CD133 Regulates IL-1β Signaling and Neutrophil Recruitment in Glioblastoma

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http://dx.doi.org/10.14348/molcells.2017.0089
www.molcells.org

CD133, a pentaspan transmembrane glycoprotein, is generally used as a cancer stem cell marker in various human malignancies, but its biological function in cancer cells, especially in glioma cells, is largely unknown. Here, we demonstrated that forced expression of CD133 increases the expression of IL-1β and its downstream chemokines, namely, CCL3, CXCL3 and CXCL5, in U87MG glioma cells. Although there were no apparent changes in cell growth and sphere formation in vitro and tumor growth in vivo, in vitro trans-well studies and in vivo tumor xenograft assays showed that neutrophil recruitment was markedly increased by the ectopic expression of CD133. In addition, the clinical relevance between CD133 expression and IL-1β gene signature was established in patients with malignant gliomas. Thus, these results imply that glioma cells expressing CD133 are capable of modulating tumor microenvironment through the IL-1β signaling pathway.

Keywords: CD133, glioblastoma, IL-1β signaling, neutrophil, U87MG glioma cell

INTRODUCTION

Glioblastoma (GBM) is a highly heterogeneous malignancy with aberrant regulation of cellular proliferation and differentiation (Omuro and DeAngelis, 2013). During pathological and therapeutic procedures, cancer cells continuously acquire diverse traits that help in maintaining their superior properties enabling vigorous proliferation and resistance against different forms of stress (Zahreddine and Borden, 2013). Especially, glioma stem cells (GSCs) residing in brain tumors can resist many therapeutic approaches and can modulate the tumor microenvironment by themselves (Eramo et al., 2006; Lathia et al., 2015). Although GSCs can be detected by various markers (Brescia et al., 2012), exact identification of these cells remains difficult. Despite these limitations, there is a growing interest in this specific tumor subpopulation as a useful means of targeted therapy (Chen et al., 2013; Kaiser, 2015).

CD133, a surface marker of normal stem cells, is widely used as a marker for GSCs (Lathia et al., 2015). Until now, several studies dealing with GSCs have concentrated on investigating distinct characteristics between CD133+ and CD133− cells and the proportional effect of CD133+ cells, rather than the molecular functions of CD133 (Pallini et al., 2011; Zeppernick et al., 2008). Recently, it was established that CD133+ stem cells, which possess generative capacity and mutational events, represent a major intrinsic cancer risk in multiple organs (Zhu et al., 2016). However, the precise mechanism of tumorigenesis influenced by CD133 should be elucidated.

Meanwhile, a number of studies showed the biological and molecular functions of CD133 by loss-of-function studies. For instance, depletion of CD133 in patient-derived CD133+ tumorspheres led to a decrease in the self-renewal capacity and tumorigenic potential (Brescia et al., 2013). The tyrosine residue located in the C-terminal of the CD133 protein interacts with the phosphoinositide 3-kinase (PI3K)
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regulatory subunit p85, and activates PI3K-AKT signaling in GSCs (Wei et al., 2013). These results indicate that CD133 functions not only as a biomarker of GSCs, but also as a molecule assigning unique characteristics to the GSCs. However, there are few gain-of-function studies involving CD133 from glioma cells. Therefore, in the present study, we established a U87MG glioma cell line ectopically expressing CD133 and examined its function relative to cancer progression.

MATERIALS AND METHODS

Cell culture and reagents
A glioma cell line, U87MG, was purchased from the American Type Culture Collection (ATCC, USA), and a human promyeloblast, HL-60, was gifted from Dr. Taehoon Chun (Korea University, Korea). All established glioma cell lines were maintained in Dulbecco's modified Eagle's medium (Lonza, Switzerland) supplemented with 10% fetal bovine serum, maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Lonza, Switzerland) supplemented with 1% penicillin-streptomycin, and 14 mL of 1M HEPES (HyClone™; GE Healthcare Sciences, USA). After serum starvation, the cells were treated with 10 ng/mL recombinant human IL-1β (BD Biosciences, USA) at different time intervals (12, 4, and 1 h). Cells were harvested at the same time and analyzed.

Plasmids, transfection, and lentivirus infection
Plasmids for CD133 were gifted by Dr. Young-Gyu Ko (Korea University). Third-generation lentiviral packaging vectors were transfected with PolyExpress™ (Excellgen Inc., USA) in HEK-293FT (Invitrogen, USA). Lentiviruses were concentrated with Lenti-X™ Concentrator (Clontech, Japan). U87MG cells were infected with the lentivirus produced from HEK-293FT.

To perform siRNA-mediated knockdown of IL-1β gene, two siRNAs (SASI_Hs01_00028205 and SASI_Hs02_00302835; Sigma Aldrich) were transfected in U87MG cells by using ScreenFect™-A (Wako Pure Chemical Industries, Japan), according to the manufacturer's instructions. Cells were harvested 48 h after transfection.

RNA-seq analysis
For transcriptome analysis, both U87MG-control and U87MG-CD133 cells were harvested using TRizol™ reagent (Eppendorf-5prime, USA) in 3 sets each. RNA-seq analysis was performed by Beijing Genomics Institute (BGI, China). The analyzed raw fragments per kilobase million (FPKM) data were further processed for sorting differentially expressed genes (DEGs). DEGs were defined as genes that were expressed 2 folds higher or lower in U87MG-CD133 than in U87MG-control. The significance of DEGs was calculated using probability (P) values. Data are deposited in GEO (accession GSE99385).

Patient dataset analysis and gene set enrichment analysis (GSEA)
Microarray datasets from TCGA or REMBRANDT database of the National Cancer Institute (http://caintegrator-info.nci.nih.gov/rembrandt) were classified into "IL-1β signature-high" and "IL-1β signature-low" according to the mean ± standard deviation (SD) value of the enrichment score developed using single-sample GSEA (ssGSEA: http://software.broadinstitute.org/cancer/software/gene-pattern#). IL-1β signature was derived from GSE74220, which consists of DEGs at 2-fold high expression levels as determined by comparing the mean expression values (Student's t-test: p < 0.05) (Pearson et al., 2016). GSEA (https://www.broadinstitute.org/gsea/), a knowledge-based approach for interpreting genome-wide expression profiles, was performed as described previously (Subramanian et al., 2005).

Western blotting
For western blotting, whole cell extracts were prepared with radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, and 50 mM Tris (pH 7.4)) containing 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM NaF, 1 mM Na3VO4, and protease inhibitor (Roche). Proteins were quantified using the Bradford assay reagent (Bio-Rad, USA), according to the manufacturer's instructions. They were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat milk and incubated with the following antibodies: anti-CD133 (Miltenyi Biotec; 1:10000) and anti-β-actin (Santa Cruz; 1:10000).

Quantitative reverse transcription-PCR (qRT-PCR)
qRT-PCR was performed to determine mRNA levels. Total RNA was isolated using TRizol™ reagent, according to the manufacturer's instructions. Total RNA was used as a template to synthesize cDNA by using RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific, USA). qRT-PCR analysis was performed on an iCycler IQ real-time detection system (Bio-Rad) by using the IQ Supermix with SYBR-Green (Bio-Rad). Primer sequences for qRT-PCR amplification are available in Supplementary Table 1.

Cell growth assay
For comparing cell growth, 0.15 × 10^3 cells were plated in 24-well plates and monitored by IncuCyte ZOOM™ (Essen BioScience Inc., USA). Time-lapse phase-contrast images were obtained every 4 h. Cell confluence was analyzed by the software provided with IncuCyte ZOOM™.

Limiting dilution assay (LDA)
For LDA, the indicated number of cells per well was seeded in 96-well plates, and each well was examined for the formation of tumor spheres. Stem cell frequency was calculated using the software available at http://bioinf.wehi.edu.au/software/elda.

Trans-well assay
For invasion assays, the upper chambers of the 24-well trans-well plates (Corning Costar, USA) were coated with
Matrigel (BD Biosciences) and incubated at 37°C for 3 h. The opposite side of the upper chambers was coated with 0.2% gelatin. We then added $5 \times 10^5$ BV2 microglial cells to each upper chamber. For the neutrophil migration assay, $1 \times 10^5$ dHL-60 neutrophil-like cells (derived from the differentiation of HL-60 cells after a 72-h-long culture in RPMI-1640 medium supplemented with 1.25% dimethyl sulfoxide) were added to each upper chamber. The lower chambers contained DMEM- or RPMI-1640-based conditioned medium from U87MG-control and U87MG-CD133 glioma cells. The plates were incubated at 37°C and 5% CO₂ for 24 h. For enumerating invaded microglia, the medium in the upper chambers was removed, and the filter was stained with crystal violet. dHL-60 cells beneath the upper chambers were counted microscopically.

In vivo mouse xenograft

For intracranial implantation, $10^5$ of U87MG-control as well as U87MG-CD133 cells were stereotactically injected into the brain of nude mice (BALB/c nu/nu; coordinates: 2 mm right of the bregma).

Immunofluorescence and immunohistochemistry assays

For both immunofluorescence and immunohistochemistry experiments, the paraffin-embedded sections were cleared, and the sections were incubated in 10 mM sodium citrate (pH 6.0) for antigen retrieval. For endogenous peroxidase blocking, 3% H₂O₂ in methanol was used. After washing, they were further blocked with 3% ProBumirin® (EMD Millipore, USA). Samples were incubated with the following antibodies: anti-CD133 (Miltenyi Biotec; 1:200), anti-Ly6G (BD Biosciences; 1:200) or anti-Iba1 (WAKO; 1:200). All sections were examined by optical and fluorescence microscopy (Zeiss).

Statistical analysis

All data were analyzed by student’s t-test and reported as the mean ± standard error of the mean (SEM). For statistical analysis of patient dataset, log-rank (Mantel-Cox) test and unpaired t-test were used and analyzed by using GraphPad Prism. Differences were considered to be significant if the p-value was less than 0.05.

RESULTS

Ectopic expression of CD133 induces cytokine and chemokine expression in U87MG glioma cells

Many studies have reported the correlation between CD133

Fig. 1. CD133 is highly expressed in the patients with glioblastoma (GBM) and induces the expression of IL-1β-responsive genes in U87MG glioma cells. (A) CD133 mRNA expression levels were analyzed using The Cancer Genome Atlas (TCGA) database. Patient number is indicated on the x-axis: normal (n = 10), oligodendroglioma (n = 191), astrocytoma (n = 194), oligoastrocytoma (n = 130), and GBM (n = 152). ***indicates p < 0.001. (B) The transcriptomes of U87MG-control and U87MG-CD133 glioma cells were obtained by RNA-seq analysis (n = 3). Differentially upregulated genes (DEGs) are shown on the heat map. Normalized gene expression of each DEG was calculated based on the common logarithm of fragments per kilobase million (FPKM). Among the DEGs, IL-1β-responsive genes were shown in the table. (C) Gene ontology analyses are categorized by molecular function (GOTERM_MF), cellular component (GOTERM_CC), biological process (GOTERM_BP), and Kyoto Encyclopedia of Genes and Genomes pathway (KEGG_PATHWAY). Asterisks indicate gene ontology terms that are statistically significant, as decided by the Benjamini-adjusted p-value (Benjamini p < 0.01). (D) Gene set enrichment analysis (GSEA) of IL-1β treatment-induced gene signature in the U87MG-control (Con) and U87MG-CD133 (CD133) glioma cells. NES, normalized enrichment score; FDR q-value, false discovery rate q-value.
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expression level and glioma aggressiveness (Han et al., 2016; Zeppernick et al., 2008). To confirm the clinical significance of CD133 in gliomas, we compared the expression level of CD133 in patients with different types of glioma by using The Cancer Genome Atlas (TCGA) database. The CD133 mRNA level is most significantly elevated in GBM (Fig. 1A). Therefore, to elucidate whether glioma malignancy is associated with the biological function of CD133, we ectopically expressed CD133 in U87MG cells (U87MG-CD133) and examined the transcriptome by RNA-seq analysis. Consequently, we found that the genes upregulated by CD133 expression are IL-1β, CCL3, CXCL3, CXCL5, and PI3 (Fig. 1B). We also found that only IL-1β and PI3 expressions were significantly associated with poor survival of patient with GBM (data not shown). As anticipated, gene ontology analysis using DAVID function annotation also revealed that these genes are related to chemokine receptor binding and cytokine-cytokine receptor pathway (Fig. 1C). Many studies showed that CXCL3 plays a crucial role in maintaining the properties of CD133+ GSCs (Zhang et al., 2016), and that inflammatory cytokines such as IL-1β, IL-6, and IL-8 are involved in the pathological processes of gliomas (Yeung et al., 2013). Such cytokines or chemokines are secreted from not only inflammatory cells, but also cancer cells per se, and they function as signaling molecules, altering gene expression in an autocrine or paracrine manner. In particular, gene set enrichment analysis (GSEA) revealed that IL-1β downstream genes are significantly enriched in U87MG-CD133 cells (Fig. 1D). Taken together, these results indicate that the genes upregulated by CD133 overexpression might be associated with IL-1β-induced responses.

**IL-1β signaling regulates the genes upregulated by CD133 overexpression in U87MG glioma cells**

Next, the expression levels of DEGs, including IL-1β, CCL3, CXCL3, CXCL5, and PI3, in U87MG-CD133 cells compared to U87MG-control cells were analyzed by quantitative reverse transcription-PCR (qRT-PCR) to validate the results of RNA-seq analysis. We first confirmed increased CD133 protein expression in the U87MG-CD133 cells by Western blot analysis (Fig. 2A). qRT-PCR showed that the mRNA expression of all the above-mentioned genes increased in U87MG-CD133 cells (Fig. 2B). Because the gene set regulated by IL-1β treatment was significantly enriched in U87MG-CD133 cells (Fig. 1D), we assessed whether those DEGs are regulated by IL-1β treatment. qRT-PCR analysis showed that the mRNA levels of DEGs are elevated by treatment with recombinant human IL-1β (Fig. 2C). To further examine whether DEGs are directly regulated by IL-1β, IL-1β was depleted by using small interfering RNA (siRNA). The results showed that

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**Fig. 2. CD133 and DEGs are induced by IL-1β treatment and are enriched in the tumor necrotic area.** (A) CD133 and β-actin protein levels in the U87MG-control and U87MG-CD133 glioma cells were determined by western blot analysis. β-Actin was used as the loading control. (B) IL-1β, CCL3, CXCL3, CXCL5, and PI3 (DEGs) mRNA levels in the U87MG-control and U87MG-CD133 glioma cells were examined by qRT-PCR. * indicates p < 0.05; ** indicates p < 0.01. Data are expressed as the mean ± standard error of the mean (SEM). (C) mRNA levels of DEGs in the U87MG glioma cells were examined by qRT-PCR at indicated times after treatment with recombinant human IL-1β. ** indicates p < 0.01. Data are expressed as the mean ± SEM. (D) mRNA levels of DEGs in the U87MG-control and U87MG-CD133 glioma cells, which were transfected with IL-1β siRNA, were examined by qRT-PCR. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001. Data are expressed as the mean ± SEM. (E) DEGs and CD133 mRNA levels in different histological regions of GBM tissues. Normalized gene expression was shown on the heatmap calculated by using the z-score. Bar graph represents each regional gene expression by analyzing log2 intensity generated by in situ hybridization in Ivy Glioblastoma Atlas Project database. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001. Data are expressed as the mean ± SEM.
knockdown of IL-1β markedly decreased the mRNA expression of DEGs (Fig. 2D). Taken together, these results indicate that CD133 upregulates IL-1β and its downstream genes.

**CD133 and IL-1β and its downstream genes are enriched in necrotic regions**

As the expression of cytokines, chemokines, and their receptors is often altered by inflammatory responses in the perivascularure or necrotic area (Rempele et al., 2000), we determined the tumor-specific expression of DEGs by using the Ivy Glioblastoma Atlas Project clinical and genomic database, which provides transcriptional profiles of histologically characterized regions of GBM tissue specimens (Sunkin et al., 2013). Compared with other regions, the transcription levels of DEGs are significantly higher in the perinecrotic zone and the pseudopalisading cells around the necrotic region (Fig. 2E). These results suggest that the CD133-IL-1β signaling axis plays an important role in the confined region of the GBM tissue such as the necrotic area.

**CD133 increases the recruitment of neutrophils in vitro and in vivo**

Because CD133 is one of the potent markers of cancer stem cells (CSCs), there is ample evidence for the proliferative and self-renewal properties of CD133+ cells in various types of cancers (Klonisch et al., 2008; Wu and Wu, 2009).

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Fig. 3. CD133-induced IL-1β signaling increases neutrophil recruitment both in vitro and in vivo. (A) The growth rate of U87MG-control and U87MG-CD133 cells was determined by monitoring cell confluence. ns, no significance (n = 3). (B) The tumorsphere-forming ability of U87MG-control and U87MG-CD133 cells was examined by the limiting dilution assay. Included are the representative images showing the tumorspheres of U87MG-control and U87MG-CD133 cells. Scale bar represents 5 μm. ns, no significance (n = 24). (C) Immunofluorescence images showing CD133 expression in the tumors derived by orthotopic injection of U87MG-control and U87MG-CD133 glioma cells. Scale bar represents 50 μm. (D) Representative images showing the tumor size within mouse brain injected with U87MG-control and U87MG-CD133 glioma cells (n = 3). Scale bar represents 1 mm. (E) Representative immunohistochemistry images of Iba1+ microglia in tumor samples derived by orthotopic injection of U87MG-control and U87MG-CD133 glioma cells. Scale bar represents 50 μm (left panel). Quantitative data showing the ratio of Iba1+ microglia in each tumor sample (right panel). (F) Representative immunohistochemistry images of Ly6G+ neutrophils in the tumor samples derived by orthotopic injection of U87MG-control and U87MG-CD133 glioma cells. Scale bar represents 50 μm (left panel). Quantitative data showing the ratio of Ly6G+ neutrophils in each tumor sample (right panel). (G) Microglial invasion after stimulation with the conditioned medium obtained from U87MG-control and U87MG-CD133 glioma cells was examined by an in vitro trans-well assay. ns, no significance (n = 3); CM, conditioned medium. (H) Migration of neutrophil-like cells after stimulation with the conditioned medium obtained from U87MG-control and U87MG-CD133 glioma cells was examined by an in vitro trans-well assay. ** indicates p < 0.01 (n = 3); CM, conditioned medium.
To investigate the phenotypical changes arising from CD133 overexpression, we examined cell growth and tumorsphere-forming ability in U87MG-control and U87MG-CD133 glioma cells. Unlike previous studies showing the endogenous functions of CD133 by the loss-of-function mechanism (Park et al., 2015; Wei et al., 2013), we found no significant changes in cell growth (Fig. 3A) and tumorsphere formation (Fig. 3B) caused by CD133 overexpression.

Next, to identify the tumorigenic potential of CD133, we developed an orthotopic xenograft model by injecting U87MG-control and U87MG-CD133 glioma cells into immunodeficient mice. Immunofluorescence analysis showed that CD133 expression was obviously increased in the tumors derived from U87MG-CD133 cells (Fig. 3C), but there was no apparent difference in the tumor size between the two groups (Fig. 3D). This result indicates that CD133 overexpression did not benefit tumor growth in vivo.

Because the DEGs upregulated by CD133 overexpression are associated with IL-1β signaling, chemokine-receptor binding, and interaction between cytokines and receptors (Figs. 1C and 1D), and CD133 and the DEGs are enriched in the necrotic area of GBM tissue (Fig. 2E), we examined the histological changes in the xenograft tumors of both groups, in particular the infiltration of microglia (Iba1+) and neutrophils (Ly6G+) into the tumors, by immunohistochemistry analysis. The results showed that while the number of microglia infiltrated did not change (Fig. 3E), the number of neutrophils surrounding the necrotic region was significantly higher in the tumors derived from the U87MG-CD133 cells (Fig. 3F). These results suggest that CD133 overexpression influences the recruitment of neutrophils to the necrotic area, implicating that CD133+ cells may play a role in modulating the tumor microenvironment.

Next, to ascertain the effect of CD133 overexpression on inflammatory cell infiltration, we attempted the microglia invasion and neutrophil migration assays by using the conditioned medium from U87MG-control and U87MG-CD133 glioma cells. The results showed that CD133 overexpression did not induce the invasive ability of microglia (Fig. 3G), but increased neutrophil migration (Fig. 3H). Taken altogether, our studies showed that the ectopic expression of CD133 is not associated with cellular proliferation and self-renewal, but is implicated in neutrophil migration both in vitro and in vivo.

**Clinical relevance of CD133 and IL-1β signaling**

To evaluate the clinical relevance of CD133 and the IL-1β signaling axis, we interrogated TCGA and the Repository of Molecular Brain Neoplasia Data (REMBRANDT) datasets. We first classified two groups of patients with gliomas on the basis of the expression level of IL-1β treatment-induced gene signature, and then analyzed CD133 expression in these groups. Both datasets showed that CD133 expression is significantly higher in patients with gliomas with IL-1β signature-high (Fig. 4A). Furthermore, patients with gliomas with IL-1β signature-high have worse prognosis compared to those with IL-1β signature-low (Fig. 4B). Taken together, these results suggest that the CD133-IL-1β signaling axis might contribute to glioma aggressiveness.

**Fig. 4. IL-1β signature is correlated with CD133 expression and poor prognosis in patients with gliomas.** (A) CD133 mRNA expression levels were examined in patients with gliomas with IL-1β signature-high or IL-1β signature-low. Two groups of patients with gliomas were divided by the mean ± 1 (log2-value) of the differential expression levels of IL-1β treatment-induced gene signature. Sample datasets are obtained from TCGA (upper panel) and REMBRANDT (lower panel). *** indicates p < 0.001. (B) The survival of patients with gliomas with IL-1β signature-high or IL-1β signature-low was analyzed using TCGA (upper panel) and REMBRANDT datasets (lower panel). ** indicates p < 0.01; *** indicates p < 0.001.
DISCUSSION

We delineate that the ectopic expression of CD133 activates IL-1β and its downstream gene expression in the U87MG glioma cell line. Although we did not demonstrate precise mechanisms underlying the activation of IL-1β signaling by CD133, the CD133-IL-1β regulatory axis increased chemokine expression and neutrophil recruitment in vitro in cell cultures and in vivo in xenograft tumors.

Various intracellular and extracellular factors are known to regulate CD133 expression. Among them, hypoxia promotes the expansion of CD133+ GSCs by means of the hypoxia-inducible factor-1 (HIF-1) (Soeda et al., 2009). In the U251MG glioma cell line, mitochondrial dysfunction induced by treatment with rotenone, a mitochondrial complex I (NADH dehydrogenase) inhibitor, led to an increase in CD133 expression (Griguer et al., 2008). Our results representing the region-specific expression of CD133 in areas adjacent to the necrotic area (Fig. 2E) are implicated in hypoxic response, because hypoxia is predominantly induced in such necrotic regions (Brat et al., 2004). Therefore, these results indicate that the proportion of CD133+ GSCs or CD133 gene expression can be controlled by environmental factors.

A previous study showed that the quantitative amount of IL-1β is higher in the GBM tissues than in normal tissues, and that IL-1β signaling increases the transcriptional activity of HIF-1α in a Ras- and Wnt-1-dependent manner (Sharma et al., 2011a). Chronic IL-1β exposure induces COX-2 expression, which supports the self-renewal property of GSCs derived from the U87MG glioma cell line (Sharma et al., 2011b). These results indicate that the inflammatory response mediated by IL-1β can enhance the stemness property of glioma cells and modulate their ability to adjust to hypoxia. However, in the present study, we observed that IL-1β signaling activated by CD133 did not influence the proliferation, which supports the self-renewal property of GSCs (DeAngelis, 2011a). Chronic IL-1β and HIF-1α in a Ras- and Wnt-1-dependent manner (Sharma et al., 2011a). Pseudopalisades in glioblastoma are hypoxic, express extracellular matrix proteases, and are formed by an actively migrating cell population. Cancer Res. 64, 920-927.

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