Calcif Tissue Int (2009) 85:119–126
DOI 10.1007/s00223-009-9252-8

LABORATORY INVESTIGATIONS

Lysyl Oxidase (*Lox*) Gene Deficiency Affects Osteoblastic Phenotype

N. Pischon · J. M. Mäki · P. Weisshaupt · N. Heng · A. H. Palamakumbura · P. N'Guessan · A. Ding · R. Radlanski · H. Renz · T. A. L. J. J. Bronckers · J. Myllyharju · A. M. Kielbassa · B. M. Kleber · J.-P. Bernimoulin · P. C. Trackman

Received: 19 November 2008 / Accepted: 16 April 2009 / Published online: 21 May 2009
© Springer Science+Business Media, LLC 2009

Abstract Lysyl oxidase (LOX) catalyzes cross-linking of elastin and collagen, which is essential for the structural integrity and function of bone tissue. The present study examined the role of *Lox* gene deficiency for the osteoblast phenotype in primary calvarial osteoblasts from E18.5 *Lox* knockout ($Lox^{-/-}$) and wild type (*wt*) (C57BL/6) mice. Next to *Lox* gene depletion, mRNA expression of Lox isoforms, LOXL1–4, was significantly downregulated in $Lox^{-/-}$ bone tissue. A significant decrease of DNA synthesis of $Lox^{-/-}$ osteoblasts compared to *wt* was found. Early stages of osteoblastic apoptosis studied by annexin-V binding as well as later stages of DNA fragmentation were not affected. However, mineral nodule formation and osteoblastic differentiation were markedly decreased, as revealed by significant downregulation of osteoblastic markers, type I collagen, bone sialoprotein, and Runx2/Cbfa1.

Keywords Lysyl oxidase · LOXL1–4 · Knockout · Osteoblast

N. Pischon (✉) · P. Weisshaupt · N. Heng · A. M. Kielbassa · B. M. Kleber · J.-P. Bernimoulin
Department of Operative Dentistry and Periodontology, CharitéCentrum 3, University School of Dental Medicine, Charité – Universitätsmedizin Berlin, Aβmannshauserstraβe 4-6, 14197 Berlin, Germany
e-mail: nicole.pischon@charite.de

J. M. Mäki · J. Myllyharju
Oulu Center for Cell-Matrix Research, Biocenter Oulu, Department of Medical Biochemistry and Molecular Biology, University of Oulu, Oulu, Finland

A. H. Palamakumbura · P. C. Trackman
Department of Oral Biology and Periodontology, Goldman School of Dental Medicine, Boston University, Boston, MA, USA

P. N'Guessan
Department of Infectious Diseases and Pulmonology, Charité – Universitätsmedizin, Berlin, Germany

A. Ding · R. Radlanski · H. Renz
Department of Experimental Dental Medicine, Charité – Universitätsmedizin, Berlin, Germany

T. A. L. J. J. Bronckers
Department of Oral Cell Biology, ACTA, Vrije Universiteit, Amsterdam, The Netherlands

Type I collagen is the principal constituent of extracellular bone matrix and a crucial determinant for mechanical properties of bone tissue [1, 2]. Posttranslational collagen modifications result in the formation of a mature functional matrix, which is essential for subsequent matrix mineralization [3–6]. Lysyl oxidase (protein-lysine 6-oxidase, LOX) is a copper-dependent enzyme that initiates cross-linking of collagen and elastin by catalyzing oxidative deamination of ε-amino groups of lysine and hydroxylysine residues [3, 7]. Several LOX isoforms, lysyl oxidase-like proteins 1–4 (LOXL1–4) have been identified [8–13], and amine oxidase activities of some of them have been demonstrated [14, 15]. In bone tissue, pyridinolines and deoxypyridinolines are the primary cross-links of mature type I collagen which provide mechanical integrity, rigidity, and strength [16, 17]; and diminished LOX enzyme activity results in an increased risk of bone deformities and fractures [18, 19].

Cells secrete 50-kDa pro-LOX, which is then processed by procollagen C-proteinases in the extracellular region, resulting in the 32-kDa mature LOX enzyme and its 18-kDa LOX propeptide [20]. LOX has been detected in the

intracellular space [21–23], and it has been suggested to regulate gene transcription and to play a dominant role in stabilizing a normal cell phenotype [24–28].

In osteoblastic cells (MC3T3-E1), we have shown that LOX seems to be specifically regulated in the course of osteoblast cell differentiation and that this regulation is required for normal collagen deposition [29]. A stage-dependent distribution of LOX in differentiating osteoblasts with colocalization with the nuclear region as well as with the tubular network was found, which could indicate a role of LOX in the regulation of osteoblast development [21].

A lack of *Lox* gene expression leads to perinatal lethality in mice [30, 31]. *Lox* knockout mice ($Lox^{-/-}$) develop to term but die soon thereafter just before or at birth [30, 31]. Due to defective collagen and elastin cross-linkage, they suffer from severe cardiovascular and pulmonary defects [30–32]. In the present study, we examined the effects of *Lox* gene deficiency on the skeletal phenotype and osteoblast development.

Materials and Methods

Histological Analysis

For alcian blue and alizarin red staining, embryonic day (E) 18.5 $Lox^{-/-}$ mice as well as wild-type (*wt*) mice (C57BL/6) were removed from the uterus, fixed, and stored in 70% ethanol. Prior to staining, skin and eyes were removed and calcified tissues were stained with alcian blue (0.3% alcian blue 8GX [EMD Chemicals, Gibbstown, NJ], 70% ethanol) and alizarin red solution (0.1% alizarin red S [Wako Chemicals, Richmond, VA], 95% ethanol, 1 volume glacial acetic acid, 17 volumes 70% ethanol) for 3 days. Samples were placed in 1% KOH for 24–72 h and then stored in 70% glycerol.

Moreover, three-dimensional histological reconstruction of two heads of each genotype was performed. Samples were fixed, and embedded paraffin sections were cut as 10-µm-thick serial sections using a rotary microtome (model 2065; Reichert-Jung, Heidelberg, Germany) in the frontal plane. Hematoxylin–eosin sections were evaluated, every eighth section was photographed, and scanned images were aligned. Calcified tissues using the contours of well-characterized landmarks (i.e., skull base structures) were reconstructed by computer software (Analysis Software; SIS, Münster, Germany) [33].

Measurement of Collagen Fibril Diameter

Tissues were fixed in Karnovsky solution (1% glutaraldehyde, 1% tannin in 0.2 M phosphate buffer, pH 7.4) and then postfixed with 1% osmium tetraoxide in 0.1 M phosphate buffer. The samples were rinsed, dehydrated, and embedded in epon/araldite 502 resin (Ted Pella, Redding, CA). Sections of 30–50 nm were stained with uranyl acetate and lead citrate, and the images were observed on a CM-12 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands). Images were recorded at ×35,000 on SO-163 electron image film (Eastman Kodak, Rochester, NY). Five hundred fibril diameters were measured in randomly chosen areas using Analysis Software.

Primary Calvarial Osteoblast Cultures

$Lox^{-/-}$ and *wt* calvariae were digested (0.2% collagenase), minced, and cultured in growth medium containing of α-MEM supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and nonessential amino acids in six-well plates as has been previously established [34, 35]. In the present study, cells from each calvaria were cultured separately because the genotype of each embryo was determined by Southern blotting at a later time point. First and second cell passages were used for the experiments.

BrdU Incorporation

Primary osteoblasts were plated on 96-well plates and cultivated for up to 48 h at 37°C in growth medium. DNA synthesis was assessed in monolayer cultures by colorimetric immunoassay (Roche, Basel, Switzerland) at 405 nm. The assay is based on measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation following 2-h labeling into newly synthesized DNA of replicating cells, by enzyme-linked immunosorbent assay (ELISA).

Cell Apoptosis

Rapid binding of annexin V to phosphatidylserine was used for the early identification of cells undergoing apoptosis, as described previously [36]. Cells, plated on 24-well plates were serum-starved for 24 h and then grown in the presence and absence of 1 µM staurosporine for 6, 16, and 24 h at 37°C. Then, cells were incubated with FITC-labeled annexin V (1 µg/ml) and propidium iodine (PI, 2 µg/ml) for 15 min at 15–25°C and analyzed by fluorescence microscopy. In addition, a photometric ELISA was applied for detection of cytoplasmatic histone-associated DNA fragments (mono- and oligonucleosomes) in apoptotic osteoblastic cells (Cell Death Detection ELISA, Roche) [37, 38]. Osteoblasts were cultivated in the presence and absence of 1 µM staurosporine for 6, 16, and 24 h at 37°C. Then, cells were washed, lysed for 30 min, and centrifuged at 15,000 rpm for 10 min. The supernatant was transferred

into a streptavidin-precoated microtiter plate and incubated with the immunoreagent (anti-histone biotin, anti-DNA peroxidase) for 2 h. After washing, substrate solution was added and absorbance was determined at 405 nm.

Also, lactate dehydrogenase (LDH) release, as a marker of cell necrosis, was determined in the supernatants by a colorimetric Cytotoxicity Dectection Kit (Roche).

Mineral Nodule Formation

Osteoblast differentiation was induced by growth medium supplementation with 5 mM ß-glycerophosphate and 50 µg/ml ascorbic acid for up to 21 days. Mineralized cultures were fixed and stained with 2% alizarin red (pH 4.0) for 20 min. For quantification, incorporated alizarin red stain was eluted with 10% cetylpyridinium chloride, and optical density was measured at 562 nm using a spectrophotometer.

Quantitative Real-Time PCR

Total cellular RNA from calvariae of E18.5 murine embryos was isolated (RNeasy Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol, and real-time PCR was performed using sequence-specific primers and the ABI Prism 7000 sequence detection system (Applied Biosystems, Lincoln, CA). Specific primers for LOX (Mm00495386_ml), LOXL1 (Mm01145734_ml), LOXL2 (Mm00804740_ml), LOXL3 (Mm00442953_ml), LOXL4 (Mm00446385_ml), bone differentiation markers bone sialoprotein (BSP, Mm00492555_ml), COL1A1 (Mm00801666_g1), Runx2/Cbfa1 (Mm00501578_ml), and $ß_2$-microglobulin (Mm00437762_m1) were Taqman probes and purchased from Applied Biosystems. $ß_2$-Microglobulin was used as internal control. The mRNA expression relative to $ß_2$-microglobulin was determined, and the fold changes were calculated using the *wt* values as calibrator by means of the $2^{-\Delta\Delta CT}$ method [39].

Statistical Methods

Statistical analyses were performed using a statistical software package (SPSS for Windows, version 14.0; SPSS, Inc., Chicago, IL). Comparisons between data of $Lox^{-/-}$ and *wt* were made using an unpaired Student's *t*-test assuming equal variances, and $P < 0.05$ was considered statistically significant.

Results

Skeletal Phenotype

Alcian blue and alizarin red staining of the $Lox^{-/-}$ compared to the *wt* mice demonstrated normal morphology of calcified structures (Fig. 1). Three-dimensional reconstructions (Fig. 2) indicated normal development of nasal, alveolar, as well as teeth-related structures in $Lox^{-/-}$ mice. However, handling of $Lox^{-/-}$ samples compared to *wt* revealed fragile tissues and high sensitivity to KOH maceration.

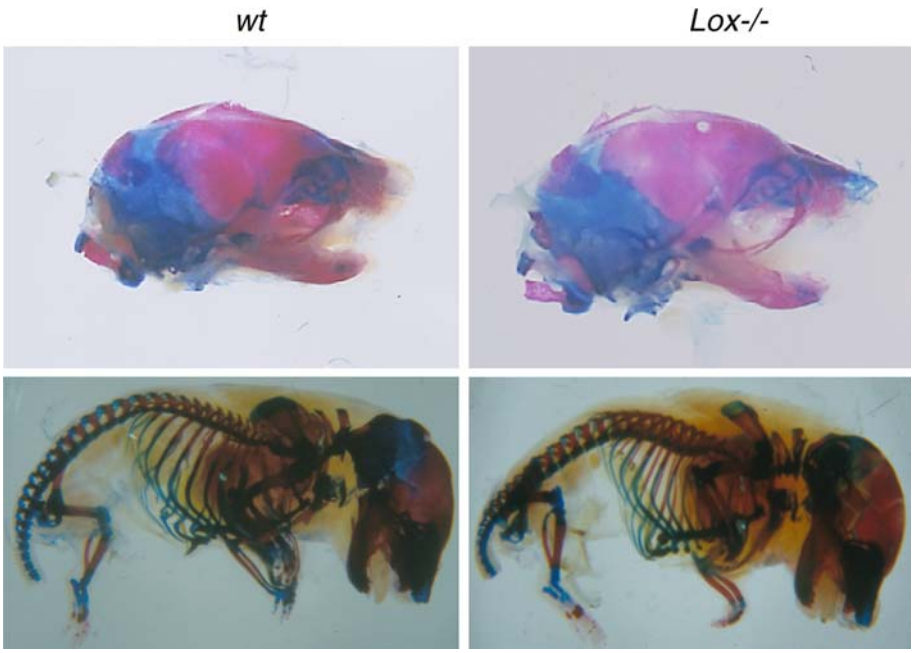

Fig. 1 Lateral views of E18.5 calcified tissues of $Lox^{-/-}$ and *wt* mice stained with alizarin red and alcian blue. Experiments were performed three times. Representative images of one experiment are shown

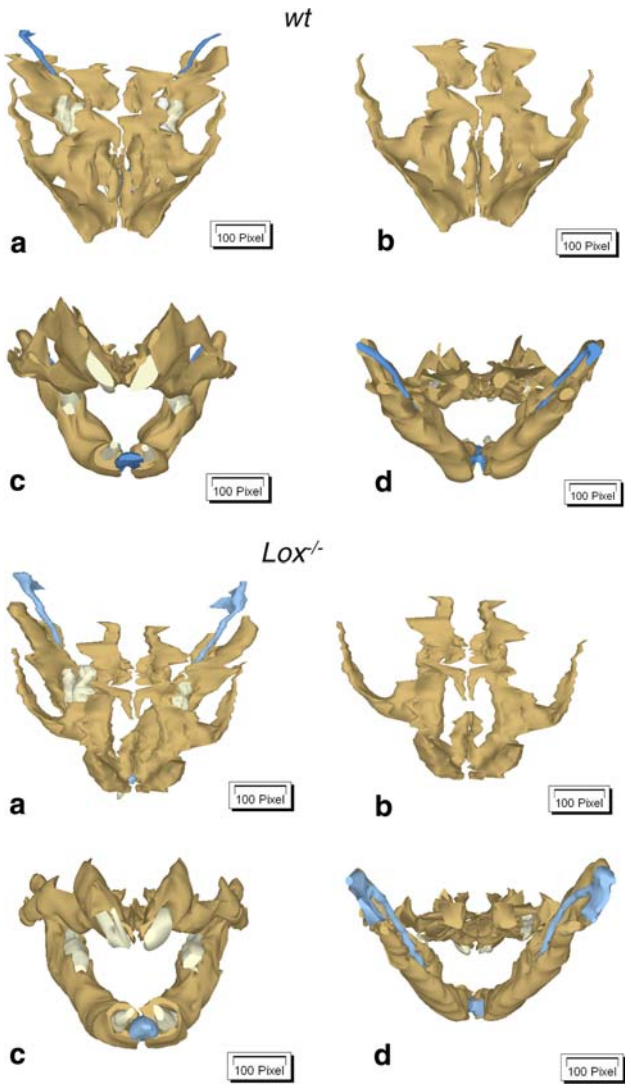

Fig. 2 Three-dimensional reconstructions of $Lox^{-/-}$ and *wt* alveolar as well as of teeth-related structures. Reconstructions were performed two times for each genotype. Representative images (**a**, **b** coronal, **c** frontal, and **d** dorsal views) of one experiment are shown. Bone tissue is presented in brown, while Meckel's cartilage and teeth are given in blue and white, respectively

Decreased Collagen Fibril Diameter

Data in Fig. 3 demonstrate decreased collagen fibril diameters in $Lox^{-/-}$ compared to *wt* bone tissues. Quantitative analyses showed that the mean fibril diameter significantly ($P < 0.001$) decreased from 34.06 ± 4.18 nm in *wt* to 31.01 ± 3.78 nm in $Lox^{-/-}$.

LOX mRNA Expression

To establish that LOX gene expression is diminished in $Lox^{-/-}$ calvarial bone tissue and to analyze mRNA expression of LOX isoforms (LOXL1–4), quantitative real-time PCR was performed. Figure 4 shows a significantly ($P < 0.05$) diminished mRNA expression of LOX and its isoforms in $Lox^{-/-}$ compared to *wt*.

BrdU Incorporation

Primary osteoblasts from $Lox^{-/-}$ and *wt* calvariae were cultivated for up to 48 h. Figure 5 demonstrates a statistically significant ($P < 0.05$) decrease of BrdU incorporation in $Lox^{-/-}$ osteoblasts compared to *wt*.

Apoptosis

Evaluation of cell apoptosis in $Lox^{-/-}$ compared to *wt* is given in Fig. 6. At 6, 16, and 24 h, no differences in annexin-V binding on outward-facing phosphatidylserine or PI binding or in the detection of cytoplasmatic histone-associated DNA fragments were found. Also, in the presence of 1 μM staurosporine, as a potent inducer of cell apoptosis, no differences in annexin-V binding and DNA fragmentation were noted between $Lox^{-/-}$ and *wt* osteoblasts (data not shown). To exclude nonspecific annexin-V binding and DNA fragmentation, we measured LDH release in the supernatants, as a marker of cell necrosis. No differences were found between $Lox^{-/-}$ and *wt* osteoblasts at any time point studied.

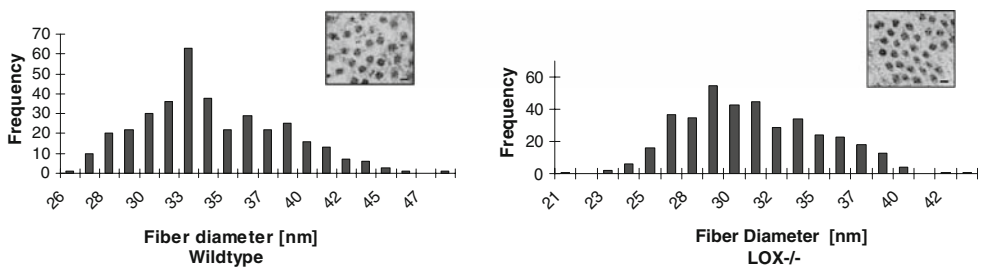

Fig. 3 Electron microscopic analysis of collagen fibril diameters of craniofacial bone tissues. For each genotype, 500 fibril diameters were measured in randomly chosen areas. Representative images are shown for $Lox^{-/-}$ and *wt*. Bar = 30 nm, magnification ×35,000

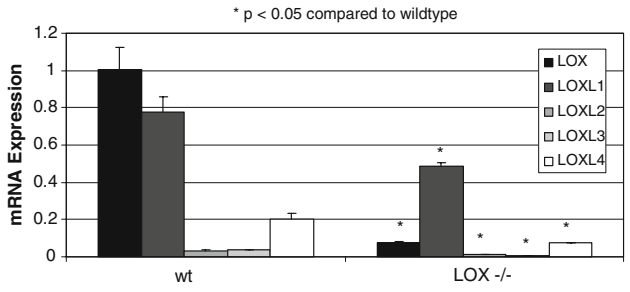

Fig. 4 Real-time RT-PCR analysis of LOX and its isoforms, LOXL1–4, from $Lox^{-/-}$ and *wt* mice. Data are presented as mean ± SD obtained from three measurements of pooled RNA samples. *Significant differences between $Lox^{-/-}$ and *wt* mice ($P < 0.05$, unpaired Student's *t*-test assuming equal variances)

Analysis of Mineral Deposition

We next examined whether decreased cell proliferation in $Lox^{-/-}$ cells affects later stages of osteoblast differentiation such as mineral nodule deposition. Significantly ($P < 0.05$) less alizarin red stain was eluted from primary $Lox^{-/-}$ osteoblast cultures compared to *wt*, indicating less mineralized nodule formation in $Lox^{-/-}$ cells at day 14 as well as at day 21 (Table 1).

Analysis of Osteoblast Differentiation Markers

Osteoblasts undergo different phases of differentiation [40]. In accordance with decreased mineralized nodule

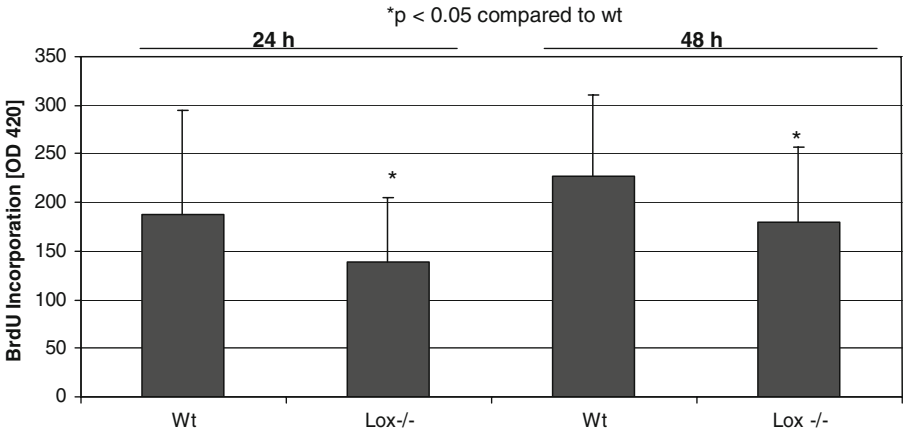

Fig. 5 BrdU incorporation in primary $Lox^{-/-}$ and *wt* osteoblastic cells after 24 and 48 h measured by ELISA at 405 nm. Data are presented as means ± SD of three experiments ($n = 6$ cultures/genotype). *Significant differences between $Lox^{-/-}$ and *wt* cells ($P < 0.05$, unpaired Student's *t*-test assuming equal variances)

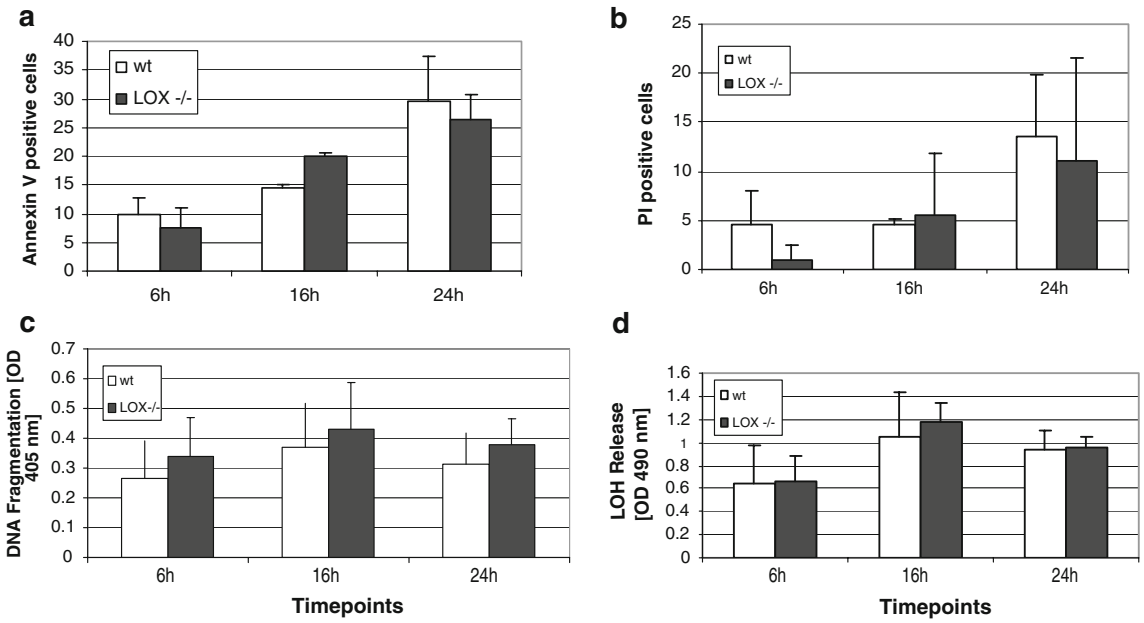

Fig. 6 Annexin V-positive (**a**) and propidium iodine (PI)-positive (**b**) cell count in primary $Lox^{-/-}$ and *wt* osteoblastic cells after 6, 16, and 24 h measured by fluorescence microscopy. Measurement of DNA fragmentation (**c**) in primary $Lox^{-/-}$ and *wt* osteoblastic cells after 6, 16, and 24 h by ELISA at 405 nm. Spectrophotometric measurement of LDH release (**d**) in primary $Lox^{-/-}$ and *wt* osteoblastic cells after 6, 16, and 24 h at 490 nm. Data are presented as means ± SD of three experiments ($n = 3$ cultures/genotype)

Table 1 Means and standard deviations of optical density (OD) of three experiments ($n = 3$ cultures/genotype)

Genotype	Day 14 OD 562 nm (mean ± SD)	Day 21 OD 562 nm (mean ± SD)
Wt	1.156 ± 0.107	1.736 ± 0.134
$Lox^{-/-}$	0.474 ± 0.066*	0.765 ± 0.087*

* Significant differences between $Lox^{-/-}$ and *wt* cells (alizarin red eluate of differentiated osteoblasts). $P < 0.05$, unpaired Student's *t*-test assuming equal variances

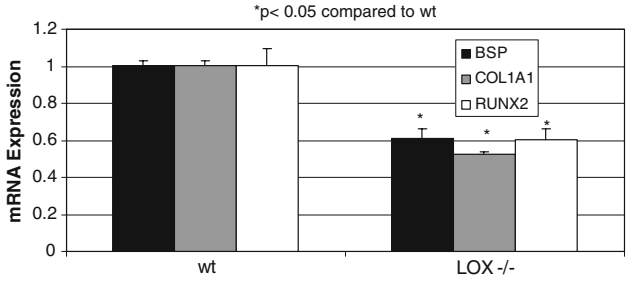

Fig. 7 Real-time RT-PCR analysis of bone markers COL1A1, bone sialoprotein (BSP), and Runx2/Cbfa1from $Lox^{-/-}$ and *wt* mice. Data are presented as mean ± SD obtained from three measurements of pooled RNA samples. *Significant differences between $Lox^{-/-}$ and *wt* cells ($p < 0.05$, unpaired Student's *t*-test assuming equal variances)

formation observed in $Lox^{-/-}$ osteoblasts, Fig. 7 shows a statistically significant ($P < 0.05$) decrease of COL1A1 as well as of BSP and Runx2/Cbfa1mRNA expression compared to *wt*.

Discussion

Extracellular posttranslational modifications of fibrillar type I–III collagens by LOX are crucial for collagen cross-linkage and for the accumulation of a functional collagen matrix in bone tissue [3, 5, 7]. In the present study, besides normal morphology of calcified structures, significant changes in collagen fibril formation as well as altered osteoblast differentiation were found in $Lox^{-/-}$ compared to *wt* mice.

Significantly diminished truncated and nonfunctional LOX mRNA expression was demonstrated in $Lox^{-/-}$ samples. In other collagen-producing cell types, such as mouse fibroblasts and vascular smooth muscle cells isolated from $Lox^{-/-}$ mice, lysyl oxidation itself has been reduced approximately by 80% [32] and the overall amount of immature collagen cross-links has been reported to be reduced by 40% in $Lox^{-/-}$ embryos [31], thus indicating that LOX's role in cross-linking is essential and may be important in osteoblasts. Also, amine oxidase activities have been demonstrated for other LOX isoforms [14, 15], indicating that multiple LOX isoforms may be involved in cross-linkage. The present data show that LOX and LOXL1 are clearly the prominent forms at the RNA level in bone tissue. On the contrary, LOXL2 and LOXL3 showed only minor expression, which may suggest that they are less relevant in bone metabolism. In MC3T3-E1 cells, it was shown that LOX, LOXL1, and LOXL4 were regulated in the course of osteoblast differentiation, whereas constituent expression of LOXL3 and no expression of LOXL2 were found [41]. It has been suggested that LOX isoforms may partly compensate for the lack of LOX [30], which may have contributed to the fact that normal bone morphology was found in $Lox^{-/-}$ bone tissues by histological means. However, in the present study, in the absence of *Lox,* other genes including LOX isoforms, LOXL1–4, were significantly downregulated as well, which may suggest regulatory interactions between different isoforms in bone tissue. Whether the effects on osteoblast development seen in the present study are primarily due to *Lox* deficiency or to its isoforms, or a combination of the two, is still unknown. Moreover, the observed effects of *Lox* depletion on osteoblasts could also potentially depend on both changes in LOX enzyme activity as well as altered functions of LOX propeptide, as recently shown in other cell types [24, 27, 28].

The formation of covalent cross-links by enzymatic LOX activity is required for the formation of mature and insoluble collagen [3, 5, 7]. In the present study, significantly decreased collagen fiber diameters were measured in $Lox^{-/-}$ tissues, indicating impaired collagen matrix accumulation. A functional collagenous bone matrix may be critical for cell–cell and cell–matrix interactions affecting cell differentiation. Previous studies suggest that LOX plays an important role in osteoblastic differentiation [21, 29, 42, 43]. The present study demonstrates for the first time that *Lox* gene deficiency affects the osteoblastic phenotype. DNA synthesis was significantly decreased in $Lox^{-/-}$ osteoblasts compared to *wt* cells. From our previous data, it is known that LOX can be localized in the intracellular space, and LOX has been suggested to regulate gene transcription [21, 24, 25, 29]. LOX binds to intracellular cell components, in particular to the microtubular network [21], which is considered a prerequisite for development of a proper mitotic spindle apparatus and normal cell cycle progression [44]. Further studies will examine involved transcriptional control of the observed effects of *Lox* depletion on cell division. In addition, the balance of osteoblast proliferation and apoptosis determines the size of the osteoblast population [45]. In the present study, decreased cell proliferation was accompanied by normal regulation of apoptosis in $Lox^{-/-}$ osteoblasts, suggesting an overall decrease of the size of the osteoblast population.

Osteoblasts pass through phenotypic changes with distinct patterns of gene expression as they differentiate [40].

The present data indicate that the *Lox* gene influences not only early but also later stages of osteoblast differentiation as decreased mineral nodule formation in $Lox^{-/-}$ cultures was noted. In addition, to gain insight into the molecular mechanisms associated with this inhibition, we determined the expression levels of osteoblast transcription factors and phenotypic markers of various stages of osteoblast differentiation. Our data show that early markers of osteoblast differentiation, such as type I collagen as well as the marker of fully differentiated osteoblasts, BSP, were significantly downregulated in bone tissue lacking the *Lox* gene. Additionally, Runx2/cbfa1, a master transcription factor for the osteoblast lineage, which positively controls the expression of type I collagen and BSP [46], was significantly decreased. Altogether, inhibition of differentiation could be a consequence of inhibited cell proliferation. Besides, *Lox* depletion seems to directly affect osteoblast differentiation, as shown by downregulation of the expression of osteoblastic differentiation markers, and thus cannot completely be explained by diminished cell density.

Further studies are warranted to examine the interaction between enzymatic collagen cross-linkage and cellular effects as well as its effects on the formation of functional bone tissue. Based on the present data, we suspect that LOX promotes bone development through mechanisms in addition to its well established role in collagen cross-linking, and further studies are in progress to address novel mechanisms of action in normal developing osteoblast cultures.

Acknowledgements We thank B. Danielowski and V. Kanitz for the technical support in the laboratory. This study was supported by grant DE140066 (to P. T.), grants 202469 from the Health Science Council and the S. Juselius Foundation (to J. M.), research stipend German Research Foundation GK-325-00 (to P.M. and N.H.), as well as short-term research fellowships from European Molecular Biology Organization and European Cooperation in Science and Technology, a habilitation stipend from the Charité – Universitätsmedizin Berlin, and research grant DFG PI 420/3 (to N.P.).

References

1. Boskey AL (1996) Matrix protein and mineralization: an overview. Connect Tissue Res 35:357–363
2. Fratzl P, Gupta HS, Paschalis EP, Roschger P (2004) Structure and mechanical quality of the collagen-mineral nano-composite in bone. J Mater Chem 14:2115–2123
3. Kagan H, Trackman P (1991) Properties and function of lysyl oxidase. Am J Respir Cell Mol Biol 5:206–210
4. Prockop D, Kivirikko K (1995) Collagens: molecular biology, diseases, and potentials for therapy. Annu Rev Biochem 64:403–434
5. Yamauchi M (1996) Collagen: the major matrix molecule in mineralized tissues. In: Anderson JJB, Garner S (eds) Calcium and phosphorus in health and disease. CRC Press, New York, pp 127–141
6. Knott L, Bailey A (1998) Collagen cross-links in mineralizing tissues: a review of their chemistry, function, and clinical relevance. Bone 22:181–187
7. Kagan H (1986) Biology and regulation of extracellular matrix: a series. In: Mecham RP (ed) Regulation of matrix accumulation, vol 1. Academic Press, Orlando, pp 321–398
8. Mäki JM, Kivirikko KI (2001) Cloning and characterization of a fourth human lysyl oxidase isoenzyme. J Biochem 355:381–387
9. Mäki JM, Tikkanen H, Kivirikko KI (2001) Cloning and characterization of a fifth human lysyl oxidase isoenzyme: the third member of the lysyl oxidase-related subfamily with four scavenger receptor cysteine-rich domains. Matrix Biol 20:493–496
10. Kenyon K, Modi WS, Contente S, Friedman RM (1993) A novel human cDNA with a predicted protein similar to lysyl oxidase maps to chromosome 15q24–q25. J Biol Chem 268:18435–18437
11. Ito H, Akiyama H, Iguchi H, Iyama K, Miyamoto M, Ohsawa K et al (2001) Molecular cloning and biological activity of a novel lysyl oxidase-related gene expressed in cartilage. J Biol Chem 276:24023–24029
12. Jourdan-Le Saux C, Le Saux O, Donlon T, Boyd CD, Csiszar K (1998) The human lysyl oxidase-related gene (LOXL2) maps between markers D8S280 and D8S278 on chromosome 8p21.2–p21.3. Genomics 51:305–307
13. Huang Y, Dai J, Tang R, Zhao W, Zhou Z, Wang W et al (2001) Cloning and characterization of a human lysyl oxidase-like 3 gene (hLOXL3). Matrix Biol 20:153–157
14. Borel A, Eichenberger D, Farjanel J, Kessler E, Gleyzal C, Hulmes DJ et al (2001) Lysyl oxidase-like protein from bovine aorta. Isolation and maturation to an active form by bone morphogenetic protein-1. J Biol Chem 276:48944–48949
15. Kim MS, Kim SS, Jung ST, Park JY, Yoon HW, Ko J et al (2003) Expression and purification of enzymatically active forms of the human lysyl oxidase-like protein 4. J Biol Chem 278:52071–52074
16. Knott L, Whitehead C, Fleming R, Bailey A (1995) Biochemical changes in the collagenous matrix of osteoporotic avian bone. J Biochem 310:1045–1051
17. Eyre DR, Dickson IR, Van Ness K (1988) Collagen crosslinking in human bone and articular cartilage: age-related changes in the content of mature hydroxypyridinium residues. J Biochem 252:495–500
18. Geiger BJ, Steenbock H, Parsons HT (1933) Lathyrism in the rat. J Nutr 6:427–442
19. Shim H, Harris ZL (2003) Genetic defects in copper metabolism. J Nutr 133:1527S–1531S
20. Uzel M, Scott I, Babakhanlou-Chase H, Palamakumbura A, Pappano W, Hong H et al (2001) Multiple bone morphogenic protein-1 related mammal metalloproteinases process pro-lysyl oxidase at the correct physiological site and control lysyl oxidase activation in mouse embryo fibroblast cultures. J Biol Chem 276:22537–22543
21. Guo Y, Pischon N, Palamakumbura AH, Trackman P (2007) Intracellular distribution of the lysyl oxidase propeptide in osteoblastic cells. Am J Physiol Cell Physiol 292:2095–2102
22. Nellaiappan K, Risitano A, Liu G, Nicklas G, Kagan H (2000) Fully processed lysyl oxidase catalyst translocates from the extracellular space into nuclei of aortic smooth-muscle cells. J Biol Chem 79:576–582
23. Li W, Nellaiappan K, Strassmaier T, Graham L, Thomas K, Kagan H (1997) Localization and activity of lysyl oxidase within nuclei of fibrogenic cells. Proc Natl Acad Sci USA 94:12817–12822
24. Palamakumbura A, Jeay S, Guo Y, Pischon N, Sommer P, Sonenshein G et al (2004) The propeptide domain of lysyl oxidase induces phenotypic reversion of ras-transformed cells. J Biol Chem 279:40593–40600
25. Jeay S, Pianetti S, Kagan H, Ge S (2003) Lysyl oxidase inhibits ras-mediated transformation by preventing activation of NF-kappa B. Mol Cell Biol 23:2251–2263

26. Giampuzzi M, Oleggini R, Di Donato A (2003) Demonstration of in vitro interaction between tumor suppressor lysyl oxidase and histones H1 and H2: definition of the regions involved. Biochim Biophys Acta 1647:245–251
27. Min C, Kirsch KH, Zhao Y, Jeay S, Palamakumbura AH, Trackman PC et al (2007) The tumor suppressor activity of the lysyl oxidase propeptide reverses the invasive phenotype of Her-2/neu-driven breast cancer. Cancer Res 67:1105–1112
28. Wu M, Min C, Wang X, Yu Z, Kirsch K, Trackman PC et al (2007) Repression of BCL2 by the tumor suppressor activity of the lysyl oxidase propeptide inhibits transformed phenotype of lung and pancreatic cancer cells. Cancer Res 67:6278–6285
29. Hong HH, Pischon N, Santana RB, Palamakumbura AH, Chase HB, Gantz D et al (2004) A role for lysyl oxidase regulation in the control of normal collagen deposition in differentiating osteoblast cultures. J Cell Physiol 200:53–62
30. Mäki JM, Räsänen J, Tikkanen H, Sormunen R, Mäkikallio K, Kivirikko KI et al (2002) Inactivation of the lysyl oxidase gene Lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice. Circulation 106:2503–2509
31. Hornstra I, Birge S, Starcher B, Bailey A, Mecham R, Shapiro S (2003) Lysyl oxidase is required for vascular and diaphragmatic development in mice. J Biol Chem 278:14387–14393
32. Mäki J, Sormunen R, Lippo S, Kaarteenaho-Wiik R, Soininen R, Myllyharju J (2005) Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. Am J Pathol 167:927–936
33. Radlanski RJ, Renz H, Klarkowski MC (2003) Prenatal development of the human mandible. 3D reconstructions, morphometry and bone remodelling pattern, sizes 12–117 mm CRL. Anat Embryol 207:221–232
34. Bellows CG, Aubin JE, Heersche JNM, Antosz ME (1986) Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcif Tissue Int 38:143–154
35. Ecarot-Charrier B, Glorieux FH, Van der Rest M, Pereira G (1983) Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. J Cell Biol 96:639–643
36. Hippenstiel S, Schmeck B, N'Guessan PD, Seybold J, Krull M, Preissner K et al (2002) Rho protein inactivation induced apoptosis of cultured human endothelial cells. Am J Physiol Lung Cell Mol Physiol 283:L830–L838
37. N'Guessan PD, Vigelahn M, Bachmann S, Zabel S, Opitz B, Schmeck B et al (2007) The UspA1 protein of *Moraxella catarrhalis* induces CEACAM-1-dependent apoptosis in alveolar epithelial cells. J Infect Dis 195:1651–1660
38. N'Guessan PD, Schmeck B, Ayim A, Hocke AC, Brell B, Hammerschmidt S et al (2005) *Streptococcus pneumoniae* R6x induced p38 MAPK and JNK-mediated caspase-dependent apoptosis in human endothelial cells. Thromb Haemost 94: 295–303
39. Livak KJ, Schnmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. Methods Enzymol 25:402–408
40. Stein G, Lian J, Stein J, Van Wijnen J, Montecino M (1996) Transcriptional control of osteoblast growth and differentiation. Physiol Rev 76:593–629
41. Atsawasuwan P, Mochida Y, Parisuthiman D, Yamauchi M (2005) Expression of lysyl oxidase isoforms in MC3T3–E1 osteoblastic cells. Biochem Biophys Res Commun 327:1042–1046
42. Pischon N, Darbois LM, Palamakumbura AH, Kessler E, Trackman PC (2004) Regulation of collagen deposition and lysyl oxidase by tumor necrosis factor-alpha in osteoblasts. J Biol Chem 279:30060–30065
43. Turecek C, Fratzl-Zelman N, Rumpler M, Buchinger B, Spitzer S, Zoehrer R et al (2008) Collagen cross-linking influences osteoblastic differentiation. Calcif Tissue Int 82:392–400
44. Jordan MA (2002) Mechanism of action of antitumor drugs that interacts with microtubules and tubulin. Curr Med Chem Anti-Cancer Agents 2:1–17
45. Hock JM, Krishnan V, Onyia JE, Bidwell JP, Milas J, Stanislaus D (2001) Osteoblast apoptosis and bone turnover. J Bone Miner Res 16:975–984
46. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89:747–754