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p130Cas promotes invasiveness of three-dimensional ErbB2-transformed mammary acinar structures by enhanced activation of mTOR/p70S6K and Rac1

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ABSTRACT

ErbB2 over-expression is detected in approximately 25% of invasive breast cancers and is strongly associated with poor patient survival. We have previously demonstrated that p130Cas adaptor is a crucial mediator of ErbB2 transformation. Here, we analysed the molecular mechanisms through which p130Cas controls ErbB2-dependent invasion in three-dimensional cultures of mammary epithelial cells. Concomitant p130Cas over-expression and ErbB2 activation enhance PI3K/Akt and Erk1/2 MAPK signalling pathways and promote invasion of mammary acini. By using pharmacological inhibitors, we demonstrate that both signalling cascades are required for the invasive behaviour of p130Cas over-expressing and ErbB2 activated acini. Erk1/2 MAPK and PI3K/Akt signalling triggers invasion through distinct downstream effectors involving mTOR/p70S6K and Rac1 activation, respectively. Moreover, *in silico* analyses indicate that p130Cas expression in ErbB2 positive human breast cancers significantly correlates with higher risk to develop distant metastasis, thus underlying the value of the p130Cas/ErbB2 synergism in regulating breast cancer invasion. In conclusion, high levels of p130Cas favour progression of ErbB2-transformed cells towards an invasive phenotype.

Introduction

The ErbB2 oncogene (also known as HER2) belongs to the epidermal growth factor-receptor family and its over-expression and activation have been detected in a large subset of mammary cancers (Hynes and Lane, 2005). Amplification or over-expression of the ErbB2 gene has been detected in many cancers, including human breast, lung and gastric cancers and is associated with increased tumour grade and shorter overall survival rates (Allgayer et al., 2000; Sharma and Settleman, 2009; Slamon et al., 1989). ErbB2 activation is associated with an increase of its tyrosine phosphorylation and results in the phosphorylation of a host of downstream molecules, which in turn activate a variety of signalling cascades, including phosphatidylinositol-3-kinase (PI3K)/Akt, Src kinase, phospholipase C gamma and the RAS/mitogen activated protein kinase cascade (Baselga and Swain, 2009; Hynes and Lane, 2005; Hynes and MacDonald, 2009; Neve et al., 2002; Yarden and Sliwkowski, 2001).

p130Cas was originally identified as a tyrosine phosphorylated protein upon transformation by v-Src and v-Crk oncogenes and for its ability to associate with Crk (Matsuda et al., 1990; Reynolds et al., 1989). p130Cas is a multifunctional adaptor protein required for embryonic development (Honda et al., 1998) and is characterised by structural motifs that enable interactions with a variety of signalling molecules. These multi-protein complexes sense and integrate signalling originating from several receptor systems (Bouton et al., 2001; Defilippi et al., 2006; O'Neill et al., 2000).

p130Cas functions as a molecular scaffold within focal adhesion complexes, and is readily phosphorylated by focal adhesion kinase (FAK) and c-Src (Mitra and Schlaepfer, 2006). For its inclusion in focal adhesion complexes, p130Cas has also been proposed to play a critical role in mediating cell migration and invasion. Indeed, fibroblasts derived from p130Cas-deficient embryos exhibit drastically altered cytoskeletal architecture (Honda et al., 1998) and after transformation with c-Src become significantly more invasive when engineered to simultaneously over-express p130Cas (Brabek et al., 2004). In addition, p130Cas association with Crk constitutes a molecular switch for cell motility by recruiting DOCK180 to integrin-containing adhesion complexes (Klemke et al., 1998). Moreover, c-Src-mediated p130Cas phosphorylation and the assembly of a p130Cas–Crk–DOCK180 scaffold drive cell migration and responses to mechanical stress (Mitra and Schlaepfer, 2006; Stupack et al., 2000)

Recent data support a role for p130Cas in the acquirement of resistance to breast cancer therapy. It has been shown that patients

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with primary breast tumours expressing high levels of p130Cas (also known as BCAR-1) experience a more rapid disease recurrence and have a greater risk of resistance to tamoxifen therapy (van der Flier et al., 2000). Moreover, over-expression of p130Cas/BCAR-1 can confer breast cancer resistance to adriamycin (Ta et al., 2008).

We have previously shown that double transgenic mice originated by crossing MMTV-p130Cas and MMTV-NeuT mice, that express the oncogenic form of the rat *neu* gene, homologous to human ErbB2 (Muller et al., 1988; Nanni et al., 2000), showed an accelerated onset of mammary tumour formation (Cabodi et al., 2006). In addition, the analysis of human breast samples revealed that tumours over-expressing both p130Cas and ErbB2 are characterised by an elevated proliferation index (Cabodi et al., 2006). We have also recently shown that p130Cas is an essential transducer element in ErbB2 transformation, demonstrating that p130Cas is necessary for ErbB2-dependent foci formation, anchorage-independent growth, *in vivo* tumour growth and lung colonisation (Cabodi et al., 2010).

The human mammary cells MCF10A.B2 that express a chimeric form of ErbB2, which can homodimerise in the presence of the synthetic ligand AP1510 (Muthuswamy et al., 2001), are a suitable *in vitro* model for the study of the ErbB2-dependent transformation in three-dimensional (3D) matrix instead of the classical monolayer culture. Here, we uncover the molecular mechanisms for p130Cas-dependent invasiveness of ErbB2-transformed cells showing that p130Cas drives invasion of ErbB2-transformed mammary acinar structures by enhancing activation of mTOR/p70S6K and Rac1.

Materials and methods

Antibodies and reagents

Mouse monoclonal antibodies to p130Cas and beta-1 integrin and rabbit polyclonal antibodies to fibronectin were produced at the MBC, University of Torino. Phospho-Src (Y416), phospho-Erk1/2 (T202/T204), phospho-Akt (S473), phospho-S6Rp (S235/236), S6Rp, Erk1/2 MAPK, Akt, phospho-p70S6K T389 and T421/S424, p70S6K antibodies were obtained from Cell Signalling (Beverly, MA), Rac1 antibody was from Millipore (Billerica, MA, USA). c-Src antibody was from Santa Cruz (Palo Alto, CA, USA). Rac inhibitor (NSC23766) was from Calbiochem (Merck KGaA, Darmstadt, Germany), MAPKK inhibitor (PD98059), Src inhibitor (SU6656) and PI3K inhibitor (LY294002) were from Sigma (St. Louis, MO, USA), Rapamycin was from Cell signalling. Matrigel and collagen I were purchased from BD Transduction Laboratories (Franklin Lakes, NY). Secondary antibodies conjugated with peroxidase were purchased from GE Healthcare. Alexa Fluor 633 phalloidin and Alexa Fluor Dye secondary antibodies were from Invitrogen (Carlsbad, CA, USA).

Cell cultures

MCF10A.B2 engineered cells were kindly provided by Dr. Muthuswamy (Cold Spring Harbor Laboratory) (Muthuswamy et al., 2001). MCF10A.B2 cells were maintained in DMEM/F12 (Gibco, BRL) supplemented with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 1 ng/ml cholera toxin, 100 μg/ml hydrocortisone, 50 U/ml penicillin and 50 μg/ml streptomycin. To generate 10A.B2Mock and 10A.B2Cas cells, we transduced MCF10A.B2 with pBabe retroviral empty vector or carrying p130Cas cDNA fused with GFP.

Three-dimensional morphogenetic assay

3D morphogenetic assays were conducted as previously described in http://muthuswamylab.cshl.edu/ml_protocols.html and in Debnath et al. (2003) and Seton-Rogers and Brugge (2004). Phase images were collected by using Zeiss microscopy at 4× magnitude.

Immunobloting analysis from 3D cultures

3D acini were released from Matrigel:Collagen using BD cell recovery solution (BD Biosciences). Protein extracts were obtained by lysing acini with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl; 1% Triton X-100, and protease inhibitors). Total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted overnight with primary antibodies at 4 °C. Blots were incubated with mouse or rabbit horseradish-peroxidase conjugated secondary antibodies for 1 h at room temperature. ECL (Euroclone) was used to detect chemoluminescent signals. Protein band intensities were determined using the Image J software developed by the NIH.

Immunofluorescence analysis of 3D acinar structures

Immunostaining of 3D cultured MCF10.B2 cells was performed as described in http://muthuswamylab.cshl.edu/protocols/IF protocol.pdf. Images were taken at HCX PL APO CS 63X 1.4 OIL Leica TCS-SP5 II confocal microscope and analysed with LASAF software.

Rac pull-down assay

Rac1 pull-down assay was performed as described in Di Stefano et al. (2007). Briefly, glutathione-coupled Sepharose 4B beads bound to recombinant GST–PAK CRIB domain fusion proteins were incubated with cell extracts at 4 °C for 45 min, eluted in Laemmli buffer and analysed for the presence of Rac1 by Western blot.

In silico analysis

The NKI dataset for early stage breast cancer containing 295 breast cancer specimens, analysed on a 25,000 spot oligonucleotide array as described previously, was used (van de Vijver et al., 2002). Only the 102 patients positive for ErbB2 expression (expression above the mean) were considered. The difference in ErbB2 expression between patients with high levels of p130Cas and with low levels of p130Cas (with respect to the mean expression level across the samples) was determined with a two-sided Mann–Whitney *U*-test.

Distant metastasis-free survival times were defined as the time to the first event. Overall survival times were based on death from any cause. Patients were censored at last follow up. Kaplan–Meier survival curves were compared using a log-rank test (R survival package).

Results

In human breast tumours p130Cas and ErbB2 co-expression correlates with metastasis and poor prognosis

Our previous data indicate that p130Cas is involved in ErbB2 tumourigenesis (Cabodi et al., 2006) and is required for lung colonisation of ErbB2-transformed cells (Cabodi et al., 2010). The clinical relevance of these findings is supported by the *in silico* analysis that we performed using publicly available microarray data from the Netherlands Cancer Institute of 295 early stage breast cancer biopsies (van de Vijver et al., 2002). On this set of data we evaluated the expression of p130Cas in patients that were positive for ErbB2 (102 out of 295). Interestingly, as shown in the box plot in Fig. 1A, the expression of ErbB2 is significantly higher in patients with high levels of p130Cas (p130Cas/BCAR1^{+}) compared to patients with low levels of p130Cas (p130Cas/BCAR1^{-})