Cells can perceive complex mechanical cues across both the micro- and nanoscale which can influence their development. While causative effects between surface topography and cellular function can be demonstrated, the variability in materials used in this screening process makes it difficult to discern whether the observed phenotypic changes are indeed a result of topographical cues alone or the inherent difference in material properties. A novel approach to directly imprint micro- and nanoscaled topographical features into the base of conventional cell cultureware is thus developed, facilitating its compatibility with standard biological techniques and methods of analysis. The utility of this technology is demonstrated by performing high-throughput screening across five distinct cell types to interrogate the effects of 12 surface topographies, exemplifying unique cell-specific responses to both behavior and cell morphological characteristics. The ability of this technology to underpin new insights into how surface topographies can regulate key image descriptors to drive cell fate determination is further demonstrated. These findings will inform the future development of advanced micro- and nanostructured cell culture substrates that can regulate cell behavior and fate determination across the life sciences, including fundamental cell biology, drug screening, and cell therapy.
Society of Cell and Gene therapy) MSC marker CD90 (cluster of differentiation 90) were significantly downregulated, highlighting the importance of the interplay between substrate topography and cell fate.

However, whilst we are currently able to demonstrate causative effects between topographical cues and cellular function, there remains a distinctive gap in our ability to effectively screen optimal surface geometries to determine the precise cues that guide these changes. Considering the conceivable number of surface geometries readily available in nature, direct replication of each possible geometry would be impractical due to their sheer number. The ability to effectively screen a select number of physical structures would ultimately allow the building of a predictive model for cellular behaviors. Consequently, recent work has striven to develop the high-throughput screening potential of these topographical platforms, with the aim of enhancing our understanding of the underlying mechanisms which govern cell-substrate surface interactions. In particular, the TopoChip system[16,17] presents a total of 2176 (mathematically defined) nanotopographies on a single surface and has been used to identify unique topographies able to modulate MSC proliferation and osteogenic differentiation. Further examples include the BioSurface Structure Array (BSSA)[18] and multiarchitecture chip (MARC)[19] systems which have been used for the expansion and differentiation of embryonic stem cells and to determine the optimal topography for directing the differentiation of primary murine neural progenitor cells, respectively. More recently, Hue et al. presented the integrated mechanobiology platform (IMP),[20] consisting of nanofabricated trench-grid structured surfaces used to investigate the influence of surface topographies in combination with immobilized anti-CD3 and anti-CD28 antibodies on T cell behavior.

Whilst these emerging technologies are indeed enabling more comprehensive analysis to be performed, and thus are enhancing our understanding of how topographical features influence cell behavior, there remains an inherent challenge in directly translating these findings to fundamental biological research which is typically performed using cells on TCP. For each of the examples described above, fabrication is conducted using combinations of conventional photolithography and etching techniques to micro-/nanopattern base substrates such as silicon, polydimethylsiloxane (PDMS), and polycarbonate. The multitude of materials used across the different systems makes it difficult to discern whether the observed phenotypic changes in each cell type are indeed comparable between materials, or if the inherent difference in material properties, including substrate stiffness, electrical conductivity, and surface chemistry may alter the phenotypic output of each cell type. Given that conventional tissue culture plastic (TCP) is made from polystyrene (PS) (which has been used for several decades as the gold standard culture platform for studying fundamental cell biology research), we believe that direct imprinting of topographies onto the TCP plate would more rapidly facilitate progress by allowing the data generated to be compared with studies performed using standard TCP plates. This will provide increased confidence that conclusions made using these screening platforms are indeed a direct result of topographical influence.

We have thus developed a novel approach to imprint both nano- and microscaled topographical features directly onto the base of TCP multiwell plates. Our platform comprises an array of customizable micro and/or nanostructured screening motifs, featured on the base of commercially available TCP multiwell plates without any modification to chemical, mechanical or optical properties of the material. We utilized 3D printing technology to create a modulator which can repeatedly transfer micro- and nanostructures using thermal nanoimprint lithography (NIL),[21,22] lending itself to both high-scalability and simple implementation into current large-scale manufacturing procedures. Furthermore, as the system uses a standard well plate, our technology retains its compatibility with advanced high-throughput screening techniques which require direct interactions with the commercial TCP plate format such as plate readers, automated imaging devices, and fluid handling robots. We have demonstrated the application of our cost-efficient and standardized TCP screening platform to high-content screening across five distinct cell types, exemplifying unique cell-specific responses to both behavior and cell morphological characteristics. As a result, this technology has the potential to enable comprehensive assessment of how cells perceive and subsequently respond to topographical cues, thus broadening its use as a discovery tool across the fields of tissue engineering, regenerative medicine, pharmacological screening, and cell therapy.

2. Results and Discussion

2.1. Substrate Composition Directly Influences Cellular Function

To assess the effects of culture material on cell morphology and function, we compared the characteristics of MSCs cultured on conventional TCP multiwell plates to those on Ormocomp substrates. Ormocomp is a polymer commonly used for microfabrication, particularly in biological applications, due to its high biocompatibility.[23–25] A comparison between MSCs cultured on TCP and flat Ormocomp (Figure 1a–i) revealed no significant difference to standard biocompatibility assays including viability, metabolic activity, and proliferation. Additionally, measurements of basic descriptors of cell and nuclear morphology were consistent between cells on the two substrates. However, a more nuanced analysis of cell behavior discerned...
Figure 1. Characterisation of MSC phenotypes across TCP and flat Ormocomp substrates. a–d) Show representative live/dead staining (green/red), cell viability, metabolic activity, and proliferation, respectively. Scale bars: 50 µm. e–i) Quantification of cell and nuclear image descriptors between MSCs cultured on TCP and flat Ormocomp substrates. j) Representative immunofluorescence staining for the mechanosensitive transcription factor YAP, k) alongside associated localisation quantification, Scale bars: 5 µm. l) Western blotting analysis of lamin A/C, β-tubulin and H3K9 expression profiles, respectively, across TCP and flat Ormocomp substrates. m) RT-PCR analysis of vinculin, LMNA, FAK, YAP, Rac-1, ROCK, Rho A, and TAZ gene expression across flat Ormocomp substrates (normalised to TCP). All graphs show mean ± SD for three independent MSC donors. For morphology, viability and proliferation quantification, a total of 300 cells per condition were analyzed. RT-PCR data are presented as mean ± SD relative to TCP control samples. Samples were analysed by one-way ANOVA with Tukey post hoc testing. Statistically different samples are denoted by **p < 0.01, ***p < 0.005, and ****p < 0.001.
significant differences in the cellular response to the two substrates. For example, nuclear localization of the mechanosensitive transcription factor YAP (Figure 1j) showed a near nine-fold reduction in cells cultured on Ormocomp compared to TCP (Figure 1k). Analyses of protein and gene expression profiles revealed further variation between those MSCs cultured across Ormocomp and TCP substrates (Figure 1l,m), in which cells cultured across Ormocomp presented significant downregulation of lamin A and lamin C (lamin A/C) protein expression, alongside increased gene expression profiles for YAP, TAZ, Rac-1, and RhoA when compared to TCP counterparts.

Together the data indicates that there are substantial differences in the molecular biology of the cells on the two substrates, despite appearing similar from more superficial analysis. Only when a more in-depth analysis on cell metabolism, gene expression and protein expression (Figure 1j–m) was conducted, did we observe significant changes to cell behavior as a result of material influence. It is likely that many of these changes are important for cell function, for example, whilst we observed no significant changes to vinculin, LMNA (Lamin A/C gene), FAK, or ROCK gene expression, we did identify an increase for YAP and TAZ, two key effectors of the Hippo signaling pathway. As the nuclear localization of YAP is well documented to enhance mechanosensitive signaling events within the cell. Furthermore, our observations of increased RhoA and Rac-1 gene activity further support this theory due to their critical role in safeguarding cellular tension and mechanosignaling through the regulation of actin polymerization. Interestingly, the data also indicate that while the two materials are broadly similar in mechanical properties (TCP, 1.18–1.39 GPa,[24] and Ormocomp, 3.3 GPa), there are still significantly different cellular responses, likely related to differences in the surface chemistry, roughness, and subsequent protein adsorption.[24]

We suggest that such a mismatch due to the difference in material composition, as exemplified here by the comparison of Ormocomp and TCP, poses a major challenge, both in elucidating the effects of topographical features on cell behaviors and in linking the findings to previous biological studies using TCP multiwell plates. This is a major limitation for the future use of topographical patterns in high-throughput screening, as these underlying cell changes in response to varied substrate properties will confound interpretation.

2.2. Direct Fabrication of Micro- and Nanotopographies in TCP Multiwell Plates

We developed a technology that enables a direct route for the fabrication of microstructures onto the base of multiwell plates, allowing us to: i) bypass the problem of varying cellular responses on different substrate materials and ii) to permit investigation into the effects of substrate topography, independent of material composition (chemical identity). In this format, we are not only able to isolate key cellular responses from the topographical cues, but the standardized design of multiwell plates also enables compatibility with current screening technologies, specifically designed to accommodate for their precise dimensions and material properties.

To do this, we selected the use of thermal NIL which is a scalable, well-established technique for the patterning of thermal polymers such as PS. Using the NIL process with PS requires the careful balancing of three independent parameters (heat, pressure, and time) to obtain high fidelity structures. To effectively transfer both micro- and nanoscaled topographies directly into multiwell plates, we developed a 3D printed, customizable modulator (Figure 2a), which can be designed and fabricated to fit any format of multiwell plate with a flat base. This modulator enables the transfer of precisely controlled pressure from the top pressing plate of the hot embosser to the bottom of the multiwell plate, ensuring high fidelity transfer of topographical features deep into the wells.

As with any NIL process, the selection of molding material is also a critical step in obtaining efficient design transfer to the PS substrate. PDMS is often not considered an optimal material for thermal NIL as its soft nature typically results in poor transfer fidelity.[32] However, in our case, demolding happens at the bottom of the wells, and a rigid mold becomes extremely challenging to separate from the patterned substrate. Thus, a relatively soft material was preferred. To simultaneously enable both high-fidelity transfer and ease of demolding, a basecuring agent ratio of 5:1 (w:w) was used to obtain a more stiff version of conventional PDMS (typically obtained with a 10:1 ratio). A cross-sectional illustration of the embossing process can be seen in Figure 2b, in which the optimized PDMS molds are positioned in the wells of the multiwell plate whilst the modulator transfers and distributes the applied pressure to the well surface during the NIL process (detailed in the experimental section). As demonstrated in Figure 2c, this process was successfully able to transfer both micro- and nanoscaled topographies from the PDMS mold to the bottom of the multiwell plates whilst maintaining high resolution across both lateral and vertical planes (Figure S1a,b, Supporting Information). Importantly, this high-fidelity transfer was maintained over a large surface area of the multiwell plate (Figure 2d–f) thereby ensuring the continued compatibility with many biological screening assays, often designed for use with the precise dimensions of commercial tissue culture plates. From this point on, we refer to TCP multiwell plates which have been processed through NIL as TCP arrays.

Interestingly, although we have successfully achieved both high-resolution and consistent topographical structure transfer across both the micro- and nanoscale, we observe a significant decrease in transfer efficiency for features presenting gradual changes in vertical dimensions (such as those observed in Si nanowires) and features below 100 nm (Figure S1c and S1d, Supporting Information respectively). We believe that these limitations result from the selection of PDMS as our molding material for use in the thermal NIL processing. Whilst our modifications to the working composition of PDMS allowed us to overcome the complication of demolding following NIL processing, the soft nature of PDMS appears to limit its use for those features below 100 nm in scale. We therefore believe that to achieve successful transfer of features below the 100 nm range, further optimization of the mold material selection would be required in future studies.
We then assessed whether the NIL process affected the surface characteristics of the TCP (Figure 3). Due to the innate hydrophilicity of TCP, we compared surface wettability between pristine TCP (i.e., plates taken straight from the packet) and plates that had been through the imprinting process using a flat PDMS stamp to retain an even surface. As both changes to surface chemistry and topography are known to influence surface hydrophilicity, we performed static contact angle (CA) measurements, demonstrating a small decrease for plates post-NIL, changing from 71° ± 5° for pristine TCP to 58° ± 5° after NIL (Figure 3a). Subsequent X-ray photoelectron spectroscopy (XPS) suggested that this may arise from a subtle increase in O1s and Si2p on imprinted surfaces (12.5 ± 0.3 and 3.6 ± 0.5 at%, respectively) in comparison to pristine TCP (8.5 ± 0.7 and 0.3 ± 0.2 at%, respectively) (Figure 3b). We believe that these findings can be attributed to the direct contact between PDMS and PS substrate during the NIL process, resulting in the direct transfer of molecular components of PDMS. In contrast to our findings, the direct transfer of these PDMS components should increase the hydrophobicity of the PS surface, however additional AFM screening of surface roughness (Ra) revealed a further decrease from 2.3 ± 0.5 nm on pristine TCP to 0.7 ± 0.3 nm on flat imprinted samples, suggesting that subtle modifications to surface topography characteristics are evident.
which may be contributing to the increased hydrophilicity of imprinted surfaces observed through CA assessment.\textsuperscript{[34]} Further AFM analysis was also conducted on microstructured samples following NIL processing, in which comparable surface roughness measurements to that of flat imprinted samples were observed across both the top and between transferred microstructures (Figure S1a,b, Supporting Information), being 0.9 ± 0.3 and 0.85 ± 0.02 nm respectively. Interestingly, these changes to surface characteristics following embossing do not change cell spreading behavior when compared to pristine conditions (Figure S2, Supporting Information).

To further determine whether the optical properties of the embossed plates were maintained, we compared images of the Monash University logo through the TCP arrays, and found

**Figure 3.** NIL processing demonstrates similar surface characteristics to that of pristine TCP. a) XPS surface comparison between pristine TCP and imprinted flat TCP. b,c) Quantification of contact angle and surface roughness, respectively, between pristine and imprinted TCP surfaces, supplemented with comparative AFM images ((d) and (e)). f) Comparative analysis of optical transparency for brightfield (I and II), MSC immunofluorescence staining for actin (green) and nuclei (blue) (III and IV) and transmittance spectra measurements (V) between pristine and imprinted multiwell plates. Scale bar: 5 µm). g) Cluster analysis of gene expression profiles for RAC1, LMNA, YAP, vinculin, TAZ, ROCK, and FAK between pristine TCP, imprinted flat TCP and flat Ormocomp, supplemented with graphical representation of Pi variance (calculation of differential expression) as a function of fold change (or normalized expression) from TCP, where \( p \leq 0.01 \) (h and i). All graphs show mean ± SD for three independent MSC donors relative to TCP samples. Samples were analyzed by one-way ANOVA with Tukey post hoc testing.
no visual differences in image clarity (Figure 3f-I,II). We further detected no difference in fluorescence microscopy image quality using conventional fluorescence microscopy or high-content image platforms for cells stained against the actin cytoskeleton and nuclear compartment between imprinted and pristine TCP substrates (Figure 3f-III,IV (MSCs) and Figure S3, Supporting Information). Quantification of transmittance across the visible light spectrum further confirmed that there were no significant changes, with only a negligible decrease in transmittance at 600 nm (Figure 3f-V). Overall this demonstrated that the NIL process had no substantial effect on the optical properties of the TCP arrays.

Finally, we compared the expression of several genes in MSCs cultured on pristine and imprinted TCP, as well as those on Ormocomp. We found negligible differences in expression profile for genes associated with cell-substrate interactions and mechanotransductive processes between pristine and imprinted TCP samples. In stark contrast, the expression in cells on Ormocomp used here as a positive control were significantly different (Figure 3g). Notably, a cluster analysis of these data showed the strong similarity of expression profiles on the pristine and imprinted TCP, whereas the cells on the Ormocomp clustered separately. Analysis by Pi value which considers both the magnitude of changes in gene expression and the variability of different replicates (Figure 3h,1), further confirmed that no significant changes in gene activity were observed between the pristine and imprinted substrates. Overall these data support the use of this imprinting technology as a means to create micro- and nanostructures at high fidelity across large areas, without affecting the characteristics of cells cultured on the imprinted TCP compared to pristine TCP.

2.3. TCP Arrays Facilitate High-Throughput Screening of Interactions Between Cells and Topographically Patterned Surfaces

Having confirmed that the NIL process did not induce any substantial changes to the TCP properties or biological response, we next utilized our platform to screen MSCs cultured on varying microtopographies to gain an insight into how their implementation can influence cellular events. Cluster analysis was performed on MSCs cultured across either pillars (P), grooves (G), or complex (C) microtopographies. For pillars and grooves, the spacing between each structure was maintained equal to the width of the structure, being 5, 7.5, 10, and 15 µm (denoted as 1, 2, 3, and 4, respectively). C1, C2, C3, and C4 refer to the complex geometries which each have distinctly unique topographical features. The selection of pillared and grooved structures for use within this study stemmed from extensive literature demonstrating both their consistent use across a range of fabrication techniques,[14,17] alongside their ability to modulate cell properties such as morphology,[38-40] behavior, and functional outputs.[5,44]. The precise geometries and dimensions were selected to emulate the most prominent features and scales observed throughout the literature.[13,42,44] Representative SEM image for each structure presented in this study can be seen in Figure S4, Supporting Information. After 72 h, MSCs were stained for the actin cytoskeleton and cell nucleus (Figure S4, Supporting Information), then using CellProfiler, images were analyzed in an unbiased manner to obtain quantitative measurements of 130 unique image descriptors covering cell and nuclear size, shape and texture. The values were normalized and scaled to a reference distribution, by robust Z-scoring to cells grown on flat control substrates. After removal of inactive and highly correlated measurements, each cell could be described by a final library of 130 unique image-based descriptors (defined in Figures S5–S7, Supporting Information).

Unbiased hierarchical clustering, based on this library of image descriptors, was used to interrogate the morphological characteristics of the MSCs and any relationships that were evident in response to the varying size and geometry of the topographies (Figure 4a,b). At feature sizes from 5–10 µm, the MSCs clustered according to the geometry of the structures and we observed strong similarity in the image-based phenotypic signatures of the cells on the pillars, grooves, and complex structures. Interestingly, MSCs cultured on complex geometries presented the highest degree of similarity, despite each structure within the group (C1-4) being significantly different in shape. This contrasts to that of pillared and grooved geometries which maintained identical shapes of sequentially increased size and spacing from patterns P1-P4 and G1-4, respectively. Cells on the largest feature size of 15 µm clustered separately to feature sizes ranging from 5–10 µm, with P4 and G4 clustering as a separate branch rather than together with the other sizes of the same geometry. This indicates that the shape of the topographical feature has less of an influence above a certain threshold size, potentially denoting that the feature size is then more readily perceived as a flat substrate. This can be supported by our cluster analysis denoting limited variation in image descriptors between the large feature sizes (P4 and G4) and that of the flat substrates (Figure 4a). An alternative explanation may be that selective control of spacing between substrate attachment sites influences cell phenotypes as documented in the literature.[46] Thus we can also speculate that patterns exhibiting feature spacing ≥15 µm may signify a critical point at which MSCs change their substrate attachment phenotypes to more closely match that of flat substrates. However, further investigation would be needed to fully validate this effect.

To determine which image descriptors were most representative of the cellular phenotype in response to changes in the topographical features, we further narrowed our cluster analysis to the top 27 image descriptors which contributed most heavily to clustering within each topographical design (Figure 4c). Interestingly, the image descriptor that contributed most to the clustering of cells on the grooved structures was that of nuclear shape, encompassing quantitation of nuclear compactness, eccentricity, extent, and solidity. For pillars and complex structures however, the distinctive clustering resulted from subtle changes to actin entropy, which denotes the level of complexity of actin stress fiber alignment within the cell.[45] When combined with the immunofluorescence analysis of cytoskeletal organization (Figures S4 and S8, Supporting Information), this provides a more comprehensive picture of the implications for distinctive changes to these image descriptors. For example, we observe that across grooved structures, MSC alignment was directed by the structure orientation (Figure 4d), whereas for the pillared structures, this high degree of control was lost (Figure 4e). This observation demonstrates how
Figure 4. MSC cluster analysis reveals pattern specific cell morphology characteristics. a) Cluster analysis presenting 130 differentially expressed z-score values for cell and nuclear shape descriptors. b) Representative immunofluorescence analysis for actin (red), lamin A/C (green), and nuclei (blue) of MSCs cultured across each micropatterned design. Scale bar, 10 µm. c) Condensed cluster analysis of the top 27 features which contributed the most heavily to MSC clustering across the TCP array. d,e) Comparative analysis of MSC alignment across grooved and pillared structures, respectively. Colors denote different orientations within the cell. Scale bars: 10 µm. All cell and nuclear shape/texture descriptors are defined in Figures S5–S7, Supporting Information.
precise control of the 1D/2D nature of substrate topography can influence cellular alignment, which in turn influences nuclear image descriptors including compactness, eccentricity and solidity. With growing evidence suggesting new insights into how nuclear architectural changes can regulate chromatin organization, gene expression, and ultimately cell fate determination,[43] we suggest that the observed pattern of changes in cell and nuclear phenotypes between distinct topographical features may serve as a fingerprint to predict cellular responses.

To further determine whether the changes in MSC morphology would lead to a change in function and whether certain image phenotypes would correlate with this, we assessed MSC differentiation potential on the TCP arrays using the designs previously characterized in Figure 4. MSCs were cultured on TCP arrays in osteogenic media to identify any change in osteogenic differentiation efficiency, and in a 1:1 mixture of osteogenic and adipogenic media to establish whether the topographies would establish a bias in the lineage specification of the cells. The data revealed significant variation in both differentiation potential and lineage bias as a result of the microtopographical features present (Figures 5a and 5b, respectively, Figure S9, Supporting Information). Staining for the early osteogenic marker alkaline phosphatase showed significantly more positive cells on the pillared structures when compared to flat controls, with an optimum pillar size of 5 µm in height, width and spacing which correlates closely with our previous report of enhanced mineralization on these structures.[43] Further quantification revealed a significant increase in osteogenic capacity for MSCs cultured across pillared structures (most notably P1, P2, and P3, presenting feature width and spacing of 5, 7.5, and 10 µm, respectively, at a height of 5 µm). In contrast, we observed a significant decrease in osteogenic capacity of MSCs cultured on grooves when compared to flat controls, as well as a significant increase in the osteogenic:adipogenic ratio under switch conditions. These findings contradict that observed previously, in which microgroove patterns of a similar scale have been observed to enhance the osteogenic capacity of MSCs across materials such as titanium and PLLA.[46–47] Interestingly, however, each of these materials have also been demonstrated to enhance the osteogenic capacity of MSCs irrespective of microtopographic features,[48,49] and thus we suggest that our use of a more standardized cell biology material (TCP) enables the comprehensive investigation of cell behavior in response to microtopographical influence, irrespective of material properties. For G1 and G2 structures, the observed increase in osteogenic:adipogenic ratio was attributed to a significant reduction in the propensity for adipogenic differentiation, which functions to artificially bias the ratio towards osteogenesis, despite the fact that the overall efficiency of osteogenic potential was low.

When further combined with the cell and nuclear shape clustering, it was evident that phenotypic fingerprints for cells cultured on both grooved and pillared structures correlated with the differentiation (Figure 5c). For example, surfaces on which the osteogenic capacity of the cells was highest (P1, P2, P3, C2, and C4) correlated with those that showed lower Nuclear Solidity (termed Nuc_Shape_8, Figures S5–S7, Supporting Information). Conversely, patterns on which the cells showed both low osteogenic capacity and low osteogenic:adipogenic ratios (e.g., G3), also displayed a significant increase in Nuclear Compactness and decreased Nuclear Extent (termed Nuc_Shape_2 and Nuc_Shape_4 respectively, Figures S5–S7, Supporting Information). Together these correlative fingerprints link reduced osteogenesis with an increased cell aspect ratio and decreased nuclear circularity (Figure 5d,e, respectively). These findings are in good accordance with the literature, where high cell aspect ratios have been reported to enhance MSC osteogenic capacity.[41,50–52] We can therefore determine that the distinctive changes to cell morphology as a result of surface topography can provide a useful insight into the phenotypic changes which correlate to different cellular outcomes. These fingerprints could in turn be used to better understand the mechanisms which drive cell behavior, and in the future, provide a predictive model for generating desired cell responses.

2.4. TCP Arrays Can Determine Cell Type-Specific Morphological Changes in Response to Substrate Topography

To further exemplify the potential utility of this platform across multiple cell types, and to determine whether there were common features in the cellular response to topographical cues, we screened a further four cell types on the TCP arrays. Cell types were selected from a variety of different tissue types, spanning origins across different germ layers, consisting of both epithelial and mesenchymal cells types and presenting distinct growth patterns from cells ranging between 10–50 µm in diameter to those which grow either individually or as colonies; (human umbilical vein endothelial (HUVEC) cells, C2C12 (mouse muscle) cells, HaCaT (human keratinocyte) cells, and L929 (mouse fibroblast) cells. As per the MSC analysis, each cell type was cultured on our platform for 72 h, stained for actin and nuclear compartments and subjected to high-throughput image analysis (Figure 6a and Figures S10–S21, Supporting Information). Validating the need for a technology to rapidly screen the cellular response to substrate topography, the resulting data revealed unique cell-specific characteristics associated with each topographical design and with varying responses to the sizing of the features. These data permitted an analysis of the response of each cell type individually as well as a comparison between the cell types, allowing unique differences and common image signatures to be identified.

For example, across each topographical structure tested, L929 cells exhibited the highest degree of Actin Texture Entropy (termed Cel_Text_22, Figures S5–S7, Supporting Information) for all cell types tested, signifying a high degree of complexity for actin distribution within the cell. The consistent nature of this profile suggests that it is a cell type specific response to the change in topography due to the distinct increase in z-score values to that of the flat control samples. Furthermore, the L929 cells also exhibited a unique decrease in Cell Zernike factors (Cell_Zernike_73) and a stark increase in Actin_SumAverage, denoting a uniform fluorescence intensity distribution across the actin cytoskeleton for only the complex pattern types (CI-3). These expression patterns demonstrate topography-driven cellular events which could signify unique fibroblast specific responses to substrate topography which if further understood, could be harnessed to direct cell-substrate interactions to more accurately depict that of the in vivo scenario.
In contrast, C2C12 cells (mouse muscle cell type) present the majority of their differential expression profiles across Actin Eccentricity, Actin Formfactor and Actin Zernike 6.0 (termed Cel_Shape_3, Cel_Shape_5 and Cel_Shape_25, respectively, Figures S5–S7, Supporting Information) for grooved and complex structures, signifying a specific impact on cell circularity, spreading, and actin distribution. For pillared structures, limited variation was observed in either nuclear or cell image descriptors, suggesting a unique cellular response to these features regardless of their size or spacing. HUVEC cells on the other hand presented the most subdued changes, with only minor variation to nuclear image descriptors for select pillared (P1 and P2) and complex structures (C3).

Figure 5. MSC fate determination can be influenced using topographical features. a) Quantification and example images of MSC osteogenic differentiation capacity using alkaline phosphatase staining (blue). Scale bar: 20 µm. b) Quantification of preferential MSC fate towards osteogenic (blue, alkaline phosphatase) or adipogenic (red, Oil red O) lineages using a switch assay. Scale bar: 20 µm. c) Heat map depicting z-scores for cell and nuclear image descriptors across each micropatterned design tested. d) Cell aspect ratio and e) associated nuclear circularity quantification of MSCs cultured across each micropatterned design tested. Graphs present the mean ± SD from 300 cells for three MSC donors. Samples were analyzed by one-way ANOVA with Tukey post hoc testing. Statistically different samples are denoted by *p < 0.05, **p < 0.01, ***p < 0.005 and ****p < 0.001. All cell and nuclear shape/texture descriptors are defined in Figures S5–S7, Supporting Information.
Interestingly, for each cell type screened we observed distinct clustering for pattern P4 (denoting pillared structures with the largest spacing and feature sizes (15 µm)) (Figures S10–S21, Supporting Information). Although the z-scores differed between each cell type, the consistent segregation of this topography suggests a potential conservation of cell behavior. In contrast, for grooves of 15 µm feature size and spacing (G4), this segregated clustering was only evident for MSCs, suggesting a unique response for this cell type.

A further observation was the similarity between MSC and HaCaT clustering, in which comparable profiles were observed for nuclear shape descriptors across both pillared and complex topographical designs (most notably P3 and C3, respectively) (Figure 6a). Despite the significant variation in cell behavior under standard culture conditions between MSCs (isolated growth) and HaCaTs (colony forming cells), the mirrored nuclear morphological changes in both cell types may indicate similarities in how both cell types interact with the underlying topography. Through further in-depth experimental analysis of nuclear phenotypes (Figure 6b–d), the data also revealed similarities in nuclear area and aspect ratio for the P3 topographical features (the pattern for which screening revealed the highest degree of overlap in nuclear shape descriptors between the two cell types). As it is well documented that both HaCaT cells and MSCs are highly mechanosensitive in nature, able to adapt to modulations in environmental properties to direct cell fate changes,[53–56] our observations denote a potentially conserved morphological response to topography which denotes mechanosensitivity.

Figure 6. High throughput screening across multiple cell types can be used to identify conservation in cellular responses to changing topographical cues. a) Z-score analysis of the top cell and nuclear image descriptors between MSC, L929, HUVEC, C2C12, and HaCaT cells following 72 h culture on pillared, grooved, and complex topographies. Comparative analysis of b) nuclear area, c) nuclear circularity, and d) nuclear aspect ratio between MSC and HaCaT cells cultured across pillared topographical features. Graphs present the mean ± SD from 300 cells for three MSC donors. Samples were analyzed by one-way ANOVA with Tukey post hoc testing. Statistically different samples are denoted by **** p < 0.001. All cell and nuclear shape/texture descriptors are defined in Figures S5–S7, Supporting Information. White boxes denote selected profiles of interest, demonstrating similarities in signatures for MSC and HaCaT cells in nuclear image descriptors and a unique profile for L929 cell image descriptors. The shape descriptors with additional color coding have been identified within the top 5 image descriptors that contributed to clustering in response to topographical cues (Figure S19, Supporting Information).
3. Conclusion

Although many micropatterned substrates have been demonstrated to modulate cellular function, they are often accompanied by a distinct difference in surface properties to that of conventional TCP used across different disciplines in life science research and development. This discrepancy makes direct links between topographical features and fundamental biological processes difficult to establish. To overcome this problem, we have generated a novel NIL method which enables the direct fabrication of micro- and nanotopographical features on standard PS-based multiwell plates. The quality, reproducibility and comparable surface chemistry of our TCP arrays enables systematic investigation of the effects of microtopographical designs on cell morphology and fate. Furthermore, the ability to directly pattern existing conventional cell cultureware, without the requirement for difficult-to-handle culture inserts, opens up the technique to mainstream biological work by providing a format that can be readily used in conjunction with existing protocols. We also demonstrate that the format is also amenable to integration with the pipelines for high-throughput screening, thereby opening up the possibility of including topographic substrates into drug discovery platforms. The technology is likely to underpin essential insights into how cell and nuclear morphological features can be screened across varied topographical designs to decipher key image descriptors that contribute to cellular fate determination. Interestingly a comparison of multiple cell types also revealed cell-specific changes as well as conservation of distinct topographical responses between cell types which correlate to cellular mechanosensitivity, suggesting that through the identification of key fingerprint cell responses to topographical features, we can potentially determine the influence of substrate properties on cellular function. Together these findings demonstrate a highly versatile platform which is compatible with current screening technologies, and its ability to facilitate the advancement of our fundamental understanding of how cells are influenced by substrate topology. We expect that this platform will open up exciting applications across the life sciences, including fundamental cell biology, tissue engineering, regenerative medicine, drug screening, and cell therapy; and will reduce the need for expensive soluble biological factors that are used to direct cell function.

4. Experimental Section

Master Stamp Fabrication: For micro- and nanostructured libraries, SU8 master stamps were fabricated using UV-photolithography as described previously.[43] Briefly, a polished 4-inch silicon (Si) wafer (University Wafer, MA, USA) was sonicated in acetone and isopropanol for 5 min. SU-8 3005 (Microchem, MA, USA) was then spin-coated at 3400 rpm for 45 s to achieve a 4.9-5.1 µm thick layer. For the development process, samples were dipped in ZED-N50 (n-aryl acetate) developer for 60 s, then rinsed with Isopropanol alcohol (IPA) and finally blow dried with N2. Reactive ion etching (RIE) was then used to transfer the micro- and nanoscale features to the Si, at a SF6:CF8 gas flow rate of 50:90 sccm and a ICP and RF power of 2000 and 25, respectively. Etching was performed for 90 s to obtain 250 nm depth.

PDMS Mold Fabrication: Micro- and nanostructured master substrates were exposed to O2 plasma treatment using a diener nano reactor for 5 min followed by a 2 h salinization step using (1H,1H,2H,2H-perfluorooctyl-trichlorosilane in a vacuumed desiccator) to form an anti-adhesion silane layer. PDMS prepolymer and curing agent were then mixed in a ratio of 5:1 (w/w) and poured over the patterned wafer to generate a negative replica of the structures. The deviation from the conventional PDMS:curing agent ratio of 10:1 resulted from evidence suggesting poor transfer fidelity during NIL processing due to the soft nature of resulting PDMS.[33] To mitigate this issue, a ratio of 5:1 was used to increase the Young’s modulus of PDMS from 580 kPa (10:1) to 1000 kPa (5:1). The PDMS was then degassed for 30 min and cured for 120 min at 70 °C. Once cured, the PDMS mold was carefully peeled off the silicon master ready for use.

NIL of Multiwell Plates: For NIL processing of multiwell culture plates (24 welled plate, ThermoFisher) an EVC-520HE hot embossing system was used. PDMS molds were first placed into each well, following which the chamber pressure was set to 10⁻³ mbar. The base of the multiwell plate was then heated to 145 °C, followed by the application of 1.2 kN force onto the PDMS stamp using the 3D printed modular for 30 min. Samples were then left to cool gradually to 45 °C, at which point the PDMS stamp was gently removed from the well, leaving a direct replication of topographical imprints in the PS substrate. The total process time was between 40 and 45 min, with follow on replication accuracy validated through direct comparison with the dimensions of the PDMS stamp to test for feature fidelity and transfer efficiency.

Surface Characterization—Scanning Electron Microscopy: SEM imaging was carried out using a FEI NovaNanoSEM 430 SEM operated at 2–5 kV in “immersion” mode. Obtained images were analysed using ImageJ V.1.46 (open source software).

Surface Characterization—Contact Angle Measurements: Contact angle (CA) measurements were measured using the sessile drop method. Briefly a 1 µl droplet of Milli-Q water was deposited onto the various imprinted and nonimprinted substrates at ambient temperature. A Panasonic super dynamic WBP550 closed circle TV camera was then used to acquire images of the droplets, following which measurements were performed using ImageJ V.1.46 drop analysis plug-in. Each measurement was performed in triplicate across three experimental repeats to account for sample-to-sample and spot-to-spot reproducibility.

Surface Characterization—X-Ray Photoelectron Spectroscopy: XPS analysis was performed using an AXIS Nova spectrometer (Kratos Analytical Inc). For sample analysis, a monochromated Al Kα source was used at a power of 180 W. System pressure was maintained between 10⁻⁸ and 10⁻⁹ mbar. For survey scans the pass energy was maintained at 160 eV with a step size of 0.5 eV. Data processing was performed using CasaXPS processing software version 2.3.15. The atomic concentrations of detected elements were calculated using integral peak intensities and the sensitivity factors supplied by the manufacturer. Binding energies were referenced to the C 1s peak at 284.7 eV for aromatic hydrocarbon.

Surface Characterization—Surface Roughness Analysis: To measure surface roughness of pristine and flat imprinted TCP substrates, a Bruker Dimension Icon atomic force microscope (AFM) was used. Samples were imaged at ambient temperature using a silicon tip on a nitride cantilever, with a spring constant (k) of 0.4 N m⁻¹. All pictures were obtained in ScanAsyst measurement mode at a pixel resolution of
512 × 512. The results were then further analyzed using the nanoscope analysis 1.8 software.

Surface Characterization—Optical Transmission Spectra Measurement: Transmission spectra of pristine and imprinted TCP were recorded using an Ocean Optics S-1000 spectrometer, fitted with a diffractured fibre optic probe. A tungsten light source was focused onto the center of the sample surface with a spot size of 1 mm. Spectra were recorded with a CCD detector in the wavelength range 400–1000 nm.

Cell Culture: Human Bone marrow derived MSCs were cultured in DMEM-low glucose [1 mg mL⁻¹] supplemented with 100 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin (DMEM/PS) and 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. These MSCs were tested and certified to meet the required ISCT defined criteria for stem cells and were free from tested pathogens. All experiments were conducted using passage six of these cells. L929, HaCaT, MCF7, and C2C12 cell lines were cultured using DMEM-high glucose [4.5 mg mL⁻¹] supplemented with 100 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin (DMEM/PS), and 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. For the culture of human umbilical vein endothelial cells (HUVEC), Endothelial Cell Growth Medium BulletKit (Lonza) was used following the supplier’s instructions. All cultures were routinely tested for mycoplasma every three months. Prior to all experiments, cells were serum-starved overnight in their respective culture medium supplemented with only 0.25% FBS.

MSC Differentiation: MSCs were plated onto the appropriate substrate at 5 × 10⁴ cells cm⁻² and treated with either osteogenic or mixed (1:1 adipogenic:osteogenic) inductive medium, with medium changes

Immunofluorescence Staining: Cells grown on TCP arrays were fixed in 4% PFA diluted in PBS for 15 min followed by permeabilization with 0.5% Triton X-100 for 10 min. Fixed samples were blocked in 5% bovine serum albumen (BSA) for 30 min and incubated with primary antibodies (diluted in BSA) for 1 h at RT. The following antibodies were used throughout this investigation: β-actin [1:1000] (Sigma A5316), lamin A/C (Jol2) [1:500] (ImmuQuest IQ608), H3K9 [1:1000] (Abcam 176 916), YAP [1:1000] (Thermo PAI-46189) and Ki67 [1:1000] (Abcam). Cells were extensively washed in PBS and then incubated with the relevant secondary antibodies for 1 h, which were conjugated with Alexa Fluor 488 or Alexa Fluor 555. DNA was counterstained with Hoechst 33 342 [1:1000] for 20 min. All samples were analyzed by conventional fluorescence microscopy using a Nikon Eclipse Ti microscope (A1 HD25).

Bioincompatibility Screening: To ensure cell phenotype changes did not arise from negative responses to the base Ormocomp substrate, bioincompatibility was tested as described previously. Briefly, cell viability was assessed using Live/Dead staining assay (ThermoFisher) following manufacturers protocol. Proliferation was monitored using KI67 fluorescence staining as detailed in Western Blotting. Finally, changes to metabolic activity were probed using the MTS assay (Promega) following the manufacturer’s protocol. Each viability assay was conducted 72 h post cell seeding.

High-Content Image Analysis: High content immunofluorescence images stained for nuclei (Hoechst) and actin were obtained as outlined in Immunofluorescence Staining. Images were then processed using CellProfiler 3.0.0 (VCFC pipelines) and analyzed in the coding programme R. Briefly, raw cell data was aggregated into per-image data and the aggregated values were then normalized, centered, and scaled by robust Z-Soring to the flat control on a per-cell line, per-feature basis. Technical replicates were then averaged by calculating the per-cell line, per-pattern type median for each image descriptor, or feature. Feature reduction was then performed in which columns with low variance across samples (“inactive” features), containing Inf, NA, and NaN values, or a high degree of correlation (redundant features) were removed, leaving a total of 130 features remaining.

Unbiased hierarchical clustering was used to organize the pattern types into groups of similarity (clusters) based on this final set of 130 features, using Pearson’s correlation as the distance metric and average linkage as the method. The output was displayed in a dendrogram, which was used to determine the allocation of clusters. The degree to which the dendrogram faithfully preserved the pairwise distances between the original unmodeled data points was estimated by calculating the cophenetic correlation coefficient (result = 0.929).

Statistical Analysis: All graphical data is presented as mean ± standard deviation across three separate MSC donors (n = 9) unless otherwise stated. A Kolmogorov-Smirnov test was used to test data for Normal distribution and Levene’s test used to determine homogeneity of variance. Data with a Normal distribution were analyzed by one-way ANOVA and Tukey (equal variance) or Games–Howell (unequal variance) post hoc tests. Non-parametric data were analyzed by Kruskal–Wallis test. All statistical analysis was performed using GraphPad Prism 8.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
J.C. and H.H.A. contributed equally to this work. This study was financially supported by the Australian Research Council (DP190100129),
Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords
high-throughput screening, micro/nano-topography, nanoimprint lithography, tissue culture plastic

Received: January 27, 2021
Revised: March 12, 2021
Published online: April 21, 2021