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Effects of *in vitro* maturation of monkey oocytes on their developmental capacity

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Abstract

The study of *in vitro* maturation (IVM) of rhesus monkey oocytes has important implications for biomedical research and human infertility treatment. *In vitro*-matured rhesus monkey oocytes show much less developmental potential than IVM oocytes of other species. Since about 1980 when rhesus monkey IVM, *in vitro* fertilization (IVF) and *in vitro* embryo culture (IVC) systems were established, numerous efforts have been made to improve the developmental competence of oocytes and to understand the mechanisms regulating oocyte maturation. This review describes recent progress in this area, particularly the effects of factors such as steroid hormones, energy substrates, amino acids, ovarian follicle status, maternal age and breeding season on the developmental competence, gene expression patterns and genome integrity of rhesus IVM oocytes.

Keywords

Rhesus monkey; Oocyte *in vitro* maturation; Embryo development

1. Introduction

Acquisition of oocyte meiotic potential and especially developmental ability requires mutual interactions between the oocyte and its surrounding somatic cells (follicular mural granulosa and cumulus oophorus) throughout oogenesis. The intimate associations between these two cell types are established through gap junctions as well as paracrine interactions and persist until ovulation (Atef et al., 2005; Dekel, 2005; Jamnongjit and Hammes, 2005). Many signal pathways, such as the BMP, Wnt and Notch pathways, have been implicated in the crosstalk between oocytes and their surrounding somatic cells during oogenesis (Wang et al., 2004; Hahn et al., 2005; Zheng et al., 2006). Because these bilateral interactions play such essential roles in oogenesis, the interruption that occurs when oocytes are released from follicles and cultured *in vitro*, will compromise the meiotic and developmental competence of IVM oocytes. The extent of damage resulting from this separation depends on many factors, including the intrinsic properties of follicles (e.g., size and atresia) and components of the *in vitro* culture environments.

Oocyte maturation encompasses both nuclear and cytoplasmic programs. Nuclear events endow oocytes with the competence to resume and complete meiosis (i. e., extrude the second

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polar body during fertilization), whereas cytoplasmic programs enable the oocyte to be fertilized successfully and support embryo development to term. In general, nuclear maturation is viewed only morphologically, and for this reason may be mistakenly considered to be normal after IVM. In contrast, cytoplasmic maturation of oocytes is evaluated from a developmental viewpoint, and while this provides unequivocal information about the success of oocyte maturation, details of the underlying processes are difficult to determine. Meiotic and cytoplasmic maturation can proceed in some degree as independent processes (Sun and Moor, 1991), but the full developmental competence is conferred only when these two processes are closely integrated. The completion of cytoplasmic maturation, which lags behind meiotic maturation, requires synthesis and storage of RNAs and proteins, post-translational regulation of proteins, relocation of cytoplasmic organelles and alterations in membrane transport systems. Failure to complete cytoplasmic maturation can block development at fertilization, embryonic genome activation, blastocyst formation or even post-implantation.

The technology of IVM of mammalian oocytes combined with *in vitro* fertilization (IVF) and embryo culture (IVC) has been widely used in basic research studies as well as for *in vitro* production of embryos of domestic animals and nonhuman primates, as well as in the clinical treatment of human infertility. There are several important reasons for the clinical application of oocyte IVM. First, the routine hormonal stimulation protocols used for induction of ovulation in human IVF are not suitable for some infertile patients at risk of ovarian hyperstimulation syndrome (e.g., women suffering from polycystic ovarian syndrome; Trounson et al., 1994; Russell, 1998). The IVM of oocytes from unstimulated or mildly stimulated follicles of these patients is an attractive alternative to hormone stimulation (Chian et al., 2004a, b, c). Second, IVM combined with ovary or oocyte cryopreservation could provide reproductive opportunities to women who undergo chemotherapy cancer treatment (Rao et al., 2004). Third, clinical ovulation induction protocols still have many indirect problems, including the high cost of hormones and the side-effects of hormonal stimulations. Because of all these advantages, many infertility clinics have attempted to use IVM to treat patients, and while there have been a few notable successes, the clinical outcomes are generally poor (Chian et al., 2004c). Some severe problems remain in IVM oocytes, such as a great incidence of spindle abnormalities and chromosome defects (Piquette, 2006). Although many efforts have been made to improve the developmental potential of human oocytes matured *in vitro*, a dearth of knowledge about primate oocyte biology, together with ethical concerns about using fertilized human oocytes as experimental material, have severely limited progress. An appropriate experimental model is needed to advance knowledge about mechanisms controlling primate oocyte maturation, and the rhesus monkey is the ideal animal for this purpose. Studies on rhesus monkey oocyte maturation have important practical implications for treatment of human infertility, and can also increase the numbers of viable non-human primate embryos available for biomedical research (Schramm and Bavister, 1999a; Brenner et al., 2004). During the last decade, considerable progress has been made in improving the developmental potential of IVM rhesus monkey oocytes. This review describes the salient achievements.

2. Serum-free chemically defined medium for IVM of rhesus monkey oocytes

Chemically-defined, serum-free culture media provide a good starting point for studies on IVM. In many investigations into IVM or IVC, blood serum, co-culture with somatic cells or tissues, or cell conditioned media have been included to enhance culture performance. Although these culture systems have sometimes provided good results, the drawbacks are obvious. Use of serum or co-culture risks contamination by toxic components or pathogens such as viruses, which could become potential problems in the clinical application of IVM or IVC (Bavister, 1995). Inclusion of serum or co-culture often makes results highly variable, making accurate repetition of experiments difficult. Furthermore, the undefined components of serum or conditioned media make it very difficult or impossible to elucidate factors having

specific effects on the performance of oocytes. For these reasons, chemically defined, serum-free culture media are the preferred approach for IVM and IVC.

Chemically defined media have been developed for IVM and IVC in rodents and domestic animals (McKiernan et al., 1991; Keskinetepe and Brackett, 1996; McKiernan and Bavister, 2000; Kishida et al., 2004; Oyamada et al., 2004). Recent work reported the success of two chemically defined media (CMRL-1066 and HECM-10) in supporting maturation of rhesus monkey oocytes *in vitro* (Zheng et al., 2001a). Rhesus oocytes cultured in serum-free and serum-containing media displayed similar potentials to complete meiotic maturation and to develop to the morula and/or blastocyst stages. The blastocysts derived from oocytes cultured in serum-free or in serum-containing media did not differ in their morphology or rate of formation. These observations indicated that serum is unnecessary for rhesus monkey oocyte IVM. The use of a simple, homemade medium such as HECM-10 instead of the complex, commercial medium CMRL-1066 allows the depletion or addition of components to identify key factors affecting oocyte maturation.

3. Effects of estradiol and progesterone in IVM medium on developmental competence of rhesus monkey oocytes

Estradiol and progesterone are important intrafollicular factors affecting many aspects of ovarian function including the acquisition of oocyte meiotic maturation and fertilization competence (Zelinski-Wooten et al., 1994), ovulation (Hibbert et al., 1996), and luteinization and maintenance of luteal structure and function (Armstrong et al., 1991; Hibbert et al., 1996). A recent study provided evidence implicating these hormones in cytoplasmic maturation of rhesus monkey oocytes (Zheng et al., 2003). Inclusion of certain concentrations of estradiol and/or progesterone in IVM medium could significantly improve the embryo developmental competence of IVM rhesus oocytes. Without these hormones present during IVM, only about 15% of IVM oocytes from nonstimulated (no exogenous FSH or hCG) adult monkeys could develop into morulae, and fewer still could form blastocysts. However, the inclusion of these steroid hormones in the IVM medium significantly increased the rates of morula and blastocyst formation to ranges of 40 to 45% and 14 to 20%, respectively. These percentages are similar to those obtained using *in vitro*-matured oocytes from FSH-stimulated adult females during the breeding season (Zheng et al. 2001b). The similarity of embryo developmental potential between oocytes from these two different sources could be explained by a requirement of steroid hormones for oocyte maturation that are either provided *in vitro* or *in vivo* after FSH priming of the donor monkeys.

Steroid hormones function via two mechanisms with distinct characteristics. The classical or so-called genomic theory has been held for many years. In this model, intracellular nuclear receptors and transcriptional modulation of the target genes are involved (Christ et al., 1999; Haseroth et al., 1999; Gerdes et al., 2000). The non-genomic model of steroid action was identified in recent years and its characteristics have been intensively discussed in many papers (Christ et al., 1999; Haseroth et al., 1999; Gerdes et al., 2000). The non-genomic steroid action does not require the events of transcriptional activation. Instead, the signal is initiated by plasma membrane receptors and calcium plays a critical role in signal transduction. In rhesus monkeys, the mechanism by which steroid hormones enhance oocyte embryonic developmental competence remains unclear. Several studies have provided evidence against the genomic mode of estradiol or progesterone action on oocyte cytoplasmic maturation. In those studies, the expression of nuclear receptors for estradiol and progesterone was not detected in somatic cells of follicles prior to the LH surge (Chandrasekher et al., 1994). Studies with other species including humans (Tesarik and Mendoza, 1995), chickens (Morley et al., 1992), fish (Das and Thomas, 1999; Zhu et al., 2003) and *Xenopus* (Morrison et al., 2000), however, implicated the non-genomic mechanism for the actions of progesterone and estradiol on the maturation of

oocytes. Clarification of the mechanisms involved in the action of steroid hormones on maturation of primate oocytes will most likely come from studies using the rhesus monkey as the experimental model.

4. Effect of energy substrates on development potential of rhesus monkey IVM oocytes

Energy substrates have been found to affect oocyte maturation and embryo development in a wide variety of mammals (McKiernan et al., 1991; Rose-Hellekant et al., 1998; Zheng et al., 2001c). Glucose, lactate and pyruvate are designated as the “energy substrate triad” in cellular metabolism (Bavister, 1995). Glucose can be metabolized by three pathways, which include glycolysis, the tricarboxylic acid cycle (TCA) and the pentose phosphate pathway, to provide cells with ATP and other essential molecules. Pyruvate has many metabolic fates, primarily its oxidation via the TCA cycle to produce energy. It can also be transaminated to alanine, while in the liver, it can be carboxylated to oxaloacetate or malate and be transformed into glucose by gluconeogenesis. Lactate is a side-product of glycolysis, formed by reduction of pyruvate in the cytosol. The first step for lactate utilization as an energy source is its reconversion to pyruvate. This ready inter-conversion between pyruvate and lactate may help cells to maintain intracellular pH, itself an important regulator of cell functions, as well as to regenerate NADP. Interestingly, Gott et al. (1990) reported that cultured human embryos produce copious amounts of lactic acid, which may indicate that their intracellular pH is perturbed *in vitro*. This would radically alter many cell functions including energy production, but this possibility has been virtually ignored in the search for improved culture media for oocyte maturation as well as for embryo culture.

During IVM of rhesus monkey oocytes, supplementation of the culture medium with different energy substrates produced oocytes with significantly different developmental competence (Zheng et al., 2001c). Among the energy substrate triad, glucose alone was most effective in supporting cytoplasmic maturation of oocytes. Absence of glucose in the IVM medium, regardless of the presence of lactate and pyruvate, resulted in a dramatic reduction of development beyond the 8-cell stage, when the major zygote genome activation occurs in the rhesus monkey (Schramm et al., 2003), as it does in humans (Braude et al., 1988). These observations suggested the essential role of glucose in the events required for successful maternal to embryonic genome control transition. The beneficial action of glucose on oocyte cytoplasmic maturation, however, was partially inhibited by pyruvate via unknown mechanisms (Zheng et al., 2001c).

It would be intriguing to know the metabolic pathways by which glucose exerts its beneficial action on oocytes leading to successful embryo development potential. The glucose metabolism pathway can be illustrated by incubation of oocytes or embryos in culture medium containing a specifically radiolabeled glucose (Rieger et al., 1992a, b). In this way, the metabolic fate of glucose has been investigated in bovine, ovine and murine oocytes and embryos (Thompson et al., 1991; Rieger et al., 1992a, b; Waugh and Wales, 1993; Khurana and Niemann, 2000). In striking contrast, little information is available regarding the metabolism of glucose by primate oocytes or embryos. During rhesus oocyte IVM, lactate and especially pyruvate, which are readily utilized in the TCA cycle and produce much more ATP per mole than glucose, still could not substitute for glucose. This indicates that conversion of glucose into pyruvate is not enough to support optimal cytoplasmic maturation; possibly, excessive conversion of glucose-derived pyruvate into lactate as suggested above may restrict entry of this pyruvate into the TCA cycle. In contrast, “flooding” the oocyte with exogenous pyruvate from the culture medium may overcome this limitation and provide enough pyruvate to supply the TCA cycle. It could be informative to measure pyruvate uptake and lactate output by monkey oocytes during IVM culture, as reported for human embryos (Gott et al., 1990).

Alternatively, the pentose phosphate pathway and glycolysis might play important roles during rhesus monkey oocyte maturation. In the mouse and cow, glucose is metabolized mainly via the pentose phosphate pathway and glycolysis is performed by both cumulus cells and oocytes during oocyte maturation (Downs et al., 1997, 1998; Downs and Utecht, 1999; Krisher and Bavister, 1999; Cetica et al., 2002; Krisher, 2006).

The first step in glucose utilization is its transport into cells. There are two general mechanisms mediating glucose transport. One is via sodium-coupled glucose transporters (sodium-glucose cotransporters, SGLTs) and the other is sodium-independent facilitative glucose transporters (GLUTs) (Wakisaka et al., 1995). Facilitative glucose transporters comprise a family of structurally related, membrane-spanning glycoproteins, which play a crucial role in regulating glucose metabolism. Thirteen members have been identified, including GLUT1-12 and HMIT (Uldry and Thorens, 2004). Some of these glucose transporters (GLUT4 and GLUT8) are regulated by insulin (Carayannopoulos et al., 2000; Uldry and Thorens, 2004), but effects of insulin on primate IVM do not appear to have been examined. The expression of certain types of GLUT genes was reported in rodent and human oocytes and embryos. For example, GLUT1 was detected in both mouse and human oocytes (Aghayan et al., 1992; Dan-Goor et al., 1997) and preimplantation stage embryos (Hogan et al., 1991; Aghayan et al., 1992; Dan-Goor et al., 1997). The GLUT2 gene was expressed in mouse and human preimplantation embryos (Hogan et al., 1991; Aghayan et al., 1992). The expression of GLUT1 and GLUT4 genes were also observed in granulosa cells and theca cells in sheep (Williams et al., 2001). However, GLUT4 was undetectable in oocytes and through the blastocyst stage or in early postimplantation stage embryos (Hogan et al., 1991; Aghayan et al., 1992). The consumption of glucose by cumulus-oocyte complexes (COCs) of rhesus monkeys during maturation implies the existence of some sort of glucose transporters in oocytes and/or cumulus cells. Fortunately, by using the Primate Embryo Gene Expression Resource (PREGER) established by Dr. Keith E. Latham at Temple University (Zheng et al., 2004a), data regarding the expression of glucose transporters have been collected in rhesus monkey oocytes and preimplantation embryos (Latham et al., unpublished data).

5. Effect of amino acids on the developmental potential of rhesus monkey IVM oocytes

In rodents, domesticated animals and humans, oocyte maturation and embryo development can be profoundly influenced by the type of amino acid provided in the culture medium (Pinyopummintr and Bavister, 1996; Kito and Bavister, 1997; Rose-Hellekant et al., 1998; Devreker and Englert, 2000; Hong et al., 2004). Amino acids function as substrates for protein synthesis (true for most of the amino acids), energy substrates (e.g., glutamine), neurotransmitters, synaptic modulators, signal transducers (Martin et al., 2003), regulators of intracellular pH (Edwards et al., 1998) and organic osmolytes (e.g., glycine) (Palacin et al., 1998; Steeves and Baltz, 2005). The effect of amino acids depends on the developmental stage examined as well as the presence or absence of the other amino acids, reflecting the stage-specific requirement for amino acids during development and the complexity of amino acid interactions (Liu and Foote, 1995; Lane and Gardner, 1997a). In rhesus monkeys, supplementation of oocyte IVM medium with different amino acids or their combinations produces embryos exhibiting variable development potential (Zheng et al., 2002). Among different groups of amino acids including Eagle's essential amino acids, Eagle's non-essential amino acids and glutamine, the non-essential amino acids as well as glutamine displayed beneficial effects on oocyte cytoplasmic maturation, whilst the essential amino acids group did not (Zheng et al., 2002). However, inclusion of all these amino acids (essential and non-essential amino acids and glutamine) in IVM medium conferred the greatest developmental ability on the oocytes. The detrimental effect of essential amino acids when used alone is not unique to IVM of rhesus monkey oocytes. Inhibitory actions of this group of amino acids were

also observed on bovine preimplantation embryo development (Liu and Foote, 1995) and on hamster zygote development *in vitro* (McKiernan et al., 1995). Similarly, beneficial roles of nonessential amino acids and glutamine were demonstrated on oocyte maturation and embryo development of several species including mouse, bovine and human (Pinyopummintr and Bavister, 1996; Lane and Gardner, 1997b; Devreker et al., 2001). Due to the shortage of rhesus monkey oocytes and the complexity of amino acid interactions, the action of individual amino acids on oocyte IVM is still far from clear in the rhesus monkey. An enhanced and more precise physiological approach would be to examine uptake and efflux of amino acids when used as a complete group, as demonstrated for human embryos (Houghton et al., 2002) but apparently this has not been done for primate oocytes during IVM.

Amino acids are transported into cells by amino acid transporter proteins. Based on the functional characteristics, amino acid transporters are classified into many different systems (Palacin et al., 1998). In light of the uptake of amino acids by oocytes and embryos, the presence of some amino acid transporters has been detected in mouse COCs and preimplantation embryos. Examples include GLYT1 and SLC38A3. The GLYT1 protein, a transporter of glycine that functions in cell volume control under conditions of normal osmolarity, was detected in mouse and human cleavage-stage embryos (Steeves et al., 2003; Steeves and Baltz, 2005); SLC38A3, a neutral amino acid transporter protein with substrate preference for glutamate, histidine and alanine, was detected in granulosa cells of small antral follicles and in cumulus cells of large antral follicles of the mouse. The synthesis of the SLC38A3 protein in surrounding somatic cells was under the control of oocytes and mediated the uptake of amino acids by oocytes via gap junctions (Eppig et al., 2005). The significant effects of amino acids on rhesus monkey oocyte IVM (Zheng et al., 2002) implied the existence of amino acid transporters in cumulus cells and/or oocytes. This was confirmed by recent studies utilizing the PREGER resource which have documented the detection of some amino acid transporters in oocytes and preimplantation embryos of rhesus monkey (Latham et al., unpublished data).

6. Effect of follicle status on oocyte IVM in rhesus monkeys

In addition to the *in vitro* culture conditions, the intrinsic characteristics of ovarian follicles are critical factors determining the developmental potential of oocytes. Among these intrinsic factors, follicle size and follicle health status are of utmost importance. Developmental competence is acquired sequentially during folliculogenesis (Schramm and Bavister, 1999a). Oocytes from larger follicles display a greater ability to complete meiotic maturation and embryo development than those from smaller follicles in almost all mammalian species examined, including primates. The molecular causes for poor developmental capacity of oocytes are likely to be very complex (Donnison and Pfeffer, 2004). Acquisition of developmental competence involves the synthesis and storage of a wide range of molecules during oocyte growth, including mRNA and proteins, followed by the reprogramming and ordered utilization of these stored products during maturation, fertilization and early embryogenesis (Moor et al., 1998). An increasing number of oocyte-specific genes that affect early embryogenesis have been identified and characterized. These include *Zar1* and *Mater*, deficiencies in which lead to arrest of development at the 1-cell and 2-cell stages in mouse embryos (Tong et al., 2000; Wu et al., 2003).

Oocytes from small follicles may not complete the accumulation of a sufficient store of mRNAs and proteins (Pavlok et al., 1992; Pavlok et al., 1993). In bovine oocytes, genes for some transcripts have been reported to be specifically or highly expressed in oocytes from large (≥ 5 mm) but not in small follicles (≤ 2 -mm) (Robert et al., 2000; Donnison and Pfeffer, 2004). The transcriptional activity of bovine oocytes drops dramatically after the follicle has grown to 3 mm and remains nearly quiescent thereafter (Fair et al., 1995; De Sousa et al., 1998). It was therefore suggested that follicles smaller than 3 mm have not yet attained the full RNA

complement required for developing beyond the stage or time when embryonic transcription is initiated.

In rhesus monkeys, the follicular size at which oocytes acquire their full mRNA complement required for successful embryo development is still uncertain. The enhanced incidence of condensed chromatin configuration (60 to 85%) was investigated in oocytes from small antral follicles with diameters of approximately 1 mm (Schramm et al., 1993; Zheng and Latham, unpublished data). Chromatin condensation is highly associated to the loss of transcriptional activity (Wansink et al., 1993; Aoki et al., 1997; Bouniol-Baly et al., 1999). The correlation between chromatin condensation and silencing of oocyte transcription was confirmed in the rhesus monkey in a recent study (Zheng and Ji, unpublished data). Thus, it seemed that a large proportion of rhesus monkey oocytes stopped transcriptional activity at an early stage of follicle development. However, it is not clear if rhesus monkey follicles larger than 1 mm have attained the full mRNA complement required for developing beyond the 8-cell stage.

The correlations between follicle atresia and oocyte developmental competence have been well demonstrated in the bovine model, in which healthy follicles were highly correlated with oocytes exhibiting greater developmental potential whilst atretic follicles contained oocytes with lesser development capacity (Bilodeau-Goeseels and Panich, 2002; Boni et al., 2002). Similarly, our recent studies found that the health status of rhesus monkey follicles, as assessed morphologically by the degree of cumulus investment, had a significant influence on the *in vitro* maturation of oocytes (Zheng and Ji, unpublished data). Oocytes with compact cumulus cell layers (from healthy follicles) showed greater meiotic maturation and embryo development competence than oocytes with fully expanded cumulus (from heavily atretic follicles).

7. Effect of maternal age and breeding season on embryo development from rhesus monkey IVM oocytes

The developmental competence of IVM oocytes can be influenced by the oocyte donor age and the breeding season during which the oocytes are recovered. In cattle and pigs, oocytes from prepubertal donors display poor meiotic maturation and embryo development compared to oocytes from adult females (Marchal et al., 2001; Salamone et al., 2001; de Matos et al., 2003; Grupen et al., 2003; Sherrer et al., 2004). Consistent with their lesser meiotic and developmental potential, oocytes from sexually immature females display morphological, biochemical and physiologic alternations or anomalies that could partially account for their reduced competence. Examples of these anomalies include aberrations in microtubule and microfilament organization, cortical granule distribution, the activities and/or concentrations of maturation-promoting factor (MPF), mitogen-activated protein kinase (MAPK), and inositol 1,4,5-trisphosphate receptor (IP(3)R), and protein synthesis activity during maturation (Gandolfi et al., 1998; Khatir et al., 1998; Ledda et al., 2001; Salamone et al., 2001; Velilla et al., 2004, 2005). As in other species, the effect of maternal age on rhesus monkey oocyte competence is obvious. Oocytes from prepubertal monkeys produced fewer morulae than those from adult females, indicating the inferior quality of oocytes. However, the impact of age on oocyte developmental competence can be profoundly affected by *in vivo* priming of females with FSH. After 8 days of FSH stimulation, oocytes from prepubertal monkeys (around 2 years of age) surprisingly displayed greater developmental capacity (54% blastocyst formation) after IVF than those from adults (16% blastocyst formation) (Zheng et al., 2001b). The morphology of IVF blastocysts using oocytes obtained from primed prepubertal monkeys was similar to that of blastocysts derived from oocytes collected from adults. However, it is not known if these blastocysts from prepubertal and adult donors possess similar potentials to implant and develop after embryo transfer. Unlike the situation in rhesus monkeys, oocytes from sexually immature animals of other species (e.g., cattle, sheep and mouse) did not show such a striking increase in their developmental potential after FSH priming *in vivo*, although a certain degree

of improvement in meiotic maturation or embryo development was observed (Eppig et al., 1992; Presicce et al., 1997; Ledda et al., 1999). The mechanisms responsible for the beneficial effect of *in vivo* FSH stimulation on prepubertal rhesus monkey oocyte development competence remain unclear, and warrant further study because of the implications for production of viable IVF embryos.

Rhesus monkeys in captivity breed during the winter (approximately from September to February), but usually remain responsive to FSH stimulation until May, although their precise breeding season varies with latitude. Seasonal effects on several aspects of reproductive function in rhesus monkeys are well known. During the non-breeding season, rhesus monkeys exhibit reduced ovulatory and mating frequencies and fewer fertile copulations. The ovarian follicular development and hormonal profiles of adult females are different between the breeding and non-breeding seasons (Walker et al., 1984; Hutz et al., 1985). However, inconsistent with the above observations, oocytes from unstimulated females did not show any differences in meiotic and developmental competence between the breeding and non-breeding seasons (Zheng et al., 2001b). A seasonal effect on oocyte developmental potential, however, was observed in females stimulated with FSH. FSH-stimulated oocytes collected during the non-breeding season showed poor embryo development when compared to those collected during the breeding season. Because somatic cells surrounding the oocyte modulate the actions of FSH and because communication between oocyte and cumulus cells plays a critical role in oocyte growth and maturation, the different effects of FSH priming on rhesus oocyte competence during breeding and non-breeding seasons might be attributed to molecular changes in somatic cells, such as down-regulation of FSH receptors, and the interruption of oocyte-somatic cell communications. Indeed, a deficiency of gap-junction mediated communications between oocytes and cumulus cells was observed in more than half of horse cumulus-oocyte complexes collected during the non-breeding season, whereas the vast majority of oocytes (90%) collected during the breeding season had fully functional communications with their surrounding cells and better developmental competence (Colleoni et al., 2004).

8. Effect of IVM on overall gene expression profiles in rhesus monkey oocytes and derived embryos

There is considerable interest in understanding the molecular mechanism governing long-term oocyte developmental competence. By using differential screening methods and loss of function studies, encouraging information has been gained in the cattle and mouse models regarding oocyte-stored molecules that play critical roles in early embryo development. For example, investigations on gene expression in developmentally competent and incompetent bovine oocytes have revealed the identity of a number of genes associated with oocyte developmental competence. A striking perturbation of gene expression was also observed in oocytes with poor developmental competence (Donnison and Pfeffer, 2004).

In rhesus monkeys, systematic comparison of gene expression in oocyte populations with different quality has not yet been performed. However, with the PREGER project (Zheng et al., 2004a, b, 2005a, b, www.preger.org), more than 100 genes have been examined for their expression patterns in oocytes and preimplantation embryos have been assessed from three different sources: oocytes from non-stimulated small follicles, oocytes from FSH-stimulated large follicles, and oocytes from both FSH- and LH-stimulated follicles and matured *in vivo*. The first source of oocytes represents the population with least developmental competence, the third one represents oocytes with greatest developmental potential and the second represents those with intermediate potential. By analyzing the extent of gene expression (e.g., housekeeping genes, transcription factors, and chromatin remodeling factors) among the three groups, several interesting findings were reported. First, IVM metaphase II (MII) oocytes from

small unstimulated follicles displayed an overall greater abundance of some maternal transcripts than those matured *in vivo*. There were two possible explanations for this difference. One possibility was that oocytes of the least developmental competence produced more of these transcripts than oocytes of good quality. Alternatively, the two populations of oocytes might have different processing of poly (A) RNA during meiotic resumption. During oocyte growth, mRNAs are produced for both immediate use and for storage (Eichenlaub-Ritter and Peschke, 2002). Stable storage of mRNA involves not only variable length of the 3' poly-A tail, but also different association with specific RNA binding proteins, especially the masking proteins (Gray and Wickens, 1998; Groisman et al., 2000; Groisman et al., 2001). In cattle, mouse and *Xenopus* oocytes, the amount of polyadenylated RNA was reduced by more than half during the maturation of oocytes by a combination of mRNA degradation and deadenylation (Bachvarova and De Leon, 1980; Sagata et al., 1980; Lequarre et al, 2004). The adenylation and deadenylation processes during maturation are believed to play important roles in stable storage of the transcripts. In the rhesus monkey, the reduced amplification of some mRNAs, when reverse transcription was performed with oligo(dT), in oocytes matured *in vivo* could be due to the deadenylation of mRNAs. The greater amount of some mRNAs in oocytes of lesser developmental competence might reflect a deficiency in mRNA deadenylation, which could lead to mRNA instability in these oocytes. This proposal was supported by the second finding that will be subsequently described.

The second exciting finding was that the abundance of many maternal mRNAs showed a dramatic decrease in 2-cell stage embryos derived from *in vitro*-matured oocytes from unstimulated small follicles compared with *in vivo*-matured oocytes or *in vitro*-matured oocytes of FSH-stimulated large follicles. The reduced abundance of these mRNAs at the 2-cell stage probably reflected the degradation of the mRNAs, implying a deficiency in the mRNA stabilization mechanism in IVM oocytes from small follicles. Combining these two findings, we proposed that oocytes from small nonstimulated ovarian follicles could not successfully deadenylate their mRNAs during maturation for stable storage (Lequarre et al, 2004). These mRNAs thereafter were prone to degradation. In contrast, the mRNAs in oocytes matured *in vivo* were processed for deadenylation for stable storage during maturation and these stored mRNAs were then available for use in early cleavage. Consequently, by oligo(dT) reverse transcription and PCR, we detected an overall greater amount of mRNAs and much less mRNA in oocytes and 2-cell embryos, respectively, from small nonstimulated follicles when compared to oocytes matured *in vivo*. Because the maternal store of mRNAs or proteins plays a crucial role in governing success of the maternal to embryonic transition, the precocious degradation of many maternal mRNAs could compromise the developmental potential of IVM oocytes from unstimulated small ovarian follicles, and thus explain why embryos derived from such oocytes fail to progress beyond the 8-cell stage when the major genome transition events occur in primates (Braude et al., 1988; Schramm and Bavister, 1999b; Schramm et al., 2003).

Oocytes from FSH-stimulated large follicles and IVF embryos derived from them resembled the *in vivo*-matured oocyte group in overall gene expression patterns more than the non-stimulated group. Thus, it seemed that mRNA stabilization competence of oocytes was acquired during the prolonged period of follicle growth rather than during meiotic maturation.

Maternal mRNAs are required not only for the survival of embryos prior to embryo genome activation, but also for the proper activation of the zygotic genome. In IVM oocytes, aberrations in many of the maternal RNA profiles did lead to the further aberration of gene expression in 8-cell stage embryos and beyond. Altogether, these gene expression perturbations observed in IVM oocytes and their derived embryos could partially account for the reduction in developmental potential imposed by *in vitro* maturation of oocytes under culture conditions that are clearly inadequate for this purpose.

9. Possible effects of IVM on oocyte genome integrity

One concern with IVM is the possibility of DNA damage imposed by a suboptimal culture system. Integrity of the oocyte genome, and the zygote genome following fertilization, is essential for successful embryogenesis. Endogenous and exogenous damage may arise during the prolonged periods required for oocyte growth and maturation in primates. For example, oxidative DNA damage can be induced by reactive oxygen species (ROS) production which is a normal consequence of cell metabolism (Cetica et al., 2001). This damage needs to be repaired to minimize the mutation load. Failure to recognize DNA damage and perform damage repair will lead to mutagenesis or cell death. In general, IVM and IVC expose the oocytes and embryos to suboptimal conditions. *In vitro* environments usually increase cellular production of ROS, which has been implicated as a primary cause of DNA damage. Oxidative stress-induced DNA damage has been reported in somatic cells as well as in oocytes and embryos cultured *in vitro*. For instance, oxidative DNA damage was observed in bovine and porcine oocytes and embryos cultured in greater concentrations of O₂ (Kitagawa et al., 2004; Tatemoto et al., 2004). Oxidative DNA damage also occurred in human cells cultured in medium with a greater glucose concentration (Shimoi et al., 2001). In rhesus monkeys, little information is available regarding the effect of oocyte maturation conditions on oocyte DNA damage. Again, by using the PREGER resource, the amount of mRNA present as a result of expression of DNA repair genes in rhesus monkey oocytes and preimplantation embryos was examined and the extent of gene expression in cultured blastocysts as compared with those flushed from the uterus were compared (Zheng et al., 2005b). Interestingly, most of the genes involved in DNA damage sensing (examples include genes that sense DNA single-strand breaks, double-strand breaks, and replication fork collapse) and DNA damage repair (e.g., mismatch repair, base excision repair, nucleotide excision repair, double-strand break repair and post-replication DNA repair) were expressed, indicating that oocytes and embryos were endowed with the requisite DNA damage repair machinery. The other interesting finding was that *in vitro*-cultured blastocysts displayed striking aberrations in expression of nearly half of the DNA damage response genes examined when compared to their putatively normal, *in vivo*-flushed counterpart embryos. Most of the affected genes had an enhanced expression in cultured embryos. The affected genes ranged from DNA damage sensing components to repair proteins and cell cycle checkpoint proteins. The tremendous scale of aberrations probably reflected the detrimental effect of culture conditions on the genomic integrity of embryos. Alternatively, the disruptions in gene expression may reflect a lack of correct gene regulation rather than a response to DNA damage, which would actually compromise the ability of these embryos to respond to DNA damage. Based on these observations with blastocysts, it is reasonable to propose a similar adverse effect of IVM culture conditions on oocyte DNA integrity, which could raise concerns about the wide utilization of IVM technology for producing viable oocytes. Clearly, the IVM and IVC culture conditions need to be improved, and equally clearly, the PREGER system approach can be invaluable for detecting gene expression defects induced by artificial culture environments. By studying such defects, it may be possible to refine IVM culture media to produce more normal and developmentally competent oocytes and embryos.

10. Future prospects

Although the developmental potential of IVM primate oocytes has greatly improved during the past decade, the mechanisms controlling oocyte cytoplasmic maturation are still far from clear. Better understanding of the factors controlling primate oocyte maturation would no doubt lead to improved culture media for IVM, and consequently assist the production of more developmentally competent embryos from IVM oocytes. This in turn would improve the adoption of IVM for routine clinical use in treating human infertility, as well as for preserving the reproductive capacity of women undergoing chemotherapy for cancer by rescuing oocytes from excised ovarian segments. An additional use for oocyte IVM in nonhuman primates would

be in assisted reproductive technology (ART) for endangered species, such as chimpanzees, either for aiding propagation efforts in captive animals or for gene banking. For all of these purposes, more studies are needed using experimental primate models.

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