Human oocyte maturation is

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Introduction

- Oocyte re-entry into the meiotic cell cycle and completion of maturation activation of two distinct programs of terminal differentiation in mural granulosa cells (mGCs) and cumulus cells (CCs) surrounding the oocyte, pave the way to the ovulatory process.

- These events are followed by the rupture of the follicle and extrusion of a mature cumulus–oocyte complex (COC) competent for fertilization.

- All the events are triggered by a rapid increase in circulating levels of the gonadotrophin LH.

- LH/hCG receptors are present on theca, on mGCs as well as on cumulus granulosa cells in some species

- Recent studies: LH signaling at the time of final oocyte maturation involves activation of the small G protein Ras as well as activation

- In multiple species, LH receptor activation causes up-regulation of the epidermal growth factor (EGF)-like growth factor family *(Ashkenazi et al., 2005; Fru et al., 2007; Yamashita et al., 2007; Li et al., 2009)*.

- This is a potential mechanism for transducing the LH signal to the oocyte, since these growth factors are known to interact with the CCs which are in intimate contact with the developing oocyte.

- In the mouse, amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) have been show to be mediators of LH signaling in the follicle (*Park et al., 2004)*.

- Further work : an impairment in female mouse fertility following genetic disruption or pharmacological blockade of LH-mediated transactivation of the EGF signaling network *(Hsieh et al., 2007)*.

- The three EGF-like ligands *AREG*, *EREG* and *BTC* were discovered within the past two decades *(Shoyab et al., 1988; Shing et al., 1993; Toyoda et al., 1995)*.

- AREG and the other family members are synthesized as transmembrane precursors which are then cleaved to release the active growth factor which can interact with different members of the EGF receptor family *(Sanderson et al., 2006)*.

- The purpose of our study is to provide a detailed characterization of EGF-like growth factors in human FF and their regulation.

- provide evidence that **bioactive AREG accumulation is induced by hCG** and **that**

Materials and Methods

(Source and collection of human FF samples)

- ART : standard ovarian stimulation protocols
- each patient had either 1 or 2 follicle aspirates collected at the time of OR 36 h after hCG from mature-sized follicles (≥16 mm diameter).
- Pre-hCG FF was obtained from mature-sized follicles

- mGCs were obtained by aspiration from FF after microscopic identification.
- CCs were obtained by mechanically separating a portion of the cumulus from the oocytes at the time of ICSI.
- The decision for conventional insemination or ICSI fertilization was made by the clinician based on the presence of a component of male factor infertility.
- The study was approved by the institutional review board at University of California, San Francisco (UCSF) and informed consent was obtained from all patients involved in this study.

(Granulosa cell RNA extraction and quantitative RT-PCR)

- Total RNA from CCs or mGCs was isolated using the RNeasy Micro Kit following the manufacturer's instructions.

- RNA quality was determined with the Bioanalyzer 2100 and RNA 6000 Pico LabChip assay (Agilent Technologies Inc., Palo Alto, CA, USA).

(Quantification of EGF-like growth factor levels)

- AREG protein levels were quantified using a commercially available ELISA kit according to the manufacturer's instructions

- EREG levels were quantified by ELISA using a mouse monoclonal anti-human primary antibody and a biotinylated goat anti-human secondary antibody with similar conditions to the AREG ELISA

(Immunodepletion of human FF)

- Immunodepletion of FF was performed by incubating FF supplemented with protease inhibitors and either goat IgG anti-human AREG or goat IgG antihuman EREG antibodies or control IgG and Protein A/G beads, and tumbling overnight at 48C.

(Statistical analyses)

- Analysis of variance (ANOVA) was performed to compare levels of AREG between multiple groups.
- Where the ANOVA was significant, Student's t-test with Bonferroni correction was performed for pairwise comparisons between groups.
- Logistic regression analyses were used to determine any significant relationship between levels of AREG and other relevant clinical parameters (female age, follicular size, follicular hCG).
- Tests were declared statistically significant for a

Results

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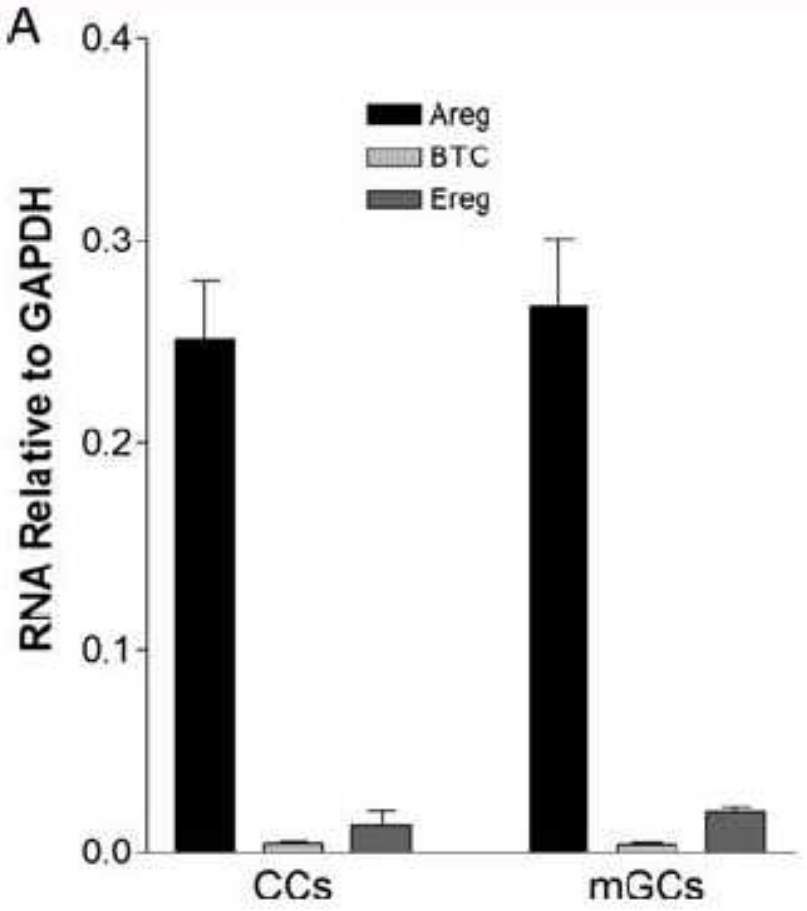
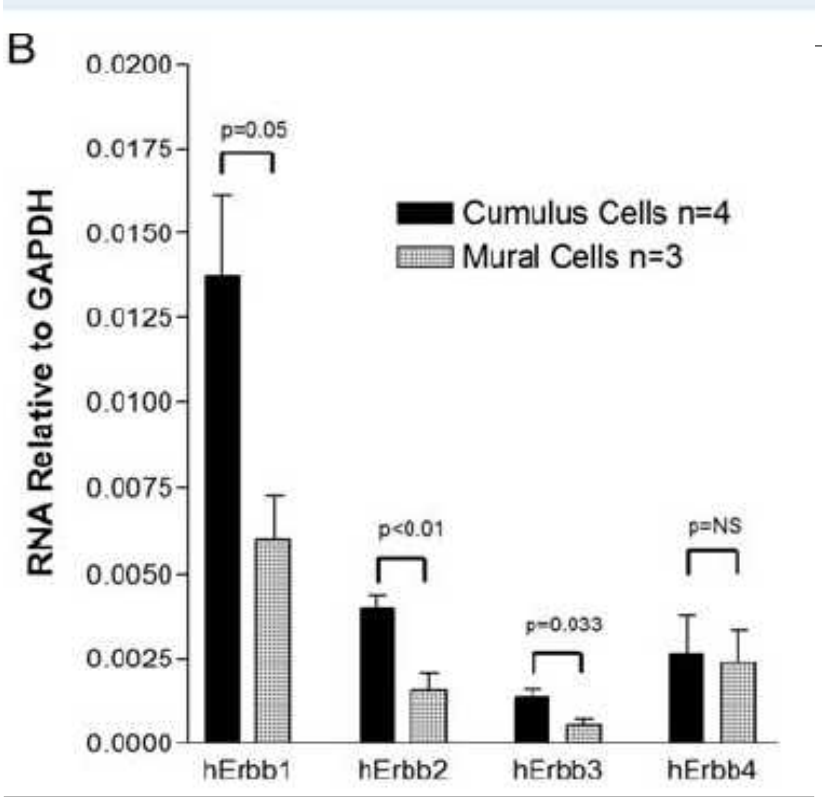


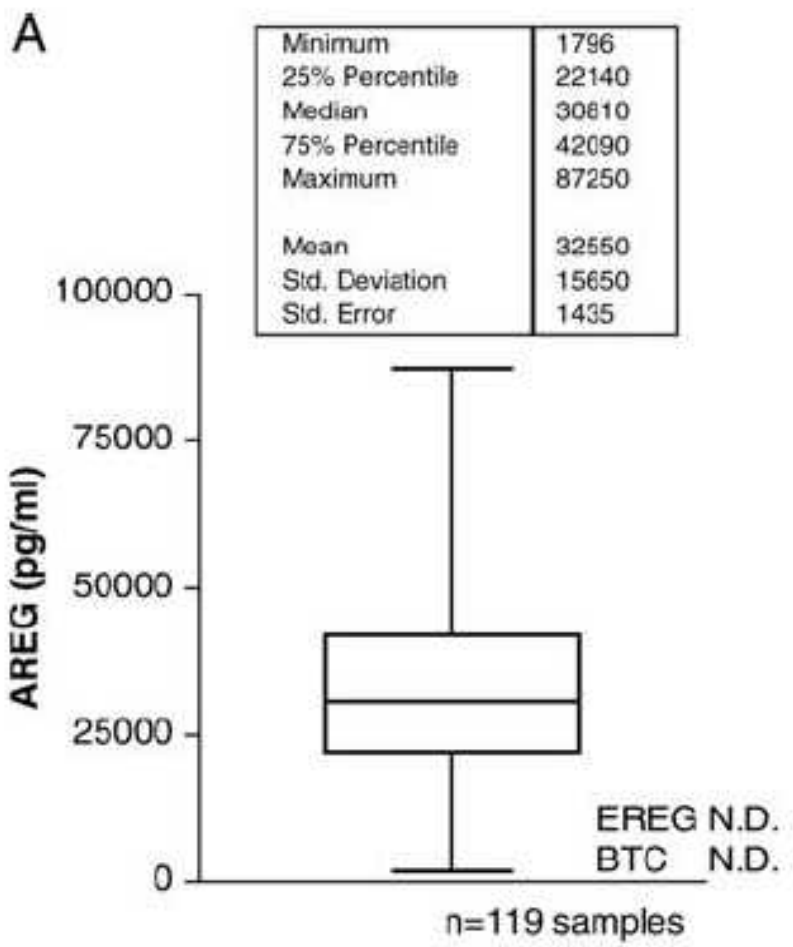
Areg mRNA was 10-fold higher than Ereg mRNA and 60-fold higher than BTC mRNA (P<0.001)

mRNA levels of Erbb1 (receptor shared by these three factors)



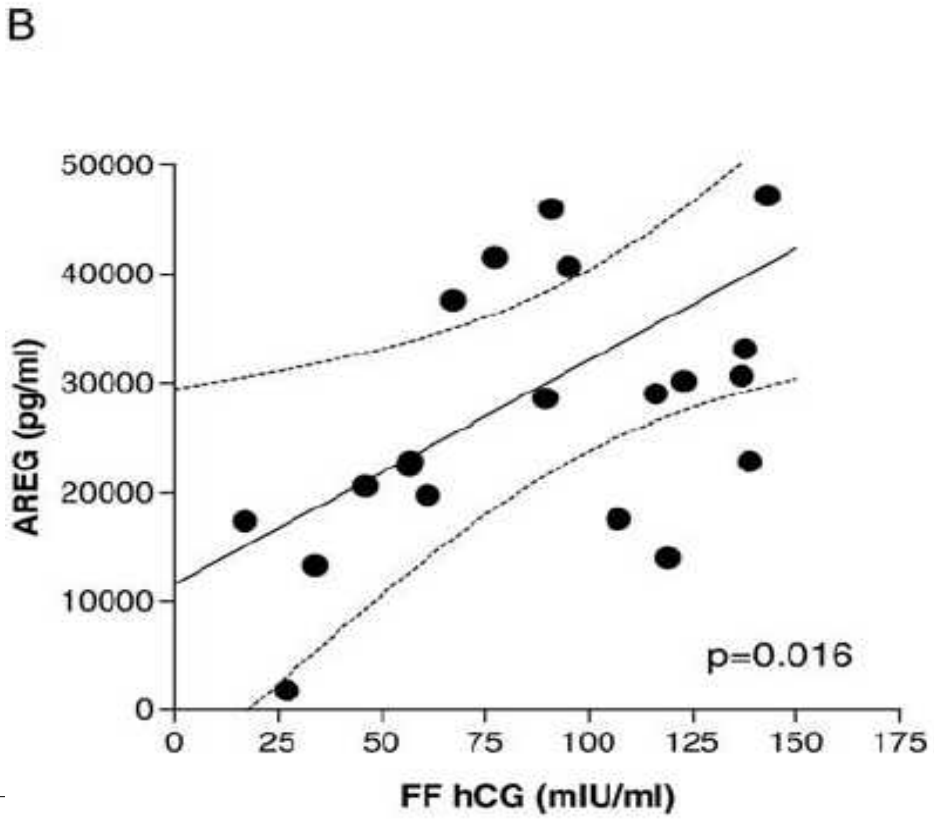
higher concentrations were detected in CCs compared with mGCs CCs are a sensitive target for EGF-like growth factors

- the protein levels of these three EGF-like ligands in human FF.
- High levels of AREG p both pooled FF and ir
- AREG concentration r 000 pg/ml (Fig. 2A).



In the five patients available where FF was obtained prior to hCG administration AREG was undetectable.

A significant correlation between hCG concentration in the FF and AREG accumulation was established by comparing AREG and hCG levels within individual follicles (P =0.016)



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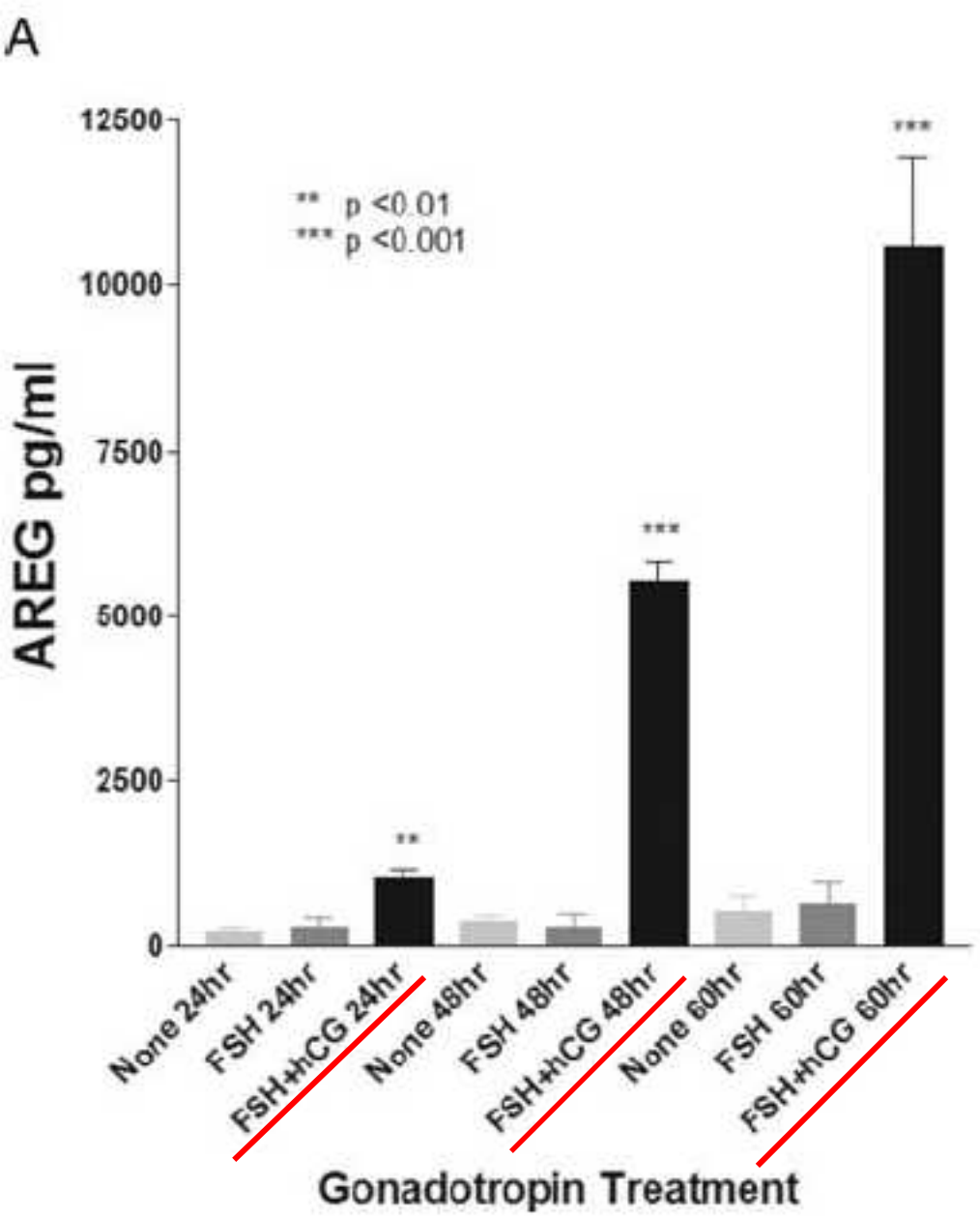

- The FSH alone did no
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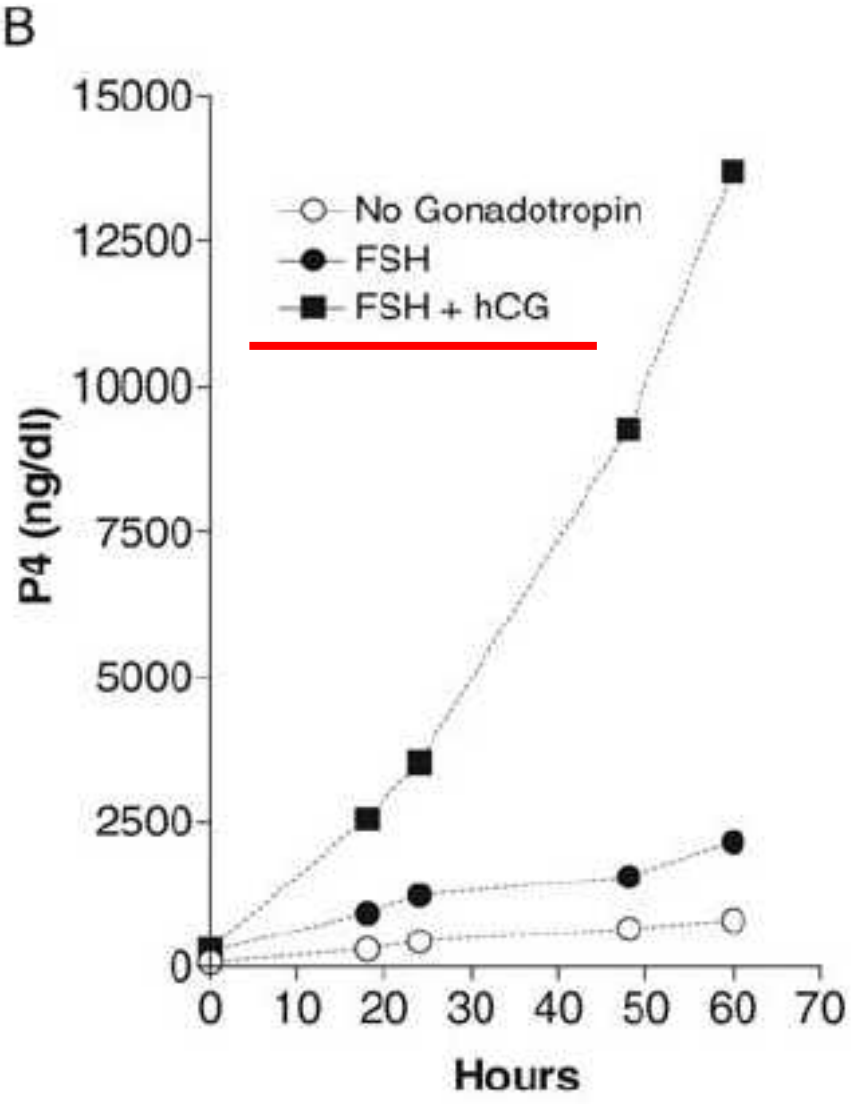

- serum AREG levels were measured in 5 patients on the day prior to hCG administration and 36 h after hCG.
  - Serum AREG levels were three orders of magnitude lower than in the FF and showed no significant change after hCG administration
- A human EREG ELISA was developed with a sensitivity of 200 pg/ml and employed to assay 20 individual FF samples.
  - Significant EREG accumulation was detected in only a single sample at a level of 400 pg/ml.
- A commercially available ELISA for BTC with a sensitivity of 15 pg/ml was used to assess four pooled samples of FF with known high levels of

(AREG in human FF is biologically active)

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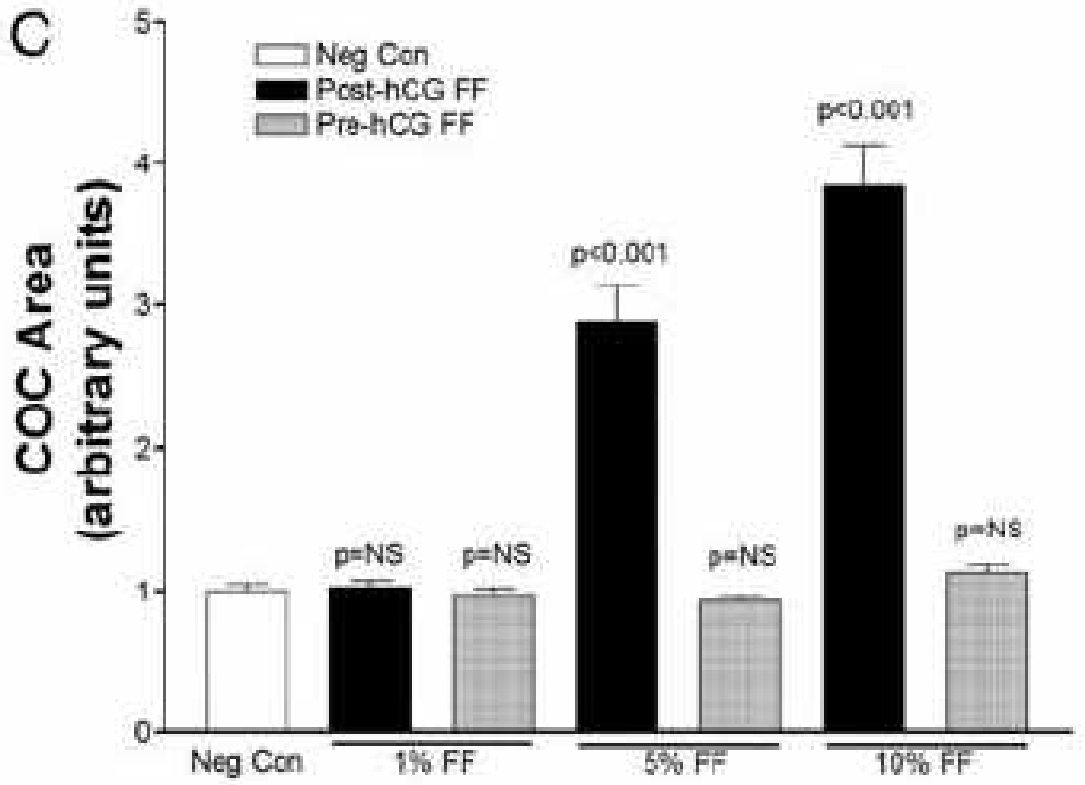
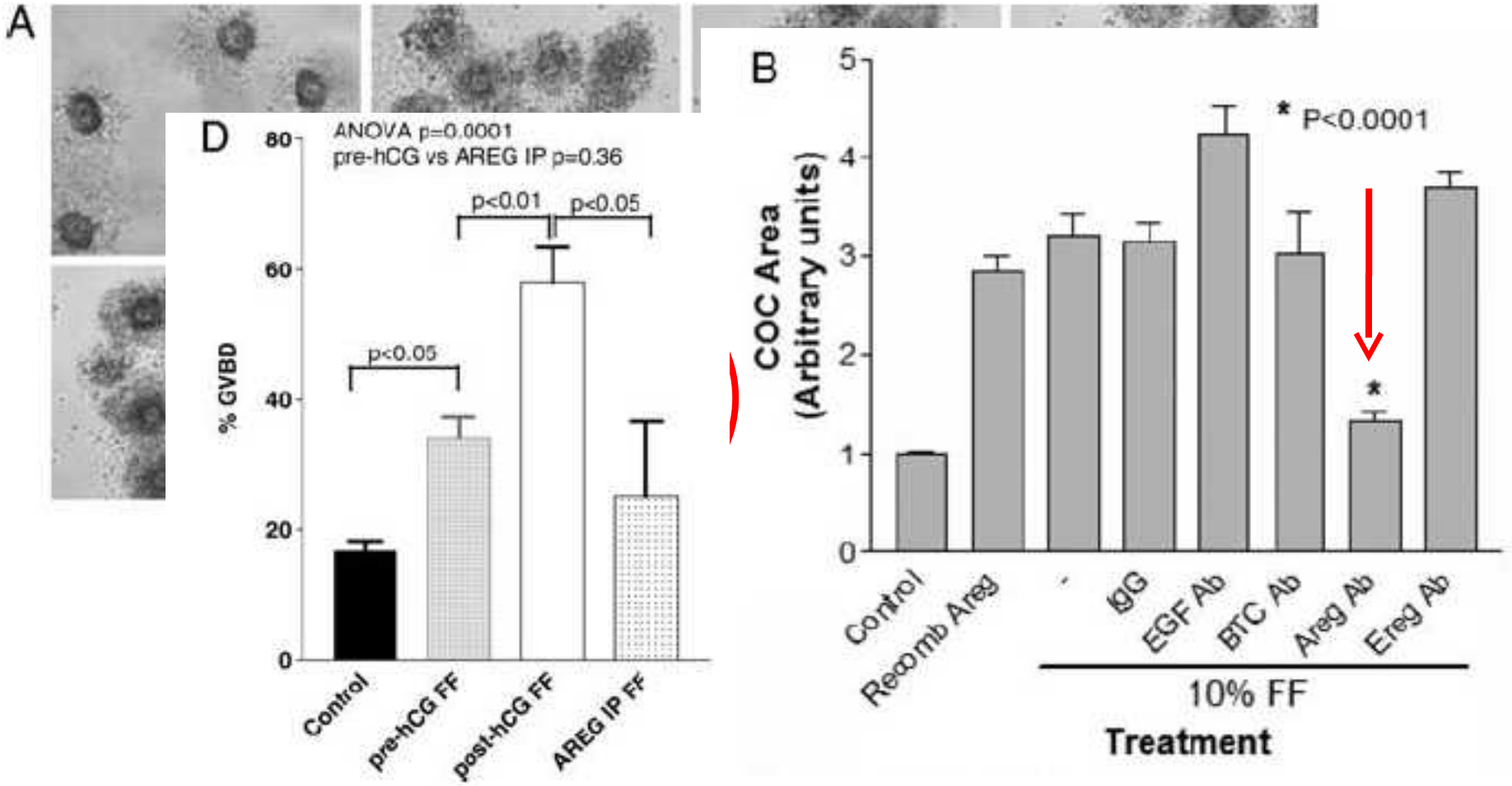


- Immunodepletion of AREG, but not EGF or other EGF-like growth factors, blocks the ability of post-hCG FF to cause this expansion (Fig. 4A and B).



- This stimulation was maximal with FF obtained after hCG trigger and was greatly reduced after immunodepletion of AREG.

- These results confirm the presence of biologically active AREG in human FF and suggest that **AREG is a physiological mediator of oocyte maturation and CC expansion.**

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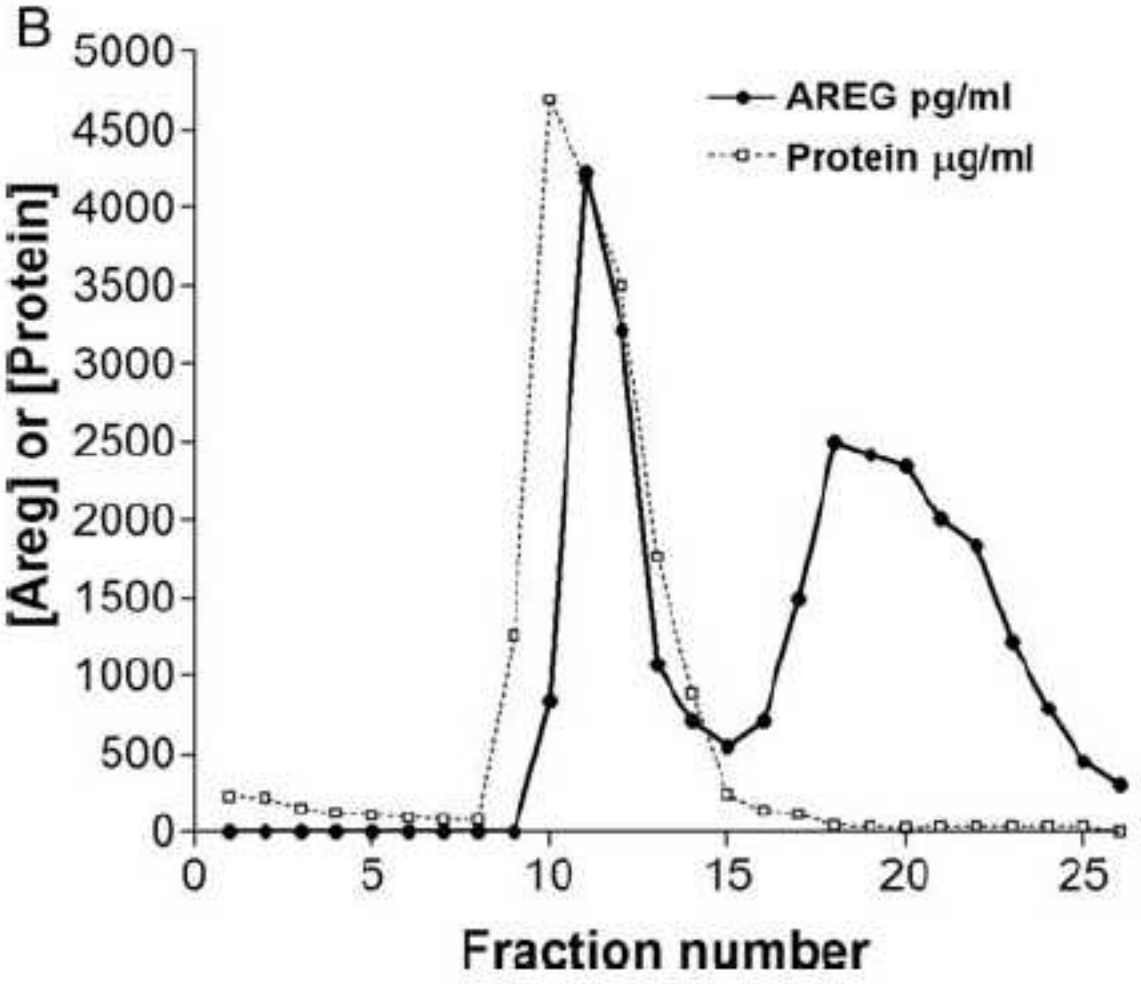


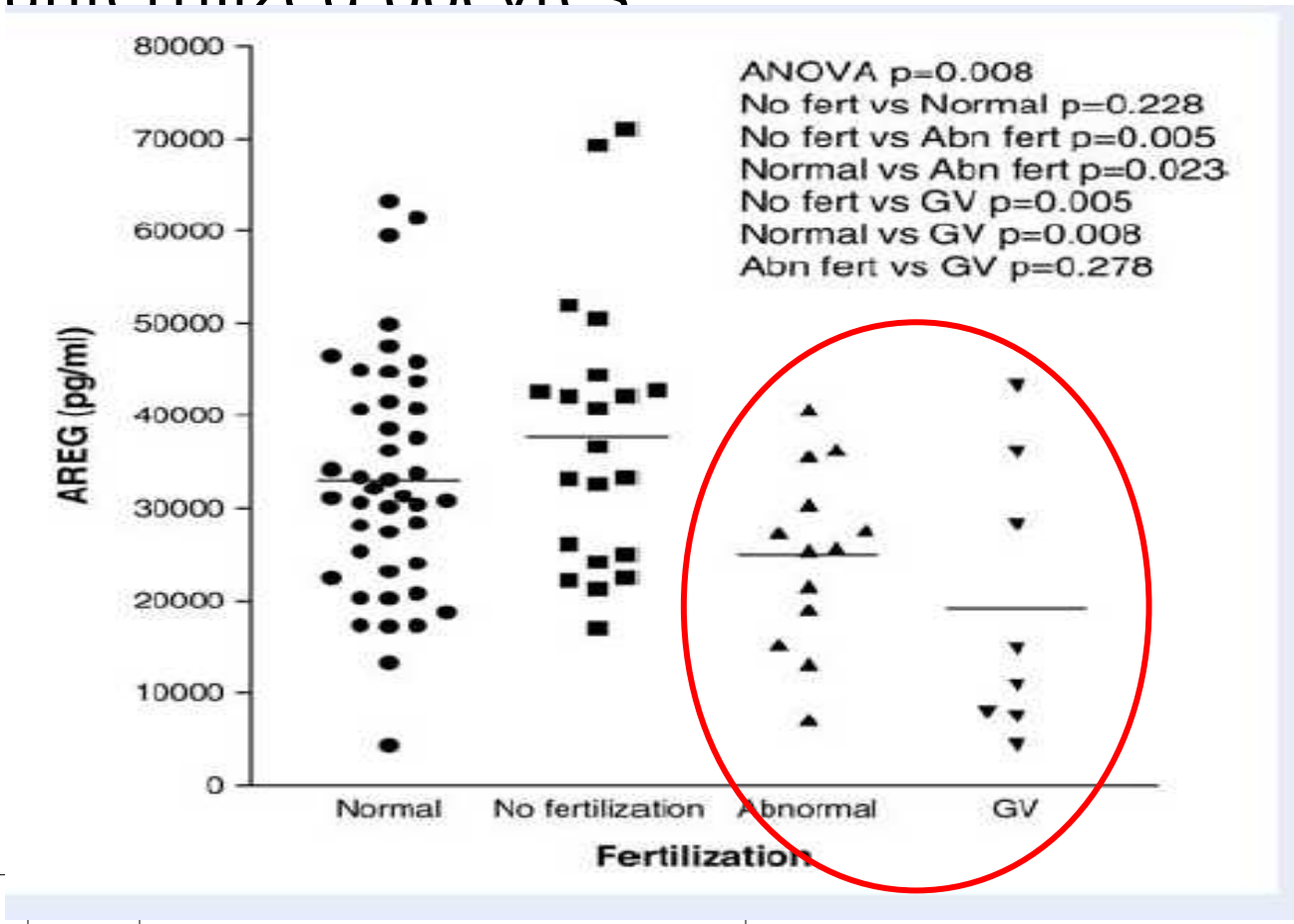
- The fractions were then analyzed in the COC expansion assay
  - the first AREG peak showing some activity for CC expansion
  - the second peak fractions caused oocyte degeneration, *(possibly related to the higher salt concentrations in the second peak fractions).*

(AREG levels as a predictor of oocyte quality)

- We examined AREG levels in follicles yielding oocytes that
  - did not fertilize
  - fertilized normally
  - fertilized abnormally with ICSI
  - follicles yielding immature oocytes

- Follicles producing immature GV oocytes have lower AREG levels than follicles producing nuclear mature metaphase II (MII) oocytes (P =0.0071).

- Figure 6 shows that follicles yielding GV oocytes or abnormally fertilized oocytes *(defined as any fertilization other than two pronuclei)* have significantly lower levels of AREG than follicles yielding either normal or unfertilized oocytes



Discussion

- we provide evidence that **gonadotrophin stimulation of the human ovulatory follicle produces massive accumulation of the EGF-like growth factor AREG in FF**.
  - no immunoreactive AREG is detected in FF prior to the LH surge
  - LH/hCG stimulates AREG secretion in vitro
  - bioactive AREG appeared in FF after hCG stimulation
  - positive correlation is established between hCG levels and AREG concentration in the FF of the mature-sized follicle.

- follicles yielding an immature oocyte or an oocyte supporting aberrant embryo development showed significantly lower levels of AREG.

- Furthermore, we show that 36 h after hCG administration, in both CC and mGCs the predominant EGF-like growth factor mRNA expressed is AREG, with much lower levels of EREG and BTC mRNAs.

- This is in contrast to murine studies, where up-regulation of all three EGF-like growth factors was seen after LH administration *(Park et al., 2004)* which could either reflect species-specific differences, such as differential LH receptor signaling, or be related to the sampling timing, since we do not have available FF at different times after hCG administration.

- One possibility is that EREG or BTC is expressed earlier than 36 h after hCG.

- we tested whether AREG was detectable in serum and if this could be used as a correlate for FF AREG levels, but we were unable to find any measurable clinical differences in serum level.

- This result is not necessarily surprising since the production of AREG would only occur for 36 h and it may not have had time to diffuse into the serum, or it could reflect degradation.

- The AREG accumulating in FF is biologically active, because it supports both oocyte maturation and cumulus expansion
- Our study suggests that AREG is physiologically a critical ligand for murine CC expansion.
- Under our experimental conditions, recombinant AREG alone is sufficient to cause COC expansion
- Alternatively, the potencies of the native and recombinant forms of AREG are different.
- The recombinant protein is expressed in E. coli and therefore may have aberrant post-translational modifications.

- in several animal models, previous work has examined the effects of EGF on human IVM conditions and shown that addition of this growth factor can improve IVM outcomes *(Das et al., 1991; Gomez et al., 1993)*.

- The addition of physiological concentrations of EGF to IVM culture media promote both nuclear and cytoplasmic maturation of human GV oocytes

- Additional work in rhesus monkeys has shown that AG1478 (a chemical EGF receptor inhibitor) decreased oocyte maturation, cumulus expansion and blastocyst formation in vitro, highlighting the critical importance of EGFR activation for proper oocyte maturation in vitro *(Nyholt de Prada et al., 2009)*.

- Previous studies have shown that AREG is synthesized as a 252 amino acid transmembrane precursor with multiple N-linked and O-linked glycosylation sites *(Shoyab et al., 1988; Sanderson et al., 2006)*.

- Differential proteolytic cleavage is thought to generate two different AREG isoforms of slightly different molecular weights (78 and 84 amino acids).

- Our chromatography studies suggest the presence of either two isoforms of AREG, or a free and bound species of AREG in human FF.

- For technical reasons, we could not directly compare the biological activities of these two

- Future work will focus on the potencies of these two different chromatographic species:
  - whether they are structurally different isoforms or represent AREG complexed with an as yet undefined binding partner
  - how this may differ in different cell populations.

- we examined levels of AREG in individual follicular aspirates to determine if there was any correlation with certain patient characteristics or oocyte-related outcomes, such as fertilization and early embryo growth.
  - We did not find a significant correlation between AREG and female age or follicle size.
- We did find that GV oocytes and abnormally fertilized oocytes with ICSI had significantly lower AREG
- We had hypothesized that given the importance of AREG for COC expansion, a subset of unfertilized oocytes from conventional IVF would

- Taken together, our findings strongly support a critical role for AREG during the peri-ovulatory period as an intrafollicular signal.

- However, the function of this growth factor may extend beyond oocyte maturation and cumulus expansion.

- Together with the demonstration that additional mitogens are present in the FF, the high concentration of AREG indicates that factors with high levels of mitogenic activity are released in the peritoneal cavity during each ovulation.

- The levels of AREG in FF are at least 100-fold higher than the levels reported in the serum of cancer patients with tumors expressing AREG, or in conditioned media from human cancer cell lines known to express *AREG (Ishikawa et al., 2005; Yotsumoto et al., 2008)*.

- Thus, the **massive peri-ovulatory AREG accumulation** may establish a **functional link between repetitive ovulations and increased**

Thanks for your attention!