Int. J. Dev. Biol. 44: 327-330 (2000)

Short Contribution

Expression patterns of follistatin and two follistatin-related proteins during mouse development

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ABSTRACT We compared the expression patterns of follistatin and two follistatin-related proteins (FRP and m7365) during early mouse development. m7365 is expressed continuously during preimplantation development, in contrast to FRP and follistatin. At early postimplantation stages, follistatin and 7365 are expressed from E6.0, while FRP is detected from E7.5 onwards. Although there is some overlap between the expression of these genes in the primitive streak and somites, their overall expression patterns are distinct.

KEY WORDS: *follistatin, m7365, FRP, in situ hybridization, mouse development*

Follistatin (Nakamura *et al.*, 1990) is a secreted protein, which functions as an antagonist of activin and bone morphogenetic proteins (BMP), members of the transforming growth factor β superfamily (Nakamura *et al.*, 1990; Iemura *et al.*, 1998). FS contains three highly conserved domains, FS modules, which are thought to function in growth factor binding (Patthy and Nikolics, 1993). Although the expression pattern of *FS* suggests a function in early mouse development (Albano *et al.*, 1994; Feijen *et al.*, 1994), FS-deficient embryos were phenotypically normal through gastrulation (Matzuk *et al.*, 1995). This suggests that FS is not important at these stages or that maternally derived FS or functional homologues rescue an early embryonic phenotype.

Recently, two novel proteins have been identified that possess FS-related structural motifs. One of these proteins, called TSC-36, was originally identified as a secreted, $TGF\beta_1$-inducible protein from mouse osteoblast cells (Shibanuma *et al.*, 1993). The recently cloned human and rat *follistatin-related protein (FRP)* (Zwijsen *et al.*, 1994), *Xenopus FRP* (Okabayashi *et al.*, 1999) and avian *follistatin-like (Flik)* (Amthor *et al.*, 1996; Patel *et al.*, 1996) genes, appear to be the orthologues of the mouse *TSC-36* gene. The second protein, called 7365, is a transmembrane protein containing a signal peptide, two FS modules, a unique epidermal growth factor (EGF) domain and a short cytoplasmic tail (Eib and Martens, 1996). The *X7365* gene is expressed during *Xenopus laevis* development from the late blastula stage onwards and predominantly in the adult brain. Comparison of the *Xenopus* gene with the human and mouse orthologues, *h7365* and *m7365*, demonstrates that *7365* is highly conserved (Eib and Martens, unpublished results). Because of the presence of a FS-related structural motif, we investigated whether *FS, m7365* and *FRP* had comparable expression patterns.

In preimplantation development, of the three genes studied, only *m7365* is detectable by RT-PCR between the two cell and blastocyst stages, although both FS and FRP transcripts are detectable in plated blastocysts cultured for 5 days on gelatin-coated tissue culture plastic (Fig. 1; Table 1) (Roelen *et al.*, 1998; Veltmaat *et al.*, 2000). This is in contrast with the results of Albano and Smith (1994) who have detected FS throughout preimplantation development. We did, however, detect FS in zygotes (data not shown).

In situ hybridization shows that *FS* expression is restricted to specific sites in the early postimplantation embryo (Fig. 2). At E6.0, FS mRNA expression is detected in the putative primitive streak (Fig. 2A). The deciduum contains high levels of FS transcripts (Fig 2A,C). At E7.5 the primitive streak remains positive while the mesoderm adjacent to the headfold is also positive (Fig. 2B). Parietal endoderm is already a site of high expression from E6.0 (Fig. 2A-E), as we and others have shown previously (Albano *et al.*, 1994; Feijen *et al.*, 1994). The allantois and amnion are negative (Fig. 2C). At E8.5 the forebrain, extraembryonic mesoderm and ectoderm, the visceral endoderm as well as the heart are negative (Fig. 2D,E). The hindbrain and paraxial mesoderm are strongly positive (Fig. 2D,E). In E9.5 embryos, FS is

Abbreviations used in this paper: FRP, follistatin-related protein; FS, follistatin; Flik, follistatin-like; BmP, Bone morphogenetic protein; TGFβ, transforming growth factor β.

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0214-6282/2000/$20.00

Printed in Spain
www.ehu.es/ijdb

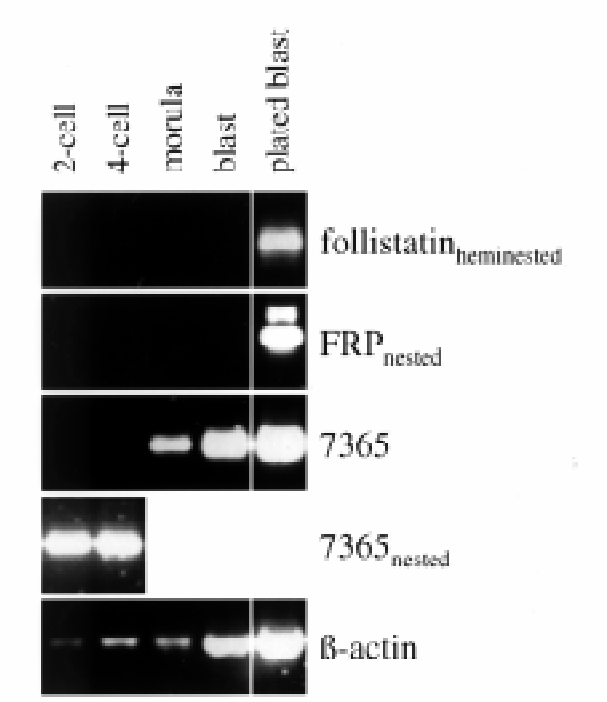

Fig. 1. RT-PCR on pre-implantation embryos. *For every stage an amount of cDNA equivalent to one embryo was used. The nested PCR to detect FRP in the plated blastocyst shows two bands. The upper band is probably produced by a combination of the nested forward and the reverse primer, which creates a product 60bp longer. Controls without RT are negative.*

expressed in the mesencephalon and diencephalon, but not in the telencephalon (Fig. 2F). In addition, high expression is observed in the somites (Fig. 2F).

FRP mRNA is not expressed as early as FS mRNA, and could not be detected in E6.5 embryos by *in situ* hybridization. Expression is detected at E7.5 at similar sites as FS, in the primitive streak (ectoderm and embryonic mesoderm; Fig. 3A) and in the mesoderm adjacent to the headfold. In avian and amphibian embryos, the FRP homologues Flik and xFRP are expressed in Hensen's node (Patel *et al.*, 1996; Amthor *et al.*, 1996) and Spemann's organizer (Okabayashi *et al.*, 1999), respectively, tissues equivalent to the anterior part of the primitive streak in the mouse. As for FS, the neurepithelium, the visceral yolk sac and the ectoplacental cone are negative while the deciduum and somites of late E7.5 mouse embryos are positive (Fig. 3B). However, in contrast to FS, FRP is present in the allantois and the mesodermal component of amnion and chorion. FRP is not detectable in the parietal endoderm (Fig. 3A,B). At later stages, FRP is expressed in the somites, the floor plate, the foregut, notochord and heart (Fig. 3C-E). The forebrain and hindbrain, as well as the first branchial arch are negative (Fig. 3C-E). Part of the ectoplacental cone is positive at this stage (data not shown). A striking site of expression at E9.5 is the nephric duct (Fig. 3H,I). Furthermore, FRP transcripts are found in the diencephalon, the second branchial arch, wall of the arch arteries, Rathkes pouch and the aortic wall (Fig. 3F-H).

The mRNA encoding the transmembrane protein m7365 is expressed very strongly in E6.0 mouse embryos (Fig. 4A). In contrast to FS, m7365 mRNA expression is ubiquitous. In the deciduum a strongly expressing zone around the uterine lumen was observed (Fig. 4A). In E7.5 mouse embryos, m7365 mRNA is strongly expressed in all embryonic and extraembryonic tissues, except for the parietal endoderm (Fig. 4B-D). At E8.5 the pattern of expression becomes more restricted. Neural crest tissue, branchial arches, somites, lateral plate mesoderm and ectoplacental cone are highly positive, whereas the heart, the neurepithelium in the forebrain and hindbrain regions (Fig. 4E-F) and the blood islands have lower expression (data not shown). At later stages of development (E9.5) m7365 shows additional intriguing sites of expression. Figure 4G-J shows that m7365 mRNA is strongly expressed in different types of neurons, including motor neurons in the ventral neural tube lateral to the floor plate, the dorsolateral visceral motor neurons, and the superficial neurons in the mid- and hindbrain regions. FS mRNA expression has not been observed in these neuronal cell types (Feijen *et al.*, 1994). The *m7365* expression pattern resembles that of the transcription factor Islet *1 (Isl1)*, a homeobox gene which is involved in the generation of embryonic motor neurons and part

TABLE 1

RT-PCR ON PRE-IMPLANTATION EMBRYOS

	2-cell	4-cell	Morula	Blastocyst	Plated blastocyst
FS heminested	-	-	-	-	+
FRP nested	-	-	-	-	+
M7365 nested	+	+	+	+	+

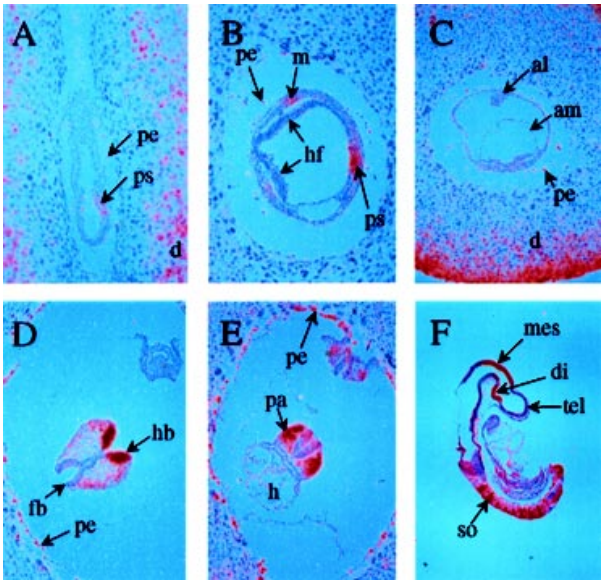

Fig. 2. Expression of follistatin mRNA in E7.5 (A-C), E8.5 (D,E) and E9.5 (F) mouse embryos. (A,F) *Sagittal sections.* **(B-E)** *Transverse sections. al, allantois; am, amnion; ba, branchial arch; baa, branchial arch artery; ch, chorion; d, deciduum; di, diencephalon; dvm, dorsolateral visceral motor neurons; epc, ectoplacental cone; fb, forebrain; fg, foregut; fp, floor plate; h, heart; hf, headfold; hb, hindbrain; lpm, lateral plate mesoderm; m, mesoderm; mes, mesencephalon; mn, motor neurons; mv, mesencephalic vesicle; nc, neural crest; no, notochord; pa, paraxial mesoderm; pe, parietal endoderm; ps, primitive streak; sn, superficial neurons; tel, telencephalon; ys, yolk sac.*

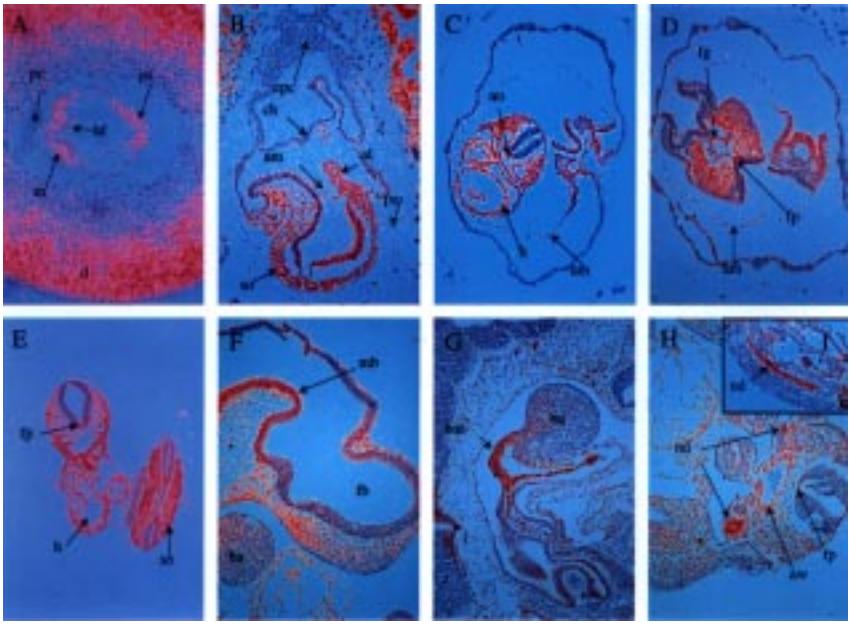

Fig. 3. Expression of FRP in E7.5 (A,B), E8.5 (C-E) and E9.5 (F-I) mouse embryos. (B,F,G,I) *Sagittal sections*; **(A,C-E,H)** *transverse sections. Abbreviations as in Figure 1.*

of the sonic hedgehog signaling cascade (Pfaff *et al.*, 1996; Dutton *et al.*, 1999).

Comparison of the mRNA expression patterns of FS, FRP and 7365 in early mouse development leads to the conclusion that the primitive streak and the somites are common sites of expression, most of the other sites being unique. Unlike FS, FRP apparently had no direct neuralizing effect when its RNA was microinjected into *Xenopus* embryos (Okabayashi *et al.*, 1999). This result might be due to the fact that FRP does not bind to activin A, TGFβ1, inhibin A and BMP4/7 (Mashimo *et al.*, 1997). However, recently it has been suggested that Flik, the orthologue of FRP in the chick, modulates the activity of signaling pathways of BMPs or TGFβ-related ligands, possibly in a FS-like way (Towers *et al.*, 1999; Connolly *et al.*, 2000) For 7365, which is a transmembrane protein, it is still not clear whether it has functional characteristics resembling those of FS.

Although all three proteins share a FS module, there is no direct evidence that they have a common function. If they are functional homologues, there are only a few regions where there could be functional redundancy between the proteins. The results presented here indicate that there is some overlap in the mRNA expression patterns of *FRP, 7365* and *FS*, but their overall expression patterns are distinct.

Experimental Procedures

Postimplantation mouse embryos were collected between embryonic days 6.0 and 9.5 (E6.0-E9.5) from C57BL6xCBA females, as described previously (Feijen *et al.*, 1994). E6.0-8.5 embryos were processed in the deciduum and E9.5 day embryos were dissected free from their membranes. Preimplantation embryos for RT-PCR were collected as described previously (Roelen *et al.*, 1997). Zygotes were isolated and cultured in M16 medium to the 2-cell, 4-cell, morula and blastocyst stages, or collected as blastocysts and plated in DMEM/20%FCS for 5 days on gelatin coated tissue culture plastic.

In situ hybridization analysis was carried out as described previously (Feijen *et al.*, 1994). The following probes were used: for FS, a 324bp PCR fragment, spanning nucleotides 512 and 832, cloned into pBluescript SKII$^-$ (Feijen *et al.*, 1994). For FRP, a 324bp AvaII-EcoRI fragment, spanning nucleotides 557 and 897 cloned into pBluescript KSII$^-$ (Zwijsen *et al.*, 1994). For 7365, an approximately 800bp probe in the protein coding region (Eib and Martens, 1996). RNA probes were generated by transcription of the T3 or T7 RNA polymerase promoter in the presence of α^{35}S UTP (Amersham).

RNA isolation and RT-PCR was carried out as previously (Roelen *et al.*, 1997). For PCR, cDNA equivalent to one embryo was used. Primers used: Follistatin: fwd.: 5'-ATACGGATCCTTTTCTGTCCAGGC-AGCTCCAC-3'. rev.: 5'-ATAGGAATTCGTCAACACTGAACATTGGTGG-

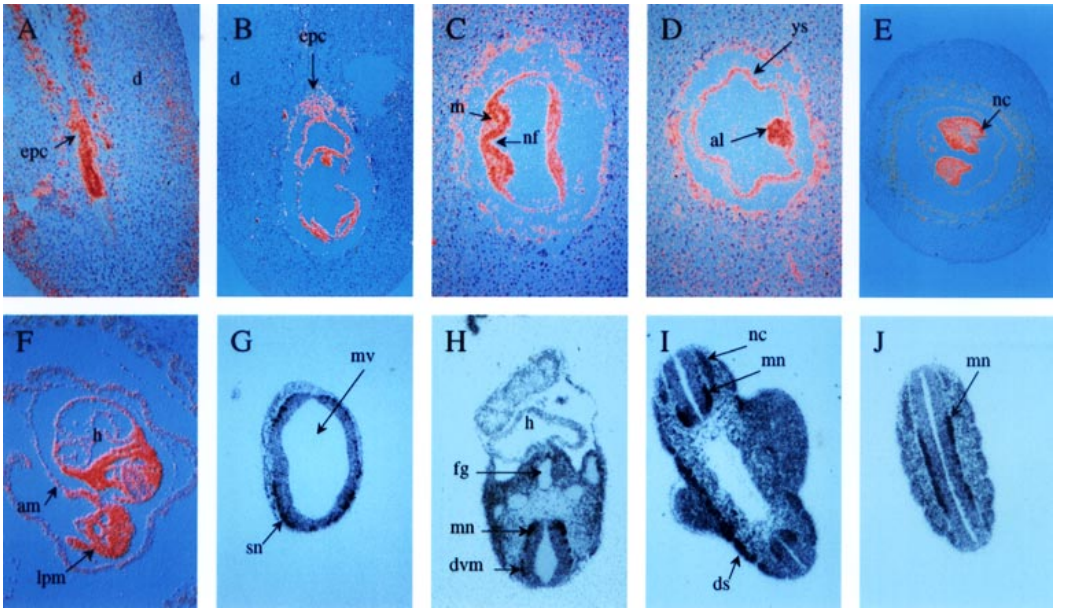

Fig. 4. Expression of m7365 in E6.0 (A), E7.5 (B-D), E8.5 (E-F) and E9.5 (G-J) mouse embryos. (A,B) *Sagittal sections*; **(C-J)** *transverse sections,* **(G-J)** *brightfield only.* **(G)** *Dorsal side on top. Abbreviations as in Figure 1.*

3'. nested rev.: 5'-ATAGGAATTCACTTACTGTCCGGGCACAGCTCA-3'. FRP: fwd.: 5'-TAAGAAGAGGCACAGAGC3'. Nested fwd.: 5'-CCACAACCTTCACCTTGTA-3'. Rev.: 5'-GCTGTGTGTTCAGGTGAT-3'. Nested rev.: 5'-CATGCTGACAGTGGTGAC-3'. m7365: fwd.: 5'-GCCACTGCACAGACACAGATGA-3'. Nested fwd.: 5'-TATCGGAAGC-CACATGCCTTGC-3'. Rev.: 5'-ATGGCGATCTGTACTGCTCCAA-3'. Nested rev.: 5'-GTTGAGCTTCTGCCTACTTGGT-3'.

All photographs of the *in situ* hybridizations were made by combining a brightfield image (blue filter) with a darkfield image (red filter), unless stated otherwise.

Acknowledgments

We are grateful to Dr. C.L. Mummery and to Dr. L. Defize for critical reading of the manuscript. We also thank Ferdinand Vervoordeldonk and Jaap Heinen for their assistance with the production of the figures. The Graduate School for Developmental Biology supported the present study.

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Received: January 2000
Accepted for publication: March 2000