Expression of Human Endogenous Retrovirus-K Coincides with that of Micro-RNA-663 and -638 in Germ-cell Tumor Cells

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Abstract. *Background/Aim: The cell line GH was established from germ-cell tumor tissue; human endogenous retrovirus-K (HERV-K) expression was detectable after prolonged culture of the cells, particularly in cells that formed domes and vesicles. In addition, keeping GH cells in culture at high cell densities increased HERV-K expression. Here, we studied whether this inducible HERV-K expression is accompanied by differences in microRNA (miRNA) expression patterns of GH cells. Materials and Methods: The global miRNA expression pattern of GH cell samples (HERV-K high versus low) was analyzed by miRNA arrays. Results: Two miRNAs were found to be differentially regulated and to exhibit expression parallel to that of HERV-K. The identified miRNAs-663 and -638, have been reported to be involved in multiple processes, including cellular senescence. However, induction of HERV-K expression did not change the cellular senescence status of GH cells. Conclusion: The expression of these two miRNAs might be useful as novel diagnostic and prognostic markers in patients with tumors.*

Human endogenous retroviruses (HERVs) are relics of evolutionary ancient viral infection events into the germ-line which have been since transmitted vertically. These retrovirus genomes are chromosomally integrated in all the cells of an individual and their sequences comprise about 8% of the human genome. HERVs are classified by the single-letter amino acid code for the tRNA-specific to the primer binding site (PBS)-used to initiate reverse transcription. At present, 11 distantly, related HERV groups with a tRNA lysine (K) PBS are known (1). One of these, the HERV-K/HML-2(hom) group, is the only known endogenous retrovirus group encoding all viral structural and enzymatic proteins [proteins encoding the viral core (Gag), UTPase/protease (Prt), polymerase (Pol), RNaseH, integrase (Int), and envelope (Env)] and the accessory protein Rec with functional similarity to the HIV Rev protein (2). For simplicity, HERV-K/HML-2(hom) is abridged to HERV-K hereafter.

In general, HERV-K gene expression is repressed. However, re-activation of HERV-K pro-viruses coding for all viral proteins is well-established for human teratocarcinomas (3) as well as for melanomas (4) and ovarian cancer (5, 6). Furthermore, elevated *HERV-K env* mRNA levels have been found in breast cancer tissues (7, 8) and prostate cancer (9).

Although full-length genomic mRNA and viral particles are detectable in some patients, HERV-K does not form infectious particles and the viral genome is only transmitted *via* the germ-line. However, expression of viral particles is able to induce an HERV-K-specific immune response. For example, it is well-documented that patients with germ-cell tumors (GCT) frequently develop an immune response against the HERV-K Gag and Env proteins (10, 11). HERV-K seropositivity is both a diagnostic and a prognostic marker for GCTs, as data show a strong association of HERV-K antibodies with the clinical manifestation of the disease, as well as with therapy failure, and significant seronegativity for healthy individuals (12). In addition, it was demonstrated that the immune response to HERV-K gene products significantly correlated with reduced survival probability of patients with melanoma (13). These data link HERV-K expression and malignancies, but they do not explain whether HERV-K expression is the cause of, or a consequence of, cell transformation. HERV-K expression may be a tumor marker by coincidence, because of the changed epigenetic status of malignant cells, or HERV-K genes may contribute to or cause the neoplastic transformation of cells. Some mechanisms through which HERV-K proteins might be oncogenic have been identified and suggest that HERV-K might be the cause of malignancies. Transgenic mice expressing the HERV-K Rec protein exhibited disturbed germ-cell development and by 19 months of age, some exhibited changes reminiscent of carcinoma *in situ*, the precursor lesion of classical seminoma in humans (14). These data indicate that HERV-K gene expression might have a direct role in cell transformation by

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creating a pre-cancerous state. In addition, the Rec protein has been shown to interact with the promyelocytic leukemia zinc finger protein (PLZF) (15, 16). PLZF is a transcriptional repressor implicated in certain types of human leukemia and has been shown to be essential for the self-renewal of spermatogonial stem cells in mice (17, 18). In transient reporter assays, expression of Rec by passed the natural PLZF-mediated repression of c-Myc and thereby counteracted PLZF-induced cell-cycle arrest and apoptosis (16). The inactivation of PLZF might also indicate a direct influence of HERV-K Rec on the development of GCTs by interfering with regulatory processes during spermatogenesis. The impact of other HERV-K proteins on cell transformation is not known, to date.

We decided to break new grounds by shedding light on the cause or consequence question of HERV-K in cell transformation and analyzed the global microRNA (miRNA) expression pattern after onset of HERV-K expression in the GCT cell line GH. miRNAs are regulatory, non-coding RNAs of about 22 nucleotides in length and have critical functions in various biological processes such as proliferation, differentiation and cell death. miRNAs are transcribed as primary miRNA transcripts (pri-miRNAs) which are then processed within the nucleus by a complex consisting of the RNAse III enzyme Drosha [named after *Drosophila*, the host firstly isolated from (19)] and the double-stranded RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR 8) into pre-miRNAs. These are exported from the nucleus into the cytoplasm by exportin-5. In the cytoplasm, the pre-miRNA enters the Dicer [named after its "dicing" activity (20)] pathway and finally binds to mRNA and induces mRNA cleavage, translational repression, and/or cleavage-independent mRNA decay (21). The expression of oncogenic miRNAs (oncomirs) that can repress tumor suppressor genes and the loss of tumor-suppressive miRNAs leading to enhanced expression of oncogenes are well-documented mechanisms of miRNA-induced cell transformation (22).

While global miRNA expression patterns of many embryological, physiological, and oncogenic processes have been described, the role of miRNAs in connection with HERV-K has not been determined. Investigating this ancient virus HERV-K with latest state-of-the art science might contribute to our understanding of the interplay of HERV-K and cell transformation.

Materials and Methods

Cell culture. GH cells were cultured in Improved Chemically Defined Basal medium (CMRL 1969, Sigma Aldrich, Hamburg, Germany) containing 5% fetal bovine serum, 1.5 mM NaOH, 15 mM bicarbonate and 2 mM L-glutamine and were propagated by standard techniques (23).

Western blot. Western blot analysis was performed as described previously (24). Proteins separated by Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) were blotted onto Polyvinylidenfluorid (PVDF, Roth, Karlsruhe, Germany) membranes with 50 mM sodium borate pH 9.0, 20% methanol, and 0.1% SDS at 100 mA per membrane for 75 min. Afterwards, membranes were washed with water, stained with Ponceau S (Sigma Aldrich, Hamburg, Germany), and blocked with Roti-Block™ (Roth, Karlsruhe, Germany). Proteins were then detected with α-HERV-K capsid monoclonal antibodies [HERMA-mix (25)] and the ECL detection system (Amersham, Freiburg, Germany).

miRNA array analysis. RNA was isolated from GH cells using standard RNA extraction protocols (TRIzol®, Life Technologies, Darmstadt, Germany) and an additional RNA purification step using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Sample labeling was performed according to the unpublished miRXplore™ user manual (Miltenyi, Köln, Germany). Subsequently, the fluorescently labeled samples were hybridized overnight to miRXplore™ microarrays using the a Hyb™ hybridization station (Miltenyi, Köln, Germany). In general, control samples are labeled with Hy3 and experimental samples are labeled with Hy5. The control, the miRXplore Universal Reference (UR), was provided by the service provider (Miltenyi, Köln, Germany) and represents a defined pool of synthetic miRNAs for comparison of multiple samples. Fluorescence signals of the hybridized miRXplore™ microarrays were detected using a laser scanner from Agilent Technologies (Agilent Technologies, Kronberg, Germany). Mean signal and mean local background intensities were obtained for each spot of the microarray images using the ImaGene® software (Biodiscovery, Hawthorne, CA, USA). Low-quality spots were flagged and excluded from data analysis. Unflagged spots were analyzed with PIQOR™ Analyzer software (Miltenyi, Köln, Germany). The PIQOR Analyzer allows automated data processing of the raw data text files derived from the ImaGene® software. This includes background subtraction to obtain the net signal intensity, data normalization, and calculation of the Hy5/Hy3 ratios for the species of interest. As an additional quality filtering step, only spots/genes that have a signal equal to or higher than the 50% percentile of the background signal intensities are taken into account for the calculation of the Hy5/Hy3 ratio. In addition, the software calculates all normalized mean Hy5/Hy3 ratios of the four replicates per gene. Furthermore, the miRXplore Universal Reference Service uses the miRXplore UR for comparison of multiple samples with the control(s). By calculating the ratio of signals from samples *versus* UR to the ratio of control *versus* UR, the resulting so-called re-ratio indirectly reflects the ratio of samples *versus* control. miRNAs that are >2-fold up- or down-regulated are putative candidate miRNAs.

Northern blot. RNA was isolated from GH cells using TRIzol® and 13 μg RNA per lane was loaded onto a 15% denaturing acrylamide gel containing 8 M urea, electrophoresed and transferred to nylon membranes at 15 V for 60 min at 4˚C, using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Freimann, Germany). The RNA was crosslinked to the membrane using a UV-Stratalinker 1800 (Stratagene, Heidelberg, Germany) at 120,000 mJ for 60 min. Membranes were pre-hybridized with PerfectHyb™ Plus Hybridisation Buffer (Sigma-Aldrich) for 1 h at 37˚C. Hybridizations were performed in the same buffer with digoxigenin

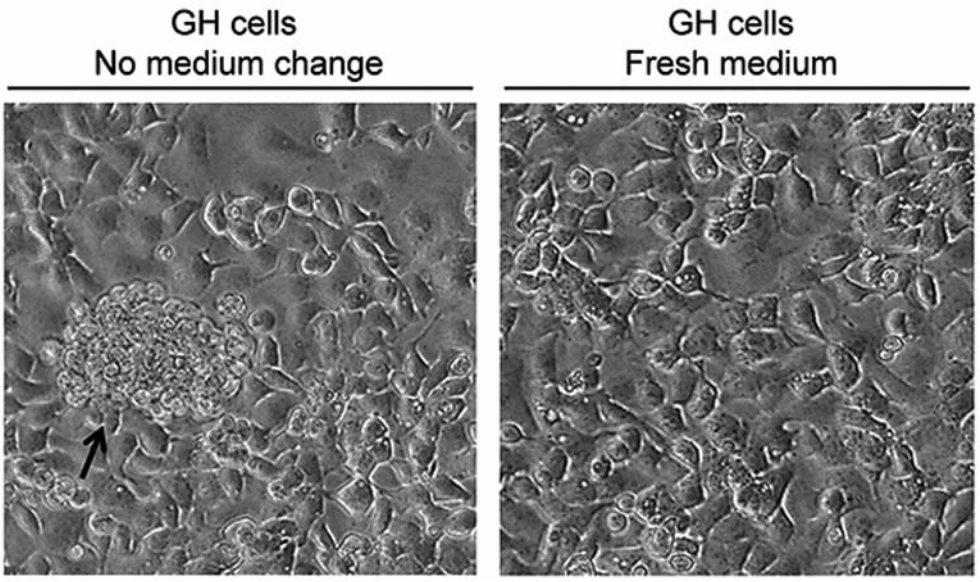

Figure 1. *Bright-field images of GH cells. Bright-field images of GH cells cultured without renewal of the culture medium (no medium change) or with daily medium change (fresh medium) for one week. A dome of GH cells is indicated by an arrow.*

(DIG)-labeled locked nucleic acids (LNA); Exiqon, Vedbæk, Denmark)-modified oligonucleotides complementary to the mature miRNAs of choice (0.5 nmol) overnight at 37˚C. Membranes were washed and signals were detected using the DIG Luminescent Detection Kit for Nucleic Acids (Roche, Mannheim, Germany), following the manufacturer's protocol. Signals were detected as chemoluminescence using the Fusion FX7 Spectra imaging system (Roche).

β-Galactosidase staining. Cells were washed with phosphate buffered saline (PBS), fixed for 15 min with 0.05% glutharaldehyde, washed twice with PBS and incubated with staining solution (β-Galactosidase Staining Kit, New England Biolabs, Frankfurt, Germany) overnight at 37˚C. The proportion of blue-stained cells was determined by microscopy and images were taken.

Results

Cell culture conditions influence HERV-K expression by GH cells. The cell line GH was established in the early 1980s from surgical testicular cancer material and exhibited a limited capacity to differentiate *in vitro* (3). GH cells were cultured at high cell density with only weekly medium replacement. Under these conditions, the cells form areas of high cell density, which look like domes and vesicles (Figure 1). These domes were continuously passaged to generate the GH cell line. Initial electron microscopic studies of GH cells revealed that HERV-K particles budded predominantly from cells growing in domes and vesicles (3).

In our approach, we studied the HERV-K expression of GH cells under two different cell culture conditions. GH cells were either cultured with daily renewal of medium or were left untouched in the same medium for one week. Cells were harvested after seven days and cell lysates were analyzed by western blot with an anti-HERV-K Gag antibody [HERMA mix (25)]. Figure 2 shows that HERV-K Gag expression was only detectable when the culture medium was not renewed (Figure 2, lane 2).

Differential miRNA expression by GH cells under altered culture conditions. The inducible HERV-K expression was further analyzed by comparison of the global miRNA expression with or without renewal of the cell culture medium. Total RNA was isolated from GH cells, either cultured with daily medium changes or after seven days without medium change, and was subjected to miRNA profiling. Differential HERV-K expression was confirmed by western blot analysis and immunofluorescence staining (data not shown). Global miRNA expression was studied in duplicates using miRXplore™ microarrays. GH samples were compared to the miRXplore UR, a defined pool of 954 synthetic miRNAs in equimolar concentrations. This enabled cross-referencing of the GH cell samples and allowed for the absolute quantification of miRNAs. miRNAs that were >2-fold up- or down-regulated were considered as putative candidate miRNAs. Using these filtering methods, only two miRNAs were detected that were differentially expressed in significant amounts, miRNA-663 and 638. Their expression positively correlated with HERV-K expression and was high in GH cells grown without medium change for one week

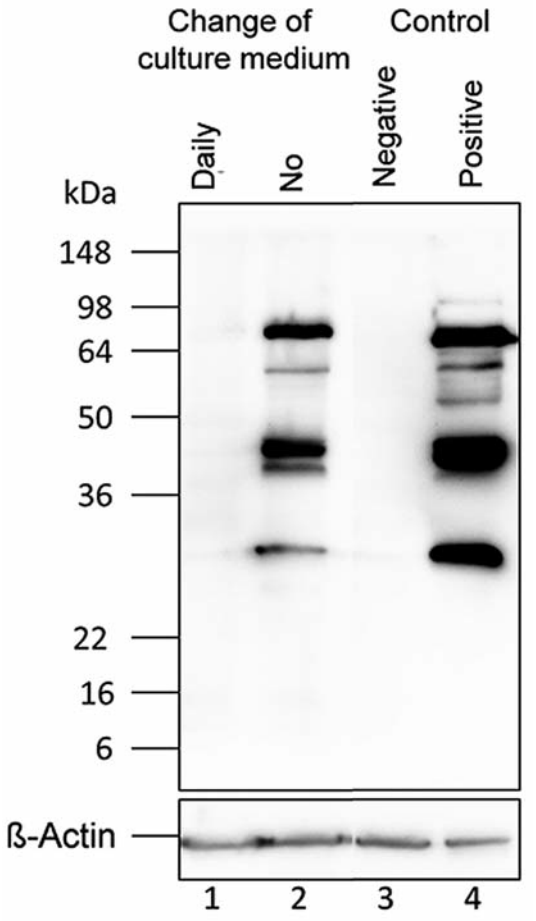

Figure 2. *Western blot analysis of GH cell lysates. GH cells without renewal of the culture medium (no) or with daily medium change (daily) for one week were analyzed. Lysates of uninfected and MVA-HKcon (expressing HERV-K Gag)-infected 293T cells (24) were loaded as negative and positive controls, respectively. HERV-K was detected with an antibody directed against HERV-K Gag (25). The Gag precursor as well as cleavage products are detectable with the antibody (24). Detection of β-actin served as a loading control.*

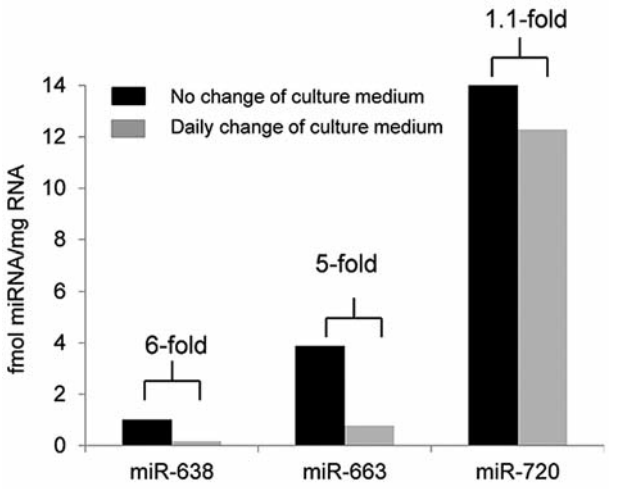

Figure 3. *Quantitative analysis of the microRNA (miR) array data. The miR levels found in GH cells with or without renewed medium are shown. The values are the average of two samples. The fold induction of each is indicated above the columns.*

(Figure 3). miRNA-720 was expressed at very high rates under both conditions and was further used as a loading standard (Figure 3).

Validation of deregulated miRNA-663 and -638 expressions by northern blot analysis. The accuracy of the miRNA microarray data was validated by northern blot analysis. Total cellular RNA of GH cells was electrophoresed, blotted and hybridized with custom-made LNA-modified oligonucleotide probes corresponding to the three tested miRNAs. RNA from GH cells treated with daily changes of the culture medium and those left without medium change are abbreviated to GH-daily and GH-none, respectively. Figure 4B and C demonstrate that the expressions of miRNA-638 and 663 were elevated in GH cells cultured without medium change, in line with the microarray data. miRNA-720 (Figure 4A), which served as a control standard, was highly but not differentially expressed and also confirms the microarray data. The overall expression rates also well-reflected the microarray data, with expression of miRNA-720 > miRNA-663> miRNA-638.

HERV-K expression has no influence on the growth-arrested state of GH cells. miRNA-663 and -638 have previously been described to be up-regulated in growth arrested human fibroblasts compared to fibroblasts in a replicating state (26). Normal human fibroblasts cultured *in vitro* have a limited lifespan and eventually reach a growth-arrested state referred to as replicative senescence. Both miRNAs were up regulated in quiescent, senescent and H_2O_2-induced prematurely senescent cells. To exclude the possibility that the miRNA expression profiles represent only a side-effect of replicative senescence, we analyzed GH cells for indications of senescence possibly caused by high cell density during passage without replenishment of culture medium. Replicative senescence in fibroblasts is typically observed as a flattened and enlarged morphology of the cells, with over 80% β-galactosidase-positive cells in the monolayer. Therefore, GH cell were again cultured for one week either with or without daily renewal of the culture medium. As illustrated in Figure 5, no difference in β-galactosidase positivity or flattened morphology was observed. Our findings indicate that GH cells exhibit a generally high level of β-galactosidase activity which is unaffected by the two culture conditions.

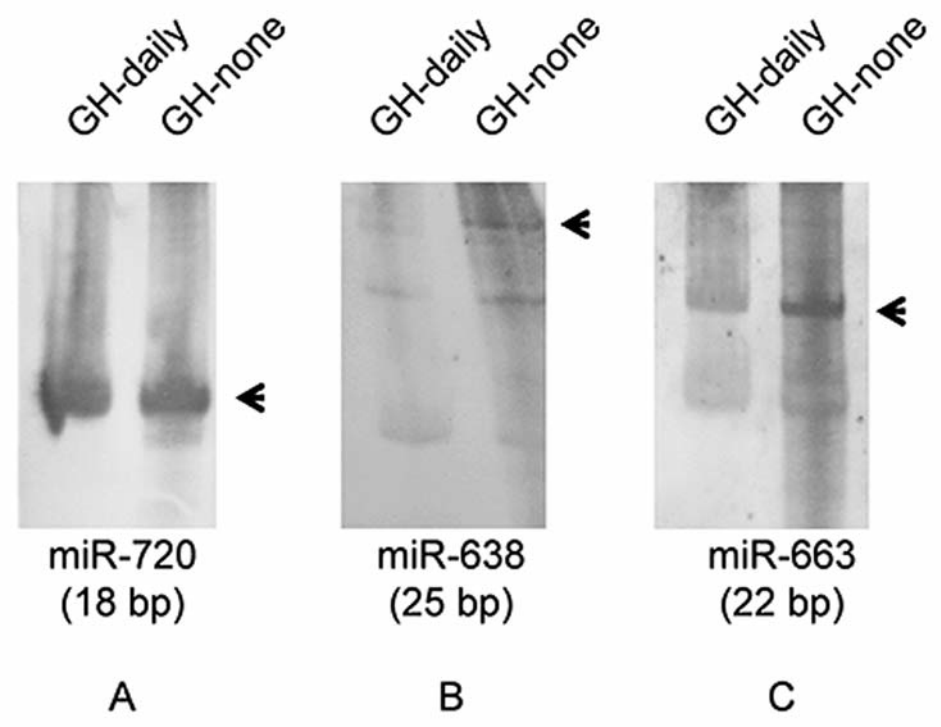

Figure 4. *Northern blot analysis of microRNA (miR) expression in GH cells. RNA was isolated from GH cells with TRIzol®, 13 μg RNA per lane size, were separated, transferred to nylon membranes and hybridized with digoxigenin (DIG)-labeled locked nucleic acids (LNA) (Exiqon)-modified oligonucleotides. Signals were detected using the DIG Luminescent Detection Kit. miRs are indicated by an arrow. A: miR-720; B: miR-638; C: miR-663.*

Discussion

It is well-established that HERV-K expression is frequently detected in various tumor types and its expression is of diagnostic and prognostic value, particularly for GCTs. Furthermore, the detection of antibodies directed against HERV-K proteins correlated with the survival probability of patients with melanoma (13). However, the factors that trigger HERV-K expression and its consequences are not understood. HERV-K particles are not infectious, so insertional mutagenesis, as a cause of transformation is very unlike. However, HERV-K gene products, such as the Rec protein, might have oncogenic effects by blocking anti-oncogenic pathways (11, 27).

To obtain insight into the molecular consequence of HERV-K expression, we investigated the expression of miRNAs in association with HERV-K expression. RNA interference by miRNAs is a regulatory pathway that controls many cellular processes, including cell proliferation and differentiation. Surprisingly, we found that the expression of only two miRNAs, miRNA-663 and -638, were altered in the germ cell line GH in the presence of high HERV-K expression. We induced high HERV-K expression state in GH cells by leaving the cells untouched for one week. In

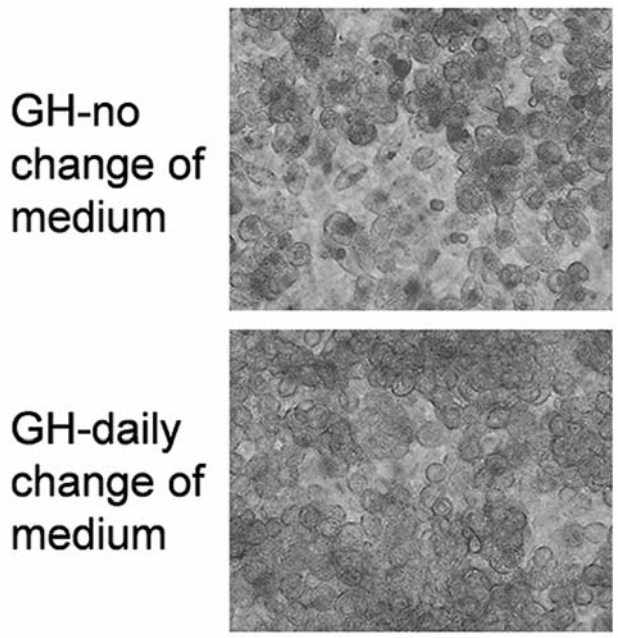

Figure 5. *β-Galactosidase staining of GH cells. Cells were either untreated for one week (GH-no) or the cell culture medium was renewed daily (GH-daily). Cells were then stained for 24 h for β-galactosidase. No difference in staining was detectable.*

contrast, daily changes of the culture medium circumvented HERV-K expression and also resulted in a lower expression of the two miRNAs.

Both miRNAs have been implicated in various biological functions, but only a few of these are related to cell proliferation. Down-regulation of miRNA-663 has been found in human gastric cancer cell lines compared to healthy cells. miRNA-663 overexpression induced morphological changes, suppression of cell proliferation and up-regulation of cyclin-B (28). These data would rather suggest that miRNA-663 expression is antiproliferative. However, this could be a cell-type-specific effect, since miRNA-663 was found to be up-regulated in nasopharyngeal carcinoma (NPC) cells compared with human immortalized nasopharyngeal epithelium cells. Inhibition of miRNA-663 impaired the proliferation of NPC cells *in vitro* and NPC tumor growth of xenografts in nude mice. The authors claimed that miRNA-663 directly targets the cyclin-dependent kinase inhibitor 1A (p21 or WAF1/CIP1) protein to promote the cell cycle and classified *miRNA-663* as an oncogene (29). Therefore, it is possible that miRNA-663 might also have oncogenic functions in GCTs.

Recently, co-expression of miRNA-663 and miRNA-638 in various human physiological conditions has been described. Marques *et al*. found down-regulation of both miRNAs in the renal cortex of untreated hypertensive white male patients, compared to normotensive patients (30). The authors further demonstrated that miRNA-663 binds renin (REN) and the apolipoprotein E (APOE) 3' untranslated region in human embryonic kidney (HEK293) cells. Consequently, the down-regulation of miRNA-663 leads to REN overexpression, which might cause disease in patients (31). The degree of change in miRNA expression correlated with the severity of clinical disease. On the other hand, up-regulation of miRNA-663 and -638 was detected in denatured dermis when compared to normal skin (32). In addition, miRNA-638 and -663 were up-regulated in peripheral blood mononuclear cells (PBMCs) of patients with *lupus nephritis* compared to unaffected controls (33). As HERV-K expression is also frequently described in patients with autoimmune disease (34, 35), our findings of up-regulated miRNAs in HERV-K-expressing cells might fit this scenario.

Finally, the up-regulation of miRNA-663 and -638 in growth-arrested human fibroblasts compared to fibroblasts in a replicating state has been described (26). When we analyzed GH cells for induction of senescence we found no difference in senescence-associated β-galactosidase staining, excluding a senescence-related function of these miRNAs in GH cells.

However, the most relevant connection between the expression of miRNA-638 and -663 and HERV-K might emanate from epigenetics. Both miRNAs contain upstream CpG islands and were found to be up-regulated by the de-methylating agent 5-azacytidine in human immortalized myelogenous leukemia K-562 cells (36). Transcriptional activation by DNA de-methylation has also been described for HERV-K. Treatment of cells with a combination of iododeoxyuridine, dexamethasone and dimethylsulfoxid (DMSO) or with 5-azacytidine led to a transient increase in HERV-K particle release, suggesting that HERV-K expression is inducible by removal of DNA methylation (3). In addition, in five human melanoma cell lines which exhibited very high levels of HERV-K mRNA expression, the HERV-long terminal repeat (LTR)-promoter activity was silenced by DNA methylation. Treatment of cell lines with the demethylating agent 5-azacytidine resulted in increased levels of HERV-K expression in cells, which suggests that demethylation of the 5'LTR increases the promoter activity and causes HERV-K expression in melanoma (37). Therefore, it is tempting to speculate that HERV-K expression caused by unaltered medium conditions might be the result of the general DNA demethylation often observed in transformed cells. Data from patients with ovarian carcinoma are in line with this (38). The authors analyzed the methylation status of retroelements in ovarian clear cell carcinomas and reported a significantly higher de-methylation status of HERV-K elements in patients with platinum-resistant disease, compared to the platinum-sensitive collective. Platinum resistance confers a poor prognosis for the patient. This indicates that the extent of genome de-methylation correlates with increased or reactivated HERV-K expression (38). Therefore, the two newly-identified miRNAs, miRNA-663 and -638, might have diagnostic or prognostic value for a multitude of cancer types. Screening of patients with cancer for expression of miRNA-663 and -638 should be performed to confirm this theory. As shown for HERV-K expression, this miRNA expression might correlate with disease progression and may also reflect the DNA methylation status of cancer cells. Some reports even suggest that serum levels of miRNAs can be used as biomarkers for diseases (39, 40). Perhaps in the future, serum levels of miRNA-663 and -638 can be used as a simple tool for diagnostics or disease prognostics in patients with cancer.

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