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REVIEW

Potential of dental pulp stem cells and their products in promoting peripheral nerve regeneration and their future applications

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Abstract

Peripheral nerve injury (PNI) seriously affects people's quality of life. Stem cell therapy is considered a promising new option for the clinical treatment of PNI. Dental stem cells, particularly dental pulp stem cells (DPSCs), are adult pluripotent stem cells derived from the neuroectoderm. DPSCs have significant potential in the field of neural tissue engineering due to their numerous advantages, such as easy isolation, multidifferentiation potential, low immunogenicity, and low transplant rejection rate. DPSCs are extensively used in tissue engineering and regenerative medicine, including for the treatment of sciatic nerve injury, facial nerve injury, spinal cord injury, and other neurodegenerative diseases. This article reviews research related to DPSCs and their advantages in treating PNI, aiming to summarize the therapeutic potential of DPSCs for PNI and the underlying mechanisms and providing valuable guidance and a foundation for future research.

Key Words: Dental pulp stem cells; Peripheral nerve injury; Regenerative medicine; Neural regeneration; Schwann cells; Stem cells engineering

Core Tip: This article reviews the potential applications of dental pulp stem cells (DPSCs) and their derivatives in the field of nerve regeneration. First, this paper describes the current status of stem cell therapies for peripheral nerve injury (PNI) and discusses the advantages of DPSCs in this field. Then, the status of research on the neuroregenerative ability of DPSCs and their derivatives is reviewed. Finally, the potential of DPSCs in treating PNI and the underlying mechanism are summarized, with an aim to provide valuable guidance and a basis for future research.

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INTRODUCTION

It has been reported that approximately 2.8% of trauma patients suffer from peripheral nerve injury (PNI), resulting in permanent disabilities[1]. PNI can lead to muscle function loss, sensory impairment, and painful neuropathy[2]. While autologous nerve transplantation is a standard treatment option, its drawbacks, such as the difficulty in obtaining donor nerves, the need for surgical procedures to acquire donor nerves, secondary deformities at the donor site, and potential mismatch issues, limit its widespread use[3,4]. With advancements in biomaterials and tissue regeneration, significant progress has been made in nerve regrowth techniques[5]. The restoration of function after injury is of paramount importance, and experiments on stem cells have shown that they can accelerate nerve regeneration[6]. Compared to other types of stem cells, dental pulp stem cells (DPSCs) are more advantageous because they can be obtained *via* noninvasive operation, can be preserved at low temperature long-term, are simple to use, are associated with few ethical problems, and have low immunogenicity, so they are ideal materials for tissue engineering[7]. Human DPSCs (hDPSCs) exhibit remarkable self-renewal, multilineage differentiation, and cloning capabilities[8]. Schwann cells play a critical role in nerve regeneration, and stem cells with the same embryonic origin as Schwann cells are suitable tools for PNI treatment. DPSCs originate from neural crest cells[9] and share homology with Schwann cells[10]. Therefore, due to DPSCs' unique neural differentiation and nerve regeneration abilities, DPSCs-derived Schwann cells are viable tools for achieving nerve regeneration after PNI *in vitro*[11]. To keep abreast of the latest developments in this field, articles published in PubMed between 2010 and 2023 were screened using the following search terms: "peripheral nerve", "tooth-derived stem cells", "exosomes", and "dental stem cells". The primary aim of this review is to provide information on the recent applications of DPSCs in PNI treatment, with a specific focus on both cellular therapies and noncellular therapies involving DPSCs. Additionally, the review discusses the therapeutic effects of DPSCs and their potential future applications in treating PNI.

PNI

PNI is a prevalent clinical condition often resulting in long-term functional impairments. The effectiveness of surgical treatments is frequently unsatisfactory[12], and the restoration of nerve function is often not optimal[13]. Peripheral nerve regeneration is a complex process involving Wallerian degeneration, axon sprouting, and myelin regeneration[14]. While nerves may regenerate over relatively short distances after mild nerve injury, the outcomes of nerve regeneration are often unsatisfactory. In cases of peripheral nerve amputation, a series of molecular and cellular changes occur, known as Waller's degeneration[15,16]. Subsequently, monocytes and macrophages migrate to the nerve stump to clear axon fragments and myelin sheaths at the damaged end, while the proliferation of Schwann cells results in the formation of longitudinal cell columns, known as Bungner bands[17,18]. According to the literature, various factors, such as scaffolds for axonal migration, supporting cells (including Schwann cells and macrophages), growth factors, and the extracellular matrix, play crucial roles in regeneration after PNI[2].

Schwann cells originate from neural crest cells[19] and play a crucial role in the regeneration of peripheral nerves, influenced by neurotrophic factors (NTFs) such as Krox-20, Oct-6, and Sox-10[18]. These cells play an instrumental role in nerve regeneration by selectively promoting axonal regrowth of both motor and sensory nerves[20-22]. After nerve injury, Schwann cells may undergo dedifferentiation, a process mainly regulated by the negative regulatory factor c-Jun. This dedifferentiation is vital because it can help nerve survival and facilitate axonal regeneration[23]. Researchers have explored the effect of transplanting Schwann cells isolated from peripheral nerves into rat sciatic nerve injury models and found that these cells promote the regeneration of nerve axons, proving the potential of autologous stem cell transplantation in treating PNI[24]. However, such regenerative strategies are associated with challenges, as collecting Schwann cells is difficult and their survival rate after transplantation is low[25]. In recent studies, DPSCs-derived conditioned medium (DPSCs-CM) was shown to promote the proliferation of Schwann cells and increase the production of myelin-associated proteins[26]. This may provide insight for the development of a new method for the treatment of peripheral nerve diseases.

STEM CELL THERAPY FOR PNI

Earlier studies have revealed that allogeneic stem cell transplantation has a favorable effect on nerve regeneration and thus warrants further investigation[27]. Acellular nerve allografts (ANAs) promote axon regeneration through Schwann cell proliferation[28]. However, the therapeutic efficacy of ANAs diminishes over time after nerve injury, likely due to the limitations of Schwann cell function in the host nerve. Therefore, there is an urgent need to explore alternative or more effective therapies for Schwann cell replacement.

An increasing number of studies have demonstrated the remarkable potential of stem cells in promoting neural regeneration. Adipose-derived stem cells have been shown to differentiate into Schwann-like cells and effectively promote nerve regeneration[29-31]. Additionally, Shimizu *et al*[32] reported that human bone marrow mesenchymal stem cells (MSCs) can serve as Schwann cell substitutes, making them a viable option for nerve regeneration applications. Furthermore, muscle-derived stem/progenitor cells have shown the ability to differentiate into myelinated Schwann cells *in vivo*, thereby promoting axonal regeneration[33]. Al-Zer and Kalbouneh[11] successfully induced the differentiation of DPSCs into Schwann cells by utilizing retinoic acid, mercaptoethanol, and neuromodulin β1. Among stem cell sources, dental stem cells have garnered significant attention due to their excellent nerve regeneration capabilities and ease of availability.

Recent studies have revealed that during tooth development, glial cells related to the peripheral nervous system produce a significant number of MSCs, including dental pulp cells and odontoblasts[34]. DPSCs, stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), stem cells from the apical papilla, and dental follicle progenitor cells all fall under the category of dental stem cells[35]. These dental tissue-derived MSCs exhibit the ability of multidirectional differentiation and hold great potential in immunomodulation and tissue regeneration[36]. Moreover, MSCs derived from hair follicles, the dental pulp, and nipples show similar biological characteristics as those from the teeth of the same donor and display osteogenic, adipogenic, and chondrogenic differentiation capabilities[37].

Apical pulp-derived cells and coronal pulp cells have demonstrated the ability to differentiate into nerve cells *in vitro* and express markers associated with neural crest cells (p75, Snail, and Slug) as well as neural stem cell markers (Nestin and Musashi1)[38]. SHEDs can differentiate into many types of cells, including nerve cells[39]. Pereira *et al*[40] utilized polyglycolic acid tubes to induce SHEDs to differentiate into Schwann cells. Human PDLSCs have exhibited the ability to promote axonal regeneration after optic nerve injury, potentially through the secretion of brain-derived neurotrophic factor (BDNF)[41]. The ERK1/2 signaling pathway plays a role in the differentiation of PDLSCs into Schwann cells[42], thus making them a viable alternative source for autologous Schwann cells[43]. Ng *et al*[44] successfully induced adult PDLSCs to differentiate into retina-like cells with the biological characteristics of nerve cells. Dental embryonic stem cells have the potential to achieve nerve tissue regeneration due to their common origin with the nervous system[5,14], making them a promising source of stem cells for nerve tissue regeneration[45]. The neurological potential of DPSCs makes them a viable candidate cell type for the treatment of peripheral nerve diseases[46]. DPSCs can differentiate into neuron-like cells, and after 5 d of neuron differentiation, Tub3 is activated, accompanied by increased Nestin expression[47]. Insulin-like growth factor binding protein 5 promotes the formation of neurospheres by DPSCs. Angiogenic markers such as vascular endothelial-derived growth factor (VEGF), platelet derived growth factor subunit A, and angiopoietin-1 and neurogenic markers such as neural cell adhesion molecule, Nestin, βIII-tubulin, and tyrosine hydroxylase are upregulated in DPSCs, reflecting the vascular and neurogenic differentiation potential of DPSCs[48]. Additionally, DPSCs can secrete NTFs that support nerve cell function[49].

Compared with other stem cells, DPSCs have stronger multidifferentiation potential, self-renewal ability, and colony formation ability[8]. The Eph/ephrin interaction indicates that unlike that of other stem cells, the formation of DPSCs involves the neural crest[50,51]. Because of their spinal origin, DPSCs have the ability to differentiate into other spinal cord-related cells[52]. Unlike bone marrow MSCs (BM-MSCs), DPSCs are heterogeneous, and they also express markers of endothelial cells (vascular cell adhesion molecule 1 and MUC-18), smooth muscle (a-smooth muscle actin), bone (alkaline phosphatase, type I collagen, osteonectin, osteopontin, and osteocalcin), and fibroblasts (type III collagen and fibroblast growth factor 2)[39,53]. DPSCs express the neural precursor and glial cell markers nestin and glial fibrillary acidic protein (GFAP), which indicates that DPSCs are similar to BM-MSCs and have the potential to differentiate into neural cells[8]. Compared with BM-MSCs, DPSCs can more strongly inhibit the proliferation of PHA-stimulated T cells and exert immunosuppressive effects[54]. Compared with umbilical cord stem cells, the secretion of vascular endothelial growth factor-A and follistatin in DPSCs is more obvious, while umbilical cord stem cells tend to secrete vascular endothelial growth factor-C, which does not produce angiogenic effects. The levels of vascular endothelial growth factor-A and vascular endothelial growth factor-D secreted by DPSCs are higher than those secreted by BM-MSCs[55]. DPSCs can survive for a long time under extreme stress conditions[56]. DPSCs have the same immunomodulatory effect as BM-MSCs in the treatment of nervous system diseases. DPSCs are easier to obtain than other stem cells, have high multidifferentiation potential and a strong proliferation ability, and are not carcinogenic. They are a good substitute for stem cells in the treatment of PNI[5].

Moreover, compared with dental follicle- and papilla-derived stem cells, DPSCs have a stronger Na^+ current, indicating that they have a higher potential for neural differentiation[57]. Both DPSCs and BM-MSCs implanted in the vitreous body secrete nerve growth factor (NGF), BDNF, and neurotrophin 3 (NT-3), and the amount of NGF and BDNF secreted by DPSCs is significantly higher than that secreted by BMSCs, which support nerve survival and axonal regeneration[58]. Adipose tissue-derived stem cells also have the potential for nerve regeneration but mainly play an active role in stimulating endogenous stem cells by releasing NTFs[59]. Wang *et al*[60] successfully induced vascular endothelial cells and BM-MSCs to differentiate into neurons with tricyclodecane-9-yl xanthate. Cryopreserved DPSCs were also shown to be able to differentiate into cholinergic neurons by tricyclodecane-9-yl xanthate[61]. By comparing stem cells from

different sources, Isobe *et al*[62] found that BM-MSCs and synovial fluid-derived stem cells showed significant osteogenic effects. An increase in alkaline phosphatase and osteocalcin levels suggested that synovial fluid-derived stem cells had the highest cartilage formation ability. Reverse transcription-polymerase chain reaction showed increased expression of class III β-tubulin and microtubule-associated protein 2, suggesting that DPSCs and human deciduous tooth stem cells have the potential for neural regeneration. In addition, studies by Isobe *et al*[62] showed that DPSCs have stronger neural differentiation potential than pluripotent stem cells isolated from bone marrow and synovial fluid.

In light of these findings, DPSCs have emerged as excellent candidate stem cells for treating PNI, with stronger tissue regeneration potential than other types of stem cells (Figure 1). Consequently, the focus of this article will be on research progress related to the effectiveness of DPSCs in treating PNI.

EFFECT OF DPSCS IN THE TREATMENT OF PNI

Teeth originate from the cranial neural crest[63]. Chai *et al*[64] were the first to confirm the involvement of cranial neural crest cells in the formation of dental pulp cells. In recent years, there has been a growing interest in research on utilizing DPSCs for nerve repair[65]. Janebodin *et al*[52] demonstrated that DPSCs originate from spinal nerves and possess the potential to differentiate into tissues derived from other neural crest-derived structures. Gronthos *et al*[53] provided evidence that the dental pulp contains cells with the ability to form clones, proliferate, and regenerate tissue, classifying them as stem cells. The embryonic origin of DPSCs endows them with the potential to serve as stem cells for neural tissue engineering.

DPSCs have been shown to effectively promote axonal regeneration both *in vivo* and *in vitro*[66] (Tables 1 and 2). Typically, DPSCs exhibit several characteristics of pluripotent stem cells and display high proliferation rates, expressing CD44, CD90, and CD166[65]. Moreover, DPSCs express embryonic stem cell markers such as Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, along with several other MSC markers[67]. DPSCs have the remarkable capability to differentiate into several stromal-derived cell types (Figure 2). These cell types include osteoblasts[68-72], neurons[73], adipogenic cells[69] chondrogenic cells[69,70], smooth and skeletal muscle cells[74], dental pulp cells[75], Schwann cells [76], pancreatic cell lineage cells[70,77], blood vessel cells[78], and hepatocytes[79,80]. These findings strongly support the potential of DPSCs to be applied for tissue engineering[7].

Subsequent studies have revealed that a large number of dental MSCs originate from glial cells associated with peripheral nerves[34]. Long-term cryopreserved DPSCs retain their potential to differentiate into cholinergic nerves, indicating their suitability for prolonged preservation[61], and they have demonstrated beneficial anti-inflammatory effects in nerve injury[81]. Paes *et al*[82] conducted a pioneering comparison between monolayer and spheroid cultures of hDPSCs, demonstrating that both exhibit promising nerve regeneration potential through different mechanisms. Furthermore, hDPSCs were found to be effective in treating diabetic polyneuropathy[83]. In a groundbreaking study, Omi *et al*[84] treated neuropathy in diabetic rats through DPSCs transplantation for the first time and observed nerve function recovery that was potentially attributed to basic fibroblast growth factor (bFGF), VEGF, NGF, and NT-3 secreted by DPSCs. As an effective therapeutic tool for treating PNI, DPSCs are anticipated to be widely used in clinical settings in the future. With the deepening of research (Figure 3), it has been confirmed that DPSCs can be used to treat PNI *via* many mechanisms.

CELL THERAPEUTIC ALTERNATIVE FOR DPSCS

DPSCs can differentiate into nerve-like cells, making them be of great significance in the field of nerve regeneration. Dental pulp MSCs exhibit strong expression of nerve and glial cell markers and can be induced to adopt a nerve-like morphology[5]. DPSCs can be induced to differentiate into cells that exhibit a neuronal morphology and express neuronal-specific markers, such as the immature neuron markers Nestin and PSA-NCAM, by a neural induction regimen *in vivo* and *in vitro*[85]. Moreover, DPSCs transplantation has been shown to promote macrophage polarization toward the anti-inflammatory M2 phenotype[86]. Saez *et al*[87] demonstrated that DPSCs can effectively promote peripheral nerve regeneration when used to treat unilateral nerve crush injury in rats.

The peripheral nerve transection model is widely utilized in the study of peripheral PNI, and DPSCs have demonstrated good nerve regeneration ability in this model[88]. Stocchero *et al*[89] transplanted dental pulp cells into Wistar rats with sciatic nerve defect and observed significant promotion of nerve regeneration during the first 2 wk. Combining dental pulp cells with biodegradable materials is a promising approach to avoid secondary surgery[90]. Additionally, Sasaki *et al*[91] embedded dental pulp cells in silicone tubes to repair facial nerve defects in rats, and the results indicated the formation of blood vessels and myelin sheath tissue by dental pulp cells. DPSCs have exhibited a robust capacity to promote nerve regeneration in peripheral nerve transection models, and optimizing material combination schemes to fully unleash DPSCs' potential in nerve regeneration remains a prominent research focus in this field.

As previously reported, DPSCs, being derived from the neural ridge, possess a strong ability to differentiate into neuronal lineages. Numerous studies have explored various methods to induce the neural differentiation of DPSCs, and these cells have been applied to treat PNI[92]. After neural differentiation induction, DPSCs generate neural progenitor cells, expressing the neural markers Nestin, TuJ-1, and GFAP[93]. Additionally, inner ear neurotrophins, such as BDNF and NT-3, along with glial cell-derived neurotrophic factor (GDNF), have been found to promote the differentiation of DPSCs into spiral ganglion neuron-like cells[94]. Notably, when Schwann cells differentiated from human DPSCs were

Table 1 Progress in application of dental pulp stem cells in treating models of peripheral nerve injury

Publication year	Cell source	Induction program	Pretransplantation procedure	Material(s)	Dose/concentration	Disease model	Experiment duration	Treatment effect	Ref.
2022	Human	DMEM + 10% (FBS + P/S)	3D culture	Fibrinogen, calcium chloride, and thrombin-like protein	3×10^5 cells	Avulsion of spinal motor roots in rats	12 wk	The transcription of TNF-α, IL-1β, IL-6, and IL-17 and the expression of anti-inflammatory cytokines (TGF-β, IL-4, IL-10, and IL-13) were increased; the animals in the reimplantation + 2D group showed the best functional recovery	[82]
2021	Human	α-MEM + 20% (FBS + P/S)	Collected and resuspended in GelMA-bFGF	10% GFD in a CSM tube	1×10^6 cells/mL	15 mm defect of the sciatic nerve in rats	12 wk	Cell based therapy repaired large gap defects in peripheral nerves; the differentiation of DPSCs into nerve cells and Schwann-like nerve cells and the formation of myelinated nerve fibers were observed	[98]
2021	Human	DMEM + 10% (FBS + P/S + NEAA)	NLCs differentiated from DPSCs	-	1×10^5 NLCs		12 wk	Two weeks after transplantation, approximately 75% of the transplanted cells differentiated into platelet-derived growth factor receptor alpha + OPCs expressing p75NTRd; transplantation promoted axon growth and improved nerve function	[145]
2021	Human	α-MEM + 15% FBS	Exosome collection	-	200 μg/100 μL	Mouse model of spinal cord injury	4 wk	Inhibited the ROS-MAPK-NFκB P65 signaling pathway to reduce M1 macrophage polarization, suppress the inflammatory response, and alleviate neurological damage	[121]
2020	Human	DMEM + 20% (FBS + P/S)	Preparation of scaffold-free cell sheets by coculture with FGF2	-	2×10^6 cells/cell sheet	Rat model of facial nerve crush injury	3 wk	Cell sheets promoted axonal regeneration and functional recovery through continuous delivery of neurotrophic factors such as BDNF and GDNF	[116]
2020	Human	α-MEM + 10% (FBS + P/S + NEAA)	Induction of DPSCs differentiation into N-DPSCs; induction of DPSCs differentiation into N-DPSCs	-	-	Rat model of sciatic nerve crush injury	1 mo	Both DPSCs and N-DPSCs promoted peripheral nerve repair through the expression of neurotrophic factors such as NGF, BDNF, and GDNF; the nerve repair effect of N-DPSCs was longer lasting	[146]
2019	Human (children)	DMEM + 15% (FBS+P/S)	-	-	5×10^5 cells in 4 μL DMEM	Unilateral facial nerve crush injury in rats	6 wk	Immature DPSCs promoted nerve regeneration and the formation of new myelin; the expression of nerve growth factor and anti-inflammatory cytokines (IL-6 and IL-10) increased significantly 7 d after treatment, and there was a decrease in the levels of soluble proinflammatory factors such as IL-2, IL-4, TNF-α, and IFN-γ	[87]

2019	Human	-	Induction of DPSCs differentiation into nerve cells	A PDO-based cell carrier	7.5×10^5 cells	6 mm defect of the sciatic nerve in rats	12 wk	Multiperforated PDO tubes were effective biomaterial carriers; delivery of DPSCs impacted the inflammatory environment and promoted nerve regeneration and functional recovery	[89]
2018	Human	-	Isolation of STRO-1+/c-Kit+/CD34+ cells	Collagen scaffolds	5×10^5 cells/animal	6 mm defect of the sciatic nerve in rats	4 wk	Nerve fiber regeneration and myelination and many myelinated axons were observed; DPSCs grafted into the sciatic nerve defect expressed the typical Schwann cell marker S100B and were positive for human NeuN	[12]
2018	Human	ADMEM + 10% FBS	Differentiated into neuronal cells (DF-DPSCs)	A conduit made from a Lyoplant membrane	-	7-8 mm defect of the sciatic nerve in rats	12 wk	DPSCs relieved neuropathic pain and inhibited inflammation in rats earlier than DF-DPSCs; at 12 wk after the operation, the expression of pAMPK/SIRT1 in DF-DPSCs and DPSCs increased, the expression of proinflammatory cytokines decreased, and the expression of NFκB decreased	[81]
2018	Human	ADMEM + 10% (FBS + P/S)	Differentiation into cholinergic neurons by adding D609	Biodegradable tubule and fibrin glue	1×10^6 DF-chNs	5 mm defect of the sciatic nerve in rats	8 wk	Transplanted DF-chNs promoted motor nerve regeneration and axon growth and expressed nerve growth factor receptor (p75NGFR)	[61]
2018	Human	-	-	An absorbable hemostat filled with human DPCs containing 1% atelocollagen, fibronectin, and laminin	3×10^5 cells	Crush injury of the sciatic nerve in rats	2 wk	DPCs stimulated Schwann cell differentiation and promoted peripheral nerve regeneration	[114]
2017	Human	Standard: α-MEM + 10% (FBS + NEAA + P/S). Differentiation: Standard + forskolin + bFGF + PDGF-AA + HRG1-β	Differentiation into Schwann-like cells (d-hDPSCs)	NeuraWrap™ conduits	-	15 mm defect of the sciatic nerve in rats	8 wk	Growth of axons, myelinated nerve fibers, and blood vessels; DPSCs still exerted strong angiogenic effects after differentiating into Schwann-like cells	[102]
2017	Human	α-MEM + 15% (FBS + AA + P/S + NEAA)	-	Fibrin conduits	2×10^6/20 μL	10 mm defect of the sciatic nerve in rats	2 wk	Promoted nerve and axon regeneration; the transplanted cells expressed BDNF near the cell body, and the expression level of caspase-3 decreased	[45]
2017	Human	ADMEM + 10% FBS	Induction of DPSCs differentiation into nerve cells	Fibrin glue scaffold and collagen tubulation	1×10^6 cells	5 mm defect of the sciatic nerve in rats	12 wk	Both hDPSCs and DF-hDPSCs promoted nerve regeneration and functional recovery; they could directly differentiate into nerve cells or facilitate nerve cell differentiation	[99]
2015	Human	α-MEM + 10% (FBS +	Transfection with Olig2	-	2×10^5 cells	Mouse model of local	6 wk	Recovery of sciatic nerve function; DPSCs	[108]

		P/S/AmB)	gene *via* a tetracycline (Tet) inducible system			sciatic nerve demyelination		differentiated into oligodendrocyte progenitors, and specific markers of oligodendrocyte progenitors and oligodendrocytes were expressed	
2015	Human	DMEM + 10% FBS	G-CSF-induced stem cell mobilization (mobilized DPSCs and MDPSCs)	Collagen conduits	3.0×10^5 MDPSCs	5 mm defect of the sciatic nerve in rats	5 wk	MDPSCs secreted neurogenic/angiogenic factors and promoted peripheral nerve regeneration	[112]
2015	Human	Culture dishes containing essential medium (alpha modification) + 10% (FBS + P/S + amphotericin B)	Induction of DPSCs differentiation into OPCs by transfection with a plasmid containing the human Olig2 gene		2×10^5 cells	Sciatic nerve demyelination in mice	6 wk	DPSCs differentiated into OPCs, and transplantation promoted myelin sheath formation and peripheral nerve function recovery	[107]
2015	Human	DMEM + b-ME; DMEM + 10% (FBS + RA); DMEM + 10% (FBS + FSK + b-FGF + PDGF + HRG)	Differentiation of hDPSCs into Schwann-like cells	Cells combined with a pulsed electromagnetic field (PEMF)	1×10^6 cells/10 mL/rat	Crush injury of the peripheral nerve in rats	3 wk	Schwann-like cells derived from DPSCs exhibited the characteristics of glial cells, expressing CD104, S100, GFAP, laminin, and p75NTR; application of a PEMF promoted peripheral nerve regeneration after cell transplantation	[147]
2012	Human	DMEM + 10% FBS	-	-	1×10^6 cells	Rat spinal cord transection model	8 wk	DPSCs promoted axonal growth, differentiated into oligodendrocytes to treat spinal cord injury, and protected the nerve by inhibiting apoptosis and paracrine signaling	[105]
2018	Human	α-MEM + 10% (FBS + NEAA + P/S)	Application of fresh medium containing vitamin C cells reached approximately 80% confluence	-	-	Patients diagnosed with a traumatized permanent incisor	12 mo	HDPSCs transplantation promoted the regeneration of pulp tissue including neuronal tissue, and the neuron marker NeuN was expressed	[75]
	Human	PBS + P/S	Collection of hDPSCs aggregates	The root canals of human teeth	-	Immunocompromised mice	8 wk	Dental pulp tissue containing sensory nerves and blood vessels regenerated after HDPSCs transplantation	
	Human	α-MEM + 10% (FBS + NEAA + P/S)	-	-	3×10^5 cells	Rats injected into the dorsal root ganglion	2 mo	HDPSCs exhibited the morphology of neurons and expressed TRPV1 and TRPM8	
	Pig	PBS + P/S	Collection of hDPSCs aggregates	-	-	Permanent incisors of young female minipigs	3 mo	Pig DPSCs resulted in the 3D regeneration of dental pulp with neural function	
2015	Pig	Culture medium + 10% (FBS + L-AA -2-P + P/S)	-	Fibrin membrane	-	Porcine intercostal nerve transection model	6 mo	DPSCs alleviated nerve injury and express NSE; neuroelectrophysiological evaluation showed that neurological function was restored	[88]
2020	Rat	α-MEM + 20% FBS	-	-	1×10^6 cells/rat	Diabetic rats	4 wk	Multiple factors secreted by DPSCs increased the nerve conduction velocity and blood flow to nerves	[118]

2019	Rat	α-MEM + glucose + 20% FBS	Collection of DPSCs-CM	-	1 mL/rat	Diabetic rats	4 wk	DPSCs-CM ameliorated peripheral neuropathy by exerting neuroprotective, angiogenic, and anti-inflammatory effects	[132]
2017	Rat	α-MEM + 20% FBS	-	-	1×10^6 cells	Streptozotocin-induced diabetes rat model	4 wk	Sensory disturbance was alleviated, the thickness and area of the myelin sheath increased, the transplanted DPSCs secreted multiple factors such as angiogenic factors, neurotrophic factors, and immunosuppressive factors	[84]
2015	Rat	α-MEM + glucose + 20% FBS	-	-	1×10^6 cells	Diabetic rats	4 wk	DPSCs transplantation relieved diabetic polyneuropathy by inhibiting inflammation, exerting immunomodulatory effects, and secreting neurotrophic factors	[86]
2015	Rat	α-MEM + 20% FBS	-	-	1×10^6 cells/limb	Diabetic rats	8 wk	DPSCs increased the nerve conduction velocity and blood flow to nerves and promoted an increase in the number of nerve fibers in diabetic rats	[83]
2013	Rat	DMEM + 10% (FBS + P/S)	-	-	1.5×10^5 cells	Crush injury of the optic nerve in rats	3 wk	Transplantation of DPSCs significantly increased the survival rate of retinal ganglion cells in rats and promote axonal regeneration	[58]
2007	Rat	-	Embedded in 10 mL type I collagen gel	10-mm silicone tube	1×10^5 cells	7 mm defect of the facial nerve in rats	2 wk	Regeneration of axons, blood vessels, and Schwann cells; Tuj1-positive axons and S100-positive Schwann-like supportive cells were found in regenerated nerves	[91]
2018	Rabbit	DMEM + 10% FBS	Construction of an acellular nerve graft for nerve regeneration	Xenogenic acellular nerve matrix	6×10^5 cells per graft	10 mm defect of the sciatic nerve in rabbits	3 mo	Regeneration of nerve space showed that acellular nerve grafts containing DPSCs treated with myroilysin had a strong neural induction effect	[148]

AA: Ascorbic acid; AmB: Amphotericin B; ADMEM: Advanced Dulbecco's modified Eagle medium; bFGF: Basic fibroblast growth factor; b-ME: Beta-mercaptoethanol; BG: Beta-glycerophosphate; Dex: Dexamethasone; CSM: Cellulose/soy protein isolate composite membrane; DPSCs-CM: DPSCs-conditioned medium; DMEM: Dulbecco's modified Eagle medium; DF-chNs: Differentiated cholinergic neurons; DF-DPSCs: Neuronal cells differentiated from DPSCs; FSK: Forskolin; bD609: Tricyclodecane-9-yl-xanthogenate; FBS: Fetal bovine serum; G-CSF: Granulocyte-colony stimulating factor; GFD: 10% GelMA hydrogel, recombinant human basic fibroblast growth factor and DPSCs; HRG1-β: Heregulin-β-1; L-AA-2-P: L-ascorbic acid-2-phosphate; NGF: Nerve growth factor; N-DPSCs: Neural-induced DPSCs; NSE: Neuron-specific enolase; NEAA: Nonessential amino acids; NLCs: Neural lineage cells; OPCs: Oligodendrocyte progenitor cells; P/S: Penicillin and streptomycin; PDGF-AA: Platelet-derived growth factor AA; PBS: Phosphate-buffered saline; PDGF: Platelet-derived growth factor; RA: All-trans retinoic acid; TGNCs: Trigeminal ganglion neuronal cells; IL: Interleukin; TNF: Tumor necrosis factor; TGF: Transforming growth factor.

employed to treat a rat sciatic nerve defect model, significant regeneration of blood vessels and nerve processes was observed, highlighting the crucial role of revascularization in supporting nerve regeneration and survival[95]. Moreover, Zheng *et al*[96] demonstrated that coculture of chitosan scaffold with bFGF can enhance the neural differentiation of DPSCs. The ERK signaling pathway, a classical MAPK pathway, plays a pivotal role in this process. As a downstream effector of bFGF, DPSCs are actively engaged in neural differentiation. Numerous neural induction protocols exist for DPSCs; however, an optimal scheme that is simple, efficient, and quick is currently lacking.

Table 2 Research illustrating the ability of dental pulp stem cells to promote nerve regeneration *in vitro*

Publication year	Cell source	Induction protocol	Material(s) applied	Dose/concentration	Neurocyte type	Culture duration	Results	Ref.
2022	Human	DMEM + 20% (FBS + P/S + AA + bFGF)	Dental pulp cell sheets	3200 cells/cm^2	SH-SY5Y neuroblastoma cells	3 d	Dental pulp cell sheets provided neurotrophic support by expressing NTF; the amount of neurotrophic factors produced by dental pulp cell sheets was sufficient to induce nerve regeneration *in vitro* and promote nerve repair *in vivo*; dental pulp cell sheets improved axon guidance and reduced axon branching	[113]
2020	Rat	α-MEM + 10% (FBS + P/S + NEAA)	DPSCs-CM	-	TGNCs from rats	3 wk	DPSCs-CM was found to contain significant levels of nerve growth factor, brain-derived neurotrophic factor, neurotrophic factor-3, and glial cell line-derived neurotrophic factor; DPSCs-CM increased the survival rate of primary trigeminal ganglion neurons and promoted the growth of neurites	[130]
2020	Rat	α-MEM + 10% (FBS + P/S + NEAA)	DPSCs-CM	50% DPSCs-CM	PC12 cells	8 d	DPSCs-CM was found to contain neurotrophic factors, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor, which increased the viability and differentiation of PC12 cells and played an important role in axonal growth and survival, proving that DPSCs-CM treatment is a potential cell-free therapy for peripheral nerve repair and has a stronger effect on PC12 cells than DPSCs	[115]

AA: Ascorbic acid; bFGF: Basic fibroblast growth factor; DPSCs-CM: DPSCs-conditioned medium; DMEM: Dulbecco's modified Eagle medium; FBS: Fetal bovine serum; NTF: Neurotrophic factor; NEAA: Nonessential amino acids; P/S: Penicillin and streptomycin; TGNC: Trigeminal ganglion neuronal cells.

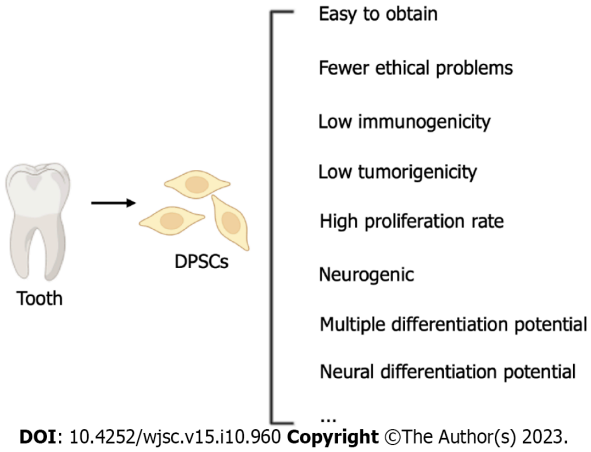

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Figure 1 Advantages of dental pulp stem cells as alternative stem cells for nerve regeneration. The figure was created with BioRender.com. DPSCs: Dental pulp stem cells.

Although significant progress has been made in the tissue field, finding a nerve conduit that can match the effectiveness of autologous transplantation remains a challenge in the treatment of PNI. Das and Bellare[97] developed a uniform bead-free nanofibrous scaffold primarily composed of polycaprolactone and gelatin A, which has been shown to support DPSCs regeneration and neural differentiation. In our previous studies, we confirmed that combining DPSCs with a third-generation nerve regeneration conduit can be used to effectively repair 15 mm long defects of the sciatic nerve in rats. Remarkably, the effect of this approach is comparable to that of autotransplantation. Furthermore, we observed that nerve tissue at the repair site mainly originated from differentiating DPSCs. This promising finding offers a potential tissue engineering strategy for the treatment of PNI[98].

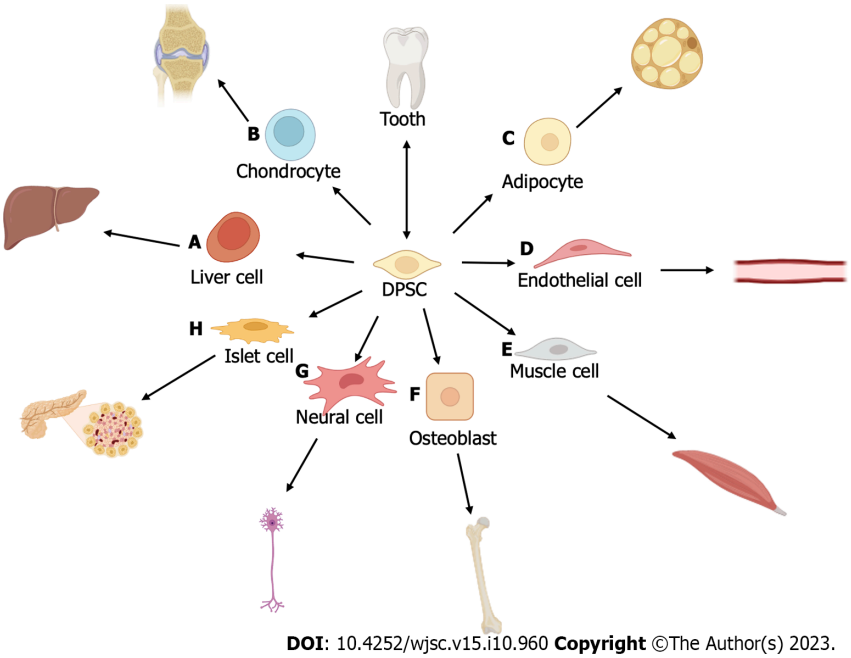

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Figure 2 Ability of dental pulp stem cells to differentiate into other types of cells. Dental pulp stem cells derived from dental pulp exhibit both self-proliferation ability and the capability to differentiate into multiple lineages *in vitro*. A: Liver stem cell; B: Chondrocyte; C: Adipocyte; D: Endothelial cell; E: Muscle cell; F: Osteoblast; G: Neural cell; H: Islet cell. The figure was created with BioRender.com. DPSC: Dental pulp stem cell.

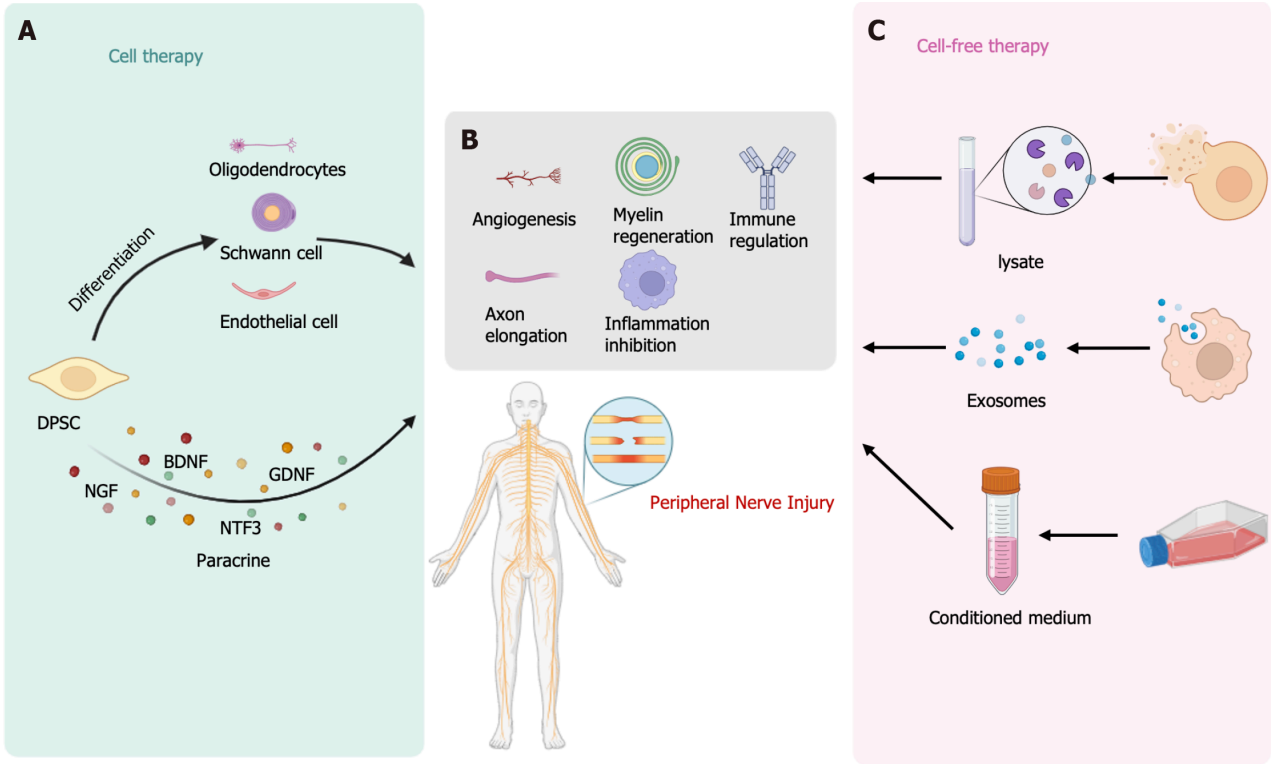

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Figure 3 Treatment of peripheral nerve injury by cell/acellular therapies involving dental pulp stem cells. A: Dental pulp stem cells (DPSCs) can directly differentiate into nerve cells or endothelial cells and can also protect nerves and promote nerve regeneration through paracrine neurotrophic factors; B: DPSCs can treat peripheral nerve injury (PNI) by promoting vascular regeneration, axonal regeneration, and myelin sheath repair, regulating the immune response, and inhibiting inflammation; C: Exosomes, lysates, and conditioned media from DPSCs are also effective in treating PNI. The figure was created with BioRender.com. DPSC: Dental pulp stem cell; NGF: Nerve growth factor; BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell-derived neurotrophic factor; NTF3: Neurotrophic factor 3.

Ullah *et al*[99] made an intriguing discovery that there was no significant difference in nerve regeneration ability between hDPSCs and differentiated neuronal cells derived from hDPSCs. More research on this topic is needed to clarify the underlying mechanisms and find a more effective treatment strategy.

Differentiation into Schwann cells

Previous studies have demonstrated that autologous adult Schwann cells have a remarkable capacity to support extensive peripheral nerve regeneration and can evade rejection[100]. Currently, Schwann cells remain a focal point of animal studies on PNI. Substitutive cell therapies bring renewed hope for nerve regeneration[20]. Schwann cells play a pivotal role in Wallerian degeneration, myelin regeneration, and supporting axon growth.

Martens *et al*[76] were the first to demonstrate that Schwann cells differentiated from hDPSCs can promote nerve regeneration. These differentiated Schwann cells express neural markers such as laminin, p75, GFAP, and CD104 and exhibit an increase in the expression of NTFs such as BDNF, b-NGF, NT-3, and GDNF. Medium conditioned by these differentiated Schwann cells was found to increase the survival of dorsal root ganglion cells and stimulate axon elongation. Subsequently, Al-Zer *et al*[101] induced the differentiation of DPSCs into Schwann cells by using forskolin, bFGF, platelet-derived growth factor, and recombinant human neuregulin-β1. The results revealed that the differentiated neural stem cells strongly expressed the Schwann cell marker Sox10. Additionally, Sanen *et al*[102] demonstrated that hDPSCs, after differentiating into Schwann-like cells, have the potential to stimulate endothelial cell migration and tubule formation. Neuronally differentiated DPSCs secrete higher levels of the angiogenic factor VEGF-A, suggesting that DPSCs retain their angiogenic ability even after differentiating into Schwann cells. However, the underlying mechanism by which DPSCs-derived Schwann cells promote endothelial cell proliferation requires further investigation. These findings collectively indicate the ability of DPSCs to differentiate into Schwann cells, making them potential candidates for tissue engineering-based approaches for treating nerve injuries. Lambrichts *et al*[103] showed that hDPSCs-derived Schwann cells could form myelin sheaths and dorsal root ganglia. Similarly, Carnevale *et al*[12] discovered that STRO-1+/c-Kit+/CD34+ hDPSCs, which originate from the neural ridge, promote axonal regeneration in an animal model of PNI and express S100b, a typical marker of Schwann cells. The differentiation of DPSCs into Schwann cells thus is a potential strategy for nerve regeneration, and the refinement of differentiation protocols and materials remains an important area of investigation.

Differentiation into oligodendrocytes

The primary function of oligodendrocytes is to form myelin, creating intricate connections between neurons in the nervous system. The transcription factor OLIG-2 and proteoglycan NG2 are markers of oligodendrocyte progenitor cells (OPCs)[104]. In a previous study, Sakai *et al*[105] transplanted SHEDs into rats with spinal cord transection and found that these cells exerted promising effects in promoting axon regeneration. This effect was achieved through the inhibition of various axon growth inhibitor signals and the differentiation of the transplanted cells into oligodendrocytes to replace damaged cells. Bagheri-Hosseinabadi *et al*[106] successfully induced DPSCs to differentiate into oligodendrocytes using cerebrospinal fluid and retinoic acid and obtained cells with a fibroblastic morphology and high adherence potential. Moreover, Askari *et al*[107] successfully induced DPSCs to differentiate into OPCs by transfecting them with a virus carrying the human Orig2 gene. The differentiated oligodendrocytes displayed a typical morphology and expressed neural markers such as GFAP, oligodendrocyte lineage transcription factor 2, and MBP. Subsequently, they applied transplanted oligodendrocytes into sciatic nerve demyelination model mice and demonstrated their effectiveness in nerve repair[108]. The nerve regeneration potential of DPSCs has been substantiated through various studies, offering hope for the treatment of peripheral nerve demyelination.

Differentiation into endothelial cells

Adequate blood supply is crucial for the survival of stem cells and nerve regeneration following injury. When MSCs differentiate into Schwann cells, they increase the secretion of angiogenic factors, including angiopoietin-1 and VEGF-A [109]. DPSCs can increase the migration of endothelial cells and promote angiogenesis *in vitro* and *in vivo*[110]. Sanen *et al* [102] also confirmed this phenomenon. Moreover, research has demonstrated that stem cells can directly differentiate into endothelial cells. In a rat facial nerve defect model, it was observed that DPSCs can directly differentiate into RECA1-positive endothelial cells, promoting nerve regeneration by increasing blood supply[91]. DPSCs were found lining the blood vessel wall of newly formed braided bone, indicating that angiogenesis occurred *in vitro*. Osteoblasts and endothelial cells were found after transplantation, and ultimately, bone-containing blood vessels were produced. Flk-1 is very important for the coupling of osteogenesis and angiogenesis. d'Aquino *et al*[69] proved that DPSCs can differentiate into Flk-1+/STRO-1+/CD44+/CD54+ endothelial progenitor cells. Sasaki *et al*[91] found that regenerated nerves contained S100-positive Schwann cells and RECA1-positive endothelial cells derived from dental pulp 14 d after DPSCs transplantation into rats with facial nerve defect. Newborn blood vessels are composed of endothelial cells from both recipient and donor sources. It has been suggested that DPSCs can differentiate into nerve cells and endothelial cells at the same time to promote nerve and vascular regeneration to treat PNI[91]. Subsequently, Maraldi *et al*[71] also proved that transplanted DPSCs can differentiate into endothelial cells *in vivo*. The ability of DPSCs to differentiate into endothelial cells further increases their potential for nerve regeneration.

Paracrine action

PNI triggers the dedifferentiation of Schwann cells and induces the formation and secretion of protogranules, which play a crucial role in nerve repair and the promotion of axonal growth[111]. Yamamoto *et al*[112] discovered that mobilized DPSCs (MDPSCs) treated with a granulocyte-colony stimulating factor (G-CSF) gradient express a variety of NTFs. These

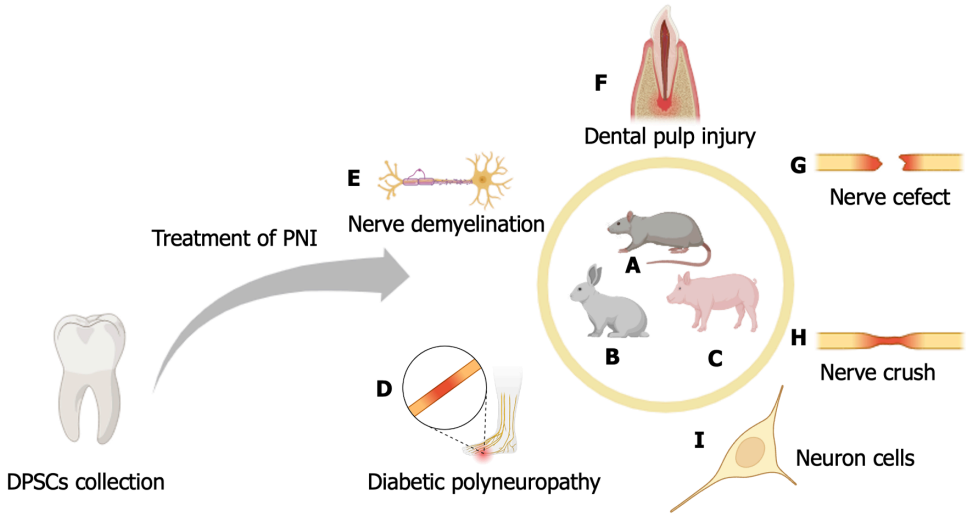

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Figure 4 Main models used to study the effect of dental pulp stem cells in treating peripheral nerve injury at present. A: Rats; B: Rabbits; C: Pigs. D: Diabetic neuropathy; E: Nerve demyelination injury; F: Dental pulp injury; G: Nerve defect injury; H: Nerve crush injury; I: Nerve cells cultured *in vitro* are used. DPSC: Dental pulp stem cell. The figure was created with BioRender.com.

NTFs not only stimulate Schwann cells but also regulate their apoptosis and proliferation. Additionally, the differentiation of hDPSCs into Schwann cells increases the expression of glial markers and the secretion of NTFs, including BDNF, GDNF, NGF, NTF3, ANGPT1, and VEGFA[45,76]. Furthermore, linearly arranged dental pulp cell slices have been shown to guide and support axonal regeneration, and the abundant NTFs produced by the cells were found to make a significant contribution to this phenomenon[113]. Implantation of DPSCs into the vitreous body can effectively treat retinal ganglion cell injuries in adult rats, with the secretion of NTFs being a critical factor. The neuroprotective effect of DPSCs is weakened when K252a and Trk are blocked, indicating the importance of the NTFs NGF, BDNF, and NT-3 in this process[58]. In the repair of sciatic nerve crush injury in rat models, transplanted dental pulp cells may secrete NTFs [114]. In a rat model of sciatic nerve injury with a 10 mm defect, DPSCs were found to exert their effects on Schwann cells through paracrine signaling, leading to significant promotion of axonal regeneration[45]. The strong paracrine effect of DPSCs, coupled with their potential for nerve regeneration, makes them promising candidates for nerve tissue engineering.

CELL-FREE THERAPEUTIC ALTERNATIVES INVOLVING DPSCS

An increasing number of studies have demonstrated that odontogenic stem cells treat nerve injury through paracrine mechanisms[105]. NTFs, such as NGF, BDNF, and GDNF, can stimulate axonal growth, making DPSCs potential candidates for acellular therapy[115,116]. Kumar *et al*[117] showed that DPSCs secrete high levels of cytokines such as G-CSF, interferon gamma, and transforming growth factor (TGF)-β, which promote nerve differentiation and axonal growth. Unlike some other stem cell types, DPSCs demonstrate strong potential for nerve regeneration, supporting the use of their secreted factors for exocrine therapy for PNI. Kanada *et al*[118] found that both DPSCs and secreted factors from DPSCs (DPSCs-SFs) exerted therapeutic effects in a rat model of diabetic polyneuropathy. The effects included increases in sciatic nerve motor/sensory nerve conduction velocity and sciatic nerve blood flow. DPSCs-SFs were found to include angiogenic, neurotrophic, and immunomodulatory proteins. However, DPSCs transplantation may provide benefits over a longer duration of time[118].

With the continuous development of research on DPSCs-derived exosomes (DPSCs-Exos), DPSCs-Exos have been demonstrated to exert potential therapeutic effects in various diseases. They have shown promise for the treatment of periodontitis[119], Parkinson's disease[120], SCI[121], degenerative diseases[122], and cerebral ischemia[123] and in achieving pulp regeneration[124]. DPSCs-Exos have garnered significant attention due to their immunomodulatory properties[125], ability to promote angiogenesis[126], inhibitory effect on inflammation[127], and marked neuroregenerative and neuroprotective effects[128]. Studies have revealed that DPSCs-Exos can increase the formation of SH-SY5Y cell axons, leading to improved neuronal ultrastructure and increased expression of neural markers[129]. Research on acellular therapies involving DPSCs is still relatively limited, and the therapeutic potential of DPSCs-Exos and DPSCs lysates requires further experimentation and validation. Progress has been made in the techniques used to extract exosomes and analyze their composition, but there is still much work to be done before their clinical application can be realized.

CM derived from D-MSCs has been shown to promote axonal growth[45]. Sultan *et al*[130] conducted a study on the neuroprotective effect of DPSCs and DPSCs-CM on isolated TGNCs for the first time. The results demonstrated that DPSCs-CM can increase neuronal survival and promote axonal growth through the action of various NTFs, including GDNF, BDNF, NT-3, and CNTF[130]. CM from human dental pulp cells was found to contain bone morphogenetic

protein 7, FGF7, insulin-like growth factor (IGF)-1, FGF4, growth hormone, and VEGF-D, all of which are related to nerve regeneration and protection, vascular regeneration, and osteogenesis. Furthermore, the addition of B-27 to CM was observed to enhance the promotion of axon growth[131]. DPSCs-CM has shown the ability to alleviate polyneuropathy in diabetic rats and reduce the number of macrophages in diseased peripheral nerves[132]. DPSCs-CM holds significant promise in the treatment of peripheral nerve injuries. However, further research is needed to explore the underlying mechanisms of nerve regeneration.

Neurotrophin, BDNF, GDNF, IGF, NGF, and VEGF were detected near transplanted DPSCs 14 d after transplantation. The secretion of vascular endothelial growth factors supports angiogenesis, thus promoting nerve regeneration[112]. BM-MSCs can also secrete NTFs such as BDNF, b-FGF, and CNTF to promote peripheral nerve regeneration and thus treat peripheral nerve defects[133]. G-CSF-MDPSCs express higher levels of granulocyte-macrophage CSF, matrix metalloproteinase 3, VEGF, and NGF than BM-MSCs. The effects of G-CSF-MDPSCs in angiogenesis, neurite extension, and migration and their anti-apoptotic effects were found to be stronger than those of BM-MSCs in the same environment [134]. Compared with medium conditioned by CD31- cells derived from bone marrow and fat, medium conditioned by CD31- cells from pulp results in higher levels of angiogenesis/NTFs and exerts stronger angiogenic and neurogenic effects[135]. Kumar *et al*[117] also proved that DPSCs and their secreted factor may exert beneficial effects in treating neurological disorders and injuries. Exosomes derived from DPSCs can inhibit the differentiation of CD4+ T cells into helper T cells 17 (Th17), reduce the secretion of the proinflammatory factors interleukin (IL)-17 and tumor necrosis factor-alpha (TNF-α), promote the polarization of CD4+ T cells into regulatory T cells, and increase the release of the anti-inflammatory factors IL-10 and TGF-β. Compared with BM-derived exosomes, exosomes derived from DPSCs have a stronger immunoregulatory effect. In CD4+ T cells stimulated by exosomes derived from DPSCs, the expression levels of IL-10 and TGF-β mRNA were found to be the highest, while the transcription levels of IL-17 and TNF-α were found to be the lowest. Exosomes derived from DPSCs have stronger anti-inflammatory effects than exosomes derived from BM-MSCs[125]. Exosomes derived from DPSCs/BM-MSCs significantly decrease the activity of caspase3/7 and exert a significant antiapoptotic effect to play a neuroprotective role by upregulating endogenous expression of neuronal survival factors. Specifically, the cell survival-related PI3K-Bcl-2 pathway protects hippocampal neurons from excitotoxicity. Exosomes derived from DPSCs have stronger antiapoptotic and anti-visceral necrosis effects than exosomes derived from bone marrow stem cells[122]. Interestingly, the factors secreted by DPSCs are different in different environments[136]. There is no doubt that the neuroprotective effect of DPSCs-Exos is stronger than that of BM-MSC-derived exosomes. However, for clinical application, it is necessary to better determine the optimal composition, dosage, and culture conditions of exosomes[121].

CONCLUSION

PNI is a prevalent clinical issue that often leads to long-term pain in patients. Recent research has demonstrated the beneficial effects of Schwann cells on axonal regeneration and functional recovery after injury[137]. However, isolating and cultivating Schwann cells are challenging due to their limited availability and low proliferation rate[138]. Tissue engineering strategies involving nerve grafts consisting of physical scaffolds combined with supporting cells and/or growth factors or other biomolecules are promising approaches for treating PNI[139].

DPSCs may occupy the injury site and exert immunomodulatory effects, participate in paracrine signaling, and directly differentiate into relevant cell types, thus promoting the repair and regeneration of diseased and injured tissues. To promote the application of DPSCs and their products in nerve regeneration in the future, clinical strategies and various administration methods are being studied on a large scale[140]. A study revealed that inducing DPSCs to differentiate into oligoprogenitor cells is a potential treatment strategy for neurodegenerative diseases[141]. DPSCs have the multilineage differentiation potential and can differentiate into neurotrophoblasts. DPSCs-CM and DPSCs-Exos contain rich NTFs, and DPSCs-Exos can also cross the blood-brain barrier. In addition, DPSCs have the ability to reduce inflammation, promote axonal growth, and resist apoptosis. They have great potential in the treatment of PNI[142].

Previous *in vivo* studies have focused on the sciatic nerve and facial nerve of rats and analyzed the behavioral and pathological manifestations of rats with sciatic and facial nerve injury, but electrophysiological studies are lacking. Moreover, it is also necessary to compare therapeutic effects among different models to identify the optimal application of DPSCs. However, there are still some questions to be answered. Do DPSCs have the same effect on nerve crush injury and amputation injury? Do different treatment methods have different effects on different models. Which is better, cell therapy or acellular therapy? What are the key factors in the neuroprotective and regenerative abilities of acellular therapies? In addition, researchers should pay more attention to the function of differentiated nerve cells *in vitro*. Furthermore, organoids may become a hot research topic in the future. A DPSCs cell bank is also urgently needed[143]. It is believed that DPSCs will bring hope to patients with PNI soon.

The unique biological characteristics of DPSCs make them significant cell sources for the treatment of PNI. DPSCs exhibit neurogenic potential, are easily accessible, exhibit pluripotency, and can be preserved for extended periods, making them excellent candidates for tissue engineering applications. DPSCs (Figure 4) can be utilized directly or in combination with nerve conduits or hydrogels or used after differentiation into nerve-like cells to treat PNI. Moreover, DPSCs lysates, DPSCs-Exos, and DPSCs-CM all hold promise in the treatment of PNI. However, overcoming safety and ethical problems and avoiding tumor formation are still the keys for translating DPSCs for clinical use[144].

This review focused on the therapeutic potential of DPSCs in treating PNI, elaborating on the neuroprotective and regenerative capabilities of DPSCs through both cellular and acellular approaches. DPSCs hold significant promise for the treatment of PNI, and it is anticipated that they will play a crucial role in the clinical treatment of PNI in the future. As

research on the effect of DPSCs on PNI continues to progress, more efficient and expedient treatment protocols are expected to be developed. However, the safety of cell therapies and the efficacy of acellular therapies in treating long-gap injuries require thorough evaluation. The search for convenient and effective treatment strategies involving DPSCs and their products for PNI remains ongoing. Furthermore, the underlying mechanisms and key components of DPSCs in peripheral nerve treatment warrant further investigation. As acellular therapies are novel stem cell therapies, there is a need to refine the purification process of cell materials and establish a standardized and effective cell production plan. Comparative studies assessing the safety, efficacy, and production costs of various therapies are also essential.

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FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

MicroRNA-584-5p/RUNX family transcription factor 2 axis mediates hypoxia-induced osteogenic differentiation of periosteal stem cells

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Abstract

BACKGROUND

The hypoxic environment during bone healing is important in regulating the differentiation of periosteal stem cells (PSCs) into osteoblasts or chondrocytes; however, the underlying mechanisms remain unclear.

AIM

To determine the effect of hypoxia on PSCs, and the expression of microRNA-584-5p (miR-584-5p) and RUNX family transcription factor 2 (RUNX2) in PSCs was modulated to explore the impact of the miR-584-5p/RUNX2 axis on hypoxia-induced osteogenic differentiation of PSCs.

METHODS

In this study, we isolated primary mouse PSCs and stimulated them with hypoxia, and the characteristics and functional genes related to PSC osteogenic differentiation were assessed. Constructs expressing miR-584-5p and RUNX2 were established to determine PSC osteogenic differentiation.

RESULTS

Hypoxic stimulation induced PSC osteogenic differentiation and significantly increased calcified nodules, intracellular calcium ion levels, and alkaline phosphatase (ALP) activity in PSCs. Osteogenic differentiation-related factors such as RUNX2, bone morphogenetic protein 2, hypoxia-inducible factor 1-alpha, and ALP were upregulated; in contrast, miR-584-5p was downregulated in these cells. Furthermore, upregulation of miR-584-5p significantly inhibited RUNX2

expression and hypoxia-induced PSC osteogenic differentiation. RUNX2 was the target gene of miR-584-5p, antagonizing miR-584-5p inhibition in hypoxia-induced PSC osteogenic differentiation.

CONCLUSION
Our study showed that the interaction of miR-584-5p and RUNX2 could mediate PSC osteogenic differentiation induced by hypoxia.

Key Words: Periosteal stem cell; Osteogenic differentiation; RUNX family transcription factor 2; MiroRNA-584-5p

Core Tip: This study simulated the hypoxic environment of periosteal stem cells (PSCs) during fracture and examined the related cellular responses to the hypoxia-induced PSC osteogenic differentiation. Importantly, the microRNA-584-5p/RUNX family transcription factor 2 axis was determined to be one of the regulating mechanisms for the hypoxia-induced PSC osteogenic differentiations.

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INTRODUCTION

The periosteum is a special connective tissue membrane that covers the surface of bone. For a long time, the types of cells that mediate osteogenic/chondrogenic differentiation at the periosteum have been unknown. The lack of generated periosteal cells following periosteal-deficient periosteal injury suggests a certain type of stem cell within the periosteum [1]. Periosteal stem cells (PSCs) were identified for the first time by Debnath *et al*[2]. PCS were extracted and identified from long bones and calvarial bones in mice, confirming the multifunctional differentiation potential of these cells in inducing the formation of tissues/organs, such as bones, cartilage, and fat. PSCs are essential for successful bone healing and provide new research ideas and directions for bone repair and maintenance since they are the main source of osteoblasts (OBs) and chondrocytes at the periosteum[3,4]. Since fracture healing is accompanied by obvious tissue hypoxia, it is worth exploring the effects of hypoxia on PSC osteogenic differentiation[5].

Noncoding RNAs are considered important biomolecules modulating cellular osteogenic differentiation[6], and microRNAs (miRNAs) are some of the most common noncoding RNAs that epigenetically modulate osteogenesis-related gene expression. Generally, aberrant miRNA expression is important in regulating stem cell differentiation into OBs/cartilage and is involved in the progression of bone diseases[7]. Based on the Gene Expression Omnibus database, a bioinformatics study[8] screened out the signature miRNAs in periodontal stem cells. MicroRNA-584-5p (miR-584-5p) was shown to be differentially expressed and inhibited during the osteogenic differentiation of periodontal stem cells. Notably, miR-584-5p might be associated with PSC osteogenic differentiation.

RUNX family transcription factor 2 (RUNX2) is a key regulatory factor for osteogenic differentiation[9]. This molecule can modulate the expression of osteogenic markers in cells by binding the functional elements of osteogenesis-related genes, regulating osteogenic differentiation[10]. RUNX2 mutation or deficiency inhibits cell differentiation into OBs, resulting in osteogenic defects[11]. Most studies have revealed the osteogenic activity of RUNX2 in bone marrow-derived mesenchymal stem cells, but its involvement in the osteogenic differentiation pathway of PSCs remains to be clarified.

This study aimed to determine the effect of hypoxia on PSCs, and the expression of miR-584-5p and RUNX2 in PSCs was modulated to explore the impact of the miR-584-5p/RUNX2 axis on hypoxia-induced osteogenic differentiation of PSCs. The findings of this study could provide new mechanistic insights into the regulation of PSC osteogenic differentiation induced by hypoxia.

MATERIALS AND METHODS

Cell isolation and experiments

PSCs were extracted from 7-d-old C57BL/6J mice (Beijing Vital River Laboratory Animal Technology). Mouse periosteal tissues were collected, chopped with a surgical blade, and digested with collagenase at 37 °C for 1 h. Then, Dulbecco's modified Eagle's medium with 2% fetal bovine serum was added to terminate digestion, and the digestive solution was centrifuged for supernatant removal. The pelleted cells were suspended in DNase solution and incubated for 5 min at 37 °C. The cells were filtered using a 70-μm cell filter, and the stem cells were induced by osteogenic differentiation medium. PSCs were incubated for 3 h under 2% O_2, 10% CO_2, and 88% N_2 to induce hypoxia; they were assigned and treated under

conditions including normoxia, hypoxia, hypoxia plus negative control (NC) mimic, miR-584-5p mimic, NC shRNA, sh-RUNX2, miR-584-5p plus mimic and pcDNA3.1, and miR-584-5p mimic plus pc-RUNX2.

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) activity in PSCs was detected according to the ALP activity detection kit (P0321S, Beyotime, Shanghai, China). The procedure was as follows: PSCs were fully lysed with cell lysis buffer composed of 20 mmol/L Tris (pH = 7.5), 150 mmol/L NaCl, and 1% Triton X-100, and the supernatant was used for ALP activity detection after centrifugation. Fifty microliters of the supernatant samples was added to each well of 96-well plates, including blank controls, and the working solution was added for 5-10 min of cultivation at 37 °C. After incubation, 100 μL of stop buffer was added to each well to terminate the reaction. A microplate reader (Thermo Fisher, United States) was used to measure the absorbance at 405 nm, and ALP activity was calculated using the standard curve set up in the assay.

Alizarin red S staining

This study determined calcified nodule formation in cells using an Alizarin red S (ARS) staining kit (C0148S, Beyotime, Shanghai, China). The cells were carefully washed with phosphate buffered saline (PBS) solution once after osteogenic differentiation induction of PSCs, incubated with a fixative for 20 min, and then washed with PBS solution three times. After addition of ARS dye for 30 min of cultivation, the cells were thoroughly rinsed with distilled water. Calcified nodule formation was observed using an optical microscope (Olympus, Japan). ARS chelated Ca^{2+} to form orange-red complexes in this experiment.

Characterization of intracellular calcium ions

The calcium ion level in PSCs was measured with a calcium-content chromogenic detection kit (S1063S, Beyotime, Shanghai, China). A lysis buffer with a volume of 100 μL was added to the cell sample for thorough lysis. Then, the lysate was centrifuged (10000 × *g*, 4 °C, 3-5 min) to obtain supernatants for calcium ion characterization. The standard curve and test working solution were prepared following the manufacturer's instructions. In a 96-well plate, the supernatant sample and 150 μL of working solution were mixed, added at 50 μL per well and incubated for 5-10 min at room temperature in the dark. The color reaction was detected at an absorbance OD of 575 nm, and the calcium ion content was calculated based on the standard curve.

Bioinformatics prediction and dual luciferase reporter gene assay

StarBase was used to predict the potential binding sites between miR-584-5p and RUNX2 mRNA, and the dual luciferase reporter (DLR) gene system was used to verify the targeting relationship between them. First, the targeted wild-type (wt) and mutant fragments of RUNX2 were cloned and inserted into pGL4 plasmids (upstream of the firefly reporter gene), yielding RUNX2-wt and RUNX2-mutant (mut), respectively, based on the predicted locus sequence information. MiR-584-5p and NC mimics were transfected into cells by the liposome method. Each constructed main reporter gene vector was then cotransfected with the Renilla reporter gene vector into cells. The firefly and Renilla luciferase activities were detected, and the relative luciferase activity (firefly/Renilla) was calculated.

Quantitative polymerase chain reaction

TRIzol solution-lysed cells were used to extract total RNA. RNA was reverse-transcribed into cDNA using a reverse transcription-fluorescence quantitative polymerase chain reaction (qPCR) kit (Tiangen, Beijing, China) and PCR apparatus (Applied Biosystems, United States) for real-time fluorescence quantification (qPCR) after determining the purity of the RNA samples. The miR-584-5p sense strand primer was 5′-CCGTTATGGTTTGCCTGGG-3′, and the miR-584-5p antisense strand primer was 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCAGT-3′. The RUNX2 sense strand primer was 5′-AGATGGGACTGTGGTTACCG-3′, and the RUNX2 antisense strand primer was 5′-GGACCGTCCACTGTCACTTT-3′. The $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression, which was normalized to that of glyceraldehyde-3-phosphate dehydrogenase and U6.

Western blot

RIPA lysis buffer (Cell Signaling, United States) was used for cell lysis. After protein isolation, a BCA detection kit (Abcam, United States) was used to measure the protein concentration, and the loading amount of the protein samples was calculated. After sodium-dodecyl sulfate gel electrophoresis, the proteins were transferred to a nitrocellulose membrane (NCM; Cell Signaling, United States) with a wet membrane transfer instrument in an ice bath. Then, the NCM was sealed at room temperature with a sealing liquid containing 5% skim milk powder for 1 h. The NCM was incubated overnight at 4 °C with primary antibodies, including RUNX2 (ab236639, 1:1000, Abcam United States), bone morphogenetic protein 2 (BMP2) (ab284387, 1:1000), hypoxia-inducible factor 1-alpha (HIF-1α) (ab179483, 1:1000), ALP (ab229126, 1:2000), and b-actin antibodies (ab8226, 1:10000), and the corresponding secondary antibody (ab6721, 1:10000, Abcam, United States) was incubated at room temperature for 1 h. An enhanced chemiluminescence reagent was used to detect protein bands. Images of the protein bands were obtained by X-ray analysis, and the relative protein expression normalized to β-actin expression was calculated.

Statistics and analysis

The average of each variable was obtained, and the standard deviation was calculated after three repeated measurements. The independent sample *t* test and one-way analysis of variance were used to identify differences between groups. The statistical analysis was conducted with Statistical Package for the Social Sciences version 22.0 software, and the graph

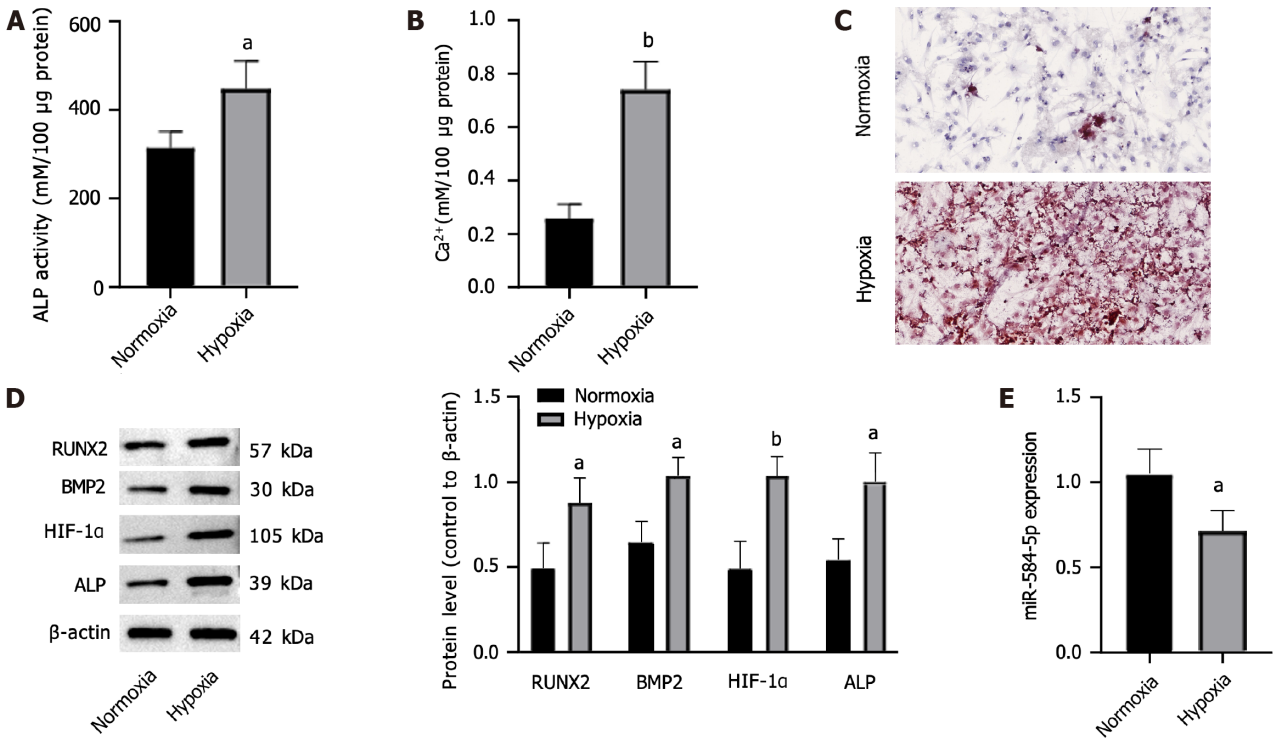

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Figure 1 Hypoxia stimulation promotes the osteogenic differentiation of periosteal stem cells. A: Alkaline phosphatase activity; B: Intracellular calcium ion level; C: Alizarin red S staining chelated Ca^{2+} form an orange-red complex; D: Osteogenic differentiation-related protein levels; E: MiRNA-584-5p expression in periosteal stem cells. One-way ANOVA and *t* tests were used to demonstrate differences between the hypoxia-induced and normal control groups. $^{a}P < 0.05$; $^{b}P < 0.01$. ALP: Alkaline phosphatase; RUNX2: RUNX family transcription factor 2; BMP2: Bone morphogenetic protein 2; HIF-1α: Hypoxia-inducible factor 1; miRNA: MicroRNA.

data were processed by GraphPad version 9.0. A 95% confidence interval was used, and a *P* value of < 0.05 indicated statistical significance.

RESULTS

Hypoxia promotes PSC osteogenic differentiation

An osteogenic differentiation medium was used to induce PSCs, and normoxic/hypoxic atmospheres were created to observe the impact of hypoxia on PSC osteogenic differentiation. ALP activity, intracellular calcium levels, and the expression of osteogenic differentiation-related factors, including RUNX2, BMP2, HIF-1α, and ALP, were measured, and ARS staining of PSCs was performed. Hypoxia significantly enhanced ALP activity (Figure 1A), Ca^{2+} levels (Figure 1B), and calcified nodule formation (Figure 1C) compared with normoxia. Hypoxia treatment increased the RUNX2, BMP2, HIF-1α, and ALP protein levels (Figure 1D). In contrast, hypoxia significantly downregulated miR-584-5p in PSCs (Figure 1E). Therefore, hypoxia could promote PSC osteogenic differentiation while downregulating miR-584-5p expression.

MiR-584-5p upregulation affects hypoxia-induced PSC osteogenic differentiation

To investigate whether aberrant miR-584-5p expression was related to hypoxia-induced PSC osteogenic differentiation, we used a miR-584-5p mimic vector to upregulate miR-584-5p in PSCs, and the NC mimic was used as a control (Figure 2A). Furthermore, PSCs were divided into two groups, transfected with NC mimic and miR-584-5p mimic, and the cells were stimulated by hypoxia and induced to differentiate into OBs to observe the role of miR-584-5p in hypoxia-induced PSC osteogenic differentiation. The results showed that upregulation of miR-584-5p lowered ALP activity (Figure 2B) and intracellular calcium deposition (Figure 2C) while suppressing calcified nodule formation (Figure 2D). Moreover, miR-584-5p overexpression significantly decreased the expression of osteogenic differentiation genes, such as RUNX2, BMP2, HIF-1α, and ALP (Figure 2E). Therefore, upregulation of miR-584-5p can inhibit PSC osteoblastic differentiation induced by hypoxia.

Targeted regulation of the RUNX2 axis by miR-584-5p

We hypothesized that miR-584-5p binds to a sequence fragment on the 3′ untranslated region of RUNX2 based on starBase (Figure 3A). The potential binding site was validated using a DLR gene assay. Relative luciferase activity was significantly reduced in the cells cotransfected with miR-584-5p mimic and RUNX2 wt but not in the cells with miR-584-

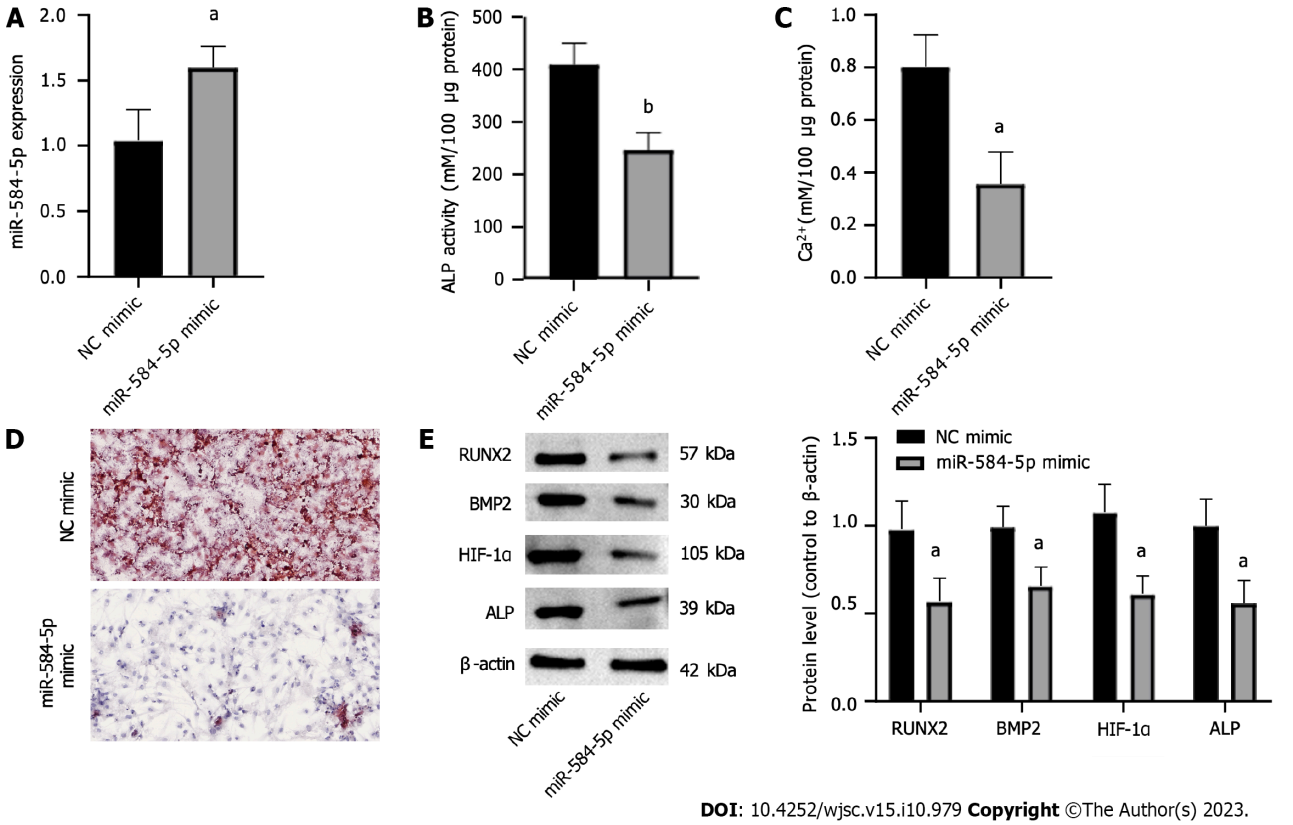

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Figure 2 Upregulation of miRNA-584-5P inhibits the osteogenic differentiation of periosteal stem cells induced by hypoxia. A: MiRNA-584-5p (miR584-5p) expression in stem cells; B: Alkaline phosphatase activity; C: Intracellular calcium ion level; D: Alizarin red S staining chelated Ca^{2+} form an orange-red complex; E: Osteogenic differentiation-related protein expression. One-way ANOVA and *t* tests were used to demonstrate differences between the miR584-5p mimic and negative control groups. $^{a}P < 0.05$; $^{b}P < 0.01$. ALP: Alkaline phosphatase; RUNX2: RUNX family transcription factor 2; BMP2: Bone morphogenetic protein 2; HIF-1α: Hypoxia-inducible factor 1; miRNA: MicroRNA; NC: Negative control.

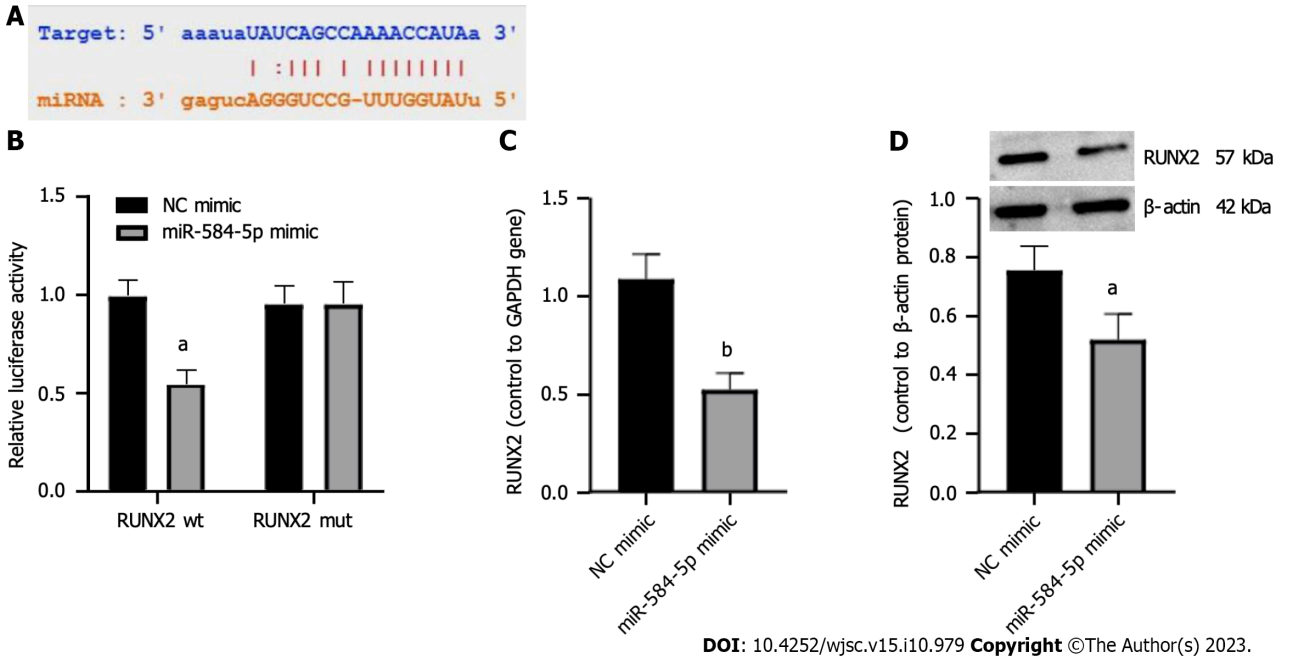

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Figure 3 Prediction of the miRNA-584-5p target. A: Potential binding sites of miRNA-584-5p and the 3'-untranslated region of RUNX family transcription factor 2 (RUNX2); B: Dual luciferase reporter gene assay; C: Quantitative polymerase chain reaction determination of RUNX2 expression; D: Western blot determination of RUNX2 protein expression. One-way ANOVA and *t* tests were used to demonstrate differences in RUNX2 responses between the miR584-5p mimic and negative control groups. $^{a}P < 0.05$; $^{b}P < 0.01$. RUNX2: RUNX family transcription factor 2; NC: Negative control; wt: Wild type; mut: Mutant; miRNA: MicroRNA.

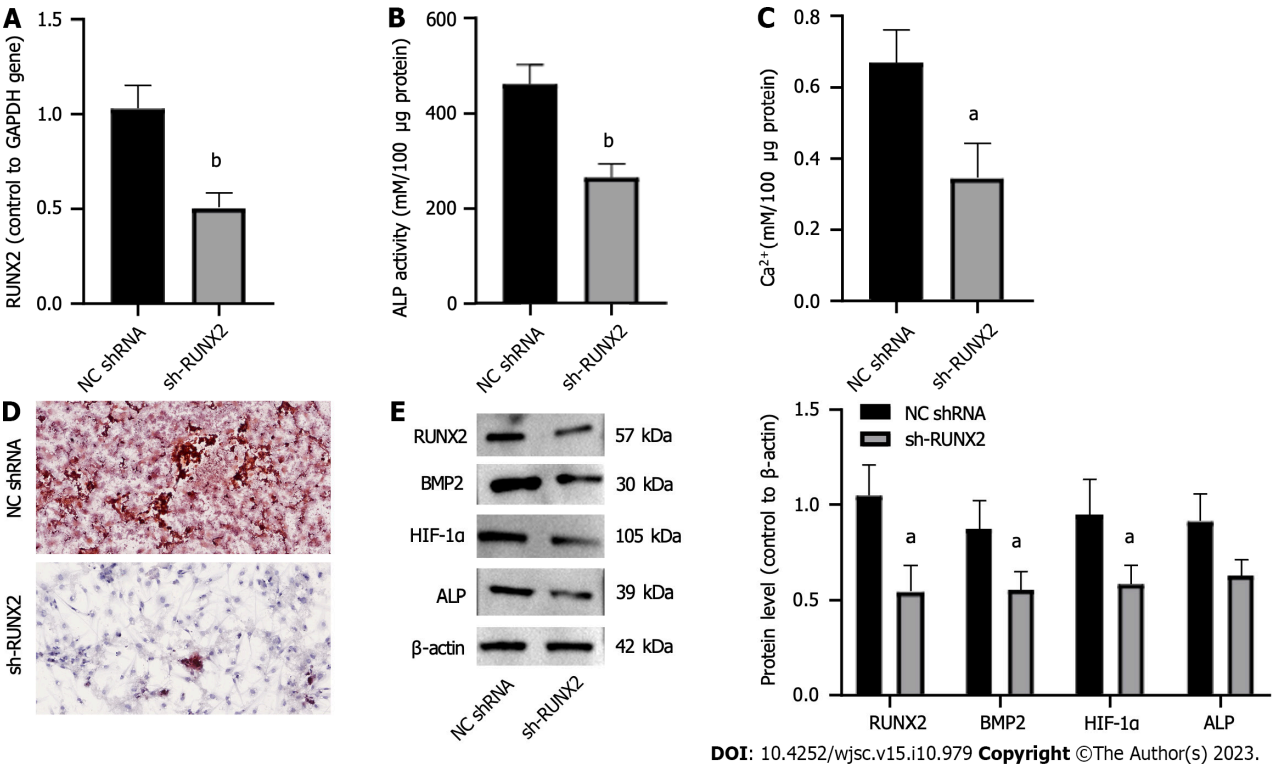

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Figure 4 Downregulation of RUNX family transcription factor 2 inhibits the osteogenic differentiation of periosteal stem cells induced by hypoxia. A: RUNX family transcription factor 2 (RUNX2) expression; B: Alkaline phosphatase activity; C: Intracellular calcium ion level; D: Alizarin red S staining chelated Ca^{2+} form an orange-red complex; E: Osteogenic differentiation-related protein expression. One-way ANOVA and *t* tests were used to demonstrate differences between the sh-RUNX2 and normal control shRNA groups. $^{a}P < 0.05$; $^{b}P < 0.01$. ALP: Alkaline phosphatase; RUNX2: RUNX family transcription factor 2; BMP2: Bone morphogenetic protein 2; HIF-1α: Hypoxia-inducible factor 1; shRNA: Small hairpin RNA.

5p mimic cotransfected with RUNX2-mut (Figure 3B). Furthermore, upregulation of miR-584-5 suppressed RUNX2 expression at both the RNA level (Figure 3C) and the protein level (Figure 3D). These results indicated that miR-584-5p could regulate RUNX2 through the predicted binding site.

Impact of RUNX2 on hypoxia-induced PSC osteogenic differentiation

To investigate the role of miR-584-5p-induced RUNX2 downregulation in hypoxia-induced PSCs, we constructed a RUNX2 inhibitory expression vector (sh-RUNX2) and a negative control shRNA (NC shRNA) (Figure 4A). Downregulation of RUNX2 significantly decreased ALP activity (Figure 4B), calcium deposition (Figure 4C), and ARS staining of osteogenic differentiation (Figure 4D); in contrast, it suppressed RUNX2, BMP2, HIF-1α, and ALP expression (Figure 4E). Thus, downregulation of RUNX2 inhibited hypoxia-induced PSC osteogenic differentiation.

MiR-584-5p regulates hypoxia-induced PSC osteogenic differentiation by targeting RUNX2

To investigate whether miR-584-5p could mediate the RUNX2 response pathway in hypoxia-induced PSC osteogenic differentiation, we established gene constructs such as NC mimic, miR-584-5p in pcDNA3.1, and miR-584-5p mimic + pc-RUNX2 and transfected them into PSCs. The results showed that miR-584-5p significantly lowered ALP activity (Figure 5A) and intracellular calcium ion levels (Figure 5B) in hypoxia-induced PSCs, and it inhibited PSC osteogenic differentiation induced by hypoxia (Figure 5C). In contrast, upregulated RUNX2 partly rescued the inhibitory responses mediated by miR-584-5p (Figures 5A-C). Moreover, upregulation of RUNX2 significantly reversed the downregulation of osteogenic differentiation-related proteins induced by miR584-5p overexpression (Figure 5D). Thus, the upregulation of RUNX2 specifically reversed the inhibitory effect of miR-584-5p overexpression on hypoxia-induced PSC osteogenic differentiation.

DISCUSSION

PSCs have a strong differentiation potential. Bone defects can activate PSCs through a series of signal transduction pathways and induce cell migration, proliferation, and differentiation into OBs/chondrocytes at the injury site[12]. However, the mechanisms underlying PSC osteogenic differentiation are poorly understood. This study simulated the hypoxic environment of PSCs during fracture and examined the related cellular responses to hypoxia-induced PSC osteogenic differentiation. Importantly, the miR-584-5p/RUNX2 axis was determined to be one of the regulatory mechanisms for hypoxia-induced PSC osteogenic differentiation.

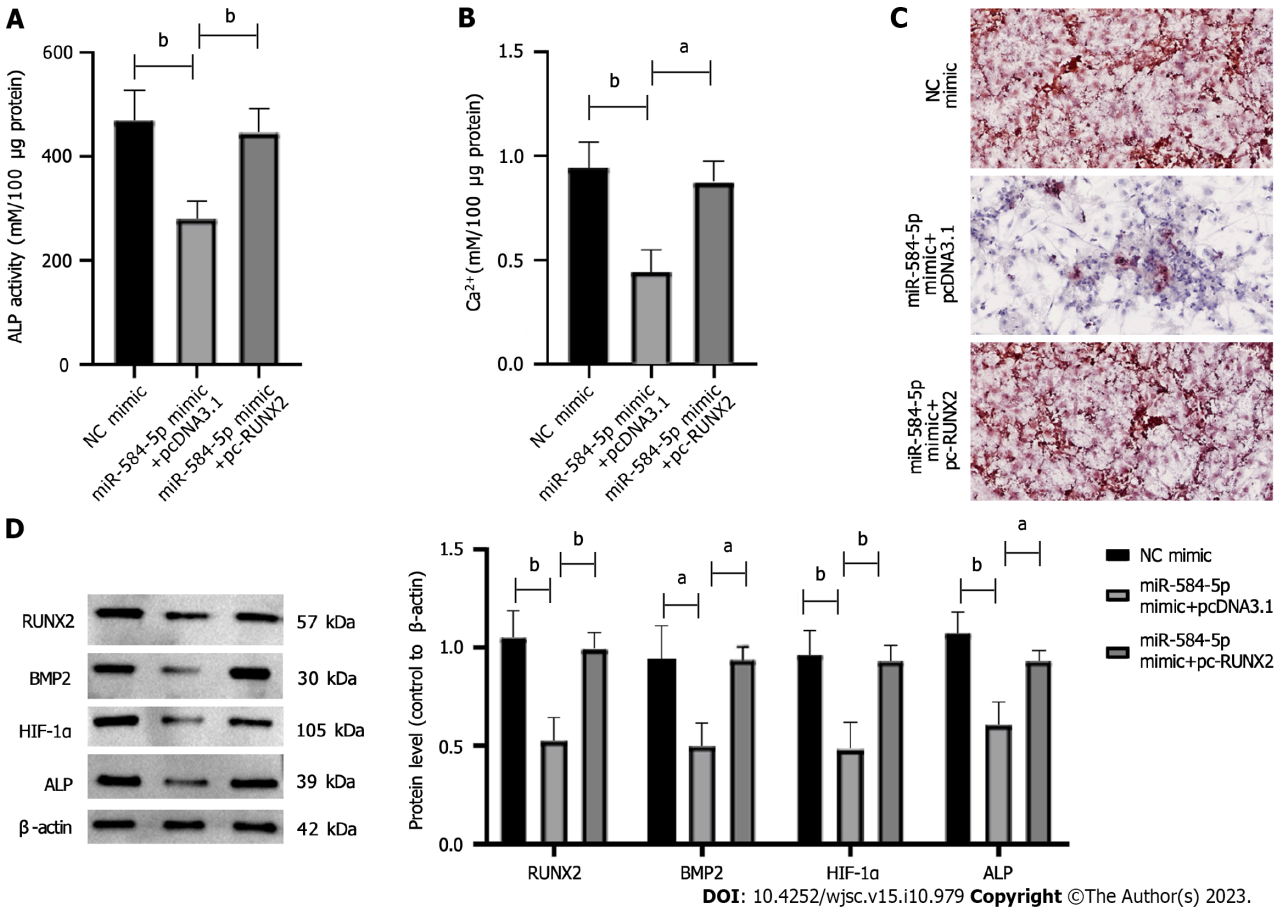

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Figure 5 MiRNA-584-5p regulates hypoxia-induced osteogenic differentiation of periosteal stem cells by targeting RUNX family transcription factor 2. A: Alkaline phosphatase activity; B: Intracellular calcium ion level; C: Alizarin red S staining chelated Ca^{2+} form an orange-red complex; D: Osteogenic differentiation-related protein expression. One-way ANOVA and *t* tests were used to demonstrate differences between the miR-581-5p mimic + pcDNA3.1-, NC mimic-, and miR-584-59 mimic + pc-RUNX family transcription factor 2-expressing constructs. $^{a}P < 0.05$; $^{b}P < 0.01$. ALP: Alkaline phosphatase; RUNX2: RUNX family transcription factor 2; BMP2: Bone morphogenetic protein 2; HIF-1α: Hypoxia-inducible factor 1; miR: MicroRNA; NC: Negative control.

Fracture healing is a complex process in which HIFs activate the osteogenic differentiation of precursor cells in response to the hypoxic environment where the fracture occurs[13]. Hypoxia is one of the main functional mechanisms of PSC osteogenic differentiation. One study emphasized that hypoxia upregulated HIF expression in PSCs, affecting the periosteal protein-mediated osteogenic differentiation pathway[14]. Similarly, we confirmed that hypoxia could activate PSC osteogenic differentiation and upregulate osteogenic differentiation-associated genes, such as RUNX2, causing cell mineralization. We also found that RUNX2 overexpression could increase osteogenic differentiation (Figure 5C). Thus, hypoxia is important for PSC osteogenic differentiation, and adjusting the oxygen concentration could induce PSC osteogenic differentiation *in vitro*.

Furthermore, we revealed that miR-584-5p was downregulated in hypoxia-induced PSCs. MiRNAs are key regulators of osteogenic differentiation and are involved in the expression of many genes in mesenchymal stem cells[15]. MiR-584-5p seems to be an important factor in bone healing and can mediate osteogenic differentiation by regulating gene expression[16-19]. Furthermore, miR-584-5p is a vital regulatory factor for osteosarcoma cell proliferation and bone homeostasis[20]. Therefore, miR-584-5p expression might be related to bone homeostasis. The significant downregulation of miR-584-5p in PSCs suggests its potential role in osteogenic differentiation. In this study, the upregulation of miR-584-5p significantly downregulated the expression of osteogenic differentiation genes such as RUNX2. This change inhibited ALP activity while lowering the degree of cell mineralization, indicating the ability of miR-584-5p upregulation to suppress PSC osteogenic differentiation. Notably, miR-584-5p plays important functions in mediating PSC osteogenic differentiation.

The biological functions of miRNAs include regulation of gene stability by binding mRNAs of downstream target genes[21]. We initially identified a sequence fragment of RUNX2 mRNA that could bind to miR-584-5p and then confirmed a binding site between miR-584-5p and RUNX2 through the DLR gene assay. We also confirmed that miR-584-5p could directly inhibit RUNX2 functions. RUNX2 is necessary for stem cell osteogenic differentiation[22]. The RUNX2 protein mediates the expression of many downstream genes, contributing to the OB phenotype[23]. RUNX2 upregulation also neutralized the regulatory effect of miR-584-5p overexpression on PSC osteogenic differentiation in our study. Therefore, miR-584-5p can affect hypoxia-induced PSC osteoblastic differentiation by regulating the RUNX2 functional pathway.

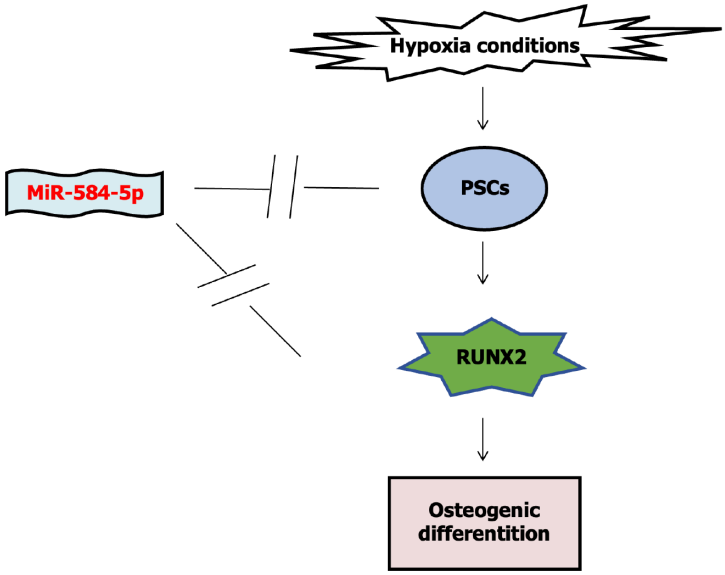

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Figure 6 The interregulation of microRNA-584-5p/RUNX family transcription factor 2 on hypoxia-induced periosteal stem cell osteogenic differentiation. The hypoxic environment induces periosteal stem cells to undergo osteogenic differentiation by activating the RUNX family transcription factor 2 (RUNX2)-related pathway; however, miR-584-5p overexpression can inhibit osteogenic differentiation by downregulating RUNX2 expression. PSC: Periosteal stem cell.

However, this study still has limitations. Skeletal reprogramming to bone regeneration requires reactivating key transcription and growth factors. The local hypoxic microenvironment can regulate cytokine synthesis. The biological performance of stem cells under hypoxia can be different from that under normoxic conditions. Thus, other key factors in the process of hypoxia-induced PSC osteogenic differentiation still need to be discovered and studied.

CONCLUSION

We revealed that miR-584-5p inhibited hypoxia-induced PSC osteogenic differentiation *via* RUNX2 (Figure 6). PSCs have good osteogenic/cartilage differentiation potential. Our findings provide a mechanism for PSC osteogenic differentiation, and modulating miR-584-5p and miR-584-5p/RUNX2 might be a new strategy for bone repair and regeneration.

ARTICLE HIGHLIGHTS

Research background

Fracture healing is accompanied by obvious tissue hypoxia, it is worth exploring the effects of hypoxia on periosteal stem cells (PSCs) osteogenic differentiation. However, the underlying mechanisms needs to be clarified.

Research motivation

RUNX family transcription factor 2 (RUNX2) is a key regulator of osteogenic differentiation, and studies have found that RUNX2 has osteogenic activity in bone marrow mesenchymal stem cells, but its role in the osteogenic differentiation pathway of PSCs is still unclear.

Research objectives

This study aimed to determine the effect of hypoxia on PSCs, and the expression of microRNA-584-5p (miR-584-5p) and RUNX family transcription factor 2 in PSCs was modulated to explore the impact of the miR-584-5p/RUNX2 axis on hypoxia-induced osteogenic differentiation of PSCs.

Research methods

PSCs were extracted from 7-d-old C57BL/6J mice. Then, PSCs were stimulated them with hypoxia, and the characteristics and functional genes related to PSC osteogenic differentiation were measured.

Research results

Hypoxia promotes PSC osteogenic differentiation. Also, miR-584-5p upregulation affects hypoxia-induced PSC osteogenic differentiation and RUNX2 axis was targeted by miR-584-59. The upregulation of RUNX2 specifically reversed

the inhibitory effect of miR-584-5p overexpression on the hypoxia-induced PSC osteogenic differentiation.

Research conclusions

MiR-584-5p inhibited the hypoxia-induced PSC osteogenic differentiation *via* RUNX2.

Research perspectives

MiR-584-5p inhibits hypoxia-induced osteogenic differentiation of PSC through RUNX2, and PSC has good osteogenic/chondrogenic differentiation potential. These results indicate that miR-584-5p/RUNX2 axis may be a key target pathway for bone repair and regeneration.

FOOTNOTES

Co-corresponding authors: Lei Zhu and Nan Lu.

Author contributions: Lu JJ, Shi XJ, Fu Q, Li YC, and Zhu L contributed equally to this work. Lu JJ, Shi XJ, Fu Q, Li YC, and Zhu L designed the research study, contributed new reagents and analytic tools, analyzed the data, and wrote the manuscript; Lu JJ, Shi XJ, Fu Q, Li YC, Zhu L, and Lu N performed the research; and all authors have read and approve the final manuscript. First, the research was performed as a collaborative effort, and the designation of co-corresponding authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. Second, the overall research team encompassed authors with a variety of expertise and skills from different fields, and the designation of co-corresponding authors best reflects this diversity. This also promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Third, Zhu L and Lu N contributed efforts of equal substance throughout the research process. The choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. In summary, we believe that designating Zhu L and Lu N as co-corresponding authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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