Identity of the renin cell is mediated by cAMP and chromatin remodeling: an in vitro model for studying cell recruitment and plasticity

Ellen Steward Pentz, Maria Luisa S. Sequeira Lopez, Magali Cordaillat, and R. Ariel Gomez

Department of Pediatrics, University of Virginia School of Medicine, Charlottesville, Virginia

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Pentz ES, Sequeira Lopez ML, Cordaillat M, Gomez RA. Identity of the renin cell is mediated by cAMP and chromatin remodeling: an in vitro model for studying cell recruitment and plasticity. Am J Physiol Heart Circ Physiol 294: H699–H707, 2008. First published November 30, 2007; doi:10.1152/ajpheart.01152.2007.—The renin-angiotensin system (RAS) regulates blood pressure and fluid-electrolyte homeostasis. A key step in the RAS cascade is the regulation of renin synthesis and release by the kidney. We and others have shown that a major mechanism to control renin availability is the regulation of the number of cells capable of making renin. The kidney possesses a pool of cells, mainly in its vasculature but also in the glomeruli, capable of switching from smooth muscle to endocrine renin-producing cells when homeostasis is threatened. The molecular mechanisms governing the ability of these cells to turn the renin phenotype on and off have been very difficult to study in vivo. We, therefore, developed an in vitro model in which cells of the renin lineage are labeled with cyan fluorescent protein and cells actively making renin mRNA are labeled with yellow fluorescent protein. The model allowed us to determine that it is possible to culture cells of the renin lineage for numerous passages and that the memory to express the renin gene is maintained in culture and can be reenacted by cAMP and chromatin remodeling (histone H4 acetylation) at the cAMP-responsive element in the renin gene.

RENIN IS THE KEY physiologically regulated hormone enzyme of the cascade that culminates in the generation of angiotensin II (ANG II), a potent vasoconstrictor that regulates blood pressure and fluid-electrolyte homeostasis. The key regulated event in the renin-angiotensin system (RAS) is the synthesis and release of renin by cells in the kidney capable of producing the hormone. In the normal adult mammalian kidney, renin is synthesized and released by juxtaglomerular (JG) cells, a small group of myoepithelioid, granulated cells located in the wall of the afferent arteriole at the entrance to the glomerulus (19, 46). During early embryonic development, renin precursor cells are present in the undifferentiated metanephric blastema before vascularization of the kidney has occurred and before the hemodynamic functions of renin are needed (41). Later in fetal life, renin-expressing cells are found along large intrarenal arteries (16, 19). As maturation continues, the number of renin-containing cells diminishes, becoming progressively restricted to a few cells in the classical JG localization found in the adult (19). If an adult animal is subjected to manipulations known to increase renin synthesis (such as when fluid electrolyte and blood pressure homeostasis are threatened), there is an increase in the number of renin-expressing cells (along the preglomerular arteries, inside the glomerulus, and in the kidney interstitium resembling the embryonic pattern) (3, 14, 17, 20). This increase in the number of renin cells (recruitment) is due to the dedifferentiation of preexisting adult cells (40) and does not involve migration or the replication of cells (3).

We showed that the cells that retained the plasticity to develop phenotypic characteristics of renin cells are the same ones that expressed renin earlier in development (40). To demonstrate this issue, we generated a mouse that expresses cre recombinase under control of the renin locus. Renin cells and all their descendants are labeled in the progeny resulting from the mating of this Ren-cre mouse with a reporter mouse (40). Our studies in these mice show that renin cells are in fact precursor cells that give rise to a number of cell types in the kidney, including a subset of renal arteriolar (RA) smooth muscle (SM) cells (RA-SMCs), mesangial cells, and interstitial cells, and that it is these cells that can reexpress renin in the adult when homeostasis is threatened (40). Thus the ability of adult kidney cells to reacquire the renin cell phenotype is determined but at the same time constrained by the developmental history of the cell (40).

The reacquisition of the renin cell phenotype may in part be regulated by cAMP. It has been shown that cAMP plays an important role at several control points for renin: release, mRNA synthesis, and mRNA stability. The constant physiological demands on renin secretion in response to changes in posture, renal perfusion pressure, sodium balance, and other factors are met by a rapid regulation of renin release (25). The intracellular production of cAMP enhances renin secretion, and cAMP is a critical second messenger that determines renin secretory rate. There seems to exist a common pathway of stimulating renin secretion via cAMP. cAMP is responsible for mediating the effects for a number of stimuli (β-adrenoreceptor agonists, low calcium) that affect renin secretion (2, 33, 37, 38). These constant demands on renin secretion require the stimulation of renin gene expression to replace the renin stores. cAMP has also been shown to play a role in regulating the expression of the renin gene to augment renin mRNA levels (11). Kidney microvessels and isolated single renal microvascular cells respond to forskolin administration by increasing the number of renin-secreting and renin-expressing cells without apparent changes in the amount of renin secreted by individual cells (11). The increase in renin release is due to the recruitment of microvascular cells secreting and synthesizing renin (11). These results in vitro corroborate many reports in whole animals demonstrating that in response to physiological stress, increased circulating renin is achieved.

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by increasing the number of cells expressing renin (3, 14, 17, 26, 48, 49).

Many questions concerning the molecular mechanisms regulating renin expression would be more readily addressed using in vitro cell culture. However, culturing and maintaining renin cells have been a major impediment to advances in the field of renin physiology. Most investigators use acute preparations of JG cells with variable degrees of purity. Enriched populations of renin-expressing cells can be isolated, but after only a few days in culture, these cells usually stop making renin and differentiate into other cell types or die (6, 8, 24). JG cells propagated on Matrigel were reported to retain the ability to express renin for numerous passages (39); however, no subsequent use of this culture method has been reported. Valuable information has been obtained using the cell line As4.1, which is derived from a kidney tumor in a mouse having simian virus (SV)-40 large T antigen expression driven by the Ren2 promoter (42). Because As4.1 cells are driven to express renin on a constitutive basis, they cannot be used to study renin cell recruitment.

We have, therefore, taken a different approach for obtaining a model cell system to study the events involved in the reexpression of renin and the reacquisition of renin cell identity. We first generated a mouse in which cells of the renin cell lineage that previously expressed renin are marked with cyan fluorescent protein (CFP) and cells currently expressing renin are marked with yellow fluorescent protein (YFP). Subsequently, we isolated RA-SMCs from the mice and cultured those dualially labeled cells. We report here that these RA-SMCs of the renin cell lineage can be maintained in culture for multiple passages, and the cells retain the memory to reexpress the renin gene in response to cAMP, an event that is mediated by chromatin remodeling at the cAMP-responsive element (CRE) of the renin gene. This model system, in turn, could be useful to understand the intracellular events that modulate renin gene expression in particular and the regulation of cell plasticity in general.

METHODS

Animals

Housing and experimental use of the mice in the reported studies conformed to the guiding principles in the care and use of animals approved by the Council of the American Physiological Society and with federal laws and regulations. All protocols have been reviewed and approved by the Animal Care and Use Committee of the University of Virginia.

Generation of Experimental Animals

Labeling renin-expressing cells: Ren1\(^c\)-YFP mice. To label renin-expressing cells, the coding sequence for YFP (EYFP; Clontech, Mountain View, CA) was cloned into the translation initiation site of the Ren1\(^c\) promoter. This promoter region contained 4.9 kb of DNA upstream of Ren1\(^c\) (a gift from Dr. H.-S. Kim, University of North Carolina). Two bases were altered adjacent to the ATG of renin (mouse A\(\text{Gatg}\) and PCR product C\(\text{Catg}\)) to introduce an Ncol restriction site to facilitate cloning. The 5' DNA was cloned into the Ncol site (blunted with Klenow polymerase) at the ATG of YFP in the plasmid pEYFPN1 (Clontech). This YFP sequence also includes a SV40 poly-A addition signal. The DNA sequence of the PCR product and the sequence across the junction with YFP were confirmed by sequencing. Transgenic mice were generated using the Ren1\(^c\)-YFP DNA. Eighteen founders were obtained, of which 16 transmitted the Ren1\(^c\)-YFP transgene. Genotyping of the mice was by PCR of DNA from tail biopsies amplified with primers in the renin promoter (\(5'\text{-TTTATCAGAGCTGCCCTGCCATG}\)) and in YFP (\(5'\text{-CGTACA-GCGAAGGTGTTGCAC}\)).

Labeling cells of the renin lineage with CFP. To label cells of the renin lineage, we bred our Ren1\(^c\)-cre animals (40) with mice carrying the ROSAloxP(CFP) reporter (43). In the resulting animals, cells of the renin lineage permanently express CFP, even if they cease expressing renin. In these animals, CFP labels the cells that previously expressed renin exactly where renin is normally expressed during ontogeny, marking the developmental history of the renin cell.

Simultaneous labeling of renin-expressing cells and cells of the renin lineage. To label the cells of the renin lineage and at the same time label cells currently expressing renin, we generated mice carrying Ren1\(^d\)-cre, a ROSAloxP(CFP) reporter (43) and Ren1\(^c\)-YFP (Fig. 1). As reported in RESULTS, these mice have bona fide expression of CFP where renin was expressed during ontogeny and bona fide expression of YFP in the sites where renin is actively being expressed.

Stimulation of the RAS in Adult Ren1\(^c\)-YFP Mice

To determine whether YFP expression in the Ren1\(^c\)-YFP mice exhibited the appropriate renin reexpression (recruitment) response, adult animals were treated for 12 days with the angiotensin-converting enzyme (ACE) inhibitor captopril (0.5 g/l) in drinking water. At the end of the treatment period, kidneys and other organs were collected for assessment of YFP expression.

Histological Analysis

Animals were anesthetized with tribromoethanol (300 mg/kg). Either the kidneys were removed directly after anesthesia or the animal was perfused through the left cardiac ventricle with cold 4% paraformaldehyde (PFA) in PBS (pH 7.2–7.4). The kidneys were removed and fixed overnight in 4% PFA in PBS (pH 7.2–7.4). Vibratome sections (30–100 \(\mu\)m) were cut or the tissue was cryopro-
ected in 30% sucrose in PBS at 4°C for 24–72 h and then frozen in NEG-50 (Richard-Allan Scientific, Kalamazoo, MI) and stored at −80°C, as previously described (17). Cryosections (10 μm) were cut with a Leica Crycut 1800 cryostat, postfixed in 4% PFA in PBS, and examined microscopically for YFP fluorescence using a Leica DMIRE2 microscope equipped with a specific YFP filter. Photographs were taken with a Retiga Exi camera. Staining for LacZ and renin in the RenIα-cre mice was performed as previously reported (40).

Culturing the Renin Cell

Isolation and culture of preglomerular arteriolar SMCs of the renin lineage was done using a modification of our protocol for microvessel isolation (11). Cells were isolated from mice described in Generation of Experimental Animals in which the renin cell lineage had been marked by CFP and renin expression was marked by YFP. Mice were anesthetized and the abdominal and thoracic cavities were opened to expose the kidneys and the heart. Each mouse was perfused through the left ventricle with a 2% solution of iron oxide in medium (DMEM-F12 + 10% FBS and penicillin, streptomycin, and amphotericin B). The kidneys were excised, decapsulated, and cut longitudinally, and the outer cortex was removed into cold Dulbecco’s PBS plus antibiotic/antimycotic (PBS−). The kidneys were minced with a scalpel and then digested with 0.25% collagenase IV in PBS− at 37°C with agitation for 10 to 20 min until no tissue chunks remained. The mixture was centrifuged to pellet the tissue, washed with medium, and pelleted again. The tissue was resuspended in medium, and the vessels were collected using a strong magnet. This procedure was repeated for four to eight times until contaminating tubules were eliminated. The vessels were cultured undisturbed for at least 7 days in a low volume of medium to encourage them to attach and allow SMCs to grow out. The initial culture was maintained until confluent and then subjected to differential plating (10) by allowing the trypsinized cells to attach to 10 to 15 min and then transferring the unattached cells to a fresh flask three times. Cells were grown to confluence, and the cultures containing the highest proportion of SMCs [as judged by CFP expression and typical morphology (spindle shape with an oval nucleus containing two or more nucleoli)] were differentially plated again. Differential plating was repeated for two to three passages, and the cultures having the highest proportion of SMCs, as judged by CFP expression, typical morphology, and PCR or staining for smooth muscle markers, were used for the studies. To enrich for cells that could respond to stimuli and reexpress renin (become YFP+), the cultures were treated with 10 μM forskolin + 100 μM IBMX for 4 days and the YFP+ cells were collected by a fluorescence-activated cell sorter (FACS). These cultures have been propagated, and they retain the ability to respond to stimuli for at least 25 passages.

RNA Extraction and RT-PCR Analysis

Cells were lysed in Tri-Reagent (Molecular Research Center, Cincinnati, OH), and total RNA was extracted according to the manufacturer’s directions. Contaminating DNA was removed using the DNA-free kit (Ambion, Austin, TX). The cDNA was prepared from 2 μg of RNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY) and an oligo(dT)15 primer according to the manufacturer’s directions. PCR was performed on 2 μl of the RT reaction as a template using Taq DNA polymerase (Promega, Madison, WI). Primer sequences for all the genes examined are listed in Table 1.

Stimulation of Renin Expression in Cultured Renin Cells

Increasing cAMP levels. Cells were treated with forskolin (10 μM forskolin + 100 μM IBMX) and the cAMP analogs dibutyryl cAMP (100 μM and 1 mM + 100 μM IBMX) and 8-bromo-cAMP (8-BrcAMP; 1 and 3 mM + 100 μM IBMX) for 3 days. Cells were examined microscopically for YFP expression, and then RNA was extracted for evaluation of renin mRNA expression. Forskolin treatment alone stimulated renin and YFP expression; however, the response was stronger in the presence of the phosphodiesterase inhibitor IBMX, so IBMX was included in most of the studies. 

Inhibiting histone deacetylation. To investigate whether the acetylation of histones plays a role in renin expression, cells were treated with the histone deacetylase (HDAC) inhibitors sodium butyrate (NaB; 5 and 10 mM) and trichostatin A (TSA; 100 and 200 nM) for 2 days and analyzed as the forskolin-treated cells described in Increasing cAMP levels.

Chromatin Immunoprecipitation

Antibodies used for chromatin immunoprecipitations (ChIPs) were anti-acetyl histone H4 (anti-acH4) and anti-acetyl histone H3 (anti-acH3) (Upstate Biotechnology, Lake Placid, NY) and normal rabbit IgG from Santa Cruz Biotechnology (Santa Cruz, CA). The ChIP experiments were performed using protocols from Upstate Biotechnology with modifications (4, 5, 29). Chromatin was prepared from untreated (control) cultured RA-SMCs and RA-SMCs treated with 10 μM forskolin + 100 μM IBMX overnight and again for 30 min with 10 μM forskolin before cross linking. Cells were rinsed with PBS, and the chromatin was cross linked by the addition of formaldehyde to 1% in PBS for 10 min at room temperature. The cross linking was stopped by the addition of glycine to a

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**Table 1. Primers used for RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
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<tbody>
<tr>
<td>ACE Forward</td>
<td>5′ ACCCTCTGGCTAACACTTC</td>
</tr>
<tr>
<td>ACE Reverse</td>
<td>5′ ACCTGCTGCAGTTCTCAA</td>
</tr>
<tr>
<td>AT1 Forward</td>
<td>5′ GCTGGGATTATGGAAGAACAG</td>
</tr>
<tr>
<td>AT1 Reverse</td>
<td>5′ GCCCAAGGAGGCTTAAAAC</td>
</tr>
<tr>
<td>ATFI Forward</td>
<td>5′ GAAAGGAGGAGGCGAAAAAT</td>
</tr>
<tr>
<td>ATFI Reverse</td>
<td>5′ TCAGGGGAGAATCCACACC</td>
</tr>
<tr>
<td>Arg Forward</td>
<td>5′ TCTGAGAGGAGGAGACTGC</td>
</tr>
<tr>
<td>Arg Reverse</td>
<td>5′ CAGAGAGAGAGGAGACAC</td>
</tr>
<tr>
<td>CREB Forward</td>
<td>5′ GCCGCTGAAATGCTGCGTA</td>
</tr>
<tr>
<td>CREB Reverse</td>
<td>5′ GCTTCAACTCTGCTACGT</td>
</tr>
<tr>
<td>CREM Forward</td>
<td>5′ CATCGGATCAGTGATAGTG</td>
</tr>
<tr>
<td>CREM Reverse</td>
<td>5′ GACAGAGGATGCTGCTGTA</td>
</tr>
<tr>
<td>GAPDH Forward</td>
<td>5′ GTCTATGTCGCTCAGTTG</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>5′ GCTGCTGTCGCTACGTGTA</td>
</tr>
<tr>
<td>Renin Forward</td>
<td>5′ ATGCCCTCTGGGCACTTTT</td>
</tr>
<tr>
<td>Renin Reverse</td>
<td>5′ GTCAAACTTGCCAGCAACTAG</td>
</tr>
<tr>
<td>α-SMA Forward</td>
<td>5′ TATGTGCTGCTGATTGAA</td>
</tr>
<tr>
<td>α-SMA Reverse</td>
<td>5′ ACATTGCTGCTGACAGAGAC</td>
</tr>
<tr>
<td>MHC Forward</td>
<td>5′ GCCTGGGCGCCTAGATTTGAA</td>
</tr>
<tr>
<td>MHC Reverse</td>
<td>5′ GAATTTAATCTGTGCTGAGTUT</td>
</tr>
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RESULTS

We generated a mouse in which cells of the renin lineage are marked with CFP, even after the cells have differentiated into nonrenin producing cells, and cells actively transcribing renin are labeled with YFP (Fig. 1). To label cells currently expressing renin, we generated a transgenic mouse in which the Ren1c promoter drives the expression of YFP. As shown in Fig. 2, these fetal (E18) and newborn (N1) mice express YFP in large arterioles in a pattern known for renin during development. In the adult, YFP expression was confined to the JG area, as we expected, the cells have downregulated expression of the renin gene and, therefore, did not contain renin mRNA (Fig. 4D). The cells can be maintained in culture for as many as 32 passages (nearly 1 yr from the time of initial isolation of the arterioles), and they retain expression of SM-MHC (Fig. 4D).

Our laboratory (40) has previously shown that a subset of kidney arteriolar SMCs is derived from the renin cell lineage, and these cells can dedifferentiate to express renin in response to homeostatic stress. To label live cells of the renin lineage and at the same time label cells currently expressing renin, we generated mice carrying Ren1c-cre (40), a ROSAloxP(CFP) reporter (43), and Ren1c-YFP (Figs. 1 and 3A). RA-SMCs from these mice were isolated using an iron oxide perfusion method and differential plating (10, 11). Figure 4A shows CFP+ cells of the renin lineage. Initially, the cells did not express YFP. RT-PCR confirmed that the cultured cells expressed α-smooth muscle actin (SMA) and SM myosin heavy chain (SM-MHC) as expected for SMCs (Fig. 4D). These RA-SMCs of the renin lineage, which are not expressing renin, can, however, be stimulated to reexpress renin under appropriate stimulation. In response to treatment with 10 μM forskolin + 100 μM IBMX, the cells responded by making renin mRNA (Fig. 4D). The transcription of renin also directs the transcription of the Ren1c-YFP transgene, resulting in the labeling of some of the CFP+ cells with YFP (Fig. 4, A and B). As the duration of the stimulus increases, the percentage of CFP+ cells becoming YFP+ increases from 0% in untreated

Fig. 2. Expression of YFP in Ren1c-YFP transgenic kidneys. Fetal (E18) and newborn (N1): YFP is expressed in large vessels. Adult: YFP is confined to the juxtaglomerular (JG) area. Adult-captopril: YFP is expressed upstream from the glomerulus in captopril-treated animals. These patterns of YFP expression are the same as the known pattern of renin expression at these different developmental stages and during recruitment with captopril. In the bright field photo, arrows indicate the YFP positive vessels or JG apparatuses.

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cells to 10%, 25%, and 33% with 1, 2, and 4 days of treatment, respectively. Thus CFP$^+$ SMCs regain the ability to express renin, as shown by the transcription of renin mRNA and the expression of YFP. These cultures were subjected to FACS (Fig. 4C), resulting in a highly purified population of cells that can reexpress renin mRNA. This cell culture system allows us to propagate and visually monitor cells of the renin cell lineage (CFP$^+$ cells) and identify them when they are expressing renin (YFP$^+$). As shown above, treatment with forskolin, which increases intracellular cAMP levels, stimulates renin mRNA expression. We tested whether other agents that altered the levels of cAMP directly had a similar effect on renin expression. Treatment with the cAMP analogs dibutyryl cAMP and 8-BrcAMP increased the expression of renin mRNA (Fig. 5A), and the stimulated cells expressed YFP$^+$ (Fig. 5B).

The cultured CFP$^+$ RA-SMCs were further characterized to determine the presence of other components of the RAS and the DNA-interacting components involved in the cAMP response. Figure 6A shows that the cells express all the components of the RAS system [angiotensinogen (Atg), ACE, and ANG II type 1 (AT$_1$) receptor]. The mRNAs for Atg and ACE were lower in abundance in the cells compared with the kidney levels, whereas AT$_1$ receptor mRNA levels were approximately equal. These cells, therefore, contain most of the components...
of the RAS and respond to various stimuli that can induce or repress renin expression.

The effect of cAMP is connected to transcription by the activation of CRE-binding protein (CREB). CREB-binding protein (CBP) was originally identified as a coactivator with CREB. CBP and its other family member p300 both bind to the phosphorylated form of CREB (31). The recruitment of the transcriptional coactivators p300 and CBP to gene promoters by DNA-bound transcription factors activates gene transcription. Figure 6B shows that Creb1, its other family members (CREM and ATF1), and the coactivators CBP and p300 are all expressed in these cells. Thus all the elements for the cAMP response are available in these cells.

The reversible acetylation of histones plays a critical role in transcriptional regulation in eukaryotic cells (50). Increased acetylation is a consistent marker of increased transcription or of the potential for transcription. To determine whether histone acetylation plays a role in renin gene activation, we inhibited the deacetylation of histones by treating cells with deacetylase inhibitors. If the level of histone acetylation at the renin locus is important for its expression, then the inhibition of the removal of acetylated marks could stimulate renin transcription. The results (Fig. 7A) show that the treatment of the cells with the HDAC inhibitors NaB or TSA stimulated the expression of renin mRNA. Treatment with both the HDAC inhibitors and forskolin resulted in the further increase in renin expression.

Fig. 5. Cultured RA-SMCs express renin mRNA and YFP in response to treatment with forskolin and cAMP analogs. A: RNA was extracted from cells treated for 2 days, and RT-PCR was done for renin and GAPDH. F, 10 μM forskolin + 100 μM IBMX; D1, 100 μM dibutyryl cAMP + 100 μM IBMX; D2, 1 mM dibutyryl cAMP + 100 μM IBMX; B1, 1 mM 8-bromo-cAMP + 100 μM IBMX; and B2, 3 mM 8-bromo-cAMP + 100 μM IBMX. The strongest response was to D2. B: YFP expression in cells after treatment for 5 days.

Fig. 6. A: components of the renin-angiotensin system are expressed in RA-SMCs in culture. RT-PCR for ACE, angiotensinogen (Atg), and angiotensin II type 1 (AT1) receptor was performed on RNA extracted from unstimulated cells (C) and K cells. B: components involved in the cAMP response are expressed in RA-SMCs in culture. RT-PCR for CREM, Creb1, ATF1, CREB-binding protein (CBP), and p300 was performed on RNA extracted from unstimulated cells.

Fig. 7. Histone acetylation is associated with increased renin expression. A: renin expression is stimulated by inhibition of histone deacetylation. Cells were treated for 2 days with the histone deacetylase (HDAC) inhibitors sodium butyrate (NaB) and trichostatin A (TSA) alone or in combination with 10 μM forskolin + 100 μM IBMX. Both NaB and TSA stimulated renin expression. The addition of forskolin further increased renin expression. None, no treatment; NaB 1, 5 mM NaB; NaB 2, 10 mM NaB; TSA 1, 100 nM TSA; and TSA 2, 200 nM TSA. B: acetylation of histones in RA-SMCs in response to forskolin stimulation. Chromatin from cells treated 2 days with 10 μM forskolin + 100 μM IBMX was immunoprecipitated with antibodies against acetylated histones H4 (acH4) and H3 (acH3). The region containing the CRE was amplified by PCR. acH4 was increased in the treated cells, whereas acH3 was unchanged. Input, 1:10 dilution of the unprecipitated chromatin. C: quantitative real-time PCR of the region containing the cAMP responsive element (CRE) from immunoprecipitation with antibody against acH4. Results from 3 independent experiments are graphed individually. Forskolin-treated samples (gray-hatched bars) had an increased level of H4 acetylation over the basal level in control samples (white bars). D: the ratio of the fold increase of forskolin to control (black bars) shows H4 acetylation in the region of the CRE element is higher in the chromatin from forskolin-treated cells. In C and D, the error bars (SE) are included to show the interassay variability.
expression, indicating a role for histone acetylation in the reexpression process.

The stimulation of renin (and other genes) via increased cAMP is mediated by the binding of CREB and its coactivator CBP to the CRE in the promoter. Among other functions, CBP has intrinsic histone acetyltransferase (HAT) activity. The results of treatment with HDAC inhibitors indicated that changes in histone acetylation play a role in the activation of the renin gene. To determine whether the histone acetylation pattern changed when the renin gene was activated, chromatin isolated from cultured cells treated with forskolin was immunoprecipitated with antibodies directed against acH4 and acH3. The immunoprecipitated DNA was amplified by PCR using primers that flank the region of the CRE element in the promoter. The gels in Fig. 7B show that there is increased acetylation of H4 in response to forskolin in the region of the CRE when renin is being expressed, whereas there was no apparent change in the amount of H3 acetylation. In additional experiments, quantitative real-time PCR (Fig. 7C) confirmed the increase in the forskolin-treated sample over the basal level of H4 acetylation in the control (untreated) cells. The ratio of forskolin-treated to control values (Fig. 7D) showed H4 acetylation in the stimulated cells increased 3.6-, 3.7-, and 8.6-fold in three separate independent experiments. The results show that in response to stimulation by forskolin, there is increased selective acetylation of one of the histones (H4) in the neighborhood of the CRE element, which is associated with the cAMP response.

**DISCUSSION**

We generated a mouse in which cells of the renin lineage are permanently labeled with CFP, and when these cells are actively transcribing renin, they are also labeled with YFP. In addition to its utility for in vivo physiological studies, this mouse is a ready source of labeled cells with the plasticity to switch the renin gene on and off. In the present study, we show that 1) cells from the renin lineage can be identified, isolated, and manipulated in culture for long periods of time; 2) as in vivo, these cells have the plasticity to reexpress the renin gene in vitro, an intrinsic capacity that is maintained over long-term culture; and 3) this plasticity is mediated by the cAMP machinery, which is facilitated by chromatin remodeling.

The cells for these studies were derived from mice having the renin cell lineage marked with CFP and cells currently expressing renin marked with YFP. The expression of cre recombinase, which deletes the strong transcriptional stop sequence (STOP) in the CFP reporter in cells of the renin lineage (40), was obtained from our mouse in which we replaced Ren1d with cre recombinase by homologous recombination (40). To retain one functional copy of Ren1d and yet be able to mark the cells currently expressing renin, we generated a transgenic animal having YFP driven by the Ren1c promoter. The present study showed that this promoter drives in vivo expression of YFP in the authentic pattern of renin during development, in adult life, and in response to physiological stress (i.e., recruitment). The expression of these markers in the dually labeled mice during development and in adults in response to captopril is in full agreement with previous studies (14, 16, 19, 20, 36, 40), showing that expression of renin switches from large arteries in the fetus to the JG cells in adults and reverts back to the fetal pattern in response to homeostatic challenges. We anticipate that these mice will be useful to investigators interested in obtaining simultaneous in vivo information on the effects of physiological manipulations on renin gene transcription and its correlation with the steady state and distribution of renin mRNA and its protein. Because it is possible to view and count the cells actively transcribing the renin gene in vivo, these mice can also be used to distinguish the recruitment of renin gene-expressing cells versus the presence of renin due to uptake in the renal vasculature (21). In addition, since in these mice the renin cell lineage is marked with CFP, they can be used to assess the effect of deletion of specific genes on renin cell fate/differentiation.

Studies of renin expression in vitro have been mainly done using the tumor cell line As4.1 (27, 28, 34, 35, 42), which expressing renin constitutively or primary cultures of JG cells (6, 8, 9) which usually cease expressing renin after a few days and do not survive well. The latter had, therefore, not been subjected to the analysis of reexpression since the cells or the expression of renin seemed to have been lost. Even if renin expression were maintained in these cultures, they would not provide easily identifiable material for addressing reexpression in potentially responsive cells since they carry no visible marker of expression. Therefore, using our mice as a source of cells, we developed an in vitro model to facilitate the study of the mechanisms underlying the reexpression of the renin gene. In this model, cells of the renin lineage are marked with CFP and cells currently expressing renin are marked with YFP. The isolated RA cells from these mice express SM-MHC and α-SMA at the beginning of culture and up to 32 passages, indicating that they are SMCs and retain this phenotype throughout the culture period. In addition to expressing CFP, indicating that they were derived from renin cell precursors (40), these cells can be stimulated to reexpress the renin gene and become YFP+. The ability to reexpress the renin gene, mimicking the in vivo recruitment response, is retained even after as many as 25 passages. These results suggest that recruitment, i.e., increasing the number of renin-expressing cells, a critical homeostatic response in vivo, is conserved in culture, intrinsic to the cell, and amenable to study in vitro.

The cells thus isolated do not express renin initially, as expected, since arteriolar SMCs distant from the glomerulus do not normally express renin in the adult and JG cells are known to stop renin expression after a few days in culture. However, the stimulation of these cells with forskolin or cAMP analogs results in a subset of the CFP+ cells beginning to express renin and YFP. Our studies show that with a longer duration of the stimulus, there is an increase in the percentage of cells expressing YFP. This again is consistent with findings in vivo that the recruitment response is graded, i.e., with increasing strength and duration of the stimulus, there is an increase in the number of cells expressing renin spreading out from the JG cells along theafferent arterioles and eventually to the larger vessels (40).

As mentioned above, the stimulation of the cells with agents that increase intracellular cAMP levels (forskolin, 8-BrcAMP, and dibutylr cAMP) causes them to express renin mRNA and marks the cells with YFP through the stimulation of the Ren1c promoter of the Ren1c-YFP transgene. These findings are in agreement with our previous work and the work of others (6, 9, 27, 34, 47), showing that cAMP stimulates renin gene expression. Our findings in these cells demonstrate that cAMP also
plays a role in renin reexpression in vitro in cultures derived from cells that have the potential to express renin but are latent until stimulated.

The effect of cAMP is connected to transcription through the activation of CREB by the phosphorylation and recruitment of the coactivator CBP/p300 (1, 31) to P-CREB. CBP/p300 acts as a bridging factor between CREB and the general transcription factor TFIIIB and mediates transcriptional activation through its association with RNA polymerase II (7, 30) complexes and through intrinsic HAT activity. The results presented here demonstrate that CREB, CBP, and p300, essential for this response, are expressed in these cells, and the cAMP pathway operates in our cell preparations to increase renin transcription in cells not currently expressing renin. Previous studies in transgenic mice (13) showed that p300 expressed under control of the human renin promoter stimulates mouse renin mRNA transcription. Furthermore, ChIP studies in As4.1 cells showed that both CREB and CBP are present on the CRE element in the Ren1’ promoter when the renin gene is active (52), confirming their role in renin regulation. CREB is also an in vivo substrate for a variety of other kinases including calmodulin kinases II and IV (45) or ribosomal S6 kinase 2 (51), implying that CREB activates transcription not only in response to cAMP but potentially through changes in Ca2+ and growth factor stimulation.

DNA sequence-specific transcriptional regulators, such as CREB, recruit cofactors to modulate the activity of the polymerase complex (32). These cofactors function at least in part by affecting chromatin structure through their associated enzymatic activities of which the HATs and HDACs have been the most characterized (23, 44). As described above, the cAMP response involves the recruitment of CREB and its coactivator CBP to the CRE element in the promoter. The HAT function of CBP and p300 very likely plays a role in the chromatin remodeling that accompanies transcriptional activation. Our studies show that in forskolin-stimulated cells, there is an increase in the acetylation of histone H4 in the region of the CRE element of the renin promoter compared with untreated cells. In addition, the inhibition of histone deacetylation stimulates renin transcription in these cells, further supporting the significance of histone acetylation in renin gene expression. To our knowledge, this is the first report that shows that increases in histone acetylation around the CRE element accompany stimulation of the renin gene. Although the memory to reexpress the renin gene in response to cAMP and HDAC inhibitors is maintained in long-term culture, further studies are necessary to determine whether long-term stimulation will elicit full-fledged phenotypic changes including granuloepoiesis and active processing and the release of renin.

In addition to its particular application to understanding the role of the cAMP-chromatin relationship to renin gene regulation, this in vitro model can potentially be used to reveal the intracellular signals and associated genes and proteins underlying the phenotypic transformation of the arteriolar SMCs into a renin-producing cell. Furthermore, since these cells also express all the components of the RAS, they are potentially useful to investigate the role of an intracrine RAS system in cell function.

In summary, we generated mice in which cells of the renin lineage are labeled with CFP and cells actively making renin are labeled with YFP. From these mice we developed an in vitro model that allowed us to determine that it is possible to culture cells of the renin lineage for numerous passages and that the memory to express the renin gene is maintained in culture and can be elicited through the cAMP pathway and histone modifications in the renin promoter.

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