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Expression of the genes for TGFα, EGF and the EGF receptor during early pig development

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Summary

Expression of mRNA for transforming growth factor-alpha (TGF-α), epidermal growth factor (EGF) and the epidermal growth factor receptor (EGF-R) during early pig development was evaluated by reverse transcription-PCR. In the unfertilised pig oocyte, maternal transcripts for EGF, but not for TGFα or the EGF-R, were detected. Pig conceptuses were analysed at days 7, 8, 10, 12, 15, 17, 18 and 22 of pregnancy. EGF-R mRNA was detected at all stages of conceptus development analysed. Interestingly, TGFα mRNA was expressed by the developing blastocyst only at days 8, 10 and 12 of pregnancy, with the highest levels apparent at day 10. In contrast, EGF mRNA was first expressed by the post-elongation conceptus at around day 15 of pregnancy with levels continuing to increase up to day 22. In the day-18 and day-22 conceptuses, this EGF message was shown to be primarily a product of the embryo-amnion and not the placental membranes. Furthermore, EGF was immunolocalised in the day-22 embryo to the developing lung bud, gut loop and amnion. In summary, the expression pattern of TGFα mRNA during early pig development is coincident with the onset of blastocyst elongation and suggests a possible role for TGFα during this period of cellular remodelling. The temporal and spatial expression of EGF mRNA and protein suggests a possible involvement for EGF in the establishment of the early organ systems.

Key words: TGFα, EGF, EGF-R, oocyte, pig.

Introduction

Development of the mammalian conceptus (embryo and associated membranes) involves a highly complex series of cellular interactions that includes proliferation, differentiation, migration and apoptosis. The expression of several peptide growth factors and their cognate receptors during early embryonic development suggests that these factors may play a role in the regulation of cell proliferation and/or specification of cell fate (see Whitman and Melton, 1989; Simmen and Simmen, 1991). Furthermore, a number of studies have indicated that endogenously synthesized growth factors are able to promote the development of preimplantation embryos in vitro (Biggers, 1971; Wiley et al., 1986; Paria and Dey, 1990).

Epidermal growth factor (EGF) and transforming growth factor-alpha (TGFα) are related growth factors that are synthesized during development of the mammalian conceptus (Werb, 1990). While EGF and TGFα bind to the same cell surface tyrosyl kinase receptor (see Burgess, 1989), different patterns of expression of the factors have been observed during embryonic development in the mouse. TGFα transcripts are detectable in the unfertilised mouse oocyte, rapidly destroyed at the two-cell stage, but are then later re-synthesized by both the pre- (Rappolee et al., 1988; Werb, 1990) and post- (Twardzik, 1985; Wilcox and Derynck, 1988) implantation conceptus. In contrast, expression of the EGF gene is not observed until after implantation has occurred and organogenesis has commenced (Kronmiller et al., 1991; Snead et al., 1989; Warburton et al., 1992). EGF receptor (EGF-R) mRNA and protein have been identified throughout mouse embryonic development, from the unfertilized oocyte (Wiley et al., 1992) through to the post-implantation embryo (Adamson et al., 1981; Adamson, 1990).

In the pig, embryo development proceeds up to the blastocyst stage in a similar manner to that of other mammalian species. However, between days 10.5 and 12 of pregnancy, the pig blastocyst undergoes dramatic cellular remodelling, being transformed from a sphere of about 4 mm in diameter to an elongated filamentous structure up to 1.5 m in length (Geisert et al., 1982). The presence of EGF receptors on the trophoderm of post-elongation pig blastocysts has been demonstrated by the specific binding of ^{125}I-labelled EGF (Corps et al., 1990), suggesting that EGF or related factors could be involved in regulating the growth and development of the early pig conceptus. In this paper, we demonstrate that EGF-R mRNA is present in both pre- and post-elongation blastocysts but that the receptor agonists, EGF and TGFα, display different temporal patterns of expression.

Materials and methods

Animals

Large White gilts were bred by natural service when detected to be in oestrus and again 24 hours later (day of first service = day 0 of gestation). Animals were taken at 7(2), 8(2), 10(4), 12(2), 15(2), 17(1), 18(3) and 22(2) days of gestation (the figures in brackets indicate the number of gilts from which samples were obtained and analysed). Reproductive tracts, removed after the animals were stunned and exsanguinated at a local slaughterhouse, were transported on ice to the laboratory within 20 minutes. Each uterine horn was cannulated at the ovarian end and flushed with 20 ml of Dulbecco's Modified Eagle's medium to recover the conceptuses. Alternatively, on day 22 of pregnancy, embryos and placental membranes were dissected out of the uteri. Total RNA was isolated from fresh tissue by the guanidine thiocyanate procedure (Chomczynski and Sacchi, 1987). RNA samples were quantified by their absorbance at 260 nm and by ethidium bromide staining of samples electrophoresed on agarose gels. Pig primary oocytes (cumulus-free) were obtained from the ovaries of pigs killed at a local slaughterhouse and were a kind gift from Dr R. Larson (Department of Molecular Embryology, Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK).

Oligonucleotide primers

The oligonucleotide primers were synthesized on a Biosearch 8750 four-column DNA synthesizer (Biosearch, San Rafael, CA, USA). All primers contained an 8 bp 5′-extension incorporating a *Bam*H1 restriction site for cloning purposes. The primer pairs were designed to span known introns so that amplification of mRNA (as reverse transcribed cDNA) could be distinguished from amplification of any contaminating genomic DNA. Primer pairs were further checked by PCR to ensure they were unable to amplify sequences from genomic DNA.

TGFα primers were designed according to conserved regions of the rat (Blasband et al., 1990) and human (Derynck et al., 1984) cDNA sequences. The upstream primer (5′-TGCCCAGATTCCCACACT-3′) is identical to nucleotides 280-297 of the rat cDNA, while the downstream primer (5′-TG(G/T)ATCAGCACACA(G/T)GTG-3′) represents the reverse complement of nucleotides 489-506. This primer pair predicts a 243 bp DNA fragment.

EGF primers were designed according to known regions of the pig EGF gene sequence (Pascall et al., 1991). The upstream primer (5′-TCTGAACCCGGACGGATTTG-3′) is identical to nucleotides 6-25 bp upstream of the 3′-end of the pig EGF gene exon 19 (JCP, unpublished data), while the downstream primer (5′-GACATCGCTCGCGAACGTAG-3′) represents the reverse complement of nucleotides 12-31 of exon 21 (Pascall et al., 1991). This primer pair predicts a 214 bp fragment.

EGF-R primers were designed according to conserved regions of the known human, chicken and mouse (Avivi et al., 1991) sequences. The upstream primer (5′-TGGAGGA(G/A)AAGAAAG-3′) represents nucleotides 260-274 of the human cDNA, while the downstream primer (5′-GAT(A/G)ATCTG(G/C)AGGTT-3′) represents the reverse complement of nucleotides 494-508. This primer pair predicts a 264 bp DNA fragment.

β-actin primers were based on the mouse sequence (Tokunaga et al., 1986). The upstream primer (5′-CTACAATGAGCTGCGTGTGG-3′) is identical to nucleotides 192-211 of the mouse cDNA, while the downstream primer (5′-TAGCTCTTCTCCAGGGAGGA-3′) represents the reverse complement of nucleotides 622-641. This primer pair predicts a 450 bp DNA fragment.

Reverse transcription-PCR (RT-PCR)

First-strand cDNA was synthesized from total RNA (5 μg) using a 'First-Strand cDNA Synthesis' kit (Pharmacia) and 50 pmol of the appropriate downstream primer in a total reaction volume of 15 μl. Reaction products were precipitated with 0.1 volumes of 4 M NaCl and 2.5 volumes of ethanol and resuspended in 10 μl of water. Half of the cDNA was subjected to PCR amplification using a Techne PHC-1 programmable Dri-block (Techne Ltd., Duxford, Cambs., UK). The amplification profile comprised 35 cycles: at 94°C for 0.3 minutes (dissociation), 55°C for 0.5 minutes (annealing) and 72°C for 1.0 minute (extension). The final cycle included a further 5 minutes at 72°C for complete strand extension. Each reaction mix contained 5 μl cDNA, 50 pmol of each primer, 5 μl 10× PCR buffer (Promega), 2 μl 5 mM dNTPs and water to 50 μl. After overlaying with 50 μl light mineral oil (Sigma), the reactions were heated to 94°C for 5 minutes prior to the addition of 2.5 u of *Taq* polymerase (Promega). All RT-PCR reactions were performed at least twice for each individual RNA sample.

Southern blot analysis, DNA cloning and sequencing

Aliquots (15 μl) of RT-PCR reaction products were separated by electrophoresis through 1.5% agarose gels and transferred to nylon membrane (Hybond-N, Amersham, UK). Probes were radiolabelled by the random-primed DNA synthesis method (Feinberg and Vogelstein, 1983). High stringency hybridisation and washing conditions were employed as previously described (Pascall and Brown, 1988). PCR products were subcloned into pBluescribe-M13$^+$ and double-stranded DNA prepared using a caesium chloride-based 'miniprep' procedure (Saunders and Burke, 1990). DNA sequencing was performed using the Sequenase Version 2.0 DNA sequencing kit (USB).

Immunocytochemical staining of embryonic EGF

Whole day-22 embryos were fixed in freshly prepared paraformaldehyde solution (4% w/v) and embedded in low-melting-point wax, and serial sections (8 μm) were cut. Sections were cleared in xylene for 15 minutes and rehydrated through an ethanol series into PBSa (138 mM NaCl, 2.8mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). Immunocytochemical staining of sections was performed as previously described (Vaughan et al., 1991), except that the pig EGF antiserum was used at a dilution of 1:100 in PBSa and sections were incubated with the primary antiserum for 6 hours.

Results

EGF and EGF-R expression

Total RNA was isolated from pig conceptuses obtained at days 7, 8, 10, 12, 15, 17, 18 and 22 of pregnancy. Using RT-PCR and EGF-specific primers, EGF mRNA was detected in total RNA isolated from post-elongation conceptuses at day 15 to day 22 of pregnancy where a single amplified band of the predicted size (214 bp) was evident (Fig. 1A). In contrast, no amplified band was detected using total RNA isolated from earlier blastocysts (up to day 12), indicating that the EGF gene is not expressed by the pre-elongation blastocyst (Fig. 1A). These results were typical and represent one of several similar analyses. The amplified product was confirmed as EGF cDNA by determining its nucleotide sequence (data not shown). As a control for RNA integrity, the same day-7 to day-22 samples were analysed by RT-PCR for β-actin expression. Using primers specific for β-actin, all samples gave an amplified product of the predicted size (450 bp) and of approximately equal intensity (Fig. 1C). It is expected that the final levels of

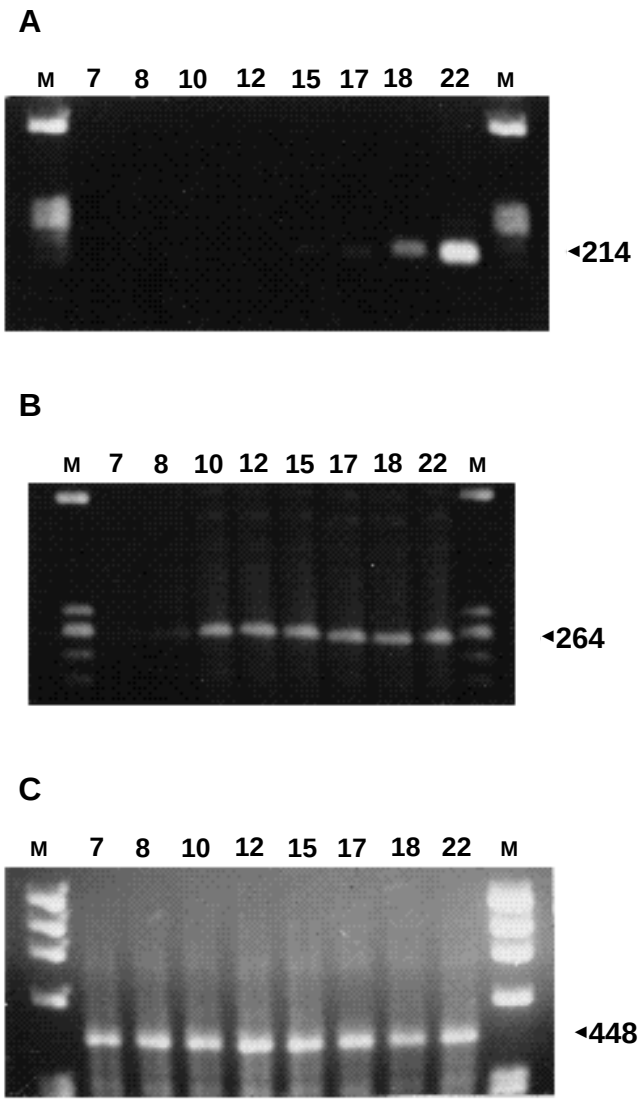

Fig. 1. Expression of EGF and EGF-R mRNA during early pig development. Total RNA was isolated from conceptuses at days 7, 8, 10, 12, 15, 17, 18 and 22 of pregnancy, submitted to RT-PCR using primers specific for (A) EGF, (B) EGF-R and (C) β-actin, and electrophoresed through a 1.5% agarose gel. Molecular weight markers (M) are φX174 DNA cut with *Hae*III. The size (bp) of the predicted amplified product in each case is indicated.

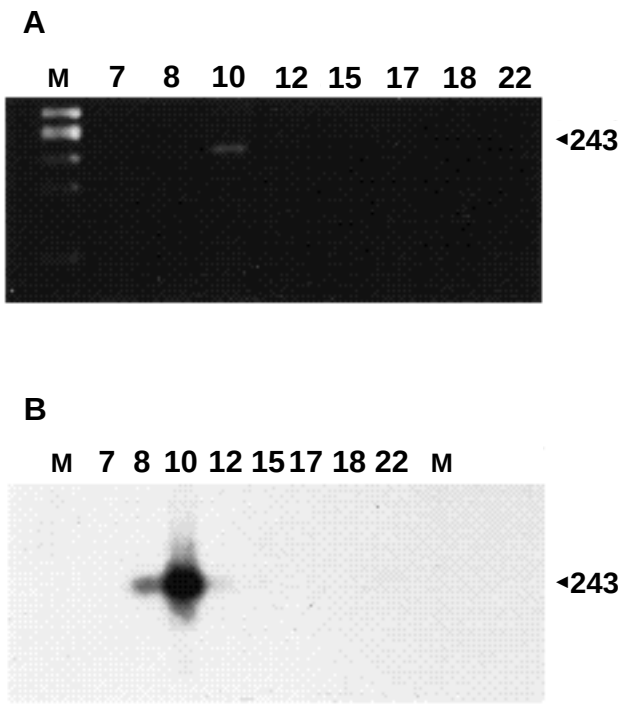

Fig. 2. Expression of TGFα mRNA during early pig development. Total RNA was isolated from conceptuses at days 7, 8, 10, 12, 15, 17, 18 and 22 of pregnancy, submitted to RT-PCR using primers specific for TGFα and electrophoresed through a 1.5% agarose gel. (A) Ethidium-bromide-stained agarose gel. (B) The gel from A was transferred to a nylon membrane and Southern analysis performed using a radiolabelled 150 bp pig genomic TGFα DNA probe. Molecular weight markers (M) are φX174 DNA cut with *Hae*III. The size (bp) of the predicted amplified product is indicated.

amplified product will reflect those of the input mRNA (as reverse transcribed cDNA) when this is sufficiently low to avoid saturation of the amplification reaction. Thus, the results shown in Fig. 1A reveal an apparent increase in the level of EGF mRNA expression as the conceptus develops from day 15 through to day 22.

We have previously demonstrated specific, saturable binding of ^{125}I-labelled EGF to trophoderm derived from day-15 to day-19 pig blastocysts (Corps et al., 1990). It has proved difficult to investigate ^{125}I-labelled EGF binding to blastocysts at very early stages of development due to the limited amounts of conceptus material available. However, using RT-PCR and EGF-R-specific primers, a single amplified product of the predicted size (264 bp) was observed in all of the RNA samples derived from day-7 to day-22 conceptuses, although the final levels of amplified product were relatively lower in the day-7 and day-8 samples (Fig. 1B). The identity of the amplified product was confirmed as EGF-R cDNA by nucleotide sequencing (data not shown) indicating that EGF-R transcripts are present in both pre- and post-elongation conceptuses.

TGFα expression

The results presented in Fig. 1 indicate that the EGF-R gene may be expressed as early as day 7 of pregnancy whereas EGF itself is not detected until day 15. For this reason, it was interesting to determine whether other ligands that interact with the EGF-R are expressed earlier than EGF in the developing blastocyst. In order to address this question, RNA samples from day-7 to day-22 conceptuses were analysed by RT-PCR using primers specific for TGFα, a second polypeptide which binds and activates the EGF-R. An amplified product of the predicted size (243 bp) was observed on an ethidium bromide stained gel only in samples from day 10 of pregnancy (Fig. 2A). The identity of this amplified product was confirmed as TGFα cDNA by nucleotide sequencing (data not shown). When the gel shown in Fig. 2A was transferred onto nylon and hybridised with a pig TGFα genomic DNA probe, a single hybridis-

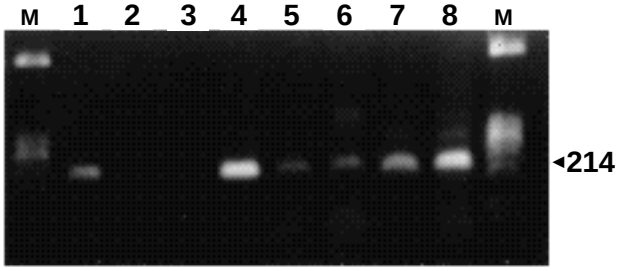

Fig. 3. Distribution of EGF mRNA expression within 18- and 22-day conceptuses. Total RNA was isolated from day-18 (tracks 1-3) and day-22 (tracks 4-8) tissues, submitted to RT-PCR using primers specific for EGF and electrophoresed through a 1.5% agarose gel. The tissues so analysed were embryo-amnion (tracks 1 and 4); allantois (tracks 2 and 5); trophoderm (tracks 3 and 6); amnion (track 7) and embryo with amnion removed (track 8). Molecular weight markers (M) are φX174 DNA cut with *Hae*III. The size (bp) of the predicted amplified product is indicated.

ing band was detected in the samples from days 8, 10 and 12 of pregnancy (Fig. 2B). These results, which were reproduced in two additional experiments, indicate that TGFα gene is expressed transiently by the pig blastocyst immediately prior to elongation.

Tissue distribution of EGF expression

In order to determine which tissues express EGF mRNA in post-elongation conceptuses, the embryo-amnion, allantois and trophoderm were individually dissected from day-18 and day-22 conceptuses. Embryos within the amnion were removed by teasing open the chorion (day 18) or chorio-allantois (day 22) and dissecting away from the placental tissues. The remaining membranes were then gently separated into trophoderm and allantois, with care being taken to avoid cross-contamination of tissues. Total RNA was isolated from the embryo-amnion, allantois and trophoderm at days 18 and 22 of pregnancy and analysed for EGF mRNA expression by RT-PCR using EGF-specific primers (Fig. 3). At day 18 of pregnancy, EGF message was expressed by the embryo-amnion (lane 1) but not by the placental membranes (lanes 2 and 3). However, at day 22 of pregnancy, although EGF mRNA remained predominately expressed by the embryo-amnion (lane 4), expression was also detected in the allantois (lane 5) and in the trophoderm (lane 6). To characterise further the sites of EGF mRNA synthesis, the amnion was carefully dissected away from the embryo of day-22 conceptuses and total RNA was isolated and analysed for expression using RT-PCR. Amplified products were derived from RNA isolated from both the amnion (lane 7) and from the embryo (lacking amnion) (lane 8). As a control for RNA integrity, these RNA samples were analysed by RT-PCR for β-actin mRNA expression. An amplified product of the predicted size (450 bp) derived from β-actin mRNA (as cDNA) was evident in all the samples analysed (results not shown).

In order to identify the sites of EGF expression in the day-22 embryo, sections of the embryo-amnion were immunostained using a rabbit polyclonal antiserum prepared against recombinant pig EGF (Pascall et al., 1991). In radioimmunoassays, this antiserum shows no cross-reactivity with a variety of other polypeptides including TGFα (Vaughan et al., 1992). EGF immunoreactivity was detected throughout the developing lung buds (Fig. 4B), the apical region of the gut loop (Fig. 4D) and in the amnion (Fig. 4F). The amnion arises as two cell layers, an outer layer of mesoderm and an inner layer of ectoderm. Interestingly, EGF appears localised in the outer, mesodermal, cell layer (Fig. 4F). The expression of EGF only in this mesodermal layer may explain our failure to detect EGF in the amniotic fluid of day-22 pig conceptuses by homologous radioimmunoassay (Vaughan et al., 1992).

Expression in the oocyte

Total RNA was isolated from pools of 50-100 pig primary oocytes (cumulus-free) and analysed for EGF, TGFα and EGF-R mRNA expression by RT-PCR. Each reverse transcription was performed using an aliquot of RNA equivalent to that extractable from ten oocytes. The results indicate that maternal transcripts for EGF, but not for TGFα or the EGF-R, are present in the pig oocyte (Fig. 5).

Discussion

In the mouse, TGFα, but not EGF, has been detected as a maternal transcript of the unfertilised oocyte. This message is then rapidly destroyed at the two-cell stage (Rappolee et al., 1988). The EGF-R is also expressed in unfertilised mouse oocytes but only at very low levels (Wiley et al., 1992). In contrast to these observations, we detected EGF, but not TGFα or EGF-R, transcripts in the unfertilised pig oocyte. Since the EGF-R gene is apparently not expressed, EGF would presumably be unable to effect a receptor-mediated response in the oocyte directly. The mammalian oocyte, however, develops both coordinately and interdependently with the surrounding somatic cumulus cells. These cells and the oocyte are able to communicate with one another via gap junctions that facilitate the exchange of low-molecular-weight, soluble factors that are the presumptive signals controlling oocyte and cumulus cell development (see Eppig, 1991). EGF could therefore represent one such factor secreted by the oocyte that is involved in regulating the differentiation and function of the surrounding cumulus cells. It will be interesting to determine whether the cumulus cells express functional EGF receptors.

TGFα mRNA was detected in the developing pig blastocyst only at days 8, 10 and 12 of pregnancy with the highest levels at day 10 where the amplified product was clearly visible on an ethidium-bromide-stained agarose gel. The expression of TGFα at day 8, but not at day 7, indicates that transcription from the TGFα gene probably commences after the blastocyst has hatched from the zona pellucida, a process that occurs around day 6 and day 7 of pregnancy (Davis, 1985). After apparently maximal expression at around day 10, TGFα mRNA fell to undetectable levels in RNA isolated from elongated blastocysts at days 15, 17, 18 or 22 of pregnancy. Thus, our results demonstrate a relatively narrow window of TGFα gene expression during early development. Of course, these results do not preclude

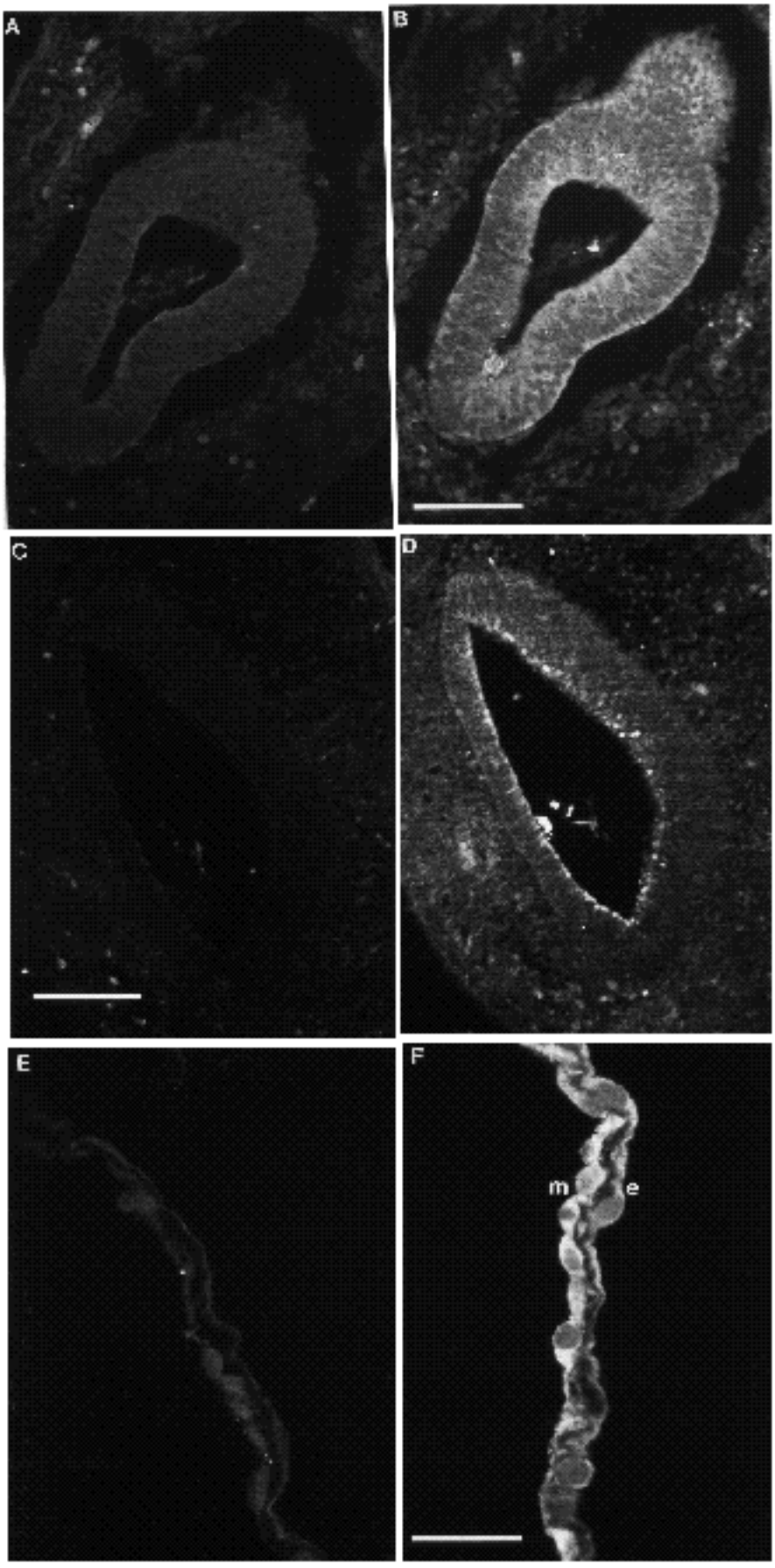

Fig. 4. Immunohistochemical localisation of EGF in day-22 embryos. Immunofluorescent staining was performed as described in the *Materials and methods* section using rabbit antiserum raised against pig EGF. EGF immunoreactivity was detected in the lung bud (B), gut loop (D) and amnion (F) of the day-22 pig embryos. In panel F, m = mesoderm and e = ectoderm. No specific staining was observed when pre-immune serum was applied to the sections instead of the primary antiserum (panels A, C, E for lung bud, gut loop and amnion, respectively). Scale bars represent 50 μm (A, B, E, F) or 100 μm (C,D).

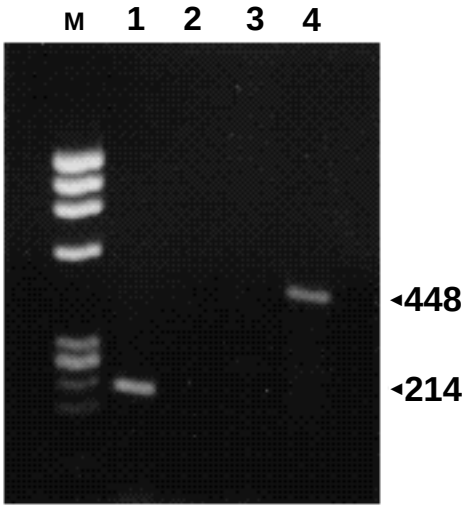

Fig. 5. Expression in the oocyte. Total RNA was isolated from pools of 50-100 primary oocytes (cumulus-free) and 10 oocyte RNA equivalents, submitted to RT-PCR using primers specific for EGF (track 1), TGFα (track 2), EGF-R (track 3) and β-actin (track 4) and electrophoresed through a 1.5% agarose gel. Molecular weight markers (M) are φX174 DNA cut with *Hae*III. The size (bp) of the predicted amplified products for EGF and β-actin are indicated.

the possibility of additional sites of TGFα expression during later development.

From days 10.5-12, pig blastocysts begin to elongate at a dramatic rate (30-45 mm/hour), initially as a consequence of cellular remodelling and, later, cell proliferation (Stroband and Van der Lande, 1990). Since our observations indicate that peak expression of TGFα apparently coincides with the onset of blastocyst elongation, it is tempting to speculate that the factor may be involved in the complex developmental reorganisation that occurs during this period. Such a role could be related to the well-known proliferative effects of TGFα, and/or to other responses required for blastocyst elongation. For example, Dardik and Schultz (1991) have recently reported that picomolar concentrations of exogenous EGF or TGFα can increase the rate of fluid uptake and blastocoel expansion in the early cavitating blastocysts of preimplantation mouse embryos. The expression of the TGFα gene during pig blastocyst elongation raises the possibility that the endogenous factor may have a role in regulating fluid transfer during this period.

The temporal pattern of EGF gene expression in early pig development is quite distinct from that of TGFα. EGF mRNA is first expressed by the post-elongation conceptus at around day 15 of pregnancy with levels continuing to rise up to day 22. We have demonstrated that this EGF mRNA is predominately present in the embryo itself and not in the placental membranes. EGF therefore appears more likely to play a role in the development of the embryo proper, particularly since activation of the EGF gene is coincident with the establishment of the early organ systems. Indeed, using immunohistochemical staining, we were able to detect EGF in the lung buds and gut loop of the day-22 pig embryo. The presence of EGF and its mRNA has been previously reported in the embryonic lungs of the post-implantation mouse conceptus (Snead et al., 1989). Furthermore, addition of exogenous EGF to embryonic mouse lungs in culture was found to stimulate branching morphogenesis, while the addition of tyrphostin (an EGF receptor tyrosine kinase inhibitor) abrogated this effect (Warburton et al., 1992). Despite a large literature concerning the role of EGF in neonatal gut maturation and in the maintenance of epithelial integrity in the gastrointestinal tract of adult animals (see Fisher and Lakshmanan, 1990), the presence of EGF in the developing embryonic gut has not, to our knowledge, been previously reported. Interestingly, EGF is found primarily at the apical surface of the embryonic pig gut loop. These results raise the interesting possibility that EGF may have an important role in regulating early lung and gut organogenesis.

In addition to the lung buds and gut loop, we were also able to identify EGF expression in the amnion of the day-22 conceptus. The amnion arises as two cell layers, an outer layer of mesoderm and an inner layer of ectoderm, that enfold the developing embryo and protect it from mechanical damage. It is well established that the amnion synthesizes prostaglandin E_2 (PGE_2) and that PGE_2 levels increase in amniotic fluid as pregnancy progresses (see Casey and McDonald, 1986). The regulation of PGE_2 biosynthesis in the amnion and the extraembryonic membranes is thought to be fundamental to the maintenance of pregnancy and the onset of parturition (see Casey and McDonald, 1986). Studies on cultured human amnion cells have demonstrated that their PGE_2 production may be stimulated from 2- to 150-fold by EGF (Casey et al., 1988). We demonstrate here the presence of immunoreactive EGF in amniotic tissue (from day-22 pig conceptuses). Although immunocytochemical staining cannot distinguish between local synthesis and sequestration from other sources, the presence of EGF mRNA in the amnion suggests synthesis by this tissue. Thus, it is possible that endogenous EGF may regulate PGE_2 biosynthesis by the amnion.

In conclusion, our studies have shown that the genes encoding EGF and TGFα maintain specific temporal patterns of expression during early pig development. Based on these observations, we propose that endogenous TGFα may play a role in blastocyst elongation and that endogenous EGF is associated with early organogenesis and with amniotic cell function.

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