Background: Endothelial cells form the inner layer of blood vessels and need to withstand considerable shear stress and mechanical forces as a result of the pulsatile blood flow. They sense and generate mechanical forces, essential for many fundamental processes such as cell adhesion, migration and proliferation. Mechanosensing is realised via signal complexes containing adhesion molecules localised at the cell-cell contacts forming the adherens junctions. Communication between the junctional proteins and the acto-myosin cytoskeleton create the chain of molecules involved in the mechanoresponse of the cells. This process not only determines cell shape, but also modulates cell-cell communication. We have found that the RAF family kinase member, RAF1 is necessary for the stabilization of VE-cadherin-based junctions between endothelial cells. The other RAF family member, BRAF has the opposite effect, since in the absence of BRAF the endothelial junctions are strengthened. Thus, both RAF1 and BRAF (through RAF1) have a role in fine tuning the amount and activity of ROKα at the junctions. The aim of the project was to study the effect of uniaxial stress on endothelial cell monolayers and **to determine how stress affects the localization of ROKα and its substrate, MLC both in the presence and in the absence of the RAF family kinases, RAF1 and BRAF, to understand the interplay between signaling and mechanics at intercellular junctions.**

Experimental protocol: The host lab has developed devices, which enable the mechanical manipulation of cell monolayers[1]. Two types of **devices have been prepared**: the first type is developed for force measurement, **the second type is suitable for high magnification microscopy to study localization changes of fluorescently labeled proteins upon stretch** (Figure 1 A and B). Two types of collagen, collagen Cellmatrix type I-A and rat tail collagen were tested as scaffolds to culture human umbilical vein endothelial cells (HUVECs). To generate suspended monolayers, cells were seeded on the temporary collagen substrate created by polymerizing a drop of collagen between the two test rods. Once cells formed a monolayer extending from one test rod to the other and covering the whole collagen substrate, as well as part of each test rod, the collagen substrate was removed by enzymatic digestion to create the suspended endothelial monolayer. During this process, however, holes were formed in the HUVEC monolayer and finally the disturbed monolayer detached from the test rods of the devices. In order to increase the attachment of the monolayer to the test rods/coverslips, they were coated with fibronectin prior to collagen preparation. This, however, still did not help to overcome the detachment of the monolayer from the test rods/coverslips after collagen digestion. This was an indication that it might be impossible to create a suspended monolayer of HUVEC cells. One reason for the unstable HUVEC monolayer might be that the cells are too dynamic and might not be able to cope with the removal of their scaffold. Therefore, we have analysed the dynamics of the endothelial cells on the collagen scaffold and we have found that indeed, endothelial cells are very dynamic, which might explain that the monolayer became unstable upon

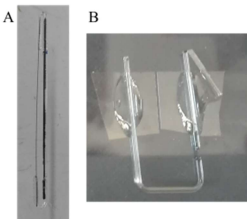

Figure 1 Stretching devices

[1] Harris AR *et al*, Generating suspended monolayers for mechanobiological studies. ***Nature Protocols***, 8(12):2516-2530. (2013)

removal of the scaffold. At this point, we have decided to keep the scaffold and stretch the monolayers in the presence of the scaffold. This is, however not possible with this type of collagen scaffold, since this gets detached immediately upon stretch and will disrupt the monolayer. Therefore, we needed a material which can be stretched without disturbing the monolayer. This material is called polydimethylsiloxane (PDMS), which can be polymerized between the two coverslips of the device (suitable for confocal microscopy, Figure 1B). I have used a modified protocol to **functionalise PDMS** to be able to **apply the scaffold of HUVECs (gelatin)** used normally for culturing these cells[2]. PDMS was functionalized with polydopamine at a concentration of 0.2 mg/ml, then 0.5 % gelatin was used to coat the functionalized PDMS surface and **finally cells were seeded on the top of the dried gelatin**. The endothelial cell shape and size was similar to the size of the cells grown on gelatin without PDMS in Petri dishes (Figure 2). This method, however, is not applicable for force measurement using the first type of device (Figure 1A), since the force of PDMS stretching is many orders of magnitude larger than the force needed to stretch the monolayer.

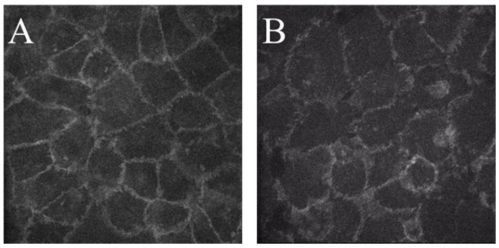

Figure 2. HUVECs on gelatin (A) and on functionalized PDMS-gelatin (B)

In order to visualize MLC and its kinase ROKα, lentiviruses were produced in HEK cells using 3rd generation lentiviral system. **These viruses were used to infect the HUVEC culture.** I have tested the expression of the **mCherry-tagged MLC**, RFP- and **GFP-tagged ROKα** in the infected HUVECs. Since the GFP-tagged ROKα virus gave a stronger expression signal, this construct was used for ROKα localization studies. MLC expression was about 80% in the HUVECs, while ROKα gave only 20% transduced cells. This might be due to the fact that ROKα has a size of 170 kD compared to the 19 kD size of MLC, thus already the transfection efficiency of HEK cells was lower with the bigger plasmid of ROKα. The combined infection with MLC and ROKα yielded less transduced cells, therefore we have decided to transduce the cells separately with the two plasmids and combine this strategy with a live cell stain, called cell mask, which labels the cell membranes (this staining was used also in the experiments shown in Figure 2).

The lentiviral transduction was combined with the silencing of the genes of BRAF and RAF1. The cells were then seeded on the device prepared with the activated PDMS-gelatin scaffold. After 72 hours of incubation, each monolayer was stretched with 30% of its size using 1%/s velocity and kept stretched for 30 minutes to monitor changes in the localization of either MLC or ROKα. After 30 minutes, the monolayers were lysed and the lysates were snap frozen in liquid nitrogen for further western blot analysis. This is important to check the silencing efficiency; furthermore the phosphorylation of MLC can also be studied from the lysates.

[2] Chuah YJ *et al,* Simple surface engineering of polydimethylsiloxane with polydopamine for stabilized mesenchymal stem cell adhesion and multipotency, **Scientific Reports**, 5:18162. (2015)

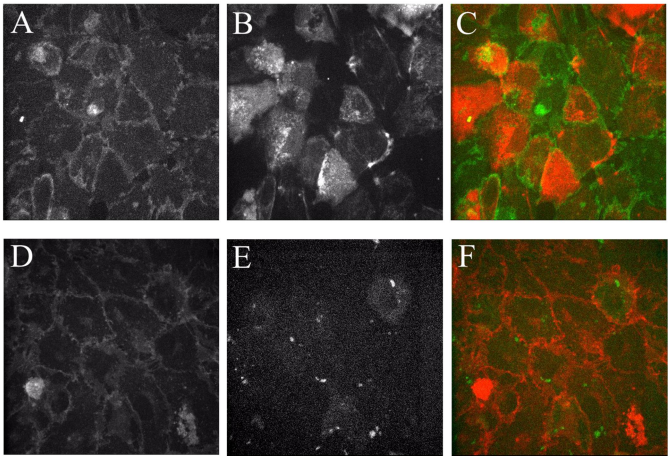

Figure 3. HUVEC monolayers expressing MLC (A-C) and ROKα (D-F). Panel A and D show the cell-cell contacts, B and E show the expression of MLC and ROKα, respectively. Panel C: green cell mask and mCherry-MLC, Panel F: GFP-ROKα and orange cell mask (detected in the far red region, red colour).

HUVEC monolayers have been monitored for 15 minutes before stretch, and followed for 30 minutes after stretch while kept stretched. From each monolayer three fields of view were monitored in the stretched region (region between the two coverslips) and three fields of view on the fixed coverslip. After stretching the HUVEC monolayer, changes in MLC localization have been observed compared to the control region. Figure 4 highlights the type of changes as an example.

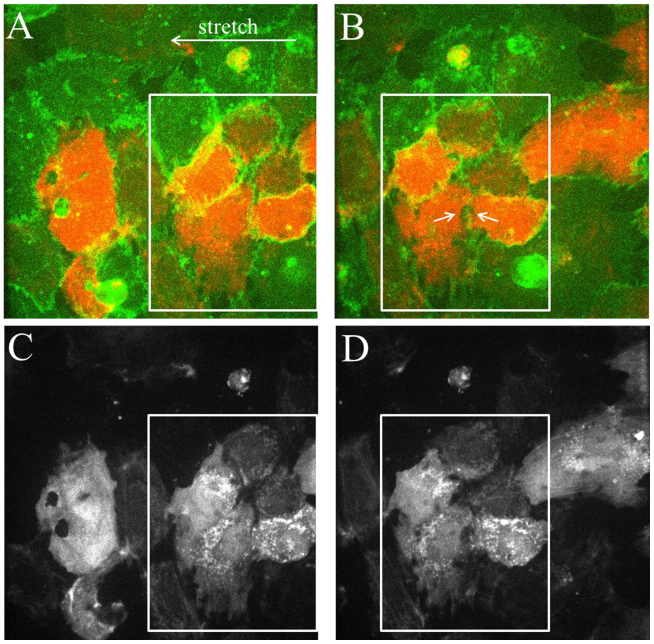

Figure 4. mCherry-MLC expressing HUVEC monolayers before (A) and after (B) stretch
Cell membranes are labelled with the cell permeable green cell mask. Panels C and D show the fine details of mCherry-MLC.

Upon stretch, the monolayer moves in the direction of the stretch and cells get also stretched (illustrated on Figure 4). **MLC is relocalized from the junctions perpendicular to the direction of the stretch** (small arrows show an example, but in all cells the same phenomenon happens to a different extent) and move away from those junctions, and in some cells a clear accumulation of MLC can be observed in the junctions parallel to the direction of the stretch. Importantly, control cells never

show this type of reorganization, MLC does not get relocalized from the junctions perpendicular to the direction of the stretch.

When the experiment was carried out with a **monolayer treated with the siRNA of BRAF, the movement of MLC happened very fast after stretch and to a lesser extent compared to the control** cells; **and MLC returned to the junctions perpendicular to the direction of the stretch after 30 minutes (at that time in the control cells the extent of MLC movement was the most remarkable).** The absence of BRAF makes the response of HUVECs faster to stretch and based on the experiments carried out so far, it seems that these "modified" cells react to lesser extent to the stretch. In order to make sure that this last statement is valid, more experiments are needed to carry out in the future.

When cells in the **HUVEC monolayer have been treated with a RAF1 siRNA**, cells failed to react to stretch as it was seen in the control cells**. No major MLC localization changes are visible upon stretch in these cells.**

In the case of the GFP-ROKα monolayers, expression of GFP-ROKα is low in most of the cells. Therefore, any changes in ROKα localization in these experiments have to be treated with caution. More experiments are needed to make an unequivocal conclusion. The present experiments are preliminary experiments showing that it is possible to follow ROKα localization during stretch in HUVEC monolayers. **Figure 5 shows an example, where an increased amount of ROKα can be found close to junctions parallel to the direction of the applied stretch (similar to MLC).** In the future experiments, however, it is advisable to increase the ROKα signal e.g. by increasing the laser power/exposure time (without damaging the live cell monolayer) and/or the detector sensitivity to get more convincing results about the changes in ROKα localization upon stretch.

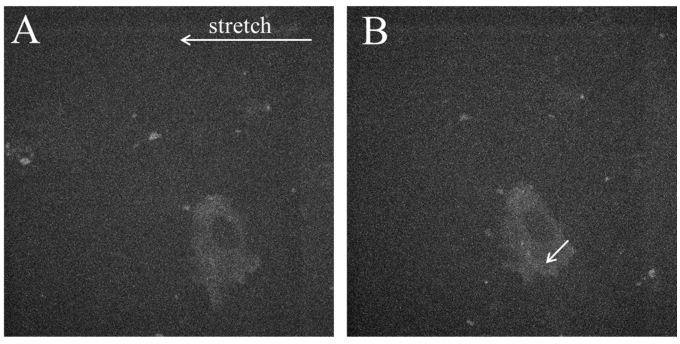

Figure 5.GFP-ROKα expressing HUVEC monolayers before (A) and after (B) stretch

Summary and outlook: HUVEC monolayers were successfully prepared on special devices appropriate for the stretching of the monolayer. In addition, a protocol was optimized for the overexpression of mCherry-MLC and GFP-ROKα in HUVEC cells. This method was used in combination with siRNA technology to study the localization changes in MLC and ROKα upon stretch. **We can conclude that upon stretch, MLC gets relocalized from the junctions perpendicular to the direction of the stretch and accumulates in the junctions parallel to the direction of the stretch.** This movement is less pronounced in BRAF knockdown cells and MLC "returns" to the junctions perpendicular to the direction of the stretch. In the absence of RAF1, movement of MLC is not visible upon stretch. All these above findings are in accordance with the fact that **in the absence of BRAF cell junctions are strengthened – react faster to changes and the junctions are re-formed fast (e.g. permeability is decreased) and the results of the experiments proved the validity of these findings also upon mechanical manipulation of the modified HUVEC monolayers. In the absence of RAF1, since the junctions are weaker, therefore it is not surprising that they are less prone to react to stretch.** In the case of ROKα-infected cells, it would be important to improve the transfection/infection efficiency of HUVECs and at the same time increase the sensitivity of the detection to be able to deduce clearly what kind of changes are happening in the absence of BRAF or RAF1. **Preliminary experiments show that similar to MLC, the amount of ROKα gets increased in the junctions perpendicular to the direction of stretch.** The NKFIH research grant made the **fruitful collaboration** with the ERC grant holder, Prof. Charras possible and opened the way for the researcher to **get insight into this new, rapidly increasing research field**, which lies between biology and physics and also **enabled the use of this powerful technique in the Department of Biophysics of Semmelweis University**. The researcher also got insight how a prestigious lab is working, helped to get **tips on how to write successful research grants** and how to **manage high-quality research** with a team of talented PhD students and postdocs.