Published in final edited form as:

Exp Cell Res. 2017 February 01; 351(1): 1–10. doi:10.1016/j.yexcr.2016.12.011.

Convergence of Eicosanoid and Integrin Biology: Role of Src in 12-LOX activation

Ashok-kumar Dilly^{a,1}, Keqin Tang^{b,2}, Yande Guo^{a,3}, Sangeeta Joshi^{a,4}, Prasanna Ekambaram^{a,5}, Krishna Rao Maddipati^a, Yinlong Cai^a, Stephanie C. Tucker^a, and Kenneth V. Honn^{a,c,*}

^aDepartments of Pathology-Bioactive Lipids Research Program, , Detroit, MI 48202

^bDepartments of Radiation Oncology, Wayne State University School of Medicine, , Detroit, MI 48202

^cDepartments of Karmanos Cancer Institute, Detroit, MI 48202

Abstract

12-Lipoxygenase (12-LOX) metabolizes arachidonic acid to 12(*S*)-hydroxyeicosatetraenoic acid, or 12(*S*)-HETE, a proinflammatory bioactive lipid implicated in tumor angiogenesis, growth, and metastasis. The mechanisms underlying 12-LOX-mediated signaling in cancer progression are still ill-defined. In the present study we demonstrate that 12-LOX phosphorylation and subsequent enzymatic activity occurs after integrin $\beta 4$ stimulation and Src kinase recruitment to the integrin subunit. Inhibition of Src activity by PP2 or Src dominant-negative mutants reduced 12-LOX tyrosine phosphorylation and 12(*S*)-HETE production in response to integrin $\beta 4$ stimulation in A431 cells. The pertinent Src-targeted residues for 12-LOX activity were mapped to Y19 and Y614, where 12-LOX mutants Y19F and Y614F showed 70% less enzymatic activity.

Furthermore, we have shown that the 12-LOX activity modulated by these residues impacts migration. To our knowledge, this is the first report that c-Src kinase activity is required for $\beta 4$ -integrin-mediated phosphorylation of 12-LOX.

Graphical abstract

*Correspondence To: Department of Pathology, 431 Chemistry Bldg. Wayne State University, Detroit, MI 48202. Tel.: (313) 577-2999; Fax: (313) 577-0798. k.v.honn@wayne.edu.

¹Present Addresses: 5177 Center Ave., Hillman Cancer Center, Department of Surgery, SCAIFE 497, Pittsburgh, PA 15232

²Present Addresses: Department of Radiation Oncology, John D. Dingell VA Medical Center, Detroit, MI, 48201

³Present Addresses: Southern Illinois University School of Medicine, Department of Medical Microbiology, Immunology, and Cell Biology, Springfield, IL 62794

⁴Present Addresses: OncoTartis, Inc., The Thomas R. Beecher, Jr. Innovation Center, Buffalo, NY 14203

⁵Present Addresses: Division of Hematology Oncology, Children's Hospital of Pittsburgh of UPMC, Department of Pediatrics, University of Pittsburgh, Pittsburgh, PA-15224 University, Detroit, MI 48202. Tel.: (313) 577-2999; Fax: (313) 577-0798.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

12-Lipoxygenase phosphorylation; $\beta 4$ Integrin; c-Src kinase; cell migration

Introduction

Integrins are heterodimeric cell adhesins that link the extracellular matrix to the cytoskeleton, where for example, the preferred extracellular ligands for integrin $\alpha 6\beta 4$ are laminin-5 and laminin-1 [1]. In addition to providing a structural, adhesive function during cellular attachment to substrata, integrins also provide a signaling scaffold at the plasma membrane and within cellular compartments to promote protein complexes that direct cell proliferation, migration, differentiation, and survival [2–4]. The $\alpha 6\beta 4$ integrin is intensively studied for its ability to promote invasion and metastases, and most recently, to direct exosomes to prepare metastatic niches [5–9]. The $\beta 4$ cytoplasmic domain can be phosphorylated by both tyrosine and serine/threonine kinases on residues whose phosphorylation state regulates integrin interaction with intracellular cytoskeleton components and signaling intermediates [10–15].

Integrins can be regulated by phosphorylation through the action of the c-Src family kinases (SFKs), non-receptor protein tyrosine kinases that mediate signal transduction during cellular differentiation, adhesion and migration [16–18]. Motivated by the observation that integrin $\beta 4$ has potential tyrosine phosphorylation sites in its cytoplasmic tail, Giancotti *et al.* revealed that $\beta 4$ functions with SFKs as a binary receptor tyrosine kinase (RTK) that affects both activation and deactivation signals [19]. Previous studies identified residue Y1494 in the $\beta 4$ cytoplasmic domain of $\alpha 6\beta 4$ as a potential regulatory site of tumor cell functions [5]. Shaw *et al.*, demonstrated that mutation of Y1494 significantly inhibited overall tyrosine phosphorylation of the $\beta 4$ subunit in response to $\alpha 6\beta 4$ ligation, suggesting that this site could be a master regulator of $\alpha 6\beta 4$ phosphorylation and signaling [14]. This is relevant as specific kinase-integrin combinations may reflect variability in downstream

signaling cascades based on cancer type. For example, in a newly published study by Leng *et al.*, it was determined that in the context of liver cancer the interaction between ITGB4 and EGFR activates AKT by using FAK instead of SRC [20].

Previously, we described an interaction between the integrin $\beta 4$ cytoplasmic domain and the eicosanoid enzyme, 12-Lipoxygenase (12-LOX; ALOX12) [21]. We subsequently validated the physiological relevance of this interaction in mammalian cancer cells, by demonstrating that stimulation of the adhesin recruits 12-LOX to integrin $\beta 4$ leading to enzymatic metabolism of arachidonic acid and subsequent 12(*S*)-HETE production [22].

12-LOX is expressed in a variety of tumor cells. Its mRNA has been detected in erythroleukemia, colon carcinoma, epidermoid carcinoma, human glioma, and breast cancer cells [23]. The enzyme is also actively expressed in cancer cell lines that produce 12(*S*)-HETE [24–30]. There is compelling evidence for 12-LOX as a key regulator of human cancer development. It is over expressed more frequently in advanced stage, high-grade cancer, which suggests an association with carcinoma progression and invasion *in vivo* [28, 31]. We demonstrated previously that 12(*S*)-HETE is involved in the proliferation of prostate carcinoma cells [32–33], and that inhibition of 12-LOX causes apoptosis of these cells [34]. In an *in vivo* mouse model, PC3 cells stably over-expressing 12-LOX promoted enhanced tumor growth and angiogenesis [28].

Like protein cytokines such as IL-6, the bioactive lipid metabolite, 12(*S*)-HETE is a fundamental pro-inflammatory activator. It signals through NF- κ B and AKT to produce proteases, promote angiogenesis, facilitate migration, and shield cancer cells from apoptosis with pro-survival programs that lead to such phenotypes as radiation resistance [22–23, 35–39].

The present study provides mechanistic insight into the regulatory functions provided by $\beta 4$ integrin ligation vis-à-vis c-Src kinase activity and 12-LOX signaling events. The biochemical activation was modeled using human epidermoid A431 cells, where the cDNA sequence of endogenous 12-LOX is identical to platelet-type 12-LOX [40]. The data demonstrate that c-Src increases 12-LOX tyrosine phosphorylation, but in an integrin-dependent manner, leading to increased lipoxygenase activity and cell migration *in vitro*. This provides additional insight into the regulation of 12-LOX by $\beta 4$ integrin and its role in cancer progression.

Materials and Methods

Materials

A431 (Human epithelial carcinoma) and HEK293 (Human embryonic kidney) cell lines were obtained from ATCC (Manassas, VA) and used at low passage within four years of purchase. HUVECs (Human Umbilical Vein Endothelial Cell) were from Lonza (Walkersville, MD). Recombinant c-Src active protein was from Millipore (Billerica, MA). 15-HETE-d8, arachidonic acid-d8 and arachidonic acid (AA) were from Cayman Chemical (Ann Arbor, MI). All other reagents were of analytical grade and from SigmaAldrich (St. Louis, MO).

Antibodies

For Western blotting and immunoprecipitation, antibodies used were as follows: 12-LOX (Oxford Biomedical Research, Rochester Hills, MI), Phosphotyrosine-20 (BIOMOL, Plymouth, PA), Phosphorylated c-Src-Y418 (pSrc) (Invitrogen), c-Src (Cell Signaling), Phospho-specific Integrin β 4-Y1494 (ECM Biosciences, Versailles, KY), β 4 integrin subunit (BD Pharmingen), and actin (Chemicon, Billerica, MA). An antibody to integrin β 4 (3E1) for immunoprecipitation and integrin stimulation [41] was also obtained from Chemicon.

SDS-PAGE and Western blotting

Cells were extracted in lysis buffer [42]. Protein was separated by 10% SDS-PAGE, and transferred to nitro-cellulose membranes. Blots were then probed with indicated antibodies and developed by enhanced chemiluminescence (Pierce) according to the manufacturer's instructions. Image intensity was quantified with AlphaEase software accompanying the Alpha Innotech Fluorchem digital imaging system (ProteinSimple San Jose, CA).

Immunoprecipitation

Whole cell, chemical lysates (typically 500 μ g protein) of A431 or HEK293 cells were pre-cleared with protein G beads before incubating with a specific antibody overnight, which was followed by the addition of protein G beads for 1h. Beads were washed with immunoprecipitation (IP) wash buffer before being boiled with SDS sample buffer and subjected to SDS/PAGE and Western blotting.

Analysis of 12-LOX phosphorylation

For metabolic radio-labeling with $^{32}\text{PO}_4$, cells were cultured in phosphate-deficient medium (GIBCO) for 1h following overnight serum starvation. After 1h, $^{32}\text{PO}_4$ was added at 1 mCi/ml and cells were incubated for an additional 2h. Cells were then stimulated with β 4 mAb (5 μ g/ml) for 5, 15, 30, 60 and 90 min. The reactions were terminated and cells were extracted with lysis buffer as described above. Samples were immunoprecipitated with 12-LOX antibody, resolved by SDS-PAGE (4–20% gradient) and transferred to PVDF membranes. Blots were exposed to X-ray film, developed, and subsequently redeveloped by immunoblot with P-12-LOX antibody for confirmation.

Measurement of c-Src phosphotransferase activity -kinase assay

After β 4 ligation, cell lysates were subjected to immunoprecipitation with β 4 integrin antibody, and associated Src activity was measured using [γ ^{32}P]-ATP with a Src-specific peptide substrate (Upstate Biotechnology, Inc., Lake Placid, NY) as per manufacturer's recommendations. Reaction products were read in a scintillation counter.

Measurement of 12-Lipoxygenase activity by LC-MS in A431 and HEK293 cells

Enzymatic activity of 12-LOX was determined by measuring 12(*S*)-HETE production by liquid chromatography-mass spectrometry (LC-MS). Parental and transfected cell lines (5×10^6 cells; A431 or HEK293, and respective transfectants) were grown to 90% confluence in 100 mm Petri dishes and serum-starved overnight prior to experimental use. Cells were washed with PBS and pretreated with different inhibitors, followed by stimulation with

either $\beta 4$ antibody (1 $\mu\text{g/ml}$), laminin (1 $\mu\text{g/ml}$), or control IgG (1 $\mu\text{g/ml}$) along with addition of 10 μM arachidonic acid in serum-free DMEM medium. After 6h, conditioned medium was collected and centrifuged. Supernatants were extracted for lipids by the method of Suzuki *et al.* [43], supplemented with an internal standard (10 ng of 15-HETE-d8), and applied to washed ODS-Silica cartridges. Lipid extracts were eluted from the cartridge with freshly redistilled ethyl acetate, evaporated under a stream of nitrogen and reconstituted in methanol. Before LC-MS detection, samples were supplemented with 50 μl of 50 mM ammonium acetate. Inhibitors baicalein and LY294002 were used at 20 μM , and PP2 at 5 μM .

Endothelial cell migration assay

Cell migration and invasion assays were performed in a modified Boyden chamber using 24-well cell culture plates fitted with 8- μm transwell filters as described [44]. Filters were coated with Matrigel (BD Pharmingen) for invasion assay. Lipid extracts from conditioned media, described above, were reconstituted with EBM-2% FBS and added to the bottom chamber. HUVEC cells that were trypsinized and suspended in 0.1% BSA (4×10^5 cells/0.5 ml) were plated onto the filters and incubated. After 18h, cells on the upper side of the membrane were removed. Cells penetrating the filter were stained with Cell Stain Solution (Chemicon) and imaged by bright field microscopy. Relative cell attachment was determined by absorbance measurements on acetic acid-extracted dye at 560 nm as per standard methods.

Site-directed mutagenesis for 12-LOX phosphorylation site

Based on the NetPhos algorithm, three putative tyrosine phosphorylation sites (Y19, Y295 and Y614) for 12-LOX were identified. Three primer pairs were designed to alter tyrosine to phenylalanine. Quik Change mutagenesis was performed using the primers listed below and according to the manufacturer's specifications (Stratagene, La Jolla, CA). The codon that was mutated is underlined:

12LOX-Y19F, 5'-ggctcttccgggtcgtacaaccgcgtgcagctttg-3';

12LOX-Y295F, 5'-atccgaggagagaagcaatacctggctgccccctcg-3';

12LOX-Y614F, 5'-gggcaccacaaagaaaatatttctcaggccccaagc-3'.

Incorporation of the mutation was confirmed by DNA sequencing at the Wayne State University Sequencing Core facility.

Transient transfection

HEK-293 cells were cultured in RPMI with 10% FBS and transiently transfected using Lipofectamine (Invitrogen) according to the manufacturer's protocols. Cells were transfected with either vector alone or the following individual plasmids encoding cDNA for: $\beta 4$, 12LOX, 12LOX-Y19F, 12LOX-Y295F, or 12LOX-Y614F in combination with a plasmid encoding c-Src or dominant-negative mutant c-Src (pCMV5 c-Src Y295R Y527F) (Addgene, MA). After transfection (48h), cells were treated with 3E1 antibody for 30 min to ligate integrin $\beta 4$, and cell homogenates were generated in cell lysis buffer for immunoprecipitation and immunoblot analysis.

Statistical analysis

Data are reported as mean $\pm$ S.D of at least three experiments. Comparisons between multiple groups were made by analysis of variance (ANOVA).

Results

Integrin ligation in A431 cells leads to tyrosine phosphorylation of integrin $\beta 4$

Previously we demonstrated that ligation of integrin $\beta 4$ either with an activating antibody (3E1) or natural ligand (laminin) prevented human epidermoid carcinoma A431 cells from undergoing apoptosis [45]. This is in large part due to the action of the arachidonate metabolite, 12(*S*)-HETE that is produced on 12-LOX recruitment and activation by the ligated integrin; a new paradigm that we characterized previously by immunofluorescence [22]. In order to understand the integrin $\beta 4$ signaling pathway further, we examined whether tyrosine phosphorylation of $\beta 4$ integrin is dependent on ligation as was observed in another system [10]. After stimulation with either laminin, 3E1 antibody, or control IgG, integrin $\beta 4$ was immunoprecipitated from A431 cell lysates and subsequently probed for phosphorylation by immunoblot with PY20 antibody. The results indicated that there was a significant difference in integrin $\beta 4$ phosphorylation between treated and untreated cells (Fig. 1A, B and C). Dutta *et al.* reported that Y1494- $\beta 4$ is likely a master regulator of integrin phosphorylation and signaling [5]. Therefore, to determine whether Y1494 is also phosphorylated in A431 cells after $\beta 4$ ligation, the integrin was again immunoprecipitated and immunoblotted with phospho-specific antibody for this phosphorylated residue (Y1494), (Fig. 1D and E). When compared to IgG control, a significant increase in tyrosine phosphorylation of integrin $\beta 4$ at Y1494 in response to integrin ligation was seen (Fig. 1F). To confirm this, we performed a reciprocal immunoprecipitation with the phospho-specific antibody to integrin $\beta 4$ (Y1494) followed by immunoblot analysis with antibody to full-length $\beta 4$ (Fig. 1G).

Ligation of integrin $\beta 4$ modulates c-Src phosphorylation and its activity

Prior reports have indicated that $\alpha 6\beta 4$ signaling is mediated by a pSFK [46]. To determine whether $\beta 4$ stimulation induces c-Src activation in A431 cells, cell lysates were immunoblotted with antibody to phospho-Src (Y418), as it has been shown that an increase in Y418 phosphorylation of c-Src is associated with its activity [47]. The antibody detected a 60 kDa band suggesting that in A431 cells, $\beta 4$ ligation induced the activation of c-Src (Fig. 2A, B, C). We next examined whether the integrin physically associates with pSrc in A431 cells after $\beta 4$ ligation by performing an immunoprecipitation with $\beta 4$ antibody followed by immunoblotting with pSrc antibody (Fig. 2D). The blots were reprobed for integrin $\beta 4$ to ensure that equal amounts of the integrin were immunoprecipitated across all samples (Fig. 2E). To verify the interaction, a reciprocal immunoprecipitation was performed on cell lysates with pSrc antibody followed by immunoblot with $\beta 4$ antibody. The results suggested that integrin $\beta 4$ stimulation augments its association with pSrc (Fig. 2F). To assay $\beta 4$ -associated c-Src activity, cell lysates were immunoprecipitated using the $\beta 4$ antibody, and integrin-associated pSrc kinase activity was measured *in vitro*. pSrc activity was significantly higher under conditions where integrin $\beta 4$ was ligated with stimulating antibody (3E1) compared to IgG control or to pSFK inhibitor, PP2 (Fig. 2G). These results

suggest that $\beta 4$ ligation induces the association between integrin $\beta 4$ and pSrc, leading to enzymatic c-Src activation in A431 cells.

pSrc-dependent phosphorylation of 12-LOX leads to increased 12(S)-HETE production

Next we examined whether integrin $\beta 4$ -mediated activation of c-Src influenced the phosphorylation status of 12-LOX. First, cell lysates from laminin-treated, 3E1 antibody-treated, or IgG control cells were immunoprecipitated with 12-LOX antibody followed by immunoblot analysis with PY20 antibody (Fig. 3A). Phosphorylation of 12-LOX was increased on integrin stimulation. Likewise, 12-LOX was more abundant in PY20 antibody immunoprecipitates under integrin ligation conditions (Fig. 3B). We have shown previously that integrin $\beta 4$ ligation activates PI3 kinase in A431 cells [45]. Therefore, to determine whether phosphorylation of 12-LOX is regulated through c-Src or the PI3 kinase pathway, we preincubated the cells in the presence of either the PI3K inhibitor, LY294002, or Src kinase inhibitor, PP2, followed by integrin $\beta 4$ ligation. The resulting immunoprecipitation and immunoblot indicated that phosphorylation of 12-LOX was inhibited by PP2, whereas LY294002 (data not shown) failed to inhibit the $\beta 4$ -ligation-induced phosphorylation of 12-LOX (Fig. 3A). This result was confirmed by reciprocal immunoprecipitation under the same conditions, and probing with PY20 antibody followed by immunoblot analysis with 12-LOX antibody (Fig. 3B). Taken together, these results suggest that increased 12-LOX tyrosine phosphorylation is dependent on c-Src kinase and is independent of PI3K kinase.

Phosphorylated 12-LOX exhibits elevated activity resulting in higher 12(S)-HETE levels [48–50]. To determine whether 12-LOX phosphorylation, resulting from integrin $\beta 4$ ligation, leads to 12-LOX activation, the enzymatic activity of 12-LOX was determined by LC-MS analysis of 12(S)-HETE. Conditioned RPMI medium from cultured A431 cells that were treated with or without the c-Src kinase inhibitor PP2 for 2h, and either control IgG, laminin, or 3E1, along with 10 μ M AA (6h) was analyzed as described in experimental procedures. A significant increase in 12-LOX phosphorylation and activity was observed in culture supernatants during integrin $\beta 4$ ligation compared to the IgG-treated control. Furthermore, the level of inhibition of 12-LOX activity by PP2 was comparable to that seen by a known 12LOX inhibitor, baicalein, and was significant compared to untreated cells (Fig. 3C). As our experimental results showed that phosphorylation of 12-LOX and its subsequent activity is inhibited by PP2, we wished to confirm the result using c-Src wild type (WT) or dominant negative (DN) expression constructs in combination with integrin $\beta 4$ and 12-LOX. Integrin $\beta 4$ ligation was induced in transfected HEK293 cells that lack endogenous $\beta 4$ and 12-LOX. Thereafter, cell lysates were immunoprecipitated using the 12-LOX antibody, followed by immunoblot with PY20 antibody and the blot was reprobed for 12-LOX. As evident, on $\beta 4$ ligation, 12-LOX tyrosine phosphorylation is significantly lower in the presence of DN-c-Src when compared to WT c-Src transfected HEK293 cells (Fig. 3D). The interaction of 12-LOX with integrin $\beta 4$ was largely unaffected by altering Src (Fig. 3E). In addition to preventing tyrosine phosphorylation of 12-LOX, DN-c-Src also inhibited its activity (Fig. 3F). Collectively, these results suggest that $\beta 4$ ligation induces c-Src-mediated tyrosine phosphorylation of 12-LOX that leads to enzymatic activation and 12(S)-HETE production, but that 12-LOX-integrin association is independent of Src.

Effect of secreted 12(*S*)-HETE from A431 β 4 ligation on HUVEC migration

12(*S*)-HETE is a known stimulus for angiogenic processes. It serves as a mitogen of endothelial cells and can induce expression of integrin α v β 3, as well as endothelial retraction and wound healing [38]. As we observed that β 4 ligation enhances 12-LOX phosphorylation and subsequent activity leading to elevated 12(*S*)-HETE production, we evaluated whether Src kinase inhibitors could alter the A431-secreted milieu vis-à-vis 12(*S*)-HETE production to impact HUVEC migration. We found that solid-phase extracted lipids from conditioned medium (CM) of β 4-ligated A431 cells did stimulate HUVEC cell migration ($P < 0.05$) when compared to supernatant extracts from cells stimulated with rabbit IgG (Fig. 4). Furthermore, lipid extracts from supernatants of A431 cells pretreated with PP2 or the 12-LOX inhibitor baicalein prior to integrin β 4 ligation were unable to induce HUVEC migration, as seen in phase contrast images (Fig. 4). These results suggest that integrin ligation in tumor cells can enhance HUVEC migration as a result of increased 12(*S*)-HETE production by phosphorylated 12-LOX, and that this paracrine effect of the tumor cell is due in part to the effects of c-Src. To test whether pSrc directly phosphorylates 12-LOX, we performed an *in vitro* phosphorylation assay. 12-LOX and pSrc were incubated in the presence of ATP and arachidonic acid-d8. The release of 12(*S*)-HETE-d8 in the presence of pSrc, but that was inhibited by PP2, provided evidence for direct phosphorylation and activation of 12-LOX by c-Src (Supplementary Fig. 1A). The outcome with the same reaction components was also examined on SDS-PAGE and immunoblot using PY-20 antibody to probe 12-LOX phosphorylation state (Supplementary Fig. 1B). These results suggest that tyrosine phosphorylation of 12-LOX by pSrc enhances its enzymatic activity, leading to enhanced production of 12(*S*)-HETE that can modulate endothelial cell migration.

Identification of 12-LOX tyrosine phosphorylation sites relevant to activity, induced by integrin β 4 ligation

The NetPhos 2.0 protein phosphorylation prediction server, accessible through the Center for Biological Sequence Analysis at the Technical University of Denmark, returned predictions of potential tyrosine phosphorylation sites in 12-LOX using the algorithm of Blom *et al.* [51]. Based on the potential phosphorylation scores, tyrosine residues at amino acids 19, 295 and 614 were changed to phenylalanine. To validate these predicted sites, we constructed 12LOX-Y19F, 12LOX-Y295F and 12LOX-Y614F point mutants, and subjected these to an *in vivo* phosphorylation assay in HEK293 transfectants. The Y19F and Y614F mutants showed a 60–70% reduction in tyrosine phosphorylation relative to wild type when compared to the 12LOX-Y295F mutant (Fig. 5A) implicating tyrosine residues 19 and 614 in 12-LOX activation. The amount of 12-LOX immunoprecipitated was consistent (Fig. 5B). Reciprocal immunoprecipitation with PY20 antibody yielded less 12-LOX in those mutants (Fig. 5C). Whole cell lysates were probed for 12-LOX, β 4 and Src (Fig. 5D–F). To examine the contribution of these two potential phosphorylation sites, we constructed a double mutant (DM) of Y19F and Y614F in 12-LOX. Expression constructs of the 12-LOX double mutant, c-Src and β 4 integrin were transfected into HEK293 cells followed by ligation of β 4 integrin. This resulted in a significant decrease in 12-LOX tyrosine phosphorylation and activity when compared to wild type 12-LOX (Fig. 5G and K, respectively). This implies that the phosphoresidues Y19 and Y614 of 12-LOX are important enzyme regulatory sites.

Glutamic acid can mimic constitutive phosphorylation [52]. However, modification of tyrosine residues Y19 or Y614 to glutamic acid, did not appear to mimic a phosphorylated state or lead to constitutive 12-LOX activation (data not shown).

To address the possible involvement of a protein tyrosine phosphatase (PTPase) in 12-LOX regulation, A431 cells were pre-treated with pervanadate prior to β 4 ligation. Pervanadate is a cell permeable tyrosine phosphatase inhibitor that increases overall protein tyrosine phosphorylation. We found that 12-LOX activity, as measured by 12(*S*)-HETE production, was sustained in A431 cells in the presence of pervanadate when compared to untreated cells (Fig. 6). This finding suggests potential involvement of PTPase(s) in 12-LOX activity.

Effect of 12-LOX tyrosine phosphorylation and its role in migration

We observed that β 4 ligation enhances 12LOX tyrosine phosphorylation and activity in A431 cells, leading to 12(*S*)-HETE production that was sufficient to induce HUVEC cell migration *in trans*. To address the role of the 12LOX-Y19 and Y614 sites in activity leading to autocrine tumor cell migration after β 4 ligation, HEK293 cells were chosen to over-express 12LOX-Y19F, -Y614F, or the double mutant (DM) Y19F/Y614F in combination with β 4 and c-Src. HEK293 migration was markedly reduced with mutant forms of 12-LOX (Fig. 7 panels d-e) compared to cells expressing wild type 12-LOX (Fig. 7 panel b). These findings demonstrate that tyrosine phosphorylation of key components of the β 4-cSrc-12LOX axis is necessary for cell migration.

Discussion

Accumulated evidence indicates that integrin β 4 has an important role in tumorigenesis and tumor progression [6, 9, 53]. Together with 12-HETE, it has been found on cancer exosomes, as has c-Src [7, 54–55], and the ITGB4 pathway appears to be regulated by micro RNAs such as miR182-5p, which is also predicted to regulate Src [56–58]. These proteins contribute to overlapping networks as well [4], where, for example, MMP9 induction can be seen by Src-mediated or ITGB4/12LOX-mediated signaling [16, 35].

Prior reports have indicated that β 4 signaling is mediated by pSFK [46]. Integrin β 4 engagement of either its natural ligand or an activating antibody leads to tyrosine phosphorylation of the β 4 cytoplasmic domain including residue Y1494 shown by Dutta *et al.* to regulate multiple signaling pathways important for tumor development [5]. Likewise, we found that pSrc associates with integrin β 4 in A431 cells in response to laminin or 3E1 stimulation thereby increasing Src kinase activity. These results support earlier findings by Gagnoux-Palacios *et al.* where integrin ligation in keratinocytes led to the association of c-Src with β 4 integrin and tyrosine phosphorylation [5, 59].

Our findings that pervanadate could sustain increased 12(*S*)-HETE production when 12-LOX was phosphorylated on key residues, suggests that a regulatory protein tyrosine phosphatase activity is still at large. The dependence on Src kinase was validated using the c-Src inhibitor, PP2 and c-Src DN mutants. ALOX12 mutants of these key Src substrate residues (Y19F and Y614F) exhibited ~ 70% less enzymatic activity as well as decreased migration when compared to wild type enzyme, which is in agreement with our previous

findings that enzymatic activity and the subsequent 12(*S*)-HETE metabolite significantly impact endothelial cell migration and proliferation [60].

This study has determined that an adaptor-type arrangement of integrin β 4-associated c-Src kinase activity [61] led to Src-dependent 12-LOX activity and subsequent production of 12(*S*)-HETE in A431 cells. Furthermore, we have demonstrated that this impacted cell migration in an autocrine and paracrine fashion, as both HEK293 cells expressing 12-LOX, and HUVEC cells exposed to 12-LOX products, respectively, were ultimately influenced by 12-LOX phosphorylation status at the Src-responsive residues. This supports a role for this axis in aiding tumor cells through paracrine effects on endothelial cells in the microenvironment.

To our knowledge, this is the first report that c-Src kinase activity is required for integrin β 4-mediated phosphorylation of 12-LOX and subsequent production of 12(*S*)-HETE. This is relevant beyond just the context of cancer growth and how tumors relate to their surrounding environment.

In virology, both the integrin and Src have been implicated in human papillomavirus (HPV) infection, where conditional integrin beta 4 knockdown in reproductive tissue demonstrably reduced the efficiency of infection [62–64]. The eicosanoids are actively being evaluated in this discipline and so the linkage to both the integrin and Src signaling are highly relevant [65]. In critical care, recent studies have found that the ITGB4 as well as the kinases are important for stretch injuries to the endothelium during ventilation, for example [66–67] where 12-HETE also figures prominently [68].

Other proteins such as syndecan-1 as well as tetraspanin CD151 that are known to regulate, relate to, or interact with ITGB4, are also implicated in diverse processes such as the examples used here of viral infection and cancer [69], and also signal through Src [70]. This cohort of proteins has likewise been isolated together with the integrin in secreted microvesicles and exosomes [71–73], where 12-HETE can also be found [54], thereby implicating this axis in tissue programming.

Studies with an *in vivo* model will help to confirm the functional significance of specific phosphorylation of 12-LOX at Y19 and Y614 in cancer development and progression and this in turn may shed light on the role of the integrin/lipoxygenase/kinase interaction in other systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institute of Health Grant CA-29997 (K.V.H.).

Abbreviations

HETE hydroxyeicosatetraenoic acid

LN	laminin
RTK	receptor tyrosine kinase
AA	Arachidonic acid
pSFK	phospho Src family kinase
AA	Arachidonic acid
PTPase	protein tyrosine phosphatase
IP	immunoprecipitation
LC-MS	Liquid chromatography-mass spectrometry

References

1. Marchisio PC. Polarized expression of integrin receptors (alpha 6 beta 4, alpha 2 beta 1, alpha 3 beta 1, and alpha v beta 5) and their relationship with the cytoskeleton and basement membrane matrix in cultured human keratinocytes. *The Journal of Cell Biology*. 1991; 112:761–773. [PubMed: 1825212]
2. Alanko J, Ivaska J. Endosomes: Emerging Platforms for Integrin-Mediated FAK Signalling. *Trends Cell Biol*. 2016; 26:391–398. [PubMed: 26944773]
3. Cabodi S, del Pilar Camacho-Leal M, Di Stefano P, Defilippi P. Integrin signalling adaptors: not only figurants in the cancer story. *Nat Rev Cancer*. 2010; 10:858–870. [PubMed: 21102636]
4. Zaidel-Bar R, Itzkovitz S, Ma'ayan A, Iyengar R, Geiger B. Functional atlas of the integrin adhesome. *Nat Cell Biol*. 2007; 9:858–867. [PubMed: 17671451]
5. Dutta U, Shaw LM. A key tyrosine (Y1494) in the beta4 integrin regulates multiple signaling pathways important for tumor development and progression. *Cancer Res*. 2008; 68:8779–8787. [PubMed: 18974120]
6. Giancotti FG. Targeting integrin beta4 for cancer and anti-angiogenic therapy. *Trends Pharmacol Sci*. 2007; 28:506–511. [PubMed: 17822782]
7. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharmed L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH, Narendran A, Schwartz GK, Healey JH, Sandstrom P, Labori KJ, Kure EH, Grandgenett PM, Hollingsworth MA, de Sousa M, Kaur S, Jain M, Mallya K, Batra SK, Jarnagin WR, Brady MS, Fodstad O, Muller V, Pantel K, Minn AJ, Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I, Peinado H, Bromberg J, Lyden D. Tumour exosome integrins determine organotropic metastasis. *Nature*. 2015; 527:329–335. [PubMed: 26524530]
8. Shin DH, Lee HJ, Min HY, Choi SP, Lee MS, Lee JW, Johnson FM, Mehta K, Lippman SM, Glisson BS, Lee HY. Combating resistance to anti-IGFR antibody by targeting the integrin beta3-Src pathway. *J Natl Cancer Inst*. 2013; 105:1558–1570. [PubMed: 24092920]
9. Stewart RL, O'Connor KL. Clinical significance of the integrin alpha6beta4 in human malignancies. *Lab Invest*. 2015; 95:976–986. [PubMed: 26121317]
10. Dans M, Gagnoux-Palacios L, Blaikie P, Klein S, Mariotti A, Giancotti FG. Tyrosine phosphorylation of the beta 4 integrin cytoplasmic domain mediates Shc signaling to extracellular signal-regulated kinase and antagonizes formation of hemidesmosomes. *J Biol Chem*. 2001; 276:1494–1502. [PubMed: 11044453]
11. Rabinovitz I, Toker A, Mercurio AM. Protein kinase C-dependent mobilization of the alpha6beta4 integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. *J Cell Biol*. 1999; 146:1147–1160. [PubMed: 10477766]

12. Rabinovitz I, Tsomo L, Mercurio AM. Protein kinase C- α phosphorylation of specific serines in the connecting segment of the β 4 integrin regulates the dynamics of type II hemidesmosomes. *Mol Cell Biol.* 2004; 24:4351–4360. [PubMed: 15121854]
13. Santoro MM, Gaudino G, Marchisio PC. The MSP receptor regulates α 6 β 4 and α 3 β 1 integrins via 14-3-3 proteins in keratinocyte migration. *Dev Cell.* 2003; 5:257–271. [PubMed: 12919677]
14. Shaw LM. Identification of insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the α 6 β 4 integrin-dependent activation of phosphoinositide 3-OH kinase and promotion of invasion. *Mol Cell Biol.* 2001; 21:5082–5093. [PubMed: 11438664]
15. Te Molder L, Sonnenberg A. PKD2 and RSK1 Regulate Integrin β 4 Phosphorylation at Threonine 1736. *PLoS One.* 2015; 10:e0143357. [PubMed: 26580203]
16. Patel A, Sabbineni H, Clarke A, Somanath PR. Novel roles of Src in cancer cell epithelial-to-mesenchymal transition, vascular permeability, microinvasion and metastasis. *Life Sci.* 2016
17. Playford MP, Schaller MD. The interplay between Src and integrins in normal and tumor biology. *Oncogene.* 2004; 23:7928–7946. [PubMed: 15489911]
18. Thomas SM, Brugge JS. Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol.* 1997; 13:513–609. [PubMed: 9442882]
19. Giancotti FG, Tarone G. Positional control of cell fate through joint integrin/receptor protein kinase signaling. *Annu Rev Cell Dev Biol.* 2003; 19:173–206. [PubMed: 14570568]
20. Leng C, Zhang ZG, Chen WX, Luo HP, Song J, Dong W, Zhu XR, Chen XP, Liang HF, Zhang BX. An Integrin β 4-EGFR unit promotes hepatocellular carcinoma lung metastases by enhancing anchorage independence through activation of FAK-AKT pathway. *Cancer Lett.* 2016
21. Tang K, Finley RL Jr, Nie D, Honn KV. Identification of 12-lipoxygenase interaction with cellular proteins by yeast two-hybrid screening. *Biochemistry.* 2000; 39:3185–3191. [PubMed: 10727209]
22. Tang K, Cai Y, Joshi S, Tovar E, Tucker SC, Maddipati KR, Crissman JD, Repaskey WT, Honn KV. Convergence of eicosanoid and integrin biology: 12-lipoxygenase seeks a partner. *Mol Cancer.* 2015; 14:111. [PubMed: 26037302]
23. Honn KV, Tang DG, Gao X, Butovich IA, Liu B, Timar J, Hagmann W. 12-lipoxygenases and 12(S)-HETE: role in cancer metastasis. *Cancer Metastasis Rev.* 1994; 13:365–396. [PubMed: 7712597]
24. Ding XZ, Tong WG, Adrian TE. 12-lipoxygenase metabolite 12(S)-HETE stimulates human pancreatic cancer cell proliferation via protein tyrosine phosphorylation and ERK activation. *Int J Cancer.* 2001; 94:630–636. [PubMed: 11745456]
25. Ding XZ, Iversen P, Cluck MW, Knezetic JA, Adrian TE. Lipoxygenase Inhibitors Abolish Proliferation of Human Pancreatic Cancer Cells. *Biochemical and Biophysical Research Communications.* 1999; 261:218–223. [PubMed: 10405349]
26. Hong SH, Avis I, Vos MD, Martinez A, Treston AM, Mulshine JL. Relationship of arachidonic acid metabolizing enzyme expression in epithelial cancer cell lines to the growth effect of selective biochemical inhibitors. *Cancer Res.* 1999; 59:2223–2228. [PubMed: 10232612]
27. Nappez C, Liagre B, Beneytout JL. Changes in lipoxygenase activities in human erythroleukemia (HEL) cells during diosgenin-induced differentiation. *Cancer Letters.* 1995; 96:133–140. [PubMed: 7553601]
28. Nie D, Hillman GG, Geddes T, Tang K, Pierson C, Grignon DJ, Honn KV. Platelet-type 12-lipoxygenase in a human prostate carcinoma stimulates angiogenesis and tumor growth. *Cancer Res.* 1998; 58:4047–4051. [PubMed: 9751607]
29. Soriano AF, Helfrich B, Chan DC, Heasley LE, Bunn PA Jr, Chou TC. Synergistic effects of new chemopreventive agents and conventional cytotoxic agents against human lung cancer cell lines. *Cancer Res.* 1999; 59:6178–6184. [PubMed: 10626810]
30. Tímár, J., Rásó, E., Honn, KV., Hagmann, W. Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury. Vol. 4. Springer Science + Business Media; 1999. 12-Lipoxygenase Expression in Human Melanoma Cell Lines; p. 617-622.
31. Guo AM, Liu X, Al-Wahab Z, Maddipati KR, Ali-Fehmi R, Scicli AG, Munkarah AR. Role of 12-lipoxygenase in regulation of ovarian cancer cell proliferation and survival. *Cancer Chemother Pharmacol.* 2011; 68:1273–1283. [PubMed: 21442472]

32. Liu B, Maher RJ, Hannun YA, Porter AT, Honn KV. 12(S)-HETE Enhancement of Prostate Tumor Cell Invasion: Selective Role of PKC JNCI. *Journal of the National Cancer Institute*. 1994; 86:1145–1151. [PubMed: 7518003]
33. Liu B, Marnett LJ, Chaudhary A, Ji C, Blair IA, Johnson CR, Diglio CA, Honn KV. Biosynthesis of 12(S)-hydroxyeicosatetraenoic acid by B16 amelanotic melanoma cells is a determinant of their metastatic potential. *Lab Invest*. 1994; 70:314–323. [PubMed: 8145526]
34. Wong BC, Wang WP, Cho CH, Fan XM, Lin MC, Kung HF, Lam SK. 12-Lipoxygenase inhibition induced apoptosis in human gastric cancer cells. *Carcinogenesis*. 2001; 22:1349–1354. [PubMed: 11532854]
35. Dilly AK, Ekambaram P, Guo Y, Cai Y, Tucker SC, Fridman R, Kandouz M, Honn KV. Platelet-type 12-lipoxygenase induces MMP9 expression and cellular invasion via activation of PI3K/Akt/NF-kappaB. *Int J Cancer*. 2013; 133:1784–1791. [PubMed: 23526143]
36. Kandouz M, Nie D, Pidgeon GP, Krishnamoorthy S, Maddipati KR, Honn KV. Platelet-type 12-lipoxygenase activates NF-kappaB in prostate cancer cells. *Prostaglandins Other Lipid Mediat*. 2003; 71:189–204. [PubMed: 14518561]
37. Lovey J, Nie D, Tovari J, Kenessey I, Timar J, Kandouz M, Honn KV. Radiosensitivity of human prostate cancer cells can be modulated by inhibition of 12-lipoxygenase. *Cancer Lett*. 2013; 335:495–501. [PubMed: 23523613]
38. Nie D, Tang K, Szekeres K, Trikha M, Honn KV. The role of eicosanoids in tumor growth and metastasis. *Ernst Schering Res Found Workshop*. 2000:201–217. [PubMed: 10943334]
39. Timar J, Silletti S, Bazaz R, Raz A, Honn KV. Regulation of melanoma-cell motility by the lipoxygenase metabolite 12-(S)-hete. *International Journal of Cancer*. 1993; 55:1003–1010. [PubMed: 8253518]
40. Hagmann W, Gao X, Timar J, Chen YQ, Strohmaier AR, Fahrenkopf C, Kagawa D, Lee M, Zacharek A, Honn KV. 12-Lipoxygenase in A431 cells: genetic identity, modulation of expression, and intracellular localization. *Exp Cell Res*. 1996; 228:197–205. [PubMed: 8912711]
41. Hessle H, Sakai LY, Hollister DW, Burgeson RE, Engvall E. Basement membrane diversity detected by monoclonal antibodies. *Differentiation*. 1984; 26:49–54. [PubMed: 6370774]
42. Dilly AK, Rajala RVS. Insulin Growth Factor 1 Receptor/PI3K/AKT Survival Pathway in Outer Segment Membranes of Rod Photoreceptors. *Invest Ophthalmol Vis Sci*. 2008; 49:4765. [PubMed: 18566464]
43. Suzuki N, Hishinuma T, Saga T, Sato J, Toyota T, Goto J, Mizugaki M. Determination of urinary 12(S)-hydroxyeicosatetraenoic acid by liquid chromatography–tandem mass spectrometry with column-switching technique: sex difference in healthy volunteers and patients with diabetes mellitus. *Journal of Chromatography B*. 2003; 783:383–389.
44. Ustach CV. A Potential Oncogenic Activity of Platelet-Derived Growth Factor D in Prostate Cancer Progression. *Cancer Research*. 2004; 64:1722–1729. [PubMed: 14996732]
45. Tang K, Nie D, Cai Y, Honn KV. The beta4 integrin subunit rescues A431 cells from apoptosis through a PI3K/Akt kinase signaling pathway. *Biochem Biophys Res Commun*. 1999; 264:127–132. [PubMed: 10527852]
46. Mariotti A. EGF-R signaling through Fyn kinase disrupts the function of integrin alpha6beta4 at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *The Journal of Cell Biology*. 2001; 155:447–458. [PubMed: 11684709]
47. Arias-Salgado EG, Lizano S, Sarkar S, Brugge JS, Ginsberg MH, Shattil SJ. Src kinase activation by direct interaction with the integrin cytoplasmic domain. *Proceedings of the National Academy of Sciences*. 2003; 100:13298–13302.
48. Khanna S, Roy S, Parinandi NL, Maurer M, Sen CK. Characterization of the potent neuroprotective properties of the natural vitamin E alpha-tocotrienol. *J Neurochem*. 2006; 98:1474–1486. [PubMed: 16923160]
49. Khanna S, Roy S, Ryu H, Bahadduri P, Swaan PW, Ratan RR, Sen CK. Molecular basis of vitamin E action: tocotrienol modulates 12-lipoxygenase, a key mediator of glutamate-induced neurodegeneration. *J Biol Chem*. 2003; 278:43508–43515. [PubMed: 12917400]

50. Khanna S, Roy S, Slivka A, Craft TK, Chaki S, Rink C, Notestine MA, DeVries AC, Parinandi NL, Sen CK. Neuroprotective properties of the natural vitamin E alpha-tocotrienol. *Stroke*. 2005; 36:2258–2264. [PubMed: 16166580]
51. Blom N, Gammeltoft S, Brunak S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *Journal of Molecular Biology*. 1999; 294:1351–1362. [PubMed: 10600390]
52. Meyer RD, Dayanir V, Majnoun F, Rahimi N. The Presence of a Single Tyrosine Residue at the Carboxyl Domain of Vascular Endothelial Growth Factor Receptor-2/FLK-1 Regulates Its Autophosphorylation and Activation of Signaling Molecules. *Journal of Biological Chemistry*. 2002; 277:27081–27087. [PubMed: 12023952]
53. Lipscomb EA, Mercurio AM. Mobilization and activation of a signaling competent $\alpha 6 \beta 4$ integrin underlies its contribution to carcinoma progression. *Cancer Metastasis Rev*. 2005; 24:413–423. [PubMed: 16258729]
54. Uchide K, Sakon M, Ariyoshi H, Nakamori S, Tokunaga M, Monden M. Cancer cells cause vascular endothelial cell (vEC) retraction via 12(S)HETE secretion; the possible role of cancer cell derived microparticle. *Ann Surg Oncol*. 2007; 14:862–868. [PubMed: 17103063]
55. DeRita RM, Zerlanko B, Singh A, Lu H, Iozzo RV, Benovic JL, Languino LR. c-Src, Insulin-Like Growth Factor I Receptor, G-Protein-Coupled Receptor Kinases and Focal Adhesion Kinase Are Enriched into Prostate Cancer Cell Exosomes. *J Cell Biochem*. 2016
56. Yao J, Xu C, Fang Z, Li Y, Liu H, Wang Y, Sun Y. Androgen receptor regulated microRNA miR-182-5p promotes prostate cancer progression by targeting the ARRDC3/ITGB4 pathway. *Biochem Biophys Res Commun*. 2016; 474:213–219. [PubMed: 27109471]
57. Chou CH, Chang NW, Shrestha S, Hsu SD, Lin YL, Lee WH, Yang CD, Hong HC, Wei TY, Tu SJ, Tsai TR, Ho SY, Jian TY, Wu HY, Chen PR, Lin NC, Huang HT, Yang TL, Pai CY, Tai CS, Chen WL, Huang CY, Liu CC, Weng SL, Liao KW, Hsu WL, Huang HD. miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database. *Nucleic Acids Res*. 2016; 44:D239–247. [PubMed: 26590260]
58. Keller, A. C.f.C. Bioinformatics. miRTargetLink-Human. Vol. 2016. Saarland University; Building E2.1, 66123 Saarbrücken, Germany: 2016. <https://ccb-web.cs.uni-saarland.de/mirtargetlink/network.php?type=miRNA&qval=hsa-miR-182-5phttps://ccb-web.cs.uni-saarland.de/mirtargetlink/manual.php>
59. Gagnoux-Palacios L. Compartmentalization of integrin $\alpha 6 \beta 4$ signaling in lipid rafts. *The Journal of Cell Biology*. 2003; 162:1189–1196. [PubMed: 14517202]
60. Tang DG, Renaud C, Stojakovic S, Diglio CA, Porter A, Honn KV. 12(S)-HETE Is a Mitogenic Factor for Microvascular Endothelial Cells: Its Potential Role in Angiogenesis. *Biochemical and Biophysical Research Communications*. 1995; 211:462–468. [PubMed: 7540838]
61. Legate KR, Fassler R. Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. *J Cell Sci*. 2009; 122:187–198. [PubMed: 19118211]
62. Szalmas A, Gyongyosi E, Ferenczi A, Laszlo B, Karosi T, Csomor P, Gergely L, Veress G, Konya J. Activation of Src, Fyn and Yes non-receptor tyrosine kinases in keratinocytes expressing human papillomavirus (HPV) type 16 E7 oncoprotein. *Virology*. 2013; 453:395–400. [PubMed: 23497302]
63. Huang HS, Lambert PF. Use of an in vivo animal model for assessing the role of integrin $\alpha 6 \beta 4$ and syndecan-1 in early steps in papillomavirus infection. *Virology*. 2012; 433:395–400. [PubMed: 22995187]
64. Fothergill T, McMillan NA. Papillomavirus virus-like particles activate the PI3-kinase pathway via $\alpha 6 \beta 4$ integrin upon binding. *Virology*. 2006; 352:319–328. [PubMed: 16781758]
65. Tam VC, Quehenberger O, Oshansky CM, Suen R, Armando AM, Treuting PM, Thomas PG, Dennis EA, Aderem A. Lipidomic profiling of influenza infection identifies mediators that induce and resolve inflammation. *Cell*. 2013; 154:213–227. [PubMed: 23827684]
66. Barabutis N, Verin A, Catravas JD. Regulation of pulmonary endothelial barrier function by kinases. *Am J Physiol Lung Cell Mol Physiol*. 2016; 311:L832–L845. [PubMed: 27663990]
67. Chen W, Epshtein Y, Ni X, Dull RO, Cress AE, Garcia JG, Jacobson JR. Role of Integrin $\beta 4$ in Lung Endothelial Cell Inflammatory Responses to Mechanical Stress. *Sci Rep*. 2015; 5:16529. [PubMed: 26572585]

68. Rossaint J, Nadler JL, Ley K, Zarbock A. Eliminating or blocking 12/15-lipoxygenase reduces neutrophil recruitment in mouse models of acute lung injury. *Crit Care*. 2012; 16:R166. [PubMed: 22973824]
69. Yang X, Li S, Zhong J, Zhang W, Hua X, Li B, Sun H. CD151 mediates netrin-1-induced angiogenesis through the Src-FAK-Paxillin pathway. *J Cell Mol Med*. 2016
70. Gesierich S, Paret C, Hildebrand D, Weitz J, Zraggen K, Schmitz-Winnenthal FH, Horejsi V, Yoshie O, Herlyn D, Ashman LK, Zoller M. Colocalization of the tetraspanins, CO-029 and CD151, with integrins in human pancreatic adenocarcinoma: impact on cell motility. *Clin Cancer Res*. 2005; 11:2840–2852. [PubMed: 15837731]
71. Sandvig K, Llorente A. Proteomic analysis of microvesicles released by the human prostate cancer cell line PC-3. *Mol Cell Proteomics*. 2012; 11:M111 012914.
72. Rana S, Yue S, Stadel D, Zoller M. Toward tailored exosomes: the exosomal tetraspanin web contributes to target cell selection. *Int J Biochem Cell Biol*. 2012; 44:1574–1584. [PubMed: 22728313]
73. Yue S, Mu W, Erb U, Zoller M. The tetraspanins CD151 and Tspan8 are essential exosome components for the crosstalk between cancer initiating cells and their surrounding. *Oncotarget*. 2015; 6:2366–2384. [PubMed: 25544774]

Highlights

- ITGB4 ligation in A431 cells regulates Src phosphorylation/activation.
- 12LOX enzymatic activity is increased by ITGB4-induced pSrc phosphorylation.
- Tyrosine residues 19 and 614 in 12LOX are pSrc targets post ITGB4 ligation.
- Phosphorylation state of 12LOX Y19 and Y614 influences cancer phenotypes.

Figure 1. Integrin β_4 becomes phosphorylated on Y1494 in A431 cells

β_4 -ligated A431 lysates were immunoprecipitated with β_4 antibody and immunoblotted with phosphotyrosine antibody, PY-20 (A). The blots were reprobed with β_4 antibody (B). Quantitative analysis of bands from respective immunoblots was performed with imaging software. The ratios of PY20/ β_4 and Y1494- β_4 / β_4 are expressed as a percentage of control (C & F). Data are mean $\pm$ SD (n=3; p<0.05). β_4 -ligated A431 cell lysates were immunoprecipitated with integrin β_4 antibody and immunoblotted with anti Y1494- β_4 (D). The blots were reprobed with β_4 antibody (E). Reciprocal immunoprecipitation of β_4 -ligated cell lysates with Y1494- β_4 antibody and immunoblotted with β_4 antibody (G). Cell lysates (30 μ g protein) were immunoblotted with β_4 antibodies to confirm equal amount of protein in each sample.

Figure 3. pSrc-dependent 12-LOX phosphorylation and activity

Cells were treated with IgG, 3E1 or laminin in the presence of PP2 (5 μ M), and lysates were subjected to immunoprecipitation with 12-LOX antibody followed by immunoblotting with PY-20 antibody (A). The blot was reprobed with 12-LOX antibody. Reciprocal immunoprecipitation with PY20 antibody and immunoblot with 12-LOX antibody (B). Cells were initially treated with or without inhibitors, followed by stimulation with β 4 antibody, or control IgG. They were then exposed to arachidonic acid (10 μ M) in serum-free RPMI for 6h. Medium was analyzed for 12(S)-HETE by LC/MS. (C). β 4 and 12LOX in combination with either WT c-Src or DN c-Src were transfected in HEK293. After β 4 ligation, cell lysates were immunoprecipitated using 12-LOX antibody and immunoblotted with PY20 antibody (D). The blots were reprobed for 12-LOX. Lanes are loaded with 20 μ g of cellular extracts from the transient transfections (E). HEK293 transfectants were stimulated and exposed to arachidonic acid (10 μ M) in serum-free RPMI. Medium was collected for analysis of 12(S)-HETE by LC/MS after 6h (F).

Figure 4. Secreted 12(S)-HETE from β 4-ligated A431 cells affects HUVEC migration
Solid-phase-extracted 12(*S*)-HETE from conditioned medium of A431 cells, +/- β 4 ligation by either laminin or 3E1 in the presence or absence of inhibitors (12LOX/baicalein; pSrc/PP2) or IgG control for 3E1, was added to the HUVEC migration assay. Migrated endothelial cells were stained, and cell density was determined by measuring the absorbance of extracted dye. The migration of endothelial cells was expressed as the percentage of medium control in which no extracted 12-HETE was added. Columns are the average percentage of migration S.D. **, $p < 0.01$; according to Student's *t* test.

Figure 5. Validation of key residues required for 12-LOX activation

HEK293 cells were co transfected with $\beta 4$ integrin and c-Src in combination with 12-LOX mutants Y19F, Y295F, or Y614F. After $\beta 4$ ligation, cell lysates were immunoprecipitated using the 12-LOX antibody and immunoblotted with PY20 antibody (A). The blots were reprobed for 12-LOX (B). Cell lysates were immunoprecipitated in reciprocal fashion using the PY-20 antibody and immunoblotted with 12-LOX antibody (C). Lanes were loaded with 30 μ g of cell extracts from the transient transfections (D, E, F, I & J). The same lysates were immunoprecipitated using the 12-LOX antibody and then immunoblotted with PY20 antibody (G). The blots were reprobed for 12-LOX (H). 12(*S*)-HETE measurement by LC/MS (K) was carried out as described under experimental procedures.

Figure 6. Effect of PTPase activity on 12-LOX enzymatic activity

A431 cells were serum starved as described in experimental procedures. Cells were pretreated with or without 0.1 mM pervanadate followed by β 4 ligation and conditioned medium was collected for 12(S)-HETE analysis by LC/MS.

Figure 7. Mutation of 12-LOX tyrosine phosphorylation sites reduces cell migration

HEK293 cells were transiently transfected with (a) vector control, (b) $\beta 4+12\text{LOX}+c\text{-Src}$, (c) $\beta 4+ [12\text{LOX Y19F}]+c\text{-Src}$, (d) $\beta 4+ [12\text{LOX Y614F}]+c\text{-Src}$, (e) $\beta 4+[12\text{LOX DM}]+c\text{-Src}$ and evaluated by transwell migration assays (A). Migrated cells were stained and quantified. Extracted stains were measured spectrophotometrically (B) and normalized to vector control.