EFFECTS OF SUBSTRATE MECHANICS AND NANOTOPOGRAPHY ON HUMAN MESENCHYMAL STEM CELLS MECHANOSOME

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Abstract

The ability of cells to perceive the mechanics and nanostructure of the extracellular matrix (ECM) relies on the activity of a number of intracellular proteins, collectively defined as mechanosome. By modifying the expression, the interaction or the localization of such proteins, the cell dynamically responds to modifications in ECM compliance and nanotopography by activating specific genetic programs. Among the intracellular proteins acting as mechanosensors, those composing the Focal Adhesions (FAs) and the effectors of Hippo pathway YAP/TAZ have been recently described as the main actors. Here, while confirming that both FAs and YAP/TAZ respond to substrate mechanics, we demonstrate that they are sensitive to dynamic modifications of substrate nanotopography by rearranging their structure or their localization. We also show that they are not sensitive to cell polarity when cell area is kept constant. In particular, the assembly of FAs, measured by image analysis through vinculin spike formation at the periphery of the cell, and YAP/TAZ nuclear shuttling to and from the cell nucleus, are proven to be sensitive to the changes in ECM nanostructure as mimicked by thermo-responsive polymers based on crosslinked poly-caprolactone (PCL). By using such a tool, we show that YAP nuclear presence - and thus its transcriptional activity - is promptly impaired by dynamic changes in substrate nanopattern. The same dynamic modifications alter FA size distribution, thus suggesting a change in their molecular composition. In doing so, we show at single cell level that the inhibition of cell spreading and of its ability to perceive ECM mechanics by either micropatterned surfaces or pharmacological inhibitors of RhoA/ROCK or Myosin IIa pathways results in the depletion of YAP/TAZ nuclear activity, the impairment of FAs formation and the regulation of genes involved in cell-matrix interaction. Altogether our results expand the current understanding of cell mechanosensing apparatus and directly connect YAP/TAZ transcriptional activity to the ability of the cell to feel the dynamic modifications of ECM composition.

Keywords: Mechanobiology, nanotopography, mechanosome, focal adhesion, Hippo pathway

1. INTRODUCTION

Cell function is controlled *in vivo* by biological and mechanical signals arising from the surrounding cells and the local extracellular matrix (ECM). These signals impact on the inner cell machinery thus regulating tissue development and organ functionality. The role of biological signals in orchestrating cell response is well described, while how physical (i.e.: mechanics and nanotopography) cues coordinate cell behavior is the subject of intense investigation. In particular, the dynamics of ECM control over cell genetic program is of great interest in order to design biocompatible structures able to control cell function.

Recently, different intracellular molecular sensors (mechanosensors) perceiving modifications in ECM mechanics and nanotopography have been described, these systems being collectively defined as mechanosome. Given the complexity of the sensing processes and the response of the cell to ECM composition, proteins laying at the interface with the ECM, or downstream effectors of the mechanosensing

apparatus can all be described as mechanosensors. Thus, the components of cell mechanosome can only be classified according to their ability to modify their structure or their function in response to mechanical stimuli arising from the ECM, while they are becoming popular as their role in determining cell function is being discovered. So far, transmembrane proteins as well as shuttling proteins and transcription factors have been shown to be responsive to modifications of cell mechanics, while a reasonable map of the interactions among the components of the mechanosensing apparatus is still lacking.

Proteins belonging to the integrin-bound focal adhesion (FA) complex like vinculin, zyxin, talin, and paxillin have been described as mechanosensors [1,2]. Their responsiveness to ECM mechanics has been highlighted. Nonetheless, these experiments unveiled that the activation of the proteins can be extremely heterogenous. For example, vinculin is known to possess a stretch-activated domain, which opens a pocket that is therefore made available to the binding with other FA components. The result of such structural transition is the maturation of FAs which are in turn able to strengthen cell-matrix interaction [3]. On the other hand, mechanical stimulation causes zyxin release from the FA site and its shuttling to the nucleus, where it exerts a number of different roles [4,5].

Among the proteins shuttling from the cytoplasm to the nucleus in response to mechanical stimulation, a special role is played by Yes-associated protein (YAP) and its paralog protein WW domain-containing transcription regulator protein 1 (WWTR1 or TAZ). These proteins are the downstream effectors of Hippo pathway, a complex intracellular axis controlling organ size and 3D organization [6]. Both proteins are known to be mostly localized in the cytoplasm following their phosphorylation on sensitive sites, when the cell is exposed to a soft environment or when the cadherin/catenin system is active. On the contrary, the proteins shuttle to the nucleus when the surface of growth is stiff or cell-cell interaction is absent [7]. YAP and TAZ act in the nucleus as transcriptional co-activators in combination with, stage- and cell-specific transcription factors. Lately our group highlighted a peculiar role for YAP but not TAZ in activating the transcription of focal adhesion genes, thus reinforcing cell-matrix interaction and the mechanical pertinence of the cell itself [8]. Other groups have independently suggested that FAs act upstream of YAP/TAZ to control their shuttling to the nucleus [7]. As such, FAs and YAP/TAZ can be considered as two flips of the mechanosensing coin acting at different levels to perceive the modifications in ECM mechanics and transduce them into a biological response. By adopting biocompatible inert materials and micropatterning technology, here we confirm that both FAs and YAP/TAZ are responsive to cell area, to substrate mechanics and nanotopography by rearranging their structure or their localization, while being insensitive to cell polarity. The assembly of FA spike at the cell periphery, and YAP/TAZ nuclear shuttling are determined by substrate mechanics. Their activation by substrate stiffness increases cell grip on the ECM and spreading. As a result, cell increases tension propagation through cell cytoskeleton. The abolishment of the tension by pharmacological inhibitors of RhoA/ROCK or Myosin IIa pathways results in the inhibition of YAP/TAZ nuclear shuttling, hinders the formation of FAs and directly change the expression of genes involved in cell-matrix interaction. Moreover, we show that a similar effect on YAP/TAZ localization and FA assembly can be obtained by mimicking cell-cell interaction at single cell level, by activating the cadherin/catenin system in adherent cells. Finally, by dynamically modifying substrate nanotopography, we demonstrate that both FAs and YAP/TAZ systems can be promptly controlled by sudden changes in ECM composition.

2. MAIN TEXT

2.1. Methods

Human mesenchymal stem cells (hMSCs) were purchased from Lonza Group Ltd. (Basel, Switzerland) and cultured according to the manufacturer's instructions. Polyacrylamide (PA) gels with stiffness values of 0.5, 10 and 40 kPa were prepared as described [9] with minor modifications. Glass round coverslips (Matsunami glass,

Japan) were amino-silanized using Aminopropyltriethoxysilane (APES), whereas squared glass slides were fluoro-silanized by the use of Fluorosilane (Trichloro (1H, 2H, 2H-perfluorooctyl)silane) (Sigma Aldrich). PA gels were prepared by the addition of the adequate proportion of 40% (w/v) acrylamide stock solution (Sigma, USA) to 2% (w/v) bis-acrylamide (N,N'-Methylenebisacrylamide) (Tokyo Chemical Industry, Japan) stock solution and PBS, towards achieving the desired stiffness (Young modulus, E), according to the reported protocol. Polymerizing catalysts were added (10% (wv) ammonium persulfate (APS) at 1% of final volume and Tetramethylethylenediamine (TEMED) at 0.1% final volume). The mixture was poured between the fluorosilanized glass slide and aminosilanized coverslip for 30 min, after which the PA gel was attached to the aminosilanized coverslip. Finally, PA gels were functionalized with collagen (100 µg/mL) by the attachment to 0.2 mg/mL sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)-hexanoate (sulfo-SANPAH; Pierce Biotechnology) covalently bound to the gel surface by photo-activation. Thermo-responsive polymers having shape-memory properties were prepared by cross-linking tetra-branched Poly-ε-caprolactone (PCL) with acrylate end-groups in the presence of linear PCL telechelic diacrylates, according to a previously reported protocol [10]. To prepare shape-memory PCL substrates with permanent surface nanopattern, the PCL macromonomer solution was cured between a nanopatterned glass mold and a flat glass slide with a 0.2 mm thick Teflon spacer for 180 min at 80 °C. Nanopatterned molding of glass substrates was performed by using an electron beam (EB) lithography system (ELS-7500EX, Elionix, Hachioji, Japan). To program temporary surface patterns, the films were compressed in a thermo chamber. A compressive stress of 0.1 MPa was applied to the samples at 37 °C and maintained for 5 min. The embossing stress was then released at 4 °C after 10 min of cooling. Samples had a temporary surface pattern (nanopattern) that could be triggered to transition to the permanent surface pattern (flat) by heating. Micropatterned glasses coated with either fibronectin or N-cadherin were purchased from CYTOO SA (CYTOO, Grenoble, France). YAP/TAZ expression and subcellular localization, vinculin expression were followed by immunofluorescence. Nuclei were counterstained with DAPI. Cell area was measured by manually highlitghing the surface of cells stained with TRITC-Phalloidin in ImageJ.

3. RESULTS AND DISCUSSION

3.1. Substrate stiffness controls focal adhesion assembly, YAP subcellular localization and modifies the expression of genes involved in cell-matrix interaction

Among the most studied components of cell mechanosome, FAs ad YAP/TAZ have been described as the most important in determining the interplay between the cell and the milieu [6]. To assess their behavior in response to substrate mechanics, human mesenchymal stem cells (hMSCs) were cultured on surfaces with increasing stiffness ranging from physiological to supra-physiological values (Young Modulus: 0.5; 10; 40; 900; 20000 kPa) and stained for vinculin and YAP/TAZ. Cells growing on very soft surfaces (0.5 kPa) fail to spread and to acquire a defined shape. On stiffer substrates (≥10kPa) hMSCs spread to acquire their characteristic shape and reach a surface area ~ 6000 μm^2. Their size is not influenced by substrate stiffness above 10 kPa (**Figure 1b**). The quantification of YAP/TAZ positive nuclei show that, while on soft surfaces the proteins are mostly localized in the cytoplasm of the round cells, on stiffer matrices the cells are predominantly stained by the antibody in the nucleus, thus testifying the shuttling of the proteins to the nucleus. As previously described, YAP/TAZ are active as transcriptional co-activators in cell nucleus. The same cells having YAP in the nucleus display the formation of vinculin-rich spikes, which confirm the assembly of focal adhesions at the periphery of the cell. As a control, round cells on 0.5 kPa surface show no spikes. Interestingly, cells cultured on substrates having different stiffness display a differential regulation of genes involved in hMSC maintenance, differentiation (d), proliferation and adhesion (e).

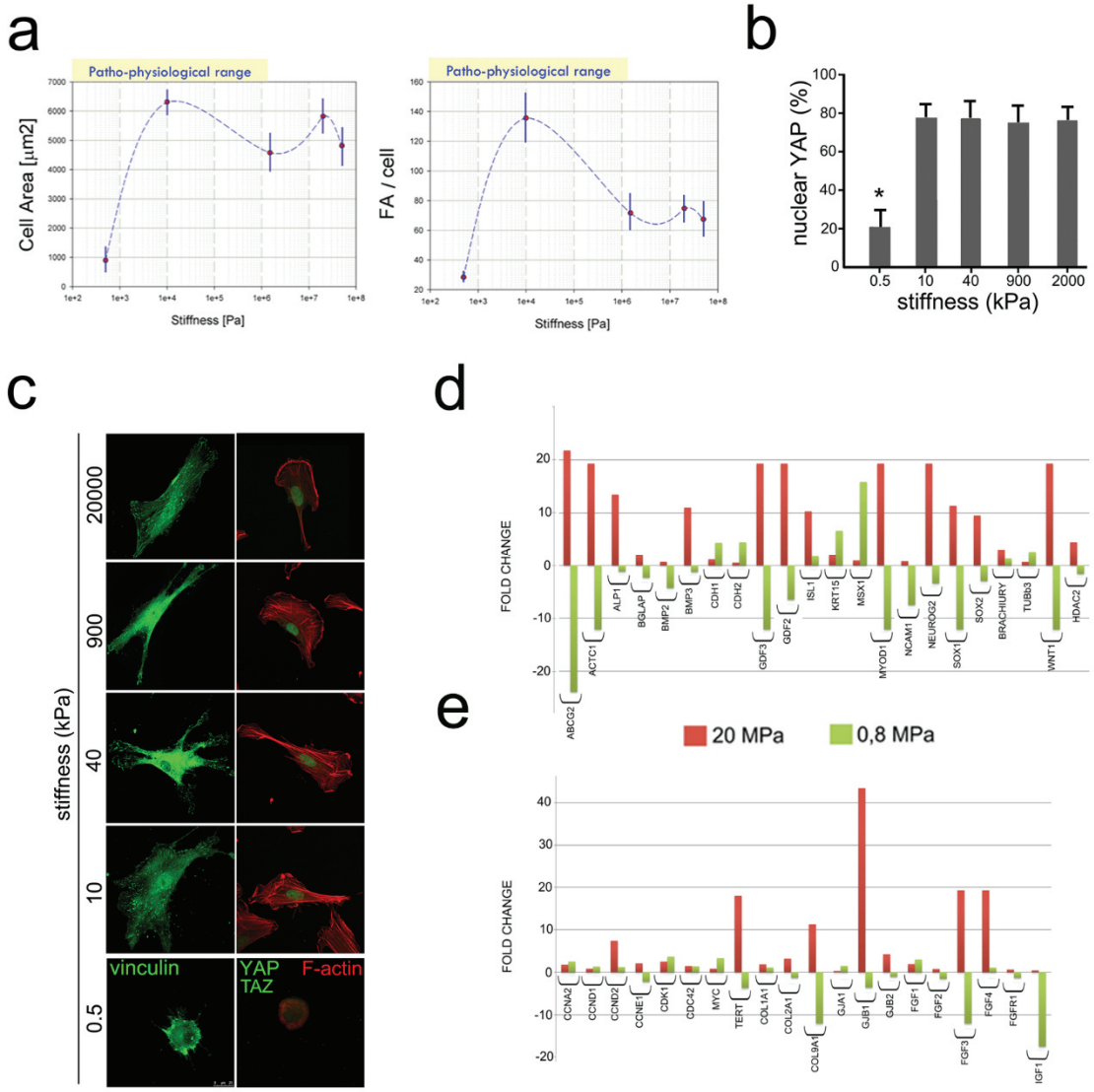

Figure 1 Substrate stiffness controls the assembly of Focal Adhesions and the sub-cellular localization of YAP transcriptional co-activator. Substrate stiffness in a pathophysiological range (0.5-10 kPa) affects cell spreading (cell area). Nonetheless, when grown onto substrates having increasing stiffness (ranging from 40 to 20000 kPa), no significant difference in cell area is reported (a). Cells grown onto soft materials (0.5 kPa) show exclusive cytoplasmic expression of Yes-associated protein (YAP, b) and a limited number of mature Focal Adhesions (FAs, here stained for vinculin in green, c) and. When grown onto stiffer substrates (≥ 10 kPa), hMSCs display a significant increase in FA formation and nuclear YAP expression, reaching a plateau on substrates stiffer than 10 KPa. Substrate stiffness differentially regulates genes involved in hMSC maintenance, differentiation (d), proliferation and adhesion (f).

3.2. Dynamic modifications of ECM nanotopography cause cell adhesion displacement by altering YAP nuclear activity and focal adhesion size distribution

Cell spreading over a stiff surface controls the formation of FAs and YAP nuclear localization, like demonstrated by single hMSCs grown onto micropatterned surfaces coated with fibronectin and imposing a constraint to cell area (**Figure 2a**). The number of FAs / cell and the fraction of YAP-positive nuclei, which could be not modified by cell polarity when cell area was kept constant (2000 μm^2), can be quantified by ImageJ software (www.imagej.net). Besides controlling YAP shuttling to the nucleus, cell area increase,

as determined by single cell spreading over micropatterned surfaces, regulates the expression of key genes involved in cell-ECM interaction (b). The treatment with inhibitors of RhoA/ROCK (cytochalasin D, CYTO D and Y27634) and Myosin IIa (blebbistatin, BLEBB) pathways, demonstrates that FA assembly, YAP nuclear localization and cell area acquisition require the transmission of tension through such pathways (**Figures 2c, d**).

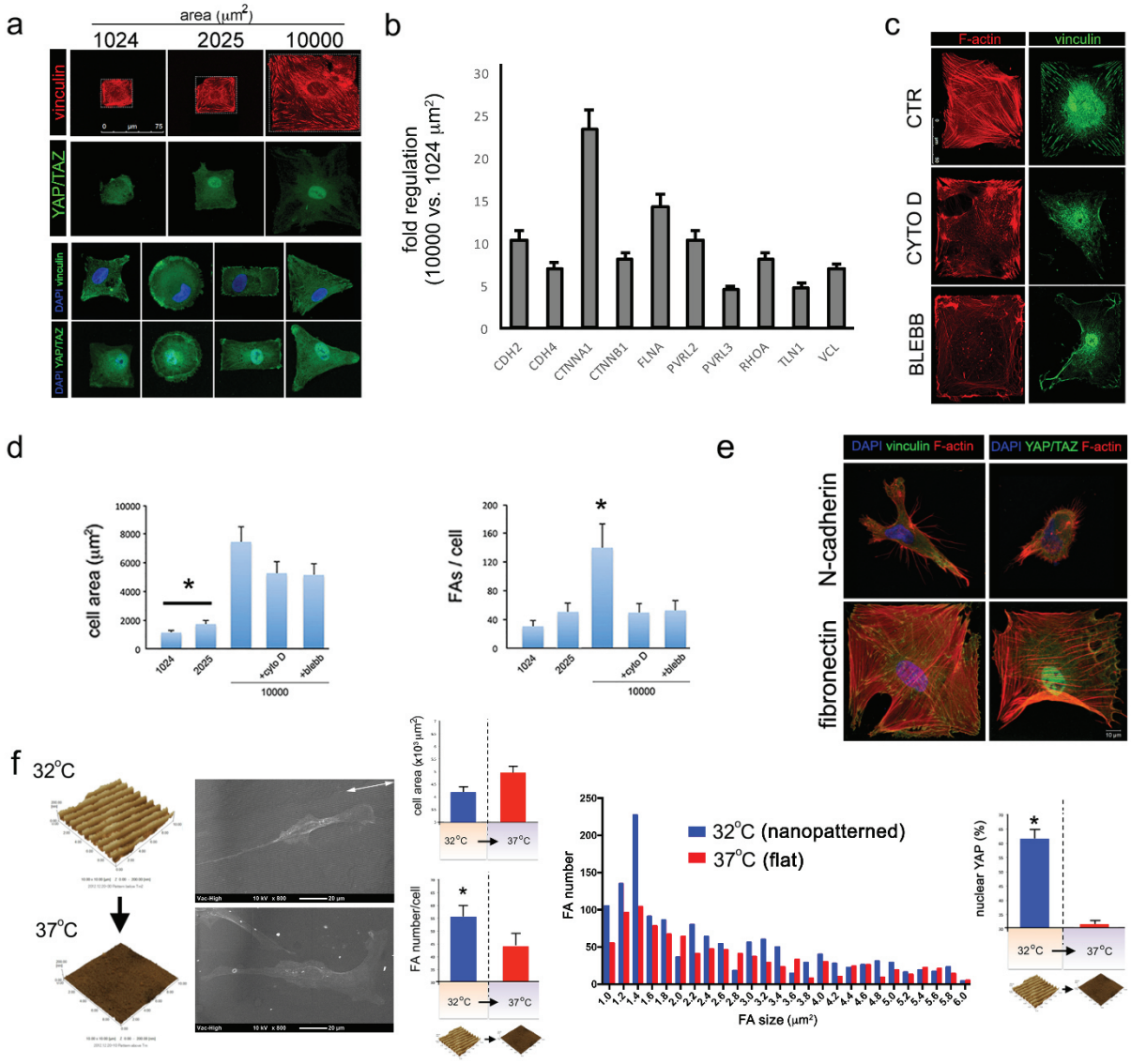

Figure 2 Mechanosome assembly and function is controlled by cell tension and ECM dynamic nanotopography remodeling. Single hMSCs grown onto fibronectin-coated micropatterns of different area display a differential regulation in FA assembly and YAP/TAZ nuclear localization. While cell area controls mechanosome function, cell polarity does not (a). Cell spreading controls the expression of genes involved in cell adhesion (b). Cell shape can be disrupted by inhibiting cytoskeleton-propagated tension through selective RhoA/ROCK and Myosin IIa pathway inhibitors (c). Tension inhibition results in the disruption of FA formation and YAP/TAZ nuclear localization (d). This phenotype can be obtained also by culturing single cells on N-cadherin, thus mimicking cell-cell interaction (e). Dynamic modifications of substrate nanotopography, as induced by thermal transition in thermoresponsive polymers, induce the cell to rearrange and alters both the number and size of FAs, while it determines the transitory shuttling of YAP/TAZ to cell cytoplasm (f).

The transmission of tension through cell cytoskeleton and cell shaping is dependent on cell-matrix interaction, since cells grown onto N-cadherin and mimicking cell-cell interaction are not able to spread and acquire a defined size. These cells show no YAP nuclear localization and no FA assembly (**Figure 2e**). Finally, the assembly of FAs and YAP nuclear shuttling are influenced by the dynamical modification of surface nanotopography: when thermo-responsive polymers are induced to a nanotopography transition, the cell is induced to rearrange its position on the surface and this process is accompanied by a change in FA number and size distribution, together with a decrease in YAP nuclear expression (**Figure 2f**).

4. CONCLUSIONS

The components of cell mechanosome - namely Focal Adhesions (FAs) and the Hippo effectors YAP/TAZ - are classified according to their ability to change their status in response to mechanical stimuli arising from the ECM. In this study, we provide evidence that dynamic modifications of substrate nanotopography directly control the size and number of focal adhesions and YAP shuttling to the nucleus. Also, we demonstrate that cell ability to spread determines the assembly of FAs and the nuclear presence of YAP/TAZ, and modifies the expression of genes involved in cell adhesion, differentiation and proliferation. Therefore, we show that given genetic programs can be promptly activated by dynamic changes in ECM structure.

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REFERENCES

[1] GULINO-DEBRAC, D. Mechanotransduction at the basis of endothelial barrier function. *Tissue Barriers,* 2013, vol. 1, no. 1, pp. e24180.

[2] KUO, J. C. Focal adhesions function as a mechanosensor. *Prog Mol Biol Transl Sci*, 2014, vol. 126, pp. 55-73.

[3] CALVO, F., EGE, N., GRANDE-GARCIA, A., HOOPER, S., JENKINS, R. P., CHAUDHRY, S. I., HARINGTON, K., WILLIAMSON, P., MOEENDABARBARY, E., CHARRAS, G., SAHAI, E. Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. Nat Cell Biol *2013,* vol. 15, pp. 637-646.

[4] CHAN, C. B., LIU, X., TANG, X., FU, H., YE, K. Akt phosphorylation of zyxin mediates its interaction with acinus-S and prevents acinus-triggered chromatin condensation. *Cell Death Differ,* 2007, vol. 14, no. 9, pp. 1688-1699.

[5] GHOSH, S., KOLLAR, B., Nahar, T., BABU, S. S., WOJTOWITCZ, A., STICHT, C., GRETZ, N., WAGNER, A., KORFF, T., HECKER, M. Loss of the Mechanotransducer Zyxin Promotes a Synthetic Phenotype of Vascular Smooth Muscle Cells. *J Am Heart Assoc* 2015, vol. 4, no. 6, pp. e001712.

[6] ZHAO, B., LI, L., LEI, Q., GUAN, K. L. The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. *Genes Dev* 2010, vol. 24, pp. 862-874.

[7] KIM, N. G., GUMBINER, B. M. Adhesion to fibronectin regulates Hippo signaling via the FAK-Src-PI3K pathway. *J Cell Biol* 2015, vol. 210, pp. 503-515.

[8] NARDONE, G., OLIVER-DE-LA-CRUZ, J., VRBSKY, J., MARTINI, C., PRIBYL, J., SKLADAL, P., PEŠL, M., CALUORI, G., PAGLIARI, S., MARTINO, F., MACECKOVA, Z., HAJDUCH, M., SANZ-GARCIA, A., PUGNO, N. M., STOKIN, G. B., FORTE, G. YAP regulates cell mechanics by controlling focal adhesion assembly. *Nat Commun* 2017, vol. 15, no. 8, pp. 15321.

[9] ENGLER, A. J., GRIFFIN, M. A., SEN, S., BöNNEMANN, C. G., SWEENEY, H. L., DISCHER, D. E. Myotubes Differentiate Optimally on Substrates with Tissue-like Stiffness: Pathological Implications for Soft or Stiff Microenvironments. *Journal Cellular Biology*, 2004, vol. 166, 877-887.

[10] MOSQUEIRA, D., PAGLIARI, S., UTO, K., EBARA, M., ROMANAZZO, S., ESCOBEDO-LUCEA, C., NAKANISHI, J., TANIGUCHI, A., FRANZESE, O., DI NARDO, P., GOUMANS, M. J., PINTO-DO-Ó, P., AOYAGI, T., FORTE, G. Hippo pathway effectors control cardiac progenitor cell fate by acting as dynamic sensors of substrate mechanics and nanostructure. *ACS Nano*, 2014, VOL. 8, 2033-2047.