Type ß transforming growth factors and activins in differentiating embryonal carcinoma cells, embryonic stem cells and early embryonic development

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ABSTRACT TGFß was originally identified on the basis of its ability to induce phenotypic transformation of non-transformed target cells while activin was discovered as a gonadal protein. They later turned out to be related and both to have possibly crucial roles in the regulation of embryonic development. Here we review the circumstantial and direct evidence for this in the context of our own studies on their expression in and effects on murine EC and ES cells and mouse embryos. Their possible interaction in development is discussed.

KEY WORDS: *activin, TGFβ, EC cells, ES cells, mouse embryo, receptors, binding proteins*

Introduction

During the first third of gestation up to early implantation, the mouse embryo develops from a single cell to a complex structure with three germ layers set aside that are the origin of both the embryo proper and most of the extraembryonic tissues. The first restriction in the developmental potency of cells in the embryo occurs at the 8-16-cell stage and follows a process known as compaction, where individual blastomeres, formed after cleavage of the fertilized oocyte, flatten, increase their contact with one another and polarize. The outer cells then form the trophectoderm (TE) lineage and the inner cells, the inner cell mass (ICM). As the embryo prepares for implantation, a second round of differentiation occurs: the formation of primitive endoderm and ectoderm. This is characterized by the appearance of an epithelial layer, the primitive endoderm on the surface of the ICM facing the blastocoelic cavity. Some of these primitive endoderm cells migrate on to a thin basal lamina that forms on the apical side of the trophectoderm and differentiate to form parietal endoderm of the yolk sac as they progress. These cells are characterized by enormous production and secretion of extracellular matrix (ECM) proteins. Primitive endoderm remaining in contact with the ICM differentiates to visceral endoderm, characterized by alphafetoprotein production and high endocytic activity. Finally at about 6.5 days of gestation, cells delaminate from the epithelial layer of the primitive ectoderm and accumulate as a layer of individual mesoderm cells between the primitive ectoderm and the visceral endoderm in a period known as gastrulation (Gardner and Rossant, 1979).

Despite this fairly thorough description of the morphology of events that occur during early development, relatively little is known about how cells orchestrate their coordinated interaction and more specifically, how particular cell-to-cell interactions are mediated to regulate growth and differentiation. The relative inaccessibility of the mouse embryo and the limited amount of material it yields have in particular hampered conventional biochemical studies. The pioneering studies of Stevens and Pierce in the sixties, however have resulted in the current availability of cell lines in culture that are pluripotent and that can be induced to form some or all of the earliest differentiated cell types present in the embryo. These first embryonal carcinoma (EC) cells isolated from spontaneous or embryo-derived teratocarcinomas (Pierce and Verney, 1961; Stevens, 1970; Damjanov *et al.*, 1971; Martin, 1975; Martin and Evans, 1975) and the later embryonic stem (ES) cells derived directly from blastocyst stage embryos (Evans and Kaufman, 1981; Martin, 1981) closely resemble those of the ICM antigenically, biochemically and in their developmental capacity. These cells provide simple access to the study of fundamental processes such as the initiation and direction of the differentiation of embryonic cells, the origin and function of the first basal laminae and the regulation of

Abbreviations used in this paper: EC, embryonal carcinoma; ES, embryonic stem; RA, retinoic acid; TGF, transforming growth factor; ECM, extracellular matrix; ICM, inner cell mass; TE, trophectoderm; MP, metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; MIS, Müllerian inhibiting substance; EDF, erythroid differentiation factor; FSH, follicle stimulating hormone; MIF, mesoderm inducing factor; FGF, fibroblast growth factor (a, acidic; b, basic; k, karposi).

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0214-6282/93/$03.00

proliferation. Interference with any of these processes may lead to the failure of an embryo to develop and implant or, in postnatal life, to tumor formation from incorrectly programmed embryonic cells. Much evidence has accumulated to suggest that polypeptide growth factors and their respective receptors regulate cell proliferation and differentiation in a variety of physiological and pathological processes. More recently it has become clear that this class of proteins are also prime candidates as mediators of cell-to-cell interaction in the early embryo on the basis of their differential expression in time and space and their known effects on various cell model systems *in vitro* (reviewed Mummery and van den Eijnden-van Raaij, 1990).

In our laboratory, the transforming growth factor ß (TGFß) family of growth factors and its role in early mouse development has been the main subject of study for several years. TGFß was first identified on the basis of its ability to stimulate anchorage-independent growth of normal fibroblasts (De Larco and Todaro, 1978; Roberts *et al.*, 1981). Anchorage-independent growth in semi-solid medium is characteristic for cells with neoplastic or metastatic potential *in vivo* (Cifone and Fidler, 1980). This transforming activity, which was entirely reversible, later appeared to consist of two components, TGFα (Derynck, 1986) and TGFß (Roberts *et al.*, 1981). TGFß itself did not induce phenotypic transformation but potentiated the action of TGFα.

Later studies showed that TGFß was in fact a misleading name since it is produced by most normal cells in culture and is multifunctional, inhibiting or stimulating cell proliferation, regulating cell migration, differentiation and the production of a variety of specific proteins (reviewed Rizzino, 1988). Four years after its first discovery, it was cloned from a human cDNA library (Derynck *et al.*, 1985) and when later other proteins with a high degree of homology were discovered, this protein was designated $TGFß_1$. There are now five, genetically distinct, known family members directly related to $TGFß_1$, which have been designated $TGFß_{1\text{-}5}$ (reviewed Mummery and van den Eijnden-van Raaij, 1990).

As important, in terms of embryonic development, as their ability to regulate the behavior of individual cells, is the ability of TGFßs to influence cell-to-cell interactions. The extracellular matrix (ECM) is a powerful component in determining the behavior of cells and commonly regulates the nature of their interaction. TGFß alters the composition of the ECM by regulating the production of its components, fibronectin (Leonard *et al.*, 1991; Wang *et al.*, 1991), laminin (reviewed Rizzino, 1988; Kelly and Rizzino, 1989), ECM degrading metalloproteinases (MP) and their inhibitors (TIMP) (reviewed Matrisian, 1990; Kubota *et al.*, 1991; Overall *et al.*, 1991). Integrins, the cell surface receptors of the ECM proteins, are also regulated by TGFßs (reviewed Ignotz and Massagué, 1987). Alterations in the ECM induced by TGFß stimulate precartilage condensation of the embryonic limb (Leonard *et al.*, 1991), inhibit mammary ductal growth (Silberstein *et al.*, 1990), increase cell-cell junctional complex formation in three-dimensional cultures of endothelial cells (Merwin *et al.*, 1990) and decrease the metastatic potential of tumors by increasing TIMP expression (Kubota *et al.*, 1991). Growth inhibition is often correlated with changes in gene expression of ECM constituents but, in general, causal relationships have not been demonstrated. Epithelia and mesenchyme communicate through basal laminae composed of specific ECM proteins so it is of note that *in situ* hybridization studies have associated TGFß expression with multiple sites of epithelial/mesenchymal interactions during embryogenesis (reviewed Akhurst *et al.*, 1990, 1992), as well as in the adult organism (see for example Robinson *et al.*, 1991). In addition TGFßs are often bound to the ECM (Fava and McClure, 1987; Mooradian *et al.*, 1989) so that the matrix may serve as a site of storage for TGFßs.

To date, few functional differences have been described between the different forms of TGFß, although exceptionally $TGFß_2$ has been shown to induce dorsal mesodermal tissues in explants of *Xenopus laevis* embryos while $TGFß_1$ is inactive unless in the additional presence of a fibroblast growth factor (FGF) (Rosa *et al.*, 1988). $TGFß_3$ is ten times more potent than $TGFß_2$ in this assay, while $TGFß_5$, derived from and expressed exclusively in *Xenopus*, is ineffective (Roberts *et al.*, 1990).

By screening cDNA libraries with (heterologous) probes and probes based on amino acid sequences of purified proteins, 19 different monomeric subunits capable of combining in at least 25 dimeric forms have been described to date (reviewed Massagué, 1990). TGFß is thus the prototype of a much larger superfamily. Based on their sequence homologies, they have been grouped into subfamilies; these include the activins and their structurally related antagonists, the inhibins, DVR (Drosophila decapentaplegic/vgl related), Müllerian inhibiting substance (MIS) and bone morphogenetic proteins (BMPs) as well as the TGFßs themselves (discussed Massagué, 1990; Ueno *et al.*, 1990; see also Burt, 1992). Of these other family members, the activins and their binding proteins, in particular their role in development, have also been a subject of intense study in our laboratory.

Activins were initially discovered as gonadal proteins that stimulated pituitary follicle stimulating hormone (FSH) production (Ling *et al.*, 1986; Vale *et al.*, 1986; Ying, 1988). Activin also modulates the secretion of growth hormone and prolactin from pituitary gland (Kitaoka *et al.*, 1988). In addition to their endocrine role, the activins have been shown to be important intragonadal regulators of ovarian and testicular function. In particular, effects of these proteins on the function and/or proliferation of gonadal theca interna cells (Hsueh *et al.*, 1987), granulosa cells (Hutchinson *et al.*, 1987; Rabinovici *et al.*, 1990), spermatogonia (Mather *et al.*, 1990), Leydig and ovary cells (González-Manchón and Vale, 1989) have been described (for a review see De Paolo *et al.*, 1991).

Growth- and differentiation-regulating effects of activins have also been noted in cell types and tissues outside the pituitary and reproductive system (Vale *et al.*, 1990). Activin was found to induce differentiation of two erythroleukemia cell lines (Eto *et al.*, 1987; Yu *et al.*, 1987) and to enhance erythropoietin-induced colony formation in bone marrow cultures (Yu *et al.*, 1987; Broxmeyer *et al.*, 1988). For this reason, activin is also known as erythroid differentiation factor (EDF). Activin has a dual effect on the proliferation of Balb/c 3T3 cells (Kojima and Ogata, 1989) and stimulates the secretion of insulin from pancreatic islands (Totsuka *et al.*, 1988) and glycogenolysis in liver cells (Mine *et al.*, 1989). Recently activin has been shown to have a potential function in the regulation of nerve cell differentiation (Hashimoto *et al.*, 1990; Schubert *et al.*, 1990; van den Eijnden-van Raaij *et al.*, 1991). One of the most exciting aspects of activin research for developmental biologists however is its possible role in early embryos as a potent mesoderm-inducing factor (Asashima *et al.*, 1990; Smith *et al.*, 1990; van den Eijnden-van Raaij *et al.*, 1990b).

The present paper gives an overview of the effects and expression patterns of activins, TGFßs and their receptors/binding proteins in EC and ES cells and in the embryo itself. The possible interaction between activins and TGFßs in development is discussed.

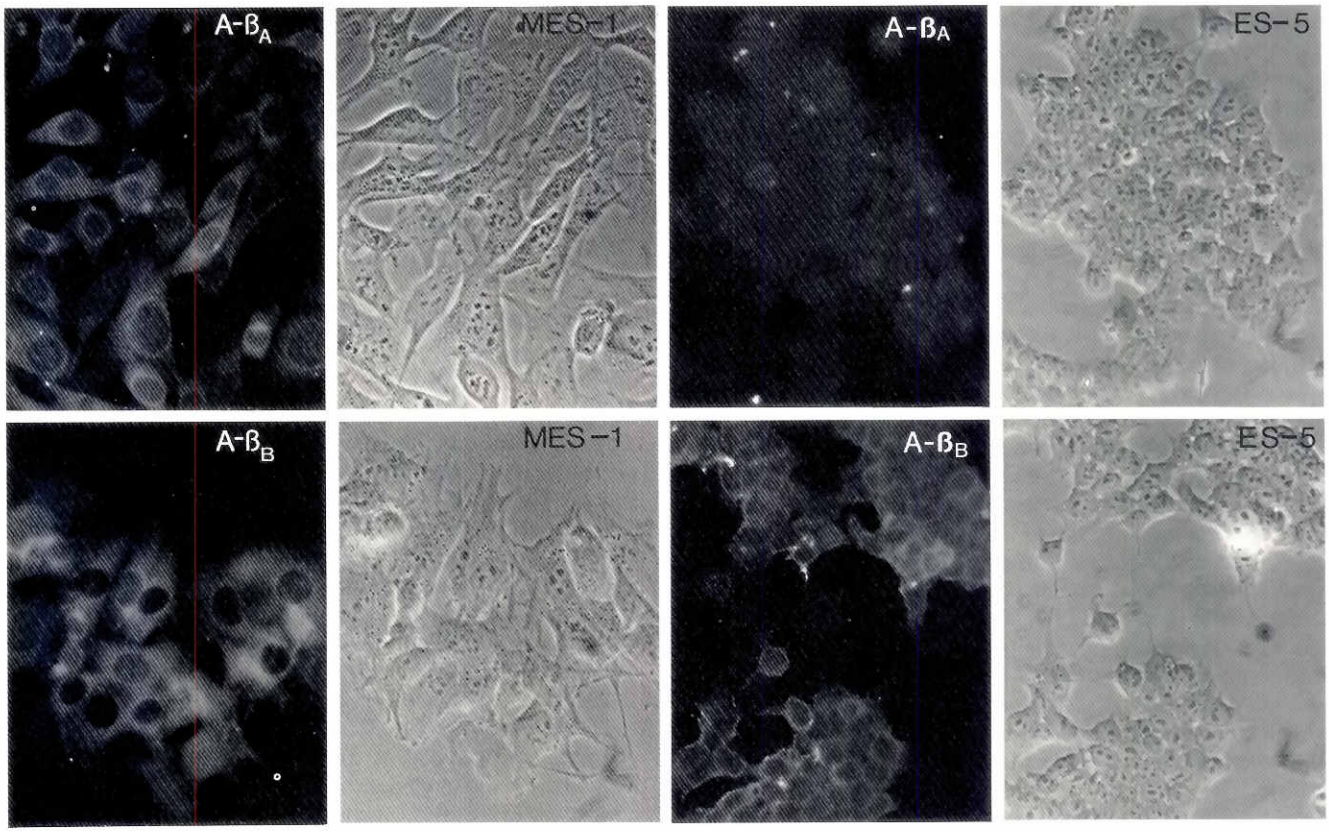

Fig. 1. Immunofluorescent staining of P19-derived (MES-1) mesodermal cells and undifferentiated embryonic stem (ES) cells with affinity-purified antiserum against the ßA (A-ßA) and ßB (A-ßB) inhibin subunits. *Data from C. Paulusma.*

Ligands, receptors and binding proteins

TGFß

TGFß is a homodimeric protein secreted by the majority of cells in culture in an inactive form (Lawrence *et al.*, 1984; Pircher *et al.*, 1986) that neither interacts with specific TGFß cell surface receptors nor elicits any of the known TGFß-induced biological responses. The active 25 kD TGFß molecule consists of two identical disulfide-linked polypeptide chains (Assoian *et al.*, 1983). The monomer is synthesized as a larger pre-pro-TGFß, which undergoes a series of cleavage and post-translational processing steps prior to secretion. Active TGFß may be generated from the latent molecule by extremes of pH, by treatment with SDS or urea (Lawrence *et al.*, 1985; Pircher *et al.*, 1986), by high temperature (Brown *et al.*, 1990) or by proteases such as plasmin (Lyons *et al.*, 1988). Recent findings that the activation of latent TGFß during co-culture of bovine endothelial cells and pericytes is due to plasmin (Sato and Rifkin, 1989) suggest that plasmin in particular may be a physiological activator. Plasmin is generated from plasminogen by the enzyme plasminogen activator. This is of interest in the context of mouse development since plasminogen activator is secreted abundantly by the trophoblast and parietal endoderm of early embryos (Strickland *et al.*, 1976).

At least twenty proteins have now been shown to bind TGFß (reviewed Lin and Lodish, 1993). These proteins can be divided into two groups: those that are soluble or associated with the extracellular matrix and those expressed on the cell surface. Of the proteins on the cell surface that bind TGFß, three have been identified in chemical crosslinking studies as likely candidates for mediating signal transduction (Massagué, 1990; Roberts and Sporn, 1990). These have been termed types I, II and III. Types I and II have high ligand affinity and low binding capacity while type III is a proteoglycan with low affinity and high capacity. The recent cloning of two of these receptors (López-Casillas *et al.*, 1991; Wang *et al.*, 1991; Lin *et al.*, 1992) has shown that the type II is a transmembrane serine/threonine kinase that can confer a growth inhibitory response to TGFß to cells deficient in this receptor (Lin and Lodish, 1993) while the type III receptor has no known signalling motif but can increase the ability of the type II receptor to bind $TGFß_1$ without affecting binding to the type I receptor (Wang *et al.*, 1991).

Activins

Activins are dimeric proteins consisting of two disulfide-linked polypeptide chains ($ß_A$ and $ß_B$), that are each processed from a larger secretory precursor (for a review see Ying, 1988). There are three forms of activin, resulting from combinations of two $ß_A$ chains (activin A), two $ß_B$ chains (activin B) or one $ß_A$ and one $ß_B$ chain (activin AB). cDNA cloning and DNA sequence analysis have shown that the predicted primary structure of the mature $ß_A$ and $ß_B$ subunits of 116 and 115 animo acids, respectively, is very highly conserved between different species (Mason *et al.*, 1985, 1986, 1989; Forage

et al., 1986; Woodruff *et al.*, 1987). The mature $ß_A$ and $ß_B$ subunits are structurally related and show 64% amino acid homology. As for other proteins of the TGFß superfamily, the position of nine cysteine residues in the carboxyl terminus is perfectly conserved. The $ß_A$ and $ß_B$ subunits are encoded by different genes that were shown to consist of two exons containing all of the coding sequences (Stewart *et al.*, 1986; Mason *et al.*, 1989).

The first forms of activin isolated from natural sources were activin A and activin AB. Although the $ß_B$-$ß_B$ homodimeric form was also believed to exist, native activin B has only recently been purified from porcine follicular fluid (Nakamura *et al.*, 1992a). The activity of the purified activin B was found to be significantly lower than those of other activins in various assay systems such as stimulation of FSH secretion, induction of erythrodifferentiation and potentiation of follicular granulosa cell differentiation. In contrast activin B was shown to have a potent mesoderm-inducing activity in *Xenopus laevis* blastula explants, comparable with that of other activins. These results suggest the existence of an activin receptor, which is specific for activin B.

Cellular activin binding sites have been identified on a number of activin-responsive cells, including erythroleukemia cells (Campen and Vale, 1988; Hino *et al.*, 1989), granulosa cells (Sugino *et al.*, 1988) and embryonal carcinoma cells (Kondo *et al.*, 1989). Chemical cross-linking studies have shown that, based on the nomenclature defined for the TGFß receptors (Massagué *et al.*, 1990), type I and type II activin receptors of 65kD and 85 kD, respectively, should exist (Hino *et al.*, 1989; Centrella *et al.*, 1991; Mathews *et al.*, 1991).

Recently the mouse activin receptor type II was successfully cloned (Mathews and Vale, 1991; Attisano *et al.*, 1992) and the nucleotide sequences of the *Xenopus* (Kondo *et al.*, 1991) and human (Matzuk and Bradley, 1992a) activin type II receptor cDNA followed shortly afterwards; these proteins are, respectively, 87% and 99% identical at the amino acid level with the mouse type II activin receptor. Cloning of the mouse gene has shown that it is encoded by 11 exons and has several putative transcription factor binding sites which may be important for its complex transcriptional regulation (Matzuk and Bradley, 1992b). In the mouse at least there is also a second type II activin receptor (type IIB), which is encoded by a different gene to the type II activin receptor (Attisano *et al.*, 1992). The type IIB receptor is expressed in four alternatively spliced forms (IIB_1-IIB_4) which bind to activin A with different affinities. Although the type II and type IIB activin receptors were assumed to be transmembrane serine/threonine-specific protein kinases, additional tyrosine kinase activity has recently been described for the activin receptor protein purified from a mouse EC cell line (Nakamura *et al.*, 1992b).

An activin-binding protein other than a cell surface receptor is follistatin, a glycosylated polypeptide isolated from porcine follicular fluid, that has inhibin-like activity on the regulation of FSH secretion (Ueno *et al.*, 1987; Shimasaki *et al.*, 1988a,b). This protein with a specific and high affinity for activin (Nakamura *et al.*, 1990a) was found to exert a neutralizing effect on activin activity in various systems, including stimulation of FSH secretion by cultured pituitary cells (Kogawa *et al.*, 1991), induction of mesodermal tissue formation in *Xenopus* animal cap explants (Asashima *et al.*, 1991a), differentiation of rat granulosa cells (Nakamura *et al.*, 1990b) and proliferation of murine osteoblastic cells (Hashimoto *et al.*, 1992). Although follistatin binds to both activin and inhibin through the common beta-subunit (Shimonaka *et al.*, 1991), binding does not affect the biological activity of inhibin (Ying *et al.*, 1987). The recent finding that follistatin associates with heparan sulfate chains of proteoglycans on follicular granulosa cells suggests that this cell-associated follistatin may regulate various actions of activin on the cell surface (Nakamura *et al.*, 1991). The wide distribution of follistatin outside the gonadal system (Shimasaki *et al.*, 1989; Kaiser *et al.*, 1990; Michel *et al.*, 1990; Tashiro *et al.*, 1991) indicates that this factor may have a significant role in extragonadal tissues as a local regulator of the biological activities of activin.

Role of TGFß and activin in development: evidence from cell model systems

TGFß

Studies on differentiating EC and ES cells in culture have provided several lines of circumstantial evidence that TGFßs have a role in the regulation of growth and differentiation in early mouse development. First of all, Rizzino *et al.* (1983) reported that F9 and PC 13 EC cells release factors into their medium that induce phenotypic transformation of a nontransformed target cell. Specific bioassays that later distinguished between TGFα and TGFß, showed that in general all EC and ES cell lines secrete TGFß while a minority, such as P19 EC also secrete TGFα (Van Zoelen *et al.*, 1989).

We have recently analyzed in detail the TGFß isoforms secreted by ES cells during their differentiation on the one hand to parietal endoderm-like derivatives expressing high levels of laminin and tissue-type plasminogen activator, or on the other, to mesenchymal derivatives expressing Brachyury T (Mummery *et al.*, 1990a; Slager *et al.*, 1993a). Serum-free conditioned media from cells at various stages of differentiation were tested in a bioassay, based on the TGFß-induced growth inhibition of mink lung epithelial cells, ML-CCL64 (Rosa *et al.*, 1988; Danielpour *et al.*, 1989). By comparing heat-treated with untreated medium and by carrying out the assay in the presence or absence of antibodies with neutralizing activity for specific TGFß isoforms, we were able to show that undifferentiated ES cells secrete latent $TGFß_1$ exclusively, their parietal endoderm-like derivatives secrete latent and active $TGFß_2$ while their mesenchymal derivatives secrete all three isoforms, $TGFß_1$ and $ß_2$ in a latent form and $ß_3$ as the active protein (Slager, 1992; Slager *et al.*, 1993a). This correlates with their differentiation-dependent expression of a 1.8 kb transcript for $TGFß_1$, four transcripts for $TGFß_2$ and high levels of expression of $TGFß_3$, described previously (Mummery *et al.*, 1990b,c). $TGFß_3$ was shown in these studies not to be associated with the extracellular matrix of the differentiated cells, in contrast to $TGFß_1$ and $ß_2$. Immunohistochemical studies have also shown distinct extracellular immunoreactivity with anti-$TGFß_1$ (Thompson *et al.*, 1989; Pelton *et al.*, 1991) and anti-$TGFß_2$ antibodies (Pelton *et al.*, 1991; Slager *et al.*, 1991), but extracellular localization of $TGFß_3$ has not been reported to date (Jakowlew *et al.*, 1991; Pelton *et al.*, 1991), supporting the results described above. Association with matrix components is likely to result in long term/short range functions (Paralkar *et al.*, 1991), while association in a latent diffusible complex may result in a short term/long range function (Massagué, 1990). $TGFß_3$ may therefore differ in its mechanism of action from $TGFß_1$ and $TGFß_2$. Interestingly, $TGFß_3$ is also predominantly (but not exclusively) expressed in cells of mesodermal origin during murine embryogenesis as well as in a number of mesenchymal cell lines (Derynck *et al.*, 1988; Millan *et al.*, 1991).

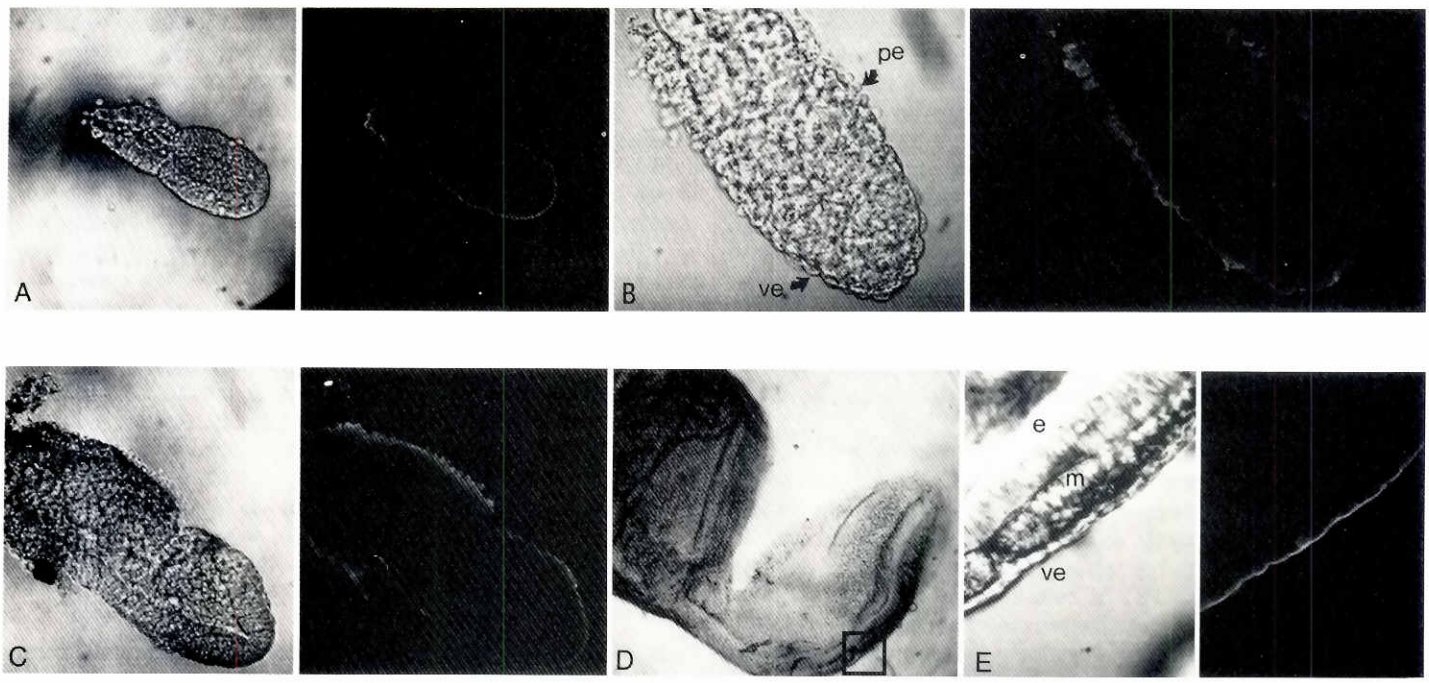

Fig. 2. Immunofluorescent staining of early postimplantation embryos for TGFß$_2$. (A) *6.0 days p.c. (20x objective). Reicherts membrane is removed. Staining is only in endoderm, not ectoderm.* **(B)** *6.0 days p.c. (40x objective). Parietal endoderm (pe) attached to remnants of Reicherts membrane is lightly stained and visceral endoderm (ve) more strongly.* **(C)** *6.5 days p.c. (20x objective). Staining in endoderm only.* **(D)** *7.5 days p.c. Embryonic (on the right) and extraembryonic (on the left) parts of the embryo have been separated to facilitate antibody access to all three of the germ layers now present.* **(E)** *7.5 days p.c. Enlargement of region, indicated in D, containing ectoderm (e), visceral endoderm (ve) and mesoderm (m). TGFß$_2$ protein is detected in endoderm only; ectoderm and mesoderm are negative. Embryos are shown in Nomarski interference optics and for fluorescence as single optical sections from a confocal laser scanning microscope. Data from V. Knecevic, E. Freund and B. Roelen.*

Binding studies with iodinated TGFß$_1$ have shown that undifferentiated EC and ES cells lack significant expression of surface receptors for TGFß (Rizzino, 1987; Mummery and van den Eijnden-van Raaij, 1990). After the induction of differentiation by retinoic acid, however, high affinity binding sites for TGFß$_1$ become detectable and all three receptor types are found after chemical cross linking; further the cells become sensitive to the growth inhibitory effects of TGFß (Rizzino, 1987; Mummery and van den Eijnden-van Raaij, 1990). A direct biological effect of TGFß on undifferentiated EC or ES cells would therefore not be expected (Rizzino, 1987). However, when EC or ES cells are triggered to differentiate by retinoic acid and/or aggregation in suspension culture, TGFßs can profoundly affect the differentiated phenotype that finally emerges (van den Eijnden-van Raaij *et al.*, 1992; Slager *et al.*, 1993b). These experiments are carried out in culture medium supplemented with fetal calf serum stripped of lipophilic substances, including retinoids, with cells forming single aggregates of 800 cells in droplets hanging from the lid of an inverted petri dish. Under these conditions the cells become exquisitely sensitive to retinoic acid (RA) so that instead of the usual 10^{-6} or 10^{-7} M RA being necessary to induce differentiation, 10^{-9}M is sufficient; mesenchymal derivatives that include beating cardiac muscle then form after the aggregates are replated on a tissue culture substrate. At 10^{-8} M RA, predominantly neural cells are formed while at 10^{-7} M RA, cells with the characteristics of endoderm emerge. TGFß$_1$ and ß$_2$ both enormously increase the rate and amount of beating muscle and myoblasts that appear; 10^{-9} M RA however completely abrogates their effects on cardiac muscle differentiation but has no effect on myoblast formation (Slager *et al.*, 1993b). These results were at first surprising since TGFß had been reported to inhibit the fusion of certain myoblast cell lines (Massagué *et al.*, 1986; Olson *et al.*, 1986) and repress transcription of the skeletal muscle specific regulatory transcription factors MyoD1 and myogenin (Vaidya *et al.*, 1989; Brennan *et al.*, 1991). However, these latter studies were carried out under mitogen-poor (serum-free) conditions; under mitogen-rich conditions TGFß in fact stimulates myoblast differentiation (Zentella and Massagué, 1992), as we found in the studies with EC and ES cells.

Taken together these results suggest that it is the subtle balance between factors such as TGFß, other polypeptide growth factors and retinoids that may determine the ultimate state of differentiation of embryonic cells and that *in vitro* models such as we describe may be useful in elucidating the mechanism by which they act.

Activin

One of the first indications that activins could be important regulators of early embryonic development came from the group of Asashima, who showed a mesoderm-inducing effect of activin A on animal cap explants from *Xenopus laevis* (Asashima *et al.*, 1990). At about the same time, the mesoderm-inducing factor (MIF) from

X. laevis XTC cells (XTC-MIF) was identified as the *Xenopus* homologue of mammalian activin A (Smith *et al.*, 1990; van den Eijnden-van Raaij *et al.*, 1990b) and was shown to have the properties of a classical morphogen, namely sharp effect thresholds and multiple stable responses (Green and Smith, 1990). In addition to activin A, the two other forms of activin (B and AB) were shown to be potent inducers of mesoderm in *Xenopus laevis* (Nakamura *et al.*, 1992a). Recently it was found that activin A also induces axial structures, including notochord and somites in the chick epiblast (Mitrani and Shimoni, 1990; Mitrani *et al.*, 1990) and newt presumptive ectoderm (Moriya and Asashima, 1992). Exposure of animal cap cells of zebrafish to activin A induces the zebrafish T gene (zf-T), which is a marker for mesodermal tissue including notochord (Schulte-Merker *et al.*, 1992). These results suggest that activin may be one of the natural inducers in a wide range of species.

Little is known about the possible function of activins and inhibins in early murine development. Using the P19 EC cell line as a model for studying mesodermal and neural differentiation in the mouse, we have recently shown that activin A itself has no mesoderm-inducing activity in this system. In fact, activin A completely inhibits differentiation of P19 EC cells into derivatives of any of the three germ layers, whether these are induced by retinoic acid (RA) at different concentrations, as described in the previous section, or by an as yet unidentified endoderm-derived factor resembling fibroblast growth factor (FGF) (Hashimoto *et al.*, 1990; van den Eijnden-van Raaij *et al.*, 1991). Effects of activin A have also been observed in monolayer cultures of P19 EC cells and appear to depend on the culture conditions used. In conventional serum-containing medium, cross-talk between activin A and RA was demonstrated by a synergistic increase in the amount of c-jun mRNA by activin A in the presence of retinoic acid (Momoi *et al.*, 1992). In addition, activin A promotes the survival, but not the division, of P19 EC cells grown under serum-free conditions on tissue culture plastic (Schubert *et al.*, 1990). However, when P19 EC cells are cultured on substrates of extracellular matrix proteins such as laminin and fibronectin, activin A appears to be a potent mitogen (Schubert and Kimura, 1991). These results together with the identification of activin-binding sites on P19 EC cells (Kondo *et al.*, 1989) suggest that activins may have a regulatory function in differentiation during early murine development.

We have examined the expression of different inhibin subunits (α, β_A, β_B), as well as follistatin and the activin type II receptor in ES cells, EC cells and their differentiated derivatives (van den Eijnden-van Raaij *et al.*, 1992). Undifferentiated ES and P19 EC cells express the β_B chain exclusively, suggesting that β_B activin subunits may be expressed as early as the blastocyst stage of development. β_B expression is unchanged as ES cells are induced to form parietal endoderm-like derivatives by RA in the presence of LIF but is greatly reduced during mesenchymal differentiation of ES cells induced by LIF deprivation. In contrast to β_B, the β_A subunit is not expressed in undifferentiated ES and EC cells, but is expressed in several differentiated cell lines derived from EC cells, including P19 EPI-7, END-2 and MES-1 (Mummery *et al.*, 1985, 1986). Using polyclonal anti-peptide antibodies specific for the β_A and β_B subunits (Paulusma *et al.*, in preparation) a good correlation between mRNA and protein expression of the ß subunits has been observed in the different *in vitro* model systems. For example ES cells stain exclusively with the anti-β_B antiserum, while MES-1 cells stain well with both anti-β_B and β_A antisera (Fig. 1).

Recently we have shown that in the MES-1 cell line, a mesodermal derivative of P19 EC, ß subunit expression is modulated by TGFβ_1 and TGFβ_2, but not significantly by other growth factors such as LIF or members of the fibroblast growth factor family (aFGF, bFGF or kFGF). β_A mRNA expression is increased while β_B expression is simultaneously decreased by TGFß (van der Kruijssen *et al.*, submitted). Furthermore, TGFß increased the amount of bioactive activin secreted by MES-1 cells. Inhibin α subunit mRNA is only present in endodermal and neuro-epithelial cells and is not affected by TGFß. These results suggest a possible role for type beta transforming growth factors as regulators of activin expression during early murine embryogenesis.

In addition to the inhibin ß subunits, expression of the activin-binding protein follistatin is developmentally regulated (van den Eijnden-van Raaij *et al.*, 1992). A change in follistatin expression occurs during the differentiation of P19 EC and ES cells. The three differentiated clones of P19, MES-1, END-2, and EPI-7 express different levels of the follistatin transcript that at least in END-2 and EPI-7 are slightly altered by TGFß (van den Eijnden-van Raaij, unpublished results). By contrast, activin receptor type II mRNA is present in undifferentiated cells and levels are not affected by any of the differentiation stimuli described above.

Role of TGFß and activins in development: evidence from embryos

TGFß

In view of the extremely wide range of biological effects reported for TGFßs, it is difficult to be comprehensive in discussing their function in embryogenesis. Comparative localization studies of protein and mRNA, however, may provide some initial clues on the relative importance of paracrine versus autocrine mechanisms in the regulation of specific growth and differentiation processes in the embryo.

It has long been known that preimplantation mouse embryos grow and differentiate in the absence of exogenous factors (Biggers *et al.*, 1971) and therefore assumed that endogenous factors must sustain the embryo during the first seven to eight cleavage divisions. The first evidence for such factors was indirect; cultured blastocysts were shown to produce transforming growth factor-like activity that promoted the anchorage-independent growth of non-transformed target cells in coculture (Rizzino, 1985). First evidence for growth factor transcripts in preimplantation embryos was provided by Rappolee *et al.* (1988) using a microtechnique for isolation of RNA followed by reverse transcription and amplification of the transcribed cDNA in a polymerase chain reaction (PCR). TGFβ_1 was expressed from the 4-8 cell stage up to at least the blastocyst; TGFα was only expressed in the blastocyst but together they could account for the TGF-like activity secreted by blastocysts. 10-90% of the cells of the blastocyst appeared to stain with an anti-TGFβ_1 antibody, so that here, RNA and protein were co-localized in the same cells.

A similar conclusion could be drawn from independent studies on TGFβ_2. Transcripts for this isoform were first detectable at the morula stage and expression was maintained through to the blastocyst (Kelly *et al.*, 1990). Using an antibody recognizing TGFβ_2 but not TGFβ_1 (van den Eijnden-van Raaij *et al.*, 1990a), the protein was shown to be present from the 4-cell stage through the blastocyst, where staining was in the trophectoderm but not inner cell mass (ICM) cells (Mummery *et al.*, 1990b; Slager *et al.*, 1991). In early postimplantation stages of development from day 6.0 p.c. to 7.5 p.c., TGFβ_2 protein was present at high levels in visceral

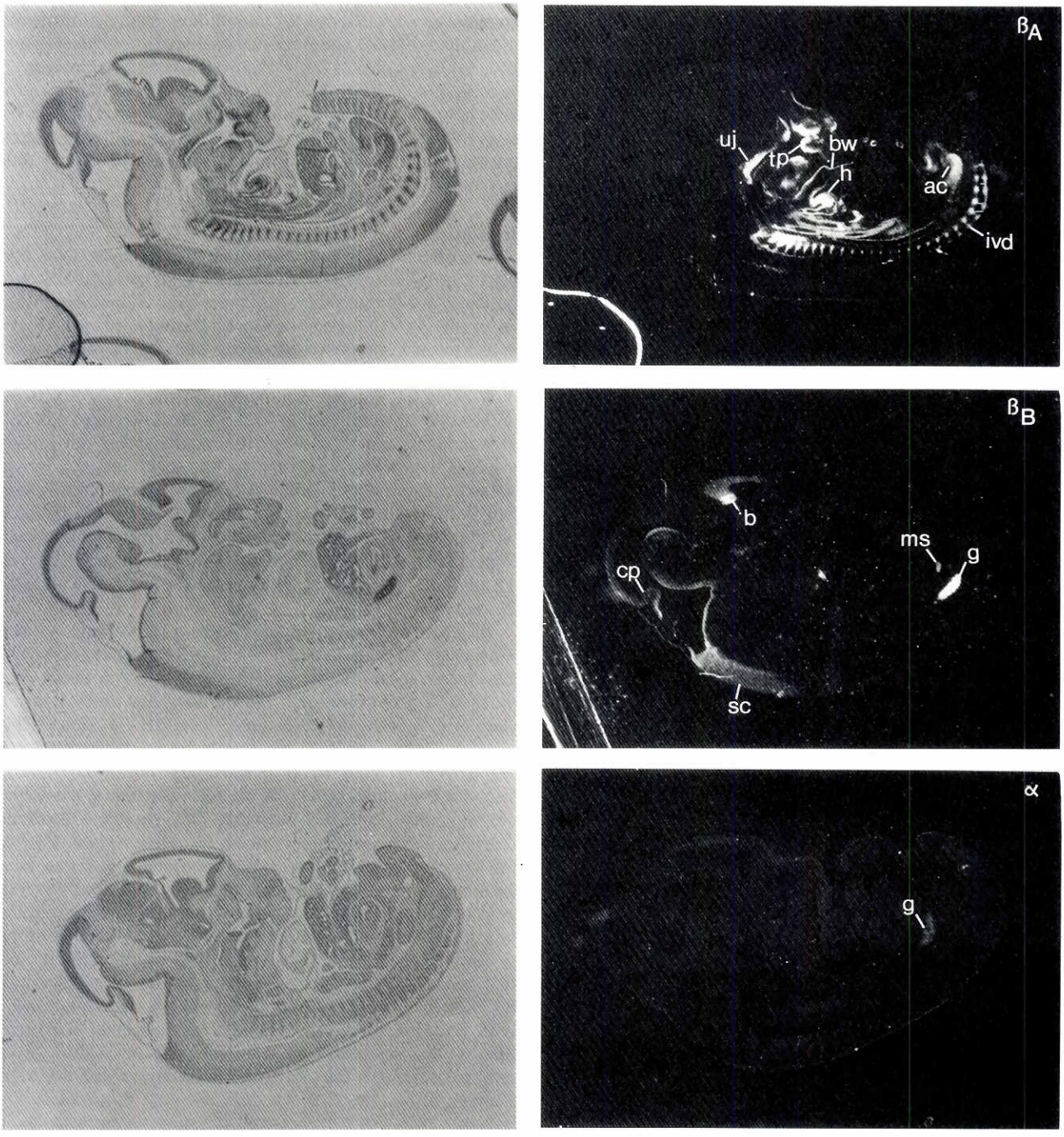

Fig. 3. Inhibin $ß_A$, $ß_B$ and α subunit RNA expression in 12.5 days p.c. mouse embryos. *Bright-field micrographs (left) and corresponding dark-field images (right). ivd, intervertebral discs; h, heart; t, tooth primordium; bw, body wall; uj, upper jaw; cp, choroid plexus; sc, spinal cord; g, primordium of gonads; ms, mucosal lining of stomach; ac, anal canal; b, brain. Data from A. Feijen.*

endoderm cells, absent in primitive ectoderm and later mesoderm (Fig.2 and Slager *et al.*, 1991) and at low or intermediate levels in parietal endoderm. Extrapolating from the mRNA expression studies in EC and ES cells described earlier, this might suggest that the primitive ectoderm does not express mRNA for $TGFß_2$ but both visceral and parietal endoderm do. However given the high degree of post-translational modification of TGFßs this may be an incorrect assumption. The protein observed in cells stained immuno-

histochemically may be the result of receptor mediated uptake while cells expressing mRNA may secrete the protein highly efficiently so that no intracellular staining is detectable. *In situ* hybridization studies of Manova *et al.* (1992) failed to detect mRNA for TGFß$_2$ in the visceral endoderm of early post-implantation embryos although it was detectable in the decidua. However, using the more sensitive PCR technique on separated germ layers from 6.5 days p.c. embryos, we have been able to detect the mRNA in visceral endoderm and not in primitive (embryonic) ectoderm (Roelen, Knecevic and Mummery in preparation). At this stage and in this case therefore, protein and RNA for TGFß$_2$ would appear to be co-localized. By contrast, in later development this is largely not the case either for TGFß$_1$ or ß$_2$. TGFß$_1$ mRNA for example was found by *in situ* hybridization in the epithelial cells overlying mesenchymal tissue containing the protein in several internal organs although mRNA and protein were co-localized in fetal bone at 10.5 to 15.5 days of gestation (Heine *et al.*, 1987; Lehnert and Akhurst, 1988). Heart development is too complex for discussion here (for a specialist review of TGFßs in heart development, see Akhurst *et al.*, 1990, 1992), but it is of note that in a detailed comparison of mRNA and protein localization in the heart between 7.0 and 9.5 days of gestation, there were also large discrepancies (Dickson *et al.*, 1993).

Taken together these data suggest that TGFßs act by both paracrine and autocrine mechanisms during development. For more definitive clues on how TGFßs might act, however, it has become essential to identify potential target cells that express cell surface receptors. Evidence for the presence of functional TGFß receptors during preimplantation development has been provided by experiments showing that TGFß$_1$ enhances the rate at which single embryos develop in culture to the blastocyst stage (Paria and Dey, 1990) and that iodinated TGFß$_1$ binds preferentially to trophectoderm at the blastocyst stage (Paria *et al.*, 1992). However, with the cloning of at least some of the important TGFß receptors, the field is open for other, more precise, analyses of receptor expression during development.

As noted earlier, few functional differences have been observed between TGFß$_1$, ß$_2$ and ß$_3$, but the promoters of these genes are strikingly different (Lafyatis *et al.*, 1990); their expression is therefore entirely independently regulated although they are frequently expressed in the same developing organ, in some cases with overlapping but distinct patterns of expression and in others with quite disparate transcript localization (Fitzpatrick *et al.*, 1990). Why there should be this apparent duplication of function is unclear. It is known that TGFß$_1$ positively regulates its own expression in normal and transformed cells (Van Obberghen-Schilling *et al.*, 1988), and although little is known about 'cross-talk' between the individual genes, one member may be induced by another. This type of interaction highly complicates interpretation of gene «knock out» experiments for example. It has been disappointing to find that mice homozygous for a knock out of the TGFß$_1$ gene, derived by homologous recombination in an ES cell line, generally survive to term and only die of chronic inflammatory syndrome after weaning (Shull *et al.*, 1992). Sufficient TGFß$_1$ may of course be derived maternally to support normal development or, alternatively, other TGFßs can substitute for TGFß$_1$ in development.

In an alternative approach we have injected neutralizing antibodies for TGFß$_2$ into blastocysts and transferred these to pseudo-pregnant females (Slager *et al.*, 1993b). This dramatically reduced the rate of implantation of embryos compared to those injected with a control antibody, and supported the hypothesis that TGFßs are of considerable importance for normal attachment of the embryo to the uterine wall and subsequent invasion of trophoblast (Tamada *et al.*, 1990; Das *et al.*, 1992; Mitchell *et al.*, 1992). Embryos that fail to receive sufficient TGFß early in development may therefore fail prior to implantation while those that do receive sufficient TGFß go on to develop normally until weaned. The loss of function approach, but with the TGFß receptors as targets, may yield more definitive conclusions.

Activin

The data on the expression of inhibin subunits, follistatin, and activin receptor type II in differentiating EC and ES cells point to a potential role of these factors in the mouse embryo. For this reason we examined whether transcripts for these factors were expressed in blastocysts and postimplantation embryos by reverse transcription-polymerase chain reaction (RT-PCR) analysis and *in situ* hybridization. Using specific primers for the ß$_A$ and ß$_B$ chains we have shown by PCR that in embryos 7.5-8 and 10.5 days p.c. ß$_A$ is the predominant subunit and that it is expressed at particularly high levels in the embryonic heart. The ß$_B$ subunit is also expressed in these post-gastrulation embryos but at relatively much lower levels in the heart than ß$_A$ (van den Eijnden-van Raaij *et al.*, 1992). These results are in close agreement with *in situ* hybridization studies of Roberts *et al.* (1991), who demonstrated high expression of ß$_A$ in 12-day-old rat embryos, particularly in the heart, and undetectable ß$_B$ expression.

In situ hybridization studies have also shown characteristic expression patterns for inhibin ß$_A$, ß$_B$ and α in mouse embryos (12.5 days p.c.) (van den Eijnden-van Raaij *et al.*, in preparation). As shown in Fig. 3, ß$_A$ is expressed in the intervertebral discs, heart, and in various mesenchymal structures, in particular around the anal canal and in the snout. ß$_B$ transcripts are predominantly located in brain, in the rapidly dividing cells in the ependymal layer around the lumen of the spinal cord, in the stomach and also in the primordium of the gonads. As the α inhibin subunit is expressed exclusively in this latter tissue, it is likely that inhibins have a specific function in the gonads during development. Indeed inhibin-deficient mice were shown to develop gonadal stromal tumors, indicating that inhibin is a critical negative regulator of gonadal stromal cell proliferation and has tumor-suppressor activity (Matzuk *et al.*, 1992). The expression patterns of the ß$_A$ and ß$_B$ subunits suggest that different forms of activin have different functions during murine development. Activin might be involved in the development of the heart, cartilage, bone, teeth and skin, while activin B might be a regulator of neuronal cell growth. The expression of the inhibin chains within the mouse embryo contradicts the results of the *in situ* hybridization studies of Manova *et al.* (1992) which demonstrated that inhibin ß$_A$ is expressed exclusively in maternal decidual tissue while ß$_B$ and α transcripts are not present in either the embryo or the decidua. The mRNA level of the inhibin subunits is probably below the detection limit of *in situ* hybridization.

In addition to sharing inhibin subunit expression in post-gastrulation embryos, PCR studies have shown that preimplantation embryos, in particular blastocysts, express ß$_B$ but that ß$_A$ transcripts are absent at this stage (van den Eijnden-van Raaij *et al.*, 1992). These results indicate a change in inhibin subunit expression during implantation, from ß$_B$ in the blastocyst to ß$_A$ as the most predominant form in the midgestation embryo. The recent observation that type beta transforming growth factors can increase inhibin ß$_A$ expression and reduce inhibin ß$_B$ expression in mesodermal cells

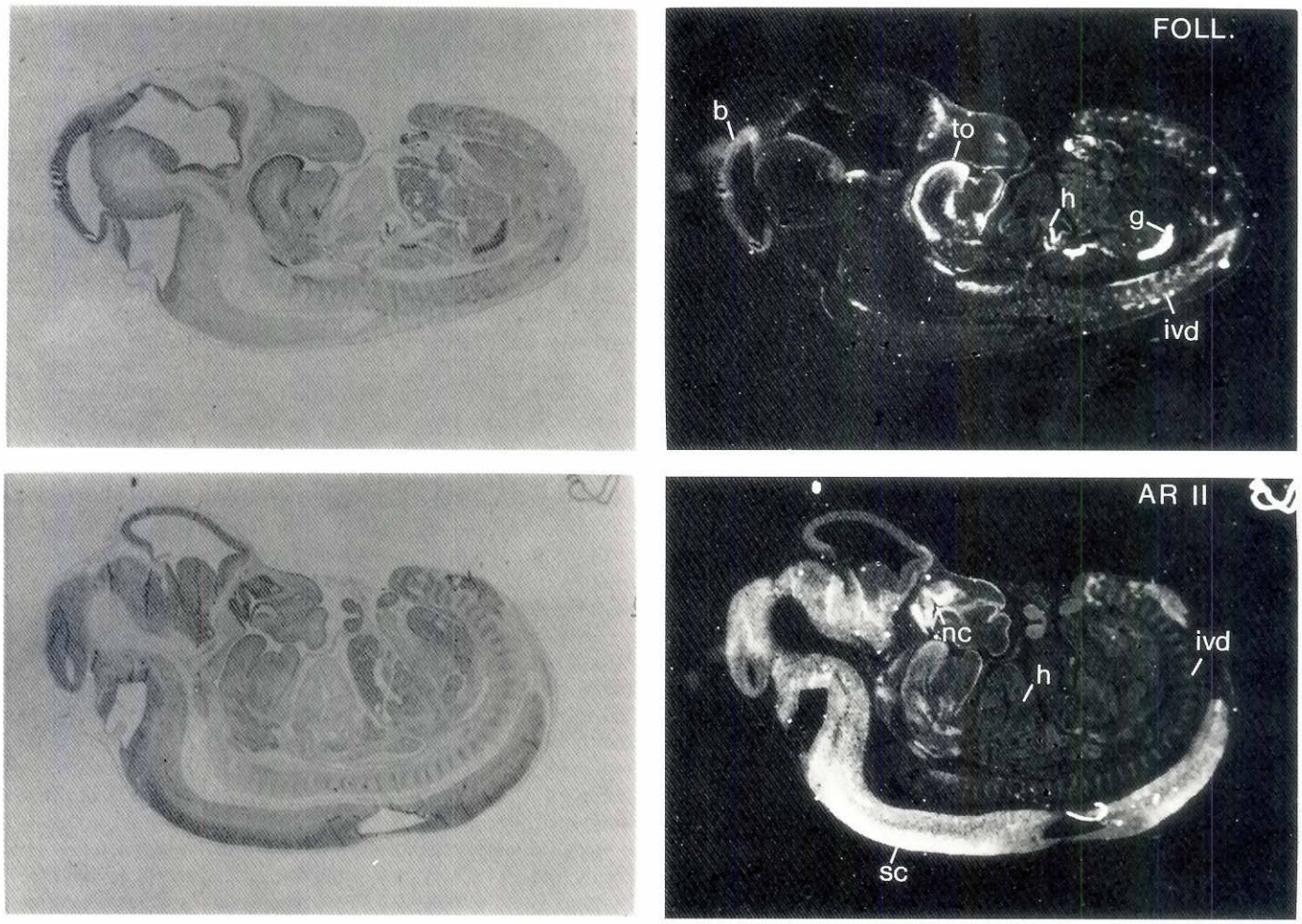

Fig. 4. Follistatin (Foll) and activin type II (AR II) receptor RNA expression in 12.5-day p.c. mouse embryos. *Bright-field micrographs (left) and corresponding dark-field images (right). sc, spinal cord; ivd, intervertebral discs; nc, nasal cavity; h, heart; g, primordium of gonads; to, tongue; b, brain. Data from A. Feijen.*

(van der Kruijssen *et al.*, submitted) suggests that one or more TGFßs might contribute to the change in expression pattern from $ß_B$ to $ß_A$ during murine development. Interestingly in *Xenopus* and chick development, the expression of $ß_A$ subunits also follows that of $ß_B$ (Thomsen *et al.*, 1990; Mitrani *et al.*, 1990). Although from these studies activin B would be implicated as the endogenous mesoderm-inducing factor, the $ß_B$ subunit is probably not expressed early enough in *Xenopus* and chick embryos for the embryonic transcript to account for the formation of mesoderm and for specifying its dorsoventral character (Stern, 1992). However, maternal activin-like proteins were recently shown to be present in the egg and early embryo, so that these and not the embryonic protein may be involved in the induction of mesoderm in amphibians (Asashima *et al.*, 1991b). Additional evidence that activins have properties of a dorsal inducer has come from the observation that injection of activin mRNA causes the formation of a partial second dorsal axis in *Xenopus* embryos (Thomsen *et al.*, 1990). The effects of activin A on the expression of the homeobox gene Xhox3 indicate that this factor might also act as a patterning morphogen involved in the establishment of the antero-posterior polarity in the frog (Ruiz i Altaba and Melton, 1989, 1990). In the early stages of murine development, no function for activins is yet known. A more detailed inventory of the inhibin subunit expression, both at the mRNA and protein levels, in the peri- and early postimplantation stages is currently in progress for a functional comparison of these factors in the amphibian, avian and mammalian systems.

Local activin signaling and biological activity during development requires that activin receptors be present on target cells but also that follistatin be absent. That activin receptors are actually present and functional during *Xenopus* development was shown by the observation that injection of a truncated activin type II receptor into *Xenopus* embryos completely prevents mesoderm induction and dorsal body axis formation (Hemmati-Brivanlou and Melton, 1992). In addition, ectopic expression of the activin type II receptor in the ventral side of *Xenopus* embryos resulted in the formation of a second body axis (Kondo *et al.*, 1991). Whereas the activin receptor appears to be expressed throughout early *Xenopus* embryogenesis, follistatin is transcribed from the stage when activin $ß_B$ subunits become first detectable, suggesting that it may indeed have a regulatory function in amphibian embryos (Tashiro *et al.*, 1991).

As shown in Fig. 4, activin type II receptors are expressed predominantly in brain and spinal cord, and to a lesser extent in the intervertebral discs and mesenchymal structures of 12.5-day mouse embryos. This expression pattern is quite similar to that of activin receptor type IIB although this also shows strong expression in the kidney (not shown). Follistatin is also present in these latter tissues, except for the spinal cord, which is completely negative (Fig. 4). In addition, follistatin is expressed in a specific area of the heart and in the primordium of the gonads. More detailed studies are necessary, both at the RNA and protein levels, to establish the exact regions of the overlapping expression between inhibin subunits, the different activin receptors and follistatin. Recently indirect evidence for the presence of activin receptor in the epiblast of 6.4-day mouse embryos has been obtained by Blum *et al.* (1992) who showed that expression of the homeobox gene goosecoid is induced after treatment of these embryos with activin A. PCR studies on early stages of murine development have shown that, at least in blastocysts and embryos 7.5-8 days and 10.5 days p.c., type II activin receptor is expressed (van den Eijnden-van Raaij *et al.*, 1992). Interestingly, follistatin transcripts are absent in 3.5-day blastocysts isolated *ex utero*, which would allow activin B to be active in early preimplantation stages of development. Expression of follistatin in plated blastocysts and midgestation embryos suggests a role for this factor as a natural regulator of activin activity in peri- and postimplantation stages (van den Eijnden-van Raaij *et al.*, 1992). Knowledge of the localization of activins/inhibins and activin-binding proteins as well as gene «knock out» studies will give further clues about their function in the early stages of vertebrate development.

Discussion

In the studies reviewed here, we have attempted to address questions on the regulation of growth and differentiation in early development and to evaluate the roles of TGFß and activin in these processes in the light of their differential regulation in time and space, the localization of their receptors/binding proteins and their effects in various *in vitro* model systems. TGFßs are clearly among the earliest proteins synthesized by the mouse embryo and the evidence would suggest that it might be involved in controlling the rate at which blastocyst formation occurs during passage of the embryo through the oviduct to the uterus. To develop beyond the blastocyst stage, the mouse embryo must implant into the uterus; the trophoblast cells then cross the basement membranes of the uterine epithelium and invade deep into the stroma in a process thought to involve proteolytic enzymes (reviewed Strickland and Richards, 1992). Although the uterus must be initially receptive to the embryo, it must also be able to limit implantation, to prevent uncontrolled invasion of the trophoblast and eventual development of malignancy. TGFß may play a role in this termination phase particularly through its control of proteases and their inhibitors. Our results with neutralizing antibodies to $TGFß_2$ provide indirect evidence that TGFßs are indeed involved in implantation.

As development of the implanting embryo proceeds, endoderm differentiates from the ICM. The endoderm that migrates over the trophectoderm, known as parietal extraembryonic endoderm, is characterized by abundant production of laminin, one of the components of Reicherts membrane. Once Reicherts membrane has formed, laminin production presumably also requires limitation. It is of interest that, in contrast to general dogma, laminin production by parietal endoderm-like cells in culture (PYS-2 and F9 EC treated with RA and dibutryl cyclic AMP) is actually decreased, rather than increased, by TGFß (Kelly and Rizzino 1989). TGFß from the trophectoderm may for example limit the production of laminin by neighboring parietal endoderm through a paracrine mechanism.

Finally, TGFß may be directly or indirectly involved in the induction of mesoderm in the early embryo. As noted earlier, TGFß can act in combination with FGFs to induce mesoderm in animal cap explants and TGFßs are present at appropriate times in mouse development to have a similar function there (see also Mummery *et al.*, 1993, and references therein). Alternatively, activin, which is one of the most potent mesoderm-inducing factors in the *Xenopus* ectodermal explants, might be the endogenous inducer in murine development. Although activin B appears to be present at the blastocyst stage, TGFß might alter activin production by endoderm at the time of gastrulation, so that activin A in turn is the true initiator of mesoderm formation. An interesting observation in this context is the switch of animal cap cells from *Xenopus laevis* to a neuronal fate after inactivation of the activin signal transduction pathway (Hemmati-Brivanlou and Melton, 1992). It is tempting to speculate that activins and the factors regulating their activity (TGFß, follistatin, inhibin) might determine the direction of differentiation in early vertebrate development.

To speculate extensively on the functions of TGFß and activin in later development is beyond the scope of the present discussion, but from the data reviewed here, it is clear that both factors are likely to have important functions in the development of organs such as the heart, lung, kidney and gonads as well as at sites of epithelial/mesenchymal transformation. In view of their multifunctionality however, it will not be straightforward to establish their function unequivocally. Loss of function approaches for ligand, receptor or binding proteins would probably only affect the first event in which these factors have an essential role. Introduction of neutralizing antibodies or anti-sense oligonucleotides to organ cultures or directly into embryos may usefully supplement this approach in the absence of technology to induce loss of function in a tissue-specific manner. Gain of function approaches directed to particular target tissues may be particularly informative where the functions of TGFß and activin may be involved in cell survival and inhibition of apoptosis. The mouse embryo *in vivo*, however, remains a difficult experimental system, since the events that are among the most interesting occur while it is least accessible to manipulation. We expect EC and ES cells therefore to continue to yield a wealth of useful information on the regulation of early differentiation.

Acknowledgments

We are grateful to Kirstie Lawson for her expert advice in the analysis of embryos and to past and present members of our group for their contributions to the studies described here, in particular Tanja van Achterberg, Alie Feijen, Eric Freund, Vladimir Knecevic (Zagreb), Corina van der Kruijssen, Coen Paulusma, Bernard Roelen, Hans Slager and Carlie de Vries. In addition, we would like to thank Barry Pierce for his encouragement during our first attempts to grow and manipulate EC cells in culture. Through his enthusiasm we learned to appreciate the value of this model system in embryology.

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