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Follistatin supplementation during in vitro embryo culture improves developmental competence of bovine embryos produced using sex-sorted semen

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Abstract

Using sex-sorted semen to produce offspring of desired sex is associated with reduced developmental competence in vitro and lower fertility rates in vivo. The objectives of the present study were to investigate the effects of exogenous follistatin supplementation on the developmental competence of bovine embryos produced with sex-sorted semen and possible link between TGF- β regulated pathways and embryotrophic actions of follistatin. Effects of follistatin on expression of cell lineage markers (*CDX2* and *Nanog*) and downstream targets of SMAD signaling (*CTGF*, *ID1*, *ID2* and *ID3*) and AKT phosphorylation were investigated. Follistatin was supplemented during the initial 72 h of embryo culture. Exogenous follistatin restored the in vitro developmental competence of embryos produced with sex-sorted semen to the levels of control embryos produced with unsorted semen, and comparable results were obtained using sorted semen from three different bulls. The mRNA abundance for SMAD signaling downstream target genes, *CTGF* (SMAD 2/3 pathway) and *ID2* (SMAD 1/5 pathway), was lower in blastocysts produced using sex-sorted versus unsorted semen, but mRNA levels for *CDX2*, *NANOG*, *ID1* and *ID3* were

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Authors contributions

Conception and design of the study (MA, LKB, JKF, GWS), acquisition of data (MA, LKB), analysis and interpretation of data (MA, JKF), drafting the article (MA), manuscript revision for important intellectual content (SR, JKF, GWS), all authors read and approved of the final article to be submitted.

Conflict of interest statement

None of the authors has any financial or other potential conflict of interest related to this manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.repbio.2018.06.004>.

similar in both groups. Follistatin supplementation restored *CTGF* and *ID2* mRNA in blastocysts produced using sex-sorted semen to levels of control embryos. Moreover, levels of phosphorylated (p)AKT (Ser-473 and Thr-308) were similar in embryos derived from sex-sorted and unsorted semen, but follistatin treatment increased pAKT levels in both groups. Taken together, results demonstrated that follistatin improves in vitro development of embryos produced with sex-sorted semen and such effects are associated with enhanced indices of SMAD signaling.

Keywords

Follistatin; TGF- β ; AKT; SMAD; Oocytes quality; Bovine; Sorted semen

1. Introduction

Use of sex-sorted semen in combination with in vitro embryo production is an effective means to obtain offspring of known sex [1,2], which can increase the profitability of livestock operations [3–5]. Frozen, sorted bovine semen has been effectively utilized in artificial insemination and in vitro fertilization (IVF) systems in cattle. The major drawbacks of using sex-sorted semen in such assisted reproductive technology (ART) applications is lower fertility rates in animals upon artificial insemination [6–8] and abnormal embryo development in IVF systems [9]. The reduced fertility rates of sex-sorted semen are attributed to the damage of the spermatozoa caused by the sorting procedure [6,10] that impairs the sperm quality [11].

Numerous studies indicated using sex-sorted semen diminishes not only the indices of early embryonic development in vitro but also the quality of embryos produced. Significant reduction in cleavage [12,13] and blastocyst formation rates [14–17] after IVF were reported for sex-sorted compared to unsorted semen. Morphological alterations were also observed for blastocysts produced with sorted semen and these alterations contribute to low developmental potential of such embryos [18]. Moreover, embryos produced with sex-sorted semen displayed lower expression of some developmentally important genes; glucose-3 transporter (Glut-3), glucose-6-phosphate dehydrogenase (G6PD), and Heat shock protein 70.1 (Hsp) [19]. Several approaches have been utilized to enhance the fertilization rate and quality of embryos produced using sex-sorted semen including different sperm gradient separation techniques [20], optimization of heparin and sperm concentrations for each bull [21,22], or by means of new sperm cryopreservation procedures [23]. However, most of the previous methods used to alleviate the effects of sorted semen require optimization for each single bull and the outcomes are not universally positive. Therefore, we hypothesized that using an exogenous embryotrophic factor, follistatin, could improve the in vitro developmental competence of bovine embryos produced with sex-sorted semen.

Follistatin, a binding protein for specific members of transforming growth factor β (TGF- β) superfamily has been established as an trophic factor for cattle and non-human primate embryos [24,25]. Exogenous supplementation of follistatin during the initial 72 h of in vitro embryo culture improved many indices of early embryonic progression including, early cleavage at 30-hour post insemination (hpi), development to 8–16 cell stage at 72

hpi and d 7 blastocyst formation rates [24]. Trophectoderm cell number per blastocyst and CDX2 expression, markers that are associated with increased pregnancy rates after transfer [26], were also increased in response to follistatin treatment of bovine embryos [24]. Moreover, exogenous follistatin treatment rescued the developmental competence of poor quality oocytes identified by brilliant cresyl blue staining [27]. Furthermore, we previously demonstrated multiple TGF- β regulated signaling pathways are linked to embryotrophic actions of follistatin [28–30]. Follistatin supplementation increased AKT phosphorylation at 24 h post treatment and rescued the effects of AKT signaling inhibition on cleavage (early, total), 8–16 cells and blastocyst rates [30]. Exogenous follistatin treatment however didn't rescue the effects of SMAD2/3 or SMAD4 knockdown on blastocyst development [28,29], suggesting that the embryotrophic effects of follistatin are potentially SMAD signaling dependent. Therefore, we hypothesized that follistatin supplementation will restore in vitro development rates in embryos produced with sex-sorted semen and that the increase in developmental capacity will be associated with changes in follistatin regulated pathways; AKT, SMAD2/3 or SMAD1/5. To test these hypotheses, we analyzed the effects of exogenous follistatin supplementation, during the initial 72 h of in vitro embryo culture, on the development of early embryos produced using sex-sorted semen. Effects of follistatin on expression of cell lineage marker genes (*CXD2* and *Nanog*) and downstream targets of SMAD signaling pathways (*CTGF*: SMAD2/3 and *ID1*, *ID2* and *ID3*: SMAD1/5) and AKT phosphorylation in early embryos produced with sex-sorted semen were also investigated.

2. Materials and methods

All chemicals and reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Ovaries, used in this study, were collected from local slaughter house in the state of Michigan. Michigan State university institutional animal care and use committee (IACUC) doesn't require approval for this study.

2.1. Oocytes retrieval and in vitro embryo production

Ovary collection, oocyte retrieval, selection, in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro embryo culture were performed as previously described [27]. IVF was done using frozen-thawed X-sorted bovine spermatozoa from three different bulls (bull A, B or C), control group was fertilized with unsorted frozen-thawed semen from different bulls. X-sorted semen was purchased from NorthStar Cooperative, Inc. Lansing, MI USA

2.2. Effects of follistatin supplementation on developmental potential of early embryos produced with sex-sorted semen

Presumptive zygotes produced with sex-sorted and unsorted semen were cultured with or without 10 ng/ml recombinant human follistatin (the maximally effective dose as previously described [24]) during the initial 72 h of in vitro embryo culture (IVC1), then 8–16-cell embryos were isolated and cultured in fresh media without exogenous follistatin (IVC2) until d 7 (25–30 presumptive zygotes/group, n = 4 replicates). Effects of follistatin supplementation on early cleavage at 30 hpi, total cleavage at 48 hpi, the development to 8–16 cell at 72 h and blastocyst stage at d7 were recorded.

2.3. Effects of follistatin supplementation on expression of cell lineage markers in blastocysts produced with sex-sorted semen

Effects of follistatin supplementation (10 ng/ml) during IVC1 on the transcript abundance for the trophectoderm (TE) marker *CDX2*, and inner cell mass (ICM) marker *NANOG* were determined by quantitative real time PCR in d 7 blastocysts (10 blastocyst/group. n = 4 replicates). Because similar responsiveness to follistatin was observed for in vitro produced embryos in above described experiments using sex-sorted semen from three different bulls, sex-sorted semen from single bull (C) was utilized for subsequent molecular analyses.

2.4. Effects of follistatin supplementation on expression of SMAD signaling pathway downstream targets in early embryos produced with sex-sorted semen

Effects of follistatin on expression of mRNA for downstream target genes of SMAD2/3; connective tissue growth factor (*CTGF*), and SMAD1/5 signaling pathway; Inhibitor of DNA Binding-1 (ID1), ID2 and ID3 were examined as described above for cell lineage markers in d 7 blastocysts (10 blastocyst/group. n = 4 replicates).

2.5. RNA Isolation, cDNA synthesis and real time PCR analysis

RNA Isolation, cDNA synthesis and real time PCR analysis were performed as previously described [24,27,29]. Primer sequences and GenBank accession numbers for all targets are listed in Supplemental Table (1).

2.6. Effects of follistatin supplementation on AKT phosphorylation in early embryos produced with sex-sorted semen

Activation of AKT requires phosphorylation on two amino acid residues: Thr308 and Ser473 [31]. To investigate the effect of follistatin supplementation during IVC1 on AKT phosphorylation in early embryos, samples were collected 10 h after follistatin supplementation [30], and subjected to Western blot analysis for phosphorylated (p) AKT-Ser473, pAKT-Thr308, total (t) AKT and actin as a loading control (20 embryo/group, n = 6 replicates/phosphorylation site).

2.7. Western blot analysis

Protein isolation, SDS-PAGE electrophoresis and membrane blotting procedures were performed as described before [30,32]. Membranes were probed sequentially starting with the primary antibody of either pAKT-Ser473 or pAKT-Thr308. After detection of pAKT, membranes were stripped (10 min/room temp) with restore Western blot stripping buffer (Thermo Scientific, Waltham, MA, USA) and re-probed with tAKT antibody. After detection of tAKT, membranes were stripped and re-probed with anti-actin antibody. HRP-conjugated anti-rabbit-IgG, and anti-mouse-IgG were used as secondary antibodies. The sources and concentrations of antibodies used in this study are listed in Supplemental Table (2).

2.8. Statistical analysis

Two-way ANOVA, with main effects of semen and follistatin treatment and their interaction, was used to determine the differences in mRNA expression and protein abundance/

phosphorylation levels using the general linear model's procedure of SAS (SAS Institute Inc., Cary, NC). For experiments studying the effects of follistatin supplementation on early embryonic development, percentage data were arcsine transformed, then analyzed by mixed linear model's analysis procedures in SAS. Differences among treatment means were compared using Fisher's protected least significant difference (PLSD) test. Data are presented as untransformed mean (\pm) standard error of mean (SEM). Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Follistatin supplementation rescued the poor developmental potential of early bovine embryos produced with sex-sorted semen

As expected, in vitro fertilization with sex-sorted semen versus unsorted semen resulted in significant reduction in all indices of early embryonic progression including early cleavage at 30 hpi, total cleavage at 48 hpi, the development to 8–16 cell at 72 hpi and d7 blastocyst formation rate (Fig. 1A–D). Exogenous follistatin supplementation (10 ng/ml) during IVC1 resulted in a significant improvement in developmental progression of bovine embryos produced in vitro using sex-sorted semen. The number of embryos cleaved within 30 hpi was increased to a level similar to the non-treated controls (embryos produced with unsorted semen) although still lower than follistatin treated control embryos. Similar patterns were observed for the rates of development to 8–16 cell and blastocyst stages. Follistatin supplementation completely restored total cleavage of embryos produced with sex-sorted semen to the levels of follistatin treated embryos produced with unsorted semen and comparable results were obtained using X sorted semen from three different bulls.

3.2. Using sex-sorted semen for IVF didn't impact the expression of cell lineage specific genes, *CDX2* or *Nanog* in early embryos

Above results showed that using sorted semen decreased the developmental competence of early embryos. To investigate the effects of using sorted semen at the molecular level, we measured the blastocyst expression level of cell lineage markers *CDX2* and *NANOG*. Quantitative real time PCR revealed that the mRNA abundance for *CDX2* and *NANOG* was similar in day 7 blastocysts produced with X- sorted and unsorted semen. Follistatin treatment (10 ng/ml) similarly increased the mRNA abundance of *CDX2* in both groups (Fig. 2A). No significant differences in the mRNA transcript abundance for *NANOG*, were observed between the blastocysts produced with sex-sorted or unsorted semen in presence or absence of exogenous follistatin supplementation (Fig. 2B).

3.3. Early bovine embryos produced with sex-sorted semen show no change in AKT signaling

To examine effects of using sex-sorted semen on AKT signaling activity in early embryos, we analyzed AKT-Ser473 and Thr308 phosphorylation in early embryos 10 h post treatment. Immunoblot analysis revealed that using sex-sorted semen did not alter AKT phosphorylation as no differences in either pAKT-Ser473 or Thr308 were observed between embryos produced with sex-sorted and unsorted semen (Fig. 3A–B). Exogenous follistatin supplementation significantly ($p < 0.05$) increased pAKT-Ser473 and pAKT-Thr308 in

embryos produced with both sorted and unsorted semen compared to untreated control embryos. No semen or follistatin effects were observed on tAKT abundance (Fig. 3C–D).

3.4. Follistatin supplementation restored the effects of using sex-sorted semen on the expression of SMAD downstream target genes

Above results suggest that using sex-sorted semen has no impact on either expression of cell lineage marker genes, *CDX2* and *NANOG*, or AKT phosphorylation in early embryos. Whereas, follistatin supplementation similarly increased *CDX2* expression and AKT phosphorylation in embryos produced with sorted and unsorted semen. To further elucidate the possible role of follistatin in modulating TGF- β signaling pathways in early embryos produced with sex-sorted semen, we examined the expression of SMAD signaling downstream target genes in the presence or absence of follistatin. Transcript analysis revealed that expression of *CTGF* (SMAD2/3 downstream target) was significantly ($p < 0.05$) reduced in embryos produced with sex-sorted semen compared to control embryos produced with unsorted semen. Follistatin supplementation restored *CTGF* expression in embryos derived from sex-sorted semen to the level of follistatin treated controls (Fig. 4A). For SMAD 1/5 downstream targets, using sex-sorted semen didn't impact the expression of *ID1* or *ID3* but significantly ($P < 0.05$) decreased the mRNA transcript abundance for *ID2* compared to control embryos produced with unsorted semen. Follistatin supplementation restored *ID2* expression in embryos produced with sorted semen to levels seen in control embryos. Follistatin treatment increased the expression of *ID1* and *ID3* (main effect $P < 0.05$) but there was no effect of semen type or interaction between follistatin and semen type (Fig. 4B–D).

4. Discussion

Using sex-sorted semen for IVF is associated with poor developmental competence of the resulting embryos. Results of the present study demonstrated that exogenous follistatin supplementation during the initial 72 h of in vitro embryo culture reversed the negative effects of using sex-sorted semen on early embryo development. Previous studies demonstrated that bovine embryos produced with sorted semen showed significant reduction in cleavage rate compared to those produced with unsorted semen [13,17,33]. In the present study, follistatin supplementation restored cleavage rates to a similar level as observed for control embryos produced using unsorted semen. Follistatin reversed the effects of using sex-sorted semen on total cleavage, possibly by accelerating the first cleavage. Using sorted semen may have delayed the first cell cycle, therefore the number of embryos reaching 2 cell-stage at 30 hpi was reduced and consequently the total cleavage rate at 48 hpi was also reduced. Previous studies demonstrated that damage to sperm DNA during sorting process impacts the subsequent development of the produced embryos rather the fertilization process itself [17,34]. Moreover, residues from the DNA labelling dye Hoechst 33,342 used during semen sorting process are retained in the spermatozoa and may affect post fertilization events including delay of timing of first embryonic cleavage [35]. Exogenous follistatin supplementation promotes the cell cycle progression and accelerates the first cleavage [24] and subsequently increased the number of embryos cleaved at 48 hpi in sex-sorted embryos. Furthermore, AKT is implicated in regulation of early cleavage, inhibition of AKT

signaling during IVC1 significantly decreased early and total cleavage rates, and follistatin supplementation increased AKT phosphorylation and rescued the effects of AKT inhibition on cleavage rates [30]. Hence, we hypothesized that AKT signaling may be altered in embryos produced with sex-sorted semen. However, AKT phosphorylation levels were not impacted by using sorted semen, at the examined timepoint. Follistatin did increase AKT phosphorylation in embryos produced with sex sorted and unsorted semen, allowing for the possibility that activation of AKT signaling is promoting cleavage in early embryos produced with sex sorted semen.

Follistatin treatment restored in vitro developmental potential of IVF embryos derived using sorted semen from three different bulls, suggesting that the embryotropic actions of follistatin on in vitro developmental potential of embryos derived from sex-sorted semen are not bull dependent. However, only bulls that showed measurable blastocyst rates greater than 5% for sex-sorted semen were used in this study. Therefore, it would be relevant to determine if follistatin can improve in vitro embryo development when sorted semen from bulls yielding even lower developmental rates is utilized.

Previous studies have found that male bovine IVF embryos grow faster than female counterparts [14,36], however other studies have demonstrated similar development rates for embryos derived from X and Y sorted semen [4,13,16,37]. Moreover, embryo sex did not affect total cell number or number TE cell number in bovine blastocysts [38]. Our results demonstrated that follistatin improved the development rate of X-sorted embryos to levels of unsorted embryos, but we cannot discount the possibility that the embryotropic effects of follistatin are due to the sex of the embryos produced. However, as follistatin has a similar effect on early embryonic development in embryos produced with unsorted semen we believe it is likely not greatly influenced by the sex of the embryo.

Follistatin supplementation during in vitro culture has been previously shown to increase *CDX2* mRNA (trophectoderm cell marker) and trophoctoderm cell numbers in resulting blastocysts [24]. Hence, we analyzed the transcript abundance of cell lineage marker genes *CDX2* and *NANOG* to determine whether sorted semen impacts the expression of these genes and if the stimulatory effect of follistatin on *CDX2* expression is still observed in embryos derived from sex-sorted semen. *CDX2* and *NANOG* transcript abundance was similar in bovine blastocysts derived from sorted versus unsorted semen, and follistatin increased *CDX2* expression in both groups. Previous studies reported that embryos produced with sex-sorted semen exhibited lower total cell number compared to those produced with unsorted semen derived from same bull, however the TE/ICM ratio was similar in both groups [33]. Another study reported no differences in total cell number between blastocysts from sorted versus unsorted semen [39]. Although we did not examine the cell number in these studies, we found that using sex-sorted semen for IVF has no impact on the expression of examined cell lineage expressed genes in early bovine embryos.

Previous studies demonstrated the essential requirement of SMAD signaling for the embryotropic actions of follistatin, as knockdown of SMAD4 or SMAD2/3 abolished the embryotropic effects of follistatin on embryos produced with unsorted semen [28,29]. To elucidate whether SMAD signaling is impacted by using sex sorted semen and the

stimulatory effects of follistatin are linked to SMAD signaling in embryos derived from sex-sorted semen, we analyzed the mRNA expression of SMAD downstream target genes; *CTGF* (SMAD2/3), *ID1*, *ID2* and *ID3* (SMAD1/5). Using sex-sorted semen for IVF resulted in decreased expression of *CTGF* and *ID2* suggesting that SMAD signaling activity is impaired in embryos produced with sex-sorted semen.

Inhibition of SMAD2 or SMAD3 diminishes the TGF- β 1-induced up-regulation of *CTGF* [40]. *CTGF* is a downstream target of TGF- β signaling that regulates multiple biological processes including follicular development and ovulation [41]. *CTGF* is a member of CCN cysteine-rich protein family [42], and in bovine blastocysts is mainly expressed in TE cells [43]. Follistatin supplementation restored the expression of *CTGF* (SMAD2/3 downstream target) to level of embryos produced with unsorted semen, implying that SMAD2/3 signaling maybe involved in the positive effects of follistatin on embryos produced with sex sorted semen.

Follistatin also restored the expression of *ID2* to levels seen in control embryos. ID genes are downstream targets of SMAD1/5 pathway regulated by TGF- β members in a cell-type-specific manner and therefore play diverse physiological roles [44–46]. *ID2* regulates trophoblast maintenance and differentiation in bovine embryos [47] and elevated levels of mRNA for *ID2* were observed in mouse blastocysts before implantation [48] suggesting its importance in embryonic development.

Taken together results demonstrate that using sorted semen affects the expression of specific downstream target genes of SMAD signaling pathways. Follistatin supplementation restored the expression of *CTGF* and *ID2* to the levels observed in embryos produced with unsorted semen, suggesting a potential role for both SMAD2/3 and SMAD1/5 signaling in mediating embryotrophic actions of follistatin in early embryos produced by sorted semen. However, further experiments will be needed to confirm the role of SMAD signaling in follistatin actions on embryos produced with sex-sorted semen and to determine further developmental potential of the resulting blastocysts.

5. Conclusions

Results of the present study demonstrate that follistatin supplementation during the initial 72 h of in vitro embryo culture increased the number of cleaving embryos at 48 hpi and restored the in vitro developmental competence of bovine IVF embryos produced with sex-sorted semen to the levels of control embryos produced with unsorted semen. Embryos produced with sex-sorted semen had reduced expression of some downstream target genes of SMAD signaling pathways (*CTGF* and *ID2*), and exogenous follistatin was able to restore expression of these genes to levels seen in control embryos, suggesting a potential role for SMAD signaling in mediating embryotrophic actions of follistatin in early embryos produced by sorted semen. Collectively, the results provide novel information about the use of follistatin to improve development of embryos produced with sex-sorted semen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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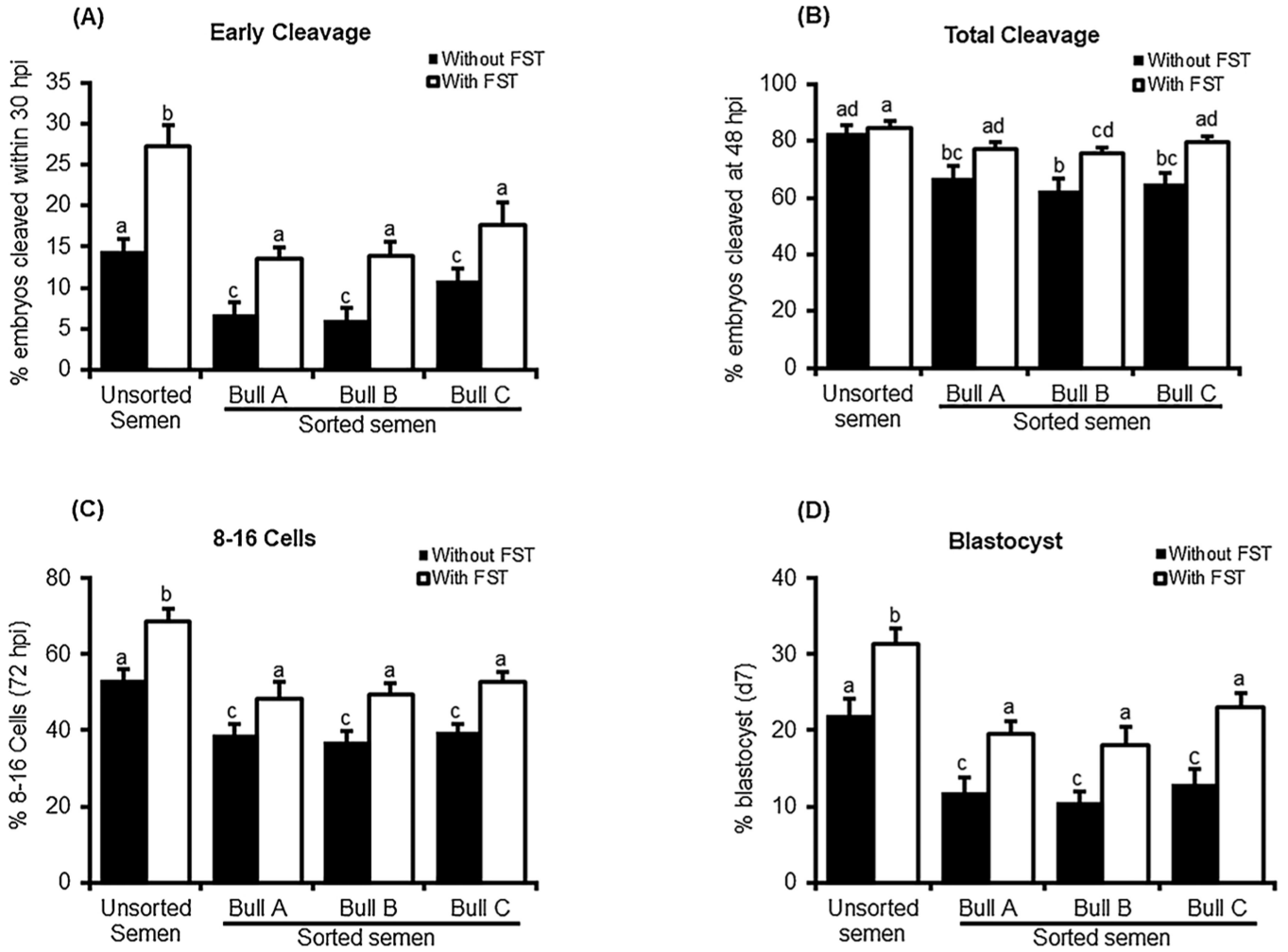


Fig. 1. Effects of follistatin supplementation during IVC1 on developmental progression of early bovine embryos produced with sex-sorted semen. Presumptive zygotes produced with unsorted or sorted semen (bull A, B and C) were cultured with or without 10 ng/ml recombinant human follistatin for 72 h, then 8–16- cell embryos were isolated, washed and cultured in fresh media without follistatin until d7 (n = 4 replicates, n = 25–30 zygote/group). Effects of follistatin on **(A)** early cleavage, **(B)** total cleavage, **(C)** development to 8–16 cell stage and **(D)** d7 blastocyst rate were determined. Data are expressed as mean ± SEM. Values with different superscripts across indicate treatment significant differences (P < 0.05).

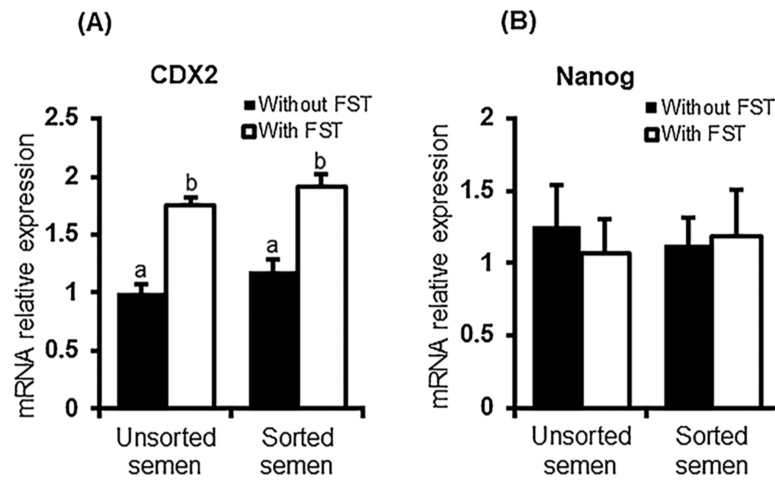
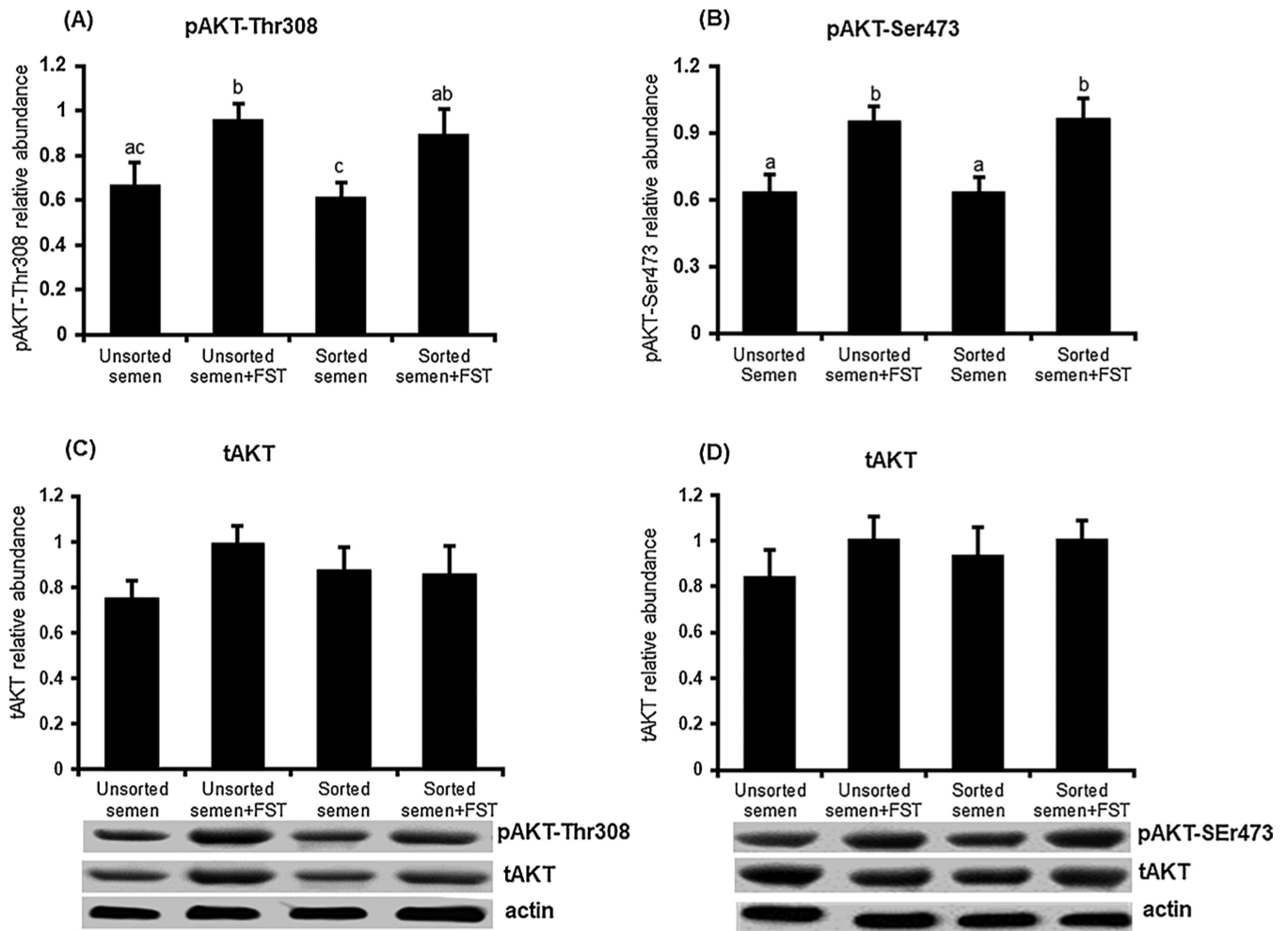


Fig. 2. Expression of cell lineage markers in bovine blastocysts produced with sex-sorted semen. The mRNA abundance for *CDX2* (A) and *Nanog* (B) in d7 blastocyst produced with sorted or unsorted semen (n = 4 replicates, n = 10 blastocysts/pool) was determined by qRT-PCR. Expression was normalized relative to the abundance of RPS18 as a housekeeping gene. Data are expressed as mean \pm SEM. Values with different superscripts across treatment indicate significant differences ($P < 0.05$).

**Fig. 3.**

Effects of follistatin treatment on AKT phosphorylation in early bovine embryos produced with sex-sorted semen. Presumptive zygotes produced with sorted or unsorted semen were cultured with or without 10 ng/ml follistatin for 10 h, then subjected to Western blot analysis for pAKT-Thr308, pAKT-Ser473, tAKT and actin ($n = 6$ replicates/phosphorylation site, $n = 20$ embryos/group). Data were normalized relative to the abundance of actin and are expressed as mean \pm SEM. Values with different superscripts across treatments indicate significant differences ($P < 0.05$). Representative Western blot images are shown.

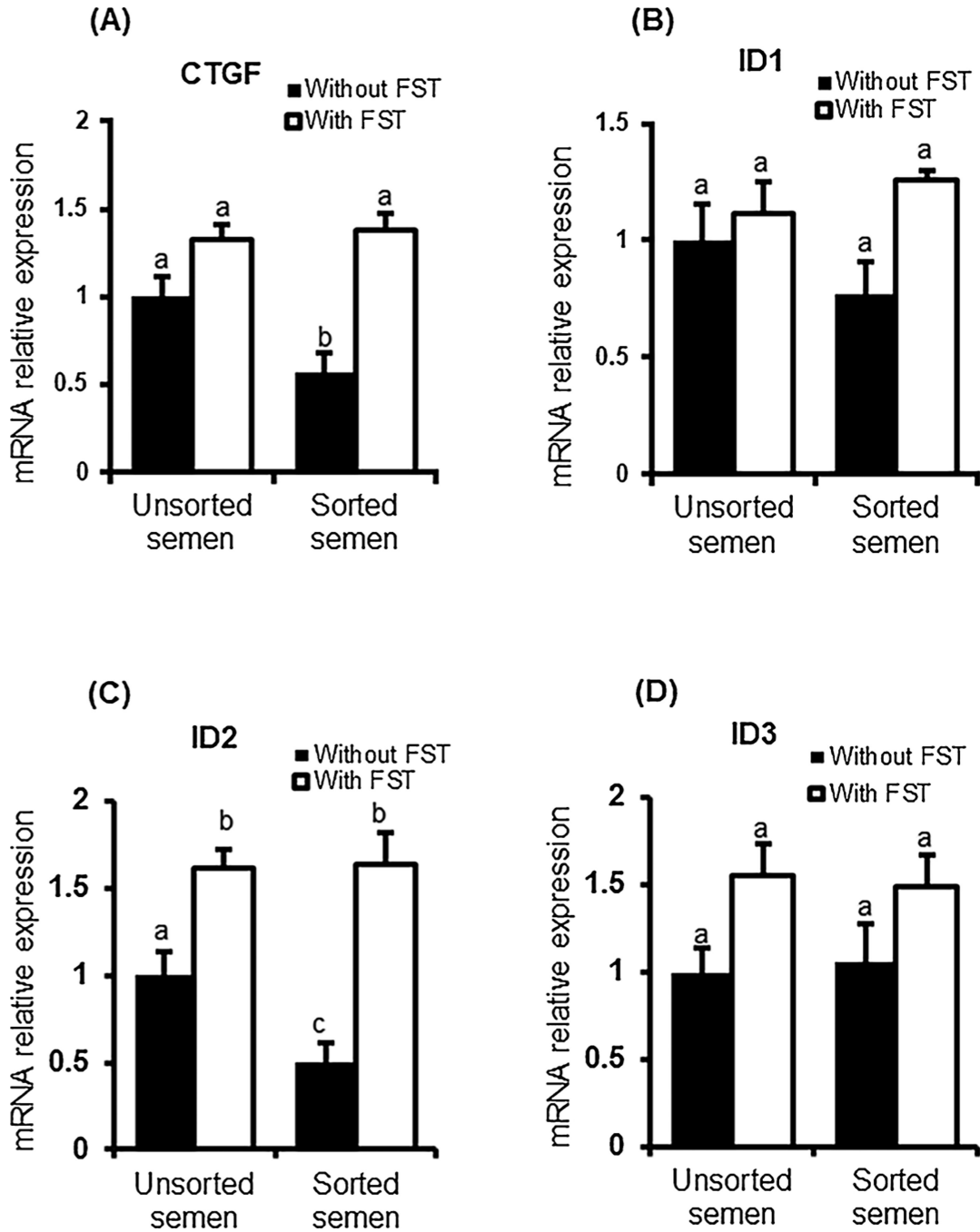


Fig. 4. Expression of downstream targets of SMAD signaling in bovine blastocysts produced with sex-sorted semen. The mRNA abundance for *CTGF* (A), *ID1* (B), *ID2* (C) and *ID3* (D) in d7 blastocyst produced with sorted or unsorted semen (n = 4 replicates, n = 10 blastocysts/pool) was determined by qRT-PCR. Expression was normalized relative to the abundance of RPS18 as a housekeeping gene. Data are expressed as mean ± SEM. Values with different superscripts across treatment indicate significant differences (P < 0.05).