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Original Research

Changes in gene expression pattern in hypoxia-induced MCF-7 breast cancer stem cells and the impact of their secretome on angiogenesis related genes in HUVECs

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Abstract

Background: Hypoxia, a hallmark of solid tumors, results from inadequate oxygen supply as tumors grow, leading to a cascade of cellular adaptations that enhance malignancy and therapeutic resistance. The research primarily investigates the hypoxia-induced gene expression changes in breast CSCs and evaluates the effects of these changes on tumor angiogenesis and metastasis. **Objective:** To characterize the changes in gene expression of hypoxia and angiogenesis pathways in both hypoxic CSCs and HUVEC cells exposed to the hypoxic CSCs conditioned medium, and to evaluate the effect of secreted factors by CSCs in the media on the angiogenic capability of Human umbilical vein endothelial cells (HUVEC). **Methods:** Chemo-resistance to doxorubicin was assessed using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) cell proliferation assay. Real-time quantitative polymerase chain reaction (qRT-PCR) assay was performed to assess gene expression pattern in hypoxia pathway in CSCs. Furthermore, the study explored the interaction between hypoxic CSCs and human umbilical vein endothelial cells (HUVEC), through assessing the expression of angiogenic pathway genes and Capillary-like tube structure formation assay in HUVECs exposed to hypoxic CSCs-conditioned medium. **Results:** Flow cytometric identification of CD44+/CD24- cells showed that that the sorted MCF7-CSCs acquired stemness character much higher than their parental cells, and declines over time. Hypoxia significantly increased the resistance of CSCs to doxorubicin, with a maximum of 4.78 folds enhancement after 22 episodes of 8-h hypoxia compared to normal MCF7 in 2D cultures. Gene expression analysis of hypoxic CSCs revealed significant changes in 14 genes of the hypoxic pathway. The expressions of 5 of these genes were significantly up-regulated, while those of 9 genes were significantly down-regulated ($p < 0.05$). Gene expression analysis of HUVECs treated with conditioned media collected from hypoxic CSCs revealed significant changes in 31 genes of Angiogenic pathway genes. The expressions of 5 of these genes were significantly up-regulated, while those of 26 genes were significantly down-regulated ($p < 0.05$). **Conclusion:** These findings underscore the critical impact of hypoxia on breast cancer progression through modulation of CSC characteristics and angiogenic responses. This study paves the way for the development of novel therapeutic approaches that could inhibit tumor progression and overcome resistance to existing treatments.

Keywords: Hypoxia; Angiogenesis; Stem cells; MCF-7; CSCs; HUVEC

INTRODUCTION

During tumor progression and expansion in the tissues, areas of low oxygen concentration develop, leading to a condition termed hypoxia, in which oxygen demand exceeds what is available. This hypoxia condition is recognized to play a crucial role in tumor progression and aggressiveness, which is clinically reflected in the form of more therapy resistance to both radiotherapy and chemotherapy. Ample evidence indicates that the effect of hypoxia on malignant progression is mediated by a series of hypoxia-induced proteomic and genomic changes, activating and promoting angiogenesis, anaerobic metabolism, and other processes facilitating cancer cells to survive or escape their low oxygen environment.[1] Under the low oxygen conditions, cancer cells adapt to the absence of exogenous mitogenic growth signals and become resistant to the anti-proliferative signals.[2] Hypoxia can induce several genetic, epigenetic, or somatic alterations as a result of increasing genomic instability caused by defects in cell cycle checkpoint controls.[3] Those changes usually result in cells with new characteristics that differ from normal cells. Examples on such characteristics are: resistance to growth inhibitory factors, proliferation in the absence of exogenous growth factors, evasion of apoptosis, limitless replication potential via the reactivation of telomerase, abnormal angiogenesis, evasion of destruction by the immune system, invasion and metastasis.[3,4] In addition, hypoxia contributes to epithelial–mesenchymal transition (EMT)-like cancer cell migration and cancer stem-cell (CSC)-like properties that are able to seed new tumors in other locations in the body.[5] Cancer stem cells, also known as tumor-initiating cells (TICs) are subcellular group of tumoral cells, characterized by self-regeneration, heterogeneity, immunosuppressive effects, and the dynamic transition between differentiation and dedifferentiation status.

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The stemness of CSCs in different tumors is thought to be maintained by the Wnt/β-catenin pathway through regulating the expression of CD24, CD44, and Oct4.[6] Overactivation of wnt/β-catenin pathway promotes cancer cells dedifferentiation and the transitions toward cancer stem cells with more aggressive and chemo/radiotherapeutic resistance properties.[7] CD44 and CD24 are surface glycoproteins, which have been used frequently to identify the presence of cancer stem cells (CSC) in breast cancers. Interestingly, their expression is differential among different breast cancer subtypes, and thought to be responsible for more aggressive phenotype of the disease. Moreover, they are responsible for higher incidence of metastasis and invasion to nucleate other organs of the body [8], which is proved by several studies where isolated CSCs from diseased rats can establish new tumors when inoculated in other rats or mice. However, the transformation of oncolytic cells into CSCs is suggested to be correlated to many factors, such as the tumor microenvironment, heterogeneity, mutations, and most importantly, metabolic reprogramming in areas undergoing hypoxia. During hypoxia, hypoxia-inducible factor 1α (HIF1-α) is upregulated, and it has been directly correlated with the increased population of CSCs in breast cancers [9], suggesting its role in the conversion of tumor cells into CSCs. However, the exact mechanisms behind cancerous dedifferentiation are still lacking and need to be further investigated.

Adaptations of tumor cells to oxygen deprivation within growing solid tumors triggers the formation of new blood vessels in a process called hypoxia induced angiogenesis, which involves the invasion and rearrangement of nearby endothelial cells in a tube-like structure in hypoxic regions. Tumoral blood vessels are characterized by structural and functional deformities which lead to a fluctuating blood flow, and extra permeable or occlusion properties. Because of the irregular vascularization feature, many areas within solid tumors are subjected to cyclic periods of hypoxia, which may extend from few minutes to hours or even longer periods of time spanning several days. Cellular responses to stressful conditions related to oxygen and nutrients reduction involve several pathways and growth factors downstream to HIF1-α transcription factor, among which are the vascular endothelial growth factor VEGF, vascular endothelial growth factor receptors 1 and 2, and the epidermal growth factor. However, accumulated evidence indicate that there is a strong correlation between cancer stem cells and a high angiogenic activities exhibited by these cells. It has been previously reported that VEGF level in the undifferentiated CSC population is higher compared to non-stem cells differentiated tumoral cells, a property that could be related to more aggressive and metastatic phenotype of cancer stem cells. Hence, the exact mechanism and cellular pathways responsible for these phenotypic properties are still under investigation and need to be further elucidated.

Since hypoxia signifies increased tumor progression and aggressiveness, better understanding of hypoxic phenomenon and dissecting out the hypoxia-inducible responses and signaling pathways including angiogenesis will grant numerous novel therapy targets in the near future. Here in this study and as a continuation of our previous work in simulating breast CSCs; an *in vitro* induced breast CSCs model will be exposed to several hypoxia intervals in a try to achieve our goal in understanding the mechanisms underlying hypoxia induced responses and more angiogenic and aggressive properties of these cells. Moreover, hypoxic CSCs secretome will be applied on endothelial cells to evaluate the effect of CSCs released factors on the invasion and enhanced angiogenic properties of CSCs. In this study, we Characterized the changes in gene expression of hypoxia and angiogenesis pathways in both hypoxic CSCs and HUVEC cells exposed to the hypoxic CSCs conditioned medium with the goal of identifying a possible biomarker for metastasis in breast cancer. In addition, we evaluated the effect of secreted factors by CSCs in the media on the angiogenic capability of Human umbilical vein endothelial cells (HUVEC) using the Capillary-Like Structure Formation Assay using CSCs.

MATERIALS AND METHODS

Mammosphere cancer stem cells (CSCs) generation and identification:

MCF7 breast cancer cell line was cultured in low adherent tissue culture flasks to convert them into cancer stem cells, they were subjected to serum-free DMEM/F12 media, supplemented with 20 ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor, 2% B27, 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, 0.4% bovine serum albumin and 2 mM L-Glutamine. After 21 days of propagation, they were tested for their stemness using the flowcytometry technique, and $CD44^+$/$CD24^-$ cells were isolated and later identified via FACS analysis.

Exposure of CSCs to hypoxia*:*

CSCs spheres were subjected to hypoxia shots using AnaeroGen Compact system that create a hypoxic condition in a tightly closed bags, generating a surrounding atmosphere with only 1% oxygen level. Low adherent flasks containing the CSCs cultured in their complete media mentioned above were placed in the bags and subjected to 22 Intermittent hypoxia shots. Intermittent (INT) shots characterized by exposure to hypoxia conditions for 8 hours three times a week. Flasks were returned to normoxic conditions after the end of each shot. Conditioned media after each shot was collected and stored at -80 °C. CSCs after shots INT.5, INT.10 and INT.22 were subjected to RNA extraction, and the extracted RNA was stored at -80 °C until being used. Normoxic CSCs were running alongside the whole experiment.

Cytotoxicity of CSCs upon exposure to doxorubicin:

Collected cells from normoxic CSCs, INT.5, INT10, INT15 and INT.22 were plated into 96 well plate with a seeding density 10×10^3. Attached cells were treated with a serial dilution of Doxorubicin starting from concentration 100 µM down ending with 50 nM. Treated cells were incubated for 72 hours and their viability were tested using MTT assay. Plates were read at 570 nm and IC50 was calculated using GraphPad prism software.

Changes of expression of human Hypoxia pathway genes of normoxic and hypoxic CSCs:

RNA extracted from normoxic, INT10, INT22 CSCs was converted to cDNA. qPCR array for Human Hypoxia pathway genes was used to investigate the changes of expression of hypoxia genes in CSCs samples exposed to hypoxia (INT10 and INT 22) in addition to normoxic CSCs. Results were analyzed using Qiagen software.

Isolation of HUVEC cells from Umbilical cords:

Human endothelial cells were isolated from the umbilical cords of delivering females at the Jordan University Hospital. Briefly, the umbilical cord was washed from the outside and inside using an antiseptic solution with a slight and continuous squeezing. Then the cord was flushed by RPMI media supplemented with 1% penicillin/streptomycin antibiotic using sterile syringes to remove excess blood and debris. The umbilical cord was digested using type I collagenase enzyme to dissociate endothelial cells, and allowed to incubate for 20 mints at 37 °C. Collagenase enzyme activity was disabled by the addition of 20 ml of RPMI media, and then again recollected with the suspended dissociated cells, then centrifuged at 1200 rpm for 6 mints. The supernatant was discarded, and the pellet was resuspended with 5 ml of endothelial cell growth medium EGM-2 media (Lonza, Walkersville, MD, USA), and then cultured in a gelatin coated tissue culture flask at 37° C overnight in a humidified incubator with 5% CO2 level. The day after, media was changes to remove nonattached cells and allowed to proliferate until reaching confluency. They were passaged and used before reaching passage number six.

Treatment of endothelial cells with CSCs conditioned media:

HUVEC cells were harvested after trypsinization and plated into 12 well plates and allowed to reach 70% confluency. Plated cells were then treated with the CSCs conditioned media. The collected conditioned media (mentioned above) that was collected from hypoxia sessions; INT.5, INT.10, INT.15, INT.22 and control CSC media (media of CSCs control spheres not exposed to hypoxia), was diluted 50% with HUVEC basal medium, and HUVEC cells were treated for 72 hrs with these groups of collected media. At the end of the incubation, HUVEC cells were harvested, and their RNA was extracted and converted to cDNA by reverse transcription reaction. The resultant cDNA was stored at -20 °C until being used.

Changes of expression of human Angiogenic pathway genes of HUVEC cells*:*

qPCR array for Human angiogenesis pathway was used to investigate the changes of expression of angiogenic genes in HUVECs treated with CSCs conditioned medium. Results were analyzed using Qiagen software.

Capillary-like tube structure formation assay of HUVECs treated with hypoxic CSCs conditioned media*:*

The effect of the factors released by hypoxic CSCs on the ability of HUVECs to rearrange themselves in a capillary like structures was evaluated *in vitro*. Briefly, 12 well plates were first coated with a cold Matrigel (with reduced factors), the matrigel was first thawed at 4° C until completely liquify, then the coated plates were incubated at 37° C to allow Matrigel to solidify. Then, starved HUVECs were seeded with a seeding density 2 × 104 cells/well and cultured with 50% hypoxic and normoxic CSCs (diluted with endothelial basal medium without serum) collected from hypoxia shots INT.5, INT.10, INT.15 and INT.22, and incubated for 6 hours at 37° C in a humidified incubator. Each treatment well was photographed at the end of the experiment and will three parameters will be measured: the average total length of the branched tubes, number of loops and the covered area. Wimasis Image Analysis software will be used for this purpose.

RESULTS

Sorting of cancer stem cells using CD44 and CD24 magnetic beads:

$CD44^+/CD24^-$ is a widely used marker for breast cancer stem cells, that was used in this experiment to test our induced mammospheres for their stemness. As shown in table 1, our model for inducing CSCs is successful and the stemness of breast cancer cells increased upon their conversion into mammospheres. Interestingly, the stemness increased and the expression of cancer stem cell markers markedly elevated after sorting and isolating these CSCs. However, the stemness was reduced overtime but the exposure of CSCs to several hypoxia shots fluctuates the stemness level. Overall, the stemness of our induced CSCs was all the ways higher than monolayer attached 2D breast cancer cell cultures.

Cytotoxicity MTT assay and resistance of CSCs to Doxorubicin after several hypoxia shots:

Induced and hypoxic CSCs were tested for their resistance to chemotherapeutic therapeutic drug; doxorubicin, since it is a common characteristic acquired by most CSCs. Our data reveals that Induced normoxic CSCs became more sensitive to doxorubicin (IC50=0.065±0.007 µM) compared to normal MCF7 in 2D cultures (0.212±0.007 µM). As they were exposed to hypoxia shots, their resistance to doxorubicin showed an increasing pattern, with the maximum resistance achieved after shot No. 22 with IC50 (0.311±0.068 µM), which is (4.78) folds the IC50 of normoxic CSCs.

Table 1: Expression of $CD44^+/CD24^-$ in cells derived from breast cancer cells subpopulations

Cells subpopulations	Day of treatment/ Hypoxia shot	$CD44^+/CD24^-$ population %
Control MCF-7 Breast cancer cells	Day 7	1.0 (±0.08%)
Starved MCF-7 "Induced mammospheres"	Day 7 post culture in ultra-low attachment flasks	36.2 (±2.5%)
Sorted CSCs	Day 3 post sorting	83.0 (±5.2%)
Sorted CSCs	Day 21 post sorting	38.4 (±3.8%)
INT.10	After hypoxia shot # 10	51.68 (±3.4%)
INT.20	After hypoxia shot # 20	39.8 (±1.4%)

qPCR Array for hypoxic pathway genes for CSCs exposed to hypoxia shots INT.10 and INT.22:

Tables 2 and **3** below summarize changes in the expression of many genes involved in the hypoxia pathway, achieved by exposing CSCs into many shots of hypoxia. Changes of expression of hypoxic groups INT.10 and INT.22 in comparison to normoxic CSCs.

Table 2: List of hypoxia pathway genes that are up regulated in hypoxia exposed groups

Up-regulated genes			
INT.10		INT.22	
Gene	Fold change	Gene	Fold change
BNIP3	2.02	HMOX1	2.51
GYS1	2	IGFBP3	2.32
P4HB	2.34	LGALS3	5.8
		LOX	2.87
		SLC2A3	2.1

Table 3: List of hypoxia pathway genes that are down-regulated in hypoxia exposed groups

Down-regulated genes			
INT.10		INT.22	
Group 1	Fold change	Group 2	Fold change
NONE		ALDOA	-2.22
		ANKRD37	-4.2
		CA9	-2.68
		EGR1	-3.41
		HIF1A	-2.67
		MAP3K1	-2.6
		PER1	-2.01
		PIM1	-4.49
		ANGPTL4	-2.74

qPCR Array for Angiogenic pathway genes for HUVECs treated with conditioned media collected from normoxic and hypoxic CSCs:

Tables 4 and **5** below summarize changes in the expression of many genes involved in the angiogenic pathway, achieved by exposing endothelial cells (HUVEC) to the conditioned media collected from normoxic CSCs and CSCs exposed to hypoxia shots, INT.5, INT.10, INT.15, INT.22. Array qPCR results of each treatment group was analyzed against the normal untreated HUVEC cell healthy group.

Capillary-like tube structure formation assay of HUVECs treated with hypoxic CSCs conditioned media:

This test evaluates the ability of endothelial cells to reorganize into tube structures, and to test if the CSCs conditioned media contains secreted factor that stimulates the angiogenesis activity. The Figure.1 below was taken after 6 hours of incubating HUVECs seeded over Matrigel coated plates with the conditioned media collected from different shots of CSCs exposure to hypoxia. As shown, the number of capillary tubes was reduced in shots INT.10 and INT.15 (C & D, respectively) compared to the positive control cells (A). Interestingly, HUVECs treated with media collected from INT.22 hypoxic CSCs noticeably rearranged into capillary like structures, suggesting that the conditioned media of CSCs of INT.22 contains

Table 4: List of Angiogenesis pathway genes that are overexpressed under exposure of HUVECs to conditioned medium collected from normoxic and hypoxic CSCs:

Up-regulated genes					
Gene	Fold change				
	Normoxic CSCs conditioned media	**INT.5**	**INT.10 conditioned media**	**INT.15 conditioned media**	**INT.22**
		Conditioned media			**Conditioned media**
ANGPTL4	19.42	12.04	11.77	10.54	6.31
CXCL6	17.95	26.83	17.9	17.09	6.37
CCL2	-	2.37	-	-	-
CXCL1	-	3.47	2.05	-	-
CXCL10	-	5.85	3.34	-	-
F3	-	2.77	-	-	-
FGF2	3.18	-	-	-	-
IFNG	2.51	-	-	-	-
IL6	5.9	-	-	-	-
CXCL8	13.61	10.76	8.7	5.86	5.51
LEP	2.07	-	-	-	-
SERPINE1	2.21	-	-	-	-
VEGFA	8	2.06	-	-	2.28

Table 5: List of Angiogenesis pathway genes that are depressed under exposure of HUVECs to conditioned medium collected from normoxic and hypoxic CSCs

Down-regulated genes					
Gene	Fold change				
	Normoxic CSCs conditioned media	**INT.5 Conditioned media**	**INT.10 conditioned media**	**INT.15 conditioned media**	**INT.22 Conditioned media**
ADGRB1	-	-	-	-2.28	-
CCL11	-	-	-	-2.06	-
CCL2	-	-	-	-	-2.71
CDH5	-	-	-	-	-2.83
COL18A1	-	-	-	-	-2.01
CCN2	-	-	-	-	-2.13
CXCL9	-	-	-	-3.39	-
EDN1	-3.88			-2.08	-6.16
EFNA1	-2.72	-	-	-	-2.81
EFNB2	-4.59	-3.91	-4.55	-5.37	-6.63
EGF	-	-	-	-2.75	-
ENG	-	-	-	-	-2.33
EPHB4	-	-	-2.13	-2.18	-2.4
F3	-	-	-	2.36	-
FGFR3	-	-	-	-3.98	-
ID1	-2.67	-2.24	-	-	-2.76
IFNA1	-	-	-	-2.02	-
IFNG	-	-	-	-2.41	-
IGF1	-	-	-	-2.46	-2.14
KDR	-2.23	-	-	-	-
CNMD	-	-	-	-2.06	-
LEP	-	-	-	-2.39	-
MDK	-	-	-	-	-3.23
MMP9	-	-	-	-2.23	-
NOS3	-2.48	-	-	-	-
PECAM1	-4.76	-	-2.31	-3.13	-3.62
PLG	-	-	-	-2.06	-
PROK2	-	-	-	-2.52	-
TGFB2	-	-	-	-	-4.17
THBS1	-2.5	-	-	-	-2.25
THBS2	-	-	-	-2.49	-
TIMP3	-	-	-	-2.04	-
TNF	-	-	-	-2.06	-

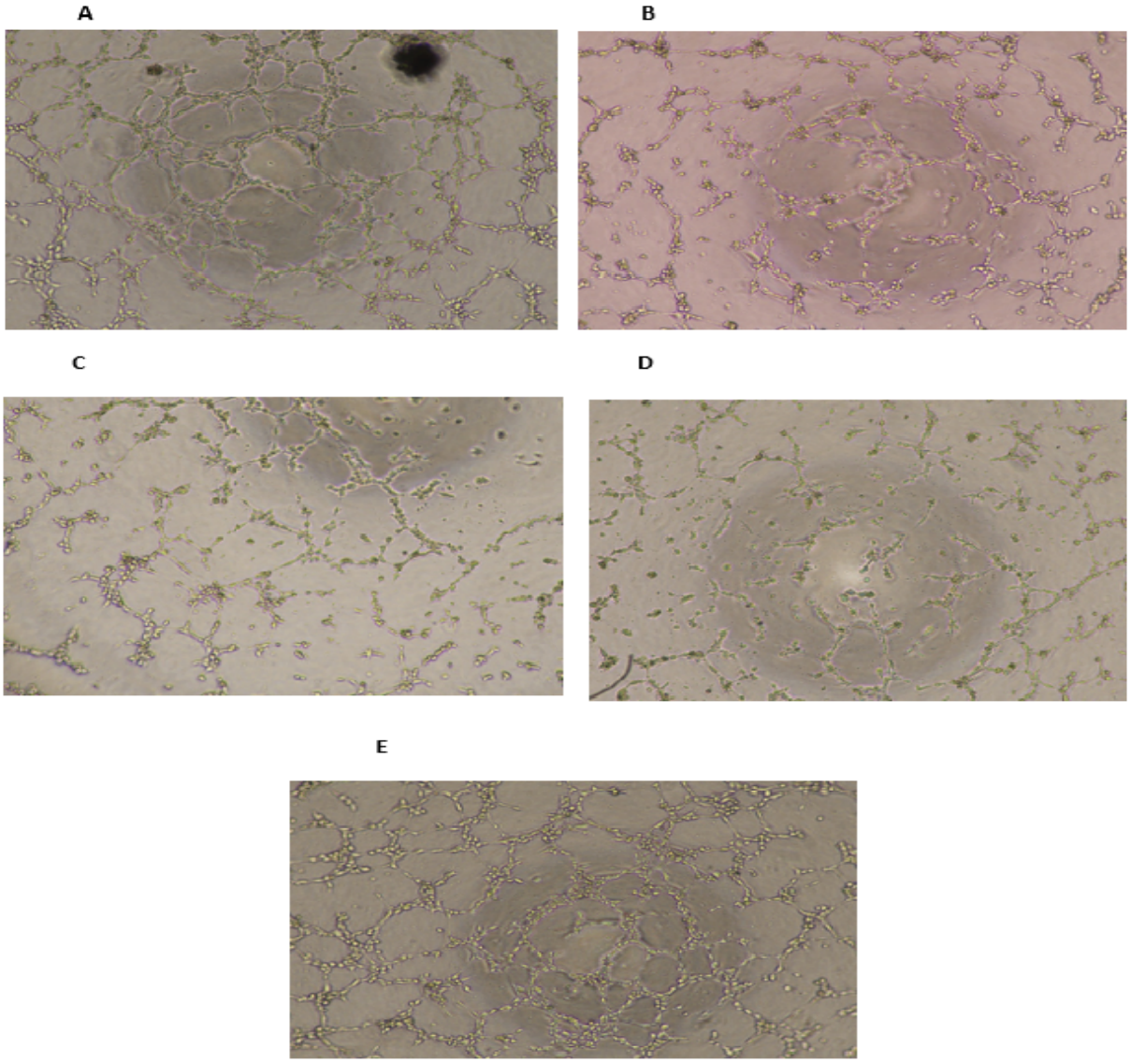

Figure 1. Capillary like tube formation of HUVECs treated with conditioned media collected from hypoxic CSCs to test the presences of secretory stimulatory or inhibitory factors released by CSCs under the hypoxia treatment. Plated HUVECs over a Matrigel coated plate were allowed to rearrange into a tube structure for 6 hours. The figure is subdivided as follow, (A) positive control HUVEC group, (B) HUVECs treated with conditioned media of **INT.5**, (C) HUVECs treated with conditioned media of **INT.10**, (D) HUVECs treated with conditioned media of **INT.15**, and (E) HUVECs treated with conditioned media of **INT.22**.

angiogenic stimulatory factors. In contrary, conditioned media of INT.10 and INT.15 is thought to have inhibitory factors or not expressing the normal levels of the stimulatory factors as in controls. (C) HUVECs treated with conditioned media of **INT.10**, (D) HUVECs treated with conditioned media of **INT.15**, and (E) HUVECs treated with conditioned media of **INT.22**.

DISCUSSION

Hypoxia and angiogenesis, with subsequent proteomic and genomic changes, can be considered two main characteristics of growing tumors and for this reason, the hypothesis motivating this study is that these two processes play a main role in determining the tumor progression and metastatic potential of CSCs. Thus, in CSCs from a primary tumor, hypoxia may link the changes in the expression of key genes in the hypoxia and angiogenesis pathways to tumor progression. Therefore, progression of transformed CSCs does not necessarily need new genetic mutations but can be acquired through a successive activation of hypoxia adaptation and changes in genes expression.

Several assays are employed to identify and quantify CSCs in breast tumor specimens and breast cancer cell lines. For

example, flow cytometric identification of CD44+/CD24- cells is a popular and useful marker of CSCs in human breast cancer cell lines. The flow cytometry data of this study have revealed a CD44+/CD24− expression for parental MCF-7 of 1.0% (Table 1). Upon the cells being cultured in a low-adherence tissue, and serum-free media with essential supplements for three days, CSCs were isolated and identified with FACS and displayed an increase of CD44+/CD24− expression to 83.0%. However, CD44+/CD24− expression was decreased to 38.4% at day 21. Thus, it is concluded that the sorted CSCs acquired stemness character much higher than their parental cells, and declines over time.

Hypoxia is considering the major factor that responsible for resistance of tumor cells to therapies [9]. The adaptation of cancer cells to hypoxia includes an interaction of genetic and biochemical factors which support cell survival, and contribute considerably to resistance and aggressiveness of the tumor cells to therapy.[10] With the purpose of finding a biomarker for hypoxia in CSCs, we have exposed CSCs to 10 and 22 of 8-hour episodes of hypoxia. Chemo-resistance toward doxorubicin was measured using MTT proliferation assay at 10 and 22 episodes of hypoxia. The findings of our study reveal that hypoxia plays a significant role in the development of a chemo-resistant phenotype in CSCs. Most prominently, distinct differences in gene expression profiling were found in cells exposed to 22 episodes of hypoxia compared to their normoxic counterparts. Exposing the CSCs to 22 episodes of 8-hour hypoxia induce chemo-resistance toward doxorubicin. This was demonstrated by a noticeable increase in the IC50 values compared to the normoxic cells by around 4.78 folds in the MTT assay. These results support the conclusions of other studies that have demonstrated that hypoxia induces chemo-resistance.[11,12] One of the speculated mechanisms by which hypoxia induces chemo-resistance is by changes brought on by hypoxia on the genomic and proteomic level of the cell.

Tumor cells employ HIF family, a family of transcription factors, to adapt to hypoxic conditions, and HIF-1 is the most studied member of this family. HIF-1 has been associated with the development of aggressiveness and chemo-resistance in different tumors.[10] On the other hand, there are many regulated pathways have also been revealed that involved in hypoxia-induced drug resistance. But, these pathways have not been fully clarified.[13] In our study, an attempt to find a proper biomarker for cancer resistance during hypoxia led to gene expression profiling when the cells showed ascending resistance after 10 episodes of hypoxia reaching to the maximum after 22 episodes of hypoxia. The findings obtained indicated that hypoxia plays an important role in the development of chemo-resistant phenotype in CSCs. Distinct differences in gene expression were showed by cells exposed to 10 and 22 episodes of hypoxia, when compared with their normoxic counterparts.

Interestingly, we have found that a gene implicated with chemo-resistance is profoundly up-regulated at 22 episodes of hypoxia. LGALS3 was found to be up-regulated by 5.8 folds and is involved in tumor cell adhesion, proliferation, differentiation, angiogenesis, and metastasis in multiple tumors. In addition, changes in this gene expression are commonly seen in cancer and pre-cancerous conditions. Other genes that are associated with metastasis and cancer progression and notably up-regulated at 22 episodes' cells is LOX and HMOX1. While HMOX1 is known to be involved in tumor cell migration, cell invasion and mediates angiogenesis, LOX helps the cells to oxidatively deaminates the ε-amino groups of lysine residues, resulting in intramolecular and intermolecular cross-linking of collagen molecules.[14] Crosslinking stabilizes collagen by assembly into fibrils and fibers, which enhance ECM tensile strength, leading to focal adhesion formation and PI3K signaling.[15] The up-regulation of the other gene IGFBP3 may have contributed significantly to the observed doxorubicin resistance. Studies have shown that IGFBP3 exhibits pro-survival and growth-promoting properties *in vitro*.

Another prominent gene in our study is ANGPTL4 which is the most markedly down-regulated gene at 22 episodes of hypoxia. ANGPTL4 is a member of the angiopoietin-like protein (ANGPTL1–7) family, which has important functions in glucose and lipid metabolism [16], especially as a suppressor of lipoprotein lipase activity. ANGPTL4 is a HIF-1 target gene that contributes to vascular infiltration.[17] The role of ANGPTL4 in the tumor progression and metastasis is complex and controversial. ANGPTL4 overexpression inhibits the migration and adhesion of invasive triple negative breast cancer cell lines and decreases the mRNA levels of extracellular matrix-related genes. Our results agree with several studies that have also shown that ANGPTL4 overexpression prevents metastasis and angiogenesis.[18]

Moreover, the results of this study also showed that another 8 genes were significantly down-regulated more than 2-fold after 22 episodes of 8-h hypoxia, including ANKRD37 and MAP3K1. These genes are most likely responsible for many cellular mechanisms. Recently, Minzi Deng et al. showed the role of subcellular localization of ANKRD37 in hypoxia-induced autophagy. The high expression of ANKRD37 reduces the survival rates of colon cancer. ANKRD37 inside the nucleus augments HIF-1α-induced autophagy which consequently increases colon cancer cell proliferation.[19] Alternatively, MAP3K1 is an important kinase that links Ras activation to MAPK signaling and play an important role in synthesis of MEK kinase 1 (MEKK1). Using in vivo nude mice model, Liu and his colleagues (2018) revealed that targeted Map3k1 with a miRNA, attenuated tumor growth and lung metastasis of breast cancer cells.[20]

It is clear that the biological effects of ANGPTL4 on cancer cells are controversial in the literature. Some studies suggested that ANGPTL4 inhibits endothelial cell migration [21] and has a regulatory role in lipid metabolism and insulin sensitivity.[22] However, other studies showed that ANGPTL4 regulates cancer progression, angiogenesis and metastasis.[23,24] One study suggested important roles for ANGPTL4 in the progression of breast cancer [25], although another reported conflicting data.[26] Interestingly, the findings of this study revealed that the expression of ANGPTL4 induced under normoxia or

hypoxia. However, the expression levels of ANGPTL4 was decreased in HUVEC cells that exposed to hypoxic conditioned media in comparison to those cells that exposed to normoxic conditioned media. These findings strongly suggest that ANGPTL4 expression is critical for angiogenesis and metastasis.

Emerging evidence has revealed that chemokines play a significant role in cancer processes, angiogenesis, lymphangiogenesis, and in the recruitment of tumor-associated cells.[27] Hypoxia/reoxygenation studies revealed an increase in CCL15/HCC-2 expression on HUVEC.[28] This chemokine increased the CC motif chemokine receptor (CCR)1-dependent expression of intracellular adhesion molecule-1 (ICAM-1) on these endothelial cells resulting in increased monocyte adhesion. Here, we show that the expressions of CXCL6, CXCL1, CXCL10, CXCL8 and CCL2 were induced in HUVEC cells more strongly under exposure to hypoxic conditioned media than under exposure of normoxic conditioned media. An increase in chemokine expression induces an increase in the expression of VEGF in HUVEC cells, which facilitate the angiogenesis.[29,30] The results of our study is consistent with the previous studies, as the expression of VEGF increase in HUVEC cells that exposed to hypoxic conditioned media in comparison to those cells that exposed to normoxic conditioned media.

EFNB2, transmembrane protein that is capable of activating EFNB2 receptors, has been shown to be both up- and down-regulated in different cancer types.[31] EFNB2 receptor ligand facilitates repulsive effects during the separation of adjacent cell population in the segmented structures during the development.[32] Likely, tumour cells need to overcome repulsive signals from the surrounding parenchyma, which is considered as an evidence to the role of repulsive interactions between EFNB2 receptors and its ligands during colon cancer progression.[33] Therefore, the inhibition of EFNB2 function may be an important factor to trigger tumour invasiveness. Indeed, we show that down-regulation of EFNB2, increases breast cancer growth and invasion. Moreover, the findings of this study revealed that EPHB4 down-regulated at 22 episodes' cells. The complex EphB4-ephrinB2 signaling can manifest into pro-tumorigenic effect as reported in several tumor models.[34] On the other hand, another gene that down-regulated at 22 episodes' cells is midkine growth factor (MDK), a modulator of angiogenesis and that it can suppress the VEGF-A-induced proliferation of human endothelial cells *in vitro* through decrease the expression of proangiogenic cytokines and increase the expression of the antiangiogenic factor.[35] This observation directly agrees with the current study, which states that MDK down-regulated at 22 episodes' cells.

This comprehensive study has elucidated the significant impact of hypoxia on breast cancer stem cells (CSCs), showcasing the intricate relationship between hypoxic conditions and the regulation of gene expression patterns that contribute to the aggressive behavior of breast cancer. Our findings demonstrate that hypoxia induces a plethora of gene expression changes, fostering an environment conducive to tumor progression, resistance to therapy, and enhanced angiogenic activities. Particularly noteworthy is the identification of upregulated genes such as LGALS3, HMOX1, and LOX under hypoxic conditions, which are implicated in tumor cell adhesion, proliferation, differentiation, angiogenesis, and metastasis. These genes, along with the observed downregulation of ANGPTL4, highlight the complex regulatory networks that underpin cancer stem cell adaptation and survival in low oxygen environments. Furthermore, the study underlines the critical role of the hypoxia-inducible factor 1α (HIF1-α) in mediating the transition of tumor cells to a more stem-like state, capable of promoting angiogenesis and metastasis. The observed alterations in gene expression not only contribute to the stemness and therapeutic resistance of CSCs but also enhance their capacity to remodel the tumor microenvironment, facilitating cancer progression. Through the application of various in vitro assays, including the Capillary-Like Structure Formation Assay, we have provided empirical evidence supporting the notion that hypoxic CSCs secrete factors that significantly influence the angiogenic capabilities of endothelial cells. This underscores the pivotal role of the tumor microenvironment in cancer progression and highlights potential targets for therapeutic intervention aimed at disrupting these critical cellular interactions.

CONCLUSION

Our study advances the understanding of how hypoxic conditions within solid tumors influence the behavior of breast cancer stem cells, emphasizing the importance of targeting hypoxia-related pathways as a promising strategy in the battle against breast cancer. By unraveling the molecular mechanisms underpinning hypoxia-induced changes in CSCs, we pave the way for the development of novel therapeutic approaches that could inhibit tumor progression and overcome resistance to existing treatments.

AUTHORS' CONTRIBUTIONS

Conceptualization: HMH, MAZ; Experimental work: MAZ, TAT; DATA analysis: HMH, MAZ, TAT, AI; Writing –original draft: HMH, TAT, AI; Writing –review & editing: HMH, MAZ, AI.

CONFLICT OF INTEREST

The authors declare that no competing financial interests or any other conflicts of interest exist.

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