

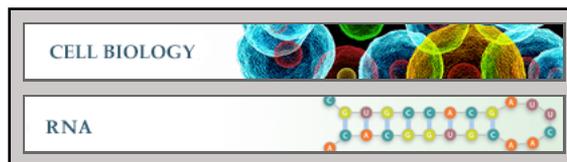
Cell Biology:

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Endothelial Cells**

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Exosomes Derived from Hypoxic Leukemia Cells Enhance Tube Formation in Endothelial Cells^{*[5]}

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Background: We recently showed communication between leukemia and endothelial cells and induction of angiogenesis via exosomes.

Results: Hypoxic leukemia cells secrete exosomal miRNA, which enhances tube formation in endothelial cells.

Conclusion: Exosomal miRNA from a tumor itself helps modulate the microenvironment of the tumor.

Significance: This study provides novel insight into the role of exosomes in cancer development.

Hypoxia plays an important role during the evolution of cancer cells and their microenvironment. Emerging evidence suggests communication between cancer cells and their microenvironment occurs via exosomes. This study aimed to clarify whether hypoxia affects angiogenic function through exosomes secreted from leukemia cells. We used the human leukemia cell line K562 for exosome-generating cells and human umbilical vein endothelial cells (HUVECs) for exosome target cells. Exosomes derived from K562 cells cultured under normoxic (20%) or hypoxic (1%) conditions for 24 h were isolated and quantitated by nanoparticle tracking analysis. These exosomes were then cocultured with HUVECs to evaluate angiogenic activity. The exosomes secreted from K562 cells in hypoxic conditions significantly enhanced tube formation by HUVECs compared with exosomes produced in normoxic conditions. Using a TaqMan low-density miRNA array, we found a subset of miRNAs, including miR-210, were significantly increased in exosomes secreted from hypoxic K562 cells. We demonstrated that cancer cells and their exosomes have altered miRNA profiles under hypoxic conditions. Although exosomes contain various molecular constituents such as proteins and mRNAs, altered exosomal compartments under hypoxic conditions, including miR-210, affected the behavior of endothelial cells. Our results suggest that exosomal miRNA derived from cancer cells under hypoxic conditions may partly affect angiogenic activity in endothelial cells.

The tumor microenvironment plays a crucial role in the pathophysiology of cancer (1, 2). Hypoxia is an important element of cancer microenvironment and is associated with

aggressive tumor phenotypes and poor patient outcomes (3, 4). Hypoxia has been shown to help maintain normal stem cells as well as cancer stem cell populations, but its roles in cancer stem cells are largely unknown (5). Under hypoxia, cancer cells secrete substances that modulate their hostile microenvironment to sustain growth and survival (1, 6, 7). In addition to conventional signaling pathways responding to hypoxia (*i.e.* direct cell-to-cell contact or VEGF signaling) (8, 9), recent studies have shown the importance of communication between tumor cells and microenvironmental factors, including angiogenesis via microvesicles or exosomes, secreted from hypoxic tumor cells (10, 11).

Exosomes are small endosome-derived vesicles (30–100 nm) containing a wide range of functional proteins, mRNAs, and miRNAs² that are actively secreted via exocytosis from different cell types, such as dendritic cells, lymphocytes, and tumor cells (12). Recent studies demonstrated that exosomes may act as mediators of cell-to-cell communication (13–16). Exosome-mediated signaling promotes tumor progression through communication between the tumor and surrounding stromal tissues (17), activation of proliferative and angiogenic pathways (18), and initiation of premetastatic sites (19). We have also demonstrated that exosomal miRNA can be transported from leukemia cells to endothelial cells and certain exogenous miRNAs modulate endothelial migration and tube formation (20).

Evidence suggests a possible role for exosomes derived from hypoxic tumor cells in modulating the tumor microenvironment (10, 11); however, the precise mechanism by which tumor progression and angiogenesis are affected has not been fully elucidated. Here we provide data showing that hypoxic tumor cells secrete exosomal miRNA, which enhances tube formation in human umbilical vein endothelial cells (HUVECs) due to inhibition of the receptor tyrosine kinase ligand Ephrin-A3.

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[5] This article contains supplemental Tables S1 and S2.

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² The abbreviations used are: miRNA, microRNA; HUVECs, human umbilical vein endothelial cells; NTA, nanoparticle tracking analysis; EFNA3, Ephrin-A3; HIF, hypoxia-inducible factor.

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EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—We used human leukemia cell line K562 for exosome-generating cells and HUVECs as exosome target cells. Cells were maintained as described previously (20). Pooled HUVECs were purchased from Lonza, Inc. (Allendale, NJ) and cultured in endothelial basal medium (EBM-2; Lonza, Inc.) supplemented with EGM-2 singleQuots and 5% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ until the third passage. K562 cells were cultured for 24–72 h under hypoxic conditions (1% O₂, 5% CO₂, 94% N₂) in a humidified gas-sorted hypoxic incubator (MCO-5M, Sanyo, Osaka, Japan) using the degassed culture mediums (AIM-V medium, Invitrogen). RPMI8226 (multiple myeloma) and SUDHL4 (diffuse large B-cell lymphoma) cell lines were also used for the tube formation assay and miRNA profiling.

Preparation of the Exosomal Fraction—K562, RPMI8226, and SUDHL4 cells were seeded at a density of 5×10^5 cells/ml and cultured for 24 h unless otherwise indicated under hypoxic conditions (1% O₂) or normoxic conditions (20% O₂) in serum-free AIM-V medium (Invitrogen). The exosomes were purified by Exoquick Exosome Precipitation Solution (System Biosciences, Mountain View, CA) as described previously (20). Exosome pellets were resuspended with 500 μ l of the serum-free AIM-V medium (Invitrogen).

Transmission Electron Microscopy—For electron microscopy analysis, exosomes were prepared, fixed with 4% paraformaldehyde and 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at incubation temperature, and placed in a refrigerator to lower temperature to 4 °C. The samples were adsorbed to a 400-mesh carbon-coated grid and immersed in 2% phosphotungstic acid solution (pH 7.0) for 30 s. The samples were observed by transmission electron microscope (JEM-1200EX; JEOL, Ltd., Japan) at an acceleration voltage of 80 kV.

Nanoparticle Tracking Analysis (NTAs) for Exosomes—NTA measurements were performed using the Nanosight LM10 system (Nanosight, Amesbury, UK) equipped with a blue laser (405 nm). Exosomes were illuminated by the laser and their movement under Brownian motion was recorded in 90-s sample videos, which were analyzed with NTA analytical software (version 2.0, Nanosight). We serially diluted all samples with PBS to reach a particle concentration suitable for analysis with NTA (1×10^8 to 2.5×10^9 particles/ml). The capture settings (shutter and gain) and analysis settings were done manually according to the manufacturer's instructions. All analysis settings were kept constant within each experiment. NTAs were averaged within each sample across the video replicates and then averaged across samples to provide total nanoparticle concentrations. The nanoparticle concentration was normalized to cell numbers at the time of harvest.

Tube Formation Assay—The formation of capillary-like structures was assessed in a 24-well plate using growth factor-reduced Matrigel (BD Biosciences). For this procedure, HUVECs (2×10^4 cells/well) were plated on top of Matrigel (280 μ l/well) and treated with exosomes derived from K562 cells cultured under hypoxic conditions (K562^{1%O₂}-exosome) or under normoxic conditions (K562^{20%O₂}-exosome). After 24 h, cells were visualized under the bright-field microscope

(BZ-8000, Keyence, Osaka, Japan). The total tube area was quantified as the mean pixel density obtained from image analysis of five random microscopic fields using ImageJ software (<http://rsb.info.nih.gov/nih-image/>).

RNA Isolation—Isolation of cellular and exosomal miRNAs was performed using the miRNeasy kit (Qiagen, Hilden, Germany). The cell or exosome pellets were dissolved with 700 μ l of QIAzol lysis reagent (Qiagen). After a 2-min incubation, 1 μ l of 1 nM ath-miR-159 (Hokkaido System Science, Hokkaido, Japan) was added to each aliquot as a spike control for losses in preparation, followed by vortexing for 30 s, and incubation on ice for 10 min. Subsequent phenol extraction and cartridge filtration were carried out according to the manufacturer's instructions.

miRNA Expression Profile—miRNA profiling in both cells and exosomes was done using a TaqMan low-density miRNA array (Applied Biosystems, Bedford, MA). To identify cellular and exosomal miRNAs universally responding to hypoxic conditions, we also performed miRNA profiles in two additional cell lines derived from hematologic malignancies (RPMI8226, multiple myeloma; SUDHL4, diffuse large B-cell lymphoma). A fixed volume of 3 μ l of RNA solution from 50 μ l of the elution was used as input in each RT reaction. An RT reaction and preamplification step were set up according to the manufacturers' recommendations. miRNAs were reverse transcribed using the Megaplex Primer Pools (Human Pools A, version 2.1) from Applied Biosystems. RT reaction products from the exosome sample were further amplified using the Megaplex PreAmp Primers (Primers A, version 2.1). The expression profile of miRNAs was determined using the Human TaqMan miRNA Arrays A (Applied Biosystems). RNU6B and a spike control (ath-miR159) were used as an invariant control for the cell and culture medium, respectively. Quantitative RT-PCR was carried out on an Applied Biosystems 7900HT thermal cycler using the manufacturer's recommended program. Finally, all of the raw data from each array were retrieved from the 7900HT and run on Data Assist Software (version 3.1, Applied Biosystems).

Transfection of K562 Cells with Cy3-Labeled Pre-mir miRNA Precursor and PKH67-labeled Exosome Transfer—Pre-mir miRNA precursor (has-miR-210; Applied Biosystems) was labeled with Label IT siRNA Tracker Cy3 Kit according to the manufacturer's instructions (Mirus, Madison, WI). K562 cells (1×10^5) were transfected with 10 nM of Cy3-labeled pre-mir miRNA precursor using HiPerFect (Qiagen) (K562/Cy3-miR-210). The day after transfection, cells were washed three times with PBS, and the medium was switched to fresh serum-free AIM-V medium (Invitrogen). After incubation for a day, the culture medium was collected and used for purification of exosome fraction by Exoquick (System Biosciences). The exosomes, including Cy3-miR-210, were incubated with 2 μ M PKH67 (Sigma Aldrich) for 5 min at 25 °C and washed four times using Amicon Ultra-0.5 (100 kDa, Millipore, Billerica, MA).

Knockdown Experiment of Exosomal miR-210—Donor cells (K562 cells; 1×10^5) or recipient cells (HUVECs; 3×10^4) were transfected with scrambled control (negative control 1, Ambion) or anti-miR-210 miRNA inhibitors (Ambion) using

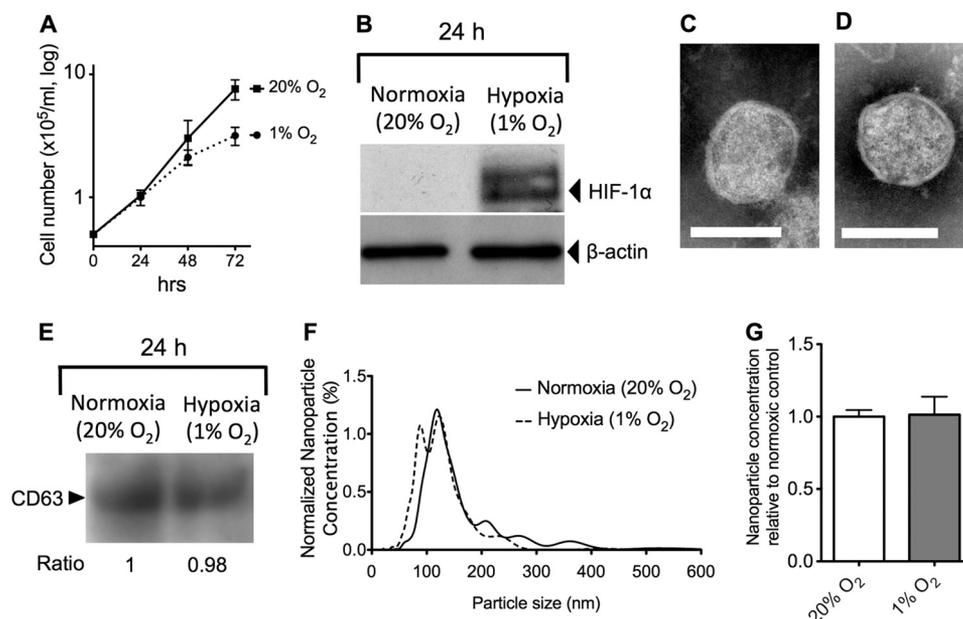


FIGURE 1. **The response to hypoxia in K562 cells.** *A*, 5×10^4 cells/ml of K562 cells were seeded, and cell growth was measured following 24, 48, and 72 h of normoxic (20% O_2) or hypoxic (1% O_2) cultivate conditions. *B*, the expression level of HIF-1 α protein in K562 cells cultured under 20% O_2 or 1% O_2 conditions for 24 h. *C*, photomicrograph of an exosome derived from hypoxic K562 cells. Scale bar, 100 nm. *D*, photomicrograph of an exosome derived from normoxic K562 cells. Scale bar, 100 nm. *E*, CD63 (exosomal marker) immunoblot of exosomes derived from K562 cells cultured under 20% O_2 or 1% O_2 conditions for 24 h. Equal amount of exosomes (300 ng) were used for the assay. *F* and *G*, the nanoparticle size distribution for the exosomes derived from K562 cells cultured under 20% O_2 or 1% O_2 conditions for 24 h were obtained by NTA (*F*), and the nanoparticle concentrations were normalized to final cell counts for 24 h under normoxic or hypoxic conditions. Each bar is presented as the mean \pm S.D. ($n = 3$) (*G*).

HiPerFect (Qiagen). The following day, after a change to fresh media, cells were cultured under hypoxic conditions for 24 h. The exosomes derived from K562^{1%O₂}/anti-miR-210 or K562^{1%O₂}/control cells were purified as described previously.

Luciferase Assay—Synthetic oligonucleotides bearing the Ephrin-A3 (EFNA3) 3'-UTR with the miR-210 complementary binding site (5'-TTTGTCTTCTGTGAAGACAGGACCTATGCAACGCACAGACACTTTTGGAGACCGT-3') were cloned into the firefly luciferase reporter plasmid pMIR-Report (Applied Biosystems) according to the manufacturer's instructions. The seed sequence of miR-210 (**ACGCACA**) is indicated by *boldface* and *underlined* type. We also generated the mutated sensor vector that replaced the seed sequence of miR-210 with CATAAC. For measuring luciferase activity, HUVECs were grown in 24-well plates until 50–60% confluence. The Luciferase plasmid (0.1 μ g) was co-transfected with 0.01 μ g of pMIR-Report β -gal control plasmid (Applied Biosystems) as control for the transfection efficiency. After incubation for a day, HUVECs were treated with the exosome derived from K562 cells cultured under hypoxic conditions (K562^{1%O₂}-exosome) or under normoxic conditions (K562^{20%O₂}-exosome), and the activity of luciferase and β -gal was assessed after 48 h with the Dual-Light System according to the manufacturer's instructions (Applied Biosystems).

Immunoblotting—The cells were lysed in lysis buffer (Roche Diagnostics) and equal amounts of protein were separated on SDS-polyacrylamide gels. Immunoblots were probed with antibodies directed against hypoxia-inducible factor 1 α (HIF-1 α ; rabbit polyclonal anti-HIF-1 α ; 1:500, NB100–134, Novus Biologicals, Littleton, CO), CD63 (rabbit polyclonal anti-CD63; 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), Ephrin-A3 (sc-733954; Santa Cruz Biotechnology), or β -actin (mouse monoclonal anti- β -actin, 1:2500, Chemicon International,

Temecula, CA). Secondary antibodies were purchased from GE Healthcare.

Immunocytochemistry—HUVECs were seeded on glass coverslips (Matsunami Glass) and cultured in normoxic conditions (20% O_2) with K562^{1%O₂}-exosomes or K562^{20%O₂}-exosomes. Ephrin-A3 was detected by using polyclonal anti-Ephrin-A3 antibody (sc-1012; Santa Cruz Biotechnology). Alexa Fluor 488-conjugated anti-mouse IgG goat serum (Molecular Probes, Invitrogen) were used as secondary antibodies. DAPI (Abbott, Abbott Park, IL) was used for nuclear staining. Analyses were performed with a fluorescent microscope (Biozero BZ-8000; Keyence, Osaka, Japan).

Statistical Analyses—Data are expressed as mean \pm S.D. Two treatment groups were compared by Mann-Whitney *U* test or Student's *t* test. Multiple group comparisons were done by analysis of variance. For statistical analysis, GraphPad Prism for Macintosh (version 5c, GraphPad, Inc., La Jolla, CA) was used. Results were considered statistically significant when $p < 0.05$.

RESULTS

Cell Growth of K562 Cells Cultured in Hypoxic Conditions—We first examined the response to hypoxia in K562 cells. The cell growth was measured following 24, 48, and 72 h of normoxic (20% O_2) or hypoxic (1% O_2) culture. K562 cell growth was not significantly different between normoxia and hypoxia for 24 h, but it was reduced by incubation under hypoxic conditions for 48–72 h (Fig. 1*A*). The expression of a master regulator of hypoxic response, HIF-1 α was remarkably up-regulated in hypoxic conditions after 24 h (Fig. 1*B*).

Amount of Exosomes Released from K562 Cells—K562 cells were cultured under hypoxic or normoxic conditions, and the exosome fraction was isolated by Exoquick after 24 h, after

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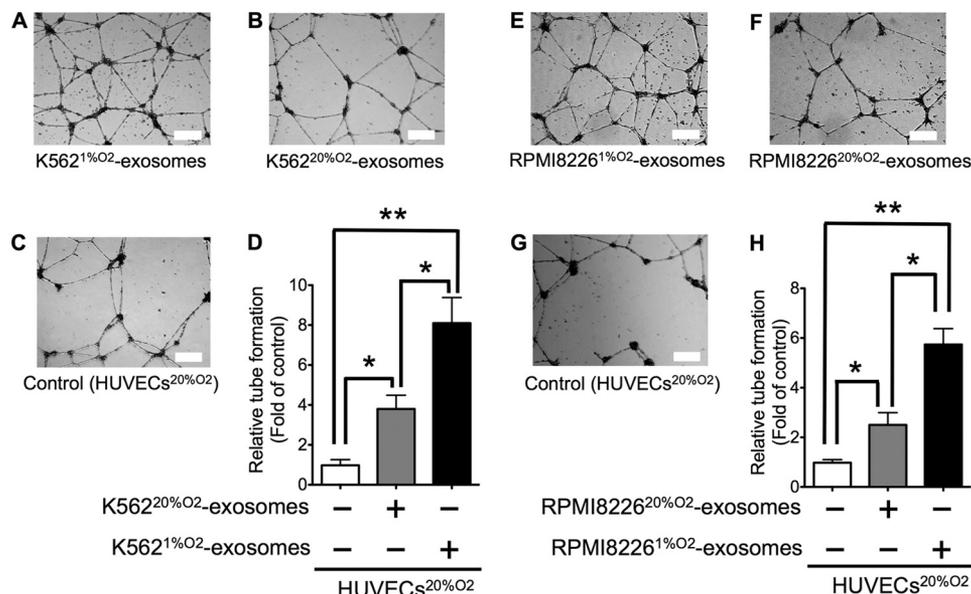


FIGURE 2. The exosomes derived from K562 cells in hypoxic conditions enhance tube formation of HUVECs. A–C, the formation of tube-like structures was observed under bright field. Endothelial tube formation of HUVECs cultured on Matrigel with K562^{1%O₂}-exosomes (A), with K562^{20%O₂}-exosomes (B), or without K562-exosomes (control) (C). The scale bar indicates 500 μ m. D, the tube-like structures determined by pixel density are significantly enhanced by the addition of K562^{20%O₂}-exosomes (*, $p < 0.01$) or K562^{1%O₂}-exosomes (**, $p < 0.001$) as compared with control (HUVECs^{20%O₂}). E–G, endothelial tube formation of HUVECs with RPMI8226^{1%O₂}-exosomes (E) and with RPMI8226^{20%O₂}-exosomes (F) or without RPMI8226-exosomes (control) (G). The scale bar indicates 500 μ m. H, the tube-like structures were significantly enhanced by the addition of RPMI8226^{20%O₂}-exosomes (*, $p < 0.01$) or RPMI8226^{1%O₂}-exosomes (**, $p < 0.001$) as compared with control (HUVECs^{20%O₂}). Values are mean \pm S.D.

which we observed the ultrastructure of exosomes using transmission electron microscopy. The size of exosomes derived from hypoxic K562 cells was similar to the exosomes derived from normoxic K562 cells (50–100 nm in diameter), and each of those vesicles showed the classical cup-shaped appearance (Fig. 1, C and D). We further checked the expression of the exosome marker CD63 by immunoblotting. The expression level of CD63 was not changed when K562 cells were cultured under hypoxic conditions for 24 h (Fig. 1E). To further investigate the size distribution profile of exosomes derived from K562 cells under normoxic conditions (K562^{20%O₂}-exosome) and hypoxic conditions (K562^{1%O₂}-exosome), we performed NTA using the Nanosight LM10 system. The nanoparticle size distribution of K562^{20%O₂}-exosomes was similar to K562^{1%O₂}-exosomes when cells were cultured for 24 h; the peaks of particle size were \sim 100 nm within the expected size of exosomes (Fig. 1F). When nanoparticle concentrations were normalized to cell numbers at the time of harvest, there were no significant differences observed between K562^{20%O₂}-exosomes and K562^{1%O₂}-exosomes (Fig. 1G). These results indicate that the size and concentration of exosomes secreted from K562 cells were not affected when cells were exposed to hypoxia for 24 h. In contrast, the nanoparticle size distribution of K562^{20%O₂}-exosomes (control) was different from that of K562^{1%O₂}-exosomes when cells were exposed to hypoxia for 72 h. We found nanoparticles of various sizes in the control sample; therefore, we could not evaluate the amount of exosome release when cells were cultured for 72 h (data not shown). Consequently, we used the exosomes isolated from culture medium following 24 h of normoxic or hypoxic conditions because culture hypoxic conditions for 24 h affected neither the cancer cell growth nor the amount of exosomes secreted into the culture medium.

The Exosomes Derived from K562 Cells in Hypoxic Conditions Enhanced Tube Formation of HUVECs—We next performed endothelial tube formation assays with the exosomes derived from K562 cells following 24 h of normoxic (K562^{1%O₂}-exosomes) or hypoxic (K562^{20%O₂}-exosomes) conditions. K562^{1%O₂}-exosomes remarkably enhanced the tube formation of HUVECs under normoxic conditions (Fig. 2, A and D; **, $p < 0.001$; *, $p < 0.01$, respectively) compared with K562^{20%O₂}-exosomes (Fig. 2, B and D) or control (HUVECs^{20%O₂}; Fig. 2, C and D). We also investigated whether or not K562^{1%O₂}-exosomes could affect growth and cell viability of HUVECs; however, we could not find any significant difference in the growth of HUVECs between K562^{1%O₂}-exosomes and K562^{20%O₂}-exosomes (data not shown). Enhanced tube formation of HUVECs also occurred with exosomes derived from hypoxic RPMI8226 cells (RPMI8226^{1%O₂}-exosomes) (Fig. 2, E–H). These results indicate that hypoxic cancer exosomes may affect tube formation rather than endothelial cell proliferation.

Cellular and Exosomal miRNA Profiling of K562 Cells Cultured in Hypoxic Conditions—To identify hypoxia-induced exosomal miRNAs, we performed miRNA profiling using the TaqMan low-density array (NCBI, gene expression omnibus (GEO) accession no. GSE45289). Based on the results obtained from the characterization of hypoxic K562 cells and the endothelial tube formation assay, we used the exosomes derived from K562 cells cultured under hypoxic or normoxic conditions for 24 h. The $2^{(-\Delta Ct)}$ values of cellular and exosomal miRNAs were ranked with a fold change > 1.5 by hypoxic conditions compared with normoxic conditions (Table 1). Two miRNAs (miR-18b and miR-210) were found among the top 10 up-regulated miRNAs under hypoxic conditions in both cells and exosomes. To clarify whether up-regulation of miR-18b and miR-210 was specific for K562 cells, we performed the

TABLE 1**Top 10 cellular and exosomal miRNAs elevated under hypoxic conditions in K562 cells**

Cellular and exosomal miRNAs, which were up-regulated >1.5-fold under hypoxic conditions, are ranked by expression level.

Rank	Cellular miRNA	Exosomal miRNA
1	hsa-miR-19a	hsa-miR-20a
2	hsa-miR-146-5p	hsa-miR-24
3	hsa-miR-454	hsa-miR-18b
4	hsa-miR-18b	hsa-miR-130b
5	hsa-miR-574-3p	hsa-miR-106b
6	hsa-miR-21	hsa-miR-224
7	hsa-miR-431	hsa-miR-210
8	hsa-miR-345	hsa-miR-652
9	hsa-miR-210	hsa-miR-379
10	hsa-miR-197	hsa-miR-185

miRNA profiles using two additional cell lines. The cellular and exosomal miRNAs with a fold change > 1.5 by hypoxic conditions compared with normoxic conditions were classified according to *Ct* value (20–25, 25–30, 30–35 cycles) and are shown in supplemental Tables S1 and S2, respectively (GEO accession nos. GSE45387 and GSE45388). Eventually, we found that miR-210 was elevated under hypoxic conditions in both cells and exosomes, in all three cell lines analyzed. Thus, we focused on miR-210 for further study.

Exosomal miR-210 Derived from K562 Cells Cultured in Hypoxic Conditions Regulates the Target Gene in HUVECs—Based on the endothelial tube formation assay, K562^{1%O₂}-exosomes containing miR-210 abundantly enhanced the capillary-like structure of HUVECs. To ascertain the association between exosomal miR-210 from K562 cells and angiogenesis under hypoxic conditions, we further investigated whether miR-210 regulated the target factor in HUVECs via exosomes.

We first visualized the transport of exosomal miR-210 derived from K562 cells into HUVECs by using the modified method that we reported previously (20). After incubation with PKH67-labeled exosomes derived from K562/Cy3-miR-210 cells, the Cy3-miR-210 signals and PKH67 signals were co-localized in the cytoplasm of HUVECs (Fig. 3, A–F).

Next, we performed a luciferase reporter assay to test whether exosomal miR-210 directly regulates the target gene, EFNA3, anti-angiogenic factor, in HUVECs. When HUVECs transduced with the reporter plasmids containing EFNA3 3'-UTR with the miR-210 complementary binding site (sensor vector) were incubated with K562^{1%O₂}-exosomes, the firefly luciferase activity was reduced as compared with K562^{20%O₂}-exosomes (*, *p* < 0.05; Fig. 3G). In contrast, the K562^{1%O₂}-exosomes did not reduce the luciferase activity using a mutated sensor vector of the miR-210 (Fig. 3H).

To demonstrate that exosomal miR-210 is a major player in promoting endothelial tube formation, we performed a knock-down experiment centered on it. We first validated the efficacy of the miR-210 inhibitor using a luciferase reporter assay. When K562 cells were transduced with the sensor vector, miR-210 inhibitor strongly inhibited miR-210 activity in K562 cells cultured under hypoxic conditions (with K562-exosome *versus* control: *, *p* < 0.05; anti-miR-210 *versus* negative control: #, *p* < 0.05; Fig. 4A). In contrast, the miR-210 inhibitor did not affect the luciferase activity when a mutated vector of the miR-210 was used (data not shown). The results of NTA suggested that there

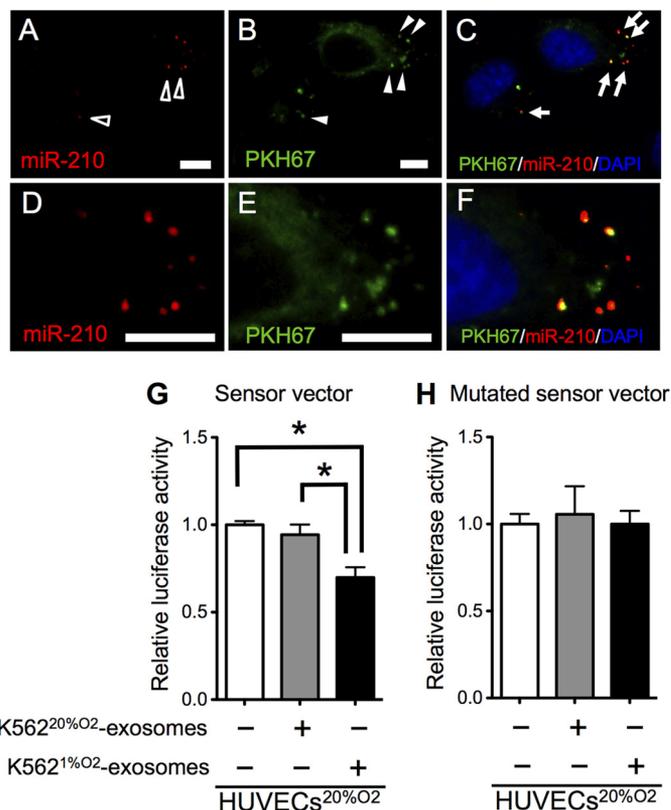


FIGURE 3. Transfer of miRNAs derived from K562 cells to HUVECs via exosomes. A–C, HUVECs were cultured with PKH67-labeled exosomes (exo.) derived from K562 cells transfected Cy3-pre-miR-210 (K562/Cy3-miR-210 cells). The Cy3-miR-210 signals were detected in the cytoplasm of HUVECs (open arrowheads) (A), and closed arrowheads indicate PKH67 signals (B). Cy3-miR-210 signals are co-localized with PKH67 in HUVECs (arrows) (C). Parts of areas in A–C are enlarged in D–F, respectively. Nuclear counterstaining was performed using DAPI (blue). The scale bar indicates 10 μ m. G, luciferase assay. The luciferase reporter vector for assessing miR-210 activity and transfection efficiency, respectively. Sensor vector: Luciferase activity of HUVECs cultured with exosomes from K562 cells cultured under hypoxic conditions (K562^{1%O₂}-exosomes) was significantly reduced compared with HUVECs only (control) or cultured with exosomes from K562 cells cultured under normoxic conditions (K562^{20%O₂}-exosomes) (**p* < 0.05; *n* = 3). Mutated sensor vector: there was no difference in luciferase activity between K562^{1%O₂}-exosomes and K562^{20%O₂}-exosomes. Mean \pm S.D. of triplicates.

was no change in the amount of secreted exosomes between K562^{1%O₂}/control (K562^{1%O₂} cells transfected with negative control miR) and K562^{1%O₂}/anti-miR-210 (K562^{1%O₂} cells transfected with miR-210 inhibitor) (Fig. 4B). In addition to affecting intercellular miR-210, the miR-210 inhibitor disrupted the activity of exosomal miR-210; K562^{1%O₂}/anti-miR-210-exosome could not reduce the luciferase activity in HUVECs transduced with the reporter plasmids (with K562-exosome *versus* control: *, *p* < 0.05; anti-miR-210 *versus* negative control: #, *p* < 0.05; Fig. 4C). Furthermore, the knockdown of miR-210 led to a decrease in endothelial tube formation via exosomes (Fig. 4, D–F, K562^{1%O₂}/control-exosome *versus* K562^{1%O₂}/anti-miR-210-exosome). These findings may indicate that exosomal miR-210 plays an important role in endothelial tube formation. However, we could not completely rule out the possibility that the major effect on the HUVECs was

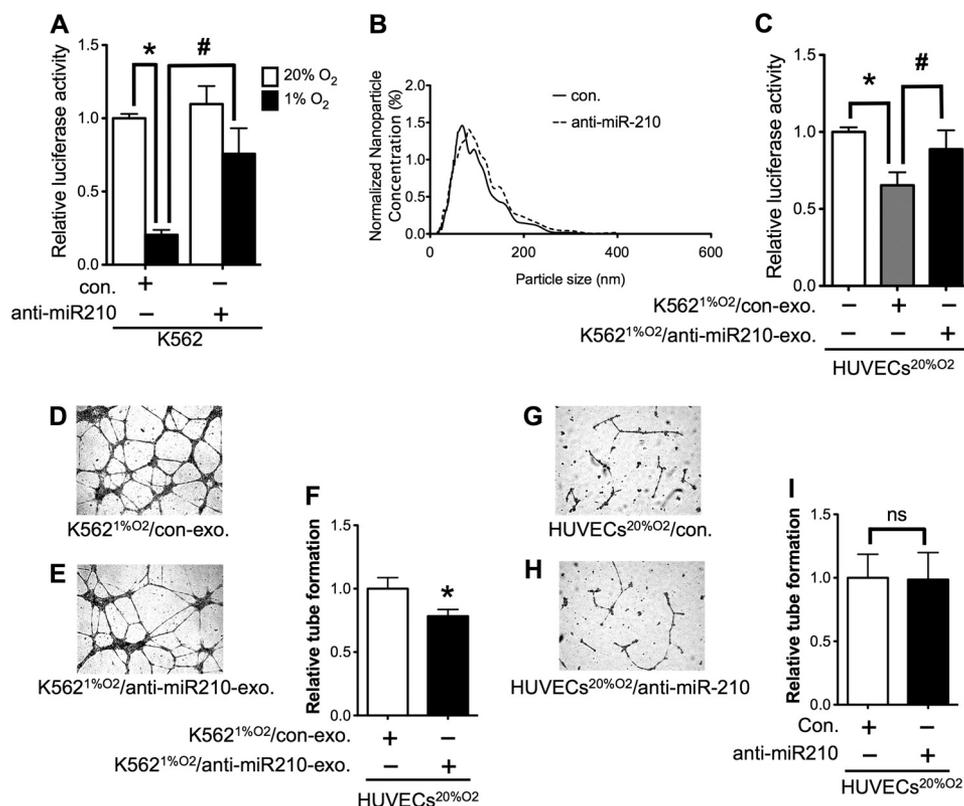


FIGURE 4. Knockdown experiment of exosomal miR-210. A, the validation of the efficacy of miR-210 inhibitor in K562 cells transfected with the luciferase reporter vector containing miR-210 sequences in EFNA3 3'-UTR. The transfection of anti-miR-210 to K562^{1%O₂} cells inhibited the induction of luciferase activity (normoxia control versus hypoxia control: *, $p < 0.05$; anti-miR-210 versus negative control: #, $p < 0.05$; $n = 3$). B, the nanoparticle size distribution for the exosomes derived from K562^{1%O₂}/control (K562^{1%O₂} cells transfected with negative control miR) and K562^{1%O₂}/anti-miR-210 (K562^{1%O₂} cells transfected with miR-210 inhibitor) were obtained by NTA. C, HUVECs^{20%O₂} were transfected with the luciferase reporter vector containing miR-210 sequences in EFNA3 3'-UTR. Luciferase activity was compared with HUVECs^{20%O₂} cultured with exosomes derived from K562^{1%O₂}/anti-miR-210 or K562^{1%O₂}/control (with K562-exosome versus control: *, $p < 0.05$; anti-miR-210 versus negative control: #, $p < 0.05$; $n = 3$). D–F, endothelial tube formation of HUVECs^{20%O₂} cultured on Matrigel with K562^{1%O₂}/control-exosomes (con-exo.; D) and with K562^{1%O₂}/anti-miR-210-exosomes (E). F, the tube-like structures determined by pixel density are reduced by the addition of K562^{1%O₂}/anti-miR-210-exosomes (*, $p < 0.01$) compared with K562^{1%O₂}/control-exosomes. G–I, endothelial tube formation of HUVECs^{20%O₂} transfected with scramble control miR (G) and with anti-miR-210 inhibitor (H). I, there was no difference in the tube-like structures with inhibition of endogenous miR-210 in HUVECs^{20%O₂} (ns; not significant; $p > 0.1$) compared with the control (con.). Values are mean \pm S.D.

likely due to another agent because miR-210 inhibitor had only a modest, albeit significant effect.

Additionally, to test whether recipient cells (HUVECs) rely on miR-210 for their response to exosomes, HUVECs^{20%O₂} were directly transfected with miR-210 inhibitor and plated on Matrigel for 24 h. We could not find any significant difference in the tube formation between HUVECs^{20%O₂}/anti-miR-210 and HUVECs^{20%O₂}/control (Fig. 4, G–I).

Exosomal miR-210 Down-Regulated the Expression of EFNA3 in HUVECs—Finally, we confirmed expression of EFNA3 in HUVECs incubated with K562^{20%O₂}-exosomes or K562^{1%O₂}-exosomes. The expression of EFNA3 was suppressed by the addition of K562^{1%O₂}-exosomes into HUVECs cultured under normoxic conditions (Fig. 5, A–C; *, $p < 0.001$). We also demonstrated the suppression of EFNA3 expression via K562^{1%O₂}-exosomes, as shown by immunoblot (Fig. 5D). The suppressed EFNA3 expression of HUVECs was also found by exosomes derived from hypoxic RPMI8226 cells (RPMI8226^{1%O₂}-exosomes) (Fig. 5, E–H). We subsequently concluded that miR-210 might be transferred into HUVECs via exosomes as a signal of hypoxic response and might induce the tubulogenesis of HUVECs under normoxic conditions.

DISCUSSION

Tumor cell-derived exosomes and their roles in intercellular communication of the tumor microenvironment is an emerging concept in cancer research (17, 18, 21). Hypoxia is one of the characteristics of the tumor microenvironment, and hypoxic tumor cells are known to acquire metastatic characteristics and drug resistance (22, 23). We therefore sought to determine the communication between hypoxic tumor cells and endothelial cells via exosomes derived from the tumor cells. This is the first report to circumstantiate the possible role of exosomal miRNA secreted from hypoxic tumor cell in the tumor angiogenic process.

Biological functions of exosomes released from cancer cells have been extensively studied (12, 24, 25); however, the quantitative alternation of exosomes released from cancer cells under a hypoxic state has not been fully elucidated. A recent study by King *et al.* (26) has clearly demonstrated that hypoxia promotes the release of exosomes in breast cancer cells; the amount of exosomes increased when cells were incubated under severe hypoxia (0.1% O₂) for 24 h, or moderate hypoxia (1% O₂) for more than 48 h. It is therefore important to take into consideration the amount of exosomes released when discuss-

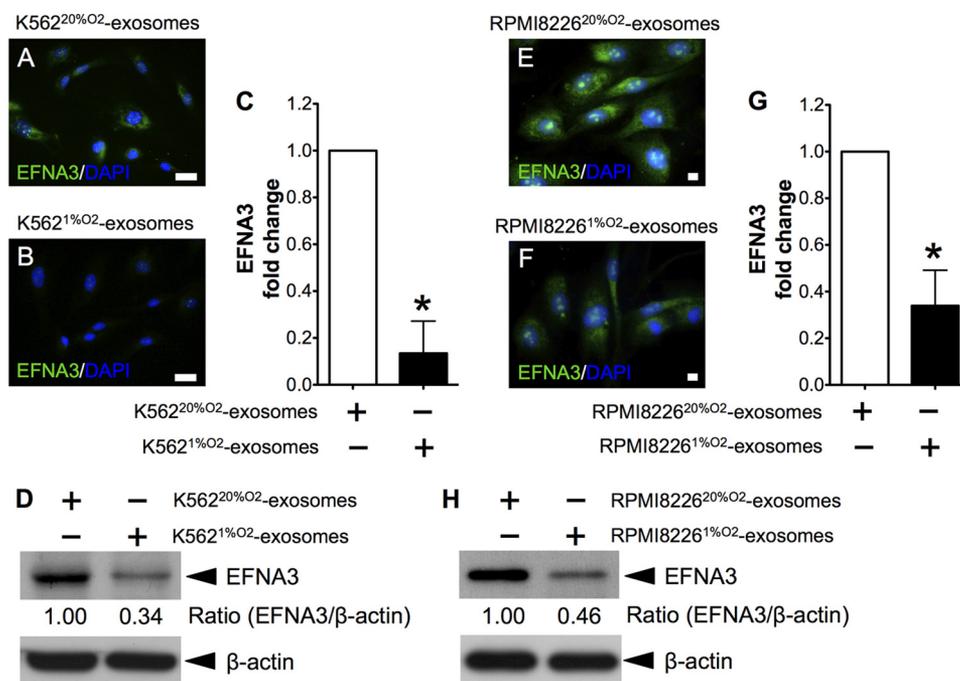


FIGURE 5. EFNA3 expression of HUVECs cultured with exosome derived from K562 cells. *A* and *B*, EFNA3 expression was inhibited by exosomes derived from hypoxic K562 cells. HUVECs were cultured under normoxic conditions with the exosomes derived from K562 cells cultured under normoxic conditions (K562^{20%O₂}-exosomes) for 24 h (*A*) and with the exosomes derived from K562 cells cultured under hypoxic conditions (K562^{1%O₂}-exosomes) (*B*). The scale bar indicates 10 μ m. *C*, EFNA3 signal was quantified using ImageJ software. EFNA3 fluorescence signals were drastically inhibited by addition of K562^{1%O₂}-exosomes (*, $p < 0.001$) as compared with K562^{20%O₂}-exosomes. *D*, expression of EFNA3 in HUVECs by immunoblot. *E* and *F*, HUVECs were cultured under normoxic conditions with the RPMI8226^{20%O₂}-exosomes for 24 h (*E*) and with the RPMI8226^{1%O₂}-exosomes (*F*). The scale bar indicates 10 μ m. *G*, EFNA3 fluorescence signals were drastically inhibited by addition of RPMI8226^{1%O₂}-exosomes (*, $p < 0.001$) as compared with RPMI8226^{20%O₂}-exosomes. *H*, expression of EFNA3 in HUVECs by immunoblot. Numbers below the panels represent the normalized EFNA3 expression signal by β -actin.

ing functional transfer of exosomes from hypoxic cancer cells to target cells. In agreement with the observation by King *et al.* (26), we found that the amount of exosomes significantly increased, and the size of exosomes measured by the Nanosight LM10 system was highly variable, when cells were cultured at 1% O₂ for more than 48 h. Although the size of exosomes from hypoxic K562 cells at 24 h was almost similar to those of normoxic K562 cells, it is still uncertain how long-term exposure to hypoxia might affect release and function of tumor exosomes. Therefore, we used exosomes derived from hypoxic tumor cells at 1% O₂ for 24 h in this study to determine the effect of exosome contents rather than the amount of exosomes.

Visualization of secreted exosome from K562 cells was recently reported by Mieno and colleagues (27). We could not visualize the three-dimensional reconstruction of imported exosomes; however, we were able to demonstrate the transfer of PKH67-labeled exosomes and Cy3-labeled exosomal miRNA from donor cells (leukemia cells) to the recipient cells (endothelial cells) by the modified method as previously reported (Fig. 3, A–F) (20).

Exosomes contain specific proteins, RNA, miRNA, and lipids. Recent reports demonstrated that the transfer of exosome-derived unique miRNAs to recipient cells is an alternative mechanism in addition to the classical mechanisms, including direct cell-cell contact or chemical receptor-mediated events, allowing gene-based communication between cells (14). Hypoxia signaling pathways are known to be regulated by HIF as a key transcriptional regulator (28). So far, several miRNAs have been implicated in regulating both upstream and down-

stream signaling of the HIF pathways: miR-199a, miR-17–92 clusters, and miR-20b regulate HIF1 α under hypoxia, and miR-23, miR-24, miR-26, miR-107, miR-210, and miR-373 have been shown to be induced by HIFs (29–31). In the current study, the miRNA signature in hypoxic exosomes was variable among the three different types of cancer cell lines analyzed. This indicates that hypoxic signaling pathways mediated by exosomal miRNA depend on the types of cancer. In addition, some of the miRNAs were expressed at higher levels in exosomes than in the cells, implying that some miRNAs may be uniquely packed into exosomes. It was not unexpected that expression of exosomal miR-210 was increased in all of the cell lines analyzed; therefore, we particularly focused on miR-210 in the current study, as we consider it to be the principal exosomal miRNA secreted from hypoxic tumor cells.

Although several miR-210 target genes have been identified to date (30), one that has been consistently reported is the receptor tyrosine kinase ligand, Ephrin-A3 (EFNA3) (32). It is known that EFNA1-EphA2 interaction plays an important role in the regulation of angiogenesis and VEGF signaling (32). Although the specific role of EFNA3 in the regulation of angiogenesis is still unknown, EphA2 has been shown to bind EFNA3 as well as EFNA1 (33). As a consequence, EFNA3 might act as a negative regulator for angiogenesis. Thus, we concluded that exosomes containing a high expression level of miR-210 down-regulate EFNA3 and thereby enhance tube formation of HUVECs. A recent investigation by Day and colleagues (34) demonstrated that the EphA3 receptor is highly expressed in a significant proportion of gliomas, particularly by the undiffer-

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entiated, tumor-initiating cells. Because tumor-initiating cells are known to exist in a hypoxic niche, targeting EFNA3 by miR-210 might be beneficial not only in inhibiting angiogenesis but also in eliminating tumor-initiating cells, although further studies are needed.

Unlike solid tumors, little is known about the exosomes for the angiogenic process in leukemia. The bone marrow of chronic myeloid leukemia patients exhibits marked neovascularization and increased numbers of endothelial cells (35). Our group and other investigators (20, 24, 27, 36) have shown that exosomes released by CML cells have the potential to influence *in vitro* and/or *in vivo* angiogenesis by directly affecting endothelial cells properties. A salient feature of the present study was that hypoxic CML cells secreted an exosomal miRNA, which was different from those secreted in a normoxic state and able to modulate angiogenesis *in vitro*.

In conclusion, hypoxic K562 cells could release exosomes containing distinct miRNAs. This has implications for how tumor cells might convey signals to its microenvironment. Hypoxic exosomes contained higher levels of miR-210, indicating a qualitative (not quantitative) difference between normoxic and hypoxic exosomes derived from hypoxic cancer cells. Although tumor angiogenesis is known to be regulated by various factors, the current pathway, noncontact cell-to-cell communication via exosomes plays an important role, at least in part, in the hypoxic tumor itself modulating the tumor microenvironment.

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