THE ENZYMATIC ACTIVITY OF LYSYL OXIDASE LIKE-2 (LOXL2) IS NOT REQUIRED FOR LOXL2 INDUCED INHIBITION OF KERATINOCTYE DIFFERENTIATION

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Running title: Non-enzymatic activities of LOXL2

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Background: LOXL2 inhibits keratinocyte differentiation. This activity was thought to require LOXL2 enzyme activity.

Results: LOXL2 mutants lacking enzyme activity nevertheless inhibit keratinocyte differentiation. The 4th SRCR domain of LOXL2 is required for this activity.

Conclusion: LOXL2 induces keratinocyte differentiation independently of its enzyme activity.

Significance: Our results suggest that LOXL2 may affect diverse biological processes independently of its enzyme activity.

SUMMARY

Lysyl-oxidase like-2 (LOXL2) induces tumor progression and fibrosis. It also inhibits the differentiation of keratinocytes promoting development of squamous cell carcinomas. Stimulation of HaCaT skin keratinocytes with exogenous LOXL2 or over-expression of LOXL2 in these cells inhibits their differentiation as manifested by inhibition of calcium or vitamin-D induced involucrin expression. The inhibition was abrogated by the LOXL2 function blocking monoclonal antibody AB0023 as well as by an anti-LOXL2 polyclonal antibody. Surprisingly, a point mutated form of LOXL2 (LOXL2Y689F) lacking enzymatic activity, as well as a LOXL2 deletion mutant lacking the entire catalytic domain, also inhibited calcium or Vitamin-D induced up-regulation of involucrin expression, suggesting that the enzymatic activity of LOXL2 is not required for this activity. This conclusion was supported by experiments which showed that beta-amino-propionitrile (BAPN), an irreversible competitive inhibitor of the enzymatic activity of all lysyl-oxidases, is unable to abolish the LOXL2 induced inhibition of HaCaT cells differentiation. The activity of LOXL2Y689F required the presence of the fourth scavenger receptor cysteine rich (SRCR) domain of LOXL2 which is also the binding target of AB0023. Epitope tagged LOXL2Y689F was internalized at 37°C by HaCaT cells. The internalization was inhibited by AB0023 and by competition with unlabeled LOXL2, suggesting that these cells may express a LOXL2 receptor. Our results suggest that agents that inhibit the enzymatic activity of LOXL2 may not suffice to inhibit completely the effects of LOXL2 on complex processes that involve altered states of cellular differentiation.

INTRODUCTION

Lysyl-oxidase like-2 (LOXL2) was identified as an upregulated gene in Werner's syndrome (1). It belongs to the family of the lysyl-oxidases and like the other four members catalyses the deamination of the ε-amino groups of lysines of collagen monomers thereby promoting the formation of covalent cross-linkages (2,3). The catalytic domain of the lysyl-oxidases is highly conserved among lysyl-oxidases as is the lysyl-tyrosyl-quinone (LTQ) cofactor domain that is absolutely required for their enzymatic activity and is unique to these copper binding enzymes (2). The enzyme activity of LOXL2 can be inhibited, like the activity of all lysyl-oxidases, by the competitive
irreversible inhibitor β-amino-propionitrile (BAPN) (4;5). LOXL2 is an inducer of lung and liver fibrosis and also promotes fibrosis in LOXL2 expressing tumors, and these activities require the enzymatic activity of LOXL2 (6-8). LOXL2 as well as lysyl-oxidase (LOX) also functions as an inducer of tumor cells invasiveness (6;9;10). These activities too were found to be dependent on the enzymatic activity of the lysyl-oxidases. It was observed that lysyl-oxidase enhanced cross-linking of collagen in the tumor microenvironment can by itself promote tumor cells invasiveness (11). Furthermore, hydrogen peroxide produced as a side product of lysyl-oxidase activity can also induce tumor cells invasiveness (12). Additional studies revealed that tumor metastasis can be induced as a result of the oxidation of the transcription factor snail by intracellular LOXL2 which subsequently leads to the induction of epithelial to mesenchymal transition (EMT) (9;13;14). Thus, all the reported effects of the lysyl-oxidases have so far been associated with their enzymatic activity.

The progression of squamous cell carcinoma of the skin is associated with enhanced expression of LOXL2 (13). Cells of such tumors are derived from keratinocyte stem cell precursors and are characterized by aberrant differentiation as manifested by their reduced expression of differentiation markers (15). Silencing LOXL2 expression in keratinocytes up-regulates the expression of genes associated with keratinocyte differentiation (16), suggesting that the LOXL2 over-expression observed in squamous cell carcinomas may contribute to tumor progression either through inhibition of the differentiation of keratinocytes derived tumor cells or through enhancement of the proliferation which in turn inhibits differentiation (13). Interestingly, the expression of LOX and LOXL2 is inversely regulated in keratinocytes as a function of their state of differentiation even though both enzymes catalyze similar enzymatic reactions and even though both oxidize lysine residues of collagen (2;7). Thus, LOX expression is induced in differentiating keratinocytes while the expression of LOXL2 is inhibited (16). Furthermore, silencing LOX expression, in contrast to the silencing of LOXL2, impairs keratinocyte differentiation (17).

Enzymes such as heparanase have been recently found to induce biological responses by mechanisms that do not require enzyme activity (18). The N-terminal domain of LOXL2 contains four scavenger receptor cysteine rich (SRCR) domains of unknown function. These domains are implicated as mediators or protein-protein interactions in a variety of systems (19168, 19169). These observations suggest that the SRCR domains of LOXL2 may serve function in as yet unexplored roles of LOXL2. Here we provide evidence suggesting that the enzymatic activity of LOXL2 is not required for LOXL2 induced inhibition of keratinocyte differentiation and show that this activity of LOXL2 requires the presence of the 4th SRCR domain of LOXL2 (2).

Furthermore, we provide preliminary evidence suggesting that keratinocytes express a functional LOXL2 receptor on their cell surface.

**EXPERIMENTAL PROCEDURES**

**Materials:** DMEM, nonessential amino acids, trypsin-EDTA solution, fungizone and fetal bovine serum were from Biological Industries, (Beit Haemek, Israel). Cell culture plates were obtained from Corning Inc. (Corning, NY). PerfectPure RNA Cultured Cell kit for RNA purification was from 5Prime (Hamburg, Germany). Verso cDNA synthesis kit and Absolute™ Blue QPCR SYBR® Green Rox Mix were from Thermo Scientific (Lafayette, CO). The polyclonal LOXL2 antibody was described previously and was purified on protein-A sepharose as previously described (6). Mouse anti-myc (sc-40) was from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Mouse anti-human β-actin (A5316) and mouse anti-human involucrin (I9018) were from Sigma (St. Louis, MO, USA). The AB0023 monoclonal antibody was previously described (5;8). ATRA, 9-cis-retinoic acid (9-cis-RA), Troglitazone and Clofibrinic acid were purchased from Sigma-Aldrich (St. Louis, MO) and Vitamin D was purchased from Biomol (Hamburg, Germany). Dynabeads protein G were from Invitrogen (Carlsbad, CA). β-aminopropionitrile fumarate (BAPN) was from Sigma (St Louis, MO). Anti-myc antibody conjugated beads were from Enco Diagnostics (Petch Tikva, Israel). BamHI, KpnI, BglIII and Xhol were from New England Biolabs (Ipswich, MA). Lopofecamine 2000 was from Invitrogen (Carlsbad, CA)

**Cell culture.** HaCaT cells, were kindly provided by Dr. Fussenig (DKFZ, Heidelberg, Germany). The cells were cultured in MEM-Eagle's medium.
supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μg/ml penicillin/streptomycin (Biological Industries, Beit Haemek, Israel) supplemented with a low (0.03 mM) or high (1.2 mM) concentration of calcium. HEK293 and HEK293T cells were purchased from the American Type Culture Collection (Manassas, VA, USA).

**Plasmids and lentiviral vector:** The NSPI-CMV-myc lentiviral expression vector was previously described (21). LOXL2 shRNA constructs were previously described (22). Plasmids used for functional analysis of the LOXL2 promoter activity were generated using the pGL2 basic vector (Promega, Madison, WI) that contains a promoter less luciferase reporter gene.

**Isolation of the LOXL2 promoter and measurements of LOXL2 promoter activity:** In order to indentify the LOXL2 promoter, we cloned a ~3.2 Kb fragment of DNA located upstream to the translation start site of LOXL2. The first untranslated intron of LOXL2 which was previously found to contain a functional hypoxia response element (HRE) (23) was not included in this fragment (Fig. 1C) due to its size (~35 kb). Thus this fragment contains 300 bp located downstream to intron 1 and 2.9 Kb from the DNA region located upstream of intron 1 of LOXL2. This promoter fragment was inserted upstream to the luciferase reporter of the pGL2 plasmid and transfected into HaCaT cells using lipofecamine 2000 according to the manufacturer's instructions. Measurements of LOXL2 promoter activity using the firefly and renilla luciferase assay were preformed as described (24).

**Quantitative RT-PCR.** Cells (5x10⁴) were seeded in 6 well dishes and grown to confluence. Total RNA was isolated using the 5-Prime kit. CDNA was synthesized from 1μg of total RNA and random hexamers using the Verso cDNA synthesis kit (Thermo Scientific, Lafayette, CO). PCR amplification of cdNA was carried out using the Absolute™ Blue QPCR SYBR® Green Rox Mix (Thermo Scientific, Lafayette, CO) using an ABI Prism 7000 sequence detection system with oligonucleotide pairs specific for human LOXL2 (5'-GGCGTCACTGACTGCAAGCAC and 5'-CGAATCCGAATGTCCTCCAC), human involucrin (5'-TGTTTCTCCTCCAGTCAATA and 5'-GGTTGGATGGGACCTCCACT), human keratin-10 (5'-GGAAGAATCAAACTATGAGCTG and 5'-ATTGTCGATCTGAAGCGAGG) or β-actin (5'-TTGCGACAGGATGCAGAAGGA and 5'-AGGTGACAGGGGAGGCGAGAT). In order to measure the expression levels of endogenous LOXL2 mRNA we used another pair of primers of which one is derived from the 3' UTR of the LOXL2 mRNA 5'-TTTGTACATCTGACGCTG and 5'-GAGTGTGACAGGGGAGGCGAGCT. To ensure the specificity of the reaction conditions, at the end of the individual runs, the melting temperature (Tm) of the amplified products was measured to confirm its homogeneity. The following conditions were used: 50°C for 2 minutes, 95°C for 15 minutes, 95°C for 10seconds, and 60°C for 1 minute for a total of 40 cycles. Each sample was analyzed in duplicate. Amplified cDNA levels were normalized using the β-actin cDNA as a reference. Products were resolved on a 2% agarose gel to confirm amplification the identity of the amplified cDNA. The target gene expression level was calculated by the 2-ΔΔct method (25). In experiments in which the expression is shown as percent of control the control was always the expression level of the gene of interest in untreated cells cultured for 3 days in the presence of high or low calcium concentrations as indicated.

**Western blot analysis:** Cell lysis and Western blot analysis were performed as described previously (26).

**Inhibition of LOXL2 expression:** ShRNA targeting LOXL2 (22) were generated in HaCaT cells using lentiviral vectors as previously described (27).

**Production and purification of Recombinant LOXL2 variants:** HEK293 or HaCaT cell lines expressing either LOXL2myc, LOXL2Y689F or various deletion mutants of LOXL2Y689F were generated using lentiviral vectors as previously described (27). Some of these recombinant LOXL2 species were purified from serum free conditioned medium as follows: HEK293 cells expressing LOXL2myc were grown to 70% confluence and incubated for 48 hrs with serum free DMEM medium. Conditioned medium (200 ml) was incubated with 1 ml of anti-myc conjugated beads for 24 h at 4 °C. The beads were then collected and washed with 20 volumes of PBS. LOXL2 was eluted with ammonium-hydroxide (0.1 M). Acetic-acid (1 M) was used to neutralize the eluant back to pH-7.
**Determination of LOXL2 enzyme activity:** The fluorimetric amplex red assay was performed essentially as described (28). Purified LOXL2_myc or LOXL2_Y689F (2.5 µg) or a corresponding volume of a similarly purified fraction from control conditioned medium were added to a reaction mix (10mM K₂HPO₄ pH 7.2, 10µM amplex red and 0.1 units Horseradish peroxidase). The substrate was a lysine rich PF4-derived peptide LYKKIKIKKLLES that we identified as a good LOXL2 substrate. This peptide was added to a final concentration of 20 µM in a final reaction volume of 100 µl. The enzymatic reaction was carried out at 37°C for 4h. Fluorescence was measured with excitation at 540 and emission at 580 nm.

**Generation of deletions in the LOXL2 SRCR and catalytic domains:** The cDNA encoding LOXL2_Y689F was used as a template for the generation of the depicted SRCR and catalytic domain deletions (Fig. 4A). For each deletion the cDNA upstream and downstream of the deletion was amplified using PfuUltra II fusion HS DNA polymerase (Stratagene, Santa Clara, CA) and the primer pairs shown (Supp. Table 1). Amplified fragments upstream and downstream from the deletions were joined using the expand long template PCR system (Roche, Mannheim, Germany), sequenced, and stitched together essentially following a previously described protocol (29). The cDNA containing the deletion was then ligated into the NSPI-CMV-myc lentiviral expression vector.

**LOXL2 internalization assay.** HaCaT cells were seeded in 10 cm dishes (16x10⁴ cells/dish) and cultured in medium containing a high calcium concentration over night. The following day the cells were supplemented with 4 µg/ml of LOXL2_myc or with LOXL2_myc that was pre-incubated for 30 min at 4°C with AB0023. After 30 min incubation at 37°C or 4°C, cells were trypsinized, washed and lysed at 4°C using western blot lysis buffer. The lysate was mixed with anti-myc antibody (200 µg/ml) for 2 hrs at 4°C. LOXL2_myc was then precipitated from the lysate using protein-G magnetic beads. The beads were washed for 3 times with PBS, boiled with SDS/PAGE, and subjected to western blot analysis using anti-myc antibodies to detect internalized LOXL2_myc.

**Proliferation assay:** HaCaT cells were seeded in 24 wells dishes (5x10⁴ cells/well) and cultured in medium containing a high calcium concentration. Wells received either no additions, elution buffer (17 µl/ml, the same volume in which LOXL2 was added), LOXL2_myc (5 µg/ml) or LOXL2_Y689F (5 µg/ml). Added factors were replenished every other day. Adherent cells were counted every other day using a coulter counter.

**Statistical analysis:** The one tailed unpaired Welch's correction student's T-test was used. Error bars represent the standard error of the mean. Statistical significance is presented in the following manner: *p<0.05, **p<0.01 and ***p<0.001. All the experiments were performed independently three times in triplicate unless otherwise stated in the figure legend. The variation between triplicates in experiments was less then 10%

**RESULTS**

The expression of LOXL2 in HaCaT cells is regulated by inducers of keratinocyte differentiation and high levels of LOXL2 inhibit the differentiation of these cells: The HaCaT cell line is a spontaneously transformed non-tumorigenic human epithelial cell line derived from adult skin, which maintains full epidermal differentiation capacity. It undergoes differentiation when exposed to calcium or to additional inducers of keratinocyte differentiation such as vitamin-D which is manifested by the up-regulation of the expression of keratinocyte differentiation markers such as involucrin, keratin-10 or filaggrin (30). HaCaT cells express the LOXL2 mRNA when cultured in medium containing a low calcium concentration (Fig. 1A). The expression of the LOXL2 mRNA is strongly down regulated when the cells are cultured in medium containing a high calcium concentration or when stimulated with vitamin-D (Fig. 1A). Likewise, the expression of LOXL2 protein is also down regulated by calcium (Fig 2B). We have also cloned a 3 kb fragment containing part of the LOXL2 promoter (Fig. 1C). This DNA fragment was able to down-regulate the expression of a luciferase reporter gene fused downstream to it when HaCaT cells transfected with this construct were exposed to high calcium concentrations or when they were stimulated with vitamin-D, suggesting that the decreased LOXL2 mRNA concentrations found in differentiating HaCaT
cells are due to inhibition of LOXL2 transcription (Fig. 1D). As expected, stimulation of HaCaT cells with high calcium concentrations or with vitamin-D upregulated the expression of the keratinocyte differentiation marker involucrin (Fig. 1E). However, HaCaT cells over-expressing recombinant LOXL2 tagged at the C-terminal with a myc epitope tag (LOXL2myc) (7) failed to up-regulate involucrin expression in response to either calcium, vitamin-D, or to several other known inducers of keratinocyte differentiation (Fig. 1F) (31). As far as we could tell, the addition of the myc epitope tag did not alter the biological properties of LOXL2 (6;22). In contrast, inhibition of endogenous LOXL2 expression in HaCaT cells cultured in the presence of low calcium concentrations by using a specific shRNA (Fig. 2A) (22) induced the expression of involucrin (Fig. 2B), suggesting that endogenously produced LOXL2 functions in keratinocytes as a gate keeper that inhibits differentiation regardless of the nature of the external stimuli. The induction of differentiation following the expression of this shRNA was not due to up regulation of LOX or other lysyl-oxidases since the expression levels of other lysyl-oxidases did not change significantly in response to this shRNA (Supp. Fig. 1B). Interestingly, the LOXL2 function blocking antibody AB0023 was also able to induce involucrin mRNA expression in HaCaT cell cultured in medium containing low calcium concentrations, suggesting that the differentiation inhibiting effect of LOXL2 was induced by secreted extracellular LOXL2 (Fig. 2C). This last conclusion was supported by another experiment in which we observed that addition of purified LOXL2myc to HaCaT cells cultured in the presence of high calcium concentration also inhibits the up regulation of involucrin. In this experiment too addition of AB0023 or addition of a polyclonal antibody directed against LOXL2 (6) inhibited the effect of LOXL2myc on involucrin expression suggesting that the inhibitory effect was transduced by extracellular LOXL2myc (Fig. 2D).

**The enzymatic activity of LOXL2 is not required for the inhibition of HaCaT differentiation:** It was previously observed that the LOXL2 function inhibiting antibody AB0023 inhibits the effects of LOXL2 on tumor progression more efficiently than BAPN (8) even though at saturating concentrations BAPN inhibits the enzymatic activity of LOXL2 "in-vitro" enzyme assays more efficiently than AB0023 (5), possibly because AB0023 targets the 4th SRCR domain of LOXL2 rather than the catalytic domain (2;5). These observations suggest that some of the biological activities of LOXL2 may not require its enzymatic activity. This hypothesis is strengthened by the results of an experiment showing that addition of BAPN to HaCaT cells cultured in the presence of low calcium concentrations fails to alleviate the LOXL2 induced repression of involucrin expression while in contrast addition of AB0023 is able to abrogate LOXL2 induced inhibition of involucrin expression (Compare Figs. 2D and 2E). The failure to restore involucrin expression was not due to cytotoxicity since the cells proliferated normally in the presence of BAPN (data not shown). This experiment also suggests that the enzymatic activity of LOXL2 may not be required for LOXL2 induced repression of involucrin expression.

In order to further examine this hypothesis, we introduced a point mutation into the conserved LTQ domain of LOXL2myc to generate LOXL2Y689F (Fig. 3A). This mutation was based on previous work showing that mutation of a conserved tyrosine residue required for the formation of the LTQ of LOX results in complete loss of enzymatic activity (32). Indeed, we found that LOXL2Y689F also lacks enzymatic activity (Fig. 3B). The expression of LOXL2myc or LOXL2Y689F in HaCaT cells did not affect significantly the expression levels of endogenously produced LOXL2 mRNA (Supp. Fig. 1A). However, in HaCaT cells cultured in the presence of high calcium concentrations expression of recombinant LOXL2Y689F inhibited the expression of the involucrin mRNA as well as the expression of the cytokeratin-10 mRNA (Figs. 3C & Supp. Fig. 1C). Furthermore, purified LOXL2Y689F that was added to HaCaT cells cultured in the presence of a high calcium concentration was able to inhibit the expression of involucrin mRNA and protein as efficiently as non-mutated LOXL2myc (Figs. 3D & 3E). Addition of LOXL2myc to the growth medium of HaCaT cells enhanced the number of adherent cells in medium containing high calcium concentrations (Fig. 3F), either because LOXL2 inhibits their differentiation or because LOXL2 promoted their proliferation directly. However, we could not detect LOXL2 induced cyclin-D1 expression in the cells nor was LOXL2 able to induce the phosphorylation of ERK1/2 (data not shown). Since loxl2 did not appear to stimulate
proliferation, we conclude that reduced differentiation in the presence of LOXL2 accounts for the apparent increase in cell numbers, since the shedding of fully differentiated cornified envelopes from the culture well will be reduced. In this experiment too LOXL2Y689F enhanced the accumulation of the cells as efficiently as LOXL2myc (Fig. 3F).

Even though the enzymatic activity of LOXL2Y689F seemed to be completely inhibited (Fig. 3B), we thought it possible that maybe some residual activity still remained, and that this residual activity may be sufficient to inhibit calcium induced expression of involucrin in HaCaT cells. To exclude this possibility we produced a deletion mutant of LOXL2Y689F that completely lacks the catalytic domain (LOXL2Y689F/-cat) (Fig. 4A). Despite the lack of a catalytic domain, expression of recombinant LOXL2/-cat in HaCaT cells cultured in the presence of high calcium concentrations was able to efficiently inhibit the expression of involucrin (Fig. 4B).

**Inhibition of involucrin expression by LOXL2 requires the presence of the fourth SRCR domain of LOXL2:** The experiments described above suggested that the enzymatic activity of LOXL2 is not required for LOXL2 induced inhibition of keratinocyte differentiation. These results suggested that other LOXL2 domains such as one of the SRCR domains may be important for that activity. To examine this possibility we produced deletion mutants of LOXL2Y689F lacking one or several SRCR domains as depicted (Fig. 4A). We then expressed the deletion mutants in HaCaT cells and determined which of these mutated LOXL2 variants are able to inhibit calcium induced induction of involucrin in HaCaT cells. These experiments indicated that inhibition of involucrin expression in HaCaT depended on the retention of the fourth SRCR domain. Loss of this fourth SRCR domain resulted in the loss of the ability to inhibit involucrin expression while the loss of any of the other SRCR domains did not affect that ability (Figs. 4C & 4D). Interestingly, AB0023, which is also able to inhibit LOXL2myc induced inhibition of involucrin expression (Fig. 2D) also binds to the 4th SRCR domain of LOXL2 (5). Thus, the finding that AB0023 also inhibits LOXL2myc induced inhibition of involucrin expression independently supports the identification of that domain as the domain that mediates LOXL2 induced inhibition of keratinocyte differentiation.

**LOXL2myc is internalized by HaCaT cells and the internalization is inhibited by an excess of unlabeled LOXL2:** The realization that inhibition of keratinocyte differentiation can be inhibited by the addition of exogenous, enzyme-dead LOXL2, suggested that LOXL2 may bind to a cell membrane anchored LOXL2 receptor that transduces its inhibitory signals. We hypothesized that if such a receptor exists, it may also internalize LOXL2 in analogy with other receptors (33;34). To examine this possibility we incubated HaCaT cells at 37°C or at 4°C with LOXL2myc. After 30 minutes we treated the cells with a mixture of proteases so as to digest any LOXL2myc exposed on the cell surface, and lysed the cells in the presence of protease inhibitors following washing to remove excess proteases. This experiment revealed that LOXL2myc is internalized by the cells, and that the internalization occurs only at 37°C (Fig. 5A). Interestingly the internalization could be inhibited by AB0023 as well as by incubation with conditioned medium containing unlabeled LOXL2, suggesting that the internalization of LOXL2myc may be mediated by a saturable receptor (Fig. 5B). Further experiments will be required in the future in order to identify this putative LOXL2 receptor.

**DISCUSSION**

The studies in which the mechanisms by which lysyl-oxidases induce fibrosis, tumor invasion and tumor metastasis were examined have found that for all of these activities the enzyme activity of the lysyl-oxidases was strictly required (9;11-13). In agreement, it was also reported that oxidation of the transcription factor snail by intracellular LOXL2 is part of the mechanism by which LOXL2 inhibits the differentiation of keratinocytes and promotes the progression of dermal malignancies such as squamous cell carcinoma of the skin (13;16).

A few observations nevertheless suggest that LOXL2 may exhibit non-enzymatic activities. The AB0023 monoclonal antibody targeting the 4th SRCR domain of LOXL2 was found to be a better inhibitor of tumor development than the irreversible general lysyl-oxidase enzyme inhibitor BAPN. Since this antibody also functions as a partial inhibitor of the enzyme activity it was thought that allosteric inhibition in the in-vivo
environment may be more efficient than direct competition for the substrate binding site by BAPN (8). However, the possibility that the 4th SCRC domain may participate directly in the induction of such LOXL2 induced functions was not investigated.

Mutation of a critical tyrosine residue in the LTQ domain of lysyl-oxidase results in complete loss of lysyl-oxidase activity (32). To determine if LOXL2 has non-enzymatic functions we introduced a similar point mutation into LOXL2 to generate LOXL2Y689F, resulting in an apparently complete loss of enzymatic activity. Nevertheless, the inhibitory effect that LOXL2 exerts on the differentiation of HaCaT keratinocytes as measured by the inhibition of the calcium induced expression of the keratinocyte differentiation marker involucrin remained unaffected by the mutation. To circumvent the possibility that the mutation may not have completely inhibited the enzyme activity we have also produced an LOXL2Y689F variant that in addition to the mutation lacks the entire catalytic domain. However, this twice "dead" LOXL2 mutant was also able to inhibit calcium induced induction of involucrin expression by HaCaT cells further suggesting that LOXL2 inhibits involucrin expression in HaCaT cells independently of its enzymatic activity.

AB0023 inhibited the effect of LOXL2Y689F on involucrin expression, suggesting that inhibition of involucrin expression by LOXL2Y689F was mediated by the 4th SCRC domain of LOXL2 which is the LOXL2 domain targeted by AB0023 (5). Indeed, the only LOXL2Y689F deletion mutants that lost their ability to inhibit involucrin expression were the ones that lacked the 4th SCRC domain, strongly suggesting that inhibition of involucrin expression by LOXL2 in HaCaT cells depends on the presence of this domain. It is intriguing that similar domains are also found in LOXL3 and Loxl4, suggesting that these lysyl-oxidases too may exhibit non-enzymatic activities and this possibility will need to be further examined.

LOXL2Y689F was also able to inhibit involucrin expression when it was added to the growth medium of the HaCaT cells, suggesting the existence of a mechanism able to transduce LOXL2 signals from the extracellular space into the cells. Indeed, our experiments indicate that HaCaT cells may express a signal transducing LOXL2 receptor on their cell surface. We found that LOXL2Y689F is internalized by HaCaT cells and that the internalization of an epitope tagged LOXL2 is can be inhibited by an excess of unlabeled LOXL2. This observation indicates that the internalization is mediated by a specific high affinity LOXL2 receptor. This possibility is also supported by experiments which showed that AB0023 can inhibit the internalization of LOXL2, lending further support for this hypothesis. The identity of this putative receptor as well as the signaling cascades that it may trigger in response to LOXL2 binding will have to be examined in greater detail in the future.
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FIGURE LEGENDS

**FIGURE 1.**

*LOXL2 inhibits involucrin expression induced by keratinocyte differentiation inducing factors: (A)*

HaCaT cells were cultured in medium containing low or high calcium concentrations. Some low calcium dished received in addition vitamin-D (10^{-8}M) as indicated. After 10 days endogenous LOXL2 mRNA levels were measured using real time qPCR. The mRNA levels of cells cultured 10 days in the presence of high calcium concentrations or in the presence of vitamin-D were compared with the levels of LOXL2 mRNA expression in cells cultured for 10 days in the presence of low calcium concentrations for 10 days. **(B)** Cell lysates were prepared from HaCaT cells cultured in low and high Ca+2 after 3 or 10 days as indicated. Aliquots containing equal protein concentrations were subjected to Western blot analysis using polyclonal anti-LOXL2 antibodies. Blots were then stripped and probed for β-actin. **(C)** Picture showing the structure of the LOXL2 promoter fragment used. The large intron containing the hypoxia response element (23) downstream to untranslated exon-1 is not included in the promoter fragment. **(D)** The luciferase activity induced by the LOXL2 promoter in HaCaT cells In response to high calcium concentrations or in response to 10^{-8}M vitamin-D was measured. The Y axis represents luciferase activity normalized to a renilla control. The data shown represent mean values of two independent experiments performed in triplicate. Means were compared to the low Ca++ control. **(E)** The effects of calcium and vitamin-D on involucrin mRNA expression were measured as described under A for LOXL2 mRNA expression. Means were compared to the high Ca++ control. **(F)** HaCaT cells cultured in medium containing high calcium concentrations were infected or not with empty lentivirus expression vector (NSPI) or with lentiviruses directing expression of LOXL2myc (LOXL2) as indicated. Some dishes containing cells infected with lentiviruses directing expression of LOXL2myc were supplemented with 10^{-8}M vitamin-D (Vit D), 2 μM ATRA (ATRA), 20 μM Troglitazone or 2 μM 9-cis-RA or 400 μM clofibrinic acids. The differentiation inducers were added every other day. Involucrin mRNA expression was measured after 10 days using real time qPCR and normalized to β-actin mRNA expression.

**FIGURE 2.**

*The lysyl-oxidase enzyme activity inhibitor BAPN fails to neutralize the inhibitory effect of LOXL2 on involucrin expression: (A)*

HaCaT cells were cultured in low calcium medium and infected with lentiviruses expressing a non-targeting shRNA (Shc) or with lentiviruses expressing a shRNA directed against LOXL2 (shLOXL2) for 10 days. The LOXL2 mRNA concentrations in the cells were then measured by real time qPCR. Means were compared to the low Ca++ (10 days) control. **(B)** The expression of involucrin mRNA was measured in HaCaT cells expressing a non-targeting shRNA (Shc) or a LOXL2 targeting shRNA (sh-LOXL2) as described under A for the LOXL2 mRNA. **(C)** HaCaT cells were cultured in medium containing low calcium concentrations in the absence of any additions (No-Add), in the presence of 5 μg/ml of a control monoclonal antibody that binds to the enzymatic site of LOXL2 but does not inhibit the enzymatic activity (AB0050) or in the presence of 5 μg/ml of a LOXL2 neutralizing antibody (AB0023) which were replenished every other day. The relative concentrations of the involucrin mRNA were measured using real time qPCR as described. Means were compared to the control. **(D)** HaCaT cells were cultured in medium containing high calcium concentrations in the absence of any additions (No-Add), 5 μg/ml LOXL2myc (LOXL2), 20 μg/ml AB0023 or 20 μg/ml of a purified anti-LOXL2 polyclonal antibody (αLOXL2). Some of the wells the received LOXL2 also received antibodies as indicated. Added proteins were replenished every other day. After 10 days the concentration if involucrin mRNA was determined by real time qPCR as described. Means were compared to the control. **(E)** HaCaT cells were cultured in medium containing high or low calcium concentrations as indicated for 10 days. Some of the low calcium wells received in addition BAPN (0.2 mM). Media and BAPN were replenished every other day. After 10 days involucrin mRNA expression levels were determined using real time qPCR. Means were compared to the high Ca++ control.
**FIGURE 3.**

*An enzyme dead LOXL2 point mutant inhibits the differentiation of HaCaT cells.* (A) A schematic presentation showing the location of the Y689F point mutation introduced into the LOXL2 LTQ domain. (B) The enzymatic activity of affinity purified LOXL2<sub>myc</sub>, LOXL2<sub>Y689F</sub> or of purified conditioned medium derived from control cells infected with empty lentiviruses and purified identically (Empty vector) were determined using the amplex red assay as described in experimental procedures. The data shown represent mean values of two independent experiments preformed in duplicate. Means were compared to the wild type LOXL2 Control. (C) HaCaT cells were cultured in medium containing high calcium and infected with empty lentiviruses (Empty vector) or lentiviruses directing expression of LOXL2<sub>myc</sub> or LOXL2<sub>Y689F</sub> as indicated. After 10 days involucrin mRNA levels were determined using real time qPCR. Means were compared to the empty vector control. (D) HaCaT cells were grown in medium containing high calcium concentration without additions (No-add), or in medium supplemented with elution buffer (control), purified LOXL2<sub>myc</sub> (LOXL2) or LOXL2<sub>Y689F</sub> (LOXL2<sub>Y689F</sub>). Added factors were replenished every other day. After 10 days involucrin mRNA levels were determined using real time qPCR. Means were compared to the control. (E) Cell lysates were prepared from cells treated as described in Fig. 3D. The presence of involucrin complex and involucrin precursor in the cell lysates was determined by western blot analysis. (F) HaCaT cells were seeded in 24 well plates (5x10<sup>3</sup> cells/well) in the presence of high calcium concentration. Wells received either no additions (No add), elution buffer (Vehicle), LOXL2<sub>myc</sub> (5 µg/ml) or LOXL2<sub>Y689F</sub> (5 µg/ml). Added factors were replenished every other day. Adherent cells were counted every other day using a coulter counter. Means were compared to the low LOXL2 and LOXL2<sub>Y689F</sub> controls respectively on day 7.

**FIGURE 4.**

The non-enzymatic effects of LOXL2 on the differentiation of HaCaT cells are mediated by the 4<sup>th</sup> SRCR domain of LOXL2. (A) A schematic presentation of the catalytic domain deletion mutant derived from LOXL2<sub>Y689F</sub> and of the various SRCR deletion mutants of LOXL2<sub>Y689F</sub>. (B, C and D) HaCaT cells grown in medium containing high calcium concentrations were infected with empty lentiviruses (Empty vector) and with lentiviruses directing expression of LOXL2<sub>Y689F-cat</sub>. (B) or lentiviruses directing expression of various LOXL2<sub>Y689F</sub> deletion mutants lacking various SRCR domains as indicated (C & D). The cells were cultured in 6 well plates for 10 days. The data shown represent mean values of two independent experiments preformed in triplicate. The expression of involucrin mRNA was then determined using real time qPCR. Means were compared to the empty vector control.

**FIGURE 5.**

Evidence for a putative LOXL2 receptor of HaCaT cells. (A) HaCaT cells were cultured in medium containing high calcium concentration to confluence in 10 cm dishes. They were then incubated with or without purified LOXL2<sub>myc</sub> as indicated for half an hour at 4°C or at 37°C. At the end of the incubation the cells were washed and trypsinized to remove cell surface associated LOXL2<sub>myc</sub>. The cells were then lysed and lysate aliquots containing equal amounts of protein analyzed by western blot using an anti-myc antibody to detect LOXL2<sub>myc</sub>. (B) HaCaT cells were cultured as described above. Upon reaching confluence the medium was exchanged with conditioned medium (high calcium) derived from HEK293 cells infected with empty lentiviruses (lane 1), with fresh HaCaT growth medium containing high calcium (lanes 2, 3), or conditioned medium from HEK293 cells producing recombinant untagged LOXL2 (lane 4). LOXL2<sub>myc</sub> (2 µg/ml) or AB0023 antibody (20 µg/ml) were added as indicated. After a 30 min. incubation at 37°C the cells were trypsinized, lysed and LOXL2<sub>myc</sub> in cell lysates visualized as described under Fig. 5A.
Figure-1

A

B

C

D

E

F

LOXL2 mRNA expression (% of control)

LOXL2+VIT D

LOXL2

Actin

Involucrin mRNA expression (% of control)

LOXL2

Actin

Luminescence (arbitrary units)

Exon 1

ATG

~ 3 Kb Promoter fragment (Without intron-1)

High Ca++

Low Ca++

High Ca++ + Vit-D

Low Ca++

High Ca++ + Vit-D

High Ca++

Low Ca++

High Ca++ + Vit-D

High Ca++

Low Ca++

High Ca++ + Vit-D

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High Ca++ + Vit-D

High Ca++

Low Ca++

High Ca++ + Vit-D

High Ca++

Low Ca++

High Ca++ + Vit-D

High
Figure-3

A

LOXL2

1 2 3 4 Y689F

Scavenger receptor cysteine rich domain (SRCR)
Copper binding domain
LTQ domain
Catalytic domain

B

Fluorescence (580 nm)
(Arbitrary units)

Empty vector
LOXL2
LOXL2_Y689F

C

High Ca^{++}

Involucrin mRNA expression (% of control)

Empty vector
LOXL2
LOXL2_Y689F

D

High Ca^{++}

Involucrin mRNA expression (% of control)

No add.
Vehicle
LOXL2
LOXL2_Y689F

E

Empty Vector LOXL2 wt LOXL2_Y689F

Involucrin complex
Involucrin precursor
Actin

F

High Ca^{++}

Cells/Well

No add.
Vehicle
LOXL2
LOXL2_Y689F

Days after seeding

0 3 5 7
Figure-4

A

B

C

D

Involucrin mRNA expression (% of control)

High Ca++

Involucrin mRNA expression (% of control)

High Ca++

Involucrin mRNA expression (% of control)

High Ca++
Figure 5

A

<table>
<thead>
<tr>
<th>LOXL2/myc:</th>
<th>+</th>
<th>-</th>
<th>+</th>
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<tr>
<td>Temp. (°C):</td>
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<td>37</td>
<td>4</td>
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B

<table>
<thead>
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<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Additions:</td>
<td>-</td>
<td>-</td>
<td>AB0023</td>
<td>LOXL2</td>
</tr>
</tbody>
</table>

[Image showing gel electrophoresis with bands labeled LOXL2/myc]
The enzymatic activity of lysyl oxidase like-2 (LOXL2) is not required for LOXL2 induced inhibition of keratinocyte differentiation
Jennie Lugassy, Shelly Zaffryar-Eilot, Sharon Soueid, Amit Mordoviz, Victoria Smith, Ofra Kessler and Gera Neufeld

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