2-20-2017

Matrix stiffness and confinement influence YAP localization in clustered epithelial cells

Samila Nasrollahi
Washington University in St. Louis

Amit Pathak
Washington University in St. Louis

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Abstract

Epithelial cell clusters reside in complex extracellular matrices (ECMs) of varying mechanical properties including stiffness, topography, dimensionality, and confinement. Through mechanotransduction, cells sense and translate the mechanical cues presented by the surrounding ECM into biochemical signals, which control fundamental aspects of cell behavior including differentiation, proliferation, and motility. While it is well established that nuclear translocation of Yes-associated-protein (YAP) in single cells serves as a key sensor of ECM stiffness, it remains unknown whether grouped epithelial cells exhibit a similar nuclear YAP localization response on stiff substrates. Moreover, the regulation of YAP activity in clustered cells in confined microenvironments has remained unexplored. In this study, we cultured epithelial cell monolayers on flat polyacrylamide (PA) gels of varying stiffness and measured nuclear and cytoplasmic localization of YAP. We found that nuclear YAP localization in grouped cells increased on stiffer gels. However, this stiffness-dependent nuclear localization of YAP was not as effective in densely packed monolayers. To understand how ECM stiffness and confinement independently influence YAP activity, we fabricated a PA-microchannels platform and cultured epithelial cell clusters in channels of tunable width and stiffness. Our measurements demonstrate that the likelihood of nuclear YAP localization increases in cell clusters confined within narrower channels. In wide channels, cells neighboring the channel walls exhibit less roundedness and more nuclear YAP compared to those in the interior of the channels. Taken together, these findings reveal that the mechanosensitive nuclear localization of YAP in clustered cells depends not only on ECM stiffness, but also on the cell density and the degree of matrix confinement.

Introduction

Cells sense their microenvironment not only through biochemical signals, but also through physical and mechanical cues present in the extracellular matrix (ECM). These mechanical cues are mainly attributed to ECM stiffness, topography, dimensionality, and porosity [1, 2], all of which are known to regulate fundamental cellular functions such as proliferation, migration, and differentiation [3-8]. In the recent years, growing evidence suggests that there is a direct link between various nuclear transcription factors and mechano-regulated cell activities [9, 10]. In particular, Yes-associated-protein (YAP) is recognized as the classical sensor that transduces mechanical signals, including ECM stiffness and cell shape, into biological outcomes [11]. These mechanosensitive functions of subcellular YAP localization are processed through the Hippo signaling pathway, which acts upstream of YAP. Upon activation of the Hippo pathway, large tumor suppressor homologue (LATS) gets phosphorylated, resulting into YAP phosphorylation [12]. Subsequently, the inactivated (phosphorylated) YAP is excluded from the nucleus and aggregated within the cytoplasm [13]. However, the inactivated form of Hippo pathway leads to de-phosphorylation of YAP and re-localization to nucleus to induce gene expression [14, 15]. Therefore, the shuttling of YAP between the nucleus and cytoplasm serves as a key mechanism through which mechanical cues are transduced to gene expression [16].

Several recent studies have demonstrated a direct relationship between ECM stiffness and the nuclear YAP activation in various cells types when cultured singly on substrates of variable stiffness [17-20]. On stiff fibronectin-coated polyacrylamide (PA) gels, mammary epithelial cells (MECs) and human mesenchymal stem cells (hMSCs) showed predominantly nuclear YAP localization. However, on soft substrates, the cytoplasmic localization was the dominant phenotype [11]. The YAP activation is also associated with osteogenic differentiation of hMSCs on stiff substrates [11, 20]. In fibrosis, higher matrix stiffness activates fibroblasts to enhance the rate of matrix synthesis, where YAP is known to be the key mediator of stiffness-dependent fibroblast activation in pulmonary fibrosis [21, 22]. Moreover, the enhanced YAP localization on stiff substrates is correlated with the higher cell spreading area [11]. Strikingly, the restriction of cell spreading led to more cytoplasmic YAP localization [23]. Although a direct correlation between substrate stiffness and cell shape with YAP activation has been identified for single cells, there is not enough evidence regarding the mechano-regulated YAP localization in epithelial clusters. Furthermore, it remains unknown how the mechanosensitive YAP activation in cell clusters depends on ECM confinement. Given that epithelial cell clusters often reside in ECMs that vary in both stiffness and topography, it is crucial to understand how

mechanosensitive YAP activity varies with the degree of confinement surrounding the clustered cells.

To understand how matrix stiffness regulates YAP localization in clustered epithelial cells, we cultured MCF-10A human mammary epithelial cells on collagen-coated flat PA gels of variable stiffness. Our results demonstrate for the first time that stiffness-dependent YAP activation holds true for epithelial cell clusters, similar to the effects previously observed for single cells [11]. While cell density is known to influence YAP activation, it is not clear how cell density affects the stiffness-dependent YAP localization in epithelial clusters. Our results indicate that the density of epithelial cells minimally affects YAP localization on soft ECMs. However, on stiff ECMs, the nuclear YAP localization is expected to be higher for lower cell density. Next, to investigate the role of ECM confinement in regulating YAP activation in epithelial clusters, we need a device that permits independent control over matrix stiffness and topography. Here, we employed a microchannels-based matrix scaffold with tunable stiffness and confinement. We examined subcellular YAP localization for epithelial clusters trapped inside the channels of varying width and stiffness. We found that the nuclear YAP localization increased in narrower channels. Our results indicate that reduced cell roundness, due to increased confinement, accompanies YAP activation in narrower channels. Surprisingly, the enhanced nuclear YAP in more confined environments persisted even in soft ECMs, which were previously shown to have inhibitory effect on YAP activation. Taken together, our findings expand the established paradigm of ECM stiffness-dependent and cell-shape dependent YAP activation in the context of clustered epithelial cells.

Materials & Methods

Polyacrylamide gels – flat and microchannels

To prepare flat hydrogels, 18mm glass coverslips were plasma cleaned, treated with Bind-Silane (GE Healthcare), rinsed with ethanol, and air-dried. The PA precursor solutions were mixed by choosing monomer:crosslinker ratios based on previous stiffness characterizations of PA gels – acrylamide:bisacrylamide (A:B) percentages of 5%A:0.2%B and 15%A:1.2%B, corresponding to PA elastic moduli of 1 and 120 kPa [5]. To make flat PA gels, the precursor solution was sandwiched between a reactive coverslip and a glass slide coated with Sigmacote (Sigma-Aldrich), and let to polymerize for 30 min. To fabricate hydrogel-microchannel PA gels, a mixed solution was polymerized against the silicon wafers, made using a photolithography technique

described previously [24]. Polymerized gels and microchannels were soaked in PBS and stored at 4°C until use. Gels surfaces were functionalized with 0.5mgml^{-1} Sulfo-SANPAH (Thermo Scientific Pierce) in HEPES buffer under 365 nm ultraviolet light for 10 min, and incubated at 4°C overnight with 0.05mg/ml of rat tail collagen I (Santa Cruz Biotechnologies).

Cell culture

Human mammary gland epithelial (MCF-10A) cells were cultured, in DMEM/F12 (Invitrogen), with 5% (v/v) horse serum (Invitrogen), 20 ng/mL epidermal growth factor (EGF, Miltenyi Biotec Inc), 0.5 mg/mL hydrocortisone (Sigma-Aldrich), 100 ng/mL cholera toxin (Sigma-Aldrich), 10 ug/mL insulin (Sigma-Aldrich), and 1% (v/v) penicillin-streptomycin (Sigma-Aldrich). A colony of MCF10A cells was seeded inside the central chamber of a PA hydrogel - microchannel device, as described previously [24], and allowed to grow out of the chamber and enter into the channels. To culture cells on flat gels, media containing enough MCF-10A cells to generate low and high density of epithelial cells was added to each well and incubated for 3 days at 37 °C and 5% CO2. Samples were fixed with 4% paraformaldehyde (Santa Cruz Technologies) in PBS, followed by permeabilization of cell membrane with 0.5% Triton-X 100 (Sigma-Aldrich) and blocking with 1% bovine albumin serum (BSA) (EMD milipore). Samples were incubated with mouse monoclonal YAP antibody (Santa Cruz Biotechnology; diluted 1:100) overnight followed by incubation with Alexa Fluor 488-labeled goat anti-mouse antibody (Invitrogen; diluted 1:500) for 1 hour. After thoroughly rinsing the substrates with PBS, 1:250 10mg/mL DAPI (Santa Cruz) was added for 30 min at RT. Finally, samples were rinsed again with PBS and stored at 4ºC before imaging.

Confocal microscopy and quantification of YAP localization

Images were taken using a laser-scanning confocal microscope (Zeiss LSM 730; Carl Zeiss MicroImaging, Germany) at 20X objective, and confocal stacks were obtained. Captured z-stacks were imported to ImageJ (NIH), and the stacks were projected with the maximum intensity. To quantify the subcellular YAP activity, the average fluorescence intensity was measured in the nucleus and the cytoplasm. Next, the nuclear/cytoplasmic ratio of YAP expression was plotted. As an alternative method, cells were examined for the nuclear inclusion/exclusion and the percentage of each type of YAP localization calculated by finding the number of cells representing the corresponding YAP localization category (nuclear or cytoplasmic) [25].

Statistical analysis

Images used for analysis were selected randomly from at least three different experiments for each condition. Results are reported as the mean ± standard error (SE), unless stated otherwise. To identify the significant differences between experimental conditions, we used two-way ANOVA followed by Tukey-Kramer HSD (honestly significant difference) for pairwise comparisons in MATLAB (Mathworks). Differences were considered to be significant for $P<0.05$.

Results

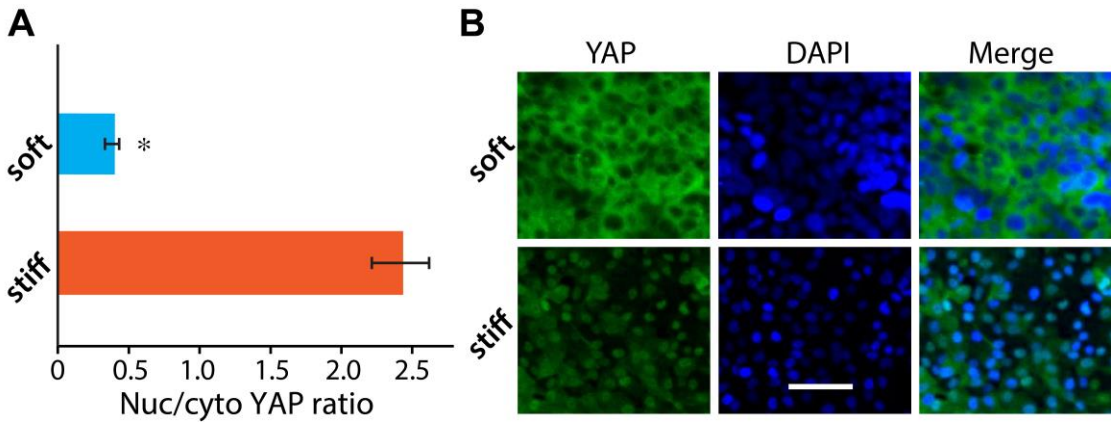

***Figure 1**: **YAP activity in clustered cells on flat gels of varying stiffness**. **(A)** Quantification of nuclear YAP localization in terms of nuclear/cytoplasmic ratio of YAP expression on soft and stiff flat PA gels. $^{*}p<0.05$ with respect to stiff ECM. N>30 cells per condition, from at least three separate experiments. **(B)** Representative immunofluorescence images of YAP (green) and DAPI (blue) in MCF-10A epithelial sheet cultured on soft (top) and stiff (bottom) ECMs. Scale bar = 50 µm.*

ECM stiffness regulates nuclear YAP localization in epithelial cell clusters

To test if YAP activity in clustered epithelial cells is regulated by ECM stiffness, we measured nuclear localization of YAP in MCF-10A cells grown on collagen-coated PA gels of either 1kPa or 120kPa elastic modulus. Following 3 days of culture in these substrates, substrates were stained for the YAP antibody. Through the analysis of immunofluorescence images, we measured

nuclear/cytoplasmic ratio of YAP expression for individual cells within epithelial cell sheets grown on soft or stiff substrates (Fig. 1B). We found that the average nuclear localization of YAP, calculated as mentioned above, in the case of soft ECM was less than $1/5^{th}$ of the value calculated for stiff ECM. These results indicate that ECM stiffness-dependent nuclear translocation of YAP that has been observed for single cells also holds true for multi-cellular epithelial sheets.

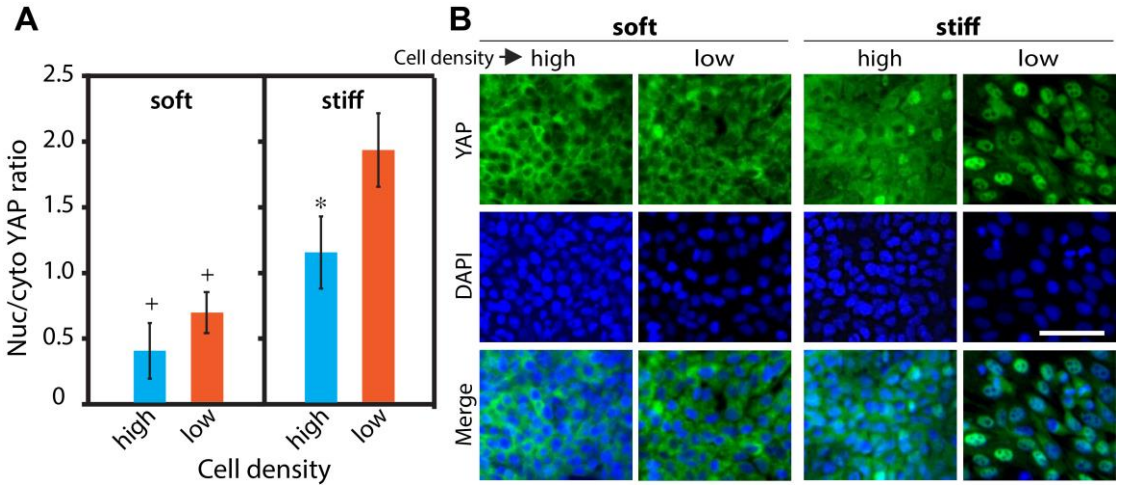

***Figure 2**: **Regulation of YAP activity by cell density. (A)** Nuclear/cytoplasmic ratio of YAP expression in cells within the low- or high-density epithelial clusters, cultured on soft and stiff ECMs. On soft ECM, the average low and high cell densities are* 2.5×10^5 *and* 8.5×10^5 *cells/cm^2, respectively. On stiff ECM, the average low and high cell densities are* 1.5×10^5 *and* 5.5×10^5 *cells/cm^2, respectively.* $^{+}p<0.05$ *with respect to stiff ECM.* $^{*}p<0.05$ *with respect to the low cell density case in either stiffness. N>30 cells per condition, from at least three separate experiments. **(B)** Confocal immunofluorescence images of YAP expression (green) and nuclei (blue) for MCF-10A cell clusters of varying cell densities, cultured on soft (left column) and stiff (right column) substrates. Scale bar = 50 µm.*

Stiffness-dependent YAP activity depends on the cell density of epithelial clusters

It is already known that cell density regulates Hippo signaling and nuclear YAP accumulation [23]. At low cell densities, weak Hippo signaling allows nuclear YAP accumulation, whereas culturing cells at high density induces strong Hippo signaling and the inhibition of nuclear YAP accumulation [23]. However, it remains unknown whether the density of cells in an epithelial

sheet could regulate YAP activation in an ECM stiffness dependent manner. To answer this question, we imaged YAP expression in epithelial clusters of different densities on both soft and stiff PA gels (Fig. 2B). Subsequently, we measured nuclear YAP localization in terms of the nuclear/cytoplasmic ratio of YAP expression, as described earlier. On soft substrates (Fig. 2A), the difference in nuclear YAP localization between low (2.5×10^5 cells/cm^2) and high (8.5×10^5 cells/cm^2) cell density conditions was small and statistically insignificant. However, on stiff ECMs (Fig. 2A), we observed an approximately 80% reduction in nuclear YAP localization in densely packed cells (5.5×10^5 cells/cm^2) compared to the cell clusters with lower density (1.5×10^5 cells/cm^2). Here, the numbers corresponding to cell densities are average densities evaluated by counting the number of cells from the DAPI images for each condition. Note that the definition of 'low' and 'high' density for soft and stiff ECMs is different because we found a sparser distribution of cells on the stiffer ECM, which could be due to increased spreading and EMT [24, 26, 27]. These results indicate that the density of cells in an epithelial cluster significantly influenced the mechanosensitive YAP localization when epithelial cells were grown on stiff substrates.

Matrix confinement regulates YAP localization through changes in cell morphology

To study the independent roles of stiffness and confinement on YAP activation in epithelial clusters, we fabricated a polyacrylamide microchannel-based matrix platform for cell culture (Fig. 3A), as we have done previously [24]. Briefly, we combined photolithography techniques and PA hydrogel synthesis to construct microchannels of varying width, embedded in PA gels of specified stiffness. In this system, an epithelial colony is seeded in the central chamber and allowed to grow beyond the central chamber to facilitate the entry of cell clusters into channels of widths ranging between 20-200 μm. Thus, our platform allows independent control over confinement and stiffness around epithelial clusters.

Cells with flat and spread morphology have been shown to exhibit nuclear YAP localization, while rounded cell morphology favors YAP cytoplasmic localization [23]. Given that cell morphology is associated with the subcellular YAP localization, we hypothesized that morphological adaptation of epithelial cells to confinement might also influence YAP distribution. First, to measure the effect of confinement on cell morphology, we quantified roundness of individual cells in epithelial clusters in channels of varying confinement and stiffness. As Fig. 3B shows, the roundness parameter is decreased with increasing confinement (decreasing channel width). We also found that the cells on soft substrate were rounder compared to the ones on stiff substrate regardless of the channel width. These results demonstrate that

confining of epithelial clusters within narrow channels reduces the roundedness of individual cell that make up the epithelial cluster (Fig. 3B).

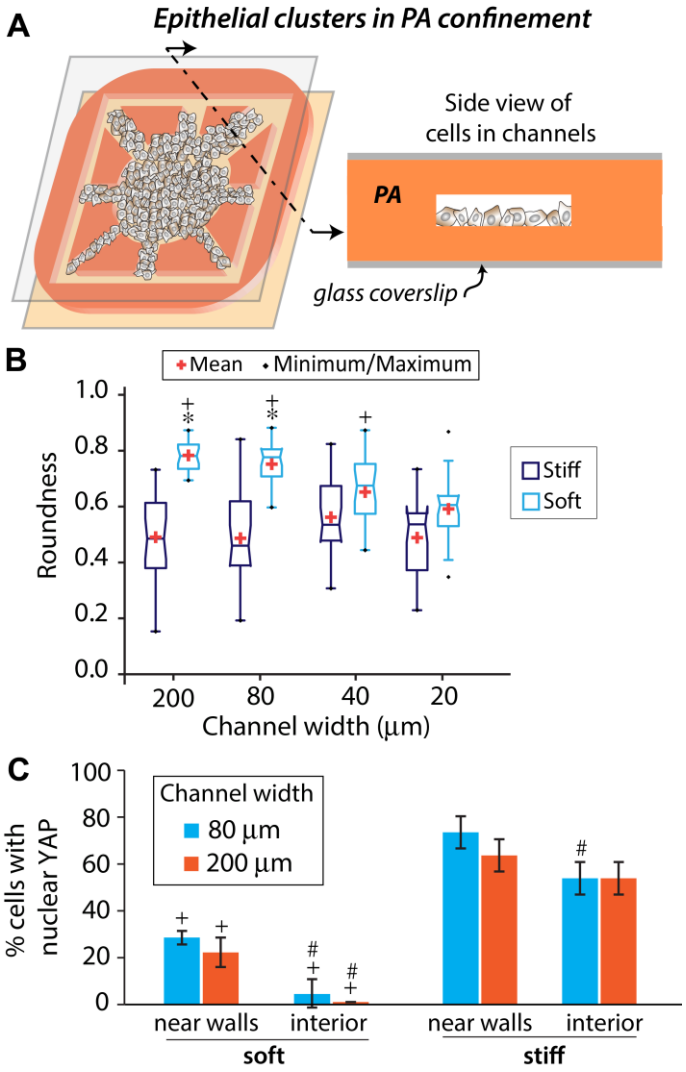

***Figure 3*: *Cell morphology and YAP activity in microchannels*. *(A)* Schematic describing epithelial cell clusters grown inside the polyacrylamide (PA)-microchannels based platform. *(B)* Cell roundness for single cells in epithelial clusters inside channels of varying widths made of soft and stiff substrates. *$p < 0.05$ with respect to stiff ECM. +$p < 0.05$ with respect to narrow (20 µm) channels. N>20 cells per condition, from at least three separate experiments. *(C)* Percentage of cells with nuclear YAP localization in wide channels (80 or 200µm channel width), in regions close or away from the channel walls. +$p < 0.05$ with respect to stiff ECM. #$p < 0.05$ with respect to 'near walls' condition. N>20 cells per condition, from at least three separate experiments.*

Next, we asked whether this change in the cell morphology accompanies a confinement-regulated YAP distribution in epithelial clusters. We examined YAP activation in epithelial clusters confined inside channels of defined properties by performing confocal microscopy and quantifying the percentage of cells with nuclear YAP localization after 5 days of cell seeding. In the widest channels (channel width of 200 μm), which are essentially the same as flat gels except with the side walls that restrict the cluster size (Fig. 3C), we compared nuclear YAP localization for clusters located in the proximity of the channel walls with those located inside the channels (Fig. 3C). We saw more nuclear YAP localization close to the channel walls in both soft and stiff substrates. Therefore, presence of confinement changes YAP distribution even in the wide channels compared to flat substrates. In both 80 and 200 μm soft channels, cell clusters showed ~25% increase in nuclear YAP along the channel wall compared to the clusters farther away from the walls (Fig. 3C). In the channels made of soft ECM, the difference in YAP activation between near and away from channel walls was even higher as compared to the difference measured in stiff channels.

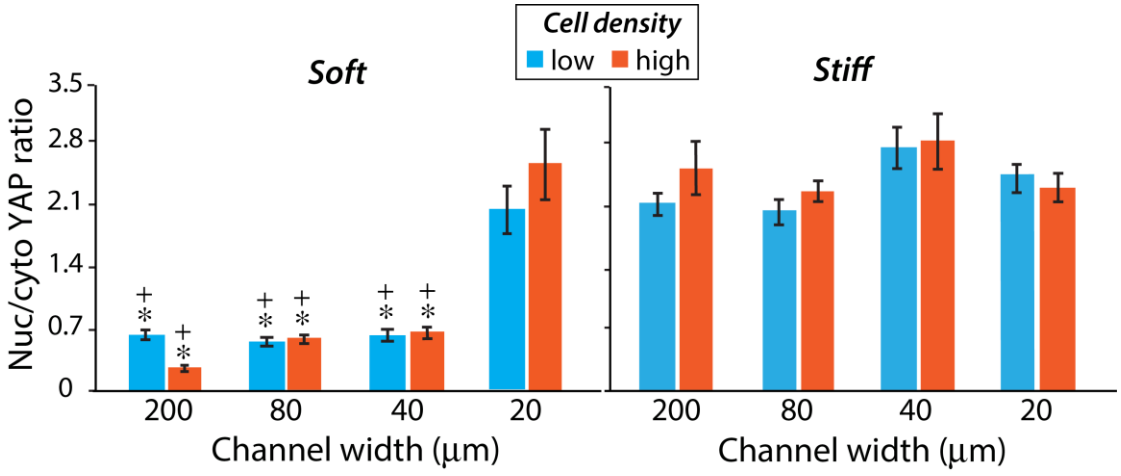

***Figure 4**: **YAP activity in cell clusters cultured in channels of varying width and stiffness**. Nuclear/cytoplasmic ratio of YAP expression in cells within the low- or high-density epithelial clusters cultured in channels of varying width made of soft (left) or stiff (right) PA gels. In soft channels, the average low and high cell densities are 5×10^5 and 15×10^5 cells/cm^2, respectively. In stiff channels, the average low and high cell densities are 2.5×10^5 and 7.5×10^5 cells/cm^2, respectively. $^+p<0.05$ with respect to stiff ECM. $^*p<0.05$ with respect to narrow (20 μm) channels. N>30 cells per condition, from at least three separate experiments.*

Greater nuclear YAP localization in stiffer and more confined ECMs

Given the previously described dependence of YAP localization on cell morphology [23] and our measurements of the influence of channel width on cell morphology (Fig. 3B), we examined the effect of confinement on YAP distribution inside the channels made of soft or stiff ECMs. Since cell density influenced mechanosensitive YAP activity on flat 2D substrates (Fig. 2), we first posited whether a similar density-dependence could occur in confined ECMs. Surprisingly, we found that nuclear YAP localization did not change significantly between low and high cell density conditions for any given channel width or ECM stiffness (Fig. 4). These results indicate that YAP activity in epithelial clusters in confinement is less sensitive to cell density as compared to the epithelial colonies on flat substrates (compare Figs. 2 and 4).

Inside soft channels, we found that nuclear YAP localization increased with decreasing channel width (for either cell density), which indicated a rise in YAP activation in more confined ECMs (Figs. 4, 5B). The epithelial clusters confined inside narrow (20 μm) channels showed an approximately three-fold increase in nuclear YAP localization compared to ones located in the wide (200 μm) channels (Fig. 4). Next, to examine the effect of ECM stiffness on YAP activation in confinement, we repeated these experiments in channels made of stiff (120 kPa) PA gels. As expected, the cell clusters inside the stiff channels showed relatively high nuclear localization regardless of the channel width (Fig. 4, 5A). Overall, in stiffer channels, we found higher nuclear YAP compared to soft substrates for any given channel width. Thus, our results demonstrate that higher matrix elasticity regulates nuclear YAP localization even for epithelial clusters trapped inside channels, which is in agreement with earlier observations on flat PA gels [11].

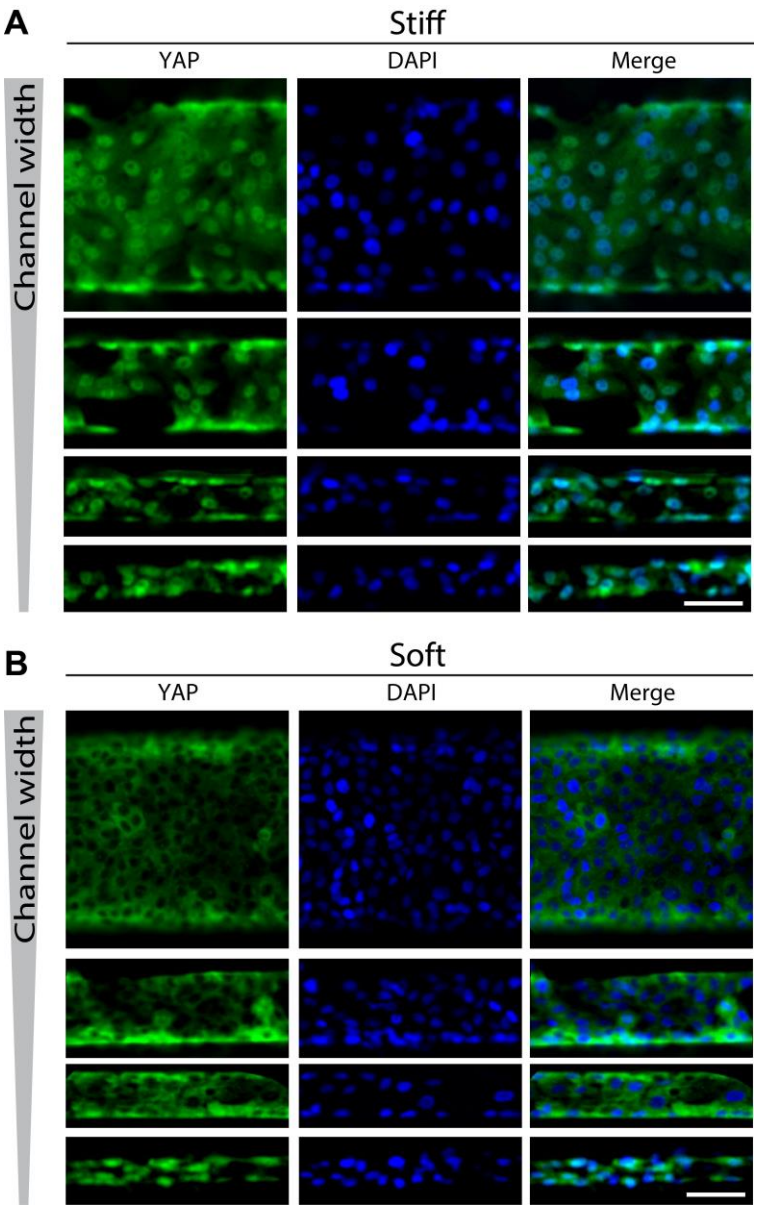

***Figure 5**: **Enhanced YAP nuclear localization in stiffer and narrower channels**. Representative immunofluorescence images of YAP (green), nuclei stained with DAPI (blue), and merged for epithelial cells embedded inside **(A)** stiff and **(B)** soft channels of varying width. Scale bar = 50 µm.*

Discussion

Mechanical signals are abundantly present in the microenvironment that surrounds the living cells. In recent years, there has been an increasing appreciation that the mechanical properties of the ECM, such as stiffness and matrix topography, impact fundamental cellular functions through cell-ECM interactions. In cancer metastasis, epithelial clusters escape from the primary tumor and pass through ECMs of varying mechanical properties before arriving at the secondary sites. It is now known that the elasticity and microstructure of the ECM surrounding the epithelial clusters can independently prepare these cells for malignant transformation [24, 28-30]. Specifically, on stiffer substrates, higher actomyosin contractility and stronger cell-ECM adhesions lead to the dissolution of cell-cell adhesions, resulting in epithelial-to-mesenchymal transition (EMT) [28]. Recent studies, including our work, have shown that MCF10A mammary epithelial cells cultured on stiff collagen-coated PA gels undergo EMT and attain invasive phenotype [24, 29]. Additionally, we have shown that ECM topography can perpetuate mesenchymal transformation independently of matrix stiffness [24], which was consistent with previous findings [30]. We also found that the elongated cell morphology in narrower channels was associated with a confinement-sensitive induction of EMT, even in softer ECMs [24].

In this study, we argue that the ECM-dependent biological response in clustered epithelial cells, e.g., stiffness- and confinement-dependent EMT [24], due to the mechanical cues presented by the ECM should be processed by signaling pathways that are transmitted through the nucleus. Since YAP is known as the classical transducer of ECM stiffness [11, 23], we shortlisted it as a potential candidate for instructing ECM-sensitive responses in epithelial clusters. While it is known that ECM stiffness and cells shape can regulate YAP distribution in single cells, it is not yet clear how these parameters might affect YAP localization in epithelial cells that typically grow in clusters with intact cell-cell junctions. Given that the disassembly of tight junctions is known to enable ZO2-mediated translocation of YAP to the nucleus, it would be important to understand the regulation of YAP activity during ECM-dependent EMT [31]. This gap in knowledge persists mainly because of an absence of matrix platforms that permit an orthogonal control over topography and stiffness of the ECM around the epithelial clusters. In this work, we addressed this challenge by utilizing a matrix platform for culturing cell clusters in PA channels of varying width and stiffness [24].

We have shown for the first time that matrix confinement alters the dependence of YAP activation on ECM stiffness. While previous studies have indicated that YAP remains inactive on soft substrates [11, 22], our results demonstrate that confining the epithelial clusters inside the narrow channels can lead to nuclear YAP localization even on soft ECMs. On stiff substrates,

most cells in the cluster showed maximal YAP activation regardless of the channel width. We attribute this result to the similar influences of ECM stiffness and confinement on cell morphology. Here, cells confined within narrow channels were forced to become less round, which was similar to the cellular elongation and simultaneous nuclear YAP localization observed on flat stiff substrates [23]. Even in wide channels, cells near the channel walls showed higher nuclear YAP localization than those in the interior of the epithelial cluster. It is possible that cells near the channel walls intimately interact with the wall surface and align themselves along the walls. This rise in spreading and elongation along the channel walls may enhance cellular mechano-activation, as we have argued in previous studies [4, 5, 24, 27], and thus lead to higher YAP activation. The current understanding of stiffness-dependent YAP activation has mainly been derived from experiments of isolated non-epithelial cells on flat surfaces. Our matrix platform of epithelial clusters in ECMs of defined stiffness and topography brings a fresh perspective to our understanding of how stiffness and topography of the ECM regulate YAP distribution in epithelial clusters. The results presented in this study, along with our previous findings [24, 27], present a novel framework for ECM stiffness- and confinement-sensitive EMT markers and YAP activity in epithelial clusters. We have also shown that nuclear YAP localization increases in sparser epithelial clusters cultured on 2D substrates, which is in agreement with another recent study [26]. However, our measurements for epithelial clusters inside channels of varying width show that the density-dependent YAP activity does not hold true in confined ECM settings. It should be noted that the presented framework of ECM- and density-dependent YAP activity in epithelial clusters has only been tested for an immortalized epithelial cell line (MCF-10A) and could potentially differ in other cell lines and primary cultures. It is likely that the influence of ECM confinement on YAP activity in clustered cells might also regulate cellular behaviors other than EMT, such as migration and differentiation, for a variety of cell types and matrices that have not been covered thus far.

Acknowledgements

This work was in part supported by grants to A.P. from the National Science Foundation (CAREER Award 1454016) and the Edward Mallinckrodt, Jr. Foundation (New Investigator Award). Lithography facilities were provided by the Institute of Materials Science & Engineering (IMSE) at Washington University in St. Louis.

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