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Short communication

Expression pattern of *STAT5A* gene during early bovine embryogenesis

Krzysztof Flisikowski[1], *Marc Boulhaeve*[2], *Fabiola P. Lopes*[2], *Eckhard Wolf*[2] *and Lech Zwierzchowski*[3]
Technische Universität München, Freising, Germany; Ludwig-Maximilians-Universität München, Oberschleissheim, Germany; and Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Magdalenka, Poland

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Summary

Growth hormone (*GH*) plays an important role in early embryo development. It has been shown to activate multiple pathways, the most comprehensively studied being the STAT/JAK (Signal transducers and activators of transcription/Janus kinase) pathway. The objective of the present study was to investigate *STAT5A* gene expression during early bovine embryogenesis. Real-time polymerase chain reaction (RT-PCR) was used to measure the abundance of *STAT5A* transcripts. The mRNA was present at all stages of preimplantation bovine embryos investigated. The most abundant *STAT5A* expression occurred at the 2-cell stage. Expression was markedly reduced between the 4-cell and 8-cell stages, coinciding with the known time of embryo genome activation and loss of maternal mRNAs. This finding suggests that the embryonic *STAT5A* gene is primarily activated by maternal gene products.

Introduction

Signal transducers and activators of transcription (STATs) are transcription factors implicated in cellular proliferation, differentiation and metabolism. After ligand binding-induced activation of cognate receptors, STAT proteins become phosphorylated, hetero- or homodimerize, and enter the nucleus (Wakao *et al*., 1994). *STAT5A* mediates, through the STAT/JAK pathway, signals of hormones with important functions in bovine reproduction – growth hormone (*GH*), leptin and prolactin (Darnell *et al*., 1994; Duncan *et al*., 1997). This signalling cascade is extraordinarily versatile and leads both to general phenomena, such as cell proliferation, and to highly specialized signalling, such as tissue-specific expression of individual genes. It is also known that *GH* exerts distinct effects on the development, differentiation and metabolism of preimplantation embryos. The development of bovine embryos cultured *in vitro* can be improved by supplementing the medium with *GH* (Kolle *et al*., 2001). *GH* and its receptor (*GHR*) mRNA are detectable from the second stage (2-cell stage) of bovine embryogenesis onwards (Kolle *et al*., 1998, 2002; Joudrey *et al*., 2003). Leptin, another possible ligand for the *STAT5* gene family, is known to regulate diverse reproductive functions, and recent studies suggest its involvement in early embryo development (Levy *et al*., 2001; Hansis & Edwards, 2003; Madeja *et al*., 2009). In the study by Madeja *et al.* (2009), the expression of leptin and its functional receptor was shown in mouse oocytes and preimplantation embryos.

The aim of the present study was to evaluate the level of *STAT5A* mRNA expression by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) in bovine preimplantation embryos at different stages. The results obtained enhance understanding of the functioning of the *GH/STAT5A* pathways during embryo development.

Materials and methods

In vitro production of bovine embryos

Bovine ovaries were collected at a local slaughterhouse (University of Munich) and washed in pre-heated

[1]All correspondence to: Krzysztof Flisikowski. Technische Universität München, Liesel-Beckmann-Str. 1, 85354 Freising, Germany. Tel. +49 8161 712036. Fax. +49 8161 712108. e-mail: flisikowski@wzw.tum.de
[2]Ludwig-Maximilians-Universität München, Hackerstrasse 27, 85764 Oberschleissheim, Germany.
[3]Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzebiec, 05–552 Magdalenka, Poland.

Table 1 Primers used for RT-qPCR determination of mRNA abundance and their localization in respective genes

Gene	Primer sequence 5′→3′	Amplified fragment length (bp)	Accession number GenBank
STAT5A	ACGGTACCTTCTTGTTGCGC CAGGTTACGGTCAGAGAGTCAAAC	85	Z72482
H2A	TTCGAAATGGCTGGCGG GGAACTGCAAACCGGCTCT	91	NM174809

phosphate-buffered saline (PBS) (30°C). Cumulus–oocyte complexes (COCs) were obtained by aspirating follicles (2–8 mm diameter) with a 20-gauge needle and a vacuum of approximately 13 kPa. Only COCs with a complete dense cumulus and dark, evenly granulated cytoplasm were selected for *in vitro* maturation. COCs were collected in a 50 ml centrifuge tube and washed twice with pre-incubated (39°C, 5% CO_2 in air) tissue culture media 199 (Invitrogen) supplemented with 10% heat-inactivated serum from cows at estrus (OCS). They were washed again in medium 199 supplemented with 10% OCS and 10 μg/ml bovine follicle stimulating hormone (FSH) and leutenizing hormone (LH) and matured in this medium for 22–24 h at 39 °C in an atmosphere of 5% CO_2 in air and maximum humidity. After maturation, COCs were maintained in Tyrode's–albumin–lactate–pyruvate (TALP) medium that contains 6 mg/ml BSA, 100 μg/ml heparin (Sigma). Motile spermatozoa were obtained by the swim-up procedure and by centrifugation of semen from the upper phase for 10 min at 700 *g* at room temperature. Viable spermatozoa (final concentration 10^6 spermatozoa/ml) were added to matured COCs for 22 h under the same conditions as used for *in vitro* maturation. Semen from the same bulls was used for all experiments.

For *in vitro* culture, cumulus cells were removed from presumptive zygotes by vortexing for 3 min. Groups of 30–40 presumptive zygotes were washed three times and cultured for 8 days in 400 μl synthetic oviduct fluid (SOF) supplemented with 10% OCS and covered with pre-equilibrated mineral oil (Sigma). Embryos were cultured in incubator chambers at 38.5 °C in an atmosphere of 5% CO_2, 10% O_2, 85% N_2. The cleavage rate was evaluated 72 h post-insemination (hpi) and the number of blastocysts scored at 186 hpi. Three replicates of each experiment were performed. Overall, high rates of maturation (82 ± 4%), fertilization (68 ± 2%), cleavage (52 ± 3%) were obtained, and a moderate rate of blastocyst formation (32 ± 3%), in the *in vitro* produced (IVP) experiments described.

Oocyte and embryo collection

Oocytes were collected 22–24 h after maturation and cumulus cells removed by vortexing. Zygotes were collected 18–20 hpi, 2-cells 26–30 hpi, 4-cells 37–39 hpi, 8-cells at day 3 (d3), 16-cells d4, morula d5, expanded d7 and hatched blastocysts d8. Embryos were stored in groups of four in 10 μl RNAlater (Qiagen) at –80 °C until extraction of RNA. Three replicates of pools of five *in vitro* matured oocytes and pools of five *in vitro* embryos were used for each developmental stage.

RNA isolation and reverse transcription

Total RNA was isolated with TriZol reagents (Invitrogen), essentially according to the method of Chomczynski & Sacchi (1987). RNA preparations were treated with 0.25 μl DNase (10 IU/μl; Invitrogen) by incubation at 25 °C for 15 min. The enzyme was inactivated by adding 1 μl 25 mM EDTA and heating at 65 °C for 10 min. Samples were chilled rapidly on ice and reverse transcription directly followed. Reverse transcription was carried out in a total volume of 40 μl according to the Superscript II reverse transcriptase protocol (Invitrogen). Samples were stored at –80 °C until quantitative PCR (qPCR).

Gene cloning

Amplification primers (Table 1) were designed according to the sequences found in GenBank and using the software Primer Express 3 (Applied Biosystems).

For qPCR, plasmid standards were established for each gene of interest with the TOPO cloning kit (Invitrogen). Prior to the cloning step PCR was performed in a total volume of 20 μl with 0.25 μl HotStarTaq (Qiagen), 1 μl of each primer listed in Table 1 (final concentration 300 nM), 1 μl cDNA, 2 μl 1 mM dNTPs (Fermentas), 1.25 μl $MgCl_2$ (0.25 mM) and 11.5 μl water. Thermal cycling conditions were as follows: 15 min at 95 °C, then 35 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, terminating with 10 min at 72 °C. Samples comprising 10% of the PCR reaction were analysed by 2.5% agarose gel electrophoresis and ethidium bromide fluorescence.

For the cloning step, a PCR aliquot of 2 μl was mixed with 1 μl salt solution, 1 μl TOPO vector and 2 μl water (Invitrogen). Reactions were incubated for 10 min at room temperature and 2 μl ligation mix added to the bacteria. Plasmids were isolated using the QIAprep® spin Mini kit (Qiagen) following the

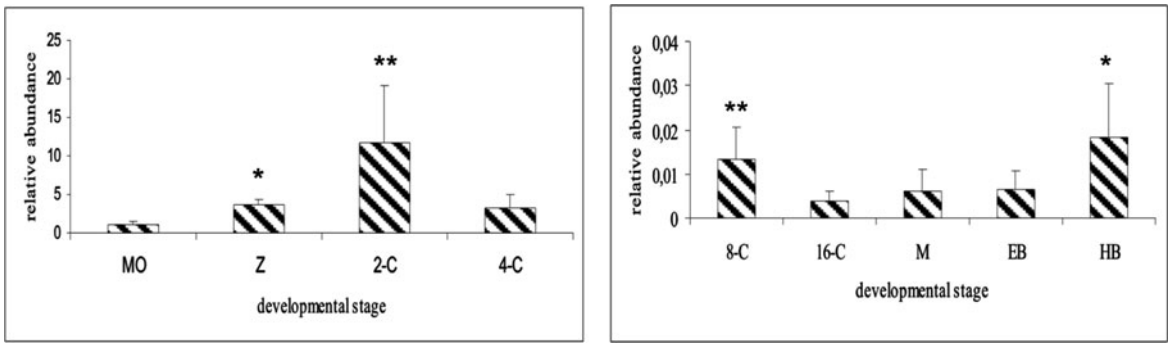

Figure 1 Relative expression levels of the *STAT5A* gene during early embryogenesis. Expression of *STAT5A* was normalized to the *H2A* gene. Mean ± standard error (SE) for nine determinations, five embryos each. Abbreviations: 2-C, 2-cell stage; 4-C, 4-cell stage; 8-C, 8-cell stage; 16-C, 16-cell stage; EB, expanded blastocyst; HB, hatched blastocyst; M, morula; MO, matured oocyte; Z, zygote. *Differences statistically significant at $P < 0.05$. **Differences statistically significant at $P < 0.01$.

manufacturer's instructions. Sequence integration was tested by digestion of 2 μl plasmid isolate with *Eco*RI (Fermentas) and gel electrophoresis.

Quantitative PCR (qPCR)

Quantification of mRNA abundance was performed by SybrGreen-based real-time PCR detection using an ABI PRISM 7000 apparatus (Applied Biosystems). Amplification mixes (25 μl) contained 2 μl cDNA, 12.5 μl SybrGreen PCR Mix, 0.25 IU AmpErase uracil *N*–glycosylase (Applied Biosystems), 300 nM each primer and 7.25 μl water.

Standard curves were obtained using plasmids prepared as described above, and seven serial dilutions (1:5) used for all selected genes, providing a range of 1 million to 64 copies. The abundance of *STAT5A* mRNA was calculated using the standard curve method, with determination of PCR amplification efficiency and normalization for histone 2A mRNA as the internal reference. qPCR was started with 2 min at 50°C for AmpErase activation and 10 min at 95 °C for denaturation. The programme continued with 45 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. Each assay included triplicates of cDNA primed separately for the two genes of interest. The significance of the differences in the expression levels of *STAT5A* was estimated using Duncan's new multiple range test using data from different embryo stages (mean ± standard deviation (SD)).

Results and Discussion

Real-time RT–PCR was performed to detect and quantify *STAT5A* mRNA expression. Real-time RT-PCR with an external calibration curve is a fully quantitative method, and thus allows an absolute comparison of the abundance of individual transcripts in RNA preparations at each embryonic stage. RT-PCR products showed single bands of the lengths predicted: 85 bp for *STAT5A* and 91 bp for *H2A* (data not shown). The abundance of mRNA encoding *STAT5A* was normalized relative to *H2A* mRNA. The C_T values obtained for *H2A* mRNA were similar in all embryonic stages (data not shown), as reported earlier (Robert *et al*., 2002).

Figure 1 shows relative expression measurements for *STAT5A*. The expression level at the mature oocyte stage was significantly lower than at zygote ($P < 0.05$) or 2-cell stage ($P < 0.01$). *STAT5A* expression reached a peak at the 2–cell stage and showed a sudden drop at the 8-cell stage ($P < 0.01$). *STAT5A* expression in hatched blastocysts appeared somewhat higher than in 8-cell, morula and expanded blastocyst stages ($P < 0.05$), but was still approximately 100-fold lower than at the 4-cell stage. Quantification of *STAT5A* expression in bovine embryos revealed that the abundance of the transcript decreased markedly at the 8-cell stage.

Low fertilization rate and embryonic mortality are significant factors contributing to failed pregnancies in cattle (Diskin & Morris, 2008). Among the genetic factors, the genes of the pituitary-specific positive transcription factor 1 (*POU1F1*) pathway play a crucial role in embryonic survival rate (Khatib *et al*., 2009a). Growth hormone, prolactin and their main mediator, *STAT5A*, belong to this pathway. Growth hormone supports mouse (Wright & Bondioli, 1981) and bovine (Kolle *et al*., 2001) preimplantation embryo development *in vitro*. Also, in both species *GH* transcripts were detected at different developmental stages of early embryos. In bovine embryos the *GH* transcript has been detected in oocytes and all pre-attachment stages including blastocysts (Kolle *et al*., 1998; Joudrey *et al*., 2003). It has also been shown that the quality of bovine blastocysts derived from non-early cleaving zygotes is improved by *GH* supplementation (Pers-Kamczyc *et al*., 2010). However,

little information is known about the major mediator of the *GH* signal, embryonic *STAT5A*. *Stat5a*$^{-/-}$ double knockout mice exhibit female infertility (Teglund *et al*., 1998), thus *STAT5A* is considered to be involved in oogenesis and early embryo development. Two studies showed upregulation of *STAT5A* expression in bovine degenerate embryos compared with blastocysts (Khatib *et al*., 2009b; Laporta *et al*., 2011).

In mice, *STAT5A* transcription decreases between MII and 4-cell stages, and then increases from the morula stage (Nakasato *et al*., 2006). The present paper demonstrates developmental changes in *STAT5A* gene expression during early bovine preimplantation embryogenesis. *STAT5A* mRNAs are present throughout the early developmental stages but fall to low levels after embryonic genome activation, which is different to mice. The bovine embryonic genome undergoes a major transcriptional activation at the 8-cell stage (66–72 hpi) and *in vitro* cultured bovine embryos can struggle to pass the so-called '8–16-cell developmental block' (Wolf *et al*., 2003). The marked drop in *STAT5A* expression after the 4-cell stage suggests that abundant *STAT5A* expression observed in the early stage embryos is a consequence of activation by maternal gene products, and the subsequent low level of *STAT5A* expression represents regulation by the embryonic genome. The different expression pattern before and after developmental block suggests different activation mechanism for *STAT5A*. It is known that mammary gland *STAT5A* expression is induced by *GH* treatment (Boutinaud & Jammes, 2004). Therefore, maternal *GH* or other cytokines may also stimulate expression of STAT5A before the 8-cell stage in cattle. Indeed, mice experiments showed that dynamic changes in the expression of cytokine receptors activate the STAT5 signalling pathway in preimplantation embryos (Nakasato *et al*., 2006). The constantly low level of *STAT5A* expression suggests that autocrine cytokine stimulation of *STAT5A* pathway may be activated at later stages in bovine embryos. It should also be mentioned that the presence of *GH* in unknown concentrations during maturation and culture could alter *STAT5A* expression. In contrast, expression of the related factor *STAT3* declines steadily from the oocyte to the 16-cell stage and then increases until hatched blastocyst (Leidenfrost *et al*., 2011). A similar expression pattern to *STAT3* has also been observed for the related *LEP* gene in *in vitro* produced bovine embryos (Madeja *et al*., 2009). In the same experiment *LEPR* mRNA expression was detected in all but 4-cell bovine embryos (Madeja *et al*., 2009). These data illustrate the complexity and dynamic nature of mRNA expression changes in preimplantation embryos.

These results provide new data regarding the embryonic *STAT5A* expression and new insight for the *GH* pathways regulation in bovine early embryonic development.

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