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TGF-β type II receptor in rat renal vascular development: Localization to juxtaglomerular cells

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TGF-β type II receptor in rat renal vascular development: Localization to juxtaglomerular cells. To further define the role of transforming growth factor-beta (TGF-β) receptors in renal vascular development, detailed immunohistochemical studies of TGF-β receptor expression were performed from gestational day 15 through adulthood. On gestational day 15, TGF-β type II receptor immunoreactivity was restricted to perirenal stromal and vascular cells. On gestational day 17 TGF-β type II receptor immunoreactive stromal cells were observed within the kidney, with the same distribution as stromal α-smooth muscle actin and renin immunoreactive cells, and intense stromal TGF-β type II receptor immunoreactivity continued through postnatal day 5. As vascular development progressed, TGF-β type II receptor, α-smooth muscle actin and renin immunoreactivity became progressively restricted to small renal arteries and arterioles. Expression of TGF-β type II receptors and renin was very intense in afferent glomerular arterioles during postnatal days 5 to 15, and then became progressively restricted only to juxtaglomerular cells in the mature kidney. TGF-β type I receptor (ALK-5, ALK-1 and ALK-2) immunoreactivity was not detected in stromal or vascular elements during development or in the mature kidney. Intense TGF-β type II receptor expression in renal stromal vascular smooth muscle cell precursors and developing blood vessels suggests a role for the TGF-β type II receptors in the formation of the renal vascular smooth muscle compartment. The continued intense expression in juxtaglomerular cells argues for a role in renin synthesis and/or release. The absence of ALK-5, ALK-1, and ALK-2 in developing vascular smooth muscle and mature juxtaglomerular cells indicates that the canonical view of TGF-β signaling may not hold in these locations.

Vascularization of the developing kidney occurs through the processes of angiogenesis and *in situ* vasculogenesis [1–3]. Angiogenesis refers to invasion by vascular sprouts that originate from formed vessels outside the kidney and grow into it alongside the ureteric bud, and vasculogenesis refers to new vessel formation *in situ* from endothelial cell precursors. Except for some capillaries, the forming blood vessels become progressively invested with smooth muscle cells or pericytes. Renin producing cells of the afferent glomerular arteriole represent specialized vascular smooth muscle cells (VSMC), and glomerular mesangial cells are pericytes.

It was long held that angiogenesis is the exclusive mechanism of renal vascularization [4]. This view was based on the findings that glomerular epithelium, but not blood vessels, can develop from unvascularized metanephric blastema *in vitro* [5], and that transplantation of unvascularized metanephric blastema onto a host vascular bed results in the formation of blood vessels and glomerular capillaries derived from the host [6, 7]. However, Abrahamson and coworkers [3] recently showed that endothelial cell precursors are present in metanephric blastema before angiogenic sprouts are formed, and that they can develop into blood vessels in the absence of angiogenesis from without [3], proving that *in situ* vasculogenesis occurs during renal vascular development.

The mechanisms that stimulate organization of endothelial cell precursors into tubes are as yet poorly understood. The angiogenic mediators VEGF, angiopoietin-1, and angiopoietin-2 [1] and transforming growth factor-beta (TGF-β) mediated signals appear to be necessary. In this regard, we found that *in vitro* capillary formation by glomerular endothelial cells requires intact signaling via TGF-β type II receptors (TβR-II) [8]. Subsequently, others reported similarly defective capillary tube formation in the yolk sac in TGF-β1 knockout mice [9] and in mice deficient in TβR-II [10].

Many endothelial cell tubes become invested with VSMC or pericytes to form arteries, arterioles, veins and specialized structures like renal glomeruli. Signaling via platelet-derived growth factor (PDGF) β receptor is critical for the development of glomerular mesangial cells, as has been shown by the failure of mesangial cell development in PDGF-B [11] and in PDGF receptor β [12] knockout mice, but arterial VSMC development is normal in these mice. In VSMC, TGF-β induces either proliferation or inhibition of growth, depending on VSMC lineage and the abundance and subtype of TGF-β receptors [13]. Further, TGF-β signals modulate PDGF effects in blood vessels in that TGF-β1 up-regulates PDGF receptors in VSMC and mesangial cells [14, 15], and stimulates PDGF-B expression by endothelial cells [16]. These effects of TGF-β suggest that there may be a role for this mediator in VSMC development.

The TGF-β receptor superfamily consists of two broad classes of receptor serine threonine kinases, type I and type II. The type I receptors are referred to as activin like kinases (ALK). Distinct type II receptors for activin, bone morphogenic proteins and TGF-β bind their appropriate ligand leading to type II receptor-ALK dimerization with consequent transphosphorylation of the respective ALK [17]. Six ALK isoforms are known: ALK-5 interacts with TβR-II [18, 19], ALK-2 and ALK-4 interact predominantly with activin type II receptors [19], and ALK-3 and

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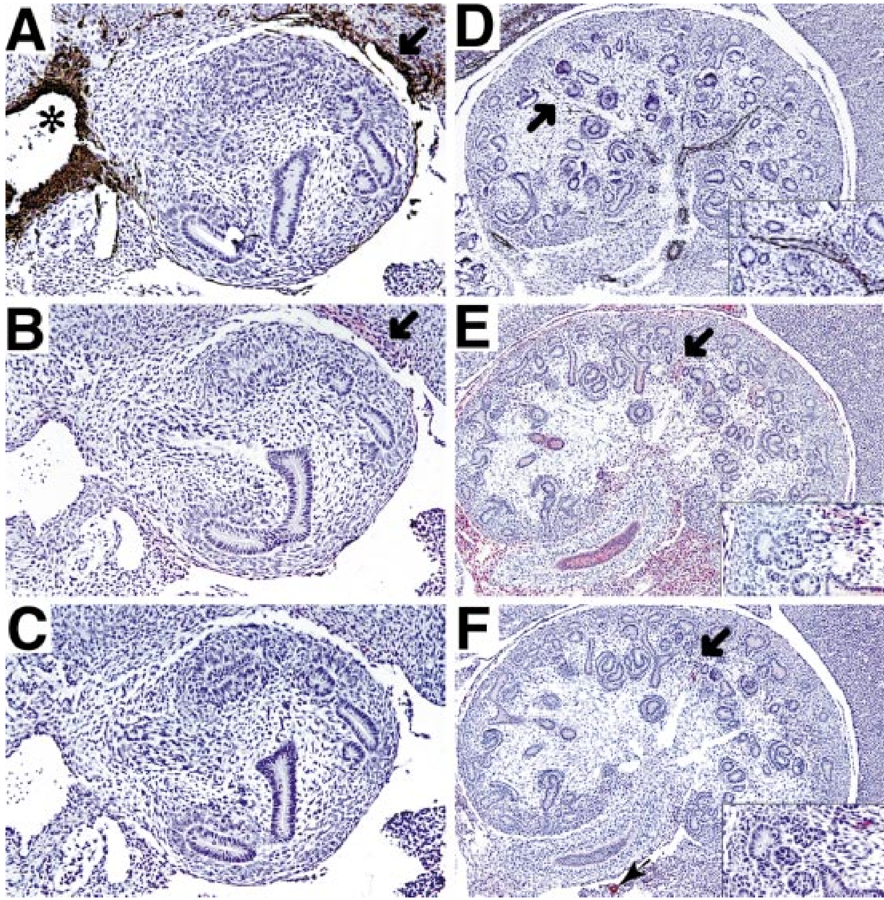


Fig. 1. Localization of α-smooth muscle (α-SM) actin, transforming growth factor-beta type II receptor (TβR-II) and renin proteins during renal development. Serial sections of rat fetal kidneys at gestational days 15 (E15; *A-C*) and 17 (E17; *D-F*) were stained with antibodies directed against α-SM actin (A, D), TβR-II (B, E) or renin (C, F). Magnifications: ×70 (A-C); ×35 (D-F); ×66 (insets in D-F). Strong α-SM actin immunoreactivity is present in aorta of E15 rats (*). Stromal staining (→) for α-SM actin, TβR-II and renin is observed in the perirenal mesenchyme at E15 (A-C), and within the kidneys at E17 (D-F). In panel F, gonadal artery staining for renin is indicated by (➜). The insets in panels E and F show co-localization of TβR-II and renin in stromal cells.

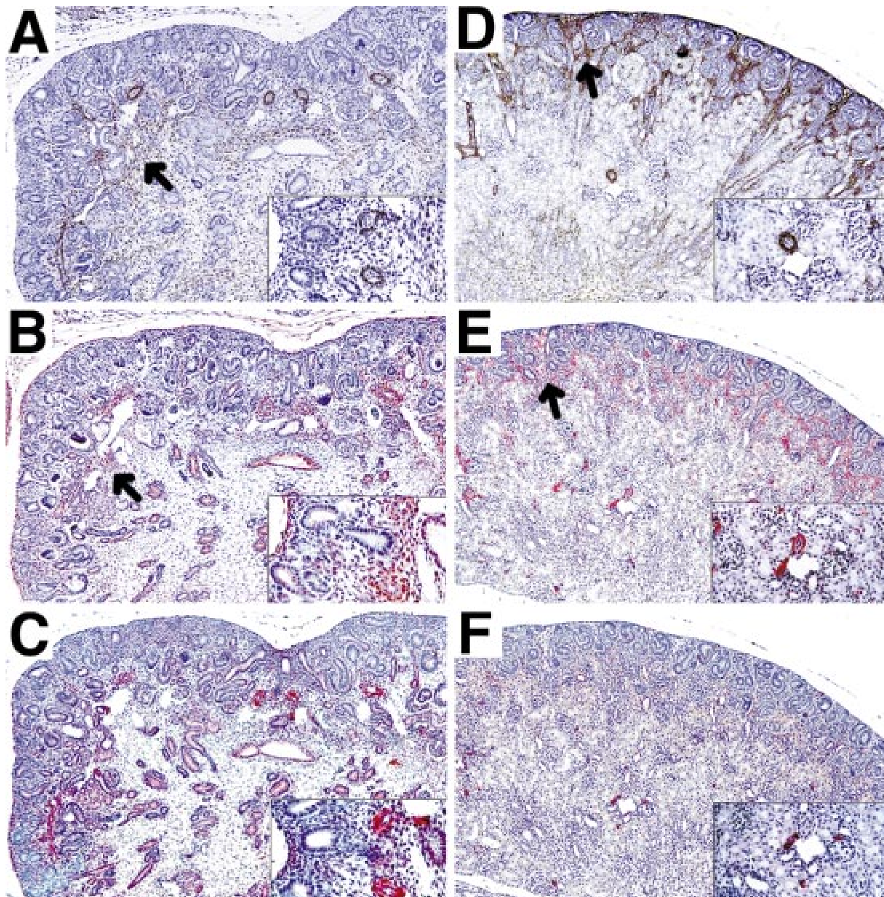


Fig. 2. Localization of α-smooth muscle (α-SM) actin, transforming growth factor-beta type II receptor (TβR-II) and renin proteins during renal development. Serial sections of rat fetal kidneys at gestational day 19 (E19; *A-C*) and postnatal day 5 (*D-F*) were stained with antibodies directed against α-SM actin (A, D) or TβR-II (B, E) or renin (C, F). Magnifications: ×35 (A-F); ×66 (insets in A-F). Alpha-SM actin, TβR-II and renin are observed in stromal cells and in renal vessels at E19 (A-C). On postnatal day 5 (D-F), α-SM actin and TβR-II are found in stromal cells and in renal blood vessels, while renin tends to be more confined to blood vessels (F).

ALK-6 are type I receptors for bone morphogenic protein [20]. We recently found much greater TβR-II and ALK-5, but not ALK-2 mRNA expression in neonatal than in mature kidney [21]. Both TβR-II and ALK-5 were heavily expressed in ureteric bud epithelial cells and in developing proximal tubule epithelium, while expression of these receptors was not detectable by immunohistochemistry in epithelial cells of the mature kidney. In the same study, intense stromal TβR-II, but not ALK-5 expression was found on neonatal day 1, though the identity of the stromal cells was not known. Since stromal cells were previously shown to express α-smooth muscle actin (α-SM actin) [22], the current study sought evidence for TβR-II expression in the renal VSMC during development. Our findings suggest that stromal TβR-II immunoreactive cells are VSMC precursors, some of which also express renin, and that they become associated with renal arterioles. As development of the renal vasculature progresses, TβR-II and renin display a remarkable level of co-localization. Strong expression of both proteins in afferent arterioles during early postnatal life later regresses, and both proteins continue to be expressed at high levels in juxtaglomerular (JG) cells of mature kidney.

METHODS

Tissue harvesting

Time-dated pregnant and adult Sprague-Dawley rats were obtained from Charles River (Wilmington, MA, USA). The day following overnight mating was defined as the first day of gestation [23]. On the day of study, rats were anesthetized with methoxyfluorane and exsanguinated. Renal tissue was taken from rats at gestational days 15, 17 and 19 (E15, E17 and E19, respectively), postnatal days 1, 5, 10, 15 and 30 (P1, P5, P10, P15, and P30, respectively) and from mature (M) rats. At each developmental stage kidneys from three to nine rats were studied.

For rat embryos, the kidneys were removed together with the other internal organs to facilitate identification. Only the kidneys were taken from newborn and adult rats. Kidneys were fixed immediately in 10% buffered formalin solution and embedded in paraffin. Six-micrometer-thick serial sections were processed for immunohistochemical analysis as described below.

Antibodies

Affinity-purified rabbit polyclonal antibody to human TβR-II receptor (L-21, catalog number sc-400; Santa Cruz, CA, USA), directed against amino acids 246-266 of the receptor was used at a dilution of 1:750. This antibody preparation is known to detect human, mouse, and rat TβR-II, and is not cross-reactive with known activin like kinases (ALKs; type I receptors for the TGF-β receptor superfamily that include activin and bone morphogenic protein receptors).

Affinity-purified rabbit polyclonal antibody to human ALK-5 (V-22, cat.# sc-398; Santa Cruz), directed against amino acids 158-179 of ALK-5 was used at a dilution of 1:1000. This antibody preparation detects human, rat and mouse ALK-5, and is not cross-reactive with type II TGF-β receptor or other ALKs. Affinity-purified rabbit polyclonal antibody, which recognizes ALK-1 and ALK-2 (T-19, cat # sc-402; Santa Cruz), directed against the carboxyterminus of ALK-1 was used at a dilution of 1:1500. This antibody preparation detects human, mouse and rat ALK-1 and ALK-2, and is not cross-reactive with other receptor serine-threonine kinases.

The highly specific rabbit polyclonal anti-rat renin antibody [24], a kind gift of Dr. T. Inagami (Vanderbilt University, Nashville, TN, USA), was used at a dilution of 1:2500.

A mouse monoclonal anti-human α-smooth muscle actin antibody (α-SM actin, clone No. 1A4; Sigma Chemical Co., St. Louis, MO, USA) was used at a dilution of 1:400. This antibody is directed against the amino terminal decapeptide sequence of α-SM actin and detects only the α-SM isoform of actin [10–13].

Immunohistochemistry

The detection methods were previously described [21]. Slides were deparaffinized with xylene followed by rehydration in descending series of ethanols. Endogenous peroxidases were quenched in 3% hydrogen peroxidase in methanol for 20 minutes. For detection of renin and TGF-β receptors, the sections were treated with 0.4% pepsin (Sigma) in 0.01 N HCl for 20 minutes at 37°C. For detection of α-SM actin, sections were microwave treated. They were then rinsed with 0.5% milk (Carnation nonfat dry milk) in Tris-buffered saline (TBS), blocked with 1% normal goat serum (NGS) in 0.5% milk/TBS for 30 minutes, and incubated overnight in a humidified chamber at 4°C with the respective primary antibodies. The sections were then rinsed in 0.5% milk/TBS followed by incubation with a 1:200 dilution of biotinylated secondary goat anti-rabbit IgG (Vector Laboratory, Burlingame, CA, USA) for polyclonal antibodies or with a 1:500 dilution of biotinylated secondary goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for monoclonal antibody for 30 minutes. Both primary and secondary antibodies were diluted in buffer containing 1% NGS in 0.5% ml/TBS. The sections were rinsed again in 0.5% milk/TBS, then incubated with the ExtrAvidin alkaline phosphatase (Sigma), diluted 1:800 in Tris buffer, pH 8.2 for 60 minutes and washed one final time in TBS, pH 8.2. The fast red alkaline phosphatase substrate was prepared fresh from fast red K salt (Sigma) according to the protocol described by Tubbs, Gephardt and Petras [25]. Unlike the fast red substrate tablets, the reaction product is not soluble in organic solvents, and produces an intense red stain. For some sections, the avidin-biotin-complex immunoperoxidase method (ABC kit was from Vector Laboratories) that uses 3,3′-diaminoanobenzidine as a substrate was used. After counterstaining with Mayer's modified hematoxylin (Polyscientific, Bay Shore, NY, USA), the sections were washed in water, dehydrated in an ascending ethanol series followed by xylene, and mounted with a coverslip using Permount (Fisher Scientific, Pittsburgh, PA, USA). The positive staining was red (or brown) on a blue background. As negative controls, the primary antibody was omitted or substituted with an irrelevant rabbit IgG. No staining occurred in the control sections.

For co-localization, serial sections were examined. Sections from three to five different stages of development (for instance, E15, P1, mature) were mounted on the same slide to control for variations in staining. Each slide was inspected for the presence, pattern of distribution, and intensity of antibody staining in the kidney. Staining intensity was graded semiquantitively from 0 to 4 (0 represents absence of staining and 4 indicates marked staining) by two independent investigators, and the scores were averaged.

RESULTS

Alpha-smooth muscle actin, TGF-β type II receptors and renin expression

Figures 1 and 2 show immunohistochemical staining for α-SM actin, TβR-II and renin in serial sections of kidney at different stages of development. Figure 3 shows representative areas under high-magnification. For each developmental stage results were similar in three to nine separate specimens.

At E15, the kidneys consisted of loosely organized blastemal cells and a few invading ureteric bud branches. At this stage of development (Fig. 1 A-C), perirenal stroma and aorta were stained intensely by α-SM actin antibodies. TβR-II immunoreactivity was observed in the same location, though TβR-II staining was much weaker than that of α-SM actin. Renin expression was also observed in some perirenal cells associated with the aorta. At E15, no α-SM actin, TβR-II or renin expression was observed within the developing kidney.

At E17 (Fig. 1 D-F), branching ureteric bud structures and several S-shaped bodies were seen in the mid-cortex. Blood vessels strongly immunoreactive with anti-α-SM actin antibody were observed alongside the developing ureter. In sections prepared from the kidneys of three separate rats, TβR-II antibodies strongly stained epithelial cells within the ureter, as well as perirenal stromal cells at this stage of development, and TβR-II immunoreactivity was present in loosely organized cells in the region of invading blood vessels, but did not appear to co-localize with α-SM actin in the blood vessels found adjacent to the ureter. At E17 α-SM actin immunoreactivity was also found in loose, lace-like stromal cells within the kidney parenchyma (Fig. 3A). TβR-II and renin immunoreactivity similarly were localized to such loosely organized stromal cells (Fig. 3 B,C). At E17, TβR-II and renin continued to be expressed in perirenal cells, and strong renin expression was consistently observed in the gonadal artery (Fig. 1F).

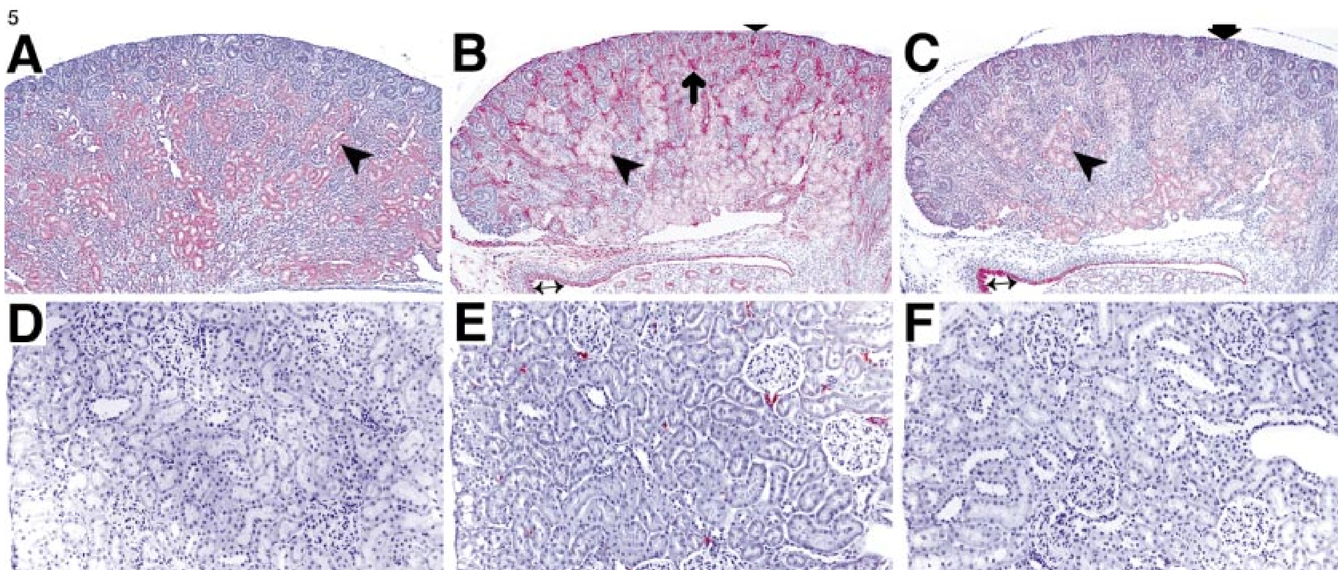
At E19 (Fig. 2 A-C), numerous S-shaped bodies and ureteric bud structures were observed in the outer cortical region of nephron morphogenesis, and some mature glomeruli were seen. Organized renal arterioles staining strongly with α-SM actin antibodies were observed just below the region of nephron morphogenesis. Such blood vessels also stained positively with TβR-II and renin antibodies. Stromal cells continued to express TβR-II, renin, and α-SM actin at this stage. S-shaped bodies were uniformly negative for α-SM actin, TβR-II and renin immunoreactivity. Glomeruli stained weakly with α-SM actin (not shown), but not with TβR-II or renin antibodies.

On postnatal day 5 (Fig. 2 D-F), α-SM actin, TβR-II and renin continued to be co-expressed in developing blood vessels, and in renal stromal cells. However, at this stage of development, renin expression tended to localize predominantly to blood vessels, while TβR-II and α-SM actin continued to be strongly expressed both in blood vessels and in the stroma. Again, S-shaped bodies and primitive as well as more mature glomeruli were not immunoreactive with TβR-II or renin antibodies; glomeruli showed weak immunoreactivity with α-SM actin antibodies (not shown). The pattern of TβR-II expression on postnatal day 1 was similar to that observed on postnatal day 5, except that stromal staining on postnatal day 1 was more intense (Fig. 5B) [21]. On postnatal day 10, α-SM actin, TβR-II and renin all were strongly expressed throughout afferent glomerular arterioles (Fig. 3 D-F), while TβR-II and renin expression in interlobular arteries and other large renal vessels was much less intense than on postnatal day 5. On postnatal day 15 (not shown), α-SM actin expression continued to be observed throughout the vasculature, TβR-II staining was still observed in afferent arterioles, albeit at a lower intensity than on postnatal day 5, and renin expression in afferent arterioles had diminished compared to that found on postnatal day 10. In kidneys from rats at postnatal day 30 (not shown), and in adult kidneys α-SM actin (not shown) expression continued to be observed in blood vessels throughout the kidney. By contrast, TβR-II and renin expression were prominent only in juxtaglomerular (JG) cells, where they co-localized (Fig. 3 G, H and 4E). In serial sections from five separate mature rats that were evaluated semiquantitatively (Fig. 4), TβR-II was observed almost without exception in JG cells of superficial and deep glomeruli if renin immunoreactivity was seen. A few intraglomerular cells immunoreactive with TβR-II antibodies were found near the vascular pole of many glomeruli (Fig. 3G).

Figure 4 summarizes the findings for intensity of α-SM actin, TβR-II and renin staining in renal stromal cells, interlobular artery, afferent glomerular arterioles and juxtaglomerular cells as a function of developmental stage. Alpha-smooth muscle actin was found in renal artery and arterioles from the earliest point of blood vessel ingrowth into the kidney, and continued to be expressed throughout development, marking blood vessels that had developed a smooth muscle cell layer. TβR-II and renin immunoreactivities were first observed to be associated with organized blood vessels at E19. Renin and TβR-II expression were most intense in afferent arterioles during days 1 to 10 of postnatal development. As development progressed, both renin and TβR-II expression in renal blood vessels became restricted to the afferent arterioles and then exclusively to JG cells. Renin expression became confined to JG cells slightly earlier than TβR-II expression.

TGF-β type I receptor expression

Since TβR-II forms a heteromeric signaling complex with type I TGF-β receptors (ALK-5, ALK-1 and ALK-2), it was of interest to further explore the potential co-expression of type I and type II TGF-β receptors. Detailed work on ALK-5 expression was previously published [21]. The ALK-5, ALK-1 and ALK-2 antibodies detected expression in proximal tubule and ureteric bud epithelial cells beginning at E17 through P10. In contrast, TβR-II immunoreactivity was greatest in the stromal cells and in the developing blood vessels. Neither ALK-5 nor ALK-1 or ALK-2 antibodies co-localized with TβR-II to stromal or vascular smooth muscle cells. Figure 5 A-C shows the distinct pattern of ALK-5, TβR-II, ALK-1 and ALK-2 immunoreactivity on postnatal day 1. As previously reported [21], ALK-5 immunoreactivity was strongest in proximal tubule epithelium, while TβR-II was found predominantly in the renal stroma, with weaker staining of proximal tubule cells. ALK-1 and ALK-2 similarly were found in proximal tubule epithelium, and in addition, strong co-localization of TβR-II, ALK-1 and ALK-2 staining was observed in the transitional epithelium of the calyceal system. Consistent with earlier studies examining mRNA expression for ALK-5, ALK-2 and TβR-II [21], adult kidneys (Fig. 5 D-I) and transitional epithelium of calyces and ureter (not shown) were uniformly negative for ALK-5, ALK-1 and ALK-2 immunoreactivity. In contrast, as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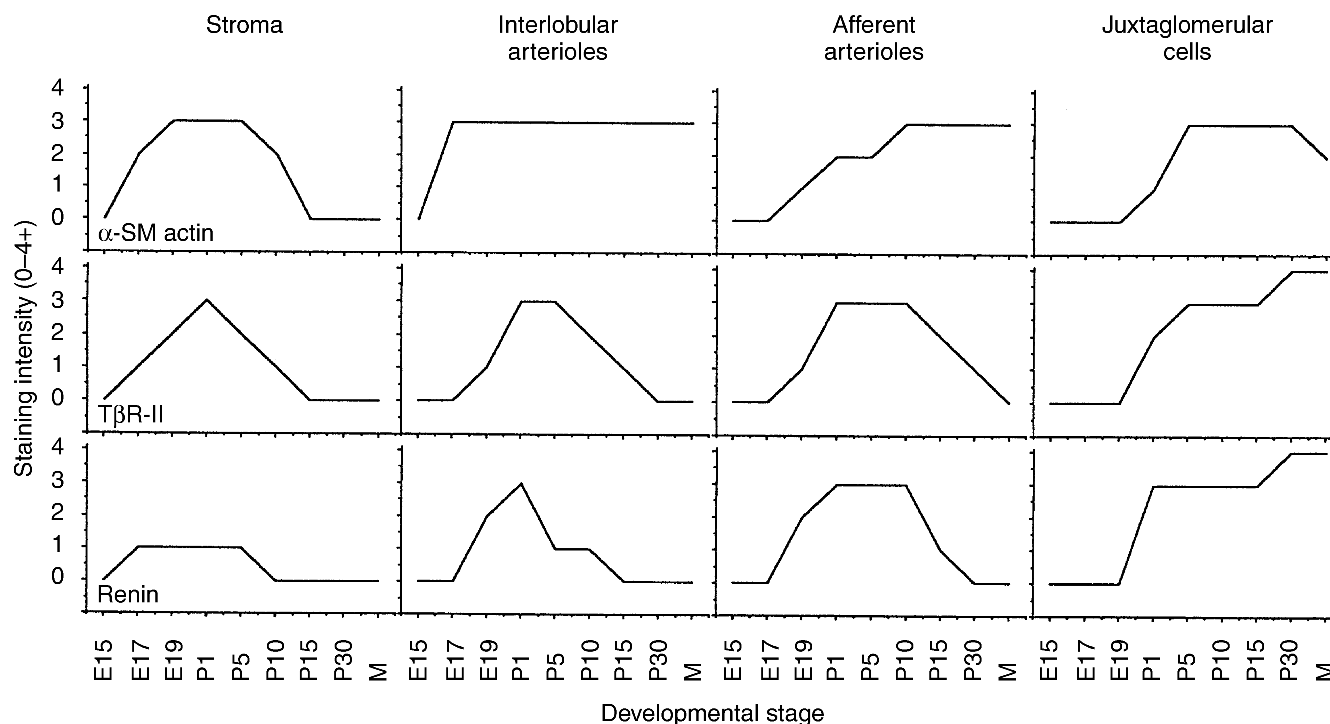

Fig. 4. Profile of staining intensity (0 to 4+) for α-smooth muscle (α-SM) actin, transforming growth factor-beta type II receptor (TβR-II), and renin in renal stroma, interlobular arteries, afferent arterioles and JG cells as a function of developmental stage. Kidneys from three to nine rats each were evaluated at gestational days 15, 17 and 19 (E15, E17, E19), on postnatal days 1, 5, 10 and 30 (P1, P5, P15, P30) and at maturity (M).

noted above, very strong TβR-II immunoreactivity was observed in JG cells of adult kidney (Fig. 5E and Fig. 2G).

DISCUSSION

The objective of this study was to explore the temporal and spatial expression pattern of TβR-II during rat renal vascular development, and to relate it to vascular α-SM actin, renin and TGF-β type I receptor expression. Beginning at E17, there was prominent TβR-II immunoreactivity within the kidney, in loosely organized stromal cells that also expressed α-SM actin. A few stromal cells also expressed renin. As development progressed, TβR-II, α-SM actin and renin immunoreactivity became progressively associated with the VSMC layer of developing blood vessels. Intense TβR-II and renin expression were observed in afferent arterioles on postnatal days 10 to 15. In the mature kidney, TβR-II was strongly expressed only in JG cells. At no time during development was ALK-5, ALK-1 or ALK-2 immunoreactivity detected in blood vessels or in renin expressing cells.

In interpreting the current findings, it is useful to first consider the value of immunohistochemical detection of TGF-β receptors. In cultured cells, type I and type II TGF-β receptors are ubiquitously expressed, and physical association between TGF-β type I and type II receptors is necessary for signal transduction [26]. Absence of functional TβR-II can lead to tumor formation in colonic cells [27] and some breast carcinoma cells [28], and absence of type I receptor abrogates TGF-β mediated signals [26]. Therefore, failure to observe TGF-β type I or II receptor immunoreactivity at some stages of development or in some cell types is more likely a reflection of low levels of receptor expression than a complete absence of receptors, though it is also possible that ubiquitous TGF-β receptor expression is an artifact of culture. Proof that TGF-β receptors are entirely absent from some cells

←

Fig. 3. Localization of α-smooth muscle (α-SM) actin, transforming growth factor-beta type II receptor (TβR-II), and renin immunoreactivity in developing and mature kidneys. Sections from gestational day 17 (E17; *A-C*), postnatal day 10 (*D-F*) and adult (*G, H*) rat kidneys were stained with α-SM actin (A, D), TβR-II (B, E, G), or renin (C, F, H) antibodies. Magnification: ×140 (A-C, E, F); ×70 (D); ×207 (G, H). Stromal staining (→) for α-SM actin, TβR-II and renin is observed within the kidneys at E17 (A-C). Afferent arteriolar immunoreactivity for α-SM actin, TβR-II and renin is observed on postnatal day 10 (D-F). Panels G and H show co-localization of TβR-II (G) and renin (H) immunoreactivity in JG cells of mature rats.

Fig. 5. Localization of activin like kinases (ALK)-5, transforming growth factor-beta type II receptor (TβR-II) and ALK-1/2 immunoreactivity in kidneys from postnatal day 1 and mature rats. Sections of kidneys from postnatal day 1 (*A-C*) and mature (*D-F*) rats were stained with ALK-5 (A, D), TβR-II (B, E), or ALK-1/2 (C, F). Magnifications: ×35 (A-C); ×140 (D-F). On postnatal day 1, ALK-5 was expressed predominantly in proximal tubules (▶) (A), some TβR-II and ALK-1/2 immunoreactivity were also seen in proximal tubule cells (▶). In the neonatal rat kidney, TβR-II staining was localized predominantly in the stroma (→); ALK-1/2 immunoreactivity was observed in and ureteric bud structures (⇒) and in calyceal transitional epithelium (↔). The latter also stained positive for TβR-II on postnatal day 1. In mature kidney, ALK-5 (D) and ALK-1/2 (F) immunoreactivity was not detected, and TβR-II expression was confined to JG cells (E).

would require demonstration that functional responses to TGF-βs are absent. Nevertheless, the extremely intense expression of TβR-II with a distinct temporal and spatial pattern in this study strongly suggests a functional role for this receptor in renal VSMC development and in JG cells, the nature of which remains to be resolved.

The distribution of α-SM actin expressing cells during renal development has previously been described in detail by Carey, Carey and Gomez [22], who found α-SM actin positive cells in the E15 rat only in the mesenchyma surrounding the kidney, not within. We similarly found α-SM actin expression in developing aorta and in perirenal stromal cells on gestational day 15. TβR-II immunoreactivity, though weaker than that of α-SM actin, was observed in the same locations in cells surrounding, but not within E15 kidney. In keeping with the absence of TβR-II in E15 kidneys, Wang et al [29] reported TβR-II mRNA expression in stromal cells surrounding the E15.5 kidney of mice, and Iseki et al [30] found no TβR-II mRNA in mouse kidney at E15. By gestational day 17, we found α-SM actin expression within the kidney in two distinct locations: in loosely organized, lace-like stromal cells (Fig. 3A), as described by Carey et al [22], and in easily discernible invading artery alongside the developing ureter (Fig. 1D). At E17, TβR-II and, to a lesser extent renin, were also found in the loose stromal cells (Fig. 3 B, C). Renin was not observed in the VSMC of invading renal artery, and TβR-II in this location was weak or absent. As development progressed, stromal TβR-II and α-SM actin immunoreactivity continued to be intense, peaking on postnatal day 1, and disappearing by postnatal day 15 (Fig. 4B and Fig. 2 D, E). The appearance of α-SM actin, TβR-II and renin immunoreactive cells first in perirenal stroma and two days later, at E17, in renal stromal cells suggests that stromal TβR-II positive cells migrate into the kidney from a perirenal location, though not as part of the VSMC layer of the invading blood vessel. Grafting of E15 or earlier kidneys into neonatal host kidneys expressing a marker such as β-galactosidase, as was done by Robert et al [3] in exploring endothelial cell precursors in the kidney, would be necessary to prove the extrarenal origin of the putative TβR-II positive stromal cells. The findings that VSMC of invading renal artery do not appear to express renin or TβR-II, whereas the stromal cells are positive, also suggest that there may be two distinct VSMC lineages that form renal blood vessels. In keeping with this hypothesis is the report by Topouzis and Majesky [13], who found two distinct VSMC lineages in chick embryos, an ectodermally-derived cell type found in large arteries, and a VSMC type derived from local mesoderm. It is of note that Topouzis and Majesky [13] observed that the ectodermally derived VSMC responded to TGF-β with proliferation, while growth of the mesodermally derived cells was inhibited, and that only immature, unglycosylated TβR-II was expressed by ectodermally derived VSMC while the functional, glycosylated TβR-II was expressed in the mesodermally derived cells. Our own findings of TβR-II positive/α-SM actin positive cells in renal stroma and TβR-II negative/α-SM actin positive cells in invading renal artery would be consistent with the view that the stromal cells represent a mesodermally-derived VSMC lineage distinct from the VSMC of the invading aorta. Alternatively, it is also possible that the VSMC of invading artery are already more fully differentiated and therefore express little or no TβR-II.

Beginning on gestational day 19, TβR-II and renin immunoreactivity were also associated with the VSMC layer of developing blood vessels located just below the zone of nephrogenesis (Fig. 1 G-I). Thereafter, arterial and arteriolar VSMC immunoreactivity for TβR-II and renin was intense, persisting in afferent arterioles through postnatal days 15 and 10, for TβR-II and renin, respectively. The progressive association of TβR-II immunoreactivity with the VSMC layer of renal arteries and arterioles is consistent with the idea that the stromal cells expressing TβR-II are VSMC precursors, which may be recruited into the appropriate location around endothelial cell tubes through as yet undefined signals. Regression of TβR-II immunoreactivity from the developing blood vessels so that expression in mature kidneys was confined only to JG cells, followed a centrifugal pattern similar to that previously described for renin [31], though disappearance of TβR-II from afferent arterioles occurred slightly later than renin. Coordinate expression of TβR-II and renin during renal vascular development and in mature JG cells implies that similar mechanisms regulate TβR-II and renin expression, or that one system positively regulates expression of the other.

Evidence that TGF-β signaling systems interact with the renin-angiotensin system has previously been reported. Horikoshi et al [32] first described markedly increased TGF-β2, but not TGF-β1 or TGF-β3 (ligands for TβR-II; Fig. 6), accumulation and co-localization with renin in JG cells and the VSMC layer of interlobular arteries in severely water-deprived mice, though TGF-β2 was expressed at very low levels in the renal vasculature of normal rats and mice. Similarly, water deprivation, dietary potassium depletion and administration of angiotensin converting enzyme inhibitors in rats are associated with JG cell hypertrophy, extension of renin expression to interlobular arteries and increased expression of TGF-β2, but not TGF-β1 or TGF-β3, in these locations [33]. Enhanced TGF-β2 expression has also been observed in infants with juxtaglomerular apparatus (JGA) hypertrophy due to renal artery stenosis and ischemic acute renal failure [34]. Though it was initially hypothesized that TGF-β2 production in the JG cells may be under the control of angiotensin II [32], the finding of enhanced TGF-β2 synthesis in the presence of an ACE inhibitor favors an angiotensin II-independent mechanisms for the regulation of TGF-β2 accumulation in JG cells [33]. Whether renin or angiotensin I might directly regulate TGF-β2 expression at these sites remains an interesting possibility.

Angiotensin II also influences the TGF-β system. Induction of TGF-β1 expression by angiotensin II in VSMC [35], mesangial cells [36] and renal epithelial cells [37] has been described, and vascular remodeling observed in cases of excess local angiotensin II may be mediated by TGF-β1 [38, 39]. However, such findings would not necessarily predict high levels of TβR-II expression in vascular compartments where angiotensin II is abundant, as TβR-II expression is markedly down-regulated by TGF-β1 [40]. Direct effects of angiotensin II on TβR-II expression have not been described so far. Nevertheless, pharmacologic inhibition of angiotensin converting enzyme in the immediate postnatal period in the rat leads to marked abnormalities in development of the vascular tree [41], and absence of angiotensin converting enzyme expression in knockout mice is also associated with abnormal vascular and JG cell development [42]. Thus, angiotensin II seems to be necessary for normal renal vascular development. The strong expression of TβR-II in developing renal VSMC and in mature JG cells found in this study, taken together with previous findings of angiotensin II-mediated TGF-β1 release and TGF-β1

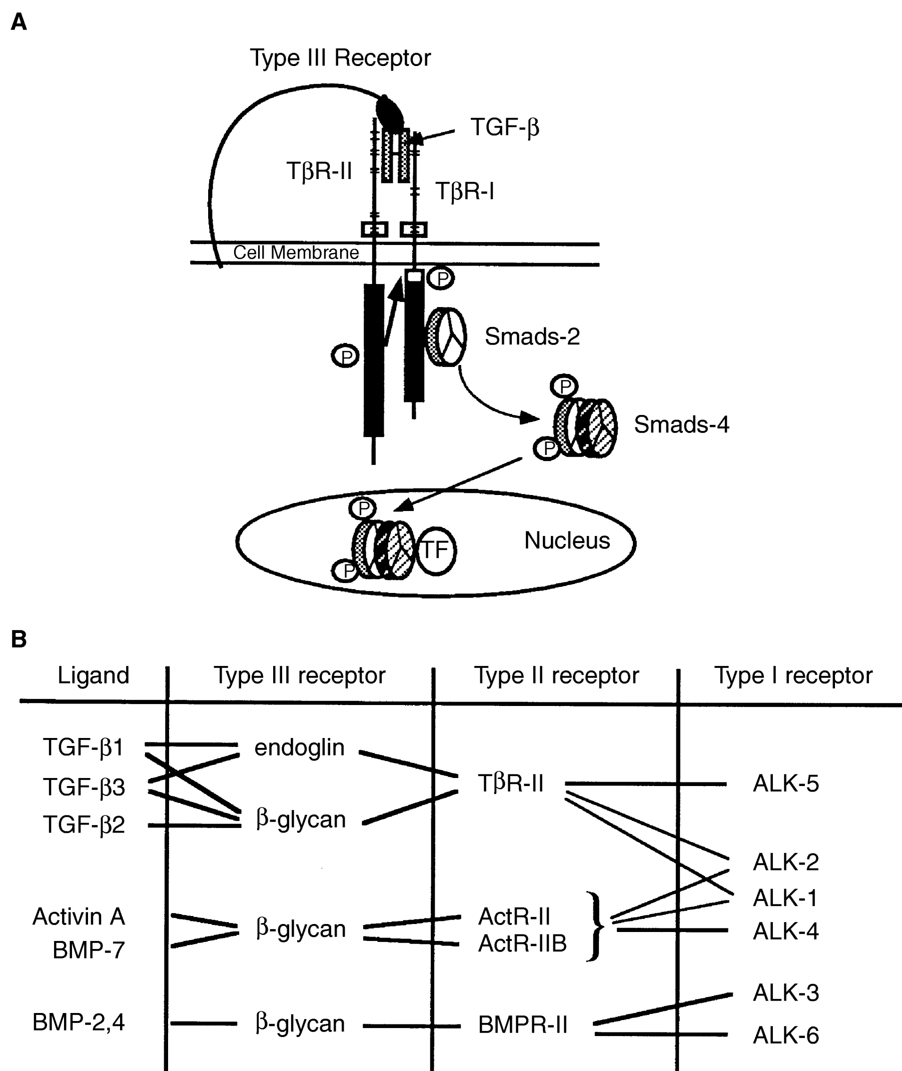

Fig. 6. Schematic representation of the transforming growth factor-beta (TGF-β) signaling cascade (*A*) and summary of ligand-receptor associations in the TGF-β receptor superfamily (*B*). (A) TGF-β ligands are homodimers that require accessory receptors (type III receptors) to bind type II receptor serine threonine kinase (TβR-II). The binding event results in recruitment of the type I receptor serine threonine kinase (TβR-I) into the complex. TβR-II kinase then phosphorylates and activates TβR-I (also known as activin like kinases, ALK). The Smad signaling molecules exist as trimers within the cell. Smad-2 trimers transiently associate with, and are phosphorylated by activated TβR-I kinase. Phosphorylated Smad-2 trimers then associate with Smad-4 trimers with consequent translocation into the nucleus and interaction with specific transcription factors (TF) [54]. (B) TGF-β, activin, and bone morphogenic proteins (BMP) belong to the same superfamily of ligands. Two distinct accessory type III receptors are known, namely β-glycan which is expressed by most cells, and endoglin, expressed by many endothelial cells. Expression of endoglin in the absence of β-glycan results in TGF-β2 unresponsiveness. Distinct type II receptors exist for the three families of ligands. Alk-5 is the predominant TβR-I; ALK-1, ALK-2 and ALK-4 interact predominantly with type II activin receptors, but can also interact with TβR-II.

effects in vascular remodeling, suggest that an interaction between angiotensin II and TGF-β signaling pathways operate in regulating the formation of the renal vasculature.

An interaction of the TGF-β system in JG cells and nitric oxide (NO) produced by macula densa cells also needs to be considered. In the kidney, neuronal NO synthase (nNOS) localizes exclusively to macula densa cells of the ascending limb of Henle and may operate to regulate renin secretion by JG cells [42–44]. TGF-β1 is known to stimulate expression of endothelial cell NOS, and strongly inhibits inducible NOS [45], though an effect of TGF-β on neuronal NOS has not been described. Further, NO may mediate some of the cellular actions of TGF-β, for instance *in vitro* capillary morphogenesis [46]. The close physical relationship between NO producing macula densa cells and TβR-II/TGF-β2 expressing JG cells raises the possibility that NO might also regulate TGF-β action in JG cells.

An interesting finding in this study is the failure of ALK-1 and ALK-2 or, as previously reported [21] ALK-5 (the predominant TGF-β type I receptor), immunoreactivity to co-localize with TβR-II in stromal, developing VSMC and JG cells. We previously noted strong co-localization of TβR-II and ALK-5 in the ureteric bud and proximal tubule epithelial cells of developing kidney [21]. Functional effects of TGF-β1 on tubule branching have previously been noted [47, 48] and were addressed in a previous study from this laboratory [21]. In the current study, developing calyceal transitional epithelial cells were strongly labeled with both TβR-II and ALK-1/2 (Fig. 5 B, C), suggesting an important role for TGF-β, acting via ALK-1 or ALK-2, on the development of transitional epithelium. The current understanding of TGF-β signaling is that it requires an association between TβR-II and a TGF-β receptor type I [49]. The receptors for activin and bone morphogenic proteins belong to the same superfamilies as the TGF-β type I and II receptors, and their signaling cascades similarly depend on dimerization between type I and type II receptors (Fig. 6). ALK-5 is the predominant type I receptor for TβR-II. In addition, ALK-1 can interact with TβR-II and activin type II receptor, while ALK-2 associates predominantly with activin type II receptors, though it too can bind and be activated by ligand-occupied TβR-II. If ALK-5, ALK-1, or ALK-2 serve as signaling partner for TβR-II in developing VSMC and JG cells,

strong co-expression of these receptors would be expected, as was observed in the epithelial cell locations. It is possible the TGF-β type I isoform receptor expressed in the stromal VSMC precursors, VSMC of developing renal vessels and mature JG cells has eluded detection because a different TGF-β type I receptor, for instance ALK-3, ALK-6, or ALK-4, even though they interact most avidly with bone morphogenic protein type II (ALK-3 and ALK-6) and activin type II (ALK-4) receptors. Another possibility is that an ALK-5 splicing variant, which fails to interact with the antibodies employed in this study, is expressed in the developing VSMC and in JG cells. Splicing variants of ALK-5 have been described [50, 51], but they do not involve the cytoplasmic region of ALK-5 against which the antibody used in the current study is directed. Another possibility, namely that the TβR-II antibody used in this study cross-reacts with activin or bone morphogenic protein type II receptors, and that type I receptors which interact predominantly with TβR-II would therefore not be found, is also not likely. Cross-reactivity of our antibody with activin and BMP receptors type II was ruled out by the manufacturer, and is furthermore unlikely given that there is $< 33\%$ sequence similarity between them and TβR-II in the region against which the antibody is directed. It is conceivable that the functional role of TβR-II in the putative stromal VSM precursors, the developing VSMC and in JG cells differs from that in cells where type I and type II receptors are expressed in similar abundance. This possibility is attractive because a similar dissociation between TβR-II and type I receptors has previously been observed in cardiac ventriculocytes in the postnatal period [52]. In that study, type I receptor mRNA was expressed at much lower levels than TβR-II. If TβR-II were expressed in vast excess of any type I receptor, it might bind ligand competitively without fruitful type I receptor interaction and thus act to inhibit TGF-β signaling. Indeed, excess expression and homodimer formation of TβR-II in cultured cells can inhibit TGF-β function through a dominant negative effect [53]. Hence, excess TβR-II by forming unproductive homodimers upon binding TGF-β could sequester the TGF-β ligand at the cell surface. The possibility that signals, perhaps triggered by TGF-β2 in renin producing cells, are transduced through TβR-II independently of any type I receptor also must be considered.

In summary, this study shows that putative stromal precursors of renal VSMC express TβR-II, and that TβR-II immunoreactive cells become associated with developing blood vessels in the kidney as a function of developmental stage. Coordinate expression and regression of TβR-II and renin in renal VSMC, and continued co-expression of TβR-II and renin in mature JG cells further suggest a functional relationship between TGF-β and angiotensin II in renal development and in differentiated JG cells. The failure to observe typical TGF-β type I receptors in these vascular locations further suggests that an as yet unknown type I TGF-β receptor or receptor isoform mediates signaling in these cells, or that TβR-II serves a distinct function at these sites.

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APPENDIX

Abbreviations used in this article are: ALK, activin like kinases; α-SM actin, α-smooth muscle actin; E number, gestational day; JG, juxtaglomerular; JGA, juxtaglomerular apparatus; M, mature; NGS, normal goat serum; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; P number, postnatal day; PDGF, platelet-derived growth factor; TβR-II, TGF-β type II receptors; TBS, Tris buffered saline; TGF-β, transforming growth factor-beta; VSMC, vascular smooth muscle cells.

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