Tumorigenesis and Neoplastic Progression

The Myxoid/Round Cell Liposarcoma Fusion Oncogene *FUS-DDIT3* and the Normal *DDIT3* Induce a Liposarcoma Phenotype in Transfected Human Fibrosarcoma Cells

Katarina Engström,^{*} Helena Willén,[†]
Christina Kåbjörn-Gustafsson,[‡]
Carola Andersson,[‡] Marita Olsson,[§]
Melker Göransson,[‡] Sofia Järnum,[‡]
Anita Olofsson,[‡] Elisabeth Warnhammar,^{*} and
Pierre Åman[‡]

From the Department of Oncology,^{*} and the Lundberg Laboratory for Cancer Research, Department of Pathology,[‡] the Gothenburg University, Sahlgrenska University Hospital, Gothenburg; the Department of Genetics and Pathology,[†] Rudbecklaboratoriet, Uppsala; and the Swegene Facility for Bioinformatics,[§] Gothenburg University/Mathematical Sciences, Chalmers University of Technology, Gothenburg, Sweden

Myxoid/round cell liposarcoma (MLS/RCLS) is the most common subtype of liposarcoma. Most MLS/RCLS carry a t(12;16) translocation, resulting in a *FUS-DDIT3* fusion gene. We investigated the role of the *FUS-DDIT3* fusion in the development of MLS/RCLS in *FUS-DDIT3*- and *DDIT3*-transfected human HT1080 sarcoma cells. Cells expressing *FUS-DDIT3* and *DDIT3* grew as liposarcomas in severe combined immunodeficient mice and exhibited a capillary network morphology that was similar to networks of MLS/RCLS. Microarray-based comparison of HT1080, the transfected cells, and an MLS/RCLS-derived cell line showed that the *FUS-DDIT3*- and *DDIT3*-transfected variants shifted toward an MLS/RCLS-like expression pattern. *DDIT3*-transfected cells responded *in vitro* to adipogenic factors by accumulation of fat and transformation to a lipoblast-like morphology. In conclusion, because the fusion oncogene *FUS-DDIT3* and the normal *DDIT3* induce a liposarcoma phenotype when expressed in a primitive sarcoma cell line, MLS/RCLS may develop from cell types other than preadipocytes. This may explain the preferential occurrence of MLS/RCLS in nonadipose tissues. In addition, development of lipoblasts and the typical MLS/RCLS capillary network could be an effect of the *DDIT3* transcription

factor partner of the fusion oncogene. (Am J Pathol 2006; 168:1642–1653; DOI: 10.2353/ajpath.2006.050872)

Myxoid/round cell liposarcoma (MLS/RCLS) is the most common subtype of liposarcoma, accounting for about 40% of all cases. The tumor cells are characterized by the chromosomal aberration t(12;16)(q13;p11), which in most cases is the only cytogenetic abnormality.¹ This translocation results in a *FUS-DDIT3* (also called *TLS-CHOP*) fusion occurring in more than 90% of cases.^{2,3} A small percentage of cases carry a variant translocation t(12;22) and an *EWSR1-DDIT3* fusion gene.⁴ None of these fusion oncogenes have been found in tumor types other than MLS/RCLS.

The fusion gene *FUS-DDIT3* encodes a protein consisting of the N-terminal half of the FUS protein juxtaposed to the DNA-binding basic leucine zipper transcription factor DDIT3 (also known as CHOP or GADD153).^{5,6} The *FUS-DDIT3* protein can be found in the nuclei of MLS/RCLS cells and maintains the capacity of DDIT3 to form dimers with other leucine zipper-possessing proteins.⁵ These and other observations suggest that *FUS-DDIT3* may act as an abnormal transcription factor.⁷

FUS (also known as *TLS*), *EWSR1*, and *TAF15* (the latter also known as *TAF2N*, *TAFII68*, or *RBP56*) are closely related genes that encode RNA-binding proteins. All three genes form fusion oncogenes with several alter-

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Address reprint requests to Prof. Pierre Åman, Lundberg Laboratory for Cancer Research, Department of Pathology, Göteborg University, Sahlgrenska University Hospital, SE-413 45 Gothenburg, Sweden. E-mail: pierre.aman@lcr.med.gu.se.

native transcription factor-encoding genes.⁸ Each fusion combination is found in a distinct tumor type.

The mechanism behind the strict tumor-type specificity of these and many other fusion genes has long been the subject of debate.^{9–11} In “instructive” models, the fusion protein controls cell fate decisions, leading to development of a particular type of tumor. Conversely, in “noninstructive” models, the translocations occur only in susceptible committed cell types as a result of a cell type-specific translocation mechanism, or the fusion protein acts as an oncogene only in a susceptible committed cell type. Secondary events are required for tumor development in both models.

In the present study, we have analyzed the effects of the *FUS-DDIT3* fusion gene and overexpression of the normal *DDIT3* and the 5' part of *FUS* in the low-differentiated human fibrosarcoma cell line HT1080. Stable transfectant clones were analyzed for tumor formation in severe combined immunodeficient (SCID) mice, and the morphology of the resulting tumors was studied. Microarray analysis was used for expression profiling of the transfected cell lines, and the cells were also tested *in vitro* for their responsiveness to adipogenic factors.

Materials and Methods

Expression Vectors

Polymerase chain reaction (PCR) fragments containing the full-length coding regions of *FUS-DDIT3* type II¹², *DDIT3*, or the sequences encoding the first 180 amino acids of *FUS* were cloned into a pEGFPN1 vector (Clontech) in-frame with the enhanced green fluorescent protein (EGFP) sequence as described previously.¹³ The resulting vectors were designated p*FUS-DDIT3*EGFP, p*DDIT3*-EGFP, and p*FUSa*EGFP. All vectors also contained a G418 resistance selection gene.

Cell Culture and Transfection

The fibrosarcoma cell line HT1080 and MLS cell lines 402-91² and MLS 2645-94 were stored in liquid nitrogen and cultivated in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 8% fetal calf serum. Cultures were split 1:3 twice every week. The cells were harvested by trypsinization, and 3×10^5 cells were seeded into 10-cm² flasks and transfected the following day using 10 μ l of FuGENE 6 transfection reagent (Roche, Indianapolis, IN) and 3 μ g of plasmid DNA per flask. The cells were transferred to 75-cm² culture flasks after 48 hours, and G418 (Sigma, St. Louis, MO) was added to a final concentration of 800 μ g/ml. After 3 days, the G418 concentration was reduced to 500 μ g/ml. Surviving cells were harvested after 10 days and seeded in 96-well microtiter plates at a density of 30 cells/plate. Clones showing nuclear EGFP fluorescence were expanded in the presence of 200 μ g G418/ml, and recombinant protein expression was confirmed by Western blot analysis.

In Vitro Adipogenesis

p*FUS-DDIT3*EGFP-transfected, p*DDIT3*EGFP-transfected, and wild-type HT1080 cells were seeded in flasks and cultured in RPMI 1640 until they reached 100% confluence, after which the medium was changed to adipogenesis induction medium (PT3004 containing human recombinant insulin, dexamethasone, indomethacin, and 3-isobutyl-L-methylxanthine [IBMX]; Cambrex, East Rutherford, NJ) or maintenance medium (MM; RPMI 1640 containing 8% fetal calf serum). The cells were treated with adipogenesis induction medium for 3 days, followed by 1 to 3 days in MM. This was repeated three times. Control cultures were fed with only MM following the same schedule. After completed cycles, the cells were cultured for 7 more days in MM with replacement of the medium every 2 to 3 days. The cells were inspected using a microscope, and accumulation of fat was assessed by staining the cells with Oil Red O after fixation with 4% buffered formalin.

Western Blot Analysis

Western blot of total proteins from cultured cells was performed after electrophoresis using precast 3 to 8% NuPage Tris-acetate gels (Invitrogen, Carlsbad, CA), followed by transfer in a Novex minigel electrophoresis apparatus (Novex, San Diego, CA) to Immobilon membranes (Millipore, Billerica, MA) according to the manufacturers' recommendations. The procedures for antibody binding and detection were as described by the suppliers of the antibodies. The *DDIT3* protein was detected using a polyclonal rabbit antiserum (GADD 153, R20, sc793; Santa Cruz Biotechnology, Santa Cruz, CA), and the *FUS* protein was detected with a polyclonal rabbit antiserum against the N terminus of *FUS*.

Genome-Wide Gene Expression Analysis

Total RNA was extracted from cultured cells using the Trizol method (Invitrogen). The RNA was further purified using a Qiagen RNeasy kit and stored at -140°C . Five micrograms of RNA was used for cDNA synthesis with Cy3- and Cy5-labeled nucleotides. cDNA labeling and hybridization to microarray glasses were performed according to the Pronto! Plus Direct Labeling v1.2 labeling and hybridization kit (Invitro, Stockholm, Sweden).

Microarrays were produced at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University (Lund, Sweden). Human array-ready oligonucleotide libraries (version 2.1, catalog no. 810516; and version 2.1.1 upgrade, catalog no. 810518), comprising approximately 27,000 unique probes representing 23,707 genes, were obtained from Operon Biotechnologies (Cologne, Germany). Probes were dissolved in Universal Spotting Solution (Corning, Acton, MA) and printed in duplicate on aminosilane-coated glass slides (UltraGAPS, catalog no. C40017; Corning) using a MicroGrid2 robot (BioRobotics, Cambridgeshire, UK) equipped with MicroSpot 10K pins (BioRobotics). After

printing, arrays were left in a desiccator to dry for 48 hours, rehydrated for 1 second over steaming water, snap-dried on a hot plate (98°C), and UV-cross-linked (800 mJ/cm²).

Image and Data Analysis

Hybridized microarrays were scanned using an Agilent G2565AA microarray scanner (Agilent Technologies, Palo Alto, CA). Fluorescence intensities were extracted using Gene Pix Pro 4.0 software (Axon Instruments Inc., Foster City, CA), and uploaded into Bio Array Software Environment (<http://base.thep.lu.se>) for further analysis. The LOWESS algorithm^{14,15} was used for normalization.

Minimum median intensity was set to 1 to avoid data loss when the ratio between the samples and the reference cell line (wild-type HT1080) was being calculated. Genes with differences of at least threefold in signal intensity between the HT1080 reference cell line and the transfected HT1080 variants were scored as up- or down-regulated. Genes with a normalized intensity below 25 in the transfected cell lines were rejected from the lists of up-regulated genes. Similarly, genes with a normalized intensity lower than 25 in the HT1080 reference were rejected from the lists of down-regulated genes. RNA from the MLS cell line 402-91 was also tested, with the HT1080 cell line as a reference.

Statistical Analysis

To estimate the statistical significance of the overlap between differentially expressed genes in 402-91 cells and differentially expressed genes in the *pFUS-DDIT3*EGFP- and *pDDIT3*EGFP-transfected lines, two sets of genes corresponding to the up- and down-regulated genes in 402-91 cells were selected at random from the total of 23,707 genes. Similarly, two sets of genes corresponding to up- and down-regulated genes in one of the transfected lines were randomly selected. The number of genes that were identical in the randomly selected groups was then observed. This experiment was repeated 10⁷ times for each pair of up- or down-regulated gene sets.

Quantitative Reverse Transcriptase-PCR Analysis

Total RNA was isolated, and cDNA was generated as described above but without labeled nucleotides. The primer sequences were as follows (F, forward; R, reverse): CTGFF, 5'-CCG TAC TCC CAA AAT CTC CA-3'; CTGFR, 5'-GTA ATG GCA GGC ACA GGT CT-3'; DNAJA1F, 5'-ACT GGA GCC AGG CGA TAT TA-3'; DNAJA1R, 5'-GAA GCC ACA CAG TGC TTC AA-3'; EFEMP1F, 5'-TGC CAT CAG ACA TCT TCC AG-3'; EFEMP1R, 5'-TGT GCG GAA GGT CCC TAT AC-3'; HSPA1F, 5'-CCG AGA AGG ACG AGT TTG AG-3'; HSPA1R, 5'-GCA GCA AAG TCC TTG AGT CC-3'; MMP3F, 5'-CAG GCT TTC CCA AGC AAA TA-3';

MMP3R, 5'-GTG CCC ATA TTG TGC CTT CT-3'; NPAS2F, 5'-GCC AGA CCG TGT TTC AAA AT-3'; NPAS2R, 5'-AGT AGC GAG TCC TGC TGC TC-3'; PBEF1F, 5'-GGA GCA TCT GCT CAC TTG GT-3'; PBEF1R, 5'-TCG CTG ACC ACA GAT ACA GG-3'; PCNAF, 5'-AAA ATT GCG GAT ATG GGA CA-3'; PCNAR, 5'-GCT GGC ATC TTA GAA GCA GTT-3'; PTX3F, 5'-CCA ATG CGT TCC AAG AAG AT-3'; PTX3R, 5'-TCC ACC CAC CAC AAA CAC TA-3'; SERPINB2F, 5'-CTC AAC AAG TGG ACC AGC AA-3'; SERPINB2R, 5'-CCA TGT CCA GTT CTC CCT GT-3'; SKP2F, 5'-CAT TTC AGC CCT TTT CGT GT-3'; SKP2R, GGG CAA ATT CAG AGA ATC CA-3'; VEGFF, 5'-CCC ACT GAG GAG TCC AAC AT-3'; VEGFR, 5'-AAA TGC TTT CTC CGC TCT GA-3'; GAPDH, 5'-GTG AAG GTC GGA GTC AAC G-3'; GAPDHR, 5'-GGT GAA GAC GCC AGT GGA CTC-3'; ONP54F, 5'-AGC CCA CTT CTT ACC ACA AG-3'; and ONP56R, 5'-CCA AAT AGG TGC ATG AGT AG-3'.

Real-time PCR was measured in an ABI 7700 sequence detector (Applied Biosystems, Foster City, CA) with SYBR Green as fluorophore. Formation of expected PCR product was confirmed by agarose gel electrophoresis and melting curve analysis. Gene expression data were normalized against ONP and GAPD by geometric averaging.¹⁶

Tumor Growth in SCID Mice

Female FOX CHASE SCID mice were used as recipients of transfected HT1080 clones and nontransfected HT1080 cells. Cultured cells were harvested by trypsinization and suspended in phosphate-buffered saline (PBS) before injection. The mice were 5 to 6 weeks old at the time of inoculation. In two separate experiments, 10 and 2 million cells, respectively, were injected subcutaneously into the flank of SCID mice. In the first experiment, three animals were injected with the HT1080 wild-type cell line, and three series of five animals were injected with *FUSa*EGFP-, *FUSD-DITT3*EGFP-, and *DDIT3*EGFP-transfected cells, respectively. The second experiment comprised two animals injected with HT1080 wild-type cells and two groups of five animals injected with *FUSD-DITT3*EGFP- or *DDIT3*EGFP-transfected cells. The animals were examined regularly for evidence of tumor growth at the site of injection. Animals with palpable tumors were sacrificed, and the tumors were excised and measured. Pieces were frozen or fixed in formalin and embedded in paraffin for histological examination. Tissue sections (5 μm thick) were deparaffinized and stained following routine protocols.

Fluorescence Microscopy

The cultures were washed twice with PBS and fixed in 4% paraformaldehyde in PBS. After two more washes in PBS, the slides were mounted in an antifade mount containing the DNA binding dye 4,6-diamidino-2-phenylindole dihydrochloride and examined in a fluorescence microscope. Fresh, living cells and tissue fragments were examined

Table 1. Growth of Transfected HT1080 Cells

Construct	GFP-positive cells 24 hours after transfection (%)	GFP-positive cells after 10 days of genetic selection (%)	GFP-positive clones 6 weeks after transfection (%)
pFUS-CHOP-EGFP	56	3	<1
pCHOP-EGFP	62	87	79
pFUSa-EGFP	60	92	94

directly in culture flasks using an inverted fluorescence microscope.

Immunohistochemistry

Series of 5- μ m tissue sections were cut from each biopsy, deparaffinized, rehydrated, and stained with the following monoclonal antibodies: rat anti-mouse CD34 (Abcam ab8158; Abcam, Cambridge, MA) and mouse anti-human CD34 (DAKO, Dakopatts A/S, Glostrup, Denmark). Bound antibodies were visualized using the LSAB second antibody streptavidine biotin peroxidase system (DAKO). Stained sections were examined with a light microscope.

Results

G418 selection for stable transfectant clones revealed a clear difference between cells transfected with *FUS-DDIT3*, *DDIT3*, and *FUSa*. All three constructs gave similar rates of transiently transfected cells, as judged by counting of cells expressing the GFP-tagged proteins by fluorescence microscopy 24 hours after transfection (Table 1). However, only a low percentage of the G418-resistant pFUS-DDIT3EGFP-transfected cells expressed the FUS-DDIT3EGFP protein after 10 days of selection, whereas more than 90% of the G418-resistant pDDIT3EGFP and pFUSaEGFP-stably transfected cells expressed DDIT3EGFP and FUSaEGFP, respectively. This observation suggests that only a small proportion of the cells expressing the FUS-DDIT3EGFP protein remained viable. We also noticed that the pFUS-DDIT3EGFP-transfected clones expressed considerably less of the GFP-tagged protein than the pDDIT3EGFP- and pFUSaEGFP-transfected cells (Figure 1). The amount of FUS-DDIT3EGFP protein was also lower compared with the levels of native FUS-DDIT3 protein seen in MLS/RCLS cell lines.

The sizes of the GFP-tagged proteins were checked by Western blot analysis, and clones showing stable expression of proteins of the correct size were selected for further analysis (Figure 1).

Tumor Growth in SCID Mice

In the first experiment, 10^7 cells were inoculated into each mouse. All mice, except one, rapidly developed large tumors, and in the following experiment, the amount of cells was reduced to 2×10^6 per animal. Judging from tumor sizes, the original HT1080 cells grew faster in SCID mice than the three transfected cell lines (Table 2).

Microscopic examination of sections from the tumors revealed that the original HT1080 cells and pFUSaEGFP-transfected cells grew as low-differentiated sarcomas, mainly with poorly defined or sinusoid blood vessels and necrotic areas. In contrast, most of the mice inoculated with the pFUS-DDIT3EGFP- or pDDIT3EGFP-expressing cells developed liposarcomas containing atypical lipoblasts of different sizes (Figure 2A). Increased proportions of extracellular matrix and small myxoid pools were observed in tumors from the two pFUS-DDIT3EGFP-transfected cell lines, but large amounts of the myxoid substance seen in many naturally occurring MLS tumors were not found. Supplemental Table S1 containing detailed information can be found at <http://ajp.amjpathol.org>.

Effects on Vascularization

In humans, most MLS/RCLSs are characterized by a typical plexiform capillary network.¹⁷ We found that the tumors grown from cells carrying pFUS-DDIT3EGFP and pDDIT3EGFP contained capillary networks with morphology similar to that found in naturally occurring MLS, whereas the original HT1080 and pFUSaEGFP-transfected tumors mainly contained sinusoid vessels (Figure 2B; Table 2). The effects on capillary morphology prompted us to stain for CD34⁺ endothelial cells in the

Figure 1. Western blot analysis of transfected HT1080 cells and MLS 2645-94 from which the FUS-DDIT3 fusion cDNA was cloned. Sample origin is indicated for each lane. A mix of anti-DDIT3 and anti-FUS antibodies were used to detect all variant proteins. **1**, the position of the recombinant FUS-DDIT3EGFP protein; **2**, the normal FUS protein present in all cells; **3**, the recombinant N-terminal part of FUS (FUSa) linked to EGFP; **4**, the native FUS-DDIT3 protein of the MLS cells from which the fusion transcript was cloned; and **5**, the recombinant DDIT3EGFP protein. Note that the EGFP tag adds around 30 kD in size to the recombinant proteins. Size markers in kilodaltons.

Table 2. Tumor Growth and Morphology in SCID Mouse

SCID mice (<i>n</i>)	Cell line	Mean tumor size (mm)	Morphology	Vascularization	
				CP	SN
Experiment 1					
3	HT1080	21 × 16	Low differentiated sarcoma with extensive necrosis	—	+
5	HT1080 FUSaEGFP Clone 1	12 × 10	Low differentiated sarcoma with extensive necrosis	—	+
5	HT1080 FUS-DDIT3EGFP Clone 1	10 × 11	Liposarcoma with abnormal lipoblasts and capillary network	+	—
5	HT 1080 DDIT3-EGFP Clone 1	11 × 14	Liposarcoma with abnormal lipoblasts and capillary network	+	—
Experiment 2					
2	HT1080	18 × 12	Low differentiated sarcoma	—	+
5	HT1080 FUS-DDIT3-EGFP Clone 1	10 × 6	Liposarcoma with abnormal lipoblasts and capillary network	+	—
5	HT1080 DDIT3-EGFP Clone 1	11 × 9	Liposarcoma with abnormal lipoblasts and capillary network	+	—

CP, capillary type; SN, sinusoid type.

tumors. Immunohistochemical staining of tumor sections with antibodies to mouse endothelial cells (anti-CD34), revealed a rich abundance of mouse CD34⁺ vessels in all tumors (Figure 2B). No expression of human CD34 was detected, indicating that the capillary networks originated from mouse cells. We also noted that the original HT1080 and *pFUSaEGFP* transfectants contained considerably more vessels than the *pFUS-DDIT3EGFP*- and *pDDIT3EGFP*-transfected tumors.

In Vitro Adipogenesis Induction Experiments

The change in tumor phenotype observed in the SCID mice may reflect an increased sensitivity to *in vivo* factors that induce adipogenic development in the *pDDIT3EGFP* and *pFUS-DDIT3EGFP* cells. To test this hypothesis, we investigated the effects of an *in vitro* adipogenesis protocol on the HT1080 cells and the transfected sublines. Treatment with adipogenic factors induced a dramatic morphological change of *pDDIT3EGFP*-transfected cells. The cells developed large vacuoles, and some cells resembled the “signet ring cell” type of adipocytes (Figure 2C). Oil Red O staining showed increased fat accumulation, but the contents of the main vacuole in each cell showed only partial staining. The *pFUS-DDIT3EGFP* transfectants showed a slight increase in fat content compared with the original HT1080 line, but this was not increased in adipogenic medium, and no morphological response or formation of large vacuoles was seen. The original HT1080 cells grew faster than the transfected variants. Adipogenic factors induced minor morphological changes, with the formation of small vacuoles in a few cells and a slightly increased accumulation of fat (Figure 2C).

Microarray Expression Analysis

Microarray-based expression analysis of HT1080 cells, *pDDIT3EGFP*- and *pFUS-DDIT3EGFP*-transfected

HT1080 cells, and MLS-derived cell line 402-91, showed that only small numbers of genes were differentially expressed in the original HT1080 line and the transfected variant cell lines. In *pDDIT3EGFP*-transfected HT1080 cells, 36 genes were up-regulated and 98 were down-regulated, and in *pFUS-DDIT3EGFP* transfectants, 51 genes were up-regulated and 82 were down-regulated, three times or more compared with HT1080 cells. In MLS line 402-91, 364 genes were up-regulated and 309 genes were down-regulated, three times or more compared with the HT1080 cells. To test whether *FUS-DDIT3* and *DDIT3* induced or repressed genes in an MLS/RCLS like pattern, we searched our microarray data for genes that were up- or down-regulated both in the transfected cell lines and in the 402-91 cell line. Many of the genes that were found to be differentially regulated in the *pFUS-DDIT3EGFP*- and *pDDIT3EGFP*-transfected HT1080 cells were also found to differ in expression between MLS 402-91 and the HT1080 cell line. (Tables 3 and 4; Figure 3). The probability of obtaining a similar overlap in up- and down-regulated genes by random selection among the 23,707 genes on the microarray is less than 10^{−7} in each of the comparisons. This shows that when *DDIT3* and *FUS-DDIT3* are expressed in HT1080 cells, their gene expression changes toward an MLS 402-91-like pattern. Supplemental data from the microarray experiments is available at <http://ajp.amjpathol.org>.

Discussion

The MLS/RCLS-specific *FUS-DDIT3* belongs to the EWS group of fusion oncogenes. All fusion oncogenes of this group contain the 5′ part of one of the closely related genes *FUS*, *EWSR1*, and *TAF15* as 5′ partner and one of many different transcription factor genes as 3′ partner.¹⁸ Each combination of fusion partners is specifically found in one or, in a few examples, two different tumor types. *FUS-DDIT3* and *EWSR1-DDIT3* are found only in MLS/RCLS.

Figure 2. **A:** Microphotographs of sections from SCID mouse tumors of HT1080 cells (**left column**) and the p*FUS-DDIT3*EGFP- and p*DDIT3*EGFP-transfected variants (**middle** and **right columns**, respectively). Note irregularly sized lipoblasts in the transfected tumors. Original magnifications, $\times 400$ and $\times 600$. **B:** Anti-D34 staining of capillaries in SCID mouse tumors from HT1080 and the two transfected variants. Note thin branching capillary vessels in the transfected tumors. Microphotographs captured at $\times 200$ magnification. **C:** *In vitro* adipogenesis of HT1080 cells and transfected variants. The upper line of photomicrographs shows the indicated cell lines after adipogenic treatment. Control cultured cells are shown in the lower series of photomicrographs. Accumulated lipids are stained red (Oil Red O staining). Original magnification, $\times 740$.

Table 3. Named Genes Up-Regulated in HT1080 Transfected with FUS-DDIT3 and DDIT3, and in MLS 402-91

Gene symbol	Function	Array data		RT-PCR		
UP >3 times in FUS-DDIT3 expressing HT1080 and MLS 402-91		HT1080 FUS-DDIT3	HT1080 FUS-DDIT3	HT1080 DDIT3	MLS 402-91	MLS 2645-94
<i>MMP1</i>	Matrix metalloproteinase 1	9	ND	ND	ND	ND
<i>SERPINB2</i>	Tissue plasminogen activator inhibitor	7	99	−2	905	175
<i>MMP3</i>	Matrix metalloproteinase 3	6	12	2	9	2
<i>IL6</i>	Interleukin 6	5	6	4	490	42
<i>FLT1</i>	VEGF receptor 1	5	ND	ND	ND	ND
<i>PTX3</i>	Inflammation-associated secreted protein	4	5	1	39	11
<i>PBEF1</i>	Nicotinamide phosphoribosyltransferase	3	5	−2	15	2
UP >3 times in DDIT3 expressing HT1080 and MLS 402-91		HT1080 DDIT3	HT1080 FUS-DDIT3	HT1080 DDIT3	MLS 402-91	MLS 2645-94
<i>HSPA1</i>	Heat shock 70-kd protein	9	2	35	46	66
<i>XTP3TPA</i>	Unknown	5	ND	ND	ND	ND
<i>SFRS2</i>	Splicing factor	5	ND	ND	ND	ND
<i>CHAF1A</i>	Chromatin assembly factor P150 subunit	4	ND	ND	ND	ND
<i>STXBP2</i>	Golgi-plasma membrane traffic	4	ND	ND	ND	ND
<i>DNAJA1</i>	DNAJ homolog, DNA repair	4	2	8	18	9
<i>IL6</i>	Interleukin 6	4	6	4	490	42
<i>FAP</i>	Fibroblast activation protein (peptidase)	4	ND	ND	ND	ND
<i>SFRS1</i>	Splicing factor	3	ND	ND	ND	ND
<i>SKP2</i>	CDK2/cyclin A-associated protein p45	3	1	7	11	11
<i>PCNA</i>	DNA binding replication and repair factor	3	1	5	15	8
<i>SFRS6</i>	Splicing factor	3	ND	ND	ND	ND
<i>UNG</i>	DNA repair	3	ND	ND	ND	ND

Expression data from microarray and quantitative PCR. Numbers show how many times up- or down-regulated the genes are in the respective cell line. The expression level in HT1080 is used as a standard and set to 1. Negative values indicate down-regulation.
 ND, not done.

FUS-DDIT3 has been previously reported to block terminal differentiation of preadipocytes *in vivo* and *in vitro*,^{19,20} and transgenic mice that expressed *FUS-DDIT3* in all tissues developed MLS/RCLS-like tumors that arose in adipose tissue.²¹ These reports lead to the hypothesis that MLS/RCLS develops from preadipocytes carrying *FUS-DDIT3* that are incapable of terminal differentiation.^{19–24}

The observation that the majority of human MLS/RCLS tumors arise in or between the large muscles of the limbs, and rarely in adipose tissues,¹ calls this hypothesis into question. In the present experiments with p*FUS-DDIT3*EGFP- and p*DDIT3*EGFP-transfected xenografted HT1080 cells, we observed a *FUS-DDIT3*- and *DDIT3*-induced switch from a low-differentiated sarcoma to an MLS/RCLS-like morphology. Microarray expression profiling showed that transfection of *FUS-DDIT3* or *DDIT3* induced a significantly changed expression pattern of HT1080 cells toward an MLS-like profile. Taken together, our morphology and microarray data show that *FUS-DDIT3* can drive a primitive mesenchymal tumor cell toward an MLS/RCLS phenotype by promoting or inhibiting expression of a limited number of genes. Thus, *FUS-DDIT3* may act as an instructive factor to induce a liposarcoma phenotype. Similar results, indicating instructive

activities of EWS group fusion oncogenes, have been published or suggested by other research groups.^{25–28} A phenotype-determining mechanism was also suggested in 1914 by Peyton Rous²⁹ for sarcomas caused in chickens by “filterable agents,” later identified as oncogene-carrying retroviruses. Our results also show that expression of the nontransforming fusion partner gene *DDIT3* induces a similar switch in morphology and expression pattern. These findings support the hypothesis that the 3' transcription factor partner genes of the EWS group of fusion oncogenes determine the tumor entity.

In vitro experiments with transfected mouse preadipocytic cells have shown that *FUS-DDIT3* blocks adipocyte differentiation, because the cells fail to exit the cell cycle.²² In contrast, *DDIT3*-expressing cells exit the cell cycle and develop into adipocytes. This is compatible with our finding that HT1080 cells carrying p*FUS-DDIT3*EGFP failed to show increased fat accumulation on treatment with adipogenic factors, and similar results were obtained with the *FUS-DDIT3*-carrying MLS cell line 402-91 (data not shown). The control-cultured p*FUS-DDIT3*EGFP-transfected cells showed a minor increase in fat content compared with the original HT1080 cell line. This may be a sign of an inter-

Table 4. Named Genes Down-Regulated in HT1080 Transfected with FUS-DDIT3 and DDIT3, and in MLS 402-91

Gene symbol	Function
Down >3 times in FUS-DDIT3 expressing HT1080 and MLS 402-91	
FN1	Extracellular matrix fibronectin
CaMKIIN α	Calcium/calmodulin-dependent protein kinase II inhibitor
KIAA0992	Palladin, microfilament-associated protein
CTGF	Connective tissue growth factor
DSIP1	Leucine zipper transcription factor
Amigo2	Transmembrane protein, cell survival
RGS4	Signal transduction regulator of G-proteins
GALNT4	Protein glycosylation
IGFBP6	Extracellular matrix protein binds IGF
EFEMP1	Extracellular matrix protein
SERPINE1	Tissue plasminogen activator inhibitor
GPC6	Extracellular plasma membrane protein
ASNS	Asparagine synthesis
STX1A	Intracellular vesicle transport
VEGF	Vascular endothelial growth factor
NPAS2	Transcription factor
SOX30	Transcription factor
LOX	Cross-linking of extracellular matrix proteins
COL4A1	Collagen type 4 A1
KLF2	Transcription factor
CNGB3	Membrane protein, potassium transport
IL1B	Interleukin 1B
Down >3 times in DDIT3 expressing HT1080 and MLS 402-91	
IL1B	Interleukin 1B
FN1	Extracellular matrix fibronectin
VEGF	Vascular endothelial growth factor
T1A-2	Plasma membrane protein
NPAS2	Transcription factor
PLAT	Tissue plasminogen activator
SPOCK	ECM proteoglycan
DSIP1	Leucine zipper transcription factor
LOC286148	Hypothetical protein
LTBP3	TGF- β binding protein
PER1	Transcription factor
THBS2	Cell-cell and cell-extracellular matrix contact
STX1A	Intracellular vesicle transport
TXNIP	Thioredoxin interacting protein
SNAI2	Snail drosophila homolog 2
GALNT4	Protein glycosylation
ITGA2	Integrin- α , extracellular matrix contact
GPC6	Extracellular plasma membrane protein
MTHFD2	Energy metabolism
ASNS	Asparagine synthesis
SH3MD4	Unknown
PCK2	Key enzyme in gluconeogenesis
JAG1	Notch1 ligand
DOCK4	Dedicator of cytokinesis 4
FNBP1L	Formin binding protein like 1
LOC152485	Unknown
LOX	Cross-linking of extracellular matrix proteins
H6PD	Hexose 6-phosphate dehydrogenase
FLYWCH1	Zink finger containing protein
DDR2	Receptor tyrosine kinase
TGM2	Transglutaminase, catalyze the cross-linking of proteins
SOX30	Transcription factor
MGC22793	Hypothetical protein
CaMKIIN α	Calcium/calmodulin-dependent protein kinase II inhibitor
SIPA1L3	Signal-induced proliferation ass.
NDRG1	N-MYC downstream-regulated
URB	Steroid controlled protein
RGS4	Regulator of G-protein signaling

Expression data from microarray and quantitative PCR. Numbers show how many times up- or down-regulated the genes are in the respective cell line. The expression level in HT1080 is used as a standard and set to 1. Negative values indicate down-regulation.
 BD, below detection; ND, not done.

Table 4. Continued

[illegible]

rupted adipogenesis, but it may also be an effect of the slower growth of the transfected cell line in adipogenic medium allowing time for more fat accumulation.

The negative *in vitro* adipogenesis results obtained with *FUS-DDIT3*-transfected 3T3-fibroblast or HT1080 cells or MLS/RCLS cell lines are in conflict with data

Figure 3. Number of genes that were at least three times up-regulated (↑) and down-regulated (↓) in DDIT3- and FUS-DDIT3-expressing HT1080 cells as compared by microarray analysis with control HT1080 cells. **Shaded parts** of bars indicate number of genes that are identical with genes up- or down-regulated in MLS 402-91 cells. Total number of genes that were represented on the CHIP was 23,707.

obtained from primary cultures of MLS/RCLS tumor cells.²⁴ However, unknown *in vivo* factors may add to the adipogenic response, and these factors may be lost after prolonged *in vitro* culture. *In vivo* factors might also explain the fact that tumors from the transfected cells and human MLS/RCLS tumors contain lipoblasts far more advanced than those obtained after adipogenic treatment of MLS/RCLS cell lines or *FUS-DDIT3*-transfected preadipocytes.

The *DDIT3*-transfected HT1080 cells accumulated large amounts of fat, and many cells developed a "signet ring" lipoblast phenotype when treated with adipogenic factors *in vitro*. The original HT1080 cells showed a much weaker response. We speculate that forced expression of *DDIT3* triggered the first steps of an adipocyte differentiation program that resulted in responsiveness to adipogenic factors.

Searches in our microarray results for *FUS-DDIT3*- and *DDIT3*-induced or -suppressed genes reported to have some role in adipogenesis identified *DSIPI*. *DSIPI* encodes a leucine zipper type of transcription factor that has been shown to inhibit adipogenesis.³⁰ The reduced expression of *DSIPI* in *FUS-DDIT3*- and *DDIT3*-transfected cells may be an important factor behind the morphological shift seen in the SCID mouse tumors.

In previous studies, only *FUS-DDIT3* (but not *DDIT3*) was found to transform normal cells or cause tumors in transgenes, thus excluding the normal *DDIT3* as a transforming gene.^{19,21,22,31} Our observation that *pDDIT3EGFP*-transfected HT1080 cells grew as myxoid liposarcomas with the capillary network and abnormal lipoblasts shows that constitutive expression of the normal *DDIT3* protein may induce an MLS-like morphology when expressed in a mesenchymal tumor. In this context, it is important to note that our *DDIT3*-carrying vector construct lacked the 5'-untranslated region of the *DDIT3*

transcript, which is known to play an important role in limiting production of the protein at a translation check-point.³² This critical part of the *DDIT3* transcript is also deleted in the MLS/RCLS fusions. Both MLS/RCLS cells and our *pDDIT3EGFP*-transfected HT1080 cells contain more of variant *DDIT3* than the levels of *DDIT3* found in normal lipoblasts or adipocytes (data not shown). Further analysis of *FUS/EWSR1-DDIT3*-negative MLS/RCLS-like tumors is required to find out whether de-regulated *DDIT3* expression may be associated with a liposarcoma phenotype.

Most MLS/RCLS tumors are characterized by a fine plexiform network of capillaries.¹⁷ A similar capillary network emerged in most tumors of *pFUS-DDIT3EGFP*- and *pDDIT3EGFP*-transfected cells (Figure 2). Tumors of original HT1080 or *pFUSaEGFP*-transfected cells showed a different pattern, dominated by poorly defined sinusoid vessels. Staining of tumor sections with anti-human and anti-mouse CD34 antibodies showed that all endothelial cells were of mouse origin. Thus, tumor-produced factors affect the angiogenesis and vessel morphology. The lower expression of *VEGF* in the *FUS-DDIT3*- and *DDIT3*-transfected cells may partially explain the differences in capillary formation. Another factor that may affect capillary morphology is interleukin-6.³³ Interleukin-6 has recently been reported to be produced by MLS/RCLS cells and up-regulated in *DDIT3*- or *FUS-DDIT3*-transfected HT1080 cells.³⁴ *DOL54*, an angiogenesis-inducing gene previously reported to be induced by *DDIT3* and *FUS-DDIT3*,³⁵ was equally expressed in HT1080 and its transfected variants (data not shown).

Our analysis showed that the expression of a limited number of genes was changed in the *DDIT3*- or *FUS-DDIT3*-containing HT1080 cells. The small overlap in differentially expressed genes between the two transfected cell lines may indicate that *DDIT3* and its oncogenic

derivative, *FUS-DDIT3*, differ with regard to their effects on target genes. This interpretation is supported by several other studies of the EWS group of fusion oncogenes, showing that addition of the N termini of EWSR1 or FUS protein to transcription factors changes their activities as transcriptional regulators. We found, however, that several of the differentially regulated genes, most notably stress-induced genes, were overexpressed both in *DDIT3*-transfected HT1080 cells and in the MLS 402-91 line. The *pFUS-DDIT3*EGFP-transfected HT1080 cells differed in this respect. It is possible that expression of *DDIT3* or *FUS-DDIT3* must exceed a threshold level before induction of stress response genes occurs and that the low level of *FUS-DDIT3* in the transfected HT1080 cells was insufficient to trigger the stress response genes and some other *DDIT3* target genes.

SERPINB2 was one of the most strongly up-regulated genes common to *pFUS-DDIT3*EGFP-transfected HT1080 cells and MLS cell lines. This gene was not induced in *pDDIT3*-transfected HT1080 cells, indicating an *FUS-DDIT3*-specific induction mechanism. Because *FUS-DDIT3*, but not *DDIT3*, is a transforming gene, *SERPINB2* may be an important factor in the transformation process. *SERPINB2*, also known as *PAI2*, encodes a plasminogen activator inhibitor that may switch between different conformations.³⁶ However, the protein has also been reported as a nuclear protein, binding to the pRB protein product of the retinoblastoma tumor suppressor gene.³⁷ The multiple roles of the *SERPINB2* protein in cell cycle control and extracellular matrix regulation make it interesting in the context of *FUS-DDIT3*-induced transformation, and this finding will be followed up in our continued research.

MLS-like tumors develop from adipose tissues in *FUS-DDIT3* transgenic mice, whereas human MLS/RCLSs most often arise inside large muscles. The differences between naturally occurring human tumors and those developing in transgenic mice suggest that the target cells and/or microenvironmental requirements for transformation by *FUS-DDIT3* may differ in the two species. We have reported previously that most *pFUS-DDIT3*EGFP-transfected human cells die shortly after the fusion protein is expressed,¹³ and the HT1080 cell line was no exception (Table 1). The single clone that we succeeded in retrieving was one of few stable *FUS-DDIT3*EGFP-expressing HT1080 clones observed in the cloning step. Sequencing of the inserted *FUS-DDIT3* and Western blot confirmation of protein size showed that the cells expressed the expected EGFP-tagged type II *FUS-DDIT3* protein but at a low level. The low level of *FUS-DDIT3*EGFP was probably of importance for the survival of this clone. The surviving clone may also have originated from a rare variant of the HT1080 cells that was compatible with expression of *FUS-DDIT3*. A similar selection mechanism may take place in the development of MLS/RCLS in humans. Observations of toxic effects have also been reported for the related *EWSR1-FLI1* fusion oncogene found in Ewing sarcoma. Mutations or silencing of *CDKN2A/p16* or *TP53* were found to increase cell survival of *EWSR1-FLI1*-transfected cells.³⁸ This suggests that cells with loss of p16 or p53 functions may be

preferential target cells for *EWSR1-FLI1* transformation. Similarly, unknown mutations or epigenetic changes may increase the compatibility with *FUS-DDIT3* expression. In this context, it is interesting to note that the HT1080 cell line carries a normal copy of the *TP53* but has deleted the *CDKN2A/p16*.

We conclude that forced expression of *FUS-DDIT3* and the normal *DDIT3* induces a switch in HT1080 cells toward an MLS/RCLS-like morphology and gene expression pattern. The results also indicate that *FUS-DDIT3* and *DDIT3* may act as instructive factors to induce a liposarcoma phenotype and that the transcription factor partner *DDIT3* of *FUS-DDIT3* is the tumor type determining part of this EWS-group fusion oncogene.

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