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Tumor Angiogenesis: A Focus on the Role of Cancer Stem Cells

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Abstract

Angiogenesis is the process of growth of new blood vessels. Tumor angiogenesis plays pivotal roles in tumor development, progression, and metastasis. The conventional notion of tumor vasculature is that new tumor blood vessels sprout from preexisting vasculature near the tumor; hence, tumor endothelial cells are derived from normal endothelial cells. However, recent evidence suggests that CD133-positive cancer stem cells (CSCs) in glioblastomas generate tumor endothelial progenitor cells, which further differentiate into tumor endothelial cells. This chapter offers an overview of current knowledge on the role of CSCs in tumor angiogenesis. Furthermore, we discuss our recent discoveries related to human hepatoblastoma stem cells. Future efforts to elucidate the characteristics of tumor angiogenesis should enable the development of effective new anti-angiogenic therapies.

Keywords: tumor angiogenesis, cancer stem cells, hepatoblastoma, cell culture

1. Introduction

Angiogenesis is an essential process by which new blood vessels are formed. In malignancies, tumor growth and metastasis are angiogenesis dependent. It is widely accepted that new tumor blood vessels sprout from preexisting vasculature. Therefore, tumor endothelial cells are considered to be derived from normal endothelial cells. However, over the last few years, different processes by which tumor vascularization occurs have been documented. It has been shown that endothelial cells in the tumor vasculature arise from a small population of tumor cells known as cancer stem cells (CSCs) or tumor-initiating cells. Since John Dick and others identified leukemia cells in 1994 [1], the presence of CSCs with similar properties to normal stem cells has been discovered. CSCs have the ability to undergo self-renewal and differentiation into diverse cancer cells and are capable of becoming malignant. CD133 (or

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Prominin1), a cell surface glycoprotein used widely as a marker for normal stem cells, is additionally recognized as a marker for CSCs. Recent reports suggest that CD133-positive CSCs in glioblastomas generate tumor endothelial progenitor cells, which further differentiate into tumor endothelial cells [2–4].

What is the origin of endothelial cells and pericytes lining tumor blood vessels? Can CSCs give rise to tumor endothelial cells? The origin of tumor endothelial cells is the main focus of this chapter. We will provide an overview of current studies and discuss the role of CSCs in tumor angiogenesis. We have investigated the relationship between tumor endothelial cells and CSCs in human hepatoblastoma, which is the most frequent type of malignant tumor to occur in the pediatric liver. Our findings are reported in this chapter.

2. Characteristics of tumor blood vessels

The field of angiogenesis research arose following a publication by Folkman in 1971. The future of anticancer therapy was emphasized in that the potential utility of angiogenesis inhibitors against cancer was identified. The term "anti-angiogenesis" was proposed by Folkman's research group to refer to the inhibition of new vessel sprouts from penetrating into an early tumor implant [5].

Tumor endothelial cells lining the inner layer of blood vessels are the main targets of anti-angiogenic therapy. It is believed that new tumor vessels generally sprout from preexisting vasculature; accordingly, new tumor vessels are considered structurally and functionally normal. However, recent studies have reported that tumor blood vessels differ morphologically and phenotypically from normal blood vessels [6]. A common feature in the architecture of the normal vasculature is a hierarchical and regular branching pattern. In contrast, tumor blood vessels are structurally distinct from normal blood vessels. Tumor endothelial cells, which are not comprised of regular monolayers, do not function as a normal barrier [7]. The pericytes form abnormally loose associations with these cells and extend cytoplasmic processes deep into the tumor tissue [8]. An inner layer of tumor blood vessels is composed of a specific phenotype of tumor-associated blood endothelial cells. Furthermore, the tumor endothelial cells are heterogeneous according to the malignancy status of tumor [9].

3. Tumor-specific angiogenesis

3.1. Cancer stem cells (CSCs) or tumor-initiating cells

The American Association for Cancer Research (AACR) defined CSCs as subpopulations of cells within a tumor that possess the capacity for self-renewal and generation of heterogeneous lineages of cancer cells that constitute the tumor [10].

The cancer stem cell theory provides an attractive explanation for tumor proliferation and progression. According to this theory, tumors retain subsets of cells with functional heterogeneity. However, the putative relationship between CSCs and tumor angiogenesis remains poorly understood [11].

3.2. The origin of the endothelial cells in the tumor vasculature

The origin of endothelial cells in the tumor vasculature is not yet known [12]. Some studies suggest that CSCs play an important role in tumor vascularization. The tumor stem cells defined as CSCs or tumor-initiating cells are considered the source of tumor cells. Moreover, novel findings, which suggest that CSCs also give rise to endothelial cells in the tumor vasculature, have been described in recent reports [2–4]. A proportion of endothelial cells that contribute to blood vessels in glioblastomas originate from the tumor itself, having differentiated from CSCs. A subset of endothelial cells that constitute tumor vessels carries genetic aberrations found in the tumor cells themselves. It has been shown that a glioblastoma cell population that could differentiate into endothelial cells and form blood vessels was enriched in cells expressing the tumor stem cell marker CD133.

3.3. The origin of pericytes in the tumor vasculature

Pericytes are mural cells that wrap around the endothelial cells of capillaries and venules. Vascular pericytes play important roles in supporting vascular structure and function. As communication between pericytes and endothelial cells has been demonstrated to occur, it is considered that pericytes may prove a novel target for tumor therapy [13]. A recent study has reported that glioblastoma stem cells give rise to vascular pericytes that support vessel function and tumor growth. These results suggest that CSCs from glioblastomas generate the majority of vascular pericytes [14].

4. Tumor angiogenesis via endothelial differentiation of human hepatoblastoma stem cells

We have investigated the relationship between tumor endothelial cells and CSCs in human hepatoblastoma, which is the most frequent type of malignant tumor that occurs in the pediatric liver.

4.1. Characteristics of human hepatoblastoma cells

4.1.1. CD133

CSCs exhibit specific cell membrane markers; CD133 is considered a stem-like cell marker in various cancers [15–17]. In human hepatocellular carcinoma (HCC) and HCC cell lines, CD133 is expressed by only a minority of the tumor cell population. CD133+ cells exhibit the ability to self-renew, produce differentiated progenies, and form new tumors [18, 19].

4.1.2. Cell culture

Human hepatoblastoma cell line (HuH-6 clone 5, well-differentiated type, JCRB0401) was procured from the Health Science Research Resources Bank (Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 μg/mL gentamicin, according to a previously described protocol [20].

4.1.3. SEM and TEM

For SEM observation, the samples were fixed in 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde for 1 h and subsequently fixed in 0.1 M phosphate buffer (pH 7.2) containing 1% OsO_4 for 1 h, dehydrated in graded ethanol, and critical-point air-dried after treatment with isoamyl acetate. The samples were sputter-coated with OsO_4 and observed under a SEM (Hitachi, S-4800; Tokyo, Japan) [21].

For TEM, the cells were fixed in 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde for 1 h, followed by fixation in 0.1 M phosphate buffer (pH 7.2) containing 1% OsO_4 for another hour. The specimens were dehydrated in graded ethanol, embedded in epoxy resin, cut into ultrathin sections, and stained with uranyl acetate and lead citrate. The stained ultrathin sections were observed under a TEM (JEM-1010; Tokyo, Japan) [21].

4.1.4. Distribution of CD133 in human hepatoblastoma cells

We investigated CD133 distribution in human hepatoblastoma cells. CD133 was mainly localized in membrane ruffles in the peripheral regions of the cell. Examination of the CD133-positive sites using SEM revealed that they coincided with filopodia and lamellipodia. TEM revealed that CD133 was preferentially concentrated in a complex structure formed by filopodia and lamellipodia [21] (**Figure 1**).

4.2. Isolation and identification of human hepatoblastoma stem cells

A key challenge in the study of CSCs is the development of reproducible and reliable methods for CSC isolation and identification. The side population (SP) assay identifies the fraction of cells that efflux Hoechst dye through ATP-binding cassette (ABC) transporters. We identified hepatoblastoma stem cells based on their ability to efflux Hoechst 33342 dye using flow cytometry, as described in Hayashi et al. [22]. A fraction of SP cells was analyzed by flow cytometry (FACS Vantage SE, BD, Tokyo, Japan) (**Figure 2**). SP cells were injected subcutaneously into immunodeficient NOD/SCID mice (male, 4-week-old: Charles River Japan Inc.), and tumor growth was evaluated. All animal experiments were approved by the Institutional Animal Care and Use Committee of Saitama Medical University.

4.3. Sphere formation assay and three-dimensional collagen gel culture system

Digested xenograft tumor fragments were cultured, and tumor sphere assay was carried out. The spheres were cultivated using three-dimensional collagen gel culture methods, referring to the previous reports [23–25]. The spheres were fixed with 4% paraformaldehyde/phosphate buffer saline (PBS) and then embedded in Technovit 8100 (T8100, Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions.

4.4. Immunohistochemical detection of CD133

The location of CD133 expression was examined in Technovit-embedded sections (**Figure 3**). Some spheres were observed to form capillary-like structures (**Figure 4**).

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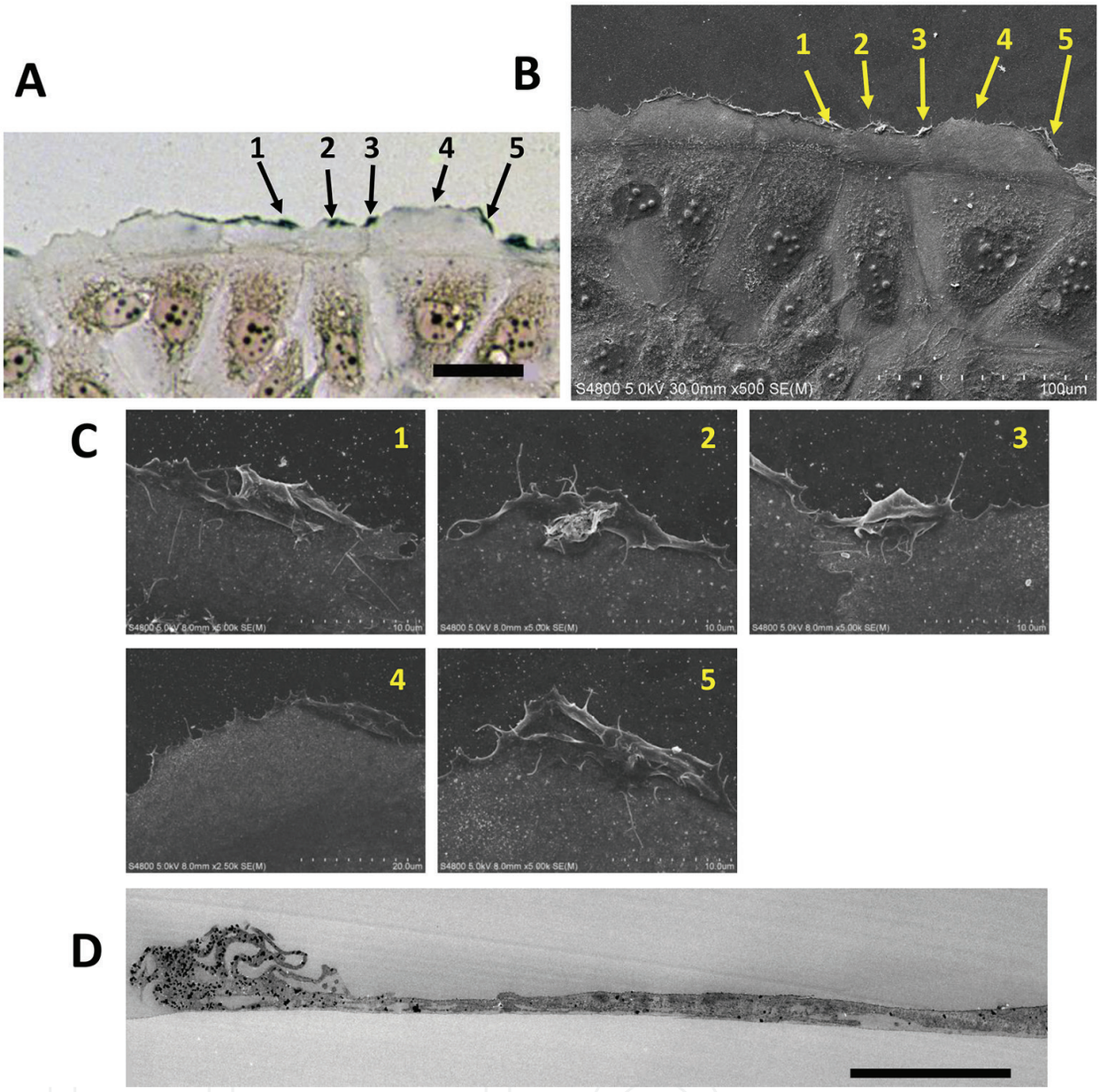

Figure 1. Immunohistochemical analyses of CD133 in hepatoblastoma cells by light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). (**A**) Intense immunolabeling of CD133 was observed at the peripheral regions of cells (arrow numbers 1, 2, 3, and 5) by light microscopy. Nanogold labeling was followed by gold enhancement for 20 min. Arrow number 4 indicated a negative site; scale bar: 50 μm. (**B**) SEM image of the sample shown in **A**; the peripheral region of cells (arrow numbers 1, 2, 3, and 5) coincided with the positive sites observed in **A**. (**C**) Higher magnification of SEM images of the positive sites shown in **B**; the positive sites were composed of a complex structure of filopodia and lamellipodia. (**D**) TEM analysis: CD133 was preferentially concentrated in the complex filopodial structure and at the leading edge of lamellipodia. The clustered particles form black spots; scale bar: 5 μm. (Modified from Akita et al. [21]).

Immunostaining for correlative observation by light microscopy and electron microscopy was performed, according to previously described methods [21], to detect the expression of CD133 in these capillary-like structures. The spheres were incubated with primary anti-CD133 antibody (Santa Cruz Biotechnology, CA). Alexa Fluor 488- and 1.4-nm nanogold-conjugated goat

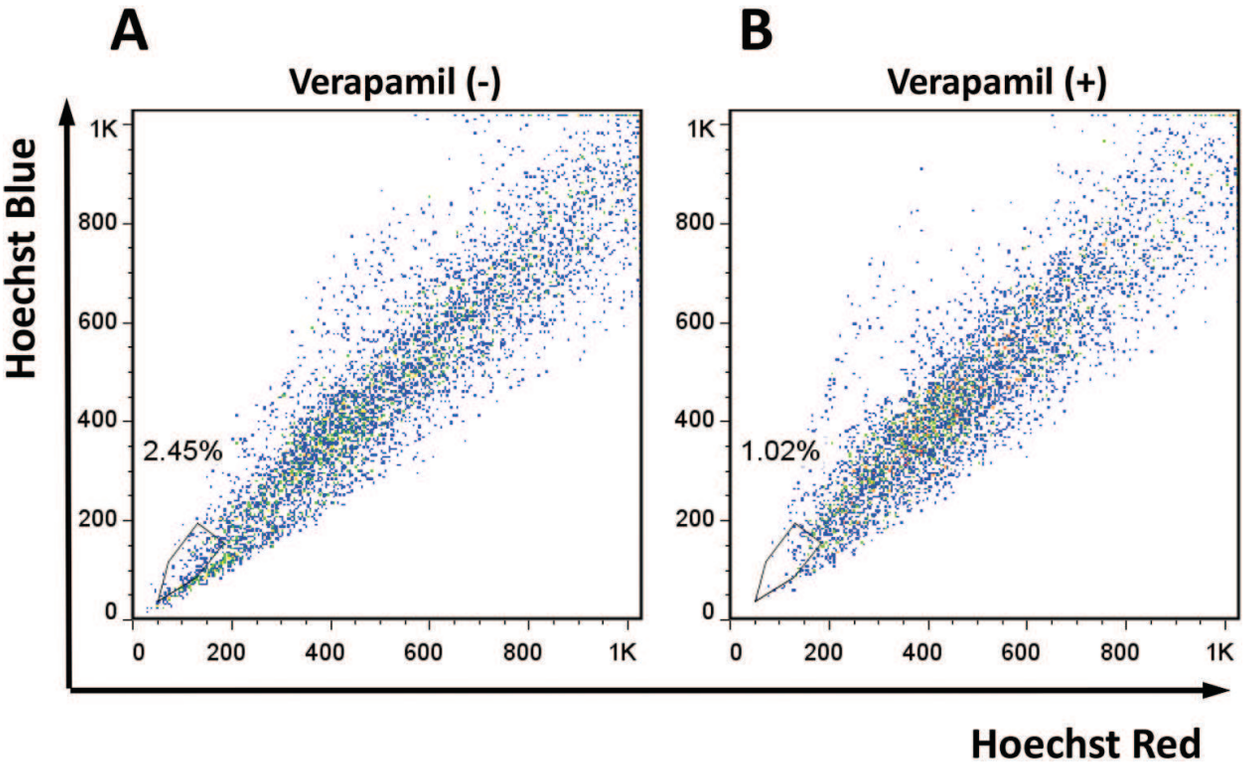

Figure 2. Identification and characterization of side population (SP) cells from a hepatoblastoma cell line. The SP cells in hepatoblastoma cells were identified by flow cytometry using a Hoechst33342-based staining procedure. (**A**) The SP cells displayed in plots show a tail-like subpopulation close to the G0/G1 phase (on the left). Representative images of dot plot analysis by FACS demonstrating the presence of 2.45% SP cells. (**B**) The ABC transporter inhibitor verapamil effectively blocks the export of the Hoechst dye, thus leading to the disappearance of the SP subpopulation. SP cells were reduced to 1.02% upon treatment with verapamil.

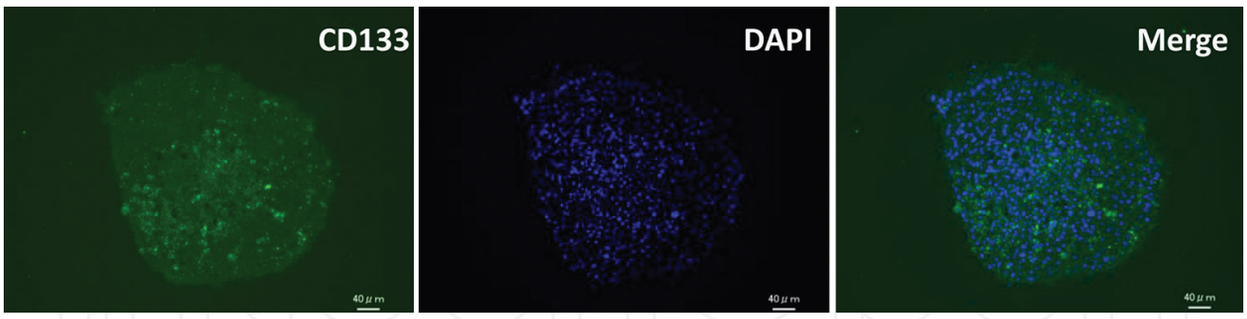

Figure 3. Immunofluorescence of hepatoblastoma tumor sphere. Immunofluorescence of tumor sphere labeled with anti-CD133 antibody (green); nuclei stained with DAPI (blue). The overlaid image is shown in the right panel (merge); scale bar: 40 μm.

anti-rabbit IgG (Nanoprobes, Yaphank, NY, USA) were used as secondary antibodies. The nanogold signal was enhanced using GoldEnhance EM (Nanoprobes) for 20 min. The spheres were analyzed by light microscopy (**Figure 5**, inset). The spheres of interest were subjected to the gold-enlargement procedure using GoldEnhance EM (Nanoprobes, Yaphank, NY, USA) to an appropriate size for TEM analysis, according to the manufacturer's instructions. Some spheres formed CD133-positive capillary-like structures. TEM imaging of these structures confirmed the presence of identifiable lumens (**Figure 5**, bottom).

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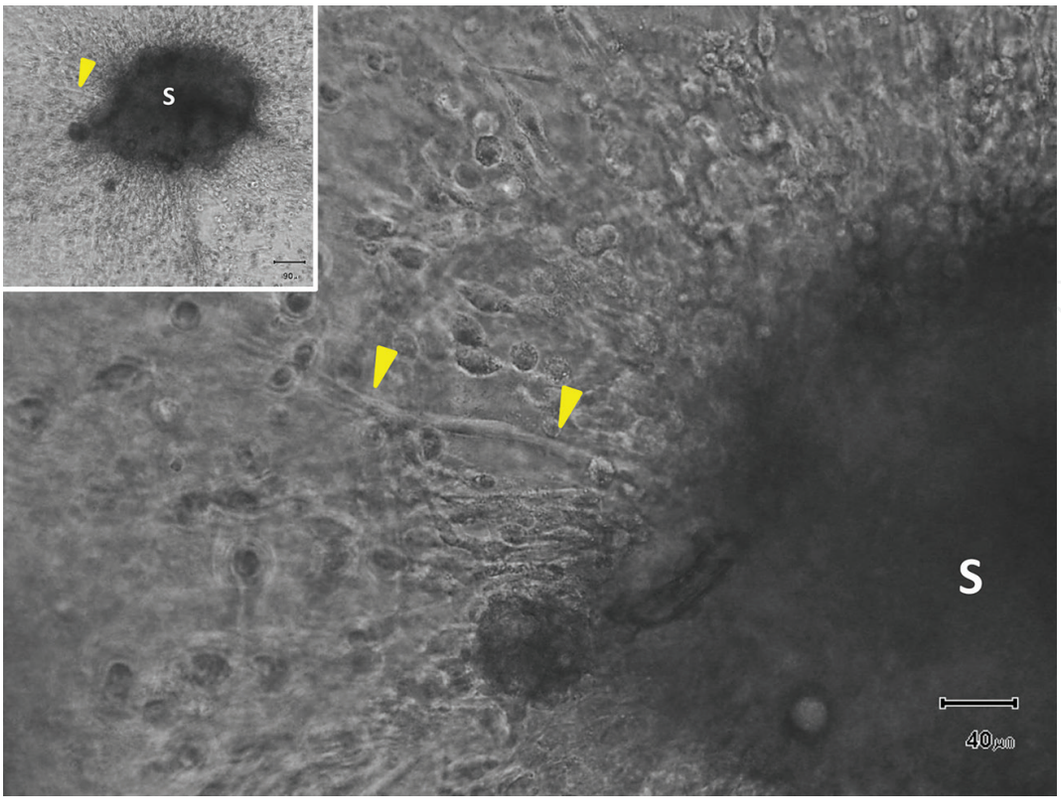

Figure 4. Tube formation assay of hepatoblastoma tumor sphere. Phase-contrast microscopy shows a tubular structure sprouting (arrowheads) from hepatoblastoma tumor sphere (S) in a three-dimensional collagen gel. Higher magnification of the inset indicated bottom; scale bar: 90 μm (inset), 40 μm (bottom).

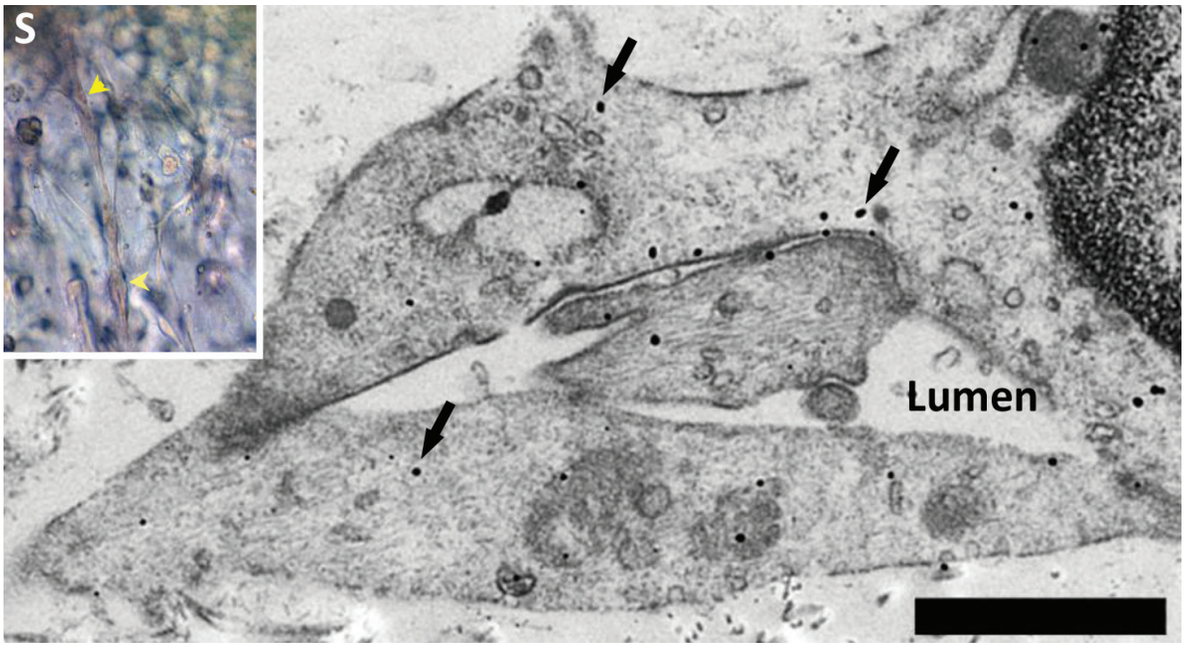

Figure 5. Immunohistochemical detection of CD133 in capillary tubes. (**Inset**) Light microscopy showed that the capillary tube formed from the hepatoblastoma tumor sphere (S) in the collagen gel was positive for CD133 (arrowheads). Nanogold labeling was followed by gold enhancement for 20 min. (**Bottom**) Transmission electron microscopy (TEM) image of the sample shown in the inset; the cross section of this tube is shown. CD133 was detected in the capillary tube. The clustered particles form black spots (arrows); scale bar: 1 μm.

5. Conclusions and perspectives

Tumor angiogenesis has been widely mentioned as a process that new blood vessels are developed from preexisting host blood vessels surrounding the tumors. However, we propose a paradigm change. Our results suggest that CD133-positive CSCs differentiate into tumor vascular endothelial cells and might be able to form tumor vessels.

Since Folkman hypothesized the notion of targeting tumor endothelial cells with anti-angiogenic therapy, numerous anti-angiogenic drugs have been discovered. Previously, we reported that CD133-positive capillary tubes were formed in vitro. Statins, which are widely used as cholesterol-lowering agents, strongly inhibited the capillary tube formation [26]. Statin diminished intraplaque angiogenesis [27] and reduced the growth and spread of many cancers [28, 29]. Khaidakov et al. suggested that statin had anti-angiogenic effects [30]. We propose that the anti-angiogenic effects of statins can be considered for the cancer therapy.

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