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Emerging modulators for osteogenic differentiation: a combination of chemical and topographical cues for bone microenvironment engineering

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Diana Jesus[#a], Ana R. Pinho[#a], Maria C. Gomes[a], Cláudia S. Oliveira[*a], João F. Mano[*a]

Bone presents an intrinsic ability for self-regeneration and repair, however critical defects and large fractures require invasive and time-consuming clinical interventions. As an alternative to current therapy, bone tissue engineering (BTE) has primarily aimed to recreate the bone microenvironment by delivering key biomolecules and/or modification of scaffolds to guide cell fate towards the osteogenic lineage or other phenotypes that may benefit the bone regeneration mechanism. Considering that bone cells communicate, in their native microenvironment, through biochemical and physical signals, most strategies fail when considering only chemical, geometrical or mechanical cues. This is not representative of the physiological conditions, where the cells are simultaneously in contact and stimulated by several cues. Therefore, this review explores the synergistic effect of biochemical/physical cues in regulating cellular events, namely cell adhesion, proliferation, osteogenic differentiation, and mineralization, highlighting the importance of the combined modifications for the development of innovative bone regenerative therapies.

Introduction

One of the main goals of bone tissue engineering (BTE) is to create platforms that modulate the fate of cells to drive and improve their activity, differentiation rate, and mineralization without the addition of artificial supplementation. The deeper understanding of the bone physiology and biology has enabled to identify crucial aspects that can be inscribed in biomaterials or biomedical devices [1, 2]. For that, numerous strategies involving biomaterials modifications, namely chemical and topographical, have emerged for the bioengineering of structures able to create ideal microenvironments for autonomous and self-regulated osteogenesis.

In fact, in their natural environment, cells simultaneously respond to biochemical and physical cues, mainly provided by the extracellular matrix (ECM), neighbouring cells, and soluble factors [3]. The ability of cells to recognize and communicate with the surrounding environment leads to the outside-in/inside-out exchange of biochemical and mechanical signals, which influences a series of physiological processes, including tissue remodelling, regeneration, healing, ageing, as well as the development of numerous bone pathological conditions [4-6]. Furthermore, at a cellular scale, cells' morphology and ability to proliferate and/or differentiate are in part regulated by the extent and strength of cell adhesion, which in turn are strongly dependent on the physical and chemical properties of the substrates [7].

Previous works have demonstrated the importance of micro-metric topographical properties, and their modifications, in the mediation of several cellular events, especially using synthetic polymers and two dimensional (2D) surfaces [8-10]. In 1964, Curtis *et al.* identified for the first time the role of microtopographic surface patterns on stem cells differentiation [11]. Following that, other studies emerged correlating specific surface topography with cell behaviour [12-16].

It is known that the biophysical features, chemical composition, and surface properties of substrates, such as their stiffness [17, 18], roughness [19, 20], and porosity [12, 21], affect stem cells mechanobiology and nuclear organization hence impacting cell attachment and migration, but also osteogenesis and matrix mineralization [9, 10, 22]. Specifically, the topography of substrates is known to influence cell morphology, migration and differentiation through integrin binding and contact guidance phenomenon [15, 22, 23]. Several topographical cues, including grooves and ridges [15, 24, 25], pillars [26], and concave/convex curvatures [27-29], have been reported to guide cells into the osteogenic lineage, improving the osteoconductivity of the scaffolds. Such effects have been also transposed to the pseudo-three dimensional (3D) space by modifying the topography of microcarriers [30].

On the other hand, the mimicking of the bone microenvironment through the delivery of biomolecules like growth factors [31-33] and

[a] *Department of Chemistry, CICECO – Aveiro Institute of Materials, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

[#] Equal contribution.
* Corresponding authors: claudiao@ua.pt and jmano@ua.pt

modulation of the surface chemistry by protein coating and/or the presence of hydroxyapatite [34, 35] in the structures is also described to impact osteogenesis and bone healing. In this regard, the integrins transmembrane protein family plays a major role in transmitting the signals by recognizing specific motifs and sensing external forces while linking the ECM and the actin cytoskeleton [36]. Upon integrin binding, these proteins will cluster to reinforce molecular links at the cell-matrix interface and undergo conformational changes, resulting in the recruitment of focal adhesion kinase (FAK) and other docking proteins that will form mature focal adhesion complexes [37-39]. Autophosphorylation of FAK results in cytoskeleton contraction, which affects several signaling pathways and impacts cell activities [37].

The concurrent combination of chemical, geometrical, and topographical modifications can improve the physiological relevance of substrates and aid the mimicking of the *in vivo* environment when compared to individual cues previously mentioned, due to the triggering of different cellular mechanisms and pathways by these signals which result in synergistic effects. However, the combination of different approaches (chemistry and topography) in a single platform has not been much explored in BTE strategies. In this perspective, we explore the combination of chemical and topographical signals for their role in regulating several cellular events, including cell adhesion, migration, cell morphology, as well as their role in inducing osteogenic differentiation and guiding bone regeneration and healing.

Improving cell attachment, proliferation, and cell communication

Over the past few decades, BTE has pursued different strategies to better mimic the native environment in anticipation of creating autonomous bioengineering platforms [2]. With increasing knowledge of the impact of chemical and biophysical signals on cells, researchers have created platforms with cues to improve cell attachment, proliferation, cell-cell communication, migration, osteogenesis, and mineralization.

In this sense, plasma treatment [40], polymer coatings [41, 42], protein immobilization [43], and peptide anchoring [44] techniques have been employed to enhance cell attachment to substrates, which in turn affects several cellular events with great chance to modulate and improve osteogenic differentiation. At the same time, surface topography is also known to impact cell adhesion, proliferation, morphology, migration, and differentiation through contact guidance phenomena, which are dependent on the cell type, topographic patterns, and culture systems, i.e., 2D or 3D conditions [23]. Alongside the myriad of chemical strategies and topographical cues, it is also possible to make a plethora of combinations towards specific outcomes.

To investigate the relative impact of topography *vs* RGD (Arginine-Glycine-Aspartate) ligand density in mediating the cellular activity of endothelial cells, one of the mature cells found in the bone microenvironment, different silicon surfaces with independently controlled roughed surfaces (nano- to micro-scaled pyramids) and RGD densities were used [45]. The number of cells attached to the substrates was dependent on the RGD density but mostly controlled by the size of the topographical features, whereas spreading, focal adhesion formation, and overall intracellular organization was governed by the RGD density.

Based on these results, the authors suggested a 2- step model where the initial contact between cells and the substrates is dominated by the surface topography, whereas the surface chemistry dictates the strength of the adhesion and the interaction with the cellular surface receptors. Besides that, the dual biofunctionalization of scaffolds with RGD and DWIVA (Aspartate-Tryptophan-Isoleucine-Valine-Alanine) peptides combined with grooved patterned surfaces showed that the microgrooved topography in dental zirconia scaffolds elicited stretching and alignment of the body of human mesenchymal stem cells (hMSCs), as well as their migration along the grooves through contact guidance [46]. At the same time, the biochemical cues enhanced cell adhesion and significantly improved the osteogenic potential, showcasing the benefits of the combined modifications. Similar results were observed for skeletal cells cultured on nanogrooved polystyrene substrates conjugated with RGD, where the grooved topography played a minor role in mediating the attachment of cells [47]. Nonetheless, the anisotropic topography played an important role in regulating cell morphology and promoting cell elongation and orientation along the grooves. In turn, the addition of RGD increased cell spreading area and facilitated their crossing over grooves.

Proteins have also been conjugated with topographical features to trigger important cellular mechanisms. Immobilization of collagen type-1 (Col1) in rough electrospun poly(lactic-co-glycolic acid) (PLGA) fibers resulted in enhanced protein adsorption and porous spider-web-like topography that leads to increased cell contact area when seeded with MC3T3-E1 cells [48]. Of note, cell adhesion and spreading benefited the most from combining both types of cues, which may be due to the enhanced adsorption of serum proteins on the PLGA fibers, but also from the 3D distribution of collagen that can result in the exposure of more active sites. Similarly, the combination of Col1 immobilization and multi-scale topographical features in PDMS substrates impacted hMSCs attachment and distribution [49]. Interestingly, when compared to flat substrates, PDMS biofunctionalized structures with surface microgrooves, micropillars, and microholes cultured with hMSCs exhibited greater cell adhesion, alignment, distribution, proliferation, and intimate cell-cell contacts. These cellular phenotypes are probably the result of cell preference to specific topographical features, namely grooves and pillars, that provide multiple junctions and anchorage points for integrin cell signaling and focal adhesion formation.

Another strategy has explored the ability of poly(ethyl acrylate) (PEA) to trigger a biomimetic assembly of fibronectin (FN) in fibrillar networks similar to *in vivo*, resulting in enhanced cell adhesion and migration [41, 42, 50, 51]. In fact, the combination of titanium (Ti) discs with nanowires topography and biofunctionalization with PEA, FN and bone morphogenetic protein-2 (BMP-2) was investigated for its osteoinductive and antibacterial properties in a co-culture system [52]. The synergy between the bactericidal action of the Ti nanowires

and the PEA/FN/BMP-2 biofunctionalization allowed the increase in MSCs adhesion while hindering bacteria biofilm formation, protecting cells from cytotoxic quorum sensing signaling molecules. The fabrication of microgrooved Ti substrates followed by the deposition of a nanostructured copper-containing tantalum layer and a pure tantalum cap layer induced the alignment of pre-osteoblasts and the anisotropic assembly of their stress actin fibers through strong contact guidance while enabling cell adhesion and anti-infection properties [53].

Moreover, taking into consideration the importance of the native ECM, namely its composition and topography in the behavior of the bone resident cells, researchers combined wrinkled polydimethylsiloxane (PDMS) scaffolds of different sizes and cell-derived matrices (CDM) [54]. After 10-days of culture with human fibroblasts, the subsequent decellularization of the scaffolds was used to study its impact in controlling the behaviour of MSCs. The CDM deposited by the fibroblasts supported a high degree of alignment while the underlying topography influenced the orientation of the MSCs. The combination of both cues also seemed to modify cell aspect ratio and cell area, resulting in a slight decrease in cell spreading which was probably influenced by the increased roughness after CDM deposition. Furthermore, the combination of wrinkled topography and CDMs also synergistically aided the osteogenic differentiation of MSCs.

Nevertheless, not always the combination of cues delivers synergistic outcomes, which are dependent not only on the conjugation of biochemical and physical signals but also on cell type. For instance, when precoated with acrylic acid polymer and FN, substrates with nanoprotusions hindered cell proliferation and the ability to retain pluripotency [55]. Cell proliferation was already negatively impacted by the smaller nanoprotusions (16 nm), which also hampered cell adhesion and spreading.

Improving mineralization and osteogenesis

Mineralization is a complex process where osteoblasts uptake the calcium and phosphate ions present in the tissue microenvironment to produce hydroxyapatite crystals (HAp, $Ca_{10}(PO_4)_6(OH)_2$). By exploring the complex mechanisms for the formation of HAp crystals, researchers have been looking for clues to create structures able to achieve mineralization similar to the bone microenvironment [56, 57]. Mineralization in these can be triggered by active osteoblasts or by self-mineralization. Plentiful chemical and topographical strategies have been introduced on the bioengineering of bioactive structures to stimulate this process.

Introducing metallic elements

In the bone microenvironment (BME), minerals like calcium (Ca), phosphorous (P), and potassium (K) are vital for bone metabolism. Concurrent, trace elements like Strontium (Sr), Copper (Cu), and Zinc (Zn) play a pivotal role in promoting osteogenic differentiation [58-60]. Bioinspired by the BME, various minerals have been included in different platforms to mediate cellular changes at a chemical level or to design a specific topography (e.g. AuNPs are immobilized on surfaces to achieve topographic gradients), granting a distinctive strategy to enhance bone formation [61, 62].

Present in the structure of biological apatite, Sr plays an important role in the stimulation of new bone tissue by increasing osteoblast proliferation and matrix deposition, while inhibiting the presence of osteoclasts [60, 63]. With a chemical similarity to Ca, Sr has been used to replace the first on the production of a new form of HAp (Sr-HAp). Upgraded bioceramics with convex HAp-based micro-patterns (~43 μm) conquered the best stimulatory effect with improved differentiation towards osteoblastic phenotype with longer and more numerous filopodia (Figure 1A) [64]. Interestingly, this effect seems to be enhanced with concave instead of convex areas. Concavities act as initiator areas for nucleation and crystal growth, and their size is an effective parameter to control the spatial position and extent of mineralization *in vitro* [65, 66]. The synergistic effect between the inclusion of Sr elements into HAp powders and micro-patterns enhanced alkaline phosphatase (ALP) activity and expression of osteogenic markers, providing a privileged environment for osteogenic differentiation. Hexagon-like microarrays and glass nanofibers have also been explored alongside the Sr-HAp strategy, providing suitable platforms for the differentiation of human stem cells with higher levels of osteogenic markers at early stages, in part due to the complex architecture presented by both physical and chemical cues [57].

Other strategies adopt the zinc (Zn) ions to stabilize rapidly degrading structures like calcium silicates, playing a crucial role in mineralization and differentiation [67]. Concurrently, the topography increased osteoblasts and osteoclasts adhesion, highlighting the importance of dual-length scale (micro and nano) topographical modification on cellular activity [68]. However, the sustained release of zinc ion also has an inhibitory effect on osteoclast activity, allowing a better modulation of osteoclastic activity [69].

The incorporation of magnesium (Mg) in mesoporous bioglass nanospheres has also been used to fashion micro/nano topographies conferring both chemical and topographic cues to previously inert structures [70]. The degradation of Mg results (among others) in the precipitation of Ca-P endorsing the formation of hydroxyapatite. Consequently, osteogenesis and cell communication improve due to its high hydrophilicity and surface energy of polyetheretherketone (PEEK) surfaces functionalized with Mg containing mesoporous bioglass nanospheres.

Improving hydrophilization of the surfaces

The surface hydrophilicity of the bioengineered structure has an active role in cell adhesion, and consequently, differentiation. With some of the structures having a hydrophobic character, researchers took advantage of film rich in amine chemical groups to promote osteoblastic differentiation by increasing the interfacial pH between cells and surface [71, 72]. Amine groups have been introduced to improve the hydrophilic character of nanotopographic surfaces bearing gold nanoparticles (AuNP). By creating a defined nano-topographic gradient designed by the presence of low or high densities of AuNPs or playing with their size and spacing, structures were successfully designed endorsing *in vitro* osteogenic differentiation [61, 62]. Nonetheless, results indicate that the topographic cues play the critical role in osteogenesis since surfaces with methyl groups

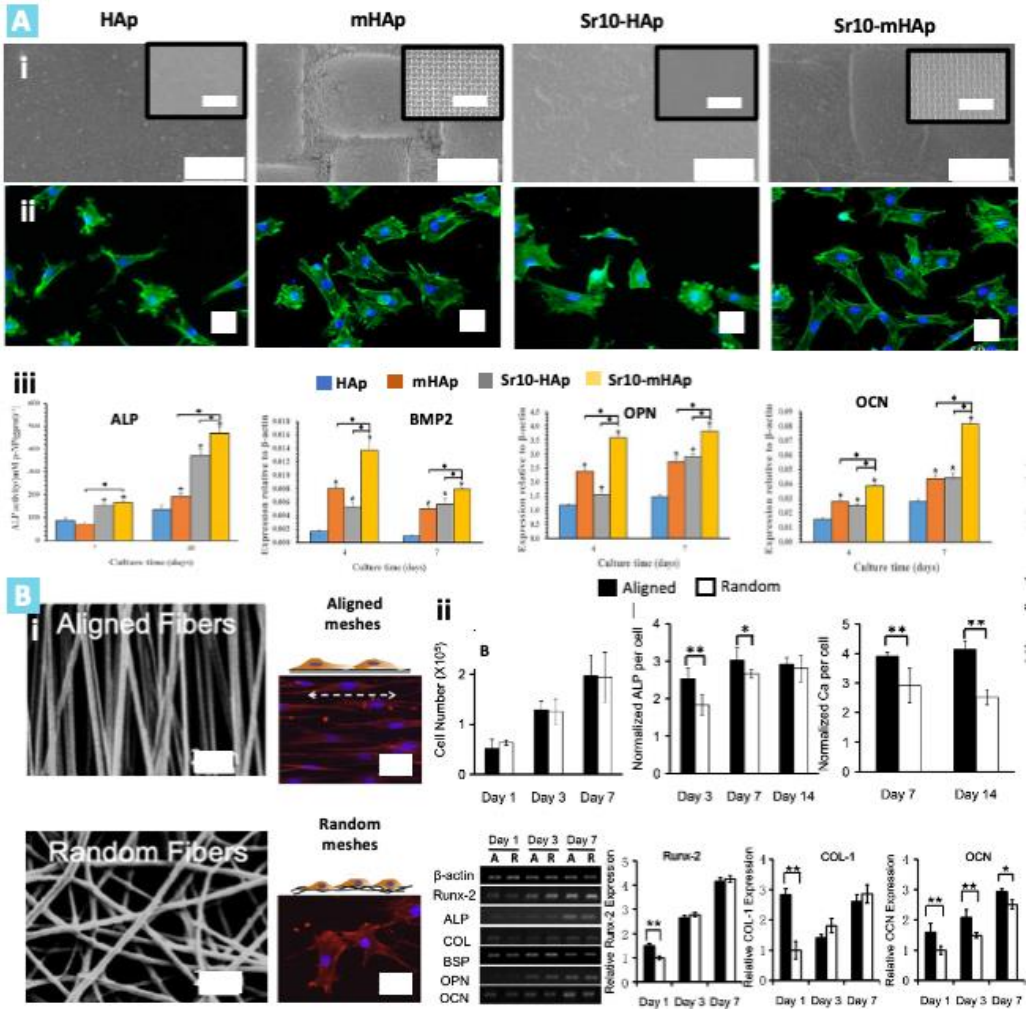

Figure 1. Synergistic effects between chemical and topographical properties in distinct substrates. A) Strontium-doped hydroxyapatite (Sr-HAp) bioceramics with an ordered micro-patterned surface: i) SEM images of flat and micro-patterned surfaces. Scale bars depict 50 µm (inset 500 µm). ii) confocal fluorescent images of cells cultured on top of each surface depicting the f-actin microfilaments (green) and nucleus (blue). Scale bar depicts 2 mm and 50 µm, iii) Evaluation of some osteogenic markers (ALP, BMP-2, OPN and OCN) [64]. Copyright 2018, Royal Society of Chemistry. B) PCL nanofibers covalently modified with collagen type-I. i) SEM images of aligned and random fibers and cell orientation evidenced by the staining of F-actin microfilaments (red) and nucleus (blue). Scale bars depict 50 µm, ii) evaluation of the fibers bioactivity towards cell proliferation and expression of some osteogenic markers [73]. Copyright 2013, Elsevier.

(hydrophobic character) also potentiated this behaviour depending on the topography [61]. Moreover, although smaller AuNPs showed higher affinity toward amines, the synergistic effect between the chemical modification and higher sizes increased osteogenic activity [62].

Additionally, various other strategies have used proteins like Col1 to decrease the hydrophobicity of surfaces, and consequently potentiate cell attachment, osteogenesis rate and mineralization. For example, Col1 is used to coat polyester substrates such as polycaprolactone (PCL) to improve its

bioactivity and promote osteoblast adhesion (Figure 1B) [73]. When combined with topographic cues of aligned and random nanofibers layers (to mimic anisotropy of bones), it is possible to regulate the behaviour of pre-osteoblasts by inducing cell polarization alongside high expression of osteogenic markers [73]. This simple strategy allowed researchers to design 3-dimensional (3D) models collecting further information about bone physiological environment.

To emulate both chemical and physical complexity present in bone environment, cutting-edge structures can be designed through reliable methodologies. The combination of hybrid microstructures **[**holes and pillars (100 µm x 100 µm), fashioned with and without microgrooves (10 x 3 µm width/depth)] in a PDMS surface chemically modified with Col-1 displayed a clear influence towards cell adhesion, expansion and morphology [49]. Micro-patterned collagen substrates promoted higher Ca mineralization during osteogenesis, while unmodified flat PDMS did not retain the cell sheet and showed shallow mineralization. Moreover, significantly higher expression levels of ALP, Col1 and osteocalcin (OCN) were found on the patterned surface emphasizing the positive influence of multi-scale elements at a micron and submicron scale coupled with the hybrid isotropic and anisotropic features. ECM proteins derived from porcine skin were also used to upgrade biphasic Ca-P granules comprised of micropores and multi-channels [74]. ECM was attached to the surface of biphasic Ca-P granules covering the micropores while multi-channels were not covered after functionalization. The produced scaffolds presented trace amounts of important growth factors (e.g., BMP-1, VEGF, TGF and FGF) for cell proliferation and differentiation. The uncovered multi-channel ensured cellular penetration and adequate diffusion, whereas high porosity was translated into higher protein adsorption promoting angiogenesis and osteogenesis. Additionally, the biphasic Ca-P ceramics already have HAp in their multi-channel granules, improving cell differentiation. However, one must advert to the extent of the coating of proteins, because although high ECM concentration translates into higher growth factor and protein content, it does not mean greater functionality. There is a threshold coating dose to avoid protein repulsion due to the limited availability of free surface and cell toxicity. Nonetheless, the synergistic effect between protein coating and topographical cues resulted in an increased *in vivo* bone regeneration, with a mature bone being observed.

Cell perception of the chemical and physical cues

In bone environment, chemical and topographical natural cues guide the biological responses of tissue by stimulating distinct cellular pathways. The main communication between cells and the bioengineered structures is through focal adhesion (FAs) points (Figure 2). These mediate the adhesion/connection between both, working as channels to drive signals from the extracellular to the intracellular environment. Alterations at this cellular level result in cytoskeleton modifications, that affect cell metabolism and the nucleoskeleton organization, causing alterations in transcription and gene expression. By modulating the cytoskeleton and gene expression, Runt-related transcription factor 2 (Runx2) is activated through different signaling pathways. Runx2 is considered as the 'master gene' for osteoblast differentiation as it regulates the expression of osteogenic genes (e.g., osteopontin, OCN, ALP), controlling the differentiation of MSCs to pre-osteoblasts [64, 75, 76]. Detected by integrins, transmembrane proteins responsible for the communication between the external and internal environment, the bioengineered structures can stimulate various regulatory pathways, namely Wnt/β-catenin, MAPK/ERK, and Rho-A/ROCK.

Topographical cues can modulate the cellular differentiation in stem cells through the MAPK/ERK signaling pathway. This pathway plays a key role in transmitting information received from cell surface receptors (e.g., integrins) to DNA. The topographical interaction with cell integrins leads to the phosphorylation and translocation to the nucleus of p38 and ERK targeting osteogenic genes [64, 77]. Collagen-coated nanofibers are able to mimic the ECM nature where collagen and the organized architecture create cell attractive cues for mineralization and cell attachment, that enhance bone tissue formation. The spatial arrangement of Col1-coated fibers highlights the importance of aligned patterns in cellular fate with higher expression of osteogenic differentiation promoters (integrin β1, α5 and αv) and suppressed osteogenic differentiation inhibitors (integrin β3), an excellent example of the synergistic effect that chemical and physical strategies have in determining together the cell fate [48, 73, 78].

The inclusion of certain elements (Ca, Zn, Mg, and Sr) and their controlled release transduces extracellular stimulation into cells and their nucleus by modulating the MAPK/ERK pathway triggering a series of biological responses, namely cell proliferation and differentiation. Mechanical forces can induce Ca release from intracellular stores or Ca extracellular entry by integrin stress-stimulus, exacerbating or not the stimulus already presented by surface functionalization with Ca [79]. Additionally, Sr and Mg also activate cell surface receptors conveying signals from the bioengineered structure through the canonical Wnt/β-catenin pathway [34–37]. Due to its chemical similarity to Ca, Sr has a similar effect translated into the inactivation of Ca-sensing receptor (CaSR), increasing Ca release and, subsequently, activation of the Wnt signaling pathway [78, 80]. Ca is an important intracellular messenger which release can regulate gene expression and cell differentiation in a fast way, acting directly at specific sites in intracellular compartments. The incorporation of certain chemical entities confers an alkaline pH to the bioengineered structures with ability to modulate the fate of stem cells by activating a signaling cascade (ERK and Wnt/β-catenin signaling pathways),

which consequently stimulate the channels to drive proliferation and differentiation of osteoblasts [71, 81-83]. Wnt/β-catenin pathway modulates the switch between chondrogenesis and osteogenesis in early MSCs [84]. Inducing Wnt/β-catenin, Runx2 gene expression is activated, while lower levels of β-catenin promote chondrogenesis. The canonical Wnt pathway activation induces β-catenin accumulation and translocation to the nucleus, interacting with TCF1. β-catenin/TCF1 complex will activate the Runx-2 promoter responsible for osteogenesis.

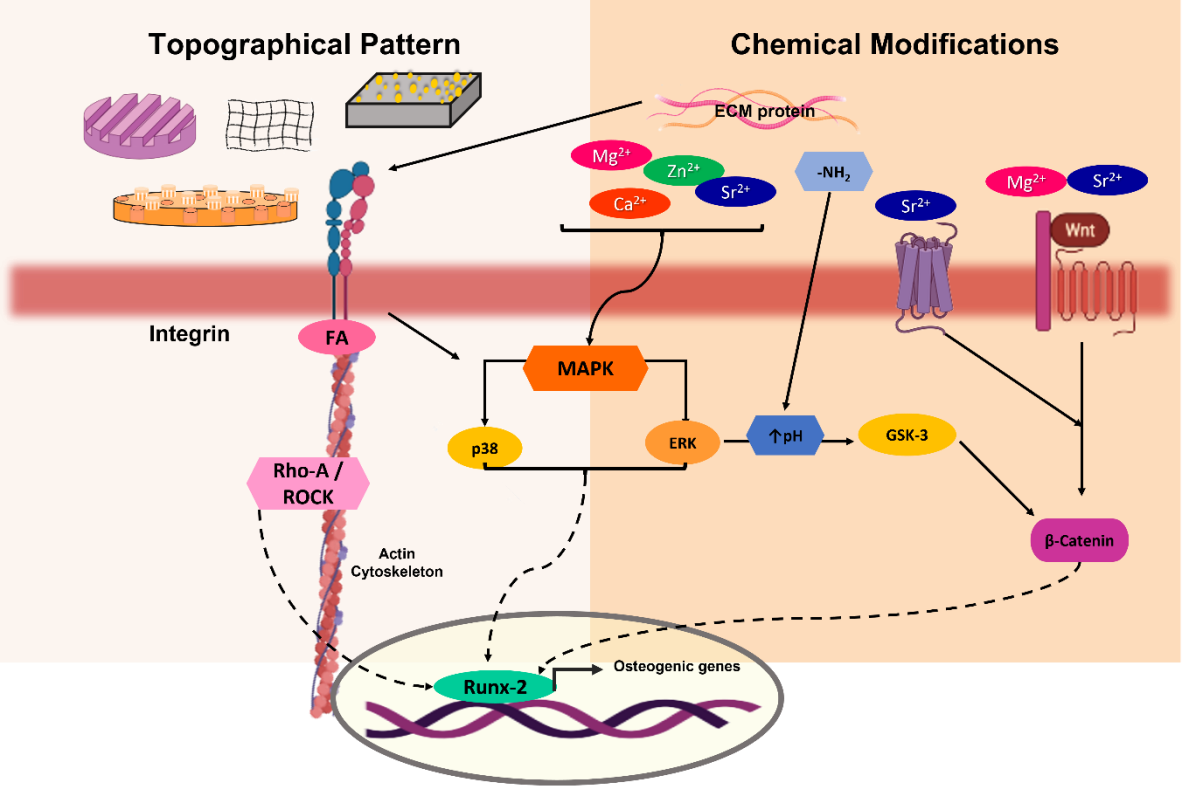

Figure 2: Chemical and topographical cues guide the intracellular signaling pathways which induce osteogenic differentiation. ECM- Extracellular Matrix; ERK- Extracellular Signal-Regulated Kinase; FA- Focal Adhesion; GSK-3- Glycogen synthase kinase-3; MAPK- Mitogen-Activated Protein Kinase; Rho-A- Ras homolog gene family; ROCK- Rho-associated protein kinase; Runx-2- Runt-related transcription factor 2.

The micro-features of certain topographic and chemically modified surfaces can create tensional forces in integrins activating the Rho-A/ROCK signaling pathway responsible for regulating cytoskeleton dynamics, modulating the cell size and morphology. The elongation of cells triggers a set of signals that mediate osteogenic differentiation through Runx-2 expression [71, 85-87]. Although cell behavior transcends the architecture or chemistry of the surface and is also highly dependent on the cell, the activated pathways are similar, and all converge towards the activation of Runx-2. Therefore, platforms for bone regeneration that consider the synergistic effect between topographical and chemical signals (Table 1), will have greater potential for application in BTE, allowing the rapid and controlled definition of cell differentiation in time and space without recourse to external stimuli.

Conclusion

The combination of topographical patterns with specific chemical modifications shows to potentiate cell adhesion, proliferation, differentiation, and mineralization. However, there is still no direct correlation on how to adapt a certain chemical modification to a certain topographical modification to achieve a specific cellular response. This review allowed the discussion of most recent advances on combinatorial strategies, highlighting their potential for cell guidance in future BTE strategies. It is important to emphasize that some studies have already been carried out on *in vivo* models that reported positive results of osteointegration and osteoconduction of the inserted chemically and topographically modified structures. These results emphasize the benefits provided by the combination of chemical/physical signals and how in the future it may be possible to achieve higher rates of *in vivo* bone regeneration. Nonetheless, more studies are essential, mainly with pre-osteoblasts, osteoblasts, and osteoclasts to describe better their cellular activity inherent to these combinations and

address the development of effective bone regeneration therapies.

In addition, it is still critical to investigate the comprehensive effects of surface chemistry and the micro-topographical signals for rational and optimal bone tissue regeneration. Most of the combined studies involving simultaneous topographical and chemical signals are performed on 2D substrates. In fact, more efforts will be needed to transpose such knowledge into the 3D models, for example by decorating the surface of porous scaffolds or by using engineered microparticles as cell carriers. The progress in the field could open new avenues of instructive biomaterials that could be used in bone regeneration, without the need of using cells, and therefore decreasing the complexity of the therapeutical solution and accelerating the translation into the clinics [2].

Author Contributions

D.J. and A.R.P. performed the bibliographic revision and wrote the manuscript. M.C.G and C.S.O. designed and provided all the necessary support during the revision phases. J.F.M. supervised and validated the manuscript.

Conflicts of interest

There are no conflicts to declare.

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