High content Image Analysis to study phenotypic heterogeneity in endothelial cell monolayers

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Abstract

Endothelial cells (EC) are heterogeneous both across different tissues and within the same tissue, reflecting distinct specialised microenvironments and functions. Microenvironment independent EC heterogeneity within the same vascular bed has been appreciated and proposed to underpin EC monolayers plasticity. Nonetheless, the underlying molecular mechanisms are elusive. Heterogeneity within the same monolayer driven by contact-dependent or short-range cell-cell crosstalk cannot be evaluated with gold standard single cell transcriptomic approaches as they don't allow extracting spatial and context information.

Dissecting the molecular mechanisms leading to EC heterogeneity is key to offers novel therapeutic targets for cancer and cardiovascular diseases and for revascularisation strategies in regenerative medicine.

Here we developed an EC profiling tool (ECPT) to enable profiling of individual EC within a monolayer providing spatial and relational information on cell behaviour (including cell proliferation, inter-endothelial Junctions and NOTCH activation). We used ECPT to characterise differential phenotypes in arterial (Aortic EC), venous (HUVEC) and microvascular (Pulmonary microvascular EC) EC populations.

We find that different EC populations display a remarkable heterogeneity in terms of cell cycle and proliferation, cytoskeleton organisation, junction stability and a previously under-appreciated single-cell NOTCH heterogeneity. We also used ECPT to correlate junction stability with different NOTCH activation states at the single cell and population levels.

In conclusion ECPT is an extremely versatile tool to study positional and relational information in EC monolayers paving the way towards pharmacodynamic screenings and synthetic biology applications and overall, a better understanding of EC biology.

Introduction

Endothelial cells (EC) form the inner layer of blood and lymphatic vessels and play major roles in tissue development, angiogenesis, inflammation and immune cell trafficking[1]. EC are functionally plastic and rapidly adapt to changes in the environment to preserve homeostasis. Local EC dysregulation is a hallmark of pathological states such as atherosclerosis, ischemia and cancer[2]. EC from different tissues and vascular beds (such as arteries, capillaries, veins) exhibit distinct metabolism, morphology and gene expression[3] and contribute in diverse ways to tissue development and regeneration[4,5]. It is well established that EC are phenotypically heterogenous not only among different tissues, reflecting specialised organ-specific functions[6], but also within the same tissue. Maintenance of endothelial homeostasis depends on new EC substituting senescent cells and the role of endothelial progenitor cells with high repopulating potential has been highlighted, in particular in large vessels endothelia[7].

Inter-endothelial adherens junctions (IEJ) are dynamically regulated by VE cadherin (VEC) shuffling between the cell membrane and intracellular compartments. This process presents variations across vascular beds[3] and involves molecular mechanisms including VEGF receptors, cytoskeletal proteins and NOTCH family members. VEC and NOTCH signalling are well studied in angiogenesis and development. Nonetheless, the role of these molecular mechanisms in EC monolayer maintenance is less clear and this knowledge is essential to understand vessel homeostasis in different organs in the human body. So far, a comprehensive analysis of EC cultures exploring and quantifying phenotypic variance has proven prohibitively difficult because of lack of adequate tools.

Single cell analysis is important to identify and characterise intermediate cell states[8,9] and to demonstrate correspondence between phenotypes and function[10] despite challenges in discriminating functional phenotypes variance from biological noise[11]. Single cell transcriptomic data is becoming available (mostly in mouse[12]) and could in principle advance the characterisation of human EC[13] and provide an overview of molecular processes in distinct EC populations. Nonetheless, spatial information is essential to map variable phenotypes to function and is also required to understand higher-level functions depending on cell-cell connectivity. Of note, EC cell cycle and rapid adaptative phenomena, such as immediate increase of endothelial permeability upon VEGF stimulation are regulated by built-in sensing mechanisms that depend on cell-cell interaction[14]. Importantly, EC monolayers maintain their integrity over years while exerting a variety of system-level functions which are emerging properties of cells in contact[15]. It has been in fact proposed that endothelial adaptability and diversity of functions within an EC population depends on cell heterogeneity[16].

Here, we set up an endothelial cell profiling tool (ECPT) based on image analysis. We present a high-content analysis (HCA) platform to characterise single EC within an endothelial monolayer capturing a wealth of cell, context- and subcellular features including extensive characterisation of IEJ. This unbiased approach allows quantification of EC heterogeneity measuring feature variance[11]. Taking advantage of machine learning based methods (Appendix 1), we addressed automated and accurate classification of IEJ using junctional VEC immunostaining, and evaluation of NOTCH activation at the single cell level. In total, we present data from >6000 images detailing 29 selected measurements from >100,000 cells including >7.5 M junction objects.

Single EC profiling at such scale has not been reported and we demonstrate the validity of this analysis to uncover key relationships across features and to characterise cell behaviour across distinct EC types.

Materials and Methods

Cell culture

HUVEC, HAoEC and HDMEC (PromoCell) were plated on 10 µg/mL fibronectin (from human plasma, Promocell)-coated flasks, grown in EGMV2 medium (Promocell) in the absence of antibiotics, detached with Accutase (Thermo Fisher Scientific, Waltham, MA), and used by passage 5. For experiments, 4×10^4 ECs per well were seeded in fibronectin-coated 96-well plates (µclear, Greiner) and cultured for 48 h under basal (EGMV2, Promocell) or activated

(EGMV2 + 50 ng/mL VEGFA, Peprotech, London, UK) conditions in triplicate. The ECs formed confluent monolayers at the time of immunostaining and image acquisition.

Immunostaining

Cells were fixed with 2% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature. Cells were blocked 1h with PBS supplemented with 1% fetal bovine serum (FBS) and permeabilised with 0.1% Triton X 100. Cells were then incubated for 1h at room temperature with primary antibodies against CDH5 (Ve-Cadherin Novusbio NB600-1409, 1 µg/mL final) and activated NOTCH (NICD, Abcam ab8925, 1 µg/mL final). CDH5 is a lineage marker for EC and cells were all CDH5+, the anti-activated NOTCH1 reveals NOTCH intracellular domain (NICD) puncta. Plates were washed and incubated 1h with 1 µg/mL secondary Alexa 488-conjugated antibody and Alexa-555-conjugated antibody (Thermo), Hoechst 33342 (1 µg/mL, Sigma) and Phalloidin-Atto 647N (Sigma).

Image Acquisition

We obtained images from slides with an Operetta CLS system (PerkinElmer, Waltham, MA) equipped with a 40×water-immersion lens (NA 1.1). In each well, 3 areas were acquired. Each area is composed of nine microscopic fields at 40× magnification (Supplementary Figure 1). We standardised acquisition parameters (led power, exposure time) throughout different experiments and used HUVEC as a standard for calibration in all experiments. We analysed an image database containing 6028 images (1507 fields in four fluorescence channels) extracted from four independent experiments conducted on EC lines from two different donor each. Three intraexperiment replicates were conducted for each experiment.

Endothelial Cell Profiling Tool

We used a combination of machine learning-aided image segmentation (ImageJ)[52] and an image-based cell profiling tool (CellProfiler)[53] to extract the phenotype of single EC in monolayers. Our workflow enables to measure EC morphology (Area, perimeter, shape descriptors, cell neighbours), NICD, VE-Cadherin and DNA intensities and to characterise inter-endothelial junctions (IEJ). In the present study, we chose to analyse only selected features with recognised functions in EC biology. Image texture features were measured and only used during the training of machine learning algorithm[54,55] which in turn were used to classify cell activation states and junction morphology. ECPT scripts and methods are detailed in Appendix 1 and available at https://github.com/exr98/HCA-uncovers-metastable-phenotypes-in-hEC-monolayers

We Imported and cleaned the results into R studio excluding artifacts and mis-segmented cell objects. We then calculated derived continuous (Junctional stability index) and categorical (Cell Cycle and NOCTH activation) parameters (Supplementary Table 1). Following guidelines suggested by Caceido et al.[55] we pre-processed the database to exclude miss-segmented cells and to normalise the measurements prior to dimensionality reduction or single factor analysis. Our final dataset was composed of measurements of 29 parameters from 104416 single cells (Supplementary Table 1)

We conducted PCA analysis on cluster of ECPT parameters (Cell morphology, intensity, neighbourhood and junctional status, Supplementary Table 1) and generated 12 PCA components which we fed into the tSNE algorithm.

We performed tSNE using different perplexity values (5, 10, 20, 40, 60, 80) and ran the algorithm with 1000 iterations, all tSNE plots displayed correspond to 10 perplexity.

We created a shiny application for interactive selection of data of interest from t-SNE scatterplots (Appendix 1). This application[56] allowed specific cluster analysis by subclustering and further analysis in the R environment[57]. All files containing the code required to reproduce the shiny application using our dataset are available on https://github.com/exr98/HCA-uncovers-metastable-phenotypes-in-hEC-monolayers.

Statistical analysis

To compare multiple groups, we used one-way ANOVA followed by Tuckey's HSD post-hoc test. We considered $p< 0.05$ (*) significative and $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$(****) highly significative. To evaluate linear correlation between continuous variables we calculated

Pearson's Correlation coefficient (*R*) and considered $p<0.001$ as highly statistically significative.

Results:

Challenging quantification of endothelial cell heterogeneity by high-content imaging.

While heterogeneity of EC has been proposed, there are few examples of studies that achieved successful quantification of phenotypic variance of EC within the same vascular bed by transcriptomic analysis[17]. We previously reported the value of specific VEC and NOTCH quantification to benchmark EC in intact monolayers[18] and we now extended our observations, including relational information between individual cells which are impossible to retrieve with current single cell transcriptomic analysis. We seeded primary HUVEC (Human Umbilical Vein EC), HAoEC (Human Aortic EC) and HPMEC (Human Pulmonary Microvascular EC) for 48 hours in the absence or presence of VEGF to show the robustness of the pipeline under different conditions. We aimed for sufficient cells to reach confluency upon adhesion and spreading (~24 h) and we cultured for a further 24 h to enable formation of stable IEJ. EC demonstrate a uniform cobblestone morphology under low magnification, confirming monolayer stabilisation.

Imaging allows the in-depth phenotyping of EC, including morphology, protein expression and junction analysis, which is of foremost importance in EC function. In analysing these cultures, we focussed on proliferation, junctions and NOTCH activation. We stained for VE-Cadherin (VEC), activated NOTCH (NICD), actin cytoskeleton and nuclei. Immunostaining or live imaging of EC monolayers have highlighted differences in cell morphology and junction patterns in response to stimuli or under distinct cell culture conditions[19,20,21,22]. In line with the literature, qualitative inspection at high magnification revealed remarkable morphological differences between the three cell types in culture. HUVEC appear smaller and display a relatively disorganised cytoskeleton (Figure 1); HPMEC in contrast are largest and often demonstrate cortical distribution of the cytoskeleton; HAoEC show irregular shapes and sizes and often present as cells with stress fibres (Figure 1).

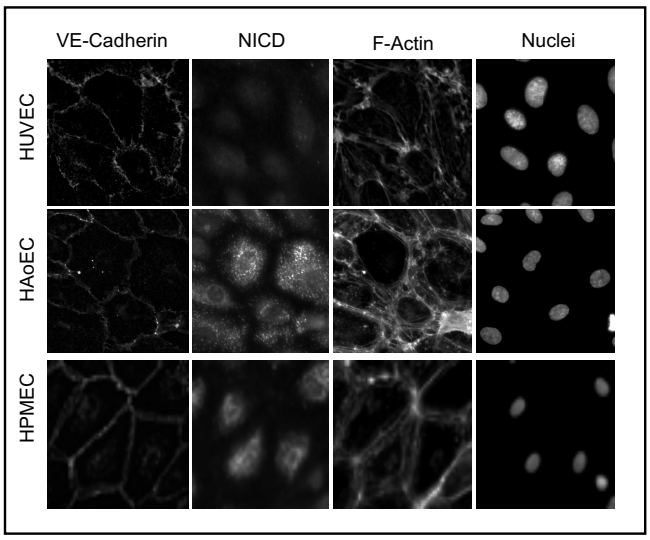

Figure 1: Overview of EC heterogeneity. Microphotographs comparing HUVECs, HAoECs and HPMECs stained for VE-Cadherin, NICD, F-actin and DNA (Nuclei).

EC junction has been linked to specific function and phenotypes[22,23], where linear and stabilised junctions are linked to mature quiescent phenotype, jagged and discontinuous junction results from proliferation, migration or immature/mesenchymal phenotype. Reticular patterns are observed in more stabilised junctions and they have been associated with immune cell transmigration[24].

Although VE-cadherin junction are dynamic, previous studies showed the possibility to characterize the junction patterning with image analysis[25, 26]. However, most work carried on

HUVEC did not study the differences in different organ-specific EC and did not study single-cell level heterogeneity.
The culture of HUVEC, HAoEC and HPMEC, 3 different primary EC from distinct locations in the body, - showed different junction patterns, cell morphology and dynamics in standard *in vitro* culture conditions. Qualitative observations led to hypothesize differences between organ-specific EC in terms of junction patterning and cytoskeleton activation. Many studies on Notch signalling have also highlighted its importance in EC function and cell junction rearrangement. Inspection of NICD intensity and repartition at the single-cell level appeared to be highly heterogeneous both intra-population and inter-population (Figure 1).
Altogether these qualitative observations point to a complex interplay of proliferation, IEJ and NOTCH that mirrors only in part previous studies referring to EC heterogeneity and distinct traits[3,27]. Measuring and scoring individual EC phenotypes to demonstrate, explain and quantify EC heterogeneity at single cell resolution is a prohibitively tedious and biased approach if not automated. Therefore, we set to assemble a dedicated automated platform that can seamlessly and comprehensively quantify variance of phenotypic parameters in EC in appropriate multidimensional space to ultimately define specific cell populations.

Creation of a semi-automated image analysis pipeline for EC phenotyping.

We developed a method to profile EC phenotypes at single cell level based on extracted high content analysis (HCA) features: Endothelial Cell Profiling Tool (ECPT, Figure 2 and Appendix 1). We used open-source software to generate an image analysis workflow able to take input of different sources, fixed or live imaging, with the aim of characterizing EC heterogeneity at single-cell level (Figure 2).

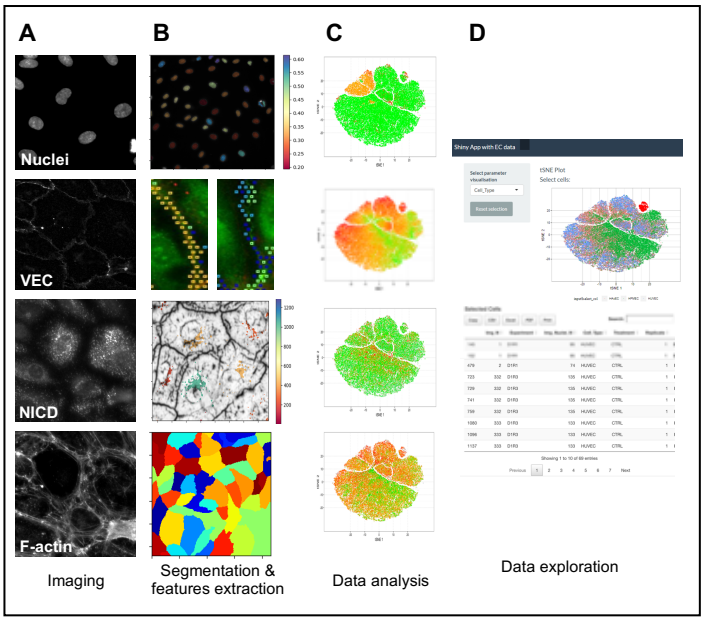

Figure 2: ECPT workflow. **A**) Acquisition of high resolution (40X OM) images on each channel (**B**) Features extraction and representation (ImageJ, Cell Profiler, Cell Profiler Analyst, R Studio). **C**) tSNE plots of features normalised and grouped by biological function. Colouring in panels (Top to bottom) correspondent to Cell cycle, STB junctions, NOTCH Activation classes and Cell activation classes. **D**) Shiny app (https://shiny.rstudio.com) enabling exploration of datasets, data extraction and iterative exploration.

A list of 29 features extending and refining our previous proof-of-principle efforts[18] was chosen including proliferation and context feature and machine learning-aided classification of junctions and of NOTCH activation (Appendix 1).
We resourced to tSNE based dimensionality reduction techniques to capture variance within our multivariate datasets and to identify discrete populations among heterogeneous cells. We first asked whether the three EC cell types analysed cluster using the chosen features. EC from different cell types formed distinct clusters (Supplementary Figure 1A) demonstrating that our parameters of choice (Supplementary Table 1) capture key differences in the three EC populations. Nonetheless, similarities across the populations are also observed (e.g., mean VEC intensity, Supplementary Figure 1B).
Altogether, tSNE enabled us to (1) specify diversities between EC populations and (2) demonstrate heterogeneity within the same populations. To understand the origin of this

heterogeneity we dissected individual sets of features dependent on the same biological mechanism. To subset our database according to tSNE clustering we also developed a dedicated user-friendly open-source interface using the shiny package within RStudio (https://shiny.rstudio.com). This shiny application allowed subsetting through interactive and iterative selection of single cells or groups of cells on tSNE scatterplots for further comparison and analysis within RStudio (Appendix 1). To understand in detail phenotypic differences, we explored our database focussing on selected traits to quantify variance in proliferation, junction stability and NOTCH activation.

Single cell Analysis of cell morphology, cell cycle and actin polymerisation to study population heterogeneity.

To characterize heterogeneity in the different EC monolayers, we first investigated differences based on morphology, cell cycle and cytoskeleton. The DNA and F-actin staining allowed the study of cell phenotypes in control conditions and in response to VEGF, after 48h of treatment, at the single-cell level.

The evaluation of EC activation state has been previously based on actin cytoskeleton organisation[28]. EC with stress fibres have been considered motile/migratory while those in which peripheral cytoskeleton overlaps with cell junctions are considered quiescent. The former EC phenotypes are usually associated with cell elongation and unstable IEJ. At simple microscopic observation, HPMEC homogeneously demonstrated features of quiescence such as cortical cytoskeleton supporting stable junctions. HAoEC and HUVEC instead presented a more heterogeneous phenotype, appeared more active and chronic VEGF treatment promoted migratory phenotypes in both cell types. Furthermore, VEGF treatment induced more stress fibres in HAoEC but not in HUVEC in contrast to previous observations under acute VEGF stimulation[29]. To substantiate these observations, we measured EC activation by ECPT. The use of supervised machine learning algorithm[30] allowed the classification of cell cytoskeleton status based on a single phalloidin immunostaining (Figure 3A). To avoid operator biases in these evaluations, we trained and used a machine-learning based software (Cell Profiler Analyst, CPA based on fast gentle boosting) to automatically classify quiescent, activated or stressed EC (Figure 3A, Appendix 1).

Under this classification, 40.6 % of HPMEC were quiescent cells, substantially more than HAoEC (20.5%) and HUVEC (16.2%) (Figure 3B). An opposite trend was observed for stressed cells (HAoEC, 25.4%; HPMEC, 13.7%; HUVEC 23.2%). In line with previous observations[18], VEGF induced elongation in all cells examined (Supplementary Figure 2) but had different effect on cytoskeleton depending on cell types. It had opposite effects on HAoEC and HUVEC, quiescent cells were reduced (-8.2%) in HAoEC and increased (+4%) in HUVEC while stressed cells were increased in HAoEC (+5.3%) and reduced in HUVEC (-7.1%). VEGF treatment also had little effects on HPMEC, save a small increase in quiescent cells (+2.2%). All EC populations had a significant proportion (46.1-55.6%) of activated cells (i.e., an intermediate, neither migratory nor fully quiescent and non-proliferating EC state characterised by reduced intercellular connectivity) further demonstrating that EC monolayers are composed of heterogeneous EC.

To evaluate cell proliferation, DNA content was analysed on the same principle as flow cytometry, based on previously published protocol[31]. The integrated Hoechst signal intensity inside nuclei was assimilated to the DNA content. The different stages of cell cycle (G1, S, G2/M) correspond to distinct DNA content and are defined via thresholding, with distinct peaks for G1 and G2/M (Supplementary Figure 3). Dividing (late mitotic, LM) cells, as shown in Figure 3A were binned in a specific category which was used to refine cell cycle analysis based on DNA intensity (Appendix 1) overcoming a specific limitation of previous approaches[31].

By culturing HUVEC, HAoEC and HPMEC under the same standardised conditions, we noticed that the three EC types present different intrinsic proliferation rates. We quantified proliferating cells within the different EC types using ECPT (Figure 3C Supplementary Figure 3). Proliferation rate was high in HAoEC and in HUVEC at baseline (average 13.6% and 11.7

% of dividing cells per field) and lower in HPMEC (average 6.8%). VEGF induced a small detectable increase in proliferation rate in all EC.

Together these data validate the use of this pipeline to characterize EC cell cycle, morphology and activation status. The shift in phenotype following VEGF treatment confirms the robustness of the image analysis and allows to highlight subtle changes at the population level and find distinct subpopulations.

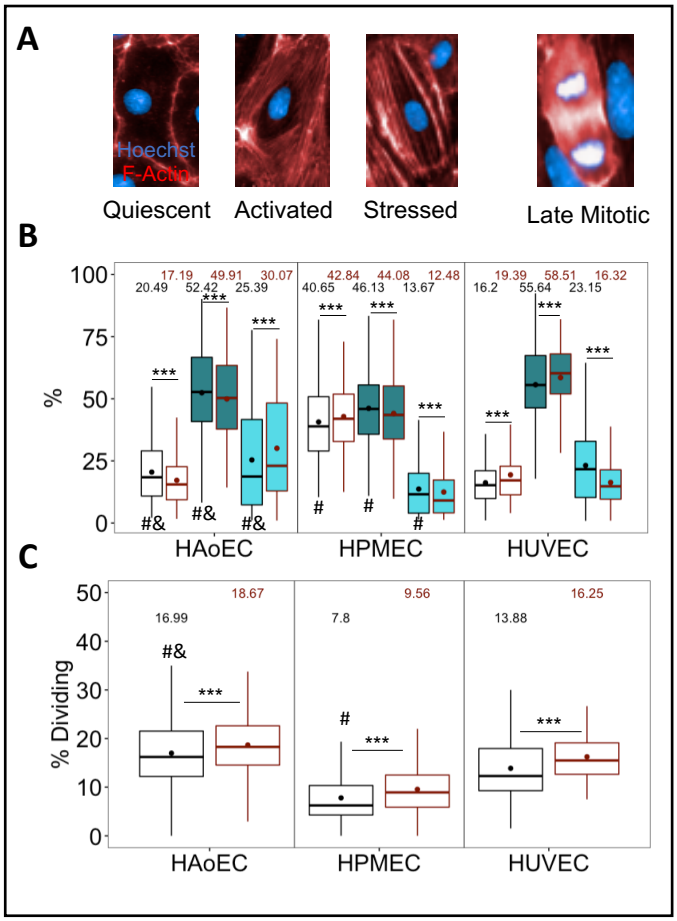

Figure 3: Cell morphology, cell cycle and cell activation status Analysis **A**) Examples of quiescent, activated, stressed and late mitotic EC counted by AI aided cell classification (CPA, Fast gentle boosting). **B**) Percentage of HAoEC, HPMEC and HUVEC in quiescent, activated or stressed categories under basal conditions (black) or upon treatment with 50 ng/ml VEGF (red). **C**) Percentage of non-proliferating cells (G0/G1) or dividing cells (S, G2/M, LM) in the populations of HAoEC, HPMEC and HUVEC under basal conditions (black) or upon treatment with 50 ng/ml VEGF (red).

*** = p<0.001, & = p<0.001 against HPMEC, # = p<0.001 against HUVEC

Image-based junction analysis to highlight EC junction heterogeneity.

The description of EC junction dynamics and morphology has been of foremost interest due to the link with vascular permeability and angiogenesis. IEJ (Inter-Endothelial Junction) morphology is an immediate index of EC activation in contrast to quiescence. In general, quiescent EC (i.e., non-proliferating, non-migrating) are also those with stabilised IEJ while migrating or proliferating EC demonstrate jagged or discontinuous junctions[24,28]. Average population measurements have been used by us and others[18,29,32] to assess stability of EC monolayers which in turn correlate with functions such as trans-endothelial permeability to large proteins[33]. Assessing IEJ at the single cell level is key to highlight subtle nuances in inter-endothelial cells connectivity within the same monolayer. Nonetheless, measuring and classifying junctional signal by image analysis is technically challenging and no currently available software is able to evaluate multiple images in a standardised way[25,26].

To overcome this obstacle, we integrated within ECPT an automated way to analyse VE-cadherin pattern and junction morphology using machine learning capabilities of CPA. Previous studies on EC junction[18,25,26] did not analyse the proportion of different junction pattern, our method subdivides the junctional inter-cellular space in several discrete areas scored using an expert-trained machine learning algorithm (CPA with Fast gentle Boosting, Appendix 1). In line with the literature in the field, we classified each junction binning in three categories: jagged, linear or stabilised (Figure 4A). Stabilised junctions are visually

reminiscent of the reticular junctions previously described[24]. To facilitate data representation, we calculated a single continuous measure accounting for the proportions of different IEJ categories in each cell (junctional stability index, STB Index, Appendix 1).

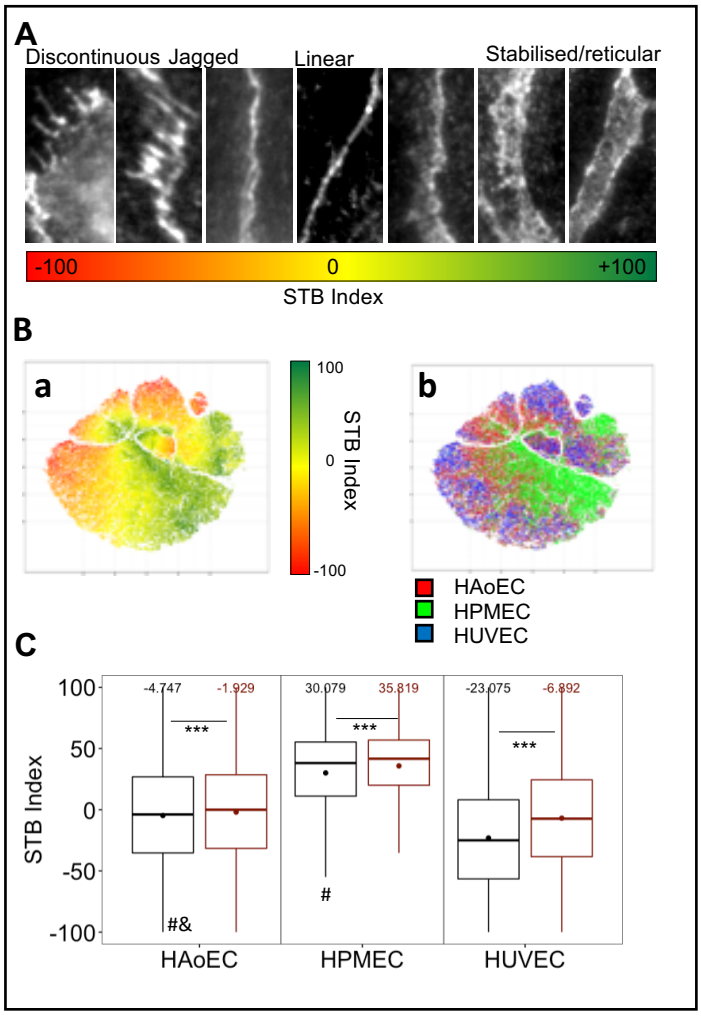

Figure 4: Image-based EC junction analysis **A**) Examples of stabilised, linear and jagged or discontinuous junctions with the scale of STB index representing junctions from really discontinuous (-100) to fully stabilised (+100). **B**) Mapping of junctional stability index (a) and cell types (b) on tSNE scatterplot. **C**) Distribution of STB Index in HAoEC, HPMEC and HUVEC either under basal conditions (black) or 50ng/ml VEGF (red).

*** = p<0.001, & = p<0.001 against HPMEC, # = p<0.001 against HUVEC

The mapping of the three EC lines STB Index (Figure 4B) shows that the different EC lines map on different areas, with a large proportion of HPMEC mapped to area with high STB Index indicating that their junctions are mostly composed of stabilised and linear junctions, in contrast to the other two lines where junction's composition was very heterogeneous. To quantify differences, we evaluated the density distribution of the STB index across the three cell types (Figure 4C). The mean STB Index was -4.7 (62.1 median IQR) for HAoEC (prevalence of linear junctions) and -23 (64.6 median IQR) for HUVEC (mixture of jagged and linear junctions). In contrast, HPMEC had prevalence of linear and stabilised junctions (30 mean) and were more homogeneous (44.2 median IQR).

The junction classifier also allowed us to highlight the response of the different lines to VEGF treatment. As for the cytoskeleton analysis, the extended exposure to VEGF had different effects on the organ-specific EC, creating a more mature junction phenotype in HUVEC and HPMEC by increasing linear and reticular junctions but inducing the opposite effect on HAoEC. Chronic exposure to supra physiologic VEGF increased STB index in all cells to different extents (+2.2, +5.6 and +16.2 in HAoEC, HPMEC and HUVEC respectively), HPMEC junctions were also more homogeneous upon VEGF treatment (36.9 median IQR) (Figure 4C).

These data demonstrate that the different EC lines are very heterogeneous in respect to IEJ, and that many cells have a composition of different junctions which might also reflect differential connectivity with different neighbours. This junctional heterogeneity that can be

picked up by quantitative observation and has been previously described can also be detected and quantified by our ECPT. It suggests that junctional stability can be regulated locally as all cells were cultured under identical experimental conditions and all individual EC within monolayers exposed to the same environment. Overall, we showed that image analysis and supervised machine learning can be used to characterize EC junctions and highlight differences at the population level. The pipeline was able to efficiently distinguish changes in junction morphology after VEGF treatment and the junctional status of single cells can be used to study intra-population heterogeneity.

Single-cell level Notch activation analysis to study heterogeneity and distinct expression levels within populations.

Notch pathway is one of the main drivers of endothelial cell heterogeneity and is linked to vascular maturation, arteriovenous specification and angiogenic sprouting[1,34,35,36]. EC junctional status depends on Notch activation via the dynamics of VE-cadherin expression, and Notch signalling is required for cell rearrangement[37]. Although Notch signalling dynamics has been studied in HUVECs and retina, it is still unclear how it affects EC heterogeneity in different organs. By analysing NICD presence in the different EC lines, we investigated the differences in Notch signalling and compared single cell expression at the subcellular level. NICD acts as a transcription factor to induce the expression of gene of interest and is therefore active in the nucleus. In line with previous results on HEY1/HEY2 expression in HUVEC[38], we hypothesised that EC within monolayer would have a homogeneous level of NOTCH signalling either low/absent in venous EC (HUVEC) and high in arterial EC as high NOTCH signalling is associated with the arterial phenotype[39]. One previous study has reported appreciable baseline NICD levels in HPMEC by western blotting[40] but not in comparison with other EC lines. Very recent single cell RNA sequencing data of human lungs has highlighted heterogeneity among arterial, venous and capillary EC and further heterogeneity within all populations and in particular microvascular EC which displayed intermediate phenotypes. Interestingly, activation of NOTCH downstream genes also seems heterogeneous across and within EC populations[41]. To distinguish “Notch-positive” cells from “Notch-negative” cells, we divided the NICD signal into nuclear and cytoplasmic signal (Figure 5A, Appendix 1). We

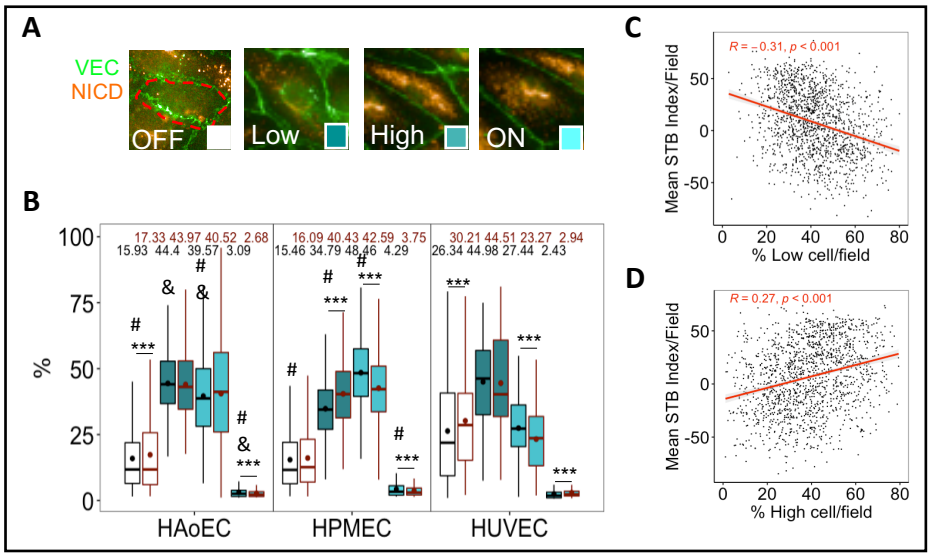

Figure 5: Notch activation status in EC populations at the single-cell level. **A**) Examples of OFF, Low, High and ON cells. **B**) Percentage of cells in the different Notch activation classes (OFF, Low, High, ON) for HAoEC, HPMEC and HUVEC either under basal conditions (black) or 50ng/ml VEGF (red). **C**) Correlation between the mean STB index per field and the percentage of LOW cells per field for the different ECs. **D**) Correlation (linear regression analysis) between the mean STB index per field and the percentage of High cells per field for the different ECs.
*** = p<0.001, & = p<0.001 against HPMEC, # = p<0.001 against HUVEC

measured the amount of NICD puncta in the cytoplasm and nuclei of single EC using ECPT. Figure 5B shows the distribution of NOTCH among four classes derived from cytoplasmic and nuclear NOTCH puncta measurements. The thresholds were selected empirically, <50 total NICD intensity reflects presence of 1-5 NICD puncta, in comparison, most highly active cells had ~50-80 NICD puncta and ~1600 total NICD intensity.

Interestingly, we found a continuous nuance of differential NICD intensities across this range (0-~1600, Supplementary figure 4). Our results indicate that irrespectively of origin, EC within monolayers are heterogeneous in NOTCH activation (Figure 5B). In line with current literature, HUVEC demonstrated higher degree of inactive cells (OFF) and lower percentage of highly active and ON cells (Figure 5B) in comparison to HAoEC and HPMEC. NOTCH activity in pulmonary microvascular EC has not been compared to other EC previously, we found that HPMEC had the highest degree of high NOTCH highly active (31.3%) and ON cells (17.7%) among the EC types examined.

To assess the functional relevance of this phenomenon we evaluated correlations between differential NOTCH activation states and the phenotypical features described in previous results. NOTCH activation has been associated with increased junctional stability in EC[37]. When we examined correlation between NOTCH activity (measured by nuclear NOTCH intensity) and junction stability at the single cell level, we find a weak evidence of positive correlation between the two phenomena (Supplementary Figure 5). Therefore, high level of NICD seems to have little effect on Junctional stability within the same (receiver) cell. We then moved our focus on cell neighbourhoods by calculating field averaged measures of Junctional stability index and the frequency of NICD classes (OFF, Low, High, or ON) and we found that the average stability index increased in fields with high frequency of High cells and decreased in fields with high frequency of Low cells (Figure 5C, D). Overall, our data indicate that sustained NOTCH signalling in one cell favours junctional stabilisation in its neighbourhood while low levels of NOTCH signalling are associated with junctional destabilisation in neighbouring cells.

As shown in Figure 5B, VEGF treatment for 48h altered the repartition of cells in the different NOTCH categories. It reduced the frequency of Low cells in HAoEC and HUVEC and was accompanied with an increase in Notch High cells in HAoEC. In HPMEC, VEGF induced an increase in Low cells and a corresponding decrease in High cells. Although treatment with chronic VEGF induced junctional stabilisation in all lines (Figure 4C), it appears to act differently on the different EC. These data demonstrate that VEGF treatment can modulate NOTCH signalling with similar effect on junctional stabilisation but to different extents and modality depending on initial conditions.

Overall, the single cell analysis of NOTCH signalling with ECPT allowed us to study intra and inter-population heterogeneity, classify different EC populations and highlight subtle changes in EC monolayers. The versatility of this tool allows the analysis of whole population phenotype and the correlation of different phenomenon such as junction and cytoskeleton stabilisation.

Discussion

EC exert an outstanding variety of specialised functions[3,6] some of which are common, some limited to specific organs and all largely reflected in EC phenotypical heterogeneity. EC diversity arises during development and is preserved throughout homeostasis. The activation of differential genetic programs interplays with microenvironmental factors[42,43]. Interestingly heterogeneity within the same vascular bed where microenvironmental factors are likely only undergoing minor fluctuations has been observed in vivo[16] and deemed crucial to understand variable functions within the same EC type.

These mechanisms require similar cells to preserve discrete diverse adaptable phenotypes and the time scale at which these changes (minutes)[44,45] is smaller than that of gene expression (hours). Thus, to justify rapid phenotype switches it is necessary to involve inherently dynamic processes such as junctional plasticity. Direct evaluation of dynamic changes in cell connectivity still poses considerable technical hurdles and despite great advances in live-microscopy, comprehensive cell phenotyping of single cells at population

level is still vastly relying end-point experiments. Although observation of fixed cells cannot provide information on the exact dynamics of the system, extensive profiling of single cells can inform about cell dynamic behaviours and help to develop hypotheses which can be evaluated experimentally or with computational methods[46].

To grasp a better understanding of endothelial cells' heterogeneity, connectivity and emerging dynamic behaviours[15,16] we developed ECPT and characterised three EC lines under standard conditions or supra-physiological (like those observed in cancer) levels of VEGF. In line with previous studies[27], our data demonstrate that Human Aortic, Umbilical Vein or Pulmonary EC present distinct phenotypes (Figure 2-5) which are maintained at steady state, independently from microenvironmental stimuli. HAoEC, HPMEC and HUVEC displayed different intrinsic proliferative potential and demonstrated diffuse ability to proliferate, in contrast to the idea that EC undergo terminal differentiation and lose proliferative capacity[7]. Our image-based analysis of cell cycle based on DNA intensity highlighted interesting differences between the different EC lines with HUVEC and HAoEC proliferating faster than HPMEC, and an overall small increase in the dividing cell number after VEGF treatment. These differences have not been reported, to our knowledge, and intrinsic organ-specific EC phenotypes could be further investigated with our pipeline at the single-cell level.

Analysis of EC activation demonstrated distinctive activation profiles in line with proliferation statuses in HAoEC, HPMEC and HUVEC with microvascular HPMEC being the most stable. At the same time, all cells demonstrated intra-population heterogeneity with intermixing of quiescent, activated and stressed cells within individual monolayers (Figure 3B) suggesting a high degree of cellular rearrangement as also suggested by time-lapse microscopy experiment showing that live EC in monolayers constantly rearrange their shape and connections (not shown).

Differential activation of EC leads to variance in IEJ which in turn chiefly regulate traffic of molecules and solutes in and out of capillaries and inhibit coagulation by preventing exposure of underlying sub-intimal layer in arteries and veins. Analysis of IEJ in three different EC populations highlighted a high degree of junctional heterogeneity in HAoEC and HUVEC, while HPMEC junctions where homogeneously stable. In line with data discussed above and literature, we show that HAoEC (arterial) had more linear (stabilised) junctions than HUVEC (venous) which instead tended to form highly dynamic jagged junctions. Importantly, HPMEC had stabilised junctions and HAoEC had mostly linear junctions and presented stress fibres. This is reminiscent of the unique role of the pulmonary and arterial vessels in tightly regulate solute exchange or adapt to high flow respectively and in line with recently reported single cells RNA sequencing data demonstrating that >40% of murine AoEC express mesenchymal genes[47] and therefore they are expected to display both stress fibres and reduced junction stability.

Distinctive features among different EC populations seem to be hard coded within cell gene expression program as cells from individual donors had vastly overlapping phenotypes and the features were maintained in all lines upon several passages in culture. Nonetheless, as discussed above analysis of single cell data highlighted extensive heterogeneity within the same population. If decision whether to enter cell cycle or to maintain stable junction were dependent on differential gene expression, we would expect to find a limited collection of homogeneous phenotypes whereas we find a continuous range of EC phenotypes within the same EC monolayer.

To interpret EC intra-population heterogeneity, we focussed on NOTCH signalling for its well-established role as a coordinator of the opposing functions of EC proliferation[48,49] and junctional complex stabilisation[37]. Fluctuations in NOTCH signalling have been hypothesised in EC monolayers and demonstrated in EC during sprouting angiogenesis where NOTCH acts as a bistable switch[50]. It has been demonstrated that leading tip cells induce NOTCH signalling in trailing stalk cells via Dll4-NOTCH1 leading to lateral inhibition of the tip cell phenotype[50] whereas EC within a stabilised monolayer cannot acquire a tip cell phenotype and all EC receive in principle similar NOTCH stimulation from neighbours.

By measuring NICD in individual EC, we found that different EC within their monolayer acquire different NOTCH activation states which can be modulated by chronic VEGF treatment and correlate with overall junctional stability of the monolayer

We also show for the first time that microvascular EC can possess high levels of NOTCH activity as demonstrated by our data on HPMEC, which cannot be explained by the currently established model positing high NOTCH activity as a mediator of arterial phenotype. Thus, despite sustained NOTCH signalling is a hallmark of arterial cells it cannot be accounted as the sole or main pathway involved in the establishment of the arterial phenotype warranting revision of protocols to generate stem cells derived arterial or venous EC[51].

Overall, our results regarding VEGF-mediated NOTCH modulation in different EC lines strongly support the idea that the net effects of chronic VEGF stimulation are sensibly dependent from initial conditions (i.e., the intrinsic baseline NOTCH signalling levels) and might lead to appreciably different responses in different EC. Ultimately, our data and observations suggest a scenario whereby EC in monolayers assume differential levels of NOTCH signalling which cannot settle on stable patterns.

Our ECPT analysis allows evaluating cell-intrinsic mechanisms of monolayer maintenance and plasticity, excluding variability caused by microenvironmental factors, in line with previous observations on blood vessel heterogeneity *in vivo* originating from EC rather than perivascular cells[17]. It will be important to elucidate in future studies how the crosstalk with perivascular cells and microenvironment can affect emerging endothelial behaviours.

We envisage that coupling our ECPT workflow with live imaging setups and single cells transcriptomic analysis will open the way to a much deeper understanding of emerging dynamic endothelial behaviours and thus help to develop novel more effective therapies for regenerative medicine, prevention of cardiovascular diseases and the treatment of cancer.

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