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Musculoskeletal Pathology

Aberrant Calreticulin Expression Is Involved in the Dedifferentiation of Dedifferentiated Liposarcoma

Masanori Hisaoka, Atsuji Matsuyama, and Mitsuhiro Nakamoto

From the Department of Pathology and Oncology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan

Liposarcomas are a representative group of soft tissue sarcomas with variably hampered adipogenesis, which is most exemplified by its dedifferentiated subtype. However, the factor(s) responsible for inhibiting adipocyte differentiation remains unknown. A recent gene expression profiling study identified several unique genes that were highly expressed in dedifferentiated liposarcoma, and the gene encoding calreticulin (*CALR*), a major Ca^{2+}-buffering protein that can inhibit adipocyte differentiation, was found to be overexpressed. Thus, we investigated the expression of calreticulin in 45 cases of liposarcomas, including 15 dedifferentiated tumors, at both the protein and mRNA levels. Immunohistochemically, calreticulin was consistently expressed in the dedifferentiated areas of dedifferentiated liposarcomas and commonly observed in atypical stromal cells and/or lipoblasts in the well-differentiated areas (87%), whereas large vacuolated adipocytic cells in either the tumors or normal fat were essentially negative. These results were further supported by the findings of Western blot and quantitative RT-PCR analyses. Although abnormalities in 19p13.1–13.2 where *CALR* is localized were uncommon in the dedifferentiated liposarcomas examined by fluorescence *in situ* hybridization, expression of miR-1257, a putative microRNA that targets calreticulin, was suppressed in the dedifferentiated subtype. The down-regulation of calreticulin by small-interfering RNA could induce adipogenesis in dedifferentiated liposarcoma cells and reduce cell proliferation. Our results therefore suggest that aberrantly expressed calreticulin in dedifferentiated liposarcoma is involved in its dedifferenitation and/or tumor progression. ***(Am J Pathol 2012, 180: 2076–2083; DOI: 10.1016/j.ajpath.2012.01.042)***

Liposarcomas are a representative group of soft tissue sarcoma and are the most common sarcoma type in adults. Liposarcomas are mainly classified into four major histologic subtypes (ie, well-differentiated, myxoid/round cell, pleomorphic, and dedifferentiated subtypes), based primarily on their morphologic features.[1] Each histologic subtype of liposarcoma differs in the extent of adipocyte differentiation, which is potentially correlated with its distinct biological behavior.[2] For instance, well-differentiated liposarcoma, which is referred to as atypical lipomatous tumor when it arises in locations amenable to surgical resection, is made up predominantly of mature adipocyte proliferation and virtually never metastasizes after complete surgical extirpation. In contrast, a nonlipogenic high-grade sarcomatous area is typically present in the dedifferentiated subtype, which often recurs locally and has been found to metastasize in 15% to 20% of cases.[1] However, the factors responsible for inhibiting the adipocyte differentiation in such liposarcoma subtypes remain unknown.

The process of adipocyte differentiation is driven by a highly coordinated cascade of transcriptional events that result in the maturation of fat cells.[3,4] Treatment with adipogenesis-inducing medium causes rapid and transient expression of the early transcriptional regulators, CCAAT enhancer-binding protein (C/EBP) β and C/EBPδ, in cultured preadipocytes, subsequently leading to the expression of other transcription factors, such as C/EBPα and proliferator-activated receptor γ (PPARγ), which are involved in adipocytic maturation, and that of late or terminal adipogenic markers [eg, adipsin, lipoprotein lipase (LPL) and aP2].[3,4] The adipocyte differentiation program can be modified by a variety of molecules or related functional pathways such as transforming growth factor-β-Smad3 and β-catenin-Wnt signal pathways, angiotensin II, neuropoietin, adrenomedullin, endothelin-1, c-jun, and calreticulin.[5–12]

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Address reprint requests to Masanori Hisaoka, M.D., Department of Pathology and Oncology, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan. E-mail: hisaoka@med.uoeh-u.ac.jp.

Notably, a recent expression profiling study of dedifferentiated liposarcoma has identified a unique set of genes significantly expressed in liposarcoma compared with normal fat, and *CALR* (encoding calreticulin) was one of the highly expressed genes.[13] The similar trend of *CALR* expression in dedifferentiated liposarcoma can be found in the data of another gene expression profiling, available at the Gene Expression Omnibus (Accession number GSE6481; available at http://www.ncbi.nlm.nih.gov/geo).[14] Calreticulin is an endoplasmic reticulum resident protein that functions as a molecular chaperone and a regulator of Ca^{2+} homeostasis, and it negatively regulates the commitment to adipocyte differentiation potentially by inhibiting the calmodulin-Ca^{2+}/calmodulin-dependent protein kinase II pathway.[12] We investigated the calreticulin status in dedifferentiated liposarcomas to address its inhibitory function in adipocyte differentiation, which is presumably involved in the development or progression of liposarcoma. As we expected, calreticulin was almost consistently overexpressed in the dedifferentiated liposarcomas in the series that we examined. The down-regulation of calreticulin could induce adipogenesis and reduce the cell proliferation *in vitro*.

Materials and Methods

Tissue Samples

Forty-five liposarcomas (15 well-differentiated, 15 myxoid, and 15 dedifferentiated subtypes), 5 lipomas, and 70 nonlipogenic soft tissue sarcomas of minimal or divergent differentiation (10 undifferentiated high-grade pleomorphic sarcomas/pleomorphic malignant fibrous histiocytomas, 10 myxofibrosarcomas, 10 leiomyosarcomas, 10 rhabdomyosarcomas, 10 angiosarcomas, 10 synovial sarcomas, and 10 Ewing sarcomas/primitive neuroectodermal tumors) were retrieved from the files of the Department of Pathology and Oncology, School of Medicine, University of Occupational and Environmental Health. Each diagnosis of the tumor was re-confirmed by two of the authors (M.H. and A.M.) according to the current criteria of the World Health Organization.[1] Nuclear expression of MDM2 and/or CDK4 was identified immunohistochemically in all of the well-differentiated and dedifferentiated liposarcomas examined. *FUS/EWSR1-DDIT3* fusion gene transcripts were detectable in the 15 myxoid liposarcomas with the use of RT-PCR. Likewise, characteristic fusion gene transcripts (ie, *SS18-SSX*, *EWSR1-FLI1/ERG*) were identified in synovial sarcoma and Ewing sarcoma/primitive neuroectodermal tumor. Normal subcutaneous fatty tissue was obtained during a mastectomy for breast tumors in five patients. Formalin-fixed, paraffin-embedded tissue from these samples was used in the following immunohistochemical analysis for calreticulin expression and fluorescence *in situ* hybridization (FISH). Snap-frozen tissue specimens of three cases of liposarcomas, lipoma, and normal fatty tissue were also included in the study, although only those of a dedifferentiated area were available for the dedifferentiated liposarcoma cases.

Cells and Culture Conditions

The human dedifferentiated liposarcoma cell line, FU-DDLS-1, was used in the study and was provided courtesy of Prof. H. Iwasaki, Fukuoka University.[15] The human monophasic synovial sarcoma cell line, HS-SY-II, was obtained from Riken BioResource Center (Ibaraki, Japan). The cells were maintained under standard culture conditions with the use of Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. To induce adipocyte differentiation, the culture medium was replaced 2 days after semiconfluence by a differentiation medium containing 1 μmol/L dexamethasone, 0.5 mmol/L methylisobutylxanthine, 5 μg/mL insulin, and 0.5 μmol/L rosiglitazone (Cayman Chemical, Ann Arbor, MI). The cells incubated with the differentiation medium 72 hours after harvesting with or without small-interfering RNA (siRNA) for calreticulin were used for the following cell growth assay and other studies, including RT-PCR, a Western blot analysis, oil red O staining, and immunohistochemistry.

Western Blot Analysis and Antibodies

Proteins were prepared from snap-frozen tissue samples by homogenization in a buffer containing 10 mmol/L K_2HPO_4, 10 mmol/L KH_2PO_4, 10 mmol/L EDTA, 6 mg/mL 3-[(3-Cholamidopropyl) dimethyl ammonio]−1-propanesulfonate and one tablet containing a complete protease inhibitor cocktail (Roche, Mannheim, Germany) per 50 mL of the buffer, followed by the addition of 50 mmol/L dithiothreitol. Cultured cells were treated with 10% trichloroacetic acid at 4°C for 30 minutes, and cell lysates were prepared by lysis in a buffer containing 9 mmol/L urea, 2% Triton X-100, and 1% dithiothreitol. Thirty micrograms of protein per sample were run on NuPage 10% Bis-Tris gels with the use of an Xcell SureLock Mini-Cell system (Invitrogen, Carlsbad, CA), and then transferred to polyvinylidene difluoride membranes (Invitrogen) by electroblotting. The membranes were probed with primary antibodies diluted in TBS-Tween buffer (50 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.1% Tween 20, 15 mmol/L sodium azide) containing 3% bovine serum albumin. Anti-calreticulin (ab22683, 1:1000; Abcam, Cambridge, MA), anti-β-actin (C4, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), anti-C/EBPα (14AA, 1:200; Santa Cruz Biotechnology), and anti-PPARγ (E-8, 1:200; Santa Cruz Biotechnology) antibodies were used as primary antibodies. Then the membranes were incubated with a peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin secondary antibody (Sigma-Aldrich, St Louis, MO), and the signals were visualized with 3,3′,5,5′ tetramethylbenzidine (Sigma-Aldrich).

Immunohistochemistry

Four-micron thick sections of the formalin-fixed, paraffin-embedded tumor tissue specimens were subjected to immunohistochemistry for calreticulin with the use of a labeled polymeric secondary antibody (Envision;

Table 1. Primer Sequences and Annealing Temperatures Used

Gene	Sense	Sequence	Annealing temperature (°C)	Product size (bp)
Adipsin	Forward	5′-CTGCTACAGCTGTCGGAGAAG-3′	56	556
	Reverse	5′-GAGATCCAATGATCCTCCCAC-3′		
aP2	Forward	5′-CAGCTTCCTTCTCACCTTGAAG-3′	56	474
	Reverse	5′-GAACTTCAGTCCAGGTCAACGT-3′		
LPL	Forward	5′-TGAGAACATCCCATTCACTCTG-3′	55	535
	Reverse	5′-AGCTTATCCTGAGCATCCTGAA-3′		
ACTA (*β-actin*)	Forward	5′-GCCCCTCCATCGTCCACCGC-3′	59	493
	Reverse	5′-GGGCACGAAGGCTCATCATT-3′		

DakoCytomation, Tokyo, Japan). After standard antigen retrieval in a pressure cooker for 10 minutes in citrate buffer (pH 6), the primary antibody was applied to the sections and incubated for 30 minutes at room temperature, followed by the incubation with the secondary antibody for 30 minutes and visualization with a 3,3′-diaminobenzidine solution. The sections were counterstained with hematoxylin. Cases with >10% positively stained cells were considered to have calreticulin expression. Human placental tissue was used as a positive control for the immunohistochemical evaluation. The immunohistochemical data were assessed by two certified pathologists (M.H. and A.M.) independently. The interobserver reliability was measured with the κ index for multiple readers as described elsewhere,[16] and the agreement was considered good, as shown by the κ value of 0.77.

RNA Extraction and Standard or Quantitative RT-PCR

Total RNA was extracted from the tissues or cultured cells with the use of the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For standard RT-PCR, 1 μg of RNA was reverse transcribed into cDNA with the use of random primers and SuperScript III Reverse Transcriptase (Invitrogen). PCR was performed using 0.5 μL of cDNA and 2.5 U of TaqDNA polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA). The primer sequences and annealing temperatures used for the detection of LPL, adipsin, aP2, and β-actin expression are shown in Table 1. For quantitative RT-PCR, the TaqMan Gene Expression assay kit for *CALR* (Hs00189032_m1; Applied Biosystems) and *GAPDH* as an endogenous control (Applied Biosystems) was used, according to the manufacturer's instructions. The mean Ct value of each sample was determined from duplicate reactions, and the relative expression level of *CALR* was calculated by the comparative Ct ($\Delta\Delta$Ct) method. To assess the expression of microRNAs (miRNAs) potentially regulating the expression of *CALR*, the expression levels of candidate miRNAs, which were identified by a computer-based and gene-related miRNA prediction analysis on the basis of the sequence information matching CALR mRNA and the strength of binding positions judged from the estimated free energy of miRNA-mRNA duplexes (miRBLAST-A; COSMO BIO, Tokyo, Japan), were evaluated by quantitative RT-PCR with the use of the TaqMan MicroRNA Assay and TaqMan MicroRNA RT kit (Applied Biosystems). RNU6B expression was measured as an internal control for the relative quantification.

Gene Silencing

FU-DDLS-1 and HS-SY-II cells were reverse transfected with 5 nmol/L Silencer Select Validated siRNA for *CALR* (5′-AUGUUGAUCAAGAAAGAUGAG-3′; Applied Biosystems) or 5 nmol/L Silencer Select Negative Control (Applied Biosystems) with the use of the siPORT NeoFX Transfection

Table 2. Immunohistochemical Results of Calreticulin Expression in Lipogenic Tumors and Other Sarcomas

	Calreticulin expression		
Tumor (*N*)	− (<10%)	+ (10% to 33%)	++ (>33%)
Lipoma (15)	11	4	0
Myxoid liposarcoma (15)	2	8	5
Well-differentiated liposarcoma (15)			
Lipoma-like (10)	4	6	0
Sclerosing (5)	0	4	1
Dedifferentiated liposarcoma (15)			
Well-differentiated area (15)	2	13	0
Dedifferentiated area (15)	1	2	12
Pleomorphic MFH (10)	2	4	4
Myxofibrosarcoma (10)	2	5	3
Leiomyosarcoma (10)	4	4	2
Rhabdomyosarcoma (10)	1	5	4
Angiosarcoma (10)	0	4	6
Synovial sarcoma (10)	8	2	0
Ewing sarcoma/PNET (10)	9	1	0

MFH, malignant fibrous histiocytoma; PNET, primitive neuroectodermal tumor.

optical densities in the cell proliferation assay were compared using the Wilcoxon rank sum test, and $P < 0.05$ was considered to be statistically significant.

FISH

Interphase FISH was performed on nuclei isolated from 50-μm thick sections of formalin-fixed, paraffin-embedded tissues prepared from dedifferentiated areas of five dedifferentiated liposarcomas with immunohistochemical overexpression of calreticulin and two lipomas. Texas Red-conjugated human BAC clones (GSP0189G01 and GSP1043C11; GSP Laboratory, Kawasaki, Japan), span-

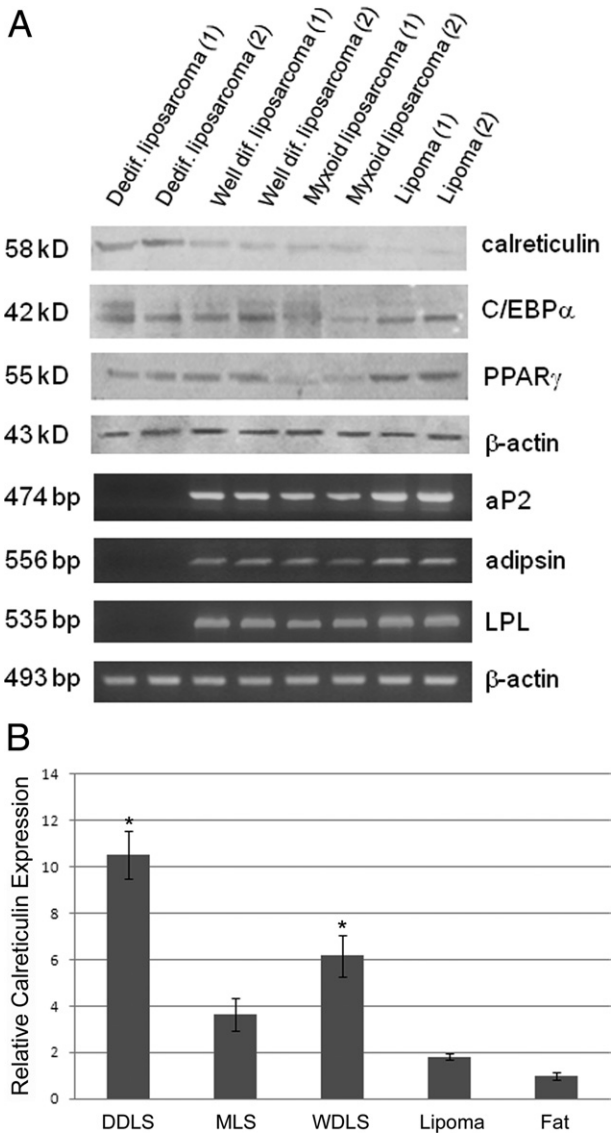

Figure 2. The expression of calreticulin and variable differentiation markers for adipogenesis (C/EBPα, PPARγ, aP2, adipsin, LPL) (**A**) in liposarcomas and lipomas. **A:** Calreticulin and adipogenic transcription factors (PPARγ, C/EBPα) were expressed in dedifferentiated liposarcoma, whereas late or terminal adipogenic markers (aP2, adipsin, LPL) were not expressed, suggesting a differentiation arrest. β-Actin served as a loading control. **B:** Calreticulin was expressed in the dedifferentiated and well-differentiated liposarcomas significantly more than in comparison with lipoma and normal fat, as measured by quantitative RT-PCR (mean $\pm$ SD of three examples). $^*P < 0.01$.

Figure 1. Immunohistochemical analysis for calreticulin expression in liposarcomas, lipoma, normal fat, and nonlipogenic sarcomas. Unequivocal expression of calreticulin could be seen in the tumor cells lacking an adipocytic phenotype in the dedifferentiated area of dedifferentiated liposarcoma (**A**) and well-differentiated sclerosing (**B**) or lipoma-like (**C**) liposarcoma. Some lipoblasts in well-differentiated liposarcoma and primitive small round or short spindle cells in myxoid liposarcoma (**D**) were also positively stained. However, the lipoma (**E**) and normal fat (**F**) were negative for calreticulin expression. Calreticulin was expressed frequently in undifferentiated high-grade pleomorphic sarcoma (**G**), angiosarcoma (**H**), and alveolar rhabdomyosarcoma (**I**). Scale bars = 50 μm.

Agent (Applied Biosystems) according to the manufacturer's instructions. The cells were cultured for 12 to 72 hours after the transfection. The efficacy of the gene silencing was confirmed by quantitative RT-PCR and immunohistochemistry with the use of the anti-calreticulin antibody and fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin antibody (DakoCytomation).

Cell Growth Assay

The proliferation of the siRNA-treated cells was assessed using Alamar Blue (BioSource, Camarillo, CA). The cells (2×10^4 cells/mL) were harvested in triplicate wells of a 96-well plate under adipocyte differentiation conditions. The Alamar Blue reagent was added into each well at a final concentration of 10%, and the optical density of the wells at 530 and 590 nm was measured with a standard spectrophotometer.

Statistical Analysis

The differences between the mean values of the relative expression levels of calreticulin and miRNA and of the

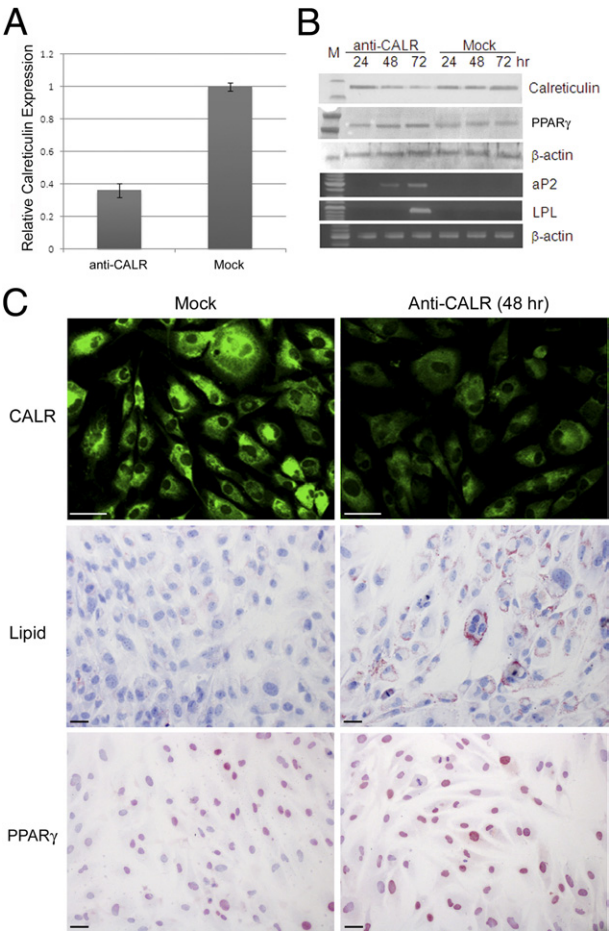

Figure 3. A calreticulin knock-down experiment in FU-DDLS-1 cells. Calreticulin expression was reduced 72 hours after the transfection of siRNA at both the mRNA (mean ± SD of duplicates) (**A**) and protein (**B**) levels. In addition to mildly enhanced expression of PPARγ (**B**), the expression of aP2 and LPL was detectable at 48 and 72 hours. **C:** Accumulated intracytoplasmic lipid droplets were shown by oil red O staining in the cells 72 hours after the gene silencing, suggesting that adipocyte differentiation was rescued in dedifferentiated liposarcoma cells by the down-regulation of calreticulin. Scale bars = 20 μm.

ning approximately 0.2 Mb and flanking CALR at 19p13.1–13.2, were hybridized to the isolated nuclei, together with an fluorescein isothiocyanate-labeled centromeric probe for chromosome 19 (CEN19q; GSP Laboratory) in a humidified chamber at 37°C overnight. The sections were counterstained with DAPI and examined with a fluorescence microscope (Eclipse E400; Nikon, Tokyo, Japan) equipped with appropriate filters and an image-capturing software program. At least 50 nuclei in each case were examined, and cases with >10% of cells displaying increased signal numbers (ie, three or more red signals) were considered to have CALR aberrations.

Results

The immunohistochemical results of calreticulin expression in lipogenic and nonlipogenic tumors are summarized in Table 2. Calreticulin was consistently and often strongly expressed in the dedifferentiated areas of the dedifferentiated liposarcomas (Figure 1A). Moreover, atypical stromal cells and multivacuolated lipoblastic cells that were present in the well-differentiated areas (87%) or in well-differentiated liposarcomas (73%) frequently expressed the protein (Figure 1, B and C). In addition to the lipoblasts, small round cells or immature spindle cells in myxoid liposarcomas were faintly positive for calreticulin (87%) (Figure 1D). Large vacuolated fat cells or mature adipocytes in liposarcomas or lipomas or subcutaneous adipose tissue were essentially negative (Figure 1, E and F). The calreticulin expression was also seen in many nonlipogenic sarcomas examined (Figure 1, G–I), although it was expressed focally in minor subsets of synovial sarcomas and Ewing sarcomas/primitive neuroectodermal tumors.

The consistent and pronounced calreticulin expression in the dedifferentiated area of dedifferentiated liposarcomas was further supported by a Western blot analysis and quantitative RT-PCR, and the expression pattern in this series of lipogenic tumors was similar to that obtained from the immunohistochemical study (Figure 2, A and B). In the dedifferentiated liposarcoma samples examined, adipogenic transcriptional regulators, C/EBPα and PPARγ, were expressed, although the mRNA expression of the late or terminal adipogenic markers (ie, aP2, adipsin, LPL) was not detectable (Figure 2A). These findings suggested that the adipocyte differentiation program had thus been arrested because of the aberrant expression of calreticulin in the dedifferentiated liposarcomas. To assess this possibility, we examined the adipocyte differentiation in dedifferentiated liposarcoma cells, FU-DDLS-1, using a siRNA technique.

Both the protein and mRNA levels of calreticulin in the FU-DDLS-1 cells were significantly reduced 48 to 72 hours after the gene silencing by siRNA (Figure 3, A–C). During this period, the late adipogenic markers (aP2 and LPL) were expressed, and intracytoplasmic lipid droplets, which were shown by oil red O staining, had accumulated in the siRNA-treated FU-DDLS-1 cells, indicating that the adipocyte differentiation had been rescued in the tumor cells (Figure 3). In addition, mildly enhanced expression of PPARγ was noted after the siRNA transfection (Figure 3, B and C). Non-siRNA-transfected cells did not spontaneously differentiate for ≥72 hours when cultured in the differentiation-inducing culture medium. Besides,

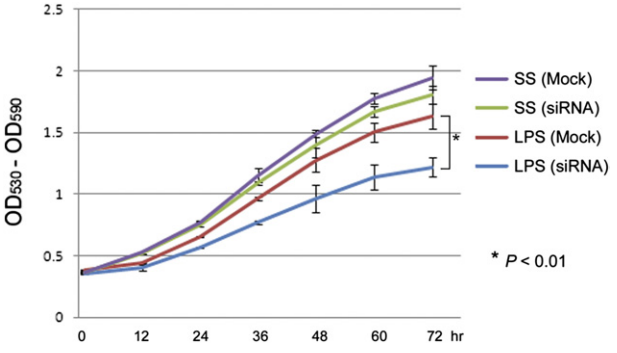

Figure 4. The effect of calreticulin knockdown by siRNA on the proliferation of dedifferentiated liposarcoma cells (LPS) and synovial sarcoma cells (SS). The down-regulation of calreticulin repressed the proliferation of LPS but did not affect that of SS (mean ± SD of triplicates).

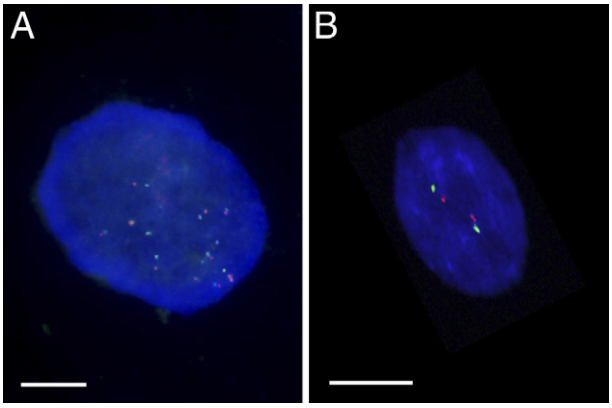

Figure 5. Interphase FISH using probes for 19p13.1–13.2 (red) and the centromere of chromosome 19 (green) in dedifferentiated liposarcoma and lipoma cases. Multiple red and green signals, suggesting polysomy of chromosome 19, were detected in the liposarcoma (**A**) but not the lipoma (**B**). Scale bars = 5 μm.

no adipocyte differentiation was induced in HS-SY-II cells after the siRNA transfection. As shown in Figure 4, the proliferation rate was lower for the FU-DDLS-1 cells treated with siRNA than for the nontreated cells, whereas the gene silencing of *CALR* did not lead to any significant change in the cell proliferation of the synovial sarcoma cells, which were used as controls.

We performed FISH to investigate the milieu of the aberrant calreticulin expression. No *CALR* alterations were identified in the samples examined, except for one dedifferentiated liposarcoma, which showed multiple *CALR* and centromeric signals, implying polysomy of chromosome 19 rather than *CALR* amplification (Figure 5, A and B). A recent study has reported that polysomy of chromosome 17 in breast cancer is not associated with increased expression of *HER2*, which is mapped to 17q21-22.[17] These findings suggested that some epigenetic mechanism(s) might have contributed to the upregulated calreticulin expression in the dedifferentiated liposarcomas. In a subsequent experiment, we attempted to identify miRNAs potentially targeting CALR. On the basis of the matched sequence between the miRNA and CALR mRNA and the assumed binding strength of the formed miRNA-mRNA duplex as determined with the use of the miRBLAST-A software program, 201 miRNAs were extracted as candidates potentially targeting CALR mRNA (see Supplemental Table S1 at *http://ajp.amjpathol.org*). We selected four of these miRNAs (miR-149*, miR-765, miR-1226*, and miR-1275) because their 5′ ends were made up of at least eight consecutive nucleotides that completely matched the nucleotide sequences located in the 3′ untranslated region (UTR) of the CALR mRNA (Table 3). By a quantitative RT-PCR analysis of three dedifferentiated liposarcoma samples, the expression of miR-1275 was found to be significantly lower than that of the other miRNAs analyzed (Figure 6A). The expression level of miR-1275 was the lowest in dedifferentiated liposarcoma, followed by myxoid liposarcoma and well-differentiated liposarcoma (Figure 6B).

Discussion

Liposarcoma subtypes show variable phenotypic features and gene expression profiles that correspond to different maturation stages of adipocyte differentiation,[18] which seems to be partly linked to characteristic genetic alterations (eg, *FUS/EWSR1-DDIT3* fusion gene in myxoid/round cell liposarcoma) as well as to the distinct biological behavior of each subtype. Despite the morphologic differences, dedifferentiated liposarcomas harbor genetic or molecular alterations, such as amplification or overexpression of *MDM2* and *CDK4*, that are identical to the well-differentiated subtype,[19] indicating that some additional molecular mechanisms are involved in the dedifferentiation phenomenon or tumor progression of the liposarcoma. We assumed that some factors that mediate the adipocyte differentiation program were the best candidates that might be involved in the process. However, recent gene expression profiling and array-based comparative genomic hybridization studies have failed to identify any changes in particular genes that regulate adipocyte differentiation, except for those of c-jun and calreticulin, which were especially overexpressed in dedifferentiated liposarcoma.[13,20] The c-jun protein, a component of the AP-1 early transcription factor complex involved in a wide range of cellular pro-

Table 3. Selected miRNAs with Homologous Sequences to CALR mRNA −3'UTR

miR ID (miR Base accession no.)	Sequence position of CALR mRNA	miRNA sequence (3′→5′) alignment targeted sequence (5′→3′)
hsa-miR-149* (MIMAT0004609)	1774–1792	3′-cguGUCGGGGGCAGGGAGGGA-5′ || ::|	|||||||| 5′-tctCATTTCAC-CCCTCCCT-3′
hsa-miR-765 (MI0005116)	1463–1486	3′-guAGUGGAAGGAA–GAGGAGGU-5′ |:: :||: || |||||||| 5′-ttTTGGTTTTGTTCCCCTCCTCCA-3′
hsa-miR-1226* (MIMAT0005576)	1545–1567	3′-ggggUAGGUCCGGACGUACGGGAGUG-5′ ||:| ||| || |||||||| 5′-tttgATTC-TCCTTCA-GCCCTCAC-3′
hsa-miR-1275 (MI0006415)	1481–1497	3′-cugucgGAGAGGGGGUG-5′ |||||||||| 5′-cctccaCTCTCCCCCAC-3′

Lowercase letters represent noncomplementary nucleotides between CALR mRNA and miRNA sequences.

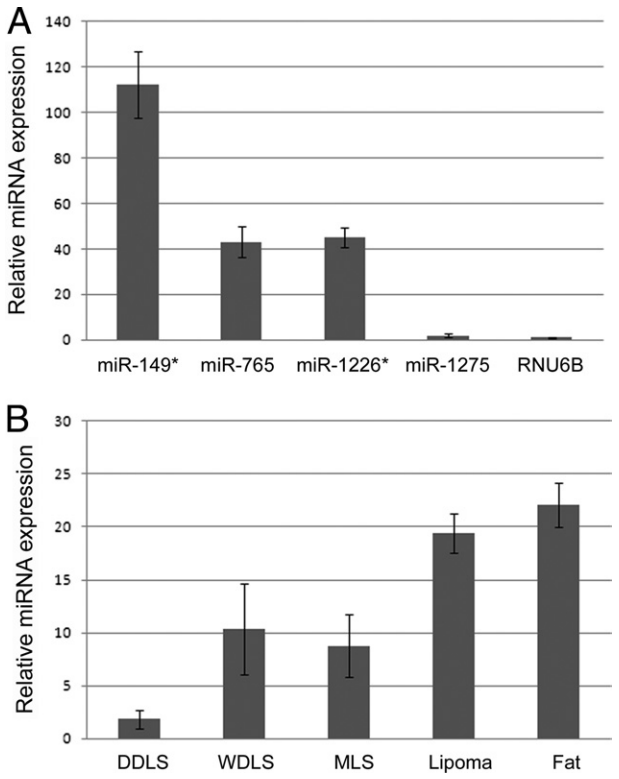

Figure 6. The relative expression levels of candidate miRNAs targeting CALR in dedifferentiated liposarcoma (**A**) and that of miR-1275 in variable lipogenic tumors and normal fat (**B**) as determined by quantitative RT-PCR (mean ± SD of triplicates). The repression of miR-1275 expression was identified in the dedifferentiated liposarcoma.

cesses such as cell proliferation, differentiation, and apoptosis, has been suggested to inhibit adipogenesis through PPARγ phosphorylation or down-regulation of the cAMP response element binding protein.[11] However, c-jun overexpression has not been shown to sufficiently inhibit the adipocyte differentiation program in liposarcoma cells.[21]

Our results confirmed that calreticulin is highly expressed in dedifferentiated liposarcoma in comparison with lipoma or mature adipose tissue at both the protein and mRNA levels. In addition, calreticulin was expressed in the tumor cells lacking an overt adipogenic phenotype or in those with less differentiated forms (ie, lipoblasts) in our immunohistochemical analysis. These findings are in line with the anti-adipogenic function of calreticulin disclosed by Szabo et al,[12] who used embryonic stem cells and 3T3-L1 preadipocytes. Calreticulin is an endoplasmic reticulum resident Ca^{2+}-binding protein and has been shown to act as a Ca^{2+}-dependent molecular switch that negatively regulates adipogenesis by down-regulating the expression and transcriptional activation of proadipogenic transcription factors.[12] In our immunohistochemical results, the overexpression of calreticulin was not confined to liposarcoma but also found in nonlipogenic sarcomas of minimal or divergent differentiation, which might reflect the multiple biological functions of this protein such as regulation of calcium homeostasis, quality control of the secretory pathway, cell adhesion, and regulation of gene expression.[22]

Adipocyte differentiation is driven by coordinated expression of multiple functional molecules and/or transcription factors, including PPARγ, which is known as a master regulator of adipogenesis. Because PPARγ is expressed in dedifferentiated liposarcoma,[23] its function in inducing adipogenesis appears to be disturbed in such tumors. Although a PPARγ-positive, yet minimal, lipid accumulation phenotype in dedifferentiated liposarcoma may suggest cells akin to brown adipocytes, such a cell linage was not identified in dedifferentiated liposarcomas immunohistochemically with the use of an antibody against its specific marker, UCP1 (data not shown).

According to Szabo et al,[12] CALR has two PPARγ-binding sites in its promoter, and PPARγ2 can stimulate the expression of calreticulin. In addition, PPARγ is down-regulated in cells in which calreticulin is overexpressed, thus suggesting that calreticulin modulates PPARγ activity through a negative feedback mechanism. Because the expression of calreticulin is tightly regulated during adipogenesis, aberrant calreticulin expression may inhibit the function of PPARγ, possibly resulting in a differentiation arrest or tumor progression in liposarcoma. This notion may be supported by our results showing that the forced down-regulation of calreticulin could rescue the arrested adipogenesis in dedifferentiated liposarcoma cells and thereby reduce their cell proliferation. However, proliferation and differentiation of liposarcoma cells may be modulated by inflammatory cells and/or stromal fibrosis possibly induced by the overexpressed calreticulin.[24] In addition to PPARγ, C/EBPα and tumor necrosis factor α have been described to alter calreticulin expression.[12,25] Thus, it seems difficult to truly represent the *in vivo* liposarcoma with the *in vitro* situation observed in this study.

In our series, less or faint calreticulin expression was also seen in other liposarcoma subtypes, yet resulting in little inhibition of expression of late adipogenic markers. This suggests that perhaps a threshold level of calreticulin expression must be achieved to induce the adipogenic block seen in the dedifferentiated liposarcoma and likely contributes to the subsequent phenotypic differences among the liposarcoma subtypes.

The milieu of the aberrant expression of calreticulin in liposarcoma had not been investigated. Therefore, we herein assessed selected tumor samples for potential *CALR* gene alterations and found that *CALR* amplification or altered 19p13.1–13.2, where *CALR* is localized, is not common in dedifferentiated liposarcoma, which seems to be in line with the recent array-based comparative genomic hybridization studies of dedifferentiated liposarcoma.[26,27] In addition, the mRNA expression level of calreticulin did not differ significantly between the liposarcomas with or without alterations of chromosome 19p13.1–13.2 in our analysis (data not shown), suggesting that other mechanisms are likely responsible for the underlying aberrant calreticulin expression.

The miRNAs are a class of evolutionally conserved, noncoding small RNAs that exert multiple biological functions by negatively regulating the expression of their target genes involved in development, differentiation, apoptosis, and cell proliferation. An altered miRNA expression level is known as a representative epigenetic mechanism

involved in oncogenesis.[28] Therefore, we speculated that some miRNAs targeting CALR may be aberrantly downregulated in dedifferentiated liposarcomas. Because miRNAs usually target the mammalian 3′ UTR,[29] we selected four miRNAs that had so-called "seed" elements (at least eight consecutive nucleotides in their 5′ termini) that matched the nucleotide sequences in the 3′ UTR of CALR mRNA as potential silencers of CALR. Interestingly, we found that the expression of miR-1275 was significantly lower than that of the other miRNAs (ie, miR-149*, miR-765, miR-1226*) examined in the dedifferentiated liposarcomas and that in other tumor types, implying that miR-1275 is a putative miRNA targeting CALR. Further functional studies of miR-1275, such as miRNA knockdown experiments that used reporter assays, are mandatory to elucidate its exact roles in the regulation of calreticulin expression and liposarcoma development.

In conclusion, our data suggest that an aberrant expression of an anti-adipogenic factor, calreticulin, induced by a potential epigenetic mechanism may result in differentiation arrest in transformed mesenchymal cells committed to an adipocytic lineage, leading to a dedifferentiated phenotype. Thus, calreticulin may be a novel molecular target for the prevention of dedifferentiation or high-grade progression of liposarcoma.

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References

1. Fltcher CDM, Unni KK, Mertens F: World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Soft Tissues and Bones. Lyon, IARC Press, 2002, pp. 35–45
2. Dalal KM, Antonescu CR, Singer S: Diagnosis and management of lipomatous tumors. J Surg Oncol 2008, 97:298–313
3. Gregoire FM, Smas CM, Sul HS: Understanding adipocyte differentiation. Physiol Rev 1998, 78:783–809
4. White UA, Stephens JM: Transcriptional factors that promote formation of white adipose tissue. Mol Cell Endocrinol 2010, 318:10–14
5. Choy L, Derynck R: Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. J Cell Biol 2003, 278:9609–9619
6. Li HX, Luo X, Liu RX, Yang YJ, Yang GS: Roles of Wnt/beta-catenin signaling in adipogenic differentiation potential of adipose-derived mesenchymal stem cells. Mol Cell Endocrinol 2008, 291:116–124
7. Schling P, Löffler G: Effects of angiotensin II on adipose conversion and expression of genes of the renin–angiotensin system in human preadipocytes. Horm Metab Res 2001, 33:189–195
8. White UA, Stewart WC, Mynatt RL, Stephens JM: Neuropoietin attenuates adipogenesis and induces insultin resistance in adipocytes. J Biol Chem 2008, 283:22505–22512
9. Harmancey R, Senard JM, Rouet P, Pathak A, Smih F: Adrenomedullin inhibits adipogenesis under transcriptional control of insulin. Diabetes 2007, 56:553–563
10. Bhattacharya I, Ullrich A: Endothelin-1 inhibits adipogenesis: role of phosphorylation of Akt and ERK1/2. FEBS Lett 2006, 580:5765–5771
11. Mariani O, Brennetot C, Coindre J-M, Gruel N, Ganem C, Delattre O, Stern M-H, Aurias A: JUN oncogene amplification and overexpression block adipocytic differentiation in highly aggressive sarcomas. Cancer Cell 2007, 11:361–374
12. Szabo E, Qiu Y, Baksh S, Michalak M, Opas M: Calreticulin inhibits commitment to adipocyte differentiation. J Cell Biol 2008, 182:103–116
13. Singer S, Socci ND, Ambrosini G, Sambol E, Decarolis P, Wu Y, O'Connor R, Maki R, Viale A, Sander C, Schwartz GK, Antonescu CR: Gene expression profiling of liposarcoma identifies distinct biological types/subtypes and potential therapeutic targets in well-differentiated and dedifferentiated liposarcoma. Cancer Res 2007, 67:6626–6636
14. Nakayama R, Nemoto T, Takahashi H, Ohta T, Kawai A, Seki K, Yoshida T, Toyama Y, Ichikawa H, Hasegawa T: Gene expression analysis of soft tissue sarcomas: characterization and reclassification of malignant fibrous histiocytoma. Mod Pathol 2007, 20:749–759
15. Nishio J, Iwasaki H, Ishiguro M, Ohjimi Y, Fujita C, Ikegami H, Ariyoshi A, Naito M, Kaneko Y, Kikuchi M: Establishment of a novel human dedifferentiated liposarcoma cell line. FU-DDLS-1: conventional and molecular genetic characterization Int J Oncol 2003, 22:535–542
16. McGinn T, Wyer PC, Newman TB, Keitz S, Leipzig R, For GG, Evidence-Based Medicine Teaching Tips Working Group: Tips for learners of evidence-based medicine, 3: measures of observer variability (kappa statistic). CMAJ 2004, 171:1369–1373
17. Vanden Bempt I, Van Loo P, Drijkoningen M, Neven P, Smeets A, Christiaens M-R, Paridaens R, De Wolf-Peeters C: Polysomy 17 in breast cancer: clinicopathologic significance and impact on HER-2 testing. J Clin Oncol 2008, 26:4869–4874
18. Matushansky I, Hernando E, Socci ND, Matos, Mills J, Edgar MA, Schwartz GK, Singer S, Cordon-Cardo Maki RG: A developmental model of sarcomagenesis defines a differentiation-based classification for liposarcomas. Am J Pathol 2008, 172:1069–1080
19. Binh MB, Sastre-Garau X, Guillou L, de Pinieux G, Terrier P, Lagacé R, Aurias A, Hostein I, Condre JM: MDM2 and CDK4 immunostainings are useful adjuncts in diagnosing well-differentiated and dedifferentiated liposarcoma subtypes: a comparative analysis of 559 soft tissue neoplasms with genetic data. Am J Surg Pathol 2005, 29: 1340–1347
20. Tap WD, Eilber FC, Ginther C, Dry SM, Reese N, Barzan-Smith K, Chen HW, Wu H, Eilber FR, Slamon DJ, Anderson L: Evaluation of well-differentiated/de-differentiated liposarcomas by high-resolution oligonucleotide array-based comparative genomic hybridization. Genes Chromosome Cancer 2011, 50:95–112
21. Snyder EL, Sandstrom DJ, Law K, Fiore C, Sicinska E, Brito J, Bailey D, Fletcher JA, Loda M, Rodig SJ, Dal Cin P, Fletcher CDM: c-Jun amplification and overexpression are oncogenic in liposarcoma but not always sufficient to inhibit the adipocytic differentiation programme. J Pathol 2009, 218:292–300
22. Michalak M, Corbett EF, Mesaeli N, Nakamura K, Opas M: Calreticulin: one protein, one gene, many functions. Biochem J 1999, 344:281–292
23. Horvai AE, Schaefer JT, Nakakura EK, O'Donnell RJ: Immunostaining for peroxisome proliferator gamma distinguishes dedifferentiated liposarcoma from other retroperitoneal sarcomas Mod Pathol 2008, 21:517–524
24. Kypreou KP, Kawadas P, Karamessinis P, Peroulis M, Alberti A, Sideras P, Psarras S, Capetanaki Y, Politis PK, Charonis AS: Altered expression of calreticulin during the development of fibrosis. Proteomics 2008, 8:2407–2419
25. Vig S, Pandey AK, Verma G, Datta M: C/EBPα mediates the transcriptional suppression of human calreticulin gene expression by TNFα. Int J Biochem Cell Biol 2012, 44:113–122
26. Horvai AE, DeVries S, Roy R, O'Donnell RJ, Waldman F: Similarity in genetic alterations between paired well-differentiated and dedifferentiated components of dedifferentiated liposarcoma. Mod Pathol 2009, 22:1477–1488
27. Rieker RJ, Weitz J, Lehner B, Egerer G, Mueller A, Kasper B, Schirmacher P, Joos S, Mechtersheimer G: Genomic profiling reveals subsets of dedifferentiated liposarcoma to follow separate molecular pathways. Virchows Arch 2010, 456:277–285
28. Calin GA, Croce CM: MicroRNA signatures in human cancers. Nat Rev 2006, 6:857–866
29. Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M: Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. Nature 2005, 434:338–345