Proenkephalin Inhibits the Osteogenic Differentiation of Bone Marrow–derived Mesenchymal Stem Cells

Xin WANG[1,#], Zhu Qing WAN[1,#], Long Wei LV[1]

Objective: *To investigate the regulatory role of proenkephalin (PENK) on the osteogenic differentiation process of human bone marrow mesenchymal stem cells (hBMMSCs).*
Methods: *PENK knockdown and overexpression in hBMMSCs were performed by using lentivirus containing the specific short hairpin RNA or whole coding region for PENK in vitro. The alkaline phosphatase level and mineralised nodule formation were observed by alkaline phosphatase (ALP) and Alizarin Red S (ARS) staining and quantification. Meanwhile, the expression levels of osteogenic-related mRNA and protein were detected by qRT-PCR and Western blot.*
Results: *Transfection of lentivirus in hBMMSCs was effectively confirmed by the green fluorescent protein-positive cells under fluorescence microscopy, and qRT-PCR and Western blot analyses verified the successful establishment of PENK knockdown and overexpression hBMMSCs. The results showed that knockdown of PENK effectively promoted the osteogenic differentiation of hBMMSCs, with enhanced ALP and ARS staining, and increased expression levels of osteogenic-related genes and proteins. Conversely, the overexpression of PENK inhibited the osteogenic differentiation of hBMMSCs.*
Conclusion: *This finding identified PENK as a potential therapeutic target for bone regeneration of oral and maxillofacial bone defects.*
Keywords: *bone regeneration, human bone marrow mesenchymal stem cells, neuropeptide, osteogenic differentiation, proenkephalin*
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The incidence of oral and maxillofacial bone defects caused by trauma, infections, tumours and other aetiological factors remains significantly high.[1] Current clinical management of these defects still faces challenges, such as limited availability of autogenous bone donors, immunogenic risks of allogeneic and xenogeneic bone grafts, as well as lack of osteoinductivity of titanium implants.[2,3] Consequently, investigation of the molecular mechanisms governing bone regeneration is expected to offer essential theoretical insights to guide the development of novel therapeutic approaches for bone defect repair.

Proenkephalin (PENK), a key precursor of endogenous opioid peptides, undergoes enzymatic cleavage to generate two enkephalins: met-enkephalin (MENK) and leu-enkephalin (LENK).[5] These neuropeptides are mainly distributed in the central and peripheral nervous systems, where they are involved in the modulation of memory, pain perception and reward-seeking behaviours by activating μ and δ opioid receptors.[4] For instance, the subpopulation of neurons in basolateral amygdala associated with long-term memory imprint-

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ing exhibited significant upregulated expression of PENK, and PENK-positive neuronal cells within the ventral part of the lateral geniculate nucleus played a role in mediating analgesic responses to green light.[5,6] Moreover, it has been shown that PENK is highly expressed and plays a crucial role in the regulation of multiple organs and tissues, including the heart and kidneys. For example, elevated PENK levels have been correlated with adverse remodelling post-myocardial infarction,[7] and have also enhanced risk stratification for renal dysfunction and mortality in patients with acute coronary syndromes.[8,9] PENK also modulates apoptosis and cell survival pathways, making it a promising biomarker for disease monitoring.[10] Notably, PENK has been identified as a diagnostic biomarker across multiple cancer types and has been shown to inhibit osteosarcoma cell migration by modulating the PI3K/AKT signalling pathway.[11-14]

In addition, previous studies indicated that PENK was expressed in bone tissues and intensively involved in the pathogenesis of bone-related diseases. For instance, PENK was identified as a key gene expressed in the pathological lesions of type 2 diabetes patients with complications caused by osteoarthritis, and it was differentially expressed in ectopic ossification.[15,16] A recent study from the present authors' research group found that bone injury stimulation significantly activated the expression level of PENK in mesenchymal stem cells (MSCs), and that PENK may play a role in pain sensing during bone regeneration.[17] However, its role in regulating bone regeneration remains unclear.

Therefore, this study investigated the effect of PENK on the osteogenic differentiation of hBMMSCs. Specifically, the present authors established PENK knockdown and overexpression hBMMSCs through lentiviral transfection. Results from ALP activity assays, ARS staining, qRT-PCR and Western blot analyses collectively demonstrated that PENK knockdown facilitated osteogenic differentiation, whereas its overexpression inhibited this process. These results demonstrate that PENK acts as a "negative regulator" of osteogenic differentiation in hBMMSCs and indicate that suppressing its function ould provide novel strategies for enhancing bone regeneration.

Materials and methods

Cell culture

hBMMSCs (obtained from ScienCell, San Diego, CA, USA) were cultured in a proliferation medium (PM) consisting of α-MEM supplemented with 10% (v/v) foetal bovine serum (FBS) and penicillin/streptomycin, under a 5% CO_2 atmosphere at 37°C. For osteogenic differentiation, cells were transitioned to osteogenic medium (OM), which included α-MEM with 10% (v/v) FBS, penicillin/streptomycin, 10 nM dexamethasone, 10 mM β-glycerophosphate and 0.2 mM ascorbic acid, once cell density reached 70%. Furthermore, the culture media were refreshed every 2 days to ensure optimal growth conditions.

Lentiviral transfection

Lentiviruses used for the PENK-knockdown experiments, including sh*PENK*-1, sh*PENK*-2 and sh*PENK*-3, along with the negative control (NC), as well as the PENK-overexpression group and control vector, were purchased from GenePharma (Shanghai, China), with the specific sequences detailed in Table 1. Once the hBMMSCs reached a fusion density of approximately 30% to 40%, a viral suspension (1~8 × 10^8 TU/mL, GenePharma) was introduced into the cell culture media with polybrene (5 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) to facilitate transduction. After 24 hours of transfection, the medium was changed and puromycin (1 μg/ml, Sigma-Aldrich) was added to select the transfected hBMMSCs.

ALP staining and quantification

hBMMSCs were plated at a density of 4 × 10^3 cells per well and cultured for 7 days in both PM and OM. On day 7, ALP staining was conducted using the BCIP/NBT alkaline phosphatase detection kit (Beyotime, Shanghai, China), and the stained images were captured with a scanner. Following this, ALP activity was assessed according to the protocol of the alkaline phosphatase assay kit (Beyotime). The ALP levels were quantified using the kit and normalised to the total protein concentration in each sample.

ARS staining and quantification

On the 14th day of culture, hBMMSCs were stained using a 1% ARS solution. Stained images were subsequently captured with a scanner. Finally, the stained cells were solubilised in 100 mM cetylpyridinium chloride (Sigma-Aldrich), and the absorbance of the resulting solution was measured at wavelength of 562 nm.

Table 1 Sequences of DNA oligonucleotides and RNA.

Name		Sense strand/sense primer (5'-3')	Antisense strand/antisense primer (3'-5')
shRNA	NC	TTCTCCGAACGTGTCACGT	-
	sh*PENK*-1	GCTTGCGTAATGGAATGTGAA	-
	sh*PENK*-2	GCAAACCGGAAGAAAGCCATT	-
	sh*PENK*-3	GGTGGATGGACTACCAGAAAC	-
Primers	*GAPDH*	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
	ALP	ATGGGATGGGTGTCTCCACA	CCACGAAGGGGAACTTGTC
	RUNX2	GAACCACAAGTGCGGTGCAA	ACTGCTTGCAGCCTTAAATGACT
	PENK	ATCCGAACAGCGTCAACTCCAT	GAGCAACAGCAGCCAAGTGC
	OCN	ACACTCCTCGCCCTATTGGC	TGCTTGGACACAAAGGCTGC
	OSX	CCTCCTCAGCTCACCTTCTC	GTTGGGAGCCCAAATAGAAA

ALP, alkaline phosphatase; NC, negative control for shPENK-1, shPENK-2 and shPENK-3; OCN, osteocalcin; shRNA, OSX, osterix; RUNX2, runt-related transcription factor 2; short-hairpin RNA; β-actin, β-cytoplasmic actin.

Quantitative real-time reverse transcription PCR (qRT-PCR)

After 7 days of osteogenic induction of transfected hBMMSCs, total RNA was isolated from cultured cells employing Trizol (Thermo Fisher Scientific, Waltham, MA, USA). The purity and concentration of the extracted RNA were evaluated with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific). Following this, total RNA underwent reverse transcription to generate complementary DNA (cDNA) using a PrimeScript RT kit (AG Scientific, San Diego, CA, USA). The resulting cDNA was then analysed through qRT-PCR employing SYBR Green Master Mix (AG Scientific). The primer sequences for the target genes can be found in Table 1, with β-actin utilised as an internal control. Lastly, gene expression levels were quantified according to the $2^{-\Delta\Delta Ct}$ method for analysis.

Western blot

Following 7 days of osteogenic induction of the transfected hBMMSCs, the protein expression levels in the cells were evaluated. Initially, the cells were lysed using a lysis buffer containing 1% protease and phosphatase inhibitors (NCM, Suzhou, China). The resulting protein extracts underwent separation by 10% SDS-PAGE, followed by transfer to polyvinylidene difluoride (PVDF) membranes. The membranes were then incubated overnight with primary antibodies targeting the nuclear markers β-actin (ABclonal, AC062, Wuhan, China), PENK (ABclonal, A6302) and RUNX2 (ABclonal, A11753). Afterwards, the membranes were treated with a peroxidase-conjugated secondary antibody at room temperature for 1 hour. The immunoreactive protein bands were subsequently visualised using an enhanced chemiluminescence detection kit (NCM).

Quantification and statistical analysis

Statistical analyses were conducted using SPSS Statistics version 24.0 (IBM, Armonk, NY, USA). Comparisons between two groups were performed using a Student *t* test. All data are expressed as mean ± standard deviation (SD) derived from three independent experimental replicates for each group. Statistical significance was considered as $^{*}P < 0.05$; $^{**}P < 0.01$.

Results

Construction of PENK knockdown hBMMSCs cell lines

To investigate the regulatory role of PENK in the osteogenic differentiation of hBMMSCs, lentiviral transfection was employed to knock down PENK expression in these cells. To minimise off-target effects, the present authors designed three distinct knockdown sequences, which resulted in the establishment of PENK knockdown hBMMSCs cell lines: shPENK-1, shPENK-2 and shPENK-3. The negative control group was named NC. The knockdown efficiency of PENK was assessed by observing the fluorescence brightness and intensity of green fluorescent protein-positive cells and the expression levels of mRNA and protein via qRT-PCR and Western blot techniques.

As illustrated in Fig 1a, the transfected cells exhibited an even distribution under the light microscope. The fluorescence images revealed a broad distribution

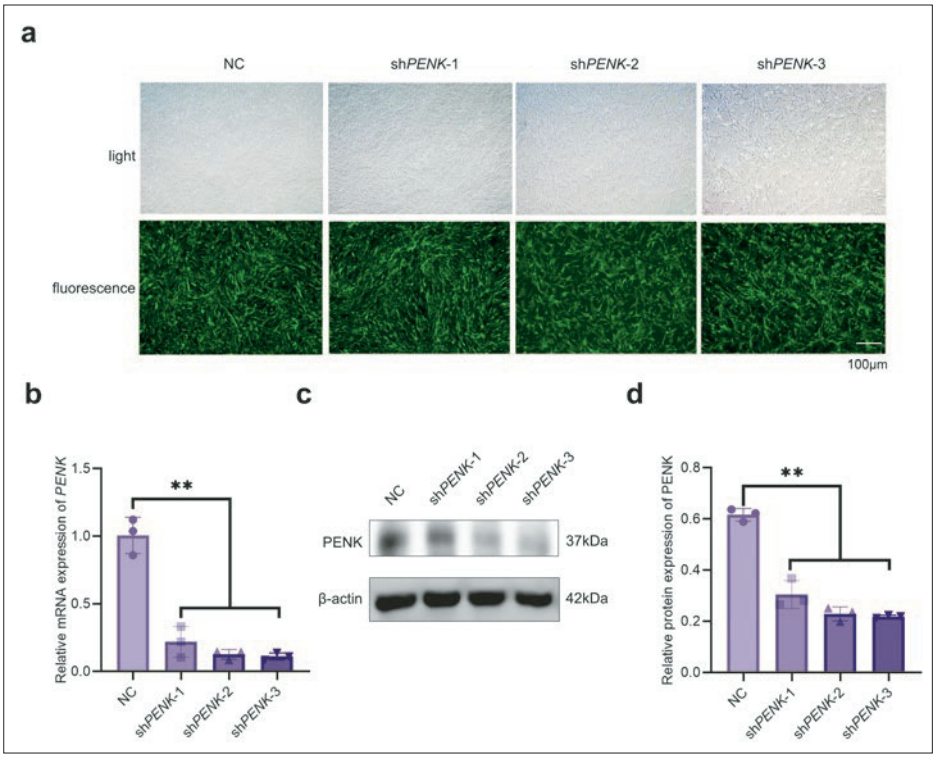

Fig 1a to d Transfection efficiency of PENK knockdown hBMMSCs cell lines. Transfection efficiency shown by fluorescence microscopy (a). Knockdown efficiency of PENK determined by qRT-PCR and Western blot **(b to d)**. $^{**}P < 0.01$.

of green fluorescent protein-positive cells, which displayed strong fluorescence intensity, indicating a high transfection efficiency. The experimental results from qRT-PCR (Fig 1b) demonstrated a significant reduction in the mRNA expression level of PENK in the PENK knockdown groups compared to the control group. Similarly, the findings from the Western blot analysis and its quantification (Fig 1c) showed a consistent trend, confirming the successful establishment of the PENK knockdown hBMMSCs.

PENK knockdown enhanced osteogenic differentiation in hBMMSCs

After culturing the transfected hBMMSCs in PM and OM for 7 days, ALP staining was performed on the PENK knockdown hBMMSCs groups (shPENK-1, shPENK-2 and shPENK-3) and the control group (NC) to investigate the role of PENK in osteogenic differentiation. The results of ALP staining (Fig 2a and b) demonstrated that following osteogenic induction, the PENK knockdown groups exhibited significantly darker staining compared to the control group, suggesting that PENK knockdown promoted the osteogenic differentiation of hBMMSCs. After 14 days of culturing the cells in PM and OM, ARS staining results (Fig 2c) revealed that the PENK knockdown groups showed darker staining and an increased number of mineralised nodules compared to the control group (NC), indicating that PENK knockdown significantly enhanced osteogenic differentiation of hBMMSCs.

The qRT-PCR results indicated that the expression levels of the ALP, OCN and RUNX2 genes were significantly higher in the PENK knockdown groups compared to the control group (Fig 3a). Western blot analysis and its quantitative results (Fig 3b and c) revealed that the protein expression level of RUNX2 was markedly elevated in the PENK knockdown groups relative to the control group, with a consistent trend observed across all three sequences. Based on these findings, it is possible to conclude that PENK knockdown effectively promoted the osteogenic differentiation of hBMMSCs.

Construction of PENK overexpressing hBMMSCs cell lines

Additionally, PENK-overexpressing hBMMSCs were established in this study. Fluorescence microscopy observations (Fig 4a) revealed that green fluorescent protein was extensively distributed in both the PENK overexpression group (PENK) and the control group (vector), indicating efficient transfection. qRT-PCR and Western

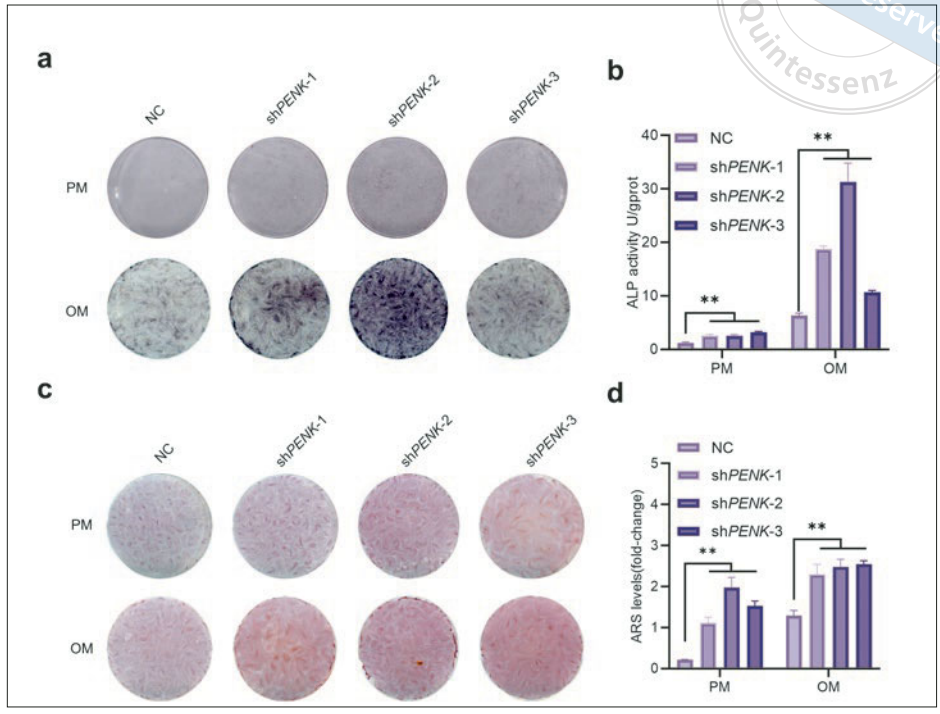

Fig 2a to d Knockdown of PENK enhanced the osteogenic capacity of hBMMSCs by ALP and ARS assays. Knockdown of PENK enhanced the ALP activity of hBMMSCs' osteogenic induction as shown by ALP staining and quantification (a and b). Knockdown of PENK accelerated mineralisation of hBMMSCs' osteogenic induction as shown by ARS staining and quantification (c and d). $^{**}P < 0.01$.

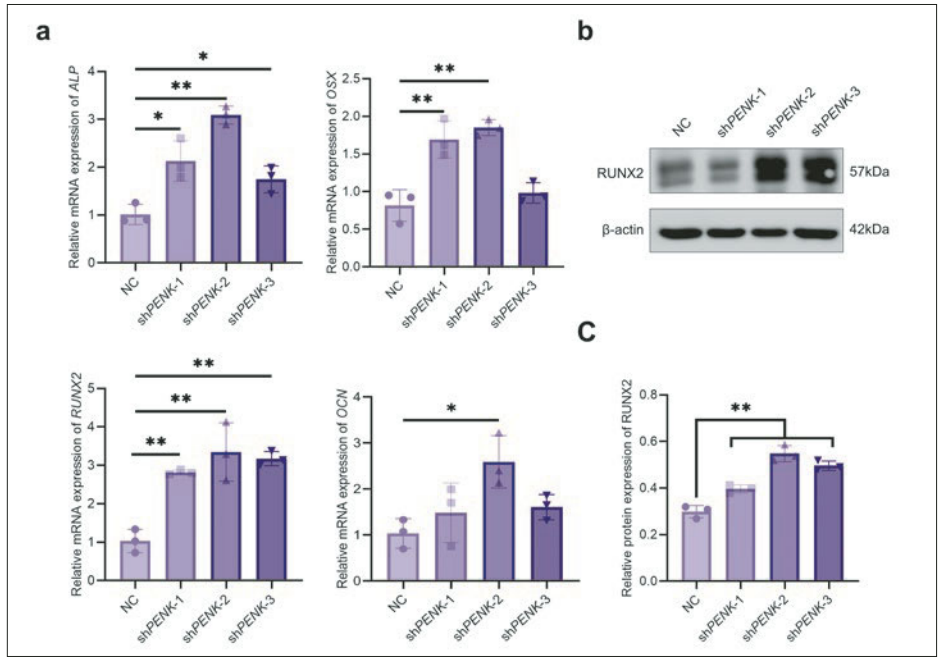

Fig 3a to c Knockdown of PENK enhanced the osteogenic differentiation of hBMMSCs by qRT-PCR and Western blot. ALP, OSX, RUNX2 and OCN mRNA expression during the osteogenic induction process tested by qRT-PCR (a). RUNX2 protein expression tested by Western blot and quantification (b and c). $^{*}P < 0.05$; $^{**}P < 0.01$.

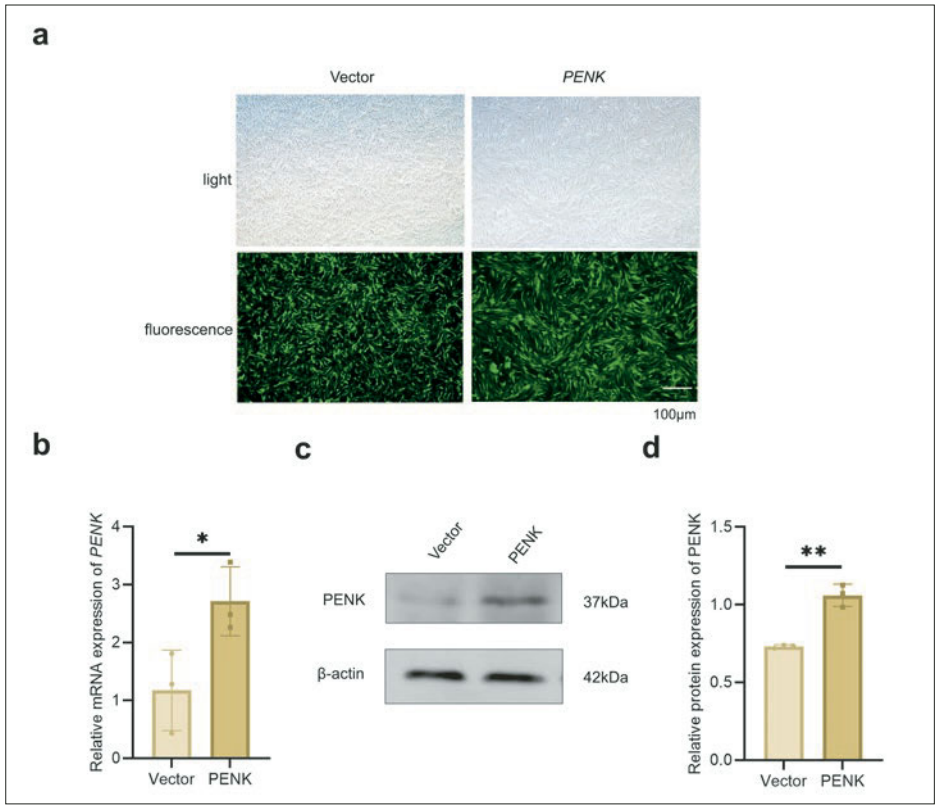

Fig 4a to d Transfection efficiency of PENK overexpression of hBMMSCs cell lines. Transfection efficiency shown by fluorescence microscopy (a). PENK mRNA and PENK protein determined by qRT-PCR, Western blot and quantification **(b to d)**. *$P < 0.05$; **$P < 0.01$.

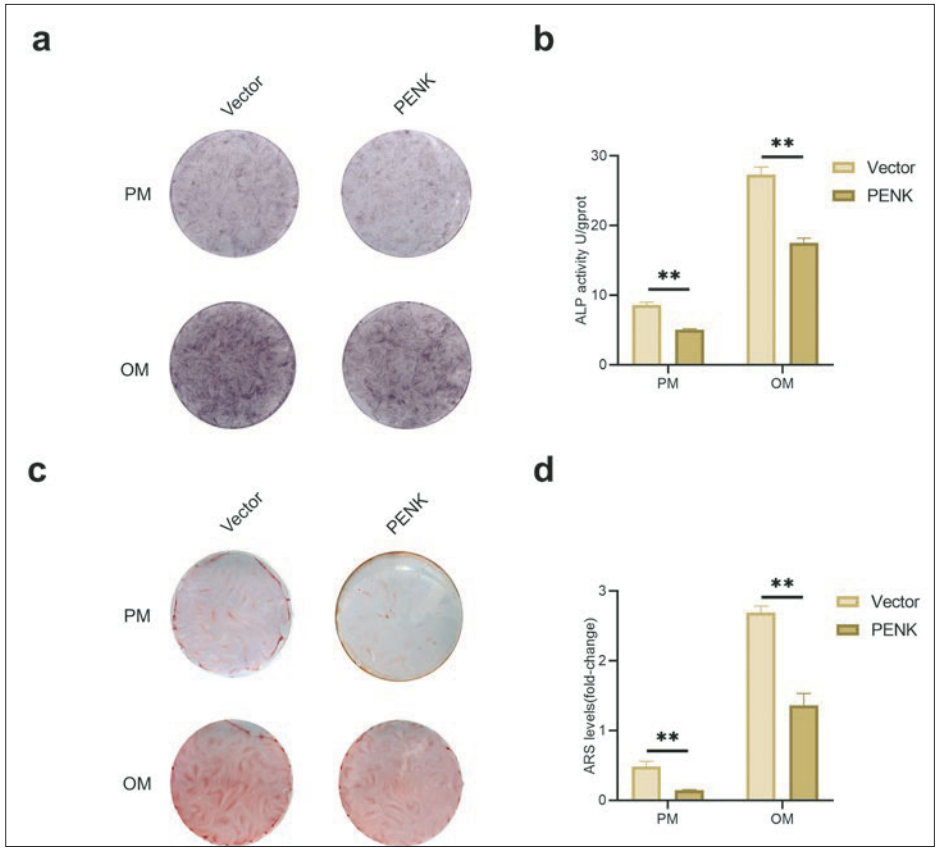

Fig 5a to d Overexpression of PENK inhibited the osteogenic differentiation of hBMMSCs by ALP and ARS assays. Overexpression of PENK inhibited the ALP activity of hBMMSCs' osteogenic induction as shown by ALP staining and quantification (a and b). Overexpression of PENK inhibited the mineralisation of hBMMSCs after osteogenic induction as shown by ARS staining and quantification (c and d). **$P < 0.01$.

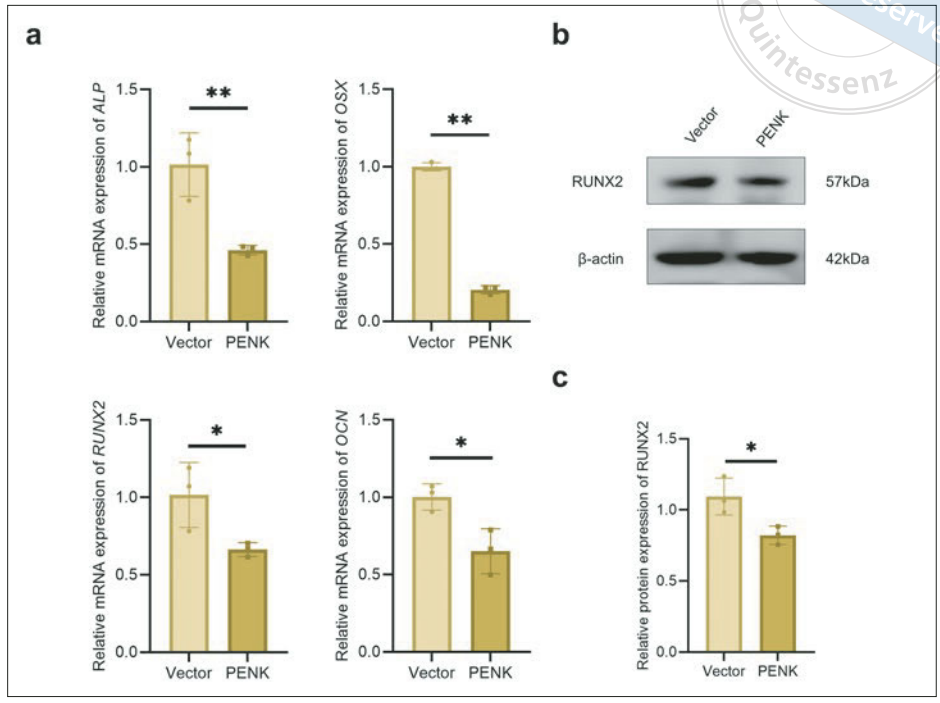

Fig 6a to c Overexpression of PENK inhibited the osteogenic differentiation of hBMMSCs by qRT-PCR and Western blot. ALP, OSX, RUNX2 and OCN mRNA expression during the osteogenic induction process tested by qRT-PCR (a). RUNX2 protein tested by Western blot and quantification (b and c). *$P < 0.05$; **$P < 0.01$.

blot analyses (Fig 4b to d) demonstrated that, compared to the vector group, the PENK group exhibited significantly elevated expression levels of both *PENK* mRNA and PENK protein. These findings confirmed the successful construction of PENK-overexpressing hBMMSCs.

PENK overexpression inhibited the osteogenic differentiation of hBMMSCs

The effects of PENK overexpression on osteogenic differentiation are presented in Fig 5 and 6. ALP staining and quantification results (Fig 5a and b) indicated that the PENK overexpression group exhibited significantly lighter staining compared to the control group. Similarly, ARS staining and quantification (Fig 5c and d) revealed a comparable trend.

The qRT-PCR analysis (Fig 6a) showed a significant reduction in the expression levels of ALP, OSX, RUNX2 and OCN of the PENK overexpression group compared to the control group. In contrast, Western blot and corresponding quantitative assessments (Fig 6b and c) showed a distinct increase in RUNX2 protein expression in the PENK overexpression group relative to the control group. Taken together, these findings suggest that PENK overexpression inhibits the osteogenic differentiation of hBMMSCs. In conclusion, these results demonstrate that PENK overexpression negatively regulates the osteogenic differentiation of hBMMSCs.

Discussion

Accelerating bone healing and restoring functional integrity within minimal timeframes remain primary objectives of oral and maxillofacial bone defect repair.[18] As a result, uncovering the molecular pathways that facilitate defect repair and developing innovative strategies to enhance bone regeneration have emerged as critical priorities in contemporary research on bone defect management.[19] Recent studies have illustrated that neuropeptides, including serotonin, neuropeptide Y and endorphins, play significant roles in promoting bone regeneration.[20-22] These findings underscore their potential as promising therapeutic targets for advancing treatment options in this field.

Endogenous opioid peptides represent a class of neuropeptides that interact with opioid receptors and are primarily derived from three precursor proteins: prodynorphin, PENK and pro-opiomelanocortin.[23,24] It is noteworthy that PENK is the earliest identified neuropeptide precursor characterised by its highest concentration in the brain, and primarily contributes to a variety of neural regulatory functions through its hydrolysis to produce enkephalins.[25-27] In recent years, studies have revealed that PENK was not only implicated in neuromodulation but may also influence the physiological and pathological processes of bone tissues through endocrine or paracrine mechanisms.

There are intricate interactions between the nervous system and bone tissue. Within this neuro-osteogenic crosstalk, PENK, as a neuropeptide precursor, may modulate bone regeneration through regulation of neurotransmitter and hormone secretion.[2]8 However, the effects of PENK on the proliferation and differentiation of bone cells and MSCs are still unclear.

Previous studies have demonstrated that multiple cell types within bone tissue could synthesise and express PENK. For instance, Kew and Kilpatrick[29] found that PENK was broadly expressed in various mesodermal cells during organogenesis, with bone and cartilage representing the mesodermal tissues with the highest concentration of PENK. Subsequently, Elhassan et al[30] utilised electron and immunofluorescence microscopy to investigate the distribution of MENK-positive cell expression in osteoblasts and neurons within joint and bone tissues, revealing comparatively lower levels in osteoblasts. These findings collectively suggest that both neuronal and osteoblastic cells within bone tissues are capable of synthesising and expressing peptides derived from PENK.

While it has been established that PENK was expressed in bone tissue, debate remains regarding its regulatory role in bone regeneration. Wan et al[28] found that PENK has tissue-specific expression in chondrocytes during murine nasal development, whereas its function for nasal capsule morphogenesis is not clear. Furthermore, research indicates that PENK expression in osteoblasts is confined to the mineralisation stage, suggesting its potential involvement in neuron-mediated bone regeneration and remodelling processes.[31] Puri et al[32] further demonstrated that pre-proenkephalin 1 (Penk1) regulated the response of the bone to mechanical uploading, indicating the interactions between neuropeptides and skeletal tissues.

The present authors' previous study revealed that bone injury stimulation activated the expression level of PENK in MSCs.[17] Thus, the present study further investigated the osteogenic regulatory of PENK in hBMMSCs. MSCs constitute a pivotal cell lineage within the realm of bone tissue engineering research, and the study focused on MSCs holds promise for pioneering innovative strategies in bone tissue engineering. Specifically, PENK knockdown and overexpression hBMMSCs were established, and their osteogenic differentiation potential was assessed. Thus, the results showed that PENK served as a "negative regulator" of osteogenic differentiation in hBMMSCs.

The present experimental results align with previous studies showing that MENK, a peptide derived from PENK, inhibited the osteoblast alkaline phosphatase activity, thereby negatively regulating osteoblast-specific gene expression.[31,33] This finding suggests that MENK may exert an inhibitory influence on the mineralisation of the bone matrix or in the bone remodelling process. Furthermore, a recent clinical study established a link between opioid use and elevated risk of fractures.[34] Mechanistically, opioids, acting via the same opioid receptors activated by PENK-derived enkephalins, reduced bone mineral density by suppressing endogenous hormone production, which consequently elevated fracture risk. In combination with these previous studies, the present study further clarified the important function of PENK in inhibiting bone regeneration, paving the way for future studies to dissect the underlying mechanisms of PENK involved in bone development and regeneration.

However, to further reveal PENK's critical role in bone regeneration, more experiments with PENK mutants and pharmaceutical treatments need to be constructed to provide strong support for the in-depth investigation of its function. Currently, studies on the role of PENK in bone regeneration remain in the early stages, and therapeutic strategies aimed at performing bone regeneration by inhibiting the function of PENK continue to face challenges. First, the mechanisms by which PENK regulates bone regeneration in vitro and in vivo have not yet been clarified. In-depth exploration of the underlying mechanisms is necessary to provide a theoretical basis for the further design of PENK-targeted treatment strategies. It also remains challenging to screen and obtain highly efficient inhibitors of PENK for bone regeneration, determine their optimal dosages and efficacy, maintain drug stability and develop appropriate delivery systems. Thus, further studies are required to explore and harness the potential role of PENK in bone regeneration in the future.

Conclusion

In this study, the present authors demonstrated that PENK knockdown enhances the osteogenic differentiation potential of hBMMSCs, whereas the overexpression of PENK downregulates the osteogenic capacity of hBMMSCs. These findings suggest that PENK plays an inhibitory role in osteogenic differentiation of MSCs, identifying it as a novel therapeutic target for craniofacial bone defect repair. Furthermore, the results provide new insights into the functional role of PENK in bone regeneration and repair, advancing the field of medical repair and reconstruction of craniofacial bone defects and highlighting its translational prospects for craniofacial bone regeneration.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Xin WANG performed the experiments and data analyses and drafted the manuscript; Dr Zhu Qing WAN performed the experiments and data analyses and revised the manuscript; Dr Long Wei LV conceived the study, supervised the project and experiments and revised the manuscript. All authors read and agreed to the final version of the manuscript.

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