Abstract. Lack of neonatal angiotensin II type 1 receptor (AT1) stimulation produces renal abnormalities characterized by papillary atrophy and impaired urinary concentrating ability, but the mechanisms involved are still unclear. DNA microarray was used to identify genes that are differentially expressed in renal medulla in response to neonatal treatment with AT1 receptor antagonist losartan (30 mg/kg per d), which commenced within 24 h after birth. The data showed that losartan treatment for 48 h downregulated 68 genes, ~30% of which encode various components of cytoskeleton and cytoskeleton-associated proteins, extracellular matrix, and enzymes involved in extracellular matrix maturation or turnover. With the use of immunohistochemistry and Western immunoblot, the microarray data were confirmed and it was demonstrated that losartan suppressed renal expression of syndecan 2, α-smooth muscle actin, MHC class II, and leukocyte type 12-lipoxygenase by day 4. In addition, losartan inhibited medullary expression of integrin α6 and caused relocalization of integrins α6 and α3. Moreover, losartan inhibited cell proliferation in medullary tubules by day 9, as detected by Ki-67 immunostaining. This study provides new data supporting the contention that a lack of AT1 receptor stimulation results in abnormal matrix assembly, disturbed cell–cell and cell–matrix interactions, and subsequent abnormal tubular maturation. Moreover, regulation of the expression of leukocyte type 12-lipoxygenase and α-smooth muscle actin by the renin-angiotensin system in the immature kidney adds new knowledge toward the understanding of renal vascular development.

Renal maldevelopment in premature infants and infants who are small for gestational age is known to be related to hypertension in adult life (1) and may be attributed to the suppressed intrarenal renin-angiotensin system (RAS) in neonates (2). In humans, nephrogenesis is completed before gestational week 36, whereas, in rodents, nephrogenesis is not completed until ~10 d after birth (3). Thus, rats and mice are born with immature kidneys, and the first 2 postnatal weeks correspond approximately to the second and third trimesters in humans. This difference provides a convenient animal model for studying mechanisms underlying the RAS-mediated kidney development in human fetus.

The importance of the RAS for normal kidney development has been demonstrated by a number of studies using gene targeting or pharmacologic interruption of the RAS (4). Thus, inhibition of angiotensin-converting enzyme activity or angiotensin II type 1 receptor (AT1) but not AT2 signaling in animals with an ongoing nephrogenesis induces kidney abnormalities (5). The most pronounced structural change is papillary atrophy, and it is associated with impaired urinary concentrating ability (5). Kidney vasculature is also affected, characterized by fewer, thicker, and shorter afferent arterioles (6).

Despite the well-recognized renal abnormalities after neonatal RAS inhibition, little is known about mechanisms by which a lack of AT1 stimulation leads to irreversible renal damage. Thus, the first aim of the present study was to use DNA microarray to identify genes involved in the RAS-mediated developmental process of renal medulla, a region where the predominant structural-functional defects are found in rats subjected to neonatal RAS inhibition. In developing renal medulla, the medullary rays and blood vessels have been shown to express AT1 receptors (7,8). We used the AT1 receptor antagonist losartan because we wanted to study expression of genes related specifically to the AT1 receptor. Our results showed that neonatal losartan treatment downregulated 68 genes and upregulated only nine genes in renal medulla.

The second aim was to validate the microarray data using immunohistochemistry and Western immunoblot. Because ~30% of the downregulated genes encode molecules involved in cell–matrix interactions, we further analyzed key molecules syndecan 2, integrin α3, and α6, which are important for cell–matrix interactions (9–11). Furthermore, three of the downregulated genes—α-smooth muscle actin (α-SMA),
MHC class II (MHC II), and leukocyte type 12-lipoxygenase (12-LO)—were examined further at the protein level. MHC II has been shown to be expressed by renal tubular epithelial cells during tubulogenesis (12). α-SMA has been shown to be expressed by developing kidney vasculature (13), whereas 12-LO metabolizes arachidonic acid to produce bioactive lipid 12(S)-hydroxyeicosatetraenoic acid, an important mediator of the effects of angiotensin II on vascular structure and function in adults (14–17). Given the tubular and vascular changes induced by neonatal RAS blockade (18,19), the identified molecules may provide new insight into the mechanisms involved in the RAS-mediated kidney development. Finally, we wanted to investigate whether neonatal AT₁ receptor blockade would affect renal growth, by determining proliferation and apoptosis.

Materials and Methods

Animals

Within 24 h after birth, Wistar rats (n = 5 per group) were treated intraperitoneally with losartan (2 × 15 mg/kg per d) or isotonic saline (2 × 10 ml/kg per d). Two days later, animals were anesthetized with pentobarbital (60 mg/kg) and kidneys were extirpated. This time point was chosen on the basis of our recent finding demonstrating renal structural abnormalities, evidenced as tubular dilation, already at such an early time after starting treatment with enalapril (20). Renal medulla was dissected from the cortex, snap-frozen in liquid nitrogen, and used for subsequent RNA preparation.

In a second series of experiments, rats (n = 8 per group) were treated with saline or losartan and kidneys were collected after 2, 4, or 9 d of treatment. One kidney was dissected into cortex and medulla, snap-frozen in liquid nitrogen, and used for Western immunoblot. The other kidney was divided into two pieces by crosscut, and one half was fixed in 4% formaldehyde and the other half was embedded in Tissue-Tek OCT compound and snap-frozen in liquid nitrogen. Paraffin sections (3 μm) were prepared for immunohistochemistry. For morphologic evaluation, paraffin sections were stained with hematoxylin and eosin. The rats were kept at the local animal department with free access to normal rat diet and tap water, controlled room temperature of 24°C, and 12-h dark/light cycle. The experiments were approved by the animal ethics committee in Gothenburg, Sweden.

RNA Preparation

Total RNA was prepared (pooled renal medulla from five pups) with a Qiagen RNeasy mini-kit according to the manufacturer’s instructions. The concentration of total RNA was determined by the optical density at 260 nm, and its purity was judged by the 260/280 ratio. The integrity of the RNA was examined on a 1% agarose gel containing 2.2 M formaldehyde.

Preparation of cRNA and Gene Chip Hybridization

Double-strand cDNA was generated from 8 μg of RNA using reverse transcription primer containing poly (dT) and a T7 RNA polymerase promoter sequence (Superscript Choice System, Life Technologies). Synthesis of biotin-labeled cRNA was carried out by in vitro transcription using ENZO BioArray HighYield RNA transcription labeling kit (Affymetrix). Triplicate cRNA were prepared from the RNA sample of saline-treated rats and duplicate cRNA from that of losartan-treated rats. These five cRNA preparations were purified, fragmented, and hybridized to five Affymetrix GeneChip arrays (RG_U34A) containing 8784 probe sets (77% known genes and 23% expressed sequence tags). The arrays were washed and stained with streptavidin-phycocerythrin conjugate and scanned by the Hewlett-Packard GeneArray Scanner. For enhancing the signals, the arrays were stained further with anti-streptavidin antibody for 30 min followed by a 15-min staining with a streptavidin-phycocerythrin conjugate.

Data Analysis

The scanned output files were inspected visually for hybridization artifacts before being analyzed by the GeneChip 3.1 software (Affymetrix). For computing comparisons of gene expression between two microarrays, the DNA microarrays were globally scaled to an average intensity of 500. Comparisons were made between the triplicate DNA microarrays used for analysis of the saline-treated groups and the duplicate DNA microarrays used for analysis of the losartan-treated group, generating a total of six comparison files. Genes whose expression differed in the losartan-treated compared with the saline-treated animals were identified by the “difference call” (Diff Call) algorithm developed by Affymetrix (GeneChip 3.1). With the Diff Call, the gene expression is classified as increased (I), marginally increased (MI), no change (NC), decreased (D), or marginally decreased (MD) using default values (Affymetrix) for the thresholds that define the boundaries among I, MI, NC, MD, and D. A Diff Call

Table 1. List of primary antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Dilution for Immunohistochemistry</th>
<th>Dilution for Western Immunoblot</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syndecan 2</td>
<td>1:100</td>
<td>1:2000</td>
<td>Santa Cruz Biototechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>Integrin α6</td>
<td>1:200</td>
<td>1:200</td>
<td>Santa Cruz Biototechnology</td>
</tr>
<tr>
<td>Integrin α3</td>
<td>1:20</td>
<td>ND</td>
<td>Developmental Studies Hybridoma Bank, Iowa</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>1:100</td>
<td>1:3000</td>
<td>Sigma, St. Louis, MO</td>
</tr>
<tr>
<td>MHC II</td>
<td>1:200</td>
<td>ND</td>
<td>Sera-lab Ltd., Crawley Down, UK</td>
</tr>
<tr>
<td>12-Lipoxygenase</td>
<td>ND</td>
<td>1:1000</td>
<td>Cayman Chemical, MI</td>
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<tr>
<td>Ki-67</td>
<td>1:20</td>
<td>ND</td>
<td>DAKO, Glostrup, Denmark</td>
</tr>
<tr>
<td>β-actin</td>
<td>1:100</td>
<td>1:5000</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

* The antibodies used for immunohistochemistry were diluted in PBS containing 1% BSA. The antibodies used for Western immunoblot were diluted in TBS-T containing 5% nonfat milk. ND, not detectable.

Polyclonal antibody.

Monoclonal antibody.
of D or MD was scored −2 or −1, respectively; a Diff Call of I or MI was scored 2 or 1, respectively. The general criteria used to select genes with increased expression in the losartan-treated compared with the saline-treated group from these six comparisons were as follows: a sum of Diff Call scores >8 (minimum four of the six comparisons classified as I), an average fold change >1.5, and a signal (Average Difference; Affymetrix) >50. Conversely, criteria for selection of genes with decreased expression were a sum of Diff Call scores <−8, an average fold change <−1.5, and a signal >50.

**Flow Cytometry for S-Phase Fraction Analysis**

Rats (n = 8 per group) were treated with saline or losartan for 4 d before they were killed and their kidneys were collected. The kidneys were treated with 3 mg/100 ml trypsin (Sigma, St. Louis, MO) in a citrate buffer for 10 min at room temperature. After filtration through a nylon mesh, the suspension was stained with propidium iodide. The cell suspensions were analyzed with a FACScan flow cytometer (Becton-Dickinson). For each sample, 15,000 cells were counted. S-phase fraction (SPF) was estimated by using a rectangular model. The number of S-phase cells was calculated by multiplying the number of channels between the G0/1 and G2/M peaks by the mean number of cells per channel in a part of the S-phase interval judged as representative by the operator. Small disturbing peaks in the S-phase region could be excluded when the SPF was calculated.

**Immunohistochemistry**

Immunostaining for syndecan 2, integrin α3, α-SMA, and MHC II was performed on cryosections, whereas immunostaining for integrin α6 was performed on paraffin sections. Sections were incubated with primary antibodies (Table 1), followed by a corresponding secondary antibody (1:100). Binding of peroxidase-labeled secondary antibody was detected by incubation with substrate 3-amino-9-ethyl-carbazole (Sigma), containing H2O2. Sections were counterstained with Mayer’s hematoxylin.

For detection of proliferation and apoptosis, paraffin sections were used. Proliferating cells were identified by incubating sections with anti–Ki-67 antibody, followed by a secondary antibody (1:100). Apoptosis was determined by *in situ* ligation of the biotinylated oligonucleotide hairpin probe (HPP) with one nucleotide overhang in the 3' end, followed by incubation with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA), according to the procedure described previously (21). Visualization was performed using diaminobenzidine solution (0.25% diaminobenzidine, 0.01% H2O2, and 0.04% NiCl) with methyl green counterstaining. For quantifying the

![Figure 1. Sequential development of the renal papilla in the control (A, C, and E) and losartan-treated rats (B, D, and F). Note that medullary tubular dilatation (arrow) is apparent at 2 d after initiation of losartan treatment (B). By day 4, cortical tubular dilatation (arrows) is observed (D). Papillary atrophy in losartan-treated rats is seen already at day 9 (F).](image)
number of proliferating or apoptotic cells, renal cortex and medulla was delineated according to Kriz and Bankir (22). Vision fields with a frame of 3070 μm² were systematically sampled with the use of a motorized stage. The number of Ki-67–positive or HPP-positive nuclei in the tubules or interstitium and of total nuclei within the frame was counted at 40× magnification. The data were expressed as a percentage of total nuclei. The number of total nuclei counted was 562 ± 82 per section.

Western Immunoblot

Tissues were homogenized in buffer that contained 250 mM sucrose, 10 mM HEPES-Tris (pH 6.95), and protease inhibitors (Complete Mini, Roche). Protein concentration was measured using a Bio-Rad Protein Assay kit. Aliquots of protein were solubilized in Laemmli sample buffer and separated by electrophoresis on 4–15% Tris-HCl gradient gel (Bio-Rad, Hercules, CA). Proteins were transferred to Hybond-P membrane (Amersham). After blocking in 5% nonfat milk with TBS-T [20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween 20], the membrane was incubated with a primary antibody (Table 1). The proteins were detected using peroxidase-linked secondary antibody and enhanced chemiluminescence detection system (Amersham) according to enhanced chemiluminescence Western blotting protocols. Nonspecific binding was evaluated by replacing the primary antibody with normal serum or IgG. Bands were visualized using a Fuji LAS-1000 cooled CCD camera/Dark Box, using the Image Reader LAS-1000 v1.1 software, and the density of the bands was analyzed using the Image Gauge software v3.45. As both microarray data and Western immunoblot data did not reveal any difference in the expression of β-actin mRNA and protein between saline-treated and losartan-treated groups (data not shown), β-actin was used as internal control and protein level was expressed as a ratio to β-actin.

Table 2. Downregulated genes encoding components of cytoskeleton and cytoskeleton-associated proteins, ECM, enzymes involved in ECM maturation or turnover, and MHC family and leukocyte type 12-lipoxygenasea

<table>
<thead>
<tr>
<th>Gene Bank Accession No.</th>
<th>Gene Description</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>M22323</td>
<td>g-enteric smooth muscle actin</td>
<td>15.9 ± 2.3</td>
</tr>
<tr>
<td>X16262</td>
<td>Smooth muscle myosin heavy chain</td>
<td>11.8 ± 1.8</td>
</tr>
<tr>
<td>X06801</td>
<td>Vascular α-actin</td>
<td>2.9 ± 1.2</td>
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<tr>
<td>AA799423</td>
<td>EST, highly similar to F-actin binding protein b-Nexilin</td>
<td>2.2 ± 0.3</td>
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<tr>
<td>S61948</td>
<td>Smooth muscle myosin heavy chain isoform SM1A</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>AF002281</td>
<td>α-actinin-2 associated LIM protein</td>
<td>4.5 ± 0.9</td>
</tr>
<tr>
<td>X05566</td>
<td>Myosin regulatory light chain</td>
<td>1.5 ± 0.4</td>
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<tr>
<td>AA894092</td>
<td>Similar to mice osteoblast specific factor 2</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>AI012030</td>
<td>Matrix GlA protein precursor</td>
<td>2.0 ± 0.1</td>
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<tr>
<td>M21354</td>
<td>Collagen type III α-1</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>J04035</td>
<td>Tropoelastin</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>M23697</td>
<td>Tissue-type plasminogen activator</td>
<td>1.9 ± 0.4</td>
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<tr>
<td>M27207</td>
<td>α-1 type I collagen</td>
<td>1.7 ± 0.2</td>
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<td>U75405</td>
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<tr>
<td>U75929</td>
<td>SPARC</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td>U09401</td>
<td>Tenascin</td>
<td>2.4 ± 1.0</td>
</tr>
<tr>
<td>S77494</td>
<td>Lysyl oxidase</td>
<td>1.6 ± 0.2</td>
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<tr>
<td>S66184</td>
<td>Lysyl oxidase</td>
<td>2.1 ± 0.7</td>
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<tr>
<td>AB010960</td>
<td>MIFR</td>
<td>1.6 ± 0.3</td>
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<td>X84039</td>
<td>Lumican</td>
<td>5.4 ± 0.7</td>
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<tr>
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<td>AI639233</td>
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<tr>
<td>X10344</td>
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<td>14.3 ± 3.0</td>
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<tr>
<td>AI235890</td>
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<td>4.1 ± 1.1</td>
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<tr>
<td>M15562</td>
<td>MHC class II RT1.u-D-α chain</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>S69383</td>
<td>Leukocyte-type 12-lipoxygenase</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

a Fold change was expressed as mean ± SD that was calculated from the six comparisons. ECM, extracellular matrix; EST, expressed sequence tag.
**Statistical Analyses**

All values are expressed as means ± SD. Statistical comparisons were made according to two-tailed t test for unpaired data. \( P < 0.05 \) was considered significant.

**Results**

**Morphology**

Medullary tubular dilation was apparent at 2 d after initiation of losartan treatment (Figure 1B). By day 4, cortical tubular dilation was found (Figure 1D). Papillary atrophy in losartan-treated rats was seen at day 9 (Figure 1F).

**DNA Microarray Data**

Neonatal losartan treatment downregulated 68 genes, ~30% of which encode various components of cytoskeleton and cytoskeleton-associated proteins, extracellular matrix (ECM), and enzymes involved in ECM maturation or turnover (Table 2).

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**Figure 2.** Expression and localization of syndecan 2 in the renal cortex and medulla. (A) A specific 90-kD band was recognized by anti–syndecan 2 antibody. Density analysis of the 90-kD bands shows a significantly decreased level of syndecan 2 in the cortex and medulla from the losartan-treated rats (\( n = 7 \) per group). \( **P < 0.01 \) and \( ***P < 0.001 \) versus saline. (B) Immunostaining of syndecan 2 is found in the tubular epithelial cells.
Renal Expression of Syndecan 2, Integrin α3, and Integrin α6

To investigate whether suppression of these genes would result in disturbed cell–matrix interaction, we determined renal expression of key molecules, syndecan 2, and integrins α6 and α3, which are important for cell–matrix interaction (9–11).

Syndecan 2 was expressed mainly by tubular structures (Figure 2B). Western immunoblot analysis revealed that the protein level of syndecan 2 was lower in both renal cortex and medulla from losartan-treated 4-d-old rats than those from controls (Figure 2A), thus confirming the microarray data (Table 2).

Losartan treatment for 4 d decreased the protein level of integrin α6 in the renal medulla but not in the cortex (Figure 3).
Figure 4. Expression and localization of integrin α3 in the renal cortex and medulla. Immunostaining of integrin α3 is found in the glomeruli and distal nephrons. The staining is localized to the basal membrane of tubular epithelial cells and is compact in the control rats (A and B). Losartan treatment for 4 d results in diffused integrin α3-staining (C and D).

Figure 5. Expression and localization of MHC class II (MHC II) protein in the renal cortex (A and C) and medulla (B and D). Kidney sections from 4-d-old rats treated with saline vehicle (A and B) or losartan (C and D) from birth until the day that the rats were killed. The tubular staining is evident in normally developing kidneys (A and B), whereas it is absent in the kidneys of rats subjected to losartan (C and D).
In the control rats, basolateral immunostaining of integrin α6 was observed in tubules (Figure 3B). Losartan altered this distribution pattern, and apical immunostaining of integrin α6 was observed in the dilated cortical tubules (Figure 3B).

Immunostaining of integrin α3 was found in the glomeruli and distal nephrons (Figure 4). In the tubular epithelial cells of the control rats, the staining was focused to the basal membrane (Figure 4, A and B). Losartan treatment for 4 d resulted in diffused integrin α3 staining (Figure 4, C and D).

Figure 6. Expression and localization of α-smooth muscle actin (α-SMA) in the renal cortex and medulla. (A) A specific 42-kD band was recognized by anti-α-SMA antibody. Density analysis of the 42-kD bands shows a significantly decreased level of α-actin in the medulla from the losartan-treated rats (n = 8 per group). *P < 0.05 versus saline. (B) Immunostaining of α-SMA is localized to the renal arterioles and peritubular capillaries.
Renal Expression of MHC II, α-SMA, and 12-LO

To validate further the microarray data, we determined the protein level of three of the downregulated genes revealed by microarray, namely MHC II, α-SMA, and 12-LO. Immunohistochemistry demonstrated MHC II staining in tubules of the medulla and cortex from normal kidneys on day 4 (Figure 5). Remarkably, the tubular staining of MHC II was absent in the renal medulla and cortex from losartan-treated rats (Figure 5).

Western immunoblot revealed decreased expression of α-SMA in the renal medulla from the losartan-treated rats on day 4 (Figure 6A). Immunostaining of α-SMA was localized to the renal arterioles and peritubular capillaries (Figure 6B). Using Western immunoblot, we found that losartan treatment for 4 d decreased the protein level of 12-LO in the renal medulla (Figure 7).

Proliferation and Apoptosis

To investigate whether neonatal AT1 receptor blockade would affect renal growth, we studied cell proliferation by determining SPF of renal cells with flow cytometry. Neonatal losartan treatment for 4 d did not change renal cell SPF (5.39 ± 1.06% versus 5.96 ± 1.5% in controls). Because this assay was performed on whole-kidney homogenates, we might have missed local changes caused by neonatal losartan treatment. To evaluate this, we used immunohistochemistry to study the spatiotemporal pattern of proliferation and apoptosis, with the help of anti–Ki-67 antibody and HPP, respectively. Quantification of the Ki-67–positive cells revealed that cell proliferation was not changed until day 9, when losartan-treated rats had a significantly lower number of proliferating cells in the medullary tubules but not in the interstitium, compared with the controls (Figure 8). In the cortex, the number of the Ki-67–positive cells was not changed by losartan. Moreover, neonatal losartan treatment did not change the number of apoptotic cells (data not shown).

Discussion

The present study demonstrates that neonatal losartan treatment for 2 d downregulates genes encoding various components of cytoskeleton and cytoskeleton-associated proteins, ECM, and different enzymes involved in ECM maturation or turnover. Moreover, neonatal losartan treatment suppresses renal expression of syndecan 2 and integrin α6 and causes relocalization of integrin α6 and integrin α3. Our data suggest that the RAS is important for normal establishment of cell–cell and cell–matrix interactions during kidney development. This is consistent with our recent findings that neonatal RAS blockade for 2 d disturbs medullary tubulogenesis and downregulates E-cadherin expression in dilated medullary tubules (20).

The ECM acts as a physical scaffold for the attachment and organization of cellular structures (23). The concomitant downregulation of different constituents of the physical scaffold, such as type I and type III collagens, decorin, and lumi can, as demonstrated in this study, points to possible changes in collagen fibril assembly. Indeed, interactions between the fibril-associated small proteoglycans and fibrillar collagen regulate collagen fibrillogenesis, which is one of the major events responsible for stabilization of tissue structure (24). Moreover, collagen fibrillogenesis could be affected further by both downregulated “matricellular” protein SPARC, which is highly regulated during development and has a collagen-binding capacity (23), and downregulated lysyl oxidase, which is a key regulator of collagen maturation (25). Taking into account that angiotensin II may regulate the expression of a variety of genes involved in ECM maintenance (26) and that cultured renomedullary interstitial cells respond to AT1 receptor stimulation with synthesis of ECM components (27), we suggest that interrupted AT1 signaling in developing kidney alters the ECM composition and structure, which are crucial to tissue integrity (28).

The information encoded in ECM is revealed by the interaction between individual ECM components and their respective receptors on the cell surface, such as integrins and syndecans (cell surface proteoglycans). The present study showed that neonatal blockade of AT1 receptors decreased renal expression of syndecan 2 and integrin α6 and caused relocalization of integrins α6 and α3. Interestingly, it has been shown that function-blocking antibody against integrin α6 blocks kidney tubulogenesis in vitro (9), whereas kidneys of integrin α3–deficient newborn mice have fewer collecting ducts within the inner medulla (10). Moreover, relocalization of integrins from the basolateral membrane to the apical membrane has been described in cultured renal tubular epithelial cells subjected to oxidative stress, which results in weakened epithelial cell attachment to ECM (29). Syndecan 2 is normally present on all adherent cells and participates in ECM assembly, regulating the signaling of heparan sulfate binding growth factors.
It is developmentally regulated and peaks during tissue morphogenesis (30). Hence, it is possible that the downregulation of syndecan 2 and integrin α6 and α3 could result in impaired cell–ECM interactions and subsequent ECM-mediated cytoskeletal organization, which are important for several cell functions, such as cell proliferation, migration, differentiation, and survival (23). This line of reasoning is supported by our finding of concomitant downregulation of various components of the cytoskeleton (Table 2).

Collectively, neonatal RAS inhibition caused a gene downregulation of components of cytoskeleton and ECM in developing kidney. This may lead to inadequate matrix assembly and disturbed cell–cell and cell–matrix interactions (Figure 9), which are known to be critical for kidney development. These data provide mechanistic support to the notion that an intact RAS is essential for the structural arrangement of the renal medulla.

In contrast to the very early changes in the tubular structure, i.e., tubular dilation on day 2 (20), the present study demon-

**Figure 8.** Effect of neonatal AT$_1$ receptor blockade on renal cell proliferation. (A) Quantification of the Ki-67–positive nuclei in the renal medulla and cortex from 4- and 9-d-old rats. Losartan treatment for 9 d decreases the number of proliferating cells in the medullary tubules ($n = 5–8$ per group). *$P < 0.05$ versus saline. (B) Immunostaining of Ki-67 in the renal medulla from 9-d-old rats treated with saline vehicle or losartan from birth until the day that the rats were killed. Immunoreactive Ki-67 is present mainly in the tubular epithelial cells.
strated that cell proliferation was not affected until day 9, when neonatal losartan treatment inhibited cell proliferation in the renal medulla. It is possible that the inhibited growth is secondary to disrupted matrix assembly and/or disturbed cell–cell and cell–matrix interactions (vide supra). It is interesting that the inhibited proliferation was confined to the tubular epithelial cells, whereas proliferation in the interstitium was not affected. In accordance with our observation, McCausland et al. (18) reported that enalapril treatment from postnatal day 3 to 10 reduces cell proliferation in the medullary rays. Thus, neonatal AT1 receptor blockade impairs tubular growth and maturation, and this is in line with the fact that neonatal RAS blockade causes abnormalities in tubular structure and function (5,31). Because the changes in proliferation are modest and cannot by themselves entirely account for the hypoplastic papillary phenotype, one may speculate that neonatal RAS blockade increases cell death. However, our data showed that neonatal losartan treatment for 4 and 9 d had no effect on apoptosis. It is interesting that we have recently shown that neonatal enalapril treatment for 9 d induces inflammatory changes in the kidney (20), which could be due to increased necrosis. Necrosis, however, was not studied in the present study because of the lack of a proper detection technique.

Keeping in mind the perturbed tubulogenesis in neonatally RAS-inhibited rats, the observed downregulation of MHC II is of interest because MHC II was shown previously to be expressed mainly by tubular epithelial cells of immature kidney (12). The function of MHC II, expressed by developing tubules, is unclear. Besides its classical antigen-presenting function, MHC II has been shown to play a role in cell-to-cell recognition and tissue organization (32). Because MHC II expression correlates highly with cellular differentiation (33), we propose that interrupted AT1 signaling could hamper the postnatal differentiation of tubular epithelial cells, as evidenced by abolished expression of MHC II in these cells, and, therefore, disturb tubulogenesis.

Another finding of the present study is that 12-LO is expressed by immature kidney and the renal expression of 12-LO is regulated by the RAS. 12-LO metabolizes arachidonic acid to produce bioactive lipid 12(S)-hydroxyeicosatetraenoic acid (HETE). It is interesting that 12(S)-HETE is an important mediator of the effects of angiotensin II on vascular structure and function in adults (14,34). Moreover, 12(S)-HETE seems to be critically involved in angiogenesis (35), a process that is needed for kidney vascularization (7). It is tempting to speculate that decreased expression of such a vasoactive substance during kidney development, as demonstrated by both microarray and Western immunoblot, may disturb renal vascular development. This notion is supported by our observation of the decreased renal expression of α-SMA after neonatal RAS inhibition because immunohistochemistry revealed α-SMA expression in the renal vasculature of the immature kidney. Indeed, neonatal RAS inhibition causes irreversible abnormalities in the renal arterial vasculature characterized by wall thickening and reduced branching (6,36).

**Conclusion**

This study provides new data supporting the contention that a lack of AT1 receptor stimulation results in abnormal matrix assembly, disturbed cell–cell and cell–matrix interactions, and subsequent abnormal tubular maturation as evidenced by decreased proliferation and loss of MHC II expression by epithelial cells. Regulation of the expression of 12-LO and α-SMA by the RAS in immature kidney, however, adds new knowledge toward our understanding of renal vascular development.

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**Figure 9.** Illustration of cell–cell and cell–matrix contacts: actomyosin bundles, cytoskeleton-associated proteins, cell surface proteoglycan, extracellular matrix (ECM), and enzymes for ECM maturation/tturnover. Listed molecules are those with decreased mRNA expression caused by neonatal losartan treatment.
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