Development of methods for detecting the fate of mesenchymal stem cells regulated by bone bioactive materials

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ABSTRACT

The fate of mesenchymal stem cells (MSCs) is regulated by biological, physical and chemical signals. Developments in biotechnology and materials science promoted the occurrence of bioactive materials which can provide physical and chemical signals for MSCs to regulate their fate. In order to design and synthesize materials that can precisely regulate the fate of MSCs, the relationship between the properties of materials and the fate of mesenchymal stem cells need to be clarified, in which the detection of the fate of mesenchymal stem cells plays an important role. In the past 30 years, a series of detection technologies have been developed to detect the fate of MSCs regulated by bioactive materials, among which high-throughput technology has shown great advantages due to its ability to detect large amounts of data at one time. In this review, the latest research progresses of detecting the fate of MSCs regulated by bone bioactive materials (BBMs) are systematically reviewed from traditional technology to high-throughput technology which is emphasized especially. Moreover, current problems and the future development direction of detection technologies of the MSCs fate regulated by BBMs are prospected. The aim of this review is to provide a detection technical framework for researchers to establish the relationship between the properties of BMMs and the fate of MSCs, so as to help researchers to design and synthesize BBMs better which can precisely regulate the fate of MSCs.

1. Introduction

In regenerative medicine, mesenchymal stem cells (MSCs) are considered to have the self-regulation ability of tissue regeneration due to their tissue-specific differentiation, which is a new promising tool for therapeutics [1]. MSCs have the characteristics of proliferation, multi-directional differentiation and natural bioactive molecule secretion to support tissue repair. The fate of MSCs can be regulated by bioactive materials to promote their adhesion, proliferation and directional differentiation, so as to achieve optimistical tissue regeneration [2].

Abbreviation

(MSCs)	mesenchymal stem cells
(BBMs)	bone bioactive materials
(MTT)	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
(CCK-8)	cell counting kit-8
(CaAM/EthD-1) assay	calein-AM/ethidium homodimer-1
(LDH)	lactate dehydrogenase
(PCR)	polymerase chain reaction
(ELISA)	enzyme linked immunosorbent assay
(HE)	hematoxylin-eosin
(DMSO)	dimethylsulfoxide
(BST)	barium strontium titanate
(β-TCP)	β-tricalcium phosphate
(hBMSCs)	human bone marrow mesenchymal stem cell
(XTT)	2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) -5 - [(pheny-lamino) carbonyl]-2H-tetrazole hydroxide;
(AM)	acetylmethoxymethyl ester
(BMP-2)	bone morphogenetic protein-2
(BSP)	bone sialo protein-II
(OP)	osteopontin
(ALP)	alkaline phosphatase
(Col-I)	collagen-I
(OCN)	osteocalcin
(RT-PCR)	reverse transcription-PCR
(cDNA)	complementary DNA
(qPCR)	quantitative real time-PCR
(RT-qPCR)	real-time RT-PCR
(hAMSCs)	human amniotic stromal cells
(PVDF)	polyvinylidene fluoride
(hADSCs)	human adipose-derived stem cells
(PVDF-TrFE)	polyvinylidene fluoride trifluoroethylene
(BCA)	bicinchoninic acid
(PL-PC)	poly-D,L-lactide/poly-ε-caprolactone
(n-CDHA/PAA)	nano-calcium-deficient hydroxyapatite/polyamino acid
(ARS)	alizarin red staning
(NHA)	natural hydroxyapatite
(HA)	hydroxyapatite;
(PCL)	poly (ε - caprolactone)
(PDLLA)	poly (D, L-lactic acid)
(RNA-seq)	high-throughput RNA sequencing
(eQTL)	expression quantitative trait loci
(GO)	gene Ontology
(KEGG)	kyoto Encyclopedia of Genes and Genomes
(IPA)	ingenuity Pathways Analysis
(SDS)	sodium dodecyl sulfate
(PAGE)	polyacrylamide gel electrophoresis
(LC)	liquid chromatography
(MS)	mass spectrometry
(iTRAQ)	isobaric tags for relative and absolute quantification
(DBM)	decalcified bone matrix
(MAPK)	mitogen-activated protein kinase
(PDA-PPy-MCs)	polydopamine polypyrrole microcapsule
(PDA)	polydopamine

Orthopaedic implants have undergone a development from inert biomaterials, which lack the ability to interact with physiological tissues [3], to bioactive materials, which have the ability to regulate the fate of MSCs to regenerate damaged tissue [4–9]. The biological properties of bioactive materials are determined by their structure and physicochemical properties [10–12]. Good biocompatibility is a prerequisite for the use of biomaterials [13–16]. The detection of cell activity is an important criterion for judging whether cells can grow normally when they interact with the biomaterials both *in vitro* and *in vivo* [17–19].

Bone bioactive materials (BBMs) are supposed to be able to promote osteogenesis by inducing MSCs to differentiate into osteoblasts [20,21]. Thus, both *in vitro* osteogenic differentiation of MSCs [22–25] and *in vivo* osteogenesis are necessary to evaluate the performance of BBMs [26–31]. The research paradigm in detecting the fate of MSCs regulated by BBMs is evolving from low-throughput experimental design to high-throughput experimental design. In traditional low-throughput characterization technology, the biological complexity of MSCs is simplified frequently when the fate of MSCs regulated by BBMs was detected and the relationship between the properties of BBMs and the fate of MSCs was established. Several "key" genes, proteins, etc. are often selected based on previous studies for the research making an overall judgement on the basis of one-sided result, which cannot fully reflect the complex biological effects of MSCs. High throughput technology refers to the technology that can analyze and process a large number of experimental objects in parallel, which has made a qualitative leap compared to traditional detection technology [32,33]. Common high-throughput technologies include high-throughput sequencing technology, high-throughput screening technology and high-throughput discovery technology [34,35]. With the development of biotechnology and computer technology, bioinformatics has been developed. The combination of high-throughput technology and bioinformatics has given birth to omics, which has the potential to characterize and analyze the complex biological systems of MSCs. Among which, transcriptomics [36–38] and proteomics [39] have been used to detect the fate of MSCs regulated by BBMs.

In this review, technologies of detecting the fate of MSCs regulated by BBMs were systematically reviewed from traditional methods to high-throughput technology which was emphasized especially (Table .1). Specifically, the latest research of traditional methods including 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), cell counting kit-8 (CCK-8), calein-AM/ethidium homodimer-1 (CaAM/EthD-1) assay and lactate dehydrogenase (LDH) were reviewed to demonstrate the way of MSCs viability and proliferation detection. The detection of osteogenic differentiation *in vitro* was reviewed by polymerase chain reaction (PCR), immunofluorescence staining and Western blot and enzyme linked immunosorbent assay (ELISA). The detection of osteogenesis *in vivo* were summarized by X-ray imaging examination, hematoxylin-eosin (HE) staining and calcium staining. The cell tracking methods for real-time monitoring the distribution of MSCs seeded on BBMs *in vivo* was reviewed. Finally while most importantly, transcriptomics including gene chip, RNA-seq and single cell RNA-seq, proteomics, high-throughput cell analysis, and high-throughput tissue analysis were introduced to review the application of high-throughput technology in detecting the fate of MSCs regulated by BBMs.

2. The latest research progress of traditional characterization methods

The so-called "bioactive materials" are materials that can provide physical or chemical stimuli to cells. With the emergence of bioactive materials, the characterization methods of bioactive materials, which include the detection of biocompatibility and cell response to stimuli, have also emerged. Herein, the latest research progress of traditional characterization methods was systematically reviewed.

2.1. Detection of MSCs viability and proliferation in vitro

To ensure the clinical safety of biomaterials, biological evaluation *in vitro* and *in vivo* are both required [40,41]. In general, *in vivo* biological

Table 1
The methods of detecting the fate of MSCs regulated by BBMs.

	Methods	Application	Advantages	Disadvantages	Citation
Traditional Characterization Methods	MTT	Cytotoxic test;Proliferation test	Relatively high sensitivity and economy	relatively high cytotoxicity and relatively low reagent stability	[51],[52]
Detection of MSCs viability and prolife -ration *in vitro*	CCK-8	Cytotoxic test;Proliferation test	Simplicity, Rapid Detection,High Sentivity,Repeatability,Low cytotoxicity	Relatively expensive	[61],[62]
	Ca-AM/EthD-1	Live/dead double staining	Living and dead cells can be observed directly	Calcein is toxic for human body	[63]
	LDH	Cytotoxic test;	Simplicity, Sensitivity,Relatively inexpensive	Many influencing factors, such as medium, pH value, temperature etc.	
Detectio of gene expression	PCR	Gene transcription detection	Rapid Detection, Accuracy	Can't detect a lot of genes at once	[64], [65]
	Immunofluo-rescence Staining	Protein expression detection	High specificity, sensitivity and Rapid Detection	Non-specific staining problem, Complex technical procedures	[66]
Detection of protein expression	Western Blot	Protein expression detection	High sensitivity and specificity	Complex technical procedures	[67-70]
	ELISA	Protein expression detection	Simple, fast, sensitive and specific operation	Poor repeatability , Many interference factors	[71]
Cell tracking	Cell Tracking	Real time monitoring of cell distribution	Repeatable, Noninvasive	Cytotoxicity	[74], [75]
Characteri-zation of Osteo-genesis *in vivo*	X-ray Imaging Examination	Detection of bone regeneration	Obversation of bone density, Cheapness	Unclear imaging, Harmful radiation	[76]
	HE Staining	Color tissues and cells	Clear staining	Complex operation	[77]
	ARS staining	Color mineralized noudles	Simple operation	False positive	[63]
High-throughput technology	Gene chip	Detection of Gene transcription at overall level	Stable and reliable	a "closed system"	[36], [88-91]
	RNA-seq	Detection of Gene transcription at overall level	A "open system"	cannot distinguish one cell from another	[37]
	scRNA-seq	Detection of Gene transcription at overall level of each cell	can distinguish one cell from another	Problems of amplification of single cell whole transcriptome	[94-100]
	Proteomics	Detection of protein expression at overall level	Comprehensive protein expression information	High technical requirements	[39], [103-107]
	High-throughput cell analysis	Simultaneous detection of cells	High efficiency	High technical requirements	[108-110]
	High-throughput tissue analysis	Simultaneous detection of tissues	High efficiency	High technical requirements	[111-112]

evaluation will be carried out after *in vitro* biological evaluation is qualified, and finally clinical research could be carried out. MSCs viability test can evaluate the cytocompatibility of materials by evaluating the viability of MSCs on bioactive materials [42–45]. MSCs proliferation could be regulated by bioactive materials. Because of the positive correlation between MSCs viability and MSCs number, the detection method of MSCs viability could be introduced to detect cell proliferation.

2.1.1. MTT assay

MTT is a widely exploited approach for measuring cell survival and growth [46–50]. Succinate dehydrogenase in mitochondria of living cells can reduce exogenous MTT to water-insoluble blue violet crystal formazan and deposit them in cells, but dead cells can't. Dimethylsulfoxide (DMSO) can dissolve formazan in cells, and its light absorption value could be measured at 570 nm by enzyme-linked immunosorbent assay, which can indirectly reflect the number of living cells [51]. Since its invention, MTT has been widely used to measure the cytotoxicity of biomaterials and the growth of cells due to its high sensitivity and economy. For example, Mozafari et al. measured the MSCs viability on the 3D-printed barium strontium titanate (BST)/β-tricalcium phosphate (β-TCP) composite scaffolds by MTT [52]. Specifically, human bone marrow mesenchymal stem cell (hBMSCs) were seeded on the scaffold for 1, 3 and 7 days. The MTT results demonstrated that the BST/β-TCP scaffold had no cytotoxicity on hBMSCs. However, MTT has relatively high cytotoxicity and relatively low reagent stability. Therefore, if the detection requirements of proliferation or cytotoxicity are not very precise, MTT can be selected.

2.1.2. CCK-8 assay

CCK-8 is a highly sensitive and non-radioactive colorimetric assay for measuring the number of living cells in cell proliferation or cytotoxicity experiments [53–56]. The reagent of CCK-8 contains WST-8 whose chemical name is 2-(2-methoxy-4-nitrophenyl) -3-(4-nitrophenyl) -5-(2,4-bisulfonate benzene) -2H-tetrazole monosodium salt, which can be reduced to highly water-soluble formazan by dehydrogenase in mitochondria under the action of electronic carrier (1-methoxy-5-methylphenazine dimethyl sulfate). The number of produced formazan is proportional to the number of living cells, which can be indirectly reflected by measuring the light absorption value at 450 nm wavelength by enzyme-linked immunosorbent assay. Compared with MTT assay, the advantage of CCK-8 assay is that its measurement results are more accurate, flexible and simple. So CCK-8 has become one of the most widely used methods to measure MSCs proliferation and cytotoxicity in recent years [57–60], even in 3D culture. For example, Naito et al. used collagen hydrogel as scaffold, in which rat-MSCs was cultured in three and two dimensions respectively, and the cell numbers was successfully detected by CCK-8 after 4 weeks of culture [61]. Although CCK-8 is an excellent method to detect cell viability, there are still some limitations. Jiao et al. [62] used MTT and CCK-8 assay to evaluate the toxicity of graphene, respectively. The results showed that graphene induced adsorption, optical interferences, as well as electron transfer can prevent to appropriate evaluate graphene toxicity, which means that both methods have limitations in this situation. Furthermore, other dye reagents such as 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazole hydroxide (XTT), neutral red, trypan blue, Coomassie blue, alamar blue, hematoxylin and eosin, actin tracking green and propidium iodide would affect the accuracy of the CCK-8, which suggested that CCK-8 assay should be avoided under these conditions. Despite these avoided conditions, CCK-8 is recommended to be used if the detection of MSCs proliferation or cytotoxicity requires a very high sensitivity and conveniency, when the research funds permit.

2.1.3. Ca-AM/EthD-1 assay

Calcein-AM(Ca-AM) is a commonly used cell staining reagent for fluorescent labeling of living cells, with green fluorescence (Ex = 490 nm, Em = 515 nm). Because of the introduction of acetylmethoxymethyl ester (AM) group on the basis of traditional calcein, the hydrophobicity of AM was increased, so that it could easily penetrate the living cell membrane. Once penetrating into the cell, Ca-AM (not itself fluorescent) is hydrolysed by esterases in the cell yielding calcein (which is fluoerescent and membrane impermeable), which is then trapped in the cell and emits strong green fluorescence. Compared with other similar reagents due to the extremely low cytotoxicity, Ca-AM is suitable for fluorescent probes for living cell staining, and does not inhibit any cell functions, such as proliferation and lymphocyte chemotaxis. Due to the lack of esterase in dead cells, Ca-AM is only used for cell viability test and short-term labeling of living cells. Therefore, Ca-AM is often used in combination with dead cell fluorescent probes, such as ethidium homodimer-1 (EthD-1), to double stain living cells and dead cells. EthD-1 can not pass through the cell membrane of living cells, but can only pass through the disordered area of dead cell membrane and reach the nucleus, and embed into the DNA double helix of cells to produce red fluorescence (Ex = 490 nm, Em = 617 nm). Since both calcein and EthD-1 can be excited by 490 nm, both living and dead cells can be observed by fluorescence microscopy at the same time. Moreover, this kit is suitable for fluorescence microscopy, fluorescence enzyme labeling, flow cytometry and other fluorescence detection systems.

2.1.4. LDH assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that exists in all cells and will be rapidly released into the cell culture medium when the cell membrane is damaged. LDH activity is catalyzed by two enzymes: LDH oxidizes lactate to pyruvate, then pyruvate and tetrazolium salt react to form formazan crystallization. The increase of formazan crystallization in culture medium is directly related to the increase of lysed cells. Formzan crystalline dye is water-soluble and can be detected by spectrophotometer at 500 nm wavelength. By detecting the activity of LDH in cell culture supernatant, the degree of cell damage can be judged. This analysis is sensitive, convenient and accurate, and can be applied to many kinds of cytotoxicity analysis. For example, Yang et al. [63] used LDH and CCK-8 assay to evaluate the cytotoxicity of silica nanoparticles with different concentrations to hMSCs. The results of CCK-8 showed that concentration of silica nanoparticles had no significant effect on cell viability. The results of LDH release were consistent with those of CCK-8, which provided another evidence for the cytocompatibility concentration range of silica nanoparticles. This research demonstrated that several different cell viability detection methods can be combined to detect the viability of MSCs, in order to obtain more comprehensive cell viability information.

2.2. Characterization of osteogenic differentiation in vitro

With the development of materials science, more and more bioactive materials have been developed as bone biomaterials. *In vitro* osteogenic differentiation is a necessary step in evaluating the fate of MSCs regulated by BBMs. Herein, the detection of gene expression and protein expression of differentiation markers will be introduced to evaluate the effect of BBMs.

2.2.1. Detection of gene expression by PCR

The expression of several genes, such as bone morphogenetic protein-2 (BMP-2), bone sialo protein-II (BSP), osteopontin (OP), alkaline phosphatase (ALP), collagen-I(Col-I), osteocalcin (OCN), has been proved to be able to evaluate the level of osteogenic differentiation. Herein, PCR, the main traditional detection method of gene expression, will be introduced.

The principle of PCR is to control the decomposition and recombination of DNA double strands by changing temperature, designing primers as promoters, and adding DNA polymerase and other

substances to complete the replication of specific genes *in vitro*. Reverse transcription-PCR (RT-PCR) refers to the reverse transcription of RNA into complementary DNA (cDNA), which is then used as a template for DNA amplification through PCR. Quantitative real time-PCR (qPCR), also known as real time-PCR, refers to the real-time recording of data in each cycle of the PCR process, so the number of initial templates can be accurately analyzed. Real-time RT-PCR (RT-qPCR) is a kind of reverse transcription polymerase chain reaction, which combines fluorescence quantitative technology. First, the reverse transcription of RNA is used to obtain the cDNA (RT-PCR), and then the quantitative analysis (qPCR) is carried out by Real-time PCR. Real-time RT-PCR has been widely used to detect gene expression related to osteogenic differentiation. For example, Oliver et al. [64] used real-time RT-PCR to study how the expression of typical osteoblast-related genes in hBMSCs was regulated. The results showed that real-time RT-PCR quantified mRNA levels of bone morphogenetic protein-2 (BMP-2), bone sialo protein-II (BSP), osteopontin (OP) could be used as markers for monitoring hBMSCs osteogenic differentiation *in vitro*. Using these markers, they further demonstrated that only a few subpopulations of hBMSCs showed enhanced osteogenic differentiation following fibroblast growth factor-2 and dexamethasone (FGF/Dex) expansion. Mauney et al. [65] also used RT-PCR to analyze the mRNA levels of bone-specific protein include ALP, OP, OCN and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to study the effect of mechanical stimulation on osteogenic differentiation of hBMSCs on three-dimensional partial demineralized bone scaffolds *in vitro*. All of these researches demonstrated that PCR can be used to detect the effect of BBMs on osteogenic differentiation.

2.2.2. Detection of protein expression

As the result of gene detection shows the trend or ability of cell behavior, while the result of protein detection shows the real result of cell behavior, the detection of osteogenic differentiation related protein expression is considered to be a reliable method to evaluate osteogenic differentiation. Usually, mutual corroboration between the result of gene detection and protein detection can provide more information. Herein, three commonly used protein expression detection methods, including immunofluorescence staining, Western blot, ELISA, will be introduced.

2.2.2.1. Immunofluorescence staining. The binding of antigen and antibody is highly specific. Without affecting the activity of antigen and antibody, immunofluorescence staining of antibody (or antigen)

and observation using a fluorescence microscope can realize the tracing of antigen (or antibody). Therefore, specific proteins related to osteogenic differentiation can be labeled by immunofluorescence staining, and then observed by laser confocal microscope. For example, in order to explore the potential of human amniotic stromal cells (hAMSCs) as seed cells of bone tissue engineering, Mei et al. [66] used immunofluorescence staining to detect the expression of Col-I and ALP after culturing hAMSCs in osteoblast differentiation medium for one week. After immunofluorescence staining, the nucleus, Col-I and ALP will be dyed to be blue, green and red respectively. The merged image of these staining results could show the secreted Col-I and ALP around hAMSCs, which indicated the osteoblast differentiation of hAMSCs. Therefore, immunofluorescence staining can detect the expression of osteogenic differentiation protein directly and clearly.

2.2.2.2. Western blot. Western blot is an important technique in cell and molecular biology, which is often used in research to isolate and identify proteins [67]. In this technology, a mixture of proteins is separated by molecular weight based on gel electrophoresis, and the separated total proteins were then transferred to solid-phase support cellulose nitrate or polyvinylidene fluoride (PVDF) membranes to produce each protein band [68]. Subsequently, the membrane was incubated with the labeled antibody specific to the target protein, and the unbounded antibody was washed away, leaving only the binding antibody of the target protein, whose amount will be detected according to the thickness of the band by setting standards. In the study of osteogenic differentiation of MSCs, Western blot assay is often used to detect ALP, OCN, OPN, BMP-2 and other markers of osteogenic differentiation [69]. For example, Ho et al. [70] used alizarin red staining (Fig. 1) to determine matrix mineralization and alkaline phosphatase (ALP) activity (Fig. 2C) to evaluate the *in vitro* effect of Aln (1–10 μM) on the osteogenic ability of human adipose-derived stem cells (hADSCs). Real time PCR (Fig. 2A) and Western blot analysis (Fig. 2B) were used to measure the expression of BMP-2. The results showed that 5 μM Aln was sufficient to enhance BMP-2 expression, ALP activity and mineralization in hADSCs.

2.2.2.3. ELISA assay. ELISA refers to the qualitative and quantitative detection method of immune response by combining soluble antigen or antibody with solid-phase carrier such as polystyrene. With the appearance of the ELISA kit corresponding to osteogenic differentiation marker protein, ELISA has gradually become one of the common methods to study osteogenic differentiation of MSC
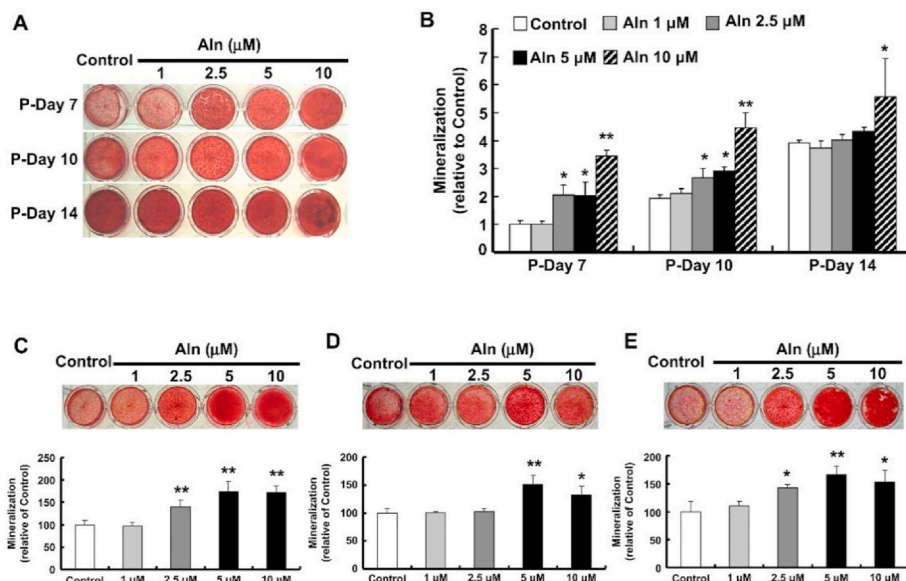

Fig. 1. Aln enhances the mineralization of hADSCs *in vitro*. Representative photomicrographs (A) and quantified data (B) of alizarin red staining from hADSC cultures of Donor A after being cultured in OIM (P-Day) for 7–14 days. The hADSCs were untreated (control) or treated with increasing doses of Aln (1–10 μM) for five days and then were placed in OIM. The representative photomicrographs and quantified data of alizarin red staining of hADSCs cultures of Donor B (C), Donor C (D) and Donor D (E) after being cultured in OIM for ten days. All experiments were performed at least three times. *p < 0.05, **p < 0.01 compared with the control. Reprinted with permission from Ref. [70].
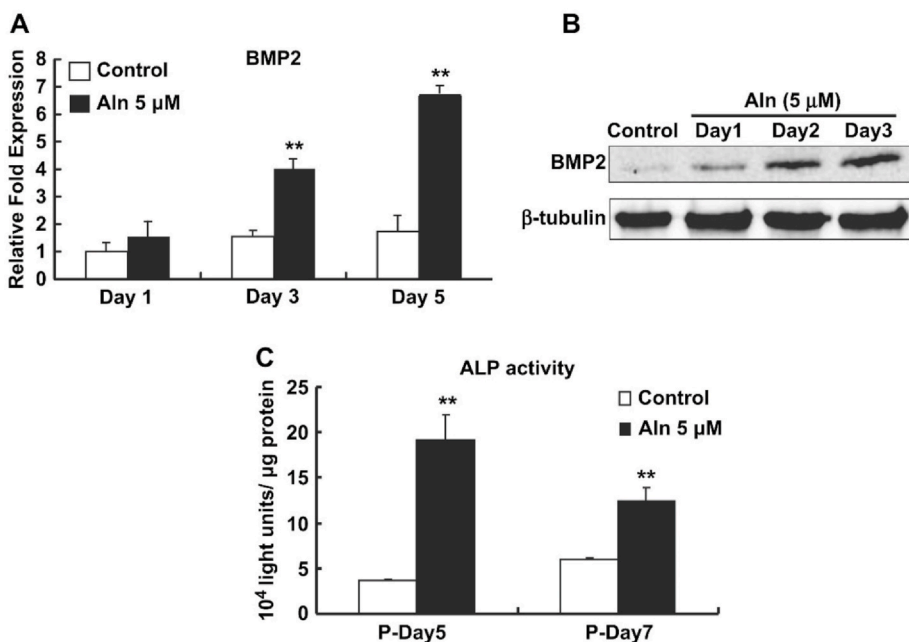
Fig. 2. Aln increases the expression of BMP2 and ALP activity *in vitro*. (A) The mRNA expression of BMP2 was increased in hADSCs after Aln treatment in a time-dependent manner. Cultured hADSCs were untreated (control) or treated with 5 μM Aln in bone medium for five days. (B) BMP2 expression was detected by Western blot. Cultured hADSCs were untreated (control) or treated with Aln (5 μM) in bone medium for three days. Membranes were immunoblotted with anti-tubulin to show equal protein loading. (C) Aln enhanced ALP activity in hADSCs. Cultured hADSCs were untreated (control) or treated with 5 μM Aln in bone medium. Post-cultured in OIM, on days 5 (P-Day 5) and 7 (P-Day 7), cells were harvested, and ALP activity was assessed. All experiments were performed at least three times. *p < 0.05, **p < 0.01 compared with the control. Reprinted with permission from Ref. [70].
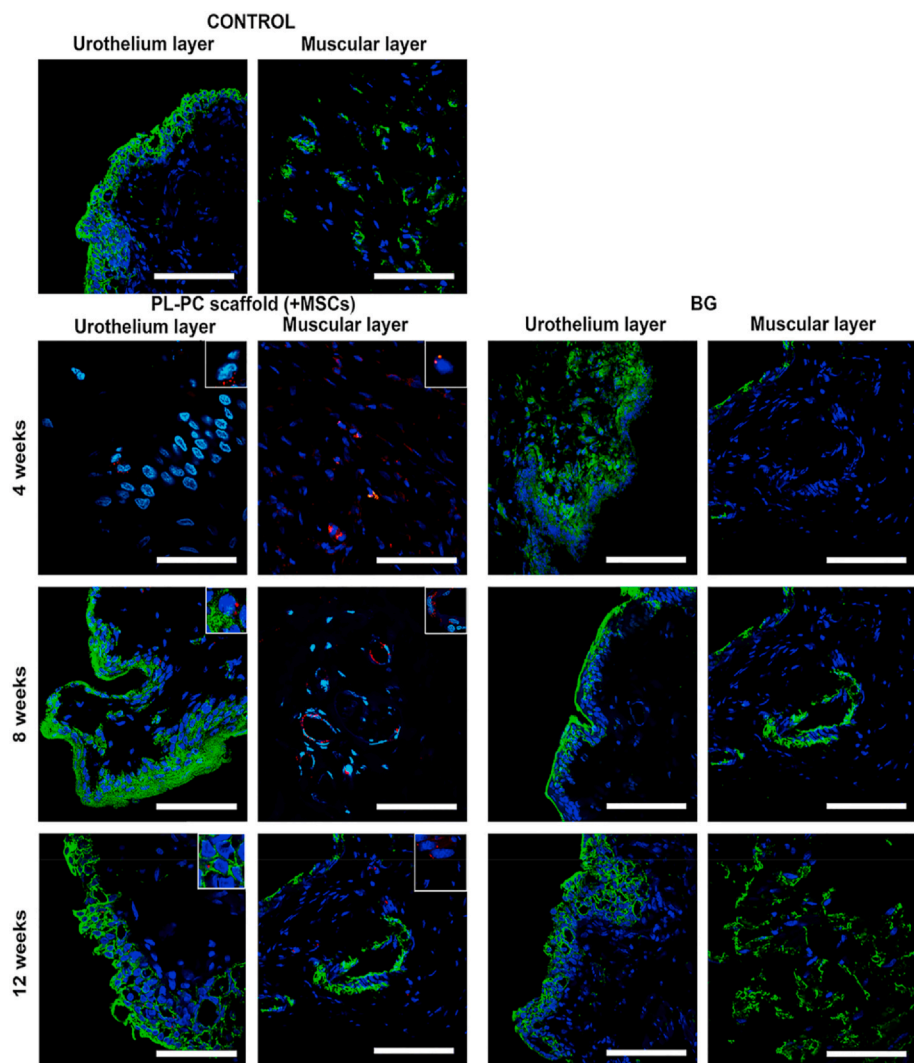

Fig. 3. Assessment of the SPION-labeled MSCs in the reconstructed urethra wall. Confocal microscopy image of the urothelium and muscular layers following application of the PL-PC scaffold seeded with nanoparticle-labeled MSCs or autologous buccal graft (BG). Intact urethra wall was used as a control. Nuclei were stained with DAPI (blue) and detected using a diode laser (405 nm). SPIONs were detected by reflected laser scanning at 504 nm (red). Labeled MSCs were additionally stained by specific antibodies (Cytokeratin AE1/AE3) with secondary FITC-labeled antibodies (green) and detected using a diode laser (488 nm) (left column, scale bar, 50 μm) or anti-αSMA antibodies (right column, scale bar, 100 μm). Autologous buccal graft (BG) and control (scale bar, 100 μm). Reprinted with permission from Ref. [74].

regulated by BBMs. For example, in order to study the effect of the surface potential of biomaterials on the osteogenic differentiation, Wang et al. [71] polarized polyvinylidene fluoride trifluoroethylene (PVDF-TrFE) through a large number of electric fields to obtain the surface potential in the range of − 3 to 915 MV MC3T3-E1 cells were cultured on different PVDF-TrFE. Then, they detected the absorbance of Col-I and OCN at 450 nm with ELISA assay kit, and the total protein content with bicinchoninic acid (BCA) protein assay kit. The results demonstrated that CoL-I and OCN have the highest expression on PVDF film with 391mv surface potential [71].

2.3. Cell tracking

Biochemical and histological analysis of cell proliferation, protein synthesis and gene expression can not provide sufficient time and space information. More and more researches seeded MSCs on BBMs to get better therapeutic effect. In order to observe the growth and migration of transplanted MSCs *in vivo*, MSCs should be labeled *in vitro* in advance. There are many techniques for cell tracking, including radiolabeled imaging, bioluminescence imaging, paramagnetic iron oxide nanoparticle magnetic resonance imaging, ultrasound-guided photoacoustic imaging, graphene quantum dot imaging [72,73]. However, only a few studies have reported that the tracking technique is used to monitor MSCs seeded on bioactive materials. For example, Natalia et al. [74] seeded superparamagnetic iron oxide nanoparticles-labeled MSCs to poly-D,L-lactide/poly-ε-caprolactone (PL-PC) scaffold, and monitored the distribution of MSCs *in vivo* after implantation (Fig. 3). Liu et al. [75] used biological activated carbon quantum dots to guide the osteogenic differentiation of hBMSCs, and observed the distribution of cells by laser confocal microscopy. These studies show that cell tracking technology has great potential to monitor the growth, migration and distribution of MSCs seeded on BBMs. However, the tracking method of the osteogenic differentiation of MSCs is still absent.

2.4. Characterization of osteogenesis in vivo

BBMs can promote osteogenesis *in vivo* and accelerate the repair and regeneration of bone tissue defects by regulating adhesion, proliferation and differentiation of MSCs. Osteogenesis characterization is a direct and reliable assay for assessing the fate of MSCs regulating by BBMs *in vivo*.

2.4.1. X-ray imaging examination

X-ray can pass through tissue structures of human body due to its penetrability, fluorescence effect and photosensitive effect. As there are differences in density and thickness between human tissues, the amount of X-ray passing through the different tissues will be different to varying degrees. In this way, images of different light and shade or black and white contrast can be formed on the screen or X-ray film, which is suitable for the detection of bone regeneration after implantation of BBMs. For example, Li et al. [76] cultured BMSCs in porousβ-TCP scaffolds to construct tissue-engineered bone, with the β-TCP scaffolds as the control group. X-ray imaging was performed 8 weeks and 16 weeks after implantation to evaluate stent degradation and bone repair. The density of β - TCP in the tissue engineering group was lower than that in the scaffold group, which indicated that the degradation of β-TCP in the tissue engineering group was faster than that in the scaffold group. In addition, the bone defect repair of tissue engineering group was better than that of scaffold group, which indicated that β-TCP seeded with BMSCs was beneficial to bone regeneration. X-ray can not only be used to evaluate the regeneration of bone defects, but also to evaluate the degradation of scaffolds, so it has great potential in the evaluation of bone regeneration based on BBMs.

2.4.2. Histopathological examination

Tissue slices need to be destructively extracted and made into sections. Hematoxylin-eosin (HE) staining can stain the nucleus and cytoplasm at the same time, which is the most commonly used method to observe the cells in tissue slices. On the other hand, calcium staining is often used to characterize the osteogenesis since calcium nodules are produced in the process of osteogenic differentiation of cells.

2.4.2.1. HE staining. In HE staining, the stain can dye the chromatin and nucleic acid in the nucleus into purple blue, while the components in the cytoplasm and extracellular matrix into red. Dai et al. [77] used HE staining to evaluate the biocompatibility of novel nano-calcium-deficient hydroxyapatite/polyamino acid (n-CDHA/PAA) composite biomaterials with bone and muscle tissue models *in vivo*. At 4 weeks, a small amount of inflammatory cells were found around the implant material, and the fibrous tissue around the biomaterial was slightly thinner. At 12 weeks, the inflammation disappeared and the fibrous tissue continued to be thinner and the peripheral muscles of the implanted materials were arranged normally. It demonstrated that HE staining can be used to analyze the repair of tissue around the implant at the cellular level, which can further reflect the osteogenesis.

2.4.2.2. ARS staining. MSC were cultured, adhered and proliferated on the surface of BBMs, and mineralized nodules would be formed after osteogenic differentiation of MSC. In the late stage of osteogenic differentiation, tiny nodules formation is another important indicator of osteogenic differentiation efficiency [78]. Alizarin red staning (ARS) is a kind of anthraquinone derivative. It can form orange red complex with calcium in the form of chelation so that the calcium salt composition in tissue or cell can be identified clearly [79–81]. Therefore, the degree of osteogenic differentiation can be evaluated by ARS staining both *in vivo* and *in vitro*. For example, Yang et al. [63] used ARS staining to identify the formation of mineralized nodules after 21 days osteoinduction of hMSCs cultured with silica nanoparticles. Specifically, the hMSCs were stained by ARS after being fixed by 4% paraformaldehyde, and then were photographed under phase-contrast microscope. To quantify the orange red complex, the adsorbance was measured after the addition of cetylpyridinium chloride monohydrate dissolved in sodium phosphate buffer [63].

3. The latest research progress of high throughput technology

High throughput technology is emerging quickly as a competing and/or mutually supportive role compared with the traditional methods. High throughput technology provides great convenience for fast and comprehensive characterization of the fate of MSCs regulated by BBMs since it can analyze and process a large number of experimental objects in parallel. Herein, the application of high-throughput technology in the detection of the fate of MSCs regulated by BBMs was systematically reviewed from transcriptomics, proteomics, high throughput cell technology, and high-throughput tissue technology.

3.1. Transcriptomics

In the process of gene expression, transcription of DNA into mRNA is a necessary and critical step. Transcriptomics, which combines gene chip or high-throughput RNA sequencing with bioinformatics analysis, refers to the study of gene transcription and regulation in cells at the overall level. Genomics refers to collectively characterizing and quantifying all genes of an organism, and studying the relationship between them and their impact on the organism. However, genomics and transcriptomics have been confused frequently, and the study of gene expression profiles is often mistakenly called genomics, which should be transcriptomics.

3.1.1. Gene chip

The principle of gene chip sequencing is hybridization sequencing method, that is to determine the nucleic acid sequence by hybridization

with a group of nucleic acid probes with known sequences. The probes of known target nucleotides are fixed on the surface of a substrate. When the fluorescent labeled nucleic acid sequence in the solution is complementary to the corresponding nucleic acid probe on the gene chip, a set of probe sequences with complete complementary sequence can be obtained by determining the position of the probe with the strongest fluorescence intensity. The target nucleic acid sequence can be recombined in this way. There are two main methods for the preparation of microarray: in situ synthesis and spotting samples method.

Microarray technology has changed the method of biological research, making it possible for rapid and high-throughput analysis of micro samples. It has rapidly expanded from the study of single gene to the research of system biology of the whole genome. Microarray technology has helped biological research to enter the era of post genome. Gene chip has been approved by FDA for clinical disease classification and prognosis research. With the development of gene chip, it can be used to detect small RNA, which plays an important role in the development of human diseases, especially tumors. Therefore, gene chip can be used for tumor typing, and has made great progress in searching for molecular markers of lung cancer, prostate cancer, breast cancer and other diseases. Transcriptomics combining gene chip with bioinformatics analysis has been used to study the signal pathway mechanism of material composition and topography on the fate regulation of MSCs [82–87]. For example, Lv et al. [36] extracted the total RNA of MSCs cultured on natural hydroxyapatite (NHA) and synthetic hydroxyapatite, respectively, and used Agilent Mouse Whole Genome Oligo Microarrays containing 43,000 genes to detect the gene expression profiles of MSCs in each sample. Subsequently, statistically significant differentially expressed genes were filtered by volcano map, and differentially expressed genes of each sample were identified by 2-fold change screening. DAVID and GoSurfer was used to screen the genes related to proliferation and ostogenic differation. After that, cluster analysis (Cluster and TreeView software), GO functional classification analysis (DAVID software), biological pathway (GenMAPP software) and interaction network analysis (Ingenuity Pathway Analysis, IPA) was used to analyze the osteogenic differentiation related genes. By the combination of gene chip and bioinformatics, this study successfully revealed that NHA induced a series of gene differential expression and its regulatory signal pathways, and ultimately promoted the osteogenic differentiation of MSCs.

Barradas et al. [88] have successfully analyzed the signaling pathways of different fates of hBMSCs caused by β-TCP and HA through transcriptomics, and further revealed their intrinsic molecular mechanism. Dalby et al. [89] compared the gene expression profiles of MSCs cultured in two different nanostructures with those of MSCs treated with dexamethasone. The results showed that the osteogenic differentiation induced by nanostructures could achieve the same effect as that induced by chemical treatment, and further revealed their respective internal mechanism of action. This study demonstrated that the morphology of materials plays an important role in the fate of MSCs [89]. More recently, Baker et al. [90] studied the signaling pathways of MSCs regulated by 3D scaffolds and 2D films, and the signaling pathways of MSCs regulated by poly (ε - caprolactone) (PCL) and poly (D, L-lactic acid) (PDLLA) respectively (Fig. 4). In addition, tissue culture polystyrene with and without osteogenic complement was used to induce phenotype analysis. The results demonstrated that biochemical and physical signals regulate similar signaling pathways, indicating that these signals have similar mechanism in regulating MSCs fate [90].

Transcriptomics has the potential to link the transcription environment that regulates cell fate with biomaterial design parameters. Groen et al. [91] parameterized the material properties (micropores, ionic composition, protein adsorption) of a group of artificial bone induced and non induced CaP ceramics, which were related to the transcriptome characteristics of osteoblasts grown on the materials *in vitro*. Based on these data, a genetic network was constructed to control bone formation induced by biomaterials. By separating the complex material

properties into a single parameter test condition, it is confirmed that a subset of these genes is indeed controlled by the surface morphology and the ions released from the ceramics, respectively. This work provides insight into the composition and engineering of biomaterials for bone gap filling, and can be used as a strategy to explore the interface between biomaterials and tissue regeneration.

3.1.2. High throughput RNA sequencing

The disadvantage of gene chip is that it is a "closed system", which can only detect the characteristics (or limited variation) of known sequences. On the contrary, the advantage of high-throughput gene sequencing is that it is an "open system", and its ability to discover and find new information is essentially more advanced than that of gene chip technology. But gene chip has the ability of high-throughput, relatively low-cost and rapid detection of known gene information. So gene chip and high-throughput sequencing can complement each other.

The first generation of gene sequencing technology used chain termination method initiated by Sanger and Coulson in 1975 or chemical method (chain degradation) invented by MAXAM and Gilbert in 1976–1977. In the past few decades, the first generation sequencing technology has been used as the standard of gene diagnosis. It plays an important role in the diagnosis of gene diseases, and other diseases such as self destructing face syndrome, phenylketonuria etc. However, the first generation of gene sequencing technology have the limitations of low-throughput and high cost. With the increasing of the flux and the decreasing of sequencing price due to the development of second generation of gene sequencing technology, the second generation high-throughput sequencing based on synthetic sequencing has been widely used, among which high-throughput RNA sequencing (RNA-seq) is one of the most important applications. The main applications of RNA-seq include identification and analysis of differentially expressed genes, annotation of gene structure and identification of new genes, exploration of gene mutation and search for expression quantitative trait loci (eQTL), fusion gene and identification of non coding RNA. In the detection of the fate of MSCs regulated by BBMs, RNA-seq and bioinformatics analysis can be used to identify and analyze the differentially expressed genes. Specifically, reverse transcriptase is used to reverse the enriched mRNA into cDNA and the corresponding RNA sequence, structure and expression amount can be obtained by building and sequencing the cDNA library. Subsequently, the difference of gene
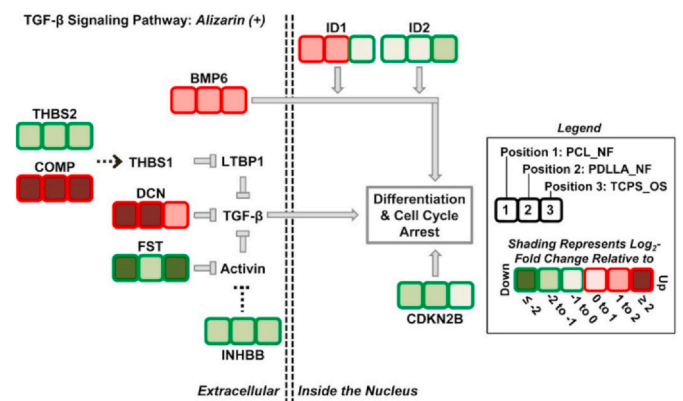

Fig. 4. Schematic of the KEGG "TGF-β Signaling Pathway" showing differentially regulated genes for the Alizarin (+) treatments (TCPS_OS, PCL_NF, PDLLA_NF). Values are from median normalization to 6-treatments (PCL_NF, PCL_SC, PDLLA_NF, PDLLA_SC, TCPS, TCPS_OS). Red boxes indicate up-regulation and green boxes indicate down-regulation of genes relative to TCPS. Shading of the boxes indicates the magnitude of the log2 fold change relative to TCPS using values calculated in Fig. 3 (determined as follows: log2(Treatment) – log2(TCPS)). Arrows indicate activation and T-blocks indicate inhibition of a downstream component. Dashed arrows and dashed T-blocks indicate less recognized associations. Reprinted with permission from Ref. [90].

expression is analyzed by monochrome method and dichroism method. The up-regulated gene or down-regulated gene selected from them may be the difference gene required for research. After that, the differentially expressed genes screened by RNA-seq in cells from different groups are analyzed by means of bioinformatics. Generally, Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis are employed. GO analysis is a commonly used biological database, which can be divided into three parts:

molecular function, biological process and cellular component. Each gene has its own sequence annotation, which can be used to find the corresponding GO Term, i.e. functional category or cell localization. Generally, through functional enrichment analysis, the p value of selected differential genes is calculated, and the hypergeometric distribution of specific branches in GO classification is related to the statistical significant enrichment of GO Term (p value is small). This function or location may be related to the research content. KEGG
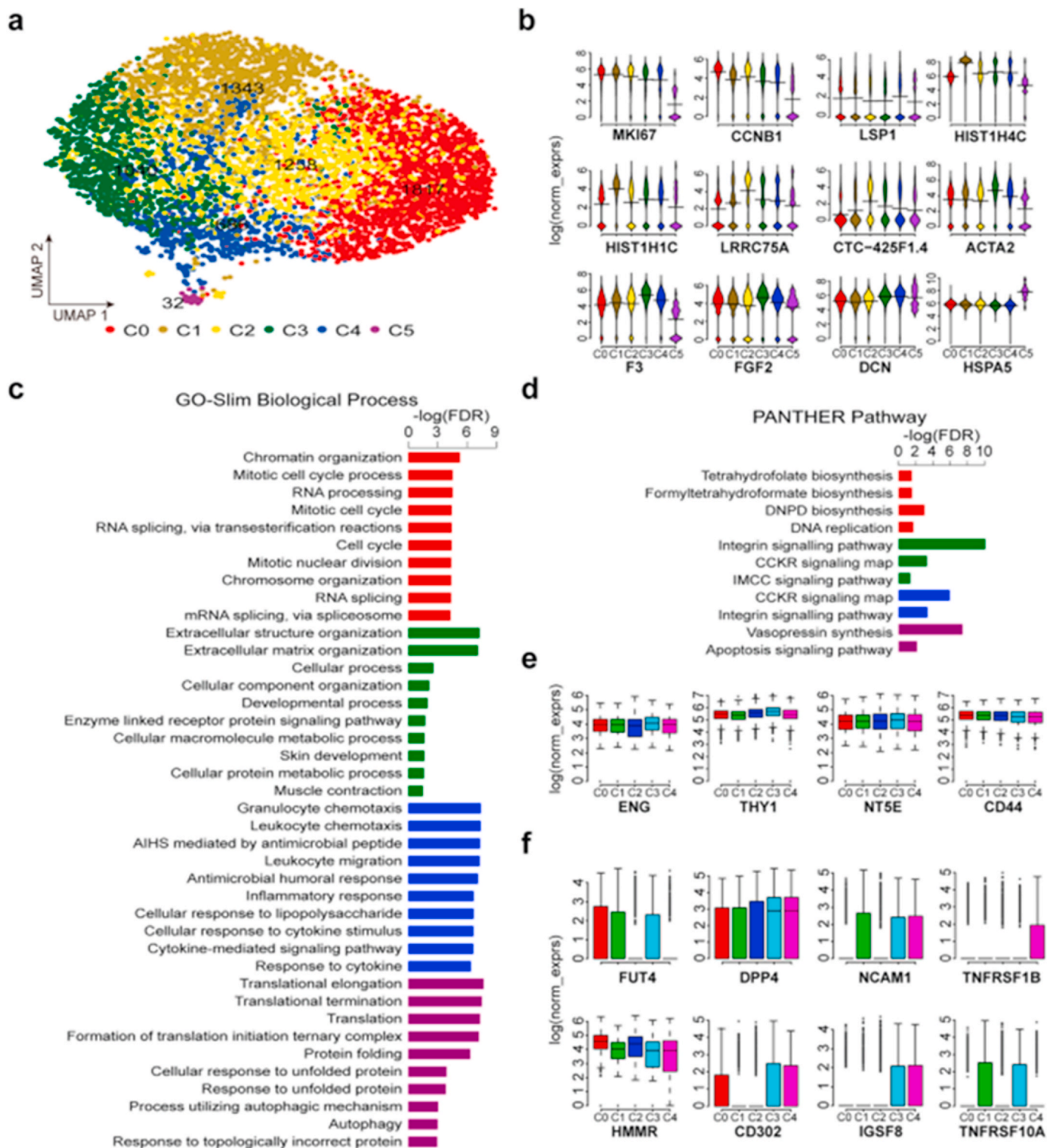

Fig. 5. Candidate subpopulations with different functional characteristics in WJMSCs. Reprinted with permission from Ref. [95].

pathway analysis is one of the most commonly used bioinformatics databases in the world. It integrates transcriptomics, chemical and systemic functional information, and links the catalogue of genes from the genome that has been sequenced to higher-level system functions at the cellular, species and ecosystem levels [92]. The application of RNA-seq in biomaterial analysis is mainly through the comparison of different genes. Genes are compared one by one between the two materials (generally, the difference of p value is more than two times), and the genes are located on GO Term or gene pathway by functional enrichment analysis, so as to analyze which related cell activities are affected by biomaterials. Cluster analysis is required for the analysis of various materials, which can reduce the dimensionality of different materials to several important factors, so as to analyze which factors will have greater impact on cell activity. Carinci et al. [93] screened several genes that were significantly up-regulated or down-regulated in osteoblasts regulated by Bio-Oss. The functions of these genes include signal transduction, transcription, cell cycle regulation, vesicle transport, apoptosis, immunity and so on.

By means of transcriptomics combining RNA-seq with bioinformatics, the fate of MSCs regulated by BBMs can be characterized at the overall transcriptional level and its potential signaling pathway can be revealed. For example, to explore the effect of graphene on gene expression profile of mesenchymal stem cells and reveal the mechanism of graphene induced osteogenic differentiation, using Truseq RNA Library Prep kit V2 (Illumina), zhou et al. [37] conducted mRNA-seq on human adipose-derived mesenchymal stem cells (hASCs) cultured on single-layer graphene-coated titanium disks or titanium disks in proliferation medium (control) or osteoinduction medium for 7 days. Subsequently, pathway and GO analyses was conducted on DAVID Bioinformatics Resources 6.8 (NIAID/NIH) and network analysis was conducted on Ingenuity Pathways Analysis (IPA). Signaling pathway analysis showed the five most affected pathways. GO analysis revealed the significant effects of cell adhesion, calcium signal transduction and epigenetic regulation. IPA network analysis showed a significant impact on inflammation related pathways, and the downstream factors of histone H3 and H4 also changed, especially in the presence of bone induction medium. The results indicate that graphene can promote the osteogenic differentiation of hASCs mainly by affecting cell adhesion, the interaction between cytokines and cytokine receptors, and inflammatory reaction, and may affect histone H3 and H4 through epigenetic regulation.

3.1.3. Single cell highthroughput RNA sequencing

The methods mentioned before, which have been proven useful in most cases, offer global views of detecting the fate of BBMs regulating by BBMs. However, they cannot distinguish one cell from another, and their depth of detection is limited, which makes them likely to neglect details. This heterogeneity may result from differences of the micro-environment in which each cell resides, and may indicate different roles of various subgroups of cells [94]. In clinical scenarios, individual differences among patients make bulk analysis methods less suitable to provide customized information on healing/disease progress and advice for better repair.

Therefore, we believe the single-cell RNA sequencing (scRNA-seq) would be beneficial for investigating the repair fate mechanisms of MSCs regulating by MSCs at a deeper level. This methodology analyses every cell in a sample as an individual and each cell's RNA is labeled separately and sequenced. It focuses on the heterogeneity within a sample. It is an excellent tool for finding rare subtypes or states of cells [94,95], which are likely to be neglected in bulk analysis. Furthermore, for samples in which cells gradually change their state over time during a certain biological progress, such as normal development [96,97] or disease progression [98], single-cell sequencing methods can help define a pseudo-temporal axis of the progress [99], and scatter cells along it to find those at different stages.

In the case of MSCs-mediated tissue repair and regeneration, scRNA-seq has been applied mostly for revealing intrinsic heterogeneity in MSCs cultured *in vitro*. Freeman et al. [100] discovered multiple lineage priming in mouse BMSCs with scRNA-seq, showing that although BMSCs express multipotency-related genes in a relatively consistent manner, different subgroups with distinct lineage-defining marker genes exist, including osteogenic, chondrogenic, adipogenic, neurogenic and vascular smooth muscle differentiation-related ones. Sun et al. [95] compared human primary Wharton's jelly-derived MSCs (WJMSCs) cultured *in vitro* and public data of adipose-derived MSC (ADMSCs) using scRNA-seq, and by gene ontology (GO) analysis discovered distinct subpopulations of WJMSCs with characteristic gene expression related to proliferation, development, and inflammation response, while WJMSCs and ADMSCs portrayed similarities in highly variable genes despite their different origins (Fig. 5). Furthermore, they discovered a CD142$^+$ subgroup of WJMSCs that also highly express genes with functional enrichment results of skin development and wound healing. They then sorted CD142$^+$ cells and confirmed its higher wound healing potency but lower proliferation capacity than CD142$-$ cells, indicating possible clinical applications of this special subtype. Application of scRNA-seq *in vivo* is to attempt to harvest MSCs from tissue which are being repaired by implanting MSCs-laden biomaterials. However, no research using this method have been reported to date, possibly due to lack of appropriate methods to extract enough viable implanted MSCs from regenerated tissue. Work in this field is being carried out in our group and publication is being expected.

3.2. Proteomics

There are three levels of regulation in the process from DNA to protein: transcription level regulation, translation level regulation and
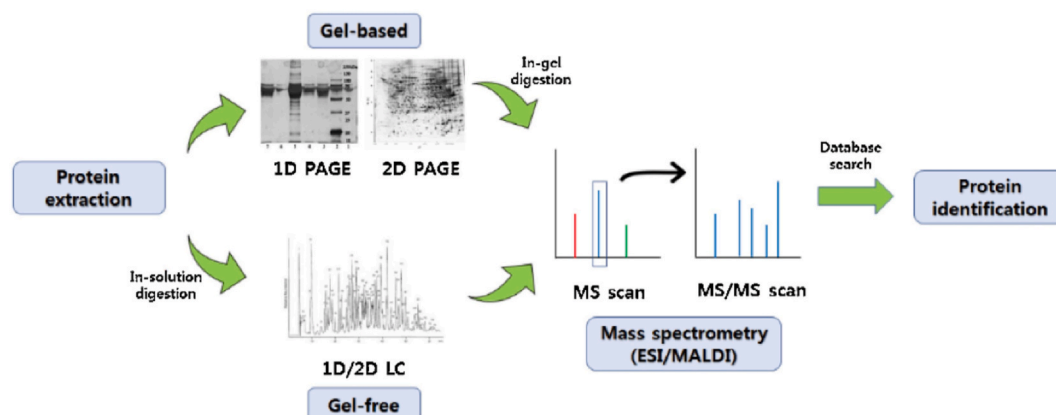

Fig. 6. A schematic showing the sequence of proteomic analysis using gel-based or gel-free proteomics. Reprinted with permission from Ref. [102].

post translation level regulation. The research object of transcriptomics is mRNA, which belongs to the regulation of transcription level, and may not represent the level of protein expression absolutely. It has also been proven that the correlation between mRNA abundance and protein abundance may be not high, especially for low abundance proteins. More importantly, the complex post-translational modification of proteins, subcellular localization or migration of proteins, and protein-protein interaction can hardly be judged from mRNA level. As is known to us, protein is the executor of physiological function and the direct embodiment of life phenomenon. The study of protein structure and function will directly clarify the mechanism of life changes under physiological or pathological conditions. The existing forms and activities of proteins, such as post-translational modification, protein-protein interaction and protein conformation, still depend on the direct study of proteins. Although the special properties of protein, such as variability and diversity, make protein research technology much more complex and difficult than nucleic acid technology, it is these properties that participate in and affect the whole life process.

The traditional technology of single protein research has been unable to meet the requirements of the post genome era. There are several reasons: (1) the occurrence of life phenomenon is often influenced by many factors, and it inevitably involves many proteins. (2) The participation of multiple proteins is interwoven into a network, or occurs in parallel, or in a cascade of cause and effect. (3) The expression of proteins is diverse and dynamic when they perform physiological functions, which is not basically fixed as genome. Therefore, in order to have a comprehensive and in-depth understanding of the complex activities of life, it is necessary to study proteins at global, dynamic and networked level. Finally, in the mid-1990s, proteomics, a new discipline, had been developed by combining high-throughput protein sequencing with bioinformatics and can essentially separate and analyze the global proteins at a large-scale parallel pattern [101].

The solubilized protein mixture derived from cells or tissues is then applied to the gel strip, which separates the protein on the first dimension based on the isoelectric point of the protein (Fig. 6). Followed that, the strip is applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE gel, where the protein are denatured and separated according to its size in the second dimension. Subsequently, the gel was fixed and visualized through dyeing. After staining, the spots of interest were cut and digested in the gel with trypsin. Instead of using a PAGE gel, the gel free method needs to be digested by 1D or 2D Liquid chromatography (LC) prior to mass spectrometry (MS). Then the peptide mixture of each protein is analyzed by mass spectrometer, and the mass spectrum of peptide is obtained. Finally, in both cases, proteins were identified by peptide molecular weight analysis or amino acid sequencing by MS/MS using a protein database search program [102].

Proteomics has been used to detect the fate of MSCs regulated by BBMs. For example, using isobaric tags for relative and absolute quantification (iTRAQ) -coupled two-dimensional liquid chromatography-tandem mass spectrometry (2D LC-MS/MS) , Xu et al. [103] conducted proteomics on plane HA and carbon nanotube (CNT) enhanced HA, respectively. The results showed that cytoskeleton proteins, metabolic enzymes, signal transduction and cell growth proteins were differentially expressed on these two surfaces. The levels of these proteins were generally higher in the cells adhering to HA surface, indicating that the proliferation level of these cells was high. In order to clarify the interaction between materials and cells, Othman et al. [39] used proteomics to detect serum proteins adsorbed on biomaterials and proteins expressed in cells. y Specifically, they analyzed the serum proteins adsorbed on two kinds of CaP ceramics (osteoinductive β- TCP and non osteoinductive HA) by proteomics. They also analyzed the protein expression profiles of hMSCs on the two ceramics, and further analyzed the mechanism of the two ceramics on hMSCs. It suggested that proteomics can help to understand the effects of biomaterial properties on cells.

More recently, proteomics have been used to detect the fate of MSCs regulated by BBMs *in vivo*. For example, Chen et al. [104] applied proteomics to analyze the mechanism of bone regeneration and re-modeling induced by implantation of 3D decalcified bone matrix (DBM)/fibrin gel scaffold in New Zealand white rabbits. Isolated BMSCs were seeded in prepared scaffolds after incubation for 28 days *in vitro*, and then implanted into New Zealand white rabbits bone defect. After 12 weeks, hematoxylin–eosin (HE) staining, ALP staining, and Osteo-pontin immunofluorescence detection was applied to observe new bone formation. It was found that BMSCs/DBM/fibrin glue group had more new bone formation than control group. The results of serum proteomics showed that TTR protein, ALB protein and RBP4 protein expressed better than the control group. They may participate in gene replication, repair and expression through neuroendocrine system. Moreover, osteopontin was significantly higher in experimental group, which might be responsible for more new bone formation.

When mass spectrometry based proteomics technology is used to detect low abundance proteins, some proteins may not be detected. To figure out the exact number and quantity of growth factors in COLLOSS®E, Nienhuijs et al. [105] analyzed the growth factors in COLLOSS®E by Elisa, Western blot and mass spectrometry. The aim of this study was to identify the growth factors in COLLOSS®E and to correlate these with osteogenic induction. Interestingly, BMP-2 and BMP-7 were identified by ELISA, but not by MS and Western blot. Several growth factors including TGF - β 1, TGF - β 2 and BMP-3 were identified by LC-MS/MS. The results were confirmed by Western blot except BMP-3 which could not be identified by this method. It can be further inferred that COLLOSS®E may contain proteins that cannot be detected by these three detection techniques. This study indicates that the combination of traditional protein detection technology and proteomics technology can improve the accuracy of detection results. However, even if different protein detection techniques including traditional protein detection technology and proteomics technology are used, there is still room for improvement in identifying single low abundance proteins. Therefore, it is still necessary to further improve the resolution of protein detection technology [106].

The multidisciplinary approach with well-integrated "omics," i.e., transcriptomics, proteomics, etc. can provide more comprehensive and systematic information for detecting the fate of MSCs regulated by BMMs. For example, Lv's group [107] studied the mechanism of osteogenesis of mice BMSCs induced by HA by using expression profile chip technology in transcriptomics and iTRAQ-coupled 2-D LC-MS/MS analysis in proteomics. It was found that natural HA could affect some key genes, such as Bmp2, Bmp4, Tgfb2, Bmp1, Bmpr1a, Bmp2k, Spp1, Tcfe3 and Vdr to activate TGF-β signaling pathway and regulate osteogenic differentiation of MSCs. The combined analysis of proteomics and genomics results showed that mitogen-activated protein kinase (MAPK) signaling pathway played an important role in the process of osteogenic differentiation of MSCs induced by natural HA. Differentially expressed genes and proteins were distributed in the upstream and downstream of the pathway, and the up-regulated or down-regulated expression of genes and proteins were in good agreement.

3.3. High-throughput cell analysis

In recent years, the emerging fluorescence detection technology of microfluidic and laser scanning has also been used to study the fate of cells regulated by BBMs in a large number of ways. By using this method, the fluorescence of parallel arrays in a large field of vision can be detected and collected. For example, Griffiths et al. [108] used high-throughput microdroplet-based microfluidic system to screen and analyze cells. It took only 2–6 h to screen 10^6 cells, and the release or secretion of proteins from each single cell was realized. It has the characteristics of microregionalization and the limitations of traditional flow cytometry and fluorescence-activated cell sorting could be overcome. This system can be used to quantify the catalytic and regulatory

effects of various intracellular, surface and secreted proteins. Our group [109] developed a polydopamine polypyrrole microcapsule (PDA-PPy-MCs) with electrical response and conductivity (Fig. 7). A High-throughput screening array was constructed with surface structure and stimulation voltage as variables. The synergistic effects of surface results and stimulation voltage on the activity of BMSCs were investigated and the morphology of cells was characterized by laser confocal technique. The results showed that BMSCs had the highest proliferation and differentiation ability under the optimal microstructures, polydopamine (PDA) content and electrical stimulation signals. Our group [110] designed a high-throughput method to study the effect of polydopamine-partially reduced graphene oxide-polyacrylamide (PDA-pGO-PAM) hydrogel on cell proliferation. The hydrogel encapsulating BMSCs was placed in 96-well plate. The effect of pGO concentration and applied voltage on BMSCs was stimulated by changing the concentration of GO in the hydrogel. Finally, the viability of BMSCs was detected by a large number of enzyme labels at one time.

3.4. High-throughput tissue analysis

In vivo high-throughput characterization technology plays an important role in the detection of the fate of MSCs regulated by BBMs. However, few *in vivo* high-throughput characterization technologies have been reported [111]. In 2013, Wu et al. [112] quantitatively measured the effect of laser on soft tissue injury using 3D digital microscopy, which may be also promising. Compared with traditional tissue processing technology, it is faster and more effective. It provides a high-throughput controllable comparative analysis method to characterize the arrangement of laser groups. This study combines an automatic programmable laser instrument with a 3D digital microscope to analyze tissue ablation and carbonization using a fast and non-invasive method. On the basis of preserving the integrity of tissue samples, this technique can quantitatively analyze the whole lesion location, and then can be applied in immunohistology.

4. Conclusion remarks

In this review, the methods of detecting the fate of MSC regulated by BBMs were systematically reviewed, and the application of high-throughput technology has been emphasized to provide guidelines for researchers who study the fate of MSC regulated by BBMs. The application of high-throughput technology can greatly improve the comprehensiveness and efficiency here. High-throughput technology can be used to analyze gene and protein expression separately, and obtain comprehensive gene and protein expression profiles. By further analysis, the expression pathways at gene and protein levels can be obtained respectively, thus revealing the intrinsic mechanism of the interaction between biomaterials and cells.

Although high-throughput technology has made preliminary progress in the detection of cell fate. However, there are still many challenges to be solved:

(1) Importantly, the results of high-throughput detection for detecting the fate of MSCs regulated by BBMs *in vitro* can not fully reflect the situation *in vivo*. However, there are few reports on the application of high-throughput technology for detecting the fate of MSCs regulated by BBMs *in vivo* detection. Therefore, more high-throughput detection technology for detecting the fate of MSCs regulated by BBMs *in vivo* needs to be explored so as to achieve more clinical applications.

(2) Protein is the executor of life activities, so the characterization of protein level can reflect MSCs fate more directly and accurately than that of gene level. However, due to the limitation of protein sequencing technology, the clinical application of high-throughput gene sequencing technology is more extensive than that of high-throughput protein sequencing technology. It is urgent to develop new protein detection technology and improve the efficiency and accuracy of protein sequencing.

(3) scRNA-seq can help us to understand the transcriptional status of each cell, so as to reveal the mechanism of MSCs fate regulated by BBMs. However, due to the mini amount of mRNA contained in a single cell, it does not meet the requirements of the current mainstream Illumina sequencing platform for the construction of a library, so one-step full transcriptome amplification is needed. There are still some problems in the amplification of single cell whole transcriptome. In the subsequent bioinformatics analysis, how to effectively remove amplification bias and how to measure gene expression will be an important aspect.

(4) Single omics can hardly reveal the intrinsic mechanism of the fate of MSCs regulated by BBMs. The multidisciplinary approach with well-integrated omics i.e., transcriptomics, proteomics and materiomics etc. can be provide more comprehensive and systematic information for detection the fate of MSCs regulated by BMMs. Furthermore, it can be expected that the design and synthesis of BBMs which can precisely regulate the fate of MSCs will be realized by converging transcriptomics and/or proteomics with materiomics.

(5) High throughput technology can overcome the disadvantage of traditional technology that only small amount of information can be obtained at one time. However, due to the low cost and stability of traditional technology, it also has the irreplaceable role of high-throughput technology. Therefore, high-throughput technology and traditional technology complement each other in detecting the fate of MSCs regulated by BBMs.

(6) As mentioned above, high throughput technology can provide great convenience for fast and comprehensive characterization, although it still has some limitations. In order to make high-throughput technology more widely used in detecting the fate of MSCs regulated by BBMs, it is necessary not only to improve the high-throughput technology itself, such as sensitivity and stability, but also to develop bioinformatics to obtain more comprehensive information based on the detected data.

Declaration of competing interest

No conflict of interest exsits.
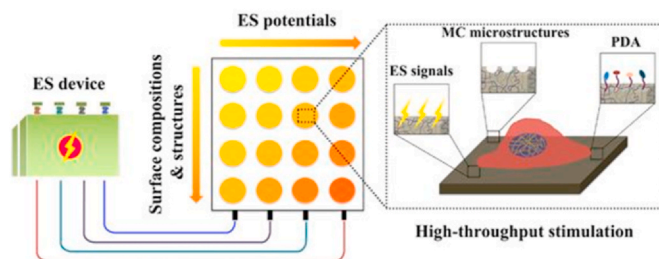
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Fig. 7. High-throughput stimulus schematic. BMSCs was stimulated at high-throughput on different surfaces with different surface components, structures and ES potentials. Reprinted with permission from Ref. [109].

References

[1] R. Rajasekaran, V.S. Seesala, K.C. Sunka, et al., Role of nanofibers on MSCs fate: influence of fiber morphologies, compositions and external stimuli, Mater. Sci. Eng. C 107 (2020) 110218.

[2] K. Metavarayuth, P. Maturavongsadit, X. Chen, et al., Nanotopographical cues mediate osteogenesis of stem cells on virus substrates through BMP-2 inter-mediate, Nano Lett. 19 (12) (2019) 8372–8380.

[3] W. Strober, Trypan blue exclusion test of cell viability, Curr. Protoc. Im. 111 (A3) (2015).

[4] Z. Chn, T. Klein, R.Z. Murray, et al., Osteoimmunomodulation for the development of advanced bone biomaterials, Mater. Today 19 (2016) 304.

[5] R.J. Miron, Bosshardt D.D. OsteoMacs, Key players around bone biomaterials, Biomaterials 82 (19) (2016).

[6] W. Zhao, D. Michalik, S. Ferguson, et al., RapidTY evaluation of bioactive Ti-based surfaces using an in vitro titration method, Nat. Commun. 10 (2019) 2062.

[7] C. Gao, S. Peng, P. Feng, et al., Bone biomaterials and interactions with stem cells, Bone Res 5 (2017) 17059.

[8] J.R. Xavier, T. Thakur, P. Desai, et al., Bioactive nanoengineered hydrogels for bone tissue engineering: a growth-factor-free approach, ACS Nano 9 (2015) 3109.

[9] L. Jiang, Y. Wang, Z. Liu, et al., 3D printing and injectable conductive hydrogels for tissue engineering application, Tissue Eng. B Rev. 25 (5) (2019) 398–411.

[10] Ž. Mitić, A. Stolić, S. Stojanović, et al., Instrumental methods and techniques for structural and physicochemical characterization of biomaterials and bone tissue: a review, Mat Sci Eng C-Mater 79 (2017) 930.

[11] W. Chen, Y. Liu, H.S. Courtney, et al., In vitro anti-bacterial and bio-logical properties of magnetron co-sputtered silver-containing hydroxy-apatite coating, Biomaterials 27 (2006) 5512.

[12] M.T. Chorsi, E.J. Curry, H.T. Chorsi, et al., Piezoelectric biomaterials for sensors and actuators, Adv Mater 31 (2019) 1802084.

[13] Z. Liu, S.S. Liow, S.L. Lai, et al., Retinal-detachment repair and vitre-ous-like-body reformation via a thermogelling polymer endotamponade, Nat Biomed Eng 3 (2019) 598–610.

[14] H. Xu, Z. Fang, W. Tian, et al., Green fabrication of amphiphilic quaternized β-chitin derivatives with excellent biocompatibility and antibacterial activities for wound healing, Adv Mater 30 (2018) 1801100.

[15] H. Song, X. Li, Z. Zhao, et al., Reversal of osteoporotic activity by endothelial cell-secreted bone targeting and biocompatible exosomes, Nano Lett. 19 (5) (2019) 3040–3048.

[16] C. Ma, L. Jiang, Y. Wang, et al., 3D printing of conductive tissue engineering scaffolds containing polypyrrole nanoparticles with different morphologies and concentrations, Materials 12 (15) (2019) 2491.

[17] L. Tan, J. Li, X. Liu, et al., Rapid biofilm eradication on bone implants using red phosphorus and near-infrared light, Adv Mater 30 (2018) 1801808.

[18] S.L. Harper, J.L. Carriere, J.M. Miller, et al., Systematic evaluation of nanomaterial toxicity: utility of standardized materials and rapid assays, ACS Nano 5 (2011) 4688.

[19] S. Gurunathan, J.H. Kim, Synthesis, toxicity, biocompatibility, and biomedical applications of graphene and graphene-related materials, Int. J. Nanomed. 11 (1927) 2016.

[20] G.L. Koons, M. Diba, A.G. Mikos, Materials design for bone-tissue engineering, Nat Rev Mater 5 (2020) 584–603.

[21] A.K. Gaharwar, I. Singh, A. Khademhosseini, Engineered biomaterials for in situ tissue regeneration, Nat Rev Mater (2020), https://doi.org/10.1038/s41578-020-0209-x.

[22] J. Xing, Y. Lu, Y. Cui, et al., A standardized and quality-controllable protocol of constructing individual tissue-engineered grafts applicable to treating large bone defects, Tissue Eng. C Methods 25 (2019) 137.

[23] A. Cheng, Z. Schwartz, A. Kahn, et al., Advances in porous scaffold design for bone and cartilage tissue engineering and regeneration, Tissue Eng. B Rev. 25 (14) (2018).

[24] Y. Wei, Z. Liu, X. Zhu, et al., Dual directions to address the problem of aseptic loosening via electrospun PLGA@ aspirin nanofiber coatings on titanium, Biomaterials 257 (2020) 200237.

[25] M. Zhang, R. Lin, X. Wang, et al., 3D printing of Haversian bone–mimicking scaffolds for multicellular delivery in bone regeneration, Sci. Adv. 6 (12) (2020) eaaz6725.

[26] W. Pei, T.B. Feyerabend, J. Rössler, et al., Polylox barcoding reveals haemato-poietic stem cell fates realized in vivo, Nature 548 (2017) 456.

[27] D. Tang, R.S. Tare, L.Y. Yang, et al., Biofabrication of bone tissue: approaches, challenges and translation for bone regeneration, Biomaterials 83 (2016) 363.

[28] L.O. Duchamp, C. Colnot, Periostin in bone regeneration, Adv. Exp. Med. Biol. 1132 (2019) 49.

[29] W.L. Grayson, B.A. Bunnell, E. Martin, et al., Stromal cells and stem cells in clinical bone regeneration, Nat. Rev. Endocrinol. 11 (2015) 140.

[30] N.J. Shah, M.N. Hyder, J.S. Moskowitz, et al., Surface-mediated bone tissue morphogenesis from tunable nanolayered implant coatings, Sci. Transl. Med. 5 (191) (2013) 191ra83-191ra83.

[31] K. Zhang, Y. Zhou, C. Xiao, et al., Application of hydroxyapatite nanoparticles in tumor-associated bone segmental defect, Sci. Adv. 5 (8) (2019) eaax6946.

[32] G. Thalji, L.F. Cooper, Molecular assessment of osseointegration in vitro: a review of current literature, Int. J. Oral Maxillofac. Implants 29 (2014).

[33] J.G. Caporaso, J. Kuczynski, J. Stombaugh, et al., QIIME allows analysis of high-throughput community sequencing data, Nat. Methods 7 (2010) 335.

[34] Robert P. Hertzberg, Andrew J. Pope, High-throughput screening: new technology

[35] for the 21st century, Curr. Opin. Chem. Biol. 4 (4) (2000) 445–451.

[35] Jason A. Reuter, Damek V. Spacek, Michael P. Snyder, High-throughput sequen-cing technologies, Mol. Cell 58 (4) (2015) 586–597.

[36] Xiaoying Lü, J. Wang, B. Li, et al., Gene expression profile study on osteoinductive effect of natural hydroxyapatite, J. Biomed. Mater. Res. 102 (8) (2014) 2833-28, 1.

[37] L.W. Lv, Y.S. Liu, P. Zhang, et al., Transcriptomics and functional analysis of graphene-guided osteogenic differentiation of mesenchymal stem cells, Chin. J. Dent. Res. 21 (2) (2018) 101–111.

[38] A. Zeisel, A.B. Muñoz-Manchado, S. Codeluppi, et al., Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq, Science 347 (6226) (2015) 1138–1142.

[39] Z. Othman, R.J.C. Mohren, B. Cillero-Pastor, et al., Comparative proteomic ana-lysis of human mesenchymal stromal cell behavior on calcium phosphate ceramics with different osteoinductive potential, Mater. Today Bio 7 (2020) 100066.

[40] M.O. Wang, C.E. Vorwald, M.L. Dreher, et al., Evaluating 3D-Printed biomaterials as scaffolds for vascularized bone tissue engineering, Adv Mater 27 (2015) 138.

[41] O.S. Fenton, K.J. Kauffman, J.C. Kaczmarek, et al., Synthesis and biological eva-luation of ionizable lipid materials for the in vivo delivery of messenger RNA to B lymphocytes, Adv Mater 29 (2017) 1606944.

[42] F. Bonnier, M.E. Keating, T.P. Wrobel, et al., Cell viability assessment using the Alamar blue assay: a comparison of 2D and 3D cell culture models, Toxicol. Vitro 29 (2015) 124.

[43] A. Del Grosso, S. Antonini, L. Angella, et al., Lithium improves cell viability in psychosine-treated MO3. 13 human oligodendrocyte cell line via autophagy ac-tivation, J. Neurosci. Res. 94 (2016) 1246.

[44] W. Strober, Trypan blue exclusion test of cell viability, Curr. Protoc. Im. 111 (2015) A3. B. 1.

[45] L.R. Arias, C.A. Perry, L. Yang, Real-time electrical impedance detec-tion of cel-lular activities of oral cancer cells, Biosens. Bioelectron. 25 (2010) 2225.

[46] A.A. Stepanenko, V.V. Dmitrenko, Pitfalls of the MTT assay: direct and off-target effects of inhibitors can result in over/underestimation of cell viability, Gene 574 (2015) 193.

[47] F. Angius, A. Floris, Liposomes and MTT cell viability assay: an incompatible af-fair, Toxicol. Vitro 29 (2015) 314.

[48] A. Van Tonder, A.M. Joubert, A.D. Cromarty, Limitations of the 3-(4, 5-di-methylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays, BMC Res. Notes 8 (2015) 47.

[49] P. Senthilraja, K. Kathiresan, In vitro cytotoxicity MTT assay in Vero, HepG2 and MCF-7 cell lines study of Marine Yeast, J. Appl. Pharmaceut. Sci. 5 (2015) 080.

[50] M. Houdkova, J. Rondevaldova, I. Doskocil, et al., Evaluation of antibacterial potential and toxicity of plant volatile compounds using new broth microdilution volatilization method and modified MTT assay, Fitoterapia 118 (2017) 56.

[51] F. Angius, A. Floris, Liposomes and MTT cell viability assay: an in-compatible affair, Toxicol. Vitro 29 (2015) 314.

[52] Tara Tariverdian, et al., 3D-printed barium strontium titanate-based piezoelectric scaffolds for bone tissue engineering, Ceram. Int. 45 (11) (2019) 14029–14038.

[53] X. Fu, H. Li, C. Liu, et al., Long noncoding RNA AK126698 inhibits proliferation and migration of non-small cell lung cancer cells by targeting Frizzled-8 and suppressing Wnt/β-catenin signaling pathway, OncoTargets Ther. 9 (2016) 3815.

[54] H.X. Ren, Q.C. Tang, L. Yan, et al., Evodiamine inhibits gastrointestinal motility via CCK and CCK1 receptor in water-avoidence stress rat model, Life Sci. 209 (2018) 210.

[55] D. Wen, M. An, H. Gou, et al., Cholecystokinin-8 inhibits methamphetamine-in-duced neurotoxicity via an anti-oxidative stress pathway, Neurotoxicology 57 (2016) 31.

[56] Y. Motojima, M. Kawasaki, T. Matsuura, et al., Effects of peripherally administered cholecystokinin-8 and secretin on feeding/drinking and oxytocin-mRFP1 fluores-cence in transgenic rats, Neurosci. Res. 109 (2016) 63.

[57] H. Fan, J. Hui, Z. Duan, et al., Novel scaffolds fabricated using oleuropein for bone tissue engineering, BioMed Res. Int. 2014 (2014) 652432.

[58] X. Xiao, P. Zhao, K. Zhong, et al., Comparison of the cytotoxic activi-ty of CIK cells by CCK-8 and LDH assay, J. Modern Oncol. 25 (2017) 2530.

[59] L. Jin, J.H. Lee, O.S. Jin, et al., Stimulated osteogenic differentiation of human mesenchymal stem cells by reduced graphene oxide, J Nanosci Nanotechno 15 (2015) 7966.

[60] K. Tiwari, M. Wavdhane, S. Haque, et al., A sensitive WST-8-based bioassay for PEGylated granulocyte colony stimulating factor using the NFS-60 cell line, Pharm. Biol. 53 (2014) 849.

[61] Hiroshi Naito, et al., The advantages of three-dimensional culture in a collagen hydrogel for stem cell differentiation, J. Biomed. Mater. Res. 101 (10) (2013) 2838–2845.

[62] G. Jiao, X. He, X. Li, et al., Limitations of MTT and CCK-8 assay for evaluation of graphene cytotoxicity, RSC Adv. 53240 (2015) 2015.

[63] X. Yang, Y. Li, X. Liu, et al., The stimulatory effect of silica nanoparticles on os-teogenic differentiation of human mesenchymal stem cells, Biomed. Mater. 12 (1) (2016) 015001.

[64] O. Frank, M. Heim, M. Jakob, et al., Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro, J. Cell. Biochem. 85 (4) (2002) 737–746.

[65] J.R. Mauney, S. Sjostorm, J. Blumberg, et al., Mechanical stimulation promotes osteogenic differentiation of human bone marrow stromal cells on 3-D partially demineralized bone scaffolds in vitro, Calcified Tissue Int 74 (5) (2004) 458–468.

[66] F. Mei, J.M. Tang, J.Y. Zhong, et al., Potential application of human amniotic mesenchymal cells for seeding cells in bone tissue engineering. Beijing da xue xue bao, J. Peking Univ. Health Sci. 41 (2) (2009) 192–195.

[67] T. Mahmood, P.C. Yang, Western blot: technique, theory, and trouble shooting, N. Am. J. Med. Sci. 4 (2012) 429.

[68] W.T. Chen, F. Zhang, X.Q. Zhao, et al., Galectin-3 and TRIM16 coregulate osteogenic differentiation of human bone marrow-derived mesenchymal stem cells at least partly via enhancing autophagy, Bone 131 (2020) 115059.

[69] L. Yuan, J. Sun, F. Cheng, et al., Acetyltransferase MORF regulates osteogenic differentiation potential of periodontal ligament stem cells, J. Practical Stomatol. 32 (2016) 778.

[70] C.Z. Wang, S.M. Chen, C.H. Chen, et al., The effect of the local delivery of alendronate on human adipose-derived stem cell-based bone regeneration, Biomaterials 31 (33) (2010) 8674–8683.

[71] B. Tang, B. Zhang, J. Zhuang, et al., Surface potential-governed cellular osteogenic differentiation on ferroelectric polyvinylidene fluoride trifluoroethylene films, Acta Biomater. 74 (2018) 291–301.

[72] M.A. Hossain, T. Chowdhury, A. Bagul, Imaging modalities for the in vivo surveillance of mesenchymal stromal cells, J. Tissue Eng. Regenerative Med. 9 (11) (2015) 1217–1224.

[73] Kerans, F.A. Fransiscus, et al., The potential of intrinsically magnetic mesenchymal stem cells for tissue engineering, Int. J. Mol. Sci. 19 (10) (2018) 3159.

[74] N.M. Yudintceva, Y.A. Nashchekina, N.A. Mikhailova, et al., Urethroplasty with a bilayered poly-D, L-lactide-co-ε-caprolactone scaffold seeded with allogenic mesenchymal stem cells, J. Biomed. Mater. Res. B Appl. Biomater. 108 (3) (2020) 1010–1021.

[75] Y. Han, F. Zhang, J. Zhang, et al., Bioactive carbon dots direct the osteogenic differentiation of human bone marrow mesenchymal stem cells, Colloids Surf. B Biointerfaces 179 (2019) 1–8.

[76] D. Li, M. Li, P. Liu, et al., Tissue-engineered bone constructed in a bioreactor for repairing critical-sized bone defects in sheep, Int. Orthop. 38 (2014) 2399.

[77] Z. Dai, Y. Li, W. Lu, et al., In vivo biocompatibility of new nano-calcium-deficient hydroxyapatite/poly-amino acid complex bio-materials, Int. J. Nanomed. 10 (2015) 6303.

[78] M. Shi, Y. Zhou, J. Shao, et al., Stimulation of osteogenesis and angiogenesis of hBMSCs by delivering Si ions and functional drug from mesoporous silica nanospheres, Acta Biomater. 21 (2015) 178–189.

[79] A. Neumann, A. Christel, C. Kasper, P. Behrens, BMP2-loaded nanoporous silica nanoparticles promote osteogenic differentiation of human mesenchymal stem cells, RSC Adv. 3 (46) (2013) 24222–24230.

[80] Z. Luo, Y. Deng, R. Zhang, et al., Peptide-laden mesoporous silica nanoparticles with promoted bioactivity and osteo-differentiation ability for bone tissue engineering, Colloids Surf. B Biointerfaces 131 (2015) 73–82.

[81] T. Moriguchi, K. Yano, S. Nakagawa, F. Kaji, Elucidation of adsorption mechanism of bone-staining agent alizarin red S on hydroxyapatite by FT-IR microspectroscopy, J. Colloid Interface Sci. 260 (1) (2003) 19–25.

[82] L.L. Zhang, N. Hanagata, M. Maeda, et al., Porous hydroxyapatite and biphasic calcium phosphate ceramics promote ectopic osteoblast differentiation from mesenchymal stem cells, Sci. Tech. Adv Mater 10 (2009) 025003.

[83] K.F. Bombonato-Prado, L.S. Bellesini, C.M. Junta, et al., Microarray-based gene expression analysis of human osteoblasts in response to different biomaterials, J. Biomed. Mater. Res. 88 (2) (2009) 401–408.

[84] X. Li, H. Wang, E. Touma, et al., Genetic network and pathway analysis of differentially expressed proteins during critical cellular events in fracture repair, J. Cell. Biochem. 100 (2) (2007) 527–543.

[85] G. Thalji, L.F. Cooper, Molecular assessment of osseointegration in vitro: a review of current literature, Int. J. Oral Maxillofac. Implants 29 (2) (2014) 171–199.

[86] Nikolaos Donos, et al., Gene expression profile of osseointegration of a hydrophilic compared with a hydrophobic microrough implant surface, Clin. Oral Implants Res. 22 (4) (2011) 365–372.

[87] Norinaga Kojima, et al., High-throughput gene expression analysis in bone healing around titanium implants by DNA microarray, Clin. Oral Implants Res. 19 (2) (2008) 173–181.

[88] A.M. Barradas, V. Monticone, M. Hulsman, et al., Molecular mechanisms of biomaterial-driven osteogenic differentiation in human mesenchymal stromal cells, Integrative Biol. 5 (7) (2013) 920–931.

[89] M.J. Dalby, A. Andar, A. Nag, et al., Genomic expression of mesenchymal stem cells to altered nanoscale topographies, J. R. Soc. Interface 5 (26) (2008) 1055–1065.

[90] B.A. Baker, P.S. Pine, K. Chatterjee, et al., Ontology analysis of global gene expression differences of human bone marrow stromal cells cultured on 3D scaffolds or 2D films, Biomaterials 35 (25) (2014) 6716–6726.

[91] N. Groen, H. Yuan, D.G.A.J. Hebels, et al., Linking the transcriptional landscape of bone induction to biomaterial design parameters, Adv. Mater. 29 (10) (2017) 1603259.

[92] P.O. Brown, D. Botstein, Exploring the new world of the genome with DNA microarray, Nat. Genet. 21 (33) (1999).

[93] F. Carinci, A. Piattelli, M. Degidi, et al., Genetic effects of anorganic bovine bone (Bio-Oss®) on osteoblast-like MG63 cells, Arch. Oral Biol. 51 (2006) 154.

[94] F. Buettner, K.N. Natarajan, F.P. Casale, et al., Computational analysis of cell-to-cell heterogeneity in single-cell RNA-sequencing data reveals hidden subpopulations of cells, Nat. Biotechnol. 33 (2) (2015) 155–160.

[95] C. Sun, L. Wang, H. Wang, et al., Single-cell RNA-seq highlights heterogeneity in human primary Wharton's jelly mesenchymal stem/stromal cells cultured in vitro, Stem Cell Res. Ther. 11 (1) (2020) 149.

[96] B. Treutlein, D.G. Brownfield, A.R. Wu, et al., Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq, Nature 509 (7500) (2014) 371.

[97] J.X. Li, H.F. Luo, R. Wang, et al., Systematic reconstruction of molecular cascades regulating GP development using single-cell RNA-seq, Cell Rep. 15 (7) (2016) 1467–1480.

[98] N. Navin, J. Kendall, J. Troge, et al., Tumour evolution inferred by single-cell sequencing, Nature 472 (7341) (2011) 90–94.

[99] C. Trapnell, D. Cacchiarelli, J. Grimsby, et al., The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells, Nat. Biotechnol. 32 (4) (2014) 381–386.

[100] B.T. Freeman, J.P. Jung, B.M. Ogle, Single-cell RNA-seq of bone marrow-derived mesenchymal stem cells reveals unique profiles of lineage priming, PloS One 10 (9) (2015) e0136199.

[101] Mike Tyers, Matthias Mann, From genomics to proteomics, Nature 422 (6928) (2003) 193–197.

[102] J. Kim, Systematic approach to characterize the dynamics of protein adsorption on the surface of biomaterials using proteomics, Colloids Surf. B Biointerfaces 188 (2019) 110756.

[103] J. Xu, K.A. Khor, J. Sui, et al., Comparative proteomics profile of osteoblasts cultured on dissimilar hydroxyapatite biomaterials: an iTRAQ-coupled 2-D LC-MS/MS analysis, Proteomics 8 (20) (2010) 4249–4258.

[104] Y. Chen, B. Bai, S. Zhang, et al., Study of a novel three-dimensional scaffold to repair bone defect in rabbit, J. Biomed. Mater. Res. 102 (5) (2014) 1294–1304.

[105] M.E.L. Nienhuijs, K. Poulsen, M.V.D. Zande, et al., Analytical assessment of the osteoinductive material COLLOSSE, J. Biomed. Mater. Res. B Appl. Biomater. 89B (2) (2009) 300–305.

[106] T. Nilsson, M. Mann, R. Aebersold, et al., Mass spectrometry in high-throughput proteomics: ready for the big time, Nat. Methods 7 (9) (2010) 81–685.

[107] X.Y. Lv, Y. Huang, Y.D. Yu, et al., Application of genomics/proteomics technologies in the research of biocompatibility of biomaterials, J. Inorg. Mater. 28 (2013) 21.

[108] L. Mazutis, J. Gilbert, W.L. Ung, et al., Single-cell analysis and sorting using droplet-based microfluidics, Nat. Protocols Erecipes Researchers 8 (2013) 870.

[109] C. Xie, P. Li, L. Han, et al., Electroresponsive and cell-affinitive polydopamine/polypyrrole composite microcapsules with a dual-function of on-demand drug delivery and cell stimulation for electrical therapy, NPG Asia Mater. 9 (2017) e358.

[110] L. Han, X. Lu, M. Wang, et al., A mussel-inspired conductive, self-adhesive, and self-healable tough hydrogel as cell stimulators and implantable bioelectronics, Small 13 (2017) 1601916.

[111] D.M. Reif, L. Truong, D. Mandrell, et al., High-throughput characteri-zation of chemical-associated embryonic behavioral changes predicts teratogenic outcomes, Arch. Toxicol. 90 (2016) 1459.

[112] Z. Dai, Y. Li, W. Lu, et al., In vivo biocompatibility of new nano-calcium-deficient hydroxyapatite/poly-amino acid complex biomaterials, Int. J. Nanomed. 10 (2015) 6303.