The Lysyl Oxidase LOX Is Absent in Basal and Squamous Cell Carcinomas and Its Knockdown Induces an Invading Phenotype in a Skin Equivalent Model

Charbel Bouez,[1,3] Caroline Reynaud,[3] Emmanuelle Noblesse,[1] Amélie Thépot,[1] Claudine Gleyzal,[3] Jean Kanitakis,[2] Eric Perrier,[4] Odile Damour,[1,3] and Pascal Sommer[3]

Abstract Lysyl oxidase initiates the enzymatic stage of collagen and elastin cross-linking. Among five isoforms comprising the lysyl oxidase family, LOX is the better studied. LOX is associated to an antitumor activity in *ras*-transformed fibroblasts, and its expression is down-regulated in many carcinomas. The aim of this work was to shed light on LOX functions within the epidermis by studying its expression in human basal and squamous cell carcinomas and analyzing the effect of its enzymatic activity inhibition and protein absence on human keratinocytes behavior in a skin equivalent. In both carcinomas, LOX expression by epidermal tumor cells was lacking, while it was up-regulated around invading tumor cells in association with the stromal reaction. Lysyl oxidase activity inhibition using β-aminoproprionitrile in a skin equivalent model prepared with both primary human keratinocytes and HaCaT cell line affected keratin 10 and filaggrin expression and disorganized the collagen network and the basement membrane. In spite of all these changes, no invasion phenotype was observed. Modelization of the invasive phenotype was only noticed in the skin equivalent developed with LOX antisense HaCaT cell line, where the protein LOX is specifically absent. Our results clearly indicate that lysyl oxidase enzymatic activity is essential not only for the integrity maintenance of the dermis but also for the homeostasis of the epidermis. Moreover, LOX protein plays a role in the skin carcinomas and invasion but not through its enzymatic activity.

Lysyl oxidase gene family comprises five members acting as extracellular modulating enzymes: *LOX*, *LOXL*, *LOXL2*, *LOXL3*, and *LOXL4* (1). The first identified and the better-studied isoform of this family is LOX. It is a copper-dependent amine oxidase that initiates the covalent cross-linking of collagens and elastin in extracellular matrices (ECM; refs. 2, 3). LOX is secreted as a glycosylated proenzyme, processed by procollagen C-proteinase into a mature active form. LOX activity modulation, due to either an increase or a decrease in its expression, induces multiple effects on the structure and major characteristics of the ECM. LOX is essential in maintaining the characteristics of blood vessels and arteries, where its activity modulation is correlated to atherosclerosis (4), aneurism (5), and human arterial dissection (6) as well as to Alzheimer disease (7). LOX down-regulation is correlated to many connective tissue disorders seen in Ehler-Danlos syndrome, cutis laxa, and Menke's syndrome (8–10). In tumors, *LOX* up-regulation is found in the stromal reaction observed around tumor foci in ductal breast carcinomas (11) and in bronchopulmonary carcinomas (12).

LOX might also have a role on nonmesenchymal cells. Indeed, LOX expression has been recently associated to differentiated keratinocytes in a skin equivalent model (13). Besides, LOX, which is expressed in healthy skin (13, 14), has not been detected in tumor cells from invading ductal breast carcinomas (11) in primary and metastatic prostate cancer cells (15). LOX is severely down-regulated in gastric carcinomas (16–18), in esophagus carcinomas (18), in head and neck squamous cell carcinomas (19), and in bronchogenic carcinomas (20). Controversially, LOX might be up-regulated in certain highly invasive/metastatic human breast cancer cell lines but not in poorly invasive/nonmetastatic breast cancer cells (21). Other lysyl oxidase isoform expression might be modified, such as the lysyl oxidase-like (LOXL), which is also present in keratinocytes (13). LOXL has been recently shown to be down-regulated in renal cell carcinomas (22). LOXL2 mRNA expression is reduced in head and neck squamous cell carcinomas (19), whereas LOXL4 mRNA is overexpressed in such carcinomas (23), and LOXL3 is overexpressed in highly

Authors' Affiliations: [1]Laboratoire des Substituts Cutanés and [2]Clinique dermatologique, Hôpital E. Herriot; [3]Institut de Biologie et de Chimie des Protéines, Centre National de la Recherche Scientifique, Université Lyon 1, Institut Fédératif de Recherches 128; and [4]Coletica, Lyon, France

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Requests for reprints: Odile Damour, Laboratoire des Substituts Cutanés, Hôpital Edouard Herriot, Pavillon I, 5 place d'arsonval, 69437 Lyon Cedex 03, France. Phone: 33-4-7211-0618; Fax: 33-4-7211-0675; E-mail: odile.damour@chu-lyon.fr.

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metastatic breast cancer (24). A recent report indicated the implication of LOXL2 and LOXL3 in carcinoma progression (25). The study of the gene expression signatures and biomarkers of noninvasive and invasive breast cancer cells led to the identification of some differentially expressed genes, such as *LOX* (26).

LOX gene has been correlated with the *ras* recision gene (*rrg*) in fibroblasts, a putative tumor suppressor isolated from nontumorigenic revertants of *ras*-transformed fibroblasts that was subsequently identified as *LOX* (27). It has been first suggested that this tumor suppressor activity is due to the formation of a dense stromal reaction modulating the cell phenotype (11, 12, 28). Then, it has been proposed that LOX works also intracellularly, as it can display a nuclear localization in smooth muscle cells and fibroblasts and can be taken off from outside the cells (29). This intracellular action of LOX in fibroblasts might involve the regulation of cyclin D1 by a catenin-dependent pathway, or the activation of the nuclear factor-κB pathway through the regulation of phosphatidylinositol 3-kinase and Akt kinases (30–32). Nevertheless, the initial mechanism explaining the tumor suppressor activity of LOX in fibroblast is yet unknown. Recent work reported that the lysyl oxidase propeptide itself directly stimulates phenotypic reversion of *ras*-transformed cells (33).

The aim of the present study is to understand the function of LOX in the epidermis. We therefore investigated whether LOX expression is modified in human basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). The implication of lysyl oxidase enzymatic activity was analyzed using a skin equivalent model, where global lysyl oxidase enzymatic activity was inhibited using β-aminoproprionitrile. LOX protein implication in skin cancer was also studied using HaCaT keratinocyte cell lines knocked down for LOX expression and used in the skin equivalent for epidermization.

Materials and Methods

Cancer specimen selection. Surgical skin samples were selected from a collection provided by the dermatology department of the Hôpital Edouard Herriot (Lyon, France). The cutaneous carcinomas selected for this study were BCC and SCC because of their difference in growing state and their invasiveness capacities (15 specimens for each tumor type). Both of these cancers are keratinocytes derived malignant neoplasms. The surgical specimens were fixed in 10% buffered formalin or in Bouin fixative and embedded in paraffin for histologic evaluation and immunoperoxidase staining.

Cell cultures. Keratinocytes and fibroblasts were isolated from human foreskin. Fibroblasts were grown in DMEM (with Glutamax-1, Life Technologies, Cergy Pontoise, France) supplemented with 10% FCS (HyClone, Logan, UT), 20 μg/mL gentamicin (Panpharma, Fougères, France), 100 IU/mL penicillin (Sarbach, Suresnes, France), and 1 μg/mL amphotericin B (Bristol Myers Squibb, Puteaux, France). For the skin equivalent, keratinocytes were grown in a 3:1 mixture of DMEM and Ham's F12 (Life Technologies), respectively, supplemented with 10% FCS (HyClone), 10 ng/mL epidermal growth factor (Austral Biologic, San Ramon, CA), 0.12 IU/mL insulin (Lilly, Saint-Cloud, France), 0.4 μg/mL hydrocortisone (UpJohn, St. Quentin en Yvelines, France), 5 μg/mL triiodo-L-thyronine (Sigma, St. Quentin Fallavier, France), and 24.3 μg/mL adenine (Sigma). For HaCaT monolayer culture, cells were cultured in DMEM supplemented with 10% FCS, 2 mmol/L L-glutamin, and 50 mg/mL gentamicin. HaCaT cells (34) were kindly provided by Dr. Arrigo (University Lyon I).

Preparation of LOX antisense HaCaT cell lines. For the preparation of the human antisense LOX construct, the −8 to +597 human LOX sequence was cloned into the *Kpn*I/*Eco*RI sites of pcDNA3 (Invitrogen, Grand Island, NY), in reverse orientation relative to the cytomegalovirus promoter. Transfection of HaCaT cell lines were made using LipofectAMINE (Invitrogen), according to the manufacturer's protocol. Ten antisense LOX clones (asLOX HaCaT) and three vehicle vector clones (VV HaCaT, transfected with pcDNA3) were selected by 1,500 μg/mL G418 disulfate salt treatment (Sigma-Aldrich, St. Louis, MO) for 3 weeks then maintained in 400 μg/mL G418 disulfate salt containing media. Genomic construct insertion was verified by PCR. The growth curve for both cell lines was obtained over a culture period of 7 days. In brief, stable transfectants were seeded at 15×10^4 cells per cell culture Petri dish, then trypsinized, and counted in duplicate at the indicated times after seeding.

Dermal and skin equivalent preparation. The skin equivalent was prepared as reported (13). Briefly, foreskin fibroblasts were seeded at a density of 250,000 cells/cm^2 onto a dermal substrate made of chitosan-cross-linked collagen-GAG matrix prepared as previously described by Duplan-Perrat et al. (35). This dermal equivalent was grown for 14 days at 37°C in a 5% CO_2 atmosphere. The fibroblast medium was supplemented with 50 μg/mL L-ascorbic acid (Sigma) and changed every day. Keratinocytes cells were seeded on the dermal equivalent on day 14, at a density of 250,000/cm^2. After 7 days of submerged culture in the keratinocyte medium, the skin equivalent was elevated at the air-liquid interface and cultured in a simplified keratinocyte medium (13). The skin equivalent samples were harvested at 35 days of culture following fibroblasts seeding (2×3 samples of each condition). In parallel, primary keratinocytes were replaced by either wild-type HaCaT (WT HaCaT), VV HaCaT, or asLOX HaCaT cell lines for epidermization. These latter skin equivalents were maintained in 400 μg/mL G418 containing media after epithelial cell seeding. When necessary, β-aminoproprionitrile (monofumarate salt, Sigma), the classic inhibitor of the lysyl oxidase activity (2), was added at the concentration of 200 μg/mL to the culture medium from the first medium change after fibroblast seeding on the dermal substrate. The IC_{50} of β-aminoproprionitrile was evaluated at 751.2 μg/mL for fibroblasts and 652.3 μg/mL for keratinocytes (evaluation by cell counting). The skin equivalent prepared with these three cell types were harvested at 30 or 37 days of culture following fibroblast seeding (2×3 of each condition).

Histologic and immunohistologic procedures. The skin equivalent were frozen or fixed in Bouin's fixative or in 10% formalin solution (Sigma) and embedded in paraffin. Five-micrometer sections were deparaffinized and whitened in glycine-HCl (100 mmol/L). Besides the homemade anti-LOX antibody (36), the following primary antibodies were used: mouse anti-filaggrin (Biomedical Technologies, Stoughton, MA), mouse anti-K10 (Novocastra, Newcastle upon Tyne, United Kingdom), mouse anti-collagen type IV, and mouse anti-collagen type VII (Sigma). The antibodies were used at the following dilution: 1:1,000 anti-$LOX^{222\text{-}356}$, 1:100 anti-filaggrin, 1:50 anti-K10, and 1:200 anti collagen type IV and VII. Secondary fluorescein-conjugated antibodies were goat antimouse and goat antirabbit used at 1:100 (Sanofi Diagnostics Pasteur, Marne-la-Coquette, France). Peroxidase-conjugated goat anti-rabbit IgG (DAKO, Trappes, France) were used to detect the immunocomplexes, using diaminobenzidine as substrate (DAKO). Counter staining was done using Harris's hematoxylin (Sigma). For controls, the primary antibody was omitted. Multiple serial sections of each specimen were processed to ensure representative samples. Only the invasion foci occurring in the central part of the skin equivalents were counted in three different skin equivalent lots of three samples each (Table 1).

Electronic transmission microscopy procedures. Samples were fixed in 2% glutaraldehyde/0.1 mol/L Na Cacodylate/HCl at pH 7.4 for 1 hour at 4°C, then post- fixed in 1% OsO_4/0.15 mol/L Na Cacodylate/HCl at pH 7.4 for 1 hour at 4°C, dehydrated in graded methanol,

Table 1. Average of invasion foci detected in skin equivalents, at days 16 and 23

	Invasion foci at day 16	Invasion foci at day 23
WT HaCaT	0	0
VV HaCaT	0	0
asLOX HaCaT	2 ± 1	4 ± 1

NOTE: The skin equivalent prepared with WT HaCaT, VV HaCaT, and asLOX HaCaT cells. Only the invasions occurring in the central part of the skin equivalents were taken into consideration. The results presented thereby represent the average of invasion foci by skin sample, counted in three different skin equivalent lots of three samples each.

and embedded in Epon. Ultrathin sections were cut with a LKB Ultratome V, contrasted with methanolic uranyl acetate (8%) and lead citrate buffer, and then examined on a JEOL 1200 EX electron microscope.

Results

LOX is absent in tumor cells from BCC and SCC. This study was carried out on sample sections from BCC and SCC to determine the global expression of LOX in these nonmelanoma skin carcinomas. The immunohistochemical detection allowed the identification of a clear LOX expression pattern: (*a*) LOX expression by tumor cells was lacking; (*b*) LOX expression was decreasing in epidermis at proximity of tumor foci; and (*c*) a strong expression of LOX was observed in relation to the stromal reaction surrounding invading tumor cells.

As previously described for the human skin and for the skin equivalent (13), LOX staining decorated the keratinocyte periphery and formed a framework around juxtaposed cells in nonpathologic conditions (Fig. 1A and B). This pattern was found in the healthy part of the skin of both BCC (Fig. 1C and D) and SCC (Fig. 1E). However, the most prevalent situation was an absence or a poor labeling of keratinocytes within the epidermis close to tumor cells of BCC (Fig. 1C and D) and SCC (Fig. 1E and H). Tumor cells from both BCC (Fig. 1C and D) and SCC (Fig. 1E-I) were not stained for LOX expression. LOX expression could be observed sometimes within the bulk of tumor foci, but this might rather correspond to the residual expression of stromal cells embedded within the tumor foci (Fig. 1D).

Interestingly, when the section passed through a budding focus of cells clearly proliferating from the epidermis, the decrease of LOX labeling could be observed from the differentiated part to the zone of nondifferentiated cells (Fig. 1E-G).

BCC and SCC displayed a stromal reaction very positive for LOX around tumor foci (Fig. 1C and E). LOX immunolabeling strongly highlighted the fibrillar framework of this reaction and could discriminate the tumor-stroma interface in areas of late densely organized desmoplasia (Fig. 1D and I), although this labeling could be absent in many regions distant from the tumor foci (Fig. 1C, E, and G). LOX is also present below the dermal-epidermal junction (Fig. 1H).

Taken together, these observations indicated that the tumor cells from epidermal origin are losing the expression of LOX, in the invading cells of both BCC and SCC, whereas the mesenchymal cells are reacting around the tumor cells by overexpressing LOX, allowing the formation of a dense stroma around tumors.

Effect of β-aminoproprionitrile lysyl oxidase activity inhibition on the structure and development of the skin equivalent. LOX was present both in the epidermis and the dermis of the normal human skin (Fig. 2A) and nontreated skin equivalent (Fig. 2B). In the dermis, LOX was detected in association with the ECM (Fig. 2A and B). Staining was more intense in the upper part of the dermis although weak beneath the dermal-epidermal junction. In the epidermis, LOX staining was restricted to the basal and spinous layers. LOX staining decorated the periphery of keratinocytes and formed a framework around juxtaposed cells (Fig. 2A and B).

To have a better understanding of LOX role in the skin, a study was achieved by studying the effect of global lysyl oxidase inhibition on the development of the skin equivalent. Treatment with 200 μg/mL β-aminoproprionitrile during 35 days had multiple effects on the dermis, the dermal-epidermal junction, and the epidermis.

In the dermis, β-aminoproprionitrile treatment allowed the formation of a skin equivalent displaying a correct global organization (Fig. 2C). We observed, by immunohistology, a well-organized dermis colonized by the fibroblasts, drowned in

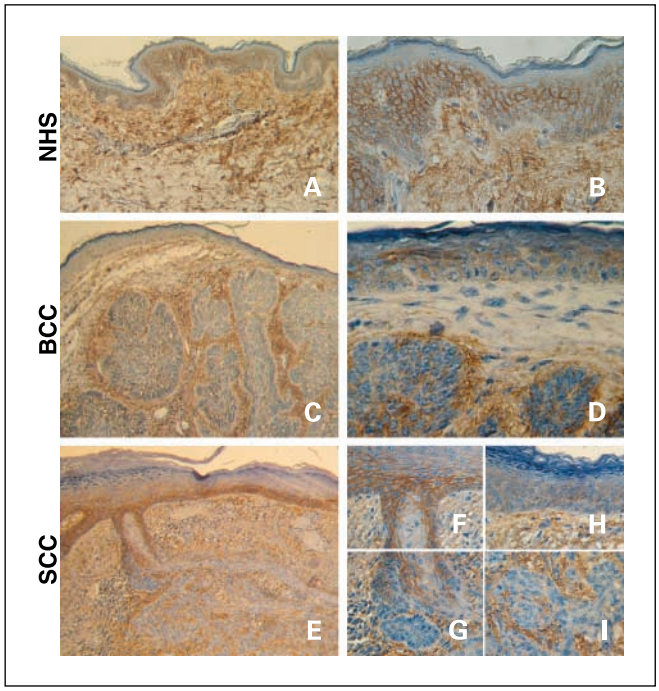

Fig. 1. Immunohistological detection of LOX in normal and tumoral human skin. LOX detection in normal human skin (*NHS; A* and *B*), in human BCC (*C* and *D*), and SCC (*E-I*). Immunodetection was made on paraffin-embedded sections using the anti-LOX antibody. The immunolabeling was indicated by a brown deposition, and the counterstaining gives the tissue the blue color. In normal human skin, LOX was localized in the dermis and the epidermis (*A* and *B*). In BCC, LOX labeling is negative in the tumor cells and highlights the fibrillar framework of concomitant stroma reaction (*C*). Keratinocytes of nonhyperproliferative regions are still expressing LOX (*C*). Labeling was obtained at the cell periphery and formed a framework around juxtaposed cells. LOX detection could be observed within some tumor foci, but this expression was not associated to tumor cells (*D*). In SCC, LOX is also not detected in tumor cells and labeled the stromal reaction around tumor foci (*E* and *I*). The epidermis irregularly expressed LOX, pericellularly as in healthy epidermis (*E*) or weakly and diffusely within the cytoplasm (*F*). This expression progressively decreased until disappearance in the keratinocytes associated to tumor invasion (*F* and *G*). Magnification, ×10 (*A, C*, and *E*), ×25 (*B, F, G, H*, and *I*), ×40 (*D*).

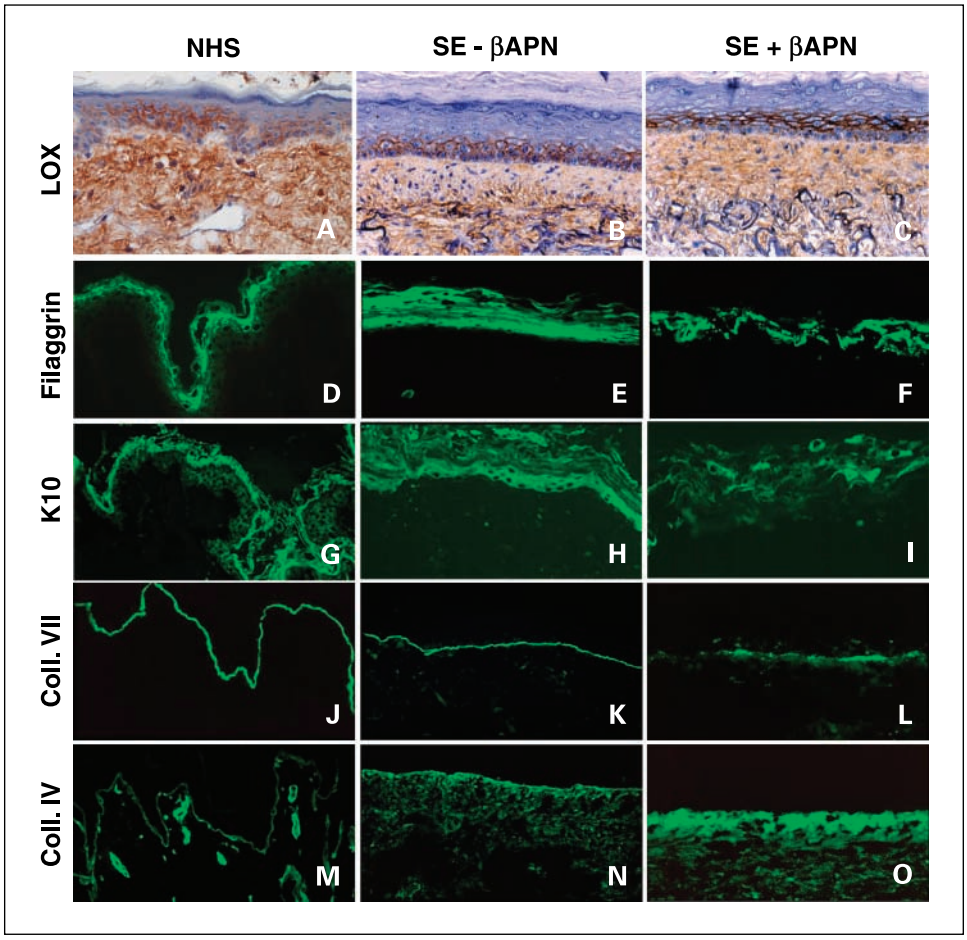

Fig. 2. Immunohistochemical analysis of the skin equivalent (*SE*) treated or not with β-aminoproprionitrile (*β-APN*). Immunodetection of LOX (*A-C*), filaggrin (*D-F*), keratin K10 (*G-I*), collagen type VII (*J-L*) and collagen type IV (*M-O*) were done on sections of NHS (*A, D, G, J*, and *M*), and skin equivalent treated or not with 200 μg/mL β-aminoproprionitrile (*B, C, E, F, H, I, K, L, N*, and *O*). LOX expression profile was not modified by the β-aminoproprionitrile treatment (*A-C*). In contrast, filaggrin disappeared locally in the upper part of the epidermis of the treated skin equivalent (*E-F*). The keratin 10 (*K10*) is expressed at different levels of the differentiating epidermis in the nontreated skin equivalent (*H*) but was sporadically expressed by keratinocytes of the suprabasal layers in the skin equivalent treated with 200 μg/mL β-aminoproprionitrile (*I*). Collagens IV and VII defined the well-formed basal lamina underneath the epidermis of nontreated skin equivalent (*K* and *N*). Once treated by β-aminoproprionitrile, the fine lining delineated by the immunodetection disappeared or was diffusely expressed (*L* and *O*). The detection was obtained by immunoperoxidase labeling for LOX and by indirect immunofluorescence for the other markers. Paraffin-embedded sections were treated with Bouin fixative for LOX detection, with a hematoxylin counterstaining that labels the nuclei. The chitosan-cross-linked collagen-GAG matrix appears at the bottom of the sections. Magnification, ×25 (*A-C*), ×10 (*D-O*).

their own neosynthesized ECM. The pattern of LOX expression was not modified (Fig. 2C). Elastin, fibrillin 1, and collagen type I and III expressions were not modified (data not shown). However, at the ultrastructural level, after 35 days, the β-aminoproprionitrile treatment induced striking modifications of the collagen fiber shapes, which seemed less regular in size and shape with fusion of adjacent collagen fibers (Fig. 3A and B).

At the dermal-epidermal junction level, β-aminoproprionitrile exerted an effect on the overall structure of the basement membrane. The basement membrane still expressed collagen VII (Fig. 2J, K, and L) and collagen IV (Fig. 2M, N, and O), but the detection is diffuse with a loss of the lamellae organization obtained in the control. At the ultrastructural level, the fine and well-defined *lamina densa* synthesized under the basal keratinocytes (Fig. 3C) was replaced by a disorganized layer in the presence of β-aminoproprionitrile (Fig. 3D), appearing like a fibrous and disorganized deposit. Hemidesmosomes, which were well present in normal skin equivalent, could also be observed in the treated skin equivalent although more rarely (Fig. 3C and D).

In the epidermis, the overall organization of the epidermal layers was conserved, in basal, spinous, and granulosa layers as seen by immunohistology (Fig. 2C). Keratinocytes were still associated in different layers throughout the epidermis, with well-formed desmosomal bonds comparing with the nontreated skin as observed at the ultrastructural level (Fig. 3E and F). The expression of Ki67, a proliferation marker of the basal layer, transglutaminase, and involucrin, was not affected, whereas E-cadherin network was scarcely disrupted (data not shown). In contrast, the expression of filaggrin was strikingly affected. It seemed weaker and disorganized (Fig. 2D, E, and F). Moreover, the differentiation marker K10 was sporadically expressed by keratinocytes of the suprabasal layers, in contrast to nontreated control (Fig. 2G, H, and I).

Therefore, although heavily affecting the dermis, epidermis, and the dermal-epidermal junction, lysyl oxidase activity inhibition was not sufficient to induce the invasive of keratinocytes in the dermis as observed in the BCC and SCC.

Development of skin equivalent with antisense LOX HaCaT cell lines. To specifically study the role of LOX protein within the epidermis of the skin equivalent, we developed skin equivalent with HaCat cell lines knocked down for LOX expression. The absence of LOX expression in monolayer culture was checked by Western blot and PCR (data not shown). The resulting asLOX HaCaT showed no anchorage independence as judged by their inability to grow on agarose gel and to invade the Matrigel (data not shown). Nevertheless, these cells present a higher rate of proliferation, reaching almost twice the rate of the control cell lines (VV HaCaT) after 1 week of culture (Fig. 4).

Before studying asLOX HaCaT cell lines behavior in the skin equivalent model, we first verified how LOX-expressing HaCaT cells (WT HaCaT or VV HaCaT cells) behave on the skin equivalent model, treated or not with β-aminoproprionitrile.

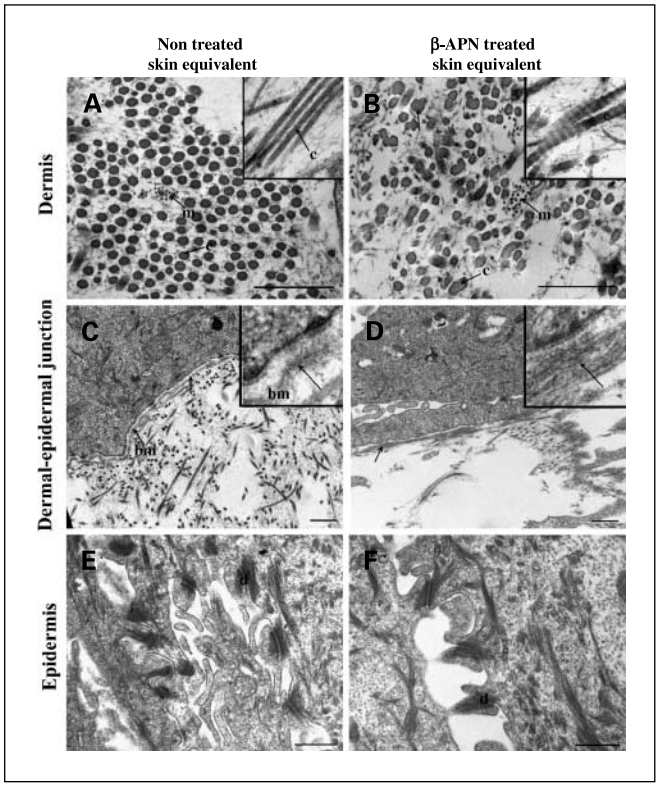

Fig. 3. Transmission electron microscopy of the skin equivalent on day 35 without (*A, C*, and *E*) or with β-aminoproprionitrile (*β-APN*) treatment (*B, D*, and *F*). Groups of densely packed and regular collagen fiber bundles are disposed in parallel around microfibrils in the nontreated skin equivalent (*A*). The treatment with 200 μg/mL β-aminoproprionitrile induced a striking modification of the collagen fiber, which are irregular in shape and displayed fusion between adjacent fibers (*B*). The well-defined basement membrane synthesized under the basal keratinocytes (*C*) was replaced by a disorganized layer in the presence of β-aminoproprionitrile (*D*). Neither the structure of the hemisdesmosomes (*C*) nor desmosomes (*E*) observed in the nontreated skin equivalent were modified by the β-aminoproprionitrile treatment (*D, F*). bm, basement membrane; c, collagen; m, microfibrils; d, desmosomes. Bar, 200 nm.

Skin equivalent prepared with WT HaCaT cells, untreated (not shown) or treated with β-aminoproprionitrile (Fig. 5A), displayed an organized multilayered epidermis, although not well differentiated as judged by the low expression of filaggrin and involucrin (data not shown).

In the skin equivalent without β-aminoproprionitrile treatment, the dermal-epidermal junction was well structured with normal deposition of collagen type IV, collagen type VII, and laminin, and the dermis was quite similar to normal skin equivalent as indicated by elastin, fibrillin 1, and collagen type I and III expression pattern (data not shown). In contrast, the skin equivalent made with VV HaCaT, without (Fig. 5B) and with β-aminoproprionitrile (Fig. 5C), displayed a nearly absent ECM deposition due to the growth impeding of fibroblasts by G418 treatment. In spite of this lack of true dermal-epidermal junction and dermal ECM, a true multilayer epidermis was built where no effect was detected.

The invasion was absent in the upper mentioned skin equivalent controls prepared with the wild-type cells or vehicle vector–transfected cells, in the absence and in presence of β-aminoproprionitrile (Fig. 5A-C), where LOX expression was not modified. The striking observation came from the asLOX HaCaT skin equivalent, where invasion of the dermis by keratinocytes was noticed (Fig. 5D). The immunohistochemical staining showed no LOX presence in the invading keratinocytes, whereas a very low expression in the noninvasive epidermis was observed (Fig. 5F), comparing with the VV HaCaT skin equivalent (Fig. 5E).

In view of all, inhibition of LOX expression but not inhibition of global lysyl oxidase activity induces an invading phenotype for keratinocyte in the skin equivalent.

Discussion

LOX has been reported not only to be involved in collagen and elastin cross-linking but also to have a tumor suppressor activity (2, 37). LOX has been discovered as a suppressor of the *ras* oncogene activity in fibroblasts (27, 28). LOX expression was down-regulated in immortalized rat fibroblasts after transformation by activated H-*ras* (38, 39). Other investigations reported a decrease of lysyl oxidase activity due to a decrease of LOX synthesis in malignantly transformed human cell lines (40), or LOX somatic mutations in colon cancer (41), or reduction of LOX mRNA expression in head and neck squamous cell carcinomas (19) and gastric cancer (16, 17). In addition, we confirm here the absence of LOX in both BCC and SCC, expanding the role of LOX absence in cancer progression.

LOX activity decreases in several malignantly transformed human cell lines is due to low quantities of LOX mRNA and a low transcription level (42). The *LOX* gene also identified as a target for the antioncogenic transcription factor IRF-1, contributing to the multistep process of malignant transformation (43). The *LOX* gene is located in chromosomal region 5q23 (41, 44), which is known to be deleted at a high frequency in many different types of cancer (45, 46). It has been already pointed out that loss of heterozygosity and LOX promoter methylation were detected in up to 30% in gastric cancers (17).

The mechanisms affected by loss of LOX expression remain to be investigated. A historical explanation for a role of LOX in cancer has been based on its extracellular function on collagen. More recently, a second explanation of LOX implication in cancer comes from its intracellular presence. Indeed, it has been

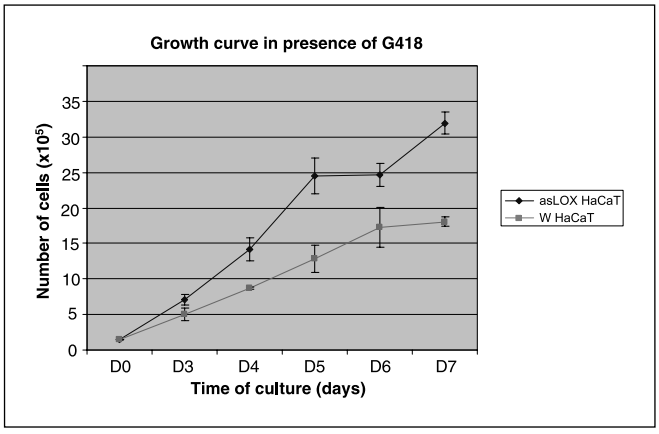

Fig. 4. Growth curve of asLOX HaCaT and VV HaCaT cells in presence of G418. The proliferation rate of asLOX HaCaT and VV HaCaT cell lines was assessed by the growth curve obtained after 7 days of culture. Cells were seeded in culture dishes at a 2×10^5 per dish. At the designated days, each cell line was trypsinized and counted in duplicate. The growth curve indicated a very high proliferation rate of asLOX HaCaT cells comparing with VV HaCaT cells. The maximum difference is observed after 7 days of culture.

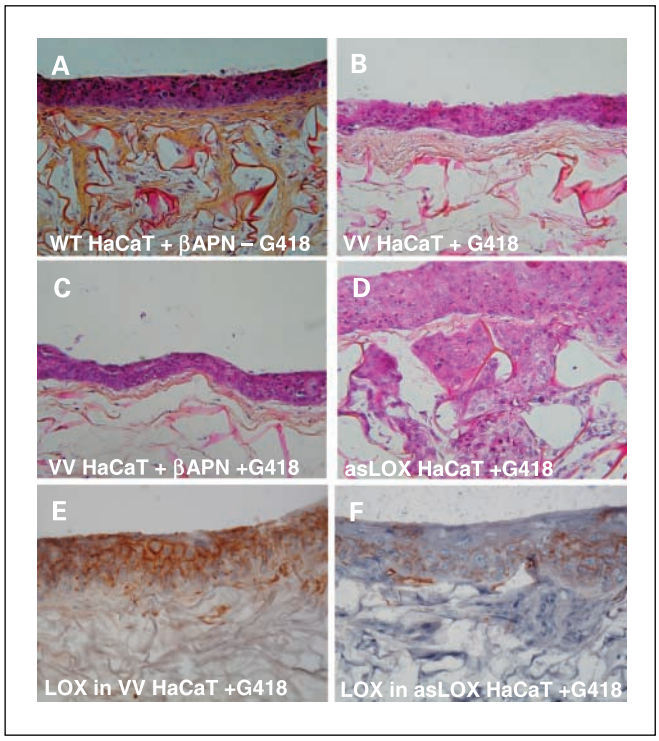

Fig. 5. Histologic analysis of the skin equivalent prepared with keratinocyte cell lines. H&E staining of skin equivalent at 35 days prepared with HaCaT (*A-D*). The skin equivalent was prepared with HaCaT cell lines seeded on human DE: WT HaCaT cells in the presence of β-aminoproprionitrile (*β-APN*) and absence of G418 (*A*), VV HaCaT cells in the presence of G418 (*B*), VV HaCaT cells in the presence of β-aminoproprionitrile and G418 (*C*), and asLOX HaCaT cells in the presence of G418 (*D*). Several invading cells were only noted in skin equivalent made with asLOX HaCaT cells. Besides the violet-colored invading cells, the chitosan-cross-linked collagen-GAG matrix appears also in violet. Immunoperoxidase labeling of LOX in the skin equivalent prepared with VV HaCaT cells (*E*) and with asLOX HaCaT cells (*F*). LOX staining, lower than in the control (*E*), was observed in asLOX HaCaT cells throughout the epidermis while the invading cells were not labelled (*F*). Magnification, ×20 (*A-D*), ×40 (*E* and *F*).

shown that extracellular LOX was able to enter into the cytosol and became concentrated into the nuclei of smooth muscle cells (29). This intracellular uptake was not altered by β-aminoproprionitrile and therefore was independent of LOX catalytic activity (47). The intracellular effect of LOX in fibroblasts has been further confirmed, involving the regulation of cyclin D1 by a catenin-dependent pathway, or the activation of the nuclear factor-κB pathway through the regulation of phosphatidylinositol 3-kinase and Akt kinases (30–32). To this regard, the pericellular observation of LOX in suprabasal keratinocytes and its disappearance in SCC and BCC constitute a model of reference to study its role within cells. Indeed, neither collagen nor elastic fibers have been observed at the same location in the epidermis, although the expression of elastin itself and elastic fiber–associated proteins have been already reported (13, 48). Therefore, other substrates should be involved. It is very likely that LOX may have intracellular substrates, even nuclear ones, which remain to be determined. For instance, it has been determined that basic fibroblast growth factor (49) or histone H1 or H2 can be substrates for LOX (50), although physiologic substrates need to be identified. One hypothesis that can be raised to explain this issue is that the LOX protein might display other functions, not related to the strict oxidative desamination inhibited by β-aminoproprionitrile and previously reported by Nellaiappan et al. (47). Indeed, LOX pro-region itself induces phenotypic reversion of *ras*-transformed fibroblasts (33). This hypothesis is reinforced here by the differences observed between the effect of the activity inhibition and the protein absence in the skin equivalent model. The invasion is only observed in the second case, although the activity inhibition induced basement membrane disruption and deregulation of filaggrin and K10 expression. The mechanisms that trigger such invasiveness are yet unknown, and further investigations are required for instance to study the gelatinolytic activity and its implication. They are not necessarily related to the basement membrane fragility or disorganization seeing that this invasion is absent in the control skin equivalent (VV HaCaT skin equivalent).

It should be noted that asLOX HaCaT cell lines invaded the dermis in the skin equivalent but were not growing in Matrigel. This discrepancy might be correlated to the presence of fibroblasts in the skin equivalent. In some cases, the presence of fibroblasts in the collagen matrix significantly increased cell proliferation and invasion in a neoplastic cell line model (51). Moreover, direct contact of fibroblasts and keratinocytes has been found to induce interleukin-1 in keratinocytes. Interleukin-1 stimulates the expression of keratinocytes growth factor and interleukin-6 in fibroblasts, inducing epithelial proliferation in a paracrine fashion (52). In our skin equivalent model, the direct contact of both cell types occurs after keratinocytes seeding. This contact might be enough to trigger the invasion in our model especially in the presence of a fragile basement membrane. Moreover, we noted an increase of LOX expression around invading cells in the BCC and SCC samples, reflecting in contrast the putative defensive role of LOX, and the subsequent collagen cross-linking in hypertrophic scar-like stromal reaction has been inferred from its expression pattern in various *in situ* epithelial cancers (11, 12, 15). This hypothesis has defined a defensive role for the neo-formed connective tissue barrier, which might be considered as a limitation against invasiveness (53). In contrast, little or no LOX expression was found within the stromal reaction of invasive carcinomas, in breast and lung cancers (11, 12, 54). This latter situation includes the decrease of LOX expression, an uncoupling with collagen synthesis, and an increase of stromal proteases that favor tumor cell dispersion and growth through non–cross-linked and loose collagen matrices (55). Such situations (*in situ* and invasive) were encountered in both BCC and SCC tumors, with a variability that might reflect dynamics of the balance between host defense and tumor progression.

In conclusion, the lack of LOX protein in tumor cells originating from keratinocytes is clearly associated to BCC and SCC skin cancers. The inhibition of the lysyl oxidase enzymatic activity in the skin equivalent induces multiple disorganization of the basement membrane and the dermis, preparing a phenotype favorable to tumor development. The loss of LOX protein adds another step towards invasion. Overall, in keratinocytes, LOX is required to maintain the homeostasis and further work is now required to identify the partners of LOX in keratinocytes.

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