Caveolin-1 Reduces Osteosarcoma Metastases by Inhibiting c-Src Activity and Met Signaling

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Abstract

Caveolin-1 (Cav-1) is highly expressed in normal osteoblasts. This article reports that Cav-1 down-regulation is part of osteoblast transformation and osteosarcoma progression and validates its role as oncosuppressor in human osteosarcoma. A survey of 6-year follow-up indicates a better overall survival for osteosarcoma expressing a level of Cav-1 similar to osteoblasts. However, the majority of primary osteosarcoma shows significantly lower levels of Cav-1 than normal osteoblasts. Accordingly, Met-induced osteoblast transformation is associated with Cav-1 down-regulation. In vitro, osteosarcoma cell lines forced to overexpress Cav-1 show reduced malignancy with inhibited anchorage-independent growth, migration, and invasion. In vivo, Cav-1 overexpression abrogates the metastatic ability of osteosarcoma cells. c-Src and c-Met tyrosine kinases, which are activated in osteosarcoma, colocalize with Cav-1 and are inhibited on Cav-1 overexpression. Thus, Cav-1 behaves as an oncosuppressor in osteosarcoma. Altogether, data suggest that Cav-1 down-modulation might function as a permissive mechanism, which, by unleashing c-Src and Met signaling, enables osteosarcoma cells to invade neighboring tissues. These data strengthen the rationale to target c-Src family kinases and/or Met receptor to improve the extremely poor prognosis of metastatic osteosarcoma. [Cancer Res 2007;67(16):7675–85]

Introduction

The frequency of primary malignant bone tumors ranks osteosarcoma second after multiple myeloma. Osteosarcoma is a high-grade malignant tumor composed of mesenchymal cells producing osteoid and immature bone with a peak incidence in the second decade of life (1). Although current treatment modalities, which include surgery and neoadjuvant multidrug chemotherapy, significantly improved the 5-year disease-free survival from 10% to 60%–70% (2,3), no significant new drugs or treatment approaches have been developed during the last 10 years, and the percentages of cure remain unacceptably low for high-risk patients (4). Drug resistance and metastasis are the major causes of treatment failure. Differently from carcinomas, molecular genetics of osteosarcoma progression is not documented and the most accepted prognostic factors are based on response to treatment (percentage of necrosis or P-glycoprotein expression; refs. 5–8) rather than on genetic signature characterizing the development and progression, with few exceptions (9–11). To search for such signature, we did a microarray analysis of genes involved in modulation of osteosarcoma malignancy by comparing osteosarcoma cell lines with different metastasis behavior and identified caveolin-1 (Cav-1) gene whose expression correlates with a lower metastasis potential (12,13). Cav-1 is the major protein component of caveolae (14), which are abundant in terminally differentiated mesenchymal cells, including adipocytes, endothelial cells, fibroblasts (15), and osteoblasts (16,17). Caveolae provide physical interaction and compartmentalization of several membrane receptors. As part of receptor signaling pathways, Cav-1 plays functions that are important in tumorigenesis and metastasis (see refs. 18–20 for reviews). However, despite significant studies have been done over the last 10 years, its functions in malignancy are very complex and still unclear, as Cav-1 has been described as either a tumor suppressor or a tumor promoter, depending on tumor type and/or stage. Although overexpression of Cav-1 is associated with, and in fact causal in metastasis and progression in some types of carcinomas, particularly prostate cancer (21,22), in breast cancer as well as fibrosarcoma, Cav-1 has been characterized as oncosuppressor (23–25). In this article, we validate the role of Cav-1 as oncosuppressor in human osteosarcoma, as we show its down-modulation in more aggressive tumors and show Cav-1 ability to suppress the invasive-metastatic ability of osteosarcoma cells in functional assays in vitro and in vivo.

Materials and Methods

Cell lines and transfection. The osteosarcoma cell lines U-2 OS, Saos-2, and MG63 were obtained from the American Type Culture Collection. IOR/OS-7 and IOR/OS-9 were obtained at the Laboratorio di Ricerca Oncologica, Istituti Ortopedici Rizzoli (Bologna, Italy) and previously characterized (26). Human osteoblasts (HOS) and their Met-transformed variants were obtained at the Laboratory of Cancer Genetics, Institute for Cancer Research and Treatment, University of Turin [Candiolo (Turin), Italy] and previously characterized (27). pcDNA3 vector was used to construct plasmids expressing mouse Cav-1 (28), which differs from human Cav-1 at only two amino acids and is functionally indistinguishable from the human protein (15), and human antisense Cav-1 (21). Stable transfectants expressing Cav-1 were obtained from U-2 OS, whereas Cav-1-deficient clones were derived from U-2 OS and Saos-2 by using calcium-phosphate transfection method. Cells transfected with the empty vector pcDNA3 were used as negative controls. Transfectants were selected in Iscove’s modified...
Dulbecco’s medium (IMDM) containing 10% fetal bovine serum (FBS) and 500 µg/ml neomycin (Sigma) and maintained in selective medium.

**Cytotoxicity analysis of Met.** Met expression was analyzed by indirect immunofluorescence using DO-24 antibody against the extracellular domain of human protein 1:50 dilution; ref. 29. A

**Analysis of growth features in monolayer conditions.** Doubling time was determined by daily harvesting of cells after seeding of 20,000 cells/cm² in IMDM 10% FBS. Cell viability was determined by trypan blue dye exclusion. Cytotoxicity of DXR on U-2 OS cells and derived clones evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (In Vitrogen) was done according to the manufacturer’s instructions.

**Soft agar assay.** Anchorage-independent growth was determined in 0.33% agarose (SeaPlaque, FMC BioProducts) with a 0.5% agarose overlay. Cell suspensions (10,000–33,000 cells per 60-mm dish) were plated in semisolid medium (IMDM 10% FBS plus agar 0.33%) and incubated at 37°C in a humidified 5% CO₂ atmosphere. Colonies were counted after 10 days.

**Poly-HEMA assay.** Six-well plates were treated with poly(2-hydroxyethylmethacrylate) (poly-HEMA; Sigma) following the Folkman and Moscona manufacturer’s instructions (Medical & Biological Laboratories). Becton Dickinson) of Annexin V-FITC–labeled cells according to the apoptotic cells was obtained by flow cytometric analysis (FACSCalibur, Becton Dickinson) and Annexin V and propidium iodide were measured on adherent cells grown on coverslips for 48 h and fixed in 4% paraformaldehyde. Cells (250,000 per well) were seeded in IMDM 10% FBS and incubated at 37°C in a humidified 5% CO₂ atmosphere. Viable and dead cells were counted after 24, 48, and 72 h. Detection and quantification of apoptotic cells was obtained by flow cytometric analysis (FACS Calibur, Becton Dickinson) of Annexin V–FITC–labeled cells according to the manufacturer’s instructions (Medical & Biological Laboratories).

**Motility assay.** Motility assay was done using Transwell chambers (Costar). Cells (10⁴) in IMDM plus 10% FBS with or without different c-Src inhibitors (PP2, 5 µmol/L; PP3, 5 µmol/L; all provided by Calbiochem) or Src activator pYEEI (100 µmol/L; Biomol Research Laboratories) were seeded in the upper compartment, whereas IMDM plus 10% FBS was placed in the lower compartment of the chamber. Experiments were done in triplicate.

**Wound-healing assay.** Monolayer wounds were made using a pipette tip on confluent cells. Cell migration was visualized, at regular intervals of time, at ×100 magnification using an inverted microscope (Nikon Diaphot) and photographed with a Nikon D70s digital camera.

**Invasion assay.** Cells (5 × 10⁵) were seeded in the upper compartment of Biocoat Matrigel invasion chambers (BD Biosciences), and assay was done according to the manufacturer’s instructions. All experiments were made in triplicate.

**Extracellular matrix adhesion assay.** Adhesive ability of U-2 OS transfectants was analyzed by using CytoMatrix cell adhesion strips coated with human collagen type I and IV, fibronectin, or laminin (Chemicon International) as described previously (30). Fluorescence on adherent fixed cells. Immunofluorescence was done on adherent cells grown on coverslips for 48 h and fixed in 4% paraformaldehyde, permeabilized with 0.15% Triton X-100 in PBS, and incubated with the following antibodies: α-Cav-1 (1:100 dilution; BD Transduction Laboratories), α-c-Src (1:10 dilution; Cell Signaling Technology), and α-h-Met (C-28; 1:10 dilution; Santa Cruz Biotechnology).

**Western blotting.** Western blotting experiments were done as described previously (13). Membranes were incubated with the following primary antibodies: anti-Cav-1 polyclonal antibody (1:10,000 dilution; BD Transduction Laboratories) and anti–phosphorylated Src Tyr Tyr145 (1:1,000 dilution; Cell Signaling Technology). Analysis of c-Src and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also done to verify the total protein as control (primary antibodies: α-c-Src, diluted 1:1,000; α-GAPDH, diluted 1:2,000; Santa Cruz Biotechnology). Anti-rabbit secondary antibody conjugated to horseradish peroxidase was from Amersham.

**Immunoprecipitation and c-Src tyrosine kinase activity assay.** Equivalent amounts of total cell lysates (800 µg) were immunoprecipitated by adding either 1.5 µg sheep anti-human polyclonal anti-p60 Src antibody (Chemicon International) and incubated overnight at 4°C. Protein G Plus/ protein A-agarose beads (Calbiochem) were then added to each lysate and incubation continued for 1 h. Beads were then collected and washed twice with lysis buffer and once in Src kinase reaction buffer (Upstate). Washed beads were used directly to evaluate c-Src tyrosine kinase activity using a nonradioactive ELISA-based assay according to the manufacturer’s instructions (Chemicon International).

**Determination of c-Src and Akt phosphorylation by cell-based ELISA.** Cells were seeded into 96-well microplates at the density of 5 × 10⁴ per well in IMDM 10% FBS. Forty-eight hours after seeding, cells were fixed in 4% formaldehyde at room temperature and thereafter used to measure Akt and Src phosphorylation using a cell-based CASE ELISA kit (SuperArray) according to the manufacturer’s instructions. To analyze the status of intracellular mediators after hepatocyte growth factor (HGF) stimulation, cells were starved for 24 h and then exposed to 40 ng/ml HGF for 30 min. Absorbance readings were normalized to relative cell number as determined by a cell staining solution and to the amount of total protein.

**Tumorigenic and metastatic ability in athymic mice.** Female athymic 5-week-old CrI-luzu/nu (CD-1) BR mice (Charles River Italia) were used. Tumorigenicity was determined after s.c. injection of 30 × 10⁵ cells, whereas metastatization was evaluated after i.v. injection of 2 × 10⁶ cells. Tumor growth was assessed once weekly by measuring tumor volume. Negative mice were checked for 6 months after cell injection. The number of pulmonary metastases was determined 2 months after cell inoculation. Experimental procedures were approved by the local ethical committee.

**Patients and quantitative PCR analysis of Cav-1.** The study includes 37 RNA samples extracted from osteosarcoma tumor specimens from primary lesions of previously untreated patients. Osteosarcoma patients were seen at the Istituti Ortopedici Rizzoli and treated with consecutive chemotherapy protocols (IOR/3a and IOR/3b) previously reported in detail (31, 32). All patients provided written informed consent according to local institutional review board. Investigation was conducted according to Declaration of Helsinki principles. Adverse events were defined as tumor recurrence at any site or death during remission. Event-free survival was calculated from the first day of chemotherapy until the date of first relapse or tumor-related death (for patients classified as relapsed) or to the date of the last follow-up examination. Overall survival was calculated from the first day of chemotherapy to death or to the last follow-up examination.

Total RNA was extracted by Trizol extraction kit (Invitrogen), and the quality of the RNA samples was determined by electrophoresis and by Bioanalyzer (Agilent). Universal Master Mix (Applied Biosystems) was used with 10 ng cDNA and with 200 nmol/L of primers for evaluation of GAPDH and Cav-1 expression, respectively. Negative controls without cDNA template were run with each assay. For GAPDH and Cav-1 specific Taqman assays were purchased from Applied Biosystems (Tagman Gene Expression Assay; Ref. Seq. Hs99999901_s1 and Hs00184697_m1).

**Statistical analysis.** The difference among groups were analyzed using Student’s t test. Fischer’s exact test was used for frequency data. Survival curves were drawn and evaluated by Kaplan-Meier and log-rank methods.

**Results**

**Met-transformed osteoblasts and osteosarcoma samples show lower Cav-1 expression than normal osteoblasts.** Osteosarcomas originate from mesenchymal cells having osteoblastic features. Osteoblasts have caveolae and express both Cav-1 and Cav-2 isoforms (16). It has been noted that faster-growing osteoblasts have less caveolae (16) and that Cav-1 expression increases along osteoblastic differentiation (12). So we wondered if osteoblast transformation was associated to Cav-1 change of expression. We took advantage of the recently established model of HOS transformation by the overexpressed MET oncogene (27). It is noteworthy that the MET oncogene is aberrantly overexpressed in ~100% of human osteosarcomas and overexpression is associated to an aggressive phenotype (33, 34). We found that Cav-1 is significantly down-regulated in HOSs transformed by the wild-type MET or the MET oncogene activated by Y1253D mutation (Fig. 1A and B; ref. 35). Interestingly, down-modulation was not reverted by the subsequent expression of a dominant-negative Met kinase, which only partially impairs the
transformed phenotype (27). These results indicate that Cav-1 down-modulation is part of the Met-induced osteoblast transformation toward a sarcomatogenous phenotype. Accordingly, we observed a reduction of the relative expression of Cav-1 evaluated by means of quantitative PCR in osteosarcomas in comparison with normal osteoblast primary cultures [median values: 1.48 (range, 0.06–14.6; n = 37) in osteosarcoma versus 4.57 (range, 4.2–6.1; n = 3) in osteoblasts; P = 0.03].

Expression of Cav-1 modulates anchorage-independent growth, migration, and invasion. To directly analyze the role of Cav-1 in osteosarcoma malignancy, we modulated this protein expression in osteosarcoma cell lines. We transfected the U-2 OS cell line with the Cav-1 gene either in direct or in reverse orientation. Three transfectants that overexpressed the molecule and three with a very low level of Cav-1 expression were chosen. Figure 1C and D shows the relative expression of Cav-1 in clones, visualized by Western blotting (C) and immunofluorescence (D).

Neither forced expression nor abrogation of Cav-1 significantly influenced growth rate of U-2 OS in monolayer cultures. All clones overexpressing Cav-1 and those lacking the protein showed doubling times similar to control, ranging from 16.9 ± 1.4 h to 20.4 ± 1.8 h (P = not significant, Student’s t test; Supplementary Fig. S1). In anchorage-independent conditions (i.e., when grown in soft agar; Fig. 2A), forced overexpression of Cav-1 reduced both number and size of colonies, whereas abrogation of Cav-1 induced the opposite effect. To test whether the different ability to grow in soft agar was caused by different sensitivity to anoikis (detachment-induced apoptosis), we examined proliferation and survival of cells grown onto dishes coated with poly-HEMA that prevents cell adhesion (30). Indeed, overexpression of Cav-1 severely reduced cell survival, whereas its silencing significantly increased the ability of U-2 OS cells to survive and proliferate in conditions where cell anchorage is prevented (Fig. 2B). No significant induction in the apoptotic rate of these cells was, however, observed by Annexin V fluorescent test. Migration and invasion were also significantly affected by Cav-1 expression. Suppression or overexpression of Cav-1 promoted or inhibited, respectively, migration (Fig. 2C) and invasion of U-2 OS cells through a reconstituted basement membrane made of Matrigel (Fig. 2D).

Loss of Cav-1 induces metastasis in experimental conditions and correlates with ominous clinical course in osteosarcoma patients. Adhesion to extracellular matrix components was severely reduced in cells lacking Cav-1 expression. When compared with parental cells, the two clones expressing antisense Cav-1 sequence showed 50% to 80% reduction of adhesion to collagen I, collagen IV, fibronectin, and laminin (Fig. 3A), whereas two clones overexpressing Cav-1 displayed similar adhesion.

The way Cav-1 influenced migration, invasion, and extracellular matrix interaction of osteosarcoma cells anticipates its role in metastasis. Indeed, we observed striking differences in the ability of

Figure 1. Expression of Cav-1 in in vitro–transformed HOSs and in U-2 OS–transfected cells. A and B, down-regulation of Cav-1 in HOSs transformed by overexpression of either wild-type (wtMET-HOS) or mutationaly activated Met receptor (mutMET-HOS). Clones shown (c15, c12, and c42) are described in detail in ref. 27. When indicated, Met-HOS clones were further transduced with dominant-negative Met (DN-MET; ref. 27), which partially impaired the Met-dependent phenotype. A, Cav-1 mRNA is quantified by measuring C_T to determine relative expression. B, Cav-1 expression by Western blotting analysis. C, Western blotting analysis of Cav-1 expression in total lysates extracted from parental cells and clones transfected either with Cav-1 cDNA in sense or antisense orientation or with plasmid vector alone (empty). D, Cav-1 expression in adherent cells. Magnification, ×600. All data and digital images were taken in identical conditions at the same time and using the same image analysis software (Quips XL genetic workstation, Abbott-Vysis).
Figure 2. In vitro biological properties of U-2 OS cells modified for Cav-1 expression. A, growth in soft agar of parental osteosarcoma cells and of clones overexpressing (Cav) or lacking (anti-Cav) Cav-1. Cells were seeded at a concentration of 10,000 to 33,000 and the number of colonies in triplicate plates was determined after 10 d of growth in 10% FBS. Columns, mean of six independent experiments; bars, SE. *, P < 0.01, Student’s t test, with respect to U-2 OS parental cells. Right, representative fields. B, survival of U-2 OS cells and derived clones on poly-HEMA–coated dishes. Points, mean of triplicate experiments; bars, SE. *, P < 0.05; **, P < 0.01, Student’s t test. Annexin V test was used to highlight apoptotic cells. C, migration of U-2 OS cells overexpressing and lacking Cav-1. Columns, mean of three independent experiments; bars, SE. *, P < 0.01, Student’s t test. Wound-healing assay. Pictures were taken at time 0 and after 18 h from wound. D, invasion ability of U-2 OS transfectants through Matrigel. Columns, mean of three independent experiments; bars, SE. *, P < 0.05; **, P < 0.01, Student’s t test. Right, representative fields. Magnification, ×100.
osteosarcoma cells to establish lung metastases depending on Cav-1 expression. Cav-1–deficient osteosarcoma cells inoculated i.v. in athymic mice produced a very high number of metastases, whereas Cav-1 overexpression abrogated even the minimal ability of parental cells to give lung colonies (Fig. 3B). The mean number of metastases was >250 metastases (range, 250 to >250; n = 7) in mice receiving cells lacking Cav-1, 2 (range, 0–16; n = 7) in mice inoculated with parental U-2 OS cells, and 0 (n = 7) in mice inoculated with Cav-1–overexpressing cells.

As metastasis is a major determinant of outcome in osteosarcoma patients, we investigated whether Cav-1 expression in human osteosarcoma samples correlated to patient outcome. We measured Cav-1 relative expression levels in clinical osteosarcoma samples by means of quantitative PCR (Fig. 3C). Patients, with a minimum follow-up of 6 years, were stratified according to median values in high (2^ΔΔCT ≥ 1.5) or low (2^ΔΔCT ≤ 1.5) Cav-1-expressing cases. Globally, overexpression of Cav-1 was observed in 19 of 37 (51%) samples. High levels of Cav-1 were associated with a favorable overall survival but not with a better event-free survival (Fig. 3D). Indeed, the incidence of Cav-1 overexpression was higher in patients still alive after 6 years than in patients who died from metastasis [16 of 25 (64%) versus 2 of 11 (18%), respectively;
P = 0.03, Fisher’s exact test], although Cav-1 overexpression was evenly distributed when the first relapse occurred (52% of positive cases in patients with no evidence of disease versus 48% in relapsed patients).

We considered that patient outcome depends on both tumor intrinsic malignancy and response to treatments, which very likely determine overall and event-free survival, respectively. Thus, we studied the possible association between Cav-1 expression and response to chemotherapy.

In vitro MTT assay indicated that Cav-1 did not significantly modulate osteosarcoma response to doxorubicin, one of the leader drugs in the treatment of sarcomas (Supplementary Fig. S2), further confirming that Cav-1 expression correlates with osteosarcoma aggressiveness rather than chemosensitivity.

Moreover, we studied three cases of patients with high-grade periosteal osteosarcoma who had wide surgery but no chemotherapy. The two patients whose tumors showed high Cav-1 mRNA expression \((2^{-\Delta\Delta C_{T}} \geq 1.5}) were disease-free after 8 years, whereas the one with a low Cav-1–expressing tumor had metastasis within 14 months from surgery. Although very limited, this small series further indicates that Cav-1 might be a marker for less malignant tumors.

c-Src and Met receptor tyrosine kinases are relevant cellular mediators of Cav-1 functions in osteosarcoma cells. Cav-1 is known to down-regulate the signaling of many proteins (20), such as kinases of c-Src family and tyrosine kinase receptors. Accordingly, we observed a significant decrease of c-Src family kinase activity in cells overexpressing Cav-1 (Fig. 4A and B), whereas the abrogation of Cav-1 induced enhancement of both expression and activity of c-Src. The latter cells also showed increased motility, likely due to Src activation, as the Src inhibitor PP2 suppressed their cell migration, whereas the inactive structural analogue PP3 was ineffective (Fig. 4C). Involvement of c-Src as mediator of Cav-1 intracellular signaling was also confirmed in U-2/Cav clones in which endogenous c-Src activity was activated using pYEEI, a peptide that competes with the negative regulatory role/s of SH2 domain (36, 37). Indeed, exposure of parental and U-2/Cav cells to pYEEI significantly increased cell motility (Fig. 4C).
In addition, kinase assay confirmed a higher c-Src enzymatic activity in cells lacking Cav-1 (Fig. 4D), whereas those over-expressing Cav-1 showed lower levels of activity according to their low migration ability. In keeping with these data, a different osteosarcoma cell line, Saos-2, showed a higher basal level of Cav-1 (Fig. 5A and B), lower amount of c-Src Y416 phosphorylation (Fig. 5B), and minor ability to grow in soft agar and invade Matrigel (Fig. 5C) than U-2 OS. Accordingly, Saos-2 cells lacking Cav-1

![Image](image_url)

Figure 5. Cav-1 expression and c-Src family kinase activity are inversely related and oppositely modulate migration and anchorage-independent growth of Saos-2 osteosarcoma cells. A, immunostaining of Cav-1 on adherent U-2 OS and Saos-2 cells. Caveolin is labeled by anti-caveolin antibody and revealed by FITC-conjugated anti-rabbit secondary antibody. B, evaluation of c-Src kinase activity in U-2 OS and Saos-2 cells by Western blotting and Src tyrosine kinase assay. C, higher expression of Cav-1 corresponds to lower Y416 c-Src phosphorylation and lower ability of Saos-2 cells to grow in soft agar and to invade Matrigel. *, P < 0.05, Student's t test. D, loss of Cav-1 in Saos-2 cells, by antisense Cav-1 transduction, confirmed the inverse correlation between Cav-1 expression and c-Src activity as well as osteosarcoma malignancy as indicated by anchorage-independent growth, migration, and invasion. *, P < 0.05; **, P < 0.001, Student's t test, with respect to parental cell line.
enhanced motility, growth, and survival in anchorage-independent conditions and invasion through Matrigel and displayed a higher level of c-Src Y416 phosphorylation than parental Saos-2 cells (Fig. 5D). In addition, Saos-2 cells expressed Met at a lower level than U-2 OS osteosarcoma cells (Fig. 6A). The inverse relationship between Cav-1 and Met expression and correlation to malignancy was confirmed in other osteosarcoma cell lines (Table 1) as also shown in Met-transformed osteoblasts (see above). In addition, U-2 OS clones overexpressing Cav-1 showed lower levels of Met expression (Fig. 6B), indicating that oncosuppressor activity of Cav-1 might account for Met functions. Moreover, coexisting with anti-Cav-1 and anti-Met antibodies showed their colocalization at plasma membrane (Fig. 6C). Therefore, we tested whether Cav-1 and Met expression by down-regulating Met signaling pathway. Both extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) are important mediators of proliferation, motility, and invasion of osteosarcoma cells in response to Met ligand (HGF) stimulation (38). In addition, c-Src signaling is activated by HGF taking part to migration, invasion, and anchorage-independent growth of other tumor cell types (39, 40). A colocalization between Cav-1 and c-Src is here shown (Fig. 6C) in agreement with previous reports (41, 42). Although we did not observe any change in ERK/MAPK activation status of Cav-1-overexpressing U-2 OS (data not shown), the latter cells showed reduced PI3K activation with respect to parental cells both in basal conditions and, more importantly, after exposure to HGF (Fig. 6D). Striking attenuation of c-Src kinase activity was also observed in the U-2 OS model, although the use of anti-Cav Saos-2 cells provided mirrored information (Fig. 6D), further confirming that Cav-1 modulates HGF signaling by negatively regulating both Src and Akt activities.

Discussion

This article investigated the role of Cav-1 in osteosarcoma malignancy. Cav-1 is highly expressed in normal osteoblasts (16). In these cells, important signaling molecules (e.g., growth receptors, heterotrimeric G proteins, Src family tyrosine kinase, and MAPK pathway) form cluster within caveolin-rich membrane complexes (16, 17). We show that Cav-1 is down-regulated in HOSs transformed by overexpressing either the wild-type Met, the tyrosine kinase receptor of HGF, or its mutationally activated version (27). This correlates with the observation that primary osteosarcomas have significantly lower levels of Cav-1 than normal osteoblasts.

When in osteosarcoma, Cav-1 expression is up-regulated, and similar to that of normal osteoblasts, patients show a significantly better outcome. An association with favorable prognosis was observed with overall, but not event-free, survival of osteosarcoma patients. This different clinical effect is, in our opinion, due to the
fact that Cav-1 is a key determinant of biological malignancy of osteosarcoma cells but does not influence their sensitivity to chemotherapeutic agents. Consistently, we did not observe any variation in chemosensitivity to doxorubicin, a major drug in the treatment of osteosarcoma, in cells deprived or abundant of Cav-1. On the contrary, modulation of Cav-1 expression in osteosarcoma cell lines greatly modifies the pattern of anchorage-independent growth, migration, invasion, adhesion to extracellular matrix components, and, more importantly, their metastatic ability. Abrogation of Cav-1 dramatically enhanced lung metastasis in athymic mice, in agreement with greater resistance to anoikis and independence from anchorage for growing, lower adhesion to fibronectin, laminin, and collagens, and a striking motile phenotype in vitro. These results show that Cav-1 suppresses osteosarcoma malignancy and are in line with previous observations in fibrosarcoma and other mesenchymal tumors (23) but not with the recent report on Ewing’s sarcoma that indicates an oncogenic role for Cav-1 (43). These contradictory findings may reflect the different origin and genetic features of osteosarcoma and other mesenchymal tumors compared with Ewing’s sarcoma, the two neoplasms being the prototypes of two distinct classes of sarcoma (44), and support the need to study each gene in its appropriate cellular context. This seems to be particularly true for Cav-1 that has been described as an oncogene or a tumor suppressor gene depending on the cell types. Particularly in prostate carcinoma (22) but also esophageal squamous cell, non–small cell lung carcinoma, and Ewing’s sarcoma (43, 45, 46), up-regulation of the protein contributes to metastatic phenotype. The opposite seems to occur not only in osteosarcoma and fibrosarcoma (23) but also in mammary (47) and colon (48) carcinogenesis. All these apparently contradictory results might be related to the role of Cav-1 in the differentiation of each cell type. More likely, Cav-1 might mainly play a permissive role by unleashing the full oncogenic or antioncogenic properties of the proteins, which are sequestered in caveolae in each tumor type.

In osteosarcomas, low Cav-1 expression is unlikely due to genetic or epigenetic alterations. In fact, although Cav-1 gene maps to a known fragile site (7q31.1) that shows deletions in a variety of malignancies, no mutation of Cav-1 gene nor evidence of its methylation within the promoter or in the first two exons has been reported in osteosarcoma cells (49). More likely, Cav-1 expression is regulated at the level of transcription, and in osteosarcoma cells, it may be also further decreased by a feedback circuit. We show that Met signaling is enhanced in osteosarcoma cells where Cav-1 is suppressed; it is noteworthy that the consistent and high expression of Met in osteosarcoma (33, 34) could consecutively contribute to Cav-1 down-modulation. In addition, Met ligand HGF is abundant in bone tumor microenvironment and might be responsible of Cav-1 down-regulation through inhibition of its promoter activity as shown in endothelia and muscle satellite cells (50–52). Conversely, Cav-1 expression might be a marker of osteoblast differentiation and low Cav-1 an intrinsic property of the osteoblast stem cell or progenitor targeted by transformation. Indeed, we observed a direct relationship between Cav-1 expression and level of osteoblastic differentiation in osteosarcoma cell lines (12) and, more recently, found evidence of physical connection between Cav-1 and CD99 (13), a molecule involved in cell-cell adhesion, migration, and apoptosis that was also implicated in osteoblastic differentiation (53, 54). A correlation between Cav-1 expression and osteoblastic differentiation was also confirmed when undifferentiated U-2 OS cells were compared with the osteoblast-like Saos-2 cells. Of note, expression of Met is inversely correlated with osteoblastic differentiation (33), reinforcing the view of Met contributing to osteoblast transformation and osteosarcoma progression in association to the down-modulation of Cav-1 and loss of differentiation. The latter hypothesis is supported by data showing that impairment of Met-dependent phenotype in transformed osteoblasts, by means of a dominant-negative Met receptor, does not directly influence Cav-1 expression.

Regardless of whether Cav-1 low expression is an intrinsic property of cells targeted by transformation or a consequence of transformation, the oncosuppressor role of Cav-1 may pass through the modulation of activity of c-Src family tyrosine kinases as well as Met signaling. Cav-1 is known to bind and sequester Src family kinases in an inactive configuration (41, 42). Furthermore, Cav-1 enhances inactivation of c-Src by CSK, therefore directly and indirectly shifting the equilibrium in favor of inactive c-Src conformation (55). Accordingly, Cav-1–deprived osteosarcoma cells exhibited marked increase in c-Src family tyrosine kinase activity, which significantly contributes to their migration and, very likely, to anchorage-independent growth. In fact, motility of cells lacking Cav-1 was strikingly suppressed when c-Src kinase activity was

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<th>Cell line</th>
<th>Cav-1 expression* (ΔΔCt)</th>
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*Relative mRNA Cav-1 expression evaluated by real-time PCR: −ΔΔCt = (Ct Cav-1 − Ct GAPDH). Higher −ΔΔCt therefore indicates a higher level of mRNA target expression.
† Cytofluorimetry evaluation of cells immunostained with DO-24 anti-Met monoclonal antibody. Data from one representative experiment.
‡ Cells (30 × 10⁶) were s.c. injected in nude mice. Incidence of positive mice/total.
inhibited. On the other hand, cells overexpressing Cav-1, which exhibited very low migration capacities, showed low levels of c-Src activity. When restored by using a Src-specific activator, cells consequently increased their migration. We also found Cav-1 overexpression associated to decrease of Met expression and signaling in U-2 OS and Saos-2 osteosarcoma cells, indicating that the oncosuppressor activity of Cav-1 also accounts for Met functions. Our hypothesis is that Met is endocytosed from the cell surface by a caveola-dependent mechanism in analogy with the Cav-1-mediated internalization process that has been described to counteract epidermal growth factor receptor signaling (56). In the presence of abundant Cav-1, cellular response to HGF is repressed. Particularly, following HGF stimulation, a reduction in activation status of PI3K and c-Src, two pathways involved in HGF-induced mitogenic and motogenic machinery, was seen (38). This indicates that Cav-1, which colocalizes with Met, might act as an inhibitor of HGF/Met signaling. To establish whether inhibitory effects of Cav-1 on c-Src and Met signaling are a reciprocal consequence or represent two separate mechanisms accounting for the oncosuppressive role of this adaptor protein is beyond the purpose of this study. However, it is tempting to speculate that Cav-1 might induce a coordinated and global inhibition of HGF-activated loop by acting both on c-Src and HGF/Met signaling. Activated c-Src increased expression of HGF mRNA and protein and cell scattering.

(57). On the other hand, Src is already known downstream mediator of HGF signaling pathway (58), thus establishing a positive regulatory feedback loop. Cav-1 by inhibiting c-Src activity via its scaffolding domain interrupts this positive regulation and by decreasing Met expression further disrupts HGF autocrine loop, therefore depriving osteosarcoma cells of a fundamental pathway for their migration and metastasis.

Overall, our results show Cav-1 as a crucial regulator of osteosarcoma malignancy and indicate that Cav-1 down-modulation might potentiate both Src family kinase and Met signaling, which provide osteosarcoma cells with the capacity of invading neighboring tissues. As a consequence, targeted therapies against c-Src family kinases and/or Met receptor may have a major role in improving the extremely poor prognosis of metastatic osteosarcoma.

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