Novel highly specific anti-periostin antibodies uncover the functional importance of the fascilin 1-1 domain and highlight preferential expression of periostin in aggressive breast cancer

Sarah Field[1,2], Catherine Uyttenhove[3,4], Vincent Stroobant[3], Paméla Cheou[4], Dominique Donckers[4], Jean-Paul Coutelier[4], Peter T. Simpson[5,6,8], Margaret C. Cummings[5,7,8], Jodi M. Saunus[5,6], Lynne E. Reid[5,6], Jamie R. Kutasovic[5,6], Anne Marie McNicol[5,8], Ba Reun Kim[9,10], Jae Ho Kim[9,10,11], Sunil R. Lakhani[5,7,8], A. Munro Neville[12], Jacques Van Snick[3,4*] and Parmjit S. Jat[2,13*]

[1]University of Oxford Branch, Ludwig Institute for Cancer Research, Old Road Campus, Off Roosevelt Drive, Oxford OX3 7DQ, UK.

[2]Department of Neurodegenerative Disease, UCL Institute of Neurology, Queen Square House, Queen Square, London WC1N 3BG, UK.

[3]Ludwig Institute for Cancer Research Ltd, Brussels Branch, Avenue Hippocrate 74, UCL, 7459, B-1200 Brussels, Belgium.

[4]de Duve Institute, Université Catholique de Louvain, Avenue Hippocrate 75, B-1200 Brussels, Belgium.

[5]The University of Queensland, UQ Centre for Clinical Research, Herston, Brisbane, QLD 4029, Australia.

[6]QIMR Berghofer Medical Research Institute, Herston 4006, Queensland, Australia.

[7]Pathology Queensland, The Royal Brisbane & Women's Hospital, Brisbane, QLD 4029, Australia.

[8]The University of Queensland, School of Medicine, Herston, Brisbane, QLD 4006, Australia.

[9]Medical Research Centre for Ischemic Tissue Regeneration, School of Medicine, Pusan National University, Yangsan 626-870, Gyeongsangnam-do, Republic of Korea.

[10]Department of Physiology, School of Medicine, Pusan National University, Yangsan 626-870, Gyeongsangnam-do, Republic of Korea.

[11]Research Institute of Convergence Biomedical Science and Technology, Pusan National University Yangsan Hospital, Yangsan 626-870, Gyeongsangnam-do, Republic of Korea.

[12]Ludwig Institute for Cancer Research, 666 Third Avenue, 28th Floor, New York, NY10017, USA.

[13]MRC Prion Unit, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK.

[*] JVS and PSJ, equal contribution.

Correspondence to: Parmjit S. Jat, Department of Neurodegenerative Disease and MRC Prion Unit, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK. Phone: 44 20 7837 3973; E-mail: p.jat@prion.ucl.ac.uk

Abstract

Periostin (POSTN), a secreted homodimeric protein that binds integrins $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 6\beta 4$, was originally found to be expressed in fetal tissues and in the adult upon injury particularly bone fractures due to its role in remodelling and repair. Recently it was found to be over-expressed in human breast cancer and a variety of other tumour types including head and neck squamous cell carcinoma, where its overexpression correlates with increased tumour invasion. Progress in studying its functional role in tumour pathogenesis has been hampered by the paucity of antibodies for its specific and sensitive detection. It has proven very difficult to obtain monoclonal antibodies (mAbs) against this highly conserved protein but we report here that combining infection of mice with lactate dehydrogenase elevating virus (LDV), a B cell activating arterivirus, with conjugation of human POSTN to ovalbumin as an immunogenic carrier, enabled us to develop 6 mAbs recognizing both human and mouse POSTN and inhibiting its binding to $\alpha v\beta 3$ integrin. Two of the mAbs, MPB4B1 and MPC5B4, were tested and found to inhibit POSTN-induced migration of human endothelial colony forming cells. All six mAbs recognized amino acids 136-51 (APSNEAWDNLDSDIRR) within the POSTN fascilin (FAS) 1-1 domain revealing the functional importance of this motif; this was further highlighted by the ability of aa 136-151 peptide to inhibit integrin-mediated cell migration. Immunohistochemistry using MPC5B4, indicated that breast tumour cell POSTN expression was a strong prognostic indicator, along with tumour size, lymph node, and human epidermal growth factor receptor 2 (HER2) status.

What's new?

We have developed monoclonal antibodies against human POSTN, a highly conserved protein, by breaching immune self-tolerance in mice. These mAbs identify the sequence APSNEAWDNLDSDIRR as essential for POSTN-binding to $\alpha v\beta 3$ integrin as well as POSTN-dependent cell migration and adhesion.

Immunohistochemistry using mAb MPC5B4 indicates that POSTN expression in breast tumour cells is a strong prognostic indicator, along with tumour size, lymph node, and HER2 status.

Introduction

POSTN, originally known as osteoblast-specific factor-2, is a 90kDa secreted glycoprotein that becomes incorporated into the extracellular matrix (ECM) (1). It has an N-terminal signal sequence, followed by a 55 amino acid Emilin (EMI) domain and four 130 amino acid FAS1 homology domains (2). The FAS1 domains have homology to the insect axon guidance protein, FAS1 (3), and are cell adhesion modules that exhibit a novel seven-stranded β-wedge with multiple α-helical folds. POSTN binds integrins $\alpha v \beta 3$, $\alpha v \beta 5$ and $\alpha 6 \beta 4$ leading to activation of the Akt/Protein kinase B (PKB) and Focal adhesion kinase (FAK) signalling pathways and promotion of cell survival, angiogenesis and resistance to hypoxia-induced apoptosis (4). It also binds to a number of ECM proteins, dependent upon the c-terminus, such as heparin, fibronectin, collagen V and tenascin C, thereby enhancing tumour cell invasiveness by affecting ECM fibrillogenesis (5).

Integrins, the main receptors for POSTN, are a family of cell surface adhesion receptors composed of non-covalently associated α and β subunits. Integrin $\alpha v \beta 3$ is an adhesion receptor that drives tumour progression (6) and has been associated with poor outcome and a higher incidence of metastasis in epithelial cancers. It is expressed in breast, lung and pancreatic cancer stem cells that are highly resistant to receptor tyrosine kinase inhibitors and recruits KRAS and RalB to the plasma membrane leading to activation of NF-κB sufficient for tumour initiation, anchorage independence, self-renewal and drug resistance (7). $\alpha v \beta 3$ is also required downstream of hormonal signalling and TGF$\beta 2$ to regulate mammary stem cell number and alveolar development during early pregnancy (8). It is also expressed

on osteoclasts and implicated in osteoclast-mediated bone resorption and promoting bone metastases (9;10).

POSTN is expressed in a wide variety of fetal tissues (5) and in the adult upon myocardial, vascular and skeletal muscle injury and bone fractures due to its role in bone/dental remodelling and tissue repair following injury (11). POSTN is a mesenchymal gene normally expressed by the stroma. Recently it was shown to be expressed by bronchial epithelial cells in response to Th2 cytokines IL-4 and IL-13 independently of TGF-β and to represent a novel component of subepithelial fibrosis in bronchial asthma (12). We originally found that POSTN RNA was over-expressed in malignant breast cancer cells and showed that expression in the cancer cells correlated with a poor outcome in estrogen receptor-positive breast cancers (13). Subsequently POSTN was reported to be expressed in other tumours including colon (14), pancreatic (15), ovarian (16;17), non-small cell lung carcinomas (18), head and neck cancer (19) and melanoma (20). Serum POSTN levels rise upon metastasis and its expression is markedly increased at the invading edge of thyroid, colon and lung cancers (21) suggesting an involvement in tumour spread. Recently, a mechanism linking POSTN expression and breast tumour progression was proposed based on the observation that POSTN is essential for cancer stem cell maintenance in metastatic niches through activation of the Wnt pathway (22). Additionally, POSTN increases angiogenesis and lymphangiogenesis by up-regulating vascular endothelial growth factor receptor-2 (VEGFR-2) (23) and promotes epithelial-mesenchymal transition (EMT) and invasion through activating lysol oxidase (LOX) (24). POSTN also has the potential to impair anti-tumour

immune responses by attracting M2 monocytes/ macrophages to the tumour niche as recently observed in experimental glioblastoma multiforme (25).

These data suggest that inhibiting the effects of POSTN could have a place in anti-tumour therapies. Our approach towards this aim has been to develop inhibitory mAbs against POSTN, a product of not only breast cancer cells themselves but also of the related stroma. Here we report the production of a panel of highly specific mouse anti-human mAbs that inhibit POSTN binding to integrin $\alpha v\beta 3$ and block POSTN-dependent migration of human endothelial colony forming cells. We further report that immunohistochemistry (IHC) analyses indicate that POSTN expression in breast cancer cells is a strong prognostic indicator along with tumour size, lymph node, and HER2 status.

Materials and Methods

Cell Culture and Transfections

Hs578T and HEK293T cells were obtained from the American Type Culture Collection (ATCC) and A375Pβ6 cells were provided by Prof. J. F. Marshall (Barts Cancer Institute, London, UK). Hs578T cells expressing lower levels of POSTN were generated by lentiviral mediated RNA silencing using GIPZ V3LHS_363447 and 363450 human POSTN lentiviral shRNAmirs (GE Healthcare Open Biosystems).

Human endothelial colony forming cells (ECFC) were isolated from human umbilical cord blood, collected in sterile pyrogen-free bags (Green Cross, Yongin, Korea) containing anticoagulant. Written informed consent was obtained from all donors

and the study was approved by the Institutional Review Board of Pusan National University Hospital. Mononuclear cells were isolated with Histopaque-1077 (Sigma-Aldrich, Switzerland) as described previously (26). ECFC were seeded on 0.1% gelatin coated culture dishes (Sigma-Aldrich) and maintained in Endothelial Growth Medium -2 Bullet Kit (EGM-2 bullet kit, Lonza AG, Switzerland). After four days non-adherent cells were removed and the adherent cells trypsinized and re-plated at a density of 1×10^6 per well until day 7 (27).

Immunization and generation of hybridomas

CBA/ca female mice were infected with LDV, a single stranded positive sense RNA enveloped arterivirus with B cell activation properties (28;29), by intra-peritoneal injection of infected plasma. One day later they were immunized in the footpads with 10μg human POSTN (Biovender Laboratory Medicine, Inc., Palackeho, Czech Republic) conjugated to ovalbumin (OVA) and emulsified in Gerbu 100 adjuvant (GERBU Biotechnik, Heidelberg, Germany). For conjugation to OVA, POSTN was first polymerized for 1 hour with 100mM N-(3-dimethylamino propyl)-N'-ethylcarbodiimide in the presence of 10mM N-hydroxysulfosuccinimide in 0.1M MES buffer pH4.8. After dialysis against 0.1M acetate buffer pH5.8, polymerized POSTN was conjugated to glutaraldehyde activated OVA (30). Mice were boosted 3 times at 2 week intervals, followed by an intravenous injection of 5µg POSTN 4 days before spleen cell fusion to SP2 plasmacytoma cells. Six anti-POSTN mAbs were selected. M444 (IgM) and M465 (IgG1) anti- 2, 4, 6-trinitrophenyl (TNP) mAbs were used as controls.

Antibodies were screened by ELISA on MAXISORP Nunc Immunoplates (Roskilde, Denmark) coated with 200ng/ml human or mouse POSTN (R&D Systems, MN) and detected using HRP-conjugated goat anti-mouse IgG (BD Bioscience, NJ). Reduction and alkylation was performed by incubating POSTN with DTT (18mM) for 1 hour, followed by iodoacetamide (150mM).

Integrin inhibition

Integrin $\alpha v\beta 3$-coated (200ng/ml) ELISA plates were incubated with a mixture of unlabelled (50μg/ml) and biotinylated (0.5μg/ml) human POSTN for 2 hours with and without anti-POSTN antibodies. After washing, streptavidin- horseradish peroxidase (HRP) was added for 2 hours. MAb dilutions were used to calculate IC50.

Mapping the Antibody Binding Sites

In vitro protein synthesis was performed using the TNT Quick Coupled Transcription/ Translation System (Promega, WI) to generate fragments of the POSTN protein. This system couples RNA transcription from a DNA template with translation of the transcribed RNA in a single reaction that utilises rabbit reticulocyte lysate. 50μl reactions assembled according to the manufacturer's guidelines were incubated at 37°C for 90 minutes and the products used for western blotting or ELISA.

Sixteen-mer POSTN peptides were used to coat ELISA plates at 10μg/ml in 50mM glycine buffer pH9.5. The peptide sequences were: 80-95**,** CPGYMRMEGMKGCPA V; 88-103, GMKGCPAVLPIDHVYG; 96-111, LPIDHVYGTLGIVGAT; 104-119, TLGIVGATTTQRYSDA; 112-127, TTQRYSDASKLREEIE; 120-135, SKLREEIEGKGSFTYF; 128-143, GKGSFTYFAPSNEAWD; 136-151,

APSNEAWDNLDSDIRR; 144-159, NLDSDIRRGLESNVNV; 152-167, GLESNVNVELLNALHS; and 160-177, ELLNALHSHMINKRMLTK. For smaller peptides, direct plate coating was not used to avoid epitope masking or irregular adsorption of some peptides to the ELISA plates. Instead, mAb (200ng/ml) was first incubated with a large molar excess of competing peptide (20μg/ml) before transfer to a POSTN-coated ELISA plate.

Adhesion Assay

To prepare POSTN coated plates, 50μl of 10μg/ml of POSTN diluted in TBS (10-20mM TrisHCl pH7.8/0.15M NaCl) was added to each well of a Nunc Maxisorp 96-well microtitration plate and incubated for 1 hour at 37°C. Wells were incubated with 5% BSA as a control. The plate was then rinsed in TBS and the remaining adherence sites blocked using 200μl 0.1% BSA for 0.5 hour. After washing the plate in TBS, either antibody or TBS were added to the wells and left to incubate for 0.5 hour before A375Pβ6 cells ($1{\times}10^4$) prepared in TBS/1mM $MnCl_2$, were added and incubated for 1 hour at 37°C. The wells were then washed 3-5 times in TBS/$MnCl_2$ and the adhered cells quantified by fluorescence using picogreen dsDNA dye (Invitrogen). The number of adherent cells was calculated using the fluorescence signal from the POSTN-coated plate and a reference plate, which contained a titration of different numbers of A375Pβ6 cells.

For ECFC adhesion assay, 96-well microculture plates (Falcon, Becton-Dickinson, Mountain View, CA) were incubated with recombinant POSTN or its FAS1-1domain (D1) at 4°C overnight, followed by blocking with PBS containing 0.2% BSA for 1 hour at 37°C. To assess mAb inhibitory activity, plates pre-coated with recombinant

POSTN or its FAS1-1 (D1) domain were pre-incubated with 1μg/ml MPB4B1(IgM) or MPC5B4(IgG1) at 37°C for 30 minutes. Cells were trypsinized and suspended in the culture media at 2 × 10^5 cells/ml, and 0.1 ml of the cell suspension was then added to each well of the plate. Analysis of cell attachment was performed as follows. After incubation for 1 hour at 37°C, unattached cells were removed by rinsing twice with PBS and the number of attached cells determined by counting under microscopy at 100x magnification after staining with hematoxylin and eosin.

Cell Migration Assay

Cell migration assay for ECFC was carried out in disposable 96-well chemotaxis chambers (Neuro Probe, Inc., Gaithersburg, MD). ECFC were harvested with 0.05% trypsin containing 0.02% EDTA, washed once, and suspended in EBM-2 at a concentration of 1x10^5 cells/ml. The lower surface of the membrane filter with 8-μm pores was coated overnight with 20μg/ml of rat-tail collagen at 4°C. A 50μl aliquot of ECFC suspension was loaded into the upper chamber, and EBM-2 supplemented with recombinant proteins such as Vascular endothelial growth factor (VEGF, 10ng/ml), recombinant human POSTN (10μg/ml, R&D Systems Inc. Minneapolis, MN), or its FAS1-1 domain (D1, 10μg/ml) were placed in the lower chamber. To test the inhibitory activity of antibodies, recombinant proteins were pre-incubated with 1μg/ml MPB4B1 (IgM) or MPC5B4 (IgG1) at 37°C for 30 minutes prior to supplementing EBM-2. Following incubation for 12 hours at 37°C, filters were disassembled and the upper surface of each membrane scraped free of cells by wiping with a cotton swab. The number of cells that had migrated to the lower surface of each membrane filter was determined by counting cells in four locations

under microscopy at 100x magnification after staining with Hoechst H 33258 (Sigma-Aldrich, Switzerland).

To investigate the involvement of integrin αvβ3 in POSTN or FAS1-1 domain-stimulated migration, ECFC were pre-incubated with 3 different POSTN peptides (10μg/ml) corresponding to amino acids 136-151, 140-150 and 112-127 of POSTN respectively, at 37°C for 30 minutes. ECFC were then loaded into the upper chamber pre-coated with rat-tail collagen, and migration determined after incubation with VEGF (10ng/ml), Vitronectin (10μg/ml; PeproTech, Inc., NJ), recombinant POSTN (10μg/ml) or FAS1 -1 domain (D1) of POSTN (10μg/ml) for 12 hours.

Immunohistochemistry and tissue microarray analysis

Cohort 1 consisted of 23 cases of invasive breast cancer together with one or more of the following: normal breast, columnar cell lesion, ductal carcinoma in situ (DCIS), lobular carcinoma in situ (LCIS). Cohort 2 consisted of invasive breast carcinomas from 449 patients diagnosed between 1987 and 1994. The median age of patients in this cohort is 60 years (range 27-88 years), and the median follow-up time is 13.2 years. All samples were collected from the Royal Brisbane and Women's Hospital (RBWH, Australia), with appropriate local ethical committee approval. Pathological and clinical data for these patients were obtained from a combination of clinical diagnostic pathology reports (Dept. of Pathology, RBWH, Australia), Queensland Cancer Registry (QCR) and internal histopathology review and analyses (SRL, MC, PS).

Tissue microarrays (TMAs) were prepared using duplicate 1.0mm cores (cohort 1) or 0.6mm cores (cohort 2) from representative, tumour-rich regions of each tumour. IHC for POSTN (mAb MPC5B4; 1/20,000 dilution of 2.8mg/ml) was performed on 4μm TMA sections. Heat antigen retrieval was performed in 0.01M sodium citrate buffer pH6.0. Staining was detected using the Mach 1 Universal HRP-Polymer Detection kit (Biocare Medical, LLC, CA).

Sections were counterstained with Mayer's Haematoxylin. For this analysis, POSTN expression was considered only in epithelial cells, since tumour stroma consistently exhibited high levels of this protein. Staining was scored as negative/weak, moderate or strong. Staining was further stratified based on whether tumour cell staining was heterogeneous or diffuse (i.e. >70% of tumour cells stained).

Associations between POSTN expression and clinico-pathological variables were analyzed using the Chi-Square test (GraphPad Prism v6). The relationship between POSTN expression and breast cancer-related survival as assessed after 25 year follow up was compared to standard prognostic indicators using the Gehan-Breslow-Wilcoxon test (GraphPad Prism v6), and those that significantly stratified outcome as single variables ($p<0.01$) were analyzed in a stepwise Cox Proportional Hazards Model (MedCalc v13.2), in which a p value of <0.05 was considered significant.

Results

Anti-human POSTN antibody production

By conjugating human POSTN to OVA as an immunogenic carrier (30) and infecting mice with LDV, a virus with immune-modulating activity (31), we successfully immunized CBA/ca mice against human POSTN (Supplementary Fig.1 shows the requirement of both OVA conjugation and LDV infection) and derived a panel of mAbs against this highly conserved protein. Specific binding titration curves for six such mAbs MPB4B1 (IgM), MPC1C3 (IgG2b), MPC4B12 (IgG2b), MPC5B4 (IgG1), MPC7A9 (IgM) and MPD9D5 (IgG2b) are depicted in Fig.1. The mAbs showed significant cross-reactivity with the mouse protein, demonstrating that the vaccination procedure had breached immune self-tolerance. Important differences in the avidities of these mAbs were observed with half maximal binding concentrations ranging from ±1ng/ml for MPC5B4 to >10µg/ml for MPB4B1. Moreover, cross-reactivity with mouse POSTN showed significant differences, indicating that these antibodies were derived from distinct B cells.

Since TGFβ1 is highly homologous to POSTN, we also tested whether these anti-POSTN antibodies will cross-react to TGFβ1 by ELISA. We found that MPB4B1, MPC1C3, MPC4B12, MPC5B4 and MPD9D5 showed no reactivity whereas MPC7A9 did exhibit a low level of cross reactivity. Since MPC7A9 is an IgM antibody that has a unique property of binding to Protein A, the low level of interaction of MPC7A9 with TGFβ1 is likely to be due to a non-specific stickiness of this IgM antibody (data not shown).

Inhibition studies using anti-POSTN antibodies

The inhibitory activity of these six mAbs was first tested by evaluating their influence

on binding of human POSTN to integrin $\alpha v\beta 3$-coated ELISA plates. IC50 for

inhibiting POSTN (5μg/ml) binding ranged from 1 to 30μg/ml for the mAbs illustrated

in Fig.2A. We also tested the ability of these six mAbs to inhibit POSTN binding to

integrin $\alpha v\beta 5$-coated ELISA plates. The results were very similar but much less

reproducible (data not shown).

The inhibitory activity was also tested by inhibition of adherence of A375Pβ6

melanoma cells to POSTN-coated plastic plates. A375Pβ6 cells express integrins

$\alpha 5\beta 1, \alpha v\beta 3, \alpha v\beta 5, \alpha v\beta 6$ and $\alpha v\beta 8$ (32) which can be non-specifically activated by

preparation in a Manganese containing buffer. The ability of these cells to adhere to

POSTN-coated plates was compared to BSA coated plates. We then determined if

our anti-POSTN mAbs would block the adherence of A375Pβ6 cells to POSTN.

MPB4B1 and MPC7A9 (both IgM) were the most inhibitory but MPC5B4 (IgG1) and

MPD9D5 (IgG2b) also significantly hindered cell adhesion (Fig.2B). MPC1C3 and

MPC4B12 which inhibited POSTN binding to $\alpha v\beta 3$ were inactive in this assay.

MPB4B1 also significantly inhibited adhesion of ECFC to POSTN or its FAS1-1

domain but not to collagen (Fig. 2C). Vitronectin, an adhesive protein, that is known

to bind a variety of integrins including $\alpha v\beta 3, \alpha v\beta 5, \alpha v\beta 6$ (33) also induces adhesion of

A375Pβ6 melanoma cells. This was not inhibited by MPB4B1 (Fig. 2D),

demonstrating that the inhibitory activity of this antibody was targetted against

POSTN.

Identification of POSTN sequences recognized by the antibodies

As our goal was to develop mAbs that were highly specific for western blotting and IHC and use them to unmask POSTN sequences required for its interaction with integrins, we tested whether they would react with linearized POSTN in western blots. MPC5B4 was the only mAb that detected POSTN expression in lysates of Hs578T human breast cancer cells, a cell line previously shown to express POSTN (20). It detected a 90kDa band which was lost when POSTN was silenced by RNA interference (Fig. 3A); POSTN expression and its silencing were confirmed at the RNA level (Fig. 3B). This attested to the specificity of MPC5B4 and also indicated that it recognized a linear epitope.

To determine the site recognised by MPC5B4, POSTN was divided into three fragments (F1, F2 and F3; Fig. 3C) which were cloned into pBlueScript SK+ and *in vitro* translated using the TNT T7 Quick Coupled Transcription/Translation System. The products of the three POSTN fragments as well as the full length molecule were immunoblotted (Fig. 3D). MPC5B4 recognised only F1 and FL indicating that the epitope was located within the first 260 amino acids. To further refine the epitope location, overlapping fragments (F4 and F5; Fig. 3C) corresponding to F1 were generated and the *in vitro* translated products tested by ELISA (Fig. 3E). Fragments F1 and F5 were recognized by MPC5B4 but not F4, thereby localising the epitope to aa 94-176.

16-mer peptides with 8aa overlaps covering aa 94-176 were synthesized and tested for MPC5B4 binding by ELISA. Only peptide 136-151 (APSNEAWDNLDSDIRR) was positive (Fig. 4A). To further refine the epitope, we performed competition experiments to avoid epitope masking or poor adsorption of these small peptides to

ELISA plates. MPC5B4 was incubated overnight with competing peptides to saturate its binding sites before transfer to POSTN-coated plates. Peptide 136-151 completely inhibited binding to POSTN (Fig. 4A&B), indicating that this was the only sequence recognized by this antibody on POSTN. Further trimming of the competing peptides showed that peptide 140-150 (EAWDNLDSDIR) was still able to block MPC5B4 binding to POSTN whereas 142-151 (WDNLDSDIRR) was not inhibitory (Fig. 4B&C). This indicated that the epitope for MPC5B4 is located within aa140-150 and corresponds to the POSTN region involved in its interaction with integrin $\alpha v \beta 3$. Interestingly binding of the five other mAbs MPB4B1, MPC1C3, MPC4B12, MPC7A9 and MPD9D5 to POSTN was also inhibited by peptide 136-151 but only MPC5B4 and MPB4B1 reacted with peptide 140-150 (Fig.4C). MPC1C3 and MPC4B12 also interacted slightly with peptide 128-143 suggesting that aa136-143 were likely to be part of their epitope. Interestingly these two mAbs did not significantly block adherence of A375Pβ6 cells to POSTN (Fig. 2B). As a control, we tested OC20, the antibody described by Orecchia *et al* which binds POSTN within the FAS1-2 domain (34); none of the peptides were able to compete with the interaction between OC20 and POSTN (data not shown).

Inhibition of ECFC migration

Recombinant POSTN has been shown to promote migration of ECFC via the FAS1-1 domain (35). To assess whether mAb MPB4B1 or MPC5B4, that recognize POSTN within aa140-150 in FAS1-1, can block POSTN-induced ECFC migration, we performed cell migration assays. Full length recombinant POSTN or its FAS1-1 domain (D1, corresponding to aa 94-234) stimulated ECFC migration to a similar level as VEGF (Fig. 5A). Pre-incubation of recombinant POSTN with MPC5B4

(IgG1) or MPB4B1 (IgM) or FAS1-1 with MPC5B4 (IgG1) reduced ECFC migration to the level of negative control whereas pre-incubation of FAS1-1 with MPB4B1 also reduced ECFC migration but this was not significant. However pre-incubation of POSTN or FAS1-1 with the isotype control IgM and IgG1 antibodies, did not significantly reduce migration as compared with the POSTN specific antibodies. Pre-incubation of VEGF with MPB4B1 or MPC5B4 did not affect VEGF-induced ECFC migration. These results show that both MPB4B1 and MPC5B4 can specifically block the stimulation of migration of ECFC by full length POSTN or its FAS1-1 domain and confirmed that their epitopes were located within the FAS1-1 domain.

To determine if POSTN dependent migration of ECFC could be competed with POSTN peptides, ECFC were pre-incubated with peptides corresponding to amino acids 136-151, 140-150 and 112-127. This showed that migration mediated by POSTN or its FAS1-1 domain was specifically inhibited by the 136-151 peptide but not by the 140-150 or the 112-127 peptides (Fig. 5B). This was particularly surprising as the 136-151 peptide is only 5 amino acids longer than the aa 140-150 peptide which comprises the epitope for MPC5B4 and MPB4B1. Vitronectin also mediated migration of ECFC which was also inhibited by the POSTN 136-151 peptide but not by aa 140-150 and 112-127 peptides (Fig. 5B). Taken together our results show that the FAS1-1 domain of POSTN can induce migration of ECFC and this can be specifically inhibited by the anti-POSTN antibodies MPC5B4 and MPB4B1 or competed by the POSTN 136-151 peptide which encompasses the epitope for these two antibodies. The peptide competition data indicates that the 136-151 APSNEAWDNLDSDIRR sequence within the FAS1-1 domain of POSTN is

required for its interaction with integrins and that this peptide can prevent integrin interaction not only with POSTN but also with vitronectin.

Clinical significance of POSTN expression in breast cancer

To evaluate whether mAb MPC5B4 can be used to identify POSTN expression in human tumour tissue, we performed IHC analysis on tissue microarrays of normal breast and breast cancer tissue. POSTN was, as expected, consistently expressed in the stromal compartment of normal and tumour samples (Fig. 6). We also observed variable expression of POSTN in tumour epithelial cells and so subsequent observations relate to POSTN expression in this epithelial cell compartment.

We first analysed expression of POSTN in a small cohort comprising matched normal breast, pre-invasive lesions and invasive tumours (cohort 1) to qualitatively examine changes in POSTN expression during tumour development. POSTN staining was consistently negative in normal epithelial cells (Fig. 6A), columnar cell lesions, LCIS and in 15/16 (93.7%) DCIS (Fig. 6B) but was evident in invasive tumour cells of both lobular and ductal type (16/23 cases, 69.6%) (Supplementary Fig. 2). Comparing matched *in situ* and invasive lesions, POSTN expression was unchanged in 7/17 (41%) cases (6 were negative in both components) and yet was increased in the invasive component relative to the in situ component in 10/17 (59%) cases. Although this is a small cohort, this pattern of expression of POSTN is intriguing and implies that epithelial cell-specific expression of POSTN might be dynamically regulated as tumour cells come into direct contact with the stromal microenvironment.

Next we examined POSTN expression in 449 invasive breast carcinomas with long

term follow up (cohort 2). We found that the epithelial cell compartment of 43.3%

invasive breast tumours expressed POSTN to moderate-strong levels (Fig. 6C).

Interestingly, in cases with heterogeneous expression levels the strongest

expression was often seen in cells at the tumour-stroma interface (Fig. 6D). Chi

square analysis showed associations between tumour cell POSTN expression and

HER2 status (P=0.0004), histological grade (P=0.0021), patient age (P=0.0046),

lymphovascular invasion (P=0.002), central scarring/fibrosis (P=0.0084) and

molecular subgroup (P=0.0081) (Supplementary Fig. 3). POSTN staining intensity

was inversely correlated with overall survival (P=0.0013, 25 years post-diagnosis)

(Supplementary Fig. 4). We also analysed the prognostic significance of tumour cell

POSTN in a multivariate cox proportional hazards model, and found that after

accounting for lymph node status and tumour size, POSTN staining was the next

most significant prognostic indicator (P=0.0321; Fig. 6E).

Discussion

Here we report that a combination of POSTN coupled to OVA as an immunogen and

mice infected with LDV virus has enabled production of highly specific anti-POSTN

mAbs that inhibit the interaction between POSTN and $\alpha v \beta 3$ integrin and identify the

sequence APSNEAWDNLDSDIRR within the FAS1-1 domain as required for this

interaction. POSTN dependent migration of human ECFC is inhibited by MPC5B4

and MPB4B1, as well as the FAS1-1 domain APSNEAWDNLDSDIRR peptide.

Moreover MPC5B4 is highly sensitive and specific for detecting POSTN by western

blotting, ELISA and IHC. IHC analysis of breast cancer tissue microarrays using

MPC5B4, confirmed that POSTN expression in the cancer cells is a significant marker of poor prognosis in breast cancer patients.

Due to the high degree of conservation between mouse and human POSTN (90.4% for the mature protein), the generation of mAbs against POSTN has proven extremely difficult. Success has been reported only in POSTN -/- mice (22), in mice immunized with human POSTN fused to human Fc (36) or to mouse γ2a heavy chain (Patent US7087727: Chen LB, Dai M, Sasaki H, Auclair D; Dana Farber Cancer Institute, USA) or by multiple peptide fragments (34). One mAb, termed MZ-1, was obtained from mice immunized with POSTN coupled to keyhole limpet hemocyanin (37). We confirmed mouse immune tolerance to human POSTN since we were only successful after conjugating POSTN to OVA as an immunogenic carrier and immunizing mice that had been infected with LDV, an arterivirus, that has been reported to enhance and modify specificity of antibody responses to various antigens (31). Even with this powerful immune stimulation, the antibody response was extremely narrow, since all mAbs produced from three immunized mice recognized sequences in the same 16 aa (APSNEAWDNLDSDIRR) located at position 136-151 within the FAS1-1 domain of POSTN. Of note, the mouse equivalent of this peptide differs from the human only by a conservative D to E substitution at aa 143. This highlights that the antibody induction required a true breach of immune tolerance. Differences were however noted between these antibodies when using smaller peptides and also in the extent of reactivity with the mouse protein indicating that they were not derived from the same B cell precursor. Several of these antibodies

completely inhibited POSTN binding to αvβ3 integrin suggesting that the 136-151

POSTN sequence could be targeted for functional inhibition.

The development of highly specific antibodies was important because our original

study demonstrating POSTN expression within the cancer cells had been

undertaken with a commercial rabbit polyclonal antibody which detected other bands

in addition to POSTN on western blots (unpublished data). This anti-POSTN

antibody was subsequently used to demonstrate a positive correlation between

POSTN expression, hormonal receptors, tumour size and unfavourable outcome in a

panel of 189 breast tumour samples (38). The IHC analysis presented here, using

the highly specific MPC5B4 mAb, showed that POSTN expression in the cancer cells

was a significant prognostic indicator in both univariate and multivariate analyses.

After accounting for lymph node status and tumour size, POSTN expression was

found to be more prognostic than HER2 status and grade. Our study also suggests

that POSTN expression increases upon tumour progression but a much larger study

involving more cases is required to reach statistical significance since our data set

comprised only 23 cases. However, this hypothesis is in accordance with the recent

report linking POSTN expression and breast tumour progression showing that

POSTN is essential for cancer stem cell maintenance in metastatic niches through

activation of the Wnt pathway (22).

This study has identified mAbs that can block the interaction between POSTN and

αvβ3. We have found that all six mAbs presented here recognize epitopes in the

16aa (APSNEAWDNLDSDIRR) located at position 136-151 within the FAS1-1

domain. Moreover the APSNEAWDNLDSDIRR peptide is able to block POSTN dependent migration of ECFC. This is very surprising and not in accordance with the $\alpha v\beta 3$ and $\alpha v\beta 5$ interaction sites on βigH3, a protein that is structurally homologous to POSTN. It has been found that both $\alpha v\beta 3$ and $\alpha v\beta 5$ interact with all 4 FAS1 domains within βigH3 (39;40). The interaction site comprises a tyrosine (Y) histidine (H) motif flanked by leucine and isoleucine residues which are all highly conserved within the FAS1 domains (KELANILK**YH**MVGRRVLY) (39;40). POSTN contains two homologous YH motifs located within FAS1-2 and FAS1-4, neither of which is close to aa136-151. It is of course possible that POSTN and $\alpha v\beta 3$ do not interact at exactly the same site as βigH3 and $\alpha v\beta 3$ but it is more likely that our mAbs perturb the binding site by steric hindrance or their accessibility given the size of the antibody molecule or the interaction perturbs the structure of the YH motifs. In any case, our results identify a novel APSNEAWDNLDSDIRR sequence that can be targetted for inhibiting POSTN function.   It is interesting to note that vitronectin that is known to bind a variety of integrins including $\alpha v\beta 3, \alpha v\beta 5, \alpha v\beta 6$ (33) also does not contain a YH motif or a sequence with significant homology to APSNEAWDNLDSDIRR even though vitronectin mediated migration of ECFC was also specifically inhibited by the FAS1-1 aa 136-151 peptide.

This conundrum was also raised by the inhibition of integrin mediated cell adhesion. Four of our anti-POSTN mAbs block the adhesion of A375Pβ6 melanoma cells to POSTN. The most efficient inhibitors were MPC7A9 and MPB4B1 even though they do not have the highest affinity for POSTN based on binding in ELISA. However both are IgMs and as pentamers they are likely to have a higher avidity/affinity ratio.

Previously Orecchia *et al* (34) generated OC20, an IgM mAb, that recognizes the POSTN sequence ALMK**YH**ILNTLQCSE comprising a YH motif within the FAS1-2 domain. OC20 also inhibits the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin-mediated adhesion of human melanoma SKMEL-28 cells to POSTN and slowed SKMEL-28 growth in NOD-SCID mice (34). Our results indicate that the FAS1-1 domain is an alternative target for modulating POSTN activity.

Recent observations that POSTN increases angiogenesis and lymphangiogenesis (23), promotes EMT and invasion, and is essential for cancer stem cell maintenance in metastatic niches (22) have suggested that inhibiting the effects of POSTN could have a place in anti-tumour therapies. The inhibitory anti-POSTN mAbs may thus have a role to play in the treatment of many forms of cancer particularly breast cancer. We have begun to test the inhibitory activities of our antibodies *in vivo* but until now we have been unable to reproduce the reported inhibition of growth of SKMEL-28 cells in NOD-SCID mice (34). In the interim, however, these antibodies provide an IHC reagent with unique specificity and relevance in the prognostication of human breast cancers. Moreover, the observation that the FAS1-1 domain peptide APSNEAWDNLDSDIRR was recognized by all antibodies inhibiting POSTN activity and can inhibit POSTN and vitronectin mediated cell migration makes this sequence a novel potential therapeutic target.

Conflict of interest

The authors declare that we have no conflict of interest.

Figure Legends

Figure 1. Saturation and specificity curves of mouse anti-POSTN mAbs. Six mAbs derived from 3 different LDV-infected CBA/ca mice immunized with human POSTN-OVA conjugates were titrated on ELISA plates coated with 200ng/ml recombinant human and mouse POSTN (R&D Systems, MN) or BSA. Bound antibodies were detected with HRP-goat anti-mouse IgG (BD Bioscience, NJ).

Figure 2. Inhibition of POSTN- $\alpha v\beta 3$ interaction by MP antibodies. (A) Integrin $\alpha v\beta 3$ (200ng/ml) coated ELISA plates were incubated with a mixture of unlabelled (50μg/ml) and biotinylated (0.5μg/ml) human POSTN for 2 hours with and without anti-POSTN antibodies. POSTN bound to integrin was detected by streptavidin-horseradish peroxidase (HRP). MAb dilutions were used to calculate IC50. (B) Antibodies or TBS were incubated for 0.5 hour at 37°C with POSTN (10μg/ml in TBS) or BSA coated Nunc Maxisorp 96-well microtitration plates. A375Pβ6 cells (1×10^4) prepared in TBS/1mM $MnCl_2$ were then added for 1 hour at 37°C. Number of adhered cells was quantified by fluorescent labelling using picogreen dsDNA dye (Molecular Probes, Invitrogen) and calculated by comparing the fluorescence signal to a reference plate, containing different numbers of A375Pβ6 cells. (C) 96-well microtitre plates were incubated with POSTN (FL) or its FAS1-1 (D1) domain or collagen as a control at 4°C overnight, followed by blocking with PBS containing 0.2% BSA at 37°C for 1 hour. To assess mAb activity, the coated plates were pre-

incubated with 1μg/ml MPB4B1(IgM) or MPC5B4(IgG1) or control mAbs at 37°C for

30 minutes before ECFC (2x10^4) were added. After incubation at 37°C for 1 hour,

unattached cells were removed and the number of adherent cells determined by

counting at 100x magnification after staining with hematoxylin and eosin. Statistical

significance was determined by Student's t-test: n=6, * indicates p<0.05. (D)

Maxisorb plates were coated with POSTN or Vitronectin (7.5μg/ml in TBS) or with

5% BSA (50μl per well) overnight at 4°C. Plates were blocked with 200μl 0.1% BSA

in TBS at 37°C for 1 hour followed by incubation with control IgM or MPB4B1

(50μl/well of 250μg/ml) at 37°C for 1 hour before addition of A375Pβ6 (2.5 x 10^4)

cells prepared in TBS/1mM MnCl$_2$. Plates were incubated at 37°C for 1.5 hours.

Adherent cells were detected with the Quant-iT Picogreen dsDNA assay kit.

Figure 3. Specificity of MPC5B4. Human POSTN pGIPZ1 (V3LHS_363447 and

363450) lentiviral shRNAs were used to silence POSTN in Hs578T cells (GE

Healthcare Open Biosystems). Lower amounts of POSTN were observed at the

protein level by western blot (A) and at the RNA level by RT-PCR (B). To map the

MPC5B4 epitope, fragments of POSTN (FL and F1-5) were inserted into

pBlueScript SK+ for *in-vitro* translation using the coupled *in-vitro* transcription

translation system. This system couples RNA transcription from a DNA template with

translation of the transcribed RNA utilizing rabbit reticulocyte lysate. (C) Schematic

diagram showing the various fragments relative to POSTN. The amino acid

sequences expressed were FL = 1-836, F1 = 1-260, F2 = 244-525, F3 = 497-836, F4

= 1-93; and F5 = 1-176. (D) The *in vitro* translation products for F1, F2, F3 and FL

were western blotted with MPC5B4. The fragments are indicated above each lane.

Two separate clones of each fragment were used. (E) The *in vitro* translation

products were used to coat ELISA plates overnight. MPC5B4 was used at 1μg/ml to detect fragments; binding significantly different to BSA, is denoted by *, p<0.05.

Figure 4. Identification of peptides recognized by mAbs. (A) Sixteen-mer POSTN peptides with 8aa overlaps were synthesized and used to coat ELISA plates at 10μg/ml in 50mM glycine buffer pH9.5. ELISA plates coated with mouse and human POSTN (0.5μg/ml) or the indicated peptides (10μg/ml) were incubated with MPC5B4 at 1μg/ml and bound mAb was detected with goat anti-mouse-HRP Ab. (B) Amino acid sequence of peptide 136-151. (C) To further refine epitope identification, competition experiments were undertaken with different fragments of the 136-151 peptide and with other overlapping peptides. MPC5B4 (0.5μg/ml) was incubated with 10μg/ml of the indicated peptides before transfer to a POSTN-coated plate. Bound mAb was detected with goat anti-mouse-HRP Ab. MPB4B1, MPC1C3, MPC4B12, MPC7A9 and MPD9D5 were also examined.

Figure 5. Inhibition of ECFC migration. (A) 5x10³ ECFC suspended in EBM-2 were placed in the upper chamber, and EBM-2 supplemented with recombinant proteins such as VEGF (10ng/ml), full length POSTN (FL, 10μg/ml), or its FAS1-1 domain (D1, 10μg/ml) were loaded in the lower chamber. To test the activity of mAbs, POSTN (FL) or its FAS1-1 (D1) domain were pre-incubated with 1μg/ml MPB4B1 (IgM) or MPC5B4 (IgG1) or control IgM and IgG1 mAbs prior to supplementing EBM-2. Following incubation for 12 hours at 37°C, filters were disassembled and the number of cells that had migrated to the lower surface of each membrane filter was determined by counting cells in four locations after staining with Hoechst H 33258

(Sigma-Aldrich, Switzerland). Data presented are a mean of three experiments.

Statistical significance was determined by Student's t-test: n=6, * indicates $p<0.05$.

(B) To clarify the involvement of integrin $\alpha v \beta 3$ in POSTN (FL) or FAS1-1(D1)

domain-stimulated migration of ECFC, cells were pre-incubated with 3 different

POSTN peptides aa 136-151, 140-150 and 112-127 at 10μg/ml at 37°C for 30

minutes. The peptide-incubated cells were then loaded into the upper chamber and

migration of ECFC was determined after incubation with VEGF (10ng/ml), Vitronectin

(10μg/ml), POSTN (FL) (10μg/ml) and its FAS1-1 (D1, 10μg/ml) domain proteins for

12 hours. Statistical significance was determined by Student's t-test: n=8, * indicates

$p<0.05$.

Figure 6. Representative POSTN staining in breast epithelial cells. POSTN was

negative in normal epithelial cells (A), DCIS (B) and in 56.7% of invasive tumours

(C); positive staining was detected in 43.3% of invasive tumours, the example given

(D) shows heterogeneous staining in which tumour cells at tumour-stroma interface

exhibit the strongest expression. Note the strong staining for POSTN in the stromal

compartment surrounding normal and DCIS and admixed with tumour (A-D). (E)

POSTN expression is predictive of a poor outcome at 5 years and 25 years follow up

(n=432 cases with assessable POSTN IHC and clinical outcome data).

Supplementary Figures

Supplementary Figure 1. Induction of anti-POSTN Ab responses by OVA-

conjugation and LDV infection. CBA/ca mice were infected or not with LDV one day

before immunization with human POSTN conjugated or not to OVA. Sera were

collected after 4 immunizations and tested by ELISA on POSTN-coated plates. Plates coated with BSA used as control were negative (data not shown).

Supplementary Figure 2. POSTN and breast cancer development in Patient cohort 1. POSTN expression was assessed at different stages of tumour progression in samples from 23 cases; including in normal epithelium, various pre-invasive lesions [i.e. columnar cell lesions, lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS)] and invasive tumours [i.e. invasive lobular carcinoma (ILC) and invasive ductal carcinoma (IDC)]. The upper panel shows the frequency in which POSTN expression was positive in the different sample types and the lower panel shows how the pattern of expression changed between different sample types within the same patient.

Supplementary Figure 3. Correlation between POSTN expression and clinico-pathologic features. Chi-square analysis showing association between POSTN expression and other markers of clinical behaviour in breast cancer.

Supplementary Figure 4. Relationship between POSTN expression and breast cancer-related survival. POSTN expression was scored as a continuous variable (negative/weak, moderate and strong staining) and then the prognostic significance of expression was assessed using univariate and multivariate models. Events censored were breast cancer-related deaths only. HER2 positivity was defined primarily on the basis of CISH data or IHC 3+ where CISH data were not available. >1% tumour cell oestrogen and/or progesterone receptor positivity was considered as positive. TN = negative for ER, PR and HER2; Ki67 threshold was 20% tumour

cells stained; covariates with p<0.01 in univariate analysis were included in the

multivariate model.

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