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ORIGINAL ARTICLE

Signal Transducer and Activator of Transcription 1 (STAT1) Knock-down Induces Apoptosis in Malignant Pleural Mesothelioma

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Abstract Malignant pleural mesothelioma (MPM) is the most common primary tumor of the pleura. Its incidence is still increasing in Europe and the prognosis remains poor. We investigated the oncogenic function of signal transducer and activator of transcription 1 (STAT1) in MPM in more detail. A miRNA profiling was performed on 52 MPM tissue samples. Upregulated miRNAs (targeting SOCS1/3) were knocked-down using miRNA inhibitors. mRNA expression levels of STAT1/3, SOCS1/3 were detected in MPM cell lines. STAT1 has been knocked-down using siRNA and qPCR was used to detect mRNA expression levels of all JAK/STAT family members and genes that regulate them. An immunohistochemical staining was performed to detect the expression of caspases. STAT1 was upregulated and STAT3 was downregulated, SOCS1/3 protein was not detected but it was possible to detect SOCS1/3 mRNA in MPM cell lines. The upregulated miRNAs were successfully knocked-down, however the expected effect on SOCS1 expression was not detected. STAT1 knock-down had different effects on STAT3/5 expression. Caspase 3a and 8 expression was found to be increased after STAT1 knock-down. The physiologic regulation of STAT1 via SOCS1 is completely lost in MPM and it does not seem that the miRNAs identified by now, do inhibit the expression of SOCS1. MPM cell lines compensate STAT1 knock-down by increasing the expression of STAT3 or STAT5a, two genes which are generally considered to be oncogenes. And much more important, STAT1 knock-down induces apoptosis in MPM cell lines and STAT1 might therefore be a target for therapeutic intervention.

Keywords Malignant pleural mesothelioma · STAT signaling · miRNA · Apoptosis

Introduction

Malignant pleural mesothelioma (MPM) is the most common primary tumor of the pleura. The incidence of MPM has increased steadily over the last 30 years. Despite improvement in diagnostic methods and treatment strategies, the prognosis of MPM patients remains poor (12 to 18 month median survival from diagnosis), although exceptional cases of long-term survivors are reported in the literature [1].

Previous studies showed, that the JAK/STAT signaling pathway, a principal signaling mechanism for lots of cytokines and growth factors [2, 3], is totally deregulated in malignant pleural mesothelioma [4, 5]. Signal transducers and activators of transcription (STAT) are a highly conserved family of latent transcription factors that are activated differentially by various extracellular ligands including growth factors, cytokines and hormones [2]. Presently, seven different members of the STAT family are known: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 [6, 2, 7, 8]. While STAT3 and STAT5 are referred to act as the oncogenic STATs, STAT1 is regarded as a tumor suppressor [9]. STAT activation is tightly regulated through known physiological negative regulators: the Src homology 2 containing phosphatase (SHP), the protein

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inhibitors of activated STATs (PIAS) and the suppressors of cytokine signaling (SOCS) family, which are induced in a classical negative feedback-loop [10, 11]. However, as reported previously, STAT1 acts like an oncogene in MPM and SOCS1 is totally missing [12].

MicroRNAs (miRNA) are one class of small, non-coding RNAs, which regulate protein expression either by mRNA degradation or by preventing mRNA from being translated. They play a role as oncogenes, tumor suppressors, diagnostic markers or prognostic factors of outcome [13]. Furthermore, it has been shown recently that miRNA inhibitors can be used for disease treatment. MiR-122 was found to be associated with hepatitis C virus (HCV) infection: patients with chronic HCV genotype 1 infection showed prolonged dose-dependent reductions of HCV RNA levels when using the miR-122 inhibitor [14]. An early-stage clinical trial is currently performed with a miR-34a replacement therapy for the treatment of liver cancer and cancers with liver involvement [15]. Additionally, forced overexpression of miR-34b/c showed antitumor effects with induction of apoptosis in MPM cells [16].

Because of the well-known cancer association of miRNAs and all their expected functions the first aim of our study was to find out, if miRNAs are the reason for the missing expression of SOCS1 and if miRNA inhibition leads to an increased expression of SOCS1.

Based on our previous studies, we wanted to investigate more detailed, the oncogenic function of STAT1. Therefore our second aim was to identify apoptosis- and cell cycle related genes that are affected by STAT1 knock-down to clarify, if STAT1 has potential as therapeutic target in malignant pleural mesothelioma.

Material and Methods

Formalin-Fixed and Paraffin-Embedded Tumor Tissue Samples

Tissue Selection and Histological Staining

Fifty-two cases of epitheloid MPM were derived from surgically resected tumors, either open biopsies or pleurectomies. The cases were classified according to the World Health Organisation (WHO 2015). The patients gave informed consent and the study was approved by the local Ethics Committee of the Medical University of Graz (No. 24–135). Four μm thick sections of FFPE tissues were dewaxed with xylene and dehydrated in graded ethanol series prior to routine Hematoxylin-Eosin (H&E) staining. An H&E stained reference section from each specimen was histological verified by HHP; tumor areas (with more than 85% tumor cells) were marked with a pen on the slide and used to locate the tumor on the tissue block for further RNA extraction. The protocol for the RNeasy FFPE Kit (Qiagen, Hilden, Germany) has been optimized in a pilot study [17]. RNA concentration was measured by NanoDrop 1000 UV/Vis spectrophotometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The ratios of A260/280 and A260/230 were used to indicate the purity of total RNA.

miRNA Profiling

TaqMan® Array Human MicroRNA Cards (Life Technologies, Carlsbad, USA) have been used with 8 pooled RNA samples, generated out of the 52 FFPE tumor tissue samples. To identify extremely up- or downregulated miRNAs, the DeltaCt method was used with RNU44 as reference gene.

cDNA was generated using TaqMan® MicroRNA Reverse Transcription Kit and Megaplex™ RT Primers (both from Life Technologies) according to the manufacturer's instructions. QPCR (quantitative real-time PCR) was performed according to the manufacturer's instructions on the 7900HT Fast Real-time PCR system (Life Technologies).

Single miRNA Assays

Those upregulated miRNAs that could be linked to the STAT signaling pathway by using in silico target prediction tools were then quantified in every single sample.

QPCR was carried out using TaqMan® MicroRNA assays (Life Technologies, Assay IDs:000396, 000602, 000419) with a modified version relating to the volumes of reagents and cDNA. The analysis of relative miRNA expression was performed using DeltaCt method with RNU6B as reference gene.

Tissue Microarray (TMA) Construction and Immunohistochemistry

The H&E stained reference section of each case, used for further RNA extraction, was examined again. For each case (46/52), three to five tissue cylinders with a diameter of 0.6 mm were punched from the marked tumor areas and perforated into a new paraffin block by using a manual instrument (Beecher Instruments Sun Prairie, Wisconsin, USA). Four μm thick sections were cut from the TMA block and used for immunohistochemistry. Details for the antibodies are given in Table 1.

Protein expression levels were recorded semi-quantitatively and the staining scores were calculated by multiplying the staining intensity (from 0 to 3+, no staining to strong staining) by percentage (0–100%) of positive cells. Scores lower than 50 were considered to be negative, scores higher than 50 were positive.

Table 1 List of antibodies used for immunohistochemistry. All antibodies were validated for specificity by Western Blot analysis and/or immunohistochemistry in test-TMAs

antibody	dilution	antigen retrieval	detection
STAT1 (M-22) sc-592 Santa Cruz[a]	1:1000	Ventana	CC1/View
STAT3 (H-190) sc-7179 Santa Cruz[a]	1:100	Ventana	CC1/View
SOCS1 (38–5200) Invitrogen[b]	1:200	Ventana	CC1/View
SOCS3 (SO1) sc-51699 Santa Cruz[a]	1:50	MW 9.0	Dako K5001
Caspase 3a R&D[c]	1:50	MW 6.0	Dako K5007
Caspase 8 Novocastra[d]	1:30	MW 9.0	Dako K5001

[a] Santa Cruz Biotechnology, Santa Cruz, USA

[b] Invitrogen, Carlsbad, USA

[c] R&D Systems, Inc., Minneapolis, USA

[d] Novocastra, Leica Biosystems, Nussloch, Germany

CC1: Cell conditioning 1, Ventana (Ventana, A Member of the Roche Group, Tucson, USA)

Dako K5001: Dako REAL TM Detection System Peroxidase/ DAB rabbit/ mouse (Dako, An Agilent Technologies Company, Santa Clara, USA)

Dako K5007: Dako Real TM EnVision TM Detection System Peroxidase/DAB+, rabbit/mouse

DAB: 3,3′- Diaminobenzidine

MW: microwave

9.0/6.0: target retrieval solution, pH 9.0/6.0, Dako

Malignant Mesothelioma Cell Lines

Cell Culture

Two mesothelioma cell lines (CRL-5915, MSTO-211H) were purchased from ATCC (American Type Culture Collection, via LGC, Germany). These cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Four additional mesothelioma cell lines (Hmeso, PPM Mill (H2373), PPM Gar (H2461) and PPM Rob (H2595)) [18, 19] were obtained from Arti Shukla, PhD (Department of Pathology of the University of Vermont, Burlington, USA). These cell lines were cultured in special medium as reported previously [20]. The cell lines have been tested for the expression of mesothelioma-associated proteins (calretinin, cytokeratin 5/6 and podoplanin) by immunocytochemistry. HepG2 cells (ATCC), known for their high expression of SOCS1, were used as standard and internal control. All cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2. For time-dependent investigations, like transfections with siRNA, only the three cell lines showing similar growth rates (CRL-5915, MSTO-211H and Hmeso) have been used.

RNA Extraction, Concentration, Purity and miRNA Quantification

RNA has been extracted using the miRMeasy Mini Kit (Qiagen) with the recommended on-column DNase digestion using the RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were measured as mentioned previously. MiRNAs have been quantified using single miRNA assays as described above.

miRNA Inhibition

MiRNAs were inhibited in the cell line CRL-5915 using the transfection reagent Lipofectamine RNAiMAX (Life Technologies) and miRCURY LNA inhibitors (Exiqon, Vedbaek, Denmark): hsa-miR-19b (#410119–00), hsa-miR-30b (#4410189–00), hsa-miR-30c (#411548–00), microRNA antisense control A (#199004–00) with a concentration of up to 100 nM according to the manufacturer's instructions. Single miRNA inhibitors were used as well as combinations of inhibitors. The transfection was optimized regarding cell number, concentration of miRNA inhibitors and final incubation time to rule out biological effects as good as possible.

Cells were seeded on the day before transfection in 6-well plates. Afterwards cells were either treated with miRNA inhibitors or the miRNA antisense control A. Cells were examined under microscope for morphological changings and were harvested for further RNA or protein extraction 24 h post-transfection.

mRNA Quantification

cDNA was synthesized using RT [2] First Strand Kit (Qiagen). QPCR was performed in triplicates using RT [2] qPCR Primer Assays (STAT1: PPH00811C, STAT3: PPH00708F, SOCS1:

PPH00769C, SOCS3: PPH00763A, GAPDH: PPH00150F, Qiagen) and RT [2] SYBR Green Mastermixes (Qiagen). QPCR was performed using the 7900HT Fast real-time PCR System (Life Technologies) according to the manufacturer's instructions. A dissociation curve analysis was performed to verify PCR specificity. The analysis of relative mRNA expression was performed using DeltaCt method with GAPDH as reference gene.

siRNA

The transfection with siRNA was optimized for cell lines CRL-5915, MSTO-211H and Hmeso regarding cell number, concentration of siRNA and final incubation time to rule out biological effects as good as possible. The transfection was performed using Lipofectamine RNAiMAX (Life Technologies) and SignalSilence® Stat1 siRNA II (#6544, Cell Signaling Technology, Danvers, USA) and SignalSilence® Control siRNA (#6568, Cell Signaling Technology) according to the manufacturer's instructions. Cells were seeded on the day before transfection in 6-well plates. Afterwards cells were either treated with STAT1 siRNA or the Control siRNA. Cells were examined under microscope for morphological changings and were harvested for further RNA or protein extraction or agar block construction 24 h post-transfection.

Profiler Array

RNA was extracted as mentioned previously. cDNA was synthesized using RT [2] First Strand Kit (Qiagen). For each cell line (CRL-5915, MSTO-211H and Hmeso), two RT [2] Profiler PCR Arrays (Human JAK/STAT Signaling Pathway PCR Array, PAHS-039YC-2, Qiagen) were used. Therefore, two samples have been reversed transcribed: one sample, where cells have been transfected with STAT1 siRNA, and one sample, where the cells have been transfected with a control siRNA. The Profiler PCR Arrays were performed in duplicates. The analysis of relative mRNA expression was performed using DeltaDeltaCt method with GAPDH as reference gene.

Protein Extraction and Quantification

After the incubation time, media was removed completely and cells were washed twice with ice cold PBS buffer before they were covered with ice cold RIPA buffer including Phosphatase and Protease Inhibitors (Calbiochem® Protease Inhibitor Cocktail Set 1, Calbiochem® Phosphatase Inhibitor Cocktail Set II, Merck KGaA, Darmstadt, Germany). Cells were collected with a cell scrubber and all samples were vortexted several times to extract proteins. After centrifugation, supernatants were collected and protein concentrations were determined by Lowry Method (BioRad DC Protein Assay, Bio-Rad, Hercules, USA). Aliquots were stored at −20 °C until use.

Table 2 Results of the miRNA profiling in MPM tumor tissue samples. RNU44 was used as reference gene

microRNA	expression compared to RNU44	predicted targets
hsa-let-7b	slightly downregulated	SOCS1
hsa-miR-106a	upregulated	STAT3
hsa-miR-126	highly upregulated	
hsa-miR-145	upregulated	
hsa-miR-146a	upregulated	
hsa-miR-146b	upregulated	
hsa-miR-150	slightly downregulated	
hsa-miR-155	slightly downregulated	SOCS1
hsa-miR-16	highly upregulated	
hsa-miR-17	upregulated	STAT3
hsa-miR-191	upregulated	
hsa-miR-193b	slightly downregulated	
hsa-miR-199a-3p	slightly downregulated	
hsa-miR-19b	upregulated	SOCS1, SOCS3
hsa-miR-21	upregulated	STAT3
hsa-miR-210	slightly downregulated	
hsa-miR-214	slightly downregulated	SOCS1
hsa-miR-218	slightly downregulated	SOCS3
hsa-miR-222	upregulated	SOCS3
hsa-miR-223	highly upregulated	STAT1
hsa-miR-24	extremely upregulated	
hsa-miR-26a	slightly upregulated	
hsa-miR-29a	upregulated	
hsa-miR-30b	expressed as control	SOCS1, SOCS3
hsa-miR-30c	slightly upregulated	SOCS1, SOCS3
hsa-miR-31	slightly downregulated	
hsa-miR-320	slightly upregulated	
hsa-miR-342-3p	slightly upregulated	
hsa-miR-484	upregulated	

Western Blot

Twenty μg of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred onto nitrocellulose (Bio-Rad) or polyvinylidene fluoride-type (Hybond-P, GE Healthcare Europe GmbH, Freiburg, Germany) membranes using a semidry blotting apparatus (PEQLAB). Blocking was performed for 2 h at room temperature. The membranes were incubated with the primary antibody for 16 h at 4 °C (SOCS1(38–5200), 1:250, Invitrogen, Carlsbad, USA; GAPDH (FL-335) sc-25,778, 1:500, Santa Cruz Biotechnology, Santa Cruz, USA). Membranes were washed

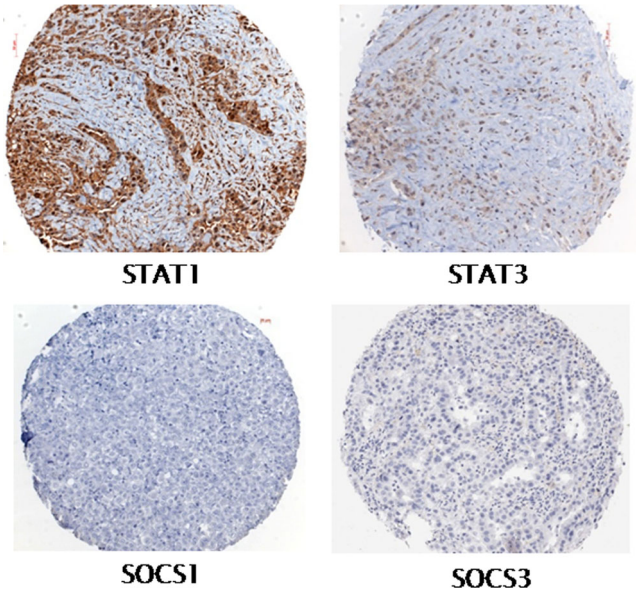

Fig. 1 Immunohistochemical staining of tissue microarray sections. Positive staining was achieved for STAT1 and STAT3. SOCS1 and SOCS3 were not detected by immunohistochemistry

and afterwards incubated with the secondary horseradish peroxidase-conjugated antibody (Swine Anti-Rabbit (#P0217), 1:1000 (Dako, An Agilent Technologies Company, Santa Clara, USA) for 90 min at room temperature. After an additional washing step, membranes were developed using ECL (GE Healthcare Europe) and Kodak film (T-MAT G film, Eastman Kodak Company, Rochester, New York). All antibodies were validated for specificity by Western blot analysis.

Agarblock

After the incubation time, another batch of cells was harvested and washed with PBS buffer before they were fixed with 6% ice-cold formalin for 10 min. The cell pellet was resuspended in 7% liquid agar and incubated at 4 °C over night. The agar-cell pellet was then formalin-fixed and paraffin-embedded according to standard protocols and was used for immunohistochemical staining.

Results

Formalin-Fixed and Paraffin-Embedded Tumor Tissue Samples

By performing the miRNA profiling it was possible to identify 29 miRNAs, which are either up- or downregulated in malignant mesothelioma. Twelve of them were predicted to target components of the STAT signaling pathway (see Table 2) by using in silico target prediction tools. MiRNAs targeting SOCS1 and SOCS3 were quantified in every single sample (45/52). MiR-19b, miR-30b and miR-30c were found to be upregulated in nearly all samples (data not shown). The immunohistochemical staining performed on tissue microarray sections showed that STAT1 is much higher expressed than STAT3 in tumor tissue samples. SOCS1 and SOCS3 expression was not detected by immunohistochemistry (see Fig. 1 and Online Supplementary Material 1).

Malignant Mesothelioma Cell Lines

The miRNA quantification data for the mesothelioma cell lines, confirm the results of the FFPE tumor tissue samples: miR-19b, miR-30b and miR-30c were found to be upregulated in all six mesothelioma cell lines and the control cell line HepG2 (see Fig. 2) compared to the reference gene RNU6B; however, the amount of each miRNA differs between the cell lines. What has

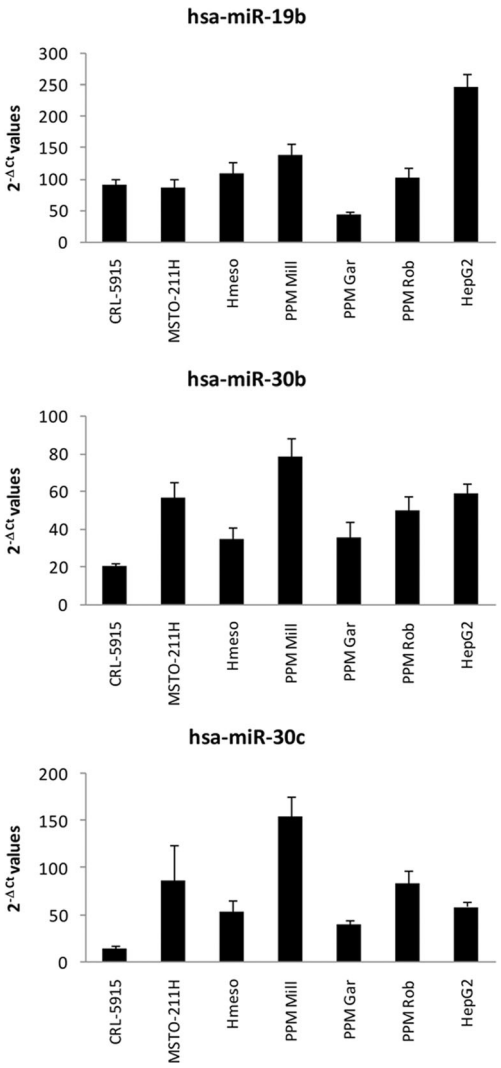

Fig. 2 Expression of miRNAs possibly targeting SOCS1 and SOCS3 in all six mesothelioma cell lines and the control cell line HepG2. RNU6B was used for normalization. $2^{-\Delta Ct}$ values are shown; error bars indicate the standard error

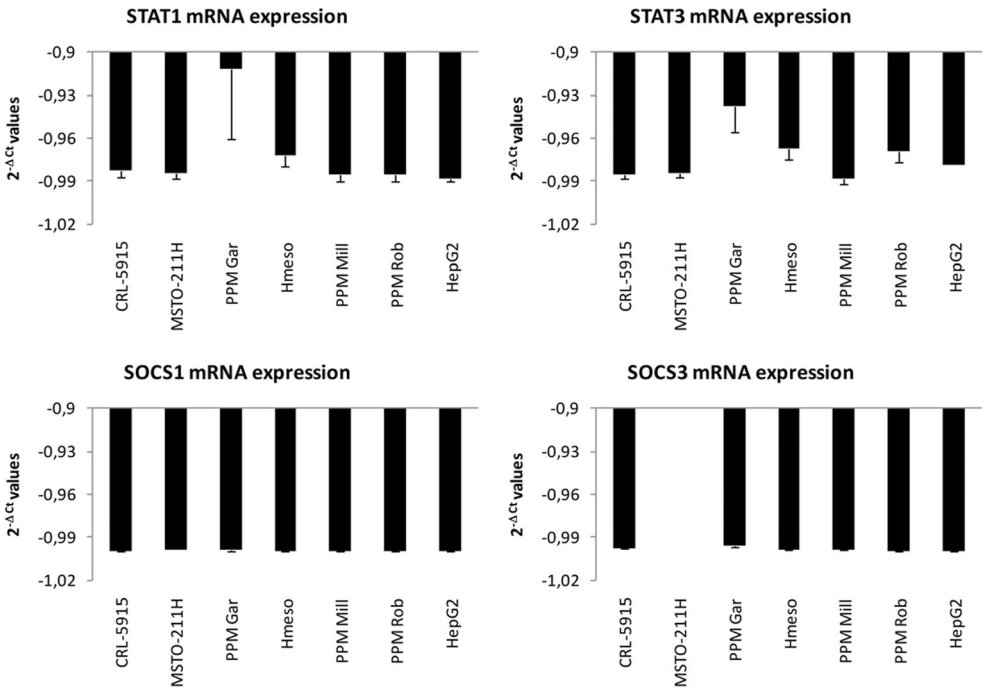

Fig. 3 STAT1, STAT3, SOCS1 and SOCS3 mRNA expression levels in all six mesothelioma cell lines and the control cell line HepG2. Expression levels are normalized to the housekeeping gene GAPDH. $2^{-\Delta Ct}$ values are shown; error bars indicate the standard error

to be pointed out is that there is no difference between the mesothelioma cell lines and the control cell line HepG2.

The results for the expression of STAT1, STAT3, SOCS1 and SOCS3 on mRNA level differ from the expression pattern on protein level: STAT1 and STAT3 mRNA is downregulated compared to GAPDH; SOCS1 and SOCS3 mRNA is downregulated as well in all six mesothelioma cell lines and the control cell line HepG2 (see Fig. 3). Therefore it is possible to detect SOCS mRNA but there is no SOCS protein in mesothelioma cell lines. And this can be caused by miRNAs. Therefore we

expected that, the higher the concentration of the miRNA inhibitor, the higher the expression of the target mRNA. SOCS1 mRNA expression – quantified by qPCR - is slightly increased when miR-19b is knocked-down, the effect of inhibitor-combinations on SOCS1 expression is even smaller (see Fig. 4). Using single miRNA inhibitors or a combination of inhibitors had no effect on SOCS1 protein expression. SOCS1 protein was only detected in an untreated sample of the control cell line HepG2 (see Fig. 5).

STAT1 was successfully knock-down by using STAT1 siRNA in the cell lines CRL-5915, MSTO-211H and Hmeso. The effect of STAT1 knock-down on the other components of the JAK/STAT signaling pathway differs between the cell lines (see Fig. 6). While CRL-5915 seems to compensate STAT1 knock-down by an upregulation of STAT5a, MSTO-211H and Hmeso show slightly upregulated expression levels of STAT3. Most of the genes included on the RT [2] Profiler PCR Array are differentially expressed in the mesothelioma cell lines investigated (see Fig. 7). Three genes were found to be either up- or downregulated in all cell lines ($2^{-\Delta\Delta Ct}$ values at least 0.5): CDKN1A, F2R and JUNB.

A positive immunohistochemical staining for Caspase 3a (see Fig. 8a) and Caspase 8 (see Fig. 8b) was detected in the

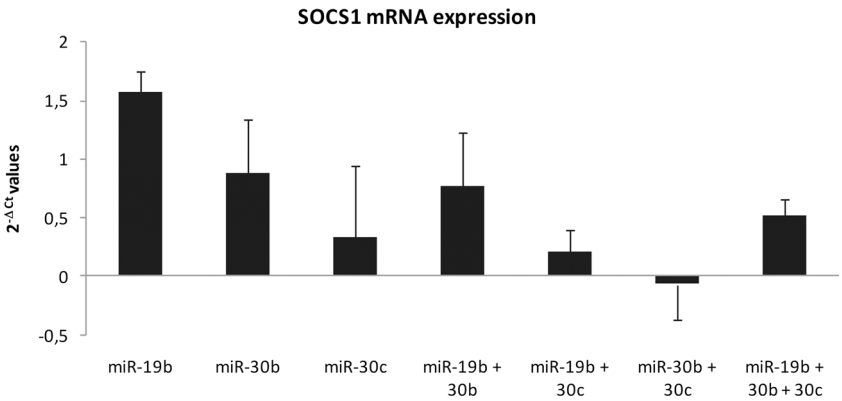

Fig. 4 SOCS1-mRNA expression levels in CRL-5915 after using single miRNA inhibitors as well as combinations of inhibitors. $2^{-\Delta Ct}$ values are shown and the expression levels were normalized to "miRNA antisense control A". Error bars indicate the standard error

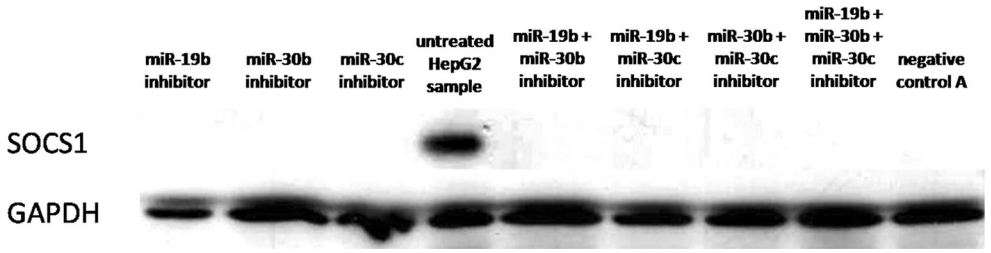

Fig. 5 Western Blot analysis showing the effect of miR-inhibition on SOCS1 protein expression in CRL-5915. Inhibitors have been used with a final concentration of 100 nM. An untreated HepG2 sample was used as positive control for SOCS1 expression. GAPDH was used as loading control

samples treated with STAT1 siRNA, whereas these Caspases were not detected in the control samples. While Caspase 3a expression was only slightly increased after STAT1 knock-down, the expression of Caspase 8 changed significantly: 100% of cells show positive immunohistochemical staining after STAT1 knock-down, which indicates that STAT1 prevents apoptosis in malignant mesothelioma cell lines.

Discussion

In our previous investigations STAT1 was found to be upregulated [5] and turned out to function like an oncogene in MPM [4]. In all MPM cases and cell lines SOCS1 protein was undetectable [12], which means that this negative feedback loop does not function, and STAT1 activation is not terminated by SOCS1 [21, 22]. Therefore we investigated the possibility that miRNAs might abolish SOCS1 expression and thus leads to constant overexpression of STAT1. In addition we studied the functional consequences of STAT1 knock-down in malignant mesothelioma cell lines.

STAT1 protein was highly expressed in all tissue samples, STAT3 was downregulated and it was not possible to detect SOCS1 and SOCS3 protein by immunohistochemistry. The expression pattern of STAT1, STAT3, SOCS1 and even SOCS3 on mRNA level differed from that on protein level: STAT1 and STAT3 were found to be downregulated compared to the housekeeping gene GAPDH in mesothelioma cell lines. It was possible to detect SOCS1 and SOCS3 mRNA – both were downregulated as well. STAT1 overexpression has been described to confer tumors resistance against radiation and cisplatin treatment [23]. Therefore better understanding of the mechanism underlying STAT1 activation and regulation could lead to successful strategies for targeting the STAT signaling pathway in MPM, probably by stimulating the negative feedback loop with analogues. It was reported previously that SOCS1 gene delivery cooperated with cisplatin plus pemetrexed to inhibit cell proliferation, invasiveness and induction of apoptosis in MPM cells. The authors hypothesize that SOCS1 gene delivery has a potent antitumor effect against MPM and that SOCS1 – in combination with cisplatin and pemetrexed – has potential for clinical use [24].

MiRNAs are often deregulated and function as oncogenes or tumor suppressors, they have potential as diagnostic markers and miRNA inhibitors can be used in disease treatment [13, 25]. MiRNAs belonging to the Oncomir-1 cluster were found to be upregulated in MPM cells. MiRNAs, known to be downregulated in several tumors (miR-21, miR-29a,

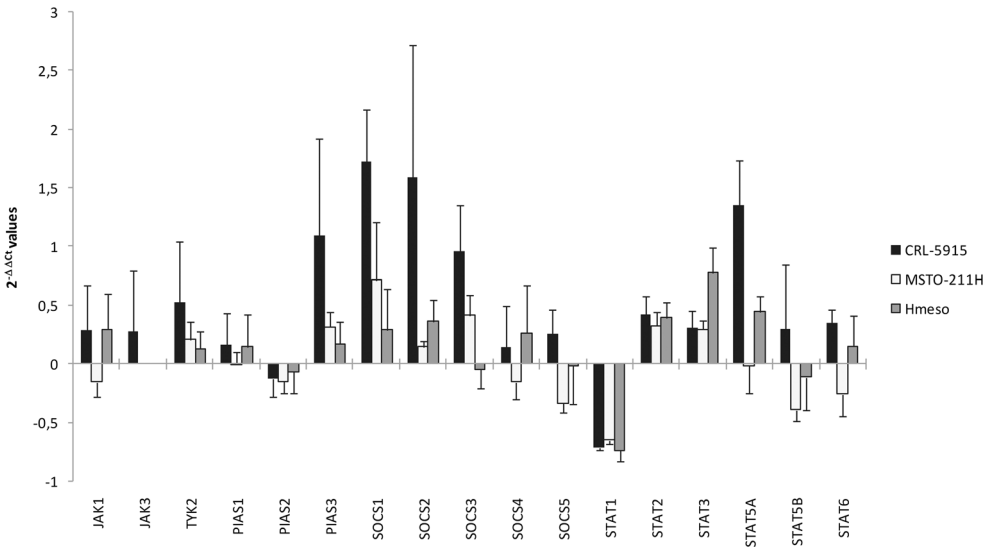

Fig. 6 mRNA expression levels of components of the JAK/STAT signaling pathway after STAT1 knock-down in CRL-5915, MSTO-211H and Hmeso cell lines. GAPDH was used as reference gene. $2^{-\Delta\Delta Ct}$ values are shown, error bars indicate the standard error

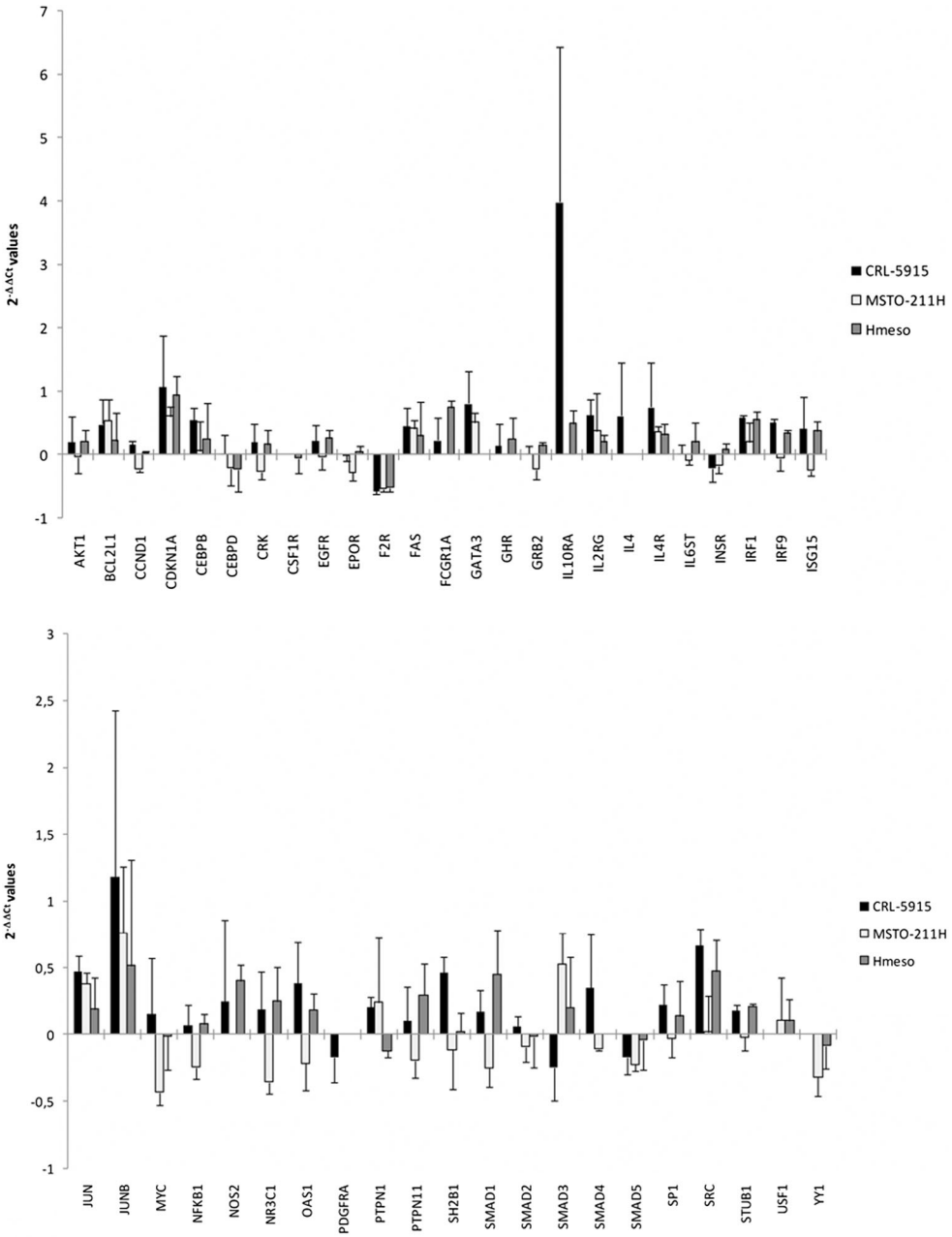

Fig. 7 mRNA expression levels of genes associated with the JAK/STAT signaling pathway after STAT1 knock-down in three mesothelioma cell lines. GAPDH was used as housekeeping gene. $2^{-\Delta\Delta Ct}$ values are shown; error bars indicate the standard error

miR-30b and miR-106a), were found to be deregulated [26]. These results go along with the findings of the present study: all mentioned miRNAs were upregulated. Subtype-related miRNAs were even correlated with better outcome: downregualtion of miR-30c was associated with better survival in MPM patients with sarcomatoid subtype, while this had no effect on epitheloid and biphasic subtypes [27]. In the present study, upregulation of miR-30c was detected in mesothelioma cell lines and epitheloid tumor tissue samples. Reid et al. reported that miR-16 is consistently downregulated in MPM tumor specimens and that restoration resulted in inhibition of tumor growth. However, they did not mention the tumor subtype [28]. In contrast, we detected an upregulation of miR-16 in our 52 epitheloid MPM tumor tissue samples which clearly shows that we may have to deal with subtype-specific expression of miRNAs and that therapeutic approaches may differ between subtypes.

Three upregulated miRNAs (miR-19b, −30b and -30c) have been identified, which are predicted to target SOCS1 and SOCS3. It was not possible to detect SOCS1/3 protein expression, but SOCS1/3-mRNA was detected in all cell lines even though the expression was downregulated in relation to the housekeeping gene GAPDH. These results suggest that the translation process might be inhibited or affected by miRNAs. Therefore high miRNA expression could definitely be the reason for downregulation or the missing expression of

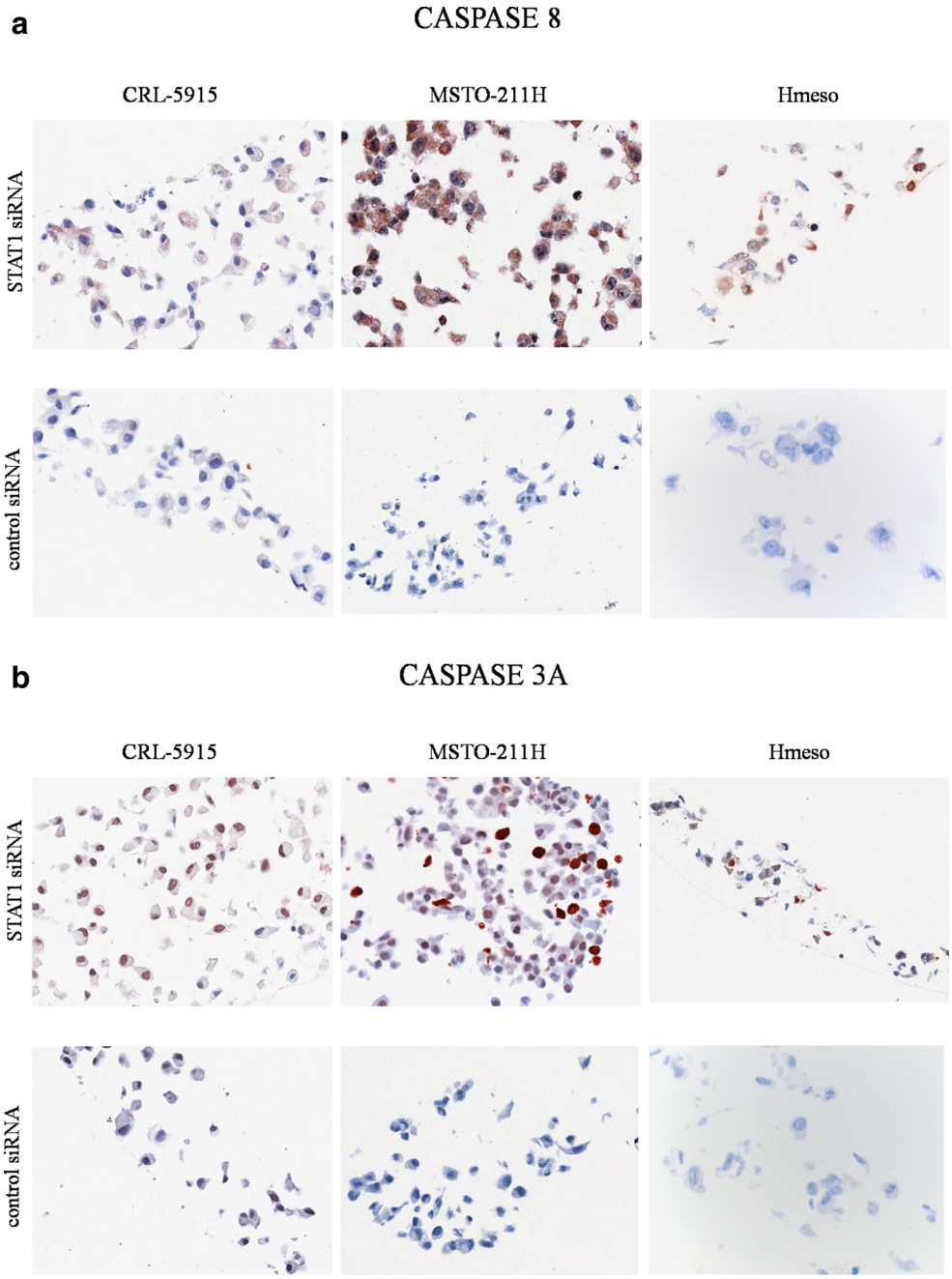

Fig. 8 Immunohistochemical staining with Caspase 3a **a** and Caspase 8 **b** antibodies on three mesothelioma cell lines (CRL-5915, MSTO-211H and Hmeso) either treated with STAT1 siRNA or control siRNA. Caspase 3a expression slightly increased when STAT1 was knocked-down. However, Caspase 8 expression changed significantly: 100% of cells show a positive staining in STAT1-siRNA treated samples

proteins. What has to be pointed out is that the same expression patterns have been detected for the control cell line HepG2: SOCS1/3-mRNA was downregulated whereas miR-19b, −30b and -30c were downregulated. Since SOCS1 protein is expressed in HepG2 cells, the identified miRNAs do not have an influence on SOCS expression in this cell line.

However, as reported previously, results differ due to different subtypes or sample source of the same tumor [29]. Therefore it is possible that the identified miRNAs do not regulate the STAT signaling pathway in HepG2 cells but have a regulatory effect in MPM cell lines. Unfortunately it was not possible to affect SOCS1 expression in MPM cell lines by inhibiting miRNA expression.

The negative regulator SOCS1 is normally translated and upregulated by STAT1, competes with STAT1 for the JAK1/2 binding site and thus abrogates the phosphorylation of STAT1 by JAK1/2 [30]. Constitutively STAT1 expression seems to provide MPM cells a selective growth and survival advantage. In order to investigate the consequences of STAT1 knock-down in malignant mesothelioma cell lines, qPCR (RT [2] Profiler PCR Array) was used to detect mRNA expression levels of all known JAK/STAT family members, the receptors

that activate them, STAT- inducible genes, negative regulators of the pathway and apoptosis and cell cycle related genes.

STAT1-mRNA was successfully knocked-down by using STAT1 siRNA in all three mesothelioma cell lines used for these experiments. The effect of STAT1 knock-down on the other components of the STAT signaling pathway differed between the cell lines: while CRL-5915 seems to compensate STAT1 knock-down by an upregulation of STAT5a, MSTO-211H and Hmeso show slightly upregulated expression levels of STAT3. Most of the apoptosis- and cell cycle related genes were differentially expressed in MPM cell lines and only three genes (*F2R*, *JUNB* and *CDKN1A*) were found to be equally expressed in all cell lines: F2R was downregulated whereas JUNB and CDKN1A were found to be upregulated. F2R belongs to a group of receptors that bind and activate JAK proteins, JUNB is known as a proto-oncogene and regulates gene expression [31, 32]. CDKN1A, also known as p21, is a regulator of cell cycle progression at the G1 phase, plays a regulatory role in S phase DNA replication and DNA damage repair and may be contributing in the execution of apoptosis [33]. Originally considered as a negative regulator of cell cycle and a tumor suppressor, it is now known that its function depends on its intracellular localization: when p21 is in the nucleus, it is a negative cell cycle regulator and tumor suppressor, whereas it acts like an oncogene by regulating migration, apoptosis and proliferation when localized in the cytoplasm [34]. *p21* was the first gene that was identified to be induced by p53 [35] and it was shown that overexpression of p21 leads to growth arrest and that the increased expression is sufficient for the negative regulation of gene expression by p53 in H1299 (human non-small cell lung carcinoma cell line) cells [36, 37].

STAT1 knock-down seems to have an effect on cell cycle progression and apoptosis by the upregulation of CDKN1A in mesothelioma cell lines used in the present study. However, no cell growth inhibition was detected microscopically after STAT1 knock-down. Therefore cells have been immunohistochemically stained with Caspase 3a and Caspase 8 antibodies and the expression of both Caspases was increased when STAT1 was knocked down. While staining for Caspase 3a was mild and not seen in all cells, Caspase 8 expression was dramatically increased in all cells, when STAT1 was knocked down which of course indicates, that apoptosis is induced. This may be explained by the fact that Caspase 8 is an initiator caspase that, once activated, cleaves and activates effector caspases like Caspase 3 [38, 39]. Possibly, the duration of STAT1 knock-down has been too short, to initiate the whole apoptotic program. However, since the effects of STAT1 knock-down differ between the three mesothelioma cell lines used and the knock-down seems to be compensated by an increased expression of STAT3 and STAT5a, all three molecules should be considered for therapeutic intervention.

The results of the present study support our previous findings that STAT1 acts like an oncogene in malignant pleural mesothelioma and provide the first evidence that STAT1, in combination with STAT3 and STAT5a, can be used as therapeutic target. Understanding the role of STATs in MPM could be the first step into the development of a targeted therapy for these tumors.

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References

1. Vogelzang NJ, Porta C, Mutti L (2005) New agents in the management of advanced mesothelioma. Semin Oncol 32(3):336–350
2. Rawlings JS, Rosler KM, Harrison DA (2004) The JAK/STAT signaling pathway. J Cell Sci 117(Pt 8):1281–1283. doi:10.1242/jcs.00963
3. Buard A, Vivo C, Monnet I, Boutin C, Pilatte Y, Jaurand MC (1998) Human malignant mesothelioma cell growth: activation of janus kinase 2 and signal transducer and activator of transcription 1alpha for inhibition by interferon-gamma. Cancer Res 58(4):840–847
4. Kothmaier H, Quehenberger F, Halbwedl I, Morbini P, Demirag F, Zeren H, Comin CE, Murer B, Cagle PT, Attanoos R, Gibbs AR, Galateau-Salle F, Popper HH (2008) EGFR and PDGFR differentially promote growth in malignant epithelioid mesothelioma of short and long term survivors. Thorax 63(4):345–351. doi:10.1136/thx.2007.085241
5. Arzt L, Kothmaier H, Quehenberger F, Halbwedl I, Popper HH (2011) STAT signalling in malignant mesothelioma: is there a regulatory effect of microRNAs? Magazine of European. Med Oncol 4(1):38–42
6. Calo V, Migliavacca M, Bazan V, Macaluso M, Buscemi M, Gebbia N, Russo A (2003) STAT proteins: from normal control of cellular events to tumorigenesis. J Cell Physiol 197(2):157–168. doi:10.1002/jcp.10364
7. Reich NC, Liu L (2006) Tracking STAT nuclear traffic. Nat Rev Immunol 6(8):602–612. doi:10.1038/nri1885
8. Sadzak I, Schiff M, Gattermeier I, Glinitzer R, Sauer I, Saalmuller A, Yang E, Schaljo B, Kovarik P (2008) Recruitment of Stat1 to chromatin is required for interferon-induced serine phosphorylation of Stat1 transactivation domain. Proc Natl Acad Sci U S A 105(26):8944–8949. doi:10.1073/pnas.0801794105
9. Stephanou A, Latchman DS (2005) Opposing actions of STAT-1 and STAT-3. Growth Factors 23(3):177–182. doi:10.1080/08977190500178745
10. Espert L, Dusanter-Fourt I, Chelbi-Alix MK (2005) Negative regulation of the JAK/STAT: pathway implication in tumorigenesis. Bull Cancer 92(10):845–857
11. Wormald S, Hilton DJ (2004) Inhibitors of cytokine signal transduction. J Biol Chem 279(2):821–824. doi:10.1074/jbc.R300030200

12. Arzt L, Kothmaier H, Halbwedl I, Quehenberger F, Popper HH (2014) Signal transducer and activator of transcription 1 (STAT1) acts like an oncogene in malignant pleural mesothelioma. Virchows Arch 465(1):79–88. doi:10.1007/s00428-014-1584-8
13. Meola N, Gennarino VA, Banfi S (2009) microRNAs and genetic diseases. PathoGenetics 2(1):7. doi:10.1186/1755-8417-2-7
14. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, van der Meer AJ, Patick AK, Chen A, Zhou Y, Persson R, King BD, Kauppinen S, Levin AA, Hodges MR (2013) Treatment of HCV infection by targeting microRNA. N Engl J Med 368(18):1685–1694. doi:10.1056/NEJMoa1209026
15. Clinicaltrials.gov (2013) A Multicenter Phase I Study of MRX34. http://clinicaltrials.gov/show/NCT01829971
16. Ueno T, Toyooka S, Fukazawa T, Kubo T, Soh J, Asano H, Muraoka T, Tanaka N, Maki Y, Shien K, Furukawa M, Sakaguchi M, Yamamoto H, Tsukuda K, Miyoshi S (2014) Preclinical evaluation of microRNA-34b/c delivery for malignant pleural mesothelioma. Acta Med Okayama 68(1):23–26
17. Arzt L, Kothmaier H, Quehenberger F, Halbwedl I, Wagner K, Maierhofer T, Popper HH (2011) Evaluation of formalin-free tissue fixation for RNA and microRNA studies. Exp Mol Pathol 91(2): 490–495. doi:10.1016/j.yexmp.2011.05.007
18. Pass HI, Stevens EJ, Oie H, Tsokos MG, Abati AD, Fetsch PA, Mew DJ, Pogrebniak HW, Matthews WJ (1995) Characteristics of nine newly derived mesothelioma cell lines. Ann Thorac Surg 59(4):835–844
19. Reale FR, Griffin TW, Compton JM, Graham S, Townes PL, Bogden A (1987) Characterization of a human malignant mesothelioma cell line (H-MESO-1): a biphasic solid and ascitic tumor model. Cancer Res 47(12):3199–3205
20. Shukla A, Hillegass JM, MacPherson MB, Beuschel SL, Vacek PM, Pass HI, Carbone M, Testa JR, Mossman BT (2010) Blocking of ERK1 and ERK2 sensitizes human mesothelioma cells to doxorubicin. Mol Cancer 9:314. doi:10.1186/1476-4598-9-314
21. Lesinski GB, Zimmerer JM, Kreiner M, Trefry J, Bill MA, Young GS, Becknell B, Carson WE 3rd (2010) Modulation of SOCS protein expression influences the interferon responsiveness of human melanoma cells. BMC Cancer 10:142. doi:10.1186/1471-2407-10-142
22. Madonna S, Scarponi C, De Pita O, Albanesi C (2008) Suppressor of cytokine signaling 1 inhibits IFN-gamma inflammatory signaling in human keratinocytes by sustaining ERK1/2 activation. FASEB J 22(9):3287–3297. doi:10.1096/fj.08-106831
23. Klampfer L (2006) Signal transducers and activators of transcription (STATs): novel targets of chemopreventive and chemotherapeutic drugs. Curr Cancer Drug Targets 6(2):107–121
24. Iwahori K, Serada S, Fujimoto M, Ripley B, Nomura S, Mizuguchi H, Shimada K, Takahashi T, Kawase I, Kishimoto T, Naka T (2013) SOCS-1 gene delivery cooperates with cisplatin plus pemetrexed to exhibit preclinical antitumor activity against malignant pleural mesothelioma. Int J Cancer 132(2):459–471. doi:10.1002/ijc.27611
25. Zhang B, Pan X, Cobb GP, Anderson TA (2007) microRNAs as oncogenes and tumor suppressors. Dev Biol 302(1):1–12. doi:10.1016/j.ydbio.2006.08.028
26. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM (2006) A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 103(7): 2257–2261. doi:10.1073/pnas.0510565103
27. Busacca S, Germano S, De Cecco L, Rinaldi M, Comoglio F, Favero F, Murer B, Mutti L, Pierotti M, Gaudino G (2010) MicroRNA signature of malignant mesothelioma with potential diagnostic and prognostic implications. Am J Respir Cell Mol Biol 42(3):312–319. doi:10.1165/rcmb.2009-0060OC
28. Reid G, Pel ME, Kirschner MB, Cheng YY, Mugridge N, Weiss J, Williams M, Wright C, Edelman JJ, Vallely MP, McCaughan BC, Klebe S, Brahmbhatt H, MacDiarmid JA, van Zandwijk N (2013) Restoring expression of miR-16: a novel approach to therapy for malignant pleural mesothelioma. Ann Oncol: Off J Eur Soc Med Oncol/ESMO 24(12):3128–3135. doi:10.1093/annonc/mdt412
29. Truini A, Coco S, Alama A, Genova C, Sini C, Dal Bello MG, Barletta G, Rijavec E, Burrafato G, Boccardo F, Grossi F (2014) Role of microRNAs in malignant mesothelioma. Cell Mol life Sci: CMLS. doi:10.1007/s00018-014-1584-5
30. Qing Y, Stark GR (2004) Alternative activation of STAT1 and STAT3 in response to interferon-gamma. J Biol Chem 279(40): 41679–41685. doi:10.1074/jbc.M406413200
31. NCBI F2R coagulation factor II (thrombin) receptor http://www.ncbi.nlmnih.gov/gene/2149
32. NCBI JUN proto-oncogene. http://www.ncbi.nlmnih.gov/gene/3725
33. NCBI CDKN1A cyclin-dependent kinase inhibitor 1A (p21, Cip1). http://www.ncbi.nlmnih.gov/gene/1026
34. Romanov VS, Pospelov VA, Pospelova TV (2012) Cyclin-dependent kinase inhibitor p21(Waf1): contemporary view on its role in senescence and oncogenesis. Biochemistry. Biokhimiia 77(6):575–584. doi:10.1134/S000629791206003X
35. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. Cell 75(4):817–825
36. Lohr K, Moritz C, Contente A, Dobbelstein M (2003) p21/CDKN1A mediates negative regulation of transcription by p53. J Biol Chem 278(35):32507–32516. doi:10.1074/jbc.M212517200
37. Wang Y, Blandino G, Givol D (1999) Induced p21waf expression in H1299 cell line promotes cell senescence and protects against cytotoxic effect of radiation and doxorubicin. Oncogene 18(16): 2643–2649. doi:10.1038/sj.onc.1202632
38. Adams JM, Cory S (2002) Apoptosomes: engines for caspase activation. Curr Opin Cell Biol 14(6):715–720
39. Earnshaw WC, Martins LM, Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68:383–424. doi:10.1146/annurev.biochem.68.1.383