Development 108, 645-656 (1990)
Printed in Great Britain ©The Company of Biologists Limited 1990

TGF beta in murine morphogenetic processes: the early embryo and cardiogenesis

ROSEMARY J. AKHURST[1]*, SIGRID A. LEHNERT[1], ANDREAS FAISSNER[2] and ELIZABETH DUFFIE[1]

[1] *Duncan Guthrie Institute of Medical Genetics, University of Glasgow, Yorkhill, Glasgow G3 8SJ, UK*
[2]*Department of Neurobiology, Im Neuenheimer Feld 364, D-6900 Heidelberg, FRG*

*To whom correspondence should be addressed

Summary

The tissue distribution of TGF beta-1 RNA was examined within whole mouse embryos from implantation to 10.5 days gestational age and, in the developing heart, up to 8 days *postpartum.* The earliest high level expression of TGF beta-1 RNA is at 7.0 days *postcoUum ip.c.)* in the cardiac mesoderm. At 8.0 days gestational age, cardiac TGF beta-1 RNA expression is limited to endocardia! cells. By 9.5 days *p.c,* this expression pattern becomes regionalised to those cells that overlie cardiac cushion tissue. High TGF beta-1 RNA levels continue to persist in endothelial cells of the heart valves until approximately one week *postpartum.*

The TGF beta-1 RNA distribution was compared with the extracellular distributions of polypeptides for TGF beta and Jl/tenascin. As previously reported, endothelial expression of TGF beta-1 RNA is correlated with mesenchymal expression of TGF beta polypeptide, suggesting a paracrine mode of action for this growth factor in cardiac development. Minor discrepancies in the distributions of TGF beta-1 RNA and the extracellular form of the TGF beta polypeptide suggest that translational or post-translational control of protein levels occurs and/or the possibility that the antibody used may also recognise other members of the TGF beta polypeptide family.

A correlation between endothelial TGF beta-1 expression and distribution of Jl/tenascin in the mesenchyme gives further support to the proposition that the biological effects of TGF beta-1 may, in part, be mediated by Jl/tenascin.

Key words: TGF beta-1, Jl/tenascin, *in situ* hybridisation, epithelial-mesenchymal interaction, epithelial-mesenchymal transformation, mouse embryo, cardiogenesis.

Introduction

It is becoming increasingly clear that polypeptide growth factors play a central role in many embryonic processes. Not only are these molecules and their receptors present within the early embryo *in vivo* (Twardzik *et al.* 1982; Heine *et al.* 1987; Rappolee *et al.* 1988), but they initiate and/or modulate a variety of cellular events which are essential to the development of the organism (Sporn *et al.* 1986; Sporn and Roberts, 1988; Roberts and Sporn, 1989). This is exemplified well by the family of growth factors related to transforming growth factor beta-1, which itself has previously been termed a 'panregulin' by Sporn *et al.* (1986).

Studies of TGF beta action on cells *in vitro* have been invaluable to our understanding of the role that these molecules might play in biological processes *in vivo,* though the interpretation of results has been confounded by the multifunctional nature of this growth factor (Sporn *et al.* 1986; Roberts and Sporn, 1989) and the multiplicity of closely related molecules within this family (ten-Dijke *et al.* 1988; Jakowlew *et al.* 1988a,*b;* Kondaiah *et al.* 1989). It is clear that the biological effects of TGF beta are dependent on the presence of other growth factors, either acting synergystically (Kimelman and Kirschner, 1987) or antagonistically (Baird and Durkin, 1986; Jennings *et al.* 1988); they are also dependent on the cell type and the nature of the culture system. This is particularly well demonstrated by the effects of TGF beta-1 on endothelial cells *in vitro,* which can modulate cellular growth, movement, adhesiveness, invasiveness and/or three-dimensional morphology, dependent on the endothelial cell source and the exact culture system (Heimark *et al.* 1986; Sato and Rifkin, 1989; Gamble and Vadas, 1988; Potts and Runyan, 1989; Madri *et al.* 1988).

For a full understanding of *in vivo* function, it is also necessary to determine the sites of synthesis and action of individual growth factors during embryological de-

velopment. TGF beta-1 and beta-2, for example, are interchangeable in *most* biological activity assays, though they clearly have different temporal and spatial profiles of gene expression during murine embryogenesis (compare Lehnert and Akhurst, (1988) and Pelton *et al.* (1989)), suggesting different *in vivo* functions. Recent reports confirm that, indeed, TGF beta-1 and -2 do show some specificity in biological action (Jennings *etal.* 1988; Ohta *et al.* 1987).

In an earlier study, we examined the global expression pattern of the gene encoding TGF beta-1 in the mid-gestation murine embryo (Lehnert and Akhurst, 1988). A novel finding of this study was that the TGF beta-1 gene is transiently expressed in epithelial cells which are involved in morphogenetic interactions in cases where TGF beta protein is localised in the underlying mesenchyme (Heine *et al.* 1987). A reasonable inference from this observation is that a major function of epitheliaUy derived TGF beta-1 is as a paracrine modulator of extracellular matrix (ECM) deposition in the underlying mesenchyme. This growth factor is known to increase accumulation of ECM material by increasing production of both matrix proteins and inhibitors of extracellular proteases, which prevent ECM degradation. It also inhibits the production of matrix-degradation enzymes (Roberts *et al.* 1986; Ignotz and Massague, 1986) and the responsiveness of cells to the ECM is increased by stimulation of the production of cell surface receptors for matrix molecules (Ignotz and Massague, 1987).

Extracellular proteins that have elevated synthesis rates include not only collagen I and fibronectin but more specialised molecules such as thrombospondin (Penttinen *etal.* 1988), osteopontin (Noda *etal.* 1988), tenascin (Pearson *et al.* 1988), osteonectin/SPARC (Noda and Rodan, 1987) and chondroitin/dermatan sulphate proteoglycans (Hiraki *et al.* 1988). The embryonic distribution of tenascin is of particular significance in this context since this is relatively restricted in the embryo compared with other ECM molecules and its distribution is almost completely correlated with the presence of TGF beta-1 RNA (Chiquet-Ehrismann *et al.* 1986; Lehnert and Akhurst, 1988). Furthermore, TGF beta is known to transcriptionally activate the tenascin gene (Pearson *et al.* 1988) and, in an *in vitro* model, epitheliaUy derived TGF beta-1 can induce synthesis of tenascin by the underlying stroma (Chiquet-Ehrismann *et al.* 1989). Tenascin could therefore mediate some of the effects of TGF beta in embryogenesis.

In our previous report, TGF beta-1 RNA was found specifically in the epithelial component of the heart valves at 12.5 days *post coitum (p.c.)* suggesting that TGF beta-1 is a modulator of septation and valve formation (Lehnert and Akhurst, 1988). In this paper, we have extended our study of embryonic TGF beta-1 gene expression to include stages from implantation to 10.5 days gestation. We have also specifically examined later stages of cardiac development, in particular with respect to accumulation of tenascin in the extracellular matrix.

Progenitor cells of the mammalian heart, the cardiac mesoderm cells, appear very early in embryogenesis, around gastrulation. These progenitors are thought to arise through induction by the underlying pharyngeal endoderm (Jacobson and Sater, 1988). Splanchnopleuric mesoderm gives rise to two primitive tubes, the endocardium and the myocardial mantle, the latter encapsulating the former. Initially, the endothelium and myocardium are separated by an extensive acellular basement membrane, the cardiac jelly. Early events leading to septation and formation of prevalvular mesenchyme include a regional differentiation of the endocardium. Endothelial cells within the region of the atrioventricular (AV) canal and outflow tract become capable of mesenchymal transformation and migrate into the underlying cardiac jelly populating the mesenchyme of the cardiac cushion tissue (Manasek, 1976).

The process of epithelial-mesenchymal transformation of endocardial cells has been extensively studied by Markwald and others in an *in vitro* collagen gel system. It involves an inductive event emanating from the myocardium. There is both regional specificity in the ability of the myocardium to produce such a signal and in the competence of the endocardium to respond (Krug *et al.* 1985; Mjaatvedt *et al.* 1987). Mjaatvedt and Markwald (1989) have recently isolated a particulate fraction that is capable of 'activating' the AV endothelium to undergo this transformation event *in vitro.*

The possibility that TGF beta may contribute to epithelial-mesenchymal cell transformation was demonstrated by Potts and Runyan (1989) who showed that, in combination with chick ventricular myocardium, TGF beta could initiate this event in atrioventricular endothelium. Furthermore, *in vitro* this process was inhibited by antibodies that block TGF beta activity. We will discuss our results on the distribution of TGF beta-1 and tenascin in the light of these observations.

Materials and methods

Mouse stocks

All embryos and neonates were obtained from Parkes females mated with NIH males (Olac). Noon on the day of the copulation plug was 0.5 days *post coitum (p.c).* All tissue was fixed overnight in ice-cold 4% paraformaldehyde in phosphate-buffered saline, then dehydrated and embedded in paraffin.

Probe synthesis

A full-length cDNA encoding murine TGF beta-1 (Derynck *et al.* 1986) was kindly provided by Dr R. Derynck (Genentech). To eliminate the possibility that this probe could cross-react with other members of the TGF beta gene family, a 600 nucleotide *Apal-Kpnl* fragment corresponding to the precursor region of the TGF beta-1 protein (amino acid residues 68-268) was subcloned into the riboprobe vector Bluescribe (Vector Cloning Systems). This removes the DNA coding sequences encoding the region of amino acid terminal homology seen between TGF beta proteins (amino acids 1-30) and the conserved mature 112 amino acid portion of the TGF beta-1 polypeptide. The resultant probe shows greater than

50% nucleotide sequence divergence from other TGF beta genes.

^{35}S-labelled sense and antisense riboprobes were generated to a specific activity of 10^9disintsmin~' $j_i g$~' using T3 or T7 polymerase. Probes were digested to an average length of 100 nucleotides by controlled alkaline hydrolysis (Cox *et al.* 1984) and used at a final concentration of 30pg^l~' in hybridisations.

In situ *hybridisation*

In situ hybridisation to 7/im tissue sections was performed essentially as described by Wilkinson *et al.* (1987) using 60% formamide in the hybridisation mix and a hybridisation temperature of 52°C. Exposure times (llford K5 emulsion) were between 3 and 15 days. After development, slides were stained in haemotoxylin and mounted. Photomicrography was performed using an Olympus BH-2 microscope and either Kodak Panatomic-X or Ektachrome EPY-50 film.

Immunohistochemistry

Polyclonal rabbit antibodies included anti-mouse fibronectin (Biogenesis Ltd); anti-alpha 1 chymotrypsin (Dakopatts); anti-TGF beta, CC, kindly supplied by Dr K. Flanders (Heine *etal.* 1987); and anti Jl-tenascin, KAF9 and KAF10 (Steindler *etal.* 1989).

Antigens were localised in 7jim tissue sections using an avidin-biotin-peroxidase system (ABC System, Dakopatts). For TGF beta detection, deparaffinised sections were pre-fixed in Bouin's fixative for 30min at room temperature, followed by extensive washing in 70% ethanol. (This was not necessary for unmasking the epitopes of the other antigens). After blocking endogenous peroxidase in hydrogen peroxide/methanol, sections were permeabilised with O.lmgml"^{1} hyaluronidase in 0.1 M sodium acetate, 0.15 M sodium chloride pH5.5 for 30min at 37°C. Non-specific antibody binding was blocked with donkey serum (0.3%), mouse serum (0.3%), gelatin (0.1%) and BSA (0.1%) in phosphate-buffered saline.

Primary antibody was applied at 20^gml~' in the blocking cocktail, overnight at 4°C. The secondary antibody was biotinylated donkey anti-rabbit (Amersham) and the Dakopatts ABC system protocol was followed using diaminobenzidine as the chromogen. Sections were lightly stained in haemotoxylin before examination using an Olympus BH-2 microscope.

In situ hybridisation and immunohistochemistry were performed on adjacent serial sections in order to compare the relative distribution of TGF beta-1 transcripts with that of TGF beta and tenascin polypeptides.

Results

In our previous *in situ* hybridisation study of TGF beta-1 RNA distribution within the midgestation murine embryo, a full-length cDNA probe against TGF beta-1 was employed (Lehnert and Akhurst, 1988). This introduced the possibility that our data may not represent TGF beta-1 transcript distribution specifically but cross-reaction to RNA of other TGF beta family members. We therefore subcloned a 600 nucleotide fragment that was known to be specific to the TGF beta-1 gene. Repetition of the original *in situ* hybridisation study using this probe produced identical results (data not shown) confirming that our original report represented hybridisation to TGF beta-1 transcripts.

Extraembryonic expression of TGF beta-1

The decidual swellings of pregnant mice were dissected from the uterus at 6.0 to 9.5 days *p. c. 7 jan* sections cut transversely with respect to the uterus were subjected to *in situ* hybridisation using the anti-sense and sense TGF beta-1 specific probes. The maternal deciduum contained abundant RNA transcripts encoding TGF beta-1. This was most clear in the decidua capsularis at 7.5 days *p.c.* with a gradient of TGF beta-1 RNA concentration across the deciduum, being highest near the uterine wall (Fig. 1A,B). By 9.5 days p.c, TGF beta-1 RNA was seen in the decidua basalis, which has developed large lacunae and sinusoids filled with maternal blood. Hybridisation was most intense in the cells lining the lacunae. The chorion also showed a signal (Fig. 1D,E).

The first appearance of detectable TGF beta-1 RNA in tissues derived from the zygote appears in the extraembryonic blood islands of the yolk sac at 7.0 days *p.c.* These cell clusters arise from mesodermal haemangioblasts, the progenitors of both haemopoietic stem cells and endothelial cells, which contribute to the extraembryonic vasculature (Pardanaud *et al.* 1989). At 7.5 days *p.c.*, the *in situ* hybridisation signal was evenly distributed over all cells of the blood island (Fig. 1G,H). The resolution of the technique did not allow us to determine whether the signal emanated from the haemopoietic or endothelial cells. However, since in later development, both haemopoietic cells and embryonic endothelial cells express TGF beta-1, we would predict that both cell types express this gene in the blood islands.

By 9.5 days *p.c,* clusters of cells that appear proerythroid in morphology appear in the blood islands as intense foci of hybridisation superimposed upon the hybridisation signal seen at 7.5 days (Fig. II). This would agree with the observations of Wilcox and Derynck (1988) who identified these TGF beta-1-expressing cells as proerythroid in nature. We have also observed hybridisation to erythroid progenitors within the fetal liver at 12.5 daysp.c. (data not shown), though by 14.5 days, the predominant liver cell type expressing abundant TGF beta-1 transcripts is the megakaryocyte (Lehnert and Akhurst, 1988).

At 7 to 8.5 days *p.c,* the allantois has high levels of TGF beta-1 RNA. The expression in this tissue is probably related to active vascularisation.

Early embryonic expression of TGF beta-1 occurs during vascularisation and angiogenesis

The cardiac mesoderm cells within the head region of the 7.0 day *p.c.* embryo were the first cell type of the embryo *per se* to express TGF beta-1 RNA. These cells were identified by their most ventral and anterior position within the head mesoderm (Fig. 2D,E). At this stage, it is impossible to discern whether these hybridising cells are endocardial precursors or progenitors of the myoepicardium. Several embryos at this stage and earlier were examined in detail by serial sectioning but no other embryonic tissue type, either ectodermal,

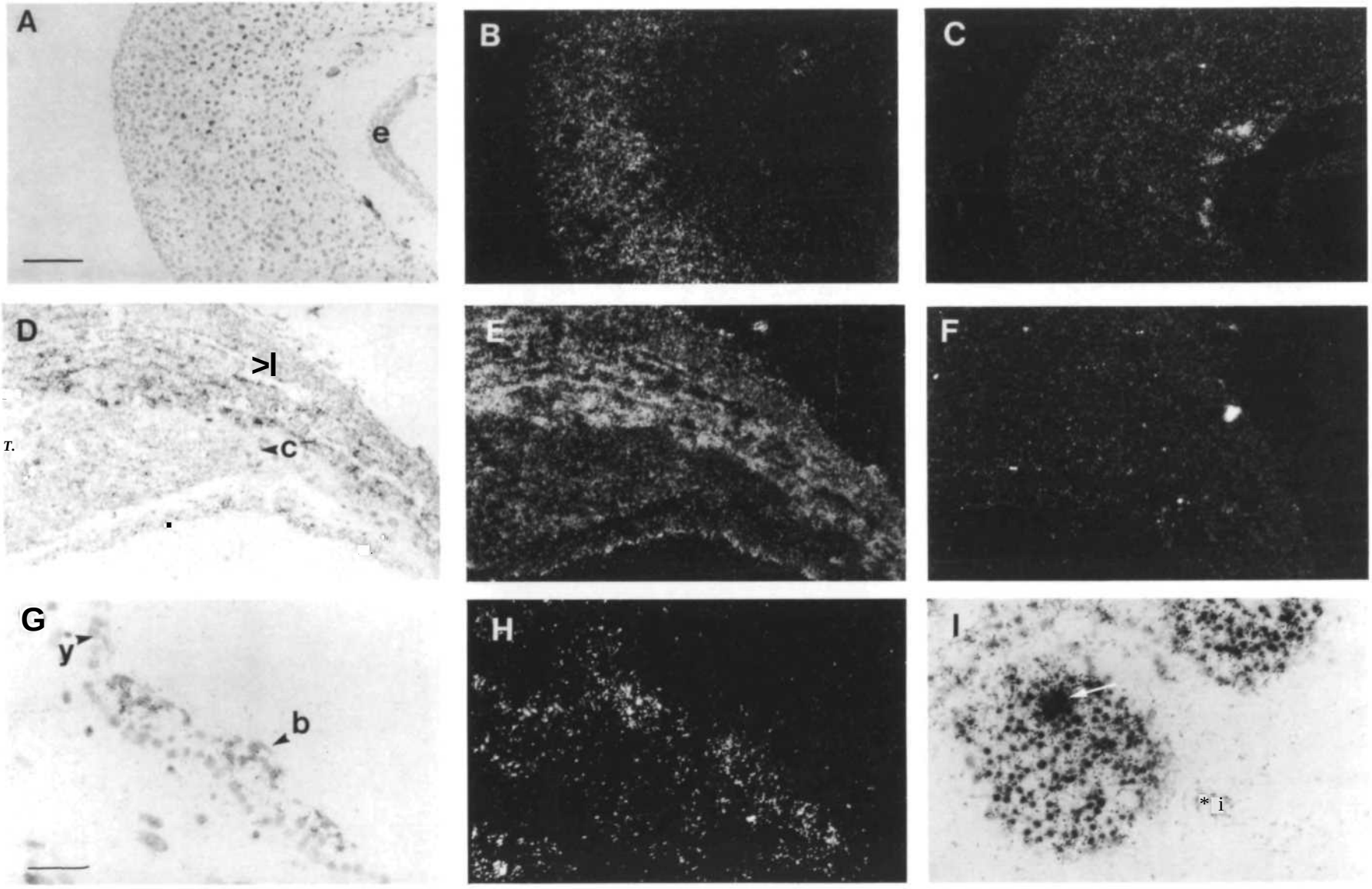

Fig. 1. TGF beta-1 expression in extraembryonic structures. (A,D,G,I) Bright-field and (B,C,E,F,H) corresponding dark-field photomicrographs showing *in situ* hybridisation to TGF beta-1-specific gene probe. (A-C) Deciduum capsularis of 7.5 dayp.c. embryo: (A, B) antisense probe; (C) sense probe. (D-F) Chorion and deciduum basalis of 9.5 day *p.c.* embryo: (D, E) antisense probe; (F), sense probe. (G, H) Yolk sac blood islands of 7.5 day *p.c.* embryo with antisense probe. (I) yolk sac blood islands of 9.5 day *p.c.* embryo showing strong hybridisation to proerthyroid progenitor (arrow), y, yolk sac endoderm; c, chorion; e, embryo; 1, lacunae; b, blood island. Scale bar in A-F=200^m. Scale bar in G-I=50/im.

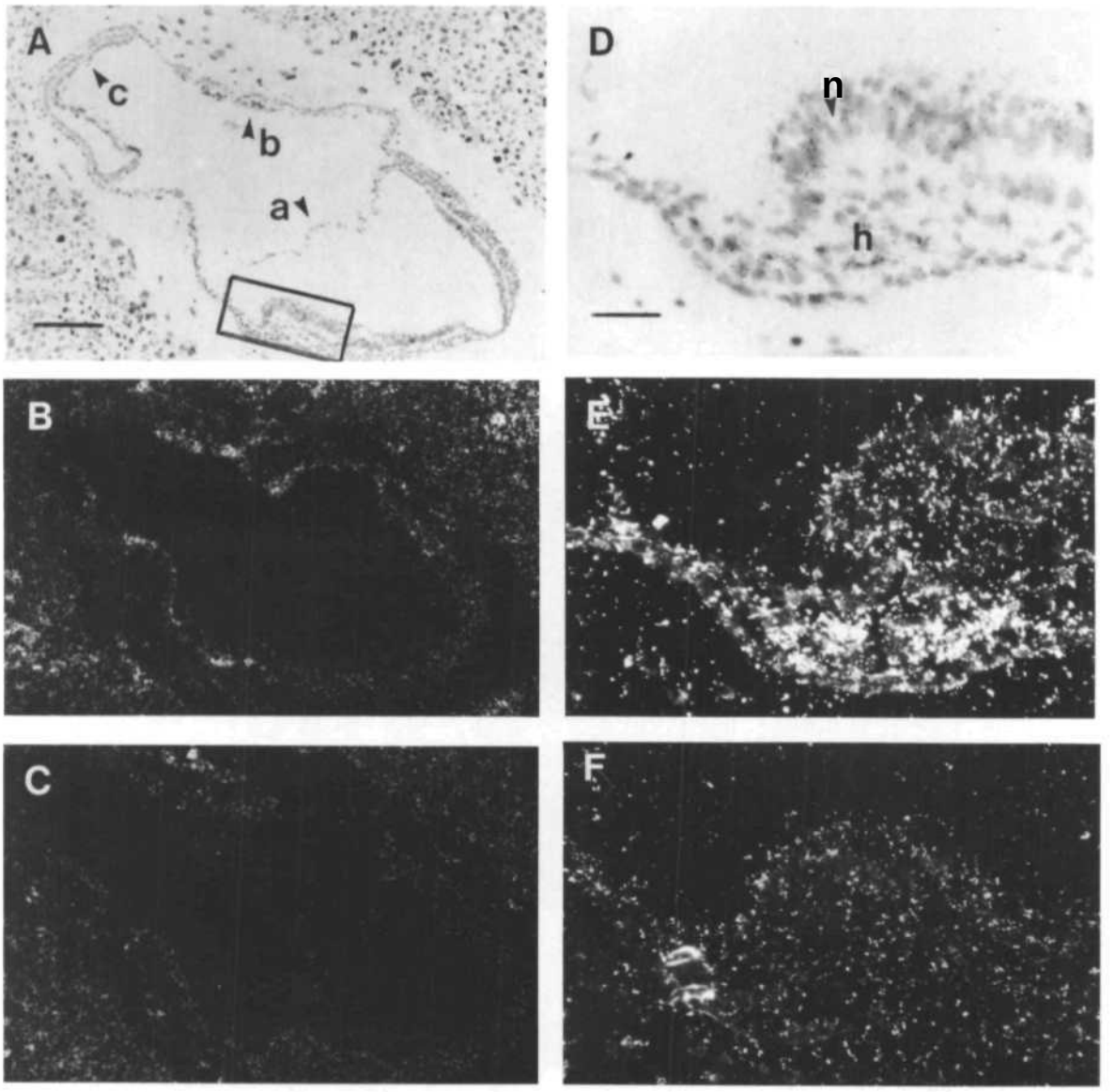

Fig. 2. TGF beta expression in the post-gastrula stage embryo. (A-C) Sagittal section through a 7.5 day *p.c.* embryo (commencement of somitogenesis). (A, D) Bright-field and (B,C,E,F) corresponding dark-field images. D-F is a high power photomicrograph of the head region of the embryo shown in A-C. (B,E) TGF beta-1 antisense probe; (C,F) sense probe. c, chorion; a, amnion; b, blood islands; n, neuroepithelium; h, heart mesoderm. Scale bar in A-C=200/<m. Scale bar in D-F=50j/m.

endodermal or mesodermal appeared to contain significant levels of TGF beta-1 RNA.

Serial sections adjacent to those subjected to *in situ* hybridisation were also examined by immunocytochemistry for TGF beta protein, as indicated by staining with the CC antibody described by Flanders *etal.* (1989). No reactivity with this antibody was observed in the heart mesoderm, despite positive staining seen in the maternal mesometrium of the same sections (data not shown). In fact, no CC immunoreactivity in the region of the heart could be detected until at least 9.5 days *p.c.*. Even then the staining was very faint compared to positive control tissues (data not shown).

By 8.0 days *p.c.* (5-7 somite stage), TGF beta-1 RNA expression in the heart is clearly limited to the endocardium. At this stage, the two cardiac cell layers are distinctly separated by the acellular cardiac jelly. All endocardial cells showed similar high levels of hybridisation whereas the myocardium was negative (Fig. 3A,B). Twelve hours later (approximately 8- to 12-somite stage), before regional transformation of endocardial cells into the cushion tissue has commenced, all endocardial cells still showed high levels of TGF beta-1 RNA expression. It was, however, noted that some endocardial cells produced a higher autoradiographic signal than others, though this was not obviously correlated with relative position within the primitive heart tube (Fig. 3C,D,F,G).

Also, at 8.5 days p.c, isolated cells within the well-developed head mesenchyme showed hybridisation to

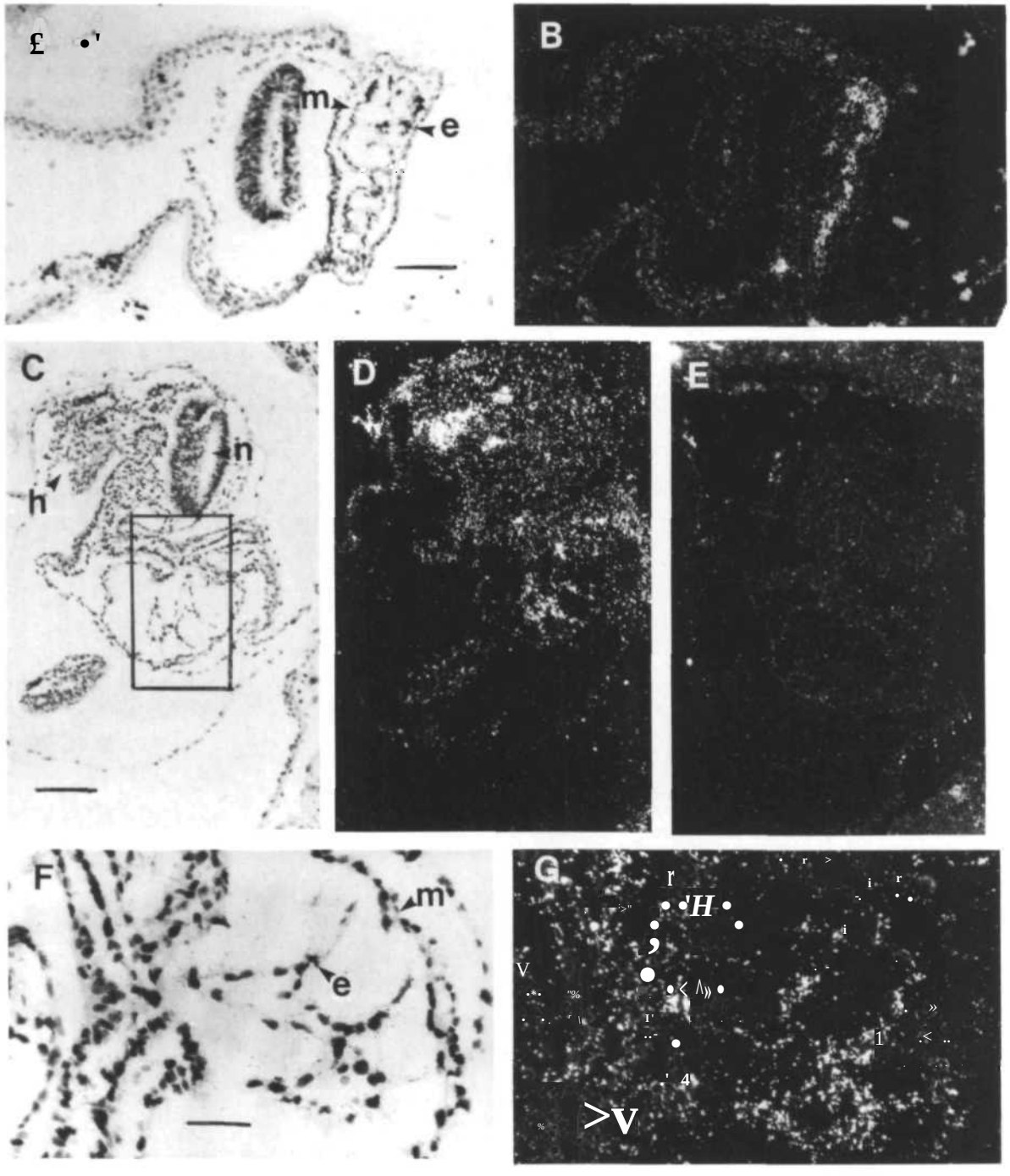

Fig. 3. TGF beta-1 in early endocardial cells. (A,C,F) Bright-field and (B,D,E,G) corresponding dark-field photomicrograph showing *in situ* hybridisation to (A,B,C,D,F,G) antisense TGF beta-1 probe, and (E) sense probe. (A,B) Transverse section through a 8.0 day *p.c.* embryo (5-7 somites). (C,D,E) Section through a 8.5 day *p.c.* embryo at a stage when the embryo is turning (7-12 somites). Boxed area in C is shown magnified in F-G. e, endocardium (positive); m, myocardium (negative); n, neuroepithelium of head; h, allantoic outgrowth. Scale bars: (A,B,F,G)=25/jm; (C,D,E)=100/tm.

the TGF beta-1 antisense probe. Similarly, by 9.5 days *p.c,* a striking pattern of positive hybridisation is seen in cells immediately adjacent to the neural tube, along the entire axis of the embryo (data not shown). We believe that these are capillary endothelial cells undergoing angiogenesis to form the plexus of blood vessels around the neural tube (Poole and Coffin, 1989).

Endothelial cells of the major blood vessels also show hybridisation to the TGF beta-1 probe at early stages of embryogenesis. These include the anterior cardinal veins, caudal veins and arteries and internal carotid artery (Fig. 4). By 11.0 days *p.c,* endothelial expression of TGF beta-1 RNA within these major vessels is extinguished though the major vessels proximal to the

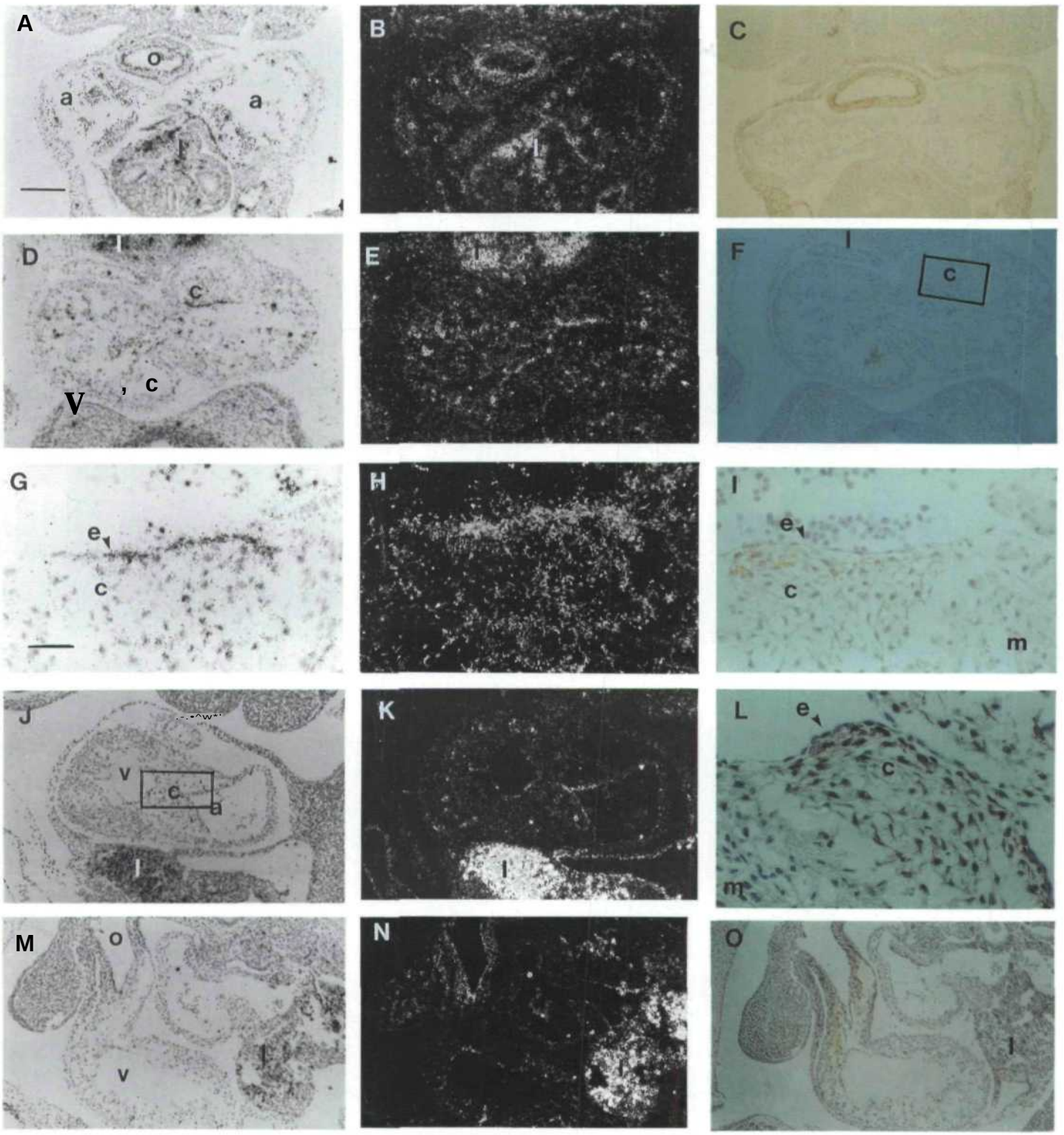

Fig. 5. Correlation between endothelial expression of TGF beta-1 and mesenchymal expression of Jl/tenascin. (A,D,G,J,M) Bright-field photomicrographs showing hybridisation to anti-sense TGF beta-1 probe, (B,E,H,K,N) corresponding dark-field photomicrographs. (C,F,I,L,O) Adjacent sections showing immunoreactivity with the KAF 9 antibody. (A-C) Transverse section through 9.5 day *p.c.* heart at the level of the atria and outflow tract; (D-F) Transverse section through the ventricular region of a 9.5 day *p.c.* heart showing the newly formed cushion tissue. Boxed area in F is shown magnified in G-I. (J-L) Sagittal section through 10.5 day p.c. heart showing atrioventricular valve region. (M-O) Sagittal section through 10.5 day p.c. heart showing ventricle and outflow tract, o, outflow tract; 1, liver; c, cushion tissue; a, atrium; v, ventricle; e, endothelium; m, myocardium. Note some of the circulating blood cells give a positive signal and the liver shows intense hybridisation in D,E,J,K,M and N. Scale bar (A,B,C,D,E,F,J,K,M,N,O)=200/nn, (G,H,I,L)=50^m.

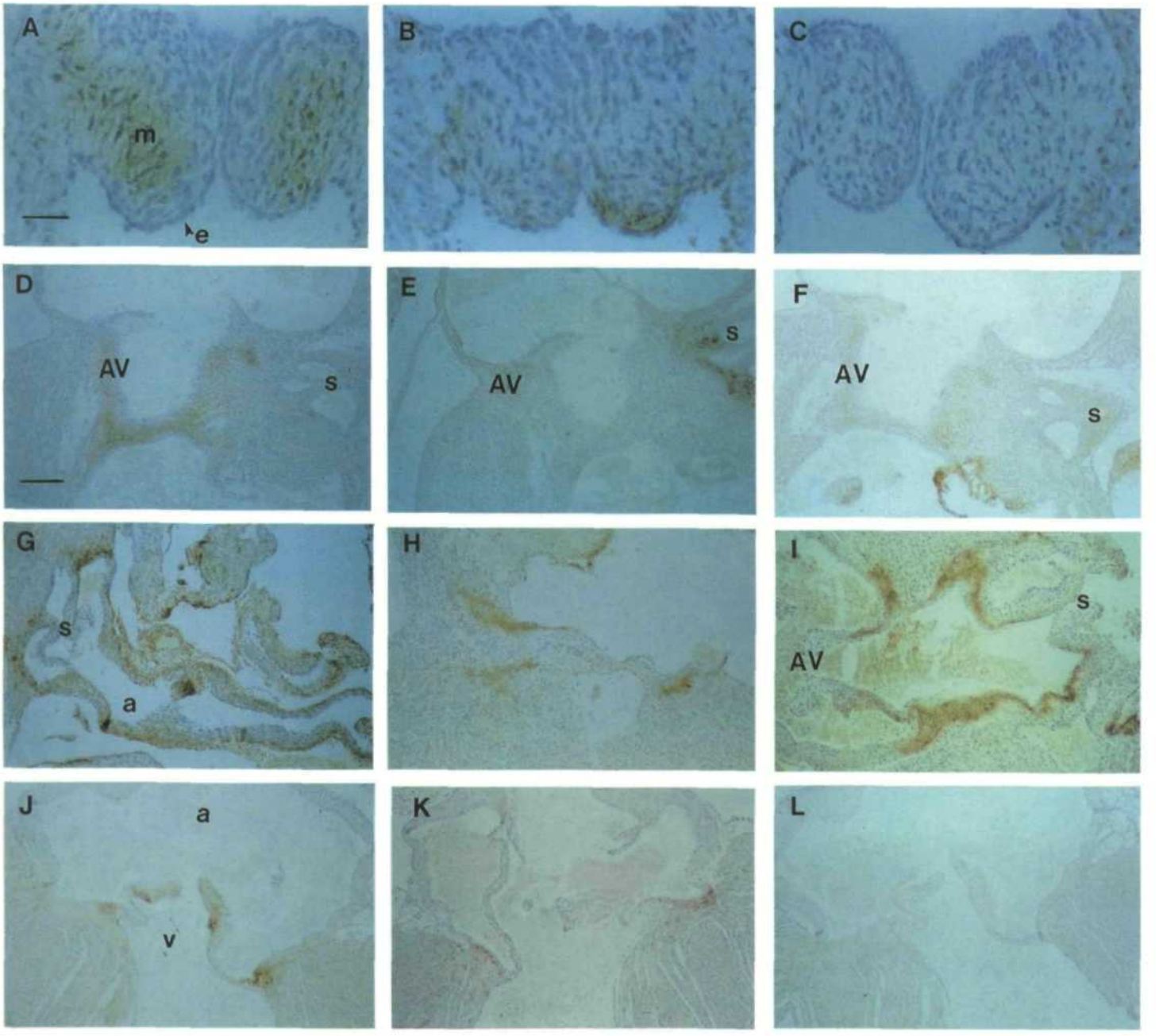

Fig. 6. Immunocytochemical localisation of TGF beta and tenascin polypeptide in developing heart valves. (A-C) Atrioventricular valves of a 12.5 day *p.c.* embryo showing staining with (A) CC antibody; (B) KAF-9 antibody; (C) anti-alpha chymotrypsin antibody. (D-F) Section through a 16.5 day p.c. heart showing the AV valve region and the semilunar valves of the pulmonary artery, stained for (D) CC antibody; (E) KAF 9; (F) anti-fibronectin antibody. (G) Section through the aorta of a newborn mouse heart stained with KAF 9. (H) KAF staining in the region of the foramen ovale of a newborn mouse. (I) KAF 9 staining in the region of the AV valves and semilunar valves of the pulmonary artery of an 8 day *postpartum* mouse. (J-L) Sections through the semilunar valves of the adult aorta showing staining with (J) CC antibody; (K) KAF 9 antibody; (L) anti-alpha chymotrypsin antibody, m, mesenchyme; e, endocardium; AV, atrioventricular valve region; s, semilunar valves; a, lumen of aorta; v, ventricular cavity. Scale bar in A-C=50/im. Scale bar in D-L=200/an.

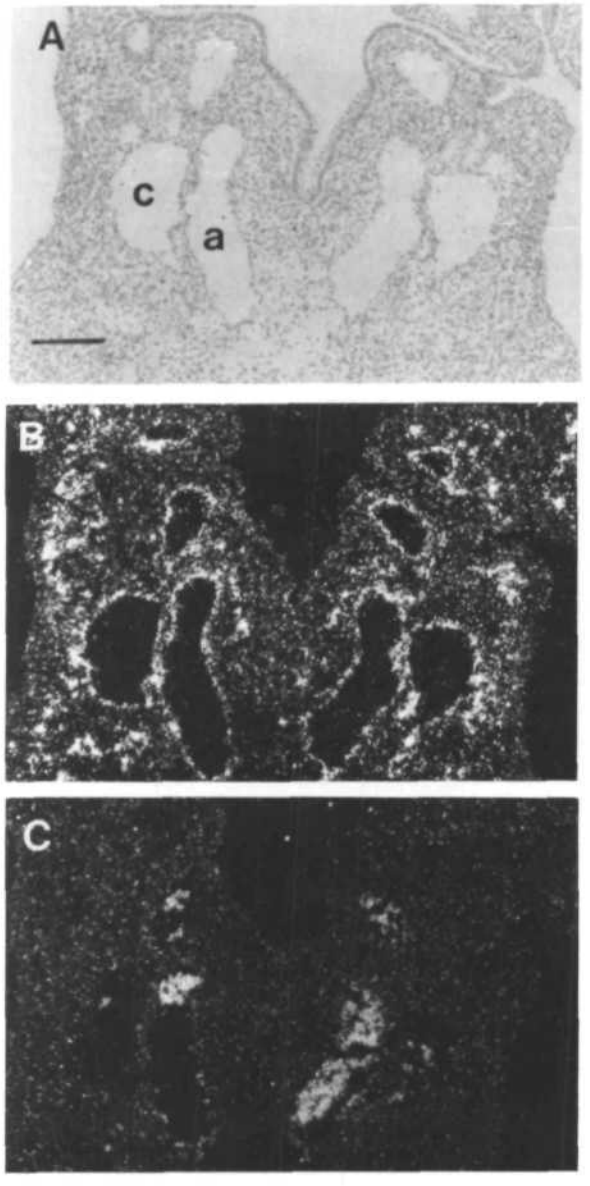

Fig. 4. TGF beta-1 expression in the endothelial cells of the major blood vessels. (A) Bright-field and (B,C) corresponding dark-field photomicrographs. (A-C) Transverse section through 9.5 day *p.c.* embryo showing *in situ* hybridisation with (A,B) antisense TGF beta-1 probe and (C) sense probe, c, cardinal vein; a, dorsal aorta. Scale bar=200^tm.

heart, including the truncus venosus and the hepatocardiac channel, showed more persistent TGF beta-1 gene expression to late fetal stages, possibly being correlated with later morphogenetic processes.

TGF beta-1 RNA expression in endocardial cushion tissue is correlated with mesenchymal expression ofJl/tenascin

The cushion tissue of the heart develops by epithelial—mesenchymal transformation of endocardial cells as these cells migrate into the underlying cardiac jelly where they proliferate to form dense mesenchymal masses. Fusion of cushion tissue plays a major role in septation of the heart and a portion of the cushion tissue contributes to valve formation (Manasek, 1976). By 9.5 days *p.c.*, transformation of endothelial cells into mesenchymal cushion tissue is commencing in the AV canal region and in the outflow tract. By this time, endocardial expression of TGF beta-1 has terminated in the cells overlying the ventricular and atrial myocardium. High TGF beta-1 RNA levels, however, still persist specifically in the endothelium of the AV region and in the outflow tract (Fig. 5A,B,D,E). This differential endothelial gene expression is apparent before the morphological appearance of mesenchymal cells (data not shown).

In all situations where we previously detected high levels of TGF beta-1 gene expression in epithelial cells (Lehnert and Akhurst, 1988), it has been reported that the ECM protein, Jl/tenascin, accumulates in the opposed mesenchymal tissue (Chiquet-Ehrismann *et al.* 1986) implying that TGF beta-1 may mediate some of its effects *via* this molecule (Chiquet-Ehrismann *et al.* 1989). To further examine this correlation, serial sections adjacent to those used for *in situ* hybridisation studies to the TGF beta-1 probe were subjected to immunohistochemistry using KAF9 and KAF10 polyclonal antibodies raised against Jl/tenascin (Steindler *etal.* 1989).

At early embryonic stages (7.0 days p.c), very weak tenascin staining was observed in the anterior mesoderm compared to posterior regions of the embryo. Prior to the appearance of mesenchymal cells in the cushion tissue, at 9.5 days p.c, staining for Jl/tenascin was also observed in the cardiac jelly of the outflow tract (data not shown). As cushion tissue develops, this staining intensified in the ECM surrounding mesenchymal cells. The localisation of Jl/tenascin staining in this tissue is clearly correlated with regions of the endothelium expressing high levels of TGF beta-1 RNA (Fig. 5).

TGF beta-1 and Jl/tenascin expression during septation and valvular maturation

A generalised observation was that throughout embryogenesis TGF beta-1 RNA expression was observed in endothelia of the heart and major blood vessels in regions where morphogenetic processes involving mesenchymal cell growth, cell migration and tissue fusion take place. In these areas, staining for Jl/tenascin and TGF beta protein was observed in the mesenchyme. This included formation of the spiral aorticopulmonary septum, formation of the interventricular septum, the atrioventricular valves and the semilunar valves of the pulmonary and aortic arteries. During septation of the atria, which does not involve cushion tissue, no endothelial TGF beta-1 expression was observed, or staining with the Jl/tenascin antibodies (Fig. 5A,B,C).

By 15.5 days, septation of the heart and the outflow tract is complete (Fananapazir and Kaufman, 1988) and the cardiac valves are easily identified as such by their characteristic shape. However, endothelial expression of TGF beta-1 RNA persists at high levels in all heart valves even to birth. By following TGF beta-1 RNA

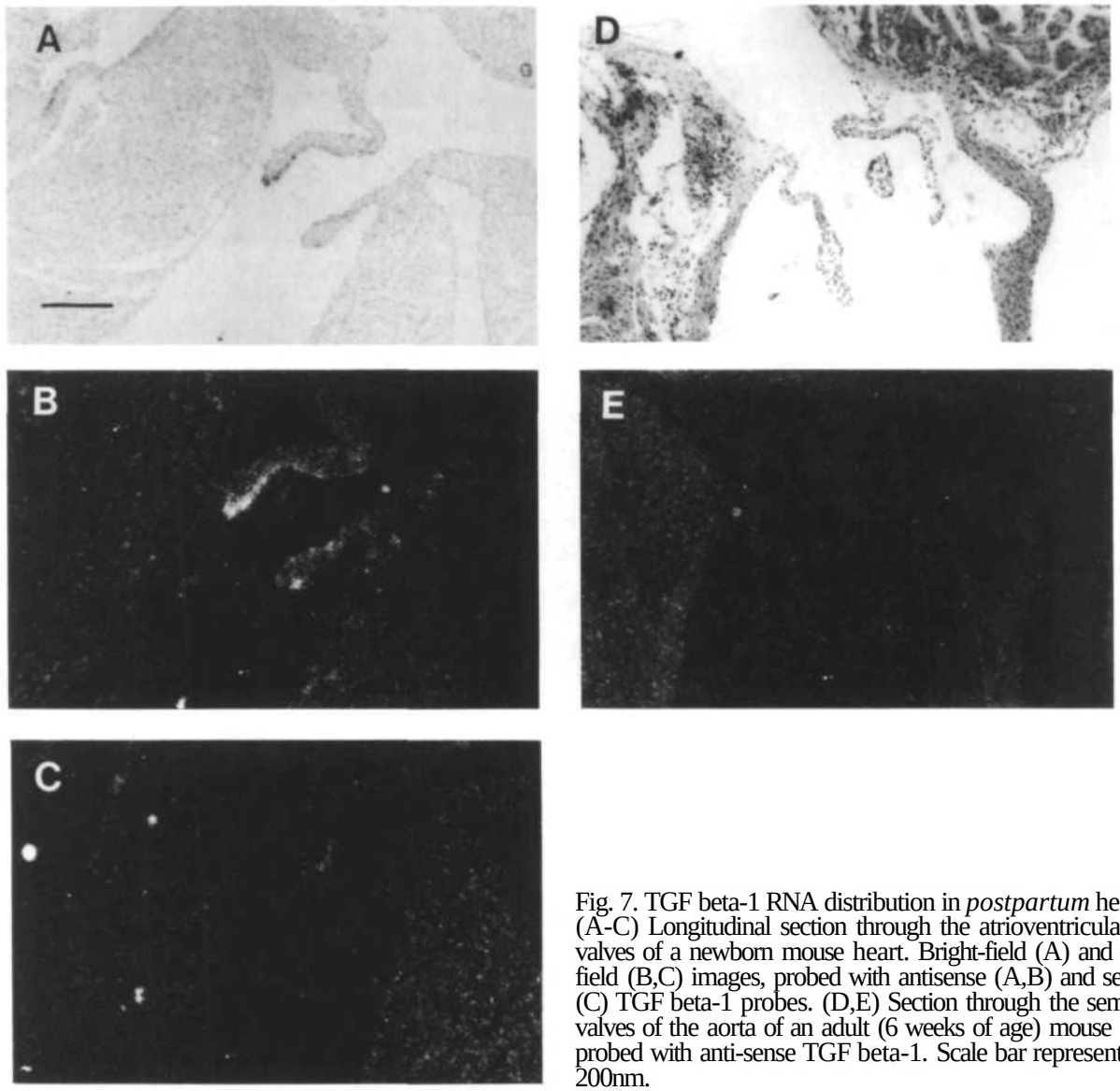

Fig. 7. TGF beta-1 RNA distribution in *postpartum* hearts. (A-C) Longitudinal section through the atrioventricular valves of a newborn mouse heart. Bright-field (A) and dark-field (B,C) images, probed with antisense (A,B) and sense (C) TGF beta-1 probes. (D,E) Section through the semilunar valves of the aorta of an adult (6 weeks of age) mouse heart probed with anti-sense TGF beta-1. Scale bar represents 200nm.

expression at 48h intervals *postpartum*, it was found that valvular TGF beta-1 RNA expression finally ceased 8 days *postpartum* (Fig. 7).

At later stages of cardiogenesis, there is little correlation between the sites and stages of TGF beta-1 RNA expression and of its encoded polypeptide, as assessed immunohistochemically. Also, tenascin distribution becomes gradually more distally located to the TGF beta-1 producing cells (compare Figs 6 and 7). At 12.5 days *p.c.*, staining for Jl/tenascin is most intense in the mesenchyme immediately adjacent to the TGF beta-1 producing endothelium (Fig. 6B) whereas CC antibody staining for extracellular TGF beta polypeptide is more prevalent in the deeper layers of the mesenchyme (Fig. 6A). By 16.5 days *p.c*, this differential distribution is reversed. Jl/tenascin staining is located at the base of the valve cusps whereas CC antibody staining is predominantly in the valve leaflets (Fig. 6D,E,J,K).

Postpartum, Jl/tenascin staining decreases both in area of distribution and in intensity. This is correlated with the decrease in TGF beta-1 gene expression. At birth, tenascin is seen in a wide area at the base of the valves, around the foramen orale and in the tunica media of the aorta (Fig. 6G,H). By eight days *postpartum*, this area of staining is reduced in size, and in the adult, tenascin staining is weak and limited to a small region at the base of the valves (Fig. 6I,K). In general, the intensity of Jl/tenascin staining correlates with the level of TGF beta-1 gene expression, though, at later stages, this extracellular matrix molecule is located somewhat distally from the endothelial cells which express TGF beta-1 RNA.

Conversely, the CC antibody showed an increasing intensity of staining as valvular maturation proceeded. Staining in the adult heart was intense and restricted to the mesenchymal portion of the valve leaflets and the base of the cusps, as previously demonstrated by Thompson *et al.* (1989) (Fig. 6J). At this stage, how-

ever, TGF beta-1 gene expression in the valvular endothelium has already been down-regulated for at least five weeks (Fig. 7).

Discussion

TGF beta-1 in vasculogenesis and angiogenesis

The first evidence of TGF beta-1 gene expression in the conceptus, as detected by *in situ* hybridisation, appears in the extraembryonic blood islands of the yolk sac. Rappolee *et al.* (1988) have shown that this gene is activated very soon after fertilisation, though the levels of expression detected in their study using the polymerase chain reaction are below the sensitivity of *in situ* hybridisation used here. Our findings are entirely consistent with those of Wilcox and Derynck (1988), who identified the TGF beta-1 expressing haemopoietic cells seen at 9.5 days *p.c.* as proerythroid in nature. We have further shown that the gene is activated, albeit at a lower level, in haemangioblasts prior to the emergence of overt erythyroid precursors. Expression in these early progenitor cells would be consistent with TGF beta-1 acting as an autocrine negative regulator of haematopoiesis (Keller *et al.* 1989).

Since members of the TGF beta gene superfamily have been reported to have mesoderm-inducing activity in an amphibian system (Kimelman and Kirschner, 1987; Rosa *et al.* 1988; Smith, 1989), we examined pregastrula- and gastrula-stage embryos for expression of TGF beta-1. In the heterologous *Xenopus* system, mammalian TGF beta-2 can induce the production of mesoderm from ectoderm (Rosa *et al.* 1988), whereas TGF beta-1 can only potentiate the mesoderm-inducing activity of fibroblast growth factor (Kimelman and Kirschner, 1987). If TGF beta-1 is an endogenous mammalian mesoderm-inducing factor (or cofactor), one might expect elevated expression of this RNA in either extraembryonic or primitive endoderm. This was not observed, making it unlikely that TGF beta-1 plays such a role. The possibility that TGF beta-1 is produced at low levels from these early embryonic cells cannot be entirely ruled out, since the sensitivity of the *in situ* hybridisation technique may be limiting.

The first embryonic expression of TGF beta-1 occurs in the cardiogenic plate, making this molecule an early embryonic marker of cardiac mesoderm. Although TGF beta-1 is clearly not an inducer of cardiac mesoderm itself, it remains a possibility that this molecule contributes to other inductive events elicited by the cardiogenic plate at later stages (Jacobson and Sater, 1988).

Due to the lack of molecular markers that differentiate endocardial from epimyocardial cells, and because of the poor resolution of the *in situ* hybridisation technique, it is impossible to say with which cell type TGF beta-1 expression is associated at this early stage. However, by the time that endocardial and myocardial cells are morphologically distinguishable, TGF beta-1 expression is clearly limited to the endothelial cell type. Early expression of TGF beta-1 in cardiac mesoderm might therefore simply reflect the vasculogenic capacity of this mesenchymal tissue.

Vascular development in vertebrates occurs by two distinct processes, 'vasculogenesis', the laying down of new blood vessels *in situ,* and 'angiogenesis', the outgrowth of vessels from pre-existing ones (Poole and Coffin, 1989; Risau *et al.* 1988). We have observed TGF beta-1 gene expression both in endothelial cells lining the major blood vessels at early stages and in capillary endothelial cells of the head and trunk region. This has also been previously demonstrated by Wilcox and Derynck (1988). It is clear, therefore, that TGF beta-1 is associated with both of these mechanisms.

In vitro, TGF beta has a number of biological effects on endothelial cells all of which are relevant to the growth and morphogenesis of vascular structures. It induces the elaboration of ECM, which in turn affects endothelial growth (Madri *etal.* 1988). It is also a potent inhibitor of endothelial cell growth (Takehara *et al.* 1987; Heimark *et al.* 1986), though this latter effect can be modulated, for example, by fibroblast growth factor (Baird and Durkin, 1986; Jennings *etal.* 1988). *In vivo,* TGF beta-1 may thus act as an autocrine negative regulator of endothelial cell growth. In this respect, it is interesting to note that, at times of rapid cell division, this gene is activated in several cell types which are known to be negatively regulated by TGF beta-1. In addition to endothelial cells, this includes haemopoietic, epithelial and lymphopoietic cells, all of which are positively regulated by other growth factors acting antagonistically to TGF beta-1 (Akhurst *et al.* 1988; Wilcox and Derynck, 1988; Lehnert and Akhurst, 1988; Akhurst *etal.* 1989). It has been suggested in these cases that the differentiated cell types are the source of TGF beta-l,which then modulates growth of the stem cells in a paracrine manner (Akhurst *etal.* 1988; Parkinson and Balmain, 1989). This type of growth regulation would obviously be very important in embryonic processes.

TGF beta-1 in epithelial-mesenchymal transformation

The early events leading to the formation of cardiac cushion tissue involve a regional differentiation of cells within the endocardium. Endothelial cells in the region of the AV canal and outflow tract become 'activated' to initiate the formation of mesenchyme, whereas the remaining endothelial cells are not competent in this process (Krug *et al.* 1985; Mjaatvedt *et al.* 1987). To our knowledge, the differential localisation of TGF beta-1 transcripts within the activated endothelial cell population is the first report of a molecular distinction between the two endothelial cell types.

Markwald and collaborators have clearly shown that the induction of epithelial-mesenchymal transformation in the chick is mediated by a complex particulate fraction synthesised by AV myocardium (Mjaatvedt and Markwald, 1989), though the developmental period over which these 'adheron-like complexes' are produced has not been defined. Whether TGF beta-1 gene expression is stimulated by these putative inducers, or whether the growth factor is itself a stimulant for

production of myocardial inducers, remains to be tested.

The fact that TGF beta-1 is expressed ubiquitously in endocardial cells at early stages would argue against a direct role in generating the regional induction signal. It is, however, possible that the AV myocardium has an active role in sustaining endothelial TGF beta gene expression specifically in the region of cushion tissue formation. TGF beta would subsequently contribute in some way to the epithelial-mesenchymal transition. In this respect, it is interesting to note that we have also observed high levels of TGF beta gene expression during the epithelial-mesenchymal transformation that occurs during growth and fusion of the secondary palate (Fitzpatrick and Akhurst unpublished). Conversely, regionalised TGF beta-1 gene expression may merely be a consequence of endogenous stimulation in response to the increased endothelial mitotic activity required to regenerate the endocardium as cells are lost to the mesenchymal layer (Fitzharris, 1981).

Recent experiments performed by Potts and Runyon suggest that TGF beta-1 or -2, in combination with other factors, can mediate the epithelial-mesenchymal transition (Potts and Runyan, 1989). They showed that chick ventricular myocardium (not normally competent to induce transformation), when supplemented with TGF beta, can mediate mesenchyme formation from AV endocardium and that this process is blocked by antibodies against TGF beta. They suggested that the source of TGF beta was the myocardium.

In the present study, it is clearly shown that the endocardium is a more plentiful source of TGF beta-1 RNA than the myocardium. In addition, our results would predict that the AV endocardium should already be producing TGF beta-1 RNA at the stage equivalent to that examined by Potts and Runyan. One would not therefore expect *additional* TGF beta to have any effect on the ability of AV endothelial cells to transform to mesenchyme in the *in vitro* system used by Potts and Runyan. Our data could be reconciled with theirs if one assumes that one major function of the AV myocardium is in maintaining this elevated TGF beta-1 gene expression in the overlying endocardium, and that this expression is then required for later events in the cellular transformation process, as discussed above.

A number of issues need to be resolved for both the mouse and chick systems before definitive conclusions may be made. First, it is now clear that there are at least five forms of vertebrate TGF beta encoded by different genes (ten-Dijke *et al.* 1988; Jakowlew *et al.* 1988a,*b;* Kondaiah *et al.* 1989). Potts and Runyon did not distinguish between these different forms in the chick whereas we have only, so far, examined the expression of one gene in the mouse. Second, there could be species differences in production and utilisation of the different molecular forms of TGF beta. Sato and Rifkin (1989) have shown, for example, that bovine aortic endothelial cells are inhibited in cell movement by TGF beta produced from bovine smooth muscle cells but not that produced from rat smooth muscle cells. Until comparative analyses on these species are performed using molecular probes to each polypeptide form, these points will remain unresolved.

TGF beta-1 in cardiac morphogenesis

Based on the data presented in this paper and on our earlier study of embryonic gene expression of TGF beta-1, we would suggest that a *major* function of TGF beta-1 in endocardial cushion tissue is not in the initial 'activation' of endothelial cells in the epithelial-mesenchymal transition *per se,* but in modulation of the activity of the underlying mesenchymal cells, particularly with respect to elaboration of the ECM. The altered composition of the ECM might then affect endothelial and mesenchymal cell phenotype. This proposition is based entirely on circumstantial evidence.

We previously showed that the TGF beta-1 gene was activated in embryonic epithelia which are undergoing specific morphogenetic events involving localised epithelial and mesenchymal cell growth and migration (Lehnert and Akhurst, 1988). We suggested that this growth factor was mainly acting *via* a paracrine mechanism on the underlying mesenchymal tissue, modulating growth, differentiation and ECM deposition. In all cases where we observed epithelial expression of TGF beta-1 RNA, there is mesenchymal localisation of the ECM molecule, tenascin (Chiquet-Ehrismann *et al.* 1986). Furthermore, TGF beta-1 is known to stimulate transcription of genes encoding ECM proteins including that for tenascin (Pearson *et al.* 1988).

We were particularly interested in comparing the distribution of tenascin with that of TGF beta-1 RNA, since this molecule has a more restricted distribution than that of other ECM molecules and might be a more specific marker for TGF beta-1-induced modulation of the matrix. A correlation between epithelial expression of TGF beta-1 RNA and mesenchymal localisation of tenascin polypeptide is seen during chondrogenesis and osteogenesis and during the development of the kidney, gut, tooth, hair follicle and salivary gland (Mackie *etal.* 1987; Aufderheide *et al.* 1987; Aufderheide and Ekblom, 1988; Chiquet-Ehrismann *etal.* 1986; Lehnert and Akhurst, 1988).

We have now extended this correlation to include morphogenesis of the heart. Indeed, we have shown that the mesenchymal distribution of tenascin polypeptide during morphogenesis of the heart valves and septae is more tightly correlated to endothelial TGF beta-1 RNA than are the distributions of collagen (Colvee and Hurle, 1981) or fibronectin (Icardo, 1985). During early cardiac valve morphogenesis tenascin localisation is immediately adjacent to the TGF beta-1 producing endothelial cells, whereas during later stages of development, and in the adult, tenascin is localised more distally from the endothelium. Clearly, many factors must contribute to induction of tenascin expression. At later stages in cardiogenesis TGF beta-1 may not play such a major role. Furthermore, morphogenetic movements may relocate extracellular molecules, such as tenascin, to sites distant from their site of synthesis.

Tenascin is known to stimulate growth of certain epithelial cells (Chiquet-Ehrismann *etal.* 1986), though no mitogenic effects on endothelial cells have been documented. It disrupts cell-substratum and cell-cell contacts and promotes cell mobility by interfering with the adhesive action of fibronectin (Chiquet-Ehrismann *et al.* 1988, 1989; Spring *et al.* 1989). These changes in cellular phenotype are all obligatory for transformation of endothelial cells into mesenchymal cushion tissue (Krug *et al.* 1985; Icardo, 1989). The effects of TGF beta-1 on endocardia! cushion formation and subsequent morphogenesis could clearly be mediated, in part, by tenascin.

High levels of both TGF beta RNA and tenascin protein in the cardiac valves are correlated with times of mesenchymal and endothelial cell growth and tissue movement. In contrast, the *intense* staining with the CC antibody against TGF beta polypeptide does not appear until fairly late stages of embryogenesis and continues to increase early *postpartum.* It therefore appears that there is lack of correlation between TGF beta-1 RNA levels, indicative of active gene expression, and TGF beta polypeptide levels. It is possible that in the adult, very low transcription levels may be translated to give much higher levels of protein. Many examples of post-transcriptional control of TGF beta have been cited (Assoian *et al.* 1987; Kehrl *et al.* 1986). In addition, the CC antibody may recognise protein products of other TGF beta genes. The antibody is known not to cross-react with TGF beta-2 polypeptide (Ellingsworth *et al.* 1986), but there have been no reports on its degree of cross-reactivity with TGF beta-3 or the putative mammalian TGF betas -4 and -5. This last point will only be resolved when gene probes and antibodies are available which show complete specificity of reactivity to individual gene products.

Thanks to Dr R. Derynck for supplying the murine TGF beta-1 cDNA probe and to Dr K. Flanders for supplying the CC antibody. Thanks also to Marion Hoggan for typing the manuscript. This work was supported in part by Birthright/ RCOG and by the MRC.

References

AKHURST, R. J., FEE, F. AND BALMAIN, A. (1988). Localized production of TGF-beta mRNA in tumour promoter-stimulated mouse epidermis. *Nature, Lond.* **331,** 363-365.

AKHURST, R. J., LEHNERT, S. A., GATHERER, D. AND DUFFIE, E. (1990). The role of TGF beta in mouse development. *Ann. N.Y. Acad. Sci.* (In Press).

ASSOIAN, R. K., FLEURDELYS, B. E., STEVENSON, H. C, MILLER, P. J., MADTES, D. K., RAINES, E. W., ROSS, R. AND SPORN, M. B. (1987). Expression and secretion of type beta transforming growth factor by activated human macrophages. *Proc. naln. Acad. Sci. U.S.A.* 84, 6020-6024.

AUFDERHE1DE, E., CHIQUET-EHRISMANN, R. AND EKBLOM, P. (1987). Epithelial-mesenchymal interactions in the developing kidney lead to expression of tenascin in the mesenchyme. *J. Cell Biol.* **105,** 599-608.

AUFDERHEIDE, E. AND EKBLOM, P. (1988). Tenascin during gut development: Appearance in the mesenchyme, shift in molecular forms, and dependence on epithelial-mesenchymal interactions. *J. Cell Biol.* **107,** 2341-2349.

BAIRD, A. AND DURKIN, T. (1986). Inhibition of endothelial cell proliferation by beta-type transforming growth factor: interactions with acidic and basic fibroblast growth factors. *Biochem. Biophys. Res. Comm.* 138, 476-482.

CHIQUET-EHRISMANN, R., KALLA, P. AND PEARSON, C. A. (1989). Participation of tenascin and transforming growth factor beta in reciprocal epithelial-mesenchymal interactions of MCF7 cells and fibroblasts. *Cancer Res.* **49,** 4322-4325.

CHIQUET-EHRISMANN, R., KALLA, P., PEARSON, C. A., BECK, K. AND CHIQUET, M. (1988). Tenascin interferes with fibronectin action. *Cell* 53, 383-390.

CHIQUET-EHRISMANN, R., MACKIE, E. J., PEARSON, C. A. AND SAKAKURA, T. (1986). Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell* **47,** 131-139.

COLVEE, E. AND HURLE, J. M. (1981). Maturation of the extracellular material of the semilunar heart valves in the mouse. *Anal. Embryol.* **162,** 343-352.

Cox, K. H., DELEON, D. V., ANGERER, L. M. AND ANGERER, R. C. (1984). Detection of mRNAs in sea urchin embryos by *in situ* hybridisation using asymmetric RNA probes. *Devi Biol.* **101,** 485-502.

DERYNCK, R., JARRETT, J. A., CHEN, E. Y. AND GOEDDEL, D. V. (1986). The murine transforming growth factor-beta precursor. *J. biol. Chem.* **261,** 4377-4379.

ELLINGSWORTH, L. R., BRENNAN, J. E., FOK, K., ROSEN, D. M., BENTZ, H., PIEZ, K. A. AND SEYEDIN, S. M. (1986). Antibodies to the N-terminal portion of cartilage-inducing factor A and transforming growth factor beta. Immunohistochemical localization and association with differentiating cells. *J. biol. Chem.* **261,** 12362-12367.

FANANAPAZIR, K. AND KAUFMAN, M. H. (1988). Observation on the development of the aortico-pulmonary spiral septum of the mouse. *J. Anat.* **u58,** 157-172.

FITZHARRJS, T. P. (1981). Origin and migration of cushion tissue in the developing heart. *SEM* **254(11),** 255-260.

FLANDERS, K. C, THOMPSON, N. L., CISSEL, D. S., VAN OBBERGHEN, SCHILLING, E., BAKER, C. C, KASS, M. E., ELLINGSWORTH, L. R., ROBERTS, A. B. AND SPORN, M. B. (1989). Transforming growth factor-beta 1: histochemical localization with antibodies to different epitopes. *J. Cell Biol.* **108,** 653-660.

GAMBLE, J. R. AND VADAS, M. A. (1988). Endothelial adhesiveness for blood neutrophils is inhibited by transforming growth factor-beta. *Science* **242,** 97-99.

HEIMARK, R. L., TWARDZIK, D. R. AND SCHWARTZ, S. M. (1986). Inhibition of endothelial regeneration by type-beta transforming growth factor from platelets. *Science* **233,** 1078-1080.

HEINE, U., MUNOZ, E. F., FLANDERS, K. C, ELLINGSWORTH, L. R., LAM, H. Y., THOMPSON, N. L., ROBERTS, A. B. AND SPORN, M. B. (1987). Role of transforming growth factor-beta in the development of the mouse embryo. *J. Cell Biol.* **105,** 2861-2876.

HIRAKI, Y., INOUE, H., HIRAI, R., KATO, Y. AND SUZUKI, F. (1988). Effect of transforming growth factor beta on cell proliferation and glycosaminoglycan synthesis by rabbit growth-plate chondrocytes in culture. *Biochim. biophys. Acta* **969,** 91-99.

ICARDO, J. M. (1985). Distribution of fibronectin during the morphogenesis of the truncus. *Anat. Embryol.* **171,** 193-200.

ICARDO, J. M. (1989). Changes in endocardia! cell morphology during development of the endocardial cushions. *Anat. Embryol.* **179,** 443-448.

IGNOTZ, R. A. AND MASSAGUE, J. (1986). Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J. biol. Chem.* **261,** 4337-4345.

IGNOTZ, R. A. AND MASSAGUE, J. (1987). Cell adhesion protein receptors as targets for transforming growth factor-beta action. *Cell 51,* 189-197.

JACOBSON, A. G. AND SATER, A. K. (1988). Features of embryonic induction. *Development* **104,** 341-359.

JAKOWLEW, S. B., DILLARD, P. J., KONDAIAH, P., SPORN, M. B. AND ROBERTS, A. B. (1988a). Complementary deoxyribonucleic acid cloning of a novel transforming growth factor-beta messenger ribonucleic acid from chick embryo chondrocytes. *Mol. Endocrinol.* 2, 747-755.

JAKOWLEW, S. B., KONDAIAH, P., DILLARD, P. J., SPORN, M. B.

AND ROBERTS, A. B. (1988fc). A novel low molecular weight ribonucleic acid (RNA) related to transforming growth factor beta messenger RNA. *Mol. Endocrinol.* 2, 1056-1063.

JENNINGS, J. C, MOHAN, S., LJNKHART, T. A., WIDSTROM, R. AND BAYUNK, D. J. (1988). Comparison of the biological actions of TGF beta-1 and TGF beta-2: differential activity in endothelial cells. *J. cell. Physiol.* **137,** 167-172.

KEHRL, J. H., ROBERTS, A. B., WAKEFIELD, L. M., JAKOWLEW, S. AND SPORN, M. B. (1986). Transforming growth factor beta is an important immunomodulatory protein for human B lymphocytes. *J. Immunol.* **137,** 3855-3860.

KELLER, J. R., SING, G. K., ELLINGSWORTH, L. R. AND RUSCETTI, F. W. (1989). Transforming growth factor beta: possible roles in the regulation of normal and leukemic hematopoietic cell growth. *J. Cell Biochem.* **39,** 175-184.

KIMELMAN, D. AND KIRSCHNER, M. (1987). Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early *Xenopus* embryo. *Cell* **51,** 869-877.

KONDAIAH, P., SANDS, M. S., SMITH, J. M., FIELDS, A., ROBERTS, A. B., SPORN, M. B. AND MELTON, D. A. (1990). Identification of a novel transforming growth factor beta (TGF beta 5) mRNA in *Xenopus laevis. J. biol. Chem.* (In Press).

KRUG, E. L., RUNYAN, R. B. AND MARKWALD, R. R. (1985). Protein extracts from early embryonic hearts initiate cardiac endothelial cytodifferentiation. *Devi Biol.* **112,** 414-426.

LEHNERT, S. A. AND AKHURST, R. J. (1988). Embryonic expression pattern of TGF beta type-1 RNA suggests both paracrine and autocrine mechanisms of action. *Development* **104,** 263-273.

MACKIE, E. J., THESLEFF, I. AND CHIQUET-EHRISMANN, R. (1987). Tenascin is associated with chondrogeneic and osteogenic differentiation *in vivo* and promotes chondrogenesis *in vitro. J. Cell Biol.* **105,** 2569-2579.

MADRI, J. A., PRATT, B. M. AND TUCKER, A. M. (1988). Phenotypic modulation of endothelial cells by transforming growth factor-beta depends upon the composition and organization of the extracellular matrix. *J. Cell Biol.* **106,** 1375-13S4.

MANASEK, F. J. (1976). Heart development: Interactions involved in cardiac morphogenesis. In *The Cell Surface in Animal Embryogenesis and Development* (edited by Poste, G. and Nichloson, G. L.) North Holland, Amsterdam: Elsevier, p. 545-598.

MJAATVEDT, C. H., LEPERA, R. C. AND MARKWALD, R. R. (1987). Myocardial specificity for initiating endothelial-mesenchymal cell transition in embryonic chick heart correlates with a paniculate distribution of fibronectin. *Devi Biol.* **119,** 59-67.

MJAATVEDT, C. H. AND MARKWALD, R. R. (1989). Induction of an epithelial-mesenchymal transition by an *in vivo* adheron-like complex. *Devi Biol.* **13<i,** 118-128.

NODA, M. AND RODAN, G. A. (1987). Type beta transforming growth factor (TGF beta) regulation of alkaline phosphatase expression and other phenotype-related mRNAs in osteoblastic rat osteosarcoma cells. *J. Cell Physiol.* **133,** 426—437.

NODA, M., YOON, K., PRINCE, C. W., BUTLER, W. T. AND RODAN, G. A. (1988). Transcriptional regulation of osteopontin production in rat osteosarcoma cells by type beta transforming growth factor. *J. biol. Chem.* **263,** 13916-13921.

OHTA, M., GREENBERGER, J. S., ANKLESARIA, P., BASSOLS, A. AND MASSAGUE, J. (1987). Two forms of transforming growth factor-beta distinguished by multipotenrial haematopoietic progenitor cells. *Nature* **329,** 539-541.

PARDANAUD, L., YASSINE, F. AND DIETERLEN-LIEVRE, F. (1989). Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. *Development* **105,** 473-485.

PARKINSON, K. AND BALMAIN, A. (1990). Chalones revisited-A possible role for TGF beta in tumour promotion. *Carcinogenesis* (In Press).

PEARSON, C. A., PEARSON, D., SHIBAHARA, S. AND HOFSTEENGE, J. (1988). Tenascin: cDNA cloning and induction by TGF-beta. *EMBO J.* 7, 2977-2982.

PELTON, R. W., NOMURA, S., MOSES, H. L. AND HOGAN, B. L. M. (1989). Expression of transforming growth factor beta-2 RNA during murine embryogenesis. *Development* **106,** 759-767.

PENTTINEN, R. P., KOBAYASHI, S. AND BORNSTEIN, P. (1988). Transforming growth factor beta increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. *Proc. natn. Acad. Sci. U.S.A.* **85,** 1105-1108.

POOLE, T. J. AND COFFIN, J. D. (1989). Vasculogenesis and angiogenesis: Two distinct morphogenetic mechanisms establish embryonic vascular pattern. *J. exp. Zool.* **251,** 224-231.

POTTS, J. D. AND RUNYAN, R. B. (1989). Epithelial-mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor beta. *Devi Biol.* **134,** 392-401.

RAPPOLEE, D. A., BRENNER, C. A., SCHULTZ, R., MARK, D. AND WERB, Z. (1988). Developmental expression of PDGF, TGF-alpha, and TGF-beta genes in preimplantation mouse embryos. *Science* **241,** 1823-1825.

RJSAU, W., SARIOLA, H., ZERWES, H-G., SASSE, J., EKBLOM, P., KEMLER, R. AND DOETSCHMAN, T. (1988). Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development* **102,** 471-478.

ROBERTS, A. B. AND SPORN, M. B. (1989). The transforming growth factor betas. *Handbook of Experimental Pharmacology,* (In Press).

ROBERTS, A. B., SPORN, M. B., ASSOIAN, R. K., SMITH, J. M., ROCHE, N. S., WAKEFIELD, L. M., HEINE, U. I., LIOTTA, L. A., FALANGA, V., KEHRL, J. H. AND FAUCI, A. S. (1986). Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro. Proc. natn. Acad. Sci. U.S.A.* **83,** 4167-4171.

ROSA, F., ROBERTS, A. B., DANIELPOUR, D., DART, L. L., SPORN, M. B. AND DAWID, I. B. (1988). Mesoderm induction in amphibians: the role of TGF beta 2-like factors. *Science* **239,** 783-785.

SATO, Y. AND RIFKIN, D. B. (1989). Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. *J. Cell Biol.* **109,** 309-315.

SMITH, J. C. (1989). Mesoderm induction and mesoderm-inducing factors in early amphibian development. *Development* **105,** 665-677.

SPORN, M. B. AND ROBERTS, A. B. (1988). Peptide growth factors are multifunctional. *Nature* **332,** 217-219.

SPORN, M. B., ROBERTS, A. B., WAKEFIELD, L. M. AND ASSOIAN, R. K. (1986). Transforming growth factor-beta: biological function and chemical structure. *Science* **233,** 532-534.

SPRING, J., BECK, K. AND CHIQUET-EHRISMANN, R. (1989). Two contrary functions of tenascin: Dissection of active sites by recombinant tenascin fragments. *Cell* **59,** 325-334.

STEINDLER, D. A., COOPER, N. G. F., FAISSNER, A. AND SCHACHNER, M. (1989). Boundaries defined by adhesion molecules during development of the cerebral cortex: The Jl/tenascin glycoprotein in the mouse somatosensory cortical barrel field. *Devi Biol.* **131,** 243-260.

TAKEHARA, K., LEROY, E. C. AND GROTENDORST, G. R. (1987). TGF-beta inhibition of endothelial cell proliferation: alteration of EGF binding and EGF-induced growth-regulatory (competence) gene expression. *Cell* **49,** 415-422.

TEN-DUKE, P., HANSEN, P., IWATA, K. K., PIELER, C. AND FOULKES, J. G. (1988). Identification of another member of the transforming growth factor type beta gene family. *Proc. natn. Acad. Sci. U.S.A.* **85,** 4715-4719.

THOMPSON, N. L., FLANDERS, K. C, SMITH, J. M.. ELUNGSWORTH, L. R., ROBERTS, A. B. AND SPORN, M. B. (1989). Expression of transforming growth factor-beta 1 in specific cells and tissues of adult and neonatal mice. *J. Cell Biol.* **108,** 661-669.

TWARDZIK, D. R., RANCHALIS, J. R. AND TODARO, G. J. (1982). Mouse embryos contain transforming growth factors related to those isolated from tumor cells. *Cancer Res.* **45,** 5413-5416.

WILCOX, J. N. AND DERYNCK, R. (1988). Developmental expression of transforming growth factors alpha and beta in the mouse fetus. *Mol. Cell Biol.* 8, 3415-3422.

WILKINSON, D. G., BAILES, J. A. AND MCMAHON, A. P. (1987). Expression of the proto-oncogene int-1 is restricted to specific neural cells in the developing mouse embryo. *Cell* **50,** 79-88.

(Accepted 29 January 1990)