kind of translocation is very rare. T(14:15) is associated with repeated spontaneous abortion and male infertility. There is no evidence of increased risk for any other type of abnormal offspring.

2.4.4 Classical embryo biopsy techniques for PGD:

The classical method to perform the embryo biopsy in PGD is using two micropipettes. One small pipette with internal diameter 5-7 μm containing acidified Tyrode's solution or acidified biopsy medium (pH 2.4), this pipette is placed close to the zona pellucida and the acidified solution is gently flushed from the pipette until the zona thins and a hole is drilled. The flow can be controlled via an oil-filled syringe (hydraulic), air-filled syringe (pneumatic) or by using a mouth pipette. Once the hole is drilled in the zona, the blastomeres are aspirated by gentle suction with a second polished pipette (internal diameter of 30-40 μm). This biopsy method is used in many PGD centers worldwide (ESHRE PGD Consortium, 1999, 2000); (Harper *et al*., 2001).

Usually there are two methods to perform the penetration of the zona pellucida of the embryo, the zona drilling (ZD) and the zona pierce.

A. Zona drilling:

-Using acidified Tyrode's Solution (pH 2.2 – 2.4)
-Using acidified biopsy medium (pH 2.2 - 2.4)
-Using laser beam

B. Zona pierce

-Using a very sharpened pipette

2.5 Mouse ovary and Ovary transplantation

Mouse ovaries are small spherical paired organs located on the dorsal abdominal wall, lateral and caudal to the kidneys, and are surrounded in abdominal fat. The round ligament suspends the ovary from the abdominal wall, and the cranial portion of the broad ligament suspends the ovary with the uterus. The ovary is completely enclosed within the ovarian bursa. The ovarian surface is smooth in prepubertal females but becomes nodular after sexual maturity because of the presence of follicles and corpora lutea. There are differences in ovarian size and weight, these are dependent on the strain and age differences. For example, NH mice have an ovarian weight of 2-3 mg, whereas the ovaries of DBA/2WyDi mice weigh 25-65 mg, but histologically no essential differences exist between the two strains (Chai & Dickie, 1966). Ovaries from DBA mice are, on average, two or three times the size of those from C57BL mice when the animals are 4 months old, although the initial adult weights are comparable (Fekete, 1946).

2.5.1 Development

The development of the embryonic gonads generally precedes that of the tubular genital tract. Day 8 of gestation, primordial germ cells, which subsequently become ova in adult mice, can first be identified in the yolk sac of the developing embryo. Days 9-10 as the paired genital ridges appear, the germ cells migrate into these ridges and increase in number by mitosis with subsequent entry into meiotic prophase. Leptotene chromosomes are already present in some germ cells at 13 days. Pachytene oocytes are numerous at 16-17 days and may continue through diplotene and enter the dictyate stage by birth. The dictyate stage persists until just before ovulation (Snell and Stevens, 1966). In the early ovary growth period, the oocytes are closely assembled in nests surrounded by a delicate stroma. In mice the transformation of these oocytes into mature follicles begins in the neonatal period. Jones and Krohn (1961) found while studying the number of oocytes at birth in four different strains of mice that the total number had the same order and magnitude (approximately 12,000-18,000). The decline rate in the number of oocytes does vary between strains like with for example CBA strain mice losing their oocytes more rapidly than others (Mohr.*et. al*., 1996).

2.5.2 Hormonal function

Gonadotropin-releasing hormone is secreted into the hypophyseal portal circulation from the hypothalamus, which stimulates the anterior lobe of the pituitary gland to release gonadotropins. As a result of gonadotropin stimulation, the ovaries synthesize and release sex steroid hormones, which affect the hypothalamus and pituitary gland in either a stimulatory (positive) or inhibitory (negative) manner, depending on the stage of the cycle. (Mohr *et al*., 1996; Davis *et al*.,1999). Sex differentiation of the hypophysis is usually realized by day 6 in males and before day 12 in females. Ovarian weight becomes responsive to exogenous follicle-stimulating hormone (FSH) by days 6-9, but follicle size cannot be altered by FSH injections much before age 12-15 days. The antral follicles are capable of producing estradiol, inhibin, progesterone and androgens (Davis *et, al*., 1999). Follicular growth is maintained by the interaction of pituitary FSH and LH, ovarian growth factors and steroid hormones produced at various stages of follicular maturation. Ovulation is stimulated by release of LH as follicular production of estradiol increases. After the oocyte is released, granulose and theca cells further differentiate to form progesterone-producing cells of the corpora lutea (CL) which sustain pregnancy if mating occurs (Peters and McNatty, 1980; Everett, 1961; Davis *et al*., 1999).

The estrus cycle is normally displayed every 4 or 5 days in mice. Normally the estrous cycle in mice is divided into four phases: diestrus, proestrus, estrus, and metestrus. Increasing irregularity in cycles with advancing age, including some rather prolonged periods of diestrus, has been reported in mice. In general, ovaries of even very old mice can respond to hypophyseal hormones (Thung et al. 1956).

2.5.3 Histology of the mouse ovary

A mouse ovary is covered by a single layer of cuboidal to columnar epithelial cells. In the immature mouse prior to puberty, the ovary is composed of follicles and interstitial and stromal tissue that contains blood vessels, nerves, and lymphatics. With the beginning of sexual maturity and ovulation the ovary will also contain corpora lutea.
The classification of mouse follicles is based upon the size of the oocyte, the follicular size and morphology according to the scheme originally proposed by Pedersen and Peters (1968), the classification groups being small, medium and large. Each main group is subclassified according to the number of granulosa cells in the largest cross-section of the follicles. Small follicles include type 1 which consist of a small oocyte (less than 20μm) with flat granulosa cells; type 2 follicles which consist of a small oocyte and few granulosa cells; and type 3b follicles which consist of a complete ring of granulose cells. Medium follicles include type 3b which consist of a growing oocyte (between 20 and 70 μm) surrounded by a complete ring of 21-60 granulosa cells; type 4 follicles which consist of two layers of granulosa cells; and type 5a follicles (a transitory stage) characterized by an oocyte which can be up to 70μm in diameter and is surrounded by 101 to 200 granulosa cells. Large follicles include type 5b which consist of a fully grown oocyte surrounded by 201-400 granulosa cells, but no antrum; type 6 which contains 401-600 granulosa cells and has the beginnings of an antrum; type 7 with more than 600 granulosa cells and a cumulos oophorus; and type 8 which is the preovulatory follicle. (Davis *et al*., 1999)

2.5.4 Ovary transplantation

The orthotopic ovary transplantation in mouse is a powerful technique in animal laboratory and core facilities, the technique provides researchers the possibility to save valuable infertile mutants when the reason for infertility is other then non germinal cells. The ovarian transplantation could also be used on animals which have low fertility. The ovary can be divided in four halves and transplanted to four recipients permitting increased chance in obtaining more offspring.
Using this technique, the ovaries could be transplanted orthotopically, unilaterally or bilaterally in the recipients. Another way to perform ovarian transplantation is to transplant them heterothopically, placing the ovary in a different site in the body of the recipient than its normal anatomical position. The ovary tissue can also be transplanted between different species, called xenografic transplantation (Snow *et al.,* 2002)

There are mutants and transgenic mouse strains which lack the ability to breed, but where the females have functional ovaries. Ovary transplantation is an important tool for maintaining and producing crosses with these non-breeding strains.

2.6 Germ-free or gnotobiotic animals and embryo transfer

Germ-free (GF): animals that are free from any microorganism and that live in a special steel or vinyl isolator environment without any contact with the outside, produced for use in biomedical research. Germ-free animals are free from all demonstrable forms of life, including bacteria, viruses, fungi, protozoa and other saprophytic or parasitic forms.
Gnotobiotic (GN): describes an animal or system in which all the life forms are known.
Conventional (CV): an animal with an uncontrolled microflora, reared under open room conditions in association with other animals of the same type. (Gustafsson & Coates 1984)

An adult mammal that lives under conventional conditions harbors normal microflora in skin and mouth mucous membranes, upper respiratory, genitourinary tracts and certain areas of the alimentary tracts. There is a lot of information supporting the concept that the body surfaces, cavities and micro-organisms found on or in the animals constitute open ecosystems obeying natural laws governing such systems (Savage, 1977b).
Micro-organisms found in the gastro-intestinal canal are recognized as either autochthonous, (native) in the communities established in the body, or allochthonous (transient) which are only passing organisms that fill in a gap temporarily when an autochthonous resident has left a vacancy. Allochthonous strains isolated from a specimen should not be regarded as members of the "normal" microflora to the region of the body from which the specimen was taken. Similarly, micro-organisms involved in disease processes should not be regarded as members of the normal flora. Autochthonous as well as allochthonous microbial strains can cause diseases in their animal hosts (Savage 1977a). Some micro-organisms of the indigenous microflora may be involved in diseases that develop over a long period, e.g. colonic cancer (Savage, 1977a). Given such possibilities, the concept of "normal microflora" is not adequate. Nevertheless, it has traditional acceptance as the common way of referring to the microflora of any animal.
The normal microflora of the exposed surfaces and alimentary cavities of an animal of any given species can be exceedingly complex in composition and function (Savage, 1977b). In addition because of anatomical, physiological, environmental or dietary reasons the microflora can differ in composition and activity from animal species to species and perhaps from individual to individual in some species, even in body regions of basic similarity in structure and function (Savage, 1983).

2.6.1 Succession (neonatal animals)

A healthy mouse fetus has a sterile gastrointestinal tract previous to its birth (Savage, 1977b). However at birth the fetus is exposed to various amounts of micro-organisms from the mother's vagina and external genitalia as well as from the external environment. On the other hand many of these micro-organisms are unable to colonize habitat in the neonatal tract and disappear from it soon after birth. There are also other microbial types, the pioneers, produced in the offspring and eventually form the climax communities in the adult. These communities are formed through succession (Savage, 1977b).
In suckling mice (Schaedler *et al*., 1965), and probably also rats (Brunel & Gouet, 1982; Raibaud *et al.,* 1966), at 1 or 2 days after birth lactic acid bacteria can be cultured from all regions of the tract but especially the stomach, where they colonize the gastric epithelium. Shortly after birth as well, populations of facultative anaerobes, such as Escherichia coli and Streptococcus faecalis, can be detected along with the lactic acid bacteria. These bacterial strains achieve high population level after they are detected initially (Lee & Gemmell, 1972; Schaedler *et al.,* 1965; Tannock, 1979) and are found at these high levels in all regions of the

tract including the stomach. When their population levels are high, usually during the second week after birth, microcolonies of these facultatives can be seen in the mucus coating the colonic epithelium in mice (Davis *et a*l., 1973; Lee & Gemmell, 1972; Savage *et al*., 1968). When the mice begin to eat solid food then only anaerobic bacteria is usually detected in the large intestine (Davis *et al*., 1973; Lee &Gemmell, 1972; Savage *et al*., 1968). The population levels of these bacteria increase rapidly and are at adult climax levels by the time the mice are weaned.

2.6.2 Conventional production of Germ-free animals

In the 19th century Pasteur's germ-free research showed to be greatly successful in the fields of artificial nursing, reproduction and long-term rearing of GF mice and rats (Gustafsson, 1959; Reyniers *et al*., 1946) as well as in the establishment of GF rearing techniques when using vinyl isolators by Texler (1959). As a result, GF animals have become one of the most important tools in biomedical research.
The production of GF animals is mostly dependent on that, the embryo that will develop in the uterus of a healthy mother, is microbiologically sterile and that it can be aseptically, either by hysterectomy or hysterotomy (caesarian section), delivered into a sterile environment.
Hysterotomy can be performed in two different ways either by closed or open technique. Both techniques are essentially similar, the main difference being that the first is conducted from within a sterile surgical isolator, while the open method is an aseptic caesarian section, with special methods for handling the fetus (Gustaffsson & Coates. 1984)

2.6.3 Problems in production of germ-free animals

The reproductive capacity of GF animals is low in many cases, rearing procedures for some species are extremely tedious and it is therefore very difficult to produce large numbers of such animals. Most often in animal experiments, about 10 animals per group are necessary to permit statistical analysis, and at least 20 animals for the experimental and control groups must be produced in each case, depending on strain, sex, date of birth, etc. For reliable experimental results, it is essential that the physiological response of the animals to treatment be as consistent and reproducible as possible. When the fetuses are removed from the uterus timing is also crucial, as well as the time when the delivery is performed, which has a major effect on the success of hand-feeding or foster nursing. It is necessary to know the exact period of gestation when deciding the time for surgical delivery but the reproductive physiology of wild animals has not been sufficiently examined in many cases, and there are slight differences even among GF mice and rats, depending on the strain and rearing environment (Gustaffsson & Coates, 1984).

2.6.4 Embryo transfer technology

Mouse embryo transfer is a technique for genetic manipulation. The principal advantage of embryo transfer is increased reproduction capacity of a valuable mutant or conventional mouse. Embryo transfer can amplify the reproductive rate of valuable donors; it can also decrease the generation interval between selection steps by having a large percentage of the progeny from young donors. Embryos also are an excellent way to move germplasm from one region to another without introducing new diseases. In some cases, embryo transfer allows mice and rats that are infertile due to disease, injury or aging to have offspring.
The standard embryo transfer procedure consists of treating a donor female with, pregnant

mare's serum (PMS) used to mimic follicle stimulating hormone (FSH) which induces the maturation of large numbers of ova, and human chorionic gonadotropin (hCG) which is used to mimic luteinizing hormone (LH) to induce ovulation (superovulation) (Hogan *et al*., 1994). After being fertilized these eggs are removed from the donor and transferred to a foster mother for gestation. Embryo transfer involves a number of steps that are simple, but require well-trained personnel and meticulous attention to detail.

3 Aims of the studies

We wanted to develop and improve three different embryo micromanipulative techniques and make them more practical in the laboratory research and in the IVF clinics, providing the personnel and the researchers with easy tools to make their work easier and more effective.

Specific aims:

- To improve embryo biopsy microtechnique so that it will allow the preimplantation genetic diagnosis (PGD) using a single-needle blastomere biopsy.

- To improve and use the ovary transplantation microsurgical technique to save valorous mutants and transgenic animals when the reason for infertility is other than non-functional germ cells, and to apply this technique in identifying the mechanism of subfertility in oestrogen receptor beta knockout (ERβ -/-) mice.

- To create a new microtechnique to allow transfer of transgenic or conventional animals to a germ- free state, through the embryo transfer into a germ free recipient.

4 Materials & Methods

All methods are described in detail in each article present in this thesis; however, the following section brings up aspects of special interest.

4.1 Single-needle embryo biopsy in PGD

The single-needle method was performed as follows: Biopsies were done on 6-10 cell embryos, in patients, early (before noon) on the third day after oocyte pick up. Tyrode's solution (pH 2.5) was loaded into the tip of the biopsy micro pipette with the use of a 1 ml syringe and rubber tubing. A hole in the zona pellucida was drilled by gently blowing the Tyrode's solution. Once a suitably sized hole was observed the acidified medium surrounding the hole was immediately removed by aspirating medium into the micro pipette- thus forcing the surrounding medium to enter the area. The medium was sucked into the micro pipette to force all the Tyrode's solution further up in the micro pipette, leaving the tip with fresh medium. Two blastomeres were immediately aspirated from each embryo by gentle suction with the same micropipette and placed in the same droplet next to the embryo. After the biopsy procedure, the embryos were swiftly moved to a fresh culture dish and kept under culture conditions until the results of the genetic analysis were finally evaluated. The biopsied blastomeres were moved to a glass slide for FISH analysis.

The basic stages involved in the PGD procedure are detailed below:

The PDG involved eight main stages:

a. Consultation of the couple

b. Hormonal stimulation of the female partner

c. The collection of the eggs

d. The collection of the sperms

e. Fertilization of the eggs in-vitro by IVF or ICSI

f. Embryo biopsy

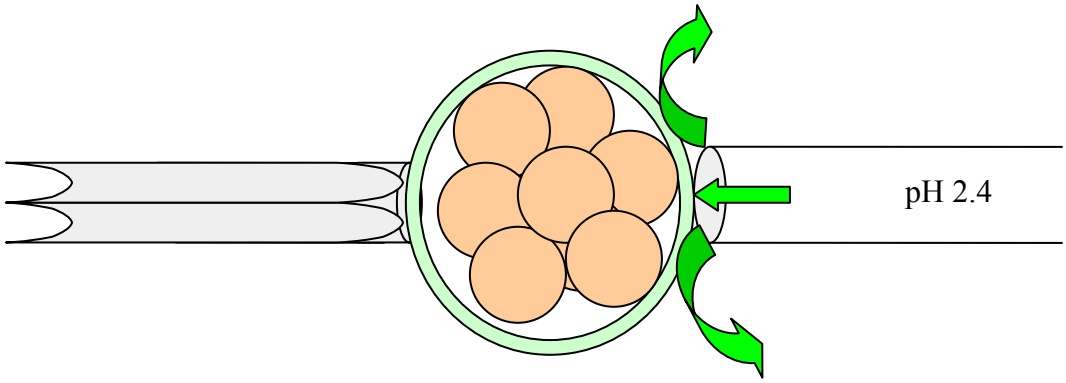
g. Analysis of blastomere by FISH or PCR

h. Transfer of the healthy embryo back into the woman.

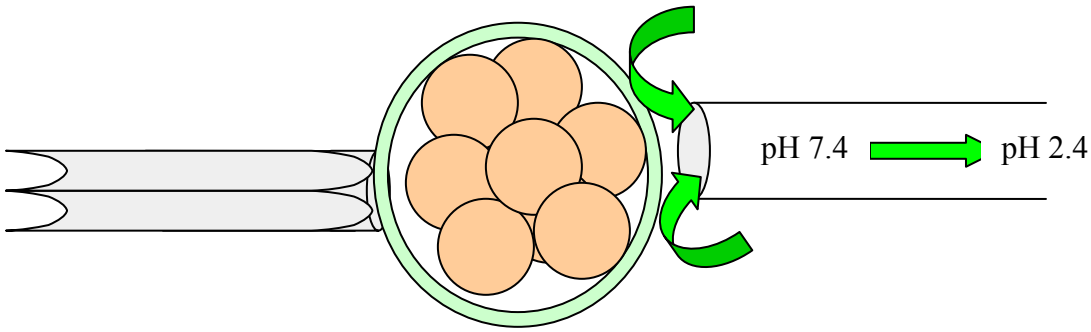
Figure 2.

Single-needle blastomere biopsy procedure

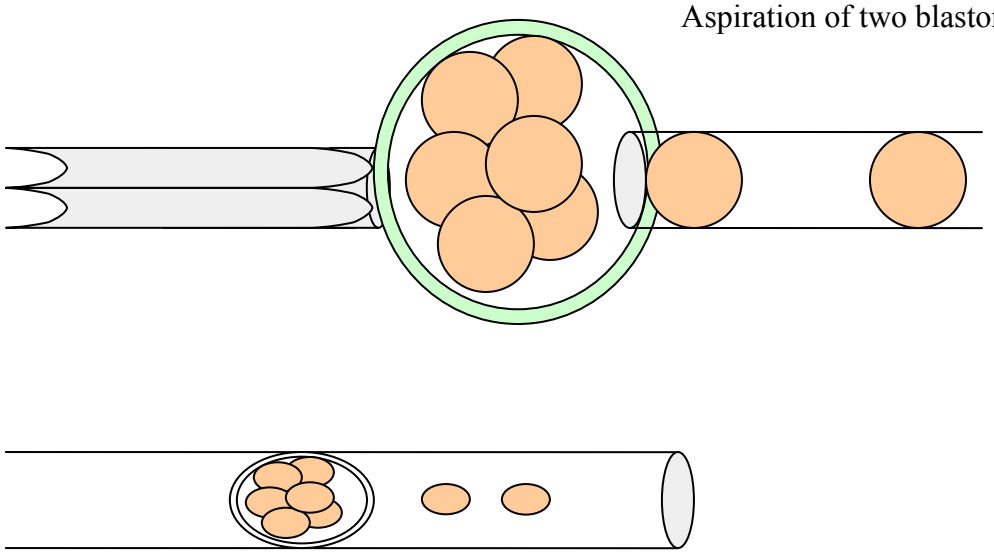
Flushing acidified Tyrode's solution pH 2.4



Aspirating back the acidified Tyrode's solution



Aspiration of two blastomeres

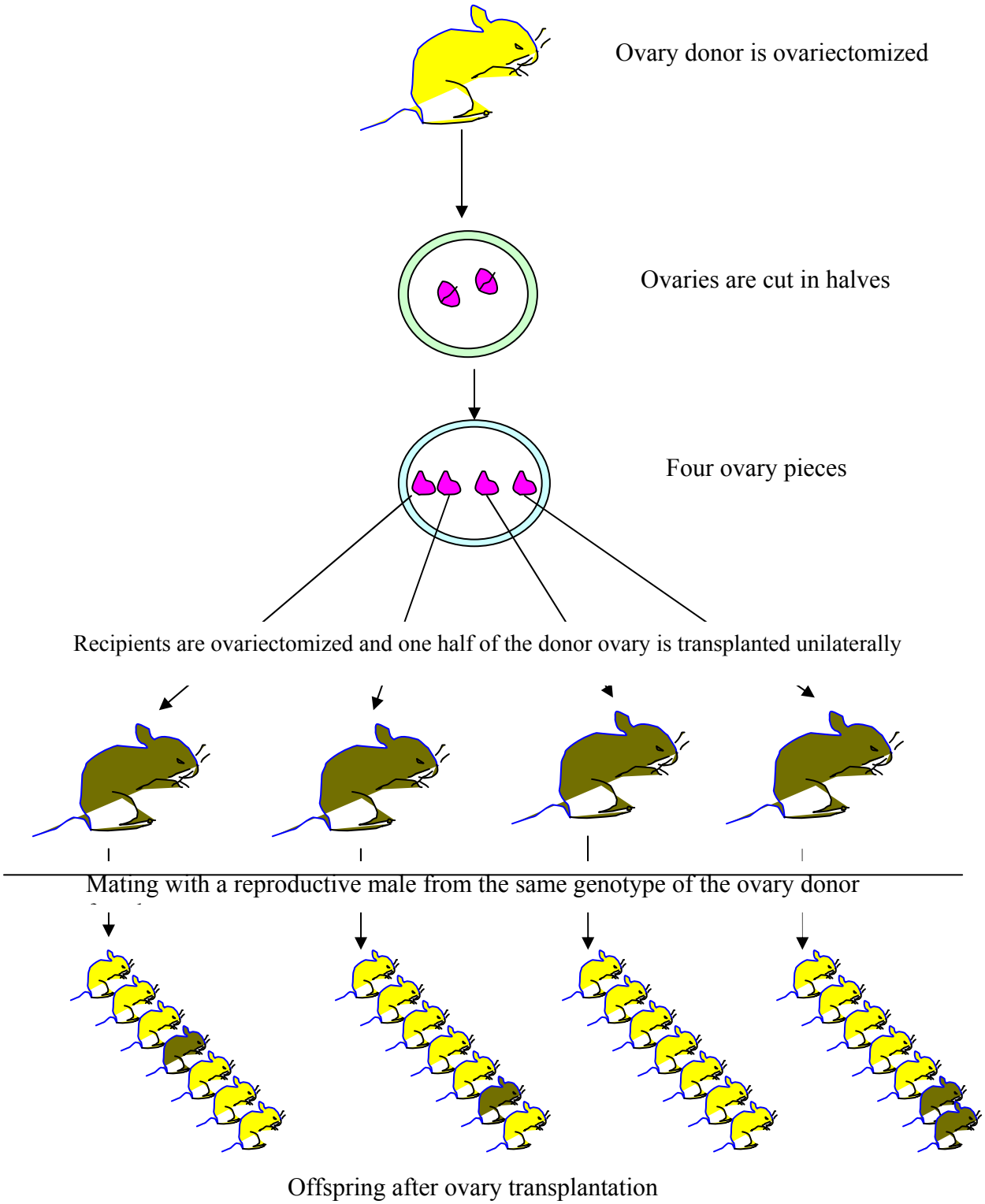


Transportation of embryo and blastomeres together to the Petri dish

4.2 Ovary transplantation procedure

The orthotopic transplantation procedure is a modified version of the technique described earlier by (Stevens 1957; Gunasena *et al*., 1997; Sztein *et al*., 1999).The donor mice were euthanized by cervical dislocation. The two ovaries from each mouse were removed under aseptic conditions. They were placed in a Petri dish containing 2 ml of M2 medium (Sigma, St.Louis MO) at room temperature, the ovaries were then cleaned and cut in halves. The recipient mice were anaesthetized with 0.2 ml/10g body weight of 2.5% tribromo-ethanol and tert-amylalcohol ("Avertin") solution. After preparing for aseptic surgery, a single incision of the skin was made at dorsal, transversally across the lumbar region, making both ovaries accessible. The ovary-attached fat pad was grasped and pulled out together with the ovary. Under a microscope, at 2 X magnification a small incision was made in the bursa, which was then peeled back over the surface of the ovary, allowing removal of the whole ovary. Both native recipient ovaries were removed at the hilum and one half of a donor-ovary was placed unilaterally into only the right bursa of the recipient mouse, the left bursa was cauterized to reduce the chance of the possible growth remains of follicles from the left native ovary.
In article III, the transplanted mice were mated with a reproductive male of strain CAST/Ei (Jackson Laboratory. USA). They were mated in 2-3 periods. The offspring were counted 12-72 hours after delivery and after approximately 2 to 3 weeks the offspring were weaning.
Typing of the haemoglobin β-chain (HHb) was performed on the whole blood (50µl) taken from the tip of the tail of each offspring mouse at 3 weeks of age. The Hbb pattern was analyzed with polyacrylamide gel electrophoresis at 50 V in electrophoresis buffer.
In article IV, the ovary transplanted mice were waiting 14 days for recovery after surgery. They were then mated with a reproductive male of the strains CBAB6F1 and C57BL/6 or ERβ-/- at the ages between 9 and 16 weeks old, they mated during 2 to 3 periods. The litters were counted and in order to confirm the progeny of the offspring, tail biopsies were taken from pups born in the second section of the ovary transplanted mice. The progeny was confirmed by Polymerase chain reaction (PCR) on DNA from the tail (Windahl *et al*., 1999). Transplanted ovaries were dissected from the recipients and fixed in 4% formaldehyde, dehydrated and subsequently paraffin embedded. Sections of 4µm were aligned in order on glass microscope slides, the sections were then stained with hematoxylin and eosin and the numbers of follicles and corpora lutea were counted. The densities were calculated by measuring the area of the image analysed programme (Easy Mätning, Bergström Instrument, Sweden) under microscope.

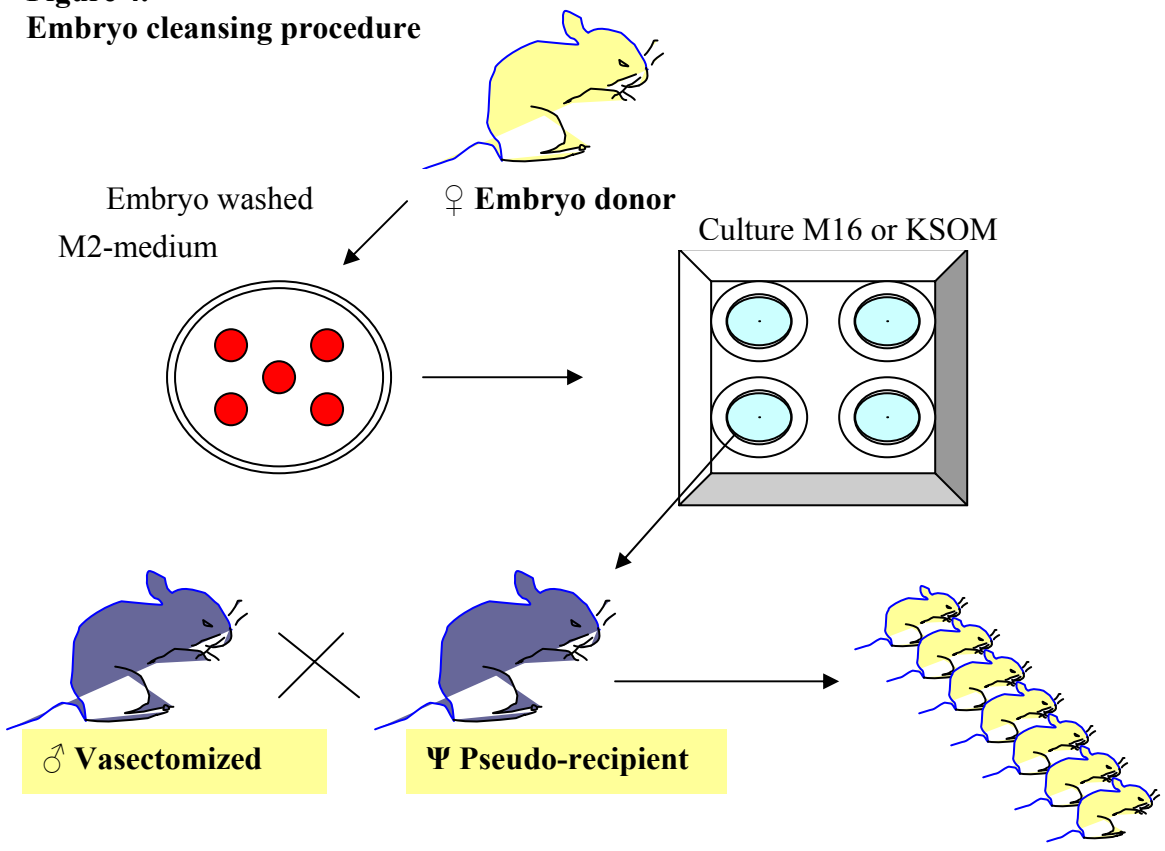
Figure 3. Orthotopic ovarian transplantation procedure in mouse



4.3 Embryo transfer to germ-free foster mothers

From a conventional or transgenic female mouse, embryos were obtained through the superovulation methods with application of intraperitonel injections of PMS (Pregnant Mare Serum Gonadotrophin) and after 48 h hCG (human Choriogonadotrophin) mated with reproductive males, and the next day the vaginal plug was checked.
The embryos were collected at the blastocyst stage (four days after the vaginal plug was checked) by flushing with M2 medium through the uterus from the cervix. The blastocysts were washed in several drops of M2 medium and loaded into the special glass capillary pipette later forged with help of a gas-flame until both ends of the pipette become hermetically closed.
The pipette with the embryos was transferred to an acid container with chromsulphuric acid to disinfect the surface of the pipette, for about 20-30 min then put into a steel isolator with the germ free pseudopreganant recipients to perform the embryo transfer procedure.
The pseudopregnant recipients were anaesthetized with Avertin solution. After preparing for surgery, a single incision of the skin was made at dorsal, transversally across the lumbar region, making both ovaries accessible. The ovary attached fat pad was grasped and pulled out together with the ovary. Under a specially constructed microscope installed over the steel isolator with 20x magnification a small incision is made in the bursa between the ovary and the oviduct to expose the infumdibulum to enable embryo transfer.
Before the embryo transfer, the pipette was cut using a diamond pen.
After 20 days the offspring were born and a germ test was made to confirm that the blastocyst had not carried any pathogen germ during the transfer.

Figure 4.
Embryo cleansing procedure



4.4 Patients

In articles I, II

The first proband was a 31-year-old woman with the typical facial appearance of DiGeorge syndrome (DGS) but otherwise no clinical symptoms. She was diagnosed after the birth of a severely affected child with tetralogy of Fallot and hypoplasia of the thymus gland. The child died at 4 months of age. A deletion in the chromosome 22q11.2 was found in the mother and her child who inherited the same deletion but was more severely affected.
The second proband was a 29-year-old woman carrying a large pericentric inversion of chromosome 5, 46,XX, inv(5)(p14q35). Her first child was diagnosed with Cri-du-Chat syndrome due to a deletion of the short arm of the chromosome 5, caused by recombination of the mother's inversion during meiosis. After the child with Cri-du-Chat syndrome was born, our proband had two spontaneous abortions during the first trimester, which may represent non-viable recombinants. The third probands were a couple where the man carried a balanced Robersonian translocation involving chromosomes 14 and 15.

4.5 Animals and husbandry

In article I, we have used B6D2F1 mice 6-8 week old from B&K Universal AB, Sweden.
In article III, females of inbred BALB/cByj mice homozygous for the spontaneous mutation megencephaly, mceph, (Jackson Laboratory, Bar Harbor,Maine) were used as donors of ovaries. These homozygous, phenotypically mutant mice do not breed but they have fully functional ovaries. Ovary recipients were females of the congenic strain C.B6-+tyr-c Hbb (Jackson Laboratory, Bar Harbor, Maine). The genome of this strain has a background of BALB/c¯+tyr-c Tyrp1b with the Hbb locus originating from C57BL/6By strain. The alleles of this locus differ between the BALB/cByJ and the C57BL/6By strain allowing them to be used as markers for determination of material germ-line in the offspring. The reproductive males used for mating with the transplanted recipient mice were of CAST/Ei strain (Jackson Laboratory, USA). They were caged in individually ventilated micro-isolators; Vent-A-Cage racks (Iwarsson & Norén, 1992).
In article IV, we used mice from the homozygous ERß-/-, heterozygote ERß +/- and wild type littermate, all mice were of mixed C57BL/6J/129 backgrounds. We also used C57BL/6 and CBAxB6 mice from B&K Universal, Sollentuna, Sweden.
ERβ-/- female mice are subfertile, they have a block in the last step in the follicle development before ovulation (Enmark & Gustanfsson, 1999; Cheng et al., 2002).
In article V, the mouse strain NMRI used in this study originates from the National Institute of Health, Bethesda, USA, and has been kept germ-free and inbred at the Karolinska Institute for close to 40 generations. Hybrid (CBAxC57/Bl6)F1 (=CBAF1) animals were generated by mating CBA/J and C57Bl/6J mice obtained from Jackson Laboratory, Bar Harbor, USA.
The transgenic mice Gfap-HSV-Tk are animals expressing mouse glial fibrillary acidic protein (Bush et al., 1998).
All animals were housed in polycarbonate plastic cages containing wood chips with free access to fresh water and comprised food pellets, in barrier animal facility and kept at 12 h light: 12 h darkness, a temperature of 21-22° C and relative humidity of 50-62%.

4.6 FISH analysis

In article I and II

After biopsy for PGD, the blastomeres were washed in phosphate-buffered saline (PBS) for 2min and transferred into a drop of spreading solution on a poly-L-lysine-coated slide. The

blastomere was observed under an inverted microscope until the nucleus was free of cytoplasm. The slides were left to air dry, then washed in PBS and dehydrated through an alcohol series. The technique of fluorescent in situ hybridization (FISH) used DNA probes that were fluorescently tagged and bind to complementary sequences on a specific chromosome. After hybridization nuclei were examined under a fluorescent microscope and the number of fluorescent signal indicated the number of chromosomes present. Several probes could hybridize simultaneously when each labelled was with a different colored fluorochrome. This enabled enumeration of more than one chromosome in a single cell.
The initial experiments using FISH on interphase blastomeres employed indirectly labelled probes (Griffin *et al*., 1991).These probes are labelled and thus linked to a fluorochrome by a specific antibody. The signal was amplified immunochemically. Improvement in the sensitivity of fluorescent detection system now means that most workers use directly labelled probes where the fluorochrome is attached to the probe itself (Harper *et al*., 1994). No signal amplification is needed, resulting in a very rapid procedure with minimal background. There are only two types of FISH that can be used in interphase nuclei: alpha-satellite or repeat sequence probes locus and unique sequence probes (Harper *et al*., 2001).

4.7 Microbial analyses

In article V, the mice were kept under specific pathogen free condition: Foster mothers after weaning of the litters, and sentinel animals (exposed to bedding from the cages housing the cleansed mice) were sent to the National Veterinary Institute (NVI), Uppsala, Sweden, for analysis.
Mice in germ-free rearing conditions were checked weekly for germ-free status by inoculating faecal samples into selected aerobic and anaerobic growth substrates at 20°C and 37°C

Aerobic growth:
-Tryptose and Glucose Broth (TGB)
(DIFCO Laboratories Detroit MI, USA and Mallinckrodt Chemical Works, St. Louis, MO, USA)
-Reinforced Clostridial Medium (RCM)
(MERCK, Darmstadt, Germany)

Anaerobic growth:
-Thioglycollate Medium (THM)
(DIFCO)
-AC Medium (ACM)
(DIFCO)

5. Results & Discussions

5.1 Preimplantation genetic diagnosis

In article I

The mouse study, the survival of the embryo after biopsy and overnight incubation was 100 % for the entire regimen tested. All blastomeres removed were intact immediately after the biopsy procedure. After one day of incubation, biopsied embryos reached the morula or blastocyst stage and were then transferred to a pseudorecipient. In the follow-up of mice born after embryo biopsy, no significant difference was observed in body weight up to four weeks of age between biopsed and control groups.

Male and female adults from the biopsed group were fertile and produced live young of expected sex ratios. In the histological examine no gross lesions were found, the histology of the necropsy was normal. The blood plasma analyses (15 biomedical parameters) do not show any difference with the control group.

In the clinical study three cycles were performed in a couple in which the woman, 31 years of age, was a carrier of a microdeletion on chromosome 22p11.2 (DiGeorge syndrome). Not many embryos were obtained in all three cycles; out of the total of eight embryos undergoing biopsy diagnosis was possible for only six. Two normal embryos were transferred but no pregnancy was achieved.

Two cycles were performed in a couple in which the 29 years old woman was a carrier of a pericentric inversion on chromosome 5. (See article II).

One cycle was performed in a couple in which the man was a carrier of a balanced Robertsonian translocation involving chromosome 14 and 15. Only four embryos were biopsied and diagnosed successfully. One embryo with balanced genotype was transferred to the woman, 29 years, leading to pregnancy and the delivery of a healthy baby.

Table II: Clinical results:

Cycles	Diagnosis	Fertilized eggs	Biopsied embryos	Analysis	Affected	Healthy	ET	Birth
A1-3	DiGeoge syndrome	14/26	8	6/8	4	2	2	No
B1-2	(Cri-du-Chat) -5p	35/48	21	19/23	10	9	2	Twins
C1	Rob.trans 14 and 15	6/8	4	4/4	3	1	1	Boy

In total, the single-needle biopsies were attempted on:

35 embryos
6 cycles
2 embryos were damaged during the biopsy
66 blastomeres were aspirated from 33 embryos
59 blastomeres (89%) were intact
29 embryos analyzed with FISH (88%)
4 embryos were not analyzed (12%)
12 embryos were affected
17 embryos were not affected
7 embryos were transferred
3 healthy babies were born (43%)

The application of the single-needle method to perform embryo biopsy in PGD is shown to be more effective concerning the rapidity and simplicity of taking biopsies. This method can ensure a better environment for the embryos, less time outside the incubator, better follow-up of the procedure, better control of the biopsy pipette, easy to set up the micromanipulator and less damaged blastomeres.

In article II
We have performed two PGD treatments to a couple in which the woman, 29 years of age, was a carrier of a pericentric inversion on chromosome 5. The treatments yielded a high number of oocytes and a total of 20 embryos were biopsied. Two embryos were damaged during the aspiration of blastomeres. In 4 out of the remaining 18 embryos one of the blastomeres was lysed during the aspiration. Both cycles led to transfer of two embryos with balanced genotype. A pregnancy was detected with raised HCG concentrations 13 days after transfer, indicating implantation, and ultrasonography confirmed the presence of two fetal hearts two weeks later. Amniocentesis was performed at 15 weeks and cytogenetic analysis confirmed two balanced inversion carrier karyotypes. After 34 weeks healthy twins, one male and one female were delivered.
This was the first rapport of an established pregnancy using PGD in order to avoid chromosome imbalance in the progeny of an inversion carrier and also the first babies born using a single-needle procedure to perform the blastomere biopsy in PGD.

5.2 Ovary transplantation

In article III,
Ten transplanted female mice from the congenic strain BALB/c.C57BL/6By HHbs, with ovaries from non-reproductive mutant BALB/cByJ, produced at least one litter. The mean litter size for all transplanted mice from the first two mating periods was 6.0± 2.7 offspring per litter. For untransplanted BALB/cJ the mean litter size is reported to be 5.2. There was no significant difference in mean litter size between the age combinations.
To determine the maternal origin of the offspring they were typed for hemoglobin β-chain variant. 76 of the total 81 surviving offspring mice, 94%, were derived from the transplanted ovaries.
The ovary transplantation used in this study shows a very high performance (94%) compared to previously published studies 33% (Cox *et al*., 1996), 48% (Parkening *et al*., 1985), 60% (Brem et al., 1990), 66% (Gunasena *et al*., 1997b), 80% (Stevens, 1957), 100% (Gunasena *et al.,* 1997a).
The mean litter size for the females was normal, despite the fact that the two native ovaries were replace by only one half of an ovary.
There is a risk of not completely removing the native ovaries during transplantation and thus a risk that the transplanted mouse produces her natural progeny. Delivery of natural progeny occurred in 6% of the offspring. Four of these 5 endogenous offspring were delivered from one and the same female, suggesting an incomplete ovary removal. However, to be sure of the genotype of the offspring, typing the progeny for the maternal germ-line is important. This can often be performed easily with coat color markers.
With this method, only one donor mouse is needed to provide ovary transplantation to four recipient mice. Surplus ovaries can be frozen for transplantation at a later time.

In article IV

After transplantation of ovary pieces of 5 ERβ -/- to 8 WT littermates and 10 normal C57BL/6 female mice, 12 litters having a total of 58 pups were born.
The litters were born to four out of 8 WT littermates and to 4 out of 10 normal mice.
When WT mouse ovaries from 6 donors were transplanted to 9 ERβ-/- recipients, 8 litters with 37 pups were born. 7 litters with a total of 31 pups were born to 4 recipients out of the 11 transplanted.
Ovaries transplanted from 1 heterozygote (ERβ+/-) mouse donor into 2 WT littermates and 2 normal C57BL/6 recipients, 24 pups were born out of a total 6 litters.
The progeny from these mice, in the second section of ovary transplantation, where 87% of the offspring originated from the transplanted ovaries and 13% from the native ovaries indicated an incomplete ovary removal in some of the recipients.

Table III: Ovary transplantation, section I and II

Donors	Nr.	Recipients	Nr.	Litters	Offspring	Comments
ERβ-/-	5	WT	8	6	23	
		C57BL/6	10	7	35	
WT	6	ERβ-/-	9	8	37	2 recipients found dead
		C57BL/6	11	7	31	
ERβ+/-	1	WT	2	4	14	
		C57BL/6	2	2	10	
Total	12	2 types	42	34	150	

When WT mouse ovaries were transplanted to knockout mice, 2 ERβ-/- recipients mice were found dead, 6 respective 31 days after transplantation. This may suggest extra-ovarian causes of subfertility and ovarian failure among the ERβ-/- mice. Such causes are not absolute and probably not the only ones, another possible cause may lay in the other organs of the ERβ-/- mice themselves. The uterus was normalized and could carry normal delivery of offspring whose endocrine disturbance was corrected by the ovarian transplantation.
We also found similar numbers of follicles and corpora lutea between ovaries from ERβ-/- mice transplanted into WT and ovaries from WT into ERβ-/-.

Table IV: Density of follicles and corpora lutea/mm³ ovarian cortex

Ovary transplantation type	Nr. of follicles	Nr. of corpora lutea
ERβ-/- ovary into WT	365	369
WT ovary into ERβ-/-	646	516
ERβ-/- control ovary- no transplanted	1055	158
WT control ovary- no transplanted	1560	282

5.3 Embryo transfer to germ-free foster mothers

In article V
For testing and evaluating the embryo cleansing technique, we performed embryo transfer to 203 pathogen free recipients mouse stocks with a verified presence of specific pathogen (s).
After weaning of the litters, the outcome of the cleansing was monitored by microbiological testing of the recipients or by use of sentinel animals. This series of transfers confirmed the

technical possibility to effectively eliminate pathogens from a large number of different mouse stocks; i.e.

Table V: List of viruses, bacteria and parasites which have been cleansed from mouse stocks

Viruses	**Bacteria**	**Parasites and protozoa**
MHV	Entamoeba	*Aspicularis tetraptera*
MVM	muris	*Myobia musculi*
Parvovirus	*Escherichia coli*	*Myocoptes musculinius*
Reovirus	*Enterobacter cloaceae*	Heminths spp
Rotavirus	Helicobacter spp	Protozoa spp
Theilers encelhlomyelitis	*Klabsiella oxytoca*	*Syphacia obvelata*
	Klabsiella pneumoniae	
	Pasteurella spp	
	Pneumocysis carinii	
	Proteus mirabilis	
	Tricomobas spp	

The efficiency of the microbial cleansing can be illustrated by finding that cleansed offspring was found to lack several different pathogens found present in the embryo donor mouse stock. An experiment in which six super-ovulated CBA/S female mice after mating to CBA/S males produced forty 8-cell embryos; these embryos were cleansed and transferred to two specific pathogen free pseudo-pregnant recipients. One recipient gave birth to five pups of which one male and two females were further bred to obtain a larger number of mice. Two male pups were kept as controls for microbiological status and the results of the analysis at the age of seven weeks, were negative concerning the presence of the indicated pathogens from the original (embryo donor) colony.
Pasteurella spp bacteria is difficult to fully diagnose, also in the putative recipient mice, therefore it is important to use Pasteurella free pseudo-recipients for the embryo transfer.

After the above result showing, the efficiency of embryo cleansing throughout the transfer of the embryos to a pathogen free pseudo-pregnant recipient, we were able to perform embryo transfer of cleaned embryos to a germ-free pseudo-recipient into a special germ-free steel isolator environment, with the intention to produce germ-free or gnotobiotic mice throughout the embryo transfer.
The embryo donor mice which were subjected to embryo cleansing were Gfap-HSV-Tk transgenic and CB6F1 strains, a total of 160 embryos were recovered and transferred to 10 pseudo-recipients (8 NMRI and 2 CB6F1 strain); seven pups were born from two CB6F1 pseudo-recipients and nine pups were born from two out of eight NMRI pseudo-recipients. The offspring was subjected to tests and were found negative for the presence of detectable microbes. For future work the results indicate that CB6F1 mice as recipients would be a more cost-effective choice. From a total of eight germ-free NMRI recipients, three produced offspring, and from those one ate their offspring. In contrast, two germ-free CB6 hybrid recipients both produced live offspring. The embryos were transfer in blastocysts or morula stage. From our previous experience, the blastocysts have a strong resistance to external factors such as temperature and poor culture conditions.

6. Conclusions

The conclusions of the present study showed that these three techniques have a potential use in reproductive biology and that they are a powerful tool in the practical application. These applications serve to overcome the limitations of classical methodology and they can pave the way for the improvement and development of new techniques based on the assumptions of the necessity to go further in the reproductive biology investigation.

In the single-needle application to perform embryo biopsy we noticed that the technique is simpler than the classical one when two needles are used to perform the blastomere biopsy.
The testing and successful application of the single-needle approach to obtain blastomere biopsies from human preimplantation embryos for preimplantation genetic diagnosis (PGD) was a completely new variant in the biopsy technique. The method was first tested in a mouse model, where it was shown to be to a great extent compatible with various in-vitro and in-vivo developments of biopsied mouse embryos. Furthermore, we showed that biopsied mouse embryos after transfer to recipient mice underwent implantation, normal development and delivery. Litters were followed through puberty and adulthood and shown to be normal with regard to sexual function and also a panel of biochemical and morphological parameters including organ histology.
Successful human preimplantation diagnosis, followed by pregnancies and birth of healthy babies, was established with two out of three couples carrying a risk to transmit chromosomal abnormalities leading to severe diseases (pericentic inversion of chromosome 5 and Robertsonian translocation of chromosome 14 and 15). This is the first report of the successful use of a single-needle approach in human PGD. Considering its simplicity, we conclude that the single-needle approach is an attractive alternative for biopsies in PGD.
The technique can be extended to analyze almost all genetic disorders including age related aneuploidies. This technique is now used as a routine in the assisting reproduction laboratory at the Huddinge University Hospital. At the present several couples have been treated in this unit using this technique.

In the attempts of transplanted ovaries we have observed that this technique is a powerful tool to overcome the limitations of breeding valuable mouse colonies when they suffer an apparent infertility or subfertility. The technique is very useful also for maintaining the genotype of strains that recessively inherit the breeding dysfunction. This allows breeding with homozygous rather than heterozygous mice resulting in twice the ratio of abnormal versus normal offspring in the litter. Using heterozygous breeding the offspring would need progeny testing to assure presence of the mutant genotype with which the strain maintenance is to be continued. In outcrosses between a normally functional strain and a breeding dysfunctional strain where an affected sex is to be used, the profit of ovary transplantation is the same as in in-cross breeding.
We showed that when we have transplanted ovaries from the female belonging to a non-reproductive BALB/cByJ mutant mouse strain, all transplanted mice, a total of ten BALB/c.C57BL/6By, produced offspring and 94 % of the progeny originated from the transplanted ovaries. The mean litter size and the mating period needed for productive mating to occur were similar to what was observed for corresponding control mice.

-We also studied mechanisms of reproductive failure in ERβ knockout (ERβ-/-) mice by transplanting ovaries of ERβ-/- mice into WT littermate and into the normal C57BL/6 mice as well as ovaries from WT littermates into ERβ-/- and into normal C57BL/6 mice.
We showed that ovary transplantation of estrogen beta-receptor knockout (ERβ-/-) ovaries

into the WT littermate and normal C57Bl/6 recipient mice, can produce similar litters as when the ovaries of WT littermate or normal C57BL/l are transplanted into WT female recipients.
The results suggest an extra-ovarian cause, probably a hypophyseal cause of subfertility in the ERβ -/- mice. The uterine abnormalities of those animals (Zhang *et al.,* 2000) are probably a consequence of ovarian dysfunction, because they have normal litters after receiving a WT ovary. Ovarian transplantation shows that an ERβ-free uterus function normalizes in mice when ovarian function is normal, and that an ERβ-free ovary function apparently normalizes once it is transferred to a normal environment. The results suggest that ERβ in granulosa cells is not essential for ovulation, but it is still not clear whether the few normal follicles of the WT recipient mice are sufficient to normalize the functions of the ERβ-/- follicles. However, despite the normalization of ovarian and uterine functions, ovarian transplantation did not improve maternal behavior of ERβ-/- mice, since some of them continued to eat their pups soon after delivery. Although there are many possible causes of the subfertility in ERβ-/- mice, the ovaries of ERβ-/- mice are clearly structurally competent for ovulation. One explanation for the recovery of normal ovarian function is that the residual normal follicles of the recipient mouse are enough to signal to the ERβ-/- ovary. Some other possible factors which could mediate such signals would be cAMP.

In the creation of a new technique to obtain germ free animals through the embryo transfer, we received positive results and showed that it was in fact possible to produce germ-free mice through the embryo cleansing and transfer techniques.
We evaluated the embryo transfer technique for the purpose of changing the microflora of valuable mutants and conventional mice. Our results showed reproducible and quality-assured conversion to gnotobiotic animals, animals without the presence of detectable microbes. Rapid and easy access to gnotobiotic mutant mice is advantageous for studies of selected microflora and their metabolic implications.
Embryo transfer is done in a steel isolator environment, allowing implantation into germ-free recipients under well-controlled germ-free conditions. The embryos were transferred in a blastocyst stage and were obtained from super-ovulated female mice belonging to the CBAxB and Gfap-HSV-Tk transgenic strains.
Compared to the Caesarean section, this method carries less risk of transmitting infections to recipients and is easy to perform. The recipient females gave normal birth and took care of the offspring as if they were their own pups, thus enhancing the survival rate. Access to advanced isolator rearing technology is, however, a prerequisite. In this study we used a specially constructed steel isolator, on which we mounted a stereo microscope used in connection with embryo transfer inside the isolator.
Using embryo transfer and advanced isolator techniques we paved the way for use of both knock-out and transgenic animals under different gnotobiotic conditions-both germ-free, mono-associated and poly-associated animals, in order to compare with conventional animals. It is shown that the flora is of crucial importance as regards phenotypic expression and in studies such as colitis models.

7. Acknowledgements

I wish to express my sincerest gratitude to:

My supervisor, professor Outi Hovatta, for helping and assisting me with valuable advice, as well as trusting and believing in me and my work.
Professor Lars Ährlund-Richter for his support, enthusiasm and his innovating ideas.

I also especially want to thank everyone who has made this thesis possible:

To the articles co-authors, Erik Iwarsson, Elisabeth Blennow, Prof. Magnus Nordenskjöld, Margareta Fridström, Björn Rosenlund, Peter Sjöblom, Torbjön Hillensjö, Prof.Jones Bernt, Prof.Tore Midtvedt, Elisabeth Norin, Ewa Österlund, Anna-Karin Persson, Mona Fartoo, Björn Rozell, Margaret Warner, Julius Hreinsson, Guojun Cheng, Prof.Jan-Åke Gustafsson, Catharina Lavebratt, Susanna Petersson, Krister Iwarsson and Prof. Martin Schalling, thank you all for your positive attitude, friendship and enthusiasm in the collaborations made together.

My special gratitude to: Prof. Britt-Marie Landgren for her support. Prof. Gabriel Fried, Prof. Åke Pousette, Agneta Blanck and Stephan Teglung for their positive disposition.

All my friends at the lab and journal club, for the great times spent in interesting discussions about science and life: to Ami Strömberg and Lusine Aghajanova for sharing the small space of the office with me and always bringing me inspiration, to Karolina Kublickiene, for her great organizational feeling, to Karin Gertow, Mia Lindeberg, Åsa Bandholtz, Catharina Maijgren-Steffensson, Mikael Andäng, Anders Rehn, Susanne Wolbank, Eimantas Svedas, Natalia Cruz, Leonid Luksha, Victoria Keros, Jennifer Scott, Inger Britt Carlsson, Pu Zhang, Heli Teerijoki and Marta Imreh for being around me every day and always being open for small talks.

All the staff from the Embryology and Genetics Unit at the Karolinska Institute: Mikael Zmarzlak, Sara Selenius, Jenny Lingren, Hanna Karlsson, Hanna Eriksson, Inna Skoglund, Arif Kisana, Jalil Polus, Marie-Luise Werner, Lena Sternmark, Elin Tühsammel, Linnea Ekwall, Anna Österland, Helena Förberg, Camilla Lund and many others, as well as to the former staff especially to Christina Karlzon for her never failing support.

All my friends at the In-Vitro Fertilization Unit at Huddunge University Hospital, to former staff members Sirpa Lundman and Mila Mikkola for being the first to use the biopsy technique. To Mauri Orava, and to all the present staff: Ejia Matilianen, Sofia Johansson, Lev Levkov, Ingalill Persson, Linda Eklund, Mirya Tolvanen, Maryam Sheikhi, Lena Möller, Ingegerd Lundqvist, Kerstin Bjuresten, Marie-Klinta Svensson, Erik Bjurulf and Ingvar Ek.

To all members of the Department of Clinical Genetics at the Karolinska Hospital and the Department of Molecular Medicine of the Karolinska Institute: to Helena Malmgren, Irene White, Sigrid Sahlén, and Maria Anvret for their great collaboration and dedication in the molecular analyses

All the members of the Microbial Ecology at Karolinska Institute in Solna: to Johannes Bergstedt, Sandra Andersson, and Halil Canay for helping me with all the small things.

All the members of the secretarial staff at the Institutions of Clinical Science: especially to Agneta Wittlock. To the department of Obstetric and Gynecology at the Huddinge University Hospital, especially to Katarina Oxelbeck, Christina Ohlson and Anita Gasperoni who have helped me with all the details. To the staff from the administration of the Clinical Research Center: Agneta Ovaskainen, Agneta Nilsson, Kirsti Törnroos, Inga-Lisa Wallgren, Lotti Fohlin, Gunilla Grunert, Åke Ellin, Mikael Gradin, Isa Inekci, Nelson Morales and Gladys Wigh.

All my friends outside the lab for never failing to encourage me while at the same time keeping my feet on the ground and reminding me that there are other things in life than science. I also want to thank José Arteaga and Maria Eugenia Cardona for their sincere friendship, to Patricia Humire, Blanca Silva and Hernan Concha, for their friendship and positive attitude.

To my family: my wife Monica and my children; Marcelo, Lisa and Mara, for love and support, and to the rest of my family as well. Thank you all for the great support, love and understanding shown to me throughout everything.

THANK YOU ALL!

These studies were supported by funds from Karolinska Institute and the Swedish Medical Research Council. E.I. is a Swedish Medical Association scholar.

8. References

Alexandre H (2001). A history of mammalian embryological research.
Int.J Dev Biol. 45 (3 spec.No): 457-67

Balkan, W., Burns, K. and Martin, R.H. (1983) Sperm chromosome analysis of a man heterozygous for a pericentric inversion of chromosome 3.Cytogenet. *Cell Genet*., **35**, 295–297.

Baskar JF, Huang ES. (1981) Infection of mouse ectoplacental cone cells with murine cytomegalovirus. *J Gen Virol.* Jun;**54**(Pt 2):415-20.

Beemer, F.A., de, F.H., Rosina, A.I. *et al*. (1984) Familial partial monosomy 5p and trisomy 5q; three cases due to paternal pericentric inversion 5(p151q333). *Clin. Genet*., **26**, 209–215.

Bras M, Lens JW, Piederiet MH, Rijnders PM, Verveld M, Zeilmaker GH. (1996). IVF lab Laboratory aspects of in-vitro fertilization.
N.V. Oregon, Ed. P.M. Rijnders.

Brem G, Baunack E, Muller M & Winnacker El (1990) Transgenic offspring by transcaryotic implantation of transgenic ovaries into normal mice.
Mol Reprod Dev. **25**, 42-44.

Brunel, A. & Gouet, P. L. (1982). Kinetics of establishment of the gastrointestinal microflora in the conventional new-born rat. Annales de Microbiologie B 133, 325-334.

Bush TG, Savidge TC, Freeman TC, Cox HJ, Campbell EA, Mucke L, Johnson MH, Sofroniew MV (1998) Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. *Cell*. Apr 17;**93(**2):189-201.

Carthew P, Wood MJ, Kirby C. (1983) Elimination of Sendai (parainfluenza type 1) virus infection from mice by embryo transfer.*J Reprod Fertil*. Sep;**69**(1):253-7.

Carthew P, Wood MJ, Kirby C. (1985) Pathogenicity of mouse hepatitis virus for preimplantation mouse embryos. *J Reprod Fertil*. Jan;**73**(1):207-13.

Cassel, M.J., Munne´, S., Fung, J. *et al*. (1997) Carrier-specific breakpointspanning DNA probes: an approach to preimplantation genetic diagnosis in interphase cells.
Hum. Reprod., **12**, 2019–2027.

Chai CK, Dickie MM (1966) Endocrine variations. In green El (ed), Biology of the laboratory mouse (2nd ed). Dove, New York, pp 387-403.

Chen, S.U., Ho, H.N., Chen, H.F., Chao, K.H., Huang, S.C., Lee, T.Y., Yang, Y.S. (1997). *A* simplified technique for embryo biopsy: use of the same micropipette for zona drilling and blastomere aspiration.*J. Assist. Reprod. Genet*., **14,** 157–161.

Cheng G, Zhang W, Mäkinen S, Mäkelä S, Saji S, Warner W, Gustafsson J-A and Hovatta O.(2002) A Role for the Androgen Receptor in Follicullar Atrasia of Receptor Beta Knockout

Mouse Ovary. *Biology of Reproduction* **66** : 77-84

Chernos, J.E., Fowlow, S.B. and Cox, D.M. (1992) Cri du chat syndrome due to meiotic recombination in a pericentric inversion 5 carrier. *Clin. Genet*., **41**, 266–269.

Colls, P., Blanco, J., Martinez-Pasarell, O. *et al*. (1997) Chromosome segregation in a man heterozygous for a pericentric inversion, inv(9) (p11q13), analyzed by using sperm karyotyping and two-color fluorescence in situ hybridization on sperm nuclei. *Hum. Genet*., **99**, 761–765.

Conn, C.M., Harper, J.C., Winston, R.M., Delhanty, J.D. (1998) Infertile couples with Robertsonian translocations: preimplantation genetic analysis of embryos reveals chaotic cleavage divisions. *Hum. Genet*., **102**(1), 117–123.

Coonen E, Hopman AHN, Geraedts JPM, Ramaekers CS. (1998) Application of in-situ hybridization techniques to study human preimplantation embryos. *Hum Reprod* (Review); 4:135-152.

Cox SL, Shaw J & Jenkin G (1996) Transplantation of cryopreserved fatal ovarian tissue to adult recipients in mice. *J Reprod Fertil*. **107**, 315-322.

Cunliffe-Bearmer TL (1983) Biomethodology and surgical techniques. In: Foster HL, JD Small & JG Fox (eds.): The mouse in biomedical research. *Academic Press*, New York. **3**: 402-430.

Dale B, Gualtieri R, Talevi R, Tosti E, Santella L & Elder K (1991). Intercellular communication in the early human embryo. *Mol Reprod Dev* **29**: 22-28

Davis, C. P., McAllister, J. S. & Savage, D. C. (1973). Microbial colonization of the intestinal epithelial in suckling mice. Infection and Immunity 7, 666-672.

Davis BJ, Dixon D, Harbert RA. (1999) Pathology of the mouse *Edited by Robet R Maronpot,* Publ. Cache River Press USA.

Dobbs, M., Overhauser, J. and Wasmuth, J.J. (1988) Molecular analysis of a case of meiotic recombination leading to cri-du-chat syndrome. Cytogenet. *Cell Genet*., **47**, 5–7.

Donahue LR., SA Cook, KR. Johnson, RT Bronson & MT Davisson (1996) Megencephaly: a new mouse mutation on chromosome 6 that causes hypertrophy of the brain. *Mamm Genome*. **7**: 871-876.

Driscoll DA, Salvin J, Sellinger B, *et. al*., (1993). Prevalence of 22q11 microdeletions in DiGeorge and velocardiofacial syndrome: implications for genetic counseling and prenatal diagnosis. *J Med Gent* 30, 813-817.

Egozcue, J. (1996) Preimplantation diagnosis in older patients. Of course not. *Hum. Reprod*., **11**, 2077–2078.

Egozcue, J. (1997) Preimplantation diagnosis in older patients.
Hum. Reprod.,**12**, 870–871.

Enmark E, Gustafsson JA.(1999). Oestrgen receptors –an overview.
J Intern Med **246**:133-138

ESHRE Preimplantation Genetic Diagnosis (PGD) Consortium (1999) Preliminary assessment of data from January 1997 to September 1998 ESHRE PGD Consortium Steering Committee. *Hum Reprod* **14**: 3138-3148.

ESHRE Preimplantation Genetic Diagnosis (PGD) Consortium (2000): Data collection II ESHRE PGD Consortium Steering Committee. *Hum Reprod* **15**: 2673-2683.

Everett J (1961). The mammalian female reproductive cycle and its controlling mechanisms. *Sex and Internal Secretions*. Young WC (ed) Williams and Williams. **1**: 497-555

Fekete E (1946) A comparative study of the ovaries of virgin mice of the DBA and C57 black strains. *Cancer Res* **6**:263-269

Festing MFW (1993a) FRAR course on laboratory approaches to aging. Genetic quality control in laboratory rodents. *Aging (Milano).* Aug;**5**(4):309-15. Review.

Festing MFW. (1993b.) International index of Laboratory Animals.
Inter. Leicester.England.

Findlay JK, Britt K, Kerr JB, O'Donnell L,Jones ME, Drummond AE, Simpson ER. (2001) The road to ovulation: the role of oestrogens. *Reprod Fertil Dev* **13**: 543-547

Findlay I, Quike P, Hall J, Rutherford A. (1996). Fluorescent PCR: a new technique for PGD of sex and single-gene defects. J Assist Reprod Genet **13**: 96-103.

Forster C, Makela S, Warri A, Kietz S, Becker D, Hultenby K, Warner M, Gustafsson JA (2002) Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium. *Proc Natl Acad Sci* USA; **99**: 15578-15583.

Fridström M, Ährlund-Richter L, Iwarsson E, Malmgren H, Inzunza J, Rosenlund B, Sjöblom P, Nordenskjöld M, Blennow E and Havatta O, (2001). Clinical outcome of treatment cycles using preimplantation genetic diagnosis for structural chromosomal abnormalities. *Prenat Diagn* **21**: 781-787.

Fries AS. (1978) Demonstration of antibodies to Bacillus piliformis in SPF colonies and experimental transplacental infection by Bacillus piliformis in mice.*Lab Anim.Jan*;**12**(1):23-6.

Garrisi, G.J., Talansky, B.E., Grunfeld, L., Sapira, V., Navot, D., Gordon, J.W. (1990). Clinical evaluation of three approaches to micromanipulation-assistedFertilization.
Fert. & Steril., **54**(4), 671–677.

Green MC & BA Witham (eds.). (1991) Handbook on genetically standardized JAX mice 4th edition, *The Jackson Laboratory*, Bar Harbor.

Griffin DK, Handyside AH, Penketh RJA, Winston RML & Delhanty JDA (1991) Fluorescent in situ hybridization to interphase nuclei of human pre-implantation embryos with X and Y chromosome specific probes. *Hum Reprod* **6**: 101-105.

Griffin, D.K., Handyside, A.H., Harper, J.C. *et al*. (1994) Clinical experience with preimplantation diagnosis of sex by dual fluorescent in situ hybridization. *J. Assist. Reprod. Genet*., **11**, 132–142.

Guichaoua MR, Devictor M, Hortung M, Luciani JM, Stahl A. (1986). Random acrocentric bivalent associations in human pachytene spermatocytes. Molecular implications in the occurrence of Robertsonian translocations
Cytogenet Cell Genet; **42** (4): 191-7

Gunasena KT, PM Villines, ES Critser & JK Critser (1997a) Live births after autologous transplant of cryopreserved mouse ovaries. *Hum Reprod*. **12**: 101-106.

Gunasena KT, JR Lakey, PM Villines, ES Critser & JK Critser (1997b) Allogenic and xenogenic transplantation of cryopreserved ovarian tissue to athymic mice.
Biol Reprod. **57**: 226-231.

Gustafsson BE. (1948) Germ-free rearing of rats. General technique.
Acta Path Microbiol Scand. Suppl **73**:130 pp.

Gustafsson BE. (1959) Lightweight stainless steel systems for rearing germ-free animals.
Annals of the New York Acad Sci. **78**: 17-28.

Gustafsson BE and Coates ME (1984). The germ-free animal in biomedical Research, Laboratory Animal Handbooks 9. *Publ. Laboratory Anim LTD*, London UK.

Gulyas BJ. (1975) A reexamination of cleavage patterns in eutherian mammalian eggs: rotation of blastomere pairs during second cleavage in the rabbit.
J Exp Zool Aug. **193** (2): 235-48

Hammer RE. (1998). Egg culture: the foundation.
Int J Dev Biol **42**(7 spec No); 833-9. Review

Handyside, A.H., Kontogianni, E.H., Hardy, K., Winston, R.M. (1990). Pregnancies from biopsied human preimplantation embryos sexed by Y-specificDNA amplification.
Nature, **344**(6268), 768–770.

Handyside AH, Pattinson JK, Penketh RJ, Delhanty JD, Winston RM, Tuddenham EG. (1989). Biopsy of human preimplantation embryos and sexing by DNA amplification.
Lancet Feb **18;** 1(8634):347-9

Handyside, A.H. (1991). Biopsy of human cleavage stage embryos and sexing by DNA amplification. In: Verlinksy, Y., Kuliev, A. *(Eds). PreimplantationGenetics*. New York: Plenum Press, 75–84.

Hardy, K., Martin, K.L., Leese, H.J., Winston, R.M., Handyside, A.H. (1990). Human preimplantation development in vitro is not adversely aVected by biopsy at the 8-cell stage.

Hum. Reprod., **5**(6), 708–714.

Harper, J.C., Coonen, E., Ramaekers, F.C. *et al*. (1994) Identification of the sex of human preimplantation embryos in two hours using an improved spreading method and fluorescent in-situ hybridization (FISH) using directly labelled probes. *Hum. Reprod*., **9**, 721–724.

Harper JC, Delhanty JDA, Handyside AH (2001) Preimplantation genetic diagnosis (ed) Harper, Delhanty,Handyside. *John Wiley & Son, Ltd*.

Hill AC, Stalley GP. (1991) Mycoplasma pulmonis infection with regard to embryo freezing and hysterectomy derivation.*Lab Anim Sci*.Dec;**41**(6):563-6.

Hogan B, Beddington R, Costantini F, Lacy E. (1994) Manipulating the Mouse Embryo, a laboratory manual. *Cold Spring Harbor Laboratory Press*, Second edition (ed.).

Hsieh YY, Tsai HD, Chang CC, Chang CC, Lo HY, Lai AC. (1999). Ultrarapid cryopreservation of human embryos: experience with 1 582 embryos.
Fertil Steril Aug; **72** (2):253-6

Iwarsson, E., Ahrlund-Richter, L., Inzunza, J., Rosenlund, B., Fridström, M., Hillensjö , T., Sjöblom, P., Nordenskjö ld, M., Blennow, E. (1998a). Preimplantation genetic diagnosis of a large pericentric inversion of chromosome 5. *Mol. Hum. Reprod*., **4**(7),719–723.

Iwarsson, E., Ahrlund-Richter, L., Inzunza, J., Fridström, M., Rosenlund, B., Hillensjo¨ , T., Sjo¨blom, P., Nordenskjo¨ ld, M., Blennow, E. (1998b). Preimplantation genetic diagnosis of DiGeorge syndrome. *Mol.Hum. Reprod*., **4**(9), 871–875.

Iwarsson K. (1990) Laboratory animal health monitoring: an introductory survey of university mouse breeding colonies in Stockholm. *Acta Physiol Scand Suppl*. **592**:139-40.

Iwarsson K & Norén L (1992). Comparison of micro-environmental conditions in standard versus forced-air ventilated rodent filter-top cages.
Scand J Lab Anim Sci. **19,** 167-173.

Jenderny, J., Gebauer, J., Rohrborn, G. *et al*. (1992) Sperm chromosome analysis of a man heterozygous for a pericentric inversion of chromosome *20*.
Hum. Genet., **89**, 117–119.

Jones EC, Krohn PL (1961) The relationship between age, numbers of oocytes, and fertility in virgin and multiparous mice. *J Endocrinol* **21**:469-495.

Katami K, Taguchi F, Nakayama M, Goto N, Fujiwara K. (1978)Vertical transmission of mouse hepatitis virus infection in mice. *Jpn J Exp Med*. Dec;**48**(6):481-90.

Kilham L, Margolis G. (1971) Fetal infections of hamsters, rats, and mice induced with the minute virus of mice (MVM). *Teratology*. Feb;**4**(1):43-61.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O. (1998).Generation and reproductive phenotypes of mice lacking

estrogen receptor beta. *Proc Natl Acad Sci* USA, vol **95**: 15677-15682.

Kunstyr I, Hartmann D. (1983) Pasteurella pneumotropica and the prevalence of the AHP (Actinobacillus, Haemophilus, Pasteurella)-group in laboratory animals.
Lab Anim. Apr;17(**2**):156-60.

Lavebratt C, Inzunza J, Petersson S, Iwarsson K, Schalling M, Ährlund-Richter L. (1998).Ovary transplantation method resulting in high reproductive performance in mice. *Scand J. Lab.Anim.Sci*. **25** (2): 53-58.

Lee, A. & Gemmell, E. (1972). Changes in the mouse intestinal microflora during weaning: role of volatile fatty acids. Infection and Immunity 5, 1-7.

Lissens, W., Sermon, K. (1997). Preimplantation genetic diagnosis: current status and new developments. *Hum. Reprod*., **12,** 1756–1761.

Martin, R.H. (1991) Cytogenetic analysis of sperm from a man heterozygous for a pericentric inversion, inv (3) (p25q21). *Am. J. Hum. Genet*., **48**, 856–861.

Martin, R.H. (1993) Analysis of sperm chromosome complements from a man heterozygous for a pericentric inversion, inv(8) (p23q22). *Cytogenet Cell Genet.,* **62**, 199–202.

Martin, R.H., Chernos, J.E., Lowry, R.B. *et al*. (1994) Analysis of sperm chromosome complements from a man heterozygous for a pericentric inversion of chromosome 1. *Hum. Genet*., **93**, 135–138.

McCance DJ, Mims CA (1977) Transplacental transmission of polyoma virus in mice. *Infect Immun*. Oct;**18**(1):196-202.

McLean SD, Saal HM, Spinner NB. *et. al*., (1993). Velo-cardio-facial syndrome. Intrafamilial variability of the phenotype. *Am J Dis Child* **147**, 1212-1216.

Midtvedt T, Bjorneklett A, Carlstedt-Duke B, Gustafsson BE, Hoverstad T, Lingaas E, Norin KE, Saxerholt H, Steinbakk M. (1985) The influence of antibiotics upon microflora-associated characteristics in man and mammals. *Prog Clin Biol Res*.;**181**:241-4.

Mims CA. (1969) *Effect* on the fetus of maternal infection with lymphocytic choriomeningitis (LCM) virus. *J Infect Dis*. 1969 Nov;**120**(5):582-97.

Miniats OP.(1984) Production of germ-free animals. Part 1. In.: The germ-free animals in biomedical research. (Eds. Coates M.E. & Gustafsson B.E., pp. 49-78. Laboratory Animal *Handbook 9, London.*

Mohanty SB, Bachmann PA. (1974) Susceptibility of fertilized mouse eggs to minute virus of mice. *Infect Immun*. Apr;**9**(4):762-3.

Mohr, L.R., Trounson, A.O. (1985). Cryopreservationof human embryos.
Ann. N. Y. Acad. Sci., **442,** 536–543.

Mohr U, Dungworth DL, Capen CC, Carlton WW, Sundberg JP and Ward JM. (1996).

Pathobiology of the Aging Mouse.
International Life Sciences Institute Press, Volume I. USA.

Mukaida T, Wada S, Takahashi K, Pedro PB, An TZ, Kasai M. (1998). Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos.
Hum Reprod Oct; **13** (10):2874-9

Munné S., Fung, J., Cassel, M.J., Marquez, C., Weier, H.U.G. (1998). Preimplantation genetic analysis of translocations—case-specific probes for interphase cell analysis.
Hum. Genet., **102**(6), 663–674.

Munné S, Magli C, Bahce M et al. (1998) Preimplantation diagnosis of the aneuploidies most commonly found in spontaneous abortions and live births,XY.13,14,15,16,18,21,22. Prenat *Diagn* **18**: 1459-1466.

Munne´, S., Dailey, T., Sultan, K.M. *et al*. (1995) The use of first polar bodies for preimplantation diagnosis of aneuploidy. *Hum. Reprod*., **10**, 1014–1020.

Munne´, S. and Cohen, J. (1997) Preimplantation diagnosis in older patients but of course. *Hum. Reprod*., **12**, 413–414.

Murillo González J (2001) Evolution of embryology: A synthesis of classical experimental, and molecular perspectives. *Clinical Anatomy* **14**:158-163

Navarro, J., Benet, J., Martorell, M.R. *et al*. (1993) Segregation analysis in a man heterozygous for a pericentric inversion of chromosome 7 (p13;q36) by sperm chromosome studies. *Am. J. Hum. Genet*., **53**, 214–219.

Niebuhr, E. (1978) The Cri du Chat syndrome: epidemiology, cytogenetics, and clinical features. *Hum. Genet*, **44**: 227–275.

Nikas G, Ao A, Winston RM, Handyside AH (1996). Compaction and surface polarity in the human embryo in vitro. *Biol Reprod* Jul; **55** (1):32-7

Nuutal GHF & Thierfelder H. (1895/86) Thierisches Leben ohne Bakterien in Verdauungskanal. *Z Physiol Chem.*. **21**:109-121

Okamoto M, Matsumoto T. (1999) Production of germfree mice by embryo transfer. *Exp Anim*. Jan;**48**(1):59-62.

Palermo G, Joris H, Devroey, Van Steirteghem AC (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte.
Lancet, Jul 4; **340**(8810):17-8

Palmiter RD, Bihringer RR, Quaife CJ, Maxwell F, Maxwell IH, Brinster RL. (1987). Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene.
Cell Jul 31; **50** (3):435-43

Parkening TA, Collins TJ & Elder FF (1985) Orthotopic ovarian transplantation in young and aged C57BL/6J mice. *Biol Reprod* **32**: 989-997.

Payne, D., McLaughlin, K.J., Depypere, H.T., Kirby, C.A., Warnes, G.M., Matthews, C.D. (1991). Experience with zona drilling and zona cutting to improve fertilization rates of human oocytes in vitro, *Hum. Reprod*., **6**(3), 423–431.

Pedersen T and Peters H (1968) Proposal for a classification of oocytes and follicles in the mouse ovary. *J Reprod Fertil* **17**:555-557.

Peschon JJ, Behringer RR, Brinster RL, Palmiter RD. (1987). Spermatid-specific expression of protamine 1 in transgenic mice. *Proc Natl Acad Sci* USA Aug; **84**(15): 531-9

Peters H and McNatty KP (1980) The Ovary. *Paul Elek*, New York

Pettersson K, Gustafsson JA (2001) Role of estrogen receptor beta in estrogen action. *Annu Rev Physiol*, **63**:165-192

Piko L, Clegg KB. (1982). Quantitative changes in total RNA, total poly (A), and ribosomes in early mouse embryos. *Dev Biol* Feb; **89** (2):362-78

Poirot C, Martelli H, Lichtblau I, Dhedin N, Abirached F, Faraguet C, Vacher-Lavenu MC. (2002). Female fertility preservation before sterilizing treatment: contribution of ovarian tissue cryopreservation. *J Gynecol Obstet Biol Reprod* (Paris) Dec; **31** (8):717-23

Raibaud, P., Dickinson, A. B., Sacquet, E., Charlier, H. & Mocquot, G. (1966). La microflore du tube digestif du rat. II.-Dénombrement de differents genres microbiens dans l'estomac et l'intestin de rats conventionnels variations quantitatives individuelles et en function de l'age. *Annales de l'Institut Pasteur* **110**, 861-876.

Reubinoff B. and Shushan A. (1996) Preimplantation diagnosis in older patients. To biopsy or not to biopsy? *Hum. Reprod*, **11**: 2071–2075.

Reubinoff, B. and Shushan, A. (1997) Preimplantation diagnosis in older patients. *Hum. Reprod*, **12**: 871.

Reyniers, J. A., Trexler, P.C. & Ervin, R. F. (1946). Rearing germfree albino rats. *Lobund Report* No. **1**, pp. 1-84. Notre Dame, Indiana: Notre Dame University Press.

Saito M & Nomura T. (1984)Production of germ-free animals. Part 1. Small Mammals In.: The germ-free animal in biomedical research (Eds. Coates M.E. & Gustafsson B.E.), pp. 33-48. *Laboratory Animal Handbooks*. 9, London.

Santalo, J., Veiga, A., Calafell, J.M., Calderon, G., Vidal, F., Barri, P.N., Gimenes, C., Egozcue, J. (1995). Evaluation of cytogenetic analysis for clinical preimplantation diagnosis. *Fert. Steril*., **64**(1), 44–50.

Sar M, Welsh F. (1999) Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary. *Endocrinology* **140**: 963-971

Savage, D.C. (1977b). Microbial ecology of the gastrointestinal tract. Annual Review of *Microbiology* **31**: 107-133.

Savage, D.C. (1977a). Interaction between the host and its microbes. In Microbiology of the Normal Gut (eds R. T. J. Clarke and T. Bauchop), pp 277-310. London and New York: *Academic Press*.

Savage, D.C. (1983). Morphological diversity among members of the gastrointestinal microflora. *International Review of Cytology* **82**, 305-334.

Savage, D.C. & Dubos, R. (1968). Alterations in the mouse cecum and its flora produced by antibacterial drugs. *The Journal of Experimental Medicine* **128**, 97-110.

Schaedler, R. W., Dubos, R. & Costello, R. (1965). The development of the bacterial flora in the gastrointestinal tract of mice. *The journal of Experimental Medicine* **122**, 59-66.

Schwanzer V, Deerberg F, Frost J, Liess B, Schwanzerova I, Pittermann W. (1975) Intrauterine infection of mice with ectromelia virus. *Z Versuchstierkd*.;**17**(2):110-20. Germany.

Snell GD, Stevens LC (1966) Early embryology. In Green El (ed), Biology of the laboratory mouse (2nd ed.). *Dover*, New York, pp 205-245.

Snow M, Cox SL, Jenkin G, Trounson A, Shaw J. (2002). Generation of live young from xenografed mouse ovaries. *Science* Sep vol.297 : 2227

Stefanou EG, Hanna G, Foakes A, Crocker M, Fitchett M. (2002) Prenatal diagnosis of Cri du Chat (5-p) syndrome in association with isolated moderate bilateral ventriculomegaly. *Prenat Diagn* Jan; **22**(1):64-6

Stevens LC. (1957). A modification of Robertson's technique of homoiotopic ovarian transplantation in mice. *Transplant Bull*, **4**: 106-107

Sutherland GR, Gardiner A.J. and Carter R.F. (1976) Familial pericentric inversion of chromosome 19, inv(19) (p13q13) with a note on genetic counseling of pericentric inversion carriers. *Clin. Genet*., **10**: 54–59.

Sztein JM, McGregor TE, Bedigian HJ, Mobraaten LE. Transgenic mouse strain rescue by frozen ovarios. *Lab Anim Sci* 1999 Feb;**49**(1);99-100

Tannock, G. W. (1979). Coliforms and enterococci isolated from the intestinal tract of conventional mice. *Microbial Ecology* **5**, 27-34.

Thung PJ, Boot LM, Mühlbock O (1956) Senile changes in the oestrous cycle and in ovarian structure in some inbred strains of mice. *Acta Endocrinologica* **23**:8-32

Trexler, P. C. (1959). The use of plastics in the desing of isolator systems. *Annals of the New York Academy of Sciences* **78**, 29-36.

Tucker MJ, Stewart RB (1976) Intrauterine transmission of Sendai virus in inbred mouse strains. *Infect Immun*. Nov;**14**(5):1191-5.

Veiga, A., Sandalinas, M., Benkhalifa, M., Boada, M., Carrera, M., Santalo, J., Barri, P.N.,

Menezo, Y. (1997). Laser blastocyst biopsy for preimplantation diagnosis in the human, *Zygote*, **5**(4), 351–354.

Verlinsky, Y. and Kuliev, A. (1996) Preimplantation diagnosis of common aneuploidies in infertile couples of advanced maternal age. *Hum. Reprod*, **11**: 2076–2077.

Verlinsky Y, Cieslak J, Freidine M. *et al*. (1996) Polar body diagnosis of common aneuploidies by FISH. *J. Assist. Reprod. Genet*., **13**, 157–162.

Warter S, Ruch JV and Lehmann M. (1973) Karyotype with chromosomal abnormality with various inherited defects in the offspring (recombination aneusomy). *Humangenetik*, **20**, 355–359.

Whittingham, D.G. (1971). Culture of mouse ova, *J. Reprod. Fert.*, **14** (Suppl.), 7–21.

Whittingghan DG, Whitten WK. (1974). Long-term storage and aerial transport of frozen mouse embryos. *J Reprod Fertil* Feb; **36**(2): 433-5

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. (1997) Viable offspring derived from fetal and adult mammalian cells. *Nuture* Feb **27**;385 (6619):810-3

Wilson TA, Blethen SL, Vallone A. *et al*. (1993). DiGeorge anomaly with renal agenesis in infants of mothers with diabetes. *Am J Med Genet* **47,** 1078-1082

William JL. (2001). Human embryology, third edition *Churchill livingstone* editor, Independence Square West. USA

Windahl SH, Vidal O, Andersson G, Gustafsson JA, Ohlsson C. (1999) Icreased cortical bone mineral content but unchanged trabecular bone mineral density in female ERß -/- mice. *J Cli Invest*. **104**: 895-901

Zhang W, Saji S, Makinen S, Cheng G, Jensen EV, Warner M, Gustafsson JA (2000) Estrogen receptor (ER) beta, a modulator of ER alpha in the uterus. *Proc Natl Acad Sci* USA , **97**: 5936-5941.