Short Contribution

Follistatin expression in ES and F9 cells and in preimplantation mouse embryos

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ABSTRACT

Activins are thought to play a role in mesoderm induction in amphibian development. Studies of the expression patterns of activin during mouse embryogenesis are consistent with the proposal that they are also involved in mesoderm formation in mammals. Activins are expressed both maternally and zygotically at preimplantation stages, and at postimplantation stages transcripts are present at high levels in the decidua, suggesting that mesoderm is induced by maternally-derived activin. The functions of activin can be modulated by follistatin. At postimplantation stages follistatin is expressed in the decidua in a pattern reciprocal to that of activin. In the embryo proper, follistatin transcripts are localized to the primitive streak region during gastrulation and later in the somites and in rhombomeres 2, 4 and 6 of the hindbrain. In this paper we show that follistatin, like activin, is expressed throughout pre-implantation mouse development. Transcripts are present at low levels in undifferentiated F9 and ES cells, but they increase greatly on differentiation of both cell types. Expression of activin mRNA is decreased in differentiated F9 and ES cells, and the simultaneous increase in follistatin may create an efficient and rapid means of decreasing levels of functional activin.

KEY WORDS: activin, follistatin, mouse embryo, ES cells, F9 cells

Activins are homo- or heterodimers of the \( \beta_A \) or \( \beta_B \) subunits of inhibin, itself a heterodimer of one of the \( \beta \) subunits together with an \( \alpha \) subunit (Ling et al., 1988). The inhibins were first isolated from mammalian follicular fluid through their ability to inhibit the release of follicle-stimulating hormone (FSH) from rat anterior pituitary cells; the activins, isolated from the same source, have the opposite effect and thus stimulate FSH release (Vale et al., 1986; Ling et al., 1988).

Further work indicates that the activins play a variety of roles in the growth and differentiation of many cell types. For example, they induce the differentiation of erythroleukemia cells (Murata et al., 1988), stimulate meiotic maturation of rat oocytes (Itoh et al., 1990), and promote the survival of P19 cells induced to differentiate by retinoic acid (Schubert et al., 1990). Most recently, they have attracted attention through their ability to act as mesoderm-inducing factors in amphibian development (see reviews by Jessell and Melton, 1992; Kimelman et al., 1992; Sive, 1993; Smith, 1993), an observation which raises the possibility that they play a similar role in mammalian development. Studies of the expression patterns of activin during early mouse development and during the differentiation of embryonic stem (ES) and embryonal carcinoma (EC) cells are consistent with this suggestion. Thus activins are expressed both maternally and zygotically in preimplantation mouse embryos (van den Eijnden-van Raaij et al., 1992; Albano et al., 1993), and although transcripts are not detected in the embryo proper at postimplantation stages, they are present at high levels in decidua (Manova et al., 1992; Albano et al., 1994). This latter result raises the possibility that maternally-derived activin acts as a mesoderm-inducing agent (Manova et al., 1992; Albano et al., 1994).

The developmental functions of activin may be modulated by the activin-binding protein follistatin (Nakamura et al., 1990), which inhibits the mesoderm-inducing activity of activin (Asashima et al., 1991). At postimplantation stages, follistatin is expressed in the decidua in a pattern reciprocal to that of the inhibin \( \beta_A \) and \( \beta_B \) chains, and transcripts are also present in parietal endoderm. In the embryo proper, follistatin is expressed in the primitive streak of the gastrulating embryo and at later stages in the somites and in rhombomeres 2, 4 and 6 of the hindbrain (Albano et al., 1994). Since the inhibin \( \beta_A \) and \( \beta_B \) subunits are not expressed in the embryo at these later stages, this suggests that the function(s) of follistatin may extend beyond inhibition of activin activity. Indeed, Shukovski et al. (1993) have recently shown that follistatin can stimulate production of progesterone by bovine thecal cells directly.

In this report we extend our analysis of follistatin expression to preimplantation stages of mouse development and investigate the regulation of follistatin during F9 EC and ES cell differentiation. Our
Follistatin is regulated during differentiation of ES cells. CCE ES cells were differentiated as embryoid bodies for 2 or 5 days as described in Experimental procedures. 20 μg RNA from each time point was analyzed by RNAase protection using probes specific for follistatin or human γ-actin. 20 μg of yeast tRNA was used as a negative control (not shown, but see Fig. 2). Exposure times were 5 h for γ-actin and 3 days for follistatin. Note an increase in follistatin expression at 5 days.

Fig. 1. Follistatin is regulated during differentiation of ES cells. CCE ES cells were differentiated as embryoid bodies for 2 or 5 days as described in Experimental procedures. 20 μg RNA from each time point was analyzed by RNAase protection using probes specific for follistatin or human γ-actin. 20 μg of yeast tRNA was used as a negative control (not shown, but see Fig. 2). Exposure times were 5 h for γ-actin and 3 days for follistatin. Note an increase in follistatin expression at 5 days.

Results show that follistatin is expressed at low levels in undifferentiated F9 and ES cells, but increases greatly on differentiation of both cell types. Expression of activin mRNA is decreased in differentiated F9 and ES cells (Albano et al., 1993), and the simultaneous increase in follistatin may create a very efficient and rapid means of decreasing levels of functional activin. Consistent with the detection of follistatin transcripts in ES cells, we find that the gene is also expressed in preimplantation mouse embryos. These results confirm and extend those of van den Eijnden-van Raaij et al. (1992), and suggest that follistatin plays a role in preimplantation mouse development.

Follistatin is expressed in ES and EC cells and is regulated on their differentiation

ES cells

ES cells provide an accessible in vitro model for the early stages of mouse development. They resemble most closely the inner cell mass (ICM) cells of 3.5-day blastocysts (Beddington and Robertson, 1989) and will differentiate into a variety of cell types, including those derived from mesoderm, when allowed to form embryoid bodies (Evans and Kaufman, 1981; Martin, 1981; Doetschman et al., 1985). Undifferentiated CCE ES cells contain low levels of follistatin mRNA (Fig. 1, lane 1), but expression increases significantly on differentiation into embryoid bodies (Fig. 1, lanes 2 and 3). This increased expression may reflect the formation of visceral and particularly parietal endoderm; parietal endoderm expresses high levels of follistatin (Albano et al., 1994). Increases in follistatin expression in ES 5 cells have similarly been noted by van den Eijnden-van Raaij et al. (1992), who induced differentiation by retinoic acid or by deprivation of leukemia inhibitory factor (LIF). In contrast to follistatin, expression of activin declines on differentiation of CCE ES cells (Albano et al., 1993).

F9 cells

Retinoic acid treatment of F9 cells provides a model for studying the differentiation of two extraembryonic cell lineages in the early postimplantation mouse embryo. When such cells are grown as a monolayer they form mainly parietal endoderm (Strickland and Mahdavi, 1978; Strickland et al., 1980), but when grown in suspension as aggregates they form mainly visceral endoderm (Hogan et al.)

Fig. 2. Follistatin is regulated during differentiation of F9 cells. F9 EC cells (Stem cells) were made to differentiate as parietal endoderm or visceral endoderm as described in Experimental procedures. Parietal endoderm-like cells were harvested at 5, 8 and 11 days. 20 μg RNA from each sample was analyzed by RNAase protection using probes specific for follistatin or human γ-actin. 20 μg of yeast tRNA was used as a negative control. Exposure times were 5 h for γ-actin and 3 days for follistatin. Note a dramatic increase in follistatin expression in parietal endoderm-like cells (compare lane 2 with lane 1), but little increase in visceral endoderm cells (lanes 3-5).
Fig. 3. RT-PCR analysis of follistatin expression during pre-implantation mouse development. Preimplantation mouse embryos were recovered from oviducts and uteri. RNA was extracted, reverse transcribed and submitted to PCR using primers specific for follistatin (see Experimental procedures). Follistatin RNA is present at all stages examined. The lower levels observed at the morula stage in the experiment illustrated here are not typical; other experiments revealed greater amounts of follistatin mRNA.

The technique of reverse-transcription polymerase chain reaction (RT-PCR) was used to study follistatin expression in oocytes, 2-cell, morula- and blastocyst-stage mouse embryos. Follistatin transcripts were detected at all these stages (Fig. 3), an observation which contrasts with the results of van den Eijnden-van Raaij et al. (1992), who were unable to detect follistatin transcripts in blastocysts. The lower levels observed in morulae in Fig. 3 are not typical; other experiments revealed greater levels of follistatin RNA at this stage.

Expression of follistatin in morulae was confirmed by in situ hybridization, which also indicated that expression was equivalent in all blastomeres at this stage (Fig. 4). This experiment also showed that follistatin is transcribed in the oviduct tissue in which these early embryos were embedded (Fig. 4A,B).

Conclusions

The experiments described in this report indicate that follistatin is expressed throughout preimplantation mouse development, from egg to blastocyst stages (Fig. 3). This conclusion contrasts in one respect with data of van den Eijnden-van Raaij et al. (1992), who were unable to detect follistatin transcripts in blastocysts. The reason for this difference is unclear. The presence of follistatin mRNA does not, of course, prove that the protein is present. We note, however, that follistatin protein is present in early Xenopus embryos of homologous stages (Fukui et al., 1994). Unfortunately, mouse embryos are too small to contemplate biochemical extraction and characterization of follistatin, as was done for Xenopus.

The role of follistatin during preimplantation mouse development is unknown. It may act to regulate the function of activin — both the inhibin $\beta_A$ and $\beta_B$ subunits are expressed during this time — or it may have functions unrelated to inhibition of activin function.
In support of this latter suggestion, it is unlikely that follistatin has a crucial role in regulating the function of activins AB or B because embryos deficient in the inhibin \(\beta_A\) subunit are viable (Vassalli et al., 1994). The phenotypes of embryos deficient for the inhibin \(\beta_B\) subunit and for follistatin will be of great interest.

Comparison of the responses of follistatin (Figs. 1 and 2) and of the inhibin \(\beta_A\) and \(\beta_B\) subunits (Albano et al., 1993) to differentiation of ES and EC cells provides an interesting example of "cooperative regulation", with the down-regulation of a ligand being accompanied by the up-regulation of an inhibitor. This might provide a way in which levels of functional ligand could be rapidly and efficiently modulated. Conventionally, one would regard the ligand as being activin and the inhibitor as follistatin, so that differentiation would result in a fall in activin activity. However, the recent report that follistatin can stimulate production of progesterone by bovine thecal cells directly (Shukovsky et al., 1993) suggests that the opposite might also be possible.

Finally, the dramatic up-regulation of follistatin expression in F9 cells induced to form parietal endoderm is consistent with the high levels of expression of follistatin in this tissue during normal embryogenesis (Albano et al., 1994). This observation may offer a useful model for understanding the regulation of gene expression in the formation of parietal endoderm.

**Experimental procedures**

**Cells**

F9 and CCE ES cells were cultured and induced to differentiate exactly as described by Albano et al. (1993).

**Mouse embryos**

Mouse embryos were obtained from matings of the outbred strain MF1 or from crosses between F1 CBAXC57/BL10 females and MF1 or F1 CBAXC57/BL10 studs. Noon on the day of appearance of the vaginal plug was taken as 0.5 days of development. Superovulation was induced by intraperitoneal injection of 5 IU of follicle-stimulating hormone (FSH) followed by 0.5 IU of human chorionic gonadotrophin (hCG) 44 to 48 h later. Embryos were recovered by flushing oviducts or uteri according to the stage desired.

**RNAase protection assays**

These were performed according to the method of Krieg and Melton (1987). RNA was obtained by the method of Chomczynski and Sacchi (1987) or by extraction with LiCl/urea (Auffray and Rougeon, 1979). The follistatin probe was as described by Albano et al. (1994), and the \(\gamma\) actin loading control was as described by Albano et al. (1995).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

RT-PCR was carried out as described by Albano et al. (1993). Approximately 150 embryos of each stage were used. Primers specific for HPRT were amplified for 32 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and then for 10 min at 72°C. The follistatin probe was described by Albano et al. (1994) and labeled with 35S-UTP.

**In situ hybridization**

Morulae and blastocysts were flushed as described above and placed inside oviducts to facilitate wax embedding. The embryos were fixed in 4% paraformaldehyde, embedded in wax and in situ hybridizations were carried out on 5 \(\mu\)m sections as described by Wilkinson and Green (1990). The follistatin probe described by Albano et al. (1994) was labeled with 35S-UTP.

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**References**


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