Hypoxia induces lineage modulation of Ewing’s sarcoma tumor cells into EWS-FLI-1+ vascular pericytes

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Introduction

Ewing’s sarcoma (ES) is the second most common bone tumor in children and adolescents. This sarcoma is characterized by a unique chromosomal translocation between chromosomes 11 and 22 leading to the formation of fusion genes that encode fusion proteins composed of the transcriptional domains of EWS and the DNA binding domain of one of the five ETS transcription factors. The most common fusion protein EWS-FLI-1 occurs in 85% of cases and functions as an aberrant transcription factor [1]. EWS-FLI-1 is necessary for the induction, progression and maintenance of the malignant phenotype [2-4]. EWS-FLI-1 regulates aberrant gene transcription and the up-regulation of multiple proteins that distinguish ES from other sarcomas. The current standard of care for patient with ES includes pre-operative and post-operative combination chemotherapy, surgical resection and radiation [5]. For patients with non-metastatic disease, this approach achieves a 70% 5-year overall survival rate. However, for the 30% of patients who experience relapse or whose cancer does not respond to front-line therapy, salvage chemotherapy protocols are ineffective, and patients

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usually die within 1 year of relapse. The outcome for patients who present with metastatic disease is even worse, with <25% surviving [5].

Increasing the dosage and frequency of chemotherapy administration has recently been shown to increase the 5-year overall survival rate of patients with non-metastatic patients to 76%, but these changes had no effect on the outcome of patients who presented with metastatic disease [6, 7]. Unfortunately, increasing the dose-intensity with interval compression adds substantial acute toxic effects and increased late effects because this involves the use of doxorubicin and high-dose ifosfamide together with etoposide. These toxic effects include male sterility, cardiomyopathy and secondary leukemia. No therapeutic approaches or targeted agents have been shown to improve the survival of relapsed patients or those that present with metastases. Clinical trials using agents that target EWS-FLI-1, the IGF-1/IGF-1R pathway and VEGF have been unsuccessful in demonstrating clinical activity [8]. New therapies are clearly needed for this cancer, which mostly affects children, adolescents and young adults.

Understanding how ES cells interact with and respond to the tumor microenvironment to support and facilitate growth has the potential to uncover new therapeutic approaches that will interfere with this symbiotic relationship. ES depends on a vascular network for growth, invasion and metastatic spread. We have shown that in ES, migration of bone marrow stem cells to the tumor site and differentiation of these cells into pericytes plays a role in the formation of new tumor blood vessels [9-15]. Interfering with tumor pericyte formation, similar to interfering with endothelial progenitor cells, significantly interfered with tumor vascular expansion, tumor growth and metastases [9-16]. These bone marrow-derived stem cells had migrated into the tumor area in response to VEGF165 and SDF-1 [14, 17, 18]. Pericytes have several important functions relating to vessel maturation and provide proliferation signals to the endothelial cells [19-23]. Without pericytes, vessels are leaky, less functional and susceptible to regression [24, 25]. We demonstrated that in ES blocking bone marrow stem cell migration and differentiation into pericyte formation resulted in reduced vessel functionality and tumor hypoxia [26, 27]. Subsequently however, the number of non-bone marrow-derived tumor vascular pericytes increased. This indicated that the tumor cells had developed a way to compensate for the loss of the bone marrow-derived pericytes. Understanding this compensatory mechanism can help in identifying a new therapeutic target.

The tumor microenvironment has been shown to stimulate vascular development. Hypoxia is often a consequence of solid tumor growth which in turn stimulates new vessel formation to provide the needed oxygen and nutrients to support tumor growth [28]. Hypoxia can induce reprogramming of human cells to become pluripotent cells with stem-like characteristics [29]. Hypoxia has also been shown to induce the differentiation of human embryonic stem cells into functional endothelium [30]. The role ES cells play in vascular mimicry, vascular expansion and remodeling is not well understood. Using a unique antibody EWS (N-18), which recognizes only the EWS-FLI-1 fusion protein, but not the wild-type EWS protein, we discovered that a portion of the Ewing’s tumor vascular pericytes were derived from tumor cells [31]. These EWS-FLI-1+ pericytes were seen in the hypoxic tumor tissue. We therefore investigated whether hypoxic condition trigger the lineage modulation of ES cells into pericytes that participate in the formation of tumor vessels.

Material and methods

I. Cell lines

Normal human osteoblasts (hOB) were purchased from Lonza Group Ltd. (Basel, Switzerland), human Pericytes from Placenta (hPC) were purchased from Promocell GmbH (Heidelberg, Germany), human vascular endothelial cells (hEC) and human mesenchymal stem cells (hMSC) were purchases from American Type Culture Collection (Manassa, VA). The cells were cultured in special medium according to manufacture instructions. TC71 human ES cells were a gift from Dr. T. Triche (University of Southern California, Los Angeles, CA). A4573 human ES cells were a gift from Dr. V. Soldatenkov (Georgetown University Medical Center, Washington, DC). Both cells were culture in Dulbecco modified Eagle medium with 10% fetal bovine serum. TC71 and A4573 cell lines were authenticated by short terminal repeat fingerprinting at the University of Texas, MD Anderson Cancer Center Cell line authentication core facility. All of the cells were Mycoplasma-free as determined by the MycoAlert Mycoplasma Detection Kit (Lonza Ltd., Basel, Switzerland).

II. Cell culture in Hypoxic Chamber

BioSpherix Xvivo Incubation Workstation (Lacona, NY) was used for cell culture in hypoxic chambers. According to the manufacturer instructions, hypoxia experiments were performed using a customized hypoxic incubator that continuously infuses a calibrated oxygen concentration of 5% with a gas mixture (5% CO2, balance N2), the oxygen concentration was monitored using an oxygen sensor. For normoxia experiments, cells were incubated in a humidified incubator with a constant supply of 21% oxygen and 5% CO2 at 37°C. Cells were exposed to hypoxic or normoxic conditions for 72 hours before the experiments were performed.

III. Hypoxia assay in vivo using Hypoxyprobe-1 (HPI)

Four to five-week-old athymic nude mice were purchased from the National Cancer Institute. The mice were maintained in a specific pathogen-free animal facility approved by the American Association for Accreditation of Laboratory Animal Care. The animal experiment protocol was approved by the Institutional Animal Care and Use Committee at MD Anderson Cancer Center. TC71 human ES cells in mid-log-growth phase were harvested by trypsinization. Cell suspensions (2 × 10⁶ cells in 0.1 ml of Hanks solution) were injected subcutaneously into the nude mice. After 4 weeks, the mice were injected with 200 µl of Hypoxyprobe solution and euthanized 2.5 hours later. Tumor tissues were collected for immunofluorescent staining using the anti-pimonidazole monoclonal antibody. The Hypoxyprobe-1 (pimonidazole HCI) kit was purchased from HPI, Inc. (Burlington, MA). Hypoxyprobe was reconstituted in phosphate-buffered saline at a final concentration of 7 mg/ml.

IV. Reverse Transcription-Polymerase Chain Reaction (PCR)

RNA was extracted from cells using TRZol reagent (Invitrogen Inc., San Francisco, CA).
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Diego, CA). cDNA was synthesized using the Reverse Transcription System (Promega, Madison, WI). The products were amplified by regular PCR using specific primers for EWS-FLI-1, Desmin, NG2, HIF-1α and Sox2. The GAPDH primers were used as internal controls. Quantitative real-time PCR was performed using SYBR Green Supermix (Bio-Rad, Hercules, CA), and β-actin (Sigma-Aldrich, St. Louis, MO).

V. Western blotting

Cells were cultured in 100-mm dishes. Cell lysate was collected after 72 hours of hypoxic or normoxic culture. The protein (100 μg) was loaded onto a 10% SDS-polyacrylamide gel. Specific protein bands were detected with the following antibodies: EWS (N-18), EWS (G-5) (Santa Cruz Biotechnology, Santa Cruz, CA); Sox-2, Oct3/4 (Cell signaling Technology Inc., Boston, MA) and β-actin (Sigma-Aldrich, St. Louis, MO).

VI. Flow cytometry analysis and isolation of CD133+ and CD133- cells using the EasySep magnet kit

Hypoxic or control TC71 ES tumor cells were incubated with CD133-PE (phycoerythrin) antibody or isotype-match control IgG antibody for 30 minutes at 4°C in 2% FBS (fetal bovine serum) in phosphate-buffered saline (PBS) (1 μl antibody per 1 x 10^6 cells). Samples were washed three times and were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). To enrich the CD133+ cells, hypoxic TC71 cells were isolated using the EasySep human PE selection kit (StemCell, Cambridge, MA) according to the manufacturer’s instructions. CD133+ or CD133- cells were seeded in 6-well plates and treated with PBS (as control), DLL4 (5μg/ml) or DLL4 with the γ-secretase inhibitor DAPT (0.5mM) for 48 hours. Cells were then immune fluorescent stained with Desmin or NG2 antibody, and Cyanine-5 was used as the secondary antibody.

VII. Immunofluorescence staining

Nine Ewing’s sarcoma patient’s tumor specimens (2 male, 7 female) were obtained from The University of Texas MD Anderson Cancer Center. The research protocol was approved by The Institutional Review Board (IRB) in The University of Texas MD Anderson Cancer Center. All animal experiments were approved by Institutional Animal Care and Use Committee in The University of Texas MD Anderson Cancer Center. ES mouse tumor tissues were collected in nude mice 4 weeks after subcutaneous injection with TC71 cells. All frozen tumor sections were fixed with acetone and chloroform, then washed with PBS. The sections were incubated with one of the following primary antibodies to human: HIF-1α, Sox-2, Oct3/4 (Cell Signaling Technology), Desman, DLL4 (Abcam, Inc., Cambridge, MA), EWS (N-18), NG2 (Santa Cruz Biotechnology), CD133(MACS Milteny Biotec, Auburn, CA), or HPI (Hypoxyprobe Inc. Burlington, MA). Cyanine 5 (Cy5)-conjugated goat anti-rabbit IgG, Cyanine 3 (Cy3) conjugated goat anti-mouse IgG, Alexa488 conjugated goat anti-mouse IgG and FITC-conjugated donkey anti-goat IgG were used as the secondary antibodies. The nuclei were stained using DAPI (Invitrogen, San Diego CA). All sections were analyzed by fluorescent microscopy (Leica, Inc.). Relative expression was quantified in at least five different microscopic fields from different samples using Simple PCI software (Hamamatsu, Sewickley, PA), and average expression was calculated. Z-stack series scans were performed on tumor vascular every 1.2μm interval layers using Zeiss LSM 510 confocal microscope.

VIII. In vitro and in vivo experiments with Des-Pro-GFP vector

A human Desmin promoter-driven vector linked to GFP (Des-Pro-GFP) was obtained from the Department of Stem Cell Biology and Regenerative Medicine, at the Cleveland Clinic [32]. TC71 and A4573 human ES cells and normal human osteoblast cells were transfected with the Des-Pro-GFP vector and cultured in a hypoxia chamber or normal incubator for 72 hours. GFP was detected under fluorescent microscopy in different cells. The experiment was repeated three times. TC71 and A4573 cells stably transfected with Des-Pro-GFP were established. Then, 2 x 10^6 cells were subcutaneously injected into nude mice (5 mice per group, repeated three times), and tumors were resected 3 weeks after injection. GFP and Desmin were detected in the tumor tissues by immunofluorescent staining.

IX. Statistical analysis

A two-tailed Student t test was used to statistically evaluate all experimental results. P < 0.05 was considered statistically significant.

Results

I. A subset of tumor vascular pericytes in ES patient tumor samples and TC71 mouse tumor express the EWS-FLI-1 fusion protein

The specific EWS (N-18) antibody was used to identify ES cells [31]. This antibody detected EWS-FLI-1 fusion protein only in TC71 and A4573 ES cells, but not in human pericytes (hPC), human endothelial cells (hEC), and human mesenchymal stem cells (hMSC) (Fig. 1A upper panel). As a control, another EWS (G-5) antibody only recognized the 90kD wild-typed EWS protein, but not the 68kD EWS-FLI-1 fusion protein (Fig. 1A bottom panel), confirming the specificity of the EWS (N-18) antibody for EWS-FLI-1. The pericyte markers Desmin and NG2 were expressed only in human pericytes (hPC), but not in TC71 or A4573 ES cells (Fig. 1B and C). These results indicated that EWS-FLI-1 is a unique tumor marker for Ewing’s sarcoma tumor cells.

However, when ES patient tumor samples were detected by pericyte marker Desmin (red) and ES tumor cell marker EWS-FLI-1 (green), the portion of pericytes were shown EWS-FLI-1 positive (yellow, as arrow indicated in Figure 2 A). 7 samples were found EWS-FLI-1/Desmin+ pericytes in 9 collected ES patient tumor samples (77.8%). These data suggest that these EWS-FLI-1/Desmin+ pericytes were derived from the ES cells, because human pericytes do not express EWS-FLI-1, ES tumor cells did not express pericyte marker Desmin (Figure 1B and C). We further examined the mouse xenograft tumors, in TC71 Ewing’s sarcoma mouse tumors EWS(N-18) (green) and Desmin (red) double positive cells (Figure 2 B, left panel, arrow) were also shown in some vessels of tumor tissues indicating that a portion of the tumor vessel pericytes were EWS-FLI-1 positive. Similar results were seen using another pericyte marker NG2 (Figure 2 B, right panel). 8 of 10 mice xenograft tumors
were found EWS-FLI-1/Desmin and EWS-FLI-1/NG2 double positive cells in tumor vessels. When HIF-1α was used to detected hypoxic areas of tumor, these Desmin/EWS-FLI-1* double positive cells (yellow area) were found in areas of hypoxic tissues (Figure 2 C, upper panel, arrows), but not in HIF-1α− non-hypoxic areas (Fig. 2 C, bottom panel), suggesting that this phenomenon was induced in response to hypoxia. The EWS (N-18)/Desmin/HIF-1α triple positive areas (white), the EWS (N-18)/Desman double positive areas (yellow) and the positive areas for Desmin (red) were quantified in 10 random fields from different tumor tissues using the SimplePCI software. The percentages of pericytes derived from ES tumor cells in the hypoxic versus non-hypoxic areas were calculated. We found that 14.7% of the total pericytes in the hypoxic tumor areas were derived from ES cells compared with < 1% in the non-hypoxic areas (Figure 2 D, P<0.01). To exclude these areas of co-localization, represent extravascular ES cells, the confocal microscope Z-stack series scans were performed in tumor vessels (Figure 2 E). The all series scan sections (every 1.2 μm) indicated that the mosaic complex Desmin/EWS-FLI-1* positive cells were inside of tumor vessels, rather than separation by red cells (Desmin positive pericytes) in upper sections and green cells (EWS-FLI-1+ tumor cells) in down sections. The confocal images further confirm that the double positive cells were the vascular pericytes derived from ES tumor cells.

Figure 1: The EWS (N-18) antibody specifically identifies EWS-FLI-1 fusion protein in Ewing’s sarcoma. (A) Western blot analysis indicated that the EWS (N-18) antibody only identified the 68kD EWS-FLI-1 fusion protein in TC71 and A4573 human ES cells, but not in human pericytes (hPC), human endothelial cells (hEC) and human mesenchymal cells (hMSC). This antibody did not recognize wild type EWS protein (90kD). By contrast, other EWS (G-5) antibody recognized only the wild-type 90kD protein and not the 68kD fusion protein, confirming the specificity of the EWS (N-18) antibody for EWS (N-18) antibody for EWS-FLI-1 (Fig 1A). (B) Immunofluorescent staining indicated that EWS-FLI-1(green) expression was only in TC71 EW tumor cells, but not in human pericytes (hPC). Pericyte marker Desmin (red) expression was only in human pericytes, but not in TC71 EW cells in normal condition. (C) RT-PCR results confirmed that EWS-FLI-1 was expressed in TC71 and A4573 ES cells but not in the hPC. Desmin and NG2 were expressed in hPC but not in TC71 and A4573 cells.
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Figure 2: EWS-FLI-1+ pericytes in human patient tumor samples and TC71 mouse xenograft tumors.

(A) Ewing’s sarcoma patient samples were analyzed by immunofluorescent staining for Desmin (red) and EWS (N-18) (green). The co-localization (yellow) indicated that a portion of pericytes expressed EWS-FLI-1. (arrows) Scale bars represent 100 µM. The middle image is with high magnification. (B) Desmin and NG2 were used to detect pericytes in TC71 xenograft mouse tumor tissues. The colocalization (yellow) of Desmin (left panel) or NG2 (right panel) with EWS (N-18) indicated that the portion of the pericytes were derived from tumor cells. Scale bars represent 50µM. (C) TC71 tumors were analyzed for Desmin (red), EWS (N-18) (green) and HIF-1α (purple). Co-localization of all three markers (white, as arrows indicated) showed that the Desmin/EWS-FLI-1+ (yellow) cells were located inside of tumor vessels to further confirm these pericytes derived from ES tumor cells.

II. Hypoxia induced sphere cell formation and the expression of stem cell markers in ES cells, but not in normal osteoblasts

The EWS-FLI-1+ pericytes were found in the hypoxic areas of the tumor. We therefore next determined whether hypoxia triggered this lineage modulation. TC71 cells were cultured under hypoxic condition for 72 hours. Hypoxia induced sphere cell formation (Figure 3 A) and up-regulated expression of stem cell markers CD133, Nanog, Sox-2 and Oct3/4 (Figure 3 B and C). Increased Sox-2, Oct3/4 and Desmin protein expression was also induced by hypoxia (Figure 3 B bottom panel). Similar results were seen using another ES cell line A4573. Hypoxia induced the expression of HIF-1α, HIF-2α, Sox-2, Oct3/4 and Nanog in A4573 cells (Figure 3 C and D). Hypoxia also induced the expression of Desmin and NG2 in both TC71 and A4573 cells (Figure 3 C). By contrast, hypoxia induced the expression of HIF-1α, but did not induce the expression of Sox-2, Desmin or NG2 in normal human osteoblasts (Figure 3 C). Although HIF-1α expression was up-regulated in TC71, A4573 and human osteoblast cells following hypoxic culture, increased expression of Sox-2 and the pericyte marker Desmin and NG2 was observed only in TC71 and A4537 ES cells (Figure 3 C). These results indicated that a hypoxic microenvironment promoted a stem-like phenotype in ES cells. In addition, these data suggest that there was lineage modulation of the ES stem cells into pericytes.

Figure 3A
Figure 3: Hypoxia-induced sphere formation and expression of stem cell markers in TC71 and A4573 ES cells, but not in normal human osteoblasts.

(A) The morphology of TC71 cells following 72 hours of culture in a hypoxic chamber or normoxia condition is shown in upper panel. Cells were analyzed by flow cytometry for the stem cell marker CD133. The percentage of CD133+ cells was increased by hypoxia. (B) CD133, Nanog, Sox-2 and Oct3/4 were analyzed by RT-PCR following hypoxic culture (upper panel). The protein levels of Sox-2, Oct3/4 and Desmin were determined by Western blot analysis (bottom panel). The relative expression in hypoxic cells was calculated in comparison with that in the normoxic control cells and adjusted with β-Actin loading control. *P<0.05. (C) Expression of EWS-FLI-1, HIF-1α, Sox-2, Desmin and NG2 was analyzed by RT-PCR in TC71, A4573 cells, and normal human osteoblasts cultured under hypoxic or normoxic conditions. Sox-2, Desmin and NG2 expression were induced by hypoxia in TC71 and A4573 cells, but not in normal osteoblasts (D) HIF-1α, HIF-2α, Sox-2, Oct3/4, Nanog and Desmin expression were determined using RT-PCR in other ES cells A4573 cultured in a hypoxic or normoxic conditions for 72 hours. Bars represent standard deviation. *P<0.05.

III. Expression of stem cell markers is increased in hypoxic areas of mouse xenograft tumor and patient tumor tissues

Mice were injected with TC71 cells. Four weeks later the specific hypoxic probe Hypoxyprobe-1 was injected prior to euthanizing the mice. Tumor tissues were collected and analyzed by immunofluorescent staining using a hypoxia probe HPI. HPI positive tissues indicated the hypoxic areas. Human Oct3/4 antibody was used to identify stem cells which were detected (green) in the hypoxic areas. By contrast, few Oct3/4+ tumor cells were detected in non-hypoxic areas (Figure 4 A). Similar results were also seen in patient samples in which HIF-1α' was used to determine the hypoxic area of the tumor tissue and human CD133 was used as the stem cell marker (Figure 4 B). Once again, stem cells were increased in the hypoxic areas of the tumor. Oct3/4' or CD133' cells in hypoxic (HPI' or HIF1α') and non-hypoxic areas (HPI' or HIF1α) in different tumor tissues were quantified using Simple PCI software. Oct3/4' (Figure 4 A right panel) or CD133' (Figure 4 B right panel) stem-like cells were significantly increased in hypoxic tumor areas compared with non-hypoxic tumor areas (P<0.01).
Figure 4: Increased expression of stem cell markers in hypoxic areas of TC71 tumors and patient samples.

(A) The hypoxia probe HPI was used to detect hypoxic cells. The tumor tissues were analyzed using human antibodies to detect HPI (red) and stem cell marker Oct3/4 (green). Oct3/4^+ cells (upper panel) were quantified in hypoxic (HPI^+) and non-hypoxic areas (HPI^-) from different tumor tissues. The expression of stem cell markers Oct 3/4 was significantly increased in the hypoxic areas of TC71 tumors samples compared with non-hypoxic areas (right panel, P<0.01). (B) Expression of HIF-1α and CD133 was analyzed in Ewing’s sarcoma patient samples by immunofluorescent staining. CD133^+ cells (green in left panel) were quantified in hypoxic (HIF-1α^+) and non-hypoxic areas. The expression of stem cell markers CD133 was significantly increased in the hypoxic areas of human patient tumor samples compared with non-hypoxic areas (right panel, P<0.01). Bars represent standard deviation.

IV. Expression of pericyte markers Desmin and NG2 is upregulated in CD133^+ but not CD133^- TC71 cells

To investigate the link between the hypoxia-induced stem cell phenotype and pericyte differentiation, TC71 cells were cultured under hypoxic conditions for 72 hours. Cells were then separated into CD133^+ and CD133^- populations by EasySep selection kit. We have previously shown that DLL4 induces the differentiation of bone marrow stem cells into pericytes [27]. Therefore, the separated cell populations (CD133^+ and CD133^- cells) were treated with DLL4 alone or DLL4 plus the γ-secretase inhibitor DAPT, which blocks DLL4 signal transduction. Hypoxia induced CD133^+ TC71 cells to express both pericyte markers Desmin (Figure 5 A) and NG2 (Figure 5 B). DLL4 can significantly increase that response indicating that the DLL4-Notch pathway controls pericyte differentiation. We previously showed that DAPT inhibited the ability of DLL4 to induce pericyte differentiation of bone marrow stem cells [27]. When CD133^+ cells were treated with DLL4 and DAPT, the expression of Desmin and NG2 were inhibited (Figure 5 A and B). These data suggest that blocking Notch signal inhibits the differentiation of ES
cells to pericytes. By contrast, DLL4 did not induce Desmin expression in the CD133\(^+\) cells (Figure 5 C). These data indicated that pericyte differentiation is limited to tumor cells with a stem-like phenotype in hypoxic microenvironment.

**Fig. 5A**

| TC1-CD133\(^+\) cells control | Desmin | Merge |
|-------------------------------|--------|-------|
| TC71-CD133\(^+\) cells with DLL4 (5µg/ml) |        |       |
| TC71-CD133\(^+\) cells with DLL4 + \(\gamma\)-secretase inhibitor DAPT(0.5nM) |        |       |

**Fig. 5B**

| TC71-CD133\(^+\) cells control | NG2 | Merge |
|-------------------------------|-----|-------|
| TC71-CD133\(^+\) cells with DLL4 (5µg/ml) |      |       |
| TC71-CD133\(^+\) cells with DLL4 + \(\gamma\)-secretase inhibitor DAPT(0.5nM) |      |       |

**Fig. 5C**

| TC71-CD133\(^+\) cells-control | Desmin | Merge |
|-------------------------------|--------|-------|
| TC71-CD133\(^+\) cells with DLL4 (5µg/ml) |        |       |

**Figure 5:** Induction of Desmin and NG2 expression in CD133\(^+\) cells but not CD133\(^-\) TC71 cells.

CD133\(^+\) and CD133\(^-\) TC71 cells were isolated by EasySep kit following 72 hours of hypoxic culture. The CD133\(^+\) cells were treated with DLL4 alone or DLL4 plus the \(\gamma\)-secretase inhibitor DAPT for 48 hours. The cells were stained for Desmin (A), NG2 (B) and DAPI (nuclear staining). Hypoxia induced expression of Desmin and NG2 in CD133\(^+\) cells, DLL4 significantly enhanced this expression (A-B, middle panel, arrows indicated examples of Desmin\(^+\) or NG2\(^+\) cells). DLL4-induced Desmin and NG2 expression was inhibited by DAPT (A-B, low panel). DLL4 did not increase Desmin expression in CD133\(^-\) cells (C). Bars represents 50µM.

**V. Hypoxia induced Desmin-promoter driven GFP expression in vitro and in vivo**

To further confirm that these hypoxia-induced pericytes were derived from ES cells, we employed the Desmin-promoter driven GFP vector (Des-Pro-GFP) [32]. GFP is expressed only in cells where the Desmin promoter has been activated. TC71 cells, A4573 cells, and normal human osteoblast cells (control cells) were transfected with Des-Pro-GFP, then cultured under hypoxic or normoxic conditions for 72 hours. GFP expression was detected only in the hypoxic TC71 (Figure 6 A, left panel) and A4573 (Figure 6 B) cells. By contrast, hypoxia did not induce normal human osteoblast cells (HOB) to express GFP (Figure 6 A, bottom panel). Quantification data indicated GFP positive cells in TC71 and A4573 EW cells were significantly higher than in normal cells HOB (Figure 6 B, right panel). The expression of GFP in TC71 and A4573 cells cultured under hypoxic conditions confirmed that ES cells can lineage modulate into pericytes in response to hypoxia. The stable transfected TC71-Des-Pro-GFP cells were also injected into mice. Tumors were excised after 4 weeks and the tumor tissues were analyzed by immunofluorescent staining for GFP and Desmin. Double positive cells (Figure 6 C, yellow area) indicated that these pericytes were derived from TC71 tumor cells. These results further confirm that ES cells are capable of lineage modulation into tumor vascular pericytes in response to the tumor microenvironment.
Figure 6: Hypoxia induced Desmin-promoter driven GFP expression in vitro and in vivo.

**Discussion**

This study demonstrated that a subset of tumor vascular pericytes in both ES xenograft tumors and human patient samples express EWS-FLI-1. These unique tumor-derived pericytes were increased in the hypoxic areas of the tumor. This lineage modulation was not seen in normal human osteoblasts. We confirmed that ES cells undergo lineage modulation into pericytes in response to hypoxia by transfecting both TC71 and A4573 ES cells and normal human osteoblasts with a Desmin-promoter GFP vector. Pericytes have several critical functions relating to vessel functionality and maturation. Pericytes protect endothelial cells, regulate endothelial cell viability and proliferation, and enable endothelial cells to form new vessels [33]. Without pericytes, vessels are leaky and poorly perfused. Our data show that ES stem cells can transdifferentiate into pericytes in response to an environmental stress such as hypoxia and that the ES-derived pericytes become part of the new tumor vasculature. Thus, ES cells can provide a portion of the needed pericyte pool to sculpt, stabilize and protect the new vessels that will bring in the needed oxygen and nutrients to support tumor growth and perhaps recovery following an insult such as chemotherapy or radiation-induced tumor cell killing, which both cause tumor hypoxia and the upregulation of HIF-1α.

Anti-angiogenic therapy, which targets only the vascular endothelial cells, has not been effective against relapsed disease [34]. The etiology of this failure is not understood but may be partially attributed to the robust pericyte layer seen around the ES vasculature [12-14]. We previously demonstrated the critical role of pericytes in the vascular development of Ewing’s tumors [26, 27]. Pericyte protection renders tumor vessels less responsive to antiangiogenic therapy. Hypoxia can trigger the plasticity of ES cells [35]. Therefore, the tumor microenvironment can trigger tumor vascular expansion and the reprogramming of the residual tumor cells left behind after therapy that can assist in the formation of efficient functional vessels that contribute to tumor perfusion and tumor cell recovery. Our data indicate that Ewing’s tumor cells, in addition to bone marrow stem cells, may provide the needed pericyte pool for the formation of new functional tumor vessels to rescue residual cells in the hypoxic tumor microenvironment. The current study is the first to demonstrate that the hypoxic microenvironment triggers the trans differentiation of ES cells into pericytes that participate in the formation of tumor vessels. The plasticity
of ES cells is stimulated by hypoxia with an increase in cells with a stem cell phenotype [28]. Our data are consistent with previously published studies showing that glioblastoma stem cells can transdifferentiate into vascular pericytes that support the formation of glioblastoma tumor vessels and that skeletal myoblasts can convert to pericytes in response to DLL4 and PDGF-BB [32, 36].

In summary, we have shown that ES stem-like cells in response to hypoxia can transdifferentiate into pericytes which contribute to the formation of new tumor vessels. We have previously demonstrated the importance of bone marrow cells, the vasculogenesis process, and specific cytokines such as SDF-1α in providing the pericytes for ES tumor vasculature expansion, blocking vasculogenesis or SDF-1 inhibits tumor neovascularization [9-14]. These data taken together suggest that if vasculogenesis is inhibited, ES cells have the capability to provide the needed pericyte pool for new tumor vessel formation which is required for tumor growth following therapy. Thus, these ES-derived pericytes may participate in the rescue of ES cells and tumor recurrence following chemotherapy and radiation. Vasculogenesis and bone marrow-derived cells were shown to be crucial for the regrowth of tumors that recur after radiation therapy [37]. Radiation is an important component of ES therapy. Understanding how tumor the microenvironment supports tumor cell recovery and the formation of new tumor vessels, as well as the pathways that control and trigger the differentiation of ES cells into pericytes, may lead to the discovery of additional agents that block tumor vessel formation and the ability of ES cells to circumvent anti-angiogenic therapy. This would be expected to inhibit recovery following therapy. Such discoveries can lead to treatment approaches that can be combined with radiation therapy or chemotherapy to prevent recurrence and increase tumor response. Because relapsed ES is usually unresponsive and patients usually die within 1 year, preventing relapse can make a significant impact on long-term survival.

**Supplemental Table ST1: PCR primer sequences.**

| PCR Primers   | Forward | Reverse |
|---------------|---------|---------|
| EWS-FLI-1     | GCCCTCATATGCAGCTCGTC | GGTGTGAAACCCCTCTGTGCTA |
| Desmin       | AACATGGCTTCCACAGGTTT | CTGGAGAGATTGGGAGACCA |
| NG2          | GCCTGCACAAACAGGTTAA | CTCAACTCTGGGCAAGAC |
| GAPDH        | AGAAAGCTGGGCTCATTGG | AGGGCCCACAGAAGTC |
| HIF-1α       | TCCATGTGACCATGAGGAAA | TATCCAGGCTGTGTCGACTG |
| Sox-2        | CCCCCGCGGGCAATAGCA | TCGGCGCGGGGAGATACAT |
| qPCR primers |          |         |
| CD133        | TCCACAGAAATTACCTACATTGG | CAGCAGAGAGCACAGACAC |
| Nanog        | TGCAAGTCGACCCCAATTCTTC | CCTAGTCGTCCTGTATTACATTAAGG |
| Oct3/4       | TCTGCAAGAAGAAGCTCGACCA | AGATGTCGCTTGGGCTGACCA |
| HIF-1α       | CAGAAATACATGAGTCCCT | CTGCTGAATACCTGTAAGT |
| HIF-2α       | AGATCAGCGCAGAGATCT | GTACGGGCTGGCCTGACCT |
| β-Actin      | CGGAACGGGTGAAGGTGACA | AAGGGACCTCTCGTAACATGCA |
| Desmin       | CGGACCAAGGCGGTCTCGAG | ACTCATGCTCGTGTG |
| DLL4         | TGCAACTGCGCCCTTATGCGTTCG | ACAATGTCGTCATGCCTGCCCTG |

**Conclusion**

The present study is the first to demonstrate that under hypoxic conditions Ewing’s sarcoma tumor cells transdifferentiated into pericytes that contribute to tumor vessel formation. As these ES-derived pericytes were seen in both xenograft and patients’ tumors in the hypoxic areas, we conclude that the hypoxic tumor microenvironment triggers ES tumor cell conversion into pericytes. These novel findings suggest that an additional therapeutic approach may involve blocking this conversion to inhibit the ES cells from contributing to the new pericyte pool that is required for tumor vascular expansion, tumor growth and metastasis.

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**Conflicts of Interest**

No conflicts of interest were disclosed.

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