Conserved roles of fibroblast growth factor receptor 2 signaling in the regulation of inner cell mass development in bovine blastocysts

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Running title: Roles of FGFR2 signaling in bovine blastocysts

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The abbreviations used are:

FGF[R], fibroblast growth factor [receptor]; GATA6, GATA binding protein 6; HNF4A, hepatocyte nuclear factor 4, alpha; ICM, inner cell mass; KD, knockdown; NANOG, Nanog homeobox; TE,

trophectoderm; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

ABSTRACT

A common process during preimplantation mammalian development is blastocyst formation, which utilizes signaling through fibroblast growth factor receptor 2 (FGFR2), yet the mechanisms through which FGFR2 signaling affect preimplantation development in bovine embryos remain incompletely understood. Here, we used RNA-interference to investigate the in vitro development, the frequency of blastomere apoptosis, and the mRNA expression of developmental marker genes in FGF receptor 2-knockdown (*FGFR2*-KD) bovine embryos. A reduction in *FGFR2* mRNA did not affect preimplantation development or the frequency of apoptotic blastomeres, but did enhanced proliferation of the inner cell mass in blastocysts ($P<0.05$) – which differs from the phenotype reported for bovine embryos using a pharmacological approach (treatment with the pan-FGFR blocker PD173074), but agrees with previous results obtained using mouse embryos. Moreover, the expression of an epiblast marker gene, *NANOG*, and a primitive endoderm marker gene, *GATA6*, remained unchanged, whereas the expression of another primitive endoderm marker gene, *HNF4A*, was significantly reduced in *FGFR2*-KD embryos. Therefore, FGFR2 signaling appears to be associated with the regulation of inner cell mass development and proliferation during blastocyst formation in cattle.

INTRODUCTION

Mammalian zygotes undergo cleavage, a series of mitotic divisions without an increase in cytoplasmic mass, soon after syngamy in the ootid stage. Cleavage of these blastomeres continues within the zona pellucida, eventually forming the morula, a spherical body whose individual blastomeres are morphologically unrecognizable. The first major developmental transition occurs beyond the morula stage. An active sodium pump in the outer layer of cells results in the accumulation of sodium ions within the embryo, resulting in fluid accumulation in the core of the morula that eventually gives rise to the fluid-filled cavity called the blastocoele; an embryo with recognizable blastocoele is a blastocyst.

The first cell-fate decisions are specified in blastocysts – namely, distinction of the surrounding trophectoderm (TE) from the inner cell mass (ICM). The TE contributes to the embryonic portion of the placenta, whereas the ICM further segregates into the epiblast and the primitive endoderm. The epiblast contributes to the embryo proper, the amnion, and the umbilical cord, whereas the primitive endoderm gives rise to the parietal and visceral endoderm of the yolk sac (Gardner 1982; Gardner 1984; Rossant and Croy 1985; Kwon et al. 2008). These hierarchical cell-fate decisions are regulated by key lineage-specific transcription factors (Nichols et al. 1998; Mitsui et al. 2003; Strumpf et al. 2005; Chazaud et al. 2006; Winger et al. 2006; Yoshikawa et al. 2006; Ralston et al. 2010). Therefore, by controlling key lineage-specific transcription factors – using inhibitors, cytokines, and growth factors – we may regulate the state of pluripotency and elucidate how specific genes direct cellular differentiation during blastocyst formation in cattle (Yang et al. 2011; Furusawa et al. 2013; Ozawa et al. 2013; McLean et al. 2014). Such knowledge would further improve embryo culture systems as well as facilitate the engineering of mammalian embryos, including embryos of farm animals.

Establishment of embryonic and extraembryonic cell lineages typically involves dual cell

characterizations (Kuijk et al. 2008; Kang et al. 2013; Bessonnard et al. 2014). The TE and ICM are specified first, followed by division of the ICM between the epiblast and primitive endoderm cell lineages. In mice, key transcription factors such as NANOG and GATA6 are expressed in most ICM cells, but this expression pattern changes on a per-cell basis as development progresses: Whereas ICM cells principally expressing *NANOG*, *SOX2*, and *FGF4* go on to form the epiblast, those cells that primarily express *FGFR2* (FGF receptor 2), *GATA4*, and *GATA6* (GATA-binding protein 4 and 6) develop into the primitive endoderm. A similar hierarchy occurs in cattle, with the exception of *GATA4* expression (Kuijk et al. 2008; Kuijk et al. 2012). Such mosaicism among ICM cells expressing either epiblast or primitive endoderm markers is referred to as a "salt-and-pepper" distribution pattern (Kuijk et al. 2008; Kang et al. 2013), which is mediated by FGF signaling in cattle (Kuijk et al. 2012).

The plasticity of lineage-dependent signaling is reflected by the ability to induce different cell fates from embryonic stem cells – even from deviation-refractory strains and animals. Indeed, researchers have applied defined conditions, known as 3i or 2i, that combine inhibitors of FGF/ERK signaling and glycogen synthase kinase-3 to enrich for specific lineages (Furusawa et al. 2013; McLean et al. 2014). In cattle, for example, preparing cultures in 2i medium improves blastocyst quality in terms of ICM cell number as well as enhancing epiblast-specific gene expression (McLean et al. 2014). Nevertheless, the role of FGF signaling during the differentiation of bovine ICM cells remains controversial because PD173074, a pan-FGFR kinase inhibitor, does not mimic the effect of 2i on ICM cell numbers that is reported for mouse embryos (Kuijk et al. 2012; McLean et al. 2014). On the other hand, FGF4 supplementation with heparin into the culture medium of bovine blastocysts resulted in an increase in the ICM cell number (Kuijk et al. 2012; Krawchuk et al. 2013).

PD173074, a synthetic compound of the pyrido [2,3-d] pyrimidine class, is a highly effective tool for investigating the role of FGF signaling in the context of other signaling pathways

(Mohammadi et al. 1998). This inhibitor exhibits both high affinity and selectivity for the FGFR family, and was used to block tumor growth in several studies (Kunstlinger et al. 2015; Saito et al. 2015), although the effects of PD173074 were also correlated with vascular endothelial growth factor receptor 2 (VEGFR2) autophosphorylation (Mohammadi et al. 1998; Buchler et al. 2007), which increases the difficulty of deciphering how FGF signaling affects bovine blastocyst development.

Cattle utilize 23 FGF family growth factors (Zimin et al. 2009) that play roles in numerous cellular processes, including cell proliferation, differentiation, and survival (Li et al. 2009; Jiang and Price 2012; Zhang and Ealy 2012; Laird et al. 2013). These signaling peptides mediate their cellular responses by binding to and activating any of 4 receptor tyrosine kinases, FGFR1–4 (Bottcher and Niehrs 2005). The various combinations of FGF ligand-receptor interactions can result in a multitude of biological responses, including the differentiation of ICM cells into primitive endoderm versus epiblast.

How the same ligands can generate such a diversity of biological responses during preimplantation development remains incompletely understood. FGFR2 appears to play the most critical roles among the various receptors because it interacts with key ligands employed during bovine preimplantation development, such as FGF2, -4, -7, and -10 (Rubin et al. 1989; Igarashi et al. 1998). FGF2 and 4 are critical for ICM differentiation, and FGF7 is associated with the differentiation and proliferation of trophoblasts in bovine embryos (Pfarrer et al. 2006). Additionally, FGF10-supplemented culture medium improves embryonic development to the blastocyst stage (Pomini Pinto et al. 2015). Therefore, we focused on signal transduction through FGFR2 in in vitro-fertilized bovine embryos, using *FGFR2* knockdown (KD), as in our previous studies (Nagatomo et al. 2015a; Nagatomo et al. 2015b), to address how this critical receptor influences preimplantation development. In contrast to what was observed using PD173074 treatment, our

FGFR2-KD embryos showed a marked increase in ICM cell number together with a downregulation of *HNF4A*, a primitive endoderm-specific gene. Our results therefore demonstrate that, as in mice, FGF signaling through FGFR2 modulates ICM development of bovine blastocysts, and imply that the sensitivity to PD173074 differs between mouse and bovine embryos. Our study further confirms that the ICM differentiation mechanisms regulated by FGF signaling are highly conserved in the bovine embryo, and thus provide evidence for species divergence in pharmacological responses.

RESULTS

Effect of FGF signaling on developmental rate of preimplantation bovine embryos

An RNA inhibition experiment in which *FGFR2* short hairpin RNA (shRNA) expression vectors were injected into in vitro-fertilized embryos was performed to examine the role of *FGFR2* expression during early bovine embryogenesis. The relative *FGFR2* mRNA abundance was significantly lower in *FGFR2*-KD blastocysts compared to controls injected with empty vector, which lacked the *FGFR2* shRNA insert ($P<0.05$) (Fig. 1A).

FGFR2 protein was detectable in both the ICM and TE of bovine blastocysts, albeit the fluorescent intensity in the TE was weaker. An obvious reduction of FGFR2 protein was observed in *FGFR2*-KD embryos at the blastocyst stage, particularly in the ICM (Fig. 1B).

The rates of cleavage and blastocyst formation on Days 2 and 8, respectively, did not differ between embryo groups (Fig. 1C). Indeed, 49.3 ± 6.3% versus 46.5 ± 7.1% of the control versus *FGFR2*-KD embryos cleaved and 19.9 ± 2.1% versus 15.8 ± 2.3% of the control versus *FGFR2*-KD embryos reached the blastocyst stage.

Effect of FGFR2 KD on apoptosis in bovine blastocysts

FGF signaling is involved in apoptosis in human endothelial and cancer cells (Katoh and

Nakagama 2014), so we asked if reduction in signaling would affect blastomere survival. The degree of apoptosis in blastocysts obtained from *FGFR2*-KD embryos was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) (Fig. 2A). The number of TUNEL-positive blastomeres relative to the total cell number did not differ between *FGFR2*-KD and control blastocysts (Fig. 2B).

Effect of FGF signaling on bovine blastocyst cell number and allocation to the ICM and TE

We previously observed ICM-enriched expression of *FGFR2* mRNA (Nagatomo et al. 2013), so we evaluated ICM and TE cell numbers in *FGFR2*-KD blastocysts using a detergent-based differential staining method (Thouas et al. 2001). The proportion of cells in the ICM versus the TE cells was significantly higher in *FGFR2*-KD blastocysts than in control blastocysts (Fig. 3A). Examination of the quantity of cells in individual blastocysts revealed that both ICM and total cell numbers increased in *FGFR2*-KD blastocysts ($P<0.05$), whereas cells of the TE did not differ between blastocyst groups (Fig. 3B).

Expression of epiblast and primitive endoderm marker genes in FGFR2-KD blastocysts

We next examined the expression of the epiblast and primitive endoderm marker genes *NANOG*, *GATA6*, and *HNF4A* in bovine blastocysts (Kuijk et al. 2012; Nagatomo et al. 2013). Although the quantity of ICM cells in *FGFR2*-KD blastocysts was higher than in controls, *NANOG* and *GATA6* mRNA abundance did not differ; by contrast, the *HNF4A* mRNA abundance was significantly decreased in the *FGFR2*-KD blastocysts ($P<0.05$) (Fig. 4).

DISCUSSION

This work provides direct evidence that FGFR2 signaling regulates ICM development and

proliferation during blastocyst formation in cattle. A reduction in *FGFR2* mRNA did not significantly impair preimplantation development or affect the frequency of apoptotic blastomeres, although it markedly enhanced the proliferation of the ICM in bovine blastocysts. This phenotype differs from previous studies in bovine conducted using a pharmacological approach – e.g. with FGFR blockers such as PD173074 – but agrees with previous results obtained using mouse embryos (Nichols et al. 2009). The expression of the epiblast marker gene *NANOG* and the primitive endoderm marker gene *GATA6* remained unchanged, whereas the abundance of the primitive endoderm marker gene *HNF4A* was significantly decreased in *FGFR2*-KD embryos (Fig. 4A).

The pluripotency transcriptome of epiblast, primitive endoderm, and TE lineages has been precisely analyzed for mice using diverse genetic approaches (reviewed in (Hermitte and Chazaud 2014). Regulation of the cell-fate decision in mouse embryos is thought to be similar to that in other mammals. The key differentiation factors expressed exclusively within murine ICM cells were identified as: *Nanog*, *Sox2*, and *Fgf4* for the epiblast; *Gata6*, *Hnf4a*, and *Fgfr2* for the primitive endoderm; and *Cdx2*, *Eomes*, and *Elf5* for the trophectoderm. Following the first segregation that defines the ICM and TE, a subset of ICM cells further differentiates into the epiblast and primitive endoderm during the second segregation event. Blastomeres expressing either epiblast or primitive endoderm markers are then sorted into adjacent layers (Guo et al. 2010). Regarding FGF signaling, expression of the epiblast marker gene *Fgf4* was not detected in *Nanog*-mutant mouse embryos (Frankenberg et al. 2010), thus *Fgf4* expression is considered to be a downstream target of NANOG. *Fgf4*-mutant mouse embryos lose their expression of primitive endoderm marker genes (Krawchuk et al. 2013), and blocking FGF signaling using specific small-molecule inhibitors results in fewer primitive endoderm cells and the absence of *Nanog*-expressing ICM cells (Nichols et al. 2009; Krawchuk et al. 2013). Collectively, these findings indicate that FGF signaling plays critical roles in ICM cell development, including primitive endoderm cell emergence, in the mouse.

The aforementioned developmental mechanisms that establish the epiblast and primitive endoderm lineages through FGF signaling appear to be mostly conserved in cattle (Kuijk et al. 2012). Both *NANOG* and *GATA6* are expressed in the ICM in the early bovine blastocyst, but this is followed by an exclusive expression pattern as development progresses. We previously observed the restriction of *FGFR2*, *FGF4*, *FGF2*, and *SOX2* expression to the ICM, and detected *HNF4A* expression in the primitive endoderm along the blastocoel surface of the ICM (Nagatomo et al. 2013) – which are consistent with those reported in the mouse embryo.

In this study, *FGFR2* mRNA abundance was approximately 40% lower in *FGFR2*-KD embryos than that in controls (Fig. 1A). This expression level is comparable to that reported for *Fgfr2* heterozygous, but not homozygous, mutant mouse embryos (Arman et al. 1998). Primitive endoderm formation and even subsequent post-implantation development were normal for *Fgfr2* heterozygous mouse embryos (Arman et al. 1998). Similarly, we did not observe any negative effects on *FGFR2*-KD pre-implantation development until the blastocyst stage. On the other hand, chemical inhibition of FGF signaling using PD173074 in bovine embryos did not affect embryo development, the number or allocations of nuclei in the ICM and TE, trophoblast gene expression, or epiblast/primitive endoderm protein expression at the blastocyst stage (Kuijk et al. 2012; Ozawa et al. 2013; McLean et al. 2014), whereas PD173074 alone markedly increased the ICM cell number in mouse blastocysts (Nichols et al. 2009). Yet, supplementation of both FGF4 and heparin into culture medium stimulated the emergence of primitive endoderm cells without affecting ICM cell number in bovine embryos, indicating that FGF signaling is active (Kuijk et al. 2012). Therefore, intrinsic differences exist between mouse and bovine embryos in regards to the signaling pathways involved in cell-lineage specification at the blastocyst stage. The underlying reason for this discrepancy between bovine and murine embryos remains unknown.

The different phenotypes associated with *FGFR2* KD versus pharmacological inhibition

using PD173074 might arise from the structural properties of the bovine FGFR2 protein. FGFRs are highly conserved at the amino acid sequence level among mammals (Miki et al. 1992): a comparison of the amino acid sequences of full-length FGFR2 from mouse (NCBI accession: NP_034337.2) and cattle (NCBI accession: NP_001192239.1) reveals >99% amino acid identity. Yet three amino acid differences exist within the tyrosine kinase catalytic domain – the 308-amino-acid-long site that is bound by PD173074 (Mohammadi et al. 1998). Although the mechanisms by which PD173074 influences FGF signaling in cultured bovine cells, including preimplantation embryos, has not been analyzed as comprehensively as in mice, this structural difference in bovine FGFR2 might underlie the observed difference in sensitivity to PD173074 between cattle and mouse.

The abundance of the primitive endoderm marker *HNF4A*, but not *GATA6*, was reduced in the *FGFR2*-KD embryos. This phenotype may have resulted from differences in the downstream response initiated by FGFR2 signaling. In human hepatocytes, pharmacological inhibition of FGF signaling significantly reduces transcription of *HNF4A,* but not *GATA6* (Twaroski et al. 2015), suggesting that *HNF4A* expression could be regulated by a mechanism independent of that regulating *GATA6* mRNA expression. Such a differential regulatory mechanism through FGFR2 might be conserved in bovine early embryos.

In summary, our results provide new insights into the functions of FGF signaling during early embryo development in cattle. The contributions of FGFR2 signaling to bovine blastocyst formation have remained disputed because of phenotypic differences observed between cattle and mice using the pharmacological inhibitor PD173074. The results of our *FGFR2*-KD approach conclusively indicate that FGF signaling is needed for normal ICM development of bovine blastocysts, which agrees with the developmental machinery described in mouse embryos. Indeed, *FGFR2* KD increased ICM cell numbers and a down-regulated the expression of the primitive endoderm gene *HNF4A*. Although such species-specific signaling differences in the molecular

wiring that governs cell differentiation might make it more challenging to derive bovine stem cells from blastocysts, clarification of the underlying differentiation mechanisms during blastocyst development should facilitate the development of alternative methods for embryonic engineering of livestock.

MATERIALS and METHODS

Preparation of in vitro fertilized embryos

Bovine embryos were prepared using in vitro fertilization, as previously described (Aono et al. 2013; Nagatomo et al. 2013). Briefly, cumulus-oocyte complexes (COCs) were aspirated from 3-to-8-mm follicles in ovaries retrieved from a slaughterhouse. COCs, including intact cumulus cells, were cultured at 38.5˚C in a humidified atmosphere of 5% CO_2 and air for 20–22 h in liquid-paraffin-covered, 100-μL droplets of TCM199 (Gibco, Grand Island, NY) containing 10 μM cysteamine (Sigma-Aldrich, St. Louis, MO), 10% (v/v) fetal bovine serum (PAA Laboratories, Pasching, Austria), 0.5 mg/mL follicle-stimulating hormone (Kyoritsu Seiyaku Corp., Tokyo, Japan), 100 U/mL penicillin (Nacalai Tesque, Inc., Kyoto, Japan), and 100 U/mL streptomycin (Nacalai Tesque, Inc.). Oocytes were then transferred to Brackett and Oliphant (BO) medium (Brackett and Oliphant 1975) containing 2.5 mM theophylline (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Freeze-thawed semen was centrifuged at 526*g* for 7 min in BO medium, and the spermatozoa were added to the COCs at a final concentration of 7.5×10^6 cells/mL. After 12 h of incubation, presumptive in vitro-fertilized zygotes were denuded by pipetting, and cultured at 38.5˚C in a humidified atmosphere of 5% CO_2 using synthetic oviduct fluid (SOF) medium supplemented with 10 μg/mL insulin (Sigma-Aldrich), 1 mg/mL polyvinyl alcohol (Sigma-Aldrich), and 10 μM cysteamine. Embryos were used for microinjection immediately after denuding. The rates of cleavage and blastocyst formation were assessed on Days 2 and 8 of in vitro culture, respectively.

Microinjection of FGFR2-shRNA expression vectors into bovine in vitro-fertilized embryos

An short hairpin RNA (shRNA) containing antisense/sense regions, an 11-bp loop (5'-GTGTGCTGTCC), and a 6-bp terminator element (5'-TTTTTT) was designed to target nucleotides 924–943 of *FGFR2* mRNA (NCBI Reference Sequence NM_001205310.1). The top and bottom strand oligonucleotides (Sigma-Aldrich) were denatured at 95˚C for 5 min, and then gradually cooled to 25˚C for annealing. The double-strand DNA was ligated downstream of the *U6* promoter in the pBAsi/mU6 Neo vector (Stratagene, La Jolla, CA). The following sequence was used: 5′- GATCCGAGCCTTATTATGGAAAGTGTGTGTGCTGTCCACACTTTCCATAATAAGG CTCTTTTTTA-3′ (the underlined and double-underlined sequences indicate the stem-loop region and terminator element, respectively). The *FGFR2* mRNA-targeting shRNA (*FGFR2* shRNA) expression vector (pBAsi/mU6/FGFR2) was prepared using EZgene EndoFree Plasmid Miniprep Kit II (Biomiga, Inc., San Diego, CA). pBAsi/mU6 Neo plasmid lacking the *FGFR2* shRNA insert (empty vector) (Andey et al. 2014; Nelson et al. 2014; Li et al. 2015) was used as a control for embryo injection.

Twelve hours after insemination, synthesized *FGFR2* shRNA or empty-vector control DNA (diluted to a final concentration of 10 ng/μL with SOF medium) was injected into the cytoplasm of denuded zygotes using a FemtoJet injection device (Eppendorf, Hamburg, Germany). These zygotes were cultured in order to examine the effect of *FGFR2* KD on subsequent embryonic development to the blastocyst stage. Blastocysts were harvested and used for quantitative reveres-transcription PCR.

Quantitative reverse-transcription PCR

Total RNA from 5 blastocysts per biological replicate was isolated using ReliaPrep

(Promega, Madison, WI), according to the manufacturer's instructions. After RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), the quantity of extracted RNA among the samples was standardized at a concentration equal to the lowest value per replicate calculated among the samples. cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan), including a mix of random (12-mer) and oligo-dT primers in a reaction solution (20 μL). Quantitative PCR was performed after preparing the reaction mixtures in THUNDERBIRD SYBR qPCR Mix (Toyobo), using the same procedure as reported previously (Nagatomo et al. 2013). The primers used for the analysis are listed in Table 1. The transcript levels were calculated relative to the transcription of the internal control *H2AFZ* (H2A histone family member Z) in each sample. The experiments were replicated three times.

TUNEL

The In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) was used to assess the presence of apoptotic cells in Day-8 blastocysts, as described previously (Balboula et al. 2010). Blastocysts were fixed in 4% (w/v) paraformaldehyde solution (pH 7.4) for 30 min, rinsed twice in phosphate-buffered saline (PBS), permeabilized for 20 min with PBS containing 0.5% Triton X-100, and then washed twice for 10 min each in PBS containing 0.2 mg/mL polyvinyl alcohol (PVA). The fragmented DNA 3′-ends in each embryo were labeled with fluorescein-dUTP for 1 h at 37˚C, following by 3 10-min washes in PBS containing 0.2 mg/mL PVA and 0.2% Triton X-100. Labeled samples were mounted onto glass slides using a mounting solution containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories. INC, Burlingame, CA). Samples were evaluated using an EVOS Cell Imaging System (Advanced Microscopy Group, Mill Creek, WA). The apoptotic cell ratio in blastocysts was calculated as the number of TUNEL-positive blastomeres divided by the total cell number of the blastocysts.

Differential staining of blastocysts

The ICM and the TE within a blastocyst were differentially stained according to the method of Thouas et al. (Thouas et al. 2001). Briefly, blastocysts were stained at room temperature for approximately 2 min using 0.1 mg/mL propidium iodide (Life Technologies, Carlsbad, CA) in PBS containing 0.2% (v/v) Triton X-100, followed by staining at 4˚C for 3 h using 25 μg/mL bisbenzimide (Sigma-Aldrich) in 99.5% ethanol. After washing with glycerol, the blastocysts were mounted on a glass slide and examined using an EVOS Cell Imaging System (Advanced Microscopy Group). The ICM and TE nuclei were differentially stained blue and pink, respectively.

Immunofluorescence and confocal microscopy

The zona pellucida of the blastocysts was removed with 0.05% (w/v) pronase (Sigma-Aldrich). The blastocysts were fixed with 4% (w/v) paraformaldehyde (Wako Pure Chemical Industries) in PBS for 60 min, and then permeabilized for 60 min with 0.2% Triton X-100 in PBS. Next, embryos were blocked for 45 min with Blocking One (1:5; Nacalai Tesque, Inc.) in 0.05% (v/v) Tween 20 in PBS (blocking buffer), followed by an overnight incubation at 4˚C with primary anti-FGFR2 antibody (H00002263-M01; Abnova, Taipei City, Taiwan) diluted 1:200 in blocking buffer. After 3 30-min washes in 0.1% (v/v) Triton X-100 and 0.3% (w/v) bovine serum albumin (Sigma-Aldrich) in PBS, the blastocysts were incubated for 30 min at room temperature with Alexa Fluor 488-conjugated anti-mouse IgG polyclonal (A11001; Life Technologies) diluted 1:400 in blocking buffer. Nuclei were labeled with 25 μg/mL Hoechst 33342 (Sigma-Aldrich) prepared in 0.2% (w/v) PVA in PBS. Fluorescence signal was visualized using a TCS SP5 II confocal laser-scanning microscope (Leica, Wetzlar, Germany).

Statistical analysis

All data were statistically analyzed using one-way analysis of variance and Fisher's post-hoc least significant difference test. StatView statistical-analysis software (Abacus Concepts, Inc., Berkeley, CA) was used for analysis. $P<0.05$ was considered significant.

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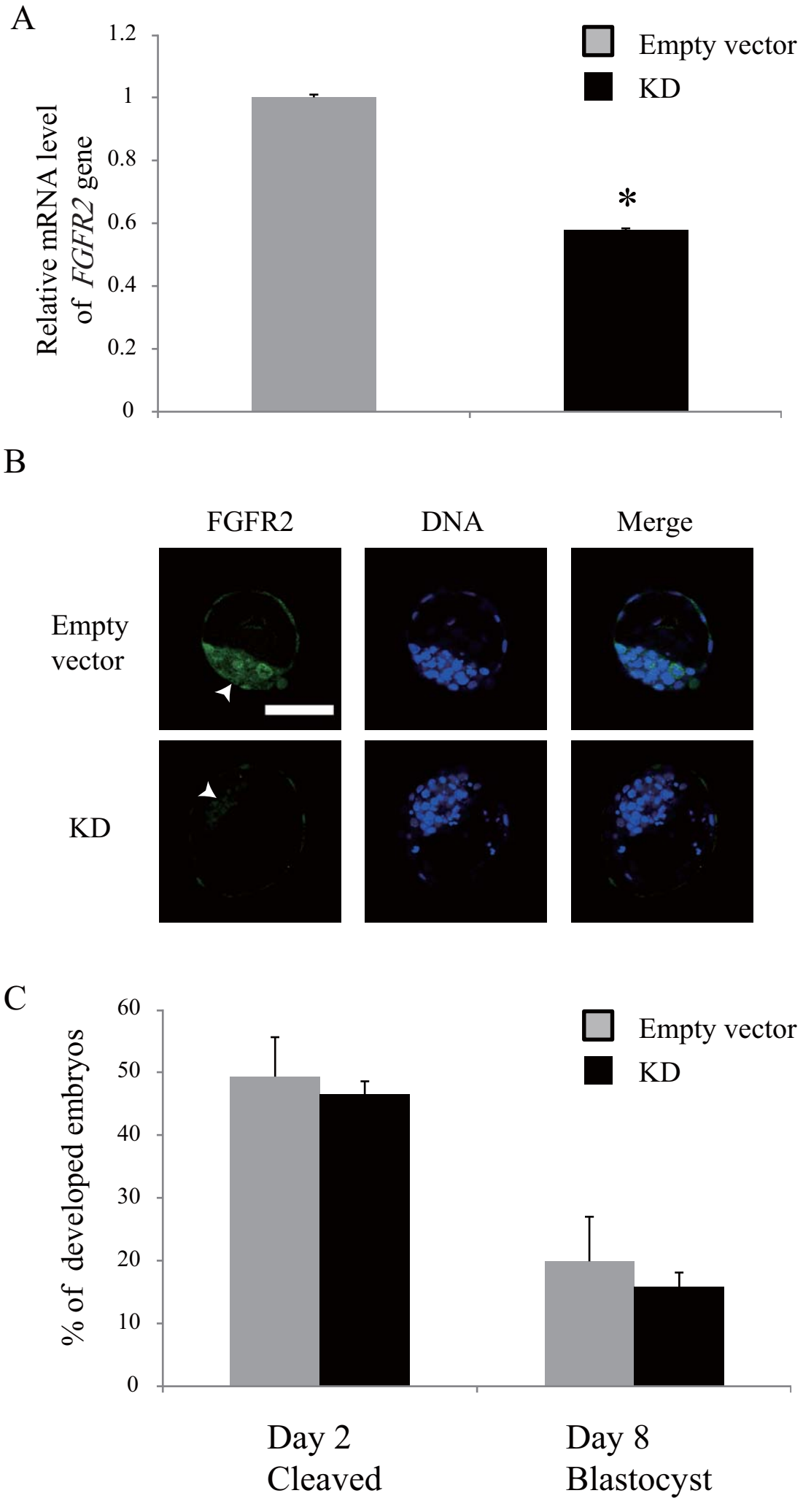
Figure legends

Figure1. Effect of *FGFR2* KD on bovine preimplantation development. A: *FGFR2* mRNA abundance determined using quantitative reverse-transcription PCR. Three independent experiments were performed using 5 blastocysts per sample. **B**: Immunofluorescence for FGFR2 at the blastocyst stage. The arrowheads indicate the ICM. FGFR2 protein is displayed in green; nuclei are in blue. Scale bar, 100 μm. **C**: Effect of *FGFR2* KD on embryonic development until the blastocyst stage. Three independent experiments were performed. Approximately 20–40 embryos were used for *FGFR2* KD and control (empty vector, e.g. the parent vector without the *FGFR2* shRNA insert) in each experiment. Values are represented as means ± standard error. *, $P<0.05$.

Figure 2. Detection of apoptosis in *FGFR2*-KD blastocysts. A: Apoptosis in control and *FGFR2*-KD blastocysts examined using TUNEL. Arrowheads indicate TUNEL-positive cells. Scale bar, 100 μm. **B**: The percentage of apoptotic cells, calculated by dividing the number of TUNEL-positive cells by the total cell number. Values are represented as means ± standard error. Eleven *FGFR2* KD and seven control (empty vector, e.g. the parent vector without the *FGFR2* shRNA insert) embryos were analyzed.

Figure 3. Cell number and allocation to the ICM and TE in *FGFR2*-KD blastocysts: Effect of *FGFR2* KD on ICM development. ICM and TE cell number in *FGFR2*-KD blastocysts were analyzed through differential staining of ICM and TE cells. **A**: The ICM-to-TE cell ratios for *FGFR2*-KD and control blastocysts. **B**: Allocations to the ICM and TE in *FGFR2*-KD and control blastocysts. Values are represented as means ± standard error. *$P<0.05$. Eleven *FGFR2* KD and twelve control (empty vector, e.g. the parent vector without the *FGFR2* shRNA insert) embryos were analyzed.

Figure 4. Quantitative-reverse-trasncription -PCR analysis for epiblast and primitive endoderm marker gene expression in *FGFR2*-KD and control blastocysts. The abundance of the epiblast gene *NANOG* and the primitive endoderm genes *GATA6* and *HNF4A* was analyzed by quantitative reverse-transcription PCR from five *FGFR2* KD or control (empty vector, e.g. the parent vector without the *FGFR2* shRNA insert) blastocysts. Three independent experiments were replicated. Values are represented as means ± standard error. Data were normalized to *H2AFZ* (H2A histone family member Z) mRNA abundance as an internal control. *: $P<0.05$; **: $P<0.01$.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1
0.8
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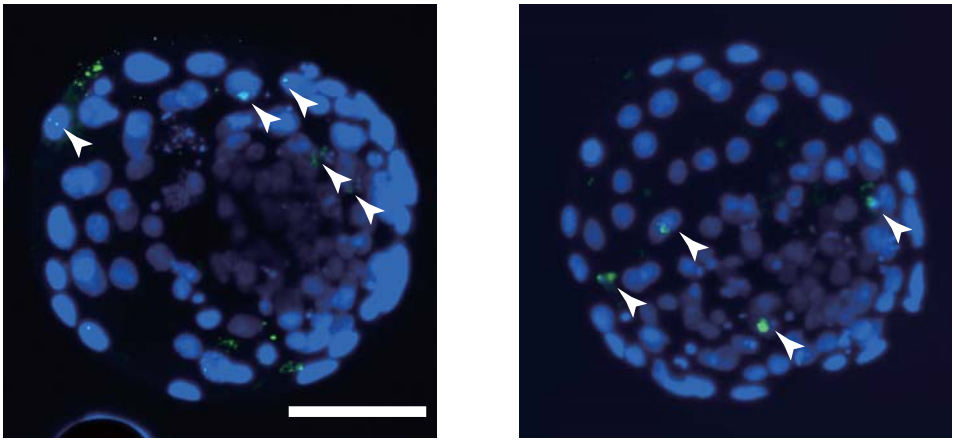
0.2
0
Relative mRNA level of FGFR2 gene
Empty vector
KD
*
B
FGFR2
DNA
Merge
Empty vector
KD
C
60
50
40
30
20
10
0
% of developed embryos
Empty vector
KD
Day 2 Cleaved
Day 8 Blastocyst

A



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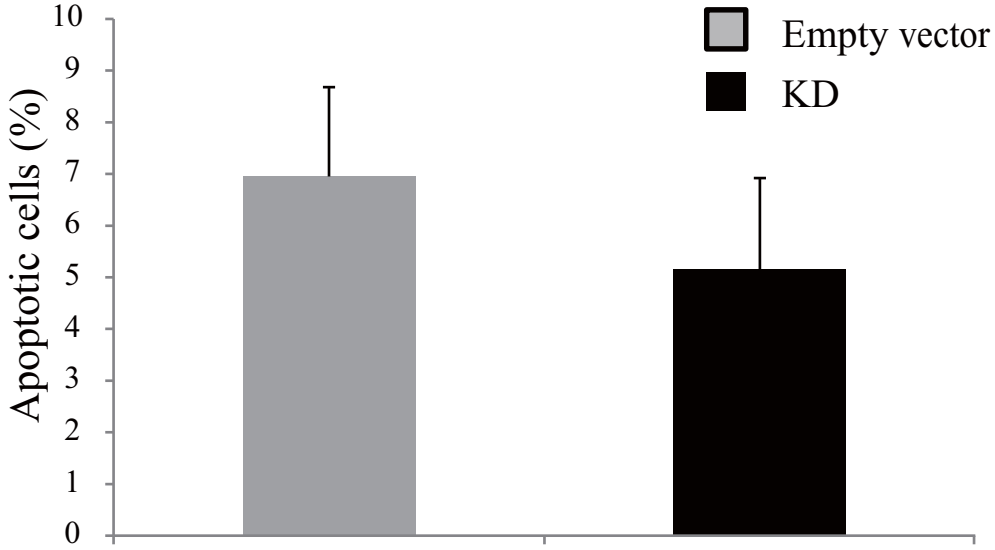

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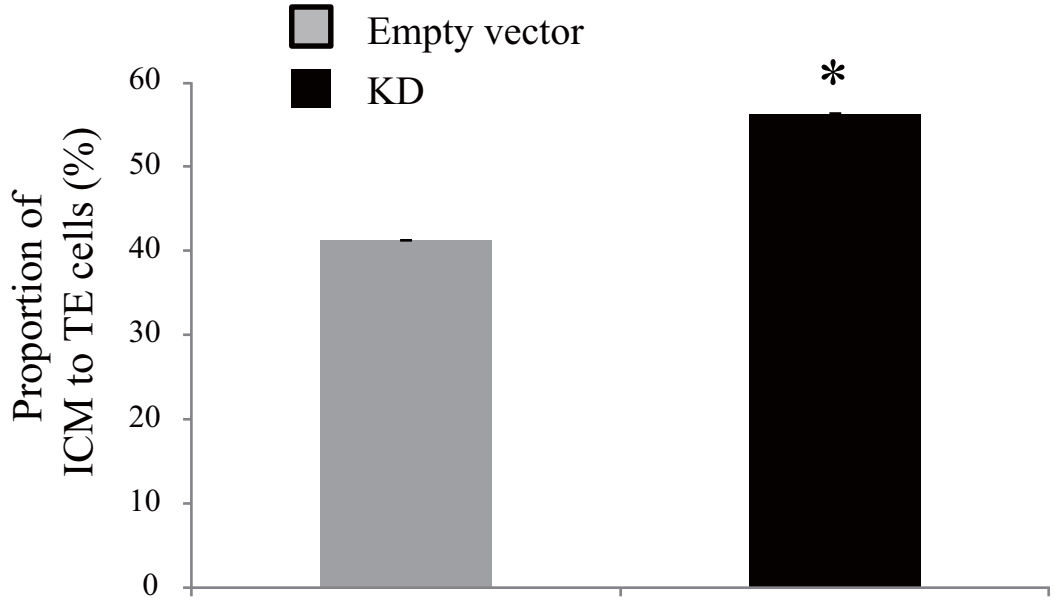
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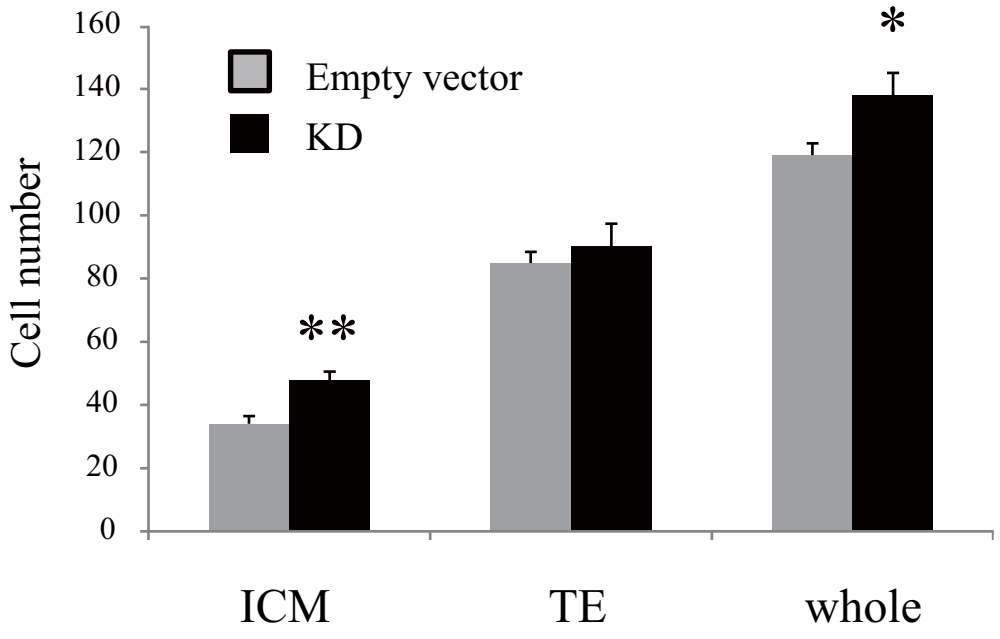
KD
Proportion of ICM to TE cells (%)
60
50
40
30
20
10
0
*

B


Empty vector
KD
Cell number
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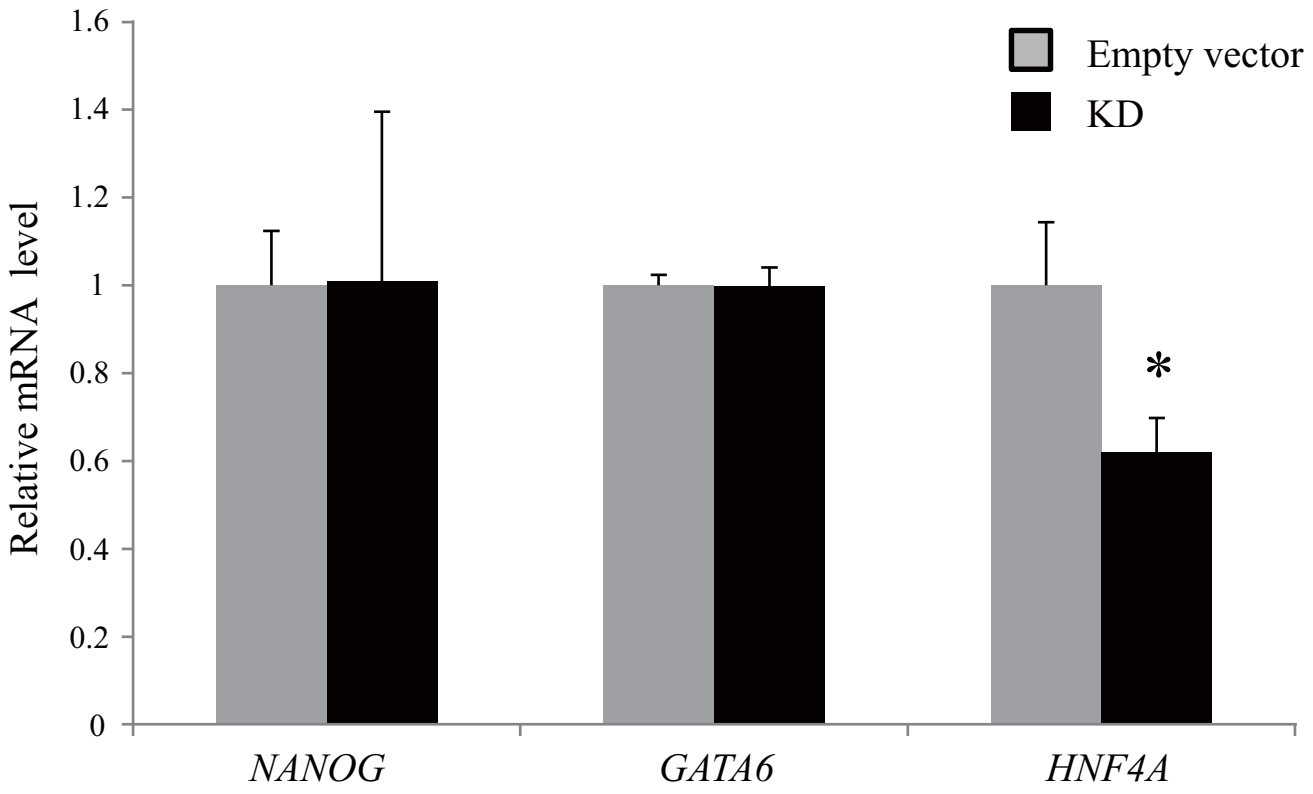
140
120
100
80
60
40
20
0
**
*
ICM
TE
whole


1.6
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0.8
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Relative mRNA level
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KD
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NANOG
GATA6
HNF4A