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Abstract

Nicastrin is an essential component of the gamma secretase (GS) enzyme complex, required for its synthesis and recognition of substrates for proteolytic cleavage. The purpose of this study was to investigate whether nicastrin has prognostic value or potential as a therapeutic target in breast cancer.

The suitability of nicastrin as a target in breast cancer (BC) was assessed using BC tissue microarrays (TMAs) (n = 1050), and its biological role *in vitro* was evaluated in BC cell lines following gene silencing. Nicastrin blocking antibodies were developed and evaluated for their suitability as potential clinical therapeutics.

TMA and cell line analysis confirmed that nicastrin expression was upregulated in BC compared to normal breast cells. In TMA patient samples, high nicastrin expression was observed in 47.5% of cases and correlated with ERα expression, patient age and tumor grade. In pre-defined subset analysis, high nicastrin expression predicted for worse BC specific survival in the ERα -ve cohort. *In vitro* gene silencing of nicastrin resulted in disruption of the GS complex and a decrease in notch 1 cleavage. This was sufficient to increase E-cadherin expression and its co-localization with p120 catenin at cell-cell junctions in MCF7 cells. Nicastrin silencing in invasive MDA-MB-231 cells resulted in loss of vimentin expression and a marked reduction in both cell motility and invasion; which was concomitant with the *de novo* formation of cell-cell junctions characterised by the colocalization of p120 catenin and F-actin. These data indicate that nicastrin can function to maintain epithelial-to-mesenchymal transistion during BC progression.

Anti-nicastrin polyclonal and monoclonal antibodies were able to decrease notch1 and vimentin expression and reduced the invasive capacity of BC cells *in vitro*. This supports our hypothesis that a nicastrin blocking antibody could be used to limit metastatic dissemination in invasive BC.

Introduction

Tumor progression and metastatic spread involves upregulation of molecular components that constitute numerous proteolytic and cell migration pathways that help drive cell invasion into the surrounding stroma [1]. Gamma-secretase (GS) is a unique multi-protein complex with activity as a cell surface protease that directly cleaves a variety of transmembrane proteins to modify their function. The GS complex comprises of nicastrin, anterior pharynx defective 1 homolog, presenilin enhancer protein-2, and the aspartyl protease presenilin [2]. The proteolytic targets of GS include the amyloid precursor protein [3] and a number of proteins that function in tumor cell proliferation, adhesion and migration: including notch 1-4, ErbB4, CD44, E-cadherin, EpCAM [4, 5].

The principal rationale behind introducing GS inhibitors (GSIs) as anti-cancer therapeutics was the discovery of activating mutations in notch 1 in >50% of T-cell acute lymphoblastic leukemia patients and later data that described the overexpression and oncogenic function of notch proteins in solid tumors, including lung, malignant melanoma and breast [6]. Activation of the notch pathway results in binding of the transcription factor CSL to the promoter of target genes, such as *Hes*, *Hey*, *Snail*, *c-myc* etc., that promote cell survival and can also regulate epithelial to mesenchymal transition (EMT) [7, 8]. E-cadherin is essential for the stabilization of epithelial cell-cell adherens junctions and its frequent loss in human cancers is associated with *Snail* overexpression [9]. Furthermore, notch proteins can maintain cancer stem cells [10] that harbour an EMT-like phenotype: $CD24^{-}/CD44^{+}$, *Snail*, *Twist* and vimentin upregulation; and loss of E-cadherin [8]. Interestingly, E-cadherin and its coregulator p120 catenin (p120ctn) directly interact with the GS complex by binding to the same domain of presenilin [11]. This indicates that their competitive binding to GS complex could have an impact on adherens junction stability [12].

Nicastrin provides critical structural support for GS complex assembly and facilitates molecular recognition of GS substrates for cleavage. Overexpression of nicastrin was sufficient to enhance GS activity without altering presenilin expression [13]. Functional sites of nicastrin have been identified in its extracellular and transmembrane domains. Mutations in the transmembrane domain (S632A and W648A) resulted in the inability of nicastrin to bind other GS components, disrupting complex formation and the proteolytic activity [14]. A Ser to Ala mutation at position 333 located in the external, DYIGS sequence of nicastrin (homologous with Domains harboring Amino Peptidase activity (DAP)), was sufficient to prevent substrate binding [14]. Targeting these functional regions of nicastrin may confer therapeutic benefit in diseases/conditions that involve nicastrin and/or GS activity.

In this study we hypothesized that nicastrin is overexpressed in breast cancer and that its large extracellular domain could represent an antigen target suitable for the development of a specific therapeutic antibody. Monoclonal antibodies (McAbs) have emerged as effective therapeutics that have demonstrated clinical benefit (reducingd tumor burden and increasing disease free and overall survival) when used alone or in combination with chemotherapy. Currently, there are four FDA approved McAbs for the treatment of solid tumors: Trastuzumab, Bevacizumab, Cetucimab and Panitumumab, and over one hundred McAbs are in early and late phase clinical trials [15] .

Our data confirm that: (1) nicastrin expression is upregulated in BC compared to normal breast tissue; (2) silencing nicastrin at the gene level can prevent breast cancer invasion; and (3) specific anti-nicastrin McAbs inhibit the invasion of breast cancer cells, which suggests that a similar approach could be used to limit metastatic dissemination in invasive breast cancer.

Materials and Methods

Cell lines. MCF-7 and MDA-MB-231 cells were from the American Type Culture Collection (ATCC). *NCSTN*$^{+/+}$ and *NCSTN*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were obtained from Dr Philip Wong (Dept. Neuropathology, Johns Hopkins University, ML, USA). Cells were grown in DMEM with 10% foetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin in 5% CO_2 at 37°C. The non-tumorigenic immortal breast cell line 184A1 (CRL-8798; ATCC) was cultured in basal mammary epithelial cell growth medium (Promocell, UK), supplemented with bovine pituitary extract (13 μg/ml), epidermal growth factor (10 μg/ml), hydrocortisone (500 μg/mI), insulin (5 μg/ml), transferrin (0.005 μg/ml), choleratoxin (1 ng/ml), penicillin (100 U/ml) and streptomycin (0.1 mg/ml).

Clinical specimens, immunohistochemistry (IHC) and statistical analysis. Patient tissue resource and patientcohort characteristics, immunohistochemistry staining protocol of TMAs and statistical analysis are described in the Online Resource 1 and are in line with the REMARK criteria [16].

RNA Interference. Cells were transfected with a final concentration of 40 nM nicastrin siRNA oligonucleotides (D-008043, Dharmacon, UK) using HiPerFect (Qiagen, UK). EGFP siRNA (AM4626, Ambion, UK) was used as a negative control.

Western Blotting. Cell lysates were lyzed using buffer (150 mM NaCl, 0.1% (w/v) sodium dodecyl sulphate, 5 mM EDTA, 10 mM Tris-HCl (pH 7.2), 1% (v/v) Triton-X and 1% (w/v) deoxycholate), containing 1 μM phenylmethanesulfonyl fluoride and 1% v/v protease inhibitor cocktail (catalogue no. P8340, Sigma UK). Further steps were as previously described, only using 10%w/v SDS-polyacrylamide gels [17]. Primary antibodies and concentrations: 1/500 rabbit polyclonal anti-nicastrin antibody (pAb) (ab2474); 1/500 mouse anti-human presenilin monoclonal antibody (mAb) (ab12272); 1/10,000 mouse anti-human β-actin (ab8229) (Abcam, UK); 1/2,000 mouse anti-human E-cadherin mAb (610182), 1/1,000 rabbit anti-human p120 pAb (610133) (BD Transduction Laboratories, UK); and 1/500 mouse anti-human vimentin mAb (sc-6260, clone V-9) (Santa Cruz, USA).

RNA isolation and quantitative RT-PCR. Isolation of total RNA was performed using the RNeasy kit (Qiagen, UK). RNA (1 μg) was reverse transcribed using oligo dT and Transcriptor First Strand cDNA Synthesis Kit (Roche, UK). Quantitative PCR was performed using the SYBR Green PCR mastermix (Applied Biosystems, UK) on a 7900HT Thermocycler (Applied Biosystems, Warrington, UK) using primers for nicastrin (*NCSTN*), E-cadherin (*CDH1*), Snail (*SNAI1*), vimentin (*VIM*), presenilin (*PSEN*), *p120ctn* and *GAPDH* (Operon, UK). The PCR cycle number that generated the first fluorescence signal above a threshold (threshold cycle, CT; 10 standard deviations above the mean fluorescence generated during the baseline cycles) was determined, and a comparative CT method was then used to measure relative gene expression.

GS activity assay. GS activity was measured using the R&D Systems Gamma Secretase Activity Kit following the manufacturer's instructions. Briefly, cells were transfected with control or nicastrin siRNA and harvested after 72h in cell extraction buffer and incubation on ice for 10 minutes. Lysates were centrifuged at 10,000 x g for 1 minute and supernatants were collected with a total protein yield of 0.5-1.0 mg/ml. Pierce BCA Protein Assay was used to

determine protein concentration. Protein (200 µg) was incubated with the GS fluorogenic substrate for 1 hour at 37°C and fluorescence was measured at 355/460nm.

Immunofluorescent staining and confocal microscopy. Immunostaining and confocal imaging were carried out as previously described [18]. 1 x 10^4 cells were seeded on 13 mm diameter glass cover slips, fixed in 4% w/v paraformaldehyde at 37°C and incubated in immunofluorescent blocking buffer (2% v/v FCS and 1% BSA) followed by incubation with 1/100 nicastrin pAb, 1/100 E-cadherin or 1/100 p120 catenin antibodies. Incubation with 1/1,000 anti-rabbit or anti-mouse-IgG Alexa Fluor®-488 or -555 or phalloidin Alexa Fluor®-633 (Molecular Probes, UK). Nuclear counterstaining was carried out using TO-PRO-3 and coverslips were mounted using Vectashield H-1200 (Vector Laboratories, UK). Images were captured using a Zeiss LSM 410 confocal microscope (63x lens; 1.40 NA oil; Leica) using Immersol 518F oil (Zeiss). Image composites of ~20 x 0.5 µm *z*-stacks were obtained using Axiovision LE software (Zeiss). Photoshop 8.0 (Adobe Software) was used for post-acquisition editing of images.

Cell migration and invasion. 1 x 10^4 cells were plated on glass bottomed 24-well plates and imaged by digital recording at a time-lapse interval of 10 min for 16 h in an environmentally controlled ImageXpress Micro microscope (Molecular Devices, UK). The average speed of cell migration (mean ± s.e.m.; n > 100 cells) was determined [19]. Cell invasion assays were performed using Matrigel-coated polycarbonate transwell filters (8 µm pore size) (Beckton Dickinson, UK). 70 µl Matrigel (diluted 1:2 in α-MEM) was added to the upper chamber and allowed to polymerise for 1 h at 37°C. Keratinocyte growth medium consisting of α-MEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B (Gibco), 1.8 × 10^{-4} M adenine, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 10 ng/ml epidermal growth factor and 1 × 10^{-10} M cholera toxin. 500 µl of this medium was placed in the lower chamber to act as a chemoattractant. Cells were plated in the upper chamber of quadruplicate wells at a density of 5 x 10^4 in 200 µl of α-MEM and incubated at 37°C for 72 hours. Cells in the lower chamber (including those attached to the under surface of the membrane) were trypsinised and counted using a Casy 1 counter (Sharfe System GmbH, Germany).

Results

Nicastrin expression in BC and correlation with relevant tumor biomarkers. Nicastrin expression was initially evaluated in a non-malignant breast cell line and four BC cells lines. A marked upregulation of nicastrin protein level was observed in four BC cell lines (MCF7, MDA-MB-231, BT-474 and SKBR3) compared to the non-tumorogenic breast cell line, 184A1 (Figure 1a). Normal breast tissue from reduction mammoplasties (n = 40) were examined by IHC. Nicastrin was absent (IHC score 0) in 30/40 cases, and expressed at very low levels (IHC score 1+) in the remaining 10 cases. Nicastrin immunostaining in BC TMAs showed predominant cytoplasmic localization with variable cell membrane staining. Informative data were obtained in 1050 tissue cores, of which 52.5% showed low (0 and 1 intensity score) and 47.5% showed high (2+ and 3+ score) expression (Figure 1b). Nicastrin expression was retained in lymph node metastasis (n = 15). Specificity of the IHC staining was verified using pre-incubation with the nicastrin blocking peptide (N164, Leinco Technologies). High nicastrin expression positively correlated with hormonal receptors, oestrogen receptor α (ER α) ($p < 0.001$), progesterone receptor (PR). There was a direct correlation with cytokeratin 18 ($p < 0.001$), and inverse with basal cytokeratines 5/6 ($p = 0.01$). Nicastrin expression showed correlated with patient age

(p = 0.031), where patients > 50 yrs were more likely to have tumors expressing high nicastrin levels. Nicastrin expression correlated with tumor grade (p<0.001). With respect to tumor type, nicastrin appeared to be significantly upregulated in tubular carcinomas (208/1050), with 61% (n = 127/208) showing high nicastrin expression, vs. 39% (n = 81/208) with low nicastrin levels (p = 0.002). In patients with invasive ductal carcinoma (624/1050), 54.3% (n = 339/624) had high and 45.7% (n = 285/624) low nicastrin levels. No correlation was observed with tumor size. Patient characteristics are summarized in Table 1.

Association of nicastrin expression with clinical outcome. Kaplan Meyer survival analysis indicated that high nicastrin expression was not predictive of BC specific survival in the whole dataset (p = 0.664) or the ERα+ve subset of patients (p = 0.183) (Figure 2a, 2b) Further subset analysis in the ERα-ve patients (275/1050) indicated that high nicastrin levels had borderline predictive value for BC specific survival at 5 years (p = 0.05) (Figure 2c). Nicastrin expression did not retain significance in a multivariate analysis model combined with tumor grade, stage and size. Nicastrin expression did not predict outcome to systemic adjuvant treatments (endocrine or chemotherapy).

Expression pattern of nicastrin in a panel of human tissues. We further studied nicastrin expression in normal and malignant tissue from other human organs. Expression of nicastrin in normal tissues is summarized in Table 2. Malignancies found to have nicastrin upregulation (2+) include colon adenocarcinoma, lung adeno- and squamous cell-carcinoma, head and neck squamous cell carcinoma, thyroid papillary and follicular carcinoma, pancreatic adenocarcinoma and liposarcoma.

Targeting nicastrin disrupts GS complex in breast cancer cells. An siRNA targeting approach was used in MCF7 and MDA-MB-231 BC cell lines, which represent luminal and basal BC phenotypes respectively [20]. A silencing efficiency of >90% was achieved for nicastrin protein (Figure 3a), and mRNA (Figure 3b), levels in both cell types (p<0.0001). Importantly, nicastrin silencing was sufficient to significantly reduce GS complex enzymatic activity (p<0.001) (Figure 3c) and transcription of its direct target in the notch signalling pathway, *Hes1* (p<0.001) (Figure 3b). In accordance with a previous report [21], the concomitant loss of presenilin protein (Figure 3a) without any change in mRNA (data not shown) suggests that loss of nicastrin is sufficient to destabilize GS complex proteins in breast cancer cells.

Nicastrin regulates breast epithelial cell-cell junctions through E-cadherin and p120ctn. The GS substrate E-cadherin is required for the formation of stable cell-cell adhesions in normal breast epithelium. To investigate whether nicastrin might impact on the stability of breast epithelium, the E-cadherin positive MCF7 cell line was transfected with nicastrin siRNA. Silencing of nicastrin in MCF7 monolayers increased the total cellular levels of E-cadherin mRNA and protein (Figure 3 a, d) and facilitated its enhanced localization to cell-cell junctions (Figure 4a). Together with the observation of reduced intercellular spacing (Figure 4a), this suggests that nicastrin plays a significant role in the regulation of BC cell-cell adherens junctions. A potential mechanism might be via the release of transcriptional repression upon *E-cadherin* by *Snail1*, since levels of this pivotal transcriptional factor in the EMT program were significantly reduced following nicastrin silencing (p<0.001) (Figure 3d).

Additional evidence suggests that nicastrin-dependent regulation of E-cadherin may involve p120ctn, a molecule that is responsible for regulating E-cadherin turn-over from the plasma membrane [22], thereby stabilizing E-cadherin localization to adherens junctions [12, 23]. This regulatory mechanism may be modulated by the presence of a stable GS complex at the plasma membrane where it can compete with p120ctn for binding to E-cadherin [23]. The increase in p120ctn protein expression (Figure 3a) and localization to MCF7 cell-cell junctions (Figure 5), following nicastrin silencing, reveals that there may be multiple levels at which this component of the GS complex can regulate the stability of luminal epithelial cell-cell adhesions.

Involvement of nicastrin in breast cancer cell migration and invasion. i) Genetic silencing of nicastrin: The finding that high nicastrin expression was borderline predictive of worse BC specific survival in ERα negative patients, led to the hypothesis that silencing nicastrin in an ERα negative MDA-MB-231 cell line, which has a typical mesenchymal-like phenotype and high metastatic potential, would inhibit their invasiveness. Genetic silencing of nicastrin using siRNA in MDA-MB-231 cells fully disrupted the GS complex, as demonstrated by the loss of presenilin protein (Figure 3a) and reduction in GS enzymatic activity (Figure 3c). Although p120ctn protein expression following siRNA targeting nicastrin was not significantly altered (Figure 3a), it displayed a marked relocalization from the cytoplasm to the sites of cell-cell contact, where it became associated with F-actin fibres (Figure 4b). This striking phenotype in MDA-MB-231 cells signifies that the loss of nicastrin and subsequent destabilisation of the GS complex was sufficient for the re-formation of cell-cell junctions in cells that have undergone progressive transformation towards a scattered single cell distribution. Genetic silencing of nicastrin was also sufficient to abolish transcription and expression of the mesenchymal marker and pro-migratory cytoskeletal protein vimentin (Figure 3a, d) [24]. Video time-lapse microscopy of MDA-MB-231 cells treated with nicastrin siRNA revealed that their marked decrease in migratory speed (Figure 4c) was consistent with a reduced invasive capacity in Matrigel (Figure 4d). To validate that the phenotypic changes associated with nicastrin silencing in BC cells were due to a direct impact on the key components of the genetic program required to maintain a mesenchymal cell phenotype, we investigated the migratory behaviour and morphology of *NCSTN*$^{-/-}$ mouse embryonic fibroblasts (MEFs) [21]. Unlike *NCSTN*$^{+/+}$ MEFs, which displayed a typical fibroblastic spindle-like morphology with a scattered distribution (Online Resource Movie 1), *NCSTN*$^{-/-}$ MEFs were rounded and formed dense aggregates (Online Resource Movie 2) with a marked reduction in their migratory speed (>60%, $p < 0.01$) (Online Resource 2). This evidence strengthens our hypothesis that nicastrin silencing is capable of restoring an epithelial and/or less invasive phenotype in BC cells that have progressed via a *bona-fide* EMT program to become more invasive. ii) Development of anti-human nicastrin blocking antibodies: A rabbit polyclonal antibody (PcAb) raised against the extracellular domain of human nicastrin protein was confirmed to bind endogenous nicastrin in MCF7 and MDA-MB-231 using FACS analysis (Online Resource 3). The efficacy of blocking nicastrin function with PcAb was confirmed by its strong inhibitory effects on MDA-MB-231 invasion (70%; $p < 0.001$) (Figure 6a). The first phase of mouse anti-nicastrin monoclonal antibody (McAb) development, resulted in the production of supernatants from twenty one clones, of which eight had strong affinity for endogenous nicastrin in MCF-7 and MDA-MB-231 cells (data not shown). Seven of the eight clones significantly inhibited the invasion of MDA-MB-231 cells (Figure 6b). The two anti-nicastrin McAb clones with the most potent anti-invasive effects significantly reduced vimentin expression and notch1 cleavage, to similar levels as anti-nicastrin PcAb (Figure 6c). None of the antibodies affected nicastrin protein levels (Figure 6d).

Discussion

Most studies to date reported altered expression and function of GS substrates, such as notch proteins, E-cadherin, CD44 and HER4 [25-27]. Limited attention has been given to investigating the expression and function of individual GS components, with a general notion that the function of all four proteins: nicastrin, anterior pharynx defective 1 homolog, presenilin enhancer protein-2, and presenilin, are united within this enzyme complex. Existing results are informative predominantly for expression of presenilin in mouse and rat tissues. Nicastrin and presenilin were shown to be upregulated in colon cancer cells as response to treatment with doxorubicine, enhancing notch activity, which indicated their involvement in developing resistance to chemotherapeutic agents [28]. One report reveals though, that levels of nicastrin and presenilin are not co-ordinately regulated, suggesting for the first time, an independent function of nicastrin in mouse smooth muscle tissue [29]. Recent work has revealed that nicastrin can indeed functions independently from its substrate recognition role within the GS complex. It was shown that nicastrin overexpression in HEK293 cells can regulate p53 mediated cell death via a mechanism that does not require GS complex formation [30].

This study reveals marked upregulation of nicastrin in human BC compared to normal breast tissue. Its expression correlated positively with ERα, PR expression and cytokeratin 18 expression, and inversely with cytokeratines 5/6, suggesting that it is predominantly expressed in non-basal like breast carcinomas. This is in line with our observation that tubular breast carcinomas are more likely to express high nicastrin levels. Tubular BC have predominantly ERα +ve phenotype and favourable prognosis, but carry a risk of developing contralateral BC of higher grade [31]. The potential disconcordance between observed co-expression of nicastrin and ERα on the one hand, and lack of impact of high nicastrin expression on survival in hormone-dependent BC, can be explained by the fact that anti-hormonal treatment given to ERα +ve patients, attenuates the adverse impact of nicastrin expression over the years. Hormone-independent BCs represent a subset of tumors currently being treated with chemotherapy as first line adjuvant systemic treatment. High nicastrin expression was observed in 38.2% of ERα -ve in the analyzed cohort where we observed a borderline impact on BC specific survival at 5 years ($p = 0.05$). Our *in vitro* data generated in the MDA-MB231, ERα-ve cell line, show that silencing nicastrin at the gene level, and blocking nicastrin with specific PcAb and McAb markedly reduced cell invasion, vimentin levels and GS activity. Taken together, these observations suggest that anti-nicastrin McAb may be used in the treatment of invasive BC to prevent cancer cell dissemination and metastasis. Also, inhibition of vimentin, a *bona fide* EMT denominator, has previously been shown to reduce cellular invasive capacity [24] during the reversal of an EMT program.

In ERα +ve, MCF7 cells, nicastrin silencing was sufficient to stabilize cell-cell junctions. The mechanism underlying this newly identified property of nicastrin appears to involve the regulation of E-cadherin and p120ctn localization to cell-cell junctions. The up-regulation of E-cadherin protein and mRNA levels, as well as the enhanced cell membrane localization upon nicastrin silencing could involve several mechanisms. The first mechanism relates to reduced GS cleavage of E-cadherin, which consequently reduces E-cadherin turn-over from the cell membrane. A secondary mechanism of regulation could occur via the activation of pro-transcriptional targets. Mechanistically, this could represent a direct nicastrin effect and/or a notch-mediated event. This is corroborated by reduced transcription of the notch target *Hes1* in MCF7 cells upon nicastrin silencing. Notch mediated effects on *Snail* were previously shown in an ovarian cancer model [7]. Nicastrin dependent E-cadherin regulation could also involve p120ctn, which stabilizes E-

cadherin localization to adherens junctions by regulating its turnover or competing with presenilin for direct binding to E-cadherin [12, 23].

Inhibitors of the GS complex used in the treatment of Alzheimer's disease [32] are currently being validated in clinical trials for a number of human malignancies. Although their exact mechanism of action remains to be elucidated, the primary target of GS inhibitors is most likely to be the catalytic component of the GS complex, presenilin [33]. Whereas in Alzheimer's disease the target is beta-amyloid, the primary targets of GSIs in human malignancies are likely to be notch proteins. Targeting the GS complex may find a role in re-sensitizing cancer cells to chemotherapy, hormonal and targeted therapies, given the upregulation of notch 1 upon treatment with oxaliplatin in colon cancer [28], and tamoxifen and trastuzumab in BC cell lines [28, 34, 35]. The lack of tissue specificity of GSIs, however, results in significant gastrointestinal toxicity and lethargy [36], limiting their clinical appeal. Targeting nicastrin may provide an alternate therapeutic way to achieve more stringent tissue specificity. Further, evidence exists to suggest that nicastrin can function independently of the GS complex [30], and therefore targeting nicastrin may represent a valid therapeutic approach. Our proof of principle studies using an anti-human nicastrin PcAb and McAbs confirm that cell surface nicastrin can be blocked to impart anti-invasive effects on BC cells. This suggests that developing a humanized McAb against nicastrin may help in the future design of personalised therapeutic strategies for the treatment of invasive BC.

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Figure legends

Figure 1. Nicastrin expression is upregulated in breast cancer compared to normal breast. **a**, Western blotting of nicastrin protein levels in a normal breast cell line 184A1 and four breast cancer cell lines (MCF7, MDA-MB231, BT474 and SKRB3); **b,** Nicastrin expression in breast cancer tissue (n = 1050) was analyzed by IHC. Representative images of nicastrin null, 1+, 2+ and 3+ staining in breast tumors. Representative image of normal breast tissue (n = 40), showing absence of nicastrin expression. Pre-incubation with competing peptide confirms the specificity of the N-1660 antibody; **c,** Nicastrin expression in a paired tumor/metastatic lymph node sample (n = 15).

Figure 2. Kaplan-Meier model comparing breast cancer specific survival in breast cancer patients showing nicastrin protein expression; **a**, Entire analyzed cohort (n = 1050) (p = 0.664); **b**, ERα positive patients (p = 0.183); **c**, *ERα negative patients (p = 0.05).

Figure 3. Nicastrin silencing in breast cancer cells disrupts GS complex, inhibits the notch signalling pathway and affects proteins that regulate cell adhesion and motility. **a**, Western blotting analysis using indicated antibodies in control and nicastrin siRNA treated cells. β-actin used as loading control. **b**, Quantitative RT-PCRs were carried out to determine fold changes in expression of nicastrin and *Hes1* in nicastrin siRNA treated samples compared with control (eGFP siRNA) samples. Data are the mean fold change in expression compared with control siRNA samples from duplicate samples in three independent experiments. **c**, GS activity assay upon nicastrin silencing in MCF7 and MDA-MB231 cells. GS inhibitor (GSI1) (Calbiochem, UK) at 1μmol concentration was used as a positive control in this experiment. Nicastrin silencing significantly inhibits GS activity ($p < 0.001$). Degree of inhibition is comparable to that of a GSI in both cell lines. d, Nicastrin silencing regulates transcription levels of E-cadherin, *Snail* and vimentin. Quantitative RT-PCRs were carried out to determine fold changes in expression of E-cadherin (*CDH1*), *Snail* (*SNAI1*) and vimentin (*VIM*) in nicastrin siRNA treated samples compared with control (eGFP siRNA) samples ($p < 0.001$). Data are the mean fold change in expression compared with control siRNA samples from duplicate samples in three independent experiments.

Figure 4. a, Nicastrin silencing affects E-cadherin in MCF7 breast cancer cells. Cells were tranfected with nicastrin siRNA and after 72 h fixed, permeabilized and stained with anti-E-cadherin mAb. The antibodies were labelled with Zenon Alexa Flour 488 (Invitrogen); **b**, Nicastrin silencing promotes formation of cell junctions in MDA-MB-231 cells. MB-MDA-231 cells were stained with anti-p120 mAb followed by Alexa Flour 488 goat anti-mouse IgG (H+L) (Invitrogen). Polymerized actin was visualized with a tetramethylrhodamine isothiocyanate-phalloidin conjugate (red). Nuclei were counterstained with TO-PRO-3 (blue). Bar = 10 μm. * Cell-cell junctions. Nicastrin silencing regulates cell-cell junction and pro-invasive proteins in breast cancer cell lines; **c,** Effect of nicastrin silencing on the migration of MDA-MB-231 cells; **d,** Effect of nicastrin silencing on invasion of MDA-MB-231 cells. Bars represent mean number of invaded cells ± SD from three separate experiments. Statistical difference from control levels, $p = 0.01$.

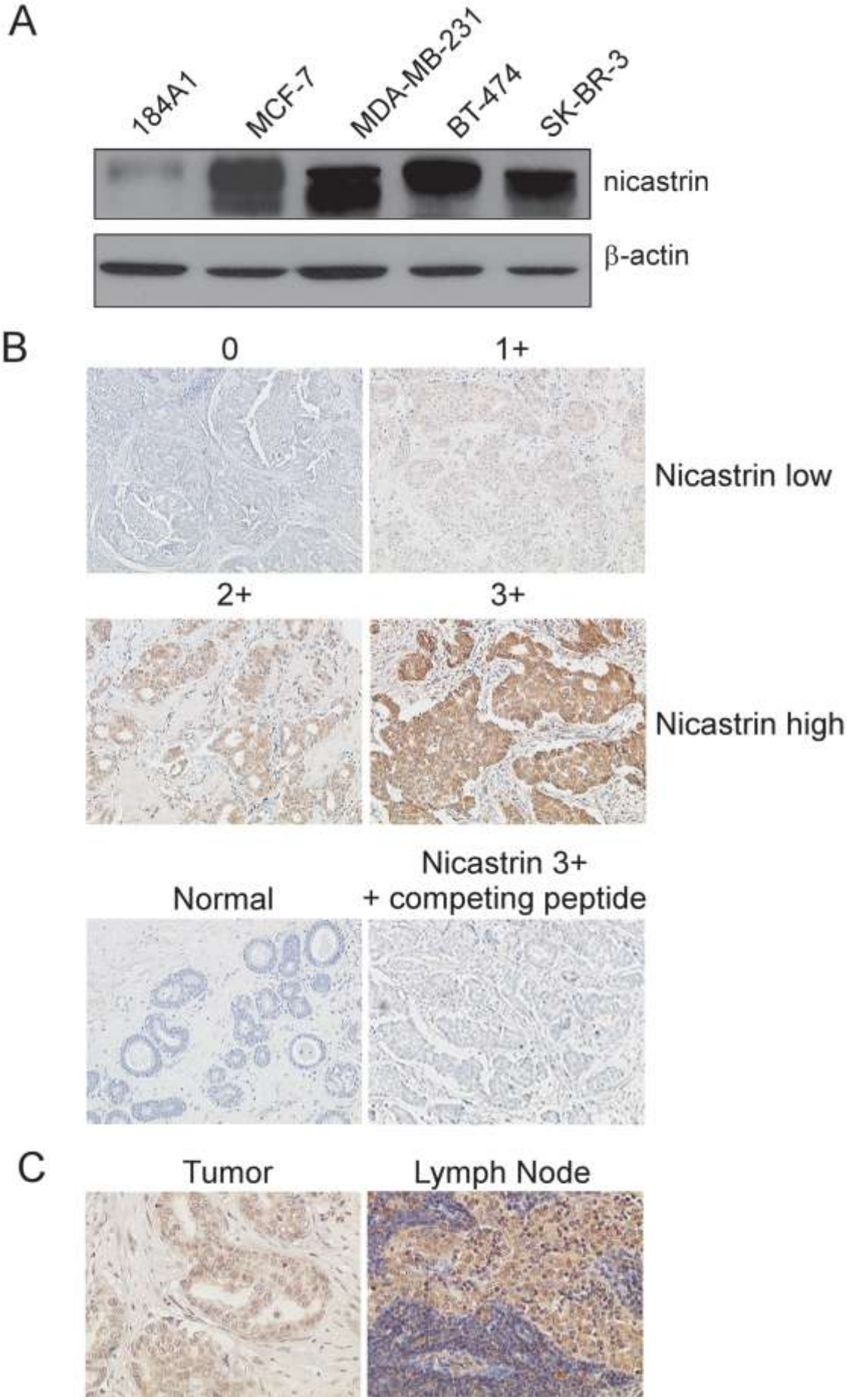
Figure 5. Nicastrin silencing regulates p120ctn in MCF7 cells. Cells were transfected with nicastrin siRNA and after 72 h fixed, permeabilized and stained with anti-p120ctn mAb followed by Alexa Flour 594 goat anti-mouse IgG (H+L) (Invitrogen). Bar = 10 μm.

Figure 6. Production of anti-nicastrin PcAb and its effects on BC cells *in vitro.* Recombinant protein for PcAb generation was produced as follows: the primers (aa261-aa512): forward: (5'ggatccgaagcctataaatacaactgggac 3'); reverse: (5' gcggccgctcactgaactgtgtcgctgaagttg 3') were used to generate the DAP-domain containing sequence of nicastrin (amino acids 261-512) by PCR (Qiagen LongRange PCR kit). This sequence was ligated into the pET-duet plasmid (Novagen) and transformed into the Rosetta host strain. His-tagged protein was isolated (soluble and inclusion bodies) using denaturing conditions (soluble protein conditions: 100 mM Na phosphate, 10 mM Tris, 8 M urea pH 8.0 with protease inhibitors; inclusion body conditions: extraction with 20 mM Tris; pH 8.0; 150 mM NaCl; 1% NP40; 1 mg/ml lysozyme; 0.01% v/v protease inhibitors), purified on a nickel column and eluted (20 mM Tris; 300 mM imidazole; 300 mM NaCl, pH 8.0). Protein was dialyzed in PBS and the precipitate collected and resuspended in PBS (1 mg/ml) then sequenced by mass-spectrometry prior to use for immunisation; **a**, Anti-nicastrin PcAb inhibits invasion of MDA-MB231 cells in a dose dependent manner. Doses of 25-100 µg/ml produce a significant inhibition (25µg/ml = 40%, $p = 0.009$; 50µg/ml = 57%, $p < 0.001$; 100µg/ml = 70%, $p < 0.001$);

b, Production of the antigen for McAb generation and anti-nicastrin McAb effects on BC cells *in vitro.* The primers: forward (5'gctagcatggctacggcagggggtggc3') and reverse (5'gcggccgctcagtatgacacagctcctggc3'), were used to generate full length nicastrin. This was cloned into pIRES-neo2 vector (Clontech) (1.07 µg/µL). Genetic immunisation was performed using proprietary immunisation plasmids (Genovac, GE). Seven McAbs inhibited invasion of MDA-MB231 cells. Clones 2H6 and 10C5 were most potent (reduction ≥60%, $*p < 0.001$). Anti-nicastrin PcAb (100 µg/µL) was used as a positive control (70% inhibition, $*p < 0.001$); **c**, MDA-MB231 cells were pre-incubated with anti-nicastrin Pc and Mc antibodies for 30 min at room temperature before plating. After 72 h in culture, cells were lyzed and protein levels were measured by western blotting. Rabbit IgG was used as a negative control for the rabbit anti-nicastrin PcAb and a serum from non-immunized rats was used as a negative control for the rat anti-nicastrin McAbs. GS inhibitor was used as a positive control, known to inhibit notch1-ICD. Anti-nicastrin Pc and Ms Abs inhibit notch1 activation equally to a GSI. Anti-nicastrin Pc and Mc antibodies also reduced vimentin protein levels, while the GSI had minimal effect: **d**, anti-nicastrin Pc and McAbs didn't affect nicastrin protein levels. Relative band intensities, normalized for loading are shown bellow blots.

Online Rosource Movie 1. *NCSTN*$^{+/+}$ MEFs. Video represents 10 h time lapse microscopy with image collected 1 frame/10 min.

Online Resource Movie 2. *NCSTN*$^{-/-}$ MEFs. Video represents 10 h time lapse microscopy with image collected 1 frame/10 min. Display rate is 10 frames per second.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MCF-7
MDA-MB-231
BT-474
SK-BR-3
nicastrin
β-actin
B
0
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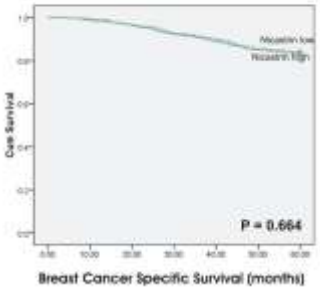
Nicastrin low
2+
3+
Nicastrin high
Normal
Nicastrin 3+
+ competing peptide
C
Tumor
Lymph Node

A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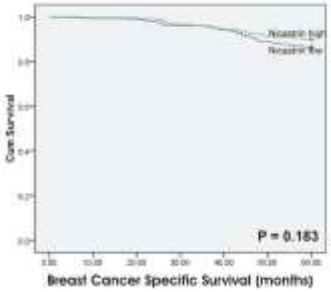
Breast Cancer Specific Survival (months)

B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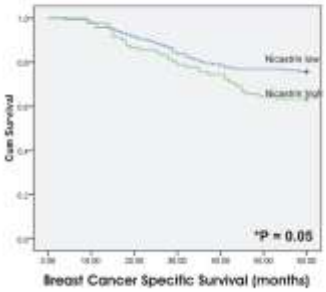
C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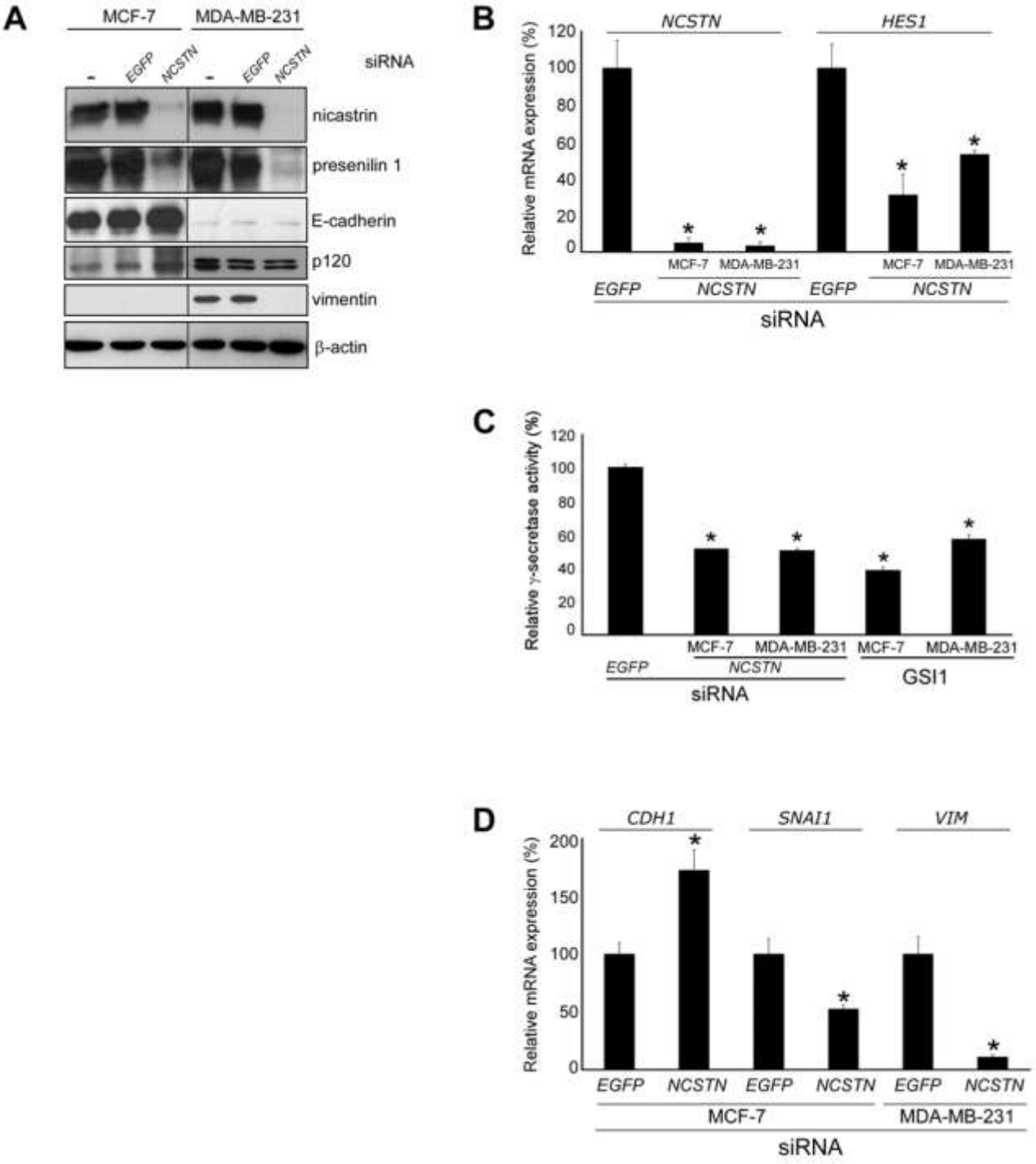
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NCSTN
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EGFP
NCSTN
nicastrin
presenilin 1
E-cadherin
p120
vimentin
β-actin
B
NCSTN
HES1
Relative mRNA expression (%)
120
100
80
60
40
20
0
MCF-7
MDA-MB-231
MCF-7
MDA-MB-231
EGFP
NCSTN
EGFP
NCSTN
siRNA
C
Relative γ-secretase activity (%)
120
100
80
60
40
20
0
MCF-7
MDA-MB-231
MCF-7
MDA-MB-231
EGFP
NCSTN
GSI1
siRNA
D
CDH1
SNAI1
VIM
Relative mRNA expression (%)
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NCSTN
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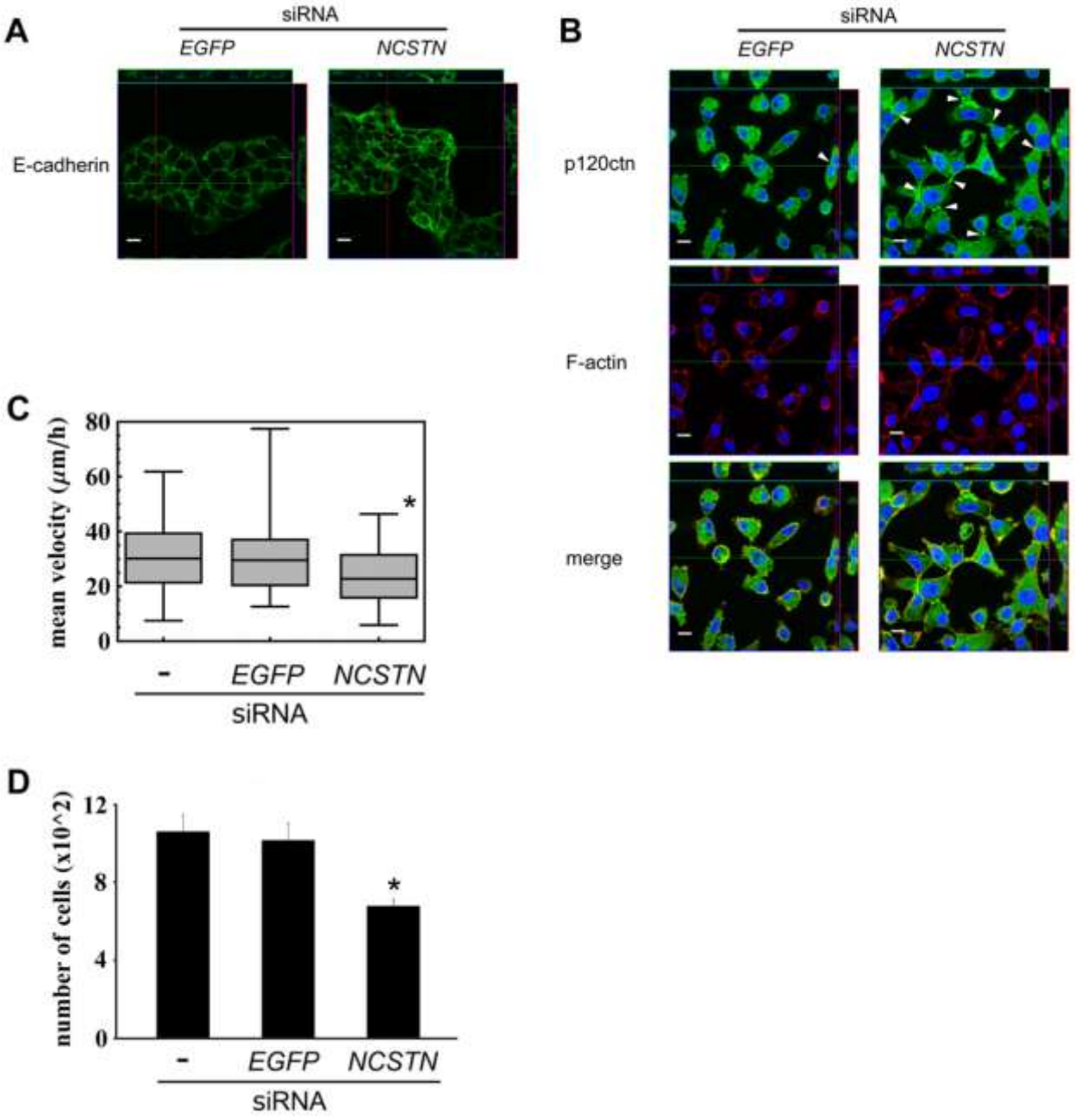
siRNA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merge
C
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60
40
20
0
*
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4
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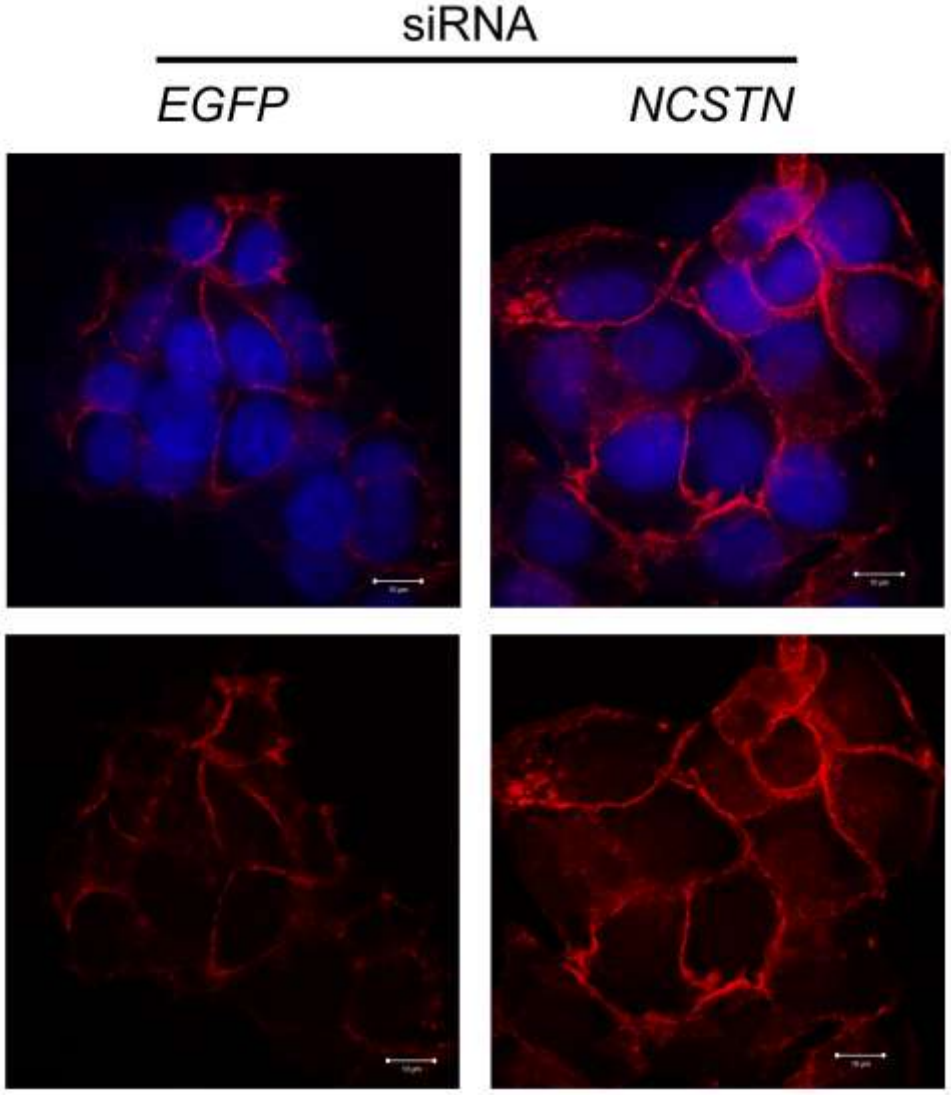
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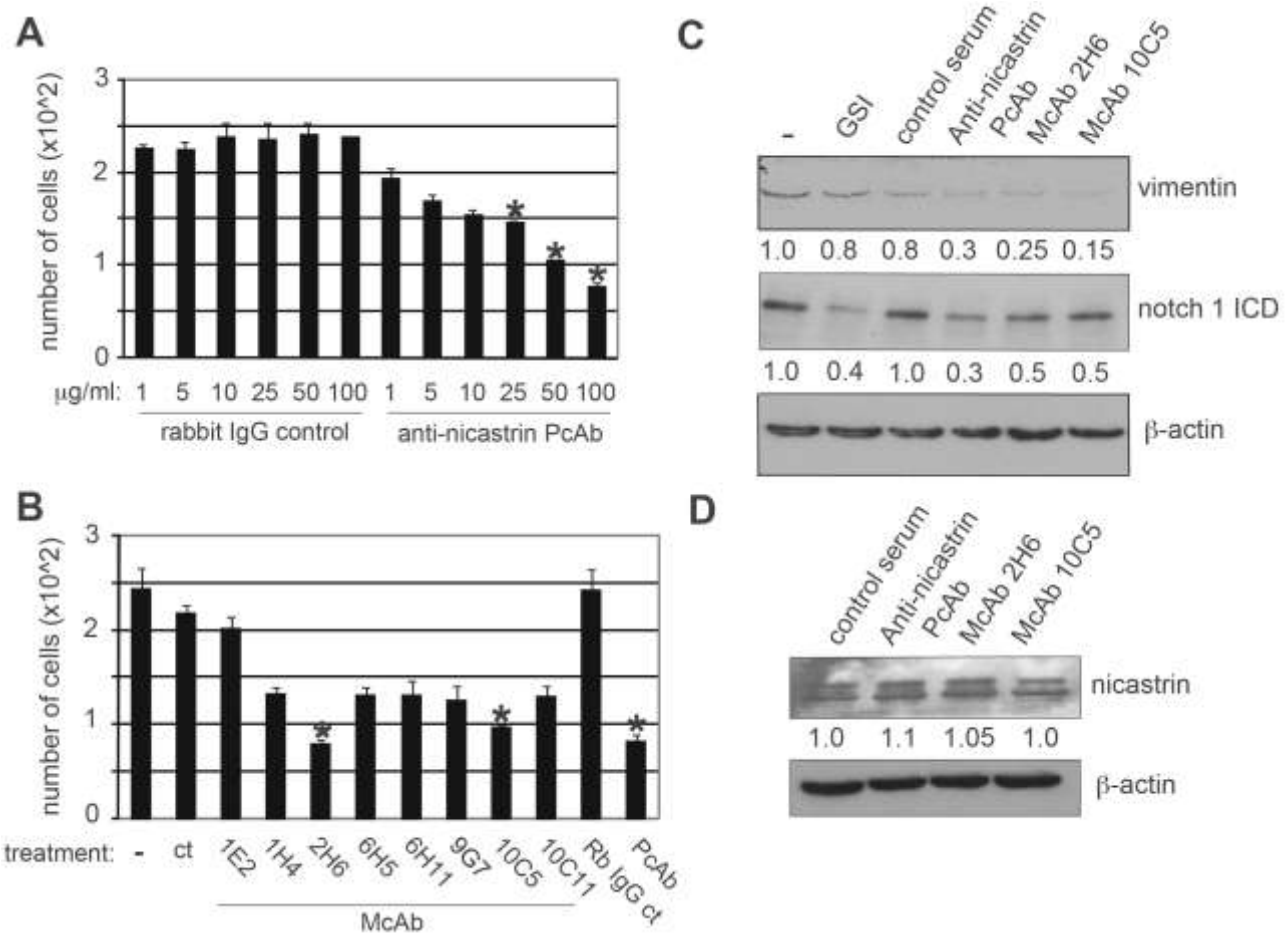

Table 1. Patients' characteristics and tumor biomarkers showing nicastrin expression

Variable/Biomarker	Nicastrin expression		p-value
Patients age (yrs)	**Low (%)**	**High (%)**	
≤ 50	**231 (41.8)**	**173 (35.3)**	**0.031**
>50	**321 (58.2)**	**325 (64.7)**	
Grade			
1	**70 (12.8)**	**96 (19.4)**	
2	**166 (30.3)**	**187 (37.7)**	**< 0.001**
3	**312 (56.9)**	**213 (42.9)**	
Stage			
1	**337 (51.8)**	**313 (48.2)**	
2	**164 (52.9)**	**146 (47.1)**	**0.827**
3	**48 (55.2)**	**39 (44.8)**	
Tumor type			
Tubular	**81 (39)**	**127 (61)**	**0.002**
Invasive Ductal	**285 (45.7)**	**339 (54.3)**	

Oestrogen receptor			
Negative	**170 (61.8)**	**105 (38.2)**	**< 0.001**
Positive	**355 (49.2)**	**367 (50.8)**	

Organ	Tissue type/ number of tissues analysed		Nicastrin IHC score
Skin	Normal	n = 12	2+
Liver	Normal	n = 7	1+/2+
Renal Tubules	Normal	n = 7	1+/2+
Renal Glomeruli	Normal	n = 7	0
Stomach	Normal	n = 5	1+/2+

Progesterone receptor			
Negative	**251 (59.6)**	**170 (40.4)**	**< 0.001**
Positive	**270 (47.7)**	**296 (52.3)**	
Her2 receptor			
0/1+/2+	**503 (53.4)**	**439 (46.6)**	**0.076**
3+	**33 (42.3)**	**45 (57.7)**	
Cytokeratin 18			
Negative	**67 (13.3)**	**437 (86.7)**	**< 0.001**
Positive	**30 (6.4)**	**439 (93.6)**	
Cytokeratin 5/6			
Negative	**432 (50.7)**	**420 (49.3)**	**0.01**
Positive	**96 (61.9)**	**51 (38.1)**	

Heart	Normal	n = 2	2+
Vaginal cervix	Normal	n = 2	2+
Prostate	Normal	n = 12	0/1+
Colon	Normal	n = 7	0/1+
Lung	Normal	n = 7	0
Ovaries	Normal	n = 5	0/1+
Testis	Normal	n = 5	0/1+
Endometrium	Normal	n = 5	1+
Cerebrum	Normal	n = 7	1+
Pancreas	Normal	n = 7	0/1+
Thyroide	Normal	n = 5	0
Lymph nodes	Normal	n = 10	1+

Table 2. Nicastrin expression in normal tissues