Opposite response to hypoxia by breast cancer cells between cell proliferation and cell migration: A clue from microRNA expression profile

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Abstract. The majority of tumors possess the features of hypoxia. It is generally accepted that hypoxia is a negative prognostic factor for cancer. Low levels of oxygen are able to modify basic cell metabolism status. Elucidating the basic response, including cell proliferation and migration, to hypoxia by cancer cells is important for understanding the role of hypoxia in the development of cancer. In the present study, $CoCl_2$ stimulation was used to simulate hypoxia. A microRNA (miRNA/miR) array was used to systematically detect the changes in miRNA expression profiles. Following treatment with $CoCl_2$ for 12 h, 15 miRNAs were markedly upregulated and 10 miRNAs were markedly decreased compared with the control. After 24 h $CoCl_2$ incubation, 15 miRNAs were increased and 3 miRNAs were decreased compared with the control. Among them, 7 miRNAs were upregulated and 2 miRNAs were downregulated at 12 and 24 h following $CoCl_2$ stimulation. The potential roles of these miRNA were reviewed and it was identified that the majority of them are associated with cell proliferation and migration. Additional experiments demonstrated that $CoCl_2$ incubation inhibited the proliferation of MCF-7 cells but promoted cell migration. miR-491 may be a key miRNA for hypoxia-inhibited cell proliferation, as it was identified that hypoxia induced the downregulation of B-cell lymphoma-extra large in a miR-491-dependent manner. As the target of miR-302a, CXCR4 may be a key protein for hypoxia-promoted cell migration. In the present study, it was identified that in the early stage of hypoxia, cell proliferation was inhibited but cell migration was promoted. These results support the hypothesis that hypoxia may be a driving force for tumor cell escape from the primary tumor site to other organs, or other sites of the same organ.

Introduction

Hypoxia is a condition in which the body or a region of the body is deprived of adequate oxygen supply. Tumor hypoxia is the situation where tumor cells have been deprived of enough oxygen. As tumor cells proliferate, the blood is no longer able to supply enough oxygen to the whole tumor. As a result, the oxygen concentration in portions of the tumor cells is significantly lower compared with that in healthy tissues. In order to support continuous growth and proliferation in challenging hypoxic environments, cancer cells have been identified to alter their metabolic pathways, including glucose and glutamine metabolism (1). Due to its contribution to chemoresistance, radioresistance, angiogenesis, vasculogenesis, invasiveness, metastasis, resistance to cell death, altered metabolism and genomic instability, hypoxia is a negative prognostic and predictive factor (2). Due to its central role in tumor progression, studies concerning the basic mechanism by which hypoxia confer to tumor growth and invasion are important. In addition, tumor hypoxia may well be considered the best validated target that has yet to be exploited in oncology.

MicroRNAs (miRNAs/miRs) are the best characterized non-coding RNA family. They have demonstrated fundamental importance in normal development, differentiation, growth control and in human diseases such as cancer (3). On one hand, miRs are involved in the regulation of key cancer-associated pathways, including cell cycle control and DNA damage response. Conversely, due to their important roles in cancer, microRNA molecules are already

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becoming diagnostic and prognostic biomarkers for patient stratification and therapeutic targets and agents (4). The microRNAs involved in hypoxia have also been studied (5) and identified to be involved in the regulation of expression of hypoxia-inducible factor 1-α (HIF-1α).

Although hypoxia and miRs in tumors have been extensively investigated, the early stage and acute hypoxia-induced tumor cell miR expression profile has not yet been examined, to the best of our knowledge. The early stages of hypoxia may reflect how tumor cells cope with the microenvironments with insufficient oxygen. The addition of hypoxia mimics such as cobalt (II) chloride to the culturing medium are able to simulate this condition. The present study sought to investigate the changes in miR expression profiles in MCF-7 cells subsequent to cobalt (II) chloride stimulation, and the association between these miRs and changes in cell behavior, including changes in cell growth and migration. The experiments from the present study demonstrated that the early stage and acute hypoxia simulated by cobalt (II) chloride was able to inhibit the cell growth, but concurrently promote cell migration dependent on different miRs. This is concordant with the hypothesis that one of the strategies for tumor cells, when faced with hypoxia, is to escape from the primary tumor site, which results in metastatic tumors.

Materials and methods

Cell culture and transfection. Human breast cancer cell lines, including low invasive MCF-7 and high invasive MDA-MB-231 cell lines were obtained from Kunming Cell Bank, Chinese Academy of Sciences (Yunnan, China), and cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA), and 2 mM L-glutamine (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO_2. The non-invasive human breast epithelial MCF-10A cell line was obtained from Shanghai Cell Bank, Chinese Academy of Sciences, and was cultured in DMEM:F12 (1:1) (Hyclone; GE Healthcare Life Science) medium supplemented with 5% horse serum (Hyclone; GE Healthcare Life Sciences), 10 μg/ml insulin (Gibco; Thermo Fisher Scientific, Inc.), 20 ng/ml epidermal growth factor (Gibco; Thermo Fisher Scientific, Inc.), 100 ng/ml cholera toxin (Sigma-Aldrich; Merck KGaA) and 0.5 μg/ml hydrocortisone (Sigma-Aldrich; Merck KGaA).

Transfection of cells was performed using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (6). miR-491 inhibitor, miR inhibitor negative control, miR-302a mimic and miR control were purchased from Guangzhou RiboBio Co. Ltd (Guangzhou, China).

All other chemicals were purchased from Sigma-Aldrich (Merck KGaA).

Cytotoxicity assay. Cytotoxicity was measured by the MTT method as previously described (7) and five repeats were performed for each sample. Exponentially growing cells were seeded at $5x10^3$ cells/well in a 96-well microplate that was supplemented with 200 μl serum-free DMEM/F12. Following overnight growth, the cells were exposed to various concentrations (50-1,600 μg/ml) of cobalt chloride (CoCl$_2$; Sigma-Aldrich; Merck KGaA). Distilled water was used to dissolve CoCl$_2$. Subsequent to incubation at 37°C of the treated cells for 24 h, 20 μl MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added and the plates were incubated at 37°C for 4 h. Then, the supernatants were carefully removed and 100 μl dimethyl sulfoxide was added to dissolve the formazan. The plates were measured at 570 nm by an Infinite 200 spectrometer (Tecan Trading Co., Ltd., Shanghai, China). The IC$_{50}$ value was determined by plotting the drug concentration vs. the survival ratio of the treated cells.

miRNA array. Total RNA from CoCl$_2$-treated or untreated MCF-7 cells, including miRNA, was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The quality and integrity of total RNA was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Small RNA was enriched using the mirVana™ miRNA Isolation kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The isolated RNA was converted to complementary DNA (cDNA) using human miRNA primer pools A & B respectively (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the cDNA was used to run the quantitative reaction in 2 human miRNA Taqman low density arrays (TLDA ABI cards version 2.0) on a 7900HT real time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95°C for 3 min followed by 45 cycles of 95°C for 10 sec and 60°C for 20 sec. The data was imported into the StatMiner® V5.0 software (Integromics, Granada, Spain) and analyzed using the default settings. The appropriate protocols were used to validate certain reactions in single-tube reaction mixes to verify expression of miRNA (Applied Biosystems protocol. Part No 4364031, Rev C 12/2009) (8). The experiment was performed two times.

Cell proliferation assay. The MCF-7 cells were seeded on a 96-well plate (1,000 cells/well), and 24 h following seeding, fresh medium supplemented with 10% FBS containing different concentrations (50-800 μM) of CoCl$_2$ was added into the plates and changed every 24 h. The number of viable cells was estimated by the MTT method every 24 h (9).

Cell migration assay. Cell migration was tested with a Boyden chamber assay, which was performed with 24-well plates (Corning Incorporated, Corning, NY, USA) and Transwell (8 μm pore; Merck KGaA) (10). The bottom side of the Transwell plates was coated with collagen type I (10 μg/ml; Sigma-Aldrich; Merck KGaA). Following starvation for 12 h with starvation medium (DMEM/F12) containing 0.25% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA), the cells were detached with cell dissociation buffer (Sigma-Aldrich; Merck KGaA), then $1x10^5$ cells were planted into the top of each chamber. Media containing 1% FBS were added into the bottom of the chamber for 16 h. Migrated cells were dyed with crystal violet (0.1% in 0.1 M borate buffer solution, pH 8.5) for 20 min. Bound crystal violet was eluted with
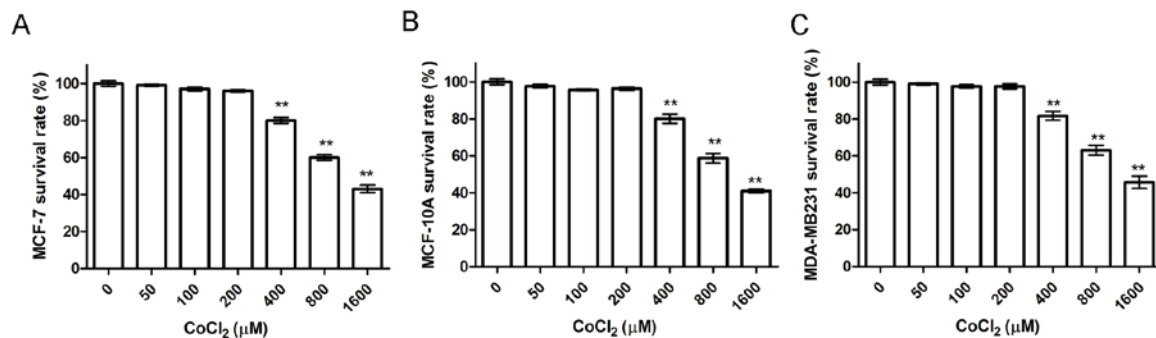
Figure 1. Toxicity of CoCl$_2$ in MCF-7, MCF-10A and MDA-MB-231 cells. (A) MCF-7, (B) MCF-10A and (C) MDA-MB-231 cells were incubated with 50-1,600 μM CoCl$_2$ for 24 h, followed by an MTT assay to assess the cell survival rate. The survival rate was calculated according to the optical density at 570 nm. [**]P<0.01 compared with the '0' group.

1 ml 10% acetic acid and the migration activity was expressed as the value monitored at 586 nm of extraction.

Western blotting. Following different stimulations, including CoCl$_2$ incubation, miRNA or miRNA inhibitor transfection, the MCF-7 cells were then collected, washed, and immediately lysed on ice in lysis buffer containing 50 mM HEPES (pH 7.4), 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 5 mg/ml aprotinin, 5 mg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. The proteins (30 μg) were electrophoresed on 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were subsequently blocked at 4°C overnight with 3% BSA and incubated at room temperature for 4 h with the appropriate primary and at room temperature for 2 h with secondary antibodies. The protein bands were visualized with Super Signal chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.), as previously described (11). The antibodies used in the present study were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The target genes of selected miRNA, including miR-491 and miR-302a, were Bcl-xL and CXCR4 as previously reported (12,13). The antibodies were as follows: Rabbit polyclonal antibody against human Bcl-xL (cat. no. sc-7195; dilution, 1:1,000); mouse monoclonal antibody against human CXCR4 (cat. no. sc-53534; dilution, 1:1,000); rabbit polyclonal antibody against human β-actin (cat. no. sc-7210; dilution, 1:2,000); mouse monoclonal antibody against ERK2 (cat. no. sc-1647; dilution, 1:1,000); mouse monoclonal antibody against human phospho-ERK1/2 (cat. no. sc-7383; dilution, 1:1,000); goat anti-mouse IgG-HRP (cat. no. sc-2005; dilution, 1:5,000); goat anti-rabbit IgG-HRP (cat. no. sc-2004; dilution, 1:5,000).

Statistical analysis. The data were analyzed with unpaired Student's t-test for variance. Experimental values expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

CoCl$_2$ demonstrates toxicity to MCF-7, MCF-10A and MDA-MB-231 in a dose-dependent manner. In vitro cytotoxicity of CoCl$_2$ in the normal breast epithelial MCF-10A cell line and breast cancer low invasive MCF-7 and high invasive

MDA-MB231 cell lines were examined. Exponentially growing cells were incubated with various concentrations of CoCl$_2$. Following 24 h incubation, the cytotoxic activity was evaluated by the MTT assay. The half-maximal inhibitory concentration (IC$_{50}$) was calculated for cobalt chloride. The CoCl$_2$ demonstrated cell toxicity in a dose-dependent manner (Fig. 1). The IC$_{50}$ value of CoCl$_2$ to MCF-7, MCF-10A and MDA-MB-231 cells were 1,257, 1,216 and 1,331 μM, respectively. No significant differences in cell survival rate were identified between the cells treated with 50, 100 and 200 μM of CoCl$_2$ in all the 3 cell lines tested. Furthermore, no significant differences in cytotoxicity were identified between MCF-7, MCF-10 and MDA-MB-231 cells in response to CoCl$_2$ stimulation.

CoCl$_2$ alters the miRNA expression profile of MCF-7 cells. miRs regulate numerous intracellular process by affecting gene expression. Thus, the changes to miRs expression may, to a large extent, reflect what happens to the cells. In the present study, the cells were incubated with CoCl$_2$ at a concentration of 200 μM for 0, 12 or 24 h. Then, miRNA expression profiles were detected by TLDA on a 7900HT real time PCR machine. At this concentration, CoCl$_2$ demonstrated no acute cytotoxicity to the cells. CoCl$_2$ induced significant upregulation of 15 miRNAs and downregulation of 10 miRNAs (Table I) following 12 h incubation with MCF-7 cells. However, following 24 h incubation, 15 miRNAs were upregulated and only 3 were downregulated (Table I). Among these miRNAs, 7 miRNAs were continuously upregulated 12 and 24 h following incubation with CoCl$_2$, and 2 miRNAs were downregulated (Table III). These miRNAs are associated with basic cell processes, including cell proliferation and cell migration (Table IV). The majority of these upregulated miRNAs appear to have the ability to suppress cell proliferation. However, their role in cell migration remains ambiguous. The downregulated miRNAs have potent abilities to inhibit cell proliferation and cell migration.

CoCl$_2$ simulates hypoxia-inhibited cell growth but promotes cell motility. Previous studies have demonstrated that CoCl$_2$ stimulation may inhibit the cell proliferation (14,15). However, other studies have suggested that CoCl$_2$-mimicked hypoxia may promote cell migration (16). The effects of CoCl$_2$ on the proliferation and migration in the MCF-7 cell line remain

Table I. miRNA expression profile of MCF-7 cells 12 h after $CoCl_2$ stimulation.

Array card name	Assay ID	Assay	miRBase_18th	Target sequence (5'→3')	Experimental group_Avg ΔCq	Control group_Avg ΔCq	Fold-change	12 h_ΔCq	0 h_ΔCq
Human A v2.1	000377	hsa-let-7a	hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU	14.68	17.00	5.00	14.68	17.00
Human A v2.1	000405	hsa-miR-26a	hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU	13.41	15.84	5.37	13.41	15.84
Human A v2.1	000411	hsa-miR-28	hsa-miR-28-5p	AAGGAGCUCACAGUCUAUUGAG	14.17	17.55	10.35	14.17	17.55
Human A v2.1	001193	mmu-miR-187	mmu-miR-187-3p	UCGUGUCUUGUGUUGCAGCCGG	17.86	14.62	-9.43	17.86	14.62
Human A v2.1	002250	hsa-miR-193a-3p	hsa-miR-193a-3p	AACUGGCCUACAAAGUCCCAGU	15.40	17.96	5.90	15.40	17.96
Human A v2.1	002363	hsa-miR-202	hsa-miR-202-3p	AGAGGUAUAGGGCAUGGGAA	17.86	8.15	-837.70	17.86	8.15
Human A v2.1	002290	hsa-miR-208b	hsa-miR-208b	AUAAGACGAACAAAAGGUUUGU	17.86	13.10	-27.19	17.86	13.10
Human A v2.1	002220	hsa-miR-216a	hsa-miR-216a	UAAUCUCAGCUGGCAACUGUGA	15.10	9.80	-39.52	15.10	9.80
Human A v2.1	000522	hsa-miR-219	hsa-miR-219-5p	UGAUUGUCCAAACGCAAUUCU	17.86	11.44	-85.38	17.86	11.44
Human A v2.1	000529	hsa-miR-302a	hsa-miR-302a-3p	UAAGUGCUUCCAUGUUUUGGUGA	17.86	11.65	-74.05	17.86	11.65
Human A v2.1	002147	hsa-miR-342-5p	hsa-miR-342-5p	AGGGGUGCUAUCUGUGAUUGA	14.85	17.96	8.64	14.85	17.96
Human A v2.1	002338	hsa-miR-483-5p	hsa-miR-483-5p	AAGACGGGAGGAAAGAAGGGAG	15.16	17.96	6.93	15.16	17.96
Human A v2.1	001630	mmu-miR-491	hsa-miR-491-5p	AGUGGGGAACCCUUCCAUGAGG	14.18	17.96	13.68	14.18	17.96
Human A v2.1	001953	mmu-miR-496	hsa-miR-496	UGAGUAUUACAUGGCCAAUCUC	14.18	17.96	13.67	14.18	17.96
Human A v2.1	002341	hsa-miR-708	hsa-miR-708-5p	AAGGAGCUUACAAUCUAGCUGGG	17.85	15.23	-6.13	17.85	15.23
Human A v2.1	002095	hsa-miR-219-1-3p	hsa-miR-219-1-3p	AGAGUUGAGUCUGGACGUCCCG	17.86	15.39	-5.56	17.86	15.39
Human B v3.0	000417	hsa-miR-30a-5p	hsa-miR-30a-5p	UGUAAACAUCCUCGACUGGAAG	17.46	12.98	-22.18	17.46	12.98
Human B v3.0	002094	hsa-miR-218-1#	hsa-miR-218-1-3p	AUGGUUCCGUCAAGCACCAUGG	17.46	9.61	-230.80	17.46	9.61
Human B v3.0	002173	hsa-miR-15b#	hsa-miR-15b-3p	CGAAUCAUUAUUUGCUGCUCUA	14.88	18.03	8.85	14.88	18.03
Human B v3.0	000567	hsa-miR-378	hsa-miR-378a-5p	CUCCUGACUCCAGGUCCUGUGU	9.92	12.42	5.64	9.92	12.42
Human B v3.0	002438	hsa-miR-21#	hsa-miR-21-3p	CAACACCAGUCGAUGGGCUGU	14.82	17.15	5.04	14.82	17.15
Human B v3.0	002768	hsa-miR-1233	hsa-miR-1233	UGAGCCCUGUCCUCCCGCAG	14.66	18.03	10.33	14.66	18.03
Human B v3.0	002818	hsa-miR-1254	hsa-miR-1254	AGCCUGGAAGCUGGAGCCUGCAGU	15.20	17.67	5.55	15.20	17.67
Human B v3.0	002854	hsa-miR-1243	hsa-miR-1243	AACUGGAUCAAUUAUAGGAGUG	8.48	10.89	5.35	8.48	10.89
Human B v3.0	002897	hsa-miR-664	hsa-miR-664-3p	UAUUCAUUUAUCCCCAGCCUACA	13.11	15.99	7.35	13.11	15.99

miR, microRNA.

Table II. miRNA expression profile of MCF-7 cells 24 h after CoCl$_2$ stimulation.

Array card name	Assay ID	Assay	miRBase_18th	Target sequence (5'→3')	Experimental group_Avg ΔCq	Control group_Avg ΔCq	Fold-change	24 h_ΔCq	0 h_ΔCq
Human A v2.1	000377	hsa-let-7a	hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU	13.24	17.00	13.61	13.24	17.00
Human A v2.1	000580	hsa-miR-20a	hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	11.36	14.12	6.76	11.36	14.12
Human A v2.1	000405	hsa-miR-26a	hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU	13.05	15.84	6.88	13.05	15.84
Human A v2.1	000411	hsa-miR-28	hsa-miR-28-5p	AAGGAGCUCACAGUCUAUUGAG	14.32	17.55	9.33	14.32	17.55
Human A v2.1	000480	hsa-miR-181a	hsa-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU	15.33	17.79	5.49	15.33	17.79
Human A v2.1	000529	hsa-miR-302a	hsa-miR-302a-3p	UAAGUGCUUCCAUGUUUUGGUGA	17.77	11.65	-69.58	17.77	11.65
Human A v2.1	002619	hsa-let-7b	hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU	8.78	12.18	10.57	8.78	12.18
Human A v2.1	002147	hsa-miR-342-5p	hsa-miR-342-5p	AGGGGUGCUAUCUGUGAUUGA	13.91	17.96	16.55	13.91	17.96
Human A v2.1	000561	hsa-miR-373	hsa-miR-373-3p	GAAGUGCUUCGAUUUUGGGGUGU	12.86	16.39	11.59	12.86	16.39
Human A v2.1	001030	hsa-miR-449	hsa-miR-449a	UGGCAGUGUAUUGUUAGCUGGU	17.77	15.03	-6.70	17.77	15.03
Human A v2.1	002208	hsa-miR-450b-3p	hsa-miR-450b-3p	UUGGGAUCAUUUUGCAUCCAUA	14.32	17.96	12.45	14.32	17.96
Human A v2.1	001630	mmu-miR-491	hsa-miR-491-5p	AGUGGGGAACCCUUCCAUGAGG	15.51	17.96	5.45	15.51	17.96
Human A v2.1	001953	mmu-miR-496	hsa-miR-496	UGAGUAUUACAUGGCCAAUCUC	11.51	17.96	87.39	11.51	17.96
Human A v2.1	002084	hsa-miR-504	hsa-miR-504	AGACCCUGGUCUGCACUCUAUC	14.29	17.96	12.72	14.29	17.96
Human A v2.1	002369	hsa-miR-515-3p	hsa-miR-515-3p	GAGUGCCUUCUUUUGGAGCGUU	15.01	17.96	7.71	15.01	17.96
Human A v2.1	002095	hsa-miR-219-1-3p	hsa-miR-219-1-3p	AGAGUUGAGUCUGGACGUCCCG	17.77	15.39	-5.22	17.77	15.39
Human B v3.0	002182	hsa-miR-939	hsa-miR-939	UGGGGAGCUGAGGCUCUGGGGGUG	14.20	17.08	7.34	14.20	17.08
Human B v3.0	002897	hsa-miR-664	hsa-miR-664-3p	UAUUCAUUUAUCCCCAGCCUACA	13.62	15.99	5.18	13.62	15.99

miR, microRNA.

Table III. Summary of upregulated and downregulated miRNAs.

miRNA	$\log_2$(Fold) (12 h)	$\log_2$(Fold) (24 h)
let-7a	5.00	13.61
miR-26a	5.37	6.88
miR-28	10.35	9.33
miR-302a	-74.05	-69.58
miR-342-5p	8.64	16.55
miR-491	13.68	5.45
miR-496	13.67	87.39
miR-219-1-3p	-5.56	-5.22
miR-664	7.35	5.18

miRNA, microRNA.

Table IV. Potential role of the miRNAs in cell proliferation, migration and invasion.

miRNA	Proliferation	Migration and invasion
Upregulated		
let-7a	A549 ↓(31), PC3 ↓(32)	HepG2 ↓(33)
miR-26a	MHCC-79L↓(34), DU145↑(35)	A549 ↓(36)
miR-28	HCT116↓(37)	HCT116↓(37)
miR-342-5p	U87MG↓(38)	ND
miR-491	U251↓(17)	ND
miR-496	ND	ND
miR-664	A375↓(39)	ND
Downregulated		
miR-302a	ND	ND
miR-219-1-3p	PDAC-derived cell lines↓(40)	PDAC-derived cell lines↓(40)

miRNA, microRNA; ↑, upregulation; ↓, downregulation; ND, not defined; PDAC, pancreatic ductal adenocarcinoma.

unclear. In the present study, MCF-7 cells were treated with different concentration of $CoCl_2$ and the cell viability were assessed by MTT assay. The results demonstrated that $CoCl_2$ inhibited cell growth of MCF-7 in a dose-dependent manner (Fig. 2A). Conversely, $CoCl_2$ promoted cell migration of MCF-7 in a dose-dependent manner between the dose of 50 and 400 μM (all P<0.01; Fig. 2B).

miR-491 is involved in $CoCl_2$ inhibited cell growth and miR-302a is involved in $CoCl_2$-promoted cell migration. As aforementioned, it is notable that $CoCl_2$ may inhibit cell growth, yet concomitantly promote cell migration. The miRNA profile is an important clue to explain this occurrence. As summarized in Table IV, these altered miRNAs are associated with cell growth and cell migration. Thus, two miRNAs, miR-491 and miR-302a, were selected for in-depth investigation.

miR-491 is significantly upregulated at 12 and 24 h following incubation with $CoCl_2$, and it was previously identified to be involved in the inhibition of cell proliferation (17). Therefore, it may serve a significant role in $CoCl_2$-inhibited cell growth. The has-miR-491-5p inhibitor was transfected into MCF-7 cells to attenuate the upregulated level of miR-491 by $CoCl_2$. The rates of cell growth and cell migration were assessed, and the results demonstrated that the miR-491-5p inhibitor significantly attenuated $CoCl_2$ inhibition on MCF-7 cell growth (P<0.05; Fig. 3A) but exhibited no significant effects on cell migration (Fig. 3B).

miR-302a was the most downregulated miRNA following $CoCl_2$ treatment. miR-302a mimics were transfected into MCF-7 cells to rescue the miR-302a downregulated by $CoCl_2$. Then, the rates of cell growth and cell migration were assessed. The results indicated that miR-302a mimics did not exhibit a significant effect on cell proliferation (Fig. 3C), but they did significantly reduce $CoCl_2$-enhanced cell migration (P<0.05; Fig. 3D).

$CoCl_2$ stimulated changes in the expression of numerous miRNAs, yet their roles in the modification of cell growth and migration in $CoCl_2$-simulated hypoxia requires additional study. However, it is clear that miR-491 is involved in $CoCl_2$-induced inhibition of cell growth, and that miR-302a is involved in $CoCl_2$-induced promotion of cell migration.

miR-491 regulates the expression of B-cell lymphoma-extra large (Bcl-xL). The aforementioned experiments demonstrated that miR-491 serves an important role in $CoCl_2$-induced inhibition of cell proliferation. It has been identified that miR-491 may target Bcl-xL, thus inducing apoptosis and inhibiting cell proliferation (18). Western blot analysis demonstrated a marked decrease in Bcl-xL expression following treatment with $CoCl_2$ compared with the control; however, treatment with miR-491 inhibitors rescued this effect (Fig. 4A).

miR-302a regulates the expression of C-X-C chemokine receptor 4 (CXCR4). miR-302a affected the $CoCl_2$-induced promotion of cell migration. The specific target gene and its mechanism of action require additional study. It has been previously revealed that CXCR4 is the target of miR-302a (12,13). In the present study, it was identified that $CoCl_2$ markedly upregulates the level of CXCR4 (Fig. 4B); however, transfection with miR-302a mimics attenuated the $CoCl_2$-induced upregulation of CXCR4. This suggested that miR-302a may affect cell migration in the MCF-7 cell line, at least partially by targeting CXCR4.

miR-491 upregulation and miR-302a downregulation mediate extracellular signal-related kinase (ERK)1/2 activation by $CoCl_2$. $CoCl_2$ stimulation markedly induced the phosphorylation of ERK1/2 (Fig. 5A), which is consistent with the results from a previous study (19). To investigate the potential role of miR-491 in $CoCl_2$-induced ERK1/2 activation, miR-491 inhibitors were transfected into MCF-7 cells, and it was identified that inhibition may moderately attenuate $CoCl_2$-induced ERK1/2 activation, which was consistent with a previous
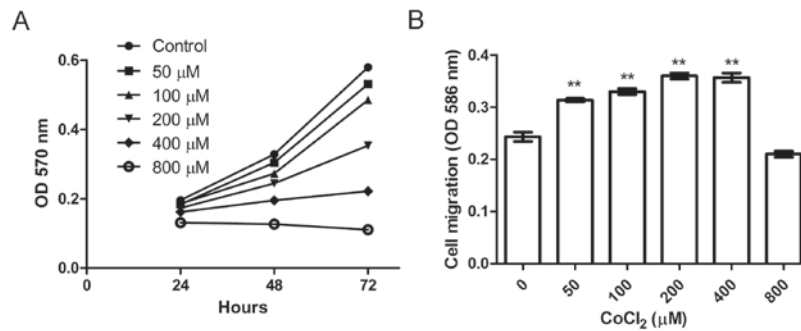
Figure 2. CoCl$_2$-simulated hypoxia inhibited cell growth but promoted cell motility. (A) Cells were incubated with different concentrations of CoCl$_2$. Cell viability was assessed by MTT assay. (B) Cells were first starved with serum-free medium for 12 h, and then pretreated with different concentrations of CoCl$_2$ for 12 h. Cell migration was assessed by Transwell assay. **P<0.01 compared with the '0' group. ns, not significant; OD, optical density.
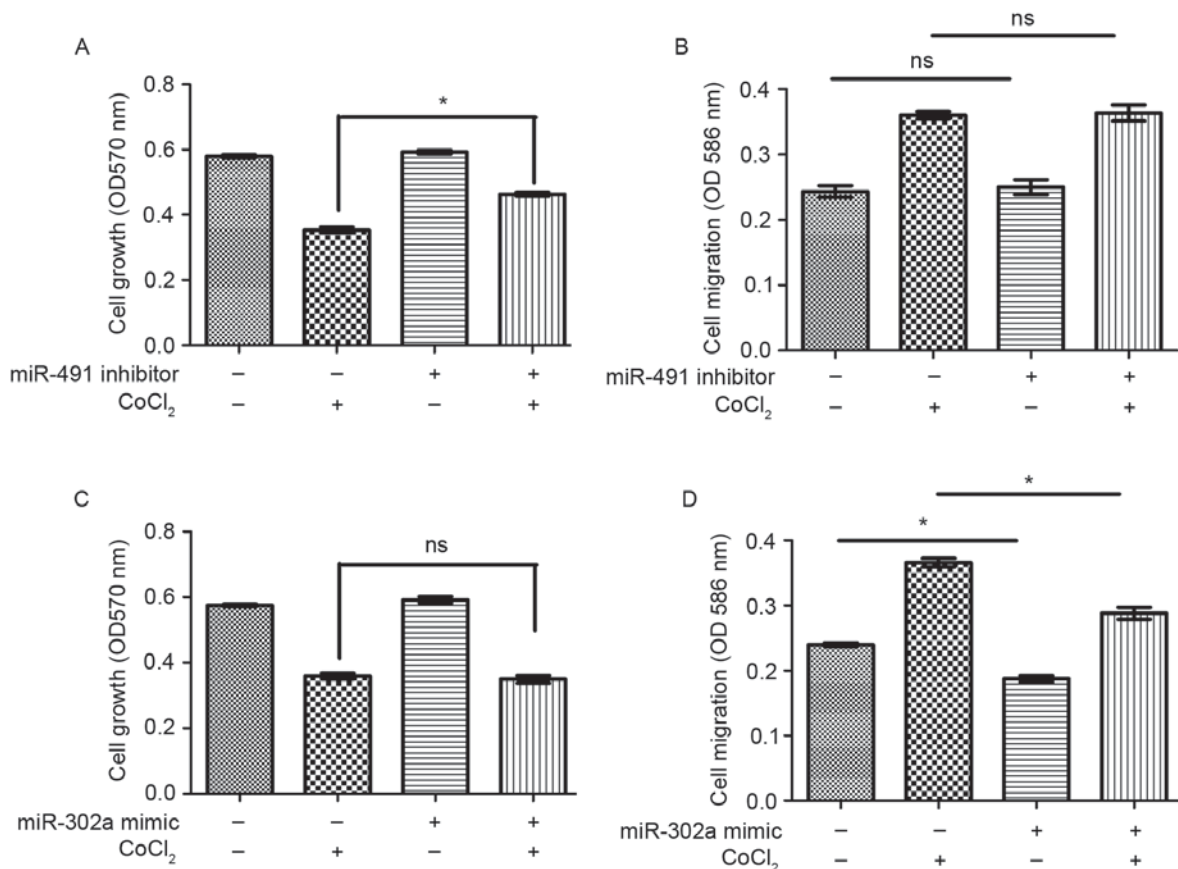

Figure 3. miR-491 is involved in CoCl$_2$ inhibited cell growth and miR-302a is involved in CoCl$_2$ promoted cell migration. (A) MCF-7 cells were transfected with a miR-491-5p inhibitor (+) or negative control (-). Following 18 h of transfection, cells were seeded onto 96-well plates. After 18 h seeding, cells were incubated with or without CoCl$_2$ for 72 h. Then, cell viability was assessed by MTT assay. (B) MCF-7 cells were transfected with a miR-491-5p inhibitor (+) or negative control (-). Cells were first starved with serum-free medium for 12 h, and then pretreated with 200 μM of CoCl$_2$ for 12 h. Cell migration was assessed by Transwell assay as aforementioned. (C) MCF-7 cells were transfected with miR-302a mimics (+) or control miRNA (-). Cell proliferation was assessed in the same way as in (A). (D) MCF-7 cells were transfected with miR-302a mimics (+) or control miRNA (-). Cell migration was assessed the same way as in (B). ns, not significant, P>0.05; *P<0.05; miR, microRNA; OD, optical density.

result (20). The present study also examined the role of miR-302a in ERK1/2 activation, and it was revealed that transfection of miR-302a mimic also resulted in the attenuation of ERK1/2 activation (Fig. 5B). ERK1/2 may be involved in the modification of cell proliferation and cell migration in hypoxia, but it requires additional study.

Discussion

Hypoxia is involved in numerous physiological and pathological processes. During previous *in vitro* studies, a number of chemicals have been used to mimic hypoxia (21). CoCl$_2$ is the most frequently used hypoxia mimic. Hypoxia is associated with
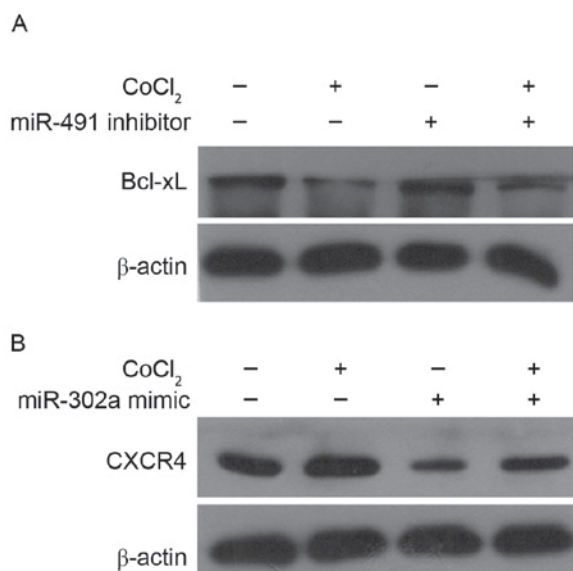
Figure 4. miR-491 targeted Bcl-xL and miR-302a targeted CXCR4, respectively, to modify cell growth and cell migration. (A) MCF-7 cells were transfected with miR-491-5p inhibitors (+) or negative control (-). After 24 h transfection, cells were incubated with or without CoCl$_2$ for 48 h. Then, the cells were harvested for western blot analysis as aforementioned. (B) MCF-7 cells were transfected with miR-302a mimics (+) or negative control (-). After 24 h transfection, cells were incubated with or without CoCl$_2$ for 48 h. Then, the cells were harvested for western blot analysis as aforementioned. Bcl-xL, B-cell lymphoma-extra large; CXCR4, C-X-C chemokine receptor 4; miR, microRNA.
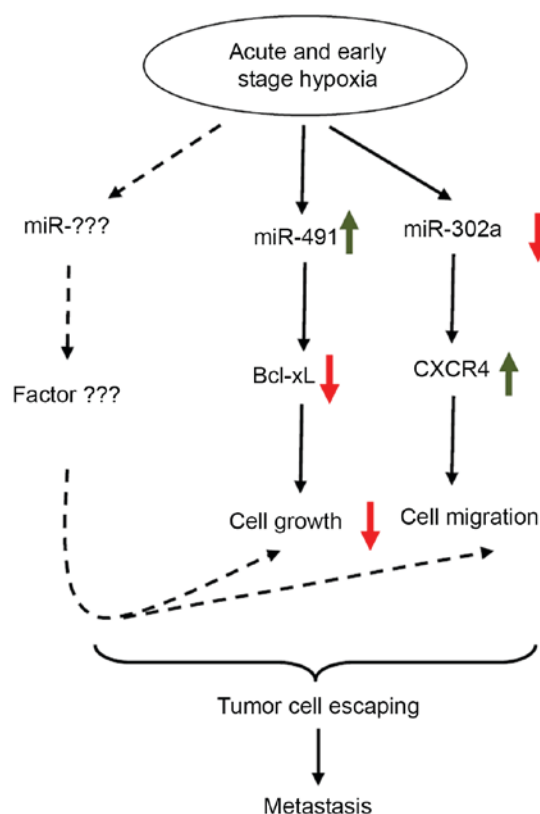

Figure 6. Proposed mechanism by miR-491 and miR-302a to modify tumor cell behavior. miR, microRNA; Bcl-xL, B-cell lymphoma-extra large; CXCR4, C-X-C chemokine receptor 4.

Figure 5. miR-491 and miR-302a were involved in CoCl$_2$ induced ERK1/2 activation. (A) MCF-7 cells were stimulated with CoCl$_2$ for 30 min, 4 or 24 h, and then the cells were lysed for the detection of phosphor-ERK1/2 (pERK1/2) by western blot analysis. The total ERK1/2 and β-actin were also detected. (B) For assessing the role of miR-491 and miR-302a, miR-491 inhibitors or miR-302a mimics were transfected 36 h prior to CoCl$_2$ stimulation. Then. the levels of p-ERK1/2 were assessed by western blotting. miR, microRNA; pERK1/2, phosphor-ERK1/2.

tumor growth (22) and metastasis (23). Numerous studies have focused on identifying the role of hypoxia in cancer occurrence and its involved mechanisms. Hypoxic conditions arise

due to the high rate of tumor cell proliferation and solid tumor formation. During the early stage of solid tumor formation, the tumor cells must become accustomed to hypoxic conditions. In *in vitro* models, the addition of hypoxia mimic chemicals, including CoCl$_2$ to the culturing medium may simulate the cellular response during the early stage of tumor growth. The present study aimed to identify what occurred to the cells during this stage.

miRNAs serve a pivotal role in tumor biology. Certain miRNAs may act as tumor suppressors or oncogenes (24). These miRNAs affect tumor growth and metastasis by targeting tumor associated protein-encoding genes. In the present study, miRNA profiles were analyzed by miRNA array. The altered miRNAs were associated with cell proliferation and cell migration, which may be important for tumor cells. Additional study of the detailed roles of these miRNAs is important for understanding CoCl$_2$-simulated hypoxia in basic tumor cell biology, including cell proliferation and cell migration. In the present study, 2 markedly upregulated or downregulated miRNAs, miR-491 and miR-302a, were selected for investigation concerning their targets and their roles in cell growth and cell migration.

It was identified that miR-491 attenuated CoCl$_2$-induced inhibition of cell growth. This may be attributed to the downregulated level of Bcl-xL by miR-491. Previous studies revealed that miR-491 may target various genes including *Bcl-xL* and G-protein-coupled receptor kinase-interacting protein 1 (*GIT1*) (13,20). *Bcl-xL* downregulation may promote cell apoptosis and inhibit cell proliferation (18), and the

downregulation of *GIT1* may inhibit cell migration (20). Hypothetically, upregulation of miR-491 will induce the downregulation of GIT1, thus inhibiting cell migration. However, CoCl₂ did not inhibit cell migration, which meant that the inhibition of cell migration induced by miR-491/GIT1 was masked by other altered genes. Of all the upregulated and downregulated miRNAs, miR-302a was the most downregulated. CXCR4 has been indicated to be the target of miR-302a (12). CXCR4 is an α-chemokine receptor specific for stromal cell-derived factor 1 (SDF-1), a molecule with potent chemotactic activity for lymphocytes. The expression of CXCR4 in healthy tissues is low or absent, however, it is expressed in a number of types of cancer, including breast cancer, melanoma and ovarian cancer (25). CXCR4 expression is associated with metastasis to tissues containing a high concentration of SDF-1, including lungs, liver and bone marrow (26). In the present study, CoCl₂-stimulated hypoxia significantly upregulated the expression of CXCR4 by lowering the miR-302a level. This is consistent with a previous study, which demonstrated that hypoxia may drive the metastasis of tumors (23).

Numerous studies have suggested that hypoxia may encourage tumor growth (22), partially mediated by HIF-1α (27). However; at the early stage of tumor cell growth, uncontrolled cell proliferation results in a lack of oxygen supply in the cells, that is, hypoxia. Logically, there are two ways for cells to manage this: i) to adjust their metabolic pathways to be accustomed to the low oxygen supply; ii) to escape from hypoxic environment, or metastasize. At the early stage of tumor growth, concomitant with the results from the present study, cell proliferation may be markedly affected by the lack of oxygen supply (28). Yet, the migration ability of these cells is markedly elevated, and this is partially mediated by CXCR4 (Fig. 6). Previous studies have been focused on the role of mitogen-activated protein kinases, including ERK1/2, in tumor cell proliferation and migration (29). It was identified that the activation of ERK1/2 generally promotes cell survival, but under certain conditions, ERK1/2 may have apoptotic functions (30). In the present study, the CoCl₂-induced activation of ERK/1/2 may be viewed as the cell response to the CoCl₂-induced hypoxia to promote cell survival. However, the role of miR-491 and miR-302a in the activation of ERK1/2 may be complicated, and requires additional investigation. Future experiments are required to explore the signal cascade involved in the hypoxia-induced inhibition of cell proliferation and promotion of migration.

Although it has been shown that hypoxia can promote tumor cell growth when cell proliferation of MCF-7 cells is inhibited, this has not been demonstrated in other cell types. Additional in-depth *in vitro* and *in vivo* experiments focused on other types of cell line are required to strengthen this hypothesis. In addition, understanding the association between the oxygen concentration of the original tumor tissue, and the rates of tumor metastasis, will also provide important evidence for this hypothesis. The different miRNA expression profiles between breast tumor and normal tissues, and also between different sites of the tumor tissues, will provide important information. In conclusion, the present study revealed that acute hypoxia may inhibit MCF-7 cell proliferation by miR-491/Bcl-xL upregulation. Conversely, the rate of cell migration was increased by CXCR4 upregulation. These results may partially demonstrate what occurs during the early stages of hypoxia. Additional clinical investigation is required to confirm these data.

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