Requirement of the transcription factor USF1 in bovine oocyte and early embryonic development

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Abstract

Upstream stimulating factor 1 (USF1) is a basic helix–loop–helix transcription factor that specifically binds to E-box DNA motifs, known *cis*-elements of key oocyte expressed genes essential for oocyte and early embryonic development. However, the functional and regulatory role of USF1 in bovine oocyte and embryo development is not understood. In this study, we demonstrated that *USF1* mRNA is maternal in origin and expressed in a stage specific manner during the course of oocyte maturation and preimplantation embryonic development. Immunocytochemical analysis showed detectable USF1 protein during oocyte maturation and early embryonic development with increased abundance at 8–16-cell stage of embryo development, suggesting a potential role in embryonic genome activation. Knockdown of *USF1* in germinal vesicle stage oocytes did not affect meiotic maturation or cumulus expansion, but caused significant changes in mRNA abundance for genes associated with oocyte developmental competence. Furthermore, siRNA-mediated depletion of *USF1* in presumptive zygote stage embryos demonstrated that *USF1* is required for early embryonic development to the blastocyst stage. A similar (*USF2*) yet unique (*Twist2*) expression pattern during oocyte and early embryonic development for related E-box binding transcription factors known to cooperatively bind USF1 implies a potential link to USF1 action. This study demonstrates that USF1 is a maternally derived transcription factor required for bovine early embryonic development, which also functions in regulation of *JY1*, *GDF9*, and *FST* genes associated with oocyte competence.

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Introduction

In mammals, oocyte-specific genes play a key role in regulation of the reproductive process and are critical to both ovarian folliculogenesis and early embryogenesis (Rajput *et al.* 2013). The products of these genes which are expressed, stored, and secreted during oogenesis control the follicular microenvironment and promote oocyte competence for successful fertilization and the subsequent embryonic development (De Sousa *et al.* 1998). Several studies have revealed that aberrant expression of these maternal transcripts is directly associated with infertility due to either complete growth arrest or compromised development of oocyte and preimplantation embryos. For example, targeted deletion of *Gdf9*, expressed throughout most stages of folliculogenesis, causes a block in follicular development at the primary follicle stage and complete infertility in mice (Dong *et al.* 1996). Oocyte expressed GDF9

protein is also an important paracrine regulator of surrounding granulosa/cumulus cell functions, which in turn affects competency of the oocyte to develop into a blastocyst (BL) after fertilization (Mottershead *et al.* 2012, 2013, Peng *et al.* 2013). Similarly, the bovine-specific protein JY1 plays a functional role in regulation of follicular function and early embryogenesis (Lee *et al.* 2014a). Supplementation of bovine granulosa cell culture media with recombinant JY1 (rJY1) protein modulates follicle-stimulating hormone (FSH)-induced steroidogenesis. Depletion of *JY1* transcripts in germinal vesicle (GV)-stage oocytes demonstrated adverse effects on nuclear maturation and expansion of surrounding cumulus cells, which retards early embryonic development after IVF. Similarly, siRNA-mediated knockdown of maternal *JY1* transcripts in zygote-stage embryos demonstrated a functional requirement for JY1 after fertilization (Betegowda *et al.* 2007). Supplementation

of rJY1 protein during oocyte and embryo culture rescued the effect of *JY1* knockdown on cumulus expansion and meiotic maturation and BL development respectively (Lee *et al.* 2014a). In addition to *JY1*, our laboratory has previously characterized the functional role of maternal (oocyte-derived) follistatin (FST) in promoting bovine early embryogenesis. Depletion of maternal *FST* in zygotes not only reduced the number of embryos developing to 8–16-cell stage and BL stage but also decreased the number of total and TE cells in BL (Lee *et al.* 2009).

Over the last decades, the functional role of numerous oocyte expressed/specific transcripts has been identified during folliculogenesis (*Nobox*, *Figla*, and *c-Kit*; Soyal *et al.* 2000, Rajkovic *et al.* 2004, Hutt *et al.* 2006), fertilization (*Zp* genes; Dean 1992), early cleavage (*NALP*, *Zar1*, and *Npm2*; Tong *et al.* 2000, Burns *et al.* 2003, Wu *et al.* 2003, Peng *et al.* 2012), and embryonic genome activation (*Brg1* and *H1oo*; Tanaka *et al.* 2001, Saeki *et al.* 2005, Bultman *et al.* 2006) using different gene knockdown strategies in mammals. The majority of oocyte-expressed genes involved in fertilization and embryonic development display highly conserved structural and functional properties among different mammalian species (Sylvestre *et al.* 2013). However, understanding of how these maternally expressed genes are transcriptionally regulated and hence fundamental mechanisms regulating oogenesis and early embryonic development remains poorly understood due to limited characterization of oocyte-expressed transcription factors in mammals. Comprehensive computational, mutational, and functional analysis of the promoters of several of these oocyte-specific genes including *GDF9*, *JY1*, *FST*, *ZP* genes, *NALP* genes, *H1oo*, and *Npm2* revealed common *cis*-acting regulatory elements known as E-box, present within 200 bp upstream of the transcription start site (TSS) and critically important for their transcriptional regulation during oogenesis (Liang *et al.* 1997, Yan *et al.* 2006, Bettegowda *et al.* 2007, Tsunemoto *et al.* 2008). Interestingly, this conserved and canonical E box (CANNTG) DNA element is a well-characterized binding site for *FIGα*, upstream stimulatory factor 1 (USF1), USF2 and TWIST2, and other transcription factors of the basic helix–loop–helix (bHLH) and leucine zipper family. The functional role of oocyte-expressed *FIGα* has been well documented in transcriptional regulation of zona pellucida genes (*ZP1*, *ZP2*, and *ZP3*) necessary for fertilization (Liang *et al.* 1997), and *NALP* family of genes (Joshi *et al.* 2007) essential for early embryonic development beyond the two-cell stage in mice (Tong *et al.* 2000). Moreover, E-box-dependent regulatory roles of USF1, USF2, and TWIST2 transcription factors have been clearly demonstrated in transcriptional regulation of genes associated with stress, immune responses, and the cell cycle and proliferation in different somatic and cancer tissues (Corre & Galibert 2005, Xue & Hemmings 2012). To our

knowledge, the temporal expression and potential functional role of USF1, USF2, and TWIST2 in oocytes and early embryos have not been reported previously.

In this study, we investigated the temporal expression of *USF1*, *USF2*, and *TWIST2* during oocyte and early embryogenesis in cattle. The functional role of *USF1* in early embryonic development and transcriptional regulation of select oocyte-expressed genes containing an E-box motif in their promoter region was analyzed using siRNA-mediated gene knockdown in cumulus-enclosed oocytes before initiation of meiotic maturation. Results support a potential regulatory role for above-mentioned transcription factors in bovine oocyte and early embryonic development.

Materials and methods

All chemicals and reagents used were obtained from Sigma–Aldrich unless specified.

In vitro oocyte and embryo culture

In vitro maturation of oocytes, IVF, and embryo culture to the BL stage were conducted as described previously (Bettegowda *et al.* 2006). Briefly, cattle ovaries were obtained from a local slaughterhouse and cumulus–oocyte complexes (COCs) were aspirated from 2 to 7 mm antral follicles. GV-stage oocytes enclosed by a minimum of three compact layers of cumulus cells and containing homogenous cytoplasm were collected immediately after aspiration. COCs were cultured for 24 h in maturation medium (Medium-199, 10% FBS; Gibco-BRL) containing 1 IU/ml FSH, 5 IU/ml luteinizing hormone (LH), and 1 µg/ml estradiol-17β to induce meiotic maturation. Matured oocytes were fertilized with Percoll-purified motile spermatozoa and incubated for 20 h at 38.5 °C under 5% CO₂ in humidified air. After fertilization, presumptive zygotes (*n*=20 per treatment; defined below) were vortexed for 6 min to completely remove the excess sperm and cumulus cells and washed zygotes cultured in potassium simplex optimization medium (KSOM; Specialty Media, Phillipsburg, NJ, USA) supplemented with 0.3% BSA at 38.5 °C in humidified air and 5% CO₂. The embryos were then transferred into fresh KSOM with 0.3% BSA and 10% fetal bovine serum at 72 h after insemination and further cultured until the BL stage (day 7).

Sample collection for RNA analysis and immunostaining

GV- and metaphase II (MII)-stage oocytes were collected immediately after aspiration and 24 h after *in vitro* maturation respectively. Both GV- and MII-stage oocytes were stripped of cumulus cells by vortexing for 5 min in HEPES–TALP containing 0.1% hyaluronidase. At ~20 h after fertilization, presumptive zygotes were denuded by vortexing, washed, and used for experimental analysis. In addition, embryos at two-cell, four-cell, eight cell, and 16-cell stage were collected at 33, 44, 52, and 72 h after fertilization respectively, and morula (MO) and BLs were collected at 5 and 7 days after fertilization

respectively. For each stage of collected oocytes and embryos, four replicates were taken for real-time PCR analysis and immunofluorescent staining.

Quantitative real-time RT-PCR

Procedures used for RNA isolation, cDNA synthesis, and quantitative real-time RT-PCR to analyze the transcript abundance in GV- and MII-stage oocytes and early embryo samples ($n=4$ pools of ten oocytes per embryo per group) were carried out as described previously (Bettegowda *et al.* 2006). *RPS18* was used as endogenous control to normalize the gene expression data. See Table 1 for the list of primer sequences used in real-time PCR. For all genes, no template controls showed no amplification and melting curves were run for all samples showing a single product.

Immunofluorescent staining

Immunofluorescent staining was performed for USF1 protein localization in oocytes and embryos according to protocols published previously (Tripurani *et al.* 2011). After fixation, permeabilization and blocking, samples were probed with a commercially available rabbit polyclonal anti-USF1 antibody (1:200 (v/v), Santa Cruz Biotechnology, sc229) and the corresponding FITC-conjugated secondary antibody (0.5% (w/v) F9887; Sigma-Aldrich). Immunostaining experiments were replicated four times using at least 10–12 oocytes/embryo per sample. Oocytes and embryos incubated without the primary USF1 antibodies were used as negative controls.

Identification of USF1 transcript origin in early embryos

To determine the origin of *USF1* transcripts in early embryos (maternal vs zygotic), presumptive zygotes were cultured in KSOM containing 0.3% BSA in the presence and absence of a transcription inhibitor (50 μ g/ml α -amanitin). After 52 h of *in vitro* culture, pools of eight-cell embryos were collected within treatment ($n=10$ embryos per pool; $n=4$ replicates for α -amanitin and untreated controls) and subjected to real-time quantification of *USF1* transcript abundance as described earlier.

Design and validation of siRNA-mediated USF1 knockdown

Microinjection of *USF1* siRNA was performed to knockdown endogenous *USF1* in bovine embryos using our procedures published previously (Bettegowda *et al.* 2007). Two distinct siRNA species were designed against the open reading frame of bovine *USF1* mRNA using publicly available siRNA design algorithm (siRNA target finder; Ambion, Austin, TX, USA; species 1: sense 5'-AATTCCTCCCTGCAACACTTCCCTGTC-TC-3' and antisense 5'-AAGAAGTGTTCAGGGAGGAACCTGTCTC-3' and species 2: sense 5'-AACATCATTGTCCA-GCTGTAGCCTGTCTC-3' and antisense 5'-AACTACAGCTG-GACAATGATGCCTGTCTC-3'). All candidate siRNA were interrogated using the basic local alignment tool program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to rule out homology off targets to any other known genes in the bovine expressed sequence tag and genomic database. The *USF1* siRNA species were synthesized with the Silencer siRNA construction kit (Ambion) by following the manufacturer's protocol.

Microinjection of *USF1* siRNA into bovine embryos and subsequent early embryo culture experiments were conducted as described previously (Bettegowda *et al.* 2007). Individual *USF1* siRNA were validated for specificity and efficacy of *USF1* mRNA and protein knockdown in early embryos. Presumptive zygotes collected at 16–18 h after insemination (hpi) were microinjected with 50 μ M cocktail of *USF1* siRNA species (*USF1*-siRNA-S1 and S2) and four-cell embryos were collected at 42–44 hpi for real-time PCR analysis of *USF1* mRNA. Uninjected embryos and embryos injected with negative control siRNA (Universal control species 1; Ambion) were used as control groups ($n=4$ experimental replicates with ten embryos per group). Efficiency of *USF1* siRNA species in reducing USF1 protein was determined by USF1 immunostaining of eight-cell embryos collected 72 hpi ($n=10$ embryos for uninjected, negative control siRNA injected, and *USF1* siRNA species injected groups).

Effect of USF1 depletion on embryonic development

To determine the effect of *USF1* ablation on early embryonic development, the proportion of *USF1* siRNA-injected presumptive zygotes that cleaved 48 hpi, reached 8–16C (72 hpi)

Table 1 Primers used for qRT-PCR

Gene name	Accession number	Primer sequence (5' → 3')
<i>USF1</i>	NM_001001161	F: TGGCCGGAAGTCTAGCACTCA R: TCCGAGGAACTGGTCCTTCTT
<i>USF2</i>	NM_001001162	F: AAGGAGAGCTCAGCATAATGAAGTG R: GAAAGCTGGACGATCCAGTTG
<i>TWIST2</i>	NM_001083748	F: TCGCCGGTCCATCTCTCA R: AAGGTTGGTCTATGAACACTCTCA
<i>JY1</i>	NM_001110098	F: TTGGAAGTCCATGGACGACC R: ATTTGCTGGTGATCCCAAGAG
<i>GDF9</i>	NM_174681.2	F: GGAATCTGAGGCTGAGACTTGG R: GTGAAAAGCCTGAGCACTTGTG
<i>FST</i>	BF774514 F	F: CAGAGCTGCAAGTCCAGTACCA R: CATGTAGAGCTGCCTGGACAGA
<i>RPS18</i>	NM_001033614	F: GTGGTGTGAGGAAAGCAGACA R: TGATCACACGTTCCACCTCATC

and BL stage (7 days after insemination) were recorded and compared with uninjected and negative control siRNA injected embryos. Each experiment was replicated six times with 50 embryos per treatment.

Effect of *USF1* depletion on oocyte maturation, cumulus expansion, and abundance of specific oocyte-expressed transcripts

To determine the functional requirement of *USF1* for meiotic maturation, cumulus expansion, and knockdown effects on mRNA abundance for select E-box-containing genes, cumulus-enclosed GV-stage oocytes were microinjected with *USF1* siRNA cocktail and pre-incubated for 48 h in 50 μ M S-roscovitine-containing maturation medium to arrest meiotic maturation and allow sufficient time for *USF1* knockdown using our procedures published previously (Lee *et al.* 2014a). Then, COCs were washed thoroughly in maturation media to completely remove the S-roscovitine and subjected to meiotic maturation for 24 h as described earlier. Uninjected and negative control siRNA-injected COCs were also subjected to the same S-roscovitine treatment and used as control groups. After 24 h of *in vitro* maturation, effects of treatments on cumulus expansion and progression to MII were determined ($n=20$ oocytes/group; $n=4$ replicates) as we described previously (Lee *et al.* 2014a,b,c). Additional oocytes were stripped of cumulus cells and used for real-time quantification of transcripts for *USF1* and related bHLH transcription factors (*USF2* and *TWIST2*) and select genes linked to bovine oocyte competence (*JY1*, *GDF9*, and *FST*; $n=10$ oocytes/group; $n=4$ replicates).

Statistical analysis

Relative expression of mRNA was analyzed in SAS using a one-way ANOVA with differences in means detected by Fisher's protected least significant difference (PLSD). Embryo development data were calculated based on the total number of zygotes and was arc-sine transformed before analysis using a one-way ANOVA. A similar statistical analysis as for embryos was used for determination of effects of treatments on meiotic maturation and cumulus expansion. Differences between means were also detected using PLSD. All data are presented untransformed.

Results

Expression characterization of *USF1* mRNA and protein at different stages of oocyte and early embryonic development

Quantitative real-time PCR was performed to characterize temporal changes in abundance of *USF1* transcripts during meiotic maturation and early embryonic development. *USF1* mRNA abundance was quite stable during meiotic maturation and pronuclear (PN) stage, increased ($P<0.001$) at two-cell stage relative to the MII-stage, and then was considerably decreased (<0.007) after the eight-cell stage to barely detectable

levels at the BL stage (Fig. 1A). To determine the origin of the *USF1* transcripts (maternal vs zygotic) present in early embryos, zygotes were cultured in the presence of the transcriptional inhibitor α -amanitin (Fig. 1B) and mRNA abundance was analyzed by real-time PCR at eight-cell stage. The abundance of *USF1* transcripts was higher in α -amanitin-treated embryos compared with control eight-cell stage embryos, indicating that transcripts detected were maternal in origin and also suggests that embryonic transcription is necessary for normal degradation of *USF1* mRNAs after eight-cell stage of embryonic development.

The abundance and localization of *USF1* protein was investigated by immunocytochemical analysis (Fig. 2), which demonstrated increased *USF1* protein at MII stage relative to GV stage and minimally decreased *USF1* protein at zygote and two-cell stage of embryo development. The abundance of *USF1* protein increased by the four-cell stage and at eight-cell stage during early embryonic development. After the eight-cell stage, abundance of *USF1* protein slightly declined at 16-cell stage and then remained stable during the stages leading up to the BL stage.

USF1 knockdown effect on bovine early embryonic development

To determine the effect of *USF1* depletion on early embryonic development, siRNA-mediated gene silencing procedures were validated in bovine embryos. Microinjection of *USF1* siRNA cocktail at the zygote stage of embryo development significantly reduced ($P<0.05$) the abundance of *USF1* mRNA by $\approx 90\%$ in the resulting four-cell stage embryos relative to the uninjected and negative control siRNA-injected embryos as determined by real-time PCR (Fig. 3A). To further confirm the efficacy of *USF1* siRNA, *USF1*

Figure 1 Expression characterization of *USF1* mRNA during oocyte and early embryonic development. (A) Relative abundance of *USF1* transcript during meiotic maturation (GV and MII stage of oocytes) and at different developmental stages of early embryos (pronuclear (PN), two-cell (2C), four-cell (4C), eight-cell (8C), 16-cell (16C), MO, and blastocyst (BL) stage). (B) Relative amount of *USF1* mRNA in eight-cell embryos cultured in the presence of α -amanitin is significantly different from that of control embryos. Data were normalized relative to abundance of endogenous control (*RPS18*) and are expressed as mean $\pm$ S.E.M. Values with different superscripts indicate significant differences ($P<0.05$).

Figure 2 Spatiotemporal expression of USF1 protein during oocyte maturation and early embryonic development. Immunofluorescent localization of USF1 protein at different stages of bovine oocyte and preimplantation embryo development. Nuclear DNA was stained with 4',6-diamidino-2-phenylindole. Negative control embryos incubated in absence of USF1 antibody.

immunostaining was performed in resulting eight-cell stage embryos. A specific reduction in USF1 protein was observed compared with both uninjected embryos and negative siRNA control-injected embryos (Fig. 3B).

Effect of the *USF1* knockdown on early embryonic development was analyzed by microinjection of validated *USF1* siRNA cocktail into bovine zygotes and analysis of subsequent effects on cleavage rate, and proportion of embryos developing to eight-cell stage and BL stages compared with uninjected and negative control siRNA-injected embryos. *USF1* siRNA injection did not affect the total cleavage rate (Fig. 4A), but reduced the proportion of IVF embryos developing to the eight-cell stage relative to uninjected and negative control siRNA-injected embryos ($P < 0.001$; Fig. 4B). A more than two-fold reduction was observed in the proportion of IVF embryos developing to BL stage for *USF1* siRNA-injected embryos compared with uninjected and negative control siRNA-injected embryos ($P < 0.001$; Fig. 4C).

Effect of USF1 knockdown in GV stage oocytes on progression to MII, cumulus expansion, and transcript abundance for select oocyte-expressed genes during bovine oocyte maturation in vitro

Given the observed effect of *USF1* siRNA knockdown on early embryonic development, we investigated the functional role of USF1 transcription factor in meiotic maturation, cumulus expansion, and regulation of *TWIST2*, *JY1*, *GDF9*, and *FST* genes, carrying USF1-binding element (E-box) in their promoter region and/or essentially required for the developmental competence of bovine oocytes. Microinjection of *USF1* siRNA into GV-stage oocytes did not affect subsequent rates of meiotic maturation and cumulus expansion following

in vitro maturation (see Supplementary Table 1, see section on supplementary data given at the end of this article). However, real-time PCR analysis demonstrated that *USF1* siRNA injection resulted in a $> 95\%$ decrease in *USF1* transcript abundance following 48 h culture in S-roscovitine and the subsequent *in vitro* maturation ($P < 0.0001$; Fig. 5A). *TWIST2* and *JY1* mRNA abundance were increased in *USF1* siRNA-injected oocytes collected at MII stage of meiotic maturation compared with uninjected and negative siRNA control-injected oocytes ($P < 0.0003$; Fig. 5B and C respectively). By contrast, *GDF9* and *FST* transcript abundance were reduced in *USF1* siRNA-injected oocytes relative to uninjected and negative control siRNA-injected oocytes collected at MII stage ($P < 0.0001$; Fig. 5D and $P < 0.003$; Fig. 5E respectively). Negative control siRNA injection resulted in a modest decrease in *GDF9* transcript abundance compared with uninjected group, suggesting a modest off-target effect of negative control siRNA on *GDF9* expression, but *GDF9* transcript abundance in *USF1* siRNA-injected oocytes was lower than both control groups (uninjected and negative control siRNA injected). These results suggest that USF1 potentially functions as a regulator of *GDF9*, *FST*, *TWIST2*, and *JY1* transcript abundance during oocyte maturation. However, abundance of *USF2* mRNA was not altered in response to *USF1* siRNA injection (Fig. 5F).

Temporal regulation of mRNA for USF2 and TWIST2 transcription factors during oocyte maturation and early embryonic development: Potential link to USF1 action

Temporal changes in *USF2* and *TWIST2* transcript abundance were established and compared with results obtained for *USF1* to determine potential association of

Figure 3 Effect of zygote *USF1* siRNA microinjection on *USF1* mRNA and protein level in resulting four- and eight-cell embryos respectively. (A) Effect of siRNA microinjection at the pronuclear stage of embryo on abundance of *USF1* transcript in resulting four-cell embryos (ten embryos per group, four replicates). Data were normalized relative to abundance of endogenous control *RPS18* and are expressed as mean $\pm$ S.E.M. Values with different letters indicate significant difference ($P < 0.05$). (B) Effect of RNAi-mediated depletion of *USF1* in bovine zygotes on *USF1* protein level in resulting eight-cell embryos as determined by immunofluorescent staining ($n = 4$). Uninjected embryos and embryos injected with a nonspecific siRNA (negative control siRNA) served as controls. Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI).

these transcription factors (Garcia-Sanz *et al.* 2013) in transcriptional regulation of *JY1*, *GDF9*, and other genes containing multiple E-box-binding elements in their promoter regions. In comparison with *USF1*, *USF2* transcript abundance displayed similar changes during oocyte and early embryonic development (Figs 1A and 6A respectively). On the other hand, temporal changes in *TWIST2* mRNA abundance were distinct from those observed for *USF1* and *USF2* (Fig. 6B). Abundance of *TWIST2* mRNA was elevated during meiotic maturation ($P < 0.0001$), decreased ($P < 0.0005$) at the pronuclear and two-cell stages relative to the MII stage, and then significantly increased ($P < 0.05$) by four-cell stage. After four-cell stage, the abundance of *TWIST2* transcripts progressively decreased through the 16C stage and was barely detectable at MO and BL stages.

Discussion

Results of this study established temporal expression of *USF1* mRNA and protein during bovine oocyte maturation and early embryogenesis and a functional requirement of this bovine oocyte-expressed *USF1* transcription factor for early embryogenesis and regulation of genes involved in oocyte competence and early embryonic development. However, results suggest that *USF1* knockdown in GV-stage oocytes does not affect meiotic maturation and cumulus expansion. To our knowledge, a functional role for *USF1* in early embryogenesis and potential regulation of genes linked to oocyte competence has not been reported previously.

USF1 is a member of the bHLH leucine zipper family, which has been implicated in tissue-specific transcriptional regulation of a wide range of genes (Littlewood & Evan 1998). Most commonly, this transcription factor binds to a pyrimidine-rich initiator (Inr) element or E-box motifs of the promoter region as homodimers or a heterodimer (*USF1/USF2*) and might act with or without association with other E-box-binding transcription factors of bHLH family (Garcia-Sanz *et al.* 2013). The *USF1/USF2* heterodimer is a major DNA-binding form present in most tissues and cell types compared with the homodimers (Fair *et al.* 1995, Rajput *et al.* 2013). In our experiments, we observed similar temporal changes in transcript abundance for *USF1* and *USF2* in bovine oocytes and early embryos, suggesting a potential functional role of *USF1/USF2* heterodimer in transcriptional regulation during oocyte and early embryogenesis. However, establishment of *USF1/USF2* protein–protein interactions and a functional role of *USF2* in bovine oocytes and early embryos will require further investigation. *TWIST2* transcription factor also functions

Figure 4 Requirement of *USF1* for bovine early embryonic development. Presumptive zygotes were microinjected (16–18 h after fertilization) with either *USF1* siRNA (cocktail) or universal negative control siRNA (negative control siRNA) species 1 (Ambion, Austin, TX, USA) or served as uninjected controls. Presumptive zygotes were then cultured in KSOM medium supplemented with 0.3% BSA (50 presumptive zygotes/group, six replicates). The 8–16-cell stage embryos were separated 72 h after insemination (hpi) and cultured in fresh KSOM medium supplemented with 0.3% BSA and 10% FBS until day 7. Effect of *USF1* depletion on (A) total cleavage rate (determined 48 hpi), (B) proportion of embryos developing to the 8–16-cell stage (72 hpi), and (C) proportion of embryos developing to the blastocyst stage (day 7). Data are expressed as mean $\pm$ S.E.M. Values with different superscripts across treatments indicate significant differences ($P < 0.001$).

Figure 5 *USF1* siRNA injection in GV-stage oocytes affects the mRNA abundance of specific oocyte-expressed genes in resulting MII stage oocytes following *in vitro* maturation. Samples of cDNA collected from MII-stage oocytes were subjected to real-time RT-PCR analysis for quantification of *USF1* (A), *TWIST2* (B), *JY1* (C), *GDF9* (D), *FST* (E), and *USF2* (F) mRNA. Data were normalized relative to abundance of endogenous control *RPS18* and are expressed as mean $\pm$ S.E.M. Values with different letters indicate statistical difference ($P < 0.0001$).

as a molecular switch to either activate or repress target genes via direct binding to conserved E-box sequences in the promoter region and recruitment of co-activator(s) or repressor(s) (Franco *et al.* 2011). High abundance of *TWIST2* transcript at MII stage in oocytes and four to eight-cell embryonic stage suggests a potential regulatory role in control of genes associated with maturation and initial cleavage divisions during early embryonic development. Interestingly, many of the mammalian target genes for *TWIST2* includes cell adhesion molecules (E-cadherin and fibronectin) and transcription factors (β -catenin (*CTNNB1*) and *STAT3*; Fang *et al.* 2011), genes involved in sperm and oocyte fusion (Takezawa *et al.* 2011), cleavage, compaction, and formation of a implantation competent embryo (Bloor *et al.* 2002).

The observed significant reduction in both *USF1* and *USF2* transcripts immediately after embryonic genome activation (occurs at eight-cell stage in bovine) suggests a maternal origin of these transcripts with potential depletion or degradation upon embryonic genome activation. Increased abundance of *USF1* transcripts in transcriptional inhibitor (α -amanitin)-treated embryos at eight-cell stage (Fig. 1B) further confirmed their maternal origin and that *USF1* transcript depletion is dependent on zygotic degradation machinery activated with the onset of embryonic genome activation. A large number of maternal transcripts are degraded by zygotic decay machinery during early embryonic development in *Drosophila* and other bilateria including zebrafish, frogs, and mice (Tadros & Lipshitz 2009, Thomsen *et al.* 2010). We have observed a similar transcription-dependent

degradation process of maternal transcripts for TGF β superfamily signaling pathway components in bovine embryos coincident with genome activation (Lee *et al.* 2014b). Results suggest that timely depletion of maternal transcription factors and signaling proteins is associated with the progression of embryo development.

We investigated the functional role of *USF1* during meiotic maturation and early embryonic development using an effective and previously optimized siRNA-based gene knockdown approach for oocytes and embryos (Bettegowda *et al.* 2007, Lee *et al.* 2014c). siRNA-based knockdown of *USF1* transcripts in fertilized embryos resulted in effective reduction in proportion of embryos reaching the eight-cell stage and the BL stage. However, no effect was observed on total cleavage rates, which suggest that *USF1* transcription factor is potentially required for later stages of embryogenesis after initial cleavage division. Results suggest that *USF1* is potentially required for development events at or after the eight-cell stage including embryonic genome activation. Moreover, *USF1* contains a nuclear localization signal that directs its transport to the nucleus to regulate a broad spectrum of targeted gene transcription (McMurray & McCance 2003, Crusselle-Davis *et al.* 2006, Anantharaman *et al.* 2011). The transcriptional regulation through *USF1* has previously been shown to be associated with its nuclear abundance in various cellular models (Zhang *et al.* 2007, van Deursen *et al.* 2008, Viswanathan *et al.* 2009). Therefore, it is likely that increased abundance of *USF1* protein in eight-cell stage embryos (Fig. 2) coincident with embryonic genome activation is of potential functional importance in the transcriptional activity of the embryonic genome.

Results of this study provide support for a functional role of *USF1* transcription factors in regulation of multiple genes linked to oocyte competence in cattle. In bovine, *JY1* and *GDF9* are widely studied oocyte-specific genes, which contain multiple E-boxes in their regulatory region and play an important role in oocyte

Figure 6 Expression characterization of *USF2* and *TWIST2* mRNA during oocyte and early embryonic development. Real-time PCR analysis for quantification of *USF2* (A) and *TWIST2* (B) mRNA during meiotic maturation (GV and MI stage of oocytes) and at different developmental stages of early embryos (pronuclear (PN), two-cell (2C), four-cell (4C), eight-cell (8C), 16-cell (16C), MO, and blastocyst (BL) stage). Transcript levels were normalized relative to abundance of endogenous control (*RPS18*) RNA and are expressed as mean $\pm$ S.E.M. Time points without a common letter (a, b, c, d, and e) are significantly different ($P < 0.05$).

competence for successful early embryonic development (Yan *et al.* 2006, Bettgowda *et al.* 2007, Roy *et al.* 2013, Lee *et al.* 2014a). Our results demonstrated that siRNA-mediated depletion of *USF1* in GV-stage oocytes significantly reduced the transcript abundance for *GDF9* gene at MII stage, which suggests a positive role in regulation of *GDF9* mRNA abundance and presumably transcription. NOBOX and E-box motifs are the critical regulatory sequences, found in the *GDF9* promoter, responsible for its oocyte specific expression in mammals (Rajkovic *et al.* 2004, Yan *et al.* 2006). Functional analysis of promoter mutations has established an essential E-box regulatory sequence (CAGCTG) at -182 to -177 bp upstream of the *Gdf9* initiation codon in mice, which was found to be conserved across the 16 mammalian species including cattle (Bettgowda *et al.* 2007). Interestingly, it has been demonstrated that *USF1* transcription factor can bind to E-box motif (CAGCTG) to regulate tissue-specific transcription in mice (Fang *et al.* 2011).

Results support potential *USF1*-dependent regulation of *GDF9* transcription in bovine oocytes, but further studies are needed to conclusively prove transcriptional regulation. In cattle, oocytes in the growth phase display the greatest transcriptional activity (Fair *et al.* 1995), but direct evidence of transcriptional activity during meiotic maturation is limited. Array analysis of the bovine oocyte transcriptome revealed increased abundance of ~10% of the mRNAs during meiotic maturation (Dalbies-Tran & Mermillod 2003). This increase in mRNA abundance was potentially attributed to transcriptional activity at the beginning of meiotic maturation of GV oocytes aspirated from >3 mm antral follicles and cultured *in vitro* (Memili *et al.* 1998, Hendriksen *et al.* 2000, Tomek *et al.* 2002). Moreover, 24 h incubation in the presence of CDK inhibitors to maintain meiotic arrest resulted in increased content of poly(A) mRNAs in bovine oocyte (Lequarre *et al.* 2004). In these studies, we incubated *USF1* siRNA-injected oocytes in the presence of the CDK inhibitor roscovitine for 48 h to allow time for siRNA-mediated depletion of *USF1* before meiotic maturation. Therefore, it is possible that *USF1* transcription factor knockdown reduced *GDF9* expression during meiotic maturation *in vitro* through interaction with E-box regulatory elements present in the *GDF9* promoter. However, further studies are required to conclusively establish *USF1*-mediated transcriptional regulation of the *GDF9* gene in bovine oocytes.

Results from our previous studies have demonstrated an important functional requirement of the bovine oocyte-specific *JY1* gene for meiotic maturation and early embryogenesis (Bettgowda *et al.* 2007). However, no effect of *USF1* knockdown on meiotic maturation and cumulus expansion was observed in the current studies. Depletion of *JY1* transcripts in zygote-stage embryos results in considerably reduced rates of development to the 8–16-cell stage and BL compared with controls.

Interestingly, five putative E-box motifs have been identified within 500 bp upstream of the *JY1* TSS (Bettgowda *et al.* 2007). Out of the five putative E-box motifs, three have CAGCTG E-box motif and the one E-box adjoining TSS has a CACCTG motif, a well-known *USF* transcription factor-binding sequence. In this study, siRNA-based knockdown of *USF1* in GV-stage oocytes resulted in a significant increase in *JY1* transcripts in MII-stage oocytes, which suggests a potential negative regulatory role for *USF1* in modulation of *JY1* transcription. Similar to other bHLH proteins, *USF1* function in both transcriptional activation and repression has been previously characterized and activity (activation vs repression) is dependent upon specific binding partner in various cellular models (McMurray & McCance 2003, Crusselle-Davis *et al.* 2006, Anantharaman *et al.* 2011). The presence of multiple E-boxes at 5' flanking region of the *JY1* promoter suggests the binding of more than one bHLH transcription factor (*BHLHE41*, *TWIST*, and *USF2*) which might be working in association with *USF1* to repress *JY1* transcription in oocytes.

Additionally, we have also analyzed the effect of *USF1* knockdown on abundance of other transcripts linked to bovine oocyte and embryo competence (*FST*) and potential *USF1*-binding partners (*USF2* and *TWIST2*) in *USF1* siRNA-injected oocyte collected at MII stage. *FST* has been previously characterized as an important maternal (oocyte-derived) factor required for early embryo cleavage, BL development, and functional determinant of BL cell allocation (Rajput *et al.* 2013). Reduced expression of *FST* in *USF1*-depleted oocytes potentially supports the observed functional role of *USF1* TF in early embryonic development via regulating the transcription of oocyte-expressed genes. Furthermore, siRNA-mediated depletion of *USF1* enhanced the mRNA abundance for *TWIST2*, whereas abundance of *USF2* mRNA remained unchanged in MII-stage oocytes. In support of *USF1* knockdown effects reported, *in silico* analysis revealed five different E-boxes within 1.3 kb upstream of the *TWIST2* mRNA TSS and two of them (CACCTG and CAGATG) were found to be highly conserved among mammals (unpublished). Moreover, two putative E-boxes were identified within the 5'-UTR region of the *FST* gene and one (CACCTG) showed high level of conservation, whereas E-box motifs were not detected upstream of the *USF2* TSS (Sandeep K Rajput and George W Smith, unpublished observations). Collectively, these results suggest that *USF1* TF potentially mediates E-box-dependent positive regulation of *GDF9* and *FST*, and negative regulation of *TWIST2* and *JY1* transcription.

In conclusion, results of this study provide novel information about the spatiotemporal expression pattern of *USF1*, *USF2*, and *TWIST* transcription factors during oocyte and early embryonic development. Results establish a functional requirement of *USF1* transcription factor in embryo development to the BL stage and

suggest an important regulatory role of USF1 transcription factor in modulating the transcription of *GDF9*, *FST*, *TWIST2*, and *JY1* genes in bovine oocytes. Moreover, siRNA-mediated depletion of USF1 transcription factor resulted in a similar adverse effect on early embryonic development as observed upon *JY1* and *FST* knockdown in fertilized embryos. Further mutation analysis of the *GDF9*, *FST*, *TWIST2*, and *JY1* promoter containing regulatory E-boxes and their interaction with USF1 and other associated bHLH transcription factors will provide further insight into precise molecular mechanisms involved in oocyte/embryo transcriptional regulation in cattle.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0445>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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