

## Article

# Vasculogenic Mimicry: A Potential Therapeutic Target for Chondrosarcoma Therapy

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## Abstract

Chondrosarcomas (ChSs) are mesenchymal chemo- and radiation-resistant tumors, representing the second most frequently diagnosed bone sarcoma after osteosarcoma and 20% of all bone sarcomas. Most of ChS patients have a good prognosis after complete surgical resection. Conversely, patients with inoperable disease, due to the tumor location or metastatic dissemination, represent a great clinical challenge due to the lack of effective therapeutic options. In this study, to the best of our knowledge, we document, for the first time in human ChS tissues, the existence of CD-31- and Podoplanin-negative vascular-like channels containing red blood cells, allowing us to hypothesize the occurrence of vasculogenic mimicry (VM) in ChSs. By using patient-derived ChS cells and a stabilized ChS cell line, we demonstrate that ChS cells are able to form in vitro tubules apparently similar to those formed by endothelial cells. Further characterization of these vessels revealed the pivotal role of the Urokinase Plasminogen Activator Receptor (uPAR) in mediating the capability of ChS cells to form VM. Finally, we provide evidence that, unlike bevacizumab, which did not exert any effect, the uPAR-derived antiangiogenic peptide RI-3 behaves as a potent inhibitor of VM.

**Keywords:** Chondrosarcoma; angiogenesis; vasculogenic mimicry; urokinase receptor (uPAR); uPAR inhibitor peptide



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## 1. Introduction

Chondrosarcomas (ChSs) are mesenchymal cartilage-producing tumors, representing the second most frequently diagnosed bone sarcoma after osteosarcoma and 20% of all bone sarcomas [1–3]. Most ChS patients have a good prognosis when a radical surgical resection of the tumor may be achieved, whereas inoperable patients represent a great clinical challenge due to the lack of effective therapeutic options [4,5]. Although originating from hypoxic, avascular cartilaginous tissues, an intense microvessel density in ChS tissues has been documented to be associated with an aggressive clinical behavior, a higher metastatic

potential and poor progression-free survival (PFS) [6,7]. In ChSs, vascularity increases with increased histologic grade, with blood vessels located either in peri- or intra-cartilage areas [6]. Some years ago, while studying the relationship between angiogenesis, tumor-associated macrophages and PFS in patients affected by ChSs [8], we noted that some vessel-like structures do not express the CD31 and CD34 endothelial markers nor the lymphatic vessel marker Podoplanin [9–11]. This observation led us to hypothesize that vasculogenic mimicry (VM) could exist in ChS tissues.

In 1999, Maniotis and coworkers [12] documented, for the first time in malignant melanoma tissues, the presence of vascular channels constituted by stem-like cancer cells trans-differentiated into an endothelial-like cell phenotype. These endothelial-like cells allow the generation of vascular networks capable of conducting fluids, supplying oxygen, transporting nutrients, and eliminating cellular debris, independently of angiogenesis and lymph angiogenesis [12]. Since then, VM has been deeply investigated, and its existence has been found in a variety of malignant tumors [13], including osteosarcoma [14], Ewing sarcoma [15], mesothelial sarcoma, and alveolar rhabdomyosarcoma [16].

VM vessels appear as hollow tubes lined by cancer cells that rest on a non-continuous basement membrane consisting of type I and IV collagens and laminin, arranged to form dense deposits which may be identified by positive staining with periodic acid–Schiff (PAS) [17] and negative staining with CD31/CD34 endothelial markers and the lymphatic marker Podoplanin. Although many efforts have been made to identify specific markers useful for distinguishing VM from canonical angiogenesis and lymph angiogenesis, to date, VM can only be identified by positive/negative staining approaches. A first positive approach is to stain pink glycoproteins of tumor cells forming the vessel-like networks by PAS staining. Another strategy consists of ascertaining by immunohistochemistry that cells forming vessels do not express the angiogenic marker CD31 nor the lymphatic vessel marker Podoplanin (negative staining approach) [10,11]. Recently, Ephrin Type-A Receptor2 (EphA2), which has been shown to drive chemoresistance and metastasis in bone sarcomas, has been suggested to be an additional marker of VM in osteosarcoma and Ewing sarcoma [18]. Considering the pivotal role of vascularization in dictating ChS progression [6,7], we decided to investigate the possibility that VM coexists with canonical angiogenesis in ChS tissues.

## 2. Materials and Methods

### 2.1. Chondrosarcoma Patients

Fifteen patients affected by chondrosarcoma (ChS), already recruited by our Institute in the last fifteen years, and three new ones were included in the study. All patients provided a written informed consent for the use of their tissue samples. The study was approved by the Ethics Committee of the National Cancer Institute of Naples. Histopathological diagnoses were reviewed on standard hematoxylin and eosin-stained slides according to the 2020 WHO Classification updated by Choi J et al. [19].

### 2.2. Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4 µm thin Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections. All staining procedures were carried out using the automated Discovery Ultra Stainer (Ventana Medical Systems-Roche, Tucson, AZ, USA), following the manufacturer's instructions. Paraffin slides were deparaffinized in xylene and rehydrated. After antigen retrieval, performed by Cell Conditioning 1 solution (pH 8.5, Ventana Medical Systems, Tucson, AZ, USA), the blocking of endogenous peroxidase and nonspecific binding sites was performed according to the manufacturer's instructions. Then, the tissues sections were exposed to the following ready-to-use primary

monoclonal anti-bodies: anti-CD31 mAb (clone JC70; Roche, Cat#05463475001) for 48 min to identify the platelet endothelial cell adhesion molecule-1 (PECAM1); anti-Podoplanin mAb (clone D2-40; Roche, Cat#05463645001) for 24 min to recognize Podoplanin, which is expressed by the lymphatic vessels; anti-Ki-67 mAb (clone MIB-1 99; Dako M7240) for 16 min to identify the proliferating cells. All staining procedures were carried out using the anti-mouse horseradish peroxidase (HRP) as secondary antibody (Roche, Cat#05269695001) and the HRP Chromogen kits: Green HRP Kit (Roche, Cat#08478295001), Purple HRP Kit (Roche, Cat#07053983001), and Chromo Map DAB Kit (Roche, Cat#05266645001). Finally, nuclei were stained with hematoxylin and the sections were mounted. Images were acquired using a light microscope and analyzed using the Carl Zeiss Axiovision 4.4 software.

Quantitative evaluation of microvessels as well as the identification and count of Ki-67-positive ChS cells were independently conducted by two pathologists who were blinded to the clinical information. Each tissue section was scored for CD31-/Podoplanin-negative microvessels and Ki-67-positive tumor cells counted in five randomly selected tumor fields per sample ( $\sim 6.9 \text{ mm}^2$ ) at  $200\times$  magnification. For each section, CD31-/Podoplanin-negative microvessels and Ki-67 positive tumor cells were scored from 1 to 4, as previously reported [8] and as detailed in Table 1.

**Table 1.** Histopathological information of enrolled patients affected by chondrosarcoma.

| Patients | Age (yr) | Gender | Site                | Size (cm)     | Histology <sup>a</sup> | Grade | Ki-67 Score <sup>b</sup> | VM Score <sup>c</sup> | Primary Cells |
|----------|----------|--------|---------------------|---------------|------------------------|-------|--------------------------|-----------------------|---------------|
| 1        | 72       | M      | Left femur          | 26 × 15 × 13  | DD ChS                 | G3    | 3                        | 3                     |               |
| 2        | 63       | F      | Right iliac wing    | 13 × 9 × 13   | DD ChS                 | G3    | 2                        | 2                     |               |
| 3        | 77       | F      | Left femur          | 14            | DD ChS                 | G3    | 3                        | 3                     |               |
| 4        | 64       | F      | Left humerus        | 13            | DD ChS                 | G3    | 3                        | 3                     |               |
| 5        | 38       | M      | Left knee point     | 23            | DD ChS                 | G3    | 2                        | 2                     |               |
| 6        | 71       | F      | Left humerus        | 14            | DD ChS                 | G3    | 3                        | 2                     |               |
| 7        | 69       | M      | Sternum             | 17 × 14       | ChS                    | G3    | 3                        | 3                     |               |
| 8        | 34       | F      | Left obturator ring | 22 × 17 × 20  | ChS                    | G3    | 3                        | 2                     |               |
| 9        | 61       | M      | Left obturator ring | 11 × 13 × 7   | ChS                    | G2    | 1                        | 1                     |               |
| 10       | 39       | F      | Right shoulder      | 9 × 4 × 6     | ChS                    | G2    | 2                        | 2                     |               |
| 11       | 75       | M      | Left femur          | 24 × 14 × 9   | ChS                    | G3    | 2                        | 2                     |               |
| 12       | 41       | M      | Left knee point     | 7 × 6 × 4     | ChS                    | G2    | 2                        | 1                     |               |
| 13       | 48       | M      | Left humerus        | 4.5 × 3.5 × 1 | ChS                    | G2    | 1                        | 1                     |               |
| 14       | 64       | M      | Left shoulder       | 20 × 16 × 15  | ChS                    | G1/G2 | 2                        | 1                     |               |
| 15       | 58       | M      | Sternum             | 18 × 15 × 8   | ChS                    | G2    | 2                        | 2                     | Sarc          |
| 16       | 69       | F      | Left scapula        | 22 × 15 × 11  | ChS                    | G2    | ND                       | ND                    | ChS-1         |
| 17       | 70       | F      | Left femur          | 3 × 9         | DD ChS                 | G3    | ND                       | ND                    | ChS-2         |
| 18       | 80       | M      | Left humerus        | 20 × 12 × 2   | DD ChS                 | G3    | ND                       | ND                    | ChS-3         |

ChS, chondrosarcoma; F, female; M, male; DD, dedifferentiated. <sup>a</sup> Histopathological diagnosis in accordance with the 2020 WHO Classification. Ki-67-positive tumor cells <sup>b</sup> and CD31-/Podoplanin-negative microvessels <sup>c</sup> were scored as 1 to 4, according to the number of proliferating tumor cells <sup>b</sup> and CD31-/Podoplanin-negative microvessels <sup>c</sup> countered as follows. VM score: 1: <5 vessels in 5 fields; 2: 5–10 vessels in 5 fields; 3: 11–20 vessels in 5 fields; 4: >20 vessels in 5 fields. Ki-67 score 1: <5 tumor cells/field; 2: 5–10 tumor cells/field; 3: 11–20 tumor cells/field; 4: >20 tumor cells/field.

### 2.3. Periodic Acid–Schiff (PAS) Staining

Glycogen, glycoproteins and proteoglycans staining in the FFPE sections from 15 ChS tissues was obtained by periodic acid–Schiff (PAS) manual staining. FFPE sections were deparaffinized in xylene, rehydrated and then rinsed with running water. Slides were incubated in 0.5% periodic acid solution (Sigma-Aldrich, Milan, Italy) for 5 min at room temperature, rinsed with running water and subsequently exposed to Schiff reagent (Sigma-Aldrich) for an additional 10 minutes, following the manufacturer's instructions. Finally, the sections were counterstained with hematoxylin and mounted. Each slide was digitized with the scanner Aperio AT2 Leica (Leica Biosystems, Wetzlar, Germany) and subsequently analyzed using the Aperio Image Scope software v12.4.6 (Leica Biosystems).

In a subset of experiments, PAS staining allowed us to identify the glycogen content in vessels formed by Chs cells when seeded on Matrigel. To this end, the newly formed

vessels were fixed for 30 min in 4% PBS-buffered formaldehyde at RT and processed for PAS staining by incubating the samples with 0.5% periodic acid solution (Sigma-Aldrich) for 10 min, with Schiff reagent (Sigma-Aldrich) for 25 min, and subsequently with an aqueous solution containing of 0.05 N HCl and 5 mg/mL sodium metabisulfite for 15 min at RT. Images were acquired using a light microscope equipped with Axiovision 4.8 software (Zeiss, Milan, Italy).

#### 2.4. Patient-Derived Primary ChS Cells and Cell Cultures

Representative fresh samples from the tumor excision (~1 cm × 1 cm) were promptly minced and enzymatically digested for 3 h with 1 mg/mL collagenase XI (Sigma-Aldrich) at 37 °C in order to obtain patient-derived primary ChS cells, as previously described [8,20]. Recovered cells were seeded and cultured in 6-well plates in Dulbecco's Modified Essential Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 100 µg/mL streptomycin and 100 U/mL penicillin, until they formed an adherent and homogeneous population. Patient-derived primary established Sarc cells obtained from Patient 15 (Table 1) and primary cells ChS-1, ChS-2, and ChS-3 obtained from Patients 16, 17, and 18, respectively (Table 1) were cultured in DMEM 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). The SW1353 chondrosarcoma cell line, provided by ATCC, was cultured in Leibovitz's L-15 Medium (Gibco) with the addition of 10% FBS, 100 µg/mL streptomycin and 100 IU/mL penicillin and maintained at 37 °C, 100% air, according to the manufacturer's instructions. Human umbilical vein endothelial cells (HUVECs) (Promo Cell), used between the third and the seventh passage, were grown in Eagle Basal Medium (EBM) supplemented with 4% FBS, 1 µg/mL hydrocortisone, 0.1% gentamicin, 12 µg/mL bovine brain extract and 10 µg/mL epidermal growth factor (Cambrex). All cells were periodically tested using the mycoplasma detection kit (MycoAlert, Lonza Bioscience, Braine-l'Alleud, Belgium) to verify the absence of mycoplasma.

#### 2.5. Tube Formation Assay

The capability of human endothelial and ChS cells to form vascular-like structures was investigated as previously described [21,22] with minor modifications. Briefly, µ-Slide 15 Well 3D (Ibidi) was coated with 10 µL Growth Factor-Reduced (GFR) Matrigel (Corning, Milan, Italy). After Matrigel polymerization, obtained at 37 °C, the cells ( $8 \times 10^3$  cells/sample) resuspended in 50 µL pre-warmed serum-free or complete medium were seeded onto Matrigel. Assays were carried out for 24 h or for the indicated times at 37 °C, 5% CO<sub>2</sub>.

When indicated, cells were preincubated with 500 µg/mL Bevacizumab (Avastin) or 10 nM retro-inverso RI-3 peptide (Ac-d-Tyr-d-Arg-Aib-d-Arg-NH<sub>2</sub>) custom-synthesized on solid-phase and purified by reversed-phase HPLC by JPT Peptide Technologies GmbH (Berlin, Germany). Each experiment was performed in quadruplicate and replicated at least three times. Images were acquired with a digital camera using the Axiovision 4.8 software (Zeiss). Quantitative analysis of newly formed tubes was assessed using the Angiogenesis Analyzer tool of ImageJ 1.51j [23]. The time-lapse imaging allowed us to monitor in real time (frame every 15 min) the formation of vascular-like structures, using an inverted phase-contrast microscope (Axiovert 200, Zeiss) equipped with a motorized stage and an incubation chamber to ensure the 37 °C and 5% CO<sub>2</sub> conditions for live cells. Furthermore, in a subset of experiments (flow cytometric and mRNA analyses), Sarc cells that formed tubes on Matrigel were recovered using Cell Recovery Solution (Corning), according to the manufacturer instructions. For these experiments, to obtain an adequate number of cells to analyze,  $1.5 \times 10^5$  cells/well were seeded on 24-multiwell plates coated with 300 µL Matrigel/well.

## 2.6. Western Blot

Cells were lysed in RIPA buffer (10 mM Tris pH 7.5, 0.1% SDS, 140 mM NaCl, 0.5% NP40, 1% Triton X-100) supplemented with a protease inhibitor cocktail (cOmplete™ Mini Protease Inhibitor Cocktail, Roche, Basel, Switzerland). Protein concentration was determined using the Bradford assay (Bio-Rad, Milan, Italy). Briefly, equal amounts (40 µg) of proteins from each cell lysate were resolved on 10% SDS-PAGE gel, under non-reducing conditions for uPAR or reducing conditions for VEGFR-2. Gels were transferred onto a nitrocellulose membrane (Amersham cytiva) and the membranes were blocked with 5% non-fat dry milk (uPAR) or 5% BSA (VEGFR-2) in TBS 0.1% Tween 20 (Fisher Scientific, Waltham, MA, USA), before probing with mAb anti-uPAR (1:1000 dilution, Abcam, Cambridge, UK, ab221680) or anti-VEGF Receptor 2 (1:1000 dilution, Rabbit mAb 55B11, Cell Signaling Technology, Danvers, MA, USA, #2479) overnight at 4 °C or 0.2 µg/mL anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA, sc-47724) at RT for 2 h. After washing, membranes were incubated with horseradish peroxidase-conjugated anti-mouse (Invitrogen, Carlsbad, CA, USA) or anti-rabbit antibody (Thermo Scientific, Waltham, MA, USA) and detected by enhanced chemiluminescence (ECL) (Amersham-GE Healthcare, Marlborough, MA, USA). Chemiluminescent signals were detected and images captured using the eBright 1500 imaging system (Thermo Fisher, Waltham, MA, USA).

## 2.7. Cell Proliferation

Cell proliferation was assessed using the xCELLigence Real-Time Cell Analysis (RTCA) technology (Acea Bioscience, San Diego, CA, USA). Briefly, Sarc cells or HUVECs ( $1 \times 10^4$  cells/well) were seeded in E-16-well plates in complete medium (DMEM 10% FBS or EBM 2% FBS) in the absence of treatments (None) or supplemented with 50 ng/mL VEGF-A (Peprotech, Rocky Hill, NJ, USA). Gold microelectrodes integrated into the bottom of the culture plates measure impedance variations, expressed as Cell Index, which proportionally correlate with the number of proliferating adherent cells. Impedance in each well was monitored over 96 h. Slopes (time range 0–96 h) were calculated from the cell-growth curves. The experiments were performed twice in quadruplicate.

## 2.8. Flow Cytometry

Cytofluorimetric analysis was performed in order to evaluate the expression levels of potential VM markers on HUVEC and ChS cell surfaces, recovered from 2D culture dishes. When indicated, Sarc cells performing VM were recovered from Matrigel using a cell recovery solution, as described above. Briefly, single-cell suspensions were incubated for 30 min at 4 °C in the dark with the following antibodies: PE-Cy7-conjugated anti-CD34 mAb (clone 8G12 –348811, BD Biosciences, San Jose, CA, USA), ABflo 488-conjugated anti-Podoplanin mAb (A24408, ABclonal, Woburn, MA, USA), ABflo 647-conjugated anti-CD31 mAb (A22509, ABclonal), ABflo 488-conjugated anti-VE Cadherin mAb (A23350, ABclonal), APC-conjugated anti-uPAR mAb (clone VIM5, Miltenyi Biotec, Bergisch Gladbach, Germany), and unconjugated anti-EphA2 polyclonal Ab (PA5-14574, Invitrogen). In the latter case, after the primary Ab, cells were incubated with the Alexa 488-conjugated F(ab')<sub>2</sub> fragment of anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) at 4 °C for 30 min in the dark. Then, the acquisition of samples was performed using the BD FACS Canto II (BD Biosciences) and data analysis was carried out using the FlowJo V10 software. Negative controls included unstained cells or omission of the primary antibody, the latter being the case when the unconjugated anti-EphA2 Ab was applied.

### 2.9. Real-Time PCR

Real-time PCR analysis was performed to assess PLAUR mRNA levels in Sarc cells cultured in 2D conditions or VM-performing Sarc cells recovered from Matrigel, as described above.

Total RNA was isolated using TRIzol solution (TRI reagent, Sigma-Aldrich) according to the manufacturer's instructions. RNA concentration and purity were measured by NanoDrop (ThermoFisher Scientific, Waltham, MA, USA), with A260/280 and A260/230 ratios used as indicators of RNA purity, and retrotranscribed (2 µg/samples) using the High-Capacity RNA-to-cDNA Kit (ThermoFisher). Quantitative real-time PCR was carried out with an Applied Biosystem QuantStudio 7 Pro System (Applied Biosystems, Foster City, CA, USA) by using TaqMan Universal PCR Master Mix (Life technologies, Carlsbad, CA, USA), uPAR gene expression assay (Applied Biosystems, PLAUR Hs00958880\_m1) and GAPDH (Applied Biosystems, Hs02786624\_g1) as reference genes. Fold change was determined by the comparative Ct method ( $2^{-\Delta\Delta C_t}$ ).

### 2.10. Knockout of uPAR in ChS Cells by CRISPR/Cas9 System

uPAR knockout (KO) cells were generated by applying the CRISPR-Cas9 technique. The CRISPR/Cas9 plasmid (sc-400666), targeting PLAUR exon 3, and the uPAR Plasmid HDR (sc-400666-HDR), providing a specific DNA repair template for a double-strand break, were obtained from Santa Cruz Biotechnology. According to the manufacturer's instructions, cells ( $1.5 \times 10^5$ /well) were seeded in 6-well plates and co-transfected with uPAR Plasmid KO CRISPR/Cas9 and uPAR Plasmid HDR for stable knockout. After 48 h, cells were cultured in complete DMEM supplemented with 1 µg/mL puromycin (Santa Cruz Biotechnology), with medium changes every two days. Surviving cells were sorted to isolate GFP-positive, uPAR-negative populations by BD FACS Melody (BD Biosciences).

### 2.11. Statistical Analysis

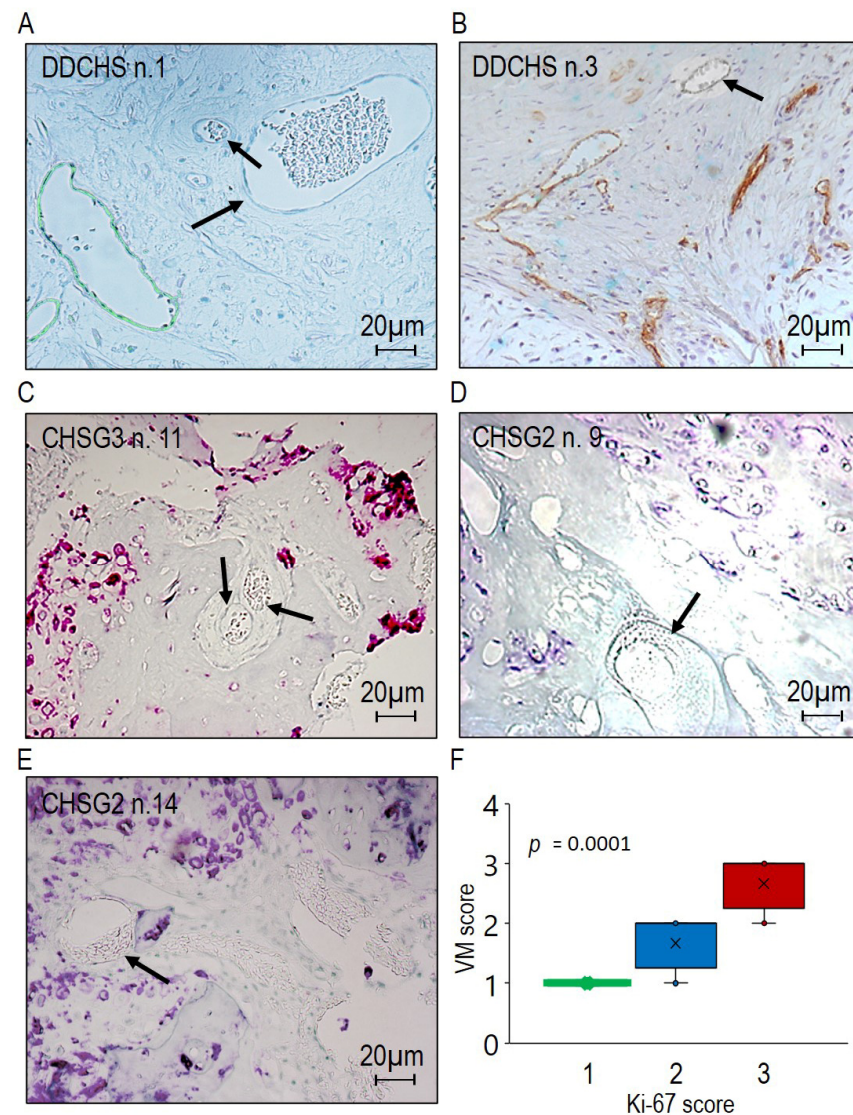
Data are presented as mean  $\pm$  SD of the indicated number of determinations. Normality was assessed using SigmaPlot 14.0 software by applying the Shapiro–Wilk test prior to the use of parametric statistical tests. The significance of the in vitro results was determined using Student's *t*-test and two-way ANOVA, with  $p < 0.05$  considered statistically significant. Pearson's correlation test was employed to evaluate the correlations between CD31, Podoplanin and Ki-67 expression levels, assessed using SPSS v20.0 software (SPSS Inc. Chicago, IL, USA).

## 3. Results

### 3.1. Evidence of Vasculogenic Mimicry in ChS Tissues

As already stated in the Introduction, some years ago, while we were studying the relationship between angiogenesis and tumor aggressiveness in patients affected by ChS [8], we observed in ChS tissues the occurrence of vascular-like structures that do not express the endothelial marker CD31 nor the lymphatic marker Podoplanin, allowing us to hypothesize that, similarly to other bone and soft tissue sarcomas [13–16], VM may occur in ChS.

To investigate whether VM effectively exists in ChS, FFPE sections from fifteen ChS patients, including six dedifferentiated ChS (DD ChS) and nine conventional ChS patients, whose clinic–pathological characteristics are summarized in Table 1, were immunostained with anti-CD31 and anti-Podoplanin Abs to identify endothelial and lymphatic vessels, respectively. As shown in Figure 1A–E, besides CD31 endothelial and lymphatic Podoplanin-positive vessels, a number of CD31-/Podoplanin-negative vascular-like channels, some of which containing erythrocytes, were observed.



**Figure 1.** Evidence of vasculogenic mimicry in human chondrosarcoma tissues. (A–E) Representative images of ChS tissues processed for IHC analysis of CD31 to identify endothelial vessels and Podoplanin to assess lymphatic vessels. Each tissue section was scored based on the average counts of CD31- and Podoplanin-negative vessels (arrows), counted in the tumor areas, in five randomly selected fields/sample ( $\sim 6.9 \text{ mm}^2$ ) at  $200\times$  magnification. (F) For each section, CD31- and Podoplanin-negative vessels (VM score) and Ki-67-positive tumor cells (Ki-67 score) were scored from 1 to 4, as detailed in Table 1. Box plots show the correlation between VM score and Ki-67 score in human ChS tissues;  $\times$  represents the mean. Pearson correlation:  $r = 0.83$ ;  $p = 0.0001$ ;  $r^2 = 0.69$  ( $n = 15$ ), indicating that higher VM scores are associated with higher Ki-67 expression levels.

Unfortunately, we were unable to identify VM vessels by periodic acid–Schiff positive staining as suggested by Imani and co-workers [17] since both tumor cells and cartilage chondroid matrix in ChS tissues are rich in PAS-positive glycans, as shown in the Supplementary Figure S1. Thus, we decided to investigate whether, besides canonical vascularization, a correlation between vasculogenic mimicry and aggressiveness in ChS effectively exists. Tissue sections from fifteen ChS tumor tissues were subjected to immunostaining with CD31, Podoplanin and Ki-67 Abs to identify and quantify CD31-/Podoplanin-negative vessels as well as the tumor proliferating cells. For each tissue sample, CD31-/Podoplanin-negative channels and Ki-67-positive cells were counted and scored from 1 to 4, depending on the number of unstained vessels and proliferating ChS cells counted in five randomly selected fields/sample ( $\sim 6.9 \text{ mm}^2$ ) at  $200\times$  magnification, as reported in Table 1. We found

that, while in DD ChS tissues VM vessels are homogeneously distributed (Figure 1A,B), in conventional ChS tissues, they are localized preferentially at the margin of cartilaginous nodules (Figure 1C–E). Interestingly, with the exception of ChS sections with VM and Ki-67 score 1, we found a statistically significant correlation between the number of CD31-/Podoplanin-negative vessels and Ki-67-positive ChS cells (Figure 1F), suggesting that, besides angiogenesis and lymphangiogenesis, high levels of VM could have a role in dictating ChS aggressiveness.

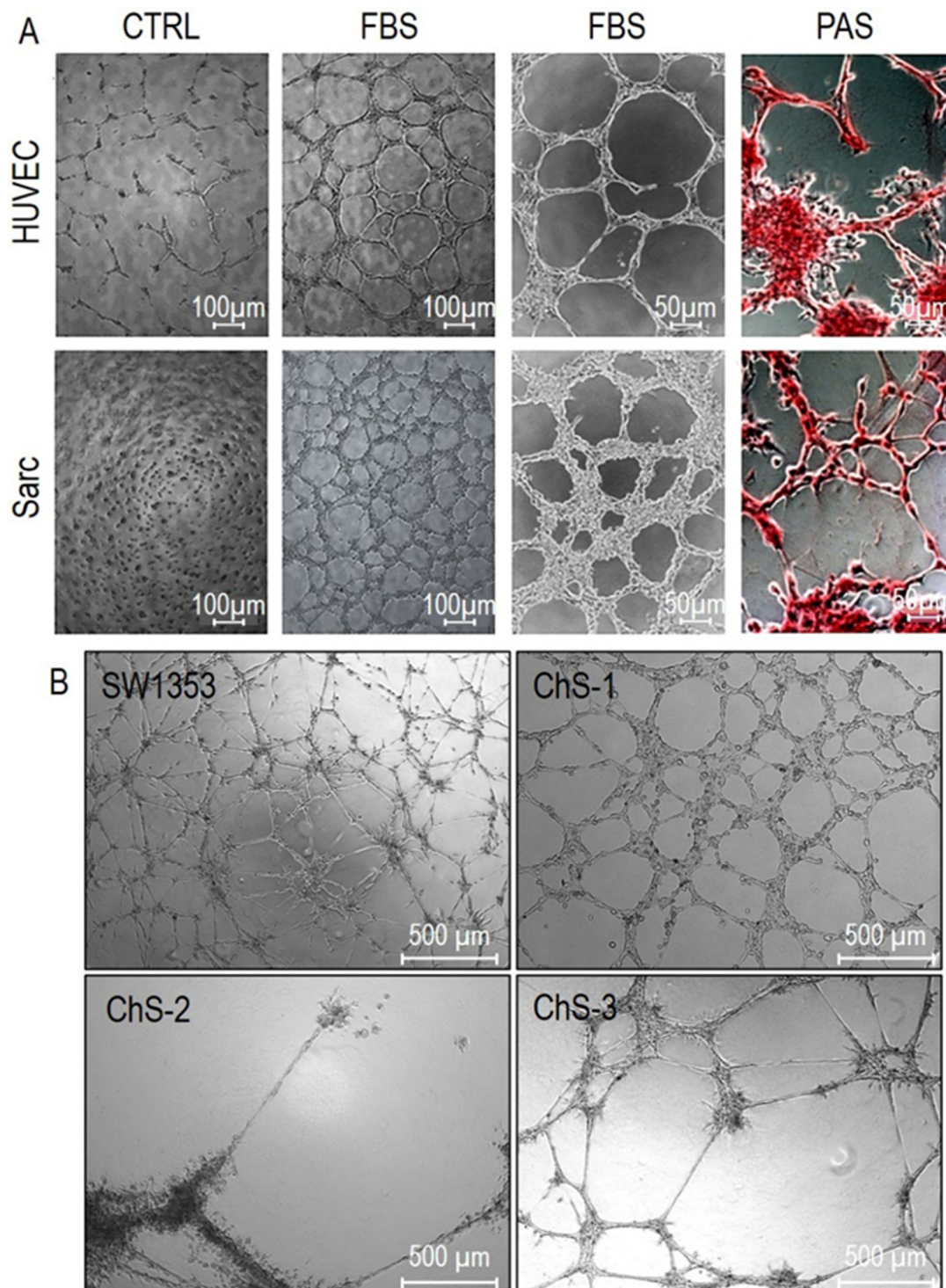
### 3.2. Vascular Channel Formation by Human Chondrosarcoma Cells

In order to have a dynamic picture of the vascular channel formation by ChS cells, stabilized primary Sarc cells were subjected to in vitro tube formation assays, and their tube formation capability was compared to that of the human umbilical vein endothelial cells (HUVECs). The formation of vascular-like structures was monitored in time lapse for 6 h and quantified after 24 h, using the Angiogenesis Analyzer tool of ImageJ software. Each experiment in which HUVECs were included as positive control was performed in quadruplicate and repeated at least three times. As shown in Figure 2A and in the time lapse movie (Supplementary Video S1), we found that, in the presence of serum, Sarc cells derived from Patient 15 (Table 1) and already stabilized in our laboratory [20] are able to form polygonal or round channels developed from cell protrusions that connect to each other, resembling those formed by human endothelial cells (Figure 2A and Supplementary Video S2). As expected, like HUVECs, Sarc cells forming vascular structures were found to be positive to PAS staining (Figure 2A). However, we have to take into the account that chondrosarcoma cells themselves are rich in glycans, as already shown in Supplementary Figure S1, thus limiting the interpretation of PAS positivity in vitro. Furthermore, with the exception of the primary ChS-2 cells that formed very few polygonal networks, SW1353, ChS-1 and ChS-3 cells were found to form cord-like structures (Figure 2B). Interestingly, quantitative analysis of nodes, junctions, meshes and segments allowed us to ascertain that SW1353, Sarc and ChS-1 primary cells, similarly to HUVECs, exhibited a high vasculogenic activity in vitro (Supplementary Figure S2).

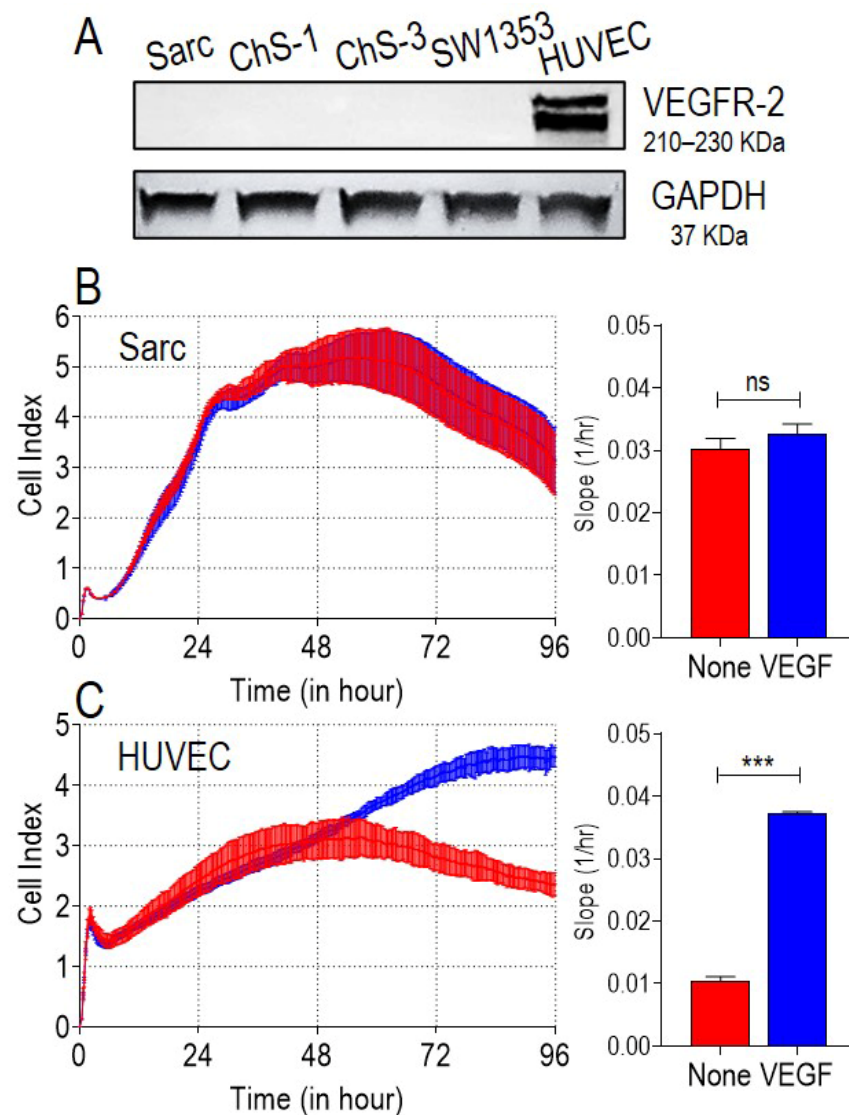
### 3.3. ChS Cell Surface Expression of VM Drivers

ChS cells were further characterized for the expression of potential VM drivers by Western blot and flow cytometry. First, the expression levels of vascular endothelial growth factor receptor 2 (VEGFR-2) were analyzed on protein extracts from ChS cells, by Western blot. As shown in Figure 3A, unlike HUVECs, which exhibit considerable VEGFR-2 expression, no analyzed ChS cells show any detectable expression of the receptor. The absence of VEGFR-2 from ChS cell surfaces was confirmed by cell proliferation experiments by which we ascertained that exposure to VEGF-A does not trigger Sarc cell proliferation (Figure 3B) [24], in contrast to the proliferative effect observed in endothelial cells (Figure 3C).

Then, the vasculogenic nature of ChS cells was investigated by cytofluorimetric analysis of CD31 (Pecam1), CD34, which has been reported to be expressed in a small subset of endothelial cells [25], and the lymphatic marker Podoplanin, using HUVECs as positive controls and Podoplanin-expressing HEK293 cells [26]. As shown in Figure 4 and the relevant quantification chart (Table 2), unlike endothelial cells that, as expected, express CD31 and CD34, no ChS cells express CD31, CD34 or Podoplanin, which is expressed only in HEK-293 cells used as a positive control (Table 2).



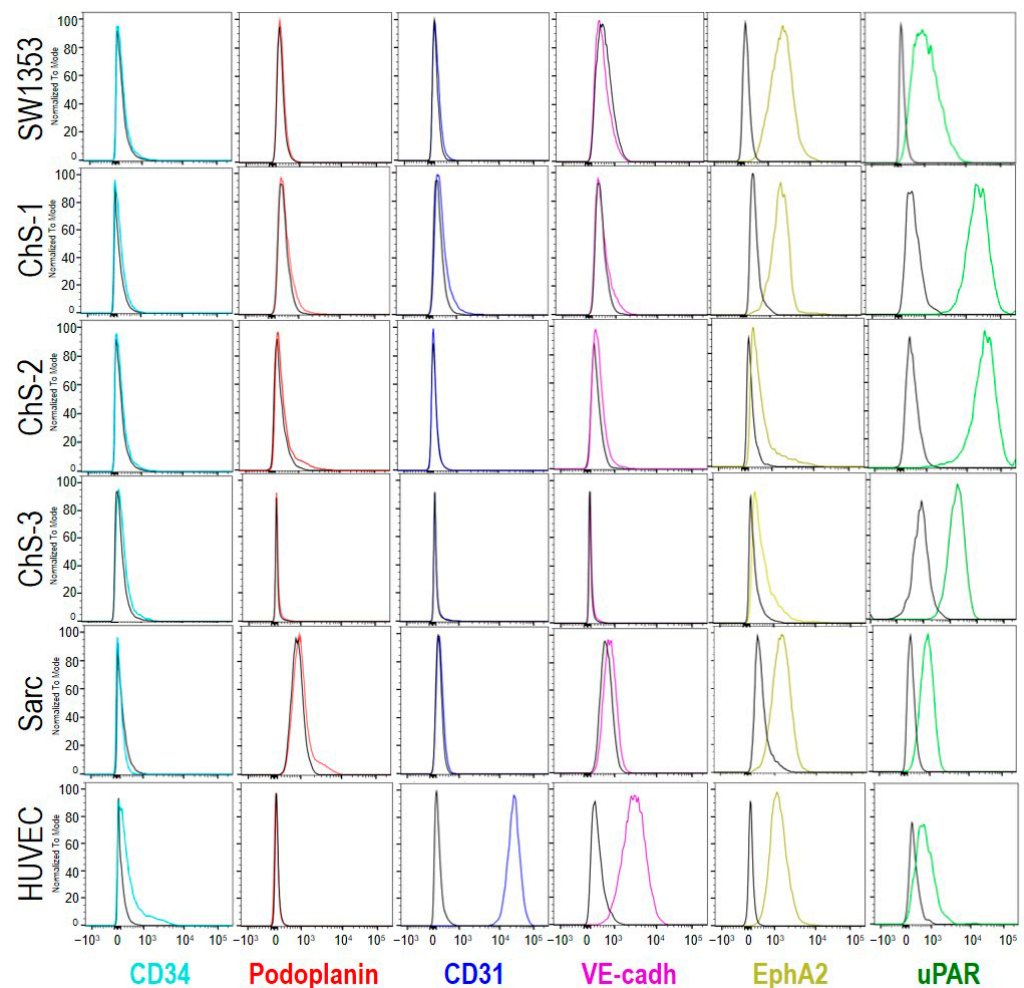
**Figure 2.** In vitro tube formation ability by chondrosarcoma cells. **(A)** Representative images of HUVEC (upper panels) or Sarc cells (lower panels) seeded onto GFR-Matrigel and allowed to form vascular tubules in vitro in the absence of serum (CTRL) or in the presence of FBS. Images acquired using an inverted phase-contrast microscope (Axiovert 200) and Axiovision 4.8 software (Zeiss). Scale bar, 100 μm or 50 μm in panels with higher magnification. Periodic acid—Schiff staining performed on HUVECs and Sarc cells (panels on the right) effectively shows that the newly formed tubes are rich in PAS-positive glycogen. **(B)** Representative images of SW1353 cell line and patient-derived ChS cells (ChS-1, ChS-2, and ChS-3), seeded onto GFR-Matrigel and allowed to form vascular-like tubules. Images acquired using an inverted phase-contrast microscope (Axiovert 200) and Axiovision 4.8 software (Zeiss). Scale bar, 500 μm.



**Figure 3.** ChS cells do not express VEGFR-2, and VEGF-A does not trigger Sarc cell proliferation. (A) Cell lysates (40 µg/samples) from the SW1353 cell line, patient-derived ChS cells, stabilized Sarc cells and HUVECs were resolved on a 10% SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane, followed by Western blot with anti-VEGFR-2 and anti-GAPDH as loading control. Chemiluminescent signals were detected and images captured using the eBright 1500 imaging system. (B,C) Time-dependent Sarc cell (B) and HUVEC (C) proliferation monitored using the xCELLigence RTCA technology. Briefly,  $1 \times 10^4$  Sarc cells (B) or HUVECs (C), seeded in E-16-well plates in the absence (None, in red) or in the presence of 50 ng/mL VEGF-A (VEGF, in blue), were allowed to proliferate over 96 h. The histograms (right panels) report the slope analysis (time range 0–96 h). Data represent mean  $\pm$  SD from quadruplicate experiments. Student's *t*-test ns, not significant; \*\*\*  $p < 0.001$ .

**Table 2.** Geometric mean values calculated for each sample and expressed as fold change over the corresponding unstained negative control.

| Cells  | CD34 | Podoplanin | CD31  | VE-Cadherin | EphA2 | uPAR |
|--------|------|------------|-------|-------------|-------|------|
| SW1353 | 1.1  | 1.1        | 1.3   | 0.9         | 10.1  | 8.8  |
| ChS-1  | 1.2  | 1.1        | 1.3   | 1.3         | 5.3   | 41.3 |
| ChS-2  | 1.1  | 1.5        | 1.0   | 1.3         | 3.3   | 72.5 |
| ChS-3  | 1.3  | 1.2        | 1.1   | 1.3         | 2.4   | 21.8 |
| Sarc   | 0.9  | 1.2        | 1.2   | 1.2         | 4.1   | 3.9  |
| HUVECs | 3.6  | 0.7        | 161.9 | 8.7         | 17.2  | 2.7  |
| HEK293 |      | 10.6       |       |             |       |      |



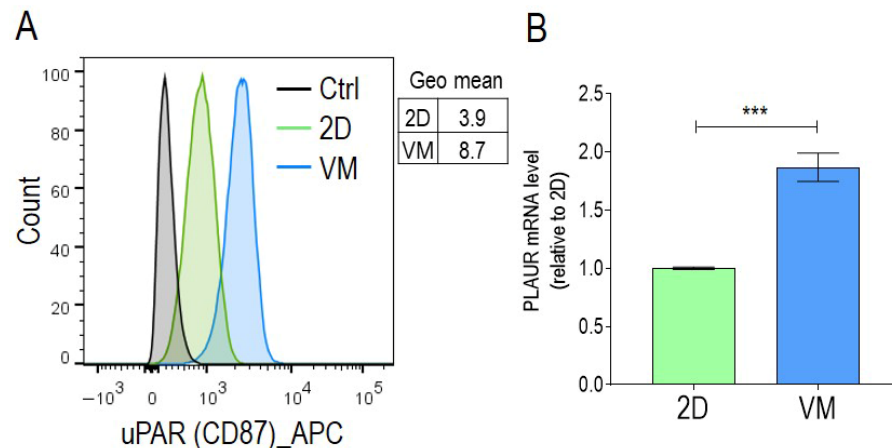
**Figure 4.** Flow cytometry analysis of vasculogenic-related markers on ChS cell surfaces. The expression levels of vasculogenic markers on SW1353, patient-derived ChS-1, ChS-2, ChS-3, stabilized Sarc ChS cells and HUVECs (with the last cell type used for comparison) were determined by flow cytometry using anti-CD34\_PE-Cy7 (sky blue), anti-Podoplanin\_ABflo 488 (red), anti-CD31\_ABflo 647 (blue), anti-VE Cadherin\_ABflo 488 (magenta), anti-EphA2-Alexa 488 (yellow) and anti-uPAR\_APC (green). Unstained cells or omission of the primary antibody (when using unconjugated anti-EphA2) were used as negative controls (black lines).

One of the endothelial-specific markers recently documented to be overexpressed by aggressive melanoma cells forming VM is vascular endothelial (VE) Cadherin, an adhesive protein that promotes homotypic cell-to-cell interactions [27], being involved in endothelial barrier function [28,29]. Flow cytometry analysis revealed that none of the ChS cells expresses VE-Cadherin compared to HUVECs used as positive control (Figure 4 and Table 2). In this set of experiments, we also found that tyrosine kinase Ephrin type-2 Receptor (EphA2), which is implicated in metastasis, self-renewal, and chemoresistance of bone sarcoma cells [30], is moderately expressed by ChS cells with the exception of the primary ChS-2 and ChS-3 cells, which retain the most limited ability to form tube-like structures (Figure 4 and Table 2).

### 3.4. The Urokinase Receptor Is an Important Driver of VM in ChS Cells

The involvement of Urokinase Plasminogen Activator Receptor (uPAR) in inducing melanoma cell mimicry has been recently demonstrated by Andreucci and collaborators [31]. Accordingly, cytofluorimetric analysis of uPAR expression revealed that all ChS cells express uPAR, although to a different extent (Figure 4 and relevant quantifica-

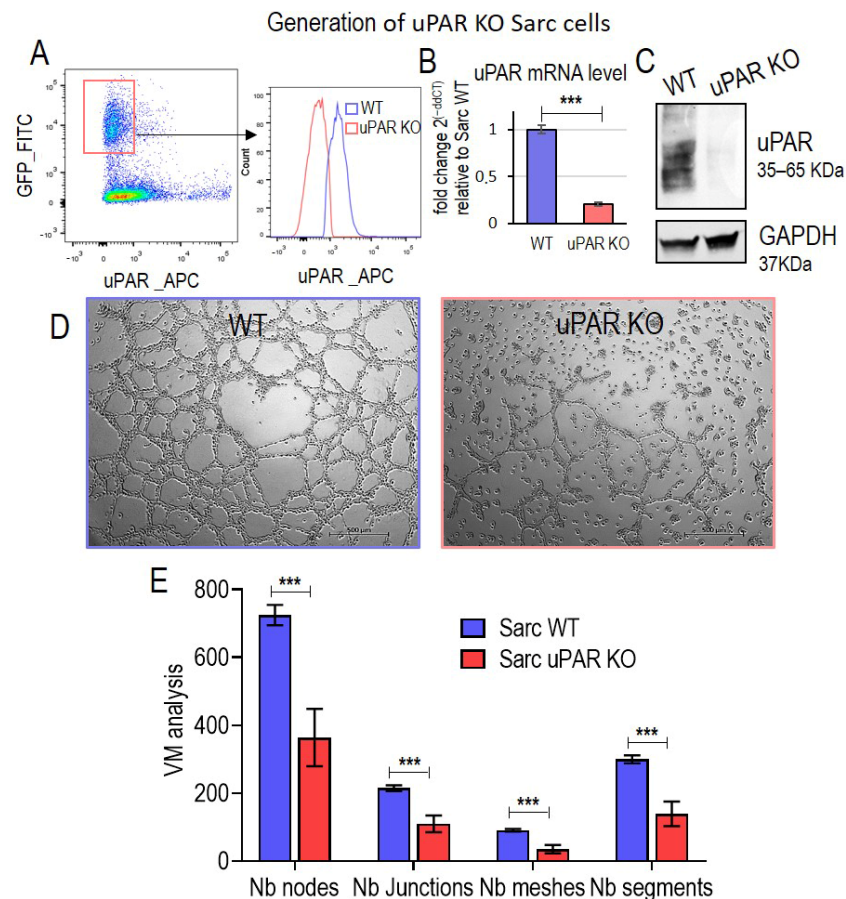
tion in Table 2). Interestingly, when we compared by flow cytometry the uPAR expression levels on the surface of Sarc cells grown in 2D to that of Sarc cells recovered after forming vascular-like structures, we found a considerable increase in uPAR levels on the surface of Sarc cells forming VM (Figure 5A). Accordingly, quantitative real-time PCR revealed that the uPAR mRNA effectively increased in Sarc cells which had already formed VM as compared to Sarc cells recovered from 2D cultures (Figure 5B), confirming the central role of uPAR in triggering the vasculogenic mimicry of ChS cells.



**Figure 5.** The cell surface and mRNA levels of uPAR increase when Sarc cells form VM. **(A)** Comparison of uPAR (CD87) levels on cell surface of Sarc cells cultured in 2D conditions (green) to Sarc cells forming VM recovered from Matrigel (blue) analyzed by flow cytometry; unstained cells were used as negative controls (black). Table reports the geometric mean values calculated for each sample and expressed as fold change over the corresponding unstained negative control. **(B)** Real-time PCR analysis of PLAUR mRNA level in Sarc cells cultured in 2D conditions or Sarc cells forming VM recovered from Matrigel. Student's *t*-test \*\*\*  $p < 0.001$ .

Driven by the interest in validating the involvement of uPAR in the VM process, we generated uPAR knockout (uPAR KO) in the Sarc cell model using the CRISPR/Cas9 gene editing system that permanently disables the PLAUR gene, encoding the uPAR protein. After puromycin selection, the uPAR knockout was preliminary verified in Sarc cells by flow cytometry (Figure 6A), real-time PCR (mRNA level, gene PLAUR) (Figure 6B), and WB (protein levels) (Figure 6C). Surprisingly, when challenged in tube formation assays, the uPAR KO Sarc cells exhibited a dramatical reduction in their ability to form vascular-like structures as compared to WT uPAR expressing Sarc cells (Figure 6D). Quantitative analysis of VM revealed a significant reduction in vascular channels formed by uPAR\_KO Sarc cells, as compared to WT Sarc cells in all the reported values: 50%, 49%, 62%, and 54% reduction in nodes (pixel with at least three neighbors), junctions (groups of nodes forming a bifurcation), meshes (area enclosed by segments), and segments (binary line link with two junctions), respectively (Figure 6E).

A large body of literature highlights the role of VM that, in virtue of its ability to provide the blood supply, may sustain tumor progression, metastasis, and poor prognosis of patients affected by malignant tumors [32–36], thus providing the rationale for the design of new agents devoted to counteracting ChS progression. Considering the important role of uPAR in inducing vascular mimicry, it is reasonable to hypothesize that inhibitors of uPAR functions should counteract VM, which would be useful for a potential treatment strategy in chondrosarcoma.

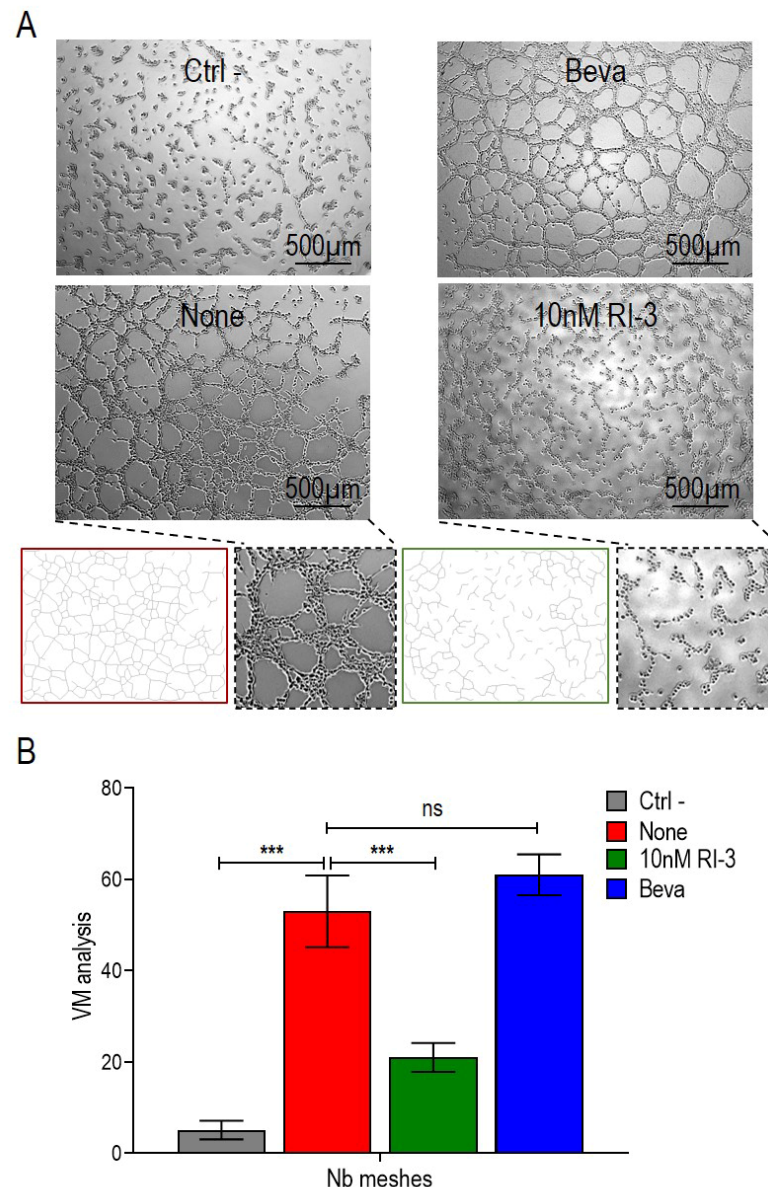


**Figure 6.** The involvement of uPAR in the VM ability of Sarc cells. (A) uPAR KO Sarc cells, generated by the CRISPR-Cas9 technique, were sorted as GFP-positive, uPAR-negative by FACS Melody (B,D); the uPAR knockout was confirmed by flow cytometry (A), real-time PCR (B) (Student's  $t$ -test \*\*\*  $p < 0.001$ ), and WB (C). (D) Representative images of VM ability by WT or uPAR KO Sarc cells suspended in 4% FBS medium and seeded onto GFR-Matrigel. Scale bar, 500  $\mu\text{m}$ . (E) Quantitative analysis of VM performed by the Angiogenesis Analyzer tool of ImageJ software. Data represent the mean  $\pm$  SD from quadruplicate experiments performed three times. Student's  $t$ -test \*\*\*  $p < 0.001$ .

### 3.5. The Retro-Inverso uPAR-Derived Peptide RI-3 Inhibits the VM Ability by ChS

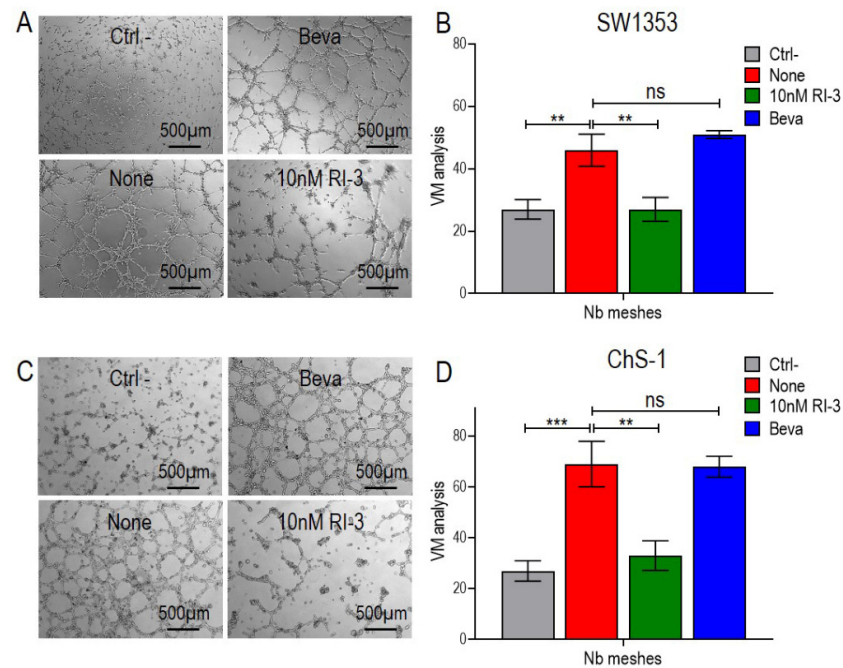
Several uPAR-derived synthetic peptides that potently inhibit signaling triggered by uPAR have been developed by our group in recent years [37]. Among these, the retro-inverso peptide Ac-(D)-Tyr-(D)-Arg-Aib-(D)-Arg-NH<sub>2</sub> named RI-3 has been revealed to be a potent inhibitor of uPAR signaling. Peptide RI-3 is stable in human serum and inhibits cell migration and tube formation of human endothelial cells, as well as the capillary sprouts originating from host vessels that invaded angioreactors implanted in nude mice [22,38]. Thus, once we identified uPAR as a driver of VM in ChS cells, we decided to test the potential inhibitory effect of the RI-3 peptide on VM of ChS cells. Results were compared to the VEGF-neutralizing monoclonal antibody Bevacizumab, usually combined with classical chemotherapy for the treatment of sarcoma patients in virtue of its ability to inhibit angiogenesis [39,40]. Firstly, Sarc cells resuspended in pre-warmed serum-free medium or 4% serum complete medium were seeded onto Matrigel in the absence or presence of 500  $\mu\text{g}/\text{mL}$  Bevacizumab (Beva), or 10 nM RI-3 peptide. The concentration of 500  $\mu\text{g}/\text{mL}$  Beva was selected based on previously reported in vitro studies [41–43] and on our preliminary dose-finding experiments, as shown in Supplementary Figure S3. As shown in Figure 7A, unlike the negative without-serum control, serum soluble factors induced Sarc cells to form vascular-like channels that were fully abrogated by 10 nM RI-3 peptide.

Conversely, Bevacizumab did not exert any inhibitory effect, as expected due to the absence of VEGFR on the Sarc cell surfaces, supporting the notion that VM in chondrosarcoma occurs with a VEGF-independent mechanism. Quantitative analysis confirmed that the uPAR inhibitor RI-3 peptide effectively reduces by 61% the extent of vascular-like channel meshes formed by Sarc cells (Figure 7B).



**Figure 7.** The inhibitor peptide RI-3 impairs the VM ability of Sarc cells. (A) Representative images of VM by Sarc cells suspended in serum-free medium (Ctrl-) or 4% FBS medium and seeded onto GFR-Matrigel, in the absence of treatment (None) or in the presence of 10 nM RI-3 peptide or 500 µg/mL Bevacizumab (Beva), and allowed to form vascular-like tubules for 24 h. Dashed box shown an inner region of interest at higher magnification; the bottom panels with red (None) and green (10 nM RI-3) outline show the representative Final Trees derived from the Angiogenesis Analyzer tool. (B) Quantitative analysis of VM formation performed by the Angiogenesis Analyzer tool of ImageJ software. Data represent mean  $\pm$  SD from quadruplicate experiments performed at least three times. Student's *t*-test ns, not significant; \*\*\*  $p < 0.001$ .

Similarly, unlike the ineffective Beva, we confirmed the inhibitory effect of the RI-3 peptide, which was able to reduce to the basal level the extent of vascular-like channel meshes in both SW1353 (Figure 8A,B) and primary ChS-1 cells (Figure 8C,D).



**Figure 8.** The inhibitor peptide RI-3 impairs the VM ability of ChS cells. (A–C) Representative images of VM by SW1353 (A) or patient-derived ChS-1 cells (C) suspended in serum free medium (Ctrl-) or 4% FBS medium and seeded onto GFR-Matrigel, in the absence of treatment (None) or in the presence of 10 nM RI-3 peptide or 500 µg/mL Bevacizumab (Beva), and allowed to form vascular-like tubules for 24 h. (B–D) The histograms report the quantitative analysis of VM formation performed by the Angiogenesis Analyzer tool of ImageJ software. Data represent mean ± SD from quadruplicate experiments performed at least three times. Student's *t*-test ns, not significant, \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

#### 4. Discussion

Emerging data from the literature show that a high vascular vessel density in tumor tissues from ChS patients associates with aggressive clinical behavior, higher metastatic potential, and poor progression-free survival [7]. Ayala G. et al. demonstrated that ChS vascularity increases with increased histologic grade and that blood vessels in ChS may be located either in peri- or intra-tumor cartilage [6]. When we investigated tumor vascularization in ChS tissues, including 12 conventional and 6 DD ChS, we found that both ChS histotypes exhibit a higher intra-tumoral microvessel density, with vessels uniformly distributed in DD ChS or mainly localized at the margin of cartilaginous nodules in conventional ChS tissues [8].

This work originates from the observation that some vascular structures in ChS tissues do not express endothelial or lymphatic markers, allowing us to hypothesize that, similarly to other bone sarcoma histotypes [14], vasculogenic mimicry may occur in ChS. Besides endothelial and lymphatic vessels, a number of CD31-/Podoplanin-negative vascular channels, some of which contain erythrocytes, were observed in tumor tissue sections from ChS patients, and their number was found to correlate significantly with the proliferation index of neoplastic cells, suggesting that VM could have a role in dictating ChS aggressiveness. However, although we cannot conclude that VM entity correlates with a poor progression-free survival (PFS), given the small number of analyzed ChS cases, we have ascertained that patient-derived ChS cells are able to form PAS-positive tube-like vessels very similar to those formed by endothelial cells when seeded on Matrigel.

Hypoxia regulates the metastatic niche formation in which cancer stem cells differentiate to form endothelial cell-like structures [44]. The hypoxic microenvironment in ChS tissue activates the hypoxia-inducible factor (HIF), which, in turn, promotes tumor growth

by allowing tumor cells to survive and adapt to low oxygen levels [5]. In this scenario, VM could be triggered by the need for cancer cells to initiate adaptive changes in an attempt to deliver oxygen and nutrients into the tumor [45]. Although the mechanisms underlying VM have not yet been fully elucidated, a large body of literature agrees that vasculogenic mimicry is due to hypoxia-induced stem-transition of tumor cells, allowing them to form new vascular-like channels [44].

Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are reported to play a crucial role in promoting angiogenesis [46]. Among them, tyrosine kinase receptor VEGFR-2 is a key regulator of angiogenesis as upon binding to VEGF on endothelial cell surfaces, it triggers a variety of responses, including the differentiation, proliferation, migration, and survival of endothelial cells [46], which makes VEGFR-2 an important target for therapeutic interventions [47].

Now, we provide evidence that ChS cells forming vessels do not express VEGFR2 and do not respond to VEGF-induced cell proliferation. Accordingly, Bevacizumab, a humanized anti-VEGF monoclonal antibody, approved by the Food and Drug Administration (FDA) as well as the European Medicines Agency (EMA) for the treatment of a variety of solid tumors, including metastatic ChS [5], failed to inhibit vasculogenic mimicry by ChS cells.

In previous years, we documented that Urokinase Plasminogen Activator Receptor (uPAR) promotes angiogenesis in a protease-independent manner, leading to new vessel formation through its chemotactic <sup>88</sup>Ser-Arg-Ser-Arg-Tyr<sup>92</sup> sequence [21,48]. Here, we show that all tested ChS cells forming VM expressed considerable levels of uPAR on cell surfaces, whereas the uPAR KO ChS cells failed to form vasculogenic channels, as already described by Andreucci and coworkers, who abrogated VM by uPAR knockdown in a model of drug-resistant melanoma cells [31].

uPAR is a glycosylated glycosyl-phosphatidyl-inositol (GPI)-anchored protein formed by DI, DII, and DIII domains connected by short linker regions [21]. Besides being responsible for focalizing uPA-mediated plasminogen activation on cell surfaces, uPAR also promotes intracellular signaling, which regulates a variety of physiological processes, including cell migration and angiogenesis [48]. Previous work from this laboratory has shown that tetra- and penta-peptides, derived from the uPAR88–92 chemotactic sequence (SRSRY) and carrying specific substitutions in the Ser90 aminoacidic residue, inhibit *in vitro* and *in vivo* cell migration of sarcoma cells [49] as well as angiogenesis [50]. Starting from this information, we developed novel and more stable peptide antagonists of uPAR functions, based on the retro-inverso concept. The retro-inverso Ac-(D)-Tyr-(D)-Arg-Aib-(D)-Arg-NH<sub>2</sub> peptide named RI-3 inhibits *in vitro* cell migration and invasion of uPAR-expressing ChS cells and angiogenesis by blocking uPAR signaling. Also, when ChS cells were subcutaneously injected in nude mice, tumor size and intra-tumoral microvessel density were significantly reduced in animals treated daily with 6 mg/Kg RI-3 as compared to animals treated with vehicle only [22,38]. Now, we show that 10 nM RI-3 prevents vasculogenic mimicry by ChS cells, which makes it a therapeutically promising candidate for ChS therapy.

The neovascularization of malignant tumors is a limiting step for tumor growth, invasion, and metastasis, and the pharmacologic control of these processes is considered one of the most promising treatments for neoplastic diseases. However, clinical benefits in PFS are frequently not accompanied by overall survival improvements [51] since several side effects have been ascribed to antiangiogenic drugs, including bevacizumab, which has been found to increase the risk of cardiac ischemic events in cancer patients [52]. With respect to the RI-3 peptide, it may be a good drug in virtue of its ability to selectively inhibit uPAR signaling, preventing both angiogenesis [22,38] and vasculogenic mimicry.

The main limitations of this study include the use of in vitro models that do not fully recapitulate the complexity of tumor vascularization in vivo, creating an opportunity for future studies to validate these findings in more physiologically relevant models and clinical samples. Furthermore, in recent years it has been documented in a variety of tumors, including osteosarcomas, that besides canonical angiogenesis and vasculogenic mimicry, alternative processes provide oxygen and nutrients to tumor cells [53,54]. It will be mandatory to investigate whether, besides vasculogenic mimicry, ChS vascularization is sustained by sprouting angiogenesis, intussusception angiogenesis or vessel co-option [54] and, if so, whether the RI-3 peptide exerts inhibitory effects on these processes.

## 5. Conclusions

This study documents for the first time the existence of Urokinase Receptor-driven vasculogenic mimicry in ChS tissues. Notably, we show that the uPAR-derived antiangiogenic peptide RI-3 prevents the formation of vascular-like structures, making it a promising candidate for anti-ChS therapies.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells15050392/s1>, Figure S1: Evidence of PAS-positive vascular microvessels in a ChS tissue; Figure S2: Quantitative analysis of VM ability by ChS cells in vitro; Figure S3: Effect of Bevacizumab treatment on cell proliferation in vitro. (A–D) Cell proliferation assessed using the xCELLigence RTCA technology. (A,B) Briefly,  $2 \times 10^3$  Sarc cells (A) or HUVECs (B) suspended in VEGF-free medium were seeded in E-16-well plates in the absence of treatments (None, red) or in the presence of increasing doses of Beva (50  $\mu\text{g/mL}$  (green), 100  $\mu\text{g/mL}$  (blue), and 500  $\mu\text{g/mL}$  (magenta)) and allowed to proliferate over 96 h. (C,D) Briefly,  $1 \times 10^4$  Sarc cells (C) or HUVECs (D), seeded in E-16-well plates in complete medium supplemented with 50 ng/mL VEGF-A (blue) or 50 ng/mL VEGF-A + 500  $\mu\text{g/mL}$  Bevacizumab (magenta), were allowed to proliferate over 96 h. The histograms (right panels) report the slope analysis (time range 0–96 h). Data represent the mean  $\pm$  SD from quadruplicate experiments; Video S1 Sarc tube formation; 6 h time lapse; Video S2: HUVEC tube formation; 6 h time lapse.

**Author Contributions:** V.I., M.M. (Michele Minopoli) and M.V.C. conceived and wrote the manuscript. V.I., R.G. and M.M. (Michele Minopoli) designed experiments. V.I., R.G., G.D.C., V.D.V., M.M. (Maurizio Maddalena) and M.C. performed the experiments and analyzed data. F.F., M.G., S.S. and G.F. provided human tissues and analyzed clinical data. V.I., C.C., M.M. (Michele Minopoli) and M.V.C. supervised and finalized the project. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of the Istituto Nazionale Tumori “Fondazione G. Pascale”—IRCCS (Quality System n. LRC 6019486/QMS/U/IT-2015 certificated in conformity with UNI EN ISO 9001:2015). The research work with primary cell lines and ChS tissues has been approved by the Institutional Ethical Committee of Istituto Nazionale Tumori “Fondazione G. Pascale”—IRCCS, Naples, Italy (protocol 258/18, December 2018 and protocol 54/22 oss, January 2023).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study. All patients provided written informed consent for the use of the tissue samples. The study was approved by the Ethics Committee of Istituto Nazionale Tumori “Fondazione G. Pascale”—IRCCS, Naples, Italy (protocol 258/18, December 2018 and protocol 54/22 oss, January 2023).

**Data Availability Statement:** All data generated during this study are available within the article and its Supporting Information. Further details are available from the corresponding author on reasonable request at: <https://doi.org/10.5281/zenodo.17951450>.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

|         |                                               |
|---------|-----------------------------------------------|
| Beva    | Bevacizumab                                   |
| BSA     | Bovine Serum Albumin                          |
| ChS     | Chondrosarcoma                                |
| DD ChS  | Dedifferentiated ChS                          |
| DMEM    | Dulbecco's Modified Essential Medium          |
| EBM     | Eagle Basal Medium                            |
| EphA2   | Ephrin Type-A Receptor 2                      |
| FBS     | Fetal Bovine Serum                            |
| FFPE    | Formalin-Fixed Paraffin-Embedded              |
| GFR     | Growth Factor-Reduced                         |
| GPI     | Glycosyl-Phosphatidyl-Inositol                |
| HUVECs  | Human Umbilical Vein Endothelial Cells        |
| IHC     | Immunohistochemistry                          |
| KO      | Knockout                                      |
| PAS     | Periodic Acid-Schiff                          |
| PFS     | Progression-Free Survival                     |
| RI-3    | Retro-Inverso Peptide                         |
| RTCA    | Real-Time Cell Analysis                       |
| uPAR    | Urokinase Plasminogen Activator Receptor      |
| uPAR KO | uPAR Knockout                                 |
| VE      | Vascular Endothelial                          |
| VEGF    | Vascular Endothelial Growth Factor            |
| VEGFR   | Vascular Endothelial Growth Factor Receptor   |
| VEGFR-2 | Vascular Endothelial Growth Factor Receptor 2 |
| VM      | Vasculogenic Mimicry                          |

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