Cells Microenvironment Engineering
Multiphoton Absorption for Muscle Regeneration Optimization

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Abstract: The membrane-substrate interactions have a topological valence and represent a level of information exchange between the cell and the extra-cellular matrix and/or between cells. The interactions can vary with boundary conditions and can be altered by varying the chemical and/or physical properties of the substrate. The alteration can presumably result in differentiation or specialization of the cells, but this fundamental aspect must still be fully understood. In such a frame, we investigated the levels of transcriptional co-activators YAP/TAZ throughout C2C12 differentiation on standard two-dimensional substrates and on polyethylene glycol-fibrinogen three-dimensional microenvironment. In detail, we observed that the use of a three-dimensional matrix permits an earlier differentiation in muscular cells when compared to standard bi-dimensional substrates. On such a basis, we want to investigate the modulation of a more regular three-dimensional pattern on cells proliferation response and we propose a matrix, generable with multiphoton absorption, with regular aligned channels in order to overcome the current limitation in muscle regeneration techniques, so a possible tool to improve the myofibers formation and alignment.

1 INTRODUCTION

Biological organisms are able to colonize different kind of environments and to live in severe environmental conditions. In particular, bio-entities are able to modify their characteristics in order to adapt successfully to unfavourable conditions. There is a poor knowledge about the effects at the cellular level of the changes of the boundary conditions, albeit the consequences on health can be of great importance. At microscopic level, modification of the cells relationship with the environment may induce modification of biological signals, thus variations of the substrate can impose different growing and differentiation conditions to the cells. There are direct connections between membrane proteins (i.e. integrins) that anchor the cell to its substrate and the extracellular matrix (ECM) determining the spatial relationship of the cell in a tissue (Miranti and Brugge 2002). The nature and the amount of the membrane-substrate interactions have a topological valence and represent a level of information exchange between the cell with the substrate and with the neighbouring cells. The same system of interaction translates modifications of these connections as intracellular signals able to modify the cell phenotype. The understanding of how cell-substrate interactions may affect the cell phenotype encases a strong theoretical and practical value: the prediction of the cells behaviour with the cellular microenvironment would allow, among possible applications, to improve the methodology associated with tissue regeneration.

Here, we report on our recent results on cell modification according to the culture substrate and, as a proof of concept, we analyse the effect of bi-dimensional and tri-dimensional environments on the growing and differentiation of a mouse myogenic C2C12 cell line. Moreover, as a feasible application, we propose new substrate processing methodologies to assist muscle regeneration. We advise that

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a multi-photon absorption (MPA) procedure can be able to control effectively the ECM physical and structural properties, thus opening the way to assist the formation of aligned myofibers for muscle regeneration.

2 MUSCLE REGENERATION

Despite remarkable results in recent years have improved the techniques for regeneration and restoration of many damaged tissues, many challenges yet remain unsolved. As an example, in the specific case of the skeletal and cardiac muscle tissue, the generation of insufficient contractile force, the low density of obtained cells, and the inadequate alignment of myofibers are important issues especially for regenerating large portion of tissues. The presence of local stimuli can likely properly direct differentiation and restore small areas of damaged muscles, however, it is difficult to re-establish the optimal muscle functionality in the case of greater damage. Regardless of the damage extension, the repair and regeneration of muscle tissue follows mainly three phases. In the first phase, the muscle fibers undergo necrosis and release factors that result in the final recruitment of inflammatory cells into the injured site (Toumi and Best 2003; Tidball 1995). In the second phase the macrophages phagocyte the necrotic muscle fibers (Novak et al. 2014) and activate the muscle progenitor cells, including satellite cells, inducing the formation and vascularization of new muscle fibers (Hawke and Garry 2001; Tidball and Villalta 2010). During the last phase, the new formed fibers reorganize and merge with the existing muscle fibers. However, if important damage occurs, the scar tissue formation rate is greater than the proliferation of myoblasts and of the formation of myotubes. As a result, the scar tissue prevents the proper myofibers formation (Turner and Badylak 2012). Therefore, in the event of extensive damage, the natural mechanisms are not sufficient to restore the original muscle functionality, so an external action is required to overcome the lack of myofibers in the injured tissue.

2.1 3D Cell Culture and Myotubes Alignment

A common methodology to support muscle regeneration is the direct cell delivery into the treatment area. However, the survival rate of the donor cells is extremely low. A possible way to increase it is to embed cells within materials that maintain the viability (Fuoco et al. 2012) while allowing the diffusion of proliferation factors: ECM implantation containing the cultured cells has a relatively good success rate in muscle regeneration. This technique, applied for injured tibial muscle of mice, induced a greater regeneration when compared to the direct injection of the same population of cells (Boldrin et al. 2007). Two-dimensional ECM technique consists in growing the cells in monolayers, followed by superimposition of the different layers. In this case, diffusion of the nutrients limits the two-dimensional stacked substrates total thickness since diffusion becomes critical upon increasing the thickness: cells that suffer from a lack of nutrient supply exhibit apoptosis. The realization of micro-patterned surfaces permitted to achieve the alignment of myotubes on each layer and the improvement in the diffusion of nutrients and oxygen to the cells, permitting to reach a maximum thickness of 384μm (Bian and Bursac 2009). Moreover, additional deposited cells were able to bind and fuse to the myotubes previously grown, and to form myofibers oriented in the direction of the underlying monolayer (Zhao et al. 2009). It was reported also that a three-dimensional collagen sponges constituted of randomly oriented tubular pores could stimulate the formation of almost aligned myotubes (Kroehne et al. 2008). Three-dimensional rather than two-dimensional arrays are to be preferred since they enable cells to have more space to proliferate, permitting the three-dimensional natural-like disposition of the cells, allowing the simple vascularization of the construct and supporting the formation of multinucleated myotubes along the walls of the structure (Kamelger et al. 2004). Thus, three-dimensional matrix, with proper geometries, stimulate cells in order to form myotubes available to repair large muscle defects. Additionally, in the case of biodegradable matrices, uniform degradation of the 3D structure permits the formation of new tissues at their place (Saxena et al. 1999).

2.2 Substrate Contribution

The microenvironment is an important regulator of cellular proliferation and differentiation. The rate of in vitro proliferation of satellite cells, that are the progenitors of muscle cells, decreases with each step (Renault et al. 2000), whilst the satellite cells on soft substrates are able to self-renew (Gilbert et al. 2010). As an example, on collagen-based substrate (elasticity of 12kPa) the cells are able to maintain their stemness (Urciuolo et al. 2013). Muscle cells integrated in fibrin matrix may increase their in vivo innervation from femoral nerve. Indeed, inserted

myoblasts in fibrin gel and transplanted near the femoral nerve in rats have demonstrated contractile forces five times greater than the control samples (Dhawan et al. 2007). Mature muscle fibers incorporated in a fibrin gel reported the induced formation of acetylcholine receptor clusters, which is the key factor for the development of neuromuscular junctions (Wang et al. 2013).

Two transcriptional co-activators, YAP and TAZ, mediate cellular response to mechanical stress and ECM properties (Low et al. 2014). Phosphorylation regulates these proteins that shuttle between the cytoplasm and the nucleus, where they interact with TEAD transcription factors that in turn activate proliferation. As an example, in human mammary epithelial cells (MEC), growing on soft matrix, the YAP/TAZ proteins are predominantly located in the cytosol. At variance, in the same cell line, growing on stiff material, the proteins migrate into the nucleus and become active (Aragona et al. 2013). It was also demonstrated that YAP phosphorylation is required for differentiation of the mouse myoblast cell line C2C12 (Watt et al. 2010).

3 MATHERIALS AND METHODS

After the seed, we cultured C2C12 myoblasts in growth medium (Dulbecco's modified Eagle's medium (DMEM) with 10% vol/vol foetal bovine serum for 24 hours. After that, we changed medium (DMEM without foetal bovine serum) to induce differentiation. Cells were maintained in differentiation medium up to 96 hours. We grew cells both on bi-dimensional polystyrene substrate and on three-dimensional substrate using a semi-synthetic hydrogel made from polyethylene glycol and fibrinogen (PF). In the three-dimensional case we resuspended cells in PF solution, then added the photoinitiator (Irgacure™ 2959; Ciba Specialty Chemicals, USA) and immediately exposed the solution to UV light (365 nm, 4mW/cm2) for 5 minutes. We performed western blotting in order to evaluate the differentiation level. Primary antibody for the detection of MyoD (1:500 #sc-760; Santa Cruz, California, USA); P-YAP antibody (1:1000 #4911; Cell Signaling Technologies); Tubulin (1:1500 #T5168; Sigma-Aldrich).

Fluorescence observations were performed with a confocal laser scanner microscope (Olympus Fluoview 1000). Samples were prepared by fixing the cells in 4% formalin solution and permeabilized with triton X-100 0.3%. We used as primary antibody P-YAP (1:100 #4911; Cell Signaling Technologies) and MyHC antibody (prepared in our lab). We choose as secondary antibody anti-mouse #sc-2785 and anti-rabbit #sc-2090 both from Santa Cruz (California, USA). We detected nuclei with 4,6-diamidino-2-phenylindole (DAPI).

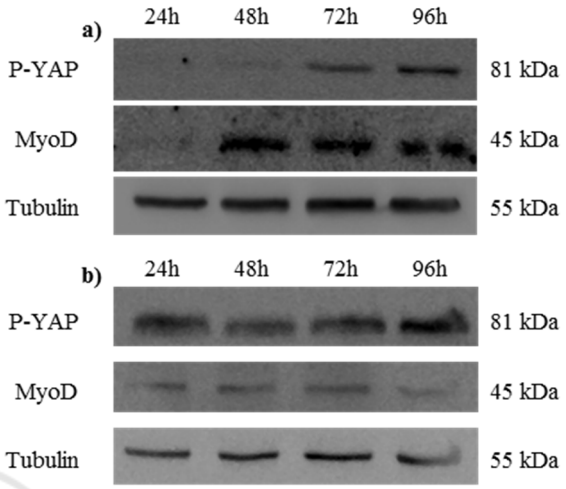

Figure 1: C2C12 cells cultured on a) bi-dimensional polystyrene and b) on three-dimensional PF hydrogels. Levels of P-Yap, MyoD and Tubulin throughout C2C12 differentiation.

4 RESULTS

We investigated the role of the substrate on the proliferation/differentiation switch of myogenic C2C12 cells analysing the expression level of two differentiation markers (i.e. the stiffness-activated YAP protein and the transcription factor MyoD). We analysed YAP phosphorylation level and MyoD expression at regular intervals of 24 hours in cells grown in standard bi-dimensional substrate or in three-dimensional semi-synthetic hydrogel. As reported in Figure 1, the time-dependent level of YAP phosphorylation changes in the cells grown on the two different substrates. Indeed, after 24 hours, the P-YAP level is well detectable in the three-dimensional hydrogel but undetectable in the bi-dimensional substrate where its expression is evident only after 72 hours. We also monitored the C2C12 ability to differentiate in muscle cells observing the time-dependent MyoD expression. As visible in Figure 1, the MyoD expression in cells, growing in three-dimensional matrix, is evident after 24 hours, but it occurs after 48 hours in cells grown in bi-dimensional conditions. The results suggest that the three-dimensional hydrogel represents a better medium to promote cells differentiation when com-

pared to the classic growth on two-dimensional substrates.

We investigated the morphological changes during the differentiation processes of C2C12 cells in three-dimensional PF matrix as shown in Figure 2. Immunofluorescence analysis of C2C12 cells cultured in PF hydrogels shows that both P-YAP (green) and the muscle-specific myosin-MyHC (red) increase as a function of time indicating the occurrence of cells differentiation. The nuclei alignment, visible using counterstaining with DAPI (blue) also indicates a time-dependent myotubes formation. We were expecting a cell growth on hydrogel matrix similar to a biological tissue since the cells of living organisms sustain a three-dimensional arrangement. However, the irregular internal network of the hydrogel induces random oriented myotubes as observed in Figure 2 (96 hours).

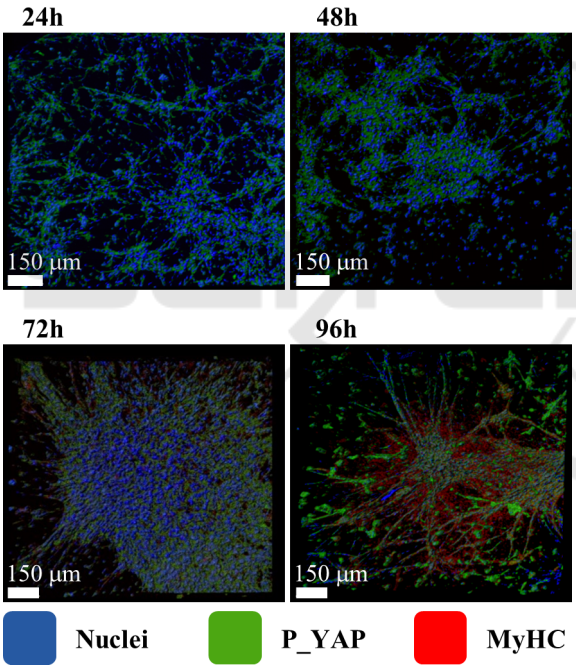

Figure 2: C2C12 cells cultured in PF hydrogels. Immunofluorescence shows P-YAP (green) and MyHC (red); nuclei counterstaining with DAPI (blue).

C2C12 cells pre-cultured in three-dimensional almost oriented pores of collagen sponges showed a greater alignment of myotubes and a better contractile force than control culture (Kroehne et al. 2008). Thus, an artificial matrix with perfectly aligned pore structures could provide favourable conditions for the optimal alignment of the forming myotubes and consequently a good integration of the generated tissue inside the natural muscle. We propose that MPA technique can generate the required three-dimensional matrix with perfectly oriented pores resulting in an enhanced alignment of myotubes.

5 FUTURE DIRECTIONS

We propose the engineering of an artificial extracellular lattice properly structured in order to encourage the cells to differentiate and to align following preferential micro-structured directions. Our expectation is that the proper alignment of the satellite cells, whose specialization generates fully functional myofibers, will form an artificial muscle similar to the natural case.

MPA permits the polymerization of photosensitive material with a manufacturing repeatability of the matrix lattice higher than other fabrication techniques. Two Photon Polymerization (2PP)-Direct Writing is a method allowing the construction of complex 2D and 3D structures. Thus precise 3D scaffold type microstructures can be produced allowing the modelling and the reproduction of the cellular microenvironment. The method is based on the interaction of femtosecond laser radiation with a monomer/photoinitiator/matrix (solvent) mixture which induces a highly localized chemical reaction leading to polymerization (Sima et al. 2013). During the process, the simultaneous absorption of two photons and the excitation of the molecules of a photoinitiator takes place. The two photon absorption process presents a quadratic dependence on the incident laser intensity (Gittard and Narayan 2010; Weiß et al. 2009), leading to a subsequently polymerization only in the vicinity of the focal point (Belfield et al. 2000). Thus, the polymerization volume is much smaller compared to the dimension of the focused laser spot (Matei et al. 2010). MPA permits to growth cells on several symmetrical three-dimensional structures and therefore to replicate the same micro-environmental conditions to all the cultures, thus permitting reliable statistical data analysis. Replicas of an elementary unit produce regular patterns that permits the cells to experience the same local conditions thus perceiving amplification of substrate-related effects. In addition, the MPA matrix fabrication resolution down to 100 nm (Sun and Kawata 2004) permits to easily realize features fully compatible with the size of the cells, since it has been observed that good alignment of C2C12 murine cell line is achievable on 100µm wide bi-dimensional micro-patches (Fuoco et al. 2015).

We expect that similar three-dimensional elongated ducts realized inside the MPA matrices, as

reported in the CAD modelling in Figure 3, can improve the cells differentiation and alignment with positive effects on their fusion helping the formation of aligned muscle myofibers. Additionally, the biodegradable material degeneration will allow the already aligned myofibers to aggregate in the space released by the dissolved matrix providing fibers sorted as in a natural muscle.

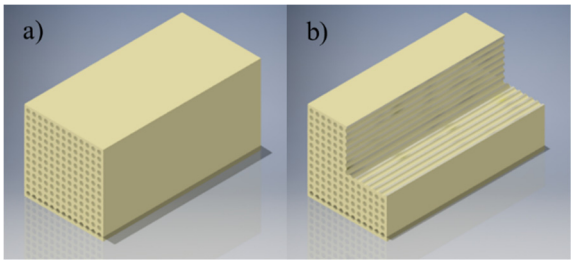

Figure 3: MPA for better alignment of myotubes. Design of three-dimensional matrix; a) orthographic view and b) sectional view.

6 CONCLUSIONS

Cells may express a different phenotype depending on the substrate properties. Proteins inside the membrane permit to modulate the interactions of the cells with the substrate: as an example, the phosphorylation of YAP (P-YAP) mediate the proliferation/differentiation processes and myogenic cells grown on different substrates exhibit different levels of P-YAP.

We investigated the levels of P-YAP during C2C12 differentiation and achieved experimental information on how the modification of the cellular microenvironment and in particular of the substrate dimensionality can influence the cells development. The C2C12 ability to differentiate in muscle cells was monitored following the MyoD expression. Our conclusion is that the use of three-dimensional matrices permits the cells to differentiate in muscular tissue earlier than the standard bi-dimensional substrates. Our results were confirmed in the three-dimensional PF matrix with immunofluorescence topographical analysis of P-YAP, MyHC and morphological analysis of nuclei counterstained with DAPI. The myofibers we obtain in this matrix are random oriented whereas is desirable to use a medium that boost cells differentiation into aligned myofibers.

We are able to engineer properly the PF physical parameters as the material stiffness, or components percentage, even if the method does not permit to exactly define the topography of the internal interconnections. Indeed, the applied diffused ultraviolet light polymerises all the volume of the PF hydrogel and creates random interconnections in the material. The MPA technique permits the polymerization of the material in a very small portion of the space corresponding to the laser focus spot. The large symmetry of the structures generated through the MPA technique permits the definition of a regular geometry of the matrix. Among feasible applications, we propose a MPA matrix with regular aligned channels in order to help the alignment of the myofibers and thus overcoming one important limit of the currently used muscle regeneration techniques.

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