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Review

Electrical stimulation: Effective cue to direct osteogenic differentiation of mesenchymal stem cells?

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ABSTRACT

Mesenchymal stem cells (MSCs) play a major role in bone tissue engineering (BTE) thanks to their capacity for osteogenic differentiation and being easily available. *In vivo*, MSCs are exposed to an electroactive microenvironment in the bone niche, which has piezoelectric properties. The correlation between the electrically active milieu and bone's ability to adapt to mechanical stress and self-regenerate has led to using electrical stimulation (ES) as physical cue to direct MSCs differentiation towards the osteogenic lineage in BTE. This review summarizes the different techniques to electrically stimulate MSCs to induce their osteoblastogenesis *in vitro*, including general electrical stimulation and substrate mediated stimulation by means of conductive or piezoelectric cell culture supports. Several aspects are covered, including stimulation parameters, treatment times and cell culture media to summarize the best conditions for inducing MSCs osteogenic commitment by electrical stimulation, from a critical point of view. Electrical stimulation activates different signaling pathways, including bone morphogenetic protein (BMP) Smad-dependent or independent, regulated by mitogen activated protein kinases (MAPK), extracellular signal-regulated kinases (ERK) and p38. The roles of voltage gate calcium channels (VGCC) and integrins are also highlighted according to their application technique and parameters, mainly converging in the expression of RUNX2, the master regulator of the osteogenic differentiation pathway. Despite the evident lack of homogeneity in the approaches used, the ever-increasing scientific evidence confirms ES potential as an osteoinductive cue, mimicking aspects of the *in vivo* microenvironment and moving one step forward to the translation of this approach into clinic.

1. Introduction

Bone is a dynamic tissue with the ability to repair and self-regenerate and many fractures heal without further complications. Nevertheless, when the defect exceeds a critical size due to trauma, tumor resection or infection, challenging medical interventions are required [1]. An autogenous bone graft from the iliac crest is the gold standard treatment, even though it involves some associated drawbacks, such as increased patient morbidity, lack of vascularization or the limited quantity and availability of healthy tissue [2,3]. Other options, including allografts or xenografts, can be related to disease transmission, immune rejection and may fail to be osteoinductive in humans [4].

Bone tissue engineering (BTE) can help to overcome these issues by promoting bone regeneration through osteoinductive, osteoconductive and osteogenic scaffolds. BTE strategies are based on the use of biomimetic cell culture supports and provide a suitable environment for osteogenic progenitors to grow and differentiate. Factors that promote healing, including biochemical and biophysical cues, can be

Abbreviations: BTE, Bone Tissue Engineering; CC, Capacitive Coupling; CCFE, Capacitively Coupled Electric Fields; CNTs, Carbon Nanotubes; DC, Direct Coupling; ECM, Extracellular Matrix; ES, Electrical Stimulation; FA, Focal Adhesion; FN, Fibronectin; GES, General Electrical Stimulation; GO, Graphene Oxide; IC, Inductive Coupling; MP, Membrane Potential; MMP, Matrix Metalloproteinase; MSCs, Mesenchymal Stem Cells; OM, Osteogenic Medium; PEDOT, Poly(3,4-ethylenedioxythiophene); PEMF, Pulsed Electromagnetic Fields; PHB, Polyhydroxybutyrate; PVDF, Poly(vinylidene fluoride); PVDF-TrFE, Poly(vinylidene fluoride-Trifluoroethylene); TC, Transformer-like Coupling.

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synergistically included to enhance these biological processes.

Mesenchymal stem cells (MSCs) are adult stem cells that reside in many organs and tissues of the body, including the bone marrow, and were first described by Friedenstein in 1974 [5]. They are the obvious choice in BTE approaches due to their self-renewal and differentiation capacity to osteoblasts, among other cell types such as chondrocytes, adipocytes and hematopoiesis supporting-stroma cells [6]. Their immunomodulatory properties and the fact that they are easy to obtain make them perfect candidates to attain suitable clinical outcomes [7].

MSCs osteogenic differentiation is a complex and orchestrated process which involves the activation of selected signaling pathways and leads to the progressive expression of osteogenic-related genes, starting with the key integrator transcriptional factor RUNX2 (Runt-related transcription factor 2). MSCs osteogenic induction *in vitro* is based on osteogenic medium (OM) containing dexamethasone, ascorbic acid and glycerophosphate. Dexamethasone is a well-known corticosteroid that can produce the undesired guidance of MSCs towards the adipogenic lineage besides inducing osteogenic differentiation [8]. Other biochemical soluble factors, such as bone morphogenetic proteins 2 or 7 (BMP-2; BMP-7), are also considered strong inducers of the osteogenic phenotype. They can trigger MSCs differentiation by binding the BMP receptors and activating signaling transducers of the Smad family [9]. Despite their extended use *in vitro*, BMPs require larger concentrations than the physiological ones to be effective *in vivo*, increasing the cost of therapy and the risk of pathological side effects [10]. Due to the reduced specificity of this type of biochemical approach, biophysical cues have been investigated as possible candidates to direct MSCs differentiation in BTE strategies, since their precise action has been demonstrated by metabolomic techniques [11]. Halim et al. [12] reviewed and summarized the recent evidence for biophysical control of MSCs differentiation, in which cyclic mechanical strain, shear fluid stress, matrix stiffness and topography, microgravity or electrical stimulation were described.

Electrical stimulation (ES) has gained attention since Yasuda described bone piezoelectricity in 1953 and was correlated with the bone's capacity to adapt to mechanical stress and self-regenerate [13–15]. From then on, hundreds of articles appeared in the scientific literature describing the effects of electrical stimulation on bone healing. Basset, Pawluk & Pilla [16] were among the first to prove the therapeutic effect of inductively coupled electromagnetic fields in canine osteotomies in 1974. Later, many clinical studies reported successful outcomes in the treatment of non-union fractures [17–19], osteoporosis [20,21] or osteonecrosis [22,23] in humans, delivering ES by means of capacitively coupled electric fields (CCFE) or pulsed electromagnetic fields (PEMF). This led to the production of medical devices able to provide electrical cues for clinically treating indicated bone defects, the first of which was approved by the US Food and Drug Administration (FDA) in 1979.

The satisfactory clinical outcomes of ES at tissue-level encouraged the scientific community to try to explain the underlying cellular mechanism. Mesenchymal stem cells are key players in *in vivo* bone regeneration. They migrate to the injury site in response to cytokines and growth factors produced by inflammatory cells in a process known as *homing*. MSCs differentiate into osteoblasts in the presence of osteogenic factors, especially members of the BMP family. Osteoblasts deposit a mineralized bone matrix in a process known as *primary bone formation* [24]. These different stages of bone repair take place in the bone niche, where MSCs find themselves subjected to an electrically active environment. This physical stimulus together with other biochemical cues may play a role in MSCs differentiation towards the osteogenic lineage, supporting the regeneration of bone tissue and enhancing the results of regenerative therapies. It can also be useful for inducing MSCs commitment in BTE strategies when combined with the appropriate scaffolds, recapitulating aspects of the *in vivo* niche.

Electrical stimulation as a biophysical cue delivered at cell culture level has been thoroughly revised in recent years [25–31]. This review focuses specifically on current evidence on the effect of *in vitro* electrical stimulation for MSCs osteogenic differentiation and offers a critical review of the data published on this matter. The different methods of providing this cue are covered, including general electrical stimulation (GES) through cell culture medium and substrate-mediated electrical stimulation delivered by means of conductive and piezoelectric cell culture supports. Several aspects are reviewed, including stimulation parameters, treatment times and cell culture media to summarize the best conditions for inducing MSCs osteogenic commitment by electrical stimulation, from a critical point of view. Possible clinical applications of an optimized stimulation protocol are proposed, as are the potential drawbacks associated with this approach. Finally, the molecular mechanisms underlying cell response are also discussed.

2. General electrical stimulation

This section discusses effects of general electrical stimulation on mesenchymal stem cell differentiation. In this type of approach an electric field is applied to cells cultured either on tissue culture plates or on non-conductive scaffolds, with the electrical stimulus transmitted through the culture medium. Three main methods of delivering this cue have been described: Direct Coupling (DC), Capacitive Coupling (CC) and Inductive Coupling (IC), all excellently reviewed in [27,28,30].

2.1. Direct coupling

In the Direct Coupling method conductive electrodes are placed inside the cell culture wells or stimulation chamber in direct contact with the cell culture medium and the MSCs. Few authors have described this approach to exploring MSCs differentiation towards the osteogenic lineage. In the reviewed literature electric fields ranging from 2 to 3300 V/m were used [32–40]. Applied stimulation parameters, treatment time, the cell culture media used and the osteogenic markers expressed during MSCs differentiation are summarized in Table 1.

Barker's group made an in-depth study of the effect of DC on MSCs differentiation by means of a stimulation chamber composed of platinum electrodes coupled to a 6-well plate lid. Their studies showed that DC ES of 100 V/m for 1 h/day, combined with osteogenic medium upregulated genes related to the osteogenic differentiation process (RUNX2, osteopontin (OPN), collagen type I (COL I)). However, it also activated different cellular mechanisms in MSCs from different sources (bone marrow and adipose tissue) [35].

This stimulation combined with osteoinductive β-tricalciumphosphate scaffolds, enhanced alkaline phosphatase (ALP) activity, as well as Transforming Growth factor β1 (TGF-β1), BMP-2 and OPN expression to a greater extent than cells cultured on tissue culture plates. Interestingly enough, calmodulin (CaM) was also up-regulated compared to the control, suggesting the involvement of the calcium/calmodulin pathway in the ES mediated differentiation process [33].

The combination of ostoinductive factors with direct coupling stimulation has also been explored by Hronik-Tupaj et al. [37]. Cells electrostimulated in OM supplemented with BMP-2 showed upregulated ALP and COL I expression compared to non-stimulated cells cultured in OM plus BMP-2. Stress markers such as heat shock protein 27 (Hsp27) were also upregulated, although the relationship between stress markers, osteogenic markers and ES is not yet fully understood.

This approach has certain disadvantages, including the production of reactive Faradic by-products from the electrochemical reactions (hydrogen peroxide, hydroxyl ions and other free radicals), changes in the pH or the oxidation of bare metallic electrodes, which can liberate traces into the cell culture medium. Also, the cells closest to the electrodes can suffer morphological changes [34]. Concerned about these facts, Srirussamee et al. [36] studied the effect of the H_2O_2 produced by platinum electrodes used for DC electrical stimulation in MSCs differentiation. Surprisingly, H_2O_2 produced by ES enhanced MSCs proliferation, without causing oxidative damage, but did not have any influence on their differentiation. However, by-products other than H_2O_2

Table 1
Summary of general electrical stimulation applied to MSCs to induce osteogenic differentiation.

Reference	Electrical stimulation type	Parameters	Treatment time	MSCs source	Cell culture media	Altered osteogenic markers
[32]	Direct coupling	Pulsed direct current Square wave pulses Frequency 500 Hz Pulse width 1 or 250 μs Voltage 70 or 210 V Electric Field 1100 or 3300 V/m	1 h/day for the duration of the culture	Human MSCs (source non-specified)	Growth and osteogenic media (10^{-8} M dexamethasone 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid)	1 μs pulses at 210 V combined with OM enhanced ALP gene expression and did not affect BMP-2, OPN nor VEGF at 7 days compared to non-stimulated OM control.
[33]	Direct coupling	Direct current Electric field 100 V/m	1 h/day for the duration of the culture	Rat AT-MSCs	Osteogenic medium (10^{-7} M dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM L-ascorbic acid-2-phosphate)	Increased expression of TGF-β1 (day 7), BMP-2 (days 3, 7, 14 and 21), OPN (days 3,7, 14) and calmodulin (day 21). No effect on RUNX2, COL I and OSX expression. Enhanced ALP activity (day 7, day 14 and day 21). Compared to non-stimulated OM control.
[35]	Direct coupling	Direct current Electric field 100 V/m	1 h/day for the duration of the culture	Rat AT-MSCs and BM-MSCs	Osteogenic medium (10^{-7} M dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM L-ascorbic acid-2-phosphate)	Increased expression of RUNX2, OPN and COL I at 7 days and enhanced calcium deposition in BM-MSCs. Increased expression of RUNX2 and OPN at 14 days in AT-MSCs. Compared to their own non-stimulated OM control.
[36]	Direct coupling	Direct current Electric field 100 V/m	1 h/day for the duration of the culture	Human BM-MSCs	Osteogenic medium (10^{-8} M dexamethasone, 10 mM β-glycerophosphate, 0.17 mM L-ascorbic acid 2-phosphate)	Enhanced expression of OPN and no effect on RUNX2. Decreased ALP activity in stimulated samples. Compared to non-stimulated OM control.
[37]	Direct coupling	Alternating current Electric field 2 V/m Frequency 6×10^4 Hz	40 min/day for the duration of the culture	Human MSCs (source non-specified)	Osteogenic medium (10^{-6} M dexamethasone, β-glycerophosphate 10 mM, 0.05 mM L-ascorbic acid 2-phosphate, 100 ng/mL BMP-2)	ALP and COL I expression upregulated at days 15 and 20 compared to non-stimulated OM control.
[38]	Direct coupling. Salt agar bridges	Direct current Electric field 600 V/m	2 or 4 h at the start of the culture	Human AT-MSCs	Growth medium	No upregulation in gene expression of osteogenic marker OPN, and upregulation of fibroblastic and vasculogenic markers compared to non-stimulated control in growth medium.
[39]	Direct coupling. Salt agar bridges	Pulsed direct current Square wave Electric field 600 V/m Frequency 50 Hz	6 h/day for the duration of the culture	Mouse AT-MSCs	Osteogenic medium (10 mM β-glycerophosphate, 100 mg/mL ascorbic acid)	Enhanced OPN, COL I and RUNX2 expression and protein production after 21 days. Enhanced ALP expression and activity after 7 days. No OCN enhancement. No differences in mineralization. Compared to non-stimulated OM control.
[40]	Direct coupling. Salt agar bridges	Direct current Electric field 600 V/m	2 h at the start of the culture	Human BM-MSCs	Osteogenic medium (10^{-7} M dexamethasone, 10 mM β-glycerophosphate, 0.05 mM ascorbic acid)	No effects of ES on calcium deposition compared to non-stimulated OM control.
[46]	Inductive coupling	Pulsed EMF Magnetic flux density 1.5 mT Pulse width 1.3 ms Frequency 75 Hz	Cells continuously exposed	Human BM-MSCs	Commercial osteogenic medium (dexamethasone, β-glycerophosphate, ascorbic acid)	Enhanced expression of RUNX2 at 3 and 7 days, Dlx5 at 3, 7, 14, 21 and 28, and OSX at 3 and 7 days. Increased ALP activity at 7, 14 and 21 days. Increased mineralization at day 21 and 28. Increased OCN production after 21 and 28 days. Compared to non-stimulated OM control.
[48]	Inductive coupling	Pulsed EMF Magnetic flux density 1.5 mT Pulse width 1.3 ms Frequency 75 Hz	Cells continuously exposed	Human BM-MSCs	Commercial osteogenic medium (dexamethasone, β-glycerophosphate, ascorbic acid) + 10 ng/mL BMP-2	Enhanced Dlx5 and RUNX2 expression at day 3, increased ALP activity after 14 days and an augmented OCN production after 28 days due to synergy of OM containing BMP-2 and stimulation. Compared to OM (without BMP-2) + PEMFs and OM + BMP-2.
[49]	Inductive coupling	Pulsed EMF Transformer-like	4 h followed by a 4 h break, for the	Human BM-MSCs	Osteogenic medium (10^{-8} M dexamethasone, 10 mM	Enhanced expression of ALP and OCN (peaks at day 14 and 28 respectively, but

(*continued on next page*)

Table 1 (*continued*)

Reference	Electrical stimulation type	Parameters	Treatment time	MSCs source	Cell culture media	Altered osteogenic markers
		coupling rectangular pulses Pulse width 7 ms Frequency 10 Hz Induced electric field 3.6 V/m	duration of the culture		β-glycerophosphate, 0.2 μM ascorbic acid)	overexpressed at both times) Increased ALP activity at day 14, 21 and 28. Compared to non-stimulated OM control.
[50]	Inductive coupling	Pulsed EMF Magnetic flux density 2 ± 0.2 mT Pulse width 1.3 ms Frequency 75 ± 2 Hz Induced electronic tension amplitude 5 ± 1 mV	10 min/day for the duration of the culture	Human BM-MSCs	Osteogenic medium (10^{-7} M dexamethasone, 5 mM β-glycerophosphate and 50 mg/mL ascorbic acid)	Enhanced ALP, COL I and OCN protein production at day 27. Negative effect on the deposition of calcium. Compared to non-stimulated OM control.
[51]	Inductive coupling	Single pulsed EMF Magnetic flux density 10^3 mT Pulse width 5 ms Frequency 75 ± 2 Hz 30 pulses	3 min/day From day 1 to day 7 or the whole duration day 1 to 25	Human BM-MSCs	Osteogenic medium (10^{-7} M dexamethasone, 10 mM β -glycerophosphate 0.2 mM L-ascorbic acid-2 phosphate)	Increased ALP activity at 3, 5 and 7 days. Enhanced mineralization at day 15 compared with OM alone in both groups (1–7 and 1–25 days). Compared to non-stimulated OM control.
[52]	Inductive coupling	Pulsed EMF 20 pulses of 4.5 ms bursts Magnetic Flux Density from 0 to 1.8 mT in 200 μs Frequency 15 Hz	8 h/day for the duration of the culture	Human BM-MSCs	Osteogenic medium (10^{-7} M dexamethasone, 10 mM β-glycerol phosphate, 0.2 mM ascorbic acid)	Early peak of RUNX2 expression at day 2. BMP-2 expression peaked later but the expression was higher, as was OCN and ALP expression at day 4. Increase in ALP activity at days 2, 3, 4 and 5. Higher mineralization at day 11. Compared to non-stimulated OM control.
[53]	Inductive coupling	Pulsed EMF Repetitive single quasi-rectangular pulses Magnetic flux density 0.13 mT Pulse width 0.3 ms Frequency 7.5 Hz Induced electric field 0.2 V/m	2 h/day for the duration of the culture	Human BM-MSCs	Osteogenic medium (10^{-7} M dexamethasone, 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid)	Earlier expression of RUNX2 and ALP (day 7) at low density cultures (1500 cells/cm^2) compared to higher density ones (3000 cells/cm^2). No differences in COL I expression in any condition.
[54]	Inductive coupling	Pulsed EMF 5 ms bursts with 5 μs pulses Magnetic flux density 0.1 mT Frequency 15 Hz	Cells continuously exposed	Human BM-MSCs	Osteogenic medium (10^{-6} M dexamethasone, 10 mM β-glycerophosphate, 0.1 mM L-ascorbic acid 2-phosphate)	BMP-2, TGF-β1, OPG, IBSP, OCN expression upregulated at day 9 No differences in COL I, OPN, Osteonectin. Upregulation of MMP 1 and 3 (indication of matrix remodeling). No differences in ALP activity. Increased mineralization at day 14. Compared to non-stimulated OM control.
[56]	Inductive coupling	Pulsed EMF 20 pulses of 4.5 ms bursts Magnetic flux density from 0 to 1.6 mT in 200 μs Frequency 15 Hz	8 h/day for the duration of the culture	Human MSCs (source non-specified)	Osteogenic medium (10^{-7} M dexamethasone and 5 mM β-glycerophosphate +40 ng/mL BMP-2)	Increase in ALP activity from day 12 to day 24 and osteocalcin production from day 6 to day 24 due to synergy of OM containing BMP-2 and stimulation. Compared to OM (without BMP-2) + PEMFs and OM + BMP-2.
[57]	Inductive coupling	Continuous sinusoidal EMF Magnetic flux density 1 mT Frequency 15 Hz	8 h/day for the duration of the culture	Rat BM-MSCs	Growth medium	Increased expression of RUNX2, BMP-2 and OCN after 6 days. Enhanced ALP activity after 3 days. Compared to non-stimulated control in growth medium.

Abbreviations: AT-MSCs, Adipose Tissue-derived Mesenchymal Stem Cells; BM-MSCs, Bone-Marrow derived Mesenchymal Stem Cells; OM, Osteogenic Medium; ALP, Alkaline Phosphatase; BMP-2; Bone Morphogenetic Protein 2; OPN, Osteopontin; VEGF, Vascular Endothelial Growth Factor; TGF-β1, Transforming Growth Factor β1; RUNX2, Runt-related Transcription Factor 2; COL I, Collagen Type I; OCN, Osteocalcin, IBSP, Integrin Binding Sialoprotein; OPG, Osteoprotegerin; MMP, Matrix Metalloproteinase; Dlx5, Distal-Less Homeobox 5.

enhanced OPN expression in electrically stimulated cells.

To overcome these issues some researchers have used different setups consisting of isolated chambers connected *via* agar salt bridges to external Ag/AgCl electrodes immersed in Steinberg's solution. In this approach short stimulation times (only 2 h at the beginning of the culture) have demonstrated that MSCs can migrate towards the anode, but the short stimulation time neither increases or reduces their osteogenic potential based on calcium deposition analysis using OM [40]. Reduced

expression of osteogenic related gene OPN was found in the presence of growth medium [38]. Longer times and continued stimulation may be needed to induce osteogenic differentiation, as described by Hammerick et al. [39], in which 6 h/day stimulation enhanced ALP, COL I and OPN expression, probably due to the increased cytosolic free-calcium and reduced cyclic adenosine monophosphate (cAMP).

2.2. Capacitive coupling

Capacitive coupling is a non-invasive electrical stimulation method in which an electric field is created between two parallel conductive layers, capacitor plates, connected to a generator. These are on the edges of the cell culture chamber or cell culture well, usually above and below the cell culture medium, but not in contact with it. A small gap of air is left between the upper conductive layer and the cell culture medium in the well. If this space between the medium and the top capacitor plate is missing, the approach is known as *semi-capacitive coupling*. The electric field created is homogeneously transmitted through the cell culture medium and the cells are evenly stimulated, whatever their position in the cell culture well [41,42].

To the authors knowledge, this approach has never been reported for MSCs stimulation to induce osteogenic differentiation, although capacitive coupling by capacitor plates not in contact with the cell culture medium to stimulate other osteogenic cell types such as osteoblasts has been described [41,43,44].

2.3. Inductive coupling

Inductive coupling stimulation is based on inducing an electric field by means of a conductive coil or a solenoid around the cell culture system. An alternating current flows through the coil generating a magnetic and an alternating electric field perpendicular to the magnetic [45]. This stimulation method avoids direct cell contact with the electrodes and eliminates the presence of undesirable by-products.

IC is the most commonly used approach in terms of ES for stimulating MSCs. There is no consensus on the optimal stimulation conditions to guide MSCs differentiation towards the osteogenic lineage, making comparison between the published research studies difficult. Different magnetic field densities, frequencies, pulse durations and stimulation times have been applied [46–57], as reflected in Table 1. However, it seems clear that osteogenic medium is required in combination with electromagnetic fields (EMF) to induce an osteogenic phenotype, although some authors have reported the effect of EMF on osteogenic differentiation using growth medium [57].

Petecchia et al. [50] found that pulsed EMF by its own was not enough to enhance ALP and COL I expression, while the combination with OM increased these early osteogenic markers through the expression of L-type Voltage Gate Calcium Channels (VGCC) and the modulation of the concentration of cytosolic free Ca^{2+}. These results agree with Bagheri et al. [46], who combined pulsed EMF with OM, describing a synergistic effect which enhanced calcium deposition, ALP production and expression of osteogenic markers (RUNX2, Dlx5, osterix (OSX)), compared to OM alone.

Martini et al. [48] went further by also adding BMP-2, proving additive effects due to the simultaneous activation of Smad 1/5/8 and p38 MAPK pathways. These results disagree with those obtained by Schwartz et al. [56], when calcium phosphate disks combined with PEMF and BMP-2 were needed to produce a synergistic effect, and not only tissue culture plates as cell substrate. Changes in the cell culture surface make MSCs more sensitive to BMP-2 and BMP-2 treated cells are more responsive to PEMF. This supports the hypothesis that PEMF can influence MSCs osteogenic differentiation, although an osteoblast-inductive stimulus combined with an osteogenic environment is required.

PEMF stimulation strategy inevitably links the presence of an electric and a magnetic field, which can also contribute to MSCs stimulation. To get over this disadvantage Hess et al. [49,58] developed a device based on Transformer-like Coupling (TC) to apply electrical stimulation without the interference of a magnetic field. Their results confirm firstly that PEMF alone cannot induce osteogenic differentiation unless combined with OM, and secondly supports Schwart's hypothesis. MSCs cultured in high-sulfated hyaluronan derivatives, which are able to present growth factors efficiently, may be acting as the BMP-2 in the supplemented medium.

3. Substrate-mediated electric stimulation

Substrate-mediated electrical stimulation uses conductive and/or electroactive supports to apply an electrical stimulus directly to cultured cells.

3.1. Conductive cell culture supports

Electrically conductive polymers are one of the most popular choices when developing cell culture supports for substrate-mediated stimulation due to their chemical, electrical, and physical tailoring possibilities. Among the generally investigated polymers for tissue engineering applications, such as polypyrrole (PPy), poly(3,4-ethylenedioxythiophene) (PEDOT) or polyaniline (PANI) PPy is one of the most intensively studied. In the context of tissue engineering, polypyrrole is mainly used in the field of neural regeneration [59–62], although it has also been applied to bone tissue engineering, specifically for inducing MSCs osteogenic differentiation *via* substrate-mediated ES [63–69].

PPy has poor mechanical properties and its processing once synthesized is by no means simple [70], which makes it difficult to use by itself so that it tends to appear in combination with other biodegradable polymers as a coating or filler, *e.g.* with poly-lactic acid (PLA) [71,72], polycaprolactone (PCL) [73,74] or chitosan [67,75,76].

In addition to electroactive polymers, the family of carbon nanomaterials such as graphene, graphene oxide (GO) or carbon nanotubes (CNTs) has gained importance in the biomedical field. In tissue engineering, their large surface area and easy functionalization with bioactive molecules have driven their use, but above all their outstanding electrical characteristics make them an effective component for designing electroactive cell culture supports [77]. As in the case of conductive polymers, carbon nanomaterials have been especially used to deliver electrical signals to well-known excitable cell types, such as neural and muscle cells. Despite this, the rise of substrate-mediated MSCs stimulation has promoted carbon nanomaterials for electroactive bone tissue engineering [78–82]. A summary of the different conductive biomaterials used for MSCs substrate-mediated stimulation can be found in Table 2.

Conductive cell culture platforms transmit the stimulus *in situ* to the cells when connected to an external supply source. A common setup for substrate-mediated ES is based on the presence of a conductive 2D biomaterial, usually in the form of a film. This cell culture support is in direct contact with the electrodes at its ends and wired to an external current source. A sealed chamber, usually made of polystyrene, polypropylene or polymethylmethacrylate is placed on the film containing the cell culture medium and limiting the space for cell seeding, as shown in Fig. 1 [64,68,80,83]. This assembly avoids direct contact between the electrodes and the culture medium. This eliminates some of the drawbacks of GES based on direct coupling, such as Faradic by-products or changes in pH due to medium electrolysis.

It is not always possible to avoid electrode immersion in the cell culture well. In these cases, the electrodes are placed in direct contact with the cell culture support to maximize the total current transmitted. In fact, some authors have shown that the current present in these setups in the cell culture medium is negligible [66]. 3D culture systems such as scaffolds or hydrogels are two examples of electrodes immersed in the medium [63,66,67,84,85]. However, they provide homogeneous stimulation regardless of the distance to the electrode while they provide a

Table 2
Summary of substrate-mediated electrical stimulation applied to MSCs to induce osteogenic differentiation.

Reference	Conductive biomaterial	Stimulation parameters	Treatment time	MSCs source	Cell culture media	Altered osteogenic markers
[63]	PLA scaffolds with chondroitin sulphate-PPy coating	Biphasic pulsed direct current Electric field 100 V/m Frequency 1 or 100 Hz	4 h/day for the duration of the culture	Human AT-MSCs	Growth medium	No differences in ALP activity at 7 and 14 days between stimulated and non-stimulated conductive scaffolds.
[64]	PPy films	Direct current Electric field 35 V/m	4 h of treatment 24 h after seeding	Rat BM-MSCs	Osteogenic medium (10^{-7} M dexamethasone, 10 mM β-glycerophosphate 100 μm L-ascorbic-2-phosphate)	Increased calcium deposition after 14 days compared with non-stimulated conductive scaffolds.
[65]	Nonwoven mats of electrospun PCL with an interpenetrating network of PPy and polystyrenesulfonate	Direct current Electric field 10 V/m	2 days without stimulation, 8 h of stimulation, no stimulation thereafter	Human MSCs (source non-specified)	Osteogenic medium (10^{-7} M dexamethasone, 10 mM β-glycerophosphate, 50 μM ascorbic acid)	Enhanced ALP activity and calcium deposition at 21 days compared with non-stimulated conductive scaffolds.
[66]	PCL scaffolds with PPy coating	Direct current Current intensity 250 μA	4 h/day for the duration of the culture	Human AT-MSCs	Growth medium	Enhanced RUNX2, OPN and OCN at 21 days. Increased ALP activity (days 7, 14 and 21) and calcium deposition (days 14 and 21). Compared with non-stimulated conductive scaffolds.
[67]	Chitosan scaffolds with PPy coating	Direct current Current intensity 200 μA	4 h/day for the duration of the culture	Human AT-MSCs	Osteogenic medium (10 mM β-glycerophosphate and 50 μg/mL ascorbic acid)	Increased calcium deposition (days 7, 14 and 21) and BMP-2 concentration in the cell culture medium (days 7 and 21) compared with non-stimulated conductive scaffolds.
[68]	PPy films	Direct current Electric field 33.3 V/m or square waves using different frequencies (from 0.001 to 100 Hz)	4 h on day 0, 2, 4, 6, 8, 10, or 12	Rat BM-MSCs	Osteogenic medium (10^{-7} M dexamethasone, 10 mM β-glycerophosphate 100 μM ascorbic-2-phosphate)	Enhanced mineralization at day 14 in samples stimulated at day 8. Elongated ALP and RUNX2 transcription and enhanced BSP and OCN (day 8 and 12) specially in stimulated samples at day 8. Compared to non-stimulated samples and stimulated at different time-points.
[69]	PLLA electrospun fibers coated with PPy	Direct current Electric field 75 V/m	3 h/day for the duration of the culture	Rat BM-MSCs	Osteogenic medium (10^{-7} M dexamethasone, 10 mM β-glycerophosphate, 50 μM ascorbate)	Increased ALP activity (days 7 and 14), COL I and calcium content (days 14 and 21). Enhanced expression of BMP-2 and COL I (day 7). Compared with non-stimulated conductive samples.
[78]	PLLA fibers with carbon nanotubes	Pulsed direct current Rectangular waveform Electric field 75 V/m 1.5 V (electrodes 20 mm apart) Frequency 100 Hz	1.5 h/day at days 1–7, 8–14 or 15–21 along the 21 day culture	Rat BM-MSCs	Osteogenic medium (10^{-8} M dexamethasone, 10 mM β-glycerophosphate, 0.05 mM ascorbic acid)	Large set of analyzed markers (see the original reference [78]).
[80]	Graphene oxide-cellulose films	Biphasic electric current square pulses Electric field 100 V/m Phase duration 1 s Interphase interval 200 ms Frequency 1 Hz Current 300 μA/cm^2	1 h/day for the duration of the culture	Human AT-MSCs	Osteogenic medium (10^{-8} M dexamethasone, 10 mM β-glycerophosphate, 50 μM L-ascorbic acid-2-phosphate)	Increased mineralization at 21 days and ALP activity at 7 and 14 days in stimulated samples in OM compared to non-stimulated ones in OM and stimulated in growth medium.
[81]	Graphene patterned surfaces	Pulsed direct current Voltage 0.1 or 0.3 V Pulse width 1 μs Frequency 1 Hz	Cells continuously exposed (72 h)	Human BM-MSCs	Growth medium	Enhanced RUNX2 protein production at 72 h, regardless the presence of the pattern in stimulated samples. OPN protein production at 72 h was only enhanced when patterned surfaces and stimulation were combined.
[85]	Silicon patterned surfaces	Alternating current	24 h at the start of the culture, no	Rat BM-MSCs	Osteogenic medium (10^{-7} M dex-amethasone, 10 mM	Enhanced expression of RUNX2, ALP, COL I, OCN and osteonectin at

(*continued on next page*)

Table 2 (*continued*)

Reference	Conductive biomaterial	Stimulation parameters	Treatment time	MSCs source	Cell culture media	Altered osteogenic markers
		Electric field 150 V/m Pulse width 2 ms Frequency 1 Hz	stimulation thereafter		β-glycerophosphate, 50 μM ascorbic acid)	7 days. Increased ALP activity and calcium deposition at 14 days. Compared to non-stimulated silicon patterned surfaces.
[86]	Hydroxyapatite-$CaTiO_3$ scaffolds	Direct current Electric field 100 V/m	10 min/day for the duration of the culture	Human MSCs (source non-specified)	Growth medium	Enhanced RUNX2 protein production at 7 days. Increased ALP activity and collagen production at day 7 and 14. Compared to non-conductive (hydroxyapatite) and conductive non-stimulated samples. Enhanced expression of RUNX2, ALP, COL and OCN at 14 days compared with stimulated non-conductive samples (hydroxyapatite).
[101]	Hyaluronic acid and chondroitin sulfate doped PPy films	Biphasic electric voltage ±0.2 V Pulse width 2.5 ms frequency 100 Hz	3 h/day for the duration of the culture	Human AT-MSCs	Osteogenic medium (5×10^{-9} M dexamethasone, 10 mM β-glycerophosphate, 250 mM L-ascorbic acid 2-phosphate)	No differences in ALP activity at 7 and 14 days between stimulated and non-stimulated conductive samples.
[102]	PANI films coated with COL I and sulfated hyaluronan derivatives	Pulsed EMF transformer-like coupling Rectangular pulses Induced electric field 0.36 V/m Pulse width 7 ms Frequency 10 Hz	4 h followed by a 4 h break, for the duration of the culture	Human BM-MSCs	Osteogenic medium (10^{-8} M dexamethasone, 10 mM β-glycerophosphate, 0.2 mM ascorbic acid)	Enhanced expression of RUNX2, COL I and ALP at 14 days in PANI-COLI-sHya stimulated samples compared with the rest of conditions. Increased ALP activity at 14 days and calcium content at 28 days compared to non-stimulated counterpart.

Abbreviations: AT-MSCs, Adipose Tissue-derived Mesenchymal Stem Cells; BM-MSCs, Bone-Marrow derived Mesenchymal Stem Cells; PLA, Poly-lactic acid; PPy, Polypyrrol; PCL, Polycaprolactone; PANI, Polyaniline; OM, Osteogenic Medium; ALP, Alkaline Phosphatase; RUNX2, Runt-related Transcription Factor 2; OPN, Osteopontin; OCN, Osteocalcin; BMP-2; Bone Morphogenetic Protein 2; BSP, Bone Sialoprotein; COL I, Collagen Type I.

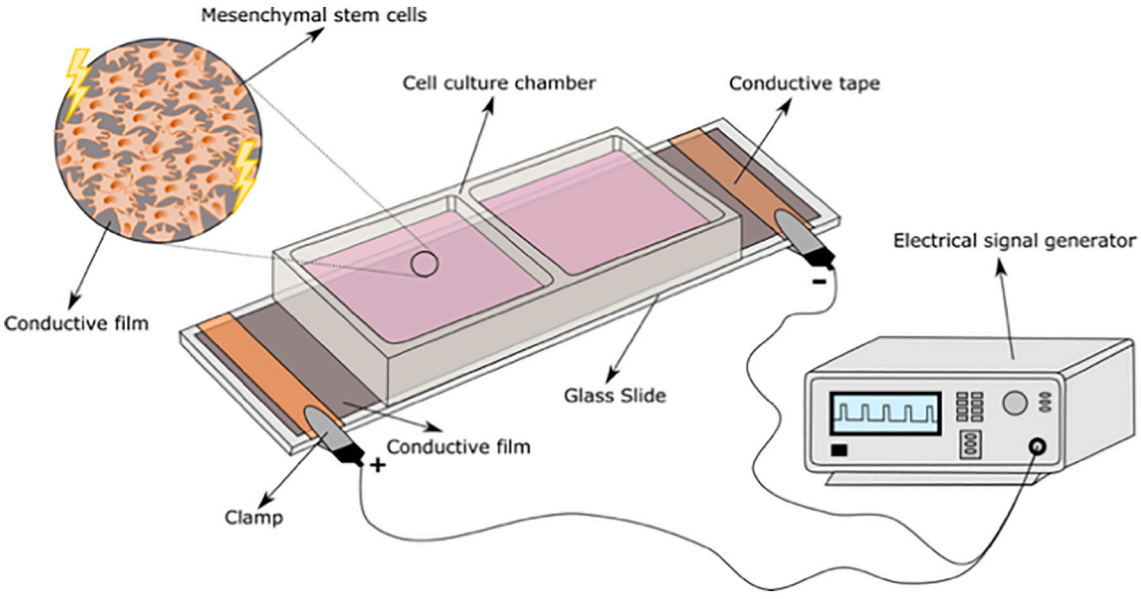

Fig. 1. Schematic representation of a common set-up for substrate-mediated electrical stimulation using 2D conductive cell culture supports. Figure not to scale.

more biomimetic environment than 2D conductive supports.

The scaffolds used to deliver substrate-mediated ES to MSCs are usually composites manufactured from biocompatible polymers and conductive materials such as PPy coatings or graphene fillers. Commonly used architectures include highly porous interconnected scaffolds with a diameter range of hundreds of microns [66,67,86]. Nonwoven mats of extruded fibers [63] or electrospinning technique [65,69,78] have also been used for MSCs stimulation. 3D printing is making its way in the field and 3D-printed conductive scaffolds are emerging as possible candidates for substrate-mediated ES [82].

The conductivity of the scaffolds produced after incorporating the electroactive coatings and fillers are between 10^{-11} and 10^{-1} S/cm [66,67,69,78,82,86], according to the type of conductive component incorporated and its concentration.

Most of the studies performed in the field support the hypothesis that an initial osteogenic stimulus from an inductive cell culture medium

containing supplements such as dexamethasone, ascorbic acid and β-glycerophosphate is necessary to trigger the effects of ES. Li et al. [80] studied the effect of electrical stimulation on MSCs osteogenic differentiation using conductive graphene oxide-cellulose films in growth and osteoinductive media. A combination of ES and osteogenic medium improved mineral deposition more than growth medium and ES together. It should be noted that osteogenic medium alone had the same effect as expansion medium combined with ES in terms of ALP expression.

Interestingly, Sayyar and collaborators [82] cultivated MSCs in OM 5 days before applying electrical stimulation on graphene/poly(trimethylene carbonate), a process they called *priming*. This induction was indeed necessary to observe the effects of electrical stimulation on osteogenic differentiation. ALP and COL I were upregulated in primed cells compared to non-primed subjected to the same stimulation parameters. Other authors have performed their studies in the presence of growth medium and failed to obtain any improvement in osteogenic differentiation combined with electrical stimulation [63,84].

The *priming* concept can be considered as a pre-condition or preparation of the cells for some specific function or lineage-specific differentiation [87]. Besides using chemical agents such as osteoinductive cell medium, MSCs have been primed with hypoxia, *in vitro* low oxygen concentrations (1–7%), that resemble the ones found in the bone marrow [88]. Nevertheless, the use of hypoxia on determining MSCs fate has a controversial role in literature. Samal et al. reflected this matter in their review [89]. Some authors have reported a significant impairment of osteogenic differentiation when cells were cultured under hypoxia [90–93], others have reported enhanced osteogenic differentiation [94–96] or even equal differentiation potential of MSCs cultured under both normoxia and hypoxia [97–99]. These diverse data are associated to a variation in the experimental design among studies. The moment when hypoxia is introduced can vary, the studies generally involving expansion in normoxia and cells differentiation under hypoxia or expansion in hypoxia and differentiation in normoxia.

The combination of this approach, as *priming* or during MSCs differentiation, with electrical stimulation has not been reported. This could be an interesting research path to follow in the future, even though the duration of exposure to hypoxia and O_2 concentration to obtain the best outcomes is yet to be analyzed, as are the best ES parameters.

The lack of an initial osteogenic stimulus provided by osteoinductive supplements can be overcome by combining ES with other physical cues such as nanopatterned surfaces. It has been proved that these have a similar efficiency to that of osteogenic medium in stimulating MSCs to produce bone mineral *in vitro* [100]. Balikov et al. [81] studied the effect of graphene patterned surfaces and ES in the absence of supplemented medium. When used alone the patterned surfaces enhanced the expression of the early marker RUNX2, although they failed to enhance late osteogenic marker OPN, unless combined with ES. The authors also investigated MSCs differentiation towards a neurogenic lineage due to the potential of stem cells for multi-lineage commitment enhancing both neurogenic and osteogenic markers. This shows the need to study markers from diverse lineages in differentiation experiments and has scarcely been addressed to avoid the presence of mixed populations.

Other research groups used coatings or osteoinductive biomaterials such as hydroxyapatite in combination with conductive cell culture supports and ES to make up for the absence of supplemented medium [69,86].

3.2. Piezoelectric cell culture supports

Bone is a piezoelectric tissue due to the collagen fibers that form the organic component of its extracellular matrix. The -CO- and -NH- units present in the amide bonds of the backbone amino acids of the protein can act as permanent dipoles, as represented in Fig. 2 I. The positively and negatively charged centers are aligned in the helical structure of the α-helix and cause significant permanent polarization. When the collagen fibers slip past each other under tension or compression, the distortion of the dipoles generates a surface charge [25,103]. This phenomenon known as the piezoelectric effect has been associated with bone's capacity to remodel in response to mechanical stress, described by Wolff's Law, providing local stimulation to bone-related cell types [14].

Since the discovery of bone's piezoelectric properties, piezoelectric materials have emerged as a possible approach to mimic the electrophysiological environment of bone tissue. These biomaterials can convert mechanical strain into electrical output and *vice versa*, a mechanical deformation is produced when a voltage is applied, known as the converse piezoelectric effect [104]. This property generates electrical charges on the surface without the need for any external electric supply, as in the case of other stimulation approaches, such as substrate-mediated ES by conductive cell culture supports. Several reviews have been published regarding the properties and use of piezoelectric polymers [103,105–108]. In this review the basic concepts of piezoelectric materials are explained to facilitate the understanding of their use as cell culture supports for MSCs electromechanical stimulation.

Piezoelectric biomaterials can be divided in two main categories, organic materials such as synthetic or natural polymers, and ceramics, which are inorganic in nature.

Ceramics with piezoelectric properties are crystalline materials with

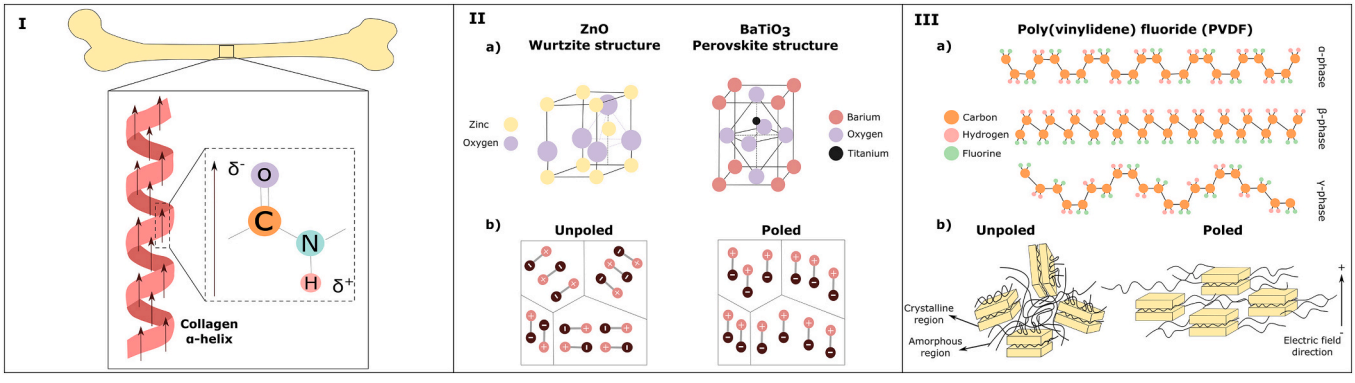

Fig. 2. Schematic representation of piezoelectric biomaterials. I. Illustration of a collagen α-helix molecule present in bone's organic extracellular matrix, responsible for the piezoelectricity due to the permanent dipoles associated to the -CO- and -NH- units present in the amide bonds. II. a) Schematic illustration of piezoelectric ceramics with wurtzite and perovskite structures. b) Crystalline structure of unpoled and poled ceramics. Dipoles are oriented after poling process. III. a) Chain conformation of α, β and γ phases of poly(vinylidene) fluoride. The electronegativity of fluorine atoms compared to hydrogen ones generates an electrical dipole moment in the monomer unit. The all trans conformation (TTT) and T_3GT_3G' of β and γ phases lead to an overall dipolar contribution of the polymer chain, while the trans-gauche–trans-gauche (TGTG′) conformation of α-phase is non-polar, conferring non-electroactive properties to this polymorph. b) Crystalline and amorphous regions of PVDF with randomly oriented dipoles before the poling process, that are oriented after it.

a non-centrosymmetric structure. Their piezoelectricity is typically based on the relative displacement of the ionic species [105]. Some of the different piezoelectric crystals also show ferroelectric properties, meaning that they exhibit an in-built spontaneous electrical polarization reversible under an applied electric field. Zinc Oxide (ZnO) and barium titanate ($BaTiO_3$) are normally used as examples of nonferroelectric and ferroelectric materials, respectively, which coincides with their crystallization form in the wurtzite or perovskite structure [107]. ZnO and $BaTiO_3$ structures are represented in Fig. 2 IIa. Barium titanate ($BaTiO_3$), lithium niobate ($LiNbO_3$) or sodium potassium niobate (K0.5Na0.5NbO3; KNN) have been used to influence MSCs fate towards the osteogenic lineage [109–114].

Regarding organic materials, synthetic polymers have emerged as an alternative to ceramics. Even though bone is a hard tissue, some applications require mechanical flexibility, while polymers are easier and less expensive to process. Their piezoelectricity is mostly based on the repositioning of molecular dipoles [105].

Poly(vinylidene fluoride) (PVDF), poly(L-lactic) acid (PLLA) or polyhydroxybutyrate (PHB) are some of the most frequently used piezopolymers for tissue engineering applications. PVDF stands out due to its high piezoelectric coefficient, reaching values up to −34 pC/N according to the processing and poling conditions [115]. PVDF has five crystalline phases, the β-phase being the most electroactive due to its net permanent dipole generated by the all-trans chain conformation (TTT). Fig. 2 IIIa shows three PVDF polymorphs, including the electroactive β and γ and the non-electroactive α-phase. The strong dipole moment is produced by the difference between the electronegativity of the fluorine and hydrogen atoms in its structure [116]. Although obtaining this crystalline phase is based on the polymer processing conditions [117], PVDF copolymer poly(vinylidene fluoride-Trifluoroethylene) (PVDF-TrFE) for specific VDF/TrFE ratios always presents the β crystalline phase, the addition of the third fluoride in the TrFE monomer unit with large steric hindrance favors the all-trans conformation and thus induces the ferroelectric β-phase regardless of the processing method [116].

Most of the piezopolymers used for BTE approaches are semi-crystalline, so that their structure can be described as randomly oriented microscopic crystals which contain the aforementioned dipoles dispersed around the amorphous regions (Fig. 2 IIIb). These dipoles can be reoriented to maximize the material's piezoelectric response in a process called poling, during which a high electric field is applied at a high temperature to align the dipoles. When the dipoles are aligned, as represented in Fig. 2 IIIb, the sample is cooled down in the presence of the electric field to maintain the dipoles' orientation [108]. Poling is directly related to a higher piezoelectric response characterized by the piezoelectric coefficient d_{ij}, which is defined as the electric polarization variation along direction i in the material per unit mechanical stress of index j applied to it or *vice versa*. d_{31} and d_{33} are the coefficients describing the electric polarization generated either in the same direction or perpendicular to the direction of the applied stress [118].

Poled piezoelectric cell culture supports do not always rely on electromechanical stimulation due to the piezoelectric effect. The polarization process and consequently the dipole alignment, implies the presence of charged positive and negative surfaces with an associated surface potential, which can also affect MSCs differentiation by maintaining an electric microenvironment. Two options arise at this point, culturing the cells either on the positively or negatively charged surface.

Parssinen et al. [119] studied the effect of poled-positive and negative surfaces of β-phase PVDF films coated with fibronectin (FN) on MSCs behavior. Polarization enhances PVDF hydrophilicity best on negatively charged surfaces favoring the adhesion of fibronectin in a more active formation, exposing RGD sequences. This enhances cell adhesion and cytoskeleton tension and is reflected in a higher number and area of focal adhesions (FAs). Cell cytoskeleton tension is related to the activation of RhoA and MAPK pathways and subsequent cell signaling cascades, which can determine MSCs fate *via* integrin mediated signaling. An increase in the number and size of FAs has been reported during osteogenic differentiation [120], although the authors simply demonstrated increased osteogenic differentiation based on ALP activity. These results agree with those obtained by Zhou et al. [121] in which negatively charged surfaces were seen to accumulate the cations present in the cell culture medium, which in turn attracted proteins such as fibronectin and the negatively charged cytomembrane of cells, favoring their adhesion and subsequent osteogenic differentiation, in agreement with the results of Parssinen et al.

Li et al. [110] used lithium niobate wafer, a ferroelectric crystal, with positive, negative or neutral surface charges to investigate their effects on MSCs fate. Positive surfaces showed greater cell areas than negative and non-charged surfaces, resulting in enhanced OPN, OCN and RUNX2 expression and ALP activity. This phenomenon is associated with the capacity of positive surfaces to accumulate negative charges due to the ionic component of the medium, and the ability of different proteins and molecules such as dexamethasone to attract positive charges, generating electrostatic interactions between the charged molecules and the charged surface. This can influence the distribution of bioactive molecules regulating MSCs fate.

It is not easy to decide whether MSCs should be cultured in positively or negatively charged surfaces given the contradictory information published in recent years. What can be extracted from the presented information is that either a positive or negative surface charge enhances protein adsorption, helping adhesion and spreading of mesenchymal stem cells. The activation of integrin mediated signaling will eventually lead to the activation of mechanosensitive genes, ultimately promoting changes in cell growth, morphology and differentiation potential.

Interestingly enough, this concept was investigated in depth by Jia et al. [122] by using PVDF-TrFE films containing Terfenol-D alloy, a magnetostrictive material, also coated with FN. These films were responsive under a magnetic field, allowing to control the surface potential by applying different intensities (0 to 2800 Oe). Positive and negative surfaces with different surface potentials were investigated. The spatial distribution of two functional sites of FN, RGD and PHSRN, which act in synergy, is affected by the surface potential. While positive charged surfaces show a tight formation of the functional sites at 55 mV, the same is true for negative surfaces at −20 mV. This tight FN formation with distances below 3.5 nm fully binds the integrin and produces the strongest integrin-mediated osteogenic differentiation.

The results obtained by Jia et al. disagree with those of Zhang et al. [123], who developed PVDF-TrFE membranes containing different concentrations of $BaTiO_3$ nanoparticles and therefore different surface potentials. Membranes with a surface potential of −76.8 mV were selected for cell culture because of their similarity to endogenous biopotential [124], comparing their performance with non-poled ones. The best osteogenic behavior was found when the cells were cultured on the negative surface with this surface potential. However, Zhang et al. [125] also studied different surface potentials of PVDF-TrFE films, varying the β-phase content and showed that lower surface potentials (−53 mV) (a range not taken into account in [123]) enhanced MSCs osteogenic differentiation more than higher surface potentials (−76 mV).

These differences can be attributed to the fact that Jia et al. used a magnetic bioreactor to modulate surface potential in the polymer matrix due to the presence of magnetostrictive nanoparticles, while Zhang et al. used different β-phase contents to do so, thus with different material surface stiffness and dynamic stimulation.

On another note, the piezoelectric properties of the cell culture supports mean that a mechanical strain needs to be applied to obtain the maximum electric response. As a matter of fact, different bioreactors compatible with cell culture conditions have been developed to deform polymeric samples and induce a change in the surface charge, as described in Fig. 3. The Lanceros-Méndez research group developed different bioreactors based on mechanical deformation using a vertical vibration module to deform the polymer matrix [126–128] in which stimulation programs were applied simulating daily human activity (16 h of stimulation and 8 h of rest) (Fig. 3 I). Magnetic bioreactors have also

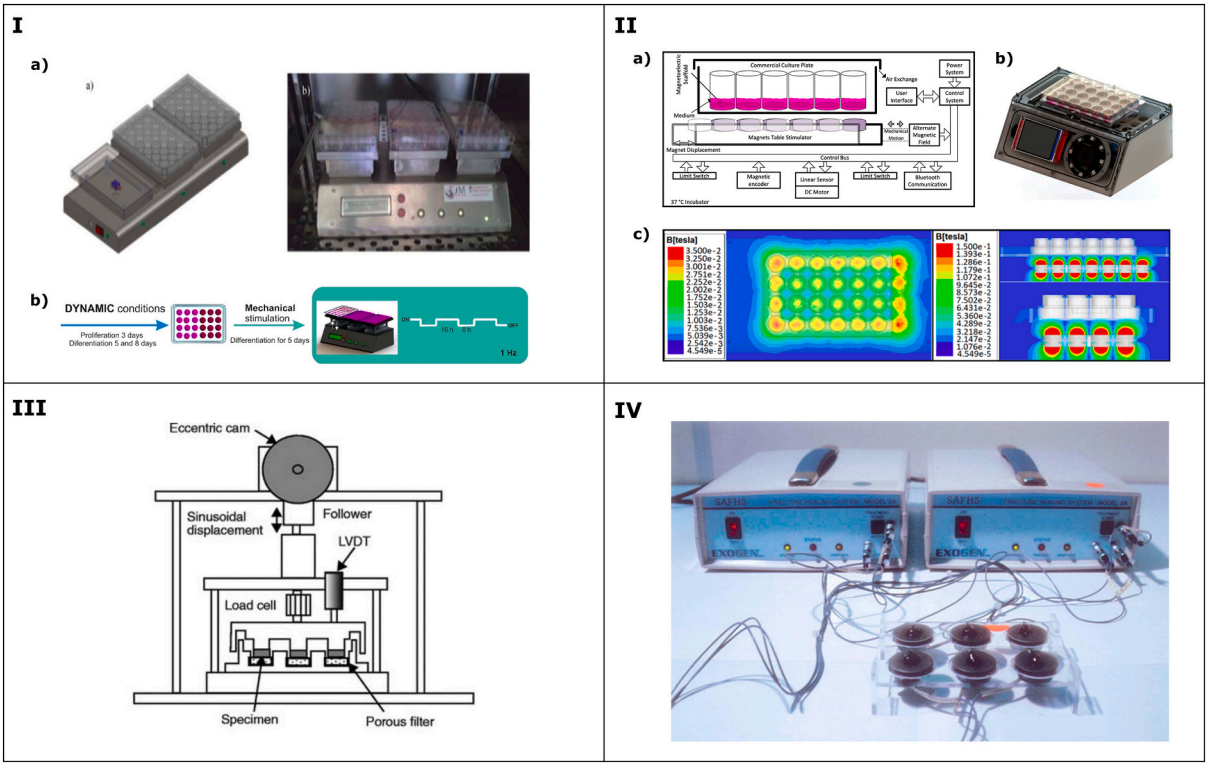

Fig. 3. Examples of bioreactors used for generating an electric response on piezoelectric cell culture supports. I. a) Schematic representation (left) and actual image (right) of a mechanical bioreactor based on a vertical vibration module. Several cell culture plates can be placed on top of the bioreactor. Reproduced from Ref. [128] by permission of the Royal Society of Chemistry, Copyright 2012. b) Diagram of a proposed stimulation program using a vertical vibration module bioreactor to reproduce daily human activity by applying 16 h of stimulation and 8 h of rest. Adapted by permission of Ref. [127], Copyright 2020 American Chemical Society. II. a) Magnetoelectric bioreactor operating principle using electrical and mechanical controls to produce an alternated magnetic field for the stimulation of biomaterials based on the combination of piezoelectric polymers and magnetostrictive nanoparticles. b) Schematic representation of the bioreactor assembled with a cell culture plate. c) Magnetic field intensity distribution at the bottom of 24-well cell culture plates (left) and magnetic field force lines simulation in frontal and side planes (right). Adapted from Ref. [129], Copyright 2020 Multidisciplinary Digital Publishing Institute (MDPI). III. Schematic representation of a cyclical compression bioreactor in which the specimens are subjected to dynamic compressive loading. A load cell and a linear variable differential transformer (LVDT) measure the load response of specimens and the imposed displacement. Adapted from Ref. [133], Copyright 2004 Wiley. IV. Ultrasound therapy unit for the application of low-intensity ultrasound *in vitro*. The therapy unit consists of two sonic accelerated fracture healing system SAFHS® devices and transducers (with coupling gel) to which the tissue culture plate can be connected. Reproduced from Ref. [134], Copyright 2001 Wiley.

been described to exploit the magnetoelectric effect when the piezoelectric polymer is combined with magnetostrictive nanoparticles [129,130]. Moving neodymium magnets below the cell culture plate generates an alternating magnetic field whose intensity depends on the position of the well, as can be seen in the simulation in Fig. 3 IIc. Other types of bioreactors based on dynamic compression [131–133] (Fig. 3 III) or ultrasound activation [107,111,113] have also been described. Commercial fracture healing systems approved by the FDA have already been used to stimulate cells with ultrasound *in vitro* by connecting them to a tissue culture plate (Fig. 3 IV) [134]. In the same setup, piezoelectric biomaterials can be placed in the wells of the tissue culture plate to electrically stimulate the MSCs activating the cell culture supports by US.

Damaraju et al. [132] used a bioreactor to apply dynamic compression to electrospun PVDF-TrFE scaffolds with different piezoelectric responses to exploit the electromechanical stimulation of piezoelectric samples combined with osteogenic and chondrogenic media. Chondrogenesis was favored with inductive medium and the less piezoresponsive scaffolds ($d_{33} = -10$ pC/N), although osteogenesis was enhanced when using osteogenic medium and the scaffolds with the highest piezoelectric response ($d_{33} = -20$ pC/N). ALP, RUNX2, OPN and OCN were significantly upregulated at day 28 in the dynamic stimulated scaffolds compared with the rest of the conditions.

These results again suggest the possibility of combining a physical stimulus with biochemical stimulation provided by soluble factors present in the differentiation medium. Their need to be combined is not so obvious as in the case of electrical stimulation using conductive cell culture supports. MSCs osteogenic fate determination has been reported as a result of using piezoelectric biomaterials as cell culture supports in the presence of growth medium [109,111,112,121,123,125,131]. These results allow speculation that electromechanical stimulation and surface charge may be stronger inducers of MSCs osteogenic differentiation than ES mediated by conductive cell culture supports, in which an initial osteogenic stimulus is needed to trigger MSCs osteoblastogenesis.

4. Defining electrical stimulation treatment time

Mesenchymal stem cell osteogenic commitment is the first step in the osteoblast differentiation pathway. These cells become preosteoblasts and acquire the characteristic osteoblastic phenotype, expressing mature markers such as osteocalcin (OCN) and osteopontin [135]. This transition is regulated by the expression of different transcription factors, starting with RUNX2 and followed by OSX in a more advanced stage [136]. Conceiving differentiation as an organized cascade of expression events involves the fact that ES application can be introduced at different time points influencing its outcomes.

Three parameters may be carefully adjusted to determine the most suitable stimulation program to induce MSCs osteogenic differentiation regarding treatment time. On the one hand, stimulation time per day, thus, the number of hours that cells are under stimulation each day. On

the other hand, stimulation period along the duration of the culture should be considered, ergo, the number of days cells are subjected to ES. Finally, the moment in which stimulation is introduced, at earlier, middle or later stages of the osteogenic differentiation process. Just few authors have considered treatment time as a determining parameter when studying the effects of ES on MSCs osteogenic differentiation, thus the number of studies covering this aspect is reduced.

Regarding general electrical stimulation, most of the approaches use a constant ES stimulation rate, usually from 3 min up to 8 h per day, for the total duration of the cell culture [32,33,35–37,39,50–53,56–58]. In few cases, cells are continuously exposed to the electric stimulus [46,48,54], and it is even more unusual the application of ES at the start of the culture with no reapplication thereafter [38,40]. Interestingly enough, Zhao et al. [40] applied ES just for 2 h at the start of the culture and no effects on calcium deposition were observed compared to the non-stimulated control, suggesting that treatment time may need to be adjusted to obtain better results.

In the case of substrate mediated stimulation, most of the published research is based on the use of ES some minutes or hours per day for the whole culture, following the pattern applied in GES [63,66,67,69,80,86,101,102]. Still, some efforts have been made aiming to elucidate the optimal treatment time.

Wechsler et al. [137] showed that for MSCs cultured in indium tin oxide-coated glass and stimulated with a 10 Hz and a current of 10 μA sinusoidal waveform the optimal stimulation time per day was 6 h rather than shorter (1−3h) and longer (24 h). Nevertheless, Zhu et al. [78] went further, and artificially divided the 21-day culture time into 7-day periods and applied ES for 1.5 h a day for the whole selected period. The authors found that the early ES (day 1–7) on MSCs improved the expressions of bone-related markers and genes more than later ES applications (day 8–14 and day 15–21). These results are in agreement with those obtained by Hu et al. [68], in which ES was only applied for 4 h on selected days (day 0, 2, 4, 6, 8, 10 and 12 after initiation with osteogenic medium). Cells stimulated on day 8 showed a higher level of mineralization after 14 days, which was supported by the upregulation of osteogenic genes, especially RUNX2 immediately after the ES treatment.

These data reveal that ES application for osteogenic differentiation induction is a time-dependent process and if optimized can only be applied at specific time-points of the differentiation process.

As mentioned in Section 3.2, the use of piezoelectric cell culture supports to provide substrate mediated ES can rely on two properties, the first one being the presence of an electroactive microenvironment due to the surface charge that may affect protein conformation and enhance cell adhesion triggering different mechanotransduction signaling pathways. The second one is related with the piezoelectric effect induced by applying a mechanical stress as depicted in Fig. 3. This kind of approach is the one allowing the application of different treatment times and their comparison with those obtained applying GES and substrate mediated ES using conductive cell culture supports.

Authors that use bioreactors to produce electromechanical stimulation by means of piezoelectric samples usually apply it for the total duration of the culture. Short periods a few times a day or continued stimulation may be chosen although, again, there is no consensus. Lanceros-Méndez group has defined a stimulation program based on reproducing human daily activity, which means 16 h of stimulation, where a short cycle of 5 min of stimulation and 25 min of rest is repeated, and 8 h of rest. This stimulation program has been applied using either magnetic or vertical vibration module bioreactors [127,130]. Damaraju et al. [132] applied dynamic compression three times a day for 1 h on and 1 h off. In the case of Zhou et al. [131] a 5 N constant dynamic cyclic force was applied. Liu et al. [114] used a pressurized culture and stimulated the cells for 1 h a day for the whole duration of the culture. In the case of piezoelectric scaffolds activated by ultrasounds, stimulation was applied three times a day for 20 s each time by Yang et al. [111] and 10 min daily for 4, 7 and 14 days in the case of Fan et al. [113].

As far as the authors know, no studies have been published on the optimization of stimulation times using piezoelectric cell culture supports. This could be a new path to explore, since osteoblastogenesis is a time-dependent process and applying stimulation at early stages of the differentiation process (days 1 to 7) may be enough to determine MSCs fate, as has been demonstrated using conductive substrate mediated ES.

Nevertheless, it is worth to mention that treatment time cannot be considered as an isolated parameter that may affect ES outcome. It influences MSCs osteogenic differentiation together with other factors such as electrical stimulation parameters, cell culture media or cell source, which may vary along the different published studies. This reflects the need of performing systematic studies regarding treatment time for different experimental approaches *in vitro* since the combination of multiple and varying factors may produce different results even if the treatment time is maintained the same.

5. Expression profile of stimulated MSCs and activated signaling pathways

Bone morphogenetic proteins are osteogenic inductive cytokines that belong to the TGF-β family and are the ligands of the BMP signaling pathway, which plays a fundamental role in the regulation of bone organogenesis. They are able to bind and bring together the serine/threonine kinase bone morphogenetic protein receptors I and II (BMPRI; BMPRII) forming the heteromeric complex required for signal propagation [138]. BMP ligand initiates a signaling cascade based on Smad proteins, the downstream effectors taking charge of transducing the signals from the cell surface to the nucleus. Once imported to the nucleus, these proteins can regulate transcription of targeted genes by directly binding to specific DNA sequences. MSCs osteogenic differentiation is based on the physical interaction of RUNX2, master regulator of the osteogenic differentiation pathway, and Smad [9]. BMP ligands can also activate mitogen-activated protein kinases (MAPK) signaling pathways, especially extracellular signal-regulated kinases 1/2 (ERK1/2) and p38, which are smad-independent, as described in Fig. 4c. MAPK pathways (ERK1/2, p38 and c-Jun N-terminal kinases (JNK)) have been reported to be activated in a time-dependent manner during MSCs differentiation in osteoinductive cultures [139]. Due to the important character of the BMP pathway on MSCs osteogenic fate determination, several authors have evaluated different molecular players of this cascade as potential targets activated by electrical stimulation.

Pulsed electromagnetic fields, either used alone or in combination with BMP-2, have been demonstrated to simultaneously activate by phosphorylation Smad 1/5/8 and the non-smad MAPK pathway p38 in MSCs. PEMFs can also enhance BMPRI (ALK2) expression in the middle-late phase of MSCs osteogenic differentiation. Inhibition of these signaling pathways resulted in a reduced expression of RUNX2, ALP activity and OCN production [48]. Similarly, Zhang et al. [67] confirmed the upregulation of BMP-2 and its receptor BMPRIA (ALK3) when MSCs were cultured and electrically stimulated on chitosan/polypyrrol scaffolds. Huang et al. [85] also used conductive silicon surfaces to stimulate osteogenic progenitors and related their differentiation to BMP-2 and 4 upregulation and phosphorylation of Smad 1/5/9. All these results show the ability of ES to promote osteogenesis *via* BMP/Smad signaling pathway, among other signaling cascades.

Yong et al. [57] corroborated the role of MAPK signaling cascades in osteogenic fate determination of MSCs when stimulated by electromagnetic fields. p38 and ERK1/2 were phosphorylated whereas JNK was found not to be activated. The authors also confirmed the involvement of the cAMP-PKA pathway, but no relation was described between both signaling cascades, bringing out the fact that electromagnetic fields could independently activate at least two signaling pathways. On the other hand, Jansen et al. [54] did not find increased ERK phosphorylation after MSCs stimulation and osteogenic differentiation using PEMFs. The use of different magnetic fields (frequencies, strengths and waveforms) leads to contradictory results, indicating that different

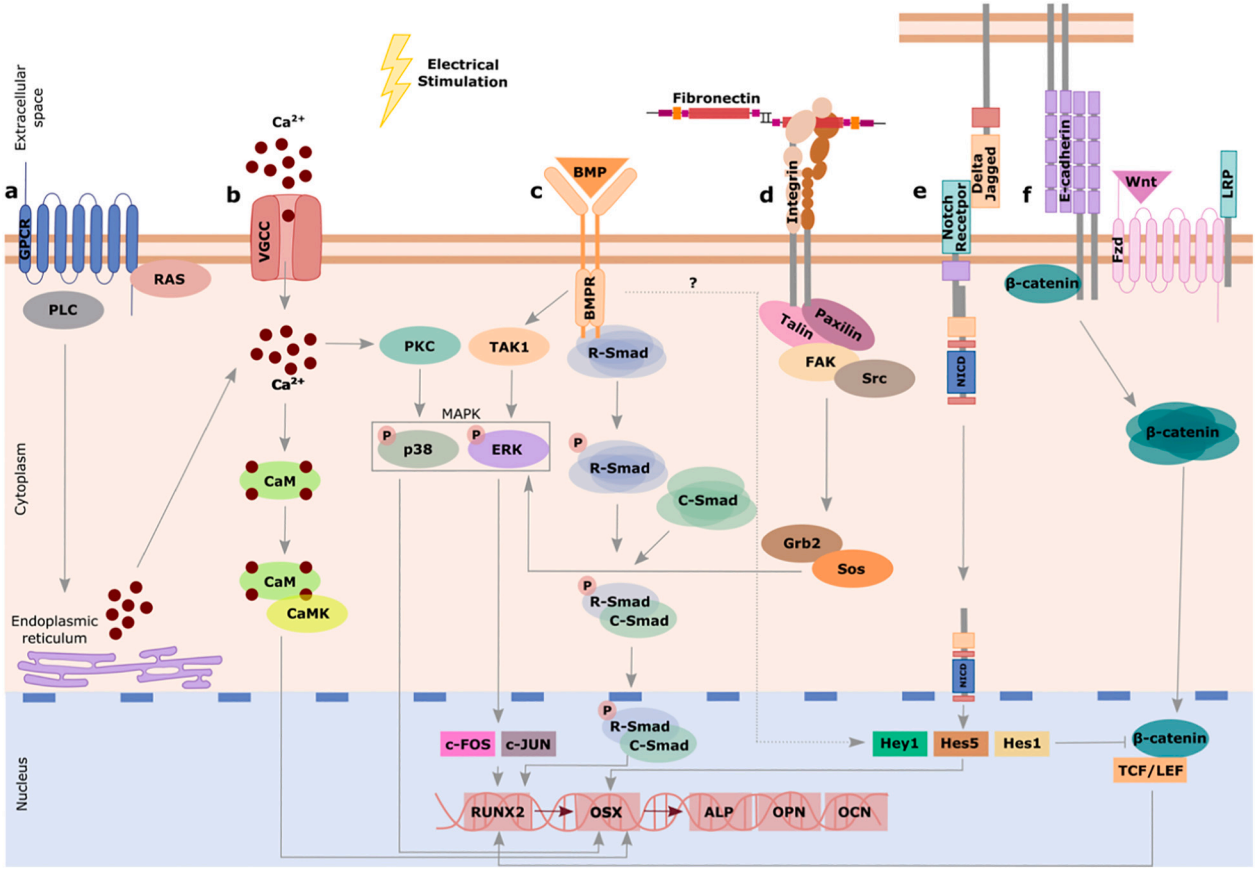

Fig. 4. Activated signaling pathways in MSCs by electrical stimulation. a) G-protein coupled receptors bind phospholipase C (PLC) liberating Ca^{2+} from the endoplasmic reticulum. The increase in intracellular calcium concentration activates protein kinase C (PKC) activating Mitogen activated protein kinases (MAPK) pathway. b) Voltage gate calcium channels (VGCC) allow the entrance of calcium in the cytoplasm, which binds to calmodulin (CaM) that interacts calmodulin-dependent protein kinases (CaMKs) promoting osterix (OSX) expression. c) Bone morphogenetic protein receptors can be activated by a combination of Bone morphogenetic protein (BMP) ligands and electrical stimulation, activating either the smad-dependent pathway, which leads to Runt-related transcription factor (RUNX2) expression or the independent one by activation of MAPK Extracellular signal-regulated kinases (ERK) and p38, which in turn can induce RUNX2 and OSX expression. d) Piezoelectric substrates with associated surface potential enhance protein adsorption and can modify their conformation, exposing adhesion domains recognized by integrins. Integrins mediate the response by activating focal adhesion kinase (FAK). e) Notch signaling pathway may be activated by electrical stimulation, promoting Hey1, Hes5 and Hes1 expression when the Notch intracellular domain (NICD) is cleaved and travels to the nucleus. f) Cell-cell connections together with piezoelectric stimulation can produce the activation of the Wnt/β-catenin signaling pathway. β-catenin liberates from E-cadherin, due to a reduction in intracellular calcium concentration, leading to its accumulation in the cytoplasm and its translocation to the nucleus, promoting T cell factor/ lymphoid enhancer factor (TCF/LEF) expression.

stimulation parameters could induce different signaling pathway activities and result in different effects.

General ES by direct coupling has also proved that the signal is transduced to the cells through MAPK pathways [36]. ES induced ERK1/2 phosphorylation within the first 30 min of treatment leading to an increase in c-FOS and c-JUN mRNA expression in the early stage of ES. Once again, JNK could not be related to osteogenic differentiation mediated by ES, narrowing the circle to p38 and ERK1/2 as main effectors of the MAPK pathway in response to electrical stimulation. Hronik-Tupaj et al. [37] went further and demonstrated the upregulation of hsp27 and hsp70 and hypothesized that hsp70 can activate the ERK1/2 pathway through Raf-1 and Bag1, enhancing the expression of RUNX2.

Intracellular calcium oscillations have also been described as potential effectors of MSCs differentiation caused by ES, since Ca^{2+} is a well-known second messenger involved in several cellular responses [140]. Osteoinductive factors, including physical stimuli, have been shown to reduce intracellular calcium spikes to a similar level to those found in terminally differentiated human osteoblasts. Electrical stimulus seems to be involved in mediating differentiation through G-protein coupled receptors (GPCR), coupling to phospholipase C (PLC) near the cell surface, which in turn liberates Ca^{2+} from the endoplasmic reticulum. It is hypothesized that PLC-mediated signaling can activate Protein kinase C (PKC) and potentially couple to the MAPK cascades, as described in Fig. 4a [141].

Calcium ion channels, such as VGCC, can also mediate Ca^{2+} influx into the cell in response to membrane depolarization and might activate ERK1/2 cascade acting downstream of Ras [142]. They have been considered as the main targets of PEMF action [143]. Petecchia et al. [50] detected an augmented expression of L-type VGCC when MSCs were stimulated with osteogenic media and PEMF for 27 days, while PEMF seemed to influence $[Ca^{2+}]_i$ after 9 days of exposure, leading to an increase of 30% compared to cells cultured in OM. Zhang et al. [66] also described the role of VGGC in MSCs osteoblastogenesis in response to substrate-mediated ES, demonstrating that matrix mineralization was mediated by an influx of Ca^{2+} and not *via* Ca^{2+} release from internal stores. They also detected the role of other ion channels (Na^+, K^+ and Cl^-) in the ES-induced enhancement of MSCs functions, but not as important as that of VGCC. Jing et al. [69] corroborated this hypothesis reporting an increase in $[Ca^{2+}]_i$ in MSCs stimulated on conductive coated PPy fibers attributed to the activation of voltage-gated Ca^{2+} channels.

This increase in intracellular calcium concentration is mainly mediated by the Ca^{2+} binding protein calmodulin, which undergoes pronounced conformational changes to activate downstream effectors [140]. CaM expression has reported to be increased in MSCs after 21 days of ES suggesting the involvement of calcium/calmodulin pathway in the differentiation process mediated by this stimulus [33]. Osteogenic differentiation activated by Ca^{2+}/CaM might be related with the activation of the osteoblast specific transcription factor Osterix. CaM is able to interact with calmodulin-dependent protein kinases (CaMKs), specially CaMKII, which in turn regulate Osx during osteoblast differentiation. Osterix proteins regulate the expression of many osteogenic factors including osteonectin, osteopontin, osteocalcin and alkaline phosphatase [144]. Piezoelectric stimulation has proven to activate the same signaling pathway. Liu et al. [114] demonstrated an increase in intracellular calcium concentration which lead to p38 phosphorylation and promotion of osterix expression, thereby achieving the osteogenic differentiation of MSCs when cultured in dynamic conditions in piezoelectric cell culture supports (Fig. 4b).

Other signaling pathways have been proposed as activated in response to ES, such as the Notch pathway. Notch receptors are activated by a ligand (Jagged-1,-2 and Delta-like (DII)-1,-3 and -4) on adjacent cells, resulting in the cleavage of the Notch intracellular domain (NICD) and its translocation to the nucleus, activating the transcription of nuclear gens of the Hes/Hey family, described in Fig. 4e [145]. Contradictory effects of Notch cascade on osteoblastogenesis have been described due to its role in the inhibition of the Wnt/β-catenin pathway, but the overexpression of NICD or ligand jagged1 enhanced mineralization in MSCs cultures [146]. Bagheri et al. [46] described the upregulation of the Notch target genes Hes5, Hes1 and Hey1 during the middle-late times of differentiation (14–21 days) in response to PEMFs. Nevertheless, the inhibition of the Notch pathway did not reduce the expression of Hey1, indicating that it is not directly modulated by Notch but might be regulated by the BMP pathway. This again highlights the idea of a synergistic activation of different signaling cascades acting together in response to electrical stimulation.

Activated signaling pathways take a different turn when culturing MSCs on piezoelectric cell culture supports. The presence of an associated surface potential, already mentioned in Section 3.2, can influence protein conformation, either from cell culture media or coatings, and exposure of adhesion motifs, modifying cell response. Fibronectin is a typically used protein to coat hydrophobic biomaterials lacking cell adhesion properties, specially the piezoelectric polymer PVDF [147–149]. It has been demonstrated that RGD adhesion domain presentation can vary depending on the presence of a surface charge [147]. It is therefore obvious to think of integrins, the principal receptors for binding extracellular matrix proteins and integrating the signals between the ECM and the cytoskeleton [150], activating mechanotransduction signaling pathways. Jia et al. [122] described the expression of α5- β1 integrin pair, a fibronectin receptor, in MSCs cultured on PVDF-TrFE films with different surface potentials coated with fibronectin. This expression was consistent with the one of focal adhesion kinase (FAK), the main protein of the integrin-mediated osteogenic differentiation signaling pathway. When FAK is recruited to focal adhesions by cytoskeletal anchor proteins such as talin and paxillin, clustered FAK molecules phosphorylate, create a phosphotyrosine docking site for members of the Src family. These Src protein bind Growth factor receptor bound 2/son of sevenless (Grb2/Sos), which in turn are able to activate ERK (Fig. 4d) [150,151]. As explained earlier, ERK activation leads to MSCs osteogenic differentiation. Other authors have described an increase in cell adhesion and consequently an enhanced osteogenic differentiation when MSCs were cultured on charged substrates, which may lead to hypothesizing about the involvement of the FAK/ERK signaling pathway [119,121].

Mitochondrial function, and therefore changes in oxygen metabolism, have also been related to the regulation of MSCs osteodifferentiation induced by electroactive materials [125]. Mitochondrial membrane potential (MP) and reactive oxygen species (ROS) can vary regarding the surface charge of the piezoelectric material and their d_{33} coefficient, indicating that the electrical environment has a dose-response relationship with bone regeneration. Nevertheless, no related signaling pathway has been proposed to be activated in response to these variations in Mitochondrial MP and ROS. Further research may be needed to elucidate the underlying mechanism.

Besides interactions with ECM proteins, cell-cell connections through connexins such as E-cadherin and their cytoplasmatic effectors, catenins, have been described as potential mechanisms of piezoelectric signaling transduction. Zhang et al. described an increase in β-catenin expression in the middle-late stage (14 days) closely related to the presence of more connected cells. This connection help to generate a stable hyperpolarization in the cell membrane potential in response to piezoelectric stimulation. In earlier stages, cells are not well connected, leading to the impossibility of reaching a stable hyperpolarized state. In response to cell membrane hyperpolarization calcium channels close, resulting in the separation of β-catenin from E-cadherin and its accumulation in the cytoplasm. Wnt/β-catenin signaling pathway is hypothesized as responsible activated signaling cascade, depicted in Fig. 4f [152]. In fact, Wnt/β-catenin can activate osteogenic differentiation program in MSCs through β-catenin binding to T cell factor/ lymphoid enhancer factor (TCF/LEF) transcription factor [153]. The involvement of the Wnt signaling pathway was also described by Li et al. [110]. Wnt4 was found to be upregulated during MSCs osteogenic differentiation cultured on piezoelectric crystal substrates. BMP2 was also overexpressed, although there is insufficient evidence to explain the relationship between TFG-β/Wnt signaling pathways and osteogenic differentiation. This work reinforces the idea of the influence of a charged surface in enhancing serum protein adsorption and therefore enhancing MSCs spreading ability.

6. From bench to clinic. Moving forward beyond *in vitro* electrical stimulation

The detailed analysis of different MSCs *in vitro* electrical stimulation methods arises an obvious question from a tissue engineering point of view. Could the described approaches be easily translated into clinic?

Bone tissue engineering has been postulated as a feasible option to replace the autogenous bone graft, the current gold standard, but its clinical application is still far from being implemented on a day-to-day basis. Although the number of pre-clinical studies using MSCs and biomaterials to treat bone defects has increased in the recent years, the approaches that made their way into clinical studies, with low number of patients, show non consistent results [154,155]. These inconsistent results may be related with MSCs limitations regarding cell selection, association of cells and biomaterials, MSCs susceptibility to compromised microenvironments and the lack of osteogenic differentiation of the implanted cells.

MSCs high sensitivity to harsh environments and their inefficient osteogenic differentiation once implanted may be overcome by using priming approaches. Pre-differentiation of MSCs prior to transplantation increases mineral deposition and results in a better integration in the damaged site compared to undifferentiated MSCs [156–158]. Biochemical approaches based on dexamethasone supplementation are currently used for MSCs priming. They generate mixed populations containing fat cells that can reduce treatment efficiency [8], moreover, MSCs osteogenic phenotype induced by biochemical pretreatment is reversible after stimuli deprivation [159]. The absence of efficient pre-differentiation approaches opens the door for the introduction of electrical stimulation protocols.

General and substrate-mediated ES by means of conductive cell culture supports can be introduced as pre-differentiation approaches for MSCs, achieving a stable osteogenic phenotype, after which they can be seeded on the appropriate scaffolds prior to implantation. These approaches require the presence of an external power supply, electrodes

that need to be in direct contact either with the cell environment (General ES) or the conductive material where cells are growing on (Substrate-mediated) making their direct implantation in the human body very difficult and reducing their clinical translation to priming strategies. Protocol standardization is the first step in a devious and long road until these approaches could be used in clinic. Ensuring a stable and non-reversible MSCs osteogenic phenotype may be the next goal for the scientific community working in the field.

Regarding piezoelectric cell culture supports, their biggest advantage relies on the piezoelectric effect itself. The absence of an external power supply is overcome by the mechanical deformation of the scaffold, produced by the movements of the human body, which generates an electrical output. Moreover, the presence of an associated surface charge provides an electrical microenvironment enhancing protein adsorption in an active conformation favoring cell adhesion, already described in Section 3.2.

Piezoelectric cell culture supports have been tested *in vivo* to treat critical size bone defects with positive results when compared with non-poled piezoelectric materials or non-piezoelectric ones [109,113,123,125,160–162]. The scaffolds are implanted in the defect without previous seeding of MSCs. When placed in the injured site cells from the surrounding healthy tissue invade the scaffold, recruited by the biomaterial electrical cues. Among the different cell types, MSCs are present and can be induced to differentiate into osteoblasts in response to the electrical stimulus, initiating the process of bone regeneration. This approach presents some drawbacks, as can be the non-degradability of the piezoelectric scaffolds, which in most cases are ceramics or non-biodegradable polymers that will reside in the body unless surgically removed. Moreover, bone microenvironment at the injured site can be compromised due to immune-mediated, inflammatory, and degenerative diseases hindering MSCs homing and thus the regeneration process. This problem may be solved by using piezoelectric biodegradable materials or piezoelectric cell culture supports as priming platforms for MSCs pre-differentiation. In the latest, once a stable phenotype is achieved, cells can be harvested and seeded onto biodegradable and already FDA approved scaffolds.

Again, priming platforms are still far ahead from being real candidates for clinical application. Once a standard protocol for MSCs pre-differentiation is achieved, these platforms must accomplish the criteria of quality cell therapy standards following good manufacturing practices (GMP), maintaining and ensuring a long-lasting osteogenic phenotype but at the same time being convenient for their commercialization.

7. Conclusions and future perspectives

This review provides a comprehensive and integrated vision of the effect of different electrical stimulation techniques on the osteogenic differentiation of MSCs, including general stimulation or substrate-mediated using conductive or piezoelectric supports. Whether ES has an osteoinductive influence in MSCs is not an easy question to answer. Ideally, this biophysical cue should be powerful enough to induce osteoblastogenesis in MSCs in the absence of biochemical soluble factors to avoid their undesired side effects. Nevertheless, the revised literature indicates that while some authors have reported successful results using expansion medium, the general trend is a combination of ES and osteogenic medium to induce a synergistic cell response. It is hard to elucidate the appropriate parameters that will lead to a standardized protocol for MSCs electrical stimulation since a variety of experimental techniques and conditions as well as biomaterials have been used. Even when the same kind of stimulation has been applied, different authors have employed diverse waveforms, electrical potentials and stimulation times. Nonetheless, it has been proved that electrical stimulation activates different signaling pathways that converge in the expression of osteogenic related genes, such as RUNX2 and OSX. These cascades are not independent, they tend to overlap at different cell levels revealing that their boundaries are not tight. Once again, the lack of uniformity in selecting stimulation parameters may be responsible for the resulting heterogeneity. Future steps may be focused on defining proper stimulation conditions, at least when using similar types of stimulation, to facilitate the integration and interpretation of the results for future ES applications in bone tissue engineering approaches.

Declaration of competing interest

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

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