Original Article

microRNA-21 is upregulated in malignant melanoma and influences apoptosis of melanocytic cells

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Abstract: Overexpression of microRNA-21 (miR-21) has been observed in various cancer types, but little is known about the role of miR-21 in melanoma. In this study, we demonstrate that levels of miR-21 are significantly increased in primary melanoma tissues as compared to benign nevi and in human melanoma cell lines as compared to melanocytic cell preparations. We show that downregulation of miR-21 in melanoma cell lines with high endogenous miR-21 expression induced apoptosis, whereas proliferation was not significantly altered. Upregulation of miR-21 in melanocytes resulted in increased proliferation and decreased apoptosis. However, in the MEWO melanoma cells with low endogenous miR-21 expression, upregulation of miR-21 had no functional effects. These findings indicate a potential pathogenetic role of miR-21 upregulation in a subgroup of melanomas.

Key words: apoptosis – cell cycle – melanoma – microRNA – proliferation

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Introduction

The pathogenesis of melanoma is poorly understood but in recent years, systematic analyses of genetic alterations contributed to the elucidation of the pathogenesis and carcinogenesis of melanoma. Mutations in the BRAF gene and less frequently several other genes, such as KIT, PTEN, CDK4, p53, MDM2, cyclin D1, AKT3, PI3Kα or N-RAS, are involved in melanoma progression (1,2). New layers of gene regulation mechanisms have been identified since the discovery of microRNAs (miRNAs). miRNAs represent a class of small single-stranded RNAs. These non-coding RNAs have emerged as important post-transcriptional regulators of gene expression. miRNAs are differentially expressed between malignant and normal cells by a complex interplay of transcriptional control by oncogenes, tumor suppressors, epigenetic mechanisms and genomic alterations. Aberrant expression of several miRNAs has been found to be associated with tumor growth, invasion and metastasis. Despite all the progress made in the identification of deregulated miRNAs in various tumor entities, to date only few upstream regulators and downstream targets of miRNAs are known. miRNA expression was reported to be constant in normal human skin (3), but there is relative little information available about the role of miRNAs in the pathogenesis and progression of malignant melanoma.

Some miRNAs are known as oncogenes and relevant in different tumor types, for example miR-21 in cancers of colon, breast, lung, pancreas, prostate, stomach and brain (4). However, to our knowledge, data on miR-21 expression in melanoma tissue are rare, and the functional role of miR-21 in melanoma is completely unclear. Therefore, we investigated the possible role of miR-21 in melanoma tumorigenesis by analysing the expression and function of miR-21 in benign melanocytic and malignant melanoma cells.

Materials and methods

Primary melanomas and melanocytic nevi

One hundred and twenty-seven patients with primary melanomas who were treated in our department (Skin Cancer Center Hannover) from the years 1998 to 2007 were retrospectively analysed. Archived formalin-fixed, paraffin-embedded (FFPE) tissue samples of primary melanomas originating from patients with clear-cut histopathological diagnosis of melanoma were analysed. FFPE tissue of histopathologically clear-cut melanocytic nevi from 12 patients treated in our department was used as samples for benign melanocytic cell proliferations.

Quantitative real-time PCR

The expression level of miR-21 was analysed in FFPE tissue of melanomas, and melanocytic nevi by quantitative real-time PCR (qRT-PCR) miRNAs of FFPE tissue were isolated using the miRNeasy FFPE Kit (Qiagen, Hilden, Germany). Reverse transcription was performed on the thermal cycler using the miScript Reverse Transcription Kit (Qiagen) following the instructions of the manufacturer. After cDNA synthesis, PCR was performed with specific primers using the miScript SYBR Green PCR Kit (Qiagen) with specific primer for miR-21 (miScript primer assay HS_miR-21_1, Qiagen) and RNU6b (miScript primer assay HS_RNU6b_1, Qiagen) according to the manufacturer's protocol on a LightCycler (Roche, Mannheim, Germany). The amplification protocol was described in the study by Satzger et al. (5).

Melanoma cell lines and melanocytes

Melanoma cell lines (WM9, WM35b, WM451, WM793, WM951, WM1205, SKMel23, SKMel113, MV3, MEWO) were cultured in RMPI1640 medium with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% non-essential amino acids (Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin.

Experimental Dermatology

Primary human melanocytes were obtained from newborn foreskin of six healthy individuals. After enzymatic digestion and trypsinization as described previously (6), single-cell suspensions were incubated in melanocyte growth medium (PromoCell, Heidelberg, Germany). Cells were passaged after confluency and detached with 2.5% trypsin/0.02% EDTA (PAN Biotech GmbH, Passau, Germany). After 3–4 passages, the resulting melanocyte preparations expressed S-100 protein (data not shown) and were used for further experiments. RNA from cultured melanoma cells and melanocytes were extracted and purified using High Pure RNA Isolation Kit (Roche) according to the instructions of the manufacturer; PCR conditions are mentioned above. miR-21 expression ΔCP level in each sample was calculated by normalization with internal control RNU6B (RelQuant Software 3.5, Roche Applied Sciences, Mannheim, Germany).

Functional analyses

Transfection of siRNA

For functional analysis, we selected the melanoma cell lines WM35 and WM951 that showed high miR-21 expression as well as MEWO and melanocytic preparation M1 with low endogenous miR-21 expression.

In vitro *downregulation of miR-21 in melanoma cell lines WM35 and WM951.* The expression of miR-21 was silenced using predesigned siRNA to miR-21 (Anti-miRTM miRNA Inhibitor, Ambion, Austin, USA) for the Amaxa system (Amaxa, Koeln, Germany).

The transfection protocol was described in the study by Satzger et al. (5). A total of 1×10^5 cells were counted for RT-PCR and Annexin V assays; 1×10^4 and 3×10^5 cells for MTT proliferation assays and TUNEL assays, respectively.

In vitro *upregulation of miR-21 in melanoma cell line MEWO and melanocytes.* In previous experiments, transfection conditions were optimized (data not shown).

For transfection, 5×10^6 cells were taken up in 100 μl Nucleofector solution (melanocytes: Amaxa NHEM-neo Nucleofector Kit; MEWO melanoma cells: Amaxa Cell Line Nucleofector Kit) containing 2 μM of the hsa-miR-21 Pre-miRTM miRNA Precursor Molecule (Ambion) and pipetted into an Amaxa cuvette suitable for electroporation. The nucleofection was performed using the desired programme (U-024 for melanocytes, U-020 for MEWO melanoma cells). In parallel, the same number of cells was transfected in the same way with Pre-miRTM miRNA Precursor Molecules – Negative Control #2 (Ambion), functioning as a negative control for pre-miR-21-transfected cells in functional assays. The cells were taken up in a suitable volume of cell culture medium, plated out for functional assays and grown under standard cell culture conditions.

Assessment of proliferation

MTT assay: To analyse the activity of miR-21 on cell proliferation, a predesigned MTT assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Mannheim, Germany) was employed (5). Thymidine incorporation assay: Proliferation was measured 48 h after transfection by incorporation of [methyl-^{3}H]thymidine (0.4 μCi/well) added for the last 24 h of culture (5).

Assessment of apoptosis

Annexin V staining. The Annexin V staining procedure was performed according to the Annexin V-FITC apoptosis detection kit I (BD Pharmingen, Heidelberg, Germany) as previously published

in the study by Satzger et al. (5). The obtained data were analysed using CellQuest™ Pro software (version 4.0.2; Becton Dickinson, Franklin Lakes, NJ, USA).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labelling (TUNEL) assay. The procedure for the analysis of suspension cells was carried out according to the DeadEnd™ Fluorometric TUNEL System (Promega). 3×10^5 cells were washed twice with 5 ml PBS, taken up in 100 μl PBS and fixed using 1 ml 1% methanol-free formaldehyde (Perbio; Thermo Scientific, Waltham, MA, USA) on ice for 20 min. The cells were incubated at 4°C for 10 min, washed twice with 5 ml PBS and resuspended in 500 μl PBS. After the addition of 5 ml ice-cold 70% ethanol, cells were frozen at −20°C for 4 h. The cells were refreezed, incubated for 10 min at 4°C, washed twice with 5 ml PBS and resuspended in 1 ml PBS. The supernatant was discarded; the cells were taken up in 80 μl equilibration buffer (Promega) and incubated for 5 min at RT. After this incubation step, the cells were centrifuged 10 min at $300 \times$ g and the resulting pellet was taken up in 50 μl rTdT buffer (Promega) and incubated at 37°C for 60 min. We avoided exposition to light from this point on. The reaction was stopped by the addition of 1 ml EDTA (Biochrom AG). After centrifugation at $300 \times$ g for 10 min, the supernatant was removed and the pellet resolved in 1 ml 0.1% Triton X-100 (Sigma, Wien, Austria) in PBS containing 5 mg/ml BSA. After an additional centrifugation step, the pellet was taken up in 0.2 ml 5 μg/ml propidium iodide (PI) solution (Promega) in PBS and incubated for 30 min at RT.

Caspase 3/7 assay. The Caspase-Glo 3/7 Assay kit (Promega) was utilized to detect the DEVDase (caspase 3/7 activity) as described in the study by Satzger et al. (5).

Western blotting

1×10^5 WM35 melanoma cells were transfected with miRNA and grown for 24 and 48 h in RPMI medium. Cells were homogenized in M-Per Mammalian Extraction Reagent (Thermo Fisher Scientific, Ulm, Germany). After cell lyses, the samples were centrifuged at 4°C, 3000 U/min, for 10 min. The concentrations of protein in the supernatants were determined using a Lowry protein assay (Bio-Rad, Munich, Germany).

A total of 35 μg of protein per sample was separated on a 5% SDS-polyacrylamide gel (Pierce, Rockford, IL, USA) under reducing conditions and transferred via semidry blotting (30 min, 17 V) onto a 0.45-μm nitrocellulose membrane (Roth, Karlsruhe, Germany). Membranes were incubated in blocking buffer composed of TBS (Bio-Rad), 0.1% Tween and 5% non-fat milk powder (2 h at room temperature; Roth GmbH, Karlsruhe, Germany). Loading control was performed using Coomassie Blue staining (Pierce). Primary antibody (mouse monoclonal IgG$_1$, Cdc25A (F-6); Santa Cruz Biotechnology, Santa Cruz, CA, USA) incubation was followed with TBS/Tween 0.1% containing 5% non-fat milk powder.

Secondary antibody (anti-mouse IgG, HRP-linked antibody; Cell Signaling Technology, Danvers, MA, USA) incubation was performed in blocking buffer (see above) for 2 h at RT. Beta-Actin antibody (Cell Signaling Technology) served as positive control, and GaPDH as a loading control (Cell Signaling Technology).

Immunoreactivity was detected using a SuperSignal Chemiluminescence kit according to the instructions of the manufacturer (Pierce). Protein levels were evaluated through densitometry analysis (Chemilmager 4400 V5.5; Alpha Innotech, San Leandro, CA, USA).

Statistical analyses

Expression levels of miR-21 were compared in melanocytic nevi versus primary melanomas as well as melanoma cell lines versus melanocytes by univariate analyses using the Mann–Whitney U-test (Sigmastat 2.0; SPSS Inc., Chicago, IL, USA). In case of a P-value < 0.05, the result was regarded as statistically significant. To evaluate the prognostic significance of miR-21, Kaplan–Meier tests were performed for univariate analysis (SPSS 13.0; SPSS Inc.). Different cut-offs for miR-21 expression levels were tested by Kaplan–Meier analyses. Functional studies were analysed using the paired t-test (MTT assay, TUNEL assay, caspase 3/7 activity, Annexin V staining, western Blotting) using GraphPad Prism, version 5 (GraphPad Software Inc., La Jolla, CA, USA). All relative expression levels were calculated as follows: pre-miR-21 expression divided by control pre-miR expression.
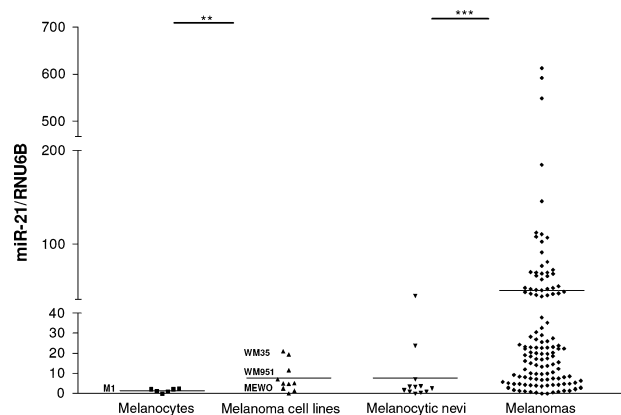
Results

Assessment of miRNA expression in primary melanoma versus melanocytic nevi and melanoma cell lines versus melanocytes

The expression level of the miR-21 was analysed in FFPE tissues of 12 clear-cut melanocytic nevi and 127 melanomas, in 6 preparations of normal melanocytes and 10 melanoma cell lines. miR-21 was significantly upregulated in melanoma tissue (***$P \leq 0.001$) compared with melanocytic nevi. The expression of miR-21 was significantly higher in melanoma cell lines compared with melanocytes (**$P \leq 0.01$). Three melanoma cell lines (WM 451, WM35 and WM951) showed high miR-21 level; two cell lines with high miR-21 expression (WM35 and WM951) and one cell line with low miR-21 level (MEWO) as well as one melanocytic preparation (M1) were chosen for further functional analyses (Fig. 1).

Assessment of prognostic relevance of miR-21 expression in primary melanoma

To assess the prognostic relevance of miR-21 expression level, the expression level of miR-21 was determined in 127 melanoma patients (Fig. 1). Expression levels of miRNA were correlated with recurrence-free survival (RFS) and overall survival (OS) in these patients. Different cut-offs were tested for miR-21 in Kaplan–

Meier analyses but did not show significant differences for RFS and OS (data not shown).

Functional effects of miR-21 downregulation on tumor cell proliferation and apoptosis

Because our data suggest that miR-21 expression is significantly increased in melanomas when compared with melanocytic nevi, we further analysed the role of miR-21 in proliferation and apoptosis of melanoma cells in vitro. Therefore, the WM35 and WM951 melanoma cell lines (showing high endogenous miR-21 expression) were transiently transfected with miR-21 inhibitor. Quantitative RT-PCR revealed a significant downregulation of miR-21 in cells transfected with miR-21 inhibitor as compared with control-transfected cells in both melanoma cell lines with high miR-21 expression (Fig. 2a). Next, the effect of miR-21 downregulation on proliferation and apoptosis of WM35 and WM951 melanoma cells was investigated. In both cell lines, anti-miR-21 transfection resulted in a significant reduction in cell proliferation after transfection as assessed by MTT assay (Fig. 2b) after 24, 48 and 72 h. Measurement of cell proliferation using the [methyl-^{3}H]thymidine incorporation showed a trend towards reduced proliferation in WM35 cells with downregulated miR-21 ($P = 0.098$) but no changes in proliferation of WM951 cells after downregulation of miR-21 (Fig. 4). Apoptosis was increased as measured by Annexin V staining (Figs 2c and S1) and TUNEL assay (Figs 2d and S2). However, we also noted a high background of Annexin V positivity in our control experiments of 40–60%, which might be explained by the transfection procedure itself (Fig. S1).

Functional effects of miR-21 upregulation on tumor cell proliferation and apoptosis

MEWO melanoma cells and melanocytic preparation M1 (showing low endogenous miR-21 expression, Fig. 1) were transiently transfected with miR-21 precursor molecules as detailed in Material and Methods. After transfection, miR-21 levels were upregulated in the melanocyte preparation M1 (Fig. 3a). miR-21 upregulation in melanocytes resulted in increased proliferation assessed by MTT assay (Fig. 3b) and [methyl-^{3}H]thymidine incorporation (Fig. 4). Apoptosis was decreased measured by Annexin V staining (Figs 3c and S3).
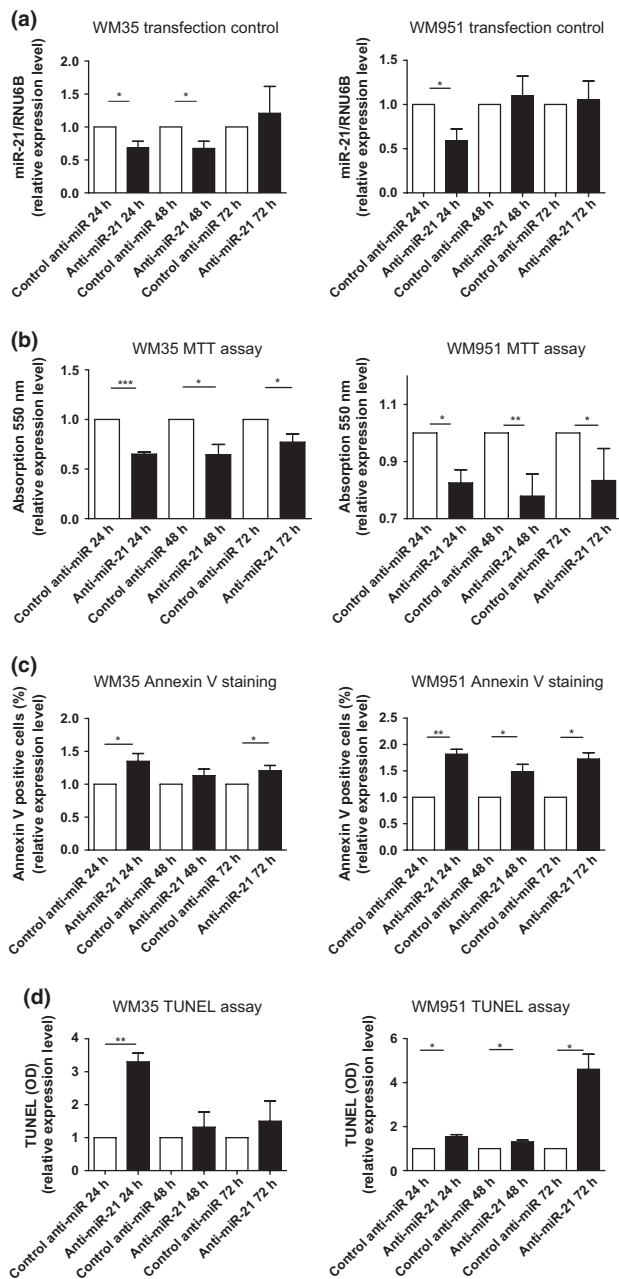
After miR-21 upregulation in the MEWO melanoma cell line (Fig. S4), proliferation and apoptosis were not altered as measured by MTT assay, [methyl-^{3}H]thymidine incorporation, caspase 3/7 assay and Annexin V staining, respectively (Figs 4, S5, S6 and S7).

Cdc25a as a potential target of miR-21

The cell division cycle 25a (Cdc25a) phosphatase functions as a critical regulator of cell cycle progression by the activation of cell cycle kinases and has been described as a direct target of miR-21 in colon cancer cells (7). Given this example, we tested the hypothesis that Cdc25a modulated by miR-21 might play a role in melanoma tumorigenesis. We quantified Cdc25a protein after downregulation of miR-21 in the WM35 and WM951 melanoma cell line. Cdc25a protein levels ($n = 6$) were not altered in cells with downregulated miR-21 expression compared with control cells 24 h after transfection in both cell lines (Fig. S8).
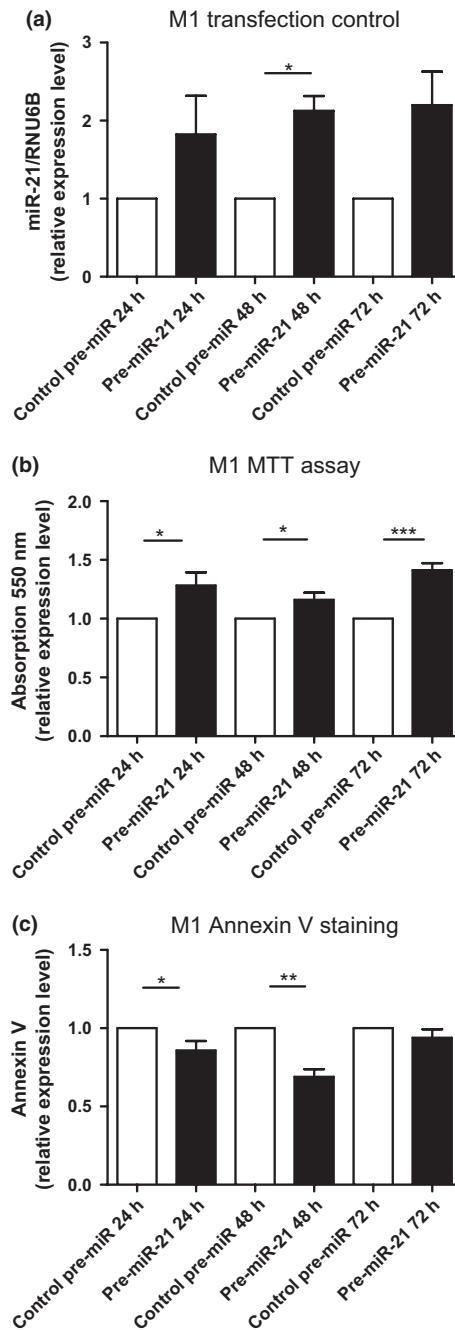
Discussion

In this study, we demonstrate that miR-21 is upregulated in malignant (primary melanomas, melanoma cell lines) as compared to benign (melanocytic nevi, melanocyte cell proliferations)

Figure 1. Comparison of miRNA expression of miR-21 between melanocytes ($n = 6$) versus melanoma cell lines ($n = 10$) and melanocytic nevi ($n = 12$) versus melanomas ($n = 127$) by real-time PCR (**$P < 0.01$, ***$P < 0.001$). Two cell lines with high miR-21 expression (WM35 and WM951) and one cell line with low miR-21 level (MEWO) as well as melanocyte preparation M1 were chosen for further functional analyses.

Figure 2. (a) miR-21 expression after transfection with anti-miR-21 siRNA. The expression level of miR-21 was significantly reduced in WM35 and WM951 melanoma cells after transient transfection of anti-miR-21 as compared with control-transfected cells (*$P < 0.05$, $n = 7$). (b) Effects on proliferation of transfected melanoma cells by MTT assay. Growth of WM35 and WM951 melanoma cells with downregulation of miR-21 and untreated cells was measured by MTT assay after 24, 48 and 72 h. Melanoma cells treated with anti-miR-21 proliferated significantly less than control-transfected cells (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $n = 7$). (c) The Annexin V staining showed increased apoptosis of WM35 and WM951 melanoma cells after downregulation of miR-21 after 24, 48 and 72 h (*$P < 0.05$; **$P < 0.01$, $n = 7$). (d) TUNEL assay showed increased apoptosis of WM35 and WM951 melanoma cells after downregulation of miR-21 after 24, 48 and 72 h (*$P < 0.05$, $n = 3$).

Figure 3. (a) Significantly upregulated miR-21 expression of melanocytic preparation M1 after transient transfection with pre-miR-21 siRNA ($P < 0.05$, $n = 3$). (b) Melanocytes treated with pre-miR-21 proliferated significantly more than control-transfected cells as measured by MTT assay (b *p < 0.05, ***$P < 0.001$, $n = 10$). (c) Annexin V staining showed decreased apoptosis of transfected melanocytes ($n = 5$).

melanocytic cells. miR-21 was significantly upregulated in melanoma tissue of 127 patients when compared with melanocytic nevi. Melanoma cell lines showed a significantly higher miR-21

expression than melanocytic preparations. Numerous previous studies reported on upregulation of miR-21 in various cancer types such as lymphoma (8), glioblastoma (9,10) and cancers of breast (11), lung (12), prostate (13), pancreas (14) but only few data exist about miR-21 expression in melanoma. In line with

Thymidin incorporation
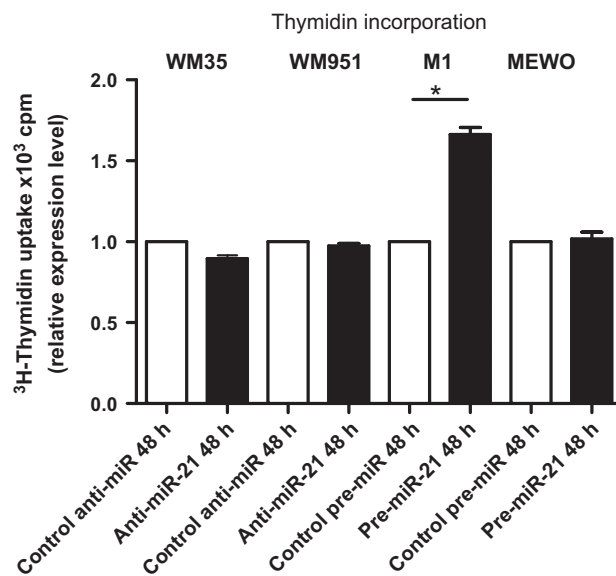
Figure 4. Effects on proliferation of transfected melanoma cells measured by [methyl-^{3}H]thymidine incorporation. WM 35 melanoma cells treated with anti-miR-21 showed a trend towards decreased proliferation compared to control-transfected cells ($P = 0.098$). Thymidine incorporation of melanocytes (M1) was significantly increased after upregulation of miR-21 for 48 h ($P < 0.01$), whereas the proliferation of MEWO and WM951 melanoma cells was not significantly altered ($n = 4$).

our findings, miR-21 was reported to be overexpressed in Mel Im melanoma cell clones, in which Snail expression was stably knocked down by an antisense-Snail construct, in comparison with the parental Mel Im cell line (15). miR-21 was reported to be significantly upregulated in a study comparing miR-21 levels of tissue from 8 benign nevi with tissue from 8 metastatic melanomas (16).

Human miR-21 is localized on chromosome 17q23.2. Zhang et al. reported that melanomas displayed genomic alterations involving many microRNA-coding genes. In this study, 45 melanoma cell lines were analysed. In 12/45 melanoma cell lines, copy number gains for the miR-21 gene region were detected (17). No melanoma cell line showed copy number losses, and thus, miR-21 overexpression might be due to genetic alterations in a subset of melanomas. 17q23.2 is known to be amplified in several other cancer types expressing high miR-21 levels, but other cancers such as gliomas with miR-21 overexpression showed no genetic alterations. This implicates that miR-21 overexpression could be due to genetic alterations as well as tumor biology (18). In line with this hypothesis, miR-21 was specified to be epigenetically regulated in prostate cancer cells (19) and ovarian cancer after treatment with 5-Aza-CdR (decitabine) possibly by DNA hypomethylation (20).

To our knowledge, functional data elucidating the role of miR-21 in melanoma are missing. In order to identify a potential role of miR-21 dysregulation in melanoma tumorigenesis, functional analyses on proliferation and apoptosis were performed. Two cell lines with high miR-21 expression (WM35 and WM951), one cell line with low endogenous miR-21 expression

(MEWO) and a melanocytic cell preparation with low endogenous miR-21 expression were chosen for functional analyses. After downregulation of miR-21 in cell lines with high endogenous miR-21 expression, pro-apoptotic effects could be detected, whereas results of proliferation assays were not consistent. While the MTT assay indicated reduced cell proliferation, the thymidine incorporation assay and Cdc25a western blot were not significantly altered. Upregulation of miR-21 in the melanocytic cell preparation with low endogenous miR-21 expression caused contrary effects on apoptosis and enhanced cell proliferation in the MTT assay and the thymidine incorporation assay. Consistent with our findings, downregulation of miR-21 was associated with pro-apoptotic effects in pancreatic and breast cancer cell lines. However, in contrast to our findings, knockdown of miR-21 resulted also in antiproliferative effects in these studies (21,22); notably, upregulation of miR-21 in the melanoma cell line MEWO with low endogenous miR-21 expression did not lead to changes detectable in our assays. Therefore, miR-21 upregulation might be relevant only for a subgroup of melanomas because miR-21 was not upregulated in all cell lines and only in a subpopulation of primary melanomas (Fig. 1). One of the reasons for these results might be the diversity of melanomas; in recent years, certain subgroups of melanomas with different genomic alterations have been described depending on the extent of UV exposure (1,23). This is consistent with the findings in pancreatic cancer. Zhang et al. (17) and Bhatti et al. (21) reported on an individual level of miRNA dysregulation when they analysed tumor tissue and cell lines, indicating the individual diversity of the disease.

Different genes and signalling molecules have been described to be direct targets of miR-21, such as PTEN, TPM1, Pdcd4, Spry2, which explains the observed functional effects of miR-21 dysregulation on proliferation, apoptosis and invasion (10,21,24,25).

Cdc25a, a cell cycle kinase, was described to be a direct target of miR-21 and miR-16 in colon cancer cells (7,26). Cdc25a must be tightly regulated throughout the cell cycle; inactivation of Cdc25a stops the cell cycle and allows repair of DNA damages or initiation of apoptosis (27). In cancer, deregulation of these processes leads to genomic instabilities. Cdc25a overexpression is correlated with poor prognosis and a more aggressive disease in cancers including breast, prostate, colorectal, ovarian as reviewed by Boutros et al. (27). However, in two melanoma cell lines with high endogenous miR-21 expression, downregulation of miR-21 had no clear effect on Cdc25a protein expression. Thus, potential targets of miR-21 in melanoma have to be determined in future analyses.

In conclusion, miR-21 was upregulated in a subset of melanoma tissues and melanoma cell lines and acted as an oncogene in such cell lines. Therefore, miR-21 may serve as a potential target of pathogenetic relevance in a subset of melanomas.

Acknowledgements

Author contributions

IS and RG contributed to study design, data collection, data analysis, statistical analysis and drafting the manuscript. UK, AM and DW contributed to data collection, data analysis and drafting manuscript. MN and AK contributed to data analysis and drafting manuscript.

Conflicts of interest

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representative experiment: Annexin V staining of melanoma cell line WM951 (corresponds to Fig. 2c).

Figure S2. Representative experiment: TUNEL assay of melanoma cell line WM951 (corresponds to Fig. 2d).

Figure S3. Representative experiment: Annexin V staining of melanocytes (corresponds to Fig. 3c).

Figure S4. Significantly upregulated miR-21 expression of melanoma cell line MEWO after transient transfection with pre-miR-21 siRNA (*$P < 0.05$, **$P < 0.01$, n = 3).

Figure S5. Proliferation of transfected cells was not altered as measured by MTT assay (n = 12).

Figure S6. Changes in apoptosis of transfected MEWO cells could not be detected by caspase 3/7 activity (n = 8) and Annexin V staining (n = 6).

Figure S7. Changes in apoptosis of transfected MEWO cells could not be detected by caspase 3/7 activity (n = 8) and Annexin V staining (n = 6).

Figure S8. Representative experiment: Quantification of Cdc25a protein by western blot analysis after down-regulation of miR-21 in WM35 and WM951 melanoma cell lines. Cdc25a protein levels were not altered after downregulation of miR-21 compared with control-transfected cells 24 h after transfection.

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