Quantification of transforming growth factor β1 (TGFβ1) mRNA expression in mouse preimplantation embryos and determination of TGFβ receptor (type I and type II) expression in mouse embryos and reproductive tract

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We hypothesized that transforming growth factor β1 (TGFβ1) and its receptors play a role in the interaction between the preimplantation embryo and the reproductive tract. To investigate this hypothesis, TGFβ1 mRNA in mouse embryos was quantified by competitive reverse transcription–polymerase chain reaction using an RNA mimic. TGFβ1 was first detected in the unfertilized oocyte, disappeared after fertilization and was expressed again at the 2-cell stage (4410 ± 1330 transcripts/embryo). Its expression increased gradually, peaked at the 8-cell stage (58 600 ± 17 300 transcripts/embryo) and declined rapidly after the morula stage reaching a concentration of 1520 ± 546 transcripts/embryo at the blastocyst stage. The mRNA levels of TGFβ1 at the 8-cell and morula stages were significantly higher than that at other cell stages (P < 0.05). The expression of TGF receptors in embryos and in the reproductive tract was also investigated. Both TGFβ1 type I (ALK-5) and type II TGFβ receptors were detected in embryos from 1-cell to blastocyst stage by immunohistochemistry. Northern hybridization and immunohistochemistry showed a constant expression of both TGFβ receptors in the oviduct from day 1 to day 4 of pregnancy, whilst in the uterus there was a marked increase in the expression of TGFβ type I receptor on day 3. Expression of TGFβ type II receptor in the uterus remained unaltered throughout the study period. This study has shown that preimplantation mouse embryos produce TGFβ1 and that both the embryos and the reproductive tract are responsive to TGFβ1 in the preimplantation period.

Key words: embryo/oviduct/receptor/TGFβ1/uterus

Introduction

The interaction between preimplantation embryos and the reproductive tract (i.e. the oviduct and uterus) is widely believed to play an integral role in preimplantation embryo development. The involvement of a growth factor signalling system between the embryos and the maternal physiology has been demonstrated (Kaye and Harvey, 1995). For example, tubal epithelial cells secrete high molecular weight glycoproteins that enhance mouse embryo development (Liu et al., 1995, 1998). Transforming growth factors and their receptors at the uterine luminal epithelium are likely to play important paracrine/autocrine roles during the peri-implantation period (Gupta et al., 1998). The embryo in turn produces transforming growth factor-β1 (TGFβ1) which induces apoptosis in the endometrium at the site of implantation (Kamijo et al., 1998). There are also indications that the intra-oviductal embryo can exert a biological effect on the uterus, enhancing endometrial receptivity (Wakuda et al., 1999). In any event, the reproductive tract provides a complex yet poorly understood environment required for gamete transport, maturation, fertilization, early pre-embryo development and implantation.

TGFβ1, one of the growth factors detected in the mouse embryo and reproductive tract, is a multifunctional polypeptide hormone that influences numerous physiological processes (Akhurst et al., 1991; Lyons et al., 1991). The expression of TGFβ1s in the ovary, testis, pre- and post-implantation uterus as well as in the embryo (Kane et al., 1997) suggest the involvement of this growth factor in various reproductive functions. Furthermore, a knockout mouse study showed that intercrosses of heterozygous animals carrying one wild type and one disrupted TGFβ1 allele resulted in a significant decrease in the production of homozygous mutant animals (Shull et al., 1992). Another knockout study showed that on a predominantly CF-1 (Albino c origin) genetic background, lack of TGFβ1 caused a pre-morula lethality in ~50% of the null embryos (Kallapur et al., 1999). Thus, TGFβ1 is believed
to play an important role in preimplantation development of embryo.

TGFβ1 exerts its effects through binding to specific cell surface receptors. Three types of TGFβ receptors, namely type I (TβRI, (ALK-5) and (Tsk 7L)), type II (TβRII) and type III (TβRIII), have been identified (Massagué, 1992; Lin and Lodish, 1993). Signal propagation for TGFβ1 is dependent upon heteromeric [TβRI (ALK-5) and TβRII] complex formation and transphosphorylation of TβRI by TβRII (Anders et al., 1998). The function of TβRII remains elusive; it has been suggested that it increases the affinity of TβRI–RII complex towards TGFβ2 (Wang et al., 1991; López-Casillas et al., 1993), or may also store and regulate the availability of TGFβ towards an unidentified TβRII subtype (Lawler et al., 1994). Studies on the expression of TGFβ receptors could manifest the capability of the cells to respond to TGFβ1 stimulation.

Quantification of transcripts in preimplantation embryos is important because it casts light on the importance of certain genes at specific stages of development. However, quantification of transcripts in the preimplantation embryo is difficult because the amount of mRNA present is scarce. Competitive reverse transcription–polymerase chain reaction (RT–PCR) using an RNA mimic (Becker-André and Hahlbrock, 1989) is one of the most sensitive methods used to quantify rare mRNA species in tissues and samples. Although TGFβ1 mRNA can be detected in mouse embryos at different stages of development (Paria et al., 1992), quantification of the message has not been demonstrated. In this study, we reported, for the first time, a sensitive quantitative competitive RT–PCR method using an RNA mimic to quantify TGFβ1 expression in preimplantation mouse embryos. We also examined the mRNA and protein expression of TGFβ receptors in preimplantation embryos. To study the role of TGFβ1 in the interaction between the embryo and the reproductive tract, the temporal expression of TGFβ receptors, at both mRNA and protein levels, in the uterus and oviduct was also investigated. Our findings, when considered together, are consistent with the hypothesis that there is a close interaction between the preimplantation embryo and the reproductive tract via TGFβ1.

Isolation of mRNA

Messenger RNA from mouse embryos was extracted by Dynabeads mRNA Direct Kit (Dynal AS, Oslo, Norway) according to the manufacturer’s protocol. In brief, the zona pellucidae of mouse embryos were dissolved by acid Tyrode treatment as we failed to extract mRNA with the mRNA extraction kit from embryos with intact zona pellucidae (unpublished data). A known number (usually 15–40) of embryos was lysed and mixed with Dynabeads oligo (dT)25 followed by repetitive washing and elution of poly(A)+ RNA. mRNA was finally eluted with 10–20 µl of diethyl pyrocarbonate (DEPC)-treated water.

Frozen oviducts (n = 3–6, for each day) and uterus were first homogenized in stainless steel grinders pre-chilled at −70°C and total RNA was prepared by Trizol Reagent (Gibco/BRL, Gaithersburg, MD, USA) according to the manufacturer’s protocol. The quality of total RNA was determined by both the ratio of A260/A280 and RNA gel electrophoresis.

Synthesis of RNA mimic for TGFβ1 (cRNA)

TGFβ1 mimic (mTGFβ1), created by inserting a 152 bp fragment from human oviduct-specific glycoprotein (accession no. U09550, nt 1067–1220) into the TGFβ1 gene, was cloned downstream to the T7 promoter of pBlueScript SK+ vector (Stratagene, La Jolla, CA, USA). RNA of mTGFβ1 (cRNA) was synthesized by in-vitro transcription using MEGAscript T7 in-vitro transcription kit (Ambion, Austin, TX, USA). cRNA was further confirmed to be free of DNA template by PCR without reverse transcription and was finally diluted to ~100 fmol/µl with glycogen (50 µg/ml) in DEPC-treated water and stored in aliquots at −70°C.

RT–PCR

Reverse transcription was done using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech., Uppsala, Sweden) according to the manufacturer’s protocol. A negative control was included by replacing Bulk First-Strand cDNA Reaction Mixes with water. All cDNA was kept on ice until used for PCR in the same day. Prolonged storage of cDNA for RT–PCR was avoided, as this was prone to give inconsistent results that may have been due to the adherence of minute amounts of mRNA to the wall of the tube.

All oligonucleotide primers, except those for TβRI (ALK-5), were designed by software Primer Premier (Premier Biosoft International, Palo Alto, CA, USA) using published cDNA sequences retrieved from GenBank. All primers were synthesized from Gibco/BRL. The sequences of primers are listed in Table I. For competitive RT–PCR between TGFβ1 and mTGFβ1, duplicated PCR reactions were performed for each concentration of RNA mimic. For each PCR reaction (final volume 50 µl), one-third of the cDNA was mixed with PCR components to a final concentration of 1× PCR buffer (10 mmol/l Tris–HCl, 1.5 mmol/l MgCl₂, 50 mmol/l KCl, pH 8.3), 0.2 mmol/l of dNTP (Boehringer Mannheim) and 0.4 µmol/l of each forward and reverse primer. After a first denaturation step at 95°C for 5 min, 2 units of Taq polymerase was added. The PCR mixture was then subjected to 40 cycles of amplification with a programme as follows: 94°C for 20 s, 60°C for 30 s and 72°C for 1 min. An extension step at 72°C for 5 min was added at the end of amplification. TGFβ receptor mRNA transcripts in embryos were amplified as above, but the reaction mixtures were cycled for 60 cycles with steps that consisted of 94°C for 45 s, 60°C for 45 s and 72°C for 45 s. PCR products for TGFβ1, TβRI (ALK-5), and TβRII were confirmed by AarII, XhoI and PstI digestion respectively. All PCR products were separated on 2.5% Nusieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME, USA) with 0.5 µg/ml ethidium bromide in 1×TBE buffer (90 mmol/l Tris–borate, 2 mmol/l EDTA, pH 8.0).

Materials and methods

Collection of embryos, oviducts and uteri

Female F1 (♀C57BL/6J×♂A2G) mice underwent ovarian stimulation with intraarterial injections of 5 IU pregnant mare’s serum gonadotrophin (Sigma, St Louis, MO, USA) followed 48 h later by 5 IU human chorionic gonadotrophin (HCG; Sigma). The animals were mated with BALB/C males. One-cell, 2-cell, 4-cell and 8-cell embryos, morulas and blastocysts were obtained by flushing the oviduct or uterus at 18–20, 42–44, 50–52, 66–72 and 90–92 h post-HCG respectively. Embryos of identical cell stage at each specified period were pooled for RNA extraction. Unfertilized oocytes were obtained from the oviduct of unplugged mice at 18–20 h post-HCG. The morning with the presence a vaginal plug was defined as day 1 of pregnancy and the mice were killed between 14:00 and 15:00 on the indicated day of pregnancy (days 1–4). The oviducts and the uteri of the stimulated animals were flash-frozen in liquid nitrogen immediately after being flushed free of embryos, and stored at −70°C until use.
Quantification of PCR products

Gel images were captured on Polaroid 667 film (Polaroid Co., Cambridge, MA, USA) after UV illumination, converted into digitized signal by a densitometer and quantified by ImageQuant Program (Molecular Dynamics, Sunnyvale, CA, USA) as described (Lee et al., 1999).

Hybridization probes

PCR products of TβRI (ALK-5), TβRII and β-actin were obtained by PCR with specific primers as listed in Table I. They were gel-purified and radiolabelled by [α-32P]dCTP with Rediprime II DNA labelling system (Amersham Pharmacia Biotech.). All radiolabelled probes were purified by spin columns (Princeton Separations, Adelphia, NJ, USA). The specific activities of the probes were ~2×10⁹ d.p.m./μg.

Northern blot analysis

Total RNA (2.0 μg) was denatured, size-fractionated on 1% agarose–2.2 mol/l formaldehyde gel electrophoresis, and transferred to Hybond N+ membrane (Amersham Pharmacia Biotech.). RNA was cross-linked to the membranes by UV irradiation (Spectrolinker XL-1000; 2.2 mol/l formaldehyde gel electrophoresis, and transferred to Hybond°). Hybridization was achieved by soaking the blots in boiled 0.1% SDS for 30 min twice. Hybridization signal was detected by autoradiography at ~70°C with intensifying screens. Autoradiographs were converted into digitized signal by a densitometer and quantified by ImageQuant Program (Molecular Dynamics, Sunnyvale, CA, USA).

Western blotting

Samples of oviduct and uterine tissue (n = 3 samples per day) were homogenized in stainless steel grinders pre-chilled at ~70°C and lysed in 1×SDS protein sample buffer (50 mol/l Tris–HCl, pH 6.8, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol, 1% β-mercaptoethanol). The lysates were denatured for 5 min at 95°C, fractionated by 12% SDS–polyacrylamide gel electrophoresis and then transferred to a PVDF membrane (Sambrook et al., 1989). The membranes were blocked with 5% skim milk in TBST (10 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 0.1% Tween 20) and probed with rabbit anti-Tβ RI, anti-Tβ RI (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse anti-β-actin antibodies (Sigma) at 1:1000 dilutions. The membranes were washed three times with TBST for 15 min each and incubated with either anti-rabbit IgG conjugated with horseradish peroxidase (HRP) or anti-mouse IgG conjugated with HRP at 1:1000 dilutions for 1 h at room temperature. The membranes were washed and the signals were visualized by enhanced chemiluminescence (ECL), according to manufacturer’s recommendations (Amersham Pharmacia Biotech.).

Immunohistochemistry

Frozen oviducts (n = 3 samples per day) and uteri (n = 6–8 samples per day) were sectioned at 6 μm and mounted onto Tespa (3-aminopropyltriethoxysilane; Fluka)-coated microscope slides. The sections were fixed in acetone at ~20°C for 20 min and then hydrated in TN (0.05 mol/l Trizma base, 0.5 mol/l NaCl, pH 8.6). After blocking for 30 min in 3% bovine serum albumin (BSA)/TNT (0.05 mol/l Trizma base, 0.5 mol/l NaCl, 0.5% Triton X-100, pH 8.6), the sections were treated with the primary anti-TβRI antibody (rabbit IgG, 4 μg/ml; Santa Cruz Biotechnology) or anti-TβR II antibody (rabbit IgG, 4 μg/ml; Santa Cruz Biotechnology), diluted in TNT overnight at 4°C. After being carefully rinsed in TNT, sections were incubated at 37°C for 1 h with the secondary antibody, sheep anti-rabbit IgG conjugated with Cy3 (Sigma), diluted 1/200 with TNT. Control sections were incubated with normal rabbit IgG (4 μg/ml; Santa Cruz Biotechnology) or primary antibody pre-neutralized with an excess of blocking peptides according to manufacturer’s protocol (February 99, Santa Cruz Biotechnology). Immunohistochemical staining with each primary antibody was repeated at least twice.

All embryos were fixed in 3.7% formaldehyde/phosphate-buffered saline (PBS) for 30 min at room temperature after thorough washing in 0.3% BSA/PBS. Embryos were then permeabilized in PBS solution containing 0.1% Triton X-100 (Sigma) for 2 min on ice. After blocking for 30 min in 3% BSA/PBS, separate embryos were allowed to react with anti-TβRI antibody (Santa Cruz Biotechnology) or anti-TβR II antibody (Santa Cruz Biotechnology) diluted in 0.3% BSA/PBS/0.1% Tween 20 (Tβ RI, 0.4 μg/ml; Tβ RII, 0.2 μg/ml) overnight at 4°C. A sheep anti-rabbit IgG conjugated with Cy3 diluted 1/1000 in 0.3% BSA/PBS/0.1% Tween 20 was used as the secondary antibody. To control for the non-specific immunofluorescence,
Figure 1. Quantification of TGFβ1 transcripts in preimplantation mouse embryos. (A) Value of TGFβ1 mRNA detected at different stage mouse embryos. The mean amount of TGFβ1 transcripts per embryo ± SD is shown for different developmental stages. +, TGFβ1 was detected, but the transcript amount was too low to be quantified by this method. –, no TGFβ1 detected. All results were repeated at least three times. Values with same superscripts: P < 0.05 between different cell stages as determined by one-way analysis of variance followed by Student–Newman–Keuls test. (B) Change in TGFβ1 transcript level at different cell-stages. The results represent the mean quantity (×10^3 ± SD).

<table>
<thead>
<tr>
<th>Cell Stage</th>
<th>Number of TGFβ1 transcripts per embryo ± SD</th>
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<tbody>
<tr>
<td>UF</td>
<td>+</td>
</tr>
<tr>
<td>1C</td>
<td>–</td>
</tr>
<tr>
<td>2C</td>
<td>4,410 ± 1,330^a,e</td>
</tr>
<tr>
<td>4C</td>
<td>12,800 ± 5,140^b,f</td>
</tr>
<tr>
<td>8C</td>
<td>58,660 ± 17,300^a,b,c,d</td>
</tr>
<tr>
<td>Morula</td>
<td>38,100 ± 6,500^c,e,f,g</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>1,520 ± 546^d,g</td>
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Figure 2. Reverse transcription–polymerase chain reaction (RT–PCR) of TGFβ receptor mRNA from preimplantation mouse embryos using Tβ RI (ALK-5) and Tβ RII specific primers. RT–PCR products were separated on 2.5% Nusieve 3:1 agarose gel in 1×TBE buffer. Lane 1, unfertilized oocytes; lane 2, fertilized oocytes; lane 3, 2-cell embryos; lane 4, 4-cell embryos; lane 5, 8-cell embryos; lane 6, morulas; lane 7, blastocysts; lane 8, negative control with water.

The insertion mutant of TGFβ1 (mTGFβ1) shares the same primer sequences with TGFβ1, with a size difference of only 152 bp. Our kinetic study showed that TGFβ1 and mTGFβ1 have similar amplification efficiencies under competitive conditions (data not shown). In this study, mRNA, extracted from a pool of embryos at the same stage of development, was divided into five equal portions and mixed with four different concentrations of mTGFβ1 (1.08, 0.36, 0.12, 0.04 amol, for 4-cell, 8-cell and morula; 0.36, 0.12, 0.04, 0.013 amol for 2-cell and blastocyst). A negative control for cDNA synthesis was included. Each competitive RT–PCR was carried out in duplicate and each experiment was repeated at least three times using different pools of embryo. The sensitivity of this assay was 7800 transcripts per reaction (unpublished data). In order to determine the minute expression level of TGFβ1 in embryos, 15–40 embryos, depending on the developmental stages, were pooled for analysis. With the use of an appropriate and reasonable number of embryos, we detected down to 1520 TGFβ1 mRNA transcript per blastocyst.

Results

Quantitative competitive RT–PCR of TGFβ1 in mouse embryos

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The calculated amount of TGFβ1 mRNA transcripts in embryos at different stages is shown in Figure 1A. TGFβ1 mRNA was first detected in unfertilized oocytes; however, the expression level was too low to be quantified by this method. No TGFβ1 mRNA was detected after fertilization at the 1-cell stage. Then TGFβ1 mRNA was detected again after first cleavage, and the expression level increased gradually and then decreased gradually. The relative intensity of images was adjusted according to their corresponding negative control.

Statistical analysis

All statistical analysis was carried out by one-way analysis of variance followed by Student–Newman–Keuls Test (Armitage and Berry, 1994). P < 0.05 was regarded as statistically significant.
reached a maximum at the 8-cell stage, then declined rapidly to the blastocyst stage (Figure 1B). The levels of TGFβ1 mRNA expression at the 8-cell and morula stages were significantly higher than at all other cell stages (P < 0.05).

Expression of TGFβ receptors in preimplantation mouse embryos

Figure 2 shows the RT–PCR products with the predicted sizes of 315 bp for TβRI (ALK-5) and 279 bp for TβRII using respective specific primers. The mRNA for TβRI (ALK-5) was detected at all cell stages studied, while mRNA expression of TβRII was only detected at the blastocyst stage. Each experiment was repeated at least three times using different pools of embryos.

TβRI and TβRII antibodies bound to the blastomeres, but not the zona pellucida, of 1-cell (Figure 3A and E), 2-cell (B and F), morula (C and G), and blastocyst stage (D and H) mouse embryos. In contrast with the restricted TβRII mRNA expression at only the blastocyst stage, TβRII immunoreactivity was detected at all the embryonic stages studied. Incubation of embryos with rabbit normal IgG (I) or primary antibody pre-neutralized with excess blocking peptide (data not shown), followed by Cy3-conjugated secondary antibody, gave no staining. Computer image analysis of the embryos that had been stained simultaneously showed that the fluorescence intensity due to TβRII immunoreactivity relative to background signal decreased as the embryo developed from the 1-cell to the blastocyst stage.

Northern blot analysis of TGFβ receptors in the mouse oviduct and uterus

The relative levels of TβRI (ALK-5) and TβRII mRNA in the oviduct and uterus were examined by Northern blot hybridization using 32P-labelled DNA probes (Figure 4A). In
Figure 4. (A) Northern blot detection of TβRI (ALK-5) and TβRII mRNA in the mouse oviduct and uterus on days 1–4 of pregnancy. Total RNA (2 µg/lane) from oviduct and uterus was extracted by Trizol Reagent, separated by formaldehyde–agarose gel electrophoresis, transferred to Hybond N+ membrane, and hybridized to 32P-labelled DNA probes. β-actin mRNA was used as internal control for the size and relative abundance of transcripts. (B) Relative levels of TβRI and TβRII mRNA in mouse oviduct (OD1–4) and uterus (UT1–4) on days 1–4 of pregnancy. Northern blots used to produce the autoradiographs shown in (A) were analysed using ImageQuant Program (Molecular Dynamics, Sunnyvale, CA, USA). The amount of mRNA relative to β-actin mRNA was calculated, and the relative amount of mRNA on day 1 was expressed as 100%.

In this experiment, the housekeeping gene, β-actin, served as an internal standard for size and relative abundance of mRNA. After correction of the expression levels of mRNA against that of β-actin, the relative abundance of mRNA at difference stages is expressed graphically in Figure 4B. A single TβRI (ALK-5) transcript (~5.4 kb) was detected in both the oviduct and uterus on days 1–4 of pregnancy (Figure 4A, the uppermost panel). The expression levels of this transcript in the oviduct remained virtually unaltered within this period (Figure 4B, uppermost panel). However, its expression in the uterus was low on day 1 of pregnancy (Figure 4B, second panel) and increased gradually on day 2. There was a nearly 3.5-fold increase in expression on day 3, but the level dropped on day 4. A single mRNA transcript of TβRII (~4.2 kb) was detected in the oviduct and uterus on days 1–4 of pregnancy (Figure 4A, second panel). As shown in the last two panels of Figure 4B, the expression levels of this transcript did not change much in either the oviduct or uterus throughout the studied period.

Protein expression of TGFβ receptors in mouse oviduct and uterus

Figure 5 shows the Western blot analysis of TβRI and TβRII in mouse oviduct and uterus. A major single immunoreactive band was observed for TβRI and TβRII respectively throughout the studied period. Although β-actin was used as a loading control, the Western blot data were interpreted only qualitatively as the amount of receptor proteins relative to β-actin fluctuated between replicated experiments.

In order to characterize the temporal and spatial protein expressions of the receptors, immunohistochemical staining was performed in the oviduct (Figure 6) and uterus (Figure 7). Control sections were incubated with normal IgG (Figures
embryos is critical in order to identify the importance of certain growth factors during development. However, there are only a few reports showing the quantification of transcripts in embryos. One study quantified the mRNA of interleukin-1 by RT–PCR using β-actin for normalization (Huang et al., 1997); however, it has been reported that expression of β-actin rapidly undergoes changes during embryonic development (Bachvarova et al., 1989). Another study used heterologous cDNA as a mimic in competitive RT–PCR (Johnson et al., 1997). This method may underestimate the transcript level for two reasons. First, using a DNA mimic neglects the suboptimal reverse transcription efficiency; usually 40–50% of mRNA is reverse transcribed (Berger et al., 1983; Bouaboula et al., 1992). Second, a heterologous sequence mimic may have different denaturation characteristics with the target and may result in a different amplification efficiency (Siebert and Larrick, 1993). In order to alleviate the above limitations, we performed competitive quantitative RT–PCR using a TGFβ1 mimic with a sequence homologous to the wild type TGFβ1. This is the first report on the use of such a technique for quantifying gene expression in mouse preimplantation embryos. The PCR products for TGFβ1 and mTGFβ1 have a similar molecular weight (only 152 bp difference) and share identical primer sequences. This should minimize the discrepancy in their amplification efficiency. In fact, our kinetics study showed that TGFβ1 and mTGFβ1 were amplified with similar efficiency (data not shown). The sensitivity of this quantification method is ~1520 transcripts per embryo.

In this study, the TGFβ1 transcript was first detected by competitive RT–PCR in unfertilized oocytes but disappeared in the 1-cell embryo. This suggests that the TGFβ1 mRNA is maternal in origin and is degraded after fertilization. However, Paria and co-workers previously reported a low but detectable TGFβ1 immunoreactivity in 1-cell embryos. This could be due to a faster degradation of mRNA than its corresponding protein (Paria et al., 1992). It is also possible that the discrepancy could be due to the use of different strains of mice in the two studies. When the zygotic genome is activated at the 2-cell stage (Telford et al., 1990; Schultz, 1993), a low level of TGFβ1 expression was found, suggesting that the observed transcript was derived from embryonic genome. The expression of TGFβ1 continued to increase through the 4-cell stage to a maximum at the 8-cell stage. The expression declined rapidly thereafter.

TβRI mRNA was detected in the oocyte as well as the embryo at all stages of development. This is consistent with the detection of TβRI immunoreactivity from 1-cell to blastocyst stage. On the other hand, TβRII mRNA was present only in the blastocyst whereas its immunoreactive signal dropped from the 1-cell to blastocyst stage. The presence of both TβRI and TβRII proteins is in line with the observation that iodinated TGFβ1 and TGFβ2 ligands bind to embryos from the 8-cell stage onward (Paria et al., 1992).

The exact reason for the discrepancy between TβRII mRNA and protein expression profiles in this study is unknown. It is possible that mRNA degrades much faster than its corresponding protein. Although we have failed to demonstrate the presence of TβRII mRNA in oocytes, and this may be due to

6F and 7L) or with primary antibodies pre-neutralized with an excess of specific blocking peptides (data not shown). These sections showed greatly reduced or no immunostaining.

In the oviduct, anti-Tß RI (ALK-5) exclusively stained the apical region of tubal epithelial cells (Figure 6B). Anti-Tß RII also intensively stained the tubal epithelial cells (Figure 6D). The intensity of staining for both receptors in the oviduct remains virtually the same from days 1–4 of pregnancy. Therefore, only a day 1 section is depicted in Figure 6.

In the uterus, weak Tß RI immunostaining was observed in the glandular epithelia on day 1 and day 2 (Figure 7E and F). More intense staining was detected on day 3 (Figure 7G), mainly restricted to the luminal epithelia. In contrast to the Western blot result, no immunopositive signal was detected on day 4 (Figure 7H). Immunoreactive Tß RI was located to the luminal and glandular epithelia of the uterus on all the days examined. As the intensity of staining for Tß RI remained similar from days 1–4, only a day 2 section is depicted in Figure 7J.

Discussion

A number of studies have reported mRNA expression of growth factors in embryos (Kane et al., 1997), but most of them have only demonstrated the expression qualitatively in an all-or-none fashion. Quantification of gene expression in

Figure 6. Immunohistochemical localization of TßRI and RII in mouse oviduct collected on days 1–4. Only results on day 1 sections are shown, as the results are indistinguishable from those for other days. (A, C and E) Bright fields of oviduct section; (B and D) immunohistochemical localization of TßRI and TßRII respectively. (F) Negative control incubated with normal IgG. Original magnification ×400.
Figure 7. Immunohistochemical localization of Tβ RI and RII in mouse uterus collected on days 1–4. All photographs were adjusted identically so that differences in brightness reflect differences in the staining intensity. (A–D) Bright fields of day 1–4 uteri respectively; (E–H) immunohistochemical localization of Tβ RI in day 1–4 uteri respectively; (I) bright field of day 2 uterus; (J) immunohistochemical localization of Tβ RII in day 2 uterus. Since day 1–4 uterine sections stained virtually the same with Tβ RII, only day 2 uterine sections are shown. (K) Bright field of negative control; (L) negative control incubated with normal IgG. Original magnification ×200.

fast degradation of mRNA at the time of oocyte collection, we are of the opinion that it is present in the oocyte for two reasons. First, Tβ RI and Tβ RII immunoreactivities are present in unfertilized human oocytes (Osterlund and Fried, 2000). Second, TGFβ has been implicated to play a regulatory role in follicular development, oocyte maturation and ovulation (Juneja et al., 1996). We believe that the maternal Tβ RII mRNA degrades rapidly after fertilization. Roelen and co-workers also reported the presence of Tβ RII mRNA only in the fertilized oocyte and blastocyst but not in other intermediate developmental stages in another strain of mouse (Roelen et al., 1998). In the present study, Tβ RII protein persisted but dropped gradually from the 1-cell to the blastocyst stage. In connection with this, it has been reported that a number of major and minor proteins persist in preimplantation embryos at all stages of development (Sasaki et al., 1999). Some of these proteins have an expression profile similar to the protein expression pattern of Tβ RII reported here. Tβ RII protein has been detected on the cell surface of 1-cell, 2-cell and blastocyst stage embryos of another mouse strain (Roelen et al., 1998). Tβ RII synthesis starts again at the blastocyst stage. Thus, the Tβ RII protein detected in the blastocyst could be of both maternal and embryonic origins.

In a knockout study, severe embryonic lethality has been shown to occur in TGFβ1 knockout embryos on a predominantly CF-1 genetic background, with ~50% of the 129xCFl Tgfb1−/− embryos dying prior to the morula stage (Kallapur et al., 1999). Furthermore, transient expression of truncated Tβ RII in fertilized oocytes stops the embryo from dividing at the 2-cell stage, an effect that can be rescued by co-injecting with constitutively active Tβ RI (Roelen et al., 1998). These findings indicate that TGFβ signalling is necessary for embryos to pass the 2-cell stage when the embryonic genome is activated. The increased production of TGFβ1 mRNA at the 8-cell stage, the presence of immunoreactive TGFβ1 (Paria et al., 1992) and the appropriate TGFβ receptors in the embryo suggest that autocrine TGFβ signalling is important in early embryo development. Furthermore, oviductal TGFβ1 may also modulate embryo development in a paracrine manner.

TGFβ1 expression in the mouse oviduct is virtually unaltered throughout the oestrous cycle in mouse (Dalton et al., 1994). We demonstrate for the first time the presence of Tβ RI and Tβ RII mRNA and protein expression in the oviduct. Similar to their ligand, their expression remains fairly constant in the first 4 days of pregnancy. These findings suggest that the oviduct is responsive to TGFβ1 and that the growth factor may have an autocrine function in the oviduct.

Early studies have shown that there is a continuously changing requirement in the embryo during early development (Leese, 1995). Alteration of oviduct biochemistry by the embryo has been demonstrated (Stein and O’Neill, 1994; Murray, 1995; Tadokoro et al., 1995). A recent report identified a number of genes, including TGF-α and TGFβ-binding protein II, that are differentially expressed in porcine oviduct containing early embryos compared with control oviduct (Chang et al., 2000). We postulate that the increased production
of TGF\(\beta_1\) by the 8-cell embryo and morula acts additionally or synergistically with the oviductal TGF\(\beta_1\) to modulate the function of the oviduct. It is known that the oviducal cells enhance mouse embryo development (Liu et al., 1995) via the production of high molecular weight glycoproteins (Liu et al., 1998). In this connection, it is possible that the paracrine action of TGF\(\beta_1\) on the oviduct may in turn induce the expression of embryotrophic factors.

In this study, we found TGF\(\beta\) receptors in the epithelium of mouse oviduct and uterus. Similar observations have been reported previously in other tissues, e.g. human Fallopian tube (Zhao et al., 1994). In all these studies, receptor immunoreactivities were localized to the cytoplasm of epithelial cells. However, one cannot exclude the presence of immunoreactivity in the membrane of these cells, as light microscopy cannot distinguish cytoplasmic immunoreactivity from membranous immunoreactivity when the former has a positive signal. Future studies using isolated membrane fractions or immunohistochemical staining at electron microscopic levels will be helpful to confirm the presence of membranous TGF\(\beta\) receptors in these tissues.

To the best of our knowledge, this is the first report on the up-regulation of T\(\beta\) RI mRNA and protein in mouse uterus on day 3 of pregnancy. The mRNA and protein levels of T\(\beta\) RI are constant for the first 4 days of pregnancy according to our Northern blot analysis and immunostaining respectively. Immunostaining localized T\(\beta\) RI and T\(\beta\) RII mainly to the luminal or glandular epithelia where TGF\(\beta_1\) and TGF\(\beta_2\) are also located (Tamada et al., 1990). Although we failed to detect T\(\beta\) RI immunohistochemically in day 4 uterus, we believe that T\(\beta\) RI protein is still expressed in this period according to the Western blot result. The discrepancy between the two experiments is probably due to the higher sensitivity of the Western blotting method. In the Western blot, all the T\(\beta\) RI molecules are concentrated in a single band, whereas it is diffused along the whole epithelium in the immunohistochemical method. Moreover, more tissue, and hence more T\(\beta\) RI, are used in the former method. Therefore, these results suggest that there is a decrease in the amount of the receptor in the day 4 uterus. The presence of embryos that were not flushed out of the uterus before protein extraction for the Western blot was unlikely to affect the present result because the amount of T\(\beta\) RI in embryos is low and would not contribute significantly to the results of the blotting analysis.

The above results, along with the previous reported increases in TGF\(\beta_1\) and TGF\(\beta_2\) protein expression in the day 3 and day 4 uterus (Tamada et al., 1990), suggest that TGF\(\beta\) modulates uterine biology during the peri-implantation period. Decreases in TGF\(\beta\) receptor expression (type I, II and III) in mouse uterus interrupts TGF\(\beta\) signalling and results in delayed implantation (Das et al., 1997). Apart from the uterine epithelium, the blastocyst in the uterine cavity is another source of TGF\(\beta_1\). Despite a decrease in mRNA expression of T\(\beta\)RI at the blastocyst stage compared with the 8-cell and morula stage, immunoreactive TGF\(\beta_1\) is still detected in the blastocyst (Rappolee et al., 1988). Mouse blastocysts have been demonstrated to produce TGF\(\beta_1\) that induces apoptosis in uterine epithelial cells (Kamijo et al., 1998). Whether the TGF\(\beta_1\) produced by the 8-cell embryos and morulas is responsible for the enhancement of endometrial receptivity by intra-oviductal embryos (Wakuda et al., 1999) remains to be investigated.

The co-expression of T\(\beta\) RI and T\(\beta\) RII at mRNA and protein levels suggests that the blastocyst is responsive to TGF\(\beta\). This is in line with the observation that TGF\(\beta_1\) promotes blastocyst outgrowth by increasing the endogenous production of parathyroid hormone-related protein (Nowak et al., 1999) and modulates gene expression of the early cavitating blastocyst (Babalola and Schultz, 1995). However whether the source of TGF\(\beta_1\) is from the embryo, from the uterus or a combination of both is unclear.

In conclusion, there is a complex interaction between preimplantation mouse embryos and the reproductive tract. TGF\(\beta_1\) derived from both the embryos and the reproductive tract may act as an autocrine/paracrine factor for embryo development and for modulating the micro-environment within which the embryo develops and implants. The detailed mechanisms of interaction remain to be elucidated.

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