The Tumor Suppressor MicroRNA-1 Exhibits Restricted Inhibition of Proliferation of Ovarian Cancer Cells

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Abstract. *Background: MicroRNAs are able to control vital tumor biological processes, such as proliferation, tissue transformation and cell migration, as well as apoptosis. One of the micro RNAs, namely miR-1, has been classified as a tumor suppressor, however, preliminary data did not confirm this finding in ovarian cancer (OC) cells. This study examined the impact of miR-1 on OC cell growth. Materials and Methods: Recombinant miR-1 was overexpressed in human OC cell lines OVCAR-3, SK-OV-3, TOV-112D, and TOV-21G. Subsequently, cell growth was analyzed. Results: After transfection, 11- to 487-fold overexpression of miR-1 was detectable in the OC cells. However, no significant differences in proliferation compared to control cells were detected, neither in transiently nor in stably transfected cells. Conclusion: In numerous cancer entities miR-1 is defined as an antiproliferative tumor suppressor. Notably, the present study demonstrated a loss of growth-inhibitory functionality of miR-1 by so far unknown mechanisms, suggesting dysregulated miR-1 signaling or effector cascades in OC cells.*

Because of its non-specific symptoms in already advanced stages, ovarian cancer (OC) is still the most common cause of death from gynecological tumors (1). This alarming fact makes it more important than ever to explore and understand the pathogenetic mechanism of this malignancy in order either to find satisfactory therapeutic approaches, or better yet, to develop markers for early detection of this deadly cancer. Former studies suggested that among others, a misguided microRNA expression pattern may be involved in tumor initiation and progression in OC (2, 3).

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MicroRNAs, small regulatory RNAs, are able to control biological tumor processes, such as proliferation, tissue transformation, cell migration and metastasis, as well as apoptosis (4-8). Regarding OC, 40 different microRNAs have been described. One of these, microRNA-1 (*miR-1*), initially found in cardiac and skeletal muscle cells (9-14), is also found in many other tissues and has become the focus in malignant diseases. Because of its anti-oncogenic properties, *miR-1* was classified as a tumor suppressor (15-17), however, these properties depend on tissue type (8). These tumor-suppressive features are characterized in various malignancies. To name a few, *miR-1* both inhibits apoptosis and cell-cycle progression in different head and neck tumors (18, 19), as well as suppresses cell proliferation in colorectal cancer (20, 21). In addition there are various studies dealing with the wide-ranging functionality of *miR-1* in different tumor tissues, including prostate cancer (22, 23), renal cell carcinoma (24), bladder carcinoma (25), osteosarcoma (26), different head and neck cancer tissues (18, 27), lung cancer (28) and colonic cancer (29).

Interestingly, the results of our preliminary study examining *miR-1* expression in established OC cell lines, however, showed new insights. Compared with previous studies characterizing *miR-1* as a tumor suppressor, the results of our study provided a basis for new diagnostic and therapeutic approaches for the treatment of OC. Cell lines that expressed high levels of *miR-1* exhibited a higher cell growth compared to cell lines whose *miR-1* levels were lower (30). In addition, our preliminary studies on patients suffering from OC even showed an increase in *miR-1* level during tumor progression (30). The assumption that *miR-1* is to be regarded as a classical tumor suppressor should be critically reviewed. It should also be considered whether in the above mentioned studies, the previously known mechanisms are actually responsible for the suppression of tumor cell growth, since it is known that microRNAs influence cell metabolism by, among others, suppressing glucose metabolism (31). In order to shed light on this topic, we investigated four established OC cell lines regarding their *miR-1* expression and extent of cell proliferation.

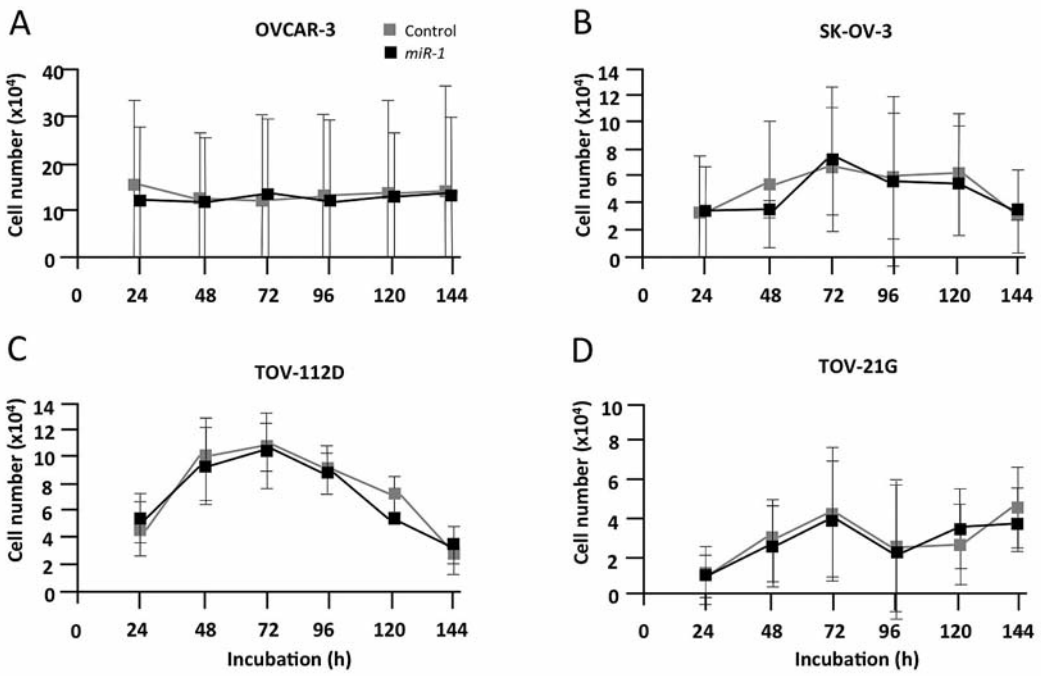

Figure 1. *Cellular growth of ovarian cancer cell lines transiently overexpressing miR-1. OVCAR-3 (A), SK-OV-3 (B), TOV-112D (C), and TOV-21G (D) cells were transiently transfected with pmiR-1 and incubated for 144 h. Cellular proliferation was assessed at different time points utilizing a CASY Cell Counter and Analyzer. Data are given as the mean±SD. Statistically significantly different at *p≤0.05, **p≤0.0 and ***p≤0.001 by the Student's t-test.*

Materials and Methods

Cell culture. Four human OC cell lines, namely OVCAR-3, SK-OV-3, TOV-112D and TOV-21G, were used for the current study. Both OVCAR-3 and SK-OV-3 (Cell Lines Service, Eppelheim, Germany) were cultivated in RPMI-1640 medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum (Biochrom), 0.125% gentamicin (Ratiopharm, Ulm, Germany) and 0.1% insulin (Novo Nordisk, Mainz, Germany), and Dulbecco's modified Eagle's medium F12 (Life Technologies, Darmstadt, Germany) supplemented with 5% fetal calf serum and 0.125% gentamicin, respectively. The other two OC cell lines TOV-112D and TOV-21G, purchased from the American Type Culture Collection (Manassas, VA, USA), were cultivated in MCDB105 (tebu-Bio, Offenbach, Germany)/Medium 199 (Biochrom) mixture containing 15% fetal bovine serum and 0.125% gentamicin. All cells were passaged twice a week in a humidified atmosphere at 37˚C with 5% CO_2.

Proliferation assay. In order to examine the cellular growth of OC cells by cell counting, a CASY Cell Counter and Analyzer Model TT (Roche Applied Science, Mannheim, Germany) was used. Adherent cells, detached by trypsin treatment, were suspended in CASYton (Roche Applied Science) as 1:100 dilution. The analyses of 400 μl of this cell suspension were replicated three times. Measurement was performed using a capillary of 150 μm in diameter and cell line-specific gate settings to discriminate between living cells, dead cells, and cellular debris: 9.00 μm/15.75 μm for OVCAR-3, 7.00 μm/15.15 μm for SK-OV-3, 6.15 μm/11.00 μm for TOV-112D, and 5.25 μm/10.15 μm for TOV-21G.

Transfection. OC cell lines OVCAR-3, SK-OV-3, TOV112D and TOV21G were used in transfection experiments. Transient and stable overexpression of *miR-1* was achieved by transfection of the *miR-1* encoding vector pmiR-1 (5) using Lipofectamine2000 reagent (Life Technologies).

RNA preparation and cDNA synthesis. In order to detect *miR-1* expression in OVCAR-3, SK-OV-3, TOV-112D, and TOV-21G OC cell lines, cells were grown in a 6-well cell culture plate to 80% confluency, and total RNA was prepared using peqGOLDTrifast Reagent (Peqlab Biotechnology, Erlangen, Germany) according to the manufacturer's instructions. According to the supplier's instructions 50-150 g of OC tumor tissue were applied to the NucleoSpin RNA/Protein Kit (Macherey-Nagel, Düren, Germany) for total RNA isolation. Subsequently, the RNA concentration was determined utilizing a Nanodrop 2000c UV/vis spectrophotometer (Peqlab Biotechnology). Finally, RNA was stored at -80˚C.

To perform the reverse transcription, 100 ng of total RNA were used with Superscript II Reverse Transcriptase (Life Technologies), according to the protocol of Chen *et al*. (19). The required stem-loop primers were designed as follows: *miR-1* stem-loop: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACATACAT-3'; *U6* stem-loop: 5'-GTCATCCTTGCGCAGG-3'.

Quantification of miR-1 by polymerase chain reaction. Quantification of *miR-1* was performed with SensiMix SYBR hi-ROX Kit (Bioline, Luckenwalde, Germany) on a CFX96 Real-Time System (Bio-Rad, München, Germany) with CFX Manager software (Bio-Rad). The sequences of the PCR primers were as follows: *miR-1*

forward: 5'-GCCCGCTGGAATGTAAAGAAGTATG-3'; *miR-1* reverse: 5'-GTGCAGGGTCCGAGGT-3'; *U6* forward: 5'-CGCTT CGGCAGCACATATAC-3'; *U6* reverse: 5'-AGGGGCCATGCTAA TCTTCT-3'. The cycling parameters were one denaturation cycle at 95˚C for 5 min and 45 amplification cycles at 95˚C for 10 s, 60˚C for 20 s, and 72˚C for 10 s, followed by a melting-curve analysis. For quantification, *miR-1* signals were standardized to *U6* RNA as reference.

Statistics. For the evaluation of the data, the graphics and statistics software Graph Pad Prism V 5.01 (GraphPad Software, La Jolla, CA, USA) was used. Statistical analyses were performed using the unpaired Student's *t*-test. Results of $p \leq 0.05$ were accepted as significant.

Results

To evaluate whether an inhibitory effect of *miR-1* can also be traced in established OC cell lines, OVCAR-3, SK-OV-3, TOV-112D and TOV-21G cells were transiently transfected with pmiR-1 and incubated for a specific time then compared with control cells. As shown in Figure 1, no significant differences in proliferation between cells transiently overexpressing *miR-1* in comparison to control cells was detected in any cell line (after 24 h compared to 144 h).

Following this, cell lines stably overexpressing *miR-1* were generated and analyzed within long-term experiments. Referring to Figure 2, overexpression of *miR-1* was detectable in each cell line after 48 h compared to control transfected cells.

Again, no significant cell-growth inhibition in the presence of high *miR-1* levels was demonstrated, as seen in Figure 3.

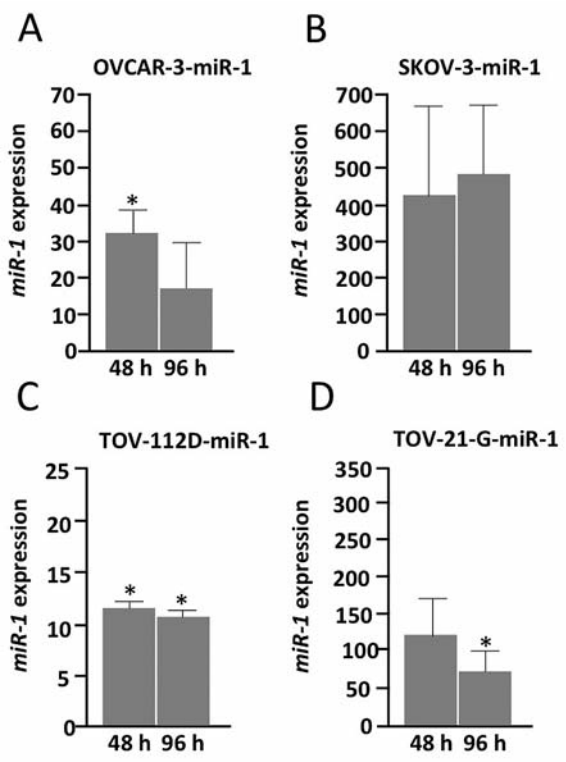

Figure 2. *Stable overexpression of miR-1 in ovarian cancer cell lines. Overexpression of miR-1 in the newly-generated stable cell lines OVCAR-3-miR-1 (A), SK-OV-3-miR-1 (B), TOV-112D-miR-1 (C), and TOV-21G-miR-1 (D) as analyzed by quantitative reverse transcription and polymerase chain reaction using miR-1-specific primers and standardized to expression level of U6 RNA. Data are given as mean±SD. Statistically significantly different at *p≤0.05, **p≤0.0 and ***p≤0.001 by the Student's t-test. Data are expressed as fold change relative to the control (=1.0).*

Discussion

The tumor-suppressive properties of *miR-1* have been investigated in a wide range of tumor tissues. In brief, cell proliferation-inhibiting features of *miR-1* have been detected in carcinoma of prostate, kidney, bladder, bone, head and neck, lung, colon and endometrium (18, 22-29, 32). Although *miR-1* has repeatedly been declared to be a tumor suppressor in numerous studies and in various tumor entities, as mentioned above, this thesis is refuted by recent studies on gastric cancer cells, in which *miR-1* was associated with a higher rate of liver metastasis (33). Additionally our preliminary study, focused on OC, gave first evidence that *miR-1* seemed to have lost its proliferation-inhibiting properties (30). With the results of our current investigations, we can confirm these new findings. Hence putting *miR-1* in the drawer of 'tumor suppressors' would appear not to be the whole truth.

Former studies reported cellular growth analysis applying *miR-1*-modulated cells merely either after one unique period of incubation (24) or by short-term growth kinetics of from 3 to up to 5 days (18, 26-29). Therefore, we performed cell proliferation studies utilizing cell lines stably overexpressing *miR-1* over a long period of time, up to 17 days. Moreover, this approach prevents the experimental limitation represented by transfection artifacts. As shown in our transfection experiments depicted in Figure 1, transient *miR-1* overexpression led to variable and poorly reproducible results. These data demonstrate a potentially serious impact of cell transfection techniques of cellular physiology and subsequent validation of the results. Accordingly, proliferation assessment applying stable cell lines overexpressing the gene of interest represents a more suitable system for this type of experiment.

In addition, differences in the method used to examine the effects on cell proliferation may have extensive consequences for the estimation of measured values. In most studies of microRNA characterization, metabolic activity-based proliferation assays were applied, *e.g.* measuring the

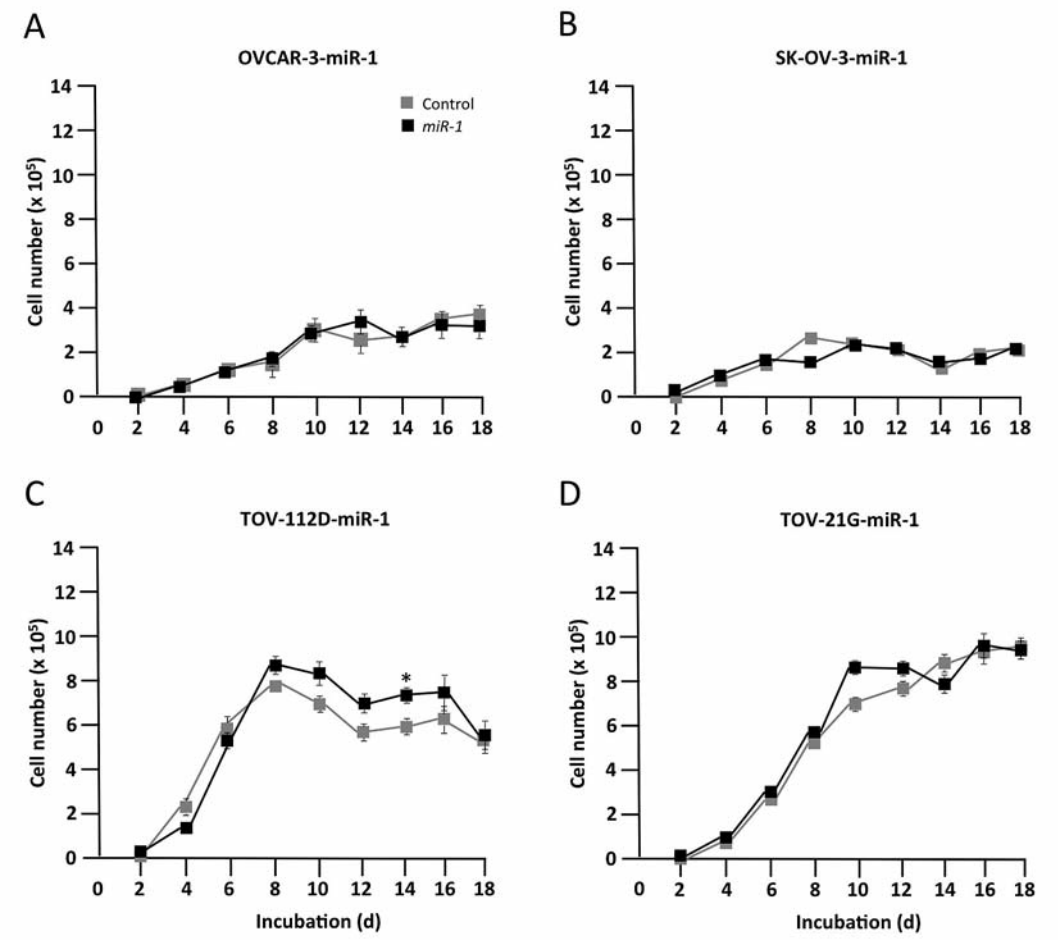

Figure 3. *Cellular growth of ovarian cancer cell lines stably overexpressing miR-1. OVCAR-3-miR-1 (A), SK-OV-3-miR-1 (B), TOV-112D-miR-1 (C), and TOV- 21G-miR-1 (D) cells stably overexpressing miR-1 were incubated for 18 days. Cellular proliferation was assessed at different time points utilizing a CASY Cell Counter and Analyzer. Data are given as mean±SD. Statistically significantly different at *p≤0.05, **p≤0.0 and ***p≤0.001 by the Student's t-test.*

enzymatic conversion of compounds such 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or derivatives by cellular metabolic enzymes (18, 23-29). For this reason, this type of assay represents a more indirect assessment of proliferation, reflecting the physiological activity of the cells, which may be due to changes in total biomass and not necessarily the effect of microRNA. On the other hand, alterations in metabolic activity may also be driven by regulatory properties of microRNAs, which may subsequently intensify or diminish metabolic enzyme activities and alter the sensitivity of proliferation assays.

Many key factors of cellular metabolism, *e.g.* enzymes and transport proteins of the amino acid and glucose pathways, are controlled by microRNAs (31, 34, 35). With regard to metabolic activity-based proliferation assays, this means that metabolic microRNAs may underlie effects on proliferation and, subsequently, result in biased outcome. Notably, mature miR-1 is derived from a bi-cistronic precursor transcript encoding two microRNAs, *miR-1* and *miR-133*. Together with other microRNAs, *miR-133* is associated with control of energy metabolism. Since both *miR-1* and *miR-133* are derived from a common, bi-cistronic gene (11, 36), the strong co-regulation of these two microRNAs may indicate similar cellular functions by targeting similar metabolic genes (37). Consequently, *miR-1* is a putative regulator of cell metabolism, which may explain the highly abundant expression of *miR-1* in muscle cells with high metabolic rates. Thus, in more specific terms, utilization of metabolic activity-based proliferation assays appears to be unsuitable in experiments examining microRNAs bearing metabolic activities. By means of the CASY Cell Counter and Analyzer Model TT, however, it is possible to determine the exact number of living cells, thereby avoiding the regulatory impact of microRNAs on cellular metabolism.

Although the underlying machinery remains unclear, the results of our study indicate a loss of tumor suppressor functionality of *miR-1* in OC cell lines stably overexpressing *miR-1*. As described by Chen *et al*., our microRNA quantification technique is specific only for mature microRNA (19) and thus regulatory functions of *miR-1* should be fully present. For this reason, the loss of antiproliferative properties of *miR-1,* as described by several other studies (22-28), may be caused by a dysregulation of downstream signaling or effector cascades. In terms of clinical relevance, however, the lack of regulatory functionality in OC cells throws the suitability of *miR-1* for OC prediction into doubt.

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