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Steroid Receptor Coactivator-1 Upregulates Integrin α_5 Expression to Promote Breast Cancer Cell Adhesion and Migration

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Abstract

Metastatic breast cancer (BC) remains a lethal disease with poorly understood molecular mechanisms. Steroid receptor coactivator 1 (SRC-1 or NCOA1) is overexpressed in a subset of BCs with poor prognosis. SRC-1 potentiates gene expression by serving as a coactivator for nuclear receptors and other transcription factors. We previously reported that SRC-1 promotes BC metastasis without affecting primary mammary tumor formation. Herein, we found that SRC-1 deficiency in mouse and human BC cells substantially reduced cell adhesion and migration capabilities on fibronectin and significantly extended the time of focal adhesion disassembly and re-assembly. In agreement with this phenotype, SRC-1 expression positively correlated with integrin α_5 (*ITGA5*) expression in estrogen receptor-negative breast tumors while SRC-1 deficiency decreased *ITGA5* expression. Furthermore, ITGA5 reduction in SRC-1 deficient/insufficient BC cells or knockdown of ITGA5 in SRC-1-expressing BC cells was associated with a disturbed integrin-mediated signaling. Critical downstream changes included reduced phosphorylation and/or dampened activation of focal adhesion kinase, paxillin, Rac1 and Erk1/2 during cell adhesion. Finally, we found that SRC-1 enhanced *ITGA5* promoter activity through an AP-1-binding site proximal to the transcriptional initiation site; both SRC-1 and c-Jun were recruited to this promoter region in BC cells. These results demonstrate that SRC-1 can promote BC metastasis by directly enhancing *ITGA5* expression and thus promoting ITGA5-mediated cell adhesion and migration. Therefore, targeting ITGA5 in SRC-1-positive BCs may result in inhibition of SRC-1-promoted BC metastasis.

Keywords

nuclear receptor; SRC-1; coactivator; ITGA5; breast cancer

Introduction

Steroid receptor coactivator 1 (SRC-1) boosts gene expression by serving as a transcriptional coactivator for nuclear hormone receptors and other transcription factors (TFs) such as estrogen receptor α (ERα), progesterone receptor (PR), PEA3, AP-1, HIF-1

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and Ets-2 (1–6). SRC-1 expression in human breast cancer (BC) positively correlates with Her2 expression, endocrine therapy resistance, and poor prognosis (5,7,8). Knockout of *SRC-1* in the MMTV-polyoma middle T antigen (PyMT) mammary tumor-prone mice dramatically suppresses lung metastasis without affecting primary tumor formation (9). These studies indicate that SRC-1 strongly promotes BC metastasis.

SRC-1 upregulates the expression of several key regulators for BC progression. In particular, SRC-1 deficiency in mouse mammary tumors reverses HER2 overexpression and reduces Akt activity (9). Knockout of *SRC-1* in these tumors suppresses the expression of colony stimulating factor (CSF-1) (9), a chemoattractant that recruits macrophages to the tumor site. In turn, the macrophages secret EGF to stimulate tumor cell motility. SRC-1 also serves as a coactivator of PEA3 to enhance Twist1 expression in BC cells. Elevated Twist1 promotes breast tumor cell epithelial mesenchymal transition (EMT), invasion and metastasis by recruiting the NuRD protein complex to repress E-cadherin expression (2,10). Furthermore, SRC-1 works with Ets-2 to induce c-Myc expression and with HOXC11 to induce calcium-binding protein S100beta expression, both of which are positively associated with acquired resistance to endocrine therapy (5,7).

Recently, we discovered that the number of mammary tumor cells in the blood of *SRC-1* wild type (WT);PyMT mice is significantly higher than that in the blood of SRC-1 knockout (KO);PyMT mice, suggesting a contribution of SRC-1 to BC cell migration and invasion from the primary tumor to the blood vessels (9). Local migration and invasion of tumor cells are early events partially induced by the tumor microenvironment in metastasis. Resident fibroblasts not only secret TGFβ to induce tumor cell EMT, but also produce abundant collagen and fibronectin (FN) extracellular matrix (ECM) proteins to provide anchorages for tumor cell adhesion and migration (11–13). Integrins consist of 18 α and 8 β glycoprotein subunits, which form 24 distinct heterodimeric transmembrane receptors. These receptors bind to ECM proteins such as FN to transport signals bidirectionally across the cell membrane, allowing cells to respond to environmental changes (14). Multiple integrins, including αvβ3, αvβ5, α5β1, α6β4, α4β1 and αvβ6, are detected in cancer cells and their expression levels are associated with tumorigenesis and cancer progression (15). In BC, integrin β4 amplifies HER2 signaling to potentiate mammary tumorigenesis (16). Activation of integrin αvβ3 supports BC cell adhesion to the vascular wall and promotes metastasis (17), while knockout of integrin β_1 inhibits mammary tumorigenesis in mice (18). In addition, integrins also regulate tumor cell survival, growth and metastasis in an anchorage-independent manner (15).

The mesenchymal integrins α_5 (ITGA5) and β_1 form heterodimers to mediate cell adhesion to FN (15). Knockout of ITGA5 in mice results in embryonic lethality (19). In human hepatocarcinoma cells, ITGA5 promotes cell adhesion and migration on FN through activating focal adhesion kinase (FAK) (20). In transformed mammary epithelial cells, ITGA5 expression is increased along with the EMT process (21). These findings indicate that *ITGA5* expression correlates with cancer progression and plays an important role to enhance cancer cell adhesion to and migration along FN.

In this study, we found that SRC-1 works with AP-1 to potentiate *ITGA5* expression. The increased ITGA5, in turn, significantly accelerates BC cell adhesion and migration on FN. The identification of *ITGA5* as a target gene of SRC-1 and AP-1 in BC cells uncovered a new molecular pathway: SRC-1 regulates *ITGA5* expression to promote BC metastasis.

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Materials and Methods

Cell adhesion and migration assays

The primary and stable *SRC-1* WT;PyMT (WT) and KO;PyMT (KO) mouse mammary tumor cell lines were generated as described previously (22). Adhesion assay was performed on FN or laminin (LN)-coated plates as described previously (23). Individual cell migration was tracked for 18 hours in 96-well plate pre-coated with fluorescent beads and track areas were analyzed using NIH image software as described previously (2,22).

Western blot analysis of human breast tumors

A total of 24 human BC specimens were collected from surgically removed tumor tissues at Luzhou Medical College Affiliated Hospital in 2009. All patients were Asian women and aged 33–65 years old. No patient survival data were available at this stage. A portion of the specimen was used for clinical diagnosis of tumor pathology and immunohistochemistry for ERα, PR and HER2. The remaining tumor tissues were immediately frozen in liquid nitrogen and stored at −80° C. Tumor tissue lysates were prepared after homogenizing the tissues in a lysis buffer containing the protease inhibitor cocktail. The tissue lysates with 50 μg protein were analyzed by Western blot using antibodies against SRC-1, ITGA5 and β-actin. Band intensities were determined by densitometry and normalized to the β-actin band intensity.

Other Methods

Immunostaining, knockdown and expression of SRC-1, focal adhesion disassembly assay, Western blotting, quantitative RT-PCR (qPCR), cell transfection, luciferase assay and chromatin immunoprecipitation (ChIP) assay were performed as descried in the Supplementary Methods.

Results

SRC-1 deficiency reduces mammary tumor cell adhesion and migration on FN-coated plate

To define the role of SRC-1 in BC cell adhesion to ECM, we compared the adhesion capabilities of two *SRC-1* KO (KO1 and KO2) to two WT (WT1 and WT2) mouse mammary tumor cell lines. As expected, SRC-1 protein was detected in both WT cell lines but was absent in both KO cell lines (Fig. 1A). After seeded in a FN-coated plate, about 90% of WT1 and 75% of WT2 cells adhered and spread within 1 hour and these percentages increased to nearly 100% by 2.5 hours. On the contrary, only about 20% of KO1 and 36% of KO2 cells adhered and spread on FN-coated plate by 1 hour, increasing to only 60% and 70%, respectively, by 2.5 hours (Fig. 1A and data not shown). After culturing overnight, both *SRC-1* WT and KO cell lines appeared to adhere and spread well on the FN-coated plate (Fig. 1A). Next, we repeated these assays using 5 WT and 5 *SRC-1* KO primary cell preparations isolated from individual mammary tumors in WT;PyMT and KO;PyMT mice. About 60–80% of WT primary cells adhered to FN-coated plate in one hour, while only 40–50% of KO primary cells adhered under the same conditions (Fig. 1B). These results demonstrate that SRC-1 is required for effective adhesion of mammary tumor cells to FN.

Next, the migration of individual *SRC-1* WT and KO cells was tracked on FN-coated plates for 18 hours. The average track areas swept by the two WT cell lines were 60,000 and 90,000 pixels, respectively, while those of the *SRC-1* KO cell lines were only 25,000 and 20,000 pixels (Fig. 1C). Knockdown of SRC-1 using siRNAs in both mouse WT cell lines effectively reduced their migration areas by 50%. (Fig. 1C). Similarly, knockdown of SRC-1 mRNA in MDA-MB-231 human BC cells using three different shRNAs also significantly reduced cell migration on FN-coated plate by 50% (Fig. 1D). These results indicate that

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SRC-1 expression facilitates the migration of mammary tumor cells along FN and SRC-1 deficiency attenuates the tumor cell migration.

SRC-1 deficiency attenuates disassembly and reassembly of focal adhesion complexes (FACs)

Focal adhesions connect the cell cytoskeleton and the ECM through integrins. In motile cells, the rate of focal adhesion assembly at the leading edge and disassembly at the trailing edge determines the speed of cell movement (24). Immunostaining of vinculin, an adaptor protein located in FACs, showed that focal adhesions distributed in a polarized pattern in most WT cells, suggesting an active migration status of these cells. However, the FAC distribution in most (~70%) of *SRC-1* KO cells was non-polarized and, instead, distributed evenly over the cell membrane. This suggested the majority of SRC-1 KO cells remained in an inactive migration status (Fig. 2A). Accordingly, F-actin filaments in *SRC-1* KO cells were much more abundant than that in WT cells (Fig. 2A).

To define the role of SRC-1 in focal adhesion turnover, we examined FAC disassembly and reassembly in WT and *SRC-1* KO cells by immunostaining vinculin. Cells were treated with nocodazole to stimulate FAC formation (Fig. 2B). After removing nocodazole, most WT cells showed FAC disassembly in 1 hour and newly formed FACs in 2 hours. However, most *SRC-1* KO cells took as long as 2 hours for FAC disassembly and 4 hours for FAC reassembly (Fig. 2B). Furthermore, FACs in WT cells transfected with non-targeting siRNAs disassembled in 1 hour and reassembled in 2 hours, while FACs in the same cells transfected with *SRC-1* siRNAs disassembled in about 2 hours and reassembled in about 4 hours (Fig. 2C). These results suggest that SRC-1 is required for faster FAC turnover in the mammary tumor cells.

Integrins are essential components of FACs. They connect ECM with their extracellular domains to cytoskeleton through interaction of their intracellular domains with multiple proteins such as talin, paxillin and FAK (14). Integrins also transfer signals across the cell membrane from both sides (14). Since SRC-1 deficiency reduced cell adhesion to FN, we measured the expression levels of integrins β_1, β_3, α_v and α_5, which form $\alpha_v\beta_3$ and $\alpha_5\beta_1$ heterodimers necessary for binding FN. We found no consensus changes in mRNA levels of integrins β_1, α_v and β_3 in WT and *SRC-1* KO cells (Fig. 2D). However, we found that both mRNA and protein levels of integrin α_5 in the two *SRC-1* KO cell lines were significantly lower than that in the two WT cell lines (Fig. 2D). These results suggest that the decreased adhesion capability of *SRC-1* KO cells may be related to the lower ITGA5 in these cells.

SRC-1 expression positively correlates with ITGA5 expression

To explore the possibility that SRC-1 may regulate *ITGA5* expression, we ectopically expressed SRC-1 in *SRC-1* KO cell lines. We found that SRC-1 restoration in these cells promoted *ITGA5* expression (Fig. 3A). Conversely, knockdown of *SRC-1* in WT cell lines dramatically reduced *ITGA5* mRNA and protein levels (Fig. 3A and data not shown). Knockdown of *SRC-1* in MDA-MB-231 human BC cells also reduced *ITGA5* expression (Fig. 3B). These results indicate that *SRC-1* expression levels positively correlate with *ITGA5* expression levels in mammary tumor cell lines.

Next, we examined *ITGA5* expression levels in *SRC-1* WT;PyMT and KO;PyMT mouse mammary tumors. In all four samples of each tumor type, ITGA5 immunoreactivity was detected on the membrane and in the cytoplasm of tumor cells. However, both the immunostaining signals of ITGA5 and the number of ITGA5-positive tumor cells in WT;PyMT tumors were much higher than those in KO;PyMT tumors isolated from 8- and 14-week-old mice (Fig. 3C and data not shown). The average ITGA5 immunoreactivity

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detected in WT;PyMT tumors (n = 12) was about two folds higher than that detected in KO;PyMT tumors (n = 13). About 40–60% of WT;PyMT tumor cells were ITGA5 positive, while only 20–30% of KO;PyMT tumor cells were ITGA5 positive. These results suggest that ITGA5 expression is also positively associated with SRC-1 expression in mouse mammary tumors.

Finally, we examined the association between SRC-1 and ITGA5 expression levels in human breast tumors by Western blot analysis. The levels of both SRC-1 and ITGA5 proteins were low in ERα- and PR-positive tumors, as well as in ERα-positive and PR-negative tumors. However, the average levels of both SRC-1 and ITGA5 proteins were significantly elevated in ERα- and PR-negative tumors (Fig. 3, D1 and Supplementary Fig. S1). In agreement with the protein levels, the expression levels of SRC-1 and ITGA5 mRNAs were also positively correlated in 264 human ductal breast carcinomas according to the data provided to Oncomine Database by M. Bittner (Fig. 3, D2 and Supplementary Fig. S2).

SRC-1 potentiates ITGA5-mediated signaling and tumor cell migration

Integrins bind to ECM and integrin clustering activates downstream signaling cascades involving phosphorylation or activation of FAK, Src, Paxillin, Rac1 and Erk1/2 (14). To examine the role of SRC-1 in the integrin-signaling pathway, we incubated *SRC-1* WT and KO cells on FN-coated plates for 0.5 or 1 hour and assessed adhesion-induced FAK activation by measuring pY397-FAK, an autophosphorylation site for Src association (14). Western blot analysis revealed that total FAK levels were similar in *SRC-1* WT and KO cell lines, while the pY397-FAK levels in the two *SRC-1* KO cell lines were significantly lower than that in the two WT cell lines at both time points examined. Knockdown of *SRC-1* or *ITGA5* in WT cells also reduced the pY397-FAK levels at both or one of the time points examined (Fig. 4A). Similarly, knockdown of *SRC-1* in MDA-MB-231 human BC cells consistently decreased the pY397-FAK levels (Fig. 4B).

It is known that a decreased pY397-FAK level should be associated with a reduced assembly of FAK and Src complex, and the formation of this tyrosine kinase complex is responsible for phosphorylating Y118-paxillin and several other phosphorylation sites of FAK including pY925 for binding GRB2 and activating Erk1/2 (14). In agreement with a lower assembly/activity of the FAK/Src complex, we found that pY118-Paxillin, active Rac1, pY925-FAK and p-Erk1/2 levels in *SRC-1* KO cells were significantly reduced when compared with WT cells at both 30- and 60-minute adhesion time points (Fig. 4, A and C). Accordingly, knockdown of *SRC-1* or *ITGA5* in *SRC-1* WT tumor cells or knockdown of *SRC-1* in MDA-MB-231 human BC cells also reduced the levels of p-Paxillin (Fig. 4, A and B). Furthermore, p-Erk1/2 levels in *SRC-1* KO cell lines were much lower than that in WT cells, which was consistent with the reduced levels of pY925-FAK for GRB2 recruitment (Fig. 4C). Taken together, these results suggest that SRC-1 deficiency and ITGA5 insufficiency disturbed integrin-mediated signaling, leading to reduced activation of FAK, Src, Rac1 and Erk1/2, the important players of cell adhesion and migration.

To assess the requirement of ITGA5 in cell migration, *ITGA5* mRNA was knocked down in the two *SRC-1* WT tumor cell lines and these cells were subjected to migration assays on a fluorescent bead-coated plate. The WT cells transfected with *ITGA5* siRNAs reduced 60–70% of cell motility on the FN-coated plate when compared with WT cells transfected with non-targeting siRNA (Fig. 4D). These results suggest that the elevated ITGA5 resulted from *SRC-1* overexpression in the mouse and human mammary tumors plays an important role in promoting BC cell migration.

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SRC-1 promotes AP-1-mediated ITGA5 expression

To examine whether SRC-1 can enhance transcriptional activity of the *ITGA5* gene, we constructed three luciferase reporter constructs containing different fragments (F1, F2 and F3) of the *ITGA5* promoter (Fig. 5A). Expression of SRC-1 in HeLa cells significantly activated all three reporters in a SRC-1 dose-dependent manner, suggesting that SRC-1 may coactivate certain TFs associated with all three fragments of the *ITGA5* promoter (Fig. 5B). Sequence analysis identified potential binding sites for several TFs known to use SRC-1 as a coactivator, including PEA3, NF-κB, C/EBPα/β?and AP-1 (2,3,25–27) (Fig. 5A). Expression of PEA3 or NF-κB, either alone or combined with SRC-1, had no significant effects on the activities of all three promoter-reporters. Expression of C/EBPα alone also showed no obvious activation of these promoter-reporters, while coexpression of SRC-1 slightly increased the activities of F1-Luc and F3-Luc promoter-reporters. Expression of C/EBPβ alone activated F2-Luc and F3-Luc promoter-reporters, but coexpression of SRC-1 only slightly increased the activities of these promoter-reporters in cells transfected with high concentrations of SRC-1 plasmids (Fig. 5C). These results suggest that PEA3, NF-κB and C/EBPα/β are not the major TFs that work with SRC-1 to enhance *ITGA5* promoter activity.

Interestingly, although expression of c-Jun alone did not increase the activity of the three *ITGA5* promoter-reporters, coexpression of c-Jun and SRC-1 robustly activated these constructs in a SRC-1 dose-dependent manner, suggesting that the transcriptional activation function of c-Jun and SRC-1 is associated with AP-1 binding site(s) located in the F3 common region of the *ITGA5* promoter (Fig. 5, A and D). Furthermore, deletion of the single AP-1 binding site at bp -9 position significantly reduced the basal activity of all three *ITGA5* promoter-reporters in cells transfected with or without c-Jun plasmids and completely abolished SRC-1-enhanced activities of these *ITGA5* promoter regions (Fig. 5D).

Since SRC-1 interacted with c-Jun (3) to activate the *ITGA5* promoter (Fig. 5D), we performed ChIP assays to examine whether SRC-1 and c-Jun are recruited to the *ITGA5* promoter. ChIP assays demonstrated that the endogenous SRC-1 protein is associated with *ITGA5* promoter regions d (bp −420 to −220) and e (bp −115 to +73) in both MDA-MB-231 and MDA-MB-435 cancer cells (Fig. 6, A and B). Region e contains the functional AP-1 site (Fig. 5A). In contrast, SRC-1 was not associated with *ITGA5* promoter regions a, b and c (Fig. 6, A and B). In agreement with the presence of the functional AP-1 binding site in region e of the *ITGA5* promoter, c-Jun was found strongly associated with region e in both MDA-MB-231 and MDA-MB-435 cells, but only weakly associated with region d in MDA-MB-435 cells (Fig. 6, A and C). These results suggest that both c-Jun and SRC-1 are recruited to the e region of the *ITGA5* promoter. The association of SRC-1 with region d might be due to either SRC-1 interaction with another TF or a cross PCR reaction from an extended template of region e, which is associated with both SRC-1 and c-Jun.

Discussion

The three transcriptional coactivators in the SRC family are gene expression amplifiers regulated by multiple signaling pathways (28). These coactivators are expressed in normal cells at limiting concentrations, so that their changes in concentration and/or activity can effectively modulate gene expression (28). These coactivators are commonly overexpressed in cancers, acting to promote carcinogenesis and/or metastasis. Specifically, *SRC-3* (*AIB1*) is amplified and overexpressed in a subset of BC and its overexpression is associated with HER2 expression and resistance to endocrine therapy (29,30). In mouse BC models, knockout of *SRC-3* suppresses oncogene and carcinogen-induced carcinogenesis and metastasis, while SRC-3 overexpression is sufficient to induce high frequency of mammary tumors (22,31–33). Recent studies also showed that SRC-3 is required for EGFR and HER2

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phosphorylation and activation in BC cells (33). SRC-3Delta4, a splicing isoform of SRC-3, also can serve as a signaling adaptor that links EGFR and FAK and promotes EGF-induced phosphorylation of FAK and c-Src to enhance cell migration (34). SRC-2 (NCOA2) is identified as an overexpressed oncogene in prostate cancer (35). SRC-1 is overproduced in a subset of BC and its expression is positively associated with HER2 expression, tamoxifen resistance and poor prognosis (6,8,28). In mouse models, knockout of *SRC-1* does not affect primary mammary tumor formation but effectively suppresses metastasis (9). These findings highlight the crucial roles of the SRC family coactivators in cancer initiation, progression and metastasis.

Because SRCs work as transcriptional co-regulators for many nuclear receptors and other TFs, it has been difficult to identify key SRC-associated TFs and their target genes responsible for promoting tumorigenesis and metastasis. Although recent studies have made progress in understanding the mechanisms responsible for SRC-1 to promote BC metastasis (2,5,9), it is just the beginning to identify key genes and gene networks regulated by SRC-1.

In this study, we have found that SRC-1 deficiency slows BC cell adhesion and migration on FN, which correlates well with the attenuated disassembly and reassembly of FACs in *SRC-1* KO and knockdown cells. Furthermore, we identified *ITGA5* as a new SRC-1 target gene based on multiple lines of evidence. First, *SRC-1* expression levels are associated with *ITGA5* expression levels in both mouse and human BC cell lines and primary tumors. Knockout or knockdown of *SRC-1* reduces *ITGA5* expression, while *SRC-1* expression enhances *ITGA5* expression. Second, SRC-1 is a known coactivator of AP-1 (3), and we found that both AP-1 and SRC-1 associate with the *ITGA5* promoter to enhance *ITGA5* promoter activity. Although experiment to knock down c-Jun was not performed to further validate the functional contribution of c-Jun to *ITGA5* expression in this study, previous studies have shown that AP-1 strongly enhances ITGA5 expression (36,37). Finally, the reduced *ITGA5* expression caused by SRC-1 deficiency or insufficiency in BC cells partially impairs the function of the FN-integrin-FAK-cell migration pathway. In this pathway, the heterodimers of α5β1 integrins bind FN to induce phosphorylation of pY397-FAk and formation of the active FAK/Src complex, followed by further phosphorylation of FAK and phosphorylation/activation of downstream signaling components including Paxillin, RAC1, and Erk1/2 (14,38–40). In agreement with an important role of SRC-1-mediated *ITGA5* expression in the FN and integrin interaction-initiated signaling and cell migration, the FAK phosphorylation at Y397, paxillin phosphorylation and activation of Rac-1 and Erk1/2 during cell adhesion process are significantly reduced in *SRC-1* knockout and *SRC-1* or *ITGA5* knockdown BC cells. Taken together, these findings demonstrate that SRC-1-mediated *ITGA5* expression is partially responsible for SRC-1-promoted adhesion, migration and metastasis of BC cells.

In the literature, the role of integrins $\alpha_5\beta_1$ in cancer is somewhat controversial. One early study showed that elevated levels of integrin $\alpha_5\beta_1$ suppressed the transformed phenotype of CHO cells (41). Some studies also reported an inhibitory effect of integrins $\alpha_5\beta_1$ on tumorigenesis and metastasis (12,42). However, multiple lines of evidence suggest that integrin $\alpha_5\beta_1$ expression positively correlates with cancer progression and metastasis. First, integrin $\alpha_5\beta_1$ expression is upregulated in malignant BC cells and its upregulation correlates with poor prognosis (43). Second, SDF1-activated CXCR4 upregulates integrin $\alpha_5\beta_1$ expression and enhances prostate tumor cell adhesion, invasion and metastasis (44). Third, a mutant p53 has been shown to drive cell invasion and metastatic behavior via activating EGFR/integrins $\alpha_5\beta_1$ signaling (45). Fourth, FN and ITGA5 are required for switching cell-cell adhesion to cell-ECM adhesion, and this switch is required for tumor cells to invade into the stromal tissue (46). Fifth, E-cadherin expression in ovarian and breast cancers negatively correlates with *ITGA5* expression, suggesting an important role of ITGA5 in EMT (47,48).

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Finally, blocking the function of integrins $\alpha_5\beta_1$ by an antibody (Volociximab) or a non-RGD-based peptide inhibitor (ATN-161) significantly inhibits BC growth and metastasis (49,50). In this study, we have demonstrated that SRC-1 upregulates *ITGA5* expression to promote BC cell adhesion and migration on FN, facilitating local invasion of these tumor cells. Based on the important contributions of ITGA5 in cancer cell migration, invasion and metastasis described above, it is reasonable to conclude that *ITGA5* functions as one of the key target genes of SRC-1 to mediate SRC-1-promoted BC cell migration and metastasis.

Since SRC-1 serves as a coactivator for multiple TFs such as AP-1, PEA3, Ets-2 and HOXC11 to upregulate the expression of multiple target genes such as *ITGA5*, *Twist1*, *CSF-1*, *c-Myc* and *S100beta* in promotion of BC metastasis (2,5,7,9), targeting SRC-1 could be a potential strategy to interfere multiple pathways involved in cancer metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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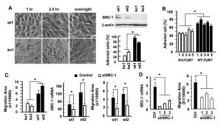

Fig. 1. SRC-1 deficiency reduces BC cell adhesion and migration on FN-coated plates

A. Phase contrast images of *SRC-1* WT1 and KO1 mammary tumor cell lines after culturing on FN-coated plates for time periods indicated (left panel), and percentages of adhered KO1, KO2, WT1 and WT2 cells at 1 hour culture time (right panel). *, $p<0.05$ by t test. Western blot results showed SRC-1 protein presence in WT1 and WT2 cells and absence in KO1 and KO2 cells. **B**. Percentages of adhered *SRC-1* KO;PyMT (n=5) and WT;PyMT (n=5) primary mammary tumor cells after culturing on FN-coated plates for 1 hour. **C**. Left panel: Migration track areas of *SRC-1* KO1, KO2, WT1 and WT2 cell lines on FN-coated plates. Average migration area was calculated from the track areas of 50 cells. Middle panel: Knockdown of *SRC-1* mRNA in WT1 and WT2 cells using siRNAs; non-targeting siRNA was used as a control. Right panel: *SRC-1* knockdown reduced the average migration areas of WT1 and WT2 cells. **D**. Left panel: knockdown of *SRC-1* mRNA in MDA-MB-231 cells using three shRNA constructs; non-targeting shRNA was used as a control. Right panel: MDA-MB-231 cells with *SRC-1* knockdown showed reduced cell migration as measured by tracing the migration areas with fluorescent beads.

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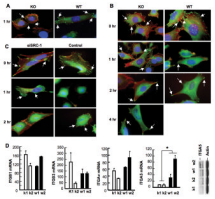

Fig. 2. The disassembly and reassembly of FACs and *ITGA5* expression in *SRC-1* KO and WT mammary tumor cells

A. Different distribution patterns of FACs and F-actin in *SRC-1* KO1 and WT1 cells as revealed by vinculin immunostaining (green) and philloidin staining (red). Cells were cultured on FN-coated plate for one hour. Arrows indicate FACs. **B**. FAC disassembly and reassembly in *SRC-1* KO1 and WT1 cells were monitored at the time points indicated by vinculin immunostaining. **C**. FAC disassembly in SRC-1 siRNA-transfected WT1 cells was slower than that in control siRNA-transfected WT1 cells. **D**. Measurements of *ITGB1*, *ITGB3*, *ITGAv* and *ITGA5* mRNA levels by qPCR, as well as ITGA5 protein levels by Western blot in *SRC-1* KO1, KO2, WT1 and WT2 cells. *, $p<0.05$ by t test.

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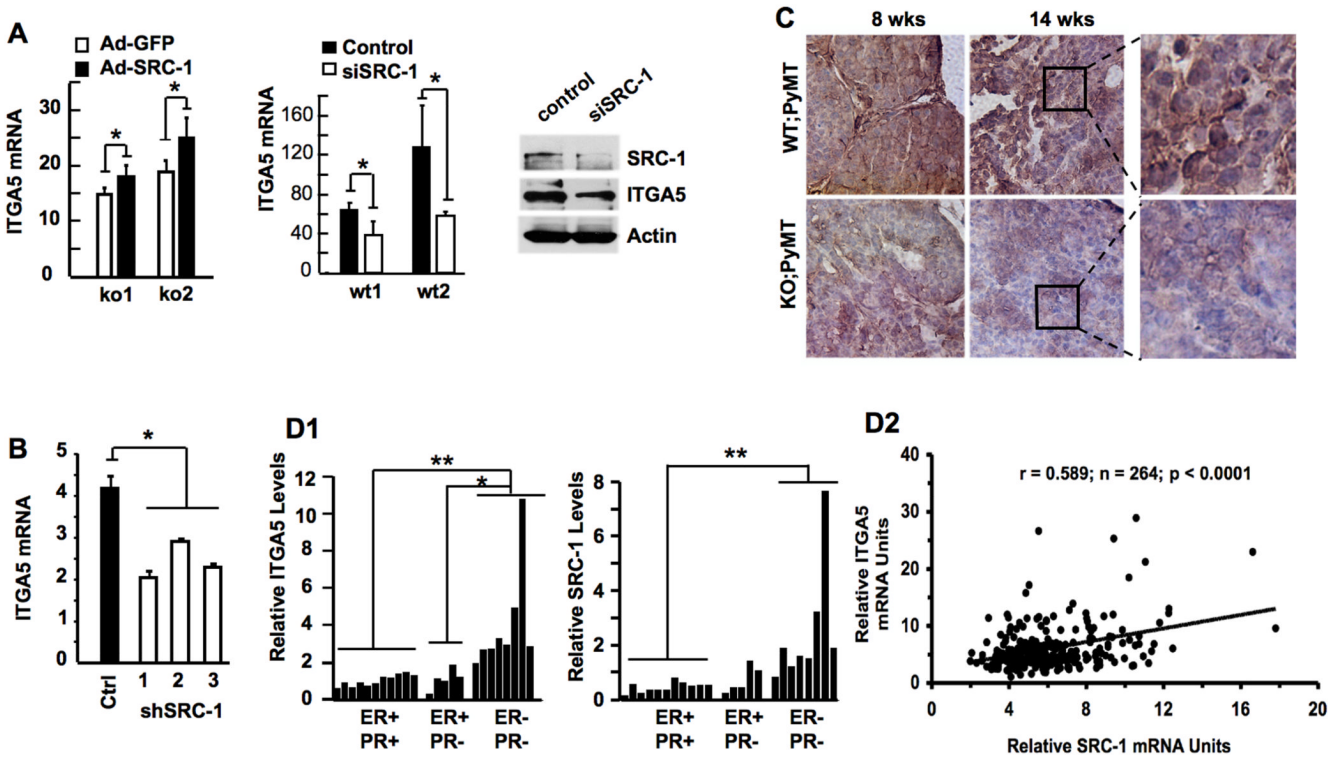

Fig. 3. *SRC-1* expression positively correlates with *ITGA5* expression

A. Adenovirus-mediated *SRC-1* expression in *SRC-1* KO1 and KO2 cells enhanced *ITGA5* expression. Adenovirus-mediated GFP expression served as a control (left panel). Conversely, siRNA-mediated knockdown of *SRC-1* decreased *ITGA5* mRNA and protein levels in *SRC-1* WT1 and WT2 cells. The non-targeting siRNA served as a control (middle and right panels). *, $p<0.05$ by t test. B. The shRNA-mediated stable knockdown of SRC-1 in MDA-MB-231 cells reduced *ITGA5* expression. **C**. Immunohistochemical staining (brown color) of ITGA5 in SRC-1 WT;PyMT and KO;PyMT tumors isolated from 8- and 14-week-old mice. Slides were counterstained with hematoxylin. **D1**. Western blot analysis of SRC-1 and ITGA5 proteins in ERα+;PR+ (n=11), ERα+;PR- (n=5) and ERα-;PR- (n=8) human breast tumors was performed as shown in Supplementary Figure 1. β-actin was assayed as a loading control. Band intensities were measured by densitometry. Relative ITGA5 and SRC-1 levels were normalized to β-actin and presented here as bar graphs. *, $p<0.05$, and **, $p<0.01$ by F test. **D2**. SRC-1 mRNA levels correlate with ITGA5 mRNA levels in human ductal breast carcinoma. The microarray data were collected by M. Bittner and downloaded from Oncomine Database. The SRC-1 and ITGA5 expression data shown in Supplementary Figure 2 were plotted and statistically analyzed by Pearson correlation. The p value indicates that the expression levels of SRC-1 significantly correlate with the expression levels of ITGA5 in the human ductal breast carcinomas.

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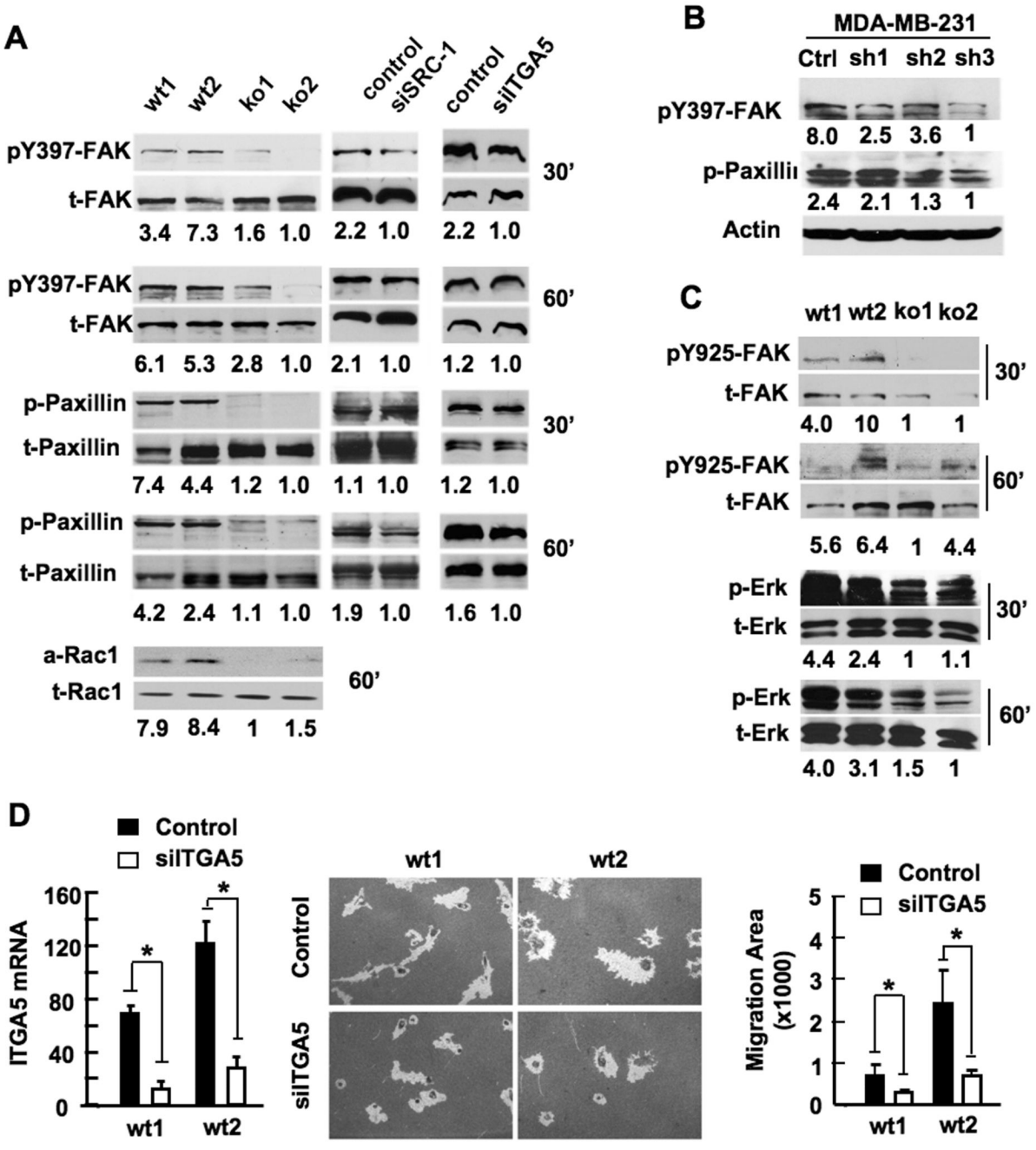

Fig. 4. Effects of *ITGA5* reduction in SRC-1 deficient cells on integrin-signaling pathways and cell migration

A. Western blot analysis of pY397-FAK, total FAK (t-FAK), p-Paxillin, t-Paxillin, active Rac1 (a-RAC1) and t-RAC1 in *SRC-1* WT1, WT2, KO1 and KO2 cells and WT1 cells transfected with siRNAs against SRC-1 (siSRC-1) or ITGA5 (siITGA5) mRNA or with non-targeting siRNA (control). Cells were allowed to adhere to FN-coated plates for 30 or 60 minutes prior to the analysis. The relative band intensities (indicated) were normalized to total protein band intensities. **B**. Western blot analysis of pY397-FAK, p-Paxillin and β-actin in MDA-MB-231 cells with stable expression of non-targeting shRNA (Ctrl) or one of the three shRNAs targeting different regions of the *SRC-1* mRNA. **C**. Western blot analysis of

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pY925-FAK, t-FAK, p-Erk1/2 and t-Erk1/2 in *SRC-1* WT1, WT2, KO1 and KO2 cells after cultured on the FN-coated plates for 30 or 60 minutes. Relative band intensities are indicated. **D**. WT1 and WT2 cells were transfected with either control siRNA or siRNAs targeting *ITGA5* mRNA. *ITGA5* mRNA levels in these cells were measured by qPCR (left panel). These cells were subjected to migration assays on FN- and fluorescence bead-coated plate (middle panel). The track areas of individual cell migration were traced and averaged from about 50 cells for each group. *, $p<0.05$ by t test.

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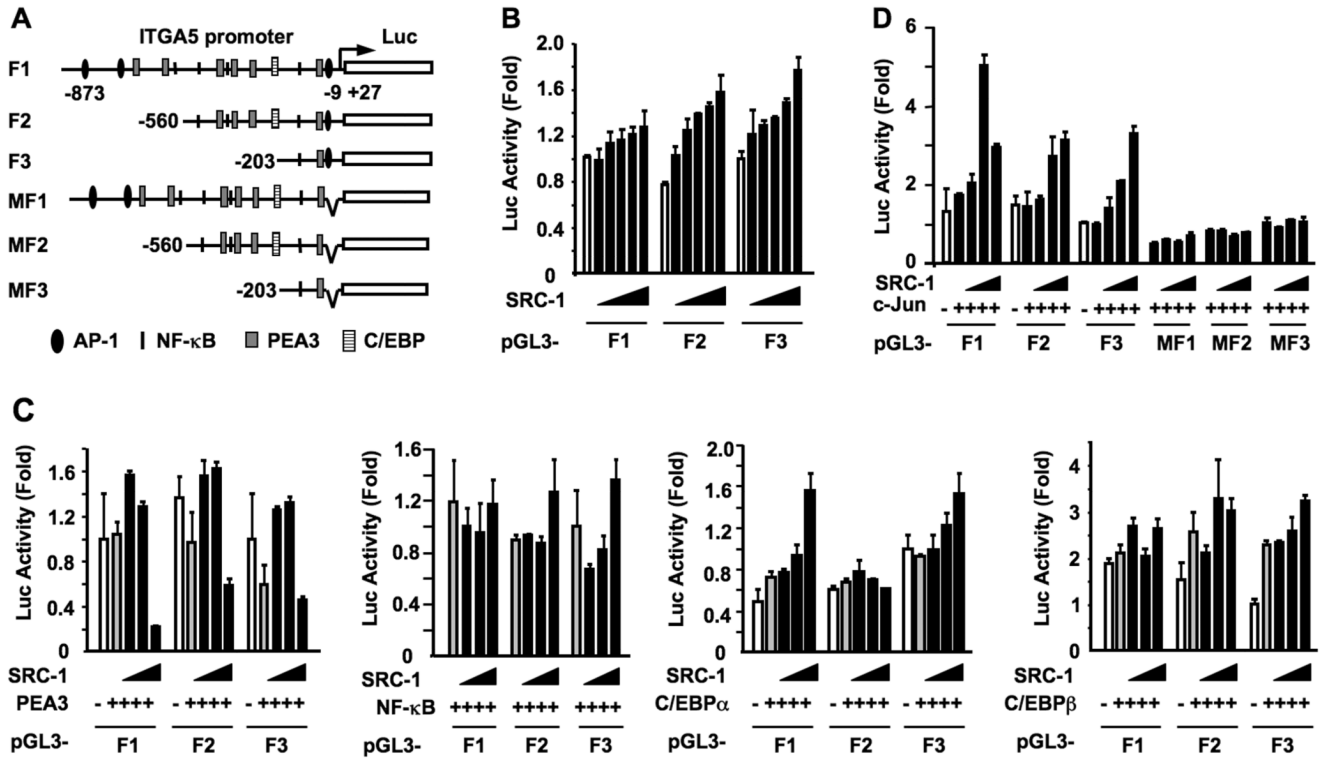

Fig. 5. SRC-1 regulates *ITGA5* promoter activity

A. The pGL3 plasmid constructs containing different *ITGA5* promoter fragments, F1, F2 or F3, which are linked to the luciferase (Luc) reporter sequence. The computer-mapped binding sites for AP-1, NF-κB, PEA3 and C/EBP are indicated. The AP-1 site at bp -9 is deleted in pGL3-MF1-Luc, pGL3-MF2-Luc and pGL3-MF3-Luc constructs. **B**. Expression of SRC-1 in HeLa cells enhances pGL3-F1-Luc, pGL3-F2-Luc and pGL3-F3-Luc activities in a dose dependent manner. **C**. Effects of SRC-1 co-expression with PEA3, NF-κB, C/EBPα or C/EBPβ on the activities of pGL3-F1-Luc, pGL3-F2-Luc and pGL3-F3-Luc in HeLa cells. **D**. Co-expression of SRC-1 and c-Jun in HeLa cells significantly enhanced the activities of pGL3-F1-Luc, pGL3-F2-Luc and pGL3-F3-Luc, but not the mutant pGL3-MF1-Luc, pGL3-MF2-Luc and pGL3-MF3-Luc.

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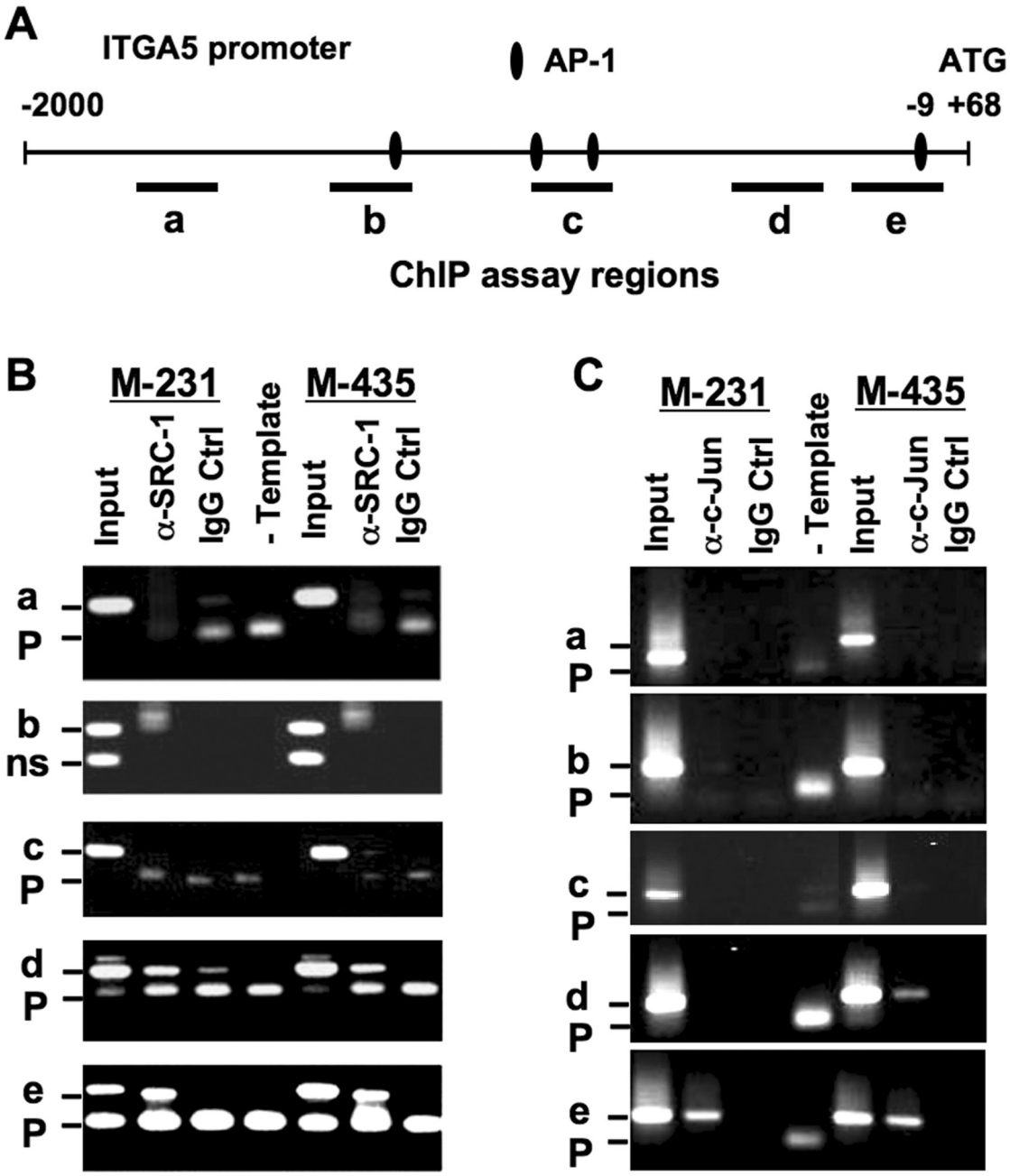

Fig. 6. SRC-1 and AP-1 are associated with a proximal region of the *ITGA5* promoter

A. ChIP assays were designed to detect the a, b, c, d and e regions of the ITGA5 promoter with specific PCR primer pairs as indicated. Predicted AP-1 binding sites are indicated. **B and C**. ChIP assays were performed with MDA-MB-231 and MDA-MB-435 cells and antibodies against SRC-1 (panel B) and c-Jun (panel C). DNA extracted from cross-linked and sonicated cell lysate was used as input control. IgG served as a control for antibody specificity in ChIP assays. No template (-template) PCR reaction served as a negative control for PCR. PCR-amplified fragments a, b, c, d and e are indicated. The P indicates the primer bands. The ns indicates a non-specific PCR product.