FEBS Letters 581 (2007) 1617–1624

The transcription factor ZEB1 (δEF1) represses Plakophilin 3 during human cancer progression

Kirsten Aigner[a], Luise Descovich[a], Mario Mikula[b], Aneesa Sultan[a], Brigitta Dampier[c], Stefan Bonné[d,e], Frans van Roy[d,e], Wolfgang Mikulits[b], Martin Schreiber[c], Thomas Brabletz[f], Wolfgang Sommergruber[g], Norbert Schweifer[g], Andreas Wernitznig[g], Hartmut Beug[h], Roland Foisner[a,*], Andreas Eger[a,*]

[a] *Max F. Perutz Laboratories, Department of Medical Biochemistry, Medical University Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria*
[b] *Department of Medicine I, Division: Institute of Cancer Research, Medical University Vienna, Austria*
[c] *Department of Obstetrics and Gynecology, Medical University Vienna, Austria*
[d] *Molecular Cell Biology Unit, Department for Molecular Biomedical Research, VIB, Ghent, Belgium*
[e] *Ghent University, Ghent, Belgium*
[f] *Department of Visceral and General Surgery, Albert-Ludwigs-University, Freiburg, Germany*
[g] *Boehringer-Ingelheim Austria, Vienna, Austria*
[h] *Research Institute of Molecular Pathology, IMP, Vienna, Austria*

Received 19 December 2006; revised 6 March 2007; accepted 9 March 2007

Available online 21 March 2007

Edited by Lukas Huber

Abstract **Plakophilin 3 (PKP3) belongs to the p120ctn family of armadillo-related proteins predominantly functioning in desmosome formation. Here we report that PKP3 is transcriptionally repressed by the E-cadherin repressor ZEB1 in metastatic cancer cells. ZEB1 physically associates with two conserved E-box elements in the PKP3 promoter and partially represses the activity of corresponding human and mouse PKP3 promoter fragments in reporter gene assays. In human tumours ZEB1 is upregulated in invasive cancer cells at the tumour–host interface, which is accompanied by downregulation of PKP3 expression levels. Hence, the transcriptional repression of PKP3 by ZEB1 contributes to ZEB1-mediated disintegration of intercellular adhesion and epithelial to mesenchymal transition.**

Keywords: Epithelial to mesenchymal transition; Invasion; Transcription; Desmosomes; Cell adhesion

1. Introduction

The functional and structural integrity of epithelial tissues is based upon apico-basal polarity of epithelial cells, which in turn depends on a number of intercellular junctions, including gap junctions, tight junctions, adherens junctions and desmosomes [1]. Desmosomes, also known as maculae adherens, are intermediate filament-attached adhering junctions which contribute to strong intercellular adhesion and confer resistance to mechanical stress [2,3]. The organization of desmosomes closely resembles that of E-cadherin type adherens junctions. Desmosomes contain clusters of cadherin-type transmembrane proteins, the Desmogleins (Dsg) and Desmocollins (Dsc), which are heavily glycosylated and mediate intercellular adhesion by homo- and heterophilic interactions [4]. The cadherin-associated plaque region contains Plakoglobin and Desmoplakin, constitutive desmosomal components mediating the linkage to the intermediate filament network.

The Plakophilins (PKPs) represent an important group of proteins integral to the inner desmosomal plaque area. They belong to the p120ctn subfamily of armadillo-related proteins sharing a characteristic series of armadillo domain repeats of about 45 amino acids [5,6]. The different members designated as Plakophilin 1–3 (PKP1–3) interact with the desmosomal cadherins as well as with the cytoskeletal linker proteins Desmoplakin and Plakoglobin, and show distinct expression patterns and effects on desmosomal adhesion [6]. Their numerous interactions within the desmosomal plaque suggest that they act as scaffolding proteins required for proper desmosome assembly. In addition, Plakophilins may also be involved in transducing signals in response to stimuli from the cellular environment, since nuclear localization has been reported for all three Plakophilins [5,7–9]. Together, these data establish the Plakophilins as critical regulators of desmosome assembly and function. However, upstream signalling molecules that regulate the expression and/or function of the different Plakophilins in epithelial tissue homeostasis remain to be identified. Here we report that ZEB1, a potent transcriptional repressor of E-cadherin directly represses the expression of PKP3 in invasive human cancer cells. ZEB1 binds to the PKP3 promoter in vivo and strongly reduces corresponding promoter activities in reporter gene assays. In human colon cancer specimens membranous PKP3 is expressed mainly in the differentiated bulk tumour region but is downregulated in invasive cells upregulating ZEB1.

2. Materials and Methods

2.1. Cell culture and Affymetrix Gene Chip™ analyses

MDA-MB-231, MCF7, and EpH4 cells were cultivated as described previously [11]. For generation of E-cadherin expressing cell clones,

*Corresponding authors. Fax: +43 1 4277 9616 (A. Eger).
E-mail addresses: roland.foisner@meduniwien.ac.at (R. Foisner), andreas.eger@meduniwien.ac.at (A. Eger).

0014-5793/$32.00
doi:10.1016/j.febslet.2007.03.026

MDA-MB-231 cells were transfected with E-cadherin-GFP and selected with 800 μg/ml Geneticin (G418; Invitrogen, Carlsbad, USA). RNA isolation, RNA quality control, RNA labeling, hybridization on Affymetrix Gene Chips™ (Human Genome U133 Plus 2.0) and data acquisition including fold-change analyses were done as described [10].

2.2. Reporter gene assays

Transient reporter experiments in human MCF7 and mouse EpH4 mammary epithelial cells were done as described previously [11].

2.3. RT–PCR

Total mRNA and cDNA was prepared as described previously [11]. PCR primers used were for PKP3: forward 5′-CTCGGAACGCTAGGAACAAG-3′, reverse 5′-AAGTCCTCCTTCCGATAGCC-3′; E-cadherin: forward 5′-TGGAGGAATTCTTGCTTTGC-3′, reverse 5′-CGTACATGTCAGCCAGCTTC-3′; Actin: forward 5′-ATCTGGCACCACACCTTCTAC-3′, reverse 5′-CAGCCAGGTCCAGACGCAGG-3′.

2.4. Antibodies

The following antibodies (Abs) were used: mouse monoclonal Ab to E-cadherin (BD Biosciences, Franklin Lakes, NJ, USA); goat polyclonal Ab to ZEB1 (ZEB-E20, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); mouse monoclonal Ab to PKP3 (23E3/4, [28]; Zymed Laboratories, South San Francisco, CA, USA); rabbit polyclonal Ab against Actin (Sigma, St. Louis, USA); secondary Abs coupled to Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR, USA), Texas Red or peroxidase (Jackson Laboratories, West-Grove, USA).

2.5. Immunofluorescence microscopy and immunoblotting

Cells were fixed in 2.5% formaldehyde (Merck Inc., Whitehouse Station, NJ, USA) and processed for immunofluorescence microscopy as described [12]. Immunoblotting of total cell lysates obtained from equal amounts of cells was performed as described previously [12].

2.6. Immunohistochemistry

Formalin-fixed, paraffin-embedded colorectal adenocarcinomas were obtained from surgical resection specimens. Tumour sections were deparaffinized in xylene, rehydrated in alcohols, and washed twice with water. Samples were boiled in 0.01 M citrate buffer (pH 6.0) for 30 min and subjected to immunohistochemistry employing the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA).

2.7. Chromatinimmunoprecipitations (ChIPs)

ChIP analyses were performed according to the instructions of the ChIP Assay Kit from Upstate Biotechnology (Lake Placid, NY, USA) as described previously [11]. Primers used were: PKP3 ChIP1 forward: 5′-CCCAAATTCCACCTTAAGCA-3′, reverse: 5′-CGCCTCCAAACCCAACTAT-3′; ChIP2 forward: 5′-ACCTGTTAGCAGCTGCAATTT-3′, reverse 5′-GAAGGGGGCGTCTGTCTG-3′.

2.8. RNA inhibition experiments

ZEB1-specific siRNA experiments were performed as described previously [11].

3. Results

3.1. Knock down of ZEB1 in invasive cancer cells causes re-expression of PKP3

Deconstruction of junctional complexes allows differentiated epithelial cells to convert to a motile fibroblastoid phenotype during embryonic development and physiological events in the adult organism [13,14]. This process, generally termed epithelial-mesenchymal transition (EMT), is also pathologically activated during cancer cell invasion and metastasis [15,16]. Recently, we have identified the transcription factor ZEB1 (δEF1, TCF8, AREB6) as a direct transcriptional repressor of E-cadherin and a potent inducer of EMT in human breast

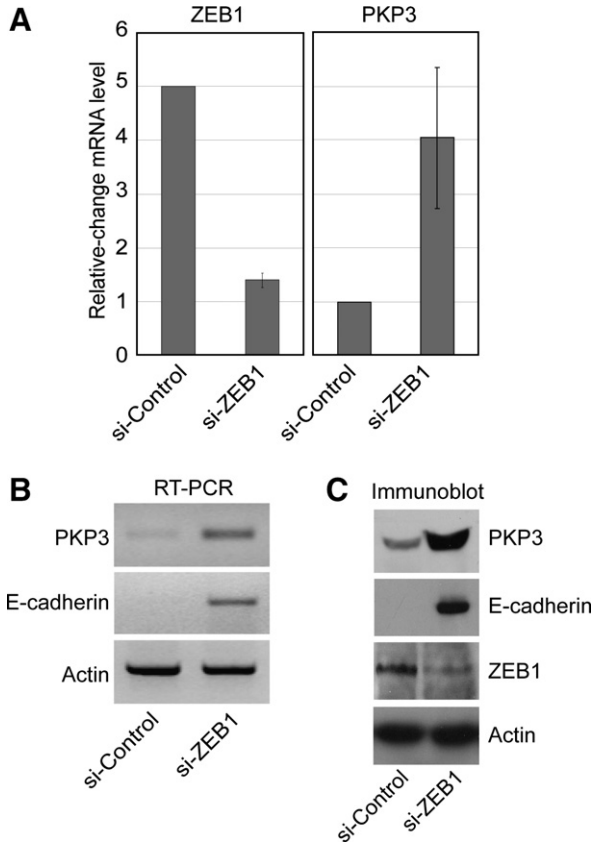

Fig. 1. Knock down of ZEB1 causes upregulation of PKP3 expression. (A) RNAi-mediated knock down of ZEB1 in MDA-MB-231 breast cancer cells. Three days after treatment with either ZEB1-specific (si-ZEB1) or unspecific scrambled (si-Control) siRNAs total RNA was harvested and processed for Affymetrix Gene Chip analysis™. Diagram shows relative-change of mRNA levels of ZEB1 and PKP3 after ZEB1-specific knock down compared to cells treated with scrambled siRNA (si-Control). (B) PKP3 and E-cadherin mRNA levels after ZEB1 knock down. mRNA levels were analysed by semi-quantitative RT-PCR three days after ZEB1 knock down (see A). Actin was included for normalization. (C) Protein levels of PKP3, E-cadherin and ZEB1 after knock-down of ZEB1. For immunoblotting cells were treated with siRNAs as indicated in (A). Total MDA-MB-231 cell lysates were separated by SDS–PAGE and immunoblots were performed using antibodies to ZEB1, PKP3, E-cadherin and Actin (loading control).

cancer cells [11] (see also Fig. 1B and C). In addition, depletion of ZEB1 in dedifferentiated and metastatic cancer cells by RNA interference not only restored E-cadherin expression but also caused the reestablishment of epithelial features including the partial formation of adherens and tight junctions [11]. These data suggest that ZEB1 may regulate various genes critically involved in the formation of epithelial adhesion complexes.

In order to determine the function of ZEB1 during cancer progression, we specifically knocked down ZEB1 in invasive and dedifferentiated MDA-MB-231 cancer cells expressing high levels of endogenous ZEB1 and lacking all junctional adhesion complexes, and identified de-repressed genes by Affymetrix Gene Chip™ analyses. Among the genes whose transcript levels were significantly upregulated upon ZEB1 depletion, we detected the desmosmal protein PKP3 (Fig. 1A). RT-PCR experiments using cDNA samples from three independent siRNA knockdown experiments confirmed