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Lysyl Oxidase Regulates Actin Filament Formation Through the p130^{Cas}/Crk/DOCK180 Signaling Complex

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Abstract We have previously demonstrated that lysyl oxidase (LOX) is expressed in invasive breast cancer cells compared to poorly invasive cells. Additionally, we have recently shown that LOX regulates cell migration, a key step in the invasion process, through a hydrogen peroxide-dependent mechanism involving the focal adhesion kinase (FAK)/Src signaling complex. Here we further elucidate the role of LOX in cell motility/migration by examining the role of LOX in actin filament polymerization. We demonstrate that inhibition of LOX leads to an increase in phalloidin staining, directly associated with an increase in actin stress fiber formation. This increase in staining was confirmed by activity assays showing an increase in Rho activity with decreased LOX activity. Additionally, Rac and Cdc42 activity decreased with the reduction in LOX activity. Taken together, these data demonstrate a loss of a motogenic phenotype with decreased LOX activity. Finally, in order to elucidate the mechanism by which LOX regulates actin polymerization, we have demonstrated that LOX facilitates p130^{Cas} phosphorylation, which allows for the binding to CAS related kinase (Crk) and formation of the p130^{Cas}/Crk/DOCK180 signaling complex. Formation of this complex leads to an increase in Rac-GTP, which decreases actin stress fiber formation and increases formation of lamellipodium. These data demonstrate that LOX regulates cell motility/migration through changes in actin filament polymerization, which involve the regulation of the p130^{Cas}/Crk/DOCK180 signaling pathway. Elucidating the role of LOX in the regulation of cell motility will allow the development of more effective therapeutic strategies to treat invasive/metastatic breast cancer. J. Cell. Biochem. 98: 827–837, 2006.

Key words: lysyl oxidase; actin; Rho GTPases; signaling; breast cancer

Lysyl oxidase (LOX) was originally described as a copper-dependent amine oxidase responsible for catalyzing the cross-linking of collagens and elastins within the extracellular matrix [Smith-Mungo and Kagan, 1998; Csiszar, 2001; Kagan and Li, 2003]. However, recent studies have shown that LOX may have several additional roles both intra- and extracellularly. Our laboratory and others have demonstrated that catalytically active LOX is localized within the nucleus and cytoplasm of cells [Li et al., 1997; Payne et al., 2005]. Specifically, LOX has been shown to have a role in the regulation of motility/migration, gene transcription, and differentiation [Nelson et al., 1988; Lazarus et al., 1995; Smith-Mungo and Kagan, 1998; Li et al., 2000; Csiszar, 2001; Giampuzzi et al., 2003a].

For years, LOX has been considered a tumor suppressor gene due to the work that was initially performed in *ras*-transformed fibroblasts. In these studies, LOX was identified as a *ras*-recision gene capable of suppressing the oncogenic activities of *ras* [Kenyon et al., 1991].

Abbreviations used: LOX, lysyl oxidase; βAPN, β-aminopropionitrile; Crk, Cas related kinase; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; FAK, focal adhesion kinase.

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Conversely, a loss of LOX transcription and activity has been demonstrated in *ras*-transformed cells [Kenyon et al., 1991; Contente et al., 1999]. Additionally, expression of LOX in *ras*-transformed cells has also been correlated with a loss of NF-κB activation and decreases in cell adhesion [Giampuzzi et al., 2003b; Jeay et al., 2003]. Giampuzzi et al. [2001] also demonstrated an increase in tumorigenicity and metastasis in nude mice injected with normal rat kidney fibroblasts transfected with antisense LOX. LOX mRNA expression has also been shown to be downregulated in a variety of spontaneous human cancers including colorectal cancer [Csiszar, 2001], bronchogenic carcinoma [Woznick et al., 2005], gastric cancers [Kaneda et al., 2004], head and neck squamous cell carcinomas [Rost et al., 2003], and primary and metastatic prostate tissues [Ren et al., 1998]. These studies demonstrate that there is a precedent to consider LOX a tumor suppressor gene. However, there are an equal number of reports demonstrating that LOX mRNA expression or activity is upregulated in human cancers such as ovarian cancer [Rae et al., 2004], renal cell carcinomas [Stassar et al., 2001], osteosarcoma [Fuchs et al., 2000; Uzel et al., 2000], and invasive breast, prostate, and melanoma cell lines [Kirschmann et al., 1999, 2002; Nagaraja et al., 2005; Payne et al., 2005]. In particular, Stassar et al. [2001] demonstrated that LOX gene expression correlates with histological grade in renal cell carcinomas. Therefore, there is also a precedent to consider LOX a metastasis promoter/enhancer. These studies demonstrate the pleomorphic nature of LOX in cancer cells.

The Rho family of GTPases is associated with changes in actin filament formation and corresponding changes in cell motility [Ridley et al., 1992; Nobes and Hall, 1995; Klemke et al., 1998]. These processes are primarily regulated through Rho, Rac, and Cdc42, which allow for the formation of actin stress fibers, lamellipodia, and filopodia, respectively, in their GTP-bound state. The Rho family GTPases is regulated by GTPase-activating proteins (GAPs) and guanosine nucleotide exchange factors (GEFs). GEFs function by facilitating the exchange of hydrolyzed GDP for GTP, leading to an active Rho family protein, while GAPs lead to the exchange of GTP for GDP, causing inactivation.

The $p130^{Cas}$/Crk/DOCK180 complex is a downstream target of the focal adhesion kinase (FAK)/Src signaling pathway. The formation and activation of the FAK/Src signaling complex allows for the phosphorylation and activation of $p130^{Cas}$ [Hamasaki et al., 1996; Sakai et al., 1997; Klinghoffer et al., 1999; Shin et al., 2004]. Upon phosphorylation, $p130^{Cas}$ is able to interact with CAS related kinase (Crk) and form a complex that will recruit DOCK180, a Rac-GEF responsible for Rac-GTP formation [Sakai et al., 1994; Kiyokawa et al., 1998a,b; Klemke et al., 1998]. The activation of Rac through this complex leads to the formation of lamellipodia and a motile phenotype.

Our laboratory previously demonstrated that LOX mRNA is highly upregulated in invasive breast cancer cells compared to poorly invasive cells [Kirschmann et al., 1999]. Additionally, we have shown that LOX activity facilitates breast cancer cell invasion [Kirschmann et al., 2002]. Recently, we reported that LOX regulates breast cancer cell motility/migration through changes in cell-matrix adhesion formation [Payne et al., 2005]. Furthermore, these changes are the result of a hydrogen peroxide-mediated mechanism involving the FAK/Src signaling pathway. In order to further elucidate the mechanism by which LOX regulates cell motility and adhesion formation, we examined the role of LOX in actin filament formation. Here we demonstrate that inhibition of LOX activity in invasive breast cancer cells leads to a corresponding increase in actin stress fiber formation as visualized using a phalloidin stain. Furthermore, LOX activity led to an increase in Rac and Cdc42 activity and a decrease in Rho activity. These changes correspond to a motile phenotype in the presence of LOX activity. Finally, we elucidate the pathway by which Rac is activated through the $p130^{Cas}$/Crk/DOCK180 signaling complex. These data demonstrate that LOX activity facilitates a motile phenotype in invasive breast cancer cells through changes in actin filament formation. A better understanding of the mechanism by which LOX facilitates cell motility/migration will allow for the design of targeted inhibitors of metastatic breast cancer.

MATERIALS AND METHODS

Cells and Culture Conditions

Breast cancer cell lines were obtained and maintained as previously described [Kirschmann et al., 2002]. MCF-7 cells were

stably transfected with LOX32-His DNA or LOX50-His DNA (a generous gift from Dr. Ben Fogelgren and Dr. Katalin Csiszar, University of Hawaii, Honolulu, HI) as previously described [Payne et al., 2005]. Cell cultures were determined to be free of *Mycoplasma* contamination using the Mycoplasma PCR ELISA kit (Roche Diagnostics, Indianapolis, IN). All cells were harvested when cultures were approximately 80% confluent.

Filamentous F-Actin-Binding

Cells were plated on fibronectin-coated coverslips (5×10^4 cells/coverslip) and allowed to attach and grow overnight at 37°C. Where indicated, cells were treated with βAPN (β-aminopropionitrile, Sigma, St. Louis, MO) or catalase (Sigma) during this time. Subsequently, media was removed and cells were washed with PBS and fixed in methanol/acetone (1:1) for 5 min on ice. Coverslips were washed in PBS twice, blocked in DAKO Protein Block Serum-Free Ready to Use (DAKO, Carpinteria, CA) for 20 min, and incubated with conjugated Texas Red-phalloidin at a 1:40 dilution for 1 h. Coverslips were washed with PBS and mounted onto glass microscope slides using gelvatol. Cells were imaged using an Axioscope 2 microscope (Carl Zeiss, Inc., Thornwood, NY) and Spot 2 camera (Diagnostic Instrument, Inc., Sterling Heights, MI) using the Zeiss Axiovision 2.0.5 software (Carl Zeiss, Inc.). Experiments were repeated three times.

Electrophoresis and Immunoblotting

Cells were plated on fibronectin-coated flasks and allowed to grow for 24 h. Where indicated, Hs578T, MDA-MB-231, and MCF-7/LOX32-His cells were treated with βAPN during this time. Whole cell lysate collection and immunoblot analysis were performed as previously described [Odero-Marah et al., 2003]. Blots were incubated with anti-Rho (1:500, BD Pharmingen, San Diego, CA), anti-Rac (1:500, BD Pharmingen), anti-Cdc42 (1:500, BD Pharmingen), anti-P-CAS ($pTyr^{249}$, 1:1,000, Cell Signaling, Beverly, MA), anti-CAS (1:1,000, BD Pharmingen), anti-Crk (1:5,000, BD Pharmingen), or anti-DOCK180 (1:250, Santa Cruz Biotechnology, Santa Cruz, CA). Equal loading was verified by staining membranes subsequent to transfer with 0.2% amido black. Experiments were repeated three times. Densitometry was performed to determine relative expression levels using Scion Image (Scion Corp., Frederick, MD). Each Western blot was repeated three times and densities of individual bands were analyzed, normalized to the corresponding loading control, and finally normalized to control cells (set at 1.0). Representative blots are shown with corresponding densitometric measurements.

Rho, Rac, and Cdc42 Activity Assays

All activity assays were performed using an immunoprecipitation technique per manufacturer protocols (Upstate, Lake Placid, NY). Prior to the addition of beads, an equal amount (10 μg) of lysate was removed from each tube and run on a separate SDS–PAGE to serve as an equal loading control. Briefly, Rho activity was measured using Rhotekin-RBD beads (30 μg beads/500 μg whole cell lysate) while Rac and Cdc42 activities were measured using PAK-1 agarose beads (10 μg beads/300 μg whole cell lysate). Rhotekin beads specifically bind to GTP-bound Rho while PAK-1 specifically binds GTP-bound Rac or Cdc42. Lysates were incubated with beads for 1 h at 4°C, centrifuged to collect beads, washed with modified RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate), and beads were collected again. Laemmli buffer (15 μl) was added to beads and boiled at 95°C for 5 min. Samples were then analyzed by SDS–PAGE and immunoblot. Experiments were repeated three times.

Immunoprecipitation

Whole cell lysate (500 μg) was pre-cleared with protein A sepharose beads (Sigma) for 15 min at 4°C. Lysates were subsequently centrifuged to remove the beads and 5 μg primary antibody was added to the pre-cleared lysate. Prior to the addition of antibody, 10 μg cell lysate was removed from each pre-cleared lysate and run on a separate SDS–PAGE to serve as a loading control. Subsequent to antibody addition, cells were allowed to rotate at 4°C for 1 h. Protein A sepharose beads (30 μl) were added to lysates and rotated for 1 h at 4°C. Samples were centrifuged to collect beads and supernatant was removed. Beads were washed three times with 500 μl modified RIPA buffer. Laemmli sample buffer (15 μl) was added to beads and samples were boiled at 95°C for 5 min. Samples were then analyzed by SDS–PAGE and immunoblot. Experiments were repeated three times.

RESULTS

LOX Activity Inversely Regulates Actin Stress Fiber Formation

We have previously demonstrated that LOX regulates breast cancer cell motility through changes in cell-matrix adhesion formation utilizing a hydrogen peroxide-mediated mechanism [Payne et al., 2005]. In order to further examine the role of LOX in the regulation of cell motility, we studied the ability of LOX to regulate actin stress fiber formation. The invasive breast cancer cell lines, Hs578T and MDA-MB-231, were treated with βAPN (an irreversible inhibitor of LOX catalytic activity) and filamentous F-actin stress fibers were visualized using Texas Red-phalloidin. Figure 1 demonstrates that as LOX activity

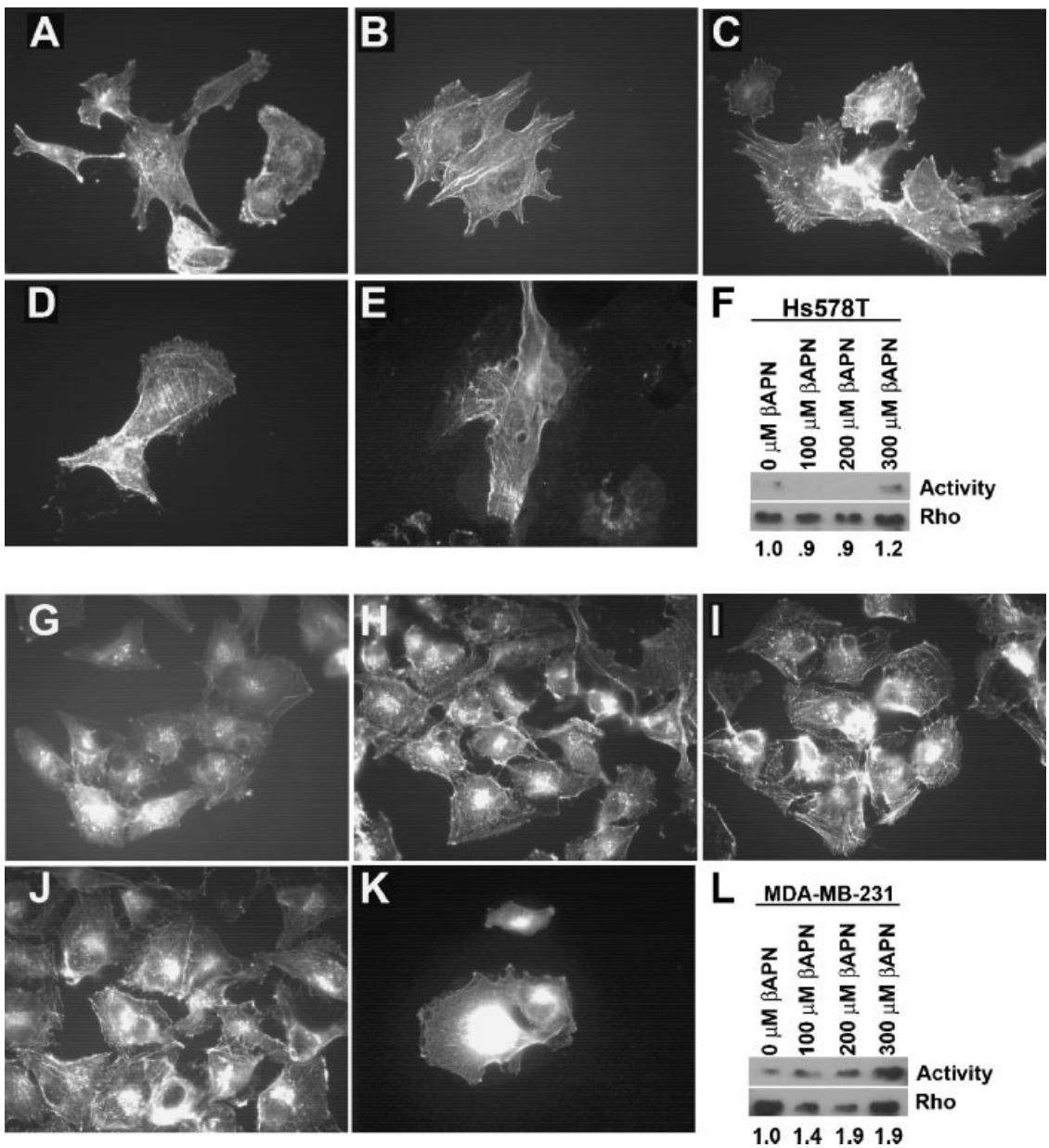

Fig. 1. Inhibition of LOX activity facilitates actin stress fiber formation and an increase in Rho activity in invasive breast cancer cells. Filamentous F-actin binding using Texas Red-phalloidin in (**A**–**E**) Hs578T and (**G**–**K**) MDA-MB-231 invasive breast cancer cells. Cells were treated with (B and H) 100 μM βAPN, (C and I) 200 μM βAPN, (D and J) 300 μM βAPN, or (E and K) 100 μM catalase. Rho activity assays of (F) Hs578T and (**L**) MDA-MB-231 invasive breast cancer cell lines are shown with total cellular Rho protein equal loading controls. Densitometric evaluation of each band normalized to its corresponding loading control and subsequently normalized to control cells (1.0) is shown.

was inhibited with increasing concentrations of βAPN, stress fiber formation increased (Hs578T, A-D; MDA-MB-231, G-J). Additionally, when cells were treated with catalase (which converts hydrogen peroxide to molecular oxygen and water), a similar increase in actin stress fibers was observed (Hs578T, 1E; MDA-MB-231, 1K). Rho activity assays were performed in order to measure the amount of GTP-bound Rho present in cell lysates that were treated with βAPN. Rho activity is measured using an immunoprecipitation with Rhotekin beads that will only bind to GTP-bound Rho. Subsequent immunoblot analysis using an anti-Rho antibody allows for visualization of Rho-GTP present in lysates. We observed an inverse relationship in which inhibition of LOX activity led to an increase in Rho activity (Fig. 1F,L).

To extend our observations regarding the role of LOX in actin stress fiber formation, we stably transfected poorly invasive MCF-7 cells with an active 32 kDa LOX enzyme (MCF-7/LOX32-His) or a catalytically inactive 50 kDa LOX proenzyme (MCF-7/LOX50-His). We have previously demonstrated that transfection of the 32 kDa LOX enzyme leads to an increase in intracellular LOX activity [Payne et al., 2005]. Conversely, transfection of the 50 kDa LOX proenzyme did not lead to a significant change in intracellular LOX activity. Additionally, extracellular LOX activity was significantly increased ($P < 0.01$) upon transfection of the 32 kDa enzyme compared to un-transfected cells (data not shown). Transfection of the 50 kDa LOX enzyme did not lead to any changes in extracellular LOX activity (data not shown).

Filamentous F-actin stress fibers were subsequently visualized in the MCF-7/LOX cells using Texas Red-phalloidin. We observed that MCF-7 cells and MCF-7/LOX 50-His cells had relatively high levels of stress fiber formation (Fig. 2A,B). However, when the 32 kDa LOX enzyme was transfected into these poorly invasive cells, very little F-actin was observed (Fig. 2C). Upon treatment with βAPN, stress fiber formation in these cells increased to normal control levels (Fig. 2D–F). Additionally, treatment of the MCF-7/LOX32-His cells with catalase led to a similar increase in actin stress fiber formation (Fig. 2G). These data were further confirmed through Rho activity assays showing a loss of Rho-GTP in MCF-7/LOX32-His cells and a corresponding increase in activity upon βAPN treatment (Fig. 2H). Together, these data demonstrate that LOX activity inversely regulates actin stress fiber formation in breast cancer cell lines, leading to an induction of a motile phenotype.

LOX Activity Regulates Rac and Cdc42 Activity Levels

To address the mechanism by which LOX regulates actin filament polymerization and cell motility, we examined the levels of GTP-bound, active, Rac and Cdc42 in cell lysates of invasive breast cancer cell lines. An increase in Rac and Cdc42 activity has previously been demonstrated to correspond with a motile phenotype due to the formation of lamellipodia and filopodia, respectively [Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995]. Thus, we hypothesized that the presence of LOX activity would correspond to an increase in Rac and Cdc42 activity. To this end, Rac and Cdc42 activity was measured in an assay similar to Rho activity assays. Immunoprecipitation with PAK-1 was performed, which specifically binds to the GTP-bound forms of Rac and/or Cdc42. Immunoblot analysis of Rac or Cdc42 is subsequently performed in order to visualize the amount of GTP-bound protein in cell lysates. Figure 3A demonstrates that when invasive breast cancer cell lines are treated with βAPN, inhibiting LOX enzymatic activity, a corresponding loss in Rac activity is observed. Conversely, MCF-7 and MCF-7/LOX50-His cells did not contain high levels of Rac-GTP. However, upon stable transfection of the active 32 kDa LOX enzyme, an increase in Rac-GTP was observed which was subsequently inhibited with βAPN treatment (Fig. 3B). Cdc42 activity levels were also measured in these lysates and Figure 3C shows a loss of Cdc42-GTP when LOX activity is inhibited by βAPN in the invasive breast cancer cell lines, Hs578T and MDA-MB-231. These data were recapitulated in MCF-7/LOX32-His cells treated with βAPN (Fig. 3D). Together, these data demonstrate that LOX activity facilitates the regulation of Rac and Cdc42 activity leading to a motile phenotype.

LOX Regulates Rac Activity Through the $p130^{Cas}$/Crk/DOCK180 Pathway

In order to further elucidate the specific mechanism by which LOX regulates Rac activity and thus changes in actin filament polymerization, we examined the $p130^{Cas}$/Crk/

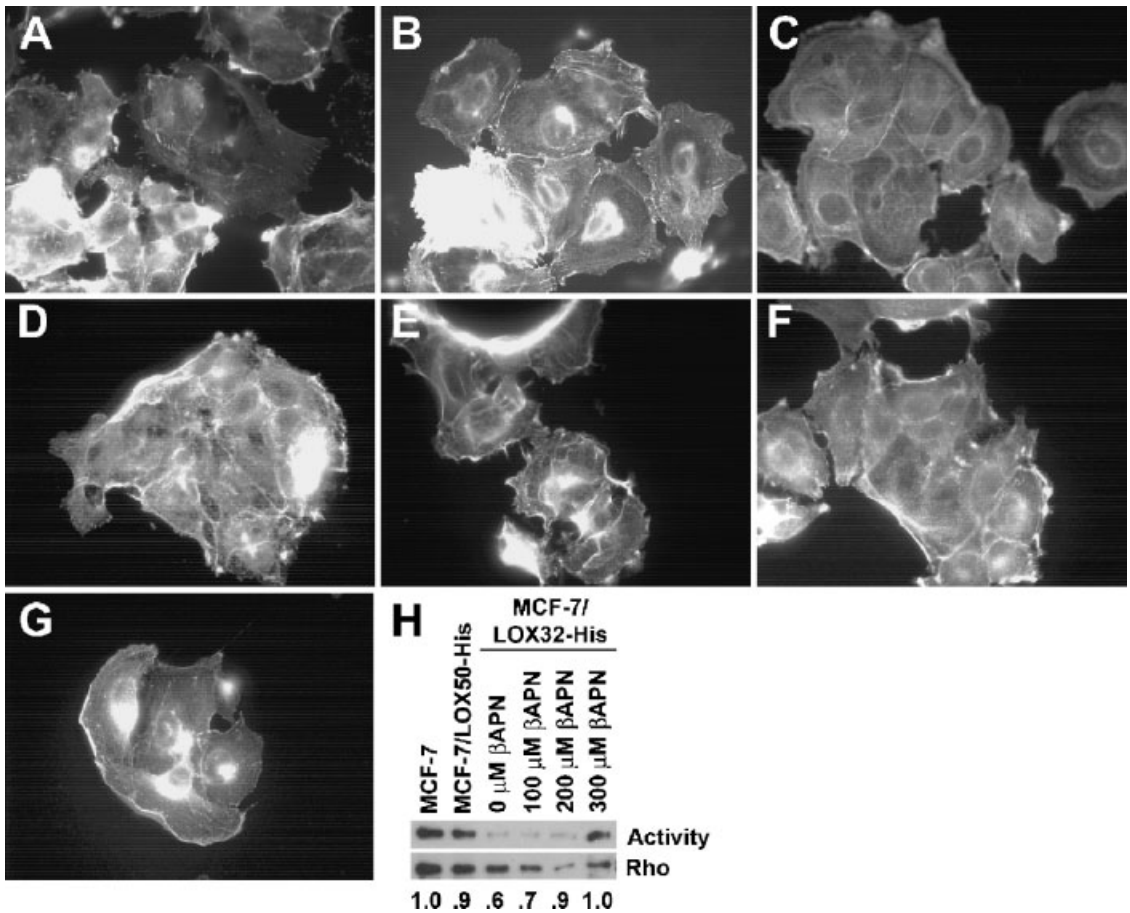

Fig. 2. LOX activity inhibits actin stress fiber formation and Rho activity in MCF-7/LOX32-His cells. Filamentous F-actin binding using Texas Red-phalloidin in (**A**) poorly invasive MCF-7 cells, (**B**) MCF-7/LOX50-His cells, and (**C**–**G**) MCF-7/LOX32-His cells. MCF-7/LOX32-His cells were treated with (D) 100 μM βAPN, (E) 200 μM βAPN, (F) 300 μM βAPN, or (G) 100 μM catalase. **H**: Rho activity assay of cells with total cellular Rho protein equal loading controls. Densitometric evaluation of each band normalized to its corresponding loading control and subsequently normalized to control cells (1.0) is shown.

DOCK180 pathway. Our previous work demonstrated that LOX regulated adhesion formation through the FAK/Src signaling complex and that LOX activity facilitated FAK and Src activity. Therefore, we examined a signaling pathway downstream of FAK/Src that leads to Rac activation: the $p130^{Cas}$/Crk/DOCK180 complex. Figure 4A demonstrates that when invasive breast cancer cells are treated with βAPN, a corresponding decrease in phosphorylated $p130^{Cas}$ is observed. This decrease is not due to a loss of CAS protein within the cells (Fig. 4A). Conversely, Figure 4B demonstrates an increase in phosphorylated $p130^{Cas}$ in MCF-7/LOX32-His cells compared to MCF-7 and MCF-7/LOX50-His cells. This increase was reversible upon βAPN treatment and was not due to changes in overall $p130^{Cas}$ expression. Equal loading was verified using a 0.2% amido black stain on membranes subsequent to transfer. In order to determine what effect the loss of CAS phosphorylation would have on the ability of the $p130^{Cas}$/Crk/DOCK180 complex to form, we immunoprecipitated Crk from these invasive cell lines treated with βAPN and measured levels of CAS and DOCK180 that were able to bind to Crk. Figure 4C demonstrates that when LOX activity was inhibited with βAPN, a corresponding decrease in CAS and DOCK180 binding to Crk was observed. These data were recapitulated in MCF-7/LOX32-His cells (Fig. 4D). Since the inability of DOCK180 to bind to Crk leads to an inhibition of Rac-GTP formation, these data demonstrate that LOX activity leads to an increase in CAS phosphorylation, which allows for the formation of the $p130^{Cas}$/Crk/DOCK180 complex to form. This complex will then lead to the conversion

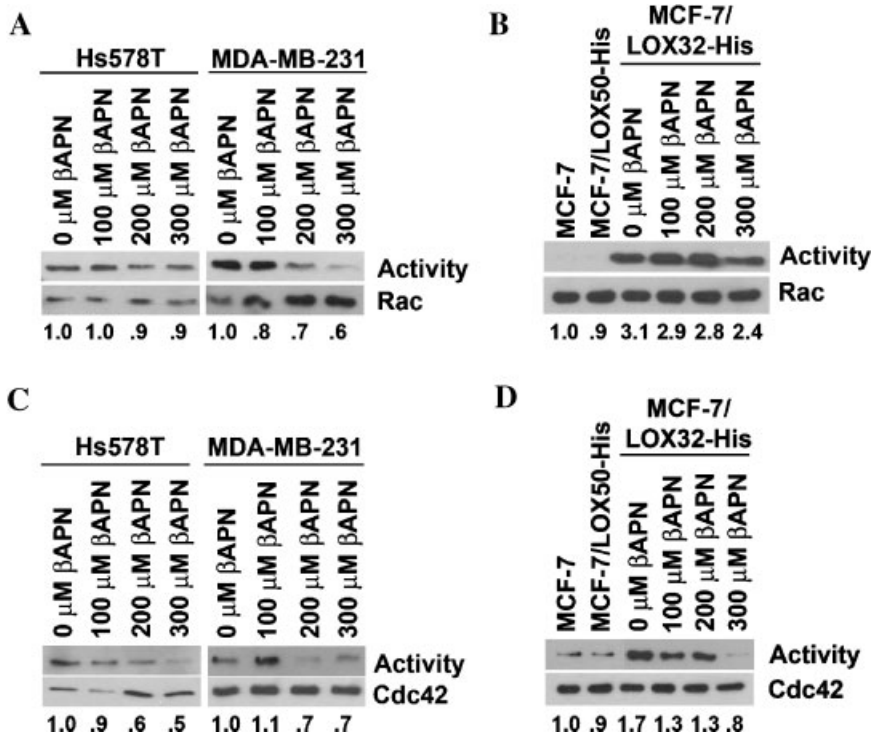

Fig. 3. LOX activity facilitates Rac and Cdc42 activity in invasive breast cancer cells. Rac activity assays with equal loading controls of (**A**) Hs578T and MDA-MB-231 invasive breast cancer cells treated with βAPN and (**B**) MCF-7, MCF-7/LOX50-His, and MCF-7/LOX32-His treated with βAPN. Cdc42 activity assays of (**C**) Hs578T and MDA-MB-231 invasive breast cancer cells treated with βAPN and (**D**) MCF-7, MCF-7/LOX50-His, and MCF-7/LOX32-His treated with βAPN. Densitometric evaluation of each band normalized to its corresponding loading control and subsequently normalized to control cells (1.0) is shown.

of Rac-GDP to Rac-GTP and the formation of lamellipodia. Consequently, cell motility is increased due to changes in actin filament formation.

DISCUSSION

Breast cancer is the second leading cause of cancer death in women in the United States [American Cancer Society, Inc., 2005]. While treatment options for women with local disease are extremely good, survival rates for women with distant metastases are significantly decreased. Therefore, it is important that we understand the molecular mechanisms of metastasis in order to better treat breast cancer patients. To this end, we have examined the role of LOX in breast cancer cell migration/invasion. Multiple reports have demonstrated a multifunctional role for LOX within the cell in addition to its classical function in the extracellular matrix [Nelson et al., 1988; Lazarus et al., 1995; Li et al., 1997, 2000, 2003; Giampuzzi et al., 2003a,b; Payne et al., 2005]. Specifically, our laboratory has recently demonstrated that LOX regulates breast cancer cell motility/migration and adhesion through an intracellular hydrogen peroxide-mediated mechanism [Payne et al., 2005]. These data have allowed us to hypothesize that LOX plays a role in tumor progression by facilitating cell motility/migration to distant sites.

The work presented here further elucidates the mechanism by which LOX regulates cell motility/migration by examining the role of LOX activity in actin filament polymerization. We demonstrate that inhibition of LOX activity corresponds with an increase in stress fiber formation, specifically through the loss of hydrogen peroxide. Additionally, LOX activity facilitates Rac and Cdc42 activity while inhibiting Rho activity. Finally, we demonstrate that LOX regulates Rac activity through the $p130^{Cas}$/Crk/DOCK180 signaling complex. Figure 5 demonstrates our hypothetical model of LOX regulation of cell migration through the Src signaling pathway. Previously, we demonstrated that LOX regulates changes in cell adhesion by phosphorylation and activation of the FAK/Src signaling pathway, which leads to

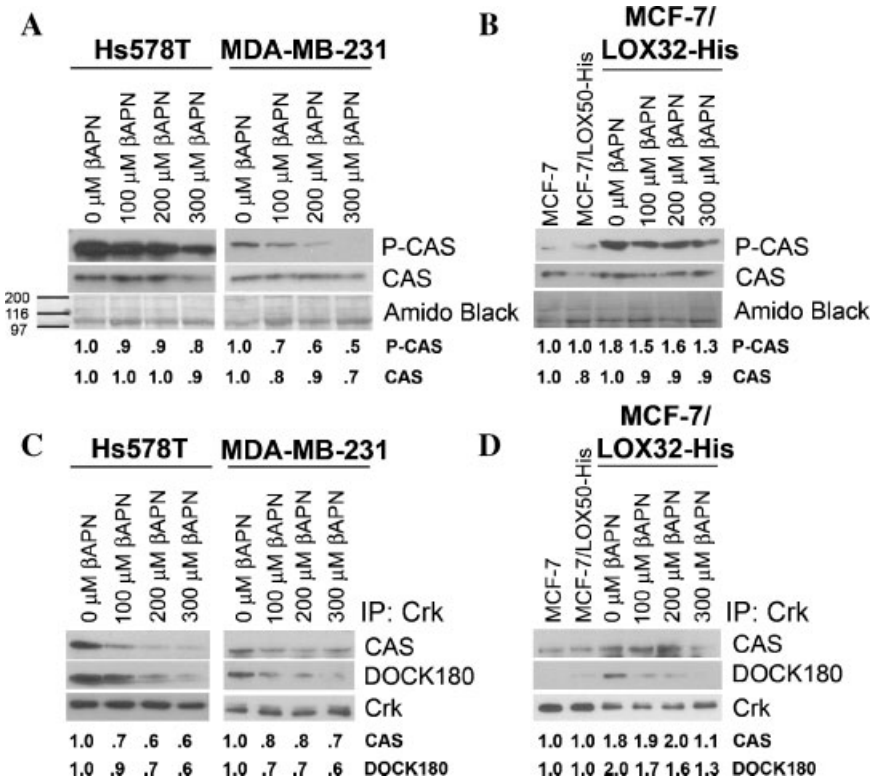

Fig. 4. LOX activity facilitates p130^{Cas} phosphorylation and the p130^{Cas}/Crk/DOCK180 signaling complex. Immunoblot analysis of p130^{Cas} phosphorylation in (**A**) Hs578T and MDA-MB-231 invasive breast cancer cells treated with βAPN and (**B**) MCF-7, MCF-7/LOX50-His, and MCF-7/LOX32-His treated with βAPN. Equal loading was verified by staining membranes subsequent to transfer with 0.2% amido black and de-staining with water. Co-immunoprecipitation of Crk with CAS and DOCK180 in (**C**) Hs578T and MDA-MB-231 invasive breast cancer cells treated with βAPN and (**D**) MCF-7, MCF-7/LOX50-His, and MCF-7/LOX32-His treated with βAPN. Densitometric evaluation of each band normalized to its corresponding loading control and subsequently normalized to control cells (1.0) is shown.

increased cell migration [Payne et al., 2005]. Here, we demonstrate a novel intracellular signaling pathway in which LOX functions to regulate actin filament polymerization and subsequent cell migration through the p130^{Cas}/Crk/DOCK180 signaling pathway, a downstream target of Src.

The Rho family of GTPases (Rho, Rac, and Cdc42) has been shown to regulate actin filament polymerization and, ultimately, cell motility [Nobes and Hall, 1995]. Specifically, Rho activity leads to the formation of actin stress fibers, typically associated with a non-motile phenotype, while Rac and Cdc42 activity are associated with the formation of lamellipodia and filopodia, respectively, which correspond with a motile phenotype. Many reports have demonstrated that as cancer cells become more invasive and motile, increases in Rac and Cdc42 activity and/or decreases in Rho activity are observed. [Odero-Marah et al., 2003; Sahai and Marshall, 2003; Kurisu et al., 2005]. To this end, the data presented here demonstrate a loss of Rho activity and an increase in Rac and Cdc42 activity in the presence of LOX activity. Furthermore, visualization of filamentous F-actin in these cancer cell lines demonstrates a loss of stress fiber formation in the presence of LOX activity. Collectively, these data corroborate the hypothesis that LOX activity facilitates a motile phenotype in invasive breast cancer cells by regulation of actin filament formation.

We further examined the mechanism by which LOX regulates Rac activity (and its corresponding changes in actin filament formation) by examining the p130^{Cas}/Crk/DOCK180 signaling complex. We had previously demonstrated that LOX regulates the FAK/Src signaling pathway through a hydrogen peroxide-mediated mechanism [Payne et al., 2005]. The p130^{Cas}/Crk/DOCK180 complex is downstream of the FAK/Src signaling complex and we

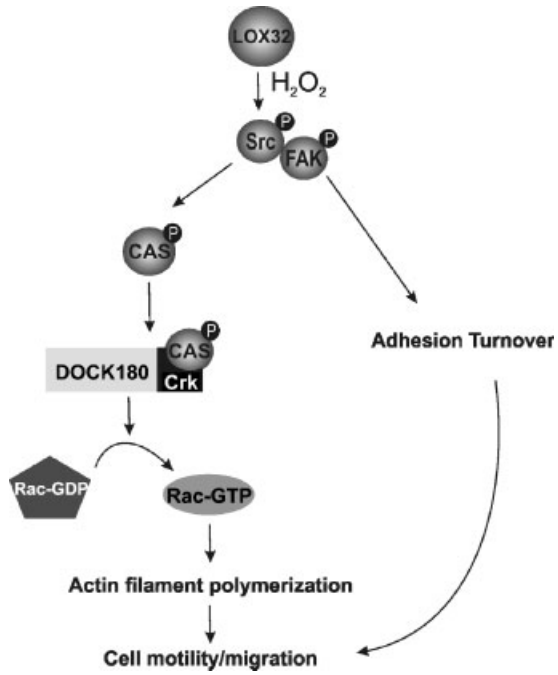

Fig. 5. Hypothetical model of LOX regulation of cell migration. LOX stimulates Src phosphorylation through a hydrogen peroxide-mediated mechanism. Subsequently, Src can phosphorylate and activate a variety of signaling proteins including FAK [Payne et al., 2005] and $p130^{Cas}$. Activation of FAK stimulates cell migration through increases in adhesion turnover while phosphorylation of $p130^{Cas}$ allows for binding to Crk. Binding of CAS and Crk allows for the recruitment of DOCK180, a Rac-GEF and leads to the exchange of Rac-GDP to Rac-GTP. Lamellipodia formation is stimulated upon activation of Rac, leading to an increase in cell motility/migration.

hypothesized that a loss in Src signaling due to inhibition of LOX activity would correspond with changes in the $p130^{Cas}$/Crk/DOCK180 complex leading to an inhibition of Rac activation. The $p130^{Cas}$/Crk/DOCK180 is formed upon phosphorylation of $p130^{Cas}$ by Src [Hamasaki et al., 1996; Sakai et al., 1997; Klinghoffer et al., 1999; Shin et al., 2004]. Phosphorylated $p130^{Cas}$ can subsequently bind to Crk. Binding of $p130^{Cas}$ and Crk leads to the recruitment of DOCK180, a Rac-GEF [Sakai et al., 1994; Kiyokawa et al., 1998a,b; Klemke et al., 1998]. Upon formation of this complex, Rac is then activated by conversion to Rac-GTP and formation of lamellipodia is stimulated. Our data demonstrate that when LOX catalytic activity is inhibited, there is a corresponding loss in $p130^{Cas}$ phosphorylation and formation of the $p130^{Cas}$/Crk/DOCK180 complex. Together, these findings demonstrate that LOX activity regulates Rac activity and actin filament formation through the $p130^{Cas}$/Crk/DOCK180 complex.

While this paper has specifically focused on the $p130^{Cas}$/Crk/DOCK180 signaling pathway, we recognize that there are multiple pathways, which can regulate Rho, Rac, and Cdc42 activities such as ROCK, WASP, and others [reviewed in Yamazaki et al., 2005]. It is possible, and highly probable, that LOX may regulate the Rho family of GTPases and cell motility in general through various signaling mechanisms. Our laboratory continues to examine the various pathways in which LOX may function intracellularly to regulate breast cancer migration.

Our previous report demonstrated increases in cell-matrix adhesion which conincided with LOX activity [Payne et al., 2005]. It has also been well documented that increases in Rho activity and/or decreases in Rac and Cdc42 activity correspond to increases in cell-matrix adhesion [Yang et al., 2001; Wang et al., 2005]. These data and our previous findings seemingly contradict each other. However, it is important to note that changes in cell-matrix adhesion that are regulated through FAK are actually changes in adhesion turnover [reviewed in Schaller, 2001]. Therefore, the increases we previously observed in cell adhesion reflect increases in adhesion turnover. A greater number of cells are able to continually attach and detach from the extracellular matrix. When cells are treated with βAPN, fewer cells attach to the matrix. However, upon attachment they form extremely stable adhesions that do not turn over/detach—phenotypes caused by an increase in Rho activity and decreases in Rac and Cdc42 activity. Therefore, the data presented here correlate with our previous report and further elucidate the mechanism(s) by which LOX regulates cell motility.

This study presents a novel intracellular signaling pathway in which LOX functions to regulate cell motility/migration through changes in actin filament formation. We show that LOX activity regulates Rho, Rac, and Cdc42 activity, as well as actin stress fiber formation. Additionally, LOX regulates changes in Rac activity through the $p130^{Cas}$/Crk/DOCK180 signaling complex. Understanding the molecular mechanisms by which LOX functions to regulate breast cancer cell motility/migration will contribute to novel anti-cancer treatment modalities.

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REFERENCES

American Cancer Society Inc. 2005. Cancer Facts and Figures 2005. www.cancer.org

Contente S, Kenyon K, Sriraman P, Subramanyan S, Friedman RM. 1999. Epigenetic inhibition of lysyl oxidase transcription after transformation by ras oncogene. Mol Cell Biochem 194:79–91.

Csiszar K. 2001. Lysyl oxidases: A novel multifunctional amine oxidase family. Prog Nucleic Acid Res Mol Biol 70:1–32.

Fuchs B, Zhang K, Bolander ME, Sarkar G. 2000. Identification of differentially expressed genes by mutually subtracted RNA fingerprinting. Anal Biochem 286:91–98.

Giampuzzi M, Botti G, Cilli M, Gusmano R, Borel A, Sommer P, Di Donato A. 2001. Down-regulation of lysyl oxidase induced tumorigenic transformation in NRK-49F cells characterized by constitutive activation of ras proto-oncogene. J Biol Chem 276:29226–29232.

Giampuzzi M, Oleggini R, DiDonato A. 2003a. Demonstration of in vitro interaction between tumor suppressor lysyl oxidase and histones H1 and H2: Definition of the regions involved. Biochim Biophys Acta 1647:245–251.

Giampuzzi M, Oleggini R, DiDonato A. 2003b. Altered adhesion features and signal transduction in NRK-49F cells transformed by down-regulation of lysyl oxidase. Biochim Biophys Acta 1647:239–244.

Hamasaki K, Mimura T, Morino N, Furuya H, Nakamoto T, Aizawa S, Morimoto C, Yazaki Y, Hirai H, Nojima Y. 1996. Src kinase plays an essential role in integrin-mediated tyrosine phosphorylation of Crk-associated substrate p130Cas. Biochem Biophys Res Commun 222:338–343.

Jeay S, Pianetti S, Kagan HM, Sonenshein GE. 2003. Lysyl oxidase inhibits ras-mediated transformation by preventing activation of NF-kappa B. Mol Cell Biol 23:2251–2263.

Kagan HM, Li W. 2003. Lysyl oxidase: Properties, specificity, and biological roles inside and outside of the cell. J Cell Biochem 88:660–672.

Kaneda A, Wakazono K, Tsukamoto T, Watanabe N, Yagi Y, Tatematsu M, Kaminishi M, Sugimura T, Ushijima T. 2004. Lysyl oxidase is a tumor suppressor gene inactivated by methylation and loss of heterozygosity in human gastric cancers. Cancer Res 64:6410–6415.

Kenyon K, Contente S, Trackman PC, Tang J, Kagan HM. 1991. Lysyl oxidase and rrg messenger RNA. Science 253:802.

Kirschmann DA, Seftor EA, Nieva DR, Mariano EA, Hendrix MJ. 1999. Differentially expressed genes associated with the metastatic phenotype in breast cancer. Breast Cancer Res Treat 55:127–136.

Kirschmann DA, Seftor EA, Fong SF, Nieva DR, Sullivan CM, Edwards EM, Sommer P, Csiszar K, Hendrix MJ. 2002. A molecular role for lysyl oxidase in breast cancer invasion. Cancer Res 62:4478–4483.

Kiyokawa E, Hashimoto Y, Kobayashi S, Sugimura H, Kurata T, Matsuda M. 1998a. Activation of Rac1 by a Crk SH3-binding protein, DOCK180. Genes Dev 12:3331–3336.

Kiyokawa E, Hashimoto Y, Kurata T, Sugimura H, Matsuda M. 1998b. Evidence that DOCK180 up-regulates signals from the CrkII-p130(Cas) complex. J Biol Chem 273:24479–24484.

Klemke RL, Leng J, Molander R, Brooks PC, Vuori K, Cheresh DA. 1998. CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. J Cell Biol 140:961–972.

Klinghoffer RA, Sachsenmaier C, Cooper JA, Soriano P. 1999. Src family kinases are required for integrin but not PDGFR signal transduction. EMBO J 18:2459–2471.

Kozma R, Ahmed S, Best A, Lim L. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 15:1942–1952.

Kurisu S, Suetsugu S, Yamazaki D, Yamaguchi H, Takenawa T. 2005. Rac-WAVE2 signaling is involved in the invasive and metastatic phenotypes of murine melanoma cells. Oncogene 24:1309–1319.

Lazarus HM, Cruikshank WW, Narasimhan N, Kagan HM, Center DM. 1995. Induction of human monocyte motility by lysyl oxidase. Matrix Biol 14:727–731.

Li W, Nellaiappan K, Strassmaier T, Graham L, Thomas KM, Kagan HM. 1997. Localization and activity of lysyl oxidase within nuclei of fibrogenic cells. Proc Natl Acad Sci USA 94:12817–12822.

Li W, Liu G, Chou IN, Kagan HM. 2000. Hydrogen peroxide-mediated, lysyl oxidase-dependent chemotaxis of vascular smooth muscle cells. J Cell Biochem 78:550–557.

Li W, Nugent MA, Zhao Y, Chau AN, Li SJ, Chou IN, Liu G, Kagan HM. 2003. Lysyl oxidase oxidizes basic fibroblast growth factor and inactivates its mitogenic potential. J Cell Biochem 88:152–164.

Nagaraja GM, Othman M, Fox BP, Alsaber R, Pellegrino CM, Zeng Y, Khanna R, Tamburini P, Swaroop A, Kandpal RP. 2005. Gene expression signatures and biomarkers of noninvasive and invasive breast cancer cells: Comprehensive profiles by representational difference analysis, microarrays and proteomics. Oncogene In press.

Nelson JM, Diegelmann RF, Cohen IK. 1988. Effect of beta-aminopropionitrile and ascorbate on fibroblast migration. Proc Soc Exp Biol Med 188:346–352.

Nobes CD, Hall A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81:53–62.

Odero-Marah VA, Khalkhali-Ellis Z, Chunthapong J, Amir S, Seftor RE, Seftor EA, Hendrix MJ. 2003. Maspin regulates different signaling pathways for motility and adhesion in aggressive breast cancer cells. Cancer Biol Ther 2:398–403.

Payne SL, Fogelgren B, Hess AR, Seftor EA, Wiley EL, Fong SF, Csiszar K, Hendrix MJ, Kirschmann DA. 2005. Lysyl oxidase regulates breast cancer cell migration

and adhesion through a hydrogen peroxide-mediated mechanism. Cancer Res 65:11429–11436.

Rae MT, Niven D, Ross A, Forster T, Lathe R, Critchley HO, Ghazal P, Hillier SG. 2004. Steroid signalling in human ovarian surface epithelial cells: The response to interleukin-1alpha determined by microarray analysis. J Endocrin 183:19–28.

Ren C, Yang G, Timme TL, Wheeler TM, Thompson TC. 1998. Reduced lysyl oxidase messenger RNA levels in experimental and human prostate cancer. Cancer Res 58:1285–1290.

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70:401–410.

Rost T, Pyritz V, Rathcke IO, Gorogh T, Dunne AA, Werner JA. 2003. Reduction of LOX- and LOXL2-mRNA expression in head and neck squamous cell carcinomas. Anticancer Res 23:1565–1573.

Sahai E, Marshall CJ. 2003. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. Nat Cell Biol 5:711–719.

Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Mano H, Yazaki Y, Hirai H. 1994. A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J 13:3748–3756.

Sakai R, Nakamoto T, Ozawa K, Aizawa S, Hirai H. 1997. Characterization of the kinase activity essential for tyrosine phosphorylation of p130Cas in fibroblasts. Oncogene 14:1419–1426.

Schaller MD. 2001. Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim Biophs Acta 1540:1–21.

Shin NY, Dise RS, Schneider-Mergener J, Ritchie MD, Kilkenny DM, Hanks SK. 2004. Subsets of the major tyrosine phosphorylation sites in Crk-associated substrate (CAS) are sufficient to promote cell migration. J Biol Chem 279:38331–38337.

Smith-Mungo LI, Kagan HM. 1998. Lysyl oxidase: Properties, regulation and multiple functions in biology. Matrix Biol 16:387–398.

Stassar MJ, Devitt G, Brosius M, Rinnab L, Prang J, Schradin T, Simon J, Petersen S, Kopp-Schneider A, Zoller M. 2001. Identification of human renal cell carcinoma associated genes by suppression substractive hybridization. Br J Cancer 85:1372–1382.

Uzel MI, Shih SD, Gross H, Kessler E, Gerstenfeld LC, Trackman PC. 2000. Molecular events that contribute to lysyl oxidase enzyme activity and insoluble collagen accumulation in osteosarcoma cell clones. J Bone Miner Res 15:1189–1197.

Wang L, Yang L, Filippi M-D, Williams DA, Zheng Y. 2005. Genetic deletion of Cdc42GAP reveals a role of Cdc42 in erythropoiesis and hematopoietic stem/progenitor cell survival, adhesion and engraftment. Blood In press.

Woznick AR, Braddock AL, Dulai M, Seymour ML, Callahan RE, Welsh RJ, Chmielewski GW, Zelenock GB, Shanley CJ. 2005. Lysyl oxidase expression in bronchogenic carcinoma. Am J Surg 189:297–301.

Yamazaki D, Kurisu S, Takenawa T. 2005. Regulation of cancer cell motility through actin reorganization. Cancer Sci 96:379–386.

Yang FC, Atkinson SJ, Gu Y, Borneo JB, Roberts AW, Zheng Y, Pennington J, Williams DA. 2001. Rac and Cdc42 GTPases control hematopoietic stem cell shape, adhesion, migration, and mobilization. PNAS 98:5614–5618.