European Journal of Cell Biology 90 (2011) 631–641

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.de/ejcb

p21^{Cip1} regulates cell–substrate adhesion and interphase microtubule dynamics in untransformed human mammary epithelial cells

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ARTICLE INFO

Article history:
Received 7 December 2010
Received in revised form 1 March 2011
Accepted 7 March 2011

Keywords:
CDKN1A
p21^{Cip1}
Focal adhesion
Microtubule
HDAC6
Mammary epithelial cells
Breast cancer

ABSTRACT

Despite its frequent inactivation in human breast cancers, the role of p21^{Cip1} (p21) in morphological plasticity of normal mammary epithelial cells is still poorly understood. To address this question, we have investigated the consequences of p21 silencing in two-dimensional (2D) morphogenesis of untransformed human mammary epithelial cells. Here we show that p21 inactivation causes a reduction of 2D cell spreading and suppresses focal adhesion. In order to investigate the cytoskeletal modifications associated with this altered morphology, we have analyzed the microtubule dynamics in interphase p21-depleted cells. Our results demonstrate that interphase microtubule dynamic instability is strongly increased by p21 silencing. This alteration correlates with severe microtubule hypoacetylation. Next, we show that these microtubule defects in p21-depleted cells can be reversed by the use of the small molecule tubacin, a specific inhibitor of the α-tubulin deacetylase HDAC6. Tubacin-induced microtubule dynamics decrease also correlates with a partial recovery of cell spreading and focal adhesion in those cells. Collectively, these data indicate that p21 regulates the morphological plasticity of normal mammary epithelial cells by modulating dynamics of key cytoskeletal components.

Introduction

The p21^{Cip1} (p21) protein, encoded by the *CDKN1A* gene, was initially identified as an inhibitor of the activity of cyclin-dependent kinases (CDK)-cyclin complexes and PCNA-dependent DNA synthesis (Chen et al., 1995; el-Deiry et al., 1993; Harper et al., 1993; Luo et al., 1995; Xiong et al., 1993). Since its discovery, it has been extensively studied as a key player in cell cycle progression and cellular proliferation due to its contribution to the G1/S checkpoint (Abbas and Dutta, 2009). These physiological functions were related to the p53-dependent transactivation of *CDKN1A* (Brugarolas et al., 1995; Deng et al., 1995; Macleod et al., 1995; Martin-Caballero et al., 2001). Besides these cell cycle inhibitory properties, several works have proposed other functions for p21 such as anti-apoptotic activities (Abbas and Dutta, 2009; Asada et al., 1999; Huang et al., 2003; Shim et al., 1996; Suzuki et al., 1999, 2000; Zhan et al., 2007) or regulation of gene transcription (Chang et al., 2000; Delavaine and La Thangue, 1999; Janicke et al., 2007; Kitaura et al., 2000; Lohr et al., 2003; Wu et al., 2002). However, the consequences of transcriptome modulation by p21 in proliferation and apoptosis are still poorly understood and seem to be highly dependent upon cellular context (Abbas and Dutta, 2009; Janicke et al., 2007).

Multiple mechanisms of p21 inactivation have been described in human cancers including loss of *CDKN1A* transactivators (*e.g.* p53), c-Myc activation, increased degradation and aberrant cytoplasmic localization related to ERBB2 activation (Abbas and Dutta, 2009; Jung et al., 2008; Mukherjee and Conrad, 2005; van de Wetering et al., 2002; Zhou et al., 2001). More specifically, functional inactivation of p21 is frequently observed in epithelial tumors (Anttila et al., 1999; Balbin et al., 1996; Caffo et al., 1996; Lu et al., 1998; Polyak et al., 1996). Despite their histological diversity, tumor progression of these malignancies is generally associated with disorganized tissue architecture, invasion and metastatic dissemination (Debnath and Brugge, 2005). Moreover, it is well documented that these processes involve major reorganization of the cytoskeleton associated with transformation of adhesion properties (Friedl, 2004; Hall, 2009; Kedrin et al., 2007; Yilmaz

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doi:10.1016/j.ejcb.2011.03.002

and Christofori, 2009). Of note, cytoplasmic p21 was proposed to be a promoter of motility in mouse transformed cells by inhibiting stress fiber assembly and favoring actin severing (Lee and Helfman, 2004; Tanaka et al., 2002). Alternatively, recent data have proposed that total p21 inhibits motility associated with Ras- and c-Myc-induced epithelial–mesenchymal transition (EMT) in human mammary epithelial cells (Liu et al., 2009). These results suggest that the role of p21 in cytoskeletal dynamics and morphological plasticity is highly dependent on the cellular context. This also points out the necessity to address this question in untransformed human epithelial cells.

In order to gain insight into the requirement of p21 for the morphological control of untransformed epithelial cells, we have investigated the effects of p21 silencing in primary and immortalized human mammary epithelial cells. Our results show that, in this cellular type, p21 inactivation induces a strong reduction of cell spreading on 2D substrate. This aberrant behavior is correlated with suppression of focal adhesion and increase of microtubule dynamics. We also demonstrate that forced microtubule stabilization allows partial rescue of adhesion in p21-depleted cells. Altogether, our results indicate that p21 controls cell–substrate adhesion in untransformed human epithelial cells and that this new function is partly mediated by regulation of microtubule dynamics.

Materials and methods

Plasmids and siRNA

PAcGFP1-Tubulin vector coding for GFP-α-tubulin was purchased from Clontech. The *PXN* coding sequence was amplified from hTERT-HMECs total cDNA using the forward primer 5′-TAATTGGTACCATGGACGACCTCGACGCCCTGCTGGCGGACTT and the reverse primer 5′-TAACGCAGATCTCTAGCAGAAGAGCTTGAGGAAGCAGTTCTGACAG. *PXN* CDS was cloned into *KpnI*/*BamHI* digested pEGFP-C1 (Clontech). The retroviral GFP-paxillin expressing vector pQC-GPXN was generated by cloning the *AgeI*/*BclI* digestion product of pGFP-PXN into *AgeI*/*BamHI* digested pQCXIN (Clontech).

The pLKO.1 *CDKN1A* shRNA lentiviral vectors (TRCN0000040123-7) were purchased from Sigma–Aldrich. The first nucleotide position of the shRNA target on the *CDKN1A* mRNA sequence (NM_000389.3) designates shRNA and their corresponding vectors. Transductions of pLKO.1 sh564 (sh5) and pLKO.1 sh669 (sh6) achieved stable p21 knock-down and pLKO.1 sh6, the most efficient vector, was used in most p21KD experiments. Data from one p21KD model was always confirmed using a second independent p21KD model. Transduction of pLKO.1 NT (non-targeting, Sigma) was used as the control.

p21-targeting siRNAs corresponding to sh5 and sh6 targeting sequences (si5 and si6) were purchased from Dharmacon. A pool of 4 independent non-targeting siRNAs (siCONTROL Non-Targeting siRNA Pool #2, siCT, Dharmacon) was used as the control in siRNA-mediated knockdown experiments.

Cell culture, transfection and infection

Primary human mammary epithelial cells (HMECs) were obtained from Lonza. Low passage immortalized human mammary epithelial cells (hTMECs, hTERT-transduced HMECs) were provided by R.A. Weinberg (Whitehead Institute, Cambridge, USA). HMECs and hTMEC-derived cell lines were cultured in MEBM basal medium (Lonza) supplemented with 10 ng/ml human EGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.4% bovine pituitary extract, 50 μg/ml gentamicin and 50 ng/ml amphotericin-B.

Plasmid transfection in hTMECs was performed using FuGENE HD (Roche). Retroviral production was performed in Phoenix Ampho cells (SD 3443, ATCC) and lentiviral production was performed in 293FT cells (Invitrogen) co-transfected by pCMVΔR8.91 (gift from D. Trono, EPFL, Lausanne, Switzerland) and phCMV-G (gift from F.-L. Cosset, ENS, Lyon, France). Stably pAcGFP1-tubulin-transfected and pQC-GPXN-transduced hTMECs were, respectively, designated as GT-hMECs and GP-hMECs, and selected under 100 μg/ml geneticin. Subclones were selected according to optimal fluorescently tagged protein expression, normal 2D mammary epithelial cell morphology and growth.

Cell lines expressing sh5, sh6 and shNT were, respectively, designated as p21KD5, p21KD6 and NT cells and selected under 0.5 μg/ml puromycin. Cells harboring stable p21 knockdown were generically designated as p21KD cells. Data presentation for one p21KD cell model indicates that similar results were obtained for second independent p21KD model. Transfection of 10–30 nM siRNAs was performed in hTMEC-derived cell lines using Lipofectamine RNAiMAX (Invitrogen). In these conditions, transfectivity was estimated to be 100% by using fluorescent control siRNA (siGLO RISC-Free Control siRNA, Dharmacon) in all hTMEC-derived cell lines used in transient siRNA-mediated knockdown experiments.

Antibodies and reagents

Mouse antibody against Ku80 (7/Ku80) and Rac1 (102) were purchased from BD Biosciences. Rabbit antibodies against FAK, phospho–tyrosine 397 (pY397) FAK, stathmin, phospho-serine 16 (pS16) stathmin were purchased from Cell Signaling Technology. Mouse antibodies against Cdc42, cyclin B1 (GNS3), p21 (CP36-CP74) and RhoA/B/C (55) were purchased from Millipore. Mouse antibodies against total β-tubulin, total α-tubulin, acetylated, tyrosinated and polyglutamylated tubulin were purchased from Sigma. FITC-conjugated monoclonal mouse anti-human α tubulin and TRITC-conjugated polyclonal rabbit anti-mouse Ig antibodies were, respectively, provided by Sigma and Dako. Hoechst 33258, thymidine, deoxycytidine, RNAse, propidium iodide, paclitaxel were purchased from Sigma. Trypsin neutralizing solution was obtained from Lonza. Oxyrase was purchased from Oxyrase. Tubacin and niltubacin were provided by R. Mazitschek, Massachusetts General Hospital, Boston, USA.

Synchronization and cell cycle analysis

Synchronization of hTMEC-derived cell lines was perfomed by the double thymidine block. Briefly, for each block, cells were treated during 16 h with 2 mM thymidine, released in fresh medium supplemented with 24 μM deoxycytidine and harvested at each time-point during 10 h. The cell cycle stage was preliminary assessed in control cells by flow cytometry and monitored by phase-contrast microscopy. In brief, collected cells were washed with PBS, fixed in 70% ethanol and stored at 4 °C until use. Cells were re-hydrated in PBS and resuspended in 1 mg/ml RNAse A and 5 μg/ml propidium iodide. The cell cycle distribution and was determined with FACScan flow cytometer and analyzed by FACS Calibur (Becton Dickinson).

Fluorescent staining and microscopy

Total α-tubulin labeling was performed on cells extracted at room temperature in microtubule stabilizing buffer (MTSB) supplemented with 0.04% Triton X-100 and 0.25 nM paclitaxel, fixed in 100% methanol at −20 °C, blocked in 3% BSA/3% FBS TBST, incubated with 1:100 FITC-conjugated mouse anti-α-tubulin antibody and counterstained with Hoechst 33258.

Acetylated-tubulin labeling was realized on cells extracted at room temperature for 1 min in MTSB (60 mM PIPES pH 6.8, 20 mM