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HCaRG increases renal cell migration by a TGF-α autocrine loop mechanism

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El Hader, Carlos, Sandra Tremblay, Nicolas Solban, Denis Gingras, Richard Béliveau, Sergei N. Orlov, Pavel Hamet, and Johanne Tremblay. HCaRG increases renal cell migration by a TGF-α autocrine loop mechanism. *Am J Physiol Renal Physiol* 289: F1273–F1280, 2005. First published July 20, 2005; doi:10.1152/ajprenal.00103.2005.—We have shown previously that the hypertension-related, calcium-regulated gene (HCaRG) is involved in the control of renal cell proliferation and differentiation (Devlin AM, Solban N, Tremblay S, Gutkowska J, Schurch W, Orlov SN, Lewanczuk R, Hamet P, and Tremblay J. *Am J Physiol Renal Physiol* 284: F753–F762, 2003). To determine whether HCaRG plays a role in kidney repair after injury, we extended our studies on the cellular function of HCaRG by comparing cell migration of two kidney cell lines [HEK293 and Madin-Darby canine kidney (MDCK)-C7] stably transfected with the plasmid alone or with a plasmid containing HCaRG cDNA. HCaRG-expressing HEK293 cells, which undergo lower proliferation, migrated faster than control cells and presented greater adhesiveness to the extracellular matrix. Faster migration was also observed for the MDCK-C7 cells, after they were stably transfected with HCaRG cDNA. HCaRG overexpression induced major morphological changes in HEK293 cells, including the formation of lamellipodia. Expression microarrays of HCaRG-expressing HEK293 cells revealed the elevated expression of several genes known to be involved in cell migration and lamellipodia formation, including transforming growth factor-α (TGF-α), galectins, autotaxins and fibronectin. These cells exhibited augmented synthesis and release of activated TGF-α. Conditioned medium from HCaRG-expressing cells stimulated the migration and induced significant morphological changes in control cells, in part, through activation of the TFG-α/EGF receptor. Together, these data support a role for HCaRG in kidney repair after injury through its effect on renal cell migration and TGF-α secretion.

hypertension-related, calcium-regulated gene; cell motility; kidney repair; lamellipodia; microarrays; kidney 293 cells; Madin-Darby canine kidney cells

ACUTE RENAL FAILURE (ARF) is of major clinical importance as it is associated with high morbidity and mortality (2). ARF may result from tubular necrosis, ischemia-reperfusion injury, or physical obstruction of renal blood flow. The kidney has the capacity to regenerate after injury, and its function may return to normal if the cause of ARF is diagnosed and corrected. The recovery of renal function parallels kidney organogenesis. It requires dedifferentiation of renal epithelial cells with a wave of proliferation, followed by the migration of cells to the damaged region of the kidney, their differentiation, and remodeling of the extracellular matrix (2, 20). For each of these steps, upregulation and downregulation of genes, protein synthesis, and entry into the cell cycle are necessary for complete kidney repair (20).

The hypertension-related, calcium-regulated gene (HCaRG) is negatively controlled by extracellular calcium and codes for an intracellular protein of 27 kDa (23). HCaRG is expressed in almost all tissues studied, with preponderance in the kidney. In situ hybridization has localized its expression in the tubular fraction of the kidney (23). We have produced two stable kidney cell lines overexpressing HCaRG to help us identify HCaRG function, the human embryonal kidney (HEK293) and the renal collecting duct-derived, Madin-Darby canine kidney (MDCK)-C7 cell lines. We have previously reported that HCaRG overexpression reduced HEK293 cell proliferation in association with cell arrest in the G2M phase of the cycle (3). Phenotypic characteristics of cellular differentiation were observed, including cell hypertrophy, microvilli, and the appearance of differentiated junctions. The transfected HEK293 cells also acquired a secretory phenotype, as demonstrated by an eightfold increase in the secretion of atrial natriuretic peptide (ANP; or urodilatin, the renal form of ANP) in HCaRG-overexpressing cells (3).

In an in vivo model of ischemia-reperfusion injury, HCaRG expression was negatively correlated with the proliferative status of the cells. HCaRG mRNA levels declined rapidly after reperfusion and increased above baseline thereafter. The expression pattern of the protooncogene c-*myc*, which is associated with proliferation of mammalian cells, was shown to be regulated in a way opposite to HCaRG (23).

Based on our previous studies suggesting a potential role of HCaRG in the proliferation and differentiation response to ischemia-reperfusion injury, the aim of this work was to investigate the effect of HCaRG on the third major event involved in kidney repair, i.e., cell migration. We examined the influence of HCaRG expression on HEK293 cell morphology, migration, and adhesion. This work led us to identify a novel role for HCaRG protein in renal cell migration.

MATERIALS AND METHODS

Stable transfection and cell culture. HEK293 and MDCK cells were transfected with control plasmid (pcDNA1/Neo; Invitrogen) or with the plasmid encoding HCaRG by a standard calcium phosphate coprecipitation method (HEK293) or by the Fugene method (MDCK), and stable transfectants were selected using 400 μg/ml G418 (HEK293) or 800 μg/ml G418 (MDCK). The clones expressing the highest levels of HCaRG, HCaRG clone 9 from HEK293, cells and

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HCaRG clone 15 from MDCK cells were used in this experiment, whereas clones transfected with vector alone, Neo clone 1 from HEK293 cells and Neo clone 3 from MDCK cells, served as controls. Stable transfectants were maintained in high-glucose DMEM$^+$ (HEK293 cells) or low-glucose DMEM$^-$ (MDCK cells) containing 10% FBS and 2 mM glutamine with 400 μg/ml G418. Wild-type HEK293 and MDCK cells were kept under identical conditions but without the selection antibiotic G418. All cell types were maintained in 5% CO_2 at 37°C.

Immunofluorescence microscopy. Glass coverslips were coated with poly-D-lysine at a final concentration of 10 μg/ml under sterile conditions. Cells were cultured in DMEM containing 10% FBS. Cells were then fixed with 4% paraformaldehyde, heated at 37°C for 15 min, and permeabilized with 0.075% saponin for 10 min. The cells were blocked with PBS containing 2% BSA and 0.2% gelatin. After labeling, the coverslips were mounted in Vectashield and viewed with a Zeiss Axioskop fluorescent microscope equipped with a ×40 Planapochromat objective and selective filters for fluorescein isothiocynate, 4′,6′-diamidino-2-phenylindole, and Texas red.

Boyden chamber assay. Migration was measured on fibronectin, collagen, or laminin matrixes in Transwell chambers with 8-μm-pore-size filters (Costar). Both the bottom and top of the filters were coated, and the lower chambers were filled with DMEM+10% FBS. An equal number of cells (1×10^5/ml) were loaded in the top chamber of each well. After 6 h, nonmigrating cells were scraped from the upper surface of the filter with a cotton swab. Cells on the lower surface were fixed with 10% formalin/phosphate and stained with 0.1% crystal violet/20% methanol for 20 min. Migration activity was quantified by computer-assisted imaging via the average density of migrated cells/4 fields.

Wound-healing assay. For wound-healing assays, stably transfected cells were seeded at a high density, 1×10^6 cells, on poly-D-lysine-coated coverslips and wounded 1 day later when the cells formed a confluent monolayer. Scraping a pipette tip through the cell monolayer created the wound. The cells were treated with mitomycin C, transforming growth factor (TGF)-α, or AG-1478 for 24 h and fixed for 15 min with 80/20% methanol/acetone at −20°C, and the coverslips were viewed with a ×4 Planapochromat objective. Quantitative wound closure measurements were recorded for control ($t = 0$) and treated wounds after 24 h. Three randomly chosen regions of a single wound (each 2 cm long) were photographed at ×4 magnification. Mean wound width was measured, and average percent wound closure was calculated.

Cell proliferation assay. Stably transfected or control cells were inoculated in DMEM containing 10% FBS in poly-D-lysine-coated plates. The counting mode served to assess cell proliferation. The cells were trypsinized and suspended in 1 ml complete medium. A portion (100 μl) of the cell suspension was subjected to counting with a hemocytometer. Four counts were taken for each data point.

Cell adhesion assay. Tissue culture plates (96 well) were coated for 2 h at 37°C with fibronectin at 10 μg/ml. Subsequently, an equal number of cells (1×10^5/ml) in a FBS-free medium were added per well and allowed to adhere for 5, 10, 20, 30, 45, and 60 min. The adherent cells were fixed with 200 μl of cold 70% ethanol, stained with 0.1% crystal violet, and then washed three times with water. Finally, the cells were incubated for 1 h with 2% SDS to solubilize crystal violet. Absorbance was measured at 562 nm.

Immunoblot analysis. Equal amounts of protein extracts in SDS-lysis buffer were subjected to 7.5 or 12% SDS-PAGE analysis and transferred electrophoretically to a nitrocellulose membrane. The lysis buffer consisted of cold PBS containing 1% SDS, 5 mM EDTA, 0.2 mM orthovanadate, 40 mM β-glycerophosphate, and protease inhibitors (1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.1 mM PMSF). The labeled bands were revealed by chemiluminescence (ECL reagent) and exposed to Kodak biomax light film.

Gene expression profiling. We identified differentially expressed genes between HCaRG9 and Neo1 cells, representing unique genes or expressed sequence tags (EST). HCaRG and Neo clones were synchronized and grown in the presence of 10% FBS for 48 h. Total RNA was isolated with a mini RNeasy kit according to the manufacturer's protocol (Qiagen). Biotinylated, amplified target cRNA was prepared and hybridized to a human U133A array (22,283 transcripts) or human U133 plus 2.0 array (54,675 transcripts) as described by Affymetrix. After hybridization, the microarray chips were washed, stained, and scanned. Chips were first normalized with GCOS1.2 using all probe sets scaling option and a target signal at 500, then normalized again to the 50th percentile employing GeneSpring 7.0 software. For each of the raw data probe sets, a perfect match vs. mismatch value was calculated, and the data were compiled into 1 value for each probe set. The final step in the filtering process was the removal of genes and EST not expressed in either of the clones being compared. The data of this analysis, which were replicated three times, have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE2555.

RT-PCR. Total RNA was isolated as described for the microarray analysis. One microgram was reverse transcribed into cDNA at 37°C for 60 min using Maloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo dT primers according to the manufacturer's protocol. PCR primers for the selected genes were designed based on published sequences in GenBank: autotaxin (sense: 5′-GTAGGTTATGGCTCAACATT, antisense: 5′-GGCTCTACCTTATCATCACA); TGF-α (sense : 5-′CTGCCATTCTGGGTACGTTG, antisense: 5′-CTGAGTGGCAGCAAGCGGTT); and fibronectin (sense : 5-′CAGTGGGAGCGGACCTACCT, antisense 5′-GCGATGGTACAGCTTATTCT). Four microliters of the RT reaction were added to 20 μl PCR reaction. For a semiquantitative analysis, Quantum RNA 18S internal standards (Ambion) were used. The linear range for each gene was determined and the optimal ratios of 18S primers: competimers were chosen. Ethidium bromide staining of the PCR product was detected with a Typhoon imager and evaluated with the Scillon image program after background correction. To calculate the gene fold-change, the value of each gene was divided by the 18S value in HCaRG and control cells and the mean of three HCaRG (gene 18S ratios) was compared with the mean of three Neo cells (gene 18S ratios).

TGF-α ELISA and drug treatment. TGF-α protein levels were evaluated with the Quantikine human TGF-α immunoassay according to the manufacturer's protocol (R&D Systems). For the drug treatment, cells were treated for 24 h with 4 μg/ml of the DNA synthesis inhibitor mitomycin C (Sigma), increasing doses of human TGF-α (Sigma), or increasing doses of the selective inhibitor of epidermal growth factor protein (AG-1478, Sigma) or 0.1 μg/ml of anti-TGF-α-neutralizing antibody (Abcam).

RESULTS

HCaRG increases the migration of HEK293 and MDCK cells. HEK293 cells were stably transfected with the control plasmid (Neo1) or with a plasmid containing rat HCaRG (HCaRG9) as described previously (3). The effect of HCaRG overexpression on cell migration was evaluated using a modified Boyden chamber assay and the wound closure test. In the Boyden chamber system, using serum as a chemoattractant, HCaRG overexpression caused a 3.5-fold increase in the migration of HEK293 cells (Fig. 1*A*). This positive influence of HCaRG on cell motility was confirmed by wound closure assay (Fig. 1*B*). After mechanical wounding, HCaRG9 migrated into the wound area, and ~50% of the wound was closed within 24 h. On the other hand, in Neo1 cultures, the wounds remained open (only 6% closure) after 24 h. This does not appear to be due to differences in cell proliferation as the addition of mitomycin C (4 μg/ml), an inhibitor of cell division, to the medium only slightly affected HCaRG9 migration or the difference between the two cell types (Fig. 1*B*).

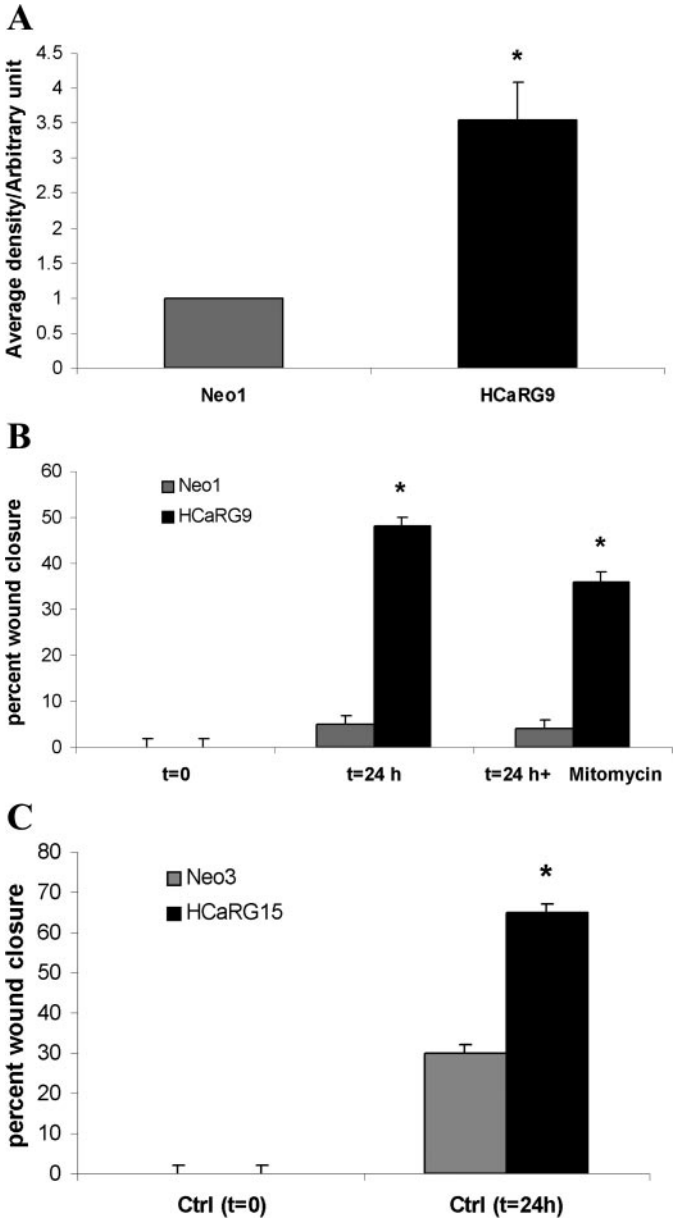

Fig. 1. Hypertension-related, calcium-regulated gene (HCaRG) activates cell migration. *A*: cells were plated at 1×10^5/insert on 8-μm-pore membranes in Transwell chambers. The membranes were coated with fibronectin (10 μg/ml). After incubation for 6 h in a CO_2 incubator, the cells that had migrated to the underside of the membranes were fixed and stained. Migration activity was quantified by computer-assisted imaging. Values are means ± SE of 4 independent experiments. $*P < 0.01$. Confluent HCaRG9 and Neo1 (*B*) and HCaRG15 and Neo3 (*C*) cultures were wounded with a pipette tip. Wound closure was monitored by microscopy. Micrographs were taken immediately ($t = 0$ h) or $t = 24$ h after wounding. Values are means ± SE of 3 independent experiments performed in triplicate. $*P \leq 0.01$.

The effect of HCaRG on cell migration was not restricted to the HEK293 cells, as the migration potential of HCaRG-expressing MDCK cells (HCaRG15) was also higher (65% closure at 24 h) than that of MDCK control cells (Neo6, 30% closure at 24 h) (Fig. 1*C*).

Figure 2*A* shows that after an initial time lag of 24 h, the wound closure of Neo1 cells (grey data points) increased with time in culture, with a closure time of 96 h. In contrast, HCaRG9 cultures presented a reduced time lag and a closure time of 48 h, indicating that HCaRG exerted its positive effect on cell migration in the initial phase of the wound repair process.

To investigate whether cell proliferation could differentially affect the wound closure of HCaRG9 and control cells, a time-dependent proliferation assay was performed. As seen in Fig. 2*B*, there was a gradual increase in cell number after 12 h for both clones, but as reported previously, we observed higher cell proliferation of Neo1 cells compared with HCaRG9 cells at 48 h (3, 23). Thus cell proliferation could contribute more to the wound closure of Neo1 cells after 48 h, whereas the more rapid wound closure of HCaRG cells, which was visible before 48 h, was mainly due to their enhanced motility.

HCaRG increases the adhesion of HEK293 cells to extracellular matrix proteins. To establish whether HCaRG is involved in the attachment step of cell migration, we evaluated the adhesion capacity of cells on fibronectin. After 5 min of culture, HCaRG-overexpressing cells showed 20% of maximal adherence, whereas none of the Neo1 cells attached to the extracellular matrix at this time point (Fig. 3*A*). Maximal attachment was observed after 20 min of culture for HCaRG9 and 120 min for Neo1. After 20 min, the number of Neo1 cells attached was only 45% of HCaRG9 cells. Similar results were obtained with collagen and laminin extracellular matrixes (data not shown). The localization of vinculin, a focal adhesion

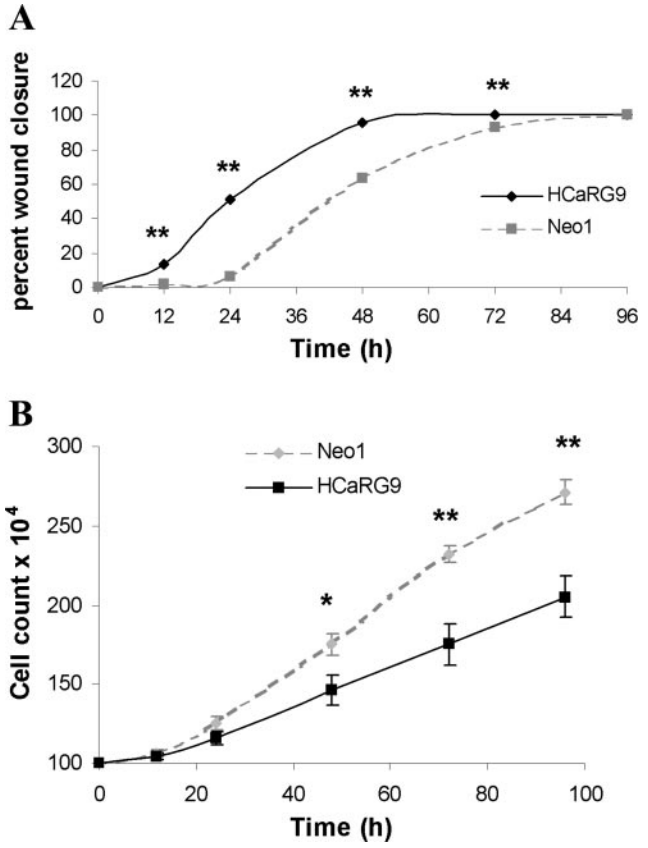

Fig. 2. Time course of Neo1 vs. HCaRG9 migration and proliferation. *A*: confluent HCaRG9 and Neo1 cultures were wounded with a pipette tip. Wound closure was monitored by microscopy. Micrographs were taken immediately and at different time points until 96 h after wounding. *B*: cells were counted at different time intervals after trypsinization. Values are means ± SE of 3 independent experiments performed in triplicate. $*P \leq 0.05$. $**P \leq 0.01$.

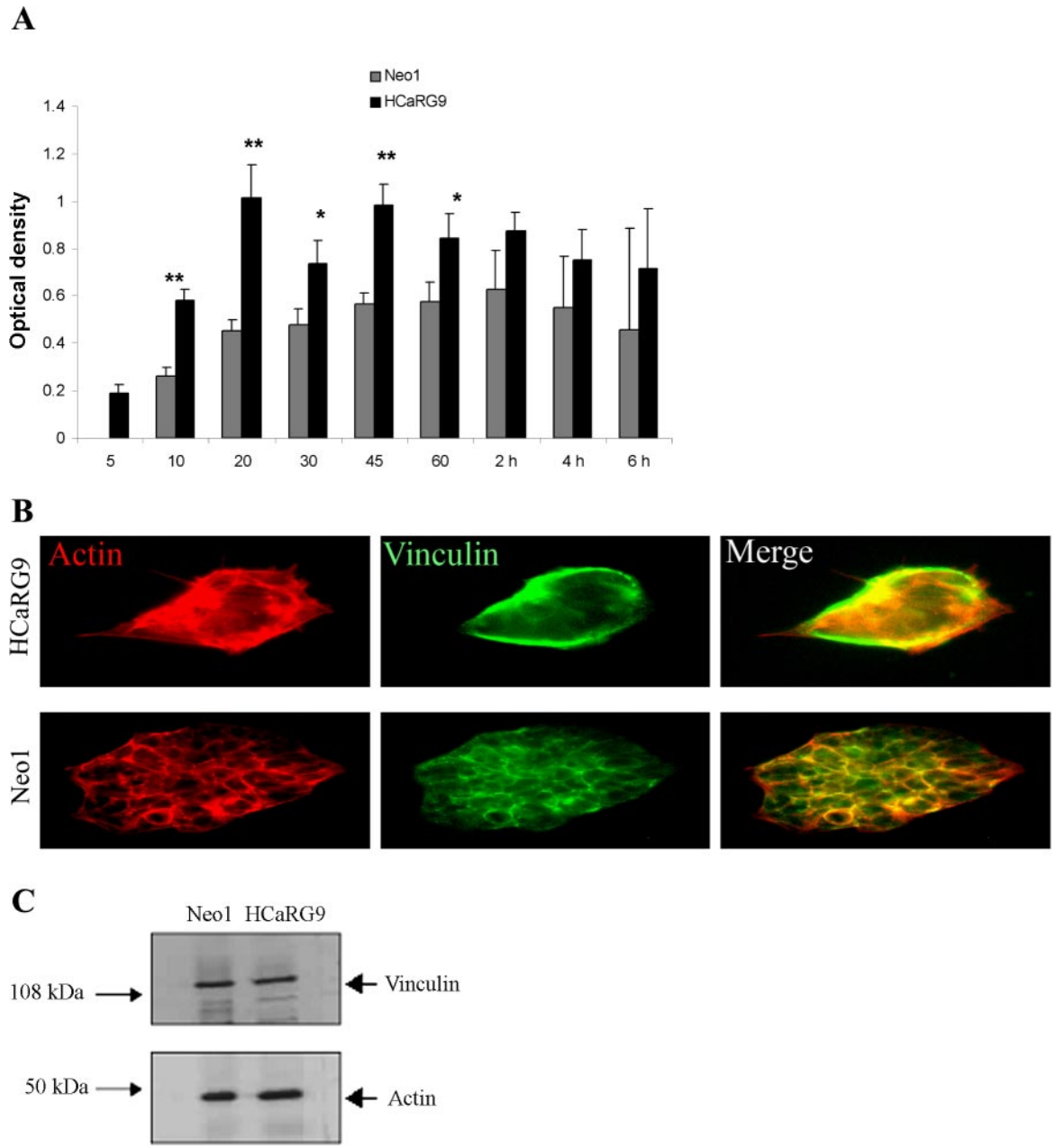

Fig. 3. *A*: effect of HCaRG on attachment of HEK293 cells. Cells were plated onto 96-well plates precoated with fibronectin (10 μg/ml). After incubation at 37°C and according to a fixed time scale, the attached cells were stained with 0.1% crystal violet, resuspended in SDS, and absorbance was measured with a microplate reader at 562 nm. The results are the average of 4–7 independent experiments ± SE, each with 3 replications. *$P < 0.05$. **$P < 0.01$. *B*: vinculin localization. HCaRG9 and Neo1 cells were cultured on coverslips for 24 h. Double immunofluorescence staining was then performed for F-actin (rhodamine-conjugated phalloidin) and vinculin (rabbit polyclonal antibody followed by FITC-conjugated goat anti-rabbit antibody). *C*: actin and vinculin levels in HCaRG9 and Neo1 cells. Equalized protein samples were subjected to reducing 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then probed with anti-vinculin (first row) and anti-actin (second row).

molecule, was also found to be modified in HCaRG-overexpressing cells. Whereas Neo1 cells displayed diffuse vinculin expression, vinculin concentrated at the cell membrane in HCaRG cells (Fig. 3*B*) with no apparent changes in its level of expression (Fig. 3*C*). Thus these results indicate that HCaRG increased the adhesiveness of HEK293 cells.

HCaRG overexpression induces lamellipodia formation. Lamellipodia are thin protrusive sheets that dominate the leading edges of many motile cells. HCaRG overexpression induced major morphological changes in HEK293 cells as seen in Fig. 4. These morphological alterations included multiple lamellipodia presentation and the formation of actin-rich domains at their tips. The morphology of parental HEK293 cells was similar to that of vector-only transfected cells (Fig. 4*A*). For both cell types, a fusiform appearance and colony formation with cell-cell contact were noted after cell synchronization in 0.2% FBS medium (Fig. 4*B*). By contrast, HCaRG-overexpressing cells exhibited lamellipodia-lamellipodia contact under the same synchronization condition. This HCaRG-dependent actin remodeling took place without any increase in actin expression (Fig. 3*C*).

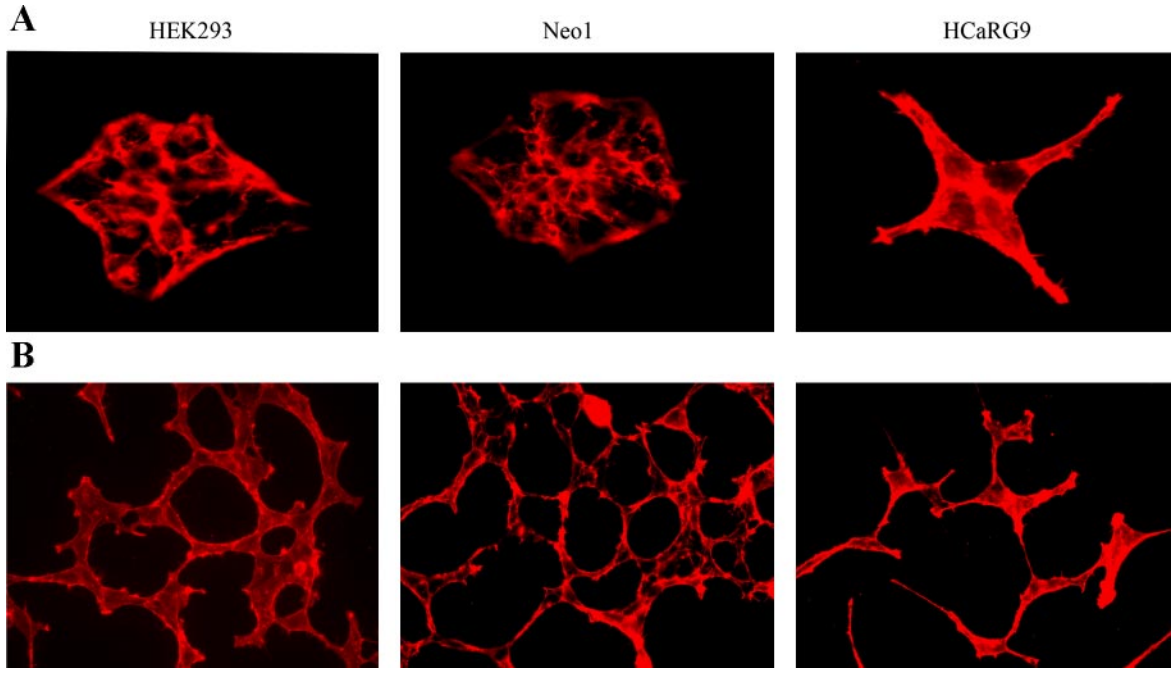

Fig. 4. Morphological changes in HCaRG-transfected HEK293 cells in 10% (*A*) or 0.2% FBS medium (*B*). The morphology of parental HEK293 cells did not differ significantly from that of empty vector-transfected cells. Morphological changes (multiple lamellipodia presentation and the formation of actin-rich domains at the tips of lamellipodia) were observed in the HCaRG-transfected cells. All cells are shown at ×40 magnification.

Identification of genes differentially expressed between HCaRG-overexpressing cells and control cells. We used Affymetrix GeneChips to compare genes differentially expressed between HCaRG-overexpressing cells (HCaRG9) and control cells (Neo1). Those genes are involved in many cellular processes, including proliferation, differentiation, metabolism, adhesion, and migration. Genes of interest to the current study are shown in Table 1, and some of them were confirmed by quantitative PCR (Fig. 5). Genes that showed consistent overexpression in HCaRG9 cells compared with Neo1 cells were autotaxin (4.25-fold), TGF-α (3.45-fold), fibronectin 1 (2.73-fold), fibronectin precursor (2.70-fold), dynein (2.51-fold), and galectin 1 (1.71-fold). These genes have been reported to contribute to cell migration and adhesion.

Conditioned medium from HCaRG cells increases migration of control cells. Because some upregulated genes in HCaRG9 cells can induce the secretion of motogenic factors, such as TGF-α, we next examined the effect of a conditioned medium of HCaRG9 on the migration of Neo1 cells. As the main difference in cell migration between HCaRG and Neo1 cells occurs during the lag phase within the first 24 h of the wound-healing assay, we added the HCaRG9-conditioned medium to Neo1 cell cultures at 12 h. Neo1 cells incubated with the HCaRG9 medium accrued morphological changes (Fig. 6*A*) and presented 18% wound closure, whereas Neo1 cells in their own medium showed only 5% wound closure (Fig. 6*B*). These results indicate that HCaRG-overexpressing cells secreted a factor(s) that accelerated the initiation of migration. One of these factors could be TGF-α, which was found to be more expressed in HCaRG9 (Table 1 and Fig. 5). TGF-α protein levels were evaluated in the culture medium from Neo1 and HCaRG9 cells, and we found that HCaRG9 cells secreted more TGF-α than did Neo1 (70 vs. 40 pg TGF-α/1,000 cells).

TGF-α is partly involved in the increased migration of HCaRG-overexpressing cells. TGF-α was shown to be overexpressed in HCaRG9 cells, suggesting that it could play a role in HCaRG-induced migration. To examine this possibility, we postulated that TGF-α could increase Neo1 cell motility. We

Table 1. *Genes known to be involved in cell migration and more expressed in HCaRG9 cells*

Gene Name	Accession No.	Neo1	HCaRG9	Fold-Increase
Autotaxin	L35594	55.57±11.62	236.47±39.85*	4.25
TGF-α	NM_003236	31.80±9.26	109.87±25.44	3.45
Fibronectin	AF_130095	1,870.67±143.04	5,114±177.28*	2.73
Fibronectin precursor	X02761	1,547.67±204.55	4,191±467.37*	2.70
Dynein	NM_004411	330.17±55.32	830.57±197.59	2.51
Galectin-1	NM_002305	3,046.40±2,458.47	5,218.67±2,062.09	1.71

Values are means ± SE. Selected genes showing a consistent upregulation in hypertension-related, calcuim-regulated gene (HCaRG)9 cells compared with Neo1 cells in 3 different RNA isolations analyzed using 3 arrays are shown in the reverse order of the fold-increase. Raw data were normalized using GeneSpring 7.0 software to the 50th percentile of all measurements on the chip. For TGF-α, dynein, and galectin, the increase was marginally significant ($P = 0.054$) by the Wilcoxon signed rank test (1-tailed). *$P < 0.05$ by paired-*t*-test (1-tailed).

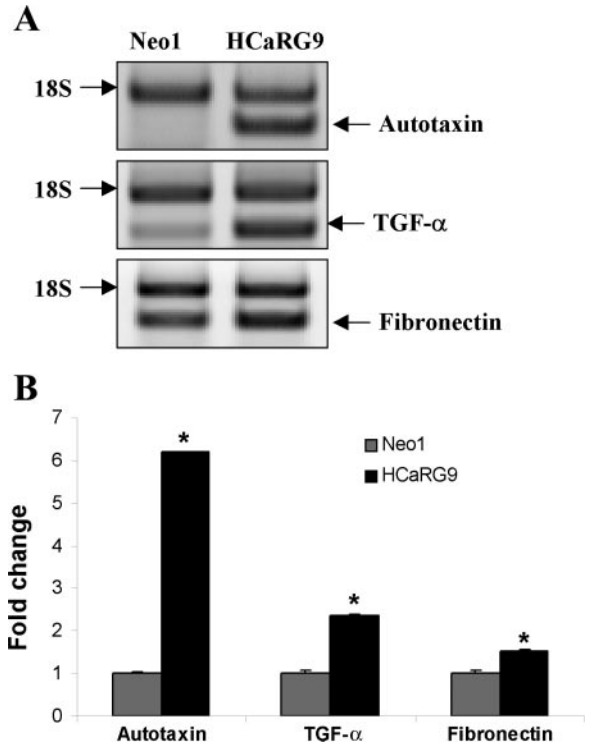

Fig. 5. RT-PCR confirmation for selected genes whose expression levels increase in HCaRG cells. Representative figures (*A*) and average of fold-change (*B*) between Neo1 and HCaRG9 for TGF-α, autotaxin, and fibronectin are shown. Values are means ± SE of 3 independent experiments. * $P \leq 0.01$.

treated both clones with increasing concentrations of TGF-α and tested the effect on the cell migratory potential and morphology. As expected, TGF-α dose dependently increased Neo1 cell motility, which thus reached 43% of wound closure after 24 h (Fig. 7*A*) but with no changes in their morphology (data not shown). In contrast, no significant effect was found on HCaRG9 cell motility after TGF-α treatment (Fig. 7*A*).

We then blocked the TGF-α effect on cell migration with the EGFR tyrosine kinase inhibitor AG-1478. The inhibitor dose dependently decreased the percentage of wound closure by HCaRG9 cells (Fig. 7*B*), whereas TGF-α blockade only slightly affected the motility of Neo1 cells in their own medium (Fig. 7*B*). Furthermore, addition of neutralizing anti-TGF-α antibody to the medium inhibited the migratory potential of both Neo1 and HCaRG9 cells with a more prominent effect on the latter. It also abolished the effect of HCaRG-conditioned medium on Neo1 cell migration (Fig. 7*C*). These results suggest that TGF-α plays an important role in the increased migration capacity caused by HCaRG overexpression most probably via its autocrine/paracrine interaction with the EGFR.

DISCUSSION

Molecular mechanisms underlying the recovery of renal cell function are complex and still poorly understood. It is proposed that after an injury, the surviving cells repolarize and/or dedifferentiate, proliferate, and migrate to the damaged region, then differentiate to restore kidney integrity and function (20). In each of the steps involved, activation or inhibition of many genes must be highly controlled to allow kidney recovery.

Our previous studies point to the involvement of HCaRG in two major processes contributing to kidney repair, i.e., control of cell proliferation and differentiation. In the present work, we tested the hypothesis that HCaRG can also be a major player in a third cellular event important for kidney regeneration: cell migration. Here, we demonstrate a positive effect of HCaRG on renal epithelial cell migration via its influence on TGF-α synthesis.

HCaRG increases HEK293 cell motility in two different paradigms, the Boyden chamber and wound-healing assays. The higher migratory potential of HCaRG-overexpressing cells was confirmed in the collecting duct-derived MDCK cells. Regulation of cell migration involves cell adhesion and actin reorganization. Adhesion is the first step in cell migration, but their association is complicated. Indeed, to have a migration phenotype, adhesion should be strong enough to permit attachment of the cell to the extracellular matrix, but, on the other hand, too strong an adhesion would inhibit motility (7, 25). HCaRG appears to increase the initial attachment of cells to the

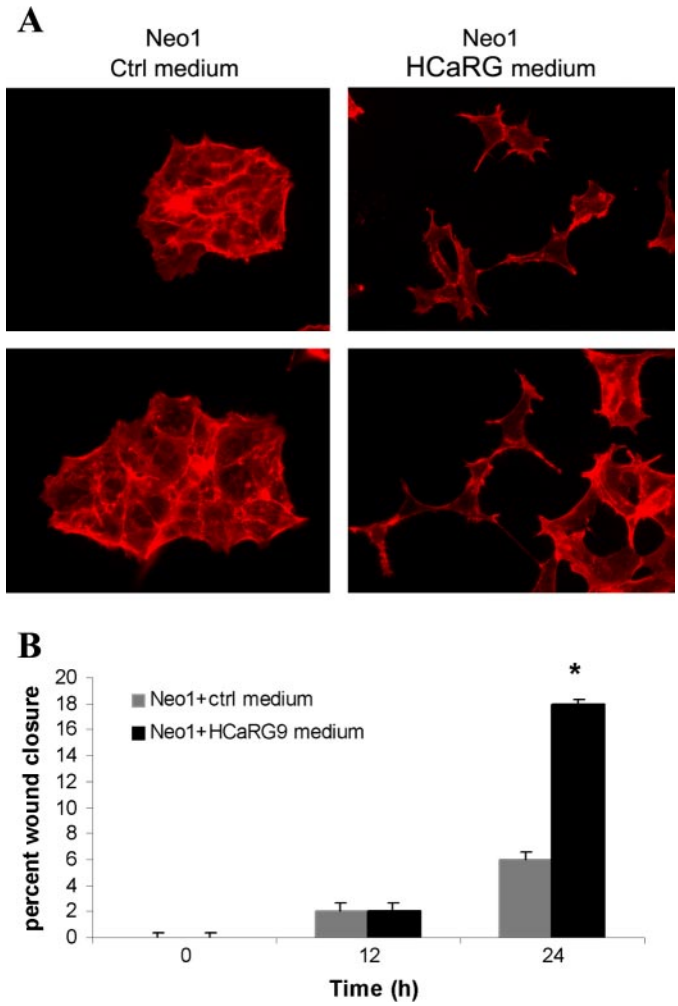

Fig. 6. Effect of conditioned medium from HCaRG9 cells on Neo1 cell morphology from 2 different fields (Neo1+ctrl medium and Neo 1+HCaRG9-conditioned medium; *A*) and migration potential (*B*). Neo1 cells incubated with control medium or conditioned medium from HCaRG cells were subjected to wound healing for 24 h. Significant cell migration is seen on addition after 12 h of HCaRG-conditioned medium. Values are means ± SE of 3 independent experiments performed in triplicate. *$P \leq 0.01$.

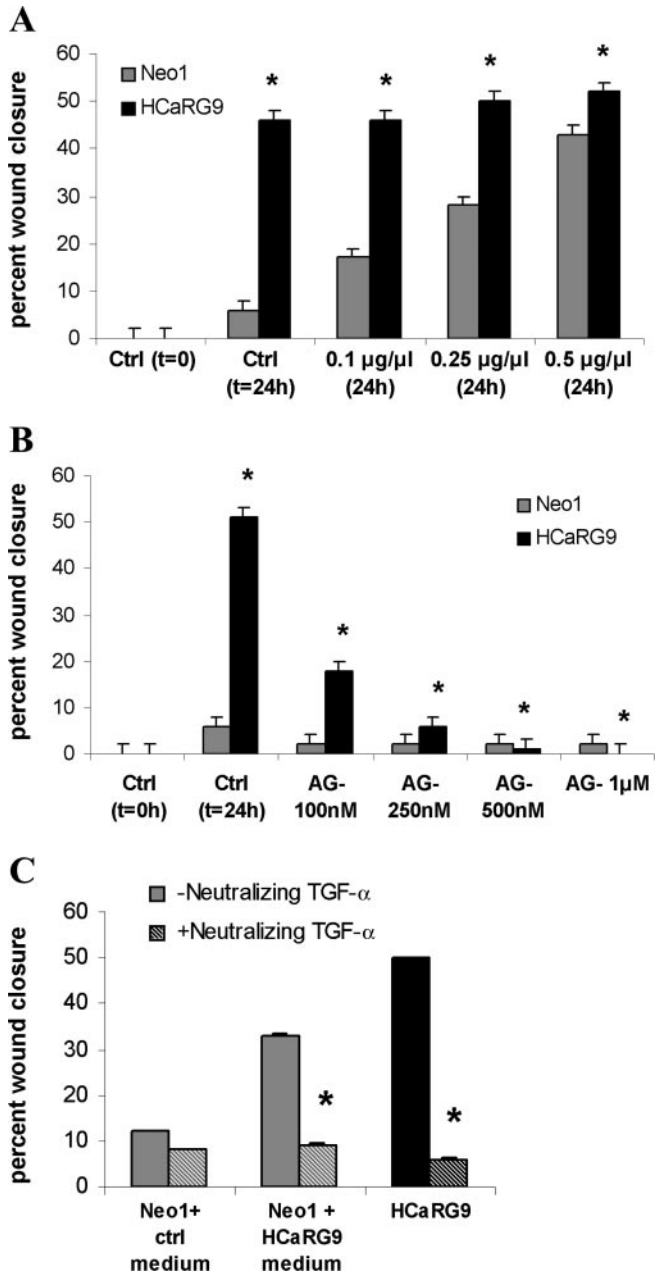

Fig. 7. Effect of TGF-α activation and neutralization and EGFR inhibition on cell migration. The migratory rate of HCaRG9 and Neo1 cells in a wounding assay was determined after 24 h of treatment by measuring wound width as a function of 0.1–0.5 μg/μl of TGF-α (*A*) or 0.1–1 μM AG-1478 (AG-; *B*) and 0.1 μg/ml anti-TGF-α-neutralizing antibody (*C*). Values are means ± SE of 3 independent experiments performed in triplicate. $*P \leq 0.01$.

extracellular matrix, giving them the strength to generate forward traction for migration.

The driving force for cellular migration is dependent on actin reorganization of the cytoskeleton. The increased motility of cells overexpressing HCaRG is accompanied by morphological changes, including lamellipodia presentation and the formation of actin-rich domains at their tips. In addition to its effect on cytoskeleton organization, we wanted to know whether HCaRG overexpression would lead to modulation of the expression of genes involved in cell motility. Microarray analysis revealed upregulation of several interesting genes in regard to their reported positive effect on cell migration, such as TGF-α, autotaxin, galectin 1, fibronectin 1, and fibronectin precursor (4, 6, 8, 10, 12–16, 19, 26). We will have to confirm that these differences are also observed at the protein level, but it is interesting to note that autotaxin is an autocrine motility factor implicated in neoplastic invasion and metastasis (24). It is involved in actin reorganization, focal adhesion turnover, and lamellipodia formation. Focal adhesion and lamellipodia formation can also be regulated by fibronectin and galectin-1 localized at the cell surface, all of them targeting FAK, which has also been linked to the PAK pathway. On the other hand, dynein is a protein responsible for short microtubule transport toward the periphery of migrating cells (1, 17). TGF-α, which is overexpressed in HCaRG9 cells, belongs to the EGF family and promotes its effects after binding to EGFR (27). Activation of EGFR either by EGF or TGF-α has been linked to the stimulation of cell motility in different cell types, such as kidney cells or breast cancer cells (11, 22).

It is not surprising that some of these genes overexpressed in HCaRG9 cells code for secreted motility factors, because our previous studies have revealed an increase in the secretory capacity of HEK293 cells overexpressing HCaRG (3). To confirm the involvement of such secreted factors in the motility phenotype of HCaRG9, conditioned medium from cells overexpressing HCaRG was tested on control cells, and its effect was evaluated on cell morphology and migration. Conditioned medium from HCaRG9 had a major impact on the morphology of Neo1 cells, giving them a migratory phenotype with the formation of lamellipodia. Moreover, the motogenic potential of Neo1 cells was augmented by 2.7-fold in the presence of conditioned medium from HCaRG9 cells. This suggests that the positive effect of HCaRG overexpression on cell motility can be partly explained by the secretion of motogenic proteins that can act as autocrine or paracrine factors.

TGF-α is involved in the early migration phase of cells overexpressing HCaRG. However, we cannot rule out the possibility that other secreted factors present in the conditioned medium can contribute to the increased migration potential of HEK293 cells overexpressing HCaRG. We have already reported a major elevation of ANP secreted from HCaRG9 cells (3). Depending on the cell type, ANP can have both positive and negative effects on cell migration (5, 9, 21). It is possible that secreted ANP promotes the motility of HCaRG9 cells and that more than one pathway contributes to the migratory phenotype.

This study shows the presence of a new player, HCaRG, in the control of cell migration. A knockout mouse for HCaRG will help us to confirm its implication in the process of kidney cell repair.

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