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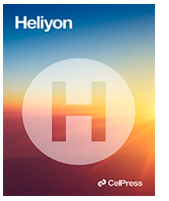

Review article

Progress in the application of graphene and its derivatives to osteogenesis

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ABSTRACT

As bone and joint injuries from various causes become increasingly prominent, how to effectively reconstruct and repair bone defects presents a difficult problem for clinicians and researchers. In recent years, graphene and its derivatives have been the subject of growing body of research and have been found to promote the proliferation and osteogenic differentiation of stem cells. This provides a new idea for solving the clinical problem of bone defects. However, as as numerous articles address various aspects and have not been fully systematized, there is an urgent need to classify and summarize them. In this paper, for the first time, the effects of graphene and its derivatives on stem cells in solution, in 2D and 3D structures and in vivo and their possible mechanisms are reviewed, and the cytotoxic effects of graphene and its derivatives were summarized and analyzed. The toxicity of graphene and its derivatives is further reviewed. In addition, we suggest possible future development directions of graphene and its derivatives in bone tissue engineering applications to provide a reference for further clinical application.

1. Introduction

At present, bone and joint damage caused by inflammation, trauma, tumors, aging, and other problems are increasingly prominent, and we have an increasing need to repair or replace bone and other damaged tissues. Traditional methods for treating bone defects include autologous bone grafting and distraction osteogenesis. However, these methods usually require a long time, have high technical requirements, and even cause new bone defects and bone nonunion at the donor site [1,2]. In recent years, with the innovation and development of biomaterials, tissue-engineered scaffolds combined with nanotechnology and stem cell technology have emerged, providing a new idea for the treatment of bone defects and a promising alternative to the existing traditional repair strategies [3,4].

Among stem cells, mesenchymal stem cells (MSCs) are widely used in medical research because of their ability to regenerate and differentiate into specific cells, such as osteoblasts, adipocytes, muscle cells, and chondrocytes [5,6]. MSCs are pluripotent stem cells derived from umbilical cord blood, peripheral blood, dermis, bone marrow, adipose tissue, fetal liver, and lung [7,8], and have the ability to self-renew and differentiate into various cell types [9]. MSCs derived from autologous or allogeneic sources have shown great superiority in many cell-mediated therapies [10]. Because MSCs can migrate to damaged sites to replace dysfunctional cells and restore

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function. Therefore, they can provide a good platform for the development of regenerative medicine, tissue repair, and other biomedical fields. Bone marrow mesenchymal stem cells (BMSCs) are the most widely used mesenchymal stem cells in the field of orthopedics. They can be isolated and cultured from the bone marrow of the femur and tibia. When induced by specific factors, they have the ability to differentiate into bone, cartilage, and muscle and can be used for the regeneration of musculoskeletal tissue [11,12].

Stem-based therapies in regenerative medicine and tissue engineering often require the presence of scaffolds to deliver cells or growth factors to the site of injury. Engineers in the field of tissue engineering build scaffolds from natural or synthetic materials. This is a new way to repair and rebuild damaged tissue, and to grow specific tissue that functions as well as or better than natural tissue [13]. Of all the cells in the human body, osteocytes are also one of the most special types because they require a carefully designed scaffold to engineer living bone [14]. After bone injury occurs, soluble factors accumulate at the site of injury and absorb MSCs, which in turn proliferate and differentiate into osteoblasts to repair the injury, and then calcify in the damaged area to form braided bone, which is then reshaped by the regeneration and absorption of osteoblasts and osteoclasts [15].

It has been found that the correct chemical composition [16] and surface properties [17] play a key role in the growth and proliferation of bone cells. Therefore, it is necessary to further study the chemical composition of scaffold surfaces, such as nanomaterials, and metal and nonmetal nanoparticles, which also play a key role in tissue engineering [18].

Among all nanomaterials, graphene and its derivatives have been the most studied in recent years. Graphene was first isolated in 2004 by the mechanical exfoliation of graphite [19]. It is a two-dimensional (2D) nanostructure made of SP^2 carbon atoms, just one atom thick, and is considered the strongest material to date [20]. Furthermore, graphene is one of the most promising and widely studied materials in nanotechnology due to its excellent electronic, mechanical, and optical properties [21]. In addition, graphene has many advantages such as extraordinary biological properties, excellent electrical conductivity, mechanical strength, large surface area, good elasticity, renewability, low cost, and easy acquisition [22–24]. In addition to graphene, many derivatives of graphene have been extensively studied due to their similar or complementary properties [25]. The main graphene derivatives include pristine graphene, graphene oxide (GO), reduced graphene oxide (RGO), graphene quantum dots (GQDs), graphene nanosheets, monolayers, multilayer graphene, and graphene-based nanocomposites [26,27]. Graphene and its derivatives have great advantages because they are low in metal impurities and do not require complex purification processes [28], and because of their high electrical conductivity, mechanical properties, and aspect ratio, they have been widely studied in many fields recently, such as water purification [29,30], electronics [31,32], sensors [33,34], composites [35,36], energy storage and conversion devices [37,38], and antibacterial and antibiotic membranes [20,39].

Various studies have found that the mechanical properties of graphene and its derivatives are affected by different interface binding modes, oxygen content, and different manufacturing processes, which makes the mechanical properties of graphene and its derivatives adjustable [40–42]. Therefore, graphene and its derivatives can theoretically mimic the mechanical properties of human bone and have broad prospects in the field of bone tissue engineering. Moreover, graphene and its derivatives have been proven to have biological activities and have shown strong bone tissue engineering capabilities, noncytotoxicity to osteoblasts, and good antibacterial and antibiofilm activity [20,28,39]. Among the graphene derivatives, GO and RGO are the most studied due to the large number of oxygen functional groups that can be used as active sites for further functionalization [43].

To obtain a more comprehensive understanding of the application of graphene and its derivatives in bone tissue engineering, this paper summarizes and reviews the related articles published in recent years. In addition, to facilitate generalization, the effects of graphene and its derivatives on orthopedic-related stem cells in solution, 2D, 3D, and in vivo will be described.

2. Graphene and its derivatives promote the proliferation and differentiation of induced bone cells in solution

Graphene and-most of its derivatives have been shown to be cytocompatible in vitro and in vivo. Furthermore, the physical and

Table 1
Effect and mechanism of graphene oxide on stem cells in solution.

Graphene	Substrate	Preparation method of solution	Cell	Outcomes	Mechanism	Reference
GO	Suspension	GO was added to the culture medium	mESCs (46C)	Maintained self-renewal	Integrin signaling pathway Decreased expression of Vinculin	[51]
GO	Suspension	Ultrasonic dispersive dissolution	BMSCs	Promoted cell proliferation	Wnt/β-catenin signaling pathway	[52]
GO	Suspension	Direct pyrolysis of citric acid	SHEDs	Promoted SHED proliferation and osteogenic differentiation	Wnt/β-catenin signaling pathway	[53]
GOQDs	Suspension	Purchased	BMSCs	Improved the osteogenic differentiation ability of BMSCs	Activating the Wnt/β-catenin signaling pathway	[54]
MGO	Suspension	Magnetic mixing of mixtures	BMSCs	Promoted osteogenic differentiation	Through the Wnt/β-catenin pathway	[55]
GO-CaP	Suspension	Mixing microemulsions	hMSCs	Promoted osteogenic differentiation	Upregulation of the Wnt/β-catenin pathway	[56]

GOQDs: Graphene oxide quantum dots; BMSCs: Bone marrow mesenchymal stem cells; hMSCs: Human mesenchymal stem cells; GH: Graphene hydrogel; SMH: Sericin methacryloyl; GO-CaP: Graphene oxide-calcium phosphates; mESCs: Mouse embryonic stem cells. MGO: magnetic graphene oxide; PEG: Polyethylene glycol.

chemical properties of graphene and its derivatives, such as shape, structure, size, surface function, concentration, and aggregation state, have important effects on cell behavior. Therefore, graphene and its derivatives play an important role in stem cell research as cell growth substrates [44]. Conventional stem cell differentiation lacks cellular interaction, so graphene and its derivatives are a good choice for regenerative medicine to provide cell adhesion to stem cell cultures [45].

The culture, proliferation, and differentiation of stem cells in solution largely depend greatly on the structure, size, and properties of graphene and its derivatives [46,47]. Among all graphene derivatives, GO has been studied the most in solution. Compared with graphene, GO is highly hydrophilic and dispersive due to the presence of a variety of oxygen-containing functional groups and is also easily modified by other functional groups [48]. Moreover, GO has many advantages, such as high strength and good electrical conductivity, and is considered an ideal biocompatibility model for exploring the interactions that occur in MSCs. GO makes stem cells pluripotent, allowing them to differentiate into a variety of cell types. Thus, this study provides a simple, reproducible method for preserving the pluripotency of stem cells for stem cell therapy and tissue engineering applications [49]. GO can further improve stem cell adhesion, growth, and differentiation [50].

In Table 1, we list the stem cells whose proliferation is promoted by GO in solution. The results show that GO can maintain the self-renewal of stem cells, promote osteogenic differentiation, and promote cartilage repair and is safe and nontoxic to cells. The mechanisms of action are diverse, and the Wnt/β-catenin signaling pathway is the most common. In Fig. 1, we have plotted the schematic of graphene and its derivatives promoting stem cell proliferation and differentiation in solution and the common mechanisms.

As shown in Table 1, graphene and its derivatives have the ability to promote the osteogenic differentiation of stem cells in solution. Therefore, in small or irregularly shaped bone defects, we can culture graphene and its derivatives and stem cells in vitro, and then inject the solution into the bone defect site, which is worth trying in the future.

3. Graphene and its derivatives promote the proliferation and differentiation of induced bone cells on a 2D plane

Bone reconstruction is a major challenge and a growing problem in medicine, and stem cell-based therapies may be an ideal solution. However the key to using stem cells to address bone remodeling is that stem cells need to rely on biocompatible platforms to promote and enhance cell survival, attachment, migration, and differentiation. This is a prerequisite to finding platforms that promote stem cell differentiation and proliferation.

Graphene and its derivatives have been widely used in various biomedical applications due to their excellent biological properties. As described above, graphene and its derivatives promoted cell proliferation and differentiation in solution, and their effect on the 2D surface was further reviewed.

The most commonly used method of obtaining 2D graphene scaffolds is to grow one or more layers of graphene by CVD on a PMMA-supported metal catalyst and then physically transfer them to the substrate under study. For example, Jangho used the technique to transfer a monolayer of graphene onto glass to investigate its possible role in promoting hMSC neurogenesis and neurite growth [57]. In addition, charge interactions are used to coat graphene and its derivatives on 2D materials. For example, Jin et al. deposited GO on the surface of Ti by electrodeposition [58], and our research team similarly coated GO on the surface of cobalt-chromium-molybdenum alloy by electrodeposition [39]. Rasch et al. found that 2D graphene scaffolds can also be obtained by vacuum filtration of a material suspension [59].

Multiple studies have found that graphene and its derivatives exhibit enhanced cell adhesion, proliferation, and differentiation on polyethylene terephthalate (PET), glass, polydimethylsiloxane (PDMS), silicon, or silica substrates [60,61]. Cardiomyocyte differentiation of ESCs on graphene substrates can also be improved due to the roughness of the graphene-based coating. Zhang et al. demonstrated that stem cells cultured on graphene-coated surfaces differentiated into more neurons and fewer glial cells and adhered significantly better than stem cells cultured on glass slides [62].

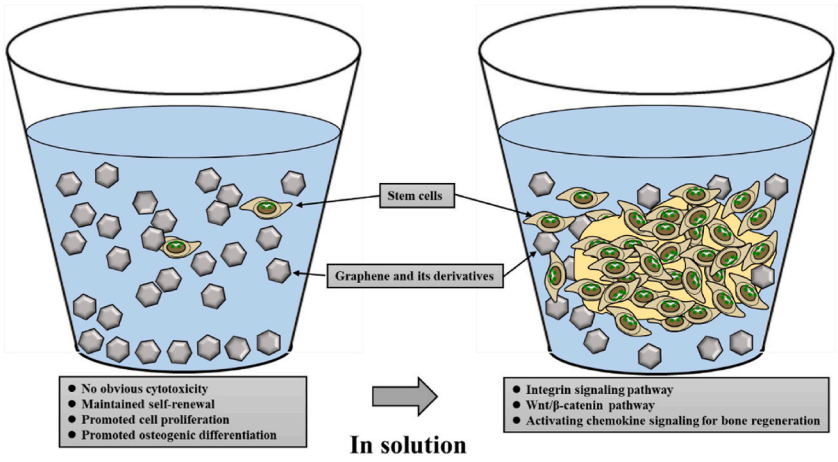

Fig. 1. Effects of graphene and its derivatives on stem cells in solution and common mechanisms.

Table 2
Effects and mechanisms of graphene and its derivatives on stem cells on 2D material surfaces.

Graphene	Fabrication method	Substrate	Coating method	Cell	Outcomes	Mechanism	Reference
G	CVD	Copper foils	CVD	DPSC	Promoted osteogenic mineralization	Wrinkles and ripples on the surface of graphene	[66]
G	CVD	PDMS	Physical transfer	MSCs	Promoted osteogenesis	The activation of the mechanosensitive integrin/FAK axis	[67]
G	CVD	Glass	Physical transfer	PDLSCs	Induced osteogenic differentiation	Physical properties of surfaces	[68]
G	CVD	PDMS	Transfer technique	BMSCs	Promoted osteogenic differentiation	Chemical binding of growth agents	[69]
G	CVD	SiO_2	Physical transfer	hMSCs and SAOS-2	Promoteds adhesion and proliferation	N/A	[70]
G	CVD	SiO_2	Physical transfer	hMSCs	Promoteds proliferation and osteoblast differentiation	N/A	[71]
G	Chemical reduction of GO	Cellulose ester membrane	Filter membrane vacuum filtration	BMSCs	Improved biocompatibility and osteoinductive	Exceptional mechanical property	[72]
G-CS	N/A	Ti6Al4V	Plasma-spraying	hMSC	Promoted hMSCs adhesion	N/A	[73]
GO	Purchased	Glass	Air-sprayed	MSCs	Promoted adhesion proliferation and osteogenic differentiation	Physical properties of GO	[74]
GO	MHM	TI	Soak (electrostatic adsorption)	BMSCs	Significantly improved osteogenic differentiation	Electrostatic and hydrophobic interactions	[75]
GO	MHM	GHA	lyophilization	hMSCs	Improved mechanical strength and osteogenic differentiation potential	N/A	[76]
GO	MHM	GA	Freeze drying technique	MG-63	Promoted cell adhesion and proliferation	N/A	[65]
GO	MHM	GA	Freeze drying technique	hMSCs	Promoted osteogenesis	N/A	[65]
GO	MHM	Glass	Oven-drying method	C3H10T1/2	Enhanced cellular adhesions, and osteogenic differentiation	N/A	[77]
GO	MHM	TI	Ultrasonic atomization spraying technique	BMSCs	Promoted cell proliferation, adhesion, diffusion and osteogenic differentiation	FAK/P38 signaling pathways	[78]
GO	MHM	MBG	High temperature calcination technique	rBMSCs)	Higher osteogenesis differentiation ability	ALP, RUNX2, OCN and COL1 gene secretion were enhanced	[79]
GO-CaP	N/A	N/A	Double-reverse microemulsion method	hMSC	Improved osteoinduction	Surface characteristics	[80]
GO/PLL	HM	Glass	Alternately assembled deposited	BMSCs	Promoted proliferation and osteogenic differentiation	Noncovalent bonding and electrostatic interaction	[81]
GO/CS	MHM	Glass	Incorporation of PDA	HEF1 fibroblast Cells and CMs	Increased cell viability and proliferation of HEF1 and CMs	Enhanced electrical communication between cells	[82]
rGONR	N/A	PDMS	Paintbrushing method	hMSCs	Promoted osteogenic differentiation	Adsorption capacity of chemical inducers and physical stress caused by nano characteristics	[83]
BC-RGO	MHM	N/A	Bacterial reduction method	hMSCs	Increased cell proliferation	N/A	[84]
rGO-Chitosan	N/A	Glass	Spin-coating	hMSCs	Enhanced adhesion and differentiation	Unique nano topography	[85]
rGO	Hydrazine hydrate reduction	Ti	MDD	hMSC	Promoted cell proliferation, ALP activity and matrix mineralization	N/A	[86]
rGO	Converted from GO	Ti	Sandblasted	hMSCs	Promoted osteogenesis	Promote the expression of matrix mineralization, osteogenesis related genes and proteins	[87]

N/A: Not applicable; MHM: Modified Hummer method; PDMS: Polydimethylsiloxane; BMSCs: Bone marrow mesenchymal stem cells; hMSCs: Human mesenchymal stem cells; GH: Graphene hydrogel; CS: Calcium silicate; GO-CaP: Graphene oxide-calcium phosphates; GHA: Gelatin–hydroxyapatite. PLL: Poly-L-lysine. GA: Gelatin-alginate; MBG: Mesoporous bioactive glass; Peptide/m-GO: Peptide-conjugated multilayer graphene oxide; WJ-MSCs: Wharton's jelly derived mesenchymal stem cells. CS: Chitosan. PDA: Mussel-inspired protein polydopamine; CMs: Cardiomyocytes; BC: Bacterial cellulose nanofibers; DPSC: Dental pulp stem cells; PDLSCs: Periodontal ligament stem cells. hTMSCs: Human tonsil-derived mesenchymal stem cells. PU: Polyurethane foam.

Studies have found that the concentration of GO can affect the colonization of cells on scaffolds [63]. In addition, the dry and wet state of the scaffold will also affect its final performance. Magaz et al. found that the metabolic activity, conductivity, and cell proliferation of scaffolds composed of silk fibroin protein and GO or RGO increased after hydration [64]. Furthermore, graphene and its derivatives can improve the mechanical strength of some scaffolds. Purohit found that the addition of GO significantly increased the compressive strength of nanocomposite scaffolds compared to alginate gelatin scaffolds without GO [65].

In Table 2, we list the proliferation of stem cells promoted by graphene and its derivatives on a 2D plane. The results show that graphene and its derivatives can not only improve the mechanical strength of 2D material surfaces but also promote the adhesion, proliferation, osteogenic differentiation, and bone induction of stem cells. Related mechanisms include the surface properties of graphene, electrostatic and hydrophobic interactions, noncovalent bonds and electrostatic interactions. Integrin/FAK pathways are also involved. In Fig. 2, we show the schematic and common mechanisms by which graphene and its derivatives promote stem cell proliferation and differentiation on 2D material surfaces. In the clinical application of orthopedics and stomatology, the osteogenesis of 2D material surfaces is very important, such as bone growth on the surface of dentures and artificial joint surfaces. Therefore, the coating of graphene and its derivatives on 2D materials has important clinical application value. Although 2D scaffolds have great potential, they also have limitations. For example, 2D environments are not suitable for reproducing natural ECM or for the diffusion of metabolic waste within a limited range. To overcome these limitations, 3D scaffolds have been increasingly studied and designed in recent years.

4. Effects of graphene and its derivatives on cells on 3D material surfaces

In our previous review, we found that graphene and its derivatives in solution and on 2D surfaces provide a good microenvironment for MSCs and can regulate cell proliferation and differentiation, but their application on 3D material surfaces in vivo is the focus of tissue engineering. Compared to cultures in solution and on the surface of 2D materials, 3D systems or 3D material surfaces are more conducive to BMSC culture. The 3D system is beneficial to maintain the natural morphology of cells in vivo, can promote proliferation according to specific materials and cell types, and can directly differentiate under the guidance of specific materials and surface topography. The generation, maintenance, and repair of tissue are closely related to the regulation of 3D structure [68,88]. In addition, the expression of differentiation-related genes and proteins can be uregulated. Therefore, it is very important to simulate the 3D culture environment of BMSC growth in vitro [89].

3D scaffolds are designed to promote cell growth and improve MSC collagen deposition, cell proliferation, and osteogenesis [90]. Therefore, such scaffolds should have a large surface area and porosity to transfer nutrients to cells [91]. Moreover, scaffolds with 3D structures have a highly interconnected porous system that can reproduce the biophysical properties of tissues, making them a better choice for use in vivo [92]. Wu et al. suggested that the high expression of Runx2, COL-I, and OCN in 3D graphene scaffolds in the absence of osteogenic differentiation-inducing mediators (OM) might be related to the spatial arrangement of the materials [93]. Lutzweiler et al. found that the shape, interconnection, curvature, porosity, and pore size of 3D scaffolds directly affect not only the migration of nutrients and waste in scaffolds but also infiltration and the communication between cells [94]. Studies have found that scaffolds with pore diameters between 100 and 750 μm are usually the best, while larger pores expose cells to a 2D-like environment that is different from the natural environment [95,96], and the simplest way to make porous scaffolds is by freeze-drying filtrate or suspension [92].

Currently, the use of stem cell-coupled 3D biomaterial scaffolds for tissue regeneration is a leading tissue engineering therapy [97]. Bone cells proliferate and differentiate in a 3D structure in the body. Graphene and its derivatives with 3D structures have appropriate biocompatibility, morphology, diverse chemical states, high physicochemical stability, appropriate flexibility, and in vivo degradation ability, and can control the differentiation of stem cells into specific lineages [21,98]. Moreover, graphene and its derivatives have conductivity similar to that of natural tissue and are considered promising scaffolds for cell proliferation and differentiation in stem cell therapy, as well as reduced cytotoxicity and improved mechanical properties [47]. Studies have shown that 3D-printed scaffolds

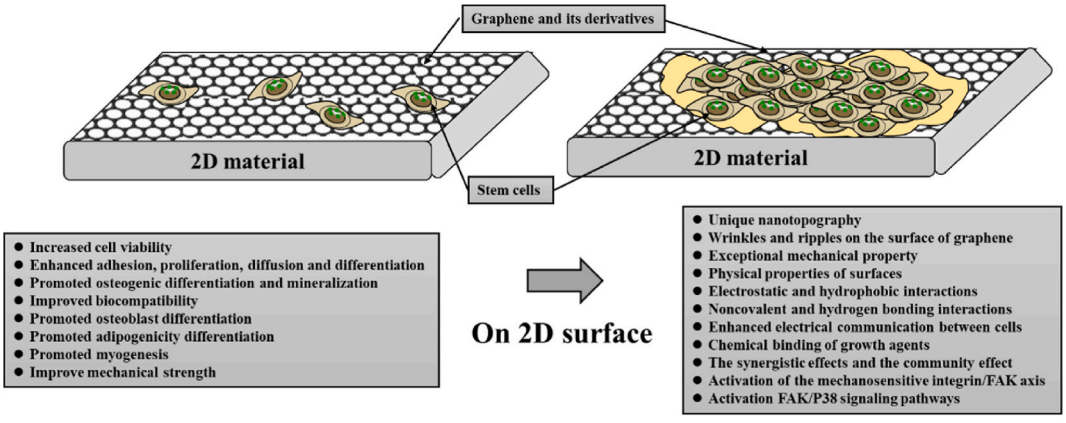

Fig. 2. Effects of graphene and its derivatives on stem cells on 2D material surfaces and common mechanisms.

containing graphene and its derivatives can not only simulate the geometry of bones but also the remodeling of bones. Therefore, graphene scaffolds are an important way to simulate the microenvironment of bone cell regeneration. For example, Wang prepared a novel 3D scaffold for G-caprolactone and found that the addition of a small amount of GO improved the mechanical properties and osteogenesis ability. Therefore, it can be used to treat bone defects and bone deposition [99]. Tapas et al. found that although graphene had no effect on cell proliferation or cell morphology, as shown by the MTT assay, graphene on the substrate was the driving force for bone formation [71].

The use of GO as a scaffold has significantly enhanced the interaction between cells and the surrounding environment, and the interaction between stem cells and GO scaffolds has been extensively studied in this framework, resulting in extensive results on stem

Table 3
Effect and mechanism of graphene and its derivatives on stem cells in 3D materials.

Graphene	Fabrication method	Substrate	Coating method	Cell	Outcomes	Mechanism	Reference
G	CVD	Ni foam	CVD	PDLSCs	Induced osteogenic differentiation	Physical properties of surfaces	[68]
3D GFs	Grows on the surface of Ni	Ni scaffold	Graphene grows on the surface of Ni	hMSC	Promoted hMSCs adhesion and viability and induce osteogenesis	N/A	[102]
G	MHM	2D GO	Hydrothermal method	BMSCs	Promoted adhesion and proliferation	N/A	[103]
G	Purchased	Hydroxyapatite	Deposition−precipitation method	MSCs	Promoted attachment and proliferation induced osteogenesis	Promote the expression of osteogenic markers	[104]
SGH	Chemical reduction of GO	Cellulose ester filter membrane	Filtration	rBMSCs	Enhanced cell adhesion, spreading, and proliferation	N/A	[72]
GO	N/A	CS	Covalent linkage	Osteoblasts	Facilitated cell attachment and proliferation and improved the stability against enzymatic degradation	The porous nature of the scaffold and charge interaction	[105]
GO	MHM	CaP	Soaking method	rBMSCs	Enhanced the adhesion and osteogenic differentiation	Activated the Erk1/2 signaling pathway, upregulated the expression of Hif-1α, increased BMP-2 expression	[106]
GO	Oxidation of graphene	PMMA	The powder is mixed and solidified	hBMSC	Promoted osteogenic mineralization	Affected the expression of BMP	[107]
GO	MHM	TCP and HA	Dipping method	BMSC	Promoted BMSC adhesion and proliferation	Nanostructure, charge balance	[108]
GO	N/A	ZnO microparticles	Solution-evaporation method	hMSCs and OEC	Improved adhesion and growth of MSC, promoted angiogenesis	Increased the capacity to bind VEGF	[109]
GO/PAA	Purchased	Hydrogel	Situ polymerization	BMSCs	Provide a more beneficial microenvironment for cell adhesion and growth	Increased adsorption of extracellular biomolecules	[110]
PEG-GO	MHM	Cryogel	Dissolved in PBS	hBMSCs	Stimulated osteogenic differentiation	Activation of adhesion kinase (FAK) signaling pathway	[111]
GO-SMH	MHM	Hydrogel	4 mg/ml GO 15 μL 255 μL SerMA Solution (15 %, w/v)	BMSCs	Induced BMSC differentiation and promoted bone regeneration	Activation of MAPK, TNF, and chemokine signaling for bone regeneration	[112]
RGO	Hydrazine hydrate reduces GO	HAP	Air dry suspension	hMSCs	Promoted osteogenic differentiation	N/A	[113]
RGO	Hydrazine hydrate reduces GO	**HAp**	Air dry suspension	MC3T3-E1	Promoted spontaneous osteodifferentiation	Significantly increased the expression levels of osteopontin and osteocalcin	[114]

MAM: Modified Arbuzov Method; 3D GFs: 3D Graphene foams. Hap: hydroxyapatite; PCL: poly(ε-caprolactone); TCP: Tricalcium phosphate; HA: Hydroxyapatite; SGH: Self-supporting graphene hydrogel; PAA: Poly (acrylic acid).

cell behavior [100]. Ali et al. prepared silicate-doped nanohydroxyapatite/GO composite-reinforced fibrous scaffolds, and found that the scaffolds significantly increased protein adsorption and had better adhesion, diffusion, proliferation, and alkaline phosphatase activities [101].

In Table 3, we list the effects of graphene and its derivatives on stem cell proliferation in a 3D structure. The results showed that graphene and its derivatives could promote stem cell adhesion, differentiation, and bone induction in 3D structures. This is related to the physical properties of the surface of graphene and its derivatives, the charge, the formed microenvironment, and the activation of the ERK1/2 pathway. We examined the effect of graphene and its derivatives on stem cells in 3D materials (Fig. 3).

In clinical work, bone defects are usually 3D, and for a large range of bone defects, 3D material filling is the best solution. We found that graphene and its derivatives can promote bone formation in 3D materials, so the application of 3D materials containing graphene and its derivatives in clinical large bone defects will greatly improve the treatment effect of bone defects.

5. Graphene and its derivatives promote cell proliferation and differentiation in vivo

In the previous section, we discussed the interaction of graphene and its derivatives with cells in solution, 2D, and 3D material surfaces, but our ultimate goal is to use these materials in vivo, so this section will elaborate on their effects on cells in vivo.

The most important application of graphene and its derivatives in vivo is its application in tissue engineering. Tissue engineering is a multidisciplinary technique that includes materials engineering, cell biology, chemistry, and immunology to develop biological materials that can restore, maintain or improve the function of tissues or organs. However, replicating a tissue or organ is extremely challenging because many different aspects must be considered. Each tissue or organ has specific mechanical and electrical properties. For example, bone regeneration medical scaffolds are relatively hard ($E > 10^9$ Pa) and neural scaffolds relatively soft ($E < 4 \times 10^2$ Pa), while muscle tissue requires a moderately stiff scaffold ($E > 10^4$ Pa) [115,116], so artificial scaffolds should be matched to the characteristics of the tissue and organ.

Bone has a remarkable ability to regenerate. However, when bone defects reach a certain size, human bone has a limited ability to regenerate itself. There are several different approaches to bone tissue engineering, the most common of which is an attempt to simulate the process of bone repair with 3D bone conduction scaffolds [47]. Scaffolds are biosubstitutes that not only enhance cellular interactions but also stimulate the differentiation of precursor cells and stem cells. In recent years, nanocomposites have been shown to effectively simulate the desired properties [92]. Graphene is one of the toughest and strongest nanomaterials ever discovered, and its addition to polymer scaffolds can improve its mechanical properties and tensile strength [117]. Graphene and its derivative scaffolds are special scaffolds made of graphene, GO, and/or RGO nanocomposites that have an excellent ability to adsorb proteins such as fibronectin, laminin, and albumin from serum and promote cell adhesion, proliferation, and differentiation [92,118].

The electrical conductivity of scaffolds plays an important role in functional electroactive tissue. Graphene and its derivatives have good electrical conductivity and their incorporation into polymer scaffolds can reduce the resistance of polymers [119]. Furthermore, graphene and its derivatives have structural characteristics and dimensions similar to many components of the extracellular environment, such as ion channels, collagen, signaling proteins, and cytoskeletal elements [120]. The use of graphene and its derivatives as scaffolds, which consist of porous networks to which cells can attach to obtain nutrients, has already provided huge therapeutic benefits for MSCs [121]. Therefore, graphene or its derivatives can be introduced into tissue engineering to give them customizable properties that match those of natural tissues.

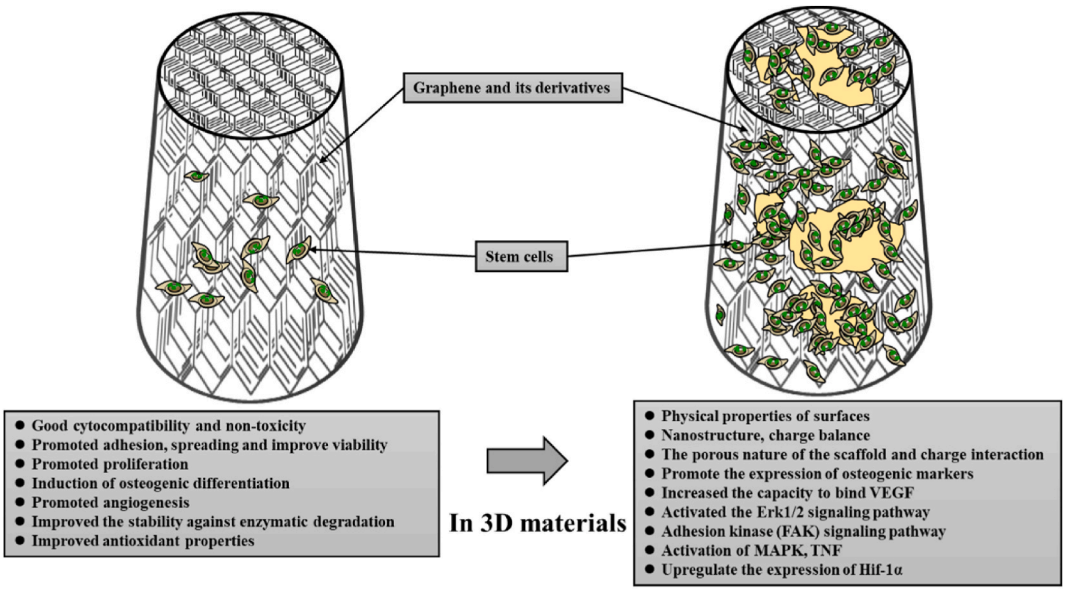

Fig. 3. Effects of graphene and its derivatives on stem cells in 3D materials and common mechanisms.

Table 4
Graphene and its derivatives promote bone formation in vivo.

Graphene	Tissue	Animal	Cell	Outcomes	Mechanism	Reference
G-caprolactone	Bone	Rat	SaOs-2 cells	No cytotoxicity and good osteogenic differentiation ability	N/A	[125]
G-caprolactone	Bone	Rat	MC3T3 preosteoblastic cells and THP-1 Human monocytic cells	Increased cell migration, new tissue formation, good calcium deposition, and bone remodeling	N/A	[125]
CHT/GO	Bone	Mice	3T3-E1 cell	Promoted new bone formation and expression of related bone morphogenetic proteins	N/A	[126]
GCs/GO	Bone	Mice	Osteoblast	Elevated osteoblast differentiation and bone formation	Enhanced the expression of runx2 and OPN	[127]
rGO-(nHA)	Bone	Rabbits	BMSCs	Promoted collagen deposition, cell proliferation, and new bone formation	N/A	[128]
Gelatin-rGO	Bone	Rat	BMSCs	Double differentiation of BMSCs was induced to achieve rapid bone repair	Regulated by Erk1/2 and AKT pathways	[129]
GOG	Bone	Mice	BMSCs	Stimulated BMSCs in accelerating bone remodeling	The activation PERK pathway	[130]
rGO	Bone	Rat	Adipose-derived MSCs	The expression of osteogenic genes was increased	N/A	[131]

PAAm: Polyacrylamide hydrogel; CHT: Chitosan; nHA: Nanohydroxyapatite; GCs: Gelatin/chitosan; GOG: Gelatin-reduced graphene oxide.

In tissue engineering scaffolds, different types and proportions of graphene and its derivatives can affect scaffold properties, such as surface roughness, cell adhesion, and interaction of scaffolds with growth factors, nutrients, and waste [122]. Thus, the mechanical properties of the tissue extracellular matrix can be simulated by adjusting the proportion of graphene in the scaffold. In addition, nanocomposite scaffolds composed of graphene and its derivatives have nanoroughness, which is not only conducive to cell anchoring but also can regulate cell morphology [92,120]. Dinesh et al. found that GO could serve as a protective layer for implants in bone tissue engineering and is an ideal scaffold for bone tissue regeneration in vivo [123]. Li et al. implanted GO-modified titanium into rat femurs and found that the GO coating promoted osseointegration and osteogenesis in vivo [78]. Akhavan et al. also found that ginseng-rGO showed great potential in differentiating NSCs into neurons [124]. GO has been used as a scaffold for stem cell growth and proliferation in cardiac surgery, urothelial surgery and urothelial surgery and as a carrier in drug delivery systems [76].

In Table 4, we list the preclinical studies of graphene and its derivatives in bone tissue engineering. The results show that graphene and its derivatives promote osteogenesis in vivo without cytotoxicity.

6. Cytotoxicity of graphene and its derivatives

As mentioned above, graphene and its derivatives have been widely used in regenerative medicine. However, the toxicity of graphene and its derivatives is an issue that cannot be ignored [132].

Several disadvantages of the use of graphene and its derivatives have been reported, including membrane damage, oxidative stress, mitochondrial disorders, genotoxicity, autophagy, and hydrophobic interactions. Graphene has sharp edges that can cause cell damage as it passes through cell membranes, so its aggregation can also cause cytotoxicity. Graphene nanosheets have been shown to disrupt the redox equilibrium of cells due to hydrophobic interactions between the nanosheets and the cell membrane [133]. Graphene derivatives can interact with a variety of biomolecules, such as proteins and nucleic acids, and these interactions can affect the physical and chemical properties of the molecules, resulting in damage to human cells, tissues, and even organs [134]. We should be aware that any byproducts or compounds from the use of graphene and its derivative materials may also have corresponding toxicity [135].

Due to the wide demand for graphene and its derivatives, a careful and comprehensive study of their toxicity is needed. Different strategies have been attempted to improve and analyze the physicochemical properties and cytotoxicity of graphene and its derivatives. For example, to study the cytotoxicity of graphene and its derivatives, researchers have used experimental parameters that induce cytotoxicity and machine learning to study cell models [136].

Mammalian cells are often used to assess the toxicity of graphene and its derivatives. However, the distribution of graphene and its derivatives in vivo depends on the surface modification of the material and the route of administration [137]. Mullick et al. considered 100 μg/mL GO-treated cells to be safe for gene delivery [138]. Similarly, a study of mammalian cells showed that 100 μg/mL is a safe concentration for therapeutic cells [139]. Liu's experiment found that RGO significantly inhibited the hatching of zebrafish embryos and reduced the hatching length of larval fish. However, GO and RGO did not change the morphology of zebrafish embryos [140]. Lu et al. implanted SGH into the subcutaneous tissue of rats and found that the host tissue reaction was mild without obvious toxicity or side effects, indicating that SGH has biocompatibility and nontoxicity in vivo [72]. Seabra found that the binding of graphene and its derivatives to BMSC showed very low cytotoxicity [141]. Kim found that rGO promoted bone regeneration, but severe cytotoxicity occurred when the rGO percentage exceeded a certain threshold level ($\geq$100 μg/mL) [142]. Lammel found that the effect of GO was time and concentration dependent, and the main toxicity mechanisms were cell membrane injury (DCM) and oxidative stress (OS). However, there was no cytotoxicity at very low concentrations ($<$4 μg/ml) [143]. Studies have shown that exposure to 50–200 μg/ml GO for 24 h induces less than 80 % cell viability through the production of reactive oxygen species (ROS), which is a common mode of toxicity of graphene and its derivatives [24].

The biodegradation of graphene and its derivatives is an important factor to be considered in clinical treatment for long-term in vivo implantation of orthopedic plants. Dextran-modified GO could be removed without toxicity after 7 days of injection, suggesting that surface modification is a key factor in graphene toxicity [144]. Askari et al. believed that the toxicity of graphene and its derivatives mainly depends on morphology, structure, and properties, which can be adjusted through functionalization [145]. The

Table 5
Toxicity of graphene and its derivatives to different cell lines.

Graphene Materials	Fabrication method	Concentration	Cell Types	Incubation Time (h)	Evaluation Method	Inhibition	Reference
G	MHM	100 μg/mL	A549	24	MTT	45.29 %	[150]
GO	MHM	50 μg/mL	HLF	24	MTT	35 %	[151]
GO	N/A	$\leq$32 μg/mL	mESCs(46C)	48	MTT	Low	[51]
GO	N/A	100 μg/mL	MH-S	8	MTT	10 %	[26]
GO	MHM	200 μg/mL	A549	24	CCK-8	20 %	[152]
GO	MHM	100 μg/mL	HBL100	24	MTT	35 %	[153]
GO	N/A	32 μg/mL	mESCs	48	CCK-8	20 %	[154]
MGO	HEBM	N/A	A431	8	MTT	20 %	[155]
GO	MHM	50 μg/mL	MC3T3-E1	48	MTT	40 %	[156]
GO/Ag	N/A	31 μg/mL	HDF	24	CCK-8	20 %	[157]
RGO/Ag	HM	20 μg/mL	MH-S/SW620	24	MTT	25 %	[158]

HEBM: High-energy ball milling method; A549: Mammalian cell line A549; HLF: Human lung fibroblast; MH-S: macrophages; HBL100: Human mammalian cell line; SW620: Colon cancer cell line; HDF: Human dermal fibroblast cells; MTT: Methyl thiazolyl tetrazolium.

preparation method of graphene and its derivatives is also closely related to its toxicity: GO prepared by Hoffman, Hummers and Tour showed significant cytotoxicity at concentrations ≥8 μg/ml, whereas GO prepared by the Staudenmaier method showed no toxicity at 125 μg/ml, which is due to the differences in the carbon–oxygen ratio of GO and the number of carbonyl groups caused by different preparation methods [24]. Akhavan et al. found that rGO nanoplatelets (rGONPs) exhibit size-dependent cytotoxicity in hMSCs [146].

There are many manufacturing methods available to prepare graphene and its derivatives with definite sizes, shapes, and surface morphologies [147]. Bellet found that covalent modification with polyethylene glycol could improve the biocompatibility and stability of graphene and reduce cytotoxicity [92]. Bengt et al. believes that low doses of graphene and its derivatives promote cell division and are therefore safe and nontoxic [148].

Several studies have found that the surface design and modification of graphene and its derivatives can effectively reduce the toxicology of the materials. Moreover, many researchers have reported the combination of various approaches to address the problem of synergistic cytotoxicity [51,141,149].

In summary, we believe that the toxicity of graphene and its derivatives is dependent on both concentration and time, and their main toxic modes are cell membrane destruction and oxidative stress. In Table 5, we summarize the cytotoxicity of graphene and its derivatives, and the results show that in most cases the toxicity is very low, especially at low doses, and the materials are relatively safe. Of course, continued research to reduce the toxic effects, especially research on the behavior and biological distribution of these materials in animal models, is needed to improve the clinical applicability.

7. Summary of the mechanisms by which graphene and its derivatives promote stem cell proliferation and differentiation

In this paper, we reviewed findings showing that graphene and its derivatives promote cell adhesion, proliferation, differentiation, etc. However, the exact mechanism is still not fully understood. In the above table, we summarize the relevant action mechanisms of graphene and its derivatives on stem cells in solution and on 2D and 3D material surfaces. However, these mechanisms are not isolated and often have much overlap. In this section, we will summarize and sort out the specific mechanisms.

The first and most important mechanism is the inherent properties of the material surface. such as matrix stiffness, micro and nanomorphology, hydrophilicity, and hydrophobicity, can profoundly affect the adhesion and proliferation of MSCs [78,159,160].

Graphene and its derivatives have excellent surface properties. For example, the surface of graphene and its derivatives is not completely flat. In the microscopic state, graphene and its derivatives have inherent roughness, and the surface is undulating, forming spontaneous folds. It was found that the height of monolayer graphene fluctuated by approximately 0.5 nm [93]. The porous folds and hard surfaces of graphene and its derivatives can produce mechanical stimulation, enabling a cascade of reactions to promote osteogenic differentiation without any chemical inducers. This rough surface also stretches cells attached to graphene and its derivatives, causing physical stimulation of the cells. Moreover, graphene and its derivatives do not preclude the use of other chemical inducers, allowing synergistic or additive effects in promoting osteogenic differentiation of MSCs. For example, Kim found that graphene or GO itself could induce osteogenic differentiation of MSCs, but the addition of growth factors or chemical inducers could further enhance this effect [77].

In addition to the physical properties of the surface, many graphene derivatives, such as GO, have a large number of oxygen-containing groups on their surfaces, such as oxygen, carboxyl, and epoxy groups, enabling them to construct ideal nanostructures with different materials through covalent or noncovalent bonds. For example, π stacking and hydrogen bonding within graphene and its derivatives can accelerate the osteogenic differentiation of MSCs due to their ability to absorb osteogenic induction factors from the medium through noncovalent binding [69,93]. In addition, the interlayer structure of graphene affects the proliferation of MSCs. For example, interlamellar covalently enhanced graphene increases surface area by regulating the interlamellar distance to provide porosity and stratified pore structure comparable to human cancellous bone, thereby promoting cell adhesion and proliferation [93,161]. Moreover, with graphene's layered structure, internalized graphene and its derivatives can interact with subcellular organelles and regulate the expression of relevant genes and proteins, thereby affecting stem cell proliferation and differentiation [137].

The excellent surface properties of graphene and its derivatives have led to increasingly widespread use in cell research. However, the exact molecular mechanisms underlying the effects of graphene and its derivatives on stem cells are still unclear.

Here we summarize the common molecular mechanisms. First, the hard surface of graphene and its derivatives can act as a physical stimulus to initiate a cascade of osteogenic signals, such as the integrity of the FAK axis. MSCs inoculated on graphene-coated PDMS showed higher expression of FAK-P397, F-actin, ROCK1, SMAD P1/5, OCN, and OPN than control cells. This reveals a possible mechanotransduction-related signaling association: the molecular properties of graphene first activate integrin-FAK transmembrane complexes, then ROCK1 and F-actin, and then stimulate phosphorylation of SMAD P1/5 and eventual osteogenic differentiation mediated by Runx, OPN, and OCN [67](Fig. 2). The number and composition of FAs directly affect the differentiation of MSCs: for example, abundant and dense FAs promote osteogenic differentiation, while loose FAs promote adipogenic differentiation. FAK is one of the characteristic markers of FAs, and other markers include, Vinculin and talin [159,162]. MHY10 may also be involved in this mechanical stimulation pathway system by interacting with FAs during osteogenic differentiation induced by the physical properties of graphene [93].

Extracellular matrix (ECM) changes, macrophage polarization regulation, and the OSM, MAPK, BMP, and Wnt/β-catenin signaling pathways are also involved in the osteogenic regulation of graphene and its derivatives [93]. For example, studies have shown that the MAPK signaling pathway is a key regulatory pathway in osteogenic differentiation mediated by graphene and its derivatives, and the ERK/MAPK pathway is the main channel for information transfer from the extracellular environment to the nucleus [112]. Zhao et al. found that graphene can promote osteogenic differentiation through long-term activation of the ERK/MAPK pathway, Ca and Mg can participate in the regulation of this pathway, and ERK pathway activation can increase ECM mineralization and osteogenic gene

expression [163] (Fig. 4). Graphene and its derivatives have also been found to be involved in osteogenic differentiation mediated by the Wnt/β-catenin signaling pathway by regulating signaling molecules [52,53]. There is considerable evidence that activation of the Wnt/β-catenin pathway is positively correlated with osteogenic differentiation [164,165]. The PI3K/Akt pathway was also found to be involved in the early stages of osteogenesis promoted by graphene and its derivatives [166,167]. Xie et al. found that ROCK1, an effector of RhoA, was highly expressed in graphene-induced osteogenic differentiation studies [67]. RhoA is an important small G protein that is expressed in osteoblasts and is involved in cell signaling and cytoskeletal organization.

In addition, graphene and its derivatives generally promote the expression of markers of osteogenesis such as alkaline phosphatase (ALP), osteocalcin, and Runx2 [107]. Srinivasa et al. suggested that GO improves biocompatibility, ALP activity, and calcium deposition levels, which are critical for bone regeneration [168]. Graphene and its derivatives can induce MSCs and promote the secretion of BMP. BMP, the largest branch of the TGF-β family, is an acidic glycoprotein widely present in the bone matrix, and different types of BMP have been shown to play a key role in bone differentiation. BMP-2 is the most important extracellular signaling molecule in promoting bone formation and inducing osteogenic differentiation. Mirza et al. found that GO could have a definite effect on BMP expression [107]. Because of their excellent BMP-2 release properties, graphene derivatives are often used as BMP-2 carriers, making them excellent scaffolds for bone defect regeneration and enhancing the osseointegration of microimplants, such as Ti/GO/BMP-2 implants [75], graphene/BMP-2, GO/SF/BMP-2 scaffolds [169]. Zou et al. also demonstrated in vivo in mice that graphene significantly enhanced bone formation in BMP-9-induced mesenchymal stem cells [170].

In the figure below (Fig. 2), we have summarized the common mechanisms by which graphene and its derivatives promote stem cell proliferation and differentiation. However, the elucidation of clear pathways and their regulation and optimization are the focus of further research.

8. Prospects

As discussed in this review, we know that graphene and its derivatives can promote the adhesion, proliferation, and osteogenic differentiation of bone tissue engineering-related stem cells, and their toxicity is extremely low, with broad prospects for use. However, much research is still needed before these materials can be applied to patients in clinical practice. In the future, we believe that the application of graphene and its derivatives in bone tissue should be developed in the following directions.

8.1. Continued optimization of modification or use in combination with other materials to promote osteogenesis

Improving the production process of graphene and its derivatives, optimizing the production parameters, or combining it with other materials to promote osteogenesis is the focus of research for a long time in the future. For example, Park enhanced the osteogenic differentiation of BMSCs using size-controlled graphene oxide flakes [171]. Sun et al. used the 3D printing of BMSC-laden highly adhesive artificial periosteum containing gelatin-dopamine and graphene oxide nanosheets to promote bone defect repair [172]. Baheti coated HA and GO on the surface of TI by electrophoretic deposition technology, to promote the proliferation and differentiation of bone marrow mesenchymal stem cells on the surface of TI implants [173]. Wang et al. synthesized A novel zwitterionic hydrogel with incorporated graphene oxide for bone tissue engineering and found that it promotes osteogenic differentiation of BMSCs [174]. In recent years, graphene-biomacromolecule nanocomposites have also been vigorously developed in medical applications [175], which is another future direction.

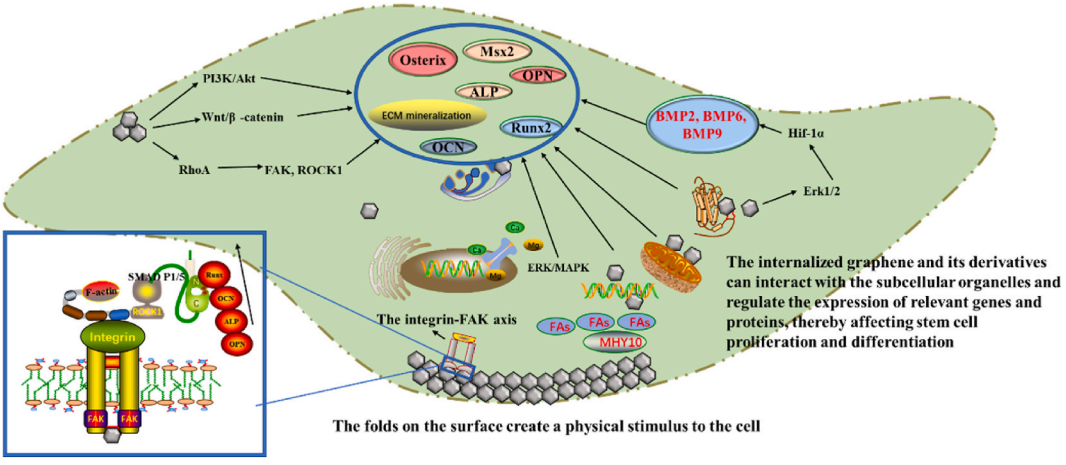

Fig. 4. Illustration of the mechanisms by which graphene and its derivatives promote stem cell proliferation and differentiation.

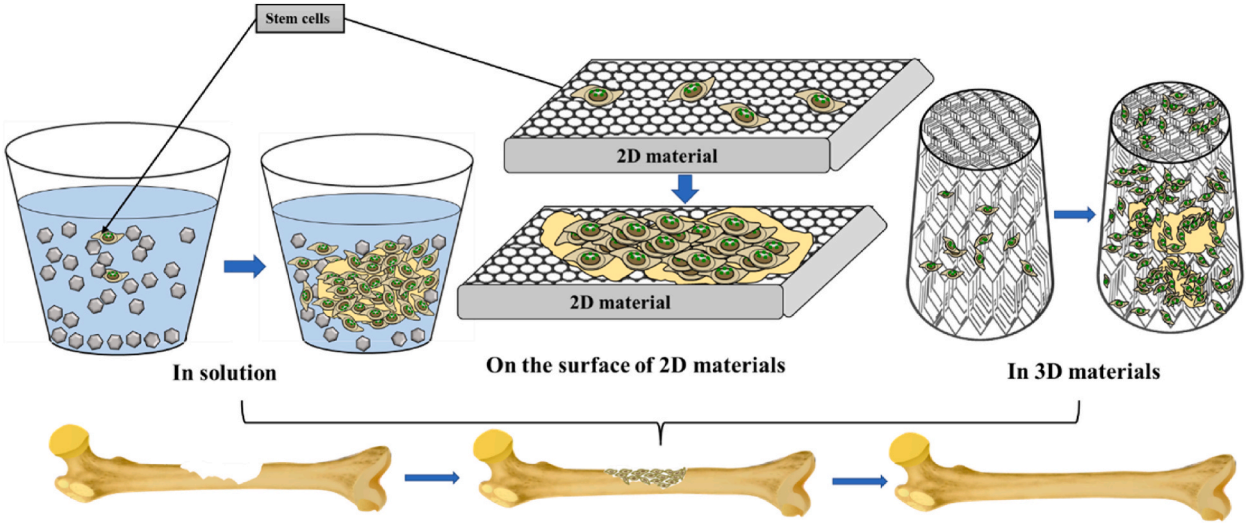

Fig. 5. Graphene and its derivatives promote the proliferation and differentiation of MSCs in solution and on 2D material and 3D material surfaces, with potential for application in bone tissue engineering.

8.2. Further study of the toxicity of graphene and its derivatives in mammals

The purity, transverse size, surface functional groups, hydrophilicity, stiffness, structural defects, and other characteristics of graphene and its derivatives all have important effects on its toxicity, especially in mammals [176]. GO surface modification and biotransformation phenomena and cell types may play key roles in the uptake of GO by mammalian cells. Furthermore, proteins present in mammalian serum may be adsorbed on GO surfaces or cause degradation of GO. Therefore, the biological characteristics of nanoparticles may change after biotransformation [177]. Ding et al. studied the current status of pathological lung events induced by graphene and its derivatives, elaborated on the damage to different biological parts (molecules, cells, tissues, and organs) and the mechanistic relationship between different toxic endpoints, and further studied the possible mechanism of lung injury caused by graphene and its derivatives [178]. At present, there are fewer than 10 studies on the toxicity of graphene and its derivatives to mammals [178], so the true biosafety of graphene and its derivatives cannot be fully elaborated. Further systematic studies on the metabolism, degradation, and excretion of graphene and its derivatives in mammals are needed to prepare for its further clinical application.

8.3. Study of the exact molecular mechanism

Previously, we reviewed the common molecular pathways of graphene and its derivatives that promote stem cell proliferation and differentiation, however, the network of osteogenic differentiation mechanisms regulated by graphene and its derivatives is very large and complex, and the effect usually stems not from a single mechanism but from the superposition and interaction of multiple mechanisms. Therefore, it is necessary to further study the exact molecular mechanisms of regulation by graphene and its derivatives and to control and optimize the effects for clinical utility.

9. Conclusion

In recent years, bone defect reconstruction has attracted increasing attention from orthopedic doctors and scientific researchers. It has been widely recognized that certain nanomaterials can promote the proliferation and differentiation of bone tissue-related stem cells. Among them, graphene and its derivatives have attracted increasing attention in bone tissue research due to their unique physical and chemical characteristics. In this paper, the interaction of graphene and its derivatives with stem cells in solution, 2D, and 3D materials was reviewed in detail, and its application in bone tissue engineering and its toxic effects were described (Fig. 5). Finally, the possible mechanisms of its promotion of stem cell proliferation and differentiation were analyzed to provide a basis and reference for better clinical solutions for bone defects.

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

Data availability statement

No data was used for the research described in the article.

Declaration of competing interest

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

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