Chapter 1

Introduction

Why Do We Bother with Embryo Selection?

Arne Sunde and Kersti Lundin

1.1 The Early Days

When fertilization in vitro was developed by the pioneers – Dr. Robert G. (Bob) Edwards, Miss Jean Purdy, and Dr. Patrick Steptoe – their primary focus was to obtain oocytes that could be successfully fertilized in laboratory conditions. Embryo culture and embryo selection were just secondary aims at that time. In the early 1970s, the first attempts to obtain a pregnancy after IVF were in cycles in which ovarian stimulation was performed by administration of human menopausal gonadotropins (hMG) [1]. This resulted in multiple follicular growth and thus more than one oocyte available for fertilization. However, the first clinical pregnancy obtained was tubal. Suspecting that an hMG-stimulated cycle increased the likelihood of an ectopic pregnancy, the next attempts were made in natural cycles. Carrying out IVF in natural cycles presented several challenges including the requirement for careful monitoring of follicular growth by repeated measurements of pituitary and steroid hormones. A single follicle, or at most two, was then aspirated laparoscopically just prior to spontaneous ovulation. However, as remains the case today, not all follicular punctures resulted in an oocyte, not all oocytes were fertilized, and not all fertilized oocytes developed into an embryo capable of implantation [1]. With only one or two oocytes available, the success rate per attempt was therefore exceedingly low and only after more than 200 unsuccessful attempts [2], Louise Brown, the first child conceived in vitro, was born [3]. Because of the complexity and the low success rate performing IVF in a natural cycle, hMG-stimulated cycles were again introduced [4]. A timeline of important events in the development of IVF with associated technologies is shown in Figure 1.1.

1.2 Gametogenesis, Fertilization, and Embryo Quality

Follicular growth is a process that takes months; a recruited primordial follicle may take more than 180 days to develop into a preovulatory follicle [5]. The great majority of growing follicles arrest at various stages during development. From the approximately 300 000 follicles present in the ovary of a newborn girl, only around 450 will ultimately ovulate. Just prior to ovulation the cohort of mature follicles are still dependent on continuous follicle-stimulating hormone (FSH) stimulation. One of the follicles in the maturing cohort then starts to express factors that will inhibit the development of the other follicles in the cohort. This follicle now becomes dominant in the sense that it suppresses the maturation of the other follicles. Usually only this follicle is then able to respond to the luteinizing hormone (LH) surge and ovulate [6]. The rationale behind hormonal stimulation of the ovary is that continuous administration of FSH in the preovulatory period will rescue some of the follicles in the maturing cohort that otherwise would have undergone atresia, thereby resulting in multiple follicles from which to retrieve oocytes (Figure 1.2).

In fetal life, the oocytes, enclosed in primordial follicles, enter meiosis but shortly thereafter become arrested at the diplotene phase of prophase I. For some oocytes, this arrest will last for many years. The last oocytes that are ovulated just prior to menopause may have been in meiotic arrest for five decades. Resumption of meiosis is triggered by the LH surge and progression to metaphase II (MII) occurs over the ensuing 36 hours or so, in preparation for ovulation or, in an IVF cycle, for retrieval. Resumption of meiosis from MII to telophase II (TII) is triggered by the sperm and is finalized when the oocyte is fertilized.

Oogenesis in humans is far from a perfect process. It is complex and many oocytes that are ovulated do not have the correct number of chromosomes. The genetic quality of human oocytes in relation to the age of the woman has a u-shaped curve, lower in young and older women. The rate of aneuploid oocytes is lowest around the age of 30, at around 20%. In

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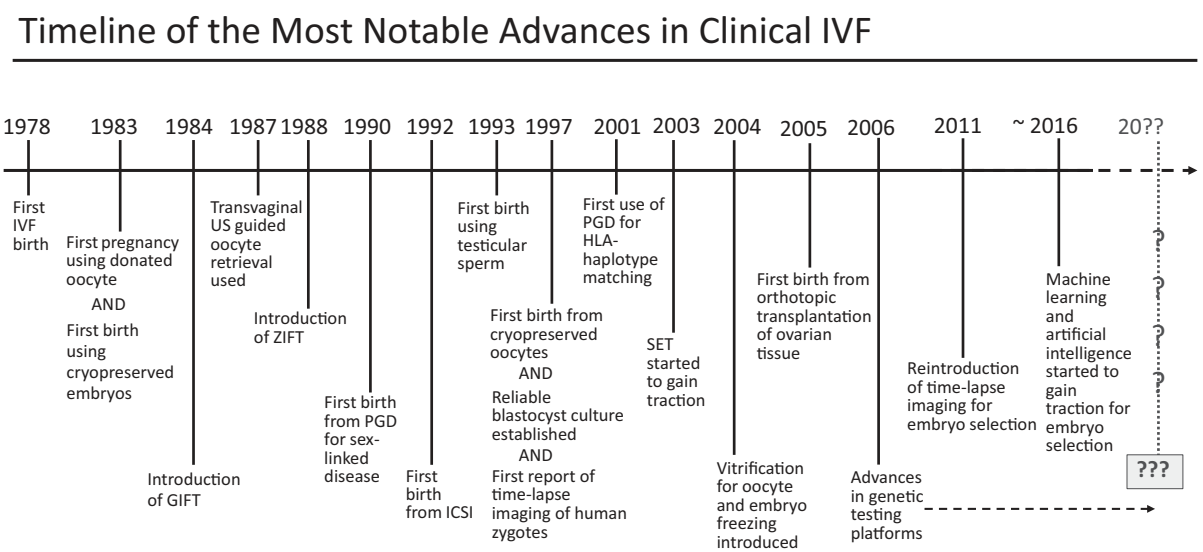
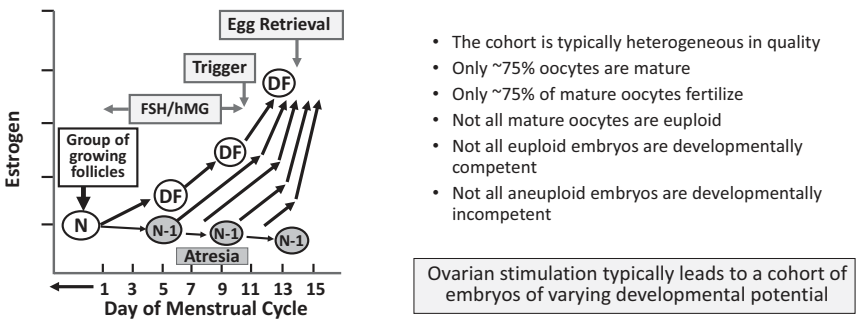


Figure 1.1 Timeline of the most notable advances in clinical IVF (Courtesy of Dr. Catherine Racowsky)



hCG = human chorionic gonadotropin; DF = dominant follicle; N = number of follicles in the cohort
FSH = follicle-stimulating hormone; hMG = human menopausal gonadotropin

Figure 1.2 Follicular maturation
Growth and maturation of human follicles is a complex process that takes over 180 days. The majority of the growing follicles will arrest and become atretic. Only 1 in 1 000 follicles in an ovary of a newborn will ever reach ovulation. Despite this seemingly strong selection of growing follicles, the frequency of aneuploid human oocytes is from 25% to 80% depending on the age of the woman. (Adapted from [7], with permission).

contrast, the rate of aneuploid MII oocytes in women who are very young is above 55% [8] and this reaches upward of 80% in women greater than 42 years of age [9]. In addition to the correct number of chromosomes, a competent MII oocyte must have undergone cytoplasmic maturation so that after fertilization it can support early embryogenesis.

Spermatogenesis takes on average 60 days from the germinal stem cell stage to ejaculated spermatozoa [10]. This is a complex process that involves several rounds of mitosis, followed by meiosis, and chromatin remodelling, and synthesis of m-RNA and proteins, all packed into a highly specialized cell, the spermatozoa. As with oogenesis, nondisjunction may occur during spermatogenesis, resulting in an aneuploid sperm cell, although the frequency of aneuploid sperm is generally lower than for oocytes. It has been shown that in men with reduced semen quality the frequency of aneuploid spermatozoa is higher than in men with normal semen quality [11]. In addition, a range of other factors such as DNA fragmentation, chromatin structure, histones, protamines, epigenetic profiles, and Y-chromosome microdeletions may influence fertilization, embryo development, and/or embryo quality [11].

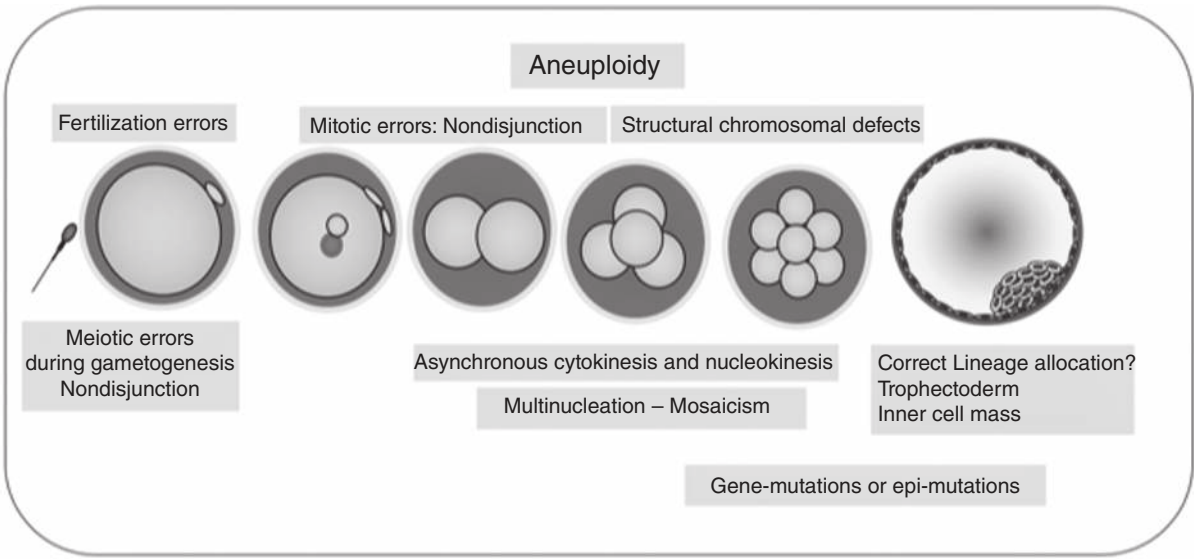


Figure 1.3 **Errors in embryo development**
Human gametogenesis is far from perfect and mature gametes may contain the wrong number of chromosomes (aneuploid). Fertilization and early cleavage may introduce new errors during mitosis (nondisjunction), frequently leading to embryos where at least one blastomere is aneuploid. Culture conditions may also have an influence on embryo viability. The embryologist working in clinical IVF is therefore faced with a situation where the embryos obtained may vary greatly with respect to implantation potential. The challenge therefore is to rank embryos so the best ones may be selected for fresh transfer and for cryopreservation.

Fertilization and early embryo development are complex processes where errors may occur that can compromise the developmental potential of the embryo (Figure 1.3). When this process takes place in vitro, iatrogenic factors can add to this. The proportion of embryos that are aneuploid or mosaic euploid/aneuploid at the cleavage stage and/or at the blastocyst stage varies according to the age of the woman and has been reported to be higher in cleavage stage embryos than in blastocysts [12]. Aneuploid embryos have been shown to have a lower implantation rate than euploid embryos [13].

Fecundity in the human is around 20% per cycle. This means that a couple with normal reproductive potential and regular intercourse have approximately a 20% chance per ovulation of obtaining a delivery. This is lower than most animal species, even when compared to the nonhuman primates such as the chimpanzee [14]. The low fecundity rate in humans may perhaps not come as a surprise when one considers that we have far from perfect gametes. There are reasons to believe that this has been the case for millennia and is part of being human and not due to a recent decline in the genetic quality of our gametes [14].

Even today, more than 40 years after the birth of Louise Brown, there are apparently still large differences in success rates between assisted reproduction technology (ART) laboratories and clinics as well as between countries. Laboratory variables such as the oxygen level, pH, osmolality, temperature control, air quality, and the culture media all have an influence on embryo development in vitro [15, 16, 17]. Suboptimal culture conditions will affect many steps of the process, and thereby reduce the likelihood of obtaining a delivery after IVF. As an example, a comparison of aneuploidy rates in embryos obtained after fertilization of oocytes from young oocyte donors show large differences between ART laboratories [18]. The cause of this is largely unknown but it cannot be ruled out that it is partly due to iatrogenic factors. However, prospective randomized studies have also shown that the composition of the culture media may influence the developmental speed and the epigenetic profile of embryos in vitro as well as have an influence on the phenotype of the children born [19, 20, 21].

1.3 Number of Transferred Embryos, Multiple Birth Rates, and Health of the Children

An attempt to obtain a delivery of a child using fertilization in vitro requires highly skilled professionals, expensive drugs, and a sophisticated laboratory. The financial burden and the emotional stress associated with assisted reproduction may be substantial. Couples that seek to be treated by assisted reproduction therefore obviously want to be offered a treatment with a high probability of success. Due to the poor results in the early days of IVF, several embryos were often transferred, to provide a reasonable chance of obtaining a pregnancy.

Reassuringly, now with more than 40 years of development of in vitro fertilization, implantation and live birth rates have been steadily improving. Data from the European Society of Human Reproduction and Embryology (ESHRE) European IVF-Monitoring Consortium (EIM) registry show an increase from a clinical pregnancy rate of 26% in 1997 (data from 18 countries; www.eshre.eu/Data-collection-and-research/Consortia/EIM/Publications) to 33% in 2018 (39 countries; [22]). This might seem like a rather modest increase for a 20-year period, but it is important to note that, during the same time, the single embryo transfer rate increased from 11% to 51%. Results from the United States reported a delivery rate of 23% for 1995 (www.cdc.gov/art/artdata/index.html) compared to 37% reported for 2019 [23].

Calculations from the ESHRE EIM datasets show that the implantation rate doubled during these years, from 12% in 1997 (mean numbers of embryos for transfer, 2.6) to 25% in 2014 (mean number of embryos transferred, 1.8). This considerable improvement can be attributed to several factors: improved stimulation strategies resulting in better oocyte quality and improved endometrial receptivity, improved handling and culture conditions for gametes and embryos, and optimization of assessment and selection of embryos. There are however considerable variations regarding results among countries, showing that there is still much room for improvement.

Unfortunately, the downside of the increased implantation rates was the dramatic increase of multiple births. The profession did not adjust the numbers of transferred embryos to the level of implantation quickly enough, and many clinics continued to transfer 3–4 or even more embryos, despite the improved implantation results. In the USA, data published by the Centers for Disease Control and Prevention (CDC) show that, in 2011, 46% of infants born from treatment with IVF were either twins or higher-order multiples (www.cdc.gov/art/artdata/index.html). The ESHRE EIM data show in the latest report (data from 2018) that although the mean multiple delivery rate has decreased from 29.6% to 12.5% for 2018, the variation is large, ranging from 1.9% to 27.4% [22]. It can be seen from these data that the multiple birth rate has a clear correlation with the number of embryos transferred, but interestingly not with improved delivery rates. The same trend has occurred in the USA where the CDC data show that the multiple delivery rate decreased to 22% in 2014, with a further decrease to 13% in 2018 without an obvious impact on live birth rates [23].

The increasing multiple delivery rates and the concurrent issues with health of the offspring have been established in many follow-up studies (e.g. [24–29]), highlighting the significant maternal, fetal, and neonatal risks associated with these pregnancies. The accumulated data finally led to regulation in many countries. In Europe, this development was led by Finland and Sweden. In 2003, the National Health Authorities in Sweden stated that transfer of a single embryo was strongly recommended, and that more than two embryos should not be transferred. This led to a dramatic decrease in national multiple rates, dropping from 25% to 5% in only four years [30]. Despite this, there has been a concurrent steady increase in live birth rates. In the USA, the Society for Assisted Reproductive Technology and the American Society for Reproductive Medicine have issued recommendations to lower the number of embryos for transfer. This has resulted in a single embryo transfer rate of 77% for 2019 in parallel with increasing live birth rates, but again with a large variation between states (www.cdc.gov/art/artdata/index.html; [23]).

The declining multiple birth rates have resulted in a positive impact for the health of the children. Follow-up studies of offspring have shown that perinatal risks for ART offspring have decreased and overall health has improved. An analysis of more than 92 000 children born from ART in Denmark, Finland, Norway, and Sweden from 1988 to 2007 found a

decline in numbers of preterm birth, low or very low birthweight, stillbirths, and perinatal death [31].

1.4 Embryo Selection and Cumulative Results

When extended culture to the blastocyst stage began to be implemented, the slow freezing technique used at that time unfortunately resulted in limited survival of blastocysts after thawing. With the introduction into clinical IVF of the vitrification technique (which had been used in veterinary medicine for quite some time), blastocyst cryosurvival rates of over 90% were reported, with much increased implantation rates. This positive development in turn led to a further increased use of blastocyst culture in many clinics and countries, and in addition to an increased possibility and willingness from both patients and clinics to aim for single embryo transfer. In a high-quality ART program, culture of all excess embryos to day 5 or 6 for blastocyst cryopreservation results in >60% likelihood of at least one cryopreserved extra blastocyst (Ahlström and Lundin, personal observation). Thus, adopting this approach provides a good chance of a subsequent frozen thaw cycle adding to the likelihood of achieving at least one pregnancy from a single cohort of embryos.

Several studies have shown that for women considered to have a good prognosis, the cumulative live birth rate after single embryo transfer, followed by the transfer of a cryopreserved embryo in a subsequent cycle, is comparable to that after double embryo transfer, but with a significantly lower risk of multiple pregnancy (e.g. [32, 33, 34]). Thus, with the current excellent survival rates and improved implantation rates for cryopreserved blastocysts, we now have the possibility of transferring the embryos from one oocyte retrieval sequentially, one by one, without risk of them failing to survive during the freeze–thaw procedures. Somewhere along the way an embryo with good potential for implantation will presumably be transferred, and the cumulative pregnancy and delivery rate will be similar irrespective of embryo selection. However, for patients having a large number of good-quality embryos, transfer of them one by one without selection might in the end lead to a high number of transfers, possibly including a series of failed implantations and/or miscarriages, causing stress, as well as being expensive and time-consuming. There is currently a lack of good-quality studies investigating the relationship between embryo selection algorithms and early miscarriage and cumulative delivery rates. However, assessment, ranking, and selection of embryos will in most cases shorten the time to live birth as well as reduce stress and save resources for both the patient and the care provider.

An additional positive aspect from the extended culture of all extra embryos is that fewer embryos are discarded at an early stage. It has been shown that around 25–35% of so-called poor-quality embryos on day 2/3 can give rise to good-quality blastocysts [35–38], with potential for implantation and live birth equal to those of blastocysts from high-quality embryos. Thereby, today embryo/blastocyst assessment is more a method of ranking potential biological quality, instead of selecting at an early stage and discarding the rest. The ranked embryos/blastocysts can then be transferred one by one, starting with the one considered to be of highest viability.

1.5 Future Challenges

There has been a shift in how we assess, select, and utilize embryos. In the early days of IVF, due to suboptimal culture conditions, the most common practice was to transfer and to cryopreserve any extra-good-quality embryos, on day 2 or 3 postfertilization. Poorer-quality embryos were discarded. Gradually, as stated above, with improved culture media and culture conditions, it has become more common to extend culture until the blastocyst stage, so as to "select" the more viable embryos. However, it might be argued that our culture conditions in vitro are perhaps still not good enough to support development of all embryos and/or there might be differences between patients. It is possible that some embryos are more sensitive to the in vitro conditions and would still have been capable of developing and implanting if transferred at an earlier stage.

Thus, the challenge of embryo selection is how far to go, and how many "add-ons" we really need – and whether they are evidence based – to find the best embryo. Assessment of embryos is still mainly performed by embryologists scoring according to developmental and morphology criteria, either all the way from the gamete stage to the transfer/cryo stage, or only at certain predetermined times. Time-lapse (TL) methodology, which allows images to be taken and stored throughout the whole culture period, has enabled

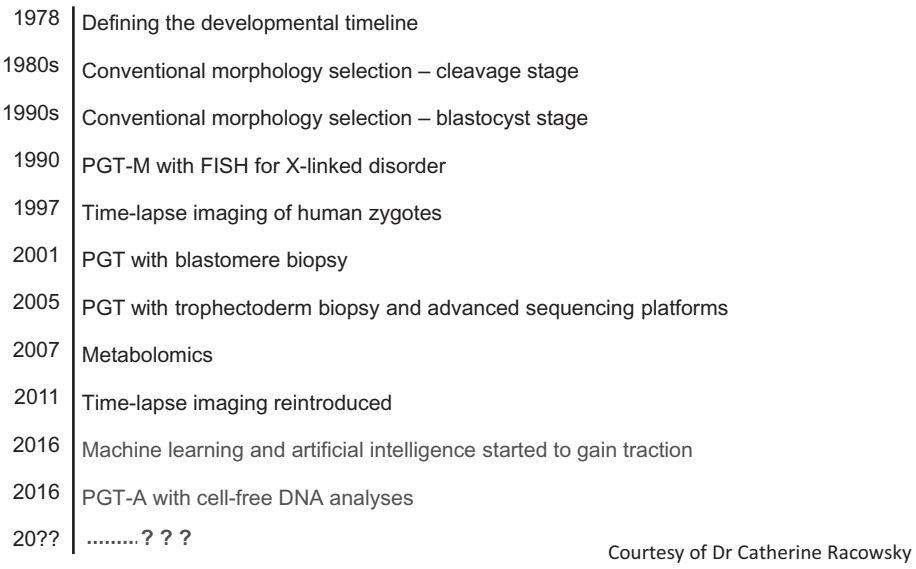


Figure 1.4 Where are we with embryo selection going forward? (Courtesy of Dr. Catherine Racowsky)

the scoring of embryos at any time point. Although the TL technology thereby facilitates the work and the logistics of the IVF lab, it has so far not been conclusively demonstrated to improve embryo selection and downstream implantation or live birth rates [39, 40]. Still, TL imaging can reveal other developmental events that may reflect compromised implantation potential such as irregular cleavage patterns [41, 42]. In addition, the technology is useful for training staff and for validating introduction of a new constituent in the IVF laboratory, such as a culture medium.

Another highly debated method of selection is PGT-A screening. This technique to select euploid embryos has evolved much during the last few years. Despite the logical reasoning behind the method, its usefulness and value in specific patient groups is still under discussion and concerns regarding interference of mosaicism in interpretation of results prevail. Furthermore, the technique is quite invasive, with cells being removed from the embryo. Noninvasive methods, involving analysis of cell-free DNA in the spent culture medium [43, 44, 45) or blastocoelic fluid [46] are underway. However, currently no such methods are routinely applied for clinical use.

There is much interest in many areas of our society in machine learning, or so-called artificial intelligence. Indeed, this has evoked great interest in ART, and research is currently ongoing using datasets from time-lapse documentation to "feed" and train computers and to try to find patterns that correlate with embryo development and live birth [47, 48, 49]. If proven to have utility and application across clinics, this would effectively remove the bias of the current subjective assessment by the embryologist. If a highly predictive model could be generated, additional "add-ons" for selection might not even be needed in the future (Figure 1.4).

1.6 Summary

Gametogenesis, fertilization, and embryo development are all complex processes, which give rise to a heterogeneous cohort of embryos, with varying potential for implantation and live birth. The use of blastocyst vitrification has dramatically increased cryosurvival rates, whereby all embryos can be transferred sequentially, and the cumulative success rates will be similar irrespective of embryo selection. However, efficient embryo selection will shorten the time to pregnancy and live birth since the embryos with the highest potential will be transferred first. Embryo culture and selection algorithms may also have an influence on the utilization rate (embryos transferred fresh and cryopreserved counted per all embryos) and thereby on the resources utilized. An efficient embryo selection and ranking system will hopefully aid in continuing to lower the multiple birth rate from IVF and improve the health of the offspring.

Key Messages

- Gamete quality and culture conditions of gametes and embryos will influence the clinical success rate.
- A proportion of embryos resulting from an IVF procedure will not have the capacity to implant or sustain a pregnancy.
- Selection of embryos will shorten the time to live birth but not increase the cumulative success rate.
- Increasing success rates and cryosurvival rates will stimulate increased use of single-embryo transfer.
- Single-embryo transfers have led to decreased perinatal risks for ART offspring and improved overall health.

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Chapter 2

Embryo Developmental Programming

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2.1 Introduction

It is over 40 years since the successful fertilization of a human egg in vitro and development of the resulting human preimplantation embryo led to the birth of a healthy baby. In the intervening years, assisted reproduction technology (ART) has become routine treatment for infertility worldwide with the birth of over 8 million babies to date [1] yet gaps remain in our understanding of the earliest stages of human embryogenesis. We know that human embryos vary in their potential to implant and lead to a healthy pregnancy, but are we confident that exposure to in vitro culture conditions does not compromise their inherent viability? Can we be certain that the embryo's intrinsic developmental program is sufficiently robust to withstand environmental insults imposed during ART, or does in vitro culture lead to genetic or epigenetic changes in the more vulnerable embryos, contributing to the known high rate of implantation failure and early pregnancy loss? May there even be long-term consequences for offspring? These questions underline the importance of detailed knowledge and understanding of the developmental program of the human embryo, and emphasize the vital need for continued study of the human preimplantation embryo in a research setting.

For obvious reasons, the human preimplantation embryo as the subject of pure research is a precious and limited resource. Consequently, while there is a wealth of knowledge derived from direct observations of human preimplantation embryos developing in vitro in a clinical setting, much of our perceived understanding of the cellular and molecular mechanisms underlying this early phase of human development is extrapolated from studies of other mammalian species, formerly the rabbit, and latterly the mouse. However, recent advances in molecular, genomic, and noninvasive imaging technologies have begun to lead to significant advances in the direct investigation, and as a result our understanding, of human development.

In the clinical setting, the varying developmental potential of individual human embryos presents clinical scientists with the challenge of developing methods for embryo selection, to enable distinction between those embryos destined to fail and those that have the potential to lead to a viable pregnancy. This chapter will summarize what is currently understood about the developmental program of the human embryo, setting the scene for an evaluation of the different approaches to embryo selection for use in ART treatment that will be considered in subsequent chapters.

2.2 Preimplantation Embryo Development

2.2.1 Fertilization

Penetration of the oocyte by a spermatozoon triggers the complex series of fertilization events that culminate in the formation of a diploid zygote from two haploid gametes. This series of events includes the establishment of the block to polyspermy, establishment of diploidy, securing the cytoplasmic constitution of the oocyte, syngamy, initiation of the developmental program, and culminates in the first cleavage division.

2.2.2 Cleavage

During the cleavage stages of development, the embryo remains the same size within the glycoprotein matrix, the zona pellucida, that forms its outer coating while its component spherical cells (totipotent blastomeres) halve in size at each division. Cleavage of the blastomeres is asynchronous, so that embryos with intermediate blastomere numbers (3-cell, and 5- to 7-cell stages) are seen before completion of the second and third cleavage divisions. During each