Knockdown of IGF-IR by siRNA injection during bovine preimplantation embryonic development

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Abstract This study aimed to assess the efficiency and effects of insulin-like growth factor receptor-1 (IGF-IR) siRNA knockdown during bovine preimplantation embryonic development. In oocytes injected with IGF-IR siRNA, the relative IGF-IR mRNA levels compared to controls were 28% and 46% at 6 and 24 h after injection, respectively. With respect to the injection of IGF-IR siRNA in zygotes, 24 h after injection the relative levels of IGF-IR mRNA and protein in the two-cell embryos were 74% and 78% of those in the controls, respectively. IGF-IR siRNA reduced blastocyst formation (23.2%) compared to siRNA controls (33.0%) and uninjected oocytes (35.4%; $P < 0.05$) and the number of viable cells per IGF-IR siRNA-treated blastocyst (64 ± 3) was significantly reduced, compared to control siRNA and uninjected blastocysts (81 ± 3 and 116 ± 4 ; $P < 0.01$). In conclusion, IGF-IR siRNA knockdown

reduces the development of bovine embryos, and microinjection in zygotes can decrease blastocyst cell number.

Keywords IGF · RNAi · In vitro · Bovine · Preimplantation embryo

Introduction

The insulin-like growth factors (IGF) are a complex system of peptide hormones (IGF-I and IGF-II), cell surface receptors (IGF-IR, IGF-IIR) and circulating binding proteins (IGF-BP 1–6; Pavelic et al. 2007). As mitogens, IGF-I and IGF-II are important growth factors, which enhance cell proliferation and regulate cell differentiation and apoptosis (Adashi et al. 1985; Kamada et al. 1992).

Culture of mouse embryos in IGF-I stimulates an increase in the total number of cells in the blastocyst, and also increases the proportion of blastocysts (Harvey and Kaye 1992; Kim et al. 2006). In vivo, IGF-II stimulates an increase in the rate of development of mouse blastocysts (Harvey and Kaye 1992) and parthenogenetic mouse embryos (Platonov et al. 2002). In bovine preimplantation embryo cultures, the proportion of blastocysts (Palma et al. 1997) and the cell number of blastocysts (Matsui et al. 1995; Byrne et al. 2002; Sirisathien and Brackett 2003) can be enhanced with IGF-I. The bovine blastocyst cell

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number also increases when cultured in presence of in IGF-II (Byrne et al. 2002).

In the mouse, IGF-II is believed to mediate growth through the IGF-IR, whereas IGF-IIR is believed to act as a negative regulator of somatic growth by limiting the availability of excess levels of IGF-II (Pantaleon et al. 2003). Our previous research has indicated that IGF-II, IGF-IR and IGF-IIR are expressed in bovine preimplantation embryos; however, IGF-I is not (Wang et al. 2009), which has also been reported in other studies (Winger et al. 1997; Yaseen et al. 2001; Moore et al. 2007). Therefore, it is hypothesized that IGF-II mediates growth through the heterologous type I IGF receptor (IGF-IR) in bovine embryos.

There have been no reports on the effect of IGF-IR downregulation in bovine embryos. The objectives of this study were to assess the knockdown efficiency of IGF-IR using siRNA, and the effect of IGF-IR knockdown during bovine preimplantation embryonic development.

Materials and methods

Oocyte collection and in vitro maturation

Bovine ovaries were obtained from a local abattoir and transported to the laboratory within 2 h in sterile saline solution (9 g NaCl/L) at 35–37 °C. Immature cumulus-oocyte complexes (COCs) were retrieved from obvious follicles (2–8 mm in diameter) with an 18-gauge needle. All oocytes which were completely surrounded by unexpanded cumulus cells were rinsed three times in maturation medium (M-199 media with Earle's Salt supplemented with 10 mM HEPES (Gibco), 1 µg E₂/mL (Sigma-Aldrich), 0.02 IU FSH/mL (Sigma-Aldrich), 10% v/v FCS (Gibco)). Cumulus-denuded oocytes were collected for injection. Oocytes after injection were cultured in maturation medium for in vitro maturation.

In vitro fertilization (IVF) and culture

Following a 22–24 h culture period, COCs were fertilized in vitro. Frozen spermatozoa were thawed and rinsed twice by centrifugation at 700g for 5 min with BO medium (Brackett and Oliphant 1975) containing 5 mg BSA/mL, and processed via the swim-up method to yield a concentration of $1\text{--}5 \times 10^6$ /mL.

COCs were rinsed three times in BO fertilization medium containing 5 mg BSA/mL, transferred in groups of 15–30 into 100 µL fertilization droplets under paraffin oil, and incubated with spermatozoa for 6 h to develop into zygotes. After in vitro fertilization, zygotes' cumulus cells were removed by vortexing for siRNA injection. Then zygotes were cultured in synthetic oviduct fluid development medium (Tervit et al. 1972) with 1 mg PVA/mL, 27.7 µM inositol, 3.4 µM citrate, 0.16 µM glutamine (SOF_{PVA}).

siRNA design

Double-stranded siRNAs were designed using top-scoring sequences identified by the siRNA Target Finder (Applied Biosystems; http://www.ambion.com/techlib/misc/siRNA_finder.html) against the IGF-IR mRNA (XM 606794) and supplied by TaKaRa. Two siRNAs against IGF-IR, siIGF-IR#1 (5'-AGGCAA UCUGCUCACUAACUU-3' against nt 606–627) and siIGF-IR#2 (5'-GGAGCAGAUGACAUUCCUGU U-3' against nt 2112–2133) were injected together. A scrambled version of siIGF-IR#1 (5'-GAUCCU ACAGCACGUACAUU-3') was used as a control siRNA: 5 to 15 picoliter (pL) of 10 µM double-stranded siRNA were injected into cumulus-denuded oocytes and zygotes, the injection volumes were estimated by observing the distance of displacement traveled by the oil front along a pipette of known internal diameter.

Real-time quantitative PCR (RT-qPCR)

Embryos were processed using a RNAiso Reagent from TaKaRa using the manufacturer's recommendations. To completely remove genomic DNA contamination from the RNA extraction, we performed the in-column DNase I optional step using Amplification grade DNase I (InvitrogenTM). A final volume of 15 µL of RNase free water was used in RNA elution. Only RNA samples with a 260/280 nm wavelength ratio above 2.00 were used for RT-qPCR assays.

Approximately 650 ng of RNA from each sample was used in subsequent reverse transcriptase reactions. Complementary DNA was synthesized using the PrimeScriptTM RT reagent Kit (Perfect Real Time, TaKaRa) according to the manufacturer's recommendations. Synthesis of the first strand was carried out at 37 °C for 15 min then a 15 s step at 85 °C for inactivation of reverse transcriptase.

Primers were designed using the Primer3 webtool with settings to generate primers with a melting temperature of ~ 60 °C. Due to the limitations of extension time in quantitative polymerase chain reactions (qPCR), primers were designed to amplify less than 200 bp of sequence when possible.

All qPCR experiments were performed using the SYBR Premix 2 $\times$ Ex Taq (TaKaRa). Reaction volumes were reduced to 20 μ L. A 7300 Real Time PCR System (Applied Biosystems) was used to quantify reactions. This was followed by one 95 °C denaturation for 30 s. Temperature cycling consisted of the following: 35 cycles of 95 °C for 5 s then 60 °C for 30 s. Melt curves (to determine if there were multiple PCR amplicons) were constructed by heating final amplification reactions from 60 to 95 °C for 15 s, 60 °C 30 s, 95 °C 15 s in single degree stepwise fashion. Primer efficiencies were calculated from readings derived from a standard curve of known DNA concentrations. Relative expression levels of target genes were calculated using the standardization as previously described (Pfaffl 2001). The β -actin was used as a reference gene to standardize relative expression in the samples. The primers of IGF-IR and β -actin are shown in Table 1. The control siRNA injection was used as control treatment to assess IGF-IR knockdown efficiency.

Western blot analysis

Two-hundred two-cell embryos of each group were collected in lysis buffer (Aprotinin, 1 μ g/mL; PMSF, 100 μ g/mL) and immediately frozen at -20 °C. Embryos were thawed, heated to 95 °C for 5 min, and then proteins were resolved using standard SDS–polyacrylamide gel electrophoresis techniques and detected with mouse anti-human IGF-IR antibody (Biosource, 1:200, 3 h) and anti-mouse IRD700 antibody (1:3,000, Rockland Inc, Pennsylvania,

USA). Mouse anti-human GAPDH (Abcam, 1:500, 3 h) was used to monitor the reference gene (GAPDH). The band intensities quantified against GAPDH using the Software of Odyssey Infrared Imaging System (LI-COR bioscience, Nebraska, USA).

Indirect immunofluorescence

Twenty-five embryos of each group were fixed in 4% paraformaldehyde for 30 min at room temperature, transferred to PBS with 0.25% Triton X-100 for 1 h at room temperature, rinsed with 2% BSA in PBS, blocked in PBS with 10% normal goat serum, 2% BSA, 2% nonfat dry milk and 0.15 M glycine overnight at 4 °C and incubated in mouse anti-human IGF-IR (Biosource; 5 μ g/mL in PBS with 2% BSA) for 2–3 h at 37 °C followed by rinsing and application of FITC-conjugated goat anti-mouse antibody (Santa Cruz; 1 μ g/mL in PBS with 2% BSA) for 2 h at 37 °C. Embryos were rinsed and stained with 5 μ g/mL propidium iodide in PBS for 15 min, rinsed and observed immediately with laser confocal scanning microscopy (Bio-Rad MRC 1024ES).

Statistical analysis

The development rate was analyzed by the χ^2 -test. Difference between mean values of the number of live cells was analyzed by oneway analysis of variance (ANOVA). P value less than 0.05 was considered as statistically significant.

Results

The knockdown efficiency of IGF-IR siRNA on the level of IGF-IR mRNA in immature oocytes

Six hours after injection, the relative level of IGF-IR mRNA in IGF-IR siRNA injected immature oocytes

Table 1 Details of primers used for real-time PCR

mRNA	Primer sequence (5'-3')	Annealing temperature (°C)	Fragment size (bp)	Genbank accession no.
IGF-IR	F-TTA AAA TGG CCA GAA CCT GAG ^a	55	314	XM606794
	R-ATT ATA ACC AAG CCT CCC AC			
β -actin	F-CAG AAG GAC TCG TAC GTG GG	56	200	AY141970
	R-TTG GCC TTA GGG TTC AGG G			

^a Primer orientation: *F* forward, *R* reverse

was 72% compared to control treatment, and after 24 h, the relative expression of IGF-IR mRNA in IGF-IR siRNA injected oocytes was 54% compared to control treatment (Fig. 1).

The effect of IGF-IR siRNA on IGF-IR mRNA and protein expression in the two-cell stage bovine embryo development

In two-cell stage embryos, produced from injected zygotes cultured in development medium for 24 h, the relative level of IGF-IR mRNA in siRNA IGF-IR injected embryos was 74% compared to the control siRNA injected (Fig. 2). A significant reduction (78%, $P < 0.05$) in IGF-IR protein expression was detected using Western blotting (Fig. 3), and also observed using immunofluorescent staining (Fig. 4) in IGF-IR siRNA injected embryos compared to control siRNA injected embryos. This was consistent with the results of the mRNA analysis (Fig. 2).

Effect of IGF-IR siRNA in bovine embryo development

Zygotes were injected with IGF-IR siRNA or control siRNA and cultured in development medium. The rate of blastocyst formation and blastocyst cell numbers are presented in Table 2 and Fig. 5.

Fig. 1 Knockdown efficiency of siRNA of IGF-IR in immature oocytes, control siRNA and IGF-IR siRNA were injected into immature oocytes cultured in maturation inhibitor respectively. After 6 or 24 h, oocytes were collected for RT-qPCR to quantify IGF-IR mRNA relative to β -actin, with 100 oocytes in each experimental group ($n = 3$)

Fig. 2 Knockdown efficiency of IGF-IR siRNA on mRNA expression in two-cell embryos. Six hours after fertilization, control siRNA and IGF-IR siRNA were injected into the cumulus-denuded zygote respectively, and after 24 h of culture in development medium, embryos in the two-cell development stage were selected for quantification of IGF-IR mRNA relative to β -actin, with 50–60 oocytes in each experimental group ($n = 5$)

Discussion

Knockdown of IGF-IR using siRNA significantly decreased IGF-IR mRNA and protein levels, the proportion of blastocysts, and the cell number of blastocysts during bovine embryo development. These results are similar to the effect of anti-IGF-IR antibody on porcine embryo culture (Kim et al. 2006). Rappolee et al. reported that IGF-I does not exist in mouse, which indicates that the main function of IGF is mediated via IGF-II. Moreover, reduction of mouse IGF-II expression using antisense IGF-II oligonucleotides decreased the rate of progression to the blastocyst stage, as well blastocyst cell number (Rappolee et al. 1992). This is similar to our research in bovine. Rappolee et al. reported that during oocyte maturation, both oocyte and cumulus cells released growth factors to support oocyte development. To study IGF function during oocyte maturation, it is technically difficult to inhibit the release of growth factors by cumulus cells, and additionally, during early preimplantation embryo development in vivo, there is paracrine regulation of IGF produced by the genital tract (Pushpakumara et al. 2002). As most IGF-I and IGF-II activity is mediated via the IGF-IR, we decided to study IGF function by inhibiting IGF-IR, avoiding the influence of paracrine or embryo secreted IGF-I or II.

Fig. 3 Knockdown efficiency of siRNA on IGF-IR protein expression in two-cell embryos. Six hours after fertilization, control siRNA (a) and IGF-IR siRNA (b) were injected into the cumulus-denuded zygote respectively. After 24 h of culture in development medium embryos in the two-cell development

stage were selected for the quantification of IGF-IR protein. The effect of IGF-IR siRNA was compared in 200 oocytes per group ($n = 5$) using Western blotting (left), and band intensities quantified against GAPDH using Quality One Software (bar-diagram on the right)

Fig. 4 Immunofluorescent staining of IGF-IR siRNA injected two-cell embryos. Control siRNA injected embryos (a) and IGF-IR siRNA injected embryos (b) were stained with anti-IGF-

IR (green) and propidium iodide (red), which denoted the nucleus. (Color figure online)

One potential mechanism to inhibit the expression of IGF-IR is RNA interference (RNAi), in which mRNA is knocked down by double-stranded RNAs that exhibit high homology to the target mRNA (Fire et al. 1998). siRNAs tend to inhibit translation of target mRNA, and mRNA expression can be knocked down if the siRNA has a high homology to the target (Olsen and Ambros 1999; Elbashir et al. 2001; Hutvagner and Zamore 2002). RNAi was first demonstrated in mouse zygotes and immature oocytes by injecting relatively long (550–650 bp) dsRNAs (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000); however, long

dsRNAs are difficult to work with, and have greater potential to induce nonspecific RNAi (Olsen and Ambros 1999; Haley and Zamore 2004). Therefore we selected and designed siRNAs to knockdown IGF-IR.

Maturation inhibition was used to quantify the IGF-IR knockdown efficiency in immature oocytes, mRNA level changes during oocyte maturation and zygote development. The relative level of mRNA reduced to 72% after 6 h and 54% after 24 h compared to injected control siRNA. Previous research has indicated the efficacy of siRNAs can be influenced by secondary structure in the transcript (Bohula et al. 2003), which

Table 2 Effect of IGF-IR siRNA knockdown on bovine embryonic development

Treatment	Number of oocytes	Number of ova cleaved (%)	Number of 8-cell embryos (%)	Number of blastocysts (%)	Mean blastocyst cell number ($\pm$ SEM)
Uninjected	270	180 (66.7)	88 (48.9)	63 (35.0) ^a	116 $\pm$ 4 ^a
Control siRNA	300	182 (60.7)	85 (46.7)	60 (33.0) ^a	81 $\pm$ 3 ^b
IGF-IR siRNA	321	193 (60.1)	75 (38.9)	45 (23.3) ^b	64 $\pm$ 3 ^c

The number of cleaved oocytes was counted 54 h after IVF and the numbers of 8-cell embryos, blastocysts and blastocyst cells were determined 8 days after IVF. Cleaved oocytes, 8-cell embryos and blastocyst numbers were analysed using the χ^2 -test, and blastocyst cell numbers were compared using oneway analysis of variance (ANOVA). Statistical significance was defined as $P < 0.05$

Number of ova cleaved and Number of 8-cell embryos were counted within 54 h post-insemination; number of blastocysts and Mean blastocyst cell number were counted within 8 days post-insemination. The same column with different superscripts means significant difference ($P < 0.05$)

Fig. 5 Bovine blastocyst labeled with Hoechst 33342 to determine the cell number. Uninjected blastocysts (a), or blastocysts resulting from control siRNA (b) and IGF-IR siRNA (c) injected oocytes were cultured for 7 days; they were then treated with PBS (pH 2.5) to remove the zona pellucida, rinsed

in SOF_{PVA}, incubated in 5 μ g/mL Hoechst 33342 (blue) and propidium iodide (red) in PBS+2% BSA for 15 min at 38.5 °C and observed under an inverted epifluorescent microscope to quantify the number of cells per blastocyst. (Color figure online)

may explain why the efficiency of our siRNA was not very high. When IGF-IR siRNA was used in embryo development, the relative level of IGF-IR mRNA was 74% of control embryos, possibly due to mRNA accumulated during oocyte maturation; however this

was sufficient to lead to a significant decrease in IGF-IR protein expression.

As oocytes are large, many siRNA molecules are needed to inhibit the function of the target mRNA. Microinjection is a good method to inject large

number of siRNAs into oocytes, with approximately $5\text{--}15 \times 10^6$ siRNA molecules injected into each oocyte in this study, which is similar to concentrations used in other studies (Svoboda, Stein et al. 2000). Oocyte maturation and preimplantation embryonic development occurs quickly, and as siRNAs need time to take effect, injection provides a good method to deliver siRNA quickly. In our study, we believe that the microinjected oocytes were stable; however, the microinjection process has a damaging effect which negatively impacts embryo development, as blastocyst cell number was significantly reduced compared to uninjected controls.

As siRNA microinjected into the oocyte can lower the expression of mRNA with minimal negative effects, RNAi may be a valuable tool for studying gene function during oocyte maturation, fertilization and egg activation and development. Additionally, this method could also be used to study gene function in early embryogenesis, as siRNA can inhibit the function of zygotically expressed genes (Wianny and Zernicka-Goetz 2000). In conclusion, siRNA can be used to knockdown the expression of IGF-IR mRNA during preimplantation embryonic development leading to a decreased expression of the IGF-IR protein, inhibiting the function of IGF, and resulting in a reduced proportion of blastocytes. In addition, the microinjection process leads also to a decreased blastocyst cell number.

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