EVII Inhibits Apoptosis Induced by Antileukemic Drugs via Upregulation of CDKN1A/p21/WAF in Human Myeloid Cells

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Abstract

Overexpression of ecotropic viral integration site 1 (EVII) is associated with aggressive disease in acute myeloid leukemia (AML). Despite of its clinical importance, little is known about the mechanism through which EVII confers resistance to antileukemic drugs. Here, we show that a human myeloid cell line constitutively overexpressing EVII after infection with a retroviral vector (U937_EVII) was partially resistant to etoposide and daunorubicin as compared to empty vector infected control cells (U937_vec). Similarly, inducible expression of EVII in HL-60 cells decreased their sensitivity to daunorubicin. Gene expression microarray analyses of U937_EVII and U937_vec cells cultured in the absence or presence of etoposide showed that 77 and 419 genes were regulated by EVII and etoposide, respectively. Notably, mRNA levels of 26 of these genes were altered by both stimuli, indicating that EVII regulated genes were strongly enriched among etoposide regulated genes and vice versa. One of the genes that were induced by both EVII and etoposide was CDKN1A/p21/WAF, which in addition to its function as a cell cycle regulator plays an important role in conferring chemotherapy resistance in various tumor types. Indeed, overexpression of CDKN1A in U937 cells mimicked the phenotype of EVII overexpression, similarly conferring partial resistance to antileukemic drugs.

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Introduction

Aberrant expression of the ecotropic viral integration site 1 (EVII) gene, which in healthy individuals is transcribed in hematopoietic stem and progenitor cells but not in mature blood cells [1,2,3,4], is associated with a poor prognosis in myeloid leukemia [5,6,7,8,9,10,11,12,13]. EVII overexpression also correlates with shorter survival in some solid tumors like ovarian carcinoma [14] and estrogen receptor negative breast cancer [15]. Despite the well documented importance of EVII in predicting and most likely causing chemotherapy resistance in human malignant diseases, knowledge about its mechanism of action is limited. EVII is thought to act mainly as a transcription factor, and a recent publication provided a comprehensive overview of genes that are directly regulated by EVII in ovarian carcinoma cells [16]. However, only a small number of EVII target genes have been functionally characterized so far. Among them, the phosphatase and tensin homolog (PTEN) gene appears to be of particular interest, as direct repression of PTEN by EVII lead to activation of the AKT/mTOR pathway in murine bone marrow cells, and rapamycin prolonged survival of mice with EVII overexpressing leukemias [17]. Increased AKT signalling is frequently observed in acute myeloid leukemia (AML) and has been reported to be associated with poor outcome [18,19]. However, recent results indicate that in fact the opposite may be true [20,21,22], so that it is presently unclear to which extent activation of the AKT pathway can explain the poor prognosis associated with EVII overexpression in AML.

Nonetheless, enhancement of AKT signalling has also been implicated in EVII mediated resistance to transforming growth factor beta (TGF-β) and taxol induced apoptosis in colon cancer cells [23]. EVII also increased cellular resistance towards ultraviolet (UV) light [24], tumor necrosis factor α (TNF-α) [24], and interferon α (IFN-α) [25]. In addition to activation of the
AKT pathway [23], inhibition of the proapoptotic jun N-terminal kinase [JNK] [24] and repression of the induction of the promyelocytic leukemia [PML] gene [25] have been reported as possible mechanisms for EVII induced apoptosis resistance. Thus, several studies have addressed the role of EVII in protecting cells from apoptotic stimuli, yet little information is available regarding a possible effect of EVII on cellular resistance to drugs used in the therapy of AML.

The protein product of the CDKN1A/p21/WAF gene, p21, is a 163 amino acid protein best known for its role in mediating p53 dependent cell cycle arrest [26]. In agreement with a tumor suppressive role of this gene, many human cancers exhibit low levels of p21 protein, and experimental ablation of CDKN1A promotes tumor formation in mice [26]. On the other hand, CDKN1A may also be overexpressed in human malignancies, and this molecular alteration is associated with therapy resistance and poor survival [26,27,28]. Accordingly, p21 protects cells from apoptosis induced by DNA damaging agents and other kinds of stress in vitro and in vivo [26,27,29,30,31,32,33,34,35,36,37]. Pre-existing p21 may play a role in increased apoptosis resistance in some cases [27,28,38]. In addition, many of the stimuli from which it protects themselves induce p21 [26,29,31,32,33,34,37,39]. The regulation of CDKN1A/p21/WAF is complex and involves a number of transcription factors in addition to p53, as well as posttranscriptional mechanisms like mRNA-mRNA interactions and phosphorylation, which, among others, affects its subcellular location [26,27,37,39]. The antiapoptotic function of p21 has often been associated with its residence in the cytoplasm [27,34], where it inhibits proapoptotic molecules like JNK and caspases [26]. However, nuclear activities of p21 also contribute to its survival promoting activity: apart from an indirect effect through cell cycle inhibition, its ability to directly bind to and inhibit the activity of transcription factors like E2F1 and MYC plays a role [26,34].

In AML, overexpression of p21 protein relative to healthy controls was found in 17/100 patient samples, and was associated with worse complete response rates and shorter overall survival [28]. Agents used for chemotherapy in AML like etoposide and chemotherapy of AML, and show that this effect may in part be mediated by upregulation of CDKN1A/p21/WAF.

### Materials and Methods

#### Ethics Statement

Animal experiments were approved by the ethics committee of the Medical University of Vienna and the Bundesministerium für Wissenschaft und Forschung Ref. II/10b (Gentechnik und Tierversuche), application Nr. BMWF-66.009/0093-II/10b/1010, and were carried out according to the Austrian and FELASA guidelines for animal care and protection in order to minimize distress for the animals. Mice were sacrificed by cervical dislocation.

#### Cell Culture, Retroviral Vectors, and Infections

The human hematopoietic cell lines U937 [42], HNT-34 [43], and HL-60 [44] were cultured in RPMI 1640 (Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Life Technologies) and 1% Penicillin/Streptomycin/Glutamine (PSG; Life Technologies) in a humidified incubator at 37°C and 5% CO₂. Phoenix-gp cells [http://www.stanford.edu/group/nolan/retroviral_systems/phx.html] were cultured in DEMEM (Life Technologies) with 10% FBS and 1% PSG at 37°C and 5% CO₂.

The full length cDNA of the human EVII gene, which codes for a 1051 amino acid protein, was cloned into the BamHI and EcoRI sites of the retroviral vector pBMIN-IRES-eGFP (Addgene, Cambridge, MA, USA) to yield pBMIN-EVII-IRES-eGFP using standard molecular biology techniques. For doxycycline inducible expression of human EVII, a codon optimized version of its cDNA (Geneart, Regensburg, Germany) was inserted into the BamHI and AsIS sites of pCCL.SIN.cPPT.TRE.IRES.eGFP.wPRE (F.H., unpublished results) and verified by sequencing. The human CDKN1A gene was amplified from cDNA from etoposide treated U937_EVII cells using primers CDKN1A_FWD (5'-CACGGAATCCGAGGCCTAGTGGACAGAC-3') and CDKN1A_REV (5'-CTGACTCGAGGGATTAGGGCCCTCCTTGGG-3') and Phusion High Fidelity Polymerase (New England Biolabs, Ipswich, MA, USA). It was cloned into the retroviral vector pMIA-II-ires-Ametrine using the BamHI and XhoI sites to yield pMIA-II-CDKN1A-IRES-Ametrine. The identity of the insert was confirmed by DNA sequence analysis. pMIA-II-IRES-Ametrine was a kind gift from Dr. Dario Vignali of the St. Jude Children’s Research Hospital, Memphis, Tennessee. Ametrine is a fluorescein marker with spectral properties similar to those of Cyan Fluorescent Protein (CFP), but brighter.

Retroviral particles were generated and U937 and their derivative cell lines infected using standard procedures [43]. Three days later, cells were sorted for enhanced Green Fluorescent Protein (eGFP) or Ametrine positivity on a FACS Aria (Becton Dickinson, Franklin Lakes, NJ, USA). Cell line authentication by short tandem repeat profiling (performed by Ingenetix, Vienna, Austria) confirmed the identity of pBMIN_EVII-IRES-eGFP and

### Table 1. Primers used for amplification and sequence analysis of the integrated, vector borne EVII gene from U937_EVII cells.

| Primer name | sequence (5’–3’) |
|-------------|------------------|
| F1-F*       | GCTCTGGATACGCGGCC |
| F1-R        | GGTCACTGCCATTTGGA |
| F2-F        | GAGGTTTTGTGAGGAGAGA |
| F2-R        | ACCACACCTTTTATGGAC |
| F3-F        | ACCACACAGGAGAGAGCC |
| F3-R        | TACGGTTCTTATGAGCAGC |
| F4-F        | AGGAACTGCTGATCGACTGTCGAG |
| F4-R        | CGGACTAGTNTGTCACGAGACATGTC |
| F5-F        | TGCTCTAGATCATATAAGCCA |
| F5-R*       | GGAGAGGGCGGGGCGATTAC |

*Primers F1-F and F5-R match the vector, rather than the EVII, sequence. doi:10.1371/journal.pone.0056308.t001
Shorty after infection and sorting (t1) as well as after another 12–15 weeks in culture (t2).

EVI1 in U937_vec, U937_EVI1, and HNT-34 cells. Dark grey histogram curves, EVI1 antibody; light grey histogram curves, isotype control. C) High-resolution gel analysis of LAM-PCR amplicons obtained from Tsp509I digested genomic DNA from U937_vec and U937_EVI1 cells. DNA was isolated shortly after infection and sorting (t1) as well as after another 12–15 weeks in culture (t2).

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Sequence Analysis

The CDKN1A cDNA in pMIA-II_ires-Ametrine was sequenced using the primers used for PCR amplification. Sequence analysis of the integrated exogenous EVI1 gene in U937_EVI1 cells was performed after amplification of 5 overlapping gene segments covering the entire EVI1 coding sequence from genomic DNA isolated from U937_EVI1 cells after several weeks in culture. Primers used for amplification of EVI1 are shown in Table 1. Capillary sequencing was performed by Eurofins-MWG-Oppron (Ebersberg, Germany).

Integration Site Analysis by LAM-PCR

500 ng genomic DNA from U937_vec and U937_EVI1 cells either shortly after infection and sorting or after 12–15 weeks in culture, were digested with Tsp509I or HinPlI and used for the analysis of integration sites by 5′-LTR-mediated linear amplification mediated (LAM)-PCR as previously described [47]. Briefly, linear PCR was performed using vector specific 5′-biotinylated primers LTRa bio (5′-TGGGTACCAGACAGATATCCTC-3′) and LTRb bio (5′-ATGCCGCTTTGGGCGCA-TATTG-3′). For the first and second exponential PCRs, vector specific primers LTR II bio (5′-GACCTTGTAGCTGAACCTTCCT-3′) and LTR III bio (5′-TTCCATGCGCTTGCAAATGGC-3′) were used in combination with linker cassette specific primers LG1 (5′-GACCCCGGAGATCTGAATTC-3′) and LC II (5′-GATCTGATTCAGTGGCGACAG-3′). 5′ of the LAM-PCR amplicons were separated on a Spreadex high-resolution gel (Elchrom Scientific, Cham, Switzerland).

For high throughput pyrosequencing (GS FLX, Roche Diagnostics, Risch, Switzerland), 40 ng per sample were prepared according to the manufacturer’s protocol. Approximately 26,000 sequence tags were obtained from each sample. They were mapped to the human genome using the UCSC blast-like alignment tool (BLAT) (GRCh37/hg19, Feb 2009, available at http://www.genome.ucsc.edu/cgi-bin/hgBlat).

Microarray Analyses

RNA for microarray analyses was extracted from two independent replicate cultures of U937_vec and U937_EVI1 cells treated or not treated with 400 nM etoposide for 48 h using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany). RNA quality control, labelling, hybridization to Human Gene 1.1 ST arrays (Affymetrix, Santa Clara, CA, USA), and primary data analysis using the Affymetrix DNA algorithm were performed at the Center of Excellence for Fluorescent Bioanalytics (KFB; Regensburg, Germany). Genes whose expression changed at least twofold in response to EVI1 (in the absence of etoposide) or to etoposide (in the absence of EVI1) in both replicate experiments were considered as regulated by the respective stimulus.

RNA Extraction, Reverse Transcription, and Real Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA for qRT-PCR was extracted using Trizol (Life Technologies, Carlsbad, CA, USA) and reverse transcribed using random hexamer primers (Life Technologies) and M-MLV reverse transcriptase (Life Technologies) according to the manufacturer’s instructions. qRT-PCR was carried out in a Step One Plus Real Time PCR system (Applied Biosystems, Life Technologies). Levels of CDKN1A and of the housekeeping gene β-2-microglobulin (B2M) were determined using the corresponding TaqMan Gene Expression Assays (CDKN1A: Hs00355782_m1, B2M: 4335766F) and the TaqMan Gene Expression Mastermix (all from Applied Biosystems, Life Technologies). All assays were carried out in triplicate. Expression values for the gene of interest relative to the...
Housekeeping gene and to a reference value were determined using the \( \Delta \Delta C_T \) method [48].

Preparation of Protein Extracts and Immunoblot Analysis

To prepare whole cell extracts for immunoblot analysis, exponentially growing cells were left untreated or treated with 400 nM etoposide for 48 h, pelleted, and boiled in SDS loading buffer (62.5 mM Tris-HCl pH 6.8, 5 mM EDTA, 2% (w/v) SDS, 10% Glycerol, 5% \( \beta \)-mercaptoethanol, and bromophenol blue). Cytoplasmatic extracts were prepared by lysis of the plasma membrane with a buffer containing 300 mM sucrose, 10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.15 mM Spermidine, 0.15 mM Spermine, 0.1% NP-40, and protease inhibitors. The remaining nuclei were then extracted with a buffer containing 20 mM Hepes pH 7.9, 420 mM NaCl, 25% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitors. An appropriate amount of 4× SDS loading buffer was added, and samples were boiled. Polyacrylamide gel electrophoresis, tank blotting onto Bio Trace PVDF membranes (Pall Corporation, Port Washington, NY, USA), and antibody hybridizations were performed using standard procedures. Primary antibodies directed against EVI1 (rabbit mAb anti-EVI1 C50E12, Cell Signaling Technology, Danvers, MA, USA) and p21 (rabbit mAb anti-p21Waf1/Cip1 12D1, Cell Signaling Technology) were used at a 1:1000 dilution; the antibody against the nuclear marker protein RCC1 (mouse mAb anti-RCC1 E-6, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:500; and the antibody against the housekeeping gene \( \beta \)-tubulin (mouse mAb anti-\( \beta \)-tubulin clone TUB 2.1, Cell Signaling Technology) were used at a 1:1000 dilution; the antibody against the nuclear marker protein RCC1 (mouse mAb anti-RCC1 E-6, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:500; and the antibody against the housekeeping gene \( \beta \)-tubulin (mouse mAb anti-\( \beta \)-tubulin clone TUB 2.1, Sigma-Aldrich, Seelze, Germany) was used at a dilution of 1:2,500. Horseradish peroxidase conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used at dilutions of 1:50,000–1:75,000 and detected using the SuperSignal.
West Pico Chemiluminescent Substrate (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) or, for faint signals, the Super Signal West Femto kit (Pierce).

**Intranuclear EVI1 Staining**

5 x 10⁵ cells per sample were fixed with 2% formaldehyde in phosphate buffered saline (PBS) for 10 min at 37°C, and permeabilized by dropwise addition of a tenfold volume of ice cold methanol, followed by a 30 min incubation on ice or storage at −20°C for up to several weeks. Cells were blocked for 15 min with 1% BSA (Sigma-Aldrich) and Beriglobin (1:80, CSL-Behring) in PBS, and incubated for 1 h with 11 ng EVI1 antibody (C50E12, Cell Signaling Technology) or isotype control (DA1E, rabbit IgG, Cell Signaling Technology) in a total volume of 60 μl. Secondary antibody incubation was for 30 min with 0.02 ng Alexa Fluor 647 F(ab')₂ fragment of goat anti rabbit IgG (H+L) (Life Technologies) in a total volume of 60 μl. FACS analysis was performed on a FACS LSR Fortessa (Becton Dickinson) using FACSDiva Software.

**Analyses of Cell Cycle Distribution, Differentiation, Viability, and Apoptosis**

For cell cycle analyses, cells were adjusted to a density of 400 cells/μl. On the next day, they were washed with PBS and incubated for 5 min in ice cold 0.5 M citrate/0.5% Tween-20. Cell membranes were disrupted mechanically before nuclei were pelleted and resuspended in PBS containing 100 μg/ml RNase A (Sigma-Aldrich) and 30 μg/ml propidium iodide (PI; Sigma-Aldrich). Nuclear DNA content was determined on a FACS Calibur (Becton Dickinson) or a FACS LSR Fortessa using ModFit software (Verity Software House) for data analysis.

To induce differentiation of U937 derivative cell lines, 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma-Aldrich) at a final concentration of 50 ng/ml was added to cells that had been adjusted to a density of 100 cells/μl. Control cultures were treated with an equivalent amount of solvent (ethanol). 5 days later, cells were stained with a phycocerythrin (PE) conjugated antibody against the monocyte/macrophage specific cell surface marker CD11b (anti-CD11b antibody D12, Becton Dickinson). Flow cytometric analyses were carried out on a FACS Calibur. The percentage of CD11b positive cells relative to the isotype control was used as a measure of differentiation. For morphological analysis of differentiation, cytospin preparations were stained according to a modified Wright technique.

To determine cellular viability/metabolic activity or apoptosis, cells were seeded into white walled 96-well plates (Greiner Bio-One, Kremsmünster, Austria) to a final density of 50 cells/μl and etoposide or daunorubicin were applied at the indicated concentrations. For HL-60 derivative cell lines, 0.5 μg/ml doxycycline was added to the cells 24 h prior to the addition of daunorubicin. After 48 h after the addition of cytotoxic drugs, metabolic activity or the activities of caspases 3 and 7 were determined using the Cell Titer Glo or Caspase-Glo 3/7 assays, respectively (both from Promega, Madison, WI, USA). Nuclear morphology after a 48 h incubation in the presence or absence of 400 nM etoposide was assessed by spinning cells onto microscopic slides and staining them with 1 μg/ml 4′,6-Diamidino-2-phenylindol (DAPI; Roche). Coverslips were mounted using Permafluor Mountant (Labvision, Thermo Fisher Scientific). Slides were inspected under a LSM 700 microscope (Zeiss, Oberkochen, Germany) and intact and apoptotic nuclei were counted by an observer blind to the identity of the samples. Nuclei were considered apoptotic if they were partially fragmented or very large and diffuse. At least 200 nuclei were counted per sample. For annexin V/propidium iodide staining, HL-60_EVI1 and HL-60_vec cells were seeded to a density of 50 cells/μl and treated with doxycycline for 24 h prior to the addition of 60 nM daunorubicin. After another 48 h, cells were stained with AnnexinV-APC (Becton Dickinson Biosciences; 1:200) and propidium iodide (AppliChem), and analyzed on a FACS BD LSR II (Becton Dickinson) using FACS Diva Software (Becton Dickinson Biosciences).

**Xenograft Experiments, Histological Analysis, and Immunohistochemistry**

Six to eight week old female CB-17 scid/scid (SCID) mice were purchased from Harlan Laboratories (San Pietro al Natisone, Italy). The animals were kept in a pathogen-free environment and all procedures were performed in a laminar airflow cabinet.

5 x 10⁶ U937_vec or U937_EVI1 cells, resuspended in 50 μl of serum free RPMI 1640 medium, were injected subcutaneously...
Figure 4. Overexpression of EVII decreases the sensitivity of U937 cells to drugs used in AML therapy. A–D) U937_vec and U937_EVII cells were treated with the indicated concentrations of etoposide (A, C) or daunorubicin (B, D) for 48 h. Cellular viability/metabolic activity was determined based on ATP content (A, B), and apoptosis was measured via caspase 3/7 activity (C, D). Data points represent the mean ± SEM from at least three independent experiments. *, p<0.05 (paired Student's t-test). E) Nuclear morphology of U937_vec and U937_EVII cells after treatment with or without 400 nM etoposide for 48 h. Apoptotic nuclei are marked by arrows. Please note difference in scale between etoposide and control treated cells. F) Quantitative assessment of nuclear morphology. Nuclei prepared as in E were counted as ‘intact’ or ‘apoptotic’ (see Methods) by an observer blinded to the identity of the samples. Data points represent the mean±SEM from 3 independent experiments. **, p<0.01; n.s., not significant (paired Student’s t-test).

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Figure 5. Inducible expression of EVI1 decreases the sensitivity of HL-60 cells to daunorubicin. HL-60_EVI1 and HL-60_vec cells were incubated with 2 μg/ml doxycycline for 24 h prior to addition of daunorubicin (DR) at the indicated concentrations for another 48 h. A) Metabolic activity was measured based on ATP content. B) The proportions of viable, apoptotic, and dead cells were determined via the annexinV (AV)/propidium iodide (PI) assay. Double negative cells were considered viable; PI− AV− cells apoptotic, and PI+ cells dead. C) Proportion of eGFP positive (eGFP+) cells in HL-60_EVI1 and HL-60_vec cells treated with various doses of daunorubicin. Data points in all panels represent the mean ± standard deviation (SD) from three independent biological replicate experiments. *, p<0.05; ***, p<0.001 (paired Student’s t-test).

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into the right flank of each mouse. Animals were controlled every day and tumor size was assessed regularly by caliper measurement. Tumor volume was calculated using the formula: (length × width²)/2. At experiment termination, mice were dissected and tumor tissue was processed for standard histological examinations. For statistical analysis, adjusted areas under curves (aAUCs) were calculated, assuming no tumor volume at day 3, and compared by nonparametric bootstrap inference [49].

Immunohistochemistry (IHC) was performed using standard procedures. 4 μm sections from xenograft tumor blocks were deparaffinized and rehydrated, and either stained by hematoxylin and eosin for histological confirmation of the presence of invasive carcinoma, or subjected to IHC. For IHC, tissue sections were heated for 10 min in 10 mM citrate buffer (pH 6.0) in a pressure cooker for epitope retrieval, and then incubated for 60 min at room temperature with rabbit mAb anti-EVI1 (clone C50E10, Cell Signaling Technology; dilution 1:200) or rabbit mAb anti-CD11b (clone EPR1344, Abcam; dilution 1:100). Antibody binding was detected by means of the UltraVision LP detection system according to the manufacturer’s recommendations (Lab Vision, Thermo Fisher Scientific). Color development was performed by 3′-3′-diaminobenzidine and counterstaining by hematoxylin.

Results

Establishment and Characterization of an in vitrO Model for Human Myeloid Leukemias Constitutively Overexpressing EVI1

In order to establish an in vitrO model for human myeloid leukemias constitutively overexpressing EVI1, the human myeloid cell line U937 was infected with a retroviral vector containing the full length human EVI1 cDNA (pBMN_EVI1-IRES-eGFP), or with empty vector (pBMN_IRES-eGFP) as a control. FACS sorting for eGFP positivity yielded the cell lines U937_EVI1 and U937_vec, respectively. Immunoblot and FACS analyses confirmed overexpression of the EVI1 protein in the bulk U937_EVI1 population and at the single cell level, respectively (Fig. 1A, B). EVI1 protein levels in U937_EVI1 cells were comparable to those in HNT-34 cells, which overexpress endogenous EVI1 due to a rearrangement of chromosome band 3q26 [43] (Fig. 1A, B), and were stable over culture periods of several weeks and during freezing and thawing of cells (data not shown). Amplification and sequence analysis of the exogenous EVI1 gene from U937_EVI1 cells confirmed that it was intact and did not contain any mutations.

To investigate whether a cooperating gene might be activated by the retroviral vector, integration site analysis was performed by LAM PCR. Shortly after being sorted for eGFP positivity, U937_vec and U937_EVI1 cells contained 25 and 10 uniquely mappable integration sites, respectively. After another 12–15 weeks of total culture time, interrupted by two freeze-thaws, the number of integration sites was reduced to 15 for U937_vec and 6 for U937_EVI1 cells. At that time, both lines contained predominant integration sites: in U937_vec cells, 79% of these mapped near the dermatan sulfate epimerase ([DSE] gene in chromosome band 6q22, and 99% of the integration sites in U937_EVI1 cells were located in the vicinity of the ubiquinol-cytochrome c reductase-Rieske iron-sulfur polypeptide 1 ([UQCRFS1]) gene in 19q12-13. However, according to gene expression microarray analysis neither of the two genes was differentially expressed between the two cell lines. Also, no other gene in band 2 of chromosome arm 6q or in 19q was upregulated by more than 3-fold in U937_vec or U937_EVI1 cells compared to the respective other cell line, indicating that retroviral integration did not cause activation of adjacent genes.

Effects of EVI1 on Proliferation of U937 Cells in vitro and in vivo

The growth rates of U937_vec and U937_EVI1 cells did not appear grossly different during regular passaging. To determine a possible effect of EVI1 on cell cycling with more precision, nuclei
from exponentially growing EVI1 overexpressing and control cells were stained with propidium iodide and subjected to FACS analysis. As shown in Fig. 2A and B, the cell cycle profiles of both lines differed only marginally, if at all.

To investigate the consequences of EVI1 overexpression in vivo, CB-17 scid/scid mice were injected subcutaneously with U937_vec and U937_EVI1 cells. U937_EVI1 tumors grew significantly more rapidly than tumors derived from U937_vec cells (Fig. 2C), and all animals with EVI1 overexpressing tumors had to be sacrificed by day 17 of the experiment versus day 22 for mice with control tumors. Immunohistochemical analysis confirmed that EVI1 protein was persistently expressed at high levels in the U937_EVI1 derived tumors (Fig. 2D). Staining with an antibody against the early myeloid differentiation marker CD11b revealed the presence of some CD11b positive cells in, but no marked differences between, U937_EVI1 and U937_vec derived tumors (Fig. 2E). In summary, even though EVI1 had only minimally enhancing effects on cellular proliferation in vitro, it greatly enhanced growth and aggressiveness of tumors in vivo.

EVI1 Interferes with Differentiation of U937 Cells

We next asked whether overexpression of EVI1 would affect the differentiation of U937 cells in response to appropriate inducers in vitro. U937_vec and U937_EVI1 cells were cultured in the absence or presence of TPA for five days and analyzed for morphological alterations as well as changes in the cell surface expression of CD11b. As expected, the vast majority of the U937_vec cells was CD11b negative in the absence of TPA, but about 70% became positive for this marker under differentiation conditions (Fig. 3A). In contrast, 30% of the U937_EVI1 cells stained positive for CD11b even in the absence of TPA, but this percentage did not increase further in the presence of the differentiation agent (Fig. 3A). Morphological analysis revealed

Figure 6. EVI1 and etoposide regulate overlapping sets of genes. cRNAs from two independent cultures of U937_vec and U937_EVI1 cells treated or not treated with 400 nM etoposide for 48 h were hybridized onto Affymetrix ST1.1 arrays. Only genes deregulated at least 2-fold in both experiments were considered as differentially expressed. Gene set enrichment analysis (GSEA) (68) was performed to evaluate concordant differences of A) etoposide regulated genes within the gene expression list ranked by average log2-fold changes between U937_EVI1 and U937_vec cells, and B) EVI1 regulated genes within the gene expression list ranked by average log2-fold changes between etoposide treated and untreated U937_vec cells. The normalized enrichment scores were 1.9 in A) and 2.2 in B); the p-values and the q-values of the false discovery rate were 0.0 in both analyses. doi:10.1371/journal.pone.0056308.g006
proportion of viable HL-60_EVI1 versus HL-60_vec cells in the presence of 60 nM daunorubicin (Fig. 5B). Finally, the proportion of eGFP positive HL-60_EVI1, but not HL-60_vec, cells increased with increasing doses of daunorubicin (Fig. 5C), indicating that EVI1 expression conferred a survival advantage during treatment with the cytotoxic drug.

EVI1 and Etoposide Alter the Expression of Overlapping Sets of Genes

In order to gain an understanding of the molecular mechanisms mediating the EVI1 induced resistance towards chemotherapeutic drugs, RNA was extracted from U937_vec and U937_EVI1 cells after 48 h of incubation in the absence or presence of etoposide, and hybridized to human gene 1.1 ST arrays. This experiment was performed in duplicate, and only genes induced or repressed at least 2-fold in both experiments were considered as regulated by the respective condition. According to this criterion, 77 unique genes were regulated by EVI1 (23 induced and 54 repressed), and 419 unique genes were regulated by etoposide (327 induced and 92 repressed). 26 genes were regulated by both EVI1 and etoposide: 14 were upregulated and 4 were downregulated by both conditions, and 8 genes were induced by etoposide and repressed by EVI1 (Table S1). Thus, while only 0.4% of the 19255 genes on the 1.1 ST array with a current RefSeq annotation were regulated by EVI1, 6.2% of the etoposide regulated genes were also regulated by EVI1 (15.5-fold enrichment, p = 9.9 \times 10^{-24}, Fisher’s exact test). Conversely, 2.2% of all genes, but 33.8% of the EVI1 regulated genes were regulated by etoposide (15.4-fold enrichment, p = 6.0 \times 10^{-21}, Fisher’s exact test) (Fig. 6).

While our results confirm the recently reported induction of CD52 by EVI1 [50], ITGA6, another presumptive EVI1 target gene [51], was not induced, but rather slightly downregulated in U937_EVI1 cells. Likewise, PTEA was not repressed by EVI1 in our system, even if the threshold for differential expression was lowered to the 1.3-fold used in the publication reporting this
In search of a molecular mechanism that could explain the partial resistance of U937_EVI1 cells to chemotherapeutic drugs, we subjected genes coding for classical apoptosis regulators to particular scrutiny. However, even with a cut-off of 1.3-fold, BCL2, MCL1, BAG3, NOXA1, BID, BIK, BAX, BAK1, and XIAP were not regulated by EVII. The survival genes BCL2A1 and BCL2L1 were induced 1.3-fold and repressed 1.6-fold, respectively, in U937_EVI1 versus U937_vec cells. In contrast, the antiapoptotic gene CDKN1A/p21/WAF was strongly induced both by EVII and etoposide.

**CDKN1A/p21/WAF Contributes to the Effects of EVII on Cellular Resistance to Cytostatic Drugs**

Because CDKN1A/p21/WAF has been shown to mediate resistance against DNA damaging agents in a number of studies, and because it was strongly regulated by both EVII and etoposide in our model system, we focussed further studies on this gene. qRT-PCR confirmed a 3.3-fold induction of CDKN1A/p21/WAF by EVII and a 20-fold induction by both etoposide and daunorubicin (Fig. 7A, B). Immunoblot analysis corroborated the regulation of p21 by EVII and etoposide at the protein level (Fig. 7C, D). To investigate the subcellular localization of p21 as

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**Figure 8. CDKN1A/p21/WAF overexpression partially mimics the chemotherapy resistance phenotype of EVII overexpression.** A) Immunoblot analysis confirming overexpression of p21 in U937_vec and U937_EVI1 cells infected with pMIA-II_CDKN1A-IRES-Ametrine. β-Tubulin was used as loading control. Detection of endogenous p21 would require longer exposure times. B) Immunoblot analysis of cytoplasmatic (cyto) and nuclear (nu) extracts from U937_vec and U937_EVI1 cells infected with pMIA-II_CDKN1A-IRES-Ametrine. The same amount of protein was loaded in each lane (corresponding to up to twice as many cell equivalents for nuclear versus cytoplasmatic extracts). The cytoplasmatic protein β-tubulin and the nuclear protein RCC1 were used as loading controls. C) Cell cycle distribution of CDKN1A overexpressing and control cells. Shown are means±SEMs from 3 independent experiments. *, p<0.05 (Student’s t-test). D) CDKN1A overexpression increases resistance to etoposide. Cells were treated with the indicated concentrations of etoposide for 48 h and ATP content was determined as a proxy for cellular viability. Shown are means±/SEM from three independent experiments. Open symbols, cells without overexpression of CDKN1A; closed symbols, cells overexpressing CDKN1A; diamonds, U937_vec derivatives; triangles, U937_EVI1 derivatives. *, p<0.05; **, p<0.01 (paired Student’s t-test; referring to the difference between the U937_vec derived cell lines); #, p<0.05 (referring to the difference between the U937_EVI1 derived cell lines).

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well as a possible effect of EVII on it, immunoblot analysis was performed on nuclear and cytoplasmic extracts from U937_vec and U937_EVI1 cells. These experiments showed that the p21 protein was predominantly (but not exclusively) cytoplasmic in both U937_vec and U937_EVI1 cells (Fig. 7D).

In order to ask whether upregulation of CDKN1A/p21/WAF was required for EVII-induced chemotherapy resistance, we attempted to knock down this gene using siRNAs, shRNAs, or antisense constructs. However, all of these experiments were unsuccessful due to a lack of efficiency and/or strong elevation of basal p21 levels under control conditions. We therefore infected U937_vec and U937_EVI1 cells with retroviral vectors containing the CDKN1A/p21/WAF cDNA (pMIA-II-CDKN1A-IRES-Ametrine) or the corresponding empty vector (pMIA-II-IRES-Ametrine) to investigate whether experimental expression of CDKN1A would mimic the phenotype caused by experimental expression of EVII. Infected cells were sorted for Ametrine positivity, and overexpression of p21 was verified by immunoblot analysis (Fig. 8A). The localization of experimentally expressed p21 resembled that of endogenous p21, being mostly, but not exclusively, cytoplasmic (Fig. 8B). FACS analysis of propidium iodide stained nuclei showed that overexpression of p21 increased and decreased the proportions of cells in G0/G1 and S phase, respectively, in both U937_vec and U937_EVI1 cells (Fig. 8C). These effects were relatively small, possibly due to the fact that exogenous p21 was mostly located in the cytoplasm. CDKN1A/p21/WAF overexpression also conferred partial resistance to etoposide as measured by the cell viability assay (Fig. 8D). This phenotype was present both in U937_vec and in U937_EVI1 cells, reflecting the fact that p21 was elevated above endogenous levels in both cell lines.

Discussion

EVII has been reported to affect the proliferation, differentiation, and apoptosis of hematopoietic cells, albeit not necessarily in a consistent manner [52]. For example, EVII accelerated, left unaffected, or retarded the cell cycle, depending on the experimental model under investigation [51,52,53,54,55,56,57,58,59]. Species differences may play a role in that the growth of murine cells was mostly stimulated by EVII [55,56], while the proliferation of human hematopoietic cells was inhibited according to several recent reports [51,53,54]. Our own earlier results showed that inducible expression of EVII in U937 cells strongly inhibited their proliferation [53], and prolonged culture of these cells under inducing conditions was associated with loss of EVII expression (T.A. Konrad, D.H., and R.W., unpublished results). Similarly, Yamakawa et al reported that EVII overexpressing U937 cells grew more slowly and accumulated in G0 to a higher extent than the corresponding control cell line [51]. In contrast, the U937_EVI1 cell line we describe here proliferated normally in vitro and sustained EVII overexpression over prolonged periods of time. When grafted into SCID mice, U937_EVI1 cells even gave rise to significantly larger and faster growing tumors than the corresponding control cells. The reasons for the difference between the in vivo and in vitro growth phenotypes of U937_EVI1 cells are likely to be related to effects of the microenvironment. Together with published data, our results suggest that the effects of EVII on cellular growth and proliferation are complex and highly context dependent, and their elucidation will require additional investigations in a variety of complementary model systems.

To address the possibility that the phenotypes of U937_EVI1 cells were partially or entirely due to transcriptional activation of genes by retroviral vector insertion rather than to expression of the EVII gene itself, LAM-PCR was performed. Even though these experiments revealed that U937_EVI1 cells were nearly monoclonal after several weeks in culture, the same was true for U937_vec cells, and there was no evidence that the predominant vector integration event activated nearby genes in either of the two cell lines. It is therefore highly unlikely that cooperation with a gene activated by retroviral insertion facilitated the continuous proliferation, or any other phenotype, of U937_EVI1 cells. Furthermore, sequence analysis of the vector-borne EVII cDNA in the nearly monoclonal state excluded the possibility that U937_EVI1 cells were able to proliferate due to a defect in the exogenous EVII gene. Because EVII has been shown to cooperate with N-RAS mutations in human and murine leukemia [60,61,62,63], we sequenced the N-terminus of the N-RAS gene in U937_EVI1 cells, but found no alterations in the mutational hotspots in exons 2 and 3. At present, the possibility that a different mutation or a stochastic change in gene expression may cooperate with EVII to allow normal proliferation of U937_EVI1 cells cannot be ruled out. In fact, the identification of such an alteration may help to explain how AMLs with overexpression of EVII are able to maintain the increased proliferation rate associated with this disease, and will therefore be attempted in future studies. We are nevertheless confident that such a secondary alteration is not the true reason - rather than EVII overexpression per se - for the most interesting phenotype of U937_EVI1 cells, namely their partial resistance to antileukemic drugs, because this phenotype could be reproduced in independently infected U937 cells (data not shown), as well as in HL-60 cells expressing EVII in an inducible manner.

EVII has previously been reported to inhibit apoptosis in response to several stimuli, including the anticancer agents taxol and IFN-γ [23,24,25]. Nevertheless, until recently no data were available regarding a role of EVII in resistance to drugs used in the treatment of AML. We now show that EVII partially protected human myeloid cells from the cytotoxic effects of etoposide and the anthracycline daunorubicin, both of which induce DNA double strand breaks and are used in AML therapy. In fact, anthracyclines are one of the two pillars of current AML regimens and accordingly were also included in all of the large studies in which EVII overexpression and/or 3q26 rearrangements were found to be associated with a poor prognosis [8,9,10,11,12,60,64,65,66,67]. For some reason, however, we did not observe an increased resistance of U937_EVI1 cells to cytotoxic araC also in suspension culture, a context in which a role of ITGA6 could be demonstrated in UCSD/AML1 cells to araC, and proposed that the cell adhesion molecule integrin α6 [ITGA6] played a role in EVII induced chemotherapeutic resistance: ITGA6 was upregulated by EVII and increased the adhesion of human hematopoietic cell lines to matrigel and stromal cells, and antibodies against ITGA6 decreased the viability of matrigel grown cell lines with high EVII expression in the presence of araC [51]. Interestingly, however, experimental downregulation of EVII enhanced cellular sensitivity towards araC also in suspension culture, a context in which a role of ITGA6 would seem less obvious [51]. Therefore, and out of the general consideration that a single downstream molecule is unlikely to fully explain the phenotype caused by the expression of a transcription factor, additional EVII targets are likely to contribute to the chemotherapy resistance induced by this oncoprotein.

Our microarray analyses revealed a substantial and highly significant overlap between gene expression changes elicited by EVII expression and etoposide treatment, suggesting that EVII overexpressing cells may be partially pre-adapted to the exposure...
to cytotoxic drugs. Among the genes that were upregulated by both EVI1 and etoposide, CDKN1A/p21/WAF appeared of particular interest. CDKN1A/p21/WAF has been previously shown to be both upregulated by cytotoxic drugs and to protect from their effects, implying that its induction under these conditions represents a cellular defense mechanism rather than a pro-apoptotic event. This has been amply demonstrated also for hematopoietic cells [29,30,31,33,35,36,37,39]. Pathological overexpression of p21 was associated with chemotherapy resistance and poor prognosis in several tumor types, including AML [26,27,28]. In addition, CDKN1A/p21/WAF has been reported to be induced in response to several leukemia associated oncogenes and to play a role in protection from DNA damage in this context [35,40,41]. According to a very recent report, CDKN1A was upregulated by EVI1 in the human osteosarcoma cell line U2OS [57]. In the experimental system presented here, CDKN1A/p21/WAF was induced by etoposide and by EVI1. Interestingly, the p21 protein was located mostly in the cytoplasm both in the absence and presence of EVI1, and even in cells experimentally expressing this protein, agreeing with the fact that the anti apoptotic activity of p21 was associated with a cytoplasmatic location in many cases [26,27,34]. Experimental overexpression of p21 in U937 vec and U937_EVI1 cells mimicked the effects of EVI1 overexpression in that it mediated partial protection from chemotherapeutic drugs. Clearly, a full understanding of the molecular mechanisms through which EVI1 confers chemotherapy resistance to AML and other malignant diseases will require further studies. The ultimate goal of these efforts is the development of new therapeutic options to improve the prognosis of patients with EVI1 overexpressing malignancies.

Supporting Information

Table S1 Genes regulated by EVI1, etoposide, or both in U937 cells.

(XLS)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: AR BS DH PH FH MF KS HG AK WB RW. Performed the experiments: AR BS DH PH FH MF KS HK SH IH IS KK. Analyzed the data: AR BS DH HH PH FH MF KS HK SH IH IS AK WB RW. Contributed reagents/materials/analysis tools: PR. Wrote the paper: RW.

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