

Concurrent and distinct transcription and translation of transforming growth factor- β type I and type II receptors in rodent embryogenesis

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ABSTRACT The transforming growth factor-betas (TGF- β s) are multifunctional regulatory polypeptides that play a crucial role in many cell processes and function through a set of cell surface protein receptors that includes TGF- β type I (RI) and type II (RII). The present study reports a comprehensive comparison of the patterns of expression of TGF- β RI and RII proteins and mRNAs in the developing mouse embryo using immunohistochemical and *in situ* hybridization analyses. Although widespread expression of both TGF- β receptors was detected throughout the embryonic development period so that many similarities occur in localization of the TGF- β receptors, TGF- β RI was expressed in a well-defined, non-uniform pattern that was different in many respects from that of TGF- β RII. Whereas higher levels of TGF- β RI compared to TGF- β RII were detected in some tissues of the embryo at the beginning of organogenesis, the level of TGF- β RII increased more dramatically than that of TGF- β RI during late organogenesis; this was especially true in many neural structures where TGF- β RI and RII were comparable by day 16. The lung, kidney and intestine, in which epithelial-mesenchymal interactions occur, showed a complex pattern of TGF- β RI and RII expression. Additionally, northern blot hybridization and reverse transcription-polymerase chain reaction (RT-PCR) amplification showed non-uniform expression of the transcripts for TGF- β RI and RII in embryonic and adult mouse and rat tissues. These data show that regulation of TGF- β RI and RII occurs concurrently, but distinctly, in a spatial and temporal manner in rodent embryogenesis which may allow control of signal transduction of TGF- β during development.

KEY WORDS: *growth factor receptor, expression, development, organogenesis, mouse embryo*

Introduction

The transforming growth factor-betas (TGF- β s) are multifunctional proteins that regulate many aspects of cellular function including proliferation, differentiation, adhesion and migration (for reviews, see Massague' 1990; Roberts and Sporn 1990). The TGF- β s exert their effects through binding to specific cell surface proteins. Three major types of TGF- β receptors, type I (RI), type II (RII), and type III (RIII), have been identified in most cells and have been defined by their ability to bind and cross-link ¹²⁵I-TGF- β (for reviews, see Derynck 1994; Massague' 1996; Hoodless and Wrana 1998). Both TGF- β RI and RII have been determined to be required for TGF- β -induced signaling (Laiho *et*

al., 1990,1991). In contrast, TGF- β RIII may be involved in presenting TGF- β ligand to TGF- β RI and RII, but does not directly mediate TGF- β signaling (Lopez-Casillas *et al.*, 1993). Molecular cloning of TGF- β RI and RII has shown that they belong to an expanding family of transmembrane serine/threonine kinases (for a review, see Hoodless and Wrana 1998). Both TGF- β RI and RII possess a cysteine-rich extracellular domain, a transmembrane domain and a cytoplasmic domain which contains all sequence character-

Abbreviations used in this paper: TGF- β transforming growth factor-beta; TGF- β RI, TGF- β type I receptor; TGF- β RII, TGF- β type II receptor; RT-PCR, reverse transcription-polymerase chain reaction; mRNA, messenger RNA; rRNA, ribosomal RNA.

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istics of serine/threonine kinases. However, several structural features distinguish TGF- β RI and RII from each other. For example, the extracellular domain of TGF- β RI is shorter than that of TGF- β RII. In addition, TGF- β RI has a highly conserved GS motif (SGSGSGLP) in the juxtamembrane region preceding the kinase domain, which is not present in TGF- β RII (Derynck 1994); deletion of this motif in TGF- β RI results in inactive receptor kinase activity and inability to transmit TGF- β signals (Feng *et al.*, 1995). The role of this motif in the activity of TGF- β RI has also been supported by loss-of-function and gain-of-function point mutations (Wieser *et al.*, 1995). TGF- β RI and RII have the ability to interact and form a tetrameric complex in the presence of ligand (Wrana *et al.*, 1992). It is this heteromeric complex that is thought to be the signaling unit responsible for mediating the biological responses of TGF- β .

The expression of TGF- β s 1, 2 and 3 has been examined in human, mouse and chicken embryogenesis in great detail and it is thought that the TGF- β ligands play a role in differentiation and morphogenesis in embryonic development (Heine *et al.*, 1987; Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990, 1991; Millan *et al.*, 1991; Schmid *et al.*, 1991; Jakowlew *et al.*, 1994). Thus far, the majority of research dealing with the TGF- β receptors in embryogenesis has focused on TGF- β RII. Expression of TGF- β RII mRNA has been examined in murine embryonic development and the expression pattern of TGF- β RII mRNA has been shown to correlate with that of TGF- β 1 mRNA (Lawler *et al.*, 1994; Roelen *et al.*, 1994; Wang *et al.*, 1995). TGF- β RI protein expression has been reported and compared with TGF- β RII protein in some mouse tissues during embryogenesis (Iseki *et al.*, 1995). However, a comprehensive study of the localization of TGF- β RI protein, and comparison with TGF- β RII protein in embryogenesis, has not been reported. In addition, no studies have been reported concerning the localization of TGF- β RI mRNA in mouse embryogenesis. Because TGF- β RI is also critically involved in the mechanism of TGF- β signal transduction, we have examined the pattern of expression of TGF- β RI mRNA and protein in rodent embryogenesis and compared it with that of TGF- β RII. Our data show that while expression of TGF- β RI precedes that of TGF- β RII in some tissues and many similarities exist in localization of TGF- β RI and RII in rodent embryogenesis, concurrent, but distinct, patterns of transcription and translation of TGF- β RI and RII occur in most tissues throughout rodent development. Our data suggest that these differences in TGF- β RI and RII expression may allow control of TGF- β signal transduction during rodent development.

Results

RT-PCR amplification of TGF- β RI and RII mRNAs in embryonic mouse

Reverse transcription-polymerase chain reaction (RT-PCR) amplification was used to examine the expression of TGF- β RI and RII mRNAs in embryonic mice of 11-, 15- and 17-days-of-age (E11, E15, E17). Figure 1 shows the 488bp and 437bp PCR products for TGF- β RI and RII, respectively, that were generated from cDNAs synthesized by reverse transcription of the mRNAs and that were detected by ethidium bromide staining with appropriate DNA molecular weight markers and confirmed by Southern blotting with antisense nested probes (data not shown). The cloned PCR products for day 17 (E17) embryos were further verified as authentic fragments of TGF- β RI and RII by nucleotide sequencing in

either direction using primers at the T7 and SP6 promoter regions (data not shown). The expected RT-PCR products for TGF- β RI and RII mRNAs were produced in all of the mouse embryos examined.

Expression of TGF- β RI and RII in mouse development

To investigate the localization of TGF- β RI protein in mouse embryo development, we examined the immunohistochemical staining pattern of TGF- β RI and compared it with that of TGF- β RII protein in several embryonic tissues ranging in age from 8- to 16-days-of-age (E8 to E16) using specific antibodies. Western blot analysis was used to demonstrate the quality of the antibodies used in our immunohistochemical analyses. Figure 2 shows a representative western blot analysis of TGF- β RI and RII proteins in extracts of a non-tumorigenic mouse cell line derived from normal mouse lung epithelium (C10). The blots show the expected 55 kDa TGF- β RI and 70 kDa TGF- β RII proteins. The specificity of the reaction was confirmed using neutralization with a 100-fold excess of the corresponding peptide antigens. Utilizing these antibodies, Table 1 summarizes a comparison of the immunocytochemical detection of TGF- β RI and RII during the organogenetic period of the mouse. Widespread expression of both TGF- β RI and RII proteins was detected throughout the embryonic development period. TGF- β RI is expressed in a well-defined, non-uniform pattern that is different in many respects from that of TGF- β RII. Throughout most of the stages of mouse development studied, the expression of TGF- β RI was generally higher than that of TGF- β RII. Differences in expression of TGF- β RI and RII proteins are also reflected in the patterns of the mRNAs for TGF- β RI and RII. The differences in the pattern and intensity of the brown immunohistochemical staining suggest that expression of both TGF- β RI and RII is regulated in a spatio-temporal manner during embryogenesis. While TGF- β RI was detected before that of TGF- β RII in some tissues including the neural tube, arterial vasculature, pituitary, dorsal root ganglia, liver hepatocytes, thymus, and the mesenchyme of the lung, intestine and lower jaw, expression of both TGF-

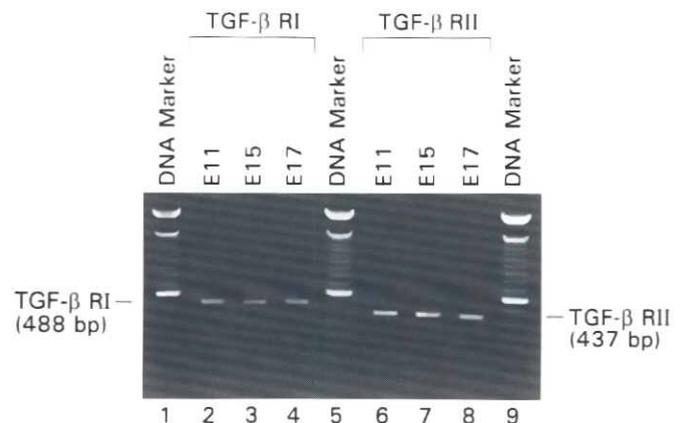


Fig. 1. RT-PCR amplification analysis of TGF- β RI and RII mRNAs in embryonic mouse. Two μ g of cDNA from E11, E15 and E17 mouse embryos were used for each PCR. The size of the amplified TGF- β RI and RII cDNA fragments is indicated based on ethidium bromide staining with molecular weight DNA markers. The gel shown is representative of 2 separate experiments.

β receptors was detected simultaneously in the brain, spinal cord, kidney, pancreas, skeletal muscle, skin, whisker follicles, liver megakaryocytes and epithelium of the lung, intestine, nose and lower jaw. Whereas higher levels of TGF- β RI compared to TGF- β RII were detected in most tissues of the embryo at the beginning of organogenesis (E8-E10) including the extraembryonic tissues, the level of immunostaining for TGF- β RII protein increased more dramatically than that of TGF- β RI in some tissues during late organogenesis (E14-E16) so that expression of both TGF- β receptors was comparable in some tissues such as the spinal cord, choroid plexus, and epithelium of the intestine, trachea and bronchioles in late organogenesis (E16). The distribution of the mRNAs for TGF- β RI and RII co-localized with the respective proteins in some, but not all, developing tissues in a spatio-temporal manner.

Expression of TGF- β RI and RII in the developing mouse placenta

Figure 3A and B shows a representative immunohistochemical staining pattern of TGF- β RI in the early placenta of a day 9 (E9) embryo and compares it with that of TGF- β RII (Fig. 3E,F). Immunostaining for TGF- β RI was detected in the early placenta, particularly in the giant trophoblastic cells, the ectoplacental cone and the maternal decidual cells; TGF- β RII was also detected in the giant trophoblastic and decidual cells, but at a considerably lower level than TGF- β RI. Intense staining for TGF- β RI was also detected in the yolk sac at the earliest stage studied (E8) (Table 1); in contrast, expression of TGF- β RII was detected in the yolk sac starting at day 10 (E10). Immunohistochemical staining for TGF- β RI and RII was completely blocked when the antibodies were preincubated with solutions of peptides against which they were raised (Fig. 3C,G). The detection of immunohistochemically stained TGF- β RI and RII was observed in a diffuse pattern of groups of primitive mesenchymal cells beginning at day 8 (E8) with the level of staining for TGF- β RI being more intense than that for TGF- β RII; the level of immunoreactive TGF- β RII increased in the mesenchyme as development proceeds as does that for TGF- β RI, although less dramatically (Table 1). Expression of TGF- β RI and RII by these groups of cells seems to follow organized patterns of distribution as the intensity of staining of the tissue varies from region to region as development proceeds. TGF- β RI immunoreactivity was also detected in the neural tube, ectoderm, endoderm and the paraxial and splanchnic mesoderm that gives rise to somites and mesenchyme of the intestine, respectively (Fig. 3D); in contrast, staining for TGF- β RII was detected only weakly in the primitive mesenchyme in the day 9 (E9) embryo (Fig. 1H). Staining for TGF- β RII was also detected in the neural tube starting in the day 10 (E10) embryo (Table 1).

To examine the tissue-specific distribution of TGF- β RI and RII mRNA expression, *in situ* hybridization was performed on mouse embryo sections using non-radioactive digoxigenin-labeled cRNA probes. High-stringency hybridization with antisense and sense (negative control) cRNA probes and washing were performed to ensure specific hybridization. Negative control sections were hybridized with sense TGF- β RI and RII cRNA probes. Figure 3I and K shows prominent hybridization as a purple precipitate to TGF- β RI and RII mRNAs in several cell types of the developing placenta, especially the giant trophoblastic cells, with hybridization being stronger for TGF- β RI mRNA than for TGF- β RII mRNA. Hybridiza-

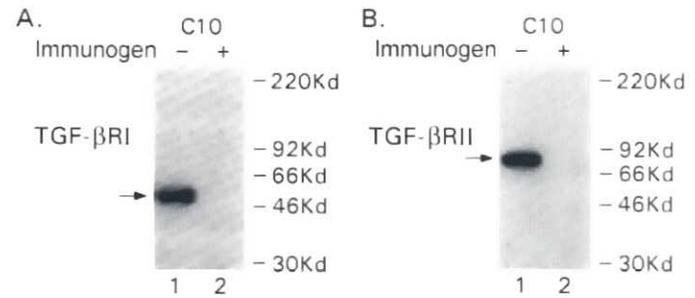


Fig. 2. Western blot analysis of TGF- β RI and RII proteins in C10 mouse cells. Fifty μ g of total protein from C10 mouse lung epithelial cells were separated by electrophoresis using a 4-20% Tris-glycine SDS polyacrylamide gel, transferred to a nitrocellulose filter and subjected to western blot analysis with (A) TGF- β RI and (B) TGF- β RII antibodies. Immunoreactive proteins are indicated by the arrows on the left of each panel. Absorption controls for (A) TGF- β RI and (B) TGF- β RII using peptide antigens are shown in lane 2 of each panel. The blots shown are representative of 3 separate experiments.

tion was also performed using digoxigenin-labeled sense TGF- β RI and RII cRNA probes as controls for specificity; Figure 3J and L shows the absence of hybridization of these probes to the early placenta.

Expression of TGF- β RI and RII in the developing mouse heart

On day 8 of development (E8), while most of the cells of the mouse embryo were not stained, TGF- β RI and RII were detected in the walls of the primitive cardiac tube (Table 1). By day 9 (E9), the myocytes of the ventricle and atrium were more intensely stained for TGF- β RI while TGF- β RII was weak in comparison (Fig. 4A,B and 4E,F). While immunoreactivity for TGF- β RI was also detected in the primitive mesenchyme at this time, staining for TGF- β RII was barely detectable. By day 10 (E10), staining for TGF- β RI continued to increase in intensity in the myocytes of the atrium as the cardiac myocytes proliferate and differentiate (Table 1 and Fig. 4C); at the same time, expression of TGF- β RII increased in the myocytes of the atrium and ventricle (Table 1 and Fig. 4G).

Staining for TGF- β RI and RII in the myocytes of the atrium and ventricle continued at these levels to at least day 16 (E16), with staining for TGF- β RI being more intense than for TGF- β RII (Table 1 and Fig. 4D,H). *In situ* hybridization analysis showed localization of both TGF- β RI and RII mRNAs in the developing mouse heart in both the endocardium and myocardium in a manner analogous to the corresponding protein immunostaining, with hybridization to TGF- β RI mRNA being greater than to TGF- β RII mRNA (Fig. 4I-L). It was noted that hybridization of TGF- β RII mRNA was detected in the primitive mesenchyme on day 9 (E9) of development, while staining for TGF- β RII protein was barely detectable at this time (Fig. 4E,J).

Expression of TGF- β RI and RII in the developing mouse nervous system

As described for the developing heart, differences in the pattern of expression of TGF- β RI and RII were also detected in the developing nervous system. In general, more intense staining and hybridization for TGF- β RI protein and mRNA compared to TGF- β

TABLE 1

TISSUE DISTRIBUTION OF IMMUNOHISTOCHEMICALLY DETECTABLE TGF-β RI AND RII IN MOUSE ORGANOGENESIS

Tissue	Localization	E8	E9	E10	E11	E12	E13	E14	E15	E16
Placenta	Ectoplacental Cone	1/1	1/1							
	Decidual (maternal) Cells	3/1	3/1 ^{1/2}							
	Giant Trophoblastic Cells	2/1	3/1							
Yolk Sac		2/1 ^{1/2}	2 ^{1/2} /1 ^{1/2}	2 ^{1/2} /2						
Primitive Mesenchyme		2/1 ^{1/2}	2/1 ^{1/2}	2 ^{1/2} /1	2 ^{1/2} /2	2 ^{1/2} /2	3/2			
Neural Tube		2 ^{1/2} /0	2/0	2 ^{1/2} /1	2 ^{1/2} /1 ^{1/2}	3/1 ^{1/2}				
Heart	Atrial Myocytes	0/0	2/1	3/2	3/2	3/1 ^{1/2}	3/2 ^{1/2}	3/2	3/2	3/2
	Ventricle Myocytes	2/1	3/1	3/2	3/2	3/2	3/3	3/2	3/2	3/2
Arterial Vasculature		2/0	2/0	2/1	2 ^{1/2} /1 ^{1/2}	3/2	3/2	2/1 ^{1/2}	2/1 ^{1/2}	3/2
Central Nervous System	Brain			2/1	2/1	2 ^{1/2} /2	2 ^{1/2} /2 ^{1/2}	3/2 ^{1/2}	3/2	3/2
	Pituitary				-/0	2/0	2 ^{1/2} /1	3/1	-/1 ^{1/2}	
	Choroid Plexus					2/1 ^{1/2}	2 ^{1/2} /2 ^{1/2}	3/2 ^{1/2}	2 ^{1/2} /2	2/2
Peripheral Nervous System	Spinal Cord				2/1 ^{1/2}	3/2	3/3	3/3	3/3	3/3
	Dorsal Root Ganglia			1 ^{1/2} /0	1 ^{1/2} /1 ^{1/2}	2 ^{1/2} /1	3/3	3/2 ^{1/2}	3/2	3/2
	Projecting Nerves			3/2	3/2	3/2	3/2	3/2	2/1	2/1
Skeletal Structures	Skeletal Muscle					2/1 ^{1/2}	3/2	3/2	3/2	3/2 ^{1/2}
	Pre-cartilaginous Blastema				2/0	1 ^{1/2} /1 ^{1/2}				
	Perichondrium						3/1 ^{1/2}	3/1	2/1 ^{1/2}	3/1 ^{1/2}
	Chondrocytes					2/1 ^{1/2}	3/1 ^{1/2}	3/1 ^{1/2}	3/1 ^{1/2}	3/1
	Hypertrophic Cartilage						2/1 ^{1/2}	2 ^{1/2} /1 ^{1/2}	2 ^{1/2} /1 ^{1/2}	1/1
Skin			2/2	2/2	2 ^{1/2} /2	2 ^{1/2} /2	2 ^{1/2} /2	3/2	3/2 ^{1/2}	
Whisker Follicles							3/1 ^{1/2}	2/1	2 ^{1/2} /1 ^{1/2}	
Intestine	Epithelium			1 ^{1/2} /1 ^{1/2}	1/1	1/1	2/2	3/2	2/1 ^{1/2} *	2 ^{1/2} /2 ^{1/2}
	Muscle Fibers						2/2	3/2	2/1 ^{1/2}	3/2
	Mesenchyme			1/0	1 ^{1/2} /0	1 ^{1/2}				
Pancreas	Exocrine					0/0	1 ^{1/2} /-	2/1	2/1	2/1
	Endocrine					2/1	3/2	3/1	1 ^{1/2} /1	2/1
Lower Jaw Epithelium				3/2	3/2	3/2 ^{1/2}	3/3	3/1 ^{1/2}	3/1 ^{1/2}	
Lower Jaw Mesenchyme			2/0	2/1	2 ^{1/2} /1 ^{1/2}	2/1 ^{1/2}	2/1 ^{1/2}	2/1 ^{1/2}	2/1 ^{1/2}	
Nose Epithelium					3/2	3/2	3/2	3/3	3/1 ^{1/2}	3/1 ^{1/2}
Liver	Hepatocytes			0/0	1 ^{1/2} /0	1 ^{1/2} /1	2 ^{1/2} /2	3/1 ^{1/2}	3/1 ^{1/2} **	3/1
	Megakaryocytes			0/0	0/0	2/1	3/2	3/1 ^{1/2}	2/1 ^{1/2}	3/1
Thymus	Cortex							2 ^{1/2} /0	2/0	3/1
	Medulla							3/0	2/0	3/1
Lung	Tracheal Epithelium							3/2	2 ^{1/2} /3	3/3
	Bronchial Epithelium			1/1 ^{1/2}	1/1	1/1	2/2	2 ^{1/2} /1	1 ^{1/2}	3/3
	Respiratory (alveolar) Epithelium						1 ^{1/2}	2 ^{1/2}	1 ^{1/2} **	1 ^{1/2}
	Mesenchyme			1/0	1 ^{1/2}	1 ^{1/2} /0	1 ^{1/2} /0	1 ^{1/2} /1 ^{1/2}	1/0	-/1 ^{1/2}
	Airway Smooth Muscle Cells						-/1 ^{1/2}	2 ^{1/2} /1	1/1	3/1 ^{1/2}
Kidney	Metanephric Duct					1 ^{1/2}	2/1 ^{1/2}	3/1 ^{1/2}	2 ^{1/2} /1 ^{1/2}	3/1
	Metanephric Duct Derivatives					1 ^{1/2} /1 ^{1/2}	2 ^{1/2} /1 ^{1/2}	3/1 ^{1/2}	2 ^{1/2} /1 ^{1/2}	3/1 ^{1/2}
	Glomeruli							2 ^{1/2}	1 ^{1/2} /1 ^{1/2}	2 ^{1/2}
Adrenal Gland						-/2	2/2 ^{1/2}	3/2	2 ^{1/2} /2	-/

Order is TGF-β RI/TGF-β RII.
 Staining intensity ranked from 0 (no staining) to 3 (very intense staining); -, not determined.
 * Stomach is intensity 2 for TGF-β RII. ** Some forming blood cells show intensity 3 staining. ***Nuclear staining.

RII protein and mRNA were detected in most neural structures at the early stages of development including the neural tube and the central and peripheral nervous systems; staining and hybridization for TGF-β RII protein and mRNA continued to increase in neural structures with development so that the levels of staining and hybridization for TGF-β RI and RII proteins and mRNAs were

comparable by day 16 (E16) in such neural tissues as the brain, spinal cord, dorsal root ganglia and choroid plexi. Immunohistochemically detectable TGF-β RI and RII were observed in the central and peripheral nervous systems by day 10 (E10); at this time, the forming brain was positively stained for TGF-β RI and RII, while the dorsal root ganglia were only reactive for TGF-β RI (Table

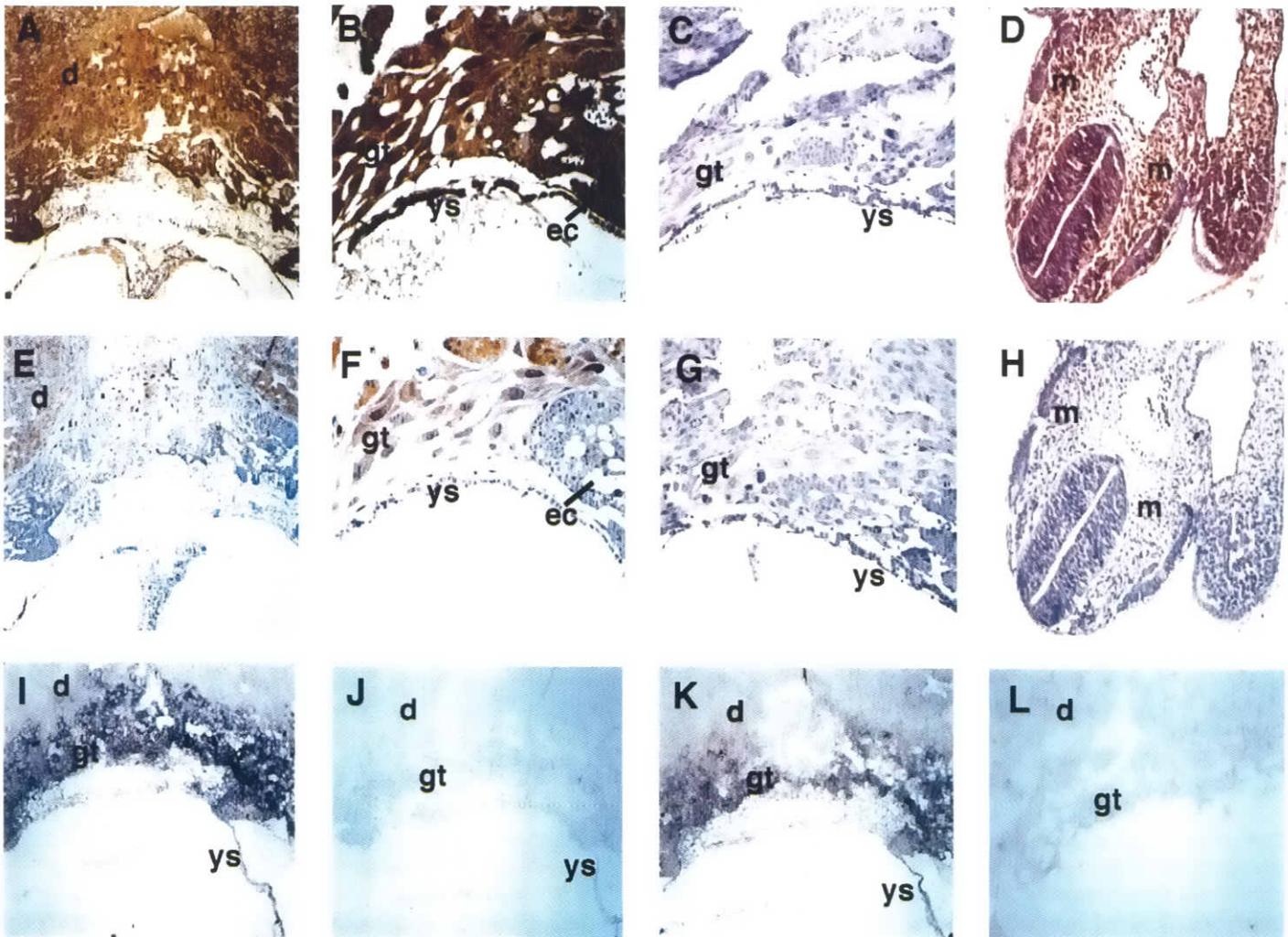


Fig. 3. Expression of TGF- β RI and RII in the E9 mouse early placenta, neural tube and mesenchyme. (A,B,D) Immunohistochemical detection of TGF- β RI. (C) Absorption control for TGF- β RI. (E,F,H) Immunohistochemical detection of TGF- β RII. (G) Absorption control for TGF- β RII. (I) *In situ* hybridization for TGF- β RI mRNA. (J) Control using a sense TGF- β RI cRNA probe. (K) *In situ* hybridization for TGF- β RII mRNA. (L) Control using a sense TGF- β RII cRNA probe. Note the intense staining for TGF- β RI in the ectoplacental cone and giant trophoblastic cells in A and B, and in the neural tube and mesenchyme in D, and the less intense staining for TGF- β RII in these cells in E, F and H and blocking in C and G. Indicated are yolk sac (ys), giant trophoblastic cells (gt), ectoplacental cone (ec), decidual cells (d), mesenchyme (m) and neural tube (n). Immunostaining and *in situ* hybridization in this and subsequent figures is representative of 3 separate experiments. Magnification: A, E, I-L $\times 40$; B, D, F-H, $\times 100$.

1). Interestingly, the projecting nerves from these ganglia were also immunoreactive for both TGF- β receptor antibodies. The spinal cord was positively stained for both TGF- β receptors by day 11 (E11), with staining for TGF- β RI being more intense than for TGF- β RII; however, by day 13 and continuing to day 16 (E13-E16), immunoreactivity for both TGF- β receptors was comparable in this region of the central nervous system (Table 1). Strong expression of TGF- β RI and RII was also detected in the ventral and marginal portions of the medulla oblongata as well as in the ventral midline cells of the hindbrain and derivatives of the metencephalon such as the pons (data not shown). Dorsal root ganglia were chosen to illustrate some changing patterns in more detail in Figure 5, but similar variations were also detected in other central and peripheral neural structures. While moderate levels of staining for TGF- β RI were detected in the developing dorsal root ganglion neurons at day 10 and day 11 (E10-E11) (Table 1 and Fig. 5A,B), minimal (Fig.

5E,F) staining for TGF- β RII was detected in these structures at day 10 (E10) and day 11 (E11), respectively. Starting at day 12 (E12), intense immunoreactivity for both TGF- β receptors was detected in the sensory neurons of the dorsal root ganglia (Figs. 5C and G) as well as for the mRNAs for TGF- β RI and RII (data not shown). Expression of the mRNAs for TGF- β RI and RII continued to be detected as development proceeds in most neural structures including the dorsal root ganglia, choroid plexi, spinal cord and ventral portions of the hindbrain and medulla oblongata, with the intensity of hybridization to TGF- β RI mRNA being more intense than that of TGF- β RII mRNA (Fig. 5D,H).

While most neural structures showed a higher level of expression of TGF- β RI than of TGF- β RII at earlier stages of development and comparable levels of both TGF- β receptors later in embryonic development, two exceptions, namely the choroid plexi and the projecting nerves from the dorsal root ganglia were observed; the

choroid plexi showed moderately intense levels of immunostaining for both TGF- β RI and RII from day 12 (E12) onwards, and the projecting nerves from the developing dorsal root ganglia showed strong immunoreactivity for TGF- β RI and RII from day 10 to 14 (E10-E14), whereupon both TGF- β receptors showed decreased immunostaining as development proceeds (Table 1).

Expression of TGF- β RI and RII in the developing mouse lung, liver and kidney

In addition to the heart, several other internal organs expressed TGF- β RI and RII during development in the mouse embryo including the lung, liver, kidney, intestine, thymus and adrenal gland (Table 1). Expression of TGF- β RI and RII was detected at the same developmental stage in epithelium of the lung (E10), intestine (E10) and kidney (E12). In contrast, expression of TGF- β RI was detected earlier than TGF- β RII in the developing liver (E11 vs E12). During organogenesis of the lung, liver and kidney, a complex pattern of expression of TGF- β RI and RII occurs. Figure 5I-L shows positive immunostaining for TGF- β RI and RII in the developing lung of day 10 (E10) and day 14 (E14) embryos. In the forming respiratory structures, while intense immunostaining for both TGF- β receptors was found in the epithelium of the forming bronchi, staining of the corresponding mesenchyme was strong for TGF- β RI by day 12 (E12) but weak for TGF- β RII throughout the stages examined. Both TGF- β receptors were also detected in the forming respiratory epithelium, which will become the alveolar compartment, but immunoreactivity for TGF- β RII was weak compared to TGF- β RI (Table 1 and Fig. 5I,J). Staining for both TGF- β receptors was comparable in the bronchial epithelium by day 14 (E14) (Fig. 5K,L). *In situ* hybridization showed expression of both TGF- β receptor mRNAs in the bronchiolar epithelium, and while TGF- β RI mRNA was also detected at moderate levels in the surrounding mesenchyme, TGF- β RII mRNA was low (data not shown). In the developing liver, increasing levels of TGF- β RI staining were found in hepatocytes and megakaryocytes starting from day 11 (E11) and day 12 (E12), respectively, and continuing at least through day 16 (E16) (Table 1). In contrast, immunostaining for TGF- β RII continued to increase up to day 13 (E13) and then decreased progressively. The intensity of staining for TGF- β RI was more intense than that of TGF- β RII at all stages of development examined in the liver (Table 1); *in situ* hybridization also showed a higher level of TGF- β RI mRNA compared to TGF- β RII mRNA in the liver as well (data not shown). In the kidney, the developing metanephric duct tubules and derivatives showed high levels of staining for TGF- β RI that increased from day 12 to day 16 (E12-E16) with staining in the mesenchyme being considerably weaker than in the ductal epithelial cells. The levels of TGF- β RII were lower than that of TGF- β RI in the metanephric duct and showed a different dynamic of change than that of TGF- β RI, reaching a peak of staining intensity at days 13 and 14 (E13-E14) and subsequently decreasing (Table 1); in addition, no immunoreactivity for TGF- β RII was detected in the surrounding mesenchyme. *In situ* hybridization showed hybridization for TGF- β RI mRNA in the forming kidney tubules and mesenchyme, while TGF- β RII mRNA was detected only in the forming tubules (data not shown).

In addition to examining the localization of TGF- β RI and RII in the epithelium and mesenchyme of the lung, liver and kidney, we extended our study to include other tissues in which epithelial and

mesenchymal interactions occur including the intestine, jaw and whiskers. While immunostaining for TGF- β RI and RII was detected in the epithelium at relatively equal intensities in the intestinal epithelium beginning at day 10 (E10), with staining for both TGF- β receptors increasing at approximately equal levels to day 16 (E16), immunostaining for TGF- β RI, but not TGF- β RII was detected at day 10 (E10) in the corresponding mesenchyme, with higher levels of TGF- β RI in the mesenchyme than in the epithelium; immunostaining for TGF- β RII could only be detected in the intestinal mesenchyme beginning at day 12 and remained at a low level to day 16 (E16) (Table 1). Moderately intense immunostaining for TGF- β RI was detected in the mesenchyme surrounding the forming intestine, pancreas and liver, with only weak staining appearing in the epithelium of the developing intestine, pancreas and liver (data not shown); more intense staining for TGF- β RI and RII was also seen in the smooth muscle surrounding the intestine as well. In contrast, only very weak staining for TGF- β RII was detected in both the epithelium and surrounding mesenchyme of the developing intestine, pancreas and liver; staining for TGF- β RII was also less than for TGF- β RI in the smooth muscle surrounding the developing intestine at day 12. By day 14 (E14), the intensity of staining for both TGF- β receptors increased in the intestinal epithelium and mesenchyme as did their corresponding transcripts. *In situ* hybridization also showed TGF- β RI and RII transcripts in the epithelial lining of the intestine and surrounding mesenchyme as well as in the smooth muscle layer (data not shown). In contrast to the intestine, the mesenchyme found in the developing upper and lower jaws showed intense staining for TGF- β RI, but weak staining for TGF- β RII throughout embryonic development (Table 1); while the epithelium also stained intensely for TGF- β RI at all stages of embryonic development, there was a marked increase in staining for TGF- β RII in both the epithelium and mesenchyme of the lower jaw between days 10 and 14 (E10-E14), followed by a rapid, continuous decrease in staining to day 16 (E16) (Table 1). Immunostaining and *in situ* hybridization for both TGF- β receptors was detected at approximately equal levels in the outer layer of the skin, the periderm, from day 10 (E10) onwards and was remarkably strong in the epidermal cells of the more differentiated skin on day 14 (E14), especially for TGF- β RI, although TGF- β RII was also detectable (Table 1). In contrast to the skin, staining for TGF- β RI was considerably more intense than that of TGF- β RII in the epithelium of the whisker follicles between days 14 and 16 (E14-E16). Staining for both TGF- β receptors was also detected in the smooth muscle and mesenchyme around the whisker follicles with the level of staining for TGF- β RI being greater than that of TGF- β RII. Transcripts for TGF- β RI and RII were also detected in the epithelium of the whisker follicles and mesenchyme (data not shown).

Detection of immunohistochemically stained TGF- β RI and RII in the developing skeletal system was not observed before day 12 (E12) (Table 1). At the E12 stage, expression of both TGF- β RI and RII was detected as the differentiation of cartilage proceeds from condensed mesenchyme and precartilage to mature chondrocytes. Whereas the intensity of staining for TGF- β RI was relatively intense and constant throughout embryonic development in the skeletal muscle, staining for TGF- β RII increased dramatically between days 12 and 13 (E12-E13) and continued at a moderate level to day 16 (E16). Staining for TGF- β RI was also intense in forming cartilage from the formation of the pre-chondrocytic blast-

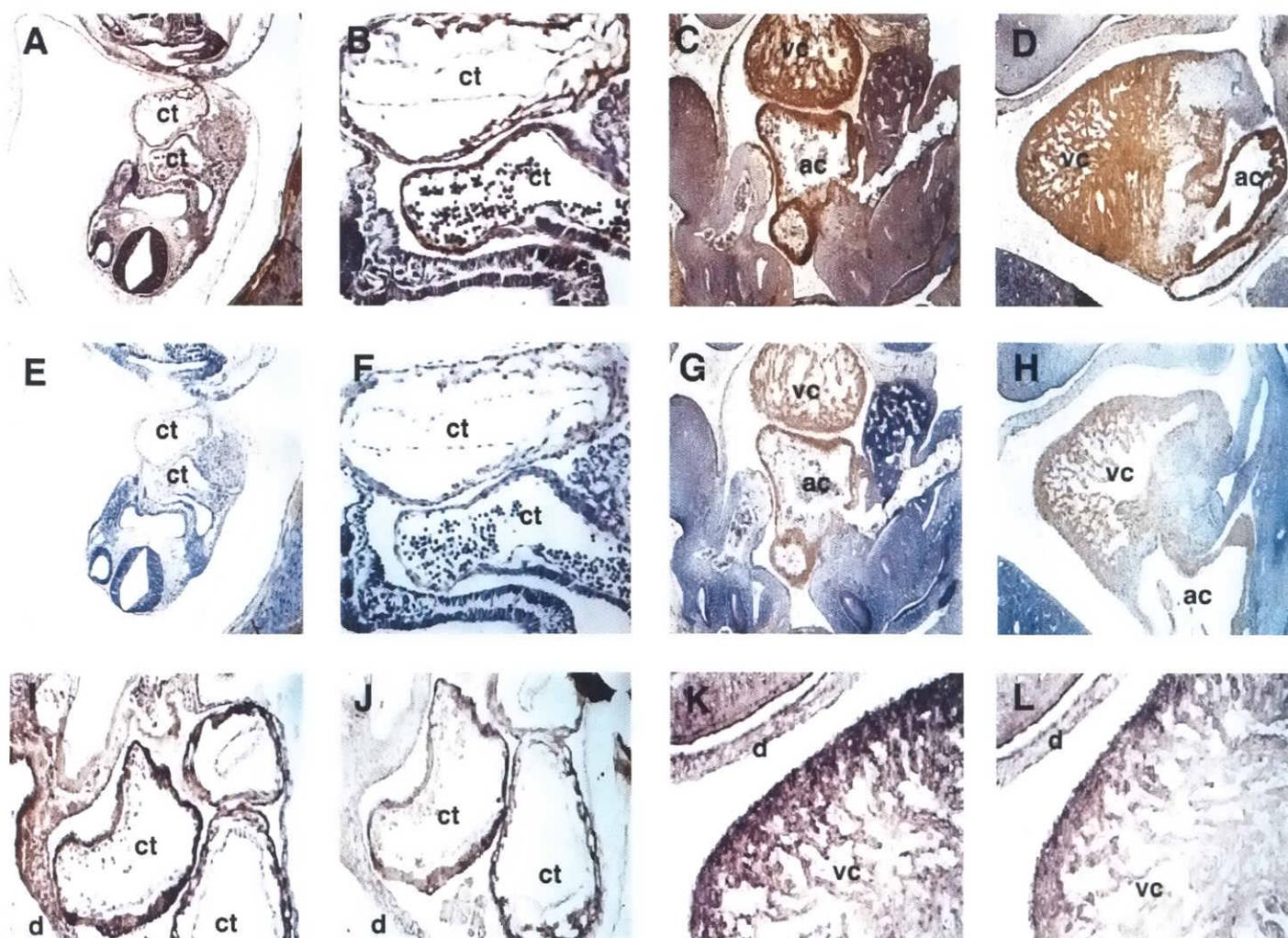


Fig. 4. Expression of TGF- β RI and RII in the developing mouse heart. (A-D) Immunohistochemical detection of TGF- β RI. (E-H) Immunohistochemical detection of TGF- β RII. (I,K) *In situ* hybridization for TGF- β RI mRNA. (J,L) *In situ* hybridization for TGF- β RII mRNA. (A,B,E,F,I,J) E9 embryos. Note that cardiac tube and some mesenchymal cells are positive for TGF- β RI. (C,G) E10 embryos. Atrium and ventricle are positive for TGF- β RI and RII. (D,H,K,L) E12 embryos. Cardiomyocytes in the ventricle are positive. Indicated are cardiac tube (ct), ventricular chamber (vc), atrial chamber (ac) and decidual cells (d). Magnification: A, C, E, G $\times 40$; B, D, F, H, I-L $\times 100$.

ema to hypertrophic cartilage (Table 1). Perichondrial cells showed higher levels of TGF- β RI than the rest of the cells of the cartilage series. In contrast, staining for TGF- β RII was very weak in the forming cartilaginous structures (Table 1). *In situ* hybridization analysis also showed the presence of TGF- β RI mRNA but not TGF- β RII mRNA in early cartilage formation and of both TGF- β receptor mRNAs in mature and hypertrophic cartilage at later stages of embryonic development (Fig. 5D,H).

Northern blot analysis of TGF- β RI and RII mRNAs in mouse and rat development

Steady-state expression of TGF- β RI mRNA was examined in several tissues in adult mouse and rat and embryonic rat using northern blot analysis and compared with TGF- β RII mRNA. Expression of TGF- β RI mRNA was readily detectable in several tissues of the 6-month-old adult mouse and rat including the brain,

heart, lung and kidney and in rat intestine (Fig. 6A). Following dehybridization of the nylon membrane and overnight exposure to film to ensure complete dehybridization, the membrane was reprobed with a TGF- β RII cDNA probe. The transcript for TGF- β RII was readily detectable in several adult mouse and rat tissues including heart, lung and kidney and rat intestine (Fig. 6B); TGF- β RII mRNA was most prominent in the lung in both adult mouse and rat. Expression of TGF- β RI and RII mRNAs could be detected in adult mouse and rat liver only after prolonged exposure (data not shown). As a control, the gel was stained with ethidium bromide and photographed to show the amounts of RNA that had been applied to the gel (Fig. 6C).

Expression of the 5.5-kb TGF- β RI mRNA was also readily detectable at varying levels in all tissues examined except the liver in the 18-day-old (E18) embryonic rat (Fig. 7A). Expression of TGF- β RI mRNA was especially prominent in the brain, while lower

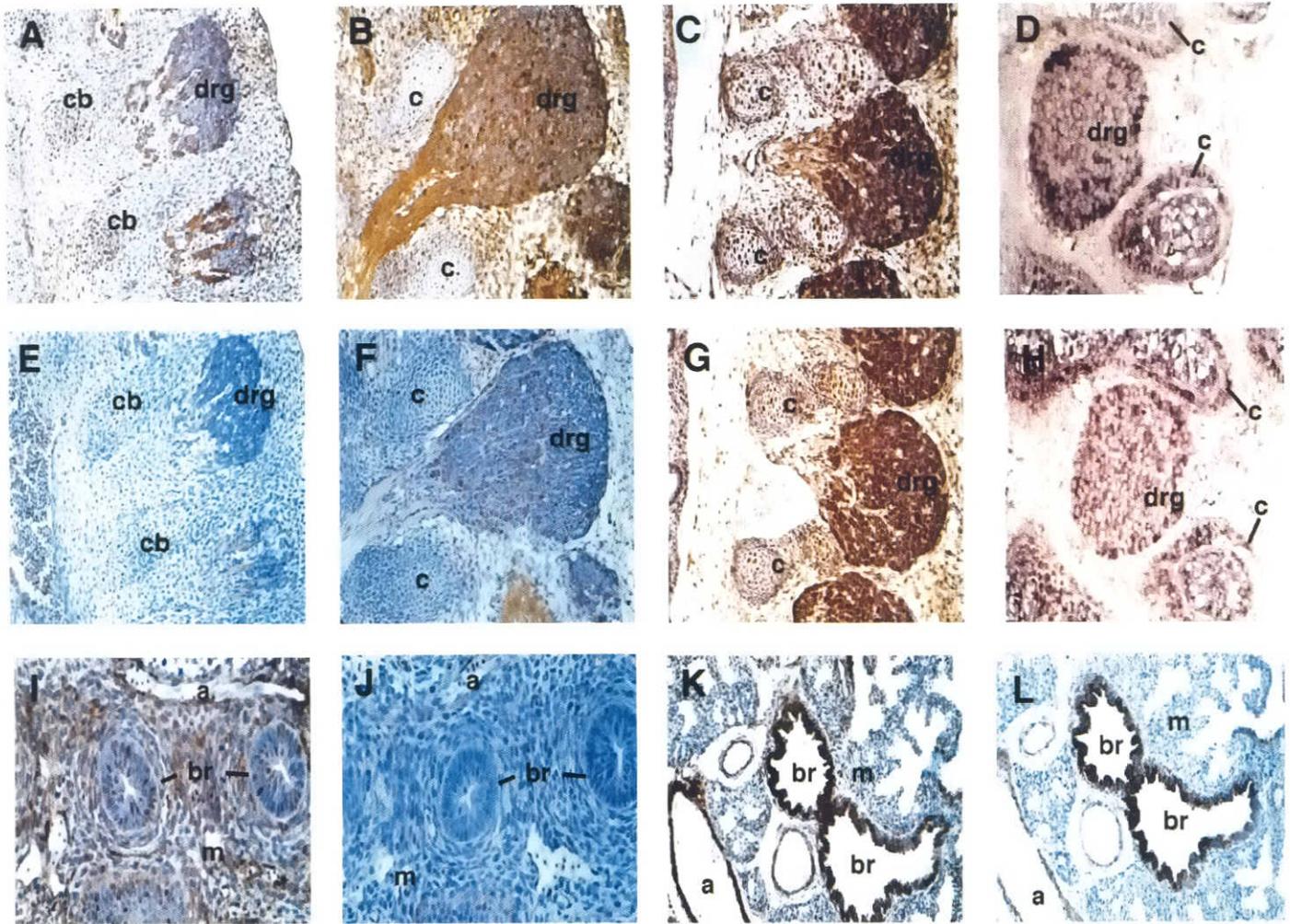


Fig. 5. Expression of TGF- β RI and RII in the developing mouse dorsal root ganglion and lung. (A-C,I,K) Immunohistochemical detection of TGF- β RI. (E-G,J,L) Immunohistochemical detection of TGF- β RII. (D) *In situ* hybridization of TGF- β RI mRNA. (H) *In situ* hybridization of TGF- β RII mRNA. (A,E) E11. Cells of the developing nerve trunks are positive for TGF- β RI and RII. The pre-cartilaginous blastema are positive for TGF- β RI. (B,F) E12. Dorsal root ganglia stain intensely for TGF- β RI and weakly for TGF- β RII. Cartilage is positive for TGF- β RI and RII. Chondrocytes and muscle of the developing vertebrae are positive for TGF- β RI but stain weakly for TGF- β RII. (C,G) E13. Note the staining of the developing nerve trunks, dorsal root ganglia and cartilage of the vertebrae for TGF- β RI and RII. (D) E15. TGF- β RI mRNA in dorsal root ganglion. (H) E15. TGF- β RII mRNA in dorsal root ganglion. The dorsal root ganglia and cartilage express TGF- β RI and RII mRNAs. (I-J) E10. Lung. Cells of the bronchioles and mesenchyme of the lung are positive for TGF- β RI but stain very weakly for TGF- β RII. (K-L). E14. Lung. The bronchial epithelium of the lung stain intensely for both TGF- β RI and RII. The lung arteries stain intensely for TGF- β RI but only moderately for TGF- β RII. Note positive staining for TGF- β RI in the lung mesenchyme but the absence of staining for TGF- β RII. Indicated are dorsal root ganglion (drg), pre-cartilaginous blastema (cb), cartilage (c), bronchiole (br), mesenchyme (m) and artery (a). Magnification: A-H,K,L x100; I-J x200.

levels of TGF- β RI mRNA were detected in the limb, heart, lung, intestine and kidney. TGF- β RI mRNA was also detected in the placenta, umbilical cord and yolk sac. Like the adult, expression of TGF- β RI mRNA could be detected in embryonic rat liver only after prolonged exposure (data not shown). Following dehybridization of the nylon membrane and overnight exposure to film to ensure complete dehybridization, the membrane was re probed with a TGF- β RII cDNA probe. Figure 7B shows the expression of the 5.5-kb TGF- β RII mRNA was especially prominent in the heart, lung, intestine, umbilical cord and yolk sac, while lower levels of TGF- β RII mRNA were detected in the brain, limb, kidney and placenta. Like the adult, expression of TGF- β RII mRNA could be detected

in embryonic rat liver only after prolonged exposure (data not shown). As a control, the gel was stained with ethidium bromide and photographed to show the amounts of RNA that had been applied to the gel (Fig. 7C). These results from northern blot analyses for TGF- β RI and RII mRNAs were also supported by RT-PCR analysis (data not shown).

Discussion

The cloning of many receptors in the TGF- β superfamily has allowed analysis of these receptors in development and insight into the resulting signal transduction processes that are involved. The

availability of TGF- β RII cDNA and the resulting analyses utilizing this cDNA in *in situ* hybridization has allowed a correlation between the mRNA for TGF- β RII and the TGF- β ligands in mouse embryogenesis (Lawler *et al.*, 1994; Roelen *et al.*, 1994; Wang *et al.*, 1995). Also, localization of TGF- β RI and RII proteins has been reported in some tissues in mouse embryogenesis (Iseki *et al.*, 1995). However, most of these reports have only made a cursory examination of a very few embryonic tissues and have not compared expression of the TGF- β RI and RII transcripts in mouse embryogenesis. Some of these reports have utilized suboptimal probes consisting of genomic DNA or DNA from heterologous species that required weeks of exposure to visualize so that the resulting localization of signal needs to be reexamined. Also, these previous reports have utilized TGF- β receptor antibodies that have generally not been readily available. In our study, we have used antibodies that are now commercially available and that have been characterized in other areas of biology including injury, disease, pathology and carcinogenesis (Horvath *et al.*, 1996; Ruifrok *et al.*, 1997; Jakowlew *et al.*, 1998; Schmid *et al.*, 1998) and corresponding mouse cRNA probes to study the localization of TGF- β RI and RII proteins and transcripts in a variety of mouse embryonic tissues. Proper localization of TGF- β RI and RII mRNAs and proteins in mouse tissues in development using these antibodies and correlation with the corresponding transcripts could provide information for their possible involvement in mouse models of disease processes. Since TGF- β RI and RII are critically involved in the mechanism of TGF- β signal transduction, we have examined the mRNA and protein expression pattern of both receptors in murine embryogenesis in detail using *in situ* hybridization and immunohistochemical analyses. In addition, we have examined the expression of the mRNAs for TGF- β RI and RII in rat embryo and adult rat tissues and compared this to the mouse using northern blot and RT-PCR analyses. Our results indicate that while expression of TGF- β RI precedes that of TGF- β RII in some tissues, overlapping patterns of TGF- β RI and RII occur in most tissues at the later stages of development and in the same cellular location in rodent embryogenesis. Furthermore, our data suggest that TGF- β RI is not ubiquitously expressed during mouse embryogenesis as has been previously reported (Iseki *et al.*, 1995), but, instead, is expressed in a distinct pattern during mouse development. This indicates a complex pattern of gene regulation at the level of transcription, translation, storage and degradation of the TGF- β receptors during development. There are at least 3 reasons to think that the differences in the patterns of expression of TGF- β RI and RII are probably not due to differences in the affinities of the antibodies used. First, the intensity of immunostaining for TGF- β RI and RII is equivalent in some tissues at certain stages of development, suggesting that the different intensities of staining for the TGF- β receptors reflect real differences in the presence or availability of the antigens. Second, western blot analysis shows detection of similar amounts of TGF- β RI and RII proteins by their respective antibodies. Third, our immunohistochemical staining results showing that the levels of TGF- β RI protein are generally higher than that of TGF- β RII protein in mouse embryogenesis parallel those of *in situ* hybridization showing higher levels of TGF- β RI mRNA compared to TGF- β RII mRNA during the same developmental time.

Both TGF- β receptors were detected simultaneously using immunohistochemical staining during mouse embryogenesis in

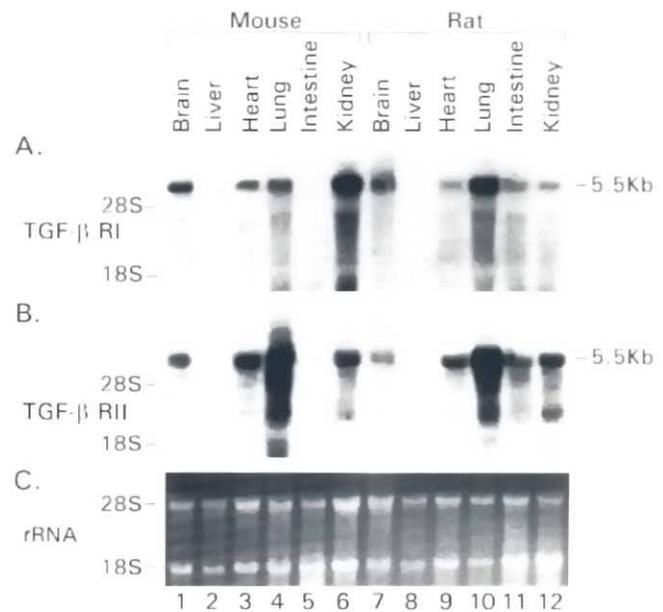


Fig. 6. Northern blot analysis of TGF- β RI and RII mRNAs in adult mouse and rat tissues. Total RNA (10 μ g) was isolated from 6-month-old adult mouse (lanes 1-6) and rat (lanes 7-12) tissues, separated by electrophoresis on a 1% agarose-formaldehyde gel, and transferred to a Nytran filter as described in Materials and Methods. Hybridization was performed with [32 P]-labeled random-primed cDNA probes for (A) TGF- β RI and (B) TGF- β RII as described in Materials and Methods. Blots for A and B were exposed for 2 days and 4 days, respectively. (C) The ethidium bromide staining pattern of the gels showing 18S and 28S rRNA. The blots shown are representative of 2 separate experiments.

several tissues including the brain, spinal cord, kidney, pancreas, skeletal muscle, skin, whisker follicles and epithelium of the lung, intestine and lower jaw. Previous reports have shown that TGF- β is frequently found at the sites of epithelial-mesenchymal interactions (Heine *et al.*, 1987; Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1991). In general, the tissue type that shows the most consistent TGF- β expression is the mesenchyme between and within many organs (Heine *et al.*, 1987; Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990, 1991). Like the TGF- β ligands, we have detected expression of the mRNAs and proteins for TGF- β RI and RII in the mesenchyme of several tissues including the heart, lung, intestine and jaw. Earlier reports have also noted TGF- β RII transcripts in the mesenchymal components of many tissues including the lung, intestine and tooth buds (Lawler *et al.*, 1994; Roelen *et al.*, 1994; Wang *et al.*, 1995). Our study has also shown expression of TGF- β RI and RII mRNAs and proteins in the epithelium of several tissues including the lung, intestine, kidney, olfactory system and lower jaw. In addition, we have detected expression of TGF- β RI and RII transcripts and proteins in the endocardium and myocardium of the developing heart, metanephric tubules, brain, smooth muscle, dermis and epidermis. In contrast to our study, Wang *et al.* (1995) reported no expression of TGF- β RII mRNA in the epithelial lining of the bronchi, intestine, and olfactory system; in addition, this report indicated strong hybridization for TGF- β RII mRNA in the endocardium, but not in the myocardium, in the metanephric mesenchyme, but not in the metanephric tubules, in the mesenchyme underlying

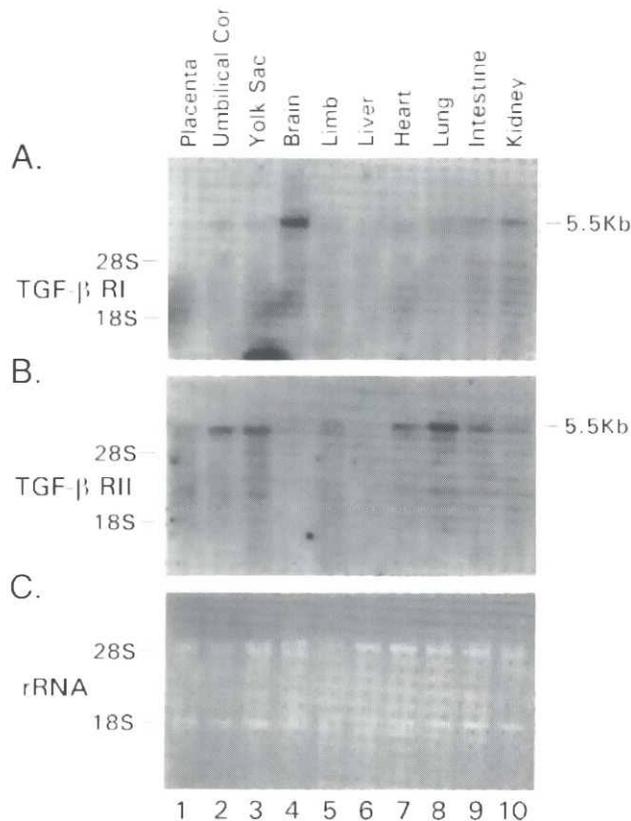


Fig. 7. Northern blot analysis of TGF- β RI and RII mRNAs in rat embryo tissues. Total RNA (2 μ g) was isolated from 18-day-old (E18) rat embryo tissues, separated by electrophoresis on a 1% agarose-formaldehyde gel, and transferred to a Nytran filter as described in Materials and Methods. Hybridization was performed with [32 P]-labeled random-primed cDNA probes for (A) TGF- β RI (B) TGF- β RII as described in Materials and Methods. Blots for A and B were exposed for 2 days and 4 days, respectively. (C) The ethidium bromide staining pattern of the gel showing 18S and 28S rRNA. The blots shown are representative of 2 separate experiments.

the periderm, but not in the periderm itself, in the dermis, but not in the epidermis, in the stroma of the intestine, but not in the smooth muscle layer, and in the choroid plexi, but not in the dorsal root ganglia. These discrepancies may be due to use of an *in situ* hybridization cRNA probe consisting of 150bp of exon 2 of mouse TGF- β RII and 350 bp of corresponding TGF- β RII genomic DNA sequence (Wang *et al.*, 1995); it is likely that this TGF- β RII cRNA probe with only 150bp complementary to TGF- β RII mRNA, may not have been of sufficient size to obtain reliable hybridization in these tissues. In support of our results, several reports of localization of TGF- β RII transcripts and protein in the epithelium of embryonic mouse tissues including the lung, kidney, intestine, tooth bud and submandibular gland have been made (Iseki *et al.*, 1995; Horvath *et al.*, 1996; Ruifrok *et al.*, 1997). In addition, positive staining for TGF- β RII in chick dorsal root ganglia has been reported by Unsicker *et al.* (1996). We noted an additional discrepancy between our data and those of Lawler *et al.* (1994) and Roelen *et al.* (1994) pertaining to TGF- β RII mRNA in the brain; while both of these reports noted the absence of TGF- β RII transcripts in mouse brain, we detected TGF- β RII transcripts and

protein in this tissue using *in situ* hybridization, northern blot, RT-PCR and immunohistochemical staining analyses. The reasons for the apparent lack of detection of TGF- β RII transcripts in mouse brain in previous reports may include the long exposure time of 5 weeks required for mRNA detection by Lawler *et al.* (1994) or the use of rat cRNA instead of mouse cRNA probe by Roelen *et al.* (1994) or the use of a probe consisting largely of genomic DNA by Wang *et al.* (1995). That TGF- β RI and RII transcripts do exist in the brain is also supported by RT-PCR and northern blot hybridization of adult rat and mouse brain in other reported studies (Tsuchida *et al.*, 1993; Lawler *et al.*, 1994; Suzuki *et al.*, 1994; Tomoda *et al.*, 1994; Bottnar *et al.*, 1996; Slotkin *et al.*, 1997). Indeed, despite the reported absence of TGF- β RII transcripts in mouse brain, Roelen *et al.* (1994) reported immunohistochemical staining for TGF- β RII in this neural tissue that could be completely competed with the immunoreactive peptide in agreement with our results. Moreover, contrary to a previous report (Iseki *et al.*, 1995), our data suggest that TGF- β RI is not ubiquitously expressed during mouse embryogenesis. For example, while moderately intense immunostaining for TGF- β RI was detected in the mesenchyme surrounding the forming intestine and pancreas, only weak staining for TGF- β RI was detected in the corresponding epithelium of these tissues. Differences in localization of TGF- β RI and RII using immunohistochemical staining and of their corresponding mRNAs by *in situ* hybridization and northern blot analyses were also observed in the lung, kidney, liver and developing cartilage, where a different dynamic of change for the two TGF- β receptors was observed. These differences indicate distinct patterns of expression for TGF- β RI and RII in mouse embryogenesis.

Our study has shown a comprehensive localization of TGF- β RI and RII mRNAs and proteins in mouse embryogenesis. The similarities in the temporal and spatial patterns of TGF- β RI and RII during rodent embryogenesis are consistent with the known requirement for the presence of both TGF- β receptors for signal transduction of TGF- β . Our data show that concurrent, but distinct, patterns of transcription and translation of TGF- β RI and RII occur in most tissues throughout mouse development. Our data suggest that these differences in TGF- β RI and RII expression may allow more precise control of TGF- β signal transduction during development. Future experiments employing TGF- β receptor mutants *in vitro* and *in vivo* model systems will be needed to determine the complex role of TGF- β RI and RII in early developmental processes.

Materials and Methods

Animals and tissue samples

Sections of NIH Swiss mouse staged embryos (Novagen, Madison, WI) were used. Adult female animals were caged with adult males overnight. The presence of a vaginal plug the following morning was designated day 0 of pregnancy. For RNA extraction, pregnant Sprague-Dawley rats (SAIC-Frederick, Frederick, MD) (18 days after the vaginal plug was found and 6-months-old) and A/J mice (Jackson, Bar Harbor, ME) (6-months-old) were used. Tissues were dissected and kept frozen at -80°C until extracted.

Western blot analysis

Cells were harvested and lysed into 0.0625 M Tris-HCl, pH 8.0, 2% SDS and 5% 2-mercaptoethanol. Equal amounts (50 μ g of protein) of cell lysates were boiled, separated by electrophoresis on 4-20% Tris-glycine gels and transferred to nitrocellulose. Blots were reacted with specific polyclonal antibodies to TGF- β RI and RII (Santa Cruz Biotechnology, Santa Cruz,

CA) and proteins were visualized by enhanced chemiluminescence for 2 min each.

Immunohistochemistry

For the immunocytochemical localization of TGF- β RI and RII in paraffin sections, the avidin-biotin peroxidase complex technique was employed (Vector Laboratories, Burlingame, CA). Affinity-purified polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and included TGF- β RI(V-22) for TGF- β RI and TGF- β RII(L-21) for TGF- β RII. After deparaffinization and blocking of endogenous peroxidase in hydrogen peroxide/methanol, the sections were blocked with 1.5% normal goat serum/0.5% BSA, incubated overnight at 4°C with the affinity-purified antisera at 0.5 μ g/ml, washed extensively, and then incubated with biotinylated goat anti-rabbit IgG and avidin-biotin-enzyme complex. Sections were stained with 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and hydrogen peroxide, and counterstained with Gill's hematoxylin. Controls include 1) using primary antisera preincubated with a 20-fold excess of the appropriate peptide for 2 h at room temperature; 2) replacing primary antisera with normal rabbit IgG. Sections from at least 3 different embryos were scored by 3 observers for each developmental stage. Scoring reflected intensity of staining as 0 = none, 1 = weak, 2 = moderate, and 3 = very intense.

Reverse transcription-polymerase chain reaction amplification

The oligonucleotide primers were synthesized using a MilliGen/Biosearch 8700 DNA synthesizer (Millipore, Marlborough, MA). Primer sets were as follows:

TGF- β RI (extracellular): 488bp product (34-521)
Sense: 5'-GTCCGCAGCTCCTCATCGTGTG-3'
Antisense: 5'-GGTGGTGCCTCTGAAATGAAAG-3'

TGF- β RII (intracellular): 437bp (1101-1537)
Sense: 5'-CCCGGGCCTCGCTCATCTC-3'
Antisense: 5'-AATTTCTGGGCGCCCTCGTCTCT-3'

RT-PCR was performed using 2 μ g total RNA and the GeneAmp RNA PCR kit according to the manufacturer's directions (Perkin Elmer/Cetus, Norwalk, CT). The reverse transcription procedure was performed using the primers with the following conditions: reverse transcription (RT) at 42°C for 50 min, inactivation of reverse transcriptase at 70°C for 15 min and RNase H digestion at 37°C for 20 min. After RT, PCR was performed using the sense primers in a Perkin Elmer 9600 thermalcycler (Norwalk, CT) as follows: 94°C for 15 sec, 60°C for 15 sec, 72°C for 1 min for 30 cycles followed by a 10 min incubation at 72°C. To visualize the PCR products, the samples were subjected to electrophoresis on 2% agarose gels containing ethidium bromide. The authenticity of the products was confirmed by Southern blot hybridization with nested internal primers and DNA sequencing.

In situ hybridization

Detection of the mRNAs for TGF- β RI and RII was performed using *in situ* hybridization. The cDNAs generated using RT-PCR as outlined previously (Jakowlew *et al.*, 1998) were ligated into the pcDNA1 vector (Invitrogen, San Diego, CA) following the manufacturer's procedures and used to generate riboprobes. The plasmids were linearized with EcoRV and BamHI and used as templates to synthesize digoxigenin-labeled sense and antisense RNA transcripts. Hybridization was performed in a moist chamber at 65°C for 20 h in a 50 μ l volume containing the antisense probes (Jakowlew *et al.*, 1998). After stringency washes, visualization of digoxigenin was performed using a digoxigenin detection kit (Boehringer Mannheim, Indianapolis, IN). Sense probes were used as controls.

RNA extraction and northern blot analysis

Total RNA was extracted from tissues by the method of Chirgwin *et al.*, (1979) using guanidine isothiocyanate and caesium chloride. For Northern blot analysis, equal amounts of total RNA (10 μ g) were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde, transferred to "Nytran"

filters (Schleicher and Schuell, Keene, NH), UV cross-linked and baked for 3 h. Ethidium bromide (33 μ g/ml) was included in both the gels and running buffers to visualize the positions of ribosomal RNAs by ultraviolet illumination after electrophoresis. Blots were hybridized with [³²P]-labeled (3000 Ci/mole, DuPont, Boston, MA) random-primed cDNA probes used for *in situ* hybridization at 65°C according to Church and Gilbert (1984) and then exposed for various times at -70°C using a DuPont Lightning Plus intensifying screen (DuPont). Densitometry of autoradiograms was performed using a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA).

Acknowledgments

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References

- BOTTNER, M., UNSICKER, K. and SUTER-CRAZZOLARA, C. (1996). Expression of TGF- β type II receptor mRNA in the CNS. *Neuroreport* 25: 2903-2907.
- CHIRGWIN J.M., PRZYBYLA, A.E., MACDONALD, R.J. and RUTTER, W.J. (1979). Isolation of biologically active ribonucleic acid from sources rich in ribonuclease. *Biochemistry* 18: 5294-5299.
- CHURCH, G.M. and GILBERT, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci. USA* 80: 1991-1995.
- DERYNCK, R. (1994). TGF- β receptor-mediated signaling. *Trends Biochem. Sci.* 19: 548-553.
- FENG, X.-F., FILVAROFF, E.H. and DERYNCK, R. (1995). Transforming growth factor- β (TGF- β)-induced down-regulation of cyclin A expression requires a functional TGF- β receptor complex. Characterization of chimeric and truncated type I and type II receptors. *J. Biol. Chem.* 270: 24237-24245.
- FITZPATRICK, D.R., DENHEZ, F., KONDAIAH, P. and AKHURST, R.J. (1990). Differential expression of TGF-beta isoforms in murine palatogenesis. *Development* 109: 585-595.
- HEINE, U.I., FLANDERS, K.C., ROBERTS, A.B., MUNOZ, E.F. and SPORN, M.B. (1987). Role of transforming growth factor- β in the development of the mouse embryo. *J. Cell Biol.* 105: 2861-2876.
- HOODLESS, P.A. and WRANA, J.L. (1998). Mechanism and function of signaling by the TGF β superfamily. *Curr. Top. Microbiol. Immunol.* 228: 235-272.
- HORVATH, L.Z., FRIESS, H., SCHILLING, M., BORISCH, B., DEFLOREN, J., GOLD, L.I., KORC, M. and BUCHLER, M.W. (1996). Altered expression of transforming growth factor- β s in chronic renal rejection. *Kidney Int.* 50: 489-498.
- ISEKI, S., OSUMI-YAMASHITA, N., MIYAZONO, K., FRANZEN, P., ICHIJO, H., OHTANI, H., HAYASHI, Y. and ETO, K. (1995). Localization of transforming growth factor- β type I and type II receptors in mouse development. *Exp. Cell Res.* 219: 339-347.
- JAKOWLEW, S.B., CIMENT, G., TUAN, R.S., SPORN, M.B. and ROBERTS, A.B. (1994). Expression of transforming growth factor- β 2 and β 3 mRNAs and proteins in the developing chicken embryo. *Differentiation* 55: 105-118.
- JAKOWLEW, S.B., MOODY, T.W. and MARIANO, J.M. (1998). Reduction of transforming growth factor- β receptor type II in mouse lung carcinogenesis. *Mol. Carcinog.* 22: 46-56.
- LAIHO, M., WEIS, F.M., BOYD, F.T., IGNOTZ, R.A. and MASSAGUE, J. (1991). Responsiveness to transforming growth factor- β (TGF- β) restored by genetic complementation between cells defective in TGF- β receptors I and II. *J. Biol. Chem.* 266: 9108-9112.
- LAIHO, M., WEIS, F.M. and MASSAGUE, J. (1990). Concomitant loss of transforming growth factor (TGF)- β receptor types I and II in TGF- β resistant cell mutants implicates both receptor types in signal transduction. *J. Biol. Chem.* 265: 18518-18524.
- LAWLER, S., CANDIA, A.F., EBNER, R., SHUM, L., LOPEZ, A.R., MOSES, H.L., WRIGHT, C.V.E. and DERYNCK, R. (1994). The murine type II TGF- β receptor has a coincident embryonic expression and binding preference for TGF- β 1. *Development* 120: 165-175.

- LOPEZ-CASILLAS, F., WRANA, J.L. and MASSAGUE', J. (1993). Betaglycan presents ligand to the TGF- β signaling receptor. *Cell* 73: 1435-1444.
- MASSAGUE', J. (1990). The transforming growth factor- β family. *Annu. Rev. Cell Biol.* 6: 597-641.
- MASSAGUE', J. (1996). TGF β signaling: receptors, transducers and Mad proteins. *Cell* 85: 947-950.
- MILLAN, F.A., DENHEZ, F., KONDAIAH, P. and AKHURST, R.J. (1991). Embryonic gene expression patterns of TGF beta-1, beta-2 and beta-3 suggest different developmental functions *in vivo*. *Development* 111: 131-144.
- PELTON, R.W., DICKENSON, M.E., MOSES, H.L. and HOGAN, B.L.M. (1990). *In situ* hybridization analysis of TGF- β 3 RNA expression during mouse development: comparative studies with TGF- β 1 and β 2. *Development* 110: 609-620.
- PELTON, R.W., SAXENA, B., JONES, M., MOSES, H.L. and GOLD, L.I. (1991). Immunohistochemical localization of TGF-beta 1, TGF-beta 2 and TGF-beta 3 in the mouse embryo-expression patterns suggest multiple roles during embryonic development. *J. Cell Biol.* 115: 1091-1105.
- ROBERTS, A.B. and SPORN, M.B. (1990). The transforming growth factor- β 's. In *Handbook of Experimental Pharmacology. Peptide Growth Factors and Their Receptors*, (ed. M.B. Sporn and A.B. Roberts) Heidelberg, FRG:Springer-Verlag, vol. 95/1, pp. 419-472.
- ROELEN, B.A.J., LIN, H.Y., KNEZEVIC', V., FREUND, E. and MUMMERY, C.L. (1994). Expression of TGF- β s and their receptors during implantation and organogenesis of the mouse embryo. *Dev. Biol.* 166: 716-728.
- RUIFROK, A.C.C., MASON, K.A., LOZANO, G. and THAMES, H.D. (1997). Spatial and temporal patterns of expression of epidermal growth factor, transforming growth factor alpha and transforming growth factor beta 1-3 and their receptors in mouse jejunum after radiation treatment. *Radiat. Res.* 147: 1-12.
- SCHMID, P., COX, D., BILBE, G., MAIER, R. and MCMASTER, G.K. (1991). Differential expression of TGF β 1, β 2 and β 3 genes during mouse embryogenesis. *Development* 111: 117-130.
- SCHMID, P., ITIN, P., CHERRY, G., BI, C. and COX, D.A. (1998). Enhanced expression of transforming growth factor- β type I and type II in wound granulation tissue and hypertrophic scar. *Am. J. Pathol.* 152: 485-493.
- SLOTKIN, T.A., WANG, X.F., SYMONDS, H.S. and SEIDLER, F.J. (1997). Expression of mRNAs coding for the transforming growth factor-beta receptors in brain regions of erythroid and hypothyroid neonatal rats and in adult brain. *Brain Res. Dev.* 99: 61-65.
- SUZUKI, A., SHIDOA, N., MAEDA, T., TADA, M. and UENO, N. (1994). A mouse TGF- β type I receptor that requires type II receptor for ligand binding. *Biochem. Biophys. Res. Commun.* 198: 1063-1069.
- TOMODA, T., KUDOH, T., NOMA, T., NAKAZAWA, A., MURAMATSU, M. and ARAI, K. (1994). Molecular cloning of a mouse counterpart for human TGF- β type I receptor. *Biochem. Biophys. Res. Commun.* 198: 1054-1062.
- TSUCHIDA, K., LEWIS, K.A., MATHEWS, L.S. and VALE, W.W. (1993). Molecular characterization of rat transforming growth factor- β type II receptor. *Biochem. Biophys. Res. Commun.* 191: 790-795.
- UNSICKER, K., MEIER, C., KRIEGLSTEIN, K., SARTOR, B.M. and FLANDERS, K.C. (1996). Expression of transforming growth factor-betas in embryonic chick spinal cord, hindbrain, and dorsal root ganglia. *J. Neurobiol.* 29: 262-276.
- WANG, Y.-Q., SIZELAND, A., WANG, X.-F. and SASSOON, D. (1995). Restricted expression of type-II TGF β receptor in murine embryonic development suggests a central role in tissue modeling and CNS patterning. *Mech. Dev.* 52: 275-289.
- WIESER, R., WRANA, J.L. and MASSAGUE', J. (1995). GS domain mutations that constitutively activate T β RI, the downstream signaling component in the TGF- β receptor complex. *EMBO J.* 14: 2199-2208.
- WRANA, J.L., ATTISANO, L., CARCAMO, J., ZENTELLA, A., DOODY, J., LAIHO, M., WANG, X.-F. and MASSAGUE', J. (1992). TGF- β signals through a heteromeric protein kinase receptor complex. *Cell* 71: 1003-1014.

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