

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 deciduum, 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 deciduum 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 β_A or β_B subunits of inhibin, itself a heterodimer of one of the β subunits together with an α 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 decidual tissue (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 deciduum in a pattern reciprocal to that of the inhibin β_A and β_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 β_A and β_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

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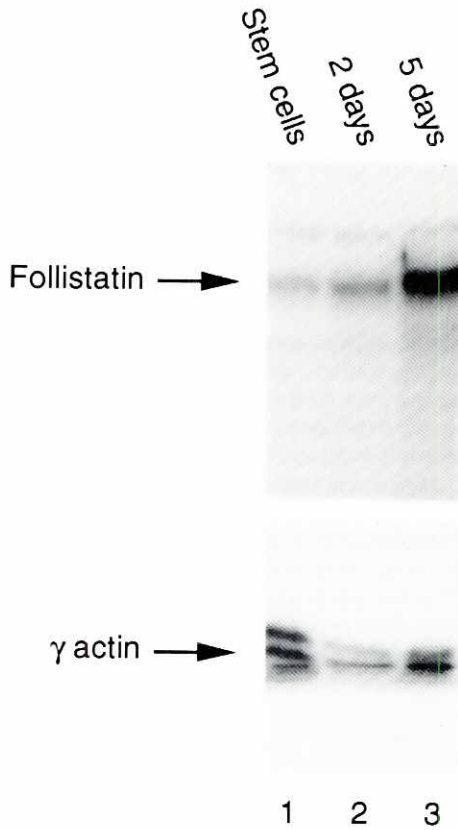


Fig. 1. Follistatin is regulated during differentiation of ES cells. CCE ES cells were differentiated as embryoid bodies for 2 or 5 days as described in Experimental procedures. 20 μg RNA from each time point was analyzed by RNAase protection using probes specific for follistatin or human γ actin. 20 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*

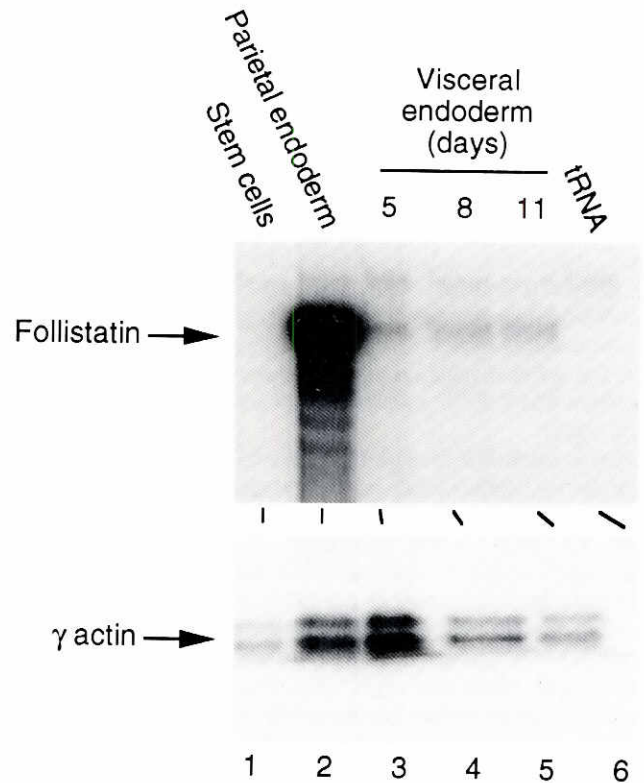


Fig. 2. Follistatin is regulated during differentiation of F9 cells. F9 EC cells (Stem cells) were made to differentiate as parietal endoderm or visceral endoderm as described in Experimental procedures. Visceral endoderm-like cells were harvested at 5, 8 and 11 days. 20 μg RNA from each sample was analyzed 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).

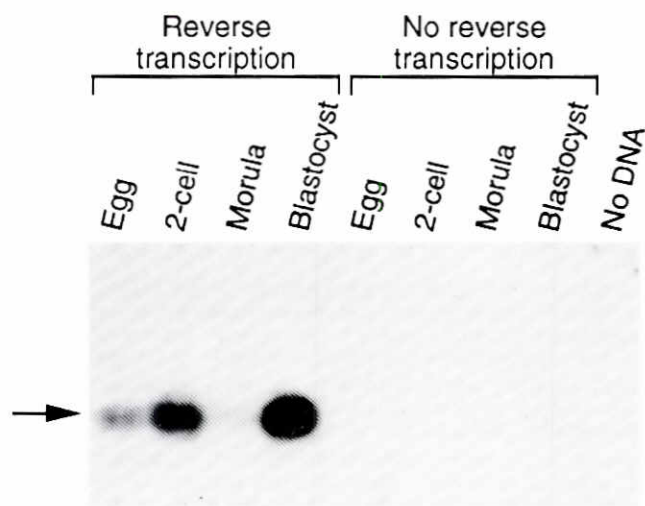


Fig. 3. RT-PCR analysis of follistatin expression during pre-implantation mouse development. Preimplantation mouse embryos were recovered from oviducts and uteri. RNA was extracted, reverse transcribed and submitted to PCR using primers specific for follistatin (see Experimental procedures). Follistatin 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.

al, 1981). Figure 2 shows that uninduced F9 stem cells express only low levels of follistatin and that differentiation into visceral endoderm results in no substantial change (Fig. 2; compare lane 1 with lanes 3-5). By contrast, differentiation into parietal endoderm causes a dramatic increase in follistatin expression (Fig. 2; compare lanes 1 and 2). This is consistent with the strong expression of follistatin in parietal endoderm cells of the normal mouse embryo (Albano *et al.*, 1994).

The marked increase in follistatin expression in EC cells induced to form parietal endoderm again contrasts with the behavior of the inhibin β_B chain, the expression of which falls sharply under these conditions (Albano *et al.*, 1993).

Expression of follistatin during preimplantation mouse development

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 3.5-day 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 β_A and β_B subunits are expressed during this time — or it may have functions unrelated to inhibition of activin function.

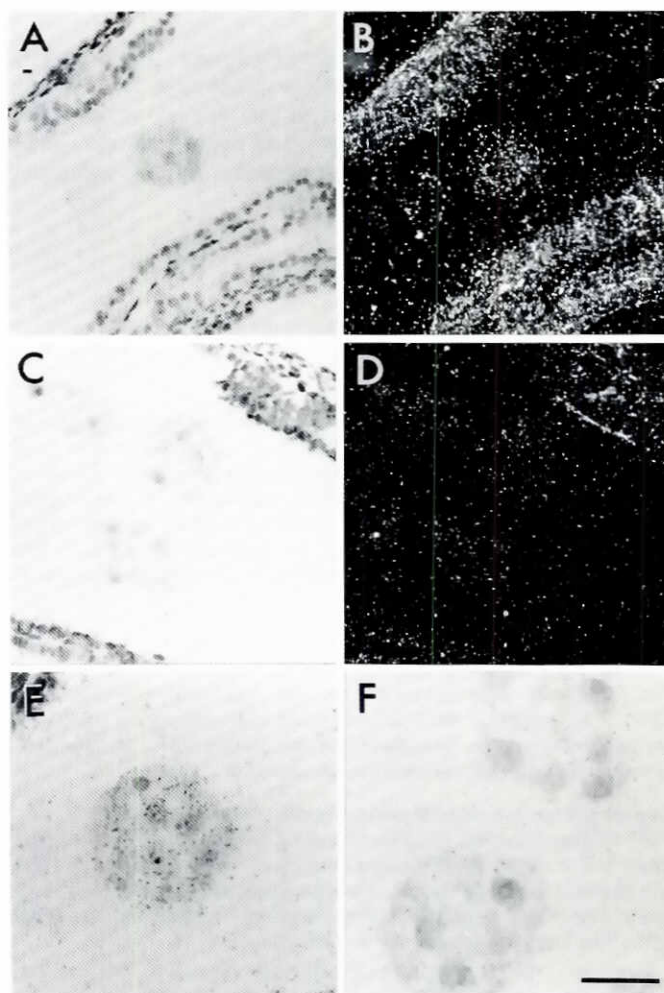


Fig. 4. *In situ* hybridization analysis of follistatin expression during preimplantation mouse development. Morulae and blastocysts were placed in oviducts, embedded in wax and sectioned at 5 μ m. *In situ* hybridization was performed using a probe specific for follistatin. (A) Bright-field and (B) dark-field view of a morula-staged embryo hybridized with an antisense follistatin probe. (C) Bright-field and (D) dark-field view of a morula-staged embryo hybridized with a sense follistatin probe. (E), (F) High-power bright-field views of morulae hybridized with an antisense (E) and a sense (F) follistatin probe. Scale bars: F, 50 μ m in A-D and 100 μ m in E and F.

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 β_B subunit are viable (Vassalli *et al.*, 1994). The phenotypes of embryos deficient for the inhibin β_A subunit and for follistatin will be of great interest.

Comparison of the responses of follistatin (Figs. 1 and 2) and of the inhibin β_A and β_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 (Shukovski *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 is taken as 0.5 days of development. Superovulation was induced by intraperitoneal injection of 5 IU of follicle-stimulating hormone (FSH) followed by 5 IU of human chorionic gonadotrophin 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 γ actin loading control was as described by Albano *et al.* (1993).

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 used to assess the success of reverse transcription prior to PCR (not shown; see Albano *et al.*, 1993). Primers specific for follistatin are shown below; they amplified a fragment of 329 base pairs.

5' primer: 5'-TGTGAGAACGTGGACTGCGGAC-3'
3' primer: 5'-CTGTTTCAGAAGAGGGGCTCT-3'

35 cycles of PCR were carried out, and the products were analyzed by agarose gel electrophoresis followed by Southern blotting and hybridization using a mouse follistatin-specific probe.

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 μ m sections as described by Wilkinson and Green (1990). The follistatin probe described by Albano *et al.* (1994) was labeled with 35 S-UTP.

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References

- ALBANO, R.M., ARKELL, R., BEDDINGTON, R.S.P. and SMITH, J.C. (1994). Expression of inhibin subunits and follistatin during postimplantation mouse development: decidual expression of activin and expression of follistatin in primitive streak, somites and hindbrain. *Development* 120: 803-813.
- ALBANO, R.M., GROOME, N. and SMITH, J.C. (1993). Activins are expressed in preimplantation mouse embryos and in ES and EC cells and are regulated on their differentiation. *Development* 117: 711-723.
- ASASHIMA, M., NAKANO, H., UCHIYAMA, H., SUGINO, H., NAKAMURA, T., ETO, Y., EJIMA, D., DAVIDS, M., PLESSOW, S., CICHOCKA, I. and KINOSHITA, K. (1991). Follistatin inhibits the mesoderm-inducing activity of activin A and the vegetalizing factor from chicken embryo. *Roux Arch. Dev. Biol.* 200: 4-7.
- AUFFRAY, C. and ROUGEON, F. (1979). Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* 107: 303-314.
- BEDDINGTON, R.S.P. and ROBERTSON, E.J. (1989). An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* 105: 733-737.
- CHOMCZYNSKI, P. and SACCHI, N. (1987). Single-step method of RNA isolation by acidic guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156-159.
- DOETSCHMAN, T.C., EISTETTER, H., KATZ, M., SCHMIDT, W. and KEMLER, R. (1985). The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87: 27-45.
- EVANS, M.J. and KAUFMAN, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292: 154-156.
- FUKUI, A., NAKAMURA, T., UCHIYAMA, H., SUGINO, K., SUGINO, H. and ASASHIMA, M. (1994). Identification of activins A, AB and B and follistatin proteins in *Xenopus* embryos. *Dev. Biol.* 163: 279-281.
- HOGAN, B.L.M., TAYLOR, A. and ADAMSON, E. (1981). Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature* 291: 235-237.
- ITO, M., IGARASHI, M., YAMADA, K., HASEGAWA, Y., SEKI, M., ETO, Y. and SHIBAI, H. (1990). Activin A stimulates meiotic maturation of the rat oocyte *in vitro*. *Biochem. Biophys. Res. Commun.* 166: 1479-1484.
- JESSELL, T.M. and MELTON, D.A. (1992). Diffusible factors in vertebrate embryonic induction. *Cell* 68: 257-270.
- KIMELMAN, D., CHRISTIAN, J.L. and MOON, R.T. (1992). Synergistic principles of development: overlapping patterning system in *Xenopus* mesoderm induction. *Development* 116: 1-9.
- KREIG, P.A. and MELTON, D.A. (1987). *In vitro* RNA synthesis with SP6 RNA polymerase. *Methods Enzymol.* 155: 397-415.
- LING, N., UENO, N., YING, S.-Y., ESCH, F., SHIMASAKI, S., HOTTA, M., CUEVAS, P. and GUILLEMIN, R. (1988). Inhibins and activins. *Vitam. Horm.* 44: 1-46.
- MANOVA, K., PAYNTON, B.V. and BACHVAROVA, R.F. (1992). Expression of activins and TGF β 1 and β 2 RNAs in early postimplantation mouse embryos and uterine decidua. *Mech. Dev.* 36: 141-152.
- MARTIN, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* 78: 7634-7638.
- MURATA, M., ETO, Y., SHIBAI, H., SAKAI, M. and MURAMATSU, M. (1988). Erythroid differentiation factor is encoded by the same mRNA as that of the inhibin β_A chain. *Proc. Natl. Acad. Sci. USA* 85: 2434-2438.
- NAKAMURA, T., TAKIO, K., ETO, Y., SHIBAI, H., TITANI, K. and SUGINO, H. (1990). Activin-binding protein from rat ovary is follistatin. *Science* 247: 836-838.
- SCHUBERT, D., KIMURA, H., LACORBIERE, M., VAUGHAN, J., KARR, D. and FISCHER, W.H. (1990). Activin is a nerve cell survival molecule. *Nature* 344: 868-870.

- SHUKOVSKI, L., DYSON, M. and FINDLAY, J. (1993). The effects of follistatin, activin and inhibin on steroidogenesis by bovine thecal cells. *Mol. Cell. Endocrinol.* 97: 19-27.
- SIVE, H.L. (1993). The frog prince-ss: a molecular formula for dorsoventral patterning in *Xenopus*. *Genes Dev.* 7: 1-12.
- SMITH, J.C. (1993). Mesoderm-inducing factors in early vertebrate development. *EMBO J.* 12: 4463-4470.
- STRICKLAND, S. and MAHDAVI, V. (1978). The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* 15: 393-403.
- STRICKLAND, S., SMITH, K.K. and MAROTTI, K.R. (1980). Hormonal induction and differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell* 21: 347-355.
- VALE, W., RIVIER, J., VAUGHAN, J., McCLINTOCK, R., CORRIGAN, A., WOO, W., KARR, D. and SPIESS, J. (1986). Purification and characterization of an FSH-releasing protein from porcine ovarian follicular fluid. *Nature* 321: 776-779.
- VANDEN EIJNDEN-VAN RAAIJ, A.J.M., FEIJEN, A., LAWSON, K.A. and MUMMERY, C.L. (1992). Differential expression of inhibin subunits and follistatin, but not of activin receptor type II, during early murine embryonic development. *Dev. Biol.* 154: 356-365.
- VASSALLI, A., MATZUK, M.M., GARDNER, H.A.R., LEE, K-F. and JAENISCH, R. (1994). Activin/inhibin β_B subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes Dev.* 8: 414-427.
- WILKINSON, D.G. and GREEN, J. (1990). *In situ* hybridization and the three dimensional reconstruction of serial sections. In *Postimplantation Mammalian Embryos: A Practical Approach* (Eds. A. Copp and D. Cockcroft). IRL Press, Oxford, pp. 155-171.

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