Hum. Reprod. Advance Access published May 16, 2012
Human Reproduction, Vol.0, No.0 pp. 1–8, 2012
doi:10.1093/humrep/des158

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

Amphiregulin promotes the maturation of oocytes isolated from the small antral follicles of the rhesus macaque

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Submitted on October 25, 2011; resubmitted on March 27, 2012; accepted on April 10, 2012

BACKGROUND: In non-primates, the epidermal growth factor (EGF) and EGF-related ligands such as amphiregulin (AREG) serve as critical intermediates between the theca/mural cells and the cumulus-oocyte-complex (COC) following the mid-cycle LH surge. Studies were designed in primates (1) to analyze AREG levels in follicular fluid (follicular fluid) obtained from pre-ovulatory follicles, as well as (2) to assess dose-dependent effects of AREG on oocytes from small antral follicles (SAFs) during culture, including meiotic and cytoplasmic maturation.

METHODS: Controlled ovulation protocols were performed on rhesus monkeys ($n = 12$) to determine AREG content within the single, naturally selected dominant follicle after an ovulatory stimulus. Using healthy COCs ($n = 271$) obtained from SAFs during spontaneous cycles ($n = 27$), *in vitro* maturation (IVM) was performed in the absence or presence of physiological concentrations of AREG (10 or 100 ng/ml) with or without gonadotrophins (FSH, 75 mIU/ml; LH, 75 mIU/ml). At the end of the culture period, oocyte meiotic maturation was evaluated and ICSI was performed ($n = 111$), from which fertilization and early embryo development was followed *in vitro*.

RESULTS: AREG levels in follicular fluid from pre-ovulatory follicles increased ($P < 0.05$) following an ovulatory bolus of hCG at 12, 24 and 36 h post-treatment. At 12 h post-hCG, AREG levels in follicular fluid ranged from 4.8 to 121.4 ng/ml. Rhesus macaque COCs incubated with 10 ng/ml AREG in the presence of gonadotrophins displayed an increased percentage of oocytes that progressed to the metaphase II (MII) stage of meiosis (82 versus 56%, $P < 0.05$) and a decreased percentage of metaphase I (MI) oocytes (2 versus 23%, $P < 0.05$) relative to controls, respectively. The percentage of either MI or MII oocytes at the end of the culture period was not different between oocytes cultured with 100 ng/ml AREG or in media alone. Fertilization and first cleavage rates obtained by ICSI of all IVM MII oocytes were 93 and 98%, respectively, and did not vary among treatment groups. Of the MII oocytes that fertilized ($n = 103$), 37 were randomly selected and maintained in culture to assess developmental potential. A total of 13 early blastocysts were obtained, with four embryos developing to expanded blastocysts.

CONCLUSIONS: These data indicate that AREG levels increase in rhesus macaque pre-ovulatory follicles after an ovulatory stimulus, and a specific concentration of AREG (10 ng/ml) enhances rhesus macaque oocyte nuclear maturation but not cytoplasmic maturation from SAFs obtained during the natural menstrual cycle. However, owing to the small number of samples in some treatment groups, further studies are now required.

Key words: oocyte maturation / amphiregulin / rhesus monkeys / small antral follicle / embryo development

Introduction

Resumption of meiosis (i.e. nuclear maturation) and as yet poorly defined intracellular processes (i.e. cytoplasmic maturation) enable the oocyte to undergo successful fertilization and embryonic development. The ability of primate oocytes to complete nuclear and cytoplasmic maturation *in vitro* is markedly inferior to that of oocytes from other species (Edwards, 1965; Schroeder and Eppig, 1984; Sirard *et al.*, 1988; Galli and Moor, 1991; Pinyopummintr and Bavister, 1996a,b; Schramm and Bavister, 1996). Moreover, the quality of primate oocytes matured *in vitro* is much less compared with their counterparts matured *in vivo* (Bavister *et al.*, 1983; Morgan *et al.*, 1991; Schramm *et al.*, 1993, 2003; Schramm and Bavister, 1994). We previously reported that rhesus macaque oocytes within cumulus-oocyte-complexes (COCs) derived from healthy small antral follicles (SAFs) of at least 0.5 mm in diameter obtained during the early follicular phase can reinitiate meiosis *in vitro* (Peluffo *et al.*, 2010). However, following fertilization *in vitro*, embryonic development arrested early and consequently oocyte competence was not fully achieved. It remains to be determined whether the failure in embryonic development in oocytes derived from these SAFs was related to suboptimal culture conditions or a requirement for factors that are typically present through the periovulatory interval.

The mid-cycle LH surge induces processes critical for fertility, including resumption of meiosis, cumulus-oocyte expansion (C-OE) and rupture of the follicle wall. It has been reported that epidermal growth factor (EGF) and EGF-related ligands serve as critical intermediates between the theca/mural cells and the COC following the mid-cycle LH surge in non-primate species (Park *et al.*, 2004; Conti *et al.*, 2006; Hsieh *et al.*, 2007; Panigone *et al.*, 2008). In rodents, LH-dependent intrafollicular expression of the EGF-like family members, such as amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) mediate or synergize with the gonadotrophin surge to promote periovulatory events including reinitiation of meiosis (germinal vesicle breakdown, GVBD) in oocytes and C-OE (Park *et al.*, 2004). Indeed, EGF-related factors mediate gonadotrophin action through the induction of steroid and prostaglandin production (Jamnongjit *et al.*, 2005; Shimada *et al.*, 2006). We reported that significant AREG, EREG and EGF receptor (EGFR) mRNA levels were also present in the rhesus macaque pre-ovulatory follicle (Xu *et al.*, 2011a). While AREG and EREG mRNA significantly increased after an ovulatory stimulus, the BTC mRNA was minimal and did not vary throughout the periovulatory interval (Xu *et al.*, 2011a). Moreover, the mRNA expression of other EGF family members, such as EGF and transforming growth factor alpha (TGFα), was minimal or undetectable (Xu *et al.*, 2011a). In agreement with these observations, follicular fluid (follicular fluid) from women undergoing IVF protocols showed higher levels of the AREG protein content than EGF, having a concentration of around 100 ng/ml in comparison with 5 pg/ml, respectively (Inoue *et al.*, 2009). However, the role that EGF signaling plays in the development and maturation of a fully competent oocyte in primates remain unknown.

Therefore, a study using adult female rhesus monkeys was designed to (1) analyze the protein content of AREG in the follicular fluid from pre-ovulatory follicles at different time points after an ovulatory stimulus; (2) assess dose-dependent effects of follicular levels of AREG on meiotic reinitiation and progression of oocytes derived from SAF COCs at different stages of the menstrual cycle in the presence or absence of gonadotrophins, including fertilization and preimplantation embryo development.

Materials and Methods

Animals

The general care and housing of rhesus monkeys (Macaca mulatta) at the Oregon National Primate Research Center (ONPRC) was previously described (Wolf *et al.*, 1990). The studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the ONPRC Animal Care and Use Committee.

To determine the AREG content in the follicular fluid from monkey pre-ovulatory follicles, a group of adult female rhesus monkeys ($n = 12$) underwent controlled ovulation (COv) protocols as previously published (Young *et al.*, 2003) to obtain a single, naturally selected dominant follicle. Follicular fluid was collected at different time points after the hCG treatment (0, 12, 24 and 36 h, $n = 3$ per time point), which was described in detail in a previous study (Peluffo *et al.*, 2011a).

For COC studies, adult female rhesus monkeys ($n = 27$; 10 years old = average age, range 4–17 years old), including those at the early follicular ($n = 10$), luteal ($n = 9$) or unknown ($n = 8$, from necropsies) phase of the menstrual cycle, were used. The first day of menses was considered Day 1 of the cycle. Ovaries were removed from anesthetized monkeys by laparoscopy, as previously described (Duffy and Stouffer, 2002). The excised ovaries were transported immediately to the laboratory in a holding medium (SAGE®, CooperSurgical, Inc., Trumbull, CT, USA) supplemented with 0.1% SPS (Serum Protein Substitute, CooperSurgical) plus gentamycin.

AREG and EREG ELISA

A commercially available enzyme-linked immunosorbent assay (ELISA) kit was used to detect and quantify the level of AREG protein in the follicular fluid from monkey pre-ovulatory follicles following the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN, USA) as previously published (Zamah *et al.*, 2010). Serial dilutions were used, with a minimum of two points within the standard curve used for each sample and each dilution was run in duplicate. EREG levels were also quantified by ELISA as previously described (Zamah *et al.*, 2010).

Recovery and *in vitro* culture of COCs

Following the dissection of SAFs from the ovarian tissue under a stereoscopic microscope using 30-gauge needles, SAF diameters were measured as previously described (Peluffo *et al.*, 2010). SAFs (devoid of dark oocytes or granulosa cells) at 0.5–2 mm in diameter were punctured and their COCs extracted. Since only healthy COCs were used for subsequent *in vitro* studies, 63% of the COCs possessing vacuolated oocytes shortly after isolation from the ovary were excluded from further analyses. Individual healthy COCs ($n = 271$) were randomly divided among six different treatment groups and cultured in 100 μl TALP (Tyrode's, albumin, lactate, pyruvate) under mineral oil (Bavister *et al.*, 1983; Alak and Wolf, 1994) for 48 h at 37°C: (1) 5% monkey serum (MS; Control Group; $n = 49$ COCs); (2) MS + recombinant human FSH + LH (FSH + LH, 75 mIU/ml each; Gonadotrophin Group, abbreviated as GNT; $n = 52$ COCs); (3) MS + FSH + LH + AREG 10 ng/ml (GNT + AREG 10 ng/ml Group; $n = 62$ COCs); (4) MS + FSH + LH + AREG 100 ng/ml (GNT + AREG 100 ng/ml Group; $n = 29$ COCs); (5) MS + AREG 10 ng/ml (AREG 10 ng/ml Group; $n = 40$ COCs) and (6) MS + AREG 100 ng/ml (AREG 100 ng/ml Group; $n = 39$ COCs). The MS

was obtained by pooling and filtering serum samples from female monkeys at a specific day of the natural cycle (Day 1 or 2 after the mid-cycle peak of estradiol). The serum was aliquotted and stored at −80°C until use. Human recombinant FSH and LH were provided by NV Organon (Oss, The Netherlands) and Ares Serono (Randolph, MA, USA), respectively. AREG was purchased from Sigma (St Louis, MO, USA). Images of the individual COCs were acquired at 0, 24 and 48 h post-treatment, using a digital camera attached to an Olympus CK40 microscope.

Oocyte maturation and immunofluorescence by confocal microscopy

At the end of the culture (48 h), COCs were treated with hyaluronidase as previously described (Peluffo *et al.*, 2010) and denuded oocytes were assessed by light microscopy to analyze their nuclear maturation stage [germinal vesicle (GV)-intact; metaphase I (MI) and metaphase II (MII)]. In addition, a small cohort ($n = 10$) of denuded MII-stage oocytes from all the treatments was randomly selected and fixed in 4% paraformaldehyde for indirect immunofluorescence as previously described (Peluffo *et al.*, 2011b; Xu *et al.*, 2011b) to assess spindle and polar body (PB) morphology after *in vitro* maturation (IVM). Oocytes were analyzed by confocal microscopy (Leica SP5 AOBS, Leica Microsystems, Heidelberg, Germany) using two different objectives (PL APO CS 20× 0.7 DRY UV and PL APO CS 63× 1.3 GLY UV). Full Z-stack data sets were collected with the 63× objective for each oocyte, with images taken every 0.5 μm.

Semen preparation, ICSI and embryo development

Semen collection from fertile male rhesus monkeys, as well as ICSI, was performed by the ART Core at the ONPRC as previously described (Lanzendorf *et al.*, 1990a,b; Mitalipov *et al.*, 2001). A cohort of MII oocytes ($n = 111$) from 16 different animals was fertilized by this technique (based on the availability of fresh, viable sperm). Randomly selected oocytes were injected and then transferred to four-well dishes (Nalge Nunc International Co, Naperville, IL, USA) containing 500 μl HECM-9 (serum free) equilibrated at 37°C in 6% CO_2, 5% O_2 and 89% N_2, and covered with tissue culture oil (SAGE®, CooperSurgical, Inc.) (Lanzendorf *et al.*, 1990b; Mitalipov *et al.*, 2001). Oocytes were examined 16 h post-insemination for the presence of two PBs and two pronuclei to confirm fertilization. Randomly selected embryos ($n = 37$) were cultured for up to 2 weeks to analyze their developmental potential. In this case, the embryos were cultured without serum for 48 h and thereafter in HECM-9 with 5% fetal bovine serum (Wolf *et al.*, 2004). The medium was changed and pictures were taken on alternating days. Acquired images were used to document embryonic development. The remaining embryos were frozen and stored for future use.

Statistical analysis

Statistical calculations for the AREG levels were performed using the Sigma Stat software package (Systat Software, Inc., Richmond, CA, USA). Differences in the AREG content among groups were analyzed using transformed data and a one-factor analysis of variance followed by comparison among means using the Holm–Sidak method. The generalized linear model and logistic regression were used to examine the role of AREG on rhesus macaque oocyte maturation, fertilization and first cleavage (fertilized oocytes that cleaved to the two-cell stage). A Generalized Estimating Equation was used to determine the significance of the different treatment conditions. Using the adjusted correlated outcome data, the odds ratios (ORs) and 95% confidence interval (CI) were calculated. Differences or ORs were considered significant at $P < 0.05$.

Results

AREG protein levels in the rhesus macaque pre-ovulatory follicle

To establish the physiological concentration of the AREG in the primate pre-ovulatory follicle, its levels were determined in follicular fluid obtained from animals undergoing a COv protocol (Fig. 1). Non-detectable levels (<15 pg/ml) of the AREG protein were observed in all samples of follicular fluid before hCG treatment (0 h). However, hCG increased ($P < 0.05$) AREG levels in follicular fluid at 12, 24 and 36 h post-hCG. AREG protein levels in follicular fluid ranged from 4.8 up to 121.4 ng/ml between the different animals at 12 h after hCG administration. In contrast, EREG levels were minimal or undetectable (<15 pg/ml) in all groups (data not shown).

Effect of physiological AREG levels on rhesus macaque oocyte maturation

Oocyte maturation was evaluated within a healthy cohort of COCs ($n = 271$) retrieved from SAFs after 48 h of culture under different treatment conditions, as detailed in Table I. The highest percentage of MII oocytes was observed in the AREG (10 ng/ml) groups cultured with or without gonadotrophins (82 and 70%, respectively) (Table I). In contrast, the control and gonadotrophins alone groups had lower percentages of MII oocytes (63 and 56%, respectively) relative to treatment with AREG (10 ng/ml) plus or minus gonadotrophins. The percentages of MII oocytes following culture with a 10-fold higher concentration of AREG (100 ng/ml) in the presence or absence of gonadotrophins (48 and 59%, respectively) were similar to those observed in the control and gonadotrophin groups. After adjusting for the correlated outcome data, oocytes from COCs treated with gonadotrophins + AREG 10 ng/ml were 3.8 times more likely to have a mature MII stage oocyte compared with the control group ($P < 0.05$, 95% CI = 2.22, 6.71). The group containing gonadotrophins treated with AREG at 10 ng/ml was 4.4 times more likely to mature to MII compared

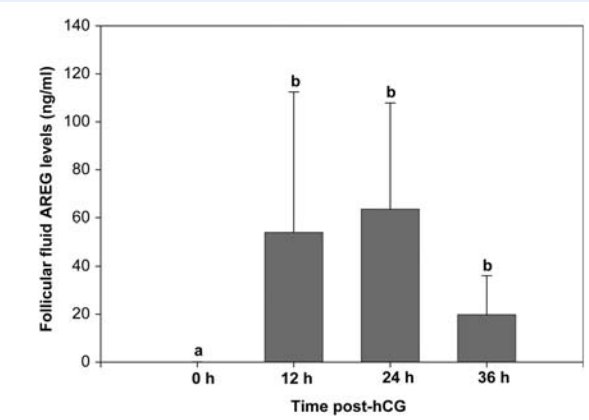

Figure 1 AREG levels in the follicular fluid of pre-ovulatory follicles. Follicles were obtained at 0, 12, 24 and 36 h post-hCG from rhesus monkeys undergoing COv protocols. Values are the mean ± SEM, $n = 3$/time point. Different letters represent significant differences among time points ($P < 0.05$).

Table I Proportion of oocytes from healthy, rhesus monkey COCs at each stage of nuclear maturation after 48 h in culture.

Treatment group	GV (%)	MI (%)	MII (%)
Control ($n = 13$)[†]	14/49 (29)	4/49 (8)[a]	31/49 (63)[A]
GNT ($n = 14$)	11/52 (21)	12/52 (23)[b]	29/52 (56)[A]
GNT + AREG 10 ng/ml ($n = 17$)	10/62 (16)	1/62 (2)[a]	51/62 (82)[B]
GNT + AREG 100 ng/ml ($n = 9$)	11/29 (38)	4/29 (14)[ab]	14/29 (48)[A]
AREG 10 ng/ml ($n = 12$)	9/40 (22)	3/40 (8)[ab]	28/40 (70)[A]
AREG 100 ng/ml ($n = 7$)	12/39 (30)	4/39 (10)[ab]	23/39 (59)[AB]

Different lower case and upper case superscripts represent significance differences ($P < 0.05$) between treatment groups for MI and MII stage oocytes, respectively.
GV, germinal vesicle; MI, metaphase I; MII, metaphase II; AREG, amphiregulin; GNT, gonadotrophins (FSH and LH, both at 75 mIU/ml).
[†]The number of animals from which SAFs and their associated COC were obtained.

with those with gonadotrophins alone ($P < 0.05$, 95% CI = 2.38, 8.10). COCs treated with gonadotrophins and AREG at 10 ng/ml were five times more likely to have oocytes that matured to MII compared with the oocytes from COCs treated with gonadotrophins and AREG at 100 ng/ml ($P < 0.05$, 95% CI = 2.18, 11.35). The oocytes from the gonadotrophins + AREG 10 ng/ml group were 2.5 times more likely to mature to the MII stage compared with the oocytes from COCs treated with 10 ng/ml AREG alone ($P < 0.05$, 95% CI = 1.18, 5.08). Finally, when comparing the results from the groups containing AREG without gonadotrophins, oocytes from COCs treated with AREG at a concentration of 10 ng/ml tended to be more likely to mature to MII oocytes when compared with the serum only control group (1.5 times, $P = 0.09$, 95% CI = 0.93, 2.55). The stage of the menstrual cycle from which the COCs were obtained did not affect the rates of oocyte meiotic resumption or progression, either in the presence or absence of AREG (data not shown). Therefore, the data presented in Table I represents COCs from all stages of the cycle within a treatment group.

When comparing the effects of the different treatment groups on the reinitiation of meiosis, the highest percentage of MI oocytes was observed in the gonadotrophins group (23%) (Table I). The addition of AREG (10 ng/ml) and gonadotrophins to the medium resulted in only 2% MI oocytes, whereas 100 ng/ml AREG and gonadotrophin-containing cultures yielded 14% MI-arrested oocytes. There was no significant difference in the percentage of MI oocytes between the control group (8%) and the AREG groups lacking gonadotrophins (8 and 10% at 10 and 100 ng/ml AREG, respectively). Those COCs treated with gonadotrophins alone were 1.2 times more likely to mature to MI when compared with the COCs treated with media alone ($P < 0.05$). The COCs treated with gonadotrophins plus AREG 10 ng/ml were 16.7 times less likely ($P < 0.05$) to be at the MI stage when compared with the COCs treated with gonadotrophins alone. However, those COCs that were treated with gonadotrophins and AREG (100 ng/ml) were 2.5 times less likely to be arrested at MI when compared with COCs treated with gonadotrophins alone.

The morphology of several of the MII oocytes obtained in the present study from the different treatment groups analyzed by immunofluorescence showed a typical barrel-shaped spindle with properly aligned chromosomes (Fig. 2B and D), suggesting that the current IVM conditions support the development of MII oocytes with an overall normal morphology. However, the PB was occasionally abnormal in shape, being elongated (Fig. 2C and D) rather than the condensed and spherical shape typically associated with *in vivo* matured oocytes. Of the MIIs within all the different treatments, 15% (27/176) displayed elongated PB from 13 out of the 27 animals used in the present study. The number of elongated PBs was too low to identify a treatment effect.

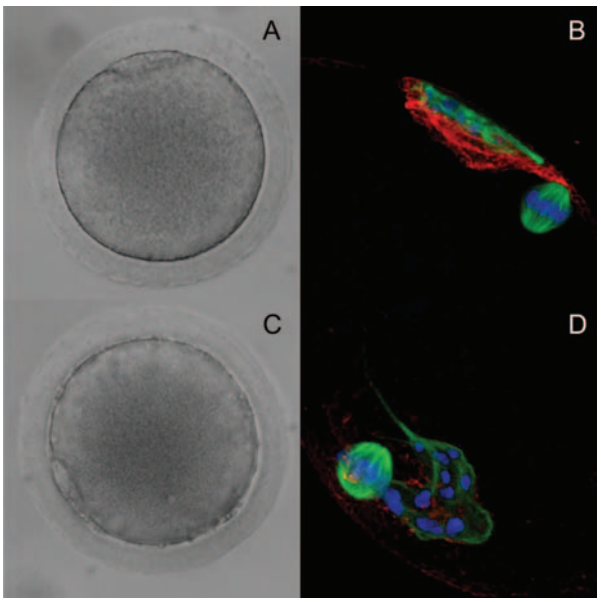

Figure 2 Representative pictures of MII oocytes from rhesus monkeys after 48 h of IVM. (**A** and **C**) Images of MII oocytes taken by light microscopy (20×). (**B** and **D**) Confocal microscopy (63×) images of the spindles and PBs from the same MII oocytes after immunofluorescence (red = F-actin, green = tubulin, blue = DNA). (B) shows a typical barrel-shaped spindle with aligned chromosomes and a normal PB, whereas (D) shows a normal-shaped spindle but with an abnormal, diffuse PB.

Table II Fertilization and first cleavage rates after ICSI for rhesus monkeys.

Treatment group[a]	MII	Fertilized (%)[b]	First cleavage(%)[b]
Control ($n = 8$)	20	18/20 (90)	17/18 (94)
GNT ($n = 12$)	26	24/26 (92)	23/24 (96)
GNT + AREG 10 ng/ml ($n = 13$)	39	35/39 (90)	35/35 (100)
GNT + AREG 100 ng/ml ($n = 7$)	14	14/14 (100)	14/14 (100)
AREG 10 ng/ml ($n = 4$)	10	10/10 (100)	10/10 (100)
AREG 100 ng/ml ($n = 2$)	2	2/2 (100)	2/2 (100)

[a]The number of animals from which MII oocytes were obtained for fertilization by ICSI.
[b]There were no significant differences in either the fertilization rate or the first cleavage rate among the different culture conditions.

Table III Embryo development in a rhesus monkey 2 days after ICSI.

Treatment group	Total embryos	2–4 cells (%)[a]	5–8 cells (%)[a]	>8 cells (%)[a]
Control ($n = 8$)[b]	17	13 (76)	4 (24)	
GNT ($n = 12$)	23	19 (83)	3 (13)	1 (4)
GNT + AREG 10 ng/ml ($n = 13$)	35	21 (60)	9 (26)	5 (14)
GNT + AREG 100 ng/ml ($n = 7$)	14	11 (79)	3 (21)	
AREG 10 ng/ml ($n = 4$)	10	5 (50)	4 (40)	1 (10)
AREG 100 ng/ml ($n = 2$)[c]	2	1	1	

[a]There were no significant differences in either the fertilization rate or the first cleavage rate among the different treatments.
[b]The number of animals from which embryos were obtained.
[c]Percentages for this category were not calculated owing to the low number of embryos.

AREG effects on oocyte fertilization and embryo development

The fertilization and first cleavage rates obtained by ICSI of the IVM MII oocytes ($n = 111$, from 16 animals) were 93 and 98%, respectively (Table II). There were no differences in either fertilization or first cleavage rates between the different culture conditions. Randomly selected embryos were cultured for up to 2 weeks to assess their developmental progression ($n = 5$ animals). Embryo development within the different treatment groups 2 days after ICSI is summarized in Table III. Overall, a total of 13 early blastocysts were obtained, with 4 embryos developing to expanded blastocysts (Fig. 3). The early blastocysts were from various treatment groups: $n = 4$ control, five gonadotrophins only, two gonadotrophins + AREG 10 ng/ml and two AREG only embryos (one from each concentration). For the four expanded blastocysts, one was from control, one from gonadotrophins only and two from the gonadotrophins + AREG 10 ng/ml group.

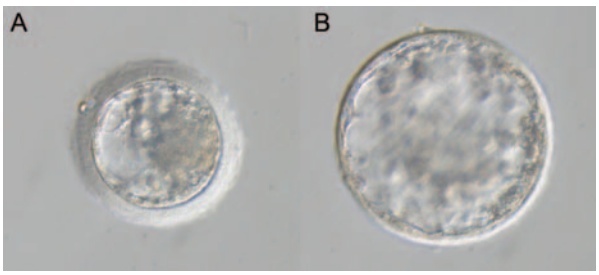

Figure 3 Representative pictures of an early (**A**) and an expanded (**B**) blastocyst obtained after ICSI of IVM MII oocytes from rhesus monkeys (20× magnification).

Discussion

The systematic characterization of differentially expressed mRNAs in the rhesus macaque follicle prior to and after an ovulatory stimulus, which is possible through standard genomic approaches, allows for the identification of the cellular and molecular events that are necessary for the formation of an oocyte capable of undergoing fertilization and early embryonic development. Using Affymetrix total genome arrays, we previously elucidated the transcriptome of the macaque pre-ovulatory follicle at different times following an hCG stimulus in a COv protocol, indicating that various members of the EGF-family, including AREG, EREG, BTC and EGFR, are expressed in the primate (Xu *et al.*, 2011a). This study utilized a COv model, which offers the unique opportunity to analyze the single, naturally selected, dominant follicle at defined intervals prior to and after exposure to an ovulatory gonadotrophin stimulus (bolus of hCG) during the menstrual cycle in rhesus macaques (Young *et al.*, 2003). While AREG and EREG mRNA significantly increased 12 h after an ovulatory stimulus, BTC

and EGFR mRNA levels did not vary. BTC mRNA, while detectable, was at very low levels relative to the other EGF-like genes. In addition, other EGF family members, such as EGF and TGFα, displayed minimal or undetectable mRNA levels. By using specific AREG and EREG ELISAs in the present study, a rapid and significant increase in the AREG protein content was observed that is in agreement with the mRNA data. Before the ovulatory stimulus, AREG was below the limit of detection. AREG protein levels in follicular fluid varied from 4.8 up to 121.4 ng/ml between the different animals at 12 h after hCG administration. This range of AREG levels is similar to that observed previously in human follicular fluid obtained from IVF samples (20–80 ng/ml) (Zamah *et al.*, 2010). A previous study reported an average of the AREG protein of 108.4 ± 6.3 ng/ml in the follicular fluid from patients undergoing IVF; however, the range was much wider (0.8–1189.4 ng/ml) (Inoue *et al.*, 2009). A possible explanation for this discrepancy may be related to the fact that the follicular fluid in the latter study was pooled from both mature and immature follicles in each ovary.

On the basis of the range of AREG levels in the rhesus macaque and human pre-ovulatory follicles, we determined if there is a specific AREG level that impacts oocyte maturation. Using COCs from SAFs with immature oocytes, AREG effects were evaluated at a low and a high concentration (10 and 100 ng/ml, respectively) with or without gonadotrophins (FSH and LH, 75 mIU/ml each). Interestingly, the highest percentage of MII stage oocytes was obtained in the AREG groups at the lowest concentration (10 ng/ml) regardless of the presence of gonadotrophins. A previous study in women also showed that supplementation of gonadotrophin-free IVM media with either AREG and EREG improves the maturation of human GV oocytes *in vitro* (Ben-Ami *et al.*, 2011). Exposure to a higher concentration (100 ng/ml) of AREG in the presence or absence of gonadotrophins had no significant effect on meiotic progression when compared with groups cultured with gonadotrophins (56%) or with MS alone (63%). Nyholt de Prada *et al.* (2009) reported that EGF, also at a concentration of 100 ng/ml, had no effects on meiotic maturation of rhesus macaques COCs *in vitro*. Moreover, the high (100 ng/ml) concentration of AREG used in our study tended to decrease the percentage of MII stage oocytes. An inverse correlation was also noted between pooled follicular fluid AREG levels in women and the fertilization rate and hCG levels during pregnancy (Inoue *et al.*, 2009). Although not yet fully characterized, the extent and the route of EGFR degradation may be dependent on EGFR ligand concentrations (Madshus and Stang, 2009). Thus, it is possible that an optimal duration and degree of EGFR signaling in primate COCs, which has been reported as being necessary for rodent C-OE and oocyte maturation (Reizel *et al.*, 2010; Zwaenepoel *et al.*, 2010), would be accommodated through a specific range of EGFR ligand concentrations. In contrast to the results of Inoue *et al.* (2009), another study noted that follicles from women undergoing IVF procedures producing immature GV oocytes had lower AREG levels in the follicular fluid (Zamah *et al.*, 2010). Such findings are in line with studies using mice, where a linear dose–response for AREG was observed (Park *et al.*, 2004). This discrepancy may be related to the fact that oocytes from SAFs may respond to EGF-like factors within a more limited range than oocytes enclosed in pre-ovulatory follicles (Yang and Roy, 2001).

While AREG at a low concentration (10 mg/ml) led to the highest rate of MII stage oocyte development, the highest percentage of MI stage oocytes was observed in the gonadotrophin group. However, the addition of low levels of AREG (10 ng/ml) to the medium significantly decreased the percentage of MI oocytes. The percentages of MI stage oocytes from the groups containing AREG (10 and 100 ng/ml) without gonadotrophins were similar to those in the control group. Thus, AREG at 10 ng/ml, in the presence of gonadotrophins, appears to accelerate or facilitate GVBD and the MI–MII transition relative to gonadotrophins alone (82 versus 56% MII, respectively).

The cellular and molecular mechanisms behind the ability of EGFR signaling to promote oocyte GVBD and subsequent progression to MII are currently unknown. Possible EGFR-dependent actions include direct (i.e. oocyte) or indirect (i.e. cumulus) effects, such as the promotion of a transcriptionally permissive state (Luciano *et al.*, 2011), loss of cumulus cell-oocyte communication (Hsieh *et al.*, 2011), changes in cyclic nucleotide levels (Conti, 2011) and the induction of paracrine-acting steroids reported to promote C-OE and oocyte maturation (Jamnongjit *et al.*, 2005; Shimada *et al.*, 2006). Investigating the specific role that EGFR signaling plays in each of these processes awaits further study.

Oocytes from human follicles that undergo IVM often display spindles with disrupted or irregular morphology, which in turn leads to chromosomal abnormalities (Gras *et al.*, 1992; Racowsky and Kaufman, 1992). Even though supplementing the IVM media can improve maturation rates, this does not necessarily result in normal spindle formation and chromosomal alignment (Christopikou *et al.*, 2010). Remarkably, the spindle morphology of the IVM-derived oocytes analyzed in this study was generally normal. Elongated PBs were noted in a minority (~15%) of MII oocytes. A normal PB size, which is dependent on the molecular pathways that determine asymmetric positioning of the meiotic spindle before cytokinesis, was reported to be under control of the MOS/MAP2K/MAPK pathway (Verlhac *et al.*, 2000; Tong *et al.*, 2003). Additional studies are required to determine if this signaling pathway is abnormal in the rhesus macaque oocytes possessing elongated PBs and, if so, whether such an anomaly is directly related to EGFR activity.

Using the present IVM media, the fertilization rate and first cleavage rates were very high and better than previously obtained (Peluffo *et al.*, 2010). In women undergoing IVF protocols, levels of AREG in follicular fluid were inversely correlated with fertilization rates (Inoue *et al.*, 2009). However, in the present study, significant differences in the fertilization rate were not observed between treatment groups, which may be related to the small number of samples in certain treatment groups; thus, additional research is required to establish an effect of any of these treatments. In women, it was shown that AREG treatment of oocytes *in vitro* increased the fertilization rates (Ben-Ami *et al.*, 2011). Moreover, De la Fuente *et al.* (1999) demonstrated that EGF enhances preimplantation developmental competence of maturing mouse oocytes. In the present study, a total of 13 early blastocysts were obtained and 4 embryos progressed to the expanded blastocyst stage. Accurate developmental rates cannot be calculated because some of the embryos from these experiments were frozen on Day 2 or 3 after ICSI. Taken as a whole, these results suggest that the MII-stage oocytes from SAFs (0.5–2 mm) after IVM are able to undergo early embryonic development after ICSI.

In summary, for the first time in non-human primates, AREG was measured in the follicular fluid from pre-ovulatory follicles and shown to be induced by an ovulatory stimulus. This study also demonstrates that a specific concentration of AREG (10 ng/ml) enhances IVM of oocytes from rhesus macaque SAFs obtained at different stages of the natural menstrual cycle. IVM is a relatively new assisted reproduction technique that needs further optimization to improve its use as an option for infertility patients (Gilchrist, 2011). Certain findings obtained in the current study are encouraging and relevant from a clinical standpoint. For example, SAFs collected at any stage of the menstrual cycle (early follicular phase, luteal phase or unknown, i.e. from necropsies) were able to provide COCs with oocytes that resume and complete meiosis upto the MII stage, as well as fertilize and undergo early embryonic development. These results are important, particularly in the context of women who wish to preserve oocytes for future use but should not delay to collect their ovarian tissue or receive a hormonal stimulation treatment, such as young women undergoing gonadotoxic chemo- or radiotherapy. The ovary is a reservoir of a large pool of competent GV-stage oocytes that represent an untapped source for female fertility preservation (Comizzoli *et al.*, 2011). Thus, COCs from SAFs warrant further research to fully define their potential as another clinical option for fertility preservation.

Acknowledgements

We are grateful for the expert contributions of the animal care staff and surgical unit of the Division of Animal Resources, and the technical support of the ART core and the Endocrine Services and Technology Laboratory at ONPRC. A special thanks to Keith Masterson for performing the ICSIs and for freezing the embryos. Recombinant human FSH (Organon) and LH (Merck Serono) were generously donated for this project. A special thanks to Melinda Murphy for her technical assistance.

Authors' role

M.C.P. contributed to the design of the study, execution, analysis and data interpretation as well as manuscript preparation; A.Y.T. helped collect the samples; A.M.Z. performed the AREG and EREG ELISAs; R.L.S., M.B.Z., M.C., and J.D.H. contributed to the design of the study, data interpretation and manuscript review. All the authors read and reviewed the manuscript.

Funding

This research was supported by the Oncofertility Consortium NIH 1 UL1 RR024926 (R01-HD058294, PL1-EB008542), the NIH/Eunice Kennedy Shriver NICHD Specialized Cooperative Centers Program in Reproduction and Infertility Research (U54 HD18185), the NIH/Eunice Kennedy Shriver NICHD Contraceptive Development and Research Center (U54 HD55744), NIH/NCRR-RR000163, NIH R01 HD052909 (MC) and a Lalor Foundation Postdoctoral Basic Research Fellowship (MCP). The use of the Leica confocal was supported by grant number S10RR024585. The statistical analyses of the data from the 27 experiments regarding oocyte maturation was performed by the statistician Byung Park with support from the ONPRC Biostatistics Unit, grant number RR000163 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH).

Conflict of interest

None declared.

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