DIVERSE EFFECTS OF PULSED ELECTRICAL STIMULATION ON CELLS – WITH A FOCUS ON CHONDROCYTE AND CARTILAGE REGENERATION

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Abstract

Biological effects of pulsed electrical stimulation (PES) on cells and tissues have been intensively studied with the aim of advancing their biomedical applications. These effects vary significantly depending on PES parameters, cell and tissue types, which can be attributed to the diverse variety of signaling pathways, ion channels, and epigenetic mechanisms involved. The development of new technology platforms, such as nanosecond pulsed electric fields (nsPEFs) with finely tuned parameters, have added further complexity. The present review systematically examines current research progress in various aspects of PES, from physical models to biological effects on cells and tissues, including voltage-sensing domains of voltage-gated channels, pore formation, intracellular components/organelles, and signaling pathways. Emphasis is placed on the complexity of PES parameters and inconsistency of induced biological effects, with the aim of exploring the underlying physical and cellular mechanisms of the physiological effects of electrical stimulation on cells. With chondrogenic differentiation of stem cells and cartilage regeneration as examples, the underlying mechanisms involved were reviewed and analyzed, hoping to move forward towards potential biomedical applications. Hopefully, the present review will inspire more interest in the wider clinical applications of PES and lay the basis for further comprehensive studies in this field.

Keywords: Electrical stimulation, electric fields, chondrocytes, chondrogenic differentiation, cartilage, stem cells.

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List of Abbreviations

ADSCs	adipose-derived stem cells
AGG	aggrecan
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BMI	body mass index
BMPs	bone morphogenetic proteins
BMSCs	bone marrow stromal cells
CaM	calmodulin
CaN-NFAT	calcineurin-nuclear factor of activated T cell
cGMP	cyclic guanosine monophosphate
COL II	collagen II
CVI	chronic venous insufficiency
Cx43	connexin 43
DNMT1	DNA methyltransferase 1
ERK	extracellular-signal-regulated kinase
FDA	Food and Drug Administration
FGF2	fibroblast growth factor 2
GAG	glycosaminoglycan
h-TENS	high-frequency transcutaneous electrical nerve stimulation
IFC	interferential current
IGF2	insulin-like growth factor II
IL	interleukin
IRS	insulin receptor substrate
l-TENS	low-frequency transcutaneous electrical nerve stimulation
JNK	c-Jun N-terminal kinase

MAPK	mitogen-activated kinase
MSCs	mesenchymal stem cells
NEUROG2	neurogenin 2
NMES	neuromuscular electrical stimulation
NIN	non-invasive interactive neurostimulation
NO	nitric oxide
nsPEFs	nanosecond-pulsed electric fields
OA	osteoarthritis
PD-ECGF	platelet-derived endothelial cell growth factor
PEG	polyethylene glycol
PES	pulsed electrical stimulation
PIP2	phosphatidylinositol 4,5-bisphosphate
ROS	reactive oxygen species
snRNPs	small nuclear ribonucleoprotein particles
TGF-β	transforming growth factor beta 1
VEGF	vascular endothelial growth factor
VOCCs	voltage-operated calcium channels
VSDs	voltage-sensing domains

Introduction

PES has long been studied in the context of clinical therapy for various diseases, including wound healing (Ud-Din and Bayat, 2014), bone and cartilage regeneration (Chao and Inoue, 2003; Ciombor and Aaron, 2005), recovery of motor neurons (Rossini *et al.*, 2015), contractile properties of infarcted heart tissues (Hirt *et al.*, 2014), and rehabilitation of muscle contractile function (Kern *et al.*, 2016), as well as tumor therapy through triggering permanent membrane lysis or loss of homeostasis (Cemazar *et al.*, 2013). Several PES devices have been approved by the US FDA for use in the treatment of various conditions, such as tumors, arthritis, and pain, with durations varying from μs to ms (Table 1). Accumulation of Joule heating energy is still a major hurdle for clinical application of electrical stimulation (Voldman, 2006; Weaver *et al.*, 1999), which in turn demands critical restrictions on PES parameters, even though mammalian cells can survive up to a 1-3 °C increase in temperature. Relatively broad or unfocused biological targets limit the exploration of the mechanistic effects of electrical stimulation, as well as their widespread applications in clinical therapy (Okino and Mohri, 1987; Yao *et al.*, 2009). Some reviews have also focused on treatment of conditions such as OA, fracture healing, and muscle pain by PES (Ebrahim *et al.*, 2014; Negm *et al.*, 2013). PES has also been used for plant and animal tissue drying processes and food preservation (Toepfl *et al.*, 2006). Furthermore, high intensity PES can be used as a highly effective process for decontamination of liquid food (Sobrino-López and Martín-Belloso, 2010).

The application of PES, with varying parameters, may lead to significantly varied outcomes under different physiological conditions (depending on cell phenotypes, attachment, and extracellular environment), which limits wider biomedical useage. For example, PES with voltages ≥ 3 V and frequencies ≥ 130 Hz has been reported to ameliorate Parkinson's disease, while PES with a frequency of 5 Hz significantly worsens akinesia (Moro *et al.*, 2002). PES has been reported to prevent atrophy of long-term denervated muscles, resulting in increased muscle cross-sectional area (Ashley *et al.*, 2007). On the other hand, PES (frequency of 20 Hz and durations longer than 2 ms) can also lead to atrophy of muscle fibers in rats after immediate sciatic nerve injury, with a decrease in muscle excitability (Gigo-Benato *et al.*, 2010).

Various methods have been used to apply PES both *in vitro* and in clinical practice (Fig. 1) (Balint *et al.*, 2013; Griffin and Bayat, 2011). The simplest way to apply PES is to arrange that electrodes are in direct contact with cells cultured *in vitro* (Fig. 1**a**). However, some by-products such as ROS could be detrimental. Capacitive coupling for *in vitro* experiments consists in subjecting cells in a Petri dish to two parallel layers of electrode plates (Fig. 1**b**). Such an approach can generate a homogenous electrical field, when compared with direct coupling. To achieve a high field strength, a gap cuvette (Fig. 1**c**) was developed for *in vitro* experiments due to its short gap width of 2 or 4 mm. Such a cuvette can be used in electroporation for cell suspension and field strengths can be in kV/cm range. However, it cannot be used for repeated electrical stimulation because cells need to be suspended. In clinical practice, electrodes could be placed at the defect sites invasively, with a power source nearby (Fig. 1**d**), or could be placed outside the skin (Fig. 1**e**).

Cartilage regeneration and OA pose a formidable healthcare challenge. Interestingly, the fact that the application of PES, with appropriate parameters, could enhance regeneration of cartilage and ameliorate OA may open the door to potential clinical applications (Haddad *et al.*, 2007). However, knowledge on the physical and cellular mechanisms of the biological effects of PES is still limited (Schoenbach *et al.*, 2007). To make sense of the multitude and complexity of data within the scientific literature relating to PES and the observed beneficial therapeutic effects, the underlying cellular and molecular mechanisms associated with the application of PES were rigorously delineated, with a focus on chondrocytes, chondrogenic differentiation, and cartilage as models.

Physical models of the effects of electrical stimulation on cells

Conformational changes to biomolecules induced by electric fields elicit further biological responses. Several physical models for electrical stimulation of living cells, using varying cell parameters and electric

Table 1. Devices for PES approved by the FDA. HDE: humanitarian device exemption.

Names of devices	Anatomical locations	Conditions	Parameters			Device class	Implanted device	Product code
			Frequency	Pulse duration	Intensity			
Bladder system	Urinary bladder	Urination	7 or 9 MHz			HDE	yes	PAT
Cochlear implant with combined electrical stimulation and acoustic amplification	Ear	Hearing		20-400 μs		3	Yes	PGQ
Dorsal root ganglion stimulator for pain relief	Dorsal root ganglion	Pain	4-80 Hz	40-1000 μs	4.6 V	3	Yes	PMP
Intended to evaluate the functionality of human pain reception and transmission of sensory pathways	Wrist	Tremor symptom	150 Hz			2	No	NTU
Implanted phrenic nerve stimulator for central sleep apnea	Phrenic nerve	Central sleep apnea	20 Hz	60/150/300 μs		3	Yes	PSR
External vagal nerve stimulator for headache	Neck	Headache	5000 Hz		24 V, 60 mA	2	No	PKR
Limited output transcutaneous piezoelectric stimulator for skin reactions associated with insect bites	Skin	Pain		30 ms	222.83 mA	2	No	OSG
External pacemaker pulse generator	Right atrium and right ventricle	Heart disease				2	No	OVJ
Stimulator, electrical, implanted, for essential tremor	Deep brain structures	Tremor	130 Hz	60 ms		3	Yes	MHY
Stimulator, electrical, implanted, for parkinsonian symptoms	Internal globus pallidus (gpi) or the subthalamic nucleus (STN)	Parkinson				3	Yes	NHI
Transcutaneous electrical nerve stimulator for pain relief	Joint	Arthritis	2-100 Hz	50~250 μs	0.2~0.5 mA/ cm^2	2	No	OCF
Transcutaneous electrical nerve stimulator for pain relief	Skin	Pain		500 μs		2	No	NUH
Stimulator, low electric field, tumor treatment	Head	Tumor	100-300 kHz		0.7 V/cm	3	No	NZK
Powered muscle stimulator	Skin	Muscle conditioning	1-120 Hz	340 μs		2	No	NGX
Transcutaneous electrical nerve stimulator to treat headache	Skin	Headache	1-100 Hz	0-250 μs		2	No	PCC
Transcutaneous electrical nerve stimulator for pain relief	Skin	Pain	100 Hz	100 μs		2	No	GZJ

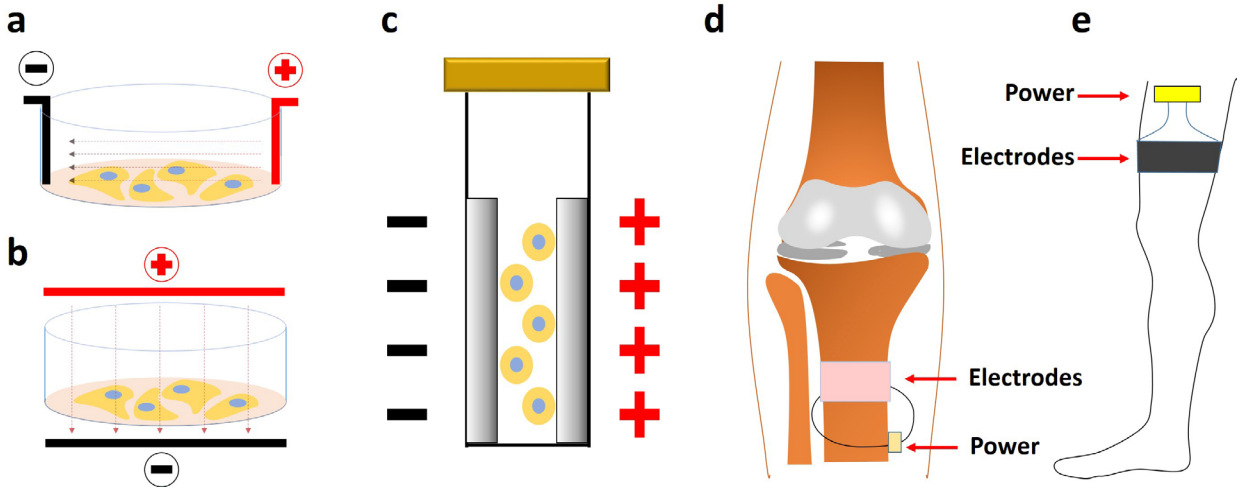

Fig. 1. Device models for PES *in vitro* and *in vivo*. (**a**) Directly applied in a Petri dish *in vitro*. (**b**) Capacitive coupling in a Petri dish *in vitro*. (**c**) Gap cuvette. (**d**) Drect current *in vivo*. (**e**) Capacitive coupling *in vivo*.

fields, have been established. Physical models of membrane potential were established as early as 1953 when elliptical cells were exposed to direct electrical currents (Fricke, 1953), while the Schwan equation, based on models of spherical cells, represents one step forward by including extracellular fluid, plasma membrane, and cytoplasmic parameters (Schwan, 1956). The Schwan equation is as follows:

$$\Delta\psi = 1.5E_{\circ}R\cos\varphi$$

where R is the cell radius, φ is the angle between the position vector and the applied electric field, E_0 is the field strength of the applied electric field, and Δψ is the change in membrane potential.

The multilayer dielectric model of spherical cells lays the foundation for a deeper understanding of the biological effects of PES, which include physical parameters of cell electrical conductivity, relative permittivity, cellular radius, and membrane thickness, as well as cell various organelles and their individual membrane thickness. Based on the multilayer dielectric model of the spherical cell, several conclusions can be drawn (Yao *et al.*, 2009):

1. membrane potentials are positively correlated with the field density of PES;
2. pores in the plasma membrane are formed within a transmembrane voltage range of about 0.5-1 V;
3. the effects of PES with a duration change of s to ps shift from plasma membranes to intracellular structures.

This, in turn, induces complex biological effects, such as activation or inhibition of signaling pathways, phosphorylation of proteins, manipulation of cell phenotypes, while longer durations or higher field strengths will induce apoptosis and necrosis (Weaver *et al.*, 2012) (Fig. 2). The upper boundary of physical parameters is mainly limited by thermal effects and cell death, while the lower boundary is limited by biological effects. Although duration and field density are two key parameters, based on these models and corresponding equations, frequency and timing of electrical stimulation are also critical due to their effect on the action area of PES (Weinberg, 2013). PES with a high frequency (10^4-10^9 Hz) would affect the inner membranes, while PES with a low frequency (< 10^4 Hz) may affect the plasma membrane (Yao *et al.*, 2009).

Field strength, duration, frequency, and time of stimulation are fundamental physical characteristics of PES. Field strength is the intensity of PES and it may range from mV/cm, V/cm, kV/cm to MV/cm for different types of application and studies. Duration of PES ranges from ps, ns, μs, m to s. Long duration plus high voltage would cause thermal effects. Frequency is an important parameter for PES, as PES with a high frequency (above 10 kHz) could affect intracellular organelles. The time span of stimulation is also important, which may be minutes to hours daily. Repeated stimulation would cause significantly different effects due to cumulative effects. For example, a pulse-number-dependent downregulation of mitochondrial function and cell numbers was observed by Hall *et al.* (2007) for nsPEFs. 3 d of PES would not cause significant cell death, while 7 d of PES would cause significant cell death (Kwon *et al.*, 2016). The formation of pores caused by PES increases due to the increase in pulse number (Yogesh, 2016). On the other hand, PES with a low field strength may require several hours daily to produce significant biological effects (Table 2).

The aforementioned physical model only works well when all individual cells have exactly the same physical properties, as well as disperse uniformly in the cell suspension (Yao *et al.*, 2009). However, distribution of the electric field on individual cells is not even, as cell suspension may not have optimal homogeneity while tissues are absolutely non-homogenous. The effects of PES are also dependent on cell attachment, as well as changes in cell morphology (Casciola *et al.*, 2017). The distribution of PES is difficult to measure, but could be simulated with commercially available software, such as HFSS

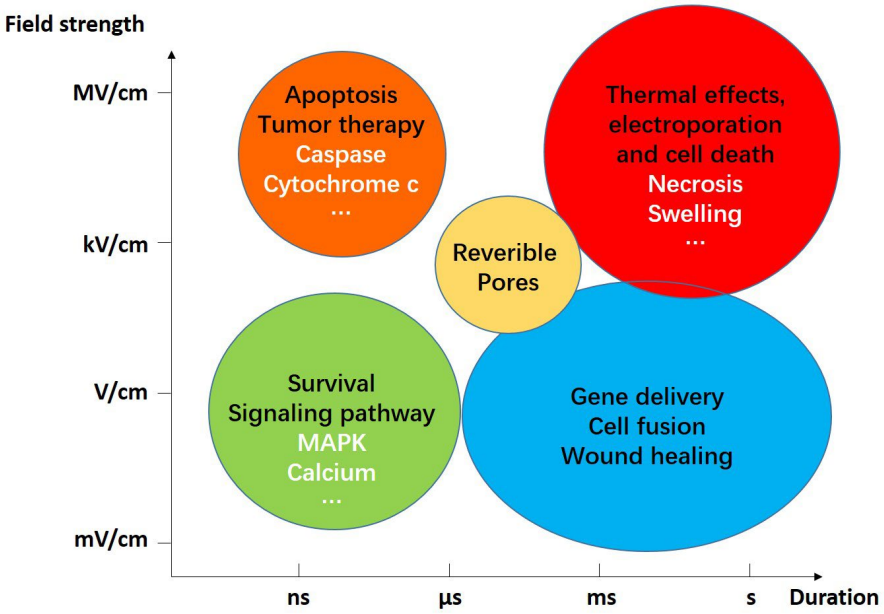

Fig. 2. Biological effects of specific PES parameters. PES can cause different biological effects depending on its parameters. PES with high field strength and long duration can cause thermal effects and cell death. Reversible pore formation can be induced by PES with low field strength and short duration. Irreversible pore formation can be induced by PES with high field strength and long duration.

ANSYS, release 15.0 (ANSYS, Canonsburg, PA, USA) and COMSOL Multi-physics (COMSOL, Burlington, VT, USA) (Buyong *et al.*, 2015; Casciola *et al.*, 2017).

Effects of electrical stimulation on cells and their microenvironment

The effects of PES on different cellular components have been explored extensively, including VSDs, plasma membrane, intracellular components, and signaling pathways, which are often interwoven. PES induces conformational changes of voltage-sensitive proteins, pore formation in the membrane, and calcium influx within seconds of its application, which, in turn, leads to activation of signaling pathways, cell migration, and cell death within minutes or hours.

VSDs

Conformational changes of proteins are early stage key biological effects of PES which are still unexplored. VSDs, key components of voltage-gated channels, usually are comprised of four transmembrane helices (S1-S4) with a pore domain (helices S5 and S6). The S4 segment of voltage-gated channels with the motif [RK]-X(2)-R-X(2)-R-X(2)-[RK] is sensitive to electric fields (Murata *et al.*, 2005). The basic residues of S4 segment are rapidly changed from hyperpolarized potentials, with an internally connected aqueous crevice, to depolarized potentials, with an externally connected aqueous crevice (Zhang *et al.*, 2012). Electrical stimulation induces high levels of intracellular Na^+ ions by the activation of Na/K ATPase, which is responsible for at least 35 % of the voltage-induced pores in the plasma membrane (Teissie and Tsong, 1980). However, electrical stimulation with a high intensity induces a negative-shift of channel open-threshold and reduces conductance of K^+ channels, which may be due to conformational changes causing supra-physiological voltage in the membrane proteins of channel-gating systems (Chen, 2004). Furthermore, PES could denature voltage-sensitive membrane proteins with charged groups in some of their component amino acids which are sensitive to the plasma membrane potential (Chen, 2005). This may be due to breakage of chemical bonds between amino acids in the voltage-dependent membrane proteins, thus reducing their ability to open potassium ion channels simultaneously. From another point of view, nsPEFs inhibit voltage-gated Ca^{2+} and Na^+ channels with reduction of the transmembrane ion gradient due to an increase in non-inactivating ''leak'' current (Nesin *et al.*, 2012). Channel subtypes should also be considered, as voltage-gated potassium channels respond to electrical stimulation variably, depending on channel kinetics (Cameron *et al.*, 2017). Reduction in the opening process of ion channels is less reversible in comparison to the process of inducing reversible pores by electroporation, which reclose within µs or ms. This mechanism is clinically significant as it helps illustrate how injuries caused by electric shock often show no signs of tissue damage but still result in malfunction (Chen, 2005). The presence of naturally occurring proteins with magnetic response hint at a possibility that there may be more proteins that have intrinsic capacity to respond to electrical stimulation (Qin *et al.*, 2016).

Membrane

PES with varying durations has selective effects on either the plasma membrane or intracellular membranes. In terms of duration of pulses, PES is classified into several groups, including µs, sub-µs, ns, and ps (Yao *et al.*, 2009). Living cells respond to PES variably since they are conceptualized as a combination of capacitors and resistors with

Table 2. Effects of PES on chondrocytes.

Model	Electric parameter	Results	References
Fetal bovine articular chondrocytes	Frequency: 60 Hz Time: 0.5 h Intensity: 20 mV/cm	*COLII* and *AGG* ↑ PG and collagen content ↑	(Wang *et al.*, 2004)
Adult bovine articular chondrocytes	Frequency: 60 kHz Intensity: 20 mV/cm Time: 4, 6 or 22 h	*COLII* and *AGG* ↑	(Xu *et al.*, 2009)
Pig chondrocytes	Frequency: 1 Hz Pulse: 5; pulse duration: 100 ns Intensity: 10 kV/cm or 20 kV/cm	Dedifferentiation *COLI* ↑, *AGG* ↓, *COLII* ↓, and *SOX9* ↓	(Zhang *et al.*, 2014)
Human chondrocytes	Frequency: 4150 Hz Time: 30 min Intensity: 0.1 to 10 mV/cm Pulse duration: 10 ms	Proliferation ↑	(Fitzsimmons *et al.*, 2008)
Human chondrocytes	Frequency: 10 Hz Current: 10 μA Time: 3 or 6 h	Chondrocyte adhesion ↑ Long-term cell densities ↑	(Khang *et al.*, 2008)
Bovine articular chondrocytes	Frequency: 60 kHz Intensity: 0-45 mV/cm Time: 24 h	15-30 mV/cm: proliferation ↑ 45 mV/cm: proliferation ↓ and proteoglycan ↑	(Armstrong *et al.*, 1988)

multilayer dielectric models (Schoenbach *et al.*, 2007; Yao *et al.*, 2009). Charging time constant is the time required to charge a plasma membrane (Deng *et al.*, 2003). The equations for charging time (τ) of the outer membrane and the inner membrane are as follows (Yao *et al.*, 2009). According to the multilayer dielectric model of a spherical cell, the constant τ_{cell} of the outer membrane (plasma membrane) can be expressed as

$$\tau_{cell} = \left(\frac{1}{2\gamma_o} + \frac{1}{\gamma_c}\right)\frac{\varepsilon_i \varepsilon_m}{d_m} R_c$$

The constant τ_{nuc} of the inner membrane (organelle membranes) can be expressed as

$$\tau_{nuc} = \left(\frac{1}{2\gamma_c} + \frac{1}{\gamma_{nc}}\right)\frac{\varepsilon_i \varepsilon_{nm}}{d_n} R_n$$

γ_o, γ_c and γ_{nc} are the conductivities of extracellular medium, cytoplasm and organelle cytoplasm, respectively. ε_i, ε_m and ε_{nm} are the permittivities of vacuum, plasma membrane and organelle membrane, respectively. d_m and d_n denotes the thicknesses of the cell and organelle membranes, respectively. R_c and R_n are the radii of the cell and organelle, respectively. According to the above equations, the transmembrane potential of the membrane is as follows. At the end of the pulse, the outer membrane transmembrane potential is

$$V_m = 1.5 R_c E \left(1 - e^{-\frac{\tau}{\tau_{cell}}}\right) cos\theta$$

and the inner membrane transmembrane potential is

$$V_n = \frac{1.5\tau_{cell} R_n E}{\tau_{cell} - \tau_{nuc}} \left(e^{-\frac{\tau}{\tau_{cell}}} - e^{-\frac{\tau}{\tau_{nuc}}}\right) cos\theta$$

E is the field intensity of the external electric field. θ is the polar angle measured with respect to the direction of the field. τ is the duration of the electric fields. Voltage of the plasma and organelle membranes are affected by the relationship of the size of τ, τ_{cell} and τ_{nuc}. τ_{cell} is in the range of hundreds of ns, while τ_{nuc} is in the range of about tens of ns. τ may be different for different cells, because different cells in different environment have different physiological parameters. If τ is much larger than τ_{cell}, then the outer membrane can be fully charged by electric fields. If τ is much smaller than τ_{cell}, then the outer membrane is poorly charged by electric fields. That is the same for the inner membrane. τ_{cell} is in the range of hundreds of ns for most cells and τ_{nuc} is in the range of tens of ns for most cells.

Based on the charge time of the plasma membrane, PES with durations of μs or longer, mainly exert effects on the cell outer membrane, while PES with a sub-μs duration exert effects on both the inner and outer membrane with a sufficient PES intensity (at the kV/cm level) (Yao *et al.*, 2009). nsPEFs with intensities up to 300 kV/cm have better penetrative capacity and mainly affect the inner cell membrane (Chopinet and Rols, 2015; Schoenbach, 2018). Theoretically, electrical stimulation with even shorter durations of ps could be endowed with even higher voltages. Ps-pulsed electrical stimulation with an intensity lower than 1 kV/cm hardly affects the plasma membrane (Yao *et al.*, 2009). The intensity of electric fields is limited to less than 1 kV/cm, while the duration ranges from μs to ms (Zimmermann, 1986). Relatively short durations and high field strengths (hundreds kV/cm) can potentially affect the intracellular membrane while restricting total energy and narrowing down the previously broad biological effects. In any case, precise control of PES is essential for various potential clinical applications and more attention should be paid to nsPEFs.

PES has the unique ability to induce reversible pore formation in plasma membranes within seconds,

including the outer plasma membrane, as well as intracellular organelles, such as mitochondria, endoplasmic reticulum, and nuclear membranes. PES induces changes in membranes immediately. Changes are reversible under PES with a low voltage and short duration, while irreversible under PES with a high voltage and long duration (Pakhomov *et al.*, 2015). Pores that form on plasma membranes are temporary and reversible when an electric field strength of about 0.1-20 kV/cm and pulse durations of 10 ns-10 ms are applied (Kirawanich *et al.*, 2010; Kotnik *et al.*, 2015). These temporary pores have been widely used in biomedical applications, including delivery of biomolecules, such as DNA, RNA, and proteins – commonly referred to as electroporation or electrofusion (Cemazar *et al.*, 2013; Schoellhammer *et al.*, 2014) – as well as drugs (Okino and Mohri, 1987). The voltage applied in electroporation can range between 200 V and 350 V, with 260 V being the most widely used (Nature Methods, 2006). The electrofusion technique is based on electroporation at 300 V pulse of the plasma membrane and is mediated by PEG (Hui and Stenger, 1993; Yu *et al.*, 2008). The effects of electrofusion on different cell types, cell sizes, and intensity of electric field have been studied. Traditionally, the duration of electric fields used for electrofusion range between 10 and 100 μs (Jordan *et al.*, 2013; Rems *et al.*, 2013), while nsPEFs increase efficiency of cell electrofusion through electroporation of the contact areas between cells (Rems *et al.*, 2014). Size of pores created by nsPEFs on the membrane are smaller than that caused by PES with longer durations (Vasilkoski *et al.*, 2006).

Intracellular components and organelles

PES affects intracellular components and organelles, such as the cytoskeleton. PES increases speed of cell migration of both inner and outer meniscus cells (Yuan *et al.*, 2014) and ROS levels in both extracellular (electrochemical) and intracellular compartments (Pakhomova *et al.*, 2012). PES [10 ns, high voltages (> 150 kV/cm)] disrupts pre-messenger RNAs by inducing nuclear speckles of snRNPs (Chen *et al.*, 2007). PES with a low field strength (2.5 V/cm) accelerates cell migration within hours (Hayashi *et al.*, 2016). PES with a duration of ns can induce disassembly of actin structures and cell swelling resulting from cell permeabilization (Pakhomov *et al.*, 2014). Whereas, PES effects on ribosomes remain unexplored.

PES can induce apoptosis through dysfunctioning mitochondria. PES with a high field strength (kV/cm) and a short duration of ns range decreases mitochondria membrane potential (Beebe *et al.*, 2012). The loss of mitochondria membrane potential may be a consequence of increased inner mitochondrial membrane permeability (Batista Napotnik *et al.*, 2012). PES with a duration of ps can induce the release of cytochrome C from mitochondria, with activation of caspase 3 and 9, resulting in apoptosis (Hua *et al.*, 2012).

PES also affects epigenetics through acetylation, histone modifications, alteration of the structural organization of chromatin, demethylation, as well as affecting RNA. PES downregulates histone deacetylase activities in HL-1 cells, with a significant reduction in Cx43 expression and cell-cell communication (Meraviglia *et al.*, 2015). PES can also reversibly downregulate the activity of histone 1 kinase in pig oocytes (Leal and Liu, 1998). PES can prevent the tumorigenic transition of embryonic stem cells through downregulation of phospho-H3 and Ki67 immuno-reactivity (Yamada *et al.*, 2007). PES promotes global DNA demethylation and downregulates *DNMT1* expression (unpublished data).

Signaling pathways

PES exerts effects on cells through activation or inhibition of various signaling pathways (Liu and Song, 2014). PES activates protein kinase C signaling pathway by inducing PIP2 hydrolysis (Tolstykh *et al.*, 2013). PES inhibits VEGF signaling pathway *via* downregulation of VEGF and PD-ECGF, thereby disrupting pro-angiogenic and anti-angiogenic balance, which may consequently inhibit cancer development and suppress tumor blood vessel growth (Chen *et al.*, 2007). PES activates MAPK signaling pathway. PES not only upregulates phosphorylation of ERK and p38 (Zhao *et al.*, 2006), but can also activate JNK, another member of the MAPK family, with fast increased phosphorylation level within minutes, which then decreases within 1 h (Morotomi-Yano *et al.*, 2011a; 2011b). As the signaling pathways activated by PES may become inactivated within minutes or hours, the effect caused by PES may last for minutes, hours, or days. PES-preconditioning promotes cartilage regeneration within weeks through enhancing differentiation of MSCs (Ning *et al.*, 2019). This is the consequence of the biological effects initiated by PES during the early stage after stimulation. Meanwhile, phosphorylation of MAPK caused by PES is reversible (Morotomi-Yano *et al.*, 2011a). PES can also induce dedifferentiation of chondrocytes through activation of Wnt/β-catenin signaling pathway (Zhang *et al.*, 2014). Activation of β-catenin signaling pathway enhances *NEUROG2* expression in brain injury after PES treatment (Matsumoto *et al.*, 2013) (Fig. 3).

PES exerts effects on calcium-related signaling pathways through the release of Ca^{2+} ions from endoplasmic reticulum compartments (Semenov *et al.*, 2013; Vernier *et al.*, 2003). PES with high field strength can induce calcium release within seconds (Zhang *et al.*, 2014). PES has been reported to induce release of Ca^{2+} leading to an increase in cytoskeletal CaM levels (Brighton *et al.*, 2001). PES can also induce calcium influx from the external medium (Bourguignon *et al.*, 1989). PES enhances proliferation of cardiac fibroblast and myocardial fibrosis through activation of the CaN-NFAT pathway, regulated by Ca^{2+}/CaM (Chen *et al.*, 2012). PES increases synthesis

of NO and production of cGMP through calcium/CaM and inhibits Ca^{2+}/CaM, thereby blocking these effects (Fitzsimmons *et al.*, 2008).

PES can also regulate the process of apoptosis and its associated biochemical markers. PES activates caspase-dependent signaling pathways in apoptosis, with the activation of caspase-12, -9, and -3, leading to release of cytochrome c, upregulation of Bax and downregulation of Bcl-2 in HeLa cells (Chen *et al.*, 2013).

Physiological microenvironment

PES can alter blood flow, temperature, and pH of tissues. Blood flow is significantly increased following application of electrical stimulation in wound healing (Ud-Din *et al.*, 2015). Increased blood flow following increasing capillary density is observed when the transcutaneous oxygen partial pressure is increased by 82.4 % during treatment of CVI (Jünger *et al.*, 1997). Thermal effects are integral in traditional PES, but on the other hand, thermal effects are dependent on tissue properties as well as stimulation parameters (*i.e.*, frequency, pulse duration, field strength) (Schoenbach *et al.*, 2004). Electrical stimulation increases temperature around the electrodes in the visual prosthesis system (Çelik and Karagöz, 2014). Temperature is increased around the electrodes (both anode and cathode) and the pH value varied from 3 (near the anode) to 12 (near the cathode) in the electrode array for local control of solid tumors (Soba *et al.*, 2018). Low-voltage electrical stimulation can promote muscle tenderness and accelerate glycolysis, with a significant fall in pH during the first 6 h *post mortem* (Polidori *et al.*, 1999).

PES has been reported to cause deleterious effects on cells, including cell death and cell fragmentation (Yang *et al.*, 2018). PES with high field strength and long duration can result in thermal effects due to Joule heating (Yarmush *et al.*, 2014), with consequent cell death, as proteins denature when the temperature reaches about 43-45 °C. Also, nsPESs with high field strength lead to cell death within minutes, which is mainly due to cell fragmentation (Ning *et al.*, 2019). PES with high field strength and long duration can also induce cell death, following both necrotic and apoptotic ways (Pakhomova *et al.*, 2013).

Effects of PES on chondrocytes, mesenchymal stem cells, cartilage explants, and OA

PES promotes cartilage regeneration and ameliorate OA through modulation of extracellular matrix and various biological factors, such as BMPs, TGF-β, and IGF2, which in turn induce anti-inflammatory and anabolic effects to improve articular cartilage regeneration together with amelioration of inflammation (Haddad *et al.*, 2007; Massari *et al.*, 2007). Cell-based repair strategies induce cartilage regeneration (Johnstone *et al.*, 2013). Chondrocytes and MSCs (or their chondrogenic progenies) are important cell sources (de Vries-van Melle *et al.*, 2014; Estes *et al.*, 2010; Guilak *et al.*, 2010). Both ADSCs and BMSCs have demonstrated their unique responses to growth-factor-induced chondrogenic differentiation, respectively to TGF-β3 and BMP6 (Diekman *et al.*, 2009). However, there are obstacles to using primary chondrocytes for cell culture applications, including limited cell numbers and dedifferentiated phenotypes during *in vitro* expansion (Chung and Burdick, 2008). Challenges faced in culturing bone-marrow-derived MSCs or ADSCs *in vitro* include low efficiency of chondrogenic differentiation and heterogeneous phenotypes (Perdisa *et al.*, 2015; Somoza *et al.*, 2014), while proper application of PES could potentially overcome these obstacles.

Chondrocytes

PES can induce varied or even opposite effects on chondrocytes, including increased proliferation and increased or decreased differentiation (represented as expression of *ColII* or ratio of *ColII*/*ColI* and GAG production). PES for 24 h at 15-30 mV/cm increases proliferation of primary chondrocytes harvested

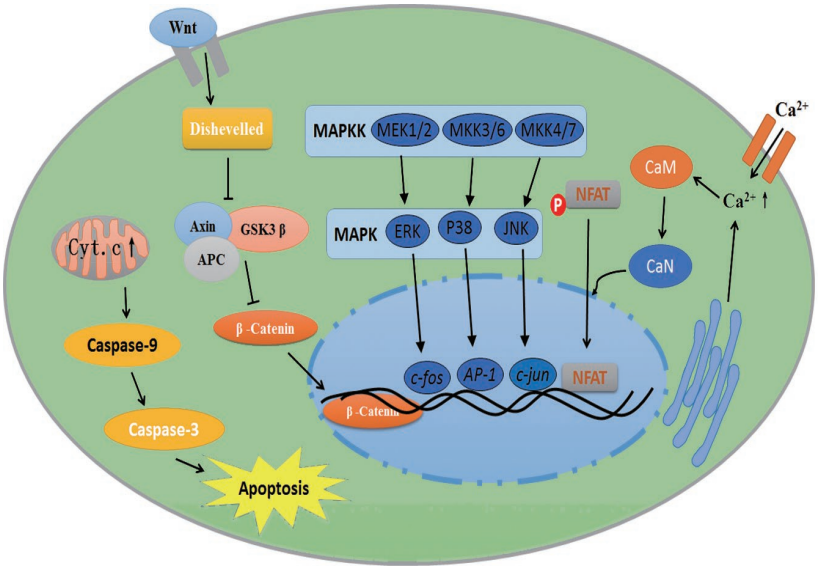

Fig. 3. Signalling pathways activated by PES for a generic cell. MAPK and its downstream signalling pathways are activated by PES. Calcium influx is induced by PES. Cell death could be induced by PES through the caspase signalling pathway. Wnt/β-catenin signalling pathway could be activated by PES.

Table 3. Effects of PES on cartilage explants.

Model	Other factors	Electrical parameters	Results	References
Calf cartilage disks		Frequency: 1, 10, 100, 10^3, and 10^4 Hz Intensity: 10-30 mA/cm^2	Protein synthesis ↑	(Macginitie *et al.*, 1994)
Bovine articular cartilage explants		Frequency: 60 Hz Intensity: 20 mV/cm Time: 1 h on and 5 h off, 4 times/d for 3 d	Production of proteoglycan and collagen ↑ *AGG* and *COLII* ↑	(Brighton *et al.*, 2006)
Human osteoarthritic cartilage explants	With or without lL-1β	Frequency: 60 kHz Intensity: 20 mV/cm Time: 1 h on and 5 h off, 4 times/d for 3 d	With IL-1β: production of proteoglycan and collagen ↑ *AGG* and *COLII* ↑	(Brighton *et al.*, 2008)

from growth plate cartilage, with upregulated [^{3}H] thymidine uptake, and enhances proteoglycan deposition, with upregulated [^{35}S] sulfate uptake, while PES for 24 h at 45 mV/cm inhibits proliferation and enhances proteoglycan deposition (Armstrong *et al.*, 1988). PES (0.5 h, 20 mV/cm, 60 Hz) can increase *COLII* and *AGG* expression in fetal bovine chondrocytes in a micromass culture system (Wang *et al.*, 2004). PES (4, 6 or 22 h, 60 kHz, 20 mV/cm) induces up-regulation of both *COLII* and *AGG* expression levels through extracellular Ca^{2+} influx through voltage-gated calcium channels rather than intracellular Ca^{2+} repositories (Xu *et al.*, 2009). PES (3 or 6 h, 10 Hz, 10 μA) enhances chondrocyte adhesion and long-term cell densities for up to 2 d (Khang *et al.*, 2008). On the other hand, nsPEFs (5 pulses, 1 Hz, 100 ns at 10 or 20 kV/cm) decreases gene expression levels of *COLII*, *AGG* and *SOX9* through activation of Wnt/β-catenin signaling pathway and intracellular calcium ion efflux (Zhang *et al.*, 2014) (Table 2).

This obvious inconsistency could be attributed to either varying parameters of PES or cell types with ever-changing intra- and inter-cellular structures. Generally, PES with low electric field strength and long duration promotes proliferation with effects mainly on calcium homeostasis, cell skeleton, or membrane, while PES with high electric field strength affects intracellular structures or more specific targets.

Chondrogenic differentiation of MSCs

PES has been optimized to promote chondrogenic differentiation of MSCs (Table 4). Piezoelectric scaffolds, exhibiting low voltage output, enhance chondrogenic differentiation of MSCs with upregulation of intense proteoglycan staining and *COLII*, while piezoelectric scaffolds with high voltage output enhance osteogenic differentiation (Damaraju *et al.*, 2017). This emphasizes the importance of PES parameters in chondrogenic differentiation. PES (20 min daily for 7 d, 20 mV/cm, 60 KHz), with or without TGF-β3, promotes chondrogenic differentiation, with enhanced *COLII* and *SOX9* expression levels (Esfandiari *et al.*, 2014). PES (20 min, 1 kHz, 20 mV/cm) leads to chondrogenic differentiation of ADSCs (Mardani *et al.*, 2016). TGF-β, BMPs and other signaling pathways have been implicated in PES treatment. PES (1, 5 or 25 V/cm, 5 Hz) promotes chondrogenic differentiation *via* upregulated *SOX9*, *COLII*, and *AGG*, with TGF-β signaling, BMP signaling and extracellular ATP signaling *via* $P2X_4$ receptors (Kwon *et al.*, 2016). Electrical stimulation induces calcium oscillations through modulation of VOCCs, which are implicated in chondrogenesis (Uzieliene *et al.*, 2018). PES (ns-scale with high intensity) enhances chondrogenic differentiation of MSCs with significantly upregulated expression levels of *SOX9*, *COLII*, and *AGG*, together with down-regulated expression of *COLX* through activation of c-Jun and Stat3 signaling pathways (Ning *et al.*, 2019). ns-PESs promote chondrogenic differentiation of MSCs through activation of MAPK signaling pathways (Morotomi-Yano *et al.*, 2011a).

PES can promote chondrogenic differentiation through upregulation of related growth factors. PES (50 and 100 mV/mm) also increases FGF2 secretion and upregulates phosphorylation levels of Smad2 and Smad3 in fibroblasts (Wang *et al.*, 2017). PES (50 mV/mm) upregulates the secretion of FGF1 and FGF2 in fibroblasts (Rouabhia *et al.*, 2013). PES (5 V, 0.1 ms, 10 min) together with a heat shock can activate insulin signaling through phosphorylation of IRS (Morino *et al.*, 2008). However, disruption of the secondary structure of insulin is observed at higher electric field strengths (above 0.25 V/nm) within 1 μs (Wang *et al.*, 2014) and the binding capacity of insulin to receptors is significantly reduced by long PES duration (0.7 V/m, 50 Hz, 20 min) (Li *et al.*, 2005). So, whether insulin is involved in chondrogenic differentiation of MSCs by PES remains to be explored. Perhaps low intensity PES can activate insulin signaling pathway to promote chondrogenic differentiation while high intensity PES can not.

Cartilage explants and OA

PES can increase matrix production and reduce matrix destruction in cartilage explants. PES (60 kHz, 20 mV/cm) increases production of proteoglycan and collagen in the absence or presence of IL-1β, while dramatically inhibiting expression of matrix

Table 4. Effects of PES on the chondrogenic differentiation of MSCs.

Model	Electrical parameter	Results	References
Piezoelectric scaffolds Human MSCs	20 mV/mm	*GAG* and *COLII* ↑	(Damaraju *et al.*, 2017)
Human ADSCs	Frequency: 60 KHz Intensity: 20 mV/cm Time: 20 min daily for 7 d	*COLII* and *SOX9* ↑	(Esfandiari *et al.*, 2014)
ADSCs	Frequency: 1 kHz Intensity: 20 mv/cm Time: 20 min	*COLII* and *SOX9* ↑	(Mardani *et al.*, 2016)
Mouse MSCs	Frequency: 5 Hz Intensity: 1, 5 or 25 V/cm	*COLII* and *SOX9* ↑ *COLI* ↓	(Kwon *et al.*, 2016)
Pig MSCs	Frequency: 1 Hz Intensity: 10 kV/cm, 20 kV/cm Duration: 10 ns, 100 ns	*COLII* and *SOX9* ↑	(Ning *et al.*, 2019)

metalloproteinase in full-thickness osteoarthritic adult human articular cartilage explants (Brighton *et al.*, 2008). Brighton *et al.* (2006) observed that PES (60 kHz, 20 mV/cm) increases collagen production and expression levels of *COLII* and *AGG* within adult bovine articular cartilage explants (Table 3).

PES used for neuromuscular stimulation increased muscle thickness and pennation angle in 45 women (age 66-75 years) with knee OA and it resulted in a significant increase in functional capacity, as measured by the 6 min walk test and Timed Up and Go Test (Melo Mde *et al.*, 2015). PES resulted in high global effectiveness of OA treatment in two patients (Fary *et al.*, 2009). However, the third patient exhibited no change, emphasizing the importance of heterogeneity. PES resulted in a significant downregulation of various OA symptoms in hands, with respect to pain, swelling, grip strength, and pinch force (Holt *et al.*, 2018).

The effects of PES on OA pain relief remains to be explored. Neuromuscular electrical stimulation reduces pain (de Oliveira Melo *et al.*, 2016). PES can alleviate pain in OA. PES (at least 6 h/d for 3 months) significantly reduced pain in patients with knee OA in clinical trials (Garland *et al.*, 2007). Although PES could improve physical functions in subjects with knee OA, it may not reduce pain. PES attenuated knee OA symptoms in a dose-response manner after 750 h in a clinical trial with 288 patients who had failed non-surgical therapy (Farr *et al.*, 2006). However, contrary results were also reported in which PES could not provide symptomatic relief for OA (Fary *et al.*, 2008; 2011). This difference may be due to limitations of the sample size and heterogeneity, such as age or BMI. Maybe the type of electrical stimulation could change the effects of PES on pain relief. Among electrical stimulation therapy, from h-TENS, l-TENS, NMES, IFC, PES to NIN, IFC seems to be the best treatment modality in terms of effectiveness, as compared with PES and other electrical stimulation modalities (Zeng *et al.*, 2015). Amplitude-modulated frequency generated by IFC can permeate more deeply, which could improve pain relief, as the main analgesic component of IFC (Johnson *et al.*, 2003). Meanwhile, mechanisms in the observed effects of electrical stimulation on OA need to be further explored to optimize the treatment, such as thermal effects, cell migration, blood flow *etc.*. Perhaps PES with a low frequency (≤ 100 Hz) is able to improve physical function but not pain intensity (Negm *et al.*, 2013), which calls for mechanistic exploration and studies using PES with high frequency and short duration or other parameters to avoid harmful changes.

Perspectives

Most research studies on PES were stalled after identification of specific signaling pathways involved, without elucidating the initiator molecule or initial conformational changes of biomolecules. Some studies have moved forward to identifying a complex array of signaling pathways involved, with the biological effects observed being just the outcome of multiple well-orchestrated processes. A comprehensive overview of biological mechanisms, as well as the corresponding physical stimuli involved, are needed to enable more extensive clinical applications by using novel toolkits. The target may be a protein subunit or some other molecular subunit that requires identification by high-throughput assays.

Integration with novel technology platforms is necessary to facilitate clinical applications of PES, *i.e.* high-throughput screening, microfluidics platforms, single-cell analysis, and bioinformatics. Novel technologies such as nsPEFs, which could precisely trigger specific activities within the cell and organelle internally, should be further investigated. PES with different parameters induces variable effects on different organelles, which in turn elicit variable biological effects on cells, tissues, and organs. The specific parameters of PES will depend on the clinical conditions of individual patients, different tissue states, and cell-types. Therefore, utilizing PES with appropriate parameters, such as specific stimulation frequencies and specific durations, must be optimized and standardized by high-throughput assays and

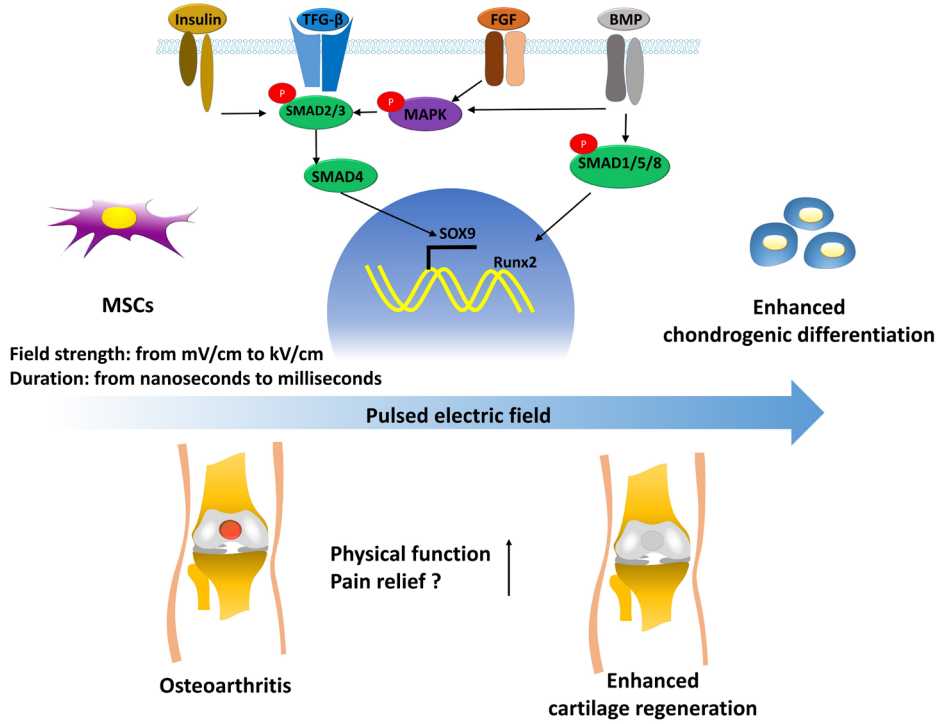

Fig. 4. Possible signalling pathways induced by PES during chondrogenic differentiation of MSCs. Parameters: field strength could range from mV/cm to kV/cm. Duration could range from ns to ms.

comprehensive biological mechanistic studies, to enable PES technology to be applied clinically for enhancing cartilage repair and regeneration. The diameter of chondrocytes is about 20 μm, while that of MSCs is 10-15 μm. This means that higher field strengths are required for chondrocyte than MSCs. Lower field strength (mv/cm), moderate duration (μs-ms), and hours-long stimulation would be preferred for cartilage repair and chondrogenic differentiation of MSCs, as ns-pulsed electric fields with high field strength may cause dedifferentiation of chondrocytes (Table 2) (Zhang *et al.*, 2014).

Wnt/β-catenin, TGF-β, MAPK, and FGF signaling pathways are critical for cartilage repair and chondrogenic differentiation of MSCs (Tang *et al.*, 2015) (Fig. 3). TGF-β/Smad signaling pathway is activated by PES during chondrogenesis of MSCs (Kwon *et al.*, 2016). Wnt/β-catenin is activated in primary chondrocytes by nsPESs (Zhang *et al.*, 2014). MAPK signaling pathway is activated in enhanced chondrogenic differentiation of MSCs induced by nsPEFs (Ning *et al.*, 2019). PES (50 mV/mm) promotes secretion of FGF-1 and FGF-2 in fibroblasts (Rouabhia *et al.*, 2013). TGF-β and FGF signaling pathways need to be further explored during chondrogenic differentiation of MSCs induced by PES, particularly for upstream and downstream signaling pathways. Possible signaling pathways need to be explored (Fig. 4).

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Editor's note: All comments/questions by the reviewers were answered by making changes in the text. Hence, there is no Discussion with Reviewers section.

The Scientific Editor responsible for this paper was Christine Hartmann.