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Micropattern size-dependent endothelial differentiation from a human induced pluripotent stem cell line

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Abstract

The multifaceted extracellular milieu presents biochemical and biophysical stimuli that influence stem cell differentiation. Two-dimensional (2D) micropatterned substrates allow the presentation of these cues in spatially defined geometries that have been demonstrated to guide stem cell fate decisions. Leveraging stem cells to reconstruct microvasculature, made up of an inner lining of endothelial cells (ECs) supported by pericytes, is critical to tissue-engineering advances; thus, methods to improve endothelial differentiation efficiency are vital to these efforts. In this study, we examine the hypothesis that the diameter of micropatterned islands influences endothelial differentiation from human induced pluripotent stem cells (hiPSCs). Comparing island diameters of 80, 140, 225 and 500 μm , we found that co-cultures of control ECs and pericytes did not yield variable ratios of cell types; however, when hiPSCs were differentiated toward a bicellular population of ECs and pericytes on these varying micropattern feature sizes, we found that smaller islands promoted EC differentiation efficiency, yielding a derived population composed of 70% ECs, which exhibited a greater sprouting propensity. Differentiation on the largest feature size exhibited a smaller EC yield, similar to that on non-patterned substrates. Taken together, these data demonstrate that micropatterned islands of varying diameters can be used to modulate EC differentiation efficiency.

Keywords

cell co-culture; endothelial cells; micropatterning; pericytes; pluripotent stem cells; tissue engineering

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Supporting information may be found in the online version of this article.

Conflict of interest

The authors have declared that there is no conflict of interest.

Supporting information on the internet

The following supporting information may be found in the online version of this article:

Supplementary 1. No effect of ECM on differentiation capability; flow-cytometry plots for VEcad of EVCs differentiated on either collagen IV or fibronectin substrates.

1. Introduction

Vascular reconstruction depends on the interplay of multiple cell types, biochemical cues and biophysical stimuli (Jain, 2003). The extracellular matrix (ECM), which has numerous functions, including presentation of instructive cues and maintenance of tissue integrity, is a key regulator in vascular development and regeneration. Advances in tissue engineering allow researchers to harness these instructive signals in precise microenvironments, offering novel therapies for vascular repair (Zhang *et al.*, 2011).

Of the multiple cell types that make up blood vessel architecture, endothelial cells (ECs) form the inner lining of the vascular system and are able to form nascent structures intrinsically. Pericytes are recruited to these structures to confer stabilization and support (Stratman *et al.*, 2009). Because all tissues, both native and engineered, require a functional blood supply for survival, rebuilding blood vessels in the laboratory continues to be a burgeoning field of study (Kusuma and Gerecht, 2010; Blinder *et al.*, 2014).

Human pluripotent stem cells (hPSCs) are an attractive starting source for this endeavour because of their ability to differentiate into all cell types of the body and to self-renew indefinitely in culture, allowing the generation of clinically relevant quantities of cell types of interest. Human embryonic stem cells (hESCs), one subclass of hPSCs, are derived from the developing blastocyst and currently make up much of hPSC research. A newer subclass of hPSCs, human induced PSCs (hiPSCs), are derived by reprogramming somatic cells, conferring patient specificity (Mali and Cheng, 2012). Our previous studies have demonstrated the derivation of a bicellular population of ECs and pericytes from hPSCs after 12 days in a two-dimensional (2D), feeder-free manner (Kusuma *et al.*, 2013). More recently, we have demonstrated that using low-oxygen (~5% O₂) culture conditions to this differentiation scheme (either over the entire 12 day differentiation or just during the first 6 days) enhances endothelial differentiation potential, yielding ECs with appropriate membrane localization of vascular endothelial cadherin (VEcad), a phenotype of mature derivatives, after just 12 days (Kusuma *et al.*, 2014; Kusuma *et al.*, 2014). In these studies, we have extensively examined the phenotype and functionality of EC and pericyte derivatives both *in vitro* and *in vivo* (Wanjare *et al.*, 2014).

Stem cell fate is predicated by stimuli in the surrounding milieu (Dickinson *et al.*, 2011; Hazeltine *et al.*, 2013). Previous studies have demonstrated a correlation between microenvironment feature size and stem cell fate decisions. Human PSCs differentiated in suspended cell aggregates, or embryoid bodies (EBs), exhibited size-dependent differentiation decisions (Hwang *et al.*, 2009); EBs with a controlled diameter of 450 µm demonstrated a preferential differentiation towards the cardiac lineage, whereas smaller EBs (~150 µm diameter) favoured endothelial lineages. Micropatterning offers a 2D approach to control cellular behaviour, using instructive cues in the form of distinct adherent geometrically defined patterns that typically consist of extracellular matrix (ECM) proteins. Mouse ESCs cultured on fibronectin circular micropatterns exhibited the greatest potential to differentiate toward cardiomyocytes on 200 µm diameters, compared to 100, 300 and 400 µm diameters (Sasaki *et al.*, 2009). On circular Matrigel patterns, cultured hESCs

differentiated toward mesoderm on larger islands (800 and 1200 μm), but toward endoderm fates on smaller geometries (200 and 400 μm) (Peerani *et al.*, 2007; Lee *et al.*, 2009). An adult stem cell population, mesenchymal stem cells (MSCs), demonstrated preferential differentiation toward adipocytes on smaller fibronectin islands, whereas larger islands promoted osteoblast differentiation (McBeath *et al.*, 2004). Taken together, these studies reveal that ECM proteins are not the lone determinant of fate specification; rather, their spatially defined presentation is also a key influence. Moreover, the influence of constrained 2D environments on endothelial differentiation from hiPSCs has not been previously examined.

In the current study, we harnessed our previous co-differentiation strategy, which guides hPSCs toward a bicellular population of ECs and pericytes under low oxygen conditions, to test the hypothesis that micropattern diameter affects endothelial differentiation potential from the hiPSC line, BC1.

2. Materials and methods

2.1. Cell culture

2.1.1. HUVECs culture—Human umbilical vein ECs (HUVECs) were cultured as previously described (Kusuma *et al.*, 2012). Briefly, HUVECs (Promocell) were cultured in endothelial cell growth medium (ECGM; PromoCell) supplemented with 2% fetal bovine serum (FBS). The medium was changed every other day. Cells were passaged every 3–4 days with 0.05 trypsin/0.1% ethylenediamine tetra-acetic acid (EDTA; Invitrogen) and maintained in a humidified incubator at 37°C in 5% CO₂ atmosphere.

2.1.2. Pericytes culture—Human placental pericytes (passages 3–5, Promocell) were cultured in the specified pericyte growth medium (Promocell) and were passaged every 3–4 days, using a detachment kit (Promocell), as previously described (Wanjare *et al.*, 2014).

2.1.3. HUVECs/pericytes co-culture—HUVECs and pericytes were seeded onto micropatterned coverslips (CYTOO) at a 1:1 ratio with a total cell number of 50 000 in endothelial growth medium 2 (EGM2; Lonza) containing 10% FBS.

2.1.4. hiPSCs culture and differentiation—A human iPSC line, BC1 (Chou *et al.*, 2011; Cheng *et al.*, 2012), was dissociated into a single-cell suspension and seeded onto collagen IV-coated dishes (Trevigen, CellStar) under 5% O₂ conditions, as described previously (Kusuma *et al.*, 2013, 2014). Briefly, differentiating cells were exposed to 5% O₂ conditions for 6 days, collected through digestion with TrypLE (Invitrogen), separated with a 40 μm strainer and reseeded on fibronectin-micropatterned coverslips (CYTOO) at 50 000 cells/coverslip in EC differentiation medium, which was made up of ECGM (Promocell) supplemented with 2% FBS (Promocell), 50 ng/ml vascular endothelial growth factor (VEGF; Roche), 10 μM SB431542 (Tocris) and 0.1% penicillin–streptomycin (Life Technologies). The medium was changed on day 3 and the samples were fixed on day 6. Cells were imaged daily using an inverted light microscope (Olympus).

2.2. Immunofluorescence

Cells were prepared for immunofluorescence as previously described (Kusuma *et al.*, 2012). Briefly, fixed cells were blocked in 1% bovine serum albumin (BSA; Sigma-Aldrich), treated with 0.1% Triton-X (Sigma-Aldrich) and incubated with mouse anti-human VEcad (1:200; Santa Cruz Biotechnology), rabbit anti-human SM22 (1:200; Abcam) or rabbit anti-human PDGFR β (1:100; Santa Cruz Biotechnology), followed by anti-mouse FITC (1:40; Sigma) or anti-rabbit IgG AlexaFluor 488 conjugate (1:1000; Molecular Probes) and DAPI (1:1000; Roche Diagnostics), all at room temperature in the dark. The immunolabelled cells were examined using a fluorescence microscope (Olympus BX60).

2.3. Quantification

Image processing and analysis were conducted using a custom-written MATLAB algorithm (see supporting information). Subsequent to preprocessing the images via a global background subtraction scheme, total nuclei were enumerated by finding regional maximum pixel intensities. Additionally, co-localized nuclei with corresponding image channels representing VEcad expression were quantified.

2.4. Graphs and statistics

All analyses were performed in triplicate samples from at least three independent experiments. A minimum of 30 patterns of each size were analysed per experiment. One-way ANOVA with Bonferroni *post hoc* test were performed to determine significance (GraphPad Prism 4.02).

3. Results

3.1. HUVECs/pericytes co-culture on circular micropatterns

We studied circular fibronectin micropatterns with diameters of 80, 140, 225 and 500 μm . To confirm preferential attachment of control cells on fibronectin micropatterns and to determine whether these cell types preferentially attach to a particular feature size, control ECs (i.e. HUVECs) and pericytes were seeded at a 1:1 ratio on patterned coverslips and cultured for 2 days, allowing for cell adhesion, spreading and the re-establishment of junctional proteins between cells. A 1:1 ratio of tissue-derived vasculogenic cells was chosen to ensure that an uneven ratio did not skew adhesion propensity, and to eliminate the chance of cellular plasticity that hPSC derivatives may exhibit. Both cell types exhibited preferential attachment to the patterned regions (Figure 1A). HUVECs, indicated by VEcad, and pericytes, indicated by SM22, co-cultured on patterns, demonstrate spread pericytes growing above as well as among the HUVECs monolayer (Figure 1A). Quantification of cell number/pattern size revealed that pattern size did not influence cell growth (Figure 1B). Co-culturing these two cell types revealed a preferential attachment of HUVECs to the patterns. Quantification of the fraction of HUVECs/pattern size showed that patterns were predominantly covered by HUVECs (Figure 1C). This finding could be due to preferential attachment of HUVECs to fibronectin surfaces or the smaller size of HUVECs compared to pericytes, which typically take on a more spread morphology. Overall, however, the fraction of HUVECs remained similar between the different pattern sizes, demonstrating that

differentiated ECs and pericytes do not demonstrate preferential attachment to any particular feature size.

3.2. Differentiation of hiPSCs toward EVCs on circular micropatterns

Next, to discern whether micropattern size influences endothelial differentiation potential from hiPSCs, we cultured differentiating cells on micropatterned coverslips. Human iPSCs were dissociated into a single-cell suspension and differentiated for 6 days on collagen IV-coated dishes under 5% O₂ conditions. Using this low-oxygen priming method, we obtained approximately 50% positive VEcad cells, on average, from three independent experiments, as previously reported (Kusuma *et al.*, 2014). In the current study, cells were differentiated for the first 6 days under 5% O₂ conditions, but on day 6 these progenitors were transferred to coverslips containing circular micropatterns with diameters of 80, 140, 225 or 500 μm. We confirmed that the second 6 days of differentiation on a fibronectin substrate does not alter VEcad composition of EVCs compared to differentiation on collagen IV, as we have utilized in our previous study (Kusuma *et al.*, 2013) (see supporting information, Figure S1). After an additional 6 days, we found that the cells demonstrated preferential attachment to the patterned regions and spread to confluence on the patterns (Figure 2A). We stained for VEcad and platelet-derived growth factor receptor-β (PDGFRβ) to determine our EC and pericyte populations, respectively. As in our bulk differentiation conditions (Kusuma *et al.*, 2014), we could observe derived ECs that took on a cobblestone-like morphology and exhibited VEcad appropriately localized to the cellular membrane. Derived PDGFRβ-positive (PDGFRβ⁺) pericytes were observed in close contact with VEcad-positive (VEcad⁺) cells. Quantification of total cell number revealed that cell growth did not depend on pattern size (Figure 2B).

3.3. Quantitative comparison of endothelial differentiation potential on varying micropattern sizes

We developed a MATLAB code to quantify the total number of VEcad⁺ cells per individual pattern. The programme produced black and white images of the VEcad stain with corresponding nuclei positive for VEcad (Figure 3A). Quantification of endothelial derivatives revealed that, unlike control HUVECs and pericytes co-culture, we could obtain different fractions of ECs on the varying pattern sizes. Differentiation on the smallest diameter, 80 μm, yielded approximately 67% VEcad⁺ cells across all patterns of this feature size (Figure 3B). Quantification over each individual pattern revealed a median value of 71% VEcad⁺ cells (Figure 3C). Differentiation on 140 μm patterns yielded both mean and median values of 58% VEcad⁺ cells. Patterns with 225 μm diameters yielded 42% VEcad⁺ cells, with a median value of 32% per individual pattern. Comparison over these three feature sizes reveals a great endothelial differentiation potential on smaller feature sizes. When we differentiated cells on 500 μm patterns; however, we obtained approximately 50% VEcad⁺ cells, closely matching our previously published results on non-patterned dishes (Kusuma *et al.*, 2014). Next, we quantified the fraction of patterns that only contained VEcad⁺ cells without any PDGFRβ⁺ cells. We could also observe that homogeneous EC patterns were more prevalent at the smallest pattern size (Figure 3D). The fractions decreased at 140 and 225 μm. Pure EC populations were not observed on 500 μm patterns.

3.4. Endothelial sprouting on varying micropattern sizes

We could also observe sprouting beyond the confined patch area from EVCs in all pattern sizes < 500 μm (Figure 4A). Sprouting is an important stage in the angiogenic cascade, initiating the growth of new blood vessels. To characterize this phenotype, we first assessed whether this behaviour was cell type-specific. Immunofluorescence analysis revealed that our VEcad⁺ ECs were the lone cell type to sprout from patterns, a phenotype that has been described in other derived EC cultures (Hwang *et al.*, 2009). Imaging patterns over time allowed us to examine the kinetics of sprouting formation (Figure 4B). We found that sprouting primarily occurred on the small micro-pattern feature sizes around day 10, but were largely absent from large micropattern feature sizes until 11–12 days. Quantification of sprouting frequency within each feature size after 12 days corroborated our EC differentiation results; sprouting frequency was much greater in smaller micropatterned islands (i.e. 80 and 140 μm diameters) compared to larger islands (i.e. 225 μm) (Figure 4C). No sprouting was observed outside of 500 μm diameter islands.

4. Discussion

The regeneration of functional blood vessels in the laboratory benefits a myriad of downstream applications, such as tissue engineering and vascular regenerative medicine; the differentiation of vascular cell types from hPSCs is vital to these efforts in order to recreate patient-specific vasculature. Toward this end, spatial presentation of ECM has been realized as an important cue in the vascular system (Raghavan *et al.*, 2010).

Previous studies on the effect of circular micropatterns on somatic cell differentiation have been primarily centred on the cardiac lineage (Sasaki *et al.*, 2009). Here we present the observation that geometric patterns can also influence the differentiation of ECs from the hiPSC line BC1. Our previous studies on non-patterned surfaces revealed that exposure to low oxygen tension augments endothelial differentiation capacity of hPSCs, yielding approximately 50% VEcad⁺ cells from the hiPSC line BC1 (Kusuma *et al.*, 2014). In the current study, we added another layer of complexity to the cellular milieu by confining the growth area to micropatterned substrates. We found that micropatterned islands with 80 μm diameters further increased differentiation to yield 70% VEcad⁺ cells. Moreover, almost 50% of these patterns were covered with ECs only, without pericyte infiltration. We suspect that this preference for small diameter size is due to the well-accepted notion of pericyte-mediated endothelial growth inhibition.

With increasing diameter size, EC differentiation yield decreased; however, differentiation on 500 μm islands, our largest tested pattern size, more closely resembled differentiation on non-patterned substrates, yielding approximately 50% VEcad⁺ cells (Kusuma *et al.*, 2014). When we quantified cell growth on the tested pattern sizes, we observed that it was independent of pattern size, suggesting that the distinct differentiation capabilities were not dependent on cell proliferation.

We anticipate that our finding that smaller diameter size promotes EC differentiation has a lower feature size limit, as previous studies have demonstrated that islands with approximately 10 μm diameter promote apoptosis (Chen *et al.*, 1997). Furthermore, we

anticipate that islands with diameters $> 500 \mu\text{m}$ will not exhibit distinct VEcad⁺ yields and rather continue to follow a 50% VEcad⁺ yield found under non-patterned differentiation conditions.

While the focus of the current study is endothelial differentiation, the system presented here could be used as a platform to further study the dynamic interactions between hiPSC-derived ECs and pericytes. First, employing a 2D substrate facilitates high-resolution imaging of homogeneous populations of ECs or pericytes, or co-cultures of both cell types. Second, because our differentiation scheme relies on the co-derivation of both vascular cell types, we anticipate that differentiation on micropatterned islands will help to facilitate the derivation of populations with specific EC:pericyte ratios, which vary based on tissue type.

Taken together, our results demonstrate an important role for ECM feature size in augmenting endothelial differentiation yield from hiPSCs in a controlled manner amenable for clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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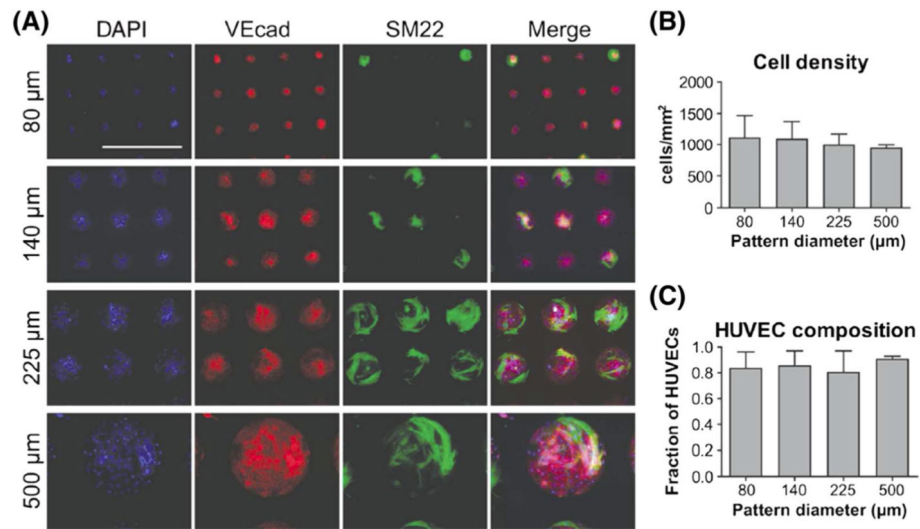


Figure 1. Co-culture of HUVECs and pericytes on circular micropatterns of varying diameter and assessed for: (A) VEcad (red) and SM22 (green) expression (nuclei in blue; scale bar = 500 μm); (B) total cell density; and (C) fraction of HUVECs on patterns

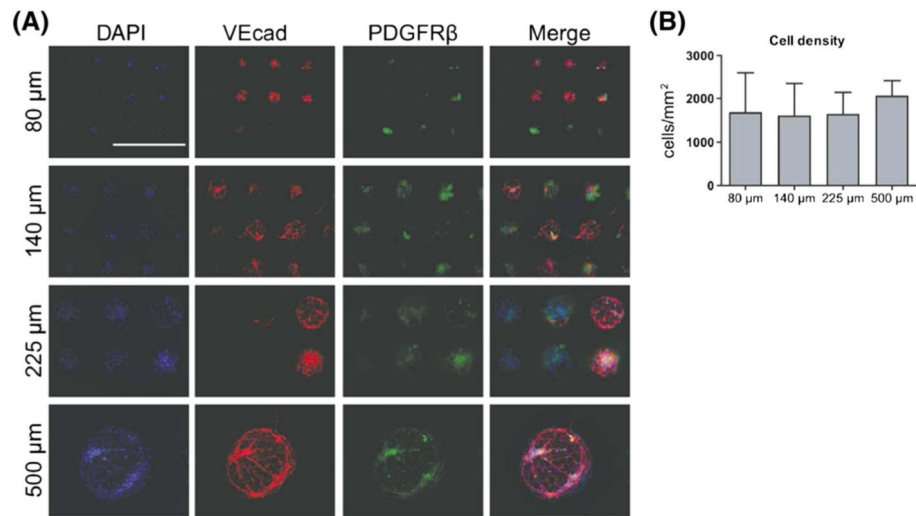


Figure 2. Co-differentiation of hiPSCs on circular micropatterns. Differentiated hiPSC-BC1 cells on micropatterns were assessed for: (A) endothelial and pericyte differentiation, indicated by VEcad (red) and PDGFR β (green), respectively (nuclei in blue; scale bar = 500 μ m); (B) cell density/pattern size

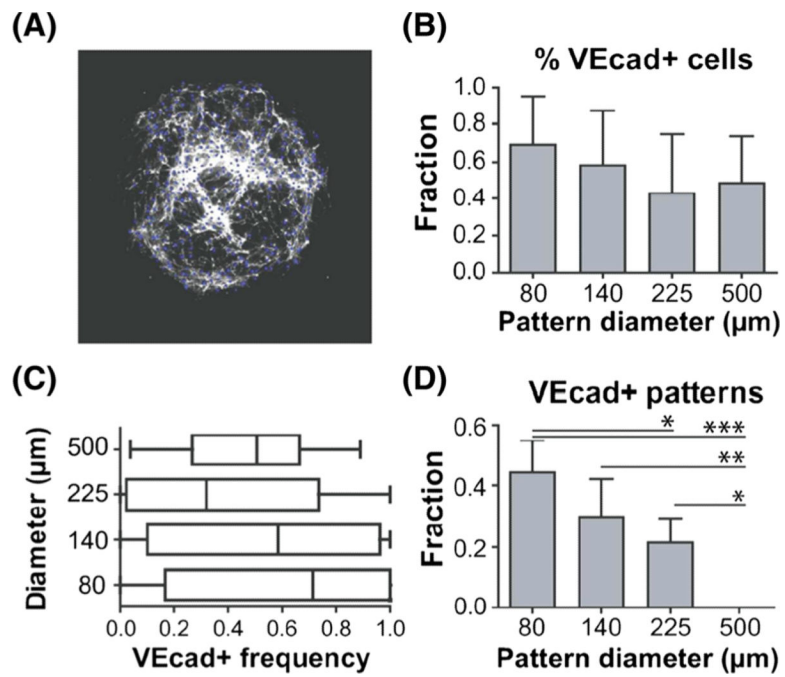


Figure 3. Quantification of endothelial differentiation potential. (A) Output of Matlab analysis and quantification of the (B) total percentage of VEcad-positive cells over all patterns of each feature size; (C) distribution of VEcad-positive cells for individual patterns; and (D) fraction of pure VEcad-positive patterns for each feature size; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

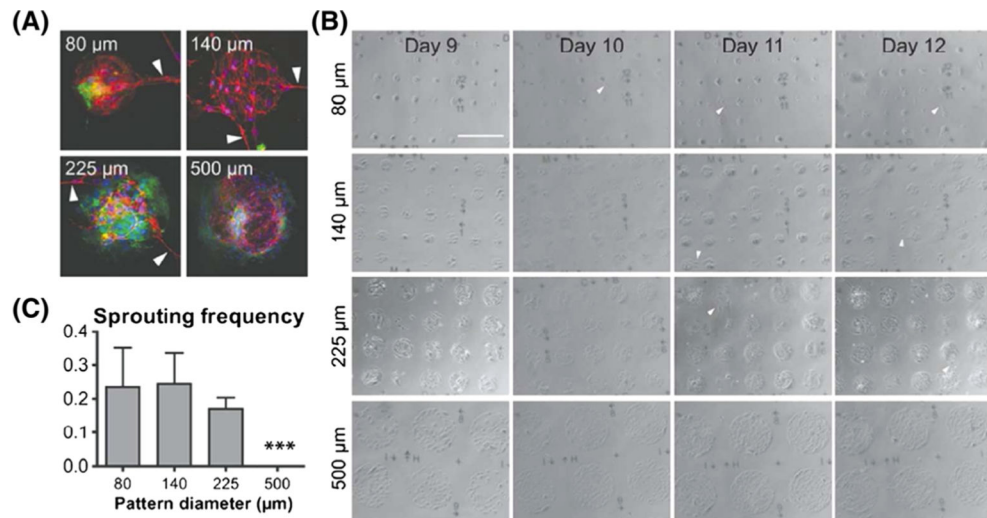


Figure 4. Characterization of sprouting propensity. (A) Immunofluorescent images of instances of sprouting on all pattern sizes; VECad in red, PDGFR β in green and DAPI in blue. (B) Light microscope images depicting emergence of sprouting over time on pattern sizes; scale bar = 500 μ m. (C) Quantification of sprouting frequency; arrowheads, instances of sprouting