MicroRNA-214 Reduces Insulin-like Growth Factor-1 (IGF-1) Receptor Expression and Downstream mTORC1 Signaling in Renal Carcinoma Cells*

Elevated IGF-1/insulin-like growth factor-1 receptor (IGF-1R) autocrine/paracrine signaling in patients with renal cell carcinoma is associated with poor prognosis of the disease independent of their von Hippel-Lindau (VHL) status. Increased expression of IGF-1R in renal cancer cells correlates with their potency of tumor development and progression. The mechanism by which expression of IGF-1R is increased in renal carcinoma is not known. We report that VHL-deficient and VHL-positive renal cancer cells possess significantly decreased levels of mature, pre-, and pri-miR-214 than normal proximal tubular epithelial cells. We identified an miR-214 recognition element in the 3′UTR of IGF-1R mRNA and confirmed its responsiveness to miR-214. Overexpression of miR-214 decreased the IGF-1R protein levels, resulting in the inhibition of Akt kinase activity in both types of renal cancer cells. IGF-1 provoked phosphorylation and inactivation of PRAS40 in an Akt-dependent manner, leading to the activation of mTORC1 signal transduction to increase phosphorylation of S6 kinase and 4EBP-1. Phosphorylation-deficient mutants of PRAS40 and 4EBP-1 significantly inhibited IGF-1R-driven proliferation of renal cancer cells. Expression of miR-214 suppressed IGF-1R-induced phosphorylation of PRAS40, S6 kinase, and 4EBP-1, indicating inhibition of mTORC1 activity. Finally, miR-214 significantly blocked IGF-1R-forced renal cancer cell proliferation, which was reversed by expression of 3′UTR-less IGF-1R and constitutively active mTORC1. Together, our results identify a reciprocal regulation of IGF-1R levels and miR-214 expression in renal cancer cells independent of VHL status. Our data provide evidence for a novel mechanism for IGF-1R-driven renal cancer cell proliferation involving miR-214 and mTORC1.

Renal cell carcinoma (RCC)* accounts for nearly 3% of all malignancies. Among the five histologic subtypes, clear cell renal carcinoma accounts for about 85% of all RCCs (1, 2). About 30% of patients show renal cancer metastasis to lung, liver, bone, and brain at the time of diagnosis, and half of the remaining patients eventually develop metastasis (3–5). Individuals bearing germ line mutation in the von Hippel-Lindau (VHL) tumor suppressor gene located on chromosome 3p have increased risk for clear cell RCC. Inherited forms of RCC occur when the remaining wild type VHL allele is lost.

Apart from inactivated VHL-driven tumorgenesis, IGF-1 signal transduction significantly contributes to the growth of RCC cells in vitro and in vivo in animal models (6, 7). In fact, increased IGF-1 mRNA and protein levels in the kidney are significantly higher in RCC in humans (8, 9). Similarly, IGF-1 receptor (IGF-1R) expression has also been shown to be significantly associated with increased risk of RCC (10, 11). Also, patients with IGF-1R-positive RCC showed significantly reduced survival rates (12, 13). The dimeric IGF-1R shares significantly high homology with insulin receptor. IGF-1R is produced as a single polypeptide, which is cleaved to form the mature α- and β-subunits. The α-protein represents the transmembrane protein with extracellular domain, whereas the β-subunit is exclusively intracellular. The IGF-1 binds to the extracellular domain of α-subunit, resulting in heterotetramerization. Upon ligand binding, conformational change in the juxtamembrane domain induces an increase in tyrosine kinase activity of the β-subunit, which autophosphorylates specific tyrosine residues in the β-subunit. Tyrosine-phosphorylated β-subunit recruits the IRS protein through binding to its N-terminal PTB domain. Receptor-bound IRS protein serves as docking sites for the Src homology 2 domain-containing proteins, which trigger signal transduction to induce tumor growth of RCC mainly by two arms, the Ras/MAPK and phosphatidylinositol 3-kinase/Akt pathways (14, 15). Because of significant homology between IGF-1R and insulin receptor, they can form a hybrid receptor, which binds IGF-1 with an affinity similar to that with IGF-1R heterotramer alone, and can elicit mitogenic signal transduction in tumor cells (14, 15). Therefore,
expression of these receptors in the RCC and availability of the ligands will influence the process of tumorigenesis. Because development of small molecular drugs for inhibition of receptor tyrosine kinases is a field of active research, it is important to consider the therapeutic strategies, which will block both IGF-1R and the hybrid receptors.

MicroRNAs (miRs) are short non-coding RNAs, which silence mRNAs post-transcriptionally in a sequence-specific manner to regulate gene expression. MicroRNAs have emerged to regulate the expression of more than 30% of mRNAs coded by the genome (16, 17). Thus, they contribute to regulation of many physiologic and pathologic processes, including oncogenesis (18). miRNAs are produced as primary transcripts (pri-miRs) by the RNA polymerase II-mediated transcription of inter- as well as intragenic regions of chromosomal DNA (19). pri-miRs are processed in the nucleus by the RNase III activity of Drosha in the microprocessor multiprotein complex to produce short hairpin pre-miRs, which are exported to the cytoplasm by the exportin 5 (19–21). The pre-miRs are then processed by the dicer exonuclease III activity in a complex containing its partner trans-activation-responsive RNA-binding protein to yield RNA duplexes. Unwinding of the duplex RNA generates the guide strand as an ∼22-nucleotide-long mature miRNA (19). Mature miRNA then interacts with the Argonaute 2 to form RNA-dependent silencing complex to bind the 3′UTR of mRNA with imperfect complementarity to induce suppression of translation and degradation.

Expression profiling of miRNAs has been extensively used to understand the progression, development, and invasion of different cancers, including renal cancer (22). Expression of miRNAs has been used to classify the malignant nature of RCC (23). Thus, targeting of specific miRNA(s) may be a therapeutic strategy in RCC. In this study, we identify increased expression of IGF-1R in both VHL-positive and -negative renal cancer cells as compared with the normal proximal tubular epithelial cells. Levels of IGF-1R negatively correlate with the expression of miR-214. We identified a functional miR-214 recognition element in the 3′UTR of IGF-1R mRNA. We report that IGF-1-stimulated Akt kinase and its substrate PRAS40 phosphorylation as well as phosphorylation of mTORC1 substrate 4EBP-1 are necessary for proliferation of renal cancer cells. Finally, we show that miR-214 prevents IGF-1-stimulated renal cancer cell proliferation by targeting IGF-1R and mTORC1.

Experimental Procedures

**Materials**—Recombinant IGF-1, Nonidet P-40, Na2VO4, phenylmethylsulfonyl fluoride, protease inhibitor mixture, and FLAG and β-actin antibodies were purchased from Sigma. Antibodies for phospho-Akt (Ser-473), phospho-Akt (Thr-308), phospho-GSK3β (Ser-9), phospho-mTOR (Ser-2448), phospho-PRAS40 (Thr-246), phospho-S6 kinase (Thr-389), phospho-4EBP-1 (Thr-37/46), phospho-4EBP-1 (Ser-65), Akt, mTOR, IGF-1R β-subunit, PRAS40, and S6 kinase were obtained from Cell Signaling, Boston. GSK3β and phospho-IGF-1R (Tyr-1165/1166) antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-HA antibody was purchased from Covance, Princeton, NJ. Plasmid isolation kit and RNA isolation reagent were obtained from Qiagen (Valencia, CA) and Invitrogen, respectively. FuGENE-HD was purchased from Roche Applied Science. PVDF membrane was purchased from PerkinElmer Life Sciences. MK-2206 was obtained from Selectek Chemicals, Houston, TX. RT2 real time SYBR Green/ROX PCR Master Mix was obtained from SuperArray Biosciences, Frederick, MD. The primers to detect miR-214, U6 (for normalization), mirVANA™ qRT-PCR miRNA detection kit were obtained from Ambion, Austin, TX. Primers to detect human IGF-1R were purchased from Qiagen. Luciferase assay kit was purchased from Promega, Madison, WI. HistoTOP-CMV-miR-214 expression vector was kindly provided by Dr. J. Q. Cheng (H. Lee Moffitt Cancer Center, Tampa, FL). Scramble shRNA vector was obtained from Addgene (plasmid 1864). pMV4EBP-1-μ (T35A, T45A, T69A, and S64A) and constitutively active mTOR (containing the following mutations in four amino acids: V2198A, L2216H, L2260P, and I2017T) plasmids have been described previously (24–26). PRAS40 T146A and shPRAS40 plasmids were purchased from Addgene. The 3′UTR-less IGF-1R expression vector was a kind gift from Dr. Douglas Yee (Masonic Cancer Center, University of Minnesota).

**Cell Culture**—The HK2 normal human proximal tubular epithelial cell has been described previously and grown in DMEM/F-12 (1:1) in the presence of 10% fetal bovine serum (27). The primary human renal proximal tubular epithelial cells (HRPTEC) were purchased from Lonza Inc., Allendale, NJ. These cells were cultured in renal epithelial cell growth medium containing 0.5% serum as suggested by the vendor (Lonza). The ACHN and 786-O renal carcinoma cells were obtained from American Type Culture Collection, Manassas, VA. These cells were grown in RPMI 1640 medium containing 10% fetal bovine serum in the presence of penicillin/streptomycin (28, 29). The A498 and RCC4 renal carcinoma cells were kindly provided by Dr. Karen Block (University of Texas Health Science Center at San Antonio). These cells were grown in DMEM in the presence of 10% fetal bovine serum. At near confluence, the cells were washed and incubated with serum-free medium for 18 h prior to addition of IGF-1 as indicated.

**DNA Synthesis and Cell Proliferation Assay**—IGF-1 was added to the serum-starved cells at the indicated concentration for 20 h. DNA synthesis was determined by incorporation of [3H]thymidine into trichloroacetic acid-insoluble material as described (30, 31). For proliferation assay, the cells were trypsinized after the indicated incubation period and counted in a hemocytometer as described (32).

**Immunoblot Analysis**—The cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM Na2VO4, 1 mM PMSF, and 0.1% protease inhibitor mixture) at 4 °C for 30 min. The cell debris was pelleted at 10,000 × g for 30 min, and protein concentration was determined for the supernatant with Bio-Rad reagent. Equal amounts of proteins present in the cell extracts were separated by SDS-PAGE. The proteins were transferred to PVDF membrane and immunoblotted with the indicated antibodies as described previously (28, 29).

**Secondary Structure Prediction for the MicroRNA Target Site**—The 3′ UTR of IGF-1R mRNA was analyzed to identify a possible miR-214 recognition element using TargetScan prediction algorithm. The secondary structure for the duplex for
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expression of IGF-1R may not correlate with the VHL status of renal carcinoma cells, we used VHL-positive ACHN and VHL-negative 786-O clear cell renal carcinoma cell lines. Levels of IGF-1R protein in both these cell lines were high as compared with that in HK2, a normal human proximal tubular epithelial cell line (Fig. 1A, left panel). This elevated expression of IGF-1R was associated with the autophosphorylation of its β-subunit (Fig. 1A, left panel), indicating receptor activation. Two other renal cancer cell lines, A498 and RCC4, also showed increased IGF-1R levels when compared with HK2 cells (Fig. 1A, middle panel). Similarly, when compared with another normal primary human proximal tubular epithelial cell, HRPTEC, the levels of IGF-1R and phospho-IGF-1R were higher (Fig. 1A, right panel). The presence of activated IGF-1R correlated with increased basal DNA synthesis and proliferation in all four RCC cell lines compared with the normal HK2 and HRPTEC cells (Fig. 1, B and C).

To examine the mechanism of increased expression of IGF-1R in renal cancer cells, we hypothesized that expression of specific miRNA may contribute to its regulation. In fact, 30% of the transcriptome is regulated by miRNAs, and 25% of miRNA recognition elements between human and mouse is highly conserved (16, 17, 37). We used prediction algorithm in TargetScan to mine the 3′UTR of IGF-1R mRNA for the miRNA recognition element. This analysis revealed the presence of an miR-214 recognition element, which is conserved in human and mouse IGF-1R mRNAs. RNA hybrid analysis showed that the predicted minimum free energy (ΔG) for binding of miR-214 with IGF-1R 3′UTR for human and mouse was comparable (human, −26.1 kcal/mol; mouse, −24.7 kcal/mol) (Fig. 1D). Furthermore, the predicted minimum free energies for the binding of seed sequence of miR-214 to IGF-1R 3′UTR of these species were identical and less than −6 kcal/mol. These data support the critical energy requirement for optimal repression of target protein expression (38). Therefore, we examined the expression of mature miR-214 in the normal HK2 and two renal cancer cell lines. Fig. 1E shows a robust reduction in miR-214 expression in ACHN and 786-O cells as compared with that in HK2 cells. Similarly, levels of pre-miR-214 were significantly decreased in the renal cancer cells (Fig. 1F). Because pre-miRs are processed from the primary transcripts (19), we tested the expression of pri-miR-214 in the renal cancer cells. As shown in Fig. 1G, the expression of pri-miR-214 was significantly decreased in the ACHN and 786-O renal carcinoma cells. Similarly, expression of mature, pre-, and pri-miR-214 was significantly lower in A498 and RCC4 when compared with HK2 and HRPTEC normal proximal tubular epithelial cells (Fig. 1, H–J). These data indicate that the expression of miR-214 in the renal cancer cells may be regulated at the transcriptional level.

miR-214 Down-regulates IGF-1R mRNA and Protein Expression—To study the role of miR-214 in IGF-1R expression, we cloned the 3′UTR of IGF-1R mRNA containing the predicted miR-214 recognition element (Fig. 2A) into the pMIR-Report vector downstream of luciferase gene (IGF-1R 3′UTR-Luc) (Fig. 2B). We examined the effect of miR-214 on the reporter activity of IGF-1R 3′UTR-Luc. Transfection of miR-214 into ACHN and 786-O renal carcinoma cells significantly increased the expression of this microRNA (Fig. 2C).
and inhibited the luciferase activity of the reporter plasmid in both these cells (Fig. 2D). To determine the specificity of the miR-214 binding to its recognition element in the IGF-1R 3’UTR, we mutated four bases in miR-214 seed-binding site in the reporter construct (Fig. 2A, indicated by arrows). Effect of expression of miR-214 was tested on the luciferase activity. Mutant IGF-1R-Luc reporter construct showed no reduction in luciferase activity when cotransfected with the miR-214 expression vector (Fig. 2D). These results suggest that miR-214 targets the 3’UTR of IGF-1R with significant specificity. Next, we tested the effect of miR-214 on IGF-1R mRNA and protein expression. Expression of miR-214 significantly inhibited both the expression of IGF-1R mRNA and protein in ACHN, 786-O, A498, and RCC4 renal cancer cells (Fig. 2, E and F). These results conclusively demonstrate that miR-214 directly regulates the expression of IGF-1R in the renal carcinoma cells.

miR-214 Regulates IGF-1-stimulated Akt Kinase Activity in Renal Carcinoma Cells—Increased circulating IGF-1 is an independent prognostic marker in patients with renal cell carcinoma (9). Our results above showed that both ACHN and 786-O renal carcinoma cells possess increased levels of IGF-1R than normal proximal tubular epithelial cells (Fig. 1A). To examine the biological function of increased IGF-1R expression

FIGURE 1. Reciprocal expression of IGF-1R and miR-214 in renal cancer cells. A, equal amounts of lysates from normal proximal tubular epithelial HK2, primary HRPTEC and ACHN, 786-O, A498, and RCC4 renal cancer cells were immunoblotted with IGF-1R, phospho-IGF-R, and actin antibodies. B, [3H]thyridine incorporation was determined as described under “Experimental Procedures.” C, proliferation of indicated cells was determined by counting the cells as described under “Experimental Procedures.” D, predicted secondary structures of mature miR-214 and its target in the IGF-1R 3’UTR of human (left) and mouse (right) are shown. Minimum free energy (mfe) for the predicted structures is shown. E–J, total RNAs from HK2, HRPTEC, ACHN, 786-O, A498, and RCC4 cells were used in real time qRT-PCR to detect mature miR-214 (E and H), pre-miR-214 (F and I), and pri-miR-214 (G and J) as described under “Experimental Procedures.” Mean ± S.E. of four measurements is shown. *, p < 0.01 versus HK2 in E–G. **, p < 0.001 versus HK2 or HRPTEC in H–J.
in renal cancer cells, we tested the effect of IGF-1 on these cells. IGF-1 increased the tyrosine phosphorylation of IGF-1R β-subunit in ACHN and 786-O renal cancer cells (Fig. 3A). Furthermore, IGF-1 significantly increased the DNA synthesis, which resulted in proliferation of these renal carcinoma cells in a dose-dependent manner (Fig. 3, B and C).

IGF-1 is known to stimulate Akt kinase, which regulates many physiologic responses, including metabolism, survival,
and proliferation of normal and cancer cells (39, 40). Incubation of ACHN and 786-O cells with IGF-1 increased phosphorylation of Akt at both Thr-246 and Ser-473 in response to IGF-1 (Fig. 3D). The Akt inhibitor MK-2206 significantly inhibited IGF-1-stimulated phosphorylation of Akt (Fig. 3E). Furthermore, MK-2206 significantly attenuated IGF-1-induced DNA synthesis and proliferation of renal carcinoma cells (Fig. 3, F and G). Next, we tested whether miR-214 affects IGF-1-stimulated Akt phosphorylation. Transfection of miR-214 expression vector into ACHN and 786-O renal cancer cells inhibited phosphorylation of Akt at Ser-473 and Thr-308 in response to IGF-1 (Fig. 3H). To confirm that enhanced phosphorylation of Akt is associated with an increase in its kinase activity, we tested phosphorylation of GSK3β, its substrate. IGF-1 increased phosphorylation of GSK3β in both ACHN and 786-O cells (Fig. 3I). Expression of miR-214 significantly decreased the phosphorylation of GSK3β in response to IGF-1 (Fig. 3I). These results demonstrate involvement of miR-214 in activation of Akt kinase by IGF-1R in renal cancer cells.

miR-214 inhibits IGF-1-stimulated PRAS40 phosphorylation to induce renal cancer cell proliferation—PRAS40 was originally identified as a substrate for Akt, which phosphorylates it at Thr-246 (41). More recently we and others have shown that phosphorylation of PRAS40 regulates protein synthesis leading to an increase in the size of normal renal cells (42–44). However, its role in renal cancer cells has not been investigated. Incubation of the two renal carcinoma cell lines with IGF-1 increased phosphorylation of PRAS40 in a time-dependent manner (Fig. 4A), corresponding to Akt phosphorylation (Fig. 3D). Use of MK-2206 to block Akt kinase activity showed inhibition of IGF-1-induced PRAS40 phosphorylation in ACHN and 786-O cells (Fig. 4B). Because Akt kinase regulates renal cancer cell proliferation (Fig. 3, F and G), we examined the role of PRAS40 phosphorylation in IGF-1-induced renal cancer cell proliferation. We used a mutant of PRAS40 where its Akt phosphorylation site Thr-246 was changed to alanine. Expression of the PRAS40 T246A mutant significantly prevented IGF-1-induced DNA synthesis in both ACHN and 786-O cells (Fig. 4C). We also counted the number of cells to determine cell proliferation directly. PRAS40T246A markedly reduced proliferation of both renal cancer cells (Fig. 4D). Phosphorylation of PRAS40 induces its inactivation (44). Therefore, to mimic its inactivation, we inhibited PRAS40 expression in renal cancer cells using shRNA, which targets PRAS40 mRNA (44). shRNA-mediated down-regulation of PRAS40 modestly but significantly increased DNA synthesis in ACHN and 786-O renal cancer cells, resulting in their proliferation (Fig. 4, E and F). However, inhibition of PRAS40 expression in the presence of IGF-1 was not sufficient to further increase the DNA synthesis as compared with that induced by IGF-1 alone (Fig. 4, E and F).

Because miR-214 regulates the phosphorylation and activation of Akt, which in turn phosphorylates and inactivates PRAS40, we examined the effect of miR-214 on PRAS40 phosphorylation. Expression of miR-214 blocked IGF-1-induced phosphorylation of PRAS40 in ACHN and 786-O renal cancer cells (Fig. 4G). These results suggest that miR-214 regulates IGF-1-stimulated phosphorylation of PRAS40 at Thr-246 in renal cancer cells. Furthermore, our data for the first time demonstrate that phosphorylation of PRAS40 in response to IGF-1 contributes to proliferation of renal carcinoma cells.

miR-214 blocks IGF-1-stimulated mTORC1 activity—PRAS40 is a negative regulator of the growth factor and nutrient sensor kinase mTORC1. In fact, PRAS40 is a component of the mTORC1 (44). Akt-mediated phosphorylation of PRAS40 at Thr-246 induces its release from mTORC1, resulting in activation of mTORC1 kinase activity (40, 44, 45). Because IGF-1 increased phosphorylation of PRAS40 at Thr-246, we tested the effect of IGF-1 on activation of mTORC1 in ACHN and 786-O renal carcinoma cells. Phosphorylation of S6 kinase at Thr-389 was used as a readout for mTORC1 activity (45, 46). IGF-1 increased phosphorylation of S6 kinase in a time-dependent manner in both renal cancer cells (Fig. 5A). It was reported that mTORC1-activated S6 kinase phosphorylates mTOR at Ser-2488 (47, 48). Therefore, we tested the phosphorylation at this site in response to IGF-1. As shown in Fig. 5B, IGF-1 increased phosphorylation of mTOR in a time-dependent fashion. Expression of miR-214 significantly inhibited the IGF-1-stimulated phosphorylation of S6 kinase and mTOR (Fig. 5, C and D). These results indicate that miR-214 regulates IGF-1-induced activation of mTORC1 in ACHN and 786-O renal tumor cells.

Next, we tested the phosphorylation of another substrate of mTORC1, the mRNA translation initiation repressor 4EBP-1. Incubation of both ACHN and 786-O renal cancer cells with IGF-1 increased phosphorylation of 4EBP-1 at Thr-37/46 and Ser-65, sites known to be phosphorylated by mTORC1, in a time-dependent manner (Fig. 6A). Recently, Dowling et al. (49) has shown that 4EBP-1 is a substrate of mTORC1 contributes to proliferation of fibroblasts. We tested its role in proliferation of renal cancer cells using a mutant in which all the phosphorylation sites for mTORC1 are changed to alanine. Expression of miR-214 inhibited phosphorylation of 4EBP-1 in both renal cancer cell lines (Fig. 6B). Total RNAs were used to detect mature miR-214 by qRT-PCR as described under “Experimental Procedures.” D, specific interaction of miR-214 with its microRNA recognition element in the IGF-1R 3′ UTR. Wild type and the miR-214 seed binding mutant IGF-1R 3′ UTR constructs were transfected into ACHN and 786-O cells along with CMV miR-214 expression vector. Luciferase activity was determined in the cell lysates as described under “Experimental Procedures.” Mean ± S.E. of four measurements is shown. For ACHN, p = 0.0006 versus Scr vector. For 786-O, p = 0.005 versus Scr vector. For A498 and RCC4, p = 0.004 versus Scr vector. F, CMV miR-214 or scramble (Scr) vector was transfected into three wells of ACHN, 786-O, A498, and RCC4 renal carcinoma cells. Total RNAs were prepared, and expression of IGF-1R mRNA was detected by real time qRT-PCR as described under “Experimental Procedures.” Mean ± S.E. of four measurements is shown. For ACHN, p = 0.0006 versus Scr vector. For 786-O, p = 0.005 versus Scr vector. For A498 and RCC4, p = 0.004 versus Scr vector. F, CMV miR-214 or scramble (Scr) vector was transfected into three wells of ACHN, 786-O, A498, and RCC4 cells. The cell lysates were immunoblotted with IGF-1R and actin antibodies.

FIGURE 2. miR-214 binds to its microRNA recognition element in the IGF-1R 3′ UTR. A, sequence complementarity between miR-214 and its target site in the 3′ UTR of human IGF-1R. Arrows indicate the mutated bases in the seed binding sequence in the 3′ UTR of IGF-1R. B, schematic showing the reporter construct containing the IGF-1R 3′ UTR downstream of firefly luciferase gene. The sequence shows the region of binding of miR-214. Underlined sequence shows the seed-binding site. C, expression of miR-214 in the ACHN and 786-O renal cancer cells. The cells were transfected with CMV-miR-214 expression vector. Total RNAs were used to detect mature miR-214 by qRT-PCR as described under “Experimental Procedures.” D, specific interaction of miR-214 with its microRNA recognition element in the IGF-1R 3′ UTR. Wild type and the miR-214 seed binding mutant IGF-1R 3′ UTR (A) luciferase constructs were transfected into ACHN and 786-O cells along with CMV miR-214 expression vector. Luciferase activity was determined in the cell lysates as described under “Experimental Procedures.” Mean ± S.E. of four measurements is shown. For ACHN, p = 0.0006 versus Scr vector. For 786-O, p = 0.005 versus Scr vector. For A498 and RCC4, p = 0.004 versus Scr vector. F, CMV miR-214 or scramble (Scr) vector was transfected into three wells of ACHN, 786-O, A498, and RCC4 cells. The cell lysates were immunoblotted with IGF-1R and actin antibodies.
DNA synthesis in both ACHN and 786-O renal cancer cells (Fig. 6B). Similarly, 4EBP-1/H9262 significantly prevented proliferation of both these cells (Fig. 6C). To examine the role of miR-214 in phosphorylation of 4EBP-1, we transfected miR-214 into these renal tumor cells followed by incubation with IGF-1. Expression of miR-214 markedly blocked the phosphorylation of 4EBP-1 at Thr-37/46 and Ser-65 in response to IGF-1 (Fig. 6D). Together, our data suggest that miR-214 inhibits mTORC1 signal transduction initiated by the IGF-1-induced phosphorylation of PRAS40.

miR-214 Prevents IGF-1-stimulated Renal Cancer Cell Proliferation by Targeting IGF-1R—The results described above demonstrate that miR-214 regulates the IGF-1R-stimulated Akt/mTORC1 signaling axis, which contributes to proliferative effects of IGF-1 on renal cancer cells. Our findings provide novel insights into the role of miR-214 in renal cancer biology and suggest potential therapeutic targets for the treatment of renal cancer.

**FIGURE 3.** miR-214 regulates IGF-1-stimulated Akt kinase phosphorylation for proliferation of ACHN and 786-O renal cancer cells. A, serum-starved ACHN and 786-O cells were incubated with 100 ng/ml IGF-1 for the indicated periods of time. The cell lysates were immunoblotted with phospho-IGF-1R and IGF-1R antibodies. B and C, ACHN and 786-O cells were incubated with indicated doses of IGF-1. [3H]Thymidine incorporation (B) and cell count (C) were determined as described under “Experimental Procedures.” Mean ± S.E. of four and six measurements is shown. *, p < 0.001 versus control; **, p < 0.001 versus control. D, ACHN and 786-O cells were incubated with 100 ng/ml IGF-1 for 15 min. The cell lysates were immunoblotted with indicated antibodies. F and G, ACHN and 786-O cells were transfected with CMV miR-214 or scramble (Scr) vector. Transfected cells were incubated with 100 ng/ml IGF-1 for 15 min. The cell lysates were immunoblotted with phospho-Akt, Akt (H) and phospho-GSK3β, GSK3β (I) and IGF-1R and actin antibodies as indicated. In parallel dishes transfected with CMV miR-214, expression of miR-214 was determined to demonstrate overexpression of miR-214 (data not shown).
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We tested the hypothesis that miR-214-targeted IGF-1R regulates this proliferation. Expression of miR-214 significantly attenuated IGF-1-induced DNA synthesis and proliferation of ACHN and 786-O renal cancer cells (Fig. 7, A and B). To examine the role of IGF-1R in miR-214 action, we used a plasmid vector that expresses IGF-1R mRNA without its 3'UTR, thus eliminating the inhibitory effect of miR-214. As a control to eliminate the overexpression artifact, we used the wild type and miR-214 recognition element mutant IGF-1R 3'UTR-Luc reporter plasmids (Fig. 2, A and B). Expression of the wild type reporter plasmid quenches the overexpressing miR-214 and thus nullifies the effect of miR-214 on proliferation.
endogenous IGF-1R expression and maintains its level (Fig. 7A, bottom panel, 5th lane). However, expression of the mutant reporter does not quench miR-214. Therefore, overexpressed miR-214 inhibits expression of endogenous IGF-1R (Fig. 7A, bottom, 6th lane). Thus, expression of the wild type reporter plasmid along with miR-214 showed increased DNA synthesis similar to IGF-1 alone (Fig. 7A, compare 5th bar with 2nd bar). In contrast, the expression of the mutant reporter along with miR-214 inhibited IGF-1-induced DNA synthesis similar to the effect found with miR-214 alone (Fig. 7A, compare 6th bar with 4th bar). Importantly, expression of the 3′UTR less IGF-1R significantly reversed the miR-214-mediated inhibition of IGF-1-induced DNA synthesis (Fig. 7A, compare 7th bar with 4th bar). Similarly, expression of IGF-1R significantly blocked the miR-214-induced suppression of IGF-1-induced proliferation of renal carcinoma cells (Fig. 7B). These results suggest that miR-214 targets IGF-1R to regulate IGF-1-stimulated renal cancer cell proliferation.

We have shown above that miR-214 inhibits the mTORC1 activity in response to IGF-1 (Figs. 5, C and D, and 6D). Therefore, we determined the role of this kinase in miR-214-regulated renal cancer cell proliferation. As expected, miR-214 inhibited IGF-1-induced DNA synthesis and proliferation of ACHN and 786-O renal cancer cells (Fig. 7, C and D). Interestingly, expression of a constitutively active mutant of mTORC1 significantly reversed the miR-214-induced inhibition of IGF-1-stimulated DNA synthesis and proliferation of the two renal carcinoma cell lines (Fig. 7, C and D). These results conclusively indicate a direct role of miR-214 in IGF-1R-mediated mTORC1 activation and proliferation of renal cancer cells by IGF-1.

Discussion

Complex interactions between the transcriptional program and protein expression contribute to the development of clear cell RCC. Inadequate therapeutic options for RCC necessitate identification of specific molecular targets, which can be utilized to prevent progression of this cancer. In this study, we identified IGF-1R as a target of miR-214. We find that reduced expression of miR-214 contributes to the increased IGF-1R expression in renal cancer cells regardless of VHL status. We show that expression of miR-214 inhibits the Akt/mTORC1
axis in the IGF-1R signaling cascade to prevent renal cancer cell proliferation.

Accumulating reports show expression profiling of multiple miRNAs in renal cancer. A recent microarray screen for identification of aberrantly expressed miRNAs in renal cell carcinoma samples showed 38 and 48 miRNAs to be up- and downregulated, respectively (50). However, experimental validation of only a few miRNAs with their target mRNAs has been reported. VHL deficiency causes increased HIF1α and HIF2α expression of which the latter contributes mainly to the development of sporadic RCC (51). However, HIF-independent function has also been reported in VHL deficiency (52). For

FIGURE 6. miR-214 regulates IGF-stimulated phosphorylation of 4EBP-1 that contributes to renal cancer cell proliferation. A, ACHN and 786-O renal cancer cells were incubated with 100 ng/ml IGF-1 for indicated periods of time. The cell lysates were immunoblotted with phospho-4EBP-1 (Thr-37/46 and Ser-65) and 4EBP-1 antibodies. B and C, ACHN and 786-O cells were transfected with 4EBP-1Δμ in which all four mTORC1 phosphorylation sites were mutated to alanine. Transfected cells were incubated with IGF-1. [3H]Thymidine incorporation (B) and cell number (C) were determined as described under “Experimental Procedures.” B, mean ± S.E. of six measurements is shown. *, p < 0.001 versus control; **, p < 0.01 versus IGF-1-treated. C, mean ± S.E. of triplicate measurements is shown. *, p < 0.001 versus control; **, p < 0.01 versus IGF-1-treated at corresponding time. The bottom panels in B and C show the expression of 4EBP-1. D, ACHN and 786-O cells were transfected with CMV miR-214 or scramble (Scr) vector. The transfected cells were incubated with 100 ng/ml IGF-1 for 15 min. The cell lysates were immunoblotted with phospho-4EBP-1, 4EBP-1, IGF-1R, and actin antibodies as indicated. miR-214 expression was examined in parallel dishes transfected with CMV miR-214 (data not shown).
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FIGURE 7. miR-214-targeted IGF-1R regulates proliferation of renal cancer cells via mTORC1. ACHN and 786-O cells were transfected with CMV miR-214 along with wild type IGF-1R 3’ UTR Luc or mutant IGF-1R 3’ UTR Luc or 3’ UTR less IGF-1R plasmid (A and B). [3H]Thymidine incorporation (A) and for cell number (B) were determined. Mean ± S.E. of triplicate measurements is shown. A, *p < 0.001 versus unstimulated; **p < 0.001 versus IGF-1-treated; @p < 0.001 versus miR-214 plus IGF-1-treated. B, 48 and 72 h, p < 0.001 versus unstimulated; **p < 0.001 versus IGF-1-treated; @p < 0.001 versus miR-214 plus IGF-1-treated. Bottom panel shows expression of IGF-1R and luciferase. C and D, ACHN and 786-O cells were transfected with CMV miR-214 and constitutively active mTORC1 plasmid as indicated. The transfected cells were incubated with 100 ng/ml IGF-1. [3H]Thymidine incorporation (C) and cell number (D) were determined as described under "Experimental Procedures." Mean ± S.E. of four measurements is shown. C, *p < 0.01 versus control; #p < 0.01 versus IGF-1-treated; @p < 0.01 versus miR-214 plus IGF-1-treated. D, mean ± S.E. of four measurements is shown. *p < 0.001 versus control; **p < 0.001 versus IGF-1-treated for corresponding time points; @p < 0.001 versus miR-214 plus IGF-1-treated. Bottom panels show expression of constitutively active (CA) mTOR and IGF-1R.

example, a recent study in renal cancer cells demonstrated the regulation of various miRNAs in a VHL-dependent manner (53). However, some of these miRNAs were HIF-dependent, although others were not.

IGF-1R can act as an oncogene when expressed in high levels in NIH 3T3 fibroblasts (54). Murine embryonic fibroblasts nullizygous for the IGF-1R gene are incapable of transformation by multiple oncogenes, including Ha-Ras and v-Src (55, 56). Apart from VHL deficiency, IGF-1R-mediated autocrine/paracrine signal transduction has been reported as an independent prognostic factor for development and progression of metastatic RCC (8, 9, 57). In line with these results, we show increased expression of IGF-1R in both VHL-positive ACHN and VHL-negative 786-O, RCC4, and A498 renal cancer cells (Fig. 1A). In breast cancer and in melanoma, amplification of the IGF-1R gene in 15q26 has been reported (58). Transcriptional mecha-
nism is established for increased expression of IGF-1R in many cancers, including renal cancer (59). Ewing sarcoma-WT1 fusion oncoprotein and Sp1 increase transcription of IGF-1R (15, 60, 61). However, tumor suppressor proteins such as p53, PTEN, and BRCA1 inhibit expression of IGF-1R (15, 62). Translational regulation of IGF-1R is also reported. The long 5′UTR of IGF-1R is a target of the RNA-binding protein HuR, which delays cap-dependent translation and inhibits the internal ribosome entry site-containing IGF-1R translational block (63, 64). In contrast, heterogeneous nuclear ribonucleoprotein C has been shown to interact with IGF-1R 5′UTR at the site of HuR binding to promote internal ribosome entry site-mediated translation of IGF-1R (65). Recently, four miRNAs, miR-7, miR-192, miR-215, and miR-145, have been identified, which repress the expression of IGF-1R by post-transcriptional mechanism in tongue squamous cell carcinoma, multiple myeloma, and VHL-deficient RCC (66–68). In this study, we identified miR-214 to target 3′UTR of IGF-1R mRNA (Fig. 2). miR-214 is increased in many cancers, including pancreatic cancer, oral squamous cell carcinoma, and malignant melanoma (69–71). In tongue squamous cell carcinoma and ovarian cancer, enhanced expression of miR-214 is associated with cisplatin resistance (72, 73). Although aberrant expression of many miRNAs has been identified in RCC, we demonstrate significant down-regulation of miR-214 in VHL-positive and VHL-deficient renal cancer cells compared with normal proximal tubular epithelial cells (Fig. 1, E–I). To our knowledge this is the first demonstration in RCC of down-regulation of miR-214, which increases the protein abundance of IGF-1R.

Enhanced phosphorylation of Akt is often detected in samples of RCC in the absence of activating mutation in Akt itself and in its upstream regulator phosphatidylinositol 3-kinase (74, 75). Lack of or reduced PTEN protein levels also increases phosphorylation of Akt, which causes prostate intraepithelial neoplasia, glioblastoma, and endometrial cancer (76). Although mutation in the PTEN gene in RCC has been reported, it is not common, and its expression is also heterogeneous in different renal cancer cells (77, 78). One mechanism of Akt kinase activation in RCC could involve activation of growth factor receptors (14, 15). Our results demonstrate that both ACHN and 786-O renal cancer cells irrespective of their VHL status possess increased levels of IGF-1R compared with the normal kidney epithelial cells (Fig. 1A). This enhanced expression of IGF-1R is associated with increased sensitivity to IGF-1, resulting in activation of Akt kinase, leading to proliferation of renal cancer cells (Fig. 3, A–G). Because increased IGF-1R levels were due to reduced miR-214 (Figs. 1 and 2), exogenous expression of miR-214 inhibited IGF-1-induced Akt kinase phosphorylation and its activation (Fig. 3, H and I).

In the renal cancer cells, we demonstrate that phosphorylation of PRAS40 is mediated by IGF-1R-stimulated Akt kinase (Fig. 4, A and B). Mutation in the Drosophila Lobe protein, the ortholog of mammalian PRAS40, results in hypoaCTive mTORC1, indicating that Drosophila PRAS40 positively regulates mTORC1 activity (79). In contrast, in mammalian cells, PRAS40 negatively regulates mTORC1 (46). Our data show that phosphorylation of PRAS40 at Thr-246 (Fig. 4A), which results in its inactivation (44), significantly increased the mTORC1 kinase activity as judged by the phosphorylation of S6 kinase and 4EBP-1 (Figs. 5A and 6A). Also, phospho-deficient mutant of PRAS40 blocked IGF-induced proliferation (Fig. 4, C and D). Furthermore, our results demonstrate that PRAS40 negatively regulates IGF-1-induced proliferation of both VHL-positive and -negative renal cancer cells (Figs. 4, E and F). Interestingly, because we found IGF-1-induced Akt activation was sensitive to miR-214, we demonstrated that expression of miR-214 significantly inhibited phosphorylation of PRAS40 in response to IGF-1 (Fig. 4G).

In Drosophila, both S6 kinase and 4EBP-1 regulate cell growth and proliferation (80, 81). However, a recent report established a significant contribution of 4EBP-1 in mammalian cell proliferation. mTORC1-mediated phosphorylation of 4EBP-1 releases elf4E, which forms the elf4F complex and regulates translation of a subset of mRNAs necessary for cell proliferation, including VEGF (82–84). Increased VEGF expression in VHL-deficient RCC is mainly mediated by stabilized Hif2α-mediated transcription (51). However, the VEGF protein level is regulated by 4EBP-1-sensitive elf4F-mediated cap-dependent translation of its mRNA (82, 83). Interestingly, Dowling et al. (49) reported that in 4EBP-deficient murine embryonic fibroblasts, inhibition of mTORC1 had no suppressive effect on the elf4F-sensitive translation of VEGF mRNA, indicating a significant role of 4EBP in mTORC1-mediated translation of this pro-tumorigenic protein. Furthermore, mTOR inhibitors did not block proliferation of 4EBP-deficient mouse embryo fibroblasts (49). The contribution of 4EBP-1 in the proliferative response of renal cancer cells has not been reported. In ACHN and 786-O renal cancer cells, we demonstrate that the phosphorylation-deficient constitutively active mutant of 4EBP-1 inhibits IGF-1R-stimulated proliferation (Fig. 6, B and C). mTORC1-mediated phosphorylation of 4EBP-1 leads to its inactivation. Thus, inhibition of its phosphorylation promotes its inhibitory activity. We show reduced IGF-1–induced phosphorylation of 4EBP-1 by miR-214, rendering this translational repressor at its active state (Fig. 6D). These data indicate that miR-214-mediated activation of 4EBP-1 may serve as a useful target for prevention of RCC proliferation.

In vitro studies demonstrated that inhibition of mTOR kinase activity blocked expression of Hif1α and Hif2α regardless of cellular oxygen levels (85). Trials using various structural derivatives of rapamycin for a variety of solid tumors, including RCC, have shown limited efficacy. An earlier phase II trial using multiple doses of temsirolimus with metastatic RCC patients showed an overall response rate of 7% (86). In a subsequent phase III trial, temsirolimus was compared with interferon-α alone and in combination. Temsirolimus alone showed significant progression-free and overall survival than that compared with interferon-α alone. But the patients with combination therapy did not show any significant difference when compared with the interferon group alone (2). Furthermore, use of everolimus in a multicenter phase III trial with metastatic RCC patients who progressed on VEGF receptor inhibitors showed a significant progression-free survival versus the placebo arm (87). On the basis of these trials and few others, the Food and Drug Administration approved temsirolimus and everolimus
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for metastatic RCC (2, 87). However, significant adverse events may limit their therapeutic efficacy (2, 87–89). Furthermore, enhanced mTORC1 activity elicits a negative feedback loop via S6 kinase-mediated phosphorylation of IRS-1 at Ser-307, which inhibits IGF-1R-stimulated phosphorylation of Akt (90, 91). Therefore, inhibition of mTORC1 alone could disrupt this negative feedback loop resulting in increased Akt phosphorylation, which can lead to maintenance of the malignant state. In fact, this feedback loop has been ascribed to be the cause for resistance to mTORC1 inhibitors in many solid tumors.

Because elevated IGF-1R signal transduction causes proliferation and survival of cancer cells, targeting this receptor has been an attractive mode to treat patients with variety of solid tumors. Several clinical trials used IGF-1R-specific antibody therapy. However, due to the presence of hybrid receptors containing IGF-1R and insulin receptor, the down-regulation of the receptor molecules resulted in adverse events in many studies, including hyperglycemia (14). Other preclinical strategies involved development of tyrosine kinase inhibitors. Due to 95% sequence homology in the ATP binding domains of IGF-1R and insulin receptor, it has been a challenge to develop specific tyrosine kinase inhibitors for IGF-1R (14). In this study, we have identified a miRNA, miR-214, which acts as an endogenous inhibitor of IGF-1R protein expression in renal cancer cells. We have shown that miR-214 expression is significantly reduced in VHL-deficient as well as VHL-positive renal cancer cells. Furthermore, expression of miR-214 in renal cancer cells significantly prevents the signal transduction pathways of IGF-1R, including Akt kinase and mTORC1. Finally, we demonstrate that miR-214 significantly blocks IGF-1-induced proliferation of both VHL-positive and -negative renal cancer cells by directly targeting IGF-1R that uses mTORC1 (Fig. 7). Therefore, use of miR-214 may represent an attractive therapy to test in preclinical models of renal cancer. Because mTORC1 inhibitors and tyrosine kinase inhibitors exhibit adverse events, it will be an added benefit to test the efficacy of combinatorial use of miR-214 with low dose mTORC1 inhibitors or with tyrosine kinase inhibitors in preclinical animal models of RCC.

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