

# Isolation and characterization of lymphatic endothelial cells from human glossal lymphangioma

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**Abstract.** Abnormal lymphangiogenesis is associated with several diseases such as tumor metastasis and lymphangioma. Human lymphangioma originated from the transformation of lymphatic endothelium is a benign malformation of lymphatic vessels and its pathogenesis has up to date not been illuminated and its cell model has also not been established. An optimized method was used to isolate lymphatic endothelial cells from human glossal lymphangioma (GL-LECs) and GL-LECs were further primarily cultured and expanded. GL-LECs were of typical cobblestone appearance when they reached confluence. The Weibel-palade body was observed in the GL-LECs cytoplasm. Almost all GL-LECs were strongly positive for specific lymphatic markers FLT-4, LYVE-1 and prox-1 by immunocytochemistry. Furthermore, three-dimension tube-like capillaries of GL-LECs resembled the lymphatic system *in vivo*, and the GL-LECs spheroids sprouted radically out to form three-dimensional buds when embedded in the cultured BME. These results indicated that high purity GL-LECs were successfully isolated and expanded. They had the abilities of tube formation and differentiation *in vitro*, which provide a favorable cell model for further uncovering the pathogenesis of human lymphangiomas.

## Introduction

The lymphatic system is made up of an extensive network of lymphatic capillaries, with functions of collecting interstitial fluid, macromolecules and even pointing leukocytes or tumor cells into the circulation system. Therefore, it plays a pivotal role in mediating tissue fluid homeostasis, tumor metastasis and immunological functions. Although the lymphatic system was discovered about 300 years ago, these functions have just been investigated in recent years due to the lack of the specific lymphatic markers. Recently, fms-like tyrosine kinase 4 (FLT-4), lymphatic endothelial hyaluronan receptor (LYVE-1), Prox-1, and podoplanin have been identified as the specific lymphatic endothelial cell markers and largely restricted to lymphatics in adult tissues (1-6), which provided new insights into the molecular mechanisms of lymphatics in physiologic functions and pathologic processes such as inflammations, tumor metastasis, lymphedema and lymphangioma. Human lymphangioma, which is a kind of benign tumor originated from lymphatic vessels, is largely diagnosed in children and has always been defined as congenital malformations. Although human lymphangiomas are asymptomatic in the early course, they develop and have characteristics of progressive growth, compression and infiltration into adjacent tissues. The most acceptable therapy for human lymphangioma is surgery at present, but it has many limitations such as the risk of complications and recurrence. The limitations of surgical treatment have awoken the interests in other therapeutic methods. The administration of sclerosing agents such as bleomycin and hypotonic salt solutions provokes an inflammation of the vascular endothelium leading to total or partial remission of human lymphangioma (7-11). These substances may extend beyond the limits of the original lymphangioma, unsatisfactory aesthetic results and increased difficulties for future surgery. As a result, a more innovative and effective treatment is highly expected. However, the pathogenesis of human lymphangioma has not been elucidated for further investigation primarily due to lack of pathological research models. Accordingly, it is imminent and necessary to construct a cell model of human lymphangioma for *in vitro* study of its molecular events via isolating and expanding lymphatic endothelial cells from human glossal lymphangiomas (GL-LECs).

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**Abbreviations:** GL-LECs, lymphatic endothelial cells from human glossal lymphangioma; FLT-4, fms-like tyrosine kinase 4; LYVE-1, lymphatic endothelial hyaluronan receptor; TEM, transmission electron microscope

**Key words:** human glossal lymphangioma, lymphatic endothelial cells, LYVE-1

Early in 1980s, investigators tried to isolate lymphatic endothelial cells from several kinds of animal macro-lymphatics including rat and bovine thoracic lymph duct, mesentery lymphatics by collagenase digestion method. Formerly, it was difficult to sort lymphatic endothelial cells from tissues because of many different types of cells such as mesenchymocytes and vascular endothelial cells. Increasing findings of specific lymphatic endothelial cell markers make it feasible to isolate pure LECs from human normal tissues for further study. Podgrabinska *et al* (11) and Mäkinen *et al* (1) used LYVE-1 antibody or VEGFR-3 antibody coated magnetic beads to sort LYVE-1 or VEGFR-3 positive lymphatic endothelial cells from dermal cell mixtures, respectively. Kriehuber *et al* (12) used multicolor flow cytometry to sort podoplanin positive lymphatic endothelial cells from human dermal cell suspensions. However, there were no pathological cell models to further study the pathogenesis of lymphatic associated diseases. Therefore, in the present study an optimized collagenase digestion method was used to isolate pure human glossal lymphangioma-associated LECs based of the fact that there are plenty of dilated lymphatics in human lymphangioma specimens, and then the primarily cultured cell lines were obtained. Molecular phenotypes and functions of glossal lymphangioma-associated LECs were further characterized by immunohistochemistry staining, tube-formation assay and sprouting assay.

## Materials and methods

**Mainly materials.** M199 culture medium, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco Co. (USA). Collagenase and collagen type I were obtained from Sigma Co. (Germany). Mouse anti human FLT-4 monoclonal antibody was from Santa Cruz (USA), rabbit anti-human LYVE-1 polyclonal antibody was from R&D Co. (USA), and rabbit antihuman Prox-1 polyclonal antibody from Abcam Co. (UK). Cultrex Basement Membrane Extract (BME) was obtained from Invitrogen Inc. (USA). Vascular endothelial growth factor-C (VEGF-C) was purchased from BD Co. (USA). The immunostaining envision systems were purchased from Dako (USA).

**Specimen preparing.** Human glossal lymphangioma specimens were obtained from Wuhan Oral Medical Hospital affiliated to Wuhan University. After surgical removal, in less than 45 min, the fresh tissues were divided into two pieces, the small one was fixed in 4% paraformaldehyde for preparing paraffin blocks, the larger one was immediately put in cold sterile M199 medium supplemented with fungizone (10  $\mu$ g/ml), penicillin (100  $\mu$ /ml), streptomycin (100  $\mu$ g/ml) and 10% fetal bovine serum and then transferred it to our laboratory for further investigation.

**Immunohistochemistry.** Immunohistochemistry was used to identify the pathological characteristics of human lymphangioma (13), paraffin-embedded sections were produced from human glossal lymphangioma specimens fixed in 4% paraformaldehyde. Sections were deparaffinized in 100%

xylene twice, followed by gradient ethanol (100, 95, 90, 80 and 70%). To expose the antigen epitopes completely, the sections were treated in a microwave in the presence of citrate buffer (pH 6.0) three times, and then incubated with primary antibody overnight at 4°C all diluted 1:200). Remaining steps were conducted as the procedure of the staining kit.

**Isolation and primary culture of GL-LECs.** The tissues were washed with Hank's solution supplemented with the antimicrobial agents as above, and the connective tissues were removed, and then the tissue block was finely minced with scissors into 0.5x0.5x0.5 mm size pieces and subjected to enzymatic digestion for 1 h at 37°C with 0.25% (w/v) collagenase/serum free DMEM. The digestion product was filtered through a 30 mm pore size filter and the filtering product was centrifuged at 1,200 rpm for 10 min, washed the cells with Hank's solution twice and centrifuged, then resuspended the cells with complete medium EGM (endothelial growth medium, M199 medium, supplemented with 20% fetal bovine serum, VEGF-C 100  $\mu$ g/ml, heparin 40 U/ml, penicillin 100 U/ml and streptomycin 100  $\mu$ g/ml) and transferred them to T25 flask and cultured in incubator. Next day, non-adherent cells were removed and discarded, whereas the adherent ones were washed three times with Hank's solution with antibiotics, and incubated with EGM until confluence. At about 80-90% confluence, the cells were digested for subculture with 0.25% trypsinase for 3-5 min at 37°C. The digested cells were transferred to a new T25 culture flask. Cell culture media were replaced 2-3 times per week. The isolated LECs can be cultured at least 10 passages and can be frozen in liquid nitrogen with 20% fetal bovine serum and 10% DMSO.

## Identification of GL-LECs

**Morphology and ultrastructure of GL-LECs.** The morphology and ultrastructure of GL-LECs were observed under a light microscope (Olympus, Japan) and transmission electron microscope (TEM) (FEITecnaiG<sup>2</sup> type 12, Holland), respectively. Cells were harvested with trypsinase digestion, centrifuged and washed in phosphate buffer solution (0.1 M, pH 7.4), and then fixed with 2.5% glutaraldehyde and post-fixed in osmium tetroxide. The samples were dehydrated with gradient ethanol 50, 70, 95 and 100% ethanol and propylene oxide prior to embedding in epoxy. The grids were stained with lead citrate and uranyl acetate. The samples were observed under transmission electron microscope (TEM), and photographed.

**Immunocytochemistry of GL-LECs.** Immunocytochemistry method was used to further identify the GL-LECs by detecting the expression of several special lymphatic endothelial cell markers. GL-LECs were seeded on cover slips overnight and fixed in 4% paraformaldehyde at room temperature. After washing twice in PBS, the cells coated on the cover slips were incubated with primary antibody overnight at 4°C all diluted 1:100). Negative control, in which cells were treated with PBS instead of primary antibody, was also used.

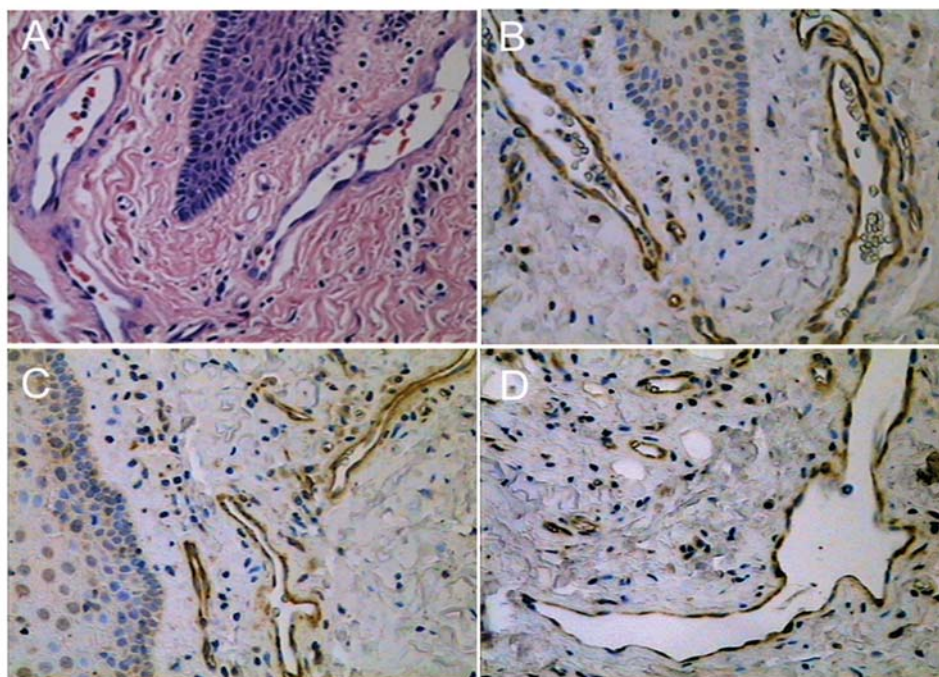


Figure 1. Characteristics of human glossal lymphangioma by immunohistochemistry. (A) H&E staining showed that there are considerable number of dilated vessels in the tissue sections of human glossal lymphangioma. (B-D) dilated vessels in the tissue sections were positive for FLT-4, LYVE-1 and prox-1 on endothelial cell membrane and cell nucleus, respectively. Original magnification, x100.

**Tube formation assay and spouting assay in vitro.** Tube formation assay was performed as described (14). Cells were harvested with trypsinase. A collagen stock solution was prepared prior to use by mixing collagen type I (Sigma, equilibrated to 2 mg/ml, 4°C; 8 vol.) with 10x DMEM (1 vol.) and 0.1 N NaOH (approx. 1 vol.) to adjust the pH to 7.4. The solution was allowed to gel for 30 min at 37°C. Then LECs were seeded on the gel and cultured in incubator at 37°C. Cells cultured in the gel were observed for 48 h under a contrast phase microscope. After at least one week, Collagen gels were fixed in 2.5% glutaraldehyde and cut into pieces, washed in phosphate buffer (0.1 M, pH 7.4) and postfixed in osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in epon. Ultrathin (50-80 nm) sections were cut and collected on copper grids, stained with uranyl acetate and lead citrate for observation by TEM. Sprouting assay was performed according to protocols in the literature (15,16). Cells (800 per well) were seeded in 0.24% methylcellulose M199 medium for overnight incubation to form a single spheroid. Spheroids were collected, centrifuged at low speed, and seeded in BME (Trevigen Inc., USA). BME was prepared prior to use by diluted with DMEM containing 0.5% (w/v) carboxymethylcellulose at 1:1. The spheroid-containing gel was rapidly transferred into pre-warmed 24-well plates and allowed to polymerize (for 1 min), after which 0.15 ml EGM basal medium was pipetted on the top of the gel. Sprout outgrowth was observed for 24 h under a contrast phase microscope.

## Results

**Characteristics of human glossal lymphangioma.** In the present study, H&E staining and immunohistochemistry

analysis were used to identify the pathological feature of human glossal lymphangioma. H&E staining revealed the fact that there were plenty of dilated vessels in the human glossal lymphangioma sections (Fig. 1A). Specific lymphatic cell markers FLT-4, LYVE-1 and Prox-1 were used to show that dilated vessels in the sections were lymphatics. The results indicated that the dilated vessels in the serial sections of the same tissues, which were positive for FLT-4 and LYVE-1 in endothelial cell membrane or cytoplasm and Prox-1 in cell nucleus, were identified as lymphatic (Fig. 1B-D) and agreed with previous reports that human lymphangiomas are rich in dilated lymphatics (17). It is evident that human glossal lymphangioma provides an available and reliable source of LECs for further isolation.

**Isolation and primary culture of GL-LECs.** The fresh tissues from human glossal lymphangiomas were processed and subjected to collagenase digestion. The periphery of the neoplasm was sheared absolutely to keep the target cells from mixing with glossal tissue cells. Bacteria and fungi adhering to their surface were removed by several washings in a solution containing high concentrations of penicillin, streptomycin and fungizone. The non-adherent cells were removed with washing solution next day and the adherent ones obtained were the primary culture cells. The population of primary culture cells was few, and the cells grew slowly as an adherent monolayer. As observed under light microscope, cells were fusiform or polygon shaped and were uniform in size, and the cell nuclei were clear and orbicular-ovate (Fig. 2A). The cell populations were propagated persistently for about two weeks until complete confluence. Cells were propagated with 0.025% trypsin at 80-90% confluence. The sub-cultured cells grew much quicker and reached



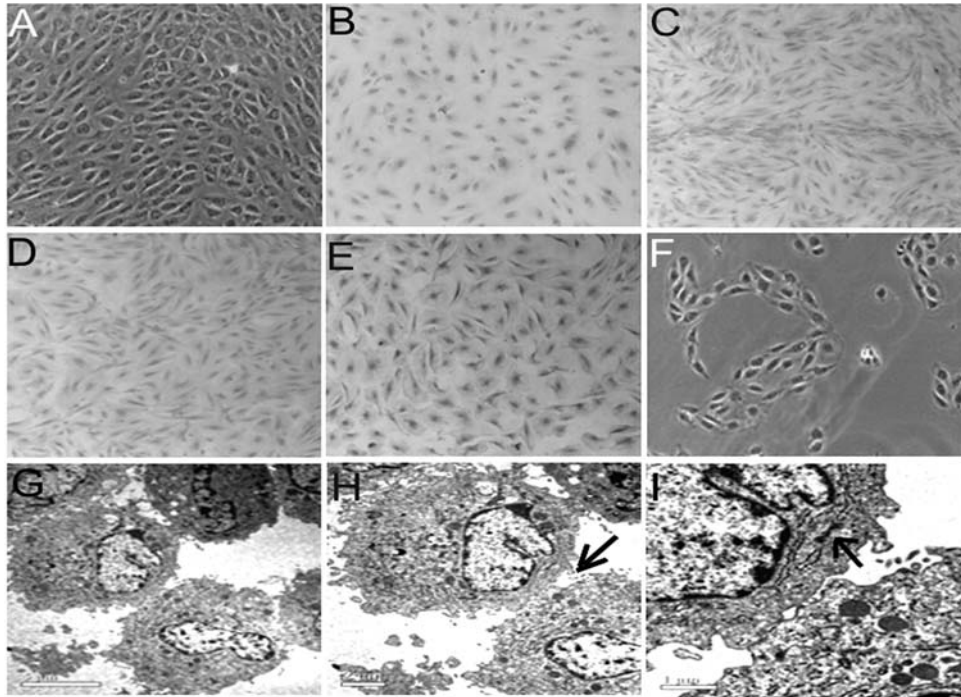


Figure 2. Identification of isolated and primarily cultured GL-LECs. (A) Phase contrast photomicrograph showed the growth pattern of conglomeration and a typical cobblestone appearance when cells reached confluence. (B) Negative control and cells nucleus were counterstained with hematoxylin. (C-E) Immunocytochemistry analysis indicated that in isolated and primarily cultured GL-LECs, almost all GL-LECs were positive for FLT-4 and LYVE-1 on cell membrane and cytoplasm and prox-1 were shown strongly positive in GL-LECs nucleus. (F) In GL-LECs, the central cells were micro size and close-up, however, the adjacent ones were macro size and irregular, and several spindle-shaped cells were seen in the periphery (original magnification,  $\times 20$ ). (G-I) Transmission electron micrograph image of GL-LECs. (G) Human LECs emerged in scale-shape and appeared as continuous monocellular layer. (H) Intercellular gap junction appeared in adjacent LECs, indicated by an arrow. (I) a higher magnification of the same region in the panel H is shown by the arrow. Under high power lens, the organoids such as Weibel-palade body (shown by the arrow I), endocytosomal reticulum, plasmalemmal vesicle and cytoplasmic processes were observed transparently. [bar in (G)= $5\ \mu\text{m}$ ; bar in (H)= $2\ \mu\text{m}$  and bar in (I)= $1\ \mu\text{m}$ ].

confluence in about 7 days, they showed typical cobblestone morphology, a specific characteristic of endothelial cells (Fig. 2A). When propagated up to confluence, the growth rate of GL-LECs slowed down, and the typical contact inhibition phenomenon of endothelial cell growth emerged.

**Identification of GL-LECs.** Ultrastructure of GL-LECs was observed by transmission electron microscopy. GL-LECs emerged scale-shaped and with monocellular layer appearance under TEM (Fig. 2G). The majority of cells existed in conglomeration, so they were discriminated from the fibroblasts which always exist in sporadic form. Gap or tight inter-cellular junctions between consecutive GL-LECs were frequently overviewed (Fig. 2H). Weibel-palade body, a typical granulation of endothelial cells was observed in cytoplasm in rod-shape in the longitudinal section with high electron density (Fig. 2I). Other specific endothelial cell organoids such as endocytosomal reticulum, plasmalemmal vesicle and cytoplasmic processes were also viewed in the cytoplasm. Although the TEM images demonstrated typical endothelial cell characteristics, lymphatic endothelial cells and blood vascular endothelial cells share common features in these ultra structures. Therefore, to further identify the GL-LECs, the immunocytochemistry method was used to detect the expression of several special lymphatic endothelial cell markers in GL-LECs. Almost all the cells were positive

for FLT-4 and LYVE-1 in cell membrane and cytoplasm, and strongly positive for Prox-1 in the cell nucleus (Fig. 2C-E), indicating that the cells isolated from human glossal lymphangioma were the lymphatic endothelial cells with more than 95% purity.

**Functional features *in vitro* of GL-LECs.** In order to reveal the function properties of cultured GL-LECs *in vitro*, tube formation assay and sprouting assay were used to observe the ability of lymphatic formation and cell differentiation, respectively. When subjected to tube-forming assay on collagen type I gel, GL-LECs began to form tube-like structures in the conditions of EGM added with VEGF-C after 48 h of incubation. When cultured in the gel longer, GL-LECs advanced to form capillary-like tubes in very different directions, and the tubes from different directions linked up to form a complex lymphatic vascular plexus, which is similar with the lymphatic system *in vivo* (shown in Fig. 3A and B). The tube-like construction ultrastructure was further verified by TEM. Cross sections of collagen gels revealed true capillary lumens and their walls were composed of lymphatic endothelial cells lacking continuous basal lamina, and then lymphatic endothelial cells typically overlapping intercellular junction was also seen in the section (shown in Fig. 3D and E). The GL-LEC spheroids formed after the cells were seeded in 0.24% methylcellulose for

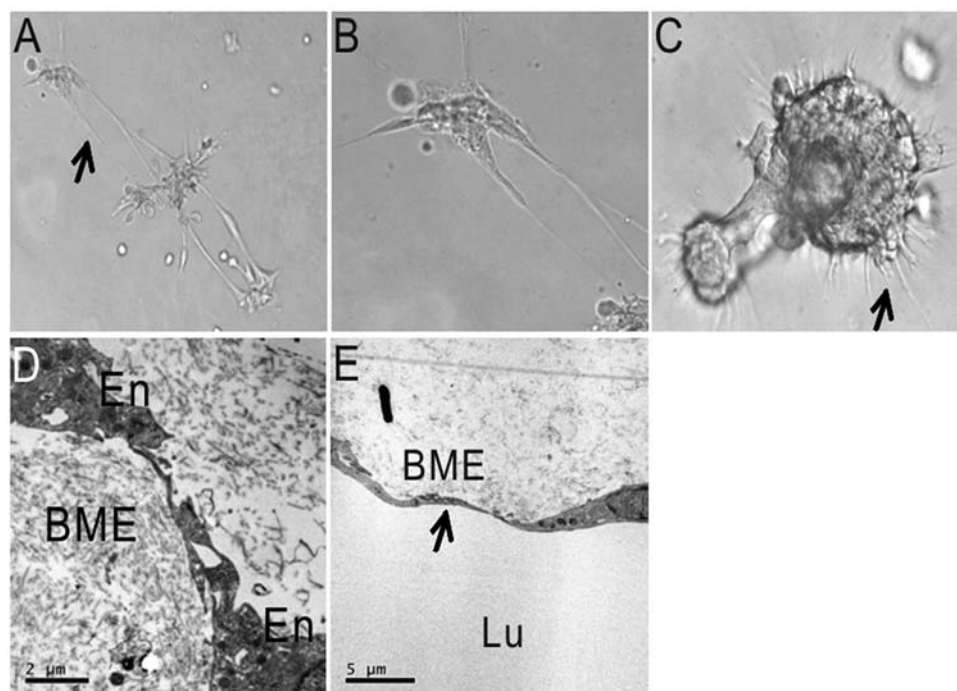


Figure 3. The abilities of differentiation and tube formation *in vitro* of human GL-LECs. (A) Human GL-LECs formed 3-dimensional capillary-like tubes and the tubes linked to each other to form a closed network. (B) A higher magnification of a certain region in the picture A. (C) In the cutlery of BME, human GL-LECs sprouted radically out of spheroplasts and formed 3-dimensional buds. (D and E) the ultrastructure of capillary-like tubes formed by human GL-LECs in collagen gel I was observed by TEM. (D) Showed a true lumen composed by several human GL-LECs. (E) Revealed a typical overlapping intercellular junction (indicated by an arrow). Lu, lumen; BME, basement membrane extracts; En, human endothelial cells.

about 24 h. The gel-embedded GL-LECs spheroids sprouted radically for the first 48 h and formed 3-dimensional buds in BME (shown in Fig. 3C). These results indicated that the abilities of differentiation and tube formation *in vitro* were conferred to GL-LECs.

## Discussion

In the present study, lymphangioma-associated LECs isolated by an optimized method could be primarily cultured and expanded more than 10 passages. Furthermore, lymphangioma-associated LECs were identified to characterize the common features of the reported LECs. Thus, we successfully developed a cell model of lymphatic endothelium for further investigating the molecular mechanism of the pathogenesis of human lymphangiomas.

Hyperplasia or abnormal growth of lymphatic vessels is associated with lymphangiomas. Lymphangiomas have been reported with the characteristics of numerous dilated lymphatic vasculatures or over-proliferation of lymphatic vessels (18,19). Although increasing cases of lymphangiomas were reported, the molecular events such as the genetic and proteomic changes are not defined. Only recently, the co-expression of VEGF and VEGFR in 29 cases of human lymphangioma were detected by RNA *in situ* hybridization, suggesting that VEGF-C and its receptors may actively participate in the formation of human lymphangioma by autocrine or paracrine regulation (19,20). However, although mouse lymphatic endothelial cells were isolated and characterized (20,21), there is no previous cell model of

human LECs to discover the molecular regulation of lymphangiogenesis in lymphatic-associated disorders. In the present study, human glossal lymphangioma was selected to be the disease model for further investigation. The fresh tissue sections stained by hematoxylin-eosin showed plenty of dilated vessels without any blood cells instead of the lymph fluid. To further confirm the fact that the dilated vessels were lymphatics, the expressions of special lymphatic endothelial cell markers such as FLT-4, LYVE-1 and prox-1 were detected by immunohistochemistry staining. We found that almost all of the dilated vessels were positive for FLT-4, LYVE-1 and prox-1. Accordingly, it naturally assured that human glossal lymphangioma was a reliable and valuable source of human LECs.

Recently, an immunomagnetic based purification method was used to isolate LECs from mixed cultures. Magnetic beads coated by LYVE-1, podoplanin, and VEGFR-3 antibodies were applied to separate the related subpopulations from gross cells, respectively (1,11,12). These methods only purified the appointed subgroups from several mixed cultures, thus this caused not only the omission of partial LECs originally included, but also partial contamination of heterogeneous cells such as blood endothelial cells. Before several novel specific lymphatic markers were reported, LECs were mainly isolated from different tissues by means of collagenase treatment. But during the primary cultures, collagenase disposal may also result in contamination of other cells including blood endothelial cells and interstitial cells. Here, we optimized the protocol of collagenase disposal. Whether we could obtain high purity of GL-LECs or not, the collage-

nase digestion time was pivotal and a key factor. Longer digested, more contaminated cells would be contained in the primary cultures. To achieve higher purity of LECs, we utilized the fact that endothelial cells were always easier to trypsinize and existed in the pattern of conglomeration (containing several endothelial cells after trypsinization) to eliminate the contaminated mesenchymal cells with serial subcultivation. Meanwhile, VEGF-C, which was recently revealed with the ability of selectively inducing survival of LECs but not blood endothelial cells, was added in the special growth medium to promote the growth and expansion of GL-LECs (11), and heparin, which enhances the growth of LECs and reduce concentrations of VEGF-C for long-term serial culture as reported by Engelmann *et al* (21), was also added in the culture medium to ensure permanent cell phenotype. When these measures were carried out, pure GL-LECs were expected.

Our results indicated that GL-LECs exhibited a cobblestone appearance at confluence, which was well received as a representative morphological feature of endothelial cells (22-24). Weible-palade body, a kind of specific granule of endothelial cells, was captured under TEM as reported (22). An abundance of cytoplasmic vesicles, which are one of the typical features of LECs (4,25-27), were seen in the ultrathin sections from human glossal lymphangioma. In addition, gap and tight intercellular junctions in adjacent cells were also displayed by TEM. These findings obviously demonstrated that GL-LECs had the characteristics of endothelial cells in morphology and ultrastructure. Almost all GL-LECs were positive for FLT-4, LYVE-1 and prox-1. The phenotypic patterns of GL-LECs were extremely similar to those observed in human glossal lymphangioma, suggesting that GL-LECs *in vitro* maintained the original molecular characterizations and a considerable high purity and then *in vitro* kept the primal information at a maximum degree. Furthermore, the functions of GL-LECs, such as forming complex tube-like capillary networks in collagen type I gel and GL-LECs spheroids sprouting to form three-dimensional buds, simulating the lymphatic system *in vivo*, thus proving their capabilities to form lymphatic vessels and *in vitro* differentiate. Taken together, GL-LECs successfully isolated through collagenase treatment were easily cultured in EGM supplemented with VEGF-C without lack of any molecular phenotype and functional activities *in vitro*.

In summary, human glossal lymphangioma provides an available source of human LECs. A convenient, facile and reliable method has successfully been developed to isolate and expand human LECs *in vitro*. GL-LECs obtained will provide a good cell model for lymphatic malformations and be useful for investigating the molecular mechanisms of human lymphangiomas.

## Acknowledgements

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