Received: 26 November 2018 | Revised: 8 March 2019 | Accepted: 21 March 2019

DOI: 10.1002/term.2869

SHORT COMMUNICATION

WILEY

Electrical pulse stimulation of skeletal myoblasts cell cultures with simulated action potentials

Paula Villanueva[1] | Sheila Pereira[1] | Alberto Olmo[2,3] | Pablo Pérez[2,3] | Yaiza Yuste[1] | Alberto Yúfera[2,3] | Fernando de la Portilla[1]

[1] Instituto de Biomedicina de Sevilla (IBIS), Campus Hospital Universitario Virgen del Rocío, Sevilla, Spain

[2] Instituto de Microelectrónica de Sevilla, IMSE, CNM (CSIC, Universidad de Sevilla), Sevilla, Spain

[3] Escuela Técnica Superior de Ingeniería Informática, Departamento de Tecnología Electrónica, Universidad de Sevilla, Sevilla, Spain

Correspondence
Alberto Olmo, Instituto de Microelectrónica de Sevilla, IMSE, CNM (CSIC, Universidad de Sevilla), Av. Américo Vespucio, s/n, Sevilla 41092, Spain.
Email: aolmo@dte.us.es

Abstract

Electrical pulse stimulation has an important effect on skeletal muscle development and maturation. However, the methodology for controlling these stimulation parameters to develop in vitro functional skeletal muscle tissues remains to be established. In this work, we have studied the effect of simulated action potentials on the growth and differentiation of skeletal myoblast cell cultures. A circuit simulating action potentials of 0.15 and 0.3 V/mm, at a frequency of 1 Hz and with a 4-ms pulse width, is proposed. Results show an important improvement of the growth rate and differentiation of myoblasts at a voltage of 0.15 V/mm. Parameters such as electrodes geometry or type of signals must be considered in the development of in vitro skeletal muscle.

KEYWORDS

electrical pulse stimulation (EPS), myoblast, skeletal muscle, stem cell differentiation

1 | INTRODUCTION

Generation of functional tissue by tissue engineering has a high impact for regenerative medicine, in many medical fields, such as urinary incontinence or aesthetic reconstruction, among others (Nikolic et al., 2017). Traditional ex vivo cultivation methods typically result in a low differentiation efficiency of stem cells and lack of contractile function (Liao & Zhou, 2009). Current tissue-engineered muscle constructs need to be upscaled to clinically relevant volumes of tissue with a good level of differentiation and morphology and with an appropriate level of contractile function (Nikolic et al., 2017).

Optimization of tissue engineering to develop functional muscle requires a complex strategy combining stimulation with soluble factors, metabolic optimization, and biophysical stimulation (Nikolic et al., 2017). Electrical stimulation from the central nervous system via the motor neurons is one of the most important signals for skeletal muscle development and maturation (Liao & Zhou, 2009). Electrical pulse stimulation (EPS) has been applied to induce cell clustering in cultured neural networks (Sang et al., 2007) or has improved skeletal muscle regeneration through satellite cell fusion with myofibers in healthy elderly subjects (Di Filippo et al., 2017). It is well known that physiological electrical impulses can be modelled in vitro by tuning EPS parameters such as voltage amplitude, pulse width, and frequency. However, methodology for controlling these stimulation parameters to develop in vitro functional skeletal muscle tissues remains to be established (Cheema, Yang, Mudera, Goldspink, & Brown, 2003).

In muscle tissue engineering, the first important EPS results were published from 2004. Radisic et al. (2004) applied EPS, with the characteristics 0.5 V/mm, 2 ms, and 1 Hz, to rat ventricular myocytes. At the end of the experiments, these cells showed alignment and an increase of the contraction amplitude. In 2007, Au, Cheng, Chowdhury, and Radisic (2007) also made use of EPS (0.23–0.43 V/mm, 1 ms, and 1 Hz) in rat cardiomyocytes resulting in elongation of the muscle fibres and alignment of the fibroblasts. Langelaan et al. (2011) published an electrostimulation study in mouse cells C2C12. In this study, they applied EPS (4 V/cm, 6 ms, and 2 Hz) on Days 0 to 3 after inducing differentiation in these cells. The results were contractions in every culture, but only the culture with EPS starting in Day 2 after differentiation showed the typical mature skeletal muscle

proteins. In 2014, Ito et al. (2014) optimized the construct skeletal muscle in vitro technic using continuous EPS in 3D skeletal muscle constructed by Mag-Te technique. They used mouse C2C12 cells and, on Day 4 of differentiation, applied EPS with various values of different parameters: 0.15, 0.3, and 0.5 V/mm; 2, 4, and 10 ms; and 0.5, 1, and 2 Hz. On Day 7, the results showed that the parameters 0.3 V/mm, 4 ms, and 1 Hz caused the highest force. At the end of the study, on Day 14, they observed in this culture an increase of force of 4.5 in relation to the control culture. It also showed an increase of myosin heavy chain and tropomyosin and striation patterns. A recent work has also shown the positive effects of an EPS protocol consisting of 2-ms pulses at 12 V, with a frequency of 1 Hz, for the induction of human skeletal muscle cell hypertrophy (Tarum, Folkesson, Atherton, & Kadi, 2017).

Variability of previous results indicate that it is still necessary to carry out more experimental work to understand the effect of electrostimulation on muscular stem cells (analysing type of electrical signal used, geometry of electrodes, or intensity and frequency used), optimizing the methodology for developing in vitro functional skeletal muscle. In this work, a new geometry for EPS electrodes and a new type of signal are proposed to optimize the growth and differentiation of skeletal myoblasts cell cultures. We base our voltage levels, frequency, and pulse duration on published works (Ito et al., 2014), but we use other electrodes geometry to stimulate cells. We also use other signals, based on the simulation of action potentials to stimulate the stem cell cultures.

2 | MATERIALS AND METHODS

For cell culture assays, commercial electrodes 8W10E PET (Applied Biophysics Inc.) were employed. To electrostimulate the muscular stem cells, an EPS circuit was implemented. An Arduino Uno

TABLE 1 Wells used in the 8W10E PET culture ware and electrostimulation voltages used

Well #	Electrostimulation applied (V/mm)
1	0 (control)
2	0.15
3	0.3
5	0 (control)
6	0.15
7	0.3

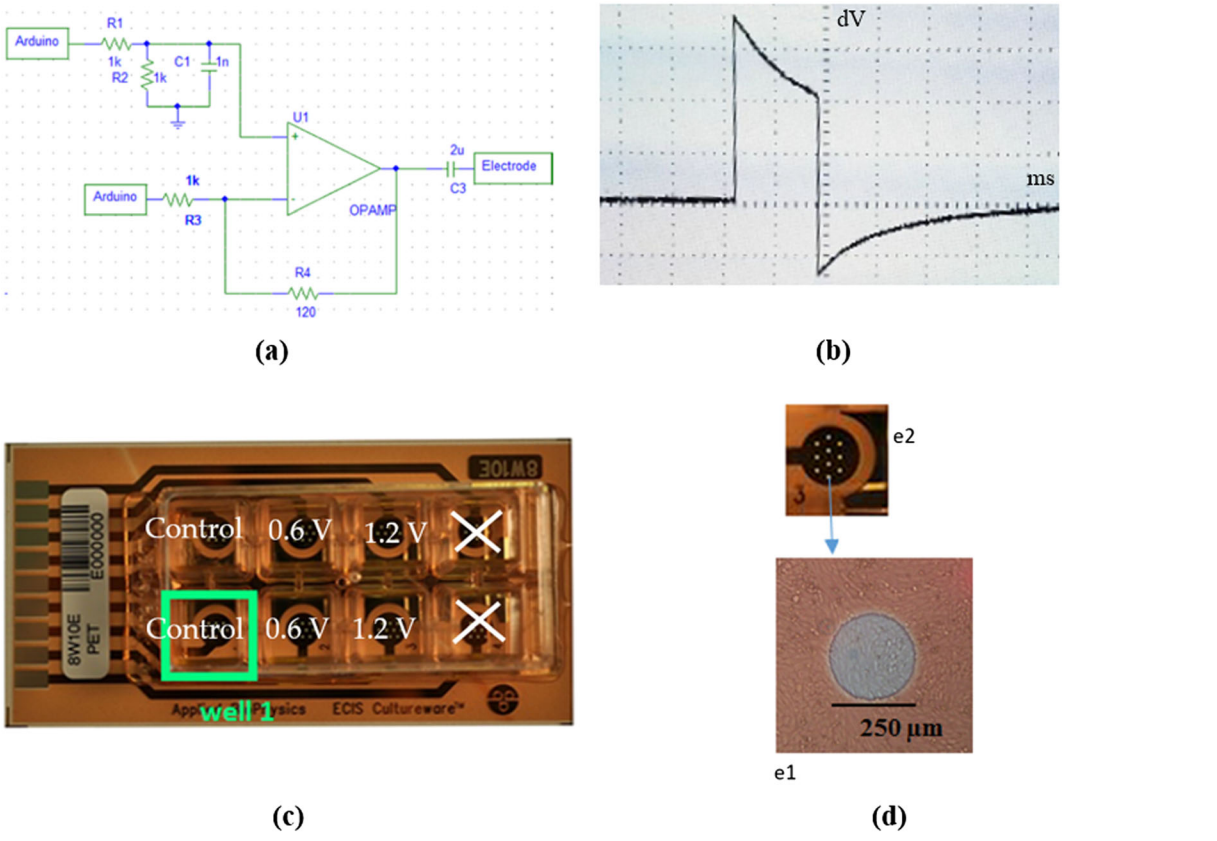

FIGURE 1 Electrical pulse stimulation circuit and signals used. (a) Block diagram of the designed and implemented circuit. (b) Detail of the electrical pulse stimulation signal obtained, simulating an action potential. (c) Detail of the eight wells from Applied Biophysics used as electrodes. (d) Detail of one of the eight wells of the 8W10E PET culture ware from Applied Biophysics that were used in the experiments, where e1 is one of the 10 circular gold electrodes and e2 is the reference or ground electrode. Each well has a side of 8 mm. We established 4 mm as the medium distance between the medium point of the electrodes and the ground electrode (so that 0.6 V corresponds to 0.15 V/mm and 1.2 V corresponds to 0.3 V/mm) [Colour figure can be viewed at wileyonlinelibrary.com]

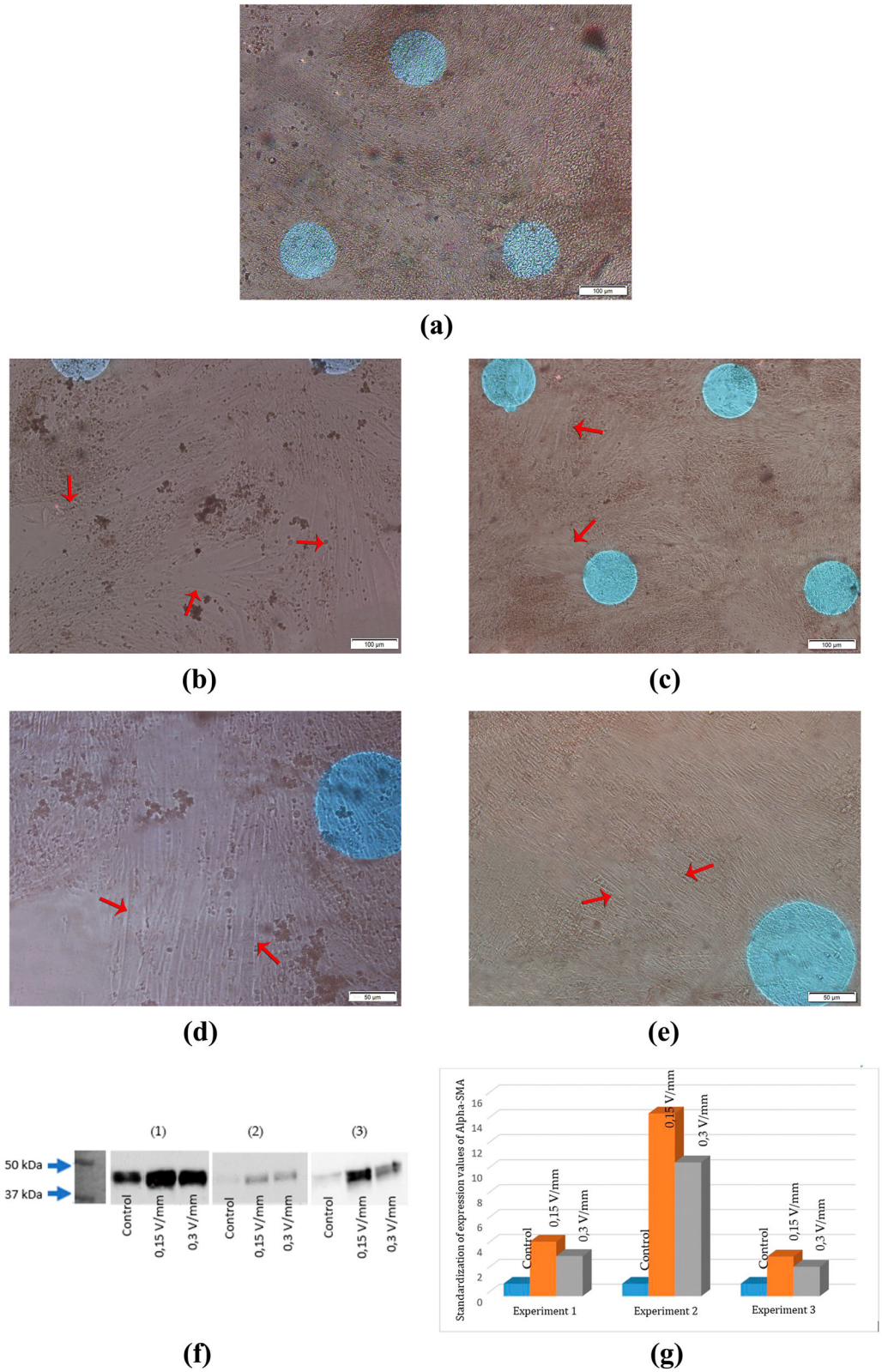

FIGURE 2 Results. (a) Microscope image 10 *g* of unelectrostimulated well. (b) Microscope image 10 *g* of electrostimulated well with 0.15 V/mm. Myotubes can be observed where the arrow points. (c) Microscope image 10 *g* of electrostimulated well with 0.3 V/mm. Myotubes can be observed like in (b). (d) Microscope image 20 *g* of electrostimulated well with 0.15 V/mm. Tubular structures corresponding to muscle myotubes can be observed. (e) Microscope image of electrostimulated well with 0.3 V/mm. Myotubes can also be observed. (f) Level of expression of the anti-alpha-smooth muscle actin antibody (ab5694) in the different wells in the three same experiments. (g) Standardization of the expression values of alpha-smooth muscle actin in the electrostimulated wells normalized with respect to the unelectrostimulated well in each experiment (control) [Colour figure can be viewed at wileyonlinelibrary.com]

microcontroller was used to generate pulses of 5 V, with a modifiable frequency and pulse duration, in this case, 1 Hz and 4 ms, respectively. An inverter operational amplifier with a high capacity condenser was implemented to generate the final signal shown in Figure 1b. This circuit modifies the pulse amplitude and form, so we finally obtain the peak voltage to be used in the experiments (0.15 and 0.3 V/mm), simulating an action potential.

Rat skeletal myoblasts were obtained from *Rattus norvegicus* L6 cell line (ATCC® CRL-1458™) and were cultured at 37°C in a CO_2 incubator at 5% on the Instituto de Biomedicina de Sevilla. The growth medium used was Minimum Essential Medium α (12571-063, Gibco) supplemented with 10% fetal bovine serum (F7524, Sigma) and 1% penicillin–streptomycin (15140-122, Gibco). After the cells reached 85–90% of confluence, they were subcultured using trypsin–EDTA at 0.05% (25300-062, Gibco) and seeded 2.500 cells in the wells 1, 2, 3, 5, 6, and 7 with growth medium. When the specific wells reached 60–70% of confluence, after rinse with phosphate-buffered saline (L0615, Linus), the medium was changed to differentiation medium, MEMα supplemented with 2% horse serum (S0910, Biowest) and 17.8mM NaHCO3 (S6297, Sigma-Aldrich). Microscope images were taken with the Olympus IX-71–inverted phase microscope.

Cells were kept at 37°C, 5% CO_2 in a humidified atmosphere. The 2,500 cells were subcultivated in each of the 8W10E PET cultureware wells (wells 1–3 and wells 5–7). All cell cultures were held in growth medium the first days. After 4 days, all cell cultures reached a confluence between 60% and 70%. The differentiation in myotubes was then initialized by treatment with differentiation medium. Two days after the change to differentiation medium, electrostimulation was started, in accordance with the optimum found by Langelaan et al. (2011). The electrostimulation voltage values used are shown in Table 1.

A western blot analysis was carried out at the end of the experiment to establish the level of expression of the anti-alpha-smooth muscle actin (SMA) antibody (ab5694) in the electrostimulated cell cultures, in comparison with unelectrostimulated ones. This antibody recognizes the alpha-SMA protein. This protein, although is typical of smooth muscle, appears in primary myoblasts and in L6 and C2C12 cells when the cultures are exposed to differentiation medium, that is, in early stages of differentiation (Springer, Ozawa, & Blau, 2002). Three sets of experiments with the setup were performed, in order to compare results.

3 | RESULTS AND DISCUSSION

In our experiments, we can observe the formation of myotubes with an increase of 10 *g* only in the electrostimulated wells (Cheema et al., 2003; Di Filippo et al., 2017; Liao & Zhou, 2009; Nikolic et al., 2017), as shown in Figure 2. The alignment patterns in these myotubes can also be observed, with a higher degree of differentiation compared with unelectrostimulated wells. The expression of alpha-SMA indicated early stages of musculoskeletal differentiation, being this expression higher in the wells that were electrostimulated with 0.15 V/mm, as it is also shown in Figure 2. This optimal value is in contrast with the optimal value of 0.3 V/mm found in other works (Ito et al., 2014).

Results show an important improvement of the growth rate and differentiation of myoblasts at a voltage of 0.15 V/mm. These experiments also indicate how important can be the geometry of electrodes or the type of signal used in electrostimulation, producing different results to other published works. It is necessary to carry out future experiments to further establish the electrostimulation optimal parameters in each different case. The electrostimulation protocol must be transferred to 3D with the objective to be used in clinical applications, optimizing parameters such as electrodes geometry or type of signals and frequency used.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Alberto Olmo https://orcid.org/0000-0001-6388-4462

REFERENCES

Au, H. T. H., Cheng, I., Chowdhury, M. F., & Radisic, M. (2007). Interactive effects of surface topography and pulsatile electrical field stimulation on orientation and elongation of fibroblasts and cardiomyocytes. *Biomaterials*, *28*, 4277–4293. https://doi.org/10.1016/j.biomaterials.2007.06.001

Cheema, U., Yang, S. Y., Mudera, V., Goldspink, G. G., & Brown, R. A. (2003). 3-D in vitro model of early skeletal muscle development. *Cell Motility and the Cytoskeleton*, *54*, 226–223. https://doi.org/10.1002/cm.10095

Di Filippo, E. S., Mancinelli, R., Marrone, M., Doria, C., Verratti, V., Toniolo, L., … Pietrangelo, T. (2017). Neuromuscular electrical stimulation improves skeletal muscle regeneration through satellite cell fusion with myofibers in healthy elderly subjects. *Journal of Applied Physiology*, *123*(3), 501–512. https://doi.org/10.1152/japplphysiol.00855.2016

Ito, A., Yamamoto, Y., Sato, M., Ikeda, K., Yamamoto, M., Fujita, H., … Kamihira, M. (2014). Induction of functional tissue-engineered skeletal muscle constructs by defined electrical stimulation. *Scientific Reports*, *4*, 4781.

Langelaan, M. L., Boonen, K. J., Rosaria-Chak, K. Y., van der Schaft, D. W., Post, M. J., & Baaijens, F. P. (2011). Advanced maturation by electrical stimulation: Differences in response between C2C12 and primary muscle progenitor cells. *Journal of Tissue Engineering and Regenerative Medicine*, *5*, 529–539. https://doi.org/10.1002/term.345

Liao, H., & Zhou, G. Q. (2009). Development and progress of engineering of skeletal muscle tissue. *Tissue Engineering. Part B, Reviews*, *15*, 319–331. https://doi.org/10.1089/ten.teb.2009.0092

Nikolic, N., Görgens, S. W., Thoresen, G. H., Aas, V., Eckel, J., & Eckardt, K. (2017). Electrical pulse stimulation of cultured skeletal muscle cells as a model for in vitro exercise—Possibilities and limitations. *Acta Physiologica*, *220*, 310–331. https://doi.org/10.1111/apha.12830

Radisic, M., Park, H., Shing, H., Consi, T., Schoen, F. J., Langer, R., … Vunjak-Novakovic, G. (2004). Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. *Proceedings of the National Academy of Sciences of the United States of*

America, *101*, 18129–18134. https://doi.org/10.1073/pnas.0407817101

Sang, B. J., Hynd, M. R., Smith, K. L., Song, J. K., Turner, J. N., Shain, W., & Kim, S. J. (2007). Electrical stimulation-induced cell clustering in cultured neural networks. *Medical and Biological Engineering and Computing*, *45*, 1015–1021. https://doi.org/10.1007/s11517-007-0218-6

Springer, M. L., Ozawa, C. R., & Blau, H. M. (2002). Transient production of α-smooth muscle actin by skeletal myoblast during differentiation in culture and following intramuscular implantation. *Cell Motility and the Cytoskeleton*, *51*, 177–186. https://doi.org/10.1002/cm.10022

Tarum, J., Folkesson, M., Atherton, P. J., & Kadi, F. (2017). Electrical pulse stimulation: An in vitro exercise model for the induction of human skeletal muscle cell hypertrophy. A proof-of-concept study. *Experimental Physiology*, *102*(11), 1405–1413. https://doi.org/10.1113/EP086581

How to cite this article: Villanueva P, Pereira S, Olmo A, et al. Electrical pulse stimulation of skeletal myoblasts cell cultures with simulated action potentials. *J Tissue Eng Regen Med*. 2019;13:1265–1269. https://doi.org/10.1002/term.2869