Tumour Plasticity and Extravascular Circulation in ECV304 Human Bladder Carcinoma Cells

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Abstract. *Background: The concepts of vasculogenic mimicry and mosaic vessels have been proposed as novel modes of tumour neovascularisation. However, the presence and significance of these types of neovascularisation remain unclear. Materials and Methods: ECV304 human bladder carcinoma cells were used to determine how tumour cells take part in tumour neovascularisation. Results: Subcutaneous ECV304 xenografts in mice showed various vessel types, including angiogenic vessels, tumour cell-related vessels and extracellular matrix networks. A tracer experiment demonstrated perfusion of beads in these structures. ECV304 cells, cultured on collagen I gels, formed tube networks with expressions of several endothelial-related markers. In coculture models of ECV304 cells and human umbilical vein endothelial cells, the two cells collaborated to form sprouts or networks. Conclusion: ECV304 cells possess an endothelial character which confers the ability to mimic and collaborate with vascular endothelial cells and facilitates the acquisition of tumour microcirculation.*

The growth and progression of solid tumours depend upon the acquisition of a microcirculation (1). Although it was believed that sprouting angiogenesis from the host vessels is the primary mechanism of tumour neovascularisation, recent studies have proposed several other concepts such as vessel cooption (2), endothelial progenitor cell-related neovascularisation (3), fluid-conducting extracellular matrix meshwork (4, 5), tumour sinuses (6), mosaic vessels (7) and vasculogenic mimicry (8). Among these new concepts, the latter three, in which tumour cells line the luminal surface of the vessels, might have a great impact on metastasis and anticancer therapy.

Vasculogenic mimicry, in particular, has been investigated intensively in terms of its presence, mechanisms and biological relevance *in vivo* and *in vitro* (9). However, the evaluation of such tumour-lined vessels *in vivo* has been somewhat controversial. Therefore, further evidence should be provided to establish appropriate methods of evaluation *in vivo*.

Tumour sinuses, mosaic vessels and vasculogenic mimicry in tumours are distinct phenomena, but they overlap in some respects. To obtain a blood flow from the host circulation, the tumour-lined vessels must have connections somewhere with the host endothelial cell-lined vessels. Hence, a common theme in these concepts is how tumour cells and host endothelial cells interact with each other. In addition, tumour plasticity, *i.e.* the expression of endothelial cell-related markers, may play a critical role in connecting tumour cell-lined vessels with the host endothelial cell-lined vessels.

In this study, a human bladder carcinoma cell line, ECV304, which was first considered to be a spontaneously transformed human umbilical vein endothelial cell line (10, 11), was utilised. Here, evidence is provided of tumour cell-lined vessels in the ECV304 xenograft. In addition, using *in vitro* models, a true-lumenised tube formation by ECV304 cells was characterised and it was demonstrated how these cells interact with endothelial cells. To our knowledge, this study is the first to demonstrate tumour cell-related vessels in ECV304 tumours and to elucidate the perfusion of bead tracers in these structures, which are immunohistochemically defined.

Materials and Methods

Cell culture. A human bladder carcinoma cell line, ECV304, was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan), and cultured in minimum essential medium (MEM; Nissui, Tokyo, Japan) containing 10% heat-inactivated foetal bovine serum (FBS, BioWhittaker, Walkersville, MD,

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USA), 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 at 37˚C. Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA, USA), and cultured in MCDB131 medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10 mM L-glutamine (Nissui), 10% FBS and 10 ng/ml human recombinant basic fibroblast growth factor (bFGF) (Wako, Osaka, Japan). The HUVECs used in this study had been passaged 5 to 8 times.

Subcutaneous xenografts in nude mice. $5x10^6$ of ECV304 cells were suspended in 0.2 ml of phosphate-buffered saline (PBS) for injection. Five-week-old male BALB/cAnCrj-nu/nu mice (Charles River, Yokohama, Japan) were given a subcutaneous injection of the tumour cell suspension into the back. The mice were sacrificed 28 days after the inoculation and the tumours were excised and prepared for further examination. All *in vivo* experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University, Japan.

Perfusion of fluorescent beads and immunohistochemistry for mouse CD31 in ECV304 subcutaneous xenografts. Fluorescent beads (FluoSpheres, F8805, 0.2 mm, Molecular Probes, Eugene, OR, USA) diluted 1:10 in heparinized saline, were used to perfuse ECV304 xenografts in mice. In brief, mice bearing day-28 tumours were euthanised with an intraperitoneal injection of 500 ml of Nembutal (5 mg/ml; Dainippon Pharmaceutical, Osaka, Japan) containing 25 mg of nitroglycerine (Cambridge Isotope Lab, Andover, MA, USA). After a midline sternotomy, a 24-gauge cannula was inserted into the thoracic aorta. The thoracic vena cava was incised to exsanguinate the animal and heparinized saline was drip-infused *via* the thoracic aorta until the discharge became clear. Subsequently, 20 ml of bead suspension was infused and then the tumour samples were excised and snap-frozen immediately. Then 10-μm-thick sections were subjected to immunostaining for mouse CD31 (MEC13.3, 1:200; BD Biosciences Pharmingen, USA).

On-gel culture and tube formation. A 300 ml volume of collagen I gel (3mg/ml, Cellmatrix Type I-A; Nitta Gelatin, Osaka, Japan) was applied to a 10-cm culture dish and polymerised for 30 min at 37˚C in a humidified 5% CO_2 incubator. Then, $1x10^6$ ECV304 cells were seeded onto the collagen-coated dish in the culture medium. The formation of tubes was observed at different time-points under an inverted microscope.

Azan histochemistry and immunohistochemistry. The xenografts were cut into blocks and fixed in 4% paraformaldehyde (PFA) and processed to make paraffin-embedded sections, then stained by Azan histochemistry (Muto Pure Chemicals, Tokyo, Japan), according to the manufacturer's instructions. To visualise the tumour vessels, the sections were immunostained with anti-mouse CD34 monoclonal antibody (RAM34, PharMingen International, San Diego, CA, USA), as described previously (12). Some sections were counterstained with haematoxylin and eosin (H&E) to highlight red blood cells.

The ECV304 cells, cultured on collagen I gels, were characterised by immunocytochemistry with anti-human CD34 mouse monoclonal antibody (QBEnd/10 Ready-to-use; No Markers, Fremont, CA, USA), anti-human CD31 mouse monoclonal antibody (JC/70A Ready-to-use; DAKO, Carpenteria, CA, USA), and anti-human von Willebrand Factor (vWF) mouse monoclonal antibody (Clone F8/86, 1:50; DAKO, Glostrup, Denmark). Briefly, after fixation and washing, the sections were incubated with the first antibodies at 4˚C overnight. The next day, the immunoreactivity was detected using the Vectastain Elite ABC Reagents and Kits. DAB was used as chromogen and the sections were counterstained with haematoxylin. Non-specific IgGs were substituted for the first antibodies in negative controls to avoid misinterpretation of the results.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from ECV304 cells cultured on the collagen I-coated dish at different time-points using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) and reverse transcribed using the First-Strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK), according to the manufacturer's instructions. Also, total RNA extracted from HUVECs and ECV304 cells cultured without collagen I was used for controls. PCR amplifications were performed with published gene-specific primers using a standard PCR protocol with TaKaRa Ex Taq (Takara Bio, Shiga, Japan), except for VE-cadherin in which the Advantage cDNA PCR Kit (BD Biosciences, Palo Alto, CA, USA) was used as per the manufacturer's instructions. Amplification reactions were performed as follows: 1 cycle of 94˚C for 2 min; 35 cycles of 94˚C for 1 min, annealing temperature (T_a) for 1 min (2.5 min for VE-cadherin) and 72˚C for 1 min; and 1 cycle of 72˚C for 10 min. As a control for contaminating genomic DNA, the reactions were performed using the RNA preparations without reverse transcription, and these reactions did not demonstrate specific bands for any of the primers. Equal loading of the samples was confirmed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control.

The oligonucleotide primer sequences, annealing temperature (T_a), and product size were as follows: KDR: forward: 5'-ATGCACGGCATCTGGGAATC-3', reverse: 5'-GCTACTGTCC TGCAAGTTGCTGTC-3', T_a: 58˚C, product size: 537 bp; human CD34: forward: 5'-GCACCCTGTGTCTCAACATGG-3', reverse: 5'-GCACAGCTGGAGGTCTTATTTTGC-3', Ta: 58˚C, product size: 380 bp; human CD31: forward: 5'-CAACGAGAAAATGT CAGA-3', reverse: 5'-GGAGCCTTCCGTTCTAGAGT-3', T_a: 53˚C, product size: 256 bp; human VE-cadherin: forward: 5'-CCGGCGCCAAAAGAGAGA-3', reverse: 5'-CTGGTTTTCCT TCAGCTGGAAGTGGT-3', T_a: 68˚C, product size: 1029 bp; human vWF: forward: 5'-AGGCAGCTCCACTCGGCACAT-3', reverse: 5'-AGCAAACAGTGGTAAGAGGAGGAC-3', T_a: 58˚C, product size: 582 bp.

Coculture of HUVECs and ECV304 cells. HUVECs and ECV304 cells were fluorescent-labelled with CellTracker Orange CMTMR or Green CMFDA (Molecular Probes, Eugene, OR, USA), as per the manufacturer's instructions.

First, 300 μl of Matrigel was applied to each well of a 24-well plate and incubated for 30 min at 37˚C to polymerise. Then, 1 ml of MCDB131-based full medium containing $1x10^4$ cells from each cell line was added to the Matrigel-coated well.

The mixture of 500 cells of each cell line was suspended in 100 μl of MCDB131-based full medium in a non-adhesive round-bottomed 96-well plate (SumilonCellTight Multiwell Plate Spheroid 96U; Sumitomo Bakelite, Akita, Japan). The day after seeding, they formed chimeric spheroids. The spheroids were collected and

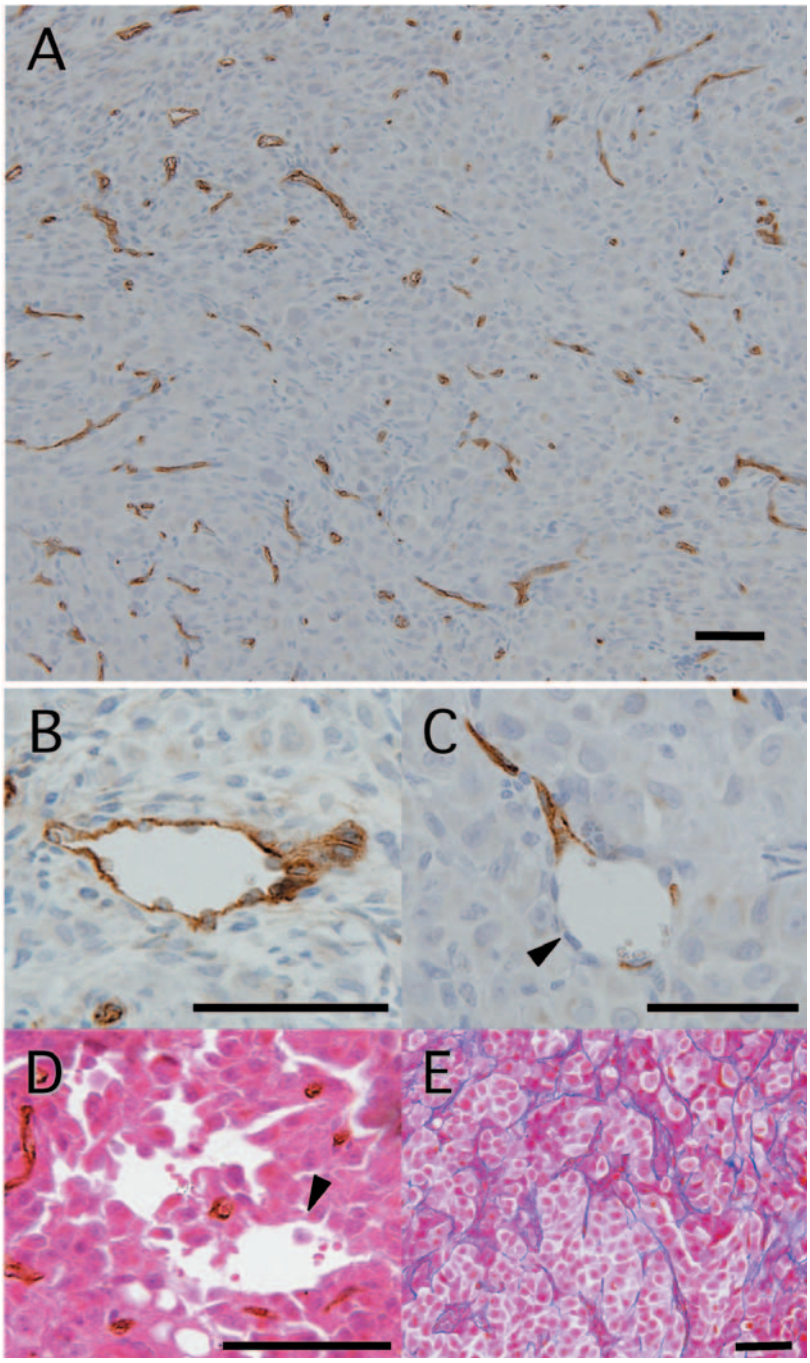

Figure 1. *Immunohistochemistry of the ECV304 xenograft for mouse CD34 (A-D) counterstained with haematoxylin (A-C), or with H&E (D). (A) A gross overview. (B) An angiogenic vessel. (C) A mosaic vessel (arrowheads indicate the vessel wall lacking immunoreactivity). (D) Tumour sinuses (arrowhead). (E) Azan staining illustrates the fine networks of the ECM (shown in blue) surrounding the tumour packets. Scale bars: 50mm.*

resuspended in MCDB131 medium supplemented with 30% FBS and 30 ng/ml bFGF, then mixed with collagen I gel to make 2 mg/ml gel and plated for the in-gel culture. The network formation on the Matrigel, or the sprouts of chimeric spheroids, were monitored with an inverted fluorescent microscope. Images were captured with a digital camera provided with the microscope, and processed using Adobe Photoshop.

Results

Various types of tumour vessels in the ECV304 xenograft. At 28 days after inoculation, the ECV304 cells had formed subcutaneous tumours approximately 10 mm in diameter in nude mice. Azan staining of the tumour sections highlighted

the fine networks of extracellular matrix (ECM) surrounding the tumour packets (Figure 1 E). To better characterise the tumour vasculature, the tumour sections were immunostained for mouse CD34 with some sections counterstained with H&E to highlight red blood cells. The anti-mouse CD34 immunostaining detected many vessels in the tumour (Figure 1 A). With close observation at higher magnification, in addition to endothelial cell-lined vessels (Figure 1 B), mosaic vessels that consisted of CD34-positive and negative cells (Figure 1 C) and tumour sinuses (Figure 1 D) were observed. To examine the actual circulation in the ECV304 xenografts, perfusion experiments were carried out by injecting the fluorescent bead suspension into tumour-bearing mice 28 days after inoculation. Immunostaining for mouse CD31 showed a similar staining pattern to that for mouse CD34 (Figure 2 A). Most of the bead-positive areas were found colocalised with CD31-positive vessels, whereas, although infrequently (<5% of the entire circulation), bead-positive channels without CD31 staining were detected (Figure 2 B, C). Fluorescent beads were also observed in tumour sinuses (Figure 2 D, E). Furthermore, some ECM networks were found perfused with tracers (Figure 2 F).

Analyses of in vitro tube formation of ECV304 cells. ECV304 cells (Figure 3 A, C), cultured on the collagen I-coated dish, formed tube structures that were similar in shape with the tubes of HUVECs (Figure 3 B). The tubes of ECV304 cells started to form 4 days after plating, and increased up to week 4 of the culture. H&E staining of the tubes demonstrated that they had true lumens and were not solid cords (Figure 3 D, E). Immunostaining for human endothelial cell-related markers revealed that the tubes formed by ECV304 cells cultured on collagen I were strongly-positive for CD34, weakly-positive for CD31 and negative for von Willebrand factor (Figure 4). Negative controls, in which the primary antibodies were substituted with the non-specific isotype-matched IgG, proved that the tube structures had functional lumens, because the lumen of the tubes physically trapped the antibody and stained positive (Figure 4 D, E, F).

To investigate the endothelial cell-related gene expression in the ECV304 cells cultured on collagen I, RT-PCR was carried out for the total RNA extracted from the cells at different time-points (Figure 5). ECV304 cells cultured without collagen I, which did not form tubes, expressed KDR, CD34 and VE-cadherin, while they expressed CD31 only weakly, and did not express the vWF. As demonstrated above, they formed tube structures when cultured on collagen I, and these structures grew up to around day 28 of the culture. The expression of KDR increased on day 14 of the culture and decreased on day 28, whereas CD34 expression basically did not change throughout the experiment. The expression of CD31 was up-regulated until day 28 of the culture. VE-cadherin expression was down-regulated from day 7 until day 28 of the culture. The expression of vWF was not detected throughout the experiment.

Cooperative interaction of HUVECs with ECV304 cells in cocultures. To investigate the mechanism by which the tumour cell-lined vessel is formed, the interaction between ECV304 cells and HUVECs was observed using cocultures (Figure 6). Fluorescent-labelled cells formed cord structures cooperatively when cocultured on Matrigel. Likewise, when they were cocultured under non-adhesive conditions, they aggregated to form chimeric spheroids. The chimeric spheroids cultured in the collagen I gels projected sprouts that were formed by both cell types.

Discussion

Hendrix *et al.* described the entire microcirculation in aggressive tumours as complex, and made up of a combination of elements such as “mosaic vessels”, “co-opted vessels”, “angiogenic vessels” and “the fluid-conducting meshwork” (9). Consistent with this remark, we have demonstrated the complexity of the tumour vasculature using a human bladder carcinoma cell line xenograft model. Among several types of tumour vasculature, tumour cell-lined vessels, *i.e.* tumour sinuses, tumour cell-lined channels and mosaic vessels, are of great importance because of their biological significance to haematogenous metastasis as well as a strategy for anticancer therapy. However, there is no clear evidence of the existence of tumour-lined vessel tubes *in vivo*. As McDonald and colleagues argue against vasculogenic mimicry (13, 14), these vessel-like structures lacking endothelial cells could be merely a blood lake or a stagnation of extravasated blood cells. It is necessary to establish well-designed *in vivo* models that show functional blood flow in well-defined tumour-lined vessels.

To address this issue, we established a method to detect tumour-cell lined vessels by combining tracer perfusion and immunohistochemistry in the ECV304 xenograft model. We demonstrated the perfusion of the bead tracer in the structures devoid of CD31 immunoreactivity. These structures possibly represent tumour cell-lined channels (Figure 2 B, C), tumour sinuses (Figure 2 D, E) and ECM networks (Figure 2 F), although it is not easy to determine whether they have significant functional circulation. Indeed, it is also possible that they represent only extravasated beads retained in interstitial spaces. However, the tracer-positive but CD31-negative structures, *i.e.* tumour cell-lined channels, tended to locate in the areas where CD31-positive vessels were rather sparse. Besides, the extravascular beads were observed strictly within

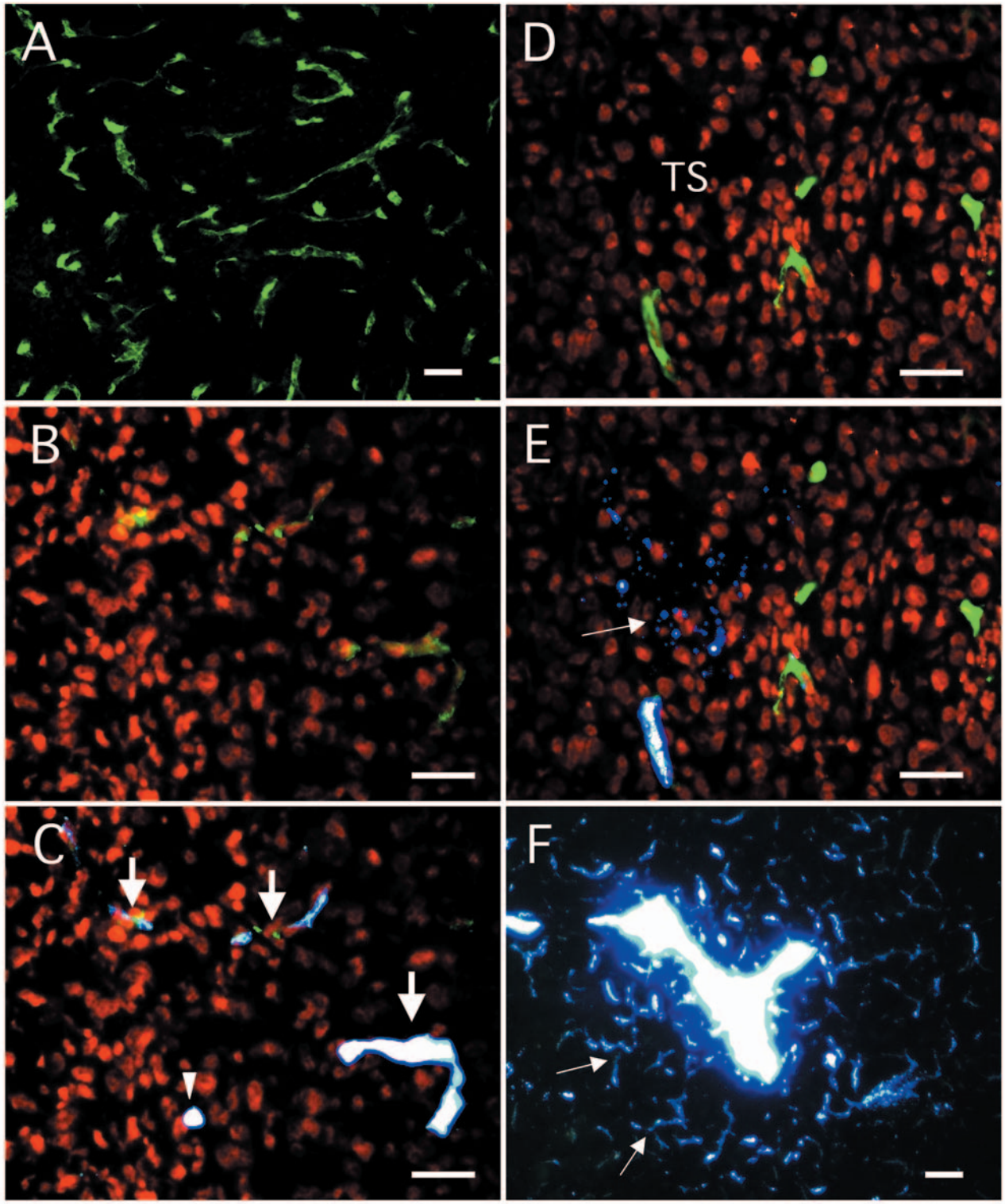

Figure 2. *Immunohistochemistry for mouse CD31 (green) and perfusion of fluorescent beads (blue-white) in ECV304 xenografts 28 days after inoculation. (A) The staining pattern for CD31. (B, C) Fluorescent images from an identical section with CD31 staining and autofluorescence of nuclei (red) in (B), and with the image of perfused beads overlaid in (C). Arrows in (C) indicate angiogenic vessels with colocalization of CD31 immunoreactivity and fluorescent beads. The arrowhead points to a channel with perfused beads without CD31 staining. (D, E) Another set of images from an identical section. In (E), beads are detected in a tumour sinus that is devoid of CD31 staining (arrow). (F) A fluorescent image from a 30-μm-thick section without immunohistochemistry. Arrows show the ECM networks perfused with beads. TS: tumour sinus. Scale bars: 30 μm.*

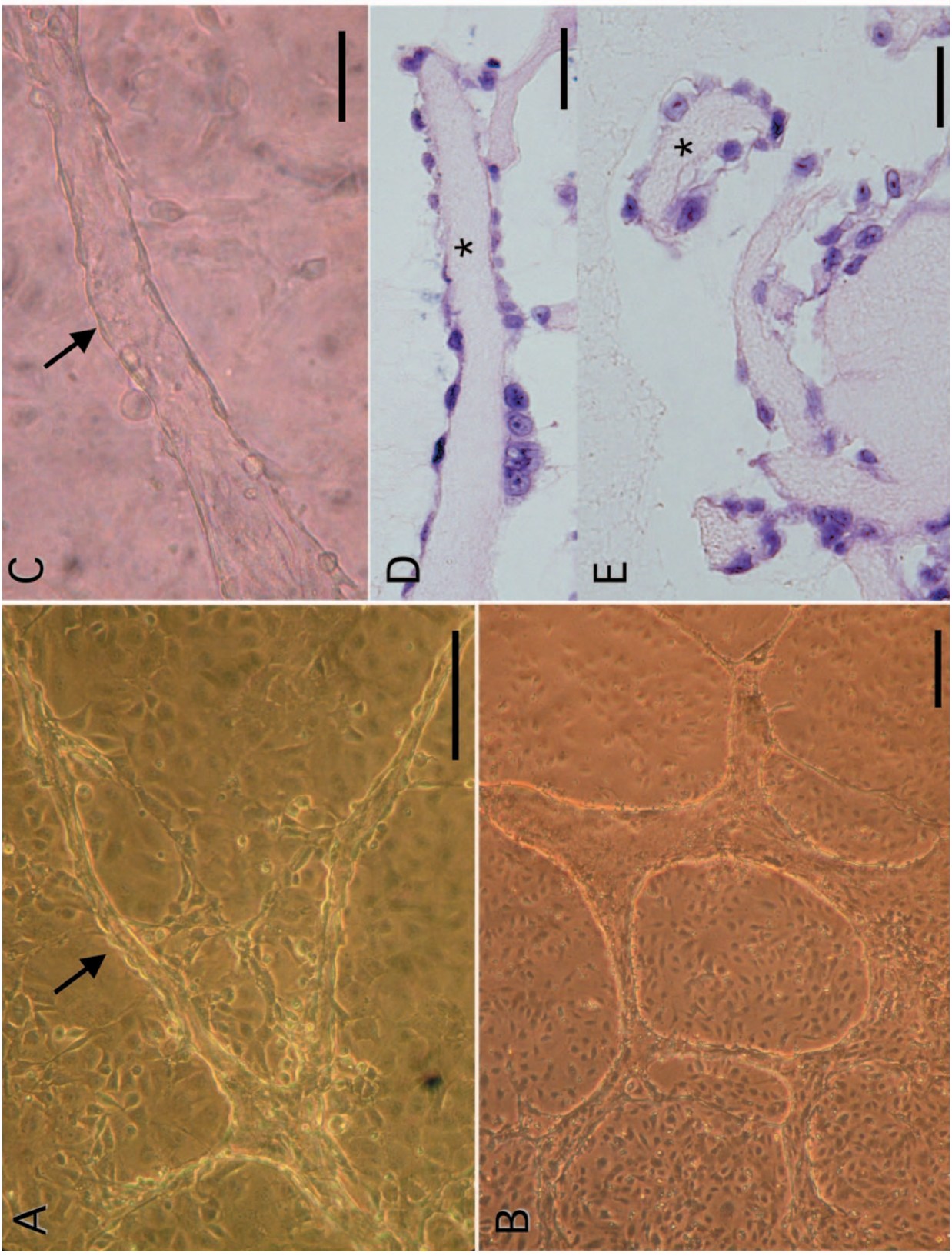

Figure 3. *Tube formation on the collagen I gel. (A, C) Representative figures of day-14 cultures of ECV304 cells forming tube structures (arrows). (B) Tube formation by HUVECs at day 7. (D, E) H&E staining of the ECV304 tubes revealed true lumens (asterisks). Scale bars: (A, B) 100 μm; (C-E) 20 μm.*

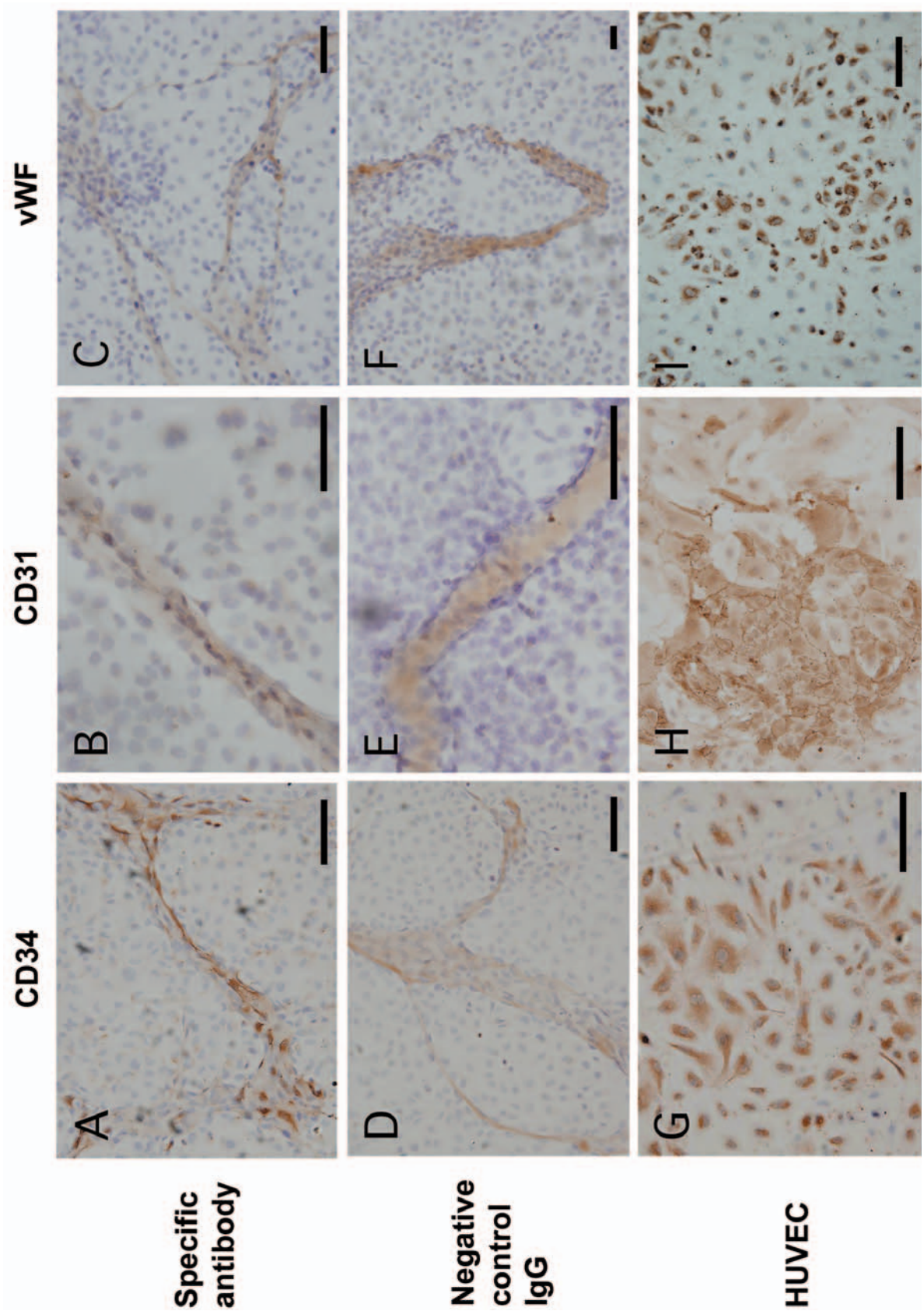

Figure 4. *Immunocytochemistry of ECV304 cells cultured on collagen I gels. The tubes formed by ECV304 cells were positive for human CD34 (A), weakly positive for human CD31 (B), and negative for human von Willebrand factor (vWF, C). Negative controls for CD34 (D), CD31 (E) and vWF (F) demonstrated positive staining inside the tubes, providing evidence that these tubes are lumenised. (G, H, I) Positive controls using HUVECs for the respective antibodies. Scale bars: 100 μm.*

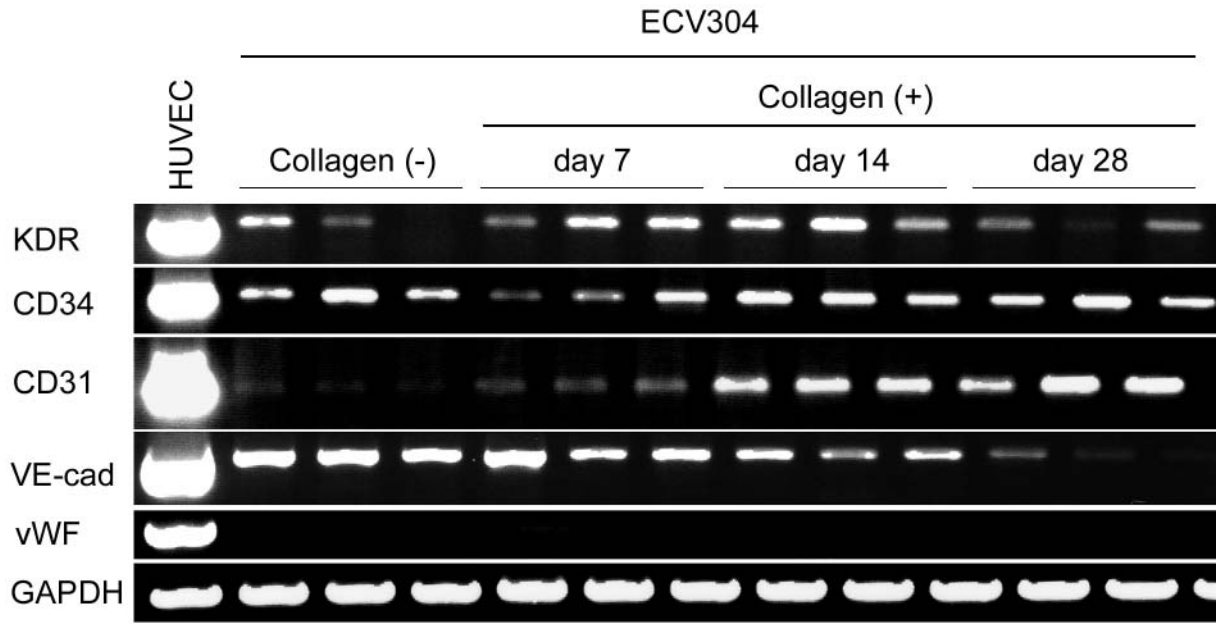

Figure 5. *RT-PCR analysis of the endothelial cell-related markers. Total RNA from three independent samples under the indicated culture conditions was subjected to analysis. 'Collagen (+) or (-)' stands for the conditions where ECV304 cells are cultured with or without collagen, respectively. HUVECs were used as positive controls. GAPDH served as a control for the equal loading of the samples.*

tumour cell-lined channels , tumour sinuses or ECM networks, while random scattering of the tracer due to leakage or artifacts was infrequent. These observations led to the speculation that tumour cell-lined vessels do exist and play some role in the circulatory system in the ECV304 xenografts.

In this study, the expression of several endothelial cell-related genes was detected in the ECV304 cells. RT-PCR revealed the expression of KDR, CD34, CD31 and VE-cadherin under the culture conditions without collagen I, where tube formation was not observed. It has been shown, in many reports, that cancer cells express endothelial cell markers in various settings. KDR, vascular endothelial growth factor (VEGF) receptor-2, is one of these markers. Several reports have demonstrated the expression of KDR on tumour cells such as ovarian carcinoma cells (15), breast tumour cells (16), prostate cancer cells (17) and ECV304 cells (18). As Dunk and Ahmed have shown that VEGF stimulated DNA synthesis and increased ECV304 cell numbers, the expression of KDR might play a role in an autocrine stimulation loop in some tumours. Similarly, the expression of CD34 has been reported in a variety of tumours, including melanoma (19), gastrointestinal stromal tumours (20), neural tumours (21) and an array of tumours of vascular origin (22). Although the functional significance of CD34 remains unclear, the coexpression of KDR and CD34 represents one of the features of haemangioblasts in the development of the haemato-vascular system (23). Furthermore, VEGFR-2-positive cells, derived from murine embryonic stem cells, can differentiate into endothelial cells, mural cells and blood cells *in vitro* (24). Therefore, although we could not provide evidence of haematopoiesis in the current *in vitro* models, the coexpression of KDR and CD34 on ECV304 cells might suggest that they have the potential to behave like haemangioblasts, provided that their lineage is not fully committed.

When cultured on the collagen I gels, the ECV304 cells formed tubes, which grew up to day 28 of the culture. Along with the morphological changes, the expression levels of CD31 (PECAM-1) and VE-cadherin altered in a reciprocal manner. It has been shown that CD31 and VE-cadherin play a major role in cell-cell contacts in vascular endothelial cells, as well as the tube formation by various types of cells. However, the discrete functions of these molecules in tube formation are quite controversial. Basically, the spatial and temporal distributions of these two molecules differ (25). Besides, although antibodies against each protein could impair the tube formation by HUVECs in a three-dimensional culture model, CD31 appeared to be required for cell elongation, while VE-cadherin seemed to play some role in intercellular lumen formation *via* the process of

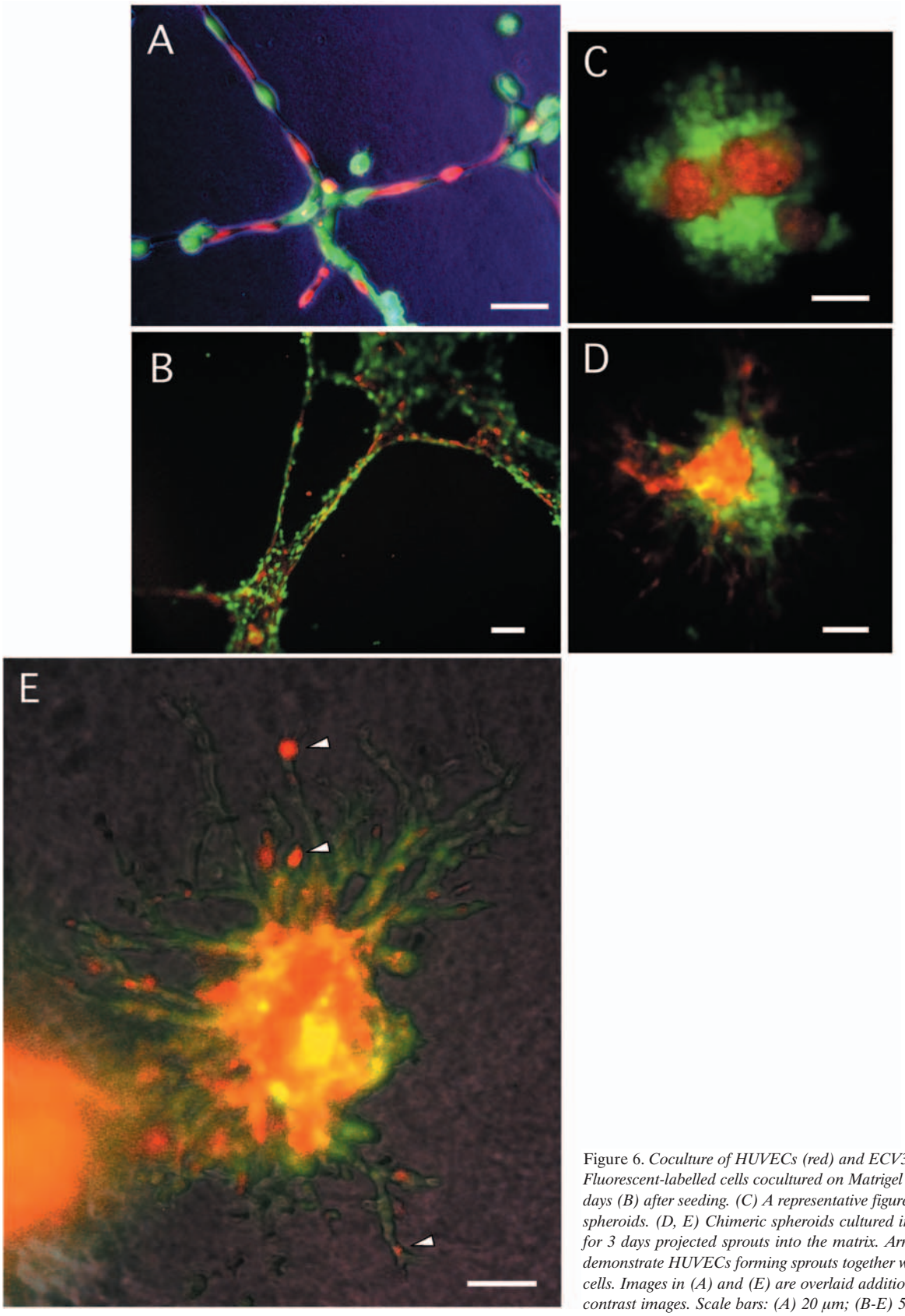

Figure 6. *Coculture of HUVECs (red) and ECV304 cells (green). Fluorescent-labelled cells cocultured on Matrigel 1 day (A) and 2 days (B) after seeding. (C) A representative figure of the chimeric spheroids. (D, E) Chimeric spheroids cultured in collagen I gels for 3 days projected sprouts into the matrix. Arrowheads in (E) demonstrate HUVECs forming sprouts together with the ECV304 cells. Images in (A) and (E) are overlaid additionally with phase contrast images. Scale bars: (A) 20 μm; (B-E) 50 μm.*

vacuolisation or vacuole fusion (26). In addition, it was reported that human mesothelioma cells, when transfected with a PECAM-1-expressing vector, obtained the ability to form tubes (27). Taken together, CD31 and VE-cadherin play a differential role in tube formation, according to the experimental model.

In this study, the interaction between HUVECs and tumour cells in cocultures was demonstrated. In the three-dimensional chimeric spheroid culture, the sprouts from the spheroids were composed of both cell types forming mosaic vessel-like structures. This finding may explain how mosaic vessels are formed in some tumours. This interaction was not exclusive to the coculture of HUVECs and ECV304 cells, because a similar network formation was observed in the model comprising HUVECs and HT1080 human fibrosarcoma cells (data not shown). Although we did not investigate the molecules responsible for the phenomena, this heterotypic contact may have resulted from some junctional molecules expressed on the cell membranes of both types of cells. With regard to the promoting effects on tumour growth and angiogenesis with the coexistence of tumour and endothelial cells, it is possible that vasculogenic mimicry has some influence on tumour growth other than providing an alternative circulation. With the transdifferentiation of a population of tumour cells to confer endothelial-like properties in a given tumour environment, the interaction between endothelial-like tumour cells and wild-type tumour cells might induce autostimulation, to give rise to a more aggressive tumour phenotype. This hypothesis could be supported by the report that the coinjection of mouse mammary carcinoma cells with endothelial cells differentiated from rhesus embryonic stem cells led to more rapid tumour growth and vascularisation (28).

In conclusion, tumours have a variety of mechanisms by which they acquire a blood circulation and, in particular, they can participate in neovascularisation by exploiting their plasticity. However, further studies are needed to clarify whether these mechanisms correlate directly with haematogenous metastasis and tumour progression.

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