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Nanoscale definition of substrate materials to direct human adult stem cells towards tissue specific populations

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Abstract The development of homogenously nano-patterned chemically modified surfaces that can be used to initiate a cellular response, particularly stem cell differentiation, in a highly controlled manner without the need for exogenous biological factors has never been reported, due to that fact that precisely defined and reproducible systems have not been available that can be used to study cell/material interactions and unlock the potential of a material driven cell response. Until now material driven stem cell (furthermore any cell) responses have been variable due to the limitations in definition and reproducibility of the underlying substrate and the lack of true homogeneity of modifications that can dictate a cellular response at a sub-micron level that can effectively control initial cell interactions of all cells that contact the surface. Here we report the successful design and use of homogenously molecularly nanopatterned surfaces to control initial stem cell adhesion and hence function. The highly specified nano-patterned arrays were compared directly to silane modified bulk coated substrates that have previously been proven to initiate mesenchymal stem cell (MSC) differentiation in a heterogenous manner, the aim of this study was to prove the efficiency of these previously observed cell responses could be enhanced by the incorporation of nano-patterns. Nano-patterned surfaces were prepared by Dip Pen Nanolithography® (DPN®) to produce arrays of 70 nm sized dots separated by defined spacings of 140, 280 and 1000 nm with terminal functionalities of carboxyl, amino, methyl and hydroxyl and used to control cell growth. These nanopatterned surfaces exhibited unprecedented control of initial cell interactions and will change the capabilities for stem cell definition in vitro and then cell based medical therapies. In addition to highlighting the ability of the materials to control stem cell functionality on an unprecedented scale this research also introduces the successful scale-up of DPN® and the novel chemistries and systems to facilitate the production of homogeneously patterned substrates (5 mm^2) that are applicable for use in in vitro cell conditions over prolonged periods for complete control of material driven cell responses.

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1 Introduction

Research into delivering on the potential of stem cells and in this case specifically human adult mesenchymal stem cells as a reliable and reproducible cell of choice for cell therapies and regenerative medicine continues to progress at great pace. Sourcing the correct stem cells is a prerequisite; however it is only the first step, with subsequent processes being critical to success. One of the key areas of research impetus in human adult stem cells is directed towards developing systems and their definition to unlock the potential of these cells such that they can be delivered reproducibly as a known entity to provide for predictable clinical outcomes. If primary isolated cells are utilised immediately in vivo it may be sufficient just to isolate a therapeutic number and deliver them appropriately, but

expansion and maintenance in vitro requires precise definition of parameters i.e. media, oxygen tension, temperature and substrate if they are to progress from the bench to the body. At present the substrate of choice for the majority of our in vitro isolation and expansion experiments is tissue culture polystyrene (TCPS), and additional control over function is often achieved using exogenous biological factors often presented to the cells in the form of media supplements, often of animal origin. The question has recently been asked is this the safest and most efficient approach for the reproducible stimulation of all cells in a population, or can the efficiency of a given reaction be increased by improving the homogeneity and stability of the stimulus provided to the cells?

Stem cells, from an increasing number of human adult tissue sources like adipose, marrow, dental pulp and blood, play a pivotal role in the developing emergent treatments of regenerative medicine. If sourced and manipulated correctly the benefits of these cell types will revolutionise medical therapies with a massive impact on global healthcare economics; clearly these significant objectives as end points justify the massive academic, commercial and public interest in stem cell and stem cell related research. The as yet undefined tissue niches which support novel sub-populations of stem/pluri-potent cells are increasing, and each discovery of a new novel population of stem/stem-like cells increases the need for a better understanding of what factors maintain, or induce specific types of cell behaviour in situ, and perhaps more importantly increases the urgency for the definition of how we can replicate these factors in vitro to produce reproducible cell populations and associated tissue engineered constructs that can be used effectively in regenerative medicine. A major component of this and a key tool to maintaining or controlling cell behaviour in vitro is the underlying substrate and the associated spatial cues that control initial protein and cell interactions.

Control over stem cell phenotype and function is urgently required to provide these cells with form and function to realise their potential and deliver therapeutic benefits, allowing efficient reproducible tissue formation and cellular function in the appropriate environment at all points in the scaffold. This has been attempted previously using a variety of methods but none work effectively with embryonic or adult stem cells [1–8]. Recently the use of chemically modified surfaces has provided encouraging results validating the hypothesis of initiating limited levels of differentiation by direct material contact, but what is actually driving the response is still unclear. The potential to control a cellular response, more specifically induce differentiation of Mesenchymal stem cells (MSC) using material driven spatial stimuli has both far reaching academic and commercial benefits. Successful incorporation of simple chemical modifications within a material that reproducibly drive a cellular response could lead to the formulation of the next generation of off the shelf smart tissue engineered scaffolds that have superior handling properties and could be manufactured to meet the requirements of a range of applications. These materials could also be chemotactic and reduce the need for extensive in vitro manipulation, once again enhancing the cost effectiveness of the materials. This study presents data that proves that MSC differentiation and functionality can be controlled by simple chemical modifications i.e. chemical modifications can be introduced using silane modification techniques to an array of base substrates that result in controlled MSC differentiation. Cell population data has provided positive indications that surfaces, glass, gold, PLLLA and PCL, modified with terminal methyl groups principally promoted and maintained the undifferentiated mesenchymal stem cell (MSC) phenotype, maintenance of STRO1 phenotype and the potential to differentiate into osteoblasts and chondrocytes when cultured in the appropriate medium [9]. Surfaces modified with amino groups triggered an osteoinductive response from MSC in both basal and osteogenic medium. Surfaces modified with hydroxy and carboxy groups preferentially induced the chondrogenic phenotype in MSCs, once again in the absence of exogenous biological stimuli, resulting in the production of collagen I. However all of these types of surfaces only directed a proportion of the complete cell population, on all base substrates tested, which currently prevents their use in producing homogeneous cell populations under controlled conditions. To realise the true potential of material induced cell functionality responses, emerging technologies which allow the precise deposition of modifications (chemical groups or biological factors) in a specific co-ordinate on the surface in a defined array need to be combined with emerging cell isolation, characterisation and maintenance protocols.

The surfaces previously reported to achieve this partial control consisted of self assembled monolayers (SAMs) or nanopatterned peptides. SAMs consisting of the chemical functionalities mentioned have been proven to trigger human MSC differentiation on a range of substrates; however this approach still lacks the necessary complete control over the entire cell population to be used as a method for directed differentiation [9]. In parallel emerging technologies associated with nanotopography and the use of nano-patterned peptide surfaces have presented extremely promising results in terms of controlling initial cellular adhesion and subsequent cell functionality [10, 11]. This research clearly demonstrates that nanotopography, and more specifically distribution of a selected stimuli, in this case an RGD peptide on the nanoscale in a controlled fashion can be used to control initial cellular responses, therefore the issue of producing nano patterned chemically modified surfaces should be addressed, that can be used to induce and control cell functionality in a reproducible and predictable manner.

Clearly if a defined and controlled nanopattern of specific chemical functionalities on a suitable surface could be produced, control of cellular adhesion and hence subsequent cell function could be assessed and engineered. In addition the knowledge of where the exact chemical groups are on a given surface would provide a tool to determine if cells respond to a chemical group, or actively avoid a chemical group, and addresses the issue of surface energy and its role in controlling or inhibiting protein adsorption and subsequent cell attachment. The combination of chemical modifications on a nano scale in a defined area (field), separated from adjacent fields of modification by a defined pitch in the nanoscale would provide control of focal contact formation from dictating chemical cues. The resultant effect on integrin clustering, focal contact formation and spacing between these events at this dimensional scale has the potential to dictate intracellular mechanotransduction via the formation of the cytoskeleton, a factor that is well known to control cell function [12–14].

Dip Pen Nanolithography, DPN®, is an emerging technology that uses an AFM tip coated via vapour or dipped into a liquid solution (an 'ink') of molecules which can then be written with nano precision onto a surface; scanning probe microscope tips are used to deliver defined molecules onto a desired surface via a water meniscus, as long as the molecules can interact with the underlying surface either by chemisorptions, physisorption, covalent or van der waals forces. This technique has proven to be a versatile lithographic method for various applications [15]. This study presents a direct comparison between bulk coated substrates and nano patterned chemical arrays to prove that firstly MSC differentiation can be induced by simple chemical groups and secondly the efficiency of the cellular response can be increased using chemical nano patterned arrays. Direct control over chemistry and pitch provide a tool that is not only invaluable for the development of fundamental research but also the production of modified surfaces that can increase the efficiency of current regenerative medicine technologies.

2 Materials and methods

2.1 Bulk coated glass

Glass coverslips (13 mm diameter, Borosilicate Glass Co. UK) were dipped into 5% NaOH solution for 1 h followed by concentrated HNO_3 for 1 h. All the coverslips were rinsed with ultra pure water followed by 100% ethanol for a total of 4 times, then dried at 120°C for 10 min, and stored in a vacuum desiccator prior to introduction of different functional groups on the surface (i.e. $–CH_3$, $–NH_2$, –SH, –OH, –COOH) by silanation.

$–CH_3$ functional groups were introduced to the coverslips via dipping into dimethyldicholorosilane (Supelco Park, USA) for 15 s, rinsed twice with toluene and three times with ethanol, dried and stored as previously described. $–NH_2$ groups were introduced via dipping into 0.5% 3-Aminopropyltrimethoxysilane (Aldrich) or (3-Mercaptopropyl)-trimethoxysilane (Sigma) isopropyl alcohol solution, then 1 ml water, refluxing for 30 min. After refluxing, the coverslips were rinsed in ultra pure water and ethanol and then dried at 120°C for 10 min and stored in a vacuum desiccator.

The –OH and –COOH functional groups were introduced on the coverslips surface by first introducing the vinyl group, Trimethoxy-vinylsilane (Fluka) using the same method as $–NH_2$ modified surfaces. For the –COOH group the vinyl surfaces were treated with an aqueous solution (0.5 mM $KMnO_4$, 9.5 mM $NaIO_4$, and 1.8 mM K_2CO_3) for 24 h with gentle stirring. Then the coverslips were rinsed with a 0.3 M $NaHSO_3$ solution, 0.1 N HCl, ultra pure water and ethanol and then dried at 120°C for 10 min and stored in a vacuum desiccator.

For the –OH group the vinyl surfaces were treated with a 1 M borane-tetrahydrofuran (Aldrich) solution for 2 h in nitrogen. The coverslips were then rinsed in anhydrous tetrahydrofuran (Fluka) for 30 min and then treated with 0.4% NaOH/30% H_2O_2 solution for 3 min., rinsed and dried as previously described. All surfaces were characterised using surface energy and contact angle measurements and were in line with previously published results [9].

2.2 Nano pattern arrays

Dip Pen Nanolithography®, NScriptor (Nanoink, Skokie, IL) system in contact mode feedback (set point = 0) was used to produce large areas of chemically defined homogenous surfaces using four different chemical functionalities deposited at the nanometre scale. DPN® provides a suitable technique for the controlled deposition of a wide range of materials such as alkyl thiols, silazanes, Au (III) complexes, and nanoparticles by using a scanning probe microscope tip to deliver defined molecules in the form of an "ink" onto a surface via a water meniscus. The size of the feature being written is related to the interaction between the tip, ink, meniscus and surface. In this study DPN® allowed a top-down nanoscale deposition of a number of inks that presented distinct chemical functionalities ($–CO_2H$, $–NH_2$, $–CH_3$, –OH). Careful selection of the materials allowed deposition in the form of isolated areas of SAMs onto surfaces via thiol linkages under low temperature and humidity conditions. In order to investigate the effect of both the nanopattern and the terminating chemical functionality, several nanopatterned gold surfaces were prepared. Each nanopattern comprised a series of parallel dots spaced by a

fixed distance (pitch, dα) and fixed diameter (dβ ~65–70 nm). Nanopatterns using each ink were prepared using differing values for dα (140, 280, 1000 nm) with dot diameter and spacing confirmed by lateral force microscopy (LFM). DPN® conditions were optimised to produce close to identical dot diameters for each ink. When conditions were optimised for small area pattern deposition larger surface areas were patterned transferring the inks to the DPN 5000 system (Nanoink, Skokie, IL). Once again conditions were optimised to ensure uniform deposition of the inks across the surface and eliminating edge effects to ensure a constant spatial stimulus to the cells.

2.3 MSC culture

Commercially available 4P human MSC (Lonza UK) were cultured in contact with the modified substrates at a concentration of 5×10^4 cells/1 ml basal medium (Lonza UK). 1 ml in 24 well plates was used for bulk coated samples and 0.5 ml in 48 well plates was used for nano patterned surfaces, these were chosen to minimise exposed TCPS in the culture wells. Cells were cultured in contact with the substrates for time periods up to and including 28 days at 37°C, 5% CO_2. At the relevant time periods samples were stained for vinculin, Collagen I, collagen II, collagen X, osteocalcin, CBFA1, aggrecan, F-actin (Oregon green Phalloidin) and DAPI (cell nuclei) and calcified extra cellular matrix using von Kossa, using previously described protocols [9, 16].

2.4 FACS analysis

MSC cultured in contact with nano patterned arrays were characterised using FACS analysis at 28 days. Cells were stained with CD29 (Cy5-PE conjugated, BD), CD90 (FITC conjugated BD), CD73 (PE conjugated, BD), CD105 (FITC conjugated, BD), CD166 (PE conjugated, BD), CD34 (FITC conjugated, BD) and CD45 (FITC conjugated, BD). Expression on modified surfaces was compared directly to levels of expression on cells cultured in contact with TCPS under the same conditions i.e. initial cell seeding density in a given surface area.

3 Results

The successful incorporation of the bulk chemistries was confirmed using contact angle and XPS measurements, the results obtained within this study were a replicate of measurements previously published [9, 16], but within this study confirmed the successful incorporation of the group of interest on the glass coverslips. This study presents a direct correlation between previously published bulk coated substrates and novel nano-patterned arrays.

When cultured in contact with bulk coated SAMs modified surfaces a distinct pattern of induced differentiation was observed. Firstly when cultured in contact with –CH_3 modified surfaces MSC maintained their characteristic phenotype i.e. no expression of markers associated with differentiation i.e. collagen II^{-ve}, $osteocalcin^{-ve}$, $aggrecan^{-ve}$ and no positive von Kossa staining, Fig. 1a–b. When cultured in contact with –OH modified surfaces MSC were positive for chondrogenic markers collagen II and aggrecan, Fig. 1c and d, but negative for markers of osteogenic differentiation and von Kossa, Fig. 1e. In contrast when cultured in contact with –NH_2 bulk coated surfaces osteogenic differentiation was induced, this was depicted by positive expression for CBFA1, osteocalcin, Fig. 1f and positive von Kossa staining, Fig. 1g. Induced differentiation into the osteogenic lineage was only observed on the –NH_2 modified surfaces, an observation confirmed by the fact that only cells cultured on these surfaces were positive for von Kossa staining after a 28 day time period in contact with basal medium. The levels of increased osteocalcin, CBFA1 and positive von Kossa staining were largely concentrated at the periphery of the modified coverslips, and with increasing time migrated towards the centre of the coverslip, a phenomena shown in Fig. 1f. This observation clearly demonstrates that factors other than surface chemistry are influencing the induced osteogenic differentiation within this experimental system. The discrete changes in surface energy associated with the edge of the coverslip are also affecting the differentiation potential of the cells, further enhancing the heterogenous nature of the induced differentiation driven by the –NH_2 group on the bulk coated surfaces.

To address the issue of "edge affects" and their role in induced differentiation and to enhance the spatial stimulus presented to the cells and increase the efficiency of the heterogenous cellular response observed on the bulk coated substrates, novel chemically nano-patterned substrates were produced using DPN® technologies. Parameters were optimised to ensure that the deposition of the chemical groups was homogenous across the surface. Substrates were characterised using AFM to ensure the optimisation of the parameters i.e. dwell time, Fig. 2. The size of the feature being written is related to the interaction between the tip, ink, meniscus and surface. The features dimensions can be controlled by varying tip surface-dwell times (the time the coated tip is in contact with the underlying surface), humidity and temperature. The combination of a large array of inks/chemical groups, nano-patterning and the development of new 2 D print array chip DPN® systems consisting of 55000 individual tips on a single chip, with the inherited AFM nano-precision have allowed the successful scale up of DPN® technologies to produce surfaces. This technology was successfully used to produce novel surfaces that

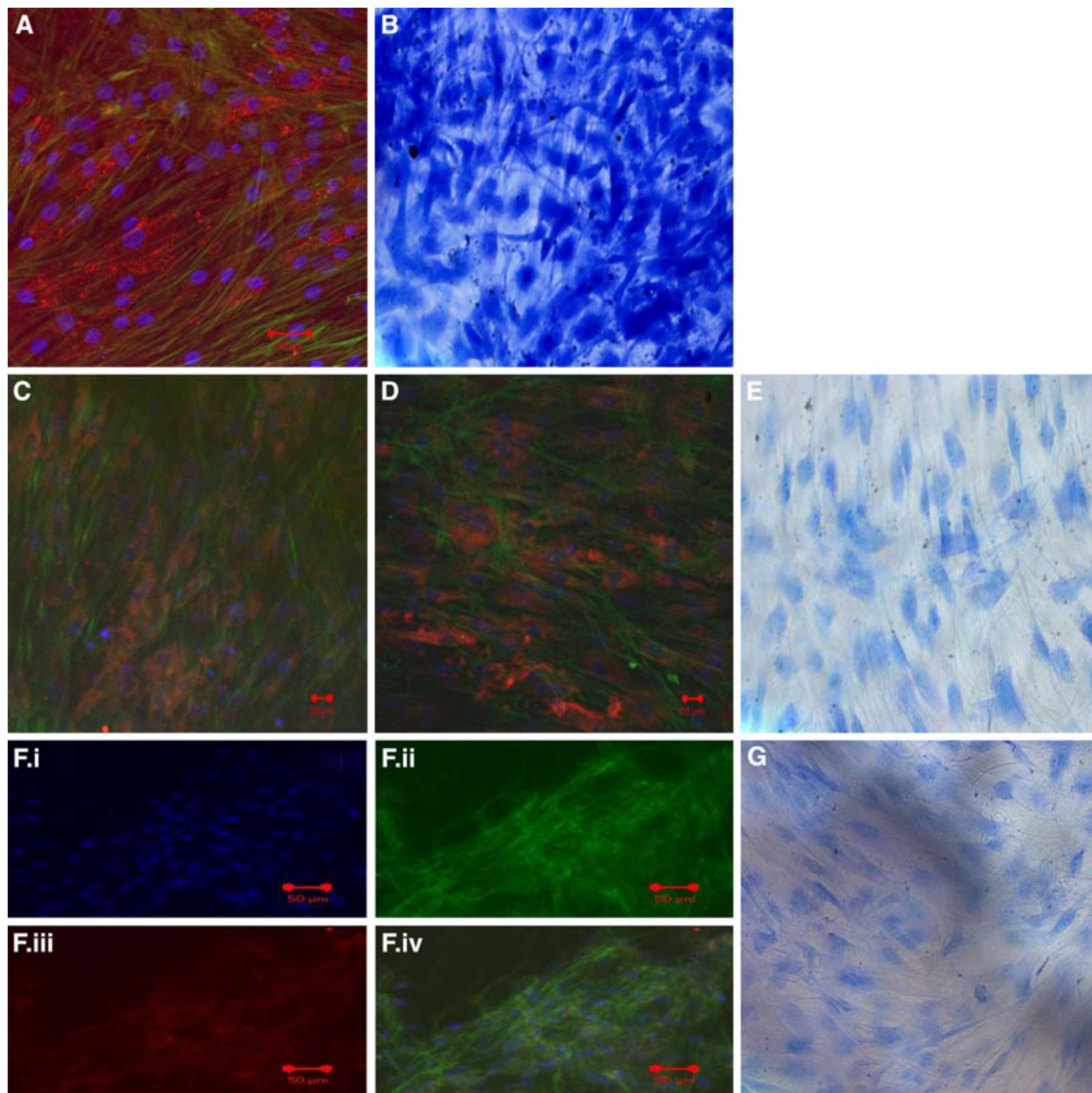

Fig. 1 Human MSC were cultured in contact with bulk coated SAMs modified surfaces in basal medium for 28 days. When cultured in contact with $-CH_3$ modified surfaces STRO-1 (**a**, STRO-1 is *red*) expression increased and the MSC phenotype was maintained and enhanced under specific test conditions, no indications of differentiation were observed and cells were negative for von Kossa (**b**) (cells were counter stained with methylene *blue*). When substrates were modified with –OH groups (**c**, **d**, **e**) chondrogenic differentiation was initiated and collagen II rich cell clusters were formed (**c**, collagen II is *red*) without the need for additional supplementation with TGF-β and other exogenous factors. The chondrogenic phenotype on areas of the surface was further confirmed by expression of aggrecan (**d**, aggrecan is *red* in colour) and negative von Kossa (**e**, only *blue* stained cells no evidence of brown calcified extra cellular matrix) When modified with $-NH_2$ surfaces induced osteogenic differentiation (**f**,**g**), as displayed by osteocalcin rich extra-cellular matrix (**f**, positive osteocalcin is *red* in colour, **f**.iii, cells were counterstained with DAPI, **f**.i and actin **f**.ii), high levels of osteocalcin expression were especially high at the periphery of the coverslip and migrated inwards towards the centre of the coverslip with increasing time. Only cells cultured in contact with $-NH_2$ modified surfaces displayed positive von Kossa staining (*brown* in colour, shown on a different focal plane to the monolayer cell culture). (Color figure online)

combined deposition of a chemical group in a defined area (70–80 nm in diameter), separated by a defined pitch in a defined pattern at the nanometre scale.

When cultured in contact with the nano patterned arrays it was evident from short time points that different combinations of chemistry and pitch had a definitive effect on initial cell adhesion. When cultured in contact with $-CH_3$ nano patterned surfaces the effect of changing the pitch from 140 nm to 280 nm was sufficient to dictate if the cells stuck to the surface. At a pitch of 140 nm no viable cell adhesion was observed, Fig. 3a, but at 280 nm a large cell cluster formed in the modified area, Fig. 3b. Additional

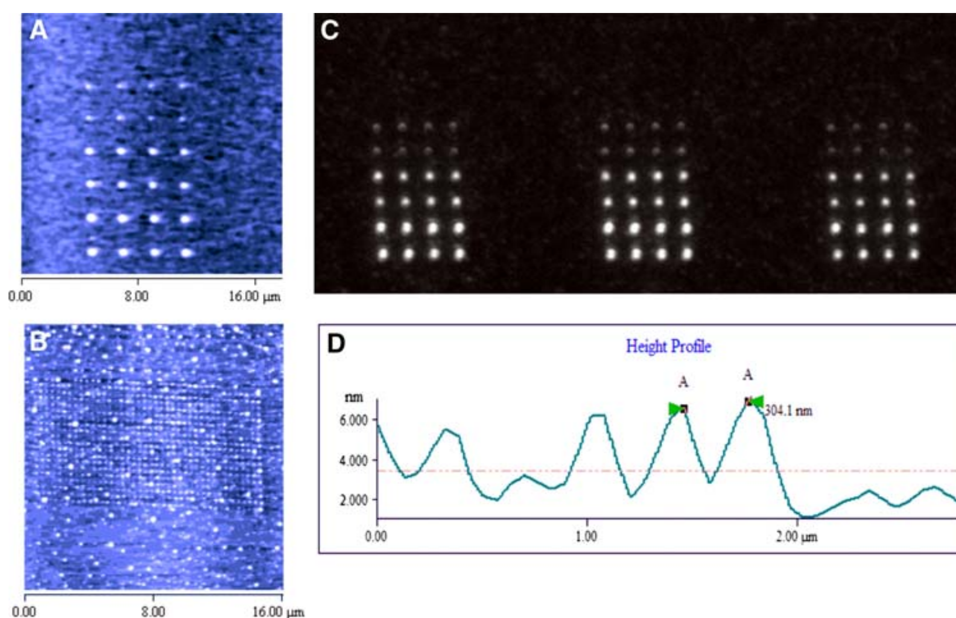

Fig. 2 (**a**,**b**) AFM and **c** Dark Field images depicting how changing dwell time can control the feature size written by the DPN system. When optimum conditions have been established for a specific ink area are prepared for QC analysis using AFM to quantify the homogenous deposition of ink across the surface and correct pitch (**d**). Surfaces used within this study have a feature size of 70–80 nm

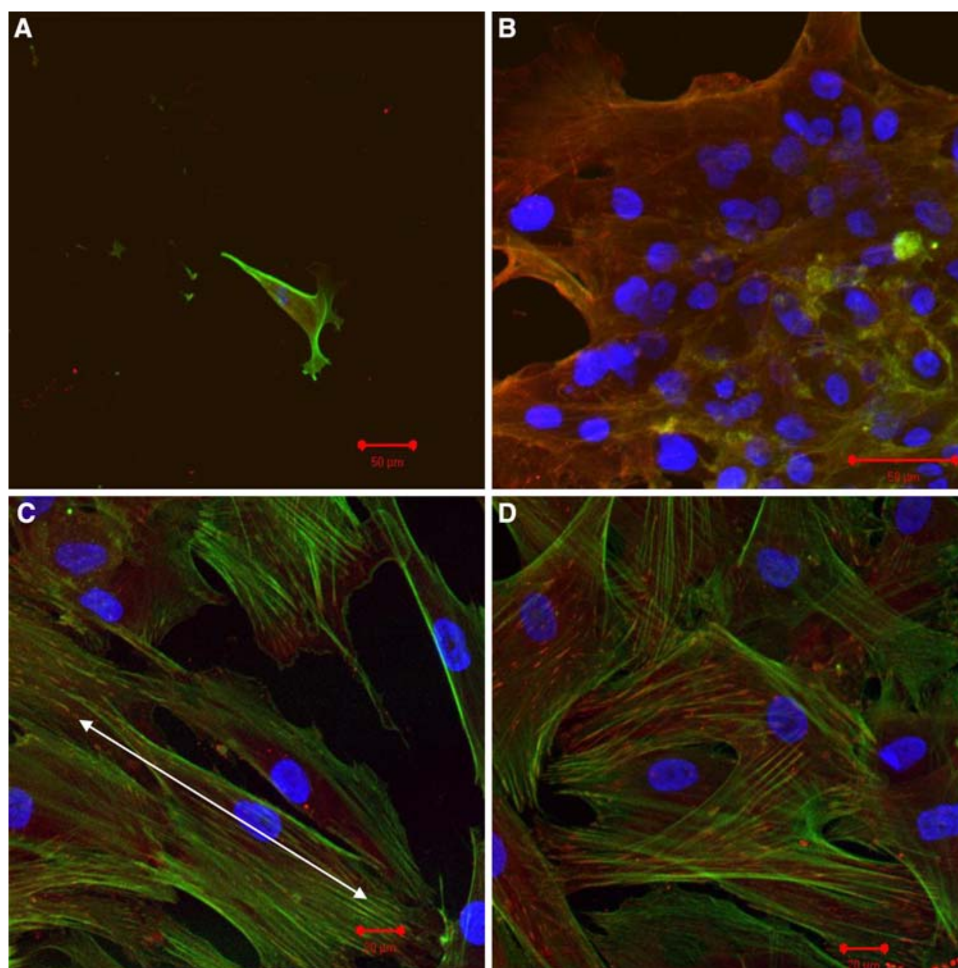

Fig. 3 Human MSC were cultured in contact with DPN® modified surfaces and stained for actin cytoskeleton (F-actin, *green*) and focal contact formation (vinculin, *red*), cell nuclei were counterstained with DAPI (*blue*). Cells did not adhere to the 140 nm –CH3 nano-pattern (**a**). When cultured in contact with surfaces modified with $–CH_3$ groups in a nano-array pattern cells actively migrated to the 280 nm square array patterned area and formed a multi-layered cluster on the patterned area (**b**). When cultured in contact with $–NH_2$ modified surfaces using the same nano-patterned array the cells showed alignment against a specific 280 nm spacing of the chemical dots (**c**), a phenomenon that was lost when the cells dimensions of the nano-pattern were changed between the $–NH_2$ dots to 1 µm (**d**). These initial experiments prove that both chemistry and pitch can be used to control initial cell adhesion. (Color figure online)

cell migration assays confirmed the chemotactic nature of the $–CH_3$ modified 280 nm pitch square array pattern, data not shown.

The effect of combining chemistry and pitch to control initial cellular responses was further confirmed when cells were cultured in contact with $–NH_2$ square array

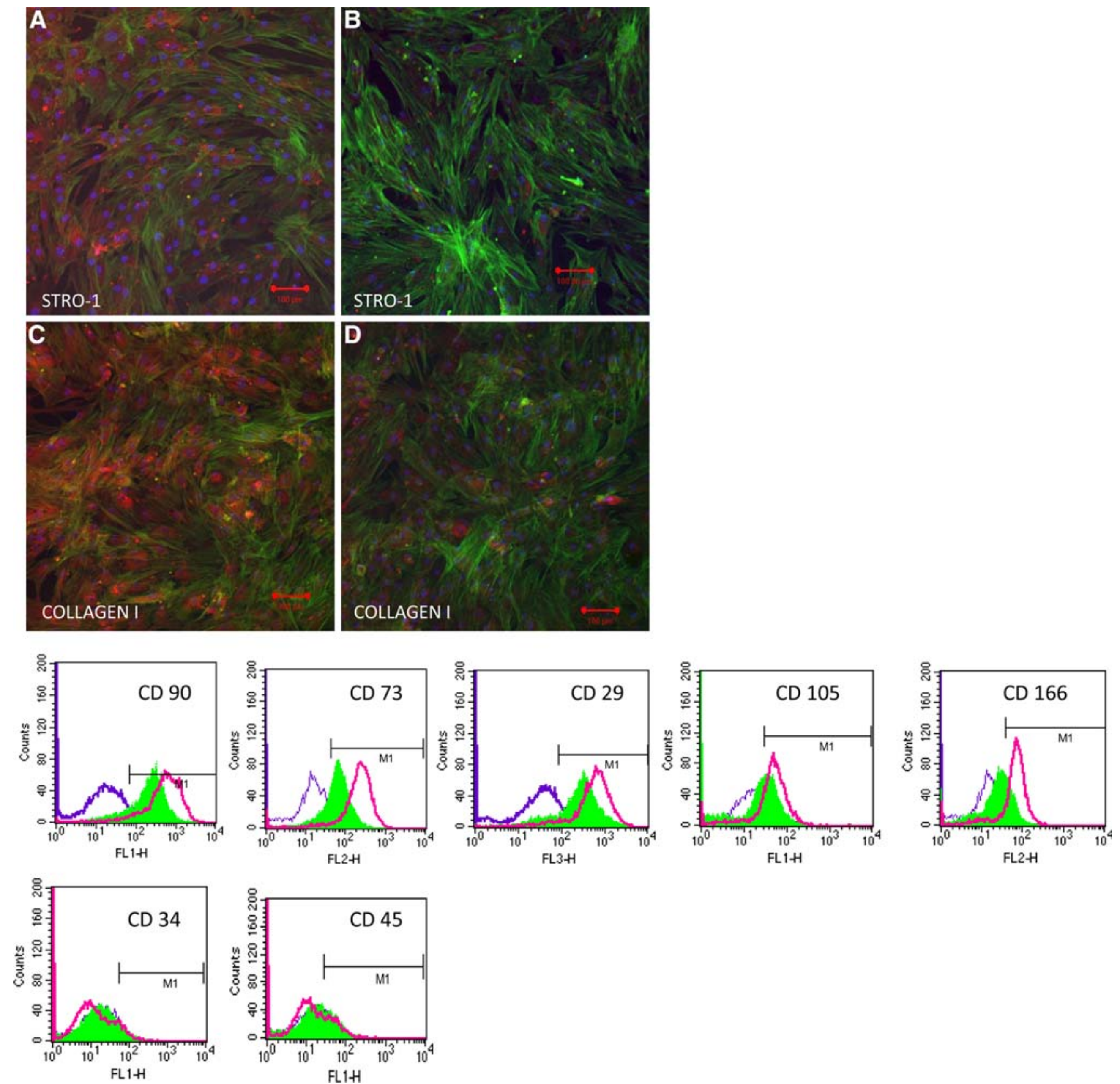

Fig. 4 Human MSC were cultured in contact with $–CH_3$ modified (**a**, STRO-1 and **c**, collagen I) ($–CH_3$ groups separated at a specified 280 nm pitch) and control (**b**, STRO-1 and **d**, collagen I) surfaces. When cultured in contact with $–CH_3$ modified surfaces MSC demonstrated an increase in expression of STRO-1 (**a**, positive *red* staining)) and collagen I (**c**) over time with no indication of differentiation, FACS analysis also confirmed the increase in expression of MSC markers (>95%) CD29, CD73, CD90, CD105 and CD166 (data not shown) compared to the control surface. For FACS analysis the appropriate isotype is depicted as the *purple* peak, control cells the *green* filled peak and cells cultured in contact with the $–CH_3$ modified substrates the *pink* peak. (Color figure online)

nano-patterns. When cultured in contact with $–NH_2$ 280 nm pitch the pattern of initial cell adhesion was completely different than observed on the $–CH_3$ 280 nm pitch. When cultured in contact with the $–NH_2$ 280 nm no cell clusters were formed, Fig. 2c, percentage of cell attachment over the same area was less on the $–NH_2$ surfaces, in addition no evidence of active cell migration after initial attachment was observed on the $–NH_2$ modified surfaces. When cultured in contact with the 280 nm $–NH_2$ surfaces there was clear evidence of cell elongation and alignment of the stress fibres, a phenomena that was not observed on other pitches tested.

To further investigate the effect of the $–CH_3$ group, 280 nm pitch on the MSC population large patterned surface areas were produced as previously described and MSC were cultured in contact with the surfaces for time periods

up to and including 28 days. MSC expressed a higher percentage of STRO-1 when cultured in contact with the nano-patterned arrays compared to the bulk coated chemistry and control surfaces (Fig. 4a). The distribution of STRO-1 across the surface was homogenous, positive STRO-1 expression was associated with >95% of cell nuclei, confirming the increase in expression attributed to the nano-pattern. Additionally the MSCs cultured in contact with the $–CH_3$ nano patterned surface also showed an enhanced expression of collagen I, once again this was found to be homogenous across the surface. The enhanced expression of markers associated with the characteristic MSC phenotype was further quantified by FACS analysis. Cells cultured in contact with $–CH_3$ surfaces maintained an increased expression of CD29, CD73, CD90, CD105, and CD166 compared with cells cultured on TCPS under the same conditions. Cells also maintained their negative CD45/34 phenotype, Fig. 4.

4 Discussion

The aim of this study was to directly compare bulk coated chemically modified substrates to substrates patterned with the same chemistry as a nano-pattern to prove that previously observed phenomena of material induced differentiation could be enhanced by using highly defined nano-patterned arrays. Control of the spatial stimuli at the nanometre scale effectively enhances the ability of the material to control initial cell attachment, in a homogenous manner and subsequent cell signalling that will drive the cell functionality responses. Within this study we have introduced DPN® as a valuable technology for the production of highly defined nano-patterned surfaces and further proved that the incorporation of the correct combination of chemistry and pitch can be used to produce surfaces that increase our control over cell populations and effectively can be used to modify our current generic cell culture protocols i.e. the utilisation of $–CH_3$ modified surfaces instead of TCPS for the efficient expansion and maintenance of an MSC population.

The fabricated surfaces with desired dimensions are suitable to support long term cell culture of populations of cells and dictate their collective response, i.e. initial attachment and functionality of human MSCs can be controlled using the correct combinations of chemical groups (inks) separated by a defined pitch, in a specific array By simply changing the pitch feature dimensions, surfaces can control cell attachment or inhibit cell adhesion in a reproducible fashion. The successful combination of DPN® technologies with the current knowledge of chemically and protein induced stem cell differentiation phenomena has provided for a new fundamental knowledge to be established at a molecular level relating to the parameters, factors and interactions that dictate and direct the substrate control of cell function over larger tissue relevant areas. Using these technologies surfaces have been produced that are chemotactic to MSCs and pro-long the MSC phenotype in vitro, enhancing expression of stem cell markers STRO-1, CD90, CD73, CD105 and CD90 compared to control surfaces.

Preliminary research has already established the role of DPN® technologies as a tool for producing precisely defined model surfaces that can control cellular interactions by delivering chemical cues to the cells at the correct spatial resolution in a homogenous pattern across the entirety of a surface, therefore allowing the collection of fundamental data that will allow the definition of exactly how combinations of chemistry and pitch can be effectively utilised to produce the next generation of tissue engineered constructs and then medical therapies. Ultimately this potentially will result in off the shelf products that will have cell selection, recruitment and functional control capabilities that could lead to the elimination of in vivo manipulation prior to implantation, resulting in a new generation of cost effective and more efficient therapies.

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