Liposarcoma

Proliferation, senescence and the role of DDIT3

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Cover illustration: NCAM –positive human muscle derived cells

To my surprise!

And to my dear, big family all over Sweden, especially my husband, Reine, and my children, Sofie and Ruben

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ABSTRACT

Lipomatous tumors comprise benign and malignant forms called lipomas and liposarcomas. Myxoid/round cell liposarcoma (MLS/RCLS) is the second most common liposarcoma and is characterized by the fusion oncogenes *FUS-DDIT3* or *EWSR1-DDIT3*. To understand the morphology of MLS 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 immune-deficient mice. 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, the fusion gene and normal *DDIT3* induce a liposarcoma phenotype when expressed in a primitive sarcoma cell line. MLS/RCLS may develop from cell types other than preadipocytes. 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. Further immunohistochemical investigation of the expression of the DDIT3 protein showed that major cell subpopulations of well differentiated tumors and MLS/RCLS tumors were found to express DDIT3 or the derived fusion protein. Our results suggest a dual, promoting and limiting, role for DDIT3 in formation of lipoblasts and liposarcoma morphology. Most liposarcoma types are characterized by genomic instability caused by impaired TP53 function. Further analysis of TP53 in MLS/RCLS with mass spectrometry, immunoblotting and immunohistochemistry show that a normal TP53 protein is produced in three of four MLS cell lines. This shows that the TP53 system is functional in the majority of MLS cases. MLS/RCLS tumors express proteins involved in cell senescence. In a study of 17 MLS/RCLS cases, large subpopulations of tumor cells expressed the RBL2 pocket protein together with senescence-associated heterochromatin binding protein 1γ and IL8 receptor β. The expression pattern suggests that MLS/RCLS tumors contain large subpopulations of senescent cells compatible with the slow growth of this tumor type.

Keywords: Liposarcoma, FUS-DDIT3, TP53, senescence

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SAMMANFATTNING PÅ SVENSKA

Cancer är en elakartad tumör som kan sprida sig i kroppen och uppstår på grund av genetiska skador (mutationer) i en normal cells arvsmassa (DNA). Arvsmassan innehåller mer än 20 000 olika gener som styr alla de processer som pågår i en cell. Det är framför allt gener som kontrollerar cellförökning som är skadade i cancer och en av de viktigaste bland dessa gener är *TP53*. Den kallas för arvsmassans väktare och det är ofta just *TP53* som har en mutation, vilket leder till en okontrollerad cellförökning och ansamling av andra mutationer. Idag vet man att det inte räcker med bara en muterad gen utan flera mutationer i olika gener krävs för canceruppkomst. De vanligaste tumörformerna utgår från hud, slemhinnor och organ. Mindre vanliga tumörformer är tumörer som växer i kroppens ben- och mjukdelar och elakartade former här kallas ofta sarkom. Fettartade tumörer, liposarkom, är den vanligaste typen av mjukdelssarkom i människa och en av de allra vanligaste är myxoida liposarkom (MLS).

I den här avhandlingen har vi undersökt olika varianter av fettumörer med tonvikt på MLS. Den här tumören har en specifik genetisk förändring där två gener smälter samman och bildar en ny gen, en fustionsgen, kallad *FUS-DDIT3*. De flesta MLS bär på denna fusionsgen. Vi har kunnat visa att fusionsproteinet FUS-DDIT3 och normalt DDIT3 har en instruerande effekt för tumörernas fettlika utseende/fenotyp. Ett onormalt uttryck av DDIT3, som del av fusionsproteinet eller som ett resultat av genetiska omkastningar, bidrar till differentieringen mot fett, viket oftast karakteriserar de här tumörtyperna. Myxoida liposarkom är långsamväxande tumörer och vi har undersökt 17 tumörer av typen MLS/RCLS med avseende på proteiner associerade med tillväxtkontroll och irreversibel vilofas (senescence). Resultaten pekar på att en stor population av tumörceller är senescenta.

Tidigare publikationer har rapporterat att mutationer av *TP53* är vanliga medan andra publikationer har rapporterat att *TP53* mutationer är sällsynta i MLS/RCLS. Här visar vi att funktionellt TP53-protein produceras i 3 utav 4 MLS-cellinjer, vilket också förklarar varför majoriteten av dessa tumörer är strålnings-känsliga. Myxoida liposarkom växer vanligen inuti muskulatur och framförallt i djupa lårmuskler. För att förstå biologin bakom uppkomsten av en tumör är det viktigt att få kunskap om ursprungscellens egenskaper. Vad gäller MLS/RCLS kan man misstänka att en i muskelvävnad vanligt förekommande celltyp, efter genetiska förändringar, ger upphov till dessa tumörer. Vi har extraherat och jämfört genetiskt material från normala odlade muskelceller och odlade MLS-tumörceller. Vi hittade att MLS-tumörceller har genetiska likheter både med förstadier till muskelceller och med mer omogna muskelderiverade celler.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Katarina Engström, Helena Willén, **Christina Kåbjörn Gustafsson**, Carola Andersson, Marita Olsson, Melker Göransson, Sofia Järnum, Anita Olofsson, Elisabeth Warnhammar, Pierre Åman. The myxoid/round cell liposarcoma (MLS/RCLS) fusion oncogene *FUS-DDIT3* and the normal DDIT3 induce a liposarcoma phenotype in transfected human fibrosarcoma cells. *Am J Pathol 2006 168:5*

II. **Christina Kåbjörn Gustafsson**, Katarina Engström, Pierre Åman. DDIT3 expression in liposarcoma development. *In revision, Sarcoma 2013*

III. **Christina Kåbjörn Gustafsson**, Anders Ståhlberg, Katarina Engström, Anna Danielsson, Ingela Turesson, Pierre Åman. Cell senescence in myxoid/round cell liposarcoma. *In revision, Sarcoma 2014*

IV. Anders Ståhlberg, **Christina Kåbjörn Gustafsson**, Katarina Engström, Christer Thomsen, Soheila Dolatabadi, Emma Jonasson and Pierre Åman. Expression of normal and functional TP53 in myxoid liposarcoma/round cell liposarcoma. *Submitted 2014*

V. **Christina Kåbjörn Gustafsson**, Anders Ståhlberg, Pernilla Grundevik, Katarina Engström, Thoas Fioretos and Pierre Åman. Myxoid/round cell liposarcoma cell of origin and human muscle derived mesenchymal stem/precursor cells. *In manuscript*

.

CONTENTS

ABBREVIATIONS

APC	Anaphase promoting complex
CCNA	Cyclin A
CEBP	CCAAT/enhancer-binding protein
CDC2	Cyklin dependent kinase 1
CDK	Cyclin dependent kinase
DDIT3	DNA-damage-inducible transcript 3
DDLS	Dedifferentiated liposarcoma
DNA	Deoxyribonucleic acid
DP1	Transcription factor dimerization partner 1
E2F	Transcription factor IIF
EWS	Ewing sarcoma breakpoint region 1
FISH	Fluorescence in situ hybridization
FUS	Fused in Sarcoma
G1	First gap phase
G2	Second gap phase
HMGA2	High-mobility group AT-hook 2
IHC	Immunohistochemistry
MAPK	Mitogen-activated protein kinases
MAX	MYC associated factor X
MDM2	Mouse double minute 2 homolog

MDSC	Muscle derived stem cell
MFH	Malignant fibrous histiocytoma
MLS	Myxoida liposarcoma
mTOR	Mammalian target of rapamycin
MYC	V-Myc avian myelocytomatosis viral oncogene homolog 1
NCAM	Neural Cell Adhesion Molecule
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PLS	Pleomorfic liposarcoma
PP	Indicates Phosphorylation
PPAR	Peroxisome proliferator-activated receptor
RB	Retinoblastoma protein
RCLS	Round cell liposarcoma
RNA	Ribonucleic acid
SASP	Senescence-associated secretory phenotype
S-phase	DNA-synthesis phase
SP	Side population
WDLS	Well differentiated liposarcoma

1 INTRODUCTION

The human body is built by tissue derived from the three early embryonic germ cell layers: the ectoderm, the endoderm, and the mesoderm [1]. The embryonic differentiation of tissues and organs is a vulnerable path where genetic conditions and genetic alterations may cause undesired effects, and even cancer. Malignant tumors is a heterogeneous group of diseases and characterized by uncontrolled growth and spread of abnormal cells. The development of cancer, carcinogenesis, is a multistep genetic process. It initiates in a single normal cell and gradually transforms its progeny into malignant counterparts by sequential genetic changes [2, 3]. Cancer causing mutations affects two main classes of genes. They activate proto-oncogenes and inactivate tumor suppressor genes [4-6].

1.1 Characteristics of cancer development

Although cancer is a group of heterogeneous diseases it has been proposed that several major alterations in cell physiology are required for cancer development [7, 8] and they are:

- Limitless replicative potential. Normal cells carry a program preventing them from limitless division.
- Resisting of cell death. A normal cell respond to signals from the intra-and extracellular environment and if abnormal signals, a programmed cell death will occur, apoptosis.
- Self-sufficiency in growth signals. A normal cell requires growth-promoting signals from the environment before they can actively proliferate.
- Insensitivity to growth suppressors. Tissue homeostasis is normally maintained by antiproliferative signals that induce cell-arrest or differentiation.
- Sustained angiogenesis. Oxygen and nutrients, as well as waste disposal, are crucial for proper cell function and survival.
- Deregulated cellular energetics. Normal cells extract their energy by oxidative phosphorylation.
- Tissue invasion and metastasis. Normal cells maintain tissue architecture and borders.

1.2 Tumor classification

Tumors can arise and develop in any tissue or organ. The most common tumors, derived from the ectoderm and classified as carcinomas, account for more than 80% of all cancer-related deaths in the Western world [9]. These tumors are further separated into two main categories, where squamous cell carcinomas are derived from the epithelia lining body surfaces, while adenocarcinomas originate from glandular epithelia, mostly in organs. The remaining malignant tumors arise from nonepithelial tissues. A group of non-epithelial cancers arise in the various cell types that make up blood-forming tissues and cells of the immune system. These are called leukemias and lymphomas respectively. Another group of nonepithelial tumors develops from cells in the central and peripheral nervous system and are termed neuroectodermal tumors. Tumors derived from the mesoderm, for example fat, muscle, cartilage, and bone, belong in the group of mesenchymal tumors, and the malignant mesenchymal tumors are often called soft tissue sarcomas [10].

Soft tissue sarcoma is a heterogeneous group of malignant tumors that constitutes less than 1% of all malignant tumors in Sweden [11]. Among these, liposarcoma is the most common type of soft tissue sarcoma in humans and constitutes about 10% - 18% of soft tissue sarcomas [12]. The two most common types are well-differentiated/dedifferentiated liposarcoma (WDLS/DDLS) and myxoid/round cell liposarcoma (MLS/RCLS). These tumors develop in different locals and in different kinds of mesenchymal tissue. The benign and most common fatty tumor is the lipoma, usually growing slowly as a lump in the subcutaneous fat, mostly in the arm, leg, or back.

1.3 Histological subtypes of liposarcoma

There are several histological subtypes of liposarcomas and the current nomenclature is based on the World Health Organization classification, (WHO), Table 1. They each have different appearance and clinical behavior [13]. The histopathological low-grade tumors constitute WDLS (grade I) and MLS with less than 5% round cells (grade II), and the high-grade group consists of MLS with more than 5% round cells (grade III or IV) and DDLS and PLS (grade IV). The higher the grade the more aggressive the behavior.

Atypical lipomatous tumors/Well-differentiated liposarcom (WDLS)
Dedifferentiated liposarcoma (DDLS)
Myxoid liposarcoma (MLS)
Pleomorphic liposarcoma (PLS)

Table 1. Liposarcoma subtypes, according to the WHO classification of 2013

1.3.1 Well differentiated liposarcoma (WDLS)

There are four different histological subtypes of WDLS with limited clinical importance, but representing 40% - 45% of all liposarcomas. The lipoma-like WDLS has a characteristic morphology with relatively mature fat cells varying in size, and where mono-or multi-vacuolated lipoblasts may be found. One sees atypical enlarged nuclei, few mitotic figures, and minimal myxoid or fibrous zones.

Most often WDLS presents as large, deep-seated lesions of the thigh followed by lesions in the retroperitoneum. They may recur locally, but do not metastasize unless they undergo dedifferentiation, which is most common in the retroperitoneum [11, 12]. Atypical lipomatous tumor is a synonymous term preferred to be used for lesions arising at surgically amenable locations whereas WDLS is preferred in reference to lesions arising in the retroperitoneum and mediastinum, because of their association with recurrence and significant mortality.

At chromosomal level these tumors usually show the presence of extra ring and/or giant marker chromosomes invariably containing amplified sequences originating from the 12q (14-15) region [12, 14]. The gene *MDM2* (12q15) is consistently amplified and overexpressed and is considered the main driver gene of the 12q amplicon. Elevated *MDM2* expression blocks TP53 function. Further discussions are in section 1.7.3. Other genes located in the 12q (14-15) region, for instance, *CDK4* and *HMGA2*, are frequently co-amplified with *MDM2*. No recurrent fusion gene is recognized.

1.3.2 Dedifferentiated liposarcoma (DDLS)

Morphologically, DDLS represents transition areas from WDLS to non-lipogenic sarcoma, which in most cases resembles high-grade fibrosarcoma or pleomorphic undifferentiated sarcoma (previously malignant fibrous histiocytoma, MFH) [12]. DDLS is a high-grade tumor most commonly

occurring in the retroperitoneum, where it is associated with a significantly worse survival.

Like WDLS, these tumors also show the presence of extra ring and/or giant marker chromosomes derived from 12q. Amplification of *MDM2* and *CDK4* is frequently seen. No specific fusion gene is recognized.

1.3.3 Myxoid liposarcoma (MLS)

About 40% of all liposarcomas consists of MLS. They have a unique morphology with hypocellular spindle cell proliferation in a myxoid background with a characteristic plexiform capillary bed. There are immature fat cells, lipoblasts around vessels or at the periphery of the tumor. The presence of hypercellular areas with undifferentiated round cell morphology is classified as mixed myxoid/round cell variant (MLS/RCLS) if the population of round cells ranges between 5% and 80%, and as pure RCLS if more than 80%. MLS is classified as a high-grade tumor, if there is a population of 5% round cells or more [12].
Classical MLS has a good prognosis with low metastatic rate, whereas round cell type with necrosis and mutated TP53 is associated with poorer prognosis [15]. Myxoid liposarcoma occurs preferentially in deep soft tissue of the extremities, especially within the musculature of the thigh.

Myxoid liposarcoma has unique genetic features [16]. More than 95% of the cases carry the *FUS-DDIT3* fusion oncogene, and in the remaining cases a variant is present carrying the *EWS-DDIT3* fusion oncogene, that is all of the FET family (Fig. 3). Further discussions are in section 1.6.

1.3.4 Pleomorphic liposarcoma (PLS)

Pleomorphic liposarcoma is an aggressive, high-grade tumor that constitutes about 10% of all liposarcoma types [12]. The morphology is of pleomorphic lipoblasts, often with hyper-chromatic, enlarged, and sometimes bizarre nuclei. It may mimic other tumors like pleomorphic undifferentiated sarcoma, and even carcinoma or melanoma, so the finding of lipogenic differentiation with lipoblasts is diagnostic. This subtype has a high risk of local recurrence and metastasis.

Cytogenetically, pleomorphic liposarcoma more closely resembles those of other pleomorphic sarcomas. There are often numerous chromosomal imbalances, and no consistent translocation or ring chromosome has been identified [17] [18].

1.3.5 Lipoma

Lipoma is a benign fatty tumor usually growing subcutaneously on the arm, leg, or back, but it can be seen wherever there is fat. The morphology of lipoma is characterized by mature fat cells growing in lobes separated by thin collagen fibers, by scattered vessels and by a thin collagen capsule that often surrounds it. There is no cellular atypia and no mitosis. Lipomas rarely recur, except for intramuscular lipoma, which has a higher grade of local recurrence [19]. There are other subgroups of lipomas, for instance, angiolipoma, consisting of more vessels; fibrolipoma, consisting of more connective tissue; and chondrolipoma, consisting of cartilage.

At chromosomal level, lipoma often shows translocations of the *HMGA2* gene, localized to 12q14.3, which plays an important role in a subset of lipomas [12]. This gene is located in the same chromosome segment as *DDIT3* at chromosome 12. Further discussions are in section 1.5 and paper II.

1.4   Stem cells

Stem cells are non-specialized cells that exist in all multicellular organisms. They have two properties that distinguish them from other cell types. They can undergo a limitless number of cell divisions with self-renewal and they have the ability to mature to several different kinds of cell types [20]. Stem cells of mammalians are divided into three groups: embryonic stem cells, stem cells from the umbilicus and adult stem cells. Adult stem cells in the grown individual have the ability to repair wounded cells and tissues, for instance, satellite cells in muscle tissue.

Since stem cells have the potential to mature to specialized cell types, they are in focus for intensive research and medical treatment using stem cells is now a reality [21-24].

1.4.1   Embryonic stem cells

These are the first cells created in the very early embryonic life of all multicellular organisms. At day four of the embryo there are, in its inner sheet, totipotent stem cells with the ability to differentiate into any cell type in the body. These cells are thus not specialized and therefore very attractive to researchers. In humans, three germ cell layers (ectoderm, endoderm, and mesoderm) have evolved in a process called gastrulation, taking place in the third week of gestation. These three layers are now programmed to give rise to different kinds of tissue [1, 25-27].

1.4.2   Mesenchymal progenitor/precursor cells

At the end of the last century there was increased research on the mesenchymal cell system meaning bone marrow-derived stroma cells as well as mesenchymal stroma cells. The mesenchymal cell system was first described by Maureen Owen [28] and over the years a massive research effort concerning tissue reconstruction, cell transplantation, hematopoietic stem cell transplantation, and gene therapy has taken place. It has been shown that there are precursor cells/stem cells in adult tissue, and more simple stem cell systems that give rise to a few specialized cell types in skin and intestinal mucosa have been described [29].

The mesenchymal cell system has been shown to consist of bone marrow-derived mesenchymal stem cells that have the ability to stimulate regeneration, and some reports show that mesenchymal stem cells may migrate and give rise to a broader differentiation than previously believed. Among cultured mesenchymal precursor cells, bone-, cartilage-, fat-, and muscle differentiation has been reported [30-32].

For the time being, it is unclear whether these multipotent abilities are a property of all neural or mesenchymal precursor cells, or if the studied populations are heterogeneous. The results suggest the occurrence of multipotent stem cells in normal tissue, and perhaps a common stem cell population that give rise to mesenchymal, neural, and hematopoietic differential pathways (Fig. 1).
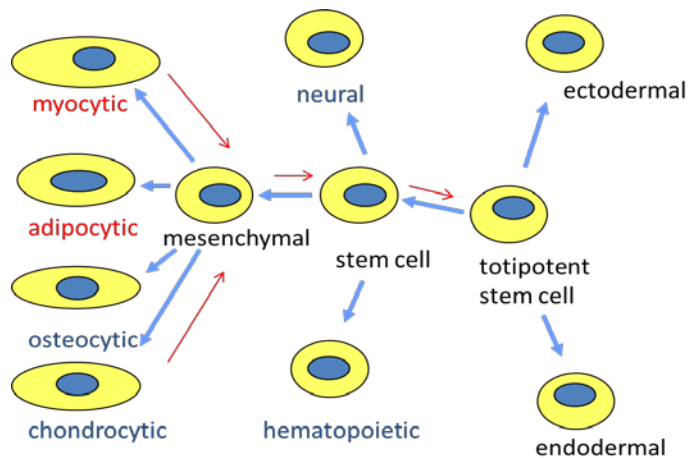
Figure 1. Schematic picture of possible paths of differentiation from a totipotent stem cell to a more committed precursor cell. Red arrows show the possibility of redifferentiation to a more totipotent stem cell.

1.4.3 Satellite cells and other progenitor cells in muscle tissue

In skeletal muscle, there is a distinct population of myogenic precursor cells called satellite cells. These cells lie on the surface of the muscle fiber beneath the basal lamina, but above the plasma membrane and are normally mitotically quiescent. They are small mononuclear cells, with virtually no cytoplasm, that have the potential to generate new muscle fibers, provide additional precursor nuclei to their parent muscle fiber, or return to a quiescent state [33, 34].

Upon activation they proliferate and give rise to daughter myogenic precursor cells that form myotubes and, subsequently, new muscle fibers. In a cell culture fragmented muscle will transform into myoblasts before undergoing myogenic differentiation with fusion of multinuclear cells. The neural-cell adhesion molecule, NCAM (CD56, Leu-19), is a protein specifically expressed on the surface of human satellite cells as well as on regenerative skeletal muscle cells [35].

Satellite cells have long been considered as committed, monopotent stem cells, but some reports claim that satellite cells may also be capable of differentiation into adipocytes and osteocytes in vitro, indicating a pluri-potent differentiation potential [33, 36-38].

Apart from satellite cells, skeletal muscle has also been reported to contain several other progenitor/stem cells such as multi-lineage stem cells including side population (SP)-cells, muscle-derived stem cells (MDSCs) and bone marrow-derived stem cells [36, 39-44]. SP-cells and MDSCs may have the potential to differentiate into several different cell types, including hematopoietic cells [45]. MDSCs have been shown to differentiate into a variety of lineages: skeletal muscle cells, glial cells, and endothelial cells. It has been suggested that the MDSC population are progenitors for satellite cells during development and regeneration [46].

A first step to separate satellite cells (muscle precursor cells) from other precursor cells may be to use the antibody CD56 (NCAM).

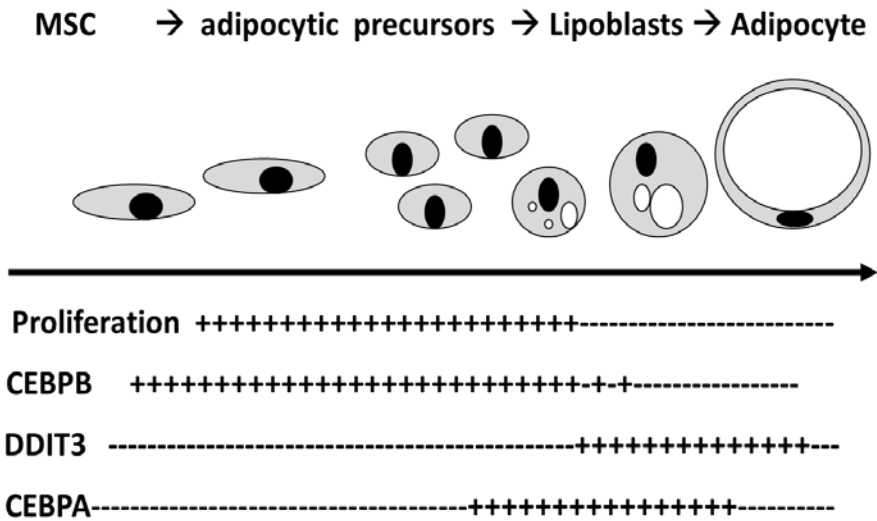# 1.5 Adipogenic differentiation

1.5.1 Normal adipogenesis

This is the process of cell differentiation by which precursor cells, preadipocytes, become adipocytes, and this process is essential for the molecular understanding of myxoid liposarcoma.

Three classes of transcription factors have been identified that directly influence fat cell development. These include PPARγ, C/EBPs, and the basic helix–loop–helix family (ADD1/SREBP1c). The PPARγ protein forms a dimer with retinoid X receptor, and this complex regulates transcription of adipocytic genes [47].

There are several isoforms of the C/EBP family, and regulated expression is seen for several of them during adipogenesis. The different isoforms show stable homodimers and heterodimers. The C/EBPs belong to the basic-leucine zipper class of transcription factors. C/EBPα expression rises after induction of PPARγ2, and these two transcription factors co-regulate. In cultured preadipocytic cell lines that have been induced to differentiate, C/EBPβ and δ mRNA and protein levels rise early and transiently [48, 49]. C/EBPα, on the other hand, is induced later in the differentiation process, slightly preceding the induction of most of the end-product genes of fat cells [47]. One of the anti-adipogenic transcription factors is DNA-damage-inducible transcript 3, DDIT3, (Fig.2).

Figure 2. Timing of CEBPB, CEBPA and DDIT3 expression in differentiation of mesenchymal stem cell to mature adipocyte.

1.5.2 The role of DDIT3 in adipogenesis

The *DDIT3* gene (also known as C/EBP-homologous protein (CHOP) or DNA-damage-inducible transcript 3, or "growth arrest" and DNA-damage-inducible protein (GADD153) encodes for a nuclear protein, which is a member of the C/EBP family. The DDIT3 protein forms stable heterodimers with the isoforms of C/EBPα and C/EBPβ [50]. Overexpression of DDIT3 has been reported to block terminal adipocytic maturation and growth arrest [51-53]. We have shown that expression of DDIT3 in a low differentiated fibrosarcoma cell line results in morphological conversion towards a liposarcoma phenotype, paper I.

The *DDIT3* gene is also a key factor in cellular stress response and was identified by its transcriptional induction upon growth arrest and DNA damage [52, 54, 55].

1.6 Molecular biology of MLS

1.6.1 Fusion genes

A common activation mechanism for oncogenes in many types of sarcomas and lymphomas/leukemias, is the formation of fusion oncogenes. Around 700 distinct fusion oncogenes are reported from human tumors [56]. A fusion gene is a hybrid gene formed from two previously separate genes where chromosome breaks coincident in time and space. It can occur as a result of: translocation, interstitial deletion, or chromosomal inversion. Often, fusion genes are oncogenes that cause cancer [57]. Chromosome translocations and gene rearrangements were first described in leukemia and lymphoma. The most famous is the Philadelphia chromosome translocation, t(9;22)(q34;q11), described by Nowell and Hungerford in 1960 [58], resulting in the formation of the *BCR-ABL* fusion gene [59, 60]. Several observations suggest that the formation of fusion oncogenes is more frequent than the tumors they are causing. Escape from stress responses leading to apoptosis or oncogene induced senescence, cell or tissue type dependence or interactions with other genetic variables are important for the final outcome of the formation of a new fusion oncogene [61-64].

1.6.2 The FET group of fusion oncogenes

The sarcoma and leukemia causing the FET family of fusion oncogenes consists of the 5' parts of one of three related genes *FUS* or *EWSR1* or *TAF15* juxtaposed with 3' partners that encode various DNA binding transcription factors, see Fig. 3 [57, 61, 64, 65].

One of the *FET* oncogenes, *FUS-DDIT3,* results from a t(12;16)-(q13;p11) chromosome translocation and has a causative role in initiation of MLS/RCLS [16, 65, 66].
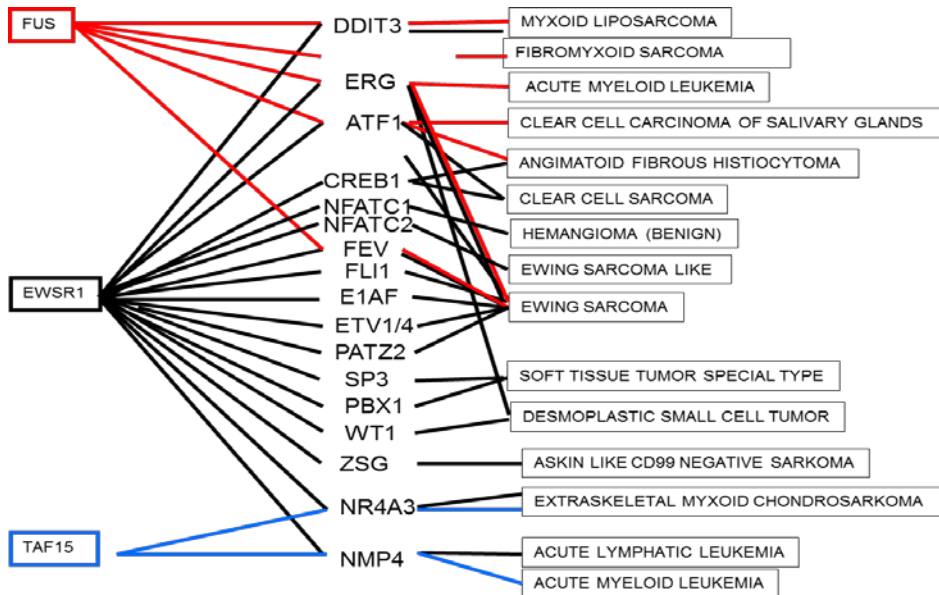

Figure 3. The FET group of fusion oncogenes. The 5´parts of the FET family genes FUS, EWSR1 and TAF15 are fused to genes encoding various transcription factors (middle column). The respective fusion genes are found in the tumor types indicated in the right column. Observe the high degree of tumor type specificity for each FET fusion gene.

1.6.3 The *FUS- DDIT3* gene

The fusion oncogene *FUS-DDIT3* t(12;16)(q13;p11) is specific for myxoid liposarcoma. The *DDIT3* gene on chromosome 12q13 fuses with the *FUS* gene on chromosome 16p11 or with *EWS* on 12q22 in MLS. *FUS-DDIT3-* and *EWSR1-DDIT3-* encoded proteins are believed to function as abnormal transcription factors interacting with differentiation and growth control of the tumor cells [12, 13, 67], (Fig.4). The protein is nuclear and considered to be an abnormal transcription factor where the N-terminal of FUS controls

DDIT3 with its strong transcriptional activation domain, fused to the dimerizing and DNA-binding elements of DDIT3 [62].
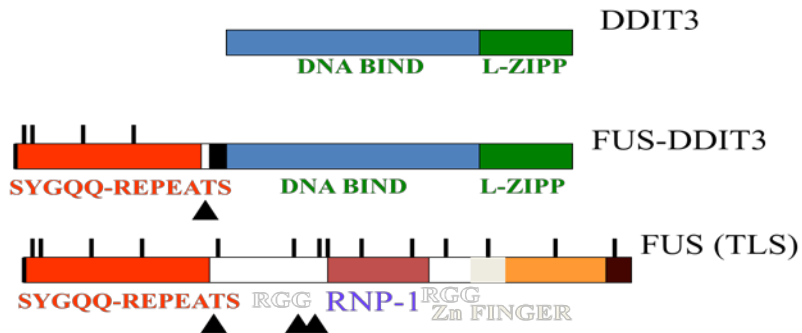

Figure 4.The t(12;16) translocation, which results from a fusion of chromosomes 12 and 16, leads to the expression of a chimeric fusion protein having the N-terminal FUS domain fused to the entire DDIT3 protein.

FUS-DDIT3 inhibits the development and the terminal adipocytic differentiation by blocking the C/EBPα activity [53]. This results in a cell population of partially committed pre-adipocytes that may progress to MLS. Experiments with transgenic mice have further demonstrated that *FUS-DDIT3* cause liposarcoma while normal *DDIT3* or normal *FUS* did not result in liposarcoma. All these three transgenes showed normal white adipose tissue histology [68, 69]. A more recent study reported that *FUS-DDIT3* caused liposarcoma only in *TP53* knockout mice [62].

1.7 Senescence

Biologically senescence means the process of ageing. On cellular level, senescence occurs when normal cells cease to divide. In cell cultures, about 50 cell divisions are possible, also known as replicative senescence-, the Hayflick phenomenon-, or the Hayflick limit [70, 71]. Telomere shortening is known to be an important mechanism in senescence [72-76]. There is a famous exception, the HeLa cells, which are the first human cells from a tumor and from which a permanent immortal cell line was created; these cells have the ability to propagate limitlessly in a cell culture [77]. HeLa cells and most tumor cells show an upregulated telomerase activity, thus providing capacity for unlimited proliferation [76, 78-80]. Most MLS/RCLS cases show an elevated telomerase expression caused by promotor mutations [81].

Cells can also be induced to senescence by stress caused by, for instance, certain toxins, irradiation, or the activation of certain oncogenes [82-84]. Senescence is thought to be an irreversible change, since chromatin is tightly packed by increased heterochromatin formation. Nuclear heterochromatin foci can be visualized by the presence of HP1γ. The pocket proteins RB1and RBL2 (P105 and P130, respectively) are important control hubs for cell cycle-proliferation driving transcription factors. RB1 is expressed in cycling cells, whereas RBL2 is expressed in resting and senescent cells [85-88].

Senescent cells has been shown to develop a senescence-associated secretory phenotype (SASP) and is characterized by the secretion of a wide range of growth factors, cytokines, extracellular matrix proteins and degradative enzymes, most of which can alter the local tissue microenvironment [89-92]. Of particular interest SASP is characterized by high level secretion of the cytokines, IL-6 and IL-8, which are key mediators of inflammation. The cytokines IL6 and IL8 are involved in induction and maintenance of senescence in human tissues [84] and these cytokines are also reported as autocrine growth or angiogenesis factors in several tumor types [89-92].
Our group has previously showed that FUS-DDIT3, through interaction with NFKB and C/EBPβ directly induce expression of IL6 and IL8 by binding to the IL6 and IL8 promoters [93, 94].
To understand the mechanisms that regulate IL-6 and IL-8 in association with senescence is important for understanding biological processes like tumor suppression and the development of cancer.

1.7.1 Cell cycle, G1/S transition, in normal cells

The cell cycle of normal cells is a series of events that takes place in a cell, leading to its division and duplication. The cell cycle consists of mitoses and interphase, divided into G1, S, and G2, during which the cell grows, duplicates its DNA and prepares for division. Cells that do not proliferate exit G1 phase and enter a quiescent stage, G0, until different growth factor signals promote the cell to re-enter the cell cycle, [6, 95, 96](Fig.5).

1.7.2 Cell- cycle arrest

This is a regulatory process that stops progression through the cell cycle during one of the normal phases. After mitosis, the cells enter G1 again, or go into G0, where they rest. At each of these phases, a checkpoint temporarily stops the cell cycle to allow the cell to decide if it should continue. The CDK inhibitors are important for this process. Some cells are programmed to duplicate infrequently, while damaged cells may need time for repair or destruction. On stimulation they re-enter the cell cycle. Radiation therapy

induces DNA damage and cells respond by activation of TP53 which induces arrest in G1, S, and G2 phases of the cell cycle, and in DNA repair [6].

Cell-cycle arrest may be reversible or result in senescence. In fact, avoidance of arrest is a common adjustment in cancer. An even more common change is the activation of growth-promoting pathways such as MAPK and mTOR, which are involved in the senescent phenotype. Activation of MAPK and mTOR makes cancer cells pro-senescent [97, 98].
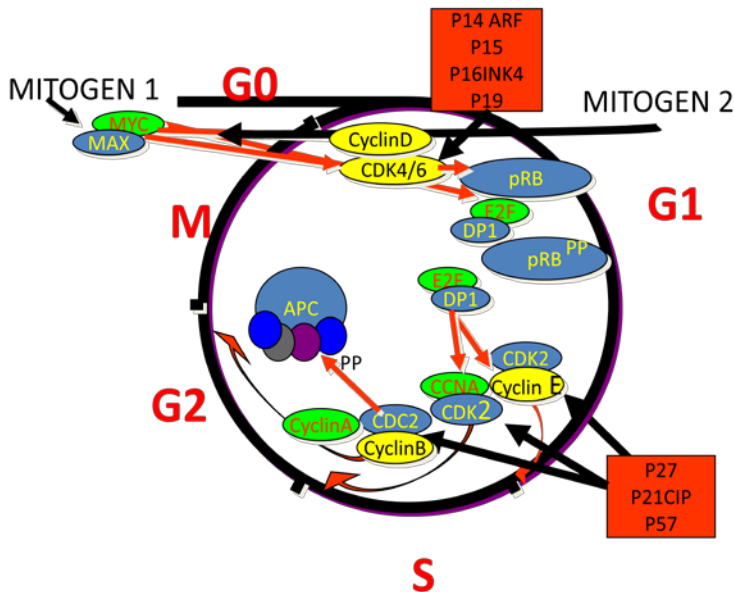

Figure 5. A complex system of cell-cycle checkpoints and regulatory protein.s Cyclins, together with enzymes, called cyclin-dependent kinases (CDKs), controls the progression through the different phases of the cell cycle. The regulatory proteins are cyclin D, cyclin E, cyclin A, and cyclin B and are required as subunits for the catalytic activity of CDKs. CDK inhibitors are shown in red boxes.

1.7.3 The role of TP53

TP53The protein TP53, also known as p53, is essential in all multicellular organisms, where it senses various cell stress signals and functions as a tumor suppressor, preventing cancer. Because of its role in maintaining genetic stability by preventing genome mutation, TP53 is also called "the guardian of the genome". The name relates to its molecular mass: it is in the 53 kilodalton fraction of cell proteins [99]. Normally, the TP53 protein is

continuously produced and degraded in the cell but stress signals and DNA damage, may trigger the increase and modification of TP53 proteins. TP53 has a major function in growth arrest, DNA repair, and apoptosis, to avoid proliferation of cells containing abnormal DNA [6, 100]. TP53 arrest growth by holding the cell cycle at the G2/S regulation point where DNA repair proteins will have time to fix the damage in the cell [100]. Activated p53 binds DNA, leading to the expression of several genes including microRNA miR-34a and WAF1/CIP1. They affect the expression of hundreds of down-stream proteins and among them p21 that binds to the G1-S/CDK (CDK2) and S/CDK complexes, see fig 5, inhibiting their activity. When p21 is in complex with CDK2, the cell is not able to continue to the next stage of cell division. If TP53 is mutated, it will no longer bind DNA effectively and the p21 protein will, as a consequence, not be able to act as the "stop signal" for cell division [101-103]. The cellular concentration of TP53 must be tightly regulated. While it can suppress tumors, high levels of TP53 may accelerate the aging process by excessive apoptosis. TP53 is also involved in stress-induced senescence [104-106].

The gene *MDM2* is the major regulator of TP53. *MDM2* is a proto-oncogene that promotes the cell cycle and encodes the nuclear phosphoprotein MDM2. In a negative feedback loop this protein is activated by TP53, and the encoded MDM2 binds to and inhibits the TP53 protein transactivation domain [107-109]. MDM2 also promotes degradation of TP53 by the ubiquitin system. If the *TP53* gene is mutated, tumor suppression is severely reduced. This may allow abnormal cells to proliferate and result in cancer [110, 111]. A large amount of all human tumors contain mutated TP53 [7, 112-114]. The majority of MLS cases carry normal TP53 genes. Compared to genetically complex sarcomas, MLS/RCLS is highly sensitive to radiation and chemotherapy suggesting a functional TP53 system [115-117]. A recent report show, however, that an *FUS-DDIT3* transgene failed to induce tumors in mice, if not introduced in a TP53-deficient background. These results suggest that an impaired TP53 function could be an important factor in MLS [62]. This report prompted the investigations presented in paper IV.

2 AIM

The objectives of this thesis were to investigate the behavior of myxoid liposarcoma and the role of the DDIT3 part of the fusion oncogene *FUS-DDIT3*.

The main aims of papers I to V were as follows:

Paper I: To examine the mechanisms behind the tumor-type specificity of MLS/RCLS.

Paper II: To analyze the morphology directing role of DDIT3-expression in different lipomatous tumors.

Paper III: To study the expression of proteins associated with growth control and senescence in MLS.

Paper IV: To investigate the TP53 protein expression and function in MLS tissues, -derived cell lines, and xenografts.

Paper V: To identify and characterize different cell populations in muscle tissue and their roles in development of MLS/RCLS.

3 MATERIALS AND METHODS

Formalin-fixed paraffin-embedded tumor tissue from 37 cases diagnosed and revalued as variant of lipomatous tumors was used for immunohistochemical analysis and RNA and FISH confirming analysis. Fresh human myoblast cell lines were used for comparison with MLS/RCLS tumor tissue from SCID-mice xenografts derived from fibrosarcoma cell line HT1080.

Immunohistochemistry and immunofluorescence microscopy

Series of 5-µm tissue sections were cut from each biopsy, deparaffinised, rehydrated, boiled in microwave oven for 10 minutes for epitope retrievment and stained with the antibodies. Bound antibodies were visualized using the LSAB second antibody streptavidin biotin peroxidase system (DAKO). Stained sections were examined on a light microscope.

For immunofluorescence analysis, cell 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 anti-fade mount containing the DNA binding dye DAPI (4,6-diamidino-2-phenylindole dihydrochloride) (Olink Bioscience) and examined on a fluorescence microscope.

FISH analysis
Interphase FISH analysis of formalin-fixed tumor tissue was performed on 1-4 µm paraffin sections. Three break-apart probes, DDIT3, FUS and EWSR1 (Vysis Inc.), were used according to protocols supplied by the manufacturer. Nuclei were counterstained with 10 µl 4´,6´,-diamidino-2´-phenylindole dihydrochloride (DAPI). The sections were analyzed and re-analyzed by two independent reviewers. At least 100 nuclei per section were scored. The interpretation of intact, fusion and split signals was based on guidelines recommended by the manufacturer and from other clinical laboratories using this method.

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 RNAeasy kit and stored at -140oC. Five µg of RNA was used for cDNA synthesis with Cy3 and Cy5-labeled nucleotides. cDNA labeling and hybridization to microarray glasses was performed according to the Pronto! Plus Direct Labeling v1.2 labeling and hybridization kit (InVitro, Sweden).
Microarrays comprising approximately 27,000 unique probes representing 23,707 genes were produced at the Swegene DNA Microarray Resource

Center, Department of Oncology, Lund University, Sweden (http://swegene.onk.lu.se).

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 (BASE) (Saal et al. 2002, http://base.thep.lu.se) for further analysis. The LOWESS algorithm 14 was used for normalization [118, 119].

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 upregulated or downregulated. Genes with a normalized intensity below 25 in the transfected cell lines were rejected from the lists of upregulated genes. Similarly, genes with a normalized intensity lower than 25 in the HT1080 reference were rejected from the lists of downregulated genes. RNA from the MLS cell line 402-91 was also tested, with the HT1080 cell line as a reference.

Statistical analysis

Statistical tests included mean value and standard deviation. To estimate the statistical significance of the overlap between differentially expressed genes in 402-91 cells and differentially expressed genes in the pFUS-DDIT3EGFP and pDDIT3EGFP transfected lines, two sets of genes corresponding to the up- and downregulated 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 downregulated 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 upregulated or downregulated gene sets.

Muscle specimen

Human skeletal muscle tissue samples were obtained from surgical excision at time of corrective surgery in conformity with Swedish legislation (S 441-03). The muscle tissues were from different muscle compartments; m vastus lateralis, m trapezius, and m Sartorius.

Each biopsy sample was collected in a sterile container containing transport medium (see below) and stored at 4˚C for up to 24 hours before processing.

Ham's F12 medium was used as transport medium. Phosphate-buffered saline (PBS) (pH 7, 2) was used for washings. Dissociation medium consisted of x% collagenase in Ham's F12. The culture medium (CM) was

composed of HAM's F12 containing natrium bicarbonate (2,2g/L) and supplemented with 20% of fetal calf serum (Life technologies), penicillin and streptomycin.

Cell culture
The muscle tissue biopsy was dissociated according to a procedure earlier described [120, 121]. After being cleaned of tendons and fat, the biopsy was cut in fragments, rinsed in PBS, incubated in 10mL of dissociation medium and incubated at 37˚C in an incubator for 1 h with gentle shaking every 10 minutes. The supernatant containing the released cells was decanted and filtered through a 100-µm nylon mesh into a tube containing 10 ml of CM.
The remaining muscle tissue was further dissociated in fresh dissociation medium (10mL for 30 minutes) before being filtered in a tube to isolate additional free cells. Free cells were collected by centrifugation at 300 g for 10 minutes and the pellet was resuspended in 5 mL of culture medium. The cells were seeded out in a 25 cm² flask in a total volume of 4 mL culture medium. The cultures were incubated at 37˚ in a humified atmosphere containing 5% CO_2. Medium was changed 24 h after primary seeding and then every 4 days. Primary cultures were trypsinized and replated after 6-8 days of incubation.

Immuno magnetic cell fractionation.
At first a fraction of the cultures (p2 and p3) was separated and incubated with NCAM in suspension and about 10-30 % of the cells were NCAM-positive indicating the existence of myoblasts in the culture.
The cells were separated by a magnetic cell sorting procedure according to Miltenvi Biotech's MACS system using Goat Anti-Mouse IgG Micro Beads. A small fraction from each separated NCAM-positive and negative pool was incubated and stained with immunofluorescens in order to evaluate the separation. The NCAM-positive fraction was seeded at a density of 0, 8 x 106 cells per 25 cm2 flask and the NCAM-negative fraction was seeded at a density of 3 x 106 cells. At second and seventh passage immunofluroscence tests on NCAM positivity was made.

In vitro adipogenic assay
Second to fourth or first frozen passages were used. The NCAM-negative and NCAM-positive populations were seeded in flaskettes and cultured in CM until they reached 70-100% confluence, after which the medium was changed to adipogenic-induction medium (Cambrex product PT3004 containing human recombinant insulin, dexamethasone, Indomethascin and IBMX) or to maintenance medium (MM; Cambrex product PT300X). The cells were treated with adipogenic induction medium for 3 days, followed by 1-3 days in MM. This was repeated three times. Control cultures were fed with MM

only following the same schedule. After completed cycles, the cells were cultured for 7 more days in MM with replacement of the medium every 2-3 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.

See the individual papers, I-V, for more detailed descriptions of the materials and other methods used. Experimental cell assays, vectors, transfections, western blot analysis, liquid chromatography-mass spectrometry, flow-cytometry and qPCR.

4 RESULTS AND DISCUSSION

4.1 Paper I

Fusion oncogene *FUS/DDIT3* and normal *DDIT3* promotes liposarcoma like phenotype

MLS tumor cells are characterized by the chromosomal aberration t(12;16)(q13;p11), which in most cases is the only cytogenetic abnormality. This translocation results in an *FUS-DDIT3* fusion occurring in more than 90% of cases [12]. 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) [16, 66]. The FUSDDIT3 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. These and other observations suggest that FUS-DDIT3 may act as an abnormal transcription factor.

To understand the role of the *FUS-DDIT3* fusion we designated vectors, p*FUS-DDIT3*EGFP, p*DDIT3*-EGFP, and p*FUSa*EGFP. Inoculation of transfected fibrosarcoma cell line HT1080 and MLS cell lines 402-912 and MLS 2645-94 in female FOX CHASE SCID mice was made, and the development of tumors was studied.

Microscopic examination of sections from the tumors revealed that original HT1080 cells and p*FUSa*EGFP transfected cells grew as low-differentiated sarcomas, mainly with poorly defined or sinusoid blood vessels and necrotic areas. In contrast, the mice inoculated with the p*FUS-DDIT3*EGFP- or p*DDIT3*EGFP-expressing cells developed liposarcomas containing atypical lipoblasts of different sizes. Increased proportions of extracellular matrix and small myxoid pools were observed in tumors from the two p*FUS-DDIT3*EGFP-transfected cell lines, but large amounts of the myxoid substance, as seen in many naturally occurring MLS tumors, were not found.

The tumors from *FUS-DDIT3*- and *DDIT3*-transfected cells exhibited a capillary network morphology that was similar to networks of MLS/RCLS. Microarray-based comparison of HT1080, the transfected cells, and MLS/RCLS-derived cell lines 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, the fusion oncogene *FUS-DDIT3* and the normal *DDIT3* induce a liposarcoma phenotype when expressed in a primitive sarcoma cell line. MLS/RCLS may thus, 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.

4.2 Paper II

DDIT3 protein expression in lipomatous tumors

Adipocytic tumors are the most frequent types of soft tissue tumors, and they are characterized by the more or less prominent presence of lipoblasts or adipocytes [12]. Some of the adipocytic neoplasms are characterized by recurrent tumor type-specific genetic rearrangements. Most WDLS/DDLS cases contain amplified segments of chromosome 12q13-15 carried as ring chromosomes or large marker chromosomes [122-124]. The amplified regions contain many tumor-associated genes, and among these, *DDIT3*. MLS/RCLS carries rearranged *DDIT3* fused to *FUS* or *EWSR1* [66, 125].

DDIT3 is normally expressed in adipocyte differentiation together with the related transcription factors CEBPB and CEBPA [47]. The timing of expression of these factors is important for normal differentiation. In the context of liposarcoma development, aberrant DDIT3 expression may open an adipocytic differentiation pathway and promotes lipoblast development in small subpopulations of the tumor cells, as seen in WDLS/DDLS and MLS/RCLS. Overexpression of DDIT3 has, however, also been reported to block terminal adipocytic maturation and growth arrest [51-53]. These seemingly conflicting reports may be explained by a crucial timing of DDIT3 expression in normal adipogenesis. DDIT3 could promote an adipocytic differentiation path in primitive mesenchymal cells but block later stages of development.

In the previous study we showed that expression of DDIT3 in a low-differentiated fibrosarcoma cell line results in morphological conversion towards a liposarcoma phenotype [126]. These observations suggest that expression of DDIT3 protein could be a common phenotype-determining factor for several types of lipomatous tumors.

In this study we tested this hypothesis by investigating the expression of the DDIT3 protein in three different subtypes of liposarcoma and in common lipoma. We further evaluated the role of DDIT3 expression by studying lipoblast formation in cultured liposarcoma cells treated with adipogenic factors.

Paraffin embedded sections were obtained from our pathology department in conformity with Swedish legislation. The material consisted of 11 lipomas, 11 PLS, 10 WDLS and 16 MLS/RCLS. The GOT3 cell line, established from a WDLS tumor was used to study adipogenic differentiation.

Our results show that DDIT3 is expressed in subpopulations of tumor cells in all 4 investigated lipomatous tumor types. There were no obvious differences between the number of DDIT3-expressing cells and the more aggressive DDLS and RCLS compared to WDLS and MLS.
Furthermore, DDIT3 was expressed at comparable levels in benign lipomas. Only a minority of DDIT3 expressing sarcoma cells responded to adipogenic conditions *in vitro*, indicating a complex role for DDIT3 as a phenotype-directing factor in lipomatous tumors.

We speculate that only a small minority of the cells would make it through the block and differentiate to lipoblasts. This would also explain the lack of correlation between numbers of DDIT3-expressing cells and numbers of lipoblasts in the investigated tumors. To test this hypothesis, the WDLS/DDLS-derived cell line GOT3 was analyzed after adipogenic treatment. This cell line carries a large chromosome 12-derived amplicon including the *DDIT3* gene and was found to express DDIT3 constitutively in almost all cells [124]. A limited accumulation of lipids in sporadic cells was seen under standard culture. Transfer to adipogenic culture conditions resulted in lipid accumulation and adipoblast development, but only in a minority of the cells.
These and our previous results show that expression of DDIT3 or FUS-DDIT3 can promote a liposarcoma phenotype in human fibrosarcoma cells [126]. *FUS-DDIT3* transfected mesenchymal stem cells cause MLS/RCLS-like tumors when injected in mice, and *FUS-DDIT3* transfection transforms 3T3 mouse fibroblasts. This shows that *FUS-DDIT3* is a powerful oncogene.

In contrast, forced expression of DDIT3 protein in mesenchymal cells or in transgenic mice gave no evidence of transformation or tumorigenic activity. The normal DDIT3 protein can thus not be considered a driving oncoprotein. Aberrantly expressed, it may, however, act as a promoting factor or/and tumor-type directing factor by interfering with the adipocyte differentiation program.

4.3 Paper III

MLS contains a major population of senescent cells

MLS is a slow-growing tumor but, 10%-15% of the cases show hyper cellular round cell morphology (RCLS) with less myxoid component that is associated with an unfavorable prognosis[12].

In the present study, we have employed immunohistochemistry (IHC) and flow cytometry, and analyzed a cohort of 17 MLS/RCLS tumors that were not previously treated with radiation or drugs, for expression of proteins associated with growth control and senescence.

Flow cytometry of two human MLS/RCLS tissues showed 94% and 96% of the cells in G1. These results are in agreement with our previous findings that that only a small percentage express cyclin A and have entered the S-phase [127]. The majority of the tumor cells are thus arrested in G1, although they express high levels of G1 cyclins, CDK4/6 and CDK2.

The pocket protein RB1 is expressed in cycling cells, whereas RBL2 is expressed in resting and senescent cells [85, 86, 88, 106]. Our IHC analysis of RB1 and RBL2 expression showed that large subpopulations of the MLS/RCLS tumor cells expressed the G0/senescence associated RBL2.

We previously showed that *FUS-DDIT,* through interaction with NFKB and C/EBP directly induced expression of IL6 and IL8 by binding to the *IL6* and *IL8* promoters [127]. These cytokines have also been implicated as autocrine growth or angiogenesis factors in various tumor types [128-131]. More recently IL6, and IL8 have been implicated in induction and maintenance of senescence in human tissues[132].

We tested MLS/RCLS tumor cell lines for growth and survival dependence of IL6 in vitro but detected no effects of treatment with IL6 receptor blocking antibodies. Instead, IL6 may, together with IL8, be a part of a senescence mechanism in MLS/RCLS. The fusion oncoprotein thus promotes a cytokine expression profile that is typical for senescent cells. The senescence-associated IL8 receptor beta (CXCR2) was also expressed on a large proportion of the tumor cells.

All together these results tell us that MLS contains a major population of senescent cells. This is also consistent with clinical behavior of this type of slowly growing tumor.

4.4 Paper IV

The MLS cells express functional TP53 protein

The vast majority of the MLS/RCLS tumors carry normal *TP53* genes, and secondary changes are few and rare, even when relapses occur after many years [116]. Compared to genetically complex sarcomas, MLS/RCLS is highly sensitive to radiation and chemotherapy, supporting the view that the tumor cells maintain a functional TP53 system [133]. A recent study shows however, that a *FUS-DDIT3* transgene fail to induce tumors in mice if not introduced into a *TP53*-deficient genetic background [62]. This indicates that impaired TP53 function could be an important factor in MLS development. These partially conflicting observations prompted us to investigate the status of the TP53 protein and function in MLS tissues, derived cell lines, and xenografts.

In the present investigation we examined the expression and function of the *TP53* gene in four MLS-derived cell lines, three with normal and one with a mutated *TP53* gene. Their genomes were searched for mutations and TP53 transcripts analyzed for splicing variants.
These results show that, except for the *TP53*-mutated DL221, the cell lines contained normal *TP53* genes. TP53 protein from one MLS-derived cell line was immune precipitated and analyzed by mass spectroscopic analysis. TP53 expression was analyzed by immunohistochemistry, and our analysis of seven MLS/RCLS tumor tissue cases showed very few expressing cells suggesting normal *TP53* genes[134].

In contrast to tumor tissues, the MLS-derived *in vitro* cultured cell lines MLS402-91, 1765-91, 2645-94, and DL221 all expressed TP53 protein, as shown by our western blot and immune-fluorescence analysis. The reason for the strong TP53 expression in cultured MLS cells is not understood. Cell stress caused by the *in vitro* culture conditions is a plausible explanation and supported by the fact that freshly explanted cells from MLS tissues rapidly start to express the TP53 protein and enter a senescent stage within a few passages.
Radiation-induced expression changes were followed by western blot and immunofluorescence analysis. Expression of P21 (also known as CDKN1A, WAF1 or CIP1) is reportedly induced by activated functional TP53 after radiation damage [135, 136]. Expression of P21 was elevated in all radiation-treated MLS derived cells, although the induction was much weaker in DL-221 and MLS2645-94.

We conclude that a normal TP53 was produced in three of four investigated MLS cell lines. Our IHC data together with investigations from other groups suggest that *TP53* mutations are uncommon in MLS/RCLS. Normal radiation-induced TP53 function was observed in two of three lines with normal *TP53* genes. The FUS-DDIT3 fusion protein had no effect on radiation-induced modifications of TP53 or TP53-induced P21 expression.

Our results suggest that most cases of MLS/RCLS contain functional TP53.

4.5 Paper V

MLS-derived cells express genes that are typical both for myotube committed satellite cells and for SP cells

Myxoid/round cell liposarcoma (MLS/RCLS) develops within large muscles, most often in the thigh, suggesting that the tumors develop from unidentified cell types of this tissue [12].

The present study is a first attempt to identify possible cells of origin for MLS/RCLS among human muscle-derived cell populations. With this aim, we isolated cells from normal human muscle tissues [121, 137], and used immunomagnetic methods to isolate satellite cells and SP cells with the antibody NCAM (CD56) [35]. The subpopulations were functionally tested by *in vitro* differentiation assays, and micro-array methods were employed to define myoblast- and SP-cell-specific expression patterns and compare them with those of MLS-derived cell lines.

Our results show a gene expression pattern of the MLS/RCLS cell lines that share features both with myoblasts and SP cells from this tissue compartment. Possible target cells could be, as yet uncharacterized, myoblast precursors, which remain NCAM negative. This would also be compatible with the capacity of the MLS/RCLS-specific fusion gene *FUS-DDIT3* to induce a liposarcoma phenotype when expressed in low-differentiated sarcoma cells.

5 CONCLUSIONS

In this thesis we have addressed the question of the tumor-type specificity of the fusion oncogene *FUS-DDIT3* and normal *DDIT3* in the context of liposarcoma.
Our results show that the genes *FUS-DDIT3* and *DDIT3* have instructive roles in the morphology of liposarcoma. They also explain why liposarcoma may arise in other compartments than adipose tissue.

Previous studies have shown peculiar pattern of growth controlling genes in MLS/RCLS. We have studied the expression of growth- and cell senescence associated proteins and concludes that MLS/RCLS tumors contain large subpopulations of senescent cells. We also show that expression of FUS-DDIT3 causes a slower growth in vitro and in vivo.

Human sarcomas can be divided into two main categories based on TP53 function leading to genetic complexity or stability. Here we conclude that most MLS/RCLS tumors have functional TP53 genes, which also explain their genetic stability and radiation sensitivity.

The majority of MLS/RCLS arise inside skeletal muscles and not in adipose tissue. This paradox is partially explained by the instructive role of the fusion oncogene. Recent studies suggest that mesenchymal stem cell may be the cell of origin for MLS/RCLS.
By comparative gene-expression analysis of muscle-derived normal cells with MLS tumor cells we conclude that MLS tumors may derive from an immature muscle precursor cell.

ACKNOWLEDGEMENT

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