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OPINION REVIEW

Mesenchymal stem cells in neurodegenerative diseases: Opinion review on ethical dilemmas

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Abstract

The treatment of neurodegenerative diseases presents a growing need for innovation in relation to recent evidence in the field of reconstructive therapy using stem cells. Understanding the molecular mechanisms underlying neurodegenerative disorders, and the advent of methods able to induce neuronal stem cell differentiation allowed to develop innovative therapeutic approaches offering the prospect of healthy and perfectly functional cell transplants, able to replace the sick ones. Hence the importance of deepening the state of the art regarding the clinical applications of advanced cell therapy products for the regeneration of nerve tissue. Besides representing a promising area of tissue transplant surgery and a great achievement in the field of neurodegenerative disease, stem cell research presents certain critical issues that need to be carefully examined from the ethical perspective. In fact, a subject so complex and not entirely explored requires a detailed scientific and ethical evaluation aimed at avoiding improper and ineffective use, rather than incorrect indications, technical inadequacies, and incongruous expectations. In fact, the clinical usefulness of stem cells will only be certain if able to provide the patient with safe, long-term and substantially more effective strategies than any other treatment available. The present paper provides an ethical assessment of tissue regeneration through mesenchymal stem cells in neurodegenerative diseases with the aim to rule out the fundamental issues related to research and clinical translation.

Key words: Mesenchymal stem cells; Neurodegenerative diseases; Stem cell research; Stem cell therapy; Ethical principles; Patient safety

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Core tip: Neurodegenerative diseases constitute a set of pathologies affecting the central nervous system whose main characteristic is a chronic and selective process of neuronal cell death. The study of stem cells and the advent of new methods able to induce neuronal differentiation, is having a significant impact in this sense in recent years, offering the prospect of transplanting healthy and perfectly functional cells, able to replace those diseased. The objective the present paper is to contribute to the construction of an ethical framework that allows a close monitoring of the scientific activity in the experimental and translational fields.

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INTRODUCTION

Modern transplant surgery presents an impressive need to explore the biological reconstructive possibilities in different branches of medicine. Hence, it is important to deepen the state of the art regarding the clinical applications and ethical implications of cell therapies in neuronal regeneration.

Over the last few years, in the field of tissue grafts in patients suffering from neurodegenerative diseases, a broad perspective has been opened, linked to the possibility of growing in the laboratory differentiated cells and tissues from isolated lines of multipotent cells grown on specific physiological substrates[1-4].

The destruction of the tissue architecture and the impairment of the function of an organ related to the death of the cells of which it is constituted are at the basis of the majority of the pathologies that afflict the population of the industrialized countries[5]. Regenerative medicine is a branch of medicine that aims to permanently recover damaged tissues and organs through the exploitation of the regenerative potential of stem cells[6-8]. Regenerative medicine, therefore, includes all the therapies that, in pursuing the goal of regeneration, use the potential of stem cells, locally stimulated both to duplicate and to differentiate, to be transferred after appropriate selection and extraction[9].

Understanding the molecular mechanisms underlying neurodegenerative disorders and the advent of methods able to induce neuronal stem cell differentiation have allowed the development of innovative therapeutic approaches offering the prospect of healthy and perfectly functional cell transplants that are able to replace sick ones[10-13].

Mesenchymal stem cells (MSCs) have interesting tissue regenerative potentialities in adult organisms[14]. MSCs are available in many tissues and have the capacity to regenerate them in part or entirely once increased in number and differentiated. The use of adult MSCs is currently one of the research areas of greatest interest in regenerative medicine[15].

In recent years, research has focused on the standardization of protocols for the isolation and expansion of MSCs from various tissue sources, on the characterization of their phenotypic and biological properties, and on the development of advanced therapies that combine MSCs with synthetic scaffolds and signalling molecules (growth factors and tissue differentiation) for the construction of hybrid constructs[16-18]. In fact, achieving full knowledge of the processes of self-replication and proliferation would allow researchers to obtain an infinite source of tissues for the treatment of degenerative diseases or of important lesions of the central nervous system (CNS).

The optimization of the therapeutic efficacy of MSCs in the treatment of neurodegenerative diseases requires researchers to overcome biological and technical challenges. Particularly, it is necessary to address the critical issues related to the dosage and routes of administration, the identification of patients able to respond to cell replacement therapy, the host's immunological response, the mechanisms of action of the grafts and the adverse effects.

The affirmation of the potential use of MSCs in regenerative medicine, currently

supported by promising scientific results, will allow researchers to meet the high expectations raised in the community only if hinged in a context of transparency and plurality of research, in full respect of different cultural and technical backgrounds[19,20]. The protection of plurality makes it possible to deal in depth with the issues of regenerative therapy to understand its mechanisms and to develop effective treatments[21]. The lack of the integration of the different perspectives would imply their subtraction from the biological foundations of clinical practice and the exaltation of an empirical medicine incapable of producing new knowledge, depriving the community of the necessary scientificity[22].

Thus, while representing a promising area of tissue transplant surgery and a great achievement in the field of neurodegenerative disease, stem cell research presents certain critical issues that need to be carefully examined from an ethical perspective[23,24].

First, the progress of research must be supported by consolidated and transparent evidence despite the enthusiasm derived from the results of preclinical and clinical trials[25]. In fact, the clinical utility of MSCs will be certain only in the presence of safe therapeutic strategies, validated in the long term, that are determined to be substantially more effective than any other available treatment. It is also necessary that the ethical, legal and commercial aspects concerning stem cell research and related clinical trials continue to be discussed on the basis of concrete objectives and through medically objective, scientifically honest and socially useful strategies[26].

In such a context, the use of appropriate methodologies in medical science is crucial for the ability to connect biology and clinical medicine as well as to offer the tools to distinguish good clinical practices from para-scientific illusions[27].

The present paper provides an ethical assessment of tissue regeneration through MSCs in neurodegenerative diseases with the aim outlining the fundamental issues related to research and clinical translation.

MSCs IN NEURODEGENERATIVE DISEASES

The diseases of the CNS represent a heterogeneous category of pathological conditions with distinct etiopathogenetic and symptomatological characteristics, for which a cure has not yet been identified[28]. Specifically, neurodegenerative diseases constitute a set of pathologies affecting the CNS whose main characteristic is a chronic and selective process of neuronal cell death. Neuronal deterioration is due to an inevitable loss of brain function that occurs, depending on the type of disease, with cognitive impairment, dementia, motor deficits, and behavioural disorders, more or less serious. Despite the partial overlaps correlated with symptomatology and pathological progression, among the different pathologies studied in tissue regeneration, it is possible to distinguish Parkinson's disease, Alzheimer's disease (AD), Huntington's disease, multiple sclerosis, and amyotrophic lateral sclerosis[29].

The treatment of neurodegenerative diseases presents a growing need for innovation in relation to recent evidence in the field of reconstructive therapy using stem cells[30,31]. Hence, it is important to deepen the state of the art regarding the clinical applications of advanced cell therapy products for the regeneration of nerve tissue. At present, there is no cure for neurodegenerative diseases, but there are pharmacological treatments available to counteract some symptoms. The effort of the scientific community is to understand the molecular mechanisms underlying these diseases to intervene with new therapeutic approaches, including genetic approaches. The study of stem cells and the advent of new methods able to induce neuronal differentiation have had a significant impact in this sense in recent years, offering the prospect of transplanting healthy and perfectly functional cells that are able to replace those that are diseased.

MSCs are present in the bone marrow, where they form the stromal counterpart of the haematopoietic stem component, but they are also present in the peripheral blood, in the umbilical cord and in other sites, including muscle tissue, adipose tissue, the synovium, and the periosteum[32]. MSCs represent a topic of growing interest and a valid therapeutic alternative in the treatment of neurodegenerative diseases. Currently, MSCs have been studied in the field of several neurodegenerative diseases and acute brain injuries, demonstrating interesting safety profiles in intravenous and intrathecal administration.

Among the different applications under investigation, substantial progress has been recorded in multiple sclerosis, where, consistent with numerous scientific contributions, the administration of human umbilical cord blood-derived MSCs or human bone marrow-derived MSCs has demonstrated an immunomodulatory effect able to provide clinical stabilization, an improvement of the symptoms and a

reduction of the onset of relapse[33-37].

The clinical application of MSCs in the treatment of Parkinson's disease presupposes, on the contrary, the overcoming of numerous challenges in relation to the results obtained in animal models and clinical trials[38-41]. The results obtained so far allow cautious optimism in relation to the demonstration of a neurodegenerative effect and a slowing of disease progression in subjects treated with MSCs[42-44].

The therapeutic use of MSCs in AD must still be developed despite extremely high expectations. At present, preclinical evidence supporting the mechanisms of action and potential therapeutic implications is abundant[45]. Because of the reliability of the preclinical results, MSC-based therapies have been approved for human trials. Early evidence on the safety and tolerability of the intrathecal administration of allogeneic human umbilical cord blood-derived MSCs, even in the absence of transplant-related adverse events, did not demonstrate significant delays in cognitive decline during follow-up. Despite these results, several other trials are underway to clarify the therapeutic relevance of MSCs and their implications for the course of AD.

Concerning amyotrophic lateral sclerosis, the injection of autologous human bone marrow-derived MSCs into the spinal cord has shown in different studies the ability to induce an improvement of functional assessment scale scores, a better response to treatment and a slowing of disease progression[46-48].

Further clinical studies have demonstrated the potential efficacy of human mesenchymal stem cell administration after ischaemic stroke. Specifically, it has been documented that hBM-MSC transplantation is capable of improving clinical outcomes and reducing mortality rates[49]. In addition, an improvement in performance in daily activities and a decrease in cerebral atrophy in patients undergoing autologous hBM-MSC transplantation were noted 6 weeks after the ischaemic event. In some trials aimed at assessing the early effects of treatment, the administration of autologous MSCs after stroke was able to determine a reduction in the size of the infarct area[50].

Again, recent studies have demonstrated that the administration of MSCs promotes recovery in traumatic brain injury due to oxidative stress reduction[51-54]. Overall, autologous mesenchymal cell transplantation has highlighted, together with high safety profiles, the ability to support the repair of neurological damage, inducing an improvement in functional performance[55,56].

Finally, as already mentioned, despite the proven potential of MSCs to trigger and regulate the process of neuronal regeneration, several factors limit their clinical translation in neurology. The application difficulties due to the lack of clinical experience, controversies concerning the results obtained and the conflicting conclusions on potential benefits or side effects can be overcome only through a careful discussion of the ethical and regulatory issues related to the subject[57-59].

ETHICAL ISSUES RELATED TO STEM CELL RESEARCH AND APPLICATIONS IN NEURODEGENERATIVE DISEASES

General principles

For other ethical issues associated with scientific research and clinical medicine, as in the case of stem cell research and applications to transplant therapy, the fundamental criterion of evaluation lies in the defence and promotion of the integral good of the human person, according to its peculiar dignity[60].

In this regard, it is worth remembering that every medical intervention on the human person is subject to limits that are not reduced to the possible technical impossibility of realization but are linked to respect for the same human nature understood in its integral meaning[61].

The most important evaluations regarding the fundamental principles must be directed to the vulnerability of the individual. Neurodegenerative diseases, in fact, determine a state of vulnerability related to the evolution of the disease or to situational factors, which makes patients more susceptible to exploitation. The condition of fragility experienced by the subjects of the experimentation imposes particular care on the part of the investigators in the planning of clinical trials that contemplate fully informed and voluntary participation[62-64]. The finding of conditions such as impaired cognitive performance, mental and motor disability, and economic and social disadvantages must lead to the introduction of further guarantees to protect patients' rights and wellbeing as well as safeguards to limit exposure to undue influences[65].

Furthermore, it is necessary to highlight how the defence of research participants' rights presuppose, together with the protection against avoidable damages, a careful evaluation of the benefits for the person and society. Professionals involved in research and clinical trials should prevent physical, psychological, economic and legal

bias with a view to maximize patient advantages.

Risk assessment and safety in clinical research

Historically, some inconsistencies found in the preclinical phase have precluded the clinical translation of some stem-cell-based therapies[66,67]. In this context, the therapeutic application of MSCs in animal models provided safety tests that favoured the approval of human clinical trials. Likewise, human experimentation was supported by the ease of the isolation and manipulation of MSCs and by evidence of efficacy related to regenerative and immunomodulatory potentials[68].

Nevertheless, several scientific contributions on the subject of cell therapies emphasize the need to address - in advance of clinical research - the issues of long-term safety, tolerability, and efficacy of the treatments under investigation[69]. The cellular product used for research purposes must meet the quality standards required by local legislation through the support of preclinical data that prove the safety of cells, the procedure, and the effective ability of MSCs to differentiate into nerve cells both in the laboratory and in the receiving host. Likewise, the use of mesenchymal stem-cell-based products must be corroborated by precise data on the dose of toxicity, reproductive toxicity, and carcinogenesis.

To date, the main problems are unwanted differentiation, the potential suppression of the anti-tumour response and the neo-angiogenetic capacity of the transplanted MSCs[70]. In fact, MSCs have shown paracrine activity with the release of growth factors and cytokines able to stimulate angiogenesis, slow down the processes of cell death and block inflammatory processes. For these reasons, safety studies must be extended to the classification and resolution of possible local complications as well as to systemic adverse effects through the provision of long follow-up periods[71]. In fact, clinical practice is the best test for evaluating the adverse effects and limitations of cell therapy regarding the functionality of grafting after transplantation. Based on these assumptions, the safety issues still under debate must be carefully discussed regardless of the promising results obtained from therapies with MSCs.

Clinical trials

MSCs have been or are currently being studied in approximately 46 phase I and II clinical trials (www.clinicaltrials.gov). However, not all trials fully meet regulatory criteria, preclinical studies are often weak and insufficient, cellular products are difficult to reproduce in a standardized manner, and results are sometimes supported by poor evidence.

In this regard, it should be noted that, as with any type of pioneering research, no one can provide guarantees of success before the availability of evidence. Hence, it is a duty to pursue every area of research on the sole condition that these are rational, verifiable and methodologically appropriate studies[72].

Therefore, the clinical testing of MSCs requires the definition of unified regulations regarding the procedures for the preparation and maintenance of cellular products with standardized methods and techniques shared between laboratories. Equally evident is the difficulty in obtaining standard reference procedures precisely because of the heterogeneity of both the patients and the original tissue samples, as well as of the expanded cultures.

As outlined in the previous section, the clinical results highlight the overall potential efficacy of MSCs in the treatment of neurodegenerative diseases. Nevertheless, it is desirable that the therapeutic profile of MSCs in the neurological field be further investigated in larger cohorts. This perspective involves meticulous scientific planning aimed at overcoming the obstacles derived from the need to respect the heterogeneity of patients and to refine the inclusion and exclusion criteria.

Moreover, the efficacy of the tested treatments must be proven through different clinical evaluation systems, objective and subjective, included in long follow-up periods in which the integration of diagnostic imaging, laboratory monitoring, functional evaluation and quality of life questionnaires are contemplated.

Finally, the obligation for experimenters in clinical trials to ethically evaluate the commitment of economic and human resources related to the organization of facilities, the clinical management of patients and the traceability of procedures should be noted. Similarly, the analysis of sustainability must include the increased use of resources related to the recruitment of a large cohort, the high turnover of coordinators and dedicated professionals, the possible extension of the study period and follow-up, and the need for the disclosure of results. Obviously, all evaluations on the sustainability and use of resources presuppose, in addition to the intervention of the professionals involved, a huge effort on the part of governments and supranational organizations that finance research and have enormous possibilities for coordination in the allocation of funds and in management of the objectives of the international scientific community.

Justice in research and treatment

The considerations related to justice and equity are issues of remarkable importance, although they are often neglected in the context of scientific research and clinical practice. In the field of new biotechnologies and, in particular, stem-cell-based treatments, the economic costs of products and interventions can be extremely high, as can the time and resources necessary for development and therapeutic use[73]. Therefore, it is crucial to focus on the costs and availability of treatments to increase sustainability and reduce inequity in access to care[74,75].

Issues related to justice in the context of MSC research and therapy can be schematically linked to production, biobanking and clinical translation.

Regarding the processing of stem cells and the manufacture of cellular products, there is widespread evidence of a greater effort to standardize and rationalize production in the field of regenerative medicine compared to other branches[76]. The development of platform technologies and large-scale production represent an important perspective that can reduce time, labour and costs with interesting ethical implications for access to treatment. However, this strategy is currently poorly practicable in the field of MSCs and "*autologous*" cell interventions that are certainly more expensive and less readily feasible due to production times. Therefore, in light of the foregoing and with a view to planning future strategies, a careful balance between the needs for cost reduction and accessibility and the implications in terms of safety and effectiveness is essential.

Similar considerations can be formulated regarding the policy and practice of biobanking. Although still at a preliminary stage in technological and regulatory development, stem cell collection, storage and use systems are an indisputable resource for regenerative medicine[77]. Ideally, the development of large-scale biobanking systems could lead to an amplification of stem cell assets and a reduction in costs of absolute utility in the implementation of tissue engineering programmes[78-80]. The structuring of sustainable systems includes the guarantee of accessibility, the regulation of use in the experimental and therapeutic fields, the refinement of consent in its different forms and the protection of information processes[81]. Therefore, large-scale biobanking requires a critical characterization based on a careful assessment of potential benefits as well as practical and ethical challenges.

Finally, it must be stated that all the efforts made in the experimental and clinical field must be supported by scientific transparency and data sharing. In fact, despite the pressures deriving from commercial competition, it is fundamental to protect patients' hopes and to avoid the feeding of false expectations or, worse, fraudulent therapies[82-84]. For these reasons, in consideration of the social and not merely scientific scope of the objectives pursued, it is necessary that the professionals involved consider the ethical implications related to justice as closely related to research and clinical practice.

POSITION STATEMENT

Stem cell research and treatment require considerations about different issues of medical, scientific, moral and ethical relevance. The field of MSCs facilitates discussion among stakeholders because it is free from the problems inherent to the use of human embryonic stem cells. However, several issues need an open and constructive debate able to support the rapid development of knowledge and the promising application of MSCs in regenerative medicine.

To prevent the onset of prejudices that can nullify the efforts of the scientific community in such a sensitive area of medical science development, it is necessary to formulate recommendations for good experimental and clinical practice[85].

First, it is desirable to carry out continuous and responsible research aimed at generating evidence on the therapeutic mechanisms of MSCs with regard to differentiation capacity and paracrine activity. The expansion of knowledge must also clarify the persistent doubts about the long-term behaviour and adverse effects of MSCs.

Second, with regard to the future objectives of the research, further studies are suggested on the epigenetics of MSCs, immunogenicity, host immune response, and the stability of the grafts.

Progress in research and therapy requires the codification of universal criteria and standards for the processing of MSCs. For example, the availability of a shared methodology for *in vitro* differentiation could eliminate the limitations resulting from the current poor understanding of the MSC profile.

Moreover, precisely for safety concerns related to the clinical use of MSCs, the

efforts of the scientific community must be aimed at the manufacture of traceable, tolerable and effective cellular products. The translation into clinical practice and the marketing of cellular products presupposes the validation of standardized operating procedures as well as a careful review of the aspects related to functionality, safety and banking.

Ultimately, a significant political and legislative commitment at the international level is needed regarding the approval of public funding able to implement research activities and support clinical translation. The fair distribution of funding and the availability of equal opportunities for researchers determine a liberalization able to accelerate the development of the regenerative approach through MSCs. Only the guarantee of equity in all phases of scientific research will make it possible to respect the principles of justice to protect patients[85].

CONCLUSION

In the heterogeneous and complicated scenario currently characterizing the treatment of neurodegenerative diseases – in terms of the functional needs of patients and research, innovation, resources, and medico-legal issues – a rigorous ethical framework and a strict surveillance of scientific activity in the experimental and translational fields are imperative. It is also necessary that ethical, legal and commercial aspects concerning stem cell research and related clinical trials continue to be discussed on concrete objectives and through strategies that always present themselves as medically objective, scientifically honest and socially useful.

Cell therapies and regenerative medicine, increasingly based on the progress of stem cell biology, have begun to lay the foundations of future clinical practice.

The progress of medical therapy based on MSCs generates multiple expectations but requires a rigorous methodological approach. In particular, a subject so complex and not entirely explored requires a detailed scientific and ethical evaluation aimed at the avoidance of improper and ineffective use rather than incorrect indications, technical inadequacies, and incongruous expectations.

Despite the enthusiasm of stem cell studies, nothing could be more wrong than the transplantation of stem cells in humans without consistent results and consolidated evidence. In fact, the clinical usefulness of stem cell transplantation strategies will be certain only if they are able to provide the patient with safe, long-term and substantially more effective treatments than any other strategy available.

Conclusively, the many challenges still open in the exaltation of the potential of MSCs in the neurological field require an integrated multidisciplinary approach aimed at the contextualization of scientific advances and responsible clinical translation of therapeutic findings. Therefore, the growing focus on the therapeutic implications of MSCs should prompt scientists, physicians, regulatory bodies and bioethicists to act in a coordinated manner to promote appropriate and evidence-based clinical applications.

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REVIEW

Mesenchymal stem cell-derived extracellular vesicles as a new therapeutic strategy for ocular diseases

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Abstract

Mesenchymal stem cells (MSCs) have attracted considerable attention for their activity in the treatment of refractory visual disorders. Since MSCs were found to possess the beneficial effects by secreting paracrine factors rather than direct differentiation, MSC-derived extracellular vesicles (EVs) were widely studied in various disease models. MSCs generate abundant EVs, which act as important mediators by exchanging protein and genetic information between MSCs and target cells. It has been confirmed that MSC-derived EVs possess unique anti-inflammatory, anti-apoptotic, tissue repairing, neuroprotective, and immunomodulatory properties, similar to their parent cells. Upon intravitreal injection, MSC-derived EVs rapidly diffuse through the retina to alleviate retinal injury or inflammation. Due to possible risks associated with MSC transplantation, such as vitreous opacity and pathological proliferation, EVs appear to be a better choice for intravitreal injection. Small size EVs can pass through biological barriers easily and their contents can be modified genetically for optimal therapeutic effect. Hence, currently, they are also explored for the possibility of serving as drug delivery vehicles. In the current review, we describe the characteristics of MSC-derived EVs briefly, comprehensively summarize their biological functions in ocular diseases, and discuss their potential applications in clinical settings.

Key words: Mesenchymal stem cells; Extracellular vesicles; Exosomes; Ocular diseases; Drug delivery

Core tip: The therapeutic potential of Mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs) has been widely studied in various diseases. In the current

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review, we summarize all the studies about the use of MSC-derived EVs in different ocular disorders, such as corneal injury, glaucoma, uveitis and retinal diseases. We also discuss the history and properties of MSC-derived EVs, the advantages of their use in treating eye diseases and their drug delivery potential. This review also provides future directions for enhancing the therapeutic effect of MSC-derived EVs in treating ocular diseases.

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INTRODUCTION

Visual impairment and blindness are global issues, leading to a significant financial and medical burden. The number of visually impaired people in 2017 was estimated to be 285 million worldwide[1]. The leading causes of moderate or severe vision impairment among the global population in 2015 were uncorrected refractive error, cataract, age-related macular degeneration, glaucoma, and diabetic retinopathy, which will not change until 2020. Among them, vision loss caused by refractive error and cataract is avoidable. However, vision loss caused by age-related macular degeneration, glaucoma, and diabetic retinopathy is sometimes preventable, but incurable and irreversible[2]. The patient's quality of life is affected considerably, imposing a serious burden on their families. At present, few effective methods are available for the treatment of retinal and neural damage caused by various ocular diseases. Hence, alternative solutions, such as regenerative cell-based therapy, are being explored[3-5].

MSCs can produce immunosuppressive, anti-inflammatory, and trophic factors, and are explored widely as therapeutic agents for regenerative cell-based therapy of ocular diseases[6]. Although MSC transplantation has shown beneficial effects in treating many refractory diseases, ethical and safety concerns after intravenous injection on undesired differentiation and their ability to promote tumor growth are still a matter of debate, while intravitreal injection could lead to severe vision loss due to proliferative vitreoretinopathy (PVR)[7,8]. Since the therapeutic effects of MSCs can be mediated primarily by the paracrine signaling of EVs[9], MSC-derived EVs, either as a therapeutic agent or as a drug delivery system, are explored widely for the treatment of ocular disorders[10]. The majority of live cells secrete EVs[11]. However, MSC is the only human cell type with a scalable ability for mass production of EVs[12]. In this review, we summarize recent studies on the role of MSC-derived sEVs in the treatment of eye diseases and discuss the possibility of future clinical application.

EVs were used to be referred to as exosomes or microvesicles (MVs) in many studies. In 2018, the International Society for Extracellular Vesicles published minimal information for studies of EVs (MISEV2018), in which the authors were urged to use operational terms for EV subtypes based on their size ("small EVs" (sEVs) [< 100 nm or < 200 nm] and "medium/large EVs" (m/lEVs) [> 200 nm]), density (low, middle, high, with each range defined) or biochemical composition ($CD63^+$/$CD81^{+-}$ EVs, annexin A5-stained EVs, *etc*.) in place of terms such as exosomes and MVs[13]. All studies that described the effect of MSC-EVs on ocular disorders were using the term of exosomes. Based on the size of the EVs mentioned in these studies, we used sEVs instead of exosomes is this review.

Characteristics of MSC-derived sEVs

MSCs are a population of non-hematopoietic stem cells with self-renewal ability. In addition to fetal tissues, MSCs can also be isolated from adipose tissue, umbilical cord blood, peripheral blood, skeletal muscle, liver, gingival and dental tissue, skin, breast milk, cartilage, and corneal limbal stroma of the eye[14]. MSCs have the potential to differentiate into mesenchymal or non-mesenchymal cell lineages, such as osteoblasts, chondrocytes, and adipocytes[15]. MSCs possess the ability to migrate to the injury sites to promote wound healing and tissue regeneration and inhibit the immune response by modulating the proliferation and function of innate and acquired immune cells. The beneficial effect of MSCs can be attributed to sEVs, soluble factor secretion, and membrane protein CD73[16-18].

MSC-derived sEVs have a narrow diameter of < 200 nm and were supposed to be mostly exosomes in earlier studies with a major peak particle size of 65-75 nm[19]. The exosomes are composed of lipid bilayer membrane and cargo of proteins, nucleic acids (mRNA, miRNAs, DNA, and long noncoding RNAs), and raft-associated lipids[20]. Their biogenesis has two steps; the first step is the inward budding of late endosomes, and the second step involves the production of multivesicular body and extracellular release[21]. After being secreted into the extracellular space, the exosomes enter various biological fluids and can travel to remote organs while protecting the inside cargo from decomposing. Due to their small size, they can easily traverse through different biological barriers, and communicate with recipient cells by releasing and transporting cargos.

The contents released from sEVs, mostly being exosomes, derived from MSCs originating from different tissues are not identical and influence their potential bioactivity. For example, CD9, CD81, CD44, and CD90 are expressed commonly on the membrane of all MSC-derived sEVs. However, bone marrow MSC-derived sEVs express CD71 and CD166, human umbilical cord MSC-derived sEVs express CK8 and HLA-II, while HLA-I and HLA-ABC are present on the membrane of adipose tissue MSC-derived sEVs[22]. Hence, they exhibit differential effects on the same disease or cell model. For example, MSC-derived sEVs from the bone marrow and umbilical cord decreased cell proliferation and suppressed tumor growth, whereas adipose tissue MSC-derived sEVs enhanced tumor cell proliferation[23]. The sEVs content also varies based on the microenvironment to which MSCs are exposed to[24]. Over 4000 gene products, miRNAs, and nearly 2000 proteins have been detected and identified in the MSC-derived sEV cargo[25,26].

The role of MSC-derived sEVs was explored initially in a mouse model of myocardial ischemia/reperfusion injury[27]. In kidney injury models, MSC-derived sEVs showed improvement in renal function through the transport of miRNA[28]. In animal neurodegeneration disease models, MSC-derived sEVs promoted neurogenesis and angiogenesis, reduced neuroinflammation, and facilitated functional recovery (increasing memory improvement and spatial learning)[29]. MSC-derived sEVs were also effective in treating brain injury through suppression of early inflammatory responses or shift of microglial M1/M2 polarization[30,31]. In liver fibrosis models, MSC-derived sEVs protected hepatocytes by inhibiting epithelial-to-mesenchymal transition[32]. MSC-derived sEVs also showed beneficial effects in the treatment of many other disease models, such as graft-versus-host disease[33], type 2 diabetes mellitus[34], tumors[35], and cutaneous wounds[36].

APPLICATION OF MSC-DERIVED SEVS IN OCULAR DISEASES

Corneal diseases

The corneal epithelium covers the outermost part of the cornea, and its integrity forms the foundation of normal corneal function. Trauma, infection, and physical abrasion can cause persistent epithelial defects, a leading cause of vision loss in different ocular surface diseases. While corneal disease treatment and protection have achieved significant progress, wound healing after severe corneal disease or injury remains challenging[37]. In recent years, MSCs were shown to aid corneal surface healing[38]. Samaeekia *et al*[39] evaluated the effect of MSC-derived sEVs on corneal wound healing and showed that human corneal MSC-derived sEVs significantly increased the proliferation of human corneal epithelial cells *in vitro*, and accelerated corneal wound closure in a murine epithelial mechanical injury model(Table 1).

Corneal stroma accounts for 90% of the corneal thickness and is important for the maintenance of corneal transparency. Severe corneal diseases affect the corneal stroma, causing a corneal scar and a significant decline in vision[40]. Currently, the conventional treatment modality is keratoplasty, and the disadvantages, especially immunological rejection, are challenging to avoid or overcome. MSC-based therapy is a promising method in prompting corneal stroma healing, which has been tested in several studies[41,42]. Recent reports showed that MSCs exert their therapeutic effect by secreting sEVs[43]. Shen *et al*[44] reported that the co-culture of corneal stromal cells (CSCs) with MSCs resulted in enhanced viability and proliferative ability along with increased plasticity. Treatment of CSCs with MSC-derived sEVs caused changes in the matrix metalloproteinases and collagen levels of CSCs and promoted extracellular matrix (ECM) synthesis and CSC proliferation. The protective effect might be exerted through promoting CSC transformation into fibroblasts or myofibroblasts. The ECM-promoting activity of MSC-derived sEVs was reported to be similar to that of MSCs, thus highlighting the potential clinical use of MSC-derived sEVs for the treatment of

Table 1 Effects of mesenchymal stem cell derived extracellular vesicles in ocular disorders

Ref.	Origin	Delivery way	Biological function
Yu *et al*[74], 2016	Human umbilical cord derived MSCs	Intravitreal injection	Ameliorate retinal laser injury
Mead *et al*[64], 2017	Human bone marrow derived MSCs	Intravitreal injection	Promote RGC survival in optic nerve crush model
Kuroda *et al*[58], 2017	Human bone marrow derived MSCs	Intravenous injection	Prevent EAU development
Moisseiev *et al*[77], 2017	Human bone marrow derived MSCs	Intravitreal injection	Decrease the severity of retinal ischemia
Bai *et al*[57], 2017	Human umbilical cord derived MSCs	Periocular injection	Inhibit inflammatory cell migration in EAU
Shen *et al*[44], 2018	Rabbit adipose derived MSCs	In vitro	Contribute to the growth and plasticity of corneal stromal cells
Samaeekia *et al*[39], 2018	Human corneal MSCs	Topical application	Accelerate corneal epithelial wound healing
Mead *et al*[67], 2018	Human bone marrow derived MSCs	Intravitreal injection	Promote neuroprotection in glaucoma model
Safwat *et al*[72], 2018	Rabbit adipose derived MSCs	Intravenous, intraocular or subconjunctival injection	Attenuate retina degeneration in diabetic retinopathy
Zhang *et al*[71], 2018	Human umbilical cord derived MSCs	Intravitreal injection	Ameliorate hyperglycemia-induced retinal inflammation
Mathew *et al*[76], 2019	Human bone marrow derived MSCs	Intravitreal injection	Protect retinal cells from cell death in retinal ischemia

MSCs: Mesenchymal stem cells; EVs: Extracellular vesicles.

corneal stromal damage[44].

Corneal endothelium, regulating stromal hydration level and maintaining corneal deturgescence, covers the posterior corneal surface[45]. The loss of endothelial cells will lead to stromal edema and severe vision loss[46]. Recently, MSCs as a potential therapeutic cell source for corneal endothelial diseases were also reported[47,48]. However, MSCs exerted the therapeutic effects on endothelial cell defect mainly through direct differentiation, and no application of MSC-derived EVs has been reported so far.

Our previous study demonstrated that MSC administration was effective in prolonging corneal allograft survival and exerted therapeutic effect against corneal allograft rejection[49,50]. Recently, we found MSC-derived sEVs acted similarly as MSCs in corneal allograft rejection (unpublished data).

Autoimmune uveitis

Autoimmune uveitis is a type of autoimmune disease involving the uveal tract and retina. It is one of leading global causes of visual disability due to severe clinical complications, including cataract, glaucoma, and retinal damage[51]. Systemic or local administration of corticosteroids combined with immunosuppressive drugs is the traditional treatment protocol for autoimmune uveitis. However, severe adverse effects limit their long-term use in the clinic[52]. The experimental autoimmune uveitis (EAU) model is used widely to understand the mechanism and new treatment options for non-infectious uveitis[53]. Our previous study showed MSCs strikingly ameliorate EAU both in mice and rats[54-56]. Recently, we proved that periocular injection of sEVs derived from umbilical cord MSCs reduced EAU severity by reducing leukocyte infiltration in the eyes of EAU rats. The *in vitro* migration of inflammatory cells such as neutrophils, NK cells, and $CD4^+$ T cells was inhibited by MSC-derived sEVs, indicating that the sEVs exert their therapeutic effect at least partially by the inhibition of leukocyte migration. The study showed the possible clinical utility of MSC-derived sEVs for the treatment of autoimmune uveitis[57]. The other study also demonstrated that MSC-derived sEVs could prevent EAU development and suppress Th1 and Th17 development in mice[58].

Glaucoma

Glaucoma is a group of optic neuropathies characterized by the degeneration of retinal ganglion cells (RGCs) and the axons. Degeneration of RGCs results in altered optic disc appearance and visual field loss[59]. Among vision disorders, glaucoma is second to cataract and also a leading global cause of irreversible vision loss. It was estimated that in 2020 the number of people with open-angle glaucoma and angle-closure glaucoma would be nearly 79.6 million[60]. Currently, ocular hypotensive

drops, laser treatment, and surgery are used to lower intraocular pressure; however, they are insufficient to rescue damaged RGCs[61]. Therefore, utilizing the neuroprotective effects of MSCs, they were shown to be effective in promoting RGCs survival in different animal models[62-64]. The MSC-derived sEVs were also tested in glaucoma models recently to avoid the potential side-effects of MSC administration. In the rodent optic-nerve crush model, the thickness of the retinal nerve fiber layer (RNFL) decreased significantly. Mead *et al*[65] showed that intravitreal injection of MSC-derived sEVs preserved RNFL thickness as measured by OCT and positive scotopic threshold response (pSTR) measured by ERG. Greater than 50% of RGC function in MSC-derived sEVs treated retina was preserved, which indicated that sEVs could protect RGC from death along with retaining their function. The Ago2 knockdown reduced microRNA quantity within the sEVs and decreased sEVs neuroprotective and neuritogenesis abilities, thus indicating the dependence of the therapeutic effect on microRNA rather than protein. DBA/2J mouse is a rodent genetic model of glaucoma. In another study, MSC-derived sEVs were injected intravitreally into DBA/2J mice once a month, from 3 mo to 1 year of age. In the treated group, the number of RGCs was higher at 12-mo and had reduced axonal damage. Concerning the RGC function, pSTR amplitudes were measured by ERG, and the pSTR amplitudes in the treated group were higher at 6-mo, but not at 9- or 12-mo, which indicated that MSC-derived sEVs might prevent RGC functional decline at an early stage, but not at late stage[66,67].

Retinal diseases

Idiopathic macular hole: An idiopathic macular hole is a common fundus disease, which causes severe vision impairment or blindness. The primary treatment is pars plana vitrectomy, and the visual recovery depends on the closure state of the hole and the function of residue photoreceptor cells in the macular area. Current treatment to achieve an ideal prognosis is challenging, especially for large or refractory holes. We previously reported a pilot clinical study, in which seven patients underwent vitrectomy combined with intravitreal injection of MSCs or MSC-derived sEVs. Among the seven patients, six achieved closure of macular holes, and five patients achieved a satisfactory improvement of visual acuity. In one patient, an epiretinal fibrotic membrane formed after MSC injection and a second surgery was performed to remove the membrane, and sEVs therapy was shown to be safer and easier to perform than MSC therapy[68].

Diabetic retinopathy: Diabetic retinopathy (DR) is currently the leading cause of vision loss and blindness in working-age people. Patients are usually asymptomatic until severe vision decline occurs in the late disease phase[69]. Blindness due to DR is preventable but irreversible and poses a substantial economic burden on the family and society. It is estimated that the blindness caused by DR will reach 3.2 million in 2020[2]. Laser therapy, anti-vascular epithelial growth factor (VEGF) agents, and vitrectomy were usually used to treat diabetic retinopathy. However, not all patients respond well to current therapies[70]. A study conducted by Zhang *et al*[71] showed that intravitreal injection of MSC-derived sEVs into the vitreous of streptozotocin (STZ) induced diabetic rats, effectively reduced the expression of inflammatory markers and adhesion molecules. MSC-derived sEVs reversed the increased expression of HMGB1 and its downstream target proteins in retinas of diabetic rats. Consistent with the *in vivo* results, the MSC-derived sEVs suppressed the inflammatory response in high glucose-stimulated human retinal epithelial cells and highlighted the critical role of microRNA126 in inflammatory regulation. The sEVs derived from microRNA126-transfected MSCs inhibited HMGB1 signaling pathway more effectively to reduce inflammation in diabetic retinopathy[71]. In another study, MSC-derived sEVs were injected by different routes (intravenous, subconjunctival, and intraocular) into rabbits with STZ-induced diabetes, and the results showed that both subconjunctival and intraocular injection of MSC-derived sEVs could protect retinal tissue structure from damage, while intravenous injection failed to ameliorate DR progression. The authors also showed an association of decreased microRNA222 expression in retinal tissues with extensive hemorrhage and severe retinal injury. MSC-derived sEVs mediated transfer of microRNA222 resulted in increased microRNA222 expression level and enhanced regenerative retinal changes[72].

Retinal injury: Retinal damage caused by ischemia, infection, or physical injury leads to photoreceptor cell degeneration or death, as well as severe vision loss. No effective neuroprotective drugs are available in the clinic to restore the damaged cells. Our research group showed that intravenous MSC transplantation was effective in alleviating photoreceptor damage[73], and further studies demonstrated that intravitreal injection of MSC-derived sEVs resulted in reduced photoreceptor

apoptosis and protection of visual function, a protective effect comparable to that of MSCs. *In vitro* experiments showed that MSC-derived sEVs could reduce heat injury-induced retinal cell loss by downregulating MCP-1[74]. We also demonstrated recently that subretinal injection of MSC-derived sEVs exhibited therapeutic effect in rat retinal detachment model by inhibiting inflammatory cytokine secretion, reducing apoptosis, and activating autophagy[75]. In a rodent ischemia-reperfusion model, intravitreal injection of MSC-derived sEVs increased retinal functional recovery after ischemic injury. After intravitreal injection, a large number of sEVs were observed in ischemic retina and were concentrated in RGCs and microglial cells. The injected sEVs could be detected in the vitreous humor up to four weeks after administration[76]. In another study of a murine oxygen-induced retinopathy model, Moisseiev *et al*[77] showed that intravitreal injection of MSC-derived sEVs decreased the severity of retinal ischemia. *In vitro* experiments showed that pretreatment of R28 cells with sEVs could protect cells against oxygen and glucose deprivation conditions.

MSC-DERIVED SEVS AS DRUG DELIVERY SYSTEM IN OPHTHALMOLOGY

With lipid bilayer membrane to protect their cargo from degradation, sEVs can travel a long distance and even traverse through biological barriers to the target cells to transfer biological message. Therefore, they are natural carriers for the transport of proteins, lipids, or RNAs to recipient cells with high biocompatibility[20], and are utilized in basic research for drug or other bioactive substance delivery[78]. MSCs are a rich source of sEVs, and MSC-derived sEVs, which have many beneficial effects for many diseases, are ideal for drug delivery and were used in studies of many diseases[12,79-81].

The nanometer size of MSC-derived sEVs facilitates their transport after intravitreal injection across the retina and choroid. Our data showed that after both periocular and intravenous injection, sEVs reach the retina rapidly (unpublished data). In contrast to the MSCs, the MSC-derived sEVs, do not cause vitreous opacity, immunologic rejection, or proliferative vitreous retinopathy[68,76]. Therefore, they could be an alternative drug delivery option for ocular disease treatment. The therapeutic substances could be loaded into sEVs by two methods: One by loading high doses of the selective therapeutic drug into MSCs and collecting the secreted sEVs, and the other is to load sEVs directly through co-culture or electroporation. Owing to the advantages of EV-based therapy, the use of MSC-derived sEVs as nanocarriers loaded with proteins, miRNAs, or other drugs hold promise for the treatment of refractory ocular disorders.

CONCLUSION

Recently, several studies showed the critical role of MSC-derived sEVs in treating ophthalmic diseases. They are also ideal nanocarriers to deliver drugs because of their high biocompatibility, bi-lipid membrane structure, and small size. With increasing evidence of their therapeutic efficacy, it is promising to transform MSC-derived sEV based therapy into clinic for treating ocular diseases in the future.

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REVIEW

Gut commensal bacteria, Paneth cells and their relations to radiation enteropathy

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Abstract

In steady state, the intestinal epithelium forms an important part of the gut barrier to defend against luminal bacterial attack. However, the intestinal epithelium is compromised by ionizing irradiation due to its inherent self-renewing capacity. In this process, small intestinal bacterial overgrowth is a critical event that reciprocally alters the immune milieu. In other words, intestinal bacterial dysbiosis induces inflammation in response to intestinal injuries, thus influencing the repair process of irradiated lesions. In fact, it is accepted that commensal bacteria can generally enhance the host radiation sensitivity. To address the determination of radiation sensitivity, we hypothesize that Paneth cells press a critical "button" because these cells are central to intestinal health and disease by using their peptides, which are responsible for controlling stem cell development in the small intestine and luminal bacterial diversity. Herein, the most important question is whether Paneth cells alter their secretion profiles in the situation of ionizing irradiation. On this basis, the tolerance of Paneth cells to ionizing radiation and related mechanisms by which radiation affects Paneth cell survival and death will be discussed in this review. We hope that the relevant results will be helpful in developing new approaches against radiation enteropathy.

Key words: Gut commensal bacteria; Paneth cell; Radiation enteropathy; Epithelial homeostasis; Gut immunity; Intestinal defense

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Core tip: In healthy individuals, Paneth cells restrict the overgrowth of commensal bacteria in the gut while killing luminal pathogenic bacteria by secreting antimicrobial peptides. Such a property protects crypt intestinal stem cells against bacterial infection, thus ensuring epithelial homeostasis in steady state. Among the active pool of intestinal stem cells, apoptosis commonly occurs as a result of ionizing irradiation. Nevertheless, the intestinal epithelium will recover its integrity after sublethal irradiation. On this basis, the mechanism by which Paneth cells provide growth signals for intestinal stem cells to facilitate epithelial regeneration is easy to understand, whereas the automatic recovery of irradiated intestine from sublethal irradiation is perplexing. Being challenged with luminal bacteria, the degranulation of Paneth cells can be stimulated in a cholinergic- or inflammatory-substance-dependent manner. Then, Paneth cells can perform an antibacterial function that influences the inflammatory milieu in irradiated intestine. Therefore, radiation-induced intestinal bacterial dysbiosis can be managed.

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INTRODUCTION

Ionizing irradiation is indeed a useful tool for treating malignant tumors. In current guidelines, radiation therapy is highly recommended for local rectal cancers with indications after preoperative or postoperative evaluations[1]. However, the standard target volume includes the iliac lymph drainage area, thus enabling a portion of the small intestine and colon to be irradiated unavoidably[2]. Clinically, the gut is regarded as an early responding organ to ionizing irradiation, and acute enteritis commonly occurs during treatment[3]. Although acute injuries can be self-limited, more severe lesions, such as intestinal obstruction, bleeding or perforation, potentially increase their morbidities in the postirradiation period among some patients, thus poorly affecting their quality of life[3].

It has been well accepted that radiation-induced intestinal injury is an independent disease, which is termed as radiation enteropathy (RE). The pathogenesis of RE is indeed complicated, and several factors are involved in this process[3]. First, radiation-induced DNA damage occurs at the initial stage of RE. As is known, the intestinal epithelium represents one tissue with fast self-renewing capability in humans, thus enabling the epithelium to be compromised by ionizing irradiation[4]. However, the cells that form intestinal tissues differ in their radiation sensitivities. For example, smooth muscle cells are more resistant to ionizing irradiation than lymphocytes or endothelial cells partially due to their inactive cell cycle[5]. Moreover, the large bowel is well tolerant to ionizing irradiation compared to the small bowel[6,7]. Apart from the potential differences between the small and large bowels in their histological structures, several other factors also account for radiation sensitivity determination. Herein, commensal bacteria have emerged as critical candidates because they function in shaping host immunity along with strengthening intestinal epithelial homeostasis[8,9]. In clinical practice, most colorectal cancer (CRC) patients undergoing radiation therapy bear tumor burdens. Critically, intestinal bacterial dysbiosis has been proven in gut carcinogenesis, as has its promotion of tumor progression[10,11]. Moreover, radiation itself is able to potentiate intestinal bacterial dysbiosis as well[8]. In this regard, although radiation therapy induces in-field tumor shrinkage, it should be argued whether radiation-induced intestinal bacterial dysbiosis further aggravates the immunological milieu, which potentially increases the risk of local or distant CRC relapse. If so, radiation-induced intestinal bacterial dysbiosis will enable radiation therapy to be contraindicated in CRC patients. In fact, it is well known that several types of cells in the gut can produce antimicrobial substances, such as secretory IgA (sIgA) by B cells or plasma cells and antimicrobial peptides by epithelial cells. Herein, Paneth cells are specialized epithelial cells of the small intestine, which provide a wider range of secretions than other epithelial cells in this process. In this regard, we hypothesize that Paneth cells are critical in regulating microbial ecology

postirradiation.

COMMENSAL BACTERIA AND GUT RADIATION SENSITIVITY

Experts in radiation-associated fields have long understood the importance of commensal bacteria in the pathogenesis of RE. To elaborate on this issue, some landmark studies should be mentioned here. Several decades ago, McLaughlin *et al*[12] reported that germfree mice were more resistant to whole-body irradiation (WBI) than conventional mice, thus confirming specific roles of commensal bacteria in determining host radiation sensitivity. Afterwards, Potten[13] identified that the numbers of crypt apoptotic cells did not differ within six hours postirradiation when using doses from 1 Gy to 10 Gy. Likewise, Beck *et al*[14] found that either 6 Gy or 14 Gy could induce a significant reduction in the number of goblet cells at the third day postirradiation, suggesting no discrimination between these doses in damaging goblet cells. Nevertheless, it is widely observed that mice can recover from sublethal irradiation even though they lack foreign interventions. To support this view, basic research revealed that intestinal injuries could be repaired automatically if irradiated using doses from 6 Gy to 12 Gy, whereas greater than 15 Gy led to an irreversible breakdown of the epithelium[15]. Moreover, although 0.01 Gy is enough to induce apoptosis in a portion of Lgr5-positive intestinal stem cells (ISCs)[16], doses less than 6 Gy barely impair epithelial structures[15]. In this case, what is the force in discriminating the biological effects between lethal and sublethal irradiation? In fact, a previous study reported that SCID mice could survive no more than two weeks if they were irradiated using doses larger than 5 Gy[17], suggesting the participation of adaptive immunity in controlling the tolerance of hosts to radiation. In general, it has been determined that ionizing irradiation can affect host immunity. After extensive exploration, it is gradually deduced that radiation therapy affects host damage and repair processes by regulating the balance between effector T (Teff) cells and regulatory T (Treg) cells or by altering the numbers of other lineage-derived promoters or suppressors infiltrating into lesioned sites[18]. According to this concept, several strategies should become potential candidates for RE treatment, such as regenerative medicine by using mesenchymal stem cells, which exhibit capacities for activating host repair responses[19]. In contrast to immunomodulatory effects, the beneficial implications of stem cell therapy in intestinal bacterial dysbiosis are rarely reported. To resolve this imbalance, bacteria-supportive care (BSC) can be used for RE because several lines of evidence from clinical trials have indicated the therapeutic efficacies of probiotics, prebiotics and symbiotics[8]. Therefore, commensal bacteria play critical roles in determining intestinal radiation sensitivity[20].

COMMENSAL BACTERIA AND GUT Th17/Treg BALANCE POSTIRRADIATION

As mentioned above, intestinal commensal bacteria shape the host immunity. Herein, small intestinal bacterial overgrowth occurs as a result of radiation[21], thus enabling the immune milieu within irradiated sites to be altered reciprocally. In this process, Treg cells and their counterparts, Th17 cells, should be highlighted here because the mutual restriction between Treg and Th17 cells certainly impacts the prognosis in various diseases, especially in autoimmune diseases[22]. In the gut, Treg and Th17 cells can be induced from $CD4^+$ naïve T cells by luminal commensal bacteria. In steady state, the human colon contains higher frequencies of commensal bacteria than the small intestine[23]. Herein, polysaccharide (PSA)–producing *Bacteroides fragilis* (*B. fragilis*) are mainly distributed in the colon, and these bacteria primarily exert the function of inducing Treg cell generation in colonic laminar propria (LP)[24]. By contrast, Th17 cells show peak numbers in the LP of the small intestine both in humans and mice[25]. Then, these cells are redistributed into other sites to defend against bacterial infection[26]. In mice, the terminal ileum was reported to contain the highest numbers of *segmented filamentous bacteria* (*SFB*) and *cytophaga-flavobacterium-bacteroidetes* (*CFB*), which specifically induced Th17 cell generation[27,28]. In contrast to mice, although commensal bacteria accounting for Th17 induction in the human gut are still unclear so far, species including enterotoxigenic *B. fragilis* and *Bifidobacterium adolescentis* are able to induce Th17 cell generation from the gut of germfree mice[25,29], while colonizing mice with feces from inflammatory bowel disease (IBD) patients also induces colonic accumulation of Th17 cells[30]. Likewise, fecal microbiota

transplantation from irradiated conventional mice into germfree mice predisposes the recipients to colitis, demonstrating that such fecal bacteria are critical agents in increasing intestinal sensitivity to radiation[31]. Nevertheless, an important question should be raised here, proposing whether intestinal bacterial dysbiosis occurrence relies on a threshold dose? To this end, it is known that intestinal bacterial dysbiosis occurs secondary to epithelial injuries because the intestinal epithelium exerts selection pressures on the gut composition of commensal bacteria by secreting antibacterial substances[32]. As previously reported, genetic depletion of the IL-17 receptor (IL-17R) resulted in a dramatic loss of α-defensins, which specifically led to the overgrowth of *SFB*[33]. Normally, IL-17R is widely expressed by intestinal epithelial cells[34]. However, radiation-induced incomplete epithelium enables IL-17R protein levels to be reduced. On this basis, intestinal tissue will be attacked by excessive *SFB*, while the infiltrated Th17 cells will become pathogenic due to high levels of Th17-polarized cytokines, such as IL-1β, IL-6 and IL-23 in lesioned sites[31,35]. However, such cytokine milieus antagonize the generation and immunosuppressive function of Treg cells[35]. Moreover, *in vitro* studies showed that irradiation using 6 Gy potentiated TRAF6 reductions in pancreatic cancer cells[36]. Originally, the expression of TRAF6 by intestinal dendritic cells (DCs) is critical for gut immune tolerance induction because intestinal DCs induce Treg cell generation by producing IL-2[37]. Conversely, 10 Gy was reported to be able to induce a significant accumulation of Treg cells in irradiated intestine, whereas these cells were impotent in immunosuppression[38]. In that way, the above results indicate that ionizing irradiation seems to establish a paradigm that favors Th17 cells rather than Treg cells. However, a previous study showed that high dose rate irradiation differed in its effect on TRAF6 expression by tumor cells compared to low dose rate irradiation[39]. At least two approaches may have different impacts on Treg cell generation in the gut. In fact, several issues remain unknown in this process. For example, which kind of cell is mostly responsible for intestinal bacterial dysbiosis formation during RE pathogenesis? In this situation, will sublethal and lethal irradiation give rise to intestinal bacterial dysbiosis with similar characteristics or exert similar radioimmune responses alternatively? Last, how does a lethal dose cause irreversible injuries or even death among irradiated hosts? These questions should be explored in future work. Nevertheless, it is hopeful that the epithelium will become a therapeutic target[40].

In steady state, DCs are potent in Th17 induction in gut of mice because the T-cell receptor (TCR) recognizes the *SFB* antigen presenting by DCs[28]; Meanwhile, MHC class II molecule on DCs can provide all essential signals for Th17 polarization[41]. Functionally, Th17 cells can stimulate synthesis of α-defensins by epithelial cells depending on IL-17/IL-17R interaction, thus protecting against *SFB* overgrowth in gut lumen[33]. However, under the irradiated condition, epithelial injuries will augment the local concentrations of IL-1β and IL-6[31,35], which functionally upregulate expression of gene encoding IL-23[35,42]. By binding with IL-23 receptor (IL-23R) on Th17 cells, IL-23 is able to stimulate Th17 cell expansion[35]. Herein, both IL-23R/IL-22 loop and IL-23/IL-17 loop are able to increase Th17 cell-mediated immune response[26,43], thus enabling the inflammation in irradiated gut to persist. In this regard, the Th17 cells are pathogenic (Figure 1). Besides, due to epithelial loss, low production of α-defensins will somewhat facilitate *SFB* overgrowth in gut lumen, thus facilitating Th17 induction as well. Collectively, Th17 cell induction will be robust in irradiated gut.

PANETH CELL AND EPITHELIAL HOMEOSTASIS

The gut possesses defensive functions in addition to nutrient absorption. Regarding the composition of the intestinal barrier, the epithelium is a critical portion[40]. In healthy adults, the intestinal epithelium is rapidly renewed, and one turnover takes about 4 to 5 d[44]. Such a capacity not only strengthens epithelial integrity but also establishes an optimal paradigm to avoid the accumulation of genetic mutations, thus protecting the gut against malformation[45]. Herein, ISCs accounts for epithelial homeostasis[44], while in their niches, Paneth cells are specialized feeders due to high secretions of epithermal growth factor (EGF), Wnt3 and Dll1/4 (Notch ligands) to neighboring ISCs[46]. Moreover, Paneth cells are derived from ISCs, and they are distributed in the basement of the crypts of Lieberkühn, tiny invaginations that line the mucosal surface all along the small intestine. The commitment of ISCs into functional Paneth cells is regulated by different signaling pathways, such as the Wnt/Sox9 and Notch/Krüppel-like factor 4 (Klf4) pathways[47]. Herein, the former promotes Paneth cell development, which can be enhanced by high-mobility group A1 (HMGA1) chromatin remodeling proteins[48]. In contrast, the retinoic acid receptor

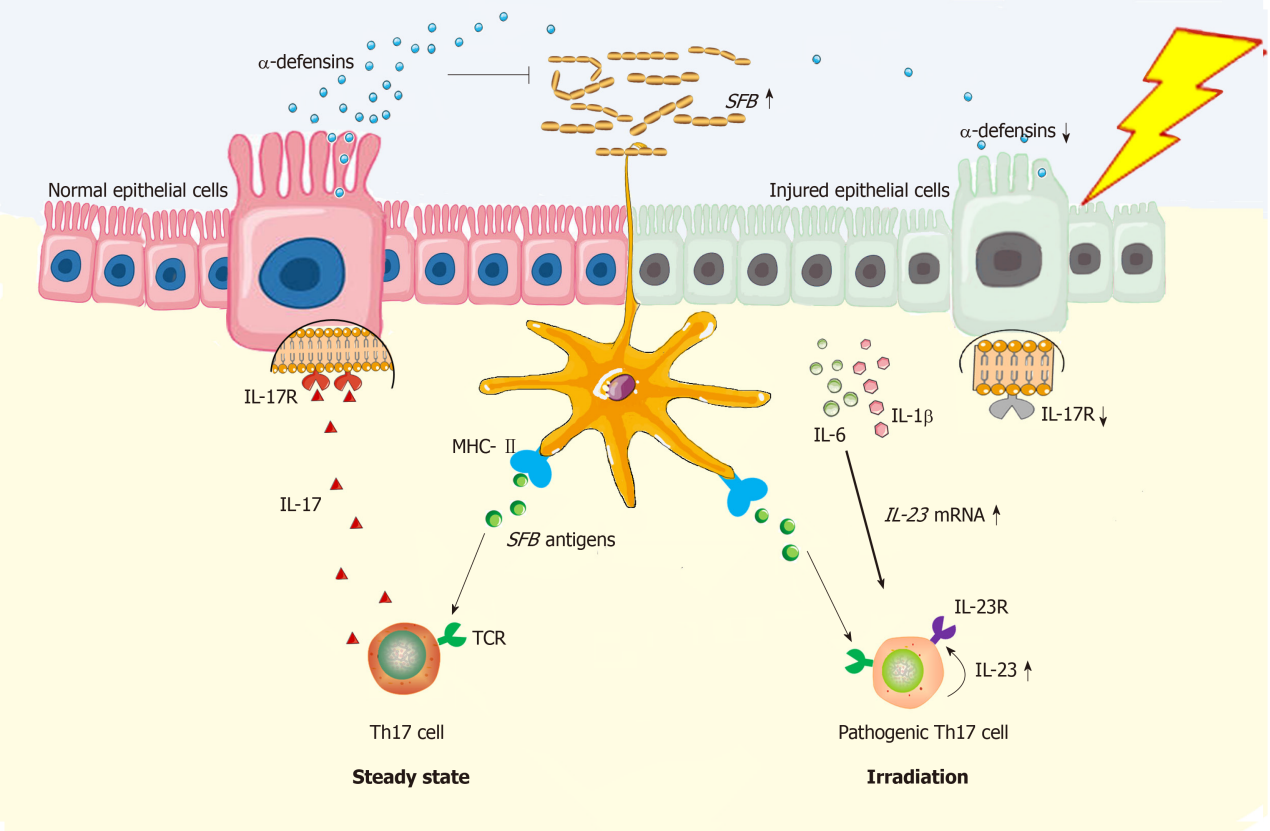

Figure 1 Schema of radiation exposure in pathogenic Th17 cell induction in gut. In steady state, dendritic cells (DCs) are potent in Th17 induction in gut of mice because the T cell receptor recognizes the *segmented filamentous bacteria* (*SFB*) antigen presenting by DCs[28]; Meanwhile, MHC class II molecule on DCs can provide all essential signals for Th17 polarization[41]. Functionally, Th17 cells can stimulate synthesis of α-defensins by epithelial cells depending on interleukin (IL)-17/IL-17R interaction, thus protecting against *SFB* overgrowth in gut lumen[33]. However, under the irradiated condition, epithelial injuries will augment the local concentration of IL-1β and IL-6[31,35], which functionally upregulate expression of gene encoding IL-23[35,42]. By binding with IL-23R on Th17 cells, IL-23 is able to stimulate Th17 expansion[35]. Herein, both IL-23R/IL-22 loop and IL-23/IL-17 loop are able to increase Th17 cell-mediated immune response[26,43], thus enabling the inflammation in irradiated gut to persist. In this regard, Th17 cells are pathogenic. Besides, due to epithelial loss, low production of α-defensins will somewhat facilitate *SFB* overgrowth in gut lumen, thus facilitating Th17 induction as well. Collectively, Th17 cell induction will be robust in irradiated gut. DCs: Dendritic cells; *SFB*: *Segmented filamentous bacteria*; MHC-II: Major histocompatibility complex class II; TCR: T cell receptor; Th17: T helper cell 17; IL-17: Interleukin-17; IL-17R: Interleukin-17 receptor; IL-1β: Interleukin-1β; IL-6: Interleukin-6; IL-22: Interleukin-22; IL-23: Interleukin-23; IL-23R: Interleukin-23 receptor.

α (RARα)/Klf4 pathway antagonizes this process, implying that retinoic acids or their precursor vitamin A serve as inhibitors during Paneth cell development[49]. In fact, several other genes downstream of Wnt and Notch jointly control the equilibrium number of Paneth cells, such as the agonists of Math1 and Gfi1, along with the antagonists of Hes1 and Elf3[47]. Through their actions, the number of Paneth cells in each crypt will be constantly maintained, thus profiting epithelial integrity and disease prevention.

PANETH CELL AND INTESTINAL DEFENSE

Paneth cells feature several characteristics. Unlike absorptive cells or other secretory lineage cells, Paneth cells are not swiftly replaced through epithelial turnover. In mice, the life span of Paneth cells is estimated as two months[46]. Such a long-lived potential ensures the stability of the number of ISCs in each crypt, which relies on Paneth cell peptides in regulating ISC development as well as in defending against luminal microbial attack. Several important peptides with anti-infective functions are derived from Paneth cells, such as α-defensins, β-defensins, regenerating islet-derived protein IIIγ (RegIIIγ), lysozyme, phospholipase A2 (PLA2) and matrix metalloproteinase 7 (MMP7)[50] (Figure 2). These peptides form a defensive network together with other lineages of cells, such as M cells in Peyer's patches (PPs), goblet cells, absorptive cells, and LP innate or adaptive immune cells. For example, goblet cells enable Paneth-cell-derived antimicrobial peptides to be well preserved in the mucus layer[51]. Moreover, α-defensins will acquire antibacterial function if processed by MMP7[52]. In this regard, Paneth cells serve as gatekeepers in the gut.

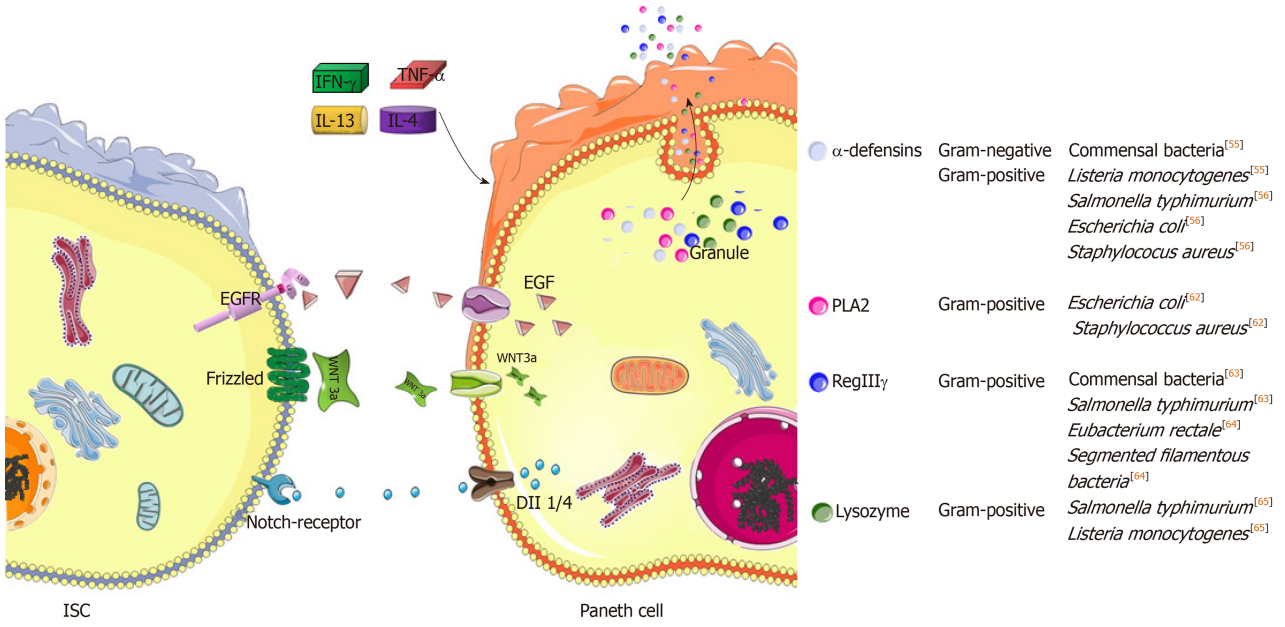

Figure 2 Specific roles of Paneth cells in maintaining epithelial homeostasis and defense. Paneth cells are critical feeders due to their high secretion of epithermal growth factor, Wnt3a or Notch ligands to neighboring intestinal stem cells (ISCs), thus driving ISC expansion[44]. Moreover, several important peptides of antimicrobial functions are generated from Paneth cells[50]. In this situation, several inflammatory cytokines, including interferon-γ, tumor necrosis factor-α, interleukin (IL)-13 and IL-4, will elicit the degranulation of Paneth cells to antagonize luminal bacterial overgrowth. growth factor receptor; Dll1: Delta-like ligand 1; Dll4: Delta-like ligand 4; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; IL-13: Interleukin-13; IL-4: Interleukin-4; PLA2: Phospholipase A2; RegIIIγ: Regenerating islet-derived protein IIIγ.

In fact, the peptides mentioned above enable Paneth cells to possess a wide antimicrobial spectrum. Herein, the degranulation of Paneth cells is one of the most critical events in defending against luminal microbiota. In addition, degranulation can be stimulated by other factors, such as inflammatory cytokines and cholinergic substances[53]. Afterwards, antimicrobial peptides achieve high concentrations on the surface of the epithelium. In general, defensins exert lethal effects on bacteria, fungi and viruses because most defensins can bind to microbes to perforate their membranes, thus leading to microbial death[54] (Figure 2). To this end, Paneth cells mainly rely on α-defensins[50]. In steady state, α-defensins potently restrict the overgrowth of commensal bacteria[55]. In addition, pathogenic bacteria, including *Salmonella typhimurium* (*S. typhimurium*), *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), are sensitive to α-defensins[56]. Likewise, their antigens, including lipid A, lipopolysaccharide (LPS) and lipoteichoic acid (LTA), are able to induce the secretion of α-defensins by Paneth cells reciprocally[56]. Herein, the secretion of α-defensins is stimulated by LPS in a concentration-dependent manner[56]. Moreover, lipid A and LTA are the most common components of gram-negative and gram-positive bacteria, respectively[57], indicating the wide antibacterial spectrum of Paneth cells by using α-defensins. In fact, among six isoforms, α-defensins 5 and 6 are the most important peptides. For example, human α-defensin 5 was tested to preferentially and powerfully defend against *S. typhimurium* infection in mice[55]. In contrast to α-defensin 5, α-defensin 6 seldom exerts bactericidal function in a straightforward manner. Herein, a previous study found that nanonets of α-defensin 6 bound luminal *S. typhimurium* to prevent infection[58]. However, unlike α-defensins, the antibacterial spectra of other peptides are relatively narrow; in particular, RegIIIγ, lysozyme and PLA2 particularly antagonize gram-positive bacteria[50]. Nevertheless, *in vivo* depletions of the α-defensins and RegIIIγ could predispose the mice to spontaneous enteritis and colitis, respectively[59,60]. In this regard, intestinal inflammation is largely attributed to intestinal bacterial dysbiosis occurring as a result of the loss of the bacterial selection pressures by these peptides[55,56,61-65].

PANETH CELL, ANTIMICROBIAL PEPTIDES AND Treg/Th17 BALANCE IN GUT

Although epithelium-derived, Paneth cells serve as critical originators of intestinal inflammation[66]. TNF-α, a prevalent cytokine regulating innate immune responses,

exists in the granules of Paneth cells[67]. Herein, the specific roles of TNF-α in intestinal inflammation have been well documented in several aspects (reviewed in[68]). For example, TNF-α is a pathogenic cytokine that facilitates the pathogenesis of Crohn's disease[69]. In this process, the endoplasmic reticulum (ER) stress of Paneth cells occurs as a result of defects in the recognition of the autophagy-related 16-like1 (ATG16L1) gene, thus impairing cell autophagy[70]. Normally, autophagy in Paneth cells is a central event against *S. typhimurium* infection[71], which potentially increases the intestinal number of Paneth cells as well[72]. In addition, ATG16L1 defects enable the granules of Paneth cells to be abnormal and hamper degranulation, proposing that ATG16L1 is essential for Paneth cell differentiation[70,71]. In fact, ATG16L1 is required for Treg cell induction in the gut[24]. Conversely, in response to *S. typhimurium* infection, the absence of ATG16L1 will increase the levels of IL-1β and IL-6 in the terminal ileum and cecum[71], the sites of which are inhabited by Th17 cells. In this regard, Paneth cells regulate Treg/Th17 balance by relying on ATG16L1 (Figure 3).

In addition to immunological participation, the antimicrobial peptides of Paneth cells also predispose the host to immune tolerance[57]. Herein, a previous study confirmed that enteric α-defensins 5 and 6 could be detected in the medullary epithelial cells of the human thymus[73]. In this situation, α-defensins 5 and 6 acted as self-reactive antigens, which could be specifically recognized by autoreactive $CD4^+$ or $CD8^+$ subpopulations[73]. Normally, through the action of negative selection in the thymus, the leakage of such cells into the periphery can be radically prevented. However, defects in AIRE, a key autoimmune regulator that normally controls the thymic expression of a set of genes encoding tissue-specific antigens, including α-defensins 5 and 6[74], will result in Th17 cell generation and spontaneous enteritis due to autoaggression targeting Paneth cells[73]. In contrast, mice overexpressing genes encoding human α-defensin 5 significantly reduced their gut frequencies of *SFB* and the numbers of Th17 cells[55]. These results further confirm the role of Paneth cells in restricting Th17 cell induction; moreover, the presence of AIRE is certainly required for Paneth cell survival. In fact, AIRE also exerts a negative impact on Paneth cell survival. For example, AIRE is required for the development of invariant natural killer T (iNKT) cells, which potentiate the degranulation of Paneth cells in an IFN-γ-dependent manner[75,76]. Herein, Paneth cells will rapidly and completely lose their granules in response to IFN-γ, which impairs the survival of Paneth cells as well[76]. In this regard, either the excessive activation or absence of AIRE seems to potentially reduce the number of Paneth cells.

Here, it is essential to mention Paneth cell degranulation in response to cytokines (Table 1). In line with IFN-γ, TNF-α, IL-13 and IL-4 cytokines induce Paneth cell degranulation as well[77,78]. In contrast to agonists of toll-like receptor (TLR) 3 & 9, oral administrations of TLR4 & 5 ligands were tested to induce Paneth cell degranulation in a TNF-α-dependent manner, thus confirming the specific role of TNF-α in this process[78]. Additionally, IL-13 receptor α1 (IL-13Rα1) is profoundly expressed by Paneth cells. The IL-13/IL-13Rα1 interaction is able to activate STAT6 and PI3K/Akt, thus upregulating the expression of lysozymes and MMP7[77]. Moreover, IL-4 is a member of the iNKT-secreted cytokines[79], further enhancing the effect of iNKT cells on inducing Paneth cell degranulation. As is known, iNKT cells are positive for CD1d, an MHC class-I-like molecule responsible for foreign antigen presentation. In addition to this function, Paneth cell degranulation is CD1d-dependent. Herein, a previous study confirmed that both cholinergic stimulation by using pilocarpine and *E. coli* infection were not able to reduce the crypt lysozyme intensities under the CD1d-absent condition[80]. Likewise, CD1d depletion also rendered the granules of Paneth cells abnormal in several aspects, mainly alterations in size, morphology and oligosaccharide content[80]. Furthermore, *SFB* overgrowth occurred if CD1d was depleted[80]. This result indicates that CD1d is required for the biosynthesis of functional α-defensins by Paneth cells because commensal *SFB* are sensitive to these peptides[33]. Similarly, when being colonized with *E. coli* or *S. aureus*, CD1d-deficient mice exhibited increased gut frequencies of these bacteria along with their translocation into the periphery compared to wild-type mice[80], further confirming the role of CD1d in mediating the protection against bacterial infections. In this process, CD1d is not a unique factor, and some other immune cells are able to assist Paneth cell degranulation or antimicrobial peptide secretion in addition to iNKT cells. Commonly, Th1 and group 1 innate lymphoid cells (ILC1s) are potent in producing IFN-γ, while IL-4 and IL-13 are typical cytokines produced by Th2 or ILC2[81,82]. Moreover, IL-4 and IL-13 potentiate the secretion of retinoic acids by intestinal DCs[83], thus potentially resulting in Paneth cell reduction by antagonizing the development process[47]. In addition to this function, retinoic acids preferentially induce the commitment of naïve T cells into Treg cells rather than Th17 cells[83]. Hereby, retinoic acids will synergize with α-defensin 5 in preventing the excessive generation of intestinal Th17 cells. Alternatively, in response to IL-23, intestinal $TCRV\gamma7^+\gamma\delta$

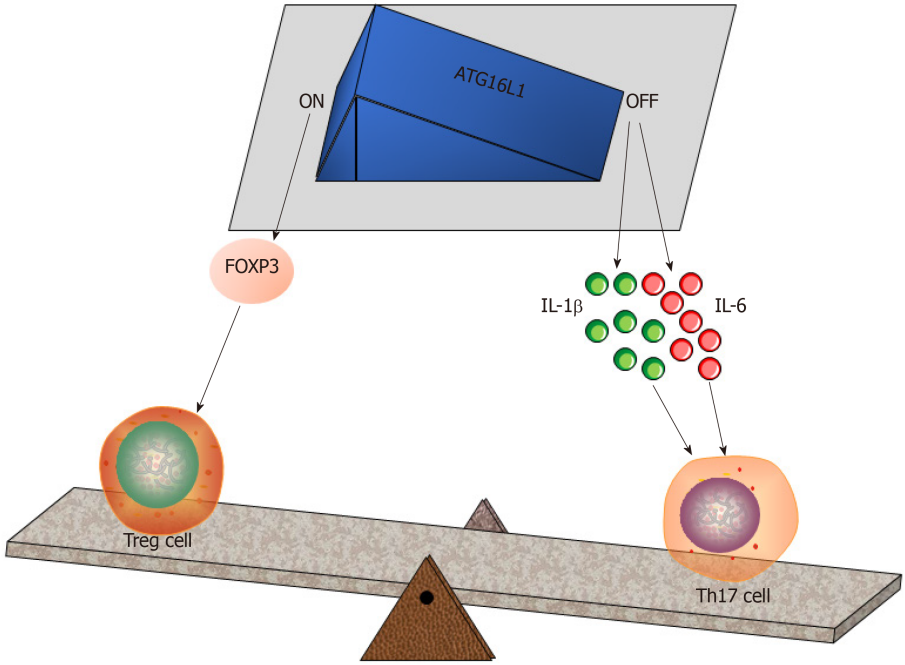

Figure 3 Autophagy-related protein 16 like protein 1 regulates the intestinal balance between Treg and Th17 cells. The autophagy-related protein 16 like protein 1 (ATG16L1)-FOXP3 axis plays a vital role in Treg cell induction[24]. Conversely, a deficiency of ATG16L1 enables Paneth cell differentiation to be hampered, while this situation will increase interleukin (IL)-1β and IL-6 in the gut[71], thus promoting Th17 commitment. Treg: Regulatory T cell; Th17: T helper cell 17; IL-1β: Interleukin-1β; IL-6: Interleukin-6; ATG16L1: Autophagy-related protein 16 like protein 1.

intraepithelial lymphocytes (IELs) can produce IL-22, which is able to induce angiogenin 4 secretion by Paneth cells to clear *S. typhimurium* infection[84]. Herein, IL-23 and IL-22 are also classified as Th17-type cytokines[85]. In this regard, Th17 cells potentially improve the anti-infective function of Paneth cells.

BACTERIAL DYSBIOSIS AND GUT CARCINOGENESIS

Although Paneth cells ensure the security of ISCs in steady state, the antimicrobial dysfunction of Paneth cells potentially enables ISCs to be attacked by luminal invaders. To address the importance of the gut microbiota in this process, it is documented that germfree mice with double depletions of genes encoding Rag2 and TGF-β exhibit no sporadic intestinal tumors, in contrast to conventional mice with the same phenotype[86]. This finding suggests that intestinal commensal bacteria independently induce gut carcinogenesis even though they lack adaptive immunity. Recently, several studies revealed that carcinogenesis in the human gut occurred as a result of intestinal bacterial dysbiosis[10,11]. In this situation, the feces could be used for human CRC screening[87,88]. Actually, it is well accepted that infection-associated chronic inflammation will drive the genomic instability of cells[61,89]. Herein, ISCs serve as major sources orchestrating gut malformation. In the process of phenotype conversion from ISCs to CRC stem cells, mutations or epigenetic alterations will accumulate in the genome[90]. In the gut, several commensal bacteria are capable of eliciting carcinogenesis. For example, the genotoxic island of polyketide synthase (pks) from the pathogenic strains of *E. coli* is required for CRC induction[91]. Instead of exerting genomic toxicity, the nonpathogenic *E. coli* K-12 strain potentiates the oncogenicity of colon epithelial cells by improving the activities of NF-κB and β-catenin[92]. Moreover, albeit indirectly, *Enterococcus faecalis* (*E. faecalis*) confers colon epithelial cells with oncogenicity by using their polarized macrophages, which induce cellular transformation along with gene mutation[93]. In addition to tumor induction, some other bacteria promote CRC progression. Herein, *Fusobacterium nucleatum* (*F. nucleatum*) improves the proliferative and invasive capacities of CRC cells by upregulating their miRNA-21 expression[94]. In addition, the Fab2 protein released by *F. nucleatum* will bind to TIGIT (T cell immunoglobulin and ITIM domain) on human T or NK cells, thus reducing their anticancer effects[95]. Similarly, enterotoxins from *B. fragilis* will increase the expression of c-Myc, an important oncogene driving CRC progression[96]. Moreover, enterotoxigenic *B. fragilis* induces Th17 cell generation[29].

Table 1 Summary of the factors regulating Paneth cell degranulation

Sort	Object	Pathway	Effect
Cytokines	IFN-γ	IFN-γ-dependent manner[75]	Impairment of the survival of Paneth cells[76]
	TNF-α	TNF-α-dependent manner	Paneth cell degranulation[78]
	IL-13	STAT6 and PI3K/Akt	Upregulation of the expressions of lysozyme and MMP7[77]
	IL-4	Antagonizing the development process[47]	Enhancing the effect of iNKT cells[79]
TLR	TLR3 / 9	TLR9 and TLR3 dependent manner	Paneth cell degranulation[78]
	TLR4 / 5	TNF-α-dependent manner	Paneth cell degranulation[78]
CD1d	iNKT cells	CD1d-dependent	Reducing the crypt lysozyme[80]
			Mediating the protection against bacterial infections[80]
Cholinergic	Pilocarpine and *E. coli*	CD1d-dependent	Crypt lysozyme intensities[80]

IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; IL-13: Interleukin-13; STAT6: Signal transducers and activators of transcription 6; PI3K/Akt: Phosphatidylinositol 3 kinase/protein kinase B; MMP7: Matrix metalloproteinase 7; IL-4: Interleukin-4; iNKT: Invariant nature killer T cell; TLR: Toll-like receptor; *E. coli*: *Escherichia coli*.

However, the infiltration of massive Th17 cells in tumors predicts a poor prognosis in CRC patients[97]. To a certain extent, Th17 cells direct CRC progression by producing IL-22, which potently activates STAT3 to increase the "stemness" of tumor cells[98]. Moreover, IL-22 elicits transient ER stress in intestinal epithelial cells[99]. In concert with ATG16L1 defects, IL-22-induced epithelial necrosis will be aggravated due to robust activation of STING-dependent type I interferon (IFN-I) signaling, thus inducing excessive TNF-α production[99]. As a result, intestinal bacterial dysbiosis will be further enhanced due to the augmented defects in the epithelial barrier.

THE MISSION OF PANETH CELLS IN RADIATION ENTEROPATHY

In steady state, Paneth cells are critical in protecting against intestinal bacterial dysbiosis. Here, the mission of Paneth cells postirradiation should be discussed. Foremost, autophagy will occur in Paneth cells in response to 9.25 Gy γ-irradiation[100]. Meanwhile, α-defensin 4 increases its production by Paneth cells[101]. Concerning the radiation sensitivity of Paneth cells, two previous studies confirmed that the phenotype conversion from the reserve pool of ISCs (Bmi1-positive) to the active pool of ISCs (Lgr5-positive) was a manifestation upon automatic recovery of the intestinal epithelium from radiation-induced damage, probably due to the interchange of their niche signals[102-104]. In this regard, it is reasonable to conceive that Paneth cells mediate this process, not only because they act as niche cells of ISCs but also because the numbers of Paneth cells are not significantly reduced in murine guts when doses are no more than 12 Gy[102,103]. Conversely, if doses are larger than 15 Gy, Paneth cells will dramatically lose their numbers[15]. Herein, it has been documented that ISCs are normally found in small intestine of mice albeit complete elimination of Paneth cells by genetic depletion of *Math1*[105]. However, *Math1*-mutant miniguts halt their growth *in vitro*[105]. This case can be translated into *Wnt3*-mutant miniguts as well, suggesting the essential role of Paneth cells in support of ISC expansion[105]. Moreover, conditional depletion of the gene encoding Frizzled-5, the receptor of Wnt3, will inactivate the MMP7/defensin maturation programme in Paneth cells of adult mice, suggesting the role of Wnt3 in eliciting antimicrobial function of Paneth cell[106]. Therefore, radiation-induced lethal effect on Paneth cells potentially impairs ISC regeneration due to loss of Paneth cell-derived niche signals and antimicrobials. In fact, Paneth cells are more resistant to ionizing irradiation than ISCs. The long-lived potential of Paneth cells is certainly attributed to their high genetic stability, while the survival of Paneth cells after irradiation can be controlled by their capacity to repair DNA lesions through nonhomologous end-joining[107]. A recent study found that mutation in Tyr4046 of DNA-dependent protein kinase, catalytic subunit with synchronous Trp53 depletion, significantly increased the sensitivity of mice to 8 Gy of WBI because such a genetic background hampered the survival of Paneth cells postirradiation[107]. To this end, it is proposed that Paneth cells press the button of controlling RE pathogenesis. In this

process, intestinal bacterial dysbiosis occurs postirradiation, thus eliciting a pro-inflammatory milieu in lesioned gut[21]. In this context, the production of antimicrobial peptides by Paneth cells can be increased to overcome intestinal bacterial dysbiosis[101]. Hence, maintaining Paneth cell survival postirradiation appears to be critical for epithelial regeneration.

THE STRATEGY AGAINST RADIATION ENTEROPATHY

In terms of RE treatment, current clinical strategies are mainly selected according to the standard classification of intestinal toxicity reported by the Radiation Therapy Oncology Group (RTOG). Herein, the principle of treatment for Grade 1 or 2 toxicity occurring during radiation therapy mainly includes anti-inflammation; symptomatic care for nausea, vomiting or diarrhea; and nutritional support[3]. Concerning Grade 3/4 toxicities or more severe complications, multidisciplinary diagnosis and treatment are highly recommended[3], yet the relevant strategies seldom support the regeneration of lesioned intestine. In fact, it has been presented that the histological features of RE overlap with those of IBD[3,8]. Herein, MSCs have been demonstrated to be effective in patients with Crohn's disease[108]. However, at the time of this writing, clinical trials with the purpose of managing RE using MSCs have still not been carried out. Nevertheless, clinical cases of prostate cancer with complications related to radiation-induced rectal injury could be well managed by using MSCs[109]. In this management, the efficacies of MSCs mainly include relieving pain, stanching bleeding or repairing fistula, indicating the perspective of such a stem cell therapy[109]. Additionally, TNF-α monoclonal antibody (infliximab) achieves good therapeutic effects in IBD patients. Therefore, this drug should be effective in RE, but this deserves further investigation. In parallel, some other issues should be addressed, particularly prior to RE treatment in clinical settings. For example, antibiotics are recommended for RE treatment only if infection occurs. As is known, long-lasting use of antibiotics will induce intestinal bacterial dysbiosis, but it is still unclear whether short-term use of antibiotics improves radiation-induced intestinal bacterial dysbiosis. Nevertheless, antibiotics exhibited potential in delaying CRC progression in an animal model[110]. This finding means that antibiotics serving as candidates for BSC therapy may be available in the defense against CRC-related intestinal bacterial dysbiosis. As mentioned above, BSC using prebiotics or probiotics is effective in relieving diarrhea[9]. In fact, BSC has become the hotspot for various diseases. To overcome bacterial dysbiosis, the administration of defensins or omega-3 polyunsaturated fatty acids is also promising in clinical settings[111,112]. Instead of BSC, the reduction in and/or the dysfunction of pathogenic cells will be available choices for RE treatment as well. Herein, RORγt antagonists against Th17 cell commitment were tested to be useful in the IBD model[113]. In this process, RORγt inhibition will potentially improve Treg cell generation because RORγt functionally antagonizes the transcriptional activity of Foxp3[114]. In particular, Th17 cells are pathogenic cells of RE and CRC as well, thus predicting the perspective of RORγt antagonists in these diseases. Collectively, targeting any critical event during RE pathogenesis should become a candidate option for RE treatment. For the development of novel treatment targets of RE, related mechanisms deserve further exploration in the future.

CONCLUSION

Pathogenesis of radiation enteropathy is highly associated with intestinal bacterial dysbiosis. Herein, Paneth cells probably control the process of bacterial dysbiosis by using their antimicrobial peptides.

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ORIGINAL ARTICLE

Basic Study

Efficient differentiation of vascular smooth muscle cells from Wharton's Jelly mesenchymal stromal cells using human platelet lysate: A potential cell source for small blood vessel engineering

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Author contributions: Mallis P designed and carried out the whole experimental procedure of this study; Papanagiotou A, Siassos G and Kassi E supervised the differentiation procedures of MSCs into VSMCs; Katsimpoulas M and Kostakis A supervised the decellularization procedures of human umbilical arteries; Michalopoulos E designed, supervised and corrected the whole manuscript; Stavropoulos-Giokas C supervised and approved the whole study.

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Abstract

BACKGROUND

The development of fully functional small diameter vascular grafts requires both a properly defined vessel conduit and tissue-specific cellular populations. Mesenchymal stromal cells (MSCs) derived from the Wharton's Jelly (WJ) tissue can be used as a source for obtaining vascular smooth muscle cells (VSMCs), while the human umbilical arteries (hUAs) can serve as a scaffold for blood vessel engineering.

AIM

To develop VSMCs from WJ-MSCs utilizing umbilical cord blood platelet lysate.

METHODS

WJ-MSCs were isolated and expanded until passage (P) 4. WJ-MSCs were properly defined according to the criteria of the International Society for Cell and Gene Therapy. Then, these cells were differentiated into VSMCs with the use of platelet lysate from umbilical cord blood in combination with ascorbic acid, followed by evaluation at the gene and protein levels. Specifically, gene expression profile analysis of VSMCs for *ACTA2*, *MYH11*, *TGLN*, *MYOCD*, *SOX9*,

of the current study was received from all mother participants.

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NANOG homeobox, *OCT4* and *GAPDH*, was performed. In addition, immunofluorescence against ACTA2 and MYH11 in combination with DAPI staining was also performed in VSMCs. HUAs were decellularized and served as scaffolds for possible repopulation by VSMCs. Histological and biochemical analyses were performed in repopulated hUAs.

RESULTS
WJ-MSCs exhibited fibroblastic morphology, successfully differentiating into "osteocytes", "adipocytes" and "chondrocytes", and were characterized by positive expression (> 90%) of CD90, CD73 and CD105. In addition, WJ-MSCs were successfully differentiated into VSMCs with the proposed differentiation protocol. VSMCs successfully expressed *ACTA2, MYH11, MYOCD, TGLN* and *SOX9*. Immunofluorescence results indicated the expression of ACTA2 and MYH11 in VSMCs. In order to determine the functionality of VSMCs, hUAs were isolated and decellularized. Based on histological analysis, decellularized hUAs were free of any cellular or nuclear materials, while their extracellular matrix retained intact. Then, repopulation of decellularized hUAs with VSMCs was performed for 3 wk. Decellularized hUAs were repopulated efficiently by the VSMCs. Biochemical analysis revealed the increase of total hydroyproline and sGAG contents in repopulated hUAs with VSMCs. Specifically, total hydroxyproline and sGAG content after the 1st, 2nd and 3rd wk was 71 ± 10, 74 ± 9 and 86 ± 8 μg hydroxyproline/mg of dry tissue weight and 2 ± 1, 3 ± 1 and 3 ± 1 μg sGAG/mg of dry tissue weight, respectively. Statistically significant differences were observed between all study groups ($P < 0.05$).

CONCLUSION
VSMCs were successfully obtained from WJ-MSCs with the proposed differentiation protocol. Furthermore, hUAs were efficiently repopulated by VSMCs. Differentiated VSMCs from WJ-MSCs could provide an alternative source of cells for vascular tissue engineering.

Key words: Vascular smooth muscle cells; Decellularized umbilical arteries; Mesenchymal stromal cells; *MYOCD*; Cardiovascular disease; Blood vessels

Core tip: In this study, mesenchymal stromal cells derived from the Wharton's Jelly tissue were differentiated into vascular smooth muscle cells (VSMCs). For this purpose, unlike the current literature, cord blood platelet lysate was used as the key element for the differentiation of mesenchymal stromal cells into VSMCs. Furthermore, the functional evaluation of VSMCs was tested. To do this, human umbilical arteries were decellularized and repopulated with the generated VSMCs.

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INTRODUCTION

Small diameter vascular grafts with inner diameter less than 6 mm are currently applied in various surgical operations, globally[1,2]. Among them, cardiovascular disease (CAD) is estimated to affect more than 18 million people[2]. Indeed, more than 500000 bypass surgeries are performed each year, worldwide[3,4]. Primary therapeutic treatment is the replacement of damaged or obstructed coronary arteries with autologous or synthetic vascular grafts. Both approaches are characterized by several limitations[3,4]. Autologous grafts, such as saphenous vein, are only available in less

than 40% of patients with CAD, and are characterized by significantly altered biocompatibility properties[3,4].

On the other hand, synthetic vascular grafts, derived from expanded polytetrafluorethylene and Dacron, are well applied for large diameter vascular applications, although small diameter graft replacement still requires further clarification[5]. Most of the time, new surgical operations are required for these patients. When more than one vascular conduit is needed, the above therapeutic strategies cannot be applied[6,7]. Moreover, small diameter vascular grafts are required for solid organ transplantation, such as kidney and liver, in order to achieve proper revascularization and nutrient supplementation[7].

Due to the broad use of small diameter blood vessels, alternative sources must be established, thus overcoming the above limitations. Vascular graft engineering, which has attracted great interest from scientific societies, could contribute to this direction[8-10]. Decellularization of vessels and their repopulation with specific cellular populations could produce properly defined tissue engineered grafts[10-12]. For this purpose, human umbilical arteries (hUAs) with inner diameters of 1-4 mm could ideally be decellularized and possibly serve as a vascular scaffold for possible seeding by cellular populations[3,4]. HUAs are contained in human umbilical cord (hUC), a tissue that is discarded after gestation[3,4]. Normally, hUC contains two arteries and one vein that playsignificant roles in fetal blood circulation[13,14]. HUAs are vessels without any branches throughout their entire length, and can be non-invasively isolated from hUCs[15].

To date, several groups have successfully decellularized hUAs, and characterized them as small diameter vascular grafts[3,16-19]. However, the repopulation of these conduits requires further evaluation. Of particular note, these decellularized vascular grafts must be repopulated with vascular cell populations, such as endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), in order to be fully functional[20,21].

In this context, VSMCs are responsible for vasoconstriction and vasodilation, and can switch from a contractile to synthetic phenotype[22]. Contractile VSMCs maintain their functional properties, such as regulation of blood pressure and blood redistribution, in response to biochemical stimuli, and are mostly found in healthy blood vessels[23]. On the other hand, synthetic VSMCs exhibit enhanced proliferation, migration, and osteochondrogenic conversion[22,23]. Synthetic VSMCs are related to vascular pathologies such as inflammation, atherosclerosis and CAD[23]. Between these two states, different genes are expressed in VSMCs. Specifically, under normal conditions, contractile phenotypes of VSMCs is regulated by the expression of *MYOCD*, *ACTA2*, *MYH11* and *TGLN*. On the other hand, under pathological conditions, a switch from a contractile to synthetic phenotype occurs, followed by *SOX9* upregulation[23]. It is known that *SOX9* expression can lead to extracellular matrix (ECM) protein synthesis[23]. Due to their significant role in vessel homeostasis, a strategy to obtain VSMCs that can be used in small diameter vascular graft engineering must be established. Unfortunately, autologous VSMCs are difficult to obtain at desired numbers from mature vessels, and their *in vitro* expansion potential is limited[23]. A large number of research groups has tried to produce VSMCs derived either from mesenchymal stromal cells (MSCs) or from induced pluripotent stem cells (iPSCs) using defined factors[24-28]. Traditional methods rely on the exogenous supplementation of biochemical induction factors[23]. However, these approaches are expensive and could cause endotoxin contamination, while iPSC technology has not been approved by the Food and Drug Administration for broad human use[23]. Taking into consideration the above data, and in order to develop functional small diameter vascular grafts, we introduced an alternative protocol for producing VSMCs from MSCs derived from Wharton's Jelly tissue (WJ-MSCs), which relied on the use of human platelet lysate (PL) from umbilical cord blood (UCB). Previous work in our laboratory conducted in UCB-PL showed significant amounts of several growth factors such as TGF-β1, PDGFA, FGF2, IFN-γ and TNF-α[29]. These growth factors have previously been used extensively in the differentiation process of MSCs to VSMCs by several groups[24-28]. In addition, UCB-PL is free of any animal-derived substances such as prions, peptides and proteins, which can cause zoonotic infections or allergic reactions. In this way, the produced VSMCs might be better tolerated by patients. Also, UCB-PL has exhibited beneficial properties as s supplement for MSC isolation and expansion[29]. The aim of this study was to produce VSMCs from WJ-MSCs using the UCB-PL, in order to serve as a potential source of cells for vascular tissue engineering. Initially WJ-MSCs were isolated, characterized according to the International Society for Cell and Gene Therapy (ISCT) standards[30], and differentiated into VSMCs. In parallel, hUAs were isolated from hUCs, decellularized, and then histologically and biochemically evaluated. Differentiated VSMCs were initially evaluated at the gene and protein levels, and then used for the repopulation of decellularized hUAs. The efficacy of repopulation was defined with both

histological and biochemical assays.

MATERIALS AND METHODS

Isolation of hUAs and WJ tissue

The hUCs (n = 10) used in this study for the isolation of hUAs and WJ tissues were derived from normal and caesarian deliveries with gestational ages 38-40 wk. Each hUC was accompanied by informed consent. The informed consent was signed by the mothers a few days before delivery, was in accordance with the Helsinki declaration, and conformed with the ethical standards of the Greek National Ethical Committee. The overall study has been approved by our Institution's ethical board (Reference No. 1440. November 20th, 2018). The hUCs were delivered to the Hellenic Cord Blood Bank in less than 48 h, and proceeded immediately to the isolation of hUAs and WJ tissues. Briefly, the hUCs were rinsed in phosphate buffer saline 1× (PBS 1×, Sigma-Aldrich, Darmstadt, Germany) for removal of excessive blood and blood clots. Sterile instruments were used for the isolation of hUAs and WJ tissues. Then, hUAs and WJ tissue were kept separately in 15 mL polypropylene falcon tubes (BD Biosciences, California, United States) at 4 °C until further use.

WJ-MSCs isolation and expansion

WJ tissues (n = 10) were trimmed, and small round segments were plated in 6-well plates (Costar, Corning Life, Canton, MA, United States) with 1 mL of standard culture medium in each well. The standard culture medium consisted of α-Minimum Essentials Medium (α-MEM, Gibco. Life Technologies, Grand Island, NY, United States) supplemented with 15% v/v Fetal Bovine Serum (Gibco. Life Technologies, Grand Island, NY, United States), 1% v/v Penicillin-Streptomycin (Gibco. Life Technologies, Grand Island, NY, United States) and 1% L-glutamine (Gibco. Life Technologies, Grand Island, NY, United States). Then, the plates were transferred in a humidified atmosphere with 5% CO_2 at 37°C, and left for 18 d. The standard culture medium was changed once per week. After 18 d, trypsinization of cells was performed using 0.025% Trypsin-EDTA (Gibco, Life Technologies, Grand Island, NY, United States) solution for 10 min at 37°C. The cells were replated in 75 cm^2 cell culture flasks (Costar, Corning Life, Canton, MA, United States). When confluency was observed (mostly after 10 d), the cells were trypsinized again and plated into 175 cm^2 cell culture flasks (Costar, Corning Life, Canton, MA, United States). The same procedure was repeated until cells reached P4.

Growth kinetics and cell viability of WJ-MSCs

The WJ-MSCs (n = 10) used in this study were evaluated for their total cell number, cell doubling time (CDT), cumulative population doubling (PD) and cell viability until reaching P4. WJ-MSCs were plated at a density of 2×10^5 cells in 75 cm^2 cell culture flasks (Costar, Corning Life, Canton, MA, United States), expanded and measured in each passage.

The CDT was calculated based on the following equation:

$$CDT = \log_{10}(N/N0) \div \log_{10}(2) \times T$$

The PD of each passage was determined with the following equation and added to the PD of the previous passages to obtain the cumulative PD.

The PD was estimated based on the equation:

$$PD = \log_{10}(N/N0) \div \log_{10}(2)$$

Where N was the number of cells at each passage, No was the number of initially plated WJ-MSCs and T was the culture duration in hours.

Total cell counting and viability estimations were performed using an automated system (Countess II FL Automated Cell Counter, Thermo Fischer Scientific, MA, United States) with Trypan blue stain (Invitrogen, ThermoFischer Scientific, MA, United States).

Additionally, cell viability was determined with Crystal Violet assays (ab232855, Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions. Briefly, 2×10^5 MSCs from passages 1 to 4 were added to each well of a 96-well plate (Costar, Corning Life, Canton, MA, United States). DMSO vehicle was used as a background control, and doxorubicin was added in a well containing MSCs as a proliferation inhibitor. All MSC samples were cultured for 72 h at 5% CO_2 in a humidified atmosphere. Then, the culture medium was removed, cultures were washed and 50 μL of Crystal Violet was added to each well for 20 min at room temperature. Then, washing was performed and repeated four times, the 96-well plate was air dried, followed by the addition of 100 μL solubilization solution to each well. Finally, absorbance at 595 nm was measured, and the % cytotoxicity was calculated

based on the determination of the optical density (OD) using the following equation:

% Cytotoxicity = [OD (DMSO) – OD (Sample)] ÷ OD (DMSO) × 100%

Where OD (DMSO) was the DMSO control after background correction and OD (Sample) was the OD of the sample after background correction.

WJ-MSCs trilineage differentiation assay

WJ-MSCs P4 (n = 5) were promoted to differentiate towards "osteocytes", "adipocytes" and "chondrocytes". For "osteogenic" and "adipogenic" differentiation, WJ-MSCs were plated at a density of 1 × 10^5 cells into 6-well plates (Costar, Corning Life, Canton, MA, United States). When the cells reached 80% confluency, differentiation was performed. WJ-MSCs were differentiated into "osteocytes" or "adipocytes" using the STEMPRO® Osteogenesis (ThermoFischer Scientific, Massachusetts, United States) or STEMPRO® Adipogenesis (ThermoFischer Scientific, Massachusetts, United States) Differentiation kits, according to the manufacturer's instructions. Evaluation of "osteogenic" or "adipogenic" differentiation was conducted with the Alizarin Red S (Sigma-Aldrich, Darmstadt, Germany) and Oil Red-O (Sigma-Aldrich, Darmstadt, Germany) histological stains, respectively. Alizarin Red S specifically stains calcium depositions, while Oil-Red-O stains produced lipid droplets. In addition, Alizarin Red S quantification assays (ECM815, Millipore, Darmstadt, Germany) were used to determine Ca^{2+} deposits, according to the manufacturer's instructions. "Chondrogenic" differentiation was performed in 3D cultures generated from seeded WJ-MSCs at a density of 5 × 10^5 cells in 15 mL polypropylene falcon tubes (BD Biosciences, CA, United States). Then, 2 mL of chondrogenic differentiation medium was added to each 3D culture. Then, 3D cultures were placed in a humidified atmosphere with 5% CO_2 at 37°C for 4 wk. The chondrogenic differentiation medium consisted of high glucose D-MEM (Sigma-Aldrich, Darmstadt, Germany) supplemented with 0.01 mmol dexamethasone (StemCell technologies, Vancouver, BC, Canada), 40 g/mL ascorbic acid-2 phosphate (StemCell Technologies, Vancouver, BC, Canada), 10 ng/mL transforming growth factor-β1 (TGF-β1, Sigma-Aldrich, Darmstadt, Germany), and 100 μL of insulin-transferin selenium liquid medium 100× (ITS 100×, StemCell technologies, Vancouver, BC, Canada). After 4 wk of differentiation, 3D cultures were fixed with 10% v/v neutral formalin buffer (Sigma-Aldrich, Darmstadt, Germany), dehydrated, paraffin-embedded and sectioned into 5 μm slices. "Chondrogenic" induction of WJ-MSCs was evaluated with Alcian blue (Sigma-Aldrich, Darmstadt, Germany) staining, which is specific for cartilage proteoglycans.

Furthermore, chondrogenic differentiation was further assessed with the Bern Score. Specifically, three independent observers evaluated chondrogenic differentiation based on a previously published protocol[31].

Immunophenotypic analysis

Immunophenotypic analysis was performed in WJ-MSCs (n = 3) P4 as has been proposed by ISCT. Specifically, WJ-MSCs were analyzed for the expression of CD90 (Thy-1), CD105 (endoglin), CD73 (ecto-5′ nucleotidase), CD29 (integrin subunit), CD19 (pan-B-cell marker), CD31 (pan-EC marker), CD45 (pan-hematopoietic cell marker), CD34 (hematopoietic stem cell marker), CD14 (TLR-4 co-receptor), CD3 (T-cell co-receptor), HLA-DR (HLA class II antigen) and HLA-ABC (HLA class I antigen). Monoclonal antibodies against CD90, HLA-ABC, CD29, CD19, CD31 and CD45 were conjugated with fluorescein isothiocyanate (FITC), while CD105, CD73, CD44, CD3, CD34 and HLA-DR were conjugated with phycoerythrin. All monoclonal antibodies were purchased from Immunotech (Immunotech, Beckman Coulter, Marseille, France). Immunophenotypic analysis was performed with a Cytomics FC 500 flow cytometer (Beckman Coulter, Marseille, France), coupled with CXP Analysis software (Beckman Coulter, Marseille, France).

VSMCs differentiation protocol

WJ-MSCs P3 (n = 5) were used for differentiation into VSMCs. Specifically, 75 × 10^5 cells were seeded into 75 cm^2 cell culture flasks (Costar, Corning Life, Canton, MA, United States) until they reached 80% confluency. Then, brief washes with PBS 1× (Sigma-Aldrich, Darmstadt, Germany) were performed. After the total removal of the remaining buffer, WJ-MSCs were cultivated in DMEM high glucose (Gibco. Life Technologies, Grand Island, NY, United States) with 20% v/v UCB-PL and 30 μmol ascorbic acid (Sigma-Aldrich, Darmstadt, Germany) for a time period of 3 wk. The above medium will be referred to as VSMC differentiation medium, and was changed biweekly. The PL was produced from UCB units that did not meet the criteria for hematopoietic stem cell isolation of the Hellenic Cord Blood Bank, and the whole procedure was performed as has been previously reported[29]. WJ-MSCs P3 (n = 3) cultured with the standard culture medium served as negative controls in this study.

Gene expression profiling

Gene expression profiling of differentiated VSMCs was performed with reverse transcription polymerase chain reaction (PCR), followed by PCR and gel electrophoresis. Total mRNA was isolated from VSMCs (n = 5) using the TRI-reagent (Sigma-Aldrich, Darmstadt, Germany) according to the manufacturer's instructions. Quantity and quality of the isolated mRNA were determined photometrically. Then, 800 ng of total mRNA was transcribed into DNA using the Omniscript reverse transcription kit (Qiagen, Hilden, Germany). PCR was performed with Taq PCR Master Mix (Cat No 201443, Qiagen, Hilden, Germany) on a Biometra T Gradient Thermoblock PCR Thermocycler (Biometra, Gottingen, Germany). The final volume of each PCR reaction was 20 μL.

The amplification program used in the current study involved the following steps: initial denaturation at 95°C for 15 min, denaturation at 94°C for 30 s, annealing at 60-62°C for 90 s and final extension at 72°C for 3 min. A total of 35 cycles was used for the amplification of genomic DNA. The specific primers used for the current assay are listed in Table 1. All PCR products were analyzed by electrophoresis on a 1% w/v agarose gel (Sigma-Aldrich, Darmstadt, Germany). Finally, comparison of gene expression of differentiated VSMCs with undifferentiated WJ-MSCs (n = 5, negative control group) was performed. For gene expression profiling, the following genes were evaluated: *ACTA2, MYH11, TGLN, MYOCD, SOX9, NANOG, OCT4*, and *GAPDH*.

Immunofluorescence of VSMCs

Indirect immunofluorescence against ACTA2 and MYH11 was performed on WJ-MSCs (n = 5) and VSMCs (n = 5). Specifically, WJ-MSCs and VSMCs were seeded at a density of 1 × 10^4 cells on culture slides (Sigma-Aldrich, Darmstadt, Germany) for 48 h. Then, the cells were washed for 1-2 min with PBS 1× (Gibco. Life Technologies, Grand Island, NY, United States) and fixed for 5 min with 10% v/v neutral formalin buffer (Sigma-Aldrich, Darmstadt, Germany). The next step of the assay involved antigen retrieval and blocking of all samples, followed by the addition of monoclonal antibody against human ACTA2 (1:500, Catalog MA1-744, ThermoFischer Scientific, Massachusetts, United States) and MYH11 (1:1000, Catalog MA5-11971, ThermoFischer Scientific, MA, United States). Secondary FITC-conjugated rabbit IgG antibody (1:100, Sigma-Aldrich, Darmstadt, Germany) was added. Finally, DAPI (Sigma-Aldrich, Darmstadt, Germany) stain was added in order for the cell nuclei to become evident, and slides were glycerol mounted. Images were acquired using a LEICA SP5 II fluorescent microscope equipped with LAS Suite v2 software (Leica, Microsystems, Wetzlar, Germany). Furthermore, mean fluorescence intensity of WJ-MSCs and VSMCs was determined using ImageJ 1.46r (Wane Rasband, National Institutes of Health, United States).

Estimation of cell proliferation using ATP assay

Cell proliferation was determined with an ATP assay (MAK190, Sigma-Aldrich, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, 1 × 10^5 WJ-MSCs or VSMCs were plated in 24-well plates (Costar, Corning Life, Canton, MA, United States). The next day, cells were lysed with 100 μL ATP assay buffer. Then, 20 μL of cell lysates were transferred to 96-well plates, followed by the addition of reaction buffer. All samples were incubated for 30 min at room temperature. Finally, the absorbance was measured by a photometer at 570 nm. Determination of ATP concentration was achieved by interpolation to a standard curve. The standard curve consisted of 0 (blank), 5, 10, 20, 50, 100, 150, and 200 nmol standards.

Decellularization of hUAs

The hUAs (n = 10, l = 2 cm) were immediately decellularized after their isolation from hUCs. Briefly, the hUAs were placed in CHAPS buffer (8 mmol CHAPS, 1 mol NaCl and 25 mmol EDTA in PBS 1×, Sigma-Aldrich, Darmstadt, Germany) for 22 h under rotational agitation. Furthermore, hUAs were transferred into SDS buffer (1.2 mmol SDS, 1 mol NaCl and 25 mmol EDTA in PBS 1×, Sigma-Aldrich, Darmstadt, Germany) for another 22 h under rotational agitation. Finally, the vessels were incubated in α-MEM with 40% v/v Fetal Bovine Serum (Sigma-Aldrich, Darmstadt, Germany) at 37°C for 48 h.

Histological analysis of hUAs

The efficacy of the decellularization protocol was evaluated by performing the following histological stains. Non-decellularized (n = 10, l = 2 cm) and decellularized (n = 10, l = 2 cm) hUAs were initially fixed with 10% v/v neutral formalin buffer (Sigma-Aldrich, Darmstadt, Germany), dehydrated, paraffin-embedded and sectioned into 5 μm slices. Hematoxylin and Eosin (H & E, Sigma-Aldrich, Darmstadt,

Table 1 Primer sets for polymerase chain reaction

Gene	Accession number	Forward primer	Reverse primer	Amplicon size
ACTA2	NM_001613	CAGCCAAGCACTGTCAGGAAT	CACCATCACCCCCTGATGTC	182
MYOCD	NM_001146312	CCACCTATGGACTCAGCCTAC	CTCAGTGGCGTTGAAGAAGAG	188
MYH11	NM_022844	CGCCAAGAGACTCGTCTGG	TCTTTCCCAACCGTGACCTTC	129
TGLN	NM_003564	ATGGCACGGTGCTATGTGAG	CCCACCCAGATTCATCAGCG	71
SOX9	NM_000346	AGCGAACGCACATCAAGAC	CTGTAGGCGATCTGTTGGGG	85
NANOG	NM_024865	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG	116
OCT4	NM_001159542	GTGTTCAGCCAAAAGACCATCT	GGCCTGCATGAGGGTTTCT	156
GAPDH	NM_001256799	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG	197

Germany), Sirius Red (SR, Sigma-Aldrich, Darmstadt, Germany) and Toluidine blue (TB, Fluka, Sigma-Aldrich, Darmstadt, Germany) were applied for the evaluation of cell and nuclear remnants, collagen and proteoglycan preservation in hUAs, respectively. Images were acquired using a Leica DM L2 light microscope (Leica Microsystems, Weltzar, Germany) and processed with ImageJ 1.46r (Wane Rasband, National Institutes of Health, United States).

In addition, indirect immunofluorescence against ACTA2 and MYH11 in combination with DAPI staining was applied. Non-decellularized and decellularized hUAs were fixed with 10% v/v formalin buffer (Sigma-Aldrich, Darmstadt, Germany), dehydrated, blocked and sectioned into 5 μm slices. Then, the slides were deparaffinized, rehydrated and blocked, followed by the addition of the monoclonal antibody ACTA2 (1:500, Catalog MA1-744, ThermoFischer Scientific, MA, United States) or MYH11 (1:1000, Catalog MA5-11971, ThermoFischer Scientific, Massachusetts, United States). Secondary FITC conjugated antibody (1:100, Sigma-Aldrich, Darmstadt, Germany) was added. Finally, DAPI (Sigma-Aldrich, Darmstadt, Germany) stain was added, the slides were glycerol mounted and processed for examination under the fluorescent microscope (LEICA SP5 II fluorescent microscope, Leica, Microsystems, Wetzlar, Germany).

Biochemical analysis

Evaluation of the decellularization procedure involved the quantification of collagen, sulphated glycosaminoglycans and DNA of non-decellularized (n = 10, l = 2 cm) and decellularized dry tissue samples (n = 10, l = 2 cm). Quantification of collagen was performed based on the measurement of hydroxyproline content, and relied on the use of the Hydroxyproline Assay Kit (MAK008, Sigma-Aldrich, Darmstadt, Germany) according to the manufacturer's instructions. Quantification of sGAGs initially involved the digestion of samples in 125 μg/mL papain buffer at 60°C for 12 h, followed by the addition of dimethylene blue dye (Sigma-Aldrich, Darmstadt, Germany). The concentration of sGAGs in each sample was estimated through interpolation to a standard curve. Chondroitin sulphate standards of 12 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL and 150 μg/mL were used for the standard curve.

DNA content was estimated in non-decellularized (n = 10, l = 2 cm) and decellularized (n = 10, l = 2 cm) hUAs after their digestion in lysis buffer. Lysis buffer contained 0.1 mol Tris pH 8, 0.2 mol NaCl, 5 mmol EDTA in PBS 1× supplemented with 20 mg/mL Proteinase K (Sigma-Aldrich, Darmstadt, Germany). Total DNA of each sample was isolated, eluted in 100 μL DNAse-free water (Sigma-Aldrich, Darmstadt, Germany) and photometrically quantified at 260 nm to 280 nm.

Repopulation of hUAs with VSMCs

Decellularized hUAs (n = 30, l = 1 cm) were repopulated with VSMCs under static seeding conditions. For this purpose, decellularized hUAs were placed into 24-well plates (Costar, Corning Life, Canton, MA, United States) with VSMCs at an average number of 3×10^5 cells. Then, 1 mL of VSMC differentiation medium was carefully added in each well. The plates were transferred to a humidified atmosphere with 5% CO_2 at 37°C for 3 wk. After the proposed time period, evaluation of the repopulation results was performed with H & E, in the same way as referred to in the previous sections (Histological analysis). In addition, indirect immunofluorescence against MYH11 in combination with DAPI staining was applied. Repopulated hUAs with VSMCs were fixed with 10% v/v formalin buffer (Sigma-Aldrich, Darmstadt, Germany), dehydrated, blocked and sectioned into 5 μm slices. Then, the slides were deparaffinized, rehydrated and blocked, followed by the addition of monoclonal antibody against MYH11 (1:1000, Catalog MA5-11971, ThermoFischer Scientific,

Massachusetts, United States). Secondary FITC conjugated antibody (1:100, Sigma-Aldrich, Darmstadt, Germany) was added. Finally, DAPI (Sigma-Aldrich, Darmstadt, Germany) stain was added, the slides were glycerol mounted and processed for examination under a fluorescent microscope (LEICA SP5 II fluorescent microscope, Leica, Microsystems, Wetzlar, Germany).

In addition, immunohistochemistry against Ki67 and proliferating cell nuclear antigen (PCNA) was performed on the repopulated hUAs. Briefly, the slides were deparafinized, rehydrated and the whole procedure was performed using the Envision Flex Mini Kit, high pH (Cat # K802421-2J, Agilent Technologies, CA, United States) according to the manufacturer's instructions. Ki67 (1:50, Cat # 305504, Biolegend, San Diego, United States) and PCNA (1:100, ab 18197, Abcam, Cambridge, United Kingdom) were used for the detection of cell proliferation in the repopulated hUAs. Decellularized hUAs served as a negative control both in the indirect immunofluorescence and immunohistochemistry assays. Furthermore, hydroxyproline and sGAG contents were quantified in the repopulated hUAs (n = 20) in the same way as referred to in the previous section (Biochemical Analysis). Decellularized hUAs (n = 20) served as the negative control group. Finally, VSMC proliferation in the repopulated hUAs was further confirmed and assessed by DNA quantification. HUAs were digested with lysis buffer as referred to previously (Biochemical Analysis), and the DNA amount was quantified photometrically at 260 nm to 280 nm.

Determination of ATP-ADP Ratio in repopulated hUAs

The evaluation of VSMC viability in repopulated hUAs was performed with an ADP/ATP assay kit (MAK189, Sigma -Aldrich, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, the light intensity, which is specific to intracellular ATP concentration, is produced with the following reaction:

ATP + D-Luciferin + O_2 → oxyluciferin + AMP + PP_i + CO_2 + light

In the next step, the ADP is converted to ATP, which further reacts with D luciferin. The second light intensity determines the total ADP and ATP concentration. The light intensity was measured using a luminometer (Lucy 1, Anthos, Luminoskan, Labsystems) and expressed as the number of relative light units (RLUs). The determination of ADP/ATP ratio is performed using the following formula:

ADP/ATP ratio = (RLU C – RLU B) ÷ RLC A

Where RLU A is the initial luminescence measurement after the addition of the ATP reagent. RLU B is the luminescence measurement after 10 min of incubation, and RLU C is the measurement of light intensity after the addition of ADP reagent.

Briefly, decellularized hUAs (n = 10) were digested with collagenase IV (Sigma-Aldrich, Darmstadt, Germany), and the lysates were supplemented with a-MEM. In addition, VSMCs at a density of 2 × 10^5 cells were seeded in 24-well plates with 1 mL of decellularized hUA lysates. Finally, cell cultures were incubated for a total of 7 d in a humidified atmosphere and 5% CO_2. VSMCs with 10% v/v DMSO were used as a positive control group, while VSMCs with non-decellularized hUAs served as a negative control group for this study. The determination of ADP/ATP ratio was performed at the end of each day.

Statistical analysis

GraphPad prism v 6.01 (GraphPad Software, San Diego, CA, United States) was used for statistical analysis. Comparison of collagen, sGAG and DNA content between samples was performed using Kruskal Wallis and Mann Whitney tests. Statistically significant difference was considered when the P value was less than 0.05. Indicated values were presented as mean ± SD.

RESULTS

Characteristics of isolated WJ-MSCs

WJ-MSCs were successfully isolated and expanded from hUCs. Specifically, spindle-shaped cells were isolated from all samples. Furthermore, WJ-MSCs retained their morphological features until P4 (Figure 1A). To better determine the WJ-MSCs characteristics, total cell number, CDT, cumulative PD and cell viability were measured. Total cell number of WJ-MSCs at P4 surpassed 12 × 10^6 cells (Figure 1B). CDT and cumulative PD at P4 were 36 ± 3 h and 6 ± 1, respectively (Figure 1C and D). Cell viability of WJ-MSCs, determined either with Trypan blue or Crystal Violet in passages 1 to 4, was above 90% (Figure 1E and Supplementary Figure 1).

WJ-MSCs of P4 successfully differentiated towards "osteogenic", "adipogenic" and "chondrogenic" lineages, as indicated by the histological stains (Figure 1F).

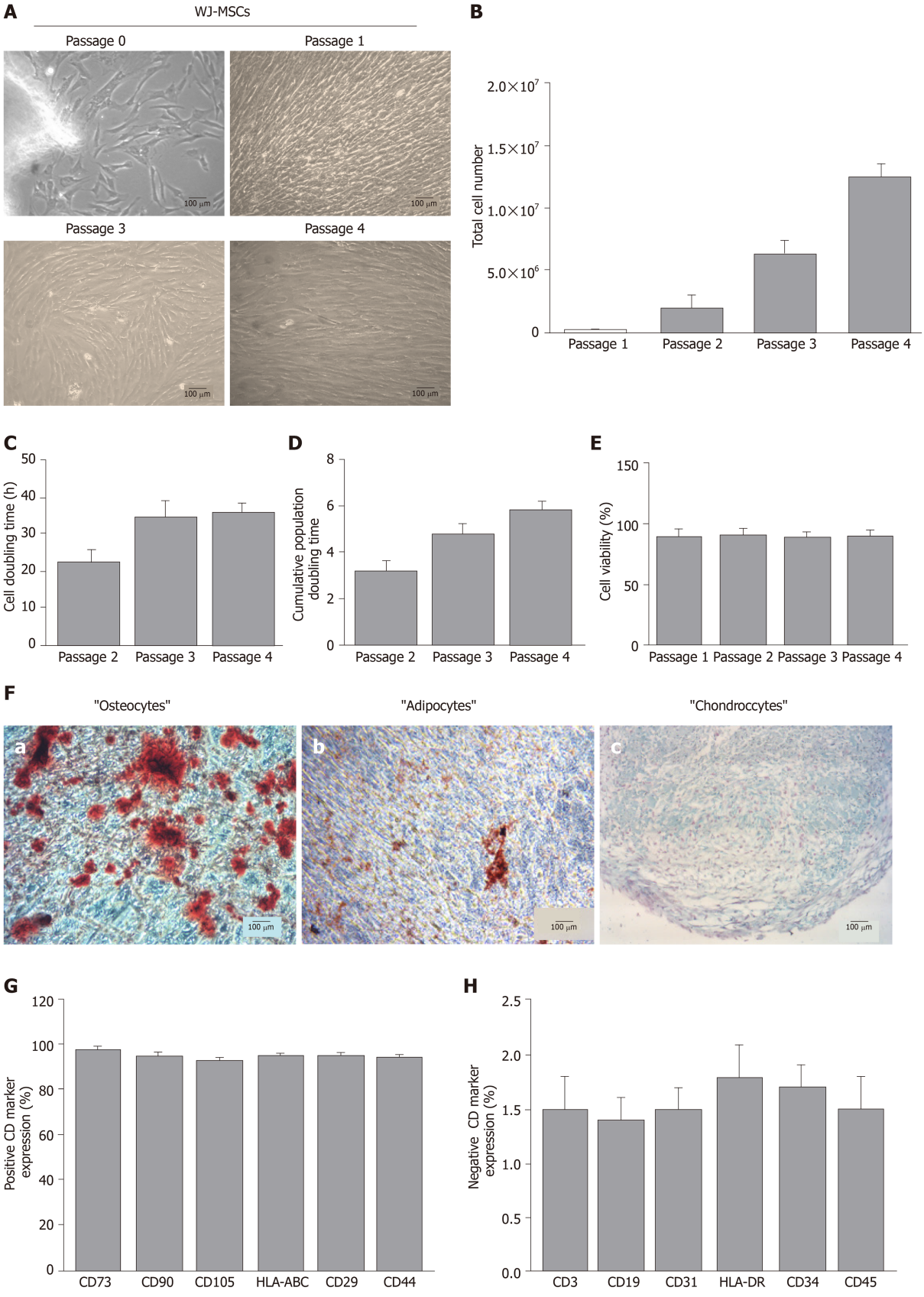

Figure 1 Evaluation of mesenchymal stromal cells derived from the Wharton's Jelly. A: Morphological features of mesenchymal stromal cells derived from the Wharton's Jelly tissue (WJ-MSCs) from P0 to P4 (A-a to A-d); B-F: Determination of total cell number (B), cell doubling time (C), cumulative PD (D) and cell viability (E) of WJ-MSCs from P0 to P4. Evaluation of tri-lineage differentiation capability of WJ-MSCs into "osteocytes" (F-a), "adipocytes" (F-b) and "chondrocytes" (F-c) as indicated by Alizarin Red-S, Oil-Red-O and Alcian blue, respectively. G, H: Positive (G) and negative (H) expression of CD markers in WJ-MSCs based on flow cytometric analysis. Images A-a to A-d and F-a to F-c were obtained with original magnification 10× and 100 μm scale bars. WJ-MSCs: Mesenchymal stromal cells derived from the Wharton's Jelly tissue.

Specifically, after 4 wk of "osteogenic" differentiation conditions, cells were characterized by calcium deposits, which stained red with Alizarin Red S (Figure 1F). Moreover, "osteocytes" produced Ca^{2+} deposits more than 0.9 mmol (Supplementary Figure 2). Under "adipogenic"-inducing conditions, WJ-MSCs successfully produced lipid vacuoles, which were visible with Oil Red O staining (Figure 1F). In regards to "chondrogenic" differentiation, 3D cultures of WJ-MSCs were characterized by the production of proteoglycan aggregations, as was indicated by Alcian blue and Bern Scores (Figure 1F and Supplementary Table 1). Flow cytometry analysis showed that WJ-MSCs were characterized by their positive expression of up to 92% for CD73, CD90, CD105, CD29, HLA-ABC and CD44, and by their negative expression below 2% for CD3, CD19, CD31, HLA-DR, CD34 and CD45 (Figure 1G and 1H).

Evaluation of VSMC differentiation

WJ-MSCs were successfully differentiated into VSMCs with the proposed differentiation protocol. Treatment of WJ-MSCs with UCB-PL in combination with ascorbic acid resulted in cells with more elongated spindle-shaped morphologies compared with undifferentiated cells (Figure 2A). VSMC markers such as *ACTA2, MYOCD, MYH11* and *TGLN* were expressed at the mRNA level in differentiated cells (Figure 2B). In addition, differentiated VSMCs also expressed *SOX9* (Figure 2B). Pluripotency-related genes, such as *NANOG* and *OCT4*, were not expressed in differentiated VSMCs (Figure 2B). On the other hand, untreated WJ-MSCs did not express the above markers, with the only exception being *ACTA2* (Figure 2B). WJ-MSCs successfully expressed pluripotency-related genes such as *NANOG* and *OCT4* (Figure 2B).

The estimation of WJ-MSC and VSMC proliferation was performed using the ATP assay. Both cellular populations were characterized by equal amounts (17 ± 3 nmol and 18 ± 3 nmol) of ATP (Figure 2C).

Further determination of successful VSMC differentiation involved the performance of indirect immunofluorescence against ACTA2 and MYH11 in combination with DAPI stain (Figure 2D and Supplementary Figure 3). Early and late VSMC-specific genes such as ACTA2 and MYH11 were successfully expressed after 3 wk (Figure 2D and Supplementary Figure 3). Untreated WJ-MSCs were characterized by low expression of ACTA2, while totally lacked MYH11 expression (Figure 2D and S3).

In addition, mean fluorescence intensity of ACTA2 and MYH11 was determined in both WJ-MSCs and VSMCs (Supplementary Figure 4). Statistically significant differences were observed in the ACTA2 ($P < 0.01$) and MYH11 ($P < 0.01$) expression levels of WJ-MSCs and VSMCs (Supplementary Figure 4), indicating the successful differentiation of VSMCs. The above results were in accordance with gene expression analysis, demonstrating the differentiation efficiency.

Decellularization of hUAs

HUAs were successfully decellularized as showed by histological analysis. Decellularized hUAs were characterized by intact ECM, without any cellular or nuclear remnants (Figure 3A). In addition, key specific ECM components, such as collagen and sGAGs, seemed to be preserved according to SR and TB stains, respectively (Figure 3A). SR stains showed that the collagen structure and orientation was preserved (Figure 3A). In addition, TB stains appeared to be less dense in decellularized hUAs compared to non-decellularized vessels. Signal detection of ACTA2, MYH11 and DAPI was evident only in non-decellularized hUAs, confirming the successful decellularization procedure (Figure 3A).

Further evaluation of the decellularization procedure in hUAs involved biochemical analysis, which included the determination of collagen (hydroxyproline), sGAG and DNA contents. Specifically, hydroxyproline content in native hUAs was 93 ± 12 μg hydroxyproline/mg of dry tissue weight, while in decellularized hUAs was 72 ± 10 μg hydroxyproline/mg of dry tissue weight (Figure 3B). Statistically significant differences in hydroxyproline content was observed between non-decellularized and decellularized hUAs ($P < 0.001$). SGAG content was significantly ($P < 0.001$) lower in decellularized hUAs compared to non-decellularized hUAs (Figure 3C). SGAG content in non-decellularized and decellularized hUAs was 5 ± 1 and 2 ± 1 μg sGAG/mg of dry tissue weight (Figure 3C). DNA content was totally eliminated in decellularized hUAs, further confirming the histological results. DNA content in non-decellularized hUAs was 1503 ± 120 ng DNA/ mg of dry tissue weight, and in decellularized hUAs was 41 ± 6 ng DNA/mg of dry tissue weight (Figure 3D). Statistically significant differences were observed in DNA content between non-decellularized and decellularized hUAs ($P < 0.001$).

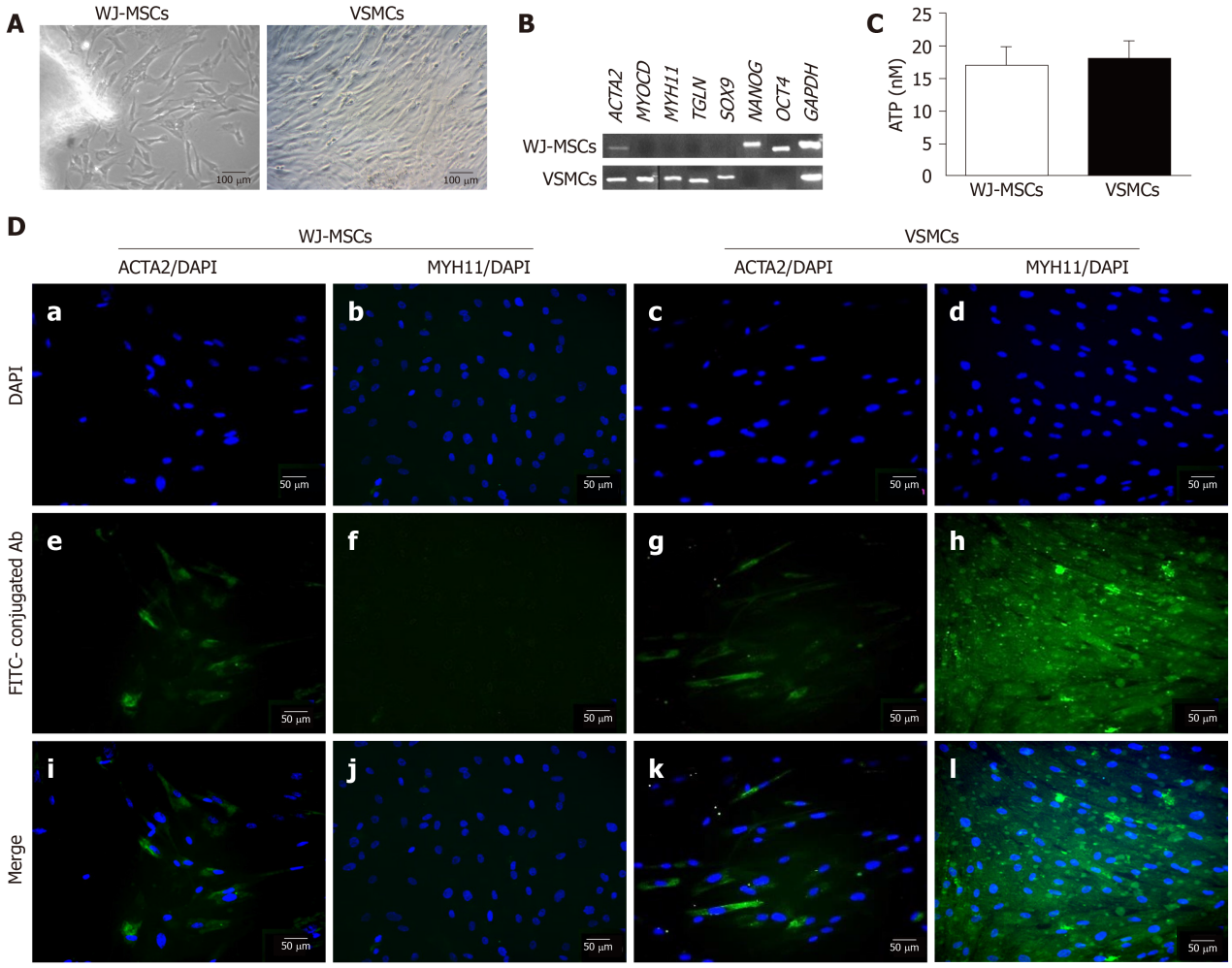

Figure 2 Differentiation of mesenchymal stromal cells derived from the Wharton's Jelly tissue into vascular smooth muscle cells. A: Morphological features of untreated mesenchymal stromal cells derived from the Wharton's Jelly tissue (WJ-MSCs) and differentiated vascular smooth muscle cells (VSMCs); B: Polymerase chain reaction results regarding the expression of VSMC-specific genes, such as *ACTA2, MYOCD, MYH11* and *TGLN*, and pluripotency-related genes, including *NANOG* and *OCT4* in untreated WJ-MSCs and differentiated VSMCs. *GAPDH* was the desired house-keeping gene for current analysis; C: Determination of WJ-MSC and VSMC proliferation by performing the ATP assay; Indirect immunofluorescence against the early VSMC marker ACTA2 and late VSMC marker MYH11 in untreated WJ-MSCs (D-a, D-e, D-i and D-b, D-f, D-j) and differentiated VSMCs (D-c, D-g, D-k and D-d, D-h, D-l) in combination with DAPI, respectively. Images A-a and A-b were presented with 10× original magnification and 100 μm scale bars. Images D-a to D-l were presented with 20× original magnification and 50 μm scale bars. WJ-MSCs: Mesenchymal stromal cells derived from the Wharton's Jelly tissue; VSMCs: Vascular smooth muscle cells.

Repopulation of hUAs with VSMCs

Decellularized hUAs were successfully repopulated by VSMCs under static seeding conditions. Indeed, VSMCs appeared in the outer layer of the vessels from the 1st wk of seeding (Figure 4A and Supplementary Figure 5). These cells were successfully expanded on vessel walls after 3 wk of repopulation (Figure 4A and Supplementary Figure 5). Indirect immunofluorescence results showed the presence of differentiated VSMCs in the outer surface of decellularized vessels, further confirming the H & E results. In addition, immunohistochemistry results indicated the expression of key proliferation markers such as Ki67 and PCNA in the VSMCs of repopulated hUAs (Figure 4A).

Further evaluation of the repopulated arteries involved the quantification of hydroxyproline and sGAG content. Specifically, total hydroxyproline content after the 1st, 2nd and 3rd wk was 71 ± 10, 74 ± 9 and 86 ± 8 μg hydroxyproline/mg of dry tissue weight, respectively (Figure 4B). Overall, total hydroxyproline content appeared to be increased within the first week of repopulation. Statistically significant differences in total hydroxyproline content were observed between the study groups ($P < 0.05$). SGAG content of the 1st, 2nd and 3rd wk was 2 ± 1, 3 ± 1 and 3 ± 1 μg sGAG/mg of dry tissue weight, respectively (Figure 4C). Statistically significant differences were observed between the study groups ($P < 0.05$). VSMCs exhibited robust proliferation in the hUAs, as was indicated by the DNA quantification results. Specifically, the DNA amount of repopulated hUAs after the 1st, 2nd and 3rd wk was 110 ± 21, 360 ± 61 and 554 ± 49 ng DNA/mg of dry tissue weight, while decellularized hUAs were

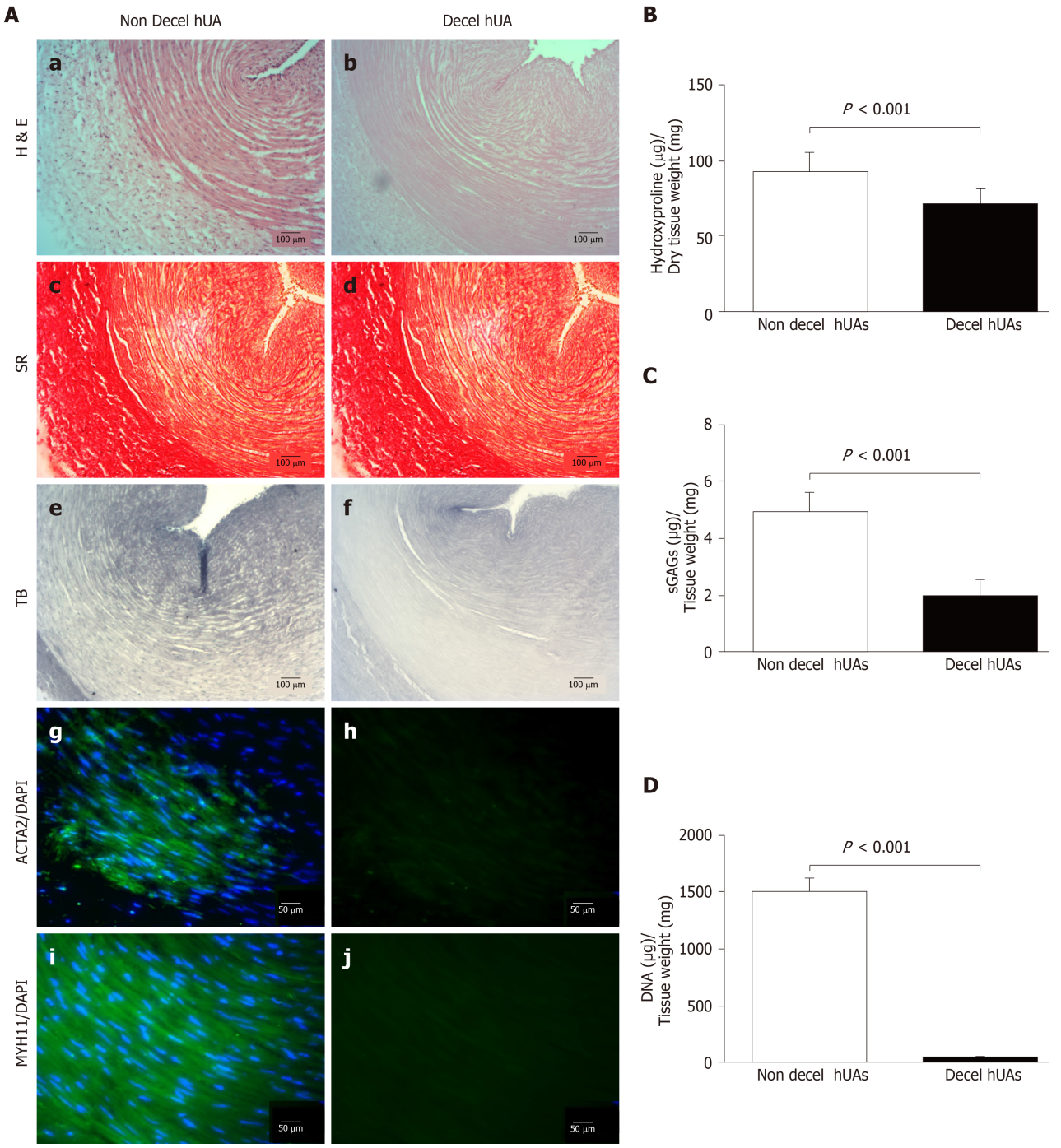

Figure 3 Histological and biochemical analysis of decellularized human umbilical arteries. A: Histological analysis with H & E (A-a, A-b), SR (A-c, A-d) and TB (A-e, A-f) in non-decellularized and decellularized human umbilical arteries (hUAs). Indirect immunofluorescence against ACTA2 (A-g, A-h) and MYH11 (A9,10) in combination with DAPI was performed in non-decellularized and decellularized hUAs; B-D: Biochemical analysis involved the determination of total hydroxyproline (B), sGAG (C) and DNA content (D) in non-decellularized and decellularized hUAs. Statistically significant differences were observed in total hydroxyproline ($P < 0.05$), sGAG ($P < 0.001$) and DNA ($P < 0.001$) content between non decellularized and decellularized hUAs. Images A-a to A-f were presented with original magnification 10× and 100 μm scale bars. Images A-g to A-j were presented with original magnification 20× and 50 μm scale bars. Non Decel hUA: Non decellularized human umbilical artery; Decell hUA: Decellularized human umbilical artery.

characterized by only 38 ± 7 ng DNA/mg of dry tissue weight (Figure 4D). Statistically significant differences were observed between the study groups ($P < 0.001$). No sign of any cytotoxic effects during VSMC proliferation in hUAs was observed, according to the determination of the ADP/ATP ratio (Figure 4E).

DISCUSSION

The development of well-defined VSMCs, which can be applied to the development

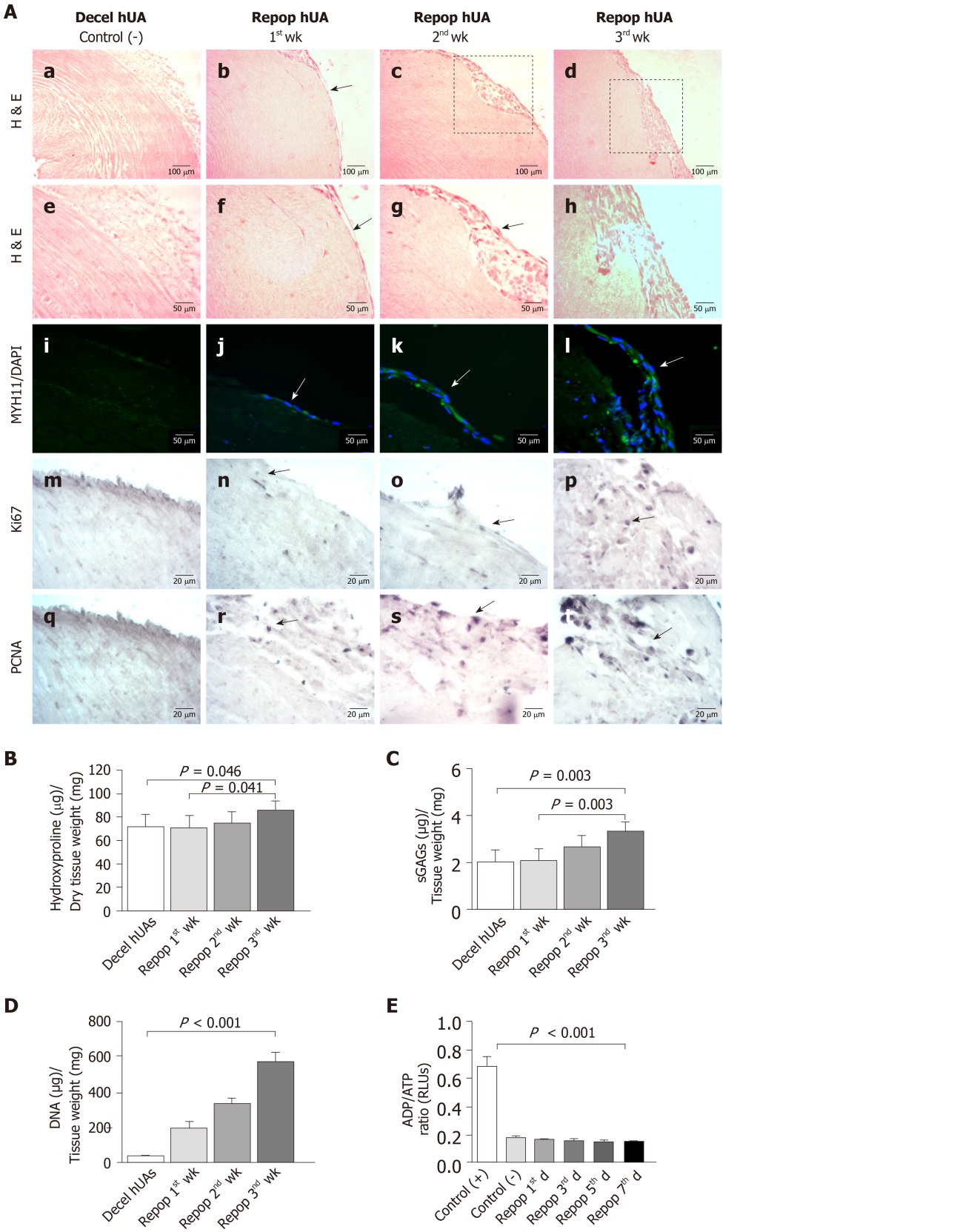

Figure 4 Repopulation of decellularized human umbilical arteries with vascular smooth muscle cells. A: Histological analysis with H & E of decellularized human umbilical arteries (hUAs) (A-a, A-e), repopulated hUAs after 1st wk (A-b, A-f), 2nd wk (A-c, A-g) and 3rd wk (A-d, A-h). Indirect immunofluorescence against MYH11 in combination with DAPI of decellularized hUAs (A-i), repopulated hUAs after 1st wk (A-j), 2nd wk (A-k) and 3rd wk (A-l). Immunohistochemistry against Ki67 and PCNA of decellularized hUAs (A-m, A-q), and repopulated hUAs after 1st wk (A-n, A-r), 2nd wk (A-o, A-s) and 3rd wk (A-p, A-t). Images A-a to d were presented with original magnification 10×, 100 μm scale bars. Images A-f to l were presented with original magnification 20× and 50 μm scale bars. Images A-i to t were presented with original magnification 40× and 20 μm scale bars; B, C: Total hydroxyproline (B) and sGAG (C) quantification of hUAs before and after repopulation with VSMCs; D, E: Determination of DNA content (D) and ADP/ATP ratio (E). Statistically significant differences in total hydroxyproline, sGAG, DNA content and ADP/ATP ratio were observed between the study groups ($P < 0.05$). Decel hUA: decellularized human umbilical artery, repop hUA: repopulated human umbilical artery.

of vascular grafts, is one of the major goals of blood vessel engineering. In order to obtain VSMCs, several sources of stem cells can be used. Unlike other stem cells, MSCs can be easily isolated from various human tissues such as bone marrow, adipose tissue and WJ tissue, and can be characterized by low immune responses, high proliferation rates and low risk of genome instability[32]. The aim of this study was the development of VSMCs through a differentiation process utilizing WJ-MSCs, for future use in small diameter vascular graft engineering.

Within this scope, WJ-MSCs were successfully isolated and expanded from WJ tissue until they reached P4. WJ-MSCs retained their spindle-shaped morphology through passages, and were characterized by high proliferation rates and cell viability. In addition, these cells were successfully differentiated into "osteocytes", "adipocytes" and "chondrocytes", as was confirmed by the presence of calcium deposits, lipid vacuoles and proteoglycan production. Immunophenotypic analysis provided evidence that WJ-MSCs positively expressed (> 90%) CD73, CD90, CD105, CD44 and CD29, while CD34, CD45, HLA-DR, CD3 and CD19 were negatively expressed (< 3%). These results indicated that the WJ-MSCs used in this study were a properly defined stem cell population, and fulfilled the minimum criteria defined by ISCT[30].

Once we established the properties of WJ-MSCs, they were then differentiated towards VSMCs. To promote the development of VSMCs from WJ-MSCs, a differentiation protocol that utilized the UCB-PL in combination with ascorbic acid was applied. UCB-PL contains significant amounts of growth factors such as TGF-β1, PDGF-A, FGF2, IFN-γ and TNF-α[29]. Among them, TGF-β1 and PDGF-A *via* their receptor are related to the activation of downstream SMADs and the MEK/ERK signaling pathway, contributing in this way to the differentiation process of VSMCs[23]. After 3 wk of differentiation, WJ-MSCs presented a more elongated spindle-shaped morphology. Moreover, these cells expressed *ACTA2, MYH11, MYOCD, TGLN* and *SOX9*. *ACTA2* is an early myogenic differentiation marker that is also expressed in WJ-MSCs. On the other hand, *MYH11, MYOCD* and *TGLN* are late myogenic differentiation markers that are especially expressed in VSMCs. Indeed, *MYH11* is required for the production of smooth-muscle myosin heavy chain, while *TGLN* is related to actin re-organization and shape change interactions[23]. *MYOCD* has a great role in the VSMC differentiation process, and its expression is restricted in smooth and cardiac muscle lineages[23]. MYOCD interacts with Serum Response Factor, thus forming a complex that binds to the CArG [CC(A/T)$_6$GG] box. CArG boxes are located in the promoters of SMC genes, regulating their transcription[32]. In our case, the myogenic differentiation conditions that were applied to WJ-MSCs induced the expression of *MYOCD*, which further regulates the transcription of contractile smooth muscle genes including *ACTA2*, *MYH11* and *TGLN*, thus promoting cell shape alterations[23,33]. Further clarification of the proper differentiation of VSMCs was provided by the indirect immunofluorescence assay against the early and late myogenic markers ACTA2 and MYH11. VSMCs were characterized by high expression of ACTA2 and MYH11, while untreated WJ-MSCs were characterized only by their low expression of ACTA2[34]. These results further confirmed our initial data at the mRNA level, indicating the successful differentiation of VSMCs. The low expression of ACTA2 in WJ-MSCs has been previously reported by other groups[35]. For this purpose, the MYH11 was used in order in our experimental approach to distinguish and characterize the differentiation state of WJ-MSCs. In addition, differentiated VSMCs expressed *SOX9*, a gene that is related to collagen production and the adaption of VSMC synthetic phenotypes[34]. *SOX9* expression may be relevant to the differentiation conditions that were applied in the current study. It is known that specific stress-strain conditions are required for the maintenance of contractile VSMC phenotypes[23]. In our study, no stress-strain conditions were applied, which may explain the *SOX9* expression in the differentiated VSMCs.

On the other hand, undifferentiated WJ-MSCs expressed only *OCT4* and *NANOG*. It is known that NANOG, in combination with other transcription factors such as OCT4, SOX2 and KLF4, establish the pluripotent state of stem cells[36]. These transcription factors block the Serum Response Factor association with CarG boxes, thus promoting *SMC* gene repression. VSMCs were not characterized by the expression of *OCT4* and *NANOG*. The repression of these genes might also contribute to the initiation of the differentiation process. Both WJ-MSCs and VSMCs were characterized by the equal production of ATP, suggesting the retention of VSMC proliferation properties.

Further work must be performed in order to obtain safer conclusions regarding the gene interplay during the VSMC differentiation process. The proposed differentiation protocol could induce the myogenic differentiation of WJ-MSCs utilizing only the exogenous supplementation of ascorbic acid. Our results seemed to be comparable with other previously published studies[37-43], where early, intermediate and late

VSMC markers such as ACTA2 and MYH11 are expressed. However, most of these studies apply more complicated and sophisticated approaches, including the use of iPSC technology, or the prolonged exogenous supplementation of growth factors[24-27]. Moreover, the use of iPSC technology in humans is under strict control. Modern research is focused on the production of safe viral-free iPSC clones for use in clinical trials. On the other hand, vascular tissue engineering demands a great number of cells, which are difficult to obtain from patients. Most of the time, vessel tissue biopsies are needed for the isolation of VSMCs. However, patient condition and age are important factors that may hamper VSMC isolation. In our study, the production of VSMCs from MSCs is proposed. MSCs can be efficiently isolated from several human patient tissues, including bone marrow and adipose tissue. Additionally, MSCs are pluripotent stem cells that can be expanded in great numbers, and can then be differentiated into the desired cell populations, such as VSMCs. Taken together, all these data propose an alternative way to obtain functional VSMCs, even from seriously diseased patients, for possible use in vascular tissue engineering.

The next step of this study was the use of VSMCs in small diameter vascular graft engineering. To date, several sources for the production of small diameter vascular grafts have been proposed, such as the use of Dacron and expanded polytetrafluorethylene conduits, and autologous vessels. Unlike these vessels, hUAs may possess an alternative source for the production of small diameter vascular grafts. In this way, hUAs were successfully decellularized as indicated by histological analysis. H & E staining revealed the preservation of ECM, without any cellular or nuclear remnants, while SR and TB showed the preservation of key ECM components such as collagen and sGAGs. Indirect immunofluorescence results indicated no presence of ACTA2 or MYH11 in decellularized hUAs, further confirming cell elimination. Biochemical analysis confirmed the presence of collagen and sGAGs, although both components were significantly reduced after the decellularization procedure. In addition, the DNA content of decellularized vascular grafts was significantly reduced, and was below 50 ng/mg of dry tissue as proposed by Crapo *et al*[44], thus further confirming the successful decellularization of hUAs. These results were in accordance with previously published studies conducted in vessels or other tissues, and the reduction of the above macromolecules were mostly attributed to SDS, a key reagent in the decellularization process[3,4,17,45]. However, hUA ECM was characterized as having the proper orientation of collagen and sGAGs, thus serving as an ideal vascular scaffold for cell repopulation.

Within this scope, the VSMCs were seeded on decellularized hUAs under static conditions. After the 1st wk of seeding, VSMCs were observed in the outer layer of the vessel. After 3 wk, the VSMCs appeared to expand onto the decellularized hUA, as was confirmed by H & E staining.

VSMCs were successfully characterized by MYH11 positivity, as indicated by immunofluorescence results after 3 wk of repopulation. Repopulated hUAs were also positive for Ki67 and PCNA, as was indicated by immunohistochemistry, confirming the successful proliferation of VSMCs. Also, an increase in DNA content was observed in repopulated hUAs, indicating the successful seeding and proliferation of VSMCs. Indeed, hUAs did not exhibit any cytotoxic effects, thus supporting the repopulation of VSMCs. For further evaluation of VSMC functionality, hydroxyproline and sGAG quantifications were performed. Total hydroxyproline content was increased even after the 1st wk of seeding. After 3 wk, the hydroxyproline content of the repopulated vessels was higher compared to the decellularized hUAs. In a similar way, sGAG content was higher in repopulated vessels after 3 wk compared to the decellularized hUAs. However, total hydroxyproline and sGAG content in repopulated hUAs was similar to the amount of native vessels. However, the further maturation of vessels is required through the use of other approaches, such as vessel bioreactors. Indeed, vessel bioreactors could contribute to a more uniform distribution of cells in vessel layers, thus inducing the proper maturation of vascular grafts, and likely the increase of total hydroxyproline and sGAG content[46].

Moreover, the above results confirmed that VSMCs retained their myogenic properties in the vascular scaffolds. Taking into consideration the expression of *SOX9* at the mRNA level in combination with the hydroxyproline production, it can be assumed that VSMCs retain their myogenic properties and contribute to the remodeling of vessel ECM. It is known that SOX9 in combination with RUNX2 and MSX2 could contribute to the synthetic conversion of VSMCs, resulting in collagen and sGAG synthesis. Although, in our study where only static seeding conditions were applied, the proper maturation of VSMCs and the adaption of contractile phenotypes onto decellularized vessels may require other approaches, such as the use of dynamic seeding conditions. Indeed, a pulsatile vessel bioreactor could mimic the blood flow of the human body with specific stress-strain conditions, and could contribute to the adaption of contractile phenotypes by VSMCs.

Future experiments will involve the use of a pulsatile vessel bioreactors for the repopulation approach in order to better define the VSMCs, and promote the maturation of vascular grafts. Moreover, a combination of VSMCs with EC is desired in order to produce fully functional small diameter vascular grafts.

In conclusion, in this study, VSMCs were successfully generated from WJ-MSCs and efficiently repopulated decellularized hUAs. Moreover, the differentiation of VSMCs relied on a protocol that utilized UCB-PL, excluding the exogenous supplementation of growth factors or ectopic expression of transcription factors[23]. Furthermore, the interaction of VSMCs *via* integrin connections such as $\alpha_v\beta_1$ with ECM proteins could maintain even more their a differentiation state. Until now, several complicated and expensive approaches are used for the production of vascular populations and small diameter vessel conduits[26,27,28,47]. Unlike these approaches, our proposal relied on the use of hUCs and their derivatives as an alternative approach for blood vessel engineering. From a material that is discarded after gestation, WJ-MSCs and hUAs can be efficiently isolated, while UCB-PL can be used for the production of myogenic differentiation medium.

The future goal will be the production and the proper maintenance of patient-specific small diameter vascular grafts under good manufacturing practice conditions, in order to be readily accessible upon demand.

ARTICLE HIGHLIGHTS

Research background

Small diameter vascular grafts can be applied in a wide variety of diseases, but mostly in cardiovascular disease (CAD). Globally, CAD affects more than 18 million people, and it is estimated that more than 500000 bypass surgeries are performed. Until now, autologous saphenous vein transplants, or conduits made of Dacron or ePTFE, represent the gold standard strategy. However, severe side effects, including impaired patency, immune reaction and intima hyperplasia, may be accompanied by their use. For this purpose, the decellularization of human umbilical arteries and the repopulation with vascular smooth muscle cells (VSMCs) in order to obtain fully functional vascular grafts, could represent an alternative approach. VSMCs are a cellular population responsible for vasoconstriction and vasodilation. Recently, the development of VSMCs has been proposed using induced pluripotent stem cell (iPSCs) technology. However, iPSCs have not been approved for broad human use. In this way, an alternative approach using platelet lysate from umbilical cord blood (UCB-PL) may be applied in the differentiation process of VSMCs from mesenchymal stromal cells (MSCs). It is known that UCB-PL contains significant amounts of growth factors such as TGF-β1, PDGFA, FGF2, IFN-γ and TNF-α, which have previously been used in several differentiation protocols. The aim of this study was to establish the differentiation process of VSMCs from MSCs derived from the Wharton's Jelly tissue (WJ-MSCs) using the UCB-PL. Then, the differentiated VSMCs were used for repopulation experiments of decellularized human umbilical arteries (hUAs) to produce fully functional small diameter vascular grafts.

Research motivation

Until now, the development of VSMCs is accomplished using exogenous supplementation of several growth factors or through iPSC technology. However, both approaches may cause allergic reactions or could even be tumorigenic. Indeed, a great number of growth factors are derived from animals. Additionally, iPSC technology has not received full approval from the Food and Drug Administration for human use, due to the use of c-Myc, which may lead to tumor development. In order to overcome these issues, the differentiation of VSMCs from MSCs using UCB-PL and ascorbic acid has been proposed. It has been shown in the past that specific growth factors, especially TGF-β1, could promote the differentiation of VSMCs. UCB-PL contains several growth factors, including TGF-β1, PDGF-A, FGF2, IFN-γ and TNF-α, and in combination with ascorbic acid may lead to the successful development of VSMCs.

Research objectives

The main objective of this study was the successful differentiation of VSMCs obtained from WJ-MSCs using UCB-PL. Secondary objectives were the production of small diameter vascular grafts in hUAs using the decellularization method. In addition, the repopulation of decellularized vessels with the produced VSMCs, which may result in functional vessels, was also evaluated in this study.

Research methods

Initially, WJ-MSCs were isolated from hUCs and expanded until they reached P4. Characterization of WJ-MSCs was performed according to the criteria of the International Society for Cell and Gene Therapy, including morphological evaluation, trilineage differentiation and flow cytometry analysis. Then, the differentiation of VSMCs was performed. To do this, WJ-MSCs were cultured in a medium containing UCB-PL and ascorbic acid for 3 wk. Gene expression profiles of VSMCs for *ACTA2, MYH11, TGLN, MYOCD, SOX9, NANOG, OCT4, and GAPDH* by RT-PCR, PCR and gel electrophoresis were evaluated. Further analysis included the indirect immunofluorescence of VSMCs using antibodies against ACTA2 and MYH11. The production of vascular grafts was performed using the decellularization of hUAs. Then

histological (H & E, SR and TB stains) and biochemical analyses (hydroxyproline, sGAG, DNA content) in decellurized hUAs were applied. Finally, the repopulation of decellularized hUAs with VSMCs through static seeding was performed. Repopulated vessels were analyzed histologically (H & E, MYH11/DAPI) and biochemically (hydroxyproline, DNA content and ADP/ATP ratio). In addition, the proliferation of VSMCs in repopulated vessels was immunohistochemically evaluated using Ki67 and proliferating cell nuclear antigen.

Research results

WJ-MSCs were successfully isolated and expanded from hUCs. Their spindle-shaped morphology was retained until they reached P4. Total cell number, CDT and PD of WJ-MSCs at P4 was > 12×10^6 cells, 36 ± 3 h and 6 ± 1, respectively. WJ-MSCs fulfilled the criteria of the International Society for Cell and Gene Therapy, indicating successful differentiation towards "osteogenic", "adipogenic" and "chondrogenic" lineages, positive expression (> 95%) for CD73, CD90 and CD105, and negative expression (< 3%) for CD34, CD45 and HLA-DR. WJ-MSCs were successfully differentiated into VSMCs using UCB-PL and ascorbic acid. Differentiated VSMCs expressed *ACTA2, MYOCD, MYH11* and *TGLN*. In addition, early and late VSMCs markers such as ACTA2 and MYH11 were evaluated according to indirect immunofluorescence analyses. HUAs were effectively decellularized and characterized by the preservation of ECM proteins, while no cell and nuclei materials were evident. Statistically significant differences were observed between non-decellularized and decellularized hUAs regarding the hydroxyproline ($P < 0.001$), sGAG ($P < 0.001$) and DNA ($P < 0.001$) content. Decellularized hUAs were successfully repopulated by the produced VSMCs, as it was indicated by histological analysis (H and E, MYH11/DAPI). Repopulated vessels were characterized by elevated levels of hydroxyproline (86 ± 8 μg hydroxyproline/mg of dry tissue weight), sGAG (3 ± 1 μg sGAG / mg of dry tissue weight), and DNA (554 ± 49 ng DNA/mg of dry tissue weight) content after 3 wk of cultivation. In addition, the key proliferation markers Ki67 and proliferating cell nuclear antigen were positively expressed by VSMCs in repopulated vessels, according to immunohistochemistry results.

Research conclusions

VSMCs can be successfully produced from WJ-MSCs using UCB-PL in combination with ascorbic acid. Unlike current approaches, including the exogenous supplementation of growth factors or the use of iPSC technology, no such approaches were applied to this study. UCB-PL contains significant amounts of key growth factors required for VSMC differentiation. In addition, ascorbic acid supplementation to the differentiation medium appears to enhance the underlying mechanism. Besides, the successful production of VSMCs and the development of functional small diameter vascular grafts were assessed. HUAs were efficiently decellularized, and could serve as potential scaffolds for blood vessel engineering. To obtain functional small diameter vascular grafts, the decellularized hUAs were repopulated with the produced VSMCs. Finally, the repopulated vessels were characterized for their similar properties to the hUAs before the decellularization process. Taking into consideration the above data, hUCs could be a rich source both for cellular populations and vessel conduits. Additionally, this study brings into light a safer differentiation process that can possibly be used for the production of patient-specific VSMCs. It is known that the circulatory system of CAD patients is primarily affected. The isolation of VSMCs from patient vessel biopsies, which can be used for vascular graft engineering, is not efficient. On the contrary, MSCs (in adults) that are presented both in bone marrow and adipose tissue, can be isolated and differentiated into VSMCs with the current protocol, and can thus potentially be used in blood vessel engineering. In this way, and unlike the complicated and expensive approaches of the past, the production of fully functional blood vessels is one step closer to its clinical application.

Research perspectives

The next step of this study will be focused on the use of the repopulated (with VSMCs) vascular grafts in an animal model, in order to better evaluate their functionality. Small blood vessel engineering is one of the milestones of personalized regenerative medicine. To this direction, the production of patient-specific small diameter vascular grafts under good manufacturing practice conditions, that can be readily accessible, will be of great importance.

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ORIGINAL ARTICLE

Basic Study

CR6-interacting factor-1 contributes to osteoclastogenesis by inducing receptor activator of nuclear factor κB ligand after radiation

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Author contributions: Li ZJ and Xiang LX conceived the study; Xiang LX designed and performed most experiments and data analysis; Chen L, Xiang Y, Li FJ, Zhang XM, Xiao YN, Zou LY, Zhong JF, Li SC, and Ran Q assisted with experiments and data analysis; Xiang LX wrote and edited the manuscript; Li ZJ supervised the study; all authors read and approved the final manuscript.

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Abstract

BACKGROUND

Radiation induces rapid bone loss and enhances bone resorption and adipogenesis, leading to an increased risk of bone fracture. There is still a lack of effective preventive or therapeutic method for irradiation-induced bone injury. Receptor activator of nuclear factor κB ligand (RANKL) provides the crucial signal to induce osteoclast differentiation and plays an important role in bone resorption. However, the mechanisms of radiation-induced osteoporosis are not fully understood.

AIM

To investigate the role of CR6-interacting factor-1 (Crif1) in osteoclastogenesis after radiation and its possible mechanism.

METHODS

C57BL/6 mice were exposed to Co-60 gamma rays and received 5 Gy of whole-body sublethal irradiation at a rate of 0.69 Gy/min. For *in vitro* study, mouse bone marrow mesenchymal stem/stromal cells (BM-MSCs) were irradiated with Co-60 at a single dose of 9 Gy. For osteoclast induction, monocyte-macrophage RAW264.7 cells were cocultured with mouse BM-MSCs for 7 d. ClusPro and InterProSurf were used to investigate the interaction interface in Crif1 and protein kinase cyclic adenosine monophosphate (cAMP)-activited catalytic

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subunit alpha complex. Virtual screening using 462608 compounds from the Life Chemicals database around His120 of Crif1 was carried out using the program Autodock_vina. A tetrazolium salt (WST-8) assay was carried out to study the toxicity of compounds to different cells, including human BM-MSCs, mouse BM-MSCs, and Vero cells.

RESULTS
Crif1 expression increased in bone marrow cells after radiation in mice. Overexpression of Crif1 in mouse BM-MSCs and radiation exposure could increase RANKL secretion and promote osteoclastogenesis *in vitro*. Deletion of Crif1 in BM-MSCs could reduce both adipogenesis and RANKL expression, resulting in the inhibition of osteoclastogenesis. Deletion of Crif1 in RAW264.7 cells did not affect the receptor activator of nuclear factor κB expression or osteoclast differentiation. Following treatment with protein kinase A (PKA) agonist (forskolin) and inhibitor (H-89) in mouse BM-MSCs, Crif1 induced RANKL secretion *via* the cAMP/PKA pathway. Moreover, we identified the Crif1-protein kinase cyclic adenosine monophosphate-activited catalytic subunit alpha interaction interface by *in silico* studies and shortlisted interface inhibitors through virtual screening on Crif1. Five compounds dramatically suppressed RANKL secretion and adipogenesis by inhibiting the cAMP/PKA pathway.

CONCLUSION
Crif1 promotes RANKL expression *via* the cAMP/PKA pathway, which induces osteoclastogenesis by binding to receptor activator of nuclear factor κB on monocytes-macrophages in the mouse model. These results suggest a role for Crif1 in modulating osteoclastogenesis and provide insights into potential therapeutic strategies targeting the balance between osteogenesis and adipogenesis for radiation-induced bone injury.

Key words: Irradiation; Osteoporosis; Bone marrow; Mesenchymal stem cells; Monocyte macrophage; Bone

Core tip: Current treatment of osteoporosis is based mainly on inhibiting bone resorption or stimulating bone generation to increase bone mass; however, the side-effects of some drugs affect long-term administration and adherence. There is still a lack of effective preventive or therapeutic method for radiation-induced bone injury. Because of the contribution of adipocytes to osteoporosis, future drug screening should target not only the regulation of the balance between bone formation and bone resorption but also the balance between osteogenic and adipogenic differentiation. Here, through screening, we identified five CR6-interacting factor-1 inhibitors targeting CR6-interacting factor-1-protein kinase cyclic adenosine monophosphate-activited catalytic subunit alpha interaction interface that could dramatically reduce receptor activator of nuclear factor κB ligand secretion and adipogenesis. Our study provides insights into potential therapeutic strategies for radiation-induced bone injury.

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INTRODUCTION

Exposure to radiation, such as accident or terrorism, radiotherapy for cancer, and astronauts on exploratory missions beyond low-Earth orbit, can cause rapid bone loss and increase the risk of bone fracture[1,2]. The risk of a hip fracture for women receiving pelvic irradiation for the treatment of carcinomas of the cervix or rectum is

three times as much as that of the population of women who do not receive radiotherapy[3]. Osteoporosis is often a long-term complication of radiotherapy, which is characterized by an imbalance in skeletal turnover with reduced bone formation and enhanced bone resorption[4]. Current treatment of osteoporosis is based mainly on inhibiting bone resorption or stimulating bone generation to increase bone mass; however, the side-effects of some drugs affect long-term administration and adherence. There is still a lack of effective preventive or therapeutic method for irradiation-induced bone injury[5].

Bone homeostasis is maintained by various types of cells, such as osteoblasts and osteoclasts, which are differentiated from different stem cells in the bone marrow. Osteoblasts are the bone-forming cells derived from bone marrow mesenchymal stem/stromal cells (BM-MSCs) and play an important role in the regulation of bone mass[6]. Meanwhile, osteoclasts are large, multinucleated cells derived from haematopoietic progenitors of the monocyte-macrophage lineage. Osteoclasts are the principal cells capable of resorbing bone and play an essential role in bone remodeling[7]. The differentiation of osteoclast is mainly regulated by macrophage colony-stimulating factor, receptor activator of nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG). Macrophage colony-stimulating factor is required for the survival and proliferation of osteoclast precursors, but RANKL and OPG play central roles in the activation of osteoclastogenesis[8]. By binding to receptor activator of nuclear factor κB (RANK) (on haematopoietic progenitors), RANKL provides the crucial signal to induce osteoclast differentiation from haematopoietic progenitor cells as well as to activate mature osteoclasts. OPG is a soluble decoy receptor that can bind to RANKL and negatively regulate RANKL binding to RANK[9]. BM-MSCs, osteocytes, osteoblasts, adipocytes, and activated T and B lymphocytes are the main sources of RANKL secretion. RANKL expression is promoted by radiation, inflammation, cytokines, hormones, and a number of other agents, including those that signal through protein kinase A (PKA), glycoprotein 130, and vitamin D receptor[10,11]. Following radiation exposure, BM-MSCs appear to preferentially differentiate into adipocytes instead of osteoblasts, which results in a reduction of bone formation and an increase in bone marrow fat accumulation[12,13]. And the irradiation-induced bone loss is also associated with increased osteoclast numbers and resorbing surfaces of osteoclasts lining trabeculae[14]. However, the molecular mechanisms of cell fate decisions in the differentiations of BM-MSCs and osteoclasts involved in irradiation-induced bone loss are still not fully understood.

CR6-interacting factor-1 (Crif1) is a multifunctional protein that can interact with many proteins to induce cell cycle arrest, modulate oxidative stress and cell radiosensitivity, and regulate transcriptional activity through interactions with the DNA-binding domains of transcription factors[15-21]. It is also the constitutive protein of the large mitoribosomal subunit required for the synthesis and insertion of mitochondrial-encoded OxPhos polypeptides into the mitochondrial membrane[22]. Crif1 deficiency in macrophages impairs mitochondrial oxidative function and causes systemic insulin resistance and adipose tissue inflammation[23]. Our previous study showed that Crif1 promotes adipogenic differentiation of BM-MSCs after radiation by modulating the cyclic adenosine monophosphate (cAMP)/PKA signaling pathway[24].

In this study, we investigated the role of Crif1 in osteoclastogenesis after radiation. Here, we showed that *Crif1* deletion caused decreases in RANKL expression and the RANKL/OPG ratio and reduced osteoclastogenesis and adipogenesis after radiation. Through screening, we also identified five compounds that could effectively inhibit RANKL expression and adipogenesis. We demonstrated that Crif1 promoted osteoclasto-genesis by inducing RANKL expression *via* the cAMP/PKA pathway. Our study suggests a role for Crif1 in modulating osteoclastogenesis and provides insights into potential therapeutic strategies targeting the balance between osteogenesis and adipogenesis for radiation-induced bone injury.

MATERIALS AND METHODS

Animals

The animal protocol was designed to minimize pain or discomfort to the animals. All animal studies performed were approved by the Laboratory Animal Welfare and Ethics Committee Of the Third Military Medical University. C57BL/6 mice (aged 12-14 wk) were purchased from Beijing HFK Bio-Technology Co. Ltd. Mice were maintained under specific pathogen-free conditions and fed standard mouse chow and water. For radiation treatment, mice (n = 6/group) were exposed to Co-60 gamma rays and received 5 Gy of whole-body sublethal irradiation at a rate of 0.69 Gy/min.

Cell culture and treatment

For *in vitro* study, mouse BM-MSCs purchased from Cyagen Biosciences were cultured in mouse mesenchymal stem cell medium (MUCMX-90011, Cyagen Biosciences) at 37 °C in an atmosphere containing 5% CO_2.

For radiation treatment, mouse BM-MSCs were irradiated with a single dose of 9 Gy Co-60 at a rate of 0.69 Gy/min. RAW264.7 cells were cultured in Dulbecco's modified Eagle medium (HyClone) supplemented with 10% fetal bovine serum.

For osteoclast induction, RAW264.7 cells (2×10^4/well) seeded in the upper well and mouse BM-MSCs (5×10^4/well) seeded in the lower well of a 12-well transwell unit (0.4 μm) were cocultured for 7 d with or without forskolin (25 μmol/L) or H-89 (20 μmol/L) treatment. After 7 d of coculture, cells were collected for real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis; meanwhile, the supernatant medium was collected for enzyme linked immunosorbent assay (ELISA).

Human bone marrow mesenchymal stem/stromal cells (H-BM-MSCs) (catalogue No. 7500, ScienCell) were cultured in mesenchymal stem cell medium (catalogue No. 7501, ScienCell) at 37 °C in an atmosphere containing 5% CO_2.

Micro-computed tomography analysis

Femurs were dissected, fixed overnight in 4% paraformaldehyde, and stored in 1% paraformaldehyde at 4 °C. Trabecular bone parameters were measured in the distal metaphysis of the femur. We started analysing slices at the bottom of the distal growth plate, where the epiphyseal cap structure completely disappeared, and continued for 95 slices (10.5 μm/slice, using SCANCO VivaCT40) towards the proximal end of the femur.

Isolation of bone marrow cells

Femurs were collected and cleaned in sterile phosphate buffered saline (PBS), and both ends of each femur were trimmed off. Bones were placed in a 0.6-mL microcentrifuge tube that was cut open at the bottom and nestled inside a 1.5-mL microcentrifuge tube. Fresh bone marrow was spun out by brief centrifugation (from 0 rpm to 10000 rpm, 9 s). Red blood cells were lysed using RBC lysis buffer (catalog No. RT122-02, TIANGEN). After centrifugation (3000 rpm, 5 min), cells in the bottom layer were collected for Western blot and RT-qPCR assays.

Crif1 knockout and overexpression in vitro

For Crif1 overexpression, mouse BM-MSCs were transfected with a Crif1 lentiviral overexpression vector (pLV[Exp]-EGFP:T2A:Puro-EF1A>mGadd45gip1[NM_183358.4]) constructed by Cyagen Biosciences (vector ID: VB180112-1182ypt) and selected with 5 μg/mL puromycin dihydrochloride (A1113803, Invitrogen). An empty vector (pLV[Exp]-EGFP:T2A:Puro-Null, vector ID: VB160420-1011mqh, Cyagen Biosciences) was included as a control.

For *Crif1* knockout, mouse BM-MSCs were first transfected with lentiCas9-Blast vector (Genomeditech) and selected with 5 μg/mL blasticidin S HCl (A1113903, Invitrogen). Then, cells were transfected with CRISPR/Cas9 M_Gadd45gip1 gRNA vector (target sequence: GCGGGGGCGCACGGTAGCTG, Genomeditech) and selected with 5 μg/mL puromycin dihydrochloride. An empty vector (LentiGuide-Puro-Scramble-gRNA, Genomeditech) was included as a control.

In vitro adipogenic differentiation

To induce adipogenesis, mouse BM-MSCs were seeded at a density of 2×10^4 cells per well in 6-well plates and cultured in mouse mesenchymal stem cell adipogenic differentiation medium (MUCMX-90031, Cyagen Biosciences). Human BM-MSCs were seeded at a density of 2×10^4 cells per well in 6-well plates and cultured in human mesenchymal stem cell adipogenic differentiation medium (HUXMA-90031, Cyagen Biosciences). After 21 d of differentiation, we preserved the supernatant medium for ELISA and fixed the cells with 2 mL of 4% formaldehyde solution for 30 min. Then, the cells were stained with 1 mL of oil red O working solution (catalog No. S0131, Cyagen Biosciences) for 30 min and visualized under a light microscope (Leica DMIRB, Heidelberg, Germany). The dye from oil red O staining was extracted using isopropanol, and the optical density at 510 nm was measured using a Varioskan FLASH microplate reader.

Western blot analysis and antibodies

Protein expression in the samples was analysed by Western blot. Briefly, total protein lysates were extracted with cell lysis buffer for Western blot and immunoprecipitation (catalog No. P0013, Beyotime) and denatured by boiling. Protein samples were resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride

membranes (Western Blotting Membranes; Roche). Membranes were blocked in PBS containing 5% (w/v) nonfat dry milk and 0.1% Tween 20 and then incubated with the appropriate primary antibodies overnight at 4 °C. Membranes were washed with Tris-buffered saline with Tween-20 three times and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at 24 °C. Immunoreactive bands were detected with the BeyoECL Plus reagent (P0018, Beyotime) using a Photo-Image System (Molecular Dynamics, Sunnyvale, CA, United States). The primary antibodies used for blotting were as follows: Crif1 (M-222) (sc-134882; Santa Cruz), RANK (H-7) (sc-374360; Santa Cruz), A-FABP (AP2, sc-18661; Santa Cruz), PPARγ (sc-7273; Santa Cruz), β-actin (sc-47778; Santa Cruz), phospho-cAMP response element-binding protein (CREB) rabbit mAb (#9198; Cell Signaling Technology), and CREB rabbit mAb (#9197; Cell Signaling Technology).

RT-qPCR

RT-qPCR was used to analyze the mRNA levels of selected genes in collected samples. Total RNA was extracted using TRIzol Reagent (catalog No. 10296010, Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from l μg of RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (catalog No. RR047A, TaKaRa). qPCR was performed in triplicate in 20-μL reactions containing SYBR Premix Ex Taq II (catalog No. RR820A, TaKaRa). The reaction protocol was as follows: Heating for 30 s at 95 °C, followed by 40 cycles of amplification (5 s at 95 °C and 30 s at 60 °C).

The sequences of the RT-PCR primers are as follows: M-Crif1-F: GAACGCTGGGAGAAAATTCA and M-Crif1-R: ATAGTTCCTGGAAGCGAGCA; M-actin-F: AGCCATGTACGTAGCCATCC and M-actin-R: CTCAGCT GTGGTGGTGAA; M-Rankl-F: GCTCCGAGCTGGTGAAGAAA and M-Rankl-R:CCCCAAAGTACGTCGCATCT; M-OPG-F: GTTCCTGCACAGCTTCACAA and M-OPG-R: AAACAGCCCAGTGACCATTC.

ELISA

The concentrations of RANKL and OPG were measured using the Mouse RANKL ELISA Kit (E-EL-M0644c, elabscience), Human Soluble Receptor Activator of Nuclear Factor-kB Ligand ELISA Kit (E-EL-H5558c, elabscience), Mouse OPG ELISA Kit (E-EL-M0081c, elabscience), and Human OPG ELISA Kit (E-EL-H1341c, elabscience) according to the manufacturer's instructions.

Tartrate-resistant acid phosphatase staining

After the 7-d coculture period, cells were washed once with PBS, fixed in 10% formalin for 10 min, and incubated with a substrate solution, naphthol AS-BI phosphate (catalog No. 387, Sigma), in the presence of 50 mmol/L sodium tartrate at 37 °C for 1 h. The resulting mononuclear and multinuclear tartrate-resistant acid phosphatase (TRAP)-positive cells were visualized by light microscopy and quantified.

Histomorphometric analysis

Femurs were dissected, fixed overnight in 4% paraformaldehyde, decalcified in 10% EDTA (pH 7.0) for 20 d, and embedded in paraffin. Longitudinally oriented sections of bone (4 μm thick), including the metaphysis and diaphysis, were processed for hematoxylin and eosin staining. Dewaxed sections were also stained for TRAP activity to identify osteoclasts. Sections were incubated in TRAP stain for 45 min at 37 °C.

Crif1 inhibitor screening

ClusPro and InterProSurf were used to investigate the interaction interface in Crif1-protein kinase cyclic adenosine monophosphate-activited catalytic subunit alpha (PRKACA) complex. Virtual screening using 462608 compounds from the Life Chemicals database around His^{120} of Crif1 was carried out using the program Autodock_vina. For inhibitor screening, H-BM-MSCs were cultured at a density of 1×10^5 cells per well in 6-well plates and pretreated with five different compounds (25 μmol/L). After 3 h of pretreatment, forskolin (25 μmol/L) was added to the medium. After 1 h of forskolin treatment, total protein lysates were extracted for CREB phosphorylation detection, and 3 d later, the supernatant medium was collected for ELISA.

Tetrazolium salt (WST-8) assay

A tetrazolium salt (WST-8) assay was carried out to study the toxicity of compounds to different cells, including human BM-MSCs, mouse BM-MSCs, and Vero cells. Cells seeded at a density of 3000 cells per well in 96-well plates were treated with five

different compounds at eight final concentrations (3.125 μmol/L, 6.25 μmol/L, 12.5 μmol/L, 25 μmol/L, 50 μmol/L, 100 μmol/L, 200 μmol/L, and 400 μmol/L). Three days later, 10 μL of cell counting kit-8 solution was added to each well. After 4 h of incubation, the absorbance at 450 nm was measured using a Varioskan FLASH microplate reader (Thermo).

Statistical analysis

The mRNA expression levels of *RANKL* and *OPG* in the tested samples were determined as the cycle threshold (CT) level, and normalized copy numbers (relative quantification) were calculated using the ΔΔCT equation as follows: -ΔΔCT = -ΔCT of the bone marrow sample -ΔCT of β-actin, and the normalized copy number (relative quantification) = $2^{-\Delta\Delta CT}$. The statistical significance of differences between the two groups was assessed using two-tailed Student's *t*-tests. The statistical significance of differences among more than two groups was assessed using one-way ANOVA with Sidak's multiple comparison tests. The statistical significance of differences between radiation treatments in mouse BM-MSCs and *Crif1* knockout BM-MSCs experiments, radiation treatments in mouse BM-MSCs and *Crif1* knockout BM-MSCs adipogenic differentiation experiments, forskolin treatments in mouse BM-MSCs and *Crif1* knockout BM-MSCs experiments, and H-89 treatments in mouse BM-MSCs and Crif1-overexpressing BM-MSCs experiments were assessed using two-way ANOVA with Bonferroni's or Sidak's multiple comparison tests. All data are expressed as the mean ± standard deviation. A *P* value less than or equal to 0.05 was considered statistically significant.

RESULTS

Radiation induces bone loss and increased Crif1 expression in mice

To confirm the extent of bone loss over the short term after irradiation, we irradiated mice with a single dose of 5 Gy, and then, 7 d later, we harvested the left femurs. Micro-computed tomography analysis of the distal femurs of both males and females at 12 wk of age revealed significant decreases in trabecular bone volume/total volume (Figure 1A and B), connectivity density (Figure 1C), trabecular number (Figure 1D), and bone mineral density (Figure 1E), as well as significant increases in trabecular spacing (Figure 1G) and structure model index (Figure 1H). There was no significant difference in trabecular thickness (Figure 1F). Hematoxylin and eosin staining of femoral sections from irradiated mice showed significantly decreased trabecular bone (Figure 1I), while increased adipocytes (Figure 1J) compared to controls. Paraffin sections of femurs showed more TRAP-positive cells in irradiated mice than in control mice (Figure 1K), indicating the increased number of osteoclasts. These results indicated that radiation-induced adipogenesis and osteoclastogenesis. Moreover, RT-qPCR data revealed dramatic increases in RANKL expression (Figure 1L) and the RANKL/OPG ratio in irradiated bone marrow cells (Figure 1M). OPG expression was not affected by radiation treatment (Figure 1L). Notably, expression of Crif1 also increased in irradiated bone marrow cells compared with control cells 7 d after irradiation (Figure 1N and O).

Overexpression of Crif1 in BM-MSCs increases RANKL secretion and osteoclastogenesis

BM-MSCs are thought to be more resistant to radiation compared with other cells in the bone marrow, such as hematopoietic stem cells and T and B lymphocytes[25]. Moreover, BM-MSCs are the progenitors of bone marrow osteoblasts and adipocytes[26]. To investigate the role of Crif1 in osteoclastogenesis *in vitro*, we transfected mouse BM-MSCs with a Crif1 lentiviral overexpression vector (Figure 2A). For osteoclast induction *in vitro*, Crif1-overexpressing BM-MSCs and RAW264.7 cells were cocultured in a 12-well transwell unit for 7 d. RT-qPCR results showed that the relative mRNA expression of *RANKL* and *RANKL/OPG* ratio both increased in Crif1-overexpressing BM-MSCs compared to controls after 7 d of coculture (Figure 2B and C). Concentrations of RANKL and OPG in coculture medium were also detected by ELISA. Crif1-overexpressing BM-MSCs produced high levels of RANKL compared to the control (Figure 2D), while there was no significant difference in OPG concentration between the two groups (Figure 2E). The RANKL/OPG ratio in Crif1-overexpressing BM-MSCs was higher than that in the control (Figure 2F). We also detected an increased number of TRAP-positive cells in RAW264.7 cells cocultured with Crif1-overexpressing BM-MSCs (Figure 2G and H). These data suggested that Crif1 could promote RANKL expression and may be involved in osteoclast differentiation.

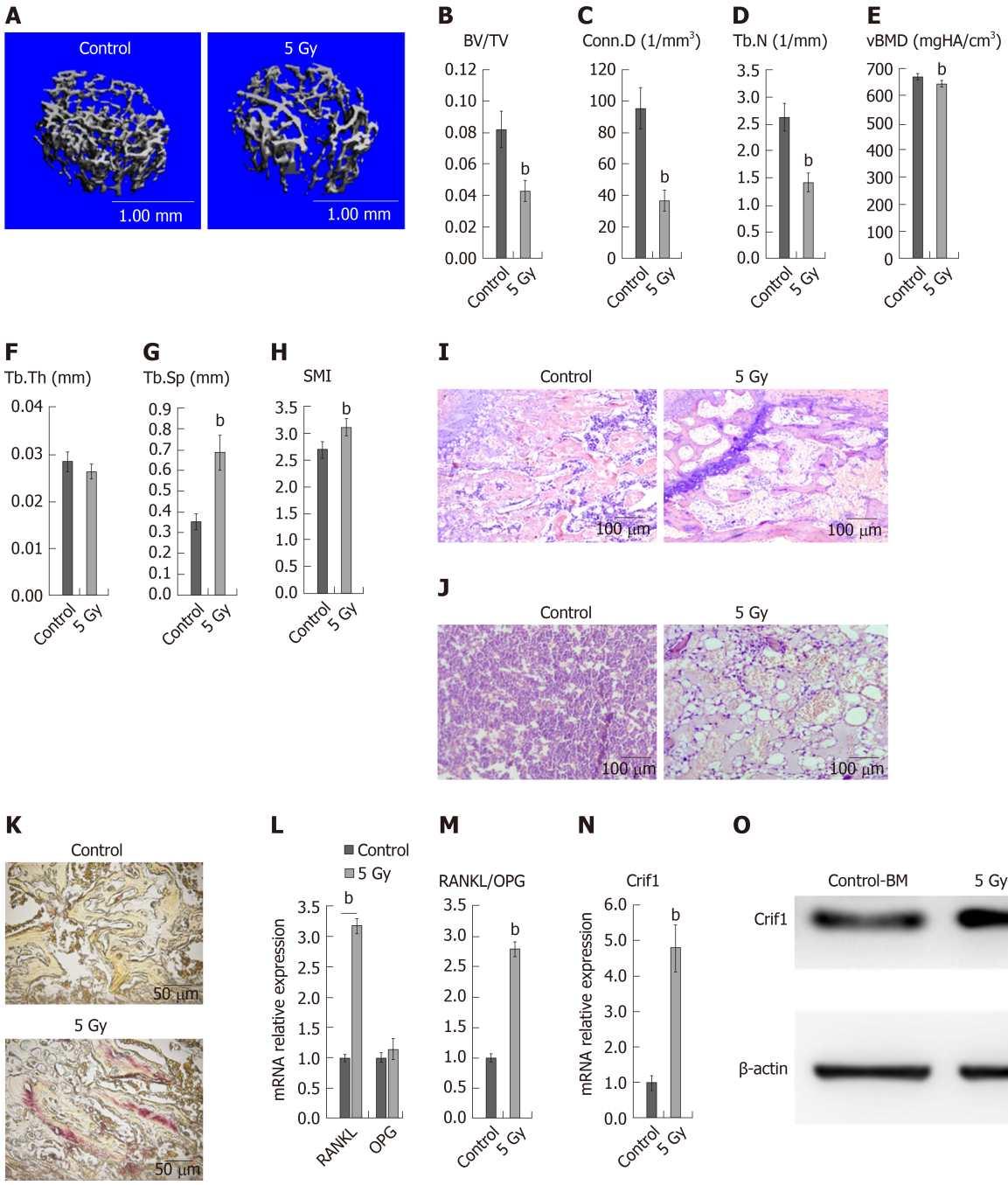


Figure 1 Radiation induces bone loss and increased CR6-interacting factor-1 expression in mice. A: Micro-computed tomography images of the distal metaphysis of the femur. Mice (n = 6/group) were exposed to Co-60 gamma rays, and received 5 Gy of whole-body sublethal irradiation at a rate of 0.69 Gy/min; B-H: Micro-computed tomography analysis of the trabecular bone volume/total volume (B), connectivity density (C), trabecular number (D), bone mineral density (E), trabecular thickness (F), trabecular spacing (G), and structure model index (H); I: Hematoxylin-eosin staining of femoral sections from irradiated mice and controls. Femoral sections from irradiated mice showed significantly decreased trabecular bone compared to controls; J: Hematoxylin-eosin staining of femoral sections from irradiated mice and controls. Femoral sections from irradiated mice showed that adipocytes increased significantly in irradiated mice; K: Tartrate-resistant acid phosphatase staining of femoral sections from irradiated mice and controls; L: Real-time quantitative polymerase chain reaction (RT-qPCR) analysis of receptor activator of nuclear factor κB ligand and osteoprotegerin mRNA expression in flushed whole bone marrow; M: Receptor activator of nuclear factor κB ligand/osteoprotegerin ratio based on RT-qPCR results; N: RT-qPCR analysis of CR6-interacting factor-1 mRNA expression in flushed whole bone marrow; O: Western blot analysis of CR6-interacting factor-1 expression in flushed whole bone marrow. $^{a}P < 0.05$, $^{b}P < 0.01$, and the bars represent the mean ± SD. OPG: Osteoprotegerin; RANKL: Receptor activator of nuclear factor κB ligand; Crif1: CR6-interacting factor-1; BM: Bone marrow; SMI: Structure model index; BV/TV: Bone volume/total volume; Conn.D: Connectivity density; Tb.N: Trabecular number; vBMD: Bone mineral density; Tb.Sp: Trabecular spacing; Tb.Th: Trabecular thickness.

Crfi1 is involved in the regulation of RANKL expression after radiation

RANKL provides the critical signal to induce osteoclast differentiation by binding to its receptor RANK (on haematopoietic progenitors, such as monocytes-macrophages)[6]. To further confirm whether Crif1 plays an important role in osteoclastogenesis after radiation, we knocked out *Crif1* in RAW264.7 cells and BM-MSCs (Figures 3A and 3D), respectively. The deletion of *Crif1* in RAW264.7 cells did

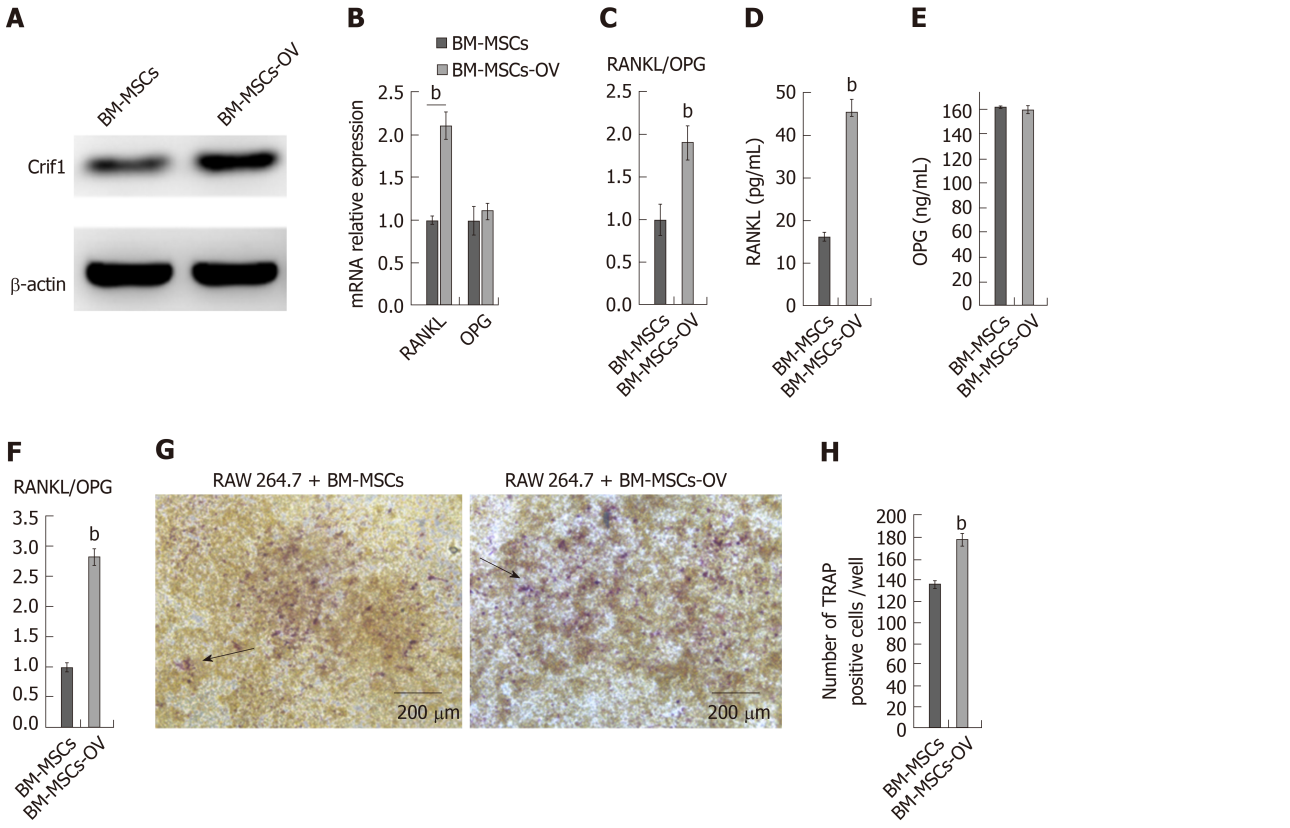


Figure 2 Overexpression of CR6-interacting factor-1 in bone marrow mesenchymal stem/stromal cells increases receptor activator of nuclear factor κB ligand secretion and osteoclastogenesis. A: Western blot analysis of CR6-interacting factor-1 (Crif1) expression in mouse bone marrow mesenchymal stem/stromal cells (BM-MSCs). Mouse BM-MSCs were transfected with a Crif1 lentiviral overexpression vector; B: Real-time quantitative polymerase chain reaction analysis of receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) mRNA expression in BM-MSCs and Crif1-overexpressing BM-MSCs. BM-MSCs and Crif1-overexpressing BM-MSCs were cocultured with RAW264.7, respectively; C: RANKL/OPG ratio based on real-time quantitative polymerase chain reaction results; D: Enzyme linked immunosorbent assay analysis of RANKL protein levels in coculture supernatant medium; E: Enzyme linked immunosorbent assay analysis of OPG protein levels in coculture supernatant medium; F: RANKL/OPG ratio in coculture supernatant medium; G: Tartrate-resistant acid phosphatase staining of RAW264.7 cells after 7 d of coculture; H: Average number of tartrate-resistant acid phosphatase-positive cells/well (arrow) from RAW264.7 cells in coculture. $^{a}P < 0.05$, $^{b}P < 0.01$, and the bars represent the mean ± SD. OPG: Osteoprotegerin; RANKL: Receptor activator of nuclear factor κB ligand; Crif1: CR6-interacting factor-1; BM-MSCs: Bone marrow mesenchymal stem/stromal cells; BM-MSCs-OV: Crif1-overexpressing BM-MSCs.

not affect the RANK expression or osteoclast differentiation (Figure 3A-C). We previously demonstrated that Crif1 expression was upregulated after radiation, and in this study, we found that RANKL expression and the RANKL/OPG ratio were also elevated after radiation (Figure 3E-G and I). Meanwhile, more TRAP-positive cells were found in RAW264.7 cells cocultured with BM-MSCs after radiation (Figure 3J and K). However, knocking out *Crif1* in BM-MSCs could significantly reduce RANKL expression and the RANKL/OPG ratio both before and after radiation (Figure 3E-G and I). OPG expression was not affected by *Crif1* deletion or radiation treatment (Figure 3E and H). Moreover, the number of TRAP-positive cells also decreased in *Crif1* knockout BM-MSCs compared to the control after 7 d of coculture with or without radiation treatment (Figure 3J and K). These results further demonstrated that Crif1 can regulate RANKL expression, especially after radiation.

Crif1 mediates adipogenesis and RANKL secretion in adipocytes

After radiation exposure, the hematopoietic red marrow gradually turns yellow, which is also known as bone marrow fatting. Moreover, excessive numbers of adipocytes are often found in the bone marrow of patients with osteoporosis, and theses adipocytes can also secrete RANKL and accelerate osteoclastogenesis[27]. To determine whether Crif1 affects RANKL expression in adipocytes, BM-MSCs were exposed to a single dose of 9 Gy and then grown in mouse mesenchymal stem cell adipogenic differentiation medium. Consistent with our previous research[24], more BM-MCSs became strongly predisposed to adipogenesis after radiation treatment. After 21 d of induction, the intensity of oil red O staining was significantly higher in irradiated BM-MSCs, and more adipocytes were found in this group. Important regulators during late adipogenesis, such as PPAR-r and AP2, both increased after radiation treatment (Figure 4A-C). Here, we also found an obvious increase in

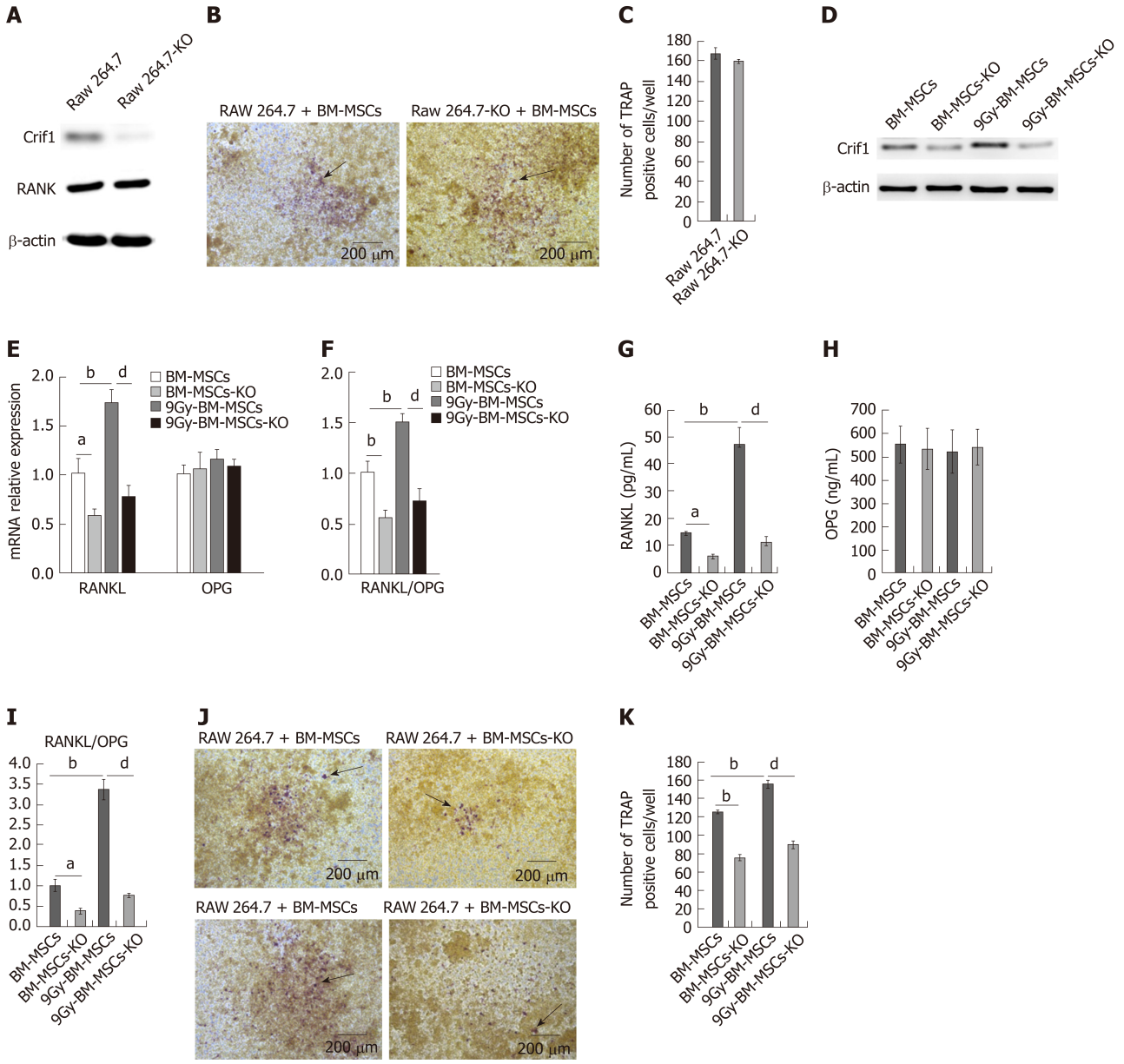


Figure 3 CR6-interacting factor-1 is involved in the regulation of receptor activator of nuclear factor κB ligand expression after radiation. A: Western blot analysis of CR6-interacting factor-1 (Crif1) and receptor activator of nuclear factor κB expression in RAW264.7 cells. *Crif1* was knocked out in RAW264.7 cells (RAW264.7-KO); B: Tartrate-resistant acid phosphatase (TRAP) staining of RAW264.7-KO and controls after 7 d of coculture with mouse bone marrow mesenchymal stem/stromal cells (BM-MSCs); C: Average number of TRAP-positive cells/well (arrow) from RAW264.7-KO and controls after 7 d of coculture with mouse BM-MSCs; D: Western blot analysis of Crif1 expression in BM-MSCs. *Crif1* was knocked out in mouse BM-MSCs (BM-MSCs-KO), and BM-MSCs-KO and controls were irradiated with Co-60 at a single dose of 9 Gy; E: Real-time quantitative polymerase chain reaction analysis of receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) mRNA expression in BM-MSCs and BM-MSCs-KO. BM-MSCs and BM-MSCs-KO were cocultured with RAW264.7; F: RANKL/OPG ratio based on real-time quantitative polymerase chain reaction results; G: Enzyme linked immunosorbent assay analysis of RANKL protein levels in coculture supernatant medium; H: Enzyme linked immunosorbent assay analysis of OPG protein levels in coculture supernatant medium; I: RANKL/OPG ratio in coculture supernatant medium; J: TRAP staining of RAW264.7 after 7 d of coculture; K: Average number of TRAP-positive cells/well (arrow) from RAW264.7 in coculture. $^{a}P < 0.05$ *vs* control (BM-MSCs), $^{b}P < 0.01$ *vs* control (BM-MSCs); $^{d}P < 0.01$ between 9 Gy-BM-MSCs and 9 Gy-BM-MSCs-KO, and the bars represent the mean ± standard deviation. BM-MSCs: Bone marrow mesenchymal stem/stromal cells; BM-MSCs-KO: *Crif1* was knocked out from mouse BM-MSCs; RAW264.7-KO: *Crif1* was knocked out from RAW264.7 cells; OPG: Osteoprotegerin; RANKL: Receptor activator of nuclear factor κB ligand.

RANKL expression and RANKL/OPG ratio in irradiated BM-MSCs after adipogenic induction (Figure 4D-F and H). However, knocking out *Crif1* in BM-MSCs reduced adipogenesis (Figure 4A-C), RANKL expression, and RANKL/OPG ratio (Figure 4D-F and H) with or without radiation treatment. OPG expression was not affected by *Crif1* deletion or radiation treatment (Figure 4D and G). These data suggested that Crif1 mediates adipogenesis and RANKL secretion in adipocytes.

Crif1 promotes RANKL secretion by modulating the cAMP/PKA signaling pathway

The regulation of bone remodeling involves many factors, such as parathyroid

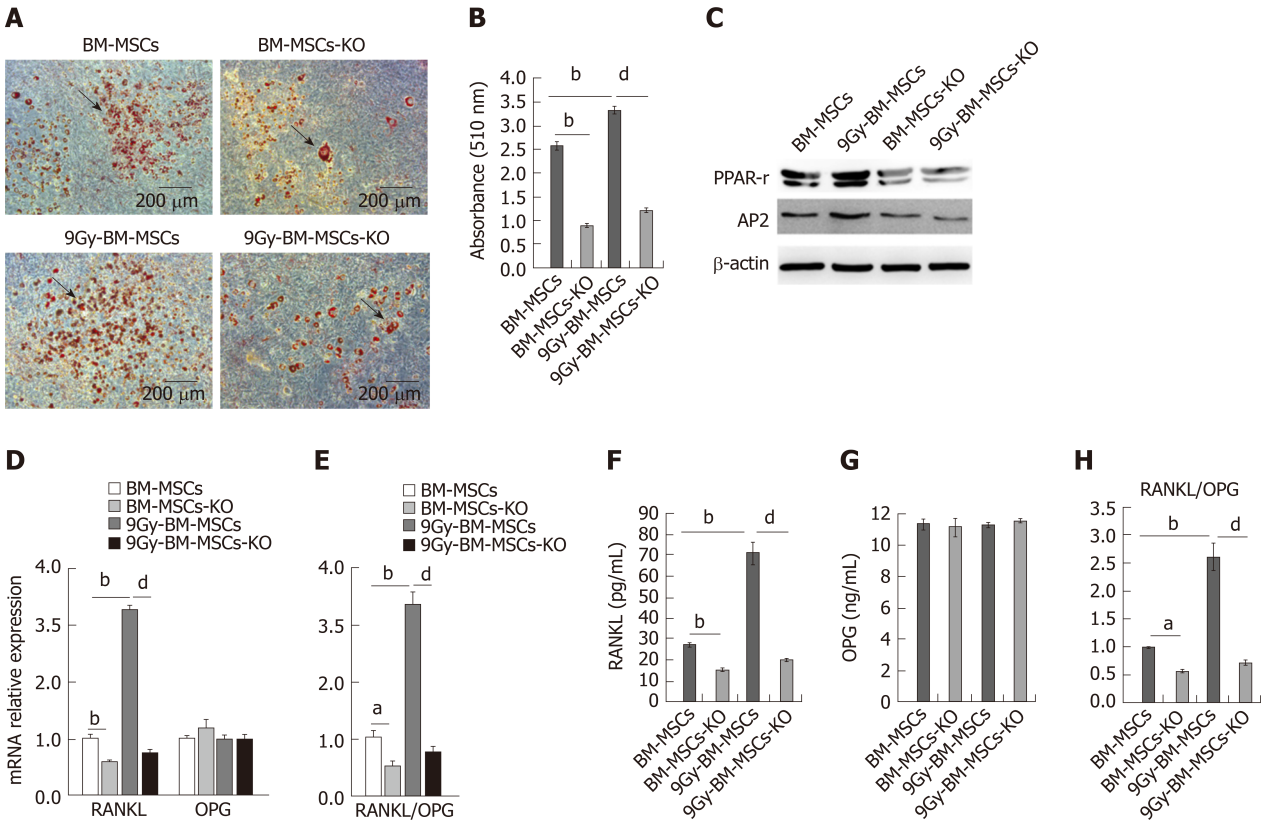
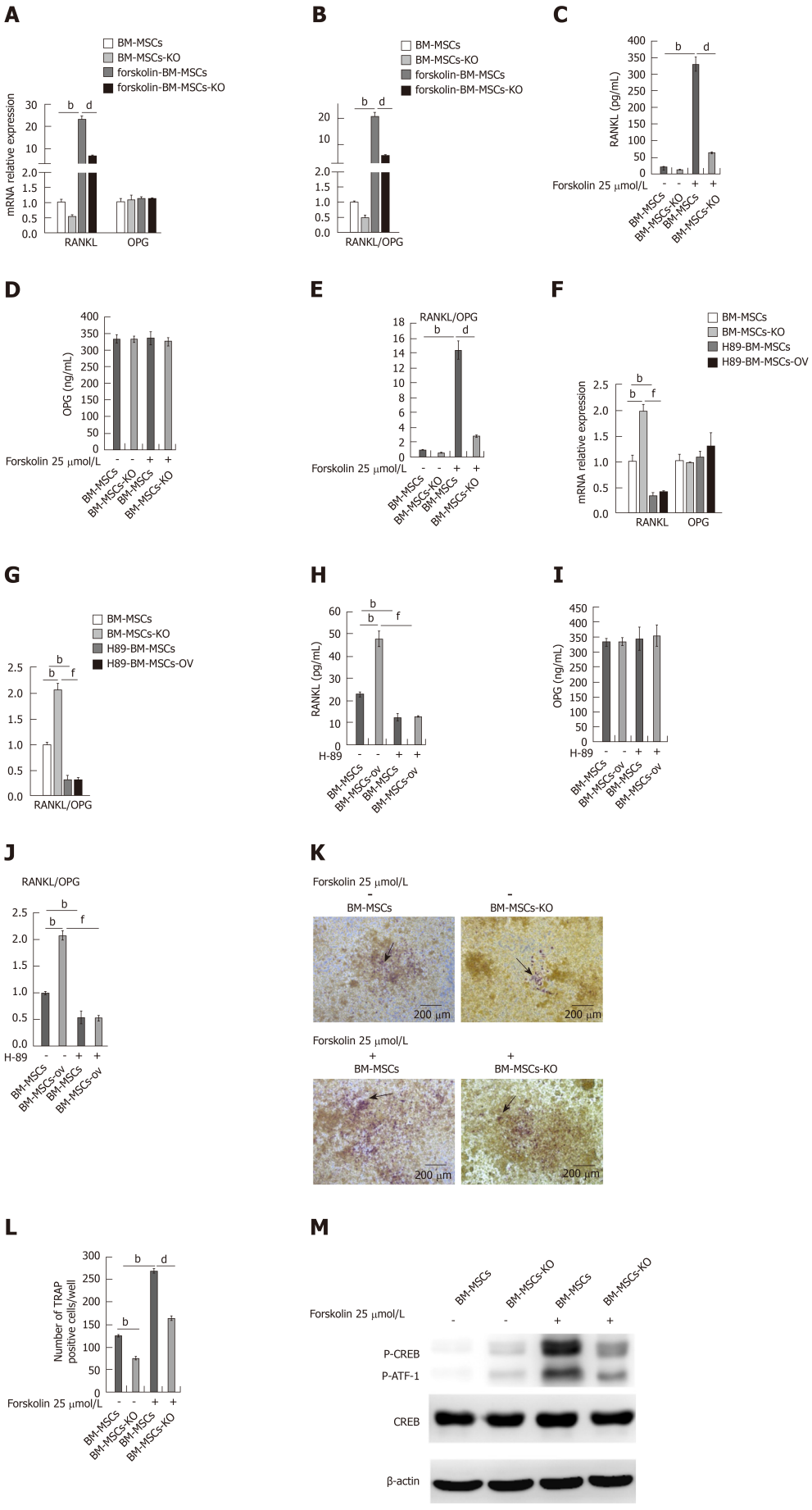


Figure 4 CR6-interacting factor-1 mediates adipogenesis and receptor activator of nuclear factor κB ligand secretion in adipocytes. A: Oil red O staining analysis of mouse bone marrow mesenchymal stem/stromal cells (BM-MSCs) after 21 d of adipogenic differentiation. *Crif1* was knocked out in mouse BM-MSCs (BM-MSCs-KO), and knockout cells and controls were irradiated with 9 Gy of Co-60, and then treated with mouse mesenchymal stem cell adipogenic differentiation medium (Ad) to induce adipogenesis; B: The dye from oil red O staining was extracted using isopropanol, and the optical density at 510 nm was measured using Benchmark Plus; C: Western blot analysis of adipogenesis-related markers and transcription factors PPARγ and AP2 in mouse BM-MSCs after 21 d of adipogenic differentiation; D: Real-time quantitative polymerase chain reaction analysis of receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) mRNA expression in BM-MSCs and BM-MSCs-KO; E: RANKL/OPG ratio based on real-time quantitative polymerase chain reaction results; F: Enzyme linked immunosorbent assay analysis of RANKL protein levels in supernatant Ad; G: Enzyme linked immunosorbent assay analysis of OPG protein levels in supernatant Ad; H: RANKL/OPG ratio in supernatant Ad. $^{a}P < 0.05$ *vs* control (BM-MSCs), $^{b}P < 0.01$ *vs* control (BM-MSCs); $^{d}P < 0.01$ between 9 Gy-BM-MSCs and 9 Gy-BM-MSCs-KO, and the bars represent the mean ± SD. BM-MSCs: Bone marrow mesenchymal stem/stromal cells; BM-MSCs-KO: *Crif1* knockout mouse BM-MSCs; OPG: Osteoprotegerin; RANKL: Receptor activator of nuclear factor κB ligand.

hormone (PTH), vitamin D3, bonemarrow-derived growth factors, and cytokines including RANKL. One of the primary mechanisms of bone remodeling is PTH-induced adenosine 3′,5′-monophosphate (cAMP), which activates PKA and leads to phosphorylation and activation of CREB resulting in RANKL production[28]. To verify the mechanism underlying Crif1-mediated upregulation of RANKL expression, PKA agonist (forskolin) and inhibitor (H-89) were added to the co-culture system. RANKL expression and the RANKL/OPG ratio were both increased remarkably after treatment with 25 μmol/L forskolin, however, these effects were significantly weakened in *Crif1* knockout BM-MSCs (Figure 5A-C and E). In addition, RANKL expression and the RANKL/OPG ratio were both decreased when Crif1-overexpressing BM-MSCs and controls were treated with 20 μmol/L H-89 (Figure 5F-H and J). OPG expression was not affected by forskolin or H-89 treatment (Figure 5A, D, F, and H). TRAP-positive cells were increased significantly by adding forskolin, the most TRAP-positive cells were found in the coculture with forskolin-treated BM-MSCs, but this effect was also reduced by *Crif1* deletion (Figure 5K and L). H-89 could inhibit osteoclastogenesis effectively, and the fewest TRAP-positive cells were found in the coculture with H-89-treated Crif1-overexpressing BM-MSCs and controls (Figure 5N and O). After the addition of forskolin, CREB phosphorylation was significantly increased in the control BM-MSCs but was dramatically inhibited in *Crif1* knockout BM-MCSs (Figure 5M). We also observed that CREB phosphorylation was suppressed in both Crif1-overexpressing BM-MSCs and controls following exposure to H-89 (Figure 5P). These results demonstrated that Crif1 promotes RANKL expression through the cAMP/PKA signaling pathway.

Crif1 inhibitors effectively suppress RANKL secretion and adipogenesis


A
BM-MSCs
BM-MSCs-KO
forskolin-BM-MSCs
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mRNA relative expression
RANKL
OPG
B
RANKL/OPG
C
RANKL (pg/mL)
Forskolin 25 μmol/L
D
OPG (ng/mL)
E
RANKL/OPG
F
H89-BM-MSCs
H89-BM-MSCs-OV
G
H
RANKL (pg/mL)
H-89
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Number of TRAP positive cells/well
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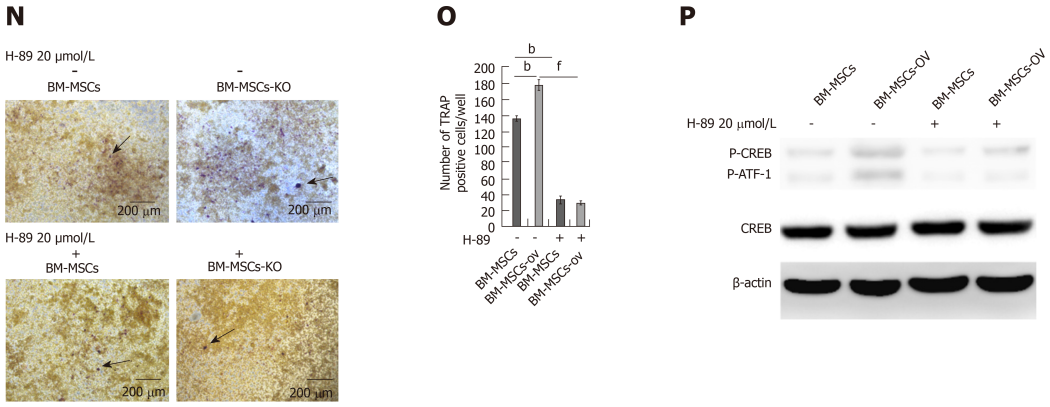

Figure 5 CR6-interacting factor-1 promotes receptor activator of nuclear factor κB ligand secretion by modulating the cAMP/PKA signaling pathway. A: Real-time quantitative polymerase chain reaction (RT-qPCR) analysis of receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) mRNA expression in bone marrow mesenchymal stem/stromal cells (BM-MSCs) and *Crif1* knockout BM-MSCs treated with 25 μmol/L forskolin in the coculture with RAW264.7; B: RANKL/OPG ratio based on RT-qPCR results; C: Enzyme linked immunosorbent assay (ELISA) analysis of RANKL levels in coculture supernatant medium treated with 25 μmol/L forskolin; D: ELISA analysis of OPG levels in coculture supernatant medium treated with 25 μmol/L forskolin; E: RANKL/OPG ratio in coculture supernatant medium treated with 25 μmol/L forskolin; F: RT-qPCR analysis of RANKL and OPG mRNA expression in BM-MSCs and BM-MSCs-OV treated with 20 μmol/L H-89 in the coculture with RAW264.7; G: RANKL/OPG ratio based on RT-qPCR results; H: ELISA analysis of RANKL levels in coculture supernatant medium treated with 20 μmol/L H-89; I: ELISA analysis of OPG levels in coculture supernatant medium treated with 20 μmol/L H-89; J: RANKL/OPG ratio in coculture supernatant medium treated with 20 μmol/L H-89; K: Tartrate-resistant acid phosphatase (TRAP) staining of RAW264.7 cells in coculture treated with 25 μmol/L forskolin; L: Average number of TRAP-positive cells/well (arrow) from RAW264.7 cells in coculture treated with 25 μmol/L forskolin; M: Western blot analysis of phospho-cyclic adenosine monophosphate response element-binding protein phosphorylation levels in BM-MSCs in coculture treated with 25 μmol/L forskolin; N: TRAP staining of RAW264.7 in coculture treated with 20 μmol/L H-89; O: Average number of TRAP-positive cells/well (arrow) from RAW264.7 in coculture treated with 20 μmol/L H-89; P: Western blot analysis of phospho-cyclic adenosine monophosphate response element-binding protein phosphorylation levels in BM-MSCs in coculture treated with 20 μmol/L H-89. $^{b}P < 0.01$ *vs* control (BM-MSCs); $^{d}P < 0.01$ between between BM-MSCs treated with 25 μmol/L forskolin and *Crif1* knockout BM-MSCs treated with 25 μmol/L forskolin; $^{f}P < 0.01$ between Crif1-overexpressing BM-MSCs and Crif1-overexpressing BM-MSCs treated with 20 μmol/L H-89, and the bars represent the mean ± SD. BM-MSCs: Bone marrow mesenchymal stem/stromal cells; BM-MSCs-KO: *Crif1* knockout BM-MSCs; OPG: Osteoprotegerin; RANKL: Receptor activator of nuclear factor κB ligand; CREB: Cyclic adenosine monophosphate response element-binding protein.

Because of the central role of RANKL in osteoclastogenesis, it is the basis for a new therapy to inhibit bone loss. We used ClusPro and InterProSurf to investigate the interaction interface in Crif1-PRKACA complex, and the results showed that Thr[197], Gly[200], Thr[201], Glu[203], and Phe[129] of PRKACA interact with Ile[132], Met[128], Ile[121], His[120], and Arg[117] in the long alpha-helical region of Crif1, forming the binding interface (Figure 6A). Virtual screening using 462608 compounds from the Life Chemicals database around His[120] of Crif1 was carried out using the program Autodock_vina. A set of 13 compounds was selected for experimental screening based on binding energy < - 12.0 kcal/mol (Supplementary Table 1). The binding pattern in the best-scored ligand−protein complexes potentially contained multiple interactions dominated by hydrophobic amino acids (Figure 6B-F). Initially, a tetrazolium salt (WST-8) assay was carried out to study the potentially toxic effects of these compounds on different cell lines, including human BM-MSCs, Vero cells, and mouse BM-MSCs (Supplementary Figures 1-3). The compounds F0382-0033, F3408-0076, F1430-0134, F3408-0031, and F1430-0130 showed low toxicity to hBM-MSCs at a concentration of 25 μmol/L (Figures 6G-6K). To determine whether these compounds could affect RANKL expression, H-BM-MSCs were pretreated with these compounds followed by treatment with forskolin. ELISA analysis of supernatant medium revealed that RANKL expression was dramatically decreased by treatment with five Crif1 inhibitors (Figure 7A). OPG expression could be significantly increased by F1430-0134, but there were no significant differences between the other four compounds and the control (Figure 7B). Moreover, RANKL/OPG ratios were also decreased by these five compounds compared with the control (Figure 7C). Crif1 is involved in adipogenesis[24], and adipocytes are also the source of RANKL in the bone marrow[27]. In order to study the inhibitory effects of these five compounds on adipogenic differentiation of BM-MSCs, H-BM-MSCs were pretreated with five different compounds (25 μmol/L) followed by adipogenic induction. Oil red O staining indicated that adipogenic differentiation of H-BM-MSCs was remarkably suppressed by the addition of these five compounds (Figure 7D and E). To further understand the mechanism, CREB phosphorylation was detected. Western blot analysis showed that CREB phosphorylation activated by forskolin was significantly inhibited by pretreatment with the five compounds (Figure 7F). These results showed that Crif1 inhibitors could effectively suppress RANKL secretion and adipogenesis by inhibiting

CREB phosphorylation.

DISCUSSION

Radiation exposure (due to radiotherapy, accidental causes, or terrorism) causes irreparable damage to tissues and organs, and both the bone marrow and bone architecture are devastated following radiation exposure. Irradiation causes rapid depletion of bone marrow, total extracted bone marrow cells in the irradiated mice, including the hematopoietic cell niches, collapsed by 65% ± 11% after 2 d, remaining at those levels through 10 d[29]. In contrast, the number of $CD90^+$, $CD29^+$, $CD45^-$, and $CD11b^-$ BM-MSCs increased relatively[24]. Irradiation also changes the bone marrow microenvironment, and adipocytes are significantly increased after radiation in the medullary cavity, which can negatively regulate the hematopoietic microenvironment, inhibit hematopoiesis, and accelerate osteoclastogenesis[30,31]. Irradiation suppresses bone formation and elevates bone resorption, disturbing the balance between them and leading to a dramatic decline in trabecular bone[32]. There is a near-immediate reduction in the number of osteoblasts, but an increased number and activity of osteoclasts post-radiation therapy[33]. In this study, we treated mice with a single dose of 5 Gy to generate a model of radiation-induced osteoporosis. RANKL expression and RANKL/OPG ratios actually increased in the surviving bone marrow cells after radiation, which was consistent with a previous study[14]. Meanwhile, expression of Crif1 and bone resorption also increased, indicating a relationship between RANKL and Crif1 in osteoporosis.

BM-MSCs, which are a major and important component of the bone marrow microenvironment, are the progenitors of both bone marrow osteoblasts and adipocytes. The balance between osteogenic and adipogenic differentiation of BM-MSCs plays a pivotal role in supporting hematopoiesis and maintaining bone homeostasis[34,35]. Crosstalk between macrophages and BM-MSCs within the bone marrow is also important for bone homeostasis. Different macrophage phenotypes exert different biological effects on the differentiation of BM-MSCs. Moreover, the modulatory effects of BM-MSCs on osteoclast progenitors, such as the monocyte-macrophage lineage, could be mediated by secretion of soluble factors[36,37]. Compared to other cells in bone marrow, BM-MSCs are more resistant to radiation, so the remaining BM-MSCs that escape radiation killing is crucial for the bone marrow microenvironment[38]. Following radiation exposure, BM-MSCs appear to preferentially differentiate into adipocytes instead of osteoblasts. This can ultimately hinder the proper bone formation and lead to disorders associated with bone loss (*e.g.*, osteoporosis) or increased adipocyte content, ultimately leading to hematopoietic progenitor cell depletion[12]. Excessive numbers of adipocytes are often found in the bone marrow of patients with osteoporosis. It is indicated that the shift of the cell differentiation of BM-MSCs to adipocytes rather than osteoblasts partly contributes to osteoporosis[39]. A recent study revealed that bone marrow adipocytes can also secrete RANKL and accelerate osteoclastogenesis[27]. We previously reported that Crif1 can promote the adipogenesis of BM-MSCs after radiation. Here, we found that Crif1 could also promote RANKL secretion by BM-MSCs after radiation, and the deletion of *Crif1* in BM-MSCs and Crif1 inhibitors can reduce both adipogenesis and RANKL expression.

RANKL functions as an osteoclast-activating factor, and its binding to RANK induces the activation of transcription factors such as c-fos, NFAT, and nuclear factor kappa B in preosteoclasts and initiates several downstream signaling pathways, especially the NF-κB pathway[40]. As RANKL is the only known ligand for RANK, and RANK and RANKL are crucial in bone metabolism, it is important to understand how the expression levels of RANKL are regulated under normal and disease conditions. RANKL expression can be upregulated by many agents, such as PTH and forskolin. Forskolin can stimulate RANKL expression through the cAMP/PKA signaling pathway[41]. It has been proved that cytokines and hormones which promote osteoclast formation act first on osteoblast lineage cells to promote the production of RANKL[42]. In this study, following forskolin exposure, RANKL expression increased significantly in BM-MSCs. However, the deletion of Crif1 from BM-MSCs impairs the promotion of RANKL expression by forskolin. Moreover, overexpression of Crif1 in BM-MSCs does not increase RANKL expression upon exposure to a PKA inhibitor. Here, we further demonstrated that Crif1 could also promote RANKL expression through the cAMP/PKA signaling pathway.

Drugs for the treatment of osteoporosis could be divided into anabolic and antiresorptive categories. Bisphosphonates (including alendronate and ibandronate, target osteoclast), estrogen, selective estrogen receptor modulators, and denosumab

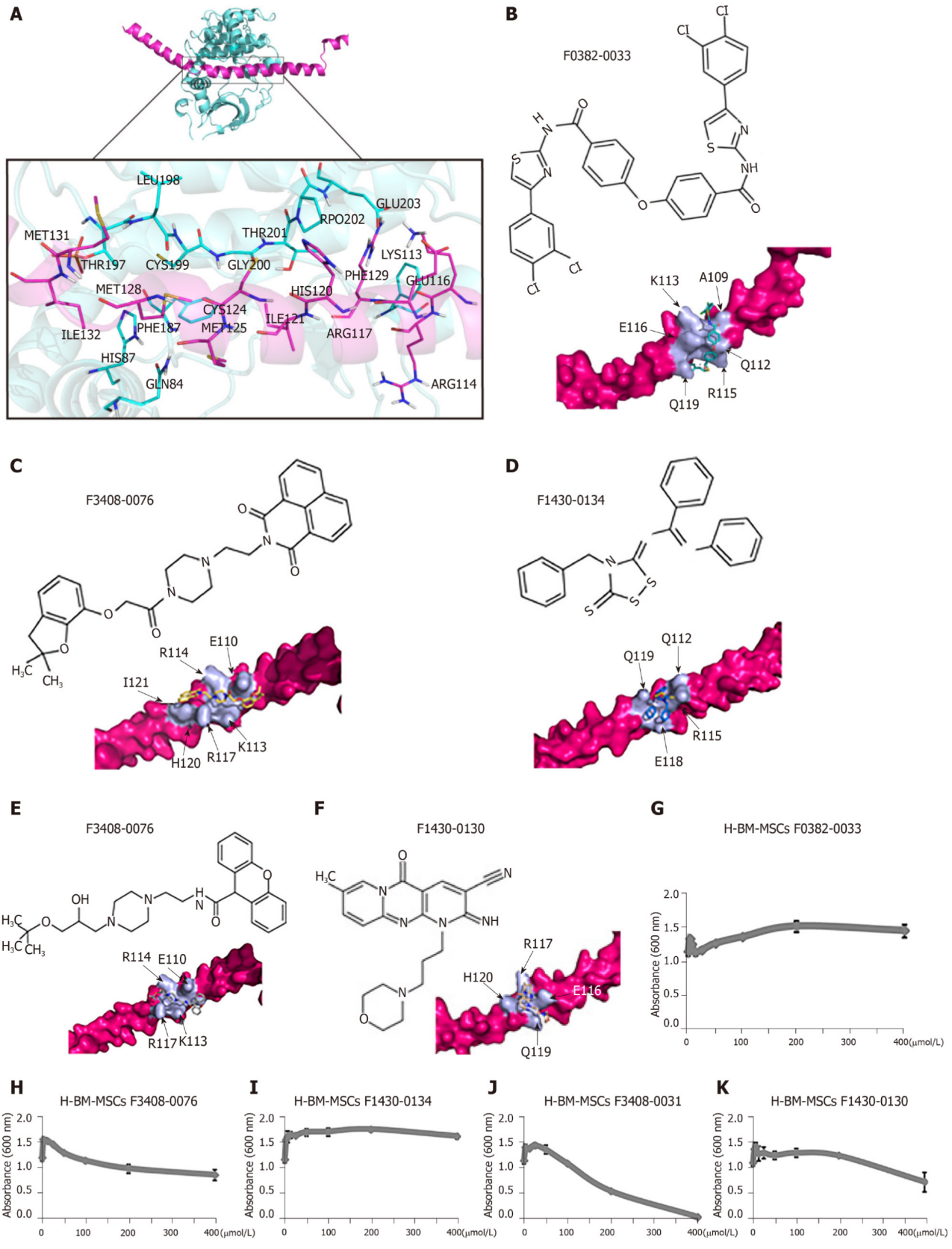

Figure 6 CR6-interacting factor-1-protein kinase cyclic adenosine monophosphate-activited catalytic subunit alpha interaction model and the inhibition potential of the lowest toxic effect of compounds. A: CR6-interacting factor-1 (Crif1)-protein kinase cyclic adenosine monophosphate-activited catalytic subunit alpha (PRKACA) interaction model showing Crif1 (colored in rose red) and PRKACA (colored in cyan). Interface amino acids are shown as sticks and colored in rose red (for Crif1) and cyan (for PRKACA) and indicated as a zoomed-in view in the inset figure; B-F: Chemical structure of each inhibitor molecule and their docked pose on Crif1 (colored in rose red, surface view). Docked molecule (stick) and the amino acids involved in the hydrophobic interactions (light purple) are shown. F0382-0033 (B), F3408-0076 (C), F1430-0134 (D), F3408-0031 (E), and F1430-0130 (F); G-K: A tetrazolium salt (WST-8) assay was carried out to study the toxic effect of compounds on the H-BM-MSCs. F0382-0033 (G), F3408-0076 (H), F1430-0134 (I), F3408-0031 (J), and F1430-0130 (K). The bars represent the mean ± standard deviation. H-BM-MSCs: Human bone marrow mesenchymal stem/stromal cells.

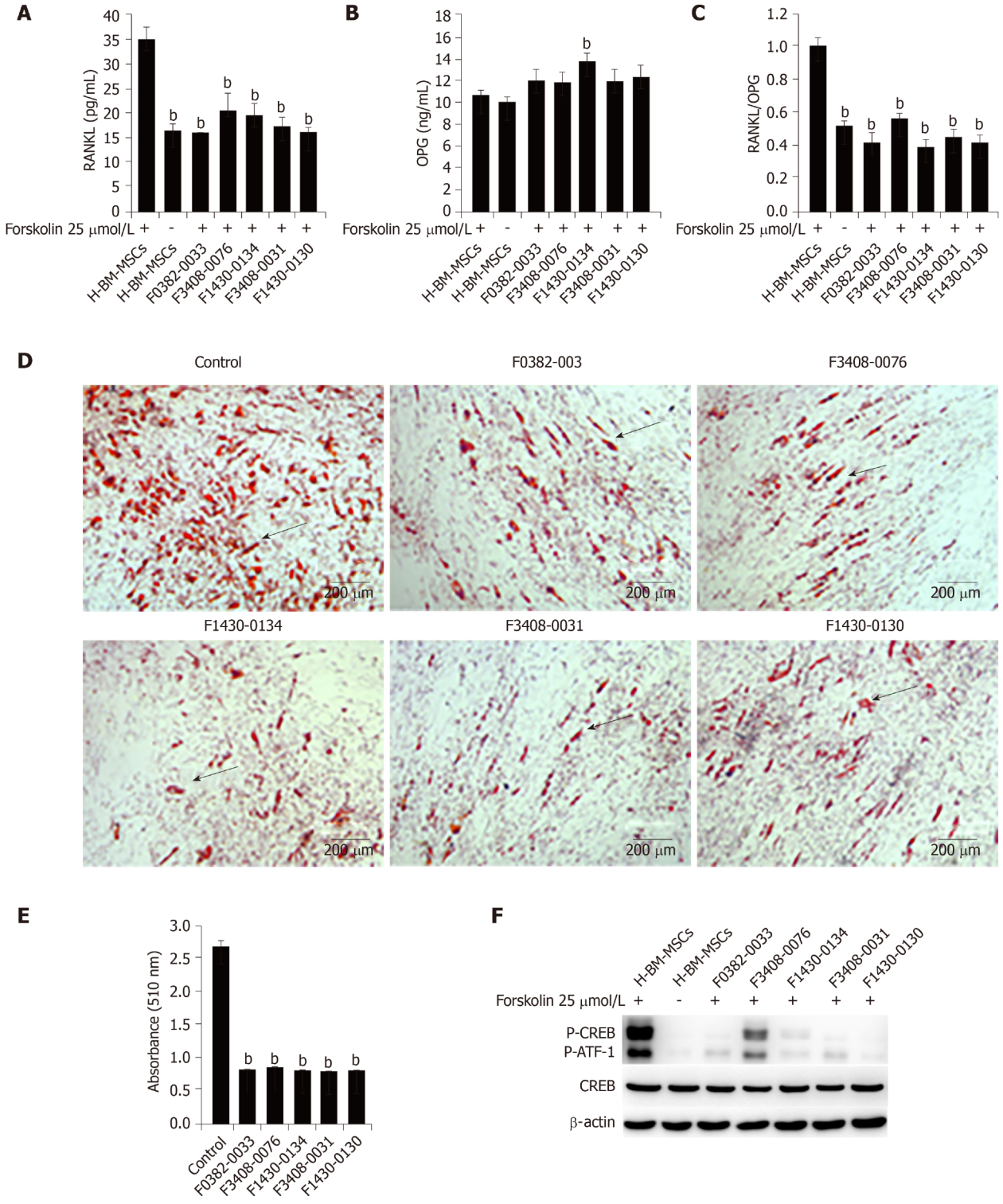

Figure 7 CR6-interacting factor-1 inhibitors effectively suppress receptor activator of nuclear factor κB ligand secretion and adipogenesis. A: Enzyme linked immunosorbent assay (ELISA) analysis of receptor activator of nuclear factor κB ligand protein levels in the supernatant medium. Human bone marrow mesenchymal stem/stromal cells (H-BM-MSCs) were pretreated with five different compounds (25 μmol/L) followed by treatment with forskolin (25 μmol/L), and supernatant medium was collected for ELISA after 3 d; B: ELISA analysis of osteoprotegerin protein levels in supernatant medium; C: Receptor activator of nuclear factor κB ligand/osteoprotegerin ratio in supernatant medium; D: Oil red O staining analysis of H-BM-MSCs after 21 d of adipogenic differentiation. H-BM-MSCs were pretreated with five different compounds (25 μmol/L) followed by adipogenic induction; E: The dye from oil red O staining was extracted using isopropanol, and the optical density at 510 nm was measured using Benchmark Plus; F: Western blot analysis of cyclic adenosine monophosphate response element-binding protein phosphorylation levels. H-BM-MSCs were pretreated with five different compounds (25 μmol/L) followed by treatment with forskolin (25 μmol/L) and total protein lysates were extracted for cyclic adenosine monophosphate response element-binding protein phosphorylation detection after 1 h. $^{a}P < 0.05$, $^{b}P < 0.01$, and the bars represent the mean ± SD. OPG: Osteoprotegerin; RANKL: Receptor activator of nuclear factor κB ligand; CREB: Cyclic adenosine monophosphate response element-binding protein; H-BM-MSCs: Human bone marrow mesenchymal stem/stromal cells.

(an antibody to RANKL) are antiresorptive drugs, whereas PTH and its analogs are

anabolic agents[43-45]. However, long-term trials showed that these drugs had no effects on the prevention of hip fractures. Moreover, the adverse effects of these anti-osteoporotic drugs should also be considered, such as hypocalcemia, arthralgia, nausea, and especially the development of breast cancer and risks of cardiovascular events and thromboembolism associated with treatment with estrogen and selective estrogen receptor modulators[46]. Mesenchymal stem/stromal cells (MSCs) are one of the favorite sources of cell-based therapy. MSCs-based preclinical studies in animal models suggest a great clinical application potential of both BM-MSCs and adipose tissue-derived MSCs for osteoporosis treatment. However, the bone marrow homing efficiency, the long-term survival, and the uncertainty of MSCs' fate after cell transplantation are the main concerns on the clinical application of MSCs for osteoporosis[47]. Therefore, it is necessary to look for alternative treatments with high efficiency but a few side effects. Because of the importance of RANKL in osteoclast differentiation, RANKL-secreting cells, which play a central role in osteoclastogenesis, are the targets of most antiresorptive agents[48,49]. BM-MSCs could be the target cells, and improving bone marrow microenvironment based on the remaining BM-MSCs in the bone marrow of patients and accelerating osteogenesis based on their fate decision to osteoblast or adipocyte could be a potential treatment for patients with radiation-induced bone injury. Our studies demonstrated that Crif1 could promote RANKL expression and adipogenic differentiation of BM-MSCs after radiation, and this may provide a potential molecular target for osteoporosis treatment. Osteogenesis and adipogenesis in the bone marrow are inversely correlated, so reduced adipogenesis results in an increase in the osteoblast pool[50,51]. Future drug screening should target not only the regulation of the balance between bone formation and bone resorption but also the balance between osteogenic and adipogenic differentiation.

In conclusion, we have demonstrated that Crif1 plays a crucial role in osteoclastogenesis by inducing RANKL expression through the cAMP/PKA signaling pathway in mice. Moreover, through screening, we have identified five Crif1 inhibitors targeting Crif1-PRKACA interaction interface that could dramatically reduce RANKL secretion and adipogenesis. But the specificity of these five compounds and their effects on bone metabolism, such as increasing bone formation and decreasing bone resorption and adipogenesis, still need further *in vitro* and *in vivo* research, and to be validated in clinical trials. This study nevertheless enriches current knowledge of the pathogenesis of radiation-induced osteoporosis and provides insights into potential therapeutic strategies for radiation-induced bone injury.

ARTICLE HIGHLIGHTS

Research background

Radiation induces rapid bone loss and enhances bone resorption and adipogenesis, leading to an increased risk of bone fracture. Receptor activator of nuclear factor κB ligand (RANKL) provides the crucial signal to induce osteoclast differentiation and plays an important role in bone resorption. However, the mechanisms of radiation-induced osteoporosis are not fully understood.

Research motivation

Current treatment of osteoporosis is based mainly on inhibiting bone resorption or stimulating bone generation to increase bone mass, however, the side-effects of some drugs affect long-term administration and adherence. There is still a lack of effective preventive or therapeutic method for radiation-induced bone injury. Therefore, it is necessary to look for alternative treatments with high efficiency but few side effects.

Research objectives

In this stduy, we aimed to investigate the role of CR6-interacting factor-1 (Crif1) in osteoclastogenesis after radiation and its possible mechanism.

Research methods

C57BL/6 mice were exposed to Co-60 gamma rays and received 5 Gy of whole-body sublethal irradiation at a rate of 0.69 Gy/min. For *in vitro* study, mouse bone marrow mesenchymal stem/stromal cells (BM-MSCs) were irradiated with Co-60 at a single dose of 9 Gy. For osteoclast induction, monocyte-macrophage RAW264.7 cells were cocultured with mouse BM-MSCs for 7 d. ClusPro and InterProSurf were used to investigate the interaction interface in Crif1 and protein kinase cyclic adenosine monophosphate (cAMP)-activited catalytic subunit alpha (PRKACA) complex. Virtual screening using 462608 compounds from the Life Chemicals database around His120 of Crif1 was carried out using the program Autodock_vina. A tetrazolium salt (WST-8) assay was carried out to study the toxicity of compounds to different cells, including human BM-MSCs, mouse BM-MSCs, and Vero cells.

Research results

Crif1 expression increased in bone marrow cells after radiation in mice. Overexpression of Crif1 in mouse BM-MSCs and radiation exposure could increase RANKL secretion and promote osteoclastogenesis *in vitro*. Deletion of Crif1 in BM-MSCs could reduce both adipogenesis and RANKL expression, resulting in the inhibition of osteoclastogenesis. The deletion of Crif1 in RAW264.7 cells did not affect the RANK expression or osteoclast differentiation. Following treatment with protein kinase A (PKA) agonist (forskolin) and inhibitor (H-89) in mouse BM-MSCs, Crif1 induced RANKL secretion *via* the cAMP/PKA pathway. Moreover, we identified the Crif1-PRKACA interaction interface by *in silico* studies and shortlisted interface inhibitors through virtual screening on Crif1. Five compounds dramatically suppressed RANKL secretion and adipogenesis by inhibiting the cAMP/PKA pathway.

Research conclusions

Crif1 promotes RANKL expression *via* the cAMP/PKA pathway, which induces osteoclastogenesis by binding to RANK on monocytes-macrophages in the mouse model. These results suggest a role for Crif1 in modulating osteoclastogenesis and provide insights into potential therapeutic strategies targeting the balance between osteogenesis and adipogenesis for radiation-induced bone injury.

Research perspectives

Because of the contribution of adipocytes to osteoporosis, future drug screening should target not only the regulation of the balance between bone formation and bone resorption but also the balance between osteogenic and adipogenic differentiation. Here, through screening, we identified five Crif1 inhibitors targeting Crif1-PRKACA interaction interface that could dramatically reduce RANKL secretion and adipogenesis. Our study provides insights into potential therapeutic strategies for radiation-induced bone injury.

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