ORIGINAL ARTICLE

Effect of CBX7 deficiency on the socket healing after tooth extractions

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Abstract
CBX7 is shown to down-regulate the expression of osteopontin (OPN) that is associated with osteoblast function. Here, we studied the role of CBX7 in the wound healing of tooth extraction socket in which osteoblast activity is critical via comparison between CBX7-knockout (CBX7$^{-/-}$) mice and their wild-type (WT) counterparts of 6 weeks old with maxillary first molar extracted. Mice were euthanized at 7, 14, and 21 days after extractions, and alveolar sockets were assessed by semi-quantitative histomorphometry for hard tissue healing, including new bone fill (Masson's trichrome staining), osteoblast activity (OPN/osterix, Osx), osteoclast activity (tartrate-resistant acid phosphatase, TRAP), and for soft tissue healing, including blood vessels (alpha smooth muscle actin, α-SMA). Also, the bone microarchitecture was evaluated by micro-CT. In radiological analysis, CBX7$^{-/-}$ mice increased bone mass significantly more than WT mice did. Consistently, both the amount of new bone fill and OPN/Osx-immunopositive cells in the extraction sockets were significantly increased in CBX7$^{-/-}$ mice at each time point with respect to their WT siblings, while osteoclast number exhibited a trend of more increase in CBX7$^{-/-}$ mice at all time points as well. In agreement with enhanced bone formation during socket healing, significantly elevated α-SMA-immunopositive area was noted in CBX7$^{-/-}$ mice in contrast to WT mice. Taken together, these data suggest that CBX7 deficiency has a positive effect on tooth extraction socket healing.

Keywords CBX7 · Socket healing · Histomorphometric analysis · Tooth extraction · Mice

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Introduction

CBX7, which encodes a polycomb protein that participates in the formation of polycomb repressive complex1 (PRC1), has been described as a controversial gene for its effect on carcinogenesis [1]. Of interest, CBX7 has been reported to retard cancer progression by blocking the expression of gene relevant to cell migration (secreted phosphoprotein, SPP1) [2]. It is noteworthy that the gene SPP1 encodes a glycoprotein known as osteopontin (OPN), which is secreted by osteoblasts and involves in osteogenesis for its positive effect on osteoblastic differentiation and proliferation [3–5]. Moreover, OPN has been demonstrated to recruit osteoclasts to the bone remodeling matrix and beneficial to bone resorption [6]. Therefore, OPN modulates both bone formation and resorption [7]. Additionally, previous study manifested that the protein levels of OPN were remarkably upregulated in mandibles of CBX7$^{-/-}$ mice with respect to their WT littermates at early development stage [8], which is in correspondence with negative regulation of CBX7 on SPP1 gene expression in preventing tumor deterioration [2]. Hence, CBX7

may affect bone formation and resorption via its impact on OPN expression level.

The socket-healing process after tooth extractions involves three sequential phases [9]. Inflammation occurs immediately after extraction, and then woven bone formation increases with osteoblasts in predominance and several blood vessels around [10]. Due to its absence of load-bearing capacity, woven bone is eventually replaced by lamellar bone or bone marrow in bone remodeling phase with the appearance of osteoclasts for bone resorption [11]. In general, the socket-healing process comprises both bone formation and resorption, which may be affected by CBX7 as we hypothesized above.

To delineate the effect of CBX7 deficiency on the socket healing after tooth extraction, differences on hard and soft tissue healing were compared between 6-week-old CBX7$^{-/-}$ mice and their WT littermates with maxillary first molars extracted via radiology, histology and immunohistology.

Materials and methods

Mice

Thirty-six male (6-week-old) mice were randomly divided into three groups of different time points ($n = 12$, six from CBX7$^{-/-}$ mice, six from their WT siblings). Actually, forty-two mice were subject to this study, while six of them were excluded for root fractures occurring in tooth extractions. The average weights were 22.61 ± 2.89 g for CBX7$^{-/-}$ mice, and 20.88 ± 2.17 g for WT mice. No significant difference was found on average weight before tooth extractions. The genotype detection and generation of mice were described in our previous studies [8]. All mice were housed in a pathogen-free barrier environment and all experimental procedures were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University (No. 10433).

Tooth extraction

Before extractions, mice were anesthetized by intraperitoneal adminstration of ketamine (100 mg/kg) and xylazine (5 mg/kg). All surgeries were performed under standard sterile condition. The upper left first molar was extracted using modified probe as tooth elevator, bent tweezer as mouth gag, and crooked steel wire (diameter 0.9 mm) as tongue fixer (available as Supplementary Material to this paper, Fig. S1). Mice were fed with sterile soft diet after extractions to gain proper wound healing.

Microcomputed tomography (micro-CT) imaging

Maxillae free of the skin and outer layer muscle were harvested at 7, 14, and 21 days after extractions, and fixed with
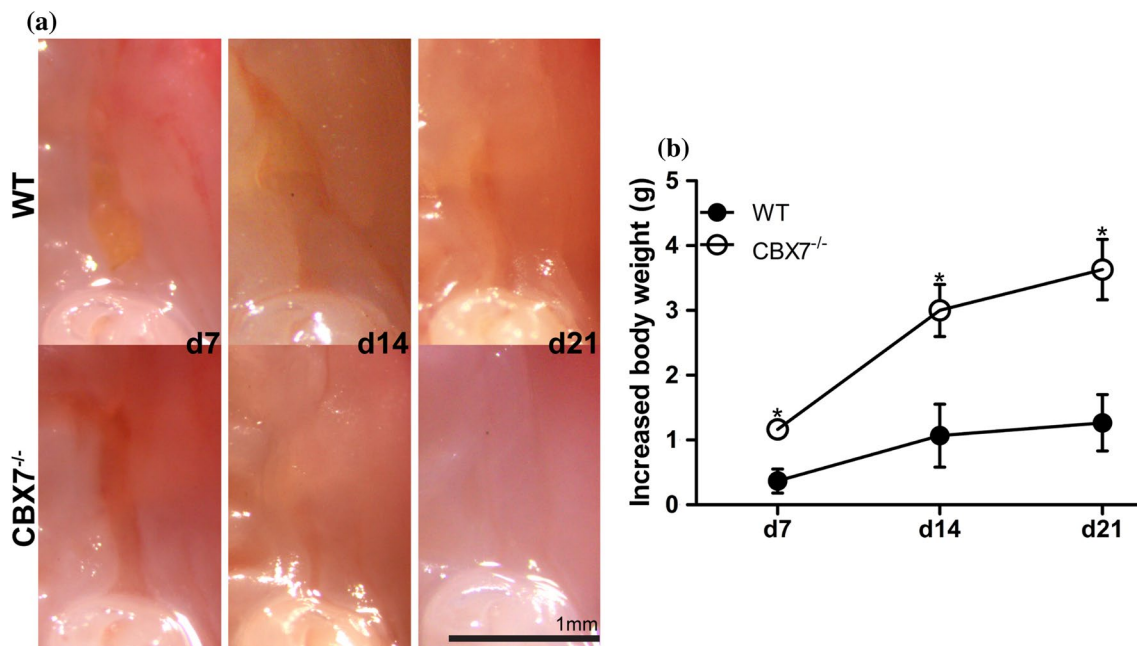
Fig. 1 Clinical observation of tooth extraction sites at different time points. **a** No visible signs of inflammation were observed and soft tissue closure of extraction sockets was formed faster and better in CBX7$^{-/-}$ mice than WT mice. Bar, 1 mm. **b** Body weight changes after tooth extractions. Values are the mean $\pm$ SD of six pairs in each time group. *$p < 0.05$ compared with WT mice

PLP fixative (2% paraformaldehyde containing 0.075 mol/L lysine and 0.01 mol/L sodium periodate) for 48 h. Micro-CT scans were taken by SkyScan 1176 scanner (SkyScan, Kontich, Belgium) at 50 kV, 456 µA, with the resolution of 18 µm. Volumetric data were reconstructed using NRecon v1.6 and CTAn v1.13.8.1 software. The region of interest (ROI) was defined to cover the whole tooth extraction socket, and a total of 30 successive images were selected from ROI for reconstruction and analysis. Bone volume (BV, mm^3), bone volume per total volume (BV/TV, %) and mean density (g/cm^3) of each extraction site were measured at all time groups.

Histomorphometric analysis

The fixed samples were subsequently decalcified for 4 weeks in EDTA–glycerol solution, embedded in paraffin and sectioned in the sagittal plane at a thickness of 4 µm. The sections were performed with Masson's trichrome staining, tartrate-resistant acid phosphatase (TRAP) staining, and immunohistochemistry for OPN, Osx and alpha smooth muscle actin (α-SMA). Masson's trichrome staining was processed as instructions provided with the kit (Leagene Biotechnology, Beijing, China). Immunohistochemistry was performed via the avidin–biotin–peroxidase complex technique with antibodies for OPN (ab8448, Abcam, UK), Osx (ab22552) and α-SMA (ab124964). The detailed steps for immunochemical and TRAP staining were described in our previous studies [8]. Histologic images were photographed with Leica DM4000 (Leica Microsystems, Mannheim, Germany) and subjected to Image-Pro Plus 6.0 software for analysis.

Statistical analysis

Results were presented as mean ± SD. Student's t test were used to quantify differences via statistical software SPSS Version 13.0 (SPSS Inc., Chicago, IL, USA). A statistical difference was considered if the p value was < 0.05.
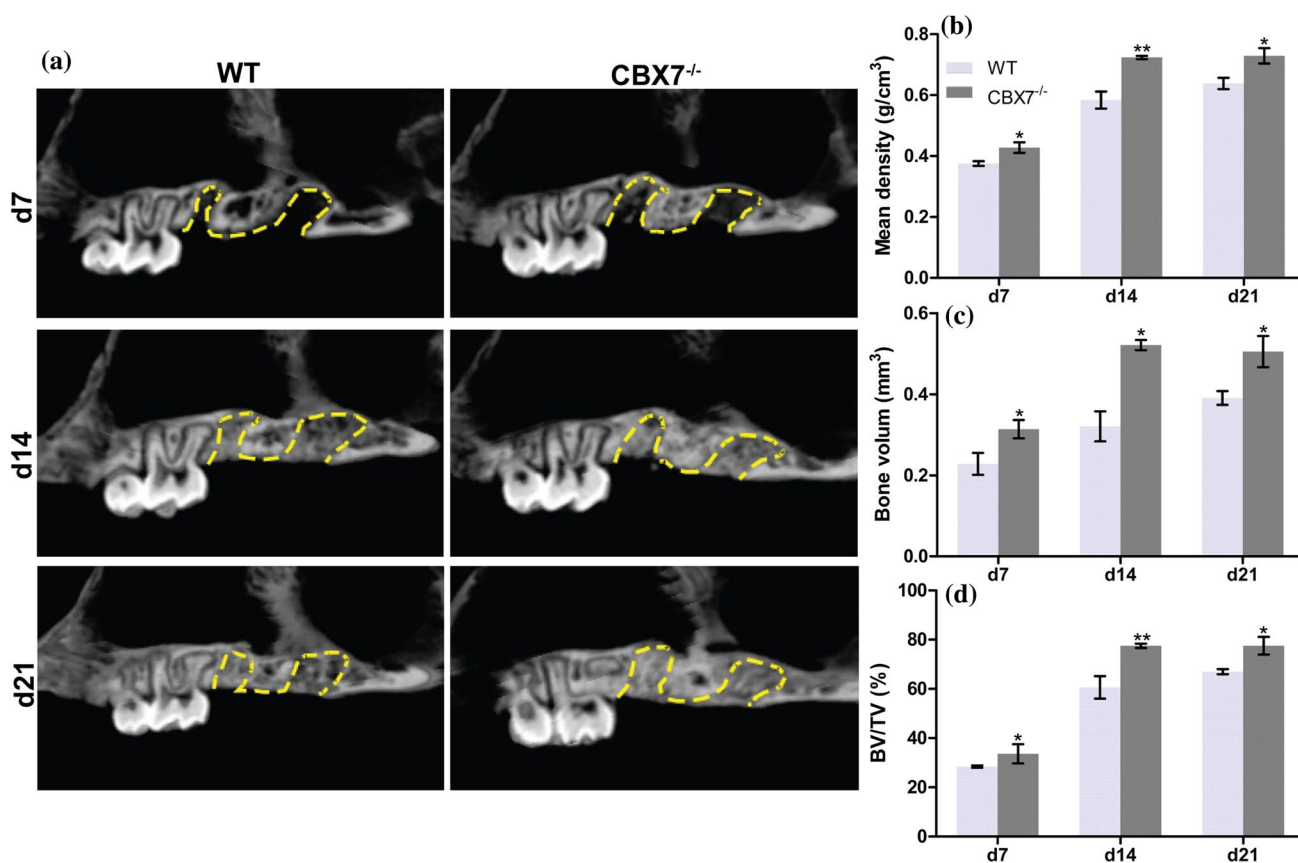
Fig. 2 Effects of CBX7 deficiency on the mean density and bone volume of tooth extraction sites at different time points. **a** Representative micro-CT scanned sections with the yellow dotted lines delineating tooth extraction sockets. **b** Mean density, **c** bone volume and **d** its ratio to total volume (BV/TV). Data are the mean ± SD of six pairs in each time group. *$p < 0.05$, $p < 0.01$ in contrast to WT mice

Results

Clinical observation

Healings after tooth extractions were uneventful at all surgical sites, where no visible inflammatory signs were observed using stereo microscope (Olympus Optical Co., Ltd., Japan). Extraction socket closure was indicated by soft tissue coverage, which formed faster and better in CBX7$^{-/-}$ mice with respect to their WT counterparts (Fig. 1a). In addition, changes in body weight were monitored as a gross evaluation of healing. Noteworthy, CBX7$^{-/-}$ mice had significantly increased body weight than their WT siblings at each time point after tooth extractions (Fig. 1b).

Radiology findings at tooth extraction sites

To measure changes of bone level in the post-extraction sockets, a series of volumetric analysis were performed by micro-CT. A significant increase in the mean density (g/cm^3) of extraction sockets was observed in CBX7$^{-/-}$ mice with respect to their WT littermates at each time point. Consistently, both the bone volume (mm^3) and its ratio to total volume (BV/TV, %) were also remarkably increased in CBX7$^{-/-}$ mice in contrast with WT mice (Fig. 2).

Histological findings on new bone fill in the sockets

Representative micrographs of Masson's trichrome-stained sections of tooth extraction sites are shown in Fig. 3a.
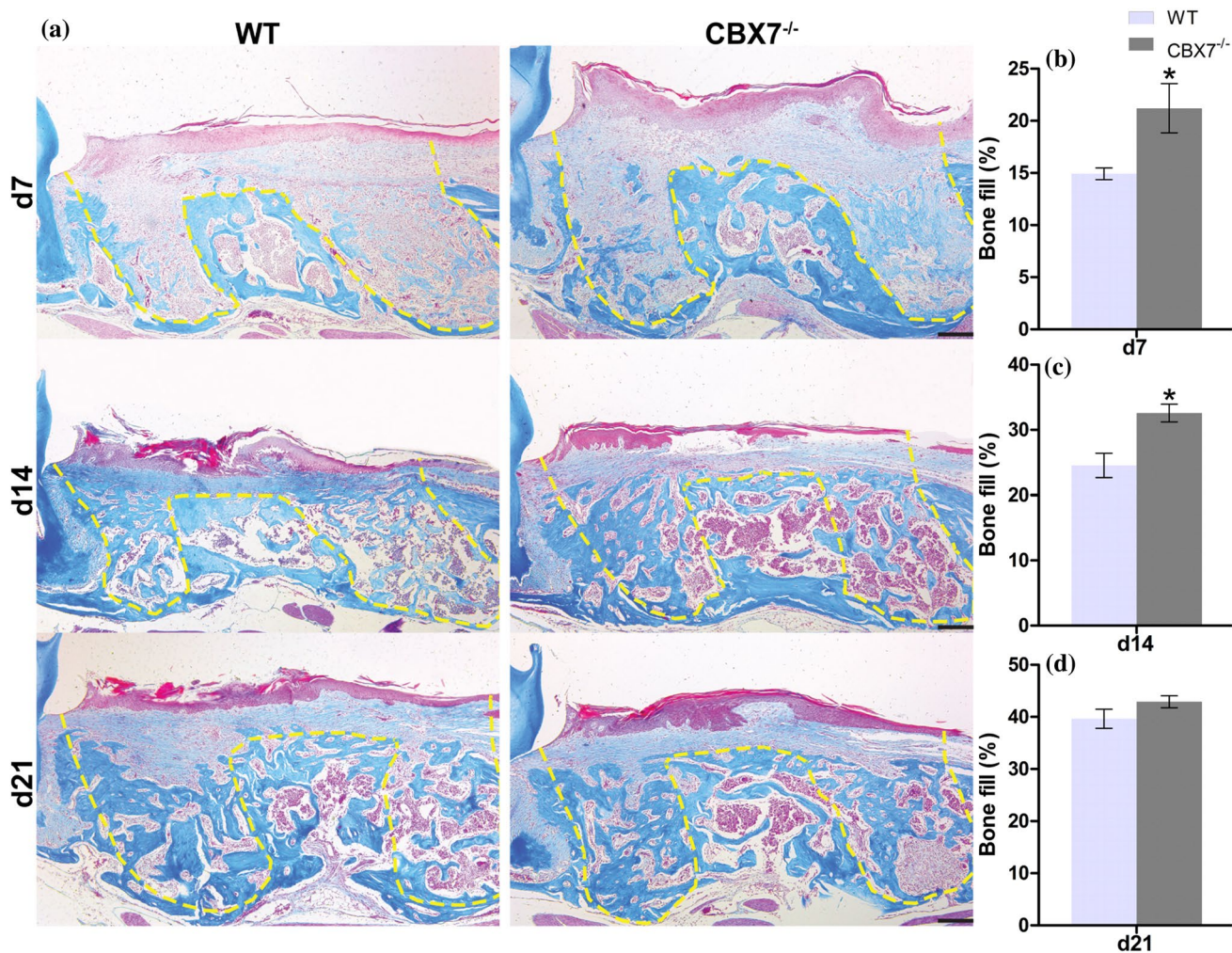
Fig. 3 Effects of CBX7 deficiency on new bone fill in the sockets at different time points. Representative micrographs of **a** Masson's trichrome-stained sections with the dotted yellow line depicting tooth extraction sockets. Bar, 200 μm. The percentage of new bone fill in tooth extraction sockets at **b** 7 days, **c** 14 days and **d** 21 days. Values are the mean ± SD of six pairs in each time group. *$p < 0.05$, with respect to WT mice

Epithelial coverage was observed in all wounds, whereas the amount of new bone fill was remarkably increased with time in both CBX7$^{-/-}$ and WT mice. Moreover, there was a trend of greater new bone formation in CBX7$^{-/-}$ mice than their WT counterparts at 7 and 14 days (Fig. 3b, c), while little increase in new bone fill was noted in comparison of two groups at 21 days (Fig. 3d).

Histological findings on osteoblastic bone formation in the sockets

To clarify whether increased new bone fill in tooth extraction sockets was associated with raised osteoblastic bone formation, paraffin-embedded sections were stained to determine the immunoreactivity of OPN. Irrespective of genotype, the percentage of OPN-positive cells increased with time (Fig. 4a); moreover, a trend of greater OPN-positive cell percentage was observed in CBX7$^{-/-}$ mice than WT mice at all time points after tooth extractions (Fig. 4b–d).

Aside from OPN, we also assessed the immunoreactivity of Osx (another specific marker of osteoblastic bone formation) in post-extraction sockets as well. Consistent with OPN, the percentage of Osx-positive cells increased with time irrespective of genotype, and a trend of more Osx expression was observed in CBX7$^{-/-}$ mice than WT counterparts at each time point (Fig. 5).

Histological findings on osteoclastic bone resorption in the sockets

Considering the ability of OPN to anchor osteoclasts to bone remodeling matrix, differences on osteoclastic bone resorption in tooth extraction sockets were measured via TRAP staining. An obvious time increase in the surface of TRAP-positive osteoclasts (the surface of osteoclasts relative to the bone surface, Oc.S/B.S) at extraction sites was shown in histochemical sections (Fig. 6a). Oc.S/B.S of CBX7$^{-/-}$ mice was significantly increased in contrast
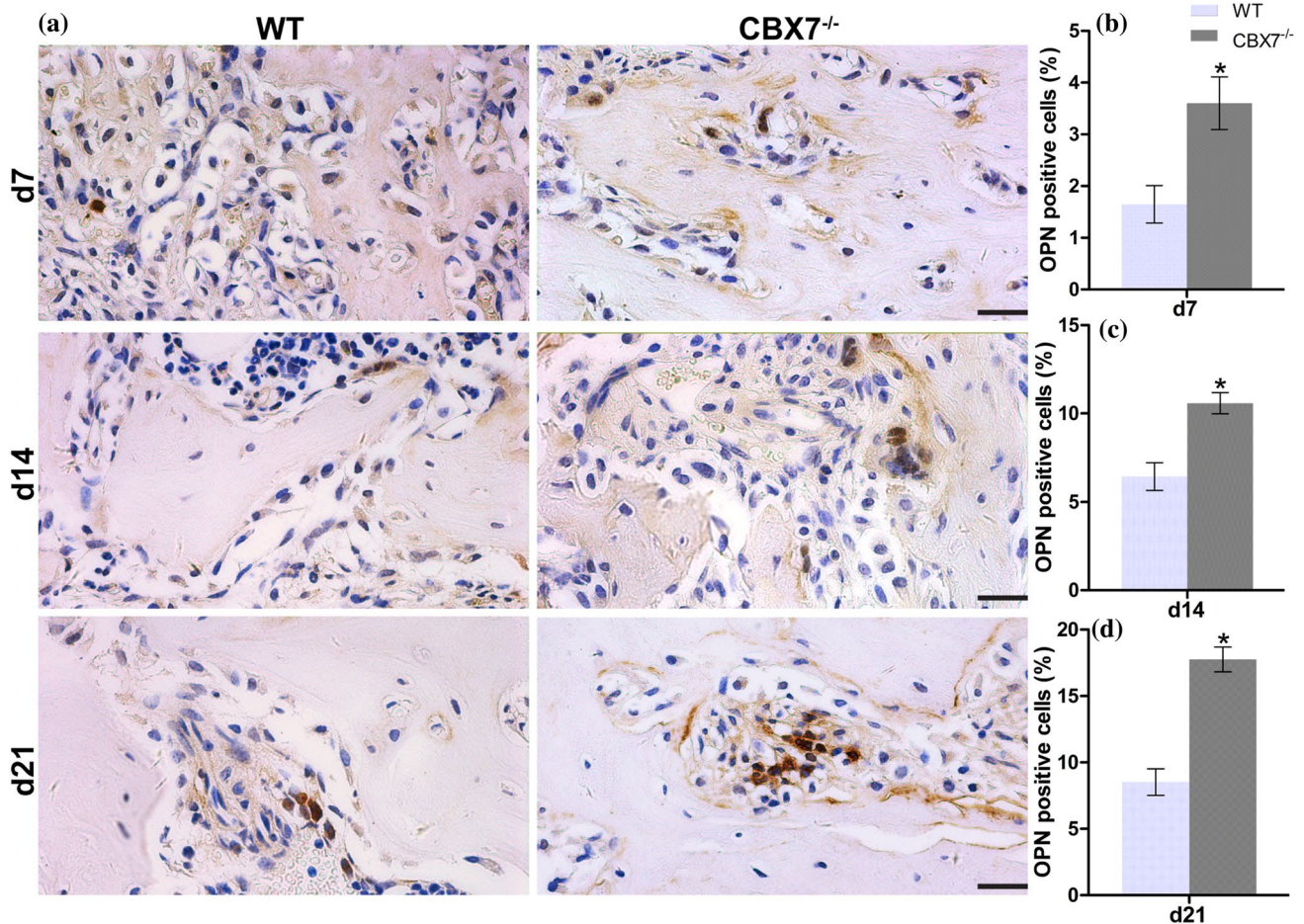
Fig. 4 Effects of CBX7 deficiency on osteoblastic bone formation in the sockets at different time points. Representative photomicrographs of extraction wound sections stained immunochemically for **a** osteopontin (OPN). Bar, 25 μm. The number of OPN-positive cells at extraction sites was determined by image analysis and the percentage of immunopositive cells relative to total cells is presented as mean ± SD of six pairs at **b** 7 days, **c** 14 days and **d** 21 days. *$p < 0.05$, in contrast to WT mice

to that of WT mice at 7 and 21 days (Fig. 6b, d), while little increase of Oc.S/B.S was observed in CBX7$^{-/-}$ mice versus their WT siblings at 14 d (Fig. 6c).

Histological findings on angiogenesis in the sockets

Blood vessel formation was assessed to evaluate soft tissue healing. In accordance with better osteogenesis after tooth extractions, increased blood vessel area (labeled with alpha smooth muscle actin, α-SMA) was noted in CBX7$^{-/-}$ mice versus their WT counterparts at any time point (Fig. 7). In addition, large blood vessels emerged earlier in CBX7$^{-/-}$ mice (around day 14) in contrast to WT mice (around day 21).

Discussion

As a member of polycomb repressive complex 1 (PCR1) [12], CBX7 exerts its role of gene transcriptional regulator by binding specific sites on the chromatin structure, thus modulating the expression of specific genes by interacting with other factors [13, 14]. Reportedly, CBX7 is able to inhibit the expression of SPP1 gene via counteracting the positive transcriptional activity of HMGA1b on the SPP1 promoter [2]. SPP1 encodes protein named osteopontin (OPN) [15], which mainly exists in osteoblasts and benefits for osteoblastic proliferation and differentiation [3–5]. On the other hand, OPN facilitates bone resorption by anchoring osteoclasts to bone remodeling matrix with its bond to vitronectin receptor [6, 16]. Consequently, OPN is involved in both osteoblastic bone formation and osteoclastic bone resorption, which might also be affected
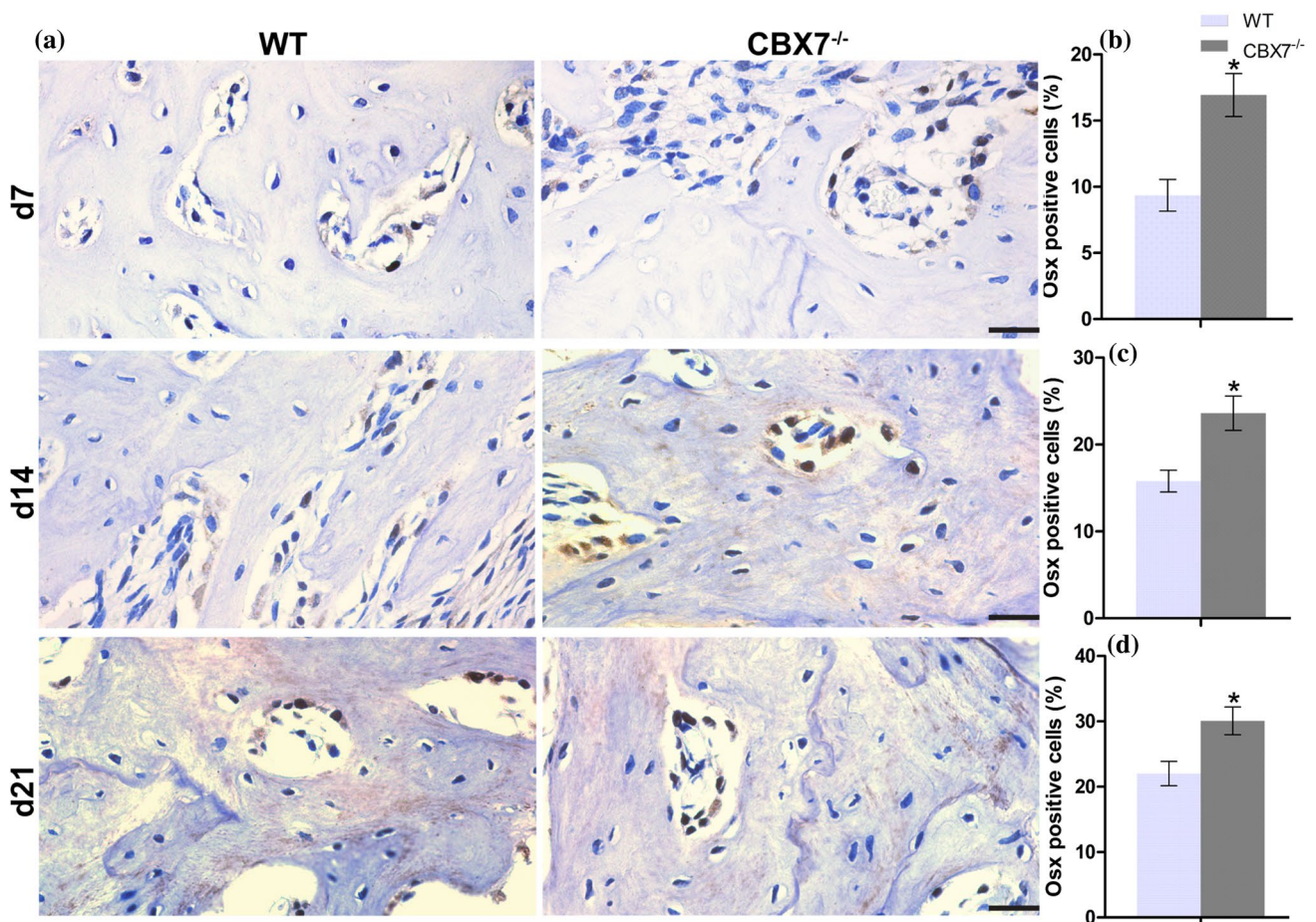
Fig. 5 Effects of CBX7 deficiency on osteoblastic bone formation in the sockets at different time points. Representative photomicrographs of extraction wound sections stained immunochemically for **a** osterix (Osx). Bar, 25 μm. The number of Osx-positive cells at extraction sites was determined by image analysis and the percentage of immunopositive cells relative to total cells is presented as mean ± SD of six pairs at **b** 7 days, **c** 14 days and **d** 21 days. *$p < 0.05$, in contrast to WT mice
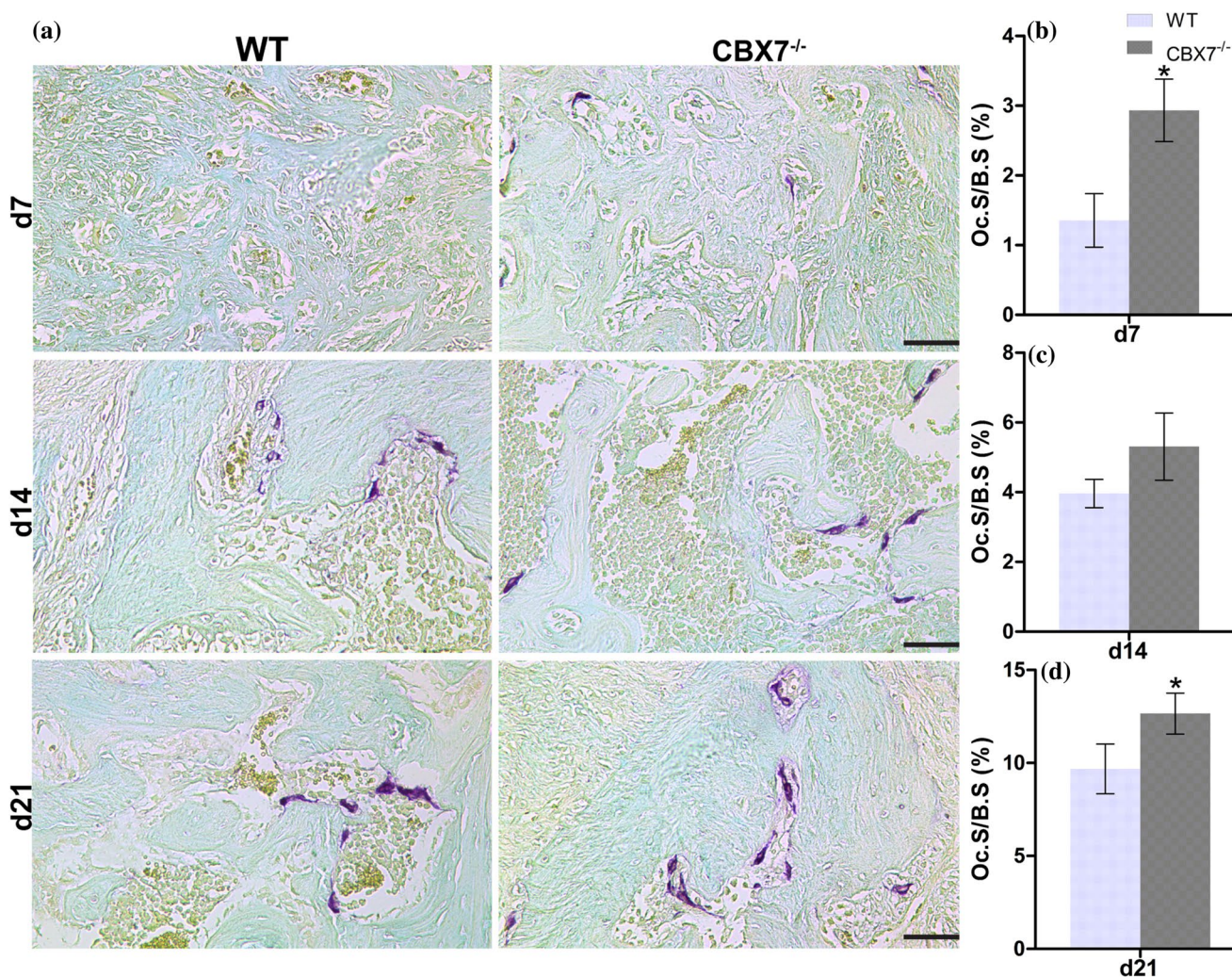
Fig. 6 Effects of CBX7 deficiency on osteoclastic bone resorption in the sockets at different time points. Representative photomicrographs of extraction wound sections stained histochemically for **a** tartrate-resistant acid phosphatase (TRAP) activity. Bar, 25 μm. The surface of osteoclasts relative to the bone surface (Oc.S/B.S) was determined in the TRAP-stained sockets at **b** 7 days, **c** 14 days and **d** 21 days. Values are the mean $\pm$ SD of six pairs in each time group. *$p < 0.05$, with respect to WT mice

by CBX7 due to its negative regulation on SPP1 expression. In a tooth extraction socket-healing scenario, woven bone formation increases with osteoblasts in predominance concomitantly with inflammation subsiding [10]. Due to its lack of load-bearing capacity, woven bone is then subject to bone remodeling with an increased number of osteoclasts [9]. Hence, we speculated that CBX7 may affect the socket healing after tooth extraction considering its potential role on bone formation and resorption via regulating SPP1 expression. In this study, CBX7$^{-/-}$ mice were employed to determine the effect of CBX7 deficiency on the socket healing (as shown in Fig. S2, CBX7 was expressed in osteoblasts/osteoclasts and epithelial cells during the healing of extraction sockets in WT mice). Our findings indicate that CBX7$^{-/-}$ mice exhibited higher bone metabolism in healing sockets with elevated osteoblastic

bone formation and osteoclastic bone resorption and displayed better soft tissue healing with increased angiogenesis at extraction sites. These results suggest that CBX7 deficiency may enhance the socket healing after tooth extractions.

The socket-healing process is clinically observed as the closure of socket entrance by firm epithelialized soft tissue and radiographic bone fill in the socket [9]. Although gross healing of extraction wounds was uneventful at all sites, a striking difference between CBX7$^{-/-}$ mice and their WT littermates was detected regarding the epithelial closure of socket entrance and the bone mass in the post-extraction socket. Visual inspection showed a rapider closure formed in CBX7$^{-/-}$ mice versus WT mice. Through micro-CT scanning, the bone mass was found to increase steadily with time regardless of genotype but a trend of higher bone level was
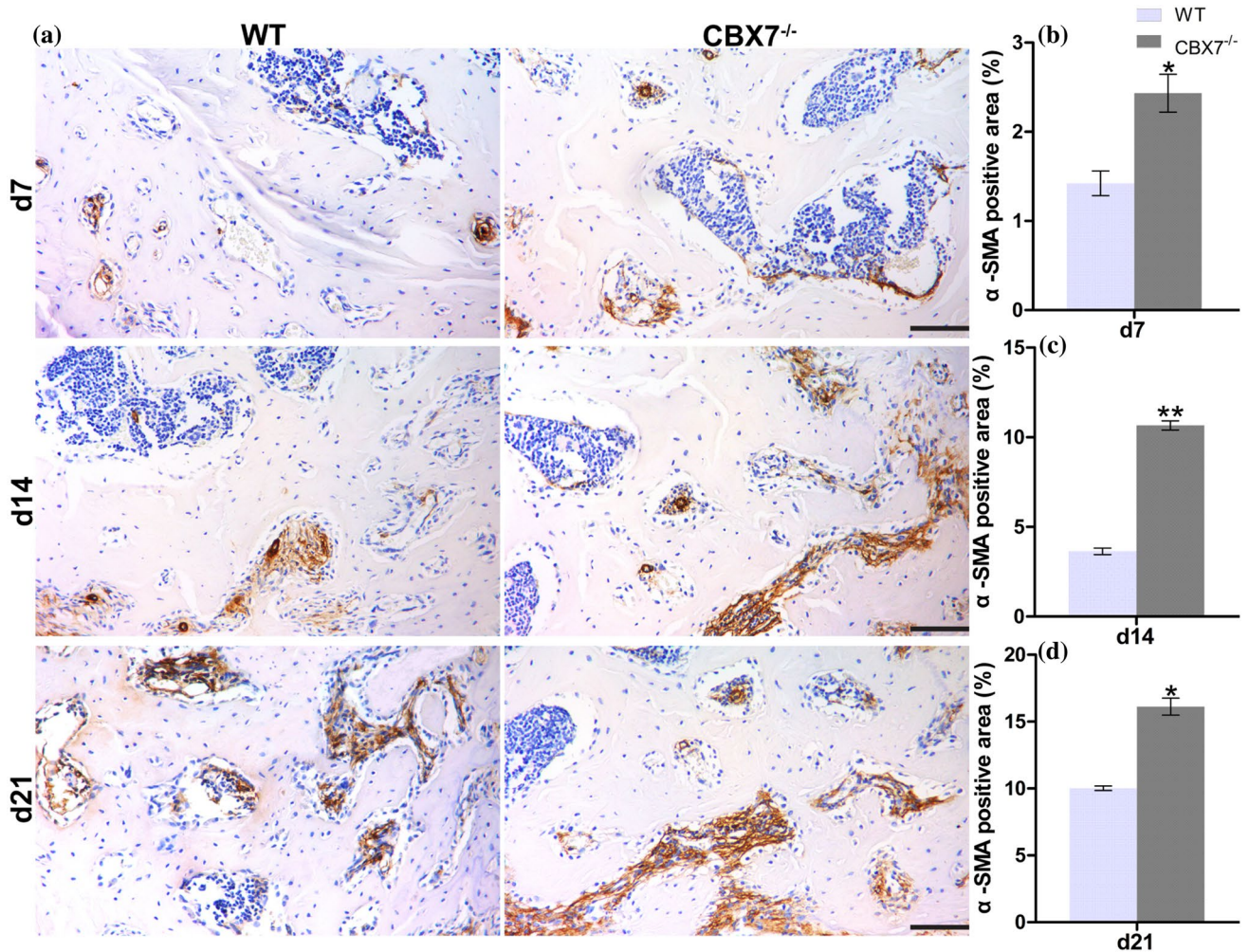
Fig. 7 Effects of CBX7 deficiency on angiogenesis in the sockets at different time points. Representative photomicrographs of extraction wound sections stained immunochemically for **a** alpha smooth muscle actin (α-SMA). Bar, 100 μm. α-SMA-immunopositive areas as a percentage of tissue areas at extraction sites are presented as mean±SD of six pairs at **b** 7 days, **c** 14 days and **d** 21 days. $*p < 0.05$, $**p < 0.01$ in contrast to WT mice

noted in CBX7$^{-/-}$ mice at each time point. Via histomorphometric analysis of Masson's trichrome-stained sections, the new bone fill in the socket exhibited the same tendency as radiological findings. Briefly, CBX7 deficiency accelerated the epithelial closure of socket entrance and increased the bone mass in the socket.

Highlighted by the increasing demand for dental esthetics, processes involved in the socket healing have been studied widely [9, 17–23]. In humans, the bone formation and remodeling activities, observed with great individual variation, are considered essential for the socket healing [11, 24]. OPN, a secreted phosphoprotein negatively regulated by CBX7 [2], is abundant in osteoblasts and mineralized tissues [25]. Moreover, it can facilitate attachment of osteoclasts to bone surface and modulate osteoclast differentiation in vitro [16]. Hence, OPN has long been implicated in bone formation and remodeling, the key portions of the socket

healing. We then evaluated the percentage of OPN-immunopositive cells in the socket and found a time increase in the percentage irrespective of genotype but a tendency of a larger percentage in CBX7$^{-/-}$ mice at each time point. Osx, as another specific marker of bone formation, exhibited the similar trend as OPN in post-extraction socket. Considering OPN's established role on osteoclasts, we thereby measured the ratio of osteoclast surface to bone surface (Oc.S/B.S), which exhibited the same trend as OPN-immunohistochemical results. These observations signify that CBX7 deficiency promoted the presence of OPN-stained cells, which benefited for bone formation and subsequently boosted TRAP-positive osteoclasts in the socket. Combined with radiographic and Masson's trichrome stain findings, it is conceivable that the net increase of bone mass in the socket following CBX7 deficiency may result from augmented bone formation surpassing augmented bone resorption.

Bone remodeling, a pivot part of socket healing, requires new blood vessel formation for supplying nutrients, oxygen, growth factors, cytokines and osteoblast and osteoclast precursors [26, 27]. As a pericyte marker, alpha smooth muscle actin (α-SMA) is widely used to label blood vessels [28]. Thus, we use α-SMA-immunohistochemical staining to depict time-dependent changes in amount of newly formed vessels involved in post-extraction wound healing. Corresponding to time-increasing bone metabolism, α-SMA-positive area was elevated with time regardless of genotype. Consistent with more vigorous bone formation and remodeling, a greater vascularization was noted in CBX7$^{-/-}$ mice at each time point. Consequently, CBX7 deficiency may promote angiogenesis in extraction sites to satisfy robust bone metabolism.

In summary, by comparing phenotypes of post-extraction sockets in CBX7$^{-/-}$ mice and their WT littermates, our results demonstrate that CBX7 deficiency leads to faster-formed epithelial closure of socket entrance and steadily increases bone mass in the socket by augmenting OPN-positive cells, which benefit for bone formation and subsequently accelerate osteoclastic bone resorption in extraction sites. To match higher bone metabolism, CBX7 deficiency enhances new blood vessel formation as well. Hence, we identify that CBX7 deficiency may facilitate the socket healing after tooth extractions.

Compliance with ethical standards

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