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c-Jun promotes cell migration and drives expression of the motility factor ENPP2 in soft tissue sarcomas

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Abstract

Genomic amplification of the *c-Jun* proto-oncogene has been identified in ~30% of dedifferentiated liposarcomas (DDLPS), but the functional contribution of c-Jun to the progression of DDLPS remains poorly understood. In previous work, we showed that knockdown of c-Jun by RNA interference impaired the *in vitro* proliferation and *in vivo* growth of a DDLPS cell line (LP6) with genomic amplification of the *c-Jun* locus. Here, we use gene expression analysis and functional studies in a broad panel of cell lines to further define the role of c-Jun in DDLPS and other soft tissue sarcomas. We show that c-Jun knockdown impairs transition through the G1 phase of the cell cycle in multiple DDLPS cell lines. We also find that high levels of c-Jun expression are both necessary and sufficient to promote DDLPS cell migration and invasion *in vitro*. Our data suggest that high levels of c-Jun enhance motility in part by driving the expression of ENPP2/Autotaxin. c-Jun overexpression has minimal effects on *in vitro* proliferation, but substantially enhances the *in vivo* growth of weakly tumorigenic DDLPS cell lines. Finally, we provide evidence that *c-Jun* genomic amplification and overexpression may have similar functional consequences in other types of soft tissue sarcoma. Our data suggest a model in which relatively low levels of c-Jun are sufficient for *in vitro* proliferation, but high levels of c-Jun enhance invasiveness and capacity for *in vivo* tumor growth. These observations provide an explanation for the selective advantage provided by *c-Jun* genomic amplification *in vivo* and

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Data from exon array and RNA-Seq experiments have been deposited in the GEO database (GSE57531).

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suggest that sarcomas with elevated c-Jun levels are likely to have a particularly high malignant potential.

Keywords

c-Jun; liposarcoma; soft tissue sarcoma; ENPP2; autotaxin

Introduction

The AP1 transcription factor is a dimeric complex comprised of members of the JUN, FOS, ATF and MAF protein families. These transcription factors possess a basic leucine zipper (bZIP) domain that is essential for dimerization and DNA binding. The major subfamilies of AP-1 are Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra1 and Fra2) proteins, which were first identified as the viral oncoproteins v-Fos and v-Jun in the Finkel-Biskis-Jenkins osteosarcoma virus and avian sarcoma virus, respectively [1–4]. c-Jun is the founding member and the most potent transcriptional activator of the AP-1 family. It is also the downstream effector of many pathways regulating development and differentiation during embryogenesis, cellular proliferation and apoptosis[5].

High levels of c-Jun are found in multiple cancer types [6–10], and c-Jun overexpression can play an important role in the development of skin, liver and nasopharyngeal cancers [11–13]. However, the role of c-Jun in tumor development can be context-dependent since it is also influenced by tumor type, genetic background and the transcriptional activation of yet-to be identified target genes by these transcription factors [14,15].

Despite the well-characterized oncogenic properties of c-Jun, genomic amplification in human tumors was only recently described in a cohort of dedifferentiated liposarcomas (DDLPS)[16]. Liposarcomas are the most common subtype of soft tissue sarcomas, comprising approximately 20% of adult sarcomas with a mortality rate of approximately 60% [17]. Liposarcomas are classified into 3 biological types based on distinct morphological and cytogenetic characteristics: (1) well differentiated/dedifferentiated (the most common type), (2) myxoid and (3) pleomorphic.

Well-differentiated liposarcoma (WDLPS) is a low-grade neoplasm that exhibits adipocytic differentiation and almost no capacity to metastasize. However, 10% of WDLPS can undergo progression and transform to DDLPS. DDLPS is an aggressive neoplasm, with a metastatic rate of 10%–20% and overall mortality of 50%–75%. WDLPS and DDLPS have supernumerary ring and/or giant chromosomes containing discontinuous 12q13-q15 amplifications with varying peaks at *CDK4*, *HMGA2* and *MDM2* [18].

Genomic amplification of the 1p32 locus (containing *c-Jun*) was first identified in DDLPS via comparative genomic hybridization [16]. Multiple studies have now demonstrated that *c-Jun* is amplified and overexpressed in ~30% of DDLPS [19–21]. Based on the absence of *c-Jun* amplification in pure WDLPS, c-Jun was proposed to block adipocytic differentiation [22]. However, *c-Jun* amplification can be found in both the well-differentiated and dedifferentiated portions of a given liposarcoma [20,23]. Thus, *c-Jun* amplification may not

be sufficient to block adipocytic differentiation in liposarcoma. Nevertheless, knockdown of c-Jun severely impaired the growth of *c-Jun*-amplified DDLPS cells *in vitro* and *in vivo*. These data raise the question of how c-Jun functions as an oncoprotein in DDLPS.

Material and Methods

Cell Culture

Cell lines were established from dedifferentiated liposarcoma (LP6, LPS141, LPS510, LPS12), well differentiated liposarcoma (T449, T778) [19], leiomyosarcoma (LMS03), unclassified sarcomas (USCS2, USCS5) and Alveolar Soft Part Sarcoma (ASPS-KY) [24]. LPS141, LPS510, LMS03 were established by author J. Fletcher. USCS2, USCS5, LPS12 were established by author E. Sicinska. T449 and T778 were a kind gift from Dr. F. Pedetour. Cells were maintained in DMEM/F-12 media with 10% fetal bovine serum. Studies were carried out in accordance with protocols approved by the Partners Health Care Institutional Review Board.

In vitro cell migration assays

Cell exclusion zone assay—In vitro cell migration was measured using the Oris™ 96-well cell migration assay kit (Platypus Technologies).

Transwell Migration/Matrigel Invasion Chamber Assays—Transwell migration and invasion assays were performed using the BD Biocoat Matrigel Migration Chamber (8-μm pore size).

Single cell tracking—50,000–200,000 cells/ml were plated at least 18 hours prior the experiment and grown on glass-bottom 6-well tissue culture dishes (MatTek). 4 different areas in each condition were imaged for 15 hours on a TE2000-E2 inverted Nikon microscope equipped with a cooled CCD camera (Orca ER, Hamamatsu) and 10x PlanFluor NA0.3 phase objective (Nikon). NIS-Elements software was used to control the microscope, shutters, and Prior x,y encoded motorized stage. Analysis was performed in NIS-Elements software with tracking module.

In vivo tumorigenesis

For c-Jun overexpression studies, LPS141 and 510 cells were infected with control or c-Jun expressing lentivirus in the presence of polybrene (5 μg/ml) and selected with blasticidin (10 μg/ml) for 5 days. Cells were sorted with BD FACS Aria II based on fluorescent reporter proteins to enrich for stably transduced cells and cultured for 2 days prior to injection. For c-Jun knockdown studies, LMS003 and ASPS-KY cells were infected with lentivirus encoding shRNA to c-Jun or GFP in the presence of polybrene (5 μg/ml) and selected with puromycin (2 μg/ml) for 2–4 days.

In all tumor studies, 5×10^5 cells were mixed with an equal volume of Matrigel (BD Biosciences) and injected subcutaneously into the flank of *Nu/Nu* female mice (Charles River) (n=5 mice per group). Volume was estimated using the formula $(0.5 * L * W * H)$, and significance was determined using the Wilcoxon Rank Sum Test. All procedures were

performed according to protocols approved by the Institutional Animal Care and Use Committee of the Dana Farber Cancer Institute.

Affymetrix Exon Array

Changes in mRNA levels were analyzed by Affymetrix Exon 1.0 ST Arrays. Pathway analysis was performed with Ingenuity Pathway Analysis software (Ingenuity® Systems).

Illumina sequencing

Libraries were prepared and samples were sequenced on Illumina GAII and HiSeq2000 platforms according to Illumina protocols.

Statistics

To assess significance, we utilized a 2-tailed student's T-test unless otherwise specified. Differences were considered significant when $p < 0.05$. See Supporting Material, Supplemental methods for additional details.

Results

Genomic amplification of *c-Jun* activates genes that promote proliferation and cell migration in liposarcoma

In previous work, we demonstrated that c-Jun is required for optimal proliferation and tumor initiation by the DDLPS cell line LP6, which harbors genomic amplification of the 1p32 locus containing *c-Jun* [25]. LP6 cells express higher levels of c-Jun mRNA and protein than several other liposarcoma cell lines that have a normal *c-Jun* copy number (Fig. 1A–B). To gain a better understanding of how c-Jun functions as an oncogene in liposarcoma, we sought to identify its target genes. We therefore performed c-Jun knockdown in both LP6 (*c-Jun* amplified) and LPS141 (non-amplified) DDLPS cells. We used lentivirus to deliver 2 control hairpins and 2 shRNAs targeting c-Jun to both cell lines and confirmed knockdown by immunoblotting (Fig. 1C).

To identify differentially expressed genes after c-Jun knockdown, we harvested total RNA from LP6 and LPS141 cells on days 4 and 7 after infection, timepoints at which we observed maximum knockdown by qRT-PCR and immunoblotting respectively (Fig. 1C, S1 and data not shown). These 16 samples were then subjected to Affymetrix exon array analysis (Fig. S2).

We performed a supervised analysis of differential gene expression within each cell line to identify the genes that consistently change at both time points in response to c-Jun knockdown. We also compared results from each cell line to identify differentially expressed genes that were unique to each line and common to both lines. Exon array analysis (Fig. S2) of LP6 cells showed that 116 genes were downregulated at least two fold on both days 4 and 7, whereas only 12 genes were upregulated at both time points. In LPS141 cells, 341 genes were upregulated and 57 were downregulated by at least two fold at both timepoints.

The first studies of c-Jun in liposarcoma hypothesized that its amplification or overexpression blocked adipocytic differentiation of DDLPS by interfering with PPAR γ and C/EBP α activity [22]. However, we did not observe an increase in the expression of adipocytic genes (such as *PPARG2*, *CFD* (adipsin) and *CEBPA*) after c-Jun knockdown by either exon array or qRT-PCR. (Fig. S3).

Furthermore, our analysis identified minimal overlap in differentially expressed genes between the two cell lines (Fig. S2), raising the possibility that c-Jun may have distinct functions in each cell line. We therefore used Ingenuity Pathway Analysis (IPA) to identify significant network functions and canonical pathways regulated by c-Jun in each cell line (Fig. 1D–E). In both cell lines, c-Jun knockdown reduced the levels of genes that promote proliferation (Fig. 1D–E). By comparison, only LP6 (*c-Jun*-amplified) cells exhibited a decline in network functions associated with cellular movement upon c-Jun knockdown (Fig. 1D). These data raise the possibility that c-Jun might promote cell migration specifically in the context of amplification or overexpression, whereas c-Jun might stimulate proliferation in most liposarcomas. We therefore performed functional studies to characterize the role of c-Jun in cellular proliferation and migration in DDLPS cell lines.

c-Jun knockdown causes G1 cell cycle arrest in liposarcoma cell lines

We assessed the effect of c-Jun knockdown (Fig. 1C, S1 and S4) on the viability of four liposarcoma cell lines (LP6, LPS141, T449, LPS510) using the CellTiter-Glo assay. Seven days after infection, the number of c-Jun knockdown cells relative to controls was significantly reduced in all cell lines, regardless of amplification status (Fig. 1F). Cell cycle analysis revealed that c-Jun knockdown caused a G1 phase arrest in four liposarcoma cell lines (Fig. 1G). c-Jun can regulate cell cycle progression by a variety of mechanisms, including repression of cyclin dependent kinase (CDK) inhibitors such as p21 [26]. Western blot analysis revealed increased expression of the cyclin-dependent kinase inhibitor p21 after c-Jun knockdown in all liposarcoma cell lines tested (Fig. 1C and data not shown), suggesting one mechanism by which c-Jun might regulate proliferation in liposarcoma cells.

Taken together, these results suggest that adequate c-Jun levels are required for liposarcoma progression through the G1 phase of the cell cycle and that promotion of cell cycle progression appears to be a common function of c-Jun in amplified and non-amplified cell lines.

c-Jun knockdown impairs cell migration in liposarcomas with *c-Jun* genomic amplification

We next investigated the possibility that c-Jun knockdown might impair cell migration in liposarcoma. We further evaluated six genes which were implicated in cell migration by IPA analysis and whose expression was reduced at both timepoints after c-Jun knockdown in LP6 cells (ENNP2, RCAN1, TGFBR3[27], ETS2[28], ERG [29] and INHBA[30]). The expression of all six genes declined by >2 fold as measured by qRT-PCR in LP6 cells after c-Jun knockdown (Fig. 2A). Importantly, levels of these genes did not change in response to shRNAs targeting the oncogenes *MDM2* and *CDK4*, suggesting that their response to c-Jun knockdown is not simply an indirect effect of proliferation arrest.

We therefore performed functional studies to determine whether liposarcoma cell migration is dependent on c-Jun expression. We focused on LP6 and LPS141 cells in order to represent DDLPS tumors with (LP6) and without (LPS141) *c-Jun* amplification. Of note, both cell lines have high-level *MDM2* amplification and they have similar in vitro growth rates.

We first used a cell exclusion zone assay to measure the effect of c-Jun knockdown on LP6 cell migration. Six days after lentiviral delivery of shRNA, LP6 cells were plated in an Oris 96-well cell migration assay plate. The next day (day 0), inserts were removed and closure of the cell-free area was monitored every 24 hours for 3 days. On day 3, the percent area closure was significantly reduced in Jun-inhibited cells (47–58% closure) compared to controls (84% closure, $p<0.04$) (Fig. 2B).

We obtained similar results using a transwell (Boyden chamber) assay to assess migration and invasion. At baseline, the migration capacity of LP6 was higher than that of LPS141 and other DDLPS cell lines (Fig. S5A). c-Jun knockdown in LP6 cell lines reduced migration capacity by 40–50 % (Fig. 2C, left, $p=0.007$), whereas no significant difference was observed in LPS141 cells (Fig. 2C, right). Similar results were obtained when the invasive capacity of these cells was assayed using Boyden chambers ($p<0.001$, Fig 2C and Fig. S5B).

Finally, we measured the impact of c-Jun knockdown on liposarcoma cell velocity during single cell migration by tracking the position of individual cells using time lapse microscopy. We found that control LP6 cells move with a significantly higher velocity than LPS141 cells (>2 fold, $p=0.002$, Fig. 2D–E). Furthermore, we observed a >2 fold decrease in the velocity of LP6 cells upon c-Jun knockdown ($p<0.0001$, Fig. 2D). In contrast, c-Jun knockdown had no effect on the velocity of LPS141 cells (Fig. 2E).

Taken together, these results indicate that c-Jun plays a critical role in promoting migration and invasion in LP6 cells, whereas these functions appear to be largely c-Jun-independent in LPS141 cells. This suggests that genomic amplification of *c-Jun* can enhance liposarcoma invasiveness.

c-Jun overexpression in non-amplified LPS cell lines enhances cell migration and invasion

We next sought to determine whether overexpression of c-Jun was sufficient to augment migration/invasion in liposarcoma. We infected four non-amplified LPS cell lines (3 DDLPS: LPS141, LPS510 and LPS12 and 1 WDLPS T449) with lentivirus expressing either c-Jun or RFP (negative control). c-Jun overexpression was confirmed both by western blot and RT-PCR (Fig. 3A). Cell viability assays demonstrated that overexpression of c-Jun in these cells did not have a significant impact on proliferation in two dimensional culture (FIG. S6).

We assayed the effect of c-Jun overexpression on gene expression in the three DDLPS cell lines using RNA-Seq (Illumina). c-Jun overexpression in LPS141, LPS12 and LPS510 significantly upregulated the expression of 351, 653 and 2283 genes and reduced the expression of 509, 891 and 1923 genes by more than two fold respectively. 58 of the upregulated and 84 of the downregulated genes were shared among the three cell lines.

Ingenuity Pathway Analysis revealed that genes implicated in cell migration were differentially expressed in c-Jun overexpressing cells relative to controls in all three cell lines (Fig. 3B). Candidate target genes identified in c-Jun knockdown experiments (ENPP2, RCAN1, ERG, ETS2, Inhibin1 and TGFBR3) showed an increase of 0.7 to ~12 fold (except ERG in LPS12) after c-Jun overexpression in the three DDLPS cell lines (Fig. 4A), which was confirmed by qRT-PCR (data not shown).

We then subjected c-Jun-overexpressing cells to a panel of cell migration assays. First, we measured LPS141 cell migration in a cell exclusion zone assay. We found that c-Jun overexpressing LPS141 cells were significantly more migratory than controls, with closure space of 93% and 45% at 3 days, respectively (Fig. 3C–D). Single cell tracking experiments demonstrated that c-Jun overexpression increased the velocity of LPS141, LPS510 and T449 cells by 2 to 4 fold (Fig. 3E). Finally, c-Jun overexpression enhanced migration and invasion of both LPS141 and T449 cells in Boyden chamber assays (Fig. 3F). Taken together, these results show that c-Jun overexpression is sufficient to enhance the migratory and invasive capacity of liposarcoma cell lines in vitro.

c-Jun drives expression of the motility factor ENPP2/Autotaxin in liposarcoma

We next sought to characterize c-Jun-responsive genes that might regulate cell migration. Autotaxin (ENPP2) is a nucleotide pyrophosphatase/phosphodiesterase [31] that is highly expressed in a number of tumor cell types [32–41], and can increase tumor invasiveness, metastasis, and neovascularization [42] [43]. Previous work has demonstrated that overexpression of v-Jun induces autotaxin in chick embryo fibroblasts [44]. c-Jun and JunD bind to a CRE/AP1-like site in the autotaxin promoter and enhance promoter activity, suggesting that autotaxin may be a direct c-Jun target [45].

Our data suggest that c-Jun also regulates autotaxin in liposarcoma. Autotaxin mRNA levels in LP6 cells are more than 130 fold higher than in LPS141 cells (non-amplified) (data not shown). Knockdown of c-Jun in LP6 cells led to decrease in autotaxin mRNA (Fig 2A). c-Jun overexpression is also sufficient to induce autotaxin mRNA and protein in two of three DDLPS cell lines (LPS141 and LPS12) (Fig. 4A–B).

We analyzed the biological effect of autotaxin on cell migration and invasion in LP6 and LPS141 cells. We found that stable autotaxin knockdown (Fig. 4C–D) significantly reduced both migration and invasion in LP6 cells but not LPS141 cells (Fig. 2C). Furthermore, single cell tracking experiments revealed that autotaxin knockdown reduced LP6 cell velocity by 30% compared to control cells, but had no effect on the velocity of LPS141 cells (Fig. 4E). In contrast, autotaxin

knockdown significantly reduced the velocity of LPS141 cells overexpressing c-Jun (Fig. 4F). We also found that overexpression of ENPP2 increased the velocity of both LPS141 and LP6 cells (Fig. S7). These data are consistent with the notion that autotaxin induction is one mechanism by which high c-Jun levels promote cell migration.

c-Jun promotes liposarcoma tumorigenicity in vivo

We have previously shown that c-Jun knockdown impairs the ability of LP6 cells to form subcutaneous tumors [20], suggesting that *c-Jun* amplification plays a role in augmenting in vivo tumorigenesis. We sought to extend these findings by testing the ability of exogenous c-Jun expression to enhance the tumorigenic potential of liposarcoma cell lines with normal *c-Jun* copy number.

Naïve LPS141 and LPS510 cell lines exhibited little growth up to 8 weeks after subcutaneous injection in nude mice (data not shown). We injected LPS141 and LPS510 cells (control and c-Jun-overexpressing) subcutaneously into the flanks of nude mice. At four weeks after injection, we observed small and pale lesions at the site where the 2 control cell lines were injected (Fig. 5A). Histologically, these small tumors consisted of liposarcoma and stromal cells scattered throughout matrigel with little evidence of proliferation. c-Jun overexpressing cells formed very cellular, high grade tumors that were larger than controls, more mitotically active (Fig. 5B, 5H and S8A), and exhibited c-Jun positivity in almost all cells (Fig 5E–F, K–L).

These data show that even though c-Jun overexpression does not significantly alter proliferation *in vitro*, it does dramatically enhance *in vivo* tumor formation. These results provide a rationale for the natural selection of *c-Jun*-amplified subclones during liposarcoma evolution *in vivo*.

c-Jun activity in other sarcoma types

To understand whether c-Jun amplification/overexpression can have similar functions in other sarcoma tumor types, we analyzed four cell lines derived from sarcomas that are histologically and genetically distinct from liposarcoma: one leiomyosarcoma (LMS03), two unclassified spindle cell sarcomas (USCS2 and USCS5) and one Alveolar Soft Part Sarcoma (ASPS-KY). SNP array analysis (data not shown) of these cell lines demonstrated that one of these cell lines (LMS03) exhibited high-level genomic amplification of *c-Jun*. Consistent with this finding, immunoblotting and qRT-PCR revealed that the levels of c-Jun protein in LMS03 cells are similar to those in LP6 cells (Fig. 6A and S8B).

c-Jun knockdown (Fig. 6B and Fig. S8C) caused a consistent reduction in cell viability in USCS2, USCS5 and ASPS-KY cells (Fig. 6C). The reduction in viability for these 3 cell lines (2.5–4 fold at day 3) was of an even greater magnitude than observed for most liposarcoma cell lines. c-Jun knockdown also impaired the migration capacity of these cell lines. Cell tracking studies demonstrated a ~2-fold decrease in the velocity of LMS03, USCS2 and ASPSKY cells upon c-Jun knockdown (Fig. 6D). Analysis of LMS03 migration in Boyden chambers revealed a decreased migratory capacity upon c-Jun knockdown (Fig. 6E). Interestingly, the velocity of USCS5 cells, whose c-Jun levels are approximately as low as LPS141 cells, was not affected by c-Jun knockdown (Fig. 6D)

We next asked whether c-Jun drives ENPP2 expression in these sarcoma cell lines. c-Jun knockdown led to a decline in ENPP2 mRNA levels in the three cell lines (LMS03, ASPS-KY, USCS2) with intermediate to high levels of basal c-Jun expression (Fig. 6F).

Furthermore, knockdown of ENPP2 via shRNA (Fig. 6G) reduced the velocity of cell migration in these three cell lines to nearly the same extent as c-Jun knockdown (Fig. 6D).

Finally, we determined whether c-Jun was critical for *in vivo* tumor growth in these sarcoma subtypes. Upon subcutaneous injection, LMS03, ASPS-KY, and USCS2 formed tumors greater than 0.5 cm within 8 weeks. Histologically, LMS03 and USCS2 were high-grade spindle cell sarcomas, whereas ASPS-KY tumors exhibited a typical organoid arrangement of neoplastic cells separated by thin walled vascular channels (Fig. 7 and S8D).

Stable knockdown of c-Jun reduced the tumorigenicity of LMS03 and ASPS-KY cells. After 4 weeks, the mean LMS03 tumor volume was 872 mm³ in control tumors and 187 mm³ in shJun cells (Fig. 7A–B). Control ASPS-KY tumors had a mean volume of 148 mm³, whereas shJun tumors averaged 59 mm³ (Fig. 7G–H). There was little difference in the histology of the tumors, except for an increased amount of residual matrigel in shJun tumors (Fig. 7). c-Jun was readily detectable by immunohistochemistry in control and shJun tumors (Fig. 7E–F, K–L), suggesting that shJun tumors were predominantly derived from cells that had escaped knockdown.

Altogether, our data suggest that high levels of c-Jun can promote cell migration and *in vivo* growth of multiple soft tissue sarcoma subtypes.

Discussion

Although *c-Jun* is genomically amplified and overexpressed in a subset of liposarcomas [20,22,23], the mechanisms by which it functions as an oncogene in these tumors have not been elucidated. A role for *c-Jun* amplification and overexpression in other types of soft tissue sarcoma has also remained unexplored. Using a panel of soft tissue sarcoma cell lines, we find that c-Jun regulates multiple processes associated with tumorigenesis, including proliferation, migration, and *in vivo* tumor growth.

Our data suggest a model in which basal levels of c-Jun regulate one set of biological processes in sarcomas, whereas high levels of c-Jun, achieved through genomic amplification or other mechanisms, promote a distinct set of biological functions. This model is suggested by the following observations:

Cellular proliferation—Knockdown of c-Jun by RNA interference impaired the *in vitro* proliferation of most cell lines tested (via G1 arrest). In contrast, c-Jun overexpression did little to augment *in vitro* proliferation rates, suggesting that only minimal levels of c-Jun are required to drive progression through the restriction point.

Cellular migration and invasion—In contrast to cellular proliferation, cell migration was not affected by c-Jun knockdown in cell lines with low basal levels of c-Jun (LPS141 and USCS5). Cell migration and invasion were significantly impaired in cells with genomic amplification (LP6 and LMS003) or c-Jun upregulation by undetermined mechanisms (ASPS and USCS2). Furthermore, c-Jun overexpression enhanced the migration of several cell lines lacking genomic amplification. These data suggest that high levels of c-Jun can

activate a motility program that is not dependent on the basal levels of c-Jun found in c-Jun-low tumors.

Our data implicate autotaxin/ENPP2 as one element of the c-Jun-regulated motility program in soft tissue sarcomas. Autotaxin is a constitutively active enzyme that possesses phospholipase D activity and hydrolyzes the head groups of lysophospholipids to lysophosphatidic acid (LPA) [46]. LPA is a multifunctional phospholipid that regulates proliferation, survival, migration and invasion of a variety of cancer types via the receptors LPA1-3 [46]. For example, LPA1 regulates cancer cell motility and metastasis [47] as well as matrix metalloproteinase expression [48]. LPA2 promotes the invasiveness of ovarian cancers by the activation of VEGF and uPA (urokinase-type plasminogen activator) [49].

In vivo tumorigenesis—Although c-Jun overexpression did not alter *in vitro* proliferation, it dramatically increased the tumorigenic potential of two cell lines with low basal c-Jun levels. These data provide a reason why *c-Jun* amplification and overexpression might confer a selective advantage during sarcoma progression. These data also suggest that high levels of c-Jun might regulate a set of genes that are required for growth in the complex *in vivo* environment but not *in vitro*. Additional studies will be needed to test this hypothesis and identify the c-Jun target genes critical for *in vivo* tumor formation.

Taken together, our data suggest that tumors with high levels of c-Jun (via amplification or other mechanisms) are likely to be more aggressive than c-Jun-low tumors, and a formal study with a large sample number is clearly warranted to address this point. Our finding that c-Jun can drive the expression of the motility factor ENPP2/autotaxin is particularly relevant given that a number of ENPP2/autotaxin inhibitors have been developed [50–53]. There is a need for additional studies to determine whether inhibitors of ENPP2 (and other c-Jun targets) display efficacy in the treatment of *c-Jun*-amplified soft tissue sarcomas. If so, the use of such inhibitors in combination with other agents might improve local control and even patient survival in this class of tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. c-Jun regulates gene networks associated with cell migration and proliferation in liposarcoma

A. Immunoblot for c-Jun on a panel of liposarcoma cell lines. Loading control: Hsp90.

B. Relative c-Jun mRNA levels in LPS cell lines. * $p < 0.05$ Data are represented as mean $\pm$ SEM from three independent experiments.

C. Immunoblot for c-Jun and p21 on liposarcoma cell lines LP6 and LPS141 after stable c-Jun knockdown by shRNA. Cells were harvested 7-days post-infection. NTC: non-targeted control.

D–E: Ingenuity Pathway Analysis (IPA) of gene expression changes in liposarcoma cell lines after c-Jun knockdown by stable RNA interference. Graphs depict the top five network functions enriched in control cells relative to c-Jun-inhibited cells. D. LP6 cells (*c-Jun* amplified). E. LPS141 cells (normal *c-Jun* copy number).

F. Quantification of cell viability using Cell Titer Glo in liposarcoma cell lines on days 4 to 9 after lentiviral delivery of shRNA. * $p < 0.04$. Data are represented as mean $\pm$ SEM from three independent experiments.

G. Cell cycle analysis of liposarcoma cell lines 7 days after lentiviral delivery of shRNA.

Figure 2. c-Jun knockdown impairs cell migration and invasion in liposarcoma cells

A. qRT-PCR validation of relative expression of genes involved in cell migration in LP6 cells. RNA was harvested 4 days post infection with lentivirus expressing the indicated shRNA. Expression levels were normalized to GAPDH. *p<0.05. Data are represented as mean $\pm$ SEM from three independent experiments

B. Cell exclusion zone assay to measure migration of LP6 cells infected with lentivirus expressing shGFP, shJUN1, shJUN3 and shJUN5. Cells were plated 6 days post infection. After 12 hours (time 0), plug was removed and cell migration was assessed daily for 3 days. Top: representative images. Bottom: quantitation of closure space over time. *p<0.04. Data are represented as mean $\pm$ SEM from three independent experiments.

C. Transwell migration and invasion assays were performed using the BD Biocoat Matrigel Migration Chamber (8- μ m pore size). The invading/migrating cells were stained with 0.04 % crystal violet. Cells were counted in three separated fields at 40x magnification and the percentage of invasion was calculated as invasion through the Matrigel membranes. *p<0.001. Data are represented as mean $\pm$ SEM from three independent experiments.

D – E. Cell velocity was assessed in LP6 (D) and LPS141 (E) cells expressing the indicated shRNA. Three days after shRNA delivery, cells were plated at low density. The migration of individual cells (n=40 per group) was then tracked for 15 hours. *p<0.0001. Data are represented as mean $\pm$ SD from two independent experiments.

Figure 3. c-Jun overexpression enhances cell migration and invasion in liposarcoma

A. Validation of lentiviral-mediated c-Jun overexpression in four liposarcoma cell lines with normal *c-Jun* copy number via immunoblotting (left) and qRT-PCR (right). $p<0.05$ for each cell line (c-Jun vs. control). Data are represented as mean $\pm$ SEM from three independent experiments.

B. IPA (Ingenuity Pathway Analysis) of c-Jun-overexpression-induced changes in gene expression. RNA-Seq analysis was performed on three pairs of liposarcoma cell lines (c-Jun overexpressing vs. controls) to identify genes whose levels significantly changed in response to c-Jun overexpression. Graphs depict the top 5 network functions associated with c-Jun overexpression in each cell line.

C–D. Cell exclusion zone assay to measure migration of 141 control and c-Jun overexpressing cell lines. Inserts were removed from wells at time 0 and the cell-free area was quantitated at 24, 48 and 72 hours. C: representative photomicrograph. D: Quantitation of closure area percentage. * $p<0.01$

E. Quantitation of cell velocity in liposarcoma cell lines overexpressing c-Jun in comparison with controls (n=40 cells/condition from 2 independent experiments, mean $\pm$ SD). * $p<0.01$.

F. Quantitation of migration and invasion via Boyden chamber assay in liposarcoma cell lines overexpressing c-Jun in comparison with controls. * $p<0.02$. Data are represented as mean $\pm$ SEM from three independent experiments.

Figure 4. ENPP2 is induced by c-Jun overexpression and promotes liposarcoma cell migration

A. Fold change in migration-related genes upon c-Jun overexpression in three DDLPS cell lines (141, 510 and LPS12) as assessed by RNA-Seq (FPKM c-Jun overexpressing cells/ FPKM control cells).

B. Immunoblot for c-Jun, RCAN1, ETS2 and ENPP2 in control and c-Jun overexpressing DDLPS cell lines. Hsp90: loading control.

C–D ENPP2 levels in LP6 and 141 cells expressing the indicated shRNA as assessed by immunoblot (C) and qRT-PCR (D, data normalized to GAPDH). Cells were harvested 7-days post-infection. * $p < 0.05$. For qRT-PCR, data are represented as mean $\pm$ SEM from three independent experiments. Actin: loading control.

E. Quantitation of cell velocity via single cell tracking in LP6 and 141 cells after stable shRNA-mediated ENPP2 knockdown. 40 cells were analyzed in 2 independent experiments and represented as mean $\pm$ SD. * $p < 0.02$

F. Quantitation of cell velocity via single cell tracking in c-Jun overexpressing 141 cell lines in the presence or absence of ENPP2 knockdown. 40 cells were analyzed in 2 independent experiments and represented as mean $\pm$ SD. * $p < 0.02$ ** $p < 0.04$

Figure 5. c-Jun overexpression enhances the growth of liposarcomas in vivo

Control and c-Jun overexpressing 141 and 510 cells were injected subcutaneously into nude mice (n= 5). Tumors were analyzed 4 weeks after injection. (A, G) Photograph of tumors. (B, H) Quantitation of tumor volume (mean $\pm$ SD). * $p < 0.01$. (C–D, H–I) H&E stain. (E–F, J–K) Immunohistochemistry for c-Jun.

Figure 6. c-Jun and ENPP2 regulate cell migration in other sarcoma subtypes

A. Immunoblot for c-Jun in a panel of human sarcoma cell lines. LMS003 and LP6 exhibit genomic amplification of *c-Jun*. HSP90: loading control.

B. Immunoblots demonstrating stable knockdown of c-Jun via shRNA in three sarcoma cell lines. HSP90: loading control.

C. Quantitation of cell viability using Cell Titer-Glo in sarcoma cells after c-Jun knockdown by shRNA. Time 0 = 4 days post infection. Data are represented as mean $\pm$ SEM from three independent experiments. * $p < 0.02$

D. Quantitation of sarcoma cell velocity via single cell tracking after stable knockdown of c-Jun and ENPP2 by shRNA. 40 cells/condition were analyzed in 2 independent experiments. Data are represented as mean $\pm$ SD. * $p < 0.001$

E. Quantitation of migration and invasion via Boyden chamber assay in LMS003 after stable knockdown of c-Jun and ENPP2 via shRNA. Cells were plated 6 days after lentiviral infection. Data are represented as mean $\pm$ SEM from three independent experiments. * $p < 0.001$.

F. Relative ENPP2 mRNA levels in sarcoma cell lines after c-Jun knockdown via shRNA. Gene expression levels are normalized to GAPDH. Data are represented as mean $\pm$ SEM from three independent experiments. * $p < 0.02$

G. Immunoblot demonstrating stable knockdown of ENPP2 via shRNA in three sarcoma cell lines. Actin: loading control.

Figure 7. c-Jun knockdown attenuates the tumor growth in other sarcomas in vivo

Control and c-Jun-inhibited LMS003 and ASPS were injected subcutaneously into nude mice 9 days post infection. Tumors were analyzed 4 weeks after injection. (A, G) Photograph of tumors. (B, H) Quantitation of tumor volume * $p < 0.01$. (C–D, I–J) H&E stain. (E–F, K–L) Immunohistochemistry for c-Jun.