universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„The role of podoplanin in cutaneous wound healing “

Verfasser

Florian Koban

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A441
Studienrichtung lt. Studienblatt:	Genetik und Mikrobiologie
Betreuerin / Betreuer:	Ao.Univ.Prof.Dr. Pavel Kovarik

Danksagung

All denjenigen gebührend zu danken, welche mir bei dieser Diplomarbeit geholfen haben hätte einen größeren Umfang als die vorliegende Arbeit selbst.
Zuallererst möchte ich Prof. Johannes Breuss und Prof. Pavel Uhrin danken, welche mir die Gelegenheit gegeben haben an diesem Projekt zu arbeiten und mich mit endloser Geduld betreut haben.

Ebenfalls gebührt mein Dank Nikolina Papac Milicevic und Judit Mihaly-Bison, die mich ermutigt, kritisiert und in meiner Arbeit vorangetrieben haben. Ich danke Revu Ann Alexander, Matthias Unseld, Alexander Stockenhuber und Gabriel Wagner, die mich abgelenkt haben.

Besonderer Dank gilt meinen Eltern, die mich in allen Hinsichten unterstützt und mir dieses Studium ermöglicht haben.

Ich danke meinem Großvater, der mich schon als Kind für logische Probleme begeistern konnte. Ich hoffe ich habe auch nur ein Fünkchen deiner Brillanz. Ich denke oft an dich!

Von ganzem Herzen danke ich meiner Paulina. Durch dich wurde Alles besser!

Contents

Abstract

The following work addresses the role of podoplanin, a 43kDa mucin-type transmembrane sialoglykoprotein, during cutaneous wound healing. The protein participates in physiological and pathological processes like development of the lymphatic system and tumour invasion. During these events podoplanin seems to increase cellular motility and tissue invasion. However, the exact biochemical function of the protein is still unclear.

In this study podoplanin knock-out and podoplanin wild-type mice were wounded using different excisional wound models. To assess the consequences of podoplanin deficiency during wound healing, I quantified the size of the wound area and the degree of wound contraction by image analyses of macroscopic images. Antibody stainings and fluorescent microscopy provide an impression of molecular significance of podoplanin expression at the wound site.

I was able to identify podoplanin in basal keratinocytes and fibroblasts, in a wide area within and around excisional wounds during the whole course of reepithelialization. Podoplanin deficiency seems to lead to delayed wound healing. However I was not able to observe any difference between podoplanin$^{-/-}$ and podoplanin$^{+/+}$ mice concerning epidermal invasion. Furthermore accelerated wound healing in podoplanin$^{+/+}$ mice is characterized by increased wound contraction. So, I assume that podoplanin has an increasing effect rather on wound contraction than on reepithelialization. This could be confirmed histologically. Preliminary experiments suggest that increased wound contraction could be the result of podoplanin dependent reorganization of the actin cytoskeleton of basal keratinocytes and wound fibroblasts.

Many further experiments will be necessary to clarify the exact role of podoplanin during wound healing processes. The investigations on this topic will definitely help us to understand the role of podoplanin during embryonic development and tumour progression.

Zusammenfassung

Die vorliegende Arbeit befasst sich mit der Funktion welche Podoplanin, ein 43kDa mucin-artiges transmembranes Sialoglycoprotein während der kutanen Wundheilung erfüllt. Dieses Protein ist sowohl an physiologischen als auch an pathologischen Prozessen, wie etwa der Entwicklung des lymphatischen Systems oder der Tumorausbreitung beteiligt. Podoplanin scheint während dieser Ereignisse sowohl die zelluläre Beweglichkeit als auch die Ausbreitung ganzer Gewebe zu fördern. Die genaue biochemische Funktion dieses Proteins ist allerdings bisher nicht aufgeklärt.

In dieser Studie wurden Podoplanin knock-out und Podoplanin Wildtyp Mäuse gewundet, wobei unterschiedliche Stanzwundmodelle eingesetzt wurden. Um die Folgen der Podoplanin-defizienz während der Wundheilung einzuschätzen, wurden sowohl die Größe des Wundareals als auch der Grad an Wundkontraktion mittels Bildanalyse makroskopischer Aufnahmen quantifiziert. Antikörperfärbungen und Fluoreszenzmikroskopie bieten einen Eindruck von der molekularen Bedeutung von Podoplanin an der Wundstelle.

Ich war in der Lage Podoplanin auf basalen Keratinocyten und Fibroblasten großflächig innerhalb der Wunden zu identifizieren. Dabei konnte die Expression dieses Proteins während des gesamten Verlaufs der Reepithelialisierung beobachtet werden.

Der Verlust von Podoplanin scheint zu einer Verzögerung im Wundheilungsprozess zu führen, charakterisiert durch eine verminderte Wundkontraktion. Überraschanderweise war ich nicht in der Lage einen Unterschied bezüglich der Einwanderung der Epidermis zwischen Podoplanin$^{-/-}$ und Podoplanin$^{+/+}$ zu beobachten. Daraus schloss ich, dass das Fehlen von Podoplanin eher einen mindernden Effekt auf die Wundkontraktion als auf die Reepithelialisierung hat. Dies konnte histologisch bestätigt werden. Erste Experimente deuten darauf hin dass die erhöhte Wundkontraktion Resultat einer podoplaninabhängigen Umstrukturierung des Aktincytoskeletts von basalen Keratinocyten und Wundfibroblasten sein könnte.

Viele weitere Experimente werden notwendig sein um die genaue Rolle von Podoplanin während der Wundheilung aufzuklären. Die Untersuchung diese Gebiets wird uns sicher helfen, die Rolle von Podoplanin während der Embryonalentwicklung und der Tumorausbreitung zu verstehen.

1. Introduction

1.1 Podoplanin

During the last decades our knowledge about and our interest in a protein called podoplanin have increased, because of its potential role in tumour invasion. Although the biological mechanism of realizing its function is hitherto not known, we have gained in knowledge about its chemical structure, expression, localization, interaction partners and the consequences of its activity.

Podoplanin was first described as a 43 kDa protein on the membrane of podocytes of the kidney by a group around Dontscho Kerjaschki[1]. Further studies by the same group revealed that the loss of podoplanin function leads to rapid flattening of these podocytes and it was postulated that podoplanin is important to for this cell type to maintain its cellular shape[2].

In the following, more and more was discovered concerning the molecular nature of podoplanin. So, it was revealed that it is a mucin-type transmembrane sialoglycoprotein which is expressed on lymphatic endothelial cells (LECs), but not in vascular endothelial cells[3]. For the development of the lymphatic system podoplanin is of tremendous importance and lymphatic endothelial cells (LECs) express podoplanin throughout the whole life of a mammalian organism. Furthermore podoplanin is found on the surface of various tumour cells, for example in tumours of the CNS[4], squamous cell carcinoma[5], Kasposi's sarcoma[6], angiosarcoma[7] or testicular germ cell tumours[8]. In all of these cancers a direct correlation can be observed between podoplanin expression and the invasion potential of the tumour, which suggests a possible connection between podoplanin and cell motility.

In fact, a huge number of studies prove a correlation between podoplanin and a spread cellular shape and a higher migratory potential. In this respect podoplanin is mainly observed during physiological processes in which a higher invasion is required. So far, not much is known about the way this rather small protein participates in processes determining cell-shape and the migratory behaviour. Podoplanin does not seem to have any enzymatic activity and to date the number of interaction partners is rather small.

Taken together, three main processes are known in which an appearance of podoplanin can be observed:

1. Separation between lymphatic and blood circulation during mammalian embryonic development.
2. Tumour invasion; in the specific types of cancer mentioned above.
3. Wound healing; at the basal layer of keratinocytes of newly formed epidermis and dermal fibroblasts around the wound area

In the following we will discuss all of these physiological processes in context of podoplanin, in order to get an image of the workings of this protein. Probably to date, the function of podoplanin is best understood during lymphatic development although most time and effort is spent on understanding the role of podoplanin in tumour progression. About podoplanin in wound healing very little is known, especially because of the fact that before podoplanin KO mice existed there was no sufficient procedure to analyze wound healing in the absence of podoplanin.

1.2 Development of the lymphatic circulation

The lymphatic vasculature consists of a network of blind-ended, thin-walled vessels traversing the whole body to drain and to recycle the protein-rich interstitial fluid, from the extracellular space back into the blood. This so called lymph fluid reaches the blood circulation via the thoracic duct, which connects the blood system with the lymphatic vasculature. Beside this function, lymphatic vessels are responsible for the transport of white blood cells and dendritic cells from the periphery to the lymphoid organs.

Both functions are guaranteed by the loose contacts between lymphatic endothelial cells (LECs) and the lack of a continuous basement membrane, which facilitates the uptake of large macromolecules and migrating cells[9].

The development of the lymphatic vasculature starts later than the development of the blood vascular system, referring to this it was worthwhile to study if the lymphatic system arises from the blood vessels, already developed in the early embryo. In fact, already 1902 it turned out that endothelial cells bud from veins to develop lymphatic sacs, which sprout their cells to form the lymphatic vasculature during embryonic development[10].

In mice the development of the lymphatic vasculature starts around day 9 with the expression of lymphatic vessel endothelial hyaluronan receptor I (Lyve1), on blood endothelial cells of the cardinal vein[11]. Interestingly, deletion of Lyve1 during embryogenesis does not affect the development of a normal lymphatic vasculature, so it is assumed that another, factor determines lymphatic development out of the blood vasculature[12].

New insights into LEC specification revealed the transcription factors COUP-TFII and Sox18 are promising candidates for the initiation of LEC development. Around embryonic day ED9.5 cells on one side of the cardinal vein begin to express prospero-related homeobox 1 (Prox1). In contrast to Lyve1, Prox1 seems to be of highest importance for the development of the lymphatic vasculature, as homozygous Prox1-deficiency leads to a complete lack of lymphatic vessels before embryos die at ED14.5[13]. Probably Prox1 is a key player in LEC-specification, as from this point Prox1 positive cells start to express various lymph-specific markers like neuropilin 2 (Nrp2) or podoplanin[14]. Development of lymphatic vessels requires guided budding of LECs out of the cardinal vein, which afterwards builds up the lymph sacs, the origins for the whole lymphatic system of the organism.

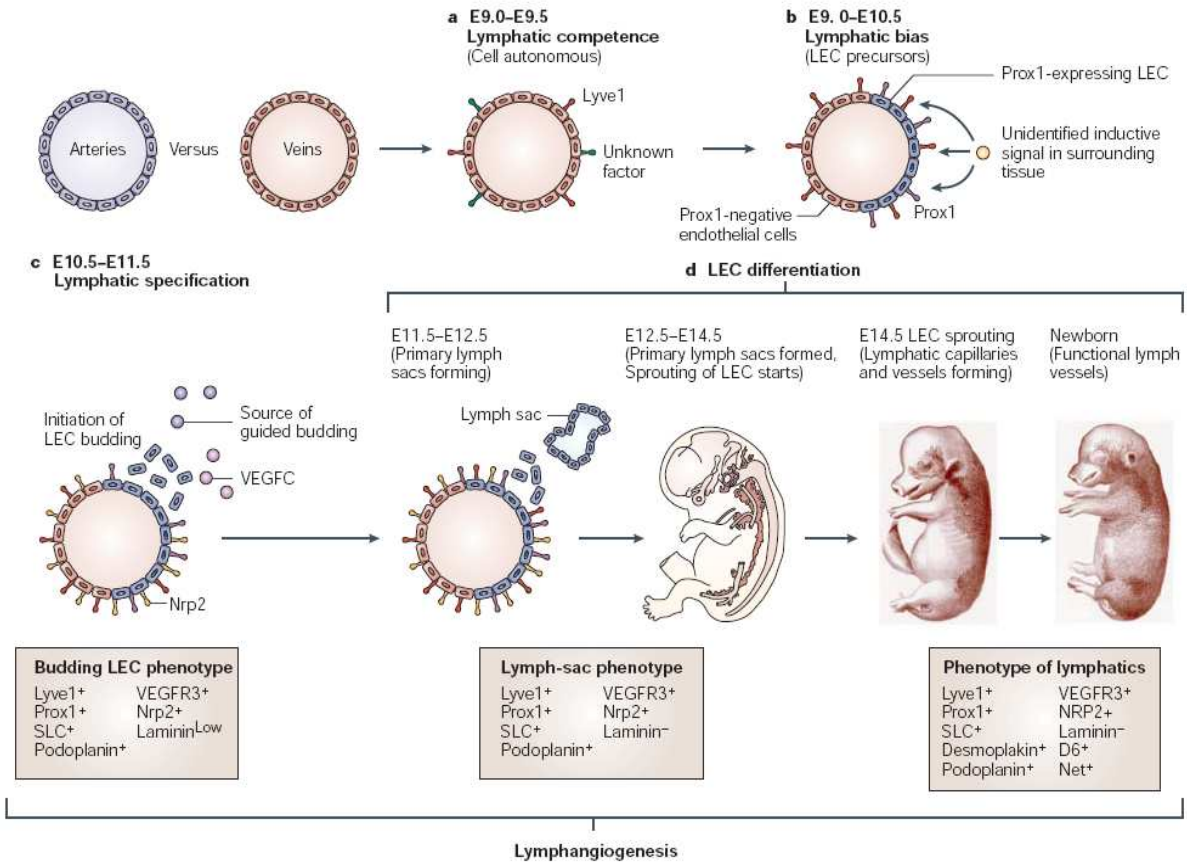

Figure 1: Development of the lymphatic vasculature[9]. a | Although Lyve1 is probably the first LEC marker expressed on cells of the cardinal vein, it does not seem to have an effect on the normal development of the lymphatic vasculature. The unknown factors in the graphic are most likely the transcription factors COUP-TFII and Sox18 which induce the expression Prox1. b,c | Expression of Prox1 on one side of the cardinal vein leads to occurrence of an LEC phenotype consisting in expression of different LEC specific proteins like podoplanin or neuropilin 2. d | On the action of VEGFC LECs bud from the cardinal vein to form primary lymph sacs, which in turn start to sprout LECs towards the periphery to act as origins for lymphatic vessels.

The action of vascular endothelial growth factor C (VEGFC) regulates the development of blood vasculature and lymphatic vasculature and requires the presentation of VEGF Receptor 3 (VEGFR3) on the surface of LECs[15,16].

Around ED12 primary lymph sacs develop consisting of LECs originated from the embryonic veins, which start to spread throughout the whole body, this leads to formation of the functional lymphatic vasculature at around ED14.5[9].

1.2.1 The role of podoplanin in the development of the lymphatic circulation

Since the time point of podoplanin expression in prospective LECs correlates with the initiation of LEC budding, one can assume that podoplanin fulfils a function during this process. In fact, mice which are deficient in podoplanin expression show blood filled lymphatic vessels which indicate a non-separation between the two circulatory systems[17].

During the studies about podoplanin and the circulatory separation it could be shown that platelets arriving from the cardinal vein aggregate between the vein and the prospective lymph sac in order to mediate their separation. This aggregation phenotype could only be observed in mice presenting podoplanin on the surface of LECs. It was suggested that interaction with podoplanin enables platelets to aggregate, which in turn leads to complete secession of the lymph sac. In this case there should be a molecular signalling mechanism, between podoplanin expressing LECs and platelets. Previously it was revealed that the C-type lectin-like receptor 2 (CLEC-2) which is specifically expressed on platelets and megakaryocytic cell lines, interacts with podoplanin[18].

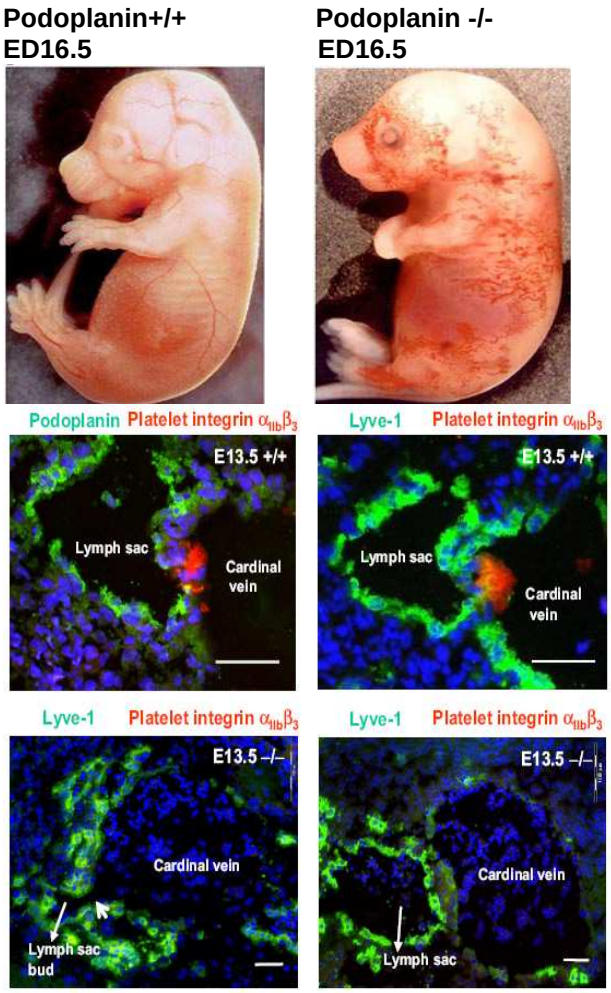

Figure 2: Podoplanin deficiency disrupts developmental separation of the lymphatic system from the blood vascular system, cognizable by the blood filled lymphatic vessels in the skin. Podoplanin expression on LECs leads to platelet aggregation at the budding position of the primary lymph sac. Podoplanin-/- mice do neither show this platelet aggregation nor sufficient separation between the lymph sac and the cardinal vein[17].

Clec-2, which was first described as a receptor for the snake toxin rhodocytin, triggers a signal transduction pathway including Syk and SLP76 following contact with this toxin[19].

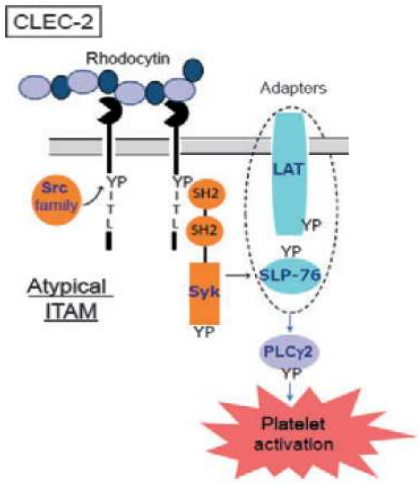

Figure 3: Rhodocytin-CLEC-2 interaction activates Platelets via Syk and SLP76. Since podoplanin is able to interact with CLEC-2 and deficiency in Syk and SLP76 disrupts lymphatic separation from the blood circulation, it is likely that podoplanin-CLEC-2 interaction triggers the same signaling pathway[20].

It is likely that interaction between podoplanin and CLEC-2 activates the same signalling cascade which finally leads to platelet aggregation at the separation site.

Taken together the role of LEC-expressed podoplanin during lymphatic development consists in activation of platelets arriving from blood of the cardinal vein, in order to aggregate and mediate lymphatic separation from the blood circulation.

The detailed physiological mechanism of this separation is hitherto not known.

1.3 The role of podoplanin during tumour invasion

The transition form a benign resting tumour to a malignant invading tumour is one of the most significant reasons for a negative clinical outcome in cancer patients. This transition of tumour cells requires changes in their capabilities to adhere, to migrate and to degrade the surrounding extracellular matrix (ECM) in order to move from the primary tumour towards peripheral tissue. Many events leading to tumour progression are at least partially mediated by the action of transmembrane proteins[53]. These molecules are responsible for functions like cell-cell-adhesion, cell-matrix-adhesion or for the activation of intracellular signal transduction pathways. Integrins for example form connections to the extracellular matrix; molecules called cadherins participate in cell-cell contacts. A specific class of cell surface proteins called matrix metalloproteinases (MMPs) are proteases which have the ability to degrade extracellular matrix. This is important for tumour invasion as well as for wound healing, since in both cases cells have to move from one position to another peripheral position[21].

During the last years, a protein of the Cadherin class, the so called E-Cadherin was moved into the focus of intensive investigation, as it turned out that its loss on the cell surface supports tissue disassembly, cell motility and in consequence tumour metastasis[22].

Several reasons are known for the inactivation of E-cadherin on the cells surface, besides mutations, the activation state of the small GTPases Rac and RhoA has effects on the adhesive functions of the protein. The activity of these GTPases can change cellular polarity and morphology; during this cellular junctions are lost[23].

Rho-GTPases fulfil their functions by reorganizing the actin cytoskeleton. Some of the effector proteins in this mechanism are the similar proteins ezrin, radixin and moesin, in short ERM. These proteins connect the transmembrane ECM receptor CD44 to the actin cytoskeleton which promotes cell motility[24].

Similar to CD44, podoplanin has the ability to interact with proteins of the ERM family intracellularly[25]. In contrast to CD44, nothing is known about any ECM binding function of podoplanin and maybe the functions of these two proteins are essentially different.

In 2006 it turned out that podoplanin, expressed in Madin-Darby canine kidney (MDCK) type-II epithelial cells, interacts with ERM proteins in order to activate RhoA and to promote epithelial-mesenchymal transition (EMT), a process during which epithelial cells acquire mesenchymal features, which results in cells with a migratory potential comparable to fibroblasts. A complete EMT is often observed in highly aggressive kinds of cancer. The group of Ester Martin-Villar pointed out, that MDCK cells which express human podoplanin down-

regulate epithelial genes like E-cadherin, catenins and cytokeratins and upregulate mesenchymal genes like N-Cadherin, vimentin or fibronectin. In consequence the migratory behaviour of these cells changes from a rather slow collective migration pattern to a faster and individualized. Furthermore it is suggested that the activation of RhoA is a consequence of the interaction between podoplanin and ezrin, the RhoA activation in turn promotes epithelial mesenchymal transition[26]. In short, podoplanin on MDCK cells seems to associate with ERM proteins at the plasma membrane, which increases the function of RhoA. RhoA activity induces cell physiological processes which, in consequence result in epithelial-mesenchymal transition (EMT), in order to support cell motility. Concerning cancer, this increased cell motility is a risk factor for tumour metastasis and invasion.

However, in contrast to the results of the group of Ester Martin-Villar in MDCK cells, we will see that during tumour invasion podoplanin obviously has the capability to promote migration in the absence of epithelial-mesenchymal transition.

In 2006 the group around Andreas Wicki used transgenic mouse lines, which co-express a potent proto-oncogene and podoplanin in the pancreas.

As expected, mice developed a pancreas carcinoma and podoplanin significantly increased the invasive potential of the primary pancreas tumours. Expression of podoplanin on tumour cells changes the cellular shape towards a mesenchymal phenotype, as observed during epithelial-mesenchymal transition. Interestingly podoplanin tumour invasion does not seem to be accompanied with the loss E-cadherin at the cell surface. This is surprising, as the loss of E-cadherin is one of the hallmarks of EMT. Obviously podoplanin enables the cell to become invasive without of down-regulation of the E-cadherin levels. As well as E-cadherin also its anchor proteins β catenin and p120 catenin, which are normally downregulated during EMT, are kept by invading tumour cells expressing podoplanin[27]. Since E-cadherin requires these catenins to fulfil its proper function, one can assume that the whole arrangement for establishing adherens junctions is maintained, following podoplanin expression.

The need for an EMT without the loss of E-cadherin remains elusive. In fact it provides the possibility to induce tumour cell invasion without dissolving epithelial adherens junctions. Otherwise it could be advantageous to dissolve and re-establish E-cadherin junctions faster in some podoplanin-dependent manner.

Similar results were obtained by Wicki et al when they stably transfected MCF7 breast carcinoma cells with a podoplanin expression construct. When these cells formed an epithelial layer *in vitro*, expression of podoplanin did not lead to loss of E-cadherin on the cell surface but to a dramatic change in cellular shape and an increased migratory behaviour.

Like others before, the group emphasizes the interaction between podoplanin and ezrin. These proteins especially co-localize at the cell membrane of filopodia. This leads to relocalization of actin to filopodia. Additionally it could be shown, that podoplanin expression raises the phosphorylation level of ezrin, suggesting that podoplanin affects the function of ezrin in a biochemical way. This in turn could be caused by functional regulation of Rho-GTPases.

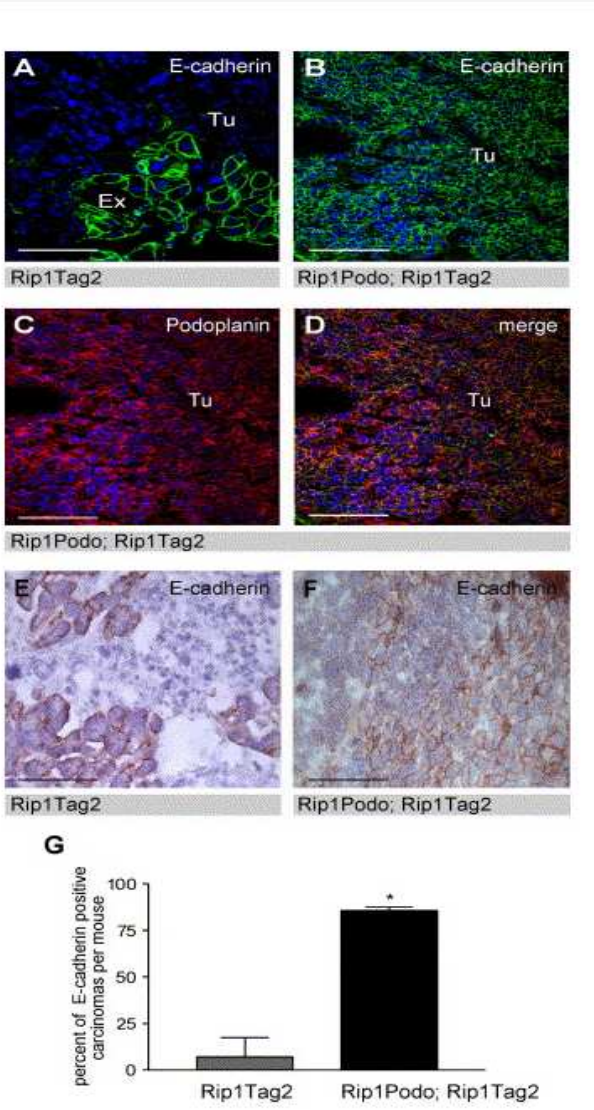

Figure 4: Podoplanin induces tumour invasiveness without loss of E-cadherin[27]

A-F| Immunohistochemical studies reveal that podoplanin expressing tumour cells (Rip1Podo;Rip1Tag2) at the invasive front do not have to downregulate E-cadherin levels in order to gain invasive properties. In contrast, no E-cadherin can be detected in podoplanin non-expressing tumours (Rip1Tag2), these tumours probably underwent full EMT. G| Significant maintenance of E-cadherin in Rip1Podo;Rip1Tag2 tumours compared to Rip1Tag2 tumours.

Tu, Tumour tissue; Ex, exocrine tissue

It is known that the concentration of actin at filopodia-like structures can be the result of decreased RhoA activity[28]. In fact it could be shown, by performance of RhoA activity assays that the expression of podoplanin leads to downregulation of RhoA activity in MCF7 cells[27]. This is insofar surprising and confusing as this result is contradictory to the results of Ester Martin-Villar's group, which revealed a podoplanin dependent *activation* of RhoA in Madin-

Darby canine kidney (MDCK) cells. At this point it is important to mention that MCF7 cells express a high intrinsic level of RhoA, whereas MDCK cells exhibit a low intrinsic activity of RhoA[29]. So, maybe podoplanin regulates RhoA to achieve a specific level of RhoA activation. In general, both groups describe the same cellular effect of podoplanin: a podoplanin-dependent support of migratory capabilities.

1.4 Cutaneous wound healing

Wound healing is a physiological process which resembles tumour progression in many aspects. Briefly, I want to outline the hallmarks of general cutaneous wound healing processes. Oversimplified, wound healing can be subdivided into 3 different phases:

- Inflammation
- Tissue formation
- Tissue remodelling

At the beginning of the first phase a fibrin clot is formed which serves as temporary wound protection and as provisional extracellular matrix for cell migration. This clot consists mainly of platelets and mesh of crosslinked fibrin fibers[30]. As a first line of defence against foreign particles, neutrophils enter the wound, followed by monocytes which become activated macrophages which fulfil their phagocytic and growth factor releasing functions[31]. These growth factors like TGFα and TGFβ or PDGF (platelet-derived growth factor) support new tissue formation[32].

During tissue formation, the most important part is the formation of new epidermis consisting of keratinocytes, a process which is called reepithelialization. As these keratinocytes are the predominant cells which express podoplanin during wound healing, this cell type is in the focus of this study. Keratinocytes are connected to neighbouring keratinocytes by adherens junctions called desmosomes and they are connected to the basement membrane by so called hemidesmosomes. Reepithelialization starts with the dissolution of desmosomes and hemidesmosomes of keratinocytes[33] and the reorganization of the actin cytoskeleton which is regulated by the activity of Rho-GTPases[34,35]. All of these events allow movement of keratinocytes into the wound area. Besides migration, keratinocytes now begin to proliferate, in order to provide new material for complete wound closure. In general, keratinocytes with a high proliferation rate remain behind the actively migrating cells[36].

During invasion of keratinocytes into the wound area, additional fibroblasts move into the wound, in order to create new granulation tissue. Macrophages continuously supply the wound with growth factors. In a process called neovascularisation new blood vessels grow into the wound to ensure the supply with oxygen and nutrients[36].

Approximately one week after injury, when tissue formation slowly ceases, the wound begins to reorganize in a process called tissue remodelling. During this fibroblasts turn into myofibroblasts by expression of α-smooth muscle actin (α SMA) which forms microfilaments along the cytoplasmic face of the plasma membrane. Myofibroblasts are capable to form

connections between other cells and the matrix, thus they are able to exert contractile forces on the wound leading to wound contraction[37]. In the end of wound contraction the granulation tissue is replaced by a scar, during a process called collagen remodelling, this is characterized large highly cross-linked collagen bundles[38].

In general a scar regains at maximum 70% if the strength of normal skin[39].

1.4.1 Podoplanin is expressed during cutaneous wound healing and other skin remodelling processes

Little is known about the cellular processes leading from resting, low-proliferative keratinocytes to migrating, high-proliferative cells at the wound margin after injury. Like mentioned above, the first steps in reepithelialization require the dissolution of connections between neighbouring cells like other keratinocytes (connected by desmosomes) and cells of the dermis (connected by hemidesmosomes). After dissociation keratinocytes enter a state of increased motility and proliferative activity. All these initial events point to a role of epithelial-mesenchymal transition in a way we have seen during tumour progression.

Indeed, there is evidence that keratinocytes at the leading edge of healing epidermis undergo a process which is reminiscent of EMT, since these cells express mesenchymal markers like vimentin or fibroblast specific protein 1 (FSP1)[40]. This raises the question if podoplanin expression by keratinocytes at the wound margin plays a role during wound healing

In 1997, a first proof was published, that podoplanin is expressed by keratinocytes in cutaneous healing. By introducing a wound into mouse skin, the group was able to stain a protein called PA2.26 antigen in the basal region of keratinocytes next to the wound. This protein was later found to be identical with podoplanin. Furthermore the group around Alberto Gandarillas found out that not only the keratinocytes of the basal skin layer but even dermal fibroblastic cells express podoplanin during tissue regeneration. Already by this point a possible relation between podoplanin and cell motility was postulated[41].

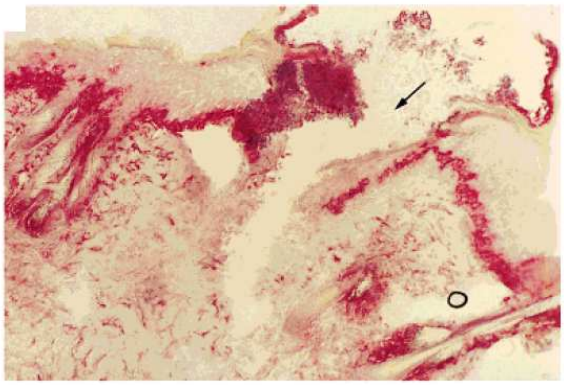

Figure 5: Immunohistochemical identification of Podoplanin in wound keratinocytes[41].
Podoplanin is solely expressed at the high-proliferative basal layer of healing epidermis and in dermal fibroblasts. Podoplanin = red, the arrow marks the site where the incision was made.

Beside the results concerning wound healing this work clearly shows a connection between podoplanin expression and skin carcinogenesis, since the work actually discusses the expression of podoplanin on keratinocytes and active fibroblasts during squamous cell carcinoma.

To sum up the results of this study, expression of podoplanin can be observed after cutaneous injury, during skin carcinogenesis and following treatment of the skin with phorbol esters like TPA. The group points out that all these processes include an increase of cell motility. However, podoplanin expression can not be observed in completely untreated skin under normal physiological conditions. This suggests a direct role of podoplanin in tissue remodelling processes[41].

However, it the role of podoplanin during wound healing was not investigated so far, as no work addressing this issue was published to date.

Nonetheless the results about podoplanin expressing keratinocytes in carcinogenesis are interesting and can be considered as the best basis to investigate the role of podoplanin during wound healing. In further studies it could be shown also for keratinocytes that podoplanin co-localizes with ERM (ezrin, radixin, moesin) family members, which leads to a massive rearrangement of the actin cytoskeleton towards a fibroblastoid cell morphology. Podoplanin obviously redistributes ezrin, to enable an interaction between these two proteins at plasma membrane projections like lamellipodia and filopodia. So, a relation between podoplanin and cell migration was highly probable[25]. To support this notion another study revealed that podoplanin which is expressed by MCA3D keratinocytes leads to destabilization of adherens junctions mediated by E-cadherin, downregulation of basal keratins, induction of vimentin and keratin K8 and the acquisition of a malignant phenotype[42]. All these events point to a function of podoplanin during epithelial-mesenchymal transition in keratinocytes, since the loss of E-cadherin and the expression of vimentin are critical hallmarks in the transition from an epithelial to a mesenchymal state. Very interestingly, RT-PCR to quantify E-cadherin gene expression shows only a slight reduction in the transcription of Cadherin mRNA. However the functional protein could not be detected performing Western immunoblotting with cell lysates, instead several smaller peptides were detected. This suggests that previously functional E-cadherin proteins were proteolytically degraded, as a result of podoplanin expression. The levels of β-catenin however were equally in podoplanin expressing cells and control cells[42].

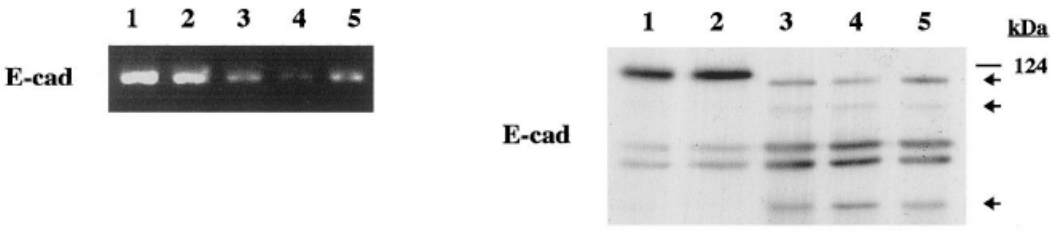

Figure 6: Left| Quantification of E-cadherin mRNA by RT-PCR. Right| Western immunoblotting of cell lysates for E-cadherin Protein.
Control cells line 1-2, podoplanin transfectants line 3-5 [42]

These results expand the data obtained by Andreas Wicki et al who discovered that podoplanin expression on invasive pancreas tumours leads to maintenance of E-Cadherin during invasion. According to this model it is possible that E-cadherin is detectable on the surface of podoplanin expressing cells, but the protein is not functional anymore. This result is confirmed by another study by a group around Ester Martin-Villar in 2005[43]**.** However, this model still allows a mechanism of rapid restoration of cell-cell-contacts, compared to cells which undergo full EMT by genetic down-regulation of adherens molecules.

Taken together, podoplanin is expressed on activated keratinocytes. The action of podoplanin influences the morphology and the migratory behaviour of keratinocytes during these processes and it seems to hold true that podoplanin supports the formation of cell-cell junctions during a mesenchymal cellular state.

By use of squamous cell carcinoma models we were able to gain much insight into the function of podoplanin expressed by keratinocytes and we hope that this knowledge will help us to understand the role of podoplanin during wound healing. Since the establishment of a podoplanin knock-out mouse line by the group around Pavel Uhrin is a very new progress in this field, it was not easy in the past to perform in vivo wound healing experiments, in which normal wound healing can be compared to podoplanin inhibited wound healing.

Next, I will briefly describe the establishment of podoplanin deficient mice, which allowed us to study the contribution of podoplanin to cutaneous wound healing.

1.5 Podoplanin KO mice

In former trials to create podoplanin null mice, the animals died immediately after birth in consequence of respiratory failure. This suggests a role of podoplanin during lung development. Indeed loss of podoplanin causes anomalies in the development of lung alveoli[44]. Nonetheless a further try by Pavel Uhrin using 129S/v and Swiss mice was finally successful creating surviving podoplanin knock-out mice, of this background.

The podoplanin knock-out mice established by Pavel Uhrin, were generated by disrupting the coding region of the podoplanin gene. More precisely, the murine gene for podoplanin consists of 6 exons which code for different parts of the protein. Using a targeting vector, the group was able to exchange the exons II, III, IV and V by a neomycin phosphotransferase cassette in 129S/v embryonic stem cells. Thus the podoplanin gene was completely disrupted. The obtained chimeric mice were crossed with C57/Bl6 mice and the disrupted podoplanin allele was partially transmitted to the offspring. The thereby generated podoplanin$^{+/-}$ heterozygous mice where intercrossed yielding podoplanin$^{-/-}$ mice[45].

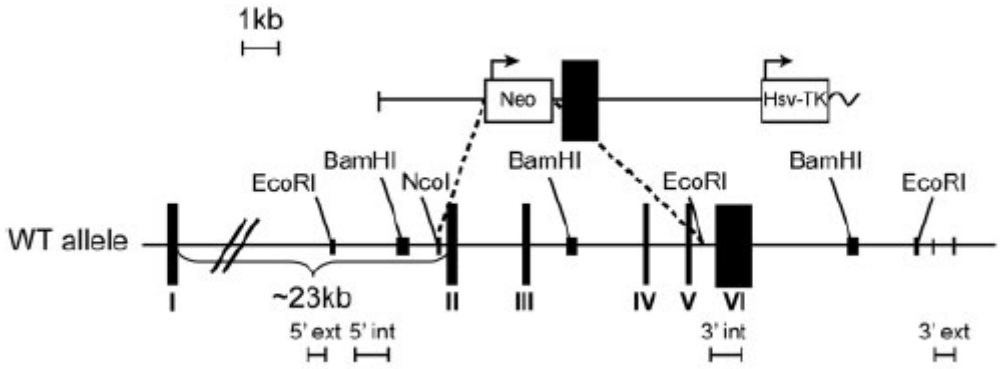

Figure 7: Disruption of the podoplanin gene by homologues recombination with the pPNT.podoplanin targeting vector. Black boxes represent exon sequences. Exons II-V are exchanged by the neo gene, upon homologues recombination[45].

As mentioned above, previous podoplanin knock-out experiments showed a neonatal death rate of 100%, probably because of respiratory failure. Also in this protocol, mice show a mortality of around 70% (40% during embryonic development, 55% during the first postnatal week). It is hitherto unclear why – in this background - around 20% of the individuals become fertile adult animals with normal life spans.

2. Aims

In the present study I want to clarify differences in the process of wound healing between podoplanin knock-out and wild-type mice. For this I am taking advantage of the podoplanin knock-out mouse strain which was generated by Pavel Uhrin, one of my supervisors. This should allow a fast and reliable approach, to reach my goals.

By macroscopic and microscopic studies I will try to assess specific properties of wound healing which are affected by podoplanin.

So far, nobody knows when podoplanin is expressed in the course of wound healing and identification of a specific time window during which podoplanin is expressed maybe points to a specific function of podoplanin.

Considering the previous results, generated during the last years, one can assume that reepithelialization in wild-type animals happens faster, because of an increased invasion potential of podoplanin expressing keratinocytes.

Summary

- Podoplanin is sialomucin typeI transmembrane glycoprotein, presented on the surface of LECs, cells of various invading tumours and keratinocytes and dermal fibroblasts during skin remodeling processes like wound healing.
- Podoplanin seems to increase cell motility as it is expressed in physiological processes during which a higher migration potential is required.
- The interaction between podoplanin and Clec-2 on platelets is necessary for separation of blood and lymphatic circulation during embryonic development.
- During tumour invasion of specific cancers, podoplanin can be detected at the invasive front. There, it is suggested to increase the invasive potential during maintenance of cell-cell junctions.
- So far, nothing is known about the mechanisms concerning podoplanin expression on basal keratinocytes and dermal fibroblasts following injury. Investigating this is in the focus of this study.
- Podoplanin interacts with the actin-cytoskeleton via proteins of ERM (ezrin, radixin and moesin) family (especially ezrin). This interaction leads to re-localization of all of its components to migratory structures like filopodia.
- Expression of podoplanin probably triggers incomplete epithelial-mesencyhmal transition which allows the formation of cell-cell-contacts via cadherins during migration processes. This could lead to collective migration of whole tissue sheets.
- Recently, podoplanin knock-out mice could be established. Although with a death rate of 70% during embryonic and early postnatal development.

Furthermore, it will be worthwhile to focus on the morphology of invading keratinocytes and the organization of the cytoskeleton, as both is obviously dramatically reorganized by the presence of podoplanin.

Herein, I will address both, the function of podoplanin on basal keratinocytes as well as on dermal fibroblasts. Since both cell types are of most critical importance for the success of wound healing, it is likely that podoplanin fulfils a crucial function during wound healing.

3. Materials and Methods

3.1 Scratch wound assay

Sterile fibronectin coated glass cover slips (Marienfeld Micro Cover Glasses) were provided by Johannes Breuss and fibroblasts were a gift from Hannes Stockinger. A circular silicone frame was applied on the cover slip using a brush and Baysilone-Paste (GE Bayer Silicones). Cover slips were put into 6-well-plates. NIH3T3 fiborblasts (NIH3T3WT) and podoplanin transfected NIH3T3 fibroblasts were diluted to final concentration of 300cells/µL in RPMI medium with 10% serum and penicillin/streptomycin. 100µL of the cell suspensions were applied into the circular frame. Cells were cultivated until confluence over night at 37°C and 5% CO_2. The cover slip was fixed onto a metal plate showing a circular recess right at the position of the circular cell layer, medium was changed. A scratch was introduced into the middle of the cell layer using a pipette tip (for 200µL) and another cover slip was fixed onto the other side of the recess. The whole construct was inserted into an Olympus AX70 microscope and images of the scratch wound were taken automatically every 8 minutes for 3 hours.

3.2 Excisional wound model

3 to 6 months old podoplanin wild-typ and knock-out mice of the same sex were transferred into separate cages, providing enough water and feed. Mice were anaesthetized 2.5 parts Ketasol (Graeub), 1 part Rompun (Bayer), and 6.5 parts sterile destilled water (Mayrhofer Pharmazeutika). For every 10 grams of mouse weight 75µL anaesthetic were injected under the abdominal skin.

Fully anaesthetized mice were shaved on the back near the forelegs using 70% Ethanol and a conventional razor blade (Gilette). Two 6mm circular excisional wounds were marked using a sterile 6mm biopsy puncher (kai medical). Skin was lifted inside the mark using sterile forceps and excised around the trace using sharp and sterile scissors.

Wounds were photographed using a stereo microscope (Olympus 3100), a Sony DSC W200 digital camera and a ruler close to the wound; mice were transferred back into the appropriate cages. Every 24 hours mice were anaesthetized by isofluoran inhalation (lasts for approximately 1 minute) and wounds were photographed as described.

In the end, mice were sacrificed softly by isofluoran inhalation, photographed and shaved again if required. Wound tissue was excised and frozen in OCT tissue tek using liquid nitrogen. Wounds were divided into two equal parts. Embedded tissue was stored on -20°C.

3.3 Tension wound model

This is a modified model of a wound model originally published by Robert D. Galiano[46].
Mice were chosen, kept, shaved and anaesthetized as described before. A hole of 6mm diameter was punched into a 0.5mm thick Press-to-seal silicone sheet (invitrogen) using a 6mm biopsy puncher, a silicone ring was circularly excised from the whole silicone sheet around 3-5mm outside of the inner hole edges, using conventional scissors. This results in a silicone torus with a hole- diameter of 6mm and a full diameter 12-16mm. Two of these silicone constructs were stitched onto the same positions the excisional wounds were introduced. For this, 8 sutures in equal distances fix the silicone ring on the back skin of the mice; thereby the skin was gently pulled from an inward position underneath the silicone toward a rather outward position, this slightly tense skin was then fixed to the silicone by a suture.
Inside each silicone structure, a 4mm circular excisional wound was traced using a sterile 4mm biopsy puncher. Skin was lifted inside the trace using *sharp* sterile forceps (because tense skin is harder grasp) and excised around the trace using sharp and sterile scissors. Mice were photographed and sacrificed as already described. The silicone torus was removed by cutting the sutures and the wound tissue was treated as described.

3.4 Histology

8µm transversal cryosections were produced starting directly at the division site of the wound, using a MICROM HM 500 OM cryostat. Sections were stored at -20°C if not immediately needed, or at least air-dried for 30 minutes for immediate use. Tissues were fixed and histological staining was performed using Hemacolor rapid staining (Merck Chemicals), according to users manual. Stained tissues were air-dried and embedded using Entellan Neu (Merck Chemicals).

3.5 Immunofluorescence

8µm cryosections which were stored at -20°C were fixed for 2 minutes in -20°C acetone. OCT Tissue Tek surrounding the tissue was washed away using mains water, for at least ten minutes. Slides were inserted into Shandon Coverplate™ apparatur and equilibrated with 50mM Tris/HCl buffer. Unspecific protein binding sites of the tissue were saturated with 2% normal goat serum (Dako) for 20 minutes. Primary antibodies were optimally diluted in Antibody Diluent (Dako) and slides were incubated with appropriate primary antibody dilutions (see table below) over night at 4°C. Slides were washed three times with 50mM Tris/HCl. If required, secondary antibodies were optimally diluted in Antibody Diluent (Dako). Slides were incubated with secondary antibody dilutions (see table below), for 30 minutes. If required, streptavidin was optimally diluted in Antibody Diluent and slides were incubated with streptavidin solutions for 5 minutes. Nuclei were counterstained with Hoechst (Molecular Probes). Samples were washed three times with 50mM Tris/HCl and mounted in Ultramount (Lab Vision Corporation)

Antibody or staining compound	**µg/mL**	**Dilution**	**Incubation**
Primary antibodies			
Hamster anti mouse Podoplanin (Acris)	3.3	1/150	o/n
Rabbit anti mouse Ki67 (NeoMarkers)	5	1/150	o/n
Biotin conjugated anti mouse CD41 (eBioscience)	5	1/100	o/n
Rabbit anti mouse Lyve1 (abcam)	5	1/200	o/n
Secondary antibodies			
Biotin conjugated rabbit anti hamster (Acris)	4	1/500	30’
Alexa Fluor 555 goat anti rabbit (invitrogen)	4	1/500	30’
Staining compounds			
Hoechst (Molecular Probes)	10	1/1000	30’
TRITC Phalloidin (Sigma-Aldrich)	5	1/200	30’
Alex Fluor 555 Streptavidin (Molecular Probes)	3	1/500	10’

3.6 Microscopy and image analysis

Microscopic images of histological and immuonfluorescent stainings were taken with a motorized Olympus AX70 microscope by 10x, 20x and 40x UplanApo air objective lenses and using a cooled F-View II Camera. Image analysis was performed using Cell^{P} imaging software (Olympus Soft Imaging Solutions).

3.7 Statistical analysis

Statistical significance was calculated by unpaired *t* test and log rank test using GraphPad Prism software. Significance was assigned to *P* values less than 0.05.

4. Results

4.1 Podoplanin leads to increased and collective cell migration

In my first experiment I wanted to know if the expression of podoplanin has any consequence on the migratory behaviour of cells. According to the literature[27] podoplanin expressing cells should show an increased and more collective motility than cells which do not express this protein. As we unfortunately failed to cultivate fibroblasts or keratinocytes from WT and podoplanin KO mice, we compared NIH3T3 wild-type fibroblasts and NIH3T3 podoplanin transfected fibroblasts in a scratch wound assay, the cells were a generous gift of Hannes Stockinger.

WT (NIH3T3) and podoplanin over-expressing (NIH3T3PodoOE) fibroblasts were seeded on a fibronectin matrix and cultivated until confluence was reached. A scratch was introduced into the cell layer using a pipette-tip and "healing" of the scratch was monitored for the following three hours.

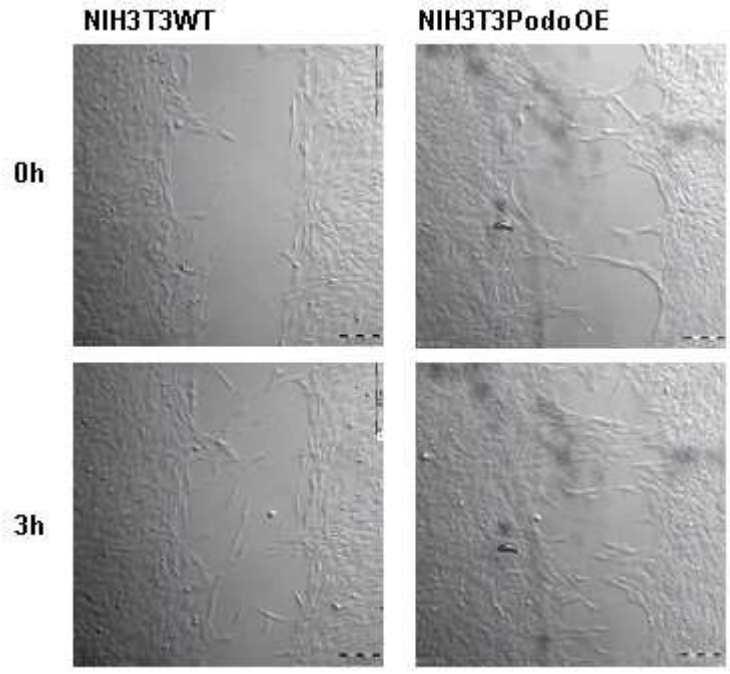

Figure 8: Scratch wound assay on fibronectin using WT and podoplanin over-expressing NIH3T3 cells. After 3 hours NIH3T3PodoOE cells show increased and more collective migration into the scratch compared to NIH3T3 WT cells.

First, we observed that scratch wounds of WT and podoplanin over-expressing (PodoOE) cells differ right from the beginning. The scratch through WT cells seems to be straight and smooth, whereas it seems to be ruffled and uneven in case of PodoOE cells. Probably podoplanin

supports intracellular cell-cell contacts already in the state of confluence. Therefore some of the cells which are affected by the scratch remain attached to the adjacent scratch margins and re-enter the scratch immediately.

Furthermore, we observed an increased and collective migration in PodoOE samples compared to WT. These results are consistent with the position that podoplanin supports cell migration and contributes to maintenance of cell-cell interactions during migration processes.

If we assume that cells which express podoplanin during wound healing behave in a similar way, it is imaginable that podoplanin has a supportive effect on the collective invasion of epidermal keratinocytes into the wound area.

4.2 Podoplanin is expressed during the full process of reepithelialization

Next, I wanted to examine when and by which cell types podoplanin is expressed, during cutaneous healing. According to the studies of Gandarillas et al[41], podoplanin is expressed at least 48 hours after wounding in basal keratinocytes and dermal fibroblasts near the wound margins. With this experiment I wanted to confirm these results and I performed a study to reconstruct a time course, for the expression of podoplanin during wound healing. For this, I sacrificed wounded WT mice every 24 hours, from day 1 until day 8 and checked the wounds for podoplanin expression, using a monoclonal antibody against this protein on 8µm tissue sections.

Fluorescence microscopy reveals, that podoplanin is expressed in basal keratinocytes, during the whole course of reepithelialization, which starts at around day 2 and lasts until around day 8. However, podoplanin seems to disappear from these cells after complete epithelialization of the wound area, which is achieved approximately at day 8 (see figure 9). This suggests a role of podoplanin which is not restricted to any distinct phase of wound healing, like the inflammatory phase in the beginning or tissue remodelling in the end of the healing process. Therefore it is worthwhile to consider a role of podoplanin during rather general aspects of healing epidermis, like keratinocyte migration, proliferation or wound contraction.

The expression of podoplanin seems to reach a peak between day 4 and day 6, during maximum proliferation and reepithelialization.

In general, we can confirm the observations of Gandarillas et al, as we obtain a positive signal for podoplanin in both, basal keratinocytes and fibroblasts within the dermis. Additionally, fibroblastoid structures within the granulation tissue were positive for podoplanin (see figure 9; day 5). This could point to a role of podoplanin in recruited fibroblasts at the site of injury in order to support development of provisional matrix and wound contraction.

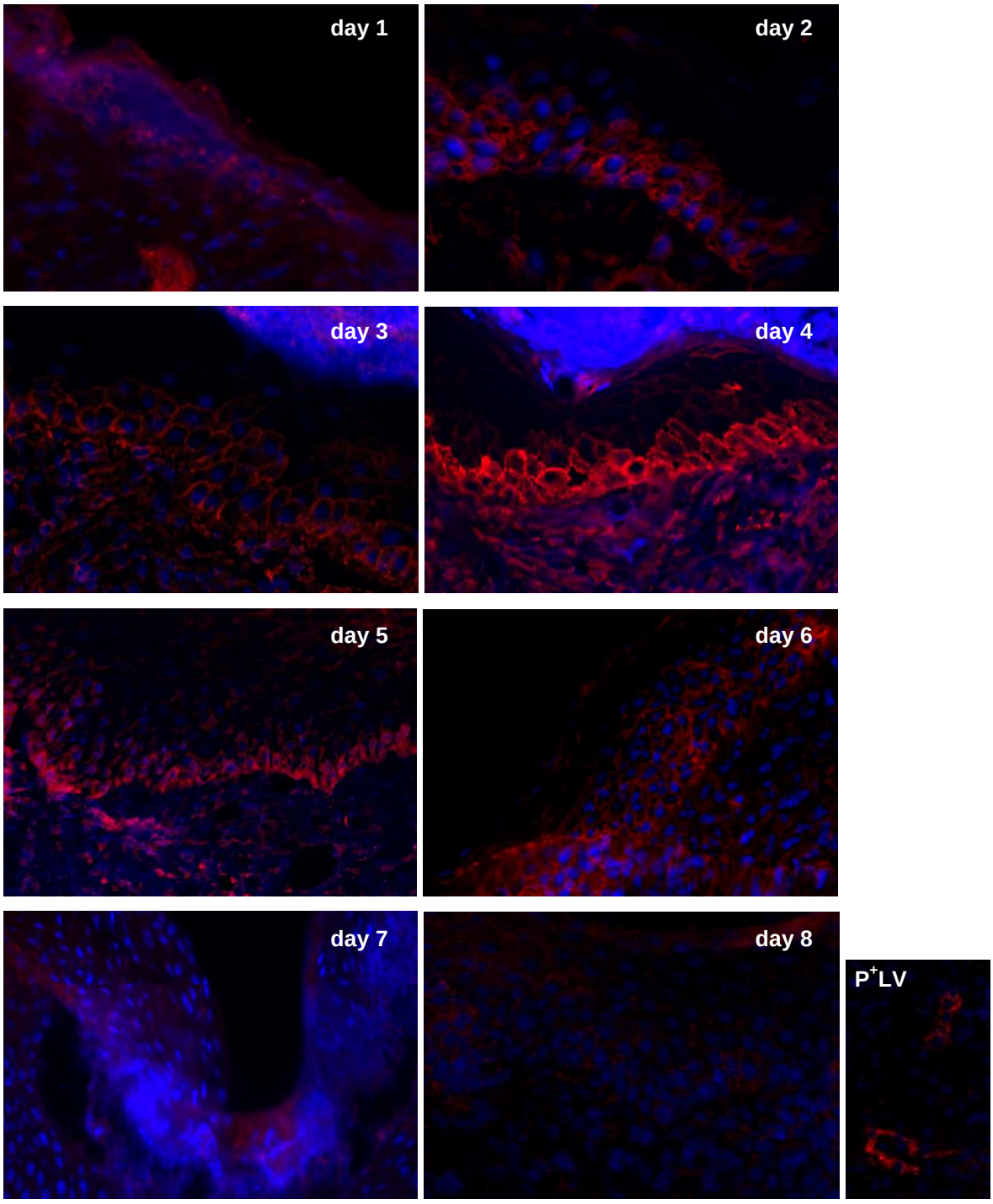

Figure 9: Podoplain is expressed in the basal keratinocyte layer of healing epidermis, throughout the whole process of reepithelialization. The figure shows podoplanin stainings for every 24 hours from day 1 until day 8. At around day 8 the whole wound area is covered with a newly formed keratinocyte layer. Podoplanin expression in basal keratinocytes of healing epidermis seems to last throughout the whole sequence of reepithelialization. At the end of epidermis formation podoplanin expression seems to cease, until it is almost completely gone at around day 8 (successful staining for podoplanin at day 8 is indicated by podoplanin$^+$ lymphatic vessels P$^+$LV in the sample)

4.3 Podoplanin is expressed in healing epidermis also distant from the invading front

Interestingly, I was able to detect podoplanin not only at the invading front of the epidermis, but along the whole migration distance, from the initial wounding site to the invading edge of the epidermis (see figure 10). This result makes it difficult to believe in a function of podoplanin which is restricted solely to the invading front of the forming epidermis, as described for invading tumours.

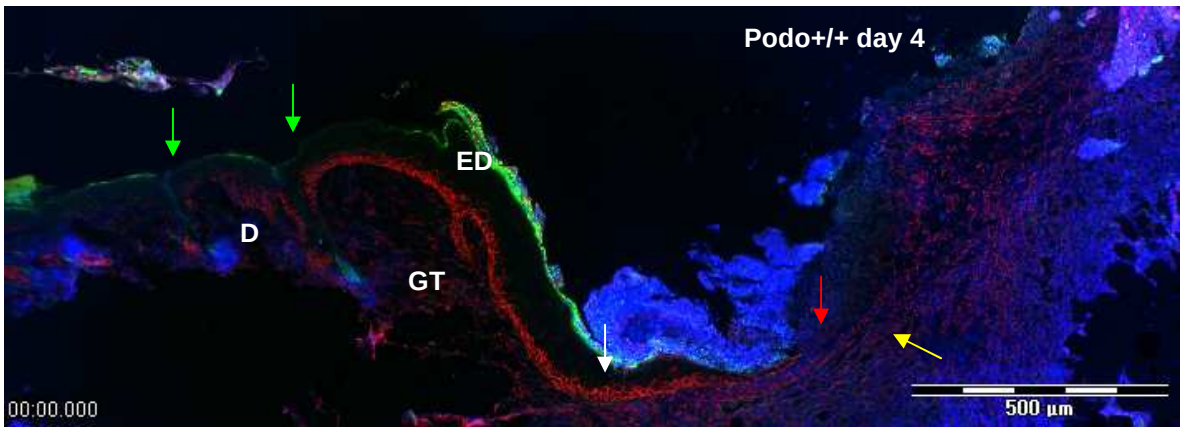

Figure 10: Day 4 wounds clearly show expression of podoplanin (red) in the basal keratinocyte layer (white arrow). The pale, green area above the basal layer shows the full thickness of the keratinocyte layer. Obviously, podoplanin is expressed even at locations far away from the actual migration front of the keratinocyte sheet (red arrow) and affects even areas between the first hair roots behind the wound area (green arrow). Podoplanin positive fibroblastoid cells within the granulation tissue are indicated by a yellow arrow.
D dermis; ED epidermis; GT granulation tissue.

Distinct expression of podoplanin can be even observed in pre-existing epidermis after wounding. The hair roots which are indicated by green arrows are parts of pre-existing epidermis at the wound edge, and we can see a clear expression of podoplanin in basal keratinocytes, between these hair roots. The epidermal areas behind these hair roots show that podoplanin expression is not a general phenomenon of basal keratinocytes, since these areas do not express the protein. So, we can assume that the signals (still unknown) which trigger the expression of podoplanin even affect areas, which do not directly belong to the invasion site (red arrow). This in turn, leads to the assumption, that podoplanin is active during general, basic rearrangements of the epidermis.

To date we do not have any explanation for the presence of podoplanin in these distant areas. As hair roots supply the wound with additional keratinocytes derived from stem cells, one possibility could be that podoplanin plays a role during keratinocyte migration or proliferation to supply the invasive front with additional cells.

4.4 Podoplanin$^{-/-}$ mice show impaired wound healing in macroscopic studies

Keratinocytes of the basal skin layer and dermal fibroblasts around healing wounds show remarkable expression of podoplanin, which we do not observe in healthy unwounded skin. The basal skin layer is the migratory and proliferative active part of the epidermis. Therefore one might hypothesize that podoplanin fulfils similar functions during wound healing as during tumour invasion and metastasis and podoplanin positive keratinocytes show a higher invasive potential into the wound area, than keratinocytes which do not express this protein.

Comparing wounds of podoplanin wild-type and knock-out mice will allow us, to assess differences in the wound healing capacity depending on the expression of podoplanin.

To get a first idea about these differences, I introduced two 6mm excisional biopsy punch wounds onto the shoulders of 7 podoplanin knock-out and 7 wild-type mice, resulting in 14 wild-type and 14 knock-out wounds. For the wound introduction, only the dermis and the epidermis were cut away. These wounds were observed for the following 13 days and pictures were taken everyday.

The first criteria I wanted to analyse, was the time the wounds need to close. Complete wound closure was defined as, the development of a bright, pale, scar-like skin surface, from a red and crustal wound surface.

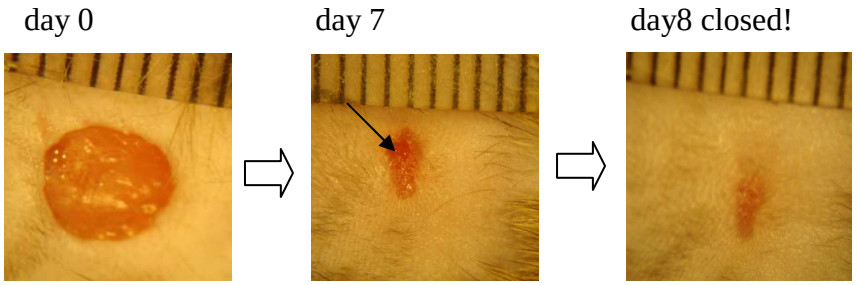

Figure 11: Wound closure in an average podoplanin Wt mouse. At day 8 a blood red wound surface was not visible anymore and the wound seems to be completely sealed.

Statistical analysis of the wound closure time for all introduced wounds revealed, that wound closure in podoplanin WT mice happens significantly faster (see figure 12). Complete wound closure in KO mice seems to be approximately 2 days delayed, as it happens earliest at day 9, compared to WT mice which show first events of wound closure already at around day 7. The whole batch of WT wounds shows complete closure between day 7 and day 11, whereas KO wounds close between day 9 and day 13.

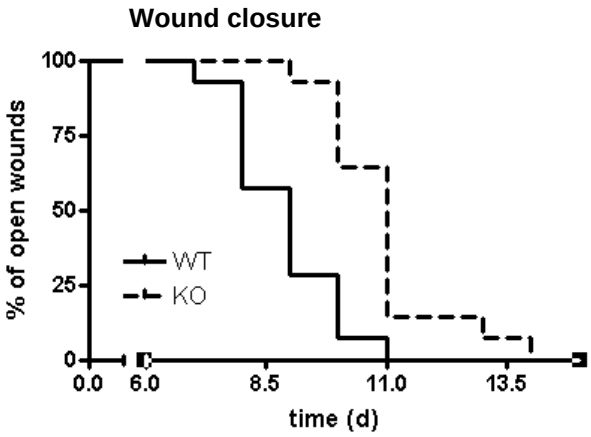

Figure 12: Statisitcal analysis of cutaneous wound closure in podoplanin KO and Wt mice. The chart shows the percentage of open wounds over time. WT wounds obviously begin to close at around day 7 and at day 11 all WT wounds are closed. KO wounds begin to close at around day 9 and even at day 13 some few wounds are not closed yet.

WT: n = 14
KO: n = 14
p = 0.0001

4.5 Podoplanin deficient mice show impaired wound contraction

Subsequently, I wanted to analyze how both types of wounds develop during the course of time. For this, two criteria were taken into account:

1. The visible wound area
2. Change of the wound shape as an indicator for wound contraction

The visible wound area was considered as the red area lacking any obvious epidermal ingrowth (brighter, pale area), the change of the wound shape as an indicator for the contraction of the wound, was measured as the ratio between the vertical and the horizontal wound extension (see Figure 14). For the macroscopic assessment of the wound contraction we hypothesized that the skin shows a higher flexibility in the axis from head to detail than around the mouse torso; therefore the wounds should become a gap-like like shape as consequence of contractile forces. The pictures of the wound healing processes in wild-type and knock-out mice suggest an impaired healing progress in podoplanin KO mice. Surprisingly healing of WT wounds seems to be characterized by a higher degree of wound contraction (see Figure 15). This is indicated by a massive decrease of the wound area (nearly 50%) and by visible distortions of the wound shape in wounds of podoplanin$^{+/+}$ mice already at day 2. Podoplanin seems to influence the initial phase of wound contraction, which basically depends on immigrated fibroblasts and the polymerization of actin into F-actin within these cells[47]. As I mentioned before, podoplanin can be detected at dermal fibroblasts during skin remodelling and it might play a role during fibroblast migration into the wound area[41].

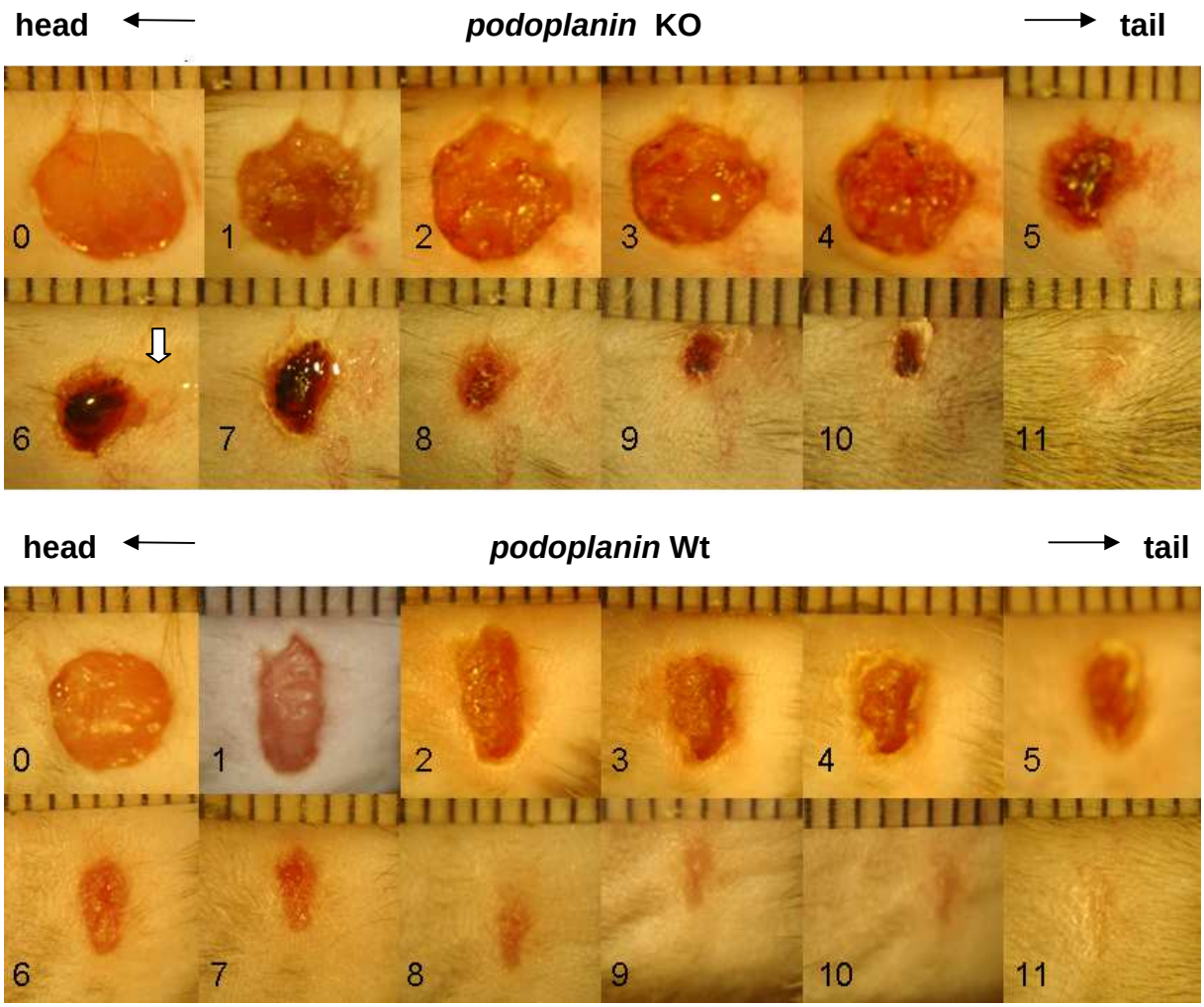

Figure 13: Comparison of wound healing in podoplanin KO and Wt mice. Mice were wounded (6mm biopsy punch wound) and healing was observed for 13 days, photos were taken every 24 hours. KO mice seem to show impaired wound healing, which could be due to decreased wound contraction. Furthermore some knock-out animals show efflux of a transparent fluid (white arrow), this could indicate malfunction of lymphatic vessels around the wound area. Numbers indicate the day after wounding.

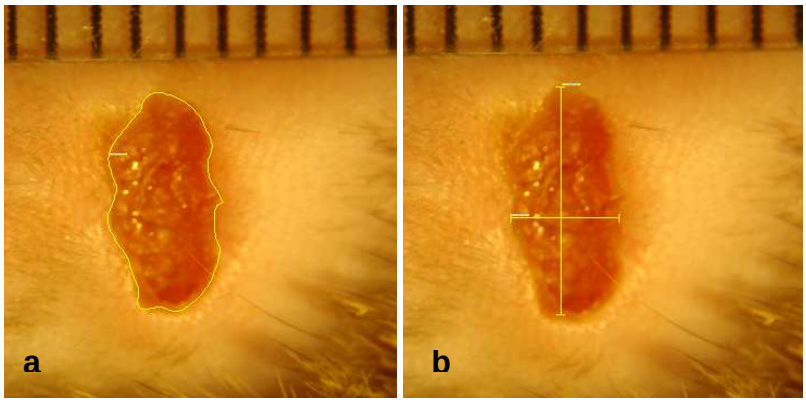

Figure 14: Image analysis of the wounds measuring the wound area (a) and the ratio between vertical and the horizontal wound extension, yellow lines indicate the measurements. µm and µm^2 are given automatically by the software.

Evaluation of all 28 wounds by image analysis and statistical analysis for every day shows that the reduction of the wound area and the appearance of wound contraction are significantly impaired in podoplanin KO mice (see Figure 15).

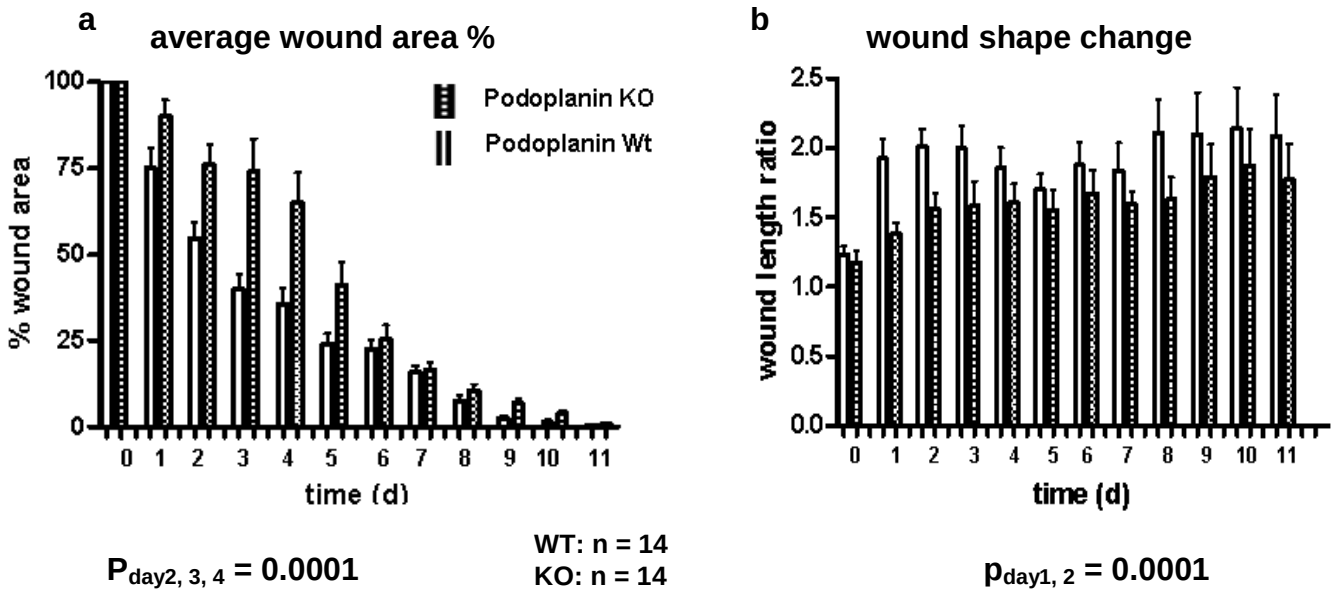

Figure 15: Statistical analysis of the reduction of the wound area in percent of the original area (a; average wound closure) and of the wound contraction. The p values of 0.0001 designate the results as significant for the days 2, 3 and 4 in the decrease of the wound area and for the days 1 and 2 in the change of the wound shape.
a: Podoplanin KO mice seem to be impaired in decreasing the size of the wound area, starting at the very beginning.
b: Wounds of podoplanin KO mice show a lesser degree of changing the wound shape which indicates impaired wound contraction, especially in the very first and the last part of wound healing.

As we can see in the left chart, the decrease of the wound area over time is homogenous in both, podoplanin knock-out and wild-type mice. This decrease is indeed faster in wild-type mice, and basically wound healing in both types of mice show a rather linear healing process. Concerning wound closure we see the biggest difference at day 4 after wounding, a time point where the level of reepithelialization and the expression of podoplanin are highest. After that time point wound healing in knock-out mice seems to catch up, until wound closure in wild-type increases again between day 8 and day 9. This increase can not be explained by reepithelialization, as the production of new epidermis is either completed or really subsided at that point (indicated in figure 9). An event during wound healing which often coincides with day 7 or day 8 is the switch from fibroblasts to myofibroblasts, which function as the contractile element during the end of wound healing[36]. So, the significant decrease of the

wound area in wild-type mice compared to knock-out mice could be due to an increased fibroblast and myofibroblast activity.

The right chart shows the effect of contractile forces on the overall shape of the wound in KO and WT mice. The degree of contraction by this measure is most prominent between day 1 and 4 and day 7 to 9, the latter phase of contraction coincides with the transformation of fibroblasts into myofibroblasts. Both podoplanin WT and KO mice show a similar time-course of wound contraction during the observation period, however in KO mice this contraction seems to happen at a lower level. The sequence of contraction events resembles the population of the wound with contractile cells, as described in the literature. During the first 5 days after wounding, fibroblasts mainly contribute to wound contraction, after that day myofibroblasts appear which leads to the highest state of wound contraction[47].

4.6 Impaired wound contraction in podoplanin$^{-/-}$ can be confirmed by histological studies

To confirm the fact that podoplanin expression contributes to wound contraction, I analyzed histological samples of day 4 excisional wounds. For this, 5 WT and 5 KO animals (1 KO animal was lost at day 2) were prepared by introduction of 2 6mm circular excisional wounds per animal. At day 4 10 WT wounds as well as 8 KO wounds were harvested and histological sections were analyzed by Hematoxylin and Eosin staining and morphometric image analysis. Wound contraction was calculated in percent, as the decrease of the distance between the margins of the dermis (see figure 19; black lines), which is 6000 µm directly after wounding and reduces during wound healing solely by wound contraction activities.

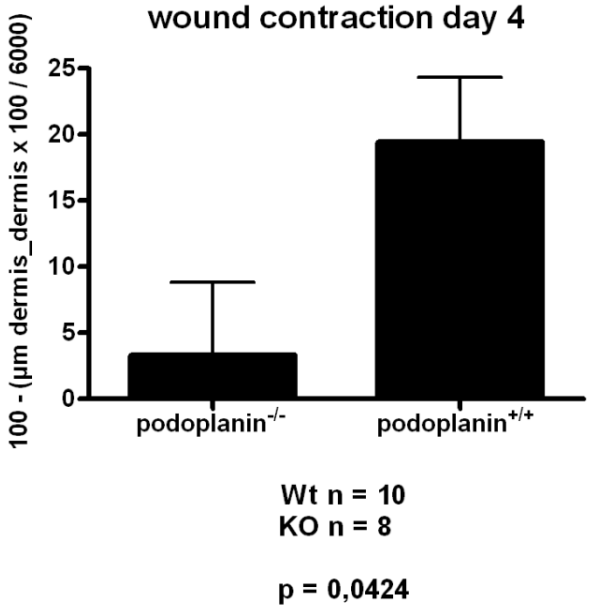

Figure 16: Histological evaluation of WT and KO mice significantly shows that podoplanin deficiency impairs wound contraction. KO wounds show an average decrease of the distance between the dermal margins of about 3%, whereas WT wounds decrease by about 20% of this distance.

By this measurement of the wound contraction, it can be shown that podoplanin deficient mice have a significantly lower wound contraction potential than podoplanin WT mice. This result is consistent with our macroscopic observations. Taken together it seems to be true that podoplanin positively influences wound contraction.

4.7 Impaired wound healing in podoplanin KO is not caused by reduced reepithelialization

To analyze the definite effect of podoplanin on reepithelialization during wound healing, I abolished the contributory effect of wound contraction.

For this I fixed two plastic rings, each onto one shoulder of one mouse. Into the middle of these rings I introduced a 4mm circular wound (see figure 17; 6mm circular wounds are not desirable because of the size of the whole construct). In consequence, the plastic rings will prevent wound contraction and the process of wound healing is solely restricted to reepithelialization.

In the following I will term these wounds as tension wounds.

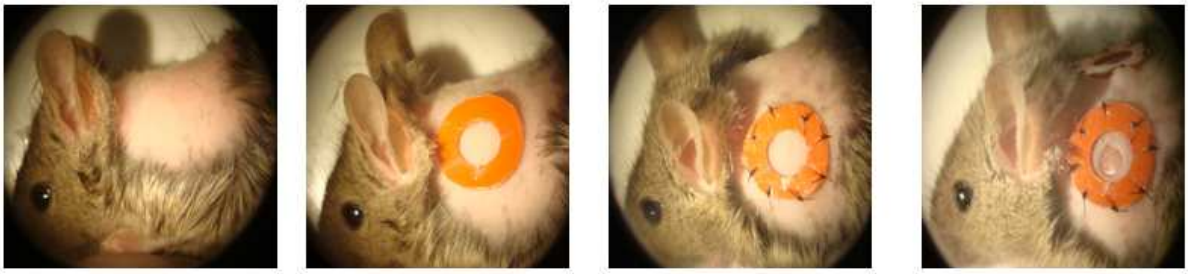

Figure 17: Generation of tension wounds to prevent wound contraction and to restrict healing to reepithelialization. The full procedure is described in Materials and Methods.

Altogether 6 podoplanin$^{-/-}$ and 6 podoplanin$^{+/+}$ mice were wounded, resulting in 12 wounds for each genotype. After wounding, the wounds were monitored for the following 4 days. Since at this time reepithelialization reaches a maximum, mice were sacrificed and the wounds were analyzed histologically.

A macroscopic estimation of the healing progress did not reveal any significant correlation between reepithelialization and podoplanin expression (see figure 18).

Day 4

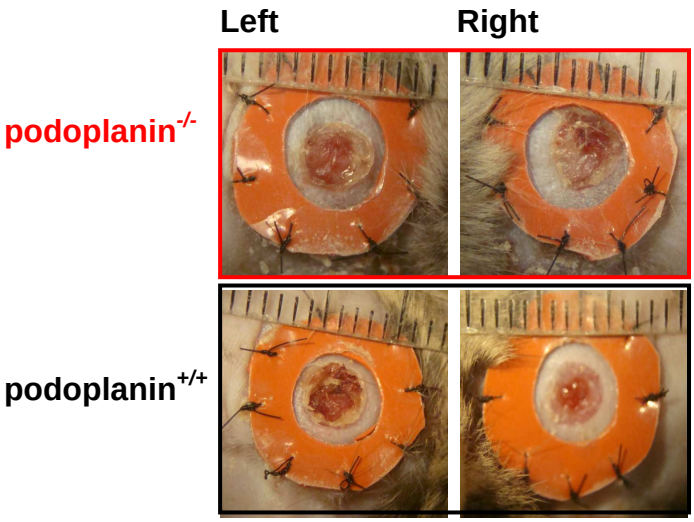

Figure 18: A macroscopic example of podoplanin$^{+/+}$ and podoplanin$^{-/-}$ tension wounds. An obvious difference between these wounds can not be observed.

To analyze reepithelialization, i.e. the development of new epidermis, I had to terminate wound healing at some specific point (day 4) to measure the formation of epidermis histologically.

Sections of podoplanin$^{-/-}$ and podoplanin$^{+/+}$ wounds were histologically stained using Hematoxylin and Eosin (see figure 19) and the newly formed epidermis was measured using image analysis. For this the newly formed epidermis was defined as the keratinocyte proliferation from the dermal margins to the outermost invasion site of the epidermis.

I was not able to detect a significant difference in the wound reepithelialization between podoplanin$^{-/-}$ and podoplanin$^{+/+}$ wounds. Although the difference is not significant, it even seems that the loss of podoplanin on the cell surface supports reepithelialization (see figure 20). If this comes true, this would be very surprising as it is broadly accepted that podoplanin supports cellular invasion, which should actually lead to the opposite effect.

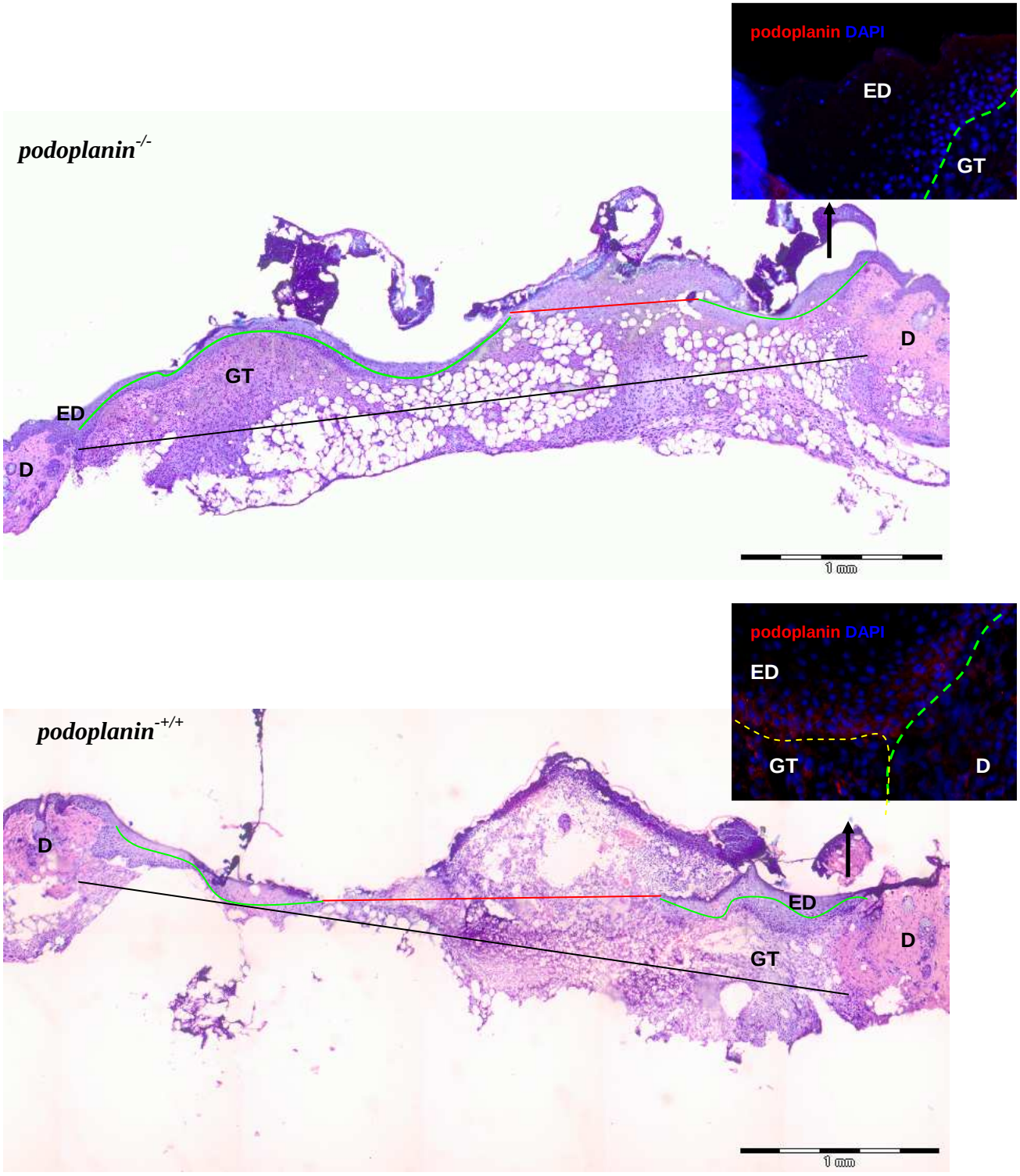

Figure 19: Histological comparison between podoplanin$^{-/-}$ and podoplanin$^{-+/+}$ wounds at day 4.
Green curves: keratinocyte migration distance; red lines: non-epithelialized area; black lines: distance between original wound margins after wounding.
Fluorescent images show podoplanin stainings (red) in the KO and the WT wound.
D dermis; ED epidermis; GT granulation tissue.

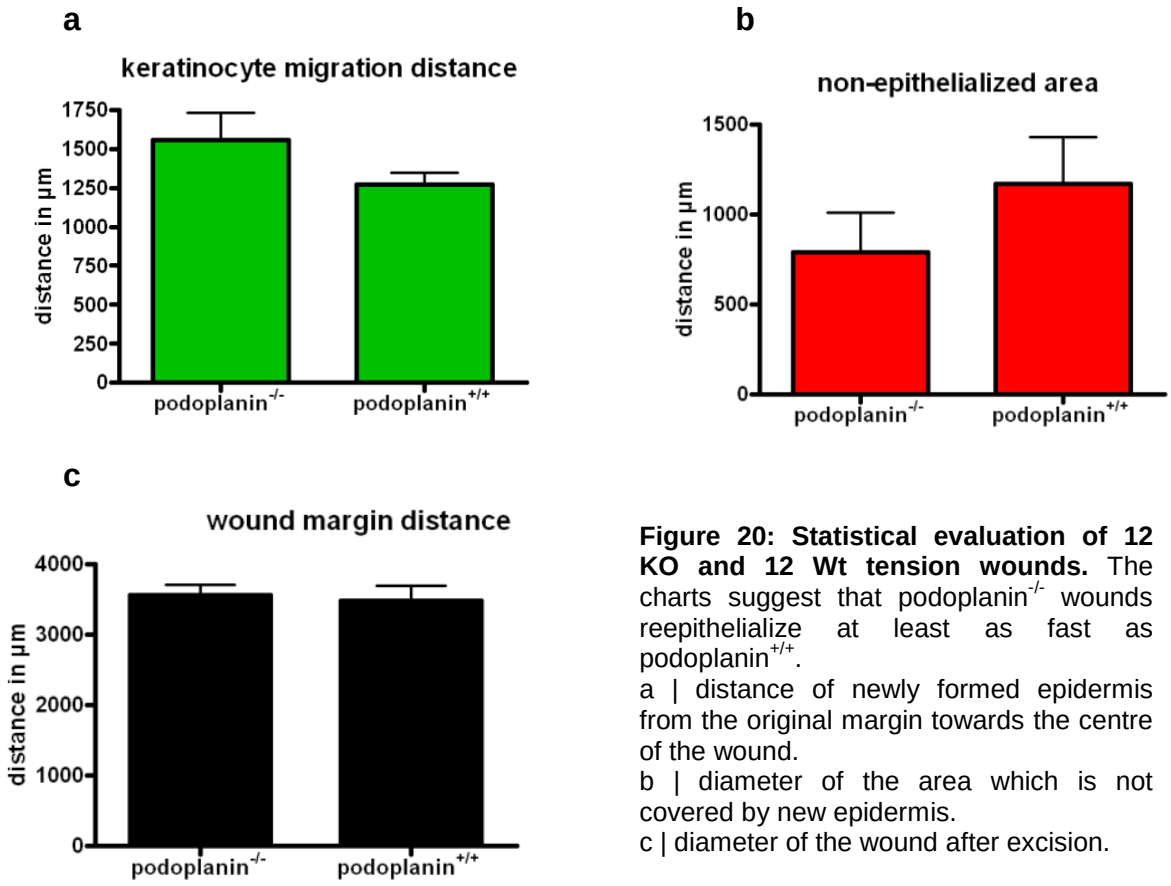

Figure 20: Statistical evaluation of 12 KO and 12 Wt tension wounds. The charts suggest that podoplanin$^{-/-}$ wounds reepithelialize at least as fast as podoplanin$^{+/+}$.
a | distance of newly formed epidermis from the original margin towards the centre of the wound.
b | diameter of the area which is not covered by new epidermis.
c | diameter of the wound after excision.

Since the statistical evaluation of all 12 KO and WT wounds does not show any significances, one can at least postulate that increased wound healing in WT mice is probably not caused by an increased level of reepithelialization. Therefore, these results suggest that the function of podoplanin in cutaneous wound healing does not consist in supporting migration of keratinocytes into the wound area.

4.8 Podoplanin does not influence keratinocyte proliferation

To assess a possible effect of podoplanin on keratinocyte proliferation I performed antibody staining for Ki-67, a protein solely expressed during the cell cycle, which is therefore an excellent marker for proliferating keratinocytes. The antibody was used to stain excisional wounds 4 days after wounding.

As indicated in figure 21, we did not see any obvious difference in the amount of proliferating keratinocytes in the basal epidermal layer.

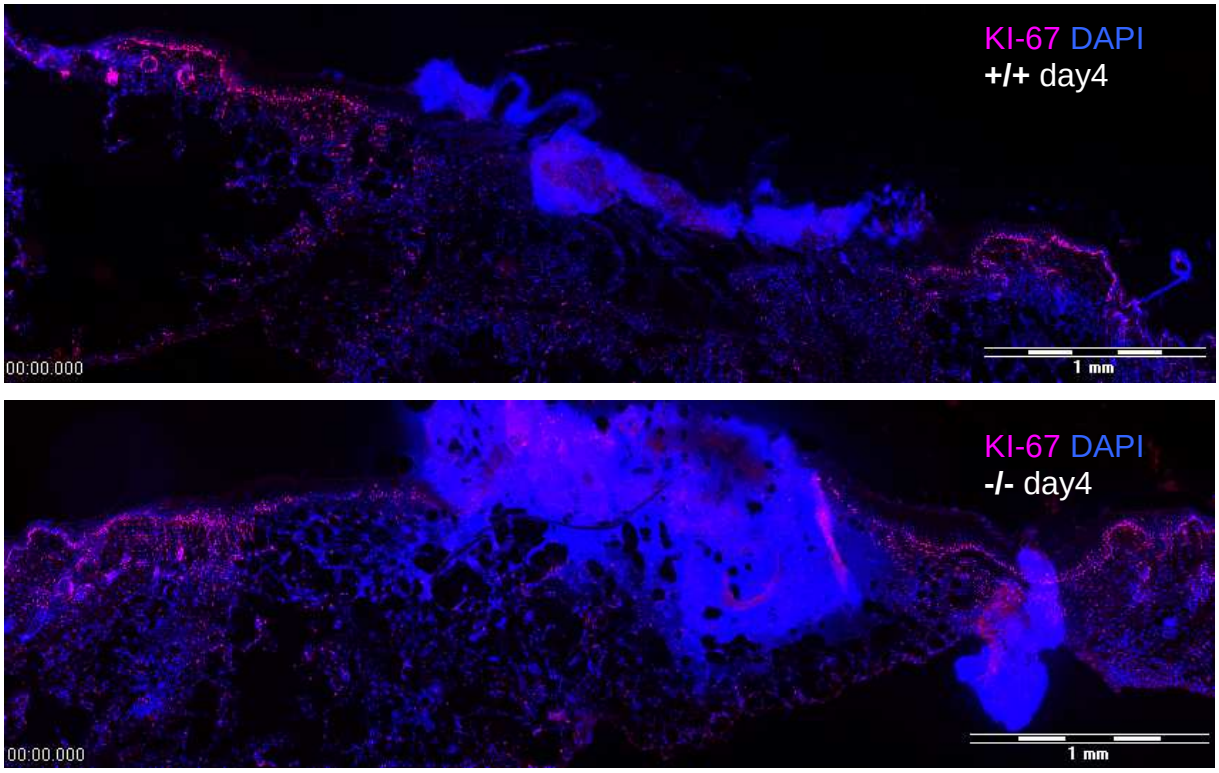

Figure 21: Ki-67 staining (red) for day 4 podoplanin WT and KO wounds. The obtained signal does not reveal any relevant difference concerning keratinocyte proliferation.

This and the previous results suggest that podoplanin does not accelerate reepithelialization. This supports the hypothesis that podoplanin supports wound healing by influencing a different physiological process, likely wound contraction.

4.9 Podoplanin co-localizes with F-actin in basal keratinocytes

As previous studies described, podoplanin is able to interact intracellularly with actin via the adapter protein ezrin[25]. This interaction triggers the appropriate cells to rearrange their actin cytoskeleton and to increase their migratory properties. Indeed, this effect was shown for carcinoma cells[27] and kidney cells[26]. In this experiment I wanted to find hints on the interaction between actin and podoplanin in epidermal wound healing. To make the two proteins visible by fluorescence microscopy, I used a monoclonal antibody against podoplanin and fluorescence labelled phalloidin, a toxin from Amanita phalloides (death cap), which specifically binds actin.

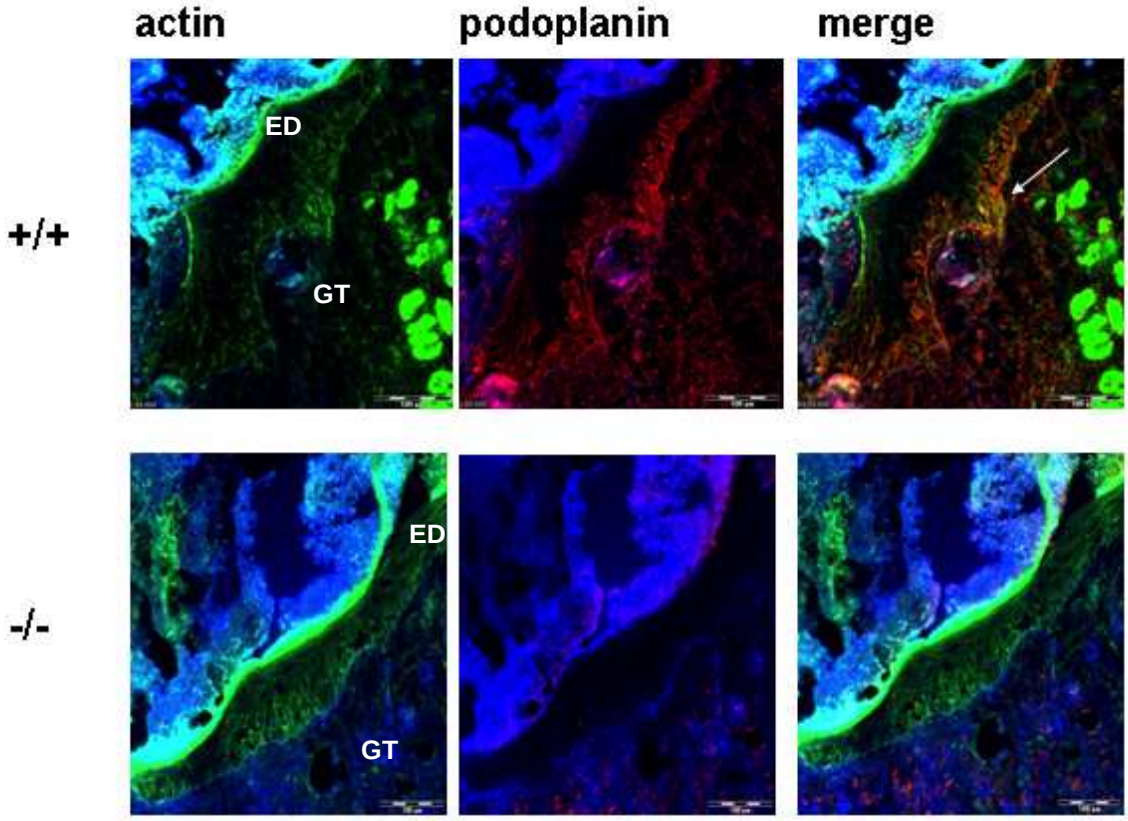

Figure 22: Podoplanin co-localizes with actin at the basal membrane of healing epidermis. Costaining of podoplanin (red) and actin (green) leads to the assumption that both proteins interact with each other, in order to rearrange G-actin towards F-Actin filaments.

The fluorescence images reveal that intense actin staining in healing podoplanin$^{+/+}$ epidermis is solely restricted to the basal epidermal area, where we expect podoplanin expression. In fact, co-staining of podoplanin and actin shows that both proteins are clearly present in the same cell layer, in wounds of podoplanin$^{+/+}$ mice.

In contrast to this, in newly formed epidermis of podoplanin$^{-/-}$, actin seems to be localized over the whole epidermal tissue and just a weak concentration of F-actin bundles can be observed in the basal region.

The notion that podoplanin affects the organization of the actin cytoskeleton of keratinocytes is supported by the fact, that in some cases podoplanin expressing solitary keratinocytes can be observed, which reside in higher epidermal layers. In these cells the actin cytoskeleton differs remarkably from the surrounding podoplanin non-expressing cells, in terms of polymerization of actin into filaments (see figure 23).

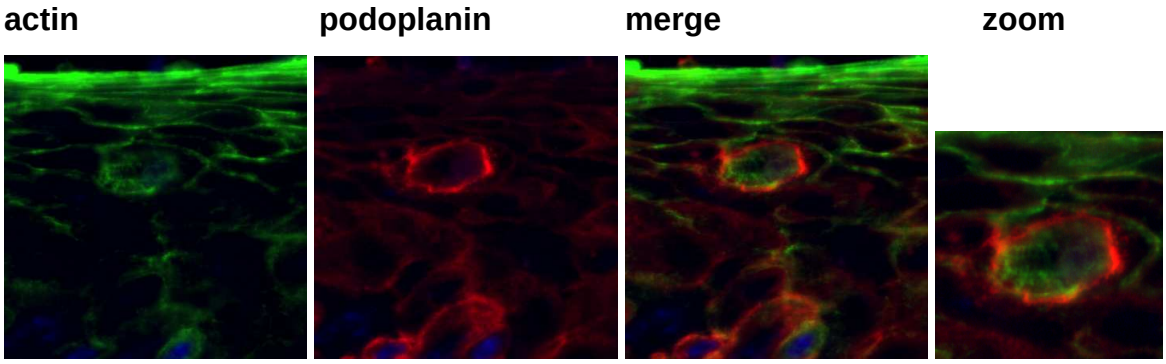

Figure 23: Podoplanin seems to lead to intracellular polymerization of actin to F-actin bundles.

According to the literature, podoplanin interacts with actin, in order to promote cell motility. Since I could not find any relation between podoplanin expression and increased cell migration of keratinocytes, it is likely that the podoplanin mediated rearrangement of the actin cytoskeleton fulfils functions beside the support of cell migration.

5. Additional Preliminary Results

5.1 Podoplanin possibly participates in interactions between keratinocytes and fibroblasts

To analyze molecular differences between podoplanin$^{+/+}$ and podoplanin$^{-/-}$ mice, concerning wound contraction, it is necessary to pay attention on the cells which are mainly responsible for this phenotype, i.e. fibroblasts within the wound tissue. The fibroblast density within the granulation tissue seems to be high 5 days after wounding. So, I analyzed appropriate excisional wounds using a monoclonal antibody against podoplanin and fluorescence labelled phalloidin, since the action of wound fibroblasts relies on the function of F-actin.

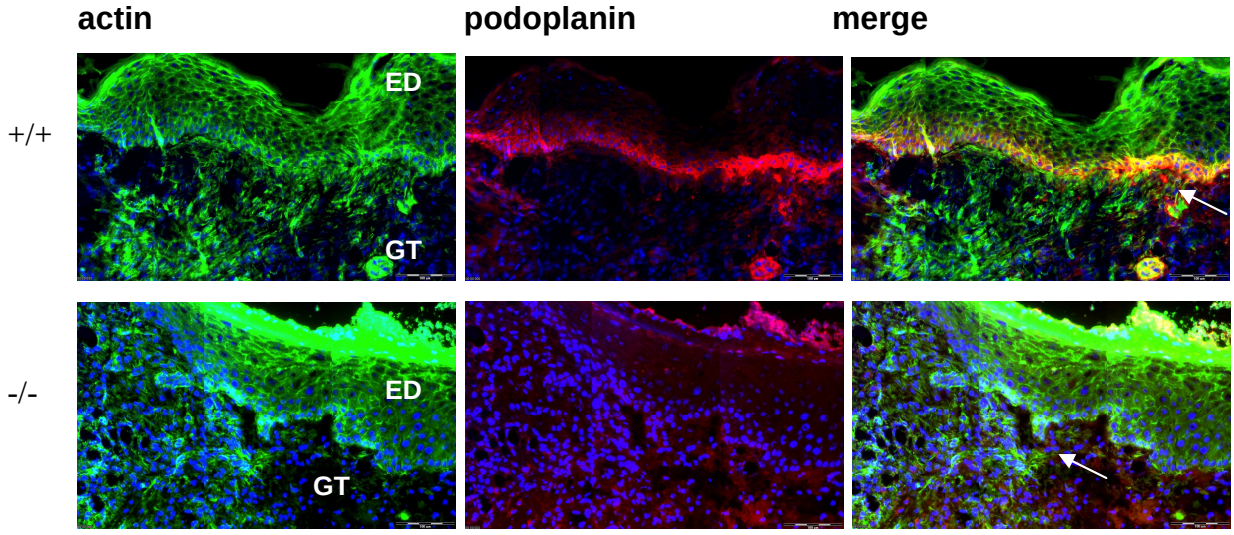

Figure 24: Actin and podoplanin staining in 5 day Wt and KO wounds. The staining shows fibroblasts (white arrows) of the granulation tissue in close vicinity to the dermis. The podoplanin positive wound is characterized by a higher amount of fibroblasts which may be orientated to connect the upper lying epidermis.

Fluorescent images were taken from the granulation tissue next to the dermis and directly below the newly formed epidermis, since this is the region to where fibroblasts migrate into the wound area. First of all, we observed a higher abundance and density of wound fibroblasts in case of podoplanin positive wounds, this suggests the possibility that podoplanin might support an increased collective migration of fibroblasts, as we have seen during my first experiment (see figure 24).

Interestingly fibroblasts seem to directly interact with cells of the epidermis, and the degree of interaction seems to correlate with the intensity of podoplanin expression. In case of podoplanin negative wounds, we observe a rather unorganized pattern of wound fibroblasts, which do not seem to be connected to epidermal cells. Consequently, these observations

suggest that podoplanin possibly plays a role during fibroblast migration into the wound area and during association of these fibroblasts with podoplanin expressing keratinocytes.

There is another possibility which could result in a phenotype which would be characterized by an increased amount of fibroblastoid structures right below the podoplanin expressing epidermal layer. It could be shown that mesenchymal cells can have an epithelial origin; these cells develop in a process called epithelial-mesenchymal transition (EMT). It is known that podoplanin supports a process which is reminiscent of EMT. Probably, development of fibroblasts out of podoplanin expressing keratinocytes

5.2 Podoplanin might play a role in lymphatic physiology after wounding

A secondary finding during this study was the observation, that some KO wounds secrete a transparent liquid (see figure 13 white arrow). This liquid really seems to arise from the wound, as it can be observed at the same position over a couple of days. These wound exudates can be caused by malfunctions of the lymphatic system. If lymphatic vessels do not become occluded after injury, interstitial fluid might extravasate from these severed vessels.
Following injury the coagulation of platelets is an important factor to occlude bleeding blood vessels. Additionally platelets have the capability to interact with podoplanin via Clec-2, as described above. As lymphatic endothelial cells express podoplanin constitutively, it is reasonable to ask, whether platelets which extravasate from severed blood vessels interact with podoplanin on severed lymphatic vessels to initiate their sealing. As we have seen in the introduction, the interaction between podoplanin on lymphatic vessels and Clec-2 on platelets leads to separation of the lymphatic system from the blood vascular system during embryonic development. This process ends up in occlusive separation of both circulatory systems. It is conceivable that a similar process is initiated by platelets, which enter disrupted lymphatic vessels following injury. Elimination of such a physiological process by podoplanin deficiency might explain the secretion of fluid by podoplanin KO wounds.
To investigate this hypothesis, I stained wound tissue sections 3 days after wounding, using antibodies against Lyve-1, a general marker for lymphatic vessels and CD41, a protein expressed on platelets.

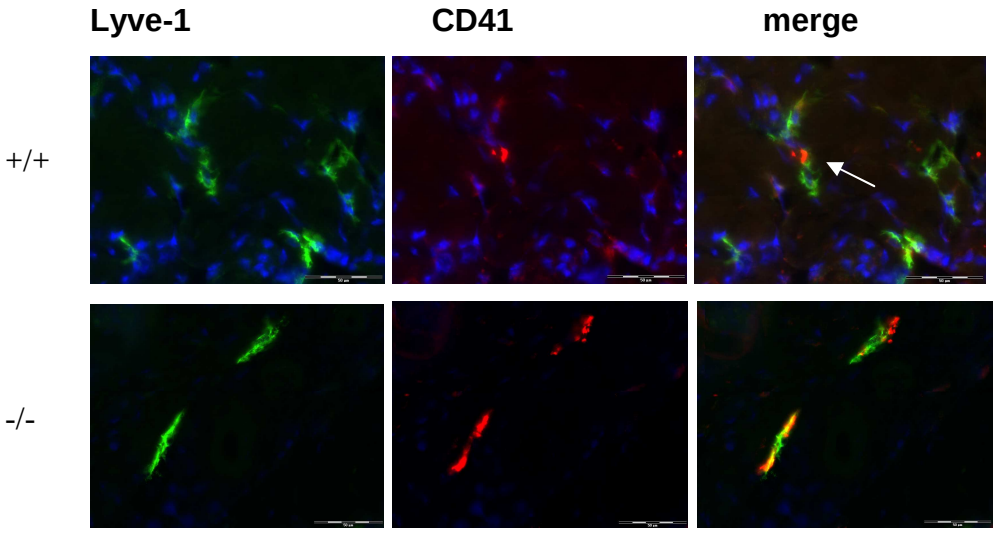

Figure 25: Lyve-1 and CD41 staining for lymphatic vessels and platelets podoplanin KO and Wt mice. The images show dermal areas right beside the wound granulation tissue, where blood from severed blood vessels might enter disrupted lymphatic vessels. Platelets possibly lead to similar separation and occlusion events in podoplanin expressing lymphatic vessels, as during embryonic development. Podoplanin negative tissue clearly shows blood filled lymphatic vessels.

The images were taken from dermal areas in close vicinity to the actual wound area, as this is the expected site where platelets could probably enter disrupted lymphatic vessels.

In fact, lymphatic vessel and platelet staining answer our expectations. Concerning podoplanin positive wounds we can see a site where platelets seem to contact a lymphatic vessel, in order to initiate lymphatic separation. In any case, we were not able to observe lymphatic vessels which were filled with platelets around podoplanin WT wounds.

A different situation is observable in podoplanin KO mice, where we obtain a clear platelet signal from the inside of lymphatic vessels around the wound area. Many of the lymphatic vessels near the wound area of podoplanin KO mice seem to be wide open and intact (not shown).

These data could be a good starting point, to investigate the interaction between platelets and lymphatic vessels during the wound healing. However, since this is not the focus of this study many things remain elusive and should be studied by further experiments.

6. Discussion

The fact that podoplanin, a 43 kDa mucin-type transmembrane sialoglycoprotein is present on the surface of basal keratinocytes and dermal fibroblasts during wound healing, was already described 1997 by Alberto Ganderillas et al[41]. Surprisingly, hitherto nobody described a specific role for this protein in cutaneous healing processes. The cellular function of podoplanin and its regulation are still unclear. However, it seems to be a key player in a variety of processes during which a higher degree of cell motility is required, for example tumour invasion and metastasis as well as developmental processes[26,27,17].

All in all, my studies confirm and expand the results obtained by the group around Alberto Ganderillas. Podoplanin is expressed during reepithelialization of cutaneous wounds by basal keratinocytes of newly formed epidermis. Also dermal fibroblasts inside the wound area and in its close vicinity induce the expression of podoplanin. Our assessment of the time course revealed strong induction of podoplanin protein expression in keratinocytes at day 3 post-wounding and the loss of expression when reepithelialization is completed. Podoplanin can not be detected in the intact, normal epithelium. Upon wounding the expression of podoplanin becomes induced in the activated epithelium around the wound margins including also nearby hair roots. However interestingly, podoplanin expression can be identified at positions of the epidermis behind the actual invasion site. This is insofar interesting as it is not consistent with previous observations of invading and metastasising tumours, which suggest, that podoplanin is expressed only where tumour cells invade tissues of the periphery. The fact that podoplanin is apparently expressed during the whole sequence of reepithelialization and the fact that it is expressed over a wide epidermal area which is affected by the wound, makes podoplanin to a protein which might participate in a general aspect of wound healing. Due to this, it is hard to believe in a restricted function of podoplanin during processes like matrix degradation directly at the epidermal invasive front, the inflammatory response in the beginning or tissue remodelling in the end of wound healing.

Considering the literature which describes podoplanin as a molecule increasing cell motility, it seems likely that keratinocytes at the wound margins - upon expression of podoplanin – might gain a higher migratory potential compared to podoplanin deficient keratinocytes and this might cause faster wound closure in case of podoplanin WT mice. Support of this expectation was provided by the result that over-expression of podoplanin in NIH3T3 cells

increased “wound healing” in the so called scratch wound assay in cell culture. This observation is consistent with the results of Andreas Wicki et al[27], which describe collective invasion of pancreas tumour cells, which are transfected with the coding region of podoplanin behind the insulin promoter. Wicki et al mention, that this phenomenon depends on the ability of podoplanin expressing cells to migrate by maintenance of cell-cell junctions (i.e. E-cadherin). This includes the possibility for the formation of new tissue on a higher level of organization, since collective migration allows the invasion of whole cellular sheets instead of single cells which have to reorganize into functional tissues. This process is highly desirable, to achieve efficient closure of cutaneous wounds.

By monitoring podoplanin deficient and podoplanin expressing wounds for a period of 2 weeks, significant differences can be observed. In this macroscopic study, WT wounds seem to be completely closed approximately 2 days earlier than podoplanin KO wounds. Corresponding to the opinion that podoplanin enhances the migratory potential, we first had hypothesized that accelerated wound healing might be caused by a higher motility of keratinocytes, which would lead to a higher reepithelialization rate.

Unexpectedly, we were unable to detect a significant difference in the migration distance of keratinocytes from the original wound margins towards the middle of the wound of podoplanin$^{+/+}$ and $^{-/-}$ animals. Nor did we note any gross difference in the organization of the newly formed epidermis.

However, the observation was made that WT wounds are characterized by a higher degree of wound contraction. Wound contraction does not depend on reepithelialization, but on the action of fibroblasts and myofibroblasts within the granulation tissue[47]. By macroscopic inspection alone it is difficult to decide whether faster wound healing of WT wounds is a result of increased development of new epidermis or of increased contraction of the wound. Evaluation of histological sections of day 4 excisional wounds confirmed that podoplanin expression corresponds to significantly enhanced wound contraction while cell proliferation did not differ between wt and knock-out animals. Thus wound contraction seems the likely explanation for accelerated wound healing in podoplanin WT mice.

A modified version of a wound healing protocol by Robert D. Galiano[46] gave me the possibility to investigate wound healing in the absence of wound contraction. A plastic ring which was fixed around the wound stabilized the wound architecture and wound closure therefore depended solely on the formation and immigration of new epithelium. Using this model I was not able assess any significant macroscopic or histological difference between podoplanin KO and WT wounds. And the trend pointed rather to an even slightly higher

reepithelialization rates in podoplanin KO wounds. This clearly contradicts our first hypothesis that accelerated wound healing depends on a faster epidermis formation. On the other hand this finding supports the interpretation that podoplanin influences wound contraction.

In addition I was not able to discern any difference concerning the keratinocyte proliferation rate, between podoplanin$^{+/+}$ and $^{-/-}$ wounds.

Taken together, podoplanin does not seem to have any direct effect on the keratinocytes during reepithelialization processes.

One of the first insights into the function of podoplanin was the observation that podoplanin interacts with the actin cytoskeleton via the adapter molecule ezrin[25]. I was able to confirm that podoplanin leads to rearrangement of the actin cytoskeleton, within basal keratinocytes of healing epidermis. However my studies do not reveal any correlation between podoplanin-dependent actin-rearrangement and increased cellular motility. A connection between the rearrangement of the actin cytoskeleton and wound contraction is imaginable, as keratinocyte at the wound margins might participate in contracting the wound.

A related question is, if there is any intercellular connection between keratinocytes of newly formed epidermis and (myo-)fibroblasts within the wound granulation tissue. In order to promote such a connection, actin rearrangement within these keratinocytes might be advantageous. In one of my preliminary results I show, that highly organized fibroblasts of the granulation tissue show a closer connection to the epidermis when podoplanin is expressed (figure 24). However more work is necessary to confirm these data.

In the literature, a theoretical basis which explains any connection between podoplanin and tissue contraction does not exist, however during the analysis of podoplanin expressing wounds in 1997, Alberto Ganderillas et al mention that podoplanin is expressed also in dermal fibroblasts. Since fibroblasts and myofibroblasts are the key players in wound contraction, a connection between podoplanin expression in fibroblasts and wound contraction seems to be plausible.

In 2008 a group around Akikazu Kawase confirmed that podoplanin is expressed by fibroblasts of stromal cancers and that these fibroblasts tend to express α-smooth muscle actin, a protein typical for myofibroblasts. The group claims that the presence of podoplanin-positive α-SMA positive fibroblasts within stromal cancers correlates with a shorter survival time for patients, as a result of invasive alterations of the tumour[49]. Whether there is a direct molecular connection between podoplanin expression and the development of contractile

fibroblasts and myofibroblasts is yet to be clarified, however there seems to be evidence that podoplanin affects these cell types, in some manner.

The function of fibroblasts and myofibroblasts during wound healing require the formation of cell-cell- and cell-matrix-interactions which are intracellularly extended by actin filaments[50]. In this respect, fibroblasts create connections to other cells or to the extracellular matrix while they remain in their extended mesenchymal appearance. Right during this process, podoplanin might play a crucial role, as the development or the maintenance of a mesenchymal phenotype without of breaking up intercellular junctions seems to be characteristic for podoplanin expressing cells[27].

Furthermore, it could be shown that fibroblasts and myofibroblasts play crucial roles during the invasion of squamous-cell carcinoma. Myofibroblast activity seems to promote collective invasion of carcinoma cells[51]. Squamous-cell carcinoma is one of the best characterized tumour models showing a clear podoplanin expression at the invasive front. It could be shown that fibroblasts within the stroma of this cancer show podoplanin expression as well[41], the biological meaning of this fact remains to be clarified. In this respect, it is worthwhile to further investigate the connection between podoplanin expression and the development of myofibroblasts during tissue remodelling processes.

If it comes true that fibroblasts which express podoplanin during wound healing rather tend to actin rearrangements, α-SMA expression and development of junctional complexes, an explanation for decreased wound contraction in podoplanin deficient wound seems to be within reach.

As a high number of studies showed before, expression of podoplanin contributes to a more fibroblastoid cellular phenotype. This is insofar interesting as the possibility exists that myofibroblasts also arise from epithelial cells which undergo epithelial-mesenchymal transition (EMT)[52]. This raises the question if keratinocytes not only interact with myofibroblasts, but turn into this cell type themselves. As podoplanin is able to initiate a process which resembles EMT[26], it could play a crucial role during such a process. A process like this would clarify why both cell types, keratinocytes of the healing epidermis and fibroblasts of the dermis and the granulation tissue are podoplanin positive. The transition from epithelial cells to myofibroblasts and the potential role of podoplanin could be part of future studies, as it would address many questions beyond wound healing.

In a second preliminary experiment, we investigated, what happens to disrupted podoplanin-positive lymphatic vessels in the wound area, which might become exposed to platelets from disrupted blood vessels. This question is related to the already described activation of

platelets by contact with podoplanin. In the setting of wound healing this interaction might serve to seal severed lymphatic vessels and could possibly also contribute to cell activation by stimulating growth factor release from the activated platelets. According to my preliminary results, platelets could work as an occlusion factor for disrupted lymphatic vessels reminiscent to the events during embryonic development.

In this work I was able to show that the expression of podoplanin contributes to accelerated wound healing. Comparison of wound healing in podoplanin WT and KO mice on a macroscopic as well as on a microscopic level allowed me to assess the effects of podoplanin deficiency on specific events during wound healing. Interestingly, it turned out that accelerated wound healing in podoplanin WT mice relies rather on increased wound contraction than on an increase of the migratory or the proliferative potential during reepithelialization. This is insofar surprising as podoplanin was described as a molecule which enhances cell motility.

Many further experiments will be necessary to conclude the herein obtained results. This work clearly indicates that future studies should focus on the effect of podoplanin expression on fibroblast function as well as on myofibroblast development, to understand the role of podoplanin during cutaneous wound healing.

7. References

1. Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G, Kerjaschki D.: Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am J Pathol. 1997 Oct;151(4):1141-52.

2. Matsui K, Breiteneder-Geleff S, Kerjaschki D.: Epitope-specific antibodies to the 43-kD glomerular membrane protein podoplanin cause proteinuria and rapid flattening of podocytes. J Am Soc Nephrol. 1998 Nov;9(11):2013-26.

3. Breiteneder-Geleff S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, Diem K, Weninger W, Tschachler E, Alitalo K, Kerjaschki D.: Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. Am J Pathol. 1999 Feb;154(2):385-94.

4. Shibahara J, Kashima T, Kikuchi Y, Kunita A, Fukayama M.: Podoplanin is expressed in subsets of tumors of the central nervous system. Virchows Arch. 2006 Apr;448(4):493-9. Epub 2006 Jan 13.

5. Schacht V, Dadras SS, Johnson LA, Jackson DG, Hong YK, Detmar M. Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. Am J Pathol. 2005 Mar;166(3):913-21.

6. Weninger W, Partanen TA, Breiteneder-Geleff S, Mayer C, Kowalski H, Mildner M, Pammer J, Stürzl M, Kerjaschki D, Alitalo K, Tschachler E.: Expression of vascular endothelial growth factor receptor-3 and podoplanin suggests a lymphatic endothelial cell origin of Kaposi's sarcoma tumor cells. Lab Invest. 1999 Feb;79(2):243-51.

7. Breiteneder-Geleff S, Soleiman A, Horvat R, Amann G, Kowalski H, Kerjaschki D.: Podoplanin--a specific marker for lymphatic endothelium expressed in angiosarcoma.
Verh Dtsch Ges Pathol. 1999;83:270-5. German.

8. Kato Y, Sasagawa I, Kaneko M, Osawa M, Fujita N, Tsuruo T.: Aggrus: a diagnostic marker that distinguishes seminoma from embryonal carcinoma in testicular germ cell tumors.Oncogene. 2004 Nov 4;23(52):8552-6.

9. Oliver G.: Lymphatic vasculature development. Nat Rev Immunol. 2004 Jan;4(1):35-45. Review.

10. Sabin F.R.: On the origin of the lymphatic system from the veins and the development of the lymph hearts and thoracic duct in the pig. 1902 May 26; 1 (3): 367- 389 American Journal of Anatomy

11. Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG.: LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Biol. 1999 Feb 22;144(4):789-801.

12. Gale NW, Prevo R, Espinosa J, Ferguson DJ, Dominguez MG, Yancopoulos GD, Thurston G, Jackson DG.: Normal lymphatic development and function in mice deficient for the lymphatic hyaluronan receptor LYVE-1. Mol Cell Biol. 2007 Jan;27(2):595-604. Epub 2006 Nov 13.

13. Wigle JT, Oliver G.: Prox1 function is required for the development of the murine lymphatic system. Cell. 1999 Sep 17;98(6):769-78.

14. Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, Jackson DG, Oliver G.: An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J. 2002 Apr 2;21(7):1505-13.

15. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K.: Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A. 1995 Apr 11;92(8):3566-70.

16. Jussila L, Alitalo K.: Vascular growth factors and lymphangiogenesis. Physiol Rev. 2002 Jul;82(3):673-700. Review.

17. Uhrin P, Zaujec J, Breuss JM, Olcaydu D, Chrenek P, Stockinger H, Fuertbauer E, Moser M, Haiko P, Fässler R, Alitalo K, Binder BR, Kerjaschki D.: Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation. Blood. 2010 May 13;115(19):3997-4005.

18. Suzuki-Inoue K, Kato Y, Inoue O, Kaneko MK, Mishima K, Yatomi Y, Yamazaki Y, Narimatsu H, Ozaki Y.: Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. J Biol Chem. 2007 Sep 7;282(36):25993-6001.

19. Suzuki-Inoue K, Fuller GL, García A, Eble JA, Pöhlmann S, Inoue O, Gartner TK, Hughan SC, Pearce AC, Laing GD, Theakston RD, Schweighoffer E, Zitzmann N, Morita T, Tybulewicz VL, Ozaki Y, Watson SP.: A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. Blood. 2006 Jan 15;107(2):542-9. Epub 2005 Sep 20.

20. Ozaki Y, Suzuki-Inoue K, Inoue O.: Novel interactions in platelet biology: CLEC-2/podoplanin and laminin/GPVI. J Thromb Haemost. 2009 Jul;7 Suppl 1:191-4. Review.

21. Gialeli C, Theocharis AD, Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FEBS J. 2011 Jan;278(1):16-27.

22. Perl AK, Wilgenbus P, Dahl U, Semb H, Christofori G. A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature. 1998 Mar 12;392(6672):190-3.

23. Price JT, Thompson EW.: Mechanisms of tumour invasion and metastasis: emerging targets for therapy. Expert Opin Ther Targets. 2002 Apr;6(2):217-33. Review.

24. Sahai E, Marshall CJ.: Rho-GTPases and cancer. Nat Rev Cancer. 2002 Feb;2(2):133-42. Review.

25. Scholl FG, Gamallo C, Vilaró S, Quintanilla M.: Identification of PA2.26 antigen as a novel cell-surface mucin-type glycoprotein that induces plasma membrane extensions and increased motility in keratinocytes. J Cell Sci. 1999 Dec;112 (Pt 24):4601-13.

26. Martín-Villar E, Megías D, Castel S, Yurrita MM, Vilaró S, Quintanilla M.: Podoplanin binds ERM proteins to activate RhoA and promote epithelial-mesenchymal transition. J Cell Sci. 2006 Nov 1;119(Pt 21):4541-53.

27. Wicki A, Lehembre F, Wick N, Hantusch B, Kerjaschki D, Christofori G.: Tumor invasion in the absence of epithelial-mesenchymal transition: podoplanin-mediated remodeling of the actin cytoskeleton. Cancer Cell. 2006 Apr;9(4):261-72.

28. Izawa I, Amano M, Chihara K, Yamamoto T, Kaibuchi K.: Possible involvement of the inactivation of the Rho-Rho-kinase pathway in oncogenic Ras-induced transformation. Oncogene. 1998 Dec 3;17(22):2863-71.

29. Wicki A, Christofori G.: The potential role of podoplanin in tumour invasion. Br J Cancer. 2007 Jan 15;96(1):1-5.

30. Martin P.: Wound healing--aiming for perfect skin regeneration. Science. 1997 Apr 4;276(5309):75-81. Review.

31. Hackam DJ, Ford HR.: Cellular, biochemical, and clinical aspects of wound healing. Surg Infect (Larchmt). 2002;3 Suppl 1:S23-35.

32. Grazul-Bilska AT, Johnson ML, Bilski JJ, Redmer DA, Reynolds LP, Abdullah A, Abdullah KM.: Wound healing: the role of growth factors. Drugs Today (Barc). 2003 Oct;39(10):787-800. Review.

33. Burgeson RE, Christiano AM.: The dermal-epidermal junction. Curr Opin Cell Biol. 1997 Oct;9(5):651-8. Review.

34. Watt FM.: Role of integrins in regulating epidermal adhesion, growth and differentiation. EMBO J. 2002 Aug 1;21(15):3919-26. Review.

35. Nobes CD, Hall A.: Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell. 1995 Apr 7;81(1):53-62.

36. Singer AJ, Clark RA.: Cutaneous wound healing. N Engl J Med. 1999 Sep 2;341(10):738-46. Review.

37. Hinz B, Gabbiani G.: Mechanisms of force generation and transmission by myofibroblasts. Curr Opin Biotechnol. 2003 Oct;14(5):538-46. Review.

38. Bailey AJ, Bazin S, Sims TJ, Le Lous M, Nicoletis C, Delaunay A.: Characterization of the collagen of human hypertrophic and normal scars. Biochim Biophys Acta. 1975 Oct 20;405(2):412-21.

39. Levenson SM, Geever EF, Crowley LV, Oates JF 3rd, Berard CW, Rosen H.: The healing of rat skin wounds. Ann Surg. 1965 Feb;161:293-308.

40. Yan C, Grimm WA, Garner WL, Qin L, Travis T, Tan N, Han YP.: Epithelial to mesenchymal transition in human skin wound healing is induced by tumor necrosis factor-alpha through bone morphogenic protein-2. Am J Pathol. 2010 May;176(5):2247-58.

41. Gandarillas A, Scholl FG, Benito N, Gamallo C, Quintanilla M.: Induction of PA2.26, a cell-surface antigen expressed by active fibroblasts, in mouse epidermal keratinocytes during carcinogenesis. Mol Carcinog. 1997 Sep;20(1):10-8.

42. Scholl FG, Gamallo C, Quintanilla M.: Ectopic expression of PA2.26 antigen in epidermal keratinocytes leads to destabilization of adherens junctions and malignant progression. Lab Invest. 2000 Nov;80(11):1749-59.

43. Martín-Villar E, Scholl FG, Gamallo C, Yurrita MM, Muñoz-Guerra M, Cruces J, Quintanilla M.: Characterization of human PA2.26 antigen (T1alpha-2, podoplanin), a small membrane mucin induced in oral squamous cell carcinomas. Int J Cancer. 2005 Mar 1;113(6):899-910.

44. Ramirez MI, Millien G, Hinds A, Cao Y, Seldin DC, Williams MC.: T1alpha, a lung type I cell differentiation gene, is required for normal lung cell proliferation and alveolus formation at birth. Dev Biol. 2003 Apr 1;256(1):61-72.

45. Mahtab EA, Wijffels MC, Van Den Akker NM, Hahurij ND, Lie-Venema H, Wisse LJ, Deruiter MC, Uhrin P, Zaujec J, Binder BR, Schalij MJ, Poelmann RE, Gittenberger-De Groot AC.: Cardiac malformations and myocardial abnormalities in podoplanin knockout mouse embryos: Correlation with abnormal epicardial development. Dev Dyn. 2008 Mar;237(3):847-57.

46. Galiano RD, Michaels J 5th, Dobryansky M, Levine JP, Gurtner GC.: Quantitative and reproducible murine model of excisional wound healing. Wound Repair Regen. 2004 Jul-Aug;12(4):485-92.

47. Hinz B, Mastrangelo D, Iselin CE, Chaponnier C, Gabbiani G.: Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. Am J Pathol. 2001 Sep;159(3):1009-20.

48. Wipff PJ, Hinz B.: Myofibroblasts work best under stress. J Bodyw Mov Ther. 2009 Apr;13(2):121-7.

49. Kawase A, Ishii G, Nagai K, Ito T, Nagano T, Murata Y, Hishida T, Nishimura M, Yoshida J, Suzuki K, Ochiai A.: Podoplanin expression by cancer associated fibroblasts predicts poor prognosis of lung adenocarcinoma. Int J Cancer. 2008 Sep 1;123(5):1053-9.

50. Hinz B, Gabbiani G.: Cell-matrix and cell-cell contacts of myofibroblasts: role in connective tissue remodeling. Thromb Haemost. 2003 Dec;90(6):993-1002.

51. Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, Sahai E.: Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol. 2007 Dec;9(12):1392-400.

52. Radisky DC, Kenny PA, Bissell MJ.: Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? J Cell Biochem. 2007 Jul 1;101(4):830-9.

53. Okegawa T, Pong RC, Li Y, Hsieh JT.: The role of cell adhesion molecule in cancer progression and its application in cancer therapy. Acta Biochim Pol. 2004;51(2):445-57. Review.

Curriculum Vitae

Florian Koban
0402500
A441

+43 650 56 107 65
kobanflorian@hotmail.com

Date and place of birth: **4th of July 1984, Vienna/Austria**

Citizenship: **Austria**

Education:

- 2003 school leaving examination
  Wirtschaftskundliches Realgymanasium Feldgasse

- Jan. 2004 – Aug.: 2004 basic military service

- 2004 – 2006: Studies on life sciences at the University of Vienna.

- 2006 current: Studies on genetics and microbiology at the University of Vienna, Max F.Perutz Laboratories (MFPL).

- 2010 current: Diploma thesis about “the role of podoplanin in cutaneous wound healing. At the “Department for Vascular Biology and Thrombosis Research”