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Identification of tumour-reactive lymphatic endothelial cells capable of inducing progression of gastric cancer

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Background: Tumour cells and stromal cells interact in the tumour microenvironment; moreover, stromal cells can acquire abnormalities that contribute to tumour progression. However, interactions between lymphatic endothelial cells (LECs) and tumour cells are largely unexamined. In this study, we aimed to determine whether tumour-specific LECs inhabit the tumour microenvironment and examine their influence on this microenvironment.

Methods: We isolated normal LECs (NLECs) from a non-metastatic lymph node and tumour-associated LECs (TLECs) from cancerous lymph nodes. We examined proliferative and migratory potency, growth factor production, and gene expression of each type of LEC. Moreover, we developed a co-culture system to investigate the interactions between gastric cancer cells and LECs.

Results: When compared with NLEC, TLECs had an abnormal shape, high proliferative and migratory abilities, and elevated expression of genes associated with inflammation, cell growth, and cell migration. NLECs co-cultured with gastric cancer cells from the OCUM12 cell line acquired TLEC-like phenotypes. Also, OCUM12 cells co-cultured with TLECs expressed high levels of genes responsible for metastasis.

Conclusions: Our results demonstrated that LECs interacted with tumour cells and obtained abnormal phenotypes that could have important roles in tumour progression.

The stromal cells in tumour microenvironments acquire abnormalities and exhibit biological properties that differ from those of stromal cell in normal tissues; for example, tumour-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) differ from normal macrophages and fibroblasts, respectively (Kalluri and Zeisberg, 2006; Joyce and Pollard, 2009). Several studies have demonstrated that tumour endothelial cells (TECs) also inhabit tumour microenvironments and that these TECs differ from normal endothelial cells with regard to gene expression and morphology (McDonald and Choyke, 2003; Ohga *et al*, 2012). Yang *et al* (2010) reported that lymphatic endothelial cells (LECs) isolated from epithelial ovarian tumours enhanced migration and invasion of a human ovarian carcinoma cell line. Additionally, when LECs were co-cultured with cells with a high potential for metastasis, these LECs secreted many cytokines and showed enhanced proliferation and tube formation (Zhuang *et al*, 2010). Therefore, we hypothesised that tumour-associated LECs (TLECs) inhabit tumour microenvironments and that these cells differ from normal LECs (NLECs) in several ways. Here, we isolated LECs from normal and cancerous lymph nodes, identified NLECs and TLECs, and demonstrated that TLECs (but not NLECs) were involved in gastric cancer progression.

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MATERIALS AND METHODS

LEC isolation and culture. Human lymph-node specimens were obtained from patients with gastric cancer whose surgical treatment was performed at Osaka City University Hospital. NLECs were isolated from non-cancerous lymph nodes that were removed from patients who showed no evidence of lymph-node metastasis; TLECs were isolated from cancerous lymph nodes. We isolated LECs as previously reported (Norder *et al*, 2012). Briefly, lymph nodes were minced and treated with collagenase (100 U ml^{-1}, Life Technologies, Polo Alto, CA, USA); each mixture was passed through a 70-μm nylon mesh (Corning Incorporated, Corning, NY, USA). Cells were then seeded onto collagen-coated dishes (Corning Incorporated) and cultured in EBM2 (Lonza, Basal, Switzerland). After 72 hours (h), non-adherent cells were removed, and adherent cells were cultured until cell monolayers were 80–90% confluent. Primary cultures were treated with trypsin and incubated with magnetic micro-beads (Militenyi Biotec, Bergish Gladbach, Germany); these micro-beads were coated with antibody recognising human Cluster of Differentiation 31 (CD31) to selectively isolate CD31-positive cells. CD31-positive cells were cultured on collagen-coated dishes in EBM2 until cell monolayers were 80–90% confluent. To isolate podoplanin-positive cells from these cultures, the cultures were then treated with trypsin and incubated for 45 min on ice with mouse anti-human podoplanin antibody (4 μg ml^{-1} Relia Tech, Wolfenbüttel, Germany), which is an antibody that binds a lymphatic-specific marker, and then washed, resuspended, and incubated with anti-mouse IgG1-coated micro-beads (Militenyi Biotec). These CD31-positive and podoplanin-positive cells were presumed to constitute the LEC population and were maintained in culture for subsequent experiments.

Flow cytometry. To confirm that these isolated cells were LECs, we performed flow-cytometric analysis. Cells were trypsinised, resuspended, and incubated with Alexa Fluor 647 mouse anti-human CD31 (BD Biosciences, San Jose, CA, USA) and Alexa Fluor 488 anti-human podoplanin (BioLegend, San Diego, CA, USA) for 30 min on ice. We then used antibodies against two lymphatic endothelial markers—PE anti-hVEGFR3 (anti-human vascular endothelial growth factor receptor 3) and APC anti-h LYVE-1 (lymphatic vessel endothelial hyaluronic acid receptor 1) (R&D Systems, Minneapolis, MN, USA)—to assess expression of other lymphatic endothelial cell-specific proteins. Flow cytometry was performed with an LSRII (BD Biosciences). FACS DIVA (BD Biosciences) software was used to analyze the data.

Tumour-LEC co-culture system and preparation of tumour-conditioned medium. The OCUM12 cell line was previously established from a scirrhous gastric cancer specimen in Osaka City University. OCUM12 cells were maintained in DMEM (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Nichirei Bioscience, Tokyo, Japan) and 20% penicillin-streptomycin (Wako, Osaka, Japan). OCUM12 cells were suspended at a density of 2×10^4 per ml, and 200 μl of this suspension was plated onto each individual Millicell cell culture insert (3 μm pore 12 mm Diameter; Merck, Darmstadt, Germany); these cultures were incubated for 24 h at 37 °C and 5% CO_2 to attach tumour cells to the filters. The filters were then transferred to the collagen-coated 24-well plates in which LECs were seeded; co-cultures were then incubated at 37 °C and 5% CO_2 for 3 days in 500 μl fresh growth factor-free EBM2. Tumour-conditioned medium (TCM) was prepared by incubating OCUM12 cultures in serum and endothelial growth factor-free EBM2. After 3 days, medium was removed from the cultures, centrifuged, passed through nylon mesh, supplemented with 2% FBS, and stored at − 80 °C until use.

Wound-healing assay. We performed a wound-healing assay to evaluate migratory ability of LECs. LECs were suspended at a density of 5×10^5 per ml, and 100 μl aliquots of suspension were placed into individual wells on 96-well plates (Essen Imagelock; Essen Instruments, Birmingham, UK). After cells were incubated for 72 h at 37 °C and 5% CO_2, a wound was scratched with wound scratcher (Essen Instruments). Wound confluence was monitored with the Incucyte Live Cell Imaging System and Software (Essen Instruments). Wound closure was observed every 3 h in three independent places for 24 h, and mean relative wound density was used to compare between NLECs and TLECs with regard to migration ability.

Cell proliferation assay. To assess proliferation of LECs, we performed MTT assays. NLECs or TLECs were suspended at a density of 1×10^5 per ml, and 50 μl aliquots of cell suspension were seeded into individual wells in 96-well plates (CELLSTAR; Greiner, Frickenhausen, Germany). Then, 50 μl of EBM2 or TCM was added to each well; these cultures were incubated for 72 h at 37 °C and 5% CO_2. Then, 10 μl of MTT solution (5 mg ml^{-1}) was added to each well, and the cultures were further incubated for 3 h at 37 °C and 5% CO_2. After incubation, medium was removed from the wells, and 100 μl of dimethyl sulfoxide (DMSO) was added to each well. Absorbance at 570 nm was then measured with a microplate reader.

Cell direct co-culture models. Analysis of direct interactions between LECs and cancer cells was used to evaluate the effect of LECs on epithelial–mesenchymal transition (EMT) of cancer cells.

Specifically, we co-cultured LECs and OCUM12 cells directly. LECs were suspended at a density of 1×10^5 per ml and seeded onto collagen-coated 24-well plates; 3 days later, 500 μl of an OCUM12 cell suspension (1×10^5 cells per ml in growth factor-free EBM2) was added to each well. After co-culture for 3 days, CD31 MicroBeads Kit (Miltenyi Biotec) was used to separate these cell populations via positive selection. RNA was then extracted from the isolated OCUM12 cells.

RNA extraction and reverse transcription reactions. RNAeasy Mini Kits (QIAGEN, Tokyo, Japan) were used according to the manufacturer's instructions to extract total RNA. ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) was used according to the manufacturer's instructions to reverse transcribe the isolated RNA and generate single-strand cDNA; this reaction was performed at 37 °C for 15 min, 50 °C for 5 min, and 98 °C for 5 min.

PCR array. Using RT^2 Profiler PCR Arrays (QIAGEN), we determined that chemokines, chemokine receptors, common cytokines, extracellular matrix molecules, and adhesion molecules were upregulated in TLECs. Total RNA was extracted from NLECs and TLECs as described above. RT^2 First Strand Kits were used according to the manufacturer's instructions to reverse transcribe single-strand cDNA from 3 μg of total RNA. Each reverse-transcription reaction was performed according to the protocol supplied by the manufacturer and analysed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Differences in gene expression levels between two groups were expressed as fold changes relative to expression levels in NLECs. Based on these results, we performed further experiments involving quantitative reverse-transcription PCR (qRT-PCR) and enzyme-linked immunosorbent assays (ELISAs).

Quantitative reverse-transcription PCR. Expression of mRNAs—interleukin 1β (IL-1β), IL-6, IL18, CXCL1, CXCL2, CXCL6, CXCL8, COLA1, MMP2, and VEGF-C in LECs and CXCR2, SNAIL, and TWIST in OCUM12—was assessed via qRT-PCR. TaqMan PCR core reagents were used for these assays; each

reaction was recorded and analysed with the ABI PRISM 7000 Sequence Detection System. After an initial denaturation for 10 min at 95 °C, each sample was subjected to 40 cycles of PCR (95 °C for 15 s, and 60 °C for 1 min, per cycle). To analyze the ratios of gene transcription levels, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression of each target gene. All experiments were performed in triplicate, and mean values were used for further calculation.

Enzyme-linked immune-sorbent assay. NLECs, TLECs, and NLECs co-cultured with OCUM12 were incubated for 3 days in growth factor-free EBM2; supernatants from individual cultures were collected, centrifuged, and filtered through nylon mesh. Quantikine human ELISA Kits (R&D Systems) were used according to the manufacturer's instructions to determine the concentrations of VEGF, VEGF-C, and inflammatory cytokine IL-1β in culture supernatants.

Western blot analysis. Aliquots containing 20 μg of total protein from cell lysates were subjected to SDS-PAGE; protein bands were then transferred to a membrane via the Trans-Blot Turbo^{TM} Transfer System (Bio-Rad, Hercules, CA, USA). Each membrane was blocked with Amersham ECL blocking agent (GE Healthcare Life Sciences, Piscataway, NJ, USA) and then incubated with each primary antibody—1:300 of anti-LYVE-1 (Abcam, Cambridge, UK), 1:300 of anti-VEGFR3 (Cell Signaling tec., Danvers, MA, USA), 1:300 of anti-Prox1 (Cell Signaling tec.), and 1:1000 of anti-β-actin (Cell Signaling tec.)—at room temperature for 15 min. Each membrane was then washed three times and incubated anti-rabbit horse radish peroxidase-conjugated secondary antibody (GE Healthcare Life Sciences) for 15 min. Protein bands were detected via Luminal Enhancers (GE Healthcare Life Sciences).

Statistical analysis. Student's *t*-tests were used to compare mean values from two groups. All *P*-values were based on two-sided testing. *P*-values <0.05 were considered as statistically significant. SPSSII (IBM Corporation, Armonk, NY, USA) software was used to perform each statistical analysis.

RESULTS

Isolation of different types of LECs from draining lymph nodes. We isolated CD31-positive, podoplanin-positive cells (LECs) from different tumour-draining lymph nodes of the stomach. Most isolated LECs have a 'cobblestone form' that is similar to standard endothelial cells. However, we found spindle-shaped cells that were like fibroblasts. We designated the cobblestone cell NLECs and the fibroblast cell TLECs (Figure 1A and B). TLECs were isolated from a metastatic lymph node. Both types of isolated cells expressed the pan-endothelial cell marker CD31 and the lymphatic marker podoplanin (Figure 1C). Representative results from two TLEC cultures as contrasted with those from an NLEC culture are shown for two TLEC lines, and the TLECs had similar shapes and gene expression profiles (Supplementary Figure 1, Supplementary Table 1).

TLEC cultures seemed to grow faster than NLEC cultures. To confirm this, we used MTT and wound-healing assays to examine cell proliferation and migration, respectively. TLECs exhibited significantly higher absorbance and wound closure than NLECs (Figure 2A–C). These results indicated that TLECs had higher proliferative and migratory abilities than NLECs.

Determining gene expression profile of NLECs and TLECs with PCR arrays. To explore the molecular characteristics of TLECs, quantitative PCR arrays were used to analyze expression of 252 genes involved in cell proliferation, chemotaxis, cell adhesion, angiogenesis, immune response, or inflammation (Supplementary Table 2). Of the 252 genes, 99 were upregulated more than two-fold in TLECs relative to NLECs. Of these upregulated genes, the most highly upregulated were IL-1β, IL-6, IL-18, VCAN, and type I collagen.

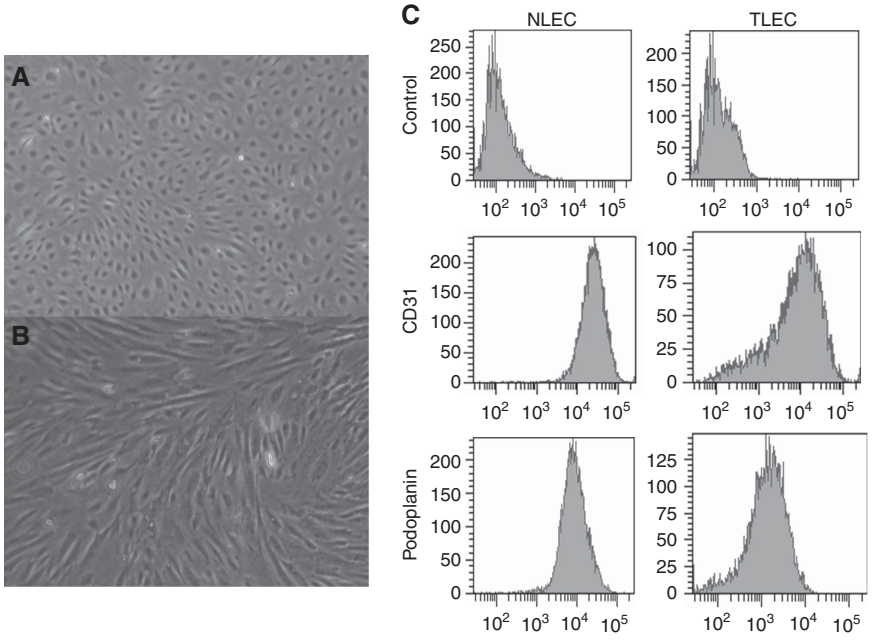

Figure 1. Isolation and identification of normal and tumour-associated lymphatic endothelial cells (LECs). CD31-positive, podoplanin-positive cells were isolated from two different lymph nodes, one normal and one containing metastatic gastric cancer cells. (**A**) Normal LECs (NLECs) isolated from the normal lymph node; NLECs had a cobblestone-like appearance (at ×40 magnification). (**B**) Tumour-associated LECs (TLECs) isolated from a lymph node with metastatic gastric cancer cells. TLECs were spindle-shaped cells, like fibroblasts (at ×40 magnification). (**C**) Both types of LECs expressed the pan-endothelial cell marker CD31 and the lymphatic marker podoplanin.

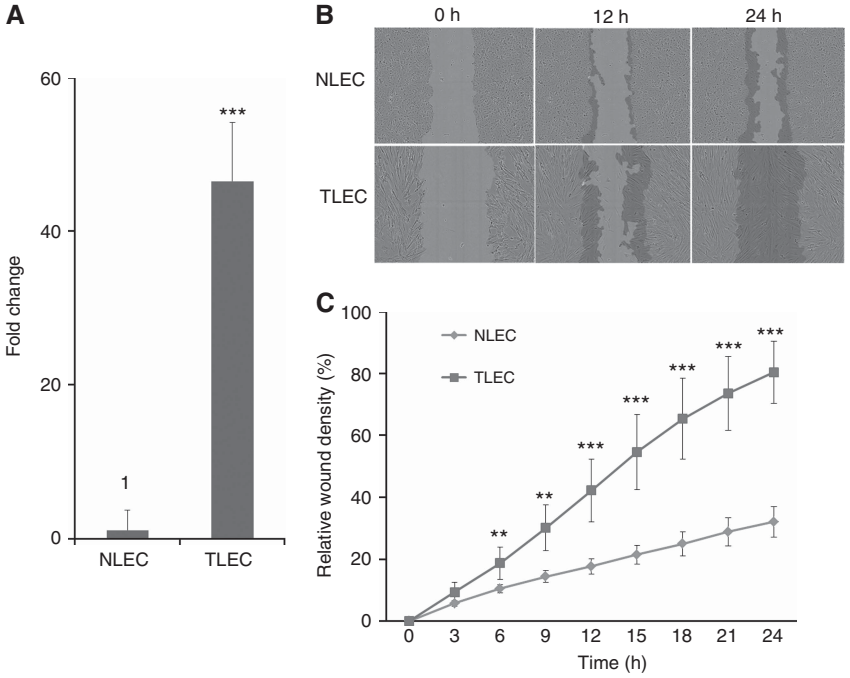

Figure 2. Comparison between NLECs and TLECs with regard to proliferation and migration. (**A**) TLECs showed markedly higher proliferative ability than NLECs (46.5 ± 7.67-fold). The data represent means of triplicate measurement ± the standard deviation (s.d.). ***$P<0.001$. (**B**) Microscope images depicting cell density and morphology in wounds at multiple time points. Cells present at the beginning of each experiment are shown in grey, and migrating cells are shown in purple. (**C**) Relative LEC density in wounds cut through NLEC or TLEC monolayers was monitored for 24 h. TLECs showed significantly greater wound closure than did NLECs. Each value represents the mean density of five wells ± s.d. **$P<0.01$, ***$P<0.001$. A full color version of this figure is available at the *British Journal of Cancer* journal online.

We used qRT-PCR to generate more precise gene expression data for NLECs and TLECs. Based on PCR array results, we selected 10 genes (IL-1β, IL-6, IL-18, CXCL1, CXCL2, CXCL6, CXCL8, COLA1, MMP2, and VEGF-C) for further analysis. Each of these genes, except MMP2 (2.30-fold), was markedly upregulated in TLECs relative to NLECs: IL-1β (3432.3-fold), IL-6 (10075.8-fold), IL-18 (3123.5-fold), CXCL1 (3011.7-fold), CXCL2 (281.7-fold), CXCL6 (4963.2-fold), CXCL8 (2987.3-fold), COLA1 (1246.7-fold), VEGF-C (51.47-fold) (Figure 3A). We used ELISA to measure levels of three proteins (VEGF-A, VEGF-C, and IL-1β) in supernatants from NLEC or TLEC cultures. NLECs produced moderate amounts of VEGF-C (184.74 ± 13.66 pg ml^{-1}); however, VEGF-C and IL-1β were not detected in supernatant of NLEC cultures. In contrast, relative to NLECs, TLECs secreted significantly higher amount of VEGF-A, VEGF-C, and IL-1β (355.61 ± 22.13 pg ml^{-1}, 3057.04 ± 45.87 pg ml^{-1}, and 4304.32 ± 112.14 pg ml^{-1}, respectively, Figure 3B).

The effect of cancer supernatant on characteristics of NLECs. LECs isolated from non-metastatic lymph nodes differed from LECs isolated from metastatic lymph nodes. We hypothesised that cancer cells might cause TLECs to promote an inflammatory environment. To test this hypothesis, we compared TCM with unconditioned medium with regard to effects on cell proliferation. Relative to unconditioned medium, TCM significantly increased the proliferative capacity of NLECs (Figure 4A). Moreover, we established a tumour-LEC co-culture system to assess the influence of tumour cells on LECs. Relative to control cells, NLECs co-cultured with OCUM12 cells exhibited significant upregulation of six genes—IL-1β (2.2-fold), IL-6 (19.5-fold), IL-18 (5.1-fold), CXCL1 (20.9-fold), CXCL2 (24.4-fold), and CXCL8 (21.9-fold), but not CXCL6 (0.7-fold), COLA1 (0.9-fold), MMP2 (0.3-fold), and VEGF-C (1.3-fold) (Figure 4B); these NLECs also secreted significantly higher concentration of three cytokines—VEGF-A, VEGF-C, and IL-1β (842.28 ± 0.95 pg ml^{-1}, 246.23 ± 3.90 pg ml^{-1}, and 314.78 ± 9.81 pg ml^{-1}, respectively, Figure 4C).

We examined expression of three LEC markers— LYVE-1, VEGF-R3, and Prox-1—in NLECs, TLECs, and NLECs co-cultured with cancer cell. As shown in Figure 5, LYVE-1, VEGF-R3, and Prox-1 were each found to be downregulated in TLECs and in NLECs co-cultured with cancer cells.

The effect of LECs on cancer cells. Thus far, we have demonstrated that TLECs generated an inflammatory microenvironment in regional lymph nodes. Inflammation is a hallmark of cancer that contributes to the development of metastasis. We hypothesised that LECs have the potential to exacerbate lymph-node metastasis of cancer cells. To determine the effects of TLECs on cancer cells, we examined the expression of mRNAs encoding a chemokine receptor (CXCR2) and two EMT-associated proteins (SNAIL and TWIST) in a gastric cancer cell line, OCUM12. Expression of CXCR2 mRNA was elevated in OCUM12 cells co-cultured with TLECs relative to those cultured without LECs; importantly, the upregulation was significantly higher in TLEC co-cultures than in NLEC co-cultures (NLEC, 6.3-fold; TLEC, 82.6-fold) (Figure 6). Moreover, mRNAs encoding SNAIL or TWIST were upregulated in OCUM12 cells co-cultured with TLECs (SNAIL, 3.2-fold; TWIST, 9.0-fold). These results indicated that LECs interact with cancer cells and induce changes in cancer-cell gene expression.

DISCUSSION

In this study, we isolated intranodal TLECs that could induce inflammation and induce cancer cells to undergo EMT-like changes; these TLEC effects might prepare some cancer cells to form metastasis.

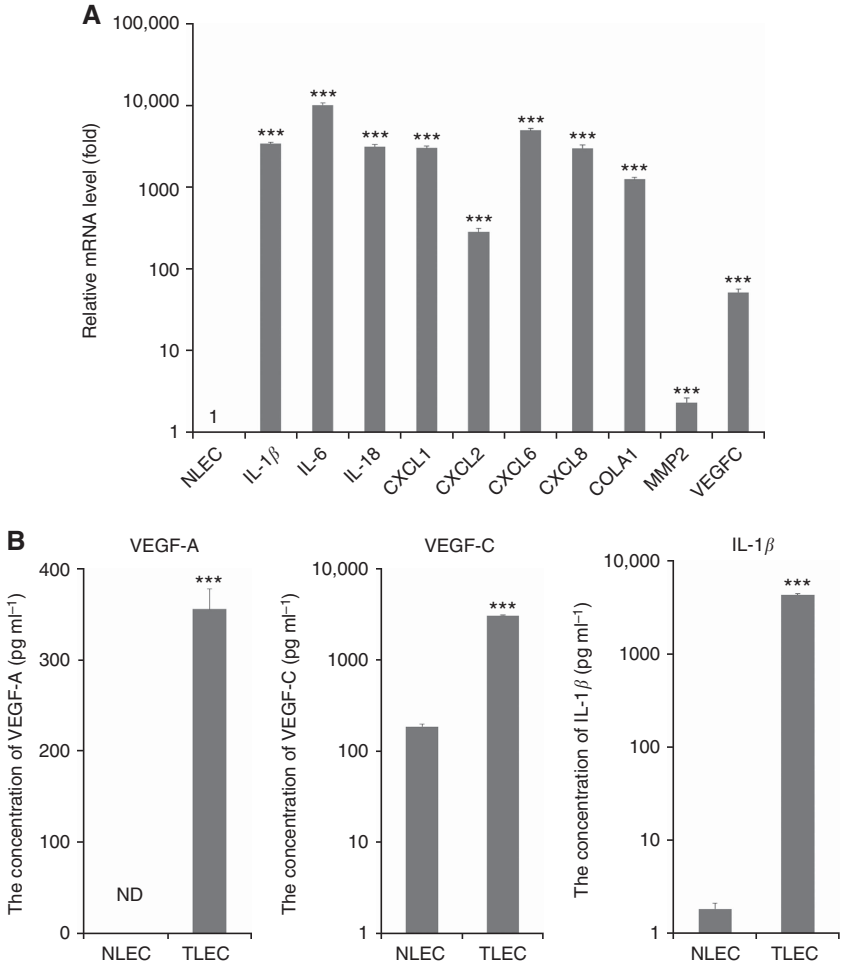

Figure 3. Comparison between NLECs and TLECs with regard to cell characteristics. (**A**) Differences in mRNA expressions between NLECs and TLECs. TLECs showed significantly higher expression of mRNAs encoding cytokines, chemokines, adhesion molecules, and growth factors than did NLECs. The relative expression levels of mRNA are shown on a logarithmic scale, and the values represent means of quintuplet measurements ± s.d. ***$P<0.001$. (**B**) Differences in production of growth factors and cytokines between NLECs and TLECs. TLECs secreted significantly higher concentrations of VEGF-A, VEGF-C, and IL-1β (355.61 ± 22.13 pg ml^{-1}, 3057.04 ± 45.87 pg ml^{-1}, and 4304.32 ± 112.14 pg ml^{-1}, respectively) than did NLECs. The concentrations are shown as pg ml^{-1} per 1×10^6 cells, and the values represent means of triplicate measurements ± s.d. ***$P<0.001$, ND, not detected.

Interactions of cancer cells with their microenvironment are important for tumour progression. Stromal cells produce cytokines, chemokines, and growth factors that, in turn, contribute to tumour progression by promoting angiogenesis, lymphangiogenesis, and metastasis. Lymphangiogenesis in a primary tumour is a poor prognostic factor for gastric cancer. Lymphatic vessels in the tumour microenvironment are important for tumour progression. Although cancer cells metastasise through lymphatic vessels to draining lymph nodes, details of the interactions between cancer cells and LECs are largely unexamined. Lymphangiogenesis and inflammation are two integral processes that promote tumour growth and progression. Ji *et al* (2014) demonstrated that the TNF-α pathway directly stimulates LECs. In this study, we isolated human TLECs derived from metastatic lymph nodes of a gastric cancer specimen.

We documented differences between TLECs and NLECs in cellular morphology. Although NLECs had regular dimensions like a rhombus, TLECs had elongated cytoplasm-filled projection and appeared morphologically similar to fibroblasts. These morphological characteristics of TLECs were similar to the EMT characteristics of cancer cells. To extend this analysis, we examined the proliferative and invasive capacity of LECs and found marked augmentation of both in the TLECs. These results indicated that LECs have the ability to alter their own function while under the influence of cancer cells.

We next compared mRNA expression profiles of TLECs and NLECs. TLECs showed exponential increases in expression of mRNAs that encode typical inflammatory cytokines, chemokines, and adhesion molecules. IL-1β, which is abundant at tumour sites, is secreted to the tumour microenvironment, and it affects tumour growth, angiogenesis, and invasiveness (Giavazzi *et al*, 1990; Voronov *et al*, 2003; Apte *et al*, 2006). IL-6 binds to soluble IL-6 receptor; this binding results in JAK (janus kinase) activation and phosphorylation of STAT3 (signal transducer and activator of transcription 3); JAK-STAT activation leads to tumour-cell survival and proliferation (Bollrath *et al*, 2009; Bromberg and Wang, 2009; Grivennikov *et al*, 2009). Interleukin-18, a member of the IL-1 family, may promote angiogenesis in gastric cancers (Kim *et al*, 2006) and cell migration and metastasis in melanoma and lung cancer (Vidal-Vanaclocha *et al*, 2000; Jiang *et al*, 2003). Furthermore, tumour-derived IL-18 upregulates PD-1 (programmed death-1) expression on T cells, which induce tumour-mediated immunosuppression; together, these effects result in metastasis *in vivo* (Terme *et al*, 2011). TLEC-induced chemokines—including CXCL1, CXCL2, CXCL6, and CXCL8 (IL-8)—are the members of the CXC chemokine family that are associated with the neutrophil

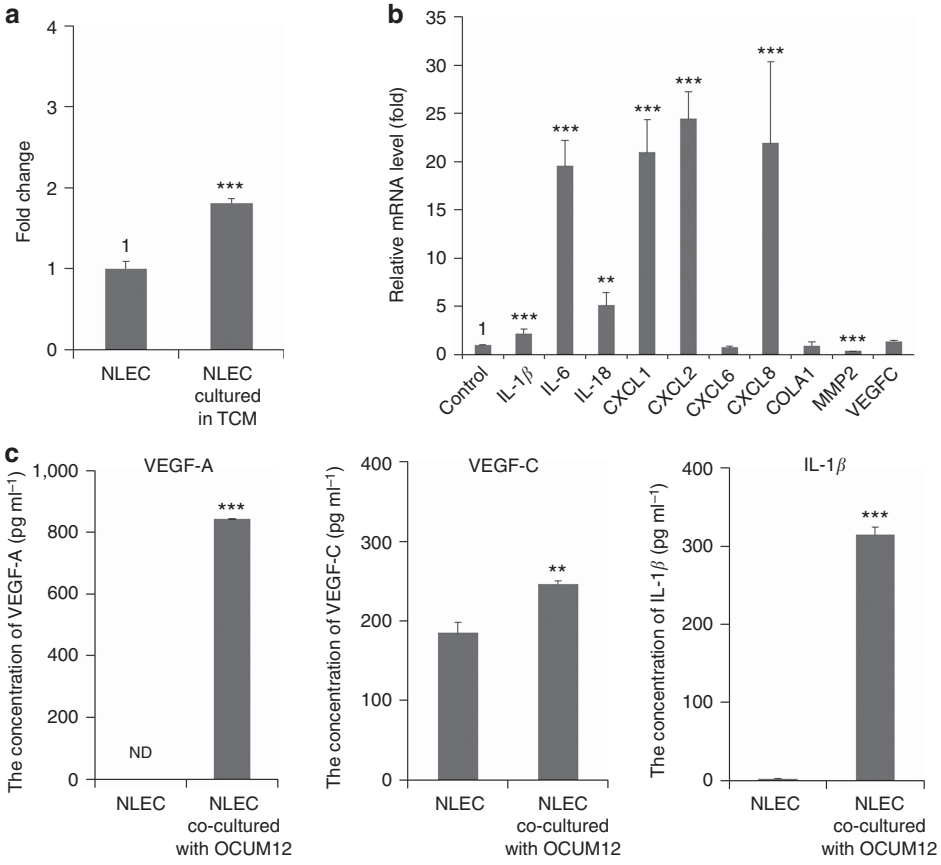

Figure 4. Phenotypic changes in NLECs co-cultured with OCUM12 cells as assessed with MTT assays, qRT-PCR, and ELISA. (**A**) The proliferation activity of NLECs was stimulated when NLECs were cultured in tumour-conditioned medium (TCM) (1.81 ± 0.02-fold). The values represent means of triplicate measurements ± s.d. ***$P<0.001$. (**B**) Changes in gene expression in NLECs co-cultured with OCUM12 cells. Expression of mRNAs encoding cytokines and chemokines in NLECs co-cultured with OCUM12 cells was significantly higher than those in NLECs cultured alone, but not mRNAs encoding CXCL6, COLA1, MMP2, or VEGF-C were not upregulated. Values represent means of quintuplet measurements ± s.d. **$P<0.01$, ***$P<0.001$. (**C**) Production of IL-1β, VEGF-A, and VEGF-C was significantly higher in NLEC co-cultured with OCUM12 cells (842.28 ± 0.95 pg ml^{-1}, 246.23 ± 3.90 pg ml^{-1}, and 314.78 ± 9.81 pg ml^{-1}, respectively) than in those cultured alone. Values represent means of triplicate measurements ± s.d. **$P<0.01$, ***$P<0.001$, ND, not detective.

chemotactic activity that has an important role in cell proliferation, angiogenesis, and immunosuppression that promote tumour progression (Gregory and Houghton, 2011; Fridlender and Albelda, 2012). The TLECs examined here also exhibited increased expression of mRNAs encoding VEGF-A and VEGF-C.

Our findings indicated that the inflammatory response induced by TLECs was likely to be important in promoting development of lymph-node metastasis. Several investigators have demonstrated that the LECs that are commercially available can secrete chemokines to promote migration of cancer cells (Zhuang *et al*, 2010). However, there is no report that LECs isolated from human lymph nodes broadly produce inflammatory cytokines in response to cancer cells.

Next, we used a co-culture system to determine whether cancer cells cause these changes in the property of LECs *in vitro*. The cancer cells induced NLECs to increase proliferative capacity and production of inflammatory cytokines or chemokines, and these NLECs appeared to become more like TLECs. Unlike in TLECs, CXCL6, COLA1, and MMP2 were not elevated in NLECs co-cultured with cancer cells. We propose that the state of co-culture system represented an early microenviromental change and that there were no changes in the abundance of molecules that are associated with cell migration because such changes occur at later stages in cancer progression. In a mouse model of gastric cancer, lymphangiogenesis was found to occur not only at the tumour site, but also in tumour-draining lymph nodes, and to correlate with distant metastasis beyond the sentinel lymph nodes (Hirakawa *et al*, 2007); these authors proposed that the process of lymph node lymphangiogenesis occurs through several coincidental mechanisms: exposure to growth factors from the tumour site or from inflamed tissue within the lymph nodes. We previously demonstrated that intranodal lymphangiogenesis occurs even before cancer spreads to lymph nodes (Watanabe *et al*, 2014). Our findings suggested that some sort of soluble factors secreted by cancer cells could convert NLECs to malignant LECs, which we now call TLECs.

We showed that expression of mRNAs encoding type I collagen or matrix metalloprotease-2 (MMP-2) was markedly higher in TLECs than in NLECs. Tumour stroma is characterised by high levels of type I and type III collagens and degradation of Type IV collagen (Kauppila *et al*, 1998; Huijbers *et al*, 2010); these characteristics promote migration of cancer cells towards interstitial ECM and metastasis. Type I collagen is a main component of ECM. The collagen fibers surrounding tumours linearise and thicken (Provenzano *et al*, 2006; Levental *et al*, 2009). This alteration in the collagen fibers promotes cell migration (Levental *et al*, 2009), leading to metastasis. Indeed, the increased expression of type I collagen correlates with the risk of metastasis (Ramaswamy *et al*, 2003; Tavazoie *et al*, 2008). MMP-2 is an enzyme that cleaves type IV collagen and is associated with tumour angiogenesis and tumour-cell migration (Kessenbrock *et al*, 2010). TLEC-mediated upregulation of type I collagen and MMP-2 may

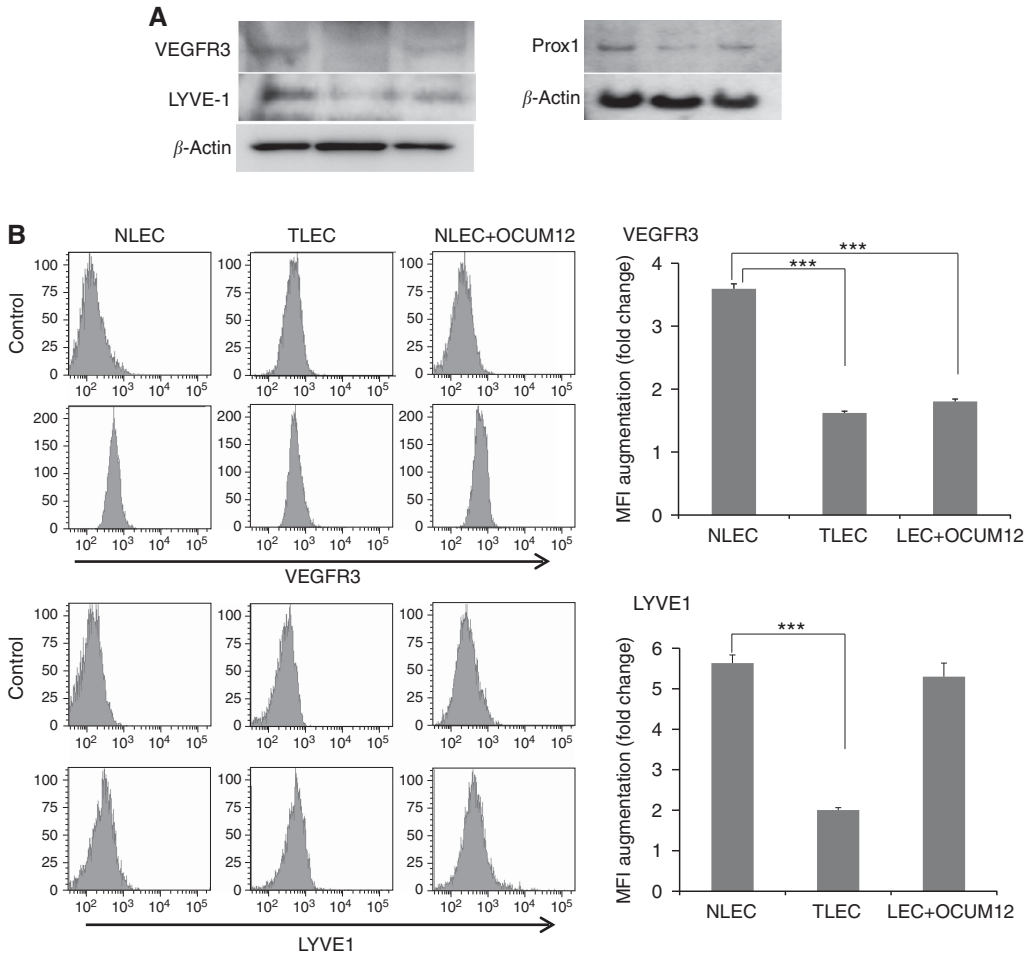

Figure 5. Differences in expression of lymphatic endothelial markers between NLECs, TLECs, and NLECs co-cultured with OCUM12 cells. (**A**) Expression of VEGFR3, LYVE-1, and Prox1 was downregulated in TLECs and NLECs co-cultured with OCUM12 cells. (**B**) NLECs expressed VEGFR3 and LYVE-1. The expression of VEGFR3 was significantly downregulated in TLECs and NLECs co-cultured with OCUM12 cells. The expression of LYVE-1 was reduced in TLECs, but no significant difference was observed in NLECs co-cultured with OCUM12 cells. Values represent means of triplicate measurements ± s.d. ***$P<0.001$. MFI, mean fluorescent intensity.

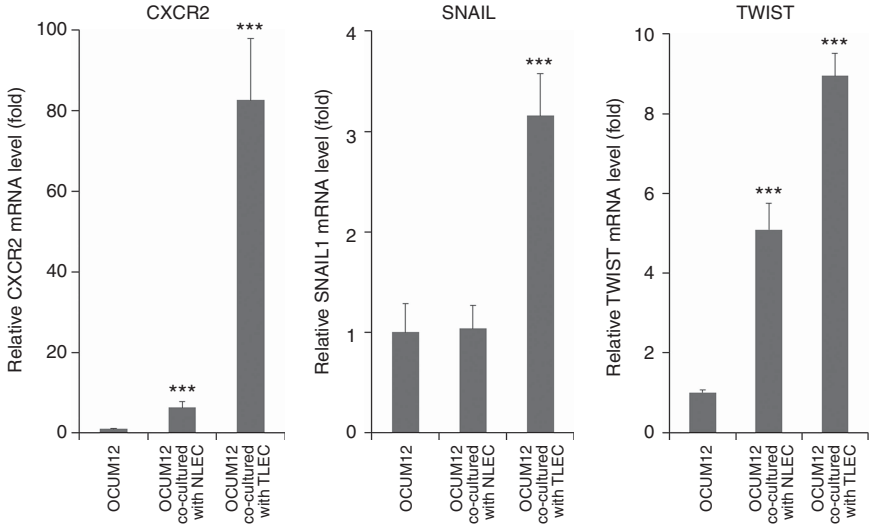

Figure 6. Alterations in mRNA expression in OCUM12 cells co-cultured with TLECs. TLECs induced increases in expression of mRNAs encoding CXCR2, SNAIL, or TWIST in OCUM12 cells co-cultured with TLECs. The values represent means of quintuplet measurements ± s.d. ***$P<0.001$.

contribute to ECM remodelling and thereby promote a milieu in which tumour cells are likely to metastasis. In this study, both the TLECs and NELCs co-cultured with cancer cells exhibited decreased expression of three LEC markers— LYVE-1, VEGF-R3, and Prox1—when compared with NLECs. Notably, inflammatory cytokines suppress expression of LYVE-1 in human dermal LECs

by (Johnson *et al*, 2007). Other investigators showed that LYVE-1 expression is decreased in intratumoral lymphatic vessels of breast cancer (Williams *et al*, 2003). Vigl *et al* (2011) demonstrated that inflammation induced by contact hypersensitivity leads to the downregulation of these three LEC-lineage markers (LYVE-1, VEGF-R3, and Prox1) in murine skin LECs. Moreover, LYVE-1 expression is reduced in the aberrant lymphatic vessels in lymphatic malformations (Wu *et al*, 2015). Prox-1 activity is required for differentiation of LECs into a more lymphatic phenotype (Wigle *et al*, 2002). Our results suggested that cancer cells might have the potential to suppress the differentiation of mature LECs and that TLECs might be a type of undifferentiated LECs.

The EMT is involved in metastatic events during cancer progression. For example, the EMT process leads to the prevention of senescence, resistance to chemotherapy, production of immunosuppressive cytokines, and activation of regulatory T cells. Co-culture with LECs did not seem to cause significant augmentation of cancer-cell proliferation (Supplementary Figure 2); however, we documented that mRNAs encoding EMT-related proteins, including SNAIL and TWIST, were upregulated in cancer cells. Sullivan *et al* (2009) reported that ectopic expression of IL-6 represses E-cadherin and maintains the EMT phenotype in breast cancer cells; these results indicate that increasing inflammatory cytokines (including IL-6) via TLEC-activated *Snail* and *Twist* transcription may induce EMT phenotypes in cancer cells. Moreover, expression of CXCR2, a receptor for CXCL1, CXCL2, CXCL6, and CXCL8, was higher in OCUM12 cells co-cultured with TLECs than in those co-cultured with NLECs or cultured alone. Reportedly, CXCL1 secreted from LECs stimulates tube formation and LEC migration by interacting with CXCR2 (Xu *et al*, 2012).

Our study has several limitations. First, we isolated only two TLEC lines from lymph nodes. This result indicated that TLECs might be rare in some tumour draining lymph nodes. However, these two TLEC lines, each derived from a different lymph node, were nearly identical to each other morphologically and functionally. The low rate of successfully isolating and culturing TLECs was probably due to interference by metastatic tumour cells or some sort of procedural issues. Second, we did not identify the key molecule that altered the properties of NLECs. Based on our results, we speculated that NLECs changed to TLECs because of factors that move through the collecting vessels. As described, we isolated intranodal TLECs from the subcapsular region around the afferent lymphatic vessels; this region was likely to be exposed to soluble factors from the primary tumour.

To the best of our knowledge, this is the first description of the identification of tumour-associated intranodal LECs with phenotypes capable of inducing metastatic characteristic in cancer cells. Our findings indicated that TLECs have a central role in the microenvironment that promotes metastasis of gastric cancer to lymph nodes. Here, we have provided a new conceptual framework for elucidating the mechanisms by which tumours spread to distant sites via lymph nodes and for designing new targeting therapies to prevent lymphangiogenesis in gastric cancers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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