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The role of extracellular matrix composition in driving collective cell migration in MDCK-II cells

Betydelsen av extracellulär matrix sammansättning i kollektiv cellmigration i MDCK-II celler

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Abbreviations

ECM = Extracellular matrix

Coll = Collagen

FN = Fibronectin

YAP-1 = Yes-Associated protein 1

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Abstract

Introduction: The extracellular matrix's (ECM) composition and rigidity is a key regulating factor for many critical cellular functions such as proliferation, differentiation and cell migration. ECM composition varies between tissues types of which fibronectin and collagen are important components affecting cell proliferation and migration. The purpose of this study was to delineate the influence of different ECM compositions on a particular mode of cell migration in our body, collective cell migration.

Methods:Immunofluorescence microscopy was used to study proteins such as YAP-1 (proliferation marker), paxillin (focal adhesion marker) and their localisation patterns in different ECM substrates with varying stiffness. Time lapse imaging was also performed to study collective cell migration patterns in real time.

Results: An overall higher ratio of nuclear YAP-1 was observed in sparse cell regions compared to dense regions, which indicates increased cell proliferation. Increased ECM stiffness also seems to correlate with an increase in nuclear YAP-1 in sparse collagen regions and dense fibronectin regions. Furthermore, focal adhesion protein paxillin seems to accumulate to the outermost edges of the cells to generate tractional force required for cell migration in sparse regions, whereas in the dense regions paxillin seem more evenly distributed throughout the cells.

Discussion: The increase of nuclear to cytoplasmic YAP-1 ratio in both dense and sparse regions at stiffer ECM could be due to increased intracellular tension. The underlying cause for this pattern is hypothesized to depend on that more cells in a defined area lowers the tension of any individual cell. The finding of focal adhesions amassing to sparse regions outer edges could be a sign of cell migration, supported by the large cell size which indicates explorative tendencies.

Svensk populärvetenskaplig sammanfattning:

Området mellan celler, även kallad extracellulär matrix (ECM), består av komponenter som utsöndras från celler. Dess sammansättning kan variera mellan vävnader. Cellerna påverkar kompositionen av ECM och ECM påverkar i sin tur cellerna. Syftet med denna studie var att undersöka hur sammansättningen av ECM kan påverka förflyttningen av en grupp av celler.

Detta uppnåddes genom intervallfotografering av celler i olika koncentrationer av kollagen (Coll) och fibronectin (FN), som är komponenter i ECM, som försökte stänga en konstlad rispa i cellagret. Dessutom odlades celler i olika koncentrationer av FN och Coll för att sedan analysera cellbeteendet i celltäta och -glesa områden. Cellanalysen gjordes genom att studera proteinerna paxillin och YAP-1, med hjälp av självlysande antikroppar som ses i mikroskopet (immunofluorescens), som på sina egna sätt kan tänkas illustrerar det eftersökta sambandet.

Resultatet från intervallfotograferingen gav ingen värdefull information då tidsramen för inspelningen var för kort. Immunfluorescens visade på skillnader av de undersökta proteinerna mellan täta och glesa cellregioner. En skillnad mellan samma regiontyp bland koncentrationer av samma ECM-protein kunde ses vid täta FN respektive glesa Coll-regioner. Där sågs en ökning av YAP-1, vilket indikerar mer cellproliferation, vid en styvare yta (lägre ECM koncentration). Vi spekulerar att styvare ECM ökar spänningen inuti cellerna. Detta kan liknas vid att celler som ligger i en stel säng måste spänna musklerna för att inte glida av, medan cellerna i en mjuk ECM kan ligga avslappnat. Reaktionen från cellerna blir att vilja öka antalet celler som kan dela på spänningen. Vidare visade paxillin att fördelningen av integriner, cellens ankare till ECM, var lokaliserade till ytterkanterna i glesa regioner oavsett ECM-protein och koncentration medan i täta regioner var fördelningen mer jämn.

För att dra slutsatser från intervallfotograferingen hade den behövts göras under en längre tid. Vid det andra experimentet sågs YAP-1 vara proportionellt mer inuti än utanför cellkärnan vid glesare regioner. Det tror vi beror på den ökade spänningen hos den individuella cellen, vilket styrks av större cellarea och integrinernas lokalisation. Dessa celler med stor yta och mycket YAP-1 i kärnan är ofta i spetsen av en gemensam migration.

ECM har med största sannolikhet en effekt på kollektiv cell migration oavsett celldensitet. Det styrks av att YAP-1 skiljde sig mellan FN-provernas täta regioner och Coll-provernas glesa regioner. För att dra mer substantiella slutsatser behöver intervallfotograferingen göras om likväl införskaffa mer data som gör diskussion möjlig om de olika utfallen hos Coll och FN berodde på deras olika egenskaper eller om ett icke representativt cellurval studerades.

Introduction

Background:

Migration of a cell is defined by the movement and processes it undergoes in order to move, or increase, its spatial position. If multiple cells (a collective) move together with a common direction, whilst they never stop adhering to each other, it is called collective cell migration.

Cell migration plays a vital role right from embryonic development, where cells move in a coordinated manner and leads to germ layer positioning and organ morphogenesis. In adults, this coordinated movement of cells is required for tissue repair, homeostasis, immune surveillance and in the pathogenesis of cancer cell metastasis. It was previously believed that cancer cells migrate as single cells from the primary tumour to the secondary sites by a process called Epithelial to Mesenchymal transition (EMT). In recent years, it has been recognized that cancer cells can also migrate in a collective manner. Properties of the extracellular matrix's (ECM) composition such as the density, stiffness and orientation are believed to be regulating factors for collective cell migration (1). ECM is the space between cells and consists of secreted molecules such as collagen and fibronectin. Studies show that ECM has a complex interplay with cells affecting their behavior (2).

The ECM composition varies across different tissue types, and also sometimes within a single tissue, and thereby its influence on nearby cells differ throughout the body (2). Collagen is an important component of the ECM because of its ability to maintain the architecture of the tissue, due to its interactions with proteins from the cell membrane, creating strong fibers. Another important ECM protein is fibronectin that aids in cellular signaling. It assists in the binding between ECM proteins such as collagen to cell surface receptors (3).

The primary cell surface receptor family that interacts with ECM are the integrin family of receptors which consists of many subtypes (4). When the integrin receptors binds with ECM ligands (e.g. fibronectin, collagen) they undergo a rearrangement in their structure (5). This integrin and ECM interaction is the basis for focal adhesions which mediate two way communication between cells and ECM (6,7). In order to facilitate cell migration, integrins will undergo a force sensitive-cycle from activation to disassembly, thus enabling cell migration to take place (8).

After integrin activation a myriad of proteins are recruited to stabilize the focal adhesion complex which is important for cellular mechanotransduction (9). An important protein in the

cascade is the cytoskeletal adaptor protein paxillin. Paxillin recruits components to focal adhesion points which amongst many functions enhances actin's ability to bind to the cell membrane (3). Another protein of importance is the transcriptional co-regulator protein Yes associated protein (YAP-1) that is part of the Hippo signaling pathway. YAP-1 is a general transcription factor, when active it promotes cell proliferation and suppresses apoptosis. Regulation of YAP-1 activity is done through shuttling the protein out of the nucleus into the cytoplasm, rendering it inactive. Previous studies have shown a correlation between high intracellular tension as well as rigid ECM and higher concentrations of nuclear YAP-1 (10).

Purpose:

The purpose is to observe how ECM composition influences collective cell migration.

Research questions:

1. How does change in ECM composition effect coordination and velocity in collective cell migration?
2. What role does integrins have in ECM in regards to collective cell migration?

Ethical reflection

MDCK immortalized cell lines bought from American Type Culture Collection (ATCC) was used. The former cell line is derived from female Cocker Spaniel dog kidney. This bypasses ethical concerns associated with the use of animals.

There are no negative ethical considerations needed to take into consideration of the study´s potential outcome, since the aim is simply to observe and understand the correlation of different circumstances. Depending on how this research field develops, ethical consideration could be needed to be taken into account when it becomes more clinical oriented.

Materials and Methods

This was an explorative and experimental study.

Cell culture and treatment:

MDCK-II (ATCC® CRL-2936™) epithelial cell line, from the dog kidney distal tubule was used in all the experiments. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplied with penicillin and streptomycin antibiotics and G418 solution for maintaining the stable expression of inherent E-cadherin-RFP. In order to collect cells from culture flasks (T-25 or T-75) trypsin was applied for approximately 20 minutes to disseminate interconnected cells from each other and the plastic wall of the tube. Thereafter centrifugation took place at 1000rpm for 5 minutes to separate live cells for extraction. The cell count was done in Bio-Rad TC20™ Automated Cell Counter and cell density of (25, 50 or 75 thousand cells) was seeded in the 12 well plate.

Preparation of ECM protein-coats:

Fibronectin (FN) (2mg/ml) and Collagen V (2.5mg/ml) was diluted in calcium and magnesium-deficient phosphate-buffered saline (PBS) to three different concentrations: 10 µg/ml, 1 µg/ml and 0.1 µg/ml of fibronectin and 50 µg/ml, 20µg/ml and 4µg/ml for collagen. Glass coverslips were coated with different FN and collagen concentrations by completely submerging the coverslips in the diluted ECM for approximately 1h in 37°C.

Immunofluorescence:

Cells were washed with 1X PBS and fixed with Paraformaldehyde (PFA) 4% for 15 mins. After fixation cells were washed for 5 mins x 3 times in 1X PBS to remove PFA. Then cells were permeabilized with TritonX100, 0.2% for 5 mins and washed for 5 mins x 3 times in PBS to remove Triton X-100. The cells were then blocked using Bovine Serum Albumin (BSA) 3% for 1h to overnight. The cells were then incubated in their respective primary antibodies anti-rabbit, Phophi-paxillin and anti-mouse YAP-1 diluted in 1% BSA overnight at 4℃.

Then the cells were washed in 0.05% Tween 20 for 3 mins x 3 times and incubated with the secondary antibodies for 1 hr 30 mins in 1% BSA using nti-mouse, alexa Fluor Plus 488 for YAP-1 and anti-rabbit, Phospho-paxillin - Alexa Fluor Plus 647. The nucleus was stained using DAPI (4′,6-diamidino-2-phenylindole). The cells were then washed 3 mins x 3 times in

1X PBS and mounted on a glass using prolong Antifade mounting medium and allowed to dry overnight at room temperature. MDCK-II were already incorporated with E-cad RFP, which makes the cell boundaries (E-cadherin) to glow red when exposed to immunofluorescence.

Nikon A1+ (Laser Scanning Confocal) microscopy, courtesy of LBIC (Lund University Bioimaging Center) was used to acquire all the immunostained images. Pre-stained E-cadherin in a red colour made it possible to visualize the cell boundaries without local staining procedure. The different stains (E-cadherin, YAP-1, paxillin, nucleus staining dye) were imaged.

Image analysis:

Image analysis was done using Fiji. By extracting individual channels and custom merging of layers, gray scaling, inverting and adjusting different parameters related to brightness and contrast. Tools such as the ROI manager and measurement tabs helped convert the cell images into quantifiable data. For presentation purposes all the four colour channels were merged. To visualise cytoplasmic to nuclear YAP-1, a merge of YAP-1 (green) and nuclei (DAPI, blue) channels, gray scaling, inversion and modification of the contrast until clarity was reached. YAP-1 mean value quantification was done by drawing a rectangle inside the nucleus in the DAPI channel, storing the coordinates in the ROI manager, and then measuring the mean fluorescence intensity value of the marked pixels and then moving that rectangle ROI outside the nucleus to the cytoplasm and repeating the measurement. The ratio was obtained by dividing the mean fluorescence intensity value inside the nucleus with the cytoplasm. FN immunofluorescence images and data quantification was not done with background subtraction whilst the collagen was performed using background subtraction. Cell area was calculated by drawing freeform around the cell boundaries. Sometimes the cell membranes were seemingly intertwined and the edges indistinguishable, in that case either a mean estimation of multiple cells was done or the cells were discarded for data quantification.

Timelapse of scratch migration assay:

Inverted Nikon Ti 2 fluorescent microscope connected to chamber to regulate heat humidity and CO2. Microscope was set to 10x magnification and pictures were taken every 30 minutes for 15 hours. Fiji was used för data quantification. Calculating the migrated distance from the beginning of the timlapse to the end at six different locations, three on each side of the

wound, in each image. The article by Ascione F et al. (11) concludes that a timelapse is a good way of quantifying cell migration.

Statistical analysis

Comparison of data between different samples or regions within a sample was done using a non-parametric and unpaired Mann-Whitney U t-test.

Results

As could be seen from Fig.1B there is an increase of nuclear YAP-1, which as mentioned in the introduction works as a proliferation marker, for the sparse regions of fibronectin. Similar patterns are seen in Fig.2B for dense collagen. Sparse regions in both Fig.1 and 2 are seen to have a larger mean cell area than dense regions, although no difference between the concentrations in the same region type.The paxillin images show that focal adhesions are more towards the edges in sparse regions while not in dense regions. Fig.3C illustrates that collagen 50 μg/ml and 4 μg/ml migrate further than the controls while migration of cells in fibronectin wells does not differ between concentrations and control.

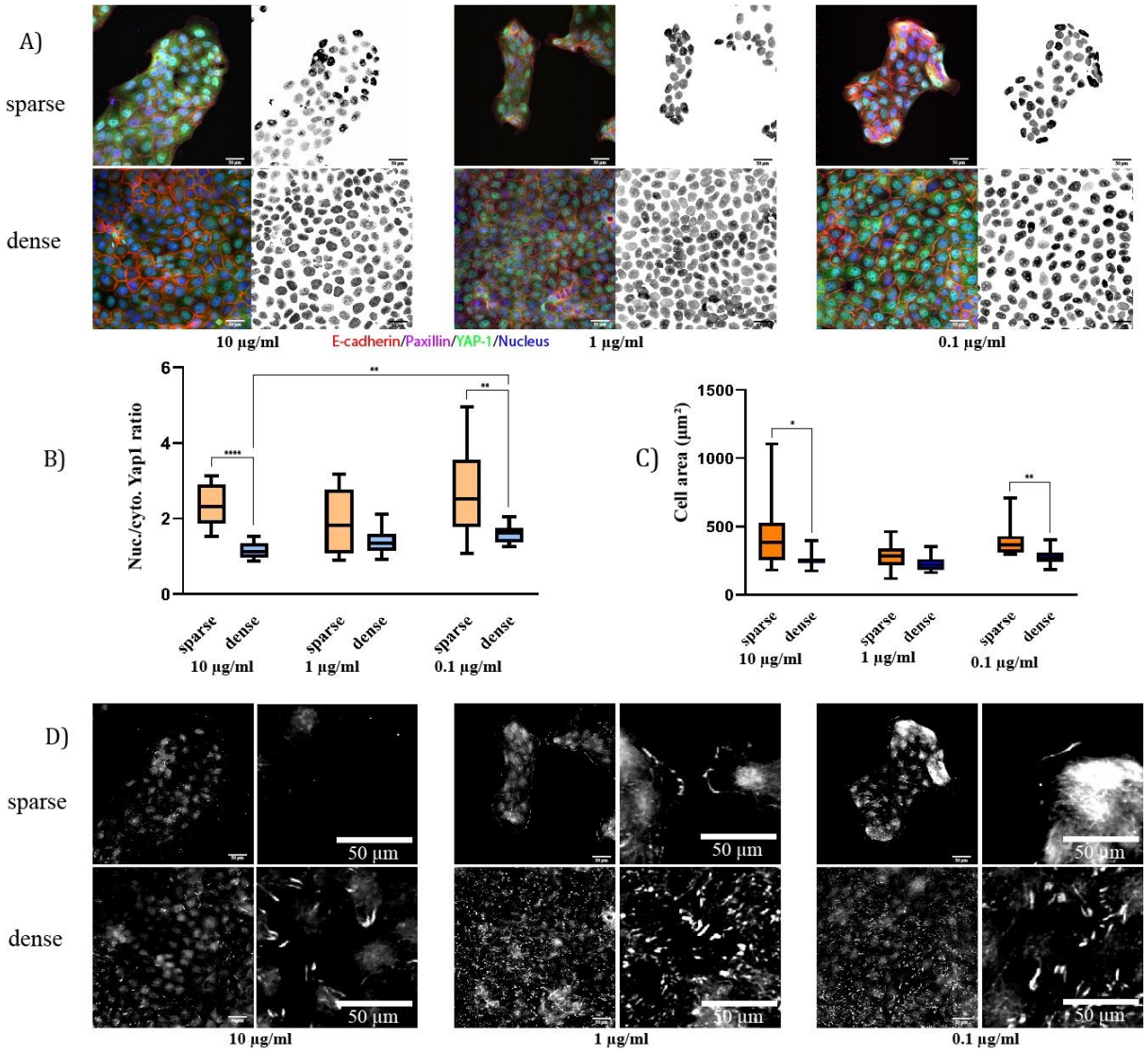

Figure 1: MDCK-E-cad-RFP cells were grown on coverslips coated with various concentrations of fibronectin such as 10, 1 and 0.1 µg/ml. The cells were stained for phopho-paxillin (magenta), YAP-1 (green), nuclei (blue) and E-cadherin (red). (A) Confocal images of cells grown in the different conditions showed in colour and also inverted gray scale images of only nuclei and YAP-1. (B) Quantitative results of the ratio between YAP-1 in nuclei and cytoplasmic. (C) Box plot histogram of the cell area. (D) Illustrates paxillin localisation, where thicker white lines depict focal adhesion. All quantitative data was based on calculations made from ten randomized cells in every sample shown above. The cells in the sparse region were randomly taken at the peripheral regions. Non-parametric and unpaired Mann-Whitney U t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

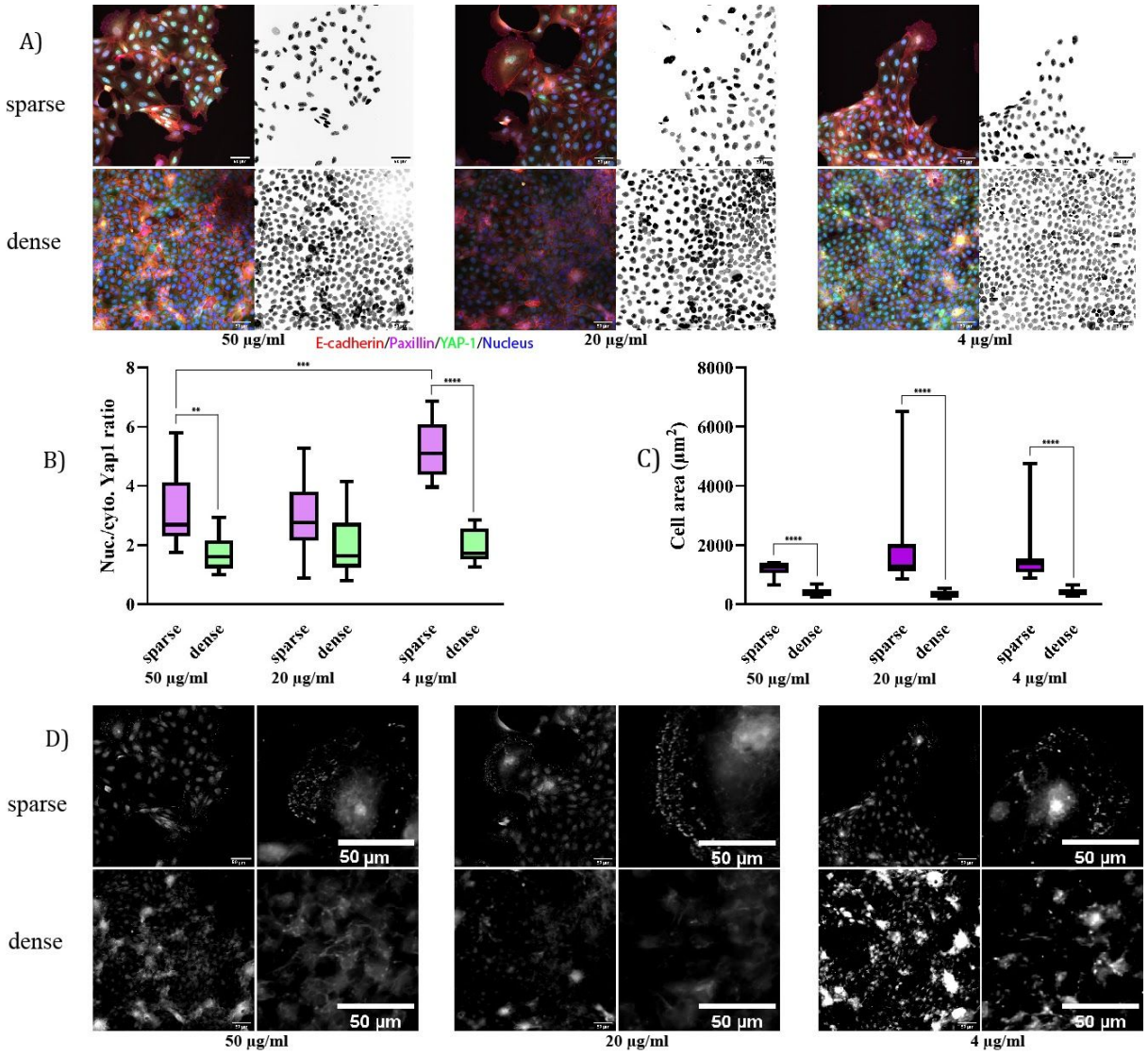

Figure 2: MDCK-E-cad-RFP cells were grown on coverslips coated with various concentrations of collagen V such as 50, 20 and 4 µg/ml. The cells were stained for phopho-paxillin (magenta), YAP-1 (green), nuclei (blue) and E-cadherin (red). (A) Confocal images of cells grown in the different conditions showed in colour and also inverted gray scale images for nuclei and YAP-1. (B) Quantitative results of the ratio between YAP-1 in nuclei and cytoplasmic. (C) Box plot histogram of the cell area. (D) Illustrates paxillin localisation, where thicker white lines depict focal adhesion. All quantitative data was based on calculations made from ten randomized cells in every sample shown above. The cells in the sparse region were randomly taken primarily at the peripheral regions. Non-parametric and unpaired Mann-Whitney U t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

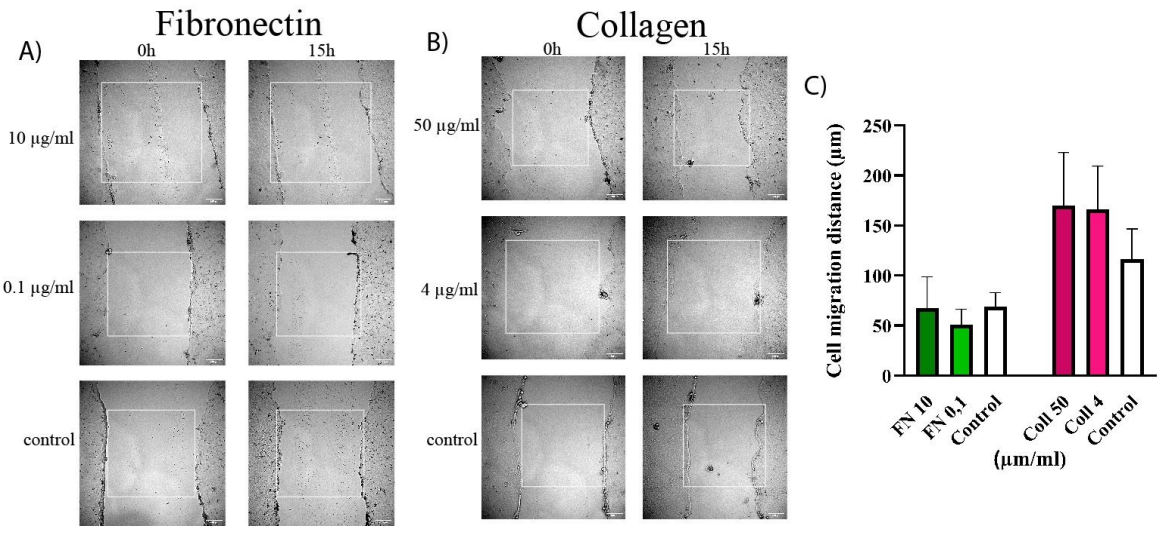

Figure 3: MDCK cells were grown on coverslips coated with two different concentrations of fibronectin and collagen as well as two non coated wells as controls. (A) Timelapse of the different fibronectin concentrations and control for about 15 hrs. (B) Timelapse of the different collagen concentrations and control for about 15 hrs. (C) Quantification of the mean distance covered by the cells in the course of 15 hours. Quantitative data was based on calculations from six random measuring points.

Discussion

The extracellular matrix in the human body consists of a multitude of proteins interconnected in different ways. This complex composition is not exactly what was replicated in the study's coats, which were based solely on fibronectin or collagen. The data presented here should therefore be audited before drawing assumptions regarding in vivo ECM-cell interactions.

ECM composition influence in collective cell migration:

By creating a timelapse imaging the scratch wounds with different concentrations of fibronectin and collagen, the cells' migration rate could be quantified and provide information about how ECM composition affects speed and coordination in collective cell migration. The article by Ascione F et al. (11) supports the fact that a timelapse could be used as a quantitative method to observe cell migration. However, as seen in A in Fig.3 none of the wounds closed at the end time point making it harder to draw significant conclusions. A longer period of timelapse recording would need to be performed, although due to a lack of resources and time the experiment could not be conducted for longer than 15 hours.

During the timelapse we noticed that in the majority of conditions the cells extended and thereafter retracted patly before the timelapse finished. This could be seen as natural cell behaviour since coordinated forward and backward motion often follow each other in a pulsating timeframe, like waves which is discussed by Burnette DT et al. (12).

However, a few hours after the timelapse finished, several dead cells were seen with the naked eye, which could indicate that apoptosis of cells might have been the reason for retraction. The reduced cell proliferation and migrations could have been affected by any number of factors, such as the heat and carbon dioxide chamber settings not being optimal and therefore amounting to cell dysregulation. A similar experiment would need to be performed where possible cautions for the collective cell retraction are studied and evaluated.

The role of integrins in ECM in regards to collective cell migration:

The results of both collagen and fibronectin show a difference in the ratio of YAP-1 inside the nuclei to the cytoplasm, as well as more defined focal adhesion points, when compared to a sparse and a dense region in the same condition. A similar pattern could be seen comparing the different concentrations of fibronectin at dense regions or collagen at sparse regions.

According to the study by Zhou DW et al. (13) a stiffer matrix results in higher intracellular tension and a softer matrix results in lower intracellular tension. Similar tendencies could be seen between the dense regions of fibronectin where there was a slight increase of YAP-1 ratio when the matrix became stiffer. This could indicate that a higher ratio of nuclear YAP-1 is due to the increased intracellular tension, since YAP-1 stimulates cellular proliferation, thus creating more cells to share the total amount of tension; decreasing the tension on individual cells. We speculate that the same mechanism occurs in the sparse regions for fibronectin but we could not see a difference in our quantitative results because the outer cells had several other events affecting intracellular tensions and thus concealing the effect of ECM stiffness.

An example of an impacting intracellular event could be how focal adhesions are distributed (D in Fig.1 and 2). In dense regions the focal adhesions seem more evenly distributed, contrary to the sparse regions where they amass to the periphery of the outermost cells. This seems to be the case for both the fibronectin and collagen coatings.

These focal adhesions are located peripherally in order to pull cells forward to cover new territory whilst the cells also want to stay connected to the collective. This could result in the

peripheral cells becoming more stretched, resulting in a bigger cell area compared with dense regions, independent of the concentration of the protein coats (C in Fig.1 and 2) - which also affects the intracellular tension. Forces specific to sparse regions but not dense could be the reason for a slight increase in YAP-1 ratio in stiffer ECM in dense but not in sparse areas. The reason why not a similar pattern is seen at the dense region at the collagen ECM could be because our sample size was too little or because collagen have other attributes compared with fibronectin. Since an increase in YAP-1 ratio is shown at collagen sparse and fibronectin dense when moving towards a stiffer matrix we suspect that it is the earlier suggestion.

A study conducted by Yeh YC et al. (14) reaches the result that ECM stiffness control **β**-1 integrin activation where a softer matrix reduced the number of integrin expressions and therefore the number of focal adhesions. Fewer focal adhesions connected to the flat substrate could mean a less flattened and stretched shape of the cell, thus decreasing intracellular tension when planted in softer ECM. The findings presented in our study neither completely dismisses nor confirms these speculations since no quantification of paxillin, and subsequent focal adhesions, were made. When visually comparing the density of focal adhesions between samples we think there is a lesser amount of visible focal adhesions in the max concentrations of fibronectin (10 µg/ml) compared to its control (0 µg/ml). The ECM becomes stiffer as the amount of ECM protein decreases, thereby pointing to a connection where softer ECM results in less focal adhesions. The same correlation is not evident in the collagen samples.

Our study could see that integrin has an important role in cell migration since the areas of focal adhesions are more intense at the leading edges of cell collectives. Speculations could be made that the intensity varies depending on the extracellular matrix's composition, although this would need to be quantified. This conclusion is supported in the article by Ilina O and Friedl P (15) which discusses how ECM regulates cell behavior by changing the integrin affinity. Integrins integrative role is an area of interest for further study to incrementally keep mapping this branch of cell behaviour.

Reflection of experimental procedures:

Three different cell seeding densities were used in conjunction with the three different coats of fibronectin or collagen. The seeding densities were thought to have an effect on cell density, e.g. the highest seed would become confluent faster compared to the lower. However we noticed that no difference in cell protein expression was seen between the different

seeding densities but rather difference was seen when comparing spare and dense regions within the same seeding densities.

Seminal studies by such as Cooke MJ et al. (16) have proven that coating the cell culture dish with ECM proteins provides a more native environment for cell growth and also proven to have influence on cell behaviour like migration and proliferation patterns**.**

Immunofluorescence images shown in the different figures throughout the paper were colour graded by multiple people at different times, and although sharing a common workflow and working in close cooperation, were without a set reference image or values. This could firstly obscure visual comparison between the different conditions within fibronectin or collagen substrates and secondly obscure visual comparison between fibronectin and collagen images. The establishment of fixed references for visual alterations of the immunofluorescence images is an area for improvement in future studies with similar methodical execution. Of note: the data is an objective mean of the light intensity within a selected area of pixels, these values are not subject to change when changing brightness and contrast of the image - these settings are simply superimposed for visual presentation.

Ten cells were chosen in each image to collect sample data. A larger sample size would have been preferable in order to increase the precision of the data about collective cell behaviour in our specific conditions. Another factor that might interfere with the validity of our data is that background subtraction was performed on the collagen samples but not on the fibronectin when calculating the ratio of YAP-1, skewing comparison between the substrates.

In dense regions cells were randomly chosen, though with probable unintentional bias. In the sparse regions peripheral cells were prioritized, most of which had larger cell areas compared to the cells in the core. No complete randomization by computer program was conducted.

Future aspects:

The purpose of this study was to explore how ECM composition influences collective cell migration. More in depth studies and analysis are needed to be performed to conclude the influence of ECM composition in collective cell migration. Better understanding of ECM-cell interconnection could become clinically impactful in treating diseases such as cardiac hypertrophy, muscular dystrophy, osteoporosis and cancer and therefore further studies are recommended to achieve better insights.

Strengths and limitations

There are both strengths and limitations of this study. A strength was that we were able to produce clear images of cells with the immunofluorescence which could have been used to produce a large amount of data, however with our time limitation we had not the time, therefore a limitation could be the sample size that we ended up analysing. Even though we only quantified a small amount of cells we managed to get significant differences. A clear limitation of the study was that the timelapse was not performed correctly to produce quantitative data, if it had been it would have helped to lay the groundwork for more conclusive findings. A drawback of our study is that the results are hard to generalize for the bigger mystery of cell-ECM interactions inside the human body, since we only used FN or Coll, which is a massive simplification of the ECM composition in vivo.

Summary:

An overall higher ratio of intranuclear YAP-1 and larger cell areas in sparse cell regions compared to dense regions could be seen which could indicate that these cells are proliferating when experiencing higher intracellular tensions. This could be related with the fact that integrins accumulate to the edges of the cells in the sparse regions, attaching to the ECM and making the cell edges protrude, indicating that integrins has an important role in driving cell migration. Tendencies of ECM increasing the YAP-1 ratio could be seen when ECM became stiffer no matter the medium. No conclusive evidence of how ECM composition influences cell migration behaviour could be made from the timelapse because the timelenght of the experiment was miscalculated. However studies such as Razinia Z et al. (17) have pointed out that cells migrate faster in stiffer ECM compared to softer**.** The immunoflourescense experiment answers questions about cell proliferation and integrin significance in different concentrations. The timelapse experiment would have given information about cell migration speed and velocity in different ECM compositions.

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Declaration of work

Both Herman and Patrik equally performed the experiments in the laboratory with the guidance of the supervisors. The written work has been divided, for instance Patrik had the main responsibility of writing the introduction while Herman wrote a larger part of the methods. However, both have gone through the entire report and have contributed equally to the finished version of the paper.