Aneuploidy screening of human IVF embryos: Cytogenetic aspects and clinical implications.
Thesis Erasmus University, Rotterdam, The Netherlands
The work presented in this thesis was performed at the Division of Reproductive Medicine (Head: Prof. dr. B.J.C.M. Fauser), department of Obstetrics and Gynaecology, Erasmus MC, Rotterdam, The Netherlands.

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Aneuploidy Screening of Human IVF Embryos: Cytogenetic Aspects and Clinical Implications

Screening voor aneuploïdie in IVF embryo's:
cytogenetische aspecten en klinische implicaties

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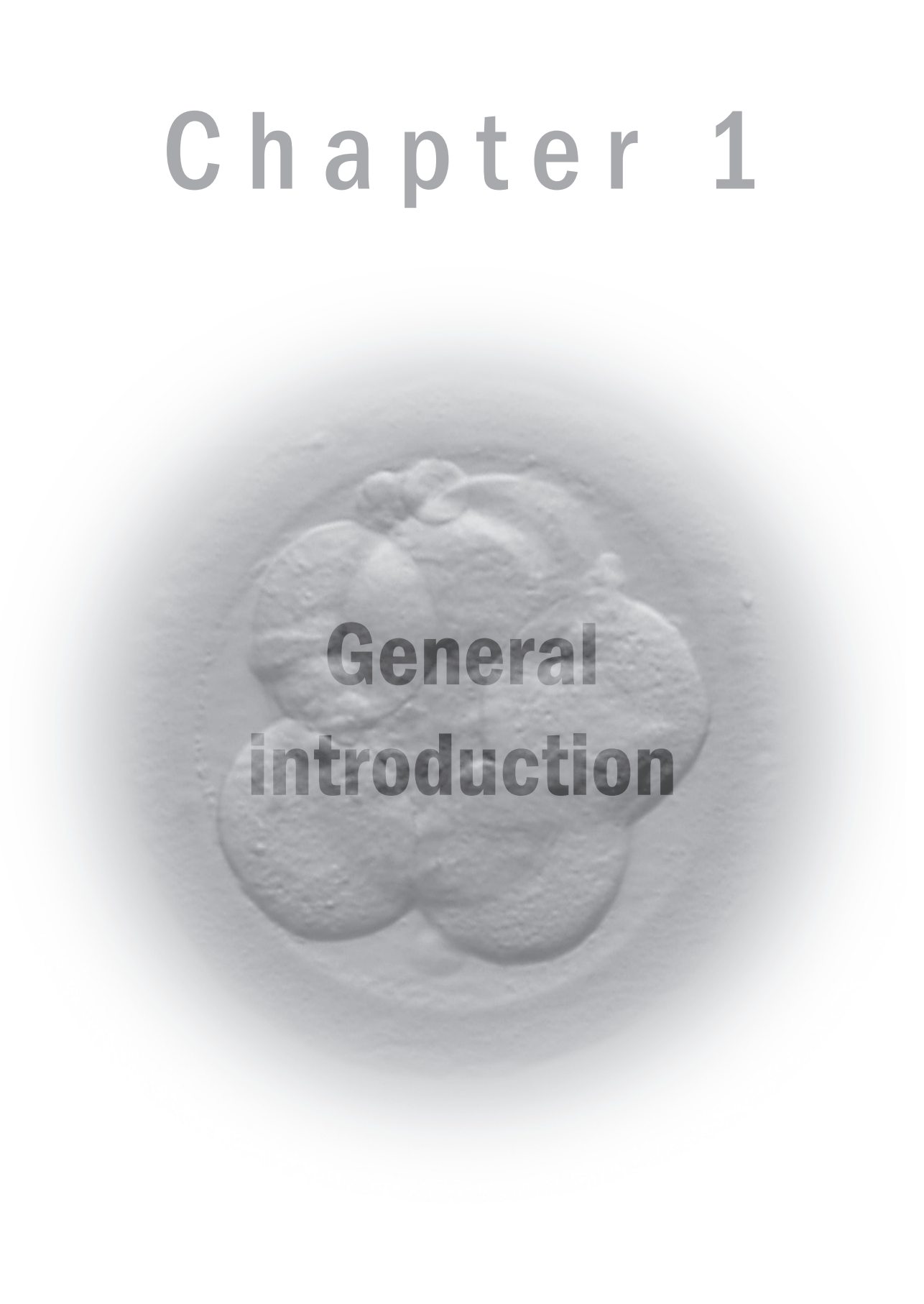
There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another which states that this has already happened.

Douglas Adams, from “The restaurant at the end of the universe”.

Contents

Chapter 1



General introduction

Introduction

Human reproduction is a relatively inefficient process (Norwitz, 2001). The chance of achieving a spontaneous pregnancy after timed intercourse is at the most 20-30% (Evers, 2002; Taylor, 2003), significantly lower than ~70% in the rhesus monkey (Ghosh, 1997), 80% in captive baboons (Stevens *et al.*, 1997) and 90% in rodents and rabbits (Foote *et al.*, 1988). Moreover, up to 30% of early human embryos is lost after recognized implantation (Wilcox, 1988), largely due to chromosomal abnormalities (Vorsanova *et al.*, 2005; Boué *et al.*, 1975). With regard to the etiology of chromosomal abnormalities in humans, increasing maternal age remains the only factor irrefutably associated with increased aneuploidy rates (Hassold and Hunt, 2001).

Subfertility is the failure to conceive after one year of unprotected regular sexual intercourse. The longer a couple has to try to conceive, the smaller the chance of spontaneous conception. If the duration of subfertility is less than three years, a couple is 1.7 times more likely to conceive than couples who have been trying for longer (Collins *et al.*, 1995). Major causes of subfertility can be grouped as ovulation disorders, male factors, tubal damage, endometrioses or it can be unexplained. An important factor negatively influencing fertility is female age. Due to social changes more couples are delaying the start of their family, leading to a reduction in their likelihood of conception.

Worldwide, the use of assisted reproductive technology (ART) as a treatment for subfertility is increasing (Andersen *et al.*, 2005; Wright *et al.*, 2005). In Europe, this technology accounts for 2 to 3% of all births (Andersen *et al.*, 2005). Among reproductive techniques, *in vitro* fertilization (IVF) is an important treatment strategy for subfertility. Shortly after the first pregnancy following *in vitro* fertilization (IVF) was reported in a spontaneous cycle (Steptoe and Edwards, 1978), evidence accumulated that overall chances of pregnancy increased if more than one embryo was transferred. Since then, ovarian (hyper)stimulation protocols have been developed, aiming at obtaining multiple growing follicles, and thus more oocytes for fertilization (Fauser *et al.*, 2005; Macklon *et al.*, 2006). Although transfer of multiple embryos results in higher pregnancy rates, it also results in a high rate of multiple gestation. The multiple pregnancy rate in relation to IVF is 26% in Europe and 35% in the United States (Andersen *et al.*, 2005; ASRM, 2004) as compared to 1.1% after spontaneous conception (Crowther, 2002). Multiple gestations are increasingly viewed as a complication of fertility treatments, as they are associated with adverse outcomes, such as serious perinatal morbidity and mortality and long term disabilities (Ombelet *et al.*, 2005). This resulted in a call for a reconsideration of the definition of "success" in ART to focus on singleton livebirth delivery (Fauser *et al.*, 2005).

By limiting the number of embryos transferred to the uterus to only a single embryo, the risk for multiple gestations can be reduced (Barlow *et al.*, 2005; Gerris, 2005). However, acceptance of this strategy is slow, for it is associated with a lower pregnancy rate per cycle (Pandian *et al.*, 2005; Gleicher and Barad, 2006). While embryos rated as high quality by standardized morphological assessment are associated with higher implantation and pregnancy rates, it is still not possible to predict with certainty which embryo will implant and has the highest potential to develop into a healthy child. An increasing body of evidence suggests that the incidence of chromosomal abnormalities in human embryos is extremely high and this may be the cause for the inefficiency of human reproduction, both *in vivo* and *in vitro* (as reviewed by Macklon *et al.*, 2002; Wilton, 2002). Embryo morphology on day 3 after fertilization is not indicative of a normal chromosomal constitution (Magli *et al.*, 2000; Munné, 2006). Therefore, current criteria for the in vitro selection of embryos for transfer are insufficient.

In order to improve the effectiveness of single embryo transfer, the ability to select the embryo with the highest potential to develop into a healthy child is of vital importance. This ability requires more insight into chromosomal competence of embryos, as this may be a crucial factor for embryo development.

Aneuploidy in human oocytes

Mechanisms of aneuploidy formation in human oocytes

Improper chromosome segregation during meiosis leads to genetically unbalanced oocytes or sperm. If these gametes participate in fertilization, the resulting embryo will be aneuploid, with either one or more chromosomes too many (trisomy) or too few (monosomy). In humans, aneuploidy can be identified in at least 5% of all clinically recognized pregnancies, making it the leading known cause of fetal wastage. Over the past fifteen years, more than 1,000 trisomic or monosomic conceptions have been studied to determine the parental origin and meiotic stage of the error (Hassold and Hunt, 2001). Although there are chromosome specific differences in incidences, most human trisomies originate from errors in maternal meiosis I, i.e. in the oocytes. This may not be surprising, as the first stage of female meiosis is initiated in the fetal ovary, and is then followed by a protracted arrest phase. This phase lasts at least 10-15 years, until the time of ovulation and resumption of the first meiotic division. In contrast, spermatogenesis is initiated at puberty and is an ongoing process that requires approximately two months from beginning to end and continues throughout the male's lifetime. Although male meiosis and spermatogenesis represent an interesting field of study onto its own, the focus of this thesis will be on oogenesis and early embryogenesis.

As mentioned before, female meiosis is initiated in the fetal ovary. During the premeiotic interphase, DNA synthesis is completed and pairing of homologous chromosomes is initiated. After an extended arrest phase, meiosis is resumed shortly before ovulation and the oocyte undergoes the two meiotic divisions (Figure 1.1). During the first meiotic division, segregation of the homologous chromosomes takes place. Errors that may occur at this stage include failure of the homologues to segregate to opposite poles of the spindle (whole chromosome non-disjunction), or loss of one homologue because of lagging behind at anaphase (anaphase lagging). The second meiotic division is completed shortly after fertilization and now the sister-chromatids divide and each migrates to an opposite spindle pole. Non-disjunction and anaphase lagging may occur at this division as well. Errors can also occur because homologues or sister chromatids separate prematurely (Angell, 1997). If this happens, proper chromosome apposition to the spindle poles is disturbed, possibly resulting in both chromosomes or chromatids migrating to the same pole.

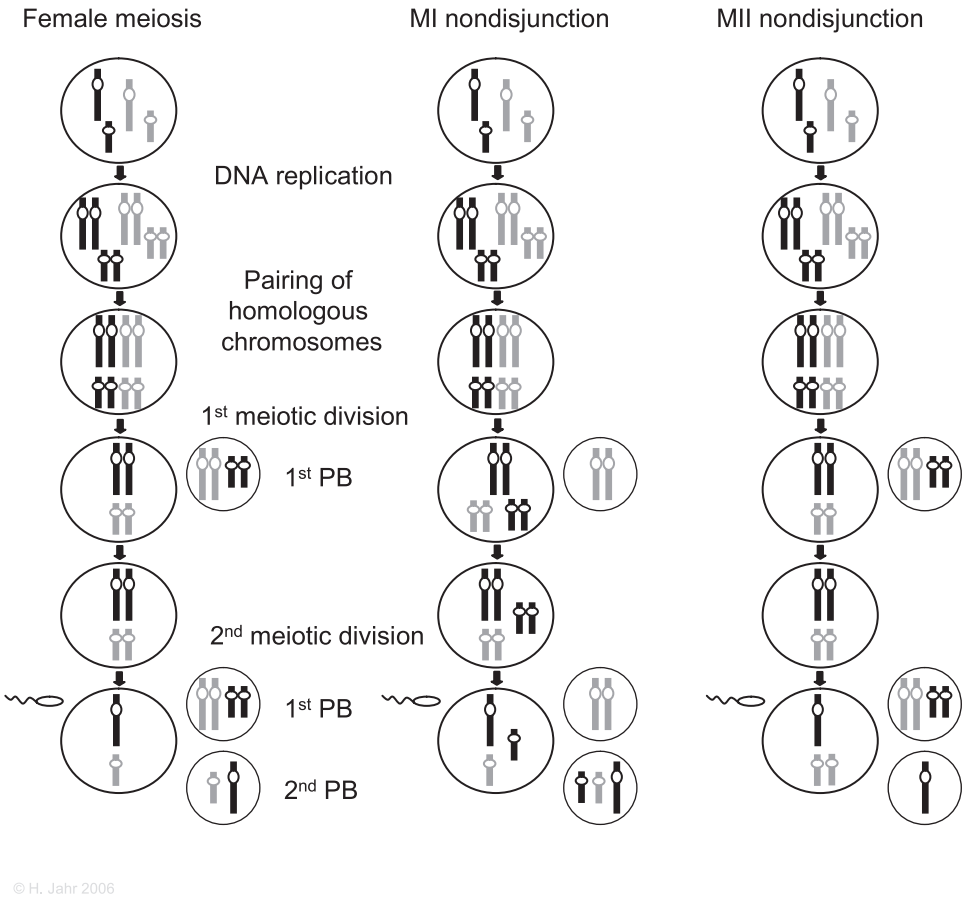


Figure 1.1. Schematic representation of normal female meiosis and abnormalities resulting from meiosis I (MI) and meisosis II (MII) non-disjunction. PB = polar body.

Cytogenetic analysis of human oocytes

Cytogenetic analysis of human oocytes can be considered as a highly valuable source of information, because it enables a direct insight into female meiosis, without the bias of viability present in studies of pregnancy losses and live borns. The advent of *in vitro* fertilization gave access to oocytes that had remained unfertilized for cytogenetic studies. Since then, more then 50 cytogenetic surveys on human oocytes have been carried out, almost all on one or two day old, unfertilized oocytes. Early studies mainly used a spreading protocol using methanol and acetic acid (Tarkowski, 1966), followed by a homogeneous Giemsa staining in order to enable karyotyping. However, due to the compact morphology of metaphase II chromosomes, they can at the most be attributed to one of seven groups, A to G (Recommendations of the ISCN Standing Commitee, London Conference, 1963). Another limitation of this spreading technique is that it can easily lead to overspreading of chromosomes, resulting in artefactual loss. Alternatively, chromosomes may not be separated enough, making it impossible to distinguish individual chromosomes. Collected data from different studies using this method showed the incidence of chromosomal abnormalities in a group of 2,434 oocytes to be highly variable (8-54%) between studies (as reviewed by Plachot, 2001; Pellestor *et al.*, 2006).

More recently, studies were performed on human oocytes using a gradual methanol/acetic acid fixation in combination with an R-banding protocol developed by Mikamo and Kamiguchi (1983). These show more consistent results regarding aneuploidy (8-33%) and a more balanced ratio between hypohaploidy and hyperhaploidy (as reviewed by Pellestor *et al.*, 2006). One large study on 1,396 unfertilized oocytes showed an aneuploidy rate of 10,8% (Pellestor *et al.*, 2002). They were able to make a distinction between whole chromosome non-disjunction (4%) and single sister-chromatid non-disjunction (6%). Also, a significant excess of non-disjunction in the E and G group chromosomes was described, whereas groups A and B displayed a lower frequency than expected. Furthermore, for the first time, a significant correlation between maternal age and the aneuploidy rate in resulting oocytes from IVF could be directly established (Pellestor *et al.*, 2003). Thus, cytogenetic studies in oocytes confirm observations in liveborns and pregnancy losses: aneuploidy arises in an age dependent manner and there are chromosome specific differences in incidences.

Origin of aneuploidy formation during oogenesis

The causes underlying aneuploidy formation are still poorly understood. However, recent observations suggest that inaccuracies of the chromosome segregation machinery in oocytes are often involved, a process influenced by maternal age (Hassold and Hunt, 2001; Champion and Hawley, 2002). During the meiotic prophase, recombination of homologous chromosomes results in a shuffling of genetic material, important for genetic variation. It also results in physical exchanges supported by proteins, serving to lock the homologues together in proper alignment until the first meiotic division (Petronczki *et al.*, 2003). In 1968, it was suggested that declining levels of recombination were associated with the maternal age effect on aneuploidy, providing the first link between recombination and human non-disjunction (Henderson and Edwards, 1968). At the time, however, it was not possible to investigate this hypothesis. Recently, techniques have become available to enable the reconstruction of the recombination events occurring in meiosis that lead to aneuploidy. By now, several laboratories have studied the relationship between recombination and human nondisjunction, comparing exchange frequency and distribution in meiotic divisions

leading to aneuploidy with those from chromosomally normal meioses. Significant reductions in recombination rates have been observed for all maternal meiosis I derived trisomies studied to date, such as trisomy 15, 16, 18, 21 and 47,XXY (Klinefelter syndrome) (Hassold *et al.*, 1991, 1995; Lamb *et al.*, 1996, 1997; Robinson *et al.*, 1998, Thomas *et al.*, 2001). Also, altered placement of meiotic recombination events has been identified to be involved in maternally derived trisomy 21 (Lamb *et al.*, 2005). In a large scale study, the effect of maternal age on recombination frequency was assessed and it demonstrated that not the frequency of recombination, but placement of the recombination site was changed in trisomy 21 in older women. A hypothesis was formed that nondisjunction may require two "hits" (Lamb *et al.*, 1997). The first hit is constituted by crossover formation between the homologues during fetal development, resulting in more or less error prone crossover configurations. The second hit then occurs many years later, around the time of ovulation as resumption of meiosis takes place. Because of age related perturbations in spindle and checkpoint function, homologues with more susceptible crossover configurations are less likely to segregate correctly, thus resulting in aneuploidy (Lamb and Hassold, 2004).

Proper attachment of chromosomes to the opposing spindle poles and subsequent biorientation is an essential requirement for normal chromosome segregation. It is suggested that cells are capable of monitoring the attachment of microtubules from the spindle to specialized structures on the chromosomes called kinetochores (Cleveland *et al.*, 2003). This surveillance mechanism is known as the spindle assembly checkpoint or SAC (Hoyt *et al.*, 1991; Li and Murray, 1991). The SAC detects defects in chromosome alignment and subsequently delays anaphase initiation until bipolar spindle attachment is achieved and each kinetochore is attached to the spindle (Wassmann and Benezra, 2001; Yu, 2002; Cleveland *et al.*, 2003). The wait signals produced once the checkpoint is activated are likely coming from unattached kinetochores (Rieder *et al.*, 1995). Several human genes have been identified as components in the checkpoint regulatory pathway, including mitotic arrest deficient (MAD) and budding uninhibited by benomyl (BUB), each encoding kinetochore-associated proteins (Li and Benezra, 1996; Ouyang *et al.*, 1998). In particular, MAD1, MAD2, BUB1, BUBR1 and BUB3 concentrate transiently at unattached kinetochores prior to metaphase, but disappear upon proper chromosome alignment (as reviewed by Nasmyth, 2005; Steuerwald, 2005).

Studies in mouse oocytes suggested that although the spindle assembly portion of the checkpoint mechanism is functional, the chromosome monitoring aspect is either ineffective or absent during mammalian female meiosis, thus explaining the high incidence of chromosome segregation errors (LeMaire-Adkins *et al.*, 1997; Woods *et al.*, 1999). However, recently localization of BUB1 to the kinetochores was observed in mouse oocytes (Brunet *et al.*, 2003) and the expression of MAD2 protein was demonstrated (Wassmann *et al.*, 2003). It was further demonstrated that mouse oocytes respond to spindle damage by a transient and reversible cell cycle arrest, consistent with checkpoint activity (Wassmann *et al.*, 2003). It appears that a cell cycle checkpoint is indeed active, but it does not always function properly. Steuerwald *et al.* (2001) hypothesized that aging in oocytes results in a breakdown in the cell cycle checkpoint signaling pathway. They investigated levels of expression of MAD2 and BUB1 during human oocyte maturation and found a decrease in levels of these transcripts in oocytes with increasing maternal age, but also after post-ovulatory aging of oocytes (Steuerwald *et al.*, 2001, 2005). This may indicate that a cell cycle checkpoint monitoring proper chromosome segregation is active in human oocytes, but its function may be compromised.

Aneuploidy and chromosomal mosaicism in early embryogenesis

If an aneuploid oocyte is fertilized, this may result in an embryo with constitutional aneuploidy. Analysis of single cells from day 3 cleavage stage embryos using interphase fluorescent *in situ* hybridization (FISH; see below), have yielded large datasets on the incidence of chromosomal abnormalities in embryos and abnormality rates of up to 73% have been reported (Munné *et al.*, 2003; Pehlivan *et al.*, 2003; Gianaroli *et al.*, 2003; Staessen *et al.*, 2004; ESHRE PGD consortium, 2005; Platteau *et al.*, 2005). Chromosomes most frequently found to be involved in aneuploidy were chromosomes 15, 16, 21 and 22 (Munné, 2006). This is in concordance with the reported higher incidence of abnormalities in the E and G group of chromosomes in oocytes. However, reported abnormality rates in preimplantation embryos after IVF are much higher than the 8-33% reported in oocytes.

The first FISH studies detecting the X and Y chromosomes in preimplantation embryos indicated that human embryos can be mosaic in their chromosome constitution (Delhanty *et al.*, 1993). Chromosomal mosaicism is caused by mitotic segregation errors, resulting in cell lines with a different chromosome constitution to be present within one embryo. Studies analyzing the entire embryo reported a highly variable incidence of chromosomal mosaicism from 2-90%, depending on developmental stage of the embryo, maternal age, patient subgroup, method of analysis, number of chromosomes analyzed and how mosaicism is defined (Jamieson *et al.*, 1994, Munné *et al.*, 1995; Almeida and Bolton, 1996; Harper and Delhanty, 1996; Delhanty *et al.* 1997; Bahçe *et al.*, 1999; Gianaroli *et al.*, 1999; Iwarsson *et al.*, 1999; Harrison *et al.*, 2000; Ruangvutilert *et al.*, 2000; Voulliare *et al.*, 2000; Wells and Delhanty, 2000; Bielanska *et al.*, 2002; Munné *et al.*, 2002, Voulliare *et al.*, 2002; Gonzalez-Merino *et al.*, 2003; Coonen *et al.*, 2004; Daphnis *et al.*, 2005). For the different chromosomes investigated so far, all chromosomes can be involved in mitotic segregation errors leading to mosaicism.

It appears that the incidence of chromosomal abnormalities and mosaicism in preimplantation embryos is far greater than reported rates in oocytes, pregnancy losses and liveborns. In the early embryo, cell cycle control is performed by maternal transcripts present in the ooplasm (Braude *et al.*, 1988; Lighten *et al.*, 1997). Only after the expression of the embryonic genome, cell cycle control becomes gradually present from the 8-cell stage onwards (Tesarik *et al.*, 1986; Braude *et al.*, 1988; Lighten *et al.*, 1997; Wells *et al.*, 2005). It is feasible that the "two hit" hypothesis used to explain failure in proper chromosome segregation during the meiotic divisions may be extended. A third, fourth and fifth "hit" may occur during the first three embryo cleavage divisions, as these divisions are under the control of the same defective cell cycle control mechanisms and rely on the same components of the chromosome segregation machinery. On the basis of this, one would expect an increase in mosaic embryos with maternal age. However, the few reports available on the effect of maternal age on the incidence of mosaicism in IVF derived embryos are conflicting (Marquez *et al.*, 2000; Munné *et al.*, 2003).

Preimplantation Genetic Screening

Preimplantation genetic diagnosis (PGD) is an early form of prenatal diagnosis, in which embryos created *in vitro* are analyzed for well-defined genetic defects. Only embryos free of the defects are subsequently replaced into the uterine cavity (Sermon, 2002). The analysis is performed on one or two blastomeres that have been removed from cleavage stage embryos (Handyside *et al.*, 1989) (Figure 1.2). After the first pregnancy following PGD was described in 1990 (Handyside *et al.*,

1990), it has been used to diagnose several monogenic diseases using single-cell PCR techniques for couples at a high risk of having a child with a genetic disease (ESHRE PGD Consortium, 2005).

The introduction of fluorescence in situ hybridization (FISH) on interphase nuclei allowed the screening of embryos for chromosomal aneuploidies (Munné *et al.*, 1993), a procedure referred to as preimplantation genetic diagnosis for aneuploidy screening (PGD-AS) or, as we prefer, preimplantation genetic screening (PGS) (Thornhill *et al.*, 2005). PGS is applied to patients who are treated with IVF with the aim of improving their chance for an ongoing pregnancy. Clinically, PGS is being advocated for older women (Munné *et al.*, 2003; Staessen *et al.*, 2004) and patients with recurrent miscarriage or repeated implantation failure (Gianaroli *et al.*, 2003; Pehlivan *et al.*, 2003; Platteau *et al.*, 2005). High rates of aneuploidy have been reported in these women.

Since its introduction, PGS has been increasingly applied over the past decade (ESHRE PGD Consortium, 2005). However, its clinical value remains uncertain. A positive effect on implantation and ongoing pregnancy rates in a group of patients with advanced maternal age has been observed in retrospective studies (Munné *et al.*, 1999; Munné *et al.*, 2003). However, recent prospective randomized studies failed to show a positive effect of PGS on clinical outcome per initiated cycle in patients with advanced maternal age (Staessen *et al.*, 2004; Stevens *et al.*, 2004). Other indications for which PGS has been proposed include recurrent implantation failure and recurrent miscarriage. Again, clinical benefits have not yet been convincingly demonstrated (Gianaroli *et al.*, 1999; ESHRE PGD Consortium Steering Committee, 2002; Pehlivan *et al.*, 2003; Rubio *et al.*, 2003; Platteau *et al.*, 2005). The conclusion from a recent Cochrane systematic review was that more properly conducted randomized controlled trials are needed to determine if PGS is an effective intervention in IVF for improving live birth rates (Twisk *et al.*, 2006).

Studies testing the efficiency of PGS have so far used clinical parameters such as implantation rates and ongoing pregnancies as outcome measures. Several factors may explain why PGS is not clinically effective. The first factor is the reliability of the FISH technique on single blastomeres. Although difficult to determine, it is estimated that about 4-6% of observed abnormalities are due to FISH artefact (Daphnis *et al.*, 2004; Munné *et al.*, 2004a). The second factor involves the choice and number of chromosomes screened. PGS analysis routinely involves the screening of the copy number of seven to ten chromosomes, mostly of those chromosomes that are involved in potentially viable trisomies (13, 18, 21 and the sex chromosomes) in combination with chromosomes often found to be trisomic in early miscarriages (such as 14, 15, 16 and 22) (Philipp *et al.*, 2003). However, chromosome aberrations can be involved in preimplantation embryos which are not found later during development, as in spontaneous miscarriage or ongoing pregnancy (Bahçe *et al.*, 1999; Fritz *et al.*, 2001; Vouillaire *et al.*, 2000; Wells and Delhanty, 2000; Phillipp and Kalousek, 2002). Therefore, it has been proposed that the efficiency of PGS could be improved by analyzing more chromosomes (Wilton *et al.*, 2002).

A third factor is the high incidence of chromosomal mosaicism in embryos, which may result in the cell(s) biopsied not being representative for the remaining embryo (Los *et al.*, 2004). It has not yet been demonstrated that the screening of one or two blastomeres obtained from an eight-cell embryo for the presence of aneuploidies will provide a reliable prediction of the chromosomal status of the remaining embryo. A fourth factor is the group of patients where PGS has been mostly applied. These are women of advanced maternal age or with an indication, such as recurrent implantation failure or recurrent miscarriage. They may have an inherent high incidence of chromosomal abnormalities, to the extent that no chromosomally normal embryos are available for transfer. Alternatively, they may have a reduced chance for implantation which is

not related to the embryos. Therefore, data are needed concerning the incidence of chromosomal abnormalities and especially mosaicism in embryos of younger IVF patients (<38 years) with no specific indication for PGS.

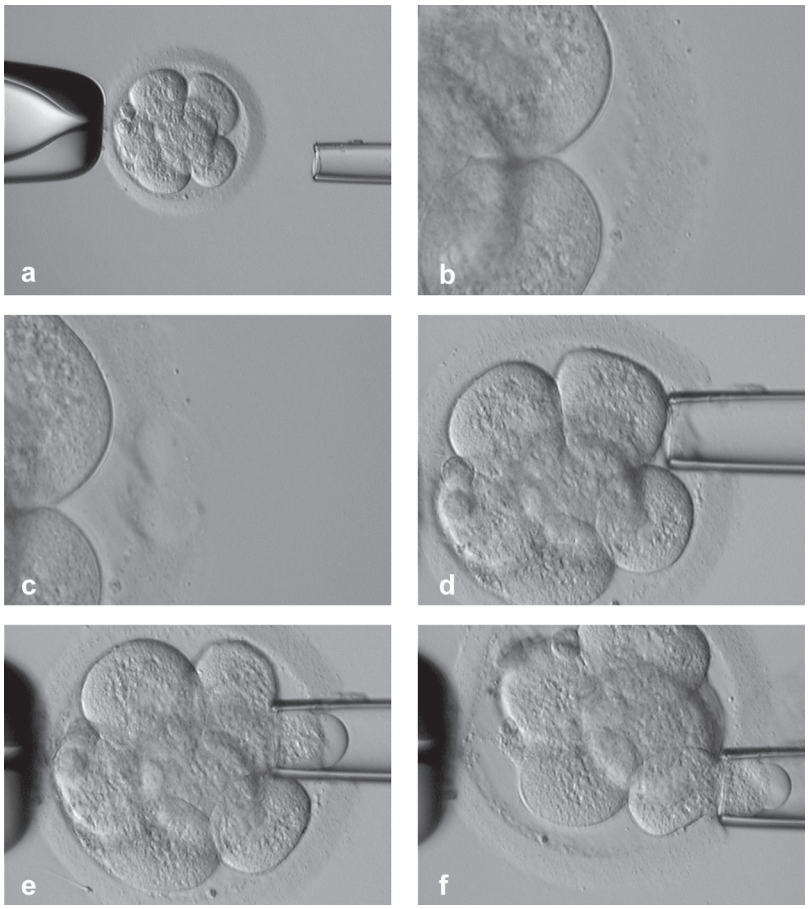


Figure 1.2. Light micrographs illustrating the embryo biopsy procedure. (a) An 8-cell embryo is attached to a holding pipette. (b) A larger magnification shows two blastomeres with visible nuclei. (c) An infrared diode lasersystem (OCTAX Laser Shot, OCTAX Microscience GmbH, Germany) with appropriate software (OCTAX EyeWare) is used to partially dissect the zona pellucida. (d-f) One by one, two blastomeres are removed by applying suction with a biopsy pipette.

Multifollicular ovarian stimulation for *in vitro* fertilization

Follicle development in the natural cycle

The initial growth of primordial follicles in the ovaries is thought to be independent of FSH and commences in a random fashion (Peters *et al.*, 1975). The majority of these primordial follicles go into atresia. Only those antral follicles that happen to be at a more advanced stage of maturation during the inter-cycle rise in follicle stimulating hormone (FSH) gain gonadotrophin dependence and continue to grow (Fauser and van Heusden, 1997; McGee and Hsueh, 2000). Within the natural menstrual cycle, gonadotrophin releasing hormone (GnRH) is released from the hypothalamus in a pulsatile manner and stimulates the synthesis and the release of luteinizing hormone (LH) and FSH by the anterior pituitary gland. In the early follicular phase serum FSH levels rise which is followed by decreasing concentrations during the mid and late follicular phase due to negative estradiol (E_2) feedback at the hypothalamic-pituitary axis (Baird, 1987). Moreover, a rise in inhibin B serum levels takes place early in the follicular phase, suggesting that it is secreted by a recently recruited cohort of follicles in response to FSH (Laven and Fauser 2004). This rapid rise in inhibin B occurs immediately after the inter-cycle rise in FSH (Groome *et al.*, 1996). It may be proposed that inhibin B limits the duration of the FSH rise through negative feedback at the pituitary level (Groome *et al.*, 1996). The decrease in FSH levels in the mid follicular phase secures the selection of a single dominant follicle (van Santbrink *et al.*, 1995), which becomes less dependent on FSH and continues to grow. Remaining follicles from the recruited cohort cease to mature and undergo atresia.

Rising levels of E_2, produced by the granulosa cells of the pre ovulatory follicle eventually result in a switch from negative feedback to a positive feedback effect, resulting in a rapid rise in LH release and the so-called LH surge. The LH surge initiates multiple events such as the commencement of oocyte meiotic maturation, granulosa cell luteinization and corpus luteum formation as well as the rupture of the dominant follicle allowing for the expulsion of the oocyte (i.e. ovulation) (Conti *et al.*, 1998; Richards *et al.*, 1998).

Follicle development in the stimulated cycle

The major goal of ovarian stimulation in IVF treatments is to induce the development and growth of multiple follicles. By administrating exogenous FSH for an extended period of time and thereby preventing the mid-follicular decrease in FSH levels, more than one follicle will gain dominance and continue to grow (Schipper *et al.*, 1998). As a result of multiple growing follicles, E_2 levels will increase more rapidly and may induce a premature LH surge and subsequent premature luteinization.

The conventional IVF regimens routinely use a GnRH agonist to prevent premature luteinization by decreasing pituitary releases of endogenous FSH and LH. Suppression of pituitary function takes around two weeks, after which high doses of exogenous FSH are administered to induce multiple follicle growth, resulting in high numbers of oocytes for retrieval. In the process, natural follicle selection is completely overruled, allowing the non-discriminate growth of many follicles.

The recent availability of GnRH antagonists has enabled the development of novel approaches in ovarian hyperstimulation. To prevent a premature LH rise, administration of a GnRH antagonist can be limited to the mid-to-late follicular phase (Fauser *et al.*, 2005). Therefore,

cyclic follicle recruitment and initial stages of dominant follicle selection can proceed within the natural cycle and the endogenous inter-cycle FSH rise can be utilized for follicle stimulation (Figure 1.3). Exogenous FSH administration can thus be limited to the mid-late follicular phase (Fauser and Van Heusden, 1997; Fauser *et al.*, 1999; Hohmann *et al.*, 2003). This constitutes a more physiological approach to ovarian stimulation and has many advantages. Treatment time and gonadotropin requirement are lower, and side-effects resulting from down-regulation with GnRH agonists are absent. However, uptake of GnRH antagonists in clinical practice has been slow (Fauser and Devroey, 2005; Griesinger *et al.*, 2005). Meta-analyses on studies comparing GnRH agonist protocols with GnRH antagonist show conflicting results (Ludwig *et al.*, 2001; Al-Inany and Aboulghar, 2002; Daya, 2005). However, it is suggested that GnRH antagonists are slightly less efficacious than GnRH agonist protocols and this has influenced clinical acceptance.

A previous RCT comparing a mild stimulation regimen using a GnRH antagonist to a conventional stimulation regimen with a GnRH agonist, showed mild stimulation to result in high quality embryos for transfer as indicated by good embryo morphology and pregnancy rates per embryo transfer (Hohmann *et al.*, 2003). Moreover, while no pregnancies were obtained in women who had produced four or less oocytes following the conventional protocol, the majority of pregnancies following mild stimulation occurred in women with four or less oocytes. It was hypothesized that a more physiological approach to ovarian stimulation, resulting in less growing follicles, may allow only the healthiest follicles and oocytes to develop.

Influence of ovarian stimulation on oocyte quality

As described before, follicles that develop to the Graafian stage are selected from a much larger pool of follicles. After becoming FSH-dependent, future dominant follicles indirectly affect the growth of the remaining follicles through their secretion of estrogens and inhibins, which feed back on the hypothalamic-pituitary system to further reduce the already falling level of circulating FSH (Zeleznik and Hillier, 1984; Fauser and van Heusden, 1997). So, although the system appears to be primarily regulated in an endocrine manner, to explain the initial emergence of future dominant follicles it has been hypothesized that direct interfollicular interactions are also important (Baker and Spears, 1999).

Oocyte maturation is interlinked with follicle development and regulating bi-directional signaling occurs between oocytes and granulosa cells (for review see Eppig *et al.*, 2001). Even during antral follicular growth, oocyte volume still increases with >10% (Eppig and O'Brien, 1996). Oocytes have to achieve both nuclear and cytoplasmic maturity in order to sustain the early stages of embryonic development (Albertini et al, 2003). Recently, experimental evidence in mice showed that disturbances in this complex interplay of signals regulating follicle growth may alter the late stages of oocyte maturation, and increase the risk for chromosome malsegregation in subsequent meiotic divisions (Hodges *et al.*, 2002). As we described before, it is likely that the early embryo depends on cell components present in the oocyte for the first cleavage divisions. Therefore, it is feasible that ovarian stimulation in humans also results in a disturbance of follicle and oocyte growth and thereby affects chromosomal competence of resulting embryos.

Although a few studies offer some indication that more profound ovarian stimulation can increase the incidence of chromosomal abnormalities (Munné *et al.*, 1997; Troncoso *et al.*, 2003; Katz-Jaffe *et al.*, 2005), it should be investigated in a prospectively randomized trial.

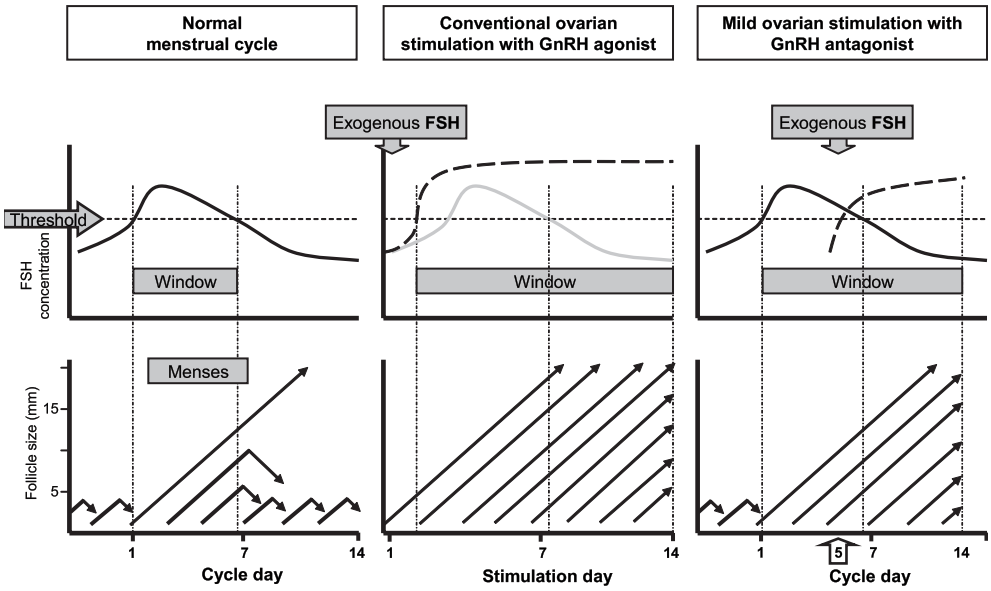


Figure 1.3. Schematic representation of levels of FSH and number and size of follicles during the follicular phase of the normal menstrual cycle, during ovarian stimulation with a GnRH agonist (conventional stimulation) and during ovarian stimulation with a GnRH antagonist and start of exogenous FSH administration on cycle day 5 (mild stimulation). The threshold level represents the concentration of FSH in serum above which ongoing, gonadotropin dependent follicle development is stimulated. The window represents the duration of time FSH concentrations are above the threshold.

Aims and outline of the thesis

To improve the effectiveness of single embryo transfer, the ability to select the embryo with the highest potential to develop into a healthy child is of vital importance. This requires more insight into chromosomal competence of embryos, as this may be a crucial factor for embryo development. In this thesis, chromosomal competence was investigated by applying PGS on fresh or cryopreserved human day 3 embryos, if possible followed by reanalysis of the biopsied embryo on day 5. Also, the chromosome constitution of good quality day 4 or 5 embryos after cryopreservation was investigated. The results of the described studies reveal cytogenetic aspects important for the reliability of PGS (Chapter 2) and have clinical implications for optimizing embryo quality (Chapter 3).

When performing PGS, only a limited time frame is available between optimal timing of embryo biopsy on day 3 after fertilization and time of transfer to the uterine cavity on day 4 or 5. To enable fast and reliable screening of as many chromosomes as possible, multi-color FISH in two rounds of hybridization was applied. Section 2.1 describes the establishment of a protocol using home-labeled DNA probes allowing the reliable screening of 10 different chromosomes within this limited time frame. This FISH protocol was applied to cryopreserved day 3 embryos donated for research purposes in Section 2.2. To investigate the role of chromosomal mosaicism on false positive or false negative results in PGS, the result obtained from a two-cell embryo biopsy on day 3 was compared to the chromosome constitution of the remaining embryo on day 5.

To confirm these results in fresh embryos, the same investigation was performed on day 3 embryos from IVF patients <38 years, where biopsied embryos considered not suitable for transfer or cryopreservation were reanalyzed. The results of this study are described in Section 2.3 and the implications of chromosomal mosaicism for PGS are discussed from a cytogenetic and a clinical point of view.

It has been proposed that the effectiveness of PGS could be improved by analyzing more chromosomes. In Section 2.4, the objective was to establish a protocol for screening 15 chromosomes in three consecutive FISH rounds and explore the added value of investigating more chromosomes for the detection of chromosomally abnormal and mosaic embryos. This study was performed on good quality, cryopreserved day 4 and day 5 embryos and the added value of screening more chromosomes for PGS is discussed.

Chapter 3 concerns with the influence of ovarian stimulation on chromosomal competence of embryos. We aimed to test the hypothesis that a more physiological approach to ovarian stimulation, may allow only the healthiest follicles and oocytes to develop, resulting in a higher percentage of chromosomally normal embryos.

In Section 3.1 the results of a randomized controlled trial are reported, comparing a mild stimulation regimen using gonadotropin-releasing hormone (GnRH) antagonist co-treatment, which does not disrupt secondary follicle selection, with a conventional high dose exogenous gonadotropin regimen and GnRH agonist co-treatment. In performing this study, we had three objectives. First, the incidence of chromosomal abnormalities in embryos from a group of relatively young IVF patients (<38 years), with no increased likelihood of producing chromosomally abnormal embryos, was studied. Second, the incidence of chromosomal abnormalities and mosaicism was determined in embryos resulting from mild versus conventional ovarian stimulation. Third, the relationship between ovarian response and the incidence of chromosomal abnormalities in resulting embryos was investigated.

Finally, in Chapter 4 the most important conclusions from the conducted studies are summarized and implications for clinical practice and future research are discussed.

Chapter 2

Cytogenetic aspects of PGS

Section 2.1 Screening for aneuploidies of ten different chromosomes in two rounds of FISH: a short and reliable protocol

Introduction

The introduction of FISH for preimplantation genetic diagnosis has enabled screening of embryos for chromosomal aneuploidies before transfer. Obviously, it would be of great use for Preimplantation Genetic Screening (PGS) to screen each blastomere for as much chromosomes as possible in a short time period. More recently, new methods have been developed to enable screening of all 23 chromosome pairs, such as Comparative Genomic Hybridization (CGH) (Wells and Delhanty, 2000; Voullaire *et al.*, 2000), spectral karyotyping (Marquez *et al.*, 1998) and nuclear conversion (Verlinsky and Evsikov, 1999). However, because of practical limitations, these techniques have not yet been widely used in clinical practice. Another promising technique, quantitative fluorescent-polymerase chain reaction (QF-PCR), was shown to be still less accurate than FISH tests in a recent comparative study (Sato *et al.*, 2003). Therefore, FISH remains at present the best choice for aneuploidy screening.

The available number of fluorescent dyes that can be individually visualised on a epifluorescence microscope has limited the number of chromosome targets that can be detected simultaneously. In order to increase the number of chromosomes that can be screened, several strategies have been used. One possibility is to screen only three chromosomes per round and increase the number of FISH rounds (Harrison *et al.*, 2000). This, however, takes time and with each FISH round there is an increasing chance for loss of DNA, resulting in poor morphology and signal loss. Another strategy is to increase the number of chromosomes that can be detected by using coded hybridization (Munné *et al.*, 1998a). Here, two or more fluorescent labels are combined to create new colours when observed with a dual or triple band pass filter. By doing so, Munné *et al.* (1998a) could include probes for nine different chromosomes in two rounds of hybridization. However, several probes share the same fluorochromes, which increases the chance for misdiagnosis due to overlap of signals.

Bahçe *et al.* (2000) published a protocol for FISH using monocolour labelled probes, introducing a ready-to-use, commercially available probe mix with five different fluorochromes. This probemix has now been widely used (Hardarson *et al.*, 2003; Silber *et al.*, 2003; Staessen *et al.*, 2003). Although this probemix was shown to be reliable and easy-to-use, it is also expensive and the choice of chromosomes is limited.

The aim of the present work was to develop a homemade probemix, which can be used in combination with a commercially available probemix, making the screening of ten chromosomes in two FISH rounds possible. We labelled repetitive DNA probes with fluorochromes that can be detected independently with specific epifluorescent filters. Furthermore, the spreading and fixation procedure for the blastomeres was further optimised for signal quality and speed of the procedure.

Materials and methods

Labelling of DNA probes

The DNA probes used in the first round were centromere probes for chromosomes 1 (pUC 1.77; Cooke and Hindley, 1979), 7 (pα7t1; Waye *et al.*, 1987), 15 (pTRA-20; Choo *et al.*, 1990), X (pBamX5; Willard *et al.*, 1983) and a Y chromosome heterochromatin probe (RPN1305X; Lau,

1985). The probes were labelled in two steps using an ARES labelling kit (Molecular Probes Europe BV, Leiden, Netherlands) according to the instructions of the manufacturers with some modifications. First, 5-(3-aminoallyl)-dUTP was incorporated into the DNA by nick translation, followed by chemical labelling of the amine-modified DNA with different fluorochromes. The nick translation reaction included: DNA (20 ng/µL), 1x buffer (50 mM Tris-HCL, pH 7.2, 10 mM $MgSO_4$, 0,1 mM DTT, Promega, Madison, USA), 40 µM of dATP, dCTP and dGTP, 10 µM dTTP, 60 µM aminoallyl-dUTP, 0,1 µg/mL Dnase I (Roche Applied Sciences, Indianapolis, USA), 0,3 U DNA polymerase I (Promega)/ µL reaction. After incubation for 2 hrs at 15°C, DNA was purified with a Qiaquick PCR purification kit (Qiagen, Valencia, USA) and subsequently precipitated with ethanol. The chemical coupling reaction was performed for one hour at RT in 10 µL 0,1 M sodiumbicarbonate and 20% DMSO or DMF, after which the DNA was again purified and precipitated. The probes for chromosomes 1, 7, 15 and Y were labelled with Pacific Blue, Alexa Fluor 350, Alexa Fluor 594 and Alexa Fluor 488, respectively. The probe for the X chromosome was labelled by adding equal amounts of Alexa Fluor 488 and Alexa Fluor 555 reactive dye to the chemical coupling reaction mixture. The labelled probes were resuspended in hybridization buffer consisting of 60% formamide and 2×SSC (pH 7.0).

The DNA probes used in the second round were centromere probes for chromosome 16 and 18, labelled with Spectrum Aqua and Spectrum Blue, combined with LSI probes for chromosomes 13, 21, and 22 labelled with Spectrum Red, Green and Gold, respectively (Multivysion PB kit, Vysis, Downers Grove, IL, USA).

Spreading and fixation of blastomeres and embryos

Preimplantation embryos for testing the protocol were obtained from couples participating in our ongoing study on PGS, a study approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO) and the local ethics review committee of the hospital. First, surplus embryos were used that were of insufficient quality for biopsy on day three and also not suitable for replacement or cryopreservation on day four. These embryos were cultured until day five before fixation. After testing the protocol on surplus embryos, the method was applied clinically on single blastomeres obtained after embryo biopsy. These were fixed as described previously (Baart *et al.*, 2004a) with some modifications. In short, the blastomere was washed once in spreading solution (0.01 N HCl/0.1% Tween 20; Coonen *et al.*, 1994; Harper *et al.*, 1994) and immediately transferred onto a clean microscopic slide (Superfrost Plus, Menzel Gläser, Braunschweig, Germany). Spreading solution was added with a finely drawn pasteur pipette. Touching the liquid with the tip of the pipette helped in isolating the nucleus and dissolving the cytoplasm. The solution was then allowed to dry and about 4 µL of freshly made fixative (methanol:acetic acid, 3:1) was dropped on top of the nucleus. After air-drying, spread blastomeres were incubated in ice-cold fixative for 4 min, washed in PBS and dehydrated through an ethanol series. Whole surplus embryos were fixed in the same way, only now the wash in spreading solution was also used to dissolve the zona pellucida, before transferring the embryo to the slide.

FISH procedure

Two rounds of five colour FISH (Table 2.1.1) were applied to single blastomeres or embryos. In the first round, FISH was performed for chromosomes 1 (aqua), 7 (blue), 15 (red), X (yellow) and Y (green). A hybridization mixture was made, containing 10 ng of each labelled probe in 50% formamide, 10% dextran sulphate, 1% Tween 20 and 2×SSC in a final volume of 10 µL. Per slide, 0.5 µL hybridization mixture was applied under a 6mm round coverslip. Blastomere and probe DNA were denatured

simultaneously at 75°C for 3 min and allowed to hybridize in a humid box (containing 50% formamide in 2×SSC, pH 7.2) at 37°C for 1 to 2 h. After hybridization, coverslips were removed and slides were washed in 2×SSC/0.05% Tween 20 for 2 min at 42°C, then in 0.1×SSC for 6 min at 60°C and in 2×SSC/0.05% Tween 20 for 2 min at RT. The slides were rinsed once in PBS, allowed to air dry and mounted in Vectashield antifade medium (Vector Laboratories, Burlingame, CA, USA), containing 1.25 ng/mL 4',6-diamidino-2-phenylindole (DAPI). After analysis of the fluorescent signals, coverslips and antifade medium were removed with two washes in 2×SSC/0.05% Tween 20 for 5 min at RT. The slides were dehydrated and the probemix for the second round was applied. The slides were denatured for 3 min at 75°C and allowed to hybridize for 2 to 4 h at 37°C. Coverslips were removed and slides were washed in 2xSSC/0.05% Tween 20 for 2 min at 42°C, then in 0.6×SSC for 6 min at 60°C and in 2×SSC/0.05% Tween 20 for 2 min at RT. After a brief wash in PBS, the slides were allowed to air dry and mounted in Vectashield antifade medium. Signals from the second round were recorded and compared with the ones from the first round to ensure they had not persisted. For enumeration of the signals after both rounds, we used scoring criteria previously published (Munné *et al.*, 1998b).

The efficiency of the FISH probes was tested on uncultured peripheral lymphocyte spreads from two men and two women with normal karyotypes. Slides were prepared according to standard protocols and they were hybridized using the same two-round FISH protocol as for the embryonic cells (see above). Signals were counted in 200 nuclei. In addition, the positions of 16 individual nuclei were recorded and images were obtained after each round to check for persisting signals.

Microscopy and interpretation of FISH results

Slides were examined with a Zeiss Axioplan 2 imaging epifluorescence-equipped microscope, using the following filters for excitation and detection (Table 2.1.1). Alexa Fluor 594 and Spectrum red: Red filter set, ID 31046; Alexa Fluor 488 and Spectrum Green: Yellow GFP BP filter set, ID 41028; Alexa Fluor 350, Spectrum Blue and DAPI: DAPI/ Hoechst/ AMCA filter set, ID 31000; Spectrum Gold and combined labelling with Alexa Fluor 488 and Alexa Fluor 555: R and B phycoerythrin Hq light source, ID 31003 (all Chroma Technology Corp., Rockingham, Vermont, USA), Pacific Blue and Spectrum Aqua: ID 0415-834 (Zeiss, Jena, Germany). Images were captured with the ISIS FISH Imaging System (MetaSystems, Altlussheim, Germany). Interpretation of the FISH results from single blastomeres after biopsy and embryos was done according to the definitions published previously (Baart *et al.*, 2004b).

Results

Labelling and multicolour FISH

The present labelling protocol allows labelling of DNA probes with four fluorescent dyes that can be detected with a specific filterset (Figure 2.1.1A-D, Appendix). The fifth, yellow fluorescent colour is created through fluorescence resonance energy transfer (FRET). By labelling simultaneously with Alexa Fluor 488 (similar to fluorescein) and Alexa Fluor 555 (spectrally similar to Tetramethylrhodamine), the fluorochromes end up so close together that, after excitation of Alexa Fluor 488 the energy is transferred to Alexa Fluor 555. This will then show sensitised fluorescence, which can be visualised as yellow with the appropriate filter set (Figure 2.1.1E, Appendix). The filter set specific for Alexa Fluor 594 does not detect Alexa Fluor 555, so only chromosome 15 is visible with this set (Figure 2.1.1C, Appendix). Some care has to be taken with the Alexa Fluor 488 signals, which indicate both the Y chromosome and, in part, the X chromosome (Figure 2.1.1D, Appendix), but these can be easily distinguished. Moreover, the X

chromosome can be specifically detected with the yellow filter set (Figure 2.1.1 E, Appendix). Bleeding occurs from the Pacific Blue fluorochrome when viewed with the blue filter set, so that these signals have to be interpreted cautiously (Figure 2.1.1A, Appendix). Good imaging software greatly helps with analysis of all the signals (Figure 2.1.1F, Appendix).

Control lymphocytes

The FISH protocol was tested for hybridization efficiency in 200 nuclei of lymphocyte spreads from two males and two females. The hybridization efficiencies for the individual probes were calculated as the percentage of nuclei showing the expected number of signals and ranged between 95-99%, depending on the individual DNA probe (Table 2.1.2). Taken together, 86% of the nuclei showed the normal amount of signals for all five probes in the first round and 90% in the second round. After comparison of the images obtained after the first and the second round, no persisting signals from the first round were observed after the second round of FISH.

Fixation of blastomere nuclei

The fixation protocol used in this study for blastomeres and embryos allows the removal of all cytoplasm. Therefore, the use of a pepsin treatment was no longer necessary. Furthermore, the additional fixation in ice-cold methanol/acetic acid ensures maintenance of nuclear morphology and specific and localized FISH signals (Figure 2.1.1, Appendix). An advantage of fixing whole embryos according to this protocol is that the HCl/Tween 20 step provides the opportunity to separate all nuclei, so overlapping of nuclei can be avoided.

When analysing the same nucleus twice by FISH, the difficulty is to both maintain good nuclear morphology and to avoid persisting signals. The spreading and fixation protocol we use in this study ensures good morphology also in the second round of FISH (Figure 2.1.1G, Appendix). The commercial probe mix incorporates labelled human placenta DNA, which provides a background colour. This background fluorescence is very persistent and it can interfere with signal detection of the homemade probes if they are used in a second round of FISH. The same goes for the signals for chromosome 16 and 13 (data not shown). We therefore use the commercial probemix in the second round only. The homemade probemix in combination with DAPI background staining never left any persisting signals after the second round of FISH.

Chromosomal analysis of embryos and single blastomeres

Nine surplus embryos from seven patients were completely fixed and analysed (Table 2.1.3). Four embryos were found to be normal. Three embryos were diagnosed as mosaic, consisting of normal cells next to abnormal ones. One embryo showed a monosomy for chromosome 15 in all its cells (embryo 4) and another embryo only had a haploid chromosome complement (embryo 7).

The protocol was applied clinically to three different patients participating in the study. In total, 15 embryos were biopsied and the results of the FISH analysis are presented in Table 2.1.4. Nine embryos were diagnosed as normal, two embryos as mosaic and three embryos were found to be aneuploid. In one embryo, the first nucleus was lost during the spreading procedure and the second nucleus was of such poor morphology that no diagnosis could be made (patient 1, embryo 5). Four normal embryos were transferred and this resulted in one ongoing pregnancy for patient no. 2.

Discussion

The protocol described here allows reliable screening of 10 chromosomes in two rounds of FISH. Furthermore, the whole process from embryo biopsy to FISH analysis can be completed within a day, which is important for PGS where time is limited.

Using the present labeling protocol in combination with the specified filter sets allows identification of five chromosome targets in one round of FISH. Performing the labelling is relatively easy and does not require any special equipment. It offers the advantage of more flexibility in choosing chromosome targets to be investigated. The commercially available probemix only screens for chromosomal abnormalities, which, if present in the embryo, still allow development and implantation. However, CGH studies have shown that all chromosomes can show abnormalities in preimplantation embryos (Voullaire *et al*., 2002, Voullaire *et al*., 2000; Wells and Delhanty, 2000) and it can therefore be useful to screen for other chromosomes. The present labelling protocol allows the screening for aneuploidies of these other chromosomes.

Acknowledgements

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Table 2.1.1: Overview of the fluorochromes and corresponding filtersets used.

FISH round	Chromosome	Fluorochrome	Filterset
I	1	Pacific Blue	Spectrum Aqua filter set, ID 0415-834
	7	Alexa Fluor 350	DAPI/ Hoechst/ AMCA filter set, ID 31000
	15	Alexa Fluor 594	Red filter set, ID 31046
	X	Alexa Fluor 488/ Alexa Fluor 555	R and B phycoerythrin Hq light source, ID 31003
	Y	Alexa Fluor 488	Yellow GFP BP filter set, ID 41028
II	13	Spectrum Red	Red filter set, ID 31046
	16	Spectrum Aqua	Spectrum Aqua filter set, ID 0415-834
	18	Spectrum Blue	DAPI/ Hoechst/ AMCA filter set, ID 31000
	21	Spectrum Green	Yellow GFP BP filter set, ID 41028
	22	Spectrum Gold	R and B phycoerythrin Hq light source, ID 31003

Table 2.1.2: Hybridization efficiency of each DNA probe after analysis of 200 lymphocyte nuclei from two males and two females with a known normal karyotype.

FISH Round	Chromosome Probe	No. of nuclei showing the expected number of signals	Hybridization efficiency
I	1	189	95%
	7	190	95%
	15	195	98%
	X	192	96%
	Y	198	99%
II	13	198	99%
	16	192	96%
	18	194	97%
	21	197	99%
	22	198	99%

Table 2.1.3: Results after fixation and FISH analysis of 9 surplus preimplantation embryos.

Embryo no.	Morphology score[1] / Cleavage stage	# cells analysed	FISH results [no. of cells]	Interpretation of FISH results
1	3 / Arrested day 4	27	2N [14]	Mosaic
			4N [4]	
			-16 [8]	
			+X [1]	
2	3 / Blastocyst	50	2N [41]	Normal
			+18 [3]	
			-18 [3]	
			-13 [1]	
			-15 [1]	
			-21 [1]	
3	4 / Early blastocyst	13	2N [10]	Normal
			4N [2]	
			-18 [1]	
4	4 / Early blastocyst	21	-15 [20]	Aneuploid
			4N -15, -15 [1]	
5	4 / Blastocyst	34	2N [23]	Mosaic
			-18 [4]	
			-13 [3]	
			+15 [3]	
			+1 [1]	
6	2 / Expanded blastocyst	26	2N [19]	Normal
			4N [3]	
			-16 [1]	
			-21 [1]	
			-22 [1]	
			-13 [1]	
7	4 / Arrested day 4	13	N [11]	Haploid
			N-X [2]	
8	3 / Blastocyst	35	2N [30]	Normal
			N [3]	
			4N [2]	
9	4 / Arrested day 3	8	2N [2]	Mosaic
			4N [2]	
			-21 [4]	

[1] Morphology score: 1= excellent; 2= good; 3= average; 4= poor quality.

Table 2.1.4: Embryo development, biopsy and FISH results of 15 preimplantation embryos from three different patients.

Patient no.	Age	No. of oocytes	No. of oocytes fertilized	Embryos biopsied	Morph. Score[1] / no. of cells on day 3	FISH results 1st / 2nd blastomere	Diagnosis	Morphology score/ cleavage stage day 4
1	35	9	8	1	2 / 8	2N / 2N	Normal	1 / Morula [2]
				2	2 / 8	2N / 2N	Normal	2 / Morula [2]
				3	2 / 9	2N / 2N	Normal	3 / Morula
				4	1 / 10	2N / +22	Mosaic	2 / Morula
				5	1 / 8	N.A. / N.A.	-	1 / Morula
				6	2 / 8	2N / -15	Mosaic	Degenerated
				7	3 / 10	2N / 2N	Normal	3 / Morula
				8	1 / 8	+22, -16 / -	Aneuploid	4 / Arrested day 3
2	26	8	3	1	4 / 8	2N / 2N	Normal	4 / Morula [2]
				2	2 / 8	+15 / +15	Aneuploid	4 / Arrested day 3
				3	4 / 5	2N	Normal	4 / Arrested day 3
3	33	8	6	1	3 / 5	2N	Normal	4 / Arrested day 3
				2	2 / 8	2N / 2N	Normal	2 / Morula [2]
				3	2 / 8	2N / 2N	Normal	4 / Arrested day 4
				4	2 / 8	4N +X,-7,-15,-13,-16 /	Aneuploid	2 / Morula
						4N –7, -15, -13, -16		

[1] Morphology score: 1= excellent; 2= good; 3= average; 4= poor quality.

[2] Embryos transferred

N.A. = not analysed

Section 2.2 Fluorescence in situ hybridization analysis of two blastomeres from day 3 frozen-thawed embryos followed by analysis of the remaining embryo on day 5

Introduction

The advent of IVF as a treatment for infertility has created the opportunity to study the chromosomal constitution of surplus human preimplantation embryos. An increasing body of evidence suggests that the incidence of chromosomal abnormalities in embryos is extremely high (as reviewed by Macklon *et al.*, 2002, Wilton, 2002). Accumulating evidence suggests that good embryo morphology does not necessarily exclude an abnormal chromosomal constitution (Magli *et al.*, 2000, Voullaire *et al.*, 2000, Wells and Delhanty, 2000, Sandalinas *et al.*, 2001). Since aneuploidies are considered the main cause of embryonic wastage and loss, this phenomenon may be primarily responsible for the relatively poor pregnancy rates reported after IVF, as well as the poor fertility performance of humans *in vivo* (Delhanty, 2001). Multiple embryos are usually transferred in IVF in an attempt to overcome low implantation rates per embryo, resulting in considerable multiple pregnancy rates (Fauser, 1999, Hunault, 2002).

Preimplantation genetic diagnosis for aneuploidy screening (PGD-AS) is proposed as an effective tool in selecting IVF embryos for transfer. Although used in over 24 IVF centres around the world, its beneficial effect remains difficult to assess. A positive effect on implantation and ongoing pregnancy rates in a group of patients with advanced maternal age was observed in retrospective studies (Munné *et al.*, 1999, Munné *et al.*, 2003). Other indications for which PGD-AS has been proposed include recurrent implantation failure and recurrent miscarriage. However, clinical benefits have not yet been convincingly demonstrated (Gianaroli *et al.*, 1999, ESHRE PGD Consortium Steering Committee, 2002, Pehlivan *et al.*, 2003, Rubio *et al.*, 2003).

An important factor affecting PGD-AS outcome is the chance for misdiagnosis. Next to technical errors, chromosomal mosaicism within an embryo can be a source of misdiagnosis. Different studies show the percentage of mosaic embryos to be highly variable. The reported range in cleavage stage embryos is 2–90%, depending on maternal age, patient subgroup, method of analysis [karyotyping, fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH)], number of chromosomes analysed and how mosaicism is defined (Jamieson *et al.*, 1994, Munné *et al.*, 1995, Almeida and Bolton, 1996, Harper and Delhanty, 1996, Bahçe *et al.*, 1999, Gianaroli *et al.*, 1999, Iwarsson *et al.*, 1999, Ruangvutilert *et al.*, 2000, Voulliare *et al.*, 2000, Wells and Delhanty, 2000, Bielanska *et al.*, 2002, Voulliare *et al.*, 2002). For the chromosomes investigated so far, and from CGH studies where all the chromosomes can be analysed, it appears that all chromosomes can be involved in mosaicism. Because of this phenomenon, analysis of a single cell may not represent the chromosomal content of the remaining embryo, leading to false positive or false negative results. It has therefore been proposed to analyse two rather than one blastomere from each embryo (Harper and Delhanty, 1996, Staessen *et al.*, 1997, Iwarsson *et al.*, 1999). Investigating two cells would provide more insight into the incidence of mosaicism of day 3 embryos. However, the impact of a 2-cell biopsy on developmental potential of the embryo and the added value for achieving a higher rate of ongoing pregnancies is still controversial and needs to be further elucidated.

So far only one comprehensive report on results after PGD-AS on two biopsied blastomeres has been published. This study considered only four embryos, analysing three chromosomes (Emiliani *et al.*, 2000). One of these four embryos showed discordant results between the two

analysed blastomeres and thus was found to be mosaic at day 3. This embryo was reanalysed at day 5 and appeared largely normal. Other studies only reanalysed those embryos that were considered not suitable for transfer due to chromosomal abnormalities diagnosed on a single blastomere (Veiga *et al.*, 1999, Magli *et al.*, 2000, Gianaroli *et al.*, 2001, Sandalinas *et al.*, 2001). Here, high rates of confirmation were described (91%, 92%, 65-100%, 50%, respectively), but very few details were given as to what was considered confirmed. In conclusion, the effect of mosaicism at the 8-cell stage on the reliability of the diagnosis and further development of the embryo is largely unknown.

We have biopsied frozen-thawed good quality embryos and performed FISH analysis for chromosomes 1, 7, 13, 15, 16, 18, 21, 22, X and Y on two blastomeres. After biopsy, the embryos were cultured until day 5 and subsequently reanalysed using the same probe panels. The current study was undertaken to assess how representative the results obtained from a 2-cell embryo biopsy on day 3 are for the chromosome constitution of the remaining embryo on day 5, when the embryo is usually transferred in a clinical setting. Moreover, additional information can be obtained regarding the chromosomal content of good quality embryos after cryopreservation.

Material and methods

Embryos

The spare cryopreserved preimplantation embryos used in this study were obtained from couples undergoing routine IVF procedures in the period between January 1993 and June 1999. The study was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO) and the local ethics review committee of the hospital. Written consent was obtained from the couples in order to confirm that the embryos could be used for research purposes.

Ovarian stimulation, ovulation induction, oocyte retrieval and IVF/intracytoplasmic sperm injection (ICSI) were performed as described previously (Huisman *et al.*, 2000). A maximum of two embryos was transferred on day 3 after oocyte retrieval according to our previously published protocol (Huisman *et al.*, 2000). Supernumerary, good quality embryos were subsequently cryopreserved. The cryopreservation procedure was performed using a slow freezing 1.5 mol/l dimethylsulphoxide (DMSO) protocol in a programmed freezer (De Jong *et al.*, 2002).

For this study, 121 frozen day 3 (4-8 cell) embryos from 25 patients were thawed and taken through consecutive washes of 1.2, 0.9, 0.6 and 0.3 mol/l DMSO for 5 min each. They were transferred to culture medium supplemented with 14.2% GPO (human plasma solution, CLB, Amsterdam, The Netherlands) and cultured for ≥ 2h. Before the biopsy procedure, the embryos were scored for quality and number of blastomeres. Embryo quality scores were assigned according to previously described criteria (Huisman *et al.*, 2000, Hohmann *et al.*, 2003). Only embryos with five or more blastomeres were biopsied, taking two cells only if the embryo consisted of seven or more blastomeres.

In total, 29 embryos from 12 patients survived the cryopreservation procedure, after storage for an average of 6.4 years (range: 10.2-3.8 years). These 12 patients (mean maternal age: 33.7 years) all had become pregnant after transfer of two embryos in the cycle where the embryos were frozen.

Biopsy procedure and spreading of blastomeres and embryos

Prior to biopsy, embryos were washed five times in calcium/magnesium-free medium (EB-10 medium; Vitrolife, Sweden) and then incubated in EB-10 medium for 5 min at 37°C. The biopsy was

performed on the heated stage of a Nikon IX-70 microscope, equipped with micromanipulation tools. An infrared diode lasersystem (OCTAX Laser Shot, OCTAX Microscience GmbH, Germany) with appropriate software (OCTAX EyeWare) was used for partial dissection of the zona pellucida prior to biopsy. After biopsy of one or two blastomeres, the embryos were removed and cultured for another 48 h under normal culture conditions. The remaining blastomeres were fixed according to the method described by Dozortsev and McGinnis (2001). In short, the blastomere was washed once in 1% sodium citrate and transferred onto a slide (Superfrost Plus, Menzel Gläser, Germany). Spreading solution (HCl/ Tween 20; 0.01 N HCl/ 0.1% Tween 20) was added until the cytoplasm was largely removed. The solution was then allowed to dry and a drop of fresh fixative (methanol:acetic acid, 3:1) was added. After drying, the slide was incubated in 100% methanol for 1 min, air-dried and stored at -20°C overnight. The biopsied embryos were analysed for cleavage stage and quality at day 5 and then spread as described above.

FISH procedure

The DNA probes used in the first round were centromere probes for chromosomes 1 (pUC 1.77; Cooke and Hindley, 1979), 7 (pα7t1; Waye *et al.*, 1987), 15 (pTRA-20; Choo *et al.*, 1990), X (pBamX5; Willard *et al.*, 1983) and a Y chromosome heterochromatin probe (RPN1305; Lau, 1985). They were labelled by nick translation with Alexa Fluor 594, Alexa Fluor 488, Alexa Fluor 350 or Pacific Blue using an ARES labeling kit (Molecular Probes Europe BV, Netherlands) according to the instructions of the manufacturers. The labelled probes were resuspended in hybridization buffer consisting of 60% formamide and 2xSSC (pH 7.0). The DNA probes used in the second round were centromere probes for chromosome 16 and 18, labelled with Spectrum Blue and Spectrum Aqua, combined with LSI probes for chromosomes 13, 21, and 22 labelled with Spectrum Red, Green and Gold, respectively (Multivysion PB kit, Vysis, USA).

Two rounds of five colour FISH were applied to each blastomere or embryo. In the first round, FISH was performed for chromosomes 1 (aqua), 7 (blue), 15 (red), X (yellow) and Y (green). A yellow signal was obtained by mixing equal volumes of green and red fluorescence probes. A hybridization mixture was made, containing 1.0 ng of each labelled probe in 50% formamide, 10% dextran sulphate, 1% Tween 20 and 2×standard saline citrate (SSC; pH 7.0) in a final volume of 10µl. Per slide, 0.5 µl hybridization mixture was applied. Blastomere and probe DNA were denatured simultaneously at 75°C for 3 min and allowed to hybridize in a humid box (containing 50% formamide in 2×SSC, pH 7.2) at 37°C for 2 h. After hybridization, the coverslips were removed and slides were washed in 2×SSC/0.05% Tween 20 for 2 min at 42°C, then in 0.1xSSC for 6 min at 60°C and in 2×SSC/0.05% Tween 20 for 2 min at room temperature. The slides were allowed to air dry and mounted in Vectashield antifade medium (Vector Laboratories, USA), or in later experiments in Vectashield containing 1.25 ng/ml 4',6-diamidino-2-phenylindole (DAPI). Slides were examined with a Zeiss Axioplan 2 imaging epifluorescence equipped microscope, using appropriate filters. Images were captured with the Powergene™ Macprobe system (Applied Imaging International Ltd., UK), or in later experiments with the ISIS FISH Imaging System (MetaSystems, Germany). After the first hybridization round, antifade medium was removed with two washes in 2×SSC/ 0.05% Tween 20 for 5 min at room temperature. The slides were dehydrated and the probemix for the second round was applied. The slides were denatured for 6 min at 69°C and allowed to hybridize overnight. Slides were washed in 2×SSC/0.05% Tween 20 for 2 min at 42°C, then in 0.6×SSC/0.05% Tween 20 for 6 min at 60°C and in 2×SSC/0.05% Tween 20 for 2 min at room temperature. The slides were allowed to air-dry and mounted in Vectashield antifade medium. Signals from the second round were recorded and compared with

the ones from the first round to ensure they had not persisted. For both rounds, we used the scoring criteria previously published (Munné *et al.*, 1998b). The efficiency of the FISH probes was tested on uncultured peripheral lymphocyte spreads from two men and two women with normal karyotypes. Slides were prepared according to standard protocols and they were hybridized using the same protocol as for the embryonic cells. Signals were counted in 200 nuclei. In addition, the positions of 16 individual nuclei were recorded and images were obtained after each round to check for persisting signals.

Interpretation of FISH signals and definitions

Based on the analysis of two blastomeres per embryo, we classed day 3 embryos as normal (both nuclei showing the normal amount of signals for the chromosomes investigated), mosaic (one normal nucleus and one abnormal or each nucleus showing a different abnormality) or aneuploid (both nuclei carrying the same abnormality). After analysis of all the cells from each embryo on day 5, we chose the following definitions on the basis of the results obtained. We classed embryos as normal (≥80% normal nuclei and <10% of the nuclei with the same chromosome abnormality), aneuploid (≥90% of nuclei showing the same abnormality) or mosaic (between 10% and 90% of the cells showing the same chromosome abnormality). Embryos with ≥90% haploid, tetraploid or triploid nuclei were classed as such. However, we considered the occurrence of some tetraploid cells as a normal phenomenon of in vitro cultured embryos (Evsikov and Verlinsky, 1998, Bielanska *et al.*, 2002) and treated them as normal cells.

A diagnosis of normal, aneuploid or mosaic on day 3 was considered to be confirmed on day 5 if normal, aneuploid or mosaic results were recovered on day 5, respectively. The diagnosis was considered cytogenetically confirmed, if the same chromosomal abnormalities seen on day 3 were observed on day 5. Furthermore, if aneuploidy was diagnosed on day 3 and a substantial proportion (~80%) of the cells on day 5 showed the same chromosomal abnormality, the diagnosis was considered cytogenetically confirmed as well.

Results

Control lymphocytes

The probes were tested for hybridization efficiency on lymphocyte spreads from two males and two females in 200 nuclei. The hybridization efficiencies for the individual probes were calculated as the percentage of nuclei showing the expected number of signals and ranged between 93 and 100%. Furthermore, 84% of the nuclei showed the normal amount of signals for all five probes in the first round and 90% in the second round. No persisting signals from the first round were observed in the second round.

Embryo biopsy and culture

After thawing, embryos were scored (average morphology score: 2.4) and 29 embryos were considered suitable for biopsy, based on the number of blastomeres. Only two embryos showed < 10% fragmentation. From 21 embryos, two blastomeres could be biopsied and spread (Tables 2.2.1 and 2.2.2). From five embryos, one of the biopsied blastomeres was lost during the spreading procedure and a further three embryos consisted of less than seven cells, so in these embryos only one cell was available for FISH analysis (Table 2.2.3). After the procedure, biopsied embryos were returned to culture. On day 5, embryos were scored for cleavage stage and morphology (Tables 2.2.1-3): 14 embryos had developed to the blastocyst stage (48%, average morphology

score: 2.1), three embryos (10%) were still at the 6-10-cell stage (arrested day 3), five embryos (14%) had arrested at compaction (arrested day 4) and seven embryos (24%) had degenerated to such an extent that no intact blastomeres were available for spreading and they were not analysed further.

FISH analysis of blastomeres and embryos

The FISH results on day 3 and day 5 and their interpretation are shown in Tables 2.2.1-3. From the 21 cases where two blastomeres were available for analysis, nine embryos showed concordant FISH results on day 3 (Table 2.2.1). From the five (24%) embryos diagnosed as normal, three of these could be confirmed after reanalysis on day 5. One embryo had degenerated, and the other embryo showed a false negative result: it was mosaic for monosomy 16. From the four embryos diagnosed as aneuploid or polyploid, the triploid embryo (case 9) had degenerated, but we could confirm the other cases. Case 6 (trisomy 1) developed into a blastocyst with the same abnormality in all but one cell (Table 2.2.1). In case 7, monosomy 7 was found in almost 80% of the cells. Although the resulting embryo was classified as mosaic, we considered the abnormality to be cytogenetically confirmed. For case 8, monosomy 18 was recovered in all but one cell of an otherwise chaotic embryo. The monosomy 18 clearly had a meiotic origin, with the other chromosomal abnormalities resulting from mitotic events.

The other 12 embryos (57%) showed different results for the two blastomeres and were considered mosaic (Table 2.2.2). Most mosaic embryos (cases 10-17) showed one normal and one abnormal blastomere. The other mosaic embryos (cases 18-21) showed two different abnormal blastomeres. One biopsied blastomere was binucleated, with both nuclei showing different abnormalities (case 18). On day 5, four of these 12 cases could be confirmed as mosaic. However, in two cases, totally different chromosome abnormalities were involved (cases 12 and 19). In case 11, monosomy X was confirmed, but the embryo also showed mosaicism for monosomy 16, which was not detected on day 3. In case 17, mosaic monosomy 1 was confirmed on day 5, but monosomy 15 was not recovered. Two embryos had degenerated (cases 18 and 21), and the other six developed into chromosomally normal embryos.

In all, 17 embryos were available with a diagnosis based on two blastomeres on day 3 and confirmatory studies on day 5. In ten of these 17 cases the initial diagnosis of normal (n = 3), aneuploid (n = 3) or mosaic (n = 4) chromosome constitution could be confirmed on day 5. However, in only six of these ten cases (three normal, one monosomy 7, one monosomy 18 and one trisomy 1) were cytogenetic results considered concordant. Besides these 10 cases with a "correct" diagnosis, there were six false positive results and one false negative, all involving mosaicism.

From the embryos where only one blastomere was available for FISH analysis, two were diagnosed as normal and six as abnormal on day 3 (Table 2.2.3). Both normal embryos were mosaic on day 5. Case 22 showed non-disjunction of chromosome 18 in about half of the cells, which was not detected on day 3. Case 23 showed non-disjunction of chromosome 16 in a low percentage of cells. Three abnormal embryos had degenerated on day 5, and after reanalysis of the other three the initial diagnosis could be confirmed only in case 24, which showed substantial mosaicism for trisomy 18. So, from the five cases where confirmatory studies were available on day 5, only one was confirmed.

Discussion

In the current study, blastomere biopsy was performed of 29 cryopreserved embryos donated for research followed by reanalysis of the remaining embryo on day 5. This offers the unique opportunity to study their chromosomal constitution on day 3 and day 5 and to investigate the developmental capacity of embryos after biopsy of two blastomeres. A good proportion of embryos (14 out of 26, 54%) developed to the blastocyst stage after biopsy of two blastomeres. This rate is similar to the blastocyst rate on day 5 of non-biopsied good quality embryos in our laboratory, as reported previously (54%, Huisman *et al.*, 2000). Although larger numbers are needed to confirm these observations, biopsy of two blastomeres does not seem detrimental to the development of the embryo.

Our data confirm previous reports on the lack of correlation between the presence of blastomeres with a chromosome abnormality on day 3 and the likelihood of development to the blastocyst stage (Magli *et al.*, 2001, Sandalinas *et al.*, 2001, Rubio *et al.*, 2003). Extended culture is no reliable means to select chromosomally normal embryos. This is well illustrated with case 6. This embryo, although morphologically scored as excellent, appeared to be uniformly trisomic for chromosome 1 (Figure 2.2.1, Appendix). PGD-AS may therefore be a useful tool in selecting embryos with the best chances for implantation and further development. The efficiency, however, depends largely on the predictive value of the results obtained on day 3.

Reanalysis of blastocysts can be a useful tool to investigate how representative the day 3 results are for the remaining embryo (Gianaroli *et al.*, 1999, Veiga *et al.*, 1999; Emiliani *et al.*, 2000, Magli *et al.*, 2000). However, if a confirmation rate should be established, it is useful to define what can be considered confirmed. From a cytogenetic point of view, confirmation would entail that the chromosome constitution of the two investigated blastomeres is exactly reflected in the cell line(s) found at the blastocyst stage. In the group of embryos with two analysed blastomeres, we found this to be the case in six out of 17 reanalysed embryos. After analysis of one blastomere, only one out of five gave the same results. Hence, cytogenetic confirmation was obtained in only 32% (7/22).

There are several explanations for this high rate of discordance. Misdiagnosis can occur because of technical problems related to the FISH procedure, especially when using more probes simultaneously in successive rounds of FISH. The most extensive FISH screen reported so far, included probes for nine different chromosomes in two rounds of hybridization (Munné at al., 1998). We used probes for ten different chromosomes in two rounds of hybridization and found an overall rate of 84% of lymphocytes to display the normal amount of signals. Although this shows that the probe panels we used can be considered efficient for simultaneous enumeration of the selected chromosomes, it has to be kept in mind that results from lymphocyte nuclei cannot be directly compared with those from blastomere nuclei. Lymphocyte nuclei are subjected to extensive fixation in methanol/ acetic acid and are smaller and more compact in appearance. This can increase the likelihood of loss of signal or signal overlap. Furthermore, since most of the probes used are alpha-satellite probes, signal size can vary due to individual variability in size of the heterochromatic region. To detect this, lymphocyte nuclei from both parents should be used as controls. In these experiments, however, that was not possible. So, although FISH artefact may offer an explanation for the cytogenetic discordance, especially since most of the abnormalities encountered involved loss of signal, there may be other explanations.

First, the abnormal cell(s) could have been removed by the biopsy procedure, thus leaving only normal cells or cells with a different chromosome abnormality. Second, from the 29 biopsied

embryos, 14 embryos showed >10% fragmentation and 13 embryos >20% at the time of biopsy. Though the origin and the mechanisms of this fragmentation process are still unclear, a strong association was established between percentage of fragmentation and chromosome abnormalities (Magli *et al.*, 2001). It has also been shown that fragments can contain chromatin (Jurisicova *et al.*, 2003) and although highly speculative, this process may provide an explanation for the high incidence of chromosomal mosaicism involving chromosome loss we encountered at the 8-cell stage.

It has been shown that significant evidence of apoptosis is not observed until the morula stage in human embryos (Juriscova *et al.*, 1996; Hardy, 1997, 1999; Hardy *et al.*, 2001). This process may be responsible for the elimination of cells carrying a chromosome abnormality making the cell less viable, such as monosomies. So it would be likely for a large part of the aberrant cells to have disappeared at the blastocyst stage, thus confounding the confirmation rate. A previous study performing reanalysis of blastocysts after PGD, indeed reported that correspondence to the day 3 diagnosis was lowest for monosomies and haploidies (Magli *et al.*, 2000). Furthermore, other studies showed that a lower percentage of embryos diagnosed with a monosomy developed to the blastocyst stage compared with normal embryos, or embryos with a trisomy (Sandalinas *et al.*, 2001, Rubio *et al.*, 2003).

A high incidence of mosaicism on day 3 (57%) and day 5 (50%) was observed in the current group of cryopreserved embryos. Others described a similar high incidence of mosaicism in cryopreserved embryos after analysis of entire day 3 embryos (Iwarsson *et al.*, 1999). The possibility that chromosome abnormalities may actually be induced by the cryopreservation procedure cannot be excluded. This may explain the poor added value of cryopreservation in IVF programs, as published recently (de Jong *et al.*, 2002). However, it should be considered that the embryos in the present study were selected in two ways. First, these embryos have survived the cryopreservation procedure, where 76% of the embryos degenerated. Second, although of good morphology, all embryos were surplus in an IVF cycle where the woman became pregnant from the fresh transfer of embryos. It is feasible that those embryos with a normal chromosomal constitution and thus the best chances for implantation were by chance selected in this cycle, leaving the embryos with chromosome abnormalities. Therefore, a direct comparison between fresh and cryopreserved embryos is needed to see if these abnormalities result from the cryopreservation procedure or are increased due to selection of a subgroup of embryos.

In the interpretation of the results, we considered the presence of some tetraploid cells to be a normal feature in preimplantation embryos as also seen in other mammalian species (Benkalifa *et al.*, 1993). A marked increase in incidence of polyploid cells from day 4 to day 6 of development was described by others, suggesting that most polyploid cells arise during blastocyst formation and that they are a hallmark of trophoblast differentiation (Bielanska *et al.*, 2002). Indeed, in most of our cases with tetraploid cells, only a few cells were involved. Tetraploid cells can even undergo correction to normal diploid cells (Staessen *et al.*, 1998). However, it cannot be excluded that a high percentage of tetraploid cells as in case 16 (Table 2.2.2) which arose during one of the first four post-zygotic cell divisions, would be detrimental. Although it has been found that tetraploid cells are almost exclusively allocated to the trophoblast compartment (Angell *et al.*, 1987, Benkhalifa *et al.*, 1993), resulting in confined placental mosaicism as detected by prenatal diagnosis in chorionic villi (Noomen *et al.*, 2001), distribution to the inner cell mass may result in a non-viable (mosaic) tetraploid fetus.

If we consider the results obtained in the present study from a clinical viewpoint, confirmation would be that an embryo diagnosed on day 3 as normal or abnormal is found to be the same after

analysis on day 5. After analysis of only one blastomere, this was the case for only one embryo out of five. After analysis of two blastomeres, the initial diagnosis was found to be most reliable if the results from both blastomeres were concordant (six out of seven). In case of discordant results, confirming the diagnosis was more complicated. Although initially we considered mosaic eight-cell embryos as abnormal, these data show that 3 out of 12 mosaic embryos became normal and morphologically good quality embryos. A recent study also showed embryos diagnosed on day 3 as mosaic to have the same developmental potential as normal embryos, but no reanalysis was performed (Rubio *et al*., 2003). More data are needed on reanalysis of mosaic embryos, since it may help in understanding the fate of specific chromosome abnormalities during embryo development. This may eventually help to determine if some mosaic embryos can be considered safe for transfer.

In conclusion, investigation of two blastomeres allows for a better differentiation between uniformly diploid, uniformly aneuploid and mosaic embryos. We show here that the chromosome constitution of mosaic embryos is subject to changes during further development of the embryo. Thus, the analysis of two blastomeres on day 3 yields a better prediction of the chromosome constitution on day 5 and the developmental potential of the embryo.

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Table 2.2.1: Embryo development, FISH results and interpretation from embryos with concordant results after analysis of two blastomeres on day 3

	Day 3			Day 5			
Case No.	Morph. Score [a] / no. of cells	FISH results	Diagnosis	Morphology score/ Cleavage stage	# cells analysed	FISH results	Interpretation of FISH results
1	2 / 8	2N / 2N	Normal	2 / Hatching	48	2N [45]	Normal [b]
				blastocyst		N [2]	
						4N [1]	
2	2 / 12	2N / 2N	Normal	2 / Hatching	59	2N [32]	Mosaic
				blastocyst		-16 [18]	
						-18 [5]	
						-X [1]	
						-X, -1 [1]	
						-7 [1]	
						-15 [1]	
3	2 / 8	2N / 2N	Normal	1 / Blastocyst	30	2N [24]	Normal [b]
						-X [2]	
						-7 [1]	
						-13 [1]	
						-18 [1]	
						-22 [1]	
4	2 / 12	2N / 2N	Normal	3 / Blastocyst	35	2N [25]	Normal [b]
						4N [3]	
						-13 [2]	
						-18 [2]	
						-1 [1]	
						+15 [1]	
						-1, -21, -18 [1]	
5	3 / 8	2N / 2N	Normal	5 / Degenerated	0	-	
6	1 / 8	+1 / +1	Aneuploid	1 / Hatching	49	+1 [44]	Aneuploid [b]
				blastocyst		+1, -18 [3]	
						+1, +18 [1]	
						4N [1]	
7	3 / 10	-7 / -7	Aneuploid	3 / Blastocyst	13	-7 [10]	Mosaic [b]
						2N [1]	
						4N [2]	
8	2 / 8	-18 / -18	Aneuploid	5 / Arrested day 3	6	+7, -16, -18 [1]	Aneuploid/
						-X, -1, +7, +13 [1]	Chaotic [b]
						+Y, +1, +15, -16, -18 [1]	
						N [1]	
						-1, -7, -18, -21 [1]	
						-16, -18, +21, +22 [1]	
9	3 / 8	3N / 3N	Triploid	5 / Degenerated	0	-	

[a] Morphology score: 1= excellent; 2= good; 3= average; 4= poor quality.
[b] Cases with cytogenetic confirmation

Table 2.2.2: Embryo development, FISH results and interpretation from embryos with discordant results after analysis of two blastomeres on day 3.

	Day 3			Day 5			
Case No.	Morph. score/ no. of cells	FISH results	Diagnosis	Morphology score/ Cleavage stage	# cells analysed	FISH results	Interpretation of FISH results
10	2 / 8	2N / +13	Mosaic	1 / Hatching	33	2N [27]	Normal
				blastocyst		4N [1]	
						-X [1]	
						+1 [1]	
						-16 [1]	
						-18 [1]	
						-16, -21 [1]	
11	2 / 8	2N / -X	Mosaic	3 / Blastocyst	26	2N [8]	Mosaic
						-16 [10]	
						-X [6]	
						-15 [1]	
						-16, +22 [1]	
12	2 / 8	2N / -1, -16	Mosaic	4 / Blastocyst	27	2N [14]	Mosaic
						-13 [4]	
						-21 [3]	
						+13, -1 [2]	
						-13, -21 [1]	
						+13 [1]	
						-22 [1]	
						+13, -22 [1]	
13	2 / 12	2N / -21	Mosaic	1 / Hatching	52	2 N [40]	Normal
				blastocyst		4N [6]	
						-18 [4]	
						+18 [1]	
						-X, -7 [1]	
14	2 / 10	2N / -21	Mosaic	1 / Hatching	175	2N [155]	Normal
				blastocyst		-18 [11]	
						-15 [7]	
						-16 [2]	
15	2 / 8	2N / +13, -16	Mosaic	4 / Arrested day 4	12	2N [9]	Normal
						4N [2]	
						-15 [1]	
16	3 / 8	2N / -16	Mosaic	5 / Arrested day 4	14	2N [8]	Normal
						4N [6]	
17	3 / 7	2N / -1, -15	Mosaic	5 / Arrested day 4	9	2N [2]	Mosaic
						4N [1]	
						-1 [5]	
						+1 [1]	
18	3 / 10	2N / -21 / -X, +16	Mosaic	5 / Degenerated	0	-	
19	3 / 8	4N /	Mosaic	3 / Blastocyst	20	2N [12]	Mosaic
		+Y, -7, -15, -22				-13 [5]	
						-18 [3]	
20	2 / 10	3N / +13, -16	Mosaic	5 / Arrested day 4	14	2N [9]	Normal
						N [1]	
						4N [3]	
						-13, -21 [1]	
21	2 / 7	-1, -16, +18 /	Mosaic	5 / Degenerated	0	-	
		-1, -7, -18, -22					

Table 2.2.3: Embryo development, FISH results and interpretation from embryos after analysis of one blastomere on day 3.

	Day 3			Day 5			
Case No.	Morph. score/ no. of cells	FISH results	Diagnosis	Morphology score/ Cleavage stage	# cells analysed	FISH results	Interpretation of FISH results
22	1 / 8	2N	Normal	1 / Hatching	98	2N [54]	Mosaic
				blastocyst		-18 [32]	
						+18 [11]	
						+X [1]	
23	2 / 10	2N	Normal	5 / Arrested day 3	17	2N [11]	Mosaic
						+16 [2]	
						-16 [2]	
						-15 [1]	
						-X [1]	
24	3 / 12	+18	Aneuploid	4 / Blastocyst	31	2N [5]	Mosaic
						+18 [22]	
						+15[1]	
						-1, +18 [1]	
						+1, +X, +18 [1]	
						-1, +15, +18 [1]	
25	3 / 8	-18	Aneuploid	5 / Arrested day 3	3	2N [2]	Mosaic
						-7 [1]	
26	3 / 7	-X, -16	Aneuploid	5 / Arrested day 4	9	2N [9]	Normal
27	3 / 5	3N	Triploid	5 / Degenerated	0	-	
28	3 / 6	-7, -16	Aneuploid	5 / Degenerated	0	-	
29	4 / 5	-X	Aneuploid	5 / Degenerated	0	-	

Section 2.3 Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF

Introduction

The advent of IVF as a treatment for infertility has created the opportunity to study the chromosomal constitution of human preimplantation embryos. An increasing body of evidence suggests that the incidence of chromosomal abnormalities in embryos is extremely high (as reviewed by Macklon *et al.*, 2002; Wilton, 2002) and good embryo morphology does not necessarily exclude an abnormal chromosomal constitution (Magli *et al.*, 2000; Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Magli *et al.*, 2001; Sandalinas *et al.*, 2001). Since aneuploidies are considered the main cause of embryonic wastage and loss, this phenomenon may be primarily responsible for the relatively poor pregnancy rates reported after IVF, as well as the poor fertility performance of humans *in vivo* (Delhanty, 2001).

The introduction of FISH for preimplantation genetic diagnosis has enabled screening of embryos for chromosomal aneuploidies before transfer. This preimplantation genetic screening (PGS) would be of special interest for couples that are thought to have a higher risk of developing chromosomally abnormal embryos, with the aim of improving their chances for an ongoing pregnancy after IVF. Although PGS is offered in many IVF centres around the world, its clinical value remains uncertain. A positive effect on implantation and ongoing pregnancy rates in a group of patients with advanced maternal age has been observed in retrospective studies (Munné *et al.*, 1999, 2003). However, a recent prospective randomized study failed to show a positive effect of PGS on clinical outcome per initiated cycle in patients with advanced maternal age (Staessen *et al.*, 2004). Other indications for which PGS has been proposed include recurrent implantation failure and recurrent miscarriage. Again, clinical benefits have not yet been convincingly demonstrated (Gianaroli *et al.*, 1999; ESHRE PGD Consortium Steering Committee, 2002; Pehlivan *et al.*, 2003; Rubio *et al.*, 2003; Platteau *et al.*, 2005).

Studies testing the efficiency of PGS have so far used clinical parameters such as implantation rates and ongoing pregnancies as outcome measures. However, it has not yet been demonstrated that the screening of one or two blastomeres obtained from an eight-cell embryo for the presence of aneuploidies will provide a reliable prediction of the chromosomal status of the remaining embryo. An important factor affecting the reliability of the diagnosis is the phenomenon of mosaicsm in embryos (Los *et al.*, 2004). Reanalysis of blastocysts can be a useful tool to investigate whether FISH results from blastomeres obtained from day 3 embryos are representative for the remaining embryo (Gianaroli *et al.*, 1999; Veiga *et al.*, 1999; Emiliani *et al.*, 2000; Magli *et al.*, 2000). In a previous study, we have biopsied frozen-thawed good quality embryos and performed FISH analysis for 10 chromosomes (Baart *et al.*, 2004b). After biopsy, the embryos were cultured until day 5 and subsequently reanalysed using the same probe panels. We observed a high percentage of mosaic embryos on day 3 (57%) and found that the chromosomal constitution of these embryos is subject to changes during development to the blastocyst stage. This yielded mostly false positive results and a low confirmation rate, confirming our suspicion that chromosomal mosaicism at the eight cell stage poses a serious problem when performing PGS.

PGS has been mostly applied to women of advanced maternal age or with an indication, such as recurrent implantation failure or recurrent miscarriage. Moreover, in most of the studies mentioned above, the diagnosis has been based on the biopsy of only one blastomere. Therefore,

only limited data are available concerning the incidence of chromosomal abnormalities and especially mosaicism in embryos of younger IVF patients (<38 yrs) with no specific indication for PGS. In order to assess the frequency of aneuploidy and mosaicism in embryos obtained from such a group of women, we performed PGS using FISH for 10 different chromosomes (1, 7, 13, 15, 16, 18, 21, 22, X and Y) on day 3 embryos after biopsy of two blastomeres. Furthermore, the impact of chromosomal mosaicism on the accuracy of the day 3 diagnosis was studied. Embryos diagnosed as normal and of morphological sufficient quality were either transferred or cryopreserved on day 4. Those embryos diagnosed as abnormal or normal embryos of insufficient quality, were cultured further until day 5 to study the developmental capacity of the embryo. The embryo was subsequently completely analysed by FISH and the reliability of the diagnosis was evaluated by comparing the results obtained on day 3 with the chromosome constitution of the embryo on day 5.

Materials and methods

Patients and embryos

Between November 2002 and August 2004, preimplantation embryos were obtained from couples participating in an ongoing study on PGS. The present study was designed to investigate the incidence of chromosomal aneuploidies in embryos from young IVF patients with no specific indication for PGS Prior to commencing the study, ethical approval was received from the Dutch Central Committee on Research Involving Human Subjects (CCMO) and the local institutional ethics committee. Only women aged <38 years and with a partner with normal semen characteristics were invited to participate in the study and written informed consent was obtained from each couple. Additional inclusion criteria included (i) a history of regular menstrual cycles, ranging from 25-35 d; (ii) a bodymass index of 19-29 kg/m^2; (iii) no known karyotype abnormalities, and (iv) no history of recurrent abortions. Couples could participate in the study for one cycle only.

Ovarian stimulation, oocyte retrieval and IVF procedures were performed as described previously (Huisman *et al*., 2000; Hohmann *et al*., 2003). Before the biopsy procedure, the embryos were scored for quality and number of blastomeres. Embryo quality scores were assigned according to previously described criteria (Huisman *et al*., 2000; Hohmann *et al*., 2003). Biopsied embryos were cultured until day 4, by which time FISH analysis was completed. Only embryos that were diagnosed as normal and of sufficient morphological quality were transferred, with a maximum of two embryos per patient. Remaining good quality, normal embryos were cryopreserved on day 4. Embryos diagnosed as abnormal or of insufficient quality were cultured until day 5, scored for morphology and the entire embryo was fixed for FISH analysis.

Biopsy procedure and fixation of blastomeres and embryos

The biopsy procedure was performed on day 3 after fertilization as described previously (Baart *et al*., 2004b). In short, embryos were washed and then incubated in EB-10 medium and later in the study, G-PGD medium (both Vitrolife, Göteborg, Sweden) for 5 min at 37°C. One blastomere was biopsied if the embryo consisted of five or six cells and two blastomeres if the embryo had at least seven cells. The biopsied embryos were returned to normal culture conditions. The removed blastomeres were fixed as described previously (Dozortsev and McGinnis, 2001) with some modifications (Baart *et al*., 2004a). In short, the blastomere nucleus was isolated with spreading solution (0.01 N HCl/ 0.1% Tween 20; Coonen *et al*., 1994; Harper *et al*., 1994) and subsequently fixed with freshly made fixative (methanol:acetic acid, 3:1). Whole surplus embryos were fixed in

the same way, only now a wash in spreading solution was also used to dissolve the zona pellucida, before transferring the embryo to the slide.

FISH procedure

A two round FISH procedure was performed as described previously (Baart *et al.*, 2004a), allowing the detection of chromosomes X, Y, 1, 7, 13, 15, 16, 18, 21 and 22. The DNA probes used in the first round were centromere probes for chromosomes 1 (pUC 1.77; Cooke and Hindley, 1979), 7 (pα7t1; Waye *et al.*, 1987), 15 (pTRA-20; Choo *et al.*, 1990), X (pBamX5; Willard *et al.*, 1983) and a Y chromosome heterochromatin probe (RPN1305X; Lau, 1985). The probes for chromosomes 1, 7, 15 and Y were labelled with Pacific Blue, Alexa Fluor 350, Alexa Fluor 594 and Alexa Fluor 488, respectively. The probe for the X chromosome was labelled with both Alexa Fluor 488 and Alexa Fluor 555, resulting in a yellow fluorescence. The DNA probes used in the second round were centromere probes for chromosome 16 and 18, labelled with Spectrum Aqua and Spectrum Blue, combined with LSI probes for chromosomes 13, 21, and 22 labelled with Spectrum Red, Green and Gold, respectively (Multivysion PB kit, Vysis, Downers Grove, IL, USA). Signals from the second round were recorded and compared with the ones from the first round to ensure they had not persisted.

As most of the probes used are repetitive DNA probes, signal size can vary due to individual variability in size of the heterochromatic region. To detect this, lymphocyte nuclei from both parents were used as controls. Slides were prepared from blood samples according to standard protocols and they were hybridized using the same two-round FISH protocol as for the embryonic cells. Signals were observed in 10 nuclei from each parent and images were obtained after each round to check for persisting signals. Probe hybridization effiency on lymphocyte nuclei was 86% for the first round of hybridization and 90% for the second round (Baart *et al.*, 2004a).

Microscopy and interpretation of FISH results

Slides were examined with a Zeiss Axioplan 2 imaging epifluorescence microscope, equipped with appropiate filters (Baart *et al.*, 2004a). Images were captured with the ISIS FISH Imaging System (MetaSystems, Altlussheim, Germany). For enumeration of the signals after both rounds, we used scoring criteria previously published (Munné *et al.*, 1998b). Interpretation of the FISH results from single blastomeres and embryos was done according to the definitions published previously, with some modifications (Baart *et al.*, 2004b). Based on the analysis of two blastomeres per embryo, we classified day 3 embryos as normal (both nuclei showing the normal number of signals for the chromosomes investigated), aneuploid (both nuclei carrying the same chromosome abnormality), abnormal/normal mosaic (one normal nucleus and one abnormal) or abnormal/abnormal mosaic (each nucleus showing one or more different chromosome abnormalities). If only one blastomere was available for diagnosis, the embryo was classed as normal or aneuploid based on the FISH result from this nucleus. If a blastomere showed aneuploidy for two or more chromosomes, it was defined as double or multiple aneuploidy respectively.

After analysis of day 5 embryos, we classified them as normal (~80% normal nuclei and, more importantly, ‹10% of the nuclei with the same chromosome abnormality), aneuploid (≥90% of the nuclei showing the same abnormality) or mosaic (›10% and ‹90% of the cells showing the same chromosome abnormality). Embryos with ≥90% haploid, tetraploid or triploid nuclei were classed as such. However, we considered the occurrence of some tetraploid cells as a normal phenomenon of *in vitro* cultured embryos (Evsikov and Verlinsky, 1998; Bielanska *et al.*, 2002; Coonen *et al.*, 2004) and treated them as normal cells.

An abnormal diagnosis made on day 3 was considered cytogenetically confirmed, if at least one of the chromosomal abnormalities seen on day 3 was recovered in >10% of the cells analysed on day 5.

Results

Clinical results

A total of 60 couples started their IVF cycle within the study period and the clinical results are summarized in Table 2.3.1. Five cycles were cancelled due to either poor response or ovarian hyperstimulation. After 55 oocyte retrievals, two cycles showed no fertilization and in seven cycles none of the embryos were suitable for biopsy on day 3. The 46 cycles where a biopsy could be performed yielded a total of 323 embryos, from which 224 embryos were suitable for biopsy. The average age of these 46 women was 33.1 years (range 25-37). This was their first (61%), second (11%), third (19%) or fourth IVF cycle (9%).

Biopsy and diagnosis on day 3

A total of 178 embryos consisted of at least seven blastomeres, enabling two cells to be biopsied (79%), and from the remaining 46 embryos, only one blastomere could be taken (21%; Table 2.3.1). From 28 embryos, blastomere(s) were lost during the spreading procedure or the FISH results were inconclusive, so no diagnosis could be made. From the other 196 embryos, a diagnosis was obtained based on two cells in 121 embryos (62%) and on one cell in 75 embryos (38%). After analysis of two blastomeres, the diagnosis was normal for 43 embryos (36%), aneuploid for 17 embryos (14%) and mosaic for 61 embryos (50%), of which 34 (28%) embryos were abnormal/normal mosaic and 27 embryos (22%) abnormal/abnormal mosaic. After analysis of only one blastomere, the diagnosis was normal for 27 embryos (36%) and 48 embryos (64%) were found to be aneuploid.

Reanalysis of day 5 embryos and interpretation of FISH results

After transfer or cryopreservation on day 4, 108 embryos were left for further culture until day 5. On day 5, 49 embryos (45%) had developed to the blastocyst stage. Twenty embryos (19%) had arrested after (arrested day 4) and 14 embryos (13%) before compaction (arrested day 3). A further 25 embryos (23%) had degenerated and could not be analysed by FISH.

In total, detailed FISH analysis was performed on 83 embryos, the results of which are presented in detail in Appendix I. After interpretation of the FISH results from the blastocysts according to the criteria described, 16 (33%) were found to be normal, 11 (22%) aneuploid and 22 (45%) mosaic. In the group of arrested embryos, five (15%) were found to be normal, 7 aneuploid (21%) and 22 mosaic (65%). The chromosomal constitution of the day 5 embryo (blastocyst or arrested) was compared to the original diagnosis on day 3 after analysis of two blastomeres (Table 2.3.2) or one blastomere (Table 2.3.3). A summary of the confirmation rates is presented in Table 2.3.4.

Cytogenetic confirmation of day 3 diagnosis

Normal day 3 diagnosis:

For the embryos diagnosed as normal on either one or two cells, we found poor confirmation rates (20 and 43%, respectively; Table 2.3.4), leading to eight cases with a false negative diagnosis (Table 2.3.2 and 2.3.3). It has to be kept in mind that these embryos, although diagnosed as

normal, were found to be unsuitable for transfer or cryopreservation on the basis of development and morphology on day 4.

Aneuploid day 3 diagnosis based on two cells

The highest confirmation rate was established for embryos diagnosed as aneuploid based on two cells. Here, we found only two false positive cases out of 11. Case 10 demonstrated a monosomy X on day 3 and was found to be normal on day 5 according to our definitions (Table 2.3.2). However, there were four cells present in the embryo with either a monosomy or a trisomy X, so a low level of mosaicism at the eight cell stage cannot be excluded, in which case we may have biopsied most of the abnormal cells.

Aneuploid day 3 diagnosis based on one cell:

From the embryos diagnosed as aneuploid based on one cell, nine embryos showed a single abnormality on day 3, and the same abnormality was recovered in the day 5 embryo in three cases (56, 57 and 72, Table 2.3.3). A further 15 embryos were diagnosed with double or multiple aneuploidy. Eleven cases could be confirmed on day 5, although in ten cases only one of the abnormalities seen on day 3 was recovered. Only in case 62 was the exact double aneuploidy present in all cells of the blastocyst.

Day 3 diagnosis of mosaicism:

After analysis of two cells on day 3, 36 embryos were found to be either abnormal/normal or abnormal/abnormal mosaics (Table 2.3.2 and 2.3.4). Of these, 18 (50%) were confirmed to be mosaic or aneuploid involving the same chromosomal abnormality in cases of single aneuploidy (e.g. cases 11 and 14) or at least one of the chromosomal abnormalities observed on day 3 in cases of double or multiple aneuploidy (e.g. cases 26 and 29). Of the 18 embryos without confirmation, 14 presented a normal chromosome constitution on day 5, from which 13 had developed into blastocysts. One embryo turned out to be triploid on day 5 (case 47) and three embryos were abnormal/normal mosaics, all involving another abnormal cell line (cases 15, 21, 22). The trisomy 7 observed on day 3 in case 22 was not confirmed on day 5, but interestingly, a cell line with a monosomy 7 was observed. It is not unlikely that the trisomy and monosomy 7 were the products of a non-disjunction event during the second or third cleavage division. Biopsy of the trisomic cell then left the embryo with the corresponding monosomic cell, next to normal cells.

If we look at the abnormal/abnormal mosaic cases in more detail (Table 2.3.2), we find that in 19 out 22 cases, multiple aneuploidy was involved. From these mosaic embryos with multiple aneuploidies, in 12 cases the two blastomeres share the same chromosome abnormality, next to other abnormalities (cases 24, 25, 27, 29, 33, 34, 35, 36, 49, 50, 51, 53). In eight of these twelve cases (67%) we were able to confirm the common aneuploidy on day 5.

Identification of the origin of chromosome aberrations observed

By comparing the day 3 and day 5 diagnosis for each embryo, valuable information on the origin of the abnormalities observed can be obtained. As mentioned above for embryo 22, there are eleven further cases where a mitotic non-disjunction event is likely to have occurred before the 8-cell stage and where the reciprocal product of such an event is recovered on day 5 (Tables 2.3.2 and 2.3.3). However, it can never be excluded that the monosomy and trisomy resulted from two separate events. With respect to embryos 46 and 71, a non-disjunction event was detected in the day 5 embryo, where both a monosomic and a trisomic cell line for the same chromosome were found. Another interesting example is embryo 33, where a cell with a monosomy 7 divided with non-disjunction for chromosomes 13, 15, 18 and 22. These daughter cells were then biopsied, probably leaving the embryo with only normal cells.

In 19 out of 83 cases (23%), a chromosome abnormality was involved that most likely originated during meiosis (Tables 2.3.2 and 2.3.3). Besides the meiotic chromosome abnormality, in all of these cases the embryo was additionally hit by one or more mitotic events, such as anaphase lagging and non-disjunction (see Appendix). An interesting example is embryo 27, where the results on day 3 and on day 5 are consistent with a chromosome constitution of XXY from a meiotic event, followed by non-disjunction of one of the X chromosomes during the second cleavage division (Figure 2.3.1).

Embryos 6 and 7 were both trisomic for chromosome 15 on day 3 and turned out to be mosaic for the same abnormality on day 5. This chromosome abnormality may, however, have originated from two different mechanisms. In case 6, trisomy 15 most likely originated postzygotically through non-disjunction, followed by loss of the monosomy 15 cell line. In contrast, in case 7 the trisomy 15 most probably arose meiotically, with a post-zygotic loss of the extra chromosome 15 resulting in only 16 % normal cells in the day 5 embryo.

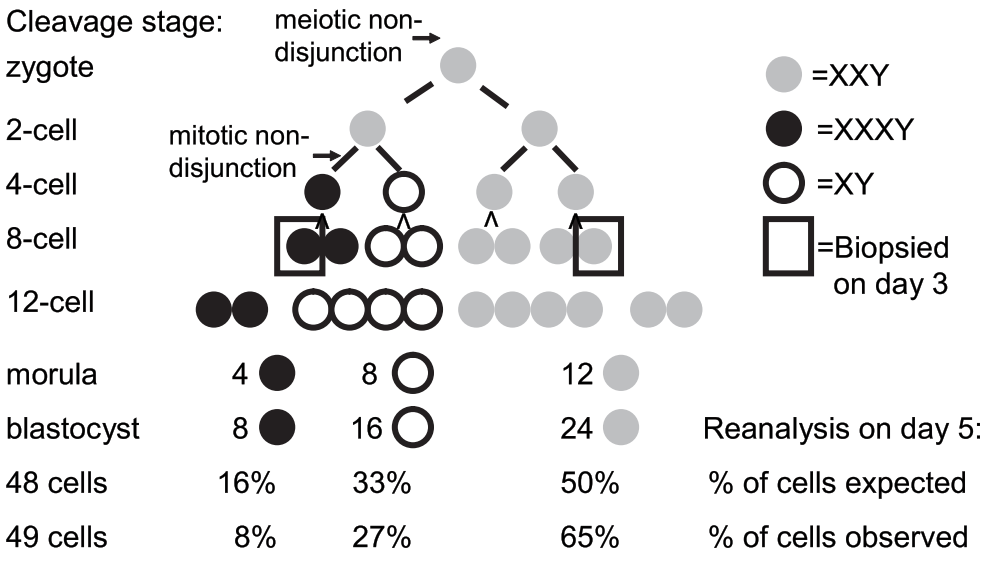

Figure 2.3.1. Schematic representation of the most likely origin of the chromosomal abnormalities observed in case 27, on day 3 and day 5 .

Discussion

Here we present the data from good quality human preimplantation IVF embryos after screening for aneuploidies of 10 different chromosomes on day 3. In addition, for embryos not suitable for transfer on the basis of the PGS results or poor morphology, subsequent analysis of the entire embryo on day 5 is also presented. Although the embryos investigated came from a group of relatively young IVF patients (mean maternal age 33.1 years, range 25-37), we found only 36% of the embryos to be normal, after analysis of both one or two blastomeres. Interestingly, Staessen *et al.*, (2004) reported the exact same percentage of normal embryos after PGS on one or two

blastomeres in a group of older aged patients. So although older women are thought to have a lower chance to produce chromosomally normal embryos, this could not be confirmed by our results. In fact, in 23% of the reanalysed embryos, we observed a chromosome abnormality that most likely originated during meiosis. Another study reported PGS analysis in a group of women aged ‹35 years (Munné *et al*., 2004b). These patients were undergoing PGS either because they were carriers of X-linked diseases, because they had two or more previous IVF failures or because of a previous aneuploid conception. After analysis of a single cell for 6-9 chromosomes, the percentages of normal embryos were found to be 52%, 47% and 29%, respectively. Comparison with our data is difficult, since we analysed two cells and more chromosomes, and a lower percentage of normal embryos would therefore be expected. Furthermore, the first group probably consisted primarily of fertile patients with no indication for IVF. Most of the patients in our study (61%) had their first IVF cycle, but our group may be more heterogeneous.

We observed a high rate of mosaic embryos after both day 3 (50%) and day 5 analysis (45% for blastocysts and 65% for arrested embryos). Artefacts of the FISH procedure resulting in misdiagnosis are one possible explanation for the high rate of chromosomal mosaicism. However, most of it will represent true abnormalities in a mosaic embryo. This has been elegantly demonstrated in a recent publication, where they used probes for two different loci on the same chromosome (Daphnis *et al*., 2004) and found an error rate caused by artefacts of 5% per nucleus. Furthermore, other studies, using techniques other than FISH, also described mosaicism in preimplantation embryos. This phenomenon was reported after performing conventional karyotyping on day 2 or 3 embryos (Jamieson *et al*., 1994). Comparative genomic hybridization (CGH) offers the advantage of allowing all the chromosomes to be analysed. Two groups using CGH on a small number of embryos confirmed the high rates of mosaicism observed by FISH (Wells and Delhanty, 2000; Voullaire *et al*., 2000, 2002). Using single cell multiplex fluorescent (FL)-PCR, mosaicism of trisomy 21 was confirmed in day 3 embryos diagnosed as aneuploid for chromosome 21 by FISH (Katz-Jaffe *et al*., 2004). This technique demonstrated a mitotic origin of trisomy 21 in half of the embryos investigated. Recently, a direct insight into the mechanisms leading to mosaicism has been provided in a study using confocal laser scanning microscopy in embryos immunolabelled with antibodies against tubulin (Chatzimeletiou *et al*., 2005). They observed various spindle abnormalities including abnormal shape and chromosome loss from the spindle, presumably leading to chromosome malsegregation to the daughter cells.

Although mosaicism is now becoming a well accepted phenomenon in preimplantation embryos, its implications for PGS require more attention. The present study provides the first detailed reanalysis data of embryos analysed by PGS and clearly demonstrates the impact of mosaicism on the reliability of the PGS diagnosis. Our results show that the chromosomal constitution of the embryo on day 3 is by no means fixed. The first cell divisions may be successively hit by mitotic events leading to chromosome loss as well as chromosome gain, as hypothesized by Los *et al*., (2004). These abnormal cell divisions can persist as long as the embryonic genome is not fully active and cell cycle control is absent. So, mechanisms such as non-disjunction and anaphase lagging are responsible for the high percentage of mosaicism as observed in 8-cell embryos and in blastocysts (Coonen *et al*., 2004; Daphnis *et al*., 2004).

Reanalysis on day 5 can be used to investigate the reliability of the day 3 diagnosis. This has been used by several groups and very high confirmation rates have been reported (Magli *et al*., 2000; Gianaroli *et al*., 2001; Sandalinas *et al*., 2001; Emiliani *et al*., 2004; Staessen *et al*., 2004). However, very few details were given as to what was considered confirmed. In the current study, we considered this from a cytogenetic point of view, so confirmation entails the chromosome

constitution of the investigated blastomeres to be reflected in the embryo after analysis on day 5. In the current group of embryos, this was the case for only 54%. Confirmation rates could also be established from a clinical viewpoint, i.e. the embryo was correctly replaced or discarded after a normal or abnormal diagnosis. However, since it is not known how many diploid cells an embryo needs to be able to develop into a healthy child, this is impossible to determine for mosaic embryos.

We found the best confirmation rate after a diagnosis based on two cells, where both blastomeres showed the same chromosomal abnormality, either as a single aneuploidy or in combination with other abnormalities. In these embryos the aneuploidy most likely arose during meiosis or fertilization. In contrast, after a mosaic diagnosis the confirmation rate was low. Especially from the 26 mosaic day 3 embryos that had developed into blastocysts on day 5, half of the embryos turned out to be chromosomally normal at that point. In line with these findings, a diagnosis based on one cell yielded poor confirmation rates, since a distinction between mosaicism and an abnormality from a meiotic origin can not be made after analysis of one cell.

Another point for consideration is the impact of the biopsy procedure itself on the confirmation rate, since the removal of blastomeres changes the constitution of a mosaic embryo. When a biopsy of two cells is performed, two blastomeres lying next to each other are removed. The biopsy is therefore not random and the chance of removing the reciprocal daughter cells is ~25%. We found several examples, in which the biopsy of two abnormal blastomeres may have "cured" the embryo, yielding a grossly normal embryo on day 5.

Because of the biological phenomenon of mosaicism, PGS at the 8-cell stage will never be fully reliable. Even if the diagnosis is based on two cells, they are removed from the embryo and the chromosomal constitution of the remaining blastomeres is not known. Moreover, because of the compromised functioning of cell cycle check points, the remaining embryo can continue to change cytogenetically until the embryonic genome becomes fully active, probably at the blastocyst stage (Wells *et al.*, 2005). The developmental potential of mosaic embryos will depend on the proportion of normal cells (Bielanska *et al.*, 2002). Although the general consensus is that embryos with <50% normal cells would be unlikely to survive beyond the implantation stage, this is impossible to assess. Therefore, no matter how many improvements are made to the technique of aneuploidy detection, it will be impossible to predict with 100% certainty the chromosomal status of the embryo at the time of transfer and beyond by performing genetic analysis at the eight-cell stage. A better understanding of the fate of mosaic embryos is needed before these embryos can be considered for transfer. Until this is resolved, PGS may result in good embryos being discarded or in chromosomally abnormal embryos being replaced.

In conclusion, reanalysis by means of FISH of the embryos on day 5 provides an improved understanding of the fate of abnormal blastomeres during embryo development and a valuable insight into the mechanisms of aneuploidy formation. We show that PGS after analysis of two blastomeres is effective in detecting abnormal embryos resulting from a meiotic non-disjunction event. Although current techniques of PGS result in limited accuracy, PGS may still offer an additional marker for embryo quality, and can thus contribute to an overall positive effect on ongoing pregnancy rates (Munné *et al.*, 1999; Munné *et al.*, 2003).

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Table 2.3.1. Clinical IVF results and FISH diagnosis based on one or two blastomeres obtained from day 3 embryos

Started cycles	60
No. of cycles with:	
Oocyte retrieval	55
Fertilization failure	2
Insufficient development of embryos	7
Biopsy	46
No. of embryos obtained	323
No. of embryos biopsied	224 (69)
No. of embryos diagnosed	196 (88)
No. of embryos diagnosed based on 2 cells	121 (62)
Normal	43 (36)
Aneuploid	17 (14)
Abnormal/ normal mosaic	34 (28)
Abnormal/ abnormal mosaic	27 (22)
No. of embryos diagnosed based on 1 cell	75 (38)
Normal	27 (36)
Abnormal	48 (64)

Values in parentheses are percentages

Table 2.3.2. Embryo development, FISH results and interpretation from embryos after analysis of two blastomeres on day 3. (For a detailed presentation of the FISH results on day 5, see appendix I).

	Day 3		Day 5			
Case no.	Morphology score[a]/ No. of cells	FISH results	# cells analysed	% of normal cells	Interpretation of FISH results	Confirmed
Embryos reaching the blastocyst stage						
		Normal				
1	3 / 10	2N / 2N	51	73%	Normal	+
2	2 / 7	2N / 2N	15	80%	Mos mon7/2N	-
3	2 / 9	2N / 2N	58	78%	Mos mon21/2N	-
		Aneuploid				
4	2 / 8	X0 / X0	18	56%	Mos monX/ 2N (XY)	+
5[e]	2 / 8	-22 / -22	41	0%	Mon 22	+
6	2 / 8	+15 / +15	43	79%	Mos tris15/2N	+
7[e]	3 / 8	+15 / +15	57	16%	Mos tris15/2N	+
8[e]	2 / 10	+22 / +22	26	0%	Tris 22	+
9	2 / 8	3N / 3N	19	0%	Triploid	+
10	2 / 8	X0 / X0	38	71%	Normal (XY)	-
		Abnormal/ normal mosaic				
11	2 / 8	-16/2N	64	84%	Mos mon16/2N	+
12	2 / 8	-16/2N	66	83%	Mos mon16/2N	+
13	2 / 8	-**16**/2N	17	47%	Mos mon15/**mon16**/mon13/ X0/2N (XY)	+
14[e]	1 / 10	+22/2N	62	10%	Tris 22	+
15	2 / 12	X0/2N (XY)	30	73%	Mos mon7/2N (XY)	-
16	1 / 8	+7/2N	76	88%	Normal	-
17	2 / 8	+15/2N	81	100%	Normal	-
18	2 / 8	+21/2N	61	89%	Normal	-
19	2 / 8	-15/2N	34	94%	Normal	-
20	2 / 8	-7,-7/2N	48	92%	Normal	-
		Abnormal/ abnormal mosaic (single aneuploidy)				
21	2 / 8	-7 / -21	38	82%	Mos mon15/2N	-
22[d]	2 / 9	+7 /-1	38	74%	Mos mon7/2N	-
23	2 / 8	+13 / -21	52	94%	Normal	-
		Abnormal/ abnormal mosaic (multiple aneuploidies)				
24[e]	2 / 8	+22, +22 / +**22**	20	10%	Mos **tris22**/tris1,22/ tris1,mon7,tris 22	+
25[e]	2 / 7	-18, -21 / -21	40	0%	Mon 21	+
26	2 / 8	+16, +22 / -**21**	27	52%	Mos tris15 and **mon 21**/tris15/2N	+[b]
27[d,e]	2 / 7	XXXY / **XXY**	49	27%	Mosaic XY/ **XXY**	+[b]
28[d,e]	2 / 8	+**1**,-15,-21 / **XXX**,+**16**,+16	23	0%	Mos **trisX,16**/**tris16**/ **tris1**,15,**16**	+[b]

29	1 / 8	X0, -**16**,-16,-22 / -1,-7,-**16**,-16	50	76%	Mos **mon16**/2N	+[b]
30	2 / 8	+15 / -18, -21	27	100%	Normal	-
31	2 / 8	X0, +15 / +22, +22	52	94%	Normal (XX)	-
32	1 / 8	XXX / 3N, -1,-15	32	63%	Normal (XX)	-
33	2 / 8	-7,-13,-15,-18,-22 /-7,+13,+15,+18,+22	127	87%	Normal	-
34	2 / 8	XXY, +15, -7,-13/ XXY, +13	103	91%	Normal (XY)	-
35	2 / 8	-7 / N,-7, -21, -13	28	82%	Normal	-
36	2 / 8	-13,-18,-18,-21 / -7,-7,-18	32	94%	Normal	-
Embryos arrested at day 3 or 4						
		Normal				
37	1 / 8	2N / 2N	22	91%	Normal	+
38	2 / 8	2N / 2N	11	82%	Normal	+
39	3 / 8	2N / 2N	6	0%	Mos Near 4N[c]/monX/mon15/mon1,7	-
40	2 / 8	2N / 2N	10	0%	Near tetraploid[c]	-
		Aneuploid				
41	3 / 7	-22 / -22	8	38%	Mos 4N/N/multiple aneuploidy (incl. **mon22** in 25%)/2N	+
42	1 / 8	3N / 3N	6	0%	Triploid	+
43 [d,e]	4 / 8	-**7**,-15, -18,-21,-**21** /-**7**,-15,-18,-21,-**21**	3	0%	**Mon7**, tris15,18, **mon21**	+[b]
44	2 / 8	+7, +13 / +7, +13	3	100%	Normal	-
		Abnormal/ normal mosaic				
45	2 / 8	-**18** / 2N	28	46%	Mos mon21/ **mon18**/mon7/2N	+
46	2 / 8	-**1**,-21/2N	9	22%	Mos mon18/ tris18/**mon1**,16/2N	+[b]
47	3 / 8	-16, -18 / 2N	10	0%	Triploid	-
48	2 / 7	-13/2N	11	73%	Normal	-
		Abnormal/ abnormal mosaic (multiple aneuploidies)				
49 [e]	2 / 8	+1 / +1, +15	19	0%	Tris 1	+
50 [d,e]	2 / 8	-7, -22/ -22	7	0%	Mos Mon 22/tris7,mon22	+
51 [e]	2 / 10	X0,-13,-18,-21,-21,-22/ X0,-7,-18,-21,-21,-22	10	0%	Mos multiple aneuploidy (same chromosome abnormalities involved)	+
52	1 / 8	-**18** /-7,+21,-13 -13,-16,-22	14	79%	Mos **mon18**/2N	+
53 [d]	3 / 8	XXY, -**15** / XXY, -**15**, +22	7	86%	Mos **mon 15**,Y0/2N (XY)	+[b]
54	1 / 10	XXY, -1 / **X0**, -21	20	70%	Mos tris1/**monX**/ 2N (XY)	+[b]

[a] Morphology score: 1 = excellent; 2 = good; 3 = average; 4 = poor quality

[b] Confirmation of at least one of the chromosome abnormalities observed on day 3 (in **bold** type).

[c] Near tetraploid = 92 ± chromosomes (ISCN, 1995)

[d] Cases with potential mitotic non-disjunction before the 8-cell stage, in which the reciprocal product was recovered on day 5

[e] These are cases where the abnormality or at least one of the abnormalities most likely resulted from a meiotic error

2N = normal copy number for the chromosomes investigated, Mos = mosaicism, mon = monosomy, tris = trisomy. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last (ISCN, 1995).

Table 2.3.3. Embryo development, FISH results and interpretation from embryos after analysis of one blastomere on day 3. (For a detailed presentation of the FISH results on day 5, see appendix I).

	Day 3			Day 5		
Case no.	Morpho-logy score[a]/ No. of cells	FISH results	# cells analysed	% of normal cells	Interpretation of FISH results	Confirmed
Embryos reaching the blastocyst stage						
		Normal				
55	4 / 5	2N	37	0%	Tetraploid	-
		Aneuploid				
56 [e]	2 / 7	-21	22	0%	Mon21	+
57 [e]	2 / 8	+16	47	0%	Tris16	+
58	3 / 6	+18	30	93%	Normal	-
59	4 / 6	-16	36	64%	Mos mon15/2N	-
60 [d]	1 / 8	+**7**	30	60%	Mos mon7/2N	-
		Double aneuploid				
61 [e]	1 / 12	-18, +**22**	86	5%	**Tris22**	+[b]
62 [e]	2 / 8	-16, -22	35	0%	Double mon16 and 22	+
63 [e]	2 / 7	-7, -**22**	57	0%	**Mon22**	+[b]
64 [d]	2 / 8	-7, -21	30	23%	Mos tris21/ tris7/ 2N	-
65 [d]	3 / 8	-15, +13	25	88%	Mos mon13,21/2N	-
		Multiple aneuploid				
66	2 / 8	X0, -16, -**21**	57	82%	Mos **mon21**/2N (XY)	+[b]
67	2 / 8	N, –1, +15, +15, -21	42	43%	Mos mon18/ 2N	-
Embryos arrested at day 3 or 4						
		Normal				
68	4 / 5	2N	12	100%	Normal	+
69	1 / 9	2N	18	77%	Mos mon22/2N	-
70	2 / 7	2N	10	90%	Mon21	-
71	4 / 6	2N	9	11%	Mos mon1/tris1/monX/trisX/2N	-
		Aneuploid				
72	3 / 6	-15	13	23%	Mos Multiple aneuploidy (incl. **mon15** in38% of cells)/2N	+
73	2 / 6	+15	10	30%	Mos triploidy/2N	-
74	3 / 6	+21	5	0%	Mos null1/ null1, monX, mon15	-
75	3 / 8	-13	6	83%	Mos mon16/2N	-
		Double aneuploid				
76 [d]	1 / 8	+22, -**16**	14	50%	Mos **mon16**/mon22/2N	+[b]
77 [e]	2 / 8	+15, -**22**	24	0%	Mos **mon22**/mon7, **22**	+[b]
78	2 / 7	-**1**, -7	15	67%	Mos **mon1**/2N	+[b]
79 [d]	1 / 8	X0, +13	24	63%	Mos mon13/2N (XX)	-

		Multiple aneuploid				
80	2 / 12	**N**, XX, +1, +15	5	0%	Mos **haploidy**/ tetraploidy	+[b]
81 [d]	3 / 8	XXX,+**13**, +16, -21	10	50%	Mos monX, **tris13**/mon7/2N	+[b]
82 [d,e]	3 / 9	**X0**,+7,+13,+13,+18,+22	1	0%	**X0**,-7,–15,-21,-22	+[b]
83	3 / 8	-1,-1,-13,-**16**,-21,-21,-22	3	33%	Mos **mon16**/2N	+[b]

[a] Morphology score: 1 = excellent; 2 = good; 3 = average; 4 = poor quality

[b] Confirmation of at least one of the chromosome abnormalities observed on day 3 (in **bold** type).

[d] Cases with potential mitotic non-disjunction before the 8-cell stage, in which the reciprocal product was recovered on day 5

[e] These are cases where the abnormality or at least one of the abnormalities most likely resulted from a meiotic error

2N = normal copy number for the chromosomes investigated, Mos = mosaicism, mon = monosomy, tris = trisomy. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last (ISCN, 1995).

Table 2.3.4. Overview of the diagnosis made on day 3 and rate of cytogenetic confirmation after reanalysis on day 5.

Diagnosis on day 3	Number of embryos reanalysed on day 5	Number of cases confirmed (%)
Based on two cells		
Normal	7	3 (43)
Aneuploid	11	9 (82)
Mosaic	36	18 (50)
Abnormal/ normal mosaic	14	6 (43)
Abnormal/ abnormal mosaic	22	12 (55)
Total	54	30 (56)
Based on one cell		
Normal	5	1 (20)
Aneuploid	24	14 (58)
Total	29	16 (55)
Overall confirmation rate	83	45 (54)

Values in parentheses are percentages

Section 2.4 FISH analysis of 15 chromosomes in human day 4 and 5 preimplantation embryos: the added value of extended aneuploidy detection

Introduction

The introduction of fluorescence in situ hybridization (FISH) for preimplantation genetic diagnosis has enabled screening of embryos for chromosomal aneuploidies before transfer in in vitro fertilization (IVF). Preimplantation genetic screening (PGS) (Thornhill et al.,2005) would be of special interest for couples that are thought to have a higher risk of developing chromosomally abnormal embryos, with the aim of improving embryo selection and thus their chances for implantation and an ongoing pregnancy. PGS analysis routinely involves the screening of the copy number of seven to ten chromosomes, mostly of those chromosomes that are involved in potentially viable trisomies (13, 18, 21 and the sex chromosomes) in combination with chromosomes often found to be trisomic in early miscarriages (such as 14, 15, 16 and 22) (Philipp et al., 2003). Although PGS is offered in many IVF centres around the world, a recent meta-analysis has questioned its efficiency (Twisk et al., 2006). This may partly be explained by the fact that the largest part of aneuploidies observed at this stage originate during the first mitotic divisions of early preimplantation development, resulting in chromosomally mosaic embryos (Coonen *et al.*, 2004; Daphnis *et al.*, 2005; Katz-Jaffe *et al.*, 2005, Baart *et al.*, 2006). Therefore, the biopsy and analysis of one or two cells may not be representative for the remaining embryo (Baart *et al.*, 2006). Another contributing factor may be that in preimplantation embryos, chromosome aberrations are involved which are not found later during development, as in spontaneous miscarriage or ongoing pregnancy (Bahçe *et al.*, 1999; Fritz *et al.*, 2001; Vouillaire *et al.*, 2000; Wells and Delhanty, 2000; Phillipp and Kalousek; 2002). Therefore, it has been proposed that the efficiency of PGS could be improved by analysing more chromosomes (Wilton et al., 2002).

We aimed to explore the added value of investigating more chromosomes for the detection of chromosomally abnormal embryos. Our approach was to investigate cryo-preserved, good quality day 4 and day 5 embryos donated for research purposes. Embryos were analyzed for the chromosomes recommended in the "best practice guidelines for PGS" (chromosomes 13, 14, 15, 16, 18, 21, 22, X and Y) (Thornhill *et al.*, 2005), next to chromosomes that are less well studied in preimplantation embryos (1, 2, 7, 6, 10 and 17). Analysis of these 15 chromosomes was performed in three consecutive FISH rounds and the proportion of aneuploid and mosaic embryos was determined. These results were compared in retrospect to what would have been observed if only the recommended probe set was analyzed.

Materials and methods

Patients and embryos

The spare cryo-preserved preimplantation embryos used in this study were donated by couples undergoing routine IVF procedures in the period between January 1993 and June 2000. Prior to commencing the study, ethical approval for the use of these embryos was received from the Dutch Central Committee on Research Involving Human Subjects (CCMO) and the local institutional ethics committee. Written consent was obtained from all participating couples.

Ovarian stimulation, oocyte retrieval and IVF procedures, including assessment of embryo morphology, were performed as described previously (Huisman et al., 2000). A maximum

of two embryos were replaced per cycle. Only good quality supernumerary embryos were cryopreserved. For day 4 embryos this means that they showed some degree of compaction and ‹ 20% fragmentation. Day 5 embryos were required to show cavitation and the presence of an inner cell mass. The cryopreservation procedure was performed in straws using a slow freezing 1.5 M DMSO protocol in a programmed freezer (De Jong *et al.*, 2002). For the present study, 29 frozen day 4 (compaction stage) and 48 day 5 (blastocyst stage) embryos were thawed. These embryos were obtained from 29 patients (median maternal age at oocyte retrieval: 32.7 (range 24-41 years). On average 1.8 embryos were donated per patient (range 1-4). Embryos had been stored for a median period of 8.2 (range 5.0-12.5 years). All donated embryos happened to be from patients who became pregnant after transfer of two embryos in the cycle where the embryos were frozen.

Fixation of embryos

Previous reports have indicated a high incidence of chromosomal abnormalities after freezing, thawing and subsequent culture of embryos (Iwarsson *et al.*, 1999; Baart *et al.*, 2004b; Salumets *et al.*, 2004). To avoid an artificial increase of chromosome abnormalities, cryopreserved embryos were fixed within 10 minutes after removal from the liquid nitrogen. Frozen-thawed embryos were fixed as described previously (Dozortsev and McGinnis, 2001), with some modifications (Baart *et al.*, 2004a). In short, embryos were transferred to a drop of spreading solution (0.01 N HCl/ 0.1% Tween 20; Coonen *et al.*, 1994; Harper *et al.*, 1994) to dissolve the zona pellucida, and subsequently transferred to a slide in a small amount (~1µl) of spreading solution. Blastomere nuclei were isolated on the slide and subsequently air-dried, followed by fixation with a drop of freshly made fixative (methanol:acetic acid, 3:1).

Labelling of DNA probes

The probes used in the first round of FISH were centromere probes for chromosome 2 (pX2, Rocchi *et al.*, 1990), 6 (D6Z1, Jabs *et al.*, 1984), 10 (pH8, Devilee *et al.*, 1988) and 17 (p17H8, Waye and Willard., 1986) and a locus specific probe for chromosome 14q13.3 (RP11-262O7, UCSC Genome Bioinformatics). The probes for 2, 14 and 17 were labelled using a random primed labelling kit (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. The probes for chromosomes 6 and 10 were labelled using nick translation as described previously (Baart *et al.*, 2004a).

The DNA probes used in the second round were centromere probes for chromosomes 1 (pUC 1.77; Cooke and Hindley, 1979), 7 (pα7t1; Waye *et al.*, 1987), 15 (pTRA-20; Choo *et al.*, 1990), X (pBamX5; Willard *et al.*, 1983) and a Y chromosome heterochromatin probe (RPN1305X; Lau, 1985). Labeling of these probes was performed as described previously (Baart *et al.*, 2004a). The DNA probes used in the third round were centromere probes for chromosome 16 and 18, combined with LSI probes for chromosomes 13, 21, and 22 (Multivysion PB kit, Vysis, Downers Grove, IL, USA).

The different fluorochromes used for labeling each of the 15 chromosome specific probes and the corresponding filter sets (Chroma Technology Corp., Rockingham, Vermont, USA) used for detection are presented in Table 2.4.1. Fluorochromes were from Molecular Probes, Invitrogen, Leiden, Netherlands, except for Coumarin-13-dUTP (Enzo Life Sciences, Inc., New York, USA) and Diethylaminocoumarine-5-dUTP (NEN Life Science Products, Inc., Boston, USA).

FISH procedure

Three rounds of five-colour FISH were applied to the embryos. The first round of FISH was performed for chromosomes 2, 6, 10, 14 and 17. A hybridization mixture was made, containing 10 ng of each labelled probe in 50% formamide, 10% dextran sulphate, 1% Tween 20 and 1 µg Human Cot-1 DNA (Invitrogen, Carlsbad, CA, USA) in 2×SSC, in a final volume of 10 µl. For the second and third round, hybridisation mixtures were prepared as described previously (Baart et al, 2004b). Per slide, 0.25 µl hybridization mixture was applied under a 4 mm round coverslip. Blastomere- and probe DNA were denatured simultaneously at 75°C for 3 min and allowed to hybridize in a humid box (containing 50% formamide in 2×SSC, pH 7.2) at 37°C for 2 h. After hybridization, coverslips were removed and slides were washed in 2×SSC/0.05% Tween 20 for 2 min at 42°C, 0.4×SSC for 6 min at 60°C and in 2×SSC/0.05% Tween 20 for 2 min at room temperature. The slides were rinsed once in PBS, allowed to air dry and mounted in Vectashield antifade medium (Vector Laboratories, Burlingame, CA, USA), containing 1.25 ng/ml 4',6-diamidino-2-phenylindole (DAPI). After analysis of the fluorescent signals, coverslips and antifade medium were removed with two washes in 2×SSC/0.05% Tween 20 for 5 min at room temperature. The slides were dehydrated and the probemix for the second round (1, 7, 15, X and Y) was applied. Nuclear and probe DNA were denatured and allowed to hybridize for 2 hrs at 37°C. The post-hybridization wash consisted of 2×SSC/0.05% Tween 20 for 2 min at 42°C, 6 min in 0.1×SSC at 60 °C and 2 min in 2×SSC at room temperature. After a brief wash in PBS, the slides were allowed to air dry and mounted in antifade medium containing DAPI. After analysis of the fluorescent signals, coverslips were again removed and the third round (13, 16, 18, 21 and 22) of hybridization was performed at 37°C for 2 hrs. The third post-hybridization wash consisted of 2×SSC/0.05% Tween 20 for 2 min at 42°C, 6 min in 0.1×SSC at 60°C and 2 min in 2×SSC at room temperature. Slides were mounted in Vectashield antifade medium, without DAPI.

The efficiency of the FISH probes was tested on uncultured peripheral lymphocyte spreads from two men and two women with normal karyotypes. Slides were prepared according to standard protocols and they were hybridized using the same three-round FISH protocol as for the embryonic cells (see above). Signals were counted in 200 nuclei. In addition, the positions of 16 individual nuclei were recorded and images were obtained after each round to check for persisting signals.

Microscopy and interpretation of FISH results

Slides were examined with a Zeiss Axioplan 2 imaging epifluorescence microscope, equipped with appropiate filters (see table 2.4.1). Images were captured with the ISIS FISH Imaging System (MetaSystems, Altlussheim, Germany). A detailed map was drawn for each embryo, allowing identification of each fixed nucleus. For enumeration of the signals, we used scoring criteria previously published (Munné *et al*., 1998b). Interpretation of the FISH results was done according to the definitions published previously, with some modifications (Baart *et al*., 2004b). After analysis, we classified the embryos as normal (approx. 80% normal nuclei and, more importantly, less than 10% of the nuclei with the same chromosome abnormality), aneuploid (at least 90% of the nuclei showing the same abnormality) or mosaic (more than 10% and less than 90% of the cells showing the same chromosome abnormality). Mosaics were classified as diploid mosaic if a normal cell line was present and as aneuploid mosaic if only abnormal cells were present. Embryos where all or a majority of nuclei showed a randomly different chromosome complement were defined as chaotic. Embryos with 90% or more haploid, tetraploid or triploid nuclei were classed as such. However, we considered the occurrence of some tetraploid cells as

a normal phenomenon of *in vitro* cultured embryos (Evsikov and Verlinsky, 1998; Bielanska *et al*., 2002; Coonen *et al*., 2004) and treated them as normal cells. If both monosomic and trisomic cells for the same chromosome were present in the embryo, it was considered to be the result of a mitotic non-disjunction event. If at least 90% of the cells showed the same abnormality, even next to other abnormalities, it was considered to be a meiotic abnormality.

Classification of embryos was first done according to FISH results of all 15 chromosomes and was then repeated by taking into account only the results from chromosomes 13, 14, 15, 16, 18, 21, 22, X and Y. Resulting percentages of normal embryos were compared.

Results

Efficiency of three consecutive FISH rounds

Initially, the hybridization of the probe set containing probes for chromosomes 2, 6, 10, 14 and 17 was performed as a third round. However, the commercial probemix (chromosomes 13, 16, 18, 21 and 22) produces a highly persistent background staining of DNA, hampering analysis of subsequent FISH rounds. With the order of FISH rounds as described here, this was not a problem. All 15 probes were tested for hybridization efficiency after consecutive rounds of FISH on lymphocyte spreads from two males and two females in 200 nuclei. The hybridization efficiencies for the individual probes were calculated as the percentage of nuclei showing the expected number of signals and ranged between 95 and 99%. Furthermore, 85%, 85% and 90% of the nuclei showed the normal amount of signals for all five probes in the first, second and third round, respectively. The recorded images showed no major persisting signals from previous rounds, although in some nuclei a remnant of chromosome 17-signal was observed in the second round. Three consecutive rounds of FISH could also be successfully applied to embryonic nuclei, with reliable signals and good nuclear morphology (Figure 2.4.1, Appendix). The procedure, including microscopic examination, could be completed within two working days.

Analysis of cryopreserved embryos for 15 chromosomes

A total of 52 embryos were successfully fixed and analysed for three rounds of FISH, immediately upon thawing (Table 2.4.2). Success rates were forty out of 48 (83%) for day 5 embryos and 12 out of 29 (41%) for day 4 embryos. From the embryos cryopreserved as good quality blastocysts, ten (25%) were found to be chromosomally normal according to our criteria. Two (5%) were found to be uniformly abnormal (monosomy 21 and triploidy), 26 (65%) were diploid mosaic and 2 (5%) were aneuploid mosaic. The embryos frozen as compacted morulas were all found to be mosaic, seven (58%) were diploid mosaic and five (42%) were aneuploid mosaic, from which two were chaotic.

In five out of 52 (10%) cryo-embryos, a chromosome abnormality was involved that most likely originated during meiosis (monosomy for chromosomes 7, 21, X and 22 and trisomy for chromosome 22) (Table 2.4.2 and 2.4.3). Chromosome abnormalities resulting from a mitotic error were detected in 40 (77%) embryos and in three of these both products of a non-disjunction event were recovered (Table 2.4.2).

Added value of the extra FISH round for the detection of chromosome abnormalities

The incidence of the investigated chromosomes to be involved in aneuploidy is listed in Table 2.4.3. Multiple aneuploidies were considered to be the result of independent events and the resulting cell line was taken into account for each chromosome involved. Aneuploidy was detected for all

chromosomes investigated, except for chromosomes 10 and 13. Aneuploidy for chromosomes 2 and 18 were most frequently encountered.

If the analysis would have been limited to only the recommended probe set for aneuploidy screening (13, 14, 15, 16, 18, 21, 22, X and Y), aneuploid cell lines or cell lines with multiple aneuploidy (i.e. chromosome aberrations of mitotic origin) would have been missed in 25 embryos. One abnormality arising from a meiotic error (monosomy 7) would have been missed in an otherwise mosaic embryo (Table 2.4.2, embryo 48). However, only seven embryos would have been misclassified as normal, instead of diploid mosaic (Figure 2.4.2 and Table 2.4.2). These included five embryos that were found to be diploid mosaic for monosomy or trisomy 2. Furthermore, the diagnosis in embryo 50 would change from aneuploid mosaic into 100% aneuploid (trisomy 22).

FISH analysis of 15 chromosomes showed that 81% (42 out of 52) of the embryos were abnormal, whereas this would have been 67% (35 out of 52) if only the nine recommended chromosomes were investigated (χ^2; P = 0.03) (Figure 2.4.2).

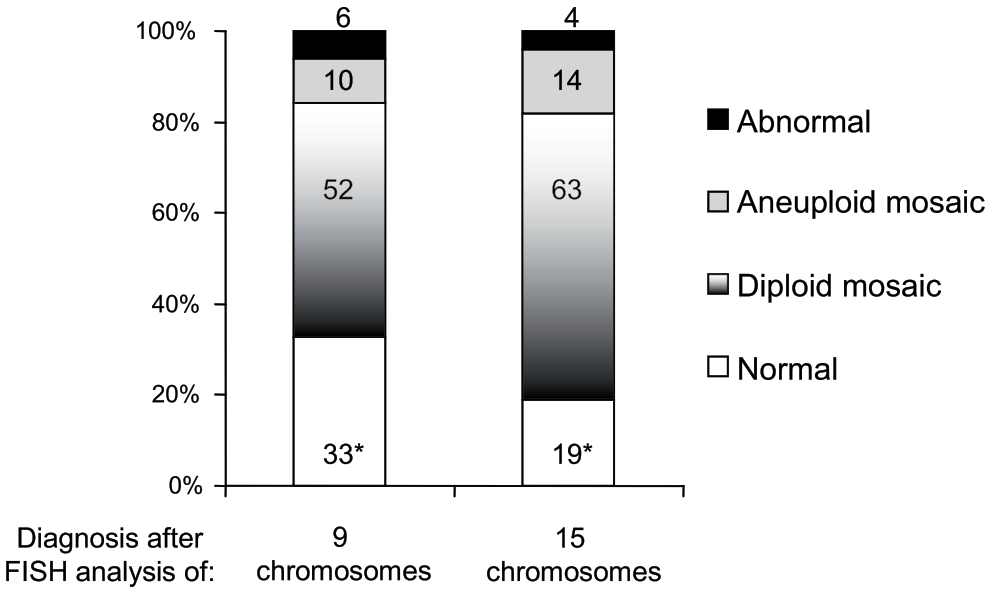

Figure 2.4.2. Cytogenetic diagnosis of day 4 and day 5 embryos (N = 52) after cryopreservation. Diagnosis of the embryo after the analysis of the recommended probe set for aneuploidy screening (13, 14, 15, 16, 18, 21, 22, X and Y) is compared to the results after analysis of 15 chromosomes (1, 2, 6, 7, 10, 13, 14, 15, 16, 17, 18, 21, 22, X and Y). * P = 0.03.

Discussion

Multicolor FISH analysis in three rounds was performed successfully on nuclei from human day 4 and 5 preimplantation embryos, allowing the analysis of the copy number of a total of 15 chromosomes (1, 2, 6, 7, 10, 13, 14, 15, 16, 17, 18, 21, 22, X and Y). Comparative genomic hybridization (CGH) allows the analysis of all chromosomes, but single cell CGH analysis of day 4 or day 5

embryos is hardly feasible due to the large number of cells. Although leaving nine chromosomes uninvestigated, FISH analysis of 15 chromosomes in three consecutive rounds was shown here to be a more practical approach, especially since it can be completed within two working days. Moreover, the successful analysis of blastomeres with minimal loss of hybridization efficiency using two or three probes at a time during five sequential hybridizations has been described previously (Harrison *et al.*, 2000). Therefore, analyzing all chromosomes in five sequential rounds of FISH could represent an alternative to CGH.

Studies concerning aneuploidy in human preimplantation embryos are always hampered by the scarcity of study material, due to obvious ethical concerns. In this study, we were able to use good quality embryos after cryopreservation, donated for research. Although this was not an inclusion criterion, all embryos donated proved to be surplus in an IVF cycle where the woman became pregnant from the fresh transfer of two embryos. This may result in patient selection and consequently, embryo selection. Those women that became pregnant after IVF treatment may be capable of producing better quality embryos. On the other hand, it is also feasible that the best quality embryos were selected for transfer and only embryos with chromosome abnormalities were left. Furthermore, it has been suggested that the cryopreservation process may induce chromosomal abnormalities after thawing and subsequent culture (Iwarsson *et al.*, 1999; Baart *et al.*, 2004b; Salumets *et al.*, 2004). To prevent any changes, embryos were fixed immediately upon thawing. In a previous study (Baart *et al.*, 2004b) cryopreserved embryos were thawed and cultured for at least 2 hours before processing, with the result that only a quarter of the embryos survived. Immediate fixation allowed 68% (52/77) of the embryos to survive and be successfully analyzed, further reducing the chances for a selection bias.

In 10% of the embryos, an abnormality resulting from a meiotic error was found. This corresponds well with the reported aneuploidy rate of 11% in a large set of unfertilized oocytes after IVF (Pellestor *et al.*, 2002). However, the rate of mitotic errors resulting in mosaic embryos was found to be much higher. In this group of good quality frozen-thawed morulas and blastocysts, the proportion of mosaic embryos was found to be 77%. Reported rates of mosaicism vary between 15% to 93%, depending on the number of chromosomes analyzed, quality and developmental stage of the embryo and how mosaic embryos were defined (Ruangvutilert *et al.*, 2000; Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Sandalinas *et al.*, 2001; Bielanska *et al.*, 2002; Baart *et al.*, 2004b; Daphnis *et al.*, 2005; Baart *et al.*, 2006). The definition used in the present study is based on criteria used in cytogenetic laboratories for the interpretation of interphase FISH results to distinguish between low-level mosaicism and FISH artifacts, if control data on the same tissue is not available.

Analysis of chromosomes 1, 2, 6, 7, 10 and 17 allowed us to detect abnormalities arising from a mitotic error in 25 embryos, although only seven of these embryos would have been misdiagnosed as being normal if only the nine recommended chromosomes were investigated. This is due to the presence of multiple abnormal cell lines and cell lines carrying multiple aneuploidy in more than half of the mosaic embryos. One meiotic chromosome aberration (monosomy 7) was detected in an embryo that otherwise would have been classified as diploid mosaic. By screening six more chromosomes in addition to the chromosomes recommended for PGS, the proportion of aneuploid and mosaic embryos was increased from 67% to 81% (χ^2; P = 0.03). This increase was mainly due to an increased detection of diploid mosaic embryos. Reassuringly, previous studies performing comparative genome hybridization (CGH) to analyze all chromosomes in blastomeres from good quality cleavage stage embryos, still reported 25% normal embryos (Voullaire *et al.*, 2000; Wells and Delhanty, 2000).

Cytogenetically, screening more chromosomes will have an added value for PGS by increasing the chances for detection of abnormal embryos. However, from a clinical viewpoint the added value is more difficult to determine. The investigation of six extra chromosomes besides the ones that are recommended for PGS, allowed the detection of mainly chromosome aberrations of mitotic origin leading to mosaicism. These embryos are capable of developing into good quality blastocysts, but little is known about their implantation potential and ability to develop into a healthy child. This will probably depend on the level of mosaicism, the type of chromosome aberration and the chromosome involved. Embryos mosaic for trisomy 21 appear to be less viable than normal embryos (Katz-Jaffe *et al.*, 2005), but for other chromosomes this is unknown. More research is needed to determine if some mosaic embryos can be considered suitable for transfer depending on the type of aneuploidy and the chromosome involved. Until this is resolved, screening of more chromosomes may result in embryos being discarded which may result in a healthy child.

Interestingly, we found a high incidence of mitotic errors for chromosome 2. Two earlier studies performing analysis of all the chromosomes in eight-cell embryos by CGH, both reported embryos with nuclei monosomic or trisomic for chromosome 2 (Voullaire *et al.*, 2000; Wells and Delhanty, 2000). Furthermore, they also reported cells with post-zygotic breakage of chromosome 2. Together, these data may indicate chromosome 2 to be frequently involved in mitotic errors, although the number of embryos is currently too small to draw any significant conclusions.

In conclusion, aneuploidy in human preimplantation embryos originates from both meiotic and, to a much greater extent, mitotic errors. Although all but one of the meiotic aneuploidies found in this study would have been detected by probe sets most frequently used in PGS clinics, we detected aneuploid cell lines originating from mitotic errors for all chromosomes, except chromosomes 10 and 13. We showed that screening of six additional chromosomes next to the nine recommended chromosomes increases the proportion of mosaic embryos. The clinical consequences remain undetermined as long as the fate of mosaic embryos after transfer is unclear. Therefore, more research into this fate is needed to make PGS more effective.

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Table 2.4.1. Overview of the fluorochromes used in each round of FISH and the filter sets used for detection.

1st FISH round		2nd FISH round		3rd FISH round		
Chromosome	Fluorochrome	Chromosome	Fluorochrome	Chromosome	Fluorochrome	Filterset
14	Alexa Fluor 594	X	Alexa Fluor 594	13	Spectrum Red	Red filter set, ID 31046
10	Spectrum Green	15	Alexa Fluor 488	21	Spectrum Green	R and B phycoerythrin Hq light source, ID 31003
2	Alexa Fluor 555	Y	Alexa Fluor 555	22	Spectrum Gold	Yellow GFP BP filter set, ID 41028
17	Coumarin-13-dUTP	1	Alexa Fluor 350	18	Spectrum Blue	DAPI/ Hoechst/ AMCA filter set, ID 31000
6	Diethylamino-coumarin-5-dUTP	7	Pacific Blue	16	Spectrum Aqua	Spectrum Aqua filter set, ID 0415-834

Table 2.4.2. FISH results from chromosomes 1,2,6,7,10,13,14,15,16,17,18,21,22,XY and interpretation of embryos after cryopreservation and immediate fixation on thawing

Embryonr.	Patientnr.	#cells analysed	% of normal cells	Interpretation of FISH results [no. of cells]
Embryos cryopreserved at the blastocyst stage (day 5)				
Normal				
1	1	122	93%	Normal (2N,XX) [114]
2	2	50	86%	Normal (2N,XY) [43]
3	3	31	81%	Normal (2N,XX) [25]
4	4	60	78%	Normal (2N,XX) [47]
5	5	100	78%	Normal (2N,XX) [78]
6	6	70	77%	Normal (2N,XY) [54]
7	1	101	75%	Normal (2N,XY) [76]
8	7	38	74%	Normal (2N,XX) [28]
9	8	60	70%	Normal (2N,XX) [42]
10	9	24	63%	Normal (2N,XX) [15]
Aneuploid or polyploid				
11 [d]	2	40	0%	Monosomy 21 [40]
12	10	30	0%	Triploidy [30]
Diploid mosaic				
13	11	18	72%	Mos tris2[2]/mon16[2]/2N,XX[13]
14	12	60	70%	Mos tris6,22[8]/2N,XX[42]
15 [b]	1	98	69%	Mos mon2[10]/2N,XY[68]
16	10	47	68%	Mos tris18[6]/2N,XY[32]
17 [b]	13	25	68%	Mos mon2[3]/2N,XX[17]
18	14	24	67%	Mos mon18[7]/2N,XY[16]
19 [b]	15	38	66%	Mos mon6[5]/2N,XY[25]
20 [b]	16	49	65%	Mos tris2[11]/2N,XX[32]
21	1	26	65%	Mos near 4N[a][3]/2N,XX [20]
22	3	57	65%	Mos near 4N[a] [11]/2N,XY[37]
23	11	46	63%	Mos tris15[6]/2N,XY[29]
24	14	19	63%	Mos mon18[3]/mon17[2]/2N,XX[12]
25	8	18	61%	Mos mon16[3]/mon15[2]/2N,XX[11]
26	5	53	60%	Mos mon15[6]/2N,XX[32]
27	2	65	58%	Mos tris18[15]/2N,XY[38]
28 [b, c]	5	34	56%	Mos tris**2**[9]/mon**2**[4]/2N,XY[19]
29	17	38	50%	Mos near 4N[a] [11]/tris15[4]/2N,XX[19]
30 [c]	18	20	50%	Mos mon**18**[3]/mon16[3]/tris**18**[2]/2N,XY[10]
31	19	22	45%	Mos multiple aneuploidy[9]/2N,XY[10]
32	19	15	40%	Mos mon16[4]/mon2[2]/mon17[2]/2N,XX[6]
33	20	18	39%	Mos near 4N[a] [7]/2N,XY[7]

34	9	38	39%	Mos tris18[7]/mon22[5]/2N,XY[15]
35	18	17	35%	Mos tris18[3]/tris2[2]/near 4N[a] [3]/2N,XX[6]
36	15	32	31%	Mos tris15[14]/monX[8]/2N,XX[10]
37 [b,c]	19	30	30%	Mos tris**1**[8]/mon**1**[4]/2N,XY[9]
38	20	18	22%	Mos mon2[5]/tris7[4]/tris7,16[2]/2N,XY[4]
Aneuploid mosaic				
39	21	8	0%	Mos near haploid[6]/tetraploid[1]/triploid[1]
40 [d]	20	20	0%	Mos monX[11]/monX,21[3]/monX,22[3]
Embryos cryopreserved at the compaction stage (day 4)				
Diploid mosaic				
41	22	20	60%	Mos monX [2]/mon2[2]/2N,XX[12]
42 b	23	14	57%	Mos mon2[2]/2N,XY[8]
43	24	8	50%	Mos tris1[2]/tris1,mon21[2]/2N,XY[4]
44	25	21	48%	Mos tris18[7]/mon15,tris18[4]/2N,XX[10]
45	22	22	41%	Mos mon18[10]/2N,XX[9]
46	26	23	22%	Mos monX[8]/monX,tris2[7]/2N,XY[5]
47	27	23	22%	Mos mon14[12]/2N,XX[5]
Aneuploid mosaic				
48 [d]	22	14	0%	Mos mon7,tris16[8]/mon7[5]
49 [d]	24	11	0%	Mos mon22[4]/mon2,22[4]/mon2,14,22[3]
50 [d]	28	13	0%	Mos tris22[6]/mon2,tris22[6]
51	29	5	0%	Chaotic
52	25	8	0%	Chaotic

[a] Near tetraploid = 92 ± chromosomes (ISCN, 1995)

[b] These embryos would have been classified as normal if only the recommended probe set (13, 14, 15, 16, 18, 21, 22, X and Y) would have been analyzed.

[c] Cases with potential mitotic non-disjunction for the chromosome in **bold**

[d] These are cases where the abnormality or at least one of the abnormalities most likely resulted from a meiotic error

2N=normal copy number for the chromosomes investigated, Mos=mosaicism, mon=monosomy, tris=trisomy. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last (ISCN, 1995).

Table 2.4.3. Number of embryos with an aneuploid cell line for each chromosome investigated, after analysis of 52 cryopreserved embryos.

		Origin				
		Meiotic		Mitotic		Total no. of embryos with aneuploid cell lines
		-	+	-	+	
Recommended probe set	13					0
	14			2 (5)		2
	15			3 (12)	3 (24)	6
	16			4 (12)	2 (10)	6
	18			4 (23)	6 (44)	10
	21	1 (40)		2 (5)		3
	22	1 (11)	1 (13)	2 (8)	1 (8)	5
	XY	1 (17)[a]		3 (25)[a]		4
Additional probes	1			1 (4)	2 (12)	3
	2			9 (42)	5 (31)	14
	6			1 (5)	1 (8)	2
	7	1 (14)			1 (6)	2
	10					0
	17			3 (17)		3

If at least 90% of the cells showed the same abnormality, it was considered to be a meiotic abnormality. Otherwise, the abnormality was considered mitotic. Values are expressed as numbers of embryos with an aneuploid cell line; values between parentheses represent the number of cells with the involved chromosome aberration.

- = monosomies; + = trisomies.

[a] Cells with XO

Chapter 3

Clinical implications of PGS

Section 3.1 Milder ovarian stimulation for in vitro fertilization reduces aneuploidy in the human preimplantation embryo

Introduction

Human reproduction is a relatively inefficient process (Norwitz *et al.*, 2001). The chance of achieving a spontaneous pregnancy after timed intercourse is 20-30% (Evers, 2002; Taylor, 2003), significantly lower than ~70% in the rhesus monkey (Ghosh *et al.*, 1997), 80% in captive baboons (Stevens, 1997) or 90% in rodents and rabbits (Foote and Carney, 1988). Moreover, up to 30% of early human embryos fail to develop into viable fetuses (Wilcox *et al.*, 1988), largely due to chromosomal abnormalities (Vorsanova *et al.*, 2005; Boue *et al.*, 1975). The incidence of embryo aneuploidy increases with maternal age (Hassold and Hunt, 2001).

In vitro fertilization (IVF) is the major treatment strategy for infertility, employing complex and costly ovarian stimulation protocols to generate multiple embryos (Fauser *et al.*, 2005; Macklon *et al.*, 2006). After ovarian hyperstimulation and *in vitro* fertilization, the best quality embryos are selected for transfer into the uterine cavity. Although embryo morphology is widely used to evaluate embryo quality, this subjective method provides only limited information concerning the chromosomal constitution (Munné, 2006). The introduction of fluorescence in situ hybridization (FISH) on interphase nuclei allowed the screening of embryos for chromosomal aneuploidies, a procedure referred to as preimplantation genetic screening (PGS) (Thornhill *et al.*, 2005). Clinically, PGS is being advocated for older women (Munné *et al.*, 2003; Staessen *et al.*, 2004) and patients with recurrent miscarriage or repeated implantation failure (Gianaroli *et al.*, 2003; Pehlivan *et al.*, 2003; Platteau *et al.*, 2005). High rates of aneuploidy have been reported in these women. Moreover, in studies where the entire embryo was analyzed, a high incidence of chromosomal mosaicism has been observed (Delhanty *et al.*, 1993; Bielanska *et al.*, 2002). The frequent occurrence of mosaicism, resulting from mitotic segregation errors (Delhanty, 1997), is also reflected in the high incidence of discordant FISH results when two blastomeres are analyzed by PGS (Baart *et al.*, 2004b; Baart *et al.*, 2006).

The mechanisms underlying aneuploidy are still poorly understood. However, recent observations suggest that inaccuracies of the chromosome segregation machinery in oocytes are often involved, a process influenced by maternal age (Champion and Hawley, 2002; Hassold and Hunt, 2001). Preliminary indications suggest that aneuploidy in embryos may also be affected by ovarian stimulation regimens employed in IVF (Munné *et al.*, 1997; Katz-Jaffe *et al.*, 2005). The conventional IVF regimens routinely use a gonadotropin-releasing hormone (GnRH) agonist co-treatment to prevent premature luteinization by suppressing pituitary releases of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Down-regulation of pituitary function takes around two weeks, after which high doses of exogenous FSH are administered to induce multiple follicle growth, resulting in high numbers of oocytes for retrieval. The recent availability of GnRH antagonists has enabled the development of novel approaches in ovarian hyperstimulation. To prevent an LH rise, the administration of a GnRH antagonist can be limited to the mid-to-late follicular phase (Fauser and Devroey, 2005), allowing the endogenous inter-cycle FSH rise to be utilized for follicle stimulation. Therefore, cyclic follicle recruitment and initial stages of dominant follicle selection can proceed within the natural cycle and exogenous FSH for inducing multiple follicle development need only be administered during the mid-late follicular phase (Fauser and Van Heusden, 1997; Fauser *et al.*, 1999; Hohmann *et al.*, 2003).

To test whether the conventional ovarian hyperstimulation protocol and the new mild

stimulation approach differentially affect the competence of oocytes and embryos for proper chromosome segregation, PGS was employed in a prospective randomized controlled trial in a group of IVF patients younger than 38 years of age.

Methods

Study design

All patients were recruited from an outpatient setting at the Erasmus Medical Center and the Medical Center "Rijnmond Zuid" from December 2002, to August 2005 and randomly assigned to undergo a mild stimulation regimen using gonadotropin-releasing hormone (GnRH) antagonist co-treatment, or a conventional high dose exogenous gonadotropin regimen and GnRH agonist co-treatment. A schematic representation of the study is outlined (Figure 3.1.1). A population of infertile couples with a regular indication for IVF was targeted, who were not at an *a priori* increased risk for chromosomally abnormal embryos. Only women below 38 years of age, with a regular indication for IVF and with a partner with a sperm count › 5 million progressively motile sperm per milliliter were invited to participate in the study. Additional inclusion criteria were: history of regular menstrual cycles (ranging from 25-35 d), body mass index between 19-29 kg/m^2, no known chromosomal abnormalities, no relevant systemic disease or uterine and ovarian abnormalities, no history of recurrent miscarriage, and no previous IVF cycles not resulting in an embryo transfer. Couples could participate in the study for one cycle only. Prior to commencing the study, ethical approval was received from the Dutch Central Committee on Research Involving Human Subjects (CCMO) and the local institutional ethics committee. Written informed consent was obtained from each couple.

Based on previous experience (Hohmann *et al.*, 2003), a higher cancellation rate before oocyte retrieval and less embryos were expected following mild ovarian stimulation. Therefore, randomization to one of the two treatment groups was performed according to a computer-generated randomization schedule in a ratio of 4:6 (conventional group: mild group; see statistical paragraph), assigned via numbered sealed envelopes. The next available numbered envelope on entry into the study was opened by the treating physician during the preparatory IVF consultation. Blood samples were drawn from each patient on cycle day 3 or 4 before the start of stimulation, to assess baseline FSH and inhibin B levels.

Multifollicular ovarian stimulation

Patients randomized to undergo conventional ovarian stimulation were treated for at least two weeks with the GnRH agonist Triptorelin (Decapeptyl®, Ferring BV, Hoofddorp, The Netherlands) 0.1 mg/day sc, starting one week before the expected menses (usually cycle day 21), to prevent a premature LH surge. Following pituitary down regulation, ovarian stimulation was commenced with a fixed daily dose of 225 IU sc recombinant FSH (Puregon®, NV Organon, Oss, The Netherlands). Patients randomized to the mild stimulation protocol were treated with a fixed dose of 150 IU recFSH sc (Puregon®) starting on cycle day 5. GnRH antagonist co-treatment (Orgalutran®, NV Organon; 0.25 mg/day sc) was initiated on the day the leading follicle reached a diameter of 14 mm, as published previously (Hohmann *et al.*, 2003). To induce final oocyte maturation, a single dose of 10,000 IU hCG sc (Pregnyl®, NV Organon) was administrated as soon as the leading follicle had reached a diameter of 18 mm and at least one additional follicle had reached a diameter of 15 mm. Oocyte retrieval was carried out 35 hours after hCG injection by transvaginal ultrasound-guided puncture of follicles.

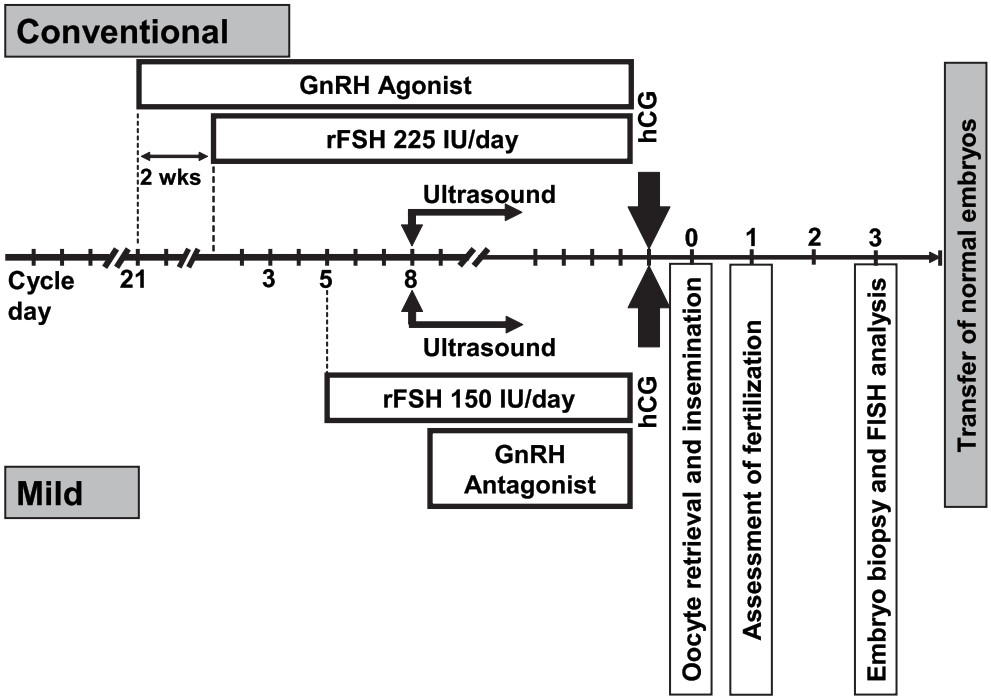

Figure 3.1.1. Schematic representation of the two ovarian stimulation protocols for IVF and laboratory procedures for PGS.

In vitro fertilization, embryo culture and biopsy

After oocyte retrieval, in vitro fertilization and embryo culture were performed as described previously (Hohmann *et al.*, 2003; Huisman *et al.*, 2000). On day 3 after oocyte retrieval, embryos resulting from normally fertilized oocytes (as evidenced by two visible pronuclei) were scored according to previously published morphological criteria (Hohmann *et al.*, 2003), blinded to the stimulation protocol. These included cell number, regularity of blastomeres, fragmentation and morphological aspects including granulation. Normal morphology was defined as embryos with timely development, ‹ 20% fragmentation, about equally sized blastomeres and small or no irregularities observed in the cytoplasm. Embryos with › 5 blastomeres were biopsied and one blastomere was removed if the embryo consisted of six blastomeres. Otherwise, two cells were removed. Embryo biopsy and fixation of biopsied cells was performed as described previously (Baart *et al.*, 2004a, 2004b).

FISH analysis and diagnosis

Fluorescent in-situ hybridization (FISH) analysis was performed to determine the copy number of ten chromosomes (1, 7, 13, 15, 16, 18, 21, 22, X and Y), as described (Baart *et al.*, 2004a, 2004b). FISH results were interpreted by two independent observers, blinded to the stimulation protocol. For enumeration of the signals on single blastomere nuclei, we used scoring criteria previously published (Munné *et al.*, 1998b). A nucleus was considered normal, if it showed the normal (diploid) amount of signals for the chromosomes investigated and abnormal if one or more of the chromosomes investigated showed more or less signals than expected. If one cell was available for diagnosis, the embryo was classified as normal or abnormal on the basis of the FISH result. In case two cells

were available, embryos were classified as normal (both nuclei normal FISH results), uniformly abnormal (both nuclei showing the same abnormality), or mosaic (one normal nucleus and one abnormal or two abnormal nuclei with each nucleus showing one or more different chromosome abnormalities). No more than two normal embryos were transferred to the patient.

As a result of chromosomal mosaicism, the definition of an abnormal embryo is different if one cell is available for analysis compared to two available cells. Also, embryos where only one cell could be biopsied differ developmentally from embryos where a two-cell biopsy was possible. To obtain uniformity for statistical analysis, we used two approaches. First, all embryos were classified in retrospect as either normal or abnormal on the basis of the FISH results obtained from the first biopsied blastomere, even if two cells were available. Second, the analysis was repeated for only those embryos with a PGS diagnosis based on two cells.

Outcome measures

Primary outcome measures were ovarian response, as assessed by the number of oocytes obtained and the proportion of chromosomally abnormal embryos per patient. This was expressed as the ratio of abnormal embryos on the number of embryos diagnosed per patient. Secondary outcome measures were the proportion of fertilized oocytes, the proportion of embryos with normal morphology and the proportion of embryos biopsied and diagnosed. All proportions were first calculated per patient and then averaged for each treatment group. As women were randomly assigned to two different ovarian stimulation protocols to detect possible differences in chromosomal abnormality rates of embryos generated, this is the correct unit of statistical analysis.

Statistical analysis

Before commencing the study, the sample size was determined. We assumed a reduction in the aneuploidy rate from 30% after conventional ovarian stimulation, to 20% after mild ovarian stimulation. We calculated that 293 embryos in each group would achieve an 80% power to detect this 10% difference at an alpha level of 0.05 with the use of a two-sided t-test. With an expected average of six embryos following conventional and four following mild ovarian stimulation and an expected drop out rate of one-third of the patients from each group, the total number of subjects to be included was 73 patients in the conventional group and 109 patients in the mild group. However, due to increasing concern regarding the safety of a two cell biopsy with respect to the implantation potential of the embryo (Cohen and Munné, 2005), an interim analysis was performed after the inclusion of 111 patients. The proportion of chromosomally abnormal embryos per patient was found to be significantly reduced after mild ovarian stimulation ($P = 0.02$), and therefore, the study was terminated.

A chi-square test was used to test for differences between the two groups in the percentage of oocyte retrieval and percentage of biopsies. A t-test was used to test for differences in continuous variables and parameters that were -per patient- averaged over oocytes or over embryos, e.g. the average morphology score of the embryos or the percentages of abnormal embryos. P-values < 0.05 were considered statistically significant.

Results

Patient and study characteristics

One-hundred and eleven patients were included. Initial screening characteristics (median and range) for all patients were: female age 33.7 (range 22-37) years, early follicular phase (cycle

day 3) FSH levels 7.5 IU/ l (range 1.3 – 18) and inhibin B levels 87 ng/ l (range 47 - 1056). There were no significant differences between the groups in demographic variables or initial screening parameters. In 73 patients (66%), treatment resulted in an IVF cycle where embryos were available for PGS. Reasons for patient drop out and exclusion from analysis are given (Figure 3.1.2).

A total of 943 oocytes were obtained in both groups, resulting in 528 (56%) fertilized oocytes. From these, 340 embryos were suitable for biopsy and 302 embryos were successfully diagnosed by FISH. In 108 embryos the diagnosis was based on one cell. For 194 embryos the diagnosis was based on two cells.

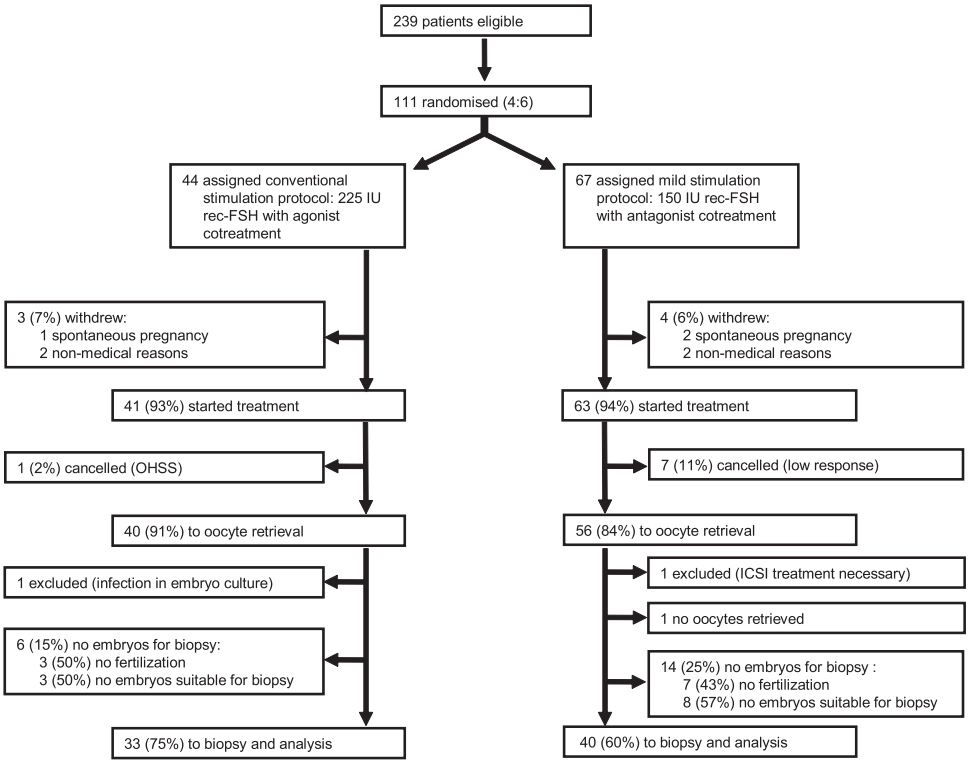

Figure 3.1.2. Trial profile and flow of patients. OHSS: stimulation was discontinued because of signs of an imminent Ovarian Hyperstimulation Syndrome; Low response: only one growing follicle observed at ultrasound; ICSI: Intra cytoplasmic sperm injection necessary due to unexpected poor semen quality on the day of oocyte retrieval.

Chromosomal competency correlates to ovarian response.

The distribution of the number of oocytes retrieved per patient is different following conventional and mild ovarian stimulation, with skewing of the curve following mild stimulation towards fewer oocytes (Figure 3.1.3a and b). For each stimulation protocol, differences in the proportion of abnormal embryos based on one cell diagnosis were correlated to ovarian response per patient (Figure 3.1.3c and d). Within the mild group, a significant positive correlation (Pearson correlation = 0.4; P = 0.006) was observed between the number of oocytes obtained and the proportion of

abnormal embryos. In the conventional stimulation group, no correlation was observed (Pearson correlation = -0.08; P = 0.679). The distribution found after mild stimulation was significantly different from the one found after conventional stimulation (P = 0.016; test for interaction in ANOVA).

Table 3.1.1 summarizes outcome measures and clinical results. Although more oocytes were obtained per patient following conventional ovarian stimulation (12.1 vs. 8.2, P = 0.001), no differences were observed in fertilization rates or percentage of embryos biopsied and diagnosed between the groups. The proportion of embryos of normal morphology was higher after mild compared to conventional ovarian hyperstimulation (51% vs. 35%; P = 0.04).

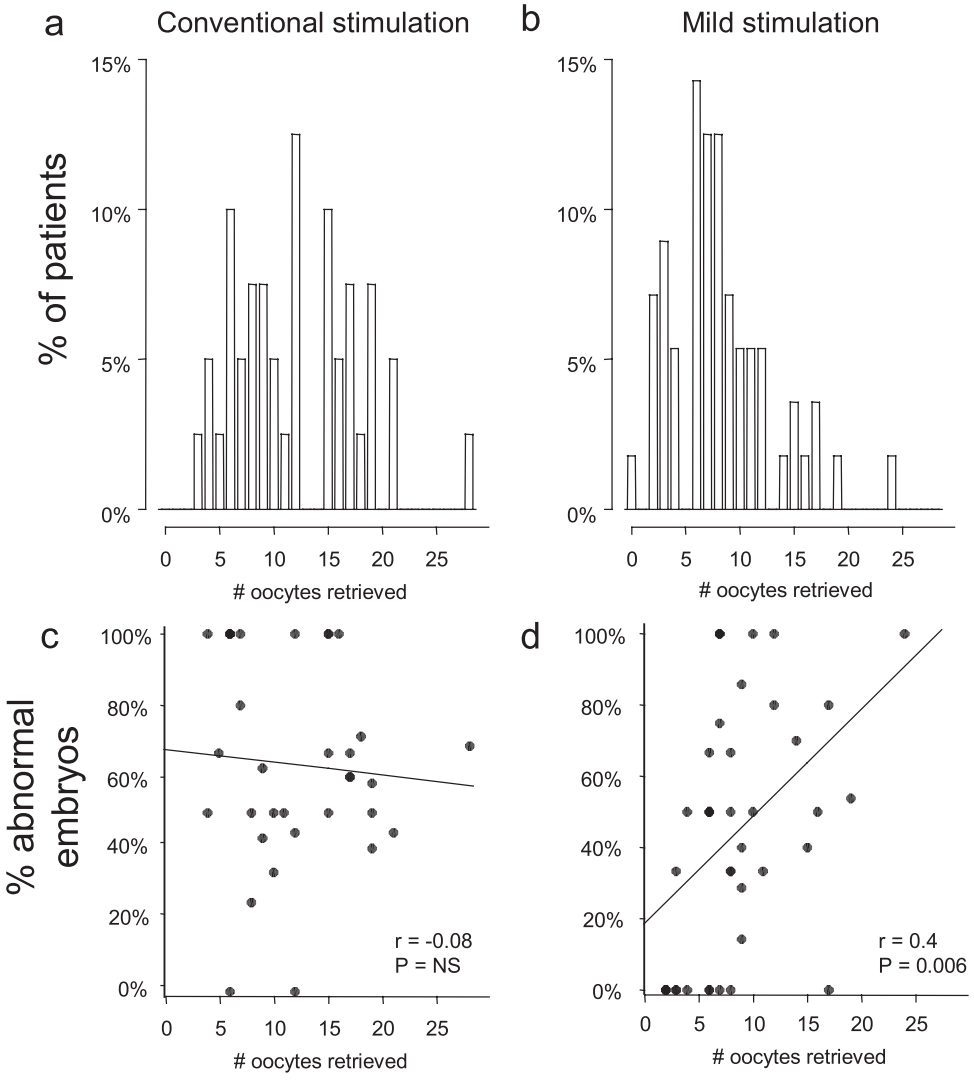

Figure 3.1.3. Distribution of number of oocytes retrieved per patient and relationship between oocyte number and percentage of abnormal embryos generated following conventional (a and c) and mild ovarian stimulation for IVF (b and d).

Mild ovarian stimulation results in a reduced proportion of abnormal and mosaic embryos.

Based on the first cell biopsied, the proportion of chromosomally abnormal embryos per patient was significantly decreased following mild stimulation (Table 3.1.1). The percentage of abnormal embryos relative to the number of embryos diagnosed was 45% following mild stimulation (40 patients), vs. 63% after conventional stimulation (33 patients; P = 0.02). Mild stimulation resulted in significantly less oocytes and embryos, but there was no difference between the two study-groups in the average number of chromosomally normal embryos (1.8) obtained per patient (Figure 3.1.4).

By analyzing the group of embryos in which two cells were available for diagnosis, insight into chromosomal mosaicism could be obtained (Table 3.1.1). In this group, the diagnosis could be normal, abnormal or mosaic. Overall abnormality rates (abnormal and mosaic embryos) were 55% following mild (38 patients) and 73% following conventional ovarian stimulation (30 patients; P = 0.046), confirming the difference in abnormality rates observed after single cell diagnosis. However, the proportion of mosaic embryos per patient was more significantly increased following conventional ovarian stimulation (37% vs. 65%; P = 0.004). This observation indicates that the increase in abnormal embryos is mainly due to an increase in mitotic segregation errors in early embryonic cleavage divisions.

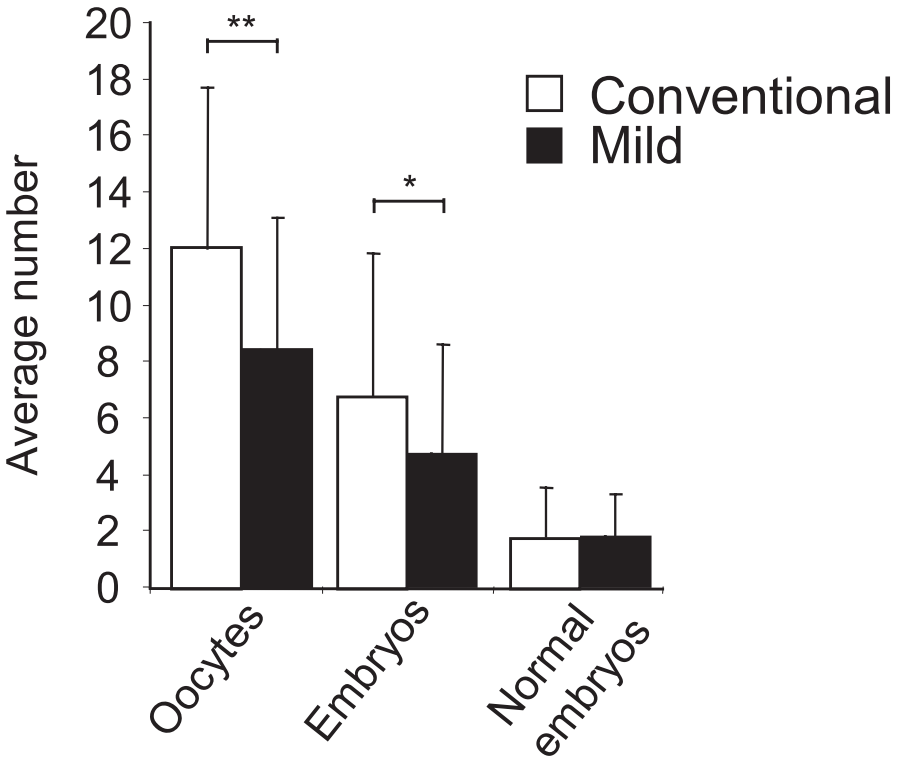

Figure 3.1.4. Oocyte and embryo yield, embryos successfully biopsied and diagnosed by FISH, and embryos diagnosed as chromosomally normal on the basis of FISH results from one cell following conventional and mild stimulation. Values are represented as mean ± SD. *P < 0.05, **P < 0.01

Patient selection does not explain observed differences in aneuploidy rate.

Although not significant (χ^2; P = 0.097), a trend was observed following mild stimulation towards a higher rate of drop out before PGS analysis, since 27 out of 67 (40%) patients were either lost before oocyte retrieval, fertilization, or embryo biopsy (Figure 3.1.2). After conventional stimulation, 11 out of 44 (25%) patients did not reach PGS analysis. The retrieval of only a few oocytes after conventional stimulation has been attributed to ovarian aging (Beckers *et al.*, 2002; de Boer *et al.*, 2002) and an age-dependent increase in chromosomal abnormalities in oocytes has been reported (Hassold and Hunt, 2001). It is therefore possible that women with more advanced ovarian aging undergoing mild stimulation were less likely to meet the criteria for oocyte retrieval. To exclude such a potential selection bias, female age and two distinct markers for ovarian aging (early follicular phase FSH and inhibine B levels) (Groome *et al.*, 1996; Creus *et al.*, 2000) were compared between patients who did and the patients who did not reach PGS following mild stimulation. No differences were observed in age (33.2 ±3.2 years vs. 32.3 ±3.4; P = 0.31), baseline serum levels of FSH (7.8 ± 2.2 IU/ l vs. 7.7 ± 3.3 IU/ l; P = 0.93) or inhibin B (110 ± 75 ng/ l vs. 108 ± 129 ng/ l; P = 0.96). Moreover, when the seven subjects with the lowest oocyte yield after conventional ovarian stimulation (Fig. 4c) were retrospectively excluded to obtain equal drop out rates, the average proportion of abnormal embryos following conventional stimulation was still significantly higher, 61% vs. 45% for conventional and mild, respectively (P = 0.037). Therefore, it is unlikely that the observed reduction in the proportion of abnormal embryos is the result of a selection after mild ovarian stimulation for women with less advanced ovarian aging.

Discussion

The recent introduction of GnRH antagonists provides the opportunity to employ ovarian stimulation for IVF without disrupting early follicular phase dynamics. In the present randomized trial, we compare the effect of a mild stimulation approach to a conventional hyperstimulation regimen by assessing chromosomal competence of embryos. We found that mild stimulation is associated with a reduction in the number of oocytes retrieved and a significantly higher proportion of chromosomally normal embryos. Consequently, the number of chromosomally competent embryos obtained per woman is similar (around two), despite a significant reduction in the total number of embryos in the mild stimulation group. In addition, analysis of two cells per embryo demonstrated that the increase in chromosomal abnormalities observed after conventional stimulation, is mainly due to an increased incidence of chromosomal mosaicism.

In the mild stimulation group, patients received lower doses of exogenous FSH. Since no down regulation of endogenous FSH production has taken place, serum FSH concentrations on cycle day 8 were shown to be equivalent to those observed in conventional stimulation (Hohmann *et al.*, 2003). Consequently, the main difference between the two stimulation protocols is effected in the early stages of secondary follicle recruitment. In the natural cycle, follicles that develop to the Graafian preovulatory stage are selected from a pool of 20-30 antral follicles. After becoming FSH-dependent, the "selected" dominant follicles indirectly affect the growth of the remaining follicles through their secretion of estrogens and inhibins, which suppress the secretion of pituitary FSH (Fauser and Van Heusden, 1997; Zeleznik and Hillier, 1984). In addition, the dominant follicle suppresses subdominant follicles through intraovarian mechanisms (Baker and Spears, 1999). In the mild stimulation protocol, these initial stages of follicle selection are not affected. In contrast, following conventional ovarian stimulation, natural follicle selection is completely overruled, allowing the non-discriminate growth of many follicles with or without competent oocytes.

However, although unlikely, the possibility that the different GnRH analogues themselves influence the chromosomal constitution of the embryos in this study cannot be ruled out.

Following recruitment into the growing pool, the oocyte expands from 35 to 120 µm in diameter which represents a 100-fold increase in volume over a period of several months (Gosden and Bownes, 1995). Oocyte growth and maturation is interlinked with follicle development, and bi-directional signaling occurs between oocytes and granulosa cells (Eppig, 2001). Oocytes have to achieve both nuclear and cytoplasmic maturity in order to sustain the early stages of embryonic development (Albertini *et al.*, 2003). Recently, experimental evidence in mice showed that disturbances in the complex interplay of signals regulating folliculogenesis may alter the late stages of oocyte growth, increasing the risk for chromosome malsegregation in subsequent meiotic divisions (Hodges *et al.*, 2002). These findings offer a rationale for our findings of an increased proportion of chromosomally normal embryos after mild ovarian stimulation. Interestingly, in the present study, the increase in the proportion of abnormal embryos was found to be mainly the result of an increase in mitotic segregation errors, leading to mosaic embryos. Since the embryonic genome does not become active until the eight cell stage (Braude *et al.*, 1988), until then the cell cycle machinery is dependent on the protein and mRNA content of the oocyte. Recently, a direct link has been established between defects in the oocyte and an increased incidence in mitotic segregation errors. An experimental mouse model, with an inactivated protein subunit of the meiotic synaptonemal complex (SYCP3) revealed not only an increased level of segregation errors at the first meiotic division but also showed a substantial increase in mitotic segregation errors during the first embryo cleavage divisions (Yuan *et al.*, 2002; Lightfoot *et al.*, 2006).

Within the mild stimulation group, we also found that a low oocyte yield is associated with a decrease in the proportion of aneuploid embryos. A previous study showed mild stimulation to result in high quality embryos for transfer as indicated by good embryo morphology, and pregnancy rates comparable to those following conventional ovarian stimulation (Hohmann *et al.*, 2003). Moreover, while no pregnancies were obtained in women who had produced four or less oocytes following the conventional protocol, the majority of pregnancies obtained following mild ovarian stimulation occurred in women where four or less oocytes were retrieved. A low number of oocytes retrieved after stimulation may therefore represent an appropriate response to mild stimulation. In contrast, a similar low response occurring after conventional ovarian stimulation is indeed indicative of ovarian ageing (Beckers *et al.*, 2002; de Boer *et al.*, 2002).

Although implantation, ongoing pregnancy and ultimately live birth are the most meaningful outcome measures of IVF, they are only partially influenced by embryo quality and can only be determined for the embryos transferred. In the current study, PGS was used as a parameter for assessing embryo quality. It revealed a significant effect of the ovarian stimulation regimen on the chromosome segregation ability of the resulting embryos. This observation supports the concept that only the follicle with the most competent oocyte is selected during the natural cycle in the mono-ovulatory human species. The present use of the mild stimulation protocol more closely simulates the natural condition resulting in developmentally more competent oocytes. This concept is also consistent with an extensive analysis of historical data showing no significant improvement of the pregnancy rate per oocytes retrieved using ovarian hyperstimulation as compared to IVF results in the early 1980s, when IVF was performed without ovarian stimulation (Inge *et al.*, 2005).

In conclusion, the present study shows that mild ovarian stimulation results in fewer oocytes and a decreased proportion of aneuploid and mosaic embryos. Future ovarian stimulation strategies should not focus on obtaining as many oocytes as possible, but should aim at inducing

a more physiological ovarian response, thus minimizing embryo aneuploidy rate and facilitating selection of the best quality embryo for transfer.

Acknowledgements

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Table 3.1.1. Outcomes after IVF and PGS diagnosis following conventional or mild ovarian stimulation.

	Conventional stimulation	MildStimulation	P*	Difference(95% CI)
No. of patients	40	55		
Oocytes retrieved (n)	12.1 ± 5.7	8.2 ±4.8	< 0.01	3.9 (1.7 to 6.0)
Fertilization rate (%)	57 ± 28	55 ± 30	0.807	1.5 (-10 to 13)
Good quality embryo rate[a] (%)	35 ± 29	51 ± 40	0.04	-17 (-32 to -1)
Percentage of embryos diagnosed (%)	40 ± 22	45 ± 23	0.376	-5 (-15 to 6)
Diagnosis based on first cell biopsied[b]:				
No. of patients	33	40		
Abnormal embryos / embryos diagnosed (%)	63 ± 28	45 ± 35	0.016	19 (4 to 34)
Diagnosis based on two cells[c]:				
No. of patients	30	38		
Abnormal embryos / embryos diagnosed (%)	73 ± 33	55 ± 42	0.046	19 (0.3 to 36)
Mosaic embryos / embryos diagnosed (%)	65 ± 37	37 ± 39	0.004	28 (10 to 47)
Clinical outcome measures				
Number of embryos / ET	1.45 ± 0.51	1.46 ± 0.51		
Ongoing pregnancy rate / started cycle (%)	7/ 41 (17)	12/ 63 (19)		
Ongoing pregnancy rate / ET (%)	7/ 31 (23)	12/ 35 (34)		

Data are expressed as mean and SD, unless otherwise stated. * P values are from a two sample t-test. ET = embryo transfer.

[a] Embryos with normal morphology were defined as embryos with timely development, <20% fragmentation, equally sized blastomeres and small or no irregularities observed in the cytoplasm.

[b] Diagnosis of normal or abnormal embryos was based on the FISH results of one cell. If two cells were available, the first cell biopsied was determined in retrospect and used for diagnosis. Rates were calculated first per patient and then averaged.

[c] Only embryos where two cells were available for diagnosis were taken into account. An embryo was considered abnormal, if at least one of the two cells showed an abnormal result.

Chapter 4

Conclusions and general discussion

Cytogenetic aspects of preimplantation genetic screening

In the first part of this thesis, the cytogenetic aspects of preimplantation genetic screening were considered. A protocol was developed which allows screening of ten different chromosomes in two rounds of FISH in a reliable fashion (Section 2.1). Furthermore, the entire process was optimized so embryo biopsy and FISH analysis could be completed within the limited time frame (1-2 days) available when performing PGS. Next to technical aspects, the choice which chromosomes to investigate was important. Comparative genomic hybridization (CGH) studies have shown that all chromosomes can show abnormalities in preimplantation embryos (Voullaire *et al.*, 2000, Wells and Delhanty, 2000; Voullaire *et al.*, 2002). We chose to combine a commercially available probe mix widely used in PGS clinics with a laboratory prepared probe mix, resulting in the analysis of the copy number of chromosomes 1, 7, 13, 15, 16, 18, 21, 22, X and Y. Probes for these chromosomes were selected since chromosomes 13, 18, 21 and the sex chromosomes can be involved in viable trisomies. Chromosomes 7, 15, 16 and 22 are often found to be trisomic in early miscarriages (Philipp *et al.*, 2003) and abnormalities for chromosome 1 can lead to implantation failure or missed abortion (Philipp *et al.*, 2002). In Section 3.4, this protocol was extended to include a second laboratory prepared probe mix, allowing the copy number of 15 chromosomes to be investigated in three consecutive FISH rounds. Probes for chromosomes 2, 6, 10, 14 and 17 were added, as these chromosomes are implicated in early miscarriage and implantation failure (Eiben *et al.*, 1990; Causio *et al.*, 2002; Philipp *et al.*, 2002, 2003; Nagaishi *et al.*, 2004), and are poorly studied in preimplantation embryos.

Most laboratories employing PGS, perform a biopsy of only one blastomere and base the diagnosis of normal or abnormal on this single cell. Aiming to investigate two blastomeres per embryo allowed us to study chromosomal mosaicism in day 3 embryos. During the initial study of biopsy and reanalysis of good quality cryopreserved embryos, 70% of the embryos were found to be mosaic (Section 2.2). Although it was hypothesized that the cryopreservation process might induce chromosomal abnormalities, comparably high rates of mosaicism were observed in the subsequent study on fresh embryos after biopsy on day 3 (Section 2.3). Although artifact of the FISH procedure resulting in misdiagnosis may explain some of the abnormalities observed, FISH error rates on blastomeres are reported to be only 5% (Daphnis *et al.*, 2004). Furthermore, studies using other techniques than FISH, also described mosaicism in preimplantation embryos (Jamieson *et al.*, 1994; Wells and Delhanty, 2000; Voullaire *et al.*, 2000; Voullaire *et al.*, 2002; Katz-Jaffe *et al.*, 2004). Therefore, mitotic errors resulting in chromosomal mosaicism are highly prevalent in human IVF derived preimplantation embryos.

By performing reanalysis of the PGS diagnosed embryos on day 5 of development, insight into mechanisms of aneuploidy formation and the accuracy of the PGS diagnosis on day 3 was obtained. Although PGS on two blastomeres appeared effective in detecting chromosome aberrations resulting from meiotic non-disjunction events, an accurate prediction of the chromosome constitution on day 5 was only obtained in about half of the cases if mitotic errors were involved. The data from Chapter 2 reveal that chromosomal mosaicism is not limited to cleavage stage embryos. The incidence of mosaicism seems to increase in embryos at the blastocyst stage, while the level of mosaicism within the embryos decreases. As Section 2.4 demonstrates, all chromosomes can be involved in mitotic segregation errors. Moreover, investigation of more chromosomes results in an even further increase in the incidence of mosaicism.

Because of the biological phenomenon of mosaicism, it will be impossible to develop PGS at the eight-cell stage into a fully reliable diagnostic test. Even if the diagnosis is based on two cells,

it remains uncertain as to what extent these results represent the chromosomal constitution of the remaining embryo. Removing cells from the embryo can also result in an alteration of the level of mosaicism in the embryo. Moreover, because of the compromised functioning of cell cycle check points, the remaining embryo can continue to change cytogenetically until the embryonic genome becomes fully active, probably until the blastocyst stage.

As mentioned before, most IVF clinics perform PGS analysis on only a single biopsied blastomere. This practice is likely to increase the chance for a false positive or false negative diagnosis due to chromosomal mosaicism. This is illustrated in a recent publication where the authors produced chromosomally normal stem cell lines from embryos diagnosed as abnormal after a single cell biopsy (Munné *et al.*, 2005). However, rather than acknowledge that this most likely resulted from a false positive diagnosis due to mosaicism, they claim that the embryo had undergone "chromosome normalization". False negative diagnoses have been reported as well. In a series of 3,747 PGS cycles resulting in 722 clinical pregnancies and 564 live births, three cases with trisomy 21 occurred (Verlinsky *et al.*, 2004). In our view, the limitation caused by the phenomenon of mosaicism explains for a large part why the clinical effectiveness of PGS still remains to be shown (Twisk *et al.*, 2005; Shahine *et al.*, 2006).

Little is known about the implantation potential of mosaic embryos and their ability to develop into a healthy child. This will probably depend on the level of mosaicism, the type of chromosome aberration and the chromosome involved. Future studies offering insight into these mechanisms will be vital to our understanding of the significance and consequences of chromosomal mosaicism. More research is needed to determine if some mosaic embryos can be considered suitable for transfer depending on the type of aneuploidy and the chromosome involved. Until this is resolved, screening of more chromosomes may result in potentially healthy embryos being discarded. Therefore, investing in advanced techniques allowing all chromosomes to be investigated, need careful consideration. Techniques combining whole genome amplification with CGH or a CGH array (le Caignec *et al.*, 2006), do not solve the problem of mosaicism and will only increase the chance for its detection, thus yielding a diagnosis with unknown clinical consequences.

Although PGS is applied in numerous IVF clinics around the world, there is an ongoing debate as to which patients would benefit from this technique. In other words, can IVF patients with an increased risk for aneuploidy be identified? In the studies described in this thesis PGS was performed on embryos derived from a group of relatively young IVF patients with no apparent increased risk. Still, only 36% of the embryos were found to be chromosomally normal. Interestingly, Staessen *et al.*, (2004) reported the exact same percentage of normal embryos after PGS on one or two blastomeres in a group of older aged patients. So although older women are thought to have a lower chance to produce chromosomally normal embryos, this could not be confirmed by our results. In fact, in 23% of the reanalyzed embryos, we observed a chromosome abnormality that most likely originated during meiosis. It may be that all women seeking infertility treatment have an increased incidence of chromosomally abnormal embryos. Alternatively, abnormality rates may be influenced by the hormonal treatment related to the IVF procedure.

Clinical implications of PGS findings

In Section 3.1 the results of a randomized controlled trial are reported, comparing a mild stimulation regimen using GnRH antagonist co-treatment (not interfering with secondary follicle recruitment), with a conventional high dose exogenous gonadotropin regimen and GnRH agonist co-treatment. In this randomized IVF study, PGS was used to assess chromosomal competence, as this is likely to reflect embryo quality. We observed that mild stimulation was associated with a reduction in the number of oocytes retrieved, but a significantly higher proportion of chromosomally normal embryos was generated. Consequently, the absolute number of chromosomally competent embryos obtained per woman was similar, despite a significant reduction in the total number of embryos generated in the mild stimulation group. In addition, analysis of two cells per embryo demonstrated that the increase in chromosomal abnormalities observed after conventional stimulation, is mainly due to an increased incidence of chromosomal mosaicism. It appears that oocyte quality is linked to ovarian stimulation and also to chromosomal competence of resulting embryos. These data show for the first time a direct relationship between ovarian stimulation and the chromosomal competence of embryos. The undisturbed process of cyclic follicle recruitment during the commencement of the menstrual cycle during mild stimulation may proof to be of fundamental significance. The possibility to minimize chromosomal abnormalities in embryos by applying milder ovarian stimulation protocols should be further explored.

Interestingly, mild stimulation did not seem to decrease the proportion of uniformly aneuploid embryos, resulting from a meiotic error. It appears that reduced oocyte quality as a result of changes in follicle growth dynamics primarily has an impact on chromosomal competence of the embryo and the incidence of mitotic errors. In contrast, ovarian aging influences the occurrence of meiotic errors. Differences in the mechanisms leading to both types of errors may underlie this observation. Meiotic errors are influenced by a predisposition of the different chromosomes due to differences in number and placement of recombination sites. This predisposition is unlikely to affect mitotic errors. More detailed studies on the incidence of chromosomal mosaicism in IVF embryos from both younger and older women in response to different stimulation protocols are needed to further elucidate causes of meiotic and mitotic segregation errors.

To improve the efficiency of single embryo selection, several strategies have been proposed. One such strategy is the transfer of a single blastocyst, as good quality blastocysts are more likely to be chromosomally normal (Staessen *et al.*, 2004, Magli *et al.*, 2000). This was recently shown to increase pregnancy rates in comparison to transfer of a single cleavage stage embryo (Papanikolaou *et al.*, 2006). However, concerns remain as to the extended culture of embryos and associated risks for epigenetic defects, which may impact on fetal growth and development (Jacob and Moley, 2005). This thesis suggests that selection of better quality embryos may also be achieved by applying milder stimulation protocols, not aiming at obtaining as many oocytes as possible, but taking advantage of processes within the natural cycle. This will have none of the risks associated with extended culture and has added benefits to the patient, such as shorter duration of treatment and less side effects from the medication. We propose that mild stimulation decreases the proportion of mosaic embryos. As mosaic embryos complicate PGS diagnosis, mild stimulation protocols may also make PGS more effective. The observation that within the mild stimulation group a low response was associated with a further decrease of the proportion of abnormal embryos, underscores the need of further development of minimal stimulation approaches.

Obviously, our findings should be confirmed by other laboratories. The clinical study undertaken by us represents the first prospectively designed randomized study focusing on a possible relationship between interference with natural follicle development and the chromosomal constitution of embryos. It should be realized that the number of women involved is relatively small. Many different stimulation protocols are currently used at different IVF clinics, as well as many different PGS methodologies. Future studies into the natural cycle and new stimulation approaches are needed to study the effect of follicle dynamics on oocyte quality and thus chromosomal competence of embryos.

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Summary

Chapter 1

The introduction of this thesis provides the reader with the necessary background information to understand the rationale behind the studies conducted. It starts with the observation that human reproduction is a relatively inefficient process and this may be largely explained by the high incidence of chromosomal abnormalities in oocytes and embryos. Furthermore, an explanation is given why selection of the embryo with the best implantation potential for transfer to the uterine cavity is becoming increasingly important for IVF treatment cycles. Mechanisms of aneuploidy formation in oocytes and embryos are discussed and the phenomenon of chromosomal mosaicism in embryos is introduced. The possibilities and problems when using preimplantation genetic screening are described. Subsequently, an outline of approaches used for ovarian(hyper) stimulation is given and possible mechanisms how ovarian stimulation can influence oocyte quality and chromosome competence of resulting embryos. The chapter then proceeds with stating the objectives and outline of the thesis.

Chapter 2

Section 2.1. The objective was to develop a DNA labeling protocol for the simultaneous detection of five different fluorescent chromosomal DNA probes within one round of hybridization. In combination with a commercial five-color probe mix for the second round of hybridization, this results in a fast and reliable fluorescent *in situ* hybridisation (FISH) protocol, enabling the detection of 10 chromosomes within a working day. This is especially of use for preimplantation genetic screening (PGS), when only single interphase nuclei are available for analysis and when time is restricted.

DNA probes were labeled with four different fluorochromes (Pacific Blue, Alexa Fluor 350, Alexa Fluor 594 and Alexa Fluor 488) using an ARES labelling kit, based on a two-step method. Aminoallyl-dUTPs were incorporated by nick translation, followed by chemical linking of the amino-modified fluorescent dye. The fifth color was achieved by using two fluorescent dyes in the chemical reaction, resulting in dual labeling of the DNA probe and fluorescence detectable with a specific filter set. These five probes were simultaneously hybridized in a first FISH round, followed by a second hybridization with a commercial five color probe mix, thus allowing the detection of chromosomes 1, 7, 13, 15, 16, 18, 21, 22, X and Y. Fixation and pre-treatment procedures of the blastomere nuclei were further optimized. Using this labeling and FISH protocol, probe hybridization efficiency, when tested on lymphocyte nuclei, is 95-99%. With the fixation protocol, blastomere nuclei maintain good morphology and show condensed and clear signals even after the second round of hybridization. This labeling method in combination with specific epifluorescence filters enables an independent detection of five different chromosomes in one round of FISH. The whole process of biopsy, fixation and two rounds of hybridization with the analysis of ten chromosomes can be completed within a day.

Section 2.2. Chromosomal mosaicism in human embryos may give rise to false positive or false negative results in PGS. Therefore, we have investigated whether the results obtained from a two-

cell biopsy of frozen-thawed embryos and FISH analysis are representative for the chromosome constitution of the remaining embryo on day 5. Cryopreserved day 3 embryos were thawed and from surviving embryos two blastomeres were biopsied. FISH analysis was performed for chromosomes 1, 7, 13, 15, 16, 18, 21, 22, X and Y. After biopsy, the embryos were cultured until day 5 and further analyzed using the same probe panels.

In all, 17 embryos were available with a diagnosis based on two blastomeres on day 3 and confirmatory studies on day 5. In 10 of these 17 cases the initial diagnosis could be confirmed. However, in only six cases cytogenetic results were concordant. Besides the ten cases with a "correct" diagnosis, there were six false positive results and one false negative, all involving mosaicism. Investigating the chromosomal constitution of two blastomere nuclei offers a good opportunity to study the incidence of chromosomal mosaicism in early embryo development. The confirmation rate of the results obtained on day 3 depends on the interpretation and is higher when considered from a clinical than from a cytogenetic point of view.

Section 2.3. In order to assess the frequency of aneuploidy and mosaicism in embryos obtained from IVF patients younger than 38 years of age, PGS was performed after biopsy of two blastomeres. Furthermore, the reliability of this diagnosis was assessed by performing reanalysis of the embryo on day 5. The copy numbers of ten chromosomes (1, 7, 13, 15, 16, 18, 21, 22, X and Y) were investigated by FISH analysis. Embryos found to be abnormal or of insufficient morphological quality were cultured until day 5 and reanalyzed. Results obtained were compared to the day 3 blastomere analysis.

After analysis of 196 embryos (one cell in 38% and two cells in 62%), only 36% of the embryos were found to be normal on day 3. After analysis of two blastomeres, 50% showed chromosomal mosaicism. Comparison of the FISH results from day 3 blastomeres and day 5 embryos yielded an overall cytogenetic confirmation rate of 54%. The rates of mosaicism and aneuploidy in these embryos from young IVF patients are similar to those published for older women. We found the best confirmation rate after a diagnosis based on two cells, where both blastomeres showed the same chromosomal abnormality. In contrast, after a mosaic diagnosis the confirmation rate was low. The present study provides the first detailed reanalysis data of embryos analyzed by PGS and clearly demonstrates the impact of mosaicism on the reliability of the PGS diagnosis.

Section 2.4. Screening for an increased number of chromosomes may improve the capability of PGS to detect the embryo(s) for transfer in IVF with the best chance to develop into a healthy child. Good quality day 4 and day 5 embryos were analyzed after cryopreservation for the nine chromosomes mostly recommended for screening (13, 14, 15, 16, 18, 21, 22, X and Y), next to six additional chromosomes which are less well studied in this context (1, 2, 6, 10 and 17). The copy numbers of 15 chromosomes were investigated by FISH in three consecutive rounds. The proportion of aneuploid and mosaic embryos was determined and compared in retrospect to results in case only the recommended probe set was analyzed.

A total of 52 embryos from 29 infertile women were analyzed. Screening the embryos for six additional chromosomes increased the proportion of abnormal embryos from 67% to 81% ($P = 0.03$), due to an increase in mosaic embryos. All but one of the meiotic aneuploidies found in this study would have been detected by the probe set most frequently used in PGS clinics. However, aneuploid cell lines originating from mitotic errors could be detected for almost all chromosomes. The added value of screening for six additional chromosomes in PGS for clinical practice remains undetermined as long as the fate of mosaic embryos after transfer is unclear.

Chapter 3

Section 3.1. The incidence of chromosomal abnormalities in human preimplantation embryos is extremely high. To test whether ovarian hyperstimulation for IVF affects oocyte quality and thus chromosome segregation behavior during meiosis and early embryo development, preimplantation genetic screening of embryos was employed in a prospective, randomized controlled trial, comparing two ovarian stimulation regimens. Infertile patients under 38 years of age were randomly assigned to undergo a mild stimulation regimen using GnRH antagonist co-treatment, which does not disrupt secondary follicle selection, or a conventional high dose exogenous gonadotropin regimen and GnRH agonist co-treatment. Following IVF, embryos were biopsied at the eight-cell stage and the copy number of nine chromosome pairs was analyzed in one or two blastomeres.

Compared to conventional hyperstimulation, significantly fewer oocytes and embryos were obtained following mild stimulation. The resulting embryos in the mild stimulation group showed a lower embryo aneuploidy rate (45 percent vs. 63 percent, $P = 0.02$), mainly due to a reduction in mitotic segregation errors. Consequently, both regimens generated a similar number (1.8) of chromosomally normal embryos. This observation supports the concept that an undisturbed process of secondary follicle selection results in the growth of only those follicles with the most competent oocytes. Future ovarian stimulation strategies should avoid maximizing oocyte yield, but focus on minimizing embryo aneuploidy rates.

Chapter 4

In this chapter the most important conclusions from the conducted studies are summarized and implications for clinical practice and future research are discussed.

Samenvatting

Hoofdstuk 1

De inleiding van dit proefschrift voorziet de lezer van achtergrond informatie, nodig om de beschreven studies te begrijpen. Het begint met de observatie dat humane voortplanting een relatief inefficiënt proces is. Dit feit kan grotendeels verklaard worden door het vaak voorkomen van chromosomale afwijkingen in eicellen en embryo's. Verder wordt uitgelegd waarom het steeds belangrijker wordt om bij een IVF-behandeling het embryo met de beste kansen op implantatie te kunnen selecteren om terug te plaatsen in de baarmoederholte. Mechanismen, die een rol spelen bij het ontstaan van aneuploïdie in eicellen en embryo's worden beschreven en het fenomeen van embryo's met een mozaïek chromosoompatroon wordt toegelicht. De mogelijkheden en problemen die pre-implantatie genetische screening met zich meebrengt, worden besproken. Vervolgens worden verschillende manieren van ovariële (hyper)stimulatie beschreven. Er wordt ingegaan op hoe ovariële stimulatie invloed zou kunnen hebben op de eicelkwaliteit en chromosomale competentie van resulterende embryo's. Tot slot bevat dit hoofdstuk de doelstellingen en de opzet van het proefschrift.

Hoofdstuk 2

Paragraaf 2.1. Het doel was een DNA labelingsprotocol te ontwikkelen, waarmee het mogelijk is vijf verschillende fluorescerende chromosomale DNA probes te detecteren binnen één hybridisatie ronde. In combinatie met een commerciële, vijf-kleuren probemix, resulteert dit in een snel en betrouwbaar fluorescent *in situ* hybridisatie (FISH) protocol. Hiermee wordt het mogelijk tien verschillende chromosomen te screenen binnen één werkdag. Dit is vooral interessant voor het gebruik bij pre-implantatie genetische screening (PGS), waarbij slechts enkele interfase kernen beschikbaar zijn voor de analyse en waarbij de tijd beperkt is.

De DNA probes werden gelabeld met vier verschillende fluorochromen (Pacific Blue, Alexa Fluor 350, Alexa Fluor 594 and Alexa Fluor 488) met gebruikmaking van een ARES labeling kit. Eerst werden aminoallyl-dUTP's ingebouwd in het DNA door middel van nick translation, waarna het fluorochroom chemisch gelinkt werd aan het amino-gemodificeerde DNA. De vijfde kleur werd verkregen door twee verschillende fluorochromen te combineren tijdens de chemische linking. Dit resulteerde in een fluorescentie, detecteerbaar met een specifieke filterset. Deze vijf probes werden tegelijkertijd gehybridiseerd in een eerste ronde FISH, gevolgd door een tweede ronde met de commerciële probemix. Op deze manier konden de chromosomen 1, 7, 13, 15, 16, 18, 21, 22, X en Y gedetecteerd worden. Ook werd de fixatie methode en voorbehandeling van de blastomeren geoptimaliseerd. Middels dit labeling en FISH protocol, kon een hybridisatie efficiëntie van 95-99% gehaald worden op kernen van lymfocyten. Met het fixatie protocol behouden de kernen van blastomeren hun morfologie en geven compacte en duidelijke FISH signalen, zelfs na de tweede hybridisatie ronde. Deze manier van labelen in combinatie met specifieke epi-fluorescente filters, levert een onafhankelijke detectie op van vijf verschillende chromosomen in één enkele FISH ronde. Het hele proces van embryo biopsie, fixatie en twee hybridisatie rondes met de analyse van tien verschillende chromosomen kan worden afgerond in één dag.

Paragraaf 2.2. Een mozaïek chromosoompatroon in humane embryo's kan leiden tot vals positieve of vals negatieve resultaten bij PGS. Daarom hebben wij onderzocht of de FISH-resultaten op dag 3 representatief zijn voor de chromosoom samenstelling van het resterende embryo op dag 5 na bevruchting. Ingevroren embryo's werden ontdooid, en uit de overlevende embryo's werden twee cellen verwijderd. FISH analyse werd gedaan voor de chromosomen 1, 7, 13, 15, 16, 18, 21, 22, X en Y. Na de biopsie werden de embryo's doorgekweekt tot dag 5, waarna alle kernen van het embryo werden geanalyseerd voor dezelfde chromosomen.

In totaal waren 17 embryo's beschikbaar met een diagnose gebaseerd op twee cellen en aanvullend onderzoek op dag 5. In 10 van deze 17 embryo's kon de initiële diagnose worden bevestigd. Echter, in slechts zes gevallen kwamen de cytogenetische bevindingen helemaal overeen. Naast de tien "correcte" diagnoses, waren er ook nog zes vals positieve diagnoses en één vals negatieve, en in deze embryo's werd steeds een mozaïek chromosoompatroon gevonden. Het onderzoeken van de chromosomale samenstelling van twee blastomeer-kernen per embryo biedt de kans om mosaicisme in het pre-implantatie embryo te bestuderen. De diagnose gevonden op dag 3 kan vaker als bevestigd worden beschouwd op dag 5, indien gezien vanuit een klinisch dan vanuit een cytogenetisch oogpunt.

Paragraaf 2.3. Om de frequentie van (mozaïek) aneuploïde IVF embryo's in vrouwen jonger dan 38 jaar te onderzoeken, werd PGS verricht na biopsie van twee blastomeren per embryo. Verder werd de betrouwbaarheid van deze diagnose onderzocht door heranalyse van het embryo op dag 5. Het kopie-aantal van tien chromosomen (1, 7, 13, 15, 16, 18, 21, 22, X en Y) werd onderzocht door middel van FISH analyse. Embryo's die afwijkend bevonden werden, of die van onvoldoende morfologische kwaliteit waren, werden doorgekweekt tot dag 5 en vervolgens volledig geanalyseerd. De resultaten verkregen op dag 5 zijn vergeleken met de resultaten van de blastomeer-analyse op dag 3.

Na analyse van 196 embryo's, waarvan in 38% één cel en in 62% twee cellen zijn onderzocht, werd slechts 36% chromosomaal normaal bevonden. Na analyse van twee cellen per embryo, werd een mozaïek chromosoompatroon gevonden in 50% van de embryo's. Na vergelijking van de FISH resultaten van dag 3 blastomeren met dag 5 embryo's, kon de diagnose cytogenetisch bevestigd worden in 54% van de embryo's. Het percentage mozaïeke of aneuploïde embryo's verkregen in deze groep jonge IVF patiënten zijn vergelijkbaar met percentages beschreven voor vrouwen boven de 35 jaar. De grootste kans op bevestiging van de diagnose vonden wij na analyse van twee cellen, waarbij beide blastomeren overeenkomstige resultaten lieten zien. Indien een mozaïeke samenstelling werd gevonden, was de kans op bevestiging op dag 5 laag. De huidige studie beschrijft de eerste gedetailleerde heranalyse data van embryo's na PGS analyse en laat duidelijk het effect zien van mosaicisme op de betrouwbaarheid van de PGS diagnose.

Paragraaf 2.4. Screening van zoveel mogelijk chromosomen zou de effectiviteit van PGS kunnen verbeteren, doordat de IVF embryo's met de beste kansen om na terugplaatsing uit te groeien tot een gezond kind beter geïdentificeerd zouden kunnen worden. Dag 4 en dag 5 embryo's van goede kwaliteit zijn geanalyseerd na cryopreservatie, voor de negen chromosomen die meestal worden aanbevolen voor screening (13, 14, 15, 16, 18, 21, 22, X en Y). Daarnaast werden nog zes andere chromosomen onderzocht (1, 2, 6, 10 en 17), waarover in deze context weinig bekend is. Op deze manier werd het aantal kopiëen van 15 chromosomen onderzocht door middel van FISH in drie opeenvolgenden hybridisatie-ronden. Het aandeel aneuploïde en mozaïeke embryo's werd hierna bepaald en dit werd retrospectief vergeleken met het resultaat dat behaald wordt indien

alleen de meest gangbare chromosomen geanalyseerd zouden zijn.

In totaal werden 52 embryo's van 29 vrouwen onderzocht. Door zes extra chromosomen te onderzoeken, nam het percentage chromosomaal afwijkende embryo's toe van 67% naar 81% (P = 0,03), voornamelijk door een toename van het aantal mozaïeke embryo's. Op één geval na zouden we alle embryo's met een meiotische chromosoomafwijking, ook gevonden hebben met de probe combinatie die meestal gebruikt wordt in PGS klinieken. Wij vonden echter afwijkende cellijnen met een mitotische oorsprong voor bijna alle onderzochte chromosomen. Het screenen van zes extra chromosomen lijkt voornamelijk de detectie van mozaïeke embryo's te verbeteren. De toegevoegde waarde hiervan is moeilijk te bepalen, zolang er geen duidelijkheid is over het ontwikkelingspotentieel van deze embryo's.

Hoofdstuk 3

Paragraaf 3.1. Chromosomale afwijkingen komen erg vaak voor in humane pre-implantatie embryo's. Om te testen of ovariële hyperstimulatie voor een IVF behandeling de eicelkwaliteit en als resultaat daarvan het chromosoom segregatie patroon tijdens de meiose en de eerste klievingsdelingen van het embryo beïnvloedt, werd PGS toegepast in een prospectief gerandomiseerde, gecontroleerde studie. Hierbij werden IVF patiënten onder de 38 jaar gerandomiseerd voor:

a) een conventionele behandeling, gebuikmakend van een GnRH agonist en een hoge dosering follikel stimulerend hormoon (FSH);

b) een behandeling met een mild stimulatie protocol gebruikmakend van een GnRH antagonist, en een lagere dosering FSH.

Dit laatste schema start op dag 5 van de menstruele cyclus, zodat het proces van secondaire follikelselectie niet wordt verstoord. Na de IVF behandeling werden de embryo's die hieruit ontstonden, in het achtcellige stadium gebiopteerd en één of twee blastomeren onderzocht op het voorkomen van numerieke afwijkingen in negen chromosoomparen.

Na milde stimulatie (b), werden significant minder eicellen en embryo's verkregen dan na de conventionele behandeling (a). Verder werd in de milde stimulatiegroep een duidelijk lager percentage chromosomaal afwijkende embryo's gezien (45% versus 63%, P = 0,02), wat vooral te wijten was aan een lager percentage mozaïeke embryo's. Dit had als resultaat dat beide stimulatieschema's gemiddeld per patient een vergelijkbaar aantal chromosomaal normale embryo's opleverde (1,8 embryo's). Deze bevinding bevestigt de hypothese dat het onverstoord laten van het proces van secondaire follikelselectie kan leiden tot groei van alleen follikels met de meest competente eicellen. In de toekomst zou ovariële stimulatie voor IVF zich niet moeten richten op het verkrijgen van zoveel mogelijk eicellen, maar zou het streven moeten zijn de kans op embryo's met chromosoomafwijkingen te minimaliseren.

Hoofdstuk 4

In dit hoofdstuk worden de voornaamste bevindingen van de beschreven studies samengevat. Vervolgens worden de implicaties van deze bevindingen voor de klinische praktijk en voor toekomstig onderzoek bediscussiëerd.

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De hele afdeling Voortplantingsgeneeskunde stond onder strikte leiding, maar op de kopkamer op 5 Noord, het rijk der onderzoekers, heerste volledige anarchie. Christien (uitgeklopte wielkasten, Prada schoenen, de rol van AMH bij dominante follikel selectie en de pianobar in Berlijn) is er sinds de ESHRE in Madrid een onderwerp waar we het nog niet over hebben gehad? Jammer dat je me niet meer elke dag kunt afleiden. Esther (Hessie), niet alleen naamgenoten, maar ook een tijdje lotgenoten, dat schept een band. Jouw vermogen om te luisteren en precies het goede te zeggen, is echt geweldig. Ik herinner mij heel veel gezellige lunches en etentjes, dat er nog maar vele mogen volgen! Annemarie, jouw werkdiscipline was indrukwekkend, maar nog indrukwekkender was je collegialiteit. Mark, jij was meer een "aquired taste", maar als je die dan eenmaal te pakken had, was de slappe lach nooit ver te zoeken. Het volgende feestje jouw promotiefeest? Femke, als "relatief groentje" jou als kamergenoot te hebben, was zowel gezellig, als leerzaam. Niet alleen op onderzoeksgebied, maar ook qua levenservaring! Emilie, jij bent echt de meest vriendelijke collega die ik ooit heb gehad. Ik hoop van harte dat jij straks ook jouw doel kunt bereiken.

Mijn nieuwe collega's van de afdeling Voortplantingsgeneeskunde en Gynaecologie van het UMC Utrecht wil ik bedanken, omdat ze mij zo hartelijk hebben opgenomen in het team en voor hun geduld als ik vaak nog met mijn hoofd bij mijn promotie-onderzoek zat.

Mijn Paranimfen: wat fijn dat jullie naast mij staan! Lieve Rein, deze promotie is het bewijs dat jij toch slimmer bent dan ik. Zo'n uitputtend traject is toch ook maar een vorm van

verstandsverbijstering, begin er nooit aan! Mireille -gooi al uw kommer op- de Heer, gelukkig was het motto van het Onafhankelijk Wagenings Dames Dispuut Mies: "het kan wèl", anders was het nooit gelukt. Wanneer zijn we ook al weer jarig? Laten we dat weer eens als vanouds gaan vieren en hopen dat de barman het rampenplan kent (staat dat pankje er nog, in dat bark?).

Mijn ouders: eigenlijk zouden jullie op de eerste plaats moeten komen, want zonder jullie positieve instelling en eindeloos vertrouwen in mijn kunnen, had ik het niet zo ver geschopt. Ontzettend bedankt voor de onvoorwaardelijke steun in de afgelopen jaren. Jullie zijn ouders uit duizenden!

Lieve, lieve Hogi, ik zou moeiteloos duizend redenen op kunnen noemen waarom ik van je hou, maar eigenlijk heb ik helemaal geen reden nodig. In een ander proefschrift las ik ooit de stelling: "Promotie-onderzoek is de ultieme relatietest." Wij hebben dit nu twee keer doorgemaakt, dus (voor zover ik dat niet toch al wist): wij kunnen niet meer stuk! Ik hoop nu weer tijd te hebben om jóuw boterhammen te smeren.

Curriculum Vitae

Esther Bianca Baart was born on November 7th 1972 in Wageningen, The Netherlands. In 1991 she graduated from secondary school (VWO-B) at the Stanislas College in Delft. From 1991 until 1997, she studied Biology at Wageningen University. The major subject, Cell Biology, included two six month training periods abroad. The first was entitled 'Chemically induced aneuploidy: Effects of Diazepam on *in vitro* maturing mouse oocytes' and supervised by Prof. Dr. U. Eichenlaub-Ritter at the Lehrstuhl für Gentechnologie/ Microbiologie, Universität Bielefeld, Germany. The second was entitled 'Female meiosis in two mutant mouse systems' and supervised by Dr. P. Hunt en Dr. T. Hassold at the Dept. of Genetics, Case Western Reserve University, Cleveland, USA. Furthermore, six months were spent at the Dept. of Genetics, Wageningen University, supervised by Dr. P. de Boer to work on a project entitled 'Inaccurate meiotic chromosome synapsis around the translocation breakpoints in male mice heterozygous for a reciprocal translocation'.

After graduation, she worked for three years at the same department under supervision of Dr. P. de Boer, researching both female and male mammalian meiosis and developing a mouse model system for studying fertilization and embryo development after ICSI. From January 2001 till November 2005, the research described in this thesis was performed at the Dept. of Obstetrics and Gynaecology and the Dept. of Clinical Genetics of the Erasmus MC in Rotterdam, under supervision of Dr. E. Martini, Dr. D. Van Opstal and Prof. B.J.C.M. Fauser. During this time, she also started her training as an embryologist at the IVF laboratory. She currently works as a researcher and an embryologist in training at the Dept. of Reproductive Medicine and Gynecology, University Medical Center, with Prof. N.S. Macklon and Prof. B.C.J.M. Fauser. She is married to Holger Jahr and they live in Delft.

Abbreviations

ANOVA	analysis of variance
ART	assisted reproductive techniques
ASRM	American Society for Reproductive Medicine
BUB	budding uninhibited by benomyl
CCMO	Dutch Central Committee on Research Involving Human Subjects
CD	Cycle day
CGH	comparative genomic hybridization
DAPI	4',6-diamidino-2-phenylindole
DMF	dimethylformamid
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
E_2	17β-oestradiol
ESHRE	European Society for Human Reproduction and Embryology
ET	embryo transfer
FISH	fluorescence *in situ* hybridization
FRET	fluorescence resonance energy transfer
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotropin
ICSI	intracytoplasmic sperm injection
IU	international unit
IVF	*in vitro* fertilization
LH	luteinizing hormone
MI	meiosis I; the first of two cell divisions resulting in haploidization of the genome, also referred to as the reduction division
MII	meiosis II, the second cell division during meiosis, also referred to as the equational division
MAD	mitotic arrest deficient
mRNA	messenger ribonucleic acid
OHSS	ovarian hyperstimulation syndrome
PB	polar body
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGD	preimplantation genetic diagnoses
PGD-AS	preimplantation genetic diagnoses for aneuploidy screening
PGS	preimplantation genetic screening
QF-PCR	quantitative fluorescent-polymerase chain reaction
RCT	randomized controlled trial
recFSH	recombinant human FSH
RT	room temperature
SAC	spindle assembly checkpoint
sc	subcutaneous
SD	standard deviation
SSC	standard saline citrate
SYCP3	synaptonemal complex protein 3
WGA	whole genome amplification

Appendix

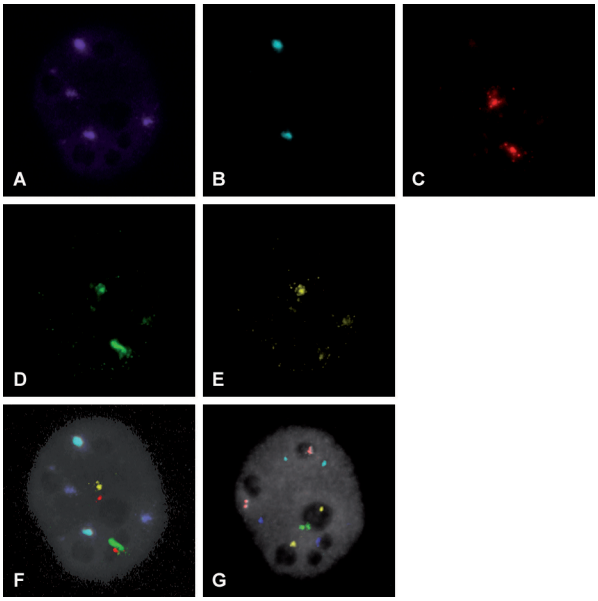

Figure 2.1.1. (A-F) Blastomere nucleus after the first round of FISH, showing the normal amount of signals for chromosome 1 (aqua), 7 (blue), 15 (red), X (yellow) and Y (green). DNA is counterstained with DAPI. (A) Image obtained after viewing with the blue filter set. The signals from the Pacific Blue fluorochrome are also clearly visible. (B, C, D, E) Images obtained after viewing with the aqua, red, green and gold filter sets, respectively. (F) Combined image of the first round of FISH after background correction. (G) Same nucleus after the second round of FISH, showing the normal amount of signals for chromosome 13 (red), 16 (aqua), 18 (blue), 21 (green) and 22 (gold).

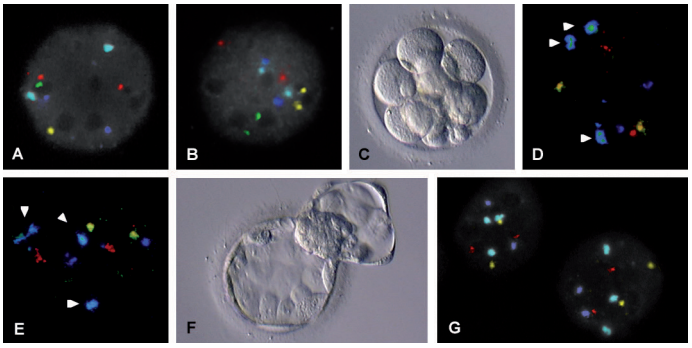

Figure 2.2.1. (A) Blastomere nucleus after the first round of FISH, showing the normal amount of signals for chromosome 1 (aqua), 7 (blue), 15 (red), X (yellow) and Y (green). DNA is counterstained with DAPI. (B) Same nucleus after the second round of FISH, showing the normal amount of signals for chromosome 13 (red), 16 (aqua), 18 (blue), 21 (green) and 22 (gold). (C) Light micrograph showing good quality day 3 embryo before biopsy. (D and E) Nuclei of two blastomeres from this embryo after biopsy and the first round of FISH, showing three signals for chromosome 1 (aqua, small arrowheads). (F) Light micrograph showing the same embryo on day 5. (G) Two representative nuclei of this embryo on day 5 after the first round of FISH. All nuclei showed an extra signal for chromosome 1 (aqua). DNA is counterstained with DAPI.

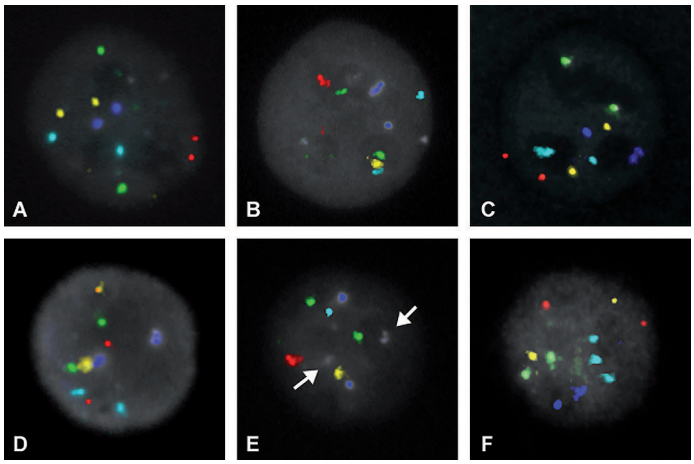

Figure 2.4.1. Nuclei from blastocyst stage embryos after cryopreservation. (A,D) Blastomere nuclei after the first round of FISH, showing signals for chromosomes 2 (yellow), 6 (aqua), 10 (green), 14 (red) and 17 (blue). DNA is counterstained with DAPI. (B,E) Same nuclei after the second round of FISH, showing signals for chromosomes 1 (aqua), 7 (blue), 15 (green), X (red) and Y (yellow). DNA is counterstained with DAPI. (C,F) Same nuclei after the third round of FISH, showing signals for chromosomes 13 (red), 16 (aqua), 18 (blue), 21 (green) and 22 (gold). (A,B,C) Male nucleus showing the expected number of signals for all the chromosomes investigated. (D,E,F) Male nucleus showing a missing signal for chromosome 7 in the second round and an extra signal for chromosome 16 in the third round. After the second round, a remnant of the signal for chromosome 17 is visible (arrows).