HCaRG is a novel regulator of renal epithelial cell growth and differentiation causing G2M arrest

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HCaRG is a novel regulator of renal epithelial cell growth and differentiation causing G2M arrest. HCaRG is a novel regulator of renal epithelial cell growth and differentiation causing G2M arrest. Am J Physiol Renal Physiol 284: F753–F762, 2003. First published December 3, 2002; 10.1152/ajprenal.00252.2002.—We recently identified a novel calcium-regulated gene, HCaRG, that is highly expressed in the kidney and maps to a chromosomal locus determining kidney weight in rats. The mRNA levels of HCaRG negatively correlate with the proliferative status of the kidney cells. To investigate its role in renal epithelial cellular growth directly, we studied the human embryonic kidney cell line (HEK-293) stably transfected with either plasmid alone or plasmid containing rat HCaRG. [3H]thymidine incorporation was significantly lower in HCaRG clones. Although HCaRG clones exhibited some enhanced susceptibility to cell death, this was not the primary mechanism of reduced proliferation. Cell cycle analysis revealed a G2M phase accumulation in HCaRG clones that was associated with upregulation of p21^Cip1/WAF1^ and downregulation of p27^Kip1^. HCaRG clones had a greater protein content, larger cell size, and released 4.5- to 8-fold more of an atrial natriuretic peptide-like immunoreactivity compared with controls. In addition, HCaRG clones demonstrated the presence of differentiated junctions and a lower incidence of mitotic figures. Genistein treatment of wild-type HEK-293 cells mimicked several phenotypic characteristics associated with HCaRG overexpression, including increased cell size and increased release of atrial natriuretic peptide. Taken together, our results suggest that HCaRG is a regulator of renal epithelial cell growth and differentiation causing G2M cell cycle arrest.

A novel gene; p21^Cip1/WAF1^; p27^Kip1^; atrial natriuretic peptide; human embryonic kidney 293 cells; hypertension-related, calcium-regulated gene

KIDNEY DEVELOPMENT AND CELLULAR differentiation are dependent on the ordered activation of a number of genes whose encoded proteins determine cell phenotype as well as functional responses (4). The novel hypertension-related, calcium-regulated gene (HCaRG) codes for an intracellular protein, which have previously shown (by using in situ hybridization) to be highly expressed in the tubular fraction of the kidney and more highly expressed in kidneys from hypertensive compared with normotensive animals (31). We have mapped HCaRG to rat chromosome 7 (30) at a locus determining kidney weight (14). In our previous studies, we also observed that HCaRG mRNA levels declined rapidly in the kidney after ischemia and reperfusion while there was a reciprocal increase in c-myc mRNA (31). The ischemia-reperfusion model of renal injury in vivo is a well-documented stress that results in dedifferentiation and mitogenesis of the surviving cells to achieve the repair of the injured kidney (10). We have also shown that HCaRG mRNA levels are negatively correlated to the proliferative status of the cell, i.e., they are lower in fetal than in adult kidneys and also downregulated in renal carcinoma. Taken together, these results suggest that HCaRG could play a role in the control of renal epithelial cell growth and differentiation for which the molecular determinants are still not completely understood.

Atrial natriuretic peptide (ANP) was originally isolated from mammalian atria and identified as a circulating hormone that is involved in blood pressure regulation (6). ANP antagonizes the pressor effects of the renin angiotensin system through its potent natriuretic, diuretic, and vasorelaxant mechanisms. Extra-atrial expression and synthesis of ANP is now well documented, and previous studies have identified ANP in the brain, ovary, pituitary, lungs (12), and kidneys (20) of several species in vivo as well as the secretion of ANP by primary cultures of neonatal and adult kidney cells (26). The developmental pattern of ANP immunoreactivity in the rat was shown to coincide with the differentiation and maturation of the tubular epithelium (20). The site of local renal ANP synthesis has been localized to the distal tubular epithelial cells of the kidney (25), and studies have identified the local ANP produced in the kidney as urodilatin,

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a 32-amino acid peptide consisting of the sequence 95–126 of the ANP prohormone (28). The human embryonic kidney cell line (HEK-293) represents an appropriate dedifferentiated embryonic renal cell line of human origin that exhibits several phenotypic characteristics of distal tubular cells, including the basal synthesis and release of an ANP-like immunoreactivity (or urodilatin) (2, 16). In the present study, we report that the novel gene HCaRG is a regulator of HEK-293 cell proliferation, cell cycle progression, and cell differentiation involving the induction of p21Cip1/WAF1 and the downregulation of p27Kip1.

MATERIALS AND METHODS

Stable transfection and cell culture. HEK-293 cells were transfected with the control plasmid (pcDNA1/Neo; Invitrogen) or with plasmid containing rat HCaRG (pcDNA1/Neo-HCaRG) by using a standard calcium phosphate coprecipitation method and then selected in 400 μg/ml G418, and rat HCaRG mRNA levels were determined as described in detail in previous studies by our laboratory (31). Clones expressing the highest levels of HCaRG, HCaRG clones 8 and 9, were used in these studies, and clones transfected with vector alone, Neo clones 1 and 6, served as controls. Stable transfectants were used at passage numbers <13 and were maintained in DMEM (high glucose) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin with 400 μg/ml G418. Wild-type HEK-293 cells were maintained in identical conditions but without the selection antibiotic G418.

Proliferation and apoptosis. Stably transfected or wild-type HEK-293 cells were inoculated in DMEM containing 10% FBS in poly-D-lysine-coated plates and then synchronized and then grown in DMEM containing 0.2% FBS. This protocol and all subsequent protocols that involved cell synchronization were conducted for 48 h in DMEM containing 0.2% FBS. Stable transfectants were then grown for 48 h in DMEM +10% FBS, and 1 μCi [3H]thymidine (ICN) was added for the last 4 h of incubation, as detailed previously (31). After synchronization, wild-type cells were treated with genistein (1–100 μM) for 48 h, and [3H]thymidine was added as above (31). Proliferation was also assessed by counting cell numbers with a haemocytometer. We used the chromatin cleavage assay (3) to measure apoptosis in stable transfectants growing in DMEM +10% FBS or after serum deprivation (0% FBS) as well as under identical conditions to those described for [3H]thymidine incorporation.

Cell cycle analysis. Stably transfected cells were synchronized and then grown in DMEM +10% FBS, and a time course was conducted by harvesting cells at 0, 12, 16, 20, 24, and 48 h for cell cycle analysis. Nonsynchronized clones growing in DMEM +10% FBS were also harvested at several time points. Growth curves were constructed for each clone from which the specific growth rate was determined by using the formula \((P_2 - P_1)/P_1(t_2 - t_1)\), in which \(P_1\) and \(P_2\) are cell density at times \(t_1\) and \(t_2\) as described in a previous study (13). Wild-type HEK-293 cells were synchronized and then treated with 50 μM genistein or vehicle for 24, 48, or 72 h. Total RNA was extracted by using the guanidinium isothiocyanate extraction technique, and HCaRG mRNA levels were determined by Northern and Dot blot hybridization using a human HCaRG probe with a correction made for GAPDH or poly-A RNA as described previously (31).

Macroarray analysis and Western blotting. HCaRG and Neo clones were synchronized, then grown in the presence of 10% FBS for 48 h, and then RNA was extracted by using the RNeasy kit (QIAGEN), treated with DNase I, purified, and used for preparation of cDNA. Two identical Atlas Human Cell Cycle Arrays (Clontech) were hybridized with human 32P-labeled HCaRG 8 or HCaRG 9, or 35S-labeled Neo 1 or Neo 6 cDNAs as probes, according to the manufacturer’s instructions. For Western blotting, cells were lysed on ice with lysis buffer (10 mM Tris · HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS plus protease inhibitors and EDTA). Cell extracts were mixed with an equal volume of sample buffer, separated by SDS gel electrophoresis, and then transferred to a polyvinylidene difluoride transfer membrane (or nitrocellulose membrane for HCaRG Western blot; Amersham Pharmacia Biotech) and incubated with antibodies to p21Cip1/WAF1 (1:500), p27Kip1 (1:2,500; Transduction Laboratories), p53 (1:2,500; Calbiochem), and HCaRG (1:500) (31), followed by horseradish peroxidase-conjugated anti-mouse, anti-goat, or anti-rabbit antibodies. Bands were visualized by using an enhanced chemiluminescence kit, and signals were scanned by densitometry and normalized to α-tubulin.

Cell size, protein content, and cell volume. Stably transfected cells were synchronized and then grown in 10% FBS, and protein concentration was measured after 12, 24, 36, 48, and 72 h with the modified Lowry method (18). Cell size was measured by using the National Institutes of Health Image program (http://rsb.info.nih.gov/nih-image/) to measure the cross-sectional area of cells at differing degrees of confluency, and the mean cell size was determined for 100 cells. Cell volume was measured directly by using the equilibrium distribution of [14C]urea to measure the intracellular volume of water per cell as described previously (22). Wild-type HEK-293 cells were synchronized and then treated with 50 μM genistein or vehicle for 24, 48, or 72 h, and the mean size of 100 cells each was also calculated.

Measurement of ANP. The level of ANP immunoreactivity released into the cell culture medium was measured as detailed previously (11). Samples were harvested into chilled tubes containing a protease inhibitor solution composed of 10−5 M EDTA, 10−5 M PMSF, and 5 μM pepstatin A and were stored at −80°C. For measurements of intracellular ANP, cells were pelleted and then resuspended in 1 ml of PBS containing inhibitor solution, and the intracellular proteins were released by conducting three freeze-thaw cycles. Appropriate buffer and incubation media were collected simultaneously and served as the control samples in each ANP radioimmunoassay.

Electron microscopy. Sample preparation for electron microscopy (EM) was carried out as described previously (29) with some modifications, and then semi-thin sections were stained with toluidine blue and an index of mitotic figures was obtained by counting cells with condensed chromosomes in 10–15 high-power fields each for confluent cells. From suitable areas, thin sections were cut, double stained on copper grids with uranyl acetate and lead citrate, and then examined with a Phillips EM 208 transmission electron microscope.

HCaRG mRNA expression. Wild-type HEK-293 cells were synchronized and then treated with 50 μM genistein or vehicle for 24, 48, or 72 h. Total RNA was extracted by using the guanidinium isothiocyanate extraction technique, and HCaRG mRNA levels were determined by Northern and Dot blot hybridization using a human HCaRG probe with a correction made for GAPDH or poly-A RNA as described previously (31).

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Statistical analysis. All experiments were conducted two to six times. Results are presented as means ± SE. Statistical analysis was performed using a Student’s unpaired t-test. \( P < 0.05 \) was considered statistically significant.

RESULTS

HCaRG-induced growth suppression is associated with changes in cell morphology. Wild-type HEK-293 cells that normally express low levels of HCaRG were stably transfected, and HCaRG clones 8 and 9, expressing moderate to high levels of HCaRG, showed a reduced proliferation rate and lower cell density at confluence compared with Neo control clones (31). We report here that the slow-growing HCaRG-transfected cells showed marked changes in morphology and size compared with the control cells (Fig. 1A). The Neo control clones, which exhibit a small, polygonal-shaped, epithelial-like morphology, continued to grow exponentially and packed together closely at confluence (see A and C in Fig. 1A), which was the growth pattern we observed for wild-type cells (not shown). However, as a result of HCaRG overexpression, the HEK-293 cells exhibited a larger surface area with the presence of very large, flattened cells (and/or cytoplasmic vacuoles; see B in Fig. 1A) and the cells did not pack together as closely at confluence (see D in Fig. 1A), consistent with reaching a lower saturation density. The overexpression of the HCaRG protein in HCaRG clones 8 and 9 was confirmed with Western blot analysis (Fig. 1B). We assessed the effect of HCaRG overexpression on cell death and found some enhanced susceptibility to apoptosis in response to complete serum deprivation (Neo, 5.5 ± 0.3%, vs. HCaRG, 11.2 ± 0.8%; \( n = 3 \); \( P < 0.05 \)). However, we did not observe any difference in the level

Fig. 1. HCaRG-overexpressing cells exhibit changes in morphology. A: HEK-293 cells stably transfected with either plasmid alone (Neo clone 1; A and C) or plasmid containing rat HCaRG (HCaRG clone 9; B and D) were photographed at low and high density in normal growth conditions. Photographs illustrate the difference in cell size and morphology (A and B) as well as the difference in cell density attained at confluence (C and D). We noted very large flattened cells and/or cytoplasmic vacuoles that were evident in HCaRG-transfected cell lines only (arrows in B and D). B: representative Western blot showing overexpression of HCaRG protein in HCaRG clones 8 and 9 compared with Neo control clones 1 and 6. HCaRG protein and molecular weight standard are as indicated. NS, nonspecific binding.
of apoptosis between Neo and HCaRG clones after synchronization in 0.2% serum for 48 h (Neo, 3.13 ± 0.33%, vs. HCaRG, 3.55 ± 0.18%; n = 8; not significant) or when returned to 10% serum for an additional 48 h (Neo, 2.73 ± 0.6%, vs. HCaRG, 4.6 ± 0.34%; n = 4; not significant), thus confirming the minimal contribution of apoptosis to the observed reduction in proliferation as observed using [3H]thymidine incorporation.

Cell cycle progression is blocked at G2M phase in HCaRG-overexpressing clones. Representative cell cycle DNA histograms for nonsynchronized actively growing cells are shown in Fig. 2, A and B, and the HCaRG cells show an accumulation in G2M phase, suggesting a putative G2M arrest. A detailed assessment of cell cycle progression after addition of serum to synchronized cells was conducted that clearly showed a significant increase in G2M phase (Neo vs. HCaRG; n = 6 at 24 and 48 h; P < 0.01) with a concomitant decrease in the G1 phase in HCaRG cells (Fig. 2, E and C). Specific growth rates for each clone under normal growth conditions showed the greatest difference between days 2 and 4 after plating (average values for Neo cells, 1.32 ± 0.02, compared with HCaRG cells, 0.85 ± 0.06; 1.6-fold difference). Cell cycle distribution in each clone after 2 and 4 days of growth was analyzed and confirmed the G1 phase reduction (Neo vs. HCaRG; 2.73 ± 0.6%, vs. HCaRG, 4.6 ± 0.34%; n = 4; not significant).

Fig. 2. The effect of HCaRG overexpression on cell cycle progression. Representative cell cycle histograms for nonsynchronized Neo control cells (A) and HCaRG cells (B) after 48 h growth are indicated. C–E: time course of cell cycle progression after synchronization. F: cell cycle distribution in nonsynchronized cells after 2 and 4 days of growth. Values are means ± SE of n = 2–4 each for Neo and HCaRG at each time point.

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HCaRG-induced growth suppression is associated with increased p21\textsuperscript{Cip1/WAF1} and decreased p27\textsuperscript{Kip1}. We compared the differential gene expression profile of 111 cell cycle regulatory genes in the stably transfected clones by using a human Atlas macroarray. Macroarray analysis was conducted in two HCaRG clones (HCaRG 8 and HCaRG 9) and in two Neo control clones (Neo 1 and Neo 6). Representative filters for Neo 1 and HCaRG 9 are presented in Fig. 3, A and B. The arrows indicate a small subset of upregulated genes in HCaRG cells. The results for differential expression in HCaRG clones 8 and 9 compared with Neo clones 1 and 6 are summarized in Fig. 3C. The gene whose expression was maximally induced in both HCaRG clones was the cyclin-dependent kinase (CDK) inhibitor p21\textsuperscript{Cip1/WAF1} (average 2.5-fold upregulation). Other genes that were upregulated include cyclin D1 (average 1.7-fold); gadd153 (average 1.8-fold); CDKL1 (average 1.9-fold); Jun-B (average 2-fold), and Big MAPK-1/ERK5 (average 1.6-fold) upregulation. Western blot analysis showed upregulation of p21\textsuperscript{Cip1/WAF1} protein levels in HCaRG clones 8 and 9 (Neo vs. HCaRG, n = 6; P < 0.005), thus confirming the results of the macroarray (Fig. 3D). Protein levels of the G\textsubscript{s}/S-specific CDK inhibitor p27\textsuperscript{Kip1} were downregulated in HCaRG clones 8 and 9 (Neo vs. HCaRG, n = 4; P < 0.005; Fig. 3E), whereas there was no difference in p53 levels as noted by both macroarray analysis and Western blotting (not shown).

HCaRG-induced growth suppression is associated with cellular hypertrophy and increased levels of ANP. HCaRG-transfected cells had a significantly greater total protein content per cell when compared with Neo control cells at several time points, and Fig. 4A presents the results after 48 h growth (Neo vs. HCaRG, n = 4–6; P < 0.005), indicating that HCaRG may be causing cellular hypertrophy. This feature was confirmed by direct measurement of cell size, which showed a significant increase in HCaRG cell size compared with Neo control cell size (Neo 1, Neo 6 vs. HCaRG 8, HCaRG 9, n = 100 cells each clone; P < 0.0001; Fig. 4B). Furthermore, the volume of the HCaRG-transfected cells measured as [\textsuperscript{14}C]urea-available space was significantly greater compared with the Neo control cells (Neo vs. HCaRG, n = 6; P < 0.0001; Fig. 4C). HCaRG cells showed a significant increase both in the intracellular content (Neo vs. HCaRG, n = 4; P < 0.05; Fig. 4D) and in the release of ANP (Neo vs. HCaRG, n = 6; P < 0.001; Fig. 4E). However, there was
no significant difference in the ratios of secreted to cellular ANP between the control and the HCaRG-transfected cell lines (extracellular ANP/intracellular ANP ratios for each clone were as follows: Neo 1, 0.04; Neo 6, 0.07; HCaRG 8, 0.03; and HCaRG 9, 0.06). This suggests that HCaRG overexpression in HEK-293 cells causes increased synthesis and release of ANP in a constitutive manner.

Features of cellular differentiation identified with EM. Sections of fixed cell preparations from confluent (Fig. 5, A and B) and subconfluent (Fig. 5, C and D) cell lines were examined by a pathologist who first confirmed the epithelial phenotype of the cells. There was a lower incidence of mitotic figures in the HCaRG-transfected cells compared with the Neo control cells (Neo, 13.6 ± 1.0, vs. HCaRG, 6.3 ± 0.3; n = 10–15 high-power fields; P < 0.0001), indicative of a specific block in G2 phase. Transmission EM ultrastructural analysis revealed the presence of more differentiated junctions (desmosome-like junctions) in the HCaRG cells only (Fig. 5B), with the Neo control cells showing the presence of tight junctions (Fig. 5A). In addition, HCaRG cells demonstrated features consistent with junctional (glandular-like) complex formation and numerous microvilli (Fig. 5, C and D).

Treatment of wild-type HEK-293 cells with genistein. Because our data suggested that HCaRG was causing growth suppression in association with the induction of a more differentiated phenotype, G2M arrest and altered cell size, we conducted the converse experiment in which we treated the wild-type HEK-293 cells with an agent, namely, genistein, that is known to act at the G2M phase to alter the differentiation status of the cells (19, 21, 24). We found that treating HEK-293 cells with 50 μM genistein for 48 h resulted in a similar decrease in proliferation (Fig. 6A) and increase in cell size (Fig. 6B) as was documented in HCaRG-transfected cells. The G2M arrest in genistein-treated cells was more marked than that observed with HCaRG overexpression, but in both models there was no polyploid population observed, as assessed by flow cytometry cell cycle DNA histogram analysis. HCaRG overexpression caused upregulation of p21^{Cip1/WAF1}, but genistein treatment did not cause any change in p21^{Cip1/WAF1} levels (not shown). Genistein treatment for 48 h caused a 1.9-fold increase in the level of ANP.
release per cell (Fig. 6C), which was less than that observed in response to HCaRG overexpression, which caused a 4.5-fold increase after 48 h. Concomitant with the increase in ANP release, wild-type HEK-293 cells exposed to genistein exhibited an increase in endogenous HCaRG mRNA levels compared with control cells (Fig. 6D).

**DISCUSSION**

In our previous studies, we have shown that HCaRG mRNA levels are negatively correlated with the proliferative cell status in the kidney in vivo (31). The purpose of this study was to investigate the cellular function and mechanisms whereby the novel gene HCaRG affects cell proliferation. To address this issue, we have characterized the effects of HCaRG overexpression in HEK-293 cells, a cell line that retains several characteristics of renal epithelial cells. We report here that the slow growth of the HCaRG clones is associated with a noticeable change in cell morphology and increased cell size, suggesting a change in the differentiation status of the embryonic kidney cells. We conducted cell cycle analysis by using synchronized and nonsynchronized cells and found that HCaRG cells accumulated at the G2M phase in both conditions. Because the specific growth rate was lower in HCaRG clones compared with controls mainly between day 2 and day 4 of growth, we analyzed the cell cycle distribution at these time points, which confirmed a G2M arrest. Cell cycle arrest in the G2M phase is frequently found to be associated with DNA endoreplication and hypertrophy (7). However, we found no evidence of polyploidy in response to HCaRG overexpression in the HEK-293 cells. It is relevant that our analysis of the HCaRG promoter has documented the presence of CHR (cell cycle gene homology region) and CDE (cell cycle-dependent element) regulatory elements (Tremblay S and Tremblay J, unpublished observations), which suggests that HCaRG may be expressed in a cell cycle-dependent fashion at the G2M phase (34).

Cell cycle-related growth suppression is frequently mediated by the induction of the CDK inhibitor p21CIP1/WAF1, which can act to cause arrest at the G1 or G2M phase of the cell cycle by p53-dependent or p53-independent means (23, 32). Although hypertrophy of renal epithelial cells is known to be associated with G1 arrest due to upregulation of p27Kip1, a previous report has shown growth suppression in HEK-293 cells due to G2M phase arrest and sustained induction of p21CIP1/WAF1 (9). To investigate the mechanisms of HCaRG-induced cell cycle arrest, we conducted macroarray differential gene expression analysis in the stably
transfected clones. The gene whose expression was upregulated the most in both HCaRG clones was p21Cip1/WAF1, which is known to be increased in association with differentiation induction. Other genes that were upregulated in both clones were cyclin D1 and gadd153, known to be involved in growth arrest in association with pathways of differentiation or apoptosis (33). Jun-B is known to be upregulated during cell growth suppression and differentiation in embryonic tissues (27), suggesting a maturation of the embryonic kidney cells in response to HCaRG overexpression. The redox-sensitive Big MAPK-1/ERK5 is upregulated in response to H2O2 and osmotic stress and has also been linked to pathways of cellular differentiation (8).

Because of the observed upregulation of p21Cip1/WAF1 mRNA, we conducted Western blotting, which confirmed that p21Cip1/WAF1 was also upregulated at the protein level in both HCaRG clones. Because of the role of p27Kip1 on G1/S phase progression, we conducted Western blotting, which showed that p27Kip1 was downregulated in both HCaRG clones, whereas macroarray and Western blot analyses showed no change in p53 levels. Therefore, elevated p21Cip1/WAF1 levels along with increased cyclin D1 expression and downregulated p27Kip1 is consistent with a successful G1/S phase transition, with growth arrest occurring at the G2M phase. The reciprocal relationship between the CDK inhibitors p21Cip1/WAF1 and p27Kip1 is interesting and possibly changes in that stoichiometry could play a pivotal role in the HCaRG-associated G2M phase arrest.

The HEK-293 cells release a basal level of ANP-like immunoreactivity (2, 16). In the present study, HCaRG overexpression caused a 4.5- to 8-fold increase in ANP levels and increased size and protein content. The increased levels of ANP may be a consequence of the induction of differentiation per se or, alternatively, may be a marker of hypertrophy, as is well known to occur in myocytes (5). The distal tubular cells of the kidney are known to produce ANP locally for subsequent release to act downstream at the inner medullary collecting duct as part of a paracrine short feedback loop (20). The upregulation of ANP that we observe in HEK-293 cells in response to HCaRG overexpression in vitro may reflect such a paracrine functional response in keeping with this in vivo role of the distal tubular epithelial cells.

Previous studies in vascular smooth muscle cells have demonstrated the role of p27Kip1 in mediating ANG II-induced hypertrophy (1). ANP has been shown to antagonize ANG II-induced renal tubular cell hypertrophy (at the G1/S phase) through mechanisms involving the downregulation of p27Kip1 (15). It is possible to hypothesize that the elevated ANP release in HEK-293 cells due to HCaRG overexpression exerts an autocrine action to offset G1/S arrest by downregulating p27Kip1 levels. In vivo, it is known that local renal ANP levels are modulated in the kidney in response to changes in the local renin angiotensin system in a blood pressure-independent manner. In the model of renal compensatory hypertrophy, increased local renal ANP was sug-
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