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PI3-K/Akt-mediated anoikis resistance of human osteosarcoma cells requires Src activation

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ABSTRACT

Considerable advances in understanding the mechanisms associated with anoikis resistance of normal and malignant epithelial cells have been made. However, little is still known about the pathways involved in anoikis resistance of non-epithelial cells such as fibroblasts and sarcomas. Our results show that Src activity contributes to anoikis resistance of human osteosarcoma SAOS-2 cells. Src was found to be upregulated in anoikis resistant SAOS cells, and pharmacological inhibition of its activity resulted in the restoration of anoikis sensitivity. A normal pattern of dephosphorylation of FAK was observed upon cell detachment of both anoikis sensitive and resistant SAOS-2 cells, suggesting that FAK activity during anoikis resistance is not essential. The activity of Akt was found to be upregulated in anoikis resistant SAOSar cells and the pharmacological inhibition of PI3-K activity restored sensitivity to anoikis resistant cells, reconfirming the critical role of PI3-K/Akt pathway in cell survival. Furthermore, pharmacological inhibition of Src resulted in a decrease of Akt phosphorylation at Ser473. Altogether, these studies indicated a survival pathway mediated by the Src-dependent activation of the PI3-K/Akt pathway in a manner independent of FAK activity.

1. Introduction

Anoikis, Greek for "homelessness", is a form of apoptosis triggered by the lack of cell survival signals generated from the interactions with the ECM.[1,2] Physiological anoikis is considered to be important in the maintenance of homeostasis and tissue architecture.[3] Although described initially in epithelial and endothelial cells, anoikis has now also been found in normal cells of non-epithelial origin such as fibroblasts, hepatocytes, and pancreatic islets.[4–9] Furthermore, we and others have shown that malignant non-epithelial cells are sensitive to anoikis as well.[10–12]

The importance of anoikis in maintaining normal cell turnover and tissue architecture is evident however, the role of cellular resistance to anoikis is not as clear. Cells that are resistant to anoikis would live longer unattached and therefore have roles in cell migration and tissue remodelling. On the other hand, aberrant regulation of anoikis resulting in pathologic anoikis resistance has been described as one of the hallmarks of malignant transformation.[13] Anoikis resistance affords transformed cells increased survival times in the absence of matrix attachment, facilitating their migration and colonization of secondary sites.[14–17] We have shown that acquisition of anoikis resistance also applies to non-epithelial malignancies such as osteosarcoma. In our studies, cyclic manipulation of adhesion receptors in human osteosarcoma can convert anoikis sensitive cells to a stable and heritable anoikis resistant phenotype.[18]

Several mechanisms associated with anoikis resistance of cells from epithelial origin have been described recently.[19]

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However, very little is known about the pathways associated with anoikis resistance of cells of non-epithelial origin, such as fibroblasts or sarcomas. Understanding the mechanisms and role of anoikis resistance during the progression and metastasis of osteosarcoma is of clinical importance since 30% to 40% of patients develop lung metastasis despite aggressive chemotherapy and surgical resection of the primary lesion.[20,21]

It has been generally accepted that survival of adhered cells is maintained by signals generated by interactions between integrins and the ECM. Such signals are in turn transmitted to the cytoplasm by components of the focal adhesions, in which FAK is the central player. The major site of FAK autophosphorylation, Tyr397, is vital for the biological and biochemical functions of FAK, and represents the docking site for SH2-containing signalling molecules such as Src, Shc and the p85 subunit of PI3-K.[22] Ultimately, cell survival is associated mostly with the downstream activation of the PI3-K/Akt pathway, and since Akt activity depends heavily on the availability of PIP3, phosphatases such as PTEN and SHIP [23] act as potent negative regulators of its activity.

Direct involvement of FAK and Src during adhesion-mediated cell survival has been documented.[24] Expression of activated FAK results in the acquisition of anoikis resistance in MDCK cells, and conversely, inhibition of FAK function results in cell detachment and apoptosis.[25–27] Moreover, the initial observation that v-Src transformed epithelial cells became anoikis resistant suggested a role for Src in adhesion-mediated survival as well.[1] This has been corroborated by studies in colon and breast carcinomas in which expression of activated Src leads to increased anoikis resistance.[28] Furthermore, a recent report has shown that in breast carcinoma cells Src acts downstream of FAK in preventing anoikis.[29] Src-mediated anoikis resistance in a FAK activation dependent manner has also been described in normal breast and gallbladder epithelial cells.[30] However, recent reports have shown that Src-mediated anoikis resistance can occur independently of FAK activation.[31] Thus, this questions the universal importance of FAK in anchorage-independent cell survival, whether different cell types use different pathways, and whether the preservation of such pathways persists during embryonic development or malignant transformation.

Therefore the purpose of our study was to identify the key mediators of anoikis resistance in human osteosarcoma cells. Our results suggest a survival pathway mediated by the Src-dependent activation of the PI3-K/Akt pathway in a manner independent of FAK activity.

2. Materials and methods

2.1. Cell culture and reagents

The parental human osteosarcoma cell line SAOS-2 (SAOSp) was obtained from the American Type Culture Collection (Manassas, VA). SAOSp cells were maintained in Eagle's MEM (BioWhittaker, Walkersville, MD), supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine, 1 mM sodium pyruvate and non-essential amino acids (Sigma, St. Louis, MO). Anoikis resistant SAOS cells (SAOSar) were generated by sequential cycles of culture on untreated (adhered) and poly-HEMA treated cell culture wells (suspended).[18] Poly-HEMA was prepared by dissolving it in 95% ethanol to a concentration of 50 mg/ml. Poly-HEMA was added to cell culture wells at a density of 5 mg/cm^2 and allowed to dry overnight, under sterile conditions in a laminar flow hood. LY294002 and wortmannin were purchased from Cell Signalling Technology, Beverly, MA, and from Sigma, St. Louis, MO, respectively. The Src inhibitors SU6656, PP2, and its control PP3 were purchased from Calbiochem, San Diego, CA.

2.2. Cell cycle analysis

Cell cycle analyses were performed using propidium iodide (PI) staining with subsequent FACS analysis. 5×10^5 cells/well of a 6 well tissue culture plate or 2×10^5 cells/well of a 12 well tissue culture plate were cultured either on plastic or poly-HEMA treated 6-well tissue culture plates for 24 hrs at 37 °C in a 5% CO_2 atmosphere. After incubation, adherent cells were detached with trypsin (0.5% trypsin/0.1% EDTA in PBS). Detached and suspended cells were harvested in complete EMEM medium and centrifuged at 500 g for 10 min. Pellets were washed with PBS and fixed with ice cold 75% ethanol overnight at 4 °C. After fixation, cells were washed with PBS and stained with 500 μl of PI solution (50 μg/ml in PBS) containing 25 μg/ml of RNase. Cells were incubated at 37 °C for 30 min and analysed by flow cytometry on an Epics Profile flow cytometer (Coulter, Miami, FL).

2.3. Western immunoblotting

Cells were lysed (10 mM Tris, 150 mM NaCl, 1% Triton X, 1mM Na_3VO_3, 1 mM PMSF, pH 7.4) on ice for 1 h. Suspended cells were centrifuged at 500 g for 5 min and pellet resuspended in lysis buffer. Adhered cells were lysed directly in the well. Both lysates were cleared by centrifugation at 14000 g for 10 min and protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Cleared lysates containing equal amounts of protein were mixed with 1X Laemmli reducing buffer and resolved by SDS-PAGE. Polypeptides were transferred to nitrocellulose membranes (0.2 μm) and probed with the relevant antibodies. The phosphorylated states of FAK, paxillin, Akt, and Src were determined by using the following phosphospecific antibodies: anti-FAK[pY^{397}], anti-paxillin[pY^{31}] (Biosource International, Camarillo, CA), anti-Akt[pS^{473}] (Cell Signalling Technology, Beverly, MA), and anti-Src[pY^{416}] (Cell Signalling Technology, Beverly, MA), which in human cells reacts against Src[pY^{418}]. Total levels of expression were determined by reprobing using antibodies directed against the non-phosphorylated form of the same molecule after gently striping of the same membrane. Striping buffer contained 62.5 mM Tris and 2% SDS at pH 6.7. The following antibodies were used: anti-FAK, anti-paxillin (Biosource International, Camarillo,CA), anti-Akt, anti-PTEN and anti-Src, (Cell Signalling Technology). Antibodies were used at a dilution of 1:1000.

2.4. Expression vectors and transfections

An eukaryotic expression vector containing a CD2-FAK construct was employed.[32] Cells were transfected using Lipofect-

amine2000® (Invitrogen, Carlsbad, CA). Briefly, monolayers were trypsinized and cell concentration adjusted to 1×10^6 cells/ml. Culture medium (100 μl/well) was added to 12-well cell culture plates. Plasmid DNA (5 μg/well) was mixed with 3 μl of Lipofetamine2000® and added to each well. Plates were incubated at room temperature for 20 min. After incubation 250 μl of cell suspension (2.5×10^5 cells/well) was added to each well. Plates were incubated at 37 °C in a 5% CO_2 atmosphere.

2.5. Akt kinase assay

Akt kinase activity was measured using a non-radioactive kinase assay. (Cell Signalling Technology, Beverly, MA). Briefly, Akt was immunoprecipitated from lysates of SAOSp and SAOSar cells cultured under adhered and suspended conditions for 24 h. The resulting immunoprecipitate was incubated with purified recombinant GSK-3 protein in the presence of ATP and kinase buffer (25 mM Tris (pH 7.5), 5 mM β-Glycerol-phosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). Phosphorylation of GSK-3 was measured by Western immunoblotting using an anti-GSK-3α/β[$pS^{21/9}$] antibody (1:1000).

2.6. PTEN phosphatase assay

PTEN activity was assayed using a colourimetric assay previously described by Maehama.[33] Briefly, PTEN was immunoprecipitated from lysates of SAOSp and SAOSar cells cultured under adhered and suspended conditions. Jurkat cells lysates were used as negative control since these cells lack expression of PTEN. Immunoprecipitates were incubated in reaction buffer (100 mM Tris.HCl pH 8.0, 10 mM DTT) containing 100 μM of diC_8PIP_3 (Echelon Research Biosciences Inc, Salt Lake City, UT). Reactions were carried out at 37 °C for 40 min and terminated with 100 μl/well of Biomol Green (Biomol, Plymouth Meeting, PA). Biomol Green is a malachite green solution that shows increased absorbance in the presence of free phosphate. The green colour was allowed to develop for 30 min, and the absorbances for the phosphatase products as well as phosphate standards were measured at 595 nm with a microplate reader.

3. Results

3.1. Resistance to anoikis of SAOSar cells involves the activation of Src

SAOSp cells undergo anoikis when proper adherence to the ECM is denied.[11] Nevertheless, an anoikis resistant subline, SAOSar, has been developed by alternating cycles of culture under suspended and adhered conditions.[18] Apoptosis was determined by PI staining followed by flow cytometry analyses of DNA content. The percentage of cells in the sub-G/0 phase is representative of apoptosis. Fig. 1A shows that when both SAOSp and SAOSar cells are allowed to adhere to cell culture treated wells very low apoptotic death is found, 1.93% and 2.8% respectively. By contrast, after culture under suspended conditions for 24 h, SAOSar cells show decreased apoptotic death (6.57%) compared with their parental population, SAOSp (38.9%).

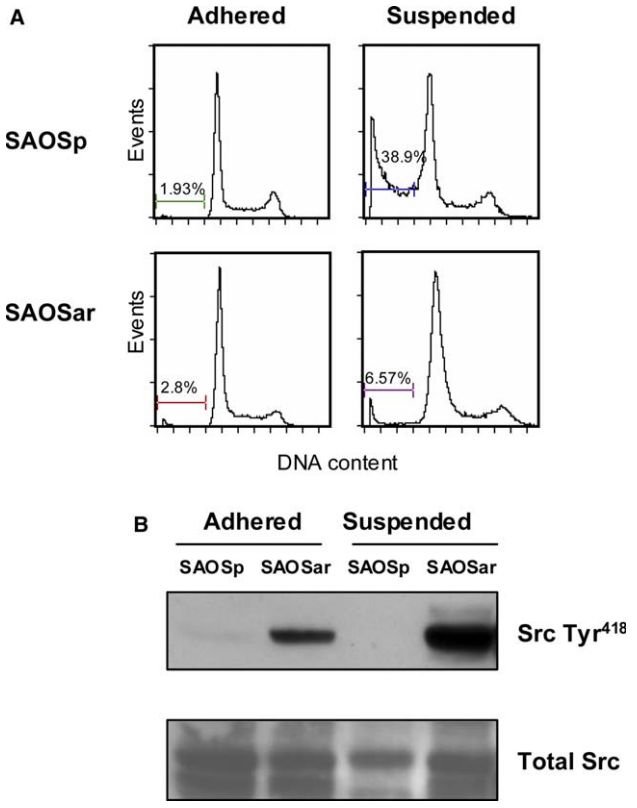

Fig. 1 – Anoikis resistance of SAOS-2 cells correlates with upregulation of Src activity. A. SAOSar cells show decreased apoptosis (6.57%) after culture under suspended conditions for 24 h when compared with SAOSp (38.9%). Percentage apoptosis was determined by PI staining followed by flow cytometry analyses. Results shown are representative of three independent experiments. B. Immunoblot analyses of phosphorylation of Src at Tyr418 after culture of SAOSp and SAOSar cells under adhered and suspended conditions for 24 h. Total levels of Src expression were assayed in the same membranes using a anti-total Src antibody.

Since upregulation of Src activity[34] has been linked to increased survival, we wanted to investigate its role during anoikis resistance of human osteosarcoma cells, SAOS-2. It was found that the levels of Src expression were similar in the lysates from SAOSp and SAOSar cells cultured under adhered and suspended conditions for 24 hours. Activated Src, as determined by phosphorylation at Tyr418, was only detected in the lysates from SAOSar cells, regardless of their culture conditions (adhered vs. suspended). There was an increase in the levels of phosphorylated Src at Tyr418 after detachment of SAOSar cells from the ECM (Fig. 1B).

In order to confirm the role of Src during resistance to anoikis, pharmacologic inhibition using PP2 was employed (Fig. 2A). Culture of SAOSar cells under suspended conditions in the presence of 50 μM of PP2 resulted in the loss of phosphorylation at Tyr418. Treatment with the same dose of PP3 (scrambled negative control), vehicle alone or medium only, did not have an effect on the phosphorylation of Src at Tyr418. Moreover, none of the above treatments had an effect on the overall expression of Src (Fig. 2A). Furthermore,

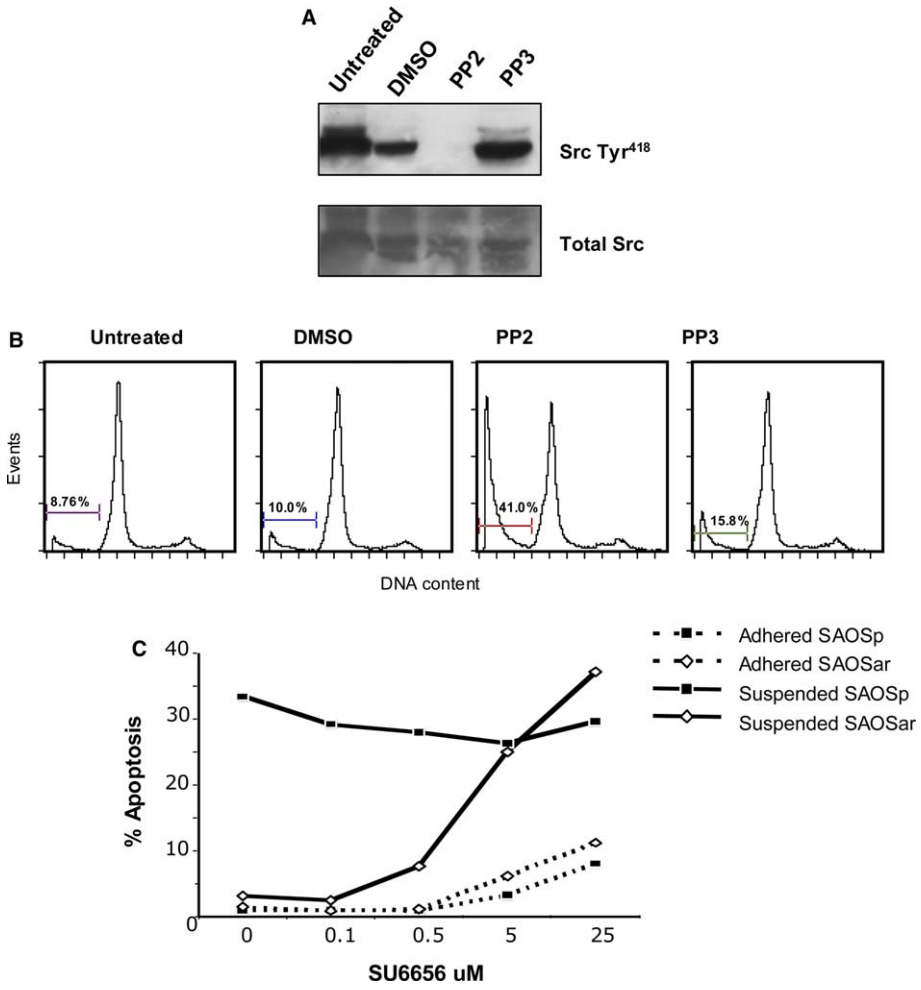

Fig. 2 – Pharmacological inhibition of Src activity restores sensitivity to anoikis in SAOSar cells. A. Immunoblot analyses of phosphorylation of Src at Tyr418 after culture of SAOSar cells under suspended conditions for 24 hours in the presence of media alone, vehicle only, PP2 or PP3. Total levels of Src expression were assayed in the same membranes using anti-total Src antibody. B. Cell cycle analyses of SAOSar cells after culture for 24 hours under suspended conditions in the presence of media alone, vehicle only, PP2 or PP3. The percentage of the cells in the sub-G/0 phase representative of apoptotic cells is marked on each histogram. Data shown is representative of three independent experiments. C. Percentage of apoptotic cells after cell cycle analyses of SAOSp and SAOSar cells cultured under adhered and suspended conditions for 24 hrs in the presence of the indicated concentrations of SU6656.

SAOSar cells cultured under suspended conditions in the presence of 50 μM of PP2, 50 μM of PP3, vehicle only or media alone were assayed for apoptosis 24 hrs later by PI staining followed by flow cytometry analyses. Fig. 2B shows that treatment of suspended SAOSar cells with the Src inhibitor PP2 for 24 hours results in the restoration of the anoikis sensitive phenotype. Treatment of adhered SAOSp or SAOSar cells with doses of PP2 ranging from 5 to 50 uM did not cause cell death (data not shown), suggesting that the increased death observed among suspended SAOSar cells was not due to cytotoxicity. Our findings were further corroborated using a different, more specific Src inhibitor, SU6656.[35] Fig. 2C shows increased anoikis among SAOSar cells in a dose response manner starting after treatment with 0.5 μM of SU6656.

The findings with SAOS-2 cells were further confirmed using a different osteosarcoma cell line, TE-85. Treatment of suspended anoikis resistant TE-85 clones with 5 μM of SU6656 resulted in a significant increase in apoptosis when compared with the untreated control (data not shown). Furthermore, we have found that normal human osteoblasts are resistant to anoikis, and that pharmacological inhibition of Src with PP2 results in anoikis sensitivity (data not shown). These findings further validate the role of Src during anoikis resistance of malignant osteosarcoma cells and suggest a potential involvement in the regulation of adhesion-dependent survival among normal osteogenic precursors as well.

3.2. *Src-mediated resistance to anoikis of SAOSar cells is independent of FAK and paxillin phosphorylation at tyrosines 397 and 31 respectively*

A direct association between Src and integrins has only been described in platelets, thus we investigated the role of FAK, as its direct association with integrins is known to promote sur-

vival.[25,36] Furthermore, activated FAK can potentially activate Src by competing with its downregulatory site.[37] Thus, we speculated that regardless of the culture conditions (adhered vs. suspended) FAK would remain phosphorylated and bind to Src, causing its activation by interfering with the conformational changes required for its inactivation,[38,39] and therefore maintaining the survival signal. Our results, however, did not support this hypothesis (Fig. 3). Antibodies directed against the phosphorylated states of FAK at Tyr397 and paxillin at Tyr31 were used to determine their activity after culture under adhered and suspended conditions in both SAOSp and SAOSar cells. Total expression of FAK and paxillin was determined by stripping the same membranes and reprobing them with an anti-total FAK or paxillin antibody respectively. Levels of expression of FAK and paxillin were similar in both SAOSp and SAOSar cells under adhered and suspended conditions. As expected, both FAK and paxillin remained phosphorylated at Tyr397 and Tyr31 respectively in adhered SAOSp and SAOSar. Dephosphorylation of both molecules was found in suspended SAOSp and SAOSar, despite the fact the SAOSar survived anoikis death (Fig. 3A). These results indicate that the major components of the focal adhesion complex are functioning normally and are not aberrantly regulated in anoikis resistant SAOSar cells. Moreover, this suggests that anoikis resistance in SAOSar cells is achieved through a mechanism independent of FAK and paxillin phosphorylation at Tyr397 and Tyr31 respectively.

Given the importance of FAK during adhesion-mediated cell survival, we wanted to further corroborate its lack of involvement during anoikis resistance in our osteosarcoma model. Both SAOSp and SAOSar cells were transfected with a plasmid vector containing transmembrane-anchored

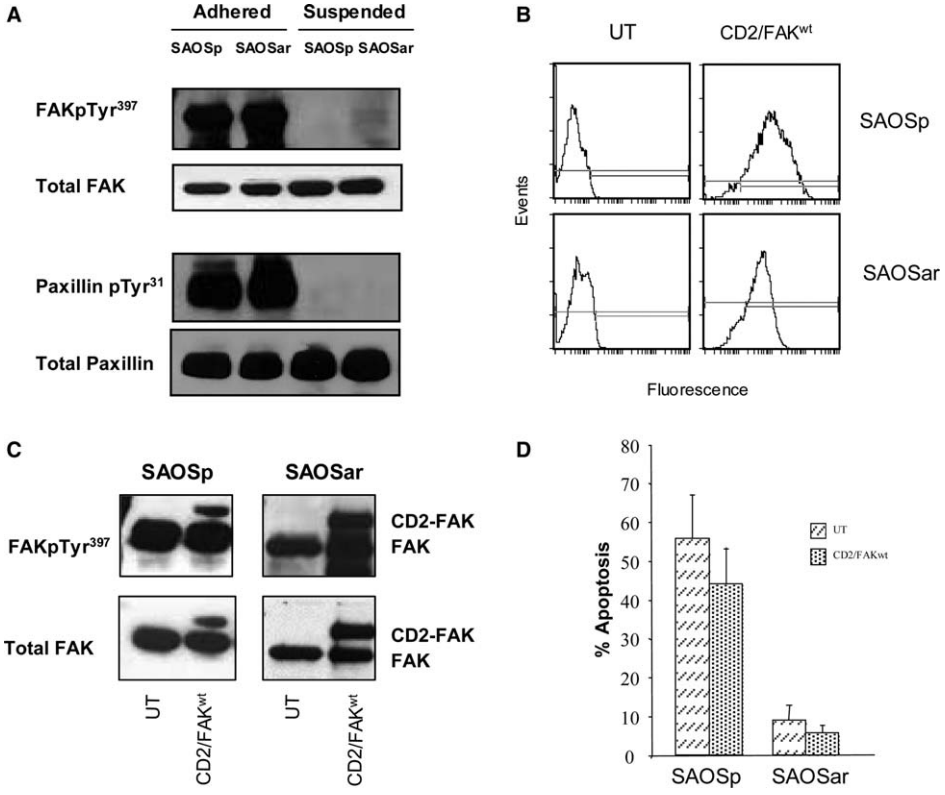

Fig. 3 – Anoikis resistance of SAOSar cells is independent of the phosphorylation of FAK and paxillin at tyrosine residues 397 and 31 respectively. A. Immunoblot analysis of phosphorylation of FAK and paxillin at tyrosine residues 397 and 31 respectively in SAOSp and SAOSar cells after culture for 24 hrs under adhered and suspended conditions. Dephosphorylation of both FAK and paxillin after detachment from the ECM was similar in SAOSp (apoptotic) and SAOSar (non-apoptotic) cells. Total levels of expression of both FAK and paxillin were determined using anti-FAK or anti-paxillin antibodies after stripping and reprobing of the same membranes. B. Expression of constitutively active FAK was determined by flow cytometry analysis of surface expression of the CD2 part of the CD2-FAK chimera. Positive expression of CD2 was found in 93.1% of SAOSp cells and 80.3% of SAOSar cells, with a MFI of 8.81 and 6.90 respectively. C. Immunoblot analysis of FAK phosphorylation at Tyr397 after transfection of SAOSp and SAOSar with constitutively active CD2-FAK. Phosphorylated FAK at Tyr397 was detected using a phosphospecific antibody. Total levels of FAK expression were assayed in the same membrane using an anti-FAK antibody. D. Cell cycle analyses after culture for 24 hours under suspended conditions of SAOSp and SAOSar cells transfected with constitutively active CD2/FAK constructs. The original anoikis sensitive (SAOSp) and resistant (SAOSar) phenotypes remained unchanged despite overexpression of constitutive active (CD2-FAK). The percentage of cells in the sub-G/0 phase representative of apoptosis was determined by PI staining followed by flow cytometry analysis. Error bars indicate ± SDEV of three independent experiments.

chimeric CD2-FAK constructs. The membrane targeting of FAK via CD2 results in constitutive activation by retaining Tyr397 autophosphorylation. Stable sublines expressing the CD2-FAK construct were generated for SAOSp and SAOSar cells. Expression of the CD2-FAK chimera was assayed by flow cytometry using an anti-CD2 FITC conjugated antibody. Positive expression of surface CD2 was found in 93.1% of SAOSp cells and 80.3% of SAOSar cells, with a MFI of 8.81 and 6.90 respectively (Fig. 3B). These results were corroborated by western immunoblot analysis using a phosphospecific antibody against FAK at Tyr397 and an anti-total FAK antibody. Both CD2-FAK and endogenous FAK were detected in the lysates from transfected SAOSp and SAOSar cells using phosphospecific anti-FAKpY^{397} and anti-total FAK antibodies. Only endogenous FAK was detected in the untransfected control using the same antibodies (Fig. 3C).

To test whether overexpression of constitutively active FAK could rescue SAOSp from anoikis, CD2-FAK transfected cells were cultured under suspended conditions (poly-HEMA treated culture wells) for 24 h and anoikis assayed by PI staining. The original anoikis sensitive (SAOSp) and resistant (SAOSar) phenotypes remained unchanged despite overexpression of constitutively active CD2-FAK (Fig. 3D). These results suggest that activation of FAK is not sufficient to prevent anoikis in SAOSp cells.

3.3. *Activation of the PI-3K/Akt pathway is required during Src-mediated anoikis resistance of SAOSar cells*

The PI-3K/Akt pathway is known to regulate apoptosis after different stimuli, including cell adhesion.[40] Moreover, it has been shown that Src can directly activate PI-3K independently of FAK.[37] This scenario may also be true for our model since we showed that the involvement of FAK seems to be dispensable. Thus, to determine the role of PI3-K we employed pharmacological inhibition using wortmannin and LY294002. Suspended anoikis resistant SAOSar cells were incubated in the presence of various doses of LY294002 or wortmannin for 24 h. After incubation, cells were harvested and either stained with PI for flow cytometry analysis or lysed for western immunoblotting. Inhibition of PI3-K activity resulted in increased anoikis in a dose dependent manner (Fig. 4).

To further corroborate the role of PI3-K/Akt pathway we determined the activity of Akt under adhered and suspended conditions in both anoikis sensitive (SAOSp) and anoikis resistant (SAOSar) cells. Total levels of Akt expression were similar in both populations under adhered and suspended conditions. However, phosphorylation at Ser 473 was found only in the lysates from SAOSar (Fig. 5A). Interestingly, this activated state was observed in both adhered and suspended culture conditions. These results suggest that activation of Akt is involved in anoikis resistance and that this activated state is constitutive. These findings were corroborated using a non-radioactive Akt kinase assay. Immunoprecipitated Akt from lysates of SAOSp and SAOSar cells cultured under adhered and suspended conditions for 24 hours was incubated with recombinant GSK-3α/β in the presence of ATP and phosphorylated GSK-3α/β at Ser21/9 was detected by western immunoblot. Using this method, Akt activity was detected in the lysates from SAOSar cells after culture under both adhered and suspended conditions. No Akt kinase activity was detected in the lysates from SAOSp cells (Fig. 5B).

PI-3 K inhibition also affected the activity of Akt. A decreased phosphorylation at Ser 473 correlated with increasing doses of LY294002 or wortmannin. When compared to the untreated control, treatment with 10 μM and 50 μM of LY294002 resulted in a 10.5% and 45.6% decrease in phosphorylation. Treatment with 1 μM and 5 μM of wortmanin resulted in a

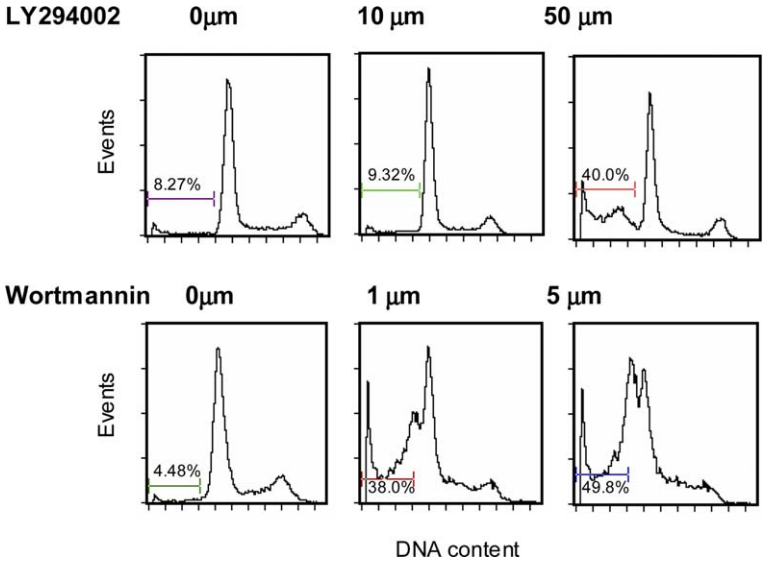

Fig. 4 – PI3-K activity is required during anoikis resistance of SAOSar cells. Pharmacological inhibition of PI3-K activity using LY294002 (upper panel) and wortmannin (lower panel) resulted in increased anoikis of SAOSar cells in a dose dependent manner. SAOSar cells were incubated with the indicated doses under suspended conditions for 24 h and analyzed by flow cytometry after staining with PI. The percentage of the cells in the sub-G/0 phase representative of apoptotic cells is marked on each histogram. Data shown is representative of three independent experiments.

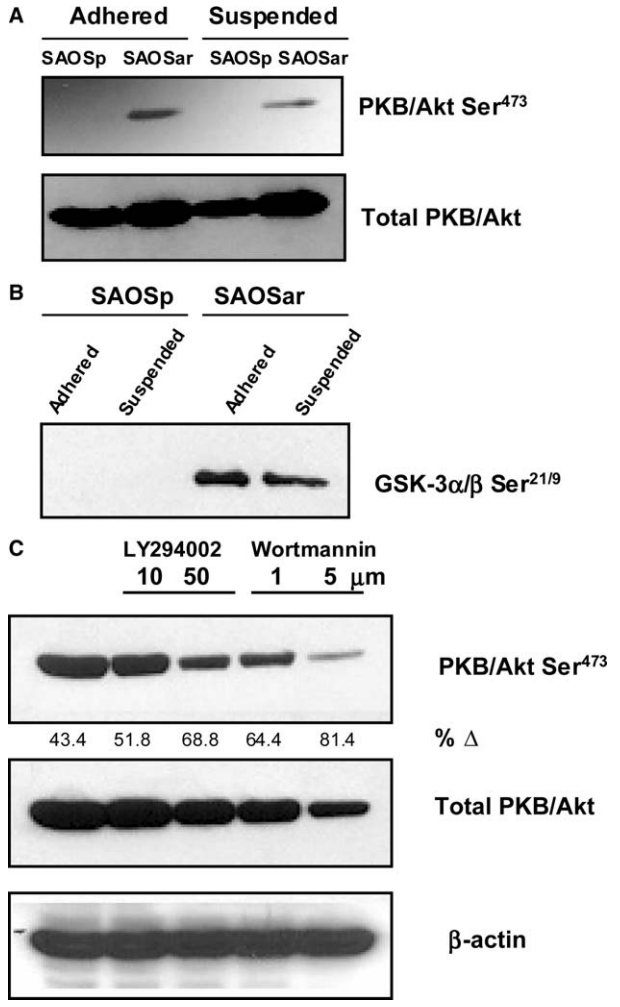

Fig. 5 – Anoikis resistant SAOSar cells show increased Akt activity. A. Immunoblot analysis of Akt phosphorylation at Ser473 of SAOSp and SAOSar after culture under adhered and suspended condition for 24 h. Phosphorylation at Ser473 was detected using a phosphospecific antibody (upper panel). Total levels of Akt expression were detected on the same membrane using an anti-Akt antibody (lower panel). Overall protein loading was determined using Ponceau S staining. B. Akt activity was determined using a non-radioactive kinase assay. Immunoprecipitated Akt from equal amounts of lysates from SAOSp and SAOSar cells incubated under adhered and suspended conditions for 24 h was mixed with its substrate GSK- 3α/β $S^{21/9}$. Phosphorylated GSK- 3α/β $S^{21/9}$ was detected using a phosphospecific antibody. C. Western immunoblot analysis of Akt phosphorylation at Ser473 (panel 1) and total expression of Akt (panel 2) after treatment with the indicated doses of LY294002 and wortmannin. Anti-β-actin antibody was used confirm equal loading (panel 3). The same membrane was reprobed with all three antibodies after gently stripping. Quantitation was performed using Scion Image® software and the percent differences between total and phosphorylated Akt are indicated in the figure. Results showed are representative of three independent experiments.

36.8% and 66.7% decrease in phosphorylation. A decrease in the total protein levels of Akt was also noted after PI3-K inhibition, thus suggesting that phosphorylation of Akt at Ser 473 contributes to increased protein stability (Fig. 5C).

The activity of PTEN was also analysed. The tumour suppressor phosphatase PTEN negatively regulates Akt activity by dephosphorylating [PI(3,4,5)P_3], the product of PI3-K. Thus, PTEN deactivation due to gene silencing or degradation could cause constitutive activation of Akt in SAOSar cells in a manner independent of upstream activators such as FAK. Western immunoblot analyses of lysates from SAOSp and SAOSar cells cultured either under adhered or suspended conditions showed no difference in the levels of expression of PTEN (Fig. 6A). Since changes in gene expression do not necessarily have an effect on overall enzymatic activity, PTEN activity was also assayed. Phosphatase activity of immunoprecipitated PTEN from lysates of SAOSp and SAOSar cells cultured under adhered or suspended conditions was also assayed. No significant difference was found among the free phosphate levels in all four lysates after reaction was stopped with

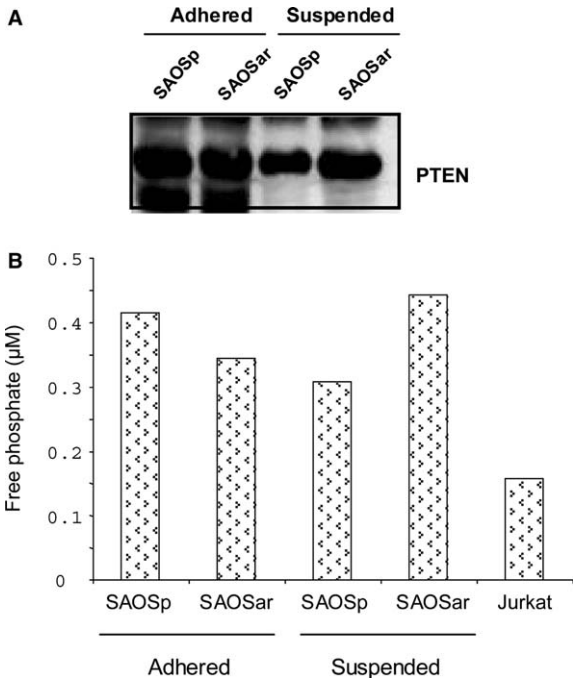

Fig. 6 – Neither PTEN gene silencing nor deactivation is responsible for the constitutive activation of Akt in SAOSar cells. A. Western immunoblot analysis of PTEN expression in lysates from SAOSp and SAOSar cultured under adhered and suspended conditions. Equal protein loading was confirmed by Ponceau red staining. B. Phosphatase activity of immunoprecipitated PTEN from lysates of SAOSp and SAOSar cultured under adhered and suspended conditions was determined by adding diC_8PIP3 and measuring the concentration of free phosphates using a malachite green solution (see "Materials and Methods"). To correct for spontaneous dissociation of phosphates from substrate all values were normalized against readings from wells containing reaction buffer and diC_8PIP3. Data shown is representative of three independent experiments.

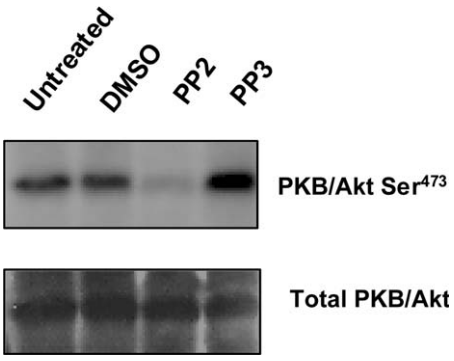

Fig. 7 – Pharmacological inhibition of Src activity results in a decrease of Akt activity. Immunoblot analysis of Akt phosphorylation at Ser473 after culture of SAOSar cells under suspended conditions for 24 hours in the presence of media alone, vehicle only, PP2 or PP3. Total Akt expression was detected on the same membrane using an anti-Akt antibody. Data shown is representative of three independent experiments.

malachite green solution. Jurkat cell lysates were included as negative controls since these cells lack expression of PTEN (Fig. 6B). These results suggest that neither PTEN gene silencing nor lack of activity is responsible for the constitutive activation of Akt in SAOSar cells.

3.4. *Pharmacological inhibition of Src results in a decrease of Akt phosphorylation at Ser 473 in suspended SAOSar cells*

We have previously shown that pharmacological inhibition of either Src or PI3-K/Akt results in the restoration of the anoikis sensitive phenotype. We then tested the effect of Src inhibition on Akt activity. Culture of SAOSar cells under suspended conditions in the presence of 50 μM of PP2 resulted in the loss of phosphorylation at Ser473. Treatment with the same dose of PP3 (scrambled negative control), vehicle alone or medium only, did not have an effect on the phosphorylation of Akt at Ser473. Moreover, none of the above treatments had an effect on the overall expression of Akt (Fig. 7).

4. Discussion

Our study focused on the mediators associated with anoikis resistance of human osteosarcoma, SAOS-2 cells. We found the activity of Src necessary for survival under suspended conditions. Since v-Src transformation results in anchorage independent growth, for which anoikis resistance would be a pre-requisite, our findings are consistent for a role of c-Src in adhesion independent survival.

Src activation in response to engagement of integrins with ECM components has been reported.[24] However, direct interaction of Src with integrin subunits has only been described in platelets.[41,42] This suggests the involvement of other mediators that would become activated upon integrin engagement and in turn activate Src. Thus it was expected that activation of FAK was a likely mechanism since it is the central component of the focal adhesion complex, contains binding sites for both integrins and Src, and its activation has been associated with anoikis resistance in other systems.[19,22,43] Our findings indicate that FAK activation is not necessary because no difference was found among the phosphorylation patterns of both FAK and paxillin between apoptotic (SAOSp) and non-apoptotic (SAOSar) cells after culture under suspension conditions that induce anoikis in sensitive cells. Furthermore, this FAK independence was corroborated by studies involving the overexpression of constitutively active FAK in which expression of CD2-FAK did not rescue the sensitive SAOSp cells from anoikis. Our findings are in contrast to other studies in which stable expression of CD2-FAK constructs rescues glomerular epithelial cells[44] and MADCK cells from anoikis. MADCK cells expressing constitutively active FAK showed signs of transformation as determined by anchorage-independent growth and tumour formation in nude mice.[45] Furthermore, it has been shown that v-Src transformed primary chicken embryo fibroblasts (CEFs) cotransfected with with FAK carrying mutations on each of the recognized Src-specific phospho-acceptor sites show decreased survival under serum deprivation and decreased proliferation and colony formation in soft agar when compared to CEFs coexpressing wt- FAK.[46] This suggests a crucial role for Src-induced phosphorylation of FAK during anchorage-independent growth. In contrast, our findings that Src-mediated anoikis resistance could be independent of FAK activation are consistent with other studies in which v-Src transformation enhanced anchorage-independence of FAK-null fibroblasts.[47] A possible explanation for this is that under such conditions other mediators are upregulated in order to compensate for the lack of functional FAK. A recent report by the same group supports this possibility. In their study, anchorage independence of FAK-null fibroblasts was mediated by the Src-dependent hyperactivation of PI3-K.[48] This is in contrast to our model in which FAK appears to function and operate normally. Under adhered conditions FAK and paxillin were found to be phosphorylated at Tyr397 and 31 respectively in both SAOSp and SAOSar cells. Moreover, detachment from the ECM resulted in their de-phosphorylation, suggesting a functional focal adhesion complex that responded normally to the signals generated during adhesion. Thus, similar to cells in which FAK expression or function is compromised, other FAK independent mediators could be utilized. Such normal pattern of dephosphorylation of FAK during Src-mediated anoikis resistance has been recently described in human lung adenocarcinoma cells and in intestinal epithelial cells.[31,49] Thus, Src mediated anoikis resistance that is FAK independent is a mechanism utilized by adherent cells of both epithelial and, as shown in our study, non-epithelial origin.

Among the pathways linked to apoptosis resistance, the PI3-K/Akt pathway stands out as the convergent point for a variety of stimuli generated at the cell surface.[40] Direct involvement of Akt during resistance to anoikis has been reported in epithelial systems.[50–52] We showed that activation of the PI3-K/Akt is also involved in anoikis resistance of human osteosarcoma cells SAOS-2, a malignancy of mesenchymal origin. In our study, pharmacological inhibition of PI3-K activity correlated with an increase in anoikis among initially resistant SAOSar cells. Moreover this pharmacological inhibition also correlated with a decrease in Akt activity suggesting

that the availability of [PI(3,4,5)P_3] is essential for constitutively active Akt, and subsequently anoikis resistance. Thus it was possible that PTEN, since it is able to reduce available [PI(3,4,5)P_3], would be abnormally downregulated in anoikis resistant SAOSar cells, resulting in increased activity of Akt. Our results showed no difference in the expression or activity of PTEN among anoikis sensitive and resistant SAOS cells. In contrast, pharmacological inhibition of Src activity had a negative effect on Akt activity, suggesting that the activation of Akt requires Src activity.

Interestingly, pharmacological inhibition of PI3-K in both SAOSp and SAOSar under adhered conditions did not cause cell death (data not shown). This implies that other mediators are involved in maintaining the survival signals generated from the ECM, and more importantly that the anti-apoptotic activity of the PI3-K/Akt pathway is crucial only under stress conditions, such as detachment from the ECM. Other death-inducing conditions known to activate Akt include the withdrawal of growth factors, oxidative and osmotic stress, irradiation, and ischemic shock.[53,54] This gives Akt a physiological role of preventing unnecessary cell death, which has been recently demonstrated in Alzheimer's and cardiovascular diseases models.[55]

Thus, from our findings we can conclude that the lack of Akt activation in response to cell detachment contributes to anoikis of SAOS-2 cells by failing to prevent apoptosis. Conversely, Src activation in suspended anoikis resistant SAOSar cells is able to maintain survival by activating Akt, circumventing the requirement for attachment-mediated integrin survival signalling. Taken together, our findings suggest a survival pathway mediated by Src that requires the activation of the PI3-K/Akt pathway in a manner independent of FAK activity.

Inhibition of anoikis resistance in malignant cells could reduce their dissemination. Our report demonstrates a crucial role for Src in Akt-mediated survival in anoikis resistant human SAOS-2 osteosarcoma cells, validating Src as an important target for therapeutic intervention. However, the fact that Src overexpression by itself is not sufficient for oncogenic transformation, therapy aimed at the inhibition of Src should be perceived as a part of a multi-target regimen. Furthermore, expression of Src and its related kinases are important in many normal physiological processes, thus systemic inhibition of Src could result in considerable adverse effects. Thus before an effective and safe Src inhibitor can reach the clinic, a deeper understanding of its biology in a malignancy-specific manner is required.

Conflict of interest statement

None declared.

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