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# Endosialin (TEM1, CD248) is a marker of stromal fibroblasts and is not selectively expressed on tumour endothelium

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**Abstract** Fibroblasts are a diverse cell type and display clear topographic differentiation and positional memory. In a screen for fibroblast specific markers we have characterized four monoclonal antibodies to endosialin (TEM1/CD248). Previous studies have reported that endosialin is a tumour endothelium marker and is localized intracellularly. We demonstrate conclusively that endosialin is a cell surface glycoprotein and is predominantly expressed by fibroblasts and a subset of pericytes associated with tumour vessels but not by tumour endothelium. These novel antibodies will facilitate the isolation and classification of fibroblast and pericyte lineages as well as the further functional analysis of endosialin.

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**Keywords:** Endosialin; TEM; CD248; Fibroblast; Pericyte; Angiogenesis

Despite their importance, fibroblast biology has lagged behind research into other cell types. In part, this reflects difficulties in studying a cell type which is phenotypically and functionally heterogeneous and plastic in its differentiation capacity [6,7]. In addition, there are surprisingly few good selective markers for monitoring and isolating fibroblast populations and/or for discriminating between the origins and differentiation of fibroblast subtypes. Our approach to this problem has been to generate a panel of monoclonal antibodies (mAbs) directed against extracellular domain epitopes of human fibroblast cell surface proteins [8] (see [Supplementary Methods](#) for further details). In this paper, we have used a series of further screens to identify mAbs which recognise proteins with a fibroblast-restricted distribution. We describe the characterization of four novel mAbs directed against a 175 kDa antigen whose expression is restricted to fibroblasts and which we have identified as endosialin/tumour endothelial marker-1 (TEM1/CD248).

## 1. Introduction

Fibroblasts are the most abundant cells of the stroma and are responsible for the synthesis and remodelling of extracellular matrix components. In addition, their ability to produce and respond to growth factors allows reciprocal interactions with other stromal cell types and with adjacent epithelial and endothelial structures. As well as playing a critical role during tissue development and homeostasis, fibroblasts also contribute to the pathology of many diseases either directly for example by overproduction of matrix components during fibrosis and/or indirectly by influencing the behaviour of neighbouring cell types [1–5].

## 2. Materials and methods

### 2.1. Antibodies and cells

The generation of four anti-endosialin mAbs (B1/22, B1/35, B1/473, 18/37) is described in [supplementary methods](#). Affinity purified antibody directed against the D2 domain of NG2 [9] was a gift of W. Stallcup (The Burnham Institute, La Jolla), mAb 23-C directed against the Golgi coat protein,  $\beta$ COP [10] was a gift of K. Willison (Institute of Cancer Research, London), mAb YRI CD31.12 was a gift of Ian Bird (Yamanouchi Research Institute, Oxford), anti-Endo180 mAb E1/183 [11] was directly labelled with Alexa488 using a Zenon labelling kit (Molecular Probes). The following antibodies were obtained commercially; FITC conjugated anti-CD31 mAb (clone WM59; BioLegend), unconjugated and FITC-conjugated anti-CD146 mAb (clone PIH12, Chemicon International), anti-CD90 mAb (clone AS02, Dianova, Germany), Alexa conjugated second layer antibodies (Molecular Probes).

Fibroblasts were isolated from synovial tissue, bone marrow, thyroid and salivary glands using standard procedures [7]. HUVEC were isolated from umbilical cords [12] and used between passages 2 and 4.

### 2.2. Other methods

Details of microscopy, flow cytometry, pulse chase labelling and mass spectrometry can be found in [supplementary information](#). Protocols for tissue collection were approved by the local ethics committee LREC number 5735 (Birmingham) and CCR/REC 2042 (London).

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**Abbreviations:** TEM, tumour endothelium marker; mAb, monoclonal antibody; HUVEC, human umbilical vein endothelial cells; SAGE, serial analysis of gene expression

### 3. Results and discussion

#### 3.1. A screen for stromal fibroblast cell surface markers

In order to identify fibroblast specific markers, a panel of mAbs generated against human fibroblast cell surface proteins were screened by cell based ELISA and by flow cytometry for reactivity against primary fibroblasts isolated from rheumatoid synovium compared to human umbilical vein endothelial cells (HUVEC). Among the novel mAbs tested, 4 of these (18/37, B1/473, B1/35, B1/22) were confirmed as reacting strongly against rheumatoid synovial fibroblasts but not HUVEC (Fig. 1A). All 4 mAbs were shown to immunoprecipitate a 175 kDa protein from  $^{125}$ I-cell surface labelled fibroblasts (Fig. 1B) and serial immunoprecipitation and limited proteolytic digestion indicated a common antigen (data not shown). To further assess the cell type distribution of the p175 antigen, a large panel of human cell lines were screened either by immunoprecipitation or flow cytometry (Fig. 1C). None of the established lines showed p175 expression including cells of epithelial (A431, MCF7, HEp2, HeLa), neural (1321N1, SK-N-SH), mesenchymal (MG63, HT1080), haematopoietic (IM9, CEM, HL60) and endothelial (HUVEC) origin. In contrast, expression was detected in three different human diploid fibroblast cultures (AG1523, Flow2000, F1084) and in a wide range of primary human fibroblasts derived from a variety of sites (Fig. 2 and data not shown). Although these studies clearly demonstrated expression of the p175 antigen on primary fibroblasts it was important to establish that this did not result from p175 upregulation when cells were cultured in vitro. To address this, cryosections from rheumatoid synovium, tonsil, salivary gland and breast were stained with mAb B1/35 to label

p175 expressing cells and with anti-CD31 mAb to label endothelial cells. In all tissues examined, p175<sup>+</sup> cells were restricted to the stromal compartments with no detectable expression on vasculature or epithelial structures (Fig. 3).

To identify the p175 antigen, Flow2000 human fibroblasts were lysed and passed over a mAb B1/35 affinity column. Bound protein was eluted and analysed by mass spectrometry. Signals observed at  $m/z$  564, 624, 705 and 720 were interpreted as deriving from the respective sequences V-D-S-L/I-V-G-A-G..., A-L/I-E-A-S..H-R,... D-D-P-H-R and ...Y-A-L/I-F-P-R. Database searching of these sequences led to an unambiguous assignment of the 175 kDa antigen as endosialin. To validate that the anti-p175 mAbs were indeed directed against endosialin, all 4 mAbs were shown to stain COS-1 cells transfected with the pcDNA3 plasmid containing a human endosialin cDNA but not cells transfected with a control plasmid (Fig. 4A).

#### 3.2. Subcellular distribution of endosialin

After cleavage of the N-terminal signal sequence, endosialin is comprised of a 78.8 kDa protein core modified by approximately 95 kDa of highly sialylated O-linked oligosaccharides [13,14]. Previously, it has been reported that endosialin constructs tagged at the C-terminus with either GFP, a Myc epitope or a 6× His epitope were located intracellularly with only a minor proportion being localized to the plasma membrane [15]. By staining non-permeabilized fibroblasts from a number of different sources (Fig. 2) or by transfecting cells with a non-tagged endosialin construct (Fig. 4A), we demonstrate here that both endogenous and exogenous endosialin are strongly expressed at the cell surface. Further, permeabili-

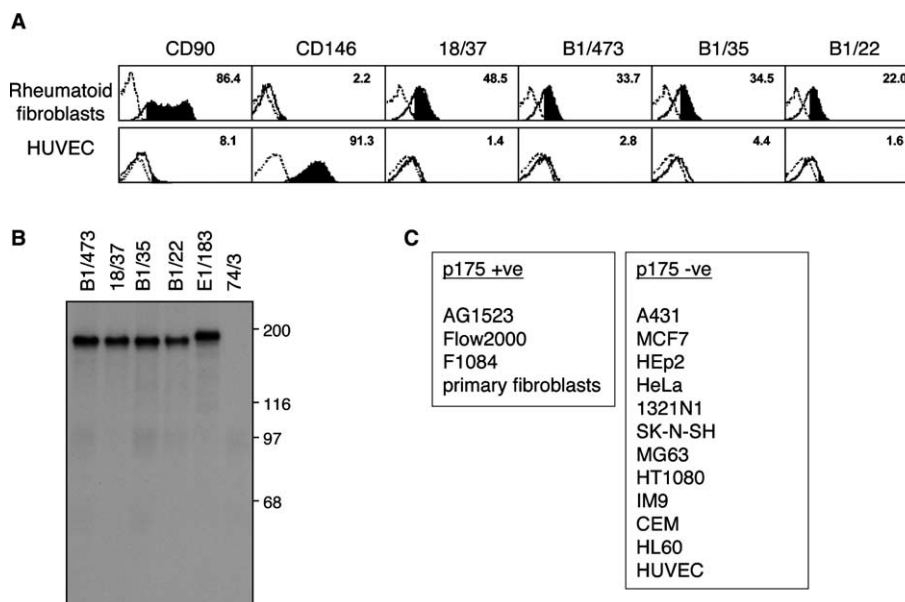


Fig. 1. Identification of 4 mAbs directed against a 175 kDa fibroblast cell surface protein. (A) Fibroblasts isolated from rheumatoid synovium and HUVEC were detached from dishes and subject to flow cytometry using 4 anti-p175 mAbs (18/37, B1/473, B1/35 and B1/22) (solid lines) or isotype-matched controls (dotted lines) followed by FITC-anti-mouse Ig. As positive controls, mAb AS02 (anti-CD90/Thy1) and mAb P1H12 (anti-CD146/MUC18) were used to label fibroblasts and HUVEC, respectively. The % of cells labelled with test antibody over controls is shown in each panel. (B) AG1523 human fibroblasts were cell surface  $^{125}$ I-labelled, lysed and subject to immunoprecipitation with the 4 anti-p175 mAbs. Lysates were resolved by 10% SDS-PAGE, the gels dried and exposed to X-ray film overnight. mAb E1/183 against the cell surface receptor Endo180 [11] and mAb 74/3 against the intracellular antigen annexin I [25] were used as positive and negative controls, respectively. Molecular size markers are in kDa. (C) Table of cell lines positive or negative for expression of the p175 antigen. Expression was assessed by immunoprecipitation following  $^{125}$ I-cell surface labelling or by flow cytometry. Primary fibroblasts were isolated from a variety of tissues as shown in Fig. 2.



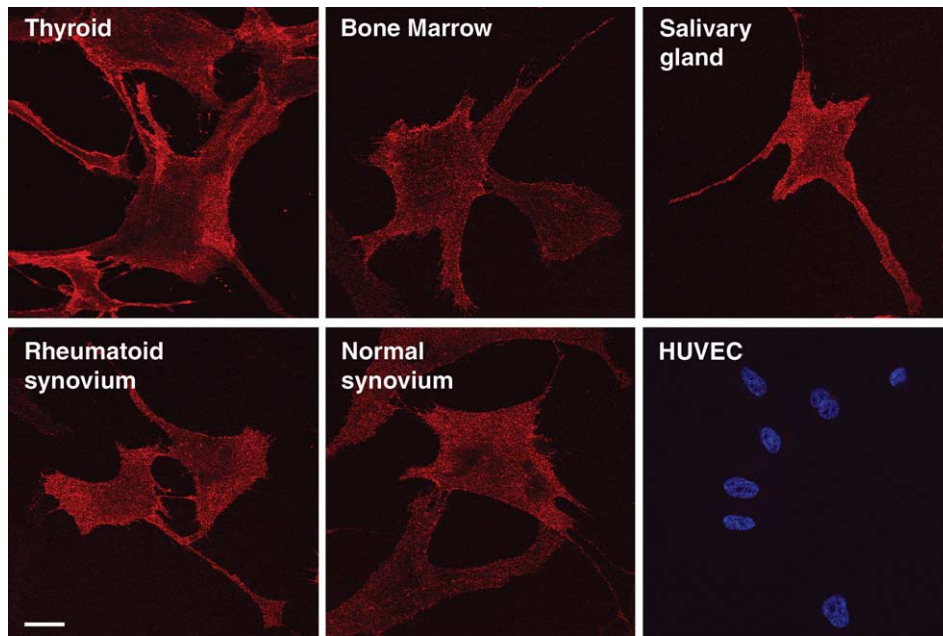


Fig. 2. Distribution of the p175 antigen on primary human fibroblasts. Fibroblasts isolated from rheumatoid and normal synovium, bone marrow, labial salivary gland and thyroid were cell surface-stained by incubating for 1 h at 4 °C with anti-p175 mAb B1/35. Cells were then fixed, permeabilized and stained with Alexa555-anti-mouse Ig. HUVEC were stained in parallel and nuclei counterstained with TO-PRO-3 (blue). Scale bar, 15  $\mu$ m.

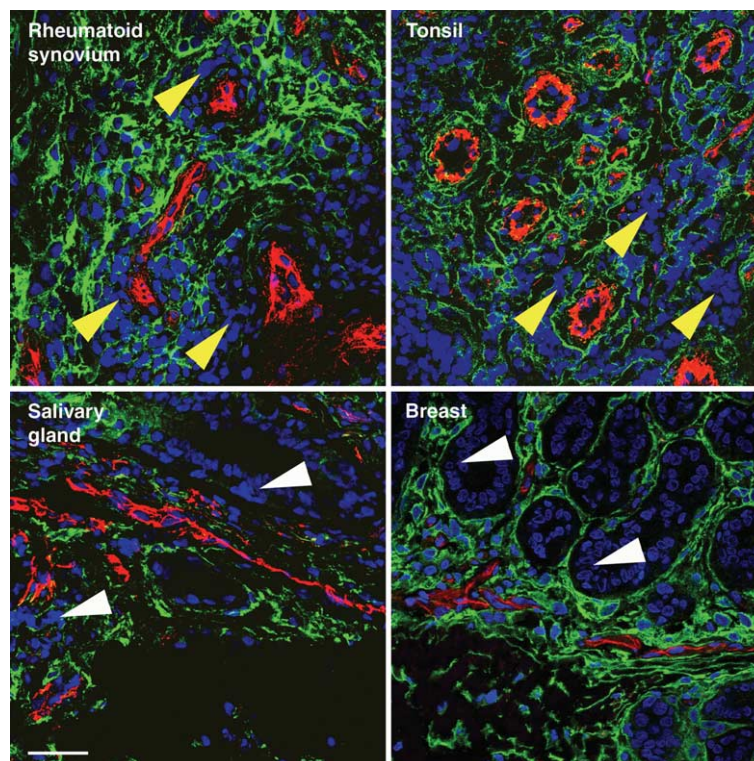


Fig. 3. p175 is expressed by fibroblasts in vivo. Cryosections of human rheumatoid synovium, tonsil, salivary gland and normal breast were stained with mAb B1/35 to detect p175 (green) and anti-CD31 to label the endothelial cells (red). Sections were counterstained with Hoechst 33258 or TO-PRO-3 (blue). White arrowheads indicate endosialin<sup>-ve</sup> epithelial cells in the salivary gland and breast lobular unit. Yellow arrowheads indicate endosialin<sup>-ve</sup> lymphocyte aggregates in perivascular cuffs of rheumatoid synovium and tonsil. Scale bar, 50  $\mu$ m.

zation of cells prior to staining revealed only a low level of intracellular perinuclear staining in a small proportion of cells. Double labelling revealed that this intracellular pool of endosi-

alin co-localises with the Golgi network but not with markers of endosomes (Fig. 4B), endoplasmic reticulum or lysosomes (data not shown). Consistent with this staining data, pulse

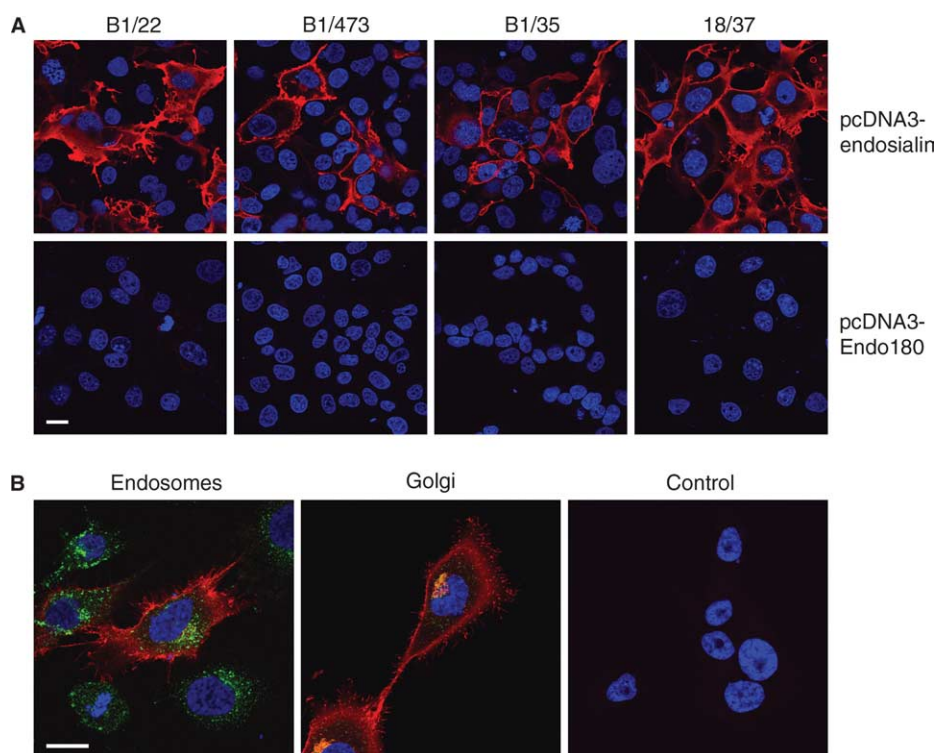


Fig. 4. The p175 antigen is endosialin. (A) COS-1 cells were transfected with pcDNA3-endosialin (see [Supplementary Methods](#) for construct design) or pcDNA3-Endo180 [26]. After 48 h, cells were fixed, permeabilized and stained with anti-p175 mAbs B1/35, B1/473, B1/22 or 18/37 followed by Alexa555-anti-mouse Ig and nuclei counterstained with TO-PRO-3. (B) MG63 cells transiently transfected with pcDNA3-endosialin were permeabilized and double-stained with mAb B1/35 followed by Alexa555-anti-mouse Ig (red) and either Alexa488 labelled anti-Endo180 mAb E1/183 to label endosomes or the anti-βCOP mAb 23-C followed by Alexa488-anti-rat Ig to label the Golgi apparatus (green). Control cells were transfected with pcDNA3 vector alone and labelled in parallel with mAb B1/35 followed by Alexa555-anti-mouse Ig. Nuclei were counterstained with TO-PRO-3 (blue). Scale bar, 20 μm.

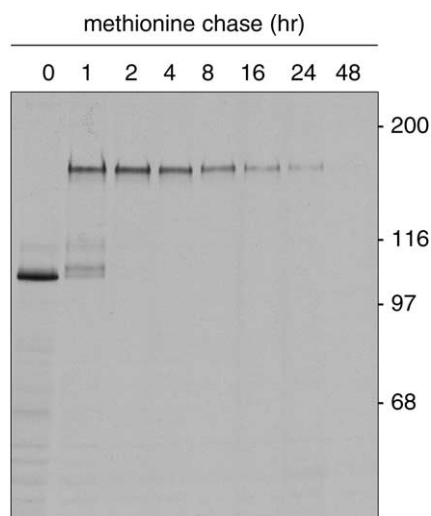


Fig. 5. Biosynthesis of endosialin. Flow2000 human fibroblasts were labelled for 15 min with  $^{35}\text{S}$ -methionine and then chased for 0–48 h in medium without  $^{35}\text{S}$ -methionine. At indicated times, cells were lysed and immunoprecipitated with mAb B1/473. Immunoprecipitates were resolved by 7% SDS-PAGE and exposed to X-ray film overnight. Molecular size markers are in kDa.

chase studies demonstrate that after 15 min of  $^{35}\text{S}$ -methionine labelling, a single immaturely glycosylated form of endosialin is observed which within 1 h of chase is converted into the ma-

ture 175 kDa form, a process that is complete within 2 h. The mature form of endosialin has a relatively short half life of approximately 3–4 h (Fig. 5). Together these data indicate that at steady state, a small proportion of endosialin will be localized to the intracellular secretory machinery and in particular in the Golgi apparatus which is the site for addition and modification of O-linked oligosaccharides. Further, we suggest that the reason for the discrepancy between the data presented here and previous reports [15] is that the presence of a C-terminal tag and/or overexpression of exogenous endosialin in the transfected cells resulted in aberrant accumulation of this heavily O-glycosylated transmembrane protein in intracellular secretory organelles. An alternative possibility is that the mAbs employed in our study recognise a variant epitope which is preferentially revealed at the cell surface. We consider this unlikely given that the 4 mAbs employed here all recognise both immature and mature forms of endosialin in pulse chase experiments and yet do not share a common epitope (data not shown).

### 3.3. Endosialin is not expressed by normal or angiogenic endothelium

As described above, our *in vitro* studies indicated that endosialin is highly restricted in its distribution to fibroblasts and is not expressed on resting HUVEC or on endothelial cells in sections of inflamed and normal tissue. This observation is at variance with previous studies. First, in a serial analysis of gene expression (SAGE) screen of endothelial cells isolated



with anti-CD146 magnetic beads, endosialin was identified as the most highly upregulated transcript in tumour endothelium compared to normal endothelium hence its alternate name of TEM1 [16]. Second, by immunohistochemistry it has been reported that endosialin is expressed on angiogenic endothelial cells but not normal tissue endothelium [13,17]. To determine whether the lack of endosialin expression in HUVEC reflected their unstimulated status, cells were stimulated with the pro-angiogenic/inflammatory factors TNF- $\alpha$ , TGF- $\beta$ 2 and IL-1. These treatments did not result in upregulation of endosialin expression (Fig. 6). However, it remained a possibility that stimulated HUVEC did not provide a suitable model for the in vivo microenvironment of tumour endothelial cells.

To address these discrepancies more directly, sections from breast tumours were stained for endosialin in conjunction with the pan-endothelial marker CD31. In all sections examined, endosialin staining was observed on stromal fibroblasts although it was notable that fibroblast expression was highly variable between the different tumours. In addition, in comparison to normal breast tissue (see Fig. 3) endosialin staining was observed on a subset of the tumour vasculature and this was particularly prominent in tumours where endosialin expression in the stromal compartment was low (Fig. 7A). However, closer examination of these sections strongly indicated that endosialin expression in the vasculature was restricted to perivascular cells intimately associated with the endothelial cells

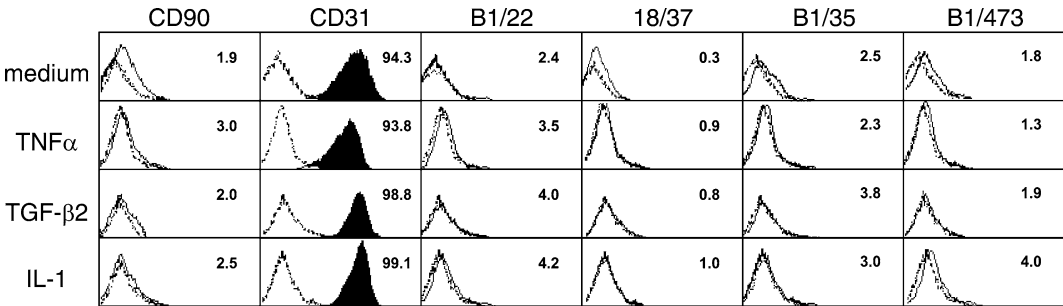


Fig. 6. Endosialin is not expressed by resting or activated HUVEC. HUVEC were treated in culture for 24 h with TNF $\alpha$  (10 ng/ml), TGF- $\beta$ 2 (2 ng/ml) or IL-1 (1 ng/ml). Cells were detached from tissue culture dishes with EDTA and analysed as described in Fig. 1A.

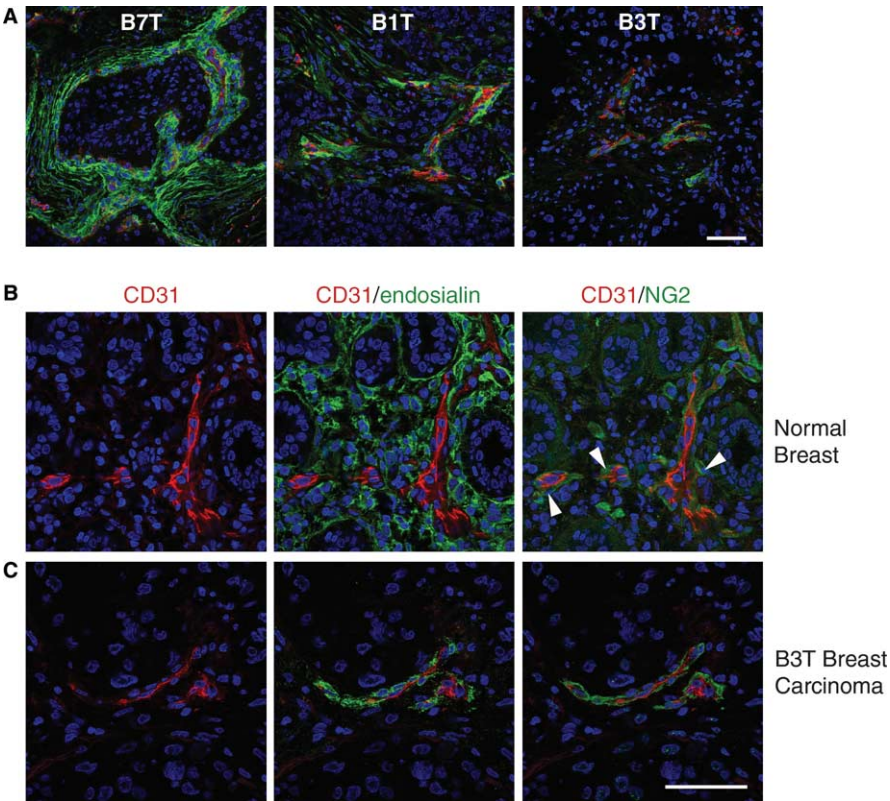


Fig. 7. Endosialin expression in breast tumours. (A) Three different breast tumours showing expression of CD31 (mAb WM59; red) and endosialin (mAb B1/35; green). Nuclei were counterstained with DAPI (blue). (B, C) Higher magnification images showing a section of normal breast and a breast tumour triple labelled for CD31, endosialin and NG2 with nuclei counterstained with DAPI (blue). Images are shown in the following combinations; CD31 alone (red); CD31 (red) and endosialin (green); CD31 (red) and NG2 (green). Arrowheads in normal breast indicate NG2<sup>+</sup><sup>ve</sup>/endosialin<sup>-</sup><sup>ve</sup> pericytes. Scale bar, 50  $\mu$ m.

rather than the endothelial cells themselves. To confirm these observations and further characterize this perivascular expression, sections of normal breast and breast tumours were labelled for endosialin, CD31 and the pericyte marker NG2 [18,19]. In all tumour sections examined, but not in normal breast tissue, there was colocalization of endosialin and NG2 expression in the perivascular cells (Fig. 7B and C) indicating that the endosialin<sup>+</sup> perivascular cells are pericytes and that expression is increased in pericytes associated with angiogenic compared to normal vessels.

These observations raised the issue as to whether the endothelial cells isolated by magnetic bead immunopurification with the anti-CD146 mAb P1H12 for the SAGE screen [16] were contaminated with non-endothelial perivascular cells which expressed CD146 or remained tightly adherent to the CD146<sup>+</sup> endothelial cells during the isolation procedure. To address this, breast and colorectal tumour sections were double labelled for CD31 and CD146 (Fig. 8). In all sections examined, strong colocalization of CD146 with all CD31<sup>+</sup> cells was observed. However, in addition, CD146 was also clearly expressed by CD31<sup>-</sup> perivascular cells which were closely associated with the angiogenic endothelial cells.

These data together indicate the following: (a) CD146, as detected by mAb P1H12, is not an endothelial specific marker but is also expressed by non-endothelial perivascular cells. It should be noted that CD146 has previously been reported to be expressed not only by small and large vessel endothelial cells but also expressed by breast epithelial cells, smooth muscle cells, myofibroblasts and melanoma cells [20], (b) although expressed on a subset of tumour vasculature, endosialin is not expressed by tumour endothelium. We suggest that the identification of endosialin as a transcript

highly upregulated in angiogenic endothelium results from the contamination of the endosialin<sup>-</sup>/CD146<sup>+</sup> endothelial population with endosialin<sup>+</sup>/CD146<sup>+</sup> perivascular cells and/or closely associated endosialin<sup>+</sup>/CD146<sup>-</sup> fibroblasts. Similarly, we would suggest that *in situ* hybridization and immunohistochemistry [13,21] cannot be used to identify endothelial specific markers without employing double labelling methods to visualise closely apposed fibroblasts and perivascular cells, and (c) the endosialin<sup>+</sup> perivascular cells also express NG2 suggesting that they are pericytes. Pericytes and other mural cells modulate the physiology of the vessels, are functionally co-dependent on the endothelial cells, are highly variable between different tissues and organs, and have been implicated in a number of disease processes including tumour angiogenesis [22–24]. Further, pericytes are notably plastic in their phenotype and the expression of endosialin in a subset of these cells suggests either that they have been recruited from the stromal fibroblast population and/or that they are undergoing fibroblast differentiation.

In conclusion, previous discussion as to the function of endosialin has focussed on its role in tumour angiogenesis and its reported intracellular distribution. The data presented here contradicts these assertions and instead we propose that endosialin is predominantly restricted to fibroblasts where it is likely to function as a plasma membrane receptor to bind extracellular ligands. In addition, although we have demonstrated that endosialin is not a TEM, it is of great interest that its expression is upregulated on pericytes in tumour but not normal tissue. Consequently, it will be important to investigate whether endosialin plays a role in tumour angiogenesis by modulating the behaviour of pericytes and surrounding stromal fibroblasts.

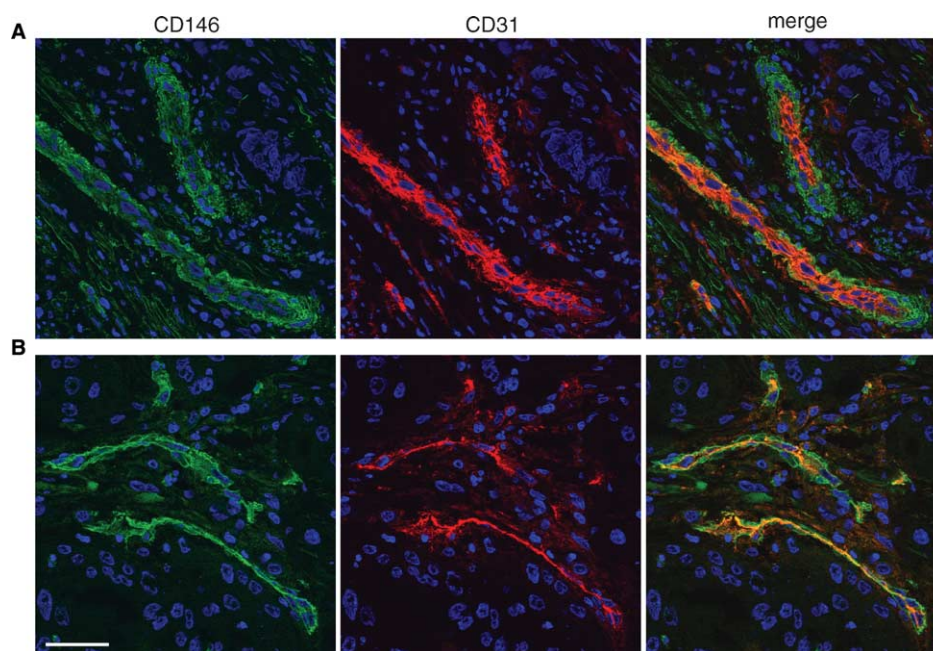


Fig. 8. CD146 is expressed by CD31<sup>+</sup> endothelial cells as well as CD31<sup>-</sup> perivascular stromal cells. Cryosections of human colorectal carcinoma (A) or breast carcinoma (B) were stained with anti-CD31 mAb YRI and Alexa555-anti-rat Ig (red) followed by FITC-anti-CD146 mAb P1H12 (green). Nuclei were counterstained with TO-PRO-3 (blue). Scale bar, 50  $\mu$ m.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2005.03.071](https://doi.org/10.1016/j.febslet.2005.03.071).

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