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Anterior gradient 2 is a binding stabilizer of hypoxia inducible factor-1α that enhances $CoCl_2$-induced doxorubicin resistance in breast cancer cells

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Key words

Anterior gradient 2, chemical hypoxia, doxorubicin resistance, hypoxia inducible factor-1α, hypoxia responsive element

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Hypoxia inducible factor-1α (HIF-1α) is associated with human breast cancer chemoresistance. Various reports have suggested that multiple pathways are involved in HIF-1α induction and that the molecular mechanisms regulating HIF-1α-induced chemoresistance are still not fully understood. Here, we report that anterior gradient 2 (AGR2), a proposed breast cancer biomarker, is an essential regulator in hypoxia-induced doxorubicin resistance through the binding and stabilization of HIF-1α. Our results show that knockdown of AGR2 in MCF-7 cells leads to the suppression of HIF-1α-induced doxorubicin resistance, whereas elevated levels of AGR2 in MDA-MB-231 cells enhance HIF-1α-induced doxorubicin resistance. AGR2 expression, in turn, is upregulated by the hypoxic induction of HIF-1α at both translational and transcriptional levels via a hypoxia-responsive region from −937 to −912 bp on the AGR2 promoter sequence. By specific binding to HIF-1α, the increased level of intracellular AGR2 stabilizes HIF-1α and delays its proteasomal degradation. Finally, we found that AGR2-stabilized HIF-1α escalates multiple drug resistance protein 1 (MDR1) mRNA levels and limits doxorubicin intake of MCF-7 cells, whereas MCF-7/ADR, a doxorubicin resistant cell line with deficient AGR2 and HIF-1α, acquires wild-type MDR1 overexpression. Our findings, for the first time, describe AGR2 as an important regulator in chemical hypoxia-induced doxorubicin resistance in breast cancer cells, providing a possible explanation for the variable levels of chemoresistance in breast cancers and further validating AGR2 as a potential anti-breast cancer therapeutic target.

Breast cancer is one of the leading causes of cancer deaths worldwide. It is the second most common cancer when ranked by cancer occurrences in both sexes.[1,2] The treatment of advanced breast cancer is currently based on multiple chemotherapeutic drugs. Doxorubicin, a topoisomerase II chemical inhibitor, is one of the most widely used chemotherapeutic drugs in cancer treatment, particularly in the treatment of HER2 positive breast cancer.[3] In addition, doxorubicin-based adjuvant therapies with cyclophosphamide,[4] paclitaxel[5] or trastuzumab[6] are often used for increased efficacy. Nevertheless, attenuation of cancer response to doxorubicin treatment in some individuals seriously restricts the success of doxorubicin-based chemotherapies. Despite concerted research efforts, the exact molecular mechanisms involved in the development of doxorubicin resistance in breast cancer cells remain poorly understood.

It has been widely reported that a major mechanism of chemoresistance is the induction of hypoxia, resulting in the increased expression of hypoxia-inducible factor-1 (HIF-1), a key player in hypoxia-induced gene expression.[7] Hypoxia is reported to prevent degradation of HIF-1 through inhibition of prolyl hydroxylase and of the von Hippel–Lindau tumor suppressor protein (VHL), a component of E3 ubiquitin ligase complex, binding to HIF-1, which rapidly degrades HIF-1 under normal oxygenation.[8,9] Induction of HIF-1 can be conveniently achieved by the treatment of cancer cells with cobalt chloride ($CoCl_2$), which abolishes VHL-HIF-1α interaction through allosteric blockade of hydroxylase activity via its metal ion binding domain. Because $CoCl_2$ is a reliable HIF-1α inducer,[10,11] and hypoxia response mimicker, this chemically induced hypoxia is widely used in hypoxia-related research.[12,13] Inhibition of HIF-1α can also be easily achieved through treatment with PX-478. As a selective HIF-1α chemical inhibitor, PX-478 has been reported to downregulate HIF-1α expression at multiple levels, including reducing HIF-1α mRNA, inhibiting HIF-1α translation and abolishing HIF-1α deubiqitination.[14]

Previous studies have shown that breast cancer cells acquire resistance to doxorubicin under both low-oxygen-induced hypoxia and $CoCl_2$-induced chemical hypoxia[9,15] and HIF-1α plays a central role in mediating this chemoresistance.[16,17] Further analyses have revealed that HIF-1α promotes doxorubicin

resistance through triggering the upregulation of Max dimerization protein 1 (MXD1),[18] carbonic anhydrase IX (CA9)[19] and multiple drug resistance protein 1 (MDR1).[17] Yet, it is still uncertain whether other factors are also involved in modulating HIF-1α signaling cascade, causing various cancer types to respond differently to chemotherapy.

Anterior gradient 2 (AGR2) is a human homologue of the Xenopus laevis cement gland protein XAG-2. AGR2 is a protein disulfide isomerase (PDI) family member with a thioredoxin domain for disulfide bond formation with substrates such as the mucin family of proteins.[20] AGR2 is both a secretory and endoplasmic reticulum protein with a KTEL C-terminal motif for endoplasmic reticulum retention.[21] AGR2 is overexpressed in several human cancer types, including estrogen receptor (ER) positive breast cancer,[22–24] and promotes breast cancer progression and malignant transformation.[25] In addition, AGR2 expression can be induced in ER-negative breast cancer cells, such as MDA-MB-231, by physiological stress, like endoplasmic reticulum stress, and hypoxic conditions.[26] HIF-1 is reported to be a major transcription factor that regulates AGR2 induction by hypoxia,[27] but the specific mechanism of AGR2 transcriptional regulation remains unexplored. Of particular importance is that AGR2 expression has been reported to be a cancer chemoresistance indicator in treatment with anti-estrogen drugs, such as tamoxifen.[28] However, the specific molecular mechanism of the AGR2 involvement in cancer chemoresistance has not been illustrated.

In this study, we report, for the first time, that HIF-1α-induced AGR2 plays a significant role in mediating hypoxia-induced chemoresistance through functional binding to and stabilizing of HIF-1α in either MCF-7 or MDA-MB-231 breast cancer cell lines. The present study provides important insight into the molecular mechanism of doxorubicin resistance in breast cancer, and indicates that AGR2 might be a potential therapeutic target to reduce chemoresistance.

Materials and Methods

Cell culture. MCF7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) were maintained in DMEM (Gibco, Grand Island, NY, US), supplemented with 10% FBS (Gibco, Grand Island, NY, US), 1% penicillin and streptomycin (Gibco, Grand Island, NY, US) at 37°C, with 5% CO_2.

MTT assay. Cells were seeded in 96-well plates and subjected to corresponding treatments for 24 h after being starved in serum-free medium for 24 h. MTT (Solarbio, Beijing, China) at 500 μg /mL was added to each well after 44 h of treatment. The system was dissolved using DMSO after incubation at 37°C for 4 h. The absorbance value at a wavelength of 570 nm was measured using a microplate reader (Thermo Fisher Scientific, Grand Island, NY, US)). The cell survival percentage was calculated by a ratio of reported value to control value.

Western blot analysis. Whole cell lysates were prepared using NP-40 lysis buffer. Proteins were separated using SDS-PAGE and transferred onto nitrocellulose membrane for antibody detection. 18A4 is a mouse monoclonal antibody against AGR2. Prepared in our laboratory and thoroughly tested, this antibody detects both human and mouse AGR2 with high specificity.[29,30] Antibodies against HIF-1α and β-actin were obtained from Santa Cruz (USA).

Immunofluorescence assay. Cells grown on coverslips were fixed with 4% formaldehyde for 10 min and blocked with goat serum for 30 min. Coverslips were incubated with the mouse monoclonal antibody against AGR2 and/or rabbit antibody against HIF-1α for 2 h and washed with PBS. The coverslips were then incubated with Dylight488 conjugated secondary antibody against mouse and/or Dylight594 conjugated antibody against rabbit (MultiSciences Biotech, Hangzhou, China). Nuclei were counterstained using DAPI (Invitrogen, Grand Island, NY, US). The fluorescence was observed and captured with laser confocal microscopy (Leica Camera, Wetzlar, Germany).

Co-immunoprecipitation assay. MCF7 cells were lysated with NP-40 lysis buffer, after incubation in DMEM with 200 μM $CoCl_2$ treatment for 6 h. After centrifugation, lysates were subjected to co-immunoprecipitation (Co-IP) using 20 μL of antibody-bound roteinG beads (Pierc, Grand Island, NY, US) at 4°C overnight. The beads were collected and washed, and boiled in protein loading buffer at 95°C for 5 min. The proteins pulled down were analyzed by western blot. The antibodies against ER-α, β-actin and HIF-1α were purchased from Santa Cruze Cell Signal Technology.

RT-PCR. Total RNA was isolated from cells after 24 h of treatment, and the RNA content was measured. Reserve transcription was performed using a ReverTra Ace qPCR RT Kit (Toyobo, Shanghai, China). Gene-specific RT-PCR, targeting AGR2 and GAPDH, was conducted using a ThunderBird SYBR qPCR Kit (Toyobo, Shanghai, China) and an Applied Biosystems Real-Time PCR Instrument (Life Technology, Grand Island, NY, US). The primer sequences were as follows:

MDR1: 5′CAACAACAGCAGCAAGACCC3′ (Forward), 5′GTAGAGCGCGGTGCAGTAG 3′ (Reverse); GAPHD: 5′TG ATGGCATGGACTGTGGTCATGAG 3′ (Forward), 5′CTCCT GCACCACCAACTGCTTAGC 3′ (Reversed).

The mRNA fold changes were calculated according to the ΔΔCt value as suggested by the manufacturer.

Plasmid construction and mutagenesis. The AGR2 luciferase plasmid was constructed with the PGL3-basic vector, inserted into the AGR2 promoter sequence. The deletion mutagenesis was performed using the KOD-Plus-Mutagenesis Kit (Toyobo). The primers designed for the mutagenesis are shown in Table 1.

Transfection and dual-luciferase assay. Transfection was performed using PEI (PolyScience, Niles, IL, US) according to CELLTECH's protocol. A reporter plasmid (2 μg) with 0.2 μg of

Table 1. Sequence of primers of different mutagenesis on anterior gradient 2 promoter

Mutagenesis	Primer sequence
Δ−1885 to −1337	Reverse: 5′ CTAGCACGCGTAAGAGCTCGGTACC 3′
	Forward: 5′ GTCATTTAATATTCAAAATGGTCCC 3′
Δ−1885 to −938	Reverse: 5′ CTAGCACGCGTAAGAGCTCGGTACC 3′
	Forward: 5′ GAATTGAAAGGAAATTCAGTATT 3′
Δ−1885 to −414	Reverse: 5′ CTAGCACGCGTAAGAGCTCGGTACC 3′
	Forward: 5′ CTCAGTTTTGAAAAATTACGTGGG 3′
Δ−1010 to −913	Reverse: 5′ CAATTCCAGTCTTTCATTTTACAGATG 3′
	Forward: 5′ GAAAGGAAATTCAGTATTTGGAGAATC 3′
Δ−910 to −780	Reverse: 5′ CTAATGAATTTATAGAAGTAATTTCTTC 3′
	Forward: 5′ CATTTTAAAAAGTCATTTATATAGG 3′
Δ−795 to −709	Reverse: 5′ GAGTTAAGGTCATAATATTTCAAAAAC 3′
	Forward: 5′ GCACACAACTTCATGAACAAAATAC 3′
Δ−705 to −611	Reverse: 5′ GCAGTCTTTAAAAGCTCAGAATGAAG 3′
	Forward: 5′ GGGAAAAAAAAACTTGGTTGCAGACC 3′
Δ−609 to −504	Reverse: 5′ CTCAAGACCATTTAATTACTCCCTG 3′
	Forward: 5′ CTGTGAAATACCTTTGAACTCTGTG 3′
Δ−510 to −283	Reverse: 5′ CTTATTTAAAGGCAAACTTTCCTGC 3′
	Forward: 5′ GACAGGAGCAGGGAAGTATTGTAG 3′

The primer sequences are used for the preparation of the AGR2 promoter deletion mutagenesis.

pRL-TK (Promega, Beijing, China) served as an internal control for each well in six-well plates. Five hours after transfection, the cells were incubated in fresh DMEM for over 12 h and transferred to 96-well plates. Cells were then exposed to the corresponding treatments for 24 h and assayed for luciferase activity with a dual-luciferase assay system (Promega). The luciferase intensity of the reporter gene and pRL-TK were measured with an automatic-sampling microplate reader (Thermo Fisher Scientific).

Statistical analysis. All values are expressed as the mean ± SD. The Origin 9.0 Pro program (OriginLab, Northampton, MA, US) was used for all statistical analyses and graph drawing. The two-tailed Students' *t*-test was used to compare measurements of pairs of samples if appropriate.

Results

Anterior gradient 2 and hypoxia inducible factor-1α are both required for the full effect of $CoCl_2$-induced doxorubicin resistance in breast cancer cells. Previous studies have reported that chemical hypoxia can induce strong doxorubicin resistance through HIF-1 upregulation in multiple human carcinomas.[26–28] Here, to further obtain insight into the role of AGR2 in chemical hypoxia-induced doxorubicin resistance in breast cancer, we constructed AGR2-knockdown and overexpression models of MCF-7 and MDA-MB-231 cell lines, respectively. We first performed AGR2-knockdown with specific shRNA transfected by lentiviruses on MCF-7 cells, which exhibited a relatively high AGR2 expression. Two stable clones with remarkable AGR2 reductions were selected for subsequent studies (shAGR2-1 and shAGR2-2). Empty vector transfected stable clones were used as control cells (shControl). We also constructed an AGR2 overexpression model, with transient transfection of an AGR2 expression plasmid (PC_AGR2) or empty vector (pcDNA3.1) into an MDA-MB-231 breast cancer cell line (MDA-MB-231/AGR2 and MDA-MB-231/PC), which normally shows minimum AGR2 expression. Western blot analysis and immunofluorescence assays confirmed both significant AGR2 level reduction in AGR2-knockdown MCF-7 cell lines and AGR2 level elevation in AGR2-overexpressed MDA-MB-231 cells (Fig. 1a,b).

To investigate AGR2 function in chemically-induced chemoresistance, we first confirmed the role of HIF-1α in $CoCl_2$-induced doxorubicin resistance by treating MCF-7 or MDA-MB-231 cells with 10 μM doxorubicin in the presence or absence of 200 μM $CoCl_2$ and PX-478, a HIF-1α chemical inhibitor (Fig. 1c,d). $CoCl_2$-induced hypoxia significantly enhanced doxorubicin resistance, whereas inhibition of HIF-1α fully abrogated this resistance in both cell lines, confirming that HIF-1α is necessary for $CoCl_2$-induced doxorubicin resistance in these cells. We then compared the effects of these same treatments on AGR2-knockdown or overexpressed cells

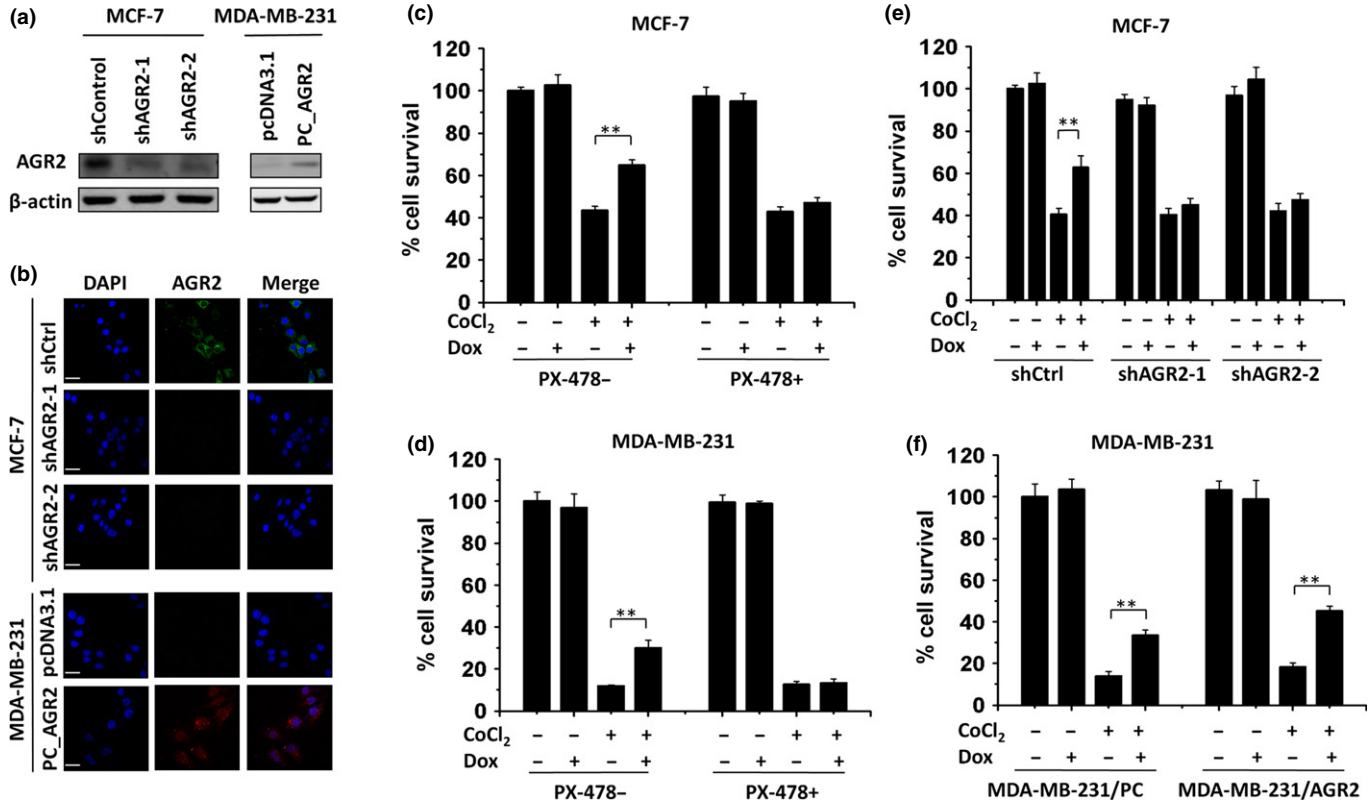

Fig. 1. Anterior gradient 2 (AGR2) and HIF-1α are both required for the full effect of $CoCl_2$-induced doxorubicin resistance in breast cancer cells. (a,b) MCF-7 cells were transfected with AGR2 shRNA or an empty vector with a lentivirus, and grown for 14 days in puromycin selection conditions. Two independent clones were selected. MDA-MB-231 cells were transfected with AGR2-pcDNA3.1 plasmid or empty vectors using PEI. Western blot of whole-cell lysates was performed using an AGR2 monoclonal antibody. β-actin was used as a loading control. AGR2 expression was also detected by immunofluorescence using confocal microscopy. The nuclei were stained with DAPI (blue) as an internal reference, and AGR2 was stained with a specific primary antibody (green in MCF-7 and red in MDA-MB-231). The original magnification was 200× (scale bar: 30 μM). (c–f) MTT assays were performed on the corresponding group of cells with 200 μM of cobalt chloride, 10 μM doxorubicin and 25 μM PX-478 for 48 h. The cell survival percentages were calculated using the ratio of treated cells to the cells of the negative control group, using the OD 570 value. Each experiment was repeated at least three times. $^{**}P < 0.01$

(Fig. 1e,f). Our results showed that knockdown of AGR2 significantly reduced $CoCl_2$-induced doxorubicin resistance in MCF-7 cells, whereas the overexpression of AGR2 led to enhancement of $CoCl_2$-induced resistance in the MDA-MB-231 cell line. These results indicate that AGR2, in addition to HIF-1α, is required for the full induction of doxorubicin resistance under conditions of chemical hypoxia.

A hypoxia-response region located at the proximal region of the anterior gradient 2 promoter is responsible for the $CoCl_2$-induced anterior gradient 2 promoter activation through hypoxia inducible factor-1α. It has been clearly recognized that both AGR2 and HIF-1α can be induced in breast cancer cells under hypoxic conditions.[26,31] To determine the relationship between AGR2 and HIF-1α, we treated both MCF-7 and MDA-MB-231 cells with increasing $CoCl_2$ levels with or without PX-478 (Fig. 2a,b). Western blot analysis revealed that both HIF-1α and AGR2 were activated by $CoCl_2$-induced chemical hypoxia in a $CoCl_2$ dose-dependent manner in each cell line. However, AGR2 induction was fully abolished by the inhibition of HIF-1α in cells treated with PX-478 in chemical hypoxia, suggesting that HIF-1α is an essential factor for AGR2 induction by hypoxia in both MCF-7 and MDA-MB-231 cell lines. To examine whether hypoxia triggers transcriptional activity on the AGR2 promoter, we constructed a 1.9-kb AGR2 promoter-luciferase reporter plasmid, and transfected it into both cell lines in the presence of different concentrations of $CoCl_2$, with or without PX-478 (Fig. 2c,d). Luciferase assays confirmed that the AGR2 promoter was markedly activated by chemical hypoxia in a dose-dependent manner, whereas abrogation of HIF-1α with PX-478 blocked the transcriptional activation of the AGR2 promoter.

To further identify the hypoxia response region on the AGR2 promoter, reporters were constructed with a series of deletions within the AGR2 promoter (Fig. 2e–h). Truncated mutation from −1885 to −413 bp abolished the hypoxic activation effect on the promoter, whereas deletions from −1885 to −1336 bp or to −937 bp maintained sensitivity towards $CoCl_2$, indicating that the response region is located between −937 and −413 bp (Fig. 2e,f). We further constructed six smaller fragments with deletions from −1011 to −282 bp (Fig. 2g,h). The results showed that deletion from −1011 to −912 bp of the promoter abolished the $CoCl_2$ activation while all other deletions showed no effect, suggesting that this sequence is indispensable for full AGR2 promoter activation by chemical hypoxia. Taken together, these results suggest that major stimulation on the AGR2 promoter by hypoxia may act through a hypoxia response region from −937 to −912 bp on the AGR2 promoter.

Anterior gradient 2 binding is required for the $CoCl_2$-induced hypoxia inducible factor-1α upregulation. To further elucidate the influence of AGR2 on HIF-1α, we used western blot assays to detect the expression level of HIF-1α in both MCF-7 AGR2-knockdown and MDA-MB-231 AGR2-overexpression cell lines under hypoxic conditions (Fig. 3a,b). The results exhibited a stronger HIF-1α upregulation by hypoxia in MDA-MB-231/AGR2 cells, compared with MDA-MB-231/PC cells. By contrast, HIF-1α upregulation was partially blocked in shAGR2 MCF-7 cells, compared with shControl cells. Furthermore, addition of doxorubicin inhibited both hypoxia-induced HIF-1α and AGR2 upregulation. These results suggest that AGR2 is necessary for the full upregulation of HIF-1α by $CoCl_2$.

We then performed a co-immunoprecipitation assay to determine whether HIF-1α is a binding target of AGR2 (Fig. 3c). We used MCF-7 whole cell lysates, with 200 μM of $CoCl_2$ administered as pre-treatment for 6 h to induce HIF-1α. The data revealed that AGR2 can be co-precipitated in MCF-7 lysates by the antibody against HIF-1α, whereas β-actin, utilized as a negative control, was unable to be pulled down by the antibody against HIF-1α. This suggests that AGR2 elevates hypoxia-induced HIF-1α levels through specific and functional binding to

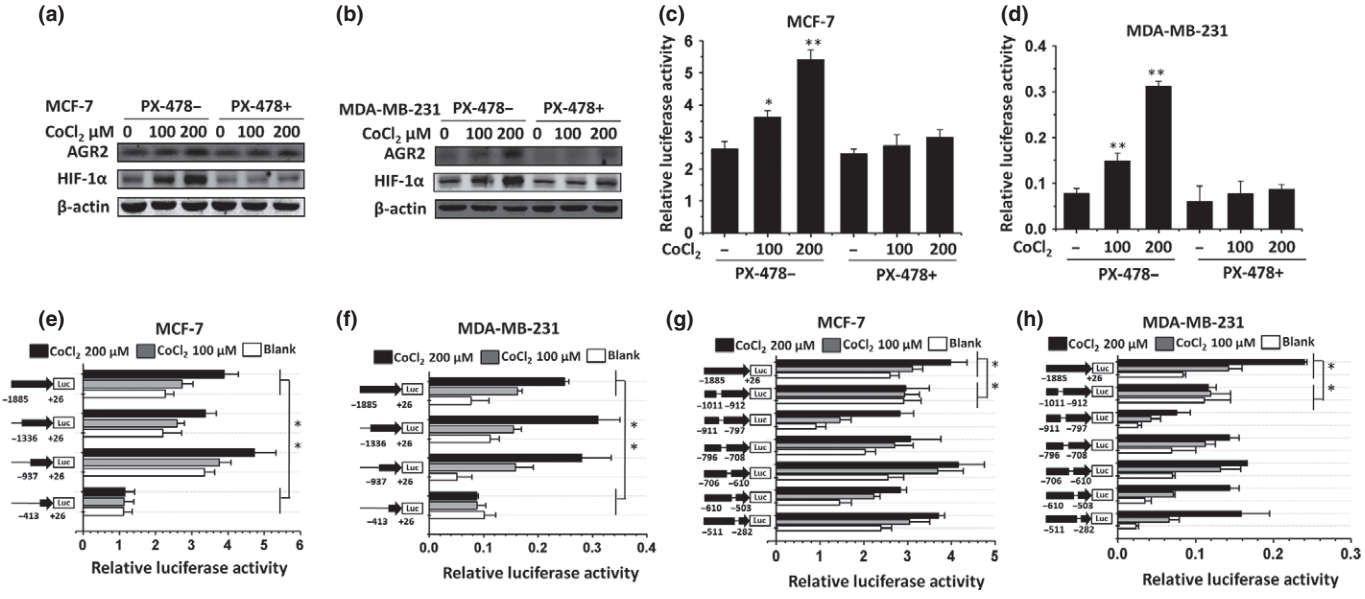

Fig. 2. A hypoxia-response region located at the proximal region of anterior gradient 2 (AGR2) promoter is responsible for the $CoCl_2$-induced AGR2 promoter activation through hypoxia inducible factor-1α (HIF-1α). (a,b) Western blot assay was performed on MCF-7 and MDA-MB-231 cell lines using corresponding concentration of $CoCl_2$ with or without 25 μM PX-478. (c,d) HIF-1 activation of the AGR2 promoter was confirmed by a 1.9 kb AGR2 promoter-luciferase reporter assay with a pRL-TK plasmid co-transfected into MCF-7 or MDA-MB-231 cells, treated with different concentration of $CoCl_2$ with or without 25 μM PX-478. The luciferase intensity was detected with a microplate reader and normalized to the intensity of Renilla. (e–h) Corresponding mutated AGR2 promoters plasmids were tranfected into MCF-7 or MDA-MB-231 cell lines using PEI. Cells were exposed to 100 or 200 μM $CoCl_2$ for 24 h and subjected to luciferase promoter assay. Each experiment was repeated at least three times. $^*P < 0.05$; $^{**}P < 0.01$

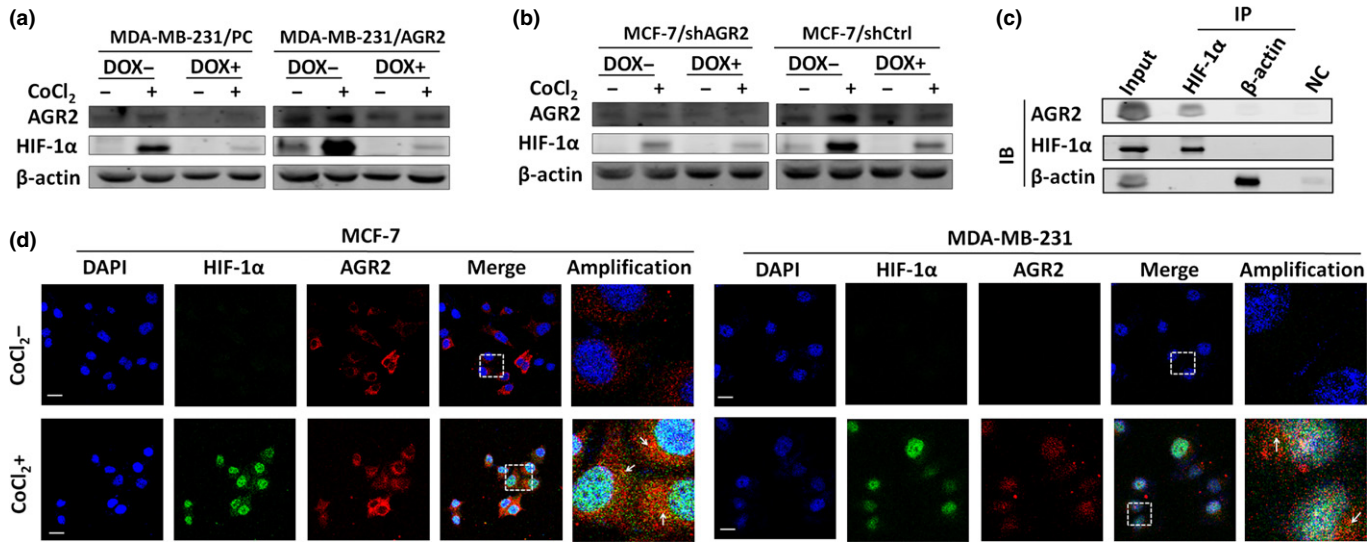

Fig. 3. Anterior gradient 2 (AGR2) binding is required for the $CoCl_2$-induced HIF-1α upregulation. (a,b) Western blot assays were performed on corresponding cell lines. The concentration of $CoCl_2$ is 200 μM and the concentration of doxorubicin is 10 μM. (c) MCF7 cells were treated with 200 μM of $CoCl_2$ for 6 h. The whole-cell lysates were then prepared and used as the input. The lysates were then mixed with a protein G-antibody solution. Western blot was performed with the corresponding antibodies. β-actin was used as a negative control (NC, a mix of the input mentioned above and the direct addition of the G protein). (d) Cells were treated with 200 μM of $CoCl_2$ for 6 h. AGR2 and hypoxia inducible factor-1α (HIF-1α) were detected by immunofluorescence using confocal microscopy. The nuclei were stained with DAPI (blue) as an internal reference. AGR2 and HIF-1α were stained in red and green, respectively. The original magnification was 200× (scale bar: 30 μM). The regions selected in the white frames are amplified, and the overlapping regions (yellow parts) are shown with white arrows. Each experiment was repeated at least three times.

HIF-1α. We further used immunofluorescence-labeled antibodies to localize AGR2 and HIF-1α in both MCF7 and MDA-MB-231 cell lines (Fig. 3d). When exposed to $CoCl_2$ for 12 h, both AGR2 (red) and HIF-1α (green) were significantly induced, with significantly enhanced co-localization (yellow, indicated by arrows) compared with the untreated cells.

Intracellular but not extracellular anterior gradient 2 stabilizes $CoCl_2$-induced hypoxia inducible factor-1α. Previous researches have uncovered that hypoxia increases HIF-1α levels by delaying rapid proteasomal degradation of HIF-1α. In the present study, proteasome inhibitor MG-132 was used to treat MCF-7 and MDA-MB-231 cells with or without $CoCl_2$, to investigate whether AGR2 enhances $CoCl_2$-induced HIF-1α upregulation by interfering with HIF-1α proteasomal degradation (Fig. 4a, b). Western blot analysis confirmed that the higher AGR2 levels resulted in the stronger HIF-1α upregulation under $CoCl_2$ treatment in both cell lines. However, combined treatment with MG-132 and $CoCl_2$ caused an equivalent level of HIF-1α upregulation independent of AGR2 levels, which implicates that AGR2 stabilizes $CoCl_2$-induced HIF-1α by affecting the proteasomal degradation process.

To distinguish whether intracellular and extracellular AGR2 share the same effect on HIF-1α, we conducted a series of protein degradation assays with cycloheximide (CHX), to inhibit protein synthesis in MCF-7 and MDA-MB-231cells, after pretreatment with 200 μM $CoCl_2$ for 6 h (Fig. 4c,d). Overexpression of AGR2 in MDA-MB-231 extended the HIF-1α half-life to 60 min, compared with 45 min in MDA-MB-231/PC cells. Similarly, knockdown of AGR2 in MCF-7 led to more rapid degradation of HIF-1α, which reached half its concentration at approximately 50 min, compared with the a half-life of approximately 75 min of shControl cells. To our surprise, addition of external AGR2 protein with $CoCl_2$ co-pretreatment did not significantly change the HIF-1α degradation rate. The quantification of HIF-1α protein levels is shown in Figure 4e and f as a function of time. An immunofluorescence assay also confirmed the $CoCl_2$-induced changes in the HIF-1α half-life of each cell line following the addition of CHX for 60 min (Fig. 4g,h). Therefore, intracellular, but not extracellular AGR2, serves as an HIF-1α stabilizer by extending its half-life.

Multiple drug resistance protein 1 is upregulated to limit doxorubicin intake through anterior gradient 2-stabilized hypoxia inducible factor-1α in MCF-7 but not MCF-7/anterior gradient cells. To further investigate whether AGR2 and HIF-1α interaction plays a key role in chemoresistance, we compared the effect of AGR2 on MDR1 in MCF-7 cells and MCF-7/ADR cells, an MCF-7-derived doxorubicin resistant cell line. We compared the cell viability curve between MCF-7 and MCF-7/ADR, using an MTT assay under an increasing concentration of doxorubicin ranging from 10^{-7} to 10^{-4} M, with or without $CoCl_2$-induced hypoxia (Fig. 5a). The data revealed that with 48 h of treatment, 10 μM doxorubicin was required to cause cell death in 50% of MCF-7/ADR cells, whereas 1 μM was required to cause cell death in 50% of MCF-7 cells. This supports stronger wild-type doxorubicin resistance of MCF-7/ADR cells. However, to our surprise, under chemical hypoxia, MCF-7 and not MCF-7/ADR cells obtained an elevated resistance. Using a western blot assay, we compared the expression levels of AGR2 and HIF-1α in both cell lines under increasing concentrations of $CoCl_2$ with or without PX-478 (Fig. 5b). In MCF-7 cells, both AGR2 and HIF-1α were detected to be induced by $CoCl_2$ in a dose-dependent manner, whereas inhibition of HIF-1α abrogated this effect. However, neither AGR2 nor HIF-1α was detected in MCF-7/ADR cells, in the presence or absence of $CoCl_2$, suggesting that MCF-7/ADR did not acquire the strong doxorubicin resistance through AGR2 or HIF-1α induction.

It has been long recognized that hypoxia can also upregulate MDR1 expression, to promote drug excretion.[32,33] Therefore, we used RT-PCR to assess the MDR1 mRNA levels in MCF-7 and MCF-7/ADR cell lines (Fig. 5c). We found that

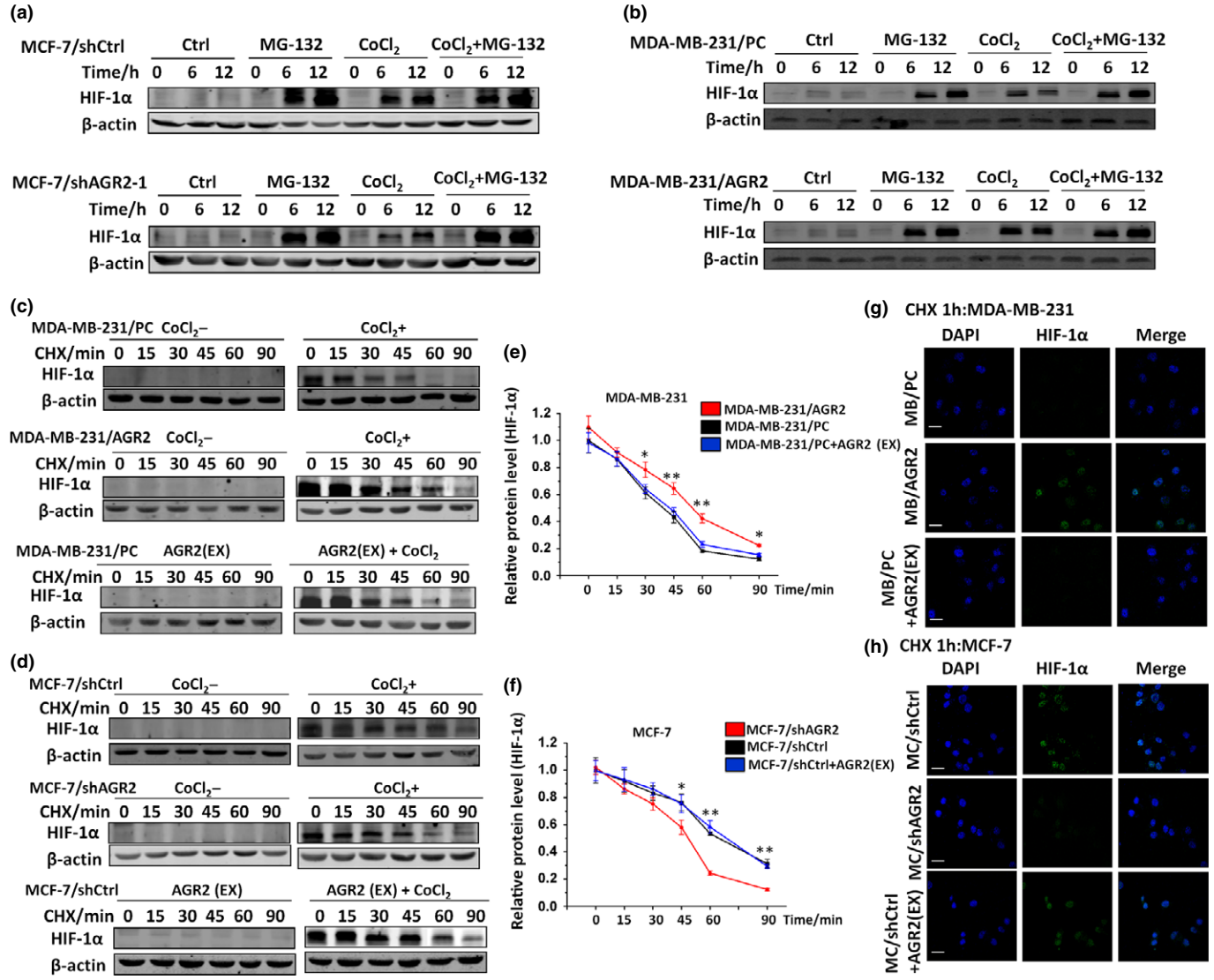

Fig. 4. Intracellular but not extracellular anterior gradient 2 (AGR2) stabilizes $CoCl_2$-induced hypoxia inducible factor-1α (HIF-1α). (a,b) Western blot was performed on corresponding cell lines with treatment of 200 μM $CoCl_2$, 20 μM MG-132 and their combination. β-actin was used as a loading control. (c,d) Cells were pre-treated with or without 200 μM $CoCl_2$ for 6 h, and subjected to 20 μM of cycloheximide (CHX). The external AGR2 protein concentration was 1 μg/mL when added with or without $CoCl_2$. Whole-cell lysates were prepared 15, 30, 45, 60 and 90 min after treatment. Western blots were performed with specific antibodies. The HIF-1α protein levels, compared to the β-actin levels at the corresponding time points, are displayed in the line chart in (e) and (f). (g,h) The same treatments were assigned to MCF-7 and MDA-MB-231 cell lines. The HIF-1α protein level at 0 and 1 h after CHX treatment is shown based on results of the immunofluorescence assay. The original magnification was 200× (scale bar: 30 μM). Each experiment was repeated at least three times. $^*P < 0.05$; $^{**}P < 0.01$

knockdown of AGR2 partially abolished hypoxia-induced MDR1 mRNA upregulation in MCF-7 cells, whereas MCF-7/ADR cells appeared to possess a pre-existing MDR1 mRNA level four-fold higher, that was not significantly affected by CoCl2 treatment, compared with MCF-7 cells. Inhibition of HIF-1α by PX-478 fully abolished the hypoxia-induced MDR1 mRNA induction in both MCF-7 and MCF-7/shAGR2 cells, but not MCF-7/ADR cells. This result demonstrates that in MCF7 cells, MDR1 upregulation is dependent on AGR2 stabilized HIF-1α under conditions of chemical hypoxia. This indicates that increased MDR1 expression may be one explanation for the effect of HIF1α-AGR2 seen in chemoresistance. To further confirm that HIF1α-AGR2 affects MDR1-regulated drug intake levels, we treated cells with different concentrations of doxorubicin with or without $CoCl_2$, in a doxorubicin cellular retention test, where the relative amount of doxorubicin absorbed in the cells could be reflected by the degree of red color in the cell pellets (Fig. 5d,e). Consistent with the above MDR1 expression results, chemical hypoxia significantly decreased the cellular doxorubicin level in MCF-7 cells compared with un-induced cells. However, AGR2 knockdown failed to reduce doxorubicin retention under conditions of chemical hypoxia, compared with shControl cells.

Discussion

The role of AGR2 expression in breast tumor development and progression has been widely studied. However, the regulation of AGR2 expression and its action in doxorubicin resistance has not been analyzed extensively. This is the first report

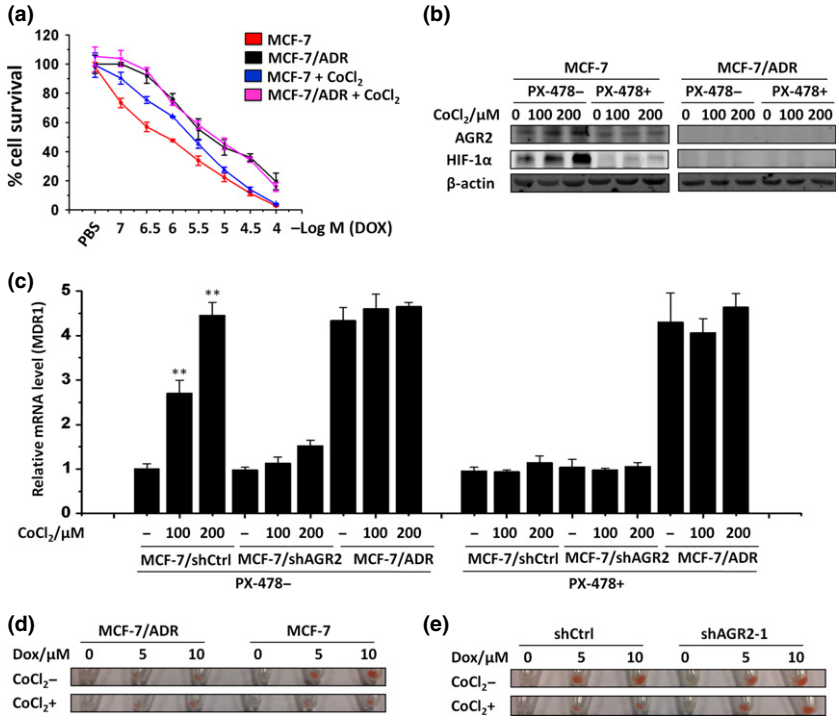

Fig. 5. MDR1 is upregulated to limit doxorubicin intake through anterior gradient 2 (AGR2)-stabilized hypoxia inducible factor-1α (HIF-1α) in MCF-7 but not MCF-7/ADR cells. (a) MCF-7 or MCF-7/ADR cells were exposed to 10^{-7} to 10^{-4} M doxorubicin with or without 200 μM $CoCl_2$ for 48 h. The cell survival percentages were calculated using the ratio of treated cells to the cells of the negative control group, using the OD 570 value. (b) Western blot was performed on MCF-7 and MCF-7/ADR cells with corresponding treatments for 12 h. β-actin was used as a loading control. (c) shControl, shAGR2 MCF-7 cells and MCF-7/ADR cells were treated with 100 or 200 μM $CoCl_2$ for 24 h and then subjected to real-time PCR assay. (d,e) Corresponding cell lines were treated with 5 or 10 μM doxorubicin with or without 200 μM $CoCl_2$ for 24 h. The cell nucleus pellets were isolated using NP-40 lysis buffer after centrifugation and subjected to photograph. Each experiment was repeated at least three times.

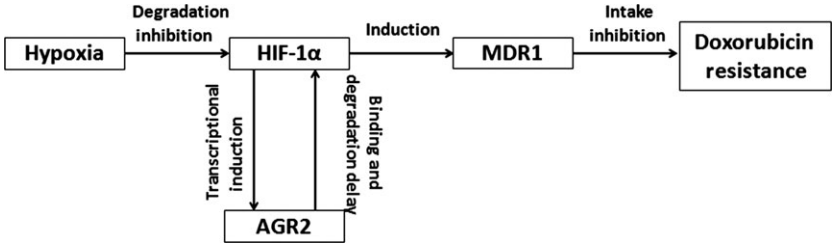

Fig. 6. Proposed role of anterior gradient 2 (AGR2) in $CoCl_2$-induced doxorubicin resistance. Hypoxia inducible factor-1α (HIF-1α) is upregulated in breast cancer cells, either under hypoxic condition or $CoCl_2$, which activates the promoter of AGR2 through a hypoxia response region. The induced AGR2 specifically binds to HIF-1α and inhibits its proteasomal degradation. The mutual effect of AGR2 and HIF-1α induces accumulation of MDR1, which further limits the intake of doxorubicin and generates drug resistance.

indicating that AGR2, working as an HIF-1α binding stabilizer, plays a significant role in hypoxia-induced doxorubicin resistance.

In our study, we constructed AGR2-knockdown MCF-7 and AGR2-overexpression MDA-MB-231 cell lines, to investigate whether AGR2 is indispensible in $CoCl_2$-induced doxorubicin resistance. Surprisingly, neither the AGR2 knockdown nor overexpression showed any significant influence on cell proliferation under normoxic or hypoxic conditions without doxorubicin treatment, compared with the fold-enhancement in cell survival with doxorubicin treatment. Similarly, in the RT-PCR analysis towards MDR1 mRNA levels, we found that AGR2 knockdown failed to change the MDR1 level in MCF-7 cells under normoxic conditions. in contrast to the significant reduction in MDR1 upregulation under chemical hypoxia conditions. Therefore, we propose that AGR2 may serve to respond to external stimulations from the cancer microenvironment, such as chemotherapeutic treatments, rather than mediate normal cell proliferation.

Besides AGR2, $CoCl_2$, as a hypoxia inducer, also showed slight and variable effects on cell proliferation (see in Fig. 1). Because $CoCl_2$ is considered a multi-factor inducer, the apparent influence on cell proliferation might be the combined results of both growth promotion and inhibition. It has been reported that induced HIF-1 activates expression of a broad range of genes that mediate tumor growth, such as vascular endothelial growth factor, which serves as a mechanism on the cell growth promotion effect. In contrast, adding $CoCl_2$ might result in cancer cell apoptosis, necrosis and DNA damage,[10,11] which contribute to cell growth suppression. Despite these limitations, chemical hypoxia mimickers, such as $CoCl_2$, remain very useful methods to investigate specific conditions such as chemical hypoxia.

Using the AGR2 promoter deletion assay, we have shown that the −937 to −912-bp sequence on the AGR2 promoter is an HIF-1α responsive element. However, no HIF-1α binding site is apparent in this region. One possible explanation is that HIF-1α binds this region with a yet unidentified sequence. Alternatively, another transcription activator may bind this region in response to $CoCl_2$ stimulation with or without HIF-1α participation. Through chromatin immunoprecipitation assay (not shown), we have identified an HIF-1α dependent regulatory mechanism controlling this element without the direct binding of HIF-1α (data not shown).

Furthermore, members of the redox protein family, such as thioredoxin-1, have been reported to exert a positive effect on HIF-1α expression,[34,35] implicating the essential role of redox protein in HIF-1α upregulation. Corresponding with the above findings, we showed that AGR2, as a PDI family member with thioredoxin domains, works as an HIF-1 stabilizer and plays a role in regulating HIF-1α levels in cancer cells.

Comparing between MCF-7 and MCF-7/ADR cells, we found that 200 μM $CoCl_2$ treatment led to a 4.5-fold increase of MDR1 mRNA level in MCF-7 cells, which is equivalent to the mRNA level in MCF-7/ADR cells. However, we also noticed that these $CoCl_2$-treated MCF-7 cells still appeared to have a weaker drug resistance than MCF-7/ADR cells under doxorubicin treatment ranging from 10^{-4} to 10^{-7} M. Hence, we propose that the strategies for acquiring doxorubicin resistance in MCF-7/ADR cells are dependent on multiple factors, including, but not limited to, the overexpression of MDR1. It has been reported that other strategies for MCF-7/ADR cells to contribute doxorubicin resistance include induction of multiple microRNA, such as miR-505,[36] and activation of several signal pathways, such as AKT[36] and p53.[37]

In summary, our study identified chemical hypoxia-induced AGR2 as a key regulator in the hypoxia-induced doxorubicin resistance of both MCF-7 and MDA-MB-231 cells. We show that AGR2 accomplishes this through the binding and stabilization of HIF-1α leading to reduced HIF-1α proteasomal degradation and upregulation of MDR1 (Fig. 6). Our study showed that AGR2 plays a significant role in breast cancer chemoresistance, suggesting AGR2 as a potentially valuable target to overcome breast cancers resistant to chemotherapy.

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Disclosure Statement

The authors have no conflict of interest to declare.

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