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CICLO

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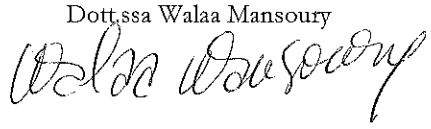
Exploring the role of PI3K/AKT signaling pathway in myxoid liposarcoma

DOTTORANDA

Dott.ssa Walaa Mansoury



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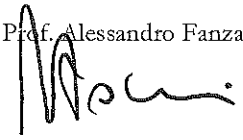

COORDINATORE DOTTORATO

Prof. Eugenio Monti

DEDICATION

In memory of those lost and those currently battling cancer.

To my parents and siblings for their unconditional love and support.

To my beloved Morocco and Italy and to our unbreakable bond.

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RIASSUNTO

Recentemente, è stato riportato che la riduzione dei livelli di proliferazione e di vitalità cellulare e la promozione dell'apoptosi sia in vitro che in vivo nel liposarcoma mixoide è il risultato dell'inibizione farmacologica della PI3K/AKT pathway (Trautmann,2019). Tuttavia, i meccanismi molecolari coinvolti non sono stati svelati fino ad oggi. Lo scopo di questo studio era di indagare su uno o più dei tanti meccanismi sottostanti. Per affrontare questa questione, abbiamo utilizzato la linea cellulare umana stabilizzata di liposarcoma mixoide MLS1765-92 caratterizzata con la variante rara tipo 8 dell'oncogeno *FUS-DDIT3*, ampiamente utilizzata nel campo della ricerca sul liposarcoma (Åman,1992). In seguito del blocco farmacologico della PI3K/AKT pathway trattando le cellule con il farmaco LY294002, un inibitore selettivo di PI3K, abbiamo osservato che i livelli di espressione della proteina Cav-1 erano sottoregolati, rivelandola come emergente downstream effettore modulando la PI3K/AKT pathway.

Il nostro gruppo ha precedentemente dimostrato che la Cav-1 svolge un ruolo di oncosoppressore in liposarcoma in condizioni indotte di differenziazione adipogenica in vitro, ma non abbiamo mai escluso un possibile ruolo di oncopromozione in contesto cellulare e condizioni sperimentali diversi (Codenotti,2016). Per approfondire la nostra comprensione dell'emergente PI3K/AKT/Cav-1 pathway e oncopromozione, abbiamo ipotizzato che la stimolazione con insulina possa sopraregolare i livelli di espressione della proteina Cav-1 e promuovere successivamente la proliferazione e la vitalità cellulare. Infatti, la stimolazione con insulina ha sopraregolato i livelli di espressione della proteina Cav-1 e ha aumentato la proliferazione e la vitalità cellulare, mentre l'esposizione al farmaco LY294002 ha abolito in modo significativo gli effetti indotti dall'insulina. Presi insieme, questi dati suggeriscono chiaramente l'esistenza di una collaborazione tra la PI3K/AKT pathway, la Cav-1 e la pathway dell'insulina e presenta la Cav-1 come oncopromotore nelle cellule proliferanti MLS1765-92 in risposta allo stimolo con insulina.

Nell'ottica del miglioramento della gestione del liposarcoma mixoide, abbiamo parallelamente investigato se la Cav-1, riportata sopraregolata in diversi tumori sottoposti alle radiazioni ionizzanti (Lin,2005), potrebbe essere coinvolta nella risposta biologica delle cellule MLS1765-92 alle radiazioni. L'analisi di immunoblotting ha dimostrato che i livelli di espressione della proteina Cav-1 erano sopraregolati in modo tempo-dipendente dopo aver somministrato una dose di 4Gy di radiazioni ionizzanti nelle cellule MLS1765-92. Questa osservazione indica che la Cav-1 potrebbe svolgere un ruolo protettivo in risposta agli attacchi ossidativi, come ad esempio le radiazioni ionizzanti tra altri (Codenotti,2021; Faggi,2014). Pertanto, per migliorare la risposta biologica delle cellule MLS1765-92 alle radiazioni, abbiamo ipotizzato che la PI3K/AKT pathway, una pathway chiave nella

radioprotezione in vari tumori maligni (Codenotti,2021), possa essere associata ai meccanismi di radioresistenza nel liposarcoma mixoide. Questa ipotesi è stata confermata come l'inibizione farmacologica della pathway PI3K/AKT utilizzando il farmaco LY294002 ha incrementato marcatamente la radiosensibilità delle cellule MLS1765-92. Questo risultato propone la PI3K/AKT pathway come un promettente bersaglio molecolare per aumentare la sensibilità del liposarcoma mixoide alle radiazioni ionizzanti, anche se sia segnalato in letteratura come più radiosensibile rispetto ad altri sarcomi dei tessuti molli, esibisce un fenotipo radioresistente nel modello cellulare MLS1765-92 (Rhomberg,2006).

In sintesi, i dati precedenti e attuali del nostro gruppo propongono la Cav-1 come un'arma a doppio taglio, agendo sia come oncosoppressore sia come oncopromotore seguendo il contesto cellulare e le impostazioni sperimentale ma anche come protettore contro le minacce dello stress ossidativo come ad esempio quelle indotte dalle radiazioni ionizzanti attraverso la modulazione della PI3K/AKT pathway nel liposarcoma mixoide. Le indagini sono in corso per la dissezione dei meccanismi molecolari che controllano l'onco-promozione e la radioprotezione che conferisci la Cav-1 tramite il coordinamento della PI3K/AKT pathway nel liposarcoma mixoide.

1. INTRODUCTION

1.1. Soft-tissue sarcomas

Soft-tissue sarcomas (STS) are rare and aggressive tumors arising from mesenchymal tissues, most commonly from the connective, muscle, adipose, neural, vascular, and lymphatic tissues (Figure 1) (Xiao,2013;Chen,2010), with a worldwide incidence of up to 3/100,000 new cases per year (Amadeo,2020). Frequent sites of occurrence include the trunk, the retroperitoneum, the head and neck, and the extremities being the most common site (Cormier,2004). Extremity STS patients typically present with a painless, enlarging mass. Core needle biopsy is the procedure of choice for establishing the tissue diagnosis. The literature describes more than fifty various types of sarcoma, and the terminology used for these rare tumors is varied and misleading. The most common STS include malignant fibrous histiocytoma, liposarcoma, leiomyosarcoma, and synovial sarcoma. STS develop in a centripetal fashion, around which a reactive pseudo-capsule is formed and consists of cellular debris, inflammatory cells, and potentially viable tumor cells. Sarcomas tend to compress nearby soft tissues and neurovascular structures as they enlarge, in contrast to carcinoma infiltrating the surrounding structures (Ramu,2017). STS are commonly aggressive neoplasms as they systemically spread, and recurrence can still occur after resection. Metastasis occurs mostly through an hematogenous dissemination to the lungs (Fong,1993). Angiosarcoma, epithelioid sarcoma, clear-cell sarcoma, synovial sarcoma, and rhabdomyosarcoma are the sarcoma subtypes most likely to spread lymphatically, representing approximately 5% of cases (Dim,2007; de Visscher,2006; Spillane,2000; Sbaraglia,2021). The American Cancer Society has reported radiotherapy (RT) used to treat other cancers, certain family cancer syndromes, damage/trauma of the lymphatic system, and exposure to toxic chemicals, as being risk factors for STS arising; however, multiple cases where patients have no apparent risk factors do exist.

Both inherited and acquired genetic aberrations affect the pathogenesis and the development of STS (Sbaraglia,2021; Strahm,2006). Chromosomal translocations have been described for different STS (Table 1) (Nakano,2018). These translocations are identified through cytogenetic analysis of the pathology specimens and can assist in diagnosis and provides prognostic information (Cormier,2004; Brennan,2018;Lazar,2006). Mutations in oncogenes and tumor suppressor genes also allow uncontrolled cell growth and are involved in the pathogenesis of STS (Sbaraglia,2021;Lazar,2006). The development of STS might occur when tumor suppressor genes *Rb* and *p53* are mutated. Li-Fraumeni syndrome, which is most often caused by inherited mutations of the *p53* gene, is characterized by a high incidence of STS and other malignancies (Sbaraglia,2021;Strahm,2006). Factors such as tumor size, depth, grade, histologic subtype, and stage are indicators of patient outcome, as well as patient age

and comorbidities informing the specific approach to management and patient outcome. Limb-sparing surgical resection is the mainstay of treatment. Radiotherapy (RT) is used for unresectable STS tumors and as a neoadjuvant or an adjuvant to surgery. The use of chemotherapy (CT) in STS is debated, and no conventional regimen has been established. Treatment of STS is complex and requires surgery and RT, with some patients demanding CT (Crago,2015;Dowli,2014).

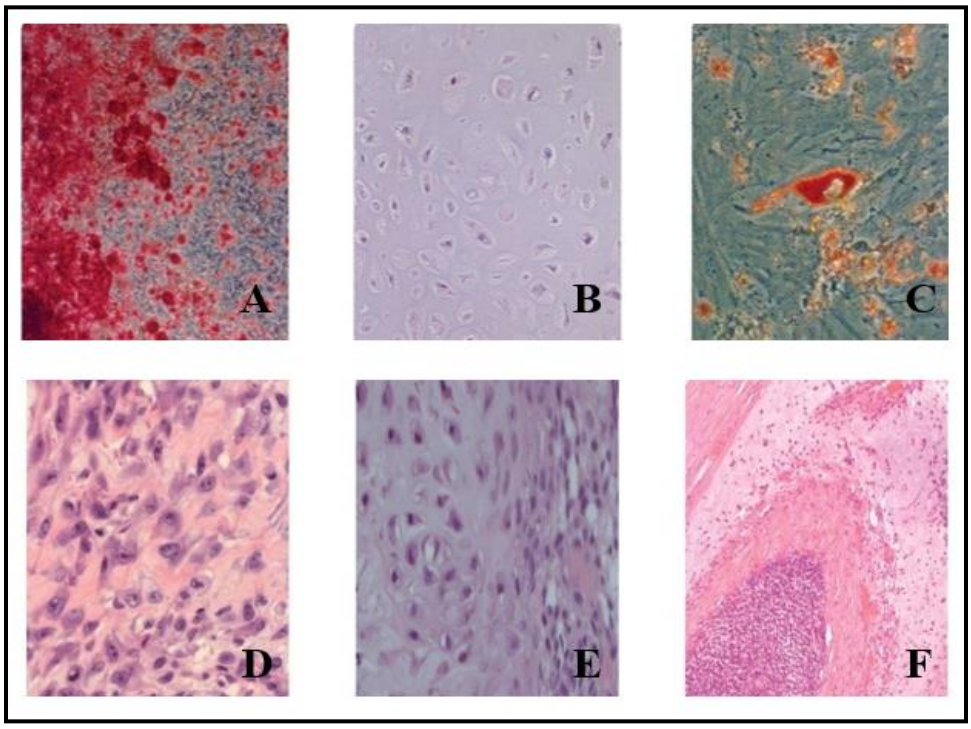

Figure 1. Neoplastic transformation of osteoblasts, chondrocytes and adipocytes into osteosarcoma, chondrosarcoma, and liposarcoma. *A: alizarin red staining for osteoblast differentiation, B: toluidine blue staining for chondrocyte differentiation, C: oil red staining for adipocyte differentiation. D: osteosarcoma, E: chondrosarcoma, F: liposarcoma. (Adapted from Xiao,2013)*

Soft-tissue Sarcoma	Translocation	Gene Product
Synovial sarcoma	t(X;18)	SYT-SSX
Myxoid liposarcoma	t(12;16)	TLS-CHOP
Ewing sarcoma/PNET	t(11;22)	EWS-FLI1
Alveolar rhabdomyosarcoma	t(2;13)	PAX3-FKHR

Table 1. STS-associated chromosomal translocations. *(Adapted from Nakano,2018)*

1.2. Liposarcoma

Liposarcoma (LPS), is a rare adipocytic neoplasm that involves deep soft tissues, including the esophagus, retroperitoneum, and popliteal fossa (Zafar,2020). LPS is the most common STS worldwide, according to the American Cancer Society (Gamboa,2020). LPS relative frequency of occurrence at different body regions depends on the LPS subtype. For instance, dedifferentiated liposarcoma (DDLPS) is much more common in the retroperitoneal areas, while myxoid liposarcoma (MLS) occurs in the lower extremities. LPS should be discriminated from benign lipoma. The cause of LPS is still under investigation (Crago,2016). The median age at diagnosis is 50 years, although cases of pediatric onset have been reported. There is no significant association with race or gender. A slight male predominance has been reported in some studies (Vocks,2000).

LPS is a complex malignancy, and its pathophysiology can be best explained by subdividing it into three subtypes (according to the World Health Organization classification of 2002). These include well-differentiated and dedifferentiated liposarcoma (WDLPS/DDLPS), myxoid and round cell liposarcoma (MLS and RCL), and pleomorphic liposarcoma (PLS) (Bill,2016). WDLPS/DDLPS are characterized by distinctive ring or giant marker chromosomes, including MDM2 and CDK4 genes amplification, leading to abnormal cell growth. For MCL and RCL, reciprocal translocation occurs between chromosomes 12 and 16, leading to the fusion of *DDIT3* (also referred as *CHOP*) and *FUS* genes that subsequently repress downstream target genes such as *PPAR-gamma2* and *C/EBP-alpha*, disrupting adipogenesis and promoting cell proliferation (Conyers,2011). PLS is the most rare and complex LPS subtype due to highly complex chromosome alterations. Tumor-suppressor genes such as *p53, NF1, RB1*, when mutated, lead to many chromosome gains, and determine the aggressiveness of this tumor (Table 2) (Keung,2019). Few studies were dedicated to PLS making it the least understood among the three LPS subtypes (Wan,2021).

Most LPS patients are asymptomatic. Symptoms develop only when the tumor grows large enough to exert a painful mass effect on surrounding structures. Other reported symptoms include paresthesia, varicose veins, fatigue, weight loss, nausea, and vomiting (Dowli,2014;Garcia,2004). Computed tomography scan and magnetic resonance imaging can assist in detecting lipomatous components. High fat percentage is associated with benign lipoma, while lower fat percentage is consistent with atypical lipoma or sarcoma. Special staining procedures are used to distinguish LPS from other STS. The mainstay of treatment is surgical excision (Lin ZC,2015). Wide and deep resection, adjuvant radiotherapy (RT), thought to decrease the rate of recurrence, and/or chemotherapy (CT) may be necessary for high-grade lesions. However, the role of adjuvant CT and RT remains controversial, and long-term follow-up is recommended due to the high rate of recurrence (Dowli,2014). Tumor site, size,

depth, histologic subtype, grade, and resection margin status determine LPS prognosis that remains poor even after complete removal of the LPS mass (Riva,2016). Low-grade WDLPS has a 5-year survival rate of 83%, while it is estimated at 20% for the high-grade DDLPS variant (Fabre-Guillevin E,2006). To date, the progress of LPS research is compromised by the scarcity of cancer models reproducing tumor growth and metastasis (Codenotti,2019).

Liposarcoma subtype	**Genomic alterations**	**Affected oncogenes**	**Grade**
Well-differentiated	12q13-15 amplification	*MDM2, CDK4*	low
Dedifferentiated	12q13-15 amplification 3p14-21 loss 11q23-24 loss 19q13 loss	*MDM2, CDK4*	high
Myxoid	FUS-DDIT3 Translocation	Unknown	low
Round	FUS-DDIT3 translocation	Unknown	high
Pleomorphic	Rb/p53 loss	Rb, p53	high

Table 2. Genetic alterations and correlated clinics in LPS. *(Adapted from Keung,2019)*

1.3. Biology and management of myxoid liposarcoma

Myxoid liposarcoma (MLS) is the second most frequent LPS subtype after WDLPS/DDLPS subtype, approximately accounting for one third of all LPS, 5-10% of all adult STS, and around 20% of all adipocytic neoplasms (Fletcher,2014). In most cases, MLS arises in younger adults below the age of 20 years. Morphologically, MLS shows a broad set of subtypes ranging from paucicellular myxoid tumors to hypercellular, round-cell high-grade sarcomas associated with higher aggressiveness (Antonescu,2001).

Genetically, the majority of MLS are characterized by a t(12;16)(q13;p11) chromosomal translocation that fuses the *FUS* gene to the entire coding sequence of *DDIT3* gene. Twelve *FUS-DDIT3* variants have been reported in the literature as a result of different breakpoints. The exon structure of each of the different *FUS–DDIT3* fusions is shown in Figure 2 (Powers,2010). Exons that are only partially retained are indicated by white areas in the rectangles. The most common variant (Type 2) occurs in 65% of cases, and fuses *FUS* exon 5 to *DDIT3* exon 2 (5-2). The first three fusion types (Types 1, 2 and 3) together represent 96% of all reported cases in a meta-analysis (Powers,2010). The resulting FUS-DDIT3 fusion protein, which acts as a transcriptional (dys-) regulator, has been shown to play an essential role in MLS pathogenesis, but its mechanism of action is still ununderstood completely (Kuroda,1997; Pérez-Losada,2000;Engström,2006;Riggi,2006).

Clinically, MLS are highly recurrent and metastases develop at 40% of patients (Dei Tos,2014). Extrapulmonary metastases, including the trunk and extremities, have a high incidence rate of 87% (Haniball,2011). The prognostic factors related to MLS metastasis are still unclear. Clearing a treatment approach for metastatic MLS has yet to be established (Spillane,2000). Long-term survival in MLS patients may be achieved through radical surgery and adjuvant radiotherapy (RT) and/or conventional chemotherapy (CT) based on anthracyclines and ifosfamide, recently supplemented by agents such as trabectedin or eribulin. Although MLS exhibits a higher sensitivity to cytotoxic agents than other LPS subtypes, patients with metastatic disease are usually incurable and CT is generally administered with palliative intent, highlighting the pressing need for identifying novel molecular targets and developing new treatment strategies (Grosso F,2007;Ratan,2016;Schöffski,2016; Jones RL,2005).

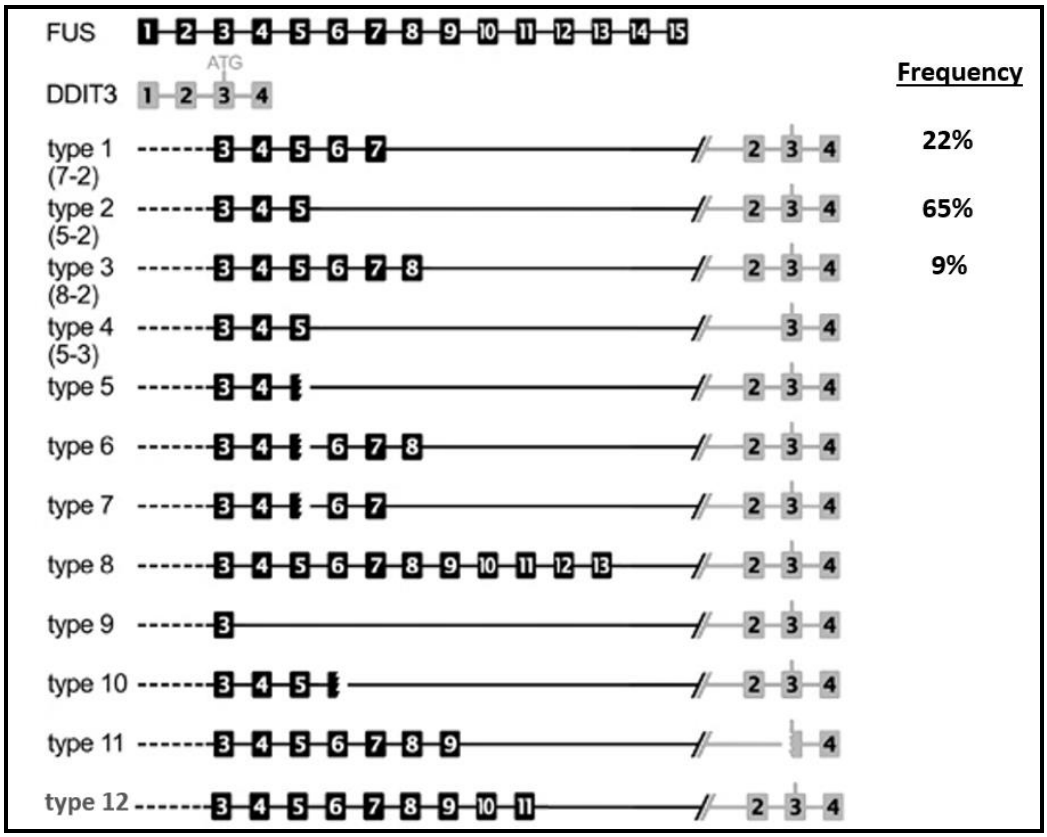

Figure 2. Fusion variants of FUS-DDIT3 fusion gene product. *(Adapted from Powers,2010)*

1.4. PI3K/AKT signaling pathway in myxoid liposarcoma

Insulin and growth factors are extracellular stimuli that bind to the extracellular region of corresponding tyrosine kinase receptors (RTKs) inducing their phosphorylation. Once activated, RTKs trigger the activation of key molecular proteins that end up building highly interconnected and complex pathways networks, such as the PI3K/AKT signaling pathway, controlling various cell processes (Schlessinger,2014).

Phosphoinositide 3-kinase (PI3K) protein is recruited to the plasma membrane upon phosphorylation of RTKs triggered by insulin and many other growth factors. Under PTEN (phosphatase and tensin homologue deleted on chromosome 10), a negative control of PI3K, PI3K activates AKT protein, that then triggers a cascade of responses, such as survival, growth and proliferation of cancer cells by a direct phosphorylation of many intracellular proteins such as the mammalian target of rapamycin (mTOR), a downstream effector of AKT (Figure 3). For these reasons, pharmacological targeting of upstream or downstream of the PI3K/AKT signaling axis constitute an anticancer therapeutic strategy

since in many cancer cells, the PI3K/AKT signaling network happens to get aberrantly disrupted, going out of control and giving rise to many cancers, including several soft-tissue-sarcomas subtypes (Demicco,2012). Aberrant AKT activation led to well-differentiated liposarcoma development in a zebrafish model (Gutierrez,2011). In a set of murine liposarcoma xenografts, exposure to either rapamycin, a mTOR inhibitor, or in combination with sorafenib, a multi-kinase inhibitor, correlated with increased lipid component and a more differentiated phenotype (Smith,2013).

Myxoid liposarcoma (MLS), an adipocytic neoplasm characterized by the fusion oncogene *FUS-DDIT3*, was reported to be presenting additional genetic abnormalities such as mutations in *PI3KCA*, *AKT1,* loss of *PTEN*, an overactivation of the insulin-like growth factor I receptor (IGF1R) and SRC signaling, that were associated with poor clinical outcomes (Trautmann,2019;Sievers,2015). Besides the fusion oncogene *FUS-DDIT3* characterizing MLS specimens and cell lines, activated PI3K/AKT signaling pathway was reported to be essential to MLS tumorigenesis. Compounds inhibiting one or more PI3K/AKT signaling pathway components decreased cell proliferation and cell viability and increased cell apoptosis both in vitro and in vivo (Trautmann,2019).

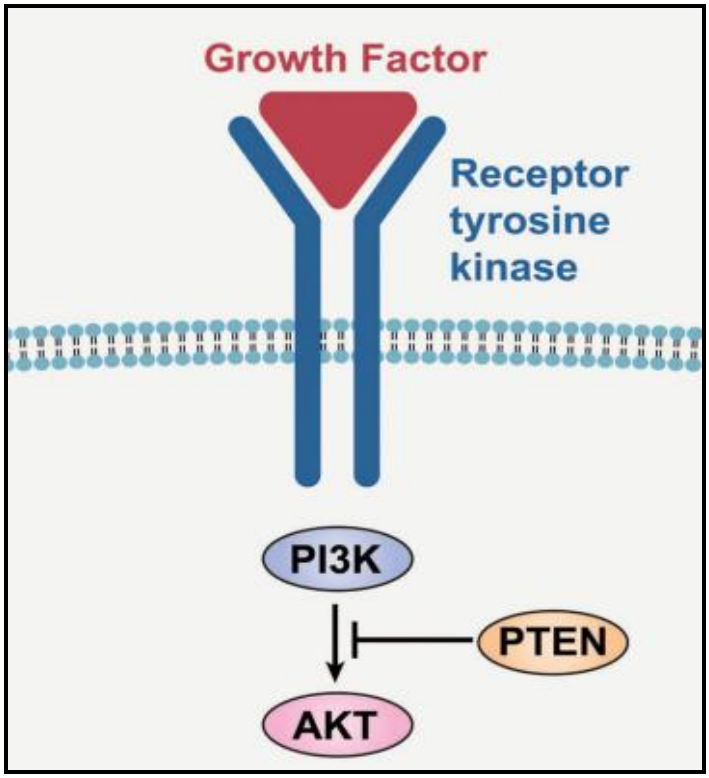

Figure 3. PI3K/AKT pathway*. (Adapted from Demicco,2012)*

1.5. Caveolae, caveolins, and cavins

1.5.1. Caveolae

Caveolae are one of the most abundant and fascinating structures of the plasma membrane (PM) of many mammalian cells. These single 50-100 nm flask-shaped invaginations of the PM, identifiable only by electron microscopy, were first visualized by G.E. Palade in 1953, then described and entitled "Caveola intracellularis" by E.Yamada in 1955. However, it took almost 40 years to identify caveolins, the main proteins conferring this singularized PM domains. In many tissues such as liver, neurons, and the kidney proximal tubule, caveolae are almost detectable while being remarkably dense in other tissues such as adipocytes and skeletal muscle, covering up to 50% of the total cell surface area. The membrane of caveolae are cholesterol-enriched with certain sphingolipids. Caveolae present also under complex structures reuniting a fistful of caveolae baptized as rosette-like structures (Figure 4) (Filippini,2020;Parton,2018).

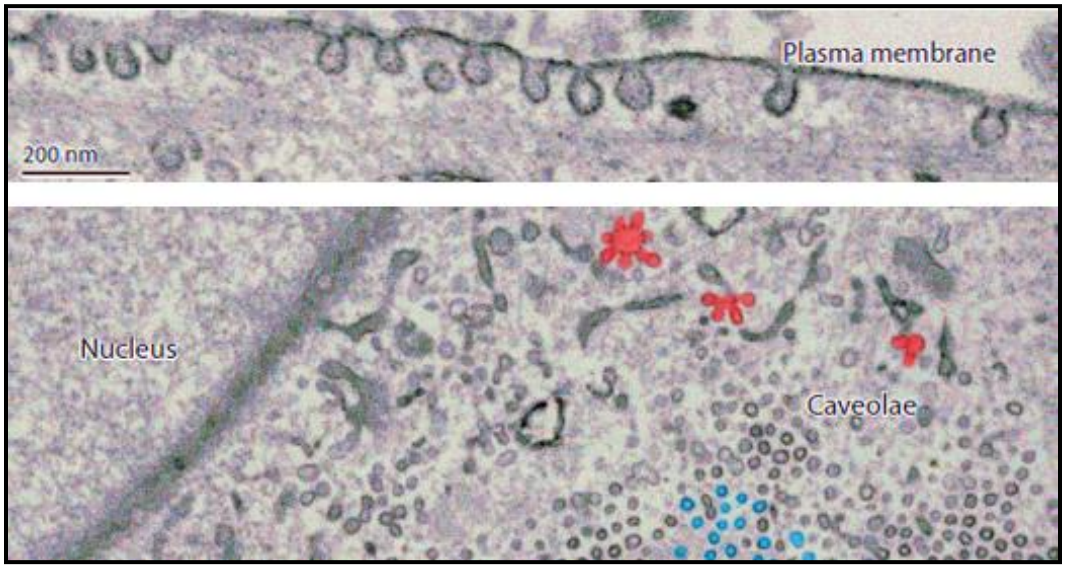

Figure 4. Transmission electron micrographs of caveolae architecture in murine 3T3-L1 adipocytes. *Single pits caveolae (blue-highlighted). Rosette-like caveolae (red-highlighted). (Adapted from Parton,2018)*

1.5.2. Caveolins and cavins

There are three mammalian caveolin genes: caveolin-1, caveolin-2 and caveolin-3. Smooth muscle expresses all three isoforms, whereas skeletal and cardiac muscle tissues express only the caveolin-3 isoform. Caveolin-1 (Cav-1) and caveolin-2 (Cav-2) isoforms are also expressed by non-muscle cells. Caveolae formation is strictly dependent on caveolin-1 or caveolin-3 (Cav-3), depending on

the tissue. Cav-2 appears to contribute to caveolae formation in some cell lines but is expendable in vivo (Figure 5) (Parton,2018). Some of the functions assigned to caveolin may not be related to caveolae that directly regulate numerous signaling pathways as caveolins also localize outside of caveolae (Williams,2004). Although the ability of caveolae to endocytose is still debated, numerous studies clearly show translocation of Cav-1 from the PM to internal compartments. The literature also suggests that cell detachment or harsh treatments, such as phosphatase inhibition, induce caveolae to translocate towards the endomembrane system, mainly in the form of clusters or rosettes (Figure 4) (Parton,2018). A second family of proteins, the cavins (a family of four proteins), has recently been shown to be important in caveolae formation (Figure 5). Reduction in the levels of Cavin-1 (also known as PTRF), Cavin-2 (SDPR) or Cavin-3 (SRBC) correlates with reduced caveolae density. Cavin-4 (MURC) is expressed predominantly in muscle and is probably a caveolae component, but its role is yet to be clarified. Although few studies have examined the role of cavins, direct or indirect interaction between cavins and caveolins appears to be important for caveolae formation, stability and possibly trafficking (Parton,2018).

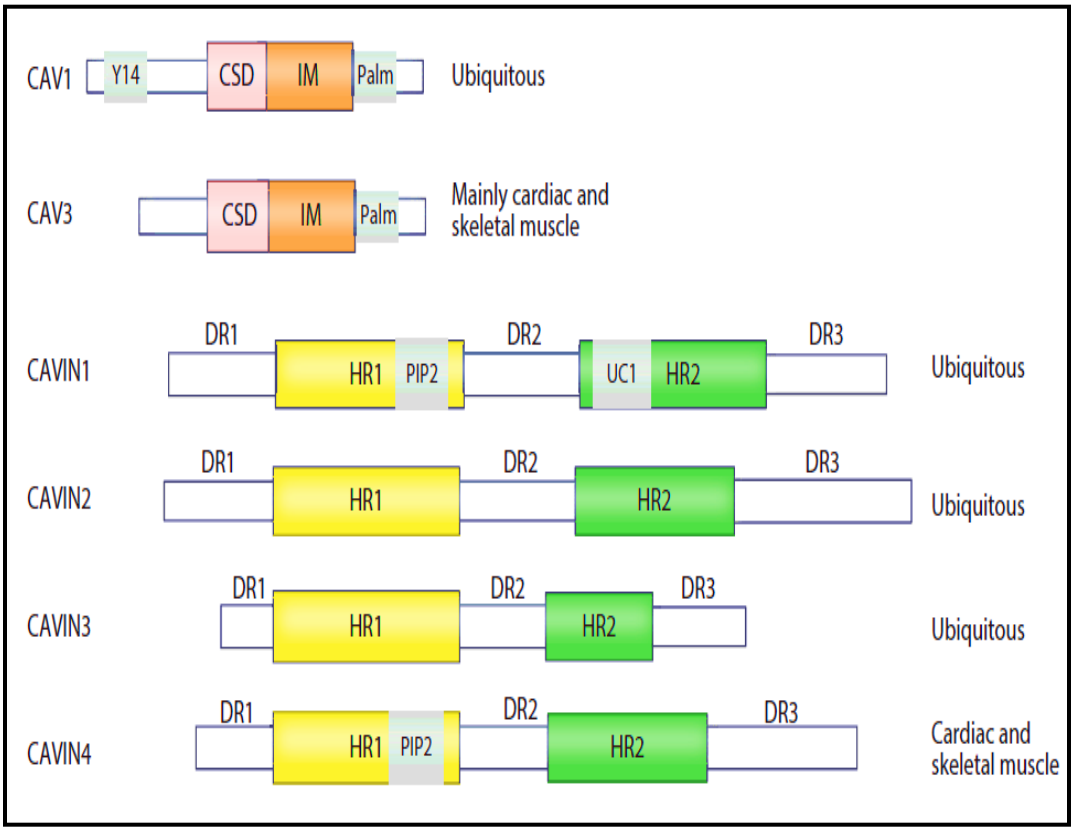

Figure 5. Domains and expression tissue of caveolins and cavins. *CSD, caveolin scaffolding domain; IM, intramembrane domain; DR, disordered region; HR, helical region; Palm, palmitoylation sites; PIP2, PtdIns(4,5)P2 binding site; UC1, undecad repeat domain. (Adapted from Parton,2018)*

1.5.3. Caveolins and cavins in liposarcoma

Previously, extensive microarray data and immunohistochemistry analyses of our group revealed a possible tumor-suppressor function of Cav-1 along with Cav-2, Cavin-1, and Cavin-2 in LPS as the expression levels of these proteins tend to be upregulated in differentiated states compared to proliferating ones. The four proteins co-express in all LPS subtypes, the highest expression being recorded in well-differentiated liposarcoma (WDLPS), the least aggressive LPS variant (Figure 6). Immunoblotting and immunoprecipitation analyses data were in line with previous findings; using the two literature widely used MLS cell lines MLS1765-92 and MLS402-91 as in vitro LPS cell models, we demonstrated that Cav-1, Cav-2, Cavin-1, and Cavin-2 are barely expressed during proliferation while being markedly incremented during induced adipogenic differentiation (Figure 7). Positive adipogenic differentiation was witnessed by murine pre-adipocytes 3T3-L1 maturation into adipocytes, highlighted by pronounced lipid droplets accumulation following oil red O staining procedure (Figure 8). Furthermore, immunofluorescence analysis revealed that Cav-1, Cav-2, Cavin-1 and Cavin-2, co-agglomerating at the PM (Figure 9,Figure 10), are reliable markers for differential diagnostic of LPS tumors exhibiting differentiated phenotypes (Codenotti,2016;Codenotti,2017).

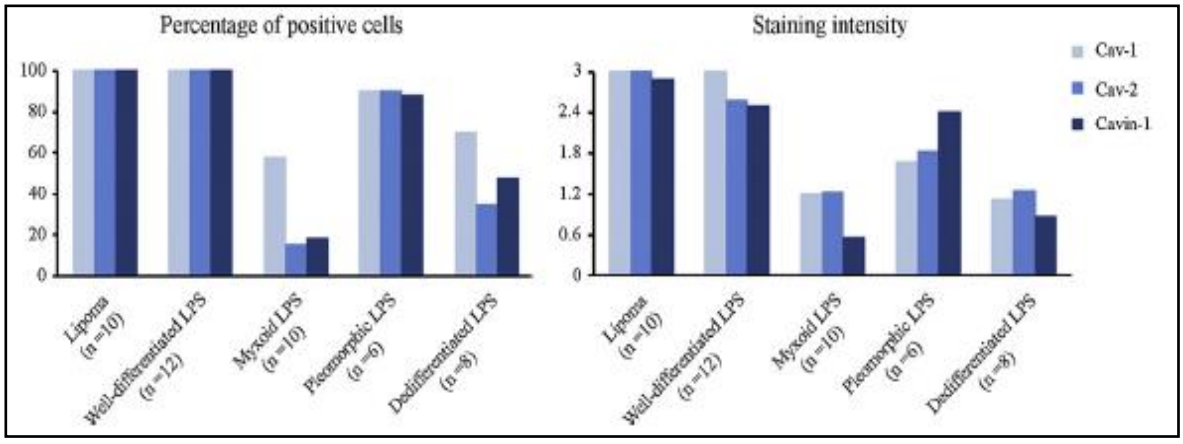

Figure 6. Immunohistochemical analysis of human LPS malignancies. *The four LPS variants were compared to benign lipoma. (Adapted from Codenotti,2016)*

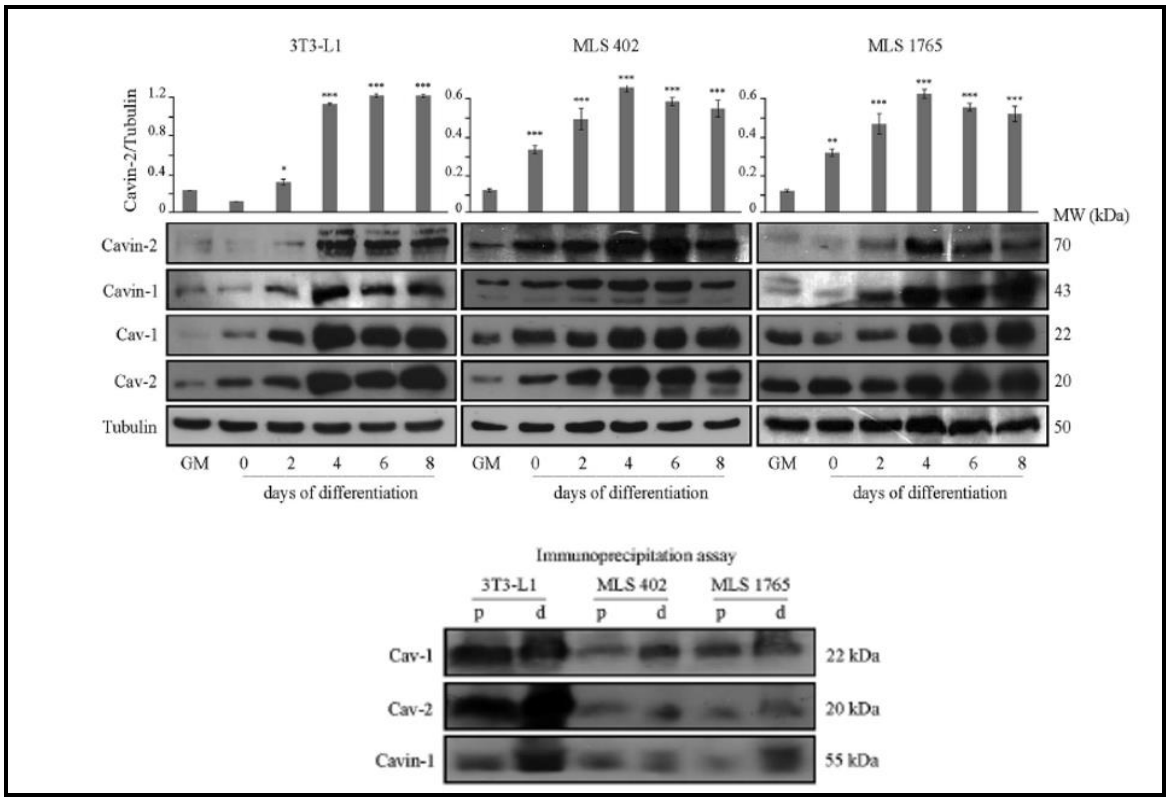

Figure 7. Immunoblotting and immunoprecipitation analyses of human myxoid liposarcoma MLS1765-92 and MLS402-91 cell lines and murine 3T3-L1 fibroblasts. *(Adapted from Codenotti,2016)*

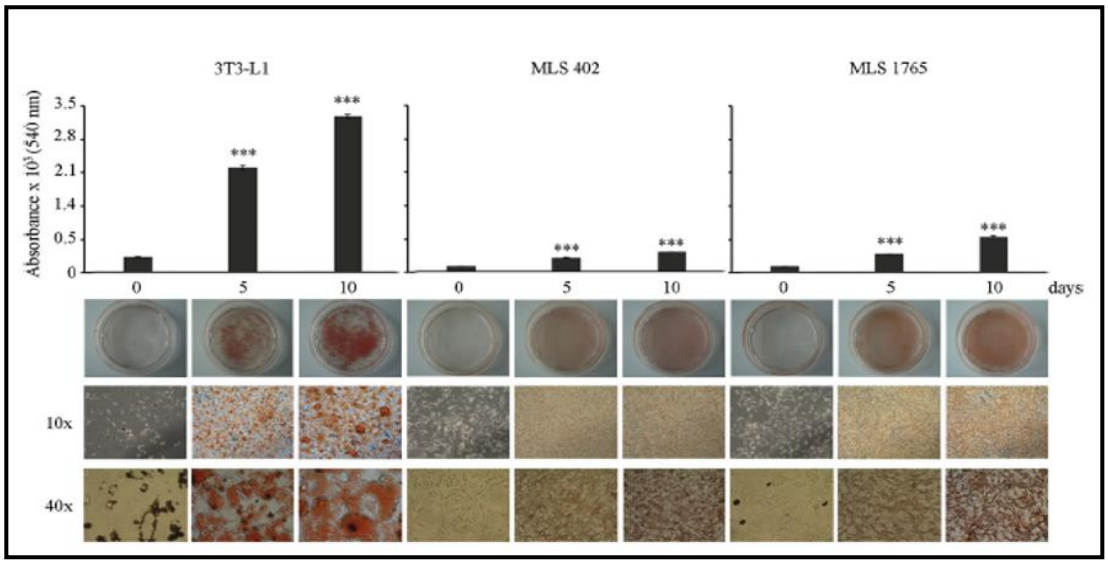

Figure 8. Oil red O staining of human myxoid liposarcoma MLS1765-92 and MLS402-91 cell lines and murine 3T3-L1 fibroblasts. *(Adapted from Codenotti,2016)*

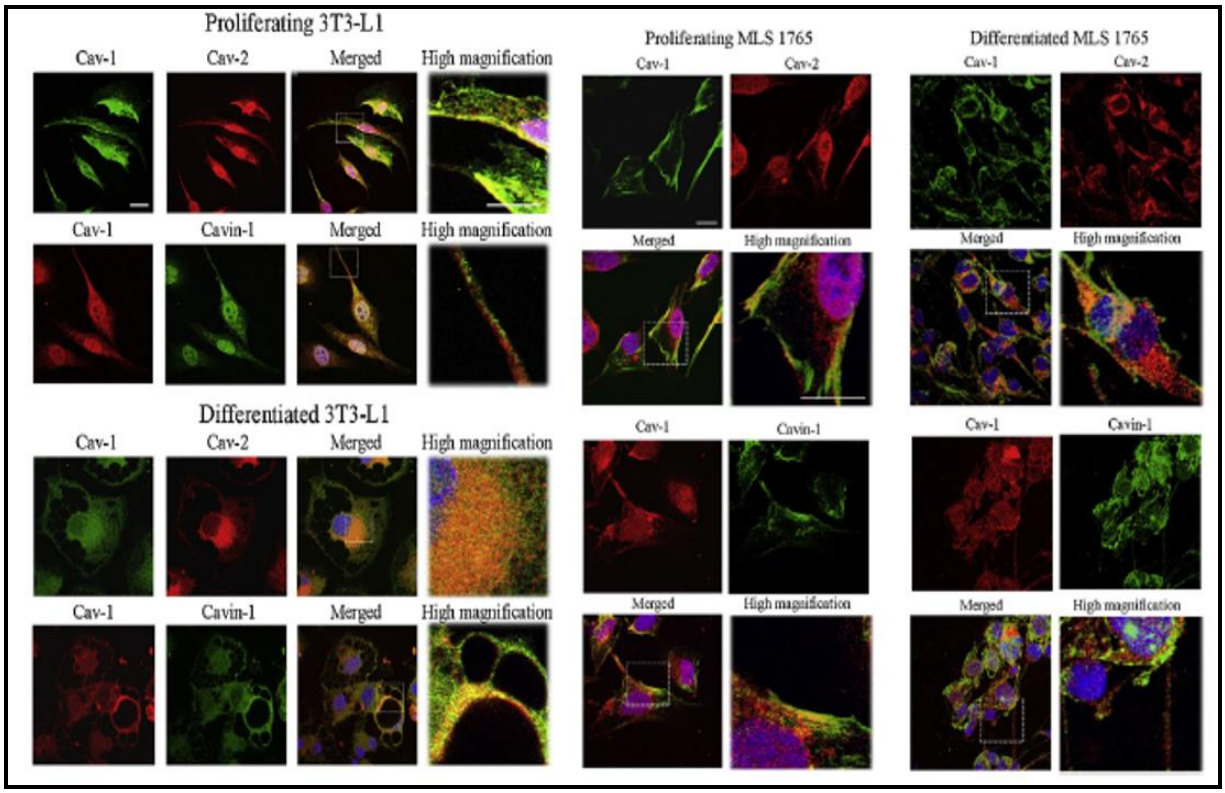

Figure 9. Immunofluorescence analysis of human myxoid liposarcoma MLS1765-92 and MLS402-91 cell lines and murine 3T3-L1 fibroblasts. *(Adapted from Codenotti,2016)*

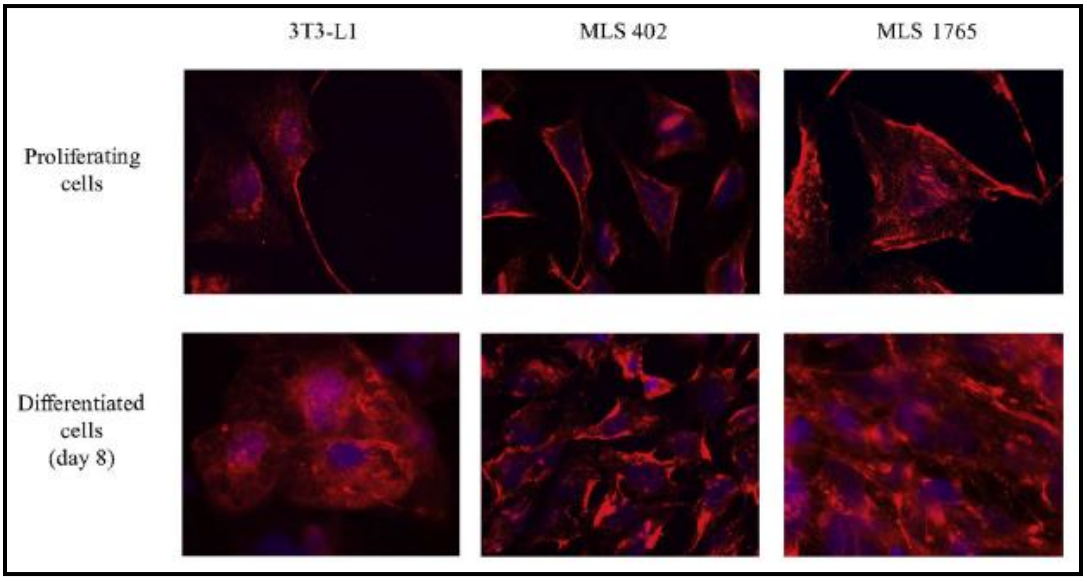

Figure 10. Immunofluorescence analysis of Cavin-2 protein in human myxoid liposarcoma MLS1765-92 and MLS402-91 cell lines and murine 3T3-L1 fibroblasts. *(Adapted from Codenotti,2017)*

1.6. Caveolin-1 in cancer Caveolin-1

Cav-1 gene is located on chromosome 7 (locus 7q31.1) and includes 3 exons: 30 bp, 165 bp and 342 bp, respectively, and two introns of 1.5 kb and 32 kb (Figure 11.A). This palmitoylated 22-kDa membrane-associated protein in caveolae has two isoforms, a full-length alpha-isoform composed of 178 amino acids, and the beta-isoform truncated by 32 amino acids at the N-terminus. These isoforms derive from two divergent translation initiation sites of the same mRNA. Suggested structure of caveolin-1 protein consists of hydrophobic amino acids stretch forming membrane spanning hairpin loop structure (amino acids 102-134), whereas both C- and N-termini are facing the cytosol. Additional structural domains are an oligomerization domain (amino acids 61 – 101) and the scaffolding domain (amino acids 82 – 101). C- and N-terminal membrane attachment domains and three palmitoyl groups at the C-terminus together with the transmembrane domain mediate attachment of Cav-1 to the PM. Phosphorylation on serine-80 residue turns caveolin-1 into the secreted protein. Tyrosine-14 is a major phosphorylation site of a-caveolin-1 upon oxidative stress and growth factor stimulation. The point mutation in the membrane-spanning domain, converting proline-132 to leucine, leads to mis-localization and intracellular retention of endogenous Cav-1 and behaves in a dominant-negative fashion (Figure 11.B) (Razani,2002).

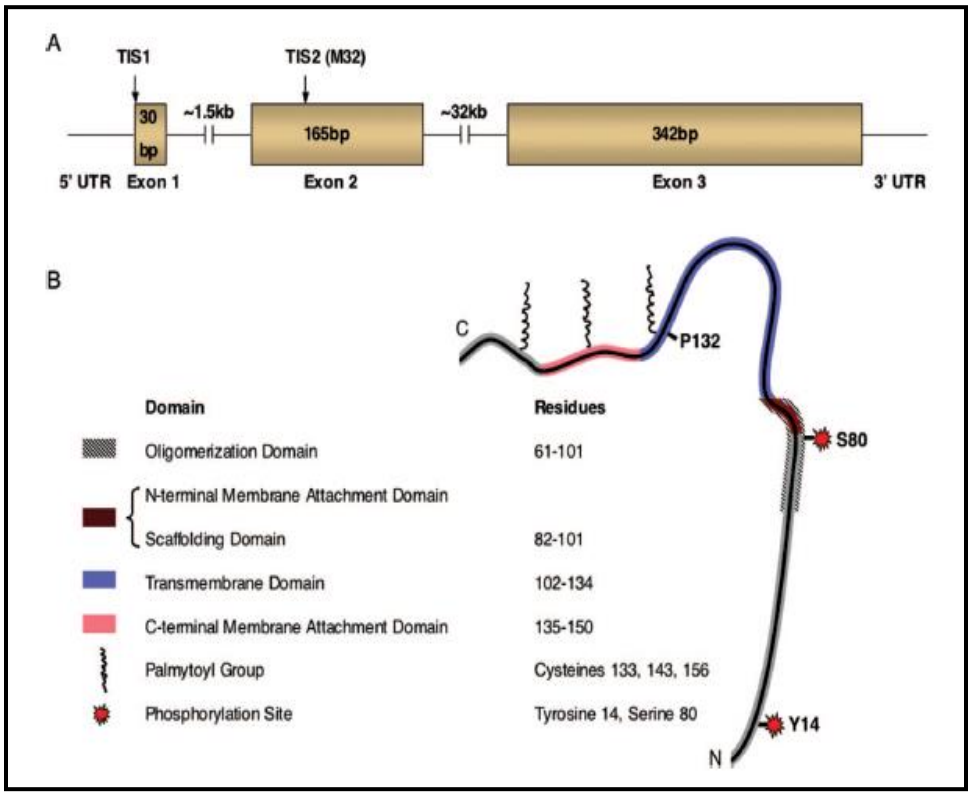

Figure 11. Representation of Caveolin-1 gene (A) and protein (B). (*Razani,2002)*

1.6.1. Caveolin-1, a double-edged sword in cancer

Cav-1 has been described as a two-sided protein functioning both as a tumor-suppressor and as a tumor-promoter depending on differentiation state and cancer stage (Williams,2004). Poorly differentiated grade malignancies and advanced tumors record Cav-1 expression levels as being higher compared to benign tissue and well-differentiated grade tumors linked to poor prognosis. Increasing evidences suggests that the higher Cav-1 levels are, the more aggressive the phenotype gets (Razani,2001).

In numerous cancers, Cav-1 is positively correlated to metastatic potential. In breast cancer, Cav-1 was reported as a tumor suppressor as its downregulation promoted cell proliferation by enhancing Ca^{2+}-activated potassium channel whose encoding gene is involved in malignancy, therefore accelerating tumorigenesis in MCF-7 cells (Du,2014), whereas parenchymal Cav-1 was shown to be acting as a tumor promoter by promoting EGFR binding to the kinase region of caveolin-binding motif, thus activating EGFR-mediated mitosis (Liang,2018). Previous work from our lab has demonstrated that embryonal rhabdomyosarcoma (ERMS) RD cells overexpressing Cav-1 acquired a higher metastatic potential. We therefore concluded that Cav-1, tightly coordinating with ERK pathway, promotes metastasis of ERMS both in vitro and in vivo (Codenotti,2019).

Beside its role as tumor promoter, Cav-1 also fills a tumor suppressor position. The genes encoding for Cav-1 are located on a putative tumor suppressor locus. Indeed, loss of Cav-1 in several tumor cells seems to induce a hyper-proliferative state, promoting cell proliferation, angiogenesis, and tumor metastatic progression, indicating that dysregulated Cav-1 is what confers the transformed phenotype. Indeed, Cav-1 levels are downregulated in many primary human cancers and cancer cell lines and in cells transformed by oncogenes such as *Bcr-Abl* and *Myc*. Under in vitro adipogenic differentiation conditions, our group has previously established a tumor-suppressor role of Cav-1 in liposarcoma (Codenotti,2016).

In summary, Cav-1 may act as a tumor suppressor protein at early stages of the malignant transformation process (Razani,2001) while in later tumor stages, a re-expression or rather upregulation and phosphorylation of Cav-1 seems to support invasion and the survival of therapy-resistant transformed tumor cells (Codenotti,2021;Codenotti,2016;Faggi,2014).

1.6.2. Caveolin-1, a therapy-resistant target in cancer

Cav-1 expression is modulated in cancer cells following exposure to cytotoxic drugs and ionizing radiation (Lin M,2005). In a previous report, we demonstrated that Cav-1 overexpression contributes to chemoresistance, whereas Cav-1 pool depletion sensitizes rhabdomyosarcoma (RMS) cells to chemotherapy drug-induced apoptosis (Faggi,2014).

Cav-1 expression was induced in human pancreatic carcinoma cell lines PATU8902, MiaPaCa2 and Panc1 following 24 hours exposure to ionizing radiation. The three cell lines were significantly sensitized to radiation after Cav-1 was knocked-down (Cordes,2007). In agreement with several reports suggesting Cav-1 as a radiotherapy-resistant target, we recently demonstrated that Cav-1 promotes radioresistance in RMS. This conclusion was made after phosphorylated levels of Cav-1 (pCav-1) on tyrosine kinase 14 were shown to be markedly increased in our embryonal rhabdomyosarcoma (ERMS) metastatic RD and alveolar rhabdomyosarcoma (ARMS) RH30 cell lines models overexpressing Cav-1 and exposed to ionizing radiation, and in RD and RH30 in vitro established radioresistant (RR) cell lines. Cav-1 was shown to be counteracting radiation-induced cell cycle arrest in the G2/M phase and protecting against cell senescence and apoptosis. Cav-1 was also demonstrated to be enhancing survival of post-irradiated cells by increasing DNA double strand breaks (DSB) repair. Increased levels of pCav-1 were correlated with incremented levels of phospho-AKT and antioxidant enzymes glutathione (GSH) activity and catalase expression in both RD and RH30 cells overexpressing Cav-1 and in RD-RR and RH30-RR cell lines. Src-kinase and AKT inhibition using PP2 and LY294002, respectively, sensitized RD and RH30 cell lines overexpressing Cav-1 and RD-RR and RH30-RR to ionizing radiation. That being said, Cav-1 levels upregulation might occur as an early event in cellular stress response and is consistent with its proposed cell protective function in terms of the acquisition and/or maintenance of the radiation-resistant phenotype under stressful conditions (Codenotti,2021). Supporting in vitro observations, Cav-1 null mice demonstrated increased sensitivity to whole body gamma-radiation (Li,2005).

1.6.3. Caveolin-1 in cancer metabolism

1.6.3.1. Caveolin-1/insulin signaling axis and glucose uptake in cancer

Benign cells switch into malignant phenotype through a highly complex process, including initiation, promotion, and progression. Cancer is initiated when DNA of cells is damaged following exposure to carcinogens, then initiated cells undergo growth stimulation before

acquiring aggressiveness and metastatic potential fed by angiogenesis (Pitot,1993). Metabolic altered states such as obesity and diabetes type 2, when combined with unhealthy diet and physical inactivity, appear to worsen cancer development (Giovanucci,2010).

Glucose is the preferred source of energy of mammalian cells and maintenance of optimal glucose levels by insulin is critical for survival under normal conditions (Kim,2018). Glucose uptake into skeletal muscle and adipose tissue requires the trafficking of vesicles transporting glucose transporter-4 (GLUT4) from the intracellular pool compartments to the plasma membrane (PM). Trafficking of vesicles transporting GLUT4 is initiated via the canonical insulin signaling cascade in skeletal muscle and adipocytes, as well as via exercise-induced contraction in myocytes (Tunduguru,2017). The insulin receptor, present on the PM of cells, is a tyrosine kinase that is activated upon insulin binding, leading to the tyrosine phosphorylation of a specific set of substrate proteins. In many cell types, insulin acts as a stimulus like other growth factors and thus shares several signaling pathways with these growth factors. In adipocytes, insulin increases glucose internalization and metabolism to hundreds of folds, whereas growth factors stimuli such as epidermal growth factor (EGF) and platelet derived growth factor alpha (PDGF1) remain ineffective on these processes (Mastick,1997). Insulin signaling plays a leading role in patients with diabetes and cancer. Increased cancer mortality was associated with diabetes (Giovanucci,2010). Antidiabetic drugs such as metformin were shown to be protective against certain cancers and possesses anti-cancer properties (Beck,2010). Previous reports showed that insulin signaling was associated with cancer cell survival and mitosis through triggering insulin receptor substrate 1 (IRS-1) that activates in turn PI3K/AKT signaling and extracellular-signal regulated kinase (ERK) signaling pathways (Lau,2012;Saltiel,2002).

Cancer cells usually grow quickly, multiplying at a fast rate, which consumes a lot of energy, meaning that their craving not only for glucose but also for other nutrients such as amino acids and fatty acids is drastically increased compared to relatively normal cells (Zhu,2019). To self-provide energy, cancer cells undergo preferably aerobic glycolysis that consists of catabolizing high levels of glucose and secreting lactate in the presence of oxygen, known as the “Warburg effect” (Potter,2016).

Glucose/insulin signaling is a complex and highly regulated cellular process that involves collaboration of many key effectors (Boucher,2014). Among these, Caveolin-1 (Cav-1) reported as a modulator of glycolytic processes (Nwosu,2016). Cav-1, the main structural and functional protein component of caveolae, mediates the insulin signaling pathway by directly

interacting with the β-subunit of the insulin receptor (IR) and increasing insulin-mediated phosphorylation of IR substrate 1 (IRS-1). Cav-1 knockout mice display insulin resistance and defective expression of IR protein in adipose tissue (Mastick,1997). Cav-1 has also been suggested to be mediating GLUT4 insertion within the PM. Cav-1 has also been linked to GLUT4 endocytosis, via two pathways, one clathrin-dependent and one dependent on cholesterol-enriched domains in adipocytes (Shigematsu,2003). Mastick *et al.* demonstrated that insulin specifically stimulates the phosphorylation of Cav-1 on tyrosine 14 in murine 3T3-L1 adipocytes. This phosphorylation involves the activation of a tyrosine kinase downstream of the IR. Even though both preadipocytes and adipocytes are expressing Cav-1 and active IR, the tyrosine phosphorylation of Cav-1 was differentiation-dependent as it was detected only in fully differentiated adipocytes (Mastick,1997).

In advanced colon cancer, high Cav-1 expression increased glucose uptake and ATP production by activating glucose transporter-3 (GLUT3) transcription, whereas loss of Cav-1 reduced cellular glucose uptake and lactate output (indicative of abolished Warburg effect), reduced intracellular ATP, and triggered autophagy through AMPK-p53 signaling activation (Nwosu,2016). In breast cancer-associated fibroblasts, caveolin-1 knockdown leads to oxidative stress activating HIF-1 and inducing glycolysis (Pavlides,2010).

1.6.3.2. Caveolin-1 and fatty acids uptake in cancer

When glucose levels drop low, fatty acids (FA) are utilized as an alternative source of energy by cells (Kim,2018). For instance, Cav-1 was reported, among many other effectors, as a key modulator tightly controlling the metabolism of fatty acids in both normal and cancer cells. Fatty acid synthase (FASN) is a crucial enzyme in FA biosynthesis catalyzing the formation of palmitate from acetyl-Coenzyme A. When dysregulated, FASN is correlated to tumorigenesis in diverse malignancies and then represent an attractive drug target in cancer. In melanoma and prostate cancers, Cav-1 and FASN are co-regulated, therefore their concomitant expression may pronounce tumorigenesis. One possible explanation could be palmitoylation, a post-translational event, in which long chain FA, mainly palmitate, bind to cysteine fragments in proteins via a thio-ester bond. Cav-1 endures palmitoylation of cysteine residues which influence the control of membrane interaction, stability, spatial configuration, and intracellular traffic. It is likely that Cav-1 and FASN co-expression reports a cooperation mechanism by which FASN provides FA availability required to palmitoylation process, while Cav-1 requires

palmitoylation of its cysteine residues to maintain membrane integrity of cancer cells. Cav-1 depletion was reported to compromise fatty acid-binding protein 7 (FABP7) that binds and facilitates long-chain FA uptake (Di vizio,2008).

2. THESIS STATEMENT

Our group has previously demonstrated a tumor suppressor function of Cav-1 in liposarcoma (LPS) (Codenotti,2016). We further characterized a likely role of Cav-1 in promoting myxoid liposarcoma (MLS) employing MLS1765-92 cell line, a human MLS established cell line extensively investigated in LPS research (Åman,1992). Indeed, Cav-1 appeared to be promoting MLS1765-92 cell proliferation and cell viability in response to insulin triggering as Cav-1 protein expression levels, cell proliferation and cell viability were significantly induced while pharmacological inhibition of the PI3K/AKT signaling pathway using LY294002 compound, a selective PI3K inhibitor, significantly impaired the insulin-triggered effects. This finding could stand as one of possibly other underlying mechanisms behind reduced cell proliferation and cell viability and induced apoptosis evidenced upon MLS exposure to PI3K/AKT signaling pathway inhibitors both in vitro and in vivo (Trautmann,2019). To improve MLS management, we studied the sensitivity of MLS1765-92 cells to ionizing radiation (IR). A radioprotective role of Cav-1 was postulated, as Cav-1 levels were markedly incremented in response to IR. Targeting PI3K/AKT signaling pathway, a crucial radioprotective pathway in several cancers including STS (Codenotti,2021) using LY294002 compound, significantly increased the sensitivity of MLS1765-92 cells to IR. Taken together, those findings suggest emerging PI3K/AKT/Cav-1 signaling pathway as a promising molecular target to overcome MLS tumors promotion and radioprotection.

3. MATERIALS AND METHODS

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (Milan, Italy). All absorbance measurements were performed using a Multiskan reader (Thermofischer Scientific, USA). Shown data represent results of at least three independent experiments.

3.1. Chemical compounds

Insulin was dissolved in 1% Glacial acetic acid in H2O. LY294002 compound, the specific PI3K inhibitor, was dissolved in DMSO. The doses of insulin used in this study were 100 nM and 500 nM, while for LY294002, 10 µM and 15 µM were used.

3.2. Cell line and cell culture

The "MLS1765-92" MLS cell line, kindly provided by Prof. Pierre Åman (University of Gothenburg, Sweden), is a patient-derived cell line that was established and immortalized by stable transfection with SV40 large T-antigen encoding sequences (Åman,1992) that expresses the rare type 8 FUS-DDIT3 variant (fusing FUS exon 13 to DDIT3 exon 2). Cells were cultured in RPMI-1640 medium, with sodium bicarbonate and without L-glutamine supplemented with 10% FBS, 1% L-Glutamine (2mM), 100 IU/mL penicillin, and 100 ug/mL streptomycin. Cells were grown in a 37ºC humidified incubator supplied with 5% CO_2. All experiments have been conducted between passage 10 and 20. Cells were confirmed mycoplasma-free using Hoechst DNA staining method.

3.3. Crystal violet cell proliferation assay

To assess cell proliferation, MLS1765-92 cells were seeded into a 24-well plate at a density of 1-1.5 x 10^4 cells/well. At the indicated time-points, the medium was aspirated followed by one 1X Phosphate-buffered saline (PBS) wash, then cells were fixed with 3% Paraformaldehyde (PFA) in 1X PBS (20 min, 4°C) and stained with crystal violet (0.2% crystal violet/20% methanol in 1X PBS). Cells were washed with deionized water and resuspended in 1% sodium dodecyl sulfate (SDS) in 1X PBS. Plates were shaken until reaching complete dissolution. Absorbance was measured by reading the plate at a wavelength of 570 nm.

3.4. Neutral red cell viability assay

Cell viability was assessed by quantitatively estimating the uptake and binding of the supravital dye neutral red (NR) by viable cells. Cells were seeded in 96-well plates at a density of 2.5 x 10^3 cells/well. At the indicated time-points, medium was aspirated and cells were incubated with pre-warmed NR medium (4 mg/mL dissolved in in RPMI-1640 medium, with sodium bicarbonate and without L-

glutamine supplemented with 5% FBS, 1% L-Glutamine (2mM), 100 IU/mL penicillin, and 100 ug/mL streptomycin) in a 37 °C humidified incubator supplied with 5% CO2 for 2h, then NR dye uptake by viable cells was assessed using a phase contrast microscope. Cells were then rinsed once with 1X PBS and the dye was dissolved by NR Desorb solution (50% Ethanol, 1% Glacial acetic acid, 49% deionized water) with shaking. Absorbance was measured by reading the plate at a 540 nm wavelength.

3.5. Immunoblotting

MLS1765-92 cells were harvested in cold RIPA lysis buffer (20 mM Tris–HCl (pH 7.6), 1% Non-idet P40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaCl), and a cocktail of protease inhibitors (Roche, Milan, Italy) plus phosphatase inhibitors (1 mM Na3VO4 and 4 mM NaF). Total homogenates were then centrifuged at 12,000g and 4°C for 10 min. Protein concentrations were measured using the Bradford protein assay. Samples resuspended in 4X Laemmli sample buffer were then boiled for 5 min at 99°C, proteins were separated by SDS-PAGE, and the proteins were transferred to polyvinylidine fluoride (PVDF) membranes (0.2 m pore size) for 1 h at 80 mA, followed by 2 h at 350 mA in 20% methanol, 192 mM glycine, 25 mM Tris, and 0.005% SDS. The membranes were blocked by incubation for 15 min in TBST (20mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20, 0.01% NP-40). The membranes were incubated on an orbital shaker overnight at 4°C with rabbit anti-total Cav-1 primary antibody (1:1000 dilution, code: sc-894, BD Biosciences, Buccinasco, Italy) and mouse anti-beta-Tubulin primary antibody (1:16000 dilution, code: MA5-16308, Thermo Scientific, Rockford, USA) both diluted in 3% BSA/0.01% Sodium azide in TBST. After three washes in TBST for 10 min each, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies anti-rabbit (code sc-2357) and anti-mouse (code sc-516102) (1:5000 dilution) both diluted in fresh TBST for 1 h at room temperature (RT) with gentle shaking, then other three washes in TBST of 10 min each were performed, and the labeled proteins were visualized using enhanced chemiluminescence reagent (GeneSpin, Milan, Italy). Band densities were measured using the Gel Pro Analyzer 4 Software (MediaCybernetics Inc., Rockville, MD, USA).

3.6. Radiation treatment and clonogenic survival assay

MLS1765-92 cells were grown to 80% confluence in 60 mm petri dishes. Dishes were placed on top of 1.5-cm-high Perspex blocks in a 20x20 cm field at 100 cm source-to-surface distance at gantry angle of 180°, then irradiated using a linear X-ray accelerator (at an average dose rate of 2 Gy/min) (in collaboration with Dr. Luca Triggiani, Radiation Oncology Department, ASST Spedali Civili di Brescia, University of Brescia, Brescia, Italy). Four hours after treatment, cells were seeded out into 6-well plates in triplicate (at a density of 3000 cells/well) to assess clonogenic survival. After 11 days,

colonies were fixed with 3% PFA in 1X PBS (20 min, 4°C) and stained with 0.2% crystal violet/20% methanol in 1X PBS for 10 min at RT. Colonies were rinsed with deionized water then photographed. The crystal violet dye was solubilized in 1% SDS in 1X PBS. Plates were shaken until reaching complete dissolution, and absorbance was measured at a wavelength of 570 nm.

3.7. Statistical analysis

The differences between the groups were analyzed by unpaired Student's t-tests and One-Way ANOVA test (with Dunnet's post-test), using Prism 4 software for Windows (GraphPad Software, SanDiego, CA, USA). Statements of significance were based on a p-value of less than 0.05.

4. RESULTS

4.1. Pharmacologically targeting PI3K/AKT signaling pathway leads to Cav-1 protein expression levels downregulation in MLS1765-92 human myxoid liposarcoma cell line

Pharmacological targeting of the PI3K/AKT signaling pathway has been demonstrated to decrease cell proliferation and cell viability and to pronounce apoptosis (Trautmann,2019) in myxoid liposarcoma (MLS). To our best knowledge, no reports of the possible underlying mechanisms were made available to date. We previously demonstrated that Cav-1 acts as a tumor suppressor in liposarcoma (LPS) under in vitro adipogenesis. We wondered, therefore, whether Cav-1 acting both as tumor suppressor and tumor promoter in multiple cancers (Williams,2004), might be playing a role in MLS promotion as well. To address this question, we started by examining whether Cav-1 protein expression levels could be modulated by pharmacologically targeting the PI3K/AKT signaling pathway in proliferating MLS1765-92 cells. Interestingly, as shown in Figure 12, Cav-1 protein expression levels, analyzed by immunoblotting, were significantly downregulated in MLS1765-92 cells after exposure to 10 µM of LY294002 compound, a selective PI3K inhibitor, for 24, 48, and 72 hours. LY294002 compound did not exhibit any cytotoxic effects. This finding postulates Cav-1 as a rising downstream effector of the PI3K/AKT signaling pathway.

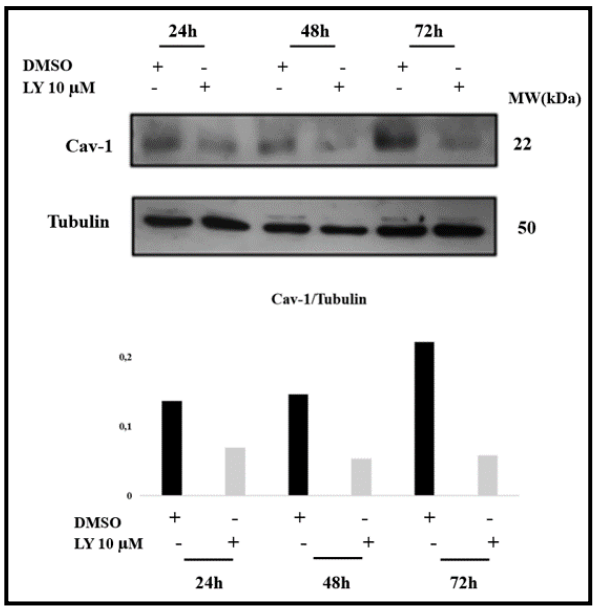

Figure 12. Cav-1 expression levels upon PI3K/AKT inhibition. MLS1765-92 cells were seeded in 60 mm dishes then treated after 24h either with DMSO (vehicle) or LY294002 10 µM. Cells were harvested in RIPA lysis buffer after 24h, 48h, and 72h; total protein extracts were analyzed by immunoblotting for Cav-1 and tubulin (used as loading control).

4.2. Insulin triggering upregulates Cav-1 protein expression levels and promotes cell proliferation and cell viability in proliferating MLS1765-92 cells *via* the PI3K/AKT signaling pathway

To further characterize Cav-1, we hypothesized that triggering MLS1765-92 cells with insulin upregulates Cav-1 protein expression levels and enhance cell proliferation and cell viability. To verify our hypothesis, we treated MLS1765-92 cells with insulin 100 nM or 500 nM for 48h. Under these experimental settings, Cav-1 protein expression levels, assessed by immunoblotting analysis, were upregulated after 48h (Figure 13). Moreover, MLS1765-92 cells exposure to insulin promoted cell proliferation and cell viability in a dose-dependent manner after 48h (Figure 14). Pharmacological blockade of PI3K/AKT signaling using 10 µM of LY294002 compound for 40 minutes prior to insulin triggering significantly impaired insulin-induced Cav-1 upregulation (Figure 13), cell proliferation and cell viability (Figure 14). Taken, together, these findings clearly suggest a possible association between insulin-triggered Cav-1 levels upregulation, cell proliferation and cell viability and PI3K/Akt signaling pathway and a possible tumor promoting role of Cav-1 in proliferating MLS1765-92 cells.

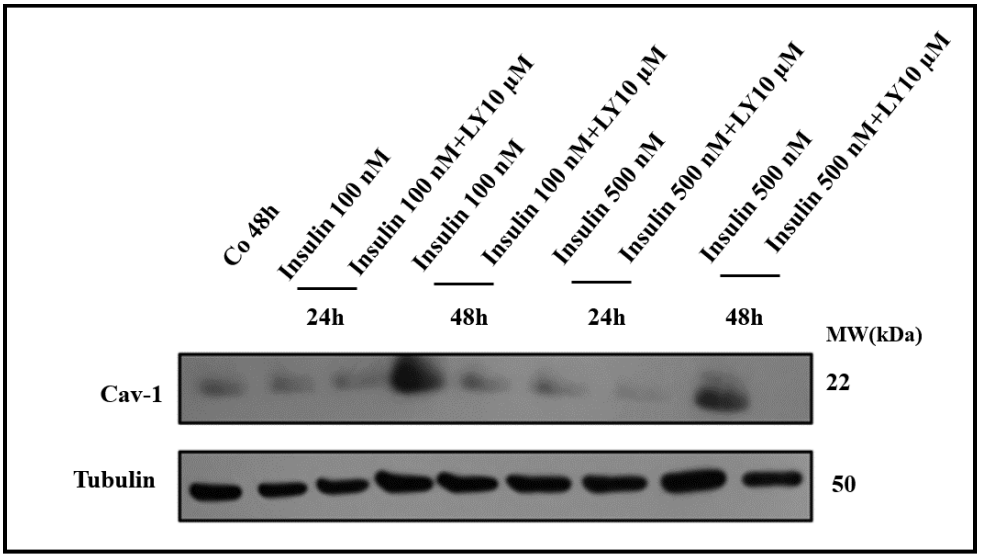

Figure 13. Cav-1 expression levels upon insulin stimulation and PI3K/AKT inhibition. MLS1765-92 cells were seeded in 60 mm dishes. After 24 hours, cells were either pre-treated with LY294002 10 µM for 40 minutes, then stimulated with insulin 100 nM and 500 nM, or treated only with insulin for 24h and 48h. Cells treated only with vehicle were harvested after 48h to better evaluate its effect compared to insulin. Cells were harvested in RIPA lysis buffer after 24h, and 48h; total protein extracts were analyzed by immunoblotting for Cav-1 and tubulin (used as loading control).

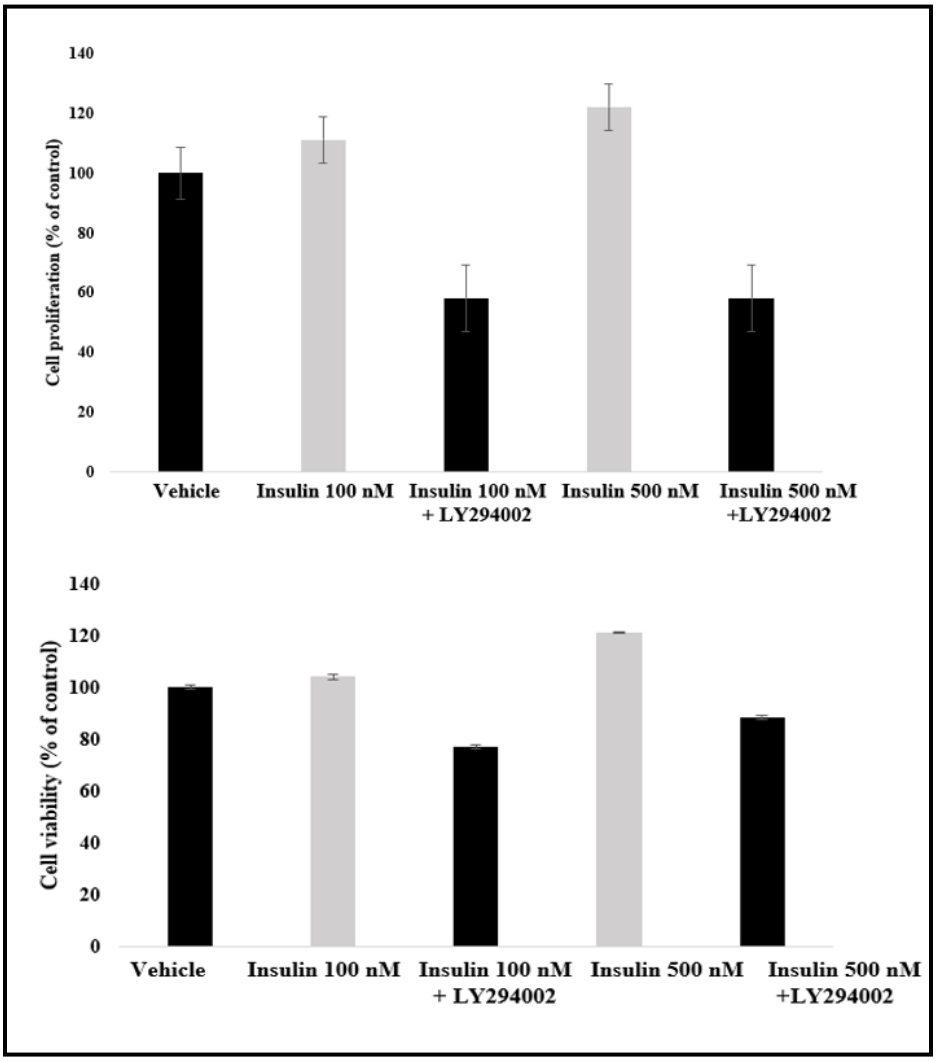

Figure 14. Effect of insulin on PI3K/AKT signaling in proliferating MLS1765-92 cell line. MLS1765-92 cells were seeded in 24 multi-well plates and 96 multi-well plates for proliferation and viability assays, respectively, then treated after 24h either with vehicle, or with insulin 100 nM and 500 nM, or pre-treated with LY294002 10µM for 40 minutes then exposed to insulin 100 nM and 500 nM. Medium was aspirated and cells were fixed with PFA after 48h from insulin exposure. Cell proliferation was measured using crystal violet assay and cell viability was quantified using neutral red cell viability assay.

4.3. Ionizing radiation upregulates Cav-1 protein expression levels in MLS1765-92 cells

Expression of Cav-1 was reported to be upregulated in many cancers exposed to ionizing radiation (Lin,2005). Immunoblotting analysis showed that an irradiation dose of 4 Gy markedly upregulated Cav-1 protein expression levels after 24 hours compared to earlier time-points in MLS1765-92 cells. This finding proposes a potential cell-protective role of Cav-1 triggered by oxidative stress induced by ionizing radiation in MLS1765-92 cells (Figure 15).

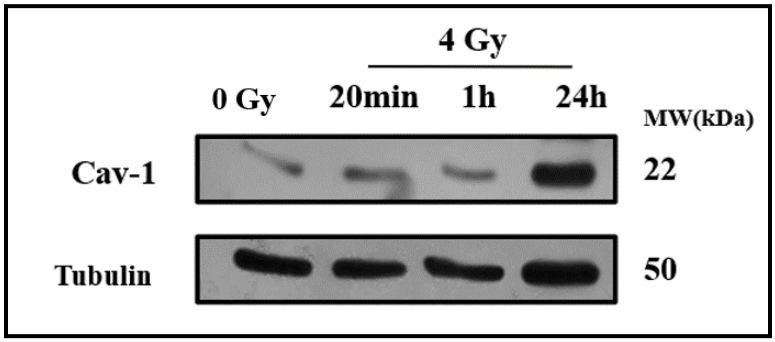

Figure 15. Cav-1 expression levels upon exposure to ionizing radiation. MLS1765-92 cells were seeded in 60 mm dishes and after 24h exposed to a dose of 4 Gy of ionizing radiation. Cells were harvested in RIPA lysis buffer at indicated time-points; total protein extracts were analyzed by immunoblotting for Cav-1 and tubulin (used as loading control).

4.4. Pharmacological blockade of PI3K/AKT signaling pathway sensitizes MLS1765-92 cells to ionizing radiation

To explore the involvement of PI3K/AKT signaling pathway in radiation response, MLS1765-92 cells were pre-treated with 15 µM of LY294002 compound for 4 hours prior to exposure to a dose of 4 Gy of ionizing radiation. LY294002 compound in combination with irradiation synergistically reduced the survival fraction of MLS1765-92 cells. Figure 16 shows the cell survival response of MLS1765-92 cells treated with LY294002 compound and ionizing radiation versus respective controls represented by absorbance. This result indicates that targeting the PI3K/AKT pathway significantly enhance the radiation response of MLS1765-92 cells.

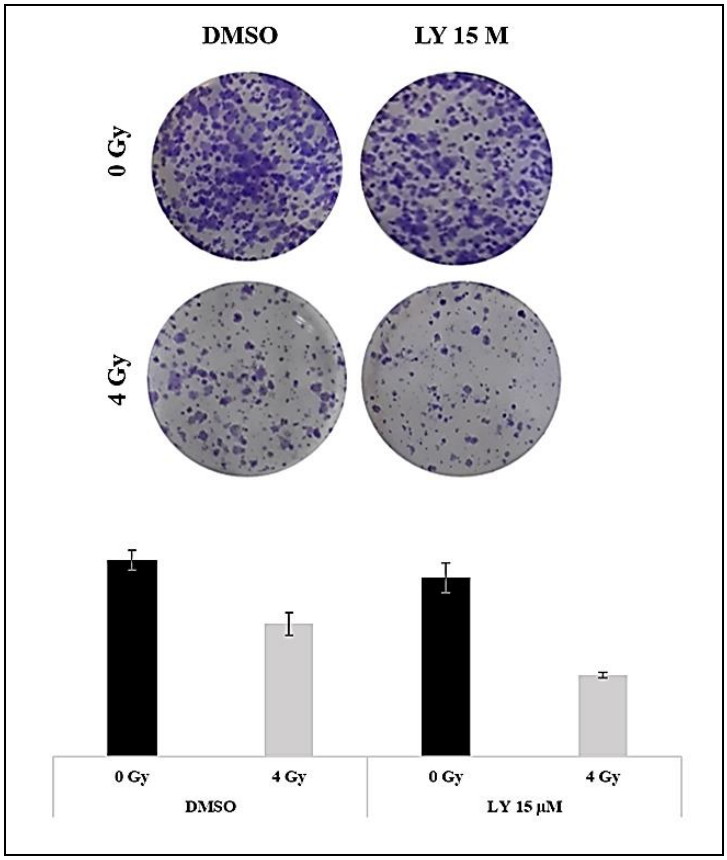

Figure 16. Pharmacological inhibition of PI3K/AKT signaling pathway enhance radiation response of MLS1765-92 cells. Cells were seeded in 60 mm dishes and after 24h were pre-treated either with DMSO or LY294002 15µM for 4 hours prior to exposure to a dose of 4Gy of ionizing radiation. After 11 days, crystal violet assay was used to quantify colonies formation.

4.5. Preliminary proposed mechanism of PI3K/AKT/Cav-1 signaling axis modulating proliferating MLS 1765-92 cell responses to insulin stimulus

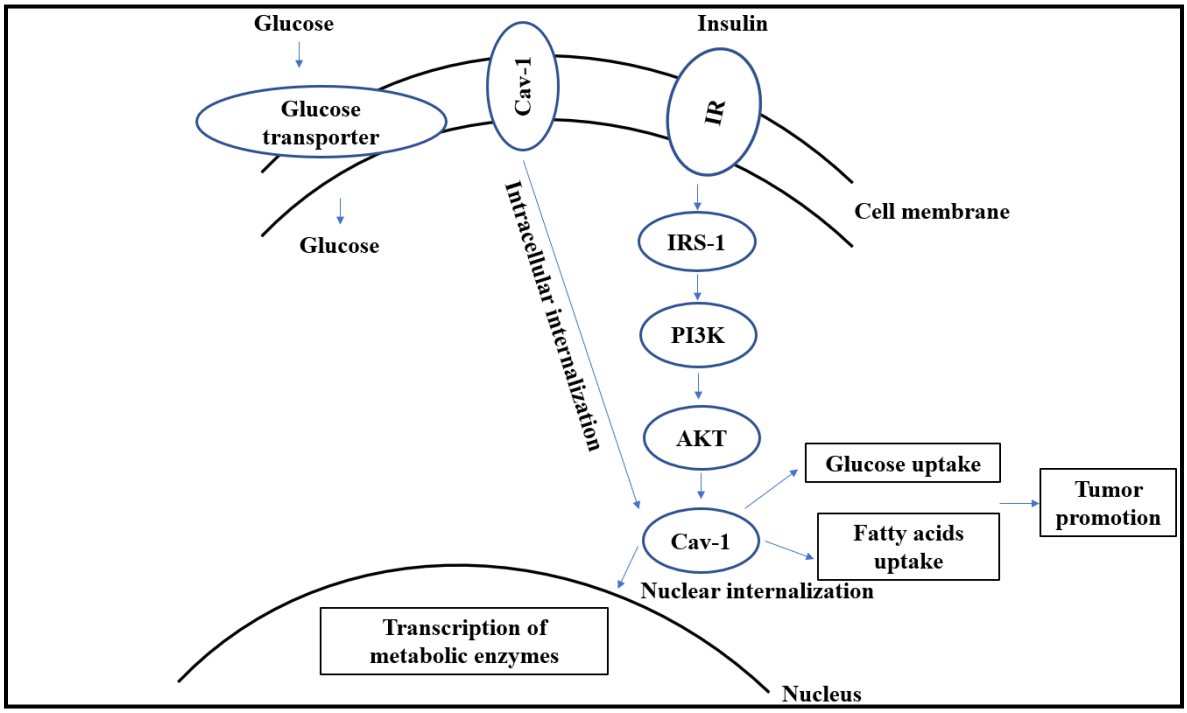

Figure 17. Preliminary proposed mechanism of PI3K/AKT/Cav-1 signaling axis modulating proliferating MLS 1765-92 cell responses to insulin stimulus. Likely, Cav-1 migrates from cell membrane and undergo a specific intracellular trafficking in response to specific extracellular triggers such as insulin and probably other growth factors. Insulin triggers a specific glucose transporter (GLUT protein) at cell membrane through which glucose enters the cell. At nuclear levels, Cav-1 might be mediating transcription of metabolic enzymes such as glycolytic and fatty acids synthesis enzymes which translates into pronounced glucose and fatty acids uptake enhancing tumor promotion in proliferating MLS1765-92 cells. IR: insulin receptor, IRS-1: insulin receptor 1.

4.6. Preliminary proposed mechanism of PI3K/AKT/Cav-1 signaling axis modulating MLS 1765-92 cell responses to oxidative stress induced by ionizing radiation

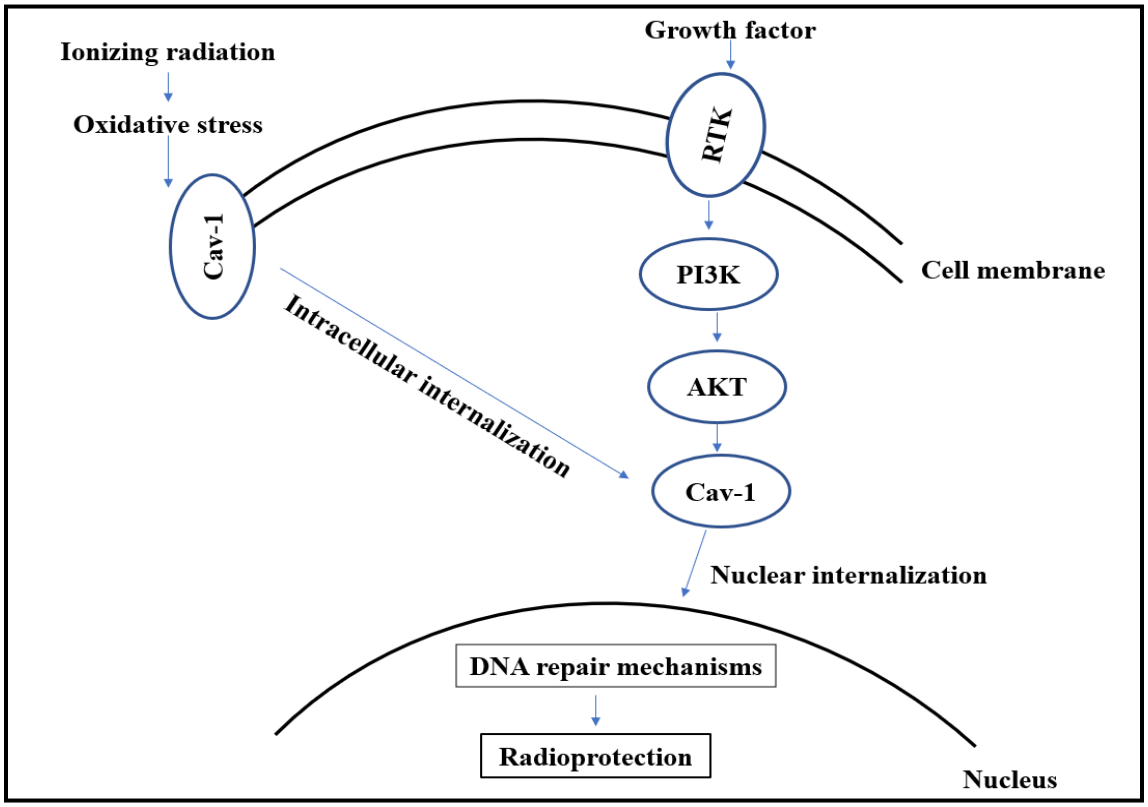

Figure 18. Preliminary proposed mechanism of PI3K/AKT/Cav-1 signaling axis modulating MLS 1765-92 cell responses to oxidative stress induced by ionizing radiation. Cav-1 is drawn to be likely migrating from cell membrane to intracellular sites interacting with PI3K/AKT signaling pathway activated effectors in response to oxidative stress induced by ionizing radiation. Cav-1 might also be acting as a mediator of radioprotective DNA repair mechanisms. RTK: receptor tyrosine kinase.

5. DISCUSSION

Myxoid liposarcoma (MLS) is a life-threatening, adipocytic cancer affecting adolescents and in some case reports, children as well (Gambora,2020;Zafar,2020). Besides characterizing *FUS-DDIT3* fusion gene, PI3K/AKT signaling pathway was reported to be playing an essential role in MLS tumorigenesis as its pharmacological targeting reduced cell proliferation and cell viability and promoted apoptosis in MLS both in vitro and in vivo (Trautmann,2019). To our best knowledge, the involved molecular mechanisms have not been unveiled to date. The aim of this study was to investigate one or more of the many underlying mechanisms. To address this question, we used MLS1765-92 cell line, a human MLS cell model widely used in liposarcoma (LPS) research, characterized by the rare type 8 variant of the *FUS-DDIT3* oncogene (Åman,1992).

Caveolin-1 (Cav-1) represents the major structural and functional protein component of caveolae, plasma membrane invaginations sites controlling many vital cellular processes, such as endocytosis and signal transduction (Parton,2018). Cav-1 was reported to be functioning either as a tumor promoter or as a tumor suppressor in several cancers (Williams,2004). Under in vitro induced adipogenic differentiation conditions, our group has previously demonstrated that Cav-1 plays a tumor suppressor role in LPS, yet we have never excluded a possible involvement in tumor promotion under different cell context and experimental settings (Codenotti,2016). Interestingly, we found that Cav-1 protein expression levels were downregulated following pharmacological blockade of the PI3K/AKT signaling pathway using the LY294002 compound, a selective PI3K inhibitor, revealing it as an emergent downstream effector of the PI3K/AKT signaling pathway.

To deepen our understanding of the PI3K/AKT/Cav-1 emerging signaling axis and tumor promotion, we hypothesized that insulin triggering might induce Cav-1 protein expression levels and promote cell proliferation and cell viability. Indeed, insulin stimulation upregulated Cav-1 protein expression levels, and increased cell proliferation and viability, while exposure to LY294002 compound significantly abrogated the insulin-triggered effects. Taken together, these data clearly suggest the existence of interconnectedness between the PI3K/AKT pathway, Cav-1 and insulin signaling and shape a tumor promoting profile of Cav-1 in MLS1765-92 proliferating cells *via* PI3K/AKT signaling pathway modulation. As preliminarily proposed in Figure 17, based on data collected so far and literature, Cav-1 might be translocating from cell membrane undergoing a specific intracellular and nuclear trafficking in response to specific extracellular triggers such as insulin and likely other growth factors. Glucose diffuse inside the cell through a specific glucose transporter that commit to cell membrane in response

to insulin stimulus (Tunduguru,2017). Cav-1 might also enhance glucose uptake and fatty acids uptake by mediating transcription of metabolic enzymes such as glycolysis and fatty acids enzymes (Tunduguru,2017;Di vizio,2008).

For the sake of improving MLS management, we parallelly investigated whether Cav-1, found to be upregulated in multiple cancers following ionizing radiation (IR) (Lin,2005), might be involved in MLS1765-92 cell responses to IR. Immunoblotting analysis showed that Cav-1 protein expression levels were upregulated in a time-dependent fashion following exposure to a dose of 4 Gy of IR. This finding indicates that Cav-1 might be playing a protective role in response to oxidative stress triggers, such as IR. Therefore, to enhance radiation responsiveness of MLS1765-92 cells, we hypothesized that PI3K/AKT signaling pathway, a crucial radioprotective pathway in multiple malignancies, including STS, may be associated as well to MLS radioresistance (Codenotti,2021). This hypothesis was confirmed as pharmacological inhibition of the PI3K/AKT signaling pathway using the LY294002 compound significantly radiosensitized MLS1765-92 cells. This finding proposes the PI3K/AKT signaling pathway as a promising molecular target to sensitize MLS malignancies to IR, although reported to be more radiosensitive compared to other STS, yet still exhibit a radioresistant phenotype (Rhomberg,2006). Based on data obtained and literature, we drew an initial network in which we sketched the possible traffic that Cav-1 might be traversing, consisting of its relocation from cell membrane to intracellular compartments cooperating with effectors of the PI3K/AKT signaling pathway. Cav-1 might also be present at nuclear levels in which it can be mediating radioprotective mechanisms such as DNA double strand breaks (DSB) repair, leading to the MLS1765-92 cells observed radioresistant phenotype (Figure 18) (Codenotti,2021).

In summary, previous and present data of our group propose Cav-1 as a double-edged sword, behaving both as a tumor suppressor and as a tumor promoter depending on cellular context and experimental settings in response to extracellular stimuli such as insulin but also as a cell-protector against threats such as oxidative stress induced by ionizing radiation through PI3K/AKT signaling pathway modulation in MLS. The investigations are ongoing to dissect Cav-1 tumor promotion and radioprotection mechanisms *via* PI3K/AKT signaling axis coordination in MLS.

6. REFERENCES

Amadeo B, Penel N, Coindre JM, et al. Incidence and time trends of sarcoma (2000-2013): results from the French network of cancer registries (FRANCIM). *BMC Cancer*. 2020;20(1):190.

Åman P, Ron D, Mandahl N, Fioretos T, Heim S, et al. (1992) Rearrangement of the transcription factor gene CHOP in myxoid liposarcomas with t(12;16)(q13;p11). *Genes Chromosom Cancer* 5: 278–285.

Antonescu CR, Tschernyavsky SJ, Decuseara R, Leung DH, Woodruff JM, Brennan MF, Bridge JA, Neff JR, Goldblum JR, Ladanyi M. Prognostic impact of P53 sta tus, TLS-CHOP fusion transcript structure, and histological grade in myxoid liposarcoma: a molecular and clinicopathologic study of 82 cases. Clin Cancer Res. 2001 Dec;7(12):3977-87.

Beck E, Scheen AJ. Activité anticancereuse de la metformine: nouvelles perspectives pour une vieille molécule [Anti-cancer activity of metformin: new perspectives for an old drug]. Rev Med Suisse. 2010 Sep 1;6(260):1601-7. French. PMID: 20853715.

Bill KL, Casadei L, Prudner BC, Iwenofu H, Strohecker AM, Pollock RE. Liposarcoma: molecular targets and therapeutic implications. Cell Mol Life Sci. 2016 Oct;73(19):3711-8.

Boucher J, Kleinridders A, Kahn CR. Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harb Perspect Biol*. 2014;6(1):a009191.

Brennan B, Zanetti I, Orbach D, Gallego S, Francotte N, Van Noesel M, Kelsey A, Casanova M, De Salvo GL, Bisogno G, Ferrari A. Alveolar soft part sarcoma in children and adolescents: The European Paediatric Soft Tissue Sarcoma study group prospective trial (EpSSG NRSTS 2005). Pediatr Blood Cancer. 2018 Apr;65(4).

Chen H, Zhang S, Wen JC, Zheng JK, Chen Q, Li WY, Wang PP, Ma L, Huang TH, Huang G, Yang LY. Several types of soft tissue sarcomas originate from the malignant transformation of adipose tissue-derived stem cells. Mol Med Rep. 2010 May-Jun;3(3):441-8.

Codenotti S, Marampon F, Triggiani L, Bonù ML, Magrini SM, Ceccaroli P, Guescini M, Gastaldello S, Tombolini V, Poliani PL, Asperti M, Poli M, Monti E, Fanzani A. Caveolin-1 promotes radioresistance in rhabdomyosarcoma through increased oxidative stress protection and DNA repair. Cancer Lett. 2021 May 1;505:1-12.

Codenotti S, Mansoury W, Pinardi L, Monti E, Marampon F, Fanzani A. Animal models of well-differentiated/dedifferentiated liposarcoma: utility and limitations. Onco Targets Ther. 2019 Jul 3;12:5257-5268.

Codenotti S, Faggi F, Ronca R, Chiodelli P, Grillo E, Guescini M, Megiorni F, Marampon F, Fanzani A. Caveolin-1 enhances metastasis formation in a human model of embryonal rhabdomyosarcoma through Erk signaling cooperation. Cancer Lett. 2019 May 1;449:135-144.

Codenotti S, Vezzoli M, Poliani PL, Cominelli M, Monti E, Fanzani A. Cavin-2 is a specific marker for detection of well-differentiated liposarcoma. Biochem Biophys Res Commun. 2017 Nov 4;493(1):660-665.

Codenotti S, Vezzoli M, Monti E, Fanzani A. Focus on the role of Caveolin and Cavin protein families in liposarcoma. Differentiation. 2017 Mar-Apr;94:21-26.

Codenotti S, Vezzoli M, Poliani PL, Cominelli M, Bono F, Kabbout H, Faggi F, Chiarelli N, Colombi M, Zanella I, Biasiotto G, Montanelli A, Caimi L, Monti E, Fanzani A. Caveolin-1, Caveolin-2 and Cavin-1 are strong predictors of adipogenic differentiation in human tumors and cell lines of liposarcoma. Eur J Cell Biol. 2016 Aug;95(8):252-64.

Conyers R, Young S, Thomas DM. Liposarcoma: molecular genetics and therapeutics. *Sarcoma*. 2011;2011:483154.

Cordes N, Frick S, Brunner TB, Pilarsky C, Grützmann R, Sipos B, Klöppel G, McKenna WG, Bernhard EJ. Human pancreatic tumor cells are sensitized to ionizing radiation by knockdown of caveolin-1. Oncogene. 2007 Oct 18;26(48):6851-62.

Cormier JN, Pollock RE. Soft tissue sarcomas. CA Cancer J Clin. 2004 Mar-Apr;54(2):94-109.

Crago AM, Dickson MA. Liposarcoma: Multimodality Management and Future Targeted Therapies. Surg Oncol Clin N Am. 2016 Oct;25(4):761-73.

Crago AM, Brennan MF. Principles in Management of Soft Tissue Sarcoma. Adv Surg. 2015;49(1):107-22.

de Visscher SA, van Ginkel RJ, Wobbes T, Veth RP, Ten Heuvel SE, Suurmeijer AJ, Hoekstra HJ. Epithelioid sarcoma: Still an only surgically curable disease. Cancer. 2006 Aug 1;107(3):606-12.

Dei Tos AP. Liposarcomas: diagnostic pitfalls and new insights. Histopathology. 2014 Jan;64(1):38-52.

Demicco EG, Torres KE, Ghadimi MP, Colombo C, Bolshakov S, Hoffman A, Peng T, Bovée JV, Wang WL, Lev D, Lazar AJ. Involvement of the PI3K/Akt pathway in myxoid/round cell liposarcoma. Mod Pathol. 2012 Feb;25(2):212-21.

Di Vizio D, Adam RM, Kim J, Kim R, Sotgia F, Williams T, Demichelis F, Solomon KR, Loda M, Rubin MA, Lisanti MP, Freeman MR. Caveolin-1 interacts with a lipid raft-associated population of fatty acid synthase. Cell Cycle. 2008 Jul 15;7(14):2257-67.

Dim DC, Cooley LD, Miranda RN. Clear cell sarcoma of tendons and aponeuroses: a review. Arch Pathol Lab Med. 2007 Jan;131(1):152-6.

Dowli A, Mattar A, Mashimo H, Huang Q, Cohen D, Fisichella PM, Lebenthal A. A pedunculated giant esophageal liposarcoma: a case report and literature review. J Gastrointest Surg. 2014 Dec;18(12):2208-13.

Du C, Chen L, Zhang H, et al. Caveolin-1 limits the contribution of BKCa channel to MCF-7 breast cancer cell proliferation and invasion. *Int J Mol Sci*. 2014;15(11):20706–20722.

Engström K, Willén H, Kåbjörn-Gustafsson C, Andersson C, Olsson M, Göransson M, Järnum S, Olofsson A, Warnhammar E, Åman P. The myxoid/round cell liposarcoma fusion oncogene FUS-DDIT3 and the normal DDIT3 induce a liposarcoma phenotype in transfected human fibrosarcoma cells. Am J Pathol. 2006 May;168(5):1642-53.

Fabre-Guillevin E, Coindre JM, Somerhausen Nde S, Bonichon F, Stoeckle E, Bui NB. Retroperitoneal liposarcomas: follow-up analysis of dedifferentiation after clinicopathologic reexamination of 86 liposarcomas and malignant fibrous histiocytomas. Cancer. 2006 Jun 15;106(12):2725-33.

Faggi F, Mitola S, Sorci G, Riuzzi F, Donato R, Codenotti S, Poliani PL, Cominelli M, Vescovi R, Rossi S, Calza S, Colombi M, Penna F, Costelli P, Perini I, Sampaolesi M, Monti E, Fanzani A. Phosphocaveolin-1 enforces tumor growth and chemoresistance in rhabdomyosarcoma. PLoS One. 2014 Jan 10;9(1):e84618.

Filippini A, D'Alessio A. Caveolae and Lipid Rafts in Endothelium: Valuable Organelles for Multiple Functions. Biomolecules. 2020 Aug 21;10(9):1218.

Fletcher CD. The evolving classification of soft tissue tumours - an update based on the new 2013 WHO classification. Histopathology. 2014 Jan;64(1):2-11.

Fong Y, Coit DG, Woodruff JM, Brennan MF. Lymph node metastasis from soft tissue sarcoma in adults. Analysis of data from a prospective database of 1772 sarcoma patients. Ann Surg. 1993 Jan;217(1):72-7.

Gamboa AC, Gronchi A, Cardona K. Soft-tissue sarcoma in adults: An update on the current state of histiotype-specific management in an era of personalized medicine. CA Cancer J Clin. 2020 May;70(3):200-229.

Garcia M, Buitrago E, Bejarano PA, Casillas J. Large esophageal liposarcoma: a case report and review of the literature. Arch Pathol Lab Med. 2004 Aug;128(8):922-5.

Giovannucci E, Harlan DM, Archer MC, et al. Diabetes and cancer: a consensus report. *Diabetes Care*. 2010;33(7):1674-1685.

Grosso F, Jones RL, Demetri GD, Judson IR, Blay JY, Le Cesne A, Sanfilippo R, Casieri P, Collini P, Dileo P, Spreafico C, Stacchiotti S, Tamborini E, Tercero JC, Jimeno J, D'Incalci M, Gronchi A, Fletcher JA, Pilotti S, Casali PG. Efficacy of trabectedin (ecteinascidin-743) in advanced pretreated myxoid liposarcomas: a retrospective study. Lancet Oncol. 2007 Jul;8(7):595-602.

Gutierrez A, Snyder EL, Marino-Enriquez A, Zhang YX, Sioletic S, Kozakewich E, Grebliunaite R, Ou WB, Sicinska E, Raut CP, Demetri GD, Perez-Atayde AR, Wagner AJ, Fletcher JA, Fletcher CD, Look AT. Aberrant AKT activation drives well-differentiated liposarcoma. Proc Natl Acad Sci U S A. 2011 Sep 27;108(39):16386-91.

Haniball J, Sumathi VP, Kindblom LG, Abudu A, Carter SR, Tillman RM, Jeys L, Spooner D, Peake D, Grimer RJ. Prognostic factors and metastatic patterns in primary myxoid/round-cell liposarcoma. Sarcoma. 2011;2011:538085.

Jones RL, Fisher C, Al-Muderis O, Judson IR. Differential sensitivity of liposarcoma subtypes to chemotherapy. Eur J Cancer. 2005 Dec;41(18):2853-60.

Keung EZ, Somaiah N. Overview of liposarcomas and their genomic landscape. *J Transl Genet Genom* 2019;3:8.

Kim SY. Cancer Energy Metabolism: Shutting Power off Cancer Factory. *Biomol Ther (Seoul)*. 2018;26(1):39-44.

Kuroda M, Ishida T, Takanashi M, Satoh M, Machinami R, Watanabe T. Oncogenic transformation and inhibition of adipocytic conversion of preadipocytes by TLS/FUS-CHOP type II chimeric protein. *Am J Pathol*. 1997;151(3):735-744.

Lau MT and Leung PC: The PI3K/Akt/mTOR signaling pathway mediates insulin-like growth factor 1-induced E-cadherin down-regulation and cell proliferation in ovarian cancer cells. Cancer Lett. 326:191–198. 2012.

Lazar A, Abruzzo LV, Pollock RE, Lee S, Czerniak B. Molecular diagnosis of sarcomas: chromosomal translocations in sarcomas. Arch Pathol Lab Med. 2006 Aug;130(8):1199-207.

Li J, Hassan GS, Williams TM, Minetti C, Pestell RG, Tanowitz HB, Frank PG, Sotgia F, Lisanti MP. Loss of caveolin-1 causes the hyper-proliferation of intestinal crypt stem cells, with increased sensitivity to whole body gamma-radiation. Cell Cycle. 2005 Dec;4(12):1817-25.

Liang YN, Liu Y, Wang L, et al. Combined caveolin-1 and epidermal growth factor receptor expression as a prognostic marker for breast cancer. *Oncol Lett*. 2018;15(6):9271–9282.

Lin M., DiVito, M. M., Merajver, S. D., Boyanapalli, M. & van Golen, K. L. Regulation of pancreatic cancer cell migration and invasion by RhoC GTPase and caveolin-1. *Mol Cancer* 4, 21, 10.1186/1476-4598-4-21 (2005).

Lin ZC, Chang XZ, Huang XF, Zhang CL, Yu GS, Wu SY, Ye M, He JX. Giant liposarcoma of the esophagus: A case report. World J Gastroenterol. 2015 Sep 7;21(33):9827-32.

Mastick CC, Saltiel AR. Insulin-stimulated tyrosine phosphorylation of caveolin is specific for the differentiated adipocyte phenotype in 3T3-L1 cells. J Biol Chem. 1997 Aug 15;272(33):20706-14.

Nakano K, Takahashi S. TranslocationRelated Sarcomas. *Int J Mol Sci*. 2018;19(12):3784.

Nwosu ZC, Ebert MP, Dooley S, Meyer C. Caveolin-1 in the regulation of cell metabolism: a cancer perspective. *Mol Cancer*. 2016;15(1):71. Published 2016 Nov 16.

Parton RG. Caveolae: Structure, Function, and Relationship to Disease. Annu Rev Cell Dev Biol. 2018 Oct 6;34:111-136.

Pavlides, S.; Tsirigos, A.; Vera, I.; Flomenberg, N.; Frank, P.G.; Casimiro, M.C.; Wang, C.; Fortina, P.; Addya, S.; Pestell, R.G.; et al. Loss of stromal caveolin-1 leads to oxidative stress, mimics hypoxia and

drives inflammation in the tumor microenvironment, conferring the “reverse warburg effect”: A transcriptional informatics analysis with validation. *Cell Cycle* 2010, *9*, 2201–2219.

Pérez-Losada J, Pintado B, Gutiérrez-Adán A, Flores T, Bañares-González B, del Campo JC, Martín-Martín JF, Battaner E, Sánchez-García I. The chimeric FUS/TLS-CHOP fusion protein specifically induces liposarcomas in transgenic mice. Oncogene. 2000 May 11;19(20):2413-22.

Pitot HC. The molecular biology of carcinogenesis. Cancer. 1993 Aug 1;72(3 Suppl):962-70.

Potter M, Newport E, Morten KJ. The Warburg effect: 80 years on. *Biochem Soc Trans*. 2016;44(5):1499-1505.

Powers MP, Wang WL, Hernandez VS, Patel KS, Lev DC, Lazar AJ, López-Terrada DH. Detection of myxoid liposarcoma-associated FUS-DDIT3 rearrangement variants including a newly identified breakpoint using an optimized RT-PCR assay. Mod Pathol. 2010 Oct;23(10):1307-15.

Ramu EM, Houdek MT, Isaac CE, Dickie CI, Ferguson PC, Wunder JS. Management of soft-tissue sarcomas; treatment strategies, staging, and outcomes. *SICOT J*. 2017;3:20.

Ratan R, Patel SR. Chemotherapy for soft tissue sarcoma. Cancer. 2016 Oct;122(19):2952-60.

Razani B, Combs TP, Wang XB, Frank PG, Park DS, Russell RG, Li M, Tang B, Jelicks LA, Scherer PE, Lisanti MP. Caveolin-1-deficient mice are lean, resistant to diet-induced obesity, and show hypertriglyceridemia with adipocyte abnormalities. J Biol Chem. 2002 Mar 8;277(10):8635-47.

Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, Macaluso F, Russell RG, Li M, Pestell RG, Di Vizio D, Hou H Jr, Kneitz B, Lagaud G, Christ GJ, Edelmann W, Lisanti MP. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. J Biol Chem. 2001 Oct 12;276(41):38121-38.

Rhomberg W. The radiation response of sarcomas by histologic subtypes: a review with special emphasis given to results achieved with razoxane. *Sarcoma*. 2006;2006(1):87367.

Riggi N, Cironi L, Provero P, Suvà ML, Stehle JC, Baumer K, Guillou L, Stamenkovic I. Expression of the FUS-CHOP fusion protein in primary mesenchymal progenitor cells gives rise to a model of myxoid liposarcoma. Cancer Res. 2006 Jul 15;66(14):7016-23.

Riva G, Sensini M, Corvino A, Garzaro M, Pecorari G. Liposarcoma of Hypopharynx and Esophagus: a Unique Entity? J Gastrointest Cancer. 2016 Jun;47(2):135-42.

Saltiel AR and Pessin JE: Insulin signaling pathways in time and space. Trends Cell Biol. 12:65–71. 2002.

Sbaraglia M, Bellan E, Dei Tos AP. The 2020 WHO Classification of Soft Tissue Tumours: news and perspectives. Pathologica. 2021 Apr;113(2):70-84.

Schlessinger J. Receptor tyrosine kinases: legacy of the first two decades. *Cold Spring Harb Perspect Biol*. 2014;6(3):a008912.

Schöffski P, Chawla S, Maki RG, Italiano A, Gelderblom H, Choy E, Grignani G, Camargo V, Bauer S, Rha SY, Blay JY, Hohenberger P, D'Adamo D, Guo M, Chmielowski B, Le Cesne A, Demetri GD, Patel SR. Eribulin versus dacarbazine in previously treated patients with advanced liposarcoma or leiomyosarcoma: a randomised, open-label, multicentre, phase 3 trial. Lancet. 2016 Apr 16;387(10028):1629-37.

Shigematsu S, Watson RT, Khan AH, Pessin JE. The adipocyte plasma membrane caveolin functional/structural organization is necessary for the efficient endocytosis of GLUT4. J Biol Chem. 2003 Mar 21;278(12):10683-90.

Sievers E, Trautmann M, Kindler D, Huss S, Gruenewald I, Dirksen U, Renner M, Mechtersheimer G, Pedeutour F, Åman P, Nishio J, Schildhaus HU, Kirfel J, Schirmacher P, Wardelmann E, Buettner R, Hartmann W. SRC inhibition represents a potential therapeutic strategy in liposarcoma. Int J Cancer. 2015 Dec 1;137(11):2578-88.

Smith KB, Tran LM, Tam BM, et al. Novel dedifferentiated liposarcoma xenograft models reveal PTEN down-regulation as a malignant signature and response to PI3K pathway inhibition. *Am J Pathol*. 2013;182(4):1400-1411.

Spillane AJ, A'Hern R, Judson IR, Fisher C, Thomas JM. Synovial sarcoma: a clinicopathologic, staging, and prognostic assessment. J Clin Oncol. 2000 Nov 15;18(22):3794-803.

Strahm B, Malkin D. Hereditary cancer predisposition in children: genetic basis and clinical implications. Int J Cancer. 2006 Nov 1;119(9):2001-6.

Trautmann M, Cyra M, Isfort I, Jeiler B, Krüger A, Grünewald I, Steinestel K, Altvater B, Rossig C, Hafner S, Simmet T, Becker J, Åman P, Wardelmann E, Huss S, Hartmann W. Phosphatidylinositol-3-kinase

(PI3K)/Akt Signaling is Functionally Essential in Myxoid Liposarcoma. Mol Cancer Ther. 2019 Apr;18(4):834-844.

Tunduguru R, Thurmond DC. Promoting Glucose Transporter-4 Vesicle Trafficking along Cytoskeletal Tracks: PAK-Ing Them Out. *Front Endocrinol (Lausanne)*. 2017;8:329.

Vocks E, Worret WI, Burgdorf WH. Myxoid liposarcoma in a 12-year-old girl. Pediatr Dermatol. 2000 Mar-Apr;17(2):129-32.

Wan, L., Tu, C., Qi, L. *et al.* Survivorship and prognostic factors for pleomorphic liposarcoma: a population-based study. *J Orthop Surg Res* 16, 175 (2021).

Williams TM, Lisanti MP. The caveolin proteins. Genome Biol. 2004;5(3):214.

Xiao W, Mohseny AB, Hogendoorn PC, Cleton-Jansen AM. Mesenchymal stem cell transformation and sarcoma genesis. *Clin Sarcoma Res*. 2013;3(1):10.

Zafar, R. & Wheeler, Y. 2020. Liposarcoma. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan. 2020 Apr 15.

Zhu J, Thompson CB. Metabolic regulation of cell growth and proliferation. *Nat Rev Mol Cell Biol*. 2019;20(7):436-450.

7. PUBLICATION

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