Experimental Cell Research xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Cellular alignment and fusion: Quantifying the effect of macrophages and fibroblasts on myoblast terminal differentiation

C. Venter, C.U. Niesler*

Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, South Africa

ARTICLE INFO

Keywords:
Cellular alignment
Co-culture
Differentiation
Myoblast
Macrophage
Fibroblast

ABSTRACT

Successful skeletal muscle wound repair requires the alignment and fusion of myoblasts to generate multi-nucleated myofibers. *In vitro*, the accurate quantification of cellular *alignment* remains a challenge. Here we present the application of ImageJ and ct-FIRE to quantify muscle cell orientation by means of an alignment index (AI). Our optimised method, which does not require programming skills, allows the alignment of myoblasts in vitro to be determined independently of a predefined reference point. Using this method, we demonstrate that co-culture of myoblasts with macrophages, but not fibroblasts, promotes myoblast alignment in a cell density-dependent manner. Interestingly, myoblast fusion was significantly *decreased* in response to co-culture with macrophages, while the effect of fibroblasts on fusion was density-dependent. At lower numbers, fibroblasts significantly *increased* myoblast fusion, whereas at higher numbers a significant *decrease* was observed. Finally, triple co-culture revealed that the effect of macrophages on myoblast alignment and fusion is unaltered by the additional presence of fibroblasts. Application of our optimised method has therefore revealed quantitative differences in the roles of macrophages versus fibroblasts during alignment and fusion: while successful myoblast alignment is promoted by increasing macrophage numbers, regenerative fusion coincides with a decreasing macrophage population and initial rise in fibroblast numbers.

1. Introduction

Skeletal muscle represents a heterogenous tissue with multiple cell types that each play distinct and important roles in wound repair [1]. Damage to skeletal muscle results in the disruption of myofibres and the extracellular matrix (ECM) that surrounds them [2]. Myogenesis, the differentiation and fusion of mono-nucleated myoblasts into multi-nucleated myofibers, is a critically important stage of muscle regeneration and serves to restore muscle structure and function [3]. Non-myogenic cell types, such as macrophages and fibroblasts, mediate the behaviour of muscle cells during wound repair by secreting an array of signalling molecules and matrix factors [4,5]. We have previously investigated the regulatory role of these non-myogenic cells on myoblast proliferation and migration. We demonstrated that macrophages promote the proliferation of myoblasts in co-culture, while fibroblasts promote migration, during in vitro wound repair; the latter pro-migratory effect was reduced when myoblasts were co-cultured in the presence of both fibroblasts and macrophages under triple co-culture conditions [6,7].

During the terminal phase of myogenesis, myoblasts align to organize themselves relative to each other and to existing myofibres [8]. This process brings the lipid bilayers in close contact with one another in order for the cells to fuse together to form functional muscle with myotubes orientated in the same direction [8]. Several strategies can be used to promote myotube *alignment,* including topographical patterning (e.g. grooved culture plates [9]), mechanical stimulation (e.g. stretch [10]) or application of magnetic/electrical fields [11,12]. In order to *quantify* the exact effect of these strategies on alignment, one has to identify the cell/nuclear outline or actin cytoskeleton, then determine the orientation of these elements and finally calculate a value that represents the extent of alignment [13]. To achieve this, cells are visually identified in images, their outlines manually selected, and their orientation ascertained by determining the angle of deviation of the longitudinal axis of the cell (in degrees) from the x-axis (set to 0°). This manual process is accurate, but arduous and time-consuming with low throughput [13]. Automated image processing techniques (e.g. Fast Fourier Transform (FFT) and local intensity gradient [14,15]) have previously been used to determine cellular orientation; however, these approaches were designed for determining the orientation of a type of pattern rather than determining the orientation of cells and therefore yield low alignment scores [13].

Xu et al. addressed the challenges of these techniques by developing the Binarization-based Extraction of Alignment Score (BEAS) method to

* Corresponding author.
E-mail address: niesler@ukzn.ac.za (C.U. Niesler).

https://doi.org/10.1016/j.yexcr.2018.07.019
Received 24 May 2018; Received in revised form 9 July 2018; Accepted 10 July 2018
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rapidly and accurately quantify the alignment of cells [13]. However, a major challenge with BEAS (and other previous methods) is that the information required to determine cellular orientation is presented as a complex image-processing algorithm, which needs to be computed in MATLAB (a costly computer programming language and computing environment). This implementation is problematic for researchers who have neither programming experience nor access to MATLAB. An easily accessible, automated method to quantify *cellular* orientation was therefore required.

ct-FIRE is a freely available, standalone and fully developed framework designed to determine the orientation of *collagen fibres;* it has not been tested for its ability to determine *cellular* orientation [16]. In order to adapt ct-FIRE to measure cellular orientation, a number of approaches can be used. Data can be organized into 10° bins and either represented as a histogram of frequency distribution (however, this gives no indication of the extent of alignment and makes significance testing problematic) or used to calculate the degree of alignment. In the latter method, cells within less than 10° of the preferred orientation are considered aligned and the percentage of cells in that particular frequency is then calculated [17,18]. Alternatively, an alignment index (AI), which determines how well cells align in a specified direction can be calculated [18–20]. An AI is generally easier to calculate if there is a set direction to which orientation can be compared (e.g. directional angle of the grooves on a plate or the average direction of the cells in a culture dish). However, under standard myoblast culture conditions, there is often no set direction to which alignment can be compared as the cells align independently of physical properties of culture plate and subsequently self-organize in response to elongating myoblasts during fusion [21].

In the current study, we present a method for determining cellular alignment; this method does not require a pre-defined reference direction. ct-FIRE is first tested for its ability to determine the alignment of elliptical shapes (representing hypothetical cells) compared to linear collagen shapes (for which the programme was developed); the orientation determined by ct-FIRE is compared to actual orientation using the AI (Fig. 1A). We then generate an alignment model, creating data sets with defined standard deviations, which represent hypothetical images of cells aligned to different extents (Fig. 1B). These hypothetical images are then rotated, and an AI calculated using either the *average* (i.e. the mean direction of cell alignment) or *preferred* (i.e. direction in which most cells are aligned) orientation (Fig. 1B). Lastly, we test our method using images of cultured myoblasts, where the image processing capability of ImageJ is first applied to automatically mark the boundaries of cells and ct-FIRE is subsequently used to analyse cellular orientation and calculate an AI (Fig. 1C) [13,16]. Once established, we then apply this protocol to assess the effect of macrophages and fibroblasts on the alignment of myoblasts during fusion. This accessible, optimised method for the analysis of cellular orientation presents a tool for analysis of alignment in vitro. Our results highlight the distinct regulatory role of non-myogenic cells on alignment and fusion during terminal skeletal muscle differentiation.

2. Materials and methods

2.1. Testing ct-FIRE on images of hypothetical cells

The programme ct-FIRE was initially developed to determine the alignment of collagen fibres; these fibres have linear shapes as opposed to elliptical shapes that are classically associated with elongated spindle-shaped cells. Linear and elliptical shapes were therefore created using Microsoft PowerPoint (2016) and orientated at 0°, 30°, 60°, 90°, 120° and 150° (Fig. 2); these orientations represent x in the equation below, where N is the total number of orientations [20,22]:

$$\text{Alignment Index}\ \left(AI\right) = \frac{1}{N}\sum_{i=1}^{N}\left(2\quad[\cos(x-y)]^{2}\ -1\right)$$

ct-FIRE was then tested to determine whether it would be able to measure the orientation of both shapes, generating the value y in the above equation. An AI value of 0 represents no agreement between x and y, while a value of 1 represents a perfect agreement (i.e. x equals y). This evaluated how well the orientation determined by ct-FIRE agrees with the actual orientation.

2.2. Alignment modelling

A set of normally distributed random data (N = 98) around a mean (set to 90) with a specified standard deviation (0, 4, 16, 64) (Supplementary Table 1) was created using the Microsoft Excel (2016) *=NORM.INV (probability; mean; standard deviation)* function with = *RAND()* as the probability (which creates a random factional value: $0 \leq \alpha \leq 1$). The values in this data set represent theoretical directions in which an "image" containing 98 cells are likely to be orientated (the smaller the standard deviation, the greater the extent of alignment, and vice versa). Using this model, a cell orientated at 180° has the same alignment as a cell orientated at 0°. Similarly, 225° equals 45°, 270° equals 90° and so on. Therefore, 180° was subtracted from values ≥ 180° and added to values < 0° in order to represent cell direction exclusively between 0° and 180°. In order to rotate hypothetical images of these cells, every theoretical cell in a data set was shifted by −90° or +37.1° and values ≥ 180° or < 0° adjusted accordingly. For every data set, the *average orientation* (an average of all orientations) and *preferred orientation* (an average of cell orientations in the frequency bin with the largest value) was calculated and used in the same alignment index equation stated earlier, where N is now the total number of cells, x is the *average* OR *preferred orientation* used as a reference point of the data set and y is the orientation of an individual cell within that data set. This equation determines how well a cell aligns along a particular direction (e.g. a fixed point of reference) or along the average/preferred direction of cells within each individual cell in a data set. The alignment index of every cell in a data set was used to calculate an average alignment index (of all the cells in that data set) between 0 and 1, where 0 represents a group of cells that are randomly orientated and 1 represents a population that is perfectly aligned [22]. The AI was first calculated using the *average orientation* (often associated with a defined reference point [17,18,20]) and this was repeated using the *preferred orientation* of the data set, in order to compare the two.

2.3. Cell culture

Mouse C2C12 myoblasts (ATCC, USA, cat. CRL-1772™; passage 10–20), LMTK fibroblasts (ATCC, USA, cat. CCL-1.3™; passage 6–25) and J774A.1 macrophages (ATCC, USA, cat. TIB-67™; passage 70–90) were cultured at 37 °C and 5% CO_2 and maintained in growth media (GM) containing Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St Louis, MO, USA, cat. D5648) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco, USA, cat. 10500) and 2% (v/v) Penicillin-Streptomycin (PenStrep, LONZA, Switzerland, cat. DE17-602E). Media was changed every 48 h.

Co-culture of macrophages and/or fibroblasts with myoblasts was established as described in Venter and Niesler [6]. Briefly, for double co-culture, macrophages or fibroblasts (0, 5, 10, 20, 40 and 80 × 10^3) were plated on the outer edge of the well of a 24-well culture plate in GM for an hour to promote adherence; C2C12 myoblasts (50 × 10^3 cells) were then plated and left to adhere in the centre of the same well for 24 h in GM. For triple co-culture, both macrophages (40 × 10^3) and fibroblasts (40 × 10^3) were plated on the outer edge and myoblasts plated in the centre as described above. Myoblasts were differentiated for 5 days in differentiation media (DM; 2% FBS in DMEM) with media

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