

# The hypoxia-mimetic agent cobalt chloride induces cell cycle arrest and alters gene expression in U266 multiple myeloma cells

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**Abstract.** Hypoxia is a common feature of tumors that occurs across a wide variety of malignancies. Multiple myeloma is an incurable malignant disorder of plasma cells in the bone marrow. Although bone marrow hypoxia is crucial for normal hematopoiesis, the effect of hypoxia on multiple myeloma is poorly understood. In this study, we demonstrated that cobalt chloride (CoCl<sub>2</sub>)-mediated hypoxia decreased cell viability and altered gene expression in U266 human multiple myeloma cells. CoCl<sub>2</sub> induced the loss of cell viability in a concentration-dependent manner. In addition, FACS analysis revealed that the loss of cell viability was related to apoptosis. Using microarray analysis, we identified mRNA expression profile changes in response to CoCl<sub>2</sub> treatment in U266 cells. Four hundred and fifty-two mRNAs exhibited >2-fold changes in expression in CoCl<sub>2</sub>-treated U266 cells compared to their expression in control cells. A follow-up bioinformatics study revealed that a great number of genes with altered expression were involved in apoptosis, cell cycle, transcription and development. In conclusion, these results provide novel evidence that CoCl<sub>2</sub>-mediated hypoxia affects the expression profiles of genes that are functionally related to apoptosis and angiogenesis in U266 multiple myeloma cells.

## Introduction

Hypoxia, defined as areas with oxygen tension values of 10 mmHg or lower, is a common feature of solid tumors that occurs across a wide variety of malignancies (1,2). Once cells encounter hypoxia, a large number of genes that stimulate adaptation to oxygen deprivation are induced. These hypoxia-inducible genes are primarily controlled by the transcription factor hypoxia-inducible factor 1

(HIF-1), including those involved in cell cycle arrest, apoptosis, erythropoiesis, angiogenesis, glycolytic metabolism, and tumor invasiveness (2,3). HIF-1 is a heterodimer consisting of a constitutively expressed HIF-1 $\beta$  subunit and a HIF-1 $\alpha$  subunit that is regulated in an oxygen-dependent manner (3). Under hypoxia, HIF-1 $\alpha$  can block its own degradation and thus induce its accumulation (3). Therefore, HIF-1 $\alpha$  is overexpressed in many cancer types and is associated with poor prognosis in cancers of the breast, brain, cervix, ovary, and uterus (3).

HIF-1 $\alpha$  activation in response to hypoxia stimulates the transcription of several of its target genes. Vascular endothelial growth factor (VEGF), which is known to promote angiogenesis, is strongly induced by HIF-1 $\alpha$  (4). In addition, matrix metalloproteinase (MMP)-1 and MMP-2 are induced to promote intravasation (5,6). Additionally, the epithelial-mesenchymal transition-inducing genes E-cadherin, SNAIL, TWIST, TCF3, and ZEB1 are up-regulated in a HIF-1 $\alpha$ -dependent manner (7-9). Interestingly, HIF-1 $\alpha$  also induces cell cycle arrest by up-regulating p53, p21, and E1B 19K/Bcl-2 binding protein Nip3 expression (10-12). These findings suggest that there are different adaptive responses to oxygen deprivation.

Cobalt chloride (CoCl<sub>2</sub>), a commonly used hypoxia-mimetic agent, artificially induces hypoxia and can block the degradation and thus induce the accumulation of HIF-1 $\alpha$  protein (13,14). Many reports have indicated that both CoCl<sub>2</sub> and hypoxia regulate a similar group of genes on a global gene expression level (15-17). In addition, the reports demonstrated that hypoxia and CoCl<sub>2</sub> induced different effects in many different types of cells. For example, CoCl<sub>2</sub> induced apoptosis in rat C6 glioma cells, human alveolar macrophages, neuronal PC12 cells, and HeLa human cervical cancer cells, although it inhibited apoptotic death in HepG2 hepatoma cells (18-22). Moreover, CoCl<sub>2</sub> induced prostate tumor cell adhesion, metastasis, and angiogenesis (23-25) and stimulated oral squamous carcinoma cell growth (26).

Multiple myeloma (MM) is a B-cell malignancy selectively localized in the bone marrow (BM) (27). Unlike other organs, the normal BM microenvironment is hypoxic. This physiological hypoxia is crucial for normal marrow hematopoiesis (28). In several studies, oxygen levels in BM in mice with MM were depressed (29,30). This hypoxia is an important microenvironmental stimulus for activities critical for MM disease progression

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such as MM cell growth, drug resistance, and angiogenesis (29,30). However, how hypoxia and MM affect each other is poorly understood. Therefore, an investigation of the effects of hypoxia on myeloma cells is necessary.

## Materials and methods

**Cell culture.** The human multiple myeloma cell line U266 was purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1640 medium containing 10% fetal bovine serum and antibiotics at 37°C in a humidified chamber containing 5% CO<sub>2</sub>. Cells were seeded into 60-mm culture dishes (4×10<sup>5</sup> cells per dish) 1 day before CoCl<sub>2</sub> (Sigma, St. Louis, MO, USA) treatment.

**Hypoxia induction.** CoCl<sub>2</sub> was dissolved in dimethylsulfoxide (DMSO; Sigma) and added to U266 cultures at the indicated final concentrations. DMSO was added to culture medium alone as a vehicle control. Cells were treated for 24 h.

**RNA preparation and cDNA synthesis.** Total RNA was extracted from cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions after the 24-h CoCl<sub>2</sub> treatment. For the microarray studies, both the quality and concentration of the RNA samples were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and an Ultrospec 3300 Pro UV/Visible Spectrophotometer (Amersham Biosciences, UK). The recommended RNA quality parameters for microarray analysis are as follows: a UV spectroscopy A260/A280 ratio of 1.8–2.0 and an A260/A230 ratio >1.8, an 18S/28S rRNA ratio of 1.8–2.1, and an RNA integrity number >8.0. To synthesize cDNA, 1 µg of RNA was incubated with oligo-dT primers at 94°C for 10 min and reverse-transcribed with reverse transcriptase (Enzymomics, Seoul, Korea) at 37°C for 1 h.

**DNA microarray analysis.** DNA microarray analysis was performed using the HumanHT-12 v4.0 Expression Beadchip kit (Illumina, San Diego, CA, USA) according to the instruction manual. Derived data were analyzed using Genespring GX 11 (Agilent Technologies). The raw data were filtered using FLAG and t-tests. Significant genes were determined using the fluorescence ratio between the control and CoCl<sub>2</sub>-treated samples, and genes displaying a >2-fold increase or decrease were selected for analysis.

**Immunoblotting and polymerase chain reaction (PCR).** Immunoblot analysis was performed as described previously (31). A primary antibody specific for HIF-1α was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin antibody was purchased from Sigma. The expression level of VEGF mRNA was determined by quantitative real-time (RT)-PCR using specific primers (forward, 5'-AAGGAGGAGGCGAGAATCAT-3'; reverse, 5'-GCTGTAGGAAGCTCATCTCT-3'). RT-PCR analysis was performed using Line-Gene K software (Bioer Technology Co. Ltd., Hangzhou, China).

**Cell viability assay and FACS analysis.** Cell proliferation was determined using the WST-1 assay (EZ-Cytox Cell Viability

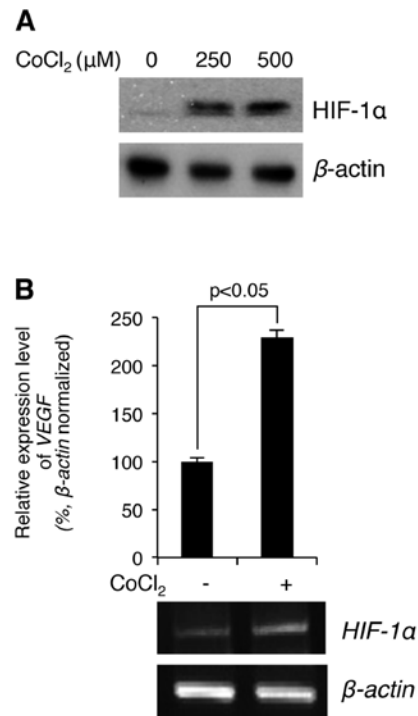


Figure 1. CoCl<sub>2</sub> induces HIF-1α accumulation and VEGF expression in MM U266 cells. (A) CoCl<sub>2</sub> up-regulated the protein expression of HIF-1α in U266 cells. U266 cells were treated with CoCl<sub>2</sub> for 4 h, and then the lysate was subjected to western blotting using the indicated antibodies. (B) CoCl<sub>2</sub> increased VEGF mRNA expression in U266 cells. The cells were treated with DMSO (control) or 250 µM CoCl<sub>2</sub> for 4 h. VEGF mRNA expression was determined by RT-PCR. The results are representative of three independent experiments (means ± SD). Statistical differences in the findings between control cells and CoCl<sub>2</sub>-treated cells were indicated by p < 0.05.

Assay kit, ITS BIO, Korea) according to the manufacturer's instructions. In brief, 4×10<sup>5</sup> cells were seeded in 60-mm dishes. The cells were cultured for 24 h and treated with the indicated concentrations of CoCl<sub>2</sub> for 24 h. At each time-point, the kit solution was added to cultured cells, which were incubated at 37°C for 30 min. Cell viability was measured using an iMark microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm by using a 620-nm reference filter. Cell cycle distribution was determined using FACS analysis. U266 cells were collected and fixed by resuspending them in 70% ethanol for 1 h, centrifuged, and washed in cold PBS. The cell pellets were resuspended in PBS containing 50 µg/ml propidium iodide (Sigma) and 100 µg/ml RNase, incubated at room temperature for 30 min, and then analyzed using a FACScalibur (BD Biosciences, San Jose, CA, USA).

**Statistical analysis.** Statistical analysis was performed using the  $\chi^2$  test or Fisher's exact test and Spearman rank correlation coefficient analysis. p < 0.05 was considered significant.

## Results and Discussion

We first examined whether CoCl<sub>2</sub> stimulation induces hypoxia in MM U266 cells. The cells were treated with the indicated concentrations of CoCl<sub>2</sub> for 4 h, and then the level of hypoxia was determined by immunoblotting against the hypoxia marker HIF-1α. As shown in Fig. 1A, the cellular protein levels of

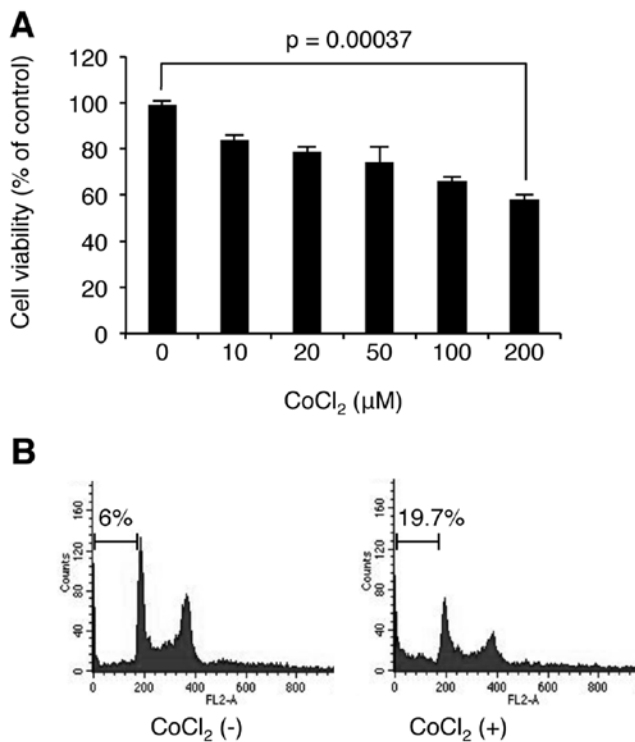


Figure 2. CoCl<sub>2</sub>-induced loss of cell viability and apoptosis in U266 cells. (A) Higher concentrations of CoCl<sub>2</sub> reduced the viability of U266 cells. The cells were treated with the indicated concentrations (μM) of CoCl<sub>2</sub> for 24 h. Cell viability was measured by the WST-1 assay. The columns and bars represent the mean ± SD, respectively, of experiments performed in triplicate. Statistical differences in the findings between control cells and CoCl<sub>2</sub>-treated cells were indicated by p<0.05. (B) CoCl<sub>2</sub> induced apoptotic cell death in U266 cells. U266 cells were treated with DMSO (control) or 100 μM CoCl<sub>2</sub> for 24 h. Cells were collected, fixed, stained with propidium iodide, and subjected to FACS.

HIF-1α were greatly increased in a CoCl<sub>2</sub> concentration-dependent manner. Further, to test whether the accumulated HIF-1α is functionally active in the cells, the expression level of a well-known HIF-1α target gene, *VEGF*, was verified by RT-PCR using its specific primers (see Materials and methods). As shown in Fig. 1B, the mRNA expression levels of *VEGF* were up-regulated by CoCl<sub>2</sub> stimulation in U266 cells. Therefore, the hypoxia-mimetic agent CoCl<sub>2</sub> induced hypoxia in U266 cells.

As hypoxia has dual roles in cell proliferation and cell death, we examined the effect of hypoxia on MM cell proliferation. U266 cells were stimulated with CoCl<sub>2</sub> at the indicated concentrations for 24 h, and then WST-1-based cell viability assay was used to determine the level of cell proliferation. As shown in Fig. 2, CoCl<sub>2</sub> decreased cell viability in a concentration-dependent manner. We next examined whether the loss of cell viability is related to cell cycle arrest or apoptosis. Interestingly, FACS analysis revealed that CoCl<sub>2</sub> stimulation induces apoptosis, but not cell cycle arrest. These results indicate that the hypoxia induced cell death in U266 cells.

Although hypoxia alters cell type-specific gene expression patterns (32), its effects have been not fully studied in MM cells. Therefore, we analyzed mRNA profiles using Illumina Human HT-12 v4.0 Beadchip kits in hypoxia-stimulated and control U266 cells. A total of 47,000 human mRNAs were selected to analyze gene expression profiles. Those human genes were continually filtered with FLAG to obtain more defined data using Agilent GeneSpring GX 11 software. We selected total 452 human mRNAs as a result of FLAG filtration, and the genes displaying >2-fold differences in expression between the control and CoCl<sub>2</sub>-treated cells are shown in Fig. 3A. In addition, the mRNA microarray was visualized on a scatter

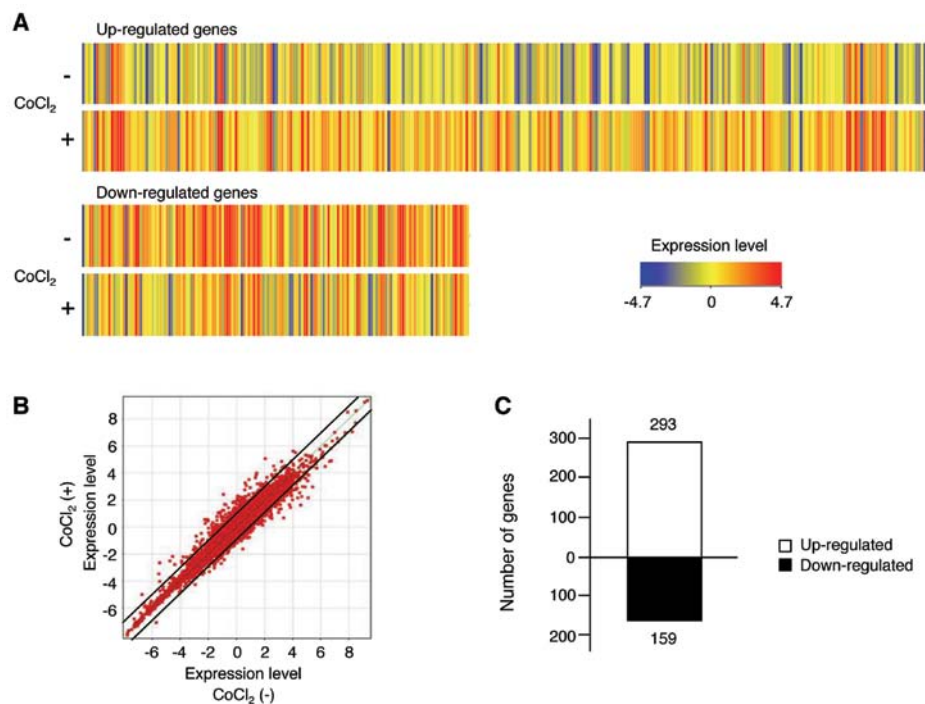


Figure 3. Gene expression profiling changes in response to CoCl<sub>2</sub>-mediated hypoxia in U266 cells. (A) CoCl<sub>2</sub> treatment altered gene expression profiles. After treatment of U266 cells with 100 μM CoCl<sub>2</sub> for 24 h, RNA samples were purified and subjected to mRNA microarray analysis. Genes displaying >2-fold changes in their expression were selected, and the degree of change in expression is represented by color. (B) Scatter plot of that 452 genes that were regulated by CoCl<sub>2</sub> in U266 cells. Scatter plot of differentially expressed genes between DMSO- and CoCl<sub>2</sub>-treated cells. The two lines parallel to the diagonal in the graph represent the 2-fold ratio of expression level. (C) The numbers of mRNAs up- and downregulated >2-fold by CoCl<sub>2</sub> treatment in U266 cells.

Table I. Top 50 genes altered by treatment with cobalt chloride.<sup>a</sup>

Up-regulated genes				Down-regulated genes			
Gene name	F.C	Gene name	F.C	Gene name	F.C	Gene name	F.C
OKL38	10.5	MT1A	4.19	HEMGN	7.56	XK	2.69
RN7SK	8.34	C17ORF91	4.12	RHOXF2B	5.29	EIF2AK2	2.66
EMP1	7.15	RAB7B	4.07	TSPAN32	5.12	ACSM3	2.61
TXNRD1	7.14	MT1X	4.01	RHOXF2	5.00	VEGFB	2.60
CDK5RAP2	6.40	CTH	3.94	TESC	4.52	SLC10A4	2.60
SRXN1	6.35	SLC3A2	3.83	ARHGAP22	4.11	BOLA3	2.56
C12ORF48	6.33	UGDH	3.81	KISS1R	3.76	C5ORF13	2.53
AGPAT9	6.03	ARRDC4	3.77	GYPA	3.75	GAD1	2.48
NDRG1	5.90	SLCO2B1	3.74	GYPE	3.67	HADH	2.48
UPP1	5.79	NOTCH1	3.52	RHAG	3.32	MYB	2.47
CTSL1	5.74	MT1E	3.48	GAD1	3.24	SPR	2.47
SLC7A11	5.44	CD53	3.38	STAT5A	3.22	TFDP2	2.46
CD44	5.32	SERPINE2	3.40	CA1	3.21	METTL7A	2.45
FLNB	5.23	ITPR1	3.37	SCARB1	3.13	ATP5G1	2.45
INHBE	5.07	RN5S9	3.32	ELOVL6	3.05	C4ORF18	2.43
GCLM	4.78	DCUN1D3	3.28	NME4	3.05	NMRAL1	2.42
FAM129B	4.78	ISG20	3.25	NFE2	3.02	BEX4	2.42
BCL6	4.74	CEBPB	3.24	CCDC34	2.99	CYP3A5	2.42
EID3	4.70	PRSS2	3.22	ATF5	2.96	CFD	2.42
MT2A	4.66	KLF6	3.18	KCNH2	2.94	SLC1A3	2.42
DUSP5	4.62	PHLDA1	3.16	STS-1	2.83	SOX21	2.41
XIRP1	4.59	PAEP	3.13	ACPP	2.82	RELN	2.41
OSGIN1	4.31	KIAA1666	3.12	ZNF121	2.73	SPIN4	2.40
SLC3A2	4.28	IER5	3.10	GATA1	2.73	EVL	2.39
ADM	4.24	LGMN	3.09	LIPH	2.71	PIK3R2	2.39

<sup>a</sup>The list was selected to show only the top 50 genes displaying >2-fold expression changes after FLAG sorting. Predicted genes were not selected in this list.

plot (Fig. 3B). The data of the scatter blot demonstrated that many genes displayed >2-fold differences in expression levels between control and CoCl<sub>2</sub>-treated cells, as indicated by their distance from the diagonal line. The numbers of up- and down-regulated mRNAs exhibiting >2-fold changes were derived from the expression patterns in each group. We found that 293 up-regulated and 159 down-regulated mRNA displayed meaningful changes in their transcription profiles in response to CoCl<sub>2</sub> treatment (Fig. 3C). The 50 genes for which their expression was most strongly altered by CoCl<sub>2</sub> are listed in Table I. To further analyze the changes in gene expression patterns, we sorted the genes displaying >2-fold changes in expression after CoCl<sub>2</sub> treatment into several groups according to common features such as homology or biochemical activity by using the Gene Set Enrichment Analysis bioinformatics tool ([www.broadinstitute.org/gsea](http://www.broadinstitute.org/gsea)), and we revealed that the sorted gene sets were categorized into transcription factors, cell differentiation markers, cytokines and growth factors, protein kinases, tumor suppressors, and oncogenes (Table II). Moreover, we

further identified the cellular process pattern of CoCl<sub>2</sub>-induced gene expression changes by using the gene ontology database. Although the analysis of cellular process pattern was firstly performed using genes with >2-fold changes in expression, the pattern results were not sufficient for elucidation because the p-values of each category were >0.05; therefore, the analysis was re-performed on genes with >1.5-fold changes in expression, and the patterns with p-values <0.05 were selected. As shown in Fig. 4A, we first identified the genomic region responsible for gene expression changes induced in response to CoCl<sub>2</sub> treatment in U266 cells by sorting the genes according to their chromosomal positions and revealed that the regions within ch11q13 and ch11q21 (up-regulated genes) and ch19p13 and ch6p21 (down-regulated genes) were the top two chromosome regions. Next, we grouped the genes according to cellular process and found that the up-regulated genes were related to apoptosis, development, cell cycle regulation, immunity, transcription, and B cell activation; however, the downregulated genes were related to cell cycle regulation, transcription, and

Table II. Genes sharing a common feature such as homology or biochemical activity.<sup>a</sup>

	Cytokines and growth factors	Transcription factors	Homeo- domain proteins	Cell differentiation markers	Protein kinases	Translocated cancer genes	Oncogenes	Tumor suppressors
Tumor suppressor	0	1 (1/0)	0	0	0	0	0	2 (1/1)
Oncogenes	0	5 (2/3)	0	0	1 (1/0)	8 (6/2)	9 (6/3)	
Translocated cancer genes	0	4 (2/2)	0	0	1 (1/0)	8 (6/2)		
Protein kinases	0	0	0	0	10 (6/4)			
Cell differentiation markers	1 (1/0)	0	0	18 (14/4)				
Homeodomain proteins	0	0	2 (0/2)					
Transcription factors	0	33 (18/15)						
Cytokines and growth factors	15 (12/3)							

<sup>a</sup>Genes displaying >2-fold expression changes after FLAG sorting were sorted according to shared biological features. Predicted genes were not selected in this list. The numbers of up- and down-regulated genes are presented in parentheses (up/down).

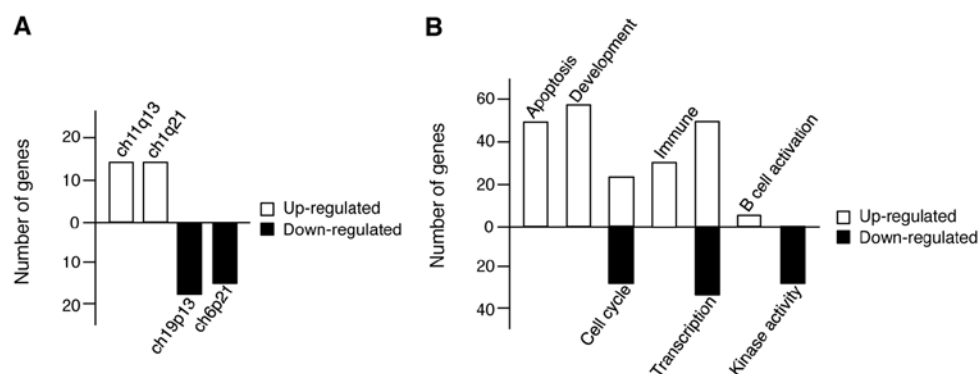


Figure 4. Categories of genes affected by CoCl<sub>2</sub> treatment. (A) The top two chromosomal regions responsible for the gene expression changes induced by CoCl<sub>2</sub>. The common regions in each chromosome in which genes expression was changed by >1.5-fold were sorted by the Gene Set Enrichment Analysis tool. (B) Biological processes of genes up- or downregulated by CoCl<sub>2</sub> treatment in U266 cells. The common biological processes were analyzed using the Gene Set Enrichment Analysis tool.

kinase activity (Fig. 4B). In particular, there are more changes among genes associated with both apoptosis and development, implying that in MM, hypoxia exerts both antitumor effects, and positively regulates cell proliferation pathways, although cell viability was decreased in hypoxic conditions.

Although both control BM and myeloma-infiltrated BM are hypoxic, the hypoxic level in myelomatous BM was revealed to

be much lower than that in native BM, as confirmed by the lower expression level of HIF-1 $\alpha$  in myelomatous BM than in native BM (29). These results imply that BM hypoxia is decreased during the progression of myeloma. Interestingly, using the 5T2 multiple myeloma mouse model, native BM hypoxia induced both apoptotic cell death of CD45<sup>-</sup> MM cells and exerted tumor-initiating effects on CD45<sup>+</sup> MM cells (29). Additionally,

blocking angiogenesis in this model restores the native BM hypoxia and results in apoptosis and suppressed proliferation of the MM cells (29). Furthermore, VEGF, a key angiogenic factor and a HIF-1 $\alpha$ -responsive gene, is induced in CD45<sup>-</sup> 5T2MM cells but not in CD45<sup>+</sup> 5T2MM cells (29). These data indicate that although BM hypoxia decreased the proliferation of CD45<sup>-</sup> MM cells, angiogenesis was induced, thus permitting outgrowth of the CD45<sup>-</sup> MM cells. Similarly, the microarray data in this study revealed that up-regulated genes were associated with both apoptosis and development. The MM U266 cells used in this study were CD45<sup>-</sup>. Therefore, although hypoxia induces U266 cell death by up-regulating apoptosis-related gene expression, resistance mechanisms for hypoxia were also induced via the up-regulation of development-related genes including VEGF and CD44.

In the microarray data, oxidative stress induced growth inhibitor 1 (OKL38) was the gene most strongly up-regulated by CoCl<sub>2</sub> treatment (fold change = 10.52). OKL38 is a tumor suppressor gene, and its overexpression inhibits tumor cell growth and induces apoptosis (33,34). Although the effect in hypoxia on OKL38 has not been researched, hypoxia-mediated cell death could be dependent on OKL38 expression. In addition, CD44 was highly up-regulated by CoCl<sub>2</sub> in U266 cells (fold change = 5.32). CD44 is an antigen glycoprotein that plays an important role in aspects of cancer progression including growth promotion, angiogenesis, and metastasis (35); however, its cellular function in hypoxia has not yet been elucidated. Therefore, the hypoxia-induced up-regulation of CD44 in U266 cells indicates that CD44 is a novel key protein controlling hypoxia resistance mechanisms including angiogenesis in MM cells.

In conclusion, we first examined the diversity of gene expression responses to hypoxia in MM cells and found significant differences in gene expression. Additionally, although hypoxia induced apoptosis, the gene expression patterns indicated the activation of both antitumor and hypoxia resistance processes. Therefore, our results may provide a useful approach to understanding cellular responses to hypoxia.

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