FEBS Letters 581 (2007) 5122–5126

ZIC1 gene expression is controlled by DNA and histone methylation in mesenchymal proliferations

Rasoul Pourebrahim[a], Kim Van Dam[a], Marijke Bauters[a], Ivo De Wever[b], Raf Sciot[c], Jean-Jacques Cassiman[a], Sabine Tejpar[a,d,*]

[a] *Department of Human Genetics, KU Leuven, Belgium*
[b] *Department of Oncology, UZ Gasthuisberg, KU Leuven, Belgium*
[c] *Department of Oncological Surgery, UZ Gasthuisberg, KU Leuven, Belgium*
[d] *Department of Gastroenterology, UZ Gasthuisberg, KU Leuven, Belgium*

Received 12 July 2007; revised 26 September 2007; accepted 27 September 2007

Available online 8 October 2007

Edited by Ned Mantei

Abstract RNA and protein analysis revealed the consistent upregulation of the neural transcription factors *ZIC1* and *ZIC4* in desmoid tumors and other fibroproliferative disorders. The 5′ flanking region of the *ZIC1* promoter was unmethylated in desmoid tumor fibroblasts, while a hypermethylated *ZIC1* promoter was found in human and mouse cell lines not expressing the gene. In addition, expressing cells showed a H3K4me2 at the *ZIC1* promoter, whereas non-expressing cells showed higher levels of H3K9me2 in the same region. To our knowledge, this is the first report describing *ZIC1* expression in mesenchymal proliferations and a role for DNA methylation in the control of *ZIC1* expression.

Keywords: Methylation; ZIC; Desmoid tumor; Fibroblasts

1. Introduction

In vertebrates, the *ZIC* gene family plays an important role in neural crest and subsequent cerebellar development [1]. Mutations in *ZIC* family members have demonstrated that they are essential for proper development of the neural tube, left-right symmetry, and for muscle and skeletal development. Although the precise cellular and biochemical activities of ZIC1 are currently unknown, it has been suggested that ZIC1 participates in the regulation of cell proliferation in the dorsal neural tube by inhibition of neural differentiation [2]. Furthermore, ZIC1 is potentially involved in the oncogenesis of undifferentiated nervous system tumors [3]. Previously, our group showed that ZIC1 was consistently upregulated in desmoid tumors compared to adjacent fascia [4]. This contrasted with the fact that *ZIC* expression was never reported in adult tissues except neural tissues and tumors. Recently the global mapping analysis of stem cells revealed that the Polycomb proteins (PcGs) regulate the expression of a set of genes involved in stem cell maintenance and differentiation, including *ZIC1* [5].

Of the five *ZIC* genes identified in humans and mice, the gene pairs *ZIC1*/*ZIC4* and *ZIC2*/*ZIC5* both are arrayed head-to-head in 30 kb genomic regions [6]. The bi-directional organization of *ZIC1* and *ZIC4* raises the possibility that the two genes are regulated by the same enhancer, controlling their expression in neural and other tissues.

Here we show *ZIC1* expression in different mesenchymal proliferations. We will focus on understanding what epigenetic mechanisms control this expression. Our investigations show differences in DNA methylation and in histone modifications at the *ZIC1* promoter which correlate with the expression levels of the gene. Moreover, we will show the co-expression of *ZIC1* and *ZIC4* while ZIC2, *ZIC3*, and *ZIC5* are not expressed in the different fibroproliferative states.

2. Materials and methods

2.1. Cell lines

Surgical resection specimens were obtained from patients undergoing surgery for a desmoid tumor at our University Hospital. Primary cell cultures were obtained by collagenase treatment and subsequently grown in D-MEM/F-12 supplied with 10% FCS. All tumor samples were diagnosed as desmoids, based on their pathology and mutational status. Biopsy material was snap frozen and stored at −70 °C. Formalin-fixed, paraffin-embedded samples of skin tissue, desmoid tumors, Dupuytren's disease and hypertrophic scars were obtained from the department of pathology of the University Hospital. The cell lines NIH 3T3, mouse embryonic fibroblast (MEF), SW480, CCL222, HCT116, Caco2, HTB185 and HTB186 were obtained from the American Type Tissue Culture Collection. For 5-aza-2′-deoxycytidine (5-aza-dC) treatments, cells were grown at low density in T-75 dishes and treated with different doses of 5-aza-dC (Sigma, Belgium) for a total duration of 3 days.

2.2. Real-time quantitative RT-PCR (TaqMan)

The PCR protocol was carried out as recommended by Applied Biosystems. Standard curves for targets and the housekeeping control gene PBGD (Porphobilinogen Deaminase) were drawn based on the Ct (threshold cycle) values, and the relative concentrations of the standards and the relative concentrations for samples were calculated from the detected Ct values and the equation of the curves. Values obtained were divided by the values of PBGD to normalize for differences in reverse transcription. The primers used in these investigations are given in the supplementary material.

*Corresponding author. Address: Department of Human Genetics, KU Leuven, Belgium. Fax: +32 16 345997.
E-mail address: sabine.tejpar@med.kuleuven.be (S. Tejpar).

Abbreviations: ZIC, zinc finger protein of cerebellum; H3K4me2, histone H3 dimethylated at lysine 4; H3K9me2, histone H3 dimethylated at lysine 9; MEF, mouse embryonic fibroblast; TSA, trichostatin A

0014-5793/$32.00
doi:10.1016/j.febslet.2007.09.061

2.3. Northern blot

Twenty μg of total RNA was denatured in a MOPS/formaldehyde/formamide buffer, run on a 1% agarose gel and transferred on Hybond-N nitrocellulose membranes (Amersham Biosciences). Specific probes for *ZIC1* and *PBGD* were generated and 32P-labelled (sequences available upon request). After prehybridization, hybridization was carried out overnight at 68 °C in ExpressHyb hybridization solution (Clontech, BD Biosciences). Membranes were washed for 1 h at 42 °C with a 2 × SSC/0.1% SDS solution and for 1 h at 62 °C with a 0.1 × SSC/0.1% SDS solution. After autoradiography, blots were stripped and hybridized with a housekeeping probe (PBGD).

2.4. Western blot

Protein samples were run on a 10% BisTris gel (Invitrogen Life Technologies) and electroblotted on Hybond-C nitrocellulose membranes (Amersham Biosciences). The following antibodies were used: polyclonal rabbit anti-ZIC1 (1/1000) (Abcam, UK), mouse monoclonal anti-GAPDH (loading control, 1/10000) (Abcam, UK).

2.5. Immunohistochemistry

Sections from patient samples (5 μm) were dewaxed and then quenched in a 0.3% hydrogen peroxide/methanol solution (30 min). Monoclonal anti-ZIC1 Ab (1:100) was diluted in PBS-BSA/10% goat serum/0.1% Triton X-100 and applied for 3 h at room temperature. Immunohistochemical staining was performed using the EnVision+ System-HRP Mouse Kit (DakoCytomation, Belgium). Nuclei were stained with hematoxylin. The monoclonal anti-ZIC1 antibody was a kind gift from Prof. Mikoshiba and Prof. Aruga [3].

2.6. Sodium bisulfite genomic sequencing

One microgram of genomic DNA was modified with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research USA). PCR primers specific to the bisulfite-modified sequences of the *ZIC1* CpG islands were generated (sequences available upon request). PCR amplicons were purified (Millipore) and sequenced on an ABI automated DNA sequencer. The methylation status of individual CpG sites was determined by taking the ratio of the amplitude of the methylated cytosine (appears as C) with that of the unmethylated cytosine (appears as T) described by Lewin et al. [7]. Normalization of peak heights of different bases was done before any ratio was determined. The normalizing factor for each base was computed by taking the average peak height of the same base across the whole sequence divided by the peak heights of all peaks.

2.7. Chromatin immunoprecipitation assay

The chromatin immunoprecipitations (CHIP) were performed as described previously with slight modifications [8]. Antibodies used were as follows: anti-di-methylated histone H3K4 (Upstate Biotechnology), anti-tri-methylated H3K27 (Upstate Biotechnology) anti-di-methylated H3K9 (Upstate Biotechnology). The relative occupancy of the immunoprecipitated factor at a locus was estimated using the following equation: 2^(Ct mock – Ct specific), where "Ct mock" and "Ct specific" correspond to the mean threshold cycles of a qPCR reaction done in duplicate on DNA samples from mock and specific immunoprecipitations.

3. Results

3.1. ZIC1 is highly expressed in desmoid tumors and other fibroproliferative states

Compared to the high expressing medulloblastoma cell line HTB186, real-time RT-PCR showed even higher *ZIC1* expression in four primary cultures and nine biopsies, whereas none of the corresponding fascia samples expressed *ZIC1* (Fig. 1A and B). *ZIC1* expression was confirmed via Western blot on the same samples. A band of 54 kDa corresponding to *ZIC1* was seen in all desmoid samples whereas fascia samples showed no ZIC1 protein (Fig. 1C). To validate the quantitative RT-PCR results, Northern blot analysis was performed on four desmoid and fascia cultures. A band at 3.1 kb for *ZIC1* was detected in all desmoid samples (Fig. 1D), but not in the fascia samples. The colon carcinoma cell lines SW480, CCL220, Caco and HCT116 as well as the mouse NIH 3T3 cell line were *ZIC1* negative, while the mouse embryonic fibroblasts, MEF cells, expressed small amounts of *ZIC1* (results not shown). Immunohistochemical staining was performed on five desmoids, five palmar fibromatoses (Dupuytren's disease) and five non-tumoral hypertrophic scars (keloids). In all samples, the majority of cells showed nuclear staining, although some cytoplasmic ZIC1 expression was present (Fig. 2).

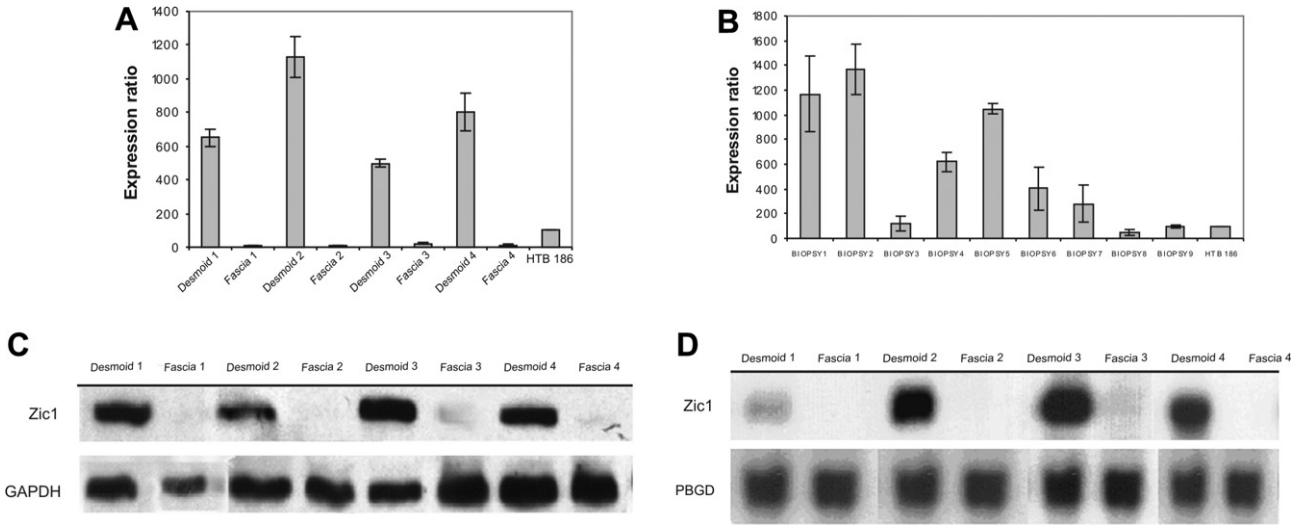

Fig. 1. *ZIC1* expression in desmoid tumors compared with adjacent fascia. (A) RT-PCR analysis of *ZIC1* expression in four desmoid, four fascia cultures and medulloblastoma cells HTB-186 used as control. The values given in ordinate are relative to HTB-186 set at 100. (B) *ZIC1* RT-PCR in nine desmoid biopsies. (C) Western blot analysis of the ZIC1 protein in the samples shown in A. (D) Northern blot analysis of *ZIC1* in the samples shown in A.