Sarcoma Cells Induce Alteration in Adipogenic Differentiation

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Abstract. *Background: Sarcomas consist of both tumor and stromal cells, and the interaction between these compartments is critical for tumor progression and metastasis. Tumor-derived factors may alter the differentiation capacity of the adjacent stromal cells. The aim of this study was to elucidate the paracrine impact of liposarcoma cells on pre-adipocytes, their adipogenic differentiation process and miRNA expression profile. Materials and Methods: 3T3-L1 pre-adipocytes were exposed to sarcoma pre-conditioned media. Following induction of adipogenic differentiation morphometrical changes were assessed. Differences in miRNA expression of conditioned and non-conditioned 3T3-L1 cells were analyzed. Results: Exposure to sarcoma pre-conditioned media substantially altered the differentiation capability of 3T3-L1 pre-adipocytes. Significant changes in the miRNA expression profile between conditioned and non-conditioned pre-adipocytes were observed. Conclusion: Sarcoma cells directly modulate pre-adipocyte differentiation. This study demonstrates that sarcoma cells influence differentiation of pre-adipocytes via paracrine factors and alter their miRNA expression.*

Malignant soft tissue tumors are rare but aggressive malignancies, with an annual incidence of up to 3/100,000 new cases worldwide (1). Liposarcoma is the most common soft tissue sarcoma in adults (2, 3). Clinically, it is characterized by a high rate of local recurrence and high metastatic potential. The mortality rate is as high as 60% after five years (4-6). At present, surgical ablation is the first choice treatment modality (7, 8). Due to the low chemosensitivity of such tumors, chemotherapy plays a limited role in the treatment of these malignancies (4, 9).

The growth of solid tumors is characterized not only by the uncontrolled proliferation of cancer cells but also by changes in the tumor microenvironment that support the growth of the neoplastic mass and the metastatic spread of cancer cells to distant sites. The tumor-stromal interaction is a complex dynamic interplay between the expanding neoplastic mass and the tumor microenvironment. In order to develop new treatment modalities and to optimize therapy, an in depth knowledge of the biology of these tumors is essential; hence an understanding of the tumor microenvironment and tumor-stromal cell interaction will provide essential clues. The local microenvironment, cell–cell, and cell-stromal interactions are inherent in serving biochemical functions. Malignant cells perpetually stimulate host stromal and vascular cells to conduct physiological invasion (10). In addition, the tumor cells create an optimum microecology which is critical for tumor progression and metastasis (11). The biochemical environment provided by the extracellular matrix (ECM) is a key determinant of normal and pathological progression in oncogenesis (12). In addition to hormones, local paracrine cell signaling molecules, such as growth factors play an intrinsic role during cell differentiation. The tumor cell's ability to interact with the local microenvironment through bi-directional channels of biochemical and biophysical information may disrupt this vital balance and enable it to create a preferable tumor bed (12). The activated stroma creates a permissive "field" for the malignant cell to grow in (10). Bone tumors are another good example in which the interaction between bone cells and tumor cells has been considered a persuasive explanation of tumor growth in bone (13). Various signaling molecules, such as cytokines, growth factors, and other proteins, are secreted by the growing tumor and these tumor derived factors modulate the local environment to enhance tumor growth (14).

MicroRNAs (miRNAs) are small non-coding RNA molecules about 19-25 nucleotides that negatively regulate gene expression post-transcriptionally by controlling mRNA

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stability and translation (15),(16). The biological role and in vivo functions of most mammalian miRNAs are still poorly understood. In general miRNAs are linked to critical biological processes such as differentiation, proliferation, cell death and metastasis (17, 18). miRNAs regulate gene expression by binding to the 3'-untranslated regions of specific mRNAs. A single miRNA can regulate anywhere from a few genes to many hundreds of genes (19). Alteration in miRNA expression of stromal cells may thus influence tumor progression and metastasis.

At our Reference Centre for Soft Tissue Sarcoma, we have observed, adipocytes to be both abnormally shaped and sized and noted the presence of adipoblasts directly adjacent to excised retroperitoneal liposarcomas (unpublished observation, Figure 1). These changes in cell shape are a morphological hallmark of differentiation. The delicate balance between the dormancy of progenitor cells and their timely proliferation and differentiation is a crucial parameter in tissue homeostasis that is often perturbed in disease. Therefore we opted to elucidate the impact of liposarcoma cells on pre-adipocytes based on a cell culture model comprising SW872 cells and 3T3-L1 pre-adipocytes which are well described as a model for adipogenesis. We hypothesized that miRNA expression changes might be caused by the sarcoma itself prior to sarcoma invasion and that these changes might provide clues to important steps in early sarcoma tumorigenesis. The aim of this study was to elucidate the paracrine impact of liposarcoma cells on preadipocytes, their adipogenic differentiation process and miRNA expression profile.

Materials and Methods

Cell culture. The preadipocyte line 3T3-L1 (ATCC-LGC Standards, Wesel, Germany) and the liposarcoma cell line SW872 (ATCC-LGC Standards, Wesel, Germany) were cultured in Dulbecco's Modified Eagle Medium (D-MEM) (PAN-Biotech, Aidenbach, Germany) containing 10% fetal calf serum (FCS) (Hyclone-Thermo Scientific, Bonn, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (PAN-Biotech, Aidenbach, Germany). Cells were incubated in a humidified incubator containing 5% CO_2. Pre-conditioned media was collected from SW872 liposarcoma cells: after the cells reached confluence, the media was exchanged and the cells were incubated for another 48 h, then the medium was collected and filtered with a 0.22 μm syringe filter and was stored at −80˚C until usage.

Adipogenic differentiation. After 3T3-L1 cells reached confluence the medium was changed. Cells were then maintained in proliferation media (D-MEM containing 2% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin) and SW872 pre-conditioned media for 48 h, respectively. Confluent 3T3-L1 cells were subsequently treated with differentiation medium [DMEM/F12 containing 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco, Invitrogen, Darmstadt, Germany), 66 nM insulin, 100 nM dexamethasone (Sigma, Taufkirchen, Germany), 0.5 mM isobutylmethylxanthine (IBMX) (Sigma, Taufkirchen, Germany), 0.1 mg/ml pioglitazone (Sigma, Taufkirchen, Germany), 1 nM triiodo-L-thyronine (Sigma, Taufkirchen, Germany), and 10 mg/ml human transferring (Sigma, Taufkirchen, Germany)]. After 5 days, the differentiation media was changed to differentiation medium lacking IBMX and pioglitazone as a control population served 3T3-L1 cells cultivated in proliferation media. The differentiation assay was repeated three times (n=4).

Oil red o staining. Differentiated 3T3-L1 cells were fixed with 3.5% para-formaldehyde for 10 min at room temperature. Fixed cells were rinsed with Phosphate Buffered Saline (PBS) and subsequently washed with 60% (v/v) isopropanol. Isopropanol pretreated cells were stained for 30 min with 0.3% (w/v) oil red (Sigma, Taufkirchen, Germany) staining solution. Excess of oil red staining solution was removed by 60% (v/v) iso-propanol followed. Oil red stained cells were counterstained with hematoxylin. Lipid drop formation was examined under light microscopy (IX71; Olympus America, Center Valley, PA, USA) at the indicated magnification.

miRNA expression analysis. RNA from 3T3-L1 cells following preconditioning with SW872 medium (n=4) and untreated 3T3-L1 controls (n=4) were extracted using a QIAGEN RNeasy kit (QIAGEN, Hilden, Germany, according to the manufacturer's protocol, respectively. Total-RNA (1000 ng) was reverse transcribed using stem-loop primer MegaPlex rodent primers pools A and B (Life Technologies, Darmstadt, Germmany). For quantitative analysis quantitative realtime poly chain reaction (qRT-PCR) were carried out using TaqMan rodent miRNA array cards A and B (Life Technologies, Darmstadt, Germmany) respectively on a ABI 7900HT Fast real time RT-PCR System ABI 7900HT Fast real time RT-PCR System. The relative miRNA expression was calculated by the comparative Ct method using U6 small nuclear RNA (RNU6) as a housekeeper.

Statistical analysis. Statistical analysis was carried out using TM4 Software Version 4.6 software (Dana-Farber Cancer Institute, 44 Binney St, Boston, MA, USA). Differences in the miRNA expression between pre-conditioned and non-conditioned cells were calculated by the rank product. Briefly, we used an α of 0.01 *p*-value cut off in a two class unpaired experimental set-up.

Results

In a first experiment the influence of sarcoma conditioned medium on the differentiation capacity of 3T3-L1 pre-adipocytes was tested (n=4). On day one all cells displayed a fibroblastic cell shape without any indication of differentiation. In contrast to the non-conditioned pre-adipocytes the 3T3-L1 cells with sarcoma pre-conditioned medium appeared more condensed and rounded (Figure 2; day 1). After three days, most of the non-conditioned 3T3-L1 cells started to differentiate spontaneously. The cells showed an increase in cytoplasm, with an even distribution of small lipid droplets, lost their fibroblastic appearance and started to be more round shaped. In the pre-conditioned 3T3-L1 cells on the other hand, the formation of lipid droplets was observed only in a small subset of cells. In contrast to the non-preconditioned control cells, these cells retained their fibroblastic phenotype (Figure 2; day 3). On day 8,

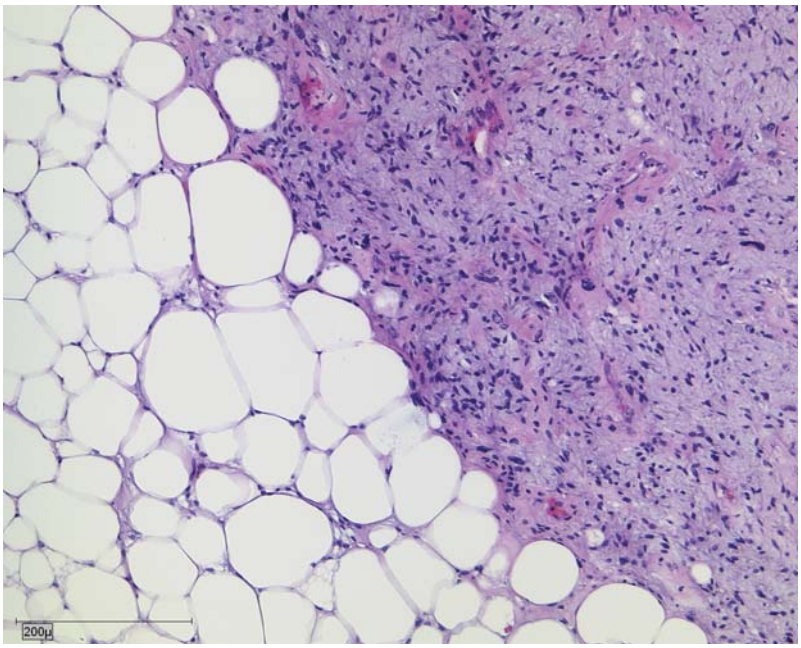

Figure 1. *Histological appearance of a retroperitoneal high grade liposarcoma. Both abnormally shaped and sized adipocytes and the presence of adipoblasts directly adjacent to the excised retroperitoneal liposarcoma are demonstrated. Changes in cell shape are a morphological hallmark of differentiation.*

nearly all of the non-pre-conditioned pre-adipocytes had changed from a fibroblastic to an adipocyte phenotype, with a distinct increase in lipid droplets which were increased in size and number. The overall number of conditioned cells decreased. The majority of these cells still displayed a fibroblastic cell shape, only a minority of cells started to round up and accumulate lipid droplets, which still remained very small (Figure 2; day 8).

Oil red staining on day 11 revealed complete differentiation of the non-pre-conditioned cells with multiple lipid droplets. The cells started to take on a ball shape and to detach from the culture dish due to the amount of incorporated lipids. The non-conditioned cells were nearly completely differentiated whereas the pre-conditioned 3T3-L1 cells displayed a heterogeneous differentiation pattern. This pattern ranged from cells with a weak formation of lipid droplets and senescence like cell shape to cells which contain a comparable amount of lipid drops as seen in the non preconditioned cells. But the number of cells with signs of differentiation was markedly reduced and the overall lipid drop formation after pre-conditioning was clearly less (Figure 2; day 11).

After having established a phenotype the second stage of the experiment was conducted to detected differences in miRNA expression of 3T3-L1 cells cultured in proliferation media and 3T3-L1 cells exposed to sarcoma-pre-conditioned media (n=4). A twofold change was noted in 18 miRNAs: in the cells that were exposed to sarcoma-pre-conditioned media 11 miRNAs were up-regulated and 7 miRNAs were down-regulated (Table I).

Discussion

This study demonstrates that sarcoma cells influence stromal cells *via* paracrine factors which influence differentiation of adipogenic precursor cells. This study has shown a statistically significant difference in expression of miRNAs in preadipocytes exposed to sarcoma pre-conditioned medium that inhibited differentiation into mature adipocytes, and those pre-adipocytes that were not exposed. In addition, sarcoma pre-conditioned medium was demonstrated to alter adipogenic differentiation and directly repress lineage commitment of pre-adipocytes.

The group of overexpressed miRNA in the cells that were exposed to sarcoma pre-conditioned medium included miR-24-1*, miR-134, miR-141, miR-200c and miR-203, which have been already reported as being amplified in various solid carcinomas of bladder, breast, colon, stomach, lung, ovary, pancreas and prostate (20-24). miRNAs have been shown to be important regulators of gene expression (25) by promoting RNA degradation or translation suppression, and can regulate development and disease processes (26). On the other hand, overexpression and silencing of specific miRNAs are associated with the development and progression of sarcoma. As a single miRNA can simultaneously target multiple genes, alterations in the amount and sequence of a

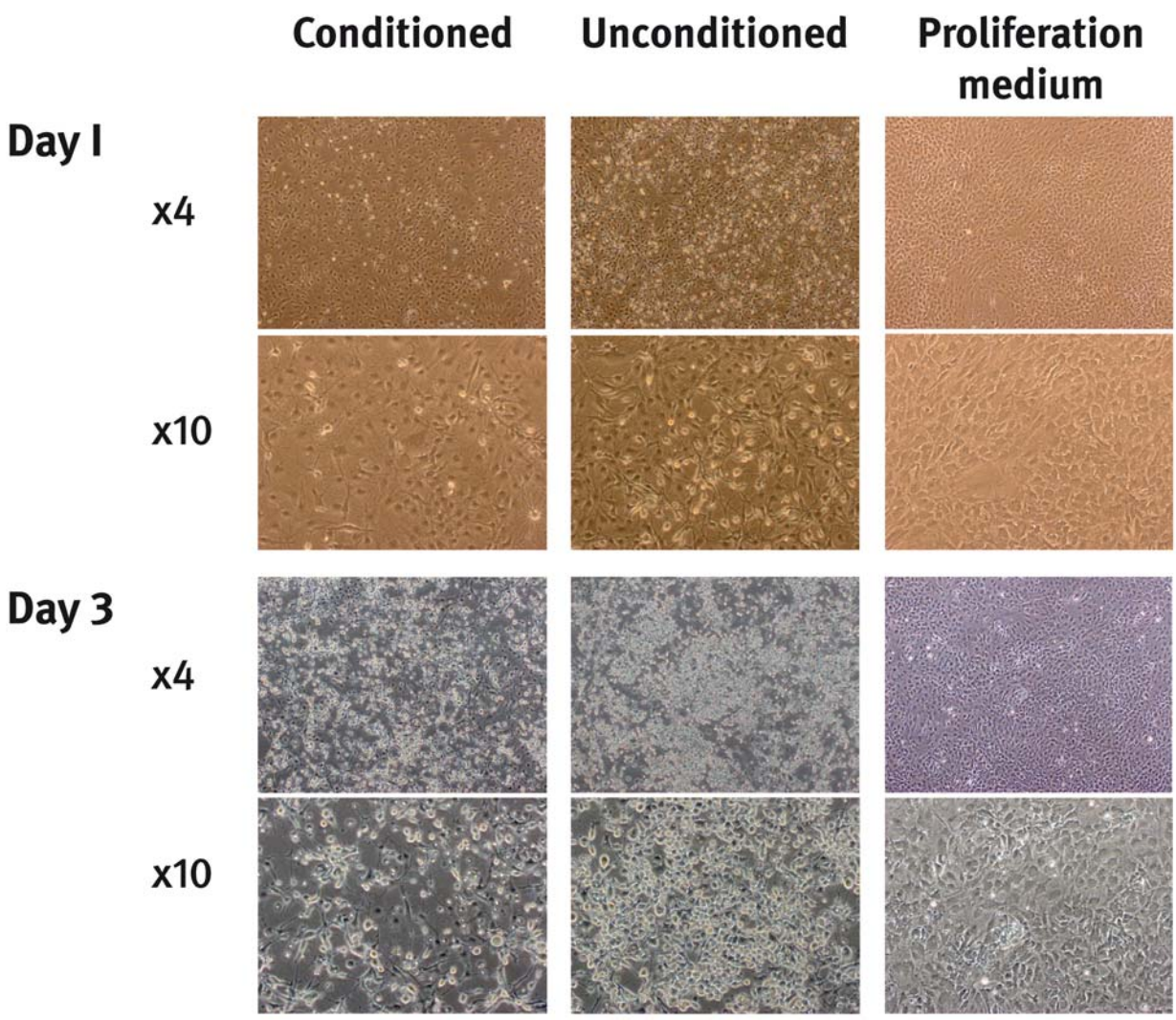

Figure 2. *Continued*

mature miRNA can have significant effects on the expression of target genes. The fact that a single miRNA can regulate multiple targets and a particular target may be regulated by various miRNAs suggests a highly complex network of miRNA-target interactions, which is only beginning to be unraveled.

This study demonstrates two phenomena, firstly the inhibition of pre-adipocyte differentiation and secondly, the changes in pre-adipocyte miRNA expression profile. Inhibition of pre-adipocyte differentiation may be reflected in modifications to their miRNA expression profile (27). Changes in miRNA expression were an effect of the sarcoma pre-conditioned medium, potentially causing activation of the stroma. A possible emerging mechanism for the control of gene expression within the tumor stroma by the mesenchymal tumor is the differential expression and transfer of miRNA species. In this study, we identified a set of miRNAs that are expressed within pre-adipocytes and found that major miRNA expression changes occur in the presence of sarcoma pre-conditioned medium, thereby suggesting a set of putative oncogenic and tumor suppressor miRNAs, that are dysregulated at the preinvasive stage of sarcoma. For example, dysregulation of six of the identified miRNAs has previously been associated with progression and invasion of solid tumors (28-32). This study identifies a potential role for these previously implicated miRNAs at an early stage of sarcoma invasion. We hypothesize that there is a coordinate mechanism of dysregulation between the abnormal expression of miRNA and target mRNA in very early sarcoma invasion. This study demonstrates an association of overexpression of these miRNAs with sarcomas. In addition, some miRNAs have been noted to be

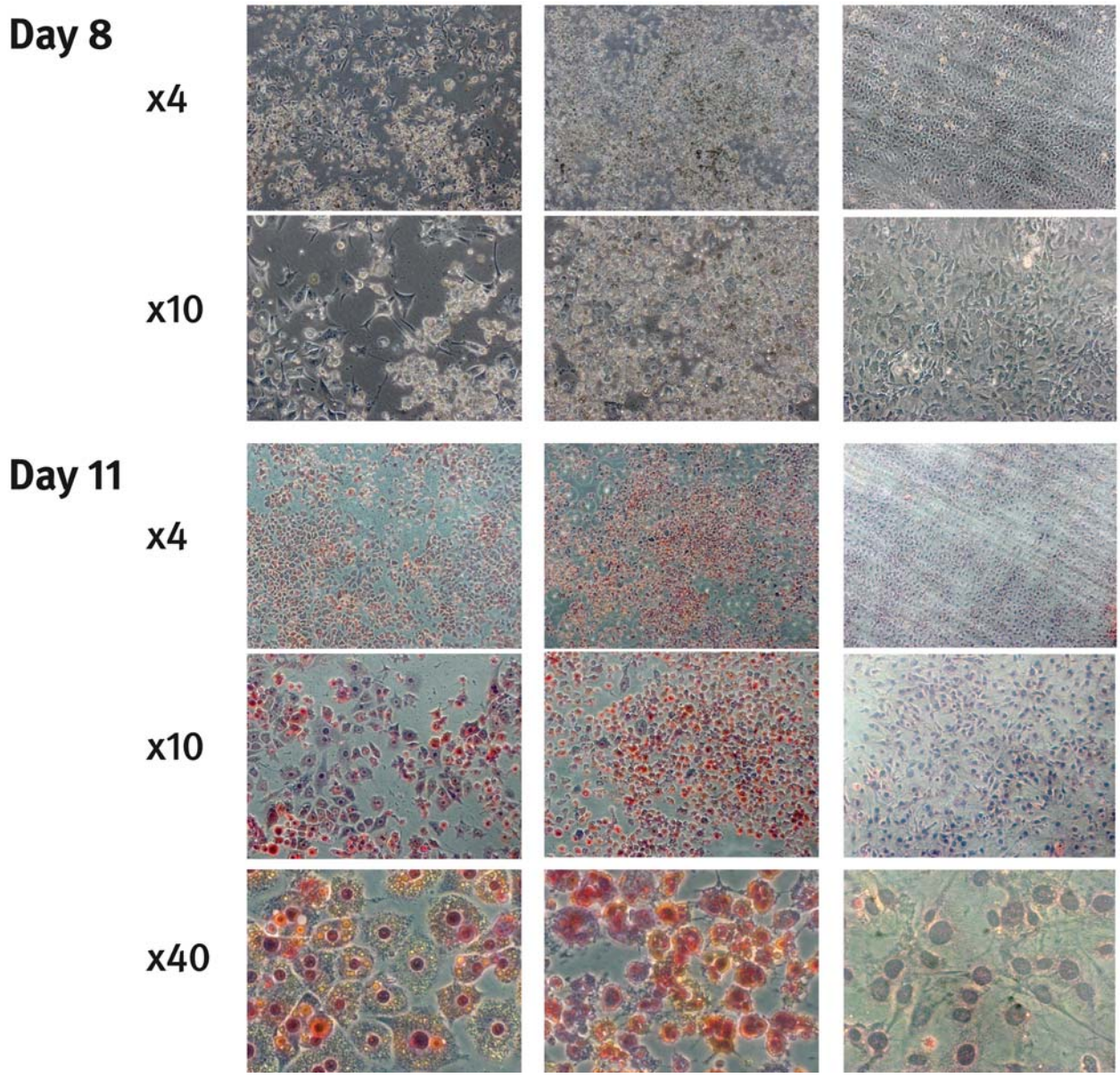

Figure 2. *Differentiation assay.*

down-regulated in sarcoma-pre-conditioned 3T3-L1 cells, including miR-295. Interestingly, miR-295 has been found to reduce efficiency of induced pluripotency by acting downstream of c-MYC (33, 34).

In solid tumours, it appears as the most common miRNA event is overexpression, whereas underexpression in cancer is a more limited event and is more tissue specific (35). The downstream consequences of such alterations would be manifested as changes in cellular physiology and phenotype. For example, if the fine-tuning of gene expression controlled by miRNAs was subtly altered, leading to enhanced rates of proliferation and/or decreased rates of apoptosis, such changes could significantly influence a person's risk of developing sarcoma over time. In the future, we aim to compare miRNA from human liposarcoma to our identified set of miRNAs to elucidate their role in tumor invasion.

Taken together, identification of such genes would be important for clarifying the mechanisms involved in tumor initiation, growth, and progression and revealing new markers for molecular diagnosis and novel targets for drug therapy. In addition to protein-coding oncogenes and tumor suppressor genes, we will have to take into account miRNAs

Table I. *Differences in the expression profile of miRNA between conditioned and non-conditioned 3T3-L1 pre-adipocytes. Rank correlation of two fold changes in miRNAs of pre-conditioned versus non conditioned 3T3-L1 cells.*

Probe	Fold change	*p*-Values (up)
Up-regulated mRNA		
mmu-miR-24-1*	2.6	0.0016
mmu-miR-409	3.4	0.0003
mmu-miR-704	2.8	0.0011
mmu-miR-376b*	3.5	0.0001
mmu-miR-299*	2.2	0.0023
mmu-miR-200c	4.4	0.0000
mmu-miR-141	2.6	0.0014
mmu-miR-134	2.9	0.0008
mmu-miR-379	2.6	0.0014
mmu-miR-297b	2.6	0.0018
mmu-miR-203	3.6	0.00003
Down-regulated mRNA		
mmu-miR-184	–1.9	0.010
mmu-miR-215	–2.6	0.007
mmu-miR-219	–2.6	0.004
mmu-miR-295	–2.4	0.007
mmu-miR-32	–2.0	0.010
mmu-miR-678	–2.2	0.007
mmu-miR-708	–2.6	0.003

and their regulatory networks in order to understand the complex processes underlying malignant transformation.

This experimental study adds to the hypothesis that altered expression of certain miRNAs, such as miR-24-1*, miR-134 and miR-295, may influence adipogenic differentiation and represse lineage commitment of pre-adipocytes. These data augment examples of miRNAs whose expression is modified in tumorigenesis.

In conclusion, our data indicate that sarcoma-stromal interaction influences differentiation of the adjacent precursor cells and leads to changes of the miRNA expression of the stromal cells. Our findings suggest that changes in miRNA expression may promote tumor expansion and invasiveness through the concurrent activity on stromal and tumor cells. Whether these changes lead to a microenvironment which accelerates tumor progression or only induces a standby state which enables alternative terminal differentiation of pre-adipocytes has to be further elucidated. This information highlights the importance of determining where miRNA alterations originate in tumor cells or the tumor stroma and establishing what factors are responsible for these altered miRNA levels. The special cellular microecology of tumors influences responsiveness to therapeutic agents and has implications for future directions in sarcoma research.

Conflicts of Interest

The authors declare that there are no financial or non-financial conflicts of interest in regards to this manuscript.

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