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Lab Note

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The isolation and characterization of endothelial cells from juvenile nasopharyngeal angiofibroma

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Juvenile nasopharyngeal angiofibroma (JNA) is an uncommon neoplasm originating between the posterosuperior and the sphenopalatine foramena in the region of the pterygoid canal [1]. Although pathologically benign, JNA has a propensity for local invasion of the nasal cavity, paranasal sinus, pterygopalatine fossa, infratemporal fossa, the orbit, and even the base of the skull [2]. Histologically, JNA is characterized as a non-encapsulated tumor composed of proliferating and irregular vascular component within fibrous stroma [3]. The etiology and pathogenesis of JNA are unknown, but angiogenesis is required for tumor development, and might be a useful therapeutic target. Vascular endothelial cells (ECs) are ideal for the study of angiogenesis, but JNA-derived ECs (JNAECs) have never been successfully isolated and cultured. We previously determined that the presence of $CD105^+$ microvascular endothelium predicts poor patient prognosis following curative resection of JNA [4]. Here, we describe, for the first time, the isolation, culture, passaging, and characterization of the phenotypic and functional properties of $CD105^+$ JNAECs from JNA.

Immunohistochemical staining of sections cut from paraffin-embedded JNA tissue showed strong, specific expressions of CD105, CD34, and von Willebrand factor (vWF) in the vascular endothelium (**Fig. 1A–D**). Samples of JNA tissue obtained from surgical specimens following resection were minced using scissors and digested for 1.5 h at 37°C in high-glucose Dulbecco's minimum essential medium (DMEM) containing 0.1% collagenase IV. After being washed in DMEM containing 10% fetal bovine serum (FBS), cell suspensions were filtered through a graded series of tissue mesh to separate cells from stroma and aggregates. ECs were isolated from single-cell suspensions by magnetic-activated cell sorting (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany) using magnetic beads coupled to anti-CD105 antibody. Cells were grown in complete EBM-2 medium supplemented with 10% FBS (**Supplementary Fig. S1**). Eight clones of $CD105^+$ JECs were obtained from eight patients using this protocol. The cellular yield depended on the amount of tissue in the sample used for isolation, but the minimum amount of tumor tissue to obtain a sufficient number of isolated cells for culturing was 2–3 cm^2. Approximately 5×10^4 $CD105^+$ cells (vascular ECs) could be obtained by MACS from a starting suspension of 1×10^6 cells. The sorted cells were inoculated on CellBIND culture dishes. When the cells reached 90% confluency, they were digested by pancreatin and passaged. **Figure 1E,F** showed that primarily isolated and cultured $CD105^+$ JEC cells had a flat appearance, with abundant cytoplasm and fibriform structures. They grew in a monolayer without the typical cobblestone-like EC morphology. The isolated and cultured $CD105^+$ JECs remained viable for up to 12 passages, and flow cytometric analysis of cell surface protein confirmed that CD105 was still highly expressed in as many as 99.7% of the cells even when passaged to 12 generations (**Fig. 1G**). $CD105^+$ JECs were further characterized by cytofluorimetric analysis for the expressions of many endothelial-specific markers, including CD31, CD34, VEGFR1, and VEGFR2 (**Supplementary Fig. S2**). The expressions of endothelial markers were tested at the first, third, and sixth passages, and similar results were obtained during cell passage, as shown in **Supplementary Table S1**.

To further characterize ECs from JNA tissues, the expressions of endothelial-specific markers including vWF and CD105 were compared with their expressions in human umbilical vein endothelial cells (HUVECs). More than 99% of JECs were positive for vWF (**Fig. 2A, B**), similar to HUVECs, which are established EC lines. In addition, more than 95% of the isolated $CD105^+$ JECs were positive for an uptake of acetylated low-density lipoprotein (Dil-Ac-LDL) (**Fig. 2E,F**). These results confirmed that the cells isolated from human JNA using anti-CD105 antibodies presented typical EC phenotype *in vitro*. More importantly, the $CD105^+$ JECs, like HUVECs, also formed

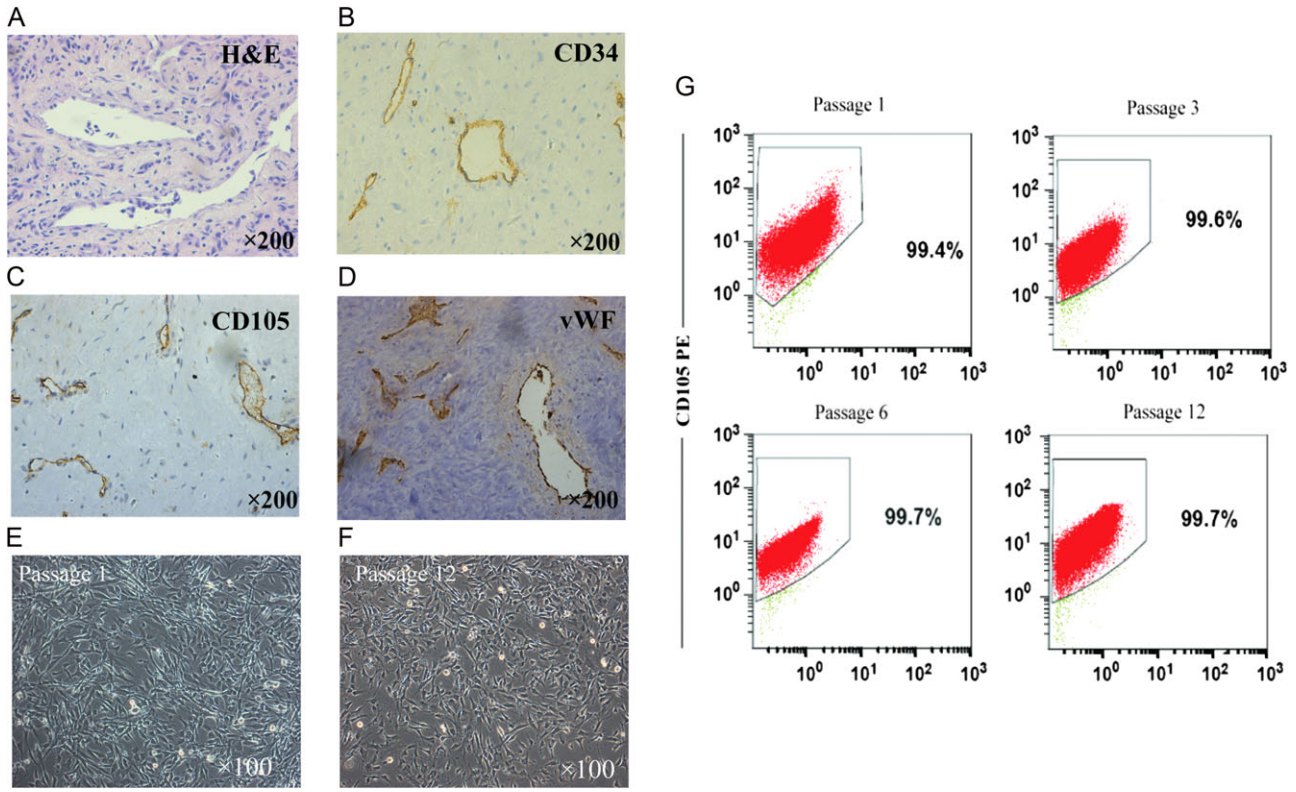

Figure 1. CD105 as a marker for isolation of JNA-derived endothelial cells (ECs) (A–D) Immunohistochemistry on paraffin sections of JNA tissue (original magnification, ×200). Sections of JNA tissue stained with hematoxylin–eosin (A). Strong and specific expression in vessel endothelium was seen for CD34 (B), CD105 (C), and vWF (D). Similar morphology of ECs isolated and cultured from JNA tissue when tested at the first (E) and 12th passages (F) (original magnification, ×100). (G) Representative flow cytometric analysis of JNA-derived endothelial cells (JNAECs) showing expression (more than 99%) of the endothelial marker CD105 is stable for more than 12 passages.

tubes when cultured on matrigel (**Fig. 2G,H**). This characteristic tube formation phenotype indicated that $CD105^+$ JECs were good models for future studies of JNA angiogenesis *in vitro*.

ECs have previously been isolated from tumor tissue and cultured successfully, but not from JNA. The JNAECs isolated and cultured in this study were more than 99% pure, and could be cultured *in vitro* for several passages without apparent loss of their key features. We chose CD105 as the marker for isolation of JNAECs for a number of reasons: (i) immunohistochemical evaluation of tumor tissue showed that CD105 was specifically expressed in vascular structures (**Fig. 1**), but not stromal cells; (ii) CD105 has previously been used to isolate ECs from human hepatocellular carcinoma and malignant human gliomas [5]; and (iii) our previous study showed that CD105 expression by microvascular ECs predicts poor prognosis of patients with JNA after curative resection [4]. Moreover, magnetic bead-based purification (which is directed against an EC-specific epitope) would not only enhance the isolation purity but also facilitate primary culture by ensuring the viability of the isolated ECs.

Angiogenesis supports tumor progression, especially in JNA, which is highly vascularized. Many studies focusing on tumor angiogenesis and EC biology have been based on established cell lines like HUVECs, which offer a reproducible and reliable *in vitro* system [6]. However, it is well documented that tumor and normal vasculature differ both morphologically and functionally [7–9], and the characteristics of ECs from different tissues can be quite different [10]. Thus, only studies with ECs derived from JNA tissue may be able to provide valid results in the biology of ECs in JNA vascularization.

In conclusion, we developed a protocol for the reliable and reproducible isolation and culture of ECs from JNA tissue. It provides a good cell culture model for *in vitro* study of the relationships between local invasion and disease-specific angiogenesis phenotypes and for studies of existing anti-angiogenic drugs.

Supplementary Data

Supplementary data is available at *ABBS* online.

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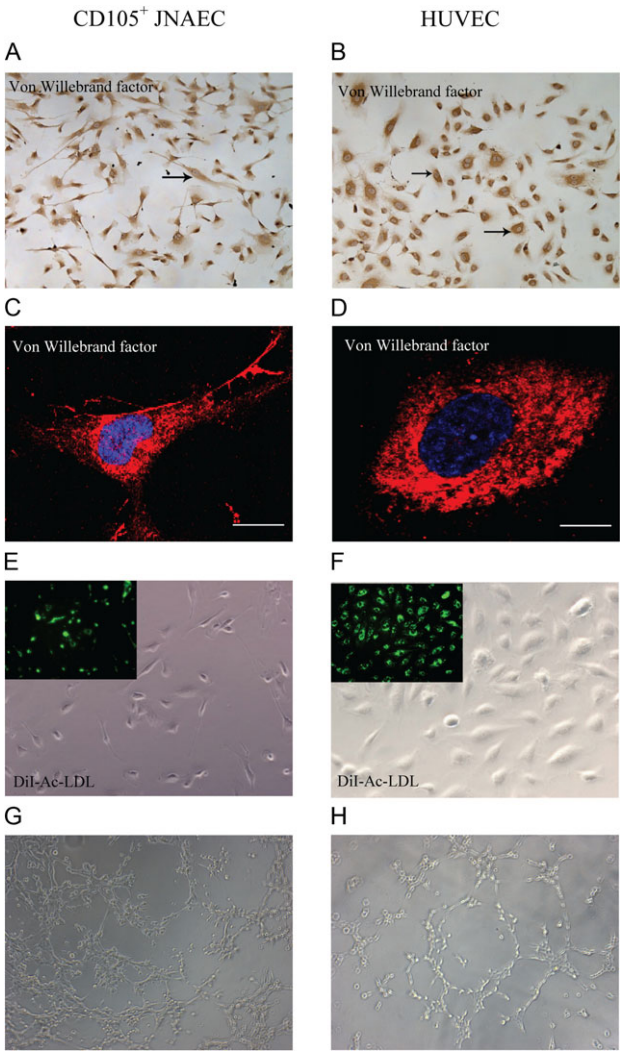

Figure 2. Identification of sorted and cultured CD105$^+$ JNAECs Immunocytochemistry (DAB coloration) showed that the sorted and cultured CD105$^+$ JNAECs (A) as well as HUVECs (B) both expressed vWF (indicated by the black arrow) (original magnification, ×100). vWF expression of CD105$^+$ JNAECs (C) and HUVECs (D) could be clearly illustrated on laser confocal microscopy (red for vWF, blue for DAPI, scale bars, 10 μm). Micrograph representative of uptake of DiI-Ac-LDL (green; original magnification, ×100) in CD105$^+$ JNAECs (E) as well as HUVECs (F). Tube-formation assay showed that CD105$^+$ JNAECs (G) have the ability to form tubes like HUVECs (H) *in vitro*.

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