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Expression of TGF-β1, TGF-β2, TGF-β3 and the Receptors TGF-βRI and TGF-βRII in Placentomes of Artificially Inseminated and Nuclear Transfer Derived Bovine Pregnancies

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Bovine nuclear transfer pregnancies are characterized by a high incidence of placental abnormalities, notably, increased placentome size and deficiencies in trophoblast cell function and establishment of placental vasculature. Alterations in gene expression during placental growth and development may contribute to the appearance of large placentomes in pregnancies derived from nuclear transfer. The placenta synthesizes a number of cytokines and growth factors, including the transforming growth factor-βs (TGF-βs) that are involved in the establishment, maintenance and/or regulation of pregnancy. All forms of TGF-β and their receptors are present at the fetal–maternal interface of the bovine placentome, where they are thought to play an important role in regulating growth, differentiation, and function of the placenta. Using real-time RT-PCR, we have examined the expression of TGF-β1, TGF-β2, TGF-β3 and the receptors TGF-βRI and TGF-βRII in placentomes of artificially inseminated (AI) and nuclear transfer (NT)-derived bovine pregnancies at days 50, 100 and 150 of gestation. TGF-β1, TGF-β2 and TGF-β3 mRNA expression increased by 2.0–2.8-fold, while TGF-βRI and TGF-βRII mRNA expression decreased by 1.7–2.0-fold in NT placentomes compared to AI controls at all gestational ages examined. These findings indicate that NT placentomes may be resistant to the growth suppressive effects of TGF-βs and could contribute to the placental proliferative abnormalities observed in NT-derived placentas. Alternatively, deficiencies in placentation may provide a mechanism whereby TGF-βs are dysregulated in NT pregnancies.
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INTRODUCTION

In the bovine placenta, initial contact between the fetal trophoblast and the maternal caruncle induces hypertrophy of the fetal chorion and the formation of a cotyledon [1]. With the advancement of pregnancy, these cotyledons progress to form much larger and more complex placentomes. In cattle, binucleate cells (BNCs) of the trophoblast play an important role in the modification of the uterine epithelium [2,3]. As BNCs migrate they temporarily fuse with a single endometrial epithelial cell to form the uterine syncytium which is critical to villous formation, expansion and the maintenance of close adhesion between microvilli and the trophoblastic cell membrane [3].

Abnormal placentation, including increased placentome size, is a major cause of pregnancy loss after transfer of cloned bovine embryos [4]. Successful placental development is dependent on temporally and spatially correct expression of a variety of growth factors. Therefore, aberrant expression of developmentally important genes may adversely affect placental development [5]. The placenta synthesizes a number of cytokines and growth factors that are involved in the establishment, maintenance or regulation of pregnancy. The transforming growth factor-β (TGF-β) superfamily consists of multifunctional growth factors that are known to be involved in many aspects of cellular growth and differentiation [6,7]. In mammalian tissues, the TGF-βs themselves exist in three highly similar isoforms termed TGF-β1, TGF-β2 and TGF-β3 [7]. These proteins share extensive regions of similarity in their amino acids, and isoforms isolated from different species

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have sequence identities on the order of 98% [6,8], suggesting the evolution of an integral regulatory function. TGF-β exerts its biological effects through binding to cell surface receptors designated types I, II and III [6,9]. The type I and II receptors are transmembrane serine/threonine kinase receptors, while the type III receptor is a membrane anchored proteoglycan with no discernable signaling motif [9]. Downstream of the TGF-β pathway, the Smads act as intracellular mediators and modulators of signaling of the TGF-β superfamily. Disruption of the TGF-β/Smad signaling pathway has been associated with many human diseases including cancer [10,11]. Alterations in receptor function and/or ratios of TGF-β receptors found in many tumor cells compromise the growth suppressive effects of TGF-β and potentiate its oncogenic functions [10]. Decreased levels of type I receptors have been observed in human breast and pancreatic cancers, while mutations in the type II receptors have been correlated with colorectal and other cancers [11–13]. Inactivating mutations in Smad proteins have also been described in a number of human cancers, while mutations in the Smad 2 gene have been associated with lung cancers, colorectal cancers and hepatocellular cancers [10,11].

All forms of TGF-β have been shown to be present at the fetal–maternal interface of the bovine placentome, where they are thought to play an important role in regulating growth, differentiation, and function of the placenta [9,14,15]. In cows, immunoreactive TGF-β1 and TGF-β2 have been localised to chorionic and endometrial epithelial cells, endometrial and allantoic stromal cells and to the allantoic epithelium [14]. Additional staining has also been noted in both uninucleated and binucleated trophoblast cells. TGF-β3, on the other hand, appears to be expressed predominantly in placentomal arcades, specifically trophoblastic and allantoic stromal cells, although some binucleated cells also appear to express the protein [14]. Spatial patterns of TGF-β immunoreactivity in the bovine placentome are consistent with their actions in cell culture and thus indicate that TGF-βs contribute to the establishment of a viable placenta [16].

Examination of gene expression in cloned cattle has been largely limited to preimplantation embryos [17,18]. In the present study we have compared expression patterns of TGF-β genes and their receptors in placentomes derived from either artificial inseminated (AI) or nuclear transfer (NT) pregnancies, in light of their involvement in placental growth, development and function. Placentomes from early to mid gestation pregnancies were investigated as this is the period of maximal placental growth in cattle.

METHODS AND MATERIALS

Nuclear transfer and artificial insemination

Investigations were conducted in accordance with the regulations of the New Zealand Animal Welfare Act of 1999. The methods used to generate the cloned (NT) embryos have been described previously [19]. In brief, an ovarian follicular cell line (EFC) derived from a 4-year-old Friesian dairy cow and demonstrated to be totipotent following NT, as described in Ref. [19], was used in this study. Serum-starved donor cells used for NT were injected underneath the zona pellucida and then electrically fused with cytoplasts. Reconstructed embryos were artificially activated 3–6 h after fusion and cultured in vitro for 7 days in a modified Synthetic Oviduct Fluid (SOF) medium, as described previously [20,21].

Tissue collection

A sample of pregnant animals from each group was slaughtered at days 50, 100 and 150 of gestation, and the reproductive tracts collected and transported to the laboratory within 1 h. The animals used in this study were the same animals referenced in Ravelich et al. [41]. Caruncles were removed and trimmed off their associated membranes. Intact placentomes were placed in tin foil, snap-frozen in liquid nitrogen or fixed in formalin in phosphate buffered saline (PBS) for subsequent analysis. As previously described [41], paraffin embedded sections were stained with haemotoxylin and eosin (H&E) and binucleate cell numbers at days 50, 100 and 150 of gestation were determined by immunostaining with bovine placental lactogen (bPL) (gifted by J.C. Byatt, Monsanto Animal Agriculture Group, St Louis, MO, USA). Cell counting was performed by a double blind procedure and random fields assigned. Six random fields were examined at 100× magnification within a 1 × 1 cm ocular grid per section.

Real-time RT-PCR

RNA isolation and reverse transcription

Frozen placental samples at days 50 (AI, $n = 4$; NT, $n = 6$), 100 (AI, $n = 4$; NT, $n = 5$), and 150 (AI, $n = 4$; NT, $n = 6$) were first homogenized with an Ultra-Turax T25 homogeniser (Janke and Kundel, Strangen, Germany). Total RNA was prepared using the TRIZol extraction method (Invitrogen, USA). The RNA was further purified with RNeasy mini kits (Qiagen, USA). Integrity of the RNA was verified by electrophoresis and ethidium bromide staining, and by optical density (OD) ratio ($OD_{260\,nm}/OD_{280\,nm} > 1.9$). To eliminate residual genomic DNA from the RNA sample prior to the reverse transcription (RT) reaction, samples were treated with 1 unit of DNaseI (Invitrogen). RT was performed using 1 μg of total RNA in a 20 μL reaction volume. Briefly, RNA, random hexamer primers and 10 mM dNTP mix were denatured by incubating at 65 °C for 10 min. The reaction mix (5× First-Strand Buffer, 0.1 mM dithiothreitol, 40 U RNase OUT RNase inhibitor) was added and the mixture incubated at 55 °C for 5 min, followed by addition of 15 U Thermoscript reverse transcriptase and subsequent incubation at 55 °C for 90 min. The reaction was stopped by heating to 85 °C for 5 min. Finally, RNA complementary to the cDNA was removed by adding 2 U *Escherichia coli* Ribonuclease H and incubating for 20 min at 37 °C. All enzymes were purchased from Invitrogen.