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

MING ZHANG1*, CHANG-E GAO2*, WEN-LIN CHEN3, YI-YIN TANG3, JIAN-YUN NIE3, LI-DA SHEN2, XIANG MA4 and DE-DIAN CHEN3

1Department of Radiation Oncology, The Third Affiliated Hospital of Kunming Medical University, Tumor Hospital of Yunnan, Kunming, Yunnan 650118; 2Department of Medical Oncology, The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650032; Departments of 3Breast Surgery and 4Orthopedics, The Third Affiliated Hospital of Kunming Medical University, Tumor Hospital of Yunnan, Kunming, Yunnan 650118, P.R. China

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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, CoCl₂ 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 CoCl₂ for 12 h, 15 miRNAs were markedly upregulated and 10 miRNAs were markedly decreased compared with the control. After 24 h CoCl₂ 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 CoCl₂ stimulation. The potential roles of these miRNAs were reviewed and it was identified that the majority of them are associated with cell proliferation and migration. Additional experiments demonstrated that CoCl₂ 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
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% CO₂. 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 5x10³ 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 (CoCl₂; Sigma-Aldrich; Merck KGaA). Distilled water was used to dissolve CoCl₂. 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 IC₅₀ value was determined by plotting the drug concentration vs. the survival ratio of the treated cells.

miRNA array. Total RNA from CoCl₂-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 (Intergenomics, 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 CoCl₂ 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 1x10⁵ 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
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 CoCl₂ 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-7210; dilution, 1:2,000); 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 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

CoCl₂ demonstrates toxicity to MCF-7, MCF-10A and MDA-MB-231 in a dose-dependent manner. In vitro cytotoxicity of CoCl₂ 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 CoCl₂. Following 24 h incubation, the cytotoxic activity was evaluated by the MTT assay. The half-maximal inhibitory concentration (IC₅₀) was calculated for cobalt chloride. The CoCl₂ demonstrated cell toxicity in a dose-dependent manner (Fig. 1). The IC₅₀ value of CoCl₂ 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 CoCl₂ 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 CoCl₂ stimulation.

CoCl₂ 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 CoCl₂ at a concentration of 200 µM for 0, 12 or 24 h. Then, mRNA expression profiles were detected by TLDA on a 7900HT real time PCR machine. At this concentration, CoCl₂ demonstrated no acute cytotoxicity to the cells. CoCl₂ 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 CoCl₂, 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.

CoCl₂ simulates hypoxia-inhibited cell growth but promotes cell motility. Previous studies have demonstrated that CoCl₂ stimulation may inhibit the cell proliferation (14,15). However, other studies have suggested that CoCl₂ mimicked hypoxia may promote cell migration (16). The effects of CoCl₂ on the proliferation and migration in the MCF-7 cell line remain
Table I. miRNA expression profile of MCF-7 cells 12 h after CoCl₂ 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 | UGAGGUAGUAGGUAGUAGUAGUU | 14.68                     | 17.00                  | -5.00       | 14.68     | 17.00   |
| Human A v2.1    | 000405   | hsa-miR-26a | hsa-miR-26a-5p | UUCAAGUAAUCCAGAGUAGCCU | 13.41                     | 15.84                  | -5.87       | 13.41     | 15.84   |
| Human A v2.1    | 000411   | hsa-miR-28 | hsa-miR-28-5p | AAGGAGCACAGCUCUAUUGAG | 14.17                     | 17.55                  | -6.12       | 14.17     | 17.55   |
| Human A v2.1    | 001193   | mmu-miR-187 | hsa-miR-187-3p | UCGUGUUCUGUGUGUUGCGCCG | 17.86                     | 14.62                  | 3.60        | 17.86     | 14.62   |
| Human A v2.1    | 002250   | hsa-miR-193a-3p | hsa-miR-193a-3p-5p | AUCUGGCCUAACAAUGGCCAGU | 15.40                     | 17.96                  | -5.60       | 15.40     | 17.96   |
| Human A v2.1    | 002363   | hsa-miR-202 | hsa-miR-202-3p | AGAGGUAGUAGGUAGGGA    | 17.86                     | 8.15                   | -9.29       | 17.86     | 8.15    |
| Human A v2.1    | 002290   | hsa-miR-208b | hsa-miR-208b-5p | AUAAGACGAAACAAAGGUUUGU | 17.86                     | 13.10                  | -4.56       | 17.86     | 13.10   |
| Human A v2.1    | 002220   | hsa-miR-216a | hsa-miR-216a-5p | UAAUCUCAGUCUGGGCAACUGUGA | 15.10                    | 9.80                   | -5.30       | 15.10     | 9.80    |
| Human A v2.1    | 000522   | hsa-miR-219 | hsa-miR-219-5p | UGAUUGGUCAAACGCAUUCU | 17.86                     | 11.44                  | -6.02       | 17.86     | 11.44   |
| Human A v2.1    | 000529   | hsa-miR-302a | hsa-miR-302a-3p | UAGUGCUUCCAGGUGUGUGUA GA | 17.86                    | 11.65                  | -14.79      | 17.86     | 11.65   |
| Human A v2.1    | 002147   | hsa-miR-342-5p | hsa-miR-342-5p-3p | AGGGGCGCUAACUGUAGUGA    | 14.85                     | 17.96                  | -4.15       | 14.85     | 17.96   |
| Human A v2.1    | 002338   | hsa-miR-483-5p | hsa-miR-483-5p-3p | AAGACCAGGAGGAAAGAGGGAG | 15.16                     | 17.96                  | -2.80       | 15.16     | 17.96   |
| Human A v2.1    | 001630   | mmu-miR-491 | hsa-miR-491-5p | AGUGGGAACCCUCUCAUGAGG | 14.18                     | 17.96                  | -3.78       | 14.18     | 17.96   |
| Human A v2.1    | 001953   | mmu-miR-496 | hsa-miR-496-5p | UAGAUAAUACAGGCAGAAUCUC | 14.18                     | 17.96                  | -3.78       | 14.18     | 17.96   |
| Human A v2.1    | 002341   | hsa-miR-708 | hsa-miR-708-5p | AAGAGCUUACAAUCUAGCGUGG | 17.85                     | 15.23                  | -2.52       | 17.85     | 15.23   |
| Human A v2.1    | 002095   | hsa-miR-219-1-3p | hsa-miR-219-1-3p-5p | AGAGUGAGUCCAGGGAGCUGCC | 17.86                     | 15.39                  | -2.87       | 17.86     | 15.39   |
| Human B v3.0    | 000417   | hsa-miR-30a-5p | hsa-miR-30a-3p-5p | UGUAACAUUCAUUCGAGCAUG | 17.46                     | 12.98                  | -4.48       | 17.46     | 12.98   |
| Human B v3.0    | 002094   | hsa-miR-218-1-3p | hsa-miR-218-1-3p-5p | AUGCUGGUCCUGAGCAACGAGA | 17.46                     | 9.16                   | -18.70      | 17.46     | 9.16    |
| Human B v3.0    | 002173   | hsa-miR-15b-3p# | hsa-miR-15b-5p-3p | CGAUAUCUUUUGCUUGCUCA    | 14.88                     | 18.03                  | -3.15       | 14.88     | 18.03   |
| Human B v3.0    | 000567   | hsa-miR-378 | hsa-miR-378a-5p | CUCUGACUCCAGCUCUCAGU | 9.92                      | 12.42                  | -2.50       | 9.92      | 12.42   |
| Human B v3.0    | 002438   | hsa-miR-21-3p | hsa-miR-21-3p-5p | CAACACGAGCUGGAGCGCGGUGU | 14.82                     | 17.15                  | -2.73       | 14.82     | 17.15   |
| Human B v3.0    | 002768   | hsa-miR-1233 | hsa-miR-1233-5p | UAGGCCCCUGUCCCGCAG     | 14.66                     | 18.03                  | -3.37       | 14.66     | 18.03   |
| Human B v3.0    | 002818   | hsa-miR-1254 | hsa-miR-1254-5p | AGCCUGGAAGCGGAGCCUGCACG | 15.20                     | 17.67                  | -2.47       | 15.20     | 17.67   |
| Human B v3.0    | 002854   | hsa-miR-1243 | hsa-miR-1243-5p | AACUGGAGCAUAUAAGAGUG    | 8.48                      | 10.89                  | -2.41       | 8.48      | 10.89   |
| Human B v3.0    | 002897   | hsa-miR-664 | hsa-miR-664-3p | UAUUCAUUUACUUCGCCACUCAC | 13.11                     | 15.99                  | -2.88       | 13.11     | 15.99   |

miR, microRNA.
Table II. miRNA expression profile of MCF-7 cells 24 h after CoCl₂ stimulation.

| Array card name | Assay ID | Assay | miRBase_18th | Target sequence (5'→3') | Experimental group_ Avg ΔCq | Control group_ Avg ΔCq | Fold-change ΔCq | 24 h_ ΔCq | 0 h_ ΔCq |
|-----------------|----------|-------|--------------|--------------------------|-----------------------------|--------------------------|------------------|-----------|----------|
| Human A v2.1    | 000377   | hsa-let-7a | hsa-let-7a-5p | UGAGGUAGUAGGUUGUAAUGUU | 13.24 | 17.00 | 13.61 | 13.24 | 17.00 |
| Human A v2.1    | 000580   | hsa-miR-20a | hsa-miR-20a-5p | UAAAGUGCUUUAAGUGCAGGUAG | 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 | AAGGAGCUCACAGUCUAAUGAG | 14.32 | 17.55 | 9.33  | 14.32 | 17.55 |
| Human A v2.1    | 000480   | hsa-miR-181a | hsa-miR-181a-5p | AACAUUAACGCUGUCGGUGAGU | 15.33 | 17.79 | 5.49  | 15.33 | 17.79 |
| Human A v2.1    | 000529   | hsa-miR-302a | hsa-miR-302a-3p | UAGUGCUUCAUUGUUAUGUGGA | 17.77 | 11.65 | -69.58 | 17.77 | 11.65 |
| Human A v2.1    | 002619   | hsa-let-7b | hsa-let-7b-5p | UAGGAGGUAGGUUGUUGGUU | 8.78 | 12.18 | 10.57 | 8.78 | 12.18 |
| Human A v2.1    | 002147   | hsa-miR-342-5p | hsa-miR-342-5p | AGGGAGCUAUCUGUGUAAUGA | 13.91 | 17.96 | 16.55 | 13.91 | 17.96 |
| Human A v2.1    | 000561   | hsa-miR-373 | hsa-miR-373-3p | GAAGGCUUCGAUUUGGUGUGU | 12.86 | 16.39 | 11.59 | 12.86 | 16.39 |
| Human A v2.1    | 001030   | hsa-miR-449 | hsa-miR-449a | UGGCAUGUAUUGUUAGCUUGU | 17.77 | 15.03 | -6.70 | 17.77 | 15.03 |
| Human A v2.1    | 002208   | hsa-miR-450-3p | hsa-miR-450-3p | UUGGAUCAUUAUUGCAUCCUA | 14.32 | 17.96 | 12.45 | 14.32 | 17.96 |
| Human A v2.1    | 001630   | mmu-miR-491 | hsa-miR-491-5p | AGUGGGAAACCUUCCAUAGAGG | 15.51 | 17.96 | 12.45 | 15.51 | 17.96 |
| Human A v2.1    | 001953   | mmu-miR-496 | hsa-miR-496 | UAGGAUAAACUGGCGCAUCUC | 11.51 | 17.96 | 17.96 | 15.51 | 17.96 |
| Human A v2.1    | 002084   | hsa-miR-504 | hsa-miR-504 | AGACCCUGUAGCGCUCACUCAU | 14.29 | 17.96 | 12.72 | 14.29 | 17.96 |
| Human A v2.1    | 002369   | hsa-miR-515-3p | hsa-miR-515-3p | GAGUGCCUCUUUUGGAGCGCU | 15.01 | 17.96 | 7.11  | 15.01 | 17.96 |
| Human A v2.1    | 002095   | hsa-miR-219-1-3p | hsa-miR-219-1-3p | AGAGUGAUGUGCGACUCGGCC | 17.77 | 15.39 | -5.22 | 17.77 | 15.39 |
| Human B v3.0    | 002182   | hsa-miR-939 | hsa-miR-939 | UGGGAGCUAGCGCGUCUCUGGUGGUG | 14.20 | 17.08 | 7.34  | 14.20 | 17.08 |
| Human B v3.0    | 002897   | hsa-miR-664 | hsa-miR-664-3p | UAUCAUUAUACCCAGCCUAACA | 13.62 | 15.99 | 5.18  | 13.62 | 15.99 |

miR, microRNA.
miR-491 is involved in CoCl₂-inhibited cell growth and miR-302a is involved in CoCl₂-promoted cell migration. As aforementioned, it is notable that CoCl₂ 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 CoCl₂, and it was previously identified to be involved in the inhibition of cell proliferation (17). Therefore, it may serve a significant role in CoCl₂-inhibited cell growth. The has-miR-491-5p inhibitor was transfected into MCF-7 cells to attenuate the upregulated level of miR-491 by CoCl₂. The rates of cell growth and cell migration were assessed, and the results demonstrated that the miR-491-5p inhibitor significantly attenuated CoCl₂ 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 CoCl₂ treatment. miR-302a mimics were transfected into MCF-7 cells to rescue the miR-302a downregulated by CoCl₂. 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 CoCl₂-enhanced cell migration (P<0.05; Fig. 3D).

CoCl₂-stimulated changes in the expression of numerous miRNAs, yet their roles in the modification of cell growth and migration in CoCl₂-simulated hypoxia requires additional study. However, it is clear that miR-491 is involved in CoCl₂-induced inhibition of cell growth, and that miR-302a is involved in CoCl₂-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 CoCl₂-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 CoCl₂ 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 CoCl₂-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 CoCl₂ markedly upregulates the level of CXCR4 (Fig. 4B); however, transfection with miR-302a mimics attenuated the CoCl₂-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 CoCl₂. CoCl₂ 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 CoCl₂-induced ERK1/2 activation, miR-491 inhibitors were transfected into MCF-7 cells, and it was identified that inhibition may moderately attenuate CoCl₂-induced ERK1/2 activation, which was consistent with a previous
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). CoCl₂ is the most frequently used hypoxia mimic. Hypoxia is associated with
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 ERK1/2 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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