Journal of Oral Biology and Craniofacial Research 16 (2026) 222–225

Contents lists available at ScienceDirect

Journal of Oral Biology and Craniofacial Research

journal homepage: www.elsevier.com/locate/jobcr

Evaluating the role of a novel homeodomain protein in periodontal regenerative therapy: An in-vitro study

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ARTICLE INFO

Keywords:
Homeodomain proteins
Regeneration
Stem cells
Cell differentiation
Wound healing
Periodontal diseases

ABSTRACT

Introduction: The goal of periodontal treatment is to halt disease progression and restore the structure and function of damaged periodontal tissues. Homeodomains—transcription factors prominently expressed during limb bud formation and craniofacial development—are abundantly present in the periosteum, and their expression continues in postnatal descendant cells, where they play a key role in bone homeostasis and fracture healing. These factors regulate stem cell differentiation critical for cementogenesis, osteogenesis, and periodontal ligament (PDL) regeneration, making them attractive targets for periodontal tissue engineering and regenerative therapies. A deeper understanding of homeodomain protein functions could lead to innovative treatments for periodontal disease and alveolar bone defects. In this context, our study investigated the role of homeodomain proteins in periodontal regeneration.

Methods: Periosteum tissue was collected from a fracture site, and protein extracts were prepared. MTT and in vitro wound-healing assays were performed using a PDL cell line co-cultured with homeodomain protein (test group) and PDL cells alone (control group).

Results: The MTT assay revealed that a concentration of 5 μg/mL yielded the highest cell viability. In the wound-healing assay, significant differences between the control and test groups demonstrated that homeodomain expression enhances cell migration and proliferation.

Conclusion: Homeodomain protein expression in the periosteum could serve as a novel biomarker for periodontal regeneration.

1. Introduction

Periodontal diseases including periodontitis, affect over 50 % of the global population, posing significant public health challenges. These conditions involve chronic inflammation triggered by bacteria, leading to deterioration of the tooth-supporting structures—namely, the periodontal ligament (PDL), cementum, and alveolar bone.[1] The primary objective of periodontal treatment is to halt disease progression and restore both the structure and function of the affected tissues. Recent advancements in periodontal research and treatment offer promising avenues for improved patient management and outcomes. Among these emerging strategies, stem cell therapy is receiving increasing attention for its potential to regenerate periodontal tissues.

Homeodomains are transcription factors that are highly expressed by mesenchymal stem cells during limb bud formation and craniofacial development.[2] Cells expressing homeodomain proteins, along with their postnatal progeny, also play a crucial role in bone homeostasis and fracture repair. This is evidenced by findings that inactivation of RANKL (receptor activator of nuclear factor kB ligand) expression in these cells causes osteopetrosis, while inactivation of BMP2 (bone morphogenetic protein-2) expression results in a low-bone-mass phenotype characterized by bones that are prone to fractures and incapable of repair.[3] The periosteum is critical for fracture repair, as its removal significantly impairs the healing process. In culture, homeodomain-expressing cells isolated from the periosteum of postnatal long bones have demonstrated the capacity to differentiate into chondroblasts and osteoblasts upon induction.[4]

Homeodomain proteins are defined by the presence of a conserved 60-amino-acid DNA-binding motif known as the homeodomain.[5] These proteins are pivotal in regulating gene expression during development, differentiation, and tissue homeostasis. Their concentrations vary within the periodontium (comprising the gingiva, PDL, cementum, and alveolar

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https://doi.org/10.1016/j.jobcr.2025.12.004
Received 28 August 2025; Received in revised form 11 November 2025; Accepted 5 December 2025
Available online 11 December 2025

bone), depending on the functional state of the tissue. Homeodomain proteins are essential for the formation, remodeling, and regeneration of these periodontal structures.

Several homeobox genes have been identified as key regulators within the periodontium. For example, Msx1 and Msx2 (muscle segment homeobox genes) are expressed in the PDL, cementoblasts, and osteoblasts, where they contribute to tooth root and alveolar bone development. Mutations in Msx1 are associated with cleft palate and tooth agenesis.[6] Dlx genes (distal-less homeobox genes) are essential for craniofacial and tooth development. Within the periodontium, Dlx5 and Dlx6 regulate osteogenesis and cementum formation, promoting mineralization. Defects in Dlx genes can result in abnormal tooth root development and periodontal defects.[7] The Barx1 gene (BarH-like homeobox gene) influences PDL fibroblast differentiation and bone remodeling, and its disruption has been shown to impair periodontium development.[8] Pitx2 (paired-like homeodomain transcription factor 2) regulates dental and craniofacial development. In the periodontium, it controls the differentiation of dental mesenchymal cells and influences cementoblast formation and fibroblast activity.[9]

Homeodomain proteins are also involved in periodontal regeneration, playing roles in wound healing, tissue repair, and stem cell differentiation. Msx and Dlx proteins have been implicated in stem cell-mediated periodontal regeneration. Targeting homeodomain proteins through gene therapy or biomaterial-based approaches represents a promising strategy for promoting periodontal regeneration. However, limited research has explored the regenerative potential of homeodomain-expressing cells and their role in the development of PDL cells. Elucidating the specific roles of these proteins in periodontal regeneration could provide critical insights for developing innovative, cell-based therapies for periodontal disease. Therefore, the aim of the present study was to assess the potential role of the homeodomain protein PRX1 in periodontal regeneration. This study seeks to isolate and quantify homeodomain proteins from tissue samples obtained from fracture sites, and to evaluate the in vitro characteristics of homeodomain proteins and PDL cells.

2. Methods

2.1. Ethical approval and sample collection

Informed consent was obtained from the subject who provided the periosteum sample. The study was approved by the Ethics Committee for Human Trials at Ramaiah University (EC-23/109-PG-FDS).

2.2. Homeodomain isolation

A 5 g periosteum tissue sample was obtained from a mandibular fracture site (para symphysis fracture) that was exposed during reconstructive surgery for fracture reduction. The tissue was weighed on an analytical balance, processed, and centrifuged to yield a supernatant containing the protein extract. The protein was then isolated, quantified using Bradford's reagent, and its concentration determined. The Homeodomain protein PRX1 was identified using ELISA kit (Abcam Human Peroxiredoxin 1 (PRX1) ELISA Kit (ab185983) and assay was run using sandwich technique on the supernatant extracted. The PDL cell line was co-cultured with the protein extract in a transwell system: the protein was placed in the upper chamber, separated by a porous membrane from the cells in the lower chamber. Protein extracts containing homeodomain proteins were prepared from source, using lysis buffer composition. The resulting lysate, hereafter referred to as the *homeodomain protein extract*, includes homeodomain-containing transcription factors among a mixture of other nuclear proteins. Further, affinity tags were used for validation.

2.3. MTT assay

PDL cells and homeodomain proteins were plated in 96-well plates at a density of 1×10^3 cells per well and induced to undergo differentiation. Cell growth was assessed over an 8-day period using the MTT assay. At each time point, 20 µL of MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to each well and incubated at 37 °C for 4 h. After incubation, the medium was replaced with 150 µL of dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader to evaluate proliferation, cytotoxicity, and cell viability in response to five different concentrations of the protein extract (5, 10, 15, 20, and 25 µg/ml). The three most effective concentrations were selected for further analysis.

2.4. Wound-healing assay

PDL cells sourced from an extracted 3rd molar were isolated by enzymatic digestion. The assay included two groups:

1. **Test Group:** PDL cell line consisting of fibroblasts co cultured with homeodomain protein (PRX1)
2. **Control Group:** PDL cell line consisting of fibroblasts.

A gap was introduced in the confluent cell layers of both groups. Cell migration, intercellular interactions, and gap-closure rates were observed at 24 h and 48 h using scanning electron microscopy (SEM). The image J software was used for assessing wound closure rate.

3. Results

3.1. Effects on PDL cell viability and proliferation

The impact of homeodomain proteins on PDL cell viability and proliferation was evaluated using the MTT assay. Concentration of 5 µg/ml significantly enhanced cell viability at all time points (Fig. 1). Test group showed higher rate of cell proliferation as compared to control group from one to three days (Fig. 2).

3.2. Effects on PDL fibroblast migration and wound repopulation

The impact of homeodomain proteins on periodontal cell migration and wound repopulation was assessed using in vitro scratch wound assays. Significant difference in wound-gap closure was observed: the test group (PDL cells co-cultured with homeodomain proteins) exhibited substantially greater cell migration and gap repopulation compared to

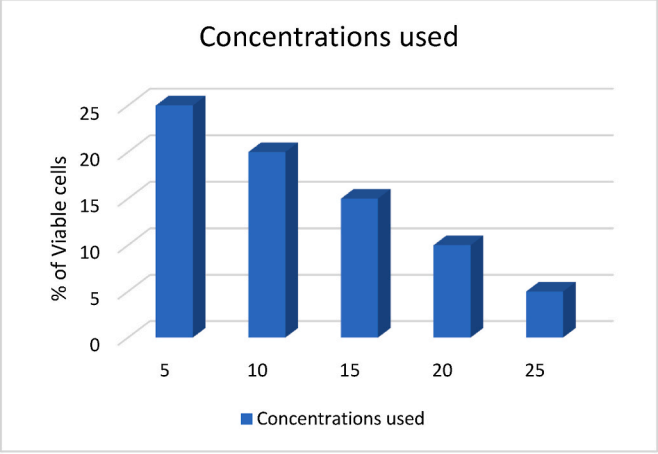

Fig. 1. Effects of homeodomain protein on PDL cell viability in both test and control groups at 5 different concentrations.

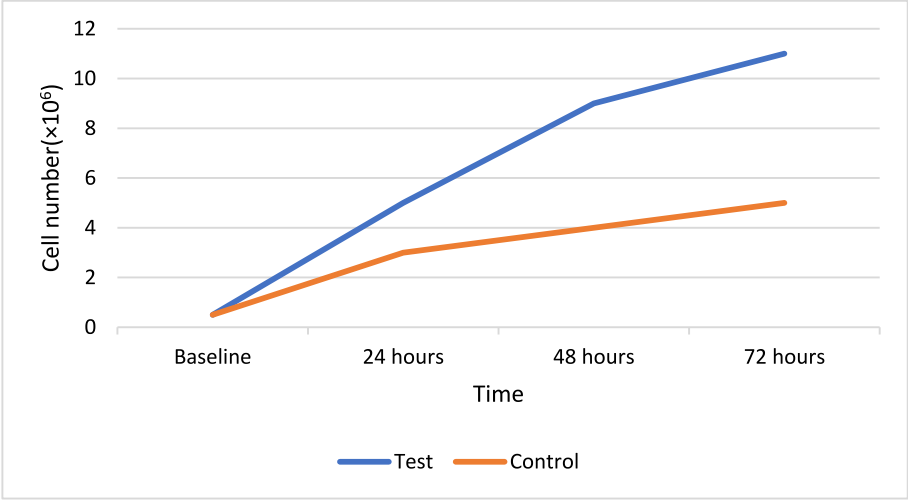

Fig. 2. Effects of homeodomain protein on PDL cell proliferation in both test and control groups at 1, 2 and 3 days.

the control group (Figs. 3 and 4).

4. Discussion

The Paired Related Homeobox 1 (PRX1) gene is a transcription factor essential for mesenchymal cell differentiation, craniofacial development, and tissue regeneration. Homeodomain proteins orchestrate PDL stem cell (PDLSC) behavior, critical for regenerating damaged periodontal tissues. For example, Lhx6 and Lhx8 promote mesenchymal-to-osteoblast and cementoblast differentiation, aiding alveolar bone and cementum regeneration. Barx1 similarly guides mesenchymal stem cell differentiation, making it a candidate in regenerative periodontal therapies, and Engrailed-1 (En1) has been investigated for modulating inflammatory signaling in periodontal tissues.[10]

Recent cell lineage tracking studies have confirmed that PRX1-expressing cells display multipotency and can form bone and connective tissue.[11] The identification of fracture-induced perivascular PRX1-expressing cells and regulation of PRX1 expression by bone morphogenetic protein 2BMP2, and in turn by C-X-C motif chemokine ligand 12 (CXCL12), in the orchestration of fracture repair, highlights a pathway to investigate defective mechanisms and therapeutic targets for fracture non-union.[12]

Gene therapy utilizing homeodomain proteins is being explored to enhance periodontal regeneration. Overexpression of PRX1 and PRX2 has been evaluated in animal models to support bone and ligament repair. PRX1-expressing cells in bone display characteristics akin to bone-derived mesenchymal stem cells (MSCs), and PRX1+ cells in the PDL exhibit stem cell–like properties. In mice, researchers isolated PRX1+ cells from PDL tissue and found high expression of MSC-associated markers. Furthermore, PRX1 has been proposed as a more reliable MSC marker in human skin compared to PDGFRA.[13] In human PDLSCs, PRX1 transcript levels were significantly higher than those of MCAM and SCX—commonly used PDLSC markers—supporting its role as a superior marker for human multipotent PDLSCs.[14–16]

Advancements in CRISPR-based gene editing now allow precise regulation of homeodomain protein expression—opening the door for personalized periodontal therapies. However, most work remains preclinical—few clinical trials exist, and challenges persist, including targeted delivery, long-term expression stability, and elucidating interactions with other signaling pathways. Homeodomain transcription factors are often low-abundance proteins which may fall below detection limits. The present study included identification with ELISA to give specific validation. Many homeodomain proteins are expressed transiently or in specific developmental windows. Sampling at wrong time gives false negatives. Hence, targeted mass spectroscopy can help with validation of protein sample. One limitation of the present study is the absence of a vehicle control group. Although a PDL control was included, it does not account for the potential effects of reagents or solvents used in the protein extraction process. Including a buffer- or solvent-only control would help confirm that observed cellular responses were attributable to the homeodomain protein content rather than to residual extraction components. Further research is needed to explore their potential combination with biomaterials, growth factors, and stem cell therapies for clinical applications.

5. Conclusion

The homeodomain proteins and its associated cells play a critical role in periodontal development and regeneration. Harnessing the regenerative capabilities of PRX1-expressing cells holds significant promise for

Fig. 3. Wound repopulation in control (a) and test (b) groups at 24 h (A and B) when viewed under scanning electron microscope.

Fig. 4. Wound repopulation in control (a) and test (b) groups at 48 h when viewed under scanning electron microscope.

advancing periodontal therapy and improving patient outcomes. In conclusion, homeodomain proteins play a crucial role in periodontal therapy by influencing various biological processes, such as stem cell regulation, tissue regeneration, and the modulation of inflammatory responses. The molecular identity and behavior of PRX1^{+} cells under injury versus steady state remains to be studied. Future research should explore combining homeodomain modulation with biomaterials, growth factors, and stem cell therapies to realize clinical applications.

Patient consent

Informed consent was obtained from the subject who provided the periosteum sample.

Ethical clearance

The study was approved by the Ethics Committee for Human Trials at Ramaiah University (EC-23/109-PG-FDS).

Sources of funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors extend thankfulness to Averin Microbiological lab, Bengaluru, India, for lab investigations and results.

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