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Paracrine effects of embryo-derived FGF4 and BMP4 during pig trophoblast elongation[☆]

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ABSTRACT

The crosstalk between the epiblast and the trophoblast is critical in supporting the early stages of conceptus development. FGF4 and BMP4 are inductive signals that participate in the communication between the epiblast and the extraembryonic ectoderm (ExE) of the developing mouse embryo. Importantly, however, it is unknown whether a similar crosstalk operates in species that lack a discernible ExE and develop a mammotypical embryonic disc (ED). Here we investigated the crosstalk between the epiblast and the trophoblast (TE) during pig embryo elongation. FGF4 ligand and FGFR2 were detected primarily on the plasma membrane of TE cells of peri-elongation embryos. The binding of this growth factor to its receptor triggered a signal transduction response evidenced by an increase in phosphorylated MAPK/ERK. Particular enrichment was detected in the periphery of the ED in early ovoid embryos, indicating that active FGF signalling was operating during this stage. Gene expression analysis shows that *CDX2* and *ELF5*, two genes expressed in the mouse ExE, are only co-expressed in the Rauber's layer, but not in the pig mural TE. Interestingly, these genes were detected in the nascent mesoderm of early gastrulating embryos. Analysis of *BMP4* expression by in situ hybridisation shows that this growth factor is produced by nascent mesoderm cells. A functional test in differentiating epiblast shows that *CDX2* and *ELF5* are activated in response to BMP4. Furthermore, the effects of BMP4 were also demonstrated in the neighbouring TE cells, as demonstrated by an increase in phosphorylated SMAD1/5/8. These results show that BMP4 produced in the extraembryonic mesoderm is directly influencing the SMAD response in the TE of elongating embryos. These results demonstrate that paracrine signals from the embryo, represented by FGF4 and BMP4, induce a response in the TE prior to the extensive elongation. The study also confirms that expression of *CDX2* and *ELF5* is not conserved in the mural TE, indicating that although the signals that coordinate conceptus growth are similar between rodents and pigs, the gene regulatory network of the trophoblast lineage is not conserved in these species.

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Introduction

The first lineage segregation in mammalian embryos gives rise to the TE and the inner cell mass (ICM). Derivatives of these two lineages contribute primarily to extraembryonic and embryonic tissues, respectively, leading to the formation of the conceptus. In domestic ungulates, where implantation begins after the second week of embryo development (from day 14 in pigs (Dantzer, 1985), 16 in sheep and 19 in cattle (Guillomot, 1995), maternal recognition of pregnancy is a pivotal event for ensuring conceptus viability. It is well known that signals produced by the trophoblast

shortly before implantation are essential in establishing foetal-maternal communication (Bazer et al., 2009; Heap et al., 1979) (Roberts et al., 2008; Wolf et al., 2003). Because the placenta of ungulates is non-invasive, the uterine histotroph is an important source of essential nutrients that support early development (Bazer, 2011; Spencer and Bazer, 2004; White et al., 2009). To best utilise these uterine nutrients the conceptus increases the surface area of the trophoblast by undergoing extensive elongation. In pigs, this remarkable process transforms the embryo from a < 5 mm sphere to an almost 1 m long thread within a few days (Anderson, 1978; Perry et al., 1976). Four major morphologically distinct conceptus sizes define the major developmental transitions during this process: spherical (< 5 mm), ovoid (5–10 mm), tubular (> 10 mm), and filamentous stages (> 100 mm) (Anderson, 1978; Geisert et al., 1982). Since the transition from a spherical to a filamentous stage occurs very rapidly (Geisert et al., 1982), embryos retrieved at days 10–12 of development can differ greatly in size (Anderson, 1978; Blomberg et al., 2010). Importantly, this period also coincides with the majority of embryonic loss in the pig (Stroband and Van der Lende, 1990), suggesting that

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smaller embryos may be compromised in their developmental capacity (Blomberg et al., 2010; Ross et al., 2009). Indeed, the changes in trophoblast size during this period are accompanied by differential gene expression, and a transient increase in synthesis of oestrogens that triggers changes in the uterine endometrium, in preparation for implantation (Ka et al., 2001).

In parallel to these remarkable changes in the trophoblast during a brief window of time, the embryonic disc (or epiblast) initiates gastrulation before the onset of implantation (Blomberg et al., 2006; Guillomot et al., 2004; Hue et al., 2001). Although there is no marked synchrony between epiblast development and trophoblast elongation during the ovoid-tubular stages, at the filamentous stage the primitive streak is always clearly visible (Blomberg et al., 2006; Vejlsted et al., 2006a) suggesting a coordinated development between the embryonic and extraembryonic compartments before implantation (Hue et al., 2007). Despite the detailed characterisation at the anatomical level, less is known about the molecular regulation of conceptus growth in this species.

Studies in mice show that the growth of the conceptus is coordinated by paracrine signals that trigger positive feed-back loops promoting cellular specification (Arnold and Robertson, 2009; Rossant and Cross, 2001; Tam and Loebel, 2007). One such signal is provided by Fibroblast Growth Factor 4 (FGF4) produced by the epiblast (Feldman et al., 1995), and was the first molecule demonstrated to play a pivotal role promoting trophoblast proliferation (Chai et al., 1998). FGF4 is essential for the development of the trophoblast stem cell (TSC) niche in the extraembryonic ectoderm (ExE), a derivative of the polar trophoblast (PT) (Guzman-Ayala et al., 2004), and is required for maintaining TSCs in culture (Tanaka et al., 1998). FGF4 signalling in the ExE is mediated by its membrane receptor FGFR2, which triggers a Ras/Erk response that stimulates *Cdx2* expression (Lu et al., 2008). *Cdx2*, together with *Eomes* and *Elf5*, have been proposed to be part of a gene regulatory network (GRN) promoting trophoblast fate in the ExE (Ng et al., 2008). *Cdx2* also promotes *Bmp4* expression (Murohashi et al., 2010), which in turn feeds-back to the epiblast to induce mesoderm differentiation (Winnier et al., 1995). These molecular interactions highlight the dynamic crosstalk between the embryonic and extraembryonic domains during trophoblast development and embryo patterning. It is however not known whether similar interactions that have been demonstrated in rodents coordinate the growth of the pig conceptus. In domestic animal embryos, and most other mammals, there is no anatomical structure equivalent to the ExE of rodents. The PT, also known as Rauber's layer (RL), is lost during the formation of the epiblast, leaving just the periphery of the ED surrounded by TE. Shortly after the disappearance of the RL, the trophoblast of pig (and cattle and sheep) embryos undergoes extensive elongation. Because of its small size at this stage, it has been suggested that the epiblast is unlikely to produce signals that can directly influence trophoblast growth (Pfeffer and Pearton, 2012; Roberts and Fisher, 2011). Instead, endometrial secretions have been suggested to play a primary role during trophoblast elongation (Ostrup et al., 2011; Spencer and Bazer, 2004; Wolf et al., 2003). The aim of the present study was to investigate how trophoblast elongation is coordinated in mammalian embryos (i.e. forming an ED). We show that FGF4 and BMP4 produced by the embryo proper signal to the TE prior to elongation. Furthermore, the response to these signals in the pig TE involves a different GRN to that described in the mouse TSC niche.

Materials and methods

Embryo collection and culture with inhibitor of exogenous ligands

All the procedures involving animals have been approved by the School of Biosciences Ethics Review Committee (University of

Nottingham, UK). Preparation of donor sows and collection was done as previously described (Rodriguez et al., 2012). Briefly, crossbred sows were artificially inseminated and embryos were collected between days 10 and 13. The oviduct and uterine horns were flushed with pre-warmed phosphate-buffered saline (PBS) supplemented with 1% foetal calf serum (FCS). Embryos were rinsed with PBS containing 1% FCS and transported to the laboratory in DMEM+0.2% BSA and 25 mM Hepes on a portable incubator at 38.5 °C. The inhibitors and growth factors were used at the following concentrations: PD161570 (FGF receptor inhibitor, Tocris) 100 nM; SB431542 (ALK5 receptor inhibitor, Tocris) 20 µM; BMP4 (R&D) 25 ng/ml, human recombinant FGF4 (Peprotech) 25 ng/ml, and heparin 1 µg/ml. DMSO was used to dissolve the inhibitors, and was maintained at equal concentrations among groups (including control groups). A minimum of three embryos per group were used for FGF4 and BMP4 stimulation experiments. Embryos were incubated with FGF4 and BMP4 for 15 min at 39 °C under 5%CO₂. FGFRi was added 1 h before treatment with FGF4.

Epiblast cultures

Epiblasts were isolated from spherical/early ovoid embryos and plated onto a feeder layer as previously described (Alberio et al., 2010). The culture medium used was: DMEM containing 0.2% BSA, and supplemented with 1% glutamine, 1% penicillin/streptomycin, 1% nonessential amino acids and 0.1 mM β-mercaptoethanol. Epiblasts were cultured in a humidified atmosphere at 39 °C under 5%CO₂. Three biological replicates were performed for these studies.

Immunohistochemistry (IHC) and in situ hybridisation (ISH)

After collection the embryos were placed in 4% paraformaldehyde (PFA) in PBS (made in DEPC-H₂O) and fixed at 4 °C overnight. Next day embryos were rinsed twice in PBS for 5 min. The embryos used for ISH were stored in 100% methanol at –20 °C, and those used for IHC were stored in 1% of PFA in PBS at 4 °C. For sections, embryos were mounted in 2–3% agarose and immediately processed using an automatic embedding machine (Leica TP1020; Leica Microsystems, Germany). After processing, the embryos and tissues were embedded in paraffin blocks and serial sections at 5–7 µm thickness were made using a microtome (Type HM355, Microm Laborgeräte GmbH, Walldorf, Germany). Sections were mounted onto SuperFrost™ plus slides (VWR), dried onto a warm plate, and oven-baked (37 °C) overnight. Embryo sections were de-waxed in xylene for 20 min and rehydrated in decreasing concentrations of alcohol solutions (100%, 90% and 70%) for 10 minutes each, and placed in 0.01 M PBS for 10 min. The sections were incubated in 10% blocking serum diluted in 0.1% BSA in 0.01 M PBS for 30 min. For wholemount immunostaining fixed embryos were permeabilised with 1% Triton solution diluted in 0.01 M PBS containing 0.1% BSA, under gentle agitation for 2–3 h at room temperature (RT). This was followed by incubation for 3–4 h with 10% non-immune blocking serum containing 0.25% BSA diluted in 0.01 M PBS. Subsequently, blocking serum was removed and the embryos were rinsed in PBS/Tween20 (PBST) on a rocker for 30 min. Embryos were incubated overnight at 4 °C with the following primary antibodies: FGF4 (Santa Cruz, SC-1361, 1:200), FGFR2 (Santa Cruz, SC-122, 1:200), pMAPK (Cell Signalling, #4376, 1:50), MAPK (Cell signalling, #9102, 1:50), BMPR2 (Santa Cruz, SC-20737, 1:100) pSMAD1/5/8 (Millipore, AB3848, 1:100), LIFR (Santa Cruz, SC-659, 1:200), CDX2 (Peprotech, 500-P236, 1:100), pSMAD2/3 (Millipore, AB3849, 1:500). After 4 washes in 0.05% PBST-Triton (PBSTT) embryos were transferred to the appropriate secondary antibody and incubated for 1 h at RT, followed by 3 washes in PBSTT. Embryos were mounted in Vectashield with

Table 1
List of primers and probes used in this study.

Gene	Primer sequence Fw Primer sequence Rv	Amplicon (bp)	Accession number
PCR primers			
CDX2	AGAACCCAGGCTCTGTCTT CAGTCCGAAACACTCCCTCACA	101	NC_010453.4
ELF5	GCATCTCCTTCCGCAATTC TGTGAGCGAATGTTCTGGAG	127	NP_001230640
GAPDH	GGGCATGAACCATGAGAAGT GTCTTCTGGGTGGCAGTGAT	162	AF017079.1
T	GCAAAAGCTTTCCTTGATGC GGCAGGATACCTCTCACAGC	204	U91519
Eomesodermin	TACCAACCAGTCTGCACAT GGAAGCGGTGTACATGAGT	210	TC251524
OCT4	GCAAAAGCTAAGCAGTGA GGTGACAGACCCGAGGAA	201	NM_001113060
CYP17A1	CCAAGGAGGTGCTTCTCAAG GTTCTCCAGCTTCAGGTTGC	188	NM_214428
β -Actin	TCCCTGGAGAAGAGCTACGA CGCACTTCATGATCGAGTTG	149	AJ312193
ISH probe primers			
CDX2	TCTCCGAGAGGCAAGTTAA CCTGCCAGCACTAAAGGAAG	541	NC_010453.4
ELF5	CGAACAAAGCCTCCAAAGTTC AGGTCACACCTCTCTCCTT	538	NP_001230640
BMP4	GAGGAAGAGCAGCCACAG CCACTCCCTTGAGGTAACGA	506	NP_001094501.1
BMP2	CTTAGACGGTCTGCGGTCTC CGGTGGGACAGAAGTTAA	390	NP_001182328.1

DAPI (4'-6-diamidino-2-phenylindole; Vector Laboratories). Antibodies were tested for their specificity in sections of pig endometrium from day 12 of pregnancy.

For ISH embryos were rehydrated in decreasing concentrations of methanol and then washed in PBST. Next embryos were stabilized in (1:1) PBST: Hybridisation buffer (HB: Formamide: 50%, 2 × SSC (pH5), EDTA (5 mM, pH8), 0.05 mg/ml Yeast RNA, 0.2% Tween20, 0.5% CHAPS, 0.1 mg/ml Heparin) for at least 10 min, and then transferred to HB before incubating at hybridisation temperature (HT) for a minimum of 2 h. After incubation, HB was replaced by the probe (see Table 1 for details on probe sequences) and incubated overnight. The following day the probe was removed and embryos were washed numerous times with wash buffer (WB: 50% Formamide, 1 × SSC (pH 5), 0.1% Tween20) at HT, and then equilibrated with (1:1) WB: MABT solution (MABT solution: 100 mM Maleic acid, 150 mM NaCl, 0.005% Tween20) before washing with MABT solution at RT. The embryos were then blocked with MABT and of 2% blocking reagent (Roche) for 1 h followed by blocking solution with MABT, 2% blocking reagent and 10% normal goat serum for at least 2 h. The blocking solution was then replaced with a 1:2000 dilution of anti-Dig-AP Fab fragments (Roche) and incubated overnight with gentle agitation at 4 °C. Embryos were rinsed in MABT several times before the development step with NBT/BCIP. After colour reaction embryos were washed with 5 × TBST (TBST: 0.7 M NaCl, 0.01 M KCl, 0.125 M Tris (pH 7.5), 0.5% Tween20) solution. The colour reaction was repeated until signal was detected. Once the colour reaction was satisfactory, the embryos were re-fixed with 4% PFA for 1 h, rinsed in PBST and observed under a microscope. For each stage at least four embryos were stained with each antibody and or processed for ISH. DNA replication was determined using the Click-iT[®] EdU kit following manufacturer's recommendations (Invitrogen). Five embryos per treatment group were stained.

Gene expression analysis

RNA isolation was carried out using RNeasy kit (Qiagen) following the manufacturer instructions. RNA reverse transcription was

performed using Omniscript synthesis kit (Qiagen). End-point PCR was performed with ReadyMix (Sigma–Aldrich) and 0.4 μ M of each primer. Quantitative RT-PCR (qRT-PCR) was performed using SYBR green mix (Roche) and 0.25 μ M of each primer. For each gene, the analysis was performed in triplicate. Melting-curve analysis to confirm product specificity was performed immediately after amplification and the amplicon size was checked by gel electrophoresis. The relative expression of the target gene was normalised with GAPDH and a calibrator sample. Sequence accession numbers and primers used in this study are listed in Table 1.

Statistical analysis

Analysis of variance was used to compare the mean differences between treatments (ANOVA with Tukey's test). Three embryos were used per treatment group (Figs. 2B and 5C). Three regions were selected and all cells (between 100 and 200 cells) counted to determine positive and negative cells after immunostaining. A $p < 0.05$ was considered significant.

Results

FGF signalling in peri-elongation pig conceptuses

FGF4 produced by the mouse epiblast signals through receptors located in the neighbouring TE cells promoting the proliferation of the ExE (Chai et al., 1998; Tanaka et al., 1998). To investigate whether this crosstalk operates during pig conceptus development we studied the expression of FGF signalling components in peri-elongation embryos. In spherical and ovoid embryos, FGF4 was detected predominantly in TE and RL cells, whereas in the epiblast (epi) the signal was faint and homogeneous (Fig. 1a–c and g–i; Suppl. Fig. 1A). In ovoid embryos, FGF4 staining varied significantly between specimens depending on the developmental status of the epiblast. Since the ovoid stage is very transient and dynamic, we studied these embryos in more detail by adapting a classification previously proposed (Vejlsted et al., 2006b). The group was subdivided into (i) early and (ii) late ovoid on the basis of the absence (PSII-E) or presence (PSII-L) of nascent mesoderm, respectively. Sections of PSII-E embryos shows that FGF4 signal localised primarily to the apical side of the TE, in contrast to the more generalised staining detected in epiblast and hypoblast cells (Fig. 1m and n). A marked increase in staining was detected in TE of PSII-L embryos, whereas in the epiblast the signal gradually decreased at this stage (Fig. 1q–r).

FGF4 signal transduction is stimulated by binding to tyrosine kinase receptors located in the plasma membrane of mouse TE cells, which triggers an intracellular response that involves MAPK phosphorylation (p42/44 ERK) (Corson et al., 2003). To study if the changes in FGF4 expression were functionally linked with TE elongation, we investigated the presence of FGF receptors and the status of MAPK signalling. In spherical embryos FGFR2 was detected predominantly in cells of the RL, and faint expression was detected in the TE (Fig. 1d–f). Later, in ovoid embryos, although the few remaining RL cells showed strong staining, a faint homogeneous FGFR2 signal was detected in the epiblast. This staining pattern was confirmed in transversal sections of PSII-E embryos, where the epiblast, the hypoblast and TE showed homogenous staining (Fig. 1o and p). In PSII-L embryos, however, the signal intensity was markedly increased in the TE compared to the epiblast and hypoblast (Fig. 1s and t). Consistent with these observations, MAPK protein was detected in most epiblast and hypoblast cells of PSII-E embryos, but was almost absent in TE (Fig. 2A, a–f). However, in PSII-L embryos, MAPK was also detected in TE cells (Fig. 2A, g–h). Phosphorylated MAPK (pMAPK) was

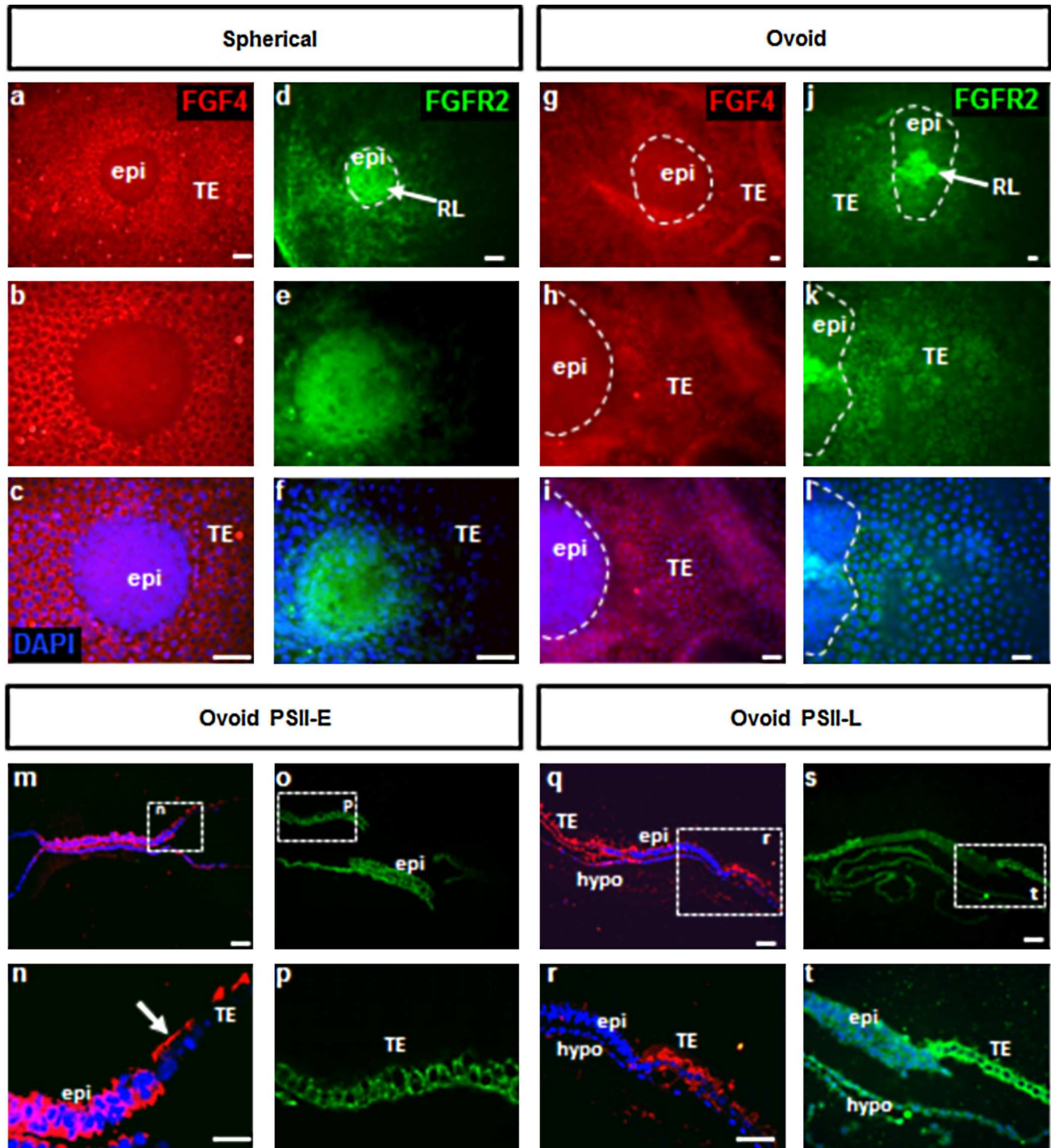


Fig. 1. Localisation of FGF4 and FGFR2 proteins in spherical and ovoid embryos. FGF4 and FGFR2 immunofluorescence of (a–l) wholemount and (m–t) transversal sections of pig spherical and ovoid embryos. (n, p, r and t) Close-up images indicated by dashed squares in (m)–(s), respectively. Four embryos are shown in the top image at different magnifications: In (a)–(c) the embryo has no RL cells and therefore the homogeneous staining of the epiblast can be clearly seen under epifluorescence. In (d)–(f) RL cells are still present, showing clear FGFR2 staining in these cells. Arrow in (n) shows the apical staining for FGF4 in the TE. epi: epiblast, TE: trophoblast, hypo: hypoblast. Dashed lines in (d), (g), (h), (i), (j), (k) and (l) demarcate the epiblast covered in part by Rauber's layer cells. Merged images are shown with DAPI staining. Scale bar: 50 μ m.

detected in the nucleus of some epiblast and hypoblast cells of PSII-E embryos, but was absent from TE cells at this stage (Fig. 2A, i–n). In contrast, in PSII-L embryos all TE cells next to the epiblast showed nuclear pMAPK signal (Fig. 2A, o–p). Wholemount immunostaining showed that the increase in pMAPK signal was particularly confined to TE cells surrounding the epiblast (Fig. 2B, control). To test whether MAPK phosphorylation was induced in

response to FGF4, freshly retrieved embryos were incubated with FGF4 (25 ng/ml) or with an FGF receptor inhibitor (FGFRI) prior to wholemount immunostaining. pMAPK was sharply reduced in the epiblast and absent in TE cells of embryos incubated with the inhibitor. In contrast, embryos stimulated with FGF4 showed a significant increase in pMAPK ($p < 0.05$), demonstrating that the MAPK response is directly linked with FGF stimulation (Fig. 2B).

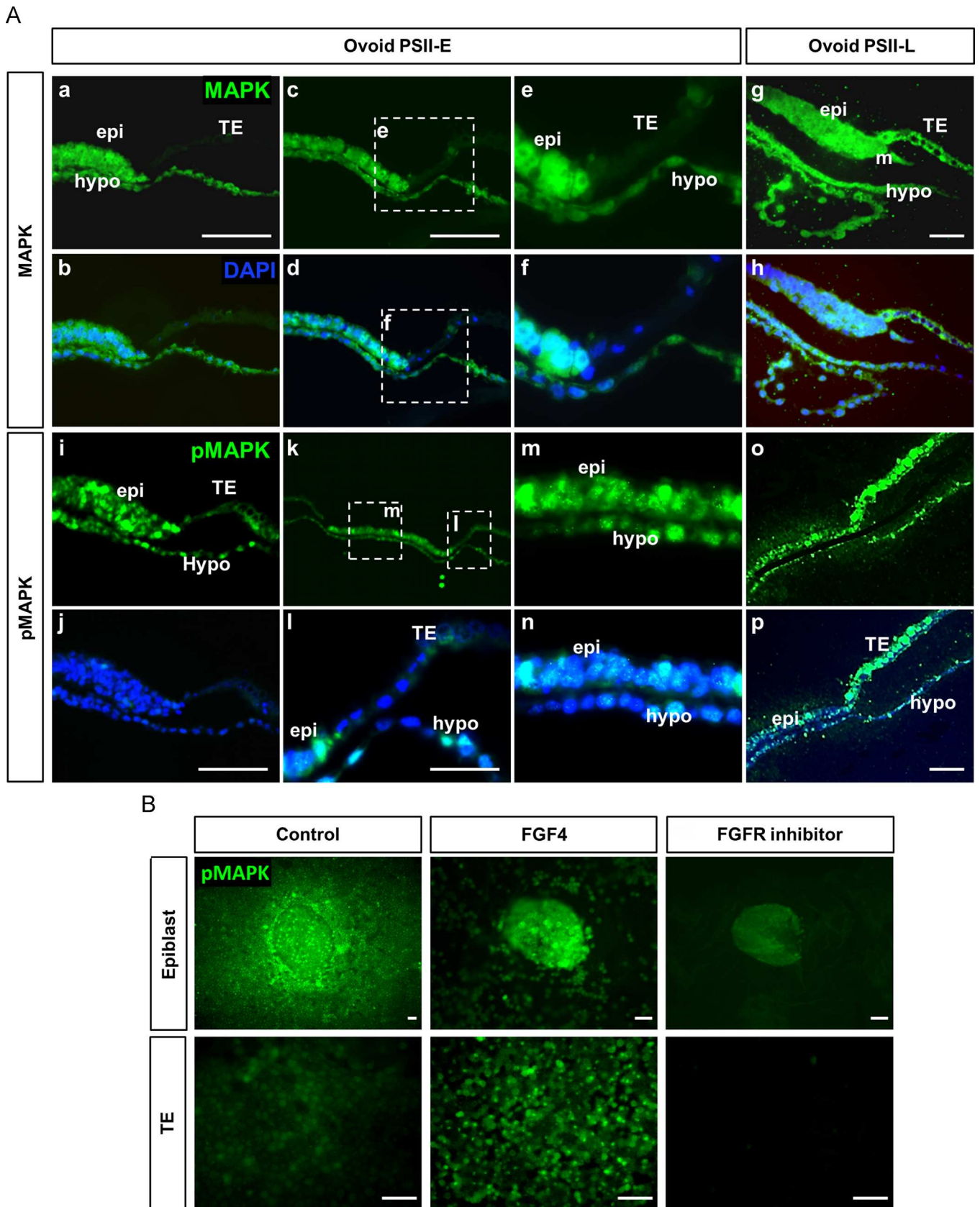


Fig. 2. MAPK kinase expression and phosphorylation in ovoid embryos. (A) Transversal sections show the localisation of MAPK (a–h) and phosphorylated MAPK (i–p) in early and late ovoid embryos. Merge images are shown with DAPI staining. (B) Ovoid embryos incubated with FGF4 or FGFRi stained for pMAPK. The images labelled TE refer to an area of the trophoblast away from the epiblast. Scale bar: 50 μ m.

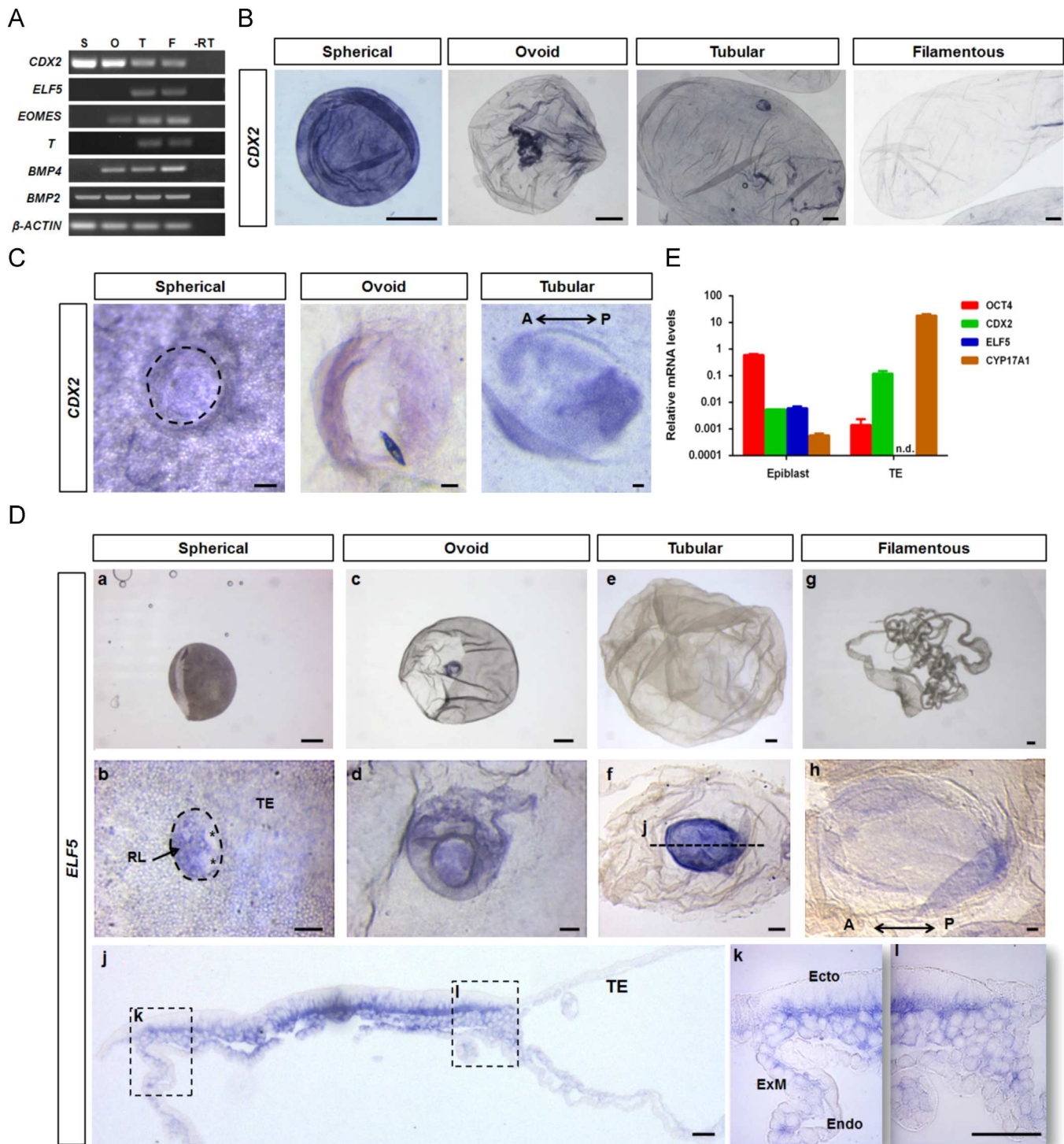


Fig. 3. *CDX2* and *ELF5* expression in peri-elongation pig embryos. (A) Gene expression analysis of embryos at the spherical (S), ovoid (O), tubular (T), and filamentous (F) stages after RT-PCR. -RT samples contained no cDNA. (B) *CDX2* expression in embryos of different stages assessed by in situ hybridisation. Scale bar: 2 mm. (C) Top views of ED from embryos in (B) are shown in detail. Dashed line in the spherical embryo delineates the ED covered by RL cells. Scale bar: 50 μ m. (D) *ELF5* expression determined by ISH in embryos of different stages and photographed (a, c, e, g) at low magnification. Scale bar: 2 mm. (b, d, f, and h) Close-up images of the same wholemount embryos with special focus on the ED. Dashed line in (b) demarcates the ED covered by positive RL cells. * marks small areas of unstained ED devoid of RL cells. (j) Transversal section through the dashed line of the embryo shown in (f). (k and l) High magnification image of dashed squares in (j). Scale bar: 50 μ m. (E) Relative *CDX2*, *OCT-4*, *CYP17A1*, and *ELF5* expression in isolated epiblasts and TE from spherical/ovoid embryos. Ecto: ectoderm, ExM: extraembryonic mesoderm, Endo: endoderm.

To investigate whether stimulation of pMAPK via FGF increased proliferation of the TE surrounding the epiblast, EdU incorporation was used to determine the proportion of cells undergoing DNA replication. Freshly retrieved spherical/ovoid embryos cultured for 10 h in medium with or without FGF4 showed no EdU

incorporation in the TE. In contrast, intense staining was detected in the epiblast and the hypoblast of these embryos, independent of FGF4 treatment (Suppl. Fig. 2). Finally, since LIF receptors are also present in these embryos (Suppl. Fig. 3; Hall et al., 2009), and LIF can elicit a MAPK response in certain cell types (Hirai et al., 2011),

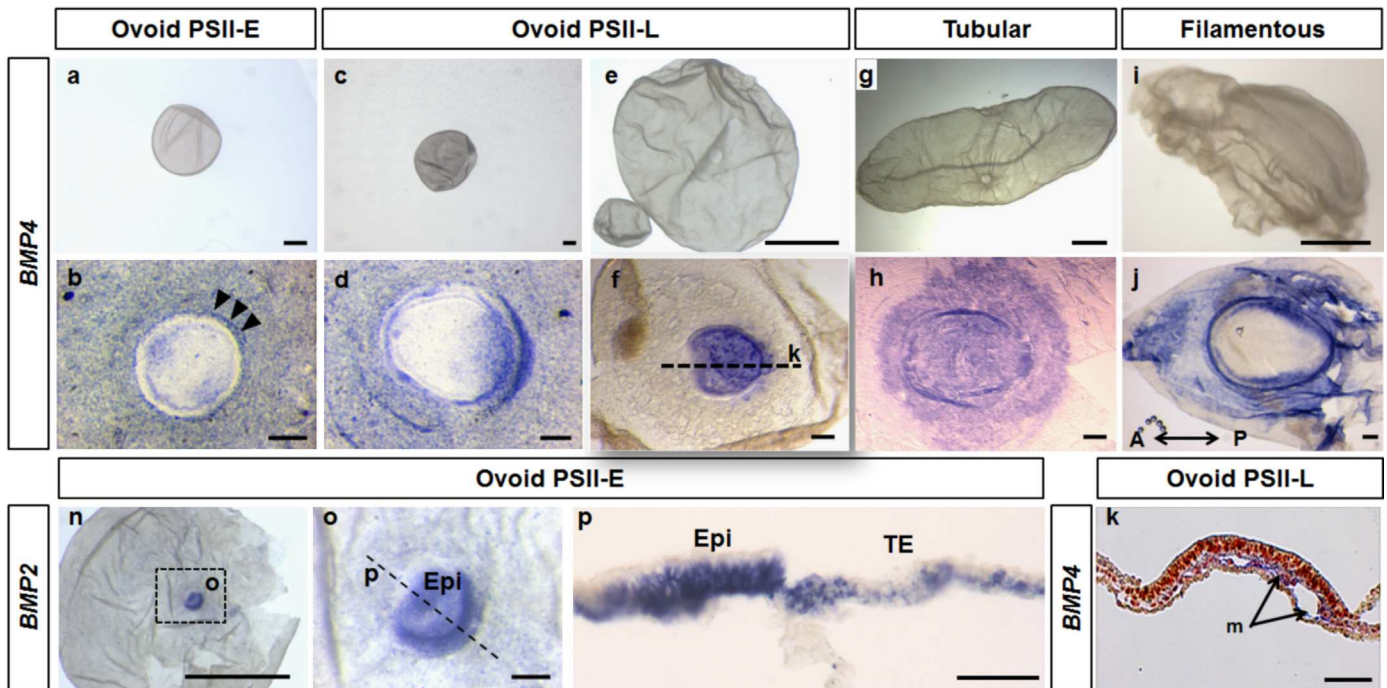


Fig. 4. *BMP4* and *BMP2* expression in peri-elongation pig embryos. (a, c, e, g, i, and n) Wholemount view of embryos at different stages before ISH, except for (n). (b, d, f, h, j, and o) Close-up images of embryos shown as wholemounts after ISH. (k) Transversal section through the dashed line of embryo shown in (f). Arrows point to the purple stained mesoderm. (p) Transversal section through the dashed line of embryo shown in (o). epi: epiblast; m: mesoderm; TE: trophoblast. Scale bar: 5 mm (a, c, e, g, i, and n), and 50 μ m (b, d, f, h, j, k, o, and p).

embryos were incubated with LIF before fixation and immunostaining. No differences in pMAPK signal were detected between treated and untreated embryos (not shown), indicating that MAPK phosphorylation in the TE of spherical/ovoid embryos is not the result of LIF stimulation.

CDX2 and *ELF5* expression during peri-elongation

The mouse TSC niche is supported by FGF4 via the regulation of *Cdx2* and *Elf5* (Ng et al., 2008). In an attempt to identify whether an equivalent cellular domain, the TSC niche, exists in the pig embryo we studied the gene expression profile of *CDX2* and *ELF5* during peri-elongation. TE isolated from spherical and ovoid embryos, from which the embryonic disc was removed, shows *CDX2* expression, but no *ELF5* (Fig. 3A). Expression of *CDX2* in the TE was also confirmed by immunostaining (Suppl. Fig. 4). In TE samples from tubular and filamentous embryos *ELF5* was detected, but *CDX2* was reduced compared to earlier stages. *T* and *EOMES* were also detected in these late stage embryos from which the ED was removed. In these advanced stages the extraembryonic mesoderm extends beyond the limits of the epiblast (see Fig. 3J), therefore it is also part of the TE samples. To determine which cellular domain expressed these genes, mRNA expression was investigated by ISH. *CDX2* mRNA was detected in TE and RL of spherical embryos, but a progressive reduction in signal intensity was observed in the TE of ovoid, tubular and filamentous embryos (Fig. 3B and C; Suppl. Fig. 5). In contrast to the reduction in the TE of advanced embryos, *CDX2* expression increased in the posterior end of early primitive streak ED of ovoid and tubular embryos (Fig. 3C).

ELF5 mRNA, however, was restricted to the RL of spherical embryos, and very faint expression was detected in the remaining TE (Fig. 3D, a–h; Suppl. Fig. 5). In late ovoid embryos, *ELF5* was detected in the basal part of epiblast cells and in the nascent mesoderm, but no signal was detected in the hypoblast (Fig. 3D,

j–l). In tubular and filamentous embryos *ELF5* was detected in the ED but not in the TE (Fig. 3D, g–h). Quantitative gene expression analysis was used to compare the levels of *CDX2* and *ELF5* to *OCT4* and *CYP17A1*, both highly expressed in the epiblast and the TE, respectively. This analysis shows that *CDX2* and *ELF5* are expressed at very low levels in the epiblast, consistent with early signs of primitive streak formation at this stage. Importantly, *ELF5* expression was not detected in the TE.

Together, these results show that *CDX2* and *ELF5* are only transiently co-expressed in the RL of peri-elongation pig embryos, but not in the mural TE.

BMP signalling in peri-elongation embryos

In mice, the crosstalk between epiblast and ExE is mediated by FGF4 and BMP4, respectively. We next sought to determine whether BMPs were produced during the peri-elongation period in the pig. ISH showed that *BMP4* is first detected in a narrow ring-like area of cells at the border between the epiblast and the TE of early ovoid embryos (Fig. 4a and b and Suppl. Fig. 6). In late ovoid embryos, the signal was more evident in the posterior epiblast, demarcating the area of nascent mesoderm (Fig. 4c–f and k), and extended beyond the limits of the ED in tubular and filamentous embryos (Fig. 4g–j). We next analysed the pattern of *BMP2* expression, since in mice it first appears in the visceral endoderm, a little after *BMP4* expression (Couvouanis and Martin, 1999). Like *BMP4*, *BMP2* was not detected in spherical embryos (not shown), but it was first observed at the early ovoid stage (Fig. 4n and o). Transversal sections show strong expression primarily in epiblast cells and some staining in the TE (Fig. 4p). RT-PCR show that *BMP2* is expressed in spherical/early ovoid samples whereas *BMP4* is first detected in ovoid stages (Fig. 3A).

To investigate whether this expression profile reflected active BMP signalling, we analysed the expression of BMP receptor type II (BMPRII) and the signal transduction proteins, phosphorylated

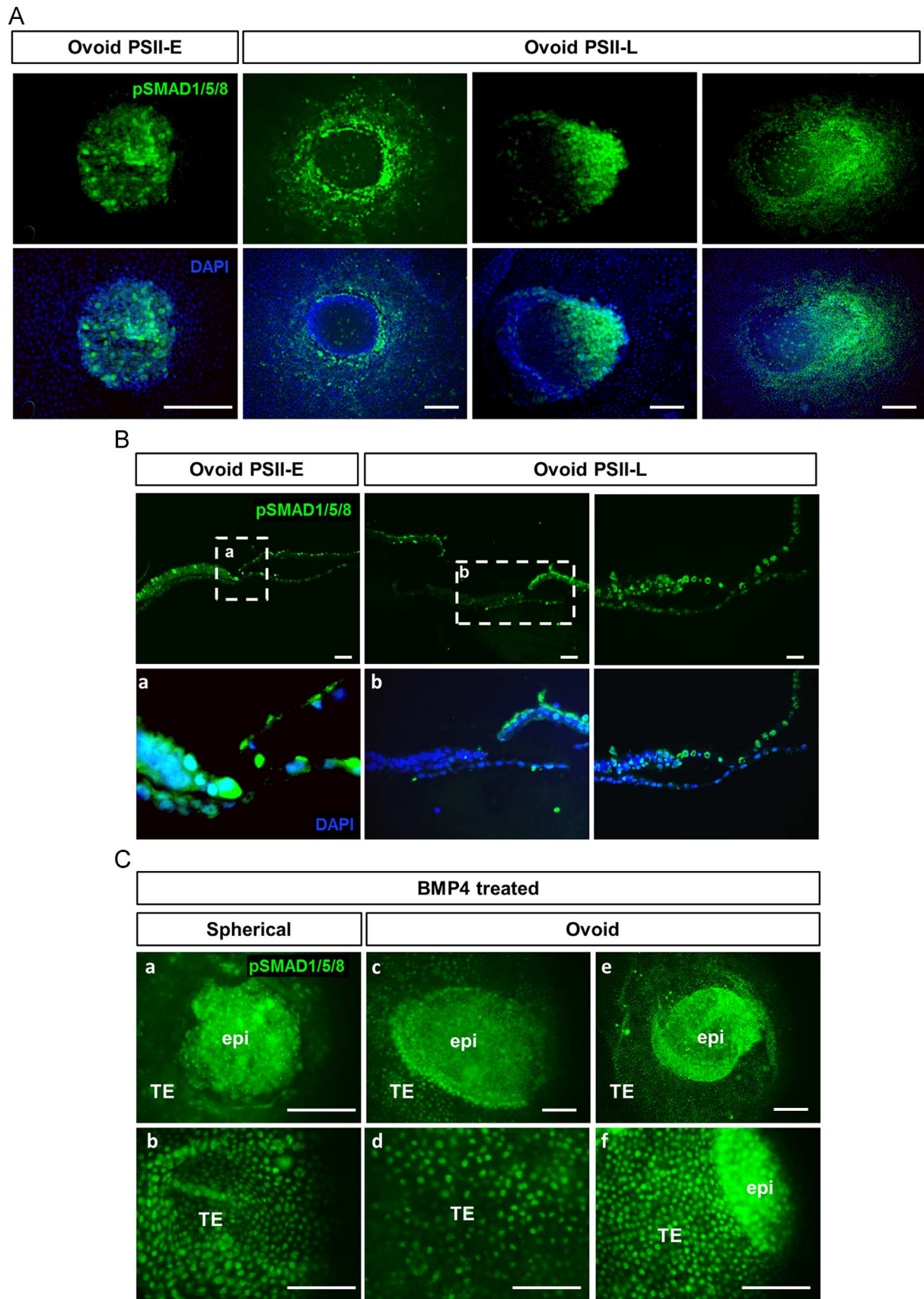


Fig. 5. Phosphorylated SMAD1/5/8 localisation in peri-elongation pig embryo. (A) Wholemout views of ED from ovoid embryos. Scale bar: 100 μ m. (B) Transversal sections of ovoid embryos showing nuclear localisation of pSMAD1/5/8. (a and b) Close-ups of dashed squares areas. Scale bar: 50 μ m. (C) Wholemout views of ED (a, c, and e) and trophoectoderm (b, d, and f) of embryos treated with BMP4 (25 ng/ml), and stained with anti-pSMAD1/5/8 antibody. Merged images are shown with DAPI staining. Scale bar: 100 μ m.

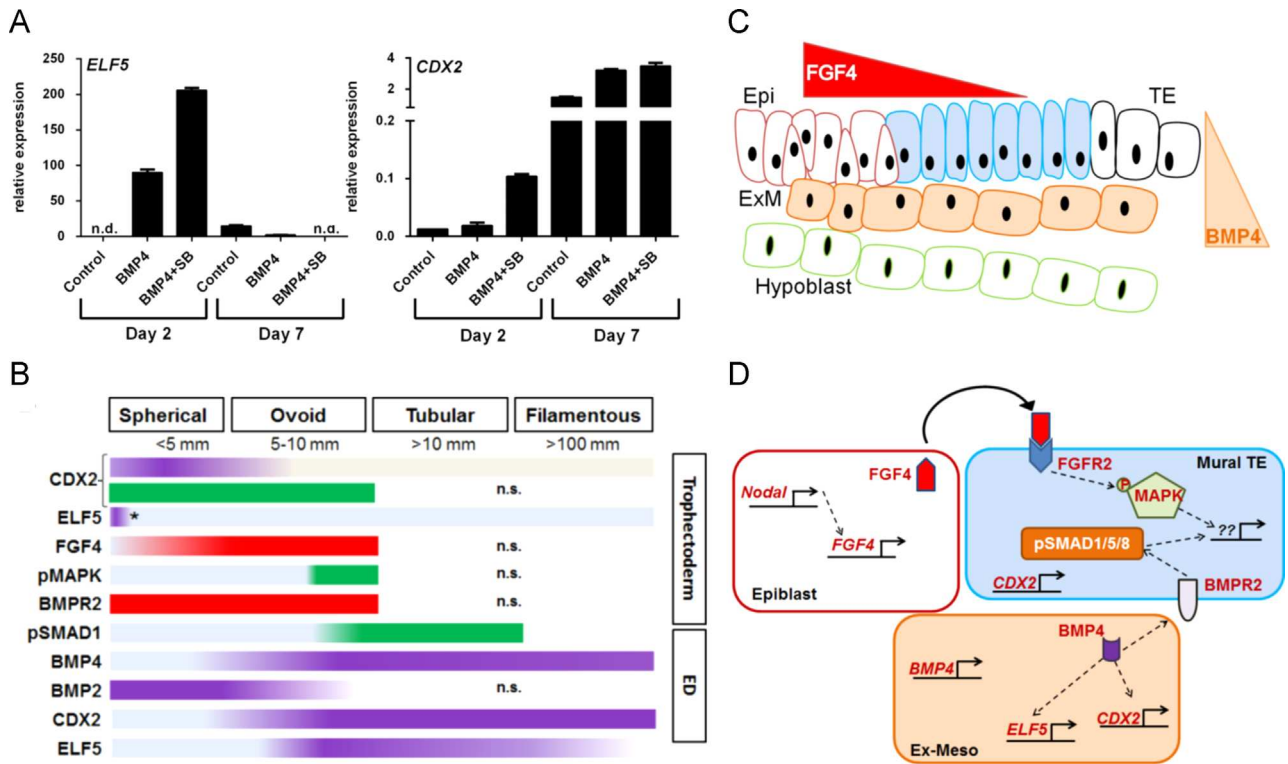


Fig. 6. Regulation of gene expression in pig peri-elongation embryos. (A) Relative expression of *ELF5* and *CDX2* in cultured epiblasts. SB: SB431542, n.d.: not detected. (B) Summary of the protein localisation (red/green lines) and mRNA expression (purple lines) profile discussed in this study. n.s.: not studied. * indicates that *ELF5* is only detected in the RL. (C) Diagrammatic model representing the paracrine effects of FGF4 and BMP4 from the ED and the extraembryonic mesoderm, respectively, on the neighbouring TE cells. Columnar TE cells (light blue) represent a presumptive TSC niche. Epi: epiblast; TE: trophoctoderm; ExM: extraembryonic mesoderm. (D) Schematic representation of the gene regulatory interactions and signalling pathways investigated in the study.

SMAD (pSMAD) 1/5/8. BMPRII was detected in the TE (including RL) of spherical and ovoid embryos, but was not found in the epiblast and hypoblast (Suppl. Fig. 7a–f). pSMAD1/5/8 was found in isolated cells in the epiblast of early ovoid embryos, but no signal was detected in the TE (Fig. 5A). PSII-L embryos showed a polarised pattern of pSMAD1/5/8 staining that mirrored *BMP4* expression in the posterior epiblast detected by ISH. Cells in the Epi-TE border stained positive in embryos with early signs of polarity. The signal shifted to the posterior epiblast in advanced embryos, extending beyond the epiblast-TE border, which reflected the expansion of the ExM (Fig. 5A). Transversal sections show that pSMADs 1/5/8 signal was particularly enriched in TE cells of PSII-L stage embryos (Fig. 5B). The epiblast of PSII-E showed less pSMAD1/5/8 than PSII-L stage embryos, in agreement with the findings of the wholemount staining (Fig. 5A). The generalised pattern of BMPR2 expression suggested that all TE cells can respond to BMP signalling. To test this possibility, embryos were incubated with BMP4 and subsequently immunostained. A significant increase in pSMAD1/5/8 was observed in the TE of treated embryos (Fig. 5C, $p < 0.05$), indicating that TE of peri-elongation embryos can respond to BMP stimulation.

The effects of BMP stimulation in the TE are not known, therefore to test whether this cytokine promoted DNA replication, EdU incorporation was assessed in embryos cultured with or without BMP4. No differences in EdU incorporation were detected in TE cells of these embryos, although cells from the epiblast and hypoblast did incorporate EdU readily (Suppl. Fig. 2). Together, these results suggest that mesoderm-derived BMP can induce a localised SMAD1/5/8 response in the TE of elongating embryos, but is not directly linked with the stimulation of cell proliferation.

Transcriptional regulation of *CDX2* and *ELF5* in ovoid embryos

The results above show that *CDX2* and *ELF5* are induced in the ED at the time when *BMP4* expression is also high. To investigate whether BMP4 signalling directly affects the activation of these genes, epiblasts from early ovoid embryos were dissected and cultured for 7 days. *ELF5* was almost undetectable in dissected epiblasts before culture and remained low in those cultured under basal conditions for 2 days (Fig. 6A). In contrast, in epiblasts cultured with BMP4, *ELF5* was strongly up-regulated. When Activin/Nodal (SB431542) signalling was inhibited in the presence of BMP4, *ELF5* expression was further increased. After 7 days of culture control embryos showed low levels of *ELF5* expression, however, in treated embryos *ELF5* expression decreased to basal or undetectable levels. *CDX2* expression did not show a strong response to BMP4 supplementation after 2 days compared to control epiblasts, however a 5-fold increase was detected when added in combination with SB431542. After 7 days culture, *CDX2* levels increased by about 100-fold compared to Day 2 cultured epiblasts in control and BMP4 treated groups. This expression profile demonstrates that BMP4 induces a robust, but transient, activation of *ELF5*, and a delayed but sustained activation of *CDX2* from differentiating epiblasts. The activation of both genes increases in the presence of an Activin/Nodal inhibitor, indicating that this pathway interferes with the effects of BMP4 in *CDX2* and *ELF5* induction from epiblast cells.

Discussion

This study provides evidence of paracrine signalling between the ED and the TE during the spherical-ovoid transition in the pig.

The distinctive profiles of FGF4, BMP4, their cognate receptors, and signalling effectors (MAPK and SMAD1/5/8, respectively) are summarised in Fig. 6B.

Paracrine FGF4 signalling during TE elongation

We first evaluated the possible role of FGF4 as a signalling mediator between the ED and the TE based on previous evidence in the mouse embryo. A recent report showed that *FGF4* is highly expressed in the epiblast but not in the TE of ovoid embryos (Fuji et al., 2013). We find that FGF4 protein, which is primarily a secreted ligand (Dailey et al., 2005), is detected primarily in the TE cells of spherical/ovoid pig embryos, and a faint cytoplasmic staining was detected in the ED. These observations suggest that FGF4 produced by epiblast cells is secreted, and subsequently sequestered by the neighbouring TE cells. Furthermore, the simultaneous increase in FGFR2 staining and MAPK phosphorylation in TE cells demonstrate the cellular response triggered by FGF4. The direct relationship between the pMAPK response and FGF4 binding was also shown in vitro, after incubation of ovoid embryos with exogenous FGF4. These dynamic interactions between the epiblast and the TE are consistent with observations in the mouse embryo showing that FGF4 secreted by epiblast cells binds avidly to the ExE (Shimokawa et al., 2011).

The epiblast, however, is not the only source of FGF for the pig conceptus during this period, since FGF7 and FGF9 are also produced by endometrial cells in pregnant sows (Ka et al., 2001; Ostrup et al., 2010). Although it has been suggested that FGF7 secreted by the endometrium in response to estrogens produced by elongating embryos can promote TE proliferation (Ka et al., 2001, 2007), there is no direct evidence demonstrating this effect in vivo.

The localised pattern of pMAPK in the TE surrounding the ED in ovoid embryos suggests that FGF4 produced by the epiblast is an important source of this growth factor that directly signals to the TE. This conclusion is also supported by two additional observations: firstly, embryos recovered from D10–12 pregnant sows differ greatly in size (Anderson, 1978; Blomberg et al., 2010; Geisert et al., 1982), suggesting that if maternally produced FGFs were the main source of this growth factor, pMAPK would be observed in the TE of all embryos from the same litter. The current data, however, confirms that only ovoid embryos have high levels of pMAPK and FGF4 staining. Secondly, maternally derived FGFs would be expected to bind to any area of TE, and induce a generalised pMAPK response (similar to the one shown in our in vitro experiments; Fig. 2B), however, a localised pMAPK response in the TE of ovoid embryos was observed. This combined pattern of pMAPK and increased FGF4 binding in TE neighbouring the epiblast suggests that FGF4 from the epiblast triggers a signalling cascade in the TE that may initiate the elongation process. The idea of paracrine, rather than endocrine, signalling inducing signalling cascades is also supported by evidence from other systems showing that FGF gradients can only spread over distances of several cell diameters (Christen and Slack, 1999; Nowak et al., 2011; Shimokawa et al., 2011). Furthermore, most secreted FGFs are sequestered to the extracellular matrix of tissues rather than being released into luminal cavities (Ornitz, 2000).

CDX2 and ELF5 expression in peri-elongation embryos

In mice, FGF4 produced by the epiblast promotes the expansion and maintenance of the TSC niche in the ExE by supporting the expression of *Cdx2* and *Eomes*. *Cdx2* is expressed in the TE of blastocysts in mammals (Berg et al., 2011; Chen et al., 2009; Kuijk et al., 2008; Strumpf et al., 2005), and functional experiments have demonstrated its critical role in regulating TE proliferation and

lineage specification (Berg et al., 2011; Ralston et al., 2010; Sritanandomchai et al., 2009). In pigs, *CDX2* is expressed in TE of blastocysts (Kuijk et al., 2008) and our results show that it is maintained in spherical embryos before it is down-regulated at tubular and filamentous stages. This expression profile also correlates with observations in cattle embryos (Berg et al., 2011; Degrelle et al., 2005). We show, however, that *CDX2* is also expressed in the nascent mesoderm of gastrulating pig embryos. Indeed, *Cdx2* is expressed in derivatives of the posterior primitive streak and regulates posterior axial growth in mice (Beck et al., 1995; Chawengsaksohak et al., 2004; Young et al., 2009). Mouse *Cdx2* mutants die between 3.5 and 5.5 days post coitum due to a failure in placenta development, however, heterozygote embryos show a variety of abnormal posterior development phenotypes (Chawengsaksohak et al., 2004). Interestingly, human embryonic stem cells (hESC) activate *CDX2* during mesodermal specification in response to *T* (Bernardo et al., 2011). These observations are consistent with the current findings showing *CDX2* expression in the gastrulating pig embryo, and suggest that *CDX2* expression during early mesoderm differentiation is conserved in mammals.

Less clear is the role of *Elf5* in mammalian trophoderm development. In mice, it is essential for maintaining undifferentiated trophoblast progenitors in the ExE and for the successful derivation of TSC (Donnison et al., 2005). In cattle, however, *ELF5* is not expressed in the TE of spherical embryos (Degrelle et al., 2005; Pearton et al., 2011). Furthermore, a transgenic approach showed that bovine *ELF5* is not expressed in the mouse TE, indicating that the role of this gene in TE development is not conserved (Pearton et al., 2011). Our results in the pig add further evidence for the lack of conservation in the function of *ELF5* during TE development. *ELF5* transcripts were detected at low levels in the RL of spherical embryos, but were not detected in the mural TE at any stage. Instead, *ELF5* was detected in the epiblast and the mesoderm of gastrulating embryos. This profile is in agreement with observations in bovine embryos, where highest expression of *ELF5* is detected in the epiblast of pregastrulation embryos (Pearton et al., 2011) and gradually disappears by day 17 (Smith et al., 2010). In mice, in contrast, except for the expression in the ExE, *Elf5* is not detected in the embryo proper until somitogenesis (Donnison et al., 2005). Together these data provide evidence for the differences in the GRN controlling trophoderm development, and shows that not only is the role of *ELF5* not conserved in TE development, but neither is its expression profile during early gastrulation.

The expression of *ELF5* and *CDX2* coincided with the increase in *BMP4* expression in early mesoderm progenitors. This prompted us to investigate the functional relationship of these events in isolated epiblasts. The results show that this tissue responds to *BMP4* by activating *ELF5* expression after 2 days of differentiation. The *BMP4* effect was augmented in epiblasts cultured with an inhibitor Nodal/Activin signalling, similar to the findings in hESC (Amita et al., 2013; Bernardo et al., 2011). Furthermore, a reduction in *ELF5* expression by 7 days of differentiation points to a transient expression of *ELF5* in differentiating epiblasts. These kinetics are consistent with the ISH results showing expression of *ELF5* in the mesoderm of early gastrulating embryos. Furthermore, a similar transient *ELF5* expression is observed in hESC differentiated with *BMP4* (Amita et al., 2013). *CDX2* expression also increased in cultured epiblasts after 2 days, particularly in response to *BMP4*+SB431542, and was further up-regulated after 7 days. The *CDX2* expression profile is also consistent with findings in cultured mouse epiblasts and hESC exposed to these differentiation regimes (Amita et al., 2013; Bernardo et al., 2011). A controversial aspect of the hESC studies is the identity of the cells produced in response to *BMP4* (Amita et al., 2013; Bernardo et al., 2011). Bernardo et al. (2011) demonstrated that *BMP4* promotes

extraembryonic mesoderm, whereas others have shown that this factor preferentially induces trophoblast differentiation (Amita et al., 2013; Li et al., 2013; Sudheer et al., 2012). The discrepancies between these studies can in part be attributed to differences in the culture conditions and the cell lines used (Amita et al., 2013). It is also possible that the lack of information on the expression of *ELF5* and *CDX2* during human epiblast differentiation may limit the interpretation of hESC differentiation studies. The present analysis of pig embryos provides unbiased evidence of *CDX2* and *ELF5* expression in the differentiating ED, which were corroborated by functional experiments, indicating that in vivo these genes are induced in the extraembryonic mesoderm in response to BMP4.

Embryo-derived BMP4 signals to the elongating TE

Expression of BMP4 identifies the ExE developing on top of the egg cylinder in the mouse. The lack of an equivalent anatomical structure in species developing from flat ED led us to study the source of this growth factor, and to determine whether it also participates in the crosstalk between epiblast and TE. BMP4 is not expressed in the ICM (Blomberg et al., 2008; Hall et al., 2009) or in the TE of pig embryos, but it is first detected in the nascent mesoderm. A similar expression pattern has been described in the rabbit embryo (Hopf et al., 2011) and in late stages of mouse development, where it localises to the extraembryonic mesoderm (Lawson et al., 1999; Winnier et al., 1995). Interestingly, *BMP2* was detected in the epiblast/hypoblast and preceded *BMP4* expression in the pig, similar to the dynamics reported in the rabbit (Hopf et al., 2011). This is in contrast to the expression dynamic in the mouse, where *BMP4* is expressed in the ICM (3.5 dpi) and the ExE (5.5 dpi), and is followed by *BMP2* expression in the visceral endoderm in 6.5 dpi embryos (Coucounanis and Martin, 1999). This spatial and temporal difference in expression of these two growth factors highlights some unique features of the development of the mouse embryo. Expression of *BMP4* has been linked with a role in promoting cellular apoptosis during cavitation of the egg cylinder (Coucounanis and Martin, 1995, 1999). The lack of *BMP4* in the pig TE is consistent with this possibility, suggesting that in species where cavitation is very transient (Barends et al., 1989; Hall et al., 2010) or non-existent, premature expression of this growth factor may be dispensable. This also suggests that in rodents which undergo cavitation premature expression of *BMP4* may have been co-opted into a novel genetic circuitry to enable formation of the egg cylinder. Our findings, however, point to a role for *BMP4* produced by mesodermal cells in triggering a paracrine signal in the neighbouring TE. Because the period in which this signal is received by the TE corresponds to the extensive elongation of the conceptus, it is conceivable that the embryo proper might be influencing these events, as suggested previously (Stroband and Van der Lende, 1990). Our results show that neither FGF4 nor *BMP4* promote DNA replication of TE cells during the spherical/ovoid transition. These observations are consistent with previous findings showing lack of cell proliferation during the early phase of TE elongation (Geisert et al., 1982), and support the suggestion that cellular remodelling is responsible for the structural changes observed during elongation (Mattson et al., 1990). In future it will be interesting to evaluate whether embryo-derived cytokines participate in the regulation of actin filament organisation of TE cells and contribute to the changes in cell shape.

In summary, the data in this study show that FGF4 and *BMP4* secreted by the ED and its derivative structures trigger a signalling response in the neighbouring trophoblast just prior to TE elongation. A model depicting how these growth factors influence the TE is shown in Fig. 6C. FGF4, which is induced by Nodal in the epiblast (Suppl. Fig. 8 and (Alberio et al., 2010)), is secreted from spherical/early ovoid embryos and induces a MAPK response in the TE. This

is followed by stimulation of the TE by *BMP4* produced by the nascent mesoderm that spreads around the ED of late ovoid embryos, delineating a domain of TE cells exposed to a gradient of both cytokines. Indeed, a domain of columnar TE cells surrounding the ED and overlaying the mesoderm was shown to have differential steroidogenic activity, and was proposed as a niche of highly proliferative TE cells (Conley et al., 1994). Our results suggest that TE proliferation is not induced in response to these cytokines in spherical/ovoid embryos. The current analysis of gene expression, however, demonstrates that neither *CDX2* nor *ELF5* are co-expressed in the mural TE when FGF4 and *BMP4* are present, suggesting that if a trophoblast stem cell niche exists, it does not express these genes (Fig. 6D).

In conclusion, our results show that paracrine signals from the embryo proper signal to the TE prior to the extensive elongation, and that the GRN represented by the FGF4-*CDX2*-*ELF5* axis described for the mouse TSC niche is not conserved in the pig.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2014.01.008>.

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