Expression pattern of \textit{STAT5A} gene during early bovine embryogenesis

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Summary

Growth hormone (\textit{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 \textit{STAT5A} gene expression during early bovine embryogenesis. Real-time polymerase chain reaction (RT-PCR) was used to measure the abundance of \textit{STAT5A} transcripts. The mRNA was present at all stages of preimplantation bovine embryos investigated. The most abundant \textit{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 \textit{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). \textit{STAT5A} mediates, through the STAT/JAK pathway, signals of hormones with important functions in bovine reproduction – growth hormone (\textit{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 \textit{GH} exerts distinct effects on the development, differentiation and metabolism of preimplantation embryos. The development of bovine embryos cultured \textit{in vitro} can be improved by supplementing the medium with \textit{GH} (Kolle et al., 2001). \textit{GH} and its receptor (\textit{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 \textit{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 \textit{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 \textit{GH/STAT5A} pathways during embryo development.

Materials and methods

\textit{In vitro} production of bovine embryos

Bovine ovaries were collected at a local slaughterhouse (University of Munich) and washed in pre-heated...
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% CO₂ 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 leutening hormone (LH) and matured in this medium for 22–24 h at 39°C in an atmosphere of 5% CO₂ 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 10⁶ spermatozoa/ml) were added to matured COCs for 22 h under the same conditions as used for in vitro maturation. Sperm 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 10% heat-inactivated serum from cows at estrus (OCS). They were washed again in medium 199 (final concentration 300 nM), 1 mM dNTPs (Fermentas), 1.25 mM MgCl₂ (0.25 mM) and 11.5 mM 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

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence 5′ → 3′</th>
<th>Amplified fragment length (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT5A</td>
<td>ACGGTACCTTCTTGTGGCCGC CAGTTACGGTCAGAGTCAACAC</td>
<td>85</td>
<td>Z72482</td>
</tr>
<tr>
<td>H2A</td>
<td>TTCGGAAATGGGCTGGCGGG GGAACCTGCAAACCGGCTCT</td>
<td>91</td>
<td>NM174809</td>
</tr>
</tbody>
</table>

**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 MgCl₂ (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.

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STAT5A embryonic expression

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 $P < 0.01$.

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 Ct 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 embryos (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 STAT3A 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.

References


