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Research paper

The requirement of integrins for breast epithelial proliferation

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ABSTRACT

Epithelial cells forming mammary gland ducts and alveoli require adhesion to the extracellular matrix for their function. Mammary epithelial cells need β1-integrins for normal cell cycle regulation. However, the role of β1-integrins in tumorigenesis has not been fully resolved. β1-integrin is necessary for tumour formation in transgenic mice expressing the Polyomavirus Middle T antigen, but it is dispensable in those overexpressing ErbB2. This suggests that some oncogenes can manage without β1-integrin to proliferate and form tumours, while others still require it. Here we have developed a model to test whether expression of an oncogene can surpass the need for β1-integrin to drive proliferation. We co-expressed the ErbB2 or Akt oncogenes with shRNA to target β1-integrin in mammary epithelial cells, and found that they show a differential dependence on β1-integrin for cell division. Moreover, we identified a key proliferative role of the Rac1-Pak axis downstream of β1-integrin signalling. Our data suggest that, in mammary epithelial cells, oncogenes with the ability to signal to Pak surpass the requirement of integrins for malignant transformation. This highlights the importance of using the correct combination therapy for breast cancer, depending on the oncogenes expressed in the tumour.

1. Introduction

Integrins are adhesion receptors that control several functions in mammary epithelium, such as differentiation, polarity and proliferation (Glukhova and Streuli, 2013). Of the integrin subunits expressed by mammary epithelial cells (MECs), the β1-subunit plays a key role during G1/S transition (Li et al., 2005; Jeanes et al., 2012). Previous studies on the role of β1-integrin in MEC proliferation have used alveolar tissue or cells isolated from pregnant mice. However, integrin-dependent pathways controlling proliferation in cells from virgin mice (ductal MECs) remain understudied. Since increased cell cycle is one of the hallmarks of cancer and most breast tumours are not pregnancy-related, it is important to understand the signalling pathways regulating proliferation in ductal MECs.

Integrin expression is altered in breast cancer. During early stages, the β1-integrin subunit is downregulated in human breast tumours (Zutter et al., 1990, 1993). Conversely in invasive breast cancers, β1-integrin is often overexpressed, and this correlates with a poor prognosis (dos Santos et al., 2012). Studies investigating whether β1-integrins are required for tumorigenesis have shown contradictory results. For example, β1-integrin is required for tumour formation in transgenic mice carrying the Polyomavirus middle T promoter (White et al., 2004). However, in a β1-integrin-null mouse strain in which activated ErbB2 is expressed, the lack of β1-integrin only delayed the formation of tumours, suggesting that β1-integrin is not necessary for cancer initiation (Huck et al., 2010). Additionally, the metastatic properties of the tumours were decreased in β1-integrin-null mice. β1-integrin may therefore be important during metastasis, but not during initial tumour formation. Furthermore, some oncogenes may overcome the need for β1-integrin to progress in the cell cycle during tumorigenesis, but it is not known which oncogenes can do this.

The fact that some oncogenes such as ErbB2 easily overcome the need for this integrin, while others (such as Polyomavirus Middle T antigen) require it, calls for a model system where the mechanistic details of these interactions can be elucidated. In this study, we have established a robust method to study both β1-integrin-dependent and independent (oncogene-driven) proliferative pathways in MECs.

Abbreviation: MECs, mammary epithelial cells.

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In normal mammary epithelium, ErbB2 regulates ductal elongation and branching during puberty (Hynes and Lane, 2005). ErbB2 has no known ligand, requires to be transactivated by another ErbB receptor, and is overexpressed in 20% of breast cancers (Slamon, 1987). This leads to the receptor no longer requiring ligand (when bound to another ErbB receptor) or receptor dimerization for activation (Penuel et al., 2002). Although $\alpha6\beta4$ integrin contributes to ErbB2-induced tumorigenesis, $\beta1$-integrin seems to be dispensable for tumorigenesis in the mammary epithelium (Guo et al., 2006; Huck et al., 2010).

The serine/threonine kinase Akt is the central effector of PI3K signalling (Viglietto et al., 2002; Zhang et al., 2011). In breast cancer, Akt is often activated due to upstream signal deregulation. Moreover, there can be somatic mutations in Akt, for example a lysine substitution at E17 occurs in 6% of breast tumours (Carpten et al., 2007; Kim et al., 2008). Other activating mutations in the pH domain of Akt (L52R, Q79K) and one in the kinase domain (D323H) are present in 4% of breast cancers (Parikh et al., 2012).

Here, we have studied the signalling pathways that are disrupted when $\beta1$-integrin is depleted in ductal MECs isolated from nulliparous mice (Kittrell et al., 1992). We found that ductal MECs require Rac1 and Pak signalling for normal proliferation. Furthermore, we used this model to test the dependence of oncogenic ErbB2 and Akt on $\beta1$-integrin to drive proliferation. We show that ErbB2, but not Akt, can surpass the need for $\beta1$-integrin to promote MEC proliferation. Our data also suggest that Akt is not required for ErbB2-mediated transformation, either in the presence or absence of $\beta1$-integrins. Thus oncogenes with the ability to signal to Pak are able to override the integrin requirement for MEC proliferation. With this work, we have established a simple model to study the dependence of oncogenes on $\beta1$-integrin to drive proliferation in ductal MECs. s

2. Materials and methods

2.1. DNA constructs

The shRNAmiR sequence for mouse $\beta1$-integrin and the high cycling L61-Rac1 were cloned in the lentivector plV-Venus (Tronolab, EPFL) and have been previously described (Jeanes et al., 2012). The plasmid pCDNA3-GFP-$\beta1$-integrin was a kind gift from Prof Bernard Wehrle-Haller (University of Geneva). To construct the human $\beta1$-integrin rescue vector, four silent point mutations were introduced in pCDNA3-$\beta1$-integrin using the following primer 5′-GAAAATCCCAGAGGGTCCAAGGATATCAAGAAGAATAAAAATGTAACC-3′ and the QuikChange Lightning Mutagenesis kit (Agilent, Cat. No. 210515-5). The plasmid was digested with Not I and Nde I, and the resulting fragment was ligated in plV-sh$\beta1$itg (previously modified to remove GFP). The EF1α promoter was replaced with the Ubiquitin promoter in order to ensure a low expression level of GFP-$\beta1$-integrin. All Pak constructs were kindly provided by Gary Bokoch (La Jolla, California).

2.2. Ethics statement

Mice were housed and maintained according to the University of Manchester and UK Home Office guidelines for animal research. Animals were bred under Home Office Project Licence 40/3155, and approved by the University of Manchester ethical review process. Experiments were conducted in accordance with the S1 killing of the Animals Scientific Procedures Act 1986.

2.3. Cell culture and transfection

Low passage FSK7 MECs (Kittrell et al., 1992) were cultured in DMEM/F-12 medium (BioWhittaker; Lonza) supplemented with 2% FBS, 5 μg/ml insulin and 10 ng/ml EGF at 37 °C in a humidified atmosphere of 5% CO_2. FSK7 MECs were transfected for 3 h by using Lipofectamine® and Plus™ Reagent (Life Technologies). After transfection, cells were cultured for 72 h before being replated at 10^5 cells/cm^2 density on glass coverslips prior to EdU labelling, fixing and staining. HEK293T cells (ATCC) were cultured in DMEM with Ultra-glutamine (Lonza) containing 10% FBS (Biosera) and 100 U/ml Penicillin/streptomycin. These cells were transfected with JetPEI™ reagent (Sigma-Aldrich). Primary MECs were isolated from 8 to 10 week old nulliparous ICR mice and grown on collagen-I coated coverslips as previously described (Pullan et al., 1996). Primary MECs were cultured in growth medium containing 5 μg/ml insulin, 1 μg/ml hydrocortisone, 3 ng/ml EGF, 10% FCS, 50 U/ml penicillin/streptomycin, 0.25 μg/ml fungizone, and 50 μg/ml gentamycin in F12 medium.

2.4. Immunofluorescence staining

Cells were fixed with 4% formaldehyde for ten minutes. After fixation, cells were permeabilised using 0.2% Triton X-100 and blocked with 10% goat serum. Cells were sequentially incubated with primary and secondary antibodies for one hour each at room temperature, protected from light. Primary antibodies used for immunostaining were: $\beta1$-integrin (MAB1997 Chemicon), $\beta1$-integrin clone 12G10 (Abcam), GFP (Life Technologies), and Paxillin (BD Transduction) and Histone3 pS10 (Millipore). Secondary antibodies used were Alexa 488 anti-mouse IgG and Alexa 647 anti-rabbit IgG (Molecular Probes), Rhodamine Red-X-AffiniPure Anti-Rat IgG and Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, Inc.). A solution of 4 μg/ml of DAPI diluted in PBS was used to stain the nuclear content. Coverslips were mounted onto glass slides with Dako Fluorescent Mounting medium. Cells were observed with a fluorescence microscope (Zeiss Axio Imager MZ HXP120C) and pictures were taken with a Hamamatsu ORCA-ER camera.

2.5. EdU proliferation assay

Cells grown on coverslips were incubated with 10 μM 5-ethynyl-2′-deoxyuridine (EdU) for 6 h (for primary MECs) or 30 min (for FSK7). Incorporated EdU was detected by incubating the cells with an Alexa Flour 647-azide that binds to EdU in the labelled cells, using the Click-iT™ EdU Alexa Fluor® 647 Imaging Kit (Invitrogen #C10340). This incubation was followed by immunostaining and DAPI counterstaining. The percentage of EdU-positive cells was calculated as a percentage of the total DAPI stained nuclei. For primary MECs, an average of 2000 cells was counted per experiment. For FSK7, a minimum of 100 transfected cells (expressing the fluorescent reporter gene) was counted per experiment. This number was compared to the percentage of non-transfected cells from the same coverslip. Statistical significance was determined by student's *t*-test. Error bars represent standard error of the mean (SEM).

2.6. Immunoblotting

Equivalent amounts of protein were resolved by SDS-PAGE and immunoblotted using the following antibodies: Akt pS473, NICD and myc tag (Cell Signaling), β-tubulin (Sigma), $\beta1$-integrin (BD Transduction), V5 (AbD Serotec), GFP (Molecular Probes). This was followed by incubation with peroxidase-conjugated anti–rabbit or anti–mouse IgG (Jackson ImmunoResearch Laboratories, Inc.). Finally, signal was detected with a chemiluminescent substrate (Super Signal West Pico reagent, Thermo Scientific) and developed in an automatic X-ray film processor JP-33 after exposing the membrane to an X-ray film (Fujifilm).