Universidad Zaragoza
1542

Trabajo Fin de Grado

Live Imaging of Stem Cells: the use of compliance substrates to determine cell's fate and shape by tracking nucleus and cytoskeleton.

Autor/es

Pablo Pérez Losantos

Director/es

Melissa Knothe Tate, Kate Poole

School of Biomedical Engineering / UNSW

2017

1. Introduction

Multipotent stem cells are crucial within the field of tissue engineering for further applications into regenerative medicine, their ability to differentiate into several cell types is one of the main reasons why they are a promising target of study. Ideal approaches imply to mimic their natural environment in order to fully understand the mechanisms underlying their developmental nature and fate. As far as we know, the context surrounding these cells modulates not only cell and nucleus shape, but also plays a key role in regulation of gene expression [1,2]. Thus, deeply comprising all the stages that stem cells go trough during mesenchymal condensation, is a crucial step towards their practical application.

It has already been reported that changes in nucleus and cell shape are one of the determinant factors that drive cells into a concrete stage [3]. Modifying seeding conditions has been one of the recent attempts to control nucleus and cell roundness, and further analysis has shown a correlation between the expression of mesenchymal condensation genes according to a particular cell and nucleus shape and thus, to specific seeding protocols.

Comprehension of cytoskeletal changes is essential in the study of cell morphology, due to the fact that the intracellular reassembly of structural components determines internal stress forces that end up interfering with gene expression and signalling pathways. Latest attempts to elucidate these changes have focused on the parallel study of both gene expression and cytoskeletal reassembly caused by mechanical stresses in live cells surroundings, where flatter nucleus disposition has been found in cells going through mesenchymal condensation and osteogenic differentiation [3]. Live cell imaging appears to be a suitable approach to study the disposition of the cytoskeleton, nonetheless, the understanding of how different mechanical forces might influence cell fate needs further research to be done.

Up to date, the study of live cells involves a huge breakthrough compared to previous research done with fixed cells. Nevertheless, moving a step closer towards an accurate understanding of how cells behave requires a better representation of their natural conditions. The culture of live cells over a flat surface might have been useful for the early study of gene expression and cytoskeletal disposition, however the mechanical forces present at their natural environment are far more complex to imitate. Previous research has shown that specific compliance substrates have higher influence over mesenchymal stem cells fate than any other biochemical signal involved with differentiation [4].

Here we suggest that culturing the cells and studying their properties over a thin homogeneous layer of PDMS, would generate a stiffness gradient that would provide a better insight about how mechanical forces act over live cells. By optimizing protocols, our aim is to reproduce previous experiments by labelling both actin and tubulin while conducting specific seeding protocols and therefore controlling nucleus and cell shape in both normal substrates and the compliance substrate of PDMS.

2. Materials and Methods

2.1 Overview

The aim of this research focuses on the ascertaining of how different stem cells adapt themselves upon mechanical stresses that cells undergo along mesenchymal condensation. The use of primary embryonic mesenchymal stem cells derived from the mesodermal core at pericondensation points would be the most suitable approach in this study, nonetheless the hurdle of collecting them in enough quantities and the fact that they suffer phenotypic drift when cultured represent the main disadvantages [5,6]. Taking this into account in addition with our prior experience [3,7,8,9], we decided to conduct all the experiments using the C3H/10T1/2 cell line of murine multipotent embryonic cells derived from the mesenchyme (CCL-226; ATCC, Manassas, VA). The C3H/10T1/2 has already demonstrated its incredible ability to differentiate following several lineage paths such as osteogenic, chondrogenic, adipogenic, smooth muscle [11,12,13] and endothelial cell fates [14].

Three target seeding densities were selected considering our previous results [7], with the purpose of achieving specific developmental contexts in which the cells might display concrete behaviours. These densities were selected by considering the growth curve of this cell line in addition to previous studies designed to create tissue templates with properties specific to pre-, peri-, and post-mesenchymal condensation events. We designed three different seeding protocols, where in addition of seeding target densities, two other attempts to reach target densities were conducted. Thus, this approach allows us to control biophysical forces acting in the environment surrounding cells [7]. We hypothesized that cells seeded at low density (LD, 16,500 cells/cm^2) would have few physical contact with other cells, in an isolated environment whereas cells seeded a very high density (VHD, 86,500 cells/cm^2) were expected to almost reach a state of confluence, since this density represents the previous stage to the plateau phase of the growth curve of the C3H/10T1/2 cells [14]. In contrast, a situation in the middle of these two opposite conditions was expected to be displayed by cells seeded at high density (HD, 35,000 cells/cm^2), where cells would still have some space to proliferate besides having physical contact between them. Apart from this initial seeding protocol, the two other ways to achieve targeted densities were either seeding cells at a very low density (5000 cells/cm^2) and give them time enough to proliferate to the target densities, or seeding cells at half targeted densities and waiting 48 hours.

Following this methodology, we aim to study the behaviour that cells might display given different time frames to develop, according to their general environment and the adjoining physical conditions that cells are exposed to, depending on the distribution of cells for the different densities designed for every single seeding protocol.

2.2 Cell Culture and Seeding Protocols

The C3H/10T1/2 cell line was cultured using growth medium (Basal Medium Eagle supplemented with 10% fetal bovine serum, 1% L-Glutamine, and 1% Penicillin/Streptomycin [Invitrogen, Carlsbad CA]) and incubated at 37℃ and 5% CO_2 in a humidified incubator until passage 3 (P3). At this stage, several vials were frozen containing these cells in a specific freeze medium containing 40% fetal bovine serum and 10% DMSO at -80℃ as a reservoir of cells. Cells were cultured in a T75 flask and then seeded on a 33mm internal diameter dishes (FluoroDish™ Cell Culture Dish - 35mm, 23mm well) after the transduction. These dishes were selected considering their optimal properties for fluorescence microscopy.

Three different seeding protocols were followed according to prior optimized results. In the first protocol, the cells were seeded at the three target densities (LD, HD and VHD) and after waiting 24 hours to let the cells settle down into the dish, they were measured after 5, 4 and 3 days in culture respectively, so they could reach a confluent state. This would give us a general idea about how the physical interactions driven by a concrete seeding density, play a role in their growth while reaching confluence. In the second designed protocol, cells were seeded at a very low density (5000 cells/cm^2) so they could reach the three target densities after 3, 4 and 5 days in culture (LD, HD and VHD respectively). Finally to generate more consistent data and fully understand the cells' behaviour, a third protocol was conducted by seeding cells at half target densities, waiting 48 hours so they could reach the three target densities. In order to achieve these concrete densities, cells were seeded in the right amount by determining cell density using an haemocytometer prior to seeding into the imaging dishes.

All the three different protocols were conducted in both PDMS substrate and into the dishes with no substrate, so we could easily determine the main differences that a stiffness gradient might generate in the cell growth, while considering not only the developmental context of the cells in culture, but also different time frames as variables.

2.3 Compliance Substrate Preparation

By providing a suitable compliance substrate, we expect the cells to display different characteristics, approaching a more natural behaviour. Applying a compliance substrate represents a feasible solution to mimic the mechanical forces that might apply within the extracellular compartment of the cells, at least until more developed three-dimensional culture methods are optimized to be done successfully.

The PDMS (polydimethylsiloxane) substrate was chosen among others due to its physicochemical properties, being innocuous to the cell development and offering a great range of stiffness gradient according to its composition. PDMS was then mixed at a ratio of 1:10, degassed and 0.15-0.17 grams were used to coat the bottom of the imaging dishes. The PDMS was then cured for 16 hours at 50

degrees. In addition, for optimal accuracy, the dishes with the PDMS layer were sterilized with UV radiation prior to cell seeding for 30 minutes.

2.4 Cell Staining

In order to determine differences within intracellular changes that might occur during cellular growth following a specific seeding protocol, actin, tubulin and the nucleus of the cells were fluorescently labelled. Hoechst was used at a final concentration of 1 μ/ml and applied 60 minutes prior to imaging, to stain the cell nucleus, allowing us to determine both nucleus shape and volume. Actin was labelled using CellLight® Actin-GFP, whereas tubulin was stained applying CellLight® Tubulin-RFP, both of them are compounds that rely on a BacMam delivery system based on viral transduction. As a result, auto-fluorescent actin and tubulin were expressed displaying green and red colours respectively when they were excited with a suitable wavelength. Each of the CellLight reagents were applied at a concentration of 15 particles per cell, since it is recommended that the total amount of particles per cell should not exceed 40, thus we calculated the required volume of CellLight reagents we should use following a given formula by the manufacturer. As a measure of optimizing prior protocols, CellLight reagents were mixed with the required amount of culture medium beforehand seeding the cells.

Taking into account that these experiments rely on the study of precise seeding densities, it is important to assure that the transduction conducted does not alter the cell growth at all, since many of the groups were studied after several days in culture. Therefore we generated a growth curve comparing the growth of this cell line once transduced, with a control group. Cells were seeded in 6 well dishes at a very low density (5000 cells/cm^2) and then we counted one well every day for the next 6 days. For generating this growth curve, the cells were transduced using CellLight® GFP control at a concentration of 30 particles per cell, equivalent to the concentration applied to the cells in the three different protocols. The nucleus was not stained with Hoechst for generating this growth curve because it was applied right before every imaging session, therefore it couldn't have time to alter the cell. As we can see in the growth curve we obtained, the staining we are using for these experiments does not slow down the cell growth.

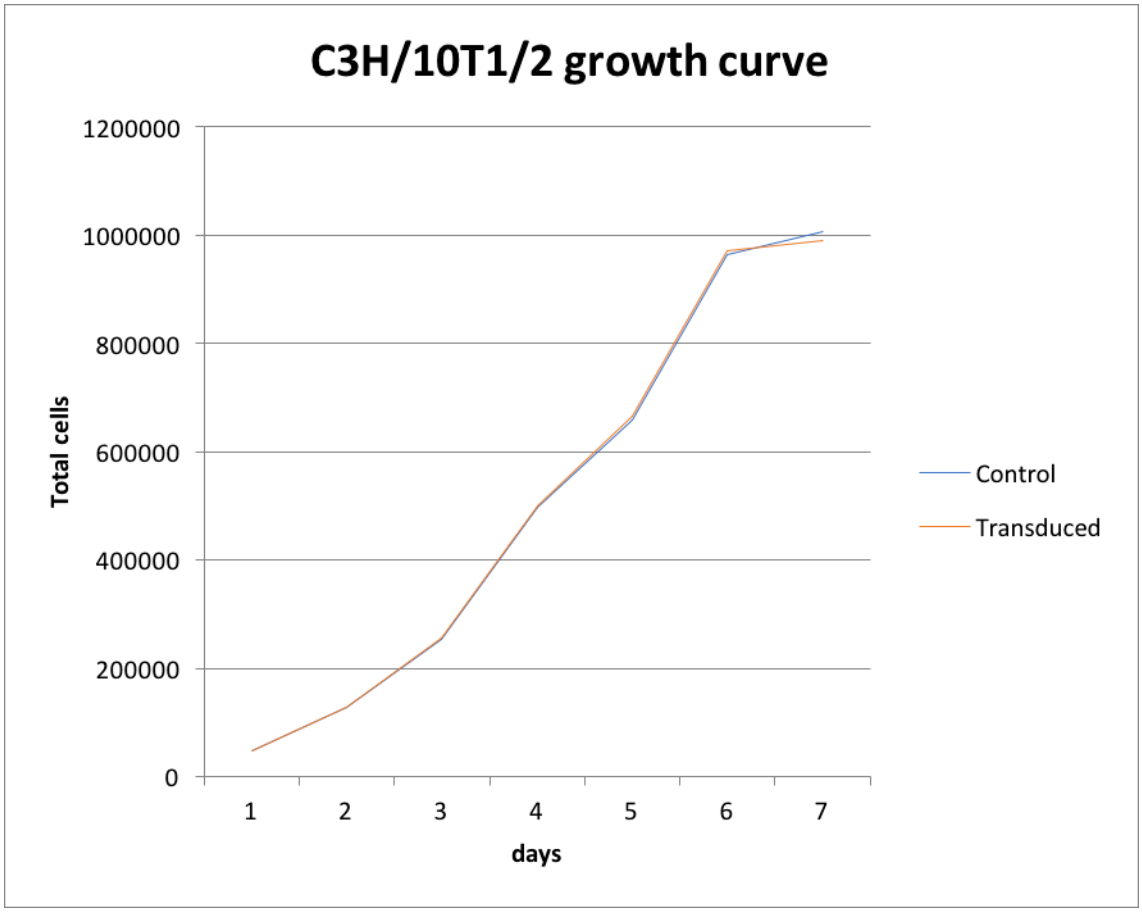

Figure 0. Growth curve of the C3H/10T1/2 cell line comparing a transduced group with a control. Cells were seeded at 5000 cells/cm^2 and further counted to confluence for the next 6 days.

2.5 Three-Dimensional Live Cell Imaging Analysis

All the imaging protocols were completed using a high-resolution confocal microscope (Zeiss LSM 880, BMIF, UNSW), where several random chosen fields of view were selected for each of the given dishes at 40x magnification. Three emission ranges were selected to collect information of every image in three different channels, green for the GFP transduced actin (499-553nm), red for the RFP transduced tubulin (409-476nm) and blue for the Hoechst in the nucleus (570-735nm). For both nucleus and cell measurements, at least three dishes were imaged for each seeding density and protocol combination. After rendering all the planar images obtained by a z-stack, general nucleus and cellular shape were reconstructed.

Once we obtained the images from the microscope, the data was analysed using Volocity 5.3.2 software (Improvision, Coventry, England). Total volume and surface area were the two main variables that were determined for both nucleus and cell measurements through intensity thresholds. The volume would give us a general idea about the size of the cells in the different developmental contexts, whereas the ratio of surface area to volume (SA/V) was used as a measurement of cell and nucleus shape [4]. Nevertheless, the value of this calculated ratio does not

equally represent the shape of all of the cells, since it depends of the volume of the cell, objects with the same shape but different volume would display a different ratio.

To solve this problem, the ratio of surface area to volume was normalized to the SA/V ratio of a sphere with the same volume. This new normalized ratio provides a better approach to figure out the real nucleus and cell shape, measuring the shape compared to a sphere, therefore a value of 1 would indicate a perfect sphere, whereas bigger values indicate more flattened objects.

2.6 Statistical and Correlation Analysis

We performed a statistical analysis to validate our results and conclude whether the differences in the shape of the cells were significant enough to make assumptions. We used both Excel (Microsoft) and JMP (SAS Institute Inc, Cary NC) to compare the data by generating a Wilcoxon rank sum test, considering that significant whenever the p-value was less than or equal to 0.05. In addition, standard error was calculated for all of the variables as the standard deviation divided by the square root of the number of samples, to assure the consistency in our data.

It had already been reported that seeding densities and the developmental context in which the cells were cultured, might have an effect in the expression of particular mesenchymal condensation markers, as well as markers of osteogenic, chondrogenic and adipogenic differentiation. In particular, the expression of *Msx2* and type I collagen (*ColIa*1) were measured, since they are makers of pre-mesenchymal and peri-mesenchymal condensation respectively [15,16]. Moreover, *Runx2* represents an osteogenic differentiation marker, and *Sox9* and type II collagen (*ColIIa1*) besides Aggrecan (AGC) had been evaluated as markers of early and later chondrogenic differentiation respectively. In addition, peroxisome proliferation activated receptor-γ2 (*Pparγ2*) was measured as marker of adipogenic differentiation [16]. Thus, a correlation analysis was previously conducted [3] in order to determine whether the expression levels of any of the mentioned seven genes was particularly relevant amongst specific values of cell and nucleus shape and volume, considering them as relevant whenever the calculated p-value was less than or equal to 0.05.

3. Results

3.1 C3H10T1/2 Growth Characteristics

The transduction of stem cells is one of the most relevant procedures to follow in these experiments, since this step would determine the capability to objectively measure and evaluate the development and behaviour of the cells under different conditions. Therefore, it is important to assure that any of the reagents applied to the cells in culture, have a significant influence on the cell growth. Our results (Figure 0) prove that cell proliferation was practically identical between

C3H/10T1/2 cells seeded at 5000 cells/cm^2 and an equivalent cohort transduced with the BacMam viral delivery system for live imaging of the cytoskeleton.

Our previous research already demonstrated that neither nucleus nor cell shape are influenced by the proliferation rate of these cells, determined by the actual cell density, but the growth process followed by a particular seeding protocol does make a difference in these parameters [3].

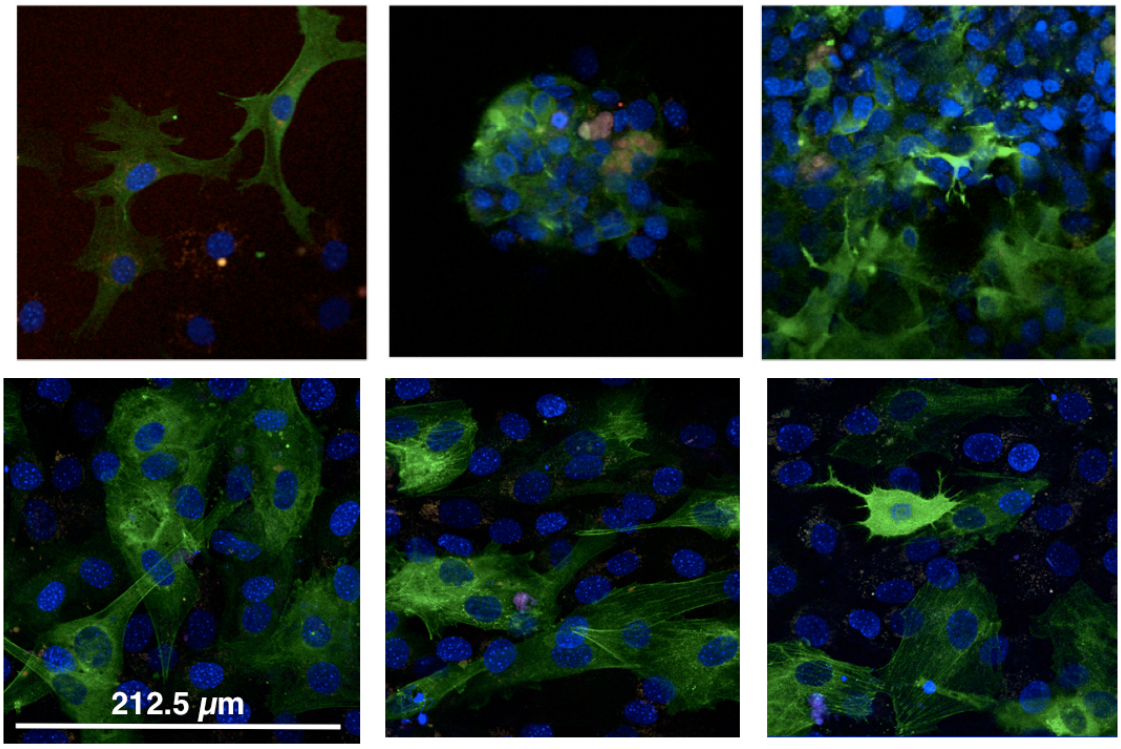

Figure 1. These three images were taken from the same dish at the same time, the cells were seeded at low density and cultured on PDMS (three at the top) and no substrate (three at the bottom) for 5 days until they reach confluency. The top pictures represent different stages of the aggregation process. In the first one, the cells modify their overall structure releasing cytoskeleton prolongations so they can make physical contact with neighbour cells. The second picture shows an early cluster of cells, whereas we can appreciate in the third one how cells have already reached a massive aggregation stage.

It is remarkable the way the development of the C3H10T1/2 cell line is influenced by the use of a compliance substrate, where cells appear to show a complete different behaviour (Figure 1) comparing to the use of no substrate. It is assumed that cells adopt a more three-dimensional disposition along the well with PDMS, and cells relying on this new substrate do not modify their proliferation rate. Nevertheless meanwhile cells seeded in dishes without PDMS tend to spread along the whole surface of the culture area, covering the biggest area possible, cells seeded on PDMS substrates display a migration phenomena. Apparently, every single cell within the PDMS substrate recognise the cells located in their surroundings and move towards each other, this is the first step that takes place during a cellular aggregation process that has been shown in all of the PDMS

dishes.

After generating several little cell sphere-like clusters along the culture dish, they keep on moving along the substrate to aggregate with other clusters. Thus, in the end after several days in culture, every single cell present in the culture dish has migrated towards the same point, where at this stage there is only one big cluster where all the cells are interacting with each other.

3.2 Cell Shape and Volume

The volume of the cells grown over a normal glass surface is initially determined by the densities in which cells are originally seeded (Figure 3A), where higher densities display smaller cell volumes. Interestingly, density no longer plays a role in cell volume whenever confluence has been reached. Cells still display smaller volumes when seeded in bigger densities in PDMS (Figure 3B), however it is noticeable how the volume of cells grown over the compliance substrate is far smaller at initial densities compared with the cell volume of the cells seeded on a glass surface. Regarding confluence, cells cultured on PDMS display bigger volumes at higher densities (at least at high and very high densities), just the opposite behaviour that these cells display when seeded on a normal substrate, where it seems like cells tend to get smaller as they grow. Therefore, in the confluent stage of cells cultured on the PDMS (Figure 3B), cell volume is manly determined by the time that cells have spent in culture, and the initial seeding densities do not have an influence on the volume of the cells, in none of the studied substrates.

According to our prior results, only the developmental context and the density conditions in which the cells are seeded regulates the cellular volume, meanwhile the cell shape is not modified, where higher seeding densities display smaller cells (Figure 3A). Thus, these volume changes are not determined by the initial seeding density, reaching similar volumes in confluence (Figure 3A). In addition, once their volume has been determined by any seeding protocol applied to them, they are able to retain that volume over time.

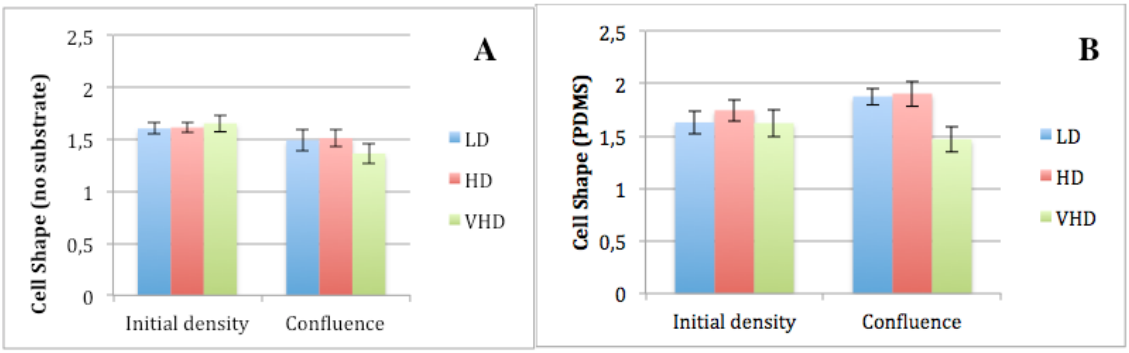

Figure 2. Comparison of cell shape at initial and confluent time points between normal substrate and PDMS compliance substrate. (A) The cell shape remains stable from initial stages to confluence in the absence of a compliance substrate.

(B) Cell shape slightly increases at confluence due to aggregation of cells into clusters.

Surprisingly, whenever we focus on shape changes, cells seeded on PDMS display a different developmental behaviour compared to the no substrate approach. The cell shape is neither influenced at all by the density conditions nor the followed seeding protocol, and all the three studied initial densities promote the same cell shape in both normal and PDMS substrates. However we can observe (Figure 2B) how cells tend to be flatter whenever they reach confluence in the PDMS, whereas in cells grown over a glass surface, only the very high density group modifies the cell shape towards a more spherical disposition at the confluent stage (Figure 2A).

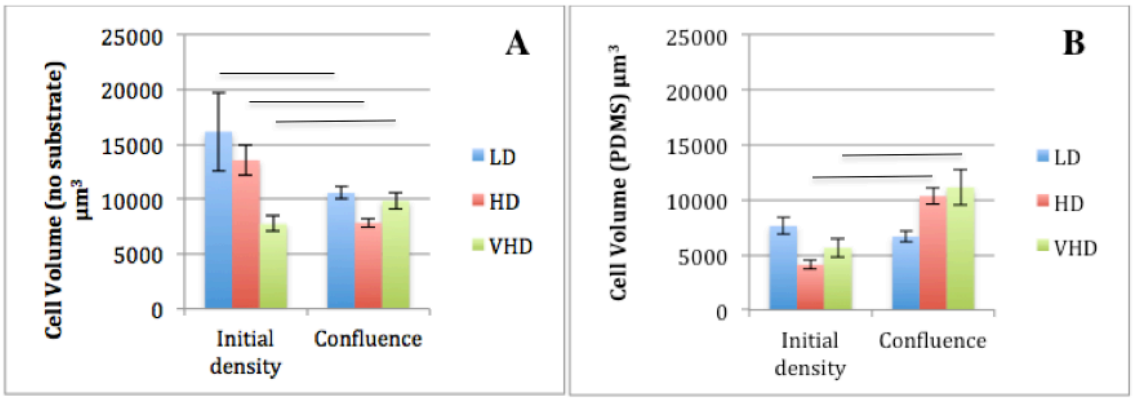

Figure 3. Comparison of cell volume at initial and confluent time points between normal substrate and PDMS. (A) The cell volume decreases with initial seeding density in normal substrate, but they reach a stable value once in a confluent stage. (B) The cells grown over the PDMS substrate display a more homogeneous disposition of the initial volumes, however in the confluent stage the bigger the density, the bigger the cell volume.

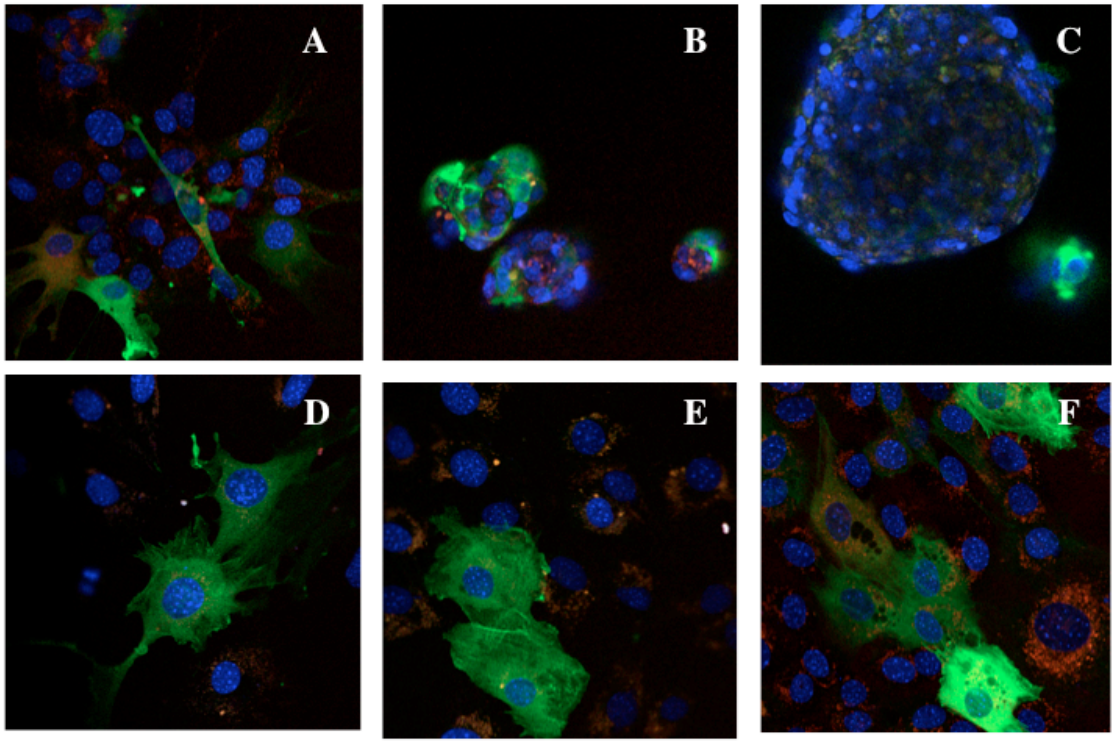

Figure 4. This image represents the protocol 3, where cells were seeded at half target densities and cultured for 2 days to reach the target densities. The 3 pictures above (A, B and C) are cells seeded at half LD, half HD and half VHD respectively in PDMS. Whereas the 3 last pictures (D, E and F) represent cells seeded at half LD, half HD and half VHD over normal substrate. In this particular experiment we can appreciate the effect the PDMS has over the cells in culture, all of the cells were in culture for the same amount of time (48 hours) and we can see how the aggregation process first takes place in the higher densities, whilst the cells seeded on normal substrate just tend to cover the whole area without aggregating.

3.3 Nucleus Shape and Volume

The study of the nucleus has shown how its shape is not influenced by the initial seeding densities (Figure 5A), but by the protocol followed in order to reach a particular density (Figure 8A) in cells seeded over no substrate. The nucleus shape seems to change towards a flatter disposition in cells grown over a glass surface as they evolve from the initial densities to the confluent stage (Figure 5A). Moreover, we can appreciate how at initial densities, the low density group display less spherical nucleus whereas at confluence is the very high density group the one that is flatter. However, whereas the nucleus shape remains stable in both initial and confluent stages (regardless the protocol followed to reach a particular density), cells that reached confluence do display smaller nucleus volumes and slightly more irregular shapes (Figure 5A) comparing to the initial stages. The PDMS also plays a major role in the nucleus shape, where flatter nucleus are found in bigger densities (Figure 5B), regardless the developmental context or protocol that cells were exposed to (Figure 8B). Interestingly, cells somehow tend to keep the initial nucleus shape even at a confluent stage, therefore the main driver that influences the nucleus shape is the density rather than the particular developmental context.

In addition there were no noticeable differences in nucleus volume among cells seeded in any of the stated densities in normal substrate (Figure 7A), whereas the developmental context to which the cells were exposed seem to have an effect in nucleus volume, where cells that grew to target densities display bigger volumes compared to those that grew to confluence. Nucleus features are also influenced by the use of PDMS as a compliance substrate. First of all, the nucleus volume appears to be slightly bigger (Figure 7B) in high and very high densities in cells who were seeded at half target densities and then proliferated to targeted densities (protocol 3), comparing to those that were seeded straight at target densities and then measured at confluence (protocol 1). Moreover, as it happens with cells seeded on normal substrates, confluent stages display smaller nucleus volumes comparing with the initial densities (Figure 6A), but not in the group initially seeded at high density. Surprisingly, the nucleus volume appears to be smaller in lower initial densities when cells are seeded over PDMS (Figure 6B), but it shrinks down as the cells grow to confluence in the higher density groups.

3.4 Actin and Tubulin expression

The expression of both actin and tubulin was assessed in order to determine major cytoskeletal changes according to a particular seeding protocol. The quantification of this parameter was considered as the fluorescence intensity normalized to the cell volume, to accurately appreciate the levels at which every single cell was expressing either GFP or RFP. Overall, the expression of actin was far more successful than tubulin (FigureA2), with minor differences regardless the cell density at which the cells were seeded.
In cells seeded over PDMS, there is a more equal expression of actin and tubulin, with slightly lower expression levels than cells seeded over no substrate.

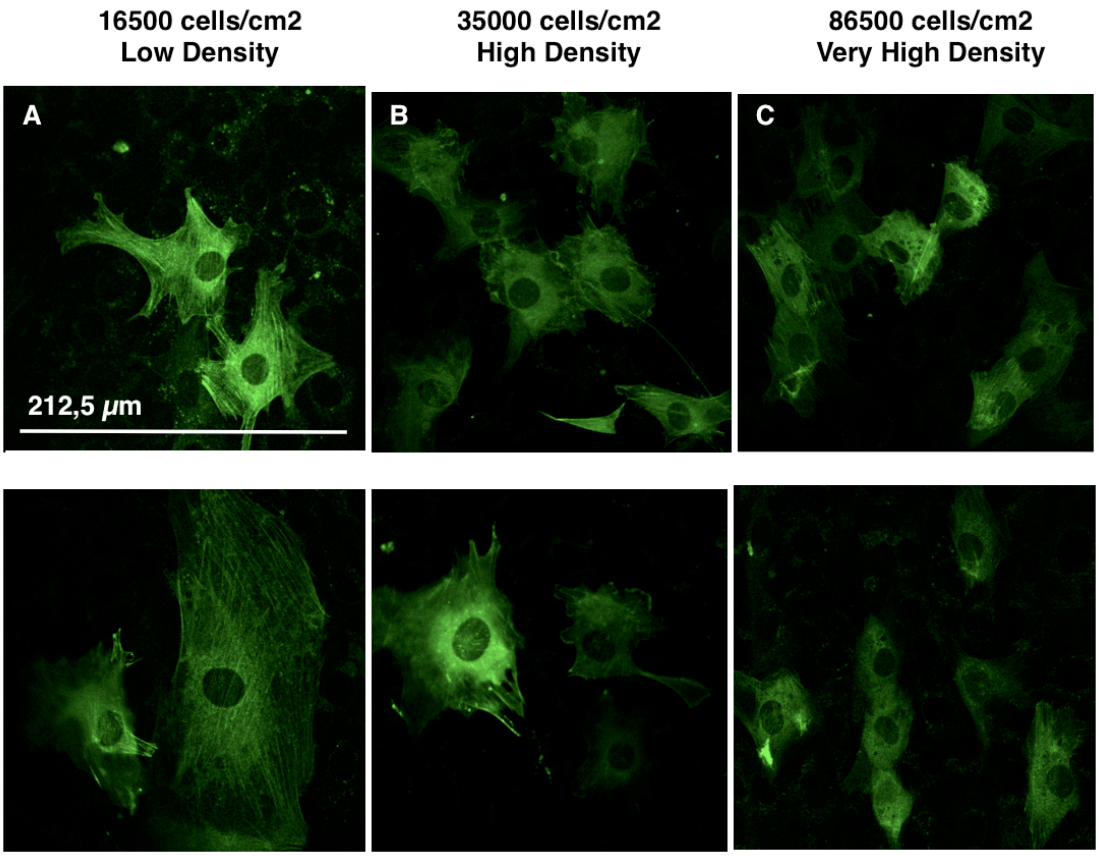

Figure A1. Seeding density influences cytoskeletal rearrangements. The pictures above represent actin expression along different seeding densities over no substrate. Cells seeded at low density (A), show more defined cytoskeletal structures tending to spread as much as possible, whereas high (B) and very high density (C) don't display such an organized actin filaments network.

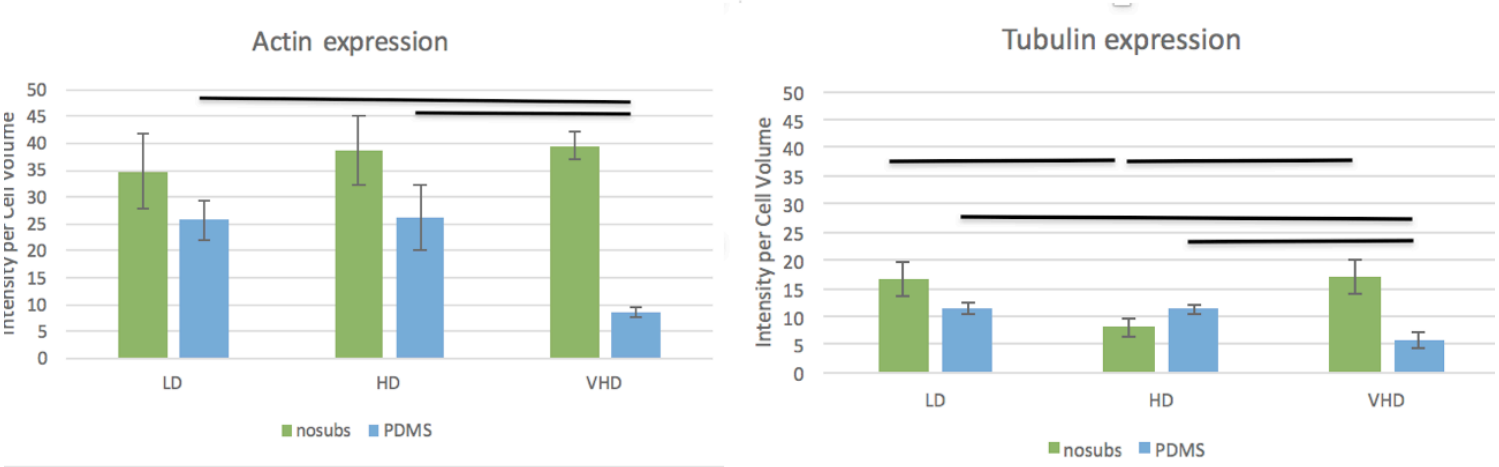

Figure A2. Comparison of actin and tubulin expression levels among different seeding densities, including measurements for both, cells seeded over no substrate and cells seeded on PDMS.

Interestingly, cells seeded at very high density show low expression levels of both actin and tubulin compared to other seeding densities, but only when the cells

were seeded on PDMS. When comparing actin and tubulin, apparently the cells display the same behaviour, showing very similar expression levels along different densities despite the very high density group in PDMS.

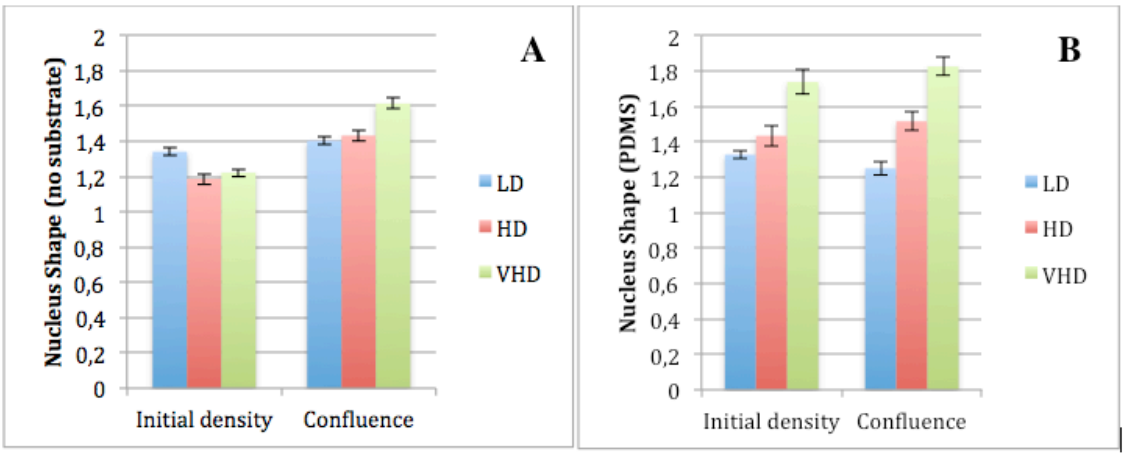

Figure 5. Comparison of nucleus shape at initial and confluence time points, between normal substrate and PDMS. (A) Initial seeding densities do not play a role in nucleus shape. In the confluence stage, only the VHD density seems to have flatter nucleus than the other two. Overall the nucleus shape is slightly flatter in the confluent state compared to the initial density. (B) The initial seeding density determines the nucleus shape, where cells keep their given nucleus shape until the confluent stage.

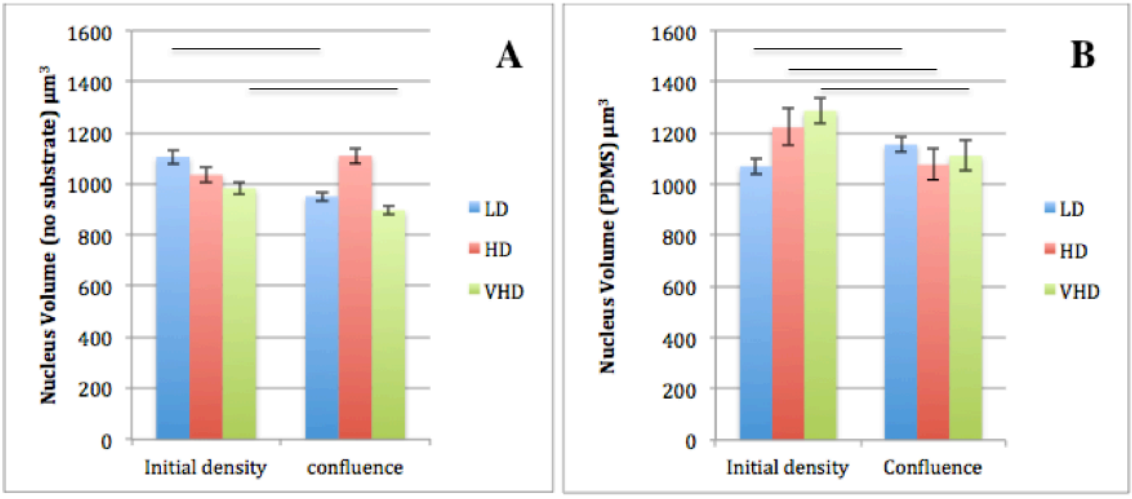

Figure 6. Comparison of nucleus volume at initial and confluence time points, between normal substrate and PDMS. (A) In cells seeded over no substrate, the nucleus is bigger in lower initial densities, shrinking down the volume as soon as they reach a confluent stage. (B) Cells seeded over PDMS display smaller nucleus in lower initial densities.

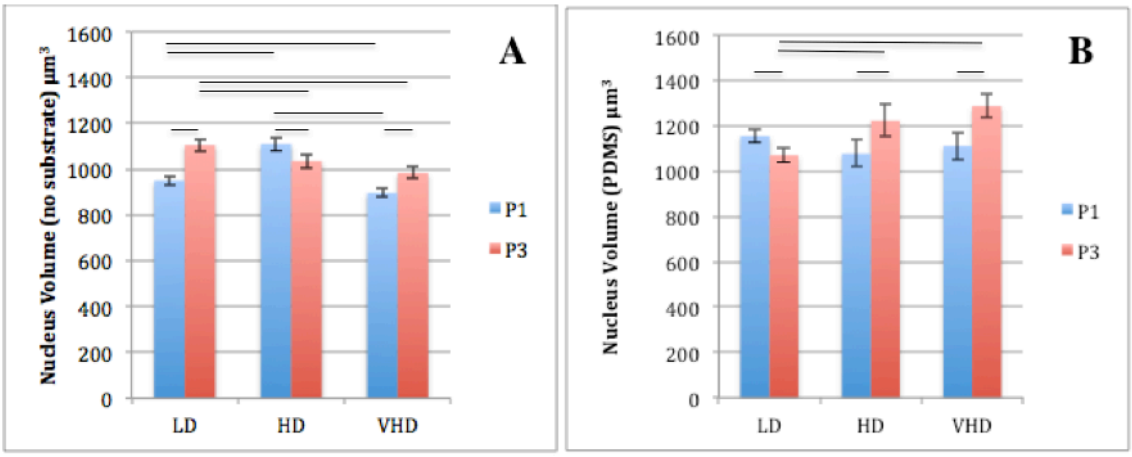

Figure 7. Comparison of nucleus volume at different seeding densities and different protocols. In protocol 1 (P1) cells were seeded at target densities and then cultured for 3, 4 and 5 days to reach a confluent stage. In protocol 3 (P3) cells were seeded at half target densities and then they reached target densities after 48 hours in culture. (A) Bigger nucleus volumes are displayed in cells that proliferated to target densities (at least LD and VHD). (B) In cells seeded over PDMS, bigger nucleus are present in cells that proliferated to density in the bigger densities.

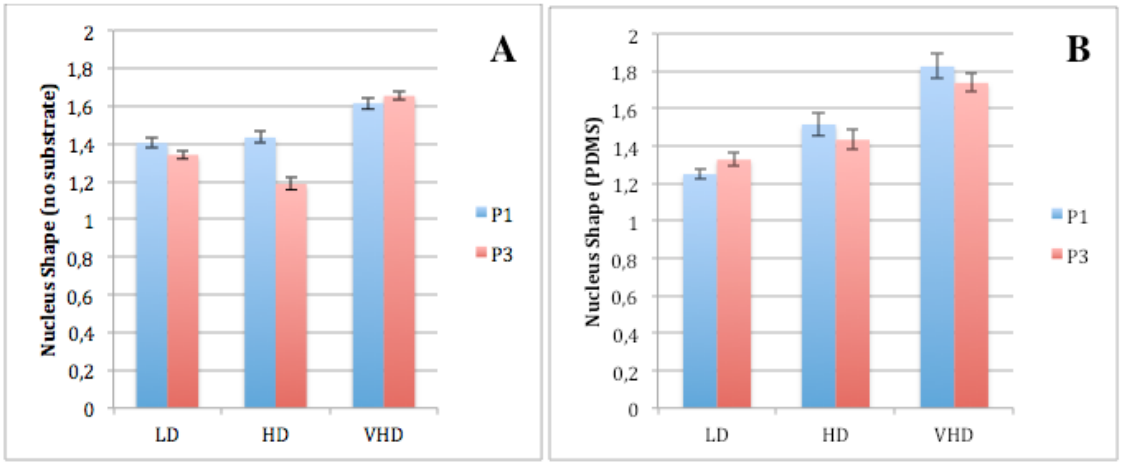

Figure 8. Comparison of nucleus shape at different seeding densities and different protocols. (A) Nucleus shape appears to be more irregular in very high density. (B) In cells cultured over PDMS, the bigger the seeding density, the flatter the nucleus is.

4. Discussion

The data showed in this article demonstrate that mechanical forces definitely play a major role regulating both nucleus and cell shape and volume. The study of biophysical strains that influence cell fate can be done by modulating not only seeding density, but also by optimizing concrete protocols and specially applying a compliance substrate. In addition, the observations made in these experiments reveal how important the features of a particular substrate are, and the potential consequences that the use of a compliance substrate such as PDMS have on the cells' fate and behaviour.

We previously conducted these experimental protocols over mesenchymal stem cells with no substrate, analysing several variables and determining how the nucleus and cell shape and volume were modulated along the different settings. Our previous studies employed fixated cells to determine both nucleus shape and volume, applying a solution of 3.7% formaldehyde as a previous step to imaging. This procedure generated big differences in the nucleus shape, where fixated cells had flatter nucleus and considerably lower volume [3]. This negative outcome of cell fixation is the leading cause that made us study these cells using live imaging protocols. Furthermore, the findings that came up with the use of a compliance substrate distant far from what we previously stated. The presence of a stiffness gradient generated by growing the cells over a bed layer of PDMS, somehow allows the cells to communicate between each other and definitively plays a major role in the behaviour that these stem cells adopt. It is assumed that the distribution of mechanical forces that apply to the cell's surface differs when growing the cells over a thin layer of PDMS. The biophysical forces that are present in a cell culture with no substrate, can only be provided by the very physical contact of the cells with their neighbours, limiting the mechanical forces that drive cells' fate to only cell to cell interactions. Nevertheless, when culturing the cells on a compliance substrate, the range of interactions that have an actual influence on cells is wider, not only there are inter-cellular interactions, but also the cells are able to disturb the surface tensions present at the compliance substrate as they grow over it. The disruption of these mechanical properties of the substrate can then be perceived by cells that are distant from other cells and thus, they can communicate with each other.

It has been shown in this study how these mesenchymal stem cells display the same behaviour of migration when they are grown on a PDMS substrate, surprisingly every single cell within the dishes undergo trough the same process since the moment they are seeded, until they reach a confluent stage. Whenever the cells are seeded into the substrate, they first adopt a more three-dimensional disposition, compared to the structure displayed by cells grown over no substrate (Figure 4A and 4D respectively). It seems like the cells located on the PDMS try to spread as most possible, elongating their filopodia so they can establish physical interactions with other cells present in the surroundings. Later on, the cells migrate towards the nearest cell present, starting a process of aggregation. Once several cells have congregated in a particular point, they start to grow and develop over each other, where they end up generating a globular-like cluster of cells. After

several days in culture, the cell clusters disrupt the surface of the surface at a sufficient level so they can be recognised by other clusters of cells, and thus at this stage, several clusters of cells aggregate to generate a single massive cellular structure. This behaviour could also be explained by the effect that an hydrophobic substrate would have over the cells, where the cells would tend to minimise the contact with the substrate, therefore maximizing the contact among cells. However, the substrate was not treated with any hydrophobic substance.

The influence that the use of PDMS have over living cells in culture, can be easily perceived by the changes in nuclear and cellular shape and volume. Whereas cell volume seem to decrease with increasing seeding density in cells cultured over no substrate, cells grown over a PDMS substrate seem to undergo big changes in their volume, as they adapt themselves through an aggregation process. Initially all the cells display a similar initial volume in the compliance substrate, and they tend to grow bigger as they develop, specially in the higher densities. This could be explained because in lower densities, the generation of cellular clusters takes more time and therefore, cells divide more often before they establish physical contact with other cells present in the surroundings. In contrast, higher densities show bigger cell volumes since the aggregation process happens close to the initial seeding. Cells that have spent longer time in culture (seeded at lower densities, so they required more time to reach the confluent stage) have smaller volumes, an adaptation that can be attributed to the previously mentioned process of migration and aggregation. Since cells tend to aggregate after several days in culture, it is not that important the fact that all of them have already reached the confluent density, but the time they have required to do so. (Even though the aggregation process has a higher pace in bigger densities (Figure 4)). Thus, all of the cells will end up shrinking their volume once they have spent enough days in culture to aggregate.

Considering cell shape, there are no significant differences between any of the seeding densities or the way the cells reach their density (either growing to target densities or seeding at target densities and waiting for confluence), but slightly less rounder cells are present near the confluent stage in cells cultured over PDMS (Figure 2B). Cells that grow over each other in the globular-like cluster, generated though the aggregation process that takes place in the compliance substrate, tend to be more irregular than those initial ones that were growing straight over the compliance substrate (Figure 2A), since cells seeded over a glass surface are more isolated and thus exposed to less compression forces (provided by neighbour cells in the aggregated mass of cells in the PDMS substrate).

Differences in nucleus does not seem as relevant as cellular changes, in the study field of mechanical forces, since they appear to have a major influence only over the external structure of the cell, rather than the inner components as the nucleus. Nevertheless, it has been proven that the volume of the nucleus can be modified by the particular developmental context applied to the cells, where smaller nucleus are shown in higher densities when the cells grew to the targeted densities, rather than when they reached confluence (Figure 7B). This can be explained due to the fact that, in an environment with more physical interactions, cells tend to

aggregate at a faster pace, so cells that were seeded at target densities have smaller nucleus volumes due to the fact that they have spent more time in culture and thus, they are already clustered after an aggregation process. One of the reasons that could explain the reason why smaller nucleus volumes are found in lower initial densities when cells are seeded over PDMS, is the fact that at the initial stages of the aggregation process, cells seeded at higher densities tend to form a cluster at a higher pace than those seeded at lower densities (Figure 4). Since cells seeded at lower densities need more time to adapt and form the cluster, they might divide faster and therefore display smaller nucleus. Another particular behaviour that cells grown over a compliance substrate have displayed, is reducing the volume as they reach confluence, but only in higher densities. This could be also related to the aggregation process, where in advance culture stages, the cluster of cells generated along the different densities would tend to generate equal nucleus sizes, regardless the initial density at which the cells were seeded.

The nucleus shape is not influenced by any of the initial seeding densities whenever cells are cultured over a glass surface, thus we can only appreciate an influence that the particular target densities have on the nucleus shape, at the confluence stage (Figure 5A). However we can actually see how in the PDMS the nucleus shape tends to be influenced by the seeding density from the very beginning (Figure 5B). This main difference could be explained due to the fact that in a compliance substrate, mechanical forces that might drive changes that occur in the nucleus shape could possibly be applied more efficiently to the cells, compared to those seeded over no substrate. Moreover, less spherical nucleus present in higher densities could simply be attributed to the stretching process that cells undergo along the aggregation phenomena.

It has also been proven that nucleus shape can be altered by applying a particular seeding density in cells grown over a PDMS substrate (Figure 8B), where higher densities display flatter nucleus. The main reason behind this fact, could be explained by the aggregation process, since cells are stacking over each other, less spherical nucleus are found in higher densities. Moreover, the seeding protocol also makes an influence on the nucleus shape, where growing cells to target densities seem to generate rounder nucleus at least in the lower densities from the cells grown over the glass surface (LD and HD in Figure 8A). This is because cells that have grown from target density to confluence (protocol 1), required more time to do so, and therefore close to confluence the mechanical forces provided by other cells are bigger, disrupting the initial rounder shape of the nucleus.

The study of cytoskeletal changes is essential to understand the cell behaviour under different conditions. By assessing actin and tubulin expression, we can have a deeper understanding of the changes that cells undergo in different processes. We can directly attribute cell shape with cytoskeletal organization, as we can see in Figure A1, in lower densities cells are more prone to have more complex actin networks, where cells tend to spread around the substrate. This behaviour is no longer shown in bigger densities, where cells don't have as much space to proliferate.

The process of aggregation also has an impact on the expression levels of both actin and tubulin (Figure A2), we can see how in very high densities on PDMS, there is a drastic reduction of the intensity levels. This could be explained due to the fact that once the process of aggregation has reached such an advanced stage (Figure 4C), cells might no longer rely as much in their individual structure, where the cytoskeleton network would rather promote the maintenance of the aggregated structure by enhancing cell adhesion.

It is therefore important to realize, that all the major observable differences present in both cell and nucleus, are essentially determined by the concrete stage of aggregation that takes place in the compliance substrate. Higher densities promote an early aggregation, nevertheless this process does not happen at the same pace along the same dishes (Figure 1). There are several aggregation points that are growing simultaneously, but not all of them have to be synchronised and display the same features, thus we can expect a higher variability in the nucleus and cells measurements compared to the data collected from the no substrate cultures. Further studies would require focusing on the particular study of this aggregation process, to accurately understand the differences shown in nucleus and cell shape.

Up to date, the elucidation of biochemical cues in the field of tissue engineering have been extensively determined, and once they were thought to be the most relevant focus of study. Nevertheless, the latest discoveries in the study of the biophysical forces that apply to cells, suggest that a mechanical approach might be the best solution towards the practical application of stem cells.

REFERENCES:

[1] Knothe Tate M.L., Gunning P., Sansalone V. (2016) *Bioarchitecture.*

[2] Engler, A. J., S. Sen, H. L. Sweeny, & Discher, D.E. (2006) *Cell* 126, 677- 689.

[3] Zimmermann JA, Knothe Tate ML. Structure - Function relationships in the stem cell's mechanical world A: Seeding protocols as a means to control shape and fate of live stem cells. MCB Molecular and Cellular Biomechanics. 2011;8(4):275-296.

[4] McBride, S. H., & Knothe Tate, M.L. (2008) *Tissue Eng*. A 14, 1561-1572.

[5] Falls,T.D.Beta-catenin's role in the mechanosensitivity of osteochondroproenitor cells. M.Sc. (Engineering) thesis. Biomedical Engineering, Case Western Reserve University, 2007. Document number: 18263005.

[6] KnotheTate,M.,T.Falls,S.K.Mishra,&Atit,R.In:Sivaloganathan,Siv(Ed.) New Perspectives in Mathematical Biology. Toronto: Fields Institute Communications, American Mathematical Society, pp. 113-134.

[7] McBride, S. H., & Knothe Tate, M.L. (2008) *Tissue Eng*. A 14, 1561-1572.

[8] McBride, S.H., T. F. Falls, & Knothe Tate, M.L. (2008) *Tissue Eng*. A 14, 1573-1580.

[9] Song, M.J., Dean, D., & Knothe Tate, M.L. (2010) *PLoS ONE* 5, e12796.

[10] Katagiri, T, A. Yamaguchi, T. Ikeda, S. Yoshiki, J. M. Wozney, V. Rosen, E.A. Wang, H. Tanaka, S. Omura, & Suda, T. (1990) *Biochem. Biophys. Res. Commun*. 172, 295-299.

[11] Singh, R., J. N. Artaza, W. E. Taylor, N.F. Gonzalez-Cadavid, & Bhasin, S. (2003) *Endocrinology* 144, 5081-5088.

[12] Taylor, S. M., & Jones, P.A. (1979) *Cell* 17, 771-779.

[13] Wang, H., G. M. Riha, S. Yan, M. Li, H. Chai, H. Yang, Q. Yao, & Chen, C. (2005) *Atertio. Thromb. Vasc. Biol*. 25, 1817-1823.

[14] McBeath R., D. M. Piron, C. M. Nelson, K. Bhadriraju, & Chen, C.S. (2004) *Dev. Cell* 6: 483-495.

[15] Ferguson, C., T. Miclau, D. Hu, E. Alpern, & Helms, J.A. (1998) *Ann NY Acad Sci*. 857, 33–42.

[16] Knothe Tate, M. L., T. D. Falls, S. H. McBride, R. Atit, & Knothe, U.R. (2008) *Int. J. Biochem. Cell Biol*. 40, 2720-2738.