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Regulation of Human Endogenous Retrovirus-K Expression in Melanomas by CpG Methylation

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Running title: METHYLATION OF HERV-K

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The overall prognosis of patients with advanced melanoma is poor due to the lack of effective treatment. A key factor for successful therapy is an early detection of disease. Therefore reliably detection methods and meaningful tumor markers are required. Expression of the human endogenous retrovirus (HERV)-K(HML-2) was found elevated in melanomas and it was shown that HERV-K supports the in vitro transition of melanoma cells from adherent to a more malignant, non-adherent phenotype. Furthermore, detection of HERV-K-specific antibodies in melanoma patients was found to correlate with reduced survival. However, the reason for HERV-K expression in melanomas still remains unclear and its use as a tumor marker needs further investigation. Therefore, the tumor-specific transcriptional regulation of HERV-K expression in melanoma was studied in detail. Human melanoma cell lines were investigated for HERV-K expression using real-time PCR. Five cell lines showed very high levels of HERV-K mRNA as a result of increased promoter activity. This promoter activity was directly silenced by DNA methylation in reporter gene experiments. Higher levels of long terminal repeat (LTR) methylation in cells not expressing HERV-K compared with cells expressing HERV-K were found using methylation-sensitive PCR and bisulfite sequencing. Treatment of cell lines with the demethylating agent 5-aza-2'-deoxycytidine resulted in increased levels of HERV-K expression in cells previously not expressing HERV-K and it was shown that this increase is not the result of transcription factor activation. These results demonstrate that increased HERV-K expression in melanomas may be due to increased promoter activity and demethylation of the 5'LTR.

INTRODUCTION

The human genome comprises approximately 60 full-length HERV-K(HML-2) proviruses and about 2500 HERV-K solo long terminal repeats (LTR) (Gifford and Tristem, 2003; Mager and Medstrand, 2003). The origin of these proviruses and LTR elements were retroviral infections of germ-line cells before and during primate evolution and subsequent amplification inside the host via retrotransposition, reinfection and complementation in trans. As a consequence of mutations, deletions and internal homologous recombination most of these retroviral elements lost their replication capability and infectivity. But open reading frames and viral protein expression can still be found and there is accumulating evidence that HERVs play a direct role in tumor induction and progression as well as in physiological processes (Boese et al., 2000; Denne et al., 2007; Serafino et al., 2009; Denner, 2010).

The family of HERV-K(HML-2) can be subdivided into type 1 and type 2 proviruses. Type 1 proviruses are characterized by a 292 base pair (bp) deletion at the *pol-env* boundary and an open reading frame (ORF) for the nonstructural protein Np9 (Armbruester et al., 2002). Type 2, the undeleted prototype, has an intact *env* sequence and encodes the accessory protein Rec (Lower et al., 1993, 1995). Both proteins were found expressed in tumors and transformed cell lines and evidence has accumulated that they play a role in cancer development. Rec and Np9 directly bind to the promyelocytic leukemia zinc finger (PLZF) protein and inhibit the transcriptional inhibiting function of PLZF. As a result the *MYC* promoter loses its repressed state and MYC expression is promoting cellular proliferation (Denne et al., 2007). In addition, mice that were transgenic for Rec showed a development of carcinoma in situ, an early marker for testicular cancer (Galli et al., 2005). Expression of HERV-K(HML-2) mRNA and protein was found in germ cell tumors (Lower et al., 1984; Herbst et al., 1998), in melanomas (Muster et al., 2003; Buscher et al., 2005, 2006) and in human breast carcinomas (Wang-Johanning et al., 2003). A matter of particular interest is the

expression of HERV-K(HML-2) in human melanomas. The presence of anti-HERV-K antibodies in early stages of the disease was shown to be a marker of reduced survival (Hahn et al., 2008). Furthermore, HERV-K was reported to support the in vitro transition of melanoma cells from adherent to a more malignant, non-adherent phenotype when exposed to stress conditions (Serafino et al., 2009).

The regulation of HERV-K(HML-2) expression in melanomas is unclear. Besides transcriptional regulation via transcription factors (TFs) (Ono et al., 1987; Akopov et al., 1998; Knossl et al., 1999; Kwun et al., 2002), an epigenetic regulation via DNA-methylation was reported in teratocarcinomas (Gotzinger et al., 1996). A correlation between methylation of the U3 region of the HERV-K(HML-2) 5'LTR and the transcriptional activity of the LTR was shown (Lavie et al., 2005).

To understand the high transcriptional activity of HERV-K(HML-2) proviruses in melanomas and to demonstrate their cancer specific expression, we investigated the regulation of these retroviral elements in more detail. A panel of human melanoma cell lines was analyzed for HERV-K-expression, promoter activity and the methylation status of the 5'LTR. Based on these data it was shown that cells with high HERV-K-expression compared to cells with basal levels of HERV-K mRNA exhibited genetic and epigenetic changes. High levels of HERV-K expression were only found in melanoma cell lines that also showed a high HERV-K promotor activity. In addition, those cell lines exhibited low levels of LTR methylation. In line with this cell lines not expressing HERV-K showed stronger methylation of 5'LTRs and expression could be stimulated by treatment with the demethylating agens 5-aza-dC. Therefore, melanoma cell lines that showed high levels of HERV-K expression must have lost methylation and, in addition, a deregulation of HERV-K specific TFs must have occurred.

MATERIALS AND METHODS

Cell Lines and Treatments

The SK-MEL-13 and SK-MEL-19 melanoma cell lines were obtained from Dr. J. Eberle (Department of Dermatology, Charité-Universitätsmedizin, Campus Benjamin Franklin, Berlin, Germany), and the SK-MEL-24 and SK-MEL-37 were a gift from Dr. Peter Walden (Department of Dermatology, Charité Campus Mitte, Berlin, Germany). The cell lines were cultured as described previously (Bredenbeck et al., 2005; Fecker et al., 2005). The human melanoma cell lines SK-MEL-28, SchM, SchW, TR, ZO, KU, RA, and ZD, the human embryonic kidney cell line 293, the human teratocarcinoma cell line GH and the isolation of PBMCs have been described previously (Buscher et al., 2005). HEK-293 and melanoma cell lines were treated with 2 µM 5-aza-dC (Sigma Aldrich, Germany) and 50 nM trichostatin A (Sigma Aldrich) and GH cells with 1 µM 5-aza-dC and 25 nM trichostatin A. The drug containing medium was renewed every day and cells were harvested after 3 days of treatment.

RNA and DNA Preparation

Total RNA and genomic DNA from cell cultures were isolated using RNeasy and DNeasy Kits, respectively, according to manufacturer’s instructions (Qiagen, Hilden, Germany). Quality and quantity of both RNA and DNA were determined by 1% agarose gels and photometry.

Quantitative Real-Time RT-PCR

500 – 2000 ng of total RNA were reverse transcribed using the RevertAid™ H Minus M-MuLV Reverse Transcriptase Kit (Fermentas, St. Leon-Rot, Germany) and Random Hexamer Primer according to manufacturer's protocols. Then the reaction products were diluted 1:10 and 1µl was used for performing quantitative real-time PCR using the Brilliant SYBR® Green QPCR Master Mix and the Mx4000® QPCR System (Stratagene, Germany).

For detection of HERV-K(HML-2) *env* mRNA the primer pair Pfor/Penv, for detection of the *rec* mRNA the primer pair Pfor/Prec and for detection of *np9* mRNA the primer pair Pfor/Pnp9 was used. Within each experiment the housekeeping gene *GUSB* was used for data normalization (for primer sequences see supplemental material Table 1).

HERV-K108 LTR Luciferase Promoter Reporter Construct

The 5'LTR reporter construct was generated by cloning a PCR fragment starting at position -283 bp upstream of the 5'LTR of HERV-K provirus K108 until position +1255 bp. Following PCR, amplicons were subcloned into the luciferase vector pGL3-Basic (Promega, Mannheim, Germany) and subclones were verified by DNA sequencing.

Dual Luciferase Reporter Gene Assay

Luciferase activity was measured after transient transfection of the pGL3 basic vector (determination of background luciferase activity), the pGL3-LTR vector (analyzing LTR-activity) and the pGL3-control vector (positive control). To normalize the transfection efficiency, a cotransfection of the renilla luciferase expression vector pRL-CMV was performed. For transfection experiments, $2x10^5$ cells were cultured overnight in 48 well tissue culture plates. Luciferase reporter plasmids were added together with Lipofectamine 2000 according to manufacturer's protocols (Invitrogen, Karlsruhe, Germany). All experiments were performed at least in triplicates. Measurement of luciferase expression was done according to manufacturer's protocols (Promega).

In Vitro DNA Methylation.

The 5'LTR/reporter construct was methylated by *Sss*I DNA methylase according to supplier's instructions (New England Biolabs, Frankfurt, Germany). Four different amounts of *Sss*I DNA methylase (0 U, 0.75 U, 2 U, 4 U) were used for generating different levels of in

vitro methylated reporter plasmids. For each reaction sample, 6 µg of plasmid-DNA was incubated in 30 µl of 1xNE buffer 2 for 4 hr at 37°C. The reaction was terminated by heat inactivation (65°C for 20 min) and the samples were purified using the Qiagen QIAquick® PCR Purification Kit (Qiagen). Control of methylation was performed with the methylation sensitive restriction enzyme *Hpa*II according to the manufacturer's manual (New England Biolabs).

Methylation Analysis by Genomic Sequencing

Sodium bisulfite treatment was performed as previously described (Frommer et al., 1992). Locus-specific nested PCR was performed using primer pairs BSS-K108-183fw/BSS-K108+1306rev and BSS-K108-40fw/BSS-K108+1121rev under conditions described elsewhere (Schenk et al., 2007). PCR amplification was performed for 32 cycles of 30 sec at 95°C, 20 sec at 55°C and 200 sec at 72°C. The second amplification step was carried out using 1/100 of the first PCR product for 32 cycles of 30 sec at 95°C, 20 sec at 55°C and 180 sec at 72°C. Then the PCR-fragments were subcloned into a TA-Cloning vector. Transformation into *E.coli* Top10 F' competent cells was performed with $CaCl_2$. Plasmid DNA was prepared from transformed clones and sequenced using M13-promotor-primers and the ABI BigDye 3.1-Mastermix (ABI Terminator Chemie, Applied Biosystems, Darmstadt, Germany).

Methylation Analysis by Methylation Sensitive Restriction Endonucleases

200 ng of genomic DNA from each cell line were digested over night in 30 µl reaction volume using the methylation sensitive endonucleases *Hpa*II, *Bsa*AI (New England Biolabs) and *Tau*I (Fermentas, St. Leon-Rot, Germany), according to the manufacturer's protocols. Each reaction batch also contained 1 U *Eco*RI (New England Biolabs) for uniformly shearing of genomic DNA (no restriction site within the HERV-K 5'LTR). For normalization of the

PCR reaction 200 ng genomic DNA were digested by *Eco*RI without the addition of methylation sensitive endonucleases. After digestion, 1 µl of the reaction volume was added to 14 µl of PCR mixture containing 1.5 µl of GeneAmp 10X PCR Gold Buffer (Applied Biosystems), 5% DMSO, 200 µM of each of the four deoxynucleoside triphosphates, 0.2 µM of HERV-K(HML-2) locus specific primers and 0.5 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems). PCR amplification was performed for 28 cycles of 30 sec at 95°C, 20 sec at 60°C and 60 sec at 72°C. The PCR reaction was then analyzed using agarose gel electrophoresis and DNA staining with ethidium bromide.

To verify the existence of *Hpa*II restriction sites, digestion was performed using the methylation insensitive *Hpa*II isochizomer *Msp*I. Due to the absence of methylation insensitive isochizomers for the restriction enzymes *Bsa*AI and *Tau*I, the verification of the presence of specific restriction sites for those enzymes was performed using PCR products of the investigated HERV-K proviruses as substrates for digestion.

To verify the assay, the 5'LTR of HERV-K 22q11 was subcloned into a TA-Cloning vector and in vitro methylation was performed by *Sss*I DNA methyltransferase as described above. The differentially methylated vector was used for digestion and PCR-amplification as also described above.

RESULTS

High Levels of Spliced HERV-K mRNA in Human Melanoma Cell Lines

In order to analyze HERV-K expression in a panel of melanoma cell lines comprising the previously analyzed cell lines SK-MEL-28, RA, TR, SchM, SchW and ZD (Buscher et al., 2005; 2006) and the cell lines SK-MEL-13, SK-MEL-19, SK-MEL-24, and SK-MEL-37 not yet investigated, a SYBR-Green real-time RT-PCR specific for HERV-K *env, rec* and *np9* mRNA was developed. This method allows the amount of spliced *env, rec* and *np9* mRNA to be estimated. The relative HERV-K expression was calculated by the 2(*-delta delta Ct*)

method (Livak and Schmittgen, 2001) in comparison with the HERV-K expression in the teratocarcinoma cell line GH known to express HERV-K mRNAs at a very high level (Buscher et al., 2005). Five of the eleven cell lines showed high levels of HERV-K *env* and *rec* mRNA expression (Fig. 1A). These cell lines also showed increased (but not high) levels of *np9* mRNA (0.2% of the expression in GH cells) (Fig. 1A). The other six cell lines showed a very low expression of HERV-K comparable with the expression of HERV-K in the human embryonic kidney cell line HEK-293.

High LTR Activity in Melanoma Cell Lines Correlated with High HERV-K Expression

To analyze the regulation of transcription initiation (one of the most important regulatory events in human gene expression) the melanoma cell lines SK-MEL-13, SK-MEL-19, and SchM (characterized by high HERV-K expression) as well as SK-MEL-24, SK-MEL-37, and RA (characterized by low HERV-K expression) were analyzed. Regulation of transcription initiation may be accomplished by two basic principles. First, by the availability of promoter specific TFs that enhance the binding of RNA-polymerases (RNAP) and second, by the presence of open promoter structures that enable the binding of the activated TFs to the promoter. To define the presence of HERV-K specific TFs in melanoma cell lines, the HERV-K 5'LTR activity was measured using a dual luciferase reporter gene assay. All cell lines (except cell line RA which showed only about 20% of luciferase activity in comparison to the other cell lines) showed nearly the same luciferase activity, when the pGL3b-control vector was used, indicating nearly equal transcriptional activities (Fig. 1B). When a luciferase expression vector was used, which contained a HERV-K 5'LTR as a promoter, all cell lines with high HERV-K expression showed a significant higher luciferase activity than cell lines with only a basal level of HERV-K transcription, indicating that HERV-K-expressing cell lines had a different level of activated HERV-K-specific TFs compared to cell lines not

expressing HERV-K (Fig. 1B). These results raised the question whether high HERV-K LTR activities were typical for melanomas or whether only a few tumor cell lines express sufficient TFs able to activate HERV-K LTRs. To answer this question a set of another ten melanoma cell lines was randomly selected without knowing their level of HERV-K mRNA expression. These cell lines were investigated for LTR activity as shown above. In none of the ten cell lines was high LTR activity detected (Fig. 1C), suggesting that high HERV-K LTR activity is not a general property of melanoma cells but may be the result of disturbed regulation during tumor progression.

The LTR Activity in Melanoma Cell Lines Depends Directly on the CpG Methylation Status of the 5'LTR.

As mentioned above, regulation of gene transcription depends not only on the availability of activated TFs but is also regulated by the accessibility of the activated TFs and the RNAP II to the promoter. It had been shown previously that the promoter activity of HERV-K(HML-2) 5'LTRs was reduced after CpG methylation in the teratocarcinoma cell line Tera-1 (Lavie et al., 2005). These data raised the question whether methylation also plays an important role in HERV-K silencing in melanoma. To answer this question, the HERV-K LTR-driven reporter gene construct pGL3-LTR was methylated in vitro using the CpG methyltransferase *Sss*I (Fig. 2A). Using increasing amounts of methyltransferase higher levels of methylation were achieved. After transient transfection of these constructs into cell lines expressing HERV-K, all analyzed cell lines showed a loss of promoter activity dependent on the level of CpG-dinucleotide methylation. Cells that were transfected with plasmids showing nearly full methylation exhibited only basal levels of luciferase expression comparable with the LTR activity measured in cell lines not expressing HERV-K (Fig. 2B). These findings indicated that the assembly of TFs and the RNAP II on the HERV-K(HML-2) 5'LTR is directly or indirectly inhibited by CpG-methylation in melanomas**.**

Cell Lines Expressing HERV-K Showed a Lower LTR Methylation Level than Cell Lines With Low HERV-K Expression

To analyze the methylation status of different HERV-K 5'LTRs in melanoma cell lines a methylation detection assay was established using three methylation sensitive restriction enzymes (see Materials and methods and supplemental Fig. 1A). To validate the assay, plasmids that contained the 5'LTR of HERV-K 22q11 methylated at increasing levels were used as a substitute of genomic DNA. Digestion of the plasmid DNA by the restriction enzymes *Hpa*II and *Bsa*AI showed that strong and intermediately methylated sites could be detected. Using *Tau*I for restriction only strong CpG methylation could be seen (supplemental Fig. 1 B). For a comprehensive analysis of the methylation status of HERV-K-5'LTRs in melanoma cell lines, specific primers for ten proviruses and two solo LTRs were designed. The 5'LTR methylation level was investigated for the HERV-K expressing cell lines GH, SK-Mel-13 and SchM and for the cell lines HEK-293, SK-MEL-24 and RA, characterized by low expression of HERV-K. Thereby, low levels of methylation were found in the cell line GH and in the melanoma cell line SK-MEL-13. High levels of methylation were found in the cell line HEK-293 and in the melanoma cell lines SK-MEL-24 and RA (Table 1). Interestingly, in the melanoma cell line SchM, which showed an increased level of HERV-K-mRNA and a strong promoter activity, demethylation was only found for two proviruses and the two solo LTRs. Furthermore, demethylation of 5'LTRs was detected for almost all 5'LTR in the cell lines GH and SK-MEL-13, indicating a genome-wide demethylation of HERV-K 5'LTRs. Noticeable, a higher methylation status of the U5-region was observed for most of the investigated 5'LTRs in each cell line.

Bisulfite Sequencing of the HERV-K108 5'LTR and 5'UTR

For a detailed analyses of the methylation level of HERV-K(HML-2) 5'LTRs the first

locus of the provirus HERV-K-108 on 7p22 was selected. Our previous studies had shown that most HERV-K mRNAs expressed in SK-MEL-28 cells were derived from HERV-K-108 (unpublished data). Using a modified bisulfite sequencing protocol, for the first time methylation in the R, U5 and 5' untranslated region of a single HERV-K(HML-2) provirus was analyzed. Whereas the cell line HEK-293 and peripheral mononuclear cells (PBMCs), both not expressing HERV-K, showed nearly total methylation of the investigated sequence (Fig. 3), strong demethylation was detected in the teratocarcinoma cell line GH. In this cell line methylation was only observed at single CpG sites in the U5 region and the 5'UTR. Whereas in cell lines expressing HERV-K demethylation of HERV-K-108 was observed in the whole investigated sequence (except SK-MEL-28 that showed some methylation of the last CpG-dinucleotides in the 5'UTR), cell lines with low levels of HERV-K mRNA showed stronger methylation of the U5 region and the 5'UTR. All melanoma cell lines not expressing HERV-K also showed demethylation in U3 and the starting R-region (Fig. 3). These data confirm differences in methylation of HERV-K(HML-2) 5'LTRs between cell lines expressing or not expressing HERV-K and that methylation negatively correlates with HERV-K expression.

5-aza-dC Stimulates HERV-K Expression in Melanoma Cell Lines.

To demonstrate that CpG methylation is an epigenetic silencer of HERV-K transcription in melanoma cell lines, control and melanoma cell lines were treated with the demethylating agent 5-aza-dC. After treatment, changes in HERV-K expression were measured by quantitative real-time RT-PCR using primers for *env, rec* and *np9*. Thereby, treatment of GH cells with 5-aza-dC resulted in a slight increase of expression of these genes (Fig. 4A). In contrast, a very strong increase of HERV-K *env* and *rec* and a slight increase of *np9* expression were detected in HEK-293 cells (Fig. 4B), in which HERV-K is normally not expressed. Similar results were obtained when treating melanoma cell lines. In the HERV-K-

expressing melanoma cell lines SK-MEL-13 and SK-MEL-19 no changes in HERV-K expression were observed after treatment with 5-aza-dC (with exception of a small increase of *np9* mRNA in SK-MEL-13). On the other hand, melanoma cell lines with a low level of HERV-K expression showed a significant increase in the levels of *env, rec and np9* mRNA. This increase was also measured in the cell line RA but it was not as strong as in cell lines, which do not express HERV-K. This finding correlates with the low promoter activity shown in RA cells. In contrast, a clear increase of HERV-K *env* and *rec* but not *np9* expression was observed after treatment of the cell line SchM, which highly expressed HERV-K. But, as shown above, the cell line SchM exhibited a much stronger LTR methylation in comparison with SK-MEL-13 (Table 1). In addition to DNA methylation, initiation of HERV-K transcription may also be regulated by complex modifications of histone molecules. Thereby acetylation of histones results in open and transcriptionally active chromatin. In order to investigate the influence of histone acetylation on the transcriptional activity of HERV-K(HML-2), melanoma cell lines were treated with the histone deacetylase inhibitor TSA. Only HEK-293 cells exhibited increased HERV-K-expression as a result of TSA treatment. Co-treatment of HEK-293 cells with 5-aza-dC and TSA showed a synergistic effect on the expression of *env, rec* and *np9*. Such effects were not observed when melanoma cell lines were treated, indicating that transcriptional regulation by histone acetylation may not be a key regulatory element in HERV-K expression.

5-aza-dC Does not Increase the Level of LTR Activating TFs.

The activation of HERV-K-expression by 5-aza-dC in cell lines with low expression of HERV-K may be due to the global demethylation of the genome and therefore due to the activation of HERV-K specific TFs or due to the demethylation of HERV-K-5'LTRs. To discriminate between both possibilities an indirect analysis was performed using transient transfection of an unmethylated LTR reporter gene construct followed by 5-aza-dC treatment.

In parallel, untransfected cells were treated with the same medium that was used for treatment of the transfected cells (containing 5-aza-dC or not). All cell lines treated with 5-aza-dC showed an increase of *rec* expression in comparison to untreated controls indicating a successful treatment with 5-aza-dC (Fig. 5B). In contrast to that, no significant changes in LTR activity was observed as measured by luciferase activity. The cell line SK-MEL-37 exhibited a slight increase in LTR activity after 5-aza-dC treatment by a factor of two (Fig. 5A), which cannot reflect the increase of HERV-K-expression measured by real-time PCR (twelvefold).

DISCUSSION

The overall prognosis of patients with advanced melanoma is very poor due to the lack of effective treatment and late detection of the tumor. Therefore reliably detection methods and meaningful tumor markers are required (Riker et al., 2007). To analyze the tumor-specific expression of HERV-K and to address HERV-K proteins as putative targets for cancer immunotherapy and as tumor markers, the transcriptional regulation of HERV-K in melanomas was investigated. Cell lines with high and cell lines with low expression of HERV-K, especially of the genes encoding the immunosuppressive transmembrane envelope protein and the two accessory proteins Rec and Np9 were identified. Using different assays, a clear correlation between the methylation level in the LTR of ten different proviral LTR and the expression of HERV-K specific mRNA was shown. The extent of methylation of the HERV-K108 5'LTR and 5'UTR was analyzed in great detail, because this provirus was found to be expressed abundantly in melanoma cells (unpublished data). Treatment with the demethylating agent 5-aza-dC resulted in increased levels of HERV-K expression in cells previously not expressing HERV-K and it was shown that this increase is not the result of transcription factor activation. High levels of HERV-K transcription were the result of a disturbed genetic and epigenetic regulation of expression. But these results also demonstrated

that high HERV-K LTR activity is not a general property of melanoma cells.

One important requirement for high levels of HERV-K(HML-2) expression in melanomas is a high promoter activity. This conclusion is based on two observations. First, none of the melanoma cell lines with a low HERV-K mRNA level showed a high luciferase expression after transfection with the unmethylated HERV-K LTR reporter gene construct. Second, none of the randomly chosen melanoma cell lines showed a strong luciferase activity after luciferase HERV-K LTR plasmid transfection – both indicating low levels of HERV-K-specific TFs in those cell lines. Therefore high promoter activities as found in cell lines with high levels of HERV-K mRNA (Fig. 1A) are most likely the result of increased activation of HERV-K specific TFs. Strong LTR activity in melanomas may be the result of higher expression of the HERV-K activating TFs YY1 (Knossl et al., 1999) or AP1 (Kwun et al., 2002). Interestingly, there are higher levels of YY1 mRNA in SK-MEL-28 cells compared with normal skin (Su et al., 2004).

In addition to a transcriptional regulation via TFs there is an epigenetic regulation by CpG-dinucleotide methylation (Lavie et al., 2005). Endogenous LTR elements are usually transcriptionally silenced by CpG methylation in somatic cells (Bestor, 2000) and strong DNA methylation was reported in fetal brain, fetal liver, placenta and adult peripheral leukocytes (Shen et al., 2006). In melanomas, HERV-K promoter activity depends directly on the methylation status of its 5'LTR (Fig. 2). This suggests that HERV-K activating TFs are sensitive to CpG methylation. For example it is known that the HERV-K activating transcription factor YY1 does not bind methylated DNA in vitro (Kim et al., 2003). Interestingly, demethylation of one or more HERV-K-5'LTRs was found in every cell line tested, but in cells with high HERV-K expression less methylation was observed than in cells with only a basal level of HERV-K expression, supporting the hypothesis of a methylation dependent regulation of HERV-K proviruses in melanoma. Especially in the cell lines GH and SK-MEL-13 with the highest HERV-K expression (Fig. 1A), demethylation was found for

nearly every tested 5'LTR indicating genome wide demethylation of HERV-K 5'LTRs. This is in line with the finding that these cell lines did not show an additional HERV-K activation after treatment with the demethylating agent 5-aza-dC. The reason for global demethylation in those cell lines could be a loss of maintaining methylation by the DNA methyltransferase-1 (DNMT1) (Bestor, 2000; Howard et al., 2008). The loss of maintaining DNA methylation and genome wide demethylation could have driven tumor progression by activation of oncogenes or chromosomal destabilization (Howard et al., 2008; Daskalos et al., 2009). Analyzing the expression and functionality of the human DNA methyltransferases DNMT1, DNMT3a and DNMT3b and in parallel the expression of HERV-K(HML-2) could give an insight into the mechanism of HERV-K demethylation in melanoma. In contrast to the hypomethylated cell lines, the melanoma cell lines RA and SK-MEL-24 showed a strong methylation of the investigated 5'LTRs and an increase of HERV-K expression after treatment with 5DC. Nevertheless, HERV-K expression did not reach the mRNA level detectable in cell lines expressing HERV-K, presumably because of a deficit in activated HERV-K specific TFs, underlining the suggested two-step activation mechanism for HERV-K transcription by transcription factor activation and 5'LTR demethylation. Of special interest is the cell line SchM, the only line showing high HERV-K mRNA levels and high LTR activity on the one hand and high methylation of many 5'LTRs on the other. Furthermore, it was the only HERV-K expressing cell line that showed an additional stimulation of HERV-K expression after treatment with 5-aza-dC. Obviously 5-aza-dC demethylates still methylated LTRs which then allow the expression of HERV-K. This cell line clearly confirms that HERV-K proviruses are silenced by DNA methylation in melanoma. These data also indicate that LTR activating TFs do not influence the methylation status of methylated LTRs directly by a recruitment of enzymes that support DNA demethylation and chromatin opening. In a reverse conclusion, HERV-K LTR demethylation may be secondary to the loss of maintaining DNA-methylation.

For the first time, using the bisulfite sequencing technique, the methylation status of a

complete HERV-K(HML-2) 5'LTR and the 5'UTR was analyzed. Demethylation of the U5 region and the 5'UTR was found only in cell lines characterized by high HERV-K expression. This suggests that these regions may contain methylation dependent regulatory elements.

Although most of the analyzes were performed using melanoma cell lines that may not reflect the entire in vivo situation in the primary tumor, in preliminary investigations a comparable high level of HERV-K expression was found in primary melanomas using real time PCR (data not shown). This indicates that the situation of HERV-K expression in melanoma cell lines is comparable to that in melanoma.

To summarize, high levels of HERV-K mRNA expression in melanomas are the result of two processes, namely upregulation of HERV-K activating TFs and a defect in maintaining LTR methylation. Both processes must occur in melanoma cells in order to induce a strong HERV-K(HML-2) expression, as seen for example in germ cell tumors. Therefore, high levels of HERV-K expression in melanomas might be a marker for genetical and epigenetical changes.

Demethylation is obviously necessary for the expression of HERV-K. Since it is unlikely that HERV-K is able to demethylate its proviruses, the loss of methylation must be the result of genetic changes in the cell, e.g., a loss of DNMT1 activity. Therefore, the activation of HERV-K expression ought to be understood as a secondary event. The increase in demethylation may enhance expression of HERV-K mRNA as well as Env, Rec and Np9 proteins. Since there is preliminary evidence that Rec and Np9 may be involved in tumorigenesis (Galli et al., 2005), they may contribute to tumor induction. In addition, the expression of the immunosuppressive Env protein may contribute to tumor progression preventing tumor rejection by the immune system (Mangeney and Heidmann, 1998; Mangeney et al., 2001, 2005; Denner, 2010). In this context it is of great interest whether HERV-K proteins are good targets for cancer immunotherapy. Such investigations will help to understand the role of HERV-K proteins, if any, in the development of melanomas.

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Figure Legends

Figure 1. Expression and promotor activity of HERV-K. (A) Expression of HERV-K mRNAs *env*, *rec* and *np9* compared with their expression in GH cells. (B) Promoter activity in melanoma cell lines as measured by a dual luciferase reporter gene assay using the promotor-less plasmid pGL3-basic, the LTR driven plasmid pGL3b-LTR and the SV40 driven plasmid pGL3b-control. (C) Relative promotor activity in a panel of ten randomly selected melanoma cell lines with unknown HERV-K expression status.

Figure 2. Silencing of HERV-K promoter activity by in vitro methylation of CpG-dinucleotides. (A) Analysis of the methylation status of the in vitro differentially methylated pGL3b-LTR plasmid using the methylation sensitive restriction enzyme *Hpa*II and the methylation insensitive isochizomer *Msp*I. Increasing amounts of *Sss*I DNA methylase (0 U, 0.75 U, 2 U, 4 U) were used for the in vitro methylation reaction to increase the extend of methylation. (B) Inhibition of promotor activity of the HERV-K(HML2) LTR by CpG methylation in GH cells and the melanoma cell lines SchM, SK-MEL-13 and SK-MEL-19.

Figure 3. Bisulfite sequencing (BSS) of the entire 5'LTR of HERV-K108. (A) Schematic presentation of the analyzed sequence, vertical lines indicate the location of CpG-dinucleotides. +1 indicates the first base pair of the 5'LTR (grey). (B) BSS of the control cell lines. Black spots indicate methylated CpG; white spots indicate

absence of methylation. (C) BSS of melanoma cell lines expressing HERV-K. (D) BSS of melanoma cell lines with low expression of HERV-K.

Figure 4. Changes of HERV-K expression in control and melanoma cell lines after treatment with epigenetic agents. Cell lines were cultured in medium (untreated control) or drug containing medium (5-aza-dC, TSA or 5-aza-dC and TSA). After 3 days cells were harvested, RNA was extracted and used for HERV-K *env*, *rec* and *np9* quantification by real-time RT-PCR. (A) Relative changes of HERV-K expression in cell lines expressing HERV-K. (B) Relative changes of HERV-K expression in cell lines not expressing HERV-K.

Figure 5. Promoter activity and rec expression in cells treated (+) or untreated (-) with the demethylating agent 5-aza-dC. (A) promotor activity of the HERV-K-5'LTR as measured by luciferase reporter gene assay. (B) Relative *rec* mRNA expression as measured by real-time RT-PCR.

TABLE 1. HERV-K 5‘LTR CpG Methylation

Genomic	Position of restriction			GH			HEK-293			SK-MEL-13			SchM			RA			SK-MEL-24		
position	*Taq*I	*Bsa*AI	*Hpa*II	*Taq*I	*Bsa*AI	*Hpa*II	*Taq*I	*Bsa*AI	*Hpa*II	*Taq*I	*Bsa*AI	*Hpa*II	*Taq*I	*Bsa*AI	*Hpa*II	*Taq*I	*Bsa*AI	*Hpa*II	*Taq*I	*Bsa*AI	*Hpa*II
1q22	304	711	854	○	○	●	●	●	●	○	○	○	●	○	●	○	●	●	●	●	●
1q23.3	n.r.	712	855		●	●		●	●		○	●		○	●	○	●	●		○	●
3q12	305	718	610	○	○	○	●	●	●	○	○	●	●	○	●	○	●	●	●	●	●
3q13	296	703	846	○	○	●	○	●	●	○	○	●	●	●	●	○	●	●	○	○	●
5q13	296	693	n.r.	○	●		●	○		○	○		●	●		●	●		●	○	
5q33	305	712	855	○	○	●	○	●	○	○	○	●	●	●	●	●	●	○	●	○	●
7p22	304	n.r.	854	○		○	●		●	○		○	●		○	○		●	○		●
11q22	304	711	854	○	○	●	●	○	●	○	○	○	○	○	○	●	●	○	●	●	●
12q11	304	711	854	●	●	●	○	●	●	○	●	●	●	●	●	●	●	○	●	●	●
22q11	304	711	854	○	○	○	●	●	●	○	○	●	○	●	●	○	●	○	●	○	●
Solo 7p21	304	711	854	○	○	●	●	●	●	○	○	●	○	○	●	●	●	●	○	●	●
Solo 19q13	304	711	854	○	○	●	●	●	●	○	○	○	○	○	●	○	●	●	○	○	○

Note: Position of restriction indicates the localisation of the restriction recognition site relative to the first base pair of the specific 5'LTR.
n.r. means no restriction recognition site

●, strong methylation; ●, methylation; ○, no methylation

SUPPLEMENTARY TABLE 1. Primers Used for RT-PCR and PCR Investigations

Name	Chromosome	Band	Position of binding			Sequence 5'-3'
Cloning of the HERV-K108 5'LTR:						
K108 fw	7	7p22.1	4606821	-	4606840	TGACGTTTGGGCTTCTATCC
K108 rev	7	7p22.1	4605303	-	4605323	CCATGGGCAAAATTGTTCTA
Detection of the methylation status of HERV-K 5'LTRs:						
1q22	1	1q22	153872281	-	153872301	GCACCCTGGAAAGTAAAGGA
1q23.3	1	1q23.3	158927019	-	158927038	CATGGGTCCTGGTAAAGTGG
3q12	3	3q12.3	102893266	-	102893285	CTTCTCAGGTGTGGGAAGGA
3q13	3	3q13.2	114235040	-	114235063	TTTCAGACTTTCCTATCTGACCTC
5q13	5	5q13.3	30532008	-	30532027	CCTGGGATGCAAGTCTTGTT
5q33	5	5q33.3	156026565	-	156026584	ACCCAACCTGCATGGTTTTT
7p22	7	7p22.1	4606596	-	4606614	TACAGGAGTGCGCCATCAC
11q22	11	11q22.1	101070927	-	101070947	CAACAAACACTTCCATGCTCA
12q11	12	12q14.1	57017040	-	57017059	AAAACCAAAGGAACGTGCTG
22q11	22	22q11.21	17306112	-	17306131	TACAGGCTGGACTGCACCTA
Solo 7p21	7	7p21.1	16203829	-	16203850	CCAACACATAATGACTCCATCC
Solo 19q13	19	19q13.13	43048582	-	43048601	CTGTGGCCCAAGCTGATACT
K108 +961 rev	multiple binding sites within the human genome					TGTGTAGGGGCAACCCAC
Real-time PCR:						
GUS-B fw	7	7q21.11	65070155	-	65070175	AAACGATTGCAGGGTTTCACC
GUS-B rev	7	7q21.11	65066803	-	65066822	GCGTTTTTGATCCAGACCCA
Pfor	multiple binding sites within the human genome					CCAACGTGGAGGCTTTTCTCTAG
PENV	multiple binding sites within the human genome					AGGGAGACTTACCACCATTGATAC
PREC	multiple binding sites within the human genome					GGGTATACCTGCAGACACCATTG
PNP9	multiple binding sites within the human genome					TGGGTACACCTGCAGACATCTC
Bisulfite-Sequencing:						
BSS-K108-183fw	7	7p22.1	4606719	-	4606740	TTGTTGTTTAGGTTGGAGTGT
BSS-K108-40fw	7	7p22.1	4606576	-	4606597	TTTTAAGTAGTTAGGGTTATA
BSS-K108+1306rev	multiple binding sites within the human genome					TTCCTTACCAATTCTTTTCCAA
BSS-K108+1121rev	multiple binding sites within the human genome					ATTTACCCCATTATCACCCTAA

Sequence information is based on www.ensemble.org 54: May 2009

Supplementary Figure 1. Analysis of the methylation status of different HERV-K(HML-2)-5'LTR (A) schematic presentation of the 5'LTR of HERV-K(HML-2). Vertical bars show the presence of a CpG dinucleotide. Horizontal arrows indicate the PCR-primer binding sites. (B) evaluation of the assay using differentially methylated plasmids containing the 5'LTR of HERV-K 22q11. Plasmids were digested with methylation sensitive endonucleases followed by PCR amplification using HERV-K 22q11 specific primers. (C) analysis of the methylation status of HERV-K 22q11 in GH and HEK-293 cells.

Fig. 1 Stengel *et al.*

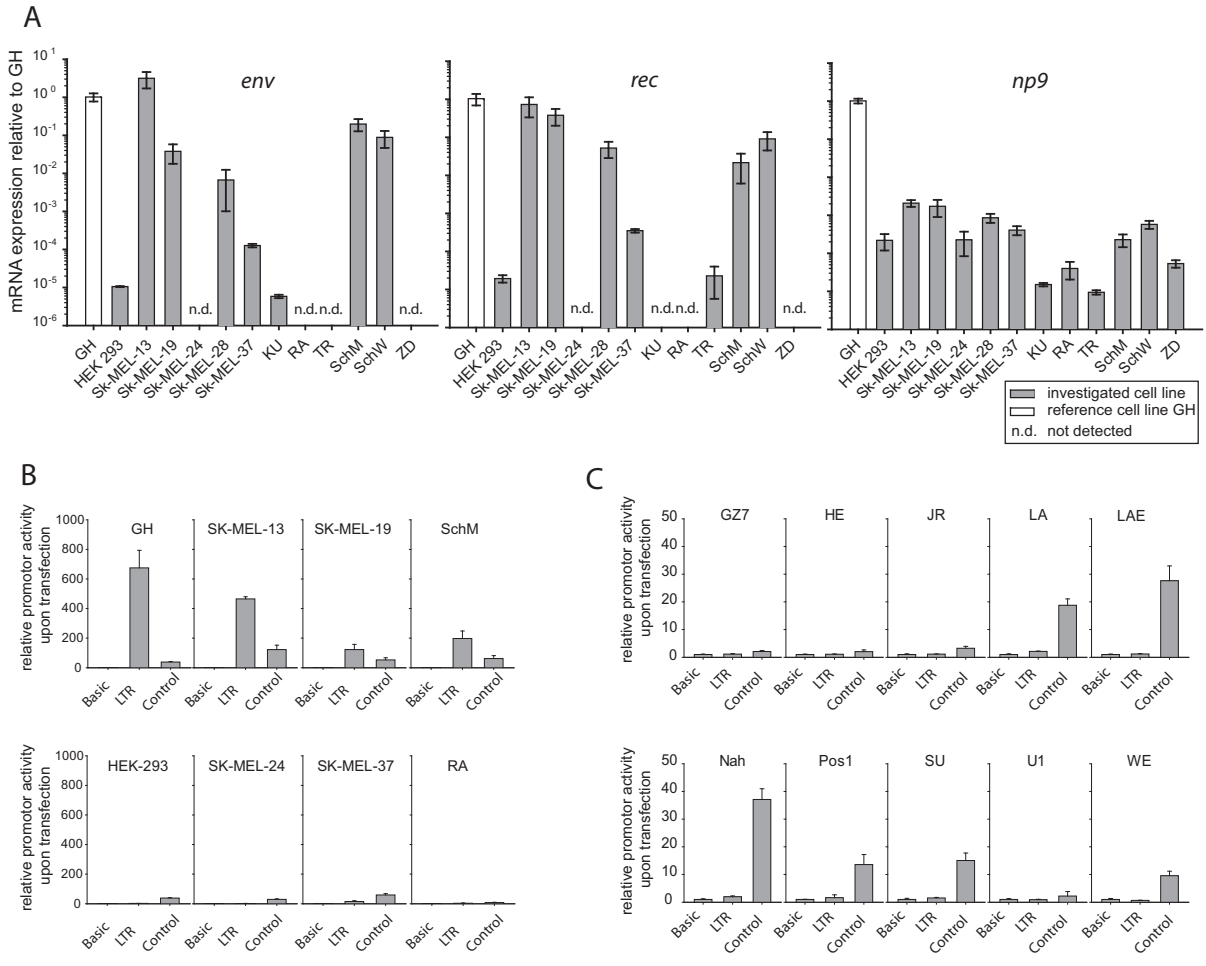


Fig. 2 Stengel *et al.*

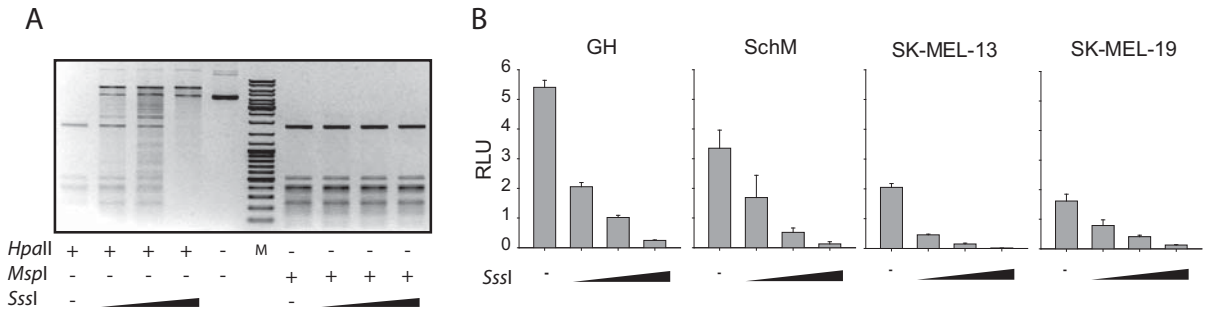


Fig. 3 Stengel *et al.*

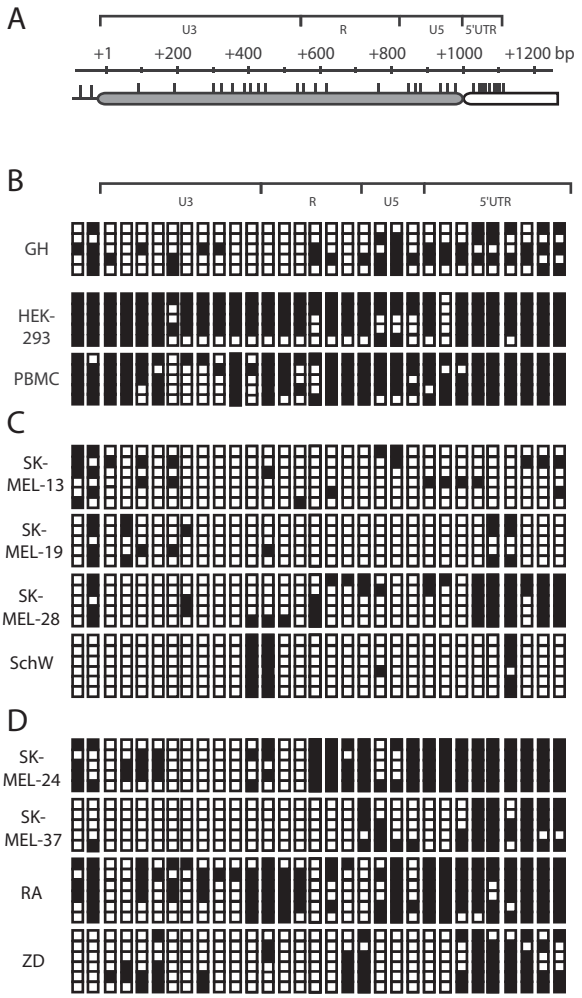


Fig. 4 Stengel *et al.*

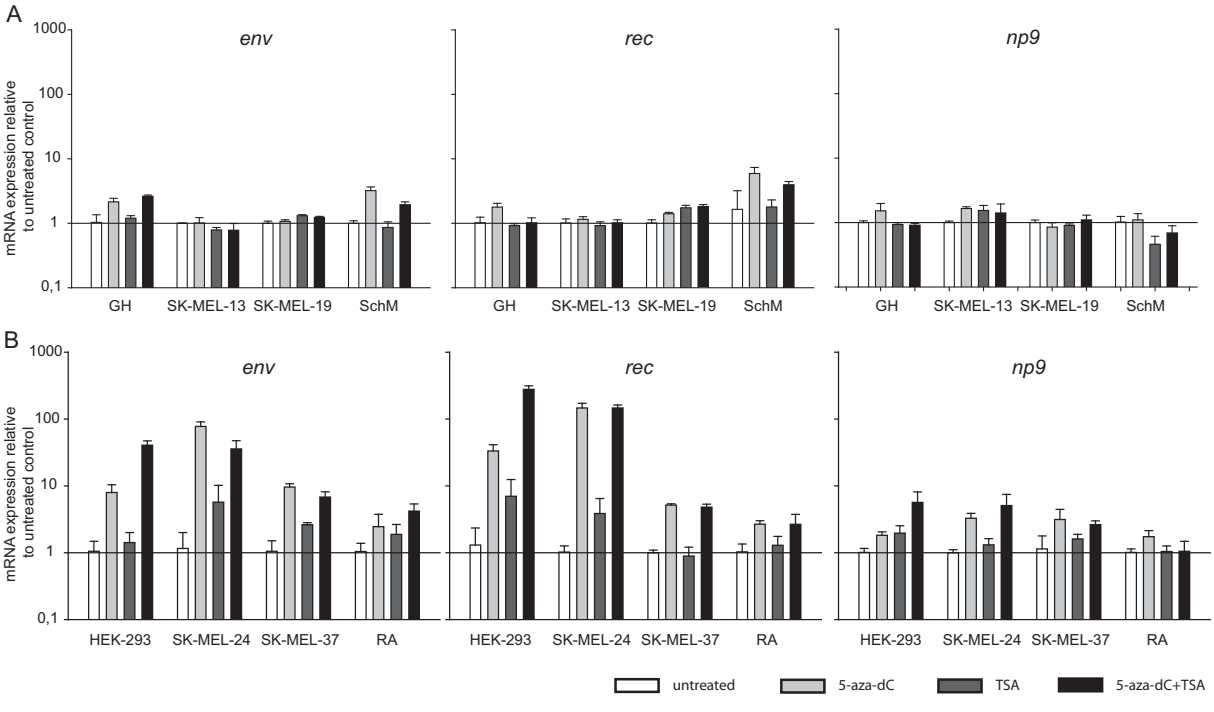
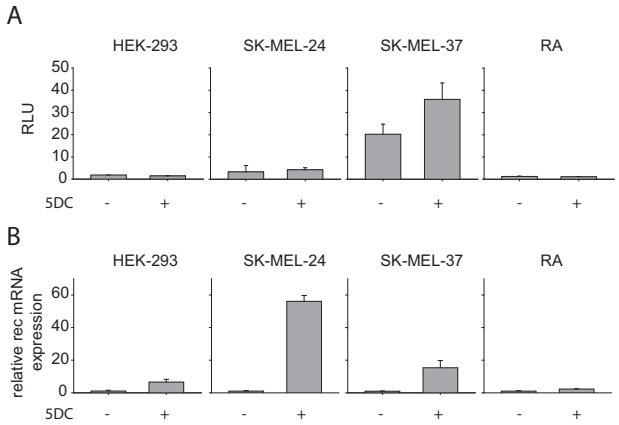


Fig. 5 Stengel *et al.*



Suppl. Fig. 1 Stengel *et al.*