Differential Effects of Follistatin on Nonhuman Primate Oocyte Maturation and Pre-Implantation Embryo Development In Vitro¹

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ABSTRACT

There is a vital need to identify factors that enhance human and nonhuman primate in vitro embryo culture and outcome, and to identify the factors that facilitate that objective. Granulosa and cumulus cells were obtained from rhesus monkeys that had either been FSH-primed (in vitro maturation [IVM]) or FSH and hCG-primed (in vivo maturation [VVM]) and compared for the expression of mRNAs encoding follistatin (FST), inhibin, and activin receptors. The FST mRNA displayed marginally decreased expression ($P = 0.05$) in association with IVM in the granulosa cells. The ACVR1B mRNA was more highly expressed in cumulus cells with IVM compared with VVM. Cumulus-oocyte complexes from FSH-primed monkeys exposed to exogenous FST during the 24-h IVM period exhibited no differences in the percentage of oocytes maturing to the metaphase II stage of meiosis compared to controls. However, embryos from these oocytes had significantly decreased development to the blastocyst stage. The effect of FST on early embryo culture was determined by exposing fertilized VVM oocytes to exogenous FST from 12 to 60 h postinsemination. FST significantly improved time to first cleavage and embryo development to the blastocyst stage compared with controls. The differential effects of exogenous FST on embryo development, when administered before and after oocyte maturation, may depend on the endogenous concentration in cumulus cells and oocytes. These results reveal evolutionary conservation of a positive effect of FST on embryogenesis that may be broadly applicable to enhance in vitro embryogenesis, with potential application to human clinical outcome and livestock and conservation biology.

embryo, gene expression, gene regulation, granulosa cells, oocyte development, ovary, rhesus macaque

INTRODUCTION

It is estimated that more than 70 million couples worldwide suffer from infertility [1]. While the problem of infertility is clearly multifactorial in nature, the success rates and prevalent

use of donor oocytes in assisted reproductive technologies [2] illustrate the significant contribution of poor oocyte quality to female infertility. The initial stages of early embryogenesis are driven by maternal (oocyte-derived) mRNAs and proteins that promote the initial cleavage divisions following fertilization, until completion of the maternal-to-zygotic transition and transfer of control of development to products of the embryonic genome [3]. The quality of an oocyte and subsequent fate of an embryo are determined by this maternal legacy [3, 4]. However, the specific molecular determinants of poor oocyte quality are not well understood. Lack of such information limits development of tools to predict oocyte quality and/or enhance efficiency of assisted reproductive technologies.

Previous functional genomics studies in the bovine model determined the characteristic RNA transcript profiles of oocytes of poor developmental competence [5]. Of particular interest was increased transcript abundance for follistatin (FST) observed in good quality oocytes compared to poor quality oocytes [5]. In addition, FST mRNA [5] and protein [6] abundances were greater in the two-cell-stage bovine embryos deemed to be of higher developmental potential. Furthermore, recent studies in bovine embryos [6] using small interfering RNA-mediated knockdown of FST demonstrated a functional requirement for maternal (oocyte-derived) FST in early embryogenesis. FST supplementation during the initial stages of bovine early embryonic development accelerated the time to first cleavage and enhanced the rate of blastocyst development. Numerous studies in humans indicate that time to first cleavage is a significant predictor of embryo developmental potential and success of assisted reproductive technologies [7–9], with up to 2.5-fold higher pregnancy rates observed in single embryo transfers with early cleaving embryos versus later cleaving embryos.

Collectively, these results suggest that maternal (oocyte-derived) FST could be an important determinant of oocyte developmental competence. Whether FST likewise contributes to primate embryogenesis has not been tested, nor have the potential effects of FST on oocyte maturation. We report here that FST treatment negatively affects in vitro maturation (IVM), but positively affects embryonic development to the blastocyst stage in rhesus monkey embryos. These studies reveal an evolutionarily conserved effect of FST on embryogenesis, and demonstrate potential translational utility of results obtained in the bovine model system to assisted reproductive technologies in primates, including humans.

MATERIALS AND METHODS

Animal Husbandry

Adult female rhesus macaques (*Macaca mulatta*) were housed at the California National Primate Research Center, as previously described [10].

¹Supported by National Institutes of Health grants RR13439 to C.A.V., RR00169 to the California National Primate Research Center, and RR15253 to K.E.L., and by the Michigan State University Reproductive and Developmental Sciences Program to G.W.S.

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Received: 27 February 2009.

First decision: 25 March 2009.

Accepted: 24 July 2009.

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eISSN: 1529-7268 <http://www.biolreprod.org>

ISSN: 0006-3363

Only females with a history of normal menstrual cycles were selected for this study. All procedures for maintenance and handling of the animals were reviewed and approved in advance by the Institutional Animal Use and Care Administrative Advisory Committee at the University of California at Davis.

Females were observed daily for signs of vaginal bleeding, and the first day of menses was assigned Cycle Day 1. Beginning on Cycle Days 1–4 recombinant human FSH (r-hFSH; Organon, West Orange, NJ) was administered (37.5 IU) twice daily, intramuscularly for 7 days total (Fig. 1). For IVM experiments, cumulus-oocyte complexes (COCs) were collected on Day 8. To obtain in vivo matured (VVM) oocytes, females were given recombinant hCG (1000 IU Ovidrel; Serono, Rockland, MA) on Treatment Day 8 in addition to the FSH treatment outlined above. COCs were removed at 28–30 h following hCG by ultrasound-guided aspiration [11, 12]. Oocytes were retrieved from aspirates as previously described [10]. Granulosa cells were obtained from the aspirates and immediately placed in buffer and stored frozen for later processing for gene expression.

IVM of Oocytes

Immature COCs were placed into 70- μ l drops of medium from one of two different treatment groups: control or FST (see below). Only oocytes with at least two layers of cumulus cells were used in these experiments, because oocytes without cumulus will not mature. The control treatment group consisted of M1A medium alone [13]. COCs were incubated in a humidified atmosphere of 5% CO₂ in air for 28–30 h at 37°C. Following incubation, COCs were prepared for in vitro fertilization (IVF).

IVF and Embryo Development

VVM oocytes or COCs after IVM were rinsed and transferred into TL-PVA medium (37°C) under oil and inseminated according to standard procedures for IVF of rhesus macaque oocytes [12]. Semen was collected from male macaques that had been trained for this procedure, as previously described [14]. Sperm were washed from seminal plasma and resuspended in TL-BSA medium [15]. The next morning, oocytes were transferred into 70 μ l drops of chemically defined, protein-free hamster embryo culture medium 9 (HECM-9) without or with FST (see below) under oil (37°C), and incubated at 37°C in a humidified atmosphere of 5% CO₂, 10% O₂, and 85% N₂ for 48 h [16]. At 30 h postinsemination, oocytes were observed for cleavage. At approximately 60 h postinsemination, noncleaved oocytes were again assessed for developmental status. Noncleaved oocytes exhibiting a polar body and all embryos were classified as having matured to metaphase II of meiosis (MII). Embryos were transferred into 70- μ l drops of HECM-9 medium with 5% bovine calf serum (Gemini Bioproducts, West Sacramento, CA) under mineral oil, and incubated as described above. Embryos were transferred to fresh medium every other day until no further development was observed. When development ceased, the percentage of embryos developing to the blastocyst stage was calculated for each treatment. The percent blastocyst development for each female was then averaged to obtain the mean for each treatment.

Blastocyst-stage embryos were fixed and stained for differential cell counting with POU5F1 (also known as OCT3/4), as previously described [10]. Optical sectioning was performed with a Delta Vision microscope using a 20 $\times$ Olympus UApo 340/0.70 water immersion objective (Olympus Optical Co., Ltd., Tokyo, Japan). A z-projection was created for each blastocyst-stage embryo. Samples were coded so that evaluation was performed without knowledge of sample identity. Manual cell counts were taken of the inner cell mass (ICM) and trophectoderm (TE) cells.

FST Treatment During IVM or Early Embryo Development

Recombinant human FST was obtained from R&D Systems (Minneapolis, MN). A concentration of 10 ng/ml FST was selected for these studies, because it was the maximal effective concentration observed in studies of embryotrophic effects in the bovine model system [6]. To determine the effects of FST on IVM of oocytes, FST was added to M1A medium, and COCs were cultured with or without FST for 28–30 h after collection. Additional COCs were cultured with activin or inhibin (10 ng/ml; PeproTech, Rocky Hill, NJ). To determine the effects of FST on early embryo development, IVM oocytes were inseminated, and presumptive embryos were cultured without or with FST from 15 to 60 h postinsemination. At 60 h, cleaved embryos were placed in fresh medium that did not contain FST.

Analysis of Temporal mRNA Expression Profiles in Oocytes and Embryos

The temporal expression studies undertaken here employed the Primate Embryo Gene Expression Resource (PREGER) (www.preger.org), which

FIG. 1. Flow diagram of experimental design.

contains a collection of reverse transcription (RT)-PCR-amplified cDNA libraries corresponding to more than 200 samples of rhesus monkey oocytes and preimplantation-stage embryos. The libraries were created by RT and exponential cDNA amplification that maintains the quantitative representation of the original mRNA population [17, 18]. The cells were lysed in a modified RT buffer, followed by oligo(dT) annealing and processing through the RT step. After amplification, aliquots of each sample library were spotted onto filters by dot blotting (quantitative amplification and dot blotting [QADB]), as previously described [19].

Blot preparation, probe preparation, hybridization, and quantitative analyses were performed as previously described [19–21]. Data were expressed as the mean $\pm$ SEM counts per minute (cpm) bound value for each stage/condition of oocytes and embryos included in the analysis. Appropriate corrections were made for DNA load to each dot and background signals, providing high sensitivity and consistency in measurement. Detailed protocols and description of the method are also available at the web site, www.preger.org. The statistical significance of differences was evaluated using the *t*-test (*P* < 0.05 considered significant).

The sensitivity, reproducibility, and quantitative reliability of the QADB method have been extensively documented, and the method applied in numerous studies of mouse and monkey embryos [19–27]. The RT and amplification method was originally developed by Iscove et al., and applied to single cells, in which subsequent array studies revealed faithful representation of mRNA abundances [17, 18]. The temporal profiles and relative differences in expression revealed by QADB are consistent with data produced in embryos by other methods, such as Northern blotting and Western blotting [20, 22–24]. The method yields smooth temporal expression profiles, generally small standard errors, and linear responses over the range of the instrument employed for phosphorimaging [20, 23]. In mouse embryos, the method allows the discernment of quantitative differences in mRNA present at as little as 500 copies/embryo [22], and even subtle quantitative differences in expression related to genomic imprinting, and was the first method to detect *Xist* RNA expression in two-cell-stage mouse embryos [22]. The method has been applied to partial blastocyst equivalents in conjunction with simultaneous analyses of embryo genotype [25], and has been applied extensively to samples of single monkey embryos and oocytes in the PREGER collection. The advantages of the QADB method are that it can be applied to as little as a single oocyte or embryo or small numbers (<100) of somatic cells, no RNA extraction procedure is required, a large number of mRNAs can be assayed on a common set of samples, and the data are quantitative over the range of the instrument employed to detect signal [23].

The isolation and culture of the oocytes and embryos during the construction of the PREGER sample set has been described in detail [19]. Between 3 and 13 samples of one to four oocytes or embryos were obtained for each stage. The embryos included in the PREGER sample set were all high quality and healthy in appearance. Samples of eight-cell and morula-stage embryos treated with the RNA polymerase II inhibitor, α -amanitin, from the pronucleate stage onward in HECM9 culture were included to evaluate transcriptional dependence of mRNA expression. Details concerning the array,

diversity, and origin of samples, and the sensitivity and quantitative reliability of the quantitative amplification and dot blotting method, have been described previously [19].

The data from the QADB method were expressed in cpm bound. These values were corrected for any background signal, and for differences in DNA load amongst the individual dots, as described previously [19, 20]. The QADB results provide sensitive measures of differences in mRNA expression that reflect differences in temporal patterns and amounts of expression observed by other methods, such as Western and Northern blotting, including small quantitative differences, and can quantify mRNAs present at as little as 500 copies/embryo for the mouse [19, reviewed in Ref. 26]. The advantages of the QADB method over other methods, such as semiquantitative RT-PCR and Northern blotting, are that the QADB method permits hundreds of mRNAs to be quantified with as little as a single oocyte as starting material.

Analysis of mRNA Expression in Cumulus and Granulosa Cells

Total RNA was isolated from cumulus or granulosa cells using the PicoPure RNA extraction kit (MDS Analytical Technologies, Sunnyvale, CA) according to the manufacturer's protocols. A total of 9 samples of cumulus cells and 13 samples of granulosa cells obtained from 9 and 13 females, respectively, were employed. For each sample, 50 ng of total RNA were employed for the QADB, as previously described [19], the same method employed previously to produce the blots for temporal expression analysis. Blot preparation and hybridization were performed using the same established methods.

Complementary DNA Probes and Hybridization

Primers were designed using primer3 software (Supplemental Data available at www.biolreprod.org). Complementary DNA probes were obtained by PCR, which was performed in 100- μ l reactions containing 4 μ l of plasmid DNA product (cDNA clones obtained from Open Biosystems, Huntsville, AL), 10 $\times$ PCR buffer containing 15 mM MgCl₂ (Roche Diagnostics, Indianapolis, IN), 10 mM dNTPs (Roche Diagnostics), 10 μ M for each of the forward and reverse primers (Supplemental Data), and 5 U/ μ l TaqDNA polymerase (Roche Diagnostics). Reactions were run on a Techne PCR machine (Techne Inc., Burlington, NJ) at 94°C for 5 min to denature, followed by 35 cycles of 94°C for 1 min, annealing at 55–60°C for 1 min, 72°C for 2 min, and a final extension for 5 min at 72°C. PCR products were resolved on 1% agarose gels. Excised PCR products were purified using a QIAquick Gel Extraction kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Purified PCR products were then used for probe labeling.

Array Analysis of FST Effects on mRNA Expression

The eight-cell-stage embryos were collected at the end of the FST treatment period, approximately 60 h postinsemination. Embryos were lysed and stored at –70°C until processing. Embryos of each kind (FST or untreated) were combined into pools of six, each pool representing embryos from at least three different females. Total RNA was isolated from four samples of control eight-cell-stage embryos and four samples of FST-treated eight-cell-stage embryos. RNAs were processed for array analysis as described previously [27]. Biotin-labeled cRNA samples were fragmented, and 10 μ g were hybridized to Affymetrix rhesus macaque genome chips in the University of Pennsylvania Microarray Facility. Microarray data were initially processed with the Microarray Analysis Suite (MAS) 5.0 (Affymetrix) algorithm using default analysis parameters and global scaling to target a mean equal to 150 units. The MAS metrics output was loaded into GeneSpring GX 10.0 (Agilent Technologies, Foster City, CA) with per-chip normalization to the 50th percentile and per-gene normalization to the median. Only the genes called “Present” in at least three out of four replicates were used for all statistical packages. In order to identify genes significantly different between FST-treated and untreated groups, the Statistical Analysis of Microarray [28] program was applied at a false discovery rate <5% and *P* value <0.05.

Statistical Analysis

All data were expressed as the mean $\pm$ SEM. Percentage of MII oocytes that cleaved 30 h after insemination and percentage of blastocyst stage embryos were compared using a two-tailed paired Student *t*-test (*n* = 6 females). Total cell count, ICM cell count, TE cell count, and percentage of ICM to TE cells in blastocyst-stage embryos were assessed with Student *t*-tests. Data were analyzed with Prism software (GraphPad Software, Inc., San Diego, CA). Differences were considered statistically significant if *P* < 0.05. For mRNA expression studies, the statistical significance of differences was evaluated using the *t*-test (*P* < 0.05 considered significant).

TABLE 1. Oocyte maturation and embryo development rate of oocytes matured in vitro in the absence or presence of follistatin.*

Treatment group	No. of animals/oocytes	Percentage MII	Percentage of embryos reaching blastocyst
Control	5/51	67.6 $\pm$ 10.7	58.0 $\pm$ 11.4 ^a
Follistatin	5/50	66.3 $\pm$ 11.9	22.4 $\pm$ 9.7 ^b

* Values are mean $\pm$ SEM.

^{a,b} Different superscripts within a column indicate a significant difference, *P* < 0.05.

RESULTS

Effects of Exogenous FST on In Vitro Oocyte Maturation

IVM yields oocytes of reduced developmental competence as compared with VVM oocytes [29]. A recent study of effects of IVM on the oocyte transcriptome revealed potential alterations in oocyte-somatic cell interactions [23]. FST inhibits the functions of activins and other transforming growth factor beta superfamily members by binding these proteins and inhibiting interaction with their respective type-I and type-II receptors, including activin receptor, ACVR1B, and the activin-related receptor, ACVR1. Given the role of FST in modulating activity of activin and related superfamily members, we investigated the expression of mRNAs encoding FST, the inhibin beta A and B subunits of activin (INHBA and INHBB), and the activin and activin-related receptors (ACVR1B and ACVR1) in the cumulus and granulosa cells obtained after VVM versus IVM (Fig. 2A). We observed significant differences between these cell types. The *ACVR1* mRNA was elevated in the granulosa cells collected in association with IVM (*P* < 0.05). The *ACVR1B* mRNA displayed marginally elevated expression in the cumulus cells with IVM (*P* = 0.069). The hybridization signal for the *ACVR1* mRNA was much higher than the hybridization signal for *ACVR1B* mRNA, indicating abundant expression in granulosa and cumulus cells. The ratio of *ACVR1* to *ACVR1B* was 65 and 13, respectively, for granulosa and cumulus cells from the IVM group and 18 and 24, respectively, for granulosa and cumulus cells from the VVM group. The *INHBB* mRNA expression was significantly increased in the granulosa and cumulus cells obtained in association with IVM (*P* < 0.05). The *FST* mRNA displayed marginally decreased expression (*P* = 0.05) in association with IVM in the granulosa cells. The *ACVR1* mRNA was more highly increased in the granulosa cells than in the cumulus cells with IVM (*P* < 0.05), but not VVM (Fig. 2A).

It is important to bear in mind that the difference in quality between IVM and VVM oocytes arises in the last 24 h of maturation. Changes in gene expression affecting the follicular environment in vivo may be essential for producing high-quality oocytes. Our observations raise the possibility that changes in granulosa and cumulus cell gene expression that affect oocyte quality may include changes in the expression of *FST* and *INHBB* mRNA. This would imply that changes in FST-modulated signaling could contribute to changes in overall oocyte quality. To determine whether exogenous FST could enhance oocyte quality by affecting IVM, we exposed COCs to exogenous FST during IVM. COCs from FSH-primed monkeys were collected and cultured in IVM medium with or without FST for 24 h (Fig. 1). COCs exposed to exogenous FST during the 24 h IVM period exhibited no differences in the percentage of oocytes maturing to the MII stage compared to controls. However, the resulting embryos from the FST-exposed oocytes displayed significantly reduced abilities to

FIG. 2. Relative expression of mRNA encoding for FST, inhibin/activin subunits, and activin receptors. **A**) mRNA expression in cumulus (Cum) and granulosa (Gran) cells stimulated with either FSH (IVM) or FSH + hCG (VVM). **B**) mRNA expression during rhesus monkeys oocyte maturation and early embryonic development. Graphs show the relative levels of expression for GV- and MII-stage oocytes and pronucleate- through hatched blastocyst-stage embryos produced by IVF of oocytes from hCG-stimulated females, and then cultured in vitro. EB, early blastocyst; HB, hatched blastocyst; GV, germinal vesicle stage oocyte; MII, MII-stage oocyte; PN, pronucleate 1 cell-stage embryo; XB, expanded blastocyst; 2 cell, two-cell-stage embryo; 8 cell, eight-cell-stage embryo; 8–16 cell aAM, 8- to 16-cell-stage cultured in α -amanitin. Expression data for the mRNAs encoding the indicated proteins (**A** and **B**) are presented as the mean cpm bound $\pm$ SEM. Statistically significant differences are indicated for some of the major changes in gene expression for comparisons between different hormonal stimulation conditions (panel **A**) and developmental stages (panel **B**) indicated at the ends of the brackets. Letters a, b, and c indicate $P < 0.05$, 0.01, and 0.001, respectively.

develop to the blastocyst stage compared with controls (Table 1). Additional COCs cultured with activin or inhibin showed no differences in oocyte maturation to MII (73.2% and 78.9%, respectively) or embryo development to blastocysts (49.4% and 46.4%, respectively) compared to controls (67.6% maturation and 58.0% blastocyst formation).

Effect of Exogenous FST on In Vitro Embryo Development

Exogenous FST enhances bovine preimplantation embryo development [6]. To determine whether FST exerts a positive effect on embryogenesis in a primate animal model, we

inseminated denuded oocytes at 6–8 h after recovery from FSH- and hCG-primed females. The following morning, at 15 h postinsemination, the inseminated oocytes were transferred to serum-free HECM9 media with or without FST. Embryos were transferred 2 days later to fresh medium containing serum and lacking exogenous FST, and monitored for developmental competence in vitro.

There were two indicators of a positive effect of FST on embryo development. First, the percentage of fertilized embryos that cleaved by 30 h after insemination was significantly higher in the FST-treated group compared with the control group (Table 2). The time to first cleavage is widely

TABLE 2. Effect of follistatin treatment during embryo culture on time to first cleavage following in vitro fertilization of MII stage oocytes.*

Treatment group	No. of females	No. of MII oocytes	Percentage cleaved at 30 h	Total percentage cleaved
Control	5	67	44.9 ± 8.3 ^a	71.9 ± 12.0
Follistatin	5	63	75.7 ± 3.2 ^b	87.5 ± 6.6

* Values are mean ± SEM.

^{a,b} Different superscripts within a column indicate a significant difference, $P < 0.05$.

utilized as an indicator of embryo developmental potential [7, 9]. The 30 h postinsemination time point was chosen so that comparison between primate and bovine studies could be made [6]. The second positive effect of FST was on the percentage of all embryos reaching the blastocyst stage (Table 3). However, no statistically significant difference was observed in blastocyst cell numbers or the ICM:TE ratio in FST-treated versus control embryos (Table 4). The difference in the percentage of embryos reaching blastocyst stage also supports the predictive value of using a 30-h cut-off for first cleavage.

To explore a possible molecular basis for the effects of FST on in vitro embryogenesis, we examined the expression of mRNAs encoding FST, inhibin/activin, activin, and activin-related receptors in rhesus monkey oocytes and early stage embryos using the PREGER resource (Fig. 2B). The *ACVR1* and *INHBA* mRNAs were not detected. Previously published array data for the rhesus monkey MII-stage oocyte revealed no detection of the *ACVR1* mRNA, and only very low, marginally detectable expression of *INHBA* mRNA [27]. The *ACVR1B* mRNA displayed prominent expression as a maternal mRNA in the oocyte- and pronuclear-stage embryos. *ACVR1B* mRNA expression diminished during development to the pronuclear ($P < 0.05$) and two-cell ($P < 0.001$) stages. The *FST* and *INHBB* mRNAs both displayed transient apparent increases in abundance upon oocyte maturation ($P < 0.05$), a change that possibly reflects an increase in maternal mRNA polyadenylation [20]. Thereafter, both mRNAs diminished in expression, were poorly detected in pronucleate-stage embryos, and displayed minimally increased levels of expression during the stages leading up to the morula stage ($P < 0.05$). The expression during this period was not reduced by treatment with alpha-amanitin (a potent inhibitor of RNA polymerase II), indicating that transcripts detected were maternal in origin. After the morula stage, both *INHBB* and *FST* mRNAs increased in abundance during blastocyst development.

Next, we sought to determine whether FST treatment significantly altered the transcriptome of developing embryos. Because FST exerts its effects during the period leading up to embryonic genome activation, we wished to determine the effect of FST treatment on gene expression immediately after gene transcription has begun. We completed an array analysis comparing the transcriptomes of treated and untreated eight-cell-stage rhesus monkey embryos produced by IVF of VVM oocytes. We received positive detection signals for a total of 21 952 Affymetrix probe IDs, encompassing a total of 16 381 IDs with annotations, which corresponds to 10 618 individual genes. Among these, we identified only three probe sets that showed significant differences in gene expression between treated and untreated embryos (Table 5). Thus, FST treatment yielded virtually no change in the overall pattern of mRNA expression, indicating that positive effects of FST on embryo development are not mediated by widespread alterations in gene expression coincident with genome activation in the rhesus monkey.

TABLE 3. Effect of follistatin treatment during embryo culture on embryo development of in vivo-matured oocytes.*

Treatment group	No. of females	No. of embryos cultured	Percentage of embryos reaching blastocyst
Control	7	60	37.0 ± 4.2 ^a
Follistatin	7	76	50.0 ± 4.0 ^b

* Values are mean ± SEM.

^{a,b} Different superscripts within a column indicate a significant difference, $P < 0.05$.

DISCUSSION

We report significant yet divergent effects of FST treatment during IVM and embryo culture on embryo development in vitro for a nonhuman primate. These results indicate a potential role for endogenous FST in regulating primate oocyte maturation and embryo development. The presence of *FST* mRNA in the rhesus monkey oocyte and observed positive effects of FST on early embryogenesis are similar to findings in the bovine model [5, 6]. FST is also expressed in human oocytes [30]. The presence of the inhibin/activin/FST system in primate follicular cells, as well as in other mammalian species, such as the cow [31], goat [32], pig [33], rat [34], and mouse [35], indicates that this system may have widespread taxonomical effects on oocyte development. However, mouse oocytes do not express FST [30]. Thus, the role of FST in follicular oocyte development appears highly conserved, but the maternal expression of FST appears to differ between rodent and nonrodent species. The *FST* mRNA is not detected by heminested PCR in mouse oocytes or preimplantation embryos, although FST-related mRNAs are detected [36]. Thus, the positive effect of FST on embryogenesis may be evolutionarily more restricted.

In this experimental model, VVM oocytes and granulosa cells have experienced a transition in the follicular environment in response to the hCG given during the 24 h after IVM follicles are aspirated. Previous studies using the rhesus model have noted that oocyte quality is improved with in vivo exposure to the maturing follicle [10, 12, 27, 29]. This study is consistent with previous studies in which changes in gene expression within all follicular cell types were reported during the last 24 h of VVM with hCG [27, 29], and with studies showing changes in oocyte gene expression related to cell-cell communication [27]. Because IVM oocytes are not experiencing that transition in the follicular environment during the final day of maturation, they may not elaborate comparable responses at the molecular level as with VVM oocytes. To understand how the intracellular signals change during VVM, and what changes may be denied to IVM oocytes, it is important to characterize the gene expression of the granulosa cells, as well as cumulus cells, from the follicles from which they are obtained. Our finding that *ACVR1B* and *INHBB* mRNAs are differentially expressed in granulosa cells from the two stages (8 day versus 9 day, recovered with IVM and VM protocols, respectively) indicates that IVM oocytes may not encounter the normal changes in this component of intracellular signaling. The potential effects of FST on gene expression could be complex during this period, and will require further study before mechanisms of action can be suggested.

The presence of *FST*, *ACVR1*, *ACVR1B*, *INHBA*, and *INHBB* mRNAs in the cumulus and granulosa cells of monkey ovarian follicles, from which IVM and VVM oocytes were obtained, indicates that FST may influence oocyte maturation. This study localized the production of the activin/inhibin system mRNAs to granulosa cells, cumulus cells, and oocytes

TABLE 4. Effect of follistatin treatment during embryo culture on differential cell counts of blastocyst stage embryos.*

Treatment group	No. of blastocysts	Total cell count	ICM cell count	TE cell count	ICM:TE ratio
Control	11	137.2 ± 16.0	30.5 ± 3.3	106.7 ± 16.5	0.35 ± 0.06
Follistatin	16	145.4 ± 17.7	28.2 ± 2.1	117.1 ± 17.1	0.28 ± 0.03

* Values are mean ± SEM.

of ovulatory follicles in a nonhuman primate. Differential expression of the inhibin/activin subunit mRNAs has been reported in the monkey ovary [37]. The inhibin alpha and *INHBA* mRNAs were found only in dominant follicles and corpora lutea, whereas *INHBB* mRNA was found in small antral follicles, and was nondetectable in the dominant follicle. Detectable expression of *INHBB* mRNA in periovulatory follicles (FSH only) in our study may be the result of FSH stimulation to maintain multiple large follicles or may be due to differences in mRNA detection procedures utilized. Previous studies demonstrated that administration of activin A and inhibin A systemically impacts luteinizing hormone and FSH levels during the follicular phase of the menstrual cycle of rhesus monkeys, a time of follicular growth [38, 39]; however, those studies did not examine endogenous production of such growth factors.

Although activin binds both ACVR1 and ACVR1B, it does not signal through ACVR1. ACVR1 binds activin, and was discovered based on its ability to bind activin when in the presence of the type-II receptor for activin [40], but it is unable to transduce the activin signal transduction cascade [41] known to be mediated via SMAD2/3 [42]. Therefore, activin binding to ACVR1 does not stimulate classical activin responses, and it is not a signaling receptor for activin. Rather, specific bone-morphogenic proteins bind to ACVR1 and signal through ACVR1, but through a different pathway (SMAD1/5/8) than activin signaling via ACVR1B [42]. Interestingly, a recent report provided indirect evidence that activin binding to ACVR1 may be inhibitory or antagonistic to activin signaling [43]. Therefore, *ACVR1* mRNA expression levels were examined in the current studies even though the potential direct relevance of ACVR1 to the observed differential effects of FST during oocyte maturation and early embryogenesis is less clear than that of ACVR1B.

The significant decrease in *INHBB* mRNA in both cumulus and granulosa cells from animals given FSH and hCG compared to those given FSH only may indicate that *INHBB* is most important prior to final maturation of the oocyte, or may reflect the changing function of granulosa and cumulus cells after the midcycle gonadotropin surge that leads to luteinization. The latter possibility is supported by the decrease in expression of *ACVR1* mRNA in granulosa cells and *ACVR1B* mRNA in cumulus cells after FSH and hCG compared with the same cell types from FSH-only cycles. The reduction in support cell levels of activin receptor and *INHBB* mRNA may explain why the addition of FST, activin,

or inhibin for the last 24 h of IVM culture did not influence the percentage of oocytes maturing to the MII stage (Table 1). However, FST treatment during IVM did have negative effects on cytoplasmic maturation, resulting in reduced embryonic development to the blastocyst stage following IVF. Although a previous study found that activin A and inhibin A improved rhesus monkey oocyte maturation, the oocytes for that study came from unstimulated ovaries, and thus follicles that were not periovulatory [44]. Moreover, such oocytes are developmentally compromised, so no study of subsequent embryo development was possible. The results of the current study indicate that treatment with exogenous FST during the final period of IVM has a detrimental effect on subsequent embryo development. This is similar to the effect that FST has during maturation of bovine oocytes [45].

Contrary to its effect on IVM, FST has a positive effect when it is present from just after insemination until the eight-cell stage of embryo development, the approximate time of embryonic genome activation. During this time of embryo development, the levels of *ACVR1B* mRNA are high in pronuclear-stage embryos, yet *FST* mRNA levels appear low (Fig. 2B). Thus, maternally supplied FST in vivo may promote primate embryogenesis. The increased number of FST-treated embryos to reach first cleavage by 30 h postinsemination also supports the use of this measurement as an early indicator of improved developmental potential. This early cleavage effect of FST was also described in bovine embryos [6]. Numerous studies in the human support a positive relationship between time to first cleavage and embryo developmental potential [7–9]. The improved developmental potential of FST-treated embryos is also evidenced by the increased percentage reaching the blastocyst stage compared with controls. The improved in vitro development of rhesus monkey and bovine embryos indicates that the inclusion of FST in embryo culture media for other species in which genomic activation is relatively late, such as the human, may be beneficial.

The different effect that FST had on IVM oocytes and early embryos may be related to the dramatically different endogenous milieu of FST to which oocytes and early embryos are exposed due to the presence (during IVM) and absence (postfertilization) of the cumulus cells. Effects of FST on early bovine embryos were dose dependent and not observed when embryos were treated with higher doses (100 ng/ml) of FST [6]. Thus, the opposite effects of the same dose of FST observed following treatment during IVM versus early embryo development may be explained by the additional contribution

TABLE 5. Genes that are differentially expressed between follistatin (FST)-treated and untreated rhesus embryos.

Gene description	Gene symbol		Ratio (untreated: FST-treated)	P value
	Rhesus	Human		
Similar to CG4699-PA, isoform A	LOC717172	KIAA1267	1.21	0.004312
Myosin, heavy polypeptide 3, skeletal muscle, embryonic	LOC717943	MYH3	1.88	0.004894
Neurofilament, heavy polypeptide 200kDa	LOC717705	NEFH	1.39	0.001283

of endogenous FST of cumulus cell origin and in close association with the oocyte during IVM. Thus, the actual concentration of FST during IVM may be much higher than the concentration of FST added to the culture medium. We also note the differential expression of activin receptor mRNAs, with the *ACVR1* mRNA predominating in granulosa and cumulus cells, and the *ACVR1B* mRNA predominating in oocytes and embryos. Whether such differences affect responses to FST requires further study.

The mechanisms involved in FST regulation of early embryogenesis are not known. FST functions as an inhibitor of action of TGFβ superfamily members in the extracellular milieu. FST binding to such growth factors inhibits receptor binding and subsequent ligand-induced signaling [46]. Of all FST actions described to date, FST is best known for its high-affinity binding and inhibition of activin activity [47]; *ACVR1B* mRNA, but not *ACVR1* mRNA was detectable in rhesus monkey oocytes and early embryos in the current studies. Although it is unknown whether the positive FST effect on rhesus monkey embryogenesis is mediated via inhibition of activin action, previous studies have reported a positive effect of activin supplementation of embryo culture medium on bovine early embryonic development [48, 49]. It is also interesting to note that stimulatory effects of FST treatment of rhesus monkey embryos were not accompanied by significant alterations in zygotic transcript profiles associated with embryonic genome activation, suggesting that FST effects are not mediated transcriptionally. Such results were not anticipated, given that FST binding to respective growth factors is known to block interaction with respective type-I and (or) type-II serine threonine kinase receptors, thus inhibiting ligand-induced *Smad* signaling and stimulation of transcription [46].

The methods applied in this nonhuman primate study closely model the protocols used in human assisted reproductive medicine. For example, the maturation of follicles is controlled with exogenous hormones; thus, the oocytes and follicular cells for this study are from follicles of known stages of maturation. Studies that rely on slaughterhouse materials do not have similar controls, and pooling samples from many animals obviously differs significantly from clinical procedures. Studies on litter-bearing animals have limited application to human, because of the lack of similar reproductive processes, such as selection of dominant follicles. Our data from a nonhuman primate, therefore, provide an important extension of results from other species that FST may play a role in regulating oocyte maturation and embryo development. The differential effects of FST on oocytes and embryos may be due to the presence of cumulus cells and endogenous FST, so that additional FST treatment inhibits oocyte maturation. The improved in vitro development of FST-treated embryos may indicate that FST also regulates embryo growth and development in vivo during the early cleavage stages. These results reveal evolutionary conservation of a positive effect of FST on embryogenesis that may be broadly applicable to enhance in vitro embryogenesis, with potential application to human clinical outcome.

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