Investigation of migration and spreading of cells expressing modified talin1 forms

Master's thesis

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August 2014

ACKNOWLEDGEMENTS

This thesis was carried out in Vesa Hytönen's research group, Protein Dynamics, in BioMediTech at the University of Tampere. I would like to thank him for this great opportunity to be part of a truly inspiring and interesting field of research. I would like to thank my supervisor M.Sc. Rolle Rahikainen for guidance and for providing solutions to problems during my everyday lab-work. I would also like to show my gratitude to our laboratory technician Ulla Kiiskinen for her patience and exceptional knowledge for almost anything I ever figured to ask.

I will also give my special thanks to Jenita Pärssinen who guided me whenever Rolle was not around and to our other group members who helped me during my work with specific and technical aspects; Teemu Ihalainen and Anssi Nurminen. But without the whole group, the atmosphere would not be as great as it was so thank you all.

I am also grateful for my family and friends for their support during this thesis and their encouragement throughout my study.

Finally, but most importantly, I want to thank my husband Tommi for giving me exceptional support and showing that you always believe in me!

PRO GRADU-TUTKIELMA

Paikka: Tampereen Yliopisto
BioMediTech
Tekijä: Taina Viheriälä
Otsikko: Migraation ja leviämisen tutkiminen soluilla, jotka ilmentävät muokattuja taliini1 -proteiineja
Sivut: 61 sivua
Ohjaajat: Dosentti Vesa Hytönen ja FM Rolle Rahikainen
Tarkastajat: Professori Markku Kulomaa ja dosentti Vesa Hytönen
Aika: Elokuu 2014

TIIVISTELMÄ

Fokaaliadheesiot (FA) ovat suuria, useiden proteiinien muodostamia komplekseja. Solut käyttävät adheesioita vuorovaikuttaakseen ympäristönsä sekä muiden solujen kanssa ja niiden avulla solut pystyvät mm. liikkumaan. Yksi tärkeistä proteiineista, joka osallistuu adheesioiden muodostamiseen ja ylläpitämiseen on taliini. Taliini muodostaa yhteyden solun pinnalla olevan integriinin ja solun sisällä olevan aktiinitukirangan välille. Tämä mahdollistaa solun vasteen ulkopuolella tapahtuvaan mekaaniseen muutokseen. Useat FA-proteiinit on tunnettu jo pitkään, mutta niiden ominaisuuksista ja tehtävistä soluissa on vielä puutteellisesti tietoa. Tässä työssä tutkittiin erilaisten lyhennettyjen taliini1 -muotojen vaikutusta solujen migraatioon ja leviämiseen. Tätä ennen täytyi kuitenkin optimoida ja pystyttää laitteisto migraatiokoetta varten. Optimointivaiheessa käytettiin kolmea eri solulinjaa; villityypin hiiren alkion fibroblasteja (WT MEF) sekä soluja, joista on poistettu taliini1:ä tai vinkuliinia koodaava geeni. Varsinaisessa migraatio- ja leviämiskokeissa käytettiin hiiren alkion fibroblasti -soluja, jotka eivät ekspressoi taliini1:ä, joten niihin pystyttiin transfektoimaan haluttua taliini1 -plasmidia; täyspitkää taliin1:ä (FL Tal1) tai neljää erilaista lyhennettyä taliini1 –muotoa. Kaikki taliiniptoteiinit ekspressoitiin fuusioituna punaiseen fluoresoivaan proteiiniin (mCherry), joten fluoresenssimikroskoopilla oli mahdollista saada näkyviin, mitkä solut olivat alkaneet ekspressoimaan transfektoitua plasmidia. Mikroskooppikuvia analysoimalla saatiin varsinaisesti määritettyä ja laskettua kyseisten plasmidien vaikutusta migraatioon ja solujen leviämiseen. Solujen kasvualustana käytettiin peitinlaseja päällystettynä fibronektiinillä, jotta saatiin luotua tunnettu ja hallittu alusta. Kokeiden avulla pystyttiin havaitsemaan kuinka FL Tal1:ä ekspressoivat solut migroituvat ja leviävät hieman nopeammin kuin muita taliini1 –muotoja ekspressoivat solut. Minitaliini4 -soluilla puolestaan havaittiin olevan hitain migroituminen ja heikoin leviäminen muihin muokattuihin taliini1 –proteiineihin verrattuna. Kokeissa määritetyt ja optimoidut laitteistot sekä olosuhteet antavat hyvän alustan jatkotutkimuksiin fokaaliadheesioproteiinien parissa.

Avainsanat: fokaaliadheesio, taliini, solujen migraatio, solujen leviäminen

MASTER'S THESIS

Place:	University of Tampere BioMediTech
Author:	Taina Viheriälä
Title:	Investigation of migration and spreading of cells expressing modified talin1 forms
Pages:	61 pages
Supervisors:	Docent Vesa Hytönen and MSc Rolle Rahikainen
Reviewers:	Professor Markku Kulomaa and Docent Vesa Hytönen
Date:	August 2014

ABSTRACT

Focal adhesions (FA) are large complexes involving numerous proteins. Cells utilize adhesions to interact with their environment and also with other cells. For example, adhesions enable cells to move. One of the major proteins participating in the formation and stabilization of adhesions is talin. It makes a link between a cell membrane protein called integrin and the actin cytoskeleton which lies inside a cell. This enables a response of a cell to the mechanical changes outside the cell. Many FA proteins have been known for a long time but their features and functions are not yet fully known. In this study we used truncated talin1 forms to see any differences in the migration and spreading of the cells. At first, we had to optimize and set up the equipment for the migration assay. Three cell lines were used in this part; wild type mouse embryonic fibroblasts (WT MEF) and cells from which either talin or vinculin genes are removed. In the actual migration assay talin1 knock-out cells were used but they were transfected with the desired talin1 plasmid: full length talin1 (FL Tal1) or with one of the four truncated talin1 forms. All the proteins are expressed with fusion to a red fluorescent protein (mCherry) in order to see which cells have started to express the transfected talin form. By analyzing the microscope images, the impact of the talin mutants on the cells' migration and spreading can be defined and measured. Fibronectin coated coverslips were used in order to gain a known and controlled substrate for the cells to grow. With the research we were able to observe how the FL Tal1 cells migrated and spread faster than the other talin1 forms. Minitalin4 cells were assumed to be the one to migrate and spread the slowest compared to the other talin1 forms used in this study. This hypothesis turned out to be right. The equipment and conditions determined and optimized give a good base for the future studies among focal adhesions proteins.

Keywords: focal adhesion, talin, cell migration, cell spreading

CONTENTS

ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
3T3	3-day transfer, inoculum 3 x 10^5 cells
AB	Antibody
ABS	Actin binding site
BSA	Bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
DDR1	Discoidin domain receptor 1
DDR2	Discoidin domain receptor 2
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DS	Dimerization domain
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FA	Focal adhesion
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FERM	Protein 4.1, ezrin, radixin, moesin
FL	Full length
Fn	Fibronectin
HUVEC	Human umbilical vein endothelial cells
IBS	Integrin binding site
MD	Membrane distal
MEF	Mouse embryonic fibroblast
MP	Membrane proximal
PBS	Phosphate buffer saline
PDMS	Polydimethylsiloxane
PFA	Paraformaldehyde
PIP2	Phosphatidylinositol 4,5-bisphosphate
PTB	Phosphotyrosine binding
RHAMM	Hyaluronan-mediated motility receptor
RT	Room temperature
Tal1	Talin1
Tal2	Talin2
TGF-β	Transforming growth factor beta
TIRF	Total Internal Reflection Fluorescence Microscopy
TLN1	Talin1 gene
TLN2	Talin2 gene
VBS	Vinculin binding site
Vh	Vinculin head
Vhd1	Vinculin head domain 1

Vin	Vinculin
vWF	von Willebrand factor
WT MEF	Wild type mouse embryonic fibroblast

1. REVIEW OF THE LITERATURE

1.1 Introduction

Focal adhesions (FA) are involved in cell-extracellular matrix (ECM) interactions with multiple different proteins with different functions. The most important proteins involved in FAs are integrin, talin, vinculin and F-actin (Lee, Kamm & Mofrad 2007). Integrin mediates the FAs by functioning as a transmembrane protein linking the ECM and the actin cytoskeleton of the cell together (Anthis, Campbell 2011). Integrin has binding sites for some proteins out of which talin works as a linker protein between integrin and the cytoskeleton. In addition, talin has binding sites for vinculin which again has binding sites for F-actin (Lee, Kamm & Mofrad 2007). Vinculin functions in the adhesion site as a stabilizer, and it reinforces the binding of talin and F-actin (Golji, Mofrad 2010). Among the linker feature talin can also activate integrins and vinculins and make integrins to form clusters in the adhesion sites (Roberts, Critchley 2009).

Cells use FAs to aid in their migration, proliferation and to their motility. There can exist different types of FAs. They can be small or large and also on different parts on the cell depending on the cell and the ECM (Geiger, Yamada 2011). In order for a cell to migrate it needs to form focal adhesions and then break them and repeat this cycle over and over. The formation and the breakage of the adhesions must happen as a continuum and in equilibrium in order for a cell to migrate. Integrin activation has a key role in formation of the focal adhesions. It can occur in two ways: inside-out or outside-in. Talin is involved in the inside-out activation and ECM proteins are involved in the outside-in activation (Anthis, Campbell 2011).

Talin is a 270 kDa cytoplasmic protein which has binding sites for integrin, vinculin and F-actin. There are two talin genes in mammalian genome, TLN1 and TLN2, and they encode for quite similar proteins talin1 and talin2, respectively. Talin1 is a more commonly expressed and more thoroughly studied protein than talin2. This study also is focused more on talin1. It consists of a globular head domain and a flexible rod domain (Figure 3). Head domain again consists of four subdomains; F0-F3 from which the F3 subdomain is involved in the binding of integrin. Other binding sites for integrin and also for vinculin are located in the rod domain and especially in its helical bundles which require to be unfolded in order for the binding sites to be accessible for vinculin (Roberts, Critchley 2009).

1.2 Extracellular matrix

Extracellular matrix (ECM) offers support and a signaling pathway for cells and it also connects the adjacent cells together. There are numerous different types of proteins in the ECM (Table 1), each with specific roles in the function of ECM, but some structural proteins are perhaps more important than others. The pattern of ECM proteins can differ between tissues: for example, collagens are the major structural proteins in connective tissue. Fibronectin (Fn) is also a certain type of structural protein. It is a glycoprotein which links collagen and integrin together so that cell adhesion and movement is enabled (Frantz, Stewart & Weaver 2010).

Table 1. Components of the ECM. (Frantz, Stewart & Weaver 2010)

Component	Type	Location	Function
Collagen	Protein	White connective tissue	Mechanical support, structural framework
Elastin	Protein	Yellow connective tissue	Impart elasticity to ECM
Keratin	Protein	Nails and hair	Mechanical support, structural framework
Laminin	Protein	Basal lamina	Anchors cell surface to basal lamina
Fibronectin	Protein	Loose connective tissue	Cell adhesion, migration
Nidogen	Protein	Basal lamina	Links laminin and type IV collagen
Thrombospondin	Protein	Fibroblasts and endothelial cells	Angiogenesis, apoptosis, activation of TGF-β and Immune regulation
Tenascin	Protein	Embryonic tissues, wound and tumors	Role in development
Vitronectin	Protein	Circulation and different tissues	Regulatory linker in cell adhesion and cell invasion
von Willebrand factor (vWF)	Protein	Circulation	Platelet adhesion, blood coagulation
Aggrecan	Proteoglycan	Cartilage, Chondrocyte	Mechanical support
Decorin	Proteoglycan	Widespread in ECM	Binds to type I collagen fibrils and TGF-β
Perlecan	Proteoglycan	Basal lamina	Structural and filtering function in basal lamina

1.3 Focal adhesions

Focal adhesions are cellular substructures making cells capable to interact and attach to the extracellular matrix (ECM). In this way the ECM can also send information to the cells so that focal adhesions are actually bidirectional communication channels. Cells use focal adhesions to communicate with their environment and to make the right responses like migration, spreading, proliferation and gene expression (Lo 2006). Focal adhesions are thus very important players in many cellular events and they can also be connected to some disease cases like cancer invasion and metastasis (Geiger, Yamada 2011). Previous studies have shown that cells make tight connections to the matrix at focal adhesion sites leaving only a ∼10-15 nm gap between the cell membrane and the substrate surface (Zamir, Geiger 2001).

There are numerous proteins, at least 180, involved in focal adhesions and they form together a complex called adhesome (Geiger, Yamada 2011). From these proteins at least 90 has been identified to locate directly at the adhesion site (Roberts, Critchley 2009). One of these proteins is the most important and it is called integrin (Lo 2006). It is a membrane protein which can mediate focal adhesions together with other proteins (Geiger, Yamada 2011). Integrin forms the direct link between cell and ECM environment (Figure 1) (Anthis, Campbell 2011). Integrins are the only focal adhesion proteins located in the ECM environment and there are several other proteins that are constituting the focal adhesions inside the cell. It has been identified that there are at least 42 proteins which bind directly to integrin (Rao, Winter 2009)(Roberts, Critchley 2009). Talin and vinculin are important proteins in the cytoplasmic side of the FA and they are among the proteins forming the linking between integrin and F-actin (Lee, Kamm & Mofrad 2007). They are also called integrin-binding proteins (Cohen et al. 2006). Cells lacking talin are shown to be unable to form focal adhesions as well as show cell spreading (Lee, Kamm & Mofrad 2007). Altogether, these proteins form sort of a linking cascade: ECM-integrin-talin-vinculin-F-actin (Figure 1) (Lee, Kamm & Mofrad 2007). All the proteins that take part in focal adhesions can be divided into three groups depending on their positions: extracellular, transmembrane or cytoplasmic and into three subclasses depending on their function: integrin-binding proteins, adaptors or/and scaffolding proteins and enzymes (Berrier, Yamada 2007). Integrins belong in the transmembrane protein group and talin belongs to the cytoplasmic group together with F-actin (Lo 2006). According to the protein function, talin belongs to integrin-

binding proteins, because it binds directly to integrin's cytoplasmic domain and therefore regulates the activation of integrin (Berrier, Yamada 2007). As the name suggests, adaptor/scaffolding proteins like vinculin and paxillin in turn link the integrin-binding proteins to other proteins (Berrier, Yamada 2007). Focal adhesion also needs a number of other enzymes that can for example phosphorylate or dephosphorylate proteins to modulate their binding properties (Anthis, Campbell 2011). All proteins involved in FAs either form a direct link to another FA protein (scaffolding interactions) or modify another FA protein (regulatory interactions) so that it can take part into adhesion (Geiger, Yamada 2011). Some proteins like talin can also interact with phospholipids (PIP2) located in the cell membrane (Wehrle-Haller 2012).

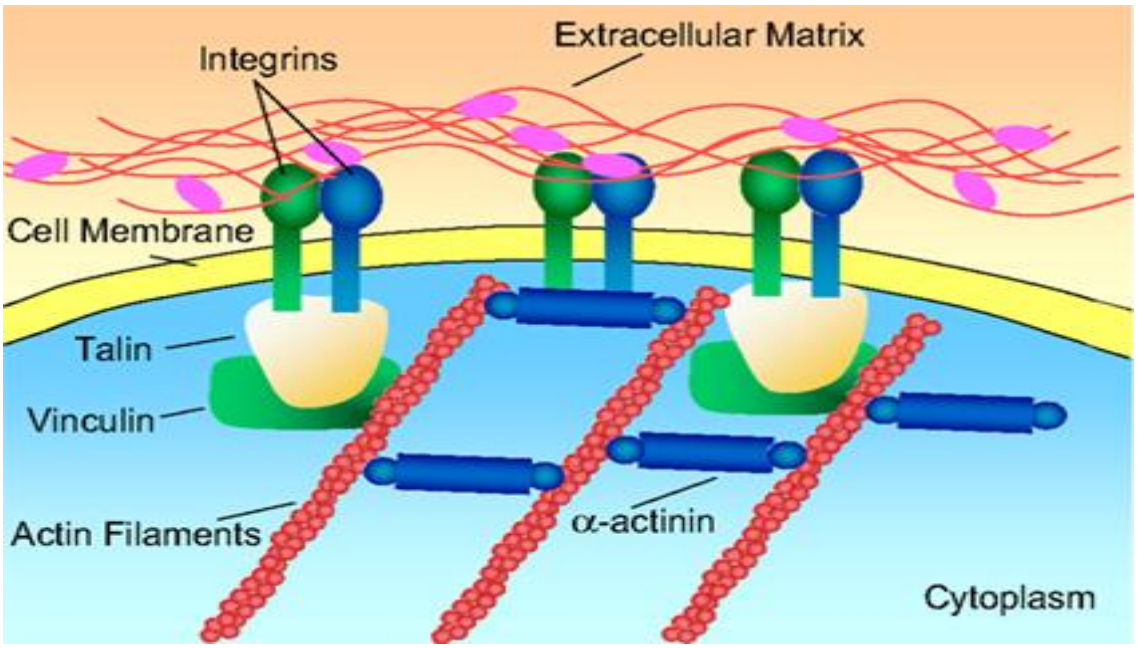

Figure 1. Figure illustrating the major proteins in the focal adhesion site. Figure adopted from (Rao, Winter 2009)

Scaffolding interactions eventually connect integrin tails to actin cytoskeleton. They consist of actin-associated molecules and adaptor proteins. The first mentioned scaffolding proteins, e.g. filamin, talin and α-actinin, interact directly with actin and regulate its organization. The second protein subclass, adaptor proteins, can then interact with the actin-associated proteins, with each other and with integrin (Geiger, Yamada 2011).

1.3.1 Different types of focal adhesions

Different types of focal adhesions can occur even in a same cell and they can be morphologically, molecularly and dynamically diverse. There are a few types of different cell adhesions that can occur in for example a migrating or spreading cell. Classical focal adhesions (1.) are the most common types, they usually appear in a flat surface and their size is about a few square micrometers (Geiger, Yamada 2011). The second type (2.) of adhesions is dotlike nascent adhesions and focal complexes which usually provide the first and early attachment sites (Berrier, Yamada 2007, Geiger, Yamada 2011). They don't survive as long as the classical form of adhesion but once stabilized they can transform themselves into the classical focal adhesions. The third type (3.) is elongated fibrillar adhesions, mainly located in the central parts of the cell. The last type (4.) of the adhesions are podosomes or invadopodia which are ringlike adhesions formed around actins (Geiger, Yamada 2011).

Usually cells utilize only one kind of adhesion type at a time. However when cells are, for example, rapidly migrating they can sometimes form different types of adhesions at the same time (Geiger, Yamada 2011).

1.3.1.1 Non-integrin mediated focal adhesions

It is good to note that not all interactions in adhesions are mediated by integrin. There are few known receptors that can play an important role in cell-matrix interactions like tyrosine kinase, which is one example of the alternative ECM receptors. It can make interactions with collagen and then take part in signaling and adhesion etc. In addition, DDR1 (discoidin domain receptor 1) and DDR2 receptors can function as integrin independent receptors and CD44 and RHAMM are receptors that can function without integrins in adhesion sites (Geiger, Yamada 2011).

1.3.2 Formation of focal adhesions

Cells can form adhesions differently depending on the type of the cell and the ECM. Adhesions can be small or large and they can be unevenly distributed within the cell. For example, there may be few small adhesions near the edge of the cell and large adhesions located in the central region of the cell (Huttenlocher, Horwitz 2011).

Some proteins involved in adhesions exist in two conformational stages (e.g. talin and vinculin); open and closed forms. Typically, open conformation represents the active form and it enables ligand binding, and closed conformation is inactive. In order to stabilize the adhesion the closed conformation needs to be switched to the open form. Closed form is usually maintained with interactions within the protein itself. Therefore the conformational change requires changes in the protein folding (Geiger, Yamada 2011).

The formation of focal adhesion sites starts from integrin activation. This can happen through outside-in or inside-out activating processes. In the outside-in activation, proteins in the ECM cause activation and in the inside-out activation, proteins inside the cell cause the activation, respectively. After integrin activation, integrins start to cluster which is triggered by the binding of cytosolic proteins to the integrin tail domains (Humphries et al. 2007). The types of integrins involved in the FAs can change during the adhesion. For example αvβ3 and α5β1 take part in formation of adhesions and signaling, but their presence in matured adhesions is substrate-dependent (Geiger, Yamada 2011). Activated integrins then allow talin to form a connection between talin and F-actin. This connection is reinforced by vinculin. When these proteins are joined together they form the initial complex of adhesion. The next step is to recruit other proteins to gain stable and functional focal adhesion complexes connecting cell and extracellular matrix (Golji, Mofrad 2010).

1.3.2.1 Chemical and physical sensing of ECM

Cells can sense ECM through chemical and also physical properties. A variety of receptors, e.g. integrins, are included in chemical sensing. Receptors can have different binding properties for specific ECM molecules such as collagen. These types of specific connections are the key for adhesion formation. The requirements for the components enabling formation of stable adhesions vary between different cell types. Integrin receptors function together with growth factor receptors to sense the properties of the ECM (Geiger, Yamada 2011).

Cells can also sense physical properties of the ECM like stiffness and ligand spacing. Although physical sensing is not yet as well understood as chemical sensing, it is known

that the geometry and topography are important for the cell to sense the ECM (Geiger, Yamada 2011).

1.3.3 Maturation of FAs

As previously described, in order to gain a stable and functional FA complex the initial focal complex needs to be changed in terms of molecular composition and rearrangement. This requires conformational changes in proteins and also changes in the assembly of the proteins. An additional step in the maturation is the formation of fibrillar adhesions. They are sometimes required, especially in fibroblasts, in the maturation of integrin-mediated FAs. This process is force-dependent as is the recognition of ECM in the assembly of focal complexes and focal adhesions (Geiger, Yamada 2011).

1.3.4 Disassembly of focal adhesions

Disassembly of focal adhesions is an important step for cells for example during cell migration. Cells need physical and biochemical interactions for releasing the connection between ECM and integrins (Petit, Thiery 2000). Integrins can either be physically broken off the membrane, they can form aggregates and move to the next adhesion site or they can stay in the cell membrane and spread along it (Palecek et al. 1996). Disassembly can occur also via a protein called calpain which can cleave the adhesion components (Geiger, Yamada 2011).

Some ECM proteins have been shown to stimulate the disassembly of the focal adhesion during the cell migration. A couple of these proteins are for example thrombospondin and tenascin (Sage, Bornstein 1991).

1.3.5 Role of FAs in cell migration

Cell migration is a complicated process which requires co-operation between adhesion proteins, signaling proteins and many other proteins. Cells need to form adhesions and then break the adhesions and again form novel adhesions to enable cellular movement (Figure 2). The best speed for migration is achieved when the formation and the release

of the adhesions are proficient. Therefore the rate for migration depends on how fast the cell can form and break the adhesions. When a cell moves it pushes its fore, central and back regions forward and this consumes lots of energy (Huttenlocher, Horwitz 2011). The migration needs reinforcement of cell polarity to create leading edge and trailing edge. The leading edge protrudes and new cell-matrix contacts are created while the trailing edge retracts, causing the cell to move forward (Berrier, Yamada 2007).

Adhesion dynamics which constitute quite a wide range of mechanisms are an important part in cell migration. Dynamics can be regulated by microtubules by destabilizing FAs by releasing the tension. Signaling proteins in the initial complex can be bound very transiently while the scaffolding proteins are making more stable interactions (Geiger, Yamada 2011).

Intgerin-mediated migration has been shown to take part in a variety of human development processes like wound healing, and it is also important for diseases such as cancer (Huttenlocher, Horwitz 2011).

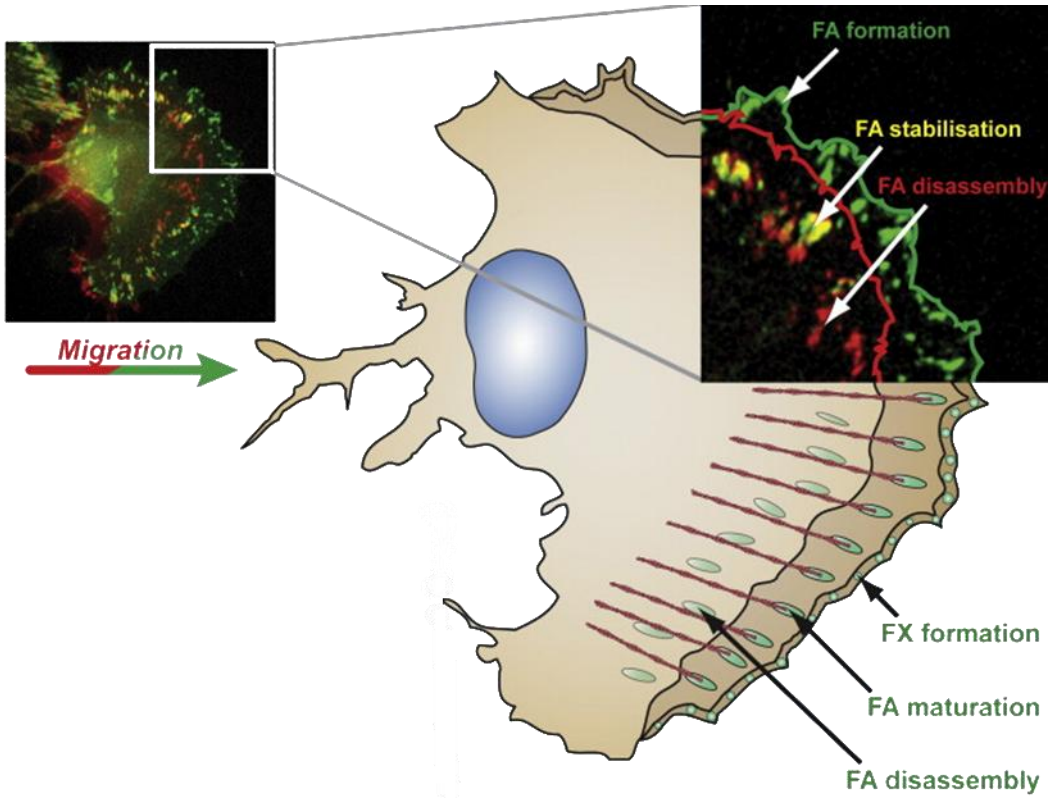

Figure 2. Picture of migrating cell shows formation and disassembly of FAs. Time elapse between the two stages of FA is 5 minutes; green color indicates the first picture and red color the second picture. Yellow color shows the FAs which were at the same spot in both pictures. Figure adopted from (Carisey, Ballestrem 2011)

1.4 Proteins in FAs

1.4.1 Talin1

Talin is a 270 kDa cytoplasmic protein consisting of N-terminal head (~50 kDa) and C-terminal rod (~220 kDa) (Figure 3) (Roberts, Critchley 2009). Rod is composed of 62 helices that form 13 (R1-R13) amphipathic 4- or 5-helix bundles (Anthis et al. 2010). Talin has 2541 amino acid residues with the head domain positioned in residues 1-400 and the rod domain in residues 482–2541 (Roberts, Critchley 2009). There are two talin isoforms in the talin protein family; talin1 and talin2, but talin1 is expressed more commonly than talin2 (Lo 2006).

Talin head contains FERM (residues 1-400) domain which in turn comprises of four subdomains (F0, F1, F2 and F3) (Critchley 2005). The F0 subdomain is not always counted as one of the FERM subdomains because it is not present in the typical FERM protein (Anthis, Campbell 2011). FERM (protein 4.1, ezrin, radixin, moesin) domains are found also in other proteins and they are often associated with biological processes such as cell adhesions and proliferation (Elliott et al. 2010). The shape of FERM domain (Figure 4) is usually cloverleaf, but in the case of talin it is found to be a linear arrangement of subdomains (Anthis, Campbell 2011). Rod and head domains take part in forming the globular and extended conformations of talin. When the rod domain is isolated, it shows extended conformation, suggesting that forming globular conformation requires interactions between the rod and the head domains (Goult et al. 2013). Rod domain is also responsible for the dimerization of the talin (Critchley 2005).

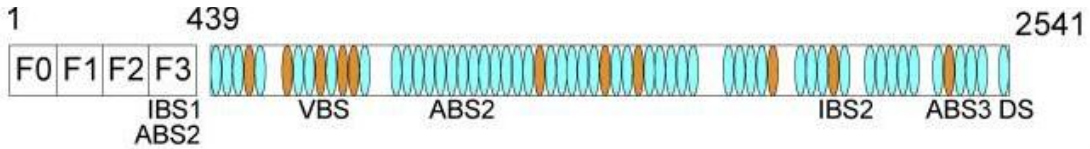

Figure 3. The schematic structure of talin with amino acid numbers. 62 α-helices are shown as ovals. Orange ovals = vinculin binding sites (VBS). ABS = actin binding site, IBS = integrin binding site and DS = dimerization domain. Figure adopted from (Wang et al. 2011)

The main function for talin is to participate to the integrin-mediated events and to act as a linker between integrin and cytoskeleton. Talin also activates integrins and vinculins whose activation is an essential process in the formation of FAs (Critchley 2005). In addition talin promotes integrin clustering and also the switch of integrins from low-affinity state to high-affinity state. But it is good to note that talin does not make all this

happen by itself, and it needs a co-operation with other proteins as well. Talin can also be said to be a recruitment protein, meaning that it recruits other proteins to join the focal adhesion (Roberts, Critchley 2009).

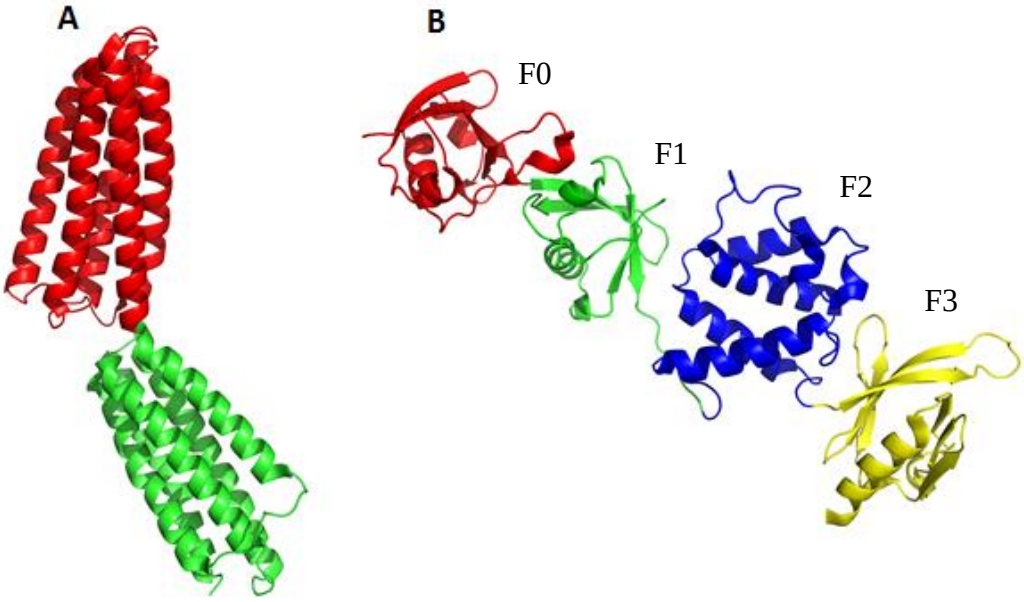

Figure 4. Crystal structures of one of the rod fragments (A) and FERM domains (B) of the talin. FERM domain contains subdomains F0-F3 (F0 is not always counted as one of the subdomains). F1 subdomain contains five β-sheets with α-helix running across it. The F2 subdomain is completely α-helical with a short linker region. The F3 subdomain is composed of two β-sheets followed by an α-helix (Calderwood et al. 2002). PDB ID 3DYJ from Gingras et al. (2009) and 3IVF from Elliott et al. (2010).

1.4.1.1 Talin2

Tln2 gene is another talin gene in the human genome (Praekelt et al. 2012). Talin1 functions in cell adhesion whereas the function of talin2 is still unclear (Debrand et al. 2009). Talin2 is expressed primarily in striated muscle and in the brain while talin1 is expressed widely in different tissues (Anthis, Campbell 2011). Talin2 also has rod and head domains as talin1 and they have sequence similarity of 74% so they encode very similar proteins (Praekelt et al. 2012). Talin2 gene (190 kb) is an approximately six times larger protein in mammals as talin1 (30 kb) and because of that talin2 also has larger introns. Due to the larger size of talin2 gene there are also more splice variants in talin2 gene and therefore it is possible that talin2 has some special functions in specific tissues (Lo 2006).

Talin2 and talin1 have different affinities for integrins and for some reason talin2 has higher affinity for β-integrin than talin1 (Wehrle-Haller 2012). They both have their

roles in FAs but their location is different; talin1 has shown to localize in peripheral focal adhesions while talin2 participates in both focal and fibrillar adhesions (Praekelt et al. 2012).

Lacking talin2 gene does not have as much of an impact for cells as lacking talin1 gene. Although when considering single cells the lack of talin1 or talin2 does not show a huge difference in their behavior but the differences become significant in the whole organism (Praekelt et al. 2012).

1.4.2 Vinculin

Vinculin is a cytoplasmic protein (116 kDa) which is associated with focal adhesions in the cytoplasmic site and contributes to the cell adhesion and motility (Carisey, Ballestrem 2011). Vinculin and talin are both among the proteins essential in formation of stable focal adhesions (Golji, Mofrad 2010). Vinculin mainly functions as stabilizing cell-ECM junctions by participating in linking integrins to the actin cytoskeleton (Debrand et al. 2009). Vinculin contains five different domains (D1, D2, D3, D4 and tail). Domains 1-4 constitute a globular head (Vh) (residues 1-850) of vinculin. Vinculin has also a third main domain besides the tail (residues 879-1066) and head and it is a proline-rich neck domain (residues 851-878) which connects head and tail domains together. Vinculin contains several binding sites for talin and F-actin. In domain D1 for example is a Vh1 subdomain which can bind to talins vinculin-binding site (VBS) (Golji, Mofrad 2010). Head domain can also bind F-actin. Tail domain in turn can bind a protein called paxillin, F-actin and also some other proteins (Lee et al. 2008).

The presence of vinculin is not as important for the formation of focal adhesions as is the presence of talin. Cells lacking vinculin can form focal adhesions, but cell spreading and cell motility are not as efficient as in cells that express vinculin (Lee et al. 2008). In vinculin deficient cells the integrin linkage to cytoskeleton is less stable and it is easily broken by mechanical force (Cohen et al. 2006). However, Xu, Baribault & Adamson (1998) published that lack of vinculin shows for some reason faster migration than the wild type cells. Lack of vinculin is also shown to be associated with cardiac diseases and with brain abnormalities (Cohen et al. 2005).

1.4.2.1 Vinculin exists in two conformations

Vinculin can exist in active and also in inactive forms (Figure 5) from which only the active form is capable working in FAs. When vinculin is in inactive form, which is actually an autoinhibitory conformation, it prevents vinculin interactions with proteins. Head, especially D1 domain, and tail domains make intramolecular connections to each other closing the binding sites for talin and F-actin so that vinculin can autoinhibit the talin-vinculin interactions and also F-actin-vinculin interactions. Therefore, in order for vinculin to be bound to talin, its conformation must be changed from autoinhitory to active. Vinculin can be activated by binding to talin and especially three VBSs are showed to have the capability to bind vinculin at this point. These VBSs are exposed to vinculin by mechanical force during talin activation and therefore it can be said that external force is needed in vinculin activation (Golji, Mofrad 2010). When vinculin is activated by this so-called force-dependent pathway it may lead at the same time to reinforcement of the actual focal adhesion (Lee et al. 2008). There is also another way to gain activated vinculin and it needs both talin and F-actin interactions with vinculin (Golji, Mofrad 2010).

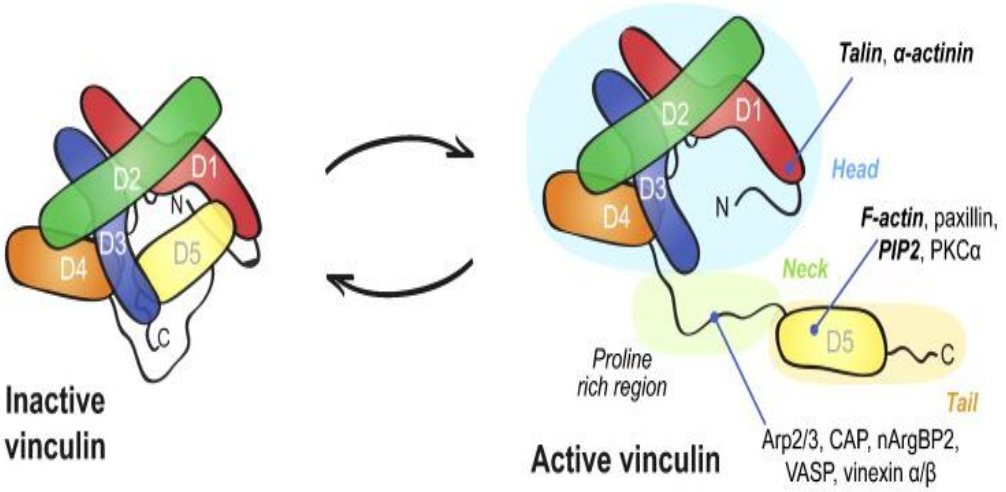

Figure 5. Picture illustrating inactive and active forms of vinculin. Domains D1-D4 constitute the head domain, between the D4 and D5 is the proline-rich neck domain and D5 is the tail domain. In the active state are the binding sites for many proteins accessible (shown in the picture) and some of these proteins (talin, α-actinin, F-actin and PIP2) can be involved in activating the vinculin. Figure modified from (Carisey, Ballestrem 2011)

1.4.3 Integrins

Integrins are large heterodimeric adhesion receptors which connect the intracellular and extracellular components. They are found in the cell membrane and they usually take part in focal adhesion sites but are found to be associated also with other cell surface receptors like growth factor receptors (Berrier, Yamada 2007). Integrins have cytoplasmic and extracellular domains and also a transmembrane helix; intracellular domains are shorter, approximately 20-50 residues, than extracellular domains (700-1000 residues) (Wegener et al. 2007). Because of these domains integrins can mediate cell-cell and also cell-ECM interactions (Roberts, Critchley 2009). Cytoplasmic domain consists of tail-regions (one α and one β region) which again consist of membrane-distal (MD) and membrane-proximal (MP) helices (Anthis et al. 2010). There are 18 different α and eight different β subunits in the human integrin family and they can combine to at least 24 different heterodimers differing from their ligand binding affinity (Anthis, Campbell 2011). Integrins even in the same cell can function with different affinities due to their numerous combinations (Geiger, Yamada 2011). Integrin α-subunit usually contains around 1000 amino acids and β-subunit contains around 700 amino acids but their size may vary (Anthis, Campbell 2011). Focal adhesions are not usually limited to a certain type of integrin receptor although exceptions occur. Also recruitment of other proteins is not usually limited in focal adhesions but in some cases only certain proteins like a protein called tensin is recruited only on some focal adhesion sites leading to the conclusion that there might be signaling specificity (Berrier, Yamada 2007).

Integrins, especially β cytoplasmic domains, form clusters in the membrane when assembling focal adhesions (Petit, Thiery 2000). This clustering activates many non-receptor proteins like focal adhesion kinase (FAK), serine/threonine kinases and lipid kinases. Usually the activation of these and other kinases causes the recruitment of proteins to adhesion sites (Berrier, Yamada 2007). Integrin uses its α-domain in focal adhesions to interact with ECM and it uses β-domain to interact with talin (Petit, Thiery 2000). In focal adhesions and in migration, integrins play a major role by linking ECM to actin cytoskeleton via talin (Anthis et al. 2010). Talin acts as a linker between integrin and F-actin and it regulates the affinity of integrins for ligands (Praekelt et al. 2012, Anthis et al. 2010). All the proteins that interact with integrins β tail can be classified into two groups: regulatory proteins and cytoskeletal proteins. Talin is an

example of a cytoskeletal protein. Numerous α and β subunits have been identified, both individual and combined (Petit, Thiery 2000).

Each α and β chain of the integrin consists of extracellular domains, a single membrane-spanning helix, and a short cytoplasmic tail (Anthis et al. 2010). Thus integrins have both extracellular and intracellular domains and they can respond to both extracellular and intracellular signals, which is quite unusual for membrane proteins (Wehrle-Haller 2012). Integrins function as glycoprotein receptors in focal adhesions, they are actually the major receptors in assembly and recognition of ECM and they facilitate cell migration to the correct tissue location (Petit, Thiery 2000, Wehrle-Haller 2012). They can also take part in some contacts between two cells and play a role in a variety of other biological processes like wound healing and immune responses (Petit, Thiery 2000). On the other hand some integrin receptors have been found to link to certain types of abnormal cell behavior like cancer. Expression of β_6 integrin is normally prevented in epithelial cells but in wound healing its expression is upregulated and therefore, if by unfortunate incident the expression of β_6 stays up, the cell can progress into a cancer cell (Berrier, Yamada 2007).

1.4.3.1 Integrin activation

As transmembrane proteins, integrins work on both sides of the membrane, and their activation can occur via inside-out or outside-in activation processes. When integrin works in focal adhesions its conformation changes throughout the activation process. In inside-out activation the affinity of ligands of ECM increases, finally leading to a step where talin is bound to the cytoplasmic tail of the integrin. Outside-in activation starts from binding to the ECM ligand and leads to intracellular changes leading to increased cell viability, proliferation and growth (Anthis, Campbell 2011).

Considering integrin activation (Figure 6), the most important tail regions are the β1 and β3 subunits. They also have a few splice variants with different activity affinity. Different β-tails have also different MP regions which affects their activation (Anthis et al. 2010). Cells that have mutations in their β1 and β3 chains have shown significant reduction in forming focal adhesions (Lyman et al. 1997, Reszka, Hayashi & Horwitz 1992).

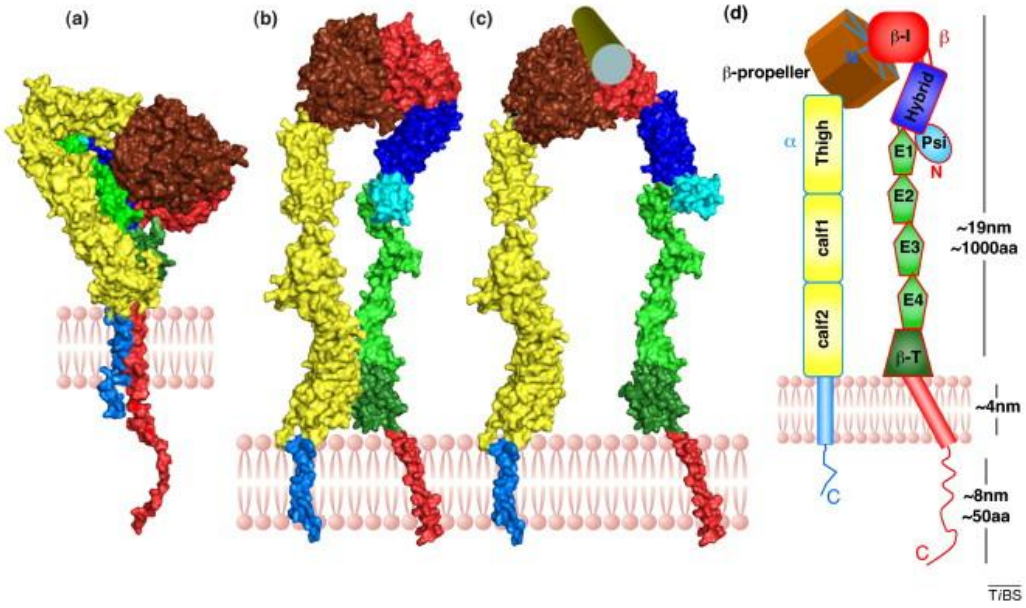

Figure 6. Picture illustrating the states of integrin. a) X-ray based extracellular structure with the cytoplasmic tails. b) Intermediate conformation in the integrin activation process. c) Active state of the integrin after ligand binding. d) Illustration of the integrin domains. E1-E4 are EGF-like domains and β-T is the β–tail domain. Figure adopted from (Anthis, Campbell 2011).

1.4.4 Talin interactions with other proteins

Talin functions as a structural linker between integrins and actin cytoskeleton and is therefore an important protein in cell adhesions (Lee, Kamm & Mofrad 2007). It can also function as a regulator for other proteins to bind to integrins (Debrand et al. 2009). Talin has binding sites for vinculin, actin and integrin (Anthis et al. 2010). Head domain contains binding site for F-actin and β-integrin in its F3 domain. The second binding site for β-integrin is located in the rod domain and there are also numerous binding sites for vinculin and at least two binding sites for F-actin in the rod (Debrand et al. 2009). R13 is the best characterized within the talin rod α-helix bundles and it interacts with F-actin. It is located near the C-terminal end and talin can therefore offer a direct link between the β-integrin tail and the actin cytoskeleton (Anthis, Campbell 2011, Gingras et al. 2007). Rod domain is also responsible for maintaining talin in its inactive form (Goult et al. 2009).

Vinculin binding sites (VBS) are located in the talin rod domain and they are consisting of individual α-helices buried in α-helix bundles. In order for the VBSs to be accessible for vinculin the bundles must unfold or at least structurally reorganize. The mechanism for the unfolding is still poorly understood but one theory is that a mechanical force can

make the bundles unfold (Roberts, Critchley 2009). The opening of the bundles under mechanical stress has been studied by using molecular dynamics simulations (Hytönen, Vogel 2008).

1.4.4.1 Talin-integrin interaction

Talin can interact with a broad range of proteins. Talin-integrin interactions are common in focal adhesions. Integrins can bind to rod and head domains of the talin (Goult et al. 2010). As shown previously, integrins play a key role in cell adhesions and talin takes part in integrin activation by interacting with the β-integrin tail (Anthis et al. 2010). In order for talin to bind integrin its integrin binding sites (IBS) must be activated. Talin can form an autoinhibited form by making a linkage between the F3 domain and a rod bundle (residues 1654-2344) so that it blocks the integrin binding site (Roberts, Critchley 2009).

Talin activates integrins by interacting with β-integrin tails (Anthis et al. 2010). The activation process requires talin F2-F3 domain pairs, but other talin domains are also needed for full integrin activation (Critchley 2000). F0 and F1 domains play their own role in this process (Elliott et al. 2010). F0 takes part in activating β3-integrin and also enhances β1-integrin activation (Critchley 2000). Integrin activation is mediated via interactions between β3-integrin NPxY motif and the talin F3 domain which has a phosphotyrosine binding (PTB) fold (Anthis et al. 2010, García-Alvarez et al. 2003). F3 also binds to integrin membrane-proximal helix (Anthis, Campbell 2011). García-Alvarez et al. (2003) showed in their research that β3-tail can be covalently attached to talin F2-F3 domains and used that fusion protein in their research (García-Alvarez et al. 2003). It has been revealed that amino acids with K324 residues in talin1 and D759 in β-integrin also contribute to talin-integrin interaction (Anthis et al. 2009). Talin-integrin interactions can be regulated by calpain proteolysis or through phosphorylation of either talin or integrin (Calderwood et al. 2002).

When integrins are activated salt bridge between α- and β-integrin tails breaks at the same time, which then affects the affinity state of the integrins for ECM (García-Alvarez et al. 2003, Tadokoro et al. 2003).

1.4.4.2 Talin-vinculin interaction

Vinculin (116 kDa) is an actin binding protein located at the adhesions sites (Roberts, Critchley 2009). Talin has numerous binding sites for vinculin in its rod domain. Vinculin can exist in two conformations; inactive and active. Inactive form is unable to bind talin. In inactive form the head and tail domains of vinculin are in close interaction with each other so that this blocks the binding site for talin. Talin itself and especially the VBS is able to release the interaction between vinculin head and tail domains. The VBS disrupts the inactive form by binding to vinculin head Vhd1 domain, and this eventually leads to the point where the tail domain breaks apart from the head domain. It is also suggested that the VBSs of talin are exposed only in the presence of Vhd1 of vinculin so that VBS helices of talin would stay in closed conformation without the stabilizing effect of vinculin itself (Cohen et al. 2006).

There are also other proteins and cell membrane lipids that can release the vinculin autoinhibition, such as α-actinin, F-actin and PIP2. Talin is more likely to be the one which breaks the head-tail interaction in vinculin because talin is usually co-localized with vinculin rather than for example with α-actinin. Talin has also been found to be one of the requirements for vinculin to localize in FA. Thus, talin-vinculin interactions have a key role in regulating focal adhesion formation (Carisey, Ballestrem 2011).

1.5 Methods used in this project

1.5.1 Scratch assay

Scratch assay (or wound healing assay) is an in-expensive and widely used method to measure cell migration *in vitro*. It is easy to use and doesn't require lots of different components. It basically works as follows: a scratch to a monolayer of cells is made and it is then measured how fast the cells invade the scratch by migration. Measuring can be made by looking at cells in different time points and taking pictures with a microscope. This makes the assay very suitable for analyzing live cells instead of fixed ones. It is important to take the first picture right after the scratch has been made in order to know how wide the scratch was (Liang, Park & Guan 2007).

When cells are migrating on the surface they are thought to migrate towards a cell-less space (Figure 7). In addition, when cells encounter another cell they can make

interactions with the cell and eventually change their migration direction. In order to avoid cell contacts too fast, it is important to note that the monolayer should not be over populated or in contrast there should not be too few cells (Liang, Park & Guan 2007).

1.5.1.1 *In vitro* scratch assay is a potential method to analyze cells *in vivo*

In vitro scratch assay is a capable tool for analyzing the cell behavior *in vivo*. By *in vitro* assay one can also regulate the migration by affecting the cell interactions with ECM and other cells. This method can be also used to detect fluorescent cells so it is capable of measuring the migration of the transfected cells in comparison to non-transfected ones (Liang, Park & Guan 2007).

There are also some disadvantages in the *in vitro* scratch assay method. It takes quite a long time to fully get the results: first it takes a few days to grow the cell monolayer and then it takes hours to days for the cells to close the scratch. It also consumes many cells so this method is not optional for studies where the amount of the cells is limited (Liang, Park & Guan 2007).

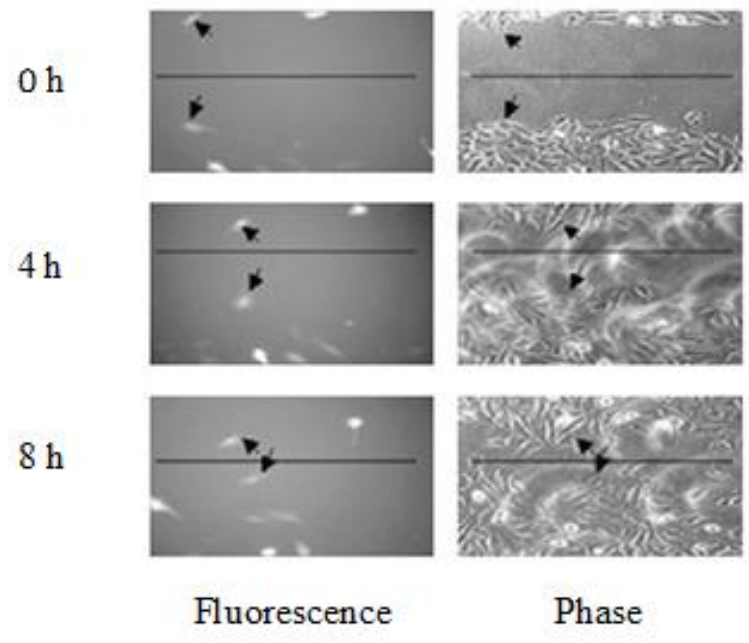

Figure 7. Figure illustrating the scratch assay method. The first picture indicates the time when the scratch has been made (0 h). The second picture is after 4 hours and the third is after 8 hours from the scratch making. The arrows are pointing to specific cells which are tracked to see how fast they migrate. Figure adopted from (Liang, Park & Guan 2007).

2. MAIN GOALS IN THE RESEARCH

The main goal of this study was to set up methodology for studying how talin and its different modified forms contribute to cell-ECM adhesions especially in terms of migration and cell spreading. This can be modelled by using talin1 knockout ($Tal1^{-/-}$) mouse embryonic fibroblasts (MEF) and then transfecting the cells with different talin constructs.

The project was basically divided into four steps: the first (1) step was to optimize the migration assay; the second (2) step was to use the assay to study how talin affects the migration. The third (3) step was to use the same cells to study cell spreading and the fourth (4) step was to do the imaging analysis from the phase and fluorescence microscope images. Transfection was done by electroporation and this step also needed some optimization to know the best conditions to gain the highest possible transfection rate. In the first step we didn't use transfected cells but instead WT MEF, $Tal1^{-/-}$ MEF and $Vin^{-/-}$ MEF cell lines.

3. MATERIALS AND METHODS

3.1 Mammalian cell culture

3.1.1 Cell strains

The cell strains were obtained as follows: mouse embryonic fibroblasts (MEF) from Dr. Wolfgang Ziegler (Hannover, Germany), WT MEF and Vin$^{-/-}$ MEF cells were both immortalized strains isolated from mouse embryos produced in heterozygous crosses of Vin$^{+/-}$ mice (more details in Xu, Baribault & Adamson 1998). Tal1$^{-/-}$ MEFs were isolated by gene disruption from embryonic stem cells (more details in Priddle et al. 1998) and they were kindly provided by Prof. Michael Sheetz (Columbia University). WT MEF and Vin$^{-/-}$ cells were frozen in liquid nitrogen but Tal1$^{-/-}$ cells were previously under maintenance of Rolle Rahikainen.

3.1.1.1 Minitalins

There are many different talin isoforms existing, from which, minitalins (Figure 8) are shortened versions of the full length talin. Minitalins are designed so that certain functional elements of the rod domain have been deleted without affecting its integrin and actin binding.

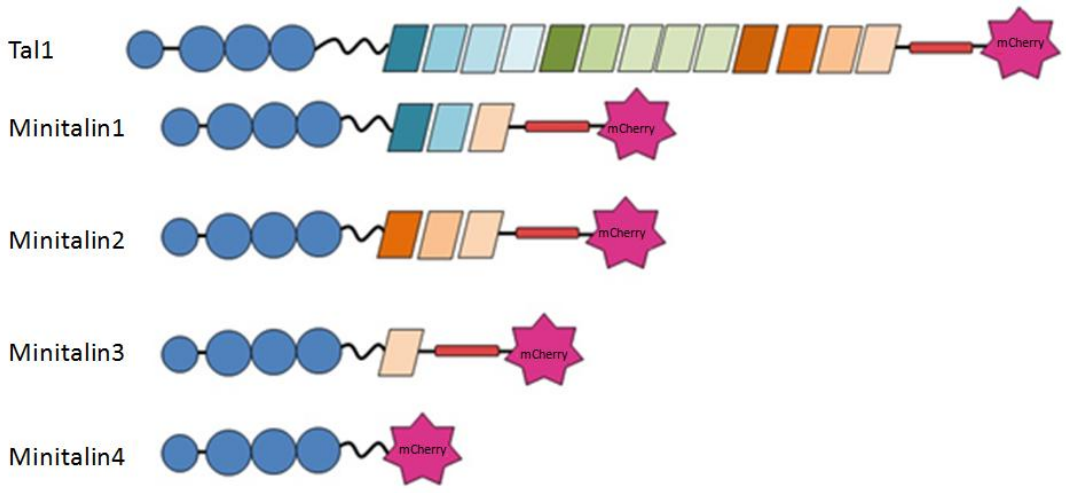

Figure 8. Full length talin and minitalins 1-4. Blue circles = F0 + FERM domain, blue/green/orange boxes = helix bundles of the rod domain, red bar = dimerization domain, red star = mCherry.

3.1.2 Maintenance of the cells

Cells were cultured in T75 cell culture flasks (Sarstedt). WT MEF and Vin$^{-/-}$ MEF cells needed medium which contained 10% FBS (fetal bovine serum) (Lonza, BioWhittaker, Cat. No.), 1% L-glutamate and 1% (v/v) β-mercaptoethanol. The rest of the medium were DMEM with 4.5 g/l glucose (Dulbecco's Modified Eagle Medium) (Lonza, BioWhittaker® Cat. No. BE12-614F). β-mercaptoethanol was added immediately after the cell culture. Tal1$^{-/-}$ MEFs required DMEM/F12 medium that contains L-glutamate. The medium was prepared by adding 15% FBS. β-mercaptoethanol was not added to Tal1$^{-/-}$ knockout cells. When the cell confluence reached approximately 70%, the cells were divided, usually twice in a week. WT MEF and Vin$^{-/-}$ MEFs divided a little bit faster than Tal1$^{-/-}$ MEFs. Subcultivation was done with preheated medium (37°C). The cells were washed with PBS (phosphate buffer saline) and the cells were removed from the bottom of the flask with trypsin-EDTA (Lonza, BioWhittaker, Cat. No. BE17-161E) (200 mg/l). The subcultivation ratio was usually 1:9 or 1:12 but sometimes even as small as 1:2, depending on when the cells were used for the experiments.

The cells which were stored in liquid nitrogen were thawed quickly to avoid cell death. The cell vial was quickly moved to water heater (37°C), the suspension was transferred to a centrifuge tube and was centrifuged (200 g, RT, 5 min) (Rotina 48R). The medium was removed and discarded, and the cell pellet was resuspended to a new medium and eventually transferred to a T75 flask.

Medium samples were taken for mycoplasma test to ensure that they were clean. This was always done when a new vial was opened. An important thing also to note is that between different cell types the laminar flow hood was cleaned with 70% ethanol so all the cell lines were handled separately. This was done to avoid cross contamination between the cells lines.

3.1.3 Fibronectin coated coverslips

Coverslips that were used in the migration and spreading tests were coated with fibronectin isolated from human serum by gelatin affinity chromatography (Finnish Red Cross, FFP8). The fibronectin concentration was 25 µg/ml when used. It was diluted in PBS.

The coating occurred so that 40 µl of the fibronectin was added to a well on a 6-well plate and a coverslip was placed on top of it. Then incubation was performed at 37ºC for 1h followed by washing with PBS three times with fibronectin side upwards. At this point the coverslips could be left on the plate immersed with PBS.

3.2 Migration assay

Migration assay was performed with two different methods: scratch assay (Figure 9) and PDMS button assay (Figure 10). Scratch assay was made with previously published protocol from Yanling Chen[1] (2011). PDMS buttons were put into a 6-well plate before adding the cells. Cells were cultured and the proper amount ($2x10^6$ or $4x10^6$) was transferred to a 6-well plate with 2-3 ml of medium. Cell counts were measured with Cell Countess (Invitrogen). Cells were left to grow (18-24 h) to obtain proper confluence (approximately 70%), after which the scratches were made or the PDMS buttons were removed. In order to get rid of the dead cells and also cells that were detached from the bottom, the wells were washed carefully with 2 ml of PBS and new medium was added. Pictures were taken with a microscope (described later in more detail) at three different time points: 0 h, 12 h and 24 h. The washing with PBS and the medium change were also done before every picture taking.

3.2.1 Optimizing migration assay with scratch assay

There were a few variables to be optimized in the scratch assay: how many cells are needed in each well, how the scratch is made, and what instrument can be or should be used to make the scratch. We also wanted to find the optimal time points (0 h, 12 h and 24 h) to see any differences and also to make sure that the scratch is not completely closed.

The first experiment was done to solve for the variables described above. It was done with WT MEF cells by transferring 200 000 cells to each of six wells and 400 000 cells to six different wells. The scratch was done with three different pipette tips to test which is the most suitable size to make the scratch. The tip sizes were 10 µl, 200 µl and 1000 µl. Two scratches were made in each well. In half of these wells the normal medium for

[1] http://www.bio-protocol.org/wenzhang.aspx?id=100; recessed 12.6.2013

the cells was used, and in the other half a starvation medium was used. The idea to use starvation medium was to stop the cells from dividing and only allow them to migrate. The scratches were imaged and saved as mosaic pictures of 1x3 images with an optical microscope (Olympus IX71 with Surveyor software) (Table 2).

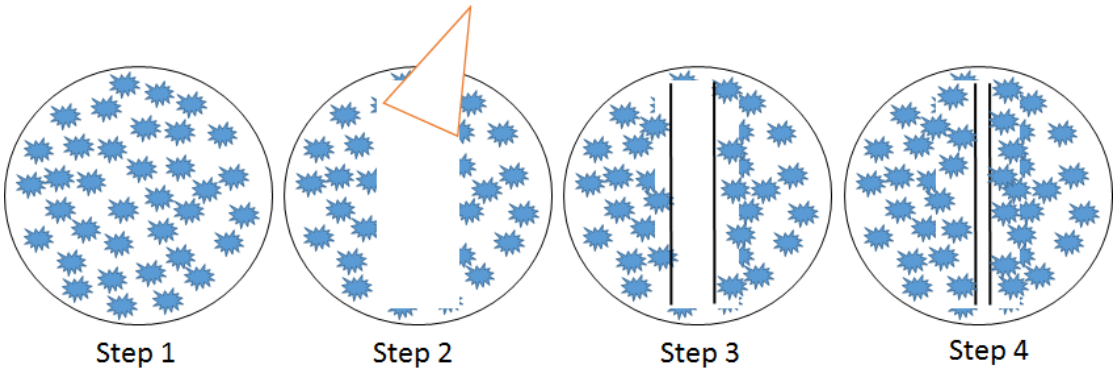

Figure 9. Scratch assay step by step. Step 1: Cells added to the plate and reached the proper confluence. Step 2: Scratch is being made with pipette point. First picture (0 h) is taken. Step 3: Cells are starting to migrate over the scratch area. The second picture (12 h) is taken. Step 4: Cells have almost closed the scratch. The third picture (24 h) is taken.

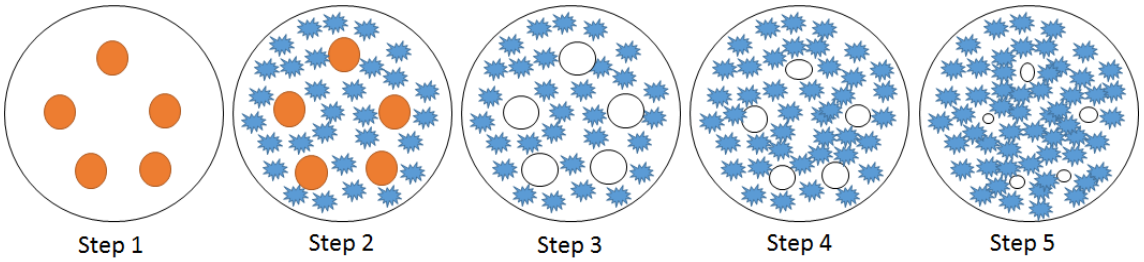

Figure 10. PDMS assay step by step. Step 1: PDMS buttons are added to the plate and sterilized. Step 2: Cells are added and they have reached the proper confluence. Step 3: PDMS buttons are removed. The first picture (0 h) is taken. Step 4 and step 5: The cells are closing the button area. Second (12 h) and third (24 h) pictures are taken.

Table 2. Settings of the microscope in the migration assay. Microscope which was in use was Olympus IX71 with Surveyor software.

Objective	10x	**Exposure**	2.846 ms
Filter	1 (no filter)	**Gain**	1.80
Phase contrast	4	**Offset**	-1028

The second experiment was done with all three cell lines: WT MEF, Tal1$^{-/-}$ MEF and Vin$^{-/-}$ MEF. This time the cell amount was only the 400 000 cells in each well, and the use of 200 000 cells per well was abandoned.

3.2.2 Optimizing migration assay with PDMS buttons

Polydimethylsiloxane (PDMS) (Dow Crowning Sylgard 184) buttons were made by mixing base (part A) and curing agent (part B) in ratio of 10:1 and pouring the mix in a plate. Air bubbles arose to the surface and the biggest bubbles were removed manually by using a pipette tip. The plates were incubated for 30-60 minutes in 57ºC and they were left to cool for the next day. On the next day the actual buttons were made with 1.7 mm wide injection needle.

Five PDMS buttons were placed in each well from a 6-well plate and they were sterilized with UV-light for 2 x 30 min. This step of the migration optimization was performed with all the three cell lines: WT MEF, Tal1$^{-/-}$ MEF and Vin$^{-/-}$ MEF and the cell amounts were the same as in the scratch assay. The cells were added after sterilization and left to grow for about 24 hours. On the next day (or when the 70% confluence was achieved) the buttons were removed with forceps. The wells were washed with 2 ml of PBS and new medium was added. At this point pictures were taken and also later at 12 h and 24 h after the removal of the buttons. The images were taken with the same optical microscope (Olympus IX71 with Surveyor software) (Table 3) as in the scratch assay but this time the images were mosaic images of 3x3 from the button area.

Table 3. Microscope settings in the PDMS button assay. Microscope which was in use was Olympus IX71 with Surveyor software.

Objective	10x	**Exposure**	7.161 ms
Filter	1 (no filter)	**Gain**	5.31
Phase constrast	4	**Offset**	-497

3.3 Transfection of the cells

In order to get the plasmids inside the cells and the cells to express the desired construct, transfection was needed. It was done with electroporation which is based on the voltage, time and pulses the machine gives. The reaction requires the cells, the DNA (plasmid) and the Neon Transfection System (Invitrogen).

One transfection reaction was done with 1 million cells and 5 µg of DNA. The DNA was in concentration of 500 ng/µl. The procedure was started with warming dishes containing 10 ml of medium in an incubator. The cells and the plasmids were mixed in a 1.5 ml tube followed by transfer into an electroporation tube where the actual reaction happened. Two types of buffers were also required for the reaction: buffer R and buffer E2.

First the cells were pooled in a tube and the cell amount was measured with Cell Countess. Then the proper amount of the cells (the amount depends on how many reactions were done) were transferred to a 15 ml tube which was then centrifuged (RT, 200g, 5 min). Medium was removed very carefully. The pellet was washed lightly with PBS and the centrifugation was repeated. The supernatant was removed properly. The buffer R was added 100 µl/reaction and the cells were resuspended. Then the resuspended cells were transferred to a transfection tube with the plasmid. The transfection tube already contained the buffer E2. The electroporation was made with the Neon Invitrogen with the optimized settings (see chapter 3.3.4). After that, the cells were transferred to the pre-warmed plates and were left in the 37ºC incubator for 24-48 hours.

3.3.1 Plasmids used in the transfections

The plasmids that were used in this project were full length (FL) Tal1$^{-/-}$ and minitalins 1-4. They all were tagged with mCherry which is a fluorescent protein. This was done so that we were able to see which cells were expressing the plasmids (those which emit the fluorescent dye) and which were not.

3.3.2 Immunostaining of the cells

This step was done after 24-48 hours from the transfection. It is important to note that too long time will kill the cells because nutrients from the medium will eventually run out. The procedure needed coverslips (24x24 mm, thickness: 1.5) to which the cells were transferred. They were washed with 70% ethanol and dH_2O before use to get rid of any dust and grease. They were then left to dry in an air flow. The coverslips were coated with fibronectin (see chapter 3.1.3); 40 µl (25 µg/ml) was added to each coverslip.

The number of cells was calculated with Cell Countess and 100 000 cells were transferred on top of coverslip which was immersed in 2 ml of medium. The cells were then left to spread and migrate over certain amount of time. For most experiments, two (0 h and 24 h) or three (0 h, 12 h and 24 h) time points were used.

After the time point was reached, the medium was taken away and the cells were washed very carefully with PBS. The cells were now ready to be fixed. The fixing was done with 4% paraformaldehyde (PFA) diluted in PBS which was added 2 ml/well and it was left to influence for 10 min at 37ºC. The fixed cells were again washed with PBS but twice this time. The cells could be left in PBS for a couple of days if necessary.

The cells were then permeabilized with 0.2% Triton x100 in PBS buffer for 5 minutes. The buffer had to be heated to room temperature. After permeabilization the samples were blocked for 20 minutes in RT in blocking buffer, which consists of 1% BSA, 5% FBS and 0.05% Triton in PBS. Blocking buffer was also used for dilution of antibodies and for washing between treatments with primary and secondary antibodies.

Antibodies (AB) were used 25 µl/coverslip and incubated in RT for 1 h. After primary antibody the coverslips were washed by dipping them into the blocking buffer, incubating them in 40 µl of the same buffer and repeating that round twice. Secondary antibody could then be added to each coverslip; 25 µl/coverslip and incubation at RT for 1 h. The same wash round was done two times after incubation. The last step was to dip the coverslips in water. The coverslips were then mounted in HardSet Mounting Medium (Vectashield, Cat. No. H-1500) with 1/50 of the DNA stain 4',6-diamidino-2-phenylindole (DAPI); 25 µl/coverslip. Samples were stored at +4˚C covered from light.

3.3.3 Antibodies used in this study

The antibodies used in the study are listed in Table 4. Primary antibody is supposed to interact with the known target molecule while the secondary antibody attaches to the primary antibody. Due to fluorescent labelling the secondary antibody gives the fluorescent color to the fixed cells.

Table 4. Antibodies used in this study. *Praekelt et al. 2012

Antibody	Primary antibody (dilution)	Secondary antibody (dilution)	Color
mCherry	SICG (Sicgen) AB0040-200 dilution (1:100) Goat	Alexafluor-594 dilution (1:500) Chicken-anti-goat	Red
Vinculin	Vin-AB (Sigma, Germany, hVIN-1, #V9131) dilution (1:1000) Mouse	Alexafluor-488 dilution (1:200) Goat-anti-mouse	Green
Talin2	Tal2 68E7 (Cancer Research Techonology)* dilution (1:200) Mouse	Alexafluor-488 dilution (1:500) Goat-anti-mouse	Green

3.3.4 Optimization of the electroporation

The first electroporation which was done in this project showed poor transfection efficiency. Therefore, optimization of the electroporation parameters was performed. The optimization happened in three settings which can be changed in the electroporation machine: voltage, time and amount of the pulses. The first reaction was made with settings of 1200 V, 30 ms and one pulse.

From these tests (Table 5), the second settings seemed to show a little better transfection rate so those were decided to be the settings for the next trials.

3.3.4.1 Evaluating the efficiency of transfection

Transfection efficiency was evaluated by Surveyor microscope by comparing the fluorescence channel and the phase channel. Another method which was used was to

use the fluorescence microscope on fixed and antibody stained cells. The microscope images were taken and the evaluation was based on them.

Table 5. Electroporation settings tested.

Voltage (V)	Time (ms)	Pulses (amount)
1200 V	30 ms	1 pulse
1200 V	40 ms	1 pulse
1350 V	20 ms	2 pulses

3.4 Microscopy

In this study different kinds of microscopes were used. In the optimization of migration assay the phase contrast implemented in the fluorescence microscope (Olympus IX71) was used. It was also used in the trials not involving transfected cells. Fluorescence microscope (AxioImager M2, Zeiss) with AxioCam using 40x oil immersion objective (Zeiss Plan NeoFluar 40x / NA 1.3) (Table 6) or 20x oil immersion objective (Table 7) was used with the transfected cells.

The former microscope contains Surveyor software which functions so that it is possible to set certain points from the sample in advance to the microscope program. This allows taking pictures from the same spots later on and measuring exactly how the cells have migrated towards the scratch area.

3.5 Image analysis

All the images during the research were analyzed by using a program called ImageJ (Rasband W., ImageJ, National Institute of Mental Health, USA). Different functions, such as circularity and threshold, inside the program were used in order to get the required information from the images.

3.5.1 Image analysis in migration assays

In the scratch assay the scratch areas were measured by calculating the widths of the scratches by drawing a line between two cells on the opposite sides of the scratches.

The measurements were taken from six different points from one image (schematically illustrated in Figure 11). The graphs were made by calculating the average widths and standard deviations from every image and adding them to match the time points. This

was done to all the time points, and at the end there should be descending trend line which tells that the cells have migrated towards the scratch area.

In the PDMS picture analysis the PDMS button areas were measured by calculating the size of the whole cell-free area (Figure 12). There should also be a descending trend which tells that the cells migrated towards the middle point of the button area.

Table 6. Fluorescence microscopy (AxioImager M2, Zeiss) with 40x oil immersion objective settings in the spreading assay

Fluorescent dye	Channel	Exposure time	Exposure power	Image panel
mCherry	596	2 s	100 %	7x7
eGFP	488	1 s	100 %	
DAPI	380	250 ms	50 %	

Table 7. Fluorescence microscopy (AxioImager M2, Zeiss) with 20x oil immersion objective settings in the migration assay.

Fluorescent dye	Channel	Exposure time	Exposure power	Image panel
mCherry	596	2 s	100 %	8x8
eGFP	488	1,5 s	100 %	
DAPI	380	250 ms	50 %	

3.5.2 Image analysis in migration assay using transfected Tal1$^{-/-}$ MEFs

The pictures were taken from the fixed cells after 20 hours from the making of the scratch so it was impossible to know anymore where the original scratch line was. The average starting widths of the scratches were thus calculated from the optimization experiments and that was thought to be a valid assumption for initial value.

The ImageJ software counted the number of the cells in the scratch area with a "threshold" and an "analyze particles" command (Figure 13). The transfected cells were counted by hand clicking every transfected cell using the "analyze particles" command.

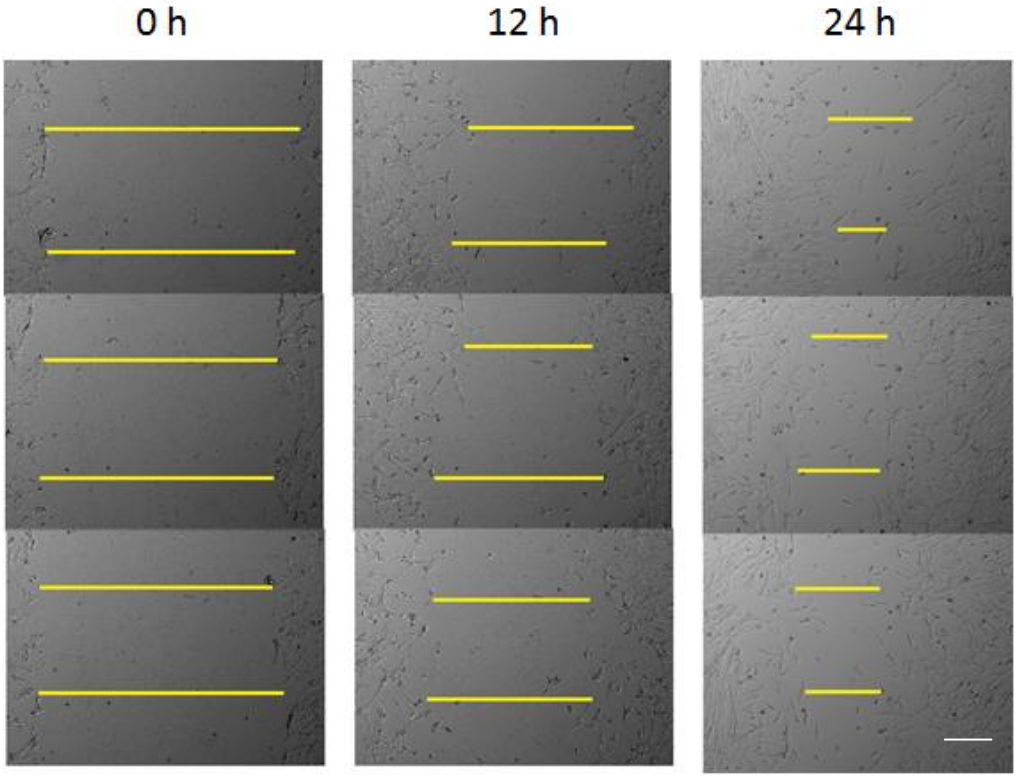

Figure 11. Image demonstrating how the widths from the scratches were measured in all the time points. The points where the widths of the scratches are measured are randomly selected. Scale bar is 200 μm.

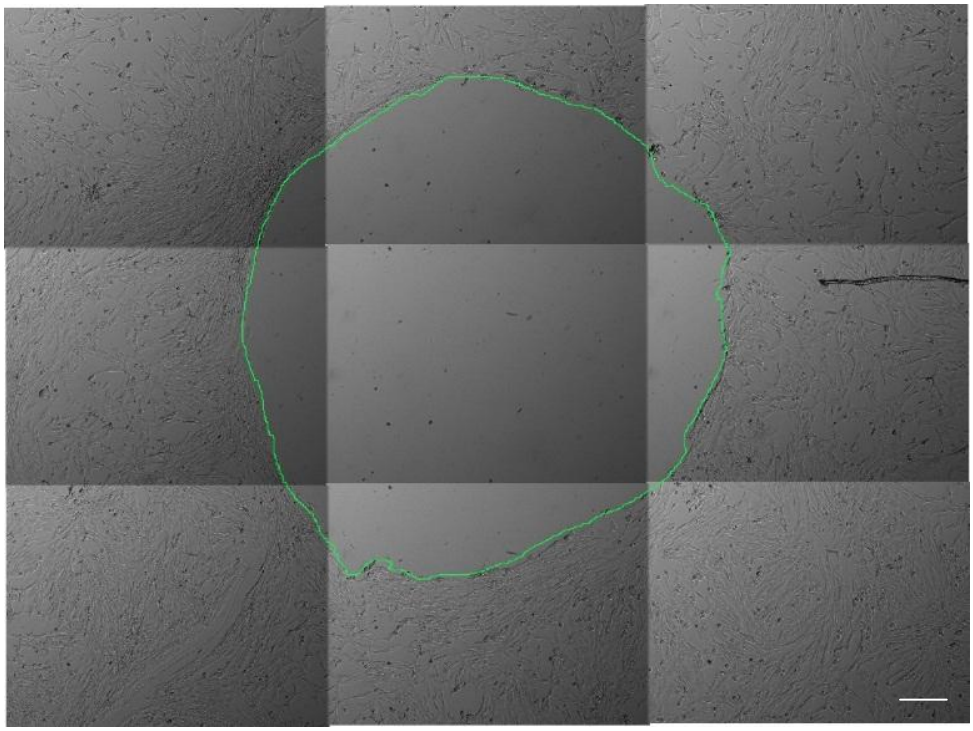

Figure 12. Image demonstrating how the PDMS area was measured. Scale bar is 200 μm.

```
run("Crop");
run("8-bit");
run("Invert");
run("Brightness/Contrast...");
setAutoThreshold("Default");
//run("Threshold...");
run("Analyze Particles...", "size=100-Infinity pixel circularity=0.05-1.00 show=Nothing display
summarize");
```

Figure 13. ImageJ script used for calculating the total amount of the cells. The size was set to start from 100 pixels to avoid cells in the edge of the image.

3.5.3 Image analysis in spreading assay

In spreading assay the cells were fixed at three different time points so that the microscope pictures were also from three different points. The transfected cell outlines were manually drawn and the circularity was measured [1] using ImageJ (Figure 14). From these results the spreading of the cells could be evaluated. It is good to note that only those cells which had one nucleus and were not surrounded by other cells were analyzed so aggregates and cell clusters were left outside the analysis.

ImageJ gave the circularity in the form of 0-1: 1 is completely circular and 0 is highly polarized. The cells were divided into three subclasses; round (circularity = 0.66-1.0), half round (0.33-0.66) and spread (0-0.33).

$$circularity = 4\pi \times \frac{area}{perimeter^2} \quad [1]$$

```
run("Set Scale...", "distance=1 known=1 pixel=1 unit=px");
run("Line Width...", "line=10");
run("Colors...", "foreground=red background=white selection=yellow");
run("Set Measurements...", "area mean min centroid perimeter fit shape redirect=None
decimal=9");
//setTool("freehand");
run("Add to Manager");
roiManager("Add");
roiManager("Select", 0);
roiManager("Split");
roiManager("Delete");
roiManager("Select", newArray(0,1));
roiManager("Draw");run("Input/Output...", "jpeg=85 gif=-1 file=.csv use_file copy_column
copy_row save_column save_row");
String.copyResults();
String.copyResults();
run("Select None");
run("Close");
run("Flatten");
```

Figure 14. ImageJ script used for calculating the circularity of the cells.

4. RESULTS

4.1 Optimization of scratch assay

The first part of this project was to optimize the migration assay. It was done with two methods: scratch assay and PDMS buttons. Three cell lines were used in both methods; Tal1$^{-/-}$ MEF, Vin$^{-/-}$ MEF and WT MEF cells.

The first experiment was done only with the WT MEF cells. The parameters to optimize were the amount of cells and the pipette tip size to be used. We also wanted to know if the pre-selected time points (0 h, 12 h and 24 h) were chosen adequately. The first experiment was done with 400 000 and 200 000 cells in one well (Figure 15) and pipette tip sizes were 10 µl, 200 µl and 1000 µl (Figure 16). The cells were left to grow for 22-24 hours in the medium. The optimal cell amount is important because the nutrients in the medium will eventually run out, but also it is important so that there are enough cells to be able to see a cell line without overcrowding the well. 200 000 cells showed to be too few because when the scratches were made there were no clear cell lines so it would be quite hard to measure how fast the cells invade the scratch. 400 000 cells on the other hand showed to be a quite good amount; the boundary between populated and empty areas was clearly visible, but the well was not too crowded.

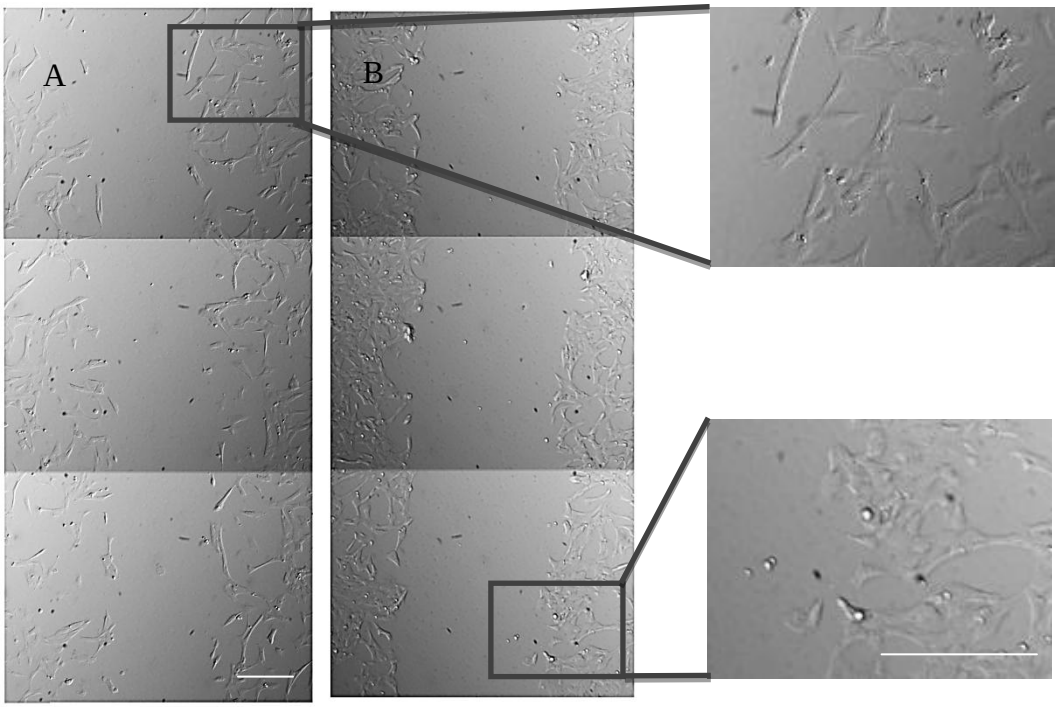

Figure 15. Scratches in plates of 200 000 cells (A) and 400 000 cells (B). The edge of the area containing attached cells is better visible in a well where $4x10^5$ cells were plated as compared to a well with $2x10^5$ cells. Scale bars are 200 µm.

The second variable was the pipette tip size. In order to get rational time points the width of the scratch should not be too small because then the cells would invade the scratch area too fast.

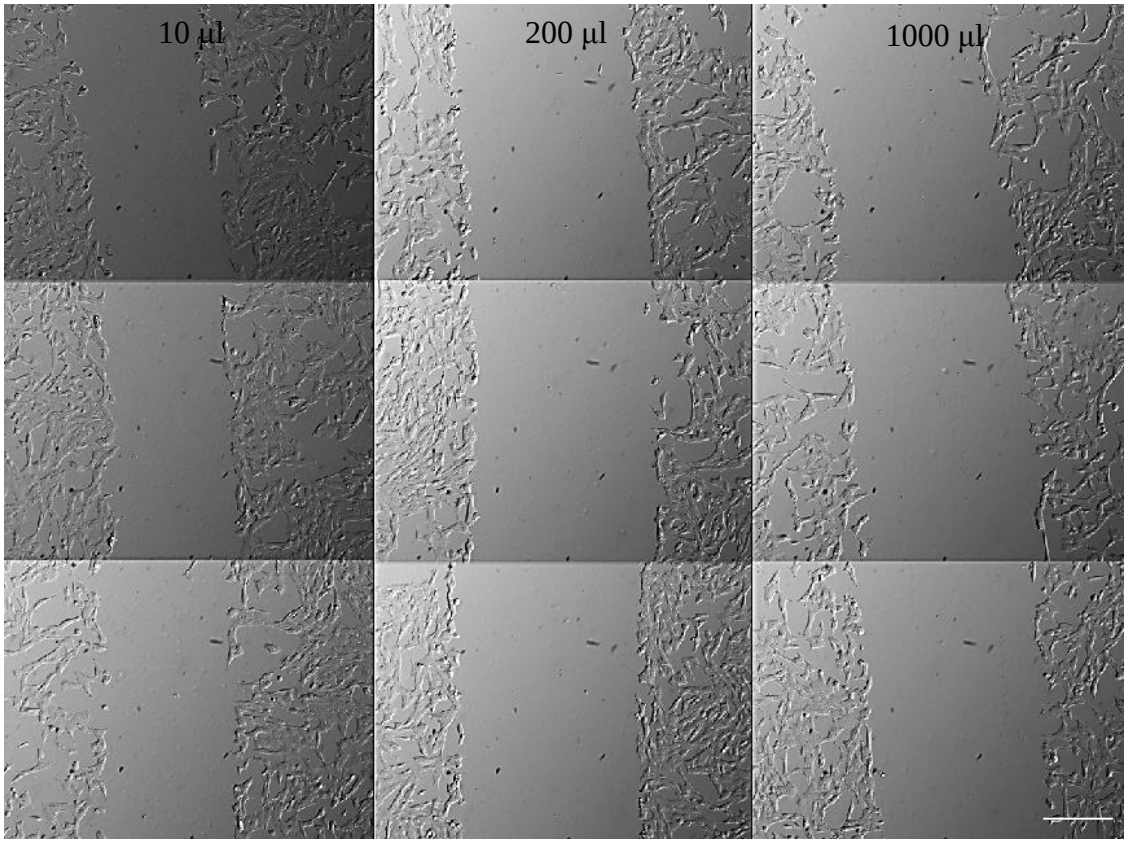

Figure 16. Scratches made with different pipette tip sizes. Left scratch is made with 10 µl, the middle scratch is made with 200 µl and the right scratch is made with 1000 µl micropipette tips. Scale bar is 200 µm.

The scratch width made with 10 µl tip was found to be too small so that after 24 hours the scratch had been completely vanished. The scratch width made with 1000 µl tip on the other hand was good in size but when comparing the width with the scratch made with 200 µl tip the widths were almost the same. That might be because it was harder to keep the right angle between the surface and tip with the 1000 µl because it was quite large, so it was easier to make the scratch with the tip size of 200 µl. Due to these facts we ended up using the 200 µl pipette tips.

Starvation media was also used along with normal media to test if it is possible to get a situation where the cells would migrate but not divide. An issue occurred during the tests; the cell amounts should be larger in order to get good confluence in the proper amount of time, so we decided not to use the starvation media.

4.1.1 Scratch assay used to study the migration of Tal1$^{-/-}$ MEF, Vin$^{-/-}$ MEF and WT MEF cells

After the optimization part, it was concluded that the cell amount to be plated is 400 000 cells/well and the pipette tip size should be 200 µl. The time points were also set up to be 0 h, 12 h and 24 h.

The next step was to study if there are any differences in the migration between all three cell lines (Figure 18). They were all plated, and after making the scratch, the plates were imaged and the images were analyzed.

WT MEF cells showed a clearly faster migration rate than Vin$^{-/-}$ and Tal1$^{-/-}$ cells (Figure 17) although Vin$^{-/-}$ cells and Tal1$^{-/-}$ showed also a clear migration. P-values comparing WT MEF cells between Tal1$^{-/-}$ or Vin$^{-/-}$ revealed a statistical significance in the difference in their migration rates.

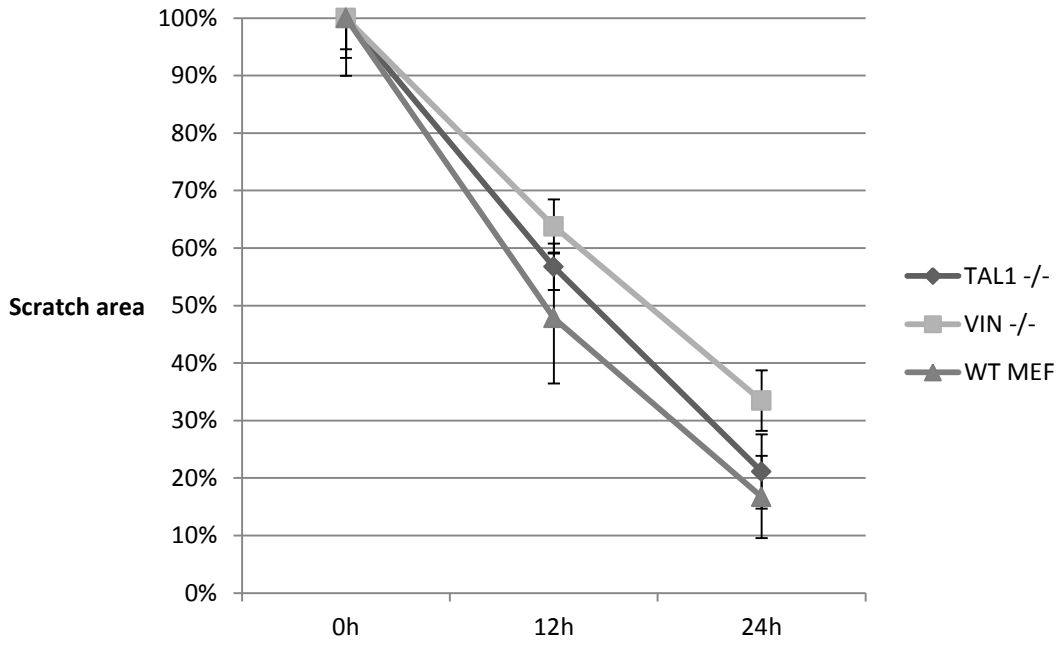

Figure 17. State of migration in time points 0 h, 12 h and 24 h. The widths of the scratches are normalized in between the experiments. 100% indicates the width of the scratch in the beginning of the experiment. The 0 h, 12 h and 24 h are the time points when the images were taken. Analysis with t-test showed the WT MEFs migrating faster than Tal1$^{-/-}$ cells (12 h, $p < 0.001$; 24 h, $p < 0.05$). Analogously, Vin$^{-/-}$ cells and WT MEF cells were found to have p-values (12 h and 24 h) < 0.001.

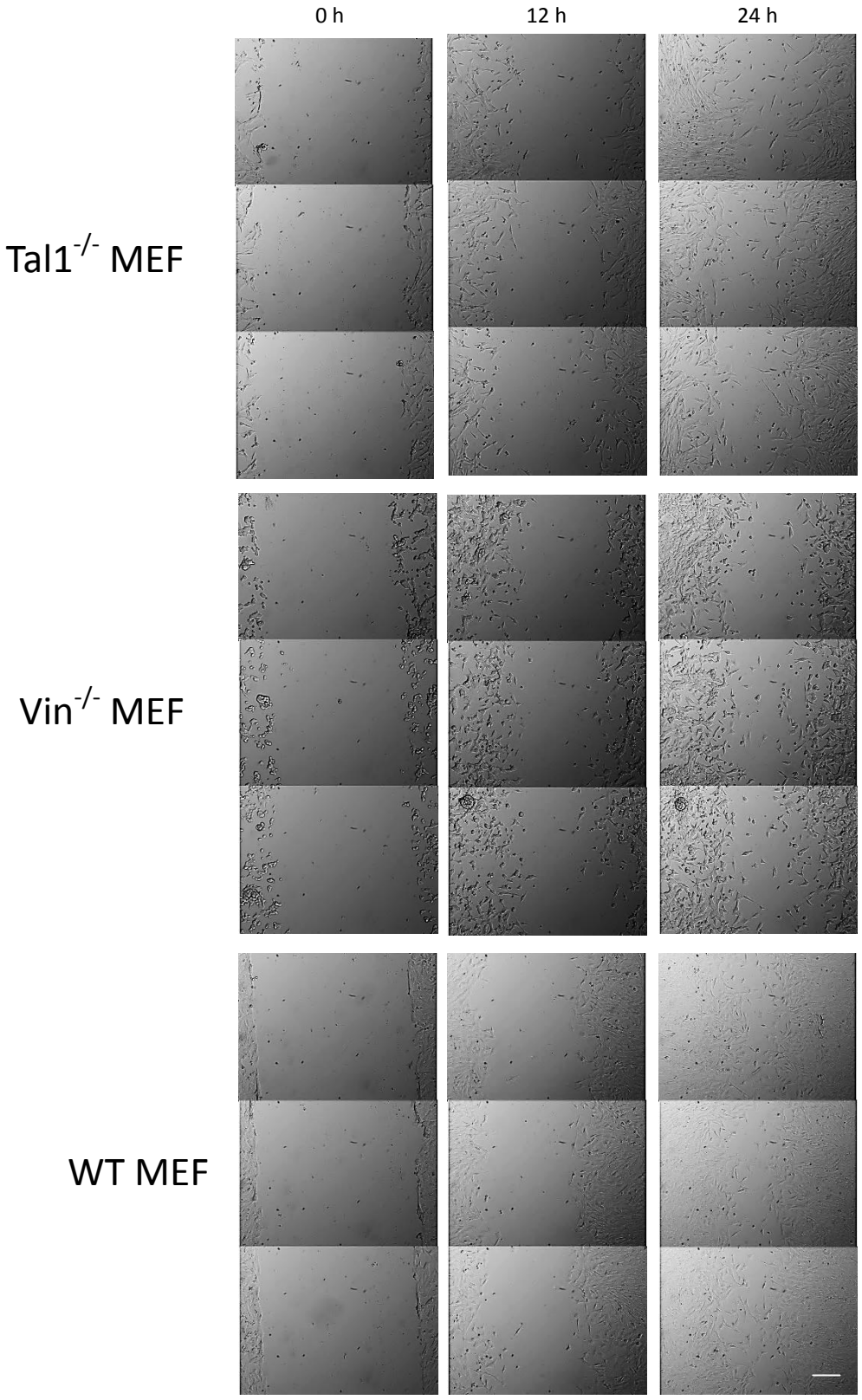

Figure 18. Migration assay for cell spreading kinetics analysis. All three cell lines and microscope images from time points 0 h, 12 h and 24 h are shown**.** The widths of the scratches are getting smaller during the time. Phase contrast microscopy (Olympus IX71) was used to take the images. Scale bar is 200 μm.

4.1.2 PDMS buttons used to study the migration of Tal1$^{-/-}$ MEF, Vin$^{-/-}$ MEF and WT MEF cells

PDMS buttons were the other method used to study the migration of the cells. It was used to see if it is a more compatible and easily repeatable method compared to the scratch assay. PDMS tests were made with all the three cell lines. Time points in this method were set up similarly to those in the scratch assay; 0 h, 12 h and 24 h.

WT MEF showed to migrate faster than Tal1$^{-/-}$ and Vin$^{-/-}$ cells ($p < 0.001$) (Figure 19) in this method also. The differences between Vin$^{-/-}$ and Tal1$^{-/-}$ were quite big even when the error bars are taken in and statistical analysis indicated $p < 0.001$. According to the graph below, the differences seem to be quite good between the cell lines and might include a statistical significance, but the problem was that the results were different in all the three times this assay was performed (data not shown in this thesis) compared to the scratch assay where the results were the same.

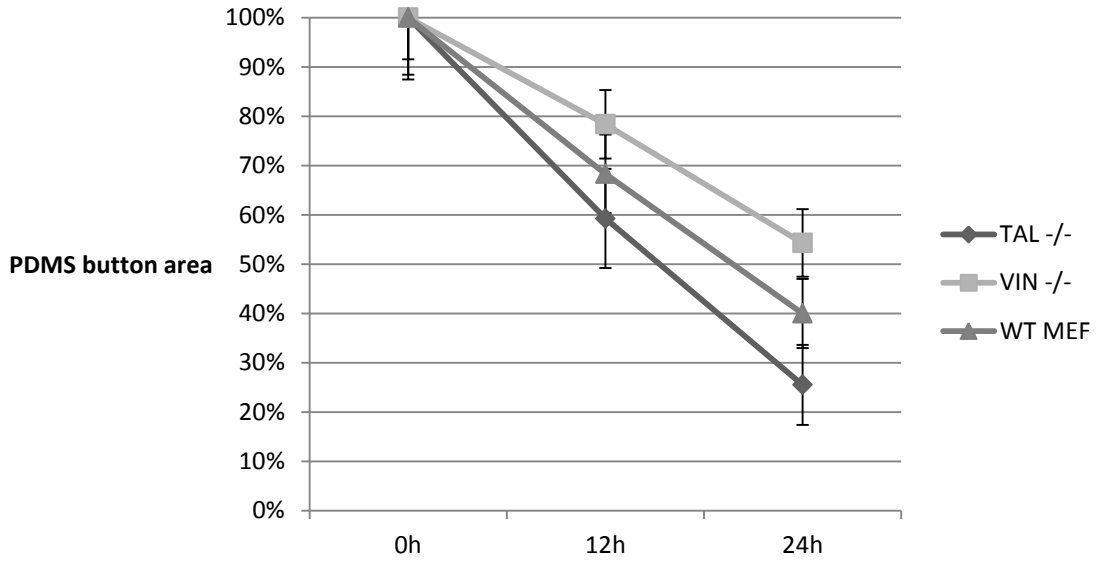

Figure 19. State of migration speed in time points 0 h, 12 h and 24 h. 100% indicates the area of the PDMS button and the areas measured are normalized according to the starting area. The graph tells how much of the area the cells have occupied as compared to the starting area of the PDMS button. Analysis with t-test showed the p-values for all the cell lines compared to each other to be < 0.001 so there is a statistical significance between the cell lines.

The method using PDMS buttons was not chosen for the future studies because it appeared not to be as repeatable as the scratch assay and also because the scratch assay is a very commonly used method in migration studies.

4.2 Migration study using cells expressing mCherry-tagged talin1 and truncated talin1 proteins

The actual migration study was done with Tal1$^{-/-}$ MEF cells by transfecting them with plasmids encoding minitalin and FL (full length) talin. 3x10^6 cells were transferred to a coverslip after about 24-48 hours from the transfection and they were left to grow on a coverslip for about 24 hours. Then the scratches were made to each coverslip. The cells were fixed after 20 hours of spreading and they were antibody stained with mCherry (produced in goat) (color: red) and talin2 (produced in mouse) (color: green) primary antibodies and Alexafluor-594 (anti-goat produced in chicken) and Alexafluor-488 (anti-mouse produced in goat) as secondary antibodies. The VectaShield included DAPI dye so the third color (blue) indicates all the nuclei.

Two scratches were made on each coverslip and 2-4 microscope images were taken from each of the scratches after 24 hours of incubation (Figure 21). One image was also taken from an area outside the scratch. The number of the cells in the scratch area was calculated and the results were compared with the value obtained from the outside of the scratch area.

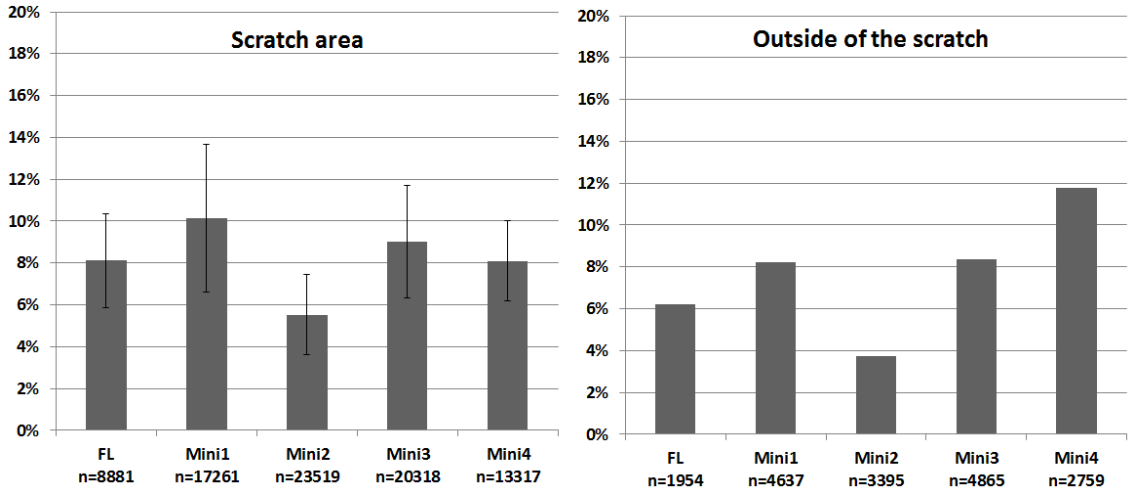

Figure 20. Transfected cells in the scratch area (left graph) and transfected cells outside of the scratch area (right graph) after 24 hours of migration. The bars indicate the number of the transfected cells which can also be considered as a measure of the transfection efficiency. n = the total number of the cells in all microscope images analyzed. Standard deviations were not used in the right graph because only one image was taken from the area outside of the scratch.

The transfection efficiency was about 10% or less (Figure 20) in all the cases. Minitalin2 sample showed to have fewer transfected cells than the other samples so the

reason for small number of cells in the scratch area might be due to a poor transfection rather than due to a slow migration.

Figure 22 shows a comparison of the number of transfected cells in the scratch versus outside of the scratch and indicates that all the constructs except minitalin4 appeared to have transfected cells concentrated in the scratch area. In the case of minitalin3 the preference was minimal.

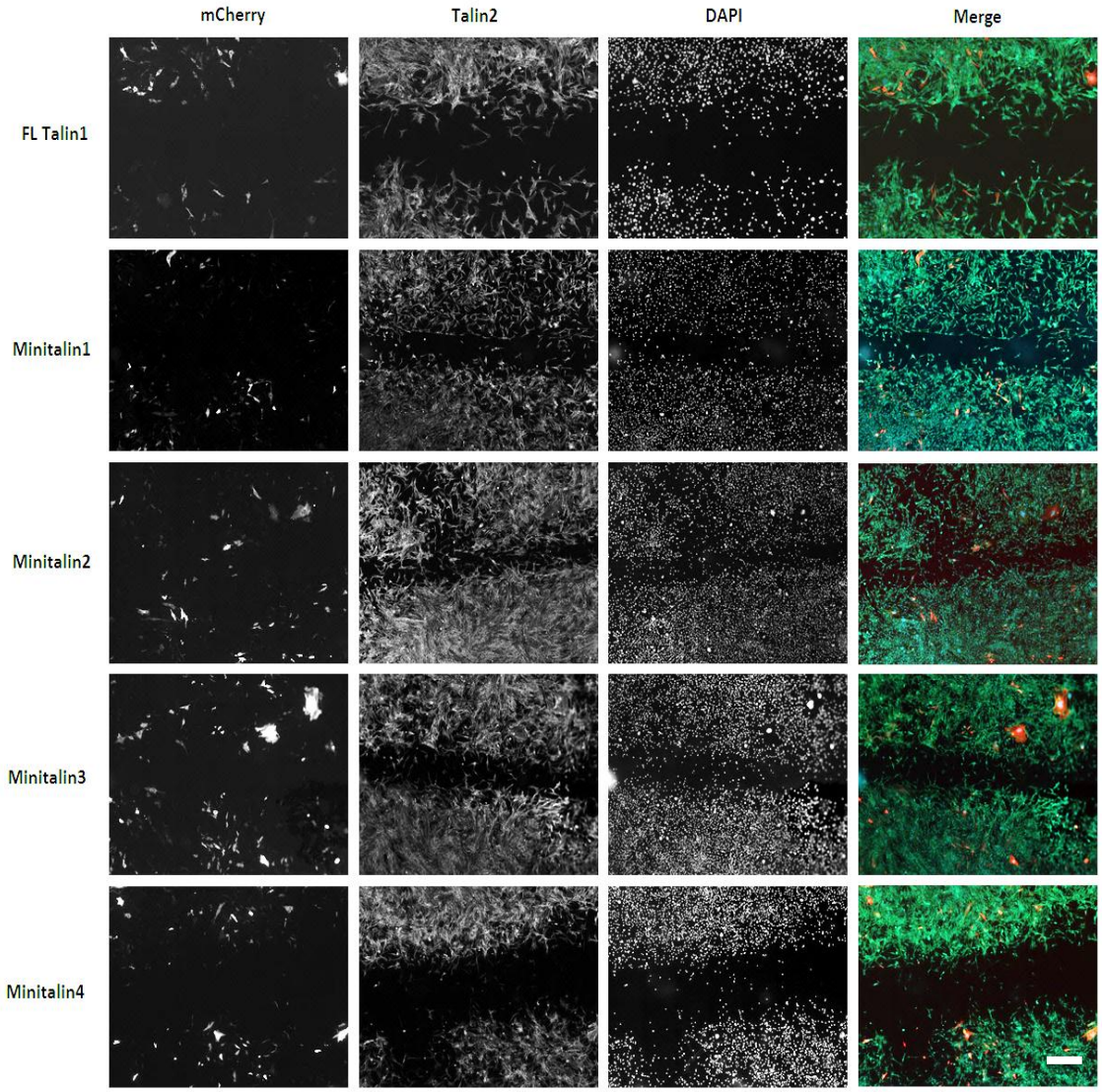

Figure 21. Immunofluorescence staining of the Tal1$^{-/-}$ cells transfected with different plasmids. Fluorescence microscopy (AxioImager M2, Zeiss) with AxioCam using 40x oil immersion objective (Zeiss Plan NeoFluar 40x / NA 1.3) was used to image these cells. Three different channels were also used: mCherry (red), vinculin (green) and DAPI (blue). The line in the middle of the images is the scratch with is closing due to the migration of the cells. Time point for the fixing was 20 hours. Scale bars is 100 μm.

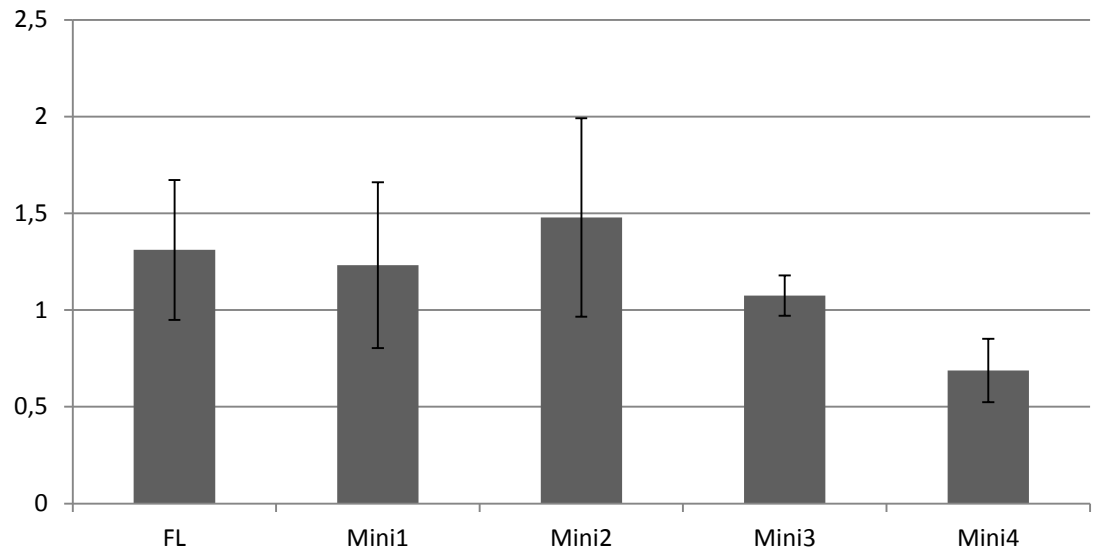

Figure 22. Fold change comparing the number of transfected cells in the scratch area to the number of transfected cells outside the scratch after 24 hours of migration.

4.3 Spreading assay using minitalin and full length talin plasmids

The spreading assay was done also with Tal1$^{-/-}$ MEF cells by transfecting them with plasmids expressing for minitalin and FL talin. In this case $3x10^6$ (20 min and 2 h time points) or $2x10^6$ (20 h time point) cells were transferred to a coverslip 24-48 hours after transfection. The cells were fixed at time points of 20 minutes, 2 hours and 20 hours. The cells were then antibody stained with mCherry and Talin2 primary antibodies and Alexafluor-594 and Alexafluor-488 as secondary antibodies. The blue color (nuclei) from DAPI in the VectaShield mounting medium also appears at this time.

Cell spreading was measured by drawing the cell outlines by hand and by using ImageJ to calculate the circularity (values from 0 to 1). The results were divided into three subclasses; spread, half round and round.

At the 20 minute time point most of the cells seemed to still be round (Figure 23 and 24). FL, minitalin1 and minitalin2 showed a little spreading. At 2 hours timepoint all the transfected cells showed signs of spreading and cells transfected with minitalin2 and minitalin3 plasmids showed more spreading than the others (Figure 24). Samples transfected with minitalin3 and minitalin4 showed approximately the same amount of the spread cells (40%) at 2 h but in the time point at 20 hours a difference was observed; minitalin3-transfected cells had spread faster than those cells expressing minitalin4 (30% compared to 55%). 25% to 55% of the cells were spread after 20 hours and only

2% to 15% of the cells were round after 20 hours. Therefore, the number of spread cells between the samples expressing different talin forms was more divergent than the number of the round cells.

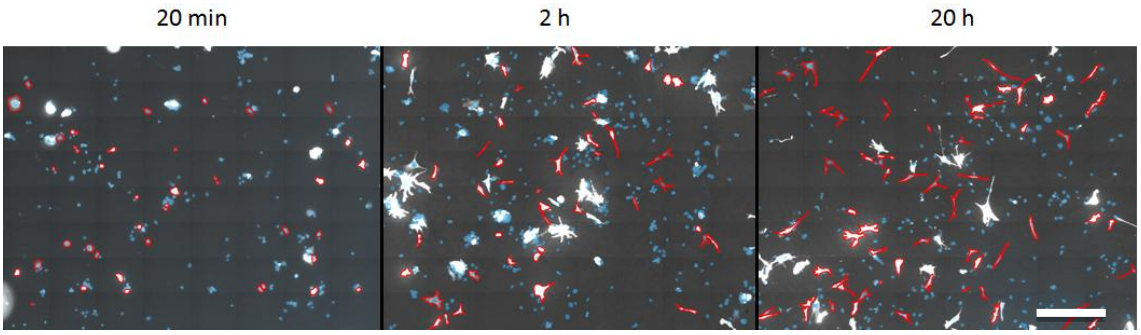

Figure 23. Cell spreading images in time points of 20 min, 2 h and 20 h. Transfected cell lines (those which are counted in) are marked with red color. Scale bars is 50µm.

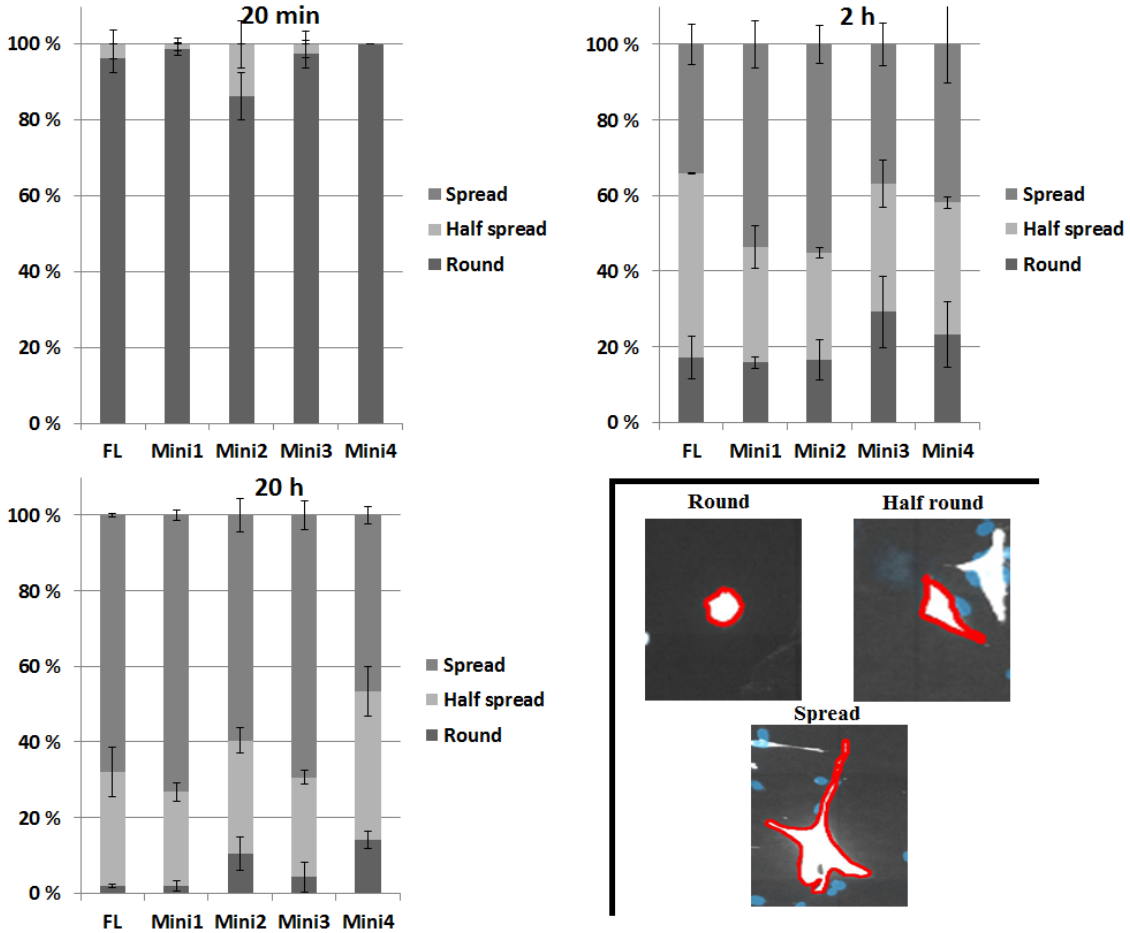

Figure 24. Graphs illustrating cell spreading in different time points. The spreading area of each cell was defined by drawing it manually and the circularity was measured by using ImageJ. The cells were divided into three subclasses (spread, half spread and round). In the right lower corner there are examples of the cells from those three categories shown.

5. DISCUSSION

Comparative analysis between two migration assays was performed in this study: scratch assay and PDMS button assay were evaluated for the performance and repeatability. Spreading assay was also performed in this study. The ultimate goal of the study was to evaluate the migration and spreading rates between cells expressing different talin1 constructs (FL talin1 and four minitalins).

5.1 Optimization of migration assay

Our results from the optimization of the scratch assay showed that the migration assay done this way is easily repeatable and image analysis can be done easily. The protocol for this method was taken from Yanling Chen (2011) though only steps 1-8 were performed. In the first part of the study we tested different methods for the preparation of the cell-free area and tried also different media to test whether it is possible to block the cell proliferation during the migration analysis. The starvation medium, containing 0.5% BSA, appeared to be not as good as we thought because it made the cells migrate slower and it did not actually completely stop the cell division. We decided to reject the starvation medium and continue only with the normal medium which included all of the necessary nutrients.

The migration assay continued with the selected equipment for testing if there is any difference in the migration between WT MEF, Tal1$^{-/-}$ MEF and Vin$^{-/-}$ MEF cells. Xu et al. (1998) published that vinculin knockout cells show faster migration than their wild type counterparts. In addition, vinculin is not necessary for the formation of FAs but it will stabilize them, and Goult et al. (2010) published that overexpression of vinculin might lead to a suppression of the migration. These two studies support each other, suggesting inhibitory role for vinculin in cell migration. It appeared that we, however, got results where the WT MEF cells migrated fastest out of the three cell lines used. WT MEF cells express all the necessary proteins in order to form stable focal adhesions with the ECM unlike the Tal1$^{-/-}$ MEF and Vin$^{-/-}$ MEF cells, which are lacking talin1 or vinculin, respectively. It is known from previous studies (Albiges-Rizo et al. 1995, Lee et al. 2007) that the lack of talin has an impact on formation of focal adhesions and that the cells will show much lower degrees of migration and cell spreading. Vinculin in turn is a protein which stabilizes adhesions and contributes to the linkage between talin and

F-actin. Therefore, the lack of vinculin might influence the adhesion turnover leading to faster adhesion formation and disassembly which again might lead to a faster migration. Therefore we were surprised to see that $Vin^{-/-}$ and $Tal1^{-/-}$ MEF cells migrated slower than the WT MEF cells. As mentioned above, cells lacking talin1 are unable to form normal talin1 consisting focal adhesions but the question is, why did the $Tal1^{-/-}$ cells show migration at all? One possible answer is talin2 protein which compensates for the lack of talin1. Talins have quite similar properties although it has been shown that the lack of talin2 does not have as much of an impact on the focal adhesions as talin1 (Praekelt et al. 2012). This supports our results that migration could still occur although talin1 was not present. Praekelt et al. (2012) also showed that talin1 was expressed in the edge of the cells whereas talin2 was more commonly expressed in the middle region of the cells.

P-values for the differences between averaged WT MEF and $Tal1^{-/-}$ cell migration states were < 0.001 (12 h) and < 0.05 (24 h) so there is significant difference in the migration between those two cell lines. P-values for the WT MEF and $Vin^{-/-}$ were both < 0.001 so there is also a significant migration difference between those cell lines. Another migration assay which was also performed (graph is not illustrated in this thesis) gave the p-value of < 0.001 for all the cell lines.

PDMS buttons were also tested in migration assay to see if they fit better for this assay. The buttons were made just for this trial in our own laboratory and they were easy to prepare, did not consume too much time and were inexpensive. Establishment of button-based assay required only a little more work than that of the scratch assay but was still reasonable. It was also thought that this method would be more repeatable due to size of the button which would always be the same on the contrary to the width of the scratch which can change due to so many variables. The assay was repeated a few times but the results were inconsistent (data not shown in this thesis) every time. On that account we concluded that PDMS assay is not repeatable with these arrangements. It should be also noted that the two figures (Figure 11 and Figure 12) should not be compared directly to each other. In case of the scratch assay (Figure 11), the width of the scratch is reported. In contrast, the PDMS button analysis (Figure 12) reports how much of the button area has been occupied by the migrating cells. Therefore, the results should be evaluated only within one assay type.

The t-test analysis showed p-values for the differences between averaged WT MEFs and Tal1$^{-/-}$ in the PDMS button analysis to be <0.001 in both time points. The p-values for the differences between WT MEF and Vin$^{-/-}$ were also < 0.001 in both time points. The assay was repeated (graph is not illustrated in this thesis) and the p-values were also < 0.001 at this time.

PDMS buttons were easy to use and showed promising results at first. However, this wasn't enough for us to choose this method for the future experiments. A slight problem occurred during this method which could explain why the results were different every time. When the cells were plated and they started to reach the confluence, they migrated and randomly collided with the PDMS button. This changed their direction of migration. Then, when the PDMS buttons were removed and the cells should have started to migrate towards the button area, we observed that they had formed kind of a wall which slowed down the migration. It thus took certain time for the cells to change their direction again. It is also possible that the cells coming from behind started to migrate across the other cells. Either way, this was a problem when studying the migration by this method. Another reason why we rejected this method was the situation when cells had migrated towards the center and they were starting to lack space. The cells start to run into each other which could eventually disrupt the free migration and results may be distorted. For these reasons and also because the scratch assay is very commonly used method in migration research this method was not selected for further studies.

5.2 Optimization of transfection

There were adversities during the research in the transfection and more precisely in its efficiency. This was however a crucial step in order to gain any information about differences between different talin1 forms. The first experiment that was done to test the transfection gave almost no transfected cells. With optimization, we managed to find settings enabling transfection efficiency in range of 10% (Figure 20). Earlier experiments (results not shown) had indicated that Tal1$^{-/-}$ MEF cells would not enable excellent transfection efficiency, so we agreed that 10% would adequate in order to continue for further studies (Table 5).

The transfected plasmids differed in size, FL talin1 was the longest (12336 bp) and the minitalin4 was the shortest (6143 bp). It is obvious that it could influence the efficiency of the transfection because the FL plasmid could have been too large for the cells to effectively take it in. In addition to this there are other possible factors influencing the transfection efficacy such as the plasmid purification method used.

There are also other ways to work with cells which are expressing the desired proteins such as stable cell lines. This means of creating cell lines which are stable and which continuously express the protein construct of interest. Although this could help to overcome the challenges in the transfection, it would have been too time consuming to produce them first.

5.3 Migration assay with transfected Tal1$^{-/-}$ MEF cells

Albegies-Rizo et al. (1995) published that cells where talin was down-regulated formed adhesions slower than their wild type counterparts. Instead of measuring the adhesion speed we measured if there were more transfected cells in the migration area (the scratch area) or in the so-called static area (area outside of the scratch). This indeed tells if the transfected cells are able to migrate more or less as compared to their nontransfected counterparts.

In the actual research we tested if there was any difference in the migration velocity in between cells transfected with different talin1 constructs. In addition, the speading of the transfected cells were evaluated. All the talin forms studied here were tagged with fluorescence marker mCherry which would help us to see which cells have started to express the proteins encoded by the plasmid. Our hypothesis was that the FL talin1 would show perhaps faster migration than the other constructs due to the fact it still contains all the necessary domains and the proteins were also intact. Minitalins on the other hand are lacking some ligand-binding sites so that the fourth minitalin has only its head domain and therefore is missing all the vinculin binding sites and also the integrin and F-actin binding sites located in the rod domain. It was estimated that the cells expressing minitalin4 would probably form fewer adhesions than the other constructs. However, it was hard to predict how adhesion formation would correlate with migration velocity.

Vinculin is not necessary for the formation of FAs but it stabilizes them. In addition, the talin-vinculin ratio in an ideal focal adhesion complex would be 1:5 or even 1:10 (Critchley 2009). Therefore, when vinculin is present it might lead to a suppression of the migration like Goult et al. published in 2010. This on the other hand suggests that the minitalin4-expressing cells would not necessarily be the slowest one to migrate although vinculin is present, because the cell is incapable of using it. The fact that minitalin4 cells are unable to use vinculin by the talin1 could eventually disturb the cell migration less than in cases of minitalin2 and minitalin3 where the vinculin is in use but not normally.

The results obtained with transfected Tal1$^{-/-}$ cells from the migration assay were that the samples treated with FL talin1 and minitalin1 and 2 appeared to have more transfected cells in the scratch area than outside of the scratch, although the large error bars should be taken into good consideration. Minitalin4-treated sample in turn showed clearly fewer transfected cells in the scratch area. This supports our hypothesis that the minitalin4-expressing cells would migrate less than the FL Tal1 –expressing cells.

It would have been advantageous if the talin1 plasmid transfected in the cells would be the only talin gene inside the cells. As previously mentioned the TLN2 gene was still present in the genome so we should not think about the results as being obtained only with the transfected plasmid. Talin1 and talin2 have a lot of sequence similarity so it is obvious that they participate in the same events. Praekelt et al. (2012) published that they both take part in the migration but occur in different parts of the cells. Talin1 is more commonly in the edge region whereas talin2 exists in the middle regions of the cells. Therefore, the significance of talin1 in the cell migration is greater than talin2. Although, in case of Tal1$^{-/-}$ cells there might occur significance chances in the role of talin2.

We measured the migration in a whole cell population. Another way would have been to study individual cells. This would have required a microscopy technique to track desired cells in real time. This way the measurement would have been different; we could have measured the speed of migration for individual cells but that would have required a lot of data in order to generalize the results for the whole population. The advantage for this is that the poor transfection efficiency would not have been such an issue as it was now when we evaluated the whole cell population.

5.4 Spreading assay with transfected Tal1$^{-/-}$ MEF cells

The role of talin in the cells has been studied by down-regulating it. Albiges-Rizo et al. (1995) published that spreading was reduced and the focal contacts were smaller when the talin was down-regulated. In the spreading assay the hypothesis was the same as in the migration assay; FL talin1 –expressing cells would spread more and perhaps faster than the cells expressing minitalins, especially the minitalin4. Here we did measure how fast the cells were spreading by measuring the size and shape of the cells in three time points. In the time point of 20 minutes the FL talin1 and also somehow surprisingly the minitalin2 construct had started to show some spreading already. Of course it is notable that 20 minutes after the cells were transferred onto a coverslip they were still quite fragile because the attachment to the ECM may have not completely occurred yet. When the medium was taken away and the fixing solution PFA was added, there was a risk that the cells might detach from the bottom, so this step required extra caution and had to be done very slowly. Even with these precautions, the risk of the detachment was not eliminated.

Talin can form an autoinhibited form by interaction between its head and tail domains. Because minitalins 1-3 contain fewer tail bundles and mintalin4 does not contain any bundles, their ability to form the autoinhibtion conformation may be disturbed. This might lead to an early integrin activation which can explain why the minitalin2 started to show some spreading already after 20 minutes. On the other hand, the lack of tail bundles might complicate the formation of stable adhesions. In the second time point, 2 hours, the cells should have formed more stable adhesions and, due to that, the risk for the cells detaching from the plate was not high anymore during PFA-medium exchange. In 2 hours all the constructs had started to spread and in the classification we used, all kinds of spread cells were found. Minitalin2 appeared to still be in the lead although it was a quite minor lead. FL talin1 was actually showing fewer cells in the spread subclass than the others. In 20 hours the cells transfected with FL talin1 construct has caught up and bypassed the others and had almost no round cells. The minitalin4-transfected cells appeared to have fewest spread cells and also the most round cells. Still the major components in every construct were the spread cells. This could be due the fact that the FL construct is able to form stable adhesions but the minitalins are possibly active only at the beginning of the cellular spreading process, leading to a good start for the spreading but not ending up as spread as in the case of the full length talin. If

considering only the last time point our hypothesis was right: FL talin1 –expressing cells had spread more than the others and the minitalin4-expressing cells less than the others. Minitalin4-transfected cells also appeared to show no change in the spread form between the last two time points. The reason might be that there is nothing but the talin2 to stabilize the adhesions so the cells just randomly form adhesions and then disassemble them. The cells still express the talin2 and it takes part in the formation of adhesions. In the case of minitalin4, it might be that the minitalin is competing with talin2 for the binding to integrin. This is supported by the publication from Lee et al. (2008) who stated that cells lacking vinculin showed to have unstable and easily breakable adhesions.

Zhang et al. (2008) reported that talin2 protein did not affect the initiation of the spreading in the talin1-null cells but instead the initiation of the spreading was driven by the integrin and F-actin. Therefore they stated that the talin is not necessary for the initiation of the cell spreading. In addition to the initiation, the spreading occurred normally when the talin2 was present and this could be due to the possibility that talin2 protein expression might have increased. They also reported that during Tal2-shRNA treatment, the cells began to become round after 48 hours. Since our research ended after only 20 hours, a longer experimental time might be a good step for the future plans. It is good to note that they used Tal2 shRNA in their experiment while we did not, so our results and their results should not be directly compared to each other. They also published that cells lacking both talin1 and talin2 became round after 28 minutes, so it is clear that our cells contained talin2 because there were no sign of the cells becoming round again, at least not in 20 hours.

Although the minitalin4 did not show any significant increase in the number of spread cells between 2 hours and 20 hours, at least the number of round-shaped cells decreased (Figure 24). Zhang et al. has described the role of vinculin in the adhesions when the cell was not expressing the talin. They did not observe the vinculin in the FA sites but they saw it localizing in the cytoplasm. This is supported by the role of vinculin which functions to link talin to F-actin. When there is no talin there is no need for vinculin. All combined, they discovered that talin depletion caused loss of FA and also cell rounding at later times, but the lack of talin did not however prevent or delay the initiation of the spreading. This is supported by the publication of Elliott et al. (2010) who used human umbilical vein endothelial cells (HUVECs) which are normally already lacking talin2.

They published that lack of both talins showed reduced spreading and FA assembly and only fewer than 50 % of those cells were spread after 24 hours. They used cells expressing talin proteins with mutated F1 and F2 domains (located in the head) and discovered that both mutants were unable to form FAs. This means that the F1 and F2 subdomains are essential in focal adhesion assembly and the head domain generally is necessary. Our cells were expressing the head domain so this explains why the FAs were assembled quite quickly. Another reason for the quick assembly could be provided by the fact that minitalins are smaller than the full length talin and they might not be that flexible to form the autoinhibited conformation. That leads to the assumption that the active head domain is greatly present during the initiation of the adhesions.

In addition, we could have identified the focal adhesion sites and how fast they assemble and again disassemble. Berginski et al. (2011) have developed a method to analyze the FA sites and how they change during time. They used 3T3 fibroblasts with Total Internal Reflection Fluorescence Microscopy (TIRF). They measured size, shape, intensity, and position of living cells. With this method it is also possible to focus on the regulation of FAs and to see how the different talin1 constructs affect the migration and spreading more precisely on the macromolecular level. It is hard to say if the individual cell tracking could be more reasonable in this case but it would be interesting to do.

5.5 Cell culture substrates used in this study

This study, as with most *in vitro* research, was performed in two-dimensional (2D) substrate although the natural environment of cells is a three-dimensional (3D) substrate. Because of this, it would be favorable to set the equipment as in a 3D model but the techniques will need a little more improvement before this can be done.

The majority of the research was done on one type of substrate; fibronectin coated coverslips. The only exception was the optimization, which was done in a 6-well plate without any coating. The optimization could have also been done to a coated plate, although the point was to optimize the method and make it repeatable so coating was not necessary at this point. Cells are known to form adhesions differently depending on the substrate (coating and ECM material) and therefore other substrates such as collagen-I and vitronectin could have also been used. In addition to the ECM materials in use, there are several other parameters available for modulation. For example, Engler

et al. (2004) showed in their publication how the stiffness of the substrate influences the spreading of myoblasts. Although the plates and the coating used differ significantly from the physiological environment, the results still give a good assumption from the cell behavior *in vivo*.

6. CONCLUSIONS

The purpose of this study was to optimize a migration assay and utilize it to study migration between WT MEF, Tal1$^{-/-}$ and Vin$^{-/-}$ cells. In the second part of the research the assay was used on Tal1$^{-/-}$ cells transfected with five different talin1 constructs. The third part comprised of a cell spreading assay with the same transfected Tal1$^{-/-}$ cells. It was essential to set up a protocol for the migration in order to get reliable and repeatable results.

The study presents how focal adhesions can be researched regarding cell migration and spreading. It also gives a good example on of how various proteins and protein parts affect forming adhesion sites in ever-changing situations in the cells. Cell migration was studied with a method called scratch assay and it was performed with talin1-null cells transfected with five different talin1 constructs. The same cell constructs were also used for the spreading assay experiments. Most of the parts in this study required optimization which therefore enables us to use the proven and suitable conditions in the future experiments as well. Some issues still require more optimization but the protocols improved here provide a good stepping stone for future studies. At the same time, many questions arose during the research, such as the migration results of Vin$^{-/-}$ cells, especially why they migrated slower than their wild type counterparts.

The results we obtained from this study give excellent future prospective opportunities for the studies focusing on focal adhesions, especially in the fields of cell migration and spreading. Due to the methodology represented, the study enables the use of mutated focal adhesion proteins while it offers the study of locating adhesion proteins in the adhesion sites.

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