The role of Matrix Stiffness induced hippo signaling pathway activation in Angiogenesis

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Abstract

The topic of Angiogenesis is a core player in the field of Regenerative Medicine and Biomedical Engineering. The formation of new capillary blood vessels from pre-existing vessels is a very important process during the development of newly engineered tissues. Previous studies have shown the involvement of the Hippo signaling pathway on organ size control, tissue regeneration and self-renewal. Thus giving the indication of the importance of the Hippo pathway in tissue homeostasis. Multiple model organisms have shined light on the Hippo pathway-YAP/TAZ as a potential player involved in angiogenesis. Studies have shown that the Hippo pathway-YAP/TAZ regulates endothelial cell proliferation, migration and survival. Subsequently, regulating vascular sprouting, vascular barrier formation and the process of vascular remodeling. The Hippo pathway-YAP/TAZ has also shown to be able to sense mechanical cues from the environment and transduce these cues into biochemical signals. This could suggest that manipulation of these mechanical cues could be introduced to stimulate Angiogenesis. Therefore, this reviews focus will be on the effect of mechanical cues from the microenvironment on the Hippo pathway YAP/TAZ and its effect on angiogenesis.

Keywords

Endothelial cells; extracellular matrix; matrix stiffness; durotaxis; hippo signaling pathway; angiogenesis;

Graphical abstract

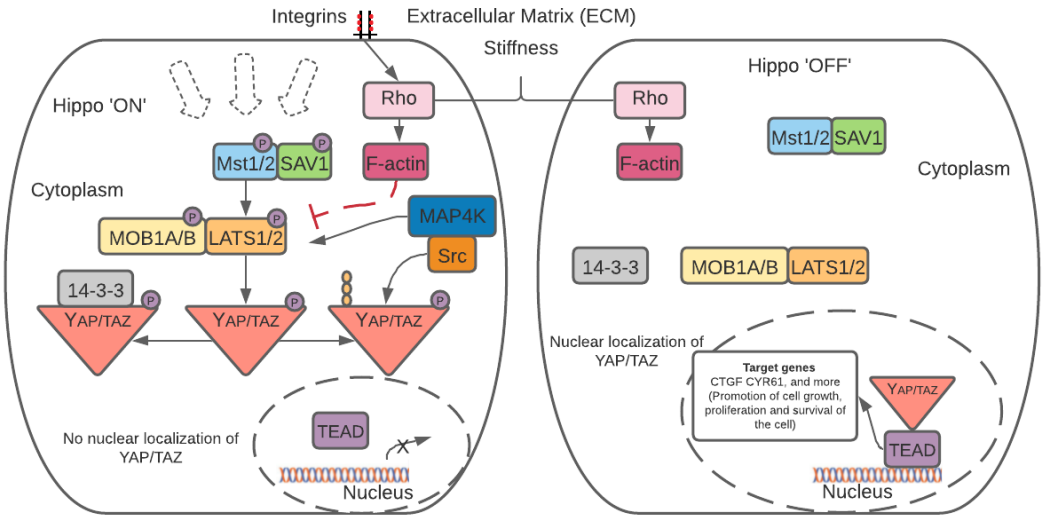

Figure 1. Overview of the Hippo signaling pathway. When Hippo is 'on', nuclear localization is inhibited and therefore TEAD does not activate target genes to promote cell growth, proliferation and survival. Normal black line shows activation whereas the red dotted line shows inhibition.

Introduction

The formation of functional blood vessels is a critical part within the field of regenerative medicine and tissue engineering. The performance of engineered tissues can be limited due to inadequate transport between them and the blood vessels. To increase the potential of success of these engineered tissues the promotion of vascular growth is a key element. Formation of blood vessels occurs through two main mechanisms: vasculogenesis and angiogenesis where in this review the focus lays on the latter mechanism.

Angiogenesis is the formation of new blood vessels from pre-existing vessels. It is regulated by a balance of both pro- and anti-angiogenic signals from the local microenvironment consisting of soluble molecules, extracellular matrices, cell-cell interactions and a diverse selection of mechanical forces (Shiu et al., 2005).

This review's focus will be on the mechanical contributions towards angiogenesis, specifically, the influence of the extracellular matrix (ECM) stiffness on the downstream pathways that affect the angiogenesis. Hereby focusing on the hippo signaling pathway, which is a pathway that has first been discovered in drosophila melanogaster and showed to influence cell size and growth. Recent discoveries have linked this pathway to the stimulation of angiogenesis, opening the possibilities for the potential use to promote vascular growth within engineered tissues (Harvey et al., 2003; Boopathy et al., 2019).

The Hippo signaling pathway is one of the central cellular signal pathways which regulates homeostasis and plays a major role in regenerative processes. Recent studies have found out that both mechanical stimuli and diffusible chemical components can regulate the Hippo pathway, this works primarily through receptors that are embedded in the plasma membrane. Its major transcriptional co-activators are YAP and TAZ, which play an important role as nuclear mechanosensors. Multiple morphologically defined structures within the plasma membrane like the cellular junction, focal adhesions, plaques, primary cilia, caveolae and clathrin-coated pits play an additional major role (Rausch et al., 2020).

An important process regarding this topic is durotaxis. Durotaxis is a form of cell migration where the guidance of cells is initiated by rigidity gradients. These rigidity gradients arise from differential structural properties of the ECM. In most cases, cells migrate up rigidity gradients (towards the direction of increased stiffness). Cells respond to mechanical input from their environment. For this they use mechanisms to both sense and translate these mechanical inputs into a cellular response. Respectively, these mechanisms are called mechanosensing and mechanotransduction, which are highly interdependent. A lot of information is still missing and there are even some cases in which some molecules can be mechanosensory as well as mechanotransductors (Espina et al., 2021).

Although a lot of information regarding the regulation of the YAP/TAZ levels and the effects of ECM stiffness is known, less is known about the mechanical regulation of the Hippo pathway signaling components. Therefore this review digs deeper into the stiffness induced activation of the downstream pathways of the Hippo signaling pathway and its effect on overall angiogenesis.
With the overall goal of combining all the known information so far, trying to find out whether manipulation of the stiffness of the ECM can positively affect the angiogenesis and therefore the possibility for a possible treatment when the stimulation of vessel formation is needed.

Major cellular components that contribute to mechanical properties of the cell

(Actin) Cytoskeleton

Dynamic network of protein fibers located in the cytoplasm of the cell; cytoskeleton. Consisting of actin filaments, intermediate filaments and microtubules. It forms the structural framework and is important in maintaining cell shape, cell shape change and the cytoplasmic organization. Endothelial cells, which are anchorage-dependent, their ability to apply cytoskeletal forces against the ECM, making use of integrin receptors, is essential for their shape stability and cell survival.

Most mammalian cells are by default spherically shaped in a suspension. Through contact with ECM components there might be rearrangements within the cells cytoskeleton to spread and form adhesion sites with the ECM. This leads to transformations of the cell shape, somewhat flattened which is common for anchorage dependent cells. Establishing and maintaining this shape requires cellular mechanical forces which are primarily generated by actin-myosin contractions. Bundles of these actin and myosin filaments form a network that span the whole cytoplasm which terminate on the basal, apical, lateral surfaces of the cell and finally on the nuclear membrane. The connections on the basal

surface consist of focal adhesions, which are needed for connection between the cell and ECM (Shiu et al., 2005).

Focal adhesions (FAs)

A combination of clustered integrins that span the whole plasma membrane and interact with specific ECM ligands on the outside and with bundles of actin connected to cytoskeletal associated proteins on the inside. This creates the ability to transmit cytoskeletal forces via integrins to the surrounding ECM. These forces act as external support to anchor the cell as well as to establish the balance of forces to maintain cell shape (Shiu et al., 2005).

An important determinant of cell shape is the resting tension of the cytoskeleton of a resting adherent cell. This affects the intracellular signal transduction as well as the gene expression. The cytoskeletal tension is not static and changes a lot over time. This due to rearrangements of cytoskeletal proteins and FAs, of on the one hand due to cellular events like cell division and migration and on the other due to external biochemical or mechanical stimuli. However, the cellular response to an external stimulus depends on the amount of initial tension, which is controlled by the interaction with the ECM and the cell's own actomyosin machinery.

This is regulated by both the Rho GTPase and its effector Rho-associated kinas (ROCK), which are important regulators of myosin activity (Shiu et al., 2005). Inactivating either Rho or ROCK diminishes the cells contractility and therefore inhibits the ability of formation of tension-dependent structures (stress fibers and FAs).
On the other hand we have intracellular contractile forces on the ECM, these are essential for the assembly of ECM fibrils and cell migration.

Mechanosensing and mechanotransduction, translating forces to cellular response

All cells in the body are subjected to mechanical forces. These forces are either self-generated or originate from the nearby environment due to physiological processes. Forces that act on a cell are also known as mechanical stress. These forces are necessary to carry out fundamental cellular events. The most prominent and known force within the cell environment is the actomyosin contractility, which is the actin-myosin complex that forms within the cytoskeleton.

Cells can sense changes in the environment due to changes of the external load on them. They respond to these changes by actively changing their internal forces to counteract the external forces. The balance between internally generated and externally applied forces determines both the cell's fate and function. These components are called respectively mechanosensing and mechanotransduction (Petridou et al., 2017). Together they receive the mechanical input and translate these stimuli into a cellular response. Even though we talk about mechanosensors and mechanotransductors as two separate elements they can still have a lot of overlaps, their interdependence is high. Some molecules can be mechanosensors as well as mechanotranductors, therefore it is challenging to determine whether a protein or structure

plays either the role of mechanosensor or mechanotransducer. Establishment of a definition to distinguish between these processes is crucial, time scales are therefore the suggested method of separating these mechanism. Next up, a deeper dive into these two elements will be given as well as the major players involved.

Mechanosensing

It is of big importance to adapt to mechanical changes within the cellular environment, migratory cells are the first to detect and respond to these cues. Distinguishing between mechanosensing and transduction can be hard, time scales are used for separation. So, mechanosensing is a relatively short term mechanism in the order of milliseconds, characterized by structural changes of specific molecules. Next up are the major components contributing to the mechanosensing of the environment.

Focal adhesions sensing aspect

Focal adhesions can act as either mechanosensors or mechanotransducers, in this part the focus will be on it mechanosensing part. Focal adhesions are the connection between cells and the ECM of their migratory substrate (Geiger et al., 2009). The overall strength of these adhesion and traction that is exerted on migratory substrates is almost all due to integrin-based signaling. Integrins are transmembrane proteins that can bind proteins from the ECM as well as the recruitment of many regulatory and adaptor proteins. The recruitment of these proteins to integrins gives the opportunity for linkage to the cytoskeleton and therefore can introduce cell shape changes and activate multiple downstream pathways (Wu et al., 2007). Being the most important force-bearing structure between the cells and the ECM, integrins play a central role in the mechanosensing of cells to mechanical changes in their environment. Other relevant FA proteins that have been shown to be recruited due to force dependent signals are vinculin and talin adaptors (Golji et al., 2011; Grashoff et al., 2010). These are molecular bridges that can transmit the actin-based force to the integrin in the adhesion sites and the other way around. Interestingly, when migrating in stiffer substrates the FAs become stretched which exposes the vinculin and talin binding domains. This promotes the cytoskeletal dynamics through downstream signaling (Haining et al., 2016). For instance, due to stretching, talin exhibits binding sites which leads to potential binding of integrins, vinculin and actin resulting in reinforcement of FA formation and dynamics (del Rio et al., 2009).

Actomyosin network sensing aspect

Due to cues in the mechanical environment the actomyosin cytoskeleton changes/adapts (Mitrossilis et al., 2010; Plotnikov et al., 2012; Raab et al., 2012). Research has shown that the ordering of the actin cytoskeleton depends on substrate rigidity (Gupta et al., 2015). Due to substrates of different stiffnesses the actin cytoskeleton can change from fluid-like to solid-like properties. It has also been suggested that, apart from actin fibers, also myosin-II isoforms cooperate to work as mechanosensors. It has been observed that stiffness can induce myosin-II accumulation (Raab et al., 2012). Giving the suggestion that multiple factors are involved when modeling the mechanosensing side through the actomyosin network.

Plasma membrane (PM) sensing aspect

The PM performs a double role, on the one hand it has to prevent rupture of the PM while on the other hand it has to maintain homeostasis (Gauthier et al., 2012; Morris et al., 2001; Sinha et al., 2011). Therefore, it has to accommodate fluctuations in tension due to changes of the environment. Most notably, physical changes of the PM are the inducers of conformational changes of multiple key signaling membrane components (Le Roux et al., 2019). These are for example, lipid arrangements (Sinha et al., 2011), ion channel openings, conformation changes on G proteins and lastly, translocation of signaling proteins. In this case, most important are the effects of cell behavior to changes in mechanically gated channels (Kefauver et al., 2020). More studies are getting focused on these channels with the suggestion being made that these channels could be involved in decision making and cell navigation in complex environments (Zhao et al., 2019).

Mechanotransduction

After sensing changes in the environment follows the translation into a biochemical signal, mechanotransduction. This process is mediated by rapid protein modifications that in return modify the cytoskeleton and alter cell behavior which eventually can generate a transcriptional response. While modifications of proteins occurs in a matter of seconds to minutes (short-term), a transcriptional response can take up to hours (long-term).

Focal adhesion transduction aspect

While the FAs can sense mechanical cues on the one hand they can also mediate downstream signaling pathways on the other hand (Stutchbury et al., 2017). This happens when proteins are recruited into FAs complexes via modulation of signaling molecules such as kinases, phosphatases and scaffold proteins. For example, integrin engagement can activate Rac1 and Cdc42 which promote cell spreading (Price et al., 1998). Research has shown that integrin-dependent adhesion can lead to rapid activation of p-21-activate kinase (PAK), which is a downstream effector of Rac1 and Cdc42, and is implicated in cytoskeleton remodeling and cell motility (Price et al., 1998; Kreis et al., 2019). As well as the Beta1 integrins being able to induce Rac1 activation while alfa1 integrins reinforce the adhesion by RhoA-mDIA activation pathway (Berrier et al., 2002; Schiller et al., 2013). Interestingly, ROCK-dependent activation of the c-Fos/c-Jun transcription complex has been shown to underpin an increase of α6-integrins when looking at fibroblasts that were cultured on stiff substrates (Chen et al., 2016). Furthermore, it has been shown that stiff substrates promote ROCK activation in fibroblasts (Zhou et al., 2013). Lastly, cells can adapt to new substrates by altering their type of integrin that they express in their FAs leading to modulation of both traction and motility (Elosegui-Artola et al., 2014).

Many other members of the FA complexes have shown to promote downstream signaling pathways in response to mechanical stimuli. With FAK, focal adhesion kinase, being one of the most studied proteins. Downstream pathways of FAK have shown to promote cell migration. This is done through formation of a complex between FAK with Src, leading to phosphorylation of p130CAS (Zhao et al., 2011). Phosphorylated p130CAS is associated with the Cas/Crk complex, which in turn is knows to play a role in migration by mediation of Rac1 activation which happens at the leading edge of motile cells (Cheresh et

al., 1999; Cho et al., 2000). Mechanical stress has been demonstrated to directly promote downstream signaling through p130CAS activity (Sawada et al., 2006). Next to its role in migration it also shows involvement in both survival and proliferation via regulation of ERK and JNK signaling (Defilippi et al., 2006).

Cell traction

Cell traction forces are the driving forces for cell movement but also other task such as cell shape, reorganizing ECM and communication with neighboring cells. Cells can migrate by push or pull against the substratum via cell/substratum contact sites, which creates a crawling movement and leads to migration. Studies have shown that tractions are organized in a centripetal pattern, with the backward and forward tractions being located in the advancing front and trailing back of the body. It was found that inhibition of ROCK decreased the traction force and therefore the migration speed of endothelial cells. This could indicate the relevance of the Rho/ROCK pathway and cell traction to regulate angiogenesis (Ting Shiu et al., 2005).

Migration explained by the Molecular clutch hypothesis

Why do cells migrate towards substrate areas of higher stiffness? Answer can be found in the molecular clutch hypothesis (Tan et al., 2020; Espina et al., 2020). New actin monomers are incorporated into the rising actin filament, at the leading edge of the lamellipodium. Integrins will bind to the ECM upon activation and form a cluster. This integrin binding will promote signaling, leading to actin polymerization and maturing of FAs, which composed of a number of proteins, making the connection between the ECM and cytoskeleton. Force will be transmitted through the ECM and the lamellipodium becomes the new leading edge of the cell. Softer substrates will cause the lamellipodia to be more unstable, which causes integrins to not be integrated properly by the ECM. This results in less actin polymerization, rapid retrograde cytoskeletal flow and no net protrusion. Thus, traction forces will not be transmitted to the ECM and therefore the cell does not move.

Deep dive into the Hippo signaling pathway

Lately, one of the most well studied long-term modifications, driven by mechanical cues, is the hippo signaling pathway with its main family of transcriptional cofactors, Yes-associated protein (YAP). Its functioning is quite interesting as it is an negatively regulating pathway of the transcriptional co-activators, YAP and TAZ. It is known that translocation of YAP to the nucleus is controlled by mechanical cues in the environment, focusing in this case on the ECM rigidity/stiffness.

Hippo pathway, a reverse on/off switch

The hippo kinase cascade uses its nuclear effectors YAP/TAZ to regulate gene expression programs. Phosphorylation of either YAP/TAZ through Hippo signaling inactivates transcriptional coactivator by exclusion from the nuclei, as well as promotion of degradation of both YAP and TAZ.
Interestingly, when hippo signaling is low, YAP/TAZ enter the nucleus to drive gene expression. In this case LATS1/LATS2 kinases are inactive, YAP/TAZ are not phosphorylated and therefore translate to the nucleus. This gene expression leads to processes involved in cell cycle, cell proliferation and suppression

of degradation, this happens due to binding of YAP/TAZ to the TEAD transcription factor family (Camargo et al., 2007; Dong et al., 2007; Zhou et al., 2009; Zhang et al., 2010).
Hippo signaling increases → YAP/TAZ no translocation to nucleus = no drive of gene expression.

Hippo pathway and Focal adhesions

The FAs are important for the regulation of the Hippo pathway. FAs are the mechanotransducing hubs that can sense and translate mechanical cues from the extracellular milieu towards the cellular cytoskeleton. Interactions between both the Hippo pathways components YAP/TAZ and the FAs are mediated through focal adhesion kinases (FAK), these frequently constitute a feedforward loop. When an mechanical cue is sensed the following pathway occurs.
First, the FAs sense that the ECM stiffness increases, this leads to activation of SRC and inhibit LATS 1/2, overall leading to YAP activation. FAK inhibition caused YAP inactivation. FAs activation causes PAK1 activation and phosphorylates (and inhibits) NF2, which causes inactivation of LATS 1/2, overall leading to YAP activation (Espina et al., 2020).
The FAs sense a decrease in ECM stiffness and relayed via the GTPase RAP2, this binds to and leads to stimulation of MAP4Ks causing LATS 1/2 activation and therefore YAP inhibition. YAP-TEAD induces THBS1, engages FAs and activates FAK. FAs (through FAK and CDC42) decrease the LATS-mediated inhibitory Ser397 phosphorylation of YAP (Espina et al., 2020).

Hippo pathway and the cytoskeleton

YAP/TAZ activation regulated RhoGAP's can remodel the cytoskeleton, consequently, this leads to initiation and maintenance of FAs (Aragona et al., 2013). Integrins also showed to regulate YAP/TAZ through Src family kinases.
Research done by Piccolo's group have showed that adaptation of the cytoskeleton due to the cell spread cause activation and nuclear translocation of YAP/TAZ and when cells are kept together into a tight space and become round shaped, YAP/TAZ are inactivated, therefore, staying localized in the cytoplasm (Dupont et al., 2011). Their work indicates YAP/TAZ as a common mechanism for regulation of mechanical signals (Piccolo et al., 2014).

Hippo signaling YAP/TAZ major downstream pathways

The core kinases of the Hippo pathway are the MST1 and MST2 Serine/Threonine kinases and the LATS1 and LATS2 Large tumor suppressor kinases. MST kinases are known, through auto-phosphorylation, to perform auto-activation. This happens on the activation loop of the MST dimer, located on the MST1 at Thr183 and at Thr180 on the MST2 kinase (Deng et al., 2003; Praskova et al., 2004). Located on the carboxyl terminus of the MST is the coiled-coil structure called the SARAH domain. The SARAH domain mediates both the homo- and heterodimerization of MST1 and MST2. The MST1/MST2 heterodimer can form a complex with the SARAH domain which contain the protein Salvador 1 (SAV1) (Callus et al., 2006).

Furthermore, MOB1A/B kinase activators form a complex with LATS1 and LATS2 kinases. MST1 and MST2 kinases can activate LATS1 and LATS2 kinases by phosphorylation at, respectively, Thr1079 and

Thr1041 (Chan et al., 2005). MOB1A/B kinases are phosphorylated by MST1/MST2 kinases at Thr35 and Thr12, this leads to promotion of the interaction between MOB1A/B to LATS1/LATS2 (Praskova et al., 2008). Phosphorylation at MOB1A/B also promotes its affinity to LATS, which leads to auto-phosphorylation of LATS1/LATS2 at the activation loop (Ser909 at LATS1, Ser872 at LATS2) (Chan et al., 2005; Praskova et al., 2008). These phosphorylation's of both MST1/2 and auto-phosphorylation of LATS are critical for the activation of LATS1/LATS2 kinases.

The key substrates of the LATS kinases are the transcription co-activators YAP and TAZ. Phosphorylation of YAP and TAZ by LATS kinases either primes to their binding with the 14-3-3 proteins, which leads to cytoplasmic sequestration of YAP/TAZ or protein degradation mediated by ubiquitin (Zhao et al., 2007; Lei et al., 2010; Zhao et al., 2010).

Following the core process, when both LATS1 and LATS2 are inactive, YAP/TAZ are not phosphorylated and will translocate towards the nucleus. In the nucleus they bind to TEAD, transcription factor family (1-4), which mediates target gene expression. These genes are for example: connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer 61 (CYR61) and many other factors that are involved in the promotion of cell growth, proliferation and survival of the cell (Chan et al., 2008; Zhao et al., 2008, 2010).

Furthermore, studies have shown the interaction of YAP/TAZ with a variety of transcription factors, for example: SMADs (1, 2/3, 7) (respectively, Alarcon et al., 2009; Varelas et al., 2008, 2010; Ferrigno et al., 2002), RUNT related factors (Yagi et al., 1999), T-box transcription factor 5 (Murakami et al., 2005; Rosenbluh et al., 2012) and p73 (Strano et al., 2001).

Lastly, research has shown that loss of any of the core components, such as MST1/MST2, SAV1, LATS1/2, MOB1A/B, show an upregulation of YAP/TAZ → TEAD target gene transcription that lead to cell proliferation and tissue growth, therefore, determining that these core proteins are crucial for the Hippo pathway (Zhou et al., 2009; Zhang et al., 2010; Lee et al., 2010).

Increased stiffness effects

Increased ECM stiffness, caused by β1-integrins activate FAK, which in response activate tyrosine kinase SRC. Next, SRC phosphorylates and activates YAP, during this process it also phosphorylates and inhibits LATS 1/2. Which leads to activation of YAP through two main pathways;

1. FAK Inhibition which leads to YAP Nuclear localization.
2. RAP2 can respond to mechanical stimuli as an upstream regulator of the Hippo signaling pathway, following the transmission of changes in the stiffness of the ECM to the cell and overall regulation of YAP/TAZ activity.

Cells that are placed on a stiff substrate present higher degree of FA assembly and therefore experience a greater level of tension. Higher rigidity causes more recruitment of FA proteins, higher rate of assembly and therefore larger FA size (Espina et al., 2020). There are more FA proteins at the front of the cell therefore creating a asymmetry between front and rear. Overall, this lead to promotion of

protrusion extension and the front and therefore, establishment of a direction of migration towards stiffer regions on the substrate.

Research done by Rosa-Cusachs and colleagues also showed the effect of direct force application to the nucleus being sufficient to cause nuclear accumulation of YAP. This due to the force causing flattening of the nucleus, stretching nuclear pores complexes (NPC) which causes mechanical resistance to molecular transport across these NPCs. Overall leading to the nuclear import of YAP (Elosegui-Artola et al., 2017).

Decreased stiffness effects

Low ECM rigidity causes activation of Rho GTPase-activating protein (RhoGAP), ARHGAP29, MAP4Ks by RAP2. These activations lead to LATS 1/2 activation as well as inhibitory phosphorylation. Research done with varying rigidities of ECM-coated polyacrylamide showed that fibroblasts that we grown on a soft substrate exert smaller traction forces, which gives the indication that the intracellular tension is lower (Wang et al., 2000; Pelham et al., 1997). This is also shown with a decrease of cell spreading area, decreased DNA synthesis and an increase in the amount of apoptosis. Usage of a myosin inhibitor as a control test also gave the same results on stiff substrates (same as the soft substrate without myosin inhibitors).

Also, α-catenin can bind to phosphorylated YAP1 which leads to prevention of YAP1 activation and therefore its nuclear translocation. It does this by being the mediator for transduction of mechanical cues from E-cadherin in fully confluent cultures, resulting in YAP/α-catenin/14-3-3 staying close to the plasma membrane and therefore prevention of YAP nuclear translocation (Schlegelmilch et al., 2011).

Discussion & Conclusion

In the last decade lots of research has been done regarding the physiological function of the Hippo signaling pathway which increased our understanding for this mechanism. First, Hippo has been discovered as a key mechanism for the regulation of organ size and tissue maintenance in Drosophila. However, research has shifted towards mammals to focus on its function on cell proliferation, migration and survival. Angiogenesis is a crucial biological process for the field of both regenerative medicine and tissue engineering. It is essential for the formation of blood vessels and required for both the transportation of both nutrients to all the parts of the body and removing waste from the body during healthy and diseased states of the body.

By default the Hippo signaling pathway is turned on and therefore the nuclear localization of YAP/TAZ is inhibited. Activation of YAP/TAZ, and therefore inactivation of the Hippo signaling pathway, is vital for the activation of target genes by TEAD. These genes, such as CYR61, CTGF and more, that are responsible for cell proliferation, migration and survival.

The downside of the Hippo signaling pathway however is that it is located everywhere in the body and YAP/TAZ playing a central role in most cellular processes. Therefore, it is crucial for the research regarding angiogenesis to specifically look at its function in endothelial cells. However, it remains a challenge to study the effects of all potential role of the Hippo pathway kinases and scaffolding proteins during regulation of these plasma membrane domains both individually and independently of YAP/TAZ.

Previously done research suggests that there is link between stiffer substrates and promotion of migration of cells. Endothelial cells are known to be able to sense mechanical cues from their environment and respond to this. Furthermore, tube formation by ECs is dependent on both the extent of cell spreading as well as the cytoskeleton tension.

For future research it would be ideal to develop tools to monitor and manipulate mechanical forces *in vivo* combined with live reporters to monitor the spatiotemporal regulation of hippo signaling in intact tissues. This would give more precise information of the effect of different kinds of stimulation and could gives additional information of the signaling events activated through the Hippo signaling pathway. Because the Hippo signaling pathway is everywhere in the body, it could be of interest to specify for ECs in the model.

Another interesting approach could be to look at the potential feedback of angiogenesis on the stiffness. Due to increased levels of MMPs (especially via MMP-13) during angiogenesis ECM degradation can be induced. This could lead to softer substrates and might cause a negative feedback loop. But also the ECM components (collagens and fibronectin) induced by YAP which can provide a feed-forward loop that leads to activation of FAK, which in return can inhibits YAP/TAZ nuclear localization as well.

The Hippo signaling pathway shows potential for sensing mechanical cues from the microenvironment, promotion of signaling effect and a positive effect on angiogenesis. However, its specificity for endothelial cells and therefore angiogenesis are still not fully known yet which gives room for further research.

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