Examining the Effect of Cell Culture Microenvironment on Stem Cell Shape and Developmental Potential

SIMS, CLIVE,HENRY,COLE

Durham University

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Clive Henry Cole Sims

Supervisor – Professor Stefan Przyborski

School of Biological and Biomedical Sciences

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Declaration

The work described herein was carried out in the School of Biological and Biomedical Sciences, Durham University between October 2013 and September 2014. All of the work is my own, except where specifically stated otherwise. No part has previously been submitted for a degree at this or any other university.

Statement of Copyright

Abstract

The cell culture microenvironment plays an important role in controlling cell shape and gene/protein expression. Conventional two dimensional (2D) cell culture provides an unnatural microenvironment for cell growth and causes cells to spread out thinly over the substrate. This change in cell shape leads to abnormal expression of genes compared with cells *in vivo* due to significant re-organisation of the cytoskeleton.

Recent developments in three dimensional (3D) cell culture allow *in vitro* cell culture to recreate similar 3D growth conditions a cell would be exposed to *in vivo*. 3D culture allows cells to maintain their more natural 3D shape which in turn leads to cells expressing more *in vivo* like levels of proteins compared to conventional 2D cell culture. This is a fundamental issue that influences the majority of cultured cells, including specialised cell types such as stem cells.

Stem cells are located within niches in the body. Stem cells grown *in vitro* are therefore far removed from their natural microenvironment. With the use of 3D cell culture, we can to recapitulate the *in vivo* niche and enhance the stem cell phenotype. By changing the shape of the cell using 3D cell culture, we alter protein expression to a more *in vivo* like state and consequently affect the developmental potential of stem cells. We show that culture of pluripotent stem cells in a 3D microenvironment leads to not only a cell shape change (acquiring a 3D morphology) but that this shape changes leads to an increased developmental potential compared with cells grown in conventional 2D culture. Our data also indicate that the protein ROCK may be involved both in the signalling process in all shape changes and in the maintenance of the new 3D phenotype.

Acknowledgements

I would like to thank many people who have helped me take my first steps into the academic world.

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Contents

1. Introduction

1.1. The relationship between cell shape and function

All cell types are different and all cells are polarised to a certain degree. Such cell polarisation is specific to the location of the cell within the body and within a tissue. For example, one edge of a cell may be in contact with a basement membrane whilst the opposite side may be in contact with stratified tissue, creating a polarised cell. Considering the way in which cells build together in tissues with each different cell type bringing its own structure and properties is vital when thinking about concepts as fundamental as cell shape and tissue structure. Cell structure and cell function are intrinsically linked – cell shape dictates cell function. The propensity of cell shape to influence cell function is evident when considering a two dimensional (2D) monolayer as an *in vitro* culture method. 2D culture creates an artificial microenvironment (defined as "local and systemic constituents surrounding a cell, including extracellular matrix (ECM), other cells, and soluble factors released locally" (Nelson and Bissell, 2006)) for cells as it causes the cells to flatten and spread out. This flattening out leads to a gene expression profile that is markedly different compared to cells *in vivo* (Birgersdotter *et al.*, 2005). This effect can be seen in Figure 1.1. as a cell detects its new 2D microenvironment and adapts by flattening.

The questions to be addressed in this thesis include: does modulating cell shape, by putting the cell into a three dimensional (3D) microenvironment, in which it has effective communication with neighbouring cells and a surrounding ECM, affect cell structure and functionality? Does this change make the cell more representative of its counterparts *in vivo*? And what is a potential signalling mechanism(s) involved in this shape change?

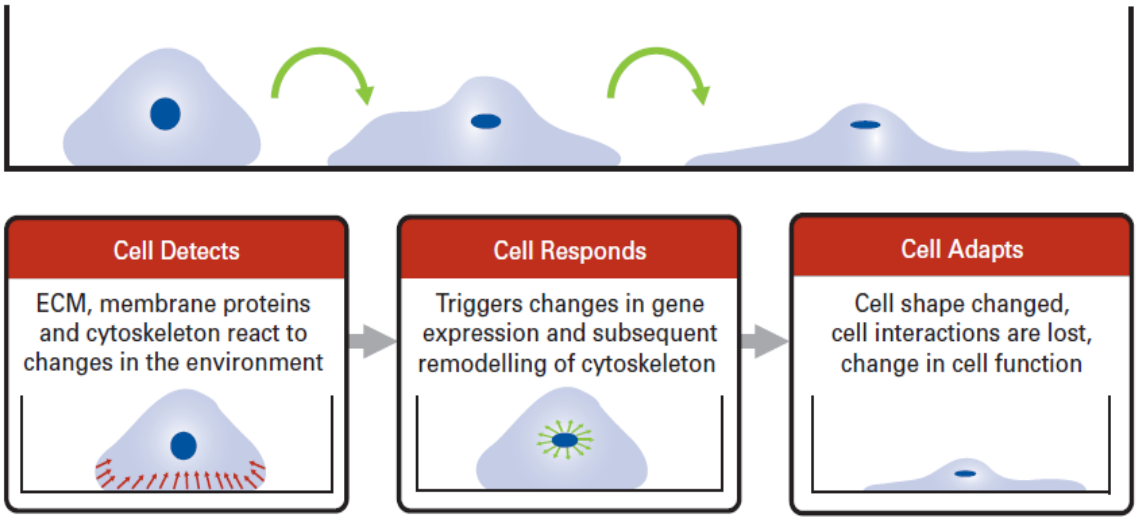


Figure 1.1. Cells flatten when responding to 2D substrates. *Cells react to the microenvironment through membrane bound proteins such as integrin which causes cytoskeletal structure changes and cell flattening. This, in turn, changes gene/protein expression of the cell and therefore cell function. This in vitro model changes cells substantially from their in vivo counterparts whereas 3D cell culture aims to reduce the difference between in vitro and in vivo cells. Image courtesy of Reinnverate Ltd.*

It has long been established that cell shape can affect cell growth and DNA synthesis in terms of anchorage of cells to a substrate (Folkman and Moscona, 1978). A seminal paper by Bissell and colleagues asked the question "How does the extracellular matrix direct gene expression?" (Bissell *et al.*, 1982). They proposed a 'dynamic reciprocity' between the ECM and the cell cytoskeleton meaning that changes in the ECM (or lack thereof) inherently alter the shape of a cell due to modified signalling with the cytoskeleton which goes on to affect gene expression (perhaps through changing the shape of the nucleus). This change in gene expression includes changes in the expression of cell adhesion molecules such as integrins in a more *in vivo* like arrangement in 3D culture microenvironments (Cukierman *et al.*, 2002). This review also states that there is considerable evidence that the expression of integrins and focal adhesion kinase – FAK – is fundamentally different between when 2D and 3D cultured cells signal with the ECM. Cell interaction with the ECM is proposed to affect gene expression and by extension, cell function.

Changes in cell shape leading to changes in cell function are demonstrated well in cells which take a specific shape *in vivo*. Hepatic carcinoma cells (HepG2 cell line) are a useful model for healthy hepatocyte cells as the cells display similar functionality and metabolism. *In vivo* hepatocytes take a cuboidal shape which is effectively mimicked by culture of these cells in 3D. It has been shown that

culture of HepG2 cells in 3D leads to an increased cell function and tolerance to toxicological insult compared with HepG2 cells cultured in conventional 2D culture (Bokhari *et al.*, 2007). This finding has important applications in pharmaceuticals and drug discovery as 3D cells seem to respond better to insult meaning certain drugs may not be as effective as first thought when only tested on 2D cultured cells. Correspondingly, a study found that only liver tissue spheroids grown in a hydrogel scaffold show diminished toxicity to nanoparticle insult (Lee *et al.*, 2009). 3D cell culture comes in many forms but all such technologies push cells to a more *in vivo* like state. Breast cancer cells cultured in a scaffold within stacks of paper have been shown to behave in a similar manner to tumours *in vivo* (Derda et al., 2009).

The culture of stem cells in 3D leads to many changes in the behaviour of the cell such as shape and potency. The composition of the 3D culture technology material itself can even help direct differentiation (Battista *et al.*, 2005) or ensure that stem cells remain potent and undifferentiated (Bajpai *et al.*, 2008). Clearly the underlying mechanisms that change stem cells which are grown in 3D are numerous and complex however the field is taking steps to identify target pathways.

Collectively, these examples support the argument for routine 3D cell culture as a more viable and relevant method of growing cells outside of the body – once robust protocols and practices have been established.

Cell shape has been shown to direct lineage commitment in human mesenchymal stem cells (hMSCs) through the interaction of RhoA-ROCK with the actin/myosin cytoskeleton (McBeath *et al.*, 2004). Rho is a family of small GTPases and RhoA is the activator of ROCK – RhoA associated protein kinase. Figure 1.2. shows how cell structure (shown by cytoskeletal arrangement) regulates gene expression and stem cell fate choice and how that then feeds back into the cytoskeleton as an output through tension. This is an example of how the architecture of a cell can fundamentally change cell function, to the point where the output is an altogether different cell type.

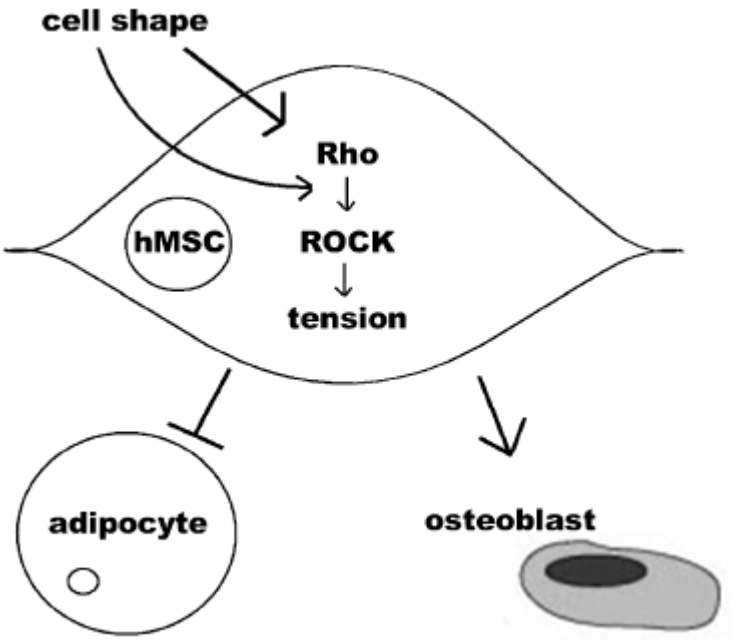


Figure 1.2. Changes in cell shape act via Rho and ROCK to influence stem cell fate choice. *This figure shows a potential molecular mechanism linking cell shape to stem cell fate choice via Rho/ROCK signalling. It shows that RhoA/ROCK signalling leads to creation of cytoskeletal tension, which initiates fate choice of human mesenchymal stem cells. Cell shape allows RhoA mediated stem cell fate choice while ROCK signalling actively creates osteoblast fates regardless of cell shape. Image adapted from McBeath et al., 2004.*

It was subsequently identified that Rac1 and N-cadherin (parts of a separate signalling pathway to RhoA/ROCK) expression are regulated by cell shape and those transcription factors in turn regulate a different fate choice of hMSCs (smooth muscle/chondrocyte) (Gao *et al.*, 2010). Evidently the mechanisms of cell shape determining cell fate choice in stem cells are multifactorial. The general field of research is now identifying target genes (and proteins) which may hold the key to understanding (and modulating) cell fate choice.

Cell structure and cytoskeletal interactions are further commented upon by Feng and colleagues in which the cytoskeleton is actively disrupted in mouse embryonic stem cells (ESCs), disrupting focal adhesion (FA) formation and causing cell rounding that leads to an adipocyte lineage choice (Feng *et al.*, 2010). This finding supports the results of McBeath and colleagues (2004) in that: cytoskeletal structure dictates cell shape which leads cell fate choice and that rounded cells tend to differentiate towards adipocytes. The above evidence feeds into the central theme that cell structure leads to cell function, which in this case is the determination of cell fate.

ROCK, as discussed, is thought to be involved in stem cell fate direction (through cell shape) however ROCK is part of a signalling cascade and feedback loop in which it is activated by RhoA. It

has similarly been presented that inhibition of ROCK (and so the negation of RhoA dependent activation of ROCK) causes a loss of cytoskeletal tension, loss of tension dependent behaviours (Kumar *et al.*, 2006) and cell flattening of human (h)ESCs in 2D (Emre *et al.*, 2010). Cell shape has recently been shown to influence pluripotency (specifically Oct4 expression) and the fate of hESCs by changing the nanotopography of substrate (Kong *et al.*, 2013). This modulation of cell fate is proposed to be due to the presence of fewer FAs with the substrate leading to increased pluripotency. This link between changes in the structure of the cytoskeleton and changes in pluripotency could have implications in clinical use of stem cells but also shows a correlative relationship between cytoskeleton structure and cell developmental potential. A recent paper hypothesised that ROCK is linked to the cytoskeleton via the pathway shown in Figure 1.3 (Seo *et al.*, 2011). Topography is the distribution of features of a surface, for example gratings or crevasses, and this study suggested that FA activation was caused by cells reacting to substrate topography via the ROCK signalling pathway and that cells grown without a topography (i.e. on flat conventional 2D culture substrates) lack the activation of this pathway.

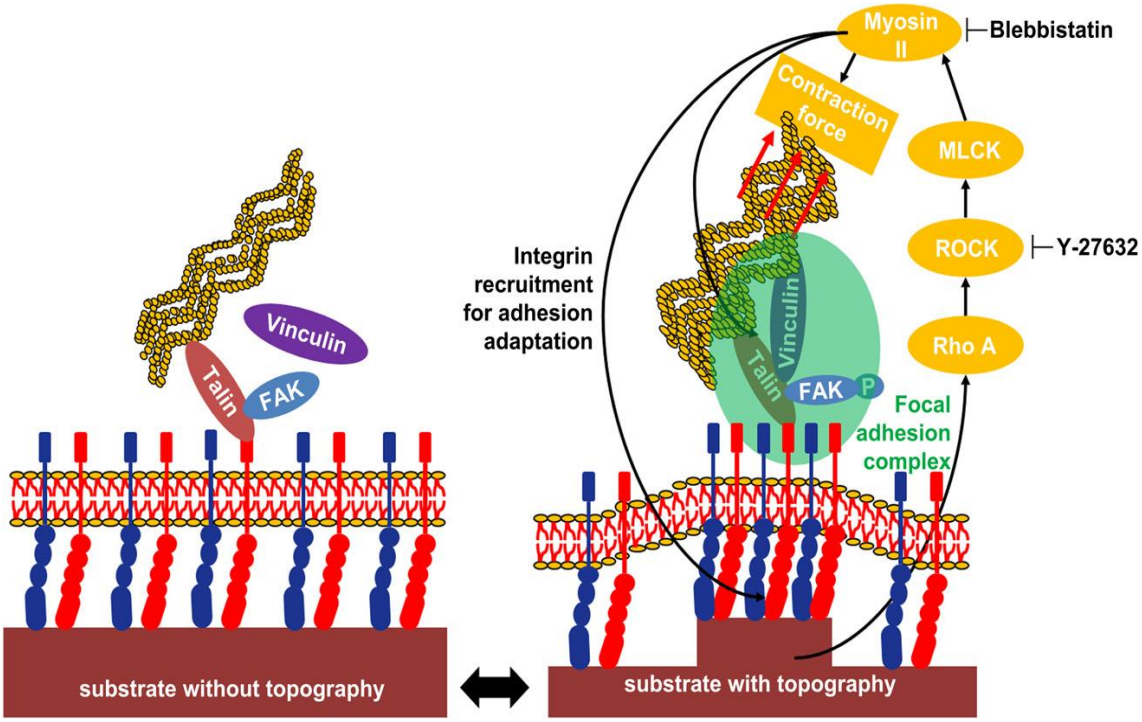


Figure 1.3. An action of ROCK as part of an ECM sensory signalling cascade in mechanotransduction. *This figure shows that when cells are grown on a substrate that has topography, i.e. is not flat and smooth, there is a change in FAs which causes a signalling cascade resulting in a change in the cytoskeleton and so the shape of the cell via changes to precursory cytoskeletal elements such as myosin. ROCK is involved in the recruitment and phosphorylation of FAK when cells are grown on surfaces with topography so when it is inhibited using Y-27632, FAK phosphorylation, actin organisation and FA formation were all significantly reduced. (MLCK – myosin light chain kinase, Blebbistatin is a myosin inhibitor). Image from (Seo et al., 2011).*

1.2. Cell culture and the growth environment

The vast majority of research currently carried out worldwide is carried out in 2D culture with cells either being grown on flat glass or plastic surfaces. 3D cell culture provides a more relevant alternative yet the idea is not a novel one and has been around for over 40 years – Elsdale and Bard showed that a collagen gel in which fibroblasts are grown causes the cells to attain a more *in vivo* like phenotype (Elsdale and Bard, 1972). Not only does the individual cell shape change but the associations between cells in 2D do not resemble tissues *in vivo*. This is a problem that 3D cell culture attempts to address by encouraging more cell-cell contact and cell adhesions. This can occur from all orientations which are not limited to the edge of cells as it is within a monolayer and is more analogous to *in vivo* cell interactions in terms of adhesion protein expression (Cukierman *et al.*, 2001). On a similar note to cell-cell contact, the role that the ECM plays and how cells interact with that ECM *in vivo* must be considered when designing and thinking about the viability of 3D models *in vitro.*

All stem cells exist in niches, from the inner cell mass from which hESCs are derived (Thomson *et al.*, 1998) to the crypt in the gut which contains intestinal adult epithelial stem cells (Barker *et al.*, 2007). Niches are a combination of the surroundings that stem cells find themselves in and the fact that there are many stem cells together in one place interacting with each other. Each stem cell niche will have a specific combination of ECM components (created by the surroundings and the stem cells secreting their own), paracrine factors (intercellular signalling) and a defined rigidity (provided by the surroundings) which is best suited for the propagation and maintenance of the stem cells within this niche (Scadden, 2006). 3D cell culture attempts to recapitulate the niche in which stem cells exist *in vivo* as much as possible. Any changes in ECM, paracrine or rigidity however may cause changes in differentiation potential, maintenance of pluripotency or apoptosis (Brizzi *et al.*, 2012). Intracellular signalling involving cell surface receptors reacting to the microenvironment primarily through integrins lead to gene expression profiles that are vastly different between 2D and 3D cultured cells (Bissell *et al.*, 2003). The main point that 3D cell culture is trying to address is that the microenvironment in which a cell exists is just as important as the genetic makeup of the cell for determining cell functionality (Weaver *et al.*, 1997). Utilising 3D cell culture is argued to be so vital to effective *in vitro* modelling that the same effects of a process may not be seen in 2D. This is a problem for much experimental data and biological hypotheses based on 2D models. If 3D models are taken as a more accurate model of representing biological processes

then certain conclusions may have previously been incorrectly drawn from 2D data. Furthermore, if 3D cell culture can be fully standardised and incorporated into everyday research, we could see more effective drug screening and a reduction in the number of animals used for toxicity assays.

1.3. Stem cells and 3D cell culture

Pluripotent stem cells are self-renewing cells with the developmental potential to produce all somatic cells in adult tissues. hESCs were first isolated from the inner cell mass of the developing embryo over 15 years ago (Thomson *et al.*, 1998) and since then have been used to drive significant advances in the fields of regenerative medicine, stem cell science and bioethics. The TERA2 cell line (Andrews *et al.*, 1984) is an embryonal carcinoma (EC) cell line and the malignant counterpart to the hESC derived from the pluripotent core of malignant teratocarcinoma tumours. Whilst being unsuitable for regenerative medicine and certain genetic comparisons (due to an irregular haplotype), EC cells serve as a useful model for human embryonic development due to their ability to differentiate in a similar manner to hESCs (i.e. the cells are similarly pluripotent) but without the ethical and legal restraints on use (Przyborski *et al.*, 2004).

The gold standard *in vi*vo assay for testing cell potency (i.e. whether a cell is pluripotent) and the one primarily used in this project is teratoma formation, an assay which attempts to reproduce aspects of normal embryonic development *in vivo*. Teratoma analysis however must not be taken too far. Teratoma formation from injecting pluripotent cells into immunodeficient mice to grow in 3D *in vivo* is an effective way of determining whether or not a cell population possesses the developmental potential to differentiate into cells from all three germ layers. Teratomas created from hESCs represent the differentiation in a 3D microenvironment and proliferation of individual cells (in communication with neighbouring cells and host tissue) rather than mimicking embryonic development *in utero* i.e. each cell differentiates and proliferates individually rather than there being a proliferative focus of the tumour (teratomas can however contain undifferentiated cells due to inefficient differentiation of cells) (Blum and Benvenisty, 2007). Quantitative over analysis may lead to assumptions being made about the developmental potential of two different populations based on inadequate criteria. Although it has been seen that the graft site of cells can hugely affect teratoma composition (Cooke *et al.*, 2006).

Additionally, the use of teratomas as an assay comes with a tangible cost of life of experimental animals. Research is continuing into finding an *in vitro* model which is equally as effective an assay through advances in tissue engineering, cell technology and bioinformatics. Until such an assay is developed, teratomas assays (and the animals which are involved in the studies) must be used but be used sparingly and with a standardised protocol which follows the 3Rs (such as that proposed by Gropp and colleagues (Gropp *et al.*, 2012)) for effective and comparable results – as reviewed by Buta and colleagues (Buta *et al.*, 2013).

Another method of differentiating stem cells in 3D involves their growth as embryoid bodies (EBs) which have been around for almost as long as isolated hESCs themselves (Itskovitz-Eldor *et al.*, 2000). EC cells can also form EBs in 3D cell culture however it was determined that not all the cells in these aggregates maintain the pluripotent stem cell phenotype indicating differentiation occurs within the EB (Martin and Evans, 1975a, 1975b). It was soon seen that cell maintenance using the EB method would not lead to major discoveries due to spontaneous differentiation (Levenberg *et al.*, 2003) and so many groups now use EB formation as a method of differentiation. A problem with using EBs for differentiation is a lack of control during the process and a random collection of end cell types. This heterogeneity could in fact be useful in allowing EBs to become a new *in vitro* pluripotency assay to replace teratoma formation (EBs can show evidence of all three germ layers (Itskovitz-Eldor *et al.*, 2000)). In general, differentiation in 3D leads to more extensive development. One of the primary problems with using EB formation as a method of stem cell differentiation into various cell types is the formation of necrotic/cystic cores, particularly following epithelial differentiation of outer cells of aggregates. Equally however, this process can be seen as merely following the correct, inherent developmental pathway of groups of hESCs as would happen *in utero* (Coucouvanis and Martin, 1995).

Maintaining stem cells in the undifferentiated state is a key requirement for the use of 3D cell culture. It has been seen that human pluripotent stem cells (ES and EC) can be grown in 3D and either maintain an undifferentiated phenotype (Knight *et al.*, 2011) or be encouraged to differentiate further than would occur in the absence of 3D culture (Baharvand *et al.*, 2006). The maintenance of an undifferentiated phenotype in 3D is something which tends to occur when stem cells are together within a stem cell niche. Signalling within the niche, be it from surrounding

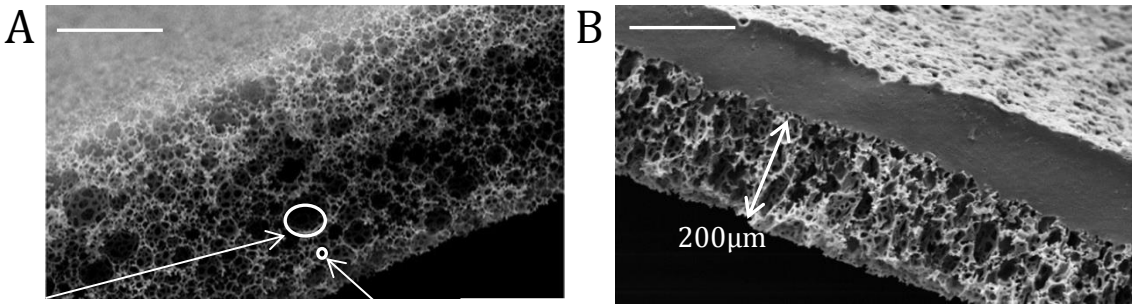
support cells or between stem cells themselves, is complex. Wnt signalling has been highlighted as an important regulator in both mouse and human stem cell niches (Nusse, 2008) as has GDF3, secreted by $Oct4^{+}$ hESCs, which supresses Smad1 and promotes maintenance of undifferentiated hESCs (Peerani *et al.*, 2007). Stem cells being in physical contact with each other has been shown to influence cell fate choice of hESCs between embryonic and trophoblastic lineages (Yu *et al.*, 2008), cell proliferation rate in human EC cells (Fox *et al.*, 2008) and the decision whether or not to differentiate at all by way of the Notch signalling pathway (Lewis, 1998). Clearly, in 3D, these interactions between cells are dramatically increased and so make for a microenvironment more similar to that which would occur *in vivo* in almost all cell types. One type of substrate which would force cells to interact in this way could improve the quality of results in all fields using cell culture.

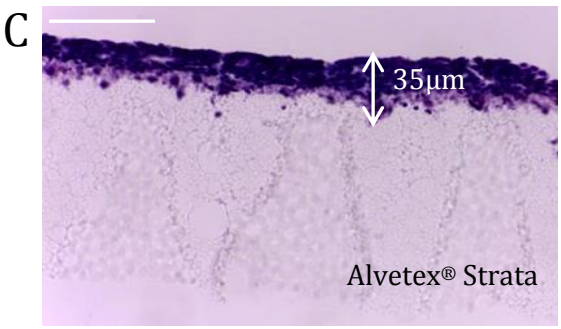
One of the most robust *in vitro* models of stem cell development in 3D involves using scaffolds and/or gels. Stem cells are influenced by the scaffold composition of these 3D models which have certain properties such as rigidity and porosity etc. Changes in these properties (e.g. by moving a cell from conventional 2D culture into a 3D scaffold or from the *in vivo* niche into 3D culture) can direct stem cells to differentiate down a particular pathway (e.g. culturing ESCs on a stiff substrate directs cells to osteogenic termini (Evans *et al.*, 2009)). The reasoning for this direction is that substrate properties directly influence cell shape/cytoskeleton. Engler and colleagues comment on the involvement of nonmuscle myosin II (as seen in Figure 1.3.) reacting to substrate rigidity in hMSC differentiation direction (Engler *et al.*, 2006). More generally, it has recently been shown that regardless of the make-up of the scaffold on/in which stem cells are grown, the gene expression and cell shape are decidedly different compared with cells grown on traditional 2D substrates with 3D cultured cells possessing both higher pluripotency marker expression and greater developmental potential than cells cultured in conventional 2D (Wei *et al.*, 2014). The eventual goal of 3D cell culture is to fully mimic the *in vivo* situation and the recent leaps forward in *in vitro* organogenesis could yield tangible results in the near future (Sasai, 2013).

1.4. Cell growth on Alvetex® Strata

Alvetex® Strata is a commercially available, non-biodegradable polymer membrane. The membrane comes as an insert which has very consistent voids and interconnects and is highly porous allowing

media diffusion throughout the entire cross section. The membrane and insert are made from chemically inert polystyrene (as tissue culture plastic is) and provides a physical topography on its surface. Cells grow on the surface of the membrane with no extra chemical interference from the growth substrate. This facilitates the ability to study the direct physical effect of the third dimension on stem cell growth.





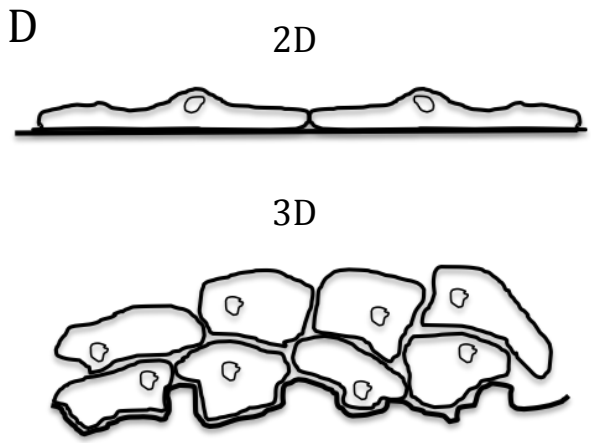


Figure 1.4. Cells grow on top of Alvetex® Strata as a thick layer of tissue. *(A): SEM of Alvetex® Strata with average void and pore sizes; (B): SEM of Alvetex® Strata with a layer of HepG2 cells growing on top; (C): H&E stain showing TERA2.cl.SP12 cells growing on top of the membrane with a thickness of 35µm. Data above show that cells grow as a thicker layer of tissue on top of Alvetex® Strata; (D): Drawing illustrating the differences in cell shape between conventional 2D cell culture and 3D cell culture on Alvetex® Strata. The pores of Alvetex® Strata are smaller than the average diameter of TERA2.cl.SP12 cells but large enough for media to penetrate as the membranes are submerged allowing cells access to media from above and below. Surface topography of Alvetex® Strata as seen in (D) facilitates cells to grow on top of each other as a thick layer as cells are unable to flatten. Scale bars - 200µm.*

As can be seen in Figure 1.4., cells grow as a thick layer of tissue like material on the surface of Alvetex® Strata membrane but in contact with media from above and below (from diffusion through the membrane) allowing all cells on the membrane to grow in 3D, not simply the cells on the top or bottom. Maintaining cells on Alvetex® Strata leads to the cells possessing a 3D phenotype. Cells cultured on Alvetex® Strata are easily accessible and are simply scraped off of the membrane surface during passaging and reseeded onto a fresh membrane (as described in the Methods Section).

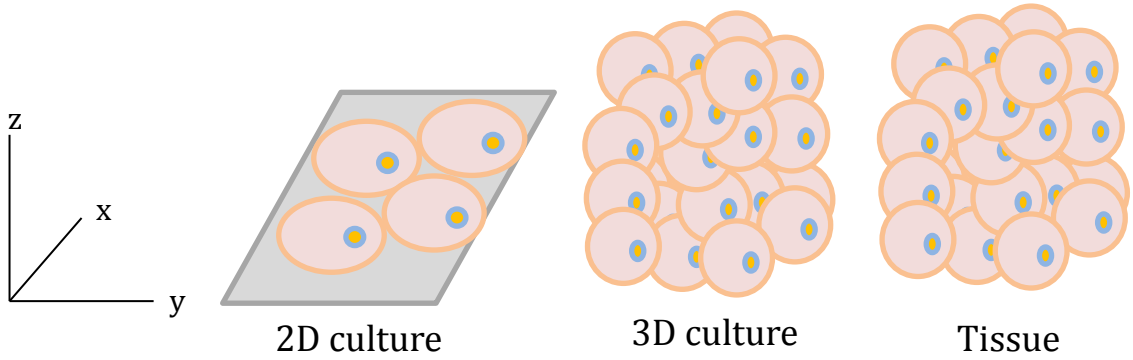


Figure 1.5. Cells grow on top of each other at all orientations in 3D. *Drawings are a representation of the three dimensions (x, y, z) and how cells are arranged in those dimensions in 2D and 3D cell culture. Cells grown in 2D cell culture flatten out and so have a small z-axis profile however cells arranged in 3D have a more spherical shape and a larger z-axis profile. Cells within tissues are arranged in three dimensions and 3D cell culture technology attempts to mimic this feature.*

The pluripotent stem cell line used in this project is the TERA2.cl.SP12 lineage derived through immunomagnetic sorting and single cell selection of SSEA-3^{+} cells (Przyborski, 2001). In traditional 2D culture, TERA2.cl.SP12 cells flatten and grow to confluency as a monolayer. In contrast, when grown on Alvetex® Strata, the cells retain a spherical 3D phenotype as the cells would possess *in vivo*. Continual propagation of this pluripotent stem cell line represents the first easily reproducible 3D long term culture of undifferentiated stem cells without need for other factors or treatments different from classical 2D cell culture.

1.5. Aims/Objectives

It is hypothesised that maintaining stem cells in a 3D microenvironment alters their phenotype and developmental potential. It is further hypothesised that the acquisition and maintenance of this 3D morphology may involve ROCK signalling.

The aims of this study are to:

- Examine the effect of cell culture microenvironment on stem cell shape and how that affects developmental potential.

- Investigate the signalling mechanisms involved when cells change shape (specifically investigating the role of ROCK).

These aims are going to be examined by the following objectives:

1) Maintain long term growth of TERA2.cl.SP12 embryonal carcinoma stem cells *in vitro* as a 2D monolayer and in 3D on Alvetex® Strata.
2) Measure differences in cell proliferation rates of 2D and 3D cell cultures.
3) Quantitatively examine stem cell surface markers to look for differences between 2D and 3D culture methods.
4) Test developmental potential of cells by induction of differentiation *in vitro* using retinoids, or by their growth as teratomas *in vivo*.
5) Compare composition/form/size of teratomas created from 2D and 3D cultured cells.
6) Investigate the underlying molecular mechanisms involved in stem cell shape change controlled by cellular microenvironment.

2. Methods

2.1. Cell culture

2.1.1. Culturing TERA2.cl.SP12 stem cells in conventional 2D culture

TERA2.cl.SP12 is a cell line isolated from TERA2 cells as previously defined (Przyborski, 2001). Cells were brought up from frozen at -140°C by rapidly defrosting in a 37°C water bath and transferring the cells into 9ml of pre-warmed Dulbecco's modified Eagle's medium (DMEM, Lonza) (with 10% heat treated FBS (Gibco, Invitrogen), 1.1ml of penicillin/streptomycin (Lonza) and 5ml of 200mM L-Glutamine (Lonza)) in a 15ml Falcon tube (Sarstedt) within a Class II laminar flowhood. Cells were centrifuged at 1000rpm for 3 minutes and media was aspirated off leaving the cell pellet which was then resuspended in 10ml of media and put in a T25 flask (BD Bioscience). Cells were then transferred to a T75 flask (BD Bioscience) once confluent. Cells were maintained in a humidified 37°C incubator in 5% CO_2.

2.1.2. Passaging TERA2.cl.SP12 stem cells in conventional 2D culture

Cells were passaged once confluent by aspirating off cell culture medium and adding 6ml of warm, fresh DMEM to the T75 flask. Acid washed glass beads (prepared by soaking in concentrated HCl overnight, washing thoroughly with de-ionised water 3 times and then left to dry in a drying oven before being autoclaved) were then added to the flask and rolled over the surface of the plastic to displace the cells. Large clumps were broken up by pipetting and 2ml of cell suspension was added to each new T75 flask (ratio 1:3 split). Each T75 flask was then topped up with 18ml of media.

2.1.3. Culturing of TERA2.cl.SP12 stem cells in 3D on Alvetex® Strata

Alvetex® Strata (Reinnverate Ltd.) was hydrated using a 70% ethanol wash then washed in PBS. Inserts were placed in a 6 well plate (Greiner) and 8ml of DMEM was added. As shown in Figure 2.1., 500µl of media containing 0.5 million cells (determined using a haemocytometer on trypsinised cells and Trypan Blue assay (Sigma)) was added to the membrane of the insert using the dispersed seeding method (Figure 2.1. Step 1.). The plate was then incubated for 24 hours at 37°C in 5% CO_2 to allow initial cell attachment. 1.5ml of media was then added to the insert to connect the two sources of media.

2.1.4. Passaging of TERA2.cl.SP12 stem cells in 3D on Alvetex® Strata

After 4 days without a media change, Alvetex® Strata membranes were carefully removed from the well inserts and washed in PBS before being placed in a new 6-well plate. 2ml of 0.25% Trypsin EDTA (Sigma) was added to each membrane and the plate was incubated for 7 minutes at 37°C in 5% CO_2 on an orbital shaker at 60rpm (Figure 2.1. Step 2.). The cells were then gently scraped off the membrane using a cell scraper (Sarstedt, flexible 2-position blade. Cat no: 83.1832, blade – 1.35cm, total length – 16cm) from the centre, outwards (Figure 2.1., Step 3). 3ml of fresh, warm DMEM was then added to each well to neutralise the trypsin. The cell suspensions were next combined in a 50ml Falcon tube (Sarstedt) having been filtered using a 100µm nylon sieve (BD Bioscience) to remove any membrane fragments. The tube was then centrifuged at 1000rpm for 3 minutes before being resuspended in 10ml of media. Cells were then counted using a haemocytometer and Trypan Blue assay and reseeded onto fresh Alvetex® Strata membrane set up as described before (Section 2.1.3. and Figure 2.1. step 3.).

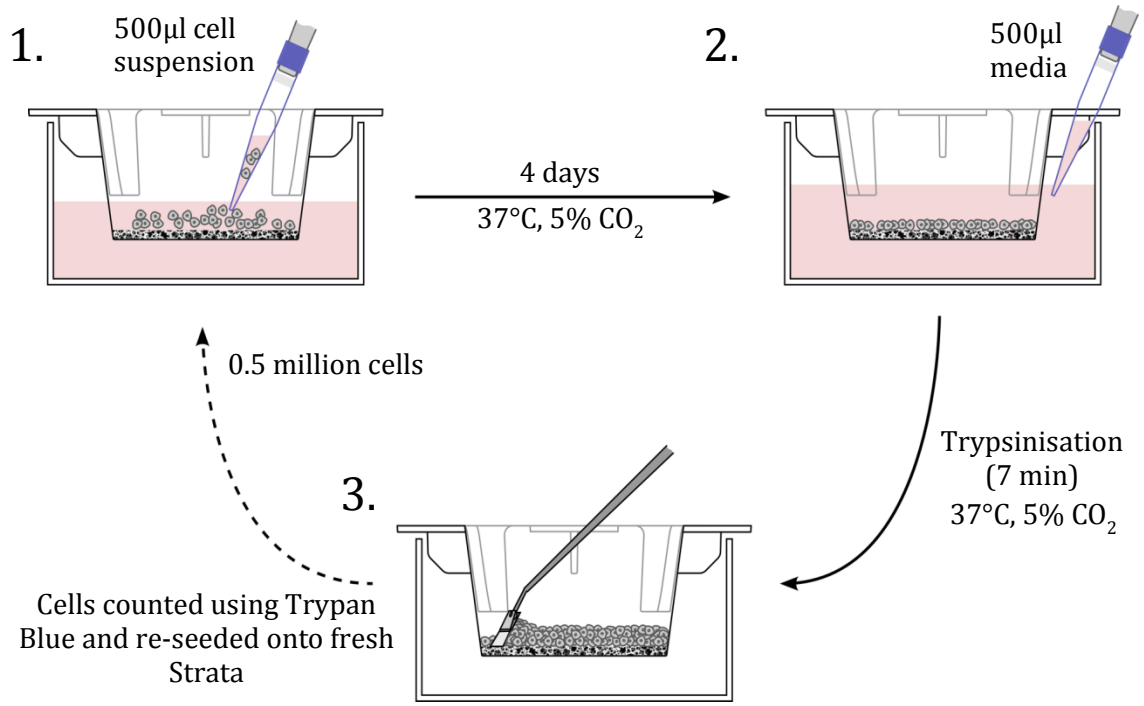


Figure 2.1. The methodology for maintaining TERA2.cl.SP12 cells on Alvetex® Strata. *This figure shows the steps involved in seeding and passaging cells using Alvetex® Strata. The details of the method are described in Sections 2.1.3. and 2.1.4.*

2.1.5. Culturing TERA2.cl.SP12 stem cells on glass coverslips in well plates

Cells were trypsinised from either 2D or 3D cultured and put into a single cell suspension as previously described. Cells were seeded on circular glass coverslips (130-170µm diameter, Fisher) or high precision width square coverslips (170µm±5µm diameter, Marienfeld) at 3 x 10^4 cells per

coverslip which were then inserted into either 12 well plates (Greiner) or 6 well plates respectively. 1ml (or 2ml for a 6 well plate) of DMEM was then added to the wells and the plates were incubated at 37°C in 5% CO_2 for up to 48 hours.

2.1.6. Cryopreservation of TERA2.cl.SP12 stem cells

Confluent cells were frozen down by aspirating off the media and washing the cells in PBS. Acid washed beads were added and rolled over the cells to cause displacement and large clusters were broken up by pipetting to ensure an even distribution of cells in suspension. Cells were transferred to a 15ml Falcon tube and centrifuged at 1000rpm for 3 minutes before the PBS was aspirated off and the pellet was resuspended in 3ml of freezing media (containing 10% heat treated FBS and 10% DMSO (Sigma)). 1ml of freezing media containing cells were added to cryovials (1:3 split) which were frozen down to -80°C and then transferred to a -140°C freezer after 24 hours.

2.1.7. Inducing differentiation of TERA2.cl.SP12 stem cells

Cells were trypsinised from either 2D or 3D cultured and put into a single cell suspension as previously described. Cells were seeded in T25 flasks at 3.5 x 10^5 cells per flask, media was topped up to 10ml and after 24 hours at 37°C in 5% CO_2, differentiation was induced using 1μM *all-trans* retinoic acid (ATRA, Sigma) which was added to media. Cells were incubated for 3 days at 37°C in 5% CO_2 during which some samples were sacrificed for flow cytometric analysis at days 1 and 3.

2.1.8. Inhibition of ROCK activity in cultures of TERA2.cl.SP12 stem cells

Cells were trypsinised from either 2D or 3D culture and put into a single cell suspension as previously described. Cells were seeded onto coverslips (as mentioned in Section 2.1.5.) with addition of selective p160 ROCK inhibitor Y-27632 (10μM from a 5mM stock, Tocris) to the media as the wells were topped up with 1ml for 12 well plates or 2ml for 6 well plates.

2.2. Flow cytometry

Media was aspirated off from the cell culture flask and the cells were washed in PBS. 2ml of 0.25% Trypsin EDTA was added to each T25 and the flasks were incubated at 37°C in 5% CO_2 for 3 minutes to detach cells. Trypsin was neutralised with 3ml of DMEM then cells were transferred to a

15ml Falcon before being centrifuged at 1000rpm for 3 minutes. Cells were re-suspended in PBS and re-centrifuged as before.

The cell pellet was re-suspended in an appropriate amount of blocking buffer (0.1% bovine serum albumin (Sigma) in PBS). An appropriate volume should contain 2 x 10^4 cells usually suspended in 200µl of buffer for each antibody required for testing (plus negative control). 200µl of the cell suspension was added to required wells of a U-bottomed 96-well plate (Costar). This plate was next centrifuged at 1000rpm for 3 minutes at 4°C. The supernatant was removed from wells by inversion. The pellets in each well were re-suspended in 50µl of the corresponding primary antibody diluted in blocking buffer (as detailed below in Table 2.1.) and incubated for 1 hour on ice. Any unbound excess antibody was removed by adding 100µl of blocking buffer and centrifugation at 4°C. Cells were re-suspended in 180µl blocking buffer and re-centrifuged twice more before incubation with 50µl of the diluted secondary antibody (antimouse-IgM (Sigma) at a dilution of 1:100 with blocking buffer) for 1 hour in the dark on ice. Cells were then washed three times in blocking buffer as previously described and finally re-suspended in 200µl blocking buffer for analysis. The now immunolabelled cells were analysed using the EasyCyte Plus system (Guava technologies) and identification of positive cells were set against the negative control mouse antibody, P3X.

Antibody	Origin	Dilution factor
P3X	Developmental Studies Hybridoma Bank	1:10
SSEA-3	Developmental Studies Hybridoma Bank	1:5
Secondary antibody - Anti-Mouse IgM (µ-chain specific)–FITC antibody produced in goat	Sigma Aldrich, UK (F9259)	1:100

Table 2.1. Details of antibodies used in flow cytometry

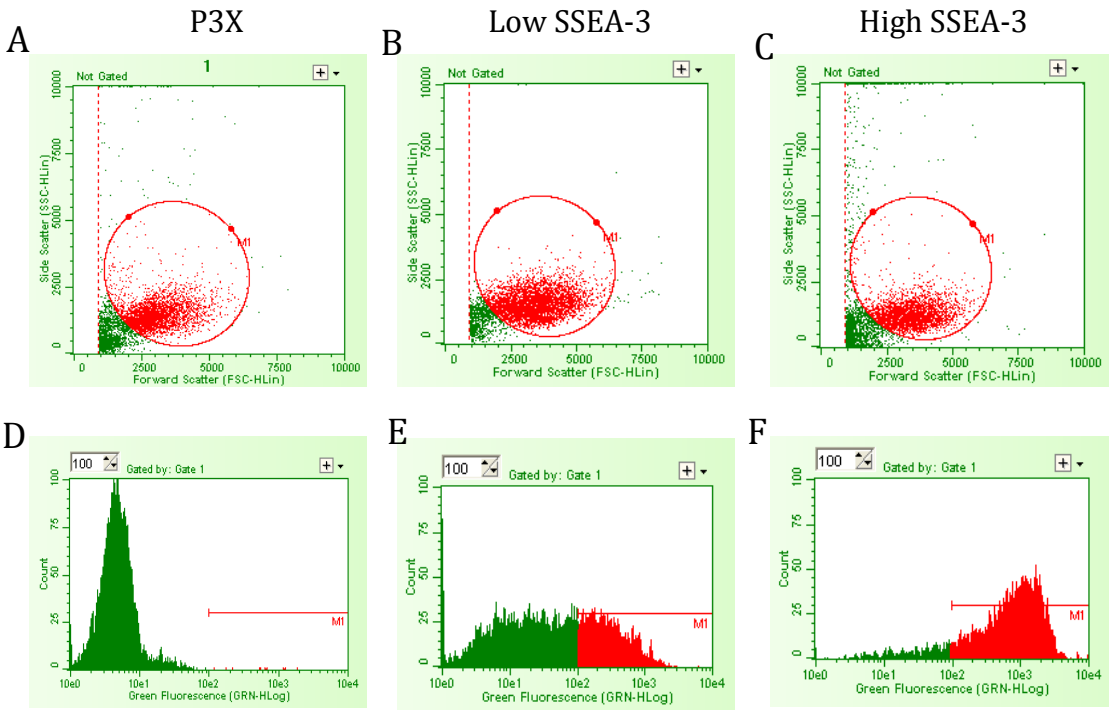


Figure 2.2. Examples of typical gating when using flow cytometry. *(A-C): Scatter graphs from TERA2.cl.SP12 cells labelled with P3X (A) and expressing low SSEA-3 (B) and high SSEA-3 (C); (D-F): Histograms of TERA2.cl.SP12 cells labelled with P3X (D) and expressing low SSEA-3 (E) and high SSEA-3 (F). Gating is set relative to the negative control marker, P3X. This is usually around 10e2 and should contain <3% of cells. The low SSEA-3 expression sample seen in (B) and (E) also possesses a low mean expression compared with (D) and (F) which may indicate that these TERA2.cl.SP12 cells could be differentiating.*

2.3. Tissue processing

2.3.1. Wax embedding of teratoma tumours

Teratomas were obtained from an animal study. All procedures involving mice were conducted in accordance with Animals (Scientific Procedures) Act 1986, the 3Rs guidelines and under the necessary Establishment, Project and Personal Licences required by the Home Office, UK.

Cells were trypsinised from either 2D or 3D culture using the methods previously described (Sections 2.1.2. and 2.1.4.). Cells were counted using the Trypan Blue assay on a haemocytometer and 0.5 x 10^6 cells were resuspended in 100µl of cell culture media. Each cell suspension was combined with 100µl of BD Matrigel (BD Bioscience) to increase the successful generation of tumours. The resultant 200µl of cell suspension was put into 1ml syringes (Fisher) with 21G needles (Fisher) and stored on ice. Cells were then injected subcutaneously into adult male nude (nu/nu) mice flanks. Each animal received bilateral grafts. 3 mice were injected with each cell type, giving the possibility of 6 tumours per cell growth condition so that statistical analysis could be performed. Mice were regularly examined and once teratoma formation was visible, the tumours

were measured at least once per week until they reached 1cm² in volume. At this point, the mice were sacrificed using approved Schedule 1 methods and the teratoma tissue was surgically removed. This was usually between 6-8 weeks after injection. This study was concluded in February 2013.

The collected teratomas were fixed in 4% PFA (Sigma) in a 50ml Falcon tube then samples were washed three times with distilled water before being washed by 30-40ml ethanol at the following concentrations, each for two hours: 70%, 80%, 90% and 95%. Samples were stored in 95% ethanol. Samples were then dehydrated in 100% dry ethanol for 2 hours at which point the ethanol was replaced and the samples were left for 24 hours. The ethanol was poured away and 30-40ml Histoclear (National Diagnostics) was added and the samples left overnight. The 50ml Falcon tubes were then topped up with paraffin wax at 60°C (ratio 1:1 with Histoclear) and kept in an oven at 60°C for 4 hours. The mixture was poured away and the 50ml Falcon tubes were filled with fresh, hot wax. Over the next 48 hours, the samples went through three changes of wax to remove any remaining Histoclear. The samples were then embedded onto processing cassettes (Thermo) and left to set.

2.3.2. Wax embedding of Alvetex® Strata 3D cultures

Cell culture medium was aspirated off from the wells and the membranes were washed three times in PBS before being fixed in 2-3ml 4% PFA and left overnight. Fixative was aspirated off and membranes were washed three times with PBS before being washed by 2-3ml ethanol at the following concentrations, each for 15 minutes: 30%, 50%, 70%, 80%, 90% and 95%. Membranes were stored in 95% ethanol.

Membranes were then dehydrated in 100% dry ethanol for 15 minutes before being transferred to a glass vial and cut in half. The ethanol was poured away and 15ml Histoclear was added for 15 minutes. The glass vials were then topped up with wax at 60°C (ratio 1:1 with Histoclear) and kept in an oven at 60°C for 30 minutes. The mixture was poured away and the glass vials were filled with fresh, hot wax and left for 30 minutes. The samples were then embedded onto coloured processing cassettes with the cut edge facing down and left to set.

2.3.3. Sample sectioning

Wax embedded sample cassettes were placed into a Leica Microtome RM2125RT, sectioned at 6μm and floated in a histology water bath at 40°C before mounting onto electrostatically charged Thermo SuperFrost+ slides and dried on drying racks.

2.4. Mayer's Haematoxylin and Eosin staining

Samples were stained for cell visualisation using Mayer's Haematoxylin and Eosin (H&E) staining. Haematoxylin stains cell nuclei purple whereas the Eosin stains the cytoplasm pink. Slides with a section of sample attached were de-waxed in Histoclear for 5 minutes then rehydrated through 100% ethanol for 2 minutes then 95% ethanol, 70% ethanol and distilled H_2O (dH_2O) for 1 minute each. Nuclei were stained using Mayer's Haematoxylin (Sigma) for 5 minutes then samples were washed in dH_2O before being blued using alkaline ethanol (30ml ammonia (Sigma) in 970ml 70% ethanol) for 30 seconds. Slides were dehydrated using 70% ethanol and then 95% ethanol for 30 seconds each then the cytoplasm was stained pink using Eosin Y (5g in 1L of 70% ethanol, Sigma) for 1 minute. Sections were washed twice for 10 seconds in 95% ethanol and then in two sets of 100% ethanol for 15 seconds and 30 seconds each. Lastly, sections were cleared in two sets of Histoclear for 3 minutes each before mounting in DPX mountant (Fisher).

2.5. Immunohistochemistry

Samples of teratoma tissue that had been fixed in 4% PFA, embedded (Sections 2.3.1. and 2.3.2.) and sectioned (Section 2.3.3.) were de-waxed and rehydrated as previously described for H&E staining (Section 2.4.). Antigen retrieval consisted of microwaving the slides for 3 x 2 minute bursts (leaving to stand in between to avoid boiling) at 800W in 10mM citrate buffer at pH6 (from 10x stock: 19.2g anhydrous citric acid in 1000ml dH_2O). Slides were left to stand in the citrate buffer for 20 minutes before being incubated for 15 minutes at room temperature in permeabilisation buffer (0.1% (v/v) Triton X-100 (Fisher) in PBS). Samples were blocked using blocking buffer (0.1% Tween-20 (Sigma) and 1% normal goat serum (Sigma) in PBS) for 30 minutes before subsequent addition of primary antibody diluted in blocking buffer (see Table 2.2. for dilutions). Sections were incubated overnight at 4°C in a humidified chamber then washed for 3 x 10 minutes in blocking buffer on an orbital shaker at 40rpm. Fluorescent-conjugated (secondary) antibodies were diluted in blocking buffer with Hoescht 33342 (Table 2.2.) to stain nuclei and were incubated on the

samples for 1 hour at room temperature in the dark. Immuno-stained slides were then washed as before for 3 x 10 minutes in blocking buffer before mounting using Vectashield mounting media (Vector Labs). Coverslips were attached and sealed using nail varnish. The slides were then examined using fluorescence microscopy.

Type of Reagent	Antibody	Origin	Dilution factor
Primary antibody	Oct4 TUJ-1 Cytokeratin 8	Abcam (ab19857) Covance (PRB-435P-100) Sigma (C5301)	1:250 1:600 1:600
Secondary antibody	Cy3-AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	1:600
Nuclear stain	Hoescht 33342	Molecular Probes (H3570)	1:1000

Table 2.2. Details of antibodies used for immunohistochemistry.

2.6. Phalloidin staining of f-actin

Samples (e.g. cells on glass coverslips as in Section 2.1.7.) that had been fixed in 4% PFA for 30 minutes and washed in PBS were submerged in blocking buffer (as described in Section 2.5.) for 30 minutes. The phalloidin stain and Hoescht 33342 were diluted in blocking buffer and 200µl was added to each sample before being incubated at room temperature in the dark for 1 hour. Samples were then washed for 3 x 10 minutes in blocking buffer on an orbital shaker at 40rpm. 50µl of Vectashield was added to each glass coverslip and the samples were then mounted face down on slides and several layers of nail varish was used to seal the sample.

The phalloidin used in this thesis was Acti-stain 488 phalloidin from Universal Biologicals (PHDG1-A) with a dilution factor of 1:600.

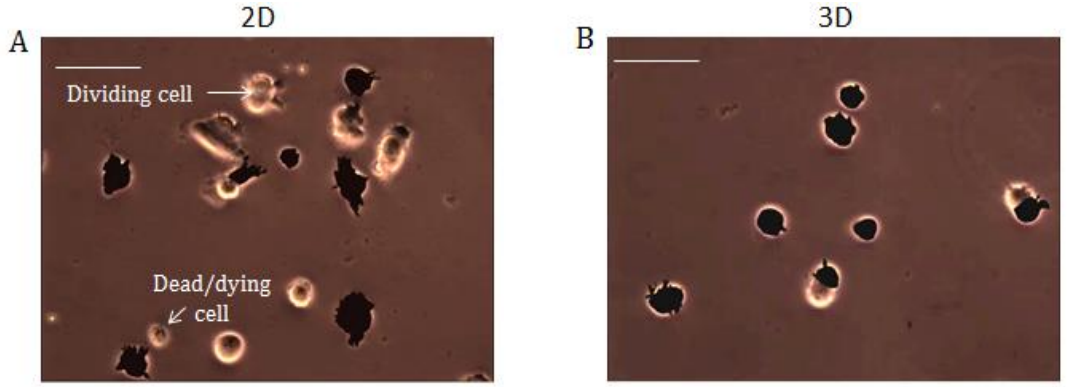
2.7. Imaging samples and data collection

Immunofluorescence images taken on Leica DMI3000B using Leica DFC310FX camera and Leica LAS v.3.7. image capture software. Histology images were taken on Leica DM500 using Leica ICC50HD and Leica LAS EZ image capture software.

2.7.1. Measurement of cell circularity, area and perimeter

Phase contrast images of individual cells were captured on the Leica DMI3000B using Leica DFC310FX camera and LAS v.3.7. image capture software. Measurements were made using ImageJ software (http://rsb.info.nih.gov/ij/). A measured scale for the computer resolution was created for pixel-micron conversion (4.34pixels/micron).

Outlines were traced around phase contrast images of the cells in culture with each cell labelled and measured using the above scale. 4 cells were measured per repeat (x3) at each time point making n=12 per time point. Means were calculated and used for graph creation. Figure 2.3. shows a graphical representation of the methodology.



C

2D								Mean
Area (µm²)	403.81	372.65	225.69	147.65	673.78	528.52	305.01	379.59 ± 67.68
Perimeter (µm)	90.54	111.68	94.80	47.60	125.86	132.22	92.16	99.26 ± 10.65
Circularity	0.62	0.38	0.32	0.82	0.54	0.38	0.45	0.50 ± 0.07
3D								Mean
Area (µm²)	425.56	304.48	216.88	377.26	223.04	199.30	287.49	287.49 ± 32.43
Perimeter (µm)	121.04	68.75	66.72	80.41	58.66	52.41	80.49	80.49 ± 8.54
Circularity	0.37	0.81	0.61	0.73	0.82	0.91	0.56	0.59 ± 0.07

Figure 2.3. Methods of measurement of changes to cell shape during adaptation to 2D cell culture. *(A): Phase contrast image of outlines of TERA2.cl.SP12 cells after 10 passages in 2D used for measurement. Also indicates dividing and dead cells not used for measurement; (B): Phase contrast image of outlines of TERA2.cl.SP12 cells after 10 passages in 3D used for measurement; (C): Table showing area, perimeter and circularity values for cells measured in (A) and (B). Scale bars - 50µm. n = 7 for this example.*

2.8. Statistical analysis

Statements of statistical relevance were calculated using SPSS software (IBM, v.2.0). The Student's *t*-test was used when comparing 2 values but Univariate Analysis of Variance (ANOVA) was carried out when comparing more than 2 values. Post-hoc analysis for comparing such samples to each other was done by Tukey's test. If results have significant statistical differences between groups,

these are shown with a number of * symbols representing level of statistical difference: * where $p < 0.05$, ** where $p < 0.01$ and *** where $p < 0.001$.

3. Results

3.1. Cell culture on Alvetex® Strata

3.1.1. Propagation of TERA2.cl.SP12 cells on Alvetex® Strata

TERA2.cl.SP12 cells are viable, proliferate and retain an undifferentiated, spherical 3D phenotype when continually maintained and propagated in 3D cell culture on Alvetex® Strata. Data as evidence for this is presented based on several criteria:

Firstly, cell proliferation rate was affected as demonstrated in Figure 3.1.1. Cells passaged in 2D have fairly high and constant proliferation compared with the initial proliferation of cells being put into 3D from 2D. On Alvetex® Strata, these cells must first adjust to the new microenvironment initially resulting in lower proliferation rate as the cell remodels its structure and adapts. A similar effect of decreased proliferation was seen in hESCs when adapting to growth on surfaces with nanogratings compared with flat surfaces (Gerecht *et al.*, 2007). Whilst this observation is not inherently an observation of a 3D phenotype, the lower proliferation during the first passages is a strong indicator that the cells are changing.

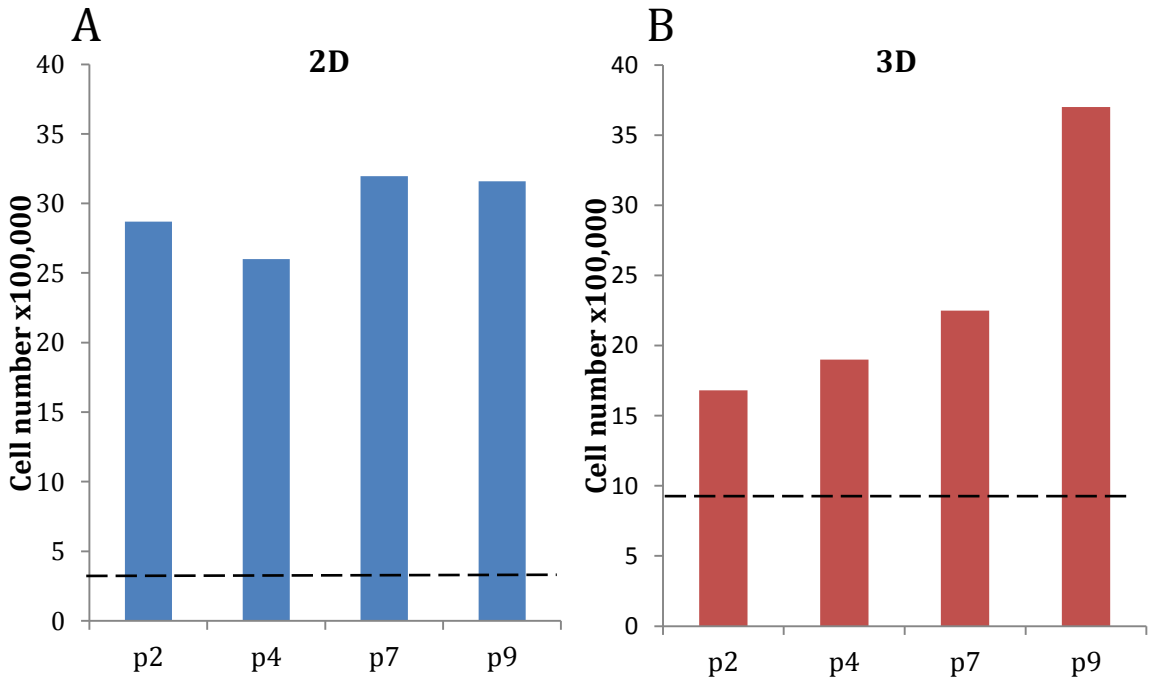


Figure 3.1.1. Cells maintained in 3D, after an initial period of adaptation, proliferate more as passage number increases. *(A): 2D cultured cells maintain a relatively constant level of proliferation; (B): 3D passaged cells firstly adapt to the new microenvironment coming from 2D culture before proliferating well (constant increase from p2 onwards). The above cell proliferation data is the raw number of cells taken off of Alvetex® Strata by the cell scraping method described. The dashed line is average cell seeding density. Data was taken from a typical study.*

3.1.2. Acquired 3D phenotype: a change toward 3D shape

Cytoskeletal structure and cell shape are clearly affected when TERA2.cl.SP12 cells are maintained in 3D, leading to the retention on the 3D phenotype. It has long been known that cells flatten out when put into a 2D microenvironment and that this change in cell shape affects gene and protein expression causing a move away from the *in vivo* arrangement. Confocal microscopy has been used to visualise and measure the shape of cells cultured in 2D and 3D (Figure 3.1.2.).

The lack of cell flattening and larger size of cells in cross section indicates that there is a morphological change occurring during the maintenance of these stem cells in 3D up to passage 10. Changes to the shape of the nucleus (Figure 3.1.2.) may also impact on changes in gene expression. As described in Figure 1.1., flattening causes changes in gene transcription and so cells which do not flatten (cells passaged in 3D, Figure 3.1.2. B) will possess a different phenotype to those that flatten (cells passaged in 2D, Figure 3.1.2. A).

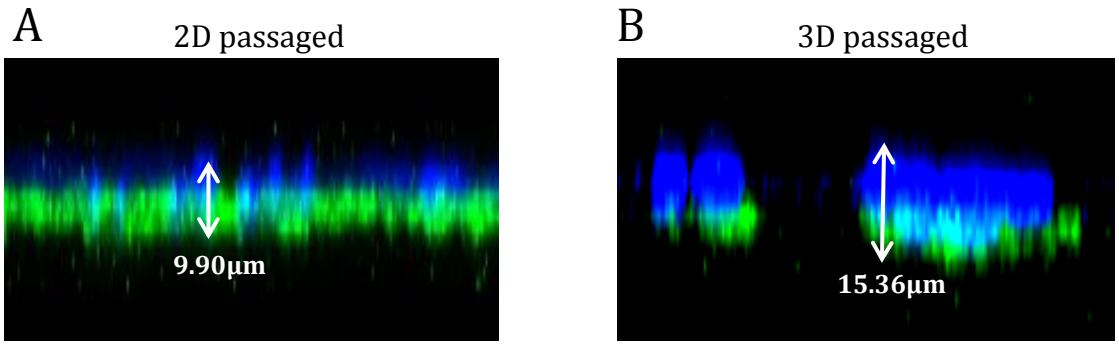


Figure 3.1.2. Cells maintained in 3D possess a more 3D structured phenotype and are less flattened than 2D passaged cells. *(A): Confocal image of TERA2.cl.SP12 cells maintained in 2D culture for 10 passages; (B): Confocal image of TERA2.cl.SP12 cells maintained in 3D culture on Alvetex® Strata for 10 passages then placed in 2D culture and imaged. The z-axes of cells from the surface of the substrate were measured. 3D passaged cells had a larger z-axis profile indicating a lack of cell flattening and the retention of the 3D phenotype. The 3D passaged cells appear to have a more spherical cell and nuclear shape. Image taken by E. Knight at Durham University.*

3.1.3. 3D maintenance of stem cells leads to increased expression of the pluripotency marker SSEA-3

EC cells in general have long been known to express stage-specific embryonic antigen-3 (SSEA-3) (Andrews *et al.*, 1982) and show pluripotency and so this marker was chosen for examination. Maintaining TERA2.cl.SP12 cells on Alvetex® Strata resulted in increased expression of SSEA-3 (Figure 3.1.3.).

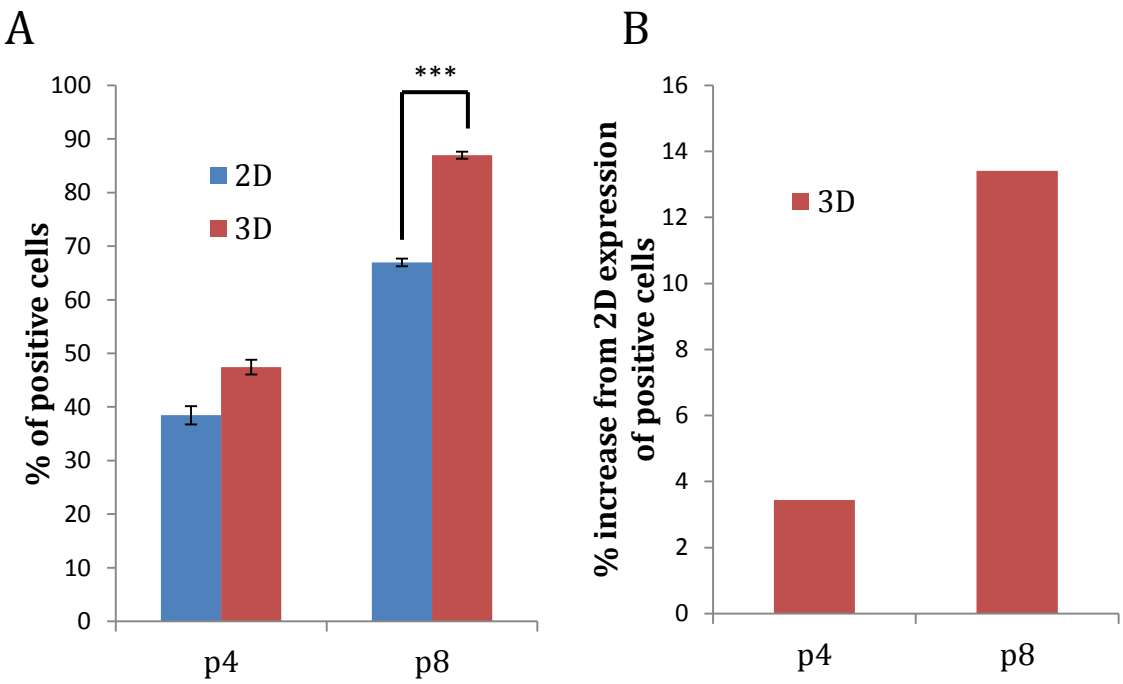


Figure 3.1.3. TERA2.cl.SP12 cells maintained in 3D express higher SSEA-3 levels after prolonged culture. *(A): Flow cytometric data showing SSEA-3 expression of 2D and 3D cultured cells; (B): Percentage increase above 2D of the 3D cells at passages 4 and 8. SSEA-3 expression is significantly higher in 3D maintained cells than in 2D counterparts after 8 passages (***p<0.001). These results may indicate that the 3D maintained cells have a more stem cell like phenotype and greater developmental potential than cells cultured purely in 2D.*

These flow cytometry data show that maintaining cells on Alvetex® Strata leads to an increased stem cell phenotype as measured by SSEA-3 expression. This increase in expression level reflects changes to the cell caused by the microenvironment in which the cells were cultured. This finding would also suggest that the cells are remaining undifferentiated as SSEA-3 expression is downregulated as cells differentiate (Brimble *et al.*, 2007).

These data suggest passaging cells in 3D on Alvetex® Strata in the long term leads to an enhanced stem cell phenotype compared with cells cultured purely on conventional 2D substrates. The acquisition of this enhanced 3D phenotype is not instantaneous when cells are put onto Alvetex® Strata. By passage 8 on Alvetex® Strata however, the cells have modified their structure and acquired enhanced expression of a well-recognised marker of stem cell phenotype.

3.2. Culture of pluripotent stem cells in 3D leads to changes in cell shape

Results indicate that the culture of cells in 3D leads to changes in initial structure of a cell compared to their growth in conventional 2D cell culture. This has been demonstrated and analysed using a range of methods as follows:

3.2.1. Maintaining cells in 3D leads to a physical change in cell structure

TERA2.cl.SP12 cells were cultured in either 2D or 3D for 3 passages. A sample of cells was then put into 2D culture on glass coverslips and imaged at hours 1, 2, 3, 4, 5 and 10. The 2D and 3D cell populations were also propagated to passage 8 at which point the cells were again put into 2D culture for 10 hours on glass coverslips and imaged at the same time points. Analysis of the cell morphology was carried out using ImageJ.

After 3 passages, both the 2D passaged and 3D passaged cells showed no significant difference in circularity (Figure 3.2.1. A), area (Figure 3.2.1. C) or perimeter (Figure 3.2.1. E) at any time point. Both samples had a very similar morphology and flattened out in 2D culture in a similar fashion.

After 8 passages, the 3D maintained cells had retained a smaller and more spherical morphology when put back into 2D culture. This 3D phenotype is potentially a result of adaptation during the course of passaging in 3D. After 5 hours in 2D culture, the 3D maintained cells are significantly different ($p<0.001$) to the 2D passaged cells in terms of circularity (Figure 3.2.1. B), area (Figure 3.2.1. D) and perimeter (Figure 3.2.1. F).

These data indicate that having been passaged in 3D for 8 passages, 3D maintained cells retain a more spherical 3D structure and do not flatten/spread over 2D substrate as much as cells cultured by conventional 2D methods.

These data also suggest that maintaining cells in 3D leads to a more rounded 3D morphology of these pluripotent stem cells which is more akin to a typical 3D *in vivo* phenotype. This further suggests that cells maintained in 3D are a more relevant model for study.

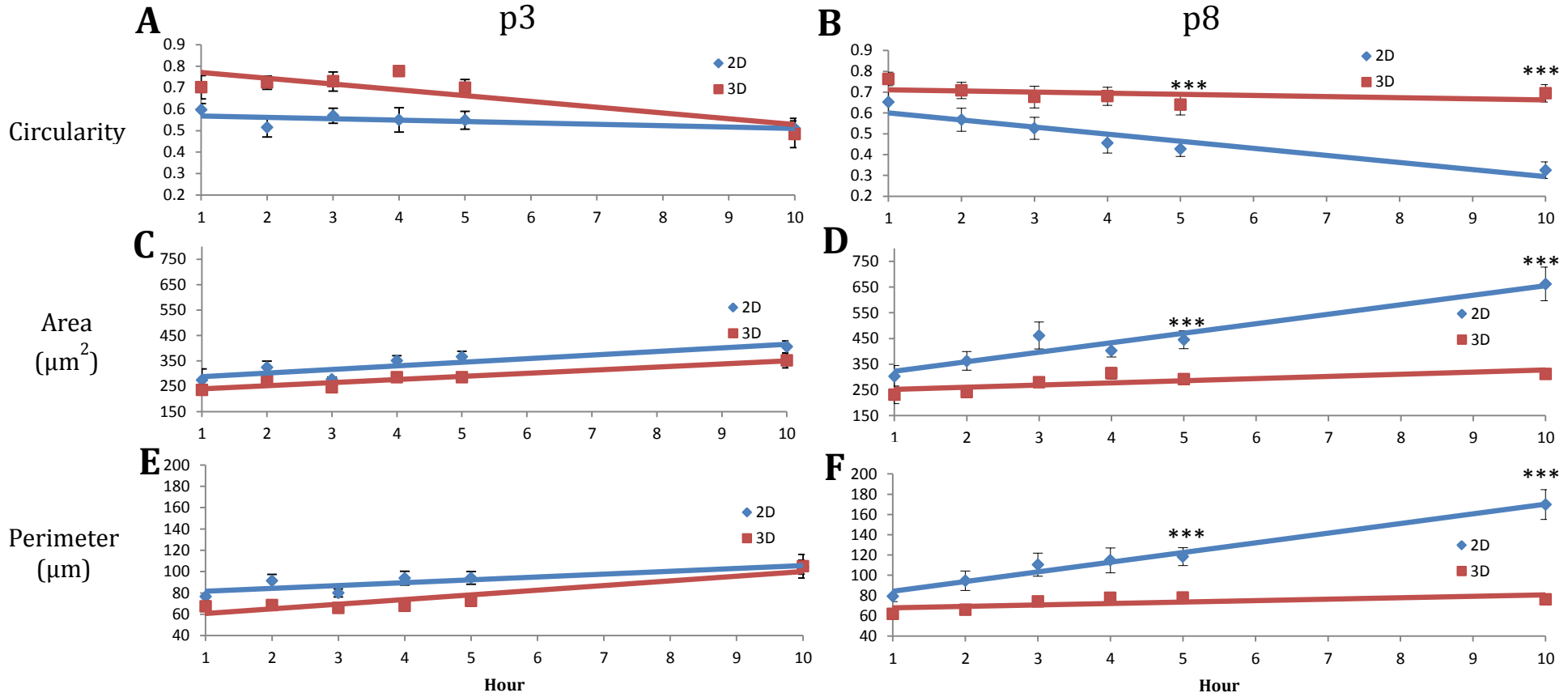


Figure 3.2.1. By passage 8, cells maintained in 3D retain a rounded, smaller phenotype as a consequence of adaptation to the 3D microenvironment. *(A), (C), (E): Graphical data showing circularity (A), area (C) and perimeter (E) of TERA2.cl.SP12 cells grown in 2D and 3D for 3 passages and placed back into 2D culture for up to 10 hours; (B), (D), (F): Graphical data showing circularity (B), area (D) and perimeter (F) of TERA2.cl.SP12 cells grown in 2D and 3D for 8 passages and placed back into 2D culture for up to 10 hours. After 3 passages, both samples had very similar morphologies at each time point however p8 cells after 4 hours in 2D, the 3D maintained cells retained a 3D phenotype (more circular and smaller than 2D cultured cells). This suggests that prolonged culture in 3D causes a phenotypic change. Data represents means ±SEM, n=12 per data point. *** indicates p<0.001.*

3.2.2. Circularity is retained after passaging cells in 3D culture

TERA2.cl.SP12 cells were cultured in 3D for 3, 6 and 8 passages and then placed into 2D culture for 10 hours on glass coverslips and imaged at hours 1, 2, 3, 4, 5 and 10. Analysis of cell circularity was carried out using ImageJ.

After 3 passages, 3D cultured cells lost circularity quickly in 2D culture between hours 1 and 10. After 6 passages, 3D cultured cells lost circularity over time in 2D culture however not as quickly as p3 3D passaged cells. After 8 passages, 3D cultured cells lost circularity slowly compared with p3 and p6 3D cultured cells (Figure 3.2.2. A).

After 10 hours in 2D culture, 3D passaged p8 cells retained a rounder phenotype than both p3 and p6 cells. Specifically, the circularity of p8 cells are significantly different from that of p3 cells ($p<0.05$) (Figure 3.2.2. B). These data indicate that having been passaged in 3D for 8 passages, 3D maintained cells keep a more rounded 3D morphology on 2D substrate longer than cells passaged in 3D culture for 3 or 6 passages.

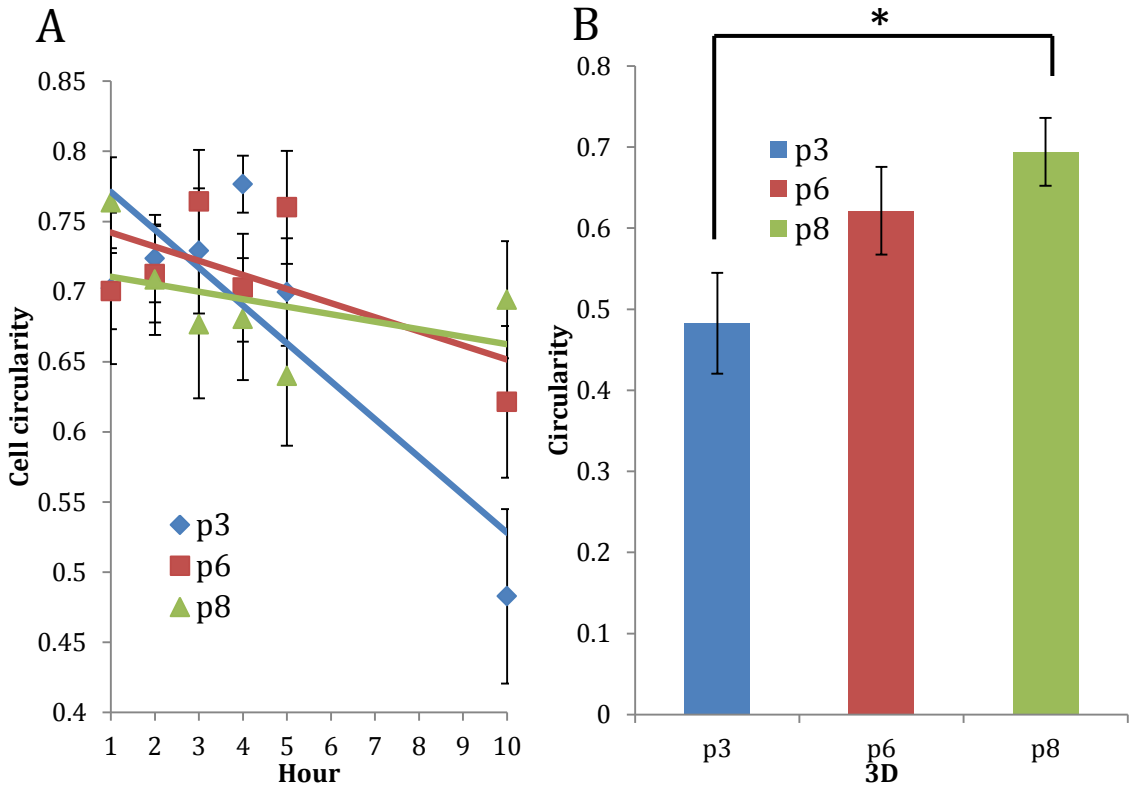


Figure 3.2.2. Cells retain circularity after maintenance in 3D. *(A): Circularity data from TERA2.cl.SP12 cells grown in 3D for 3, 6 or 8 passages and then put back into 2D on glass coverslips and cultured for up to 10 hours; (B): Graph showing mean circularity values at hour 10 of 3D maintained cells at p3, p6 and p8. Maintaining cells in 3D allows cells to retain a more rounded 3D morphology even when put back into 2D culture. Data represents mean ± SEM, n=12 per data point. Tukey's t-test shows *p<0.05 (ANOVA).*

3.2.3. Cell spreading response is weakened after passaging in 3D culture

TERA2.cl.SP12 cells were cultured in 3D for 3, 6 and 8 passages and then placed into 2D culture for 10 hours on glass coverslips and imaged at hours 1, 2, 3, 4, 5 and 10. Analysis of cell area was carried out using ImageJ.

After 3 passages, 3D cultured cells spread quickly in 2D culture between hours 1 and 10. After 6 passages, 3D cultured cells spread at a similar rate in 2D culture to cells after 3 passages. After 8 passages, 3D cultured cells flattened slower compared to both p3 and p6 3D cultured cells (Figure 3.2.3 A).

After 10 hours in 2D culture, p8 cells possessed a smaller area than both p3 and p6 cells. Specifically, the area of p8 cells are significantly different from that of p3 cells ($p<0.05$) (Figure 3.2.3. B). These data indicate that having been passaged in 3D for 8 passages, 3D maintained cells spread less distance and at a slower rate over a 2D substrate in 10 hours than cells passaged in 3D culture for 3 or 6 passages.

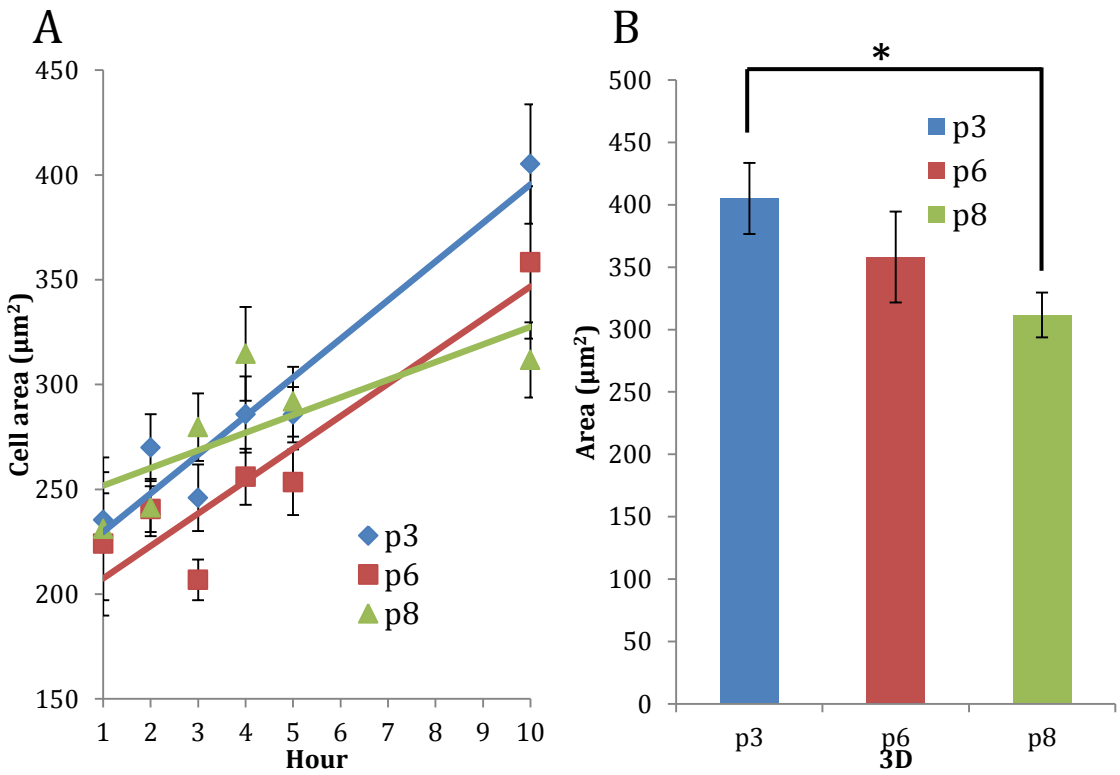


Figure 3.2.3. Cells flatten out less in 2D after maintenance in 3D. *(A): Area data from TERA2.cl.SP12 cells grown in 3D for 3, 6 or 8 passages and then put back into 2D on glass coverslips and cultured for up to 10 hours; (B): Graph showing mean area values at hour 10. Maintaining cells in 3D allows cells to retain a 3D more rounded morphology so cells do not spread as much as lower 3D passages when put back into 2D culture. Data represents mean ± SEM, n=12 per data point. Tukey's t-test shows *p<0.05 (ANOVA).*

3.2.4. The perimeter of the cell is smaller after passaging in 3D culture

TERA2.cl.SP12 cells were cultured in 3D for 3, 6 and 8 passages and then into 2D culture for 10 hours on glass coverslips and imaged at hours 1, 2, 3, 4, 5 and 10. Analysis of the cell perimeter was carried out using ImageJ.

After 3 passages, 3D cultured cells flatten quickly in 2D culture between hours 1 and 10. After 6 passages, 3D cultured cells spread over the substrate slightly slower over time in 2D culture than p3 cells. After 8 passages, 3D cultured cells spread at a slower rate compared to both p3 and p6 3D cultured cells (Figure 3.2.4. A).

After 10 hours in 2D culture, p8 cells possessed a smaller perimeter than both p3 and p6 cells. Specifically, the perimeter of p8 cells are significantly different from that of p3 cells ($p<0.05$) (Figure 3.2.4. B). These data indicate that having been passaged in 3D for 8 passages, 3D maintained cells possess a smaller profile when viewed from the z-axis perspective down on the substrate surface and flatten (thereby creating a larger profile) slower than cells passaged in 3D culture for 3 or 6 passages over 10 hours.

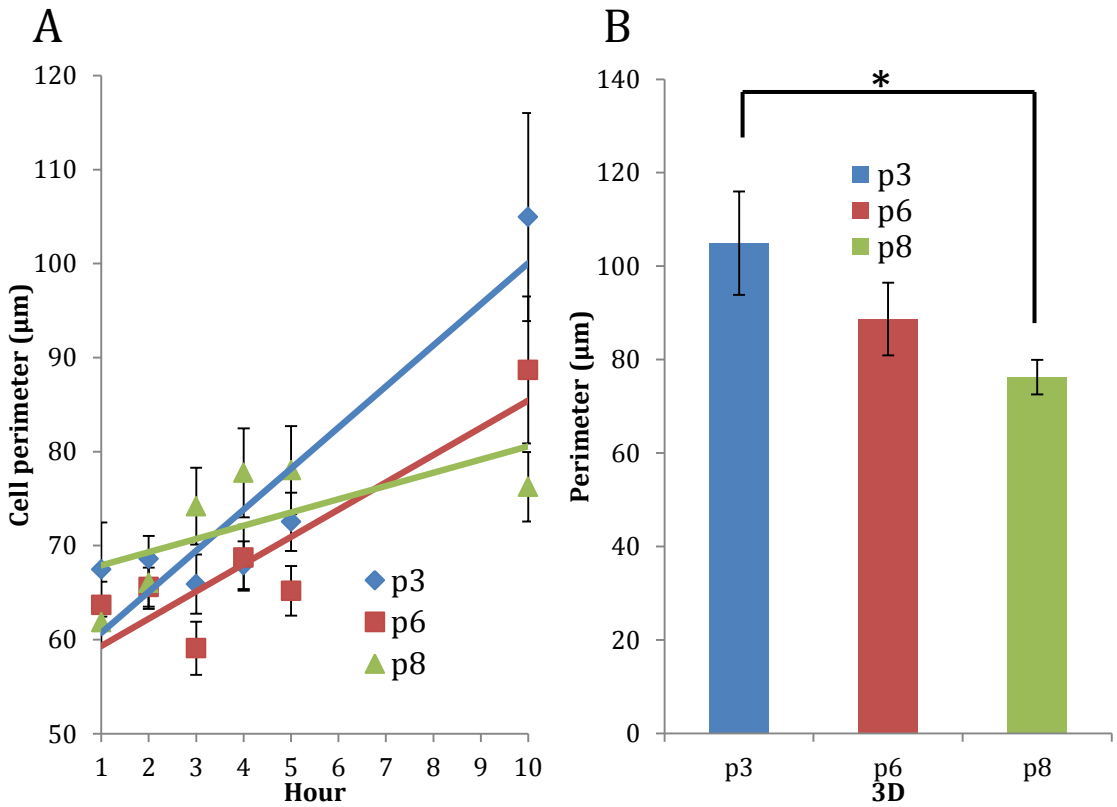


Figure 3.2.4. Cells present a smaller top-down profile when put into 2D after maintenance in 3D. *(A): Perimeter data from TERA2.cl.SP12 cells grown in 3D for 3, 6 or 8 passages and then put back into 2D on glass coverslips and cultured for up to 10 hours; (B): Graph showing mean perimeter values at hour 10. Maintaining cells in 3D allows cells to retain a 3D rounded morphology so cells do not flatten as much as lower 3D passages when put back into 2D culture. Data represents mean ± SEM, n=12 per data point. Tukey's t-test shows *p<0.05 (ANOVA).*

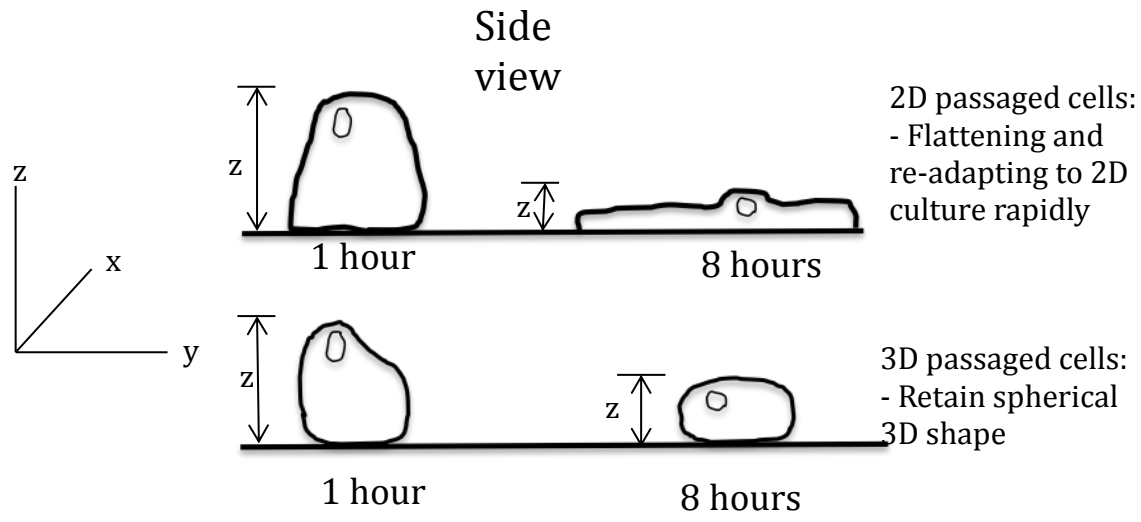


Figure 3.2.5. Cells continually maintained in 3D flatten less than those passaged in 2D when put back onto a 2D substrate. *Cartoon schematic illustrating how cells passaged in 3D flatten less than cells passaged in 2D when put back into 2D culture after 8 hours. At 1 hour after being put back into 2D culture, both 2D and 2D passaged cells have a similar 3D, spherical phenotype immediately after removing cells from culture substrate. Cells acquire a rounded, smaller morphology after prolonged culture in 3D and so when put back into 2D culture; 3D passaged cells do not flatten or spread as much as 2D passaged cells.*

3.2.5. Assessment of cytoskeletal changes in cells after 2D and 3D culture

TERA2.cl.SP12 cells were cultured in either 2D or 3D for 6 passages and then a sample of cells were put into 2D culture for 48 hours on glass coverslips and fixed at hours 6, 24 and 48. The fixed cells were then stained with phalloidin to allow visualisation of the f-actin cytoskeleton in detail using an x100 oil emersion objective.

F-actin plays an important role in maintaining basic cell shape and is subject to gradual changes caused by gene expression and microenvironment changes. After 6 hours, both the 2D passaged and 3D passaged cells showed no significant difference in circularity, area or perimeter (Figure 3.2.6. A and B). Both samples had a very similar morphology yet the 2D cultured cells appear have a slightly larger area and slightly lower circularity. Based on examination of Figure 3.2.6 A and B, it appears that after 6 hours in 2D culture, the 3D cultured samples had a smaller cell-nucleus ratio in terms of area and that these cells were more rounded than the 2D cultured counterparts (which have small filapodia). After 24 hours, the 3D passaged cells had retained a more rounded and smaller morphology when put back into 2D culture than 2D passaged cells (Figure 3.2.6. C and D). This 3D phenotype is potentially a result of adaptation during the course of maintenance in 3D, which seems to have an effect even after 6 passages in 3D. After 48 hours in 2D culture, the 3D maintained cells began to flatten and spread slightly but not to the same extent as the 2D passaged cells which have a larger area and a lower circularity. Many f-actin projections are visible after 48

hours from the 2D passaged cells (Figure 3.2.6. E) whereas there are very few from the 3D maintained cells (Figure 3.2.6. F). These data indicate that having been passaged in 3D for 6 passages, cells do not flatten/spread over 2D substrate as much as cells cultured by conventional 2D methods due to changes in f-actin structure. These data also correlate with earlier data on prolonged maintenance of cells in 3D leading to a more rounded 3D phenotype.

From this, it can be argued that maintaining cells in 3D leads to cells retaining a more rounded and smaller morphology for longer when put back into 2D culture. This is reflected by changes in f-actin structure, a major component of cell shape, as 2D and 3D maintained cells spread.

Collectively, Figures 3.2.5. and 3.2.6. build a picture of how 2D and 3D passaged cells flatten down when put back onto a 2D substrate. Figure 3.2.5. illustrates a side view of cells and Figure 3.2.6. shows the top-down profile of cultured cells.

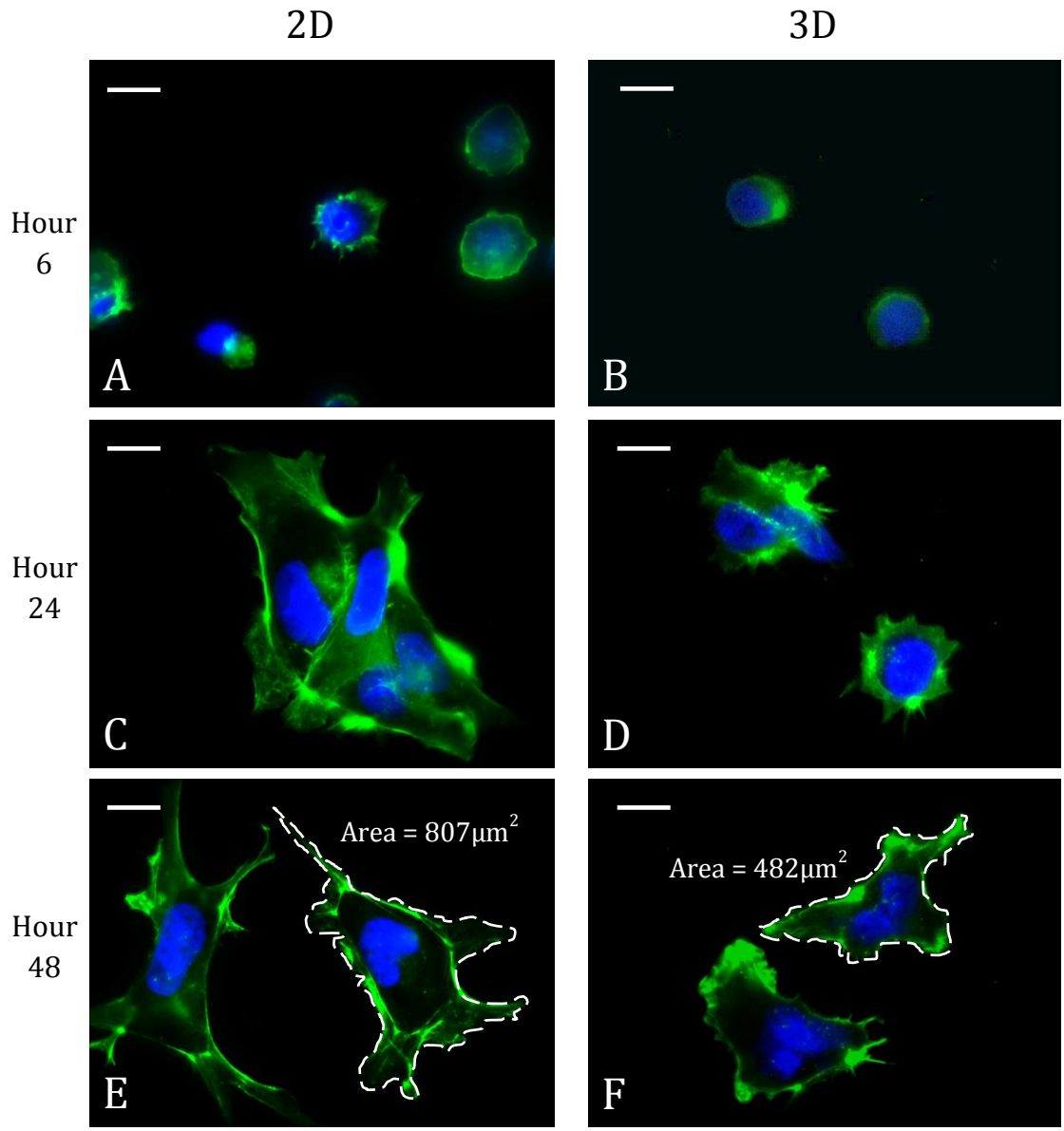


Figure 3.2.6. Staining the f-actin cytoskeleton reveals that 3D maintained cells do not appear to spread as rapidly as 2D cultured cells when put back into 2D culture. *(A), (C), (E): Images of f-actin cytoskeleton of p6 2D passaged cells grown in 2D on glass coverslips for 6 (A), 24 (C) and 48 (E) hours; (B), (D), (F): Images of f-actin cytoskeleton of p6 3D passaged cells grown in 2D on glass coverslips for 6 (B), 24 (D) and 48 (F) hours. The images above show that TERA2.cl.SP12 cells maintained in 3D retain a 3D rounded phenotype even when put back into 2D culture. This effect can be seen easily by measuring the area of each cell. There is less flattening out in the 3D samples compared with the 2D counterparts at each time point but after 48 hours, the 3D samples do begin to flatten out. Dashed lines are typical cell outlines, areas shown (E) and (F). Scale bars - 20µm.*

3.2.6. Pharmacological inhibition of ROCK leads to increased flattening of cells in 2D culture

ROCK, as described previously, is linked with cell shape change signalling and stem cell fate choice. ROCK is therefore a potential candidate for examination of the shape change of our pluripotent stem cell line. TERA2.cl.SP12 cells were cultured in either 2D or 3D for 6 passages and then cultured on a 2D substrate for 10 hours on glass coverslips with 2D and 3D cultured cells grown in

the presence of 1μM Y-27632 ROCK inhibitor and others without, as a control. Cells were stained with phalloidin and imaged at hours 1, 2, 3, 4, 5 and 10. Analysis of the cell morphology was carried out using ImageJ.

ROCK inhibition lead to cells passaged on 2D substrates possessing a lower circularity (Figure 3.2.7. A), larger area (Figure 3.2.7. C) and larger perimeter (Figure 3.2.7. E) at each time point compared with cells lacking Y-27632. For cells continually propagated in 3D on Alvetex® Strata, ROCK inhibition similarly lead to cells presenting a lower circularity (Figure 3.2.7. B), larger area (Figure 3.2.7. D) and larger perimeter (Figure 3.2.1. F) at each time point. Interestingly, Y-27632 appeared to have a rapid effect given the difference between samples in both 2D and 3D after 60 minutes. These data suggest that culturing cells in the presence of a ROCK inhibitor leads to an increased flattening response of cells when put into 2D culture regardless of how the cells had been passaged previous to that. These data also validate previous data regarding there being a difference in flattening response between 2D and 3D passaged cells, although the difference is less pronounced given that these cells are only passage 6 and so there may not yet be full adaptation to the 3D microenvironment.

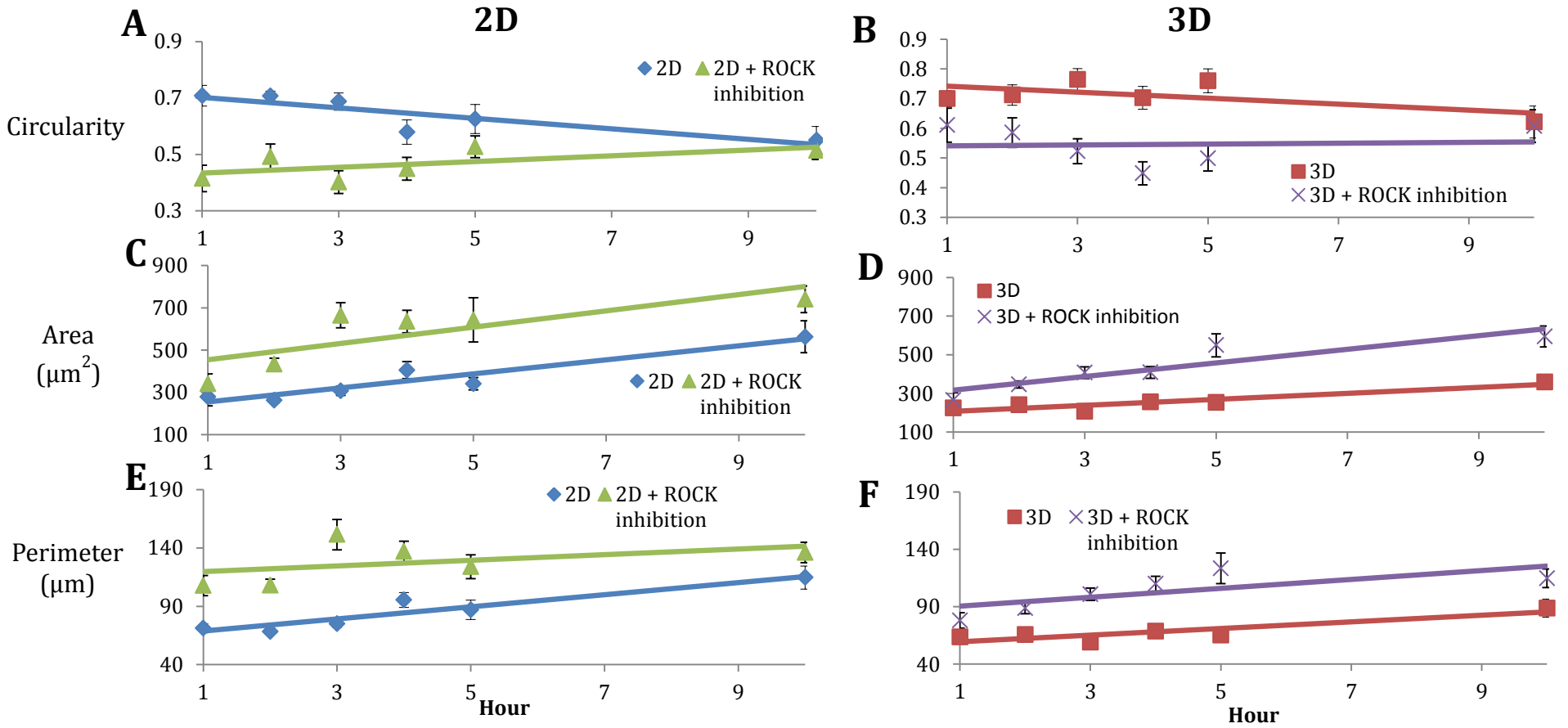


Figure 3.2.7. Inhibition of ROCK signalling caused increased cell flattening and spreading over the 2D growth substrate for both 2D and 3D passaged cells. *(A), (C), (E): Graphical data showing circularity (A), area (C) and perimeter (E) of TERA2.cl.SP12 cells grown in 2D on for 6 passages and placed back into 2D culture for up to 10 hours. Green lines indicate samples exposed to Y-27632; (B), (D), (F): Graphical data showing circularity (B), area (D) and perimeter (F) of TERA2.cl.SP12 cells grown in 3D for 6 passages and placed back into 2D culture for up to 10 hours. Purple lines indicate samples exposed to Y-27632. Inhibition of ROCK in both 2D and 3D samples caused an increased flattening/spreading response of the cell. There was no significant difference in response between 2D and 3D maintained cells to Y-26732 addition. These data suggest ROCK plays an important role in cell shape maintenance. Data represents means ±SEM, n=12 per data point.*

3.2.7. Pharmacological ROCK inhibition leads to changes in the f-actin cytoskeleton

TERA2.cl.SP12 cells were cultured in either 2D or 3D for 6 passages and then put into 2D culture for 48 hours on glass coverslips with some 2D and 3D cultured cells being grown in the presence of 1 μM Y-27632 ROCK inhibitor and others without. Cells were then fixed and stained with phalloidin to allow visualisation of the f-actin cytoskeleton in detail using an x100 objective oil emersion objective.

In 2D cultured cells, ROCK inhibition lead to increased flattening of cells compared with 2D cultured cells lacking Y-27632 treatment (Figure 3.2.8. A and B). 2D cultured cells in which ROCK has been inhibited showed a general lack of positive f-actin staining (Figure 3.2.8. B) perhaps indicating that ROCK is involved in actin polymerisation (as has been previously described (Maekawa *et al.*, 1999)). In 3D cultured cells, ROCK inhibition similarly lead to an increase in cell spreading compared with 3D cultured cells lacking Y-27632 treatment but interestingly, there is no tangible decrease in positive f-actin staining (Figure 3.2.8. C and D). This could be due to the adaptation undergone by the 3D maintained cells so that as the cells are put into 2D culture from suspension, their 3D phenotype is maintained more than in 2D cultured cells.

These data suggest that culturing cells in the presence of a ROCK inhibitor leads to an increased flattening response of cells, which in 2D may be attributed to a lack of normal f-actin structure. In 3D maintained cells which have a 3D phenotype gained from culture in the 3D microenvironment, there is increased cell spreading but it is less pronounced – this finding combined with the presence of positive f-actin staining in 3D passaged cells cultured with Y-27632 in 2D culture suggests that ROCK signalling and the f-actin cytoskeleton may be involved in the acquisition and maintenance of the 3D phenotype.

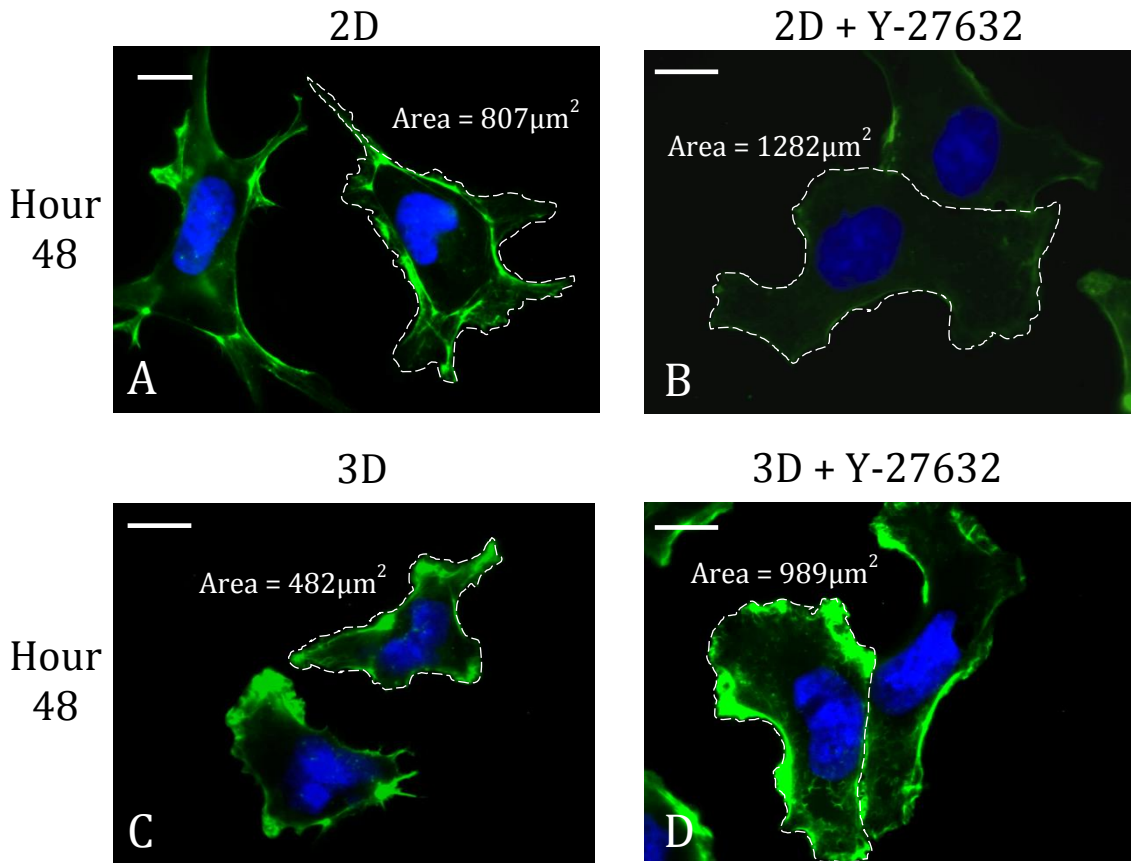


Figure 3.2.8. Inhibition of ROCK promotes cell flattening in both 2D and 3D passaged cells. *(A) and (B): Images of f-actin cytoskeleton of 2D passaged cells grown on glass coverslips for 48 hours with addition of Y-27632 (B) and without (A); (C) and (D): Images of f-actin cytoskeleton of 3D passaged cells grown on glass coverslips for 48 hours with addition of Y-27632 (D) and without (C). 2D cells cultured with ROCK inhibitor show a lack of positive f-actin staining whereas 3D maintained cells cultured with ROCK inhibitor show positive areas of f-actin staining, potentially due to 3D adaptation during the passaging in 3D. Addition of Y-27632 to 3D maintained cells causes the cells to spread out more and lose their acquired 3D phenotype, suggesting involvement of ROCK in cell structure maintenance. Scale bars - 20µm.*

3.3. Culture of pluripotent stem cells in 3D leads to changes in cell function and developmental potential

The results presented thus far clearly show that cells propagated and maintained long term in 3D culture, acquire a more 3D structural phenotype. As reviewed earlier, previous evidence suggests that culture of cells in 3D leads to changes in cell function compared with conventional 2D cell culture. In the context of stem cells this may influence their developmental potential. In this study, we examined the induction of cell differentiation *in vitro* in response to retinoic acid and we assessed the ability of cells to produce teratomas *in vivo*.

3.3.1. Maintenance of stem cells in 3D enhances the loss of pluripotency markers upon differentiation *in vitro*

TERA2.cl.SP12 cells were cultured in either 2D or 3D for 10 passages and then were cultured on conventional 2D substrates for 3 days in T25 flasks in the presence of 1μM ATRA. After days 1 and 3, cells were removed from the flasks and pluripotency marker SSEA-3 expression was measured using flow cytometry.

After 1 day of differentiation, SSEA-3 expression by 3D cultured cells was significantly lower than 2D cultured cells ($p<0.05$). Similarly, after 3 days of differentiation, SSEA-3 expression of 3D cultured cells was significantly lower than 2D cultured cells ($p<0.05$) (Figure 3.3.1.). These data indicate that having been passaged in 3D for 10 passages, 3D maintained cells decrease expression of the pluripotency marker SSEA-3 more quickly during differentiation compared to stem cells previously cultured in 2D.

These results suggest that maintaining cells in 3D enables stem cells to differentiate more rapidly. This may be due to a change in their developmental potential as a result of maintenance in a 3D microenvironment.

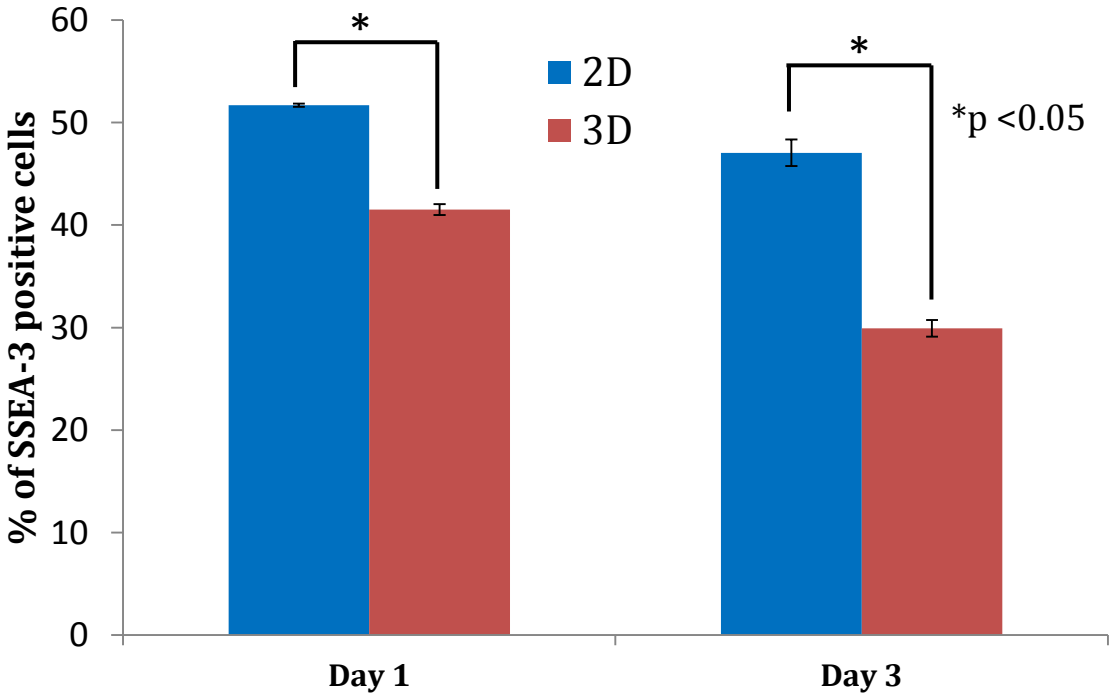


Figure 3.3.1. Cells maintained in 3D decrease SSEA-3 expression quicker than 2D counterparts during differentiation. *Flow cytometric analysis of SSEA-3 expression shows that when TERA2.cl.SP12 cells are grown in 2D and 3D for 10 passages then put back into 2D culture and differentiated using ATRA, 3D maintained cells appear to differentiate more rapidly than 2D cultured cells due to decrease in SSEA-3 expression. Data represents means ± SEM, n=3 per data point. Statistical analysis was Student's t-test.*

3.3.2. 3D maintenance of stem cells leads to greater developmental potential *in vivo*

TERA2.cl.SP12 cells were cultured in either 2D or 3D for 4, 6 and 10 passages before being transplanted subcutaneously into the flanks of immunodeficient mice. The cells then created teratomas whose growth was measured using callipers until their size was large enough for harvesting.

Cells previously passaged x10 in 3D culture produced larger teratomas than cells previously cultured in 2D (Figure 3.3.2. A). Additionally, teratomas produced from cells cultured in 3D for 6 passages created significantly heavier teratomas upon harvest than cells cultured in 2D ($p<0.05$) (Figure 3.3.2. B).

These data suggest that prolonged culture of stem cells in 3D leads to cells possessing an increased potential for growth *in vivo* as seen by the creation of larger and heavier teratomas compared with teratomas created by cells passaged in conventional 2D culture. In order to account for cyst formation, histological analysis of teratoma sections was carried out and found no qualitative difference between number and/or size of cysts between teratomas produced from 2D and 3D cultured cells. Furthermore, teratomas derived from 3D maintained cells appear structurally more heterogeneous indicating more differentiation.

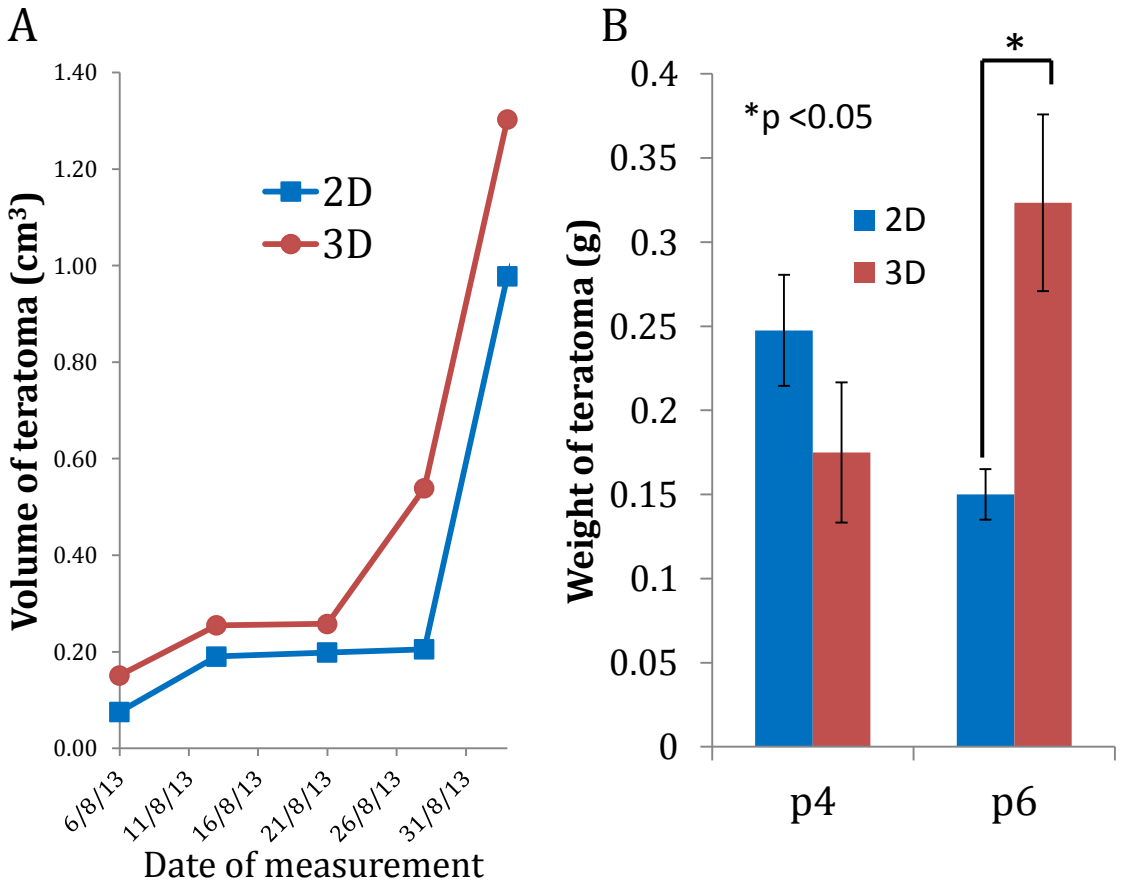


Figure 3.3.2. Cells maintained in 3D create larger and heavier teratomas in vivo. *(A): Individual tumour volume data showing teratoma growth from 2D and 3D cultured cells injected after passage 10; (B): Average weights of harvested teratomas from 2D and 3D cultured cells injected after 4 and 6 passages. Data represents means ± SEM, n=4 for p4 samples and n=3 for p6 samples. Weights below 0.1g were omitted. The above data shows that cells maintained in 3D and then transplanted into a mouse create significantly larger tumours after 2.5 weeks of growth in vivo and heavier tumours upon harvesting than 2D counterparts. This difference could be due to 3D maintained cells having adapted during 3D cell culture influencing their ability to differentiate in vivo.*

3.3.3. Teratomas created from 3D maintained stem cells lack undifferentiated cells

TERA2.cl.SP12 cells were cultured in either 2D or 3D for 4 and 10 passages before being transplanted subcutaneously into the flanks of immunodeficient mice. The derived teratomas from these procedures were processed, sectioned and stained with the stem cell marker Oct4 to visualise undifferentiated cells and Haematoxylin & Eosin to visualise tissue structure. Tissue was stained using immunohistochemistry.

Oct4 expression was not detected in teratomas derived from cells cultured in 3D for 4 and 10 passages, suggesting absence of undifferentiated cells (Figure 3.3.3. E and G). In contrast, tumours derived from cells cultured in 2D for 4 and 10 passages, both presented cells expressing Oct4 (Figure 3.3.3. A and C, white arrows).

These data suggest that culture of stem cells in 3D enhances their ability to differentiate *in vivo* as seen by the lack of positive Oct4 staining in teratomas derived from 3D cultured cells. This enhanced ability to differentiate is perhaps due to the 3D microenvironment in which the cells were initially passaged. These data correlate with the more rapid decrease in SSEA-3 expression during induction of differentiation *in vitro* (Figure 3.3.1) of 3D passaged cells.

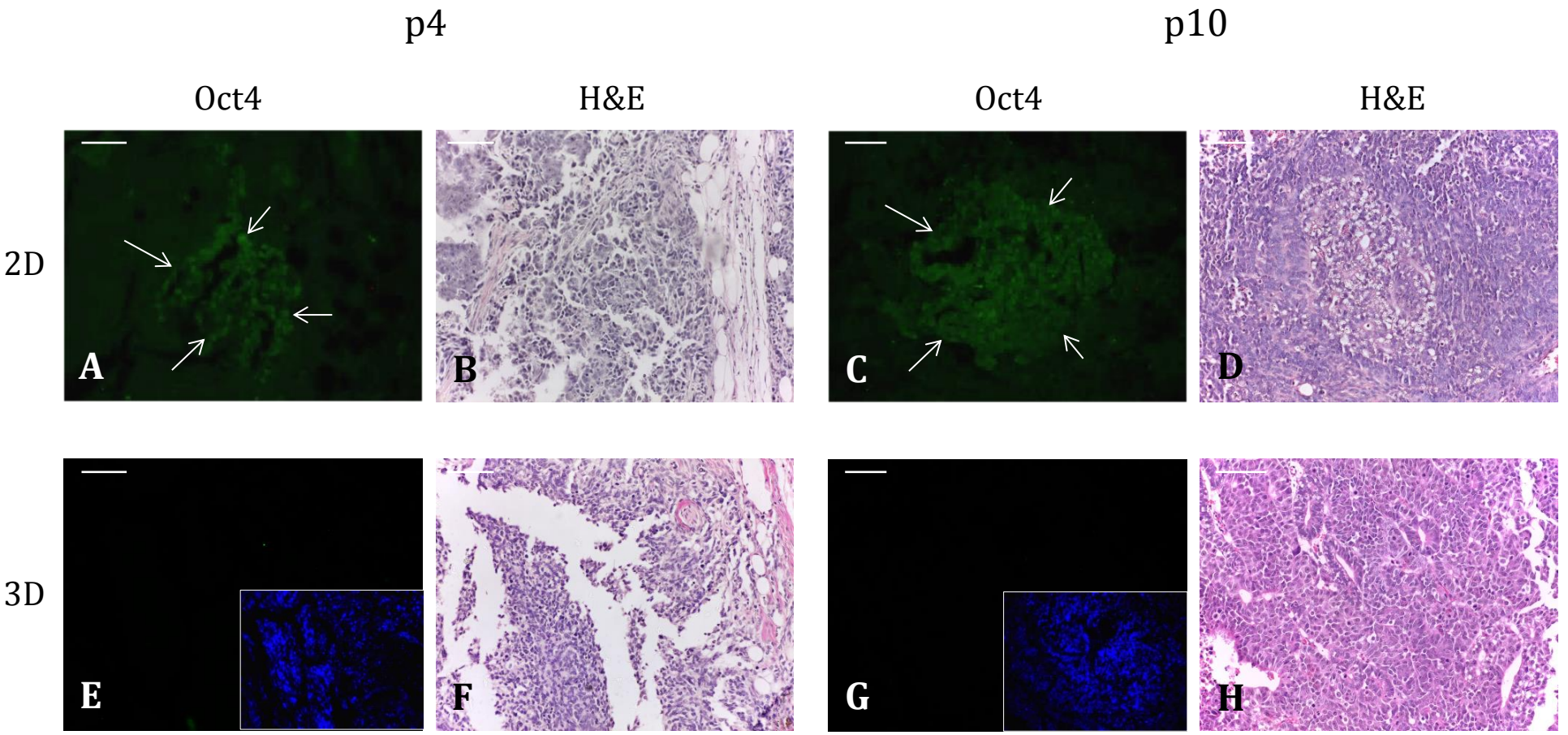


Figure 3.3.3. Teratomas from cells maintained in 3D lack Oct4 expression suggesting more advanced differentiation. *(A) and (C): Oct4 expression of teratoma sections from cells cultured in 2D for 4 (A) and 10 (C) passages with corresponding haematoxylin and eosin stained samples (B) and (D); (E) and (G): Oct4 staining of teratoma sections from cells cultured in 3D for 4 (E) and 10 (G) passages (nuclei are inset in blue stained with Hoescht 33342) with corresponding haematoxylin and eosin stained samples (F) and (H). These images show immunohistochemistry of Oct4 (a non-differentiation marker) staining from teratoma sections (6μm). Teratomas from cells maintained in 3D lack any Oct4 positive staining suggesting that the cells differentiated more fully. In contrast, 2D samples which have large areas of Oct4 positive staining (arrows). Scale bars - 100μm.*

3.3.4. Teratomas created from 3D maintained stem cells produce more definitive neural structures

Teratomas are produced from cells cultured in 3D on Alvetex® Strata or in 2D in conventional flasks as before. The teratomas from these procedures were processed, sectioned and stained with TUJ-1 to visualise neuronal structures and Haematoxylin & Eosin to visualise tissue structure. Tissue was stained using immunohistochemistry.

Cells cultured in 3D created teratomas containing more defined and robust neuronal structures than teratomas created from cells cultured in 2D (Figure 3.3.4. C, arrows). Teratomas created from cells cultured in 2D show more diffuse, lower level staining for TUJ-1 (Figure 3.3.4. A) which lacked the specific organisation seen in teratomas derived from cells cultured in 3D.

These data suggest that culture of stem cells in 3D leads to the formation of more defined neuronal structures when the cells produce teratomas. This seemingly more advanced differentiation is perhaps due to the 3D microenvironment in which the 3D passaged cells were initially grown enabling the cells to mature further *in vivo* and form more defined neuronal structures.

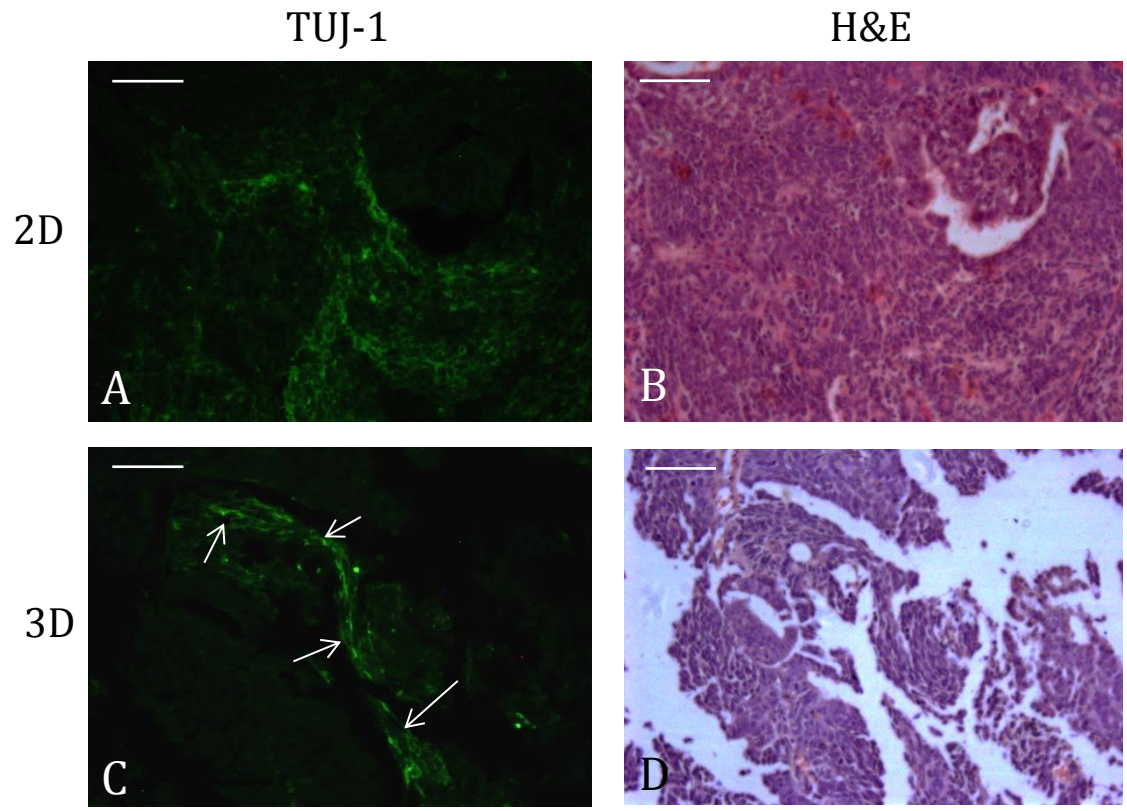


Figure 3.3.4. Teratomas from 3D maintained cells create more robust and defined TUJ-1 positive structures. *Data show typical examples of immunohistochemical localisation of the neural marker TUJ-1. (A) and (C): TUJ-1 positive staining of teratoma sections from cells cultured in 2D (A) and 3D (C) for 4 passages with corresponding haematoxylin and eosin stained samples (B) and (D). These images show immunohistochemistry of TUJ-1 (an early neuronal marker) staining from teratoma sections (6μm). Cells maintained in 3D create teratomas with large structure precursors with more definitive expression (arrows) whereas teratomas from cells passaged in 2D merely contain general areas of TUJ-1 positive staining. Scale bars - 100μm.*

3.3.5. Prolonged maintenance of stem cells in 3D creates teratomas with extensive epithelial markers

TERA2.cl.SP12 cells were cultured in either 2D or 3D for 4, 6 and 10 passages before being transplanted subcutaneously into the flanks of immunodeficient mice. The derived teratomas from these procedures were processed, sectioned and stained with cytokeratin 8 to visualise epithelial cells and Haematoxylin & Eosin to visualise tissue structure.

Cells cultured in 3D produced teratomas showing more positive staining for CK8 than cells cultured in 2D at passages 4, 6 and 8. which is qualitatively shown by the number of white arrows (Figure 3.3.5.). Furthermore, not only do teratomas derived from cells cultured in 3D contain more CK8 positive regions than their counterparts from 2D cultured cells but the higher the passage number of the cells cultured in 3D used to create the teratoma, the more positive and intense CK8 staining can be found.

These data further suggest that culture of stem cells in 3D leads to more advanced differentiation *in vivo* as seen by the larger number and generally higher CK8 staining found in teratomas derived from 3D cultured cells. This enhanced developmental potential is perhaps due to the 3D microenvironment in which the cells were initially passaged which in turn influences the ability of cells to differentiate *in vivo*.

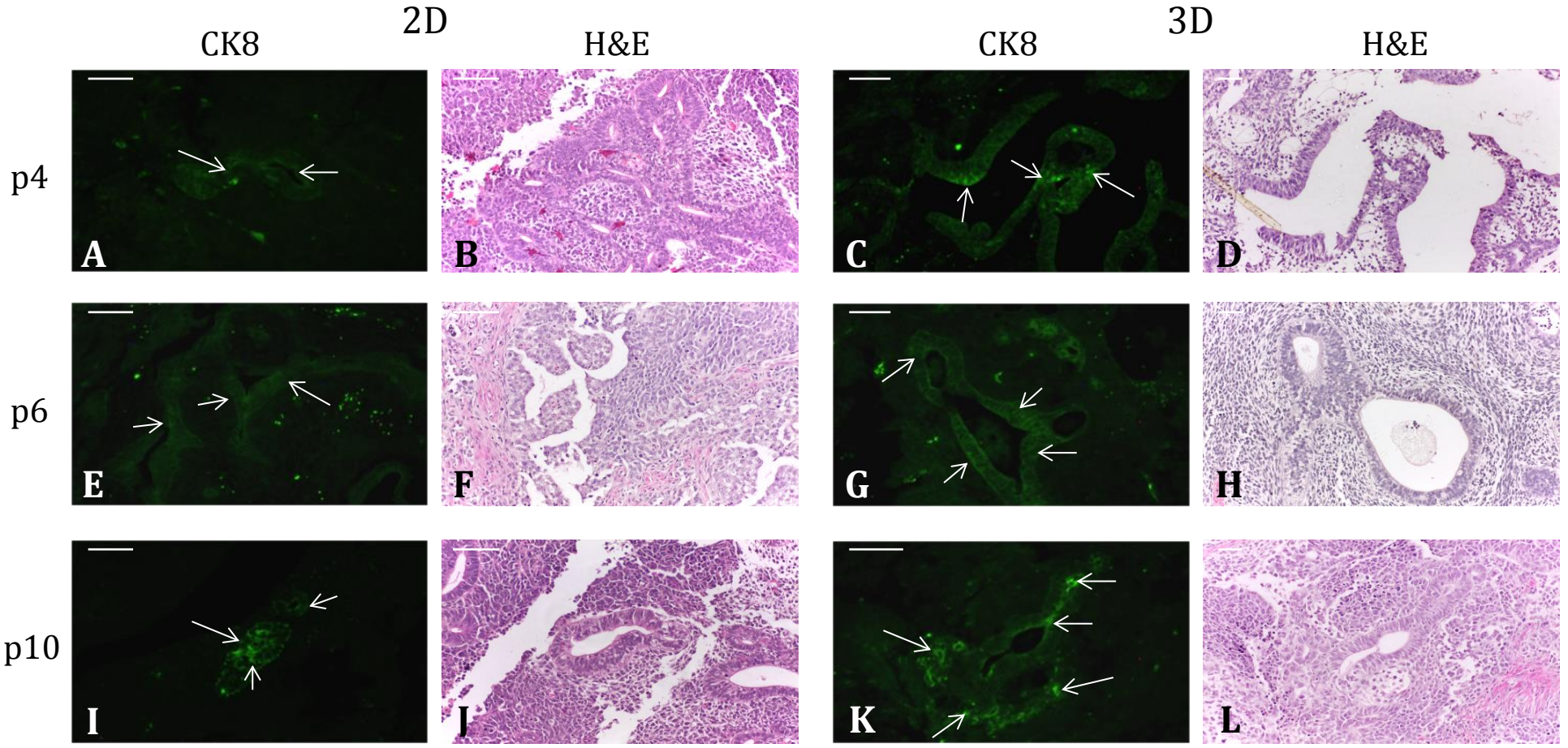


Figure 3.3.5. Teratomas derived from 3D maintained stem cells express more cytokeratin 8 than those derived from 2D passaged stem cells. *(A), (E), (I): CK8 expression of teratoma sections from cells cultured in 2D for 4 (A), 6 (E) and 10 (I) passages with corresponding haematoxylin and eosin stained samples (B), (F) and (J); (C), (G), (K): CK8 expression of teratoma sections from cells cultured in 3D for 4 (C), 6 (G) and 10 (K) passages with corresponding haematoxylin and eosin stained samples (D), (H) and (L). These images show immunohistochemistry of cytokeratin 8 (an epithelial marker) staining from teratoma sections (6μm). There is qualitatively more positive cytokeratin 8 staining (arrows) in teratomas created using 3D maintained cells at all passages compared with 2D cultured cells. Scale bars - 100μm.*

4. Discussion

The goal of 3D cell culture is simple: to mimic *in vivo* cell behaviour outside of the body. Whilst this may not yet have been achieved, research carried out using cells cultured in 3D gives more physiologically relevant results, closer to what one expects from cells *in vivo* in terms of gene expression, shape and behaviour. Furthermore, stem cells gain enhanced cell growth and differentiation as a result of 3D cell culture. Cell-cell contact, ECM establishment and intercellular signalling all differ between cells cultured in 2D and 3D with 3D cultured cells possessing an enhanced phenotype regardless of cell type. Compared against conventional 2D cell culture, cell culture in 3D allows us to obtain more accurate results and higher yields of specific cells when differentiating pluripotent cells. The effects of this work may impact upon many areas including drug discovery/toxicology and regenerative medicine (to ensuring that large numbers of mature cells can be grown for transplant).

4.1. Comparison of Alvetex® Strata with other 3D cell culture techniques

We demonstrate that long term maintenance of undifferentiated pluripotent stem cells is feasible in 3D culture. We are not aware of any other model of 3D cell culture that has achieved what Alvetex® Strata has shown.

Conventional methods encourage the growth of pluripotent stem cells as monolayers in 2D culture. Whilst this is routinely possible for EC cell lines, hESCs are normally grown as clusters/colonies. A limitation of this method is heterogeneity of cells within a colony (cells at the edges will be afforded more space to spread yet the central cells will be tightly packed together). Clearly a more efficient way of growing cells for experimental purposes is a monolayer to allow global consistency within a population. This was achieved using Accutase dissociation of hESCs colonies which did not result in apoptosis and maintained pluripotency as measured by Oct4 expression (Bajpai *et al.*, 2008). This is a stepping stone towards effective *in vitro* culture of hESCs however this study was only carried out in 2D culture and showed solely maintenance of pluripotency. Previous pilot work demonstrated that hESCs can not only be maintained in 3D in an undifferentiated state but also express increased pluripotency markers (Knight, 2013).

A precursor and sister product to Alvetex® Strata is Alvetex® Scaffold which is an identical material however the pore and void sizes are around 3x larger. Alvetex® Scaffold has already been shown to facilitate 3D growth of larger cells (HepG2 cells) (Bokhari *et al.*, 2007). This model is not suitable for long term TERA2.cl.SP12 cell propagation (mainly due to pore size) and in fact results in differentiation when cells are grown using Alvetex® Scaffold (Knight *et al.*, 2011). To retain their pluripotent stem cell phenotype during passaging, TERA2.cl.SP12 cells are grown as a mass of 3D cells on the surface of the Alvetex® Strata membrane. When grown in this way, cells are in constant and substantial contact with each other which facilitates signalling and ensures a 3D morphology is acquired and maintained.

A benefit of using Alvetex® Strata as the method of 3D propagation of pluripotent stem cells is that the substrate is chemically identical to normal tissue culture plastic, only the geometry of the plastic has been changed. Certain other 3D methods have a matrix composition that may directly affect the cells and so influence *in vitro* results and is not desirable.

A chitosan scaffold as created by Li and colleagues, which is biodegradable and so perhaps possesses a higher potential for transplantation and clinical work, was able to maintain undifferentiated hESC growth for 21 days (Li *et al.*, 2010). There may be an unknown effect on the hESCs from the chitosan makeup of the scaffold (derived from crustacean shells) particularly in terms of ECM formation and cytoskeletal signalling/structure. The reason for using this substrate was to substitute the mouse embryonic fibroblastic feeder layer on which hESCs were traditionally grown. This method in itself differs from the *in* vivo hESC microenvironment and so is not appropriate. There was no apparent change in pluripotency expression over the 3 week growth. In contrast, stem cell marker expression of cells grown in 3D on Alvetex® Strata showed an increase over time. A possible reason for the lack of increase in pluripotency marker expression on chitosan scaffolds could be an unknown effect of the matrix composition. A similar observation was reported in a paper which used chitin rather than chitosan and the pluripotency of the human stem cells did not increase after 10 passages and was similar to 2D culture with Matrigel (Lu *et al.*, 2012). Differences in scaffold components can therefore be responsible for different experimental outcomes.

The influence of the 3D microenvironment has been previously demonstrated when considering less potent cells which exist in specific niches in adult somatic tissues. Adipose tissue derived stem cells were grown on scaffolds composed of varying amounts of collagen and chitosan. These proteins in combination present a niche similar to that which adipose derived stem cells would experience *in vivo* (i.e. collagen-rich) but one that also has structural integrity (due to the chitosan component) (Zhu *et al.*, 2009). This microenvironment may not be suitable for growth of, for example, hESCs since different cell types have different niche requirements. This is especially important when attempting to grow cells *in vitro*. It is key that the microenvironment of the cell should not differ from the specific niche in which the cell would develop *in vivo* or at least be inert and not preferentially influence cell behaviour, as is the case with Alvetex® Strata. Alvetex® Strata allows for 3D growth of cells as a thick, tissue-like layer allowing cells to create their own local microenvironment through secretion of ECM components and establishing direct cell-cell contact. Such features are also important in maintaining an appropriate niche microenvironment.

Alvetex® Strata may have advantages over other methods of 3D cell culture such as hydrogels and EBs. For example, cells are easily accessible on top of the membrane on Alvetex® Strata and can be scraped off for analysis whereas in hydrogels, cells are encased within the gel. Additionally, EBs often contain central regions of necrosis.

Other promising developments in long term propagation of pluripotent stem cells centres on the coating of polymers. One specific method consisting of coating polymers with poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH) allows for long term growth of hESCs (Villa-Diaz *et al.*, 2010). The benefit of this coating is that it allows for serum free propagation of hESCs and so alleviates the need for xenogeneic culture components. This method creates a synthetic microenvironment that facilitates propagation of undifferentiated cells however the PMEDSAH coating has only been tested in 2D culture. There is potential to combine this coating with other technologies such as Alvetex® Strata providing further synergistic advantages.

There are many benefits to culturing cells in 3D. These impact on several areas of science including: basic research, drug discovery and clinical applications. The aim of using 3D cell culture is to enable

cells to mimic *in vivo* growth conditions more closely than cells cultured on conventional 2D substrates. Furthermore, as shown in this project, stem cells cultured in 3D differentiate more effectively both *in vitro* and *in vivo* which may increase yields of target cell types. In drug discovery, experiments may have led to false conclusions regarding the effectiveness of certain drugs as the compounds may or may not have worked on 2D cultured cells however such compounds could show a different effect on 3D cultured cells. For clinical applications, there may be benefits if cells acquire a more *in vivo*-like phenotype when cultured in 3D prior to implantation and therapeutic use. Preparing cells with a 3D phenotype may provide a higher chance of successful integration with patient tissues.

4.2. Continued maintenance of cells in 3D leads to adoption of a 3D phenotype

As shown in Results Section 3.2., the culturing of TERA2.cl.SP12 cells on Alvetex® Strata leads to the acquisition of a 3D rounded, small phenotype more typical of cells *in vivo*. This adaptation to the 3D microenvironment provided by growth on Alvetex® Strata is not instantaneous as shown in Figure 3.2.1. After 6-8 passages however, the 3D maintained cells have adapted and showed less spreading/flattening when put back into 2D culture. This maintenance may be as a result of changes in and re-organisation of the cytoskeletal structure over time; this feature of 3D culture adaptation may have been able to be viewed using histology/high power microscopy at various time points throughout the adaptation process.

Growth of cells on Alvetex® Strata is the first example of long term propagation of human pluripotent stem cells in 3D. This leads to an increase in pluripotency through cell shape change. These findings are novel since there is not much published work to which these data can be directly compared. Recently however a study has shown that regardless of the type of scaffold used for 3D cell culture, cells cultured in 3D lead to cells possessing higher pluripotency markers (Wei *et al.*, 2014). The group used mouse ESCs grown on scaffolds made of collagen, PLGA and chitosan. The goal was to directly compare pluripotency marker expression upon addition of the third dimension rather than scaffold composition. The results showed that introducing the third dimension to cell culture leads to cells expressing higher levels of stem cell markers indicative of a more stem cell like phenotype compared with culture on conventional 2D substrates. Scaffold composition appears to play a part in stem cell maintenance in 3D culture, as there were differences between the three

types of scaffolds used by Wei and colleagues. An advantage of 3D culture using Alvetex® Strata is that it is chemically inert and so attempts to negate this effect.

ROCK (and the Rho GTPases) has long been known to be involved in not only cytoskeletal organisation but signalling cascades and mediating gene expression changes (as reviewed by (Aspenström, 1999)). ROCK is potentially involved in acquisition of the 3D phenotype and maintenance as inhibition of ROCK by Y-27632 leads to an increase in cell spreading and loss of the 3D phenotype. Similarly, ROCK inhibition causes an increased flattening response in long term 2D cultured cells indicating that ROCK may act in maintaining general cell shape.

Qualitative results showing that inhibition of ROCK causes changes to f-actin structure is backed up by a recent paper in which it was described that use of Y-27632 does decrease the amount of actin polymerisation within a cell causing change in structural phenotype such as adaptation to the 3D cell phenotype (Seo *et al.*, 2011). There is however some debate in the literature regarding whether addition of Y-27632 ROCK inhibitor causes cell flattening. Bhadriraju and colleagues (2007) present contrary results to findings of this project. The group used pulmonary artery endothelial cells to examine cell adhesion, spreading and cytoskeletal tension but this is a cell type decidedly different from the TERA2.cl.SP12 pluripotent stem cells used in this project. Their cells have a much higher tendency towards spreading and flattening due to their natural *in* vivo microenvironment compared with TERA2.cl.SP12 cells (Bhadriraju *et al.*, 2007). Further work backing the findings of this project indicate that ROCK inhibition may lead to more phosphorylated FAK which causes increased cell adhesion (through more FAs) to the substrate surface and resulting in increased cell flattening (Wozniak *et al.*, 2003).

Cells cultured in 3D on Alvetex® Strata change their shape and cytoskeletal structure over time. The cells acquire a 3D morphology that is evident from morphological analysis and from reduced cell spreading when 3D propagated cells are put back into 2D culture. This acquisition takes time and is present after 8 passages in 3D culture but not after 3 passages. There appears to be an adaptation period critical to acquiring this modified phenotype.

4.3. Maintenance of stem cells in 3D alters their developmental potential

It is shown in Results Section 3.3. that culturing TERA2.cl.SP12 cells on Alvetex® Strata leads to changes in the ability of the cells to differentiate indicating an increase in their developmental potential. This was demonstrated by the enhanced decrease in expression of the pluripotency marker SSEA-3 in cells exposed to retinoic acid and grown in 3D culture for 10 passages than those grown in 2D culture for 10 passages upon addition of retinoid (Figure 3.3.1). A decrease in SSEA-3 expression suggests loss of the stem cell phenotype and is characteristic of induction of differentiation. This apparently more rapid induction of differentiation response *in vitro* may be due to the adaptation to 3D culture and their enhanced developmental potential.

Culture of pluripotent stem cells in 3D also led to the formation of heavier and larger teratomas *in vivo* than those produced by cells cultured in conventional 2D (Figure 3.3.2.). Teratomas produced from 3D propagated cells were more heterogeneous and contained differentiated structures that were generally more diverse, notably there was: a lack of undifferentiated cells (Figure 3.3.3.), more defined neuronal structures (Figure 3.3.4.), and more intense and concentrated cytokeratin 8 immunostaining (Figure 3.3.5.). This enhanced ability for differentiation *in vivo* of 3D propagated cells may also be a result of adaptation to the 3D microenvironment facilitated by Alvetex® Strata in which the cells were initially grown.

There is little doubt in the literature that expression of SSEA-3 is a sign of pluripotency of a cell however there is some semantic debate on whether expression of the surface marker itself is required for maintenance of pluripotency (Brimble *et al.*, 2007). That aside, it has long been shown that EC cells are pluripotent and express SSEA-3 as a marker of the stem cell phenotype which is lost as cells commit to differentiation (Andrews *et al.*, 1982). There is published work using the hESC cell line H7 (a cell line morphologically similar to EC cells) that shows a decrease of SSEA-3 expression upon differentiation (Draper *et al.*, 2002) which supports data from Figure 3.3.1. It was shown in Figure 3.1.3. that prolonged culture of cells in 3D on Alvetex® Strata leads to increased SSEA-3 marker expression yet the mean values used in Figure 3.3.1. are lower than perhaps expected for 3D maintained cells. This observation could be due to the 3D passaged stem cells adapting to the new 2D microenvironment in which they are in and differentiating in response to the retinoid at the same time. This would create a challenging environment for the cells and may explain the slight inconsistencies in SSEA-3 expression values.

Another potential reason for an increase of SSEA-3 expression seen in stem cells cultured in 3D could be down to the geometry of the cell itself. If a cell is cultured on conventional 2D substrates, much of the cell is in contact with the substrate and so the cell cannot express the membrane protein SSEA-3 throughout its membrane. If a cell is cultured in 3D, the cell does not have as much contact with the substrate surface (in cells grown on Alvetex® Strata, some in the tissue layer would have no contact) and so has more surface area on which it can express SSEA-3 protein.

The teratomas derived from cells cultured in both 2D and 3D microenvironments show positive staining for the neuronal marker TUJ-1. There is positive, diffuse staining in teratomas produced from 2D cultured cells however there are more defined structures in the teratoma produced from 3D maintained cells. A theory has been proposed recently which states that EC cells have 2 states – neuronal and non-neuronal precursors (Tonge *et al.*, 2010). This could explain the high levels of TUJ-1 positive structures in teratomas produced by 2D and 3D passaged cells alike. Regardless of the state in which the EC cells were in at the time, when the 3D passaged cells differentiated *in vivo*, the structures within teratomas formed were more mature and defined compared to teratomas derived from EC cells cultured in 2D. This again indicates more advanced differentiation.

Collectively, these data suggest that cells cultured in 3D on Alvetex® Strata possess a greater differentiation potential than cells cultured on conventional 2D substrates. In general, this can be seen by 3D maintained cells more rapidly decreasing pluripotency marker expression *in vitro* upon induction of differentiation and differentiating more thoroughly *in vivo*. This apparent increase in developmental potential of the 3D maintained cells is likely to be a result of long term propagation of the cells in 3D which also leads to increased SSEA-3 marker expression as seen in Figure 3.1.3.. This propagation leads to a cell shape change, as mentioned previously, which is likely to be responsible for the observed changes developmental potential. ROCK may be involved in the maintenance of this 3D morphology as inhibition of ROCK in 3D maintained cells is known to play a role in cells flattening out of cells across a 2D substrate.

These data implicate that it is the architecture of the cell shape which leads to the increased developmental potential of cells cultured in 3D on Alvetex® Strata. Importantly, this shape change results in maintenance of the shape of the nucleus in a more *in vivo*-like spherical structure, as can

be seen in Figure 3.1.2. which shows that before cells flatten, they possess a spherical nucleus which is distorted upon flattening. Research agrees that changing cell shape (through flattening, mechanical stretching etc.) negatively affects normal gene expression due to compression of the nucleus and disruption of normal gene transcription, transport in and out of the nucleus and nuclear matrix organisation (Thomas *et al.*, 2002). It is hypothesised that culture of cells in 3D (and specifically on Alvetex® Strata) preserves the normal 3D nuclear structure and therefore prevents this abnormal gene expression. Moreover, it is seen that based on a more 3D, spherical nucleus afforded by culture of stem cells on Alvetex® Strata (Figure 3.1.4.), cell differentiation is enhanced both *in vitro* and *in vivo* (Figure 3.3.1. and Figures 3.3.3.-3.3.5.).

In summary, the experimental data presented in this thesis suggest that cell shape and architecture play an important role in maintenance of the stem cell phenotype and their subsequent ability to differentiate. We propose that it is beneficial to culture stem cells in a more natural 3D state. This could have major implications in downstream applications of stem cells in basic research, drug discovery and therapeutic applications. Enabling stem cells to differentiate and develop mature tissues is a major challenge facing scientists. Everything needs to be done in order to create a more favourable environment for cell differentiation to occur, including maintenance of the 3D environment and state.

4.4. Future work and further experiments

ROCK inhibition experiments carried out in this project implicate a role in cytoskeletal re-organisation during adaptation to a 3D phenotype afforded by culture using Alvetex® Strata. Additional experiments are needed to further explore this concept.

The data presented in this thesis suggest that there may be a link between maintenance of a 3D phenotype leading to increased pluripotency and that inhibition of ROCK may lead to loss of this acquired phenotype. As a result of this concept, it may be further proposed that cells which gain and then lose their 3D morphology (through the inhibition of ROCK) should possess a similar developmental potential to cells cultured in 2D throughout; unless the slow adaptation and acquisition of a 3D phenotype causes epigenetic modification(s) of the genome of the cell resulting

in pluripotency that is 'hardwired'. This suggestion would fit with aforementioned evidence of phenotype being perhaps even more important than genotype (Weaver *et al.*, 1997).

To further explore the role of ROCK signalling, it could be useful to see the effect of inhibiting exclusively RhoA (the upstream activator of ROCK) and to examine whether this causes the same reaction in the cells as seen with ROCK inhibition (an increased flattening response when cells go from a 3D microenvironment onto a 2D substrate). This again can be adapted to observe whether the morphological change (adaptation to 3D) can be stopped if cells are cultured continually in the presence of RhoA inhibitor.

Perhaps the most important further experiment is to examine whether this effect of increased pluripotency and developmental potential as a result of 3D cell culture is limited to this TERA2.cl.SP12 EC cell line or if it can be seen in other pluripotent stem cell lines for example ESCs, induced pluripotent stem cells, MSCs, etc.

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