Chapter 1

Introductory Chapter: New Technologies for the Study of Embryo Cleavage

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69382

1. Introduction

After fertilization by sperm into oocyte combination, mammal embryogenesis is the process of cell division and cellular differentiation of the embryo that occurs during the early stages of development. In embryology, cleavage is the division of cells in the early embryo. This division from a one-celled zygote into 2, 4, 8, and 16 cells; morula stage; and finally into blastocyst stage until implantation in the uterus is called embryo cleavage. The zygotes of many species undergo rapid cell cycles with no significant growth, producing a cluster of cells the same size as the original zygote. The different cells derived from the cleavage are called blastomeres and form a compact mass called the morula. Cleavage ends with the formation of the blastula known as the blastocyst stage embryo that is yet to implant in the uterus and hence is also called preimplantation embryo.

In the last three decades, the development of assisted reproductive technology (ART) has created some new observations and novel discoveries in preimplantation embryos, especially during embryo cleavage. Preimplantation embryo development experiences a series of critical events and remarkable epigenetic modifications, and reprogramming of gene expression occurs to activate the embryonic genome. The alteration of these events often results in changes of embryo quality and morphology. At the cleavage stage, although morphological scores assigned using traditional criteria have little relationship with chromosome abnormalities [1], morphological evaluation is a major tool to assess embryo quality. Thus, many new observations and technologies have been developed. For example, in order to observe embryo morphology and to assess embryo quality, time-lapse imaging, and light-sheet microscopy have made it possible to visualize early mammalian development in greater detail and over longer time periods than ever before [2–4]. This book collects some new technologies and methods on the study of cleavage embryos to select high-quality embryos for transfer and to improve embryo implantation and pregnancy.

2. Observation of fertilized embryos to cleavage embryos

Since the first rabbit embryo culture was described in 1912 [5] and mouse zygote could be cultured in vitro to form blastocyst stage embryos [6, 7], embryo quality has become an important factor for pregnancy after the transfer of in vitro embryo into the uterus because embryo quality has a close correlation with transferred embryo implantation in uterus. Since the birth of the first "test-tube" baby, Louise Brown in July 1978, for which the 2010 Nobel Prize for Physiology or Medicine was awarded to Robert Edwards for developing in vitro fertilization (IVF) and embryo transfer (ET) to treat infertility in women with non-patent oviducts, in vitro embryo production (IVP) has been widely used in human infertility treatment and animal population reproduction and expansion. However, the success of assisted reproductive technology mainly depends on the production of viable embryos with high implantation potential. More importantly, choosing the best embryo for transfer has become the major challenge in IVF. In the early embryo culture, the embryo quality assessment was mainly based on the morphological criteria of the transferred embryo. Thus, performing a serial observation of embryo morphology is a common technique for embryologists to evaluate embryos and has been considered as a key predictor of implantation and pregnancy [8–10]. For a long term, embryologists performed embryo quality and morphology assessments by taking the embryos out of the incubator and placing under a microscope. Besides morphology observation, the researchers are interested in a series of studies on cell nuclear change, gene activation and expression, cytoplasmic protein expression, blastomere differentiation, and so on. However, these studies often result in the death of embryos. For example, in our early study which observed microspindle change after the sperm entry into the egg or the activation of oocyte, the fertilized zygotes or activated eggs needed to be fixed on the slide and stained with immunocytochemical fluorescein and laser confocal microscopy [11]. Our research clearly showed the alteration of microtubule and chromatin after bovine oocyte activation and introcytoplasmic sperm injection (ICSI; **Figure 1**). The sperm into oocyte or calcium ionophore and ethanol may activate oocyte and cause extrusion of the second polar body. In order to observe the time of the second polar body, we stained various stages of oocytes after activation. The result showed that after 5-hour postactivation, the second polar body may be completely extruded (**Figure 2**).

The study of gene expression often requires to isolate mRNA or protein from embryos [12–14]; hence, embryos needed to be lysed and no embryo would survive. In order to study the cell differentiation on moral and blastocyst stage embryos, a double staining with fluorescein microscopy method has been used to distinguish inner cell mass (ICM) from trophoectoderm (TE). The numbers of two different cells may be counted based on different colors (ICM as blue and TE as pink, **Figure 3**).

These research methods finally damage all embryos, and it is impossible to apply these methods to clinical practice. Thus, current embryo quality assessment is based primarily on the morphological criteria of transferred embryos, which includes three major parameters such as blastomere regularity, fragmentation, and cytoplasmic granularity [15]. Also, embryo cell numbers on different culture day and multinuclearity can be considered to evaluate embryo quality [16, 17]. Several reports have documented the association between the morphological

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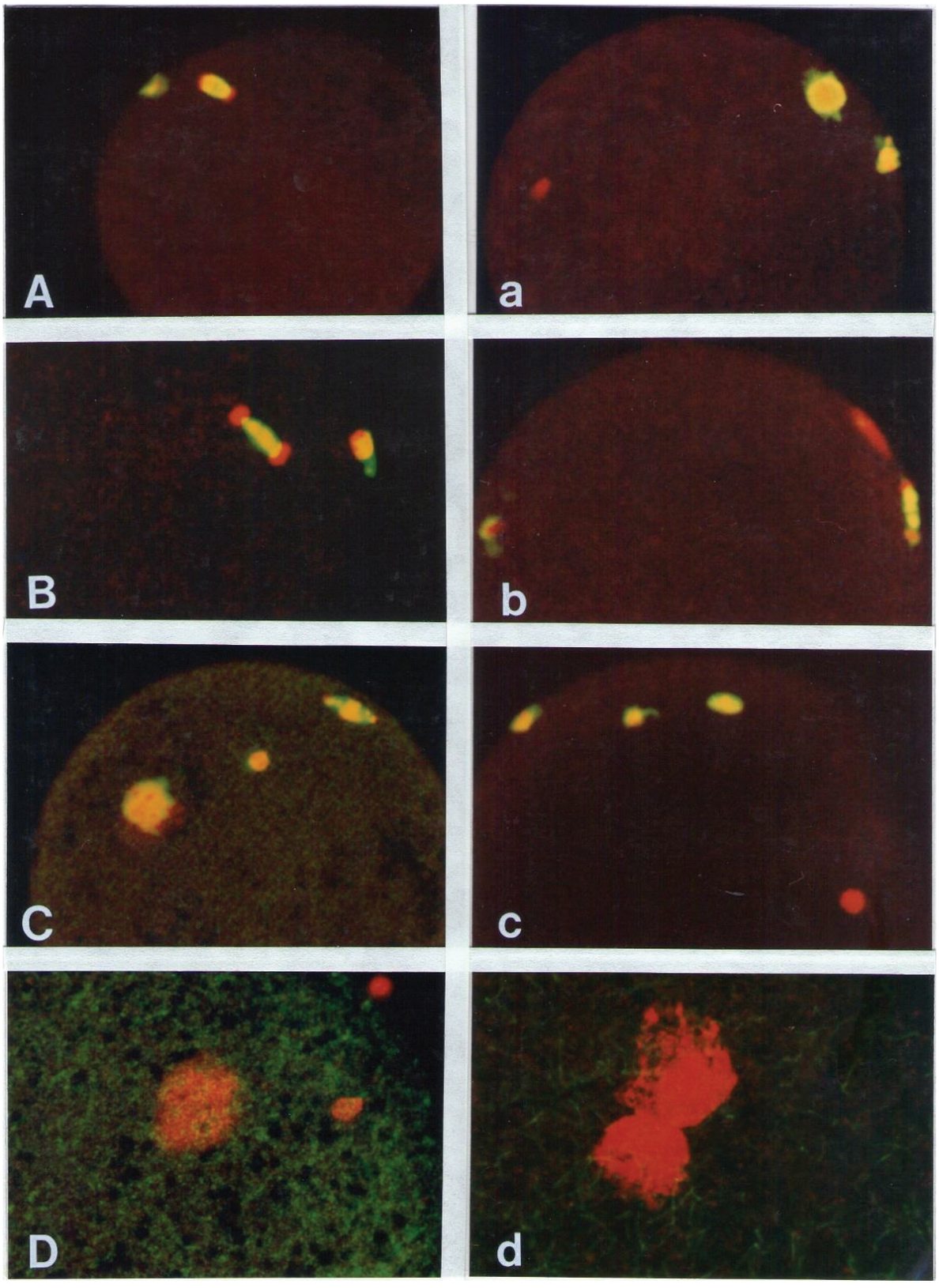

Figure 1. Laser-scanning confocal microscopy of spindle and chromatin changes at the various time post-activation and intracytoplasmic sperm injection (ICSI) in bovine. Capital letters (Left) indicate the change post-activation and small letters (Right) indicate after ICSI. A/a showed at 0.5 h, B/b is 2 h, C/c is 3 h, and D/d is 7 h post-activation or ICSI. Prenucleus in activated egg and prenuclei in ICSI egg have appeared with red color.

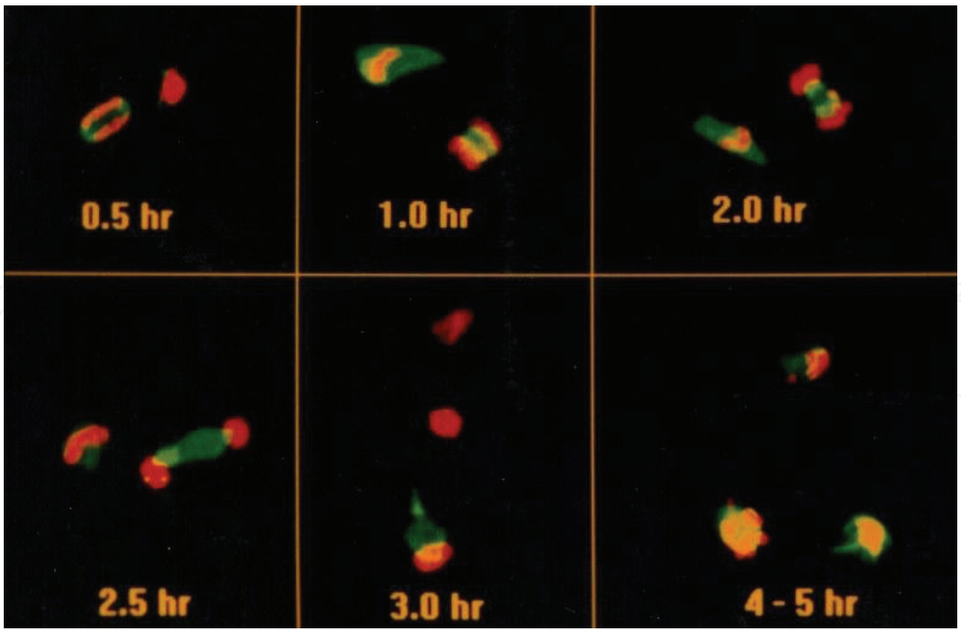

Figure 2. Laser-scanning confocal microscopy of spindle and chromatin changes at the various times post activation in bovine. At 0.5 h after activation, the chromosomes of spindle start to divide, and the completion of spindle division needs about 3 hours and the second polar body may be extruded at about 5 hours. The red and green together indicate the spindle, and the red point indicates the first polar body.

characteristics of cleavage stage embryos with pregnancy success. Thus, this is currently the basic method for embryo quality assessment in human IVF and animal in vitro embryo production. However, although this is easily practiced, it frequently takes embryos out of the incubator which leads to concerns for the safety and stability of culture conditions [18]. Also, some key points of embryonic development may be missed during observation. Evaluation of cleavage embryos during culture and before embryo transfer is an important clinical practice. Currently, the major assessment of in vitro fertilized embryos is visual observation using microscopy. In recent years, various time-lapse microscopy incubators are being used in human IVF clinic to monitor all the steps of embryo growth and development. Although preimplantation embryo diagnosis and screen (PGD/PGS) technologies have been applied in human embryo selection practice to improve pregnancy rate, these techniques are invasive for embryos. Finding another noninvasive method to select a good embryo will be very useful in human ART practice. Sallam et al. [19] reviewed noninvasive methods for embryo selection and evaluated these methods in the light of the best currently available evidence to find out whether any of them is ripe for replacing or supplementing the time-honored method of morphological assessment. Thus, we need more powerful tools to estimate the morphokinetic markers of embryos.

2.1. Embryo cleavage morphokinetics based on time-lapse imaging

For decades, researchers have attempted to follow the development of multicellular organisms from fertilized eggs into adults. While scientists had explored individual steps of this process, no method existed to enable them to model the whole process of development live. Currently, advances in light-sheet microscopy reported in two *Nature Methods* papers have

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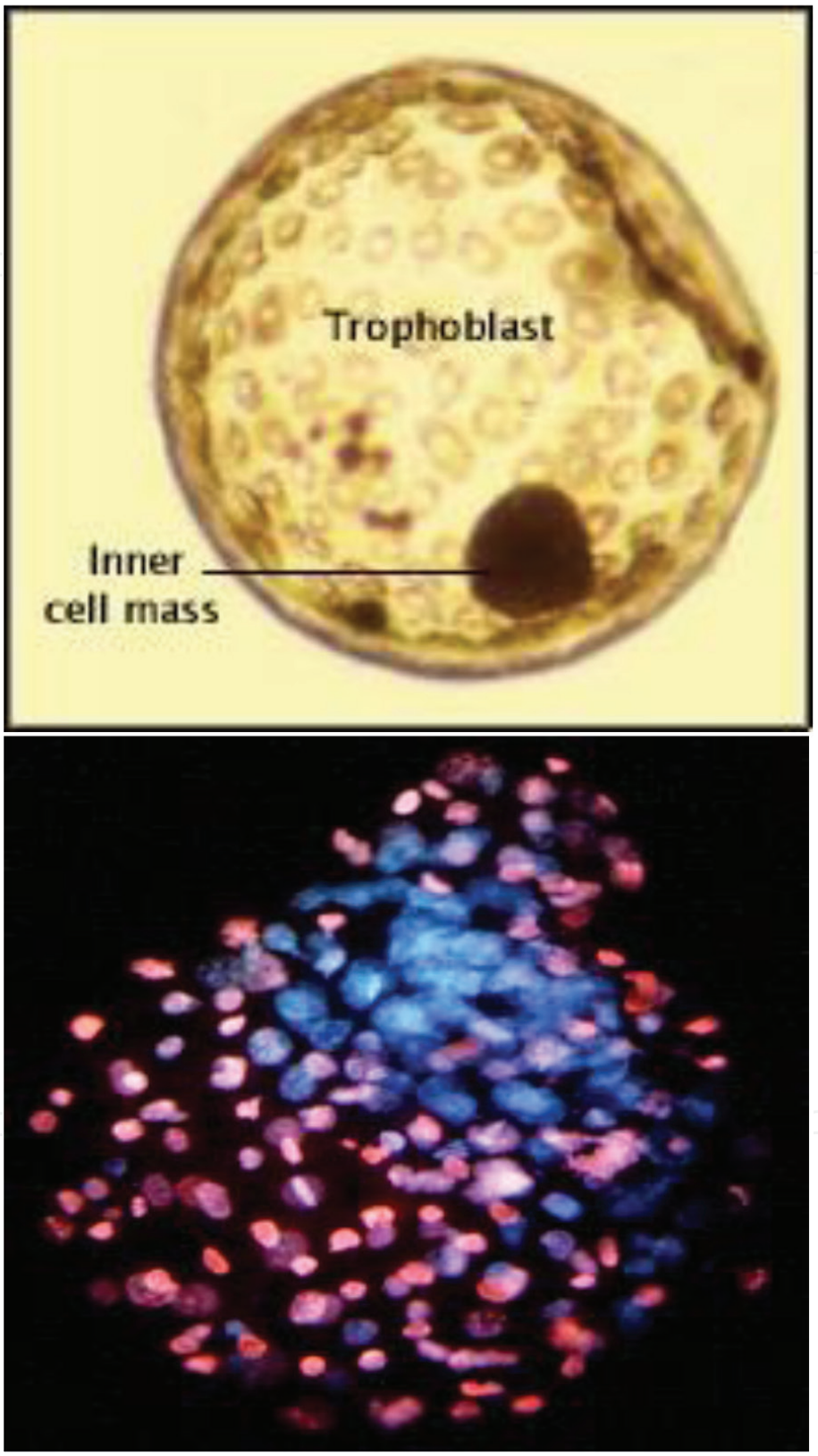

Figure 3. Distinguishing different cells in bovine blastocyst embryos with double staining. Top figure shows a blastocyst embryo with marked inner cell mass (ICM) and around trophectoderm cells (TE). Bottom figure shows double-stained bovine blastocyst embryo with blue as ICM and pink as TE cells. The picture on top is from webpage search, and the author greatly appreciates Prof. Fuliang Du's courtesy for the unpublished bottom photo.

enabled researchers to visualize early development in great detail [3, 4]. Recent light-sheet microscopes use a sheet of laser light to illuminate a thin section of a sample and capture the entire plane in one snapshot. This allows them to use much less light than confocal or two-photon microscopes. It is very fast but also very gentle to perform extremely well in multiple critical ways at the same time [20]. For imaging the development of entire embryos like those of *Drosophila*, zebrafish, and mice, this new multiview imaging technique is fantastic.

Time-lapse imaging is another noninvasive, emerging technology that allows 24-hour monitoring of embryo development, offering the possibility of increased quantity and quality of morphological information without disturbing the culture condition [21]. The time-lapse microscope is very useful for embryo development observation. In the last decade, many human IVF clinics or centers have started to use time-lapse imaging to monitor embryo growth and division during in vitro culture and finally to select good quality embryo for transfer according to record data and pictures. This technique has been reported to be able to improve transferred embryo implantation and pregnancy [22, 23]. Based on time-lapse record for embryo cleavage, normal embryo cleavage speed may be determined. Thus, in the second chapter of this book, the timing of embryo cleavage has been outlined based on morphokinetic markers by the time-lapse monitor. According to this embryo cleavage timing outline, embryologists may clearly know at which stage an embryo should be at various time points. Thus, an optimal quality embryo or a high-potential implantation embryo may be selected for transfer to obtain a higher pregnancy rate. Using time lapse continuously and frequently recording system, some morphokinetic markers can be revealed in time-lapse system. For instance, the rapid division of embryo cells at a given time often results in lower implantation rate. In the normal situation, the division from zygote into 2–3 cells requires about 10–11 hours of time, but Rubio et al. [21] found that some embryos just spend about 5 hours to complete this division, and these embryos have much lower implantation rate than normal division embryos (1.2% vs 20%). Also, embryo unequal cleavage which is defined as an abruption of one blastomere into three daughter blastomeres or an interval of cell cycle less than 5 hours often produces significant lower implantation potential [24]. Thus, we may use these more precise morphokinetic markers to distinguish the embryo quality.

The third chapter further examines and verifies whether time-lapse imaging technology is useful for the selection of "top-quality" embryos for transfer to improve ART outcome rather than conventional morphological evaluation. Interestingly, the possible correlations between the sex of the embryo, embryo fragmentation, treatment protocols, different culture media, and embryo morphokinetics have been evaluated based on some new researches on time-lapse imaging facilities. Furthermore, various algorithms and predictive models designed in ART cycles with time-lapse imaging are also discussed. For example, a lot of researches on animal and human embryonic development speed by ordinary morphology observation showed that male embryos grow faster than female embryos [25–27]. However, current time-lapse imaging observation may provide more detail and exact information on the difference in male and female embryos during early divisions. Although female embryos showed late cleavage (t8), morula (tM), and blastocyst stage morphokinetic parameters, they presented earlier expansion than males. Thus, the key time points of observation is related to embryo gender development. Interestingly, the authors designed a model according to the time of

second synchrony and morula formation with four subgroups to predict the probability of an embryo being female.

In order to further study and explore morphokinetics of embryo cleavage, the fourth chapter discusses some methods for **spatiotemporal analysis of embryo cleavage in vitro.** Automated or semiautomated time-lapse analysis of early stage embryo images during the cleavage stage can give insight into the timing of mitosis, regularity of both division timing and pattern, as well as into cell lineage. Simultaneous monitoring of molecular processes enables the study of connections between genetic expression and cell physiology and development. By time-lapse imaging data and analytical software, a four-dimensional video sequencing of embryos can be easily created so that growing embryos display new insights into temporal embryo development. In this chapter, the authors describe three methods with variations in hardware and software analysis by giving some examples of the outcomes to open a window to new information in developmental embryology, as embryo division pattern and lineage are studied in vivo.

2.2. Gene expression of cleavage embryo and noninvasive assessment of embryo viability via culture media analysis

Preimplantation embryo development experiences a series of critical events and remarkable epigenetic modifications, and reprogramming of gene expression occurs to activate the embryonic genome. In the early stages of preimplantation embryo development, maternal mRNAs direct embryonic development. Throughout early embryonic development, a differential methylation pattern is maintained, although some show stage-specific changes. Recent studies have shown that differential demethylation process results in differential parental gene expression in the early developing embryos that may have an impact on the correct development [28]. Also, noncoding RNAs, long noncoding RNAs (lncRNA), and short noncoding RNAs, microRNAs (miRNAs) have been shown to play an important role in the regulation of mRNAs, and therefore their role in preimplantation development has gained significance. Chapter Five reviews the different factors affecting gene expression during preimplantation embryo development, which includes epigenetic factors, focusing on methylation profiles, of gametes and preimplantation embryos. The effects of noncoding RNAs on gene expression were thoroughly evaluated.

Because gene expression appearance during embryo development in in vitro culture, preimplantation embryos often require rich nutrition culture media. The embryo during its growth and development needs to absorb some important nutritive components from culture medium and metabolically produce some by-products as gene expression results. From this point of view, in vitro culturing of embryos also provides a very important material for further noninvasive embryo evaluation by means of examining biomarkers in the spent embryo culture medium. Current developed methods concentrate on the measurement of metabolic compounds secreted from developing embryos. These studies mainly utilize the tools of modern analytics and proteomics. Some studies suggest that metabolic profiling of embryo culture media using optical and nonoptical spectroscopies may provide a useful adjunct to the current embryo assessment strategies and provide insight into the phenotype of embryos with increasing reproductive potential [29].

In the sixth chapter, the authors describe their new discovery, the alpha-1 chain of the human haptoglobin molecule as a quantitative biomarker of embryo viability. In a series of retrospective, blind experiments achieved more than 50% success rate. This chapter summarizes the currently available metabolic and proteomic approaches as the noninvasive molecular assessment of embryo viability. Recent studies showed that the assessment of the molecular components of nutrient media is a promising area in searching for the markers of successful embryo implantation with the subsequent development of a clinical pregnancy and the birth of a healthy baby to enhance the efficiency of treatment using ART techniques [30]. If the molecular composition of cultivation media can be used as an additional noninvasive procedure to choose an embryo for selective transfer, it will be very useful to improve human IVF pregnancy outcome.

3. Improving in vitro culture environment for embryo cleavages

Embryonic quality, cleavage speed, and gene expression have a close relationship with in vitro culture environment, including culture media, incubator type, and gas concentration [31, 32]. Thus, since starting embryo in vitro culture, many studies have concentrated on improving embryo culture condition. For many decades, optimization of culture media for the support of human and animal embryos has been a focus of considerable interest [33]. So far, many commercial embryo culture media are available for human embryo culture, and their effects on embryo culture are varied. The studies comparing these effects of culture media on embryonic development have reported contradictory conclusion. Many studies did not find a significant difference or found just a tiny difference between various culture media [34]. Recently, Mantikou et al. [35] used meta-analysis to evaluate 31 different comparisons for 20 different culture media and could not find which culture medium leads to the best success rates in IVF/ICSI.

Also, incubators in the IVF laboratory play a pivotal role in providing a stable and appropriate culture environment required for optimizing embryo development and clinical outcomes. With technological advances, several types of incubators have been applied to human IVF laboratory. Recently, Swain [32] did a comparative analysis of embryo cultural incubators in human IVF laboratories and reviewed some incubator functions and key environmental variables controlled and the technology utilized in various units. This comparison indicates that smaller benchtop/top-load incubators provide faster recovery of environmental variables, but there is no clear advantage of any particular incubator based on clinical outcomes.

However, based on last decade's IVF practical observation, Dr. Bin Wu's laboratory has found an interesting phenomenon which showed a favorable response of individual patient's embryos to media and incubators. Some patents' embryos grow very well in one kind of medium, but it does not grow well in the other medium. The seventh chapter gives a detailed report on this research result. Thus, in human IVF clinical practice, using two media and two incubators for embryo culture could significantly improve IVF/ICSI embryo quality and increase pregnancy rates.

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