

Integrins Alpha-2 and Beta-1 increase through Multiple Generations of the EDW01 Patient-Derived Xenograft Model of Breast Cancer – Markers but not Drivers of Epithelial Mesenchymal Transition

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Abstract

Background: Breast cancers acquire aggressive capabilities via epithelial to mesenchymal transition (EMT), in which various integrins/integrin linked kinase signalling are upregulated.

Methods: We investigated this in two patient-derived xenografts (PDXs) developed from breast-to-bone metastases, and its functional significance in a breast cancer cell line system. ED03 and EDW01 PDXs were grown subcutaneously in immunocompromised SCID mice through 11 passages and 7 passages, respectively. Tumour tissue was assessed using immunohistochemistry (IHC) for estrogen receptor (ER)-alpha, E-cadherin, vimentin, Twist1, beta-catenin, P120-RasGAP, CD44, CD24 and Ki67, and RT-qPCR of EMT-related factors (CDH1, VIM, CD44, CD24), integrins beta-1 (ITGB1), alpha-2 (ITGA2) and ILK. Integrin and ILK expression in epidermal growth factor (EGF) induced EMT of the PMC42-ET breast cancer cell line was assessed by RT-qPCR and Western blotting, as were the effects of their transient knockdown via small interfering RNA +/- EGF. Cell migration, changes in cell morphology and adhesion of siRNA-transfected PMC42-ET cells to various extracellular matrix (ECM) substrates was assessed.

Results: The ED03 (ER+/PR-/HER2-/lobular) and EDW01 (ER+/PR-/HER2-/ductal) PDXs were both classified as molecular subtype luminal A. ED03 xenografts exhibited mutated E-cadherin with minimal expression, but remained vimentin-negative across all passages. In EDW01, the hypoxic indicator gene CAIX and Twist1 were co-ordinately upregulated at passage 4-5, corresponding with a decrease in E-cadherin. At passages 6-7, vimentin was upregulated along with ITGB1 and ITGA2, consistent with an increasing EMT. The ED03 PDX displayed minimal change over passages in mice, for all genes examined. ILK, ITGB1 and ITGA2 were also increased in the EGF-induced EMT of PMC42-ET cells (in which E-cadherin was downregulated) although siRNA against these targets revealed that this induction was not necessary for the observed EMT. However, their knockdown significantly reduced EMT-associated adhesion and Transwell migration.

Conclusion: Our data suggest that despite an increase in integrins alpha-2 and beta-1 in the EMT exhibited by EDW01 PDX over multiple generations, this pathway may not necessarily drive the EMT process.

Introduction

Human breast cancer cell lines have been used extensively in vitro and in mice to dissect the cellular mechanisms associated with tumour aggressiveness and metastasis. However, cell line xenografts typically fail to recapitulate tumour cell heterogeneity. By contrast, human breast tumours engrafted into mice as patient derived xenografts (PDXs) generally show excellent reproducibility of morphological and genetic characteristics of the original tumour with minimal genetic drift and as such are clinically relevant platforms for preclinical studies [1–5].

Aggressive cancers are known to display epithelial-mesenchymal plasticity (EMP), through which they can fluctuate between epithelial or mesenchymal states (or degrees of these) to assist survival in

changing microenvironmental conditions [6–8]. Epithelial to mesenchymal transition (EMT) describes a phenotypic change toward a more mesenchymal state resulting in more motile and invasive cancer cells. EMT has been shown to promote progression in several cancer types including breast [9, 10], contributes to chemoresistance [11, 12], and is prominent in circulating tumour cells (CTCs) [7, 13–15]. Evidence for EMP has been demonstrated in numerous human breast cancer cell line studies and increasingly in breast cancer in vivo models [16–19] and clinical material (reviewed in [20]).

Invasive lobular carcinoma (ILC) and invasive ductal carcinoma (IDC), (also known as invasive carcinoma of no special type (IC NST)), have distinctive morphological features [21]. ILC is typified by single-file epithelial tumour cells, a finer stromal infiltration and often a minimal sclerotic tissue reaction - the combination of which makes self-detection and screen-detection (mammography) more difficult than for the typically more palpable IDC tumours, which grow as masses of epithelial cells within a desmoplastic stroma. These patterns are intimately linked to E-cadherin: ILCs do not express E-cadherin due to the presence of inactivating mutations [22, 23] or silencing via methylation [24], or copy loss [25], and hence grow as individual and linear arrays of tumour cells. By contrast, IDCs typically express E-cadherin and hence grow as cohesive tumour nests [21].

A major initiating event in the transcriptional programming of EMT is E-cadherin repression [26–28]. IDC expression of E-cadherin is downregulated when they undergo an EMT, which is associated with increased invasiveness [29, 30]. Interestingly, EMT does not occur spontaneously in ILC cells [31] and they tend to show less vimentin expression than IDC [32]. Similarly, not all IDC-derived cell lines undergo EMT upon E-cadherin silencing, while some do [18].

Integrin switching is prominent in breast cancer EMT and has been linked to tumour aggressiveness [33], and integrins have been shown to play important roles in tumour cell transmigration via EMT (reviewed in [34]). Integrins are a large family of heterodimeric cell surface receptors that play a prominent role in the adhesive interactions between cells and their surrounding extra-cellular matrix (ECM), providing adhesion for stationary cells, as well as traction during cell movement [35–38]. TGF β -induced EMT in NMuMG mouse mammary cancer cells results in the downregulation of epithelial $\alpha 6\beta 4$ integrin expression (which mediates contact with the basement membrane) through epigenetically silencing of the gene encoding integrin $\beta 4$ [39]. $\alpha 3\beta 1$ integrin, which binds laminin and also associates with E-cadherin, is required for progression through EMT in lung alveolar epithelial cells, where it integrates beta-catenin and transforming growth factor- β (TGF β)-SMAD signalling to promote myofibroblast formation and lung fibrosis [40]. Interactions between $\alpha 5\beta 1$ integrin and fibronectin have been associated with EMT in Eph4 mouse mammary cancer cells and human lung cancer cell lines [41, 42]. In pancreatic carcinoma cells, increased expression of $\alpha 1\beta 1$ or $\alpha 2\beta 1$ integrins and their interactions with type I collagen facilitate the disruption of E-cadherin complexes and the nuclear translocation of beta-catenin, and promote proliferation and motility [43].

Indeed, $\alpha 2\beta 1$ integrin is widely expressed on epithelial cells and its levels are increased in several carcinoma cells from the epithelial origin [44]. Growing evidence indicates that $\alpha 2\beta 1$ integrin can be a key

pathway in cancer pathogenesis [43, 45, 46]. Furthermore, Chen et al (1994) showed that increased expression of $\alpha 2\beta 1$ integrin is positively correlated with increased metastatic ability in human squamous cell lung cancer cells when i.v. inoculated in severe combined immunodeficiency (SCID) mice [47].

It has been demonstrated that ILK can induce a complete EMT in various epithelial cell lines, and thus be involved in the initiation of EMT in vivo, and the maintenance of the mesenchymal phenotype and disease progression [48–51]. ILK can modulate the expression of not only E-cadherin, but also other epithelial markers such as cytokeratin 18 [52] and MUC1 [53], as well as mesenchymal markers such as LEF1 [54] and vimentin [53, 54]. Therefore, ILK is able to initiate an EMT. Gain and loss of function strategies have shown that over-expression, and/or constitutive activation of ILK results in oncogenic transformation and progression to invasive and metastatic phenotypes [55].

We examined the relationship between EMT, $\alpha 2\beta 1$ integrin and ILK in serial passages of two different breast cancer PDX (ED03 and EDW01) in mice. We coupled this with an investigation into the pattern of ILK and integrin expression changes in PMC42 human breast cancer cells induced by epidermal growth factor (EGF) treatment to undergo an EMT in vitro, and assessed whether these integrin changes were necessary for EMT to occur.

Materials And Methods

Patient material and creation of xenografts

Establishment of the PDXs used in this study was described previously [56].

The ED03 xenograft was derived from a lobular breast cancer bone metastasis in a 40 year old woman, 3.5yrs after initial diagnosis of her primary tumour. The EDW01 PDX was derived from a bone metastasis of an invasive ductal carcinoma of the breast presenting as clinically overt macrometastatic deposits in a 44 year old woman.

Briefly, the tumour tissue derived initially from the bone metastasis deposits was diced into ~ 1 mm pieces, mixed with Matrigel® (BD Biosciences, Australia) and implanted bilaterally subcutaneously in SCID mice (ARC, Perth, Australia). For each passage, once tumour volumes reached 2000 mm³, mice were euthanised, and the tumours were removed. The tumour tissue was again chopped into chunks, mixed with Matrigel®, and implanted into fresh mice. This was repeated 6 times for EDW01 (total of 7 passages) and 10 times for ED-03 (total of 11 passages). Portions were snap frozen for RNA extraction and formalin fixed and paraffin embedded for immunohistochemical analyses at each passage.

Immunohistochemistry (IHC)

A tissue microarray was created of randomised duplicate 2 mm diameter cores of tumour blocks corresponding to various passage numbers through mice of the ED03 and EDW01 PDX model systems. IHC was performed using the Ventana Discovery Ultra Automated Slide Preparation system. Details of antibodies used in this study can be found in Table 1. The membrane associated proteins (E-cadherin,

beta-catenin, P120-RasGAP, CD24, CD44, carbonic anhydrase IX (CAIX)) were scored as cytoplasmic or membranous, and whether they were heterogeneous or homogeneous in these areas. Vimentin staining was scored as positive if present in the cytoplasm of cells, whereas for Twist1 and Ki67, the proportion of positive nuclei was recorded.

Table 1
Antibodies used in this study.

Antigen	Antibody	Dilution	Supplier
Vimentin ^{^,#}	Mouse Monoclonal IgG (V9)	1:750	Dako, Australia
E-cadherin ^{^,#}	Mouse Monoclonal IgG (36)	1:12500	BD Transduction Laboratories, USA
N-cadherin (A-CAM)	Mouse Monoclonal IgG (GC-4)	1:2000	Sigma-Aldrich, Australia
TWIST [^]	Mouse/ 2C1a	1:100	Abcam, England
Beta-catenin [^]	Mouse monoclonal, clone 14	1:500	BD Biosciences, Australia
P120 [^]	Mouse monoclonal 98/pp120	1:200	BD Biosciences, Australia
CD24 [^]	Mouse monoclonal SN3b	1:50	Thermo Fisher Scientific, Australia
CD44 [^]	Mouse monoclonal 156-3C11	1:75	Abcam, England
Ki-67 [^]	Mouse monoclonal MIB-1	1:100	Dako, Australia
CA-IX [^]	Rabbit monoclonal EP161	1:100	Cell Marque (Sigma Aldrich), USA
ITGB1 [#]	Mouse Monoclonal IgG	1:5000	Chemicon International (Fisher Scientific), USA
ITGA2 [#]	Rabbit Polyclonal	1:1000	Chemicon International (Fisher Scientific), USA
Integrin-Linked Kinase (ILK) [#]	Rabbit monoclonal IgG	1:4500	Cell Signaling Technologies (Danvers, MA,USA)
Pan-Actin, Ab-5 [#]	Mouse Monoclonal IgG	1:10000	Neomarkers (Invitrogen), USA
Secondary antigen	Antibody	Dilution	Supplier
Biotinylated Immunoglobulin ^{^,#}	Polyclonal Rabbit Anti-Mouse	1:200	Dako, Australia
IgG-HRP ^{^,#}	Goat Anti-Mouse	1:20000	Dako, Australia

[^] indicates antibody used for IHC

[#] indicates antibody used for WB

Antigen	Antibody	Dilution	Supplier
IgG-HRP ^{^,#}	Goat Anti-Rabbit	1:20000	Dako, Australia
^ indicates antibody used for IHC			
# indicates antibody used for WB			

Necrosis quantification and determination of cellular area in phase contrast images using ImageJ

Whole core images (4x magnification) were imported into ImageJ (v.1.52a, National Institutes of Health, USA). Necrotic area was outlined in cores and expressed as a percentage of the total core area. Since cores were sampled from the blocked tumour in a random, unbiased manner, necrotic area in the core was considered representative of the whole tumour. Assessment of cellular area in phase contrast images was determined using ImageJ after setting to 8-bit image, adjusting the thresholding such that maximal cellular area was selected in the absence of background, then using the “Analyse particles” tool. Area maps were generated to show where data was collected, which was then averaged for each microscopic view.

Reverse transcriptase- quantitative PCR

RNA was extracted using the Qiagen RNeasy Mini prep Kit (Qiagen, Doncaster, Vic, Australia). cDNA synthesis and reverse transcriptase - quantitative PCR (RT-qPCR) was performed as previously described, using a specific reverse transcriptase (RT) primer in the cDNA synthesis step [57, 58]. Expression levels of the genes of interest were normalised to ribosomal protein L32 mRNA. Human-specific primers for various genes examined in this study are detailed in Table 2.

Table 2
 QPCR primers for various genes examined in this study.

Oligonucleotide Name	Species	Sequence (5'-3')
5' Hs L32	Human	CAGGGTTCGTAGAAGATTCAAGGG
3' Hs L32	Human	CTTGGAGGAAACATTGTGAGCGATC
Hs L32 RT	Human	CAGAAAACGTGCACATGAGCTGC
5' Hs CD24	Human	GACTCAGGCC AAGAAACGTC TTCTAAA
3' Hs CD24	Human	GTTGCCTCTCCTTCATCTTG TACATGAAA
Hs CD24 RT	Human	gggcgacaaagtgagactgtctaaaa
5' Hs CD44	Human	CACAATGGCCCAGATGGAGAAA
3' Hs CD44	Human	CTTCGACTGTTGACTGCAATGCAAA
Hs RT CD44	Human	ggcaatgttgcaagggttggaagactt
5' Hs VIM	Human	CAGGCGATATATTACCCAGGCAAGAA
3' Hs VIM	Human	CTTGTAGGAGTGTCGGTTGTTAAGAA
Hs VIM RT	Human	CTAAATCTTGTAGGAGTGTCGGTTGTT
5' Hs CDH1	Human	GGCACAGATGGTGTGATTACAGTCAAAA
3' Hs CDH1	Human	GTCCCAGGCGTAGACCAAGAAA
Hs CDH1 RT	Human	ctctgtctttggctgcagcacttta
5' Hs ILK	Human	GATGCAGGACAAGTAGGACTGGAA
3' Hs ILK	Human	CAACCAGAGGCCTGCTGCTTT
Hs ILK RT	Human	GCTGGGGTAGTACCATGACTG
5' Hs TWIST1	Human	ctagagactctggagctggataactaaaa
3' Hs TWIST1	Human	cgacctcttgagaatgcatgcatgaaaa
Hs TWIST1 RT	Human	gagaaagtccatagtgatgcctttccttt
5' Hs SNAI1	Human	CCAGACCCACTCAGATGTCAAGAA
3' Hs SNAI1	Human	GGCAGAGGACACAGAACCAGAAAA
Hs SNAI1 RT	Human	cgcagacaggccagctcaggaat
5' Hs SNAI2	Human	CCCAATGGCCTCTCTCCTCTTT
3' Hs SNAI2	Human	CATCGCAGTGCAGCTGCTTATGTTT

Oligonucleotide Name	Species	Sequence (5'-3')
Hs SNAI2 RT	Human	CATCGCAGTGCAGCTGCTTATGTTT
5' Hs ZEB1	Human	GTTACCAGGGAGGAGCAGTGAAA
3' Hs ZEB1	Human	GACAGCAGTGTCTTGTTGTTGTAGAAA
Hs ZEB1 RT	Human	GACAGCAGTGTCTTGTTGTTGTAGAAA
5' Hs ZEB2	Human	CCACCTGGA ACTCCAGATGCTTTT
3' Hs ZEB2	Human	GCCTTGCCCACTCTGTGCATTT
Hs ZEB2 RT	Human	GCCTTGCCCACTCTGTGCATTT
5' Hs ITGA2	Human	GACCTATCCACTGCCACATGTGAAAAA
3' Hs ITGA2	Human	CCACAGAGGACCACATGTGAGAAAA
Hs ITGA2 RT	Human	GTCAGAACACACACCCGTTGTGTAATA
5' Hs ITGB1	Human	GACTGATCAGTTCAGTTTGCTGTGTGTTT
3' Hs ITGB1	Human	CCCTGCTTGTATACATTCTCCACATGATTT
Hs ITGB1 RT		CCCTGCTTGTATACATTCTCCACATGATTT
5' Ms ITGB1	Mouse	GCGTGTGCAGGTGTCGTGTTT
3' Ms ITGB1	Mouse	GAAGGCTCTGCACTGAACACATTCTTT
Ms ITGB1 RT	Mouse	GAAGGCTCTGCACTGAACACATTCTTT

siRNA mediated knockdown

Small interfering RNA (siRNA)-mediated knockdown of ITGB1, ITGA2, and ILK (Horizon, [formerly Dharmacon], Melbourne, Australia) was performed in PMC42-ET cells. These cells display a molecular phenotype of Basal B (E Tomaskovic-Crook and T Blick, unpublished observation), based on clustering of a limited number of the Basal B discriminator genes [19] showing reliable data in an Affymetrix U133A analysis kindly performed by the laboratory of Joe Gray, Lawrence Berkeley National Laboratory, Berkeley, California [59]. These IDC-derived cells express E-cadherin mRNA and protein but do not assemble it at the cell membrane [27]. PMC42-ET were grown in RPMI with 10% foetal bovine serum (FBS, Thermo Fisher Scientific, Australia) at 37°C with 5% CO₂.

The siRNA target sequences for ITGB1, ITGA2, and ILK are presented in Table 3. A commercial non-targeting control sequence (control siRNA) was also used (siSTABLE Non-targeting siRNA #1, Horizon, [formerly Dharmacon], Melbourne, Australia). Briefly, PMC42-ET cells were transfected using DharmaFECT4 (Horizon, [formerly Dharmacon], Melbourne, Australia) and 100 nM siRNA targeting ITGB1, ITGA2, ILK, or control siRNA. After 8 hrs, cells were then either left unstimulated, or stimulated with

10 ng/ml EGF for 72 hrs. Controls included cells alone (no transfection), transfection reagent alone, and the control siRNA. Protein and RNA were extracted 72 hrs post EGF-stimulation, and analysed by Western immunoblotting and RT-qPCR, respectively. Recombinant EGF was purchased from BD Biosciences, (Bedford, MA, USA).

Table 3
Sequences of siRNA used in the current study.

siRNA constructs	siRNA sequence
ITGB1 siRNA	AAGCTTTTAATGATAATTCAT
ITGA2 siRNA	TCGCTAGTATTCCAACAGAAA
ILK siRNA	CCTGACGAAGCTCAACGAGAA

Western blotting

Western blotting for ITGB1, ITGA2, ILK, pan-actin, Vimentin, N-cadherin and E-cadherin in siRNA transfected PMC42-ET cells +/- EGF was performed as previously described [57], with protein extracted using RIPA (radioimmunoprecipitation assay) buffer containing protease inhibitors [57]. Antibodies and their dilutions used for Western blotting are detailed in Table 1.

Cell matrix adhesion assay

Wells of 24-well plates (polystyrene, non-tissue culture treated; Nunc Inc., Naperville, IL) were coated with 100 µg/ml collagen-I, 100 µg/ml collagen-IV, 20 µg/ml fibronectin or 50 µg/ml laminin. Proteins were allowed to bind to the cells overnight at room temperature under the laminar flow hood, before the wells were rinsed with phosphate buffered saline (PBS) and non-specific interactions were blocked for 1 hr at 37 °C with 3% bovine serum albumin (BSA) in PBS, pH 7.4. PMC42-ET cells were transfected with siRNA. Eight hours later, cells were either left unstimulated, or stimulated with EGF for 72 hrs, after which the cells were detached using 0.25% trypsin and allowed to attach to the various (ECM substrate-coated plates for 1hr. Cells attached to ECM was estimated by the average of cell counts from five random high-power fields under light microscopy (counted in situ on the substrate after washing). Collagen 1, collagen IV, laminin (from Engelbreth-Holm Swarm murine sarcoma), and fibronectin (from bovine plasma) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Boyden chamber migration assay

Boyden chamber migration assays were performed as previously described [60]. Briefly, transmigration culture assays were performed using 8 µm-pore Transwell chambers (Corning, USA). Polycarbonate membranes (8 µm pore size) of the upper compartment of 24-well chambers were coated with 100 µg/ml collagen I in serum-free media (SFM; Roswell Park Memorial Institute [RPMI]-1640 medium). siRNA-transduced PMC42-ET cells (+/- EGF) harvested by trypsinisation were re-suspended in SFM supplemented with 0.2% BSA, and the cell suspension (2.5×10^5 cells suspended in 250 µl SFM) was

applied to the upper compartment in triplicate wells. The lower compartment was filled with 650 µl of chemoattractant (RPMI-1640 containing 10% FBS [Sigma-Aldrich]). After 24 hrs of incubation, the chambers were rinsed in PBS to eliminate non-adherent cells and the remaining non-migrated cells on the upper surface of the filter were removed carefully with a cotton swab. Migrated cells on the lower side of the filter were stained with 0.5% crystal violet (Sigma Aldrich, Australia) for 15 minutes. The crystal violet dye retained on the filters after washing was extracted with 10% acetic acid and cell migration was measured by reading the absorbance at 560 nm on a micro-titre plate reader (PolarStar Optima, BMG Labtech, Ortenberg, Germany).

Statistical analyses

The two-tailed Mann-Whitney test (non-parametric) and Pearson's correlation co-efficient were performed using GraphPad Prism v7 (GraphPad Software, San Diego, USA). Cell matrix adhesion and Boyden chamber migration assay results were analysed using two way ANOVA with Dunnett's multiple comparison test.

Results

Histological comparison of the ED03 and EDW01 xenografts with increasing passages through mice

The ED03 PDXs was serially passaged up to passage (p) 11, whereas the EDW01 PDX was passaged up to p7. Histologically, the ED03 xenografts displayed a diffuse growth pattern with minimal visible tumour stroma, often growing in long cords of cells, consistent of ILC. By contrast, EDW01 PDX revealed histology consistent with IDC, with clearly visible stromal septae separating growing tumour islands (Fig. 1A). In ED03 the stromal collagen was evident only under higher magnification as it was finer and more pericellular compared with EDW01, in which the thicker stromal cords separated lobules of tumour. This stroma was of murine origin, as it did not stain with human specific vimentin antibody (Fig. 1A).

Assessment of estrogen receptor (ER α) across serial passages

We investigated the expression of ER α in the ED03 and EDW01 PDX models. As shown in Fig. 1B, immuno-reactivity to ER α in original ED03 patient material was seen in approximately 99% of tumour cells, and although the ER α + proportion was lower in early (p2) passage xenografts, strong positivity was observed in later passages (p5, almost 100% of tumour cells). By contrast, the original EDW01 xenograft derived from bone metastasis appeared weakly positive (not shown), early (p2) passage material for EDW01 xenograft exhibited less than 10% of positive cells, and ER α positivity increased at p3, showing 40% ER α -positivity at this passage. Progesterone receptor (PR) expression was negative for both of the clinical samples and for both xenograft models at each passage (not shown). ED03 original tumour

material (p0) was HER-2 negative however p2 tumour material showed focal incomplete membranous staining, but only in around 10% of the cells (clinically classified as negative staining, 1+), whilst EDW01 tumours were negative at all passages examined. The clinical approximated subtypes of breast cancer (defined according to the 2011 St Gallen International Breast Cancer Conference) classifies both ED03 and EDW01 as Luminal A, since Ki67 is less than 14% in both PDXs (Fig. 2) [61].

Immunohistochemistry (IHC) and RT-qPCR quantification of EMT markers

To assess any changes in EMT status over sequential passaging, key effector molecules implicated in the EMT process (vimentin/E-cadherin, Twist1, beta-catenin, P120-RasGTPase activating protein [P120-RasGAP], CD24/CD44) and the proliferative marker Ki67 were screened in the ED03 and EDW01 PDX models across the series of passages using IHC (Fig. 2) and human-specific RT-qPCR (Fig. 3).

E-cadherin immunostaining was almost completely absent in ED03 original patient material, consistent with its lobular carcinoma derivation [23]. We subsequently confirmed a putative somatic missense variant (p.His128Asn, data not shown). Consistent with this, less than 1% of cells expressed E-cadherin in any ED03 PDX passage in mice (Fig. 2, top left panel). Similarly, beta-catenin was not detectable within the ED03 PDXs, p120-RasGAP was aberrant, with staining observed to be mostly cytoplasmic. By contrast, EDW01 PDXs displayed strong E-cadherin immunostaining (Fig. 2, top right panel) and readily detectable RNA levels (Fig. 3). However, this was accompanied by a progressive increase in human-specific vimentin mRNA expression with each passage (Fig. 3B): passage 6 material displayed an approximate 8-fold increase ($P = 0.008$), and passage 7 material displayed an approximate 12-fold increase ($P = 0.024$) in comparison to passage 3 material. E-cadherin positive tumour cells transitioning to vimentin positivity (possibly remaining E-cadherin positive, see high magnification inset, Fig. 2) were observed in EDW01 PDXs whereas vimentin mRNA and protein in ED03 were almost negligible (Fig. 2, 3B).

As shown in Fig. 2, positive nuclear Twist1 expression was seen only in EDW01 xenograft tumours, adjacent to regions of necrosis (as indicated by black arrows). Beta-catenin and P120-RasGAP was also mainly observed in EDW01 at the cell membrane, and corresponded with E-cadherin staining.

We went on to further examine the expression of breast cancer stem cell markers CD44 and CD24, as upregulation of CD44 and downregulation of CD24 is observed in breast cancer cell line EMT [19]. ED03 displayed a homogeneous CD44 IHC pattern, which was relatively consistent throughout the passages at the protein and mRNA level (Fig. 2, 3). By contrast, CD44 protein was heterogeneously expressed in EDW01 (Fig. 2). Although CD24 was upregulated with increasing passages in both models, ED03 exhibited a higher expression overall than EDW01. Within EDW01, but not ED03, there were regions of tumour cells that appeared to lack both CD24 and CD44.

A parallel study of integrin expression in the PMC42-ET breast cancer cell line induced to undergo EMT with EGF revealed that ITGA2 and ITGB1, and their downstream regulator ILK, were consistently upregulated (Supplementary Fig. 1). Hence ILK and these integrins were examined further in the PDX models. Increases in ITGA2 (p7 significantly higher than p3) and ITGB1 (p6 significantly higher than p3) were observed in ED03 xenograft material, which were maintained (Fig. 3). However, similar to the increased vimentin seen with each passage in EDW01, the levels of human ITGB1 mRNA in the xenografts increased with successive passage, demonstrating an approximately 26-fold increase ($p = 0.026$) at passage 6 in comparison to passage 3 (Fig. 3). Whilst passage 7 showed approximately a 38-fold increase in ITGB1 mRNA levels when compared to passage 3, this did not reach statistical significance. However, ITGA2 mRNA expression in EDW01 xenografts (Fig. 3) was significantly upregulated by approximately 33-fold at passage 7 material when compared to passage 3 ($p = 0.024$). ILK is activated by integrins including $\alpha 2\beta 1$, and mediates a number of signalling responses in relation to survival and proliferation in addition to induction of EMT [62]. A trend was observed towards upregulation of ILK mRNA expression in both ED03 and EDW01 xenografts (Fig. 3).

Murine (stromal) ITGB1 displayed a similar pattern of upregulation as human (tumoural) ITGB1 (Fig. 3).

These findings illustrate that with serial passage EDW01 has accrued features consistent with EMT. The co-induction of the mRNA levels of ITGB1 and ITGA2 in EDW01 implies that they may be important for the EMT process and/or phenotype, because they track with the indices of EMT (decreased E-cadherin and increased vimentin) clearly observed in this model system.

E-cadherin expressing xenograft tumours exhibit more necrosis

Cores of tumours from the EDW01 series at passage 3 exhibited noticeably more necrosis than any other passage from this line, whereas necrosis was minimal or absent in all PDXs across the various passages in the ED03 line (data not shown). We have previously demonstrated that E-cadherin expression is associated with high proliferative rate and observed an association with E-cadherin expression and the appearance of necrotic tissue in actively growing xenografts [18]. We investigated whether an association existed between E-cadherin expression in the EDW01 line and the proportion of necrosis. As shown in Fig. 4, a trend was observed such that in the cores that had detectable necrosis, E-cadherin was generally expressed at a high level, and more highly expressed than vimentin.

Further investigation of EMT drivers and markers in the EDW01 xenograft model

Hypoxia is a common driver of EMT in breast cancer, and E-cadherin repressor genes have been implicated in this process [63]. We sought to examine the pattern of Snail1, Snail2, Twist11 and Zeb1/2 expression through the serial passages in mice in the ED03 and EDW01 xenograft models, in comparison to the hypoxic indicator gene carbonic anhydrase 9 (CAIX).

As shown in Fig. 5, of the E-cadherin repressor genes examined (Snail1, Snail2, Twist11, Zeb1 and Zeb2), Twist1 was more highly expressed in the EDW01 xenograft compared with ED03 (Fig. 5A, i). Zeb1 and Zeb2 were not expressed at detectable levels in either PDX. Twist1 displayed the highest correlation with CAIX in the EDW01 xenograft model, with both exhibiting a progressive increase from p2 to p5 which was then reduced in p6 to p7 (Fig. 5A, i). Membrane intensity of CAIX (Fig. 5C) aligned with the expression data, suggesting Twist-1 may drive hypoxia-induced EMT through consecutive passages of the EDW-01 PDX.

CD24 is an epithelial-associated marker with relevance to breast cancer stem cells, where its expression is reduced in comparison to luminal breast cancer cells [19]; its expression has been shown to indirectly stimulate cell adhesion to fibronectin, collagens I and IV, and laminin through the activation of integrin activity [64]. Interestingly, the expression pattern of CD24 with ITGB1 was significantly positively correlated in the ED03 series ($R^2 = 0.9$, $p = 0.0012$) and in the EDW01 series ($R^2 = 0.96$, $p = 0.023$). CD24 was also positively correlated with ITGA2 in the ED03 series ($R^2 = 0.84$, $p = 0.0035$) and this reached near significance in the EDW01 series ($R^2 = 0.76$, $p = 0.054$) (Fig. 5C). No significant or near significant correlations were observed for CD44 with integrins in either PDX systems (Fig. 5B, iii).

Functional assessment of candidate genes ITGB1, ITGA2 and ILK in the PMC42 system

As expression of the $\alpha 2\beta 1$ integrin components were associated with the EMT observed in EDW01 xenografts over serial passages through mice, we tested whether they could be a “driver” of the EMT, using the PMC42 EMT model system.

Although already somewhat mesenchymal [27], PMC42-ET cells treated with EGF in vitro undergo a further EMT in which ILK, ITGB1 and ITGA2 are upregulated (supplementary Fig. 1), [63]. We examined the effects of siRNA knockdown of ITGA2, ITGB1 and ILK on late stage mesenchymal gene expression, cell adhesion and cell migration.

As shown in Fig. 6A, individual siRNA knockdown of ITGB1, ITGA2 or ILK resulted in the expected reduction of expression of the target genes. In addition, ILK inhibition also led to the reduction of ITGB1 protein levels by 80% (0.2 in Fig. 6A); inhibition of ITGB1 also led to reduced ITGA2 expression (0.06 in Fig. 6A)); and, ILK inhibition led to a reduction in ITGA2 expression by 60% (0.41 in Fig. 6A). Inhibition of ITGB1 and ITGA2 by siRNA did not affect ILK protein levels. These data indicate a complex interplay between these three components

To determine whether the cells with suppressed ITGB1, ITGA2 or ILK were able to undergo EMT with EGF treatments, the “classical” indicators of EMT, vimentin, E-cadherin, and N-cadherin were measured by Western immunoblotting (Fig. 6B). E-cadherin was dramatically reduced in the “cells alone” treated with EGF compared to the untreated “cells alone” control, whereas slight increases in vimentin and N-cadherin were observed in these untransfected cells, consistent with EGF-induced EMT of these cells as previously

reported [27, 63]. After 72 hours of EGF treatment, cellular morphology (Fig. 6C) revealed a clear acquisition of spindle-shapes and breaking apart of cellular islands, consistent with an EMT.

No observable differences in the responses of vimentin, N-cadherin or E-cadherin protein levels to 72hrs of EGF treatment were seen following treatment with ITGB1, ITGA2 or ILK siRNA, when compared to treatment with control siRNA (Fig. 6B). Similarly, the mesenchymal morphology caused by EGF treatment was not abrogated by any of the siRNAs (Fig. 6C). Together, this indicates that these candidates do not directly mediate the EMT induced by EGF in this breast cancer cell line.

We then investigated what effect the stepwise increase in integrin expression, observed in the EDW01 xenografts (Fig. 3) may have had on growth and invasion/motility of these tumours over serial passages in mice, inferred by parallel analyses in the PMC42-ET cells. We focused on cell adhesion to various substrates and migration, properties known to be mediated by integrins and ILK, and these were again examined in PMC42-ET cells.

PMC42-ET cells require ITGB1, ITGA2 and ILK for maximal adherence to collagen I, collagen IV, laminin and fibronectin substrates, as knockdown of these molecules significantly reduced adhesion in comparison to control siRNA (Fig. 7A-D, $p < 0.05$). When stimulated with EGF, the attachment of the PMC42-ET cells treated with ITGB1, ITGA2, and ILK siRNA was also significantly abrogated ($p < 0.001$).

Coordinated regulation of cell adhesion and adhesion complex remodelling are crucial for cell movement. ITGB1, ITGA2, and ILK silencing in PMC42-ET cells caused them to be significantly less migratory (Fig. 7E, $p < 0.001$). EGF treatment caused these cells to increase their migration. ITGB1, ITGA2, and ILK silencing each significantly reduced migration under EGF-stimulated conditions ($p < 0.001$).

Discussion

We have shown that the EDW01 PDX model displayed evidence of EMT with progressive passages through mice, which was not seen in ED03. This partial EMT was associated with a rising hypoxia leading to Twist1 expression in early-mid passages, repressing E-cadherin expression and orchestrating vimentin upregulation, and accompanied by upregulation of ITGB1 and ITGA2 expression. The mesenchymal shift appeared to then return to the epithelial direction in later passages of EDW01, however the increased integrin expression persisted. We present cell line data to support the association of integrin $\alpha 2\beta 1$ and the ILK signalling pathway with the observed EMT, but that the EMT was not mediated by these.

Although the EMT-associated ITGB1, ITGA2, and ILK are not essential for EGF-induced EMT in PMC42ET cells (Fig. 6) they are necessary for breast cancer cell adhesion to ECM-substrates (Fig. 7A-D) and cellular movement (Fig. 7E). These molecules were more significantly upregulated in increasing EDW01 passages through mice than ED03 (Fig. 3), thus they may have enabled ECM adhesion in this PDX. Indeed, previous studies demonstrate that the $\alpha 2\beta 1$ integrin is primarily a receptor for collagen and laminin [65] and expression is also associated with motility, invasiveness, and cellular differentiation of a variety of

tumours [66, 67]. This is in contrast to studies in which ITGA2 and ITGB1 have been found to suppress metastasis in models of mouse and human cancer [68]. However, Dedhar and Saulnier [69] showed that the expression of $\alpha 2\beta 1$ integrin increased in the chemically transformed human osteosarcoma cells, and this integrin was implicated in tumour progression and metastasis. Similarly, $\alpha 2\beta 1$ integrin expression accelerated either experimental metastasis or tumour dissemination of melanoma [70] and rhabdomyosarcoma [71, 72], gastric cancer [73, 74] and colon cancer cells [75]. Taken together, our data suggest that $\alpha 2\beta 1$ integrins contribute to the EMT phenotype observed increasingly in EDW01 over serial passages in mice.

Actively growing tumours acquire areas of hypoxia, a product of imperfect angiogenesis coupled with rapid growth, which can facilitate cellular invasion via the induction of EMT [76]. Vimentin and Twist1 positivity was observed in close proximity to necrotic areas in early passages and less commonly found at the centre of tumour 'islands' (Fig. 2). Of the E-cadherin repressor genes examined, Twist1, a target of HIF-1 α [77–79] displayed the strongest correlative pattern of induction with CAIX (Fig. 5B, $R^2 = 0.81$, $p = 0.04$). Induction of Twist1 coincided with the repression of E-cadherin and induction of vimentin mRNA, and therefore may be the instigator of the observed EMT in the EDW01 xenograft model. Indeed, hypoxia has been implicated in inducing EMT-related genes in another PDX model of serial transplantation. Wegner and colleagues [80] demonstrate in their cervical cancer PDX model serially transplanted in mice, that the EMT orchestrating gene Snail1 and stem cell markers were found to be increased in late compared to early passages along with hypoxic CAIX gene expression, accompanied with an increase in tumour aggressiveness and proliferative rate. Their finding, in a different cancer type (cervical) adds further support to our supposition that hypoxia was a major driving force in the observed progressive EMT in the EDW01 PDX model.

However, why did the mesenchymal shift return to epithelial in later passages of EDW01? Tumours in vivo to have been found to adapt to low oxygen environments, such as reprogramming Akt signalling in the mitochondria [81]. This coupled with the well-known ability of tumours to increase angiogenesis [82] contributes to tumour cell survival and progression. Although beyond the scope of this investigation, the EDW01 PDX model provides a means to investigate these phenomenon further, with relevance to understanding the progression of breast cancer in women.

We have previously shown that MDA-MB-468 xenografts, which express E-cadherin, exhibit hypoxia-related necrosis and subsequent EMT, features of which were lost upon E-cadherin knockdown [18]. In the current study we also observed necrosis at p4 in EDW01 but not ED03, along with the hypoxic indicator gene CAIX induction (Fig. 5A, C). IDC, the classification of EDW01 PDX, generally proliferate at a higher rate than ILC [83], of which ED03 is classified. In our previous study, E-cadherin knockdown MDA-MD-468 xenografts grew slower than their control counterparts [18]. In contrast, EDW01 (IDC) and ED03 (ILC) displayed similar Ki67 expression (Fig. 4C) and we did not find significant differences in implantation to harvest time durations between ED03 and EDW01 PDXs (data not shown). However, changes in daily growth rate cannot be ruled out between EDW01 and ED03, as apoptosis was not monitored. Regardless,

the results from the current study reinforce a direct connection between E-cadherin tumoural expression and the appearance of hypoxia.

Although many studies have associated EMT with therapy resistance [11, 84], it is important to note that the EDW01 xenograft was not challenged by therapy; the EMT progression was spontaneous. Interestingly, considerable emphasis is being placed recently on the hybrid state of EMP recently [6, 85–88], and this phenotype appears to manifest in the EDW01 xenografts (elevated vimentin and apparently only partially lost E-cadherin - Fig. 3, 4). A separate analysis of circulating tumour cells (CTCs) in the ED03 model indicate that despite the lack of any evidence of EMT in the primary xenograft tumours, the CTCs are enriched in mesenchymal gene expression, but also in epithelial genes (E-cadherin and CD24), compared to the primary tumour, indicating a dysregulation of this axis and/or possibility of hybrid cells [58].

Although the PDX models examined had varied ER α positivity in early passages, this increased in both models over serial transplantation in mice (Fig. 1). Mice used in this study for serial xenograft propagation were not administered estradiol, which is often used to foster the growth of ER α -positive xenografts, which generally have a lower engraftment rate than ER α negative tumours [89]. ER α positivity is likely to have been maintained/increased because estradiol was not administered, as ER α undergoes ligand-dependent downregulation [90]. Indeed, removal of estrogen for several weeks in PDXs has been found to increase ER α levels [89].

Tumour-stroma crosstalk plays an integral role in EMT in vivo [91], similarly, we observed key changes in murine stroma in the PDX models examined in this study. We have previously demonstrated that EDW01 evoked greater expression of MMPs (-2, -9, -11 and MT1-MMP) in the murine stroma than ED03 [56]. Furthermore, EDW01 displayed MT1-MMP and MMP-13 at the tumour-stromal boundary, but did not express these factors or MMP-2 and -9 within the tumour mass itself. As shown in the current study, the EDW01 tumours grew as islands traversed by thick collagenous stromal bands whereas the ED03 had delicate pericellular stroma dispersed throughout (Fig. 1B). This pattern of growth may be directly attributable to the pattern of MMP expression of each of these PDX models – EDW01 lacked the capacity to invade as individual cells, possibly due to the lack of induction of intratumoral MMP-2 and -9. Furthermore, the murine microenvironment (non-orthotopic) in which the EMT occurred in the EDW01 PDX over successive passages may have been conducive to this change. We found that murine (stromal) ITGB1 expression aligned with human (tumoural) expression of the same integrin, in fact for EDW01, stromal ITGB1 expression was approximately 22 fold higher than tumoural ITGB1 at passage 7 (Fig. 3). This leads to speculation as to whether the murine microenvironment was the instigator of the EMT or a responder in this process. However, given that an EMT was not observed in the ED03 line, which was passaged through mice of the same genotype (SCID), it could be postulated that drivers of EMT came from within the tumour itself, lending further support to the notion that hypoxia was an initiating event.

Decreased CD44/CD24 expression ratio in later passages in both PDX lines was an unexpected finding, at least in EDW01, as CD24^{+/high}/CD44^{low-} phenotype is associated with the epithelial phenotype despite

EMT being observed in this xenograft model. However, CD24 expression can also confer adhesive properties enabling invasion. In a meta-analysis of 16 studies of 5,697 breast cancers, CD24 was found to be significantly associated with poorer survival [92], presumably due to non EMP functions. In studies on breast cancer cell lines in vivo, CD24 was found to act as a ligand for P-selectin on the lung vascular endothelium [64]. We found that CD24, but not CD44, correlated with ITGA2 and ITGB1 in both PDX models (Fig. 5C versus 5D), providing further evidence in addition to our PMC42-ET integrin knockdown/EGF studies (Fig. 7B, C) that activation of these integrins is not necessarily intimately related to the EMT process.

Conclusion

This study illustrates a cumulative EMT in a PDX system through several passages in mice, an effect characterized by, but not orchestrated by, ILK signalling via ITGA2 and ITGB1, and possibly ILK.

Declarations

Ethics approval and consent to participate

All human tissue samples used in this study and associated approval and consent to participate was obtained in accordance with the standards of the St. Vincent's Hospital Human Ethics Committee.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing Interests

The authors declare that they have no competing interests.

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Author's contributions

RW generated 50% of the real-time PCR data and all siRNA work including associated westerns. EDW performed EDW01 PDX generation initiation and harvest and maintained records. EdeS performed all ED03 generation initiation and harvest, maintained records. PTS and JRK performed P120, Ki67 and beta-catenin IHC and PTS provided direct guidance in scoring. AER performed ED03 CDH1 mutation analyses. MW assisted in ED03 and EDW01 generation initiation and harvest. CES assisted in hormone receptor IHC interpretation and PDX morphology analysis. TB assisted in all statistical analysis. EWT and HJH provided intellectual input as to the direction of the work and HJH performed 50% real-time data, remaining IHC, and drafted the manuscript. All authors reviewed and edited the manuscript.

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Supplementary Figure

Supplementary figure 1. EGF treatment (10 ng/ml, 72 h) of PMC42-ET breast cancer cells resulted in an EMT which featured upregulation of ITGB1, ITGA2 and ILK. A. Phase contrast morphology, B. gene expression changes as assessed by RT-qPCR. Results are from one experiment, representative of two independent experiments. Error bars are standard deviation of n=3 technical replicates within 1 biological replicate (1 experiment). C. Western blotting for vimentin, E-cadherin, and actin across the EGF time course. Scale bar, 100 μ m.

Figures

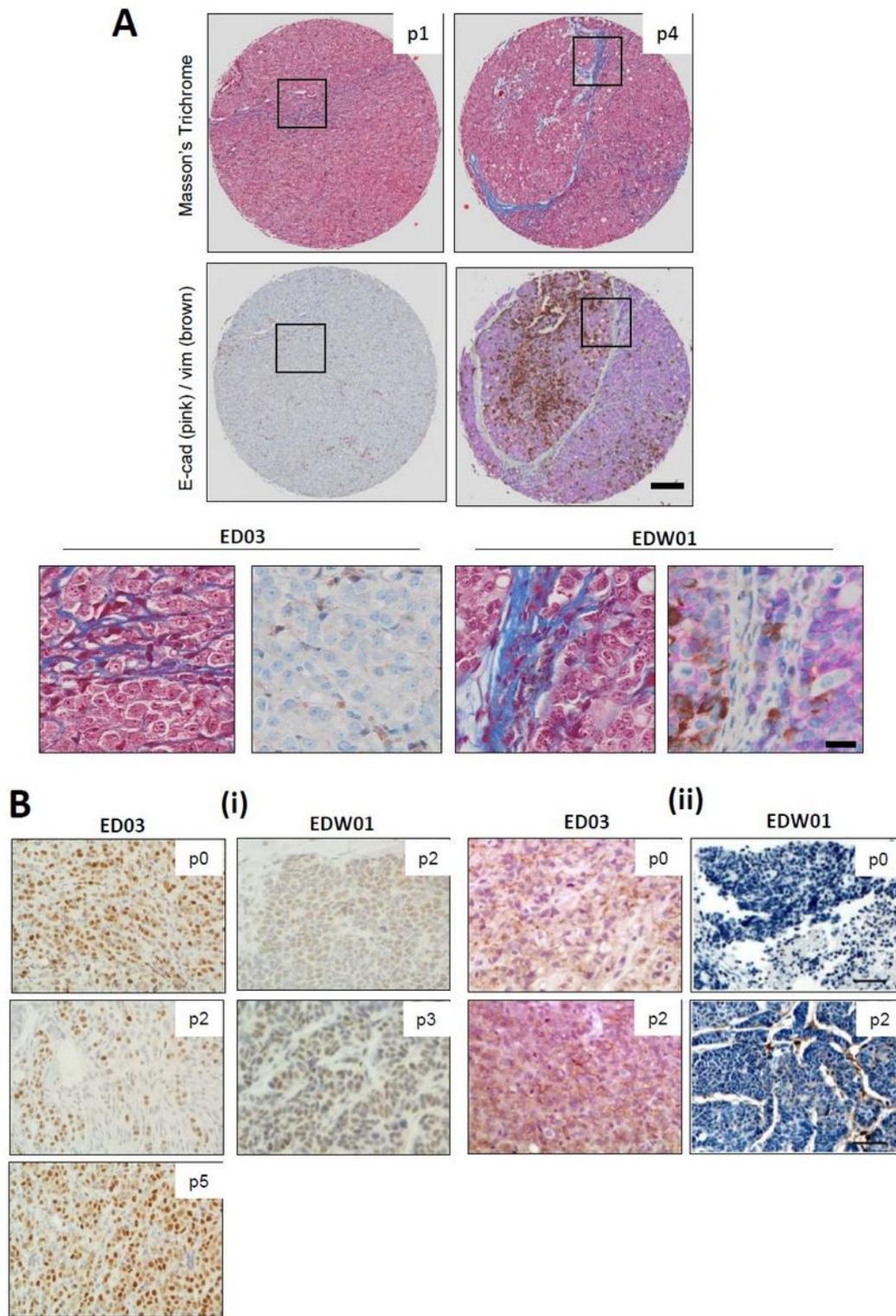


Figure 1

A. Masson's trichrome and E-cadherin (E-cad)/vimentin (vim) immunohistochemistry performed on serial section from ED03 (LHS; passage 1 (p1)), and EDW01 (RHS; passage 4 (p4)) PDX models. Boxed areas shown as higher magnification images displayed below images of whole cores. Scale bar upper panels, 200 μ m; lower panels 20 μ m. B. Immunohistochemical analysis (brown) of original tumour specimens

(passage 0 (p0)) and after passaging in mice (passage 2 (p2), passage 3 (p3), passage 5 (p5)) for (i) ER α and (ii) Her-2. Nuclei counterstained with hematoxylin. Scale bar, 100 μ m.

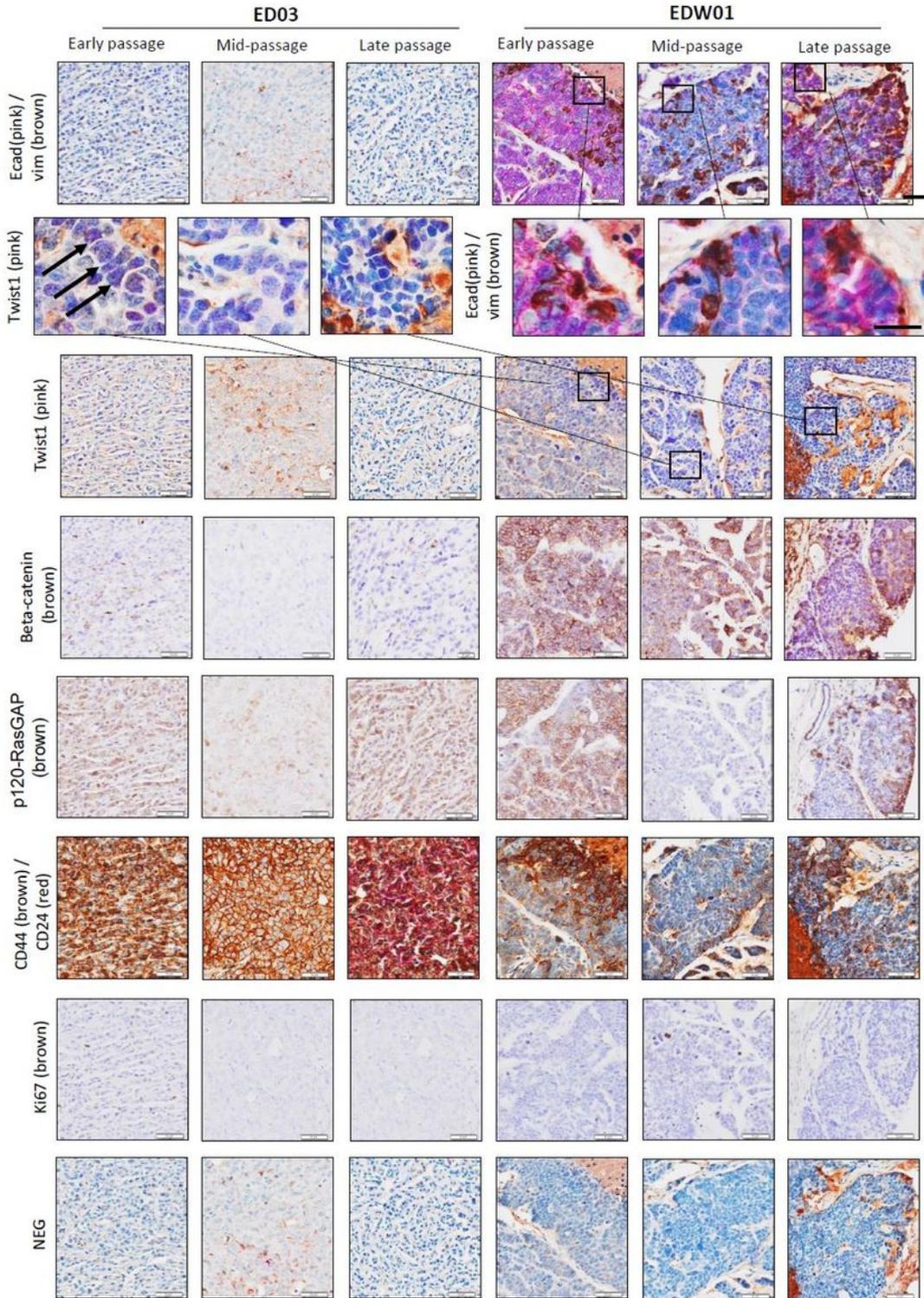


Figure 2

Epithelial and mesenchymal marker analysis of the ED03 & EDW01 PDX tumours over serial passages (ED03 early: passage 3, mid: passage 7, late: passage 11; EDW01 early: passage 3, mid: passage 5, late: passage 7). Nuclei counterstained with hematoxylin. NEG: negative control (Concentration and isotype

matched IgG substituted for primary antibody) Arrows indicate TWIST1 positive nuclei. Scale bar, 50 μ m. Row 2 selected higher magnification areas: scale bar, 20 μ m.

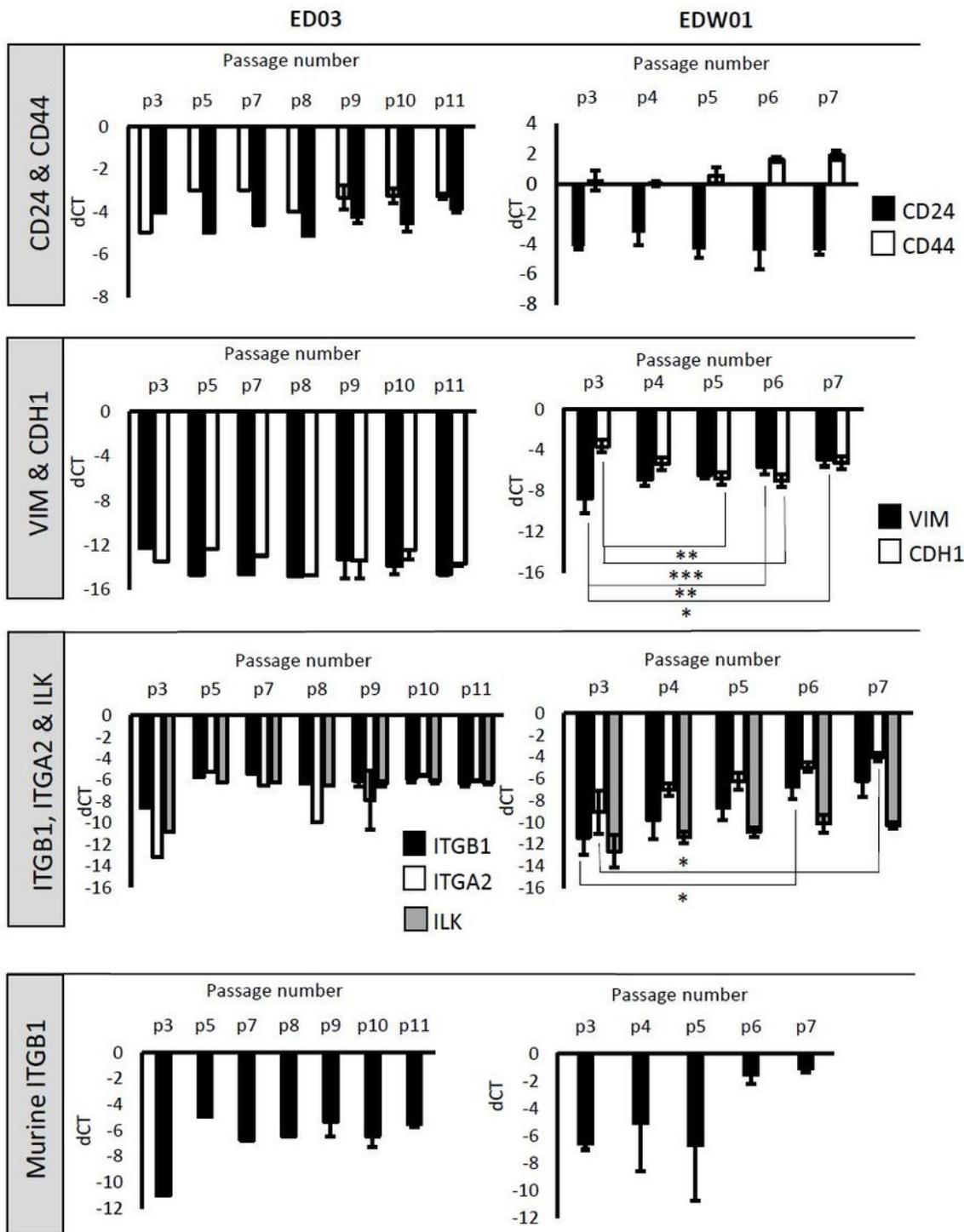


Figure 3

Gene expression (RT-qPCR) for the ED03 and EDW01 PDX models. Mean of individual xenograft tumours are shown, where $n \geq 3$ error bars are shown (standard error of the mean). For ED03: p3 $n=1$, p5 $n=1$, p7 $n=2$, p8 $n=1$, p9 $n=3$, p10 $n=3$, p11 $n=3$. For EDW01: vimentin/CDH1 and for ITGAB1/A2/ILK: p3 $n=6$, p4

n=11, p5 n=6, p6 n=11, p7 n=3; for CD24/CD44 and murine ITGB1 all passages were n=3. Statistical significance was calculated using two-tailed Mann-Whitney test (Non-parametric). * p<0.05, ** p<0.01, *** p<0.001.

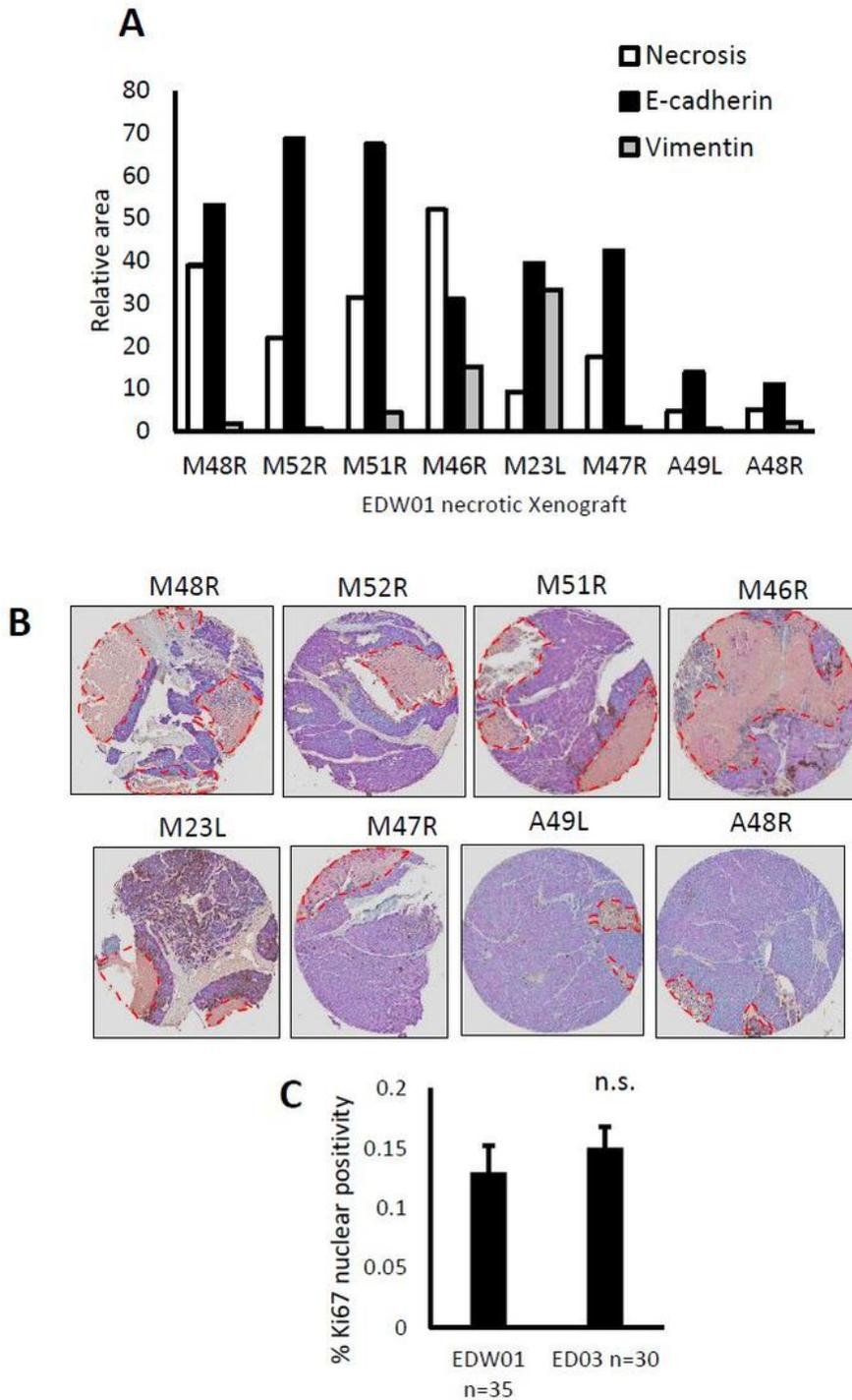


Figure 4

E-cadherin protein expression associated with necrosis in passage 3 EDW01 tumour cores. A. Average area of necrotic area (white bars), E-cadherin positivity (black bars) and vimentin positivity (grey bars) for

each individual tumour core shown in B.. B. Images of the tumour cores, matching data shown in A, dual stained with E-cadherin (pink) and vimentin (brown) with a hematoxylin (blue) nuclear counterstain. Necrotic area indicated by red dotted line. C. Proliferation, measured by Ki67 immunohistochemical nuclear positivity, was similar between the ED03 and EDW01 PDX systems. n.s., not significant (Ki67 intensity in cores from EDW01 vs ED03), as determined by Student's paired t test.

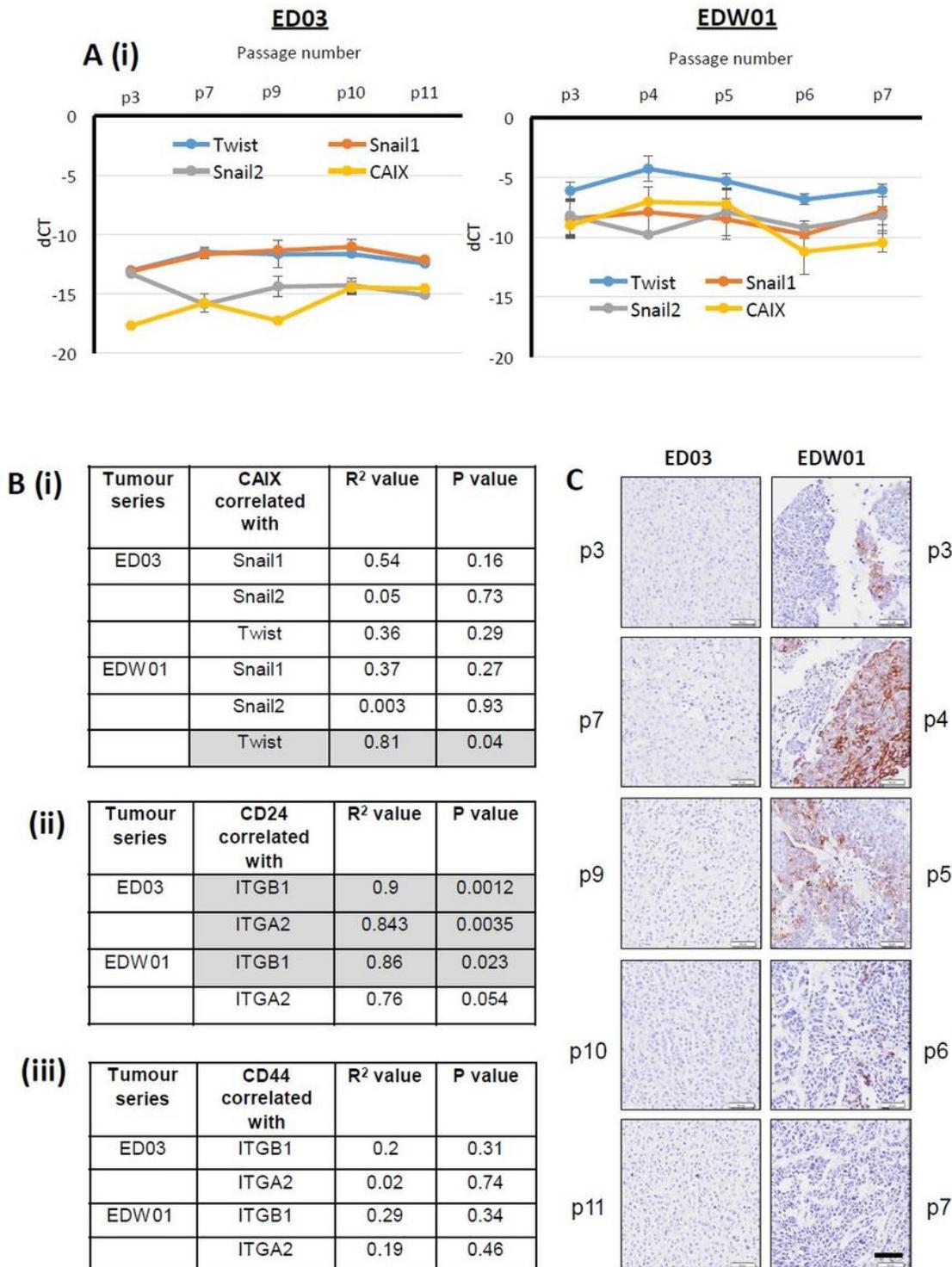


Figure 5

A. Quantitative, real time RT-PCR gene expression of various E-cadherin transcriptional repressor genes (TWIST1, SNAI1, SNAI2) and correlation with expression of the hypoxic indicator gene Carbonic Anhydrase 9 (CAIX) in RNA extracted from tumours in the ED03 and EDW01 PDX models across serial passages. Mean expression values shown, error bars are standard error from the mean; B (i) Pearson correlation statistics of data shown in A.; Pearson correlation statistics of ITGB1 and ITGA2 correlated with (ii) CD24 or (iii) CD44 in the PDX models. C. Carbonic Anhydrase 9 immunohistochemistry in the ED03 and EDW01 PDX models with passage (p) numbers shown in the vertical plane. p-value shown is two-tailed, and $p < 0.05$ defined as statistically significant and shaded grey in B. Scale bar, 50 μm .

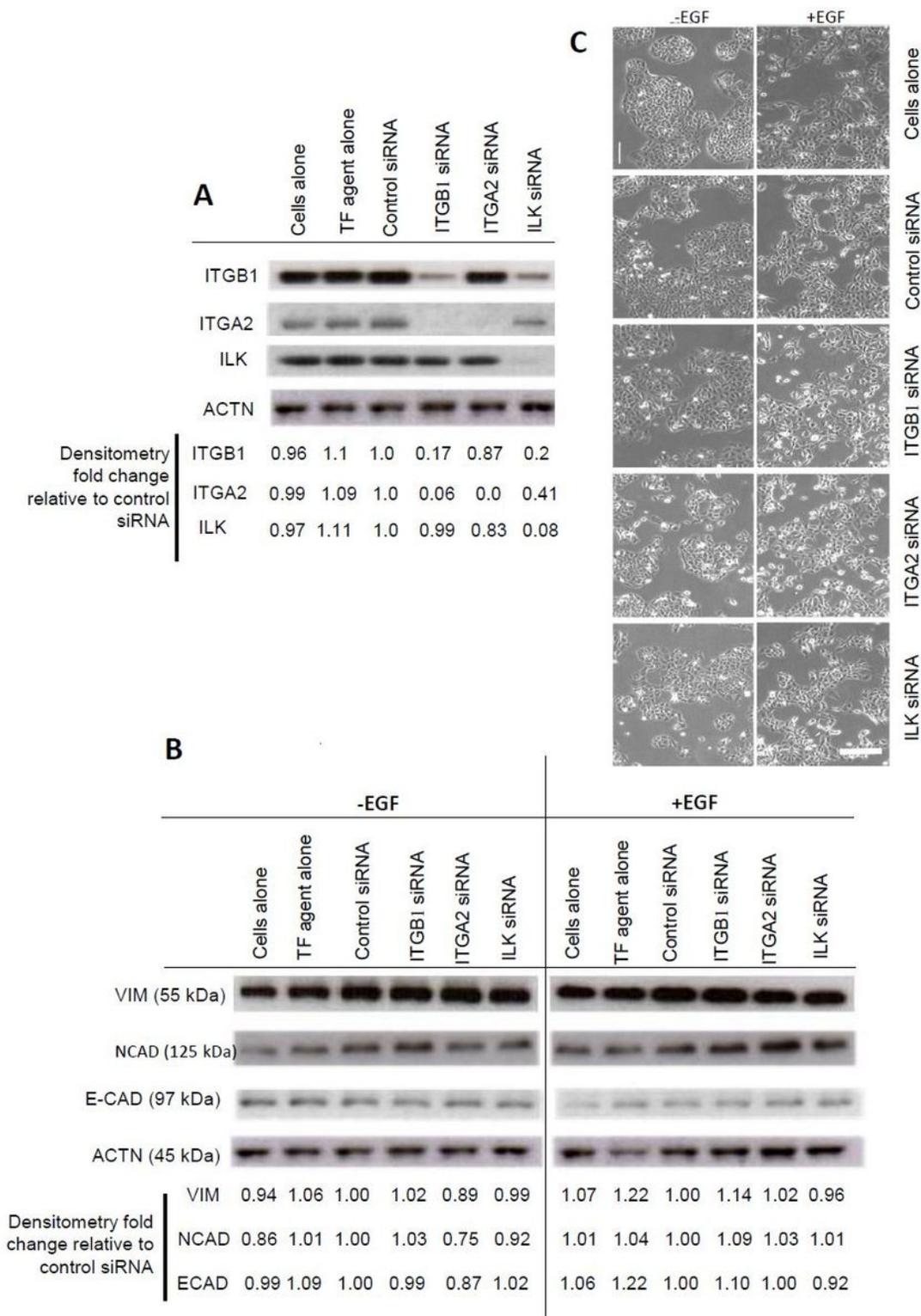


Figure 6

A. Western blots showing ITGB1 (130 kDa), ITGA2 (160 kDa), and ILK (50 kDa) versus pan-actin (ACTN, 45 kDa) protein expression of siRNA transfected PMC42-ET cells; siRNA knockdown did not affect EMT of PMC42ET cells in response to 10 ng/ml EGF treatment (+EGF) after 72 hr as shown by B. western blotting for vimentin, (53 kDa), NCAD (100 kDa) and E-cadherin (120 kDa); and C. phase contrast cellular morphology. Scale bar, 100 μ m. TF: Transfection.

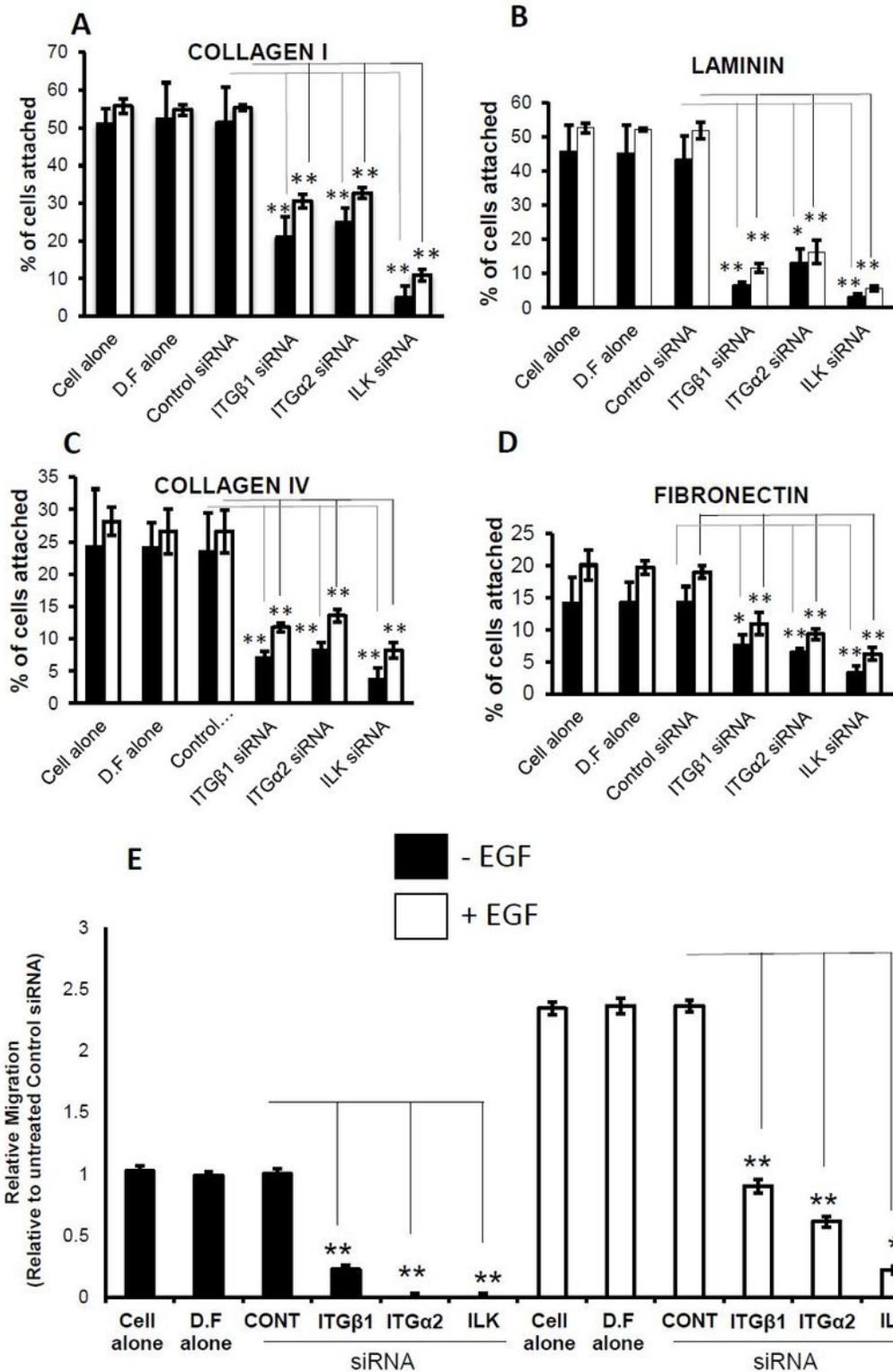


Figure 7

Inhibition of PMc42-ET breast cancer cell adhesion to A. collagen I, B. collagen IV, C. laminin, and D. fibronectin, by integrin subunit-specific and ILK siRNAs. Results are expressed as % of cells adhered and represent the mean \pm SD from 4 biological replicates. Results were analysed using two way ANOVA, with Dunnett's multiple comparison test, with P values adjusted for multiple comparisons; * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0001$.

Supplementary Files

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- [Supplementaryfigure1.pdf](#)