Molecular Determinants of Oocyte Competence: Potential Functional Role for Maternal (Oocyte-Derived) Follistatin in Promoting Bovine Early Embryogenesis

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Previous studies established a positive relationship between oocyte competence and follistatin mRNA abundance. Herein, we used the bovine model to test the hypothesis that follistatin plays a functional role in regulation of early embryogenesis. Treatment of early embryos with follistatin during *in vitro* culture (before embryonic genome activation) resulted in a dose-dependent decrease in time to first cleavage, increased numbers of blastocysts, and increased blastocyst total and trophectoderm cell numbers. To determine the requirement of endogenous follistatin for early embryogenesis, follistatin ablation/replacement studies were performed. Microinjection of follistatin small interfering RNA into zygotes reduced follistatin mRNA and protein and was accompanied by a reduction in number of embryos developing to eight- to 16-cell and blastocyst stages and reduced blastocyst total and trophectoderm cell numbers. Effects of follistatin ablation were rescued by culture of follistatin small interfering RNA-injected embryos in the presence of exogenous follistatin. To investigate whether follistatin regulation of early embryogenesis is potentially mediated via inhibition of endogenous activin activity, the effects of treatment of embryos with exogenous activin, SB-431542 (inhibitor of activin, TGF-β, and nodal type I receptor signaling) and follistatin plus SB-431542 were investigated. Activin treatment mimicked positive effects of follistatin on time to first cleavage and blastocyst development, whereas negative effects of SB-431542 treatment were observed. Stimulatory effects of follistatin on embryogenesis were not blocked by SB-431542 treatment. Results support a functional role for oocyte-derived follistatin in bovine early embryogenesis and suggest that observed effects of follistatin are likely not mediated by classical inhibition of activin activity. **(*Endocrinology* 150: 2463–2471, 2009)**

Poor oocyte competence limits reproductive success (1). Oocyte competence is defined as the ability of an oocyte to resume meiosis, cleave after fertilization, help promote embryonic development and pregnancy establishment, and bring a pregnancy to term in good health (2). The pools of oocyte mRNA and protein drive oocyte development, maturation, fertilization, and early embryogenesis and are the functional mediators of oocyte competence (3). Perturbations in accumulation of specific maternal factors key to above oocyte developmental milestones could be functionally associated with poor oocyte competence, but the specific molecular mediators of poor oocyte competence are not well understood.

We have used functional genomics approaches to identify differences in RNA transcript profiles of both the oocyte and adjacent cumulus cells associated with poor oocyte competence (4, 5) using the prepubertal calf model of poor oocyte quality (6, 7). Of particular interest from the differentially expressed genes revealed from oocyte microarray studies were genes in the regulation of hormone secretion ontology category (including follistatin and the βA and βB subunits of inhibin/activin) which

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Abbreviations: hpi, Hours post insemination; ICM, inner cell mass; KSOM, potassium simplex optimization medium; siRNA, small interfering RNA; TE, trophectoderm.

were overrepresented in the good quality oocytes harvested from adult (control) *vs.* prepubertal animals. Follistatin mRNA was also greater in two-cell bovine embryos that cleaved early and developed to the blastocyst stage at a rate 4-fold greater than their late cleaving counterparts, which displayed reduced follistatin mRNA. Given embryos were collected before completion of the maternal-to-embryonic transition and initiation of robust transcription from the embryonic genome (8), such differences likely reflect inherent differences in maternal follistatin mRNA content after fertilization and suggest a potential functional role for oocyte-derived follistatin in early embryogenesis.

Follistatin was initially isolated based on its ability to suppress FSH secretion (9) and classified as a high-affinity activin-binding protein (10). Follistatin can also bind (albeit at a lower affinity) and regulate activity of multiple additional TGF-β superfamily members (11–13). Follistatin binding blocks interactions with respective type I and (or) type II serine threonine kinase receptors, thus inhibiting ligand-induced Smad signaling (14). To our knowledge, a functional role for follistatin in mammalian early embryonic development has not been previously established. Homozygous follistatin mutant mouse embryos proceed through development, but pups display early postnatal lethality (15). However, follistatin mRNA is not expressed by mouse oocytes but is expressed by human (16) and bovine (4) oocytes. Transcriptome analysis of human oocytes indicates that multiple components of the TGF-β superfamily signaling pathway are potentially active (17). Given the above association of follistatin mRNA with oocyte quality in two experimental paradigms (4) and the key functional role that maternal mRNA and proteins play in promoting early cleavage divisions after fertilization until embryonic genome activation (8), we hypothesized that follistatin plays a key regulatory role in bovine early embryonic development.

The objective of the present studies was to determine the functional role of maternal (oocyte-derived) follistatin in regulation of bovine early embryogenesis. Results support a prominent role for follistatin in acceleration of time to the first cleavage division and in promoting development to the blastocyst stage. Results also suggest that follistatin actions early in development have a stimulatory influence on blastocyst cell allocation resulting in increased trophectoderm cell numbers and that positive effects of follistatin are not mediated by inhibition of activin activity.

Materials and Methods

Materials

Unless otherwise stated, all chemicals and reagents used were purchased from Sigma-Aldrich (St. Louis, MO).

Effect of follistatin supplementation on early embryonic development

In vitro maturation of bovine oocytes, *in vitro* fertilization, and culture of embryos to blastocyst stage were conducted as reported previously (18). After 20 h coincubation with spermatozoa, presumptive zygotes were stripped of cumulus cells and cultured in potassium simplex optimization medium (KSOM; Specialty Media, Phillipsburg, NJ) supplemented with 0.3% BSA containing 0, 1, 10, or 100 ng/ml recombinant human follistatin (R&D Systems, Minneapolis, MN), respectively (n = 25 zygotes per treatment; n = 6 replicates). At 30, 36, and 48 h post insemination (hpi), numbers of embryos cleaved were recorded and proportion of early cleaving (<30 hpi) and late cleaving embryos (30–36 hpi) and total cleavage rates (48 hpi) determined. A subset of early and late cleaving embryos were collected and processed for follistatin immunolocalization as described below (n = 15–20 embryos per group). Embryos at the eight- to 16-cell stage were separated 72 hpi, washed and cultured in fresh KSOM (minus exogenous follistatin) supplemented with 0.3% BSA and 10% fetal bovine serum until d 7 when numbers of embryos developing to the blastocyst stage were documented. Blastocysts were then subjected to differential staining using previously published procedures (19) to determine total cell numbers and allocation to trophectoderm (TE) *vs.* inner cell mass (ICM) cells.

Effects of follistatin treatment on rates of development of early- *vs.* late-cleaving embryos to the blastocyst stage were also determined. Early- and late-cleaving embryos from each treatment group (0, 1, 10, and 100 ng/ml follistatin) were separated at 30 and 36 hpi, respectively (n = 15–20 embryos per treatment; n = 4 replicates) and the above follistatin treatments continued for an additional 36–42 h. Embryos at the eight- to 16-cell stage were separated 72 hpi, washed, and cultured as described above until d 7 when numbers of embryos reaching the blastocyst stage were recorded.

Validation of small interfering RNA (siRNA)-mediated knockdown of follistatin in early embryos and effects on development

Ablation of endogenous follistatin in bovine embryos was performed via microinjection of follistatin siRNA. The publicly available siRNA design algorithm (siRNA target finder; Ambion, Austin, TX) was used to design two distinct siRNA species corresponding to exons 2 and 3 of bovine follistatin (designated as siRNA species 1 and 2, respectively). The candidate siRNA species were interrogated using BLAST (basic local alignment search tool) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to rule out homology to other known genes in the bovine expressed sequence tag (EST) and genomic databases. Individual follistatin siRNA species were synthesized using the Silencer siRNA construction kit (Ambion) following manufacturer's instructions and dissolved in nuclease-free water. The sense and antisense oligonucleotide template sequences were as follows: follistatin siRNA species 1 (sense, 5′-AACCCGTTGAAAATCATCCACCCTGTCTC-3′; antisense, 5′-AAGTGGATGATTTTCAACGGGCCTGTCTC-3′) and follistatin siRNA species 2 (sense, 5′-AACATTCGTTGCGGTAGGTTTCCTGTCTC-3′; antisense, 5′-AAAAACCTACCGCAACGAATGCCTGTCTC-3′).

Microinjection of follistatin siRNAs into bovine embryos and subsequent embryo culture were conducted using procedures described previously (20). Presumptive zygotes collected 16–18 hpi were used in all microinjection experiments. Follistatin siRNA species 1 and 2 were first validated for efficacy and specificity of follistatin ablation in early embryos and effects on development. To determine efficacy in follistatin RNA knockdown, zygotes were microinjected individually with follistatin siRNA species 1 or 2 (25 μM concentration) and four-cell embryos collected 42–44 hpi for real-time PCR analysis of follistatin mRNA. Control embryos were uninjected, injected with similar volume of negative control siRNA (25 μM, universal control species 1; Ambion), and/or subjected to a sham injection with a similar volume of water (n = 4 pools of 10 embryos per treatment). Specificity of siRNA-mediated follistatin ablation (species 1) was determined by real-time RT-PCR analysis of above samples from four-cell embryos to determine mRNA abundance for five housekeeping genes (β-actin, ribosomal protein L-19, ribosomal protein L-15, GAPDH, and cyclophilin-A) and the bovine oocyte-specific gene JY-1 (20). Efficacy of follistatin siRNA in reducing follistatin protein in early embryos was determined by follistatin immunostaining of 16-cell embryos (uninjected and follistatin siRNA species 1 injected) collected 72 hpi (n = 15–20 embryos per group). To determine effects of individual siRNA species on embryonic development, zygotes were mi-

croinjected with follistatin siRNA species 1 or 2 as described above. Sham-injected and/or uninjected and negative control siRNA-injected zygotes were used as controls (n = 25–30 embryos per treatment; n = 4 replicates). Percent development of embryos to eight- to 16-cell and blastocyst stages were determined 72 hpi and on d 7, respectively.

Effects of follistatin ablation/replacement on early embryonic development

For ablation/replacement studies, a cocktail of follistatin siRNA species 1 and 2 (25 μM) was used. Efficacy of the cocktail in ablation of follistatin RNA was verified by real-time RT-PCR analysis of follistatin mRNA in uninjected, follistatin siRNA cocktail-injected and negative control siRNA-injected embryos (four cells) collected 42–44 hpi (n = 4 pools of 10 embryos per treatment). To determine effects of follistatin ablation and/or replacement on early embryonic development, presumptive zygotes were subjected to follistatin siRNA cocktail microinjection or served as uninjected controls (n = 25–30 embryos per treatment; n = 4 replicates). After completion of microinjections, embryos in each group were cultured in the presence or absence of 10 ng/ml follistatin for 72 h and then follistatin free as described above. Minimum effective concentration of follistatin used was selected based on results of above described dose-response studies. Effects of treatments on early, late, and total cleavage rates, percent development to eight- to 16-cell and blastocyst stages, and blastocyst cell allocation (total, ICM, and TE cell numbers) were determined. A subset of blastocysts from each treatment group were pooled (n = 2 per treatment) within each replicate and subjected to real-time RT-PCR analysis of mRNA abundance for the TE marker CDX-2 and the ICM marker Nanog (21).

Effect of activin and SB-431542 treatments on early embryonic development

To determine the effect of activin treatment and treatment with SB-431542, an inhibitor of signaling through the type I receptors for activin, TGF-β, and nodal (22, 23) on early embryonic development, presumptive zygotes were stripped of cumulus cells and cultured in KSOM supplemented with 0.3% BSA containing 0, 1, and 10 ng/ml recombinant human activin A (R&D Systems; n = 25–30 presumptive zygotes per treatment, n = 4 replicates) or in the presence of 0, 2, or 4 μM SB-431542 (Sigma-Aldrich; n = 25–30 presumptive zygotes per treatment; n = 6 replicates). To determine whether effects of follistatin treatment are affected by inhibition of activin, TGF-β or nodal signaling, presumptive zygotes were cultured in the presence of medium alone, 10 ng/ml follistatin, 4 μM SB-431542, or 10 ng/ml follistatin plus 4 μM SB-431542 (n = 30 presumptive zygotes per treatment; n = 6 replicates). Embryos at the eight- to 16-cell stage were separated 72 hpi, washed, and cultured in fresh KSOM (minus exogenous activin, SB-431542, or follistatin) supplemented with 0.3% BSA and 10% fetal bovine serum until d 7. Effects of treatments on early, late, and total cleavage rates and percent development to eight- to 16-cell and blastocyst stages were determined as described above.

Quantitative real-time RT-PCR

Procedures used for RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis of mRNA abundance in early embryos have been described previously (4, 18, 20). See supplemental Table 1 (published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) for list of primer sequences. Quantification of all transcripts was done using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Relative amounts of target gene expression for each sample were calculated using the formula $2^{-\Delta\Delta C_T}$ as described elsewhere (24). Amounts of mRNAs of interest were normalized relative to abundance of an endogenous control (18S rRNA) to account for differences in total RNA concentrations between samples.

Immunofluorescent localization of follistatin in early embryos

Follistatin protein was localized in embryos according to previously published procedures (25) using a rabbit polyclonal anti-follistatin antibody (1:1500 dilution; kindly provided by Dr. D. J. Phillips, Monash Institute of Medical Research, Clayton, Victoria, Australia). Control embryos were incubated in the absence of anti-follistatin antibody or in the presence of similar concentration of follistatin antibody that had been preincubated in the presence of 10 μg/ml recombinant human follistatin, which blocked all immunoreactivity (data not shown).

Statistical analysis

Data were analyzed using one-way ANOVA. For embryo culture experiments, percent data were subjected to arc-sin transformation before analysis. Differences in treatment means were compared using Fisher's protected least significant difference test.

Results

Effect of follistatin treatment of early embryos on time to first cleavage and blastocyst development

Follistatin mRNA is lower in oocytes collected from prepubertal (model of poor oocyte competence) *vs.* adult animals and is also lower in two-cell bovine embryos (collected before embryonic genome activation) that cleave late *vs.* early-cleaving two-cell embryos with higher rates of blastocyst development (4). Such results suggest that endogenous maternal (oocyte-derived) follistatin could be a limiting factor in development to the blastocyst stage. Hence, effects of supplementation with exogenous follistatin during the first 72 h of *in vitro* culture of bovine embryos (from fertilization to embryonic genome activation) on time to first cleavage and embryonic development to the blastocyst stage were determined. As depicted in Fig. 1A, follistatin treatment resulted in a pronounced, dose-dependent increase ($P < 0.05$) in proportion of embryos that cleaved early (within 30 hpi). The increase in early cleaving embryos was maximal in response to 10 ng/ml follistatin treatment and was not observed in response to 100 ng/ml follistatin. Follistatin treatment (10 ng/ml) also decreased ($P < 0.05$) the proportion of embryos that cleaved within 30–36 hpi (late cleaving; Fig. 1B). However, no effect of treatments on total cleavage rate (determined 48 hpi) was observed (Fig. 1C), suggesting that stimulatory effects of follistatin are mediated by an acceleration of time to first cleavage rather than enhancement of total number of embryos undergoing the initial cleavage division. To further examine the relationship between maternal follistatin and time to first cleavage, immunolocalization of follistatin in early- and late-cleaving two-cell embryos was performed. Enhanced staining for follistatin was observed in the early-cleaving embryos (Fig. 1D).

Follistatin treatment also increased the proportion of embryos that developed to the blastocyst stage (determined on d 7). Similar to observed dose-dependent effects on time to first cleavage, rates of blastocyst developed were increased by up to 40% in response to 1 and 10 ng/ml follistatin treatment ($P < 0.05$), whereas treatment with 100 ng/ml follistatin had no effect (Fig. 1E).

It is well established that early-cleaving bovine embryos develop to the blastocyst stage at a greater rate than their late-

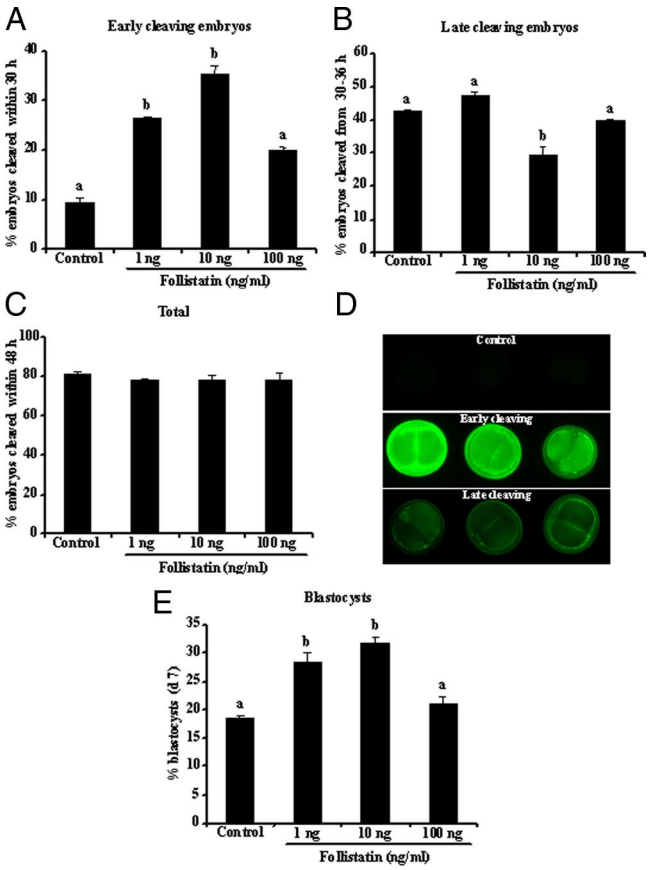

FIG. 1. Follistatin regulation of time to first cleavage and subsequent development of bovine embryos. A–C, Effects of exogenous follistatin treatment during the first 72 h of *in vitro* embryo culture on proportion of embryos that reached the two-cell stage within 30 hpi (early cleaving) (A), proportion of embryos that reached the two-cell stage from 30–36 hpi (late cleaving) (B), total cleavage rate (determined 48 hpi) (C); D, localization of follistatin protein in untreated early- *vs.* late-cleaving two-cell bovine embryos (n = 15–20 embryos per group). E, effects of exogenous follistatin treatment during the first 72 h of *in vitro* culture on proportion of embryos developing to the blastocyst stage (determined on d 7). The values (in A–C and E) are expressed as the mean ± SEM of the data collected from six replicates (n = 25–30 zygotes per treatment in each replicate). Values with *different letters* across treatments indicate significant differences ($P < 0.05$).

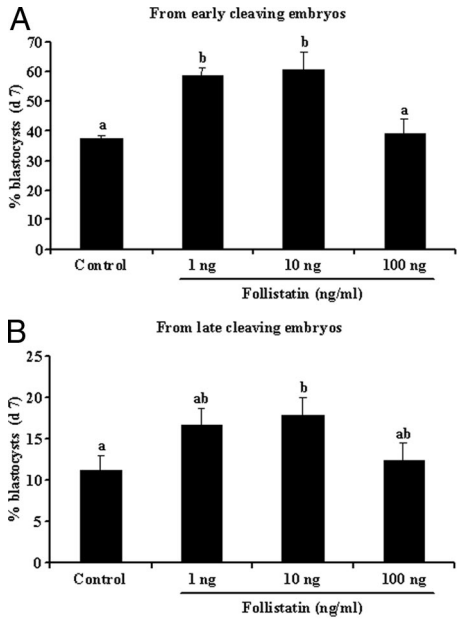

FIG. 2. Follistatin regulation of development of early-cleaving and late-cleaving two-cell bovine embryos to the blastocyst stage. A and B, Effects of exogenous follistatin treatment of early-cleaving (cleavage within first 30 hpi) and late-cleaving embryos (cleavage 30–36 hpi) during the first 72 h of *in vitro* culture on proportion of early-cleaving embryos reaching the blastocyst stage (A) and proportion of late-cleaving embryos reaching the blastocyst stage (determined on d 7) (B). The values are expressed as mean ± SEM of the data collected from four replicates (n = 25–30 zygotes per treatment in each replicate). Values with *different letters* across treatments indicate significant differences ($P < 0.05$).

cleaving counterparts (4, 26). To further examine the relationship between follistatin treatment, time to first cleavage, and subsequent blastocyst development, effects of follistatin treatment (during first 72 h of *in vitro* culture) specifically on development of early-cleaving and late-cleaving embryos to the blastocyst stage were determined. Follistatin treatment of early-cleaving embryos further increased the proportion of embryos developing to the blastocyst stage in comparison with untreated embryos that also cleaved early ($P < 0.05$), with more than 60% of early-cleaving embryos developing to the blastocyst stage in response to 10 ng/ml follistatin treatment (Fig. 2A). Follistatin treatment (10 ng/ml) of late-cleaving embryos also increased rates of blastocyst development ($P < 0.05$), although effects were less dramatic than observed after treatment of early-cleaving embryos (Fig. 2B). Collectively, results demonstrate a significant dose-dependent stimulatory effect of exogenous follistatin treatment (from fertilization to embryonic genome activation) on time to first cleavage and subsequent development of bovine embryos to the blastocyst stage and support a potential role for endogenous follistatin in regulation of early embryonic development.

Effect of exogenous follistatin treatment on cell allocation in bovine blastocysts

The effects of follistatin treatment on blastocyst TE, ICM, and total cell numbers were also determined as an indicator of blastocyst developmental characteristics. Follistatin treatment (10 ng/ml) did not influence ICM cell numbers (Fig. 3A) but did increase TE (Fig. 3B) and total cell numbers (Fig. 3C) in resulting blastocysts ($P < 0.05$). No significant effects of treatment with other doses of follistatin (1 and 100 ng/ml) were observed (Fig. 3, A–C).

Evidence supporting a requirement of maternal follistatin in bovine early embryonic development

Results of above studies support the ability of exogenous follistatin to positively influence time to first cleavage, blastocyst development, and cell allocation but do not directly establish a requirement for maternal (oocyte-derived) follistatin in early embryonic development. Thus, we validated procedures for siRNA-mediated ablation of endogenous follistatin in early bovine embryos (using two distinct follistatin siRNA species) and determined effects of follistatin siRNA injection on early embryonic development. Microinjection of follistatin siRNA 1 or 2 resulted in a more than 80% reduction ($P < 0.05$) in follistatin mRNA in four-cell embryos relative to uninjected, sham-injected, and negative control siRNA-injected controls (supplemental Fig. 1, A and B, respectively) and was accompanied by a

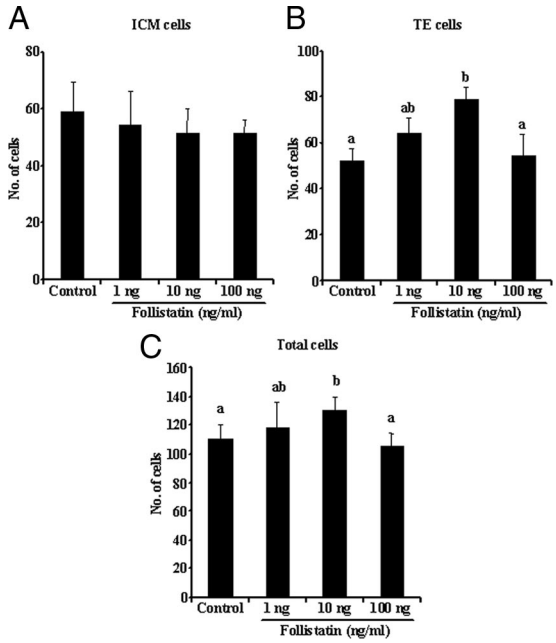

FIG. 3. Follistatin regulation of bovine blastocyst cell allocation. A–C, Effects of exogenous follistatin supplementation during the initial 72 h of *in vitro* embryo culture on number of ICM cells (A), number of TE cells (B), and total cell numbers as determined after differential staining of blastocysts collected on d 7 (C). The total number of blastocysts examined after 0, 1, 10, and 100 ng/ml follistatin treatment were 14, 22, 27, and 15, respectively. The values are expressed as the mean ± SEM of the data collected from four replicates. Values with *different letters* across treatments indicate significant differences ($P < 0.05$).

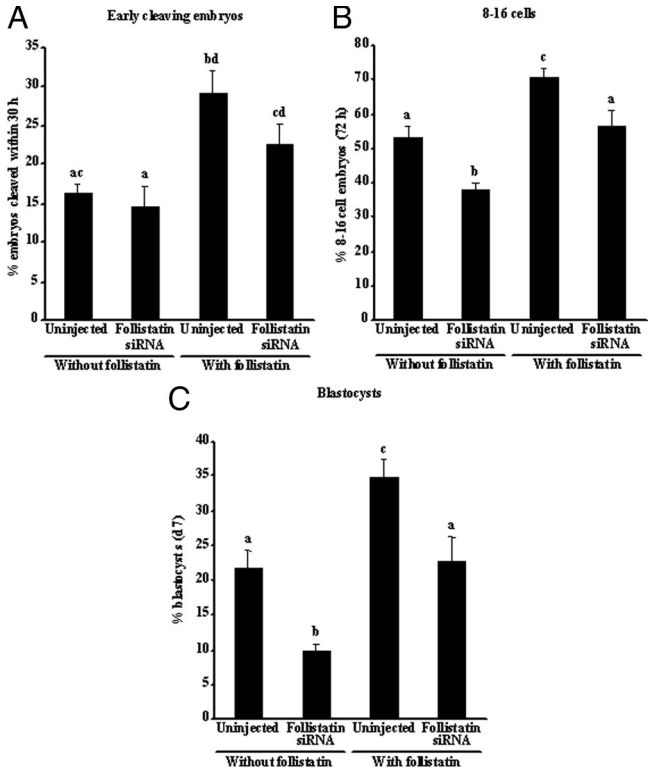

FIG. 4. Follistatin ablation/replacement studies in early bovine embryos: regulation of developmental progression. A–C, Effect of follistatin ablation and/or replacement on proportion of embryos that reached the two-cell stage within 30 h after fertilization (early cleaving) (A), proportion of embryos developing to the eight- to 16-cell stage (determined 72 hpi) (B), and proportion of embryos developing to blastocyst stage (determined on d 7) (C). Data are expressed as mean ± SEM from four replicates (n = 25–30 zygotes per treatment per replicate). Values with *different letters* across treatments indicate significant differences ($P < 0.05$).

significant reduction ($P < 0.05$) in development of follistatin siRNA-injected embryos to the eight- to 16-cell and blastocyst stages (supplemental Fig. 1, C and D). Real-time PCR analysis confirmed no off-target effects of siRNA injection on mRNA abundance for five housekeeping genes and for the oocyte-specific gene JY-1 in four-cell bovine embryos (supplemental Fig. 2). Follistatin siRNA microinjection (siRNA 1) into bovine zygotes also dramatically reduced follistatin immunostaining in 16-cell embryos (supplemental Fig. 3). Above results support specificity of follistatin siRNA effects on bovine early embryonic development.

Follistatin ablation (siRNA microinjection) and replacement (exogenous follistatin supplementation) studies were performed to conclusively establish a role for endogenous follistatin in embryonic development. Microinjection of a cocktail of follistatin siRNA 1 and 2 reduced ($P < 0.05$) follistatin mRNA levels in four-cell embryos by more than 90% relative to controls (supplemental Fig. 4) and was used to achieve a greater reduction in endogenous follistatin in early embryos. Although no effect of follistatin siRNA injection on proportion of embryos cleaving early was observed (Fig. 4A), presumably due to inadequate duration of time to deplete embryos of endogenous follistatin before time to first cleavage was measured, follistatin siRNA cocktail injection significantly reduced ($P < 0.05$) the proportion of embryos developing to the eight- to 16-cell (Fig. 4B) and blastocyst (Fig. 4C) stages, and effects of siRNA injection were completely reversed when follistatin siRNA-injected embryos were cultured in the presence of exogenous follistatin. No effects of treatments on numbers of ICM cells in resulting blastocysts were observed (Fig. 5A), but microinjection of follistatin siRNA resulted in a reduction ($P < 0.05$) in numbers of TE and total cells (Fig. 5, B and C), and siRNA effects were reversed by exogenous follistatin supplementation. To further establish a role for follistatin in regulation of cell lineage allocation, effects of above treatments on blastocyst mRNA abundance for the ICM marker Nanog and the TE marker CDX-2 were determined. No effects of treatments on Nanog mRNA abundance were observed (Fig. 5D). However, treatment of uninjected embryos with exogenous follistatin resulted in increased CDX-2 mRNA abundance ($P < 0.05$). Follistatin siRNA injection tended to reduce CDX-2 mRNA levels relative to uninjected control embryos ($P < 0.08$), and follistatin treatment of siRNA injected embryos restored CDX-2 mRNA to levels seen in uninjected control embryos (Fig. 5E). Collectively, results of ablation/replacement studies strongly support a functional role for maternal follistatin in regulation of early embryonic development and blastocyst cell allocation.

Are stimulatory effects of follistatin on early embryonic development mediated by classical inhibition of activin activity?

Follistatin is best known for its ability to bind at a high affinity and inhibit activin action (10). To determine whether above effects of follistatin treatment are potentially mediated by inhibi-

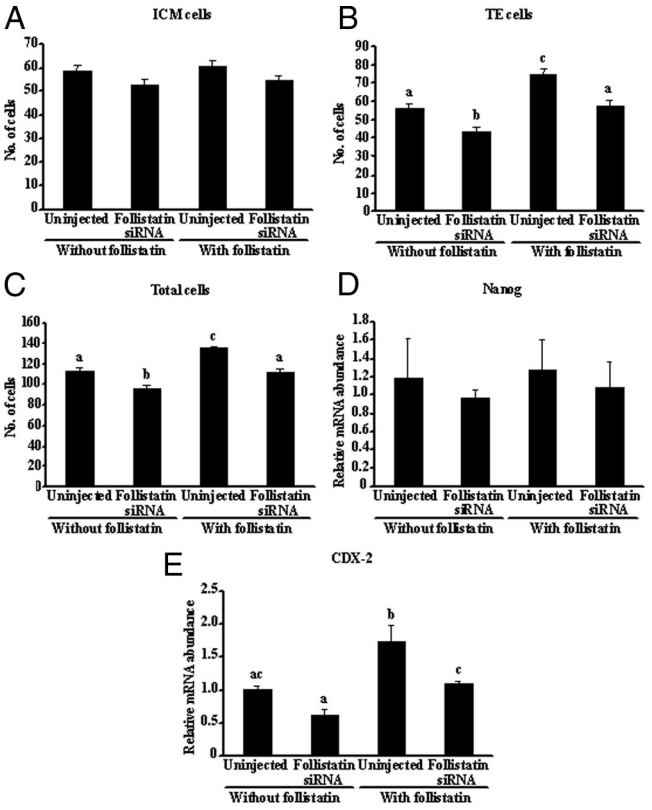

FIG. 5. Follistatin ablation/replacement studies in early embryos: regulation of bovine blastocyst cell allocation. A–E, Effect of follistatin ablation and/or replacement on number of ICM cells (A), number of TE cells (B), total cell numbers (C), RNA abundance for the ICM marker Nanog (D), and mRNA abundance for the TE cell marker CDX-2 in bovine blastocysts collected on d 7 after insemination (E). Data are expressed as mean ± SEM from four replicates (n = 25–30 zygotes per treatment per replicate). Values with *different letters* across treatments each graph indicate significant differences ($P < 0.05$).

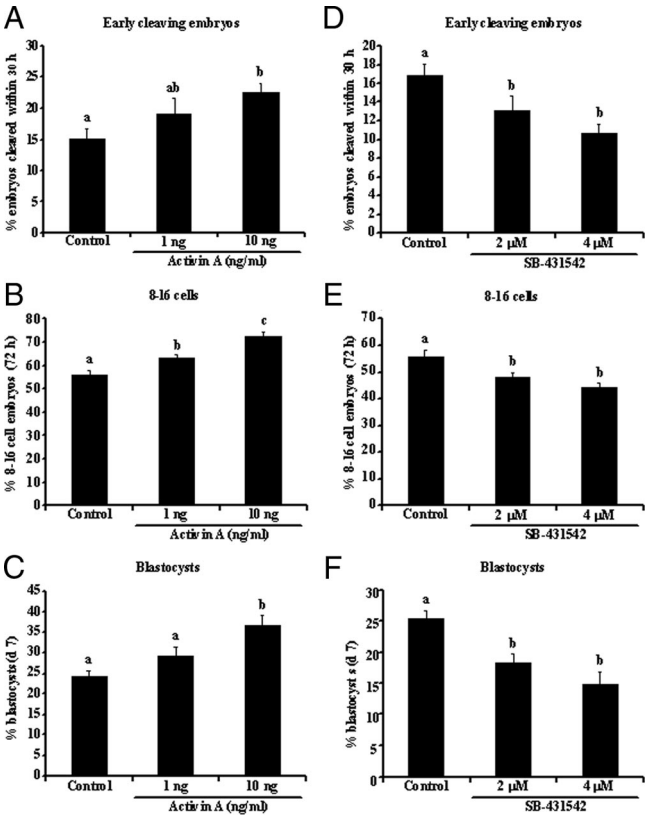

FIG. 6. Activin and activin-like kinase (Alk) 4, 5, and 7 inhibitor effects on bovine early embryogenesis. A–C, Effects of exogenous activin treatment during the first 72 h of *in vitro* embryo culture on proportion of embryos that reached the two-cell stage within 30 hpi (early cleaving) (A), proportion of embryos that reached the eight- to 16-cell stage within 72 hpi (B), and proportion of embryos reaching the blastocyst stage by d 7 after insemination (C). D–F, Effects of treatment with SB-431542 [an inhibitor of signaling through the type I receptor for activin (Alk-4), TGF-β (Alk-5), and nodal (Alk-7)] during first 72 h of *in vitro* culture of bovine embryos on proportion of embryos that reached the two-cell stage within 30 hpi (early cleaving) (D), proportion of embryos that reached the eight- to 16-cell stage within 72 hpi (E), and proportion of embryos reaching the blastocyst stage by d 7 of culture (F). Data are expressed as mean ± SEM (n = 4 replicates for A–C; n = 6 replicates for D–F; n = 25–30 zygotes per treatment per replicate). Values with *different letters* across treatments within a graph indicate significant differences ($P < 0.05$).

tion of activin action, we determined whether effects of endogenous activin A treatment of bovine embryos are in fact opposite to those observed after follistatin treatment. Treatment with activin A (10 ng/ml) increased ($P < 0.05$) the proportion of embryos that cleaved early (Fig. 6A) and the proportion of embryos developing to the eight- to 16-cell (Fig. 6B) and blastocyst (Fig. 6C) stages. Effects of treatment with 1 ng/ml activin A were less robust. Hence, effects of activin treatment on time to first cleavage and blastocyst development mimic the above stimulatory effects of follistatin treatment. To further rule out a role for potential inhibition of activin activity in the mechanism of action of follistatin, we tested effects of treatment with SB-431452, a potent inhibitor of signaling through the type I receptors for activin, TGF-β, and nodal (22, 23), on bovine early embryonic development. SB-431542 treatment reduced ($P < 0.05$) the proportion of early cleaving embryos (Fig. 6D) and embryonic development to the eight- to 16-cell (Fig. 6E) and blastocyst stages (Fig. 6F). Hence, effects of SB-431542 treatment were the opposite of those seen in response to follistatin treatment. Furthermore, the proportion of embryos cleaving early and embryonic development to the eight- to 16-cell and blastocyst stages were increased ($P < 0.0001$) in SB-431542-treated embryos supplemented with exogenous follistatin (supplemental Fig. 5). Collectively, results suggest that embryotrophic effects of follistatin are nonclassical and not mediated by inhibition of endogenous activin action on early embryos.

Discussion

Results of the present studies demonstrated for the first time that levels of follistatin resident in oocytes at time of fertilization influence not only time of first cleavage but also capacity of fertilized oocytes to develop into blastocysts and the cell allocation characteristics of such blastocysts. These novel observations may have relevance to humans because they imply that inherent amounts of follistatin in oocytes of a single ovulating species contribute to oocyte competence and thus the potential for embryo survival. A number of genes transcribed and stored during oogenesis that are obligatory for the maternal-to-embryonic transition and progression through initial stages of embryogenesis have been described to date, particularly through gene targeting studies in mice (8). Functional evidence supporting a pos-

itive role for follistatin during mammalian early embryogenesis has not been reported previously.

We previously reported a positive relationship between follistatin mRNA abundance in two-cell bovine embryos and time to first cleavage (4) and observed a greater abundance of follistatin protein in early- *vs.* late-cleaving two-cell embryos in the current studies. Furthermore, treatment with exogenous follistatin after *in vitro* fertilization significantly enhanced (in a dose-dependent fashion) the proportion of embryos that cleaved early. After *in vitro* fertilization, early-cleaving bovine (4, 26) and human embryos (27, 28) have enhanced capacity to reach the blastocyst stages compared with their later-cleaving counterparts. Numerous studies in humans indicate that time to first cleavage is a significant predictor of embryo developmental potential and success of assisted reproductive technologies (29–31), with up to 2.5-fold higher pregnancy rates observed in single embryo transfers with early- *vs.* late-cleaving embryos. Hence, levels of maternal (oocyte-derived) follistatin may influence not only rates of embryonic development to the blastocyst stage but also overall pregnancy success.

The precise window before embryonic genome activation when the beneficial effects of follistatin are manifest has not been elucidated. The beneficial effects of follistatin treatment on subsequent rates of blastocyst development could potentially be mediated solely during the first 30 h of culture and through acceleration of time to first cleavage. However, rates of blastocyst development were reduced by more than 50% in response to siRNA-mediated follistatin ablation despite no significant effect on time to first cleavage (presumably due to insufficient time between siRNA injection and first cleavage to deplete endogenous follistatin). Furthermore, rates of development of early-cleaving embryos to the blastocyst stage were further enhanced in response to follistatin treatment during the first 72 h of culture, suggesting that the beneficial effects of follistatin treatment on subsequent rates of blastocyst development are not likely mediated solely through stimulation of early cleavage. Future studies will be required to determine the specific mechanisms whereby follistatin treatment accelerates time to first cleavage.

Follistatin functions as an inhibitor of action of TGF-β superfamily members in the extracellular milieu. Follistatin binding to such growth factors inhibits receptor binding and subsequent ligand-induced signaling (14). Of all follistatin actions described to date, follistatin is best known for its high-affinity binding and inhibition of activin activity (10). However, results of the present studies suggest that the effects of follistatin on bovine early embryogenesis are nonclassical and not mediated by inhibition of activin activity. Observed effects of activin treatment of early embryos mimicked stimulatory effects of follistatin treatment on time to first cleavage and blastocyst development. Inhibition of activin receptor signaling via SB-431542 treatment yielded opposite effects to those observed in response to follistatin treatment, and SB-431542 treatment did not block the stimulatory effects of follistatin on time to first cleavage and blastocyst development. Although the inhibitory effects of SB-431542 treatment are not specific to activin signaling, because SB-431542 also inhibits signaling through type I receptors for TGF-β and nodal (22, 23), results further indicate it is unlikely that the stimulatory role of follistatin in bovine early embryonic development is mediated by an inhibition of activin activity. An inhibitory effect of SB-431542 treatment during *in vitro* oocyte maturation on subsequent blastocyst development after *in vitro* fertilization was reported previously (32). Previous studies also reported a positive effect of activin supplementation during *in vitro* embryo culture on development of bovine embryos to the blastocyst stage (33, 34). Interestingly, such studies also reported a subtle inhibitory effect of exogenous follistatin treatment on bovine early embryonic development *in vitro* (34). However, follistatin ablation/replacement studies reported here clearly support a positive role for endogenous maternal (oocyte-derived) follistatin in bovine early embryogenesis.

In contrast to the proposed inhibitory effect of follistatin treatment on endogenous activin activity, one can speculate that follistatin treatment at low levels may increase bioavailability and enhance endogenous activin activity. Administration of low *vs.* high doses of follistatin to bovine cumulus oocyte complexes increased their embryo developmental potential and did not antagonize the effects of activin A (35). The stimulatory effects of follistatin treatment on multiple indices of early embryonic development in the current studies were clearly dose dependent, and no beneficial effects were observed when embryos were cultured in the presence of the highest dose (100 ng/ml) of follistatin tested. However, crystallization of the activin-follistatin complex revealed that interaction of follistatin with activin (in a 2:1 stoichiometry) blocks binding sites on the activin molecule for both its type I and type II receptors (14), and follistatin effects in the present studies were not blocked by SB-431542 treatment, making the potential for follistatin enhancement of endogenous activin activity in early embryos unlikely. However, follistatin also binds other TGF-β superfamily members, including bone morphogenetic proteins 4, 6, and 7 (36) and myostatin (37), but at a markedly reduced affinity relative to activin binding. Further studies will be required to determine whether the actions of follistatin on bovine embryos are mediated by inhibition of activity of other TGF-β superfamily members.

Results of the present studies also indicate that maternal (oocyte-derived) follistatin is a functional determinant of cell allocation in bovine blastocysts. Treatment with exogenous follistatin during embryo culture results in an increase in total cell numbers in bovine blastocysts mediated specifically by an increase in TE cells and described loss-of-function experiments also support a role for endogenous follistatin in regulation of blastocyst cell allocation. Although the relationship between blastocyst cell allocation and embryo survival/pregnancy success has not been directly investigated, retrospective microarray analysis of bovine blastocyst biopsies indicated that increased blastocyst mRNA for the TE-specific transcription factor CDX-2 is associated with embryos that resulted in calf delivery after embryo transfer (38). In the present studies, increased blastocyst CDX-2 mRNA was observed in response to follistatin treatment during the initial 72 h of embryo culture. Hence, follistatin treatment may also enhance pregnancy potential of resulting blastocysts, but further research is needed to support the hypothesis.

The mechanisms whereby follistatin treatment during the first 72 h of *in vitro* embryonic development or follistatin abla-

tion impact blastocyst cell allocation are not clear. It is believed that differentiation of the TE lineage is initiated coincident with compaction at the eight- or 16-cell stage of embryonic development in the mouse (21). Little information is currently available on timing of initiation of TE lineage determination in bovine embryos. However, it is curious that follistatin treatment for the first 72 h of *in vitro* embryo culture (well before compaction) has stimulatory effects on bovine blastocyst cell numbers that are mediated specifically by an increase in TE cells rather than an increase in both TE and ICM cells. Other evidence from the bovine model system indicates that treatments early in development can specifically impact TE cell numbers in resulting blastocysts. Studies of coculture of bovine cumulus oocyte complexes with denuded oocytes during *in vitro* maturation demonstrated that oocyte-secreted factors can not only enhance rates of blastocyst development but can also stimulate an increase in cell numbers mediated specifically by an increase in TE cells (32).

In summary, results of the current studies strongly support a functional role for follistatin in control of time to first cleavage, blastocyst development, and blastocyst cell allocation in bovine embryos and suggest the stimulatory effects of follistatin are not mediated classically by inhibition of activin activity. Collectively, results suggest that follistatin may be an important functional determinant of oocyte competence. Future studies of interest will be to determine whether stimulatory effects of follistatin treatment are mediated by inhibition of activity of other TGF-β superfamily members in early embryos, particularly known TGF-β superfamily members of oocyte origin (*e.g.* growth differentiation factor-9 and bone morphogenetic protein-15), and to determine the specific signaling pathways and downstream targets that mediate observed effects of follistatin on embryonic development.

Acknowledgments

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