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Issue Editors

Warren Zimmer
Texas A and M University, United States

Steven Richard Goodman
University of Tennessee Health Science Center (UTHSC), United States

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*CORRESPONDENCE
Warren Zimmer,
wezimmer@tamu.edu

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Experimental Biology and medicine conference thematic issue introduction

Warren Zimmer*

Medical Physiology, College of Medicine, Texas A&M University Health Science Center, College Station, TX, United States

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Editorial on the Research Topic Experimental
Biology and medicine conference thematic issue introduction

The Society for Experimental Biology and Medicine (SEBM) sponsored a scientific meeting called Experimental Biology and Medicine Conference (EBMC 2024) on 13–16 October 2024. This was a transition for the Society from being a part of the larger Experimental Biology meeting to a meeting completely organized by SEBM and was the inaugural national meeting for the society. A committee of resolute members planned for over 2 years to have an in person meeting in Orlando, Florida. While planning touched on almost all contingencies, the track of Hurricane Milton through central Florida and Orlando necessitated a change of plans. In a tour de force SEBM changed the meeting format to online in just under 48 h to avoid any weather-related issues.

Even though EBMC moved to an online format, SEBM, a premier supporter of basic biomedical interdisciplinary research, maintained true to its mission of the dissemination of innovative translational research engaging basic and clinical scientists as well as promoting the career development of trainees and early career scientists. Over 50 scientists presented their cutting-edge research at EBMC and the Society, in collaboration with the Alliance for Cell Therapy, held a tribute to Dr. Arnold (Arnie) Caplan, the discoverer of Mesenchymal Stem Cells (MSCs) and largely regarded as the "father" of modern stem cell research therapies.

With input from SEBM membership, it was decided to have 3 themes for the meeting which were: Disorders of the Nervous System, Cardiovascular Disease and Regenerative Medicine: Stem Cell Based Therapies. Each theme was chosen to be an umbrella which sessions had content from basic science to clinical science. Each speaker was given the opportunity to submit a manuscript to the Journal (EMB) that summarized their work. The program committee chose the following papers to highlight in a special issue of the journal, termed *Experimental Biology and Medicine Conference Thematic Issue*, that represents at least one study from each area of interest from EBMC 2024.

Cardiovascular Diseases (CVDs) are a collection of disorders of the heart and blood vessels and remain a leading cause of death worldwide. One major issue is the inability of

heart cells to be repaired after injury. This has led to many studies of stem cell therapies for heart repair, however there is limited positive outcomes from this approach. The studies described by [1] show that there is significant reprogramming of heart cells (cardiomyocytes) *in situ* through limited injection of synthetic mRNAs encoding two important developmental factors called STEMIN and YAPS5A. Short term expressions of these factors lead to a reprogramming and subsequent repairing of the once senescent cardiomyocyte. This approach represents a major leap forward in potential therapies of cardiac injuries such as myocardial infarction and heart failure.

A second principal CVD issue involves changes in vessels carrying blood from the heart to the periphery, largely referred to as Atherosclerosis. These changes are responsible for a range of cardiovascular and cerebrovascular diseases, such as heart attack, heart failure, and stroke and are a major contributor to the global burden of cardiovascular disease. The studies reported here by [2] describe the capability of reversing many of the debilitating changes of atherosclerotic disease through restoring copper homeostasis in the system. Thus, it is possible that new, improved therapies for reversing atherosclerosis can be had by simply repairing metal physiology to the affected cells and tissues.

Like cardiomyocytes, neural cells are particularly intransigent to regeneration and repair. This is especially important in treatment of issues such as spinal cord injury (SCI). As discussed in the review by [3] there have been huge discoveries in treating SCI. However, there remains barriers in developing therapies for such injuries. As discussed in their review there is great hope with the continued progress in the field aimed at enhancing quality of life and functional outcomes for patients with debilitating spinal cord injuries.

As mentioned above, EBM dedicated a session to a tribute for Dr. Arnie Caplan, the recognized founder of stem cell therapies. A theme of the Regenerative Medicine talks centered around the ability to generate stem cells sufficiently from patients to be used in attacking a specific disease or injurious issue. This is a major problem and leads to treating patients with non-autologous cells leading to important issues of rejection of the added cells by the hosts, sometimes leading to greater complications than the initial lesions. The studies reported here by [4] at the University of Tennessee Health Science Center shows that induced mesenchymal stem cells (MSCs) can be isolated from periodontal ligament tissues in numbers that allow for regenerative medicine treatments. Thus, these authors indicate that we may be on the threshold of designing therapies using cells from the patients themselves. Clearly a breakthrough in the ability of treating many diseases and improving world health.

The papers presented here in the *Experimental Biology and Medicine Conference Thematic Issue* represent a small sample of the cutting-edge research presented at EBM 2024. Although we did switch to a virtual format, the presentations provoked much discussion and have led to enhanced collaborations among scientists that might not have come together except for listening and participating in the diverse talks.

Respectfully,

Warren Zimmer, PhD, SEBM Past President

Author contributions

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*CORRESPONDENCE
Robert J. Schwartz,
drbob6649@aol.com

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STEMIN and YAP5SA, the future of heart repair?

Nada Bejar[1], Siyu Xiao[1], Dinakar Iyer[1,2], Azeez Muili[1], Adeniyi Adeleye[1], Bradley K. McConnell[2] and Robert J. Schwartz[1]*

[1]Department of Biology and Biochemistry, University of Houston, Houston, TX, United States, [2]Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX, United States

Abstract

This review outlines some of the many approaches taken over a decade or more to repair damaged hearts. We showcase the recent breakthroughs in organ regeneration elicited by reprogramming factors OCT3/4, SOX2, KLF4, and C-MYC (OKSM). Transient OKSM transgene expression rejuvenated senescent organs in mice. OKSM transgenes also caused murine heart cell regeneration. A triplet alanine mutation of the N-terminus of Serum Response Factor's MADS box SRF153(A3), termed STEMIN, and the YAP mutant, YAP5SA synergized and activated OKSM and NANOG in adult rat cardiac myocytes; thus, causing rapid nuclear proliferation and blocked myocyte differentiation. In addition, ATAC seq showed induced expression of growth factor genes *FGFs*, *BMPs*, *Notchs*, *IGFs, JAK, STATs* and non-canonical *Wnts*. Injected STEMIN and YAP5SA synthetic modifying mRNA (mmRNA) into infarcted adult mouse hearts, brought damaged hearts back to near normal contractility without severe fibrosis. Thus, STEMIN and YAP5SA mmRNA may exert additional regenerative potential than OKSM alone for treating heart diseases.

KEYWORDS

stem cell factors, OCT3/4, SOX2, KLF4, and C-MYC (OKSM), serum response factor, STEMIN, YAP5SA

Impact statement

The induction of reprograming factors, OCT3/4, SOX2, KLF4, and C-MYC (OKSM), truly stands out from a myriad of regeneration studies, for their rejuvenation of senescent organs, such as the adult heart. However, long term treatment of OKSM, as with adenoviral expression, elicited cancers. Short term transfections of a regenerative cocktail STEMIN, and YAP5SA synthetic mmRNA induced OKSM plus Nanog, and rejuvenated infarcted hearts. Short-term treatments with STEMIN and YAP5SA mmRNA delivery may become a safer strategy to treat debilitating human cardiac diseases.

Introduction

We showcase breakthroughs in stem cell factor [1, 2] and STEMIN and YAP5SA [3, 4] in heart regeneration. Human adult heart lacks the intrinsic regenerative capacity to self-repair after cardiac injury, such as a myocardial infarction (MI). Many of the patients with ischemic heart disease not only undergo the acute phase of MI, but also develop ischemic cardiomyopathy, due to the loss of cardiomyocytes, and decreased cardiac function culminating in heart failure [5]. Due to the low regenerative capacity of cardiomyocytes, the damaged myocardium is replaced by fibrotic scar tissue, which further reduces pumping and circulatory function of the heart. Subsequently, the cardiac remodeling process results in further fibrosis, loss of cardiomyocytes, decrease cardiac function, and eventually resulting in heart failure, the leading cause of death worldwide [6].

Protecting the heart from progression to fatal heart failure continues to be focus of treating ischemic heart diseases [7, 8]. Cardiac intervention via revascularization by thrombolysis, and bypass surgeries to improve blood supply can salvage the injured ischemic myocardium. Medications such as angiotensin-converting enzyme inhibitors, angiotensin receptor-neprilysin inhibitors, mineralocorticoid-receptor antagonists, and β-blockers were proven to be effective on decreasing heart failure mortality [6, 9, 10]. Patients could benefit from these cardioprotective therapies targeting the remodeling process in the failing hearts. However, efficacious therapies for advanced cardiac remodeling in the later stages of heart failure are limited [11]. Mechanical support therapies such as cardiac resynchronization therapy and the application of left ventricular assist devices show beneficial contributions to end-stage heart failure patients [12], but the only treatment to end-stage heart failure with definitive effects is heart transplantation, which is limited by the lack of donor hearts [13].

Virtually the complete supply of human cardiomyocytes is established within the first month of life, and there is a dramatic drop in regenerative capacity within the first few days after birth [14]. Naqvi et al. [15] showed that the IGF-1/IGF-1-R/Akt pathway can be activated by a thyroid hormone surge in juvenile mice and initiated a brief but intense cardiomyocyte proliferative burst. Cardiomyocyte proliferation contributes to developmental heart growth in children. The number of cardiomyocytes in the left ventricle increased 3.4-fold between the first year and 20 years of age [16]. Adult human myocytes still maintain the ability to renew at approximately 1% per year, which was revealed by carbon-14 dating experiments [17]. Therefore, the poor regenerative capacity of adult human cardiomyocytes severely limits myocardial repair after a cardiac scenario. This review will survey potential therapies for the promotion of cardiomyocyte endogenous regenerative capacity towards cell replacement and cardiac repair.

Cell cycle regulation

Cell cycle regulators were among the first factors reported to be sufficient for driving adult cardiomyocyte through cell cycle, long before the trans-differentiation methods were published. In 2004, CNNA2 was reported to induce cardiac enlargement by cardiomyocyte hyperplasia, when expressed from embryonic day 8 into adulthood [18]. Intramyocardial delivery of adenoviral vector expressing CNNA2 could induce myocardial regeneration and enhance cardiac function in injured heart [19] and constitutive expression of CNNA2 could limit ventricular dilation while enhancing cardiac function [20]. Besides CNNA2, other cyclins such as CNND1, CNND2, and CNND3 were also proved to promote cardiomyocyte cell cycle activity [21, 22]. A discrete combination of cell cycle regulators besides cyclins were reported to efficiently unlock the proliferative capacity in cardiomyocytes that have terminally exited the cell cycle. Overexpression of four factors cyclin-dependent kinase 1 (CDK1), CDK4, CNNB1, and CNND1 indicated as 4F could drive robust cell proliferation in post-mitotic mouse, rat, and human cardiomyocytes, whereas CDK1 and CNNB can be substituted by small molecules SB431542 and MK1775 [23].

Growth factor stimulants

Growth factors were also described to have the ability to stimulate mature cardiomyocytes entry into cell cycle. FGF1/p38 MAP kinase inhibitor treatment after acute myocardial infarction in 8 to 10-week-old adult rat could increase cardiomyocyte mitosis. FGF1/p38 MAP kinase inhibitor treatment of 4 weeks resulted in reduced scar tissue and improved heart function [24]. However, a randomized clinic trial did not support the strategy of p38 MAPK inhibition in patients hospitalized with myocardial infarction. Losmapimod, a selective, reversible, competitive inhibitor of p38 MAPK, did not reduce the incidence of recurrent major adverse cardiovascular events in patients hospitalized with acute myocardial infarction [25]. In a swine model, IGF-1/HGF therapy was able to improve cardiac function in chronic myocardial infarction heart, and further increases can be observed by using an improved new delivery method, UPy hydrogel [26]. Nevertheless, treatments of growth factors not only stimulate the capacity of cardiomyocytes to re-enter cell cycle, but also fibroblasts to enter the cell cycle [26].

Manipulate signaling pathways

Signaling pathways involved in cardiogenesis and cardiomyocyte maturation were also investigated for their ability to promote cardiomyocyte regeneration. Meis1 deletion

in mouse cardiomyocytes was sufficient to extend the proliferative window of postnatal cardiomyocytes and reactivate cardiomyocyte mitosis in adult mouse heart without deleterious influences [27]. Paracrine factors such as Fgf16 were also reported to be potential regulatory factors in promoting myocardial repair [28]. GATA4 regulates neonatal heart regeneration through regulating expression of FGF16, and overexpression of FGF16 via adeno-associated virus in Gata4-ablated mice heart could partially rescue cardiac hypertrophy and improve cardiac function after injury. Tbx20 overexpression in adult cardiomyocyte directly represses cell-cycle inhibitory genes Meis1, Btg2, and p21, hence promotes adult cardiomyocyte proliferation and preserves cardiac function after myocardial infarction [29]. Hippo signaling pathway has appeared to be a key regulator of cardiomyocyte proliferation [30–33]. MicroRNAs such as miR302-367 cluster have been shown to regulate cardiomyocyte proliferation [34]. miR590 and miR199a were reported to act as key regulators of cardiomyocyte proliferation [35].

Cell reprogramming

In the past decade, with the advent of iPSC technology, numerous cell differentiation methodologies have been developed [36–38]. Somatic cell reprogramming of adult murine cardiac fibroblasts into beating cardiac-like myocytes *in vitro* were first established by the introduction of four transcription factors, GATA4, HAND2, TBX5, and MEF2C [39]. Also, microRNAs were proven to mediate somatic cell transdifferentiation into cardiomyocyte-like cells. For example, a combination of microRNAs (miR-1, miR-133, miR-208, and miR-499) could induce direct cellular reprogramming of fibroblasts to cardiomyocyte-like cells both *in vitro* and *in vivo*. [40] The authors demonstrated that a single transient transfection of the miRNAs was able to mediate reprogramming confirmed by expression of mature cardiomyocyte markers, exhibition of cardiomyocyte spontaneous calcium flux characteristic, and sarcomeric organization. Wang et al. [40] demonstrated that the introduction of "GMT" factors Gata4, Mef2c, and Tbx5 could mediate the resident non-cardiomyocyte in the murine heart to be reprogrammed into cardiomyocyte-like cells *in vivo*. Islas *et al.* [41] reported that mammalian mesoderm posterior (MESP) homolog and v-ets erythroblastosis virus E26 oncogene homolog 2 (ETS2) can reprogram primary human dermal fibroblasts into cardiac progenitor cells, whereas Nam *et al.* [42] showed that four human cardiac transcription factors, GATA4, Hand2, T-box5, myocardin, and two microRNAs, miR-1 and miR-133, can activate cardiac specific marker expression in both neonatal and adult human fibroblasts. Purely chemical means by introduction of small molecules and chemical cocktails were soon discovered to conduct direct reprogramming of fibroblasts to functional cardiomyocytes. Treatment of a combination of nine compounds termed 9C to can reprogram human fibroblasts to uniformly contracting induced cardiomyocyte-like cells [43]. Bypassing the use of viral-derived factors, automatically beating cardiomyocyte-like cells could be generated from mouse fibroblasts only by addition of chemical cocktails instead of transcription factors [44]. The studies of purely chemical means replacing viral-derived factors laid foundations for potential safer treatment for heart failure.

Reprograming factors, OKSM

Recently, short-term *in vivo* transgene induction of reprogramming factors OCT3/4, SOX2, KLF4, and C-MYC (OKSM) for less than a week generated partial reprograming, rejuvenated senescent organs, and extended mouse lifespans [1]. Transgenic expression of OSKM *in vivo* improves recovery from metabolic disease and muscle injury in older wild-type mice. Partial reprogramming may, lead to rejuvenating effects in different tissues, such as the kidney and skin [45]. The rejuvenating effects were associated with reduced expression of genes involved in inflammation, senescence and stress response pathways. Mechanistically, epigenetic chromatin remodeling occurs during shorter term OKSM treatment which coincides with anti-aging. But, long term transgene expression by adenoviruses may cause tumorgenesis [45].

Indeed, a recent study showed that *in vivo* expression of OKSM transgenes caused murine heart cell regeneration [2]. Short-term expression of OKSM did not cause cancer but was sufficient to induce cell replication and rejuvenation. However, long term treatment of OKSM, as with adenoviral expression, elicited cancer like transformation. Thus, to rejuvenate senescent myocytes and expand their number after a cardiac infarct, adult myocytes may need to be taken backwards to a primitive replicative state driven by stem cell factors for short term expression. Avoidance of long term expression from viral vectors provide a strong rationale of the use of synthetic mode RNA for short term transfections into cardiac myocytes.

Synthetic RNA delivery to cardiac myocytes

The idea of gene transfer by mRNA as a method to transfer somatic genes into mammalian tissue was first introduced, by Bhargava and Shanmugam [46]. Wolff *et al.* [47] injected vectors expressing mRNA encoding luciferase, chloramphenicol acetyltransferase, and β-galactosidase into mouse skeletal muscle *in vivo*. Protein expression was detected for all the genes, which marked the opening for the use of mRNA as a method to somatic gene transfer method into mammalian tissue. However, this method had limited use because of the immune

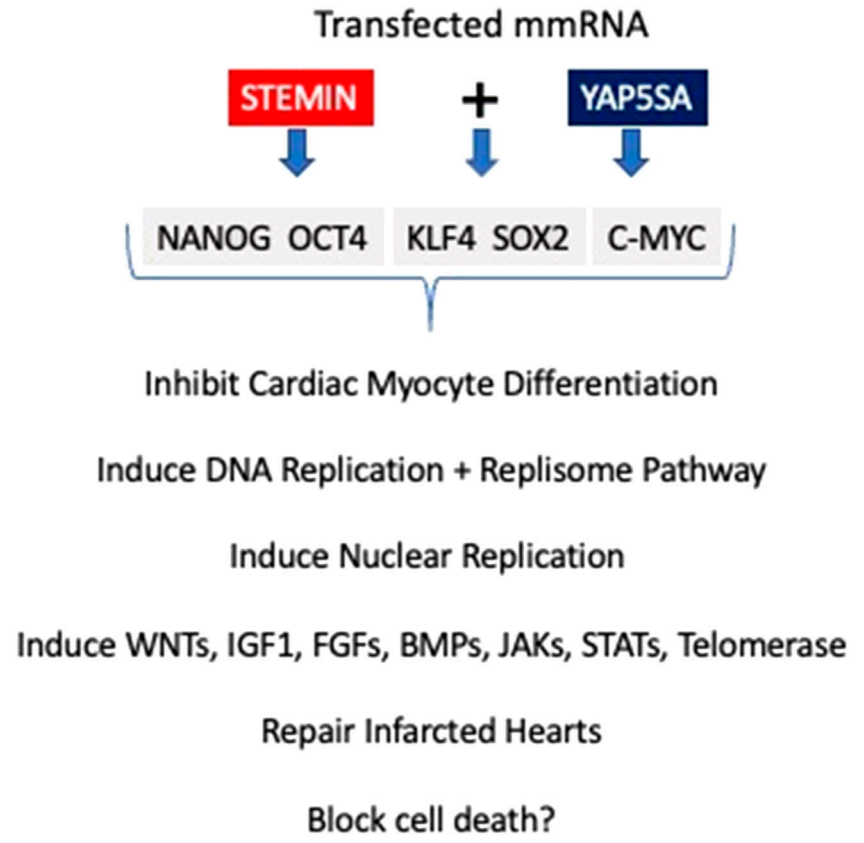

FIGURE 1
Schematic diagram of STEMIN and YAP5SA synthetic mmRNA induction of the cardiac myocyte regeneration pathway. STEMIN and YAP5SA synergize by the activation of the stem cell factors OCT4, KLF4,SOX2 and C-MYC (OKSM) + Nanog. Evidence provided by Chen et al. [2] and Xiao et al. [3, 4] showed that OKSM treatment of adult cardiac myocytes has a fundamental role in inducing replication and the inhibition of myocyte differentiation, taking cardiac myocytes backwards to a more primative developmental state. In addition, Xiao et al. [3, 4] showed that STEMIN and YAP5SA growth factor pathways plus telomerase maintence gene activities repaired infarcted mouse hearts and the potential for blocking cell death.

response that mRNA elicited [48]. Unmodified mRNAs can be recognized by the innate immune system of the cells via toll-like receptors [49], thus promoting the degradation of the unmodified mRNA. Fortunately, modified mRNA was made to bypass toll-like receptors. Modifying mRNA's (mmRNA) secondary structure, substituting uridine with pseudouridine, and replacing cytosine with 5-methyl-cytosine can all lead to less recognition by nucleases and toll-like receptors [49].

STEMIN and YAP5SA induced OKSM

A schematic diagram of STEMIN and YAP5SA synthetic mmRNA induction of the cardiac myocyte regeneration pathway. STEMIN and YAP5SA synergize by the activation of the stem cell factors OCT4, KLF4,SOX2 and C-MYC (OKSM) + Nanog, shown in Figure 1. Evidence provided by Chen et al. [2] and Xiao et al. [3, 4] showed that OKSM treatment of adult cardiac myocytes has a fundamental role in inducing replication and the inhibition of myocyte differentiation, taking cardiac myocytes backwards to a more primative developmental state. Xiao et al. [3] discovered that a triplet alanine mutation of N-terminus of SRF's MADS box SRF153 (A3), termed STEMIN, showed powerful activation of stem cell factors, and inhibited the induction of sarcomere assembly factors and cardiac myocyte specific genes. The triplet alanine mutation at aa153, aa154, and aa155 of the N-terminus of SRF's MADS box blocked the interaction of Nkx2.5 and GATA4 required for facilitating SRF DNA binding to CArG boxes; thus, blocking myocyte differentiation. Xiao *et al.* [3] showed the ability for STEMIN to be the "myogenic driver" was completely abrogated in the SRF null ES cells. The mutation of aa154 lysine to an alanine in the MADS box severely weakened SRF153(A3) transcription of many CArG-dependent cardiac-specified

genes. Rescue of SRF null ES cells with lentiviral expressed triplet SRF mutant, STEMIN inhibited the induction of several cardiac myocyte specific genes, such as those encoding sarcomeric actins, heavy and light chain myosins, ion channels, and structural proteins. And caused powerful activation of stem cell marker genes, such as *Egr1*, *Rex1*, *Nanog*, *Oct4*, *Zic3*, *Dppa2*, *Dnmt1*, *Dnmt2*, and *proliferin* [3].

Constitutive YAP1 activity by mutant YAP5SA

Transcription co-activator YAP can be an effective target to manipulate due to its function, as the key regulator in Hippo signaling pathway. Zhao *et al.* [50] generated and active form of YAP, termed YAP5SA, by mutating all the LATS1/2 phosphorylation sites. The phosphorylation sites mutation of YAP prevents 14-3-3 binding, thus preventing YAP protein degradation. YAP5SA enters nucleus and binds with TEAD to regulate nuclear targets. Recently, YAP5SA has been proven to partially reprogram the highly differentiated adult mouse cardiomyocytes to a more primitive proliferative state [51].

The mutual role of STEMIN and YAP5SA synthetic mmRNA was tested in adult rodent cardiomyocytes. Xiao at al [3]. showed adult cardiomyocytes entered the mitotic cell cycle 24 h post-transfection. Their synthetic mmRNA declined by at least 90% within 24 h and was undetectable by 48 h supported the notion of the rapid turnover of mmRNA. We then asked, how does STEMIN and YAP5SA activate nuclear replication so quickly? Azeez Muili, a recent doctoral student, discovered that transfection of neonatal rat ventricular myocytes (NVRM) with STEMIN mmRNA for 24 h revealed the induction of NANOG by anti-NANOG staining, and significant induction of NANOG and OCT4 RNA, but not KLF4, SOX2 and C-MYC transcripts assayed by quantitative PCR and by RNA sequencing [3]. In fact, in comparison to transfected YAP5SA, NANOG, and OCT4 transcripts were induced to a greater extent with STEMIN, while YAP5SA upregulated C-MYC. Together STEMIN and YAP5SA synergized and induced KLF4 and SOX2 and the stem cell program similar to short term OKSM transgenic expression [2].

Next, the expression of cyclins appeared to be repressed in murine ES cells in the absence of SRF. Rescue with wild-type SRF caused activation of cyclins, CNNB1, CNND1, CNNC, and CNNE1, while STEMIN strongly induced CNNA2, CNNB1, and CNNE1. Note the induction of CNNA2 fostered myocardial regeneration and enhance cardiac function in injured heart [19, 20]. Most of the crucial genes involved in DNA replication in the replisome pathway, such as ORC2, MCM2, CDC45, and CLASPIN, were significantly increased by STEMIN and YAP5SA mmRNA in the G1 phase of the cell cycle. Mitotic genes such as, *Bub1*, *Bub1b*, *Cenpe*, *Ndc80*, *CcnB1*, and *Dync1* was observed by 32 h and the appearance of DNA packaging genes, which mark the S phase of the cell cycle, including histone 1 genes, such as *Hist1h1a*, *Hist1h1b*, and *Hist1h2ba*, by 40 h post transfection. Upregulation of crucial cell cycle genes such as Plk1 and Anln suggested that STEMIN and YAP5SA promoted several steps of cell-division cycle of cardiomyocyte. In addition, DIAPH3 was localized to multiple regions between and surrounding dividing nuclei [3, 4]. DIAPH3 marks anaphase of the cell cycle and induced F-actin to help assemble a contractile ring during cytokinesis. By 40 h post-STEMIN and YAP5SA treatment, many cardiac-specified genes including *Actc1*, *Myh6*, *Myocd*, and *Mef2C* were downregulated. Thus, STEMIN and YAP5SA mmRNA is a potent activator of stem cell gene activity of OKSM plus Nanog, cell replication and inhibitor of cardiac-specific gene activity.

A new molecular technology named ATAC seq (Assay for Transposase-Accessible Chromatin using sequencing) accesses remodeled open chromatin DNA with an hyperactive mutant Tn5 Transposase that inserts sequencing adapters into open regions of the genome [52]. Sequencing TnT5 bound DNA revealed regions of increased accessibility and maps transcription factor binding sites. To identify the underlying mechanism of how STEMIN works as a novel transcription factor, we used ATAC-seq to create a bioinformatics topography of interactomes of STEMIN, wildtype SRF, and YAP5SA. Xiao et al. [3] findings suggest a complementary effect of YAP5SA and STEMIN interactions with known and novel co-factors.

SRF has several tissue-specific regulatory cofactors, such as Nkx2.5 and GATA4, that control SRF activity by interacting with SRF's MADS box [3]; whereas, YAP does not directly bind to DNA or bind directly to SRF [53]. ETS factors bind well to wildtype SRF as previously shown [54–56] and to mutant STEMIN [3]. TEAD1 or TEF1, one of SRF's cofactors shown by our previous studies [57, 58] to physically interact with SRF, may also serve as a bridge between YAP5SA and STEMIN to implement their synergy. STEMIN's interactome prefers recruitment by ETS factors, and CTCF, SP1, RBPJ, NFAT5, and TEAD1. In addition, we found many new YAP5SA cofactor associations with DNA binding cofactors ETS1, SP2, SP1, JUNB, FOS, CTGF, IRF3, MEF2C, and RBPJ, as well as its well-known cofactors RUNX1, SMAD3, and TEAD1 [3]. YAP5SA interactomes also revealed considerable association with SRF and its cofactors, previously not shown. Thus, STEMIN and YAP5SA share interactive associations with many more transcription factors than previously imagined, providing a powerful spectrum of transcription regulators that are strongly pro-replicative.

ATAC-seq also revealed chromatin remodeling of many growth factors and signaling pathway genes, including *FGFs*, *BMPs*, *Notchs*, and *Wnts*. [3] Activation of non-canonical WNT5A/B and WNT11, stimulates cardiomyogenic proliferation [59–61]. WNT5A/WNT11 inhibits

CTNNB1 signaling and promotes cardiac progenitor development in differentiating embryonic stem cells. Signaling pathways that express STATS and JAKS, such as STAT5 and JAK3, have key roles in cellular growth [62]. In addition, YAP signaling has a strong impact on inducing IGF1, IGF2, and their binding proteins and gene remodeling to enhance cell growth and resist apoptosis [63].

Co-expression of STEMIN and YAP5SA repaired infarcted adult mouse hearts *in vivo*

We tested the treatment combination of STEMIN and YAP5SA mRNA *in vivo* by injecting directly into the left ventricles of adult mice after myocardial infarction [4]. The mmRNA injection method with the co-transfectant agent, Lipofectamine MessengerMAX, delivered STEMIN and YAP5SA mmRNA together into 5 precise injection sites surrounding the infarct in the mouse left ventricle proved to be an effective, precise, and leak-free method. In the short-term experiments, we were able to detect incorporated 5-ethynyl-2′-deoxyuridine (alpha-EdU) into DNA of transfected myocytes, which co-stained with anti-SRF and anti-YAP antibodies, around the needle tracts in the mRNA treatment groups. Co-staining with Tnnt and pH3 antibodies marked replicated cardiac myocyte nuclei in response to STEMIN and YAP5SA mRNA injection [4]. Bioinformatic analysis revealed the upregulation of multiple cell cycle gene clusters with co-expression of STEMIN and YAP5SA, while gene clusters associated with cardiomyocyte differentiation (GO: 0055007), sarcomeric assembly and cardiac muscle contraction (GO: 0060048) were profoundly down regulated. We further illustrated the improvement in mouse cardiac function in long-term experiments for 4 weeks. Mice cardiac function evaluated by echocardiography, revealed improved cardiac pumping function by STEMIN and YAP5SA mRNA co-injection.

STEMIN and YAP5SA may block cardiac apoptosis

Induced myocyte proliferation may not be the only program responsible for the maintenance and or growth of cardiac mass; could the concomitant STEMIN and YAP5SA-induced upregulation of pro-survival and anti-apoptotic miRNAs, as observed from our ATAC-sequencing data [3], be responsible? Preliminary studies revealed transfected STEMIN and YAP5SA mRNAs alone and or in combination in cardiac myocytes for 24 h significantly inhibited CASP3 transcripts by over 65%–90% and inhibited TP53 transcripts primarily with YAP5SA by over 50% (study in preparation). Thus, chromatin remodeling data directed us to hypothesize that the inhibition of cell death may also come into play in the viability of the cardiomyocytes. Studies are underway to determine whether STEMIN and YAP5SA might induce anti-apoptotic miRs through the induction of OKSM.

Conclusion

Finally, synthetic mRNA may be used as a safe and efficient gene delivery vehicle in adult hearts. Compared to viral vectors, the transient gene expression that mmRNA provides is far more controllable, which makes the mmRNA gene-delivery method a safer option to deliver therapeutic factors for cardiac regeneration. In fact, adenovirus delivery of stem cell factors is initially curative for regenerating cardiac function, but it causes cardiac rhadomyosarcomas in the long term [64]. Given the post-transcription nature of mRNA, mmRNA does not require transfer to the nucleus to get the expression of the target protein. Besides, mmRNA-based gene delivery can deliver gene combinations with different ratios specifically tailored to patients with a different course of the disease. Our data suggest that synthetic mmRNA may be used to deliver STEMIN and YAP5SA into adult cardiac myocytes both *in vitro* and *in vivo* to achieve high transfection efficiency with little biosafety concern. Inducing tissue regeneration by short-term treatments with STEMIN and YAP5SA mRNA may become a useful and safer strategy to treat debilitating human cardiac diseases.

Author contributions

All authors contributed to the writing and editing of this review article. NB, SX, DI, AM, AA, BM, and RS contributed to previous published studies and studies in preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

RS is a founder of Animatus Biosciences. This review was underwritten in part by a grant from Animatus Biosciences LLC, in which there is a financial interest. A management plan, reviewed and recommended by the University of Houston Conflict of Interest Committee as acceptable to address the conflict.

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*CORRESPONDENCE
Y. James Kang,
james-kang@tasly.com

†These authors have contributed equally to this work

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Reversal of atherosclerosis by restoration of vascular copper homeostasis

Xiao Zuo[1†], Xueqin Ding[2†], Yaya Zhang[1] and Y. James Kang[1,2*]

[1]Tasly Stem Cell Biology Laboratory, Tasly Biopharmaceutical Co., Tianjin, China, [2]Regenerative Medicine Research Center, West China Hospital, Sichuan University, Chengdu, Sichuan, China

Abstract

Atherosclerosis has traditionally been considered as a disorder characterized by the accumulation of cholesterol and thrombotic materials within the arterial wall. However, it is now understood to be a complex inflammatory disease involving multiple factors. Central to the pathogenesis of atherosclerosis are the interactions among monocytes, macrophages, and neutrophils, which play pivotal roles in the initiation, progression, and destabilization of atherosclerotic lesions. Recent advances in our understanding of atherosclerosis pathogenesis, coupled with results obtained from experimental interventions, lead us to propose the hypothesis that atherosclerosis may be reversible. This paper outlines the evolution of this hypothesis and presents corroborating evidence that supports the potential for atherosclerosis regression through the restoration of vascular copper homeostasis. We posit that these insights may pave the way for innovative therapeutic approaches aimed at the reversal of atherosclerosis.

KEYWORDS

atherosclerosis, copper, macrophages, inflammation, reversal therapy

Impact statement

Recently advanced understanding of pathogenesis of atherosclerosis transformed the disease treatment approach from slowing its progression to promoting the regression of atherosclerosis. Copper plays a critical role in the regulation of structural integrity and lipid metabolism of vascular tissue. However, copper is deficient in the atherosclerotic vasculature, and contrarily elevated in the blood of atherosclerotic patients. Experimental restoration of copper homeostasis between the vessel wall and the circulation reverses the established atherosclerosis in animal models. It is predictable that the time is coming for therapeutic reversal of atherosclerosis.

Introduction

Atherosclerotic disease is a chronic inflammatory condition that affects the wall of arteries, resulting in a buildup of plaque and subsequent narrowing or blockage of blood

vessels [1, 2]. Atherosclerosis is responsible for a range of cardiovascular and cerebrovascular diseases, such as heart attack, heart failure, and stroke. Atherosclerosis has been a major contributor to the global burden of cardiovascular disease, which remains one of the leading causes of mortality worldwide [3].

Currently, atherosclerosis is widely considered as a progressive and irreversible disease [4, 5]. As summarized in Figure 1, clinical therapies primarily involve antagonistic therapy approaches that focus on inhibition of factors contributing to the occurrence and progression of the disease, with the aim of delaying the progression of the disease and reducing the risk of cardiovascular events due to plaque rupture [6].

Primary therapeutic interventions for atherosclerosis includes lifestyle modifications, such as regular exercise, a healthy diet, smoking cessation, and management of hypertension, diabetes, and high cholesterol levels [7, 8]. Such alterations in lifestyle can exert preventative effects at the nascent stages of the disease, potentially restoring some patients to a state of health [9–11]. However, these lifestyle modifications fail to reverse the condition once the disease has progressed to the phase characterized by intimal thickening within the blood vessels [12]. At this juncture, pharmacological interventions become the cornerstone of atherosclerosis management.

Statins are commonly prescribed for effective reduction of blood cholesterol levels and lowering the risk of plaque formation [13, 14]. Furthermore, antiplatelet agents, such as aspirin, are employed to inhibit blood clot formation within arteries [15, 16]. Moreover, medications including angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) are prescribed to address hypertension, which often concomitantly exist with atherosclerosis, thus contributing to a comprehensive management strategy for the condition [17].

Inflammation is known to play a decisive role in the progression of atherosclerosis. Therefore, inhibition of inflammation is currently considered as the most important treatment option [18–21]. While early administration of broad-spectrum anti-inflammatory and anticoagulant drugs, such as aspirin and colchicum, can reduce the risk of cardiovascular events, these approaches, however, do not have

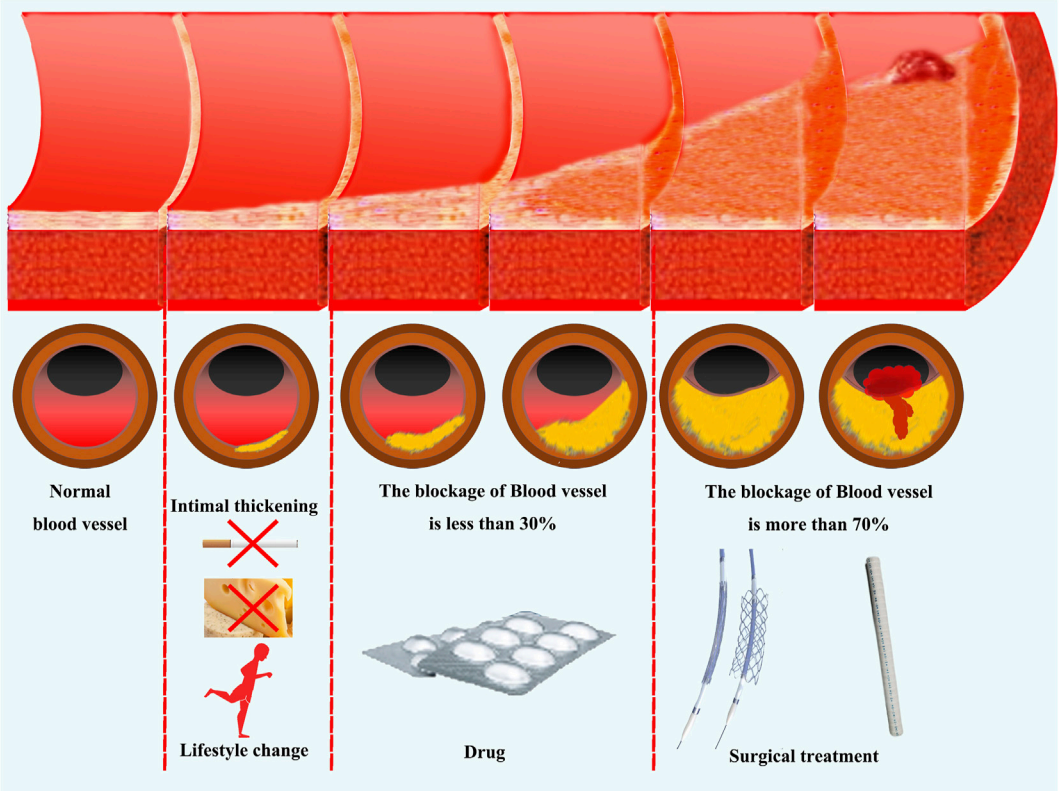

FIGURE 1
Current clinical approaches for the treatment of atherosclerosis. Antagonistic therapy against pathogenic factors is the mainstay of currently clinical treatments for atherosclerosis. In the early stages of vascular lesions, disease risk factors can be reduced by changing one's lifestyle. When the thickening of the vascular endothelium and the degree of vascular obstruction is less than 30%, the disease progression can be controlled through drug therapy. When vascular obstruction exceeds 70% or patients exhibit significant clinical symptoms, revascularization treatment can be performed through surgery.

a significant effect on regression of the disease condition [22]. In addition, there are considerable risks associated with broad-spectrum anti-inflammatory drugs, such as tumorigenesis [23].

It appears that, although current drug therapy can delay the progression of atherosclerosis to some extent, the diseased vessels remain prone to blockage or atherosclerotic plaque rupture. Surgical treatment is typically reserved for patients with advanced stage of atherosclerosis, particularly when the vascular obstruction surpasses 70%, or when patients exhibit significant clinical symptoms including pain or functional impairments in the distal extremities, indicative of inadequate blood flow [24]. The most common surgical procedures for treating advanced atherosclerosis include angioplasty, stent placement, and coronary artery bypass grafting (CABG).

Angioplasty entails the introduction of a balloon catheter into a constricted artery, followed by balloon inflation to expand the arterial passage [25]. In stent placement is to use a small metal mesh tube implanted within the artery to hold it open [26]. CABG involves the transplantation of a healthy blood vessel to circumvent the obstructed segment of the artery [27]. These interventions aim to restore blood flow by physically clearing the blockages. However, despite the effectiveness of these efforts, the issue of progressive stenosis and re-occlusion of blood vessels remains a significant challenge, putting patients at risk of secondary surgery or acute vascular occlusion [28, 29].

Therefore, combating atherosclerosis is a challenging task. There is an urgent need for a new approach focusing not only on delaying the progression of the disease, but also on fundamentally restoring the structure and function of the diseased blood vessels. To achieve this goal, it is crucial to establish new treatment strategies from the perspective of promoting vascular repair and regeneration. These strategies should be based on more advanced understanding of the pathological mechanisms of atherosclerosis.

In this review, we summarize the advanced knowledge of the pathogenesis of atherosclerosis, drawing attention to the role of copper metabolism in maintaining vascular homeostasis. We analyzed the potential of restoring copper homeostasis in atherosclerotic plaque tissue to reverse the progression of atherosclerosis. We also provided an overview of the current research in this area and discussed the challenges that need to be addressed for future development of clinically feasible treatments for the reversal of atherosclerosis.

The pathogenesis of atherosclerosis

Atherosclerosis is a progressively pathological process characterized by chronic inflammation as its primary pathogenic mechanism [21, 30]. The stages of its initiation and development are outlined below and diagrammed in Figure 2.

Endothelial cell dysfunction and lipid particles accumulation

The vascular endothelium plays a critical role in regulating homeostatic network of the cardiovascular system [31]. Endothelial cell dysfunction (ECD), which marks the initial alteration in the trajectory of atherosclerotic lesion progression, is typified by the activation of endothelial cells (ECs) and their shift from an anti-inflammatory to a pro-inflammatory state [32, 33]. Recent studies suggest that the hemodynamic change is the primary cause of ECD [34].

Under undisturbed laminar flow, ECs exhibit an up-regulation of Kruppel-like factor 2 (KLF2), a transcription factor integral to maintain vascular equilibrium. This upsurge in KLF2 expression precipitates an increase in endothelial nitric oxide synthase (eNOS) gene expression, catalyzing the synthesis of nitric oxide (NO). NO, a small lipid soluble molecule, traverses cell membranes to influence a wide array of cell functions within the bloodstream and the vascular endothelium [35–37]. NO's effects include the inhibition of platelet activation, adhesion, and aggregation [38], a reduction in leukocyte adhesion to the endothelium [39], a facilitation of vasorelaxation through dephosphorylation of myosin light chain in vascular smooth muscle cells [40], and an enhancement of the oxygen delivery capacity of red blood cells [41].

Steady laminar flow promotes the alignment of ECs [34] and inhibits signal transduction by pro-inflammatory stimuli such as TNF and interleukin-1 (IL-1) [42]. This hemodynamic condition diminishes IL-6 induced progression of cell cycle in ECs [43], and protects against ECs apoptosis, ensuring the endothelial integrity [44].

Blood flow irregularities trigger ECs activation, leading to a consequential decrease in eNOS gene expression and the stimulation of nuclear factor kappa B (NF-κB) signaling pathway [45, 46]. NF-κB plays a pivotal role in the initiation of pro-inflammatory responses within the endothelium. These responses include up-regulation of ECs surface adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1); the release of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and Fractalkine; and the production of pro-thrombotic mediators including tissue factor (TF), von Willebrand factor (vWF), and plasminogen activator inhibitor-1 (PAI-1), in both their soluble and membrane-bound forms [47–49].

Activated ECs cause an increased production of reactive oxygen species (ROS) [50]. The resulting oxidative stress, in addition to prolonged inflammation, disrupts adherent junctions, such as VE-cadherin and gap junctions, primarily due to the reduction in NO levels [51]. This disruption leads to an increased accumulation of sub-endothelial atherogenic apolipoprotein B (ApoB)-containing lipoproteins, including LDL, VLDL and chylomicrons [52, 53]. Importantly, the increased ROS facilitates oxidative modification of ApoB-containing lipoproteins [54], which act as dual-function agents in the immune response: serving as antigens that initiate the

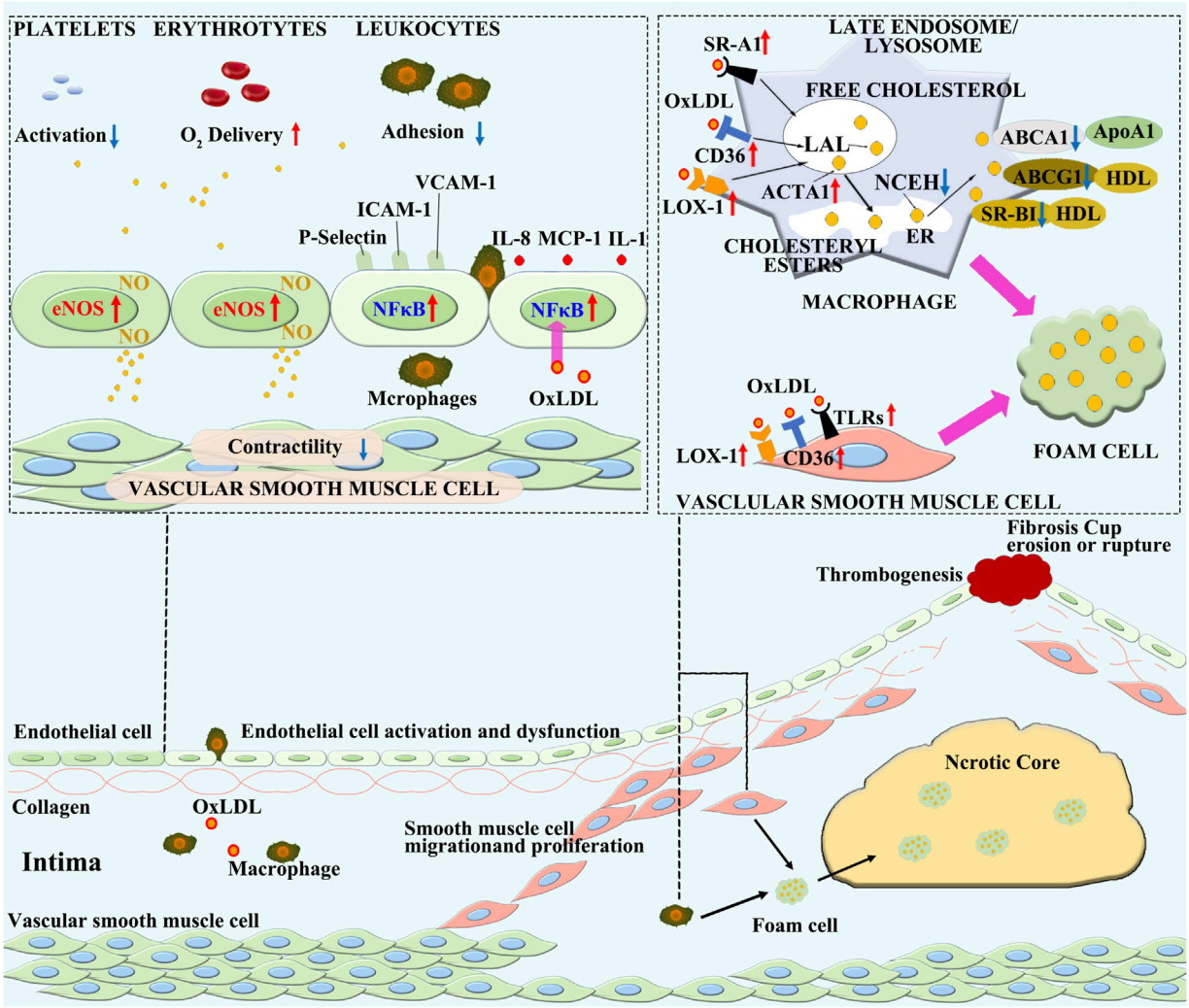

FIGURE 2
The pathogenesis of atherosclerosis. Various pathological factors in the blood cause dysfunction of ECs, leading to lipid deposition in the vascular intima. The oxidation of lipids to oxidized lipoproteins constitutes an initial inflammatory microenvironment, which further stimulates the activation and inflammatory transformation of ECs. Monocytes in the blood are attracted into the vascular intima by inflammation, where they differentiate into macrophages. In the inflammatory microenvironment, the steady state lipid metabolism in macrophages is disrupted, leading to the engulfment of oxLDL without restriction. On the other hand, the ability of macrophages to excrete lipids is decreased, resulting in lipid accumulation within macrophages and the formation of foam cells. Inflammatory macrophages exacerbate further development of inflammation, and with the accumulation of a large number of foam cells, cell necrosis occurs, forming a necrotic core. Smooth muscle cells are activated by inflammatory stimuli and form a fibrous cap on the surface of the plaque by migration, proliferation, and secretion of collagen, leading to the formation of a stable plaque. As inflammation continues to intensify, the necrotic core continues to enlarge, smooth muscle cells undergo massive necrosis, and the collagen fibrous cap becomes thinner and vulnerable. Ultimately, the plaque ruptures, leading to thrombosis.

adaptive immune response and as adjuvant molecules that stimulate the innate immune system [55–57].

Lifestyle modifications and pharmacological interventions can improve the blood microenvironment by lowering lipid levels, restoring EC's function. If ECs function is not restored, the lipid deposition within the vessel wall will persist. The retention of oxidized low-density lipoprotein (oxLDL) in the intima of blood vessels acts as a constant source of inflammatory stimulus, continuously activating ECs and triggering inflammatory responses [58, 59]. Therefore, it is crucial to remove oxLDL to suppress the pathogenesis of atherosclerosis.

Immune cell recruitment and foam cell formation

Macrophages are the major immune cells that respond to inflammation triggered by oxLDL, playing a pivotal role in the

formation and progression of atherosclerotic lesions [55, 57]. Macrophages mainly originate from myeloid progenitor cells in the bone marrow. Myeloid progenitor cells develop into circulating monocytes, which can infiltrate into atherosclerotic lesions from the bloodstream or from the spleen that acts as a reservoir for monocytes in mice [60]. The recruitment of monocytes to atherosclerotic lesions is mediated by the activation of ECs.

Activated ECs initiate monocyte recruitment by enabling their initial rolling on the endothelium through P-selectin engagement, followed by the strengthening of monocyte adhesion via interactions with immunoglobulin-G family proteins, VCAM-1 and ICAM-1. Subsequent monocyte infiltration into the subendothelial layer is driven by chemokines such as MCP-1 and IL-8 [35, 61, 62]. Once penetrating the intima of blood vessels, monocytes encounter a pro-inflammatory milieu constructed by activated ECs and oxidized lipid particles. This environment fosters the transformation of monocytes into pro-inflammatory macrophages, actively up-taking lipids [63, 64].

Macrophages play a crucial role in regulating plasma lipoprotein content and metabolism [65]. Under normal conditions, macrophages recognize native LDL via the LDL receptor (LDLR). The LDL is then endocytosed and transported to lysosomes, where the cholesteryl ester (CE) is hydrolyzed to free cholesterol (FC) by acid lipase [66, 67]. The FC is then transferred to the endoplasmic reticulum (ER) to be esterified by acyl CoA:cholesterol acyltransferase 1 (ACAT1). The CE produced by ACAT1 is stored in cytoplasm as lipid droplets, undergoing a continual cycle of hydrolysis to FC by neutral cholesterol esterases and re-esterification by ACAT1 [68, 69].

Neutral cholesteryl ester hydrolase 1 (NCEH1) processes CE, releasing FC that is transported out of the cell through ATP-binding cassette (ABC) transporters, including ABCA1 and ABCG1 and scavenger receptor class B1 (SR-BI) [70]. Apolipoprotein A-1 (ApoA-1) acts as a receptor for cholesterol transported by ABCA1, while high-density lipoprotein (HDL) accepts cholesterol transferred by ABCG1 and SR-BI. This machinery is tightly regulated under normal conditions to maintain cholesterol homeostasis. An increase in FC in an ER regulatory pool triggers a signaling cascade that down-regulates the LDL receptor, preventing foam cell formation in hypercholesterolemia. Thus, these proteins ensure an effective control of LDL and cholesterol content in peripheral blood under normal conditions [71–73].

In atherosclerosis, the process of macrophage-dependent cholesterol handling is disrupted. In addition to an increase in the production of oxLDL, macrophages are stimulated by multiple inflammatory factors and express various scavenger receptors, including SR-A1, CD36, and lectin-like oxLDL receptor-1 (LOX-1), which all have an affinity for oxLDL [74, 75], leading to an excessive uptake of oxLDL transforming macrophages to foam cells [76]. Simultaneously, the activity of ACAT is elevated, resulting in an overproduction of CE that accumulate in the endoplasmic reticulum. Furthermore, the expression of NCEH, ABCA1, and ABCG1 is decreased in atherosclerosis, further exacerbating intracellular cholesterol accumulation and foam cells formation [77, 78].

In addition to monocyte-derived macrophages, vascular smooth muscle cells (VSMCs) also significantly contribute to the foam cell population [79]. Clinical studies found that over 50% of foam cells may be derived from VSMCs in human atherosclerotic lesions [80]. Intracellular cholesterol accumulation inhibits VSMC gene expression (including α-smooth muscle actin (α-SMA), smooth muscle myosin heavy chain (SMMHC), and smooth muscle 22α (SM22α) and induces the expression of pro-inflammatory and macrophage markers [81]. VSMCs uptake oxidized LDL mainly through LDLR family and SR family [75, 82, 83]. Compared to macrophages, VSMCs are inefficient at lysosomal processing and cholesterol trafficking, with a much low expression of ABCA1 [84], contributing to an impaired cholesterol efflux [85]. In addition, ECs and dendritic cells have also been reported to participate in the formation of foam cells [86].

In short, lipids infiltrated in the vascular intima are engulfed by macrophages, smooth muscle cells, and other cells, and eliminated through lipid metabolism pathways. However, in the pathogenesis of atherosclerosis, the balance between lipid engulfment and elimination by the effector cells is disrupted. The excessive engulfment of oxLDL leads to lipid accumulation in these cells transforming these cells to foam cells. This progression pushes the atherosclerosis lesion towards a more severe and uncontrollable direction.

Necrotic core formation and plaque rupture

During the process of intravascular adipose streaks formation or pathological intimal thickening, inflammatory ECs and macrophages attract more monocytes to infiltrate the intima by secreting chemokines such as CCR2, CCR5, CXCR1, and CXCR2 [87, 88]. In the early stage of vascular intimal lesions, monocyte infiltration and aggregation are the main driving factors, while in later stages, macrophage proliferation becomes more important [89]. Inhibition of macrophage proliferation has been shown to reduce the size of plaque [90].

Within the inflammatory milieu, macrophages and other immune cells further release inflammatory factors, such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF) isoforms, matrix metalloproteinases (MMPs), fibroblast growth factor (FGF), and heparin-binding epidermal growth factor (HB-EGF), activating VSMCs located in the arterial wall media [91, 92]. Activated VSMCs gain the ability to proliferate, migrate, and secrete various extracellular matrix

(ECM) proteins, and are attracted to the lesion to form a fibrous cap that stabilize lipid plaques [93].

The formation of the fibrous cap makes the atherosclerotic lesion less reversible. The persistence of inflammation leads to the accumulation of apoptotic cells that cannot be cleared by macrophages in a timely manner, causing secondary necrosis. This in turn further promotes inflammation, oxidative stress, and death of adjacent cells, forming a necrotic core [94–96].

The growth of the necrotic core causes a thinning of the fibrous cap. The accumulation of inflammatory cytokines and oxidative products in the vascular lesion provoke uncontrolled accumulation of adjacent VSMCs and, consequently, decreased synthesis of ECMs [96–98]. The components of the ECMs are degraded by macrophage-derived MMPs [99, 100], elastase, and tissue protease [101]. Under this condition, the production of TGF-β is reduced, leading to a decrease in collagen production in healthy VSMCs [102, 103]. In combination, these factors accelerate the thinning of the fibrous cap.

Core necrosis and fibrous cap thinning transform stable fibrous plaques to vulnerable plaques. Plaque rupture abruptly exposes the plaque interior to circulating pro-coagulant factors and platelets, leading to thrombosis [104, 105]. Atherosclerosis-associated clinical events are mainly attributed to thrombus detachment, causing acute vascular occlusion in major organs, leading to myocardial infarction, pulmonary embolism, and stroke.

Copper regulation of vascular metabolism and function

Copper (Cu) is an essential mineral nutrient that participates in cellular metabolism and function as a component of a number of cuproenzymes, an integrated structural element, and a regulatory agent [106–108]. However, Cu also catalyzes the production of highly reactive oxygen species (ROS), which have the potential to cause oxidative damage to lipids, proteins, DNA and other molecules [109–111]. Therefore, either Cu deficiency or excess can lead to diseases or affect the progression of diseases including atherosclerosis. Understanding the complexity of the role of Cu in vascular homeostasis is helpful in designing targeted therapies for reversal of atherosclerosis.

Cu promotion of angiogenesis

The involvement of Cu in angiogenesis has been known for more than 40 years [112, 113]. In 1980s, studies using rabbits demonstrated that Cu and Cu-binding proteins significantly induced angiogenesis in the cornea [113]. It was further found that $CuSO_4$ alone stimulated the expression of VEGF in human keratinocytes in a dose-dependent manner [114]. Following these observations, a series of studies revealed that Cu is instrumental in regulating various EC functions, including proliferation, migration, and tube formation [115–117]. Cu-binding proteins play a critical role in VSMCs migration [118–120] and blood vessel maturation [115, 121]. Mechanistic understanding of the role of Cu in angiogenesis during last two decades revealed that Cu promotion of angiogenesis acts through its regulation of hypoxia-inducible factor-1 (HIF-1) in multiple cell types [122, 123].

The processes of Cu trafficking between intracellular organelles are depicted in Figure 3, including the indications of Cu regulation of HIF-1 transcriptional activity for angiogenetic gene expression. The initial evidence of Cu

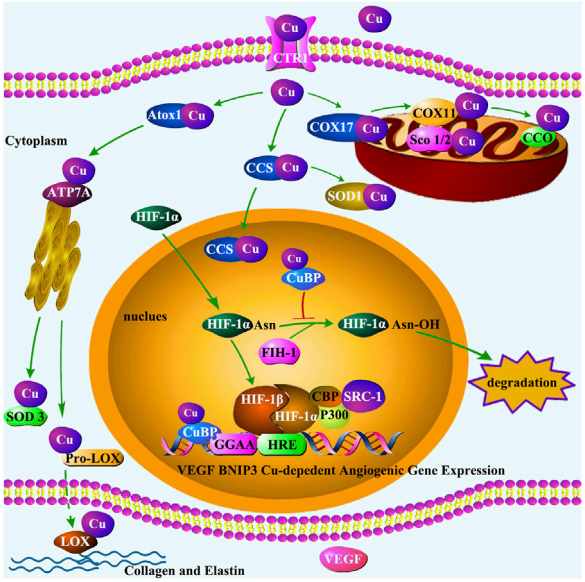

FIGURE 3
Cu trafficking in the cell and its regulation of HIF-1 transcriptional activity. The transportation of Cu by CTR1 enables Cu chaperones to acquire Cu, transferring Cu to Cu-containing enzymes and proteins. Three primary pathways have been identified for Cu chaperones: (1) delivering Cu to CCO in mitochondria by Cox17 and Sco1/Sco2 proteins; (2) delivering Cu to SOD1 in the cytosol and mitochondrial intermembrane by CCS; and (3) delivering Cu to secretory Cu enzymes such as extracellular SOD3 and LOX by Atox1 via Cu transporter ATP7A located in the trans-Golgi network. SOD3 protects cells by scavenging ROS. LOX regulates the formation of ECM, playing an important role in maintaining vascular homeostasis. In addition, under hypoxia condition, CCS transports Cu to the nucleus, where HIF-1α dimerizes with HIF-1β. The HIF-1 heterodimer recruits cofactors such as p300/CBP and SRC-1 to form transcriptional complex, a process may be inhibited by FIH-1. Cu-binding proteins (CuBP) act as an inhibitor for FIH-1activation to ensure the formation of HIF-1 transcriptional complex. The interaction of HIF-1 with HRE requires Cu to initiate the Cu-dependent expression of genes such as VEGF and BNIP3. VEGF plays an important role in promoting endothelial cell proliferation. The core base "GGAA" (the core motif of the ETS family) is a crucial motif in the binding site of Cu-dependent genes.

interaction with HIF-1 was reported by Jiang et al., who explored how dietary Cu supplementation mitigates pressure overload-induced cardiac hypertrophy in mice [124]. Sustained cardiac pressure overload leads to reduced myocardial Cu and VEGF levels, and diminished angiogenesis. Cu replenishment increases VEGF and promotes angiogenesis in the hypertrophic hearts, leading to regression of cardiac hypertrophy. Further studies found that in cultured human cardiomyocytes, Cu chelation blocks insulin-like growth factor (IGF)-1- or Cu-stimulated VEGF expression, which is relieved by addition of excess Cu. Both IGF-1 and Cu activate HIF-1α. Consequently, HIF-1α gene silencing blocks IGF-1- or Cu-stimulated VEGF expression. In addition, HIF-1α coimmunoprecipitates with a Cu chaperone for superoxide dismutase-1 (CCS-1), and gene silencing of CCS-1 prevents IGF-1- or Cu-induced HIF-1α activation and VEGF expression [124]. Thus, Cu promotion of angiogenesis is mediated by HIF-1α activation of angiogenic gene expression with the aid of CCS-1.

Cu regulates HIF-1 transactivation of angiogenic gene expression in multiple mechanisms of action. Under hypoxic conditions, Cu enters the nucleus in both CCS-1-dependent and -independent processes [125]. In the cytoplasm, Cu stabilizes HIF-1α, the rate-limiting component of HIF-1, leading to its accumulation and promoting its entrance to the nucleus [126–128]. In the nucleus, Cu inhibits the activity of asparaginyl hydroxylase factor inhibiting HIF-1 (FIH-1) and ensures the formation of HIF-1 transcriptional complex [129]. Importantly, Cu selectively regulates the binding of HIF-1 to the HRE elements of target angiogenic genes by affecting the interaction between the transcription factor and the promoter region of the angiogenic genes [130, 131].

Cu selectively regulates the process of HIF-1 transactivation of angiogenic gene expression. It is important to note that not all of the HIF-1 regulated genes require Cu for expression [130, 132]. This was first demonstrated by an *in vitro* study in which the treatment of HUVECs with a Cu chelator, tetraethylenepentamine (TEPA), suppressed the expression of a group of HIF-1 target genes such as BNIP3 and VEGF, but did not affect other HIF-1 target genes such as IGF-2 [132]. This Cu selective regulation of the expression of HIF-1-controlled genes was further defined in studies of monkey model of HIF-1 regulation of angiogenesis in ischemic myocardium [133]. During the acute phase of ischemic injury, angiogenesis was activated in the injured heart along with an increase in angiogenic factors. In the chronic phase of myocardial ischemia, sustained accumulation of HIF-1α was observed. However, the accumulation of HIF-1α was not accompanied by the expression of HIF-1-controlled angiogenic factors, including VEGF, tyrosine-protein kinase receptor Tie-2, angiopoietin-1 (Ang-1), and FGF-1 in the ischemic myocardium [133]. On the other hand, an up-regulation of HIF-1-controlled non-angiogenic gene expression such as IGF-2 was associated with HIF-1α accumulation [133].

This paradoxical phenomenon, HIF-1α accumulation being accompanied by suppression of HIF-1 target angiogenic gene expression, is now recognized to be ascribed to the reduced Cu concentrations in the ischemic heart [133]. In response to ischemic insult, Cu content in the heart is significantly decreased, along with a significant increase in serum Cu concentrations [134–136]. A recent study using ChIP-sequencing and RNA-sequencing identified 218 Cu-dependent and 10 Cu-independent HIF-1 target genes across the genome under hypoxic conditions [131]. Cu efflux from the heart under hypoxic conditions leads to suppressed expression of Cu-dependent HIF-1 target genes, but does not change the expression of Cu-independent HIF-1 target genes.

The mechanism by which Cu selectively regulates the binding site of the HIF-1 target genes was recently revealed by a study in HUVECs [130]. In this study, Cu deprivation by TEPA completely suppressed the binding of HIF-1α to HRE site of BNIP3 along with a complete inhibition of BNIP3 mRNA expression, but the binding of HIF-1α to the HRE site of IGF-2 or the expression of IGF-2 mRNA was not affected under hypoxic conditions. Furthermore, *de novo* motif analysis of all 218 Cu-dependent and 10 Cu-independent HIF-1 target genes further revealed that the core bases “GGAA” and “TTCC,” previously identified as the core motifs for E26-transformation-specific (ETS) family [130] constitute the critical motifs for the binding sites of Cu-dependent genes, while there is no specific motif found in Cu-independent genes except the motif for HIF-1α [130]. The difference in the binding loci and pattern between all the Cu-dependent and Cu-independent HIF-1 target genes indicate that Cu, by selectively affecting the binding of HIF-1α to the critical motifs in the promoter and putative enhancer regions of HIF-1-regulated genes, selectively regulates the expression of HIF-1-controlled angiogenic genes.

Cu regulation of endothelial cells (ECs)

The regulatory action of Cu on ECs, as depicted in Figure 4, promotes angiogenesis. Cu at physiologically relevant levels stimulated vessel tube formation from HUVECs cultured in Matrigel [115], indicating the involvement of ECs proliferation, migration and integration in this process. ECs migration is a critical process in the vessel tube formation. Cu stimulation of ECs migration was confirmed using HUVECs for wound healing and transwell migration assays [115]. In the process of Cu stimulation of ECs migration, a Cu transporter-1 (CTR-1) is critically involved. Gene silencing by siRNA targeting CTR-1 in HUVECs significantly suppressed Cu entrance to the cells along with an inhibition of ECs migration [115].

Cu promotes ECs proliferation that is essential for the maturation of new blood vessels. Recent studies showed that

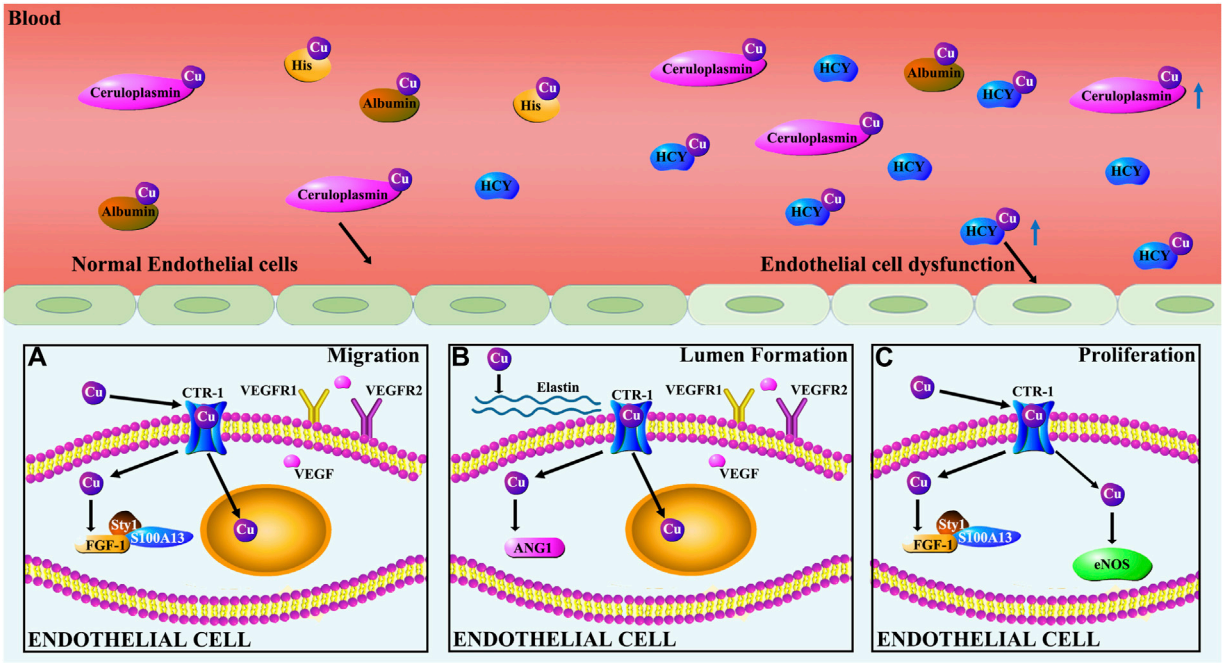

FIGURE 4
Cu regulation of endothelial cells. **(A)** Cu induces migration of ECs by regulating the expression of FGF-1 and VEGF. Specifically, Cu stimulates the release of FGF-1 from ECs by promoting FGF-1 binding to S100A13 and p40Syt1. Cu promotes HIF-1 transcriptional activity leading to increased VEGF gene expression. VEGF, in turn, regulates the dynamic transition of ECs by controlling the relative levels of VEGFR1 and VEGFR2. **(B)** Cu modulates EC-involved lumen formation by promoting the action of ANG-1 and elastin. ANG-1 is involved in vasculature stability, tightening cell contacts, inducing pericyte adhesion, and preventing alterations in vessel permeability. **(C)** Cu promotes proliferation of ECs by activating eNOS and FGF-1.

Cu stimulates ECs proliferation in a concentration-dependent fashion within the effective concentration range [115–117]. NO generated by eNOS plays a critical role in regulating ECs proliferation, angiogenesis and vascular homeostasis [117]. Cu stimulation of ECs proliferation is eNOS-dependent [117]. Li et al found that Cu increased the expression of eNOS in HUVECs and that siRNA targeting eNOS blocked Cu stimulation of ECs proliferation [117].

Exposure of ECs to excessive Cu stimulates the expression of pro-inflammatory cytokines in the cells, leading to endothelial dysfunction [35, 137]. Excessive Cu accumulation in the blood was found in the pathogenesis of atherosclerosis. It is important to note that under the condition of atherosclerosis, Cu is deficient in the atherosclerotic lesion walls, but Cu concentrations are increased in the circulation [138–142]. The endothelial dysfunction is closely related to the disturbance in Cu homeostasis between the vascular wall and circulation, being the critical event in the initiation and progression of atherosclerosis [32, 33, 143].

Serum Cu elevation is closely associated with hyperhomocysteinemia [144]. Experimental and clinical studies over the last decade have shown that the elevation of blood homocysteine (Hcy) levels is linked to increased risk of atherosclerosis [145, 146]. Patients with homocystinuria were associated with high plasma Cu concentrations [144, 147, 148]. A correlation between plasma concentrations of total Cu and Hcy was identified [149, 150]. Addition of small amounts of Cu significantly enhanced the inhibitory effect of Hcy on ECs function, thus suppressing angiogenesis in isolated endothelial tissues in culture [151]. Cu and Hcy complexes have been identified *in vitro* and their exposure to cultured ECs elicited remarkable changes in relation to atherogenic activities [152–155].

Cu regulation of lipid metabolism

There is increasing evidence that indicates a strong correlation between Cu homeostasis and lipid metabolism. Systemic Cu alterations appear to be inversely correlated to the level of lipids and lipid-transporting lipoproteins in the peripheral circulation [156, 157]. It was observed that Cu-deficient diet feeding induced Cu deficiency in organ systems in rats along with increased levels of circulating HDL and LDL, and an increase in total cholesterol, triglyceride and phospholipid levels [158, 159]. The total volume of HDL components in the blood was elevated in Cu deficient Sprague-Dawley rats, accompanied by a corresponding increase in the cholesterol and protein content of the HDL and LDL fractions [159, 160]. In Cu deficient rats, the triglyceride content of circulating LDL

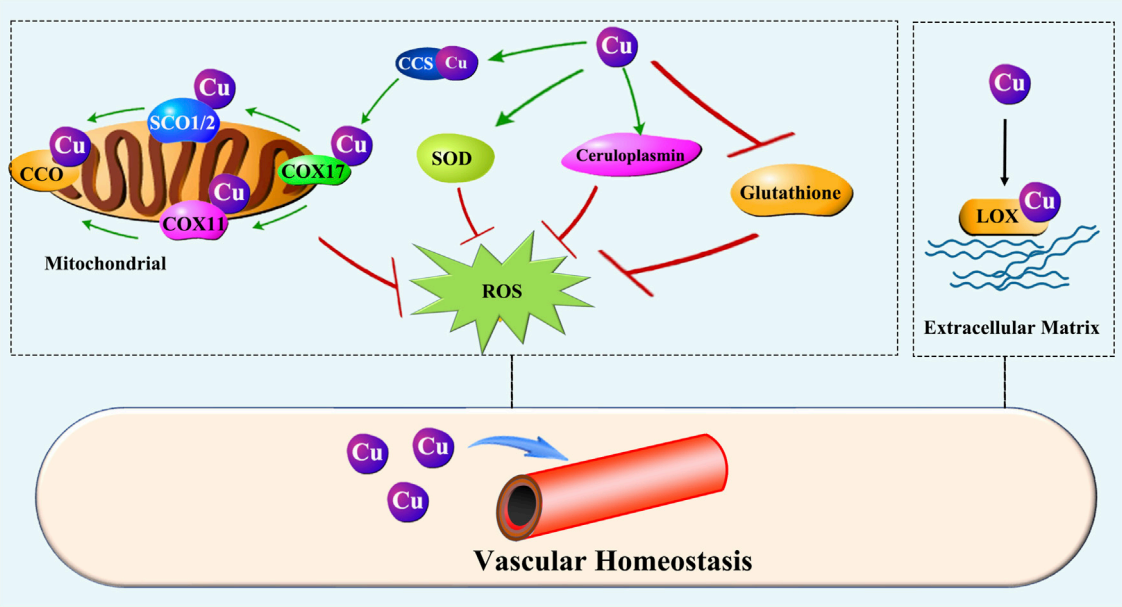

FIGURE 5
Cu regulation of vascular homeostasis. Cu prevents oxidative injury in the vascular system by ensuring the activity of key enzymes such as SOD, ceruloplasmin, and CCO. Moreover, Cu is essential for LOX activity, which is responsible for ECM remodeling during initiation and maturation of vascularization.

and VLDL was increased, as was the ApoE content of HDL [159, 160], however, the concentration of liver cholesterol was reduced [161]. Systemic Cu deficiency is associated with an increased production of HDL and an increased turnover of HDL cholesterol esters [159].

The polarity of macrophages affects the role that they play in the regulation of lipid metabolism [162]. Cu in its Cu^{2+} ionic form was found to play an important role in the regulation of macrophage polarization [163]. The concentrations of Cu^{2+} lower than 10 μM promoted the expression of M2 related genes in macrophages. However, higher concentrations of Cu^{2+} (100 μM) stimulated pro-inflammatory marker expression [163]. This bipolar regulation of macrophages by Cu ions is of a significant impact on the role of macrophages in the initiation and progression of atherosclerosis. In particular, the elevation of Cu concentrations in the blood in the progression phase of atherosclerosis would affect the severity of atherosclerotic lesions.

The oxidative modification of LDL is a key event in human atherosclerosis. Cu ions catalyze oxidative modification of LDL *in vitro* [164–166]. It was also reported that Cu participates in the oxidation of LDL *in vivo* [167]. It was found that high cholesterol feeding leads to Cu deficiency in the vessel tissue and Cu elevation in the plasma [138]. The increase in plasma Cu associated with high cholesterol feeding would promote oxidative modification of LDL, thus promoting atherosclerosis. HDL is more sensitive to oxidation by Cu than LDL [168, 169]. Dose-dependent oxidative damage to HDL and protective effect of vitamin E against oxidation of HDL was observed in the studies of Cu incubation with HDL [170].

Cuproptosis, a newly identified Cu-induced cell death, occurs via Cu binding to lipoylated enzymes in the tricarboxylic acid cycle, leading to subsequent protein aggregation, proteotoxic stress, and eventual cell death [171]. It is possible that high levels of plasma Cu under the condition of atherosclerosis cause cuproptosis of macrophages and other immune cells in the circulation, leading to unbalanced immunological responses in the circulation. However, cuproptosis may not take place in the endothelial cells because they cells are Cu deficient under the condition of atherosclerosis [138, 139]. This phenomenon, high circulation versus low tissue Cu levels, underscores the critical role of Cu homeostasis in health and diseases, as discussed in several recent reviews [172–174].

Cu regulation of extracellular matrix

The maintenance of vascular homeostasis depends on the dynamic stability of the cells and ECM that constitute blood vessels. Cu is involved in the regulation of vascular cells and the ECM via many factors and enzymes, as depicted in Figure 5.

Oxidative stress disrupts vascular homeostasis by impairs ECs function and vascular wall. Cu is a constituent of superoxide dismutase (SOD) and ceruloplasmin (CP), both are importantly involved in preventing oxidative injury [175–178]. It was also

shown that low dietary Cu intake reduces glutathione peroxidase activity [179, 180]. Cu is required for mitochondrial cytochrome c oxidase (CCO) activity, rendering it essential for oxidative phosphorylation [181]. Cu deficiency leads to depressed activity of CCO [124, 182, 183]. These abovementioned enzymes or proteins are so crucial for maintaining the homeostasis of vascular system [184–189].

Several *in vitro* studies have examined Cu redox activity [190, 191]. Cu is redox active and involved in ROS generation [191]. Exposure to elevated levels of Cu significantly decreases glutathione levels [192]. The depletion of glutathione may enhance the cytotoxic effect of ROS and allow the metal to be more catalytically active, thus producing higher levels of ROS [193]. However, it has been demonstrated that there is virtually no free Cu in the biological system [194]. Therefore, the redox injury generated from free Cu *in vitro* studies may not extrapolated to the *in vivo* conditions. A general belief is that Cu-related generation of ROS is more related to Cu overload [195–197], but it is Cu deficiency that causes severe oxidative stress partially resulting from mitochondrial respiration defects due to CCO depression [182].

The ECM is an important component of the blood vessel wall and is essential for maintaining the structural integrity of blood vessels. Cu is essential for the synthesis and maturation of ECM. Fibronectin, an avascular elongation promoter [198], was observed to be increased in cultured ECs in exposure to trace amounts of Cu [199]. Fibronectin mats were strengthened when a small amount of Cu was present [200]. Lysyl oxidase (LOX) is a critical enzyme involved in the ECM remodeling, and Cu is required for the LOX activity [201]. Elastin is required for the lumen formation and maintenance. Studies conducted in swine found that Cu is associated with aorta elastin and essential for the function of the vessels [202–204].

Therefore, Cu is essential for vascular homeostasis through its action on multiple enzymes or proteins involved in the regulation of vascular cells and ECM. In most cases, the disturbance of vascular homeostasis would result from Cu deficiency, although Cu overload also causes severe consequences in vascular homeostasis. In terms of atherosclerosis, Cu deficiency in the vascular tissue and Cu overload in circulation are often observed concomitantly, leading to a double damage to the vessel wall.

Disturbance of Cu homeostasis in atherosclerosis

Cu concentrations were significantly reduced in atherosclerotic vascular walls [142]. High dietary cholesterol feeding causes hypercholesterolemia and atherosclerosis in animal models [139, 205]. During the process of fatty substances deposition and vascular wall hardening in large and medium-sized arteries [206], Cu concentrations in the atherosclerotic wall became significantly reduced compared to that in the normal aortic wall [139, 142]. This reduction in Cu concentrations leads to decreased proliferation and migration of endothelial cells, and inhibits the synthesis of ECM. Therefore, Cu deficiency has been postulated to be a triggering event of atherosclerosis in high cholesterol-fed animals, alongside multiple other hypotheses on the etiology of atherosclerosis induced by high dietary cholesterol [207–209].

Many studies from animal models to human clinical data reported that plasma Cu levels are significantly elevated along with hypercholesterolemia in atherosclerotic subjects [140, 141]. Two cross-sectional clinical studies with apparently healthy subjects showed that serum Cu was inversely associated with low-density lipoprotein cholesterol (LDL-C), suggesting that a higher or adequate serum Cu level is linked to a better lipid metabolic state [210]. It was interesting to note that the reduction of Cu in the atherosclerotic lesion vessels is associated with an increase in the serum Cu concentrations [138]. Although the reason for Cu loss in the atherosclerotic wall and increase in the serum are unknown, a recent study clearly demonstrated an inverse correlation between Cu concentrations in vascular plaque and the severity of atherosclerotic lesions [138].

In brief, Cu plays a crucial role in the process of vascular development and homeostasis. Maintaining Cu homeostasis is essential for maintaining vascular stability, including its regulation of ECs function and lipid metabolism, as well as combating oxidative stress. Numerous studies have shown that Cu homeostasis in vascular tissue is disturbed during the process of atherosclerosis, with Cu being lost from atherosclerotic plaques but increased in the plasma. This process is directly related to the severity of vascular disease. Therefore, we hypothesize that reversing treatment of atherosclerosis can be achieved by restoring Cu homeostasis in the diseased blood vessels.

Reversal of atherosclerosis by restoring vascular Cu homeostasis

There have been numerous exploratory studies on the use of Cu supplementation for the treatment of atherosclerosis, although the results generated are uncertain and sometimes controversial. David et al [211] found that dietary Cu supplementation reduces atherosclerosis in the cholesterol-fed rabbit. Eman et al. found that Cu supplementation reduced cholesterol diet-induced atherosclerosis in rabbit [209]. However, a meta-analysis of 176 randomized controlled clinical trials showed no effect of Cu supplementation on lipid levels [212]. Similarly, another study found that dietary Cu supplementation had no significant effect on atherosclerosis or serum lipid levels in rats [213].

We made an effort to clarify possible differences among these confounding results. As shown in Figure 6, we fed laboratory rabbits a high-fiber diet supplemented with 1% (w/w) cholesterol for 12 weeks to create an atherosclerosis model [214]. Rabbits fed a cholesterol-supplemented diet had higher serum cholesterol

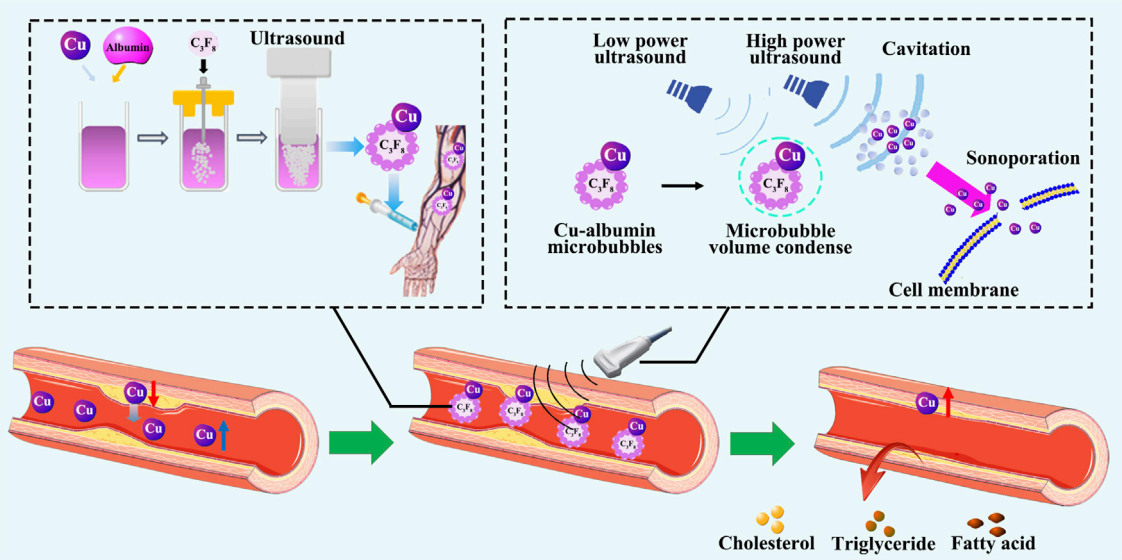

FIGURE 6
Reversal of atherosclerosis by restoring Cu homeostasis. Cu and albumin are prepared to make Cu-albumin microbubbles through ultrasonic vibration, and then injected by intravenous infusion. Under high-energy ultrasound, the microbubbles are broken, and Cu enters the diseased vascular tissue. After treatment with Cu-albumin microbubbles, the area of atherosclerotic plaques significantly decreases, Cu content in the diseased tissue significantly increases, the lipid content significantly decreases, the apoptosis of endothelial cells is reduced, and the stability of the plaque is not affected.

levels and developed atherosclerosis. Cu concentrations in the cholesterol-fed rabbits were increased in the serum and kidney but decreased in the atherosclerotic lesion walls and multiple organs, including heart, liver, spleen, and lungs [138]. These results indicate that the body as a whole is not deficient in Cu during the pathogenesis of atherosclerosis caused by high cholesterol, but Cu homeostasis is altered, leading to unbalanced distribution of Cu to different organ systems [138]. Therefore, if a simple dietary Cu supplementation would only increase Cu levels in the blood, but would not help to replenish Cu content in the Cu deficient organ systems, including the atherosclerotic vessel walls.

There are studies showing that the increase in serum Cu levels can exacerbate LDL oxidation, which may lead to worsened atherosclerosis [215]. Thus, a simple dietary Cu supplementation approach would not be a reasonably feasible approach to replenish Cu in the Cu-deficient vessel wall due to the risk of adverse effects of further serum Cu elevation. The fundamental problem to be solved in the reversal of atherosclerosis by Cu supplementation is how to supplement Cu to the Cu deficient target organ systems.

To solve this problem, we developed an ultrasound-assisted Cu-albumin microbubbles (Cu-MB-US) target-specific Cu delivery procedure [215]. In this procedure, Cu was first reacted with albumin to form a Cu-albumin complex, followed by a microbubble formation through ultrasonic vibration. The Cu-albumin microbubbles were then injected intravenously into rabbits with atherosclerotic vessel lesions. High-energy ultrasound was used to irradiate the atherosclerotic lesion area. As the Cu-albumin microbubbles flowed through the area with blood, the high-energy ultrasound induced the collapse of the microbubbles, causing instant cell cavitation-directed penetration of Cu into the lesion tissue. This process achieved atherosclerotic lesion-specific Cu delivery.

The treatment with Cu-MB-US resulted in an average of 24.2% reduction in lesion surface area (from an average of 79.0% without treatment to an average of 54.8% after treatment). The use of pure albumin microbubbles demonstrated no therapeutic effect [215]. Furthermore, the treatment with Cu-MB-US did not increase Cu levels in plasma, but did significantly increase Cu levels in the diseased vascular tissue [215]. There was an inverse correlation between Cu concentration and the size of the atherosclerotic lesion [215]. Histopathological examination demonstrated that the reduction in atherosclerotic plaques was associated with a decrease in lipid content within the arterial wall after the Cu-MB-US treatment. Moreover, Cu repletion significantly reduced the cholesterol and phospholipid contents in the lesion tissue [215]. There was a positive correlation between cholesterol and phospholipid levels and the size of the atherosclerotic lesion. Consequently, Cu levels were inversely correlated with the levels of cholesterol or phospholipid in the lesion. Importantly, treatment with Cu-MB-US did not decrease the stability of atherosclerotic plaques. Cu-MB-US significantly reduced apoptosis of endothelial cells in the atherosclerotic lesion area. LOX

activity and VSMC contents in the lesion were not altered after the treatment. Cu repletion did not alter the collagen content or the ratio of collagen I to collagen III in the lesion [138, 215].

In brief, it appears that Cu efflux from the atherosclerotic lesion walls during the pathogenesis of atherosclerosis leads to Cu deficiency in the injured vessel tissue and Cu elevation in the circulation. These double injuries generated from Cu deficiency in the affected organs and Cu overload in the circulation would not be recovered by a simple dietary Cu supplementation. Therefore, some confounding results from Cu supplementation on atherosclerosis, particularly between animal studies and human studies, would be explained at least partially by differential disease stages at which Cu supplementation was administrated. In the acute phase, Cu supplementation may show some beneficial effects, but in the late phase of chronic development, it may not be beneficial, or it may be adverse. It is thus reasonable to observe the beneficial effect of target-specific Cu delivery to the Cu-deficient lesion vessels, as reported recently [138, 215–217].

Challenges for the reversal of atherosclerosis

Reversal of atherosclerosis is a new challenge in clinical practice. Studies in the last two decades have shifted the focus of treatment for atherosclerosis from on the cholesterol and thrombotic material deposition in the arterial wall to on the multifactorial inflammatory interactions. Based on the advanced understanding of the pathogenesis of atherosclerosis, it should be recognized that rejuvenating the self-repair mechanism of the vascular tissue is an appealing approach for the treatment of atherosclerosis [218]. Recent studies have shown that in the early stages of atherosclerotic lesions, the body has the ability to self-repair by removing the deposited lipids and repairing the injured vasculature; this has been demonstrated in many early clinical studies. However, as the pathological microenvironment of the diseased vascular tissue continues to deteriorate, the body gradually loses its self-repair ability, and lipid metabolism pathways become imbalanced, leading to further aggravation of the lesion and falling into a vicious cycle. Along with this process, Cu homeostasis is disturbed, further worsening the severity of atherosclerosis.

Restoring Cu homeostasis in the vessel wall may present a feasible treatment strategy for reversing atherosclerosis. By delivering Cu to the Cu-deficient lesion vascular tissue, it effectively reduced the area of atherosclerotic plaques [215]. While this procedure has proved to be successful in animal studies, its extrapolation to humans will face safety issues and adaptation modifications.

The treatment time window of atherosclerosis is crucial. Some patients can restore vascular health with exercise alone in the early stages of the disease. Studies showed that once vascular tissue lesions progress to foam cell formation and lipid stripes appearance, the disease often progresses irreversibly [219]. Inflammation-induced foam cell accumulation, cell apoptosis, necrosis, and subsequent formation of necrotic cores further aggravate the inflammatory response. Existing drug and surgical treatments are often applied until the disease progresses to a specific late stage, which cannot fundamentally change the progression of the disease. However, implantation of early treatment would require the development of new technologies instead of application of the existing technologies in the early stage of the disease, demanding innovations.

Vascular tissue lesions are a manifestation of the imbalance between organ injury and regeneration [220]. Treating only the factors that cause damage to the vessels has been proven to have limitations in the expected outcome. The development of future technologies should focus more on the restoration of regenerative capacity, including reversing the inflammatory phenotype of ECs and promoting the efflux of lipids from the vascular wall, which has already been carried out in many preclinical studies. From the perspective of promoting the restoration of the injury-repair mechanism and rebuilding the body's autonomous regenerative capacity, the development of earlier intervention treatment for atherosclerosis would achieve better reversal treatment results. This not only requires continuous breakthroughs in new technologies but also requires a shift in the medicinal concept from disease treatment to health rejuvenation. The reversal of atherosclerosis is possible, but we have to face new challenges for its fulfilment in the future.

Author contributions

XZ: Investigation, Writing–original draft, Writing–review and editing. XD: Writing–review and editing, Methodology, Writing–original draft. YZ: Writing–original draft, Writing–review and editing. YK: Conceptualization, Project administration, Supervision, Writing–review and editing.

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Conflict of interest

Authors XZ, YZ, and YK were employed by Tasly Biopharmaceutical Co.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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*CORRESPONDENCE
Michael G. Fehlings,
michael.fehlings@uhn.ca

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Bridging the gap: a translational perspective in spinal cord injury

Omar Imad Hassan[1,2], Soichiro Takamiya[1], Azam Asgarihafshejani[1] and Michael G. Fehlings[1,2,3,4]*

[1]Division of Genetics and Development, Krembil Brain Institute, University Health Network, Toronto, ON, Canada, [2]Institute of Medical Science, University of Toronto, Toronto, ON, Canada, [3]Division of Neurosurgery, Krembil Neuroscience Centre, Toronto Western Hospital, University Health Network, Toronto, ON, Canada, [4]Division of Neurosurgery and Spine Program, Department of Surgery, University of Toronto, Toronto, ON, Canada

Abstract

Traumatic spinal cord injury (SCI) is a devastating and complex condition to treat with no curative options. In the past few decades, rapid advancements in our understanding of SCI pathophysiology as well as the mergence of new treatments has created more optimism. Focusing on clinical translation, this paper provides a comprehensive overview of SCI through its epidemiology, pathophysiology, currently employed management strategies, and emerging therapeutic approaches. Additionally, it emphasizes the importance of addressing the heavy quality of life (QoL) challenges faced by SCI patients and their desires, providing a basis to tailor patient-centric forms of care. Furthermore, this paper discusses the frequently encountered barriers in translation from preclinical models to clinical settings. It also seeks to summarize significant completed and ongoing SCI clinical trials focused on neuroprotective and neuroregenerative strategies. While developing a cohesive regenerative treatment strategy remains challenging, even modest improvements in sensory and motor function can offer meaningful benefits and motivation for patients coping with this highly debilitating condition.

KEYWORDS

spinal cord injury, neuroregeneration, animal models, clinical trials, pathophysiology

Impact statement

Despite advancements in medical, surgical, and rehabilitation management for traumatic spinal cord injury (SCI), there remains a critical need for neuroprotective and neuromodulatory treatment strategies. By providing an overview of the current state of SCI understanding and management strategies, this paper aims to bridge the gap between current therapeutic limitations and emergent treatments. It also examines the challenges in treating and studying SCI due to the complexities in the heterogeneity of the disease. Emphasizing the integration of patient feedback and emergent therapies, this paper advocates for the development of tailored approaches that are crucial for advancing SCI care and inclusivity. Ultimately, the goal is to provide insights and guidance that will

enhance recovery and quality of life outcomes for SCI patients, benefiting researchers, healthcare professionals, policymakers, and caregivers alike.

Introduction

Traumatic spinal cord injury (SCI) remains a debilitating condition, but over the past century rapid growth has been made to uncover its pathophysiology and translate preclinical research to patient care. This paper provides an overview of SCI pathophysiology, epidemiology, currently employed management strategies, and emerging therapeutic approaches. It also highlights the quality of life (QoL) challenges faced by patients as well as their desires, providing a basis for caretakers to tailor more patient-centric forms of care. This review underscores the heterogeneous nature of SCI both in disease presentation and individual patient needs, having profound effects on treatment effectiveness. By delving into the wide range of strategies to manage SCI, both established in the clinic and emerging approaches, this paper examines their therapeutic potential and limitations. Furthermore, this paper discusses the frequently encountered barriers in translation from preclinical models to clinical settings. Although the need remains urgent for novel and effective SCI treatments, there is great hope with the continued progress in the field aimed at enhancing QoL and functional outcomes for patients.

Epidemiology

Of those that survive the initial injury, most will have persisting neurological deficits [1]. Direct costs incurred by SCI due to permanent disability are large, estimated to be between 1.1 and 4.6 million USD per patient in the United States [2]. The World Health Organization estimates that 250,000 to 500,000 people suffer a new SCI each year [3] but direct comparisons are shrouded by a lack of an international standard for SCI reporting. Despite challenges surrounding SCI reporting, commonalities can still be drawn from regionally reported data. Within developed countries, SCIs are primarily caused by motor vehicle accidents (MVAs), yet there is a shift towards an increase in fall-related injuries [1, 4, 5]. For example, in the United States, 38.1% of injuries were caused by MVAs from 2010 to 2014, with falls as the close second cause at 31.0% [6].

In regard to sex, males make up the highest distribution of SCI at 79.8% as opposed to female SCI cases at 20.2% [6]. Within the elderly population, this disparity between sex decreases as the age at which SCI occurs in females tends to be later [7, 8]. Preclinical models for SCI assessing the role of gonadal hormones do not have an established consensus [7]. Clinically, the higher incidence of male SCI as well as disparities in the cause and types of injuries make sex-based comparisons difficult.

With an aging global population, the average age of injury is increasing from patients in their late 20s to those in their early 40s [2]. This increase in age remains true for most causes of SCI, with the exception of violence, as it predominantly occurs in younger individuals (16–30 years of age) [6]. In comparison to younger patients, individuals over 50 have greater rates of cervical injury leading to paraplegia than their younger counterparts [9].

There are also variations in injury trends between countries, predominantly related to economic status. Developing countries primarily report falls as the leading cause of SCI, while MVAs dominate SCI cases in wealthier nations [5, 10–12]. However, there are exceptions to this trend. Prevention efforts have reduced MVAs, work-related SCIs, and driving-related injuries in high-income countries. Unfortunately, MVAs and work-related SCIs remain significant issues in low and middle-income countries. Advances in acute surgical, medical, and rehabilitation care have disproportionately benefited high-income countries [5, 10–12].

Despite its stronger economic position in the world, falls are beginning to dominate the SCI landscape in Japan due to the large elderly population [13]. Violence also contributes to a greater proportion of SCI cases in developing regions [14]. Even within a nation, variations in urbanization, economic status, and occupation have different outcomes [15].

Mortality and quality of life in patients with SCI have improved but remain lower than in healthy, age-matched controls in the global population [16]. In the first-year post-injury, the mortality rate is close to 3.8%, followed by 1.2% the next year and an increased rate of 1.2% per annum over the next 10 years [16]. The most significant indicators of mortality in the time surrounding the injury are the severity of the SCI as well as the level of the SCI and the age of the patient [16–18]. Major risks that consistently place patients at higher long-term mortality rates are a loss of autonomy as well as reduced social engagement and support [19, 20].

Pathophysiology

SCI is a heterogeneous and multifaceted condition that threatens the physical, social, and vocational well-being of patients. It is one of the leading causes of paralysis worldwide [1, 21]. SCI begins with an external mechanical trauma that causes contusion and compression of the spinal cord (Figure 1). This leads to the generation of toxic debris and disruption of vasculature, which initiates the secondary injury cascade [22]. In the acute phase (<48 h post-injury), inflammation is initiated accompanying the activation of microglia into a proinflammatory phenotype, which leads to glutamate excitotoxicity and nitric oxide production [1, 23, 24].

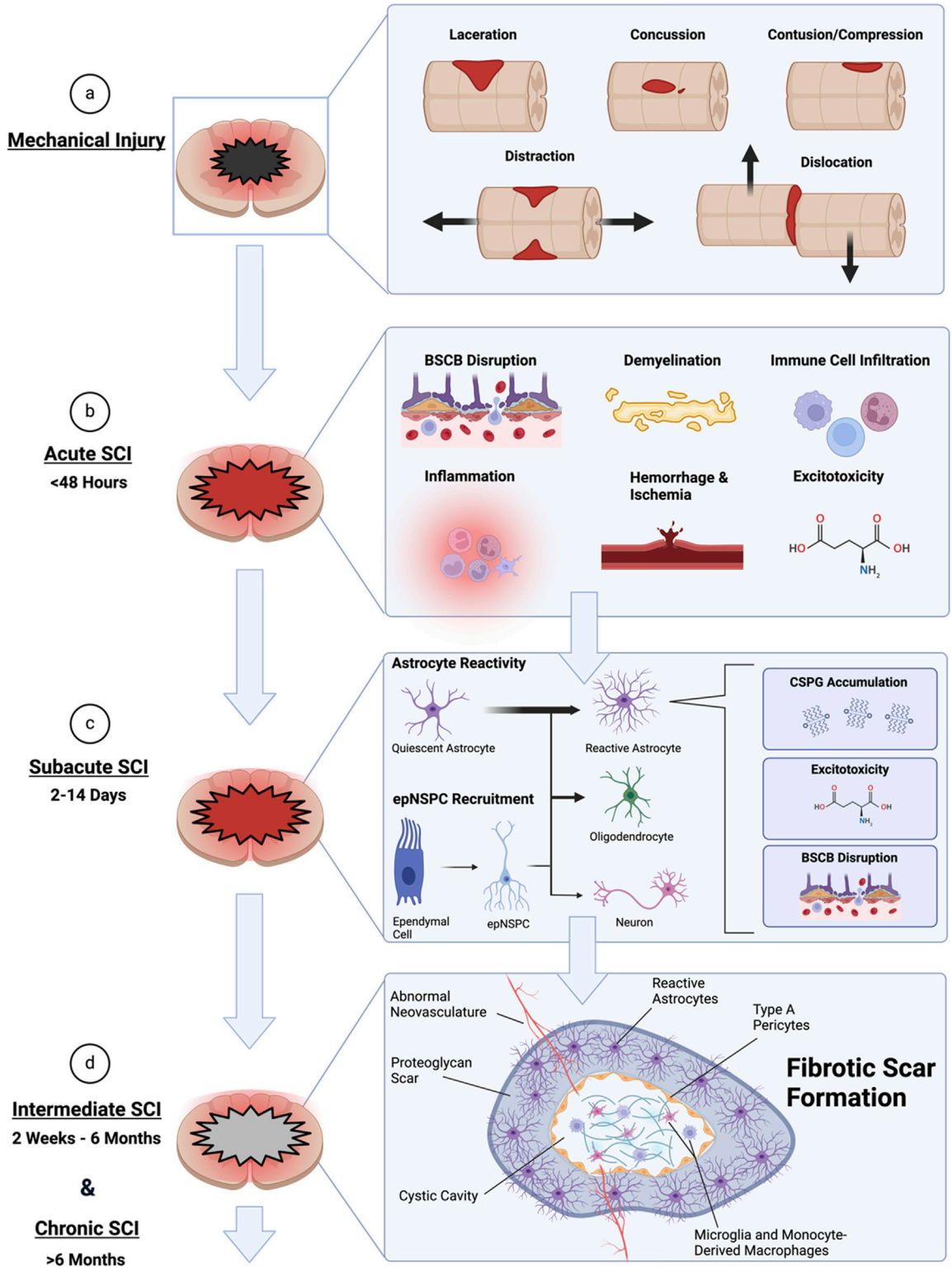

FIGURE 1
Timeline of the pathophysiological developments of spinal cord injury (SCI). **(A)** The initial mechanical forces that contribute to lesion formation and necrosis, initiating SCI. **(B)** The acute phase of injury occurs following the initial injury. It is characterized by inflammation, ischemia, blood-spinal cord barrier (BSCB) disruption, immune cell infiltration and recruitment, demyelination, as well as excitotoxicity. This leads to further damage of the parenchyma beyond the initial lesion. **(C)** The subacute phase sees the recruitment of astrocytes from their quiescent state to reactive.
(*Continued*)

FIGURE 1 (Continued)
Astrocytes are also derived from resident ependymal cells through neural stem/progenitor cell (epNSPC) differentiation. Predominantly epNSPCs differentiate into astrocytes, with few becoming oligodendrocytes and even less becoming neurons. The reactive astrocytes then contribute to further disruption of the BSCB, reduced glutamate uptake involved in excitotoxicity, and chondroitin sulfate proteoglycan (CSPG) deposition. Inflammation and ischemia also persist in this phase. **(D)** During intermediate and chronic phases of SCI, the reactive astrocyte border and fibrotic scar is formed and consolidated. The fibrotic scar contains type A pericytes, abnormal vasculature growth, and CSPG deposits. The scarring and cystic formation inhibits recovery. Created with Biorender.com.

Furthermore, blood-spinal cord barrier (BSCB) disruption, hemorrhage, ischemia, as well as demyelination contribute to greater neuronal and glial cell death [25, 26]. The subsequent subacute phase (2–14 days post-injury) sees sustained inflammation and ischemia as well as the recruitment of resident astrocytes into reactive astrocytes [27, 28]. These astrocytes have impaired glutamate reuptake contributing to excitotoxicity, disrupted BSCB contact and maintenance, as well as formed chondroitin sulfate proteoglycan (CSPGs) deposits that disrupt regeneration [25]. Ependymal cells undergo significant alterations after SCI. This involves activating specific signaling pathways in the spinal cord that promote self-renewal, proliferation, and differentiation. An orchestrated regulation of receptor and ion channel expression fine-tunes and coordinates the activation of ependymal cells after SCI or cell transplantation [29]. While ependymal cells have been proposed as adult neural stem cells, controversy remains as to whether they provide a significant portion of scar-forming astrocytes to protect tissue and function after SCI [30]. Infiltrating microglia is also a key cellular component in orchestrating the glial scar that develops after SCI to protect neural tissue [31].

Resident ependymal cells are recruited during this period and form neural stem/progenitor cells (epNSPCs) that predominantly differentiate into astrocytes [32, 33], contributing to the upregulated population of reactive astrocytes. In the intermediate (2 weeks-6 months post-injury) and chronic phases of SCI (>6 months post-injury), a fibrotic scar core consisting of type A pericytes, abnormal neovasculature, and CSPGs is formed within a reactive astrocyte encasing border [34]. Scarring, cystic cavity formation, as well as limited remyelination and axon regrowth act in concert to greatly stunt recovery. This causes devastating and often permanent neurological deficits with complex barriers to treatment.

Targets for potential SCI treatment

BSCB disruption

The BSCB is a special structure within the spinal cord parenchyma that mediates the exchange of compounds between the blood and the parenchyma while maintaining a regulated chemical balance and homeostasis crucial for neural function [35–37]. Preserving the integrity of the BSCB may enhance spinal cord repair and functional improvement, therefore, the BSCB plays a role in the pathophysiology of SCI progression [34–36].

Morphological and functional changes in the BSCB after SCI include vascular changes, increased permeability of the BSCB, edema, and cavity formation [38]. The initial mechanical damage, combined with compression, laceration, and distraction, contributes to disruption of the neurovascular system [35]. The adverse environment then rapidly results in neuropil damage, swollen neurovascular unit cells, and membrane structure disruption [32, 36, 37]. The morphological alterations accompanying BSCB disruption are instrumental in the progression of SCI because the disrupted BSCB allows the immune cells to enter the injured sites [35, 39, 40].

Lymphocytic infiltration mediates inflammation, reactive astrogliosis, scar formation, and neutrophils leading to demyelinating as well as neuroinflammatory events [39, 40]. The BSCB alterations following SCI lead to altered permeability, which commences several minutes after injury, persists for up to 4 weeks, and may extend over a longer duration, often accompanied by cavity formation [41, 42]. In addition, following SCI, edema begins within several minutes, intensifies rapidly, and persists for up to 15 days, affecting both the lesion site and adjacent segments [38, 39]. Progressive cavity formation causes deficits in neurological function and neuropathic pain [43–45].

Inflammation

Although inflammation serves as a vital defense mechanism in removing pathogens, clearing debris, and facilitating wound healing in the context of SCI, it also accentuates detrimental effects [45, 46] Following SCI, the inflammatory response leads to the production of toxic molecules which, instead of aiding in healing, cause further damage on otherwise intact tissues. While inflammation is required for repair, the response that follows SCI is often exaggerated and leads to further damage and cell loss [47]. Further adding to the complexity of SCI, the infiltration of immune cells non-resident to the CNS (central nervous system) also plays a critical role in inflammation and signaling molecules, affecting the progression of the disease. These infiltrating immune cells are guided by cytokines produced from astrocytes, microglia, peripherally derived macrophages, and endothelial cells [48, 49].

Following SCI, Microglia change their cellular morphology and protein expression profiles [49–51]. Under normal physiological conditions, microglia have long, thin processes that extend from the central cell body to sample the extracellular environment [50, 51]. After SCI, microglia retract their processes and assume an amoeboid shape, primed for phagocytosis and debris clearance [49–51]. In the first hours after injury, microglia, astrocytes, and neurons synthesize pro-inflammatory cytokines [49, 52]. Chemokines drive the increased expression of selectins and cell adhesion proteins on endothelial cells, facilitating integrin-mediated adhesion of circulating immune cells and the subsequent leakage of monocytes and neutrophils into the spinal cord [53, 54]. As the injury response progresses, microglia proliferate extensively during the first 2 weeks, accumulating around the lesion site. These activated microglia position themselves at the interface between infiltrating leukocytes and astrocytes, orchestrating glial scar formation by releasing factors such as IGF-1 [31].

Infiltrating macrophages provide proteolytic enzymes, reactive oxygen species, and inflammatory cytokines to the injury microenvironment but also perform the necessary functions of debris clearance, cellular remodeling, and producing pro-regenerative factors [55, 56]. Preclinical studies have shown that while macrophages increase axon regeneration and neuronal function, they can also worsen tissue destruction. The dual beneficial and reparative functions of macrophages make understanding their role in the injury response difficult [55, 56].

Ischemia and hemorrhage

Mechanical damage from SCI leads to the disruption of capillaries and the BSCB, which creates a harsh microenvironment for spinal cord parenchyma [57]. A direct rupture of the local capillaries induces bleeding into the parenchyma of the spinal cord, which could cause increased release of cytokines and chemokines from macrophages, microglia, and astrocytes into the extracellular space [57]. The presence of red blood cells in the parenchyma is likely to induce free radicals and consequently lead to edema [58, 59]. On the contrary, neural tissue edema can also increase interstitial pressure, which presses the neighboring vessels and causes ischemia [60]. The lack of adenosine triphosphate caused by ischemia and ion channel defects results in an ion imbalance [60, 61].

Demyelination and re-myelination

Oligodendrocytes oversee the generation and maintenance of myelin segments, which is crucial to maintaining the integrity of axons and eases axon signal conduction [62, 63]. After SCI, mechanical damage and the imbalance of local microenvironment factors leads to demyelination [64, 65]. The apoptosis of oligodendrocytes is potentially the leading cause of axonal demyelination [64, 65]. The level of oligodendrocyte apoptosis at the epicenter of the lesion peaks within a week of the injury; however, uninjured axons around the lesion remain myelinated [64, 65]. The presence of myelin debris inhibits remyelination, thus the extent and quality of remyelination are limited [66].

Mechanical injury, ischemia, inflammatory cytokines, oxidative stress, excitotoxicity, and autophagy can cause the death of oligodendrocytes because of demyelination and remyelination imbalance [67–69]. Molecules involved in demyelination are potential inhibitors of axon regeneration, thus the process of demyelination inhibits the regeneration of axons [67–69].

Following a SCI, remyelination mainly involves replacing oligodendrocytes, with the primary source of these new cells being progenitor oligodendrocytes and endogenous neural stem cells [67]. Endogenous neural stem cells remain inactive in normal conditions and become activated upon spinal cord damage; these cells mainly differentiate into astrocytes and to a lesser degree into oligodendrocytes [67, 70]. This suppression of differentiation into oligodendrocytes is mainly due to the lack of growth factors that switch the balance toward differentiation into oligodendrocytes [67, 70].

Hyperexcitation (switch from KCC2 to NKCC1)

NKCC1 and KCC2 are members of the SLC12 cation-chloride co-transporter (CCC) family, which participate in physiological and pathophysiological processes by regulating intracellular and extracellular chloride concentrations, and in turn the GABAergic system [71, 72]. NKCC1 transports Cl^- into cells while KCC2 transports Cl^- out of cells, thereby regulating chloride balance and neuronal excitability. An imbalance of NKCC1 and KCC2 after SCI will disrupt CI^- homeostasis, resulting in the transformation of GABA neurons from an inhibitory to an excitatory state, which leads to abnormal conditions such as spasticity and neuropathic pain [73–75].

After SCI, the segment below the injury site presents a state similar to upregulation of NKCC1 seen in the early stages of development [75]; therefore, the expression of KCC2 was reported to be downregulated at the injury site, followed by a transient upregulation of NKCC1 expression levels, and this altered expression trend was consistent with the post-neuropathic pain occurrence [76].

Inflammation or injury can inhibit the expression and function of KCC2 in the dorsal horn and advance the development of neuropathic pain [77, 78]. GABAA receptors (GABAARs) are involved in the regulation of tonic inhibition in the dorsal horn, sustaining the relative balance of inhibition and

excitation in the central nervous system [79]. After SCI, the function of GABAARs changes and their activation can cause a depolarizing shift as well as the disclosure of nociceptive sensitization [80]. Therefore, improving the abnormal Cl– concentration gradient in the dorsal horn through targeting KCC2 and NKCC1 represents a promising therapeutic direction for restoring the inhibitory function of the GABAergic system and relieving or improving neuropathic pain [81–83]. Moreover, disruption of Cl– homeostasis after SCI, especially the downregulation of KCC2 in motor neurons, depolarizes the Cl– equilibrium potential and decreases the strength of postsynaptic inhibition [74].

Numerous studies have confirmed the therapeutic effect of NKCC1 and KCC2 in neuropathic pain, spasticity, and motor functional recovery post SCI, and these co-transporters are expected to become key targets in future SCI treatments [74, 84]. However, KCC2 and NKCC1 are distributed throughout the nervous system and methods to achieve localization, orientation, and quantitative regulation of their levels may be the main obstacle to their clinical application in the treatment of SCI.

Patient-centric approaches

The concept of patient-centered care (PCC) is crucial for bridging the gap between patients' desires and what healthcare professionals consider beneficial for patients. Gerteis et al. identified that patients defined PCC as having the following dimensions: 1) respect for patient's values, preferences and expressed needs; 2) coordination of care and integration of services within an institutional setting; 3) communication between the patient and providers; 4) dissemination of accurate, timely and appropriate information; 5) education about the long-term implications of disease and illness; 6) physical care, comfort and the alleviation of pain; 7) emotional support and alleviation of fears and anxiety; 8) involvement of family and friends; and 9) transition and continuity from one locus of care to another [85]. This section will delve into specific dimensions of PCC relevant to patients with SCI, with a focus on translational perspectives.

Respect for patients' desires

Healthcare professionals generally strive to provide the best treatment for patients with SCI in accordance with clinical practice guidelines [86]. However, therapeutic strategies for SCI do not always align with patient satisfaction. According to a qualitative study on decision-making regarding bladder drainage methods after SCI, conducted by Engkasan et al., some patients felt that they were forced to accept their doctor's decision [87]. Additionally, Scheel-Sailer et al. reported in their qualitative interview-based study that patients with SCI often experience difficulties making decisions during the initial rehabilitation phase due to physical, psychological, and environmental factors [88]. Thus, it appears that patients' opportunities for decision-making in therapeutic strategies for SCI might be limited in certain contexts.

Importantly, patients' treatment preferences might differ from the actual treatments, potentially undermining respect for their desires. Bowers et al. conducted a survey to clarify SCI patients' preferences regarding methylprednisolone sodium succinate (MPSS) treatment for acute SCI and found that most SCI patients considered MPSS treatment important, even if it offered only minor neurological benefits and carried a risk of complications [89]. However, the 24-hour administration of high-dose MPSS to adult patients within 8 h of SCI is still controversial, with only a weak recommendation in the 2017 AO Spine Clinical Practice Guidelines [86]; therefore, not all patients wishing to receive this treatment may be able to, depending on the physicians' decision.

Looking forward, as novel therapeutic strategies for SCI emerge, they will initially lack robust evidence to guide evidence-based decision-making. In such situations, it will be important for physicians to respect patient's desires and provide them with opportunities to make decisions about their treatments.

Accessibility of information

Patients with SCI have a keen interest in health-related information; hence, the accessibility of such information is crucial for them [90]. According to the interest assessment survey performed by Edwards et al. in the early 2000s, 64% of Canadian chronic SCI patients reported using the Internet to obtain research information [91]. In a more recent 2020 study by Farrehi et al., 89% of participants with SCI in the United States reported sourcing information about experimental therapies online [92]. This trend suggests that access to medical information, including emerging therapies, will continue to grow in the future. However, SCI patients tend to deem information from SCI specialists as more reliable [92]. Therefore, it is equally important to enhance accessibility to SCI specialists, and there are opportunities to leverage emerging areas, such as Telemedicine, to improve access for patients in rural areas [93].

Moreover, improving accessibility for research information also benefits researchers by aiding in the recruitment of participants for clinical trials, as individuals with SCI are willing to participate in translational research [94].

Enhancing quality of life for patients

Among the various neurological symptoms experienced after SCI, pain is the most prevalent issue of SCI as highlighted by patient feedback, followed by bowel and bladder dysfunction, spasticity, and sexual dysfunction [95, 96]. It significantly affects

patients' quality of life by interfering with sleep and daily activities [95]. A survey by Jensen et al. found that pain was both the most common (experienced by 84% of individuals) and the most severe symptom among participants [96]. According to a meta-analysis regarding the prevalence of neuropathic pain following SCI, the pooled point prevalence rate was 53% [97]. However, a postal survey by Finnerup et al. indicated that only a small number of patients received treatment with antidepressants or anticonvulsants, which are considered to be most effective for neuropathic pain [98]. This suggests that there is significant potential for enhancing the quality of life in SCI patients.

In preclinical studies, behavioral assessment tests for sensory function are less commonly utilized than those for motor function. A systematic review regarding animal models of SCI showed that sensory tests, such as the von Frey filament test, were used in only 16.3% of studies, compared to 89.2% for locomotor tests [99]. This discrepancy might be due to most researchers focusing primarily on motor function. However, considering the clinical application and the impact on patients' quality of life, there may be merit in the inclusion of sensory assessment as well as locomotor assessment.

Currently employed strategies in the management of SCI

Early surgical decompression

Surgical decompression of the spinal cord within the first 24 h after injury limits tissue damage by restoring compromised blood flow and reducing the extent of ischemia-related secondary injuries. A recent pooled analysis to evaluate the efficacy of early decompressive surgery for SCI demonstrated that the American Spinal Injury Association (ASIA) motor score in the early (within 24 h of SCI) surgery group was significantly higher than that in the late (after 24 h of SCI) surgery group (23.7 points vs. 19.7 points; $p = 0.0006$) at 1 year after injury [100]. According to the latest meta-analysis regarding timing of decompressive surgery for acute SCI, patients were 2 times more likely to recover by ≥2 grades on the ASIA Impairment Score at 6 months and 1 year after SCI (risk ratios: 2.76 [95% CI: 1.60–4.98] and 1.95 [95% CI: 1.26–3.18]) if they underwent decompressive surgery within 24 h after injury [101]. Based on this evidence, the recommendation for early surgical decompression (within 24 h after SCI) was upgraded in the recently published AO Spine-Praxis Clinical Practice Guidelines from "Quality of Evidence: Low; Strength of Recommendation: Weak" in 2017 [86] to "Quality of Evidence: Moderate; Strength of Recommendation: Strong" in 2024 [101]. Although the evidence has become stronger, early surgical decompression for acute SCI remains a significant challenge in low- and middle-income countries due to limited logistical and infrastructural resources [102].

As for ultra-early surgical interventions (within 4, 5, 8, and 12 h after SCI), it is difficult to draw firm conclusions on their efficacies compared to early surgical decompression, due to the inconsistency in results observed so far [101]. Just as the evidence for early surgical decompression has been established, the evidence for ultra-early surgical intervention is expected to be solidified as more clinical findings become available.

Recently, a phase III RCT, Duroplasty for Injured Cervical Spinal Cord with Uncontrolled Swelling (DISCUS) (NCT04936620), was initiated. This ongoing trial compares laminectomy with duroplasty to laminectomy alone for treating acute cervical SCI. It is expected to reveal the optimal surgical procedure for acute SCI in the near future.

Blood pressure augmentation

Hemodynamic management following acute SCI is crucial, as ischemia and hypoperfusion can exacerbate secondary injury. Preclinical research has indicated that maintaining arterial pressure can improve spinal cord blood flow and, consequently, electrophysiological function [103, 104]. Accordingly, the 2013 guidelines from the American Association of Neurosurgical Surgeons (AANS) and the Congress of Neurological Surgeons recommended maintaining a mean arterial pressure (MAP) of 85–90 mmHg for the first 7 days post-SCI [105]. However, considering the strict MAP target range of 5 mmHg and newer literatures since the 2013 AANS/Congress of Neurological Surgeons guidelines, the 2024 AO Spine Guideline now recommends that MAP should be maintained between 75 and 80 mmHg as a lower limit and not exceed 90–95 mmHg at the higher range during the first 3–7 days post-SCI [106]. A phase III, randomized, controlled trial (RCT), the Randomized Trial of Early Hemodynamic Management of Patients Following Acute Spinal Cord Injury (TEMPLE) (NCT02232165), was initiated in 2017. This ongoing trial aims to compare augmented blood pressure management (targeting MAP of 85–90 mmHg) with conventional management (65–70 mmHg), and may provide additional evidence on the benefit of blood pressure augmentation for acute SCI.

Additionally, spinal cord perfusion pressure (SCPP), which has recently emerged as a more relevant parameter to predict functional outcomes as compared to MAP, is recommended to be maintained above 50 mmHg [107]. It is anticipated that the ongoing Canadian-American Spinal Cord Perfusion Pressure and Biomarker Study (CASPER) (NCT03911492) will soon provide further evidence to support this approach.

Methylprednisolone sodium succinate (MPSS)

MPSS is a corticosteroid that inhibits lipid peroxidation of the neuronal membrane and prevents secondary damage of SCI [108]. The National Acute SCI Study (NASCIS) trials were

representative trials of MPSS for SCI. In the NASCIS-2 trial, the primary analysis did not show significant motor recovery in the MPSS group; however, secondary analyses demonstrated that patients who had received high-dose MPSS within 8 h post-SCI improved motor scores compared to the control group at 6 months post-SCI (16.0 points vs. 11.2 points; $p = 0.033$) [109]. Additionally, the NASCIS-3 trial suggested that patients who received MPSS within 3 h post-SCI should be maintained on the 24-hour treatment regimen, whereas those who received 3–8 h after SCI should be maintained on the 48-hour therapy [110]. Although there are some controversies from a perspective of complications [111], the side effects of steroids are much less relevant in modern times with improved general medical care and the avoidance of steroids in medically compromised individuals. Currently, a 24-hour infusion of high-dose MPSS should be offered to adult patients with acute SCI (<8 h post-injury) as a treatment option [86].

Challenges in translation

Generally, the process from technology initiation to FDA approval in translational science takes a considerable amount of time. McNamee et al. reported that the median interval from technology initiation to establishment was 25 years, to the start of clinical trials was 29 years, and to the first FDA approval was 36 years among new molecular entities approved by FDA between 2010 and 2014 [112]. Broadly, clinical and translational research encompasses the following five phases: T0, basic research (pre-clinical research); T1, translating basic research to humans (phase I clinical trials); T2, translating findings to patients (phase II/III clinical trials); T3, translating research to general practice care (phase IV clinical trials); and T4, translating research to populations or communities [113]. This section will focus on animal models for phase T0, highlight key clinical trials for phase T1–2, and examples of advanced translation.

Animal models and clinical relevance

In translational research, multiple animal models should be used to verify the effectiveness of potential treatments and establish proof of concept. Utilizing a variety of models enhances the robustness and translatability of the research findings [114]. When considering differences among preclinical SCI models, it is important to note the animal species, injury mechanisms, and injured level.

Animal species

Rodent models are the predominant model in SCI research. Rats are most commonly used (72.4%) in SCI preclinical research, followed by mice at 16% [99]. Rodent models also have distinct phases of SCI pathophysiology that are clinically relevant and, given their rapid reproduction cycle and small size, can allow for greater sample sizes. This is especially useful in drug studies where multiple groups are needed to test a range of dosages for safety and efficacy. The preference for rat models is due to their long-term usage as a robust and reliable model for assessing even incremental improvements [115]. Immunodeficient rats that lack T-cell presence have allowed for cell transplantation therapy experiments without the risk of host-vs-graft disease. As an example, human pluripotent stem cell lines have been applied in these models with success [116]. The main caveat to this model, however, is that immunosuppression of SCI patients to allow for transplantation would be through immunosuppressant drugs rather than genetic alterations. This hinders translatability but also reduces variability from the many possible tailored immunosuppressant regimes. For more extensive genetically modified animal research, mouse models are utilized due to their widespread usage in knockout studies. Immunodeficient mice with engrafted human hematopoietic stem cells have shown promise as a translatable model for human immune responses after SCI [117]. However, they are less resilient to SCI induction with higher mortality rates and have species-specific timelines in SCI less reflective of human patient timelines. Where rats have T-cell infiltration peak at 3–7 days post-injury, closer to the human timeline of 7–9 days post injury [49], mice do not have significant T-cell infiltration until after 14 days post injury [49].

Despite the advantages of rodent models, there is a need for large animal and non-human primate models from a translational perspective [118, 119]. When comparing SCI in rodents and humans, functional recovery after injury in rodent models tends to be much faster compared to humans, which seems to be associated with various neural pathways. For example, the rubrospinal tract has been reported as an alternative pathway to improve motor function after corticospinal tract injury in rodent models, which is not observed in humans [120, 121]. Moreover, the size of the spinal cord and its surrounding environment, including the cerebrospinal fluid, differ considerably in rodents, potentially affecting the distribution of locally delivered therapies [122]. This holds especially true for the development of surgical interventions, which are limited when applied to the small stature of rodent models. By examining both rodent and large preclinical animal models, more robust findings can be obtained prior to clinical trials. To date, various large animal SCI models have been utilized, including those involving pigs [123], dogs [124], cats [125], and monkeys [126].

Injury mechanisms

Based on the mechanisms of injury, SCI models can be classified as contusion, transection, compression, and distraction/dislocation models. Contusion models are the most commonly used (43.4%), followed by transection (34.4%) and compression models (20.5%) [99].

Contusion models are created using weight-drop apparatuses or electromagnetic impactors, such as the New York University impactor [127] and the infinite horizon impactor [128]. Compression models are generated by compression using a modified aneurysmal clip [129], forceps [130], or balloon [131]. Both contusion and compression models effectively reflect the pathophysiology of SCI; in particular, clip contusion models not only cause compression but also contusion and hypoperfusion, closely mimicking clinical situations [118]. Transection models, which include complete and partial transection, are advantageous for investigating axonal regeneration following SCI. However, they do not fully represent the complex pathophysiology of SCI, as the spinal cord is seldom sharply transected in clinical settings [118].

Injured levels

Although approximately 60% of SCIs occur at the cervical level [132], only 12% of preclinical research studies have utilized cervical models, with the majority (over 80%) employing thoracic SCI models [99]. This discrepancy may stem from the challenge of postoperative care in cervical SCI models, including manual bladder expression and feeding as well as daily fluid administration, which is necessary to maintain low mortality rates [133]. While challenging to implement, it is essential to validate therapeutic effects in cervical models, as the pathophysiology of cervical SCI differs from that of thoracic SCI due to anatomical and physiological variations [134].

Even though the animal models can be sophisticated from a clinical relevance perspective, a large gap between preclinical studies and early-phase clinical trials remains due to issues of poor validity and reproducibility, often caused by improper study designs. The lack of alignment in design between basic research and clinical trials, including the dosage of drugs and timing of administration, makes it difficult to predict the effectiveness of novel therapies in human trials [135]. To maximize clinical translation, we should refine study designs as well as using clinically relevant animal models.

Regenerative strategies under research

Stem cells and associated growth factors

Endogenous stem cells

Cellular replacement strategies are necessary to restore disrupted neural signaling pathways following the extensive parenchyma loss after SCI. Oligodendrocyte progenitor cells (OPCs) are the majority of progenitor cells that proliferate and differentiate in response to SCI [136]. OPCs are unipotent and neural stem/progenitor cells (NSPCs) are fewer, underlying the spinal cord's limited neurogenic potential [137]. Of the NSPCs, the largest population responding to SCI are ependymal derived neural/stem progenitor cells (epNSPCs) in the spinal canal. They display multipotent properties and are capable of self-renewal, responding to SCI in the acute phase through proliferation and migration to the site of injury. Although they have been shown *in vitro* to have the capability to differentiate into neurons, astrocytes, and oligodendrocytes, *in vivo* studies have displayed them to be particularly biased towards an astrocytic fate post-SCI, with a small potential for becoming oligodendrocytes and an even lower potential to form neurons [137, 138].

Strategies to bias epNSPCs towards neuronal cell fates are emerging. Bioartificial scaffoldings to bias NSPCs towards neuronal cells such as those developed by Zhang et al. [139] have been shown to facilitate neural stem cells to differentiate into neuron-like cells. As an alternative approach, biasing through changes to the microenvironment has also been displayed. Ohori et al. [140] injected fibroblast growth factor 2 and epidermal growth factor within the lesion site in a rat SCI model to promote immature markers of neuronal cells in NSPCs. As a caveat, they used NSPCs that were genetically manipulated by a retrovirus, pMXIG, to express Neurogenin2 (NGN2) and Mash1, transcription factors that bias towards neurons and oligodendrocytes.

Induced pluripotent stem cells

Induced pluripotent stem cells (IPSCs) can be biased as neural progenitor cells (NPCs) for transplantation and integration into the spared parenchyma to enhance local circuits and aid in motor recovery [141]. These cells can be made in an autologous fashion from fibroblasts, circumventing the need for immunosuppressants when using exogenous cells as well as the ethical concerns of their origins. Fibroblasts are exposed to the factors Oct4, Sox2, Klf2, and c-Myc in accordance with the work done by Yamanaka and Takahashi on mice in 2006 [142] and in human fibroblasts by Takahashi et al. in 2007 [143]. This comes at the cost of time, however, for the development and biasing of IPSCs, limiting their application to later stages of injury.

Generating IPSCs also comes with added risks, particularly related to tumorigenicity. When generating the IPSCs, residual undifferentiated cells can proliferate and form tumors [144], thus emphasizing the need for stringent quality control.

One of the factors used to generate IPSCs from fibroblasts, c-Myc, is protooncogenic and often overexpressed in a majority of human cancers, contributing to over 40% of tumor formations [145, 146]. Retroviral c-Myc introduction has also shown an increased tumorigenicity in mouse models [147]. Clinically this carries a large risk. Using alternative factors reduces the efficiency and speed of IPSC induction but makes the IPSCs clinically acceptable [148].

The first IPSCs generated by Yamanaka and Takahashi through retrovirus transduction resulted in random integration [142, 143] at start sequences with increased likelihoods of loss of function effects [149]. Alternative

TABLE 1 Summary of leading completed and ongoing clinical trials regarding neuroprotective therapies for spinal cord injury.

Drug	Treatment Type	Study design	Study period	Stage of SCI	Sample size	Outcome	References
Minocycline	Anti-apoptosis	Phase II, RCT	2004–2008	Acute (<12 h of SCI)	52	A trend toward motor improvement in cervical SCI patients treated with minocycline was observed No significant difference in motor function in thoracic SCI patients treated with minocycline was observed	Casha et al. [156]
Riluzole	Anti-excitotoxicity	Phase IIb/III, RCT	2014–2021	Acute (<12 h of SCI)	193	All subgroups of cervical SCI patients treated with riluzole showed significant gains in functional recovery on the *post hoc* analyses	Fehlings et al. [157]
G-CSF	Anti-apoptosis, anti-inflammation	Phase III, RCT	2015–2019	Acute (<48 h of SCI)	88	A trend toward motor improvement in the G-CSF group was observed	Koda et al. [158]
KP-100 (HGF)	Anti-apoptosis, cell growth	Phase I/II, RCT	2014–2018	Acute (2–5 days post-SCI)	43	KP-100 contributed to motor improvement	Nagoshi et al. [159]
		Phase III, open-label, single-arm	2020–2023	Acute (<72 h of SCI)	25	not published yet	NCT04475224

Abbreviations: SCI, spinal cord injury; RCT, randomized controlled trial; G-CSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor.

strategies have arisen with lowered tumorigenicity. Sendai and adeno-associated viruses, as well as plasmid integration, have been used to potentially lower teratogenicity but are highly inefficient [150–152]. Sustained delivery of synthetic mRNA that encodes for the reprogramming factors used by Yamanka's group are more efficient and avoid the heritable tumorigenicity of cellular DNA modification [153, 154]. Direct reprogramming of adult somatic cells without viral vectors through the transient expression of Msi1, Ngn2, and MBD2 by Ahlfors et al. has shown high reprogramming efficiency, no tumorgenicity in murine models, and low-cost [155]. It remains hopeful for clinical application.

Overview of current significant clinical trials

In the currently employed therapies for SCI, there are not enough neuroprotective and neuro regenerative approaches. To address this issue, numerous clinical trials have been performed. In this section, we provide an overview of clinical trials examining neuroprotective (Table 1), regenerative (Table 2), and other strategies for SCI.

Neuroprotective therapy

Minocycline

Minocycline is a tetracycline antibiotic used clinically as an antimicrobial agent. It also exhibits anti-apoptotic characteristics through the inhibition of caspase-1 and –3 [160, 161]. A phase II RCT (NCT00559494) conducted from 2004 to 2008 demonstrated safety and a trend toward motor function improvement, as measured by the ASIA motor score, in cervical SCI patients treated with minocycline (14 points; 95% CI: 0–28; $p = 0.05$) [156]. Based on these promising results, a phase III RCT, Minocycline in Acute Spinal Cord Injury (MACS) (NCT01828203), was initiated in 2013. However, this trial was discontinued, and its results have not yet been published.

Granulocyte colony stimulating factor (G-CSF)

G-CSF, known as a growth factor for hematopoietic cells, has also demonstrated neuroprotective characteristics for SCI through angiogenesis, inflammation suppression, and apoptosis inhibition in preclinical research [162–164]. An open-label phase I/IIa trial of G-CSF for acute SCI conducted from 2008 to 2010 revealed no severe adverse events related to G-CSF administration [165]. Another open-label, non-randomized controlled phase II trial carried out between 2009 and 2011 by the same group found a significantly greater improvement in the ASIA motor score in the G-CSF group compared to the control group [166]. Encouraged by these promising results, a phase III RCT, the G-CSF mediated spinal cord injury recovery induction trial (G-SPIRIT) (UMIN000018752), was initiated in 2015. Although this trial reported no significant differences in the primary efficacy endpoint, measured by changes in the ASIA motor score at 3 months post-intervention between the G-CSF and control groups, those at 6 and 12 months showed a trend towards better improvement in the G-CSF group [158].

TABLE 2 Summary of leading completed and ongoing clinical trials regarding regenerative therapies for spinal cord injury.

Cell-based approach								
Cell type		Treatment type	Study design	Study period	Stage of SCI	Sample size	Outcome	References
NP/PCs		Anti-apoptosis, anti-inflammation (acute phase) Remyelination, axonal regrowth (chronic)	Phase I/IIa, open-label, single-arm	2021– (ongoing)	Subacute (7–60 days post-SCI)	5 (estimated)	Not completed yet	NCT04812431
			Phase I, open-label, single-arm	2020– (ongoing)	Subacute (14–28 days post-SCI)	4 (estimated)	Not completed yet	jRCTa031190228
OPCs		Anti-apoptosis, anti-inflammation (acute phase) remyelination, axonal regrowth (chronic)	Phase I/IIa, open-label, single-arm	2015–2018	Subacute (21–42 days post-SCI)	25	Two grade 3 serious adverse events (CSF leakage and bacterial infection) were observed 24/25 participants experienced functional recovery	Fessler et al. [168]
MSCs	Bone marrow-derived	Anti-apoptosis, anti-inflammation (acute phase) remyelination, axonal regrowth (chronic)	Phase II, open-label, single-arm	2014–2017	Subacute (26–54 days post-SCI)	13	No serious adverse events were observed 12/13 participants experienced functional recovery	Honmou et al. [169]
			Phase II/III, RCT, delayed-start	2022– (ongoing)	subacute (6–10 weeks post-SCI)	16 (estimated)	Not completed yet	NCT03935724
	Umbilical cord-derived		Phase I/II, RCT	2022– (ongoing)	Acute (<7 days post-SCI)	80 (estimated)	Not completed yet	NCT05693181
	Adipose tissue-derived		Phase I, open-label, single-arm	2017–2021	chronic (2–12 months post-SCI)	10	No serious adverse events were observed 7/10 participants experienced functional recovery	Bydon et al. [170]
	Muse cells		Phase II, open-label, single-arm	2019–2023	Subacute (<2 weeks post-SCI)	10	Not published yet	jRCT1080224764
Non-cell-based approach								
Drug		**Treatment Type**	**Study Design**	**Study Period**	**Stage of SCI**	**Sample Size**	**Outcome**	**References**
C3 transferase		Axonal growth, regeneration	Phase IIb/III, RCT	2016–2018	Acute (<72 h of SCI)	67	No significant difference in motor function in the cethrin group was observed	Fehlings et al. [171]
Anti-Nogo-A antibody		Axonal growth, regeneration	Phase II, RCT	2019–2023	Acute (4–28 days post-SCI)	129	Not published yet	NCT03935321
Endothelin B receptor agonist		Anti-apoptosis, enhances neuronal differentiation	Phase II, RCT	2019– (ongoing)	Acute (<48 h of SCI)	40 (estimated)	Not completed yet	NCT04054414
Anti-RGMa antibody		Axonal growth, regeneration	Phase II, RCT	2020– (ongoing)	Acute (<24 h of SCI)	54 (estimated)	Not completed yet	NCT04295538
			Phase II, RCT	2021– (ongoing)	Acute	72 (estimated)	Not completed yet	NCT04683848

Abbreviations: SCI, spinal cord injury; RCT, randomized controlled trial; NP/PCs, neural stem/progenitor cells; OPCs, oligodendrocyte progenitor cells; CSF, cerebrospinal fluid; MSCs, mesenchymal stem cells; RGMa, repulsive guidance molecule A.

Hepatocyte growth factor

Hepatocyte growth factor (HGF) is secreted by mesenchymal stem cells and regulates cell growth and cell motility by activating a tyrosine kinase signaling cascade through binding to the c-Met receptor. In preclinical research using primate models, HGF enhanced motor neuron survival and reduced cavitation at the injured site [167]. A phase I/II RCT using recombinant human HGF (KP-100IT) for acute SCI (NCT02193334) was conducted starting in 2014. It demonstrated safety and suggested improvements in motor function as evaluated by the ASIA motor score [159]. Based on these results, an open-label, single-arm phase III trial (NCT04475224) was conducted. However, it appears not to have achieved its primary efficacy endpoints, potentially influenced by variations in patients' baselines due to the COVID-19 pandemic. Detailed results, including any *post hoc* analyses, are to be published in the near future.

Regenerative therapy (Table 2)

Cell-based therapy

Cell-based therapies are a promising strategy for the treatment of SCI, offering a variety of therapeutic mechanisms. Supported by substantial pre-clinical evidence, numerous clinical trials have been conducted.

Neural stem/progenitor cells (NS/PCs)

NS/PCs, capable of self-renewal and differentiating into neurons and glial cells, have been utilized in several clinical trials. Two clinical trials include an open-label phase I/II trial for chronic thoracic SCI (NCT01321333) and a single-blinded phase II RCT for chronic cervical SCI (NCT02163876), where human fetal brain-derived NS/PCs were transplanted into the spinal cord around the epicenter. These trials indicated no serious adverse events related to the intramedullary injection or additional spinal cord damage; however, they failed to demonstrate the efficacy anticipated by the sponsor [172–174]. Another open-label, single-arm phase I trial with the intramedullary transplantation of human spinal cord-derived NS/PCs for chronic thoracic SCI (NCT01772810) began in 2014, with 5-year follow-up results recently reported. According to this report, no serious adverse events were directly attributed to cell transplantation [175]. Currently, there are ongoing clinical trials using human embryonic stem cell (ESC)-derived NS/PCs and human induced pluripotent stem cell (iPSC)-derived NS/PCs. An open-label, single-arm phase I/IIa trial with human ESC-derived NS/PCs (NCT04812431) is targeting subacute cervical SCI and is estimated to be completed by 2028. Another open-label, single-arm phase I trial using human iPSC-derived NS/PCs (UMIN000035074, jRCTa031190228) is targeting subacute cervical or thoracic SCI [176]. This trial is expected to be completed by 2024 and aims to address ethical concerns associated with deriving NS/PCs from human ESC or fetuses.

Oligodendrocyte progenitor cells (OPCs)

OPCs are also self-renewing, multipotent cells that preferentially differentiate into oligodendrocytes, as opposed to NS/PCs. Preclinical studies have demonstrated their capability to secrete neurotrophic factors, suppress inflammation, remyelinate axons, and spare tissues [177–180]. An open-label, single-arm phase I trial (NCT01217008) was conducted from 2010 to 2013, involving the direct transplantation of OPCs into the injured epicenter in patients with subacute thoracic SCI. This study confirmed their safety for up to 10 years post-SCI [181]. Based on this safety profile, an open-label, single-arm phase I/IIa trial for subacute cervical SCI (NCT02302157) took place from 2015 to 2018. Results from this trial indicated not only the safety of OPCs but also functional improvements as assessed by the International Standards for Neurological Classification of Spinal Cord Injury examination at 1-year post-SCI [168]. Consequently, a phase III trial to confirm their efficacy is now warranted.

Schwann cells (SCs)

SCs have shown the ability to promote remyelination, improve axonal sparing, and reduce the inflammatory response in preclinical studies [182–184]. Two open-label, single-arm phase I trials of SCs for SCI have been conducted by the Miami Project to Cure Paralysis. The first one (NCT01739023) was performed between 2012 and 2015 and enrolled six patients with subacute thoracic SCI [185]. Another trial (NCT02354625) was conducted from 2015 to 2019 and enrolled six patients with chronic (more than 1 year) thoracic SCI [186]. In both trials, SCs were harvested from the sural nerve of the participants, and autologously transplanted into the epicenter of SCI, with no serious adverse events being reported. However, no evidence of its efficacy has been reported to date.

Mesenchymal stem cells (MSCs)

MSCs can exert immunomodulatory, anti-inflammatory, neuroprotective, and angiogenic effects by secreting numerous trophic factors [187], This secretion improves the local environment of the injured spinal cord. Due to their ability to migrate to the injured lesion [188], MSCs can be transplanted directly into the injured site or via intravenous injection, offering a less invasive option for patients.

MSCs can be derived from multiple sources, including bone marrow (BM), umbilical cord (UC), adipose tissue (AD), Wharton's jelly, and amnion. Their efficacy has been demonstrated in several preclinical studies [189–193]. Based on these sources, numerous clinical trials have been conducted. For instance, autologous BM-MSCs were intravenously injected in an open-label, single-arm phase II trial for subacute cervical SCI (JMA-IIA00154). This trial

reported no serious adverse events related to the cell injection and showed neurological improvement [169]. Based on the results, the MSC product (Stemirac®) has been approved through the conditional early approval program in Japan, although further evaluations are required to determine its efficacy. Additionally, a double-blinded, placebo-controlled, and delayed-start phase II/III trial, Stem Cells in Spinal Cord Injury (SCI2) (NCT03935724), began in 2022. In this trial, patients with subacute cervical and thoracic SCI are intrathecally injected with the BM-MSC product (Neuro-Cells). The trial is expected to be completed by 2024.

Regarding UC-MSCs, allogeneic UC-MSCs were administered intravenously in an open-label phase I/IIa RCT for acute SCI (NCT04331405). This trial showed only two mild adverse events (transitory mild hyperthermia after cell infusion) and most patients experienced neurological improvement, although the results from the phase IIa trial are still pending [194]. Currently, the same group is conducting a single-blinded phase I/II RCT for acute SCI, Systemic Umbilical Cord Blood Administration in Patients with Acute Severe Contusion Spinal Cord Injury II (SUBSCI II) (NCT05693181), which began in 2022 and is estimated to be completed by 2025.

As for AD-MSCs, an open-label, single-arm phase I trial, the Adipose Stem Cells for Traumatic Spinal Cord Injury (CELLTOP) (NCT03308565), was conducted at the Mayo Clinic from 2017 to 2021. In this trial, participants with subacute and chronic SCI intrathecally received AD-MSCs. Recent results indicated no serious adverse events, with 7 out of 10 participants showing improvement in AIS grade post-injection [170]. Another phase I/II RCT for acute thoracic SCI (NCT02917291) involved transplanting allogenic AD-MSCs (FAB117-HC) into the injured spinal cord. This trial was expected to be completed by 2023, though its current status is unknown.

Multilineage-differentiating stress-enduring (Muse) cells, identified as stress-tolerant pluripotent stem cells within MSCs, are promising for SCI therapy [195]. Muse cells recognize injured sites with sphingosine-1-phosphate (S1P) via the S1P receptor 2 and migrate accordingly. A preclinical study showed that they reduced cystic cavities and preserved axons [196]. An open-label single-arm phase II trial using Muse cells (CL2020) for acute/subacute cervical SCI (jRCT1080224764) was conducted in Japan. The trial was completed in 2023, and the results are expected to be reported in the near future.

C3 transferase

C3 transferase inhibits Rho signaling, consequently promoting axonal growth and regeneration [197, 198]. An open-label, single-arm, phase I/IIa trial (NCT00500812) was conducted from 2005 to 2009, and Cethrin, a recombinant C3 transferase, was applied to the surface of the dura mater overlying the injured lesion during decompressive surgery [199]. No serious adverse events were reported, and then, a phase IIb/III RCT, SPinal Cord Injury Rho INhibition InvestiGation (SPRING), was conducted from 2016 to 2018 (NCT02669849). Unfortunately, this trial was terminated because the interim efficacy results, evaluated by the upper-extremity motor score, did not show a significant difference between the C3 transferase group and the placebo [171].

Anti-Nogo-A antibody

Nogo-A is one of the myelin-associated proteins that inhibit neuronal growth by activating the Rho/ROCK pathway upon binding to the Nogo receptor [200]. Therefore, the anti-Nogo-A antibody has the potential to improve axonal regrowth by mediating Rho/ROCK signaling, as demonstrated in preclinical research using primate models [201]. An open-label phase I trial utilizing recombinant anti-Nogo-A antibody (ATI355) for acute traumatic SCI (NCT00406016) was performed from 2006 to 2011 and found no drug-related serious adverse events [202]. A phase II RCT trial using anti-Nogo-A antibody (NG-101) for acute cervical SCI, Nogo Inhibition in Spinal Cord Injury (NISCI) (NCT03935321), was initiated in 2019 and recently completed in 2023. In this trial, the anti-Nogo-A antibody treatment did not show a statistically significant benefit for the primary efficacy endpoint. The detailed results, including *post hoc* analysis, have not yet been published. Similarly, a phase I/II trial using a soluble Nogo-Receptor-Fc decoy (AXER-204) for chronic cervical SCI, ReNetX Safety Efficacy and Tolerability of AXER-204 for Chronic SCI (RESET) (NCT03989440), was conducted from 2019 to 2022. It demonstrated safety; however, no significant differences were observed for secondary efficacy endpoints between the AXER-204 group and the placebo group [203].

Endothelin B receptor agonist

Sovateltide, also known as IRL-1620 or PMZ-1620, is an endothelin B receptor agonist that has enhanced neuronal differentiation and reduced apoptosis in animal models of cerebral infarction [204, 205]. Following the promising results of a RCT with Sovateltide for acute cerebral ischemic stroke [206], a phase II RCT of PMZ-1620 for acute SCI (NCT04054414) was initiated in 2019. The trial is currently ongoing and is estimated to be completed in 2024.

Anti-repulsive guidance molecule A (RGMa) antibody

RGMa is a protein that activates the RhoA-Rho kinase pathway and consequently inhibits axonal regeneration [207]. In preclinical research using a primate model of SCI, an RGMa antibody facilitated the recovery of manual dexterity by enhancing the penetration of corticospinal tract fibers into laminae VII and IX [208]. To date, a few clinical trials are ongoing. Phase II RCT using a human anti-RGMa monoclonal antibody, known as Elezanumab (ABT-555), for acute SCI (NCT04295538) was initiated in 2020 and is estimated to be completed in 2026. Additionally, the expanded

access program for Elezanumab has been approved (NCT04278235). Another phase II RCT using MT-3921 for acute cervical SCI (NCT04683848) was started in 2021 and is estimated to be completed in 2025.

Others

CSF drainage

As SCPP is determined by the difference between MAP and intraspinal pressure, CSF drainage through a lumbar intrathecal catheter placed into the subarachnoid space is another method to maintain spinal cord blood flow. This procedure is commonly utilized for patients undergoing thoracoabdominal aortic aneurysm repair surgery, which potentially has a risk of spinal cord ischemia due to hypoperfusion from the important segmental artery connected to the anterior spinal artery [209]. A phase I clinical trial for acute SCI (NCT00135278) that commenced in 2006 indicated that CSF drainage did not result in any significant adverse events. However, it failed to demonstrate a significant difference in the ASIA motor score, likely due to the small sample size [210]. Following this trial, a phase II RCT was conducted from 2015 to 2019 (NCT02495545) to compare outcomes between MAP maintenance with CSF drainage and MAP maintenance alone. The results of this trial have not yet been published.

Therapeutic hypothermia

Therapeutic hypothermia is used in various medical scenarios to minimize secondary damage to the central nervous system. For instance, the 2020 American Heart Association Guidelines for cardiopulmonary resuscitation recommends that the target temperature for patients who achieve return of spontaneous circulation should be maintained between 32°C and 36°C for at least 24 h [211].

Therapeutic hypothermia is also regarded as a neuroprotective strategy for acute SCI. Several preclinical and clinical studies have suggested that this procedure might improve behavioral outcomes [212, 213]. A RCT comparing systemic hypothermia with standard treatment (NCT02991690) was initiated in 2017 and is expected to be completed by 2024. According to the interim report published in 2022 [214], preliminary data indicated that modest systemic hypothermia (33°C for 48 h) following acute SCI was not associated with an increased risk of complications. The results are anticipated to clarify the efficacy of therapeutic hypothermia.

Examples of therapies at the advanced stages of translation

Riluzole

Riluzole, a benzothiazole approved by the FDA for the treatment of amyotrophic lateral sclerosis, acts as a neuroprotective agent. It blocks sodium channels and reduces glutamate-associated excitotoxicity by decreasing glutamate release from the presynaptic terminal, preventing glutamate receptor hypofunction, and stimulating glutamate uptake [215]. Following SCI, voltage-sensitive sodium channels are constitutively activated, leading to increased intracellular sodium concentration, cellular swelling, and intracellular acidosis [216]. Additionally, the increase in intracellular sodium facilitates the influx of calcium ions through the Na^+/Ca^{2+} exchanger, resulting in the extracellular release of excess glutamate and localized cell death. Riluzole is well-suited to inhibit these processes involved in secondary injury.

Based on promising results from several pre-clinical studies supporting the effectiveness of riluzole for acute SCI [217, 218], a phase I clinical trial for demonstrating the safety of riluzole in acute SCI (NCT00876889) was conducted between 2010 and 2012. This trial demonstrated that there were no serious adverse events associated with riluzole [219]. Additionally, patients with cervical SCI treated with riluzole had a significantly higher mean ASIA motor score at 90 days post-SCI compared to matched patients in the North American Clinical Trials Network SCI Registry (31.2 points vs. 15.7 points; $p = 0.021$). These encouraging results led to a double-blind phase IIb/III RCT, the Riluzole in Acute Spinal Cord Injury Study (RISCIS) (NCT01597518), initiated in 2014. Originally planned to enroll 351 patients, the trial was terminated in 2021 with 193 participants due to the COVID-19 pandemic. The primary efficacy outcome of the Upper Extremity Motor score at 180 days post-SCI showed no significant difference between the riluzole and control groups, likely due to insufficient power [157]. However, *post hoc* analysis revealed some hopeful results; for instance, the Upper Extremity and Total Motor score in the AIS C population treated with riluzole at 180 days post-SCI were significantly better than those without, according to multivariate linear regression models. Although this trial could not definitively determine the efficacy of riluzole, Fehlings et al. concluded that riluzole could be considered as one of therapeutic options in the clinical settings, given the lack of alternative pharmacological treatments for severe SCI. Currently improved techniques for trial design and handling the heterogeneity of patients will enhance future results.

Additionally, a phase III RCT for chronic cervical SCI (NCT01257828) was also conducted between 2012 and 2017. This trial did not show significant difference between the riluzole and control groups in the primary efficacy endpoint measured by the change in the modified Japanese Orthopaedic Association score at 6 months post-intervention [220]; however, the latest secondary analyses using a global statistical test showed a significant functional improvement at 1-year post-intervention in the riluzole group compared to the control group (in press). Riluzole remains a promising pharmaceutical treatment for SCI.

TABLE 3 Summary of neurorehabilitation and stimulation strategies on spinal cord injury patients.

Approach	Treatment type	Study design	Stage of SCI	Sample size	Main outcome	References
Kinesiotherapy and rTMS in patients after Incomplete Cervical or Thoracic SCI	rTMS enhanced the corticospinal synaptic transmission	Clinical Research	Incomplete SCI at the C2–Th12 levels	26	Neurophysiological recordings produced significantly better MEP parameters in the K + rTMS group. This effect was sustained for at least 5 months	Wincek et al. [229]
5 Hz rTMS on sensory, motor, and autonomic function	Decreased motor cortical excitability	Clinical Research	Chronic SCI	15	Active motor threshold for the most caudally innervated hand muscle was increased with slightly improved hand function	Kuppuswamy et al. [224]
rTMS of the motor cortex on central pain after SCI	Depression scores were reduced	Clinical Research	Thoracic SCI	11	Long-term clinical effect on central pain. Pain scores were reduced and continued to improve at follow-up	Defrin et al. [226]
SCS on spasticity	Enhancing pre- and post-synaptic spinal inhibitory mechanisms	Clinical Research	Chronic SCI	12	Spasm was significantly reduced immediately after SCS, and spasticity measures were improved by 2 h post-induction	Hofstoetter, 2020 [230]
Non-invasive spinal cord electrical stimulation for arm and hand function in chronic tetraplegia	Improved the recovery of sensory function Decrease in the frequency and severity of muscle spasms Reduced pain	Clinical Research	Chronic Cervical SCI	65	Safe and effective for improving hand and arm function	Moritz et al. [231]
Exercise program on the rehabilitation of patients with SCI	Improve resistance and muscular strength	Clinical Research	Thoracic SCI	13	Positive impact on physical function	Durán et al. [232]
Targeted stimulation for restoration of motor and autonomic function in individuals with SCI	NA	Clinical Research	Thoracic SCI cervical SCI	47	Effective strategies for the concurrent recovery of the various effects associated with severe chronic SCI	Angeli et al. [233]
Targeted neurotechnology restores walking in SCI patients	Adaptive control of paralyzed muscles during overground walking, locomotor performance improved, regained voluntary control over paralyzed muscles and walk or cycle in ecological settings	Clinical Research	Chronic cervical SCI	3	Technological framework for improving neurological recovery and supporting the activities of daily living after SCI	Wagner et al. [234]
Recovery of overground walking after chronic motor complete SCI	Recovery of walking, standing, and trunk mobility	Clinical Research	Chronic SCI C4–T4	4	Intentional over-ground walking ability years after SCI	Angeli et al. [235]
Activity-dependent spinal cord neuromodulation rapidly restores trunk and leg motor functions after complete paralysis	Sufficient improvement to restore activities	Clinical Research	Chronic SCI	3	Activity-specific stimulation programs improved stand, walk, cycle, swim, and control trunk movements	Rowald et al. [236]
Walking naturally after SCI using a brain-spine interface	BSI enables natural control over the movements of legs to stand, walk and climb stairs	Clinical Research	Chronic SCI	1	Neurorehabilitation supported by the BSI improved neurological recovery	Lorach et al. [237]
Robot-assisted gait training improves walking function and activity in SCI	Improvements in gait distance, leg strength, and functional level of mobility	Clinical trials	Incomplete SCI	502	RAGT treatment is a promising technique to restore functional walking and improve locomotor ability	Nam et al. [238]

(Continued on following page)

TABLE 3 (*Continued*) Summary of neurorehabilitation and stimulation strategies on spinal cord injury patients.

Approach	Treatment type	Study design	Stage of SCI	Sample size	Main outcome	References
Robotic assisted gait training on ambulation and functional capacity in patients with SCI	Improvement in the walking index and functional independence measure scores	Clinical Research	Complete and Incomplete SCIs	88	Robotic-assisted gait training combined with conventional therapy is superior to conventional therapy in terms of gait function and level of disability	Yıldırım et al. [239]

Abbreviations: SCI, spinal cord injury; SCS, transcutaneous spinal cord stimulation; rTMS, repetitive transcranial magnetic stimulation; scES, spinal cord epidural stimulation; BSI, brain–spine interface; RAGT, robot-assisted gait training.

Neuromodulation and stimulation

The main goal of rehabilitation strategies after SCI is to enhance functional recovery [221]. One possible way to achieve this goal is to strengthen the efficacy of the residual neuronal pathways [222, 223]. Electrical and magnetic neural stimulation induces significant and long-lasting neuroplastic effects that involve neuroplasticity markers [222, 223].

Transcranial magnetic stimulation

Non-invasive repetitive transcranial magnetic stimulation (rTMS) has been applied to target sensory and motor function impairments, spasticity, and neuropathic pain [222]. The influence of rTMS in patients with SCI may confirm the hypothesis about the significance of the propriospinal system and other residual efferent pathways in the recovery of motor control [224]. Moreover, rTMS targeted at the motor cortex has suggested therapeutic potential in alleviating chronic neuropathic pain [225–228], indicating its beneficial effects in evaluating and enhancing motor function in SCI patients [224, 229] (Table 3).

Electrical stimulation

Electrical stimulation can accelerate axonal growth and myelination [240], stimulate neurons to discharge bioelectric signals to strengthen muscle contraction, and reconnect the neural network of the spinal cord [241]. Therefore, the baseline excitability of neural circuits is regulated by electrical stimulation, leading to action potentials within and between neural circuits by adjusting excitability to a precise level [242]. Electrical stimulation also enhances neurotransmitter release capacity via recruiting local neurotransmitters across synaptic sites by stimulating the amount of neurostimulation of afferent nerve fibers [242]. Moreover, epidural electrical stimulation leads to greater recovery of motor output after a severe SCI, and with intensive training and electrical stimulation, recovery of walking, standing, and trunk mobility can occur years after SCI [235]. Within a single day, activity-specific stimulation programs have enabled standing, walking, cycling, swimming, and control of trunk movements [236]. In addition, Transcutaneous Spinal Cord Stimulation (SCS) shows promise in reducing spasticity [230]. The application of this non-invasive spinal cord electrical stimulation technique is safe and effective for improving hand and arm function in individuals with cervical SCI (Table 3) [231].

Electrical stimulation and neuromodulation strategies show promise for enhancing motor function in SCI patients. Despite their potential, the use of electrical and magnetic stimulation in SCI rehabilitation faces several considerations and limitations, necessitating significant validation through clinical trials. Additionally, methodological variability across studies complicates the interpretation of outcomes. Standardization of stimulation parameters, patient selection criteria, and outcome measures are essential to ease meaningful comparisons and robust conclusions regarding stimulation effectiveness in SCI rehabilitation.

Neurorehabilitation

Various types of motor training such as bicycling, swimming, and locomotor training decrease the inflammatory response, increase neurotrophins, and may strengthen spared functions and guide spinal reorganization [83]. Exercise has been shown to preserve muscle mass [243], restore motor and sensory function [232, 244, 245], induce synaptic plasticity [246], increase the concentration of neurotrophic factors in spinal and muscle tissue [247, 248], and reduce inflammation around the injured site [245] (Table 3).

Studies investigating the timing of exercise post-injury suggest it may yield advantageous or adverse consequences on the recovery outcomes [249–251]. Despite numerous studies, several questions remain unanswered regarding therapeutic tools, such as optimal rehabilitation timing, the most suitable intensity, duration, and frequency, as well as the best use of task-specific training for recovery of various functional modalities.

Robot rehabilitation and brain–computer interfaces

In recent years, neural interfaces such as Brain-computer interfaces (BCIs) have used physiological brain activity to control external devices, thereby enabling severely disabled patients to interact with the outside environment [252]. Invasive and non-invasive BCI approaches have been used to promote neural control of a robotic arm [253] for patients with severe paralysis. This system has the potential to provide a new way of controlling their wheelchair [238, 239, 254].

All approaches to adaptive technology for patients with SCI have aimed to provide patients with control of their paralyzed limbs [233, 234, 237]. However, the possibility that similar systems could bring neuroplastic alterations that contribute to functional rehabilitation remains unclear. The technologies in combination with current pharmacological and neurostimulation approaches may offer a crucial pathway to motor recovery following SCI [255, 256].

Ethical considerations

While the medical concerns of SCI such as neuropathic pain and spasticity have received significant attention, a smaller number of works have focused on ethical issues related to treatment and research in SCI, such as voluntary consent, patient welfare, transparency, medical decision-making, and the patient-physician relationship [257, 258]. The perspectives, priorities, and experiences of individuals living with SCI are influenced by social, environmental, clinical, and injury-associated aspects [259]. In addition, living with SCI is aggravated by a fair treatment concern and sociocultural factors at a systems level, such as a lack of accessible support, information, and rehabilitation as well as healthcare services, which create hindrance to reunification of research and clinical studies [260].

SCI research raises crucial ethical questions concerning participant welfare and the use of funding [257–260]. The approach of a consistent clinical trial plays a very important role in the reliability of the research, especially the inclusion and exclusion criteria, ethical issues, treatment uniformity, and informed consent [258, 259]. The inclusion and exclusion criteria to control the consistency of the trial must be developed based on the specific research content [259, 261]. Informed consent for clinical trials as well as the standardization of the operation procedures and rehabilitation treatment is necessary [261].

However, within SCI research, the concept of data sharing, meta-analysis, and the application of new statistical techniques such as recursive partitioning and the global statistical test show promise. A notable challenge is that publications focusing on preclinical research often present only a fraction of the generated data [262]. This limitation may be attributed, in part, to the constraints imposed by journals on word counts as well as the number of figures and tables allowed. Nonetheless, in recent years, various stakeholders within the SCI research community have actively advocated for publication standards and promoted the sharing of experimental data. Initiatives like The Open Data Commons for Spinal Cord Injury (ODC-SCI.org) have emerged, facilitating data sharing and enabling pooled data-driven discoveries while appropriately acknowledging the contributors of valuable SCI data [261].

Research involving individuals with SCI must prioritize patient well-being and minimize any potential harm or discomfort related to experimental procedures [263–265]. In addition, clear communication about the risk factors and potential benefits of research is essential; therefore, SCI patients should have access to information about the research process and its implications. Moreover, transparency regarding funding, prioritizing research subjects with the greatest potential to improve quality of life and develop new treatments, adhering to established standards of scientific integrity, accurately reporting findings, avoiding data manipulation, and community engagement to promote ethical conduct are key factors in clinical trial studies [263–265].

The burden of SCI and inequality in the international context

The challenges faced by individuals with SCI across different countries and regions around the world include issues such as access to healthcare, rehabilitation services, and supporting devices. In many parts of the world, there is a lack of resources and support systems for people living with SCI, leading to increased vulnerability and inequality. Understanding and addressing these issues on a global scale is crucial for improving the quality of life and outcomes for individuals with SCI worldwide [266].

Unaddressed healthcare needs are significant for SCI patients, where people in low-income groups tend to be more affected. Among the barriers to meeting healthcare needs are healthcare cost, transportation, and service availability [266]. To improve the situation, a combination of measures from the health and social systems are required. Improving access to healthcare to ensure individuals with SCI have access to affordable and appropriate assistive technologies, specialized medical care, and rehabilitation services. This can improve functional outcomes and quality of life for all SCI patients regardless of their socioeconomic status. Providing equal access to education and employment opportunities for individuals with SCI and promoting public awareness to reduce stigma contributes to creating a more equitable and inclusive world for individuals living with SCI globally [266, 267].

Future directions: combinatorial therapies

As each therapy seeks to target aspects of SCI, combinatorial approaches are now emerging to match the disease timeline and improve functional outcomes. Exemplifying the notion of combinatorial approaches to SCI can be done with the strategies currently available. Rehabilitative as well as FES (functional electrical stimulation) therapies rely on the presence of remaining neurons and parenchyma for the establishment of new synapses and subsequent functional recovery. Complementing rehabilitation and FES are early surgical intervention and stem cell therapies. Early surgical intervention prevents further secondary injury through reperfusion of the injured spinal cord, increasing the net preserved CNS tissue for synapse recruitment as well as reducing inflammation and scarring hostile to recovery. Similarly, stem cell therapy can be used for the replacement of lost cells in the CNS as well as creating a microenvironment conducive to repair through reduced inflammation. This potentially gives greater neuronal and synapse recruitment for better functional outcomes and impact from rehabilitation and FES.

Pharmaceutical options are currently limited but follow a similar trajectory, with each drug potentially playing a different role dependent on which aspect of SCI injury is targeted. The growing movement towards personalized medicine is highly applicable to the heterogeneous nature of SCI. There is a great degree of variability in effectiveness and perceived outcomes between treatments across patients. This has been highlighted particularly well by patient perceptions on the effectiveness of different treatments for chronic pain. Preferences have been shown for different opioid medications, diazepam, rehabilitative exercise, as well as massage – all to differing degrees [268, 269].

Conclusion

There have been remarkable advancements in medical, surgical, and rehabilitative treatments for SCI. However, despite these advances, opportunities exist to develop reparative and regenerative approaches to enhance outcomes. Although it remains true that the outcomes in SCI improve in a very incremental fashion due to the complex nature of SCI pathophysiology, the advancements in techniques and strategies are impressively thorough and creative. By incorporating patient-centric approaches, insight into individual differences can guide current and emergent treatment as well as provide better autonomy to patients. Adapting each regenerative approach into a cohesive strategy in concert remains a difficult task yet even modest improvements in sensory and motor function returning to patients can be both meaningful and motivating in the face of a highly debilitating disease.

Author contributions

AA: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing–original draft, Writing–review and editing. MGF: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing–original draft, Writing–review and editing. OH: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing–original draft, Writing–review and editing. ST: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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*CORRESPONDENCE
Johnson Rajasingh,
rjohn186@uthsc.edu

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Induced mesenchymal stem cells generated from periodontal ligament fibroblast for regenerative therapy

Hemanathan Vembuli[1], Sheeja Rajasingh[1], Patrick Nabholz[1], Jefferson Guenther[1], Brian R. Morrow[1], Margaret M. Taylor[1], Marziyeh Aghazadeh[1], Vinoth Sigamani[1] and Johnson Rajasingh[1,2,3]*

[1]Department of Bioscience Research, University of Tennessee Health Science Center, Memphis, TN, United States, [2]Department of Medicine-Cardiology, University of Tennessee Health Science Center, Memphis, TN, United States, [3]Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, United States

Abstract

Bone fractures and bone loss represent significant global health challenges, with their incidence rising due to an aging population. Despite autologous bone grafts remain the gold standard for treatment, challenges such as limited bone availability, immune reactions, and the risk of infectious disease transmission have driven the search for alternative cell-based therapies for bone regeneration. Stem cells derived from oral tissues and umbilical cord mesenchymal stem cells (MSCs) have shown potential in both preclinical and clinical studies for bone tissue regeneration. However, their limited differentiation capacity and wound healing abilities necessitate the exploration of alternative cell sources. In this study, we generated induced pluripotent stem cells (iPSCs) using a safe, nonviral and mRNA-based approach from human periodontal ligament fibroblasts (PDLF), an easily accessible cell source. These iPSCs were subsequently differentiated into MSCs, referred to as induced MSCs (iMSCs). The resulting iMSCs were homogeneous, highly proliferative, and possessed anti-inflammatory properties, suggesting their potential as a superior alternative to traditional MSCs for regenerative therapy. These iMSCs demonstrated trilineage differentiation potential, giving rise to osteocytes, chondrocytes, and adipocytes. The iMSC-derived osteocytes (iOSTs) were homogeneous, patient-specific and showed excellent attachment and growth on commercial collagen-based membranes, highlighting their suitability for bone tissue regeneration applications. Given their promising

characteristics compared to traditional MSCs, PDLF-derived iMSCs are strong candidates for future clinical studies in bone regeneration and other regenerative dental therapies.

KEYWORDS

mesenchymal stem cells, induced pluripotent stem cells, differentiation, osteocytes, regenerative therapy

Impact statement

We introduced a new and easily accessible homogeneous induced mesenchymal stem cells from dental tissue, which can be readily obtained during routine tooth extractions or surgeries for personalized regenerative dentistry. These stem cells represent a unique alternative form of stem cells distinct from traditional MSCs, with better proliferation activity, immunomodulatory function, and wound-healing properties, making them a promising candidate for advancing regenerative dental therapies.

Introduction

Bone fractures and bone loss are significant healthþ problems worldwide, and their incidence is increasing due to the aging population [1]. These problems arise from the slowing down of the bone remodeling process and the reduction of minerals in the extracellular matrix. Congenital abnormalities, trauma, infectious diseases and cancer contribute to the growing number of patients needing bone reconstruction [2, 3]. Despite autologous bone grafts being considered the gold standard, surgeons and patients face various challenges with current treatments. These include the shortage of bone sources, immune reactions, transmission of infectious diseases, and difficulties in graft harvesting [4, 5]. These challenges have limited the development of cell-based therapies for bone regeneration. Dental-derived stem cells, obtained from extracted, impacted, and exfoliated teeth, are a promising source for regenerative medicine due to their availability and potential for multilineage differentiation. Numerous preclinical studies have demonstrated their potential in treating medical conditions including ischemic disease [6, 7], neural tissue injuries [8, 9], diabetes [10, 11], skin and hair injuries [12, 13], muscular dystrophy [14–16] and cartilage defects [17, 18]. Dental-derived cells have also been successfully used for bone tissue regeneration in several preclinical and clinical studies [19–23].

Umbilical cord mesenchymal stem cells (UC-MSCs) are another source of multilineage stem cells with properties such as high self-renewal and low immunogenicity. They are isolated and cultured from umbilical cord [24]. For years, the storage of UC-MSCs has been utilized worldwide as a source of cells for future therapy. In some countries, at the time of delivery, most parents are advised to preserve the umbilical cord stem cells or umbilical blood in a stem cell bank for potential use in autologous stem cell therapy. However, this option is only available at the time of birth and is very expensive, making it impractical for the majority of people [25]. Mesenchymal stem cells (MSCs) from bone marrow, adipose tissues and umbilical cord blood have been shown to be promising alternative sources for MSCs and have become important in regenerative medicine [26–29]. However, these cells from human body are not easily accessible due to invasive and discomforting procedures. Moreover, these cells are categorized as adult stem cells, and their differentiation capacity is limited compared to embryonic stem cells. Access to embryonic stem cells is not feasible, so scientists are striving to develop an alternative method to generate induced pluripotent stem cells (iPSCs), a promising cell sources for treating human diseases.

This iPSC technology provides a basic platform for generating patient-specific pluripotent stem cells and subsequently differentiating them into specific cell lineages for cell therapy due to their remarkable proliferation ability and immune compatibility. However, the reprogramming approach for deriving iPSCs needs improvement in terms of technique, efficiency, and availability of primary autologous cell sources. Initially, these cells were developed using retro- and lentiviruses to deliver vectors containing genes required for cell reprogramming. One major disadvantage of this method is the uncontrolled integration of viral DNA into host cell genomes [30]. To address this limitation, different methods have been developed. Our non-viral and mRNA-based reprogramming method, utilizing mRNA of pluripotent genes and a cocktail of miRNAs, has demonstrated high efficiency and the production of high-quality autologous iPSCs with high replicative and differentiation potentials, suitable for regenerative therapy [31]. These iPSCs present new opportunities for understanding embryogenesis and a great impact on drug screening and toxicological tests [26].

Human periodontal ligament fibroblasts (PDLF) are easily accessible cells that can be obtained from extracted teeth. In this study, we utilized PDLF as an optimal source for generating iMSCs which is similar to UC-MSCs and evaluated the efficacy of PDLF derived iMSCs in regenerative therapy.

Materials and methods

Antibodies and reagents

Primary antibodies for OCT4, NANOG, SOX2 (Cell Signaling Technology), β-actin, TRA1-60, SSEA4, VE-Cadherin, α-fetoprotein

(AFP) (Santa Cruz Biotechnology, Inc.) and Nestin (R&D Systems) were used to perform *in vitro* protein analysis (Supplementary Table S1). Secondary antibodies APC. TRITC, PE and FITC-conjugated anti-donkey, anti-mouse, anti-goat and anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.) were used respectively. Culture media including NutriStem (NS) medium, Mesencult medium (Stem Cells), reprogramming kits (Repro cell) and DAPI stain (Life Technologies) were used in this study. RNA of cells was collected using TRIzol reagent (Ambion by Life Technologies), and the quantification of the samples was evaluated by the NanoDrop 8000 Spectrophotometer (Thermo Fisher). The list of the primers for specific gene expression is mentioned in Supplementary Table S2. The isolated protein samples were quantified by Bradford's method using the AccurisTM instrument SmartReader and the absorbance read at 595 nm.

PDLF and UC-MSC cell culture

Human PDLF cell line from Lonza (Walkersville, MD; cc-7049) was gifted by Dr. Ammaar Abidi. Cells were grown in a 60-mm or 100-mm dish containing SCBM fibroblast culture medium (Lonza, United States) supplemented with 15% FBS, and 1% penicillin/streptomycin antibiotics and maintained at 37°C in a 5% CO_2 incubator. The human UC-MSCs (referred to as MSCs) were purchased from ScienCell Research Laboratories. The cells were cultured in Mesencult medium (Reprocell, United States) and maintained as per supplier's instruction. When the cells become 80% confluent, they were sub-cultured and used for reprogramming experiments.

Non-viral reprogramming of human PDLF cell-derived iPSCs (referred to as iPSCs)

PDLF cells were sub-cultured in NS medium (Stemcell Technologies, Canada) in a 6-well plate coated with iMatrix (Reprocell USA Inc). At 80% confluency, the cells were reprogrammed with the mRNA of OCT4, NANOG, SOX2, KLF4, MYC and LIN28 along with a cocktail of microRNAs (Reprocell USA Inc) using Lipofectamine RNA iMAX transfection agent [32, 33]. After 8 days, several iPSC-granulated colonies were generated which resembled human embryonic stem cell colonies. These colonies were characterized for pluripotency using real time quantitative PCR, Western blot, and immunofluorescence analyses. TRA1-60, a marker of pluripotent stem cells, was employed to identify positive colonies. These colonies were manually picked from day thirteen onwards and subsequently cultured and maintained on Matrigel-coated plates in NS medium.

Trilineage differentiation of iPSCs

To evaluate the trilineage differentiation potential of iPSCs, we differentiated them into three distinct cell lineages: mesoderm, endoderm, and ectoderm [32].

For mesoderm differentiation, specifically endothelial cells (ECs), iPSCs were cultured in NS medium in a 30-mm culture dish. When the cells reached 70%–80% confluency, the NS medium was replaced with mesodermal medium (DMEM supplemented with 1X B27, 1X N2, 5 μM CHIR, 25 ng BMP4) and cultured for 3 days. After 3 days, mesodermal medium was replaced with StemPro34 medium for 4 days, followed by the addition of endothelial EGM2 medium to promote the development of matured ECs. The generated ECs were characterized for endothelial-specific genes VE-cadherin, and CD31by qRT-PCR analysis and protein expressions by immunofluorescence analysis.

For endoderm differentiation, iPSCs were cultured in NS medium. When the iPSCs reached 70%–80% confluency, cells were cultured with Stemdiff definitive endoderm medium (Stemcell Technologies). Twelve days post culture, we observed that these cells exhibited a cuboid shaped morphology representing primary hepatocytes. The cells were harvested and subjected to qRT-PCR analysis to evaluate the gene expression of hepatocyte specific markers, including apolipoprotein A1 (APOA1) and α–fetoprotein (AFP). Additionally, immunofluorescence analysis was performed to access AFP protein expression.

For ectodermal differentiation, iPSCs were similarly cultured in NS medium, when the cells reached 70%–80% confluency, the NS medium was replaced with the neuronal induction medium (Stemcell Technologies). After 10 days, we observed the morphological changes indicative of successful differentiation, with the cells adopting shapes resembling neuronal cells. These cells were further analyzed for neuronal-specific gene expression, including OLIG2 and MAP2 by qRT-PCR analysis and protein expressions of GFAP and Nestin by immunofluorescence staining.

Differentiation of iPSCs into iMSCs

For the differentiation of iPSCs into iMSCs, we adhered to the protocol previously developed in our lab [31]. Briefly, the iPSCs were cultured in a 30-mm culture dish with NS medium. When these cells reached 80% confluency, NS medium was replaced with mesenchymal induction medium (STEMdiff-ACF, Stem Cell Technologies) and incubated for 4 days followed by culturing in MesenCult ACF Plus medium for 21 days. By day 21, the cells acquired the matured MSC morphology. This was confirmed through analysis of mRNA and protein expression using qRT-PCR, immunostaining, Western blot and flowcytometry analyses. The characterized

cells were further sub-cultured and maintained in MesenCult ACF plus medium.

Trilineage differentiation potential of iMSCs

To evaluate the trilineage differentiation capacity of the developed iMSCs, the cells were seeded at the density of 7.5×10^5 cells in a 30-mm culture dish containing MesenCult ACF Plus medium. They were subsequently induced to differentiate into chondrocytes, adipocytes, and osteocytes following our established differentiation protocol [31].

To induce osteocyte differentiation, once the iMSCs reached 80% confluency, osteocyte differentiation medium containing Dexamethasone and BMP4 was introduced. After 7 days, the medium was substituted with osteocyte mineralization medium and maintained for 2 weeks. Finally, the induced osteocytes (iOSTs) were evaluated for expression of osteogenic specific markers using qRT-PCR analysis. Alizarin red (ALZ) staining was employed to detect osteocyte mineralization. Later the cells were seeded onto a commercially available collagen-based scaffold to evaluate their adherence to available dental membranes, paving the way for potential clinical applications.

For chondrocytes differentiation, when the iMSCs attained 80% confluency, chondrocyte differentiation medium (Thermo Fisher) was added and cultured for 17 days. The differentiated chondrocytes (iCHON) were characterized by the expression of chondrocyte specific mRNA along with Alcian blue staining.

For adipocyte differentiation, once the iMSCs reached 80% confluency, adipocyte differentiation medium (Thermo Fisher) was introduced. After 11 days, the induced adipocytes (iADIPO) were harvested and characterized through qRT-RCR for adipocyte specific mRNA expression and adipocyte lipid droplets were visualized using Oil red staining.

Anti-inflammatory assay

To compare the immunomodulatory properties of iMSC and MSC, the cells were treated with 1 μg/mL of LPS for 24 h. The LPS stimulated cells were then harvested and analyzed for the inflammatory and anti-inflammatory marker expression via qRT-PCR following our previously described method [31].

Cell migration assay

The scratch test is a convenient method for assessing cell migration *in vitro*. The steps include creating an artificial gap by using an insert and seeding a cell monolayer on both sides. The insert is used to provide a homogenous, cell-free space in the middle of attached cells. Then the migration of the cells toward the scratch is quantified by comparing the images at various time points. This migration leads to covering the area and cell to cell contact in the scratch. The major advantage is that it mimics cell migration in tissues and organs [34]. In this study, the cell migration potential of iMSCs and MSCs were assessed and compared by scratch test. iMSC and MSCs were seeded at a concentration of 2×10^4 in the insert placed in 30 mm cell culture plates. After 24 h, the insert was removed (T0) and the percentage of covered area was evaluated after 8 hours (T8) by ImageJ software (NIH).

Colony formation assay

To determine the capacity of individual iMSCs and MSCs to proliferate and form colonies through clonal expansion, we performed a colony forming assay. This property was assessed by seeding the cells into 30-mm plates at a density of 6.25×10^4 in MesenCult proliferation medium (MesenCult™Proliferation Kit, Stemcell Technologies) for 2 weeks. After a week small-to medium-sized colonies were observed. At the end of 2 weeks culture, the plates were stained by crystal violet. The cells were placed on ice, washed with cold PBS and fixed with 100% ice-cold methanol for 10 min. They were then incubated with 0.05% crystal violet solution in 25% methanol for 20 min. Finally, the cells were washed five times with water, and images were captured using EVOS phase-contrast microscope. The intensity of crystal violet staining was analyzed with ImageJ software.

Assessment of differentiated osteocytes growth on collagen-based membranes

To evaluate the clinical applicability of iMSC cells, they were differentiated into osteocytes, as described earlier [31]. The osteocytes were then seeded onto a commercial collagen-based membrane (Zimmer BioMend absorbable collagen membrane). The membrane was prepared by cutting it into 8 × 12 mm pieces and inserting in a ring within a 30 mm culture plate. Osteocyte differentiation medium was added to the ring, followed by the addition of 10,000 osteocytes drop wise onto the membrane. After 20 minutes 2 mL of medium was added to the plate, and the cells were incubated for 2 weeks with medium changes every 3 days. After 2 weeks, the membrane was removed and rinsed with PBS. It was then fixed in 2.5% glutaraldehyde for 12 hours at 4°C. The membrane was subsequently sectioned into two pieces to evaluate the osteocyte adhesion and morphology by DAPI staining followed by Scanning Electronic Microscopic analysis. For nuclear staining, the membrane was washed with PBS and DAPI stain solution was added to cover the membrane for 10 minutes. The membrane was washed again, and the images were captured using fluorescent microscope.

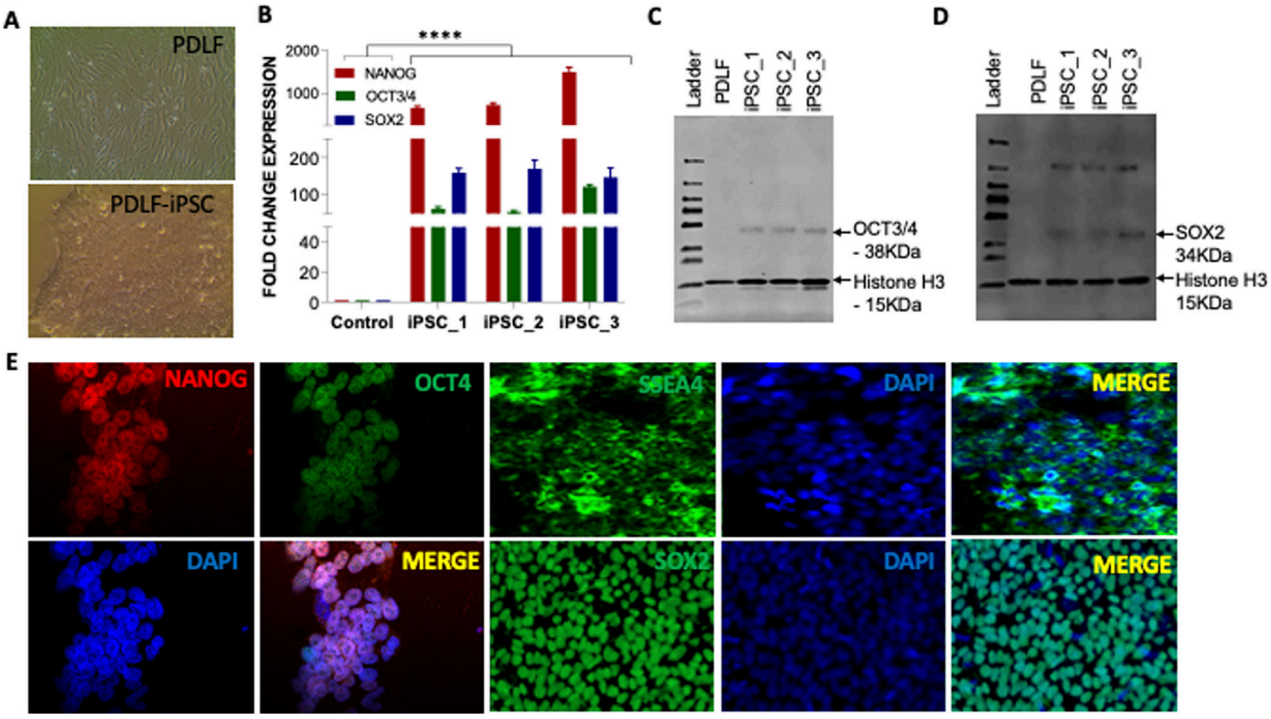

FIGURE 1
Characterization of the pluripotency of PDLF-iPSCs. **(A)** Microscopic picture of PDLF and PDLF-iPSCs. **(B)** qRT-PCR analysis for the expression of pluripotent genes NANOG, OCT3/4, and SOX2 in three clones of iPSCs on day 11. PDLF cells served as control. The relative mRNA expression was normalized to the 18S gene. Results were expressed as fold change, and the values were calculated as the ratio of induced expression to control expression (****$p < 0.00001$). **(C)** Western blot analysis showing OCT3/4 and **(D)** SOX2 protein expression levels in iPSC clones derived from PDLF compared to the parent PDLF cells. Histone H3 was used as loading control. **(E)** Pluripotency-specific protein expression, including OCT4, NANOG, SSA4, and SOX2, was assessed via immunofluorescence staining. DAPI staining was performed to visualize the nucleus.

Scanning electron microscopy (SEM)

For SEM analysis, the membrane was first fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C, followed by rinsing with 0.2 M sodium cacodylate buffer (pH 7.4) every 30 min for a total of 1.5 h. Subsequently, the membrane was rinsed with water and subjected to sequential dehydration with increasing concentrations of ethanol (50%, 75%, 95% for 15 min each, and 100% ethanol for 20 min). It was then immersed in hexamethyldisilane (HMDS, Sigma) for 10 minutes and air-dried at room temperature. Before SEM imaging, the membrane was mounted on aluminum stub using carbon adhesive tabs and coated with a gold nanoparticle at ~100 Å, and then imaged.

Immunofluorescent staining

We performed immunostaining analysis to quantify the specific proteins within the cells, as described previously [31, 32]. The cells were grown in 4-well chamber slides, washed three times with Dulbecco's phosphate-buffered saline (DPBS), and then fixed with 4% paraformaldehyde for 5 min. After an additional three washes with DPBS, the cells were permeabilized with 0.1% TritonX-100 in DPBS for 3 min. The cells were then blocked with blocking buffer for 30 min and incubated with primary specific antibodies for overnight at 4°C, followed by a 1-hour incubation with secondary antibodies at 37°C. After washing with DPBS three times, these cells were stained with DAPI, and the staining was analyzed using either a confocal or immunofluorescence microscope.

Western blot analysis

We conducted Western blot analysis to quantify the proteins of interest as described in our earlier studies [31, 32]. Briefly, the cells were washed with PBS, and lysis buffer was added for protein isolation. The samples were then centrifuged at 12,000 g for 20 min at 4°C. The supernatant was carefully transferred to a new vial and quantified using Bradford's method with an Accuris™ SmartReader 96-well microplate absorbance reader at 595 nm. These protein samples were uses for Western blot analysis.

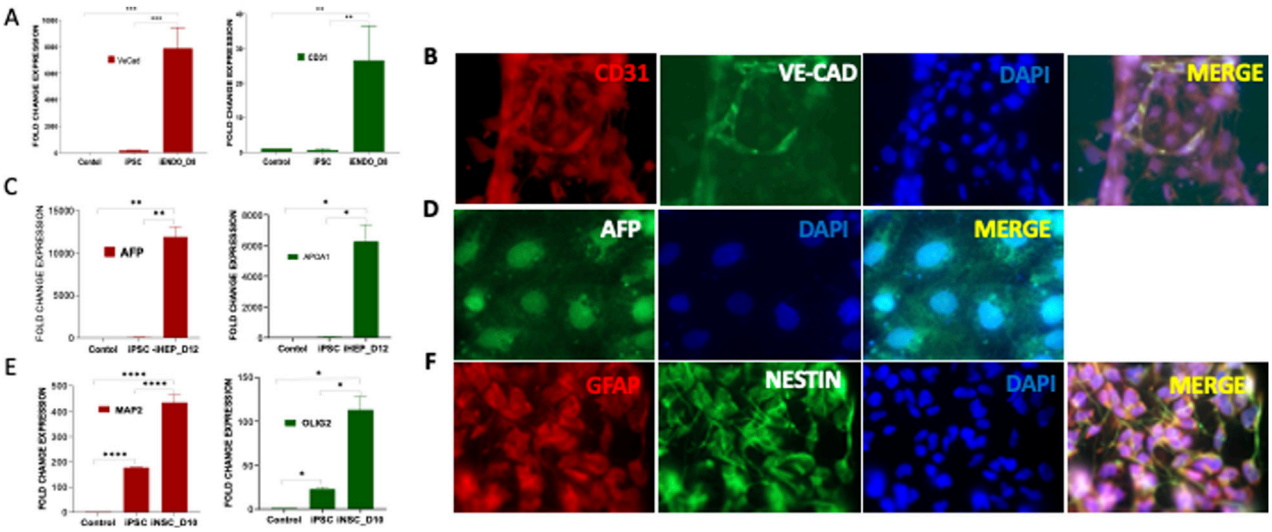

FIGURE 2
In vitro trilineage differentiation of PDLF-iPSCs (iPSCs) **(A)** iPSCs were cultured in mesodermal medium and differentiated to endothelial cells (iENDO). After 8 days (D8) the mRNA expression of VE-Cadherin (VE-Cad) and CD31 was evaluated as an endothelial-specific gene. The significant alterations in the expression of these genes in differentiated cells and control cells (PDLF and iPSCs) (**p < 0.001, ***p < 0.0001) were examined. **(B)** Protein expression of endothelial cell surface markers CD31 (Red), and VE-Cad (Green) were detected by immunofluorescence staining. DAPI (Blue) was used to visualize the nucleus. **(C)** iPSCs were cultured in endodermal differentiated medium and differentiated to Hepatocytes (iHEPO). Hepatocytes specific genes were evaluated for endoderm differentiation. APOA1 and AFP gene expressions were assessed at day12 (D12) (*p < 0.01, **p < 0.001). **(D)** Hepatocyte surface marker AFP (Green) was analyzed using immunofluorescent analysis. **(E)** iPSCs were differentiated to neural cells in neuronal induction medium for 10 days (D10) and the mRNA expression of neuronal specific genes including OLIG2 and MAP2 in the differentiated cells was evaluated (*p < 0.01, ****p < 0.00001). **(F)** Immunofluorescence staining demonstrating the expression of neuronal cell surface markers GFAP (red) and NESTIN (green) in the differentiated cells. Nuclei are counterstained with DAPI (blue). The colocalization of red and green signals indicates the co-expression of GFAP and NESTIN in the cells.

Software and statistical analysis

To account for any experimental bias, each experiment was conducted in triplicate. The findings are expressed as Mean ± SD. Comparisons were carried out using ANOVA (GraphPad Prism) or the T-test, with a probability value of <0.05 deemed statistically significant. Image analysis was performed using ImageJ software (NIH). Flow cytometry analysis was conducted using FlowJo software.

Results

Generation of iPSCs from PDLF: a milestone for dental applications

The reprogramming of PDLF into iPSCs was accomplished through mRNA transfection of reprogramming factors combined with a miRNA cocktail, as outlined in our previous articles [32, 33]. During reprogramming, alterations in cell morphology were monitored throughout the process. These changes were sequentially imaged using a phase-contrast microscopy (Supplementary Figure S1). Starting from day 8, several colonies of granulated iPSCs were observed. By the end of day 10, cells with large nuclei that occupied most of the cytoplasm with well-defined round and smooth boarder colony are the typical morphology of pluripotent stem cells also evident (Figure 1A; Supplementary Figure S2). Positive colonies were identified using live TRA1-60 antibody staining and selectively picked and cultured for generating the pure population of PDLF iPSCs. Our findings indicate that iPSCs were successfully generated from PDLF for dental applications.

Characterization of iPSCs: pluripotency assessment and lineage differentiation

We proceeded with further characterization of the iPSCs by examining the mRNA and protein levels across different clones. Our findings demonstrated a significant elevation in mRNA levels of pluripotent genes OCT3/4, SOX2 and NANOG compared to PDLF (Figure 1B). Moreover, Western blot analysis confirmed notable protein expression of OCT3/4 and SOX2 (Figures 1C, D), while immunofluorescence staining also showed an increased expression of SSEA4, OCT3/4, SOX2 and NANOG markers, respectively (Figure 1E).

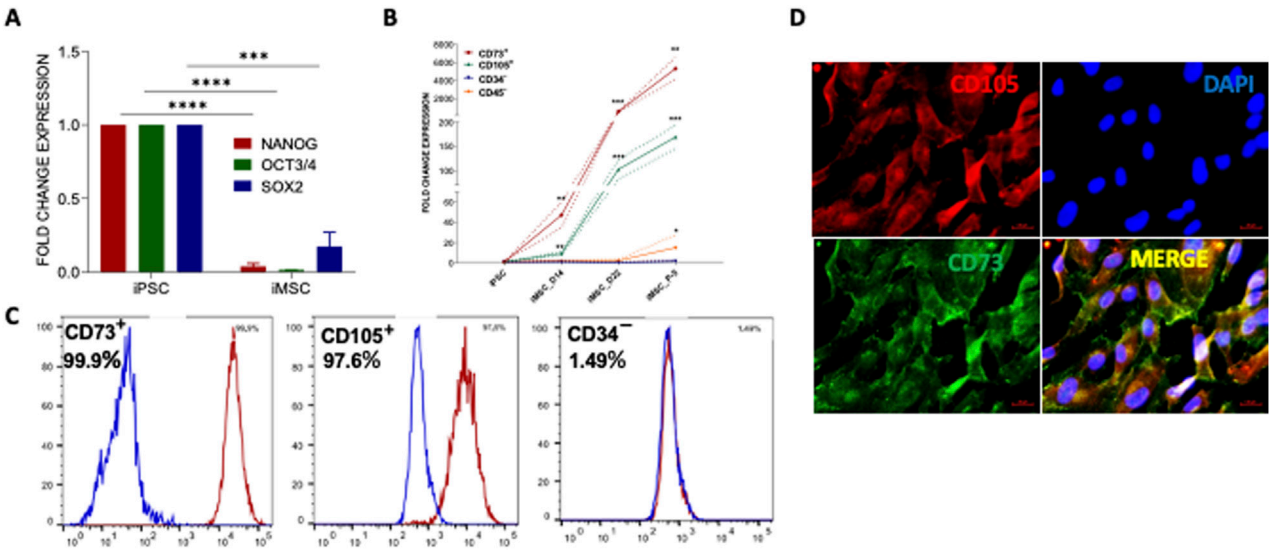

FIGURE 3
Generation and characterization of PDLF-iMSCs. **(A)** The expression of iPSC markers OCT4, NANOG and SOX2 was significantly decreased in the differentiated cells (PDLF-iMSCs). The mRNA expression levels were normalized to 18s rRNA. **(B)** Expression analysis of mesenchymal-specific genes and hematopoietic markers in PDLF-iMSCs. The differentiated cells (PDLF-iMSCs) showed a significant increase in the expression of mesenchymal-specific genes CD73 and CD105 at different time points compared to iPSCs (**$p < 0.001$, ***$p < 0.0001$). In contrast, the expression of hematopoietic markers CD34 and CD45 was negligible or significantly lower in PDLF-iMSCs compared to iPSCs (*$p < 0.01$). **(C)** Flow cytometric analysis revealing over 95% cell expression of mesenchymal surface markers CD73 and CD105, alongside non-expression of CD34. Isotype control antibodies served as negative controls to validate specificity. **(D)** The Protein expression of mesenchymal markers, CD105 and CD73 in iMSCs was significant and confirmed by immunofluorescence staining.

Pluripotency of PDLF-iPSCs were further validated through *in vitro* trilineage differentiation into endothelial cells (mesoderm), hepatocytes (endoderm), and neuronal cells (ectoderm). During endothelial differentiation, qRT-PCR analysis revealed a significant upregulation in mRNA expression of endothelial-specific genes, including VE-Cadherin (VE-CAD) and CD31, compared to the control (PDLF) and iPSCs (Figure 2A). This was further confirmed by immunofluorescence staining, which showed the expression of VE-CAD and CD31 proteins (Figure 2B). Following endodermal differentiation, the cells exhibited elevated mRNA levels of hepatocyte-specific markers, including α-fetoprotein (AFP) and Apolipoprotein A1 (APO1) (Figure 2C). Immunostaining further confirmed strong AFP protein expression (Figure 2D), affirming their successful differentiation into the endodermal lineage. During neuronal differentiation, the differentiated cells showed enhanced expression patterns of neuronal specific genes, including oligodendrocyte transcription factor 2 (OLIG2) and microtubule associated protein 2 (MAP2) than the iPSCs and PDLF cells (Figure 2E). Moreover, the expression of Nestin and GFAP in the differentiated cells was evident in through immunofluorescence staining (Figure 2F). Overall, these findings affirm the pluripotent nature of the reprogrammed iPSCs, demonstrating their ability to differentiate into all three germ layers of the embryo.

Comprehensive characterization and differentiation potential of iPSCs into iMSCs

After confirming the pluripotent nature of PDLF-derived iPSCs, they were further differentiated into iMSCs through our standard lab protocol as described earlier [31]. Morphological changes during the differentiation process were observed and documented (Supplementary Figure S3). To fully access the differentiation of iPSCs to iMSCs, we examined the expression of iPSC-specific genes, including OCT3/4, Nanog, and SOX2. Meanwhile, the iMSC clones displayed decreased expression pattern of pluripotent genes indicating the loss of embryonic properties during differentiation (Figure 3A). To characterize the resulting iMSCs, qRT-PCR analysis was performed for specific mesenchymal genes, including CD73 and CD105 as positive markers and CD34 and CD45 as negative markers. The results indicated an elevated expression of positive markers along with a lower expression of negative markers (Figure 3B). Interestingly, we found that the iMSCs exhibited a greater level of maturation, with heightened expression of MSC markers in comparison to iPSCs. The protein expression of some of these markers was confirmed by flow cytometric analysis, and immunostaining. Flowcytometric analysis demonstrated that over 95% of the cells expressed the mesenchymal cell surface markers CD73 and CD105. Conversely, the expression of the negative marker CD34 was lowered (Figure 3C). This was

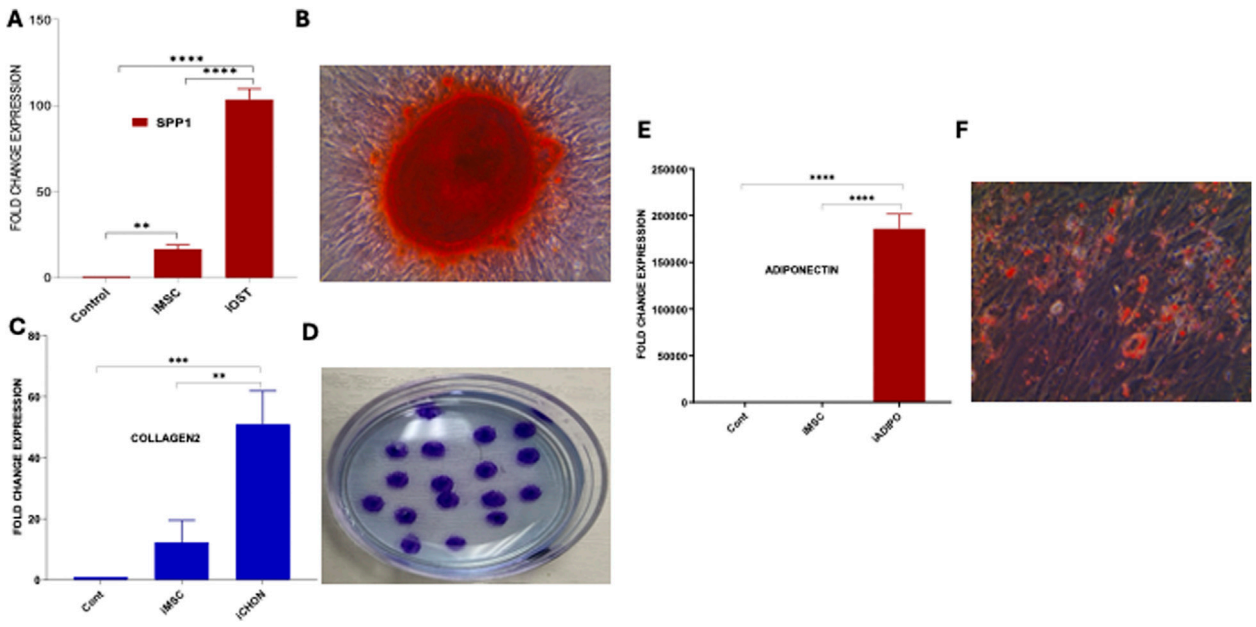

FIGURE 4
Trilineage differentiation of iMSCs. **(A)** Comparative analysis of SSP1 expression in iOSTs, iMSCs, and control cells. iOSTs exhibited significantly higher expression of the osteocyte-specific gene SSP1 compared to iMSCs and control cells (****$p < 0.00001$). **(B)** The calcium depositions of these cells were prominently stained by Alizarin Red. **(C)** The expression of the chondrocyte-specific marker collagen-2 was significantly higher in induced chondrocyte differentiated cells (iCHON) compared to iMSCs and control cells (**$p < 0.001$, ***$p < 0.0001$). **(D)** Alcian blue staining of iCHON cell colonies, revealing several stained areas on the culture plate, indicative of chondrocyte-specific matrix production. **(E)** In adipocyte differentiation, the induced adipocyte (iADIPO) cells showed significantly higher expression of the adiponectin gene compared to iMSCs and control cells (****$p < 0.00001$). **(F)** Induced adipocyte (iADIPO) cells positively stained with Oil Red O, indicating the presence of lipid droplets characteristic of adipocytes.

corroborated by the high protein expression levels of CD73 and CD105 markers in immunofluorescent staining (Figure 3D).

Trilineage differentiation potential of iMSCs into osteocytes, chondrocytes, and adipocytes

Furthermore, these iMSCs were evaluated for their multipotent differentiation potential into induced osteocytes (iOSTs), induced adipocytes (iADIPO), and induced chondrocytes (iCHON). For osteocyte differentiation, iMSCs were cultured in iMSC medium and then replaced with osteocyte differentiation medium. Subsequently, they were cultured in osteocyte mineralization medium for 2 weeks. The morphological changes of the cells during the osteocyte differentiation process were observed under phase-contrast microscopy (Supplementary Figure S4). The qRT-PCR analysis showed the mRNA expression of the osteocyte-specific gene SSP1 was significantly increased in the iOST (Figure 4A). Furthermore, the presence of calcium deposits in the iOSTs was clearly highlighted by Alizarin Red staining, indicating their role in bone mineralization and calcium regulation, which are essential for bone strength and structure (Figure 4B). The iMSCs were further differentiated into iCHONs. The differentiated iCHONs showed significantly increased expression levels of the chondrocyte-specific marker collagen-2 compared to the undifferentiated iMSC control (Figure 4C). Multiple stained areas were visible on the cultured plate when subjected to alcian blue staining, which specifically highlights chondrocytes (Figure 4D). The iMSCs also demonstrated their ability to differentiate into adipocytes. The resulting adipocytes displayed high expression of the adipocyte-specific marker adiponectin, confirming pure clones (Figure 4E). Furthermore, the deposition of fat and lipid droplets in iADIPO cells was confirmed by oil red O staining (Figure 4F). Overall, our data clearly indicate that iMSCs possess markedly superior multipotential differentiation abilities, similar to UC-MSCs, highlighting their greater potential for diverse clinical applications.

Comparative analysis of anti-inflammatory characteristics of iMSCs with standard MSCs

The anti- inflammatory cytokines play a crucial role in regulating the immune response and counteracting the effects of pro-inflammatory cytokines [35]. In the present study, we

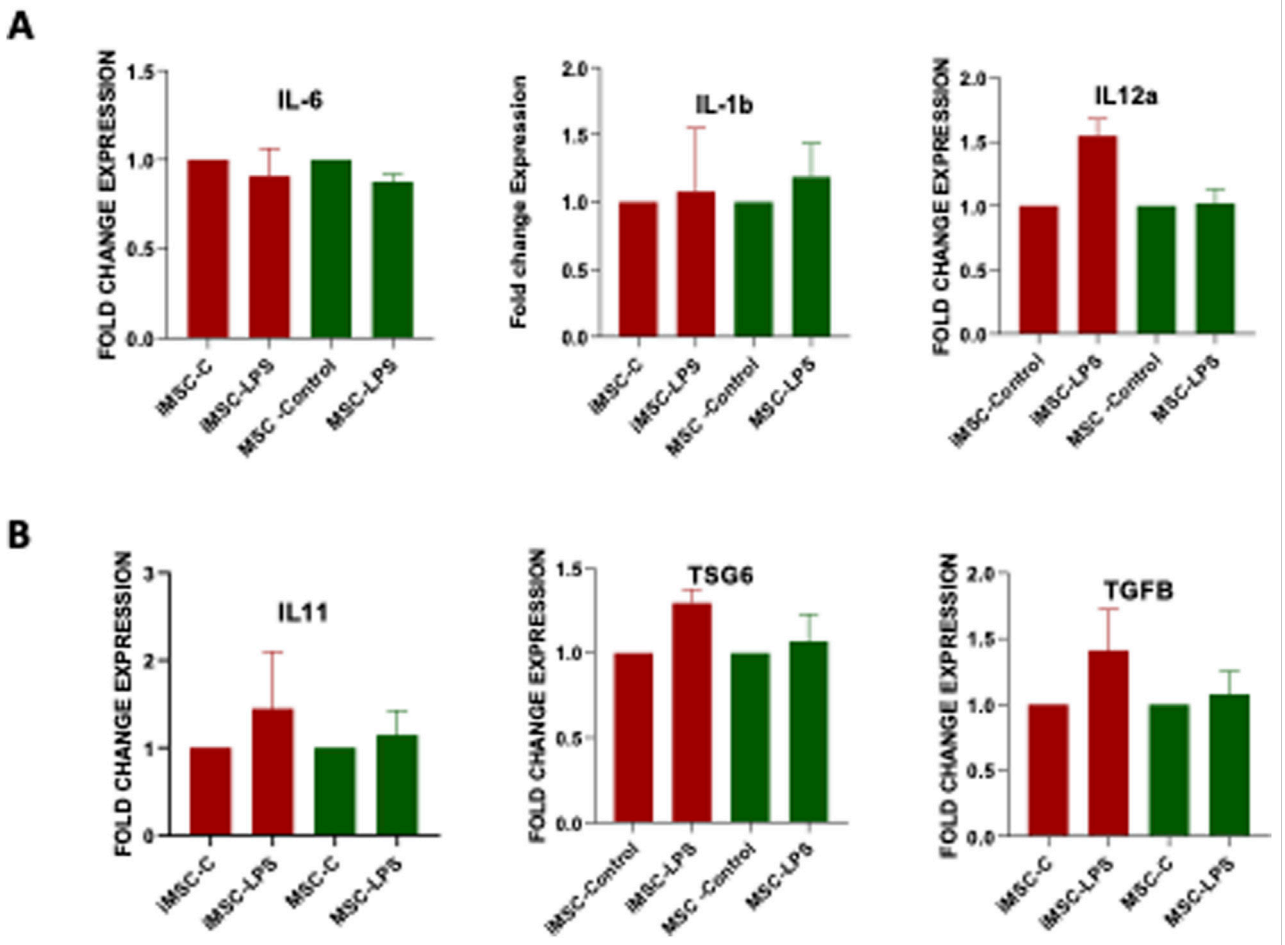

FIGURE 5
Comparative analysis of **(A)** pro-inflammatory (IL-1β, IL-6, IL-12α) and **(B)** anti-inflammatory (IL-11, TGF-β, TSG-6) gene expression in PDLF-iMSCs and UCMSCs after 24 h of LPS stimulation, showing no significant differences in mRNA expression levels between the two cell types.

aimed to evaluate the anti-inflammatory potential of iMSCs by comparing them to MSCs as the control group. To assess the anti-inflammatory properties, both iMSCs and MSCs were treated with lipopolysaccharides (LPS-Sigma-Aldrich). After 24 h, we examined the gene expression levels of major anti-inflammatory cytokines such as IL-11, TGF-β and TSG-6, as well as pro-inflammatory cytokines such as IL-6, and IL1β, using qRT-PCR analysis. The results indicated that there was no significant difference in the expression of these cytokines between iMSCs and MSCs. Both cell types exhibited similar gene expression patterns in response to the inflammatory stimulus. Importantly, iMSCs showed a slightly higher expression of IL-11, TGFα, and TSG6, which are known to be anti-inflammatory markers (Figure 5A). On the other hand, the expression of pro-inflammatory markers, including IL-1β, IL-6, and IL-12α was relatively lower in iMSCs compared to MSCs, although these differences were not statistically significant (Figure 5B). The proinflammatory gene expression is lower because of anti-inflammatory nature of iMSCs. These findings suggest that iMSCs possess comparable anti-inflammatory properties to MSCs, as evidenced by their ability to express anti-inflammatory cytokines in response to an inflammatory stimulus. While there were subtle differences in the expression levels of certain cytokines, overall, iMSCs demonstrated similar immunoregulatory capabilities to MSCs in the context of inflammation.

Enhanced migratory and proliferative abilities of iMSCs compared to MSCs

Scratch assay, an *in vitro* model of cell migration, was used to assess the migratory capacity of iMSCs compared to MSC. The results revealed that iMSCs covered approximately 60% of the scratch area, demonstrating a significantly higher migratory capacity (three times greater than that of MSCs) 8 hours after

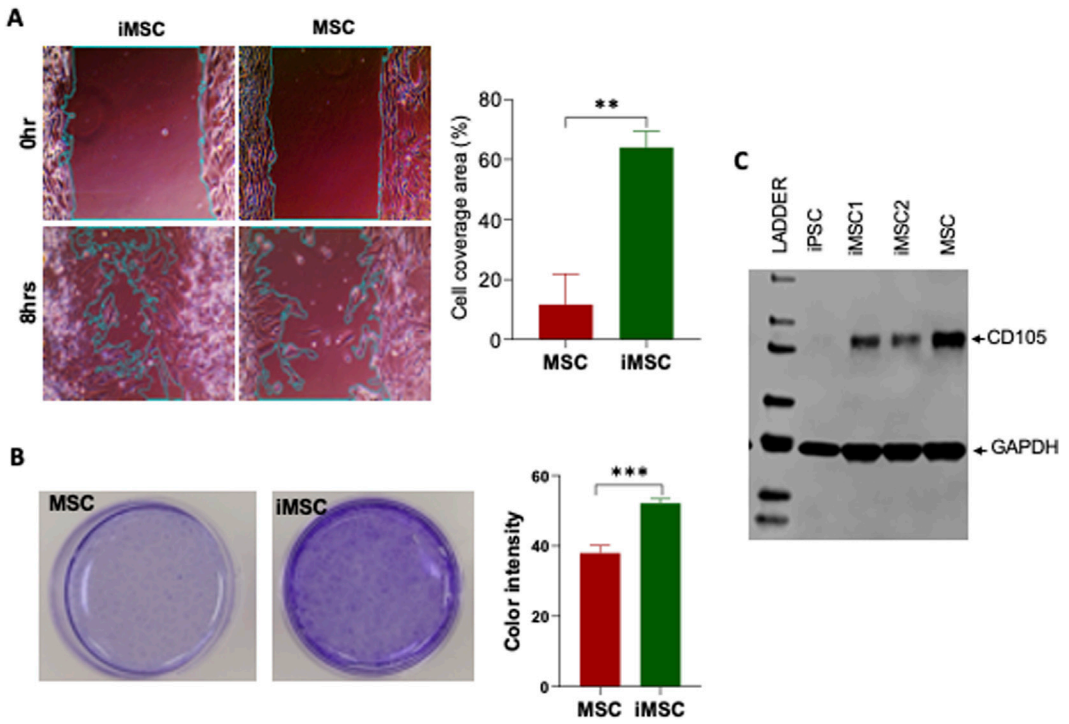

FIGURE 6
The migration and colony formation potential of PDLF-iMSCs compared to UCMSCs. **(A)** Scratch assay showing cell migration after 8 h. The covered area in the PDLF-iMSCs is significantly larger than in the UCMSCs (**$p < 0.001$). **(B)** Colony formation of PDLF-iMSCs and UCMSCs after 2 weeks. PDLF-iMSCs formed more colonies with increased blue staining intensity compared to UCMSCs (***$p < 0.0001$). **(C)** Western blot analysis demonstrating comparable CD105 (71 KDa) protein expression in PDLF-iMSCs and MSCs. GAPDA (36 KDa) protein serves as a loading control.

the scratch was made (Figure 6A). These findings highlight the superior migration ability of iMSCs in terms of covering the scratch area. To evaluate cellular survival, differentiation ability, and growth potential, a clonogenic assay was performed on iMSCs and MSCs. This assay is an *in vitro* method that measures how effectively a single cell can proliferate into a substantial colony through clonal expansion. The results demonstrated that iMSCs were capable of significantly forming more colonies compared to MSCs (Figure 6B). This indicates that iMSCs have a higher proliferation capacity than MSCs. Apart from migratory and proliferative characteristics, iMSCs also exhibited CD105 protein expression similar to MSCs, as demonstrated through western blot analysis (Figure 6C). These properties position iMSCs as an alternative autologous source and a favorable option for clinical applications compared to MSCs.

iMSCs for regenerative dentistry: applications on collagen membranes

For regenerative dentistry applications, we programmed our iMSCs to differentiate into terminally matured osteocytes. The mRNA expression levels of early marker Podoplanin, mineralizing marker DMP1, and mature or late markers associated with osteocyte differentiation namely BSP, FGF23, SOST, and SPARC were notably elevated in these differentiated cells (Figure 7A). Furthermore, these cells were positively stained with fluorescent antibodies for osteocalcin and DMP1, confirming their osteocyte-like characteristics (Figure 7B). APL staining and Alizarin Red staining revealed prominent calcium deposits in the iOSTs (Figures 7C, D). These findings collectively manifest that PDLF-iOSTs closely replicate the properties of human osteocytes.

Subsequently, we transferred these cells PDLF-iOSTs onto a collagen membrane (Figure 8A). After fixation and preparation, cell adhesion was assessed using DAPI staining via immunofluorescence microscopy and the ultrastructure was examined with scanning electron microscopy (SEM). DAPI staining displayed a substantial number of cells attached to the membrane (Figure 8B), and SEM images provided insights into the cell morphology and adhesion (Figure 8C). Overall, these findings illustrate that PDLF-iOSTs successfully adhered to the collagen membrane, underscoring their promising potential for applications in regenerative medicine and tissue engineering.

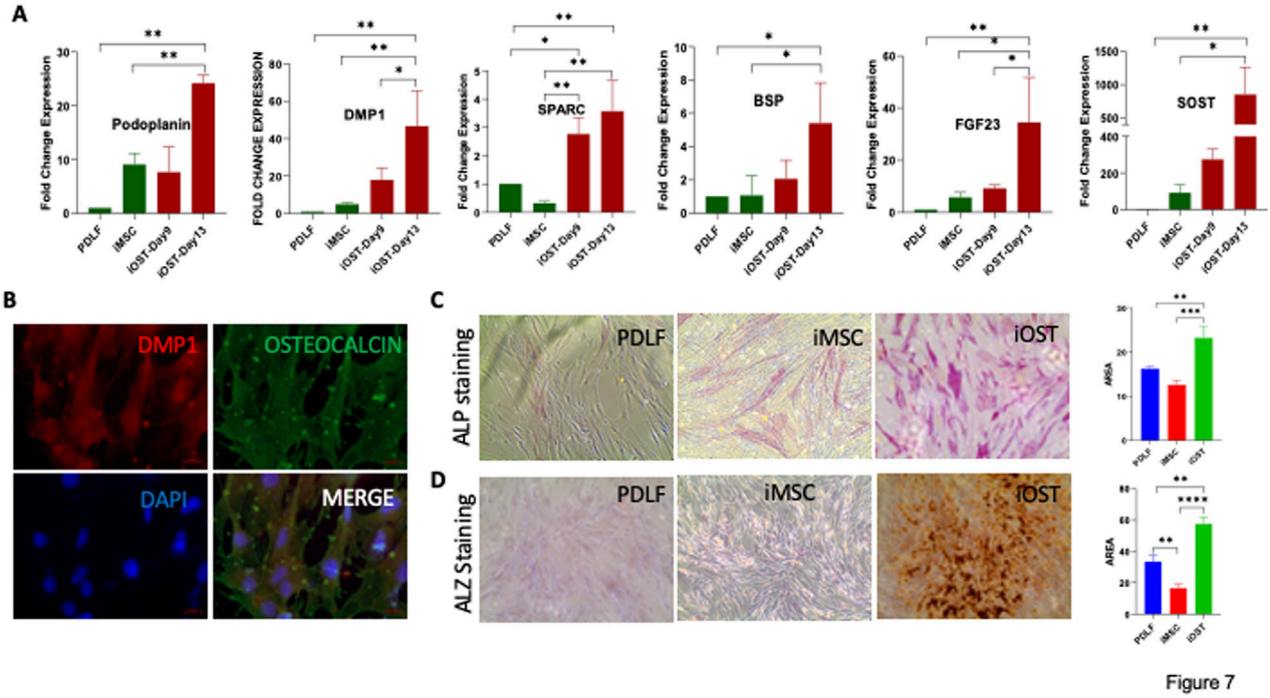

FIGURE 7
Osteocyte differentiation potential of iMSCs. **(A)** Expression levels of osteocyte-specific genes in PDL-iOSTs at days 9 and 13, compared to PDLF and PDLF-iMSCs. Significant increases in gene expression in PDL-iOSTs over time are indicated (*p < 0.01, **p < 0.001, ***p < 0.0001, ****p < 0.00001). **(B)** Immunofluorescence analysis of PDLF-iOSTs demonstrating the expression of DMP1 (red) and osteocalcin (green) proteins. Mineralization and calcium deposition in PDLF-iOSTs detected by **(C)** ALP and **(D)** Alizarin Red (ALZ) staining. The intensity of staining, indicative of mineralization, was measured using ImageJ software to assess the area of staining.

Discussion

Regenerative dentistry has emerged as a promising approach for repairing and replacing lost oral tissues and organs. Advances in molecular biology and the understanding of tissue development genes have paved the way for developing functional and biocompatible oral tissues. Teeth and their supporting tissues, such as PDLF, are easily accessible source of stem cells, making them highly relevant for personalized regenerative therapies [36, 37]. The PDLF cells can be conveniently isolated during routine procedures, such as tooth extractions, third molar removal, makes them a compelling option for regenerative treatments, particularly in the field of personalized dentistry [38]. Studies have shown that MSCs are the most used cell type in regenerative dentistry [39]. However, MSCs face challenges such as limited survival and proliferation in harsh environments [40–42], immune rejection risks [43], donor age-related cell senescence [44] and oncogenic potential due to prolonged *in vitro* culture [45].

To address these challenges, our study utilized a novel, viral- free reprogramming strategy [31] combining messenger RNA (mRNA) and microRNA (miRNA), to generate iPSCs from PDLF cells. This method eliminates the safety concerns associated with viral vectors and resulted in iPSCs with robust regenerative properties and trilineage differentiation potential, a crucial factor for their therapeutic use. The PDLF-derived iMSCs demonstrated strong differentiation capacities into osteocyte, adipocyte, and chondrocyte, comparable to other sources of MSCs but with the advantage of a minimally invasive collection process. These findings align with previous research on urinary epithelial-derived iMSCs, further validating the regenerative capabilities of iMSCs from various sources [31].

iMSCs have demonstrated superior immune suppression compared to MSCs, as supported by previous studies [46]. To further explore this, we subjected both iMSCs and MSCs to LPS to assess their inflammatory properties. Our findings revealed that the iMSCs showed mild inflammatory and high anti-inflammatory responses when exposed to LPS which is comparable to those of MSCs, indicating that iMSCs maintain immune regulatory functions. LPS is indeed a potent stimulator of immune cells; however, in the context of iMSCs, it exhibits a more moderate stimulatory effect. iMSCs are known for their anti-inflammatory properties, which could mitigate the typical

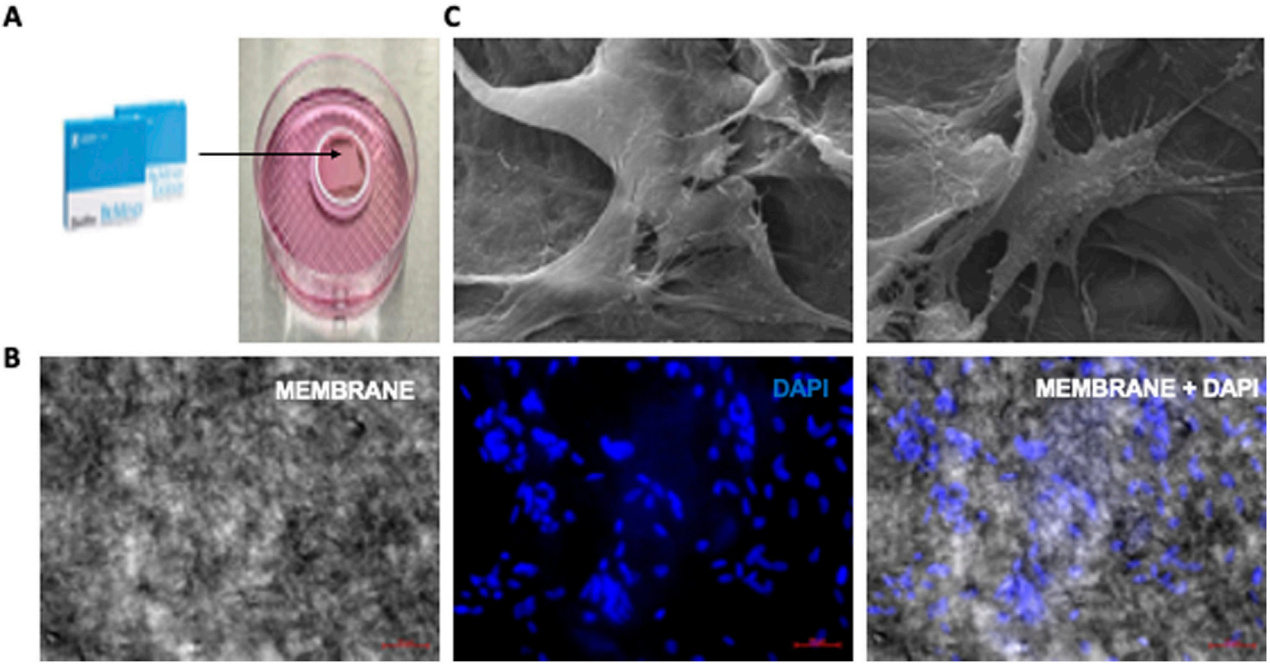

FIGURE 8
Attachment of PDLF-iOSTs to a commercial collagen-based membrane. **(A)** A piece of collagen membrane placed in a ring-shaped instrument. **(B)** DAPI staining and fluorescent imaging of PDLF-iOSTs attachment on a collagen-based membrane. The left image shows the membrane alone, the center image highlights the nuclei of attached and proliferated cells, and the right image displays the membrane with attached cells. A substantial number of cells remained on the membrane following the preparation process. **(C)** SEM images of PDLF-iOSTs adhered to the collagen-based membrane through their extended processes.

proinflammatory response to LPS. This suggests that iMSCs retain the immune-regulatory functions typical of iMSCs, making them a promising candidate for treating inflammatory conditions, not only in dental applications but also in broader clinical settings.

In our study, iMSC-derived osteocytes successfully adhered to commercially available collagen-based resorbable membranes, demonstrating their suitability for bone regeneration and guided tissue repair. The ability of iOSTs to adhere to a commercial collagen membrane is significant, as it demonstrates that these cells can attach, migrate, and potentially proliferate, key characteristics for their application in tissue engineering, particularly in bone regeneration and wound healing. The chemically crosslinked collagen membrane used in this study effectively supports guided bone regeneration by providing a scaffold conducive to cell attachment and growth. Additionally, its slower degradation rate helps maintain the structural integrity and stability of the injury site, enhancing effective healing while minimizing the risk of deformities [47]. Importantly, the use of iOSTs on such membranes can serve a dual purpose: aiding in bone regeneration prior to or during dental implant placement and creating a favorable environment for successful guided bone regeneration surgery. This approach holds promise for addressing gaps in current regenerative strategies.

Our findings demonstrate that the iMSCs derived from PDLF not only possess superior regenerative properties but also offer a more accessible and cost-effective cell source for regenerative therapies. The advantage of PDLF derived iMSCs is that PDLFs will have periodontal niche which will elucidate better periodontal regeneration and craniomaxillofacial regeneration and they also have low immunogenicity compared to blood and skin cells [48]. Moreover, the use of iMSCs derived from dental tissues for clinical applications, such as bone regeneration and soft tissue repair, could significantly improve the outcomes of regenerative dental procedures, such as sinus lifts, bone augmentation, and guided tissue regeneration. Our study also highlights the advantages of iMSCs compared to umbilical cord-derived MSCs, which are widely stored and used in clinical trials. In many developing countries, there is a growing trend of storing umbilical cord-derived MSCs at the time of delivery, resulting in significant financial gains for stem cell storage companies. Through this research, we aim to raise awareness that iMSCs can be generated from adult body cells, such as PDLF, can provide an equally effective or even potentially superior to MSCs. This comparison underscores the feasibility of utilizing easily accessible body-derived cells for therapeutic purposes. While we acknowledge the limitation of donor variability and

the small sample size, the intent of this study is to explore this alternative approach and establish a basis for further, more extensive research in the future.

In conclusion, PDLF-derived iMSCs offer a cost-effective, accessible, and viable cell source with superior regenerative properties for dental and craniofacial applications. Further studies should focus on refining these techniques, addressing and explore their clinical application in a broader range of therapeutic contexts.

Author contributions

JR, SR, HV, and MA contributed to conceptualization and literature collections; SR, HV, PN, JG, MT, BM, MA, and VS contributed to methodology, validation and formal analysis; SR, HV, and MA, contributed to manuscript writing-original draft preparation; SR, HV, and JR contributed to editing; SR and JR contributed to supervision of the project; JR contributed to project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

Data availability

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

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Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.ebm-journal.org/articles/10.3389/ebm.2025.10342/full#supplementary-material

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