Anterior gradient 2 promotes tumorigenesis through upregulation of CCAAT-enhancer binding protein beta and hypoxia-inducible factor-2α and subsequent secretion of interleukin-6, interleukin-8, and vascular endothelial growth factor in the Caki-1 clear cell renal cell carcinoma cell line

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Abstract
It has been previously established that hypoxia leads to tumor development, treatment resistance, and a poor prognosis. Under oxygen deprivation, hypoxia-inducible factors (HIFs) are stimulated to activate the genes necessary for tumor development in a low-oxygen environment. These genes encode regulators of angiogenesis, epithelial-mesenchymal transition, and cellular metabolism. A disulfide isomerase, anterior gradient 2 (AGR2), has been shown to increase hypoxia-inducible factor 1, alpha subunit (HIF-1α) stability in breast cancer. Our goal was to determine if AGR2 affects the level of transcription factor hypoxia-inducible factor 2, alpha subunit (HIF-2α). As a model, we used the clear cell renal cell carcinoma (ccRCC) cell line Caki-1. The cells were transduced with lentiviral vector (Tet-On) encoding AGR2. After induction of AGR2 expression, cells were grown under either hypoxic (0.5% O2) or normoxic (21% O2) conditions. Our data showed that AGR2 upregulated both HIF-1α and HIF-2α expression in Caki-1 cells increasing the expression of HIF-activated genes (glucose transporter 1, phosphoglycerate kinase 1, vascular endothelial growth factor A, and transforming growth factor-alpha) under the hypoxic conditions. Under the normoxic conditions, AGR2 strongly activated CCAAT-enhancer binding protein beta (C/EBPβ). Upregulation of C/EBPβ correlated with increased expression and secretion of the interleukin-6 and interleukin-8, inducing angiogenesis and inflammation in Caki-1 cells. In

Abbreviations: AGR2, anterior gradient protein 2; C/EBPβ, CCAAT-enhancer binding protein beta; CCND1, cell proliferation upregulating cyclin D1; EF2, elongation factor-2; GATA3, GATA binding protein 3; GLUT1, glucose transporter 1; HIF-1α, hypoxia-inducible factor 1, alpha subunit; HIF-2α, hypoxia-inducible factor 2, alpha subunit; IL-1β, interleukin 1beta; IL-6, interleukin-6; IL-8, interleukin-8; MCPIP1, monocYTE chemotactrant protein-1-induced protein-1; NF-kB, nuclear factor kappa B; PDI, protein disulfide isomerase; PGK1, phosphoglycerate kinase 1; RPL28, ribosomal protein L28; STAT3, signal transducer and activator of transcription 3; TGF-α, transforming growth factor-alpha; VEGFA, vascular endothelial growth factor A; VEGFC, vascular endothelial growth factor C; VHL, von Hippel–Lindau protein.

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summarized, our studies revealed that AGR2 has essential functions in ccRCC progression through upregulation of C/EBPβ and HIF-2α expressions, which affects cell signaling and metabolism.

**KEYWORDS**
anterior gradient 2 protein, C/EBP-beta, carcinogenesis, hypoxia, hypoxia-inducible factors, inflammation

1 | **INTRODUCTION**

Protein folding and assembly into functional complexes is frequently dependent on the activity of molecular chaperones and enzymatically active proteins such as protein disulfide isomerases (PDIs) and peptidylprolyl isomerases.²⁴ Anterior gradient 2 (AGR2) is a PDI predominantly localized within the endoplasmic reticulum (ER).²³ AGR2 levels are elevated in various tumors including prostate, breast, pancreas, and gastric carcinomas.³⁴ The expression of AGR2 is driven by the reaction to ER stress induced by an increase in unfolded proteins. AGR2 was initially identified as bound to nascent proteins from ER ribosomes.³⁵ Despite the primary localization of AGR2 in the ER, this protein is also secreted to the extracellular matrix. It has been shown that secreted AGR2 promotes tumor invasiveness and metastasis.⁶ AGR2 influences signaling pathways regulating cell cycle and inflammation.⁴ Through its PDI activity, extracellular AGR2 facilitates the formation of active dimers of fibroblast growth factor 2 and vascular endothelial growth factor (VEGF).¹⁰

Patients with advanced clear cell renal cell carcinoma (ccRCC) have low survival. The 5-year survival time for metastatic ccRCC cases is below 32%.¹¹ Microarray data analysis has revealed that AGR2 is moderately expressed in both normal renal tissue and renal carcinoma tissue, although some renal tumor patients have substantially elevated expression of AGR2.⁵,¹² Single-cell RNA-sequencing studies of human fetal renal cells showed that AGR2 is predominantly localized in the kidney collecting tissues and that its induction is combined with coexpression of the transcription factors GATA binding protein 3 (GATA3) and ETS-related transcription factor.¹³ Similarly, expression of AGR2 significantly overlaps with the induction of GATA3 and ERα in breast tumor cells.¹⁴ Consistently, previous studies have demonstrated the roles of AGR2 in the induction of mucinous phenotypes in many tumors and in the production of intestinal and stomach mucin.²,¹⁵ Interestingly, recent analyses of single-cell transcriptomes from zebrafish kidneys have identified a novel type of mucin-secreting renal cells that highly express agr2 and mucus/gel-forming (muc2.4).¹⁶

Our latest studies revealed that overexpression of monocyte chemoattractant protein-1-induced protein-1 (MCPIP1) significantly reduced the AGR2 transcript level in the Caki1 ccRCC cells.¹⁷ Moreover, expression of the RNase MCPIP1 contributed to the inhibition of the development and metastasis of ccRCC by reducing the mRNA levels of glucose transporter 1 (GLUT1), VEGFA, interleukin-6 (IL-6), and interleukin-8 (IL-8).¹⁷,¹⁸ However, there is no information regarding the significance of AGR2 expression in the promotion of ccRCC or other types of renal tumors. Therefore, we investigated the function of AGR2 in the Caki-1 ccRCC cell line. Here, we uncover the importance of AGR2 in the secretion of inflammatory cytokines and proangiogenic factors (IL-6, IL-8, and VEGF) induced by pathways that involve the transcription factors CCAAT-enhancer binding protein beta (C/EBPβ) and hypoxia-inducible factor (HIF)-2α.

2 | **MATERIALS AND METHODS**

2.1 | **Cell culture**

Caki-1 cells (ATCC) were grown in Eagle’s minimal essential medium (Lonza) containing 10% fetal bovine serum (Biowest) and penicillin–streptomycin (100 U/ml, Lonza). Cells were grown at 37°C with 5% CO₂ and passed 2–3 times a week. For stable expression of AGR2, we used a doxycycline-activated Tet-On overexpression system. An empty lentiviral vector (pLIX-PURO) was used as a negative control. For transduction, Caki-1 cells were seeded in 6-well plates (8 × 10⁴ per well) and transduced with lentiviral vectors at a multiplicity of infection of 50 and cultured for 24 hr. Then, the virus-free medium was added, and cells were grown for another 24 hr. Transfected cells were selected for 10 days with a medium supplemented with 1 μg/ml puromycin (InvivoGen). AGR2 overexpression was induced using doxycycline (BioShop) at a final concentration of 1 μg/ml.
2.2 | Hypoxia

Caki-1 cells were seeded in 6-well plates \((1.8 \times 10^5 \text{ cells per well})\). After 24 hr, AGR2 overexpression was induced with doxycycline (BioShop) at a final concentration of \(1 \mu \text{g/ml}\). After an additional 24 hr, the Caki-1 cells were placed in a hypoxic chamber (0.5% \(\text{O}_2\)) or an incubator with atmospheric conditions (21% \(\text{O}_2\)). After 24 and 48 hr of incubation, the medium was collected; subsequently, total protein and RNA were isolated.

2.3 | RNA isolation and reverse transcription quantitative polymerase chain reaction

The guanidium isothiocyanate method was used to isolate total RNA. The total RNA concentration and purity were determined with the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). For reverse transcription, oligo d(T) primers (Promega) and M-MLV reverse transcriptase (Promega) were used. For real-time polymerase chain reaction (PCR), 10-fold dilution of cDNA was used. A SYBR Green-based master mix (A&A Biotechnology) and the Applied Biosystem QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) were used to perform quantitative PCR. The expression level was determined by technical replicates and normalized to the geometric average of the expression of elongation factor-2 (\(\text{EF2}\)) and ribosomal protein L28 (\(\text{RPL28}\)). Figure S3 shows the comparison of the expression of \(\text{EF2}\) and \(\text{RPL28}\) in the Caki-1 cell line. The relative expression was determined by the \(2^{-\Delta \text{Ct}}\) method. List of primer sequences is to be found in Table S1.

2.4 | Western blot analysis

Total protein was isolated using radioimmunoprecipitation assay (RIPA) buffer containing Protease Inhibitor Cocktail (Sigma) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche). The protein concentrations were quantified with a bicinchoninic acid assay. The samples were resolved by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS/PAGE; 10% Bis-Tris acrylamide gels) at a 120 V. The sample's transfer was performed using a polyvinylidene difluoride (PVDF) membrane (Millipore) at a constant voltage of 30 V overnight at 4°C. To block the membranes, 5% powdered milk dissolved in Tris-buffered saline containing 0.05% Tween 20 (BioShop) was used. Next, the membranes were incubated with primary antibodies for 16 hr at 4°C. Then, the membranes were incubated for 1 hr at room temperature using horseradish peroxidase (HRP)-conjugated secondary antibodies. Detection of chemiluminescence was performed using an Immobilon Western HRP substrate (Millipore); signals were measured with the ChemiDoc System (BioRad). The following antibodies were used: anti-AGR2 (1:1,000; NBP2-27393, Novus Biologicals), anti-hypoxia-inducible factor 1, alpha subunit (HIF-1\(\alpha\)) (1:1,000; 14179, Cell Signaling), anti-hypoxia-inducible factor 1, alpha subunit (HIF-2\(\alpha\)) (1:1,000; 7096, Cell Signaling), anti-phospho-nuclear factor kappa B (NF-\(\kappa\)B) p65 (1:1,000; 3033, Cell Signaling), anti-NF-\(\kappa\)B p65 (1:2,000; 4764, Cell Signaling), anti-phospho-signal transducer and activator of transcription 3 (STAT3) (1:2,000; 9145, Cell Signaling), anti-STAT3 (1:2,000; 4904, Cell Signaling), anti-C/EBP\(\beta\) (1:1,000; 3,082, Cell Signaling), and anti-\(\beta\)-actin (1:5,000; A1978, Sigma-Aldrich). Pictures of the full blots are presented in the Supporting Information (Figures S6 and S7).

2.5 | Enzyme-linked immunosorbent assays

To determine the levels of proteins secreted by Caki-1 cells (IL-8, IL-6, VEGF, and transforming growth factor-alpha [TGF-\(\alpha\)]), DuoSET enzyme-linked immunosorbent assays (ELISAs) were used (R&D Systems) according to the vendor's protocol. Absorbance was measured using the Tecan SPECTRAFluor Plus Microplate Reader. Four biological replicates in technical duplicates were performed.

2.6 | Statistical analysis

GraphPad Prism 8.0.1 was used for statistical analyses. The results of statistical analyses are depicted as the mean ± SEM of four independent experiments. Multiple datasets were analyzed by two- or three-way analysis of variance followed by Tukey’s HSD post hoc test. Significant \(p\)-values are marked as: \(*p < .05\); \(**p < .01\); \(***p < .001\); and \(****p < .0001\).

3 | RESULTS

3.1 | AGR2 upregulates HIF-1\(\alpha\) and HIF-2\(\alpha\) in Caki-1 cells

To evaluate AGR2 endogenous expression in renal cell carcinoma (RCC), we used the A498, Caki-1, and Caki-2 human cell lines. We also investigated AGR2 levels in the human lung adenocarcinoma cell line A549 (Figure S1), which was previously described as a cell line characterized by high AGR2 level. To determine the effects of
AGR2 on HIFs in Caki-1 cells, we used an overexpression system with a lentiviral vector encoding AGR2 (pLIX-AGR2) or an empty vector (pLIX-PURO), which was used as a negative control. Following 24 hr of incubation in the medium containing doxycycline, cells were cultured for another 24 or 48 hr in normoxia (21% O2) or hypoxia (0.5% O2). Using a Tet-On overexpression system, we observed a significant increase in AGR2 transcript levels in Caki-1 cells cultured in the normoxic or hypoxic environment (Figure 1a). Furthermore, the amount of AGR2 protein in pLIX-AGR2 cells was higher under the normoxic conditions than under the hypoxic conditions (Figure 1c).

To evaluate HIF-1α and HIF-2α transcript levels, we performed real-time PCR. This analysis showed a 5.2-fold upregulation of the HIF-1α mRNA level in cells expressing AGR2 after 24 hr in normoxia, although it was not statistically significant. Changes after 48 hr under the same conditions and for both time points under the hypoxic conditions were not statistically significant. In contrast, the HIF-2α transcript level was 2.9-fold higher after 48 hr under the hypoxic conditions in AGR2-expressing cells than in pLIX-PURO cells (Figure 1b).

Next, we investigated HIF protein level in Caki-1 cells overexpressing AGR2. As expected, HIF-1α and HIF-2α proteins were observed only under hypoxic conditions (Figure 1c). The HIF-1α levels were 3.6 and 3.6-fold higher in AGR2-expressing cells (pLIX-AGR2) compared with pLIX-PURO cells after 24 and 48 hr, respectively (Figure 1d). These differences were statistically significant. Subsequently, HIF-2α protein levels in AGR2-expressing cells were 1.6 and 1.9 times higher than those in pLIX-PURO cells after 24 and 48 hr under hypoxic condition (Figure 1d). Changes in the protein level observed in cells expressing AGR2 under 48 hr of hypoxic conditions were also significant statistically. We further investigated the function of AGR2 in the regulation of HIF-2α level in a human liver cancer cell line, HepG2. We observed that AGR2 has the same impacts on HIFs in HepG2.

**FIGURE 1** The influence of AGR2 on HIF1-α and HIF-2α expression in the human ccRCC cell line Caki-1. Caki-1 cells overexpressing AGR2 (pLIX-AGR2) and control cells (pLIX-PURO) were cultured under normoxic (21% O2) or hypoxic conditions (0.5% O2) for 24 or 48 hr. (a) AGR2 mRNA levels after the induction of AGR2 expression with 1 μg/ml doxycycline in pLIX-PURO and pLIX-AGR2 Caki-1 cells. (b) HIF-1α and HIF-2α mRNA levels under normoxic (21% O2) or hypoxic (0.5% O2) conditions in pLIX-PURO and pLIX-AGR2 Caki-1 cells. The mRNA level in pLIX-PURO-expressing Caki-1 cells cultured in normoxia for 24 hr was set to 1. (c) Western blots representative of four independent experiments showing AGR2, HIF-1α, and HIF-2α expression in pLIX-PURO and pLIX-AGR2 Caki-1 cells. β-actin was used as a loading control. (d) HIF-1α and HIF-2α protein levels under hypoxic conditions (0.5% O2) in pLIX-PURO and pLIX-AGR2 Caki-1 cells. The results are normalized to β-actin protein expression. Graphs represent the mean ± SEM from four independent experiments. The p-values were estimated by two- (d) or three-way (a, b) analysis of variance followed by Tukey’s HSD post hoc test (*p < .05; **p < .01)
cells and Caki-1 cells (Figure S2). Moreover, we investigated time-dependent expression of HIF-1α and HIF-2α in the Caki-1 cell line under hypoxic conditions (Figure S3). We observed time-dependent decrease in the expression of HIF-1α. HIF-2α was quite high after 4 hr of culture under hypoxia in cells expressing AGR2; then, the change after 24 hr was minimal relative to control cells and at 96 hr of culture the increase was already significant (5.4-fold higher in AGR2-expressing cells) (Figure S3). Moreover, we studied HIFs regulating enzyme, von Hippel–Lindau protein (VHL) (Figure S4). We observed a significant decrease in the VHL expression in pLIX-PURO cells, as well as, in AGR2-expressing cells after 24 and 48 hr under hypoxic conditions. However, we did not observe significant differences in the VHL levels between the control (pLIX-PURO cells) and AGR2-expressing (Figure S4).

**FIGURE 2** AGR2 induces the expression and secretion of IL-8 and IL-6 in Caki-1 cells via C/EBPβ upregulation. Caki-1 cells overexpressing AGR2 (pLIX-AGR2) and control cells (pLIX-PURO) were cultured under normoxic (21% O2) or hypoxic conditions (0.5% O2) for 24 or 48 hr. (a) C/EBPβ mRNA expression estimated by real-time PCR. The mRNA levels in pLIX-PURO Caki-1 cells cultured in normoxia for 24 hr was set to 1. (b) C/EBPβ protein levels under normoxic or hypoxic conditions in Caki-1 cells overexpressing AGR2. The results are normalized to β-Actin protein expression. (c) Western blots representative of four independent experiments showing AGR2 and C/EBPβ protein levels in pLIX-PURO and pLIX-AGR2 Caki-1 cells. (d) IL-8 and IL-6 mRNA expression estimated by real-time PCR. The mRNA level in pLIX-PURO Caki-1 cells under normoxic conditions for 24 hr was set to 1. (e) IL-8 and IL-6 secreted protein levels determined by ELISA. Graphs represent the mean ± SEM of four independent experiments. The p-values were estimated by three-way analysis of variance followed by Tukey’s HSD post hoc test (*p < .05; **p < .01; ***p < .001; ****p < .0001)
3.2 AGR2 induces the expression and secretion of the proangiogenic and proinflammatory cytokines IL-8 and IL-6 in Caki-1 cells by increasing the C/EBPβ level

We observed that AGR2 induces C/EBPβ expression, particularly in normoxia (Figure 2a–c). The C/EBPβ mRNA level was 8.8-fold higher in AGR2-expressing cells compared with pLIX-PURO cells after 48 hr in normoxia. C/EBPβ protein levels in AGR2-expressing cells were 4.1-fold and 3.3-fold higher than those in pLIX-PURO cells after 24 and 48 hr, respectively (Figure 2b). To uncover the roles of AGR2 in inflammation and angiogenesis, we examined the cytokines IL-8 and IL-6 in Caki-1 cells cultured in normoxia or hypoxia. We observed that AGR2 strongly upregulated the expression of IL-8 and IL-6 (Figure 2d), predominantly in normoxia. After 24 and 48 hr under the normoxic conditions, the IL-8 transcript level increased by 30.6-fold and 19.5-fold in cells that express AGR2 compared with control cells (Figure 2d). In AGR2-expressing cells, the IL-8 transcript level was 4.8-fold higher after 48 hr in normoxia than after 24 hr in normoxia (Figure 2d). Compared with pLIX-PURO, pLIX-AGR2 induced an increased expression of IL-6 by 57.6-fold and 16.9-fold after 24 and 48 hr in normoxia, respectively (Figure 2d); however, it was not statistically significant. In AGR2-expressing cells in normoxia, IL-6 expression was increased by 2.3-fold after 48 hr compared to 24 hr (Figure 2d), although, it was not statistically significant. To test whether AGR2-expressing cells secrete IL-8 and IL-6, we performed ELISAs. AGR2 significantly increased IL-8 and IL-6 secretion in Caki-1 cells (Figure 2e), and the highest expression and secretion were observed under the normoxic conditions. We observed 40.7-fold and 15.9-fold increases in IL-8 secretion in AGR2-expressing cells compared to control cells after 24 and 48 hr in normoxia, respectively (Figure 2e). In AGR2-expressing cells under the normoxic conditions, we noticed a significant 2.3-fold growth in IL-8 secretion after 48 hr compared to 24 hr (Figure 2e). AGR2 upregulated soluble IL-6 levels by 24.2-fold and 15.3-fold after 24 and 48 hr in normoxia, respectively (Figure 2e). In AGR2-expressing cells in normoxia, IL-6 secretion was increased by 2.3-fold after 48 hr compared to after 24 hr (Figure 2e). The increased expression of IL-6 in normoxia could not be the result of changed HIF activity, as HIF transcription factors were not present in the cells in normoxia (Figures 1c and 2d). However, under normoxic conditions, AGR2 induces upregulation of the expression of the transcription factor C/EBPβ, which binds to the promoter sites of the genes encoding IL-8 and IL-6.19 An increased C/EBPβ level highly corresponded with elevated secretion of IL-8 and IL-6 (Figure 2a–e).

3.3 AGR2 contributes to the induction of the expression and secretion of the VEGF and TGF-α and alters cell metabolism and proliferation by upregulating GLUT1, phosphoglycerate kinase 1, and cell proliferation upregulating cyclin D1 expression

As high VEGF expression is reported in the majority of ccRCC cases,20 we further examined the impact of AGR2 on the expression and secretion of this proangiogenic factor. We observed that AGR2 significantly promoted VEGFA expression in hypoxia (Figure 3a). ELISA analyses revealed that cells overexpressing AGR2 displayed increased secretion of VEGF after 48 hr in normoxia (2.6-fold) or hypoxia (2.8-fold) (Figure 3b). TGF-α has been described as a proangiogenic factor.21 We noticed that AGR2 strongly induced TGF-α expression in hypoxia (Figure 3a). The promotive role of AGR2 in TGF-α secretion was observed in normoxia (Figure 3b). We observed a significant 15.1-fold increase in TGF-α secretion in AGR2-expressing cells compared with pLIX-PURO cells after 24 hr in normoxia (Figure 3b). The TGF-α protein level in hypoxia was increased 9-fold and 6.8-fold in AGR2-expressing cells compared with pLIX-PURO cells after 24 and 48 hr, respectively (Figure 3b). Next, we studied whether AGR2 impacts cell metabolism. We analyzed GLUT1 and phosphoglycerate kinase 1 (PGK1) mRNA expression in Caki-1 cells. We observed, as expected, increase in GLUT1 and PGK1 expression in hypoxia (Figure 3c). Compared with pLIX-PURO, pLIX-AGR2 increased GLUT1 expression by 1.4-fold and 1.9-fold in hypoxia after 24 and 48 hr, respectively. Compared with pLIX-PURO, pLIX-AGR2 upregulated PGK1 expression by 2.7-fold after 48 hr in hypoxia; however, it was not statistically significant. Moreover, we observed that AGR2 influenced cell proliferation upregulating cyclin D1 (CCND1) expression under the normoxic conditions (Figure 3c).

3.4 AGR2 regulates cell signaling through activation of NF-κB and suppression of STAT3 pathways

As shown in Figure 2, AGR2 strongly upregulates IL-6 expression. Therefore, we investigated the NF-κB and STAT3 pathways in Caki-1 cells overexpressing AGR2. Our study showed that AGR2 promoted the phosphorylation of NF-κB p65 in hypoxia but not in normoxia (Figure 4a,b). In AGR2-expressing cells in hypoxia, NF-κB p65 activation was 1.8-fold and 2.4-fold higher compared with pLIX-PURO cells after 24 and 48 hr, respectively (Figure 4b). Next, we investigated the
expression of IL-1β and tumour necrosis factor alpha (TNF-α), which are the elements of the pathway of NF-κB activation. We did not observe significant changes in the expression of TNF-α and IL-1β in the AGR2-expressing cells (Figure 4c). We further studied the role of AGR2 in the STAT3 pathway. Compared with pLIX-PURO, pLIX-AGR2 reduced the STAT3 protein level by 1.7-fold after 24 hr in normoxia (Figure 4a,d). Our data showed diminished activation of STAT3 in both control and AGR2-expressing cells in hypoxia. We observed that AGR2 decreased STAT3 activation under the normoxic conditions (Figure 4a,d).

4 | DISCUSSION

AGR2 is a disulfide isomerase which participates in folding, trafficking, and assembly of various proteins. It
has been previously described that AGR2 expression is elevated in distinct tumors.4–7,23 Our results demonstrate that the expression of AGR2 in the Caki-1 cells affects cellular pathways including the induction of inflammation, angiogenesis, the regulation of metabolism, and the response to oxygen deficiency. In an environment with a regular oxygen concentration, the destabilization of HIF-1α and HIF-2α through the ubiquitin-proteasome pathway is initiated by hydroxylation of HIF prolyl residues and subsequent ubiquitination by prolyl hydroxylases and the VHL ubiquitin ligase complex.24–26 However, the common characteristics of renal carcinomas are frequent mutations in VHL and therefore dysfunction of the mechanism of HIFs degradation by the proteasome.27 Mutations of VHL gene are present in 89.2% of ccRCC cases and lead to high VEGF expression and enhanced tumor vascularity, which significantly reduce the survival of ccRCC patients.20 Here, we exposed that AGR2 overexpression had a significant enhancing impact on the protein level of the HIF-2α in hypoxia. Accordingly, to the known targets of the transcription factor HIF-2α, we observed that AGR2 contributed to the induction of the expression of VEGFA, GLUT1, IL-8, IL-6, CCND1, and TGF-α.

Many HIF targets, such as VEGFA, GLUT1, IL-8, IL-6, and lactate dehydrogenase A, are controlled by either HIF-1α or HIF-2α.25,28,29 Although the expression of some genes, such as TGF-α and CCND1, is specifically activated only by HIF-2α,29 HIF-1α is solely responsible for the activation of PGK1 expression.30 Elevated HIF-2α expression is related to the decrease of metastasis- and progression-free survival of patients.31 Moreover, stabilization of HIF2α promotes the progression of ccRCC by inducing the expression of both VEGFA and VEGFC.32 numerous studies indicate that for the development of ccRCC, HIF-2α is more relevant than HIF-1α.33 Fifteen different ccRCC cell lines that were collected from patients were enriched in HIF-2α.34 Surprisingly, inhibiting HIF-2α is sufficient to suppress tumors that contain VHL mutations.35 Considering the potential
significance of HIF-2α in ccRCC progression and metastasis, we hypothesize that high AGR2 expression will lead to a poor survival prognosis in solid renal carcinomas.

The ER stress induced by a rise in the level of unfolded proteins leads to the activation of AGR2 expression. Interestingly, stabilization of HIF-1α contributes to the activation of the AGR2 promoter. Previous studies have revealed the stabilization of the HIF1-α protein level by AGR2 after cell stimulation using cobalt chloride. Thus, there is a positive feedback loop between the expression of AGR2 and HIF-1α. Immunoprecipitation data show that in breast cancer cells, AGR2 stabilizes HIF-1α through a direct interaction. Additionally, HIF-1α is responsible for the upregulation of AGR2 expression via hypoxia-response elements that are localized in the AGR2 promoter. Concordantly, a recent publication reports that AGR2 is responsible for the HIF-1 stabilization in the pulmonary system that induces enhanced secretion of the gel-forming molecule Mucin5ac.

Induction of angiogenesis occurs at the primary stage of tumorigenesis and precedes the progression to invasive cancer. IL-6 and IL-8 induce multiple biological activities, including proliferation, angiogenesis, and metastasis. Therefore, these cytokines promote tumor progression. Our results show that in the Caki-1 cell line, AGR2 is responsible for the upregulation of C/EBPβ expression and significant activation of NF-κB p65 that are followed by increases of the proinflammatory cytokines IL-6 and IL-8 and the proangiogenic factor VEGF. The promoters of IL-6 and IL-8 are recognized by both C/EBPβ and NF-κB and these transcription factors act synergistically to induce transcription of various genes committed to the inflammatory and acute-phase response. The results presented in this publication reveal that C/EBPβ is highly activated by AGR2 expression in Caki-1 cells cultured in normoxia. Therefore, we hypothesize that C/EBPβ activation might be an important event in the initiation of tumorigenesis, tumor progression, and angiogenesis. Interestingly, an increased level of C/EBPβ has been reported to promote tumor invasiveness in RCC. Increased C/EBPβ level driven by AGR2 strongly correlated with the induction of IL-8 and IL-6 secretion in normoxia. Surprisingly, our results showed a reduced level of the C/EBPβ protein in hypoxia; therefore, under hypoxic conditions, other activators of IL-6 and IL-8 are presumably stabilized by AGR2. The expression of both IL-6 and IL-8 is activated by the NF-κB transcription factor. Therefore, a possible mechanism that clarifies the increase of IL-6 and IL-8 in hypoxia is enhanced NF-κB phosphorylation. Furthermore, previous results indicate that AGR2 promotes tumor metastasis by directly binding VEGFA and activating the NF-κB. Other publications have shown that AGR2 promotes a proinflammatory microenvironment through the induction of the IL-6 gene as a response to ER stress, as indicated by measuring the level of X-box binding protein 1, that is one of the proteins connected to the unfolded protein response; therefore, through stabilization of the proinflammatory niche, AGR2 is important in the initiation of pancreatic ductal adenocarcinoma. Additionally, oxygen deprivation is a factor that contributes to the upregulation of IL-6 and IL-8 cytokine secretion in RCC (the 786-O and RCC4 cell lines). IL-6 is a crucial mediator of inflammation, as well as one of the activators of STAT3.

In our studies, we observed a reduced phosphorylation level of STAT3 during hypoxia; therefore, we excluded the possibility that the induction of IL-6 expression is driven by STAT3 activation.

Here, we observed that AGR2 induces TGF-α expression in the Caki-1 ccRCC cell line cultured under hypoxic conditions. Supporting our results from the renal cell line Caki-1, RNA-sequencing data for samples from patients with the EGFR-mutant type of human lung adenocarcinoma reveal that a high level of TGF-α positively correlates with elevated expression of AGR2 and reduction of IL-6. Conclusively, AGR2 plays a crucial role in the development of ccRCC.
survival times. Interestingly, lung adenocarcinomas with an enhanced level of AGR2 have a mucinous histological phenotype.

Effective treatment of renal tumors is a complex task due to the different histologies and phenotypes of kidney tissues, above 10 distinct histological subtypes of renal cancers have been identified so far. High secretion of IL-6 correlates with a short life expectancy in patients with metastatic RCC and negatively affect the effectiveness of applied treatments. Furthermore, serum detection of tumor markers: VEGF, IL-6, IL8, and hepatocyte growth factor is linked with low survival in cases of renal tumors. Interestingly, we observed that the secretion of VEGF, IL-6, and IL8 was induced through AGR2 overexpression in Caki-1 cells. Therefore, we hypothesize that the development of AGR2 inhibitors may lead to the implementation of new therapy for treatment of some types of renal carcinomas characterized by an upregulated AGR2 level.

5 CONCLUSIONS

In summary, our data reveal that AGR2 is a significant modulator of ccRCC, primarily by the upregulation of the proinflammatory cytokines IL-6, IL-8, and proangiogenic factor VEGF (Figure 5). Taken together, our findings show that AGR2 induces conditions that favor tumorigenesis. We demonstrated that AGR2 expression was linked with the upregulation of C/EBPβ expression in the Caki-1 ccRCC cell line (Figure 5). Additionally, for tumor tissues experiencing oxygen deficiency, AGR2 is a stabilizer of HIF-2α and HIF-1α. Interestingly, inflamed tissues are frequently linked with hypoxic conditions; thus, the transcription factors C/EBPβ, HIF-2α, and HIF-1α may cooperate in the induction of angiogenesis and prolong inflammation in some types of renal tumors.

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CONFLICT OF INTEREST

The authors declare no conflicts of interests.

AUTHOR CONTRIBUTIONS

Kinga Pajdzik, Mateusz Wilamowski, and Jolanta Jura designed the research. Kinga Pajdzik, Kinga B. Stopa, Michal Nodzyński, Dariusz Żurawek, and Agata Kalita performed the experiments. Kinga Pajdzik, Mateusz Wilamowski, Dariusz Żurawek, and Jolanta Jura analyzed the data. Kinga Pajdzik and Mateusz Wilamowski wrote the manuscript. Kinga Pajdzik, Mateusz Wilamowski, Dariusz Żurawek, and Jolanta Jura commented on the manuscript.

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