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Claudin-16 Reduces the Aggressive Behavior of Human Breast Cancer Cells

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ABSTRACT

Claudin-16 (Paracellin-1) is a transmembrane tight junction (TJ) protein originally described as having a critical role in the re-absorption of magnesium and calcium in the kidney. This study examined expression of Claudin-16 in human breast cells and tissues to identify a possible link between expression and aggressiveness in cells and between Claudin-16 levels and patient prognosis. Insertion of the Claudin-16 gene into MDA-MB-231 human breast cancer cells resulted in cells that were significantly less motile and invasive in behavior, with increased adhesion to matrix. These cells also exhibited significantly increased TJ functionality and "tighter" colony morphology. Moreover, growth rates were reduced in both in vitro and in vivo assays ($P < 0.002$). Frozen sections from breast cancer primary tumors (matched tumor 124 and background 33) were immuno-stained. RNA was reverse transcribed and analyzed by Q-PCR (standardized using β-actin, normalized with cytokeratin-19 levels). Levels of expression of Claudin-16 were significantly decreased in node positive tumors compared to negative ($P = 0.016$). Expression was significantly lower in patients with node positive tumors ($P = 0.016$) and in those who had died from breast cancer or had general poor prognosis ($P < 0.015$). Immunohistochemical staining showed decreased expression of Claudin-16 in tumor sections ($P < 0.00001$). In conclusion, forced expression of Claudin-16 in breast cancer cells resulted in a less aggressive phenotype and reduced in vivo tumor volume. Claudin-16 expression was reduced in human breast cancer, particularly in patients with aggressive tumors and high mortality. This suggests that Claudin-16 plays a role beyond that of an initial metastasis repressor in this cancer type. J. Cell. Biochem. 105: 41–52, 2008.

KEY WORDS: CLAUDIN-16; TIGHT JUNCTIONS; BREAST CANCER; PROGNOSIS; METASTASIS SUPPRESSOR

Most cancers, including breast cancer, originate from epithelial tissues and are characterized by aberrant growth control, and loss of differentiation and tissue architecture. The mutual adhesiveness of cancer cells is significantly weaker than that of normal cells and so reduced cell–cell interaction results in a loss of normal tissue architecture. Loss of contact inhibition, which reflects disorder in the signal transduction pathways that connect cell–cell interactions are typical of both early (loss of cell polarity and growth control) and late (invasion and metastasis) stages of tumor progression. Recent studies have shown that several tight junction (TJ) components are directly or indirectly involved in breast cancer progression and metastasis [Morin, 2005; Grone et al., 2006; Hewitt et al., 2006; Martin et al., 2006, 2007; Oliveira and Morgado-Diaz, 2007].

The TJ is the most apical element of the junctional complex in epithelial and endothelial cells. This complex includes the TJ, adherens junction, desmosomes and gap junctions. The TJ forms a barrier to paracellular movement of substances, keeping the apical and basolateral fluid compartments on opposite sides of the epithelial cell layer distinct. The TJ also acts in the maintenance of apical versus basolateral plasma membrane compositional asymmetry by restricting the movement of lipids and integral membrane proteins within the plane of the membrane [Wittchen et al., 1999]. Claudins comprise a multi-gene family consisting of more than 20 members; heterogeneous Claudin species (and also occludin) are co-polymerized to form individual TJ strands as heteropolymers [Hamazaki et al., 2002]. Between adjacent cells Claudin molecules adhere to each other in both homotypic and heterotypic manners. Claudins are integral membrane proteins of around 22 kDa, structurally related, with four transmembrane domains, but no sequence similarity to occludin [Morita et al., 1999; Tsukita and Furuse, 1999]. Claudins generally have a valine residue at their COOH termini, suggesting that they strongly attract PDZ-containing proteins, such as ZO-1, -2 and -3. MUPP1 (multi-PDZ domain

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protein 1) is also a binding partner for Claudins at the COOH termini [Hamazaki et al., 2002]. The distinct permeability properties observed in different epithelia and endothelia seemingly result from the restricted tissue expression, variability of the homopolymer and heteropolymer assembly, regulated transcription and translation, and the subcellular localization of Claudin family proteins [Heiskala et al., 2001]. In conditions where the cell adhesion function contributed by TJs is essential, such as in altered paracellular transport, in proliferative diseases and morphogenesis, the Claudins provide the molecular basis for the uniqueness of TJs and emerge as targets for intervention [Heiskala et al., 2001].

Claudin-16 has been described as being expressed in kidney epithelial cells. The gene encodes a 305 amino acid, 36 kDa protein that has four transmembrane domains and intercellular N- and C-termini [Simon et al., 1999]. As its sequence and structural similarity to Claudins is obvious, it has been termed Claudin-16. Defects in the Claudin-16 gene have been shown to cause the autosomal recessive inherited disorder familial hypomagnesemia, hypercalciuria and nephrocalcinosis. Claudin-16 is the Claudin protein responsible for tubular re-absorption of magnesium and calcium [Satoh and Romero, 2002]. Claudin-16 regulates the paracellular transport of magnesium ions in the kidney tubule, and was the first report of a TJ protein that is involved in paracellular ion conductance [Blanchard et al., 2001]. The disease mutations documented in Claudin-16 may result in an increase in the resistance of the electrical seal of TJs, with a concomitant decrease in epithelial ionic permeability.

Our previous work investigating the expression of TJ molecules in breast cancer led to this current study examining the effect of Claudin-16 over-expression in human breast cancer cells and the expression and distribution of Claudin-16 in human breast cancer tissues (Martin et al., 2004a, 2004b). The loss or reduction of TJ molecules in cancer is becoming a frequently found occurrence. That Claudins are key in the maintenance of TJ function begs investigation into the changes that can occur during the metastatic process. In vitro studies revealed that forced expression of Claudin-16 in the aggressive human breast cancer cell line MDA-MB-231 resulted in cells with decreased invasive phenotype, increased TJ function and reduced in vivo growth. Although expressed at low levels, we discovered that Claudin-16 expression is significantly reduced in patients with node positive tumors, reduced with increasing NPI status and in those patients with poor outcomes. Moreover, there was significantly reduced expression of Claudin-16 in ERβ negative tumors. We can thus conclude that Claudin-16 acts as a tumor inhibitor in human breast cancer, and demonstrates additional evidence that maintenance of correct TJ architecture and function could be a key process in the prevention of cancer cell dissociation and spread.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Anti-Claudin-16 (sc-10178) and actin (sc-8432) antibodies were purchased from PharMingen International (San Diego, CA). Anti-Claudin-1 (18-7362) antibody was purchased from Zymed (Invitrogen Ltd., Paisley, UK). Peroxidase-conjugated anti-mouse, anti-rabbit IgG for Western blotting were from Sigma–Aldrich Ltd. (Poole, UK). FITC- or TRITC-conjugated anti-mouse, anti-goat and anti-rabbit IgG were from Sigma–Aldrich Ltd. FITC-conjugated Dextran (40 kDa) were obtained from Molecular Probe Inc. (Eugene, OR). Carbonate filter inserts with pore size of 0.4 μm (for 24-well plates) were from Greiner Bio-One Ltd. (Stonehouse, Glos, UK).

CELL LINES

The human breast cancer cell line MDA-MB-231 was routinely maintained in Dulbecco's Modified Eagle's medium (DMEM; Sigma–Aldrich Ltd.) supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin (Sigma–Aldrich Ltd.).

CONVENTIONAL PCR

Conventional PCR mix (ABgene, Epsom, Surrey, UK) was used to screen a number of human breast, prostate, colorectal and bladder cancer cell lines. Normal human kidney cDNA was used as a positive control. Cycling conditions were as follows: enzyme activation 95°C for 5 min, 1 cycle, followed by 36 cycles of denaturing: 95°C for 15 s; annealing: 55°C for 15 s; extension: 72°C for 2 min. Amplified products were visualized on agarose gels.

CLONING AND EXPRESSION OF THE CLAUDIN-16 GENE

Normal human breast tissue was screened for endogenous expression of Claudin-16. An invasive cell line, MDA-MB-231 (MDA^{WT}) which was Claudin-16 negative was chosen for introduction of the Claudin-16 gene. The gene, after amplification from normal breast tissue cDNA (using primer set Paracell F11 atgacctccaggaccccact and Paracell R11 cacccttgtgtctacagcat) was T-A cloned into an NT GFP-TOPO (Invitrogen Ltd.) plasmid before electroporation into the breast cancer cells. Expression of the gene was confirmed by RT-PCR, with cells positive for Claudin-16 designated $MDA^{Cl\text{-}16}$. Stably transfected cells were then used for subsequent assays (after 6 weeks of culture and confirmation of expression).

IN VITRO ASSAYS ANALYZING CLAUDIN-16 GENE TRANSFECTED BREAST CANCER CELLS

Six assays were used to determine what, if any, effect over-expression of Claudin-16 would have on the human breast cancer cell line MDA-MB-231. HGF at 25 ng/ml was used as a stimulator of motility and invasive behavior.

TRANS-EPITHELIAL RESISTANCE (TER)

TER was measured with an EVOM voltohmmeter (EVOL, World Precision Instruments, Aston, Herts, UK), equipped with a pair of STX-2 chopstick electrodes (WPI, Sarasota, FL), as we previously reported [Martin et al., 2004b, 2007]. Briefly, MDA-MB-cells were seeded into the 0.4 μm pore size insert (Greiner Bio-One Ltd.) and allowed to reach full confluence, after which fresh medium was replaced for further experiments. Inserts without cells, inserts with cells in medium and inserts with cells with HGF (25 ng/ml) were tested for a period of 2 h. Electrodes were placed at the upper and lower chambers and resistance measured with the voltohmmeter.

TRANS-EPITHELIAL CELL PERMEABILITY

This was determined using fluorescently labeled dextran FITC-Dextran 40 (Sigma–Aldrich Ltd.) molecular weight being 40 kDa [Martin et al., 2004b, 2007]. Human breast cancer cells were prepared and treated as in the TER study, but with the addition of Dextran-40 to the upper chamber. Medium from the lower chamber was collected for intervals up to 2 h after addition of HGF. The relative fluorescence from these collections was read on a multi-channel fluorescence reader (Denly, Sussex, UK).

CYTODEX-2-BEAD MOTILITY ASSAY

Cells are pre-coated onto cytodex-2 carrier beads (Sigma–Aldrich Ltd.) for 2 h in complete medium [Jiang et al., 2001]. After the medium is aspirated and the cells washed (2× in complete medium), they are aliquoted into wells of a 96-well plate in triplicate (300 μl/well). I/Se/GLA with/without 17-β-estradiol (Sigma–Aldrich Ltd.) was added and the cells incubated over-night. The beads are washed off in medium, and the cells that migrated onto the floor of each well fixed (4% formaldehyde) and stained with crystal violet. The cells are then counted microscopically (40×, 10 fields of view).

INVASION ASSAY

Invasiveness of MDA-MB-231 breast cancer cell line was assessed using the following in vitro assay. Transwell chambers equipped with 6.5 mm diameter polycarbonate filter (pore size 8 μm) (Becton Dickinson Labware, Oxford, UK) were pre-coated with 50 μg/membrane of solubilized basement membrane in the form of Matrigel (Collaborative Research Products, Bedford, MA). After membrane re-hydration, 15,000 cells were aliquoted into each insert with/without HGF. After 96 h co-culture non-invasive cells were removed with cotton swabs. Invaded cells on the underside of the insert were fixed and stained with crystal violet, followed by microscopic counting (20 fields/insert).

CELL MATRIX ADHESION ASSAY

The cell–matrix attachment assay was carried out as previously reported [Hiscox and Jiang, 1999]. Briefly, Matrigel (10 μg/well) was added to a 96-well plates, which were incubated for 24 h to allow binding of matrix protein to the surface of the well. The plates were then washed and blocked with 5% bovine serum albumin (BSA, Sigma–Aldrich Ltd.). Cells were added at 104/well for 30 min, followed by aspiration and washing. The number of attached cells was determined by direct counting under microscope (7 counts per experimental setting).

GROWTH ASSAY

Standard growth assays using crystal violet were carried out over 5 days to determine whether the insertion of the Claudin-16 gene changed the growth rate of the MDA-MB-231 breast cancer cells.

IMMUNOFLUORESCENT STAINING OF HUMAN BREAST CANCER CELLS

For immunofluorescence staining, cells were grown in 16-well chamber slides (LAB-TEK International, Sussex, UK) (30,000 cells/well) and incubated in a 37°C/5% incubator for a set period of time (0–24 h). After incubation, the culture medium was aspirated, the wells rinsed with balanced salt solution (BSS) buffer and the cells fixed in methanol for 20 min at −20°C. After fixation the cells were washed twice using BSS buffer and permeabilized by the addition of 200 μl of 0.1% Triton X-100 (Sigma–Aldrich Ltd.) detergent in Phosphate buffered solution (PBS) for 5 min at room temperature. Cells were rinsed twice with BSS buffer and 200 μl of blocking buffer (10% horse serum in TBS) was added to each well and the chamber slide incubated for 40 min at room temperature on a bench rocker. The wells were washed once with wash buffer (3% horse serum in TBS buffer containing 0.1% Tween20) and 100 μl of primary antibodies prepared in wash buffer was added to the appropriate wells. The chamber slide was incubated on the rocker for a further 60 min at room temperature. Wells were washed twice with TBS buffer (with 0.1% Tween20) and cells were incubated in 100 μl of secondary antibodies (fluorescein isothiocyanate (FITC) or tetramethyl rhodamine iso-thiocyanate (TRITC) conjugates, Sigma–Aldrich Ltd.) (diluted in the same manner as the primary antibodies) for 50 min. The chamber slide was wrapped in foil to prevent light reaching the conjugate. Finally, the wells were rinsed twice with wash buffer, once in BSS buffer mounted with FluorSave (Calbiochem-Novabiochem Ltd., Nottingham, UK) reagent and visualized using an Olympus BX51 microscope with a Hamamatsu (Welwyn Garden City, Herts, UK) Orca ER digital camera at X 100 using oil immersion lens.

SDS-PAGE AND WESTERN BLOTTING

Total cell lysates and tissues were prepared as follows: cells were pelleted or tissue was macerated and lysed in HCMF buffer plus 0.5% SDS, 0.5% Triton X-100, 2 mM $CaCl_2$, 100 μg/ml phenylmethyl-sulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 10 mM sodium orthovanadate for 40 min, sample buffer was added and the protein boiled at 100°C for 5 min before clarification at 13,000*g* for 10 min. Equal amounts of protein from each cell sample were added onto an 8% polyacrylamide gel. Following electrophoresis, proteins were blotted onto nitro-cellulose sheets and blocked in 10% horse serum for 60 min before probing with specific primary antibodies, following with peroxidase-conjugated secondary antibody (1:2.000). Protein bands were visualized with Supersignal West Dura Extended Duration Substrate chemi-luminescent system (Perbio Science UK Ltd., Cramlington, UK) and detected using a CCD UVIprochemi system (UVItec Ltd., Cambridge, UK).

IN VIVO GROWTH ASSAY ANALYZING CLAUDIN-16 TRANSFECTED BREAST CANCER CELLS

The in vivo tumor progression model was adapted from similar previously described protocols [Martin et al., 2003; Jiang et al., 2005]. Briefly 2×10^6 cells in 100 μl were mixed in a 0.5 mg/ml Matrigel suspension and sub-cutaneously injected into the left and right flanks of 4–6-week-old athymic nude mice (CD-1; Charles River Laboratories, Kent, England, UK) and allowed to develop. The mice ($n = 5$) were maintained in filter top units and were weighed and the tumor size measured weekly using Vernier calipers under sterile conditions. Humane end points were (a) mice which suffered 25% weight loss, (b) development of tumors exceeding 1 cm^3 (subject to schedule 1 method according to the United Kingdom Home Office and the United Kingdom Coordinating Committee on

Cancer Research (UKCCCR) guidelines). At the conclusion of the experiment, the tumors (n = 10) were measured, the mice humanely killed under schedule 1 and the tumors removed and weighed. Tumor volume was determined using the following formula; tumor volume $mm^3 = 0.523 \times width^2 \times length$.

TISSUE COLLECTION AND PREPARATION

Breast tissue samples (124 tumor and 33 matched background (Martin et al., 2004a) were collected and immediately frozen in liquid nitrogen before processing – a portion of each sample for quantitative-PCR analysis, a portion for immunohistochemical analysis and a portion for routine histological examination. RNA was isolated from tissue samples using standard RNA-zol procedures. For RT-PCR, cDNA was synthesized in a 20 μl reaction mixture using 1 μg RNA, as described in the protocol (AB Gene Reverse Transcription System, Surrey, UK).

QUANTITATIVE-PCR

The Q-PCR system used the Amplofluor™ Uniprimer™ system (Intergen Company Oxford, UK) and Thermo-Start® (ABgene). Specific primer pairs for Claudin-16 were designed by the authors using a Beacon Designer software and manufactured by Invitrogen, each amplifying a region that spans at least one intron (primer details given in supplement 1), generating an approximately 100 base pair product from both the control plasmid and cDNA. PCR primers: ParazF agccacgttactaatagcag and ParazR actgaacctgaccgtacaattgtgcaaaaccaaagtag.

Using the Icycler IQ system (Bio-Rad), which incorporates a gradient thermocycler and a 96-channel optical unit, the plasmid standards and breast cancer cDNA were simultaneously assayed in duplicated reactions using a standard hot-start Q-PCR master mix. Q-PCR conditions were as follows: enzyme activation 95°C for 12 min, 1 cycle, followed by 60 cycles of Denaturing: 95°C for 15 s; Annealing: 55°C for 40 s; extension: 72°C for 25 s. Using purified plasmids as internal standards, the level of cDNA (copies/50 ng RNA) in the breast cancer samples were calculated. Q-PCR for β-actin was also performed on the same samples, to correct for any residual differences in the initial level of RNA in the specimens (in addition to spectrophotometry). Results were then normalized using Cytokeratin-19 levels in the same tissues. The products of Q-PCR were verified on agarose gels (not shown).

IMMUNOHISTOCHEMISTRY

Cryostat sections of frozen tissue were cut at 6 μm, placed on Super Frost Plus slides (LSL UK, Rochdale, UK), air dried and fixed in a 50:50 solution of alcohol:acetone. The sections were then air dried again and stored at −20°C until used. Immediately before commencement of immuno-staining, the sections were washed in buffer for 5 min and treated with horse serum for 20 min as a blocking agent to non-specific binding. Sections were stained using Claudin-16 antibody (Insight Biotechnology, Wembley, Cambridgeshire, UK). Negative controls were used where necessary. Primary antibodies were used at 1:100 dilution for 60 min and then washed in buffer. The secondary biotinylated antibody at 1:100 dilution (Universal secondary, Vectastain Elite ABC, Vector Laboratories Inc., Burlingham, CA) was added (in horse serum/buffer solution) for 30 min, followed by numerous washings. Avidin/biotin complex was added for 30 min, again followed with washes. Diaminobenzadine was used as a chromogen to visualize the antibody/antigen complex. Sections were counterstained in Mayer's hematoxylin for 1 min, dehydrated, cleared and mounted in DPX. Following this, the sections were analyzed for staining intensity as previously described [Martin et al., 2004a, 2004b].

FLUORESCENT STAINING OF HUMAN BREAST TISSUES

Tissues were prepared as for immune-staining. Immediately before commencement of fluorescent staining, the sections were washed in buffer for 5 min and treated with horse serum for 20 min as a blocking agent to non-specific binding. Sections were stained using Claudin-16 and Claudin-1 antibodies (Insight Biotechnology, Wembley, Cambridgeshire, UK) and Hoescht stain (5 μg/ml) (Sigma–Aldrich Ltd.). Negative controls were used where necessary. Primary antibodies were used at 1:100 dilution for 60 min and then washed in buffer. The secondary fluorescently labeled antibodies (FITC- or TRITC-conjugated, Sigma–Aldrich Ltd.) at 1:100 dilution was added (in horse serum/buffer solution) for 30 min, followed by numerous washings. Tissues were then mounted in FluorSave (Calbiochem-Novabiochem Ltd.) reagent and visualized using an Olympus BX51 microscope with a Hamamatsu (Welwyn Garden City) Orca ER digital camera at 40× using oil immersion lens.

STATISTICAL ANALYSIS

Statistical analysis was performed by MINITAB version 13.32 (Minitab Inc., State College, PA) using a two-sample Student's *t*-test and the non-parametric Mann–Whitney confidence interval and test or Kruskal–Wallis, where appropriate. In addition, Microsoft Excel Professional was used to calculate correlation coefficients.

RESULTS

EXPRESSION OF CLAUDIN-16 IN HUMAN CANCER CELL LINES

The Claudin-16 gene was successfully amplified from normal breast tissue (Fig. 1A) and cloned into a suitable vector for subsequent work. Following transformation of the breast cancer cell line MDA-MB-231 with the Claudin-16 gene, the expression of the gene was demonstrated by the presence of mRNA (by RT-PCR). Figure 1A shows the expression of Claudin-16 in the transfected cells ($MDA^{Cl\text{-}16}$) with no signal for the wild type. Cells were also transfected with control plasmid (MDA^{Plas}) as a standard control. The expression of Claudin-16 was also demonstrated at the protein level with successful Western blotting (Fig. 1B. actin used as control). Moreover, immunofluorescence showed that the Claudin-16 protein was expressed and located to the correct location of the TJ in $MDA^{Cl\text{-}16}$ cells, as shown in Figure 1C. There was a distinct change in morphology of the transfected cells, as they became more tightly packed together, exhibiting obvious junctional formation, in contrast to the wild type and plasmid control cells. Claudin-16 was correctly targeted at the cell membrane as observed by its co-localization with Claudin-1 (Fig. 1D).

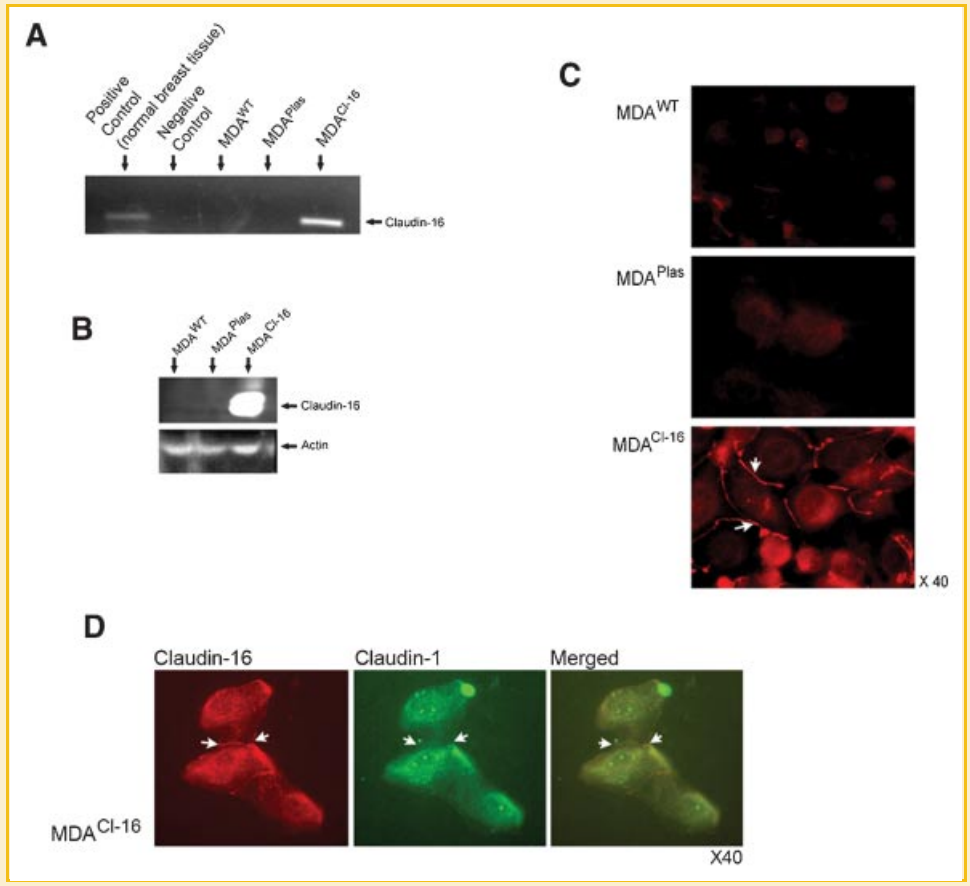

Fig. 1. A: RT-PCR showing origin and successful cloning of Claudin-16 from normal human breast tissue and forced expression in the invasive human breast cancer cell line MDA-MB-231. B: Western blotting confirming the correct expression of the Claudin-16 protein in transfected MDA-MB-231 human breast cancer cells (actin shown for loading efficiency). C: Immunofluorescent imaging of Claudin-16 location at the TJ of transfected MDA-MB-231 human breast cancer cells. The change in morphology is evident as is the lack of expression for Claudin-16 in both wild type and plasmid control cells. D: Co-localization of Claudin-16 and Claudin-1 in transfected MDA-MB-231 cells. White arrows show localization of both proteins to the junctional region. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

IN VITRO ASSAYS ANALYZING CLAUDIN-16 GENE TRANSFECTED BREAST CANCER CELLS

All six assays used to determine the effect of over-expression of Claudin-16 in the human breast cancer cell line MDA-MB-231 demonstrated a change cellular behavior and function, particularly in conjunction with treatment by HGF to stimulate motility and invasive behavior.

CLAUDIN-16 CONFERS INCREASED TRANS-EPITHELIAL RESISTANCE (TER) IN TRANSFECTED BREAST CANCER CELLS

TER was used to assess the effect of inserting the Claudin-16 gene on TJ functionality in human breast cancer cells. $MDA^{Cl\text{-}16}$ cells showed increased TER (Fig. 2A) over 2 h in comparison to MDA^{WT} cells (change in TER: 30 min MDA^{WT} 0 ± 3.6 vs. $MDA^{Cl\text{-}16}$ 5.6 ± 3; 60 min MDA^{WT} -2 ± 5 vs. $MDA^{Cl\text{-}16}$ 6 ± 3; 120 min MDA^{WT} -8.3 ± 1.5 vs. $MDA^{Cl\text{-}16}$ 7.3 ± 4, $P < 0.01$). This was more striking after addition of HGF (at 25 ng/ml) which reduces TER in MDA^{WT} cells but had less effect on $MDA^{Cl\text{-}16}$ transfected cells (change in TER with HGF: 30 min MDA^{WT} -22 ± 5 vs. $MDA^{Cl\text{-}16}$ -6 ± 2.5; 60 min MDA^{WT} -35 ± 5.7 vs. $MDA^{Cl\text{-}16}$ -7 ± 2.1; 120 min MDA^{WT} -58 ± 4.7 vs. $MDA^{Cl\text{-}16}$ -6.7 ± 2.6), which was significant by 30 min treatment ($P < 0.005$) and continued to be so at 2 h treatment ($P < 0.001$).

CLAUDIN-16 CONFERS REDUCED PARACELLULAR PERMEABILITY (PCP) IN TRANSFECTED BREAST CANCER CELLS

PCP was used to assess the change in TJ function in the transfected breast cancer cell lines, via the paracellular pathway (Fig. 2B). MDA^{WT} cells showed the usual reduction in PCP over time (a consequence of low TJ formation in these active cells). This reduced PCP was negated in $MDA^{Cl\text{-}16}$ cells over 2 h (Change in PCP: 30 min MDA^{WT} -27 ± 2 vs. $MDA^{Cl\text{-}16}$ 53 ± 3; 60 min MDA^{WT} -129 ± 7 vs. $MDA^{Cl\text{-}16}$ 1 ± 3; 120 min MDA^{WT} -176 ± 9 vs. $MDA^{Cl\text{-}16}$ -9 ± 3). Again, as with TER measurements, insertion of the Claudin-16 gene enabled a negation in the effect of HGF on these human breast cancer cells when compared to the control cells (change in PCP with HGF: 30 min MDA^{WT} 19 ± 3 vs. $MDA^{Cl\text{-}16}$ 46 ± 5; 60 min MDA^{WT} -157 ± 8 vs. $MDA^{Cl\text{-}16}$ 34 ± 4; 120 min MDA^{WT} -65 ± 4 vs. $MDA^{Cl\text{-}16}$ -37 ± 2).

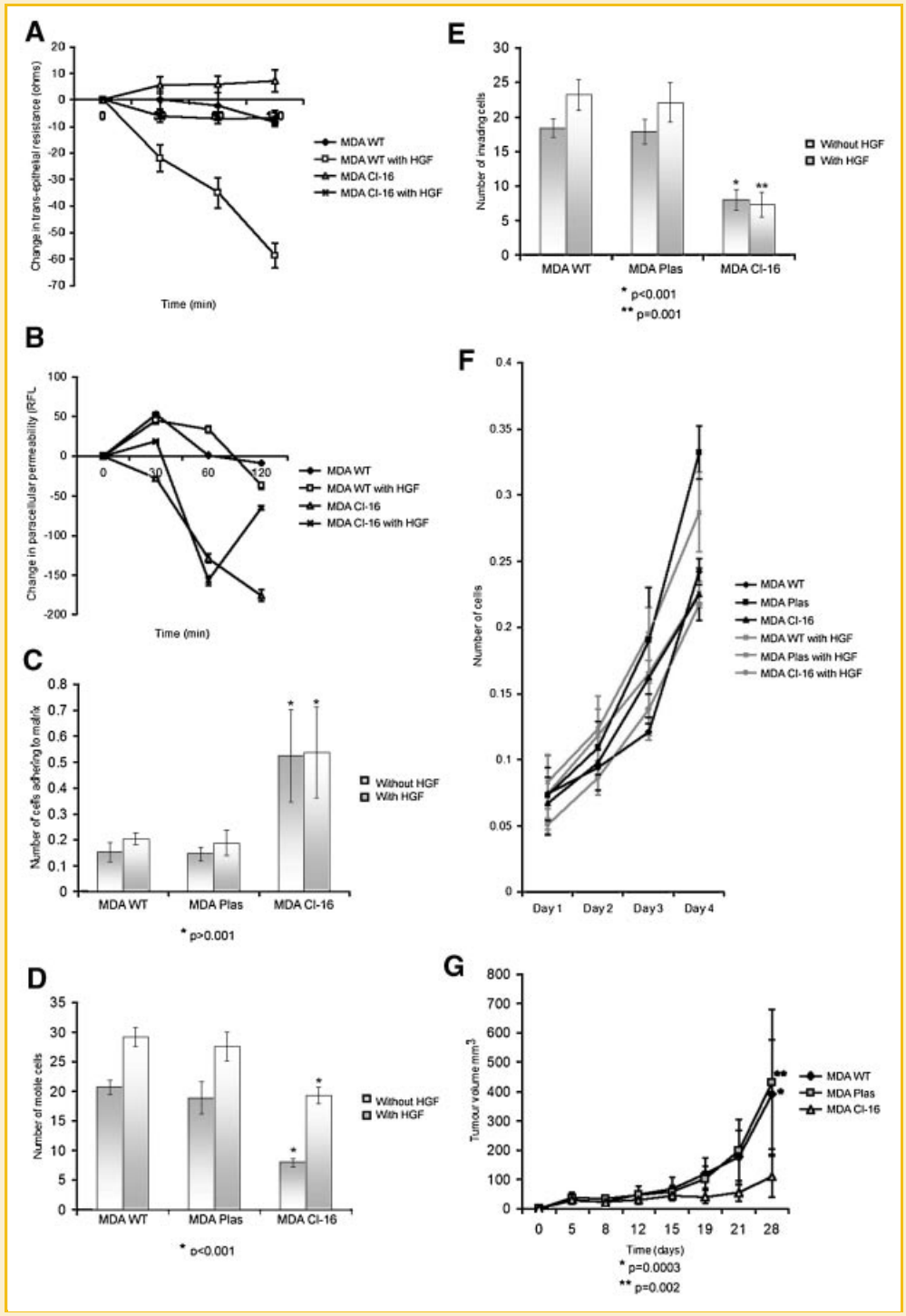

Fig. 2. Assays demonstrating that Claudin-16 transfected MDA-MB-231 cells have a less aggressive phenotype than wild type cells. A: Graph depicting the change in trans-epithelial resistance of these cells and to their response to HGF (25 ng/ml). B: Graph showing the change in paracellular permeability of these cells. C: Adherence to matrix. D: Motility. E: Invasiveness. F: In vitro growth assay. G: In vivo tumor growth over a 28 day period (mm^3).

CLAUDIN-16 CONFERS REDUCES THE INVASIVE PHENOTYPE OF HUMAN BREAST CANCER CELLS

The ability of MDA^{Cl-16} cells to adhere to matrix was assessed (Fig. 2C). There was a significant difference between the adherence of MDA^{Cl-16} and MDA^{WT} and MDA^{Plas} with MDA^{Cl-16} cells being more adherent to matrix (RFU: MDA^{Cl-16} 0.524 ± 0.08 vs. MDA^{WT} 0.151 ± 0.04 and MDA^{Plas} $0.145 \pm 0.0.03$, $P < 0.001$). After treatment with HGF, MDA^{Cl-16} cells showed no change in adhesion to

matrix, compared with MDA^{WT} and MDA^{Plas} cells both of which exhibited elevated adhesion ($MDA^{Cl\text{-}16}$ 0.536 ± 0.17 vs. MDA^{WT} 0.203 ± 0.02 and MDA^{Plas} 0.187 ± 0.05).

Motility studies revealed that $MDA^{Cl\text{-}16}$ cells were significantly less motile than either the wild type or plasmid control cells (average number of motile cells/field of view: $MDA^{Cl\text{-}16}$ 8 ± 0.67 vs. MDA^{WT} 20.67 ± 1.2 and MDA^{Plas} 18.9 ± 2.8, $P < 0.001$), Figure 2D. This also held even after treatment with the know motogen HGF (with HGF: $MDA^{Cl\text{-}16}$ 19.33 ± 1.4 vs. MDA^{WT} 29.25 ± 1.6 and MDA^{Plas} 27.6 ± 2.4, $P = 0.001$).

$MDA^{Cl\text{-}16}$ cells were significantly less invasive than MDA^{WT} cells and MDA^{Plas} (number of cells invading: $MDA^{Cl\text{-}16}$ 8 ± 1.5 vs. MDA^{WT} 18.4 ± 1.4 or MDA^{Plas} 17.9 ± 1.3, $P < 0.001$, Figure 2E. There was little difference in $MDA^{Cl\text{-}16}$ when cells were treated with HGF at 25 ng/ml (HGF treated $MDA^{Cl\text{-}16}$ 7.33 ± 1.8 vs. HGF treated MDA^{WT} 23.2 ± 2.3, MDA^{Plas} 22.9 ± 2.9, $P = 0.001$).

There was some small reduction in the in vitro growth rate of the $MDA^{Cl\text{-}16}$ cells compared to MDA^{WT} or MDA^{Plas} cells (Fig. 2F); this became more apparent with the addition of HGF.

CLAUDIN-16 REDUCES THE IN VIVO TUMOR GROWTH OF HUMAN BREAST CANCER CELLS

The tumors induced in the athymic nu/nu female mice were measured over 28 days. During the growth period and at the experimental end point, the $MDA^{Cl\text{-}16}$ tumors were consistently slower growing compared to the MDA^{WT} and MDA^{Plas} control cells (Fig. 2G). After 28 days, the tumor volume of the $MDA^{Cl\text{-}16}$ cells was significantly smaller than both control cell groups, indicative of an inhibitory effect of Claudin-16 in these human breast cancer cells (final tumor volume mm^3: $MDA^{Cl\text{-}16}$ 110.33 ± 70.16 vs. MDA^{WT} 390.72 ± 185.69; $P = 0.0003$ and MDA^{Plas} 431.84 ± 247.479; $P = 0.002$).

DISTRIBUTION AND EXPRESSION OF CLAUDIN-16 IN TUMOR AND BACKGROUND CANCER TISSUES

We carried out an immunohistological study to assess the location, distribution and the degree of staining of Claudin-16 using 10 pairs of matched samples (tumor and background tissue). In normal mammary tissues, Claudin-16 appeared as strong staining in the epithelial cells lining vessels (black arrows) and ducts with no staining of cells in the stroma (grey arrows) (Fig. 3A, background). The staining for Claudin-16 within the tumor sections was, however, greatly decreased in the both the cancer and surrounding cells, including those lining vessels (Fig. 3A, tumor). This is illustrated by the transition seen between tumor and associated normal/background tissue as shown (Fig. 3A). Moreover, the staining distribution within cells from background tissues was correctly located at the junctional regions of cells, plainly at the TJ (black arrows). No such distribution was observed in cells from tumor sections, where even the weakly staining cells showed diffuse cytoplasmic staining for Claudin-16 that was not located at the TJ. Thus, over-all staining intensity was reduced in the tumor cells, compared to the normal/background sections as assessed using image analysis (tumor: mean $\pm$ SD 98.06 ± 11.61 vs. background: 160.63 ± 29.71, $P < 0.00001$). Confirmation of the correct location for Claudin-16 in breast tissues was carried out using dual staining of Claudin-16 and Claudin-1 in the same tissue sections (Fig. 3B). It can be clearly seen that both Claudins are located at the same position in epithelial staining lining a vessel (as shown). Hoescht staining was used as a control. Comparison of three paired tumor and normal breast tissues by Western blotting also confirmed that the expression of Claudin-16 protein is reduced in tumor tissues (Fig. 3C).

When Claudin-16 expression was examined at mRNA level using quantitative PCR, the levels were not significantly different between tumor and background tissue (tumor: mean $\pm$ SD 1.008 ± 10.634 vs. background: 0.01 ± 0.02, $P = 0.3$). However, a significant difference in expression was apparent in node positive tumors compared to node negative tumors (median value node positive 0.00003; 1.9 ± 1.8 vs. median value node negative 0.0025; 0.062 ± 0.031, $P = 0.01$ (with range Q1–Q3 node positive 0–0.0037; node negative 0–0.02) (Fig. 4A).

CORRELATION OF CLAUDIN-16 WITH PROGNOSIS AND STAGING

The Nottingham Prognostic Index (NPI) was used as one parameter to assess the prognosis of the patients, in that patients with NPI value less than 3.4 (NPI) were regarded as with good prognosis, 3.4–5.4 (NPI-2) moderate prognosis, and NPI > 5.4 (NPI3) with poor prognosis. The formula for NPI being: NPI = [0.2 × size cm] + grade grade (1–3) + nodal status (1–3). There were higher levels of Claudin-16 expression seen in patients with good prognosis (Fig. 4B), i.e. NPI-1 tumors (NPI < 3.4), although this did not reach significance (NPI-1 1.85 ± 1.83; NPI-2 0.552 ± 0.031; NPI-3 0.0787 ± 0.0787; $P = 0.34$); however, median values showed that NPI-1 tumors expressed significantly higher levels of Claudin-16 than NPI-3 tumors ($P = 0.0023$). The levels of the molecules were also analyzed against TNM status of tumors (TNM-1 1.77 ± 1.72; TNM-2 0.0353 ± 0.017; TNM-3 0.0019 ± 0.0015; $P > 0.17$, Fig. 4C). Levels of Claudin-16 showed some decrease with TNM-2 and -3 status, but significance was not reached. No trend was observed with grade of tumor.

Interestingly, Claudin-16 expression was considerably lower in lobular cancers compared to ductal type (lobular: 0.0269 ± 0.0109; ductal: 1.3 ± 1.260) although significance was not reached ($P = 0.39$). Conversely, there was increased expression of the receptor in lobular cancers compared to other types (lobular: 0.0269 ± 0.0109; others: 0.018 ± 0.01) although this, again, did not reach significance.

Correlation with ER status indicated that Claudin-16 was reduced in ER-positive tumors (ER-negative 1.6 ± 1.6 vs. ER-positive 0.037 ± 0.017, $P = 0.32$) with a striking reduction in expression in ERβ-negative versus ERβ-positive tumors (1.4 ± 1.3 vs. 0.0101 ± 0.0088 respectively, $P = 0.31$; medial values 0.00125 and 0.00002, respectively, $P = 0.04$).

EXPRESSION OF CLAUDIN-16 AND CLINICAL OUTCOME FOLLOWING 10 YEAR FOLLOW-UP

Figure 4D shows data comparing patients with different clinical outcomes after a median follow-up of 120 months. Those patients with metastatic disease had reduced levels of Claudin-16 expression compared to patients without (metastatic disease: 0.009 ± 0.006 vs. disease free: 1.41 ± 1.36) although this did not reach significance.

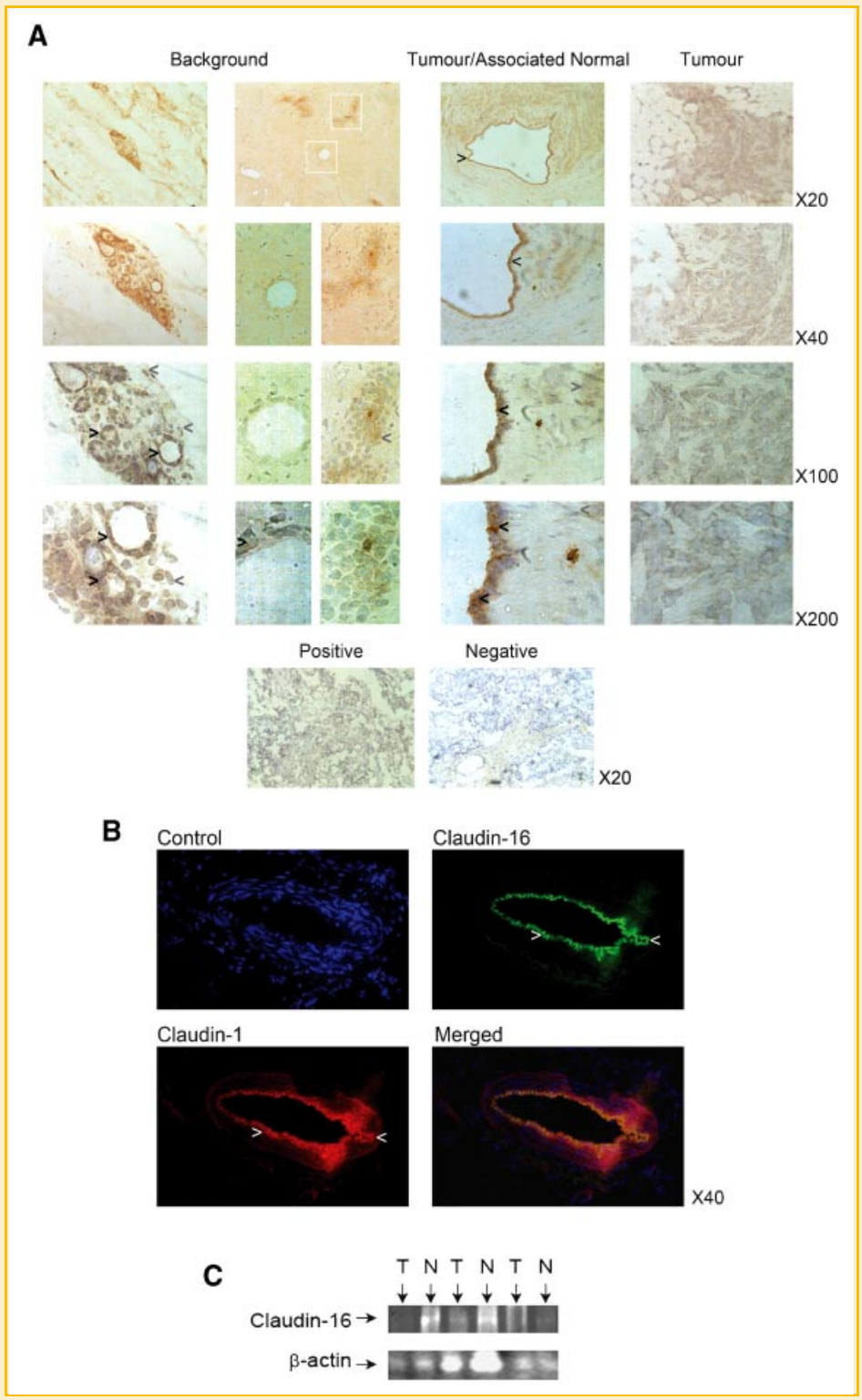

Fig. 3. A comparison of the expression of Claudin-16 in background and tumor breast tissues ($n = 10$) is shown in consecutively increasing magnification in (A) for background and tumor tissues. Regions of Claudin-16 expression located at the TJ area are indicated by black arrows (indicating epithelial cells lining vessels and residual breast epithelia). Stromal cells are indicated by grey arrows, showing little/no staining. The positive and negative controls are also shown. Dual staining of Claudin-16 and Claudin-1 (white arrows) showing co-localization at the junctional area of epithelial cells lining vessels in human breast tissue is shown in (B). C: Western blotting of 3 pairs of tumor and background tissues shows the reduced expression of Claudin-16 in tumor tissues (β-actin as loading control). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

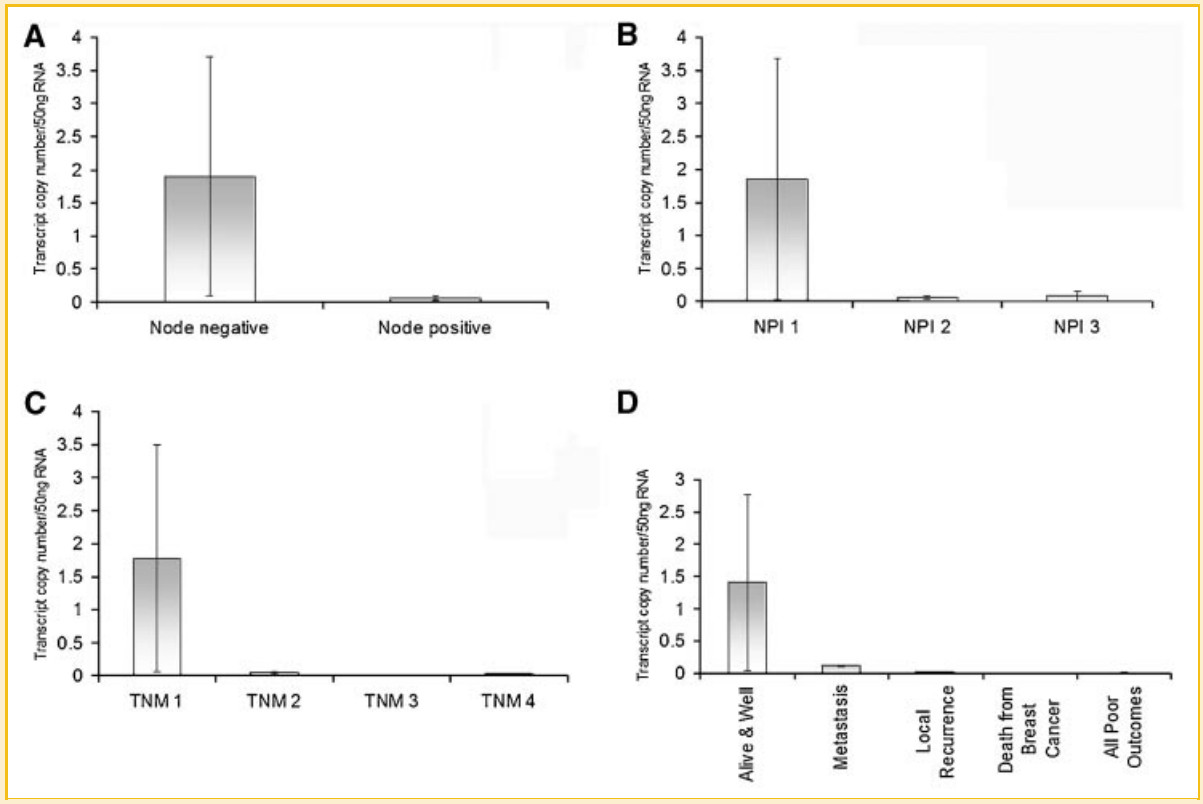

Fig. 4. mRNA levels of the total patient cohort are shown in (A) for correlation to nodal status, (B) correlation to NPI status, (C) correlation to TNM status and (D) correlation with patient outcome. Levels are expressed as transcript copy number/50 ng RNA.

Patients who had local recurrences also showed decreased Claudin-16 expression when compared to those who remained disease free (local recurrence: 0.011 ± 0.009, Fig. 4D). Patients who had died of breast cancer had significantly reduced levels of Claudin-16 compared to patients with who remained alive and well (0.001 ± 0.009, $P = 0.015$). It appears from this data, that reduced Claudin-16 expression is associated with poor prognosis and outcome in patients with breast cancer and is negatively correlated with poor outcome, particularly in those patients who died from the disease.

In addition, survival curves (over 120 months) showed that reduced levels of Claudin-16 transcript levels were associated with poor survival in patients with breast cancer. Survival was analyzed using the Cox-proportion-Hazardous test and showed a 0.03 probability of low Claudin-16 levels indicating low survivability.

DISCUSSION

This study has demonstrated for the first time that forced expression of Claudin-16 (Paracellin-1) in the aggressive human breast cancer cells line MDA-MB-231, resulted in cells with strengthened TJ function and "tighter" colony morphology. There was also a concurrent reduction in aggressive, invasive phenotype with these cells being less motile, less invasive, and more adherent and showing retarded in vivo growth. We have also shown for the first time that Claudin-16 is expressed in human breast tissue, and that this expression is reduced in breast cancer. This was shown at the protein and mRNA levels. Moreover, from our cohort of patient samples ($n = 124$) it is evident that this reduced expression is associated with poor prognosis for patients with breast cancer, *sic*: reduced in node positive tumors and with increasing NPI and TNM status. Interestingly, Claudin-16 expression is significantly reduced in ER positive tumors, particularly those that are ERβ negative. Overall poor survival of the patients is thus correlated with reduced Claudin-16 expression (Cox-proportion Hazardous test).

Cell adhesion to adjacent cells and the extracellular matrix is key not only to the organization of epithelium into a tissue but also to the regulation of cellular processes such as gene expression, differentiation, motility and growth [Chlenski et al., 1999]. Cell adhesion molecules, transmembrane receptors and cytoskeletal all of which are organized into multi-molecular complexes and the activation of signaling pathways, mediate these regulatory functions. Studies suggest that some of the cell adhesion and cytoskeletal proteins may subserve an additional and important function, namely, suppression of the malignant phenotype of cells in tumorigenesis [Chlenski et al., 1999].

TJs are hallmark structures of one-layered epithelia and endothelia and are of central biological importance as intramembranous "fence" and as hydrophobic "barriers" between lumina represented by liquid or gas filled spaces on one hand, and the

mesenchymal space on the other [Langbein et al., 2003]. The TJs of epithelia of different tissues have different permeability properties to ions and molecules [Wong et al., 1999]. These differences in permeability properties appear to be important for the respective physiological function of each tissue. Tight junctions are regulated in various physiological processes such as leukocyte transmigration across endothelium. During such dynamic processes, the TJ permeability barrier is temporarily disrupted, but subsequently resealed, relatively quickly (usually within 1 h). This resealing is accomplished by assembly of pre-existing elements, rather than resynthesis [Wong et al., 1999]. It is increasingly clear that the TJ and its constituent molecules play a role in a series of pathophysiological events, including cancer progression and development [Oliveira and Morgado-Diaz, 2007]. We have also previously demonstrated that the expression of ZO-1, ZO-2 and ZO-3, and MUPP-1 (multi-PDZ domain protein 1), peripheral/plaque proteins that function in maintaining tight junction integrity and in transducing regulatory signaling have low levels of expression in breast cancer and are associated with poor patients prognosis [Martin et al., 2004a].

Claudin-16 is a member of the Claudin protein family. Mutations in the Claudin-16 gene cause familial hypomagnesemia with hypercalciuria and nephrocalcinosis, a rare autosomal recessive tubular disorder that eventually progresses to renal failure [Kang et al., 2005]. Claudin-16 with its associated proteins forms an intracellular pore permitting the paracellular passage of Mg^{2+} and Ca^{2+} down their electrochemical gradients [Simon et al., 1999]. It has been suggested that one mechanism of Claudin-16 dysfunction leads to loss of cation selectivity [Hou et al., 2005]. Claudin-16 localizes to the TJ via its association with ZO-1. One of the mutations in human involves inactivation of a PDZ-domain binding motif, thereby disabling the association of the TJ scaffolding protein ZO-1 with Claudin-16. In contrast, the mutant no longer localizes to TJs in kidney epithelial cells, but instead accumulates in lysosomes [Muller et al., 2003]. In addition, it has been demonstrated that protein kinase A-dependent phosphorylation of Ser217 in Claudin-16 is essential for its localization in the TJ and subsequent transepithelial Mg^{2+} transport [Ikari et al., 2006].

Previous work has highlighted the differential expression of Claudins in human cancers [Oliveira and Morgado-Diaz, 2007]. In comparison to a recent mRNA-only based study [Hewitt et al., 2006], we show here for the first time that Claudin-16 is expressed in human breast tissues. Historically however, Claudins that initially exhibited tissue specific distribution have subsequently been demonstrated to be expressed in many different tissue types for example Claudin-1 and -5. This discrepancy may be due to differences in sensitivity of the techniques used. The reduction on expression in tumor tissues shown in this study is clearly associated with indicators of poor prognosis in patients with breast cancer. Claudins have long been shown to be associated with this disease. Human Claudin-1 (SEMP-1) expression has been observed in human mammary epithelial cells, but was observed to be at low or undetectable levels in a number of breast tumors and breast cancer cell lines [Kramer et al., 2000]. This indicates a possible tumor suppressor function. In sporadic and hereditary breast cancer, there were no genetic changes, implying that regulatory or epigenetic factors may be involved in the downregulation of the Claudin-1 gene during breast cancer development [Kramer et al., 2000].

It is interesting to note that SAGE analysis of human ovarian tumors revealed that Claudin-16 was over-expressed in this tissue type [Rangel et al., 2003], underlining the complex role that Claudins have in different tissue types. A comprehensive study by Hewitt et al. [2006] showed that most Claudin genes appear decreased in cancer, while CLDN3, CLDN4, and CLDN7 are elevated in several malignancies such as those originating from the pancreas, bladder, thyroid, fallopian tubes, ovary, stomach, colon, breast, uterus, and the prostate. However, this was a study of limited numbers of samples.

Kominsky et al. [2003] have shown that Claudin-7 loss correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. The study showed that the expression of Claudin-7 was lost in both preneoplastic and invasive ductal carcinoma of the breast occurring predominately in high grade lesions. Expression was also frequently lost in DCIS correlating with the increased cellular discohesion observed in DCIS. Additionally, the majority of IDC cases displaying a low Claudin-7 expression had a positive (lymph node status). Such findings suggest that the loss of Claudin-7 may aid in tumor cell dissemination and augment metastatic potential. Moreover, silencing of Claudin-7 expression correlated with promoter hypermethylation in 3/3 breast cancer cell lines but not in invasive ductal carcinomas (0/5). In addition, HGF treatment (10 ng) resulted in disassociation of MCF-7 and T47D cells in culture, and a loss of Claudin-7 expression within 24 h.

Tokes et al. [2005] compared levels of protein and mRNA expression of three members of the Claudin family in malignant breast tumors and benign lesions. Altogether, 56 sections from 52 surgically resected breast specimens were analyzed for Claudin-1, -3 and -4 expression by immunohistochemistry and mRNA was also analyzed using real-time PCR. Claudins were rarely observed exclusively at tight junction structures. Claudin-1 was present in the membrane of normal duct cells and in some of the cell membranes from ductal carcinoma in situ, and was frequently observed in eight out of nine areas of apocrine metaplasia, whereas invasive tumors were negative for Claudin-1 or it was present in a scattered distribution among such tumor cells (in 36/39 malignant tumors). Claudin-3 was present in 49 of the 56 sections and CLDN4 was present in all 56 tissue sections. However, Claudin-4 was highly positive in normal epithelial cells and was decreased or absent in 17 out of 21 ductal carcinoma grade 1, in special types of breast carcinoma (mucinous, papillary, tubular) and in areas of apocrine metaplasia. Claudin-1 mRNA was down regulated by 12-fold in the tumor group. Claudin-3 and -4 mRNA exhibited no difference in expression between invasive tumors and surrounding tissue. The significant loss of Claudin-1 protein in breast cancer cells suggests that this protein may play a role in invasion and metastasis. The loss of Claudin-4 expression in areas of apocrine metaplasia and in the majority of grade 1 invasive carcinomas also suggests a particular role for this protein in mammary glandular cell differentiation and carcinogenesis.

We have also shown here for the first time the forced expression of Claudin-16 in aggressive human breast cancer cell lines leads to cell with a less aggressive phenotype, slower growth and increased

TJ functionality. These results concur with work of others investigating the expression and role of TJ molecules in human cancer, that TJ constituent proteins are key elements in the prevention of cancer cell dissociation and hence metastasis. Our previous study using the current breast tissue cohort revealed that levels of ZO-1 were also reduced in tumor tissues [Martin et al., 2004a]. This has a direct bearing on the currently observed mislocalization of Claudin-16 cancer cells in tumor tissue sections; that is, less ZO-1 is available in tumor cells for correct binding of Claudin-16, and hence to localization to the cell periphery. It is clear that this interesting area requires continued investigation. We had also previously shown that HGF, a cytokine secreted by stromal cells, is capable of modulating expression and function of TJ molecules in human breast cancer cell lines, particularly Claudin-1 and occludin (Martin et al., 2004b). This suggested that HGF disrupts TJ function in human breast cancer cells by effecting changes in the expression of TJ molecules at both the mRNA and protein levels. The conclusion was that regulation of TJs could be of fundamental importance in the prevention of metastasis of breast cancer cells. The work presented here demonstrates how this disruption of TJ structure and function can be overcome by the forced expression of Claudin-16, providing some insight as to how TJ of breast cancer cells might be modulated; if the induction of expression of one Claudin can affect the function and behavior of aggressive breast cancer cells so significantly, then this underlines the importance of the TJ in cancer progression. It is unfortunate that in the current study we were unable to provide direct comparison between the level of Claudin-16 in the transfected cell line and in human tissues to inherent differences in properties of the two. We can also assume that Claudin-16 may act in a similar fashion to other Claudins in clinical breast cancer. This can be seen from the study of Hoevel et al. [2004]. A retro-viral system was used to reintroduce Claudin-1 into MDA-MB-361 human breast cancer cells. Interestingly, a more prominent cell membrane localization of Claudin-1-positive cultures correlated with a pronounced increase in apoptosis in tumor spheroids. In parallel, inhibition of paracellular flux was observed. This report suggests that Claudin-1 has a potential role in restricting nutrient and growth factor supplies in breast cancer cells and indicate that the loss of the cell membrane localization of Claudin-1 in carcinomas may be a crucial step toward tumor progression.

From the data presented it here, we can say that the in vitro forced expression of Claudin-16 in human breast cancer cells shows a tantalizing glimpse into the possible therapeutic value of TJ proteins. Moreover, Claudin-16 has potential as a prognostic indicator in human breast cancer, particularly with relevance to patient survival and outcome.

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