Multiple Functions of Lysyl Oxidase Like-2 in Oral Fibroproliferative Processes

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Abstract

Gingival overgrowth is a side effect of certain medications, including calcium channel blockers, cyclosporin A, and phenytoin. Phenytoin-induced gingival overgrowth is fibrotic. Lysyl oxidases are extracellular enzymes that are required for biosynthetic cross-linking of collagens, and members of this enzyme family are upregulated in fibrosis. Previous studies in humans and in a mouse model of phenytoin-induced gingival overgrowth have shown that LOXL2 is elevated in the epithelium and connective tissue in gingival overgrowth tissues and not in normal tissues. Here, using a novel LOXL2 isoform-selective inhibitor and knockdown studies in loss- and gain-of-function studies, we investigated roles for LOXL2 in promoting cultures of human gingival fibroblasts to proliferate and to accumulate collagen. Data indicate that LOXL2 stimulates gingival fibroblast proliferation, likely by a platelet-derived growth factor B receptor-mediated mechanism. Moreover, collagen accumulation was stimulated by LOXL2 enzyme and inhibited by LOXL2 inhibitor or gene knockdown. These studies suggest that LOXL2 could serve as a potential therapeutic target to address oral fibrotic conditions.

Keywords: cell biology, collagen, extracellular matrix, fibroblasts, matrix biology, receptors

Introduction

Lysyl oxidase-like 2 (LOXL2) belongs to the family of lysyl oxidases. Like the other 4 members of this family (lysyl oxidase [LOX] and lysyl oxidase-like 1, 3, and 4 [LOXL1, LOXL3, and LOXL4]), LOXL2 catalyzes the extracellular oxidative deamination of the ϵ -amino groups of some lysine residues in collagens. The resulting aldehydes participate in the formation of covalent cross-linkages required for collagen biosynthesis (Csiszar 2001; Kagan and Li 2003).

Excess levels of lysyl oxidases participate in the elevated accumulation of the extracellular matrix in fibrotic pathologies. For example, LOXL2 is among the 10 genes that are related to proliferation, migration, and invasion in hepatocellular carcinoma and cancer-associated fibroblasts (Lin and Chuang 2013). Studies have identified different roles for LOXL2 in proliferation and migration of human umbilical vein endothelial cells (HUVECs) (Bignon et al. 2011). Increased levels of LOXL2 are present in hepatocytes of patients with Wilson disease or primary biliary cirrhosis, which are fibrotic liver diseases (Vadasz et al. 2005). LOXL2 is elevated in renal tubulointerstitial fibrosis from experimental unilateral ureteral obstruction and human renal samples of diabetic nephropathy, IgA nephropathy, and hypertensive nephrosclerosis (Higgins et al. 2007). LOXL2 is overexpressed in phenytoin-induced human gingival overgrowth and recapitulated in a mouse model. LOXL2 was found to be expressed in both epithelial cells and fibroblasts (Assaggaf et al. 2015). Earlier studies reported abnormally high levels of specific cytokines in gingival overgrowth tissues, including platelet-derived growth

factor B (PDGF-B) (Iacopino et al. 1997), fibroblast growth factor 2 (FGF-2), transforming growth factor β 1 and β 2 (TGF- β) (Saito et al. 1996), and connective tissue growth factor (CCN2 or CTGF) (Uzel et al. 2001). Phenytoin has been shown to increase the production of platelet-derived growth factor (PDGF), a cytokine involved in connective tissue growth and repair, in rat peritoneal macrophages and human blood monocytes (Dill et al. 1993). Similar to phenytoin, cyclosporin A causes an increase in PDGF-B messenger RNA (mRNA) in gingival overgrowth tissues compared to nonovergrowth controls (Plemons et al. 1996).

The goal of this study was to investigate novel mechanisms by which LOXL2 could contribute to gingival overgrowth and other oral fibrotic conditions, focusing on fibroblast proliferation and collagen accumulation in vitro. Data indicate that LOXL2 optimizes PDGF signaling, cell proliferation, and collagen accumulation by human gingival fibroblasts.

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Materials and Methods

Reagents

Lysyl oxidase like-2 antibody was from Genetex (GTX105085). Affinity purified LOX antibody was previously described (Khosravi et al. 2014), while LOXL1-, LOXL3-, and LOXL4-validated antibodies were purchased from Santa Cruz. Cell Signaling supplied antibodies for PDGFR β (clone 28E1), rabbit mAb (catalogue 3169), and phospho-PDGFR β (Tyr751) (clone C63G6) rabbit mAb (catalogue 4549S). Recombinant human PDGF-BB (100-14B) was purchased from PeproTech, ECL Western blotting detection reagents were from Amersham Biosciences, Dulbecco's modified Eagle medium (DMEM) and phosphate-buffered saline (PBS) were from Gibco. PXS-S1C was the generous gift of Pharmaxis Ltd; BAPN fumarate was purchased from Sigma-Aldrich. Recombinant active purified human LOXL2 was either purchased from R&D Systems (Rodriguez et al. 2010) or was purified from conditioned medium of LOXL2 lentivirus-transduced CHO-K1 cells. The LOXL2 construct contained a C-terminal His₆ tag and was purified on a nickel affinity column (BioRad) according to methods employed previously for the lysyl oxidase propeptide (Vora et al. 2010), except that protein was eluted with 8 M urea, 0.1 M imidazole, 0.01 M sodium phosphate, pH 8.0. Both commercial and in-house preparations resulted in consistent data with specific activity greater than 0.300 nmol/ μ g/30 min assay (Palamakumbura and Trackman 2002) and exhibited LOXL2 immunoreactive bands at 100 and 75 kDa, as expected (Rodriguez et al. 2010; Iftikhar et al. 2011).

Lysyl Oxidase Inhibitor Specificity

Native human LOX and LOXL2 were obtained from the conditioned medium of IMR90 cell cultures, after 3 d in DMEM (0.1% fetal bovine serum [FBS], no phenol red, supplemented with nonessential amino acids and 10 μ M CuSO₄). The supernatant was concentrated and buffer exchanged to 4 M urea and 50 mM sodium borate buffer, pH 8.2, by means of Amicon 10-kDa centrifuge filters (Millipore). The concentrated supernatant was then fractionated with Amicon filters with a higher cutoff (50 kDa), separating LOXL2 in the portion at a higher molecular weight and collecting the 30-kDa LOX in the lower molecular weight portion and further concentrated and buffer exchanged with Amicon filters to 1.2 M urea and 50 mM sodium borate buffer, pH 8.2. The enzymes were subsequently verified by Western blot. In some experiments specified in the figure legends, purified human LOXL2 was purchased from R&D Systems. Recombinant human LOXL1 and LOXL4 were respectively expressed in 293 cells, and concentrated conditioned media were employed as enzyme sources. LOXL3 was purchased from R&D Systems. All lysyl oxidase family enzyme activity assays were performed as described (Palamakumbura and Trackman 2002). Recombinant human semicarbazide-sensitive amine oxidase (SSAO/VAP1), diamine oxidase (DAO), and monoamine oxidase A and B (MAO-A and MAO-B) assays were performed as previously described (Schilter et al. 2014).

Gingival Fibroblast Cell Cultures

Gingival tissues were obtained from 3 adult subjects undergoing routine periodontal treatments and who did not have gingival overgrowth (Hong et al. 1999). Donors were 3 females between the ages of 25 and 35 y. Human subject protocols were approved by a Boston University Medical Center institutional review board, and informed consent was obtained from all donors. After second passage, cell stocks were stored in liquid nitrogen. For experiments, cells were grown from frozen stocks at passage 3 in 100-mm cell culture plates and cultured at 37°C (Black et al. 2007). The fibroblasts grown from frozen stocks were passaged twice for expansion before being plated for experimental treatments at an initial concentration of 50,000 cells per well in 6-well plates.

LOXL2 Short Hairpin RNA

Lentiviral plasmid constructs were bought from Open Biosystems, except for the NonTarget construct (Sarbasov et al. 2005), which was obtained via Addgene. The short hairpin RNA (shRNA) sequences for reference LOXL2 sequence and the NonTarget sequence were as follows. NM_002318:

Virus 195: (TRCN0000046195) CCGGCGATTACTCC
AACAACATCATCTCGAGATGATGTTGTTGGAGT
AATCGTTTTTG

Virus 193: (TRCN0000046193)-CCGGCCAGATAGAG
AACCTGAATATCTCGAGATATTCAGGTTCTCTAT
CTGGTTTTTG

NonTarget (plasmid 1864): CCTAAGGTTAAGTCGCC
CTCGCTCGAGCGAGGGCGACTTAACCTTAGG
(Bais et al. 2009; Iftikhar et al. 2011)

Lentiviruses were produced in 293T cells as previously described (Bais et al. 2009). Lentivirus shRNA virus particles were employed to knock down LOXL2 and NonTarget shRNA for control and supplemented with fresh media after 12 h (Bais et al. 2009). The samples were collected after addition of fresh media for 24 h, and knockdown of LOXL2 was confirmed by Western blot analysis. In growth curve experiments, primary human gingival fibroblasts were transduced with LOXL2 shRNAs or NonTarget shRNA at 80% to 90% visual confluence for 24 h. The medium was then refreshed with 10 mL of media containing 0.5 μ g/mL puromycin for 48 h to select for transduced cells. After 3 d, transduced cells were seeded as described for each experiment.

Western Blot

Freshly transduced primary gingival fibroblast cells were grown in 10-cm plates with DMEM (ATCC cat. 30-2003). Cell layers were extracted into sample buffer (0.5 M Tris [pH 6.8], glycerol, 10% sodium dodecyl sulfate [SDS], 5% β -mercaptoethanol) for each condition, boiled for 5 min, and stored at -20°C and subjected to Western blotting and analyses as we have described previously (Bais et al. 2012).

DNA Synthesis Assays

The CyQuant cell proliferation reagent assay (C7026) from Invitrogen was used to measure DNA accumulation over a 24-h period. Freshly transduced and selected cells were seeded at a density of 5,000 cells per well in each of eight 24-well plates per experimental group and grown under standard cell culture conditions. Cells were placed in serum-free medium containing 0.1% bovine serum albumin (BSA) for 18 h. Media were removed from 4 wells, washed with PBS, and then stored at -80°C (day 0). After an additional 24 h of culture, media were removed from the remaining 4 plates and washed with PBS, and plates were stored at -80°C . Measurements were carried out as indicated by the CyQuant (Molecular Probes) instructions, with fluorescence of samples read at an excitation of 420 nm and an emission of 535 nm. Cell numbers were derived from a standard curve of trypsinized cell suspensions counted by hemocytometer versus CyQuant fluorescence.

Growth Curve

Primary human gingival fibroblast cells transduced with LOXL2 shRNA plasmids or control shRNA plasmid were seeded at a density of 5,000 cells per well in 24-well plates overnight in medium containing 5% serum. Cells were detached by trypsin–ethylenediaminetetraacetic acid and were counted using a hemocytometer. The number of cells at this point was designated as day 0 to establish that the number of cells in the control and knockdown groups at the beginning of the experiment was equivalent. Media were refreshed every second day throughout the remainder of the experiment. Cells in triplicate wells were then counted and subjected to CyQuant assays each day for the remainder of the experiment. Plots of the number of cells as a function of postseeding days were generated to compare the growth of LOXL2 knockdown cells compared to controls.

Collagen Accumulation

The validated Sirius Red dye-binding assay for measuring collagen accumulation in gingival fibroblasts was performed (Heng et al. 2006). Optical density was then measured at 540 nm against 0.1 N NaOH as blank using a Berthold Technologies LB940 Plate Reader.

Exogenous LOXL2

To evaluate the effect of exogenously added LOXL2 on stimulation of DNA synthesis, primary gingival fibroblast cells were seeded at a density of 5,000 cells per well in each of eight 24-well plates per experimental group and grown under standard cell culture conditions and then serum-depleted overnight. Cells were then stimulated with the indicated concentrations of LOXL2 or vehicle (PBS) and cultured for 24 h. Plates were then processed for the CyQuant assay described above.

Statistical Analysis

Analysis of variance with Tukey's, Sidak's, or Dunnett's multiple comparisons post hoc tests were performed using GraphPad Prism software version 7 for Windows as indicated in the figure legends; data were considered significant at $P < 0.05$.

Results

Identification of Secreted Forms of Lysyl Oxidase by Human Gingival Fibroblasts

To determine which lysyl oxidase family members are secreted by human gingival fibroblasts, conditioned media were collected from cultured cells obtained from 3 separate human donors and concentrated, and equal amounts of protein were subjected to Western blotting. Data in Figure 1A indicate that LOXL2 and LOXL4 are the predominant lysyl oxidases secreted by human gingival fibroblasts. Both proteins appear to have lost SRCR domains by proteolytic processing, based on apparent molecular weights of about 72 kDa and 60 kDa proteins on SDS–polyacrylamide gel electrophoresis (PAGE) (Lopez-Jimenez et al. 2017; Okada et al. 2017).

LOXL2/LOXL4 Pharmacological Inhibitor Inhibits Cell Proliferation in Primary Human Gingival Fibroblasts

Lysyl oxidases are multifunctional proteins, and nonenzymatic functions for LOXL2 have been reported (Cuevas et al. 2014). To assess whether proliferation of human gingival fibroblasts could depend on LOXL2 or LOXL4 enzyme activity, a DNA synthesis assay was performed in cells in the presence or absence of a novel potent inhibitor that selectively targets these isoforms. The Table provides inhibition constants for the inhibitor (PXS-S1C) employed measured against all lysyl oxidase isoforms and other amine oxidases. Data demonstrate high selectivity of PXS-S1C for LOXL2.

Gingival fibroblasts were cultured and then serum deprived for 18 h and subsequently cultured in the presence or absence of 1 μM PXS-S1C for an additional 24 h. DNA content was then determined employing the CyQuant assay. Results (Fig. 1B) indicate that inhibition of LOXL2/LOXL4 enzyme activity attenuates DNA accumulation in primary gingival fibroblasts. Data suggest that LOXL2 and/or LOXL4 are required for optimal human gingival fibroblast DNA synthesis.

LOXL2 Knockdown Inhibits Cell Proliferation by Human Gingival Fibroblasts

To investigate the specific importance of LOXL2 in particular in gingival fibroblast cell growth, primary human gingival fibroblasts were transduced with lentiviral particles containing LOXL2 shRNA(195), LOXL2 shRNA(193), or NonTarget

Figure 1. (A) Lysyl oxidases secreted by human gingival fibroblasts. Conditioned medium was collected under serum-free conditions for 24 h from cultured primary human gingival fibroblasts obtained from 3 different donors and concentrated 1:20 by Millipore/Centricon ultrafiltration. Equal amounts of protein were subjected to Western blotting using antibodies specific for each lysyl oxidase protein. Data shown are Western images taken from the same blot, in which lanes were separated before probing with respective relevant antibodies. Data are consistent for all 3 subjects tested. (B) LOXL2/LOXL4 enzyme activity is required for optimal cell primary human gingival fibroblast DNA synthesis. LOXL2 inhibitor (PXS-S1C) at different concentrations was added to serum-depleted cultures, and relative DNA synthesis over a 24-h period was assessed by CyQuant assay. Data are expressed as fold change compared to the dimethyl sulfoxide (DMSO) control induced by PXS-S1C. Data are presented as means taken from 3 different subjects with 6 replicates per condition. Data are presented as $\pm$ SEM ($n = 3$), with 1-way analysis of variance ($P < 0.0001$), followed by Tukey's post hoc test ($*P < 0.001$) compared to each DMSO or 0 μ M PXS-S1C controls.

shRNA (control) and then cultured in medium containing 5% serum, refreshed every 2 d. Results indicate that LOXL2 knockdown using 2 different LOXL2 shRNAs inhibits primary gingival fibroblast cell growth (Fig. 2A, B). Knockdown of LOXL2 was confirmed by Western blot of cell layers (Fig. 2C, D). Data indicate that LOXL2 is required for optimal primary human gingival fibroblast cell growth.

Recombinant LOXL2 Protein Promotes a Proliferative Response in Primary Human Gingival Fibroblasts

To assess for effects of LOXL2 on gingival fibroblast cell proliferative responses using a gain-of-function approach, primary human gingival fibroblasts were treated with or without active recombinant LOXL2, and DNA synthesis was then assessed by the DNA accumulation assay. Primary human gingival fibroblasts were seeded at 5,000 cells per well in a 24-well plate on day -1, and on day 0, cells were refed with serum-free medium. After 16 h, cells were treated with different concentrations of the active recombinant LOXL2 (rLOXL2) and cultured for 24 h. DNA accumulation was assayed using the CyQuant assay. Data (Fig. 3A) indicate that addition of active rLOXL2 increased cell proliferation in primary gingival fibroblasts.

Recombinant LOXL2 Increases Collagen Accumulation in Cultures of Human Gingival Fibroblasts

Phenytoin-induced gingival overgrowth is a fibrotic condition (Uzel et al. 2001; Assaggaf et al. 2015; Trackman and Kantarci 2015). To investigate whether LOXL2 can modulate collagen

accumulation by human gingival fibroblasts, we carried out a collagen accumulation assay as we have previously described and validated (Heng et al. 2006) in the presence or absence of added recombinant LOXL2 protein at different concentrations. Data (Fig. 3B) show that under these conditions, LOXL2 promotes collagen accumulation by human gingival fibroblasts.

LOXL2 Regulates Cell Proliferation upon Activation of PDGFR β by PDGF Ligand

Data suggest that LOXL2 activity is required for proliferation and subsequent collagen accumulation by primary gingival fibroblast cells. PDGF is a strong mitogenic factor. Moreover, PDGF has been reported to be highly elevated in human gingival overgrowth tissues (Iacopino et al. 1997). We therefore assessed whether LOXL2 could positively modulate PDGFR β -activating phosphorylation in human gingival fibroblasts. Human gingival fibroblasts were treated under serum-free conditions with 10 ng/mL PDGF-BB with or without 1 μ g/mL rLOXL2 and the novel LOXL2-selective inhibitor PXS-S1C at 1 μ M or with 500 μ M β -aminopropionitrile (BAPN), which inhibits all LOX isoforms. As shown in Figure 3C, data indicate that both inhibitors reduced PDGF-BB-induced activating phosphorylation of PDGFR β , whereas rLOXL2 added only in the presence of PDGF-BB upregulated ligand-induced receptor activation above that induced by PDGF-BB alone under these conditions of short-term PDGF-BB treatment. We conclude that LOXL2 enhances the response of human gingival fibroblasts to PDGF-BB.

LOXL2 Enhances Human Gingival Fibroblast Proliferation Mediated by PDGFR Signaling

To further confirm that LOXL2-enhanced proliferation is mediated by PDGFR signaling, the effect of a PDGFR inhibitor on DNA synthesis of LOXL2-stimulated cells was measured. First, the effect of 10 ng/mL PDGF-BB on gingival fibroblasts was measured in gingival fibroblasts obtained from 3 different individuals over a 24-h period. As shown in Figure 4A, PDGF-BB-stimulated gingival fibroblast DNA synthesis was attenuated by PDGFR inhibitor Tyrphostin AG1296 as expected. Interestingly, LOXL2 addition alone to cells for 24 h stimulated gingival fibroblast DNA synthesis to a lesser degree compared to PDGF-BB but was significantly attenuated by 5 μ M Tyrphostin AG1296 (Fig. 4B). Taken together, data support the hypothesis that LOXL2 optimizes the response of human gingival fibroblasts to endogenous PDGF ligands.

Discussion

Oral fibrosis occurs as a side effect of therapy with the antiseizure medication phenytoin and is characterized by accumulation of fibrogenic cells and collagens, with relatively little inflammation (Uzel et al. 2001). Other oral fibroproliferative disorders include oral mucositis progressing to oral fibrotic lesions observed as a consequence of radiation therapy for oral cancer and in response to smoking or betel nut chewing habits

Table. Amine Oxidase Inhibition Profile of PXS-S1C.

	LOX	LOXL1	LOXL2	LOXL3	LOXL4	DAO	MAO-A	MAO-B	SSAO
IC ₅₀ (μM)	8.6	1.9	0.076	0.23	0.098	3.8	>30	>30	8.2
pIC ₅₀	5.1	5.8	7.1	6.7	7.0	5.4	<4.5	<4.5	5.1
SD	0.16	0.33	0.09	0.14	0.082	0.12			0.19

Data are averages of at least 3 repeats. PXS-S1C is a small-molecule (molecular weight = 415 Da) mechanism-based inhibitor of lysyl oxidases of balanced polarity (logD [pH 7.4] = 0.3) and moderate protein binding (16% unbound in human plasma). Enzymes employed were those described in the "Lysyl Oxidase Inhibitor Specificity" section, and identities of each respective enzyme were confirmed by Western blotting. DAO, diamine oxidase; IC₅₀, concentration of inhibitor that results in 50% inhibition; LOX, lysyl oxidase; LOXL1, lysyl oxidase-like 1; LOXL2, lysyl oxidase-like 2; LOXL3, lysyl oxidase-like 3; LOXL4, lysyl oxidase-like 4; MAO-A, monoamine oxidase A; MAO-B, monoamine oxidase B; pIC₅₀, negative logarithm of half-maximal inhibitory concentration; SSAO, semicarbazide-sensitive amine oxidase.

(Murray et al. 2010; Sroussi et al. 2017). We have previously reported high levels of LOXL2 in human phenytoin-induced gingival overgrowth and here investigated the mechanisms by which LOXL2 could participate in the control of the phenotype of human oral fibroblasts and thereby promote fibrotic lesions. In both oral and nonoral tissues, high LOXL2 levels are strongly associated with a variety of cancers. For example, LOXL2 is highly associated with carcinoma of the breast and is upregulated in colon, gastric, esophageal squamous cell carcinoma, and oral squamous cell carcinoma (Kirschmann et al. 2002; Fong et al. 2007; Peinado et al. 2008; Peng et al. 2009; Barker et al. 2012; Li et al. 2012). LOXL2 expression is significantly upregulated in 4 types of hepatic cell carcinoma cell lines, and inhibition of LOXL2 expression in the SMMC-7721 cell line led to decreased cell growth (Lin and Chuang 2013). Interestingly, the mechanisms by which LOXL2 could contribute to cell proliferation were not identified. Here we have focused on investigating mechanisms by which LOXL2 could promote oral fibroblast proliferation as a way to assess for tissue-specific mechanisms that may be relevant to oral fibroproliferative disorders.

The present study demonstrated the presence of secreted LOXL2 and LOXL4 by primary gingival fibroblasts, while knockdown of only LOXL2 mimicked the attenuation of DNA synthesis by an inhibitor that blocks the enzyme activity of both LOXL2 and LOXL4. This finding emphasizes the specific importance of LOXL2 in promoting human gingival fibroblast proliferation. Our laboratory previously reported that lysyl oxidase activity is enhanced by CCN2/CTGF in gingival fibroblast cultures (Hong et al. 1999). Since lysyl oxidase mRNA levels were not regulated by CCN2/CTGF, this increased lysyl oxidase enzyme activity appeared to depend on lysyl oxidases derived from isoforms encoded by 1 or more members of the more recently discovered *LOXL* genes (Csiszar 2001). Here, human gingival fibroblasts cultured in the presence of added active LOXL2 significantly stimulated collagen

Figure 2. Lysyl oxidase like 2 (LOXL2) knockdown attenuates cell growth in primary human gingival fibroblast cells. Primary human gingival fibroblast cells were transduced with lentiviral particles containing LOXL2 short hairpin RNA (shRNA) 193 (A), LOXL2 shRNA 195 (B), or NonTarget shRNA as indicated. Western blots (C, D) demonstrate successful knockdown of LOXL2. Nontransduced cells were eliminated using puromycin. Transduced cells were then seeded at 5,000 cells per well in 24-well plates for the growth curve analysis. Cells from 3 independent donors were evaluated. Data are presented as means $\pm$ SEM, using 2-way analysis of variance ($P < 0.0003$) followed by post hoc Sidak's test to compare the means in each group at each time point, which are all significant ($*P < 0.05$) except for day 0.

accumulation, while specific LOXL2 inhibition blocked collagen accumulation. These studies taken together suggest that LOXL2 is the predominant functional lysyl oxidase isoform in oral fibroblasts.

PDGF may play a key role in fibrosis. Furthermore, studies have shown in different cells the critical role of PDGF-BB in promoting cell proliferation and matrix synthesis. For example, sustained delivery of PDGF-BB promoted cell proliferation and extracellular matrix synthesis in both vivo and in vitro flexor tendon fibroblasts in canine models (Thomopoulos et al. 2007). Studies presented here indicate that LOXL2 activity optimizes the proliferative response of primary human gingival fibroblasts to PDGF-BB. This results in both the proliferation of primary gingival fibroblast cells and increased collagen accumulation in primary gingival fibroblasts. LOXL2 down-regulation or inhibition of enzyme activity may ultimately provide a new avenue for treatment of oral fibrosis, not only by

Figure 3. Recombinant lysyl oxidase like-2 (rLOXL2) protein (**A**) promotes DNA synthesis, (**B**) increases collagen accumulation, and (**C**) enhances platelet-derived growth factor receptor β (PDGFR β) activation in primary human gingival fibroblast cultures. (**A**) Primary human gingival fibroblasts were treated with different concentrations of active rLOXL2 for 24 h. Purified human rLOXL2 employed was purchased from R&D Systems. Relative DNA accumulation was determined using the CyQuant assay. Results indicate that addition of active rLOXL2 upregulates cell proliferation in primary gingival fibroblasts. Data presented are means taken from 3 different subjects and 3 replicates per condition. Data are expressed as means $\pm$ SE ($n = 3$) and 1-way analysis of variance ($P < 0.001$), followed by Dunnett's multiple post hoc comparison test (* $P < 0.01$) showing significance between the no rLOXL2 control and each level of LOXL2 added. (**B**) Seven-day confluent human gingival fibroblasts were cultured for an additional 7 d, with 3 media changes, each in the continuous presence of 50 μ g/mL ascorbate containing either vehicle or human rLOXL2 enzyme at different concentrations. Purified human rLOXL2 employed was purchased from R&D Systems. Each treatment condition consisted of 3 wells of cells from each of 3 individual donors. Cultures were then fixed and stained with Sirius Red and the eluted dye samples quantitated by measuring absorbance at 540 nm as described (Heng et al. 2006). Sirius Red elutions (top) and quantitation of data (bottom). Data are presented as means taken from 3 different subjects and 3 replicates per condition per cell isolate. Data are expressed as means $\pm$ SE ($n = 3$) and 1-way analysis of variance ($P < 0.0002$), followed by Dunnett's multiple comparisons post hoc test compared to no added rLOXL2 (* $P < 0.02$). (**C**) Human gingival fibroblasts were cultured and treated with inhibitors or vehicles at the indicated concentrations for 1 h. Purified active lysyl oxidase like-2 (LOXL2) or vehicle was then added, followed by platelet-derived growth factor BB (PDGF-BB). rLOXL2 employed was purified from transduced CHO-K1 cells as indicated in the Methods section. Cell layers were extracted after 15 min and subjected to Western blotting for activating phosphorylation of PDGFR β . Data are from 1 subject's cells, performed 4 times with the same outcome. LOXL2 inhibition with the LOX family pan inhibitor β -aminopropionitrile (BAPN) and the LOXL2 selective inhibitor PXS-S1C at the indicated concentrations were found to reduce PDGF-BB-induced activating phosphorylation of PDGFR β , whereas rLOXL2 addition upregulated PDGF-BB-induced receptor activation. PDGFR β -P, blot probed with anti-PDGFR β phosphorylated at Tyr755; PDGFR-T, blot probed with anti-nonphosphorylated PDGFR β ; β -actin, blot probed with anti- β -actin as a loading control.

Figure 4. Platelet-derived growth factor BB (PDGF-BB) and lysyl oxidase like-2 (LOXL2) stimulation of human gingival fibroblast proliferation are mediated by platelet-derived growth factor receptor (PDGFR). Gingival fibroblasts from 3 different donors were cultured under serum-free conditions in the presence or absence of (**A**) PDGF-BB or (**B**) recombinant LOXL2 (rLOXL2) (1 μ g/mL) $\pm$ Tyrphostin AG1296 (5 μ M) for 24 h, and relative DNA accumulation was determined by CyQuant assays. Data are derived from 4 replicate assays per donor and are expressed as fold change of means compared to vehicle controls $\pm$ SE, $n = 3$, analysis of variance ($P = 0.0019$, panel A; $P = 0.0034$, panel B), followed by Tukey's multiple comparisons test (* $P < 0.02$), which are significant compared to all other groups (* $P < 0.02$).

reducing collagen cross-linking and deposition but also by attenuating PDGF-driven proliferation of the mesenchymal cells that ultimately produce fibrosis. Interestingly, this collaboration between LOXL2 and PDGF signaling is shared with LOX, in that PDGF signaling has previously been demonstrated to be optimized in the presence of active LOX in primary aortic smooth muscle cells and in megakaryocytes. This occurs by LOX oxidizing lysine residue(s) on PDGFR β , thereby enhancing the receptor response to PDGF ligands, although the specific lysine residues have not yet been identified (Lucero et al. 2008; Eliades et al. 2011). It will be of

importance to determine if members of the lysyl oxidase family synergize with other mitogenic or fibrogenic pathways or directly modulate the activity of other cell surface receptors by oxidizing critical receptor lysine residues to understand their critical role in fibrosis and cancer.

Author Contributions

D. Saxena, contributed to design, data acquisition, and analysis, drafted and critically revised the manuscript; F. Mahjour, contributed to design, data acquisition, and interpretation, drafted and critically revised the manuscript; A.D. Findlay, E.A. Mously, contributed to design and data acquisition, drafted and critically revised the manuscript; A. Kantarci, contributed to design and data analysis, drafted and critically revised the manuscript; P.C. Trackman, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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