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Differential Expression of Beta Transforming Growth Factors (TGFβ1, TGFβ2, and TGFβ3) and Their Receptors (Type I and Type II) in Peri-implantation Porcine Conceptuses[1]

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ABSTRACT

Beta transforming growth factors (TGFβ1, TGFβ2, and TGFβ3) and type I and II TGFβ receptors were immunohistochemically localized in peri-implantation porcine conceptuses (embryos and associated membranes) collected on Day 10 through Day 14 of gestation. Our results indicate specific immunolocalization of TGFβ isoforms and their receptors in conceptuses during these gestational days. In parietal endoderm, TGFβ1 immunoreactions were weak to undetectable, TGFβ2 immunoreactions were intense, and TGFβ3 immunoreactions were intermediate in intensity to TGFβ2 and TGFβ1. In contrast to immunoreactions in endoderm, TGFβ1 and TGFβ3 immunostaining in trophectoderm (Tr) was intense. Differences in TGFβ2 immunostaining of Tr were observed from Days 10 to 14 of gestation. A drastic decrease in cytoplasmic immunostaining of ectoderm and mesoderm was detected from Days 12 to 14 for all TGFβ isoforms and type II receptor; however, type I receptor immunoreactions were consistently detected between Days 10 and 14. Concurrent expression of both type I and type II receptors in the peri-implantation conceptuses suggests that porcine conceptuses are capable of binding and responding to TGFβs during this period. Differential expression of the three TGFβ isoforms suggests different roles for TGFβs 1, 2, and 3 in conceptus development. Our results suggest possible roles for TGFβs in early growth and differentiation of the embryo, differentiation of the Tr, and implantation.

INTRODUCTION

Members of the transforming growth factor beta (TGFβ) superfamily mediate many key events in normal growth and development [1–3]. TGFβ1, TGFβ2, and TGFβ3 are 12.5-kDa mammalian isoforms that are differentially expressed and are encoded for by distinct genes on separate chromosomes. Regulatory response elements in the promoters of the three different genes differ markedly and may explain their differential regulation during embryonic development [4]. All three isoforms are synthesized as large precursor molecules with an amino-terminal signal sequence and a pro-domain of varying size. This precursor molecule is cleaved off at a dibasic or Arg-X-X-Arg site to release a mature carboxyl-terminal segment of 110–140 amino acids [5].

TGFβs signal via specific high-affinity cell surface receptors that transduce signals through a serine-threonine kinase pathway. The TGFβ receptors designated as type I (50 kDa) and type II (80 kDa) are believed to be critical for signal transduction [6–8]. These receptors are found on most cells, although their ratio varies among different cell types and they bind with TGFβ isoforms with varying affinity [9].

Little is known about the effects of TGFβs on mammalian conceptus development, especially on conceptuses of domestic species. These growth factors are involved in mesoderm induction and body patterning in *Xenopus* and *Drosophila* embryos [10–12]. TGFβ1 immunoreactive molecules have been identified in the early stage of avian embryonic development and appear to influence cell differentiation during gastrulation by altering extracellular matrix (ECM) deposition and by modulating epithelial/mesenchymal transformations [13]. The different isoforms of TGFβs (TGFβ1, TGFβ2, and TGFβ3) exhibit differential expression during mammalian embryogenesis [14]. Transcripts of TGFβ1 and TGFβ1 protein were found in mouse peri-implantation blastocysts [15]. Immunoreactive TGFβ1 and TGFβ2 were found in first-trimester and term human decidua and chronic villi [16] and in ovine placenta [17]. Type I and type II TGFβ receptors were recently immunolocalized in epithelial and mesenchymal tissues during mouse organogenesis [18].

In this study, we have investigated the immunolocalization of TGFß isoforms (TGFβ1, TGFβ2, and TGFβ3) and their type I and II receptors in porcine conceptuses during the peri-implantation period, from Days 10 to 14 of gestation. Our results indicate specific expression of these isoforms and their receptors as gestation progresses.

MATERIALS AND METHODS

Sample Collection

Crossbred gilts were observed daily for estrous behavior and were mated when detected in estrus, and at 12 and 24 h after the onset of estrus. On Days 10, 11, 12, 13, or 14 of gestation, gilts were hysterectomized using sterile techniques. Animal handling and surgical procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Conceptuses were segmentally flushed from the uterus using sterile saline, and individual conceptuses were measured. Conceptuses were collected from three to five gilts on each day of gestation (Days 10–14). A greater number (five to seven) of litters were collected on Day 11 to account for different morphological forms of conceptuses present at this time. Conceptuses were fixed in 4% paraformaldehyde for 8–10 h, washed with 70% ethanol, and embedded in paraffin.

Immunohistochemistry

Fixed and embedded conceptus tissues were cut into 4-μm sections and mounted on slides coated with 2% aminopropyltriethoxysilane (Sigma Chemical Company, St. Louis, MO). Sections were deparaffinized through xylene

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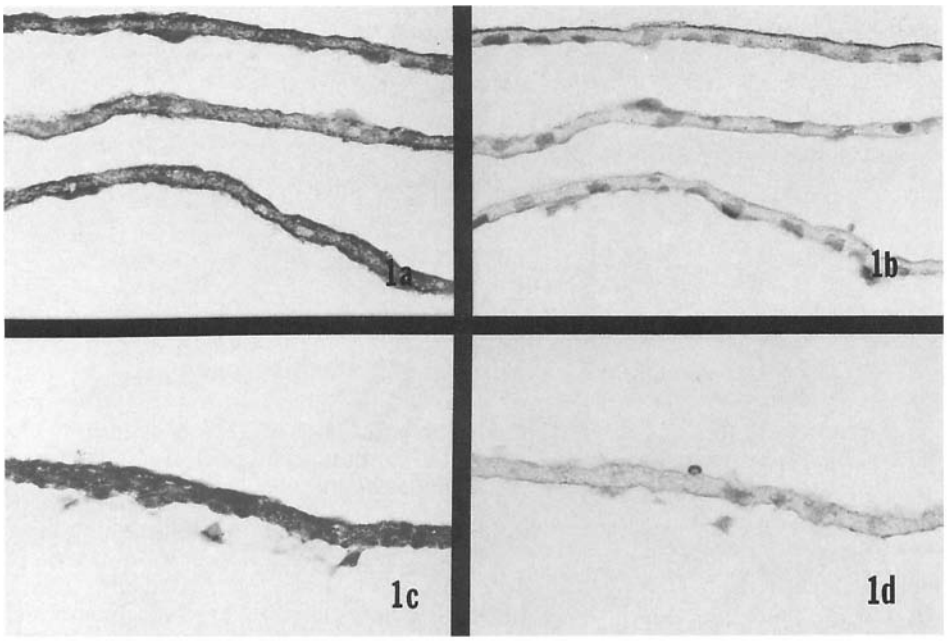

FIG. 1. Representative sections of conceptus Tr (collected at Day 10 of gestation) demonstrating immunoreaction specificity. Sections were counterstained with aqueous hematoxylin. **a**) Anti-TGFβ3; **b**) anti-TGFβ3 preincubated with its control peptide; **c**) anti-TGFβR-I; and **d**) anti-TGFβR-I preincubated with its control peptide. Note the inhibition of immunostaining in (**b**) and (**d**). Similar results were obtained for all other antibodies used in this study (data not shown). ×160.

and graded alcohols and washed with PBS (10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 136.9 mM NaCl, 2.68 mM KCl) and were then blocked for endogenous peroxidase activity. Nonspecific avidin-biotin binding was blocked by sequential application of avidin and biotin, according to the Vector Avidin/Biotin Blocking Kit protocol (Vector Labs., Inc., Burlingame, CA). This was followed by additional blocking with 10% normal goat serum. Sections were incubated with the primary antibody (diluted in PBS containing 2% BSA, pH 7.4) for 14–16 h at 4°C, and then with biotinylated secondary antibody (diluted in PBS/2% BSA, pH 7.4, and 5% normal goat serum) at room temperature for 2 h. Bound antibody was detected using an avidin-biotin complex, peroxidase-linked detection system (Vectastain Elite ABC kit, Vector Labs.) as directed by the manufacturer. Diaminobenzidine was used as the chromogen. For each antibody, in every experiment, representative sections from each stage of conceptus development were run together to allow for comparisons between them. All slides were developed for 30–60 sec. Sections were counterstained with aqueous hematoxylin, and immunoreactivity was assessed by light microscopy.

For controls, primary antibody was replaced by 1) PBS/2% BSA, 2) IgG of the same species, class, and concentration as the primary antibody, or 3) primary antibody that had been preincubated with control peptide for 2 h before use.

Isoform-specific, affinity-purified rabbit polyclonal primary antibodies for TGFβs and their receptors were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TGFβ peptide antibodies were raised against the carboxyl terminal of human TGFβ1 (amino acid residues 328–353), human TGFβ2 (amino acid residues 352–377), and human TGFβ3 (amino acid residues 350–375). Homology between the human peptides used to generate the antibodies and the corresponding porcine sequences is 95–100% [19–22]. The TGFβ receptor antibodies were raised against human TGFβ receptor (R)-I (amino acids 246–266) and human TGFβR-II (amino acids 158–179), which are the conserved kinase domains in rodents, humans, and other species [7]. These receptor antibodies have been tested to work in human, rat, ovine, and mouse paraffin embedded or frozen tissues, as indicated by Santa Cruz Biotechnology. Because of the limited availability of conceptus tissues, immunoblotting and quantitative analyses of the immunoreactive molecules in the porcine conceptuses were not performed.

Antibodies were used at a concentration of 1 μg/ml (TGFβ1, cat. #sc-146), 1 μg/ml (TGFβ2, cat. #sc-90), 0.5 μg/ml (TGFβ3, cat. #sc-82), 0.5 μg/ml (TGFβR-I, cat. #sc-398), and 1 μg/ml (TGFβR-II, cat. #sc-400). Control peptides (Santa Cruz Biotechnology) for each of these antibodies were used at 10 times higher concentration of that of the primary antibody. Rabbit IgG was obtained from Sigma Immunochemicals. Biotinylated secondary antibody (Vector Labs.) was used at a concentration of 5 μg/ml.

RESULTS

Expression of immunoreactive TGFβ isoforms and type I and type II receptors was detected in porcine conceptuses collected from Days 10 to 14 of gestation. Isoform-specific differences in immunostaining were detected as gestation progressed. Among Day 11 conceptuses, no differences were found in immunostaining between spherical and tubular forms. No background immunostaining was detected in sections incubated with IgG (see Figs. 3b, 3e, 4e, 5c, and 6b), or with PBS/2% BSA (secondary antibody control) (data not shown) or with primary antibody preincubated with control peptide (Fig. 1, b and d) for any of the TGFβ isoforms and their receptor antibodies.

Immunolocalization of TGFβs

Embryonic disc/ectoderm. TGFβ1, TGFβ2, and TGFβ3 intracellular, cytoplasmic immunoreactions were intense in embryonic discs on Days 10 and 11 of gestation (Figs. 2a, 3a, and 4a). An apparent decrease in cytoplasmic immunostaining was detected beginning on Day 12 (Figs. 2b and 3c), and immunostaining continued to decrease on Days 13 (data not shown) and 14 (Figs. 3d and 4c). On Day 14, the diffuse immunoreaction of the ectoderm cells was no longer visible, and immunostaining was concentrated near the pe-

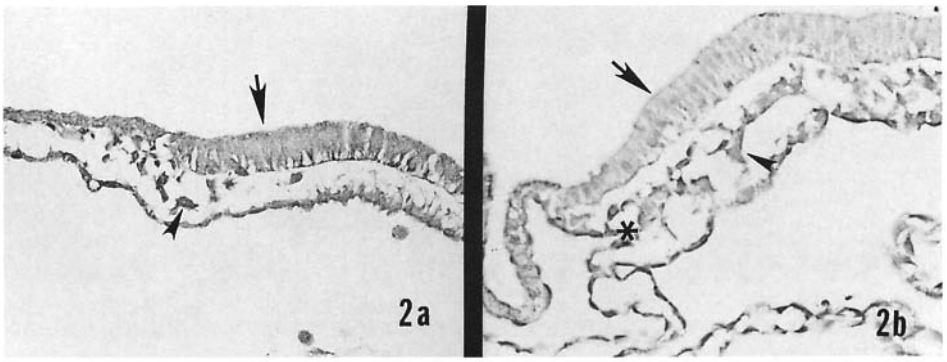

FIG. 2. Immunohistochemical localization of TGFβ3 in Day 11 (**a**) and Day 12 (**b**) conceptuses. Sections were counterstained with aqueous hematoxylin. Embryonic ectoderm is the uppermost layer. Note the decrease in immunostaining in embryonic ectoderm (arrows) from Day 11 to Day 12. Mesoderm cells (arrowheads) were first apparent in Day 11 conceptuses. Somatic and splanchnic mesoderm (asterisk) were first visible in porcine conceptuses at Day 12 of gestation. ×60.

riphery of the cells (Figs. 3d and 4c). No differences in the pattern of expression of TGFβ1, TGFβ2, and TGFβ3 were detected.

Mesoderm. On Day 11, when mesoderm was first distinguishable, intense cytoplasmic TGFβ1, TGFβ2, and TGFβ3 immunostaining was detected in mesoderm (Fig. 3a). Decreases in immunostaining were first observed on Day 12 (Fig. 3c), and continued through Day 14 (Fig. 3, d and f). On Day 14 of gestation, mesodermal cells in close association with the ectoderm and the endoderm had light to moderate cytoplasmic immunoreactions, in contrast to the more central portions of the visible mesoderm in which immunoreactions were markedly decreased or absent (Fig. 3, d and f). This pattern of immunostaining was the same in both embryonic and extra-embryonic mesoderm, and for all three TGFβ isoforms.

Endoderm. Isoform- and location-(parietal vs. visceral endoderm) specific immunostaining was detected in endoderm. Expression of TGFβ1 protein was weak to undetectable in parietal endoderm (Fig. 4f), whereas light cytoplasmic immunopositive reaction was present in visceral endoderm (data not shown). This pattern of immunostaining was consistent for conceptuses between Days 10 and 14 of gestation. Intense TGFβ2 immunostaining was detected in both visceral and parietal endoderm on Days 10 and 11 (Fig. 4, a and b); from Days 12 to 14, this cytoplasmic immunostaining decreased in visceral endoderm (Fig. 4c) but remained intense in parietal endoderm (Fig. 4d). The endoderm staining pattern for TGFβ3 (Fig. 4h) was similar to that of TGFβ2; however, the staining was less intense. Yolk sac was immunopositive for all three TGFβ isoforms (Fig. 5).

Trophectoderm (Tr). Isoform-specific immunostaining was also detected in Tr. Intensely immunopositive TGFβ1 (Fig. 4f) and TGFβ3 (Fig. 4h) immunoreactions in Tr were present on Days 10 to 14 of gestation. Staining for TGFβ2 in Tr was very light on Day 11 (Fig. 4b). On Day 14, TGFβ2 immunostaining was intense in Tr most distal to the embryonic disc (Fig. 4d) but remained weak to undetectable in Tr near the embryonic disc (data not shown).

Immunolocalization of TGFβ Type I and Type II Receptors

Embryonic disc/ectoderm. Type I receptor immunostaining was detected in embryonic discs between Days 10 and 14 (Fig. 6c). This embryonic disc immunostaining was diffuse within the cytoplasm and was more intense on apical cell surfaces. Diffuse cytoplasmic immunoreactivity for type II receptor was detected from Days 10 to 12 (Fig. 6a); cell membrane immunostaining was more intense. On Day 13, type II receptor immunostaining decreased (data not shown), and was undetectable on Day 14 (Fig. 6d).

Mesoderm. Diffuse cytoplasmic immunostaining in both embryonic and extra-embryonic mesoderm was detected for both TGFβ receptor types; as expected, this reaction was more intense on cell membranes. This membranous staining pattern for the type I receptor was present on Days 10 to 14 of gestation (Fig. 6c). Immunostaining for the type II receptor in mesoderm decreased between Days 12 and 14 and was weak to undetectable on Day 14 (Fig. 6, a and d).

Endoderm. Type I and type II TGFβ receptor immunostaining was intense in visceral endoderm between Days 10 and 12 (Fig. 6a) and decreased from Days 13 to 14, when both negative and positive cells were present (Fig. 6, c and d). In parietal endoderm, type I and II receptors were immunopositive only on Days 12 to 14 (data not shown). The yolk sac was immunopositive for both receptor types (data not shown).

Tr. Diffuse, cytoplasmic immunopositive reactions for type I and II receptors were detected between Days 10 and 14 (Fig. 1c). This reaction was more intense in apical regions of Tr, and was especially prominent in conceptuses recovered on Days 13 and 14 of pregnancy (data not shown).

DISCUSSION

Porcine blastocysts begin rapid elongation (30–40 mm/h) and mesoderm differentiation around Day 11 of gestation. At this time, they begin to change from spherical (5–6 mm) to tubular forms (10–50 mm), and then to filamentous forms, which may be > 200 mm in length [23, 24]. On Day 13 of gestation, conceptuses begin attachment to the uterine surface. Results of the present study indicate that immunoreactive TGFβ isoforms (TGFβ1, TGFβ2, and TGFβ3) and their type I and type II receptors are present in porcine conceptuses during the peri-implantation period.

This is the first report of the temporal expression of TGFβ3 protein in the peri-implantation period in domestic species. In agreement with our findings, TGFβ3 mRNA expression in porcine blastocysts from Days 10 to 12 of gestation was reported in an abstract by Yelich et al. [25]. Interestingly, these results are different from those for ovine conceptuses, in which TGFβ3 protein and mRNA were not detected [17]. This could be due to species differences in embryonic development and implantation, or possibly due to the use of different antibodies and probes for detection. TGFβ3 was also reported to be absent from mouse placen-

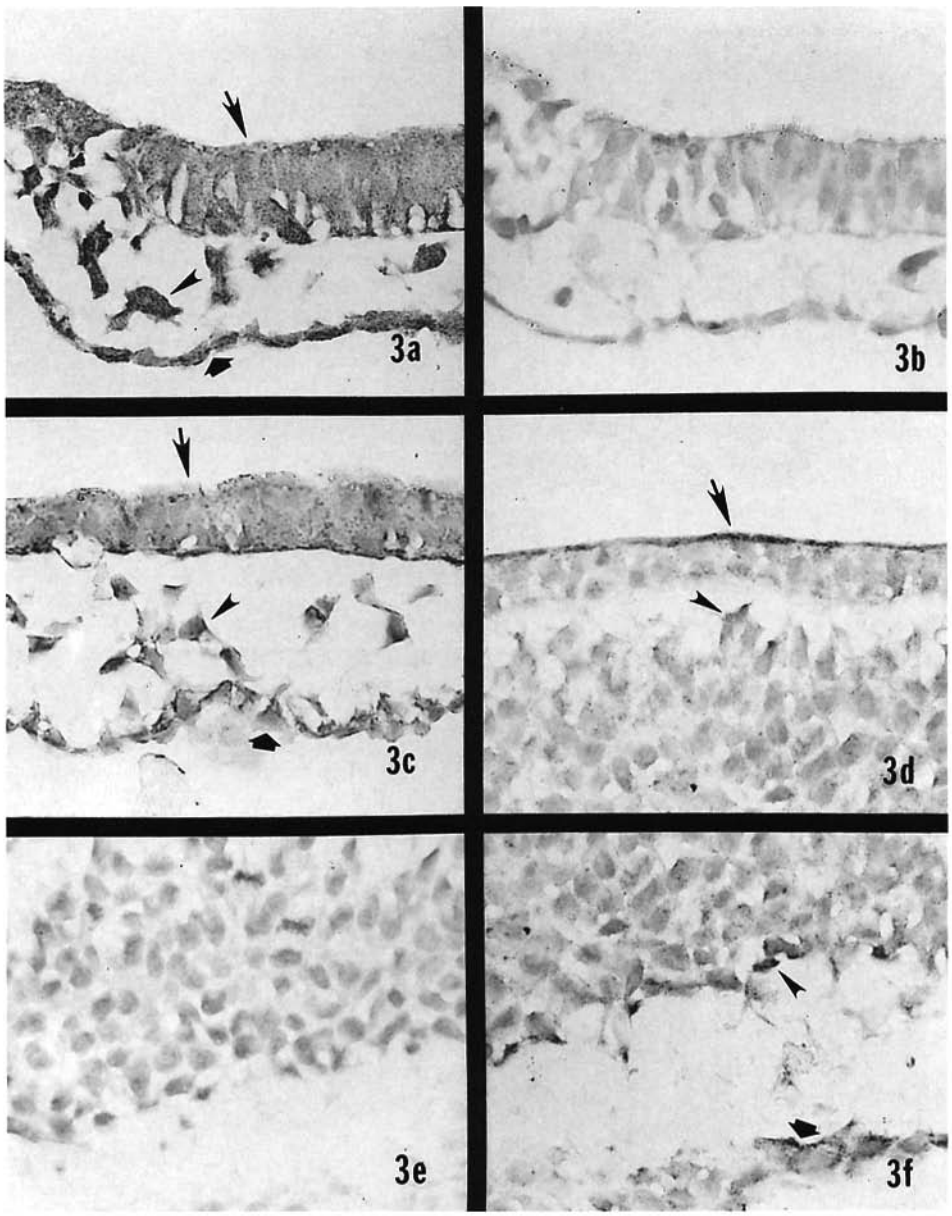

FIG. 3. TGFβ1 immunostaining in conceptuses collected at Days 11, 12, and 14 of gestation. Sections were counterstained with aqueous hematoxylin. Embryonic ectoderm is the uppermost layer, endoderm the lowermost layer, and mesoderm the middle layer. **a**) Day 11 conceptus; **b**) IgG control for **a**; **c**) Day 12 conceptus; **d**) Day 14 conceptus illustrating embryonic ectoderm (arrow) and mesoderm (arrowhead); **e**) IgG control for **f**; and **f**) Day 14 conceptus illustrating mesoderm (arrowhead) and endoderm (broad arrow). Panels **d** and **f** are part of the same section. Note strong immunostaining in mesoderm cells (arrowhead) in **a** and weaker immunostaining in **c, d, f**. In Day 14 conceptuses, immunostaining is evident in mesoderm cells in close association with embryonic ectoderm (**d**) and endoderm (**f**). Additionally, embryonic ectoderm (arrow) immunostaining, which was prominent in Day 11 conceptuses (**a**) decreased in intensity from Days 12 (**c**) to 14 (**d**) of gestation. Decreases in the immunostaining intensity of visceral endoderm (broad arrow) from Day 11 (**a**) to Days 12 (**c**) and 14 (**f**) of gestation were also detected. TGFβ2 and TGFβ3 immunostaining patterns (not shown) for mesoderm and embryonic ectoderm were the same as above. ×160.

ta, although both TGFβ1 and TGFβ2 were detected [26, 27].

The diffuse cytoplasmic immunoreactions of the TGFβs in the present study demonstrate that these proteins are present and may be functional in the different layers of conceptus. This is supported by results from our receptor localization, which indicated diffuse cytoplasmic reactions accompanied by stronger reactions on the cell membrane. However, TGFβs are produced as latent forms, and activation of TGFβs to biologically active forms is under post-translational control [28]. Thus, immunoreactive molecules detected in this study may not represent biologically active peptides in all cells or at all stages in which they were observed. Additionally, we cannot definitively determine the source of the conceptus TGFβs from our current data. Although embryonic mRNAs for TGFβs have been reported to be present in blastocysts of other species [10, 13, 15], TGFβs are also known to be present in endometrium of peri-implantation mouse uterus [27, 29] and human first-trimester decidual tissue [16]. Preliminary immunohistochemical data from our laboratory indicate that TGFβs are present in porcine endometrium during the peri-implantation period (unpublished data); however, the availability of these factors to the developing conceptuses is not known.

Intense TGFβ1 immunoreactions in Tr suggest that TGFβ1 may influence Tr differentiation and attachment. In human Tr, TGFβ1 stimulates synthesis of oncofetal fibronectin [30], an ECM protein implicated in implantation. In addition, TGFβ1 has been shown to stimulate synthesis of various integrin dimers ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$), which are ECM protein receptors [31, 32] that have been implicated as important for Tr differentiation [33]. The integrin subunits α_4, α_5, and β_1 have been detected on the apical surface of peri-implantation porcine Tr (personal communication, J. Bowen and R.C. Burghardt), and TGFβ1 may thus influence porcine conceptus differentiation and implantation through modulation of Tr integrins or ECM proteins. Immunolocalization of type I and type II TGFβ receptors in Tr also suggests that these cells could respond to TGFβ.

Interestingly, embryonic disc and visceral endoderm showed a high level of expression of all three TGFβ isoforms on Days 10 and 11, but showed decreases in im-

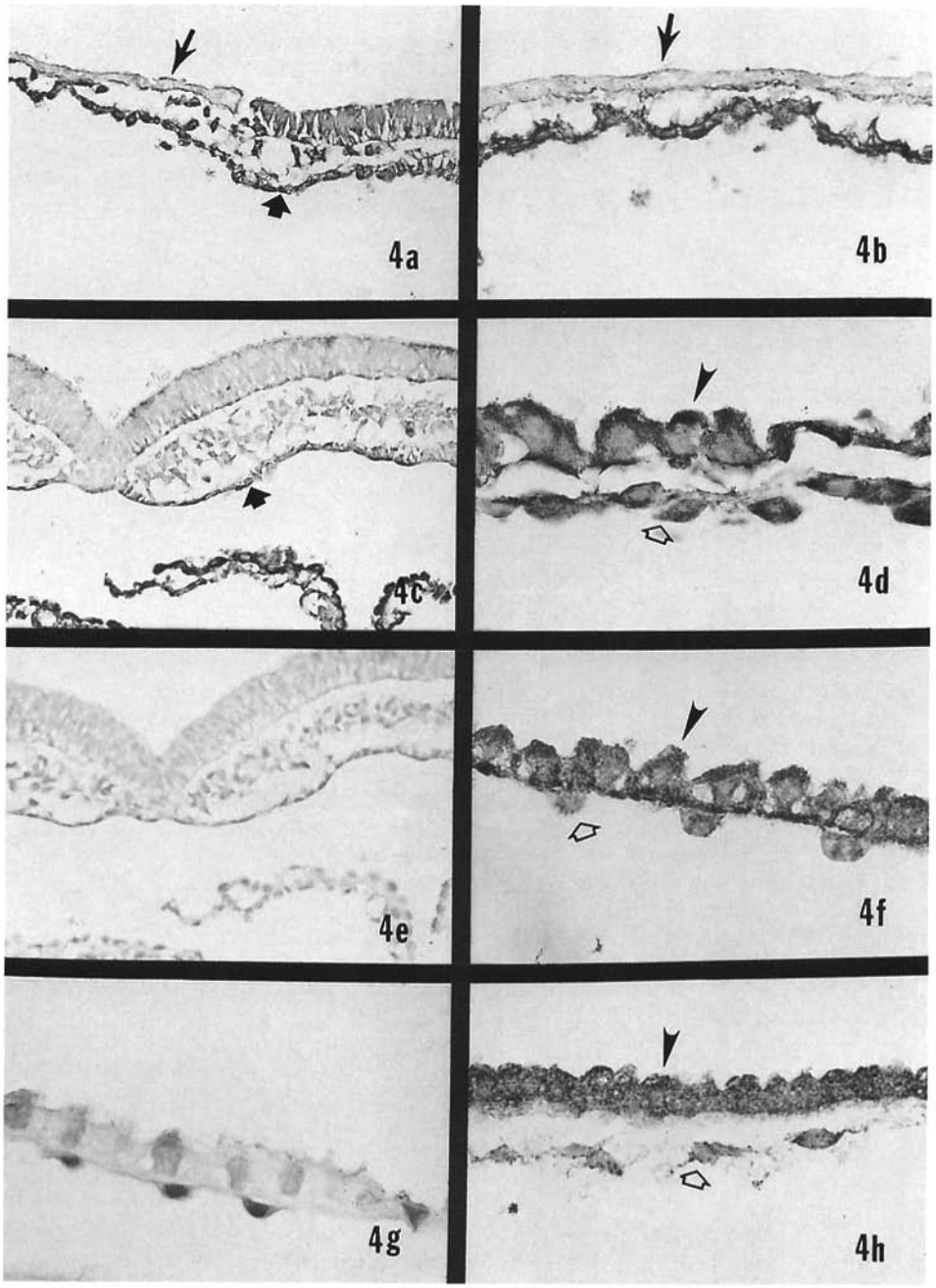

FIG. 4. Immunohistochemical localization of TGFβs in Tr and endoderm in porcine conceptuses from Days 11 to 14 of gestation. Sections were counterstained with aqueous hematoxylin. **a**) TGFβ2 immunostaining in Day 11 conceptus. Note intense visceral endoderm staining (broad arrow) and a weak to undetectable immunostaining in Tr (pointed arrow) close to the embryonic disc (×60). **b**) Higher magnification of Tr (pointed arrow) and endoderm near the embryonic disc of Day 11 conceptus shown in **a** (×160). **c**) TGFβ2 immunostaining in Day 14 conceptus. Compare to **a** and note the decreased immunostaining of visceral endoderm (broad arrow) (×60). **d**) TGFβ2 immunostaining in Day 14 conceptuses; note the intensely immunoreactive parietal endoderm (open broad arrow) and Tr (arrowhead) most distal to the disc (×160). **e**) IgG control for **c** (×60). **f**) TGFβ1 immunostaining in Day 11 conceptus. Note the intensely immunopositive Tr (arrowhead) and weak to undetectable immunostaining in parietal endoderm (open broad arrow) (×160). **g**) IgG control for **f** (×160). **h**) TGFβ3 immunostaining in Day 11 conceptus; immunoreactions were detected in Tr (arrowhead) and parietal endoderm (open broad arrow) (×160).

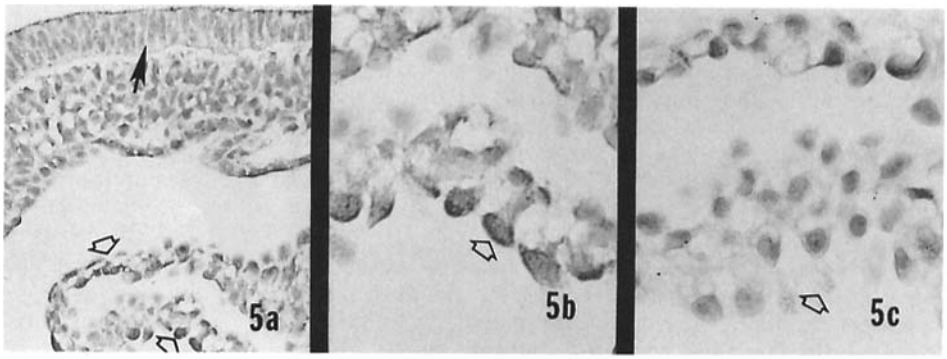

FIG. 5. Immunohistochemical localization of TGFβ1 in yolk sac (porcine conceptus at Day 14 of gestation). Sections were counterstained with aqueous hematoxylin. **a**) Day 14 conceptus with embryonic disc (arrow) and yolk sac (open broad arrow) (×60); **b**) higher magnification of yolk sac in **a** (×160); and **c**) IgG control for **b** (×160). Note immunopositive yolk sac cells in **a** and **b** and the absence of immunostaining in the IgG control. Yolk sac was also immunopositive for TGFβ2, TGFβ3, TGFβR-I, and TGFβR-II (not shown).

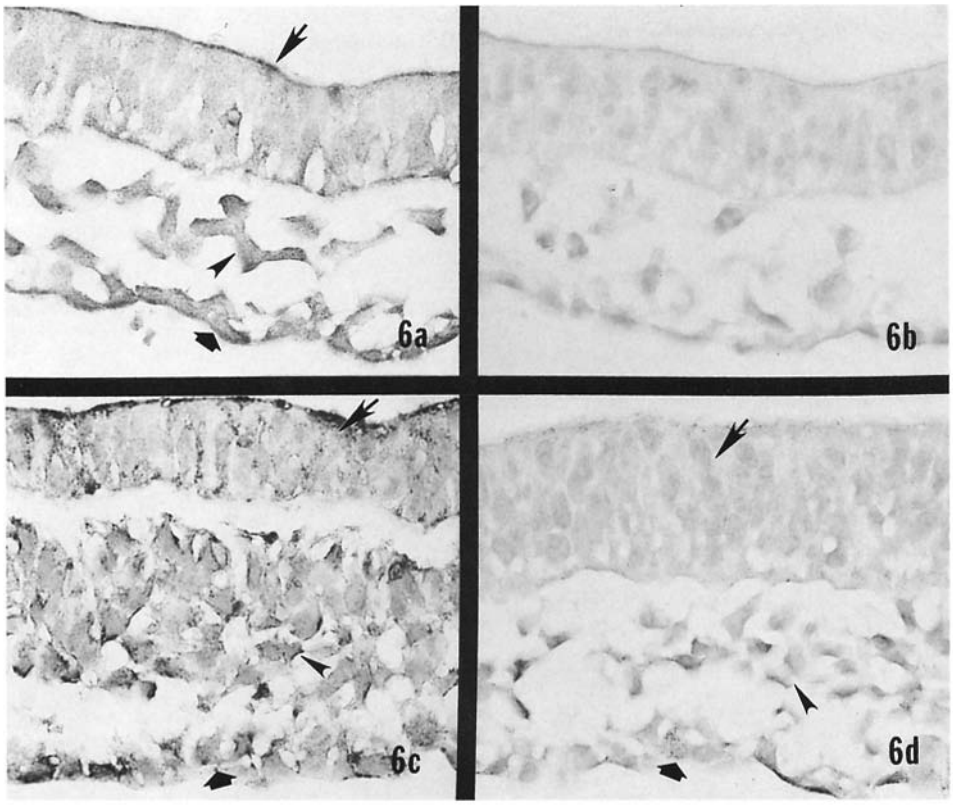

FIG. 6. Immunohistochemical localization of TGFβR-I and TGFβR-II. Sections were counterstained with aqueous hematoxylin. Embryonic ectoderm (arrow) is the uppermost layer, endoderm (broad arrow) the lowermost layer, and mesoderm (arrowhead) the middle layer. **a**) TGFβR-II, Day 12; **b**) IgG control for **a**; **c**) TGFβR-I, Day 14; and **d**) TGFβR-II, Day 14. In Day 12 conceptuses, immunostaining patterns were the same for TGFβR-I (not shown) and TGFβR-II (**a**). On Day 14 of gestation, TGFβR-I immunostaining remained intense (**c**), while immunostaining for TGFβR-II was very weak to undetectable (**d**). ×160.

munostaining from Days 12 to 14 of gestation (Figs. 2, 3, 4a, and 4c). This observation was correlated with decreased type II TGFβ receptor immunoreactivity on Days 13 and 14 (Fig. 6d), although the type I receptor appeared to be constitutively expressed from Days 10 to 14 (Fig. 6c). The exact mechanisms for regulation of expression of TGFβs and their receptors are not known. Changes in the patterns of immunostaining suggest several roles for TGFβs in porcine early conceptus development. First, during Days 10 and 11, TGFβ may modulate or up-regulate expression of ECM proteins between embryonic disc and endoderm. Endoderm, which later contributes to development of extraembryonic (placental) membranes, has been implicated in the production of the ECM proteins laminin and fibronectin in the elongating porcine blastocyst [34]. In cultured murine embryos, these proteins increased mesodermal cell migration by providing suitable substrates [35], and may also contribute to interactions between the endoderm and adjacent Tr in the developing conceptuses. TGFβs have been shown to regulate the synthesis of specific ECM components and integrins [36] in various cell lines, and results of the present study suggest that TGFβs could be involved in initiation or progression of mesoderm migration in porcine conceptuses.

TGFβs have been linked to immunosuppressive activity and thus maternal tolerance of the semi-allogenic fetus [37–39]. High rates of porcine conceptus mortality occur between Days 8 and 16 of gestation [40] and may result, in part, from immunological rejection of the Tr. Therefore, another role for TGFβs in porcine conceptuses during the peri-implantation period may be to suppress maternal immune reactions.

Our results also indicate that TGFβ1, TGFβ2, and TGFβ3 and type I and type II receptor immunoreactive proteins are present in the yolk sac. Dore et al. [17] also reported that TGFβ1 and TGFβ2 protein and mRNA expression was detected in yolk sac and extra-embryonic membranes. The yolk sac serves as the initial embryonic hematopoietic organ, and the presence of TGFβs in yolk sac supports its previously known function as a potent hematopoietic and angiogenic agent [41, 42].

In conclusion, results of the present study indicate that specific TGFβ isoforms and their receptors are expressed by porcine conceptuses during the peri-implantation period and can be temporally associated with specific embryo differentiation and implantation processes. Additional experimentation is ongoing to clarify the role of these growth factors in conceptus development and differentiation, implantation, and early embryonic survival.

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