



PRL2/PTP4A2, a modulator of lymphatic functions and inflammation

Rarahu Magaut

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PRL2/PTP4A2, a modulator of lymphatic functions and inflammation

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Abstract

Lymphatic vessels are essential for tissue fluid homeostasis, immune cell trafficking, and dietary fat absorption. Dysfunction of the lymphatic vasculature is linked to serious diseases such as lymphedema and metastatic cancer. It has been recently shown that the protein tyrosine phosphatase PRL2 (PTP4A2) modulates blood vascular development¹. Based on the close relationship between lymphatic and blood vasculatures, we hypothesized that PRL2 likely plays an essential role in lymphatic vessels morphogenesis and function.

In chapter 2, we demonstrate that silencing PRL2 in lymphatic endothelial cells (LECs) induces a drastic decrease in cell proliferation, migration, and sprouting. PRL2 critically modulates signaling in LECs. Under VEGF-C or VEGF-A stimulation, PRL2 silencing modulated ERK and Akt signaling. Moreover, Notch1/DLL4 downstream signaling is also inhibited in these cells.

In chapter 3, a mouse model with an inducible specific deletion of PRL2 in LECs is described in order to study the role of PRL2 lymphatic vessels morphogenesis. With a secondary lymphedema model in these mice, edema was more pronounced in LEC-PRL2-deleted mice. Immune cell infiltration, especially of T-cells, was also modified. Already without lymphedema, CD4⁺ and CD8⁺ T-cells were more abundant in the skin of LEC-PRL2-deleted mice, which suggests a deregulation in immune cells recruitment.

In chapter 4, we describe how PRL2 deletion in LECs affects tumor development. While we did not observe a significant difference in tumor growth, there were significantly more metastatic sites in the lymph nodes when PRL2 expression was reduced in LECs. Similar to the lymphedema results, an increased number of immune cells were present in tumors, composed mainly of macrophages and T-cells. This increase in immune cell infiltration and PD-L1 expression favor an immunosuppressive tumor microenvironment.

All in all, the work presented in this thesis identifies PRL2 as a modulator of lymphatic functions and inflammation which are implicated in several diseases.

Résumé

Les vaisseaux lymphatiques sont essentiels pour la circulation des fluides, le transport des cellules immunitaires et l'absorption des lipides alimentaires. Un dysfonctionnement du système lymphatique vasculaire est relié au développement de graves maladies telles que le lymphoedème ou les cancers métastatiques. Récemment, il a été montré que la protéine tyrosine phosphatase PRL2 (PTP4A2) régule le développement vasculaire sanguin¹. Étant donné les similarités entre les vaisseaux lymphatiques et sanguins, nous avons émis l'hypothèse que PRL2 pourrait également jouer un rôle important dans les vaisseaux lymphatiques.

Dans le chapitre 2, nous démontrons que la réduction d'expression de PRL2 dans les cellules endothéliales lymphatiques (CELs) induit une diminution radicale de la prolifération, la migration et le bourgeonnement des cellules. PRL2 régule de façon critique les voies de signalisation in CELs. Après une stimulation avec VEGF-C ou VEGF-A, l'absence de PRL2 régule la signalisation de ERK et Akt. De plus, la signalisation en aval de Notch1/DLL4 est également inhibée dans ces cellules.

Dans le chapitre 3, une souris avec une délétion inductible et spécifique de PRL2 dans les CELs est décrite dans le but d'étudier le rôle de PRL2 dans la morphogenèse des vaisseaux lymphatiques. Avec un modèle de lymphoedème secondaire chez ces souris, l'œdème est plus prononcé chez les souris avec une délétion de PRL2 dans les CELs. L'infiltration des cellules immunitaires, particulièrement des cellules T, a été modifiée. Même sans lymphoedème, les cellules T CD4⁺ et CD8⁺ sont plus abondantes dans la peau des souris avec une délétion de PRL2 dans les CELs, ce qui suggère un dérèglement au niveau du recrutement des cellules immunitaires dans la peau.

Dans le chapitre 4, nous décrivons comment la délétion de PRL2 dans les CELs affecte le développement d'une tumeur. Bien que nous n'ayons observé aucune différence significative

pour la croissance de la tumeur, il y a statistiquement plus de métastases dans les ganglions lymphatiques lorsque l'expression de PRL2 est réduite dans les CELs. De façon similaire aux résultats sur le lymphoedème, un nombre plus important de cellules immunitaires était présent dans les tumeurs, composées principalement de macrophages et de cellules T. Cette augmentation d'infiltration des cellules immunitaires et l'expression de PD-L1 favorise un microenvironnement tumoral immunosuppresseur.

Dans l'ensemble, le travail présenté dans cette thèse identifie PRL2 comme un modulateur des fonctions lymphatiques et de l'inflammation qui sont impliquées dans plusieurs maladies.

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Contribution to original knowledge

My thesis provides the following contributions to the field of medicine, particularly in lymphatic vessels, lymphedema and cancer research:

- 1) The phosphatase PRL2 plays a role in the proliferation, migration and sprouting of LECs. PRL2 can modulate the phosphorylation of Akt and ERK 1/2, the formation of focal adhesion, and the downstream signaling of Notch1 cleavage.
- 2) PRL2 is involved in lymphatic function. By generating a new mouse model with an inducible and specific deletion of PRL2 in LECs, we studied the role of PRL2 *in vivo*. With a lymphedema model, we demonstrated that its deletion in LECs might affect the draining function of lymphatic vessels, but also the trafficking of immune cells.
- 3) PRL2 modulates the inflammation surrounding LECs. Its deletion could change the tumor microenvironment, especially the accumulation of immune cells, and increase lymph node metastasis formation. This study also showed that PRL2 expression can modify the production of cytokines and chemokines to promote a pro- or an anti-inflammatory response. Additionally, PRL2 can affect the activation of NF κ B and the expression of PD-L1 in LECs, which suggest again a role in modulating the inflammation.

Contribution of authors

Chapter 2

I designed, performed and analyzed most of the experiments. Claire Peghaire performed and analyzed two proliferation assays (cell counting, MTT) and the sprouting assay. Barbara Garmy-Susini provided critical advice on the project with her expertise in lymphatic vasculature. Andreas Bikfalvi and Michel L. Tremblay conceived and supervised the project and provided all resources and funding.

Chapter 3

I managed the mouse colony, designed the timeline for mouse experiments, performed the qPCR and analyzed the data. Barbara Garmy-Susini generously gave 2 male Prox1-CreERT2 mice. Our mouse model PRL2^{fl/fl}-Prox1Cre⁺ was generated and maintained by the animal facility in Bordeaux (Marie-Paule Algeo and Coralie Leroy). Mouse surgery and care were all performed in the animal facility in Bordeaux (Marie-Alix Derieppe and Julie Martineau). Wilfried Souleyreau helped with the design and the organization of experiments, mouse surgery and sacrifice. Halyna Loi performed and analyzed all post-sacrifice experiments (lymphography, tissue staining, flow cytometry). Claire Peghaire performed the VE-cadherin staining of *in vitro* LECs transfected with siRNAs. Barbara Garmy-Susini provided her expertise on the lymphedema model and all resources for the post-sacrifice experiments. Andreas Bikfalvi and Michel L. Tremblay participated with ideas and comments on data analysis. They also provided funding for mouse production, surgery and care.

Chapter 4

I managed the mouse colony, designed the timeline for mouse experiments, performed all *in vitro* experiments and analyzed the data. Mouse production was done by the animal facility in

Bordeaux (Coralie Leroy). Mouse surgery and care were all performed in the animal facility in Bordeaux (Marie-Alix Derieppe and Julie Martineau). Wilfried Souleyreau helped with the design and the organization of experiments, mouse surgery, and sacrifice. Halyna Loi performed and analyzed all post-sacrifice experiments (tissue staining). Barbara Garmy-Susini provided her expertise on lymphatic vasculature and all resources for the post-sacrifice experiments. Andreas Bikfalvi and Michel L.Tremblay participated with ideas and comments on data analysis. They also provided resources and funding for *in vitro* experiments and funding for mouse production, surgery and care.

Rationale and objectives

Lymphatic vessels are essential for immune cell trafficking, fluid homeostasis and fat absorption. In the past 20 years, there has been a growing interest for this vasculature. Lymphatic vessels have been shown to be involved in many diseases and may represent a potential therapeutic target. As a regulator of immune cell trafficking, lymphatic vessels could help to enhance or decrease the immune response. Stimulation of lymphangiogenesis increases the absorption of extracellular fluid, which may be important to reduce lymphedema. On the other hand, inhibition of lymphangiogenesis may be crucial to limit metastatic spread. Thus, understanding how lymphatic vessels are formed and unraveling the molecular mechanisms more precisely is important for health and disease.

The PRLs-protein tyrosine phosphatases are intracellular molecules that have multiple roles in physiology and pathology, notably in cancer. Their role within the tumor microenvironment remains to be understood.

In a previous work, it has been demonstrated that PRL2 is a modulator of blood vascular development. As lymphatic vessels and blood vessels share similarities, we hypothesized that PRL2 could also play an essential role in lymphatic vessel formation, and thus directly or indirectly involved in lymphedema and tumor dissemination through lymphatic vessels.

Hypothesis:

- PRL2 plays an important role in the formation of new lymphatic vessels
- PRL2 expression in lymphatic endothelial cells modulates lymphedema and the metastatic process

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List of abbreviations

Ang 1/2 = Angiopoietin 1/2

BSA = Bovine Serum Albumin

CCL = Chemokine Ligand

CLEC-2 = C-type Lectin-like receptor 2

CNNM = cyclin and CBS domain divalent metal cation transport mediator or cyclin M

COUP-TFII = Chicken Ovalbumin Upstream Promoter Transcription Factor II

CXCL = CXC chemokine ligand

CXCR = CXC chemokine receptor

DLL4 = Delta-Like Ligand 4

ECBM MV2 = Endothelial Cell Basal Medium MV2

ECGB MV2 = Endothelial Cell Growth Medium MV2

EDTA = EthyleneDiamineTetraacetic Acid

ERK = Extracellular signal-Regulated Kinase

FAK = Focal Adhesion Kinase

FBS = Fetal Bovine Serum

FCS = Fetal Calf Serum

FDA = Food and Drug Administration

FLT3 = FMS-like tyrosine kinase 3

HCl = Hydrogen Chloride

HES = Hairy/Enhancer-of-Split

HEV = High Endothelial Venule

HEY = Hairy/Enhancer-of-split related with YRPW motif protein

HGF = Hepatocyte Growth Factor

HUVECs = Human Umbilical Vein Endothelial Cells

ICAM = InterCellular Adhesion Molecule

IL = Interleukin

KO = Knock-Out

LECs = Lymphatic Endothelial Cells

LMCs = Lymphatic Muscle Cells

LYVE1 = Lymphatic vessel endothelial hyaluronan receptor 1

NaCl = Sodium Chloride

NF κ B = Nuclear Factor Kappa B

NICD = Notch1 Intracellular Cleaved Domain

PBS = Phosphate-Buffered Saline

PCNA = Proliferating Cell Nuclear Antigen

PD-L1 = Programmed Death Ligand 1

PDPN = Podoplanin

PI = Propidium Iodide

PI3K = PhosphoInositide 3-Kinase

PNAd = Peripheral Node Adressin

PRL = Phosphatase of Regenerating Liver

Prox1 = Prospero homeobox 1

PTEN = Phosphatase and TENsin homolog

PTP = Protein Tyrosine Phosphatase

qPCR = quantitative real-time Polymerase Chain Reaction

SCF = Stem Cell Factor

SDS = Sodium Dodecyl Sulfate

siCTL = non-targeting/control siRNA

siPRL2 = siRNA against PRL2

siRNA = small interfering RNA

SMCs = Smooth Muscle Cells Sox18 = SRY-Box transcription factor 18

TRAIL = Tumor-necrosis-factor Related Apoptosis Inducing Ligand

TRPM7 = Transient Receptor Potential cation channel, subfamily M, member 7

VCAM = Vascular Cell Adhesion Molecule

VCP = Valosin-Containing Protein

VEGF = Vascular Endothelial Growth Factor

VEGFR = VEGF Receptor

Chapter 1: Introduction - Literature review

1.1. Lymphatic vessels

Lymphatic vessels have a close relationship with blood vessels. Lymphatic vessels run through the whole body, intertwined with blood vessels. Both vasculatures share some structural and functional similarities as veins are the origin of lymphatic vessels during development (see 1.1.1.3). However, they also have their distinctive characteristics which make it possible to differentiate them^{1,2}.

1.1.1. Structure

The lymphatic system is unidirectional and is divided into 3 types of vessels: capillaries, pre-collectors, and collecting vessels^{2,3}.

Lymphatic capillaries or initial lymphatic are blind-ended and thin-walled as they only have a single layer of lymphatic endothelial cells (LECs) (Figure 1.1). Compared to blood capillaries that are closed vessels, lymphatic capillaries have an oak-leaf shape with discontinuous basal membrane and “button-like” junctions between cells. These openings in the vessels make them highly permeable and allow cells and fluid to enter into the lumen and go to pre-collector vessels.

Pre-collector vessels are also composed of a single layer of LECs, but with a complete basal membrane and a sparse coverage of lymphatic muscle cells (LMCs). They mainly serve as a connection between lymphatic capillaries and collecting vessels as they present features of both vessels.

Collecting lymphatic vessels are the most complex lymphatic vessels, though they still have fewer layers than blood vessels. They have a layer of LECs with tight junctions covered by a

complete basal membrane and a layer of LMCs. This difference in layer could be explained by the difference in pressure due to their respective functions. In this aspect, collecting lymphatic vessels are more similar to small veins as lymph propulsion depends on intrinsic contractility of LMCs, contraction of surrounding skeletal muscles and arterial pulsation. They also contain bileaflet valves to prevent lymph backflow. Through these vessels, cells and fluid can reach lymph nodes and bloodstream via the thoracic duct and the right lymphatic duct connected to the subclavian veins.

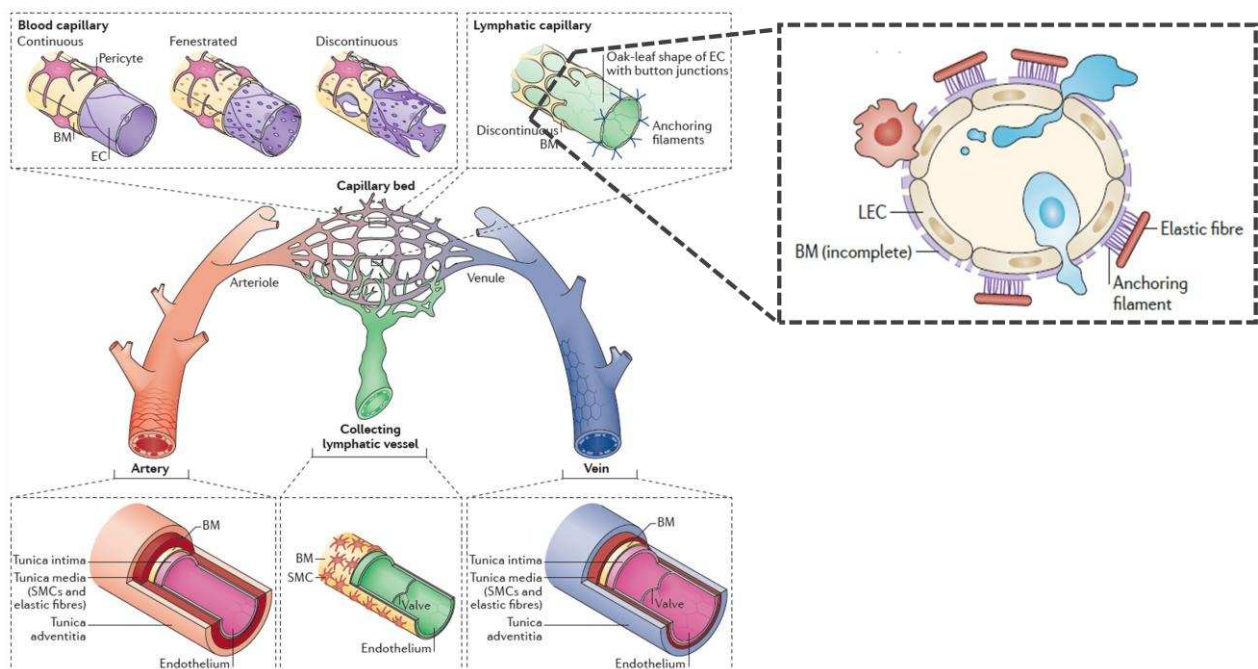


Figure 1.1: Lymphatic vasculature structure

Lymphatic vessels and blood vessels work closely together to regulate the fluid in the body. The figure shows a comparison between the lymphatic vessel and blood vessel structure. Adapted from Potente and Mäkinen (2017) and Stacker *et al.* (2014).

1.1.2. Function

Lymphatic vessels have three important functions that are essential for a well-functioning body: tissue fluid homeostasis, dietary fat absorption and immune cells trafficking. A dysfunction in one of these functions could lead to long-life diseases (see 1.1.4).

1.1.2.1. Fluid homeostasis

Similar to blood vessels, lymphatic vessels transport fluid with particles such as metabolites or proteins⁴. This fluid going through lymphatic vessels is called lymph. Its composition is similar to blood plasma, the basal component of blood, and undergoes continual change as fluid from interstitial space enters. Indeed, interstitial fluid is liquid that has exited blood circulation to get inside a tissue. Then, lymphatic vessels collect this fluid with products or waste from tissue to prevent its accumulation in the tissue.

Lymphatic capillaries are directly connected to the surrounding extracellular matrix and the uptake of tissue fluid is a passive process regulated by a difference of pressure^{5,6}. At lymphatic capillary surface, there are anchoring filaments attached to collagen fibers (Figure 1.1). When there is an excess of fluid, these anchoring filaments will induce the opening of the lymphatic vessel lumen to decrease the intraluminal pressure and increase the uptake of interstitial fluid.

1.1.2.2. Dietary fat absorption

Lymphatic capillaries inside villi of the intestine are called lacteals⁷. They are different from other lymphatic capillaries as they adapted to their function in this tissue⁸. One of their main adaptations is their proliferation state. Contrary to other LECs in classic lymphatic vessels, LECs in lacteals are in constant proliferation⁹ to follow the renewal of the intestine tissue due to mechanical and chemical stress, but also to their exposure to nutrients and bacterial products they absorb.

Another interesting characteristic of lacteals is their close interaction with villi smooth muscle cells (SMCs). The SMCs are actively involved in lymphatic functions by their muscle contraction helping with absorption and lymph flux, but they also provide growth factors such

as VEGF-C¹⁰. By producing VEGF-C, SMCs, but also macrophages¹¹, stimulate VEGFR-3 on LECs, which contribute to maintain their identity as LECs and the integrity of lacteals.

Chylomicrons are lipoprotein particles that can transport triglycerides, long-chain fatty acids, cholesterol and fat-soluble vitamins. Enterocytes, intestinal epithelial absorptive cells, absorb free fatty acids and fat-soluble nutrients to pack them into chylomicrons.^{12,13} These chylomicrons are released into the lamina propria where blood and lymphatic vessels are present. Then, chylomicrons enter lacteals through a “button-like” junction between cells^{14–16} and drained by the flux, they reach collecting lymphatic vessels inside the mesentery, followed by mesenteric lymph nodes and efferent lymphatic vessels that drive them to blood circulation. The passage of chylomicrons into lacteals depends on tight control of their size and their characteristics¹⁷, but also on regulation of LEC junctions¹⁸ and VEGFR-3 signaling¹⁹.

1.1.2.3. Immune cell trafficking

Immune cells are attracted to lymphatic vessels thanks to chemokines and cytokines produced by LECs. The main chemoattraction mechanism known between LECs and immune cells are the CCL21/CCR7 axis and the CXCL12/CXCR4 axis^{20,21}. During inflammation, LECs release CCL21 and CXCL12 to form a gradient. Immune cells with their corresponding receptors will migrate towards the lymphatic vessels where they will enter the vessels by a passive way through “button-like” junction between LECs following lymph flow, or by transmigration between LECs, involving adhesion molecules (VCAM1, ICAM1, E-selectin) in the process (Figure 1.2). Once inside the vessels, immune cells are driven towards lymph nodes where they can activate other immune cells and enhance the immune response.

However, lymphatic vessels not only transport immune cells; they can also activate them. When interstitial fluid enters lymphatic capillaries with immune cells, they can contain some pathogens such as bacteria or viruses. These pathogens can be recognized by immune cells around and initiate activation of the immune cells such as dendritic cells that will then act as antigen-presenting cells inside lymph nodes.

In the absence of inflammation, immune cells can passively enter in the lymphatic vessels using the lymph flow. Indeed, immune cells are constantly patrolling and sensing the microenvironment in order to orchestrate the immune response when necessary.

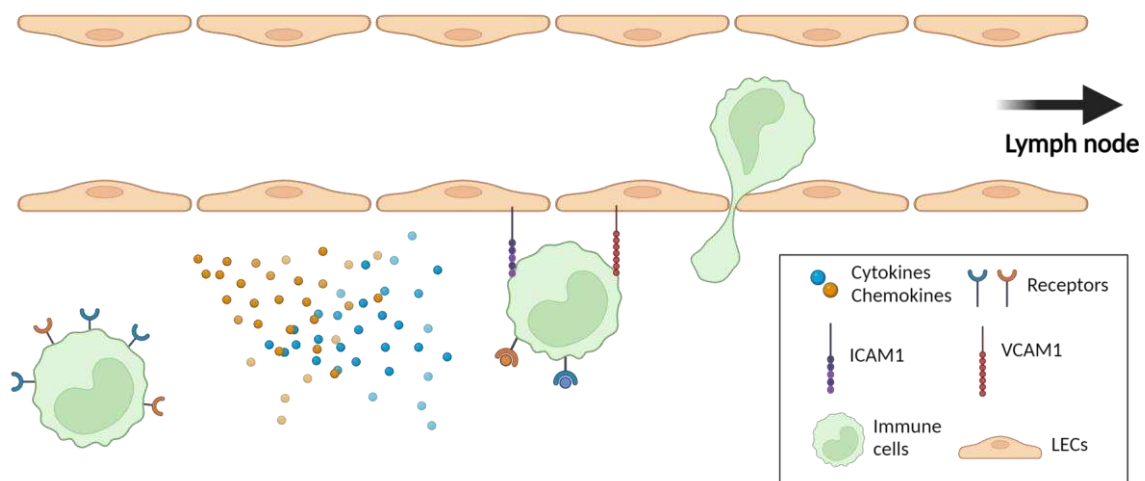


Figure 1.2: Immune cell entry into lymphatic vessel

Simplify schematic of immune cell entry into lymphatic vessel. Upon inflammation, LECs release cytokines and chemokines. With the corresponding receptors, immune cells get attracted to lymphatic vessels where they adhere to LECs with adhesion molecules such as ICAM1 and VCAM1. These adhesion molecules help immune cells to enter lymphatic capillary through a « button-like » junction between two cells. Once inside the vessel, immune cells are driven toward a lymph node. (Created with BioRender.com)

1.1.3. Lymphangiogenesis

Lymphangiogenesis is the formation of new lymphatic vessels from pre-existing vessels. This process is only observed during two phenomena: during development or during inflammation.

1.1.3.1. Development

Lymphatic vessels arise after the cardiovascular system is functional, around 6-7 embryonic weeks in human (E9.5-10.5 in mice) (Figure 1.3)^{2,22}. After arterial-venous specification, embryonic veins express a high level of Vascular Endothelial Growth Factor Receptor-3 (VEGFR-3). While this expression of VEGFR-3 is downregulated with time, only a subpopulation of endothelial cells keeps a high expression of this receptor. This subpopulation is called LEC progenitors. The differentiation of LECs is driven by the expression of Lymphatic Vessel Hyaluronan Receptor-1 (LYVE-1) which induced the expression of the transcription factor Sox18 in LECs progenitors. Then Sox18 combined with COUP-TFII, another transcription factor, activates the expression of Prox1 (prospero-related homeodomain transcription factor 1), an important lymphatic marker that plays a role in both the induction and the maintenance of LEC fate (Figure 1.3).

Prox1 allows the cells to maintain the expression of VEGFR-3 and restricts it to LECs only. Then, VEGFR-3 combined with neuropilin-2, a co-receptor, makes LECs responsive to Vascular Endothelial Growth Factor-C (VEGF-C) produced by cells from the lateral mesenchyme. The activation of VEGFR-3/VEGF-C signaling induces the budding of a group of LECs to form a lymph sac. Once the cells have budded, they start expressing podoplanin (PDPN), another lymphatic marker that can bind to the platelet receptor CLEC-2. This binding activates the downstream signaling of Syk tyrosine kinase in platelets. This interaction platelet-lymphatic will induce platelet aggregation to block the connection between the vein and the lymph sac. Once the two vasculatures are completely separated, the

lymph sac continues to grow with constant VEGF-C/VEGFR-3 axis stimulation and eventually forms a lymphatic vessel.

The final step of development is to differentiate lymphatic capillaries from collecting lymphatic vessels. The latter vessels develop intraluminal valves, recruit LMCs, tighten the cell junctions, and produce a complete basal membrane to become impermeable and ensure a unidirectional lymph flow.

Of note, a small portion of lymphatic vessels has a non-venous origin. Some supplemental dermal lymphatic vessels arise from non-venous progenitors and intestinal lymphatic vessels grow from the hemogenic endothelium. The formation of these lymphatic vessels with non-venous origins has yet to be elucidated, but the VEGFR-3/VEGF-C axis might play an important role.

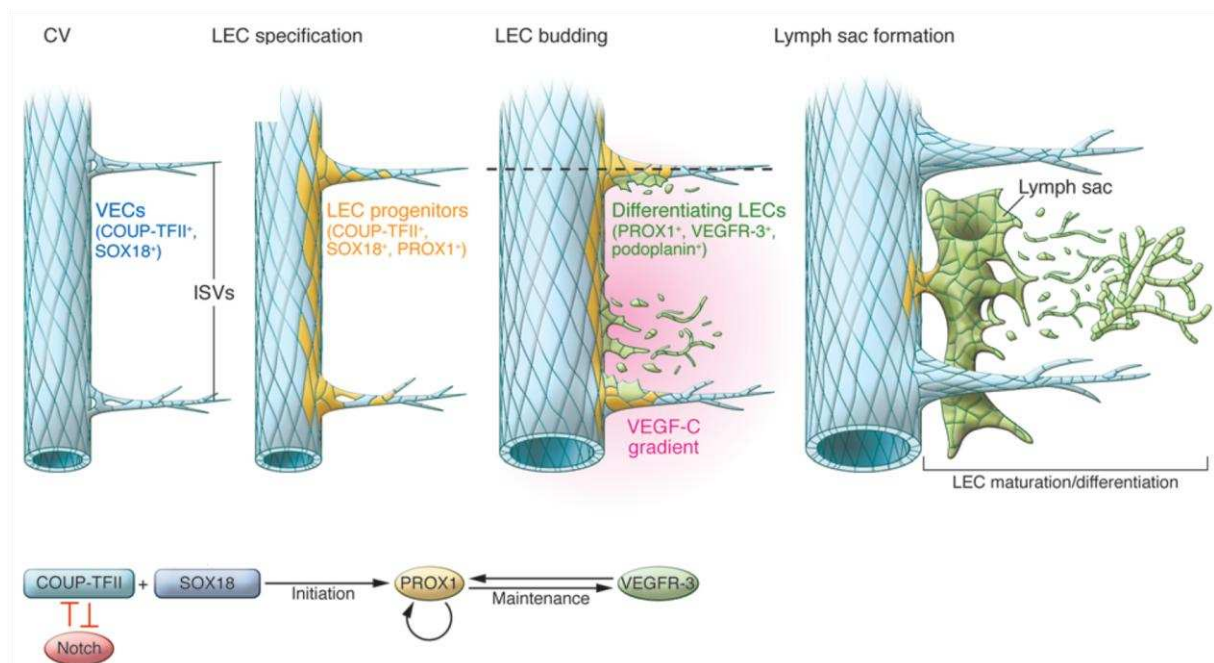


Figure 1.3: Developmental lymphangiogenesis

Lymphatic vessels arise from veins. A subset of blood endothelial cells starts expressing COUP-TFII and Sox18. These two transcription factors induce the expression of Prox1, another transcription factor that determine the cell fate and the maintenance of LECs features

such as VEGFR-3 expression. This growth factor receptor is another lymphatic marker that allows the cells to respond to the VEGF-C gradient produced by surrounding cells. From there, LECs progenitors sprout to form a lymph sac, an immature vessel that will be separated from the vein and keep growing until forming the complete lymphatic vasculature. Adapted from Yang and Oliver (2014).

1.1.3.2. Inflammation

During inflammation, lymphatic vessels play a crucial role in immune response²⁰. They form new lymphatic vessels to reach the site of infection or the wound during healing where they lead immune cells, especially the antigen-presenting cells, to lymph node where they can activate more immune cells.

VEGF-C and, to a lesser extent, VEGF-D are produced by surrounding cells, such as dendritic cells and macrophages, to induce lymphangiogenesis²³. The growth factors will bind to their receptor VEGFR-3 which homo-dimerizes or hetero-dimerizes with VEGFR-2 (Figure 1.4). The homo-dimer VEGFR-3/VEGFR-3 will preferentially activate the ERK signaling pathway and the hetero-dimer VEGFR-3/VEGFR-2 stimulates Akt as a downstream player²⁴. Akt signaling promotes the migration and the survival of LECs while ERK 1/2 signaling promotes migration as much as proliferation. Thus, these signaling pathways actively participate in the lymphangiogenesis process.

Lymphangiogenesis can also be activated by VEGF-A. This growth factor is more angiogenic, but as LECs express VEGFR-2, one of the VEGF-A receptors, they can respond to this stimulation. Consequently, activation of VEGFR-2 promotes LEC migration via Akt and ERK signaling pathways²⁴.

Stimulation by VEGF-C and VEGF-A can also stimulate the expression of Delta-Like Ligand 4 (DLL4) which is a ligand of Notch1. Once bind to its ligand, Notch1 is cleaved and the intracellular domain, called NICD, is translocated inside the nucleus to induce the expression of HEY and HES genes. The role of Notch1 signaling in lymphangiogenesis is still unclear. Previous publications have shown that Notch1 would promote cell migration and thus be an active player in lymphangiogenesis²⁵. On the contrary, others have demonstrated the inhibitor role of this pathway²⁶, suggesting that depending on the stage of development the role of Notch1 might be different. Indeed, at an early stage of development, this signaling pathway is a negative regulator of LEC specification²⁷. This divergence showed that there is more to understand about Notch1 signaling pathway in LECs.

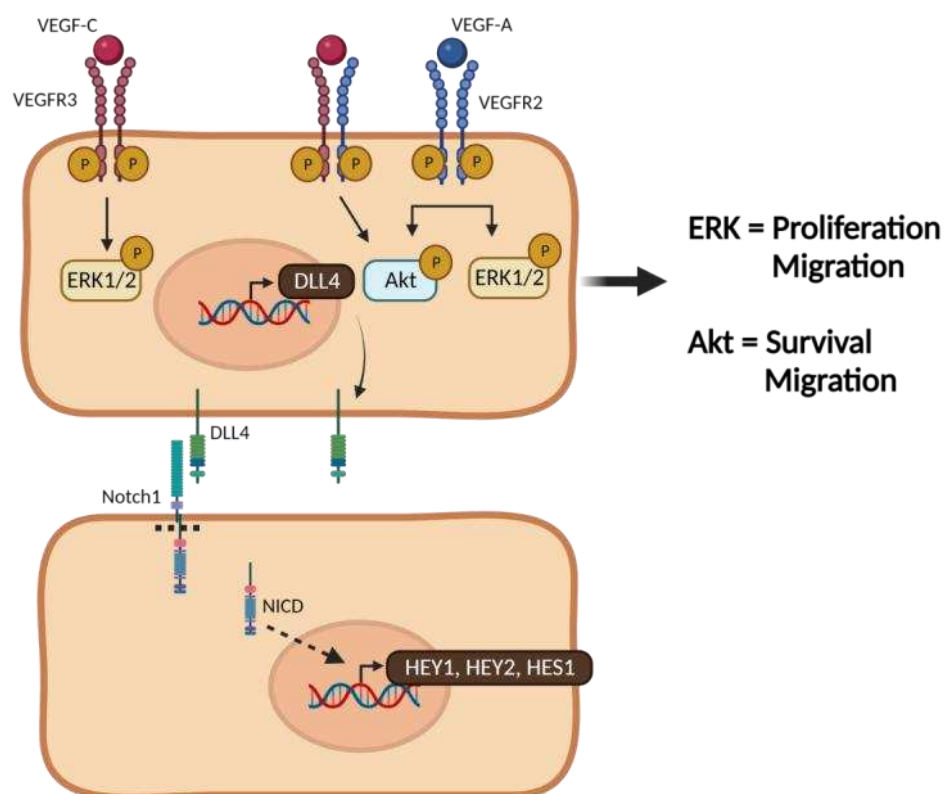


Figure 1.4: VEGF-A and VEGF-C signaling pathways

LECs express two vascular endothelial growth factor receptors: VEGFR-3 and VEGFR-2. VEGF-C can bind to VEGFR-3, but also to VEGFR-2 to form a heterodimer. VEGF-A only binds to VEGFR-2. Upon the binding to their growth factor and their dimerization, there is an

autophosphorylation of these receptors and then activation of downstream signaling pathways such as ERK1/2 and Akt. Thus, VEGF-C and VEGF-A can induce the proliferation, migration and survival of the cells. VEGFs can also increase the expression of DLL4 and then activate the Notch1 signaling pathway. (Created with BioRender.com)

1.1.4. Diseases related to lymphatic vessels

The absence of lymphatic vessels is incompatible with life in mice and dysfunction of these vessels has been involved in several diseases related to fluid accumulation, metabolism, or immune response. At first, lymphatic vessels were only related to lymphedema and cancer metastasis. It is only recently that increasing evidence show that malfunction of lymphatic functions is associated with diverse diseases (obesity, atherosclerosis, auto-immune diseases, neurological diseases, etc)⁴.

Lymphedema is an accumulation of fluid in limbs, which is noticeable by its swelling. In addition to physical impairment due to swelling and tissue fibrosis, patients with lymphedema also have impaired wound healing and more risk of infection due to reduced immune function. There are two types of lymphedema: primary lymphedema and secondary lymphedema. Primary lymphedemas are due to genetic alteration of genes involved in lymphatic vessel formation such as VEGFR-3 and Sox18^{28,29}. Secondary lymphedemas result from treatment (surgery or radiation therapy) or infection (filariasis). In both cases, fluid accumulation can be explained by lymphatic vessel malformation which impaired their fluid clearance. To this day, current management of lymphedema can control the disease, but cannot fix it. The treatments available are manual drainage, massage, and/or compression garments. They aim to reduce the swelling, although it must be done regularly to be effective.

Other than lymphedema, a defect in fluid clearance by lymphatic vessels could also be involved in neurological^{30–33} and cardiovascular diseases^{34–36}. Indeed, a defect in fluid

clearance may lead to an accumulation of components such as proteins, cells, metabolites or bacteria, which could develop into pathology. Different studies have shown that the clearance of excess fluid containing proteins, debris and immune cells can reduce neurodegenerative diseases or help with myocardial infarction healing. Lymphatic vessels also have a beneficial role in atherosclerosis by reducing cholesterol accumulation and inflammation^{37,38}. Indeed, Milasan *et al.* have demonstrated that early treatment with VEGF-C improved the lymphatic transport and prevented the formation of atherosclerosis plaque³⁹.

Obesity has also been associated with lymphatic vessel malfunction, but in a bi-directional cause and consequence. Harvey *et al.* and Escobedo *et al.* have shown in two different studies that Prox1^{+/-} pups have leaky lacteals, which results in an accumulation of chyle (lipid-rich fluid) in the intestine⁴⁰⁻⁴². This lipid accumulation leads to poor survival of pups and weight gain starting at 4 months old for the ones that survive. Following this observation, they demonstrated that free fatty acids leaking from lymphatic vessels can induce adipogenesis and lead to obesity. On the other hand, Savetsky *et al.* and García Nores *et al.* have studied the effect of obesity on lymphatic vessels^{43,44}. After inducing obesity in mice, they observed decreased lymphatic fluid clearance and increased inflammation around lymphatic vessels. Overall, these studies showed that lymphatic vessels are essential for fluid clearance and lipid transport and their dysfunction can lead to long-life diseases.

The relationship between lymphatic vessels and tumorigenesis has become a topic of great interest. This interplay has been proven to have a dual effect on the burden of cancer. On one hand, lymphangiogenesis can promote immune cell infiltration and can be associated with immunotherapy to kill tumor cells⁴⁵⁻⁴⁷. On the other hand, lymphangiogenesis can stimulate tumor growth and metastasis formation while producing an immunosuppressive

microenvironment⁴⁸. In the tumor microenvironment, immune cells or tumor cells can release lymphangiogenic growth factors to induce lymphangiogenesis (Figure 1.5). The newly formed vessels produce cytokines and chemokines to attract immune cells and increase the immune response against tumor cells. Unfortunately, tumor cells and immune cells express similar receptors. Consequently, tumor cells are also attracted to lymphatic vessels and enter the lumen to reach lymph nodes or distant organs to form metastasis. In fact, it has been shown that going through lymphatic vessels is less straining for tumor cells than going through blood vessels^{49,50}.

In conclusion, lymphatic vessels have a complex role in tumor development. In addition, regional heterogeneity (peripheral lymphatics versus intratumoral lymphatics) and differences between tumor cell types exist. This is important for the prognosis of the disease. Furthermore, lymphatics have immune-regulatory functions that contribute to tumor growth and spread⁵¹. They may have pro- or anti-inflammatory properties, thus impacting differently the tumor development⁴⁷.

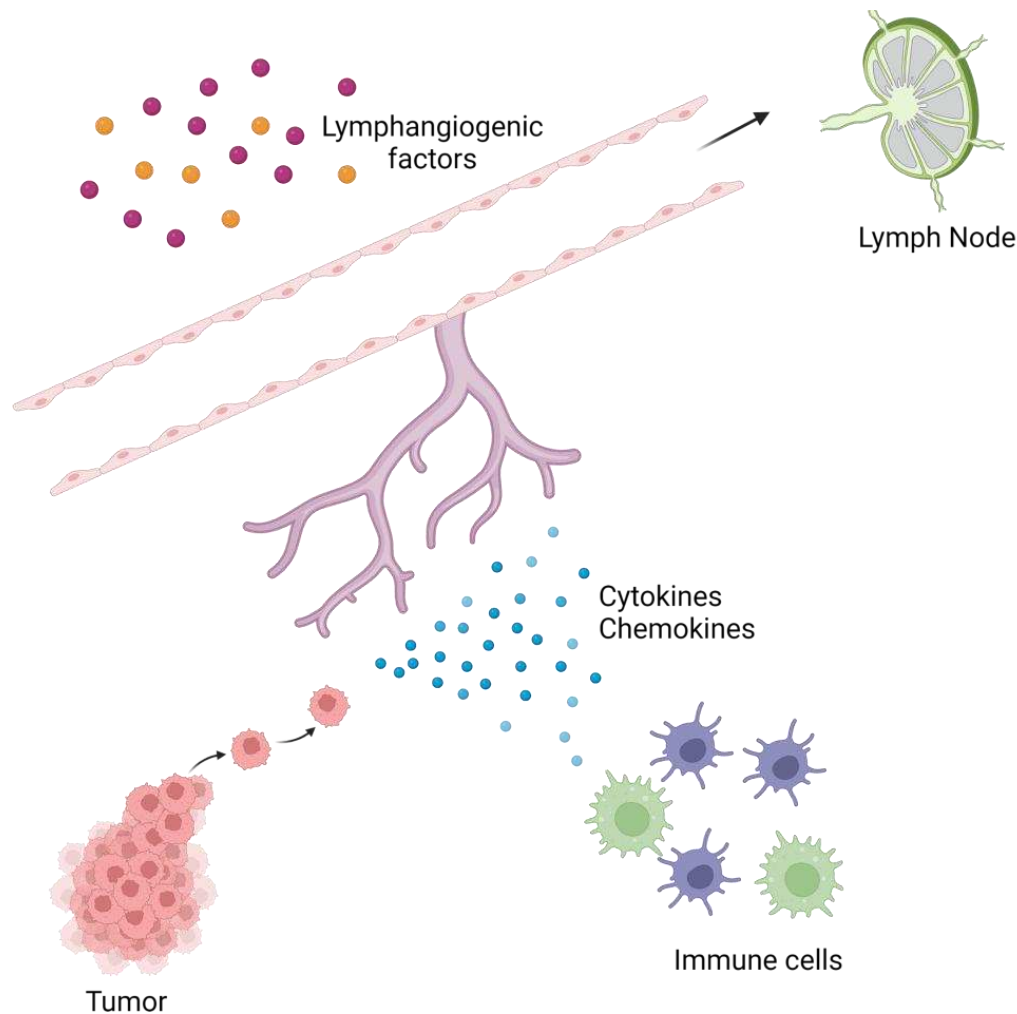


Figure 1.5: Lymphangiogenesis and its dual consequences in cancer

In cancer, some cells can release lymphangiogenic factors to induce the formation of new lymphatic vessels. These new vessels produce cytokines and chemokines to attract immune cells as it was described in inflammation (see 1.1.3.2). By recruiting immune cells, especially antigen-presenting cells, lymphatic vessels guide them to lymph nodes to activate more immune cells to fight the tumor. Unfortunately, tumor cells also respond to cytokines and chemokines. Thus, tumor cells can enter lymphatic vessels and develop metastasis in lymph nodes and distant organs. (Created with BioRender.com)

1.2. PRL2/PTP4A2

1.2.1. Protein tyrosine phosphatase (PTP) superfamily

The PTP superfamily has originally 107 members and up to 125 members in the extended family^{52,53}. A member of this superfamily is defined by one of these four characteristics:

- a PTP domain, a CxxxxxR conserved catalytic loop (C=Cysteine, x= any amino acid, and R=Arginine)
- a CxxxxxR signature motif in a non-PTP phosphatase domain
- a tyrosine phosphatase activity
- a high similarity to a member of the family

Depending on their characteristics, the PTPs are classified into four classes with most of its members in class I (Figure 1.6).

The PTPs can remove a phosphate group from their substrates to counteract the activity of kinases and thus, regulating protein activity and biological processes. Classical PTPs dephosphorylate tyrosine residue, but other family members can have a dual activity, meaning they can dephosphorylate both tyrosine and/or serine/threonine residues. Dysfunction in PTP activity could lead to the development of pathologies such as cancer, diabetes, or autoimmune dysfunction. For example, PTPN11 (MIM176876; MIM163950) and PTPN14 (MIM603155; MIM613611) were found to be mutated in patients with lymphedema and Noonan Syndrome, a disease involving lymphatic malformation⁵⁴⁻⁵⁶. PRL3 has also been correlated to lymph node metastasis and may be indirectly involved in tumor lymphangiogenesis⁵⁷⁻⁵⁹.

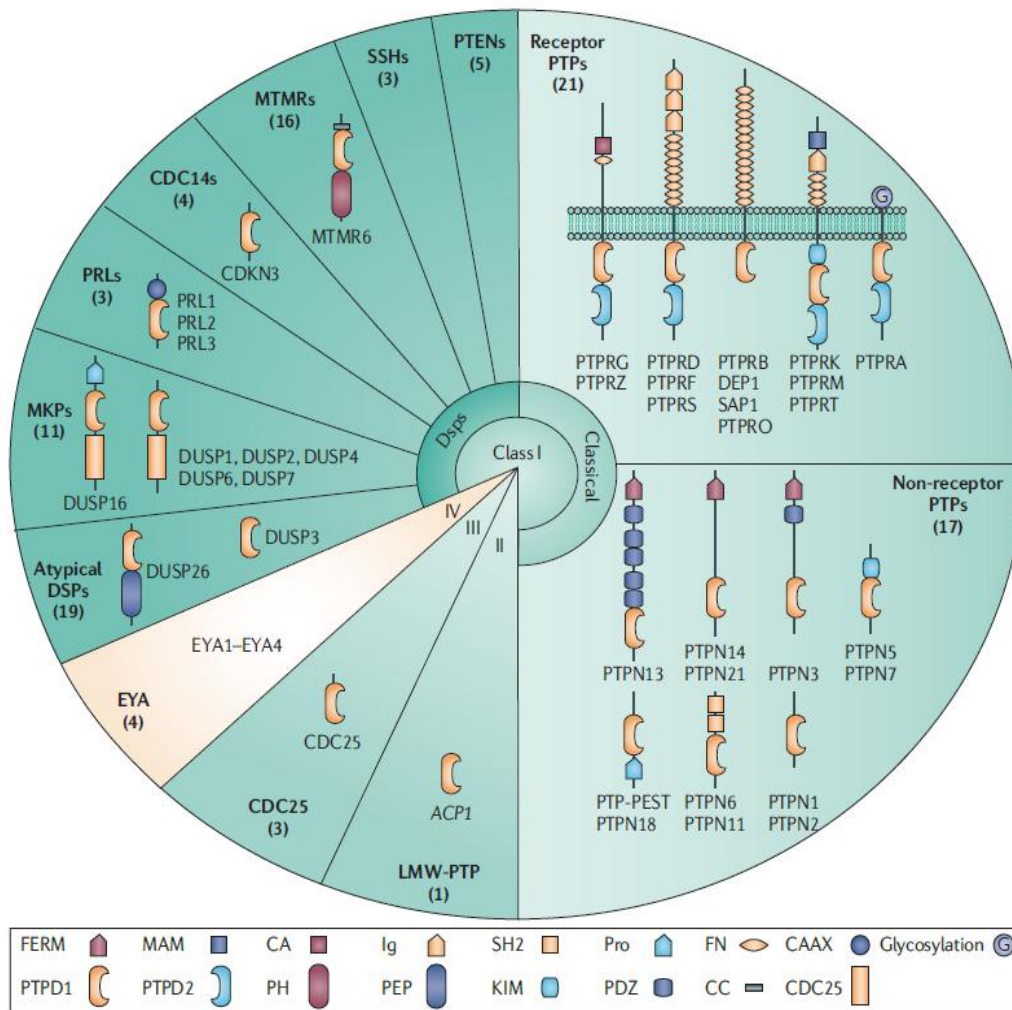


Figure 1.6: Classification of PTPs

Classification of the 107 members of PTP superfamily. PRL2 has its own sub-category with the two other PRLs in Class I, subclass II DSPs. Adapted from Julien *et al.* (2011)

Phosphatases of Regenerative Liver (PRLs) are dual PTPs in Class I PTP, part of the DSPs subclass. There are three members of the PRL sub-category: PRL1 (PTP4A1), PRL2 (PTP4A2), and PRL3 (PTP4A3). The first member to be discovered was PRL1^{60,61}. It was identified as an early gene upregulated in liver regeneration. Then, PRL2 and PRL3 were identified by homology with PRL1 as they share 75% similarity in their amino acid sequences⁶². PRL1 and PRL2 are the most expressed PRLs as they are found in almost all organs (Figure 1.7)^{63,64}. On the contrary, PRL3 is mainly expressed in heart, skeletal muscle

and prostate. As PRL2 is the highest PRL expressed in lymph node, it seems to have a more important role in lymphatic-related functions.

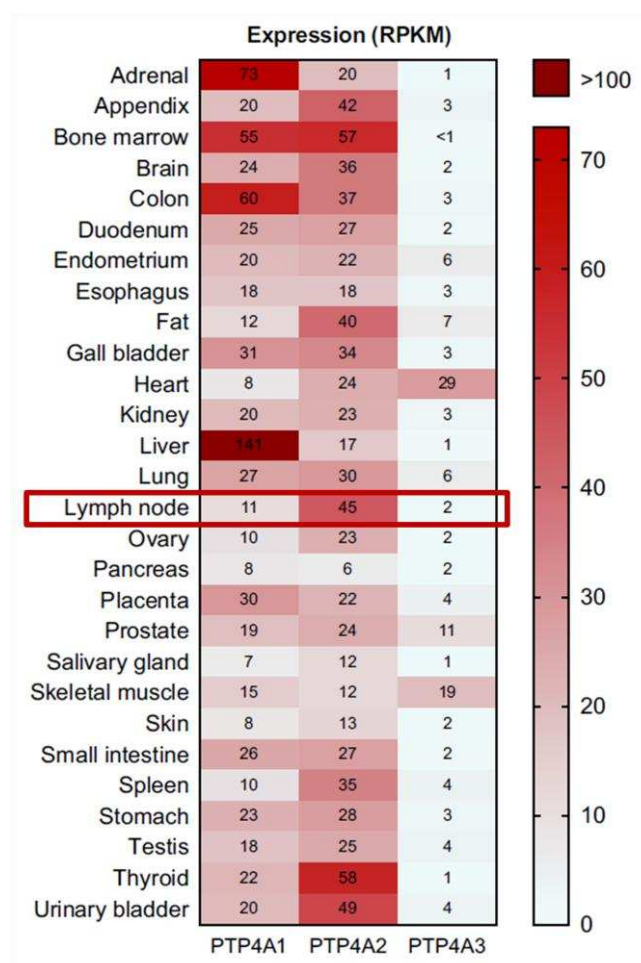


Figure 1.7: Difference in PRLs mRNA level in all organs

PRL1 and PRL2 are ubiquitously expressed, while PRL3 is mainly expressed in the heart, skeletal muscles and the prostate. Close to lymphatic vessels, we could see that the lymph node has a higher expression of PRL2 compared to other PRLs. Adapted from Hardy *et al.* (2018)

1.2.2. PRL2/PTP4A2 function

PRL2 has been shown to have a crucial role in various physiological processes. Indeed, PRL2 knock-out (KO) mice have growth retardation, splenic and testicular hypoplasia, and defects in spermatogenesis resulting in a reproductive ability impaired⁶⁵⁻⁶⁸. It was also demonstrated that the expression of PRL2 is regulated in a time-dependent manner; thus, its expression is important for circadian control of behavior and metabolism⁶⁵. To explain how PRL2 could be

involved in so many physiological processes, it has been shown that its expression is related to magnesium flux⁶⁵, an essential ion for many biological processes. When the intracellular magnesium concentration is low, the cell starts expressing PRL2 which can bind to cyclin M (CNNM) through their Bateman domain. Upon PRL2 binding, CNNM is dissociated from TRPM7, an ion channel that allows the entry of magnesium into the cell⁶⁹.

PRL2 is also a mediator of self-renewal and proliferation of hematopoietic stem cell, and is required for T-cell differentiation^{68,70}. These roles of PRL2 have been described to be associated with cell survival promoted by SCF/Kit-mediated PI3K/Akt activation. Indeed, PRL2 expression has a reverse correlation with PTEN expression^{67,71}. Zhong-Yin Zhang's team has studied this correlation between PRL2 and PTEN and recently they showed that PRL2 can dephosphorylate PTEN at the tyrosine residue 336 which allows NEDD4 to bind to PTEN and ubiquitinate it⁷¹. Following this ubiquitination, PTEN will be degraded by the proteasome, leading to PI3K/Akt signaling pathway.

In search of novel PRL2 substrates to explain his versatility, two other partners have been discovered: CBL and VCP^{72,73}. Hence, PRL2 can dephosphorylate a tyrosine residue on these two proteins (Y371 for CBL and Y805 for VCP). By dephosphorylating CBL, PRL2 inhibits its ubiquitination activity, while the dephosphorylation of VCP inhibits the formation of the complex regulating the lysophagy of the damaged lysosomes.

Both of our laboratories have recently demonstrated that PRL2 is a modulator of vascular development¹. Indeed, in the retina of PRL2 KO mice, we observed a reduced vascular outgrowth with a defect in arterio-venous differentiation (Figure 1.8A). Moreover, the

absence of PRL2 in endothelial cells (HUVECs) *in vitro* impairs cell migration and disrupts VEGFR-2 and Notch1/DLL4 signaling pathways (Figure 1.8B).

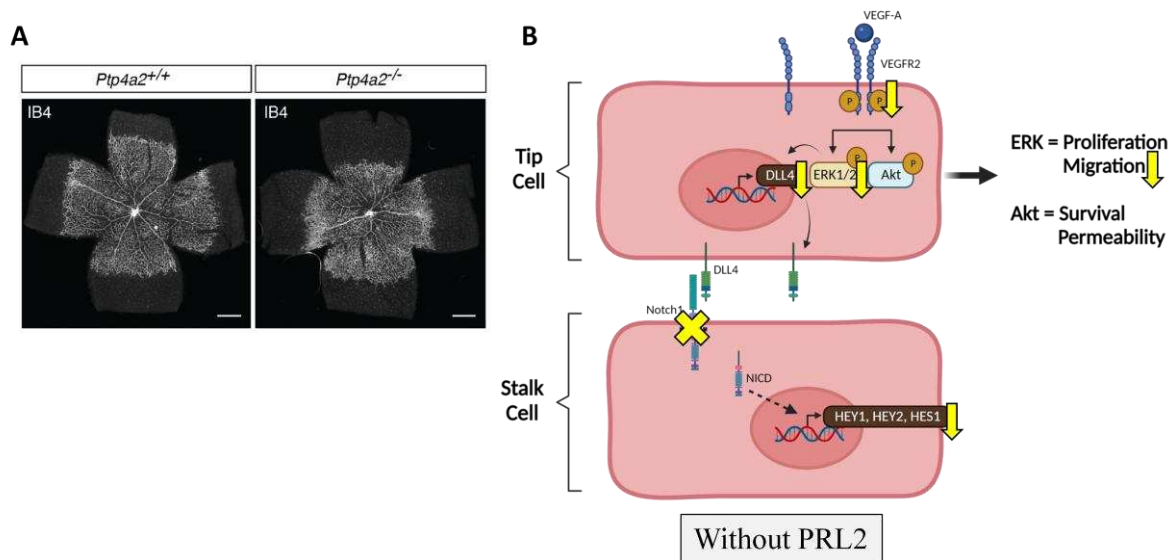


Figure 1.8: PRL2 is involved in blood vessel formation

(A) Whole-mount staining with isolectin B4 (IB4) of retina from PRL2^{+/+} and PRL2^{-/-} mice. (B) In absence of PRL2, there is a decrease of VEGFR-2 phosphorylation and ERK 1/2 phosphorylation. This is associated to a decrease in DLL4 expression and a disruption of Notch1 signaling. As Notch1 signaling pathway regulates the differentiation in tip and stalk cells, its impairment could lead to a defect in blood vessel formation. Adapted from Poulet *et al.* (2020) and created with BioRender.com.

1.2.3. PRL2 in cancer

An increase in PRL2 expression has been described in several cancers such as breast and pancreatic cancer (Figure 1.9). This increased expression has been associated with a poor prognosis and it was higher in metastatic lymph nodes^{74,75}. It appears that PRL2 is strongly involved in tumor development and metastatic spread. Thus, like the other PRLs, PRL2 is considered an oncogenic PTP and several groups are trying to determine its exact role in tumor development and if the PRLs could be an efficient therapeutic target against cancer.

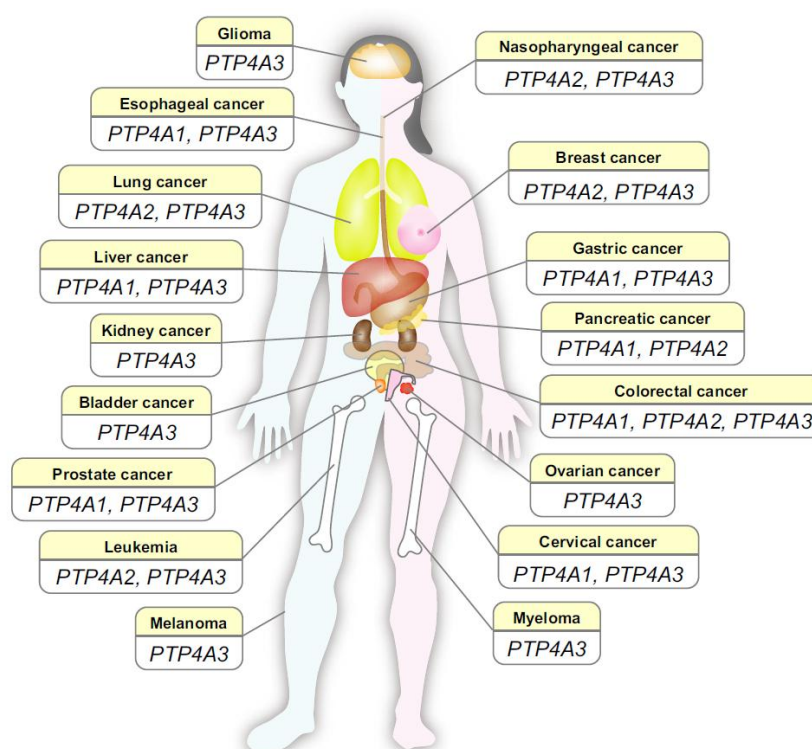


Figure 1.9: Overexpression of PRLs in cancer

mRNA and protein level of PRLs are high in several types of cancer and this overexpression has been correlated with a poor prognosis in cancer. Adapted from Hardy *et al.* (2018)

It has already been demonstrated that intracellular magnesium imbalance can be involved in several hallmarks of cancer⁷⁶. Indeed, an increase in magnesium can give metabolic advantages for cells to proliferate and spread, which is beneficial for cancer cells⁷⁷. PRL2 interaction with CNNMs to regulate magnesium homeostasis was demonstrated to play an important function in breast cancer progression⁷⁸. By using a CNNM3 mutant or a small molecules inhibitor to disrupt PRL2/CNNM3 complex, breast cancer cells proliferation was decreased, which also translated a decrease in tumor growth in the xenograft model. This demonstrates how targeting PRL2, especially PRL2/CNMMs interaction, could be an efficient target in cancer.

The inverse correlation of PRL2 and PTEN expression could also be an interesting target to reduce tumor progression. PTEN is a tumor suppressor and contrary to PRL2 is often downregulated in cancers. Zhong-Yin Zhang's team investigated the potential of inhibiting PRL2 expression in a PTEN low expression context and indeed, the ablation of PRL2 expression reduced the tumor progression and improved the survival of mice bearing-tumor^{71,79}.

As mentioned before, PRL2 is involved in self-renewal and proliferation of hematopoietic stem cells. Various groups started studying its potential role in hematological malignancies such as leukemia. Indeed, Kobayashi *et al.* demonstrated that PRL2 has an oncogenic function in acute myeloid leukemia and acute lymphoblastic leukemia^{80,81}. They concluded by suggesting that PRL2 has potential as a novel therapeutic target. More recently, a study showed that PRL2 enhances the oncogenic FLT3 signaling by dephosphorylating CBL, which prevents the degradation of FLT3⁷². By using a small molecule inhibitor of PRL2, decrease in leukemia burden and better survival have been observed in leukemia-bearing mice.

Overall, these studies on PRL2 and cancers show that depending on the cancer type, the oncogenic role of PRL2 could be distinct, and more have to be explored to fully understand how PRL2 acts in cancer cells. Therefore, targeting PRL2 function to improve cancer survival could be more challenging as it could depend on the cancer type.

Chapter 2: Deciphering the role of PRL2 in lymphatic endothelial cells

2.1. Objectives

In this chapter, we investigate the role of PRL2 in lymphatic endothelial cell biology by using a pool of siRNAs to knockdown PRL2 expression in LECs *in vitro* and we performed several functional assays to know if PRL2 takes part in the main biological processes related to lymphangiogenesis, namely proliferation, migration, and sprouting.

2.2. Materials and methods

2.2.1. List of antibodies

Table 2.1: List of antibodies used for western blot

Protein target	Antibody
Akt	Rabbit anti-Akt (pan), CST #4691
Phospho-Akt	Rabbit anti-phospho-Akt, CST #9271
Cyclin D1	Mouse anti-Cyclin D1, Santa Cruz #sc-20044
Cyclin E	Rabbit anti-Cyclin E, Santa Cruz #sc-481
DLL4	Rabbit anti-DLL4, CST #2589
p44/42 MAPK (ERK 1/2)	Rabbit anti-p44/42 MAPK (ERK1/2), CST #4695
Phospho-p44/p42 MAPK (Phospho ERK 1/2)	Rabbit anti-phospho-p44/42 MAPK (phospho-ERK 1/2), CST #4370
Notch1	Rabbit anti-Notch1, CST #3608
Cleaved Notch1	Rabbit anti-Cleaved Notch1, CST #4147
p21	Mouse anti-p21, Upstate #05-345
PCNA	Mouse anti-PCNA, CST #2586

PRL 1/2	Mouse anti-PRL1/2, EMD Millipore #05-1583
PTEN	Rabbit anti-PTEN, CST #9188
α -Tubulin	Mouse anti- α -Tubulin, Sigma Aldrich T5168
Vinculin	Mouse anti-vinculin, Sigma Aldrich V9131

2.2.2. List of primers

Table 2.2: List of primers used for qPCR

Gene	Primer forward	Primer reverse
60S ⁸²	CAGATTGGCTACCCAACCTGGT	GGGAAGGTGTAATCCGTCTCC
Cyclin D1	AGATGAAGGAGACCATCCCCC	CCACTTGAGCTTGTTACCA
Cyclin E1	CCGGTATATGGCGACACAAG	TACGCAAACCTGGTGCAACTT
DLL4	GACCACTTCGGCCACTATGT	CAGCCCGAAAGACAGATAGG
HEY1	GCATACGGCAGGAGGGAAA	CTGGGAAGCGTAGTTGTTGAGAT
HEY2	GAACAATTACTCGGGGCAAA	TCAAAAGCAGTTGGCACAAG
Notch1	CAGCCTCAACATCCCCTACAAG	GCAGCCCACGAAGAACAGAA
p21	TGCCGAAGTCAGTTCCTT	CATTAGCGCATCACAGTC
p53	GAGCTGAATGAGGCCTTGGA	CTGAGTCAGGCCCTTCTGTCTT
PCNA	GTGGAGAACTTGGAATGGAA	ACCGTTGAAGAGAGTGGAGTG
PRL1	TGCTGTTCATTGCGTTGCAG	CCACGCCGCTTTTGTCTTATG
PRL2	GGAATCCACGTTCTAGATTGGC	AACACAGCAACCTGGCTCTT
PRL3	AGCACCTTCATTGAGGACCTG	CAGCGGCGTTTTGTCATAGG

2.2.3. Cell culture

Human Dermal Lymphatic Endothelial Cells (LECs) were purchased from Promocell and cultured in Endothelial Cell Growth Medium MV2 (ECGM MV2, Promocell) on flasks or plates coated with 0.4% gelatin (PAN BIOTECH P06-25200). Cells were maintained in a humidified incubator with 5% CO₂ at 37°C and the media was renewed every 2-3 days. LECs were used between passage 4 and 7.

2.2.4. Transfection

siRNAs were purchased from Dharmacon : PTP4A2 (SMART Pool, M-009078-01-0005, GE Healthcare Europe GmbH) and negative control (SMART Pool, Non-targeting siRNA, D-001206-13-05, GE Healthcare Europe GmbH). Lipofectamine RNAiMAX kit (Invitrogen 13778075) was used for transfection, following manufacturer's protocol.

2.2.5. Stimulation with VEGF-A/-C or DLL4 coating

For VEGF-A/-C stimulation, LECs were starved overnight in Endothelial Cell Basal Medium MV2 (ECBM MV2 Promocell) supplemented with 0.2% fetal calf serum (FCS). Then, they were treated with 50 ng/ml VEGF-A (R&D Systems #293-VE), or 100 ng/ml VEGF-C (R&D Systems #9199-VC) for the indicated time.

For DLL4 stimulation, recombinant human DLL4 (R&D system #10185-D4) was diluted in PBS (1 µg/ml and 2.5 µg/ml) and coated on 6-well plates for 5h at 37°C. LECs were starved overnight in ECBM MV2 supplemented with 0.5% FCS, and then they were seeded on DLL4-coated plates and harvested at the indicated time.

2.2.6. Proliferation assays

2.2.6.1. Cell counting

LECs were seeded in 6-well plate with 100000 cells/well and allowed to attach overnight. The next day, cells were transfected with siRNAs. Cells were harvested 24h, 48h and 72h post-transfection and the number of cells was counted using a cell counter (Luna, Logos, L20001).

2.2.6.2. WST-8 test

LECs were seeded in a 96-well plate with 8000 cells/well and allowed to attach for 4h. Cells were transfected with siRNAs on the same day. Proliferation was assessed for 3 days, starting 24h post-transfection, following colorimetric cell viability kit I (WST-8) protocol (Promocell PK-CA705-CK04). After each measure, cells were washed once with PBS 1X and fresh media was added.

2.2.6.3. 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay

LECs were seeded in 96-well plates with 4000 cells/well and allowed to attach overnight. The next day, cells were transfected with siRNAs overnight. 5 mg/ml of MTT (Euromedex, 4022-B) diluted in ECGM-MV2 were added to each well 24h, 48h or 72h post-transfection and the cells were incubated for a further 4 h. The absorbance was recorded at 570 nm with a Microplate reader (Biochrom Asys, UVM340) after the addition of dimethyl sulfoxide.

2.2.7. Cell cycle analysis

LECs were seeded on dish 100 mm and allowed to grow to 90% confluence. Then, they were transfected with siRNAs for 24h and treated with 2 mM thymidine for another 24h to synchronize the cells. The next day, these cells were seeded on 6-well plates with 100000 cells/well and allowed to grow for 24h and 48h. Then, the cells were harvested and fixed in 75% ethanol for 30 min on ice. Staining was done with FxCycle™ PI/RNase Staining

Solution (Invitrogen F10797) following the manufacturer's protocol. Data were acquired on a BD LSR Fortessa and analyzed with FlowJo software.

2.2.8. Scratching assay

LECs were seeded in a 24-well plate at 80% confluence and transfected with siRNA on the same day. 24h post-transfection, cells were starved overnight with ECBM MV2 supplemented with 0.5% FCS. A vertical scratch was made across the cell monolayer with a 1000 μ l pipette tip. Floating cells were removed using two washes with PBS 1X. Then, the cells were incubated at 37°C with ECBM MV2 supplemented with 0.5% FBS and 50 ng/ml VEGF-A or 100 ng/ml VEGF-C. Image acquisition was done with a video microscope (Nikon Eclipse Ti) right after the scratch (T0) and 12h after the scratch (T12). Scratch wound area was quantified with Image J software and wound coverage was calculated following this equation:

$$\text{Wound coverage (\%)} = \frac{\text{Wound area } T0 - \text{Wound area } T12}{\text{Wound area } T0} \times 100$$

2.2.9. Single cell tracking

LECs were transfected with siRNAs for 24h and seeded on a 12-well plate with 20 000 cells/well. The cells were allowed to attach overnight. The next day, they were stimulated with 50 ng/ml VEGF-A or 100 ng/ml VEGF-C 4 hours before recording. Image acquisition was done with a video microscope (Nikon Eclipse Ti) for 4 hours with pictures taken every 5 minutes. The analysis was done with Image J using the Manual Tracking plugin.

2.2.10. Bead sprouting assay

LECs were coated on beads at a concentration of 600 cells/bead. Cells attached to the beads are transfected with siRNA overnight. Then, the beads with LECs are embedded into fibrin

gel and fibroblasts are seeded on top of the gel after polymerization. Images were acquired 72h, 96h and 120h after. Number and length of sprouts were analyzed using Image J software. For sprouting assays, LECs in suspension were incubated with collagen-coated microcarrier beads (Cytodex 3, Sigma, C3275) for 5 h (400 cells per bead). LECS-coated beads were then transferred to a Petri dish and placed in an incubator for transfection. LECS coated on beads were transfected with Antisense LNA GapmeRs (30 nM) overnight. LECS adhering to the Petri dish (and not to the beads) were collected at the end of the experiments to control LINC-PINT knock-down efficiency overtime (72h and 6 days after transfection). Next day, equal numbers of LECs-coated beads were embedded in fibrin gels in 24-well plate or lab-tek 4 well chamber slide (180 beads per well). The fibrin gel was prepared by dissolving bovine fibrinogen (2 mg.ml^{-1}) (Sigma, 341573) in PBS and clotting was induced by addition of bovine thrombin (2 U.ml^{-1}) (Sigma, T4648). After 30 min, human skin fibroblasts were plated on top of the gel and Endothelial Cell Growth Medium-2 (Lonza, CC-3162) supplemented with human VEGF-C (100 ng/ml, Peprotech, 100-20CD) was added. The formation of sprouts was imaged at 10 $\times$ magnification using a bright field microscope (Nikon Eclipse, TS100) for quantification. The percentage of beads with or without sprout, the number of sprouts per bead and the length of sprouts were quantified and counting manually at 72h, 96h and 120h after plating using ImageJ.

2.2.11. Immunofluorescence

LECs were seeded on glass coverslips coated with 0.4% gelatin. At the indicated time after treatment, cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.5% Triton for 5 minutes, then incubated with the blocking solution (2% Donkey serum, 1% BSA in PBS) for 30 minutes at room temperature. Primary antibodies were diluted in staining solution (2% Donkey serum, 1% BSA in PBS) 1:250 and incubated for 1h at room

temperature. Secondary antibodies Alexa Fluor were diluted in staining solution 1:250 and incubated for 1h at room temperature. Nuclei were visualized using DAPI (4',6-diamidino-2-phenylindole, Invitrogen #D1306) 1:1000 and cell shapes were visualized using Alexa-Fluor 594 phalloidin (Invitrogen #A12381) 1:100. Coverslips were mounted on glass slides with ProLong Gold antifade mountant (Invitrogen #P36934) and dry overnight. Images were taken using a fluorescent microscope (Leica, DM4 B). Images were analyzed with ImageJ.

For LECs-coated beads, they were fixed six days (144h) after plating with 4% paraformaldehyde for 1h at room temperature, then washed three times with PBS, incubated at room temperature for 45 min with permeabilization solution (0.5% Triton X-100 in PBS) and then with blocking solution (1% BSA-5% FBS-0,1% Tween in PBS) overnight at 4°C. The next day, cells were stained with Hoechst (Thermo Scientific, 33342) and Alexa-Fluor 488 phalloidin (Invitrogen, A12379) diluted 1:5000 and 1:500 respectively in staining solution (1% BSA-5% FBS-0,1% Tween in PBS) for 24h at 4°C. Following five washes in 0.5% Tween in PBS at room temperature on gentle rocking, images were taken at 10X magnification using a fluorescent inverted microscope (DMi8, Leica).

2.2.12. Western Blot

LECs were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche) for 30 minutes on ice, followed by a centrifugation at 10000 g for 10 minutes. Supernatants were collected and proteins were quantified following Pierce BCA protein assay kit protocol (Thermo Scientific #23225). Samples were prepared with 15 µg proteins and Laemmli buffer (300 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.5 mg/ml bromophenol blue, 10% β-mercapto-ethanol), boiled for 5 minutes at 100°C, then

separated by SDS-PAGE. After migration, proteins were transferred on a PVDF membrane. Membranes were blocked with 5% BSA for 1h at room temperature, then incubated with primary antibodies diluted in antibody solution (PBS 1X supplemented with 0.5% Tween and 0.02% sodium azide) overnight at 4°C. Secondary antibodies were diluted in antibody solution and incubated for 1h at room temperature. Membranes were scanned with an Odyssey imager (LI-COR) and image analysis was performed using Image Studio Lite 5.2.

2.2.13. RNA isolation, reverse transcription, and quantitative real-time PCR

RNA was isolated from LECs with TRI-Reagent (MRC #TR118) following the manufacturer's protocol. The only adjustment done was that RNA precipitation was performed for 4 hours at 4°C. RNA pellets were resuspended in sterile water, and then quantified with NanoDrop. 500 ng of RNA was reverse-transcribed to cDNA using the High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific #4368813) following the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed with an AriaMx Real-time PCR System (Agilent) using EurobioGreen qPCR Mix Hi-ROX (Eurobio #GAEMMX02H 0T) according to the manufacturer's protocol.

60S (RPLP0, ribosomal subunit) was used as a housekeeping gene as it was the most stable in all conditions for our experiments.

2.2.14. Statistical analysis

Statistical analyses were performed on GraphPad Prism 9 software. A Shapiro-Wilk test was performed to assess the normal distribution of the sample. Student t-test (normal distribution) or Mann-Whitney test was used to compare two groups, while two-way ANOVA, followed by Sidak or Tukey's post-hoc test, was used to compare two groups with more than one variable. The difference was statistically significant when the p-value was below 0.05.

2.3. Results

2.3.1. Validation of PRL2 knockdown in LECs

We first wanted to validate and assess the efficiency of PRL2 knock-down in LECs transfected with a pool of siRNAs against PRL2 (siPRL2) and a pool of non-targeting siRNAs (siCTL) as a control. This transfection efficiently decreased PRL2 expression at the mRNA level by more than 95% (Figure 2.1B). Moreover, at the protein level 24h post-transfection, PRL2 protein expression is reduced by 40%, while after 48h, it is more than 90% decrease (Figure 2.1D-E). Of note, the knockdown of PRL2 did not affect PRL3 expression, but an increase in PRL1 gene expression was observed as a compensatory effect (Figure 2.1A-C). However, at the protein level, PRL1 expression was low when compared to PRL2 expression in LECs (Figure 2.1D).

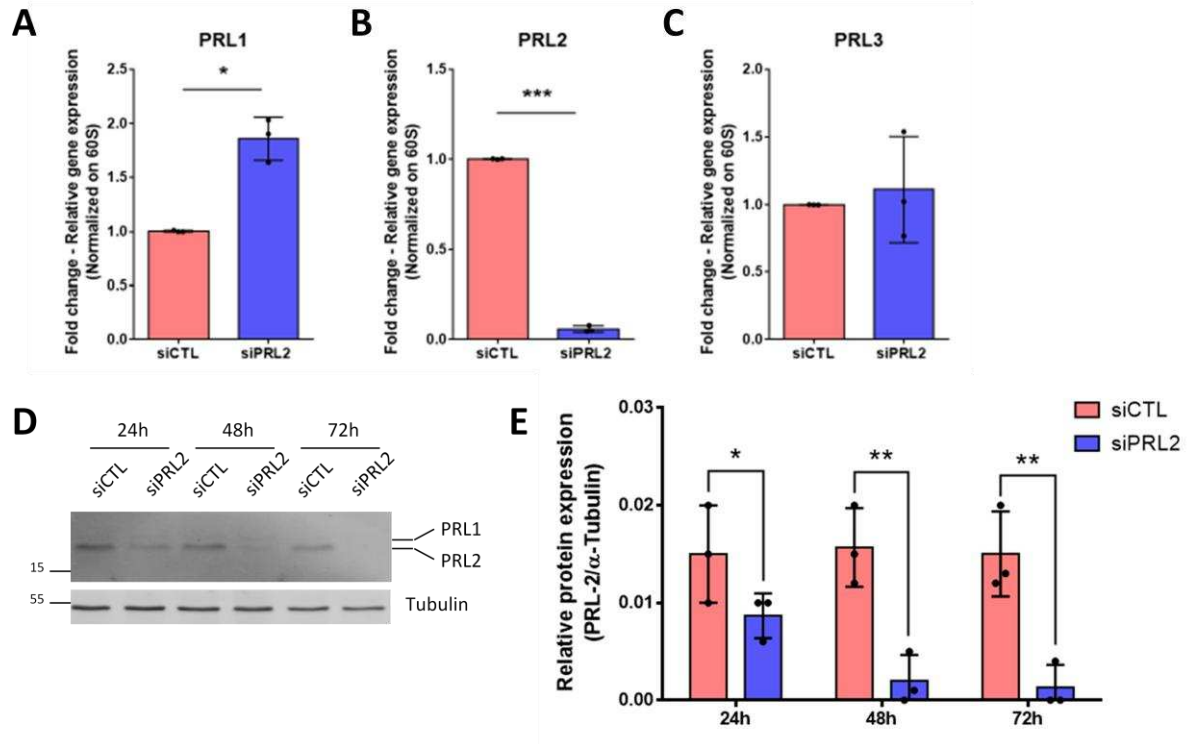


Figure 2.1: Silencing of PRL2 in LECs.

LECs were transfected with siRNA control (siCTL) or siRNA against PRL2 (siPRL2). (A-C) mRNA level of PRLs was assessed by qPCR 72h post-transfection. (n=3; Student t-test) (D-E) Western Blot of PRL2 expression at different time post-transfection and quantification of relative protein level of PRL2. (n=3; Two-way ANOVA, Sidak's post-hoc test) Graphs are represented in mean $\pm$ SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

2.3.2 PRL2 silencing slows down the proliferation of LECs

To assess the effect of PRL2 silencing on the proliferation of LECs, we performed three distinct proliferation assays: cell counting, WST-8 test, and MTT assay (Figure 2.2A-C). In all assays, we observed that siPRL2 LECs have a slower proliferation compared to siCTL LECs.

Given the results on proliferation, we hypothesized that the cell cycle could be affected by PRL2 silencing. Upon siRNA transfection, cells have an increased expression of cyclin D1 and a decreased expression of PCNA and cyclin E1 (Figure 2.2F-H). No difference was

observed for p21 and p53 gene expression (Figure 2.2D-E). These results suggest that the cells could be slowed down in their S phase as cyclin E1 and PCNA are both involved in the G1/S phase transition.

When we performed a cell cycle analysis by propidium iodide staining, we saw a significant difference in cell cycle between siCTL LECs and siPRL2 LECs (Figure 2.2I-J). 24h after releasing of the thymidine block, the difference was significant in the S phase and G2 phase, with more siPRL2 LECs in the G2 phase and less in the S phase. 48h after releasing of the thymidine block, the difference was reduced, but still significant in the G1 phase. This indicates that siPRL2 LECs were still able to pass the different checkpoints and keep on proliferating, but they were still slower than siCTL LECs, which correspond to the results of the proliferation assay. In parallel, we also performed western blot analysis to detect the protein expression of the corresponding genes that we analyzed by qPCR. Like qPCR results, p21 protein level was not different (Figure 2.2K). At 24h, we observed no difference in PCNA, cyclin D1 and cyclin E1. At 48h, we observed a lower level of cyclin D1 and a higher level of PCNA for siPRL2 LECs, but no difference for cyclin E1. These results were different from the qPCR analysis, but this could be explained by the thymidine block that synchronized the cells in these experiments.

Taken together, we could not precisely determine which processes of the cell cycle were altered. Analysis at shorter time points after releasing of the thymidine block and following the cells entering each phase after being synchronized in G1 should be helpful. More investigation remains to be done to understand how PRL2 affects the proliferation of these cells.

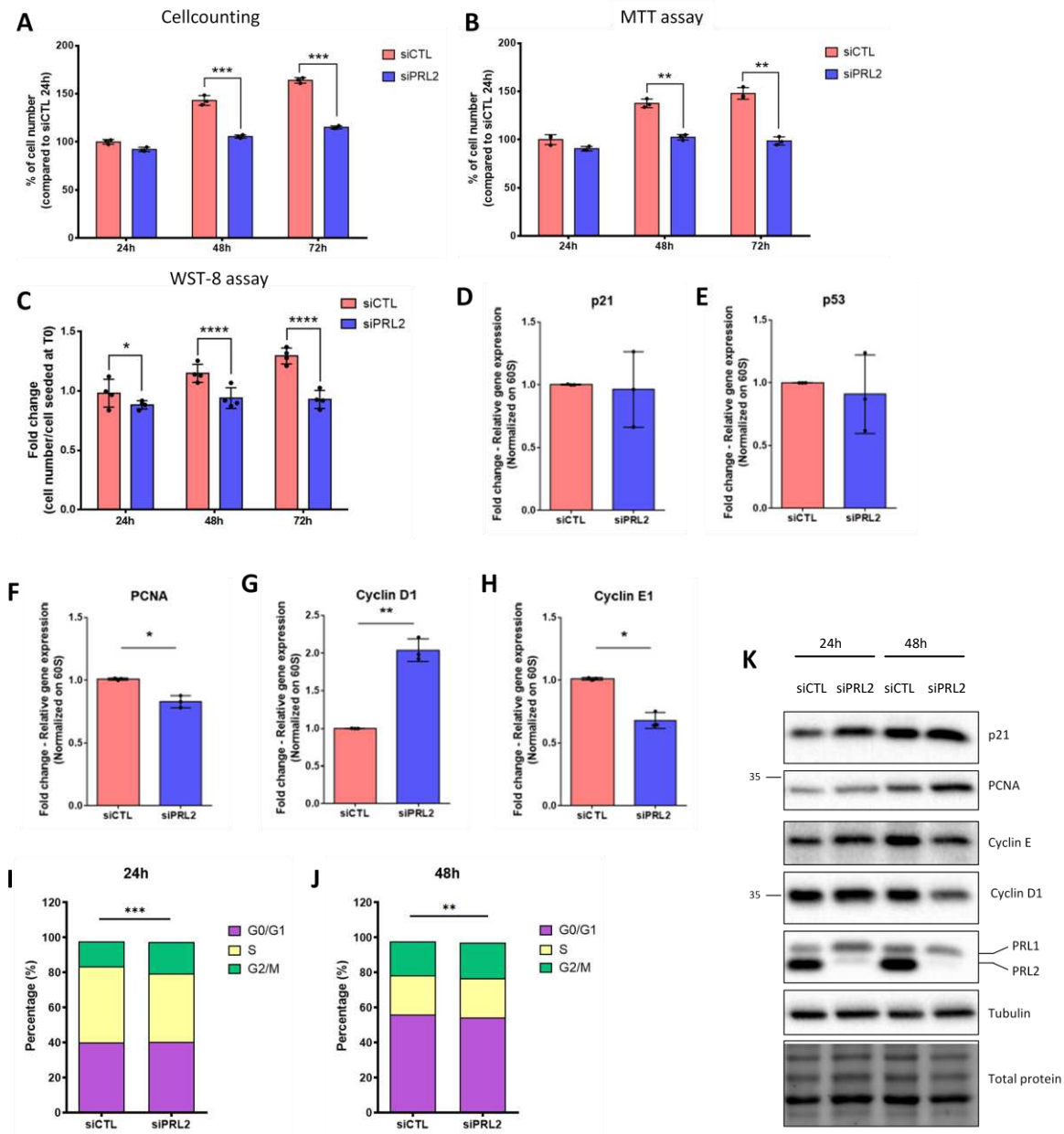


Figure 2.2: PRL2 silencing slows down the proliferation of LECs.

(A-C) Proliferation of LECs transfected with siCTL and siPRL2 was assessed by cell counting (A), MTT assay (B), and WST-8 assay (C). (n=3-4; Two-way ANOVA, Sidak's post-hoc test) (D-H) mRNA level of p21, p53, PCNA, Cyclin D1, and Cyclin E1 assessed by qPCR 72h post-transfection. (n=3; Student t-test) (I-J) Cell cycle analysis of siCTL and siPRL2 LECs by propidium iodide at the indicated time after release of thymidine block. (n=3; Two-way ANOVA, Sidak's post-hoc test) (K) Western blot of siCTL and siPRL2 LECs 24h and 48h after release of thymidine block. Graphs are represented in mean $\pm$ SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

2.3.3. PRL2 is involved in the cell motility/migration of LECs

Beside proliferation, migration is another essential process to create new lymphatic vessels. To assess this process, we performed a scratching assay. 12 hours after the scratches were done, we observed that the siCTL LECs covered 15-20% of the wound in the control condition and 25-40% when treated with VEGF-A or VEGF-C (Figure 2.3A-B). On the opposite, siPRL2 LECs barely achieved 10% of wound coverage. Even when the siPRL2 LECs are stimulated with VEGF-A or VEGF-C, they did not obtain more coverage. These results indicate that the migration capacity of the cells is affected by PRL2 knockdown.

Moreover, when single cell tracking was performed to assess cell motility, siPRL2 LECs were moving with low velocity or did not move at all compared to siCTL LECs (Figure 2.3C). VEGF-A or VEGF-C stimulation did not improve the velocity, which is compatible with the results of migration in the scratching assay.

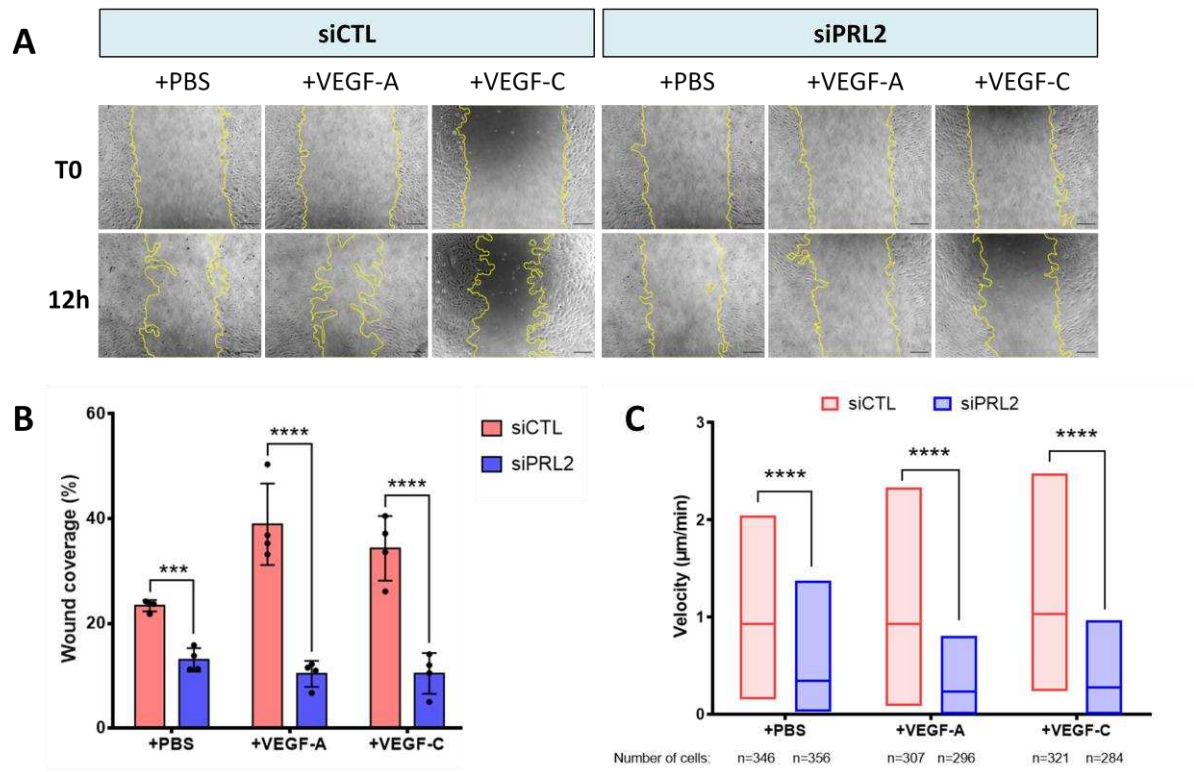


Figure 2.3 : Decrease of cell motility with PRL2 silencing in LECs.

(A) Representative images of scratching assay at T0 (when the wound was made) and 12h after. During the wound coverage, siCTL and siPRL2 LECs were treated with VEGF-A or VEGF-C. (B) Quantification of wound coverage (n=4; Two-way ANOVA, Sidak's post-hoc test) (C) Velocity of siCTL and siPRL2 LECs was assessed by a single cell tracking analysis. (Two-way ANOVA, Sidak's post-hoc test). Graphs are represented in mean $\pm$ SD; *** p<0.001; **** p<0.0001

Focal adhesions are protein-complexes regulating cell adhesion to the extracellular matrix and cell motility. To visualize focal adhesions, vinculin staining was performed. A higher number and smaller focal adhesions were present in siPRL2 LECs (Figure 2.4A-C). The organization at the cell surface was also modified as focal adhesions were distributed all over the cell surface of siPRL2 LECs, while in siCTL LECs, they were found mostly at the edge. Thus, focal adhesions higher number and patterning correlate with cell motility.

Overall, these results demonstrate that in the absence of PRL2 in LECs, cell motility/migration is dramatically affected with an increase in focal adhesions and changes in their cell surface distribution.

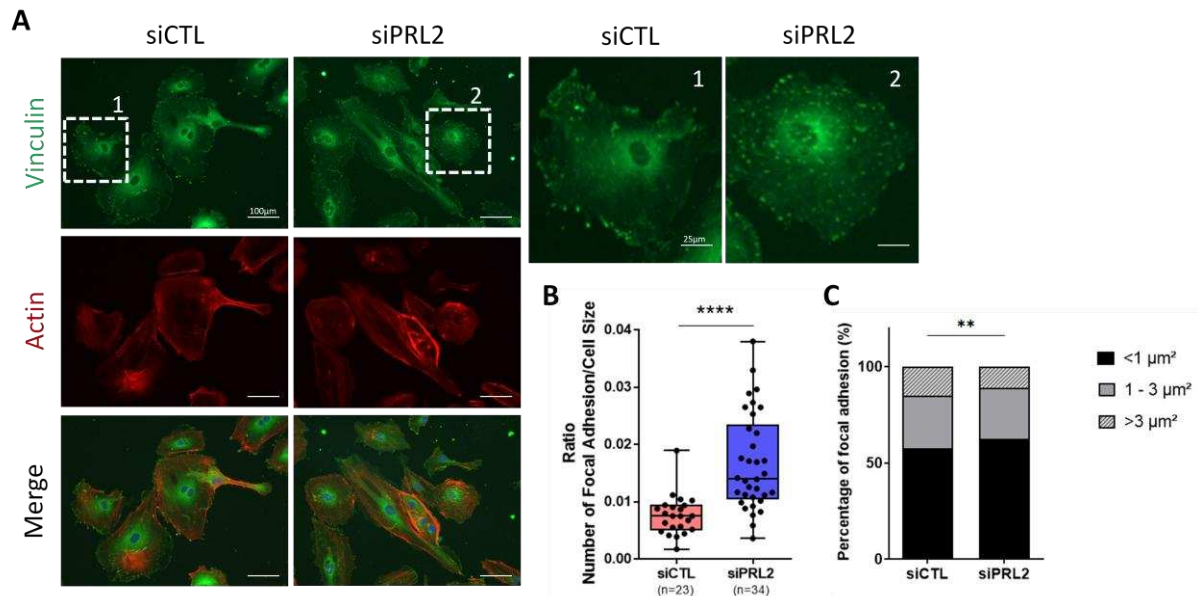


Figure 2.4 : PRL2 silencing affects the formation of focal adhesion in LECs.

(A) Representative images of focal adhesion in siCTL and siPRL2 LECs with vinculin staining. (B-C) Quantification of focal adhesion per cell size and per size for each cell (C). (Mann-Whitney test and Two-way ANOVA). Graphs are represented in mean $\pm$ SD; ** $p < 0.01$; **** $p < 0.0001$

2.3.4. Sprouting of LECs is modulated by PRL2

Sprouting is the first step of new vessel formation, combining cell proliferation and migration. Given the previous results, we expected to have less sprouting with PRL2 knockdown. To address this, a bead sprouting assay was performed. Beads coated with cells were included in a fibrin gel and sprout formation was observed for 72h, 96h and 120h. At 72h, more than 85% of beads with siCTL LECs presented sprouts, but only 53% of coated beads with siPRL2 LECs displayed few sprouts (Figure 2.5A-C). The percentage of beads with sprouts increased with time, but beads with sprouts for siPRL2 LECs were still significantly less when compared to beads with siCTL LECs. Moreover, when beads with siPRL2 LECs formed

sprouts, the average sprout number was about 1 to 3 sprouts per bead, while beads with siCTL LECs had 2 to 4 sprouts per bead on average (Figure 2.5D). Sprouts were also significantly shorter for siPRL2 LECs (Figure 2.5E). These results confirmed our hypothesis that PRL2 knockdown decreases the sprouting capacity of LECs.

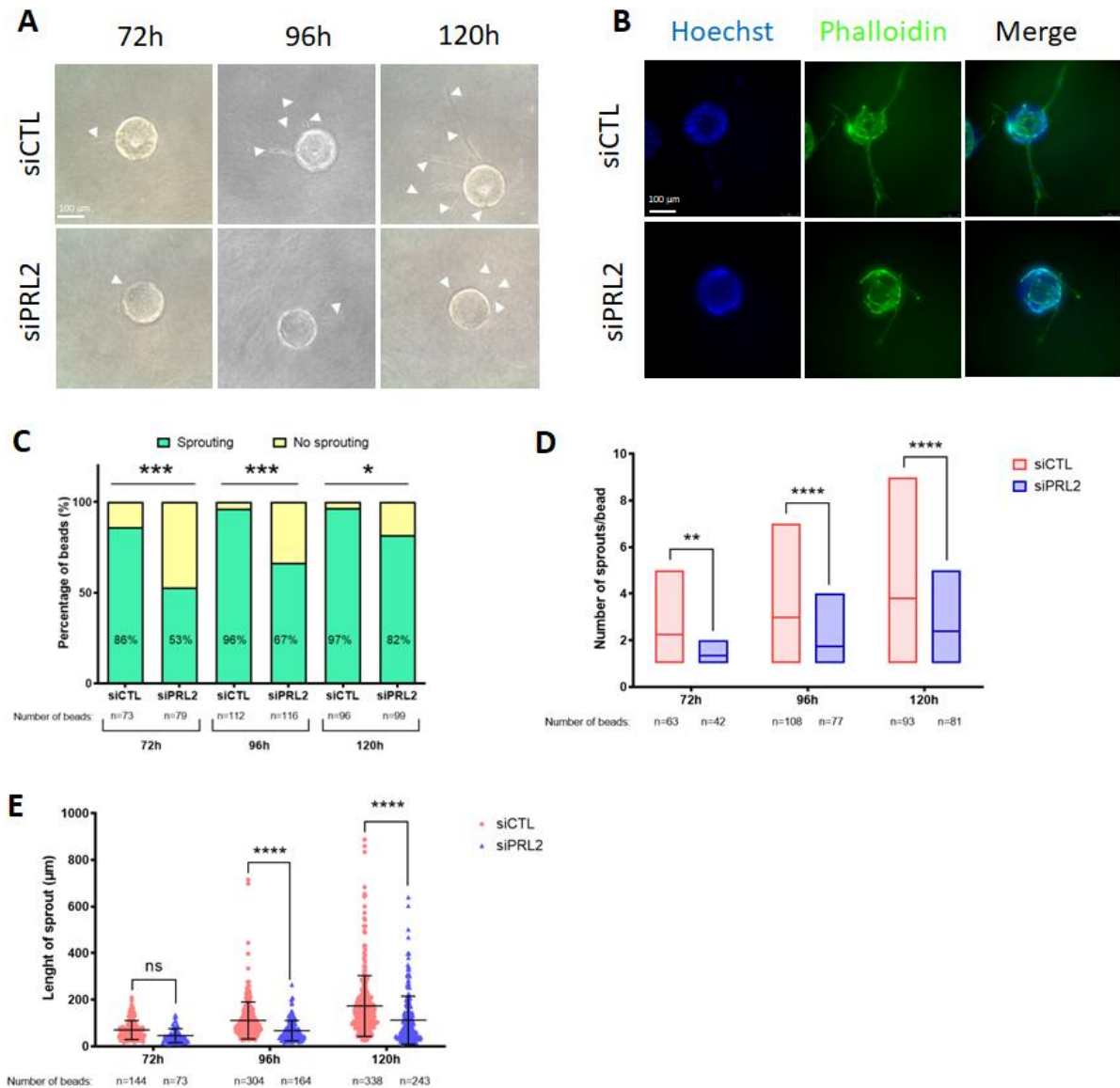


Figure 2.5: Decrease of cell sprouting with PRL2 silencing in LECs.

Bead-sprouting assay was performed with siCTL and siPRL2 LECs. (A-B) Representative images of sprouting-beads in brightfield (A) and with an actin staining (B). (C-E) Quantification of sprouting-beads (C), number of sprouts/bead (D), and length of sprouts (E). (Two-way ANOVA, Sidak's post-hoc test). Graphs are represented in mean $\pm$ SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

2.3.5. PRL2 silencing affects VEGF-A/-C signaling in LECs

Survival, proliferation, and migration of LECs are crucial for lymphangiogenesis and depend mainly on VEGFR-3 and VEGFR-2 expression and activation by VEGF-A or VEGF-C²³. Engagement of these receptors results in the activation of various classical signaling pathways including RAS/ERK1/2 and PI3K/AKT. To investigate this, cells were treated with both main lymphangiogenic factors and downstream pathways were studied. Following VEGF-C stimulation, Akt phosphorylation was increased and ERK 1/2 phosphorylation decreased in siPRL2 LECs compared to siCTL LECs (Figure 2.6A-C). Similar results were observed with VEGF-A stimulation (Figure 2.6D-F). A decrease in ERK 1/2 phosphorylation might explain the reduction in migration and the absence of response to either VEGF-C or VEGF-A in siPRL2 LECs. The increase in Akt phosphorylation was unrelated to PTEN as we saw no difference in its expression following PRL2 silencing (Figure 2.6G). This increase could be associated with cell survival of the cells.

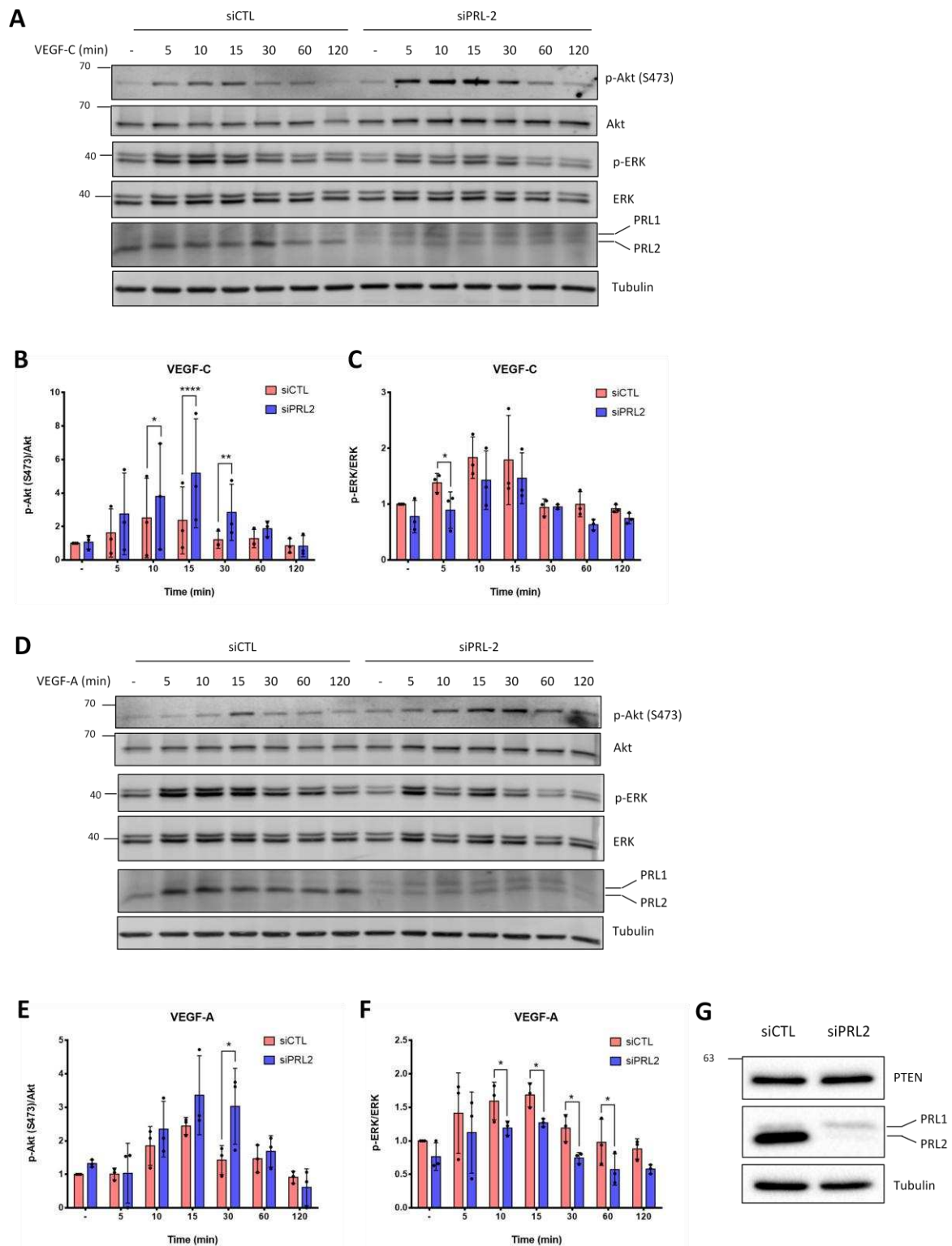


Figure 2.6: Modification in signaling with PRL2 silencing in LECs.

(A-B) Western blot of Akt and ERK 1/2 phosphorylation after VEGF-A or VEGF-C stimulation of siCTL and siPRL2 LECs. (C-D) Quantification of Akt phosphorylation and (E-F) ERK phosphorylation. (G) Western blot of PTEN protein level in siCTL and siPRL2 LECs

72h post-transfection. (n=3; Two-way ANOVA, Sidak's post-hoc test). Graphs are represented in mean $\pm$ SD; * p<0.05; ** p<0.01; **** p<0.0001

2.3.6. Notch1 signaling is disrupted by PRL2 silencing in LECs

It has been previously shown that Notch1/DLL4 signaling is impaired when PRL2 is knocked down in HUVECs¹. Thus, we investigated this pathway in LECs to see if this was also the case upon VEGF-C or VEGF-A stimulation. Notch1 expression was not altered, but DLL4 was less expressed in siPRL2 LECs at the mRNA and protein levels (Figure 2.7A-C). To avoid a difference in the activation of the Notch1 signaling pathway due to a reduced expression of DLL4 in siPRL2 LECs, we decided to pursue our experiment in the presence of DLL4 coating. After DLL4 coating, we saw an increase in Notch1 cleavage (Figure 2.7D-E), which indicated activation of the pathway. We observed the same pattern with siPRL2 LECs. However, upon DLL4 stimulation, target genes, HEY1 and HEY2, expression were downregulated (Figure 2.7F-G), which suggests that in the absence of PRL2, Notch1 signaling pathway is disrupted after the cleavage of Notch1.

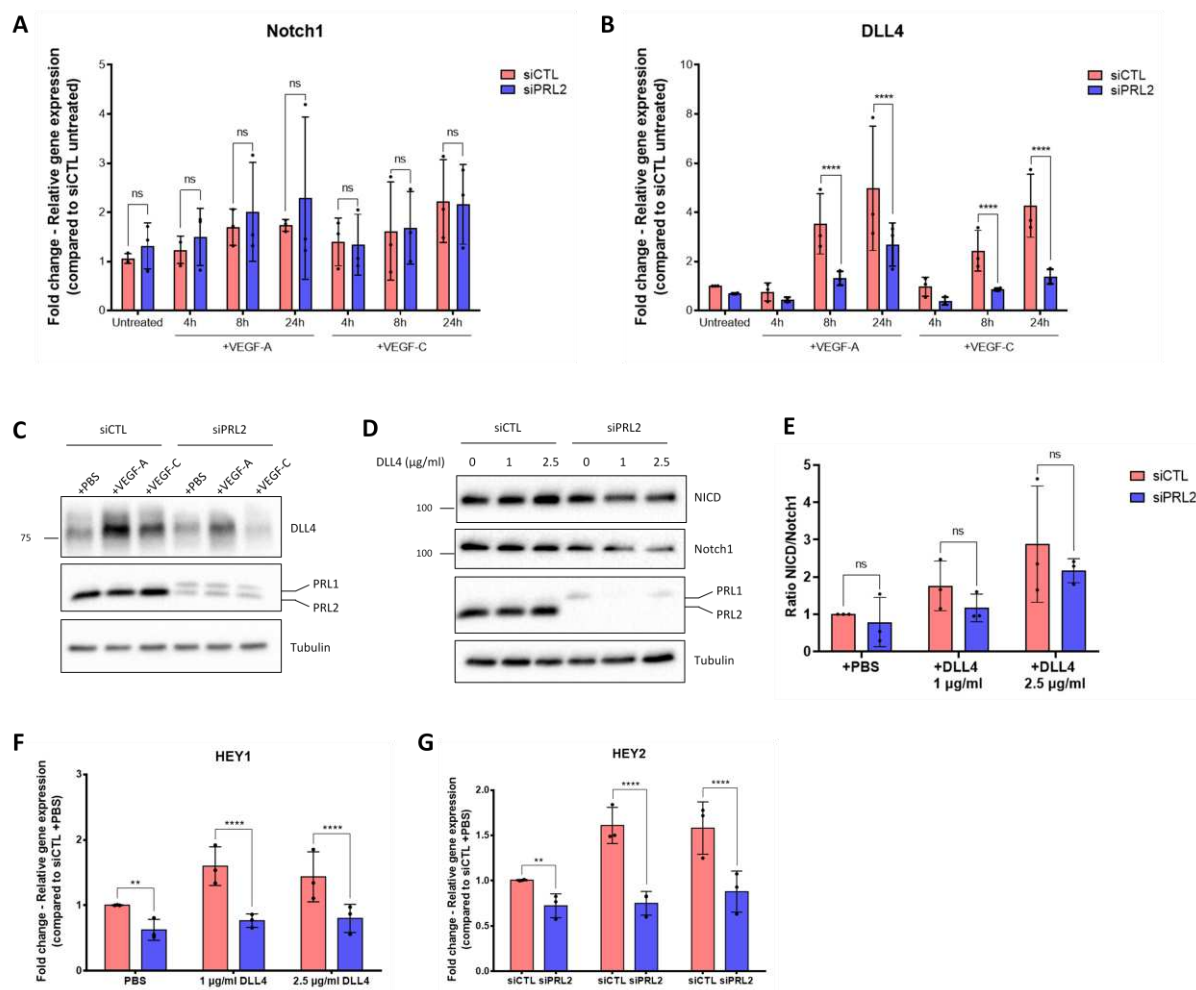


Figure 2.7: Notch1 signaling is disrupted by PRL2 silencing in LECs

(A-B) mRNA level of Notch1 and DLL4 assessed by qPCR following VEGF-A or VEGF-C stimulation at the indicated time. (n=3; Two-way ANOVA, Sidak's post-hoc test) (C) Western blot of DLL4 protein level after VEGF-A or VEGF-C stimulation for 24h. (D) Western blot of Notch1 cleavage after DLL4 stimulation for 24h. (E) Quantification of Notch1 cleavage. (n=3; Two-way ANOVA, Sidak's post-hoc test) (F-G) mRNA level of HEY1 and HEY2 assessed by qPCR after DLL4 stimulation overnight. (n=3; Two-way ANOVA, Sidak's post-hoc test). Graphs are represented in mean $\pm$ SD; ** p<0.01; **** p<0.0001

2.4. Discussion

In this chapter, we have demonstrated that PRL2 controls several important phenotypic properties of LEC, including proliferation, migration and sprouting *in vitro*. These biological

processes are the most important for lymphangiogenesis and the results point to an important role of PRL2 in the process. Thus, PRL2 is likely to play an important role not only in the blood vasculature, but also in lymphatic vessels.

As the absence of PRL2 in LECs decreased the main processes involved in lymphangiogenesis, we investigated cell signaling that could underlie these phenotypic changes. VEGFR-3 and VEGFR-2 are the main driver of lymphangiogenesis²³. When lymphangiogenic factors such as VEGF-C or VEGF-A bind to their respective receptors, they activate several signaling pathways involved in proliferation, migration and cell survival. Our results demonstrate that Akt and ERK 1/2 phosphorylation are modulated in SiPRL2 LECs following VEGF-C or VEGF-A stimulation. When PRL2 is silenced, ERK 1/2 phosphorylation is decreased which may contribute to the inhibition of migration and sprouting. Unexpectedly there was also an increase in Akt phosphorylation. It has been shown in HEK293 cell line that the absence of PRL2 increases PTEN protein expression which lead to a decrease of Akt signaling^{67,71}. However, it is the opposite in LECs as we observed an increase in Akt signaling after PRL2 silencing with PTEN expression not modified. Thus, PRL2 does not affect Akt signaling via PTEN.

Then, we investigated focal adhesion by immunolabeling and observed increased number of focal adhesion in cells with PRL2 silencing. This points to a possible link between PRL2 and focal adhesion formation. The main signaling pathway involved in the formation of focal adhesions is the Focal Adhesion Kinase (FAK) signaling pathway. Phosphorylation of FAK induces the focal adhesion formation by clustering proteins in 3 layers with FAK being on the bottom layer and vinculin in the middle layer⁸³. Focal adhesions interact directly with actin to modulate cell migration. Actually FAK associates with Src and activates cdc42, a GTP-

binding protein regulating cell migration⁸⁴. As PRL2 has been shown to minimally interact with Src and stimulate the ROCK pathway⁸⁵, it would be interesting to study this pathways upon PRL2 silencing to find a possible explanation on how PRL2 can affect the migration of LECs.

Another interesting pathway that could be affected by PRL2 is the Notch1/DLL4 signaling pathway. In HUVECs, it has been observed that the absence of PRL2 disrupts the Notch1/DLL4 signaling pathway by inhibiting Notch1 cleavage¹. In siPRL2 LECs, Notch1 cleavage was not affected, but DLL4 expression and its target genes were decreased. These results show that the Notch1 pathway is affected when PRL2 is silenced in LECs. The low level of DLL4 could already impair the signaling pathway without stimulation. Thus, in LECs, PRL2 affects Notch1 signaling by acting on DLL4 but not on Notch1 cleavage, which is different to vascular endothelial cells (HUVECs). Coating DLL4 could rescue Notch1 cleavage in siPRL2 LECs. This indicates that Notch1 cleavage is not affected by the silencing of PRL2. This is in line with inhibiting the expression of Notch1 target genes and the disruption of this signaling pathway. The absence of PRL2 could affect the translocation of cleaved Notch1 inside the nucleus.

Overall, the results of this chapter reinforce the contention that PRL2 represents a novel modulator of lymphangiogenesis by affecting several signaling pathways involved in proliferation, migration and lymphatic sprouting. The *in vivo* impact will be discussed in the following chapter.

Chapter 3: Lymphatic functions and PRL2

***in vivo*: PRL2^{fl/fl}-Prox1Cre⁺ mouse model**

3.1. Objectives

In this chapter, we investigated *in vivo* functions of PRL2 by generating a mouse model with an inducible deletion of PRL2 in lymphatic endothelial cells. This mouse model was investigated in a mouse lymphedema model by surgically removing lymph nodes in one limb. The lymphedema model in the forelimb is a well-known model to study lymphangiogenesis in lymphedema context.

3.2. Material and methods

3.2.1. List of antibodies

Table 3.1: List of antibodies used for western blot, immunostaining and flow cytometry

Protein target	Antibody
CD11c	Brilliant Violet 605 anti-mouse CD11c, BioLegend #117334
CD3	Rabbit anti-CD3, Abcam #ab16669 PE-Cy5 Anti-Mouse CD3e, BD 553065
CD4	APC/Cyanine7 anti-mouse CD4, BioLegend #100526
CD45	Alexa Fluor 488 anti-mouse CD45, BioLegend #103121
CD8	Brilliant Violet 510 anti-mouse CD8a, BioLegend #100751
F4/80	Rat anti-mouse F4/80, Biorad #MCA497G APC anti-mouse F4/80, BioLegend #123116
LYVE1	Goat Anti-LYVE1, R&D #AF2125
PRL 1/2	Mouse anti-PRL1/2, EMD Millipore #05-1583
α -Tubulin	Mouse anti- α -Tubulin, Sigma Aldrich #T5168

VE-Cadherin	Mouse anti-VE-Cadherin (F-8), Santa Cruz #sc-9989 Goat anti-VE-cadherin (C-19), Santa Cruz #sc-6458
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3.2.2. List of primers

Table 3.2: List of primers used for qPCR

Gene	Primer forward	Primer reverse
PRL2	CCAATGCGACTCTCAACAAGTTCAC	CAACACAACAGCCTGGCTCTTCAC
Prox1 ⁸⁶	CTGGGCCAATTATCACCAGT	GCCATCTTCAAAAGCTCGTC

3.2.3. Mice

PRL2^{fl/fl}-Prox1Cre⁺ mice were generated by breeding PRL2^{fl/fl} mice¹ with Prox1-CreERT2 mice⁸⁷. Dr. Barbara Garmy-Susini generously donated two Prox1-CreERT2 male mice for this breeding. For our experiment, we bred PRL2^{fl/fl}-Prox1Cre⁺ mice (heterozygous for Prox1-CreERT2 allele) with PRL2^{fl/fl}-Prox1Cre⁻ to obtain PRL2^{fl/fl}-Prox1Cre⁺ (also heterozygous for Prox1-CreERT2 allele) and their control littermates PRL2^{fl/fl}-Prox1Cre⁻. Mice of both sexes were used to verify PRL2 deletion, and then only female mice were used for the lymphedema study.

To induce the deletion of PRL2 gene, six week-old mice received six intraperitoneal injections of tamoxifen (50µg/g; Sigma Aldrich, T5648) distributed over two weeks (approximately one injection every two days). Control littermates also received the tamoxifen injections.

All protocols used on mice were approved by the Ethic Committee of Bordeaux (CE050, DAP38181).

3.2.4. Forelimb lymphedema model

Mice were anesthetized with an intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg). The surgery area was shaved and sanitized with 70% ethanol. A 3-4 mm incision was cut at the axilla of left forelimb. Axillary and brachial lymph nodes were removed, and the incision was sutured and disinfected. The right forelimb was used as a control.

Two weeks after the surgery, the forelimbs were measured with a caliper and we performed a lymphography before sacrificing the mice.

3.2.5. Lymphography

Mice were anesthetized with a subcutaneous injection of ketamine (100mg/kg) and xylazine (10mg/kg). 5 µl of FITC-Dextran was injected into the footpad of mice (70 kDa fluorescein isothiocyanate-dextran, 2 mg/ml; Sigma Aldrich) and the lymphatic drainage of the fluorescent dye was monitored with a fluorescent microscope (Leica, DMi8). Visualizing the dye in the lymphatic vessels provides clear information about lymphatic function, vessel integrity and potential leakage.

3.2.6. Immunostaining

Skin of the two forelimbs and inguinal lymph nodes from mice were fixed with 10% formalin at 4°C and included in paraffin. 5 µm and 10 µm sections were cut with a microtome before being deparaffinized and rehydrated in water. Antigen unmasking was performed using a heat antigen retrieval solution (Tris solution pH9, Vector Laboratories #H-3301) microwaved for 5 minutes three times. After cooling, slides were blocked with 5% BSA for 30 minutes. Primary antibodies were diluted in BSA 2% (1:100) and incubated overnight at 4°C. Secondary antibodies Alexa Fluor were diluted in BSA 2% (1:500) and incubated for 2h at room

temperature. Nuclei were visualized using DAPI (4',6-diamidino-2-phenylindole, Hello Bio, HB 0747). Coverslips were mounted on slides with ProLong Gold antifade mountant (Invitrogen #P36934). Images were taken using a fluorescent microscope (Leica, DMI8) and analyzed with ImageJ.

LECs were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.5% Triton before blocking with 3% BSA. Primary antibody diluted in 1% BSA was incubated at room temperature for 1 h and secondary antibody diluted in 1% BSA was incubated for 30 minutes. Nuclei were visualized using DAPI (4',6-diamidino-2-phenylindole, Hello Bio, HB 0747). Images were taken at 20X magnifications using a fluorescent inverted microscope (Leica, DMI8) and analyzed with ImageJ.

3.2.7. Flow cytometry

Skins from control and lymphedema fore limb were dissociated with Multi Tissue Dissociation Kit 1 (Miltenyi, #130-110-201) and gentleMACS Dissociator following the manufacturer's recommendations. Cell suspensions obtained were filtered through a 100 μ m cell strainer. RBC lysis buffer (eBioscience, #00-4300) was used for the lysis of erythrocytes before filtering cell suspensions through a 40 μ m cell strainer. After spinning, cell pellets were incubated with Fc-blocking antibody CD16/CD32 (eBioscience, #14-0161-82) on ice for 10 minutes. Cells were stained with antibodies (see 3.2.1. List of antibodies) on ice for 30 minutes. Flow cytometry was performed using a BD Influx cell sorter and FACSDiva software analysis.

3.2.8. Cell culture

Human Dermal Lymphatic Endothelial Cells (LECs) were purchased from Promocell and cultured in Endothelial Cell Growth Medium MV2 (ECGM MV2, Promocell) on flasks or plates coated with 0.4% gelatin (PAN BIOTECH P06-25200). Cells were maintained in a humidified incubator with 5% CO₂ at 37°C and the media was renewed every 2-3 days. LECs were used between passage 4 and 7.

3.2.9. Transfection

siRNAs were purchased from Dharmacon : PTP4A2 (SMART Pool, M-009078-01-0005, GE Healthcare Europe GmbH) and negative control (SMART Pool, Non-targeting siRNA, D-001206-13-05, GE Healthcare Europe GmbH). Lipofectamine RNAiMAX kit (Invitrogen 13778075) was used for transfection, following manufacturer's protocol.

3.2.10. Western Blot

LECs were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche) for 30 minutes on ice, followed by a centrifugation at 10000 g for 10 minutes. Supernatants were collected and proteins were quantified following Pierce BCA protein assay kit protocol (Thermo Scientific #23225). Samples were prepared with 15 µg proteins and Laemmli buffer (300 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.5 mg/ml bromophenol blue, 10% β-mercapto-ethanol), boiled for 5 minutes at 100°C, then separated by SDS-PAGE. After migration, proteins were transferred on a PVDF membrane. Membranes were blocked with 5% BSA for 1h at room temperature, then incubated with primary antibodies diluted in antibody solution (PBS 1X supplemented with 0.5% Tween and 0.02% sodium azide) overnight at 4°C. Secondary antibodies were diluted in antibody

solution and incubated for 1h at room temperature. Membranes were scanned with an Odyssey imager (LI-COR) and image analysis was performed using Image Studio Lite 5.2.

3.2.11. RNA isolation, reverse transcription, and quantitative real-time PCR

RNA was isolated from mouse lymphatic duct with TRI-Reagent (MRC #TR118) following the manufacturer's protocol. The only adjustment done was that RNA precipitation was performed for 4 hours at 4°C. RNA pellets were resuspended in sterile water, and then quantified with NanoDrop. 500 ng of RNA was reverse-transcribed to cDNA using the High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific #4368813) following the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed with an AriaMx Real-time PCR System (Agilent) using EurobioGreen qPCR Mix Hi-ROX (Eurobio #GAEMMX02H 0T) according to the manufacturer's protocol.

3.2.12. Statistical analysis

Statistical analyses were performed on GraphPad Prism 9 software. A Shapiro-Wilk test was performed to assess the normal distribution of the sample. Then, Student t-test (normal distribution) or Mann-Whitney test was used to compare two groups, while Two-way ANOVA, followed by Sidak or Tukey's post-hoc test, was used to compare groups with more than one variable. The difference was statistically significant when the p-value was below 0.05.

3.3. Results

3.3.1. Generation of a PRL2^{fl/fl}-Prox1Cre⁺ mouse

To study how PRL2 expression is important for lymphatic vessel formation, we decided to generate a mouse model with a specific inducible knock-out (KO) of PRL2 in lymphatic

endothelial cells. To generate our mouse model, we crossed a PRL2^{fl/fl} mouse with a Prox1Cre⁺ mouse to obtain a PRL2^{fl/fl}-Prox1Cre⁺ mouse named PRL2^{fl/fl}-Prox1Cre⁺ (Figure 3.1A). As control mice, we used littermate mice, without the Prox1-Cre named PRL2^{fl/fl}-Prox1Cre⁻.

PRL2 KO was induced by six intraperitoneal injections of tamoxifen distributed over two weeks (approximately 1 injection every two days). KO was verified by isolating the lymphatic duct of mice and extracting the mRNA for qPCR analysis (Figure 3.1B). To normalize gene expression of PRL2, Prox1 was used as a housekeeping gene as all LECs express it and its expression should not be modified by PRL2 KO. A decrease in PRL2 expression was observed in LECs from PRL2^{fl/fl}-Prox1Cre⁺ mice. Thus, PRL2 was partially deleted (approximately 45%) in LECs in PRL2^{fl/fl}-Prox1Cre⁺.

After induction by tamoxifen of PRL2 deletion, mouse well-being was monitored. There was no change in behavior, gross phenotype and weight between LEC-PRL2-deleted mice and control mice three days and one month post-injection of tamoxifen (Figure 3.1C-D).

To assess the functionality of lymphatic vessels after PRL2 partial deletion, lymphography was performed to investigate whether pre-existing vessels were altered. Backflow of fluorescent dye in the lymphatic network was observed in both control mice and PRL2^{fl/fl}-Prox1Cre⁺ mice, and no leakage was observed.

Overall, these results indicate that partial deletion of PRL did not affect gross morphology, behavior, and vessel function of pre-existing lymphatic vasculature.

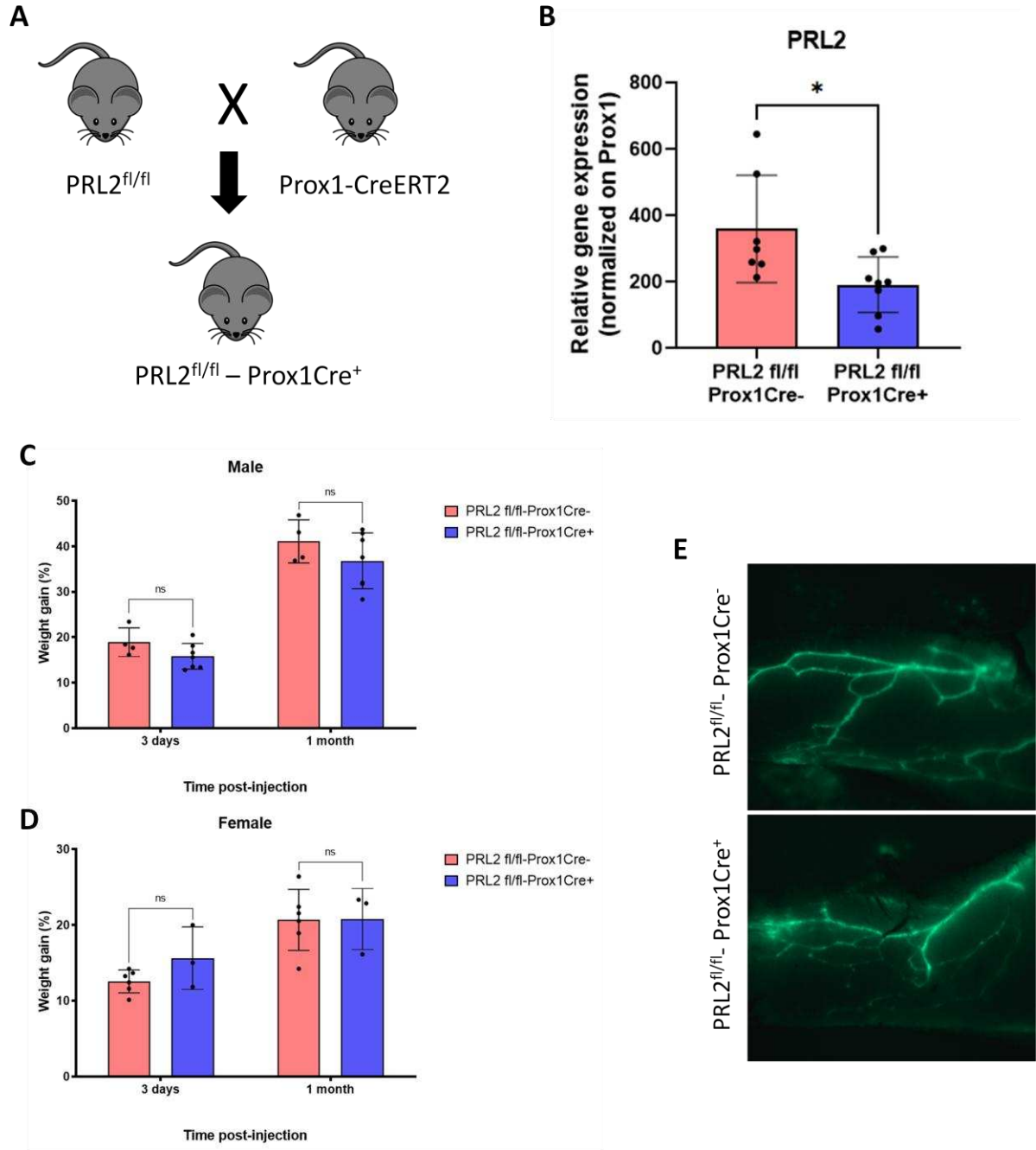


Figure 3.1: Generation of PRL2^{fl/fl}- Prox1Cre⁺ mouse

(A) Schematic of the breeding to obtain PRL2^{fl/fl}- Prox1Cre⁺ mice. (B) mRNA level of PRL2 assessed by qPCR. Each dot represents one mouse. (Student t-test) (C-D) Weight gain of male and female mice following tamoxifen injection. Each dot represents one mouse. (Two-way ANOVA, Tukey's post-hoc test) (E) Representative images of lymphography performed on PRL2^{fl/fl}-Prox1Cre⁺ mice and their control littermates PRL2^{fl/fl}-Prox1Cre⁻ 1 month after tamoxifen injection. Graphs are represented in mean ±SD; ns=non-significant; * p<0.05

3.3.2. PRL2 expression could affect lymphatic drainage in lymphedema

To get further insights into whether lymphangiogenesis was altered in PRL2^{fl/fl}-Prox1Cre⁺ mice, we took advantage of a lymphedema model. This was performed six weeks after the induction of PRL2 deletion (Figure 3.2A). In this model, lymphedema is induced by surgically removing lymph nodes in one limb of the mouse. The other limb is used as control. Lymphedema was observed by the swelling of the limb 14 days post-surgery. Measurement of limb swelling showed a significant increase in operated limb in both groups of mice (Figure 3.2B). Although, no major differences between the two groups of mice were observed, PRL2^{fl/fl}-Prox1Cre⁺ mice tended to have slightly bigger limbs (1-2 mm increase when compared to PRL2^{fl/fl}-Prox1Cre⁻ mice).

During lymphedema, new lymphatic vessels are formed to drain fluid accumulation in the operated site. This was assessed by lymphography documenting differences in fluid drainage (Figure 3.2C). In the control mice, we saw the formation of new lymphatic vessels after lymph node removal. These new capillaries were dense and showed functional dermal lymphatic backflow after injection of FITC-Dextran. On the opposite, PRL2^{fl/fl}-Prox1Cre⁺ mice did not develop new functional lymphatic vessels on the operated site as no fluorescent dye drainage was seen. This result indicates that PRL2 is involved in the function of lymphatic vessels which is related to fluid accumulation.

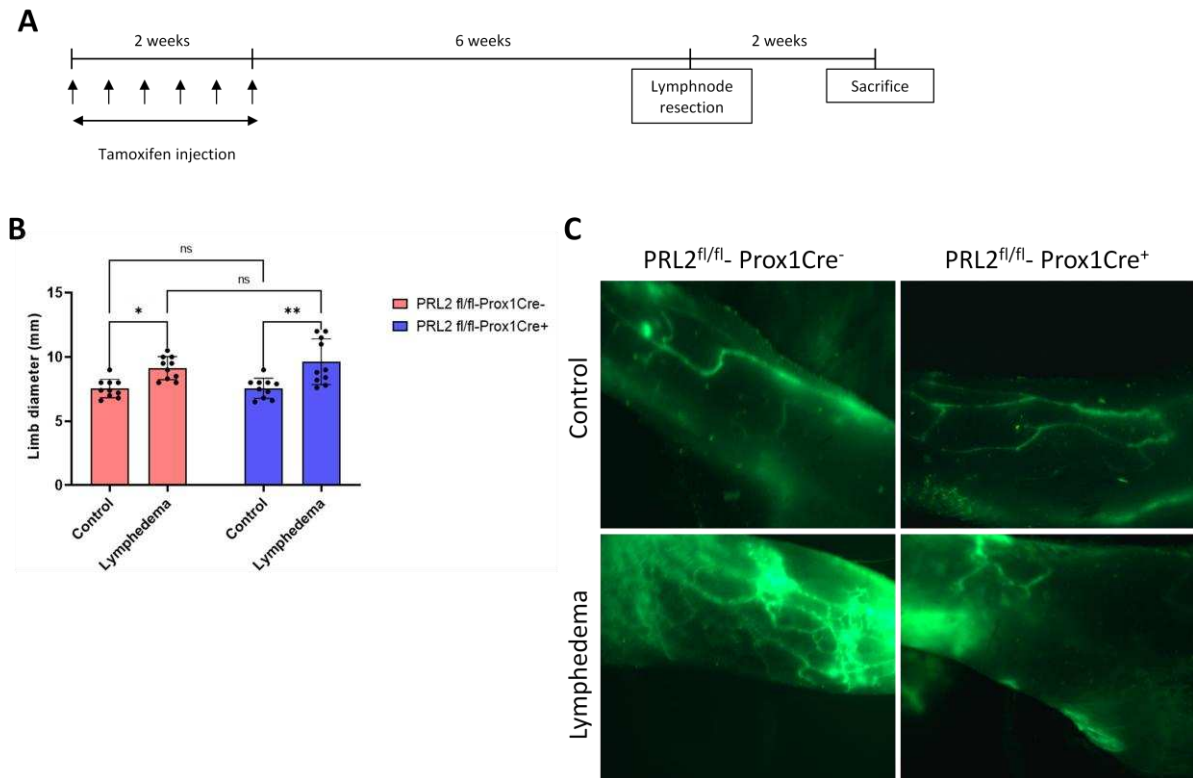


Figure 3.2: PRL2 could help draining fluid in lymphedema

(A) Timeline of lymphedema experiment. (B) Measure of limb with and without lymphedema in PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice. (n=10; Two-way ANOVA, Sidak's post-hoc test) (C) Representative images of lymphography performed on PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice 14 days after lymph node removal surgery. Graphs are represented in mean $\pm$ SD; ns=non-significant; * p<0.05; ** p<0.01

3.3.3. PRL2 could be involved in lymphedema development

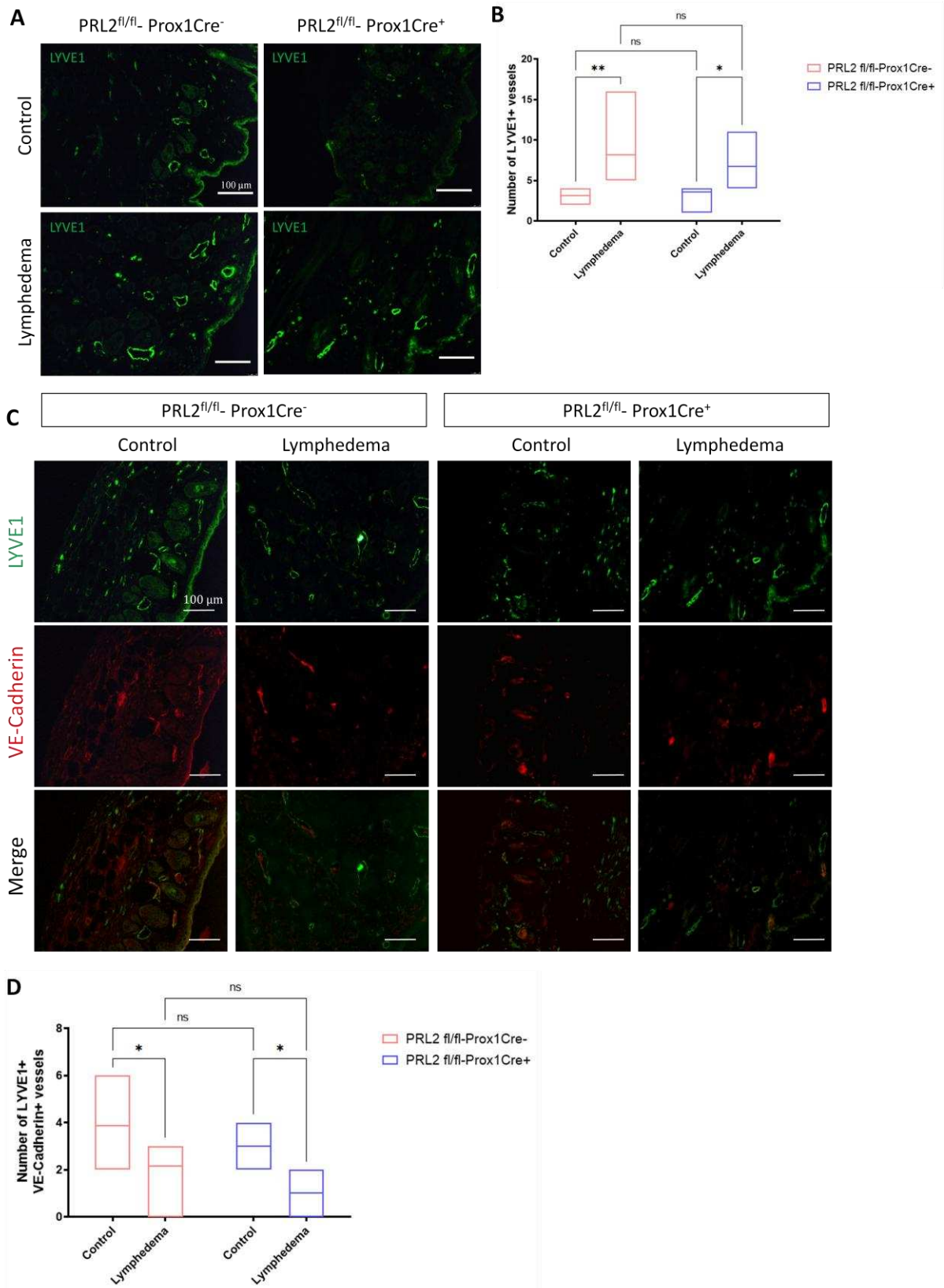
As a difference in the lymphatic drainage was observed, we collected skin samples for LYVE1 and VE-Cadherin staining. LYVE1⁺ lymphatic vessels are increased after lymphedema formation and are more dilated (Figure 3.3A-B). While PRL2^{fl/fl}-Prox1Cre⁺ mice exhibited a lower number of lymphatic vessels that were less dilated when compared to control mice.

Double staining of LYVE1 and VE-Cadherin was used to evaluate the integrity of lymphatic vessels. The integrity of the vessels is determined by the state of junction between cells. If the

junctions are tight, there will be no leakage. If the junctions are loose, the vessels will be leaky. VE-Cadherin is one of the main proteins in the complex forming junction between cells. After lymphedema, there was a decrease in double-stained vessels in tissue for both groups (Figure 3.3C-D), which indicates an impact on vessel integrity and a possible increase in permeability as the junction between cells might be weakened. Double staining of lymphatic vessels from PRL2^{fl/fl}-Prox1Cre⁺ mice revealed a lower mean number of doubled stained vessels, which is compatible with our *in vitro* observations in siPRL2 LECs where cell junctions were also disrupted (Figure 3.3E). This defect was not due to a decrease of VE-Cadherin protein, but most likely to a change in VE-Cadherin organization at cell junctions (Figure 3.3E-F). Thus, the disorganization of cell junctions affects the integrity of the vessels which could lead to a defect in fluid uptake.

Taken together, these results favor the contention that lymphatic function is perturbed in PRL2^{fl/fl}-Prox1Cre⁺ mice after lymphedema and point to a role of PRL2 in pathological lymphatic development.

Finally, Masson's trichrome staining assessed collagen accumulation as an indicator of fibrotic tissue. After lymphedema, a significant increase in collagen at the operated site was evidenced in both groups of mice, but collagen accumulation was lower in PRL2^{fl/fl}-Prox1Cre⁺ mice than in PRL2^{fl/fl}-Prox1Cre⁻ mice (Figure 3.3G). Thus, PRL2 deletion might impair the accumulation of collagen, thus reducing the risk of fibrosis.



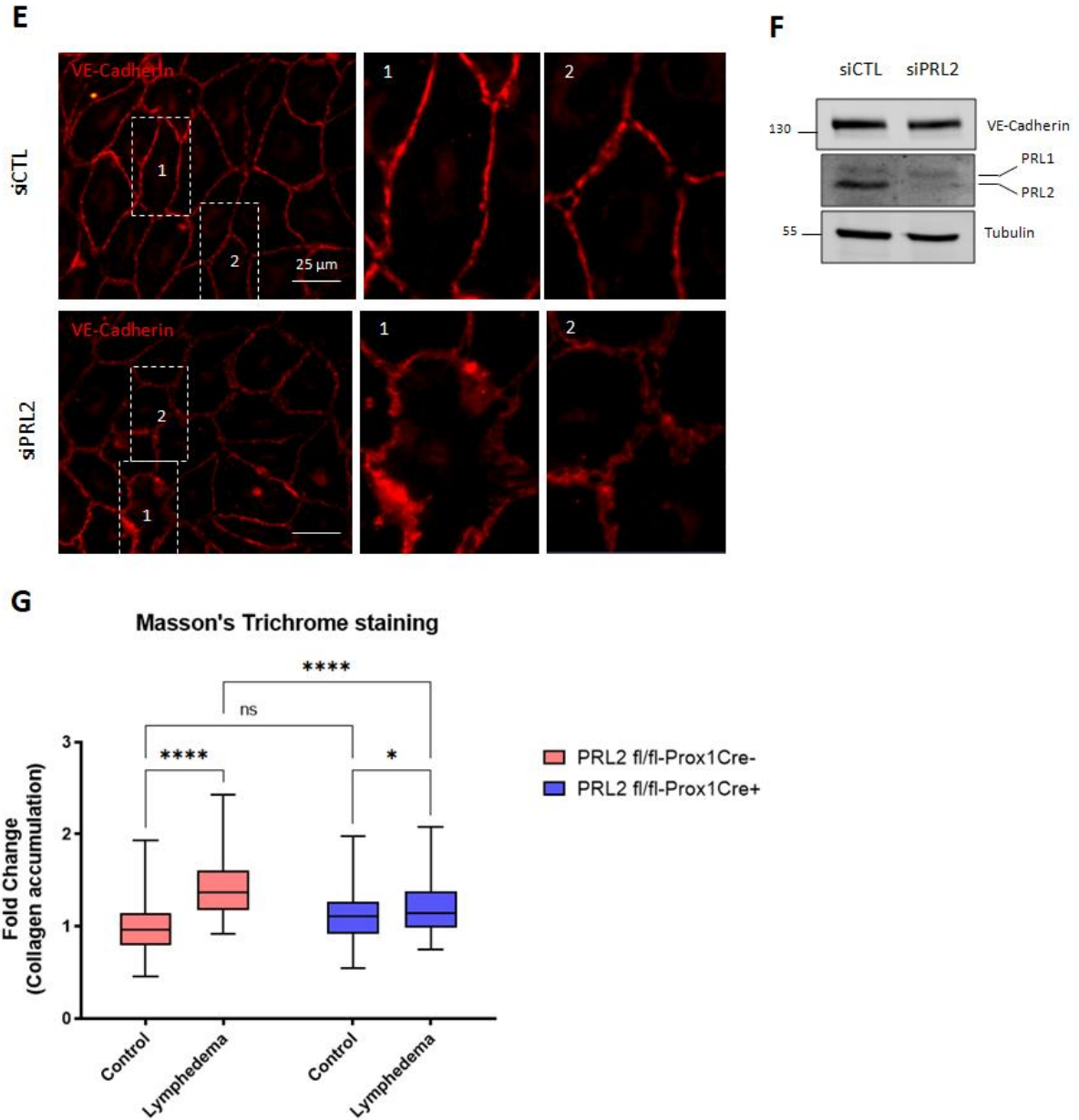


Figure 3.3: PRL2 could be involved in lymphatic vessel integrity and fibrosis

(A-D) Representative images and quantification of LYVE1 and VE-Cadherin immunostaining on the skin from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice with or without lymphedema. (n=5 mice; Two-way ANOVA, Tukey's post-hoc test) (E) VE-Cadherin immunostaining on siCTL and siPRL2 LECs 72h post-transfection. (F) Western blot of SiCTL LECs and siPRL2 72h post-transfection. (G) Analysis of collagen accumulation in the skin from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice with or without lymphedema. (n=5 mice; Two-way ANOVA, Tukey's post-hoc test). Graphs are represented in mean $\pm$ SD; ns=non-significant; * p<0.05; ** p<0.01; **** p<0.0001

3.3.4. PRL2 modulates immune cell infiltration

There is an increase in immune cell infiltration and accumulation at lymphedema site. Thus, immunostaining and flow cytometry on skin tissue were performed to identify the immune cell populations present. Immunostaining and flow cytometry analysis of the skin of PRL2^{fl/fl}-Prox1Cre⁻ mice with lymphedema showed an increased number of macrophages-F4/80⁺ cells (Figure 3.4A-D). Similarly, macrophages were also detected in the skin of PRL2^{fl/fl}-Prox1Cre⁺ with lymphedema, but no significant difference was observed when compared to skin from control mice.

The presence of dendritic cells in the skin was analyzed by flow cytometry. Akin to macrophages, the number of dendritic cells was increased in the skin of mice with lymphedema (Figure 3.4E-F). However, the difference with PRL2^{fl/fl}-Prox1Cre⁻ mice was not significant. In conclusion, PRL2 deletion seems not to affect significantly myeloid cell infiltration during lymphedema.

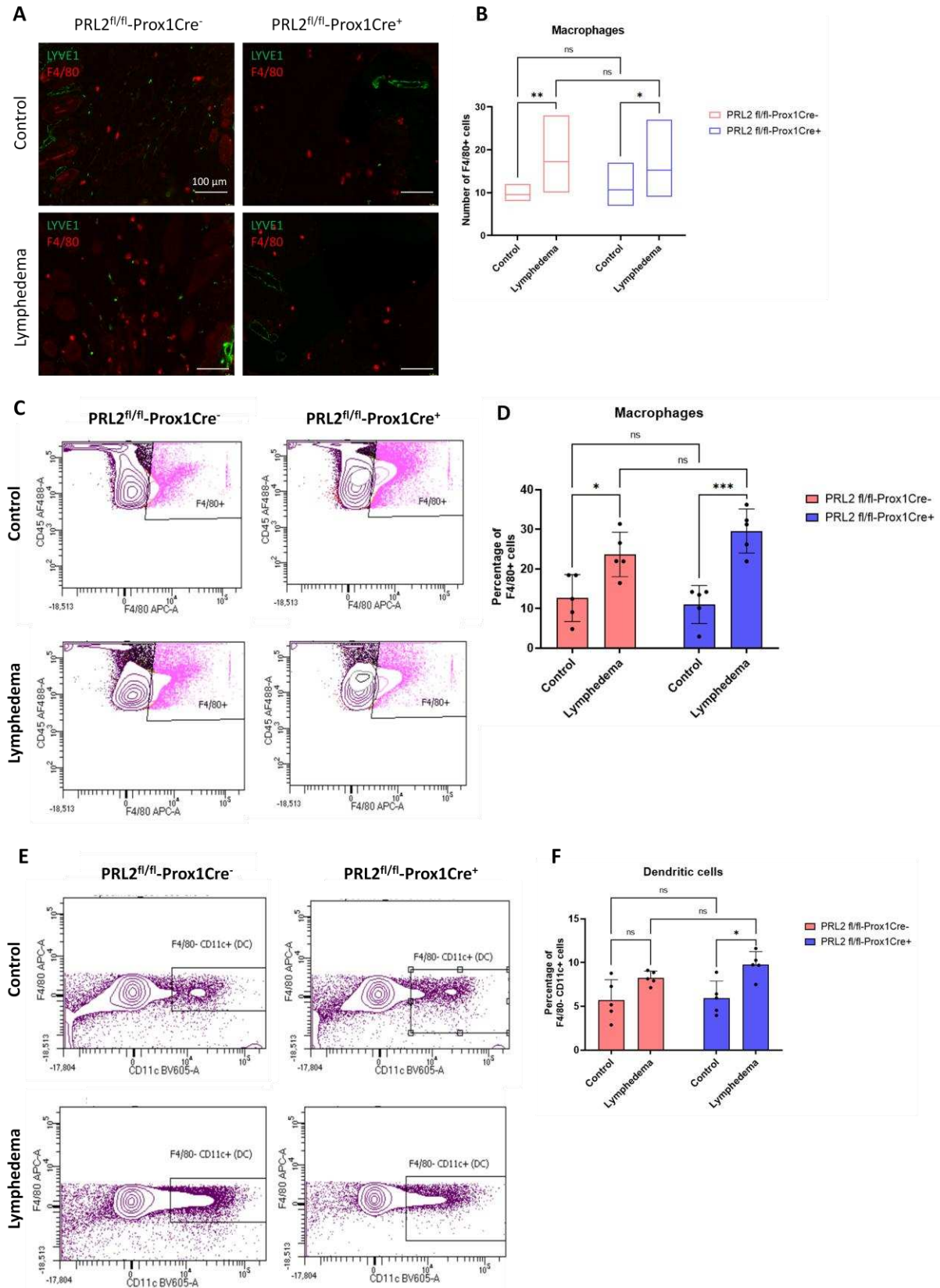


Figure 3.4: PRL2 deletion do not affect myeloid cell infiltration in lymphedema

(A) Representative images of LYVE1 and F4/80 staining on skin from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice with or without lymphedema. (B) Quantification of

F4/80+ cells in skin from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice with or without lymphedema. (n=5 mice; Two-way ANOVA, Tukey's post-hoc test) (C-F) Flow cytometry analysis and quantification of macrophages and dendritic cells infiltration in skin from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice with or without lymphedema. (n=5 mice; Two-way ANOVA, Tukey's post-hoc test). Graphs are represented in mean $\pm$ SD; ns=non-significant; * p<0.05; ** p<0.01; *** p<0.001

However, PRL2 deletion had a different effect on the T-cell population in the skin. A higher number of T-cells in the control limb of PRL2^{fl/fl}-Prox1Cre⁺ mice were observed when compared to PRL2^{fl/fl}-Prox1Cre⁻ mice (Figure 3.5A-B). However, while PRL2^{fl/fl}-Prox1Cre⁻ mice showed an increase in T-cells after induction of lymphedema, no increase was observed in PRL2^{fl/fl}-Prox1Cre⁺ mice with lymphedema.

Two different T-cell populations were identified by flow cytometry: helper T-cells (CD4⁺ T-cells) and cytotoxic T-cells (CD8⁺ T-cells). In control mice, the increase in T-cell seems to only involve helper T-cells and not cytotoxic T-cell (Figure 3.5C-E). In PRL2-deleted mice, the high infiltration of T-cell in the control limb involved both helper and cytotoxic T-cells and their percentage did not change with lymphedema (Figure 3.5C-E).

These results suggest that PRL2 deletion can increase the infiltration of T-cells in basal condition and no additional increase of T-cell infiltration is observed during lymphedema, which means that by an unknown mechanism, PRL2 seems to promote T-cell infiltration and accumulation in the skin of healthy mice. The physiological implications of this observation are, at present, not known.

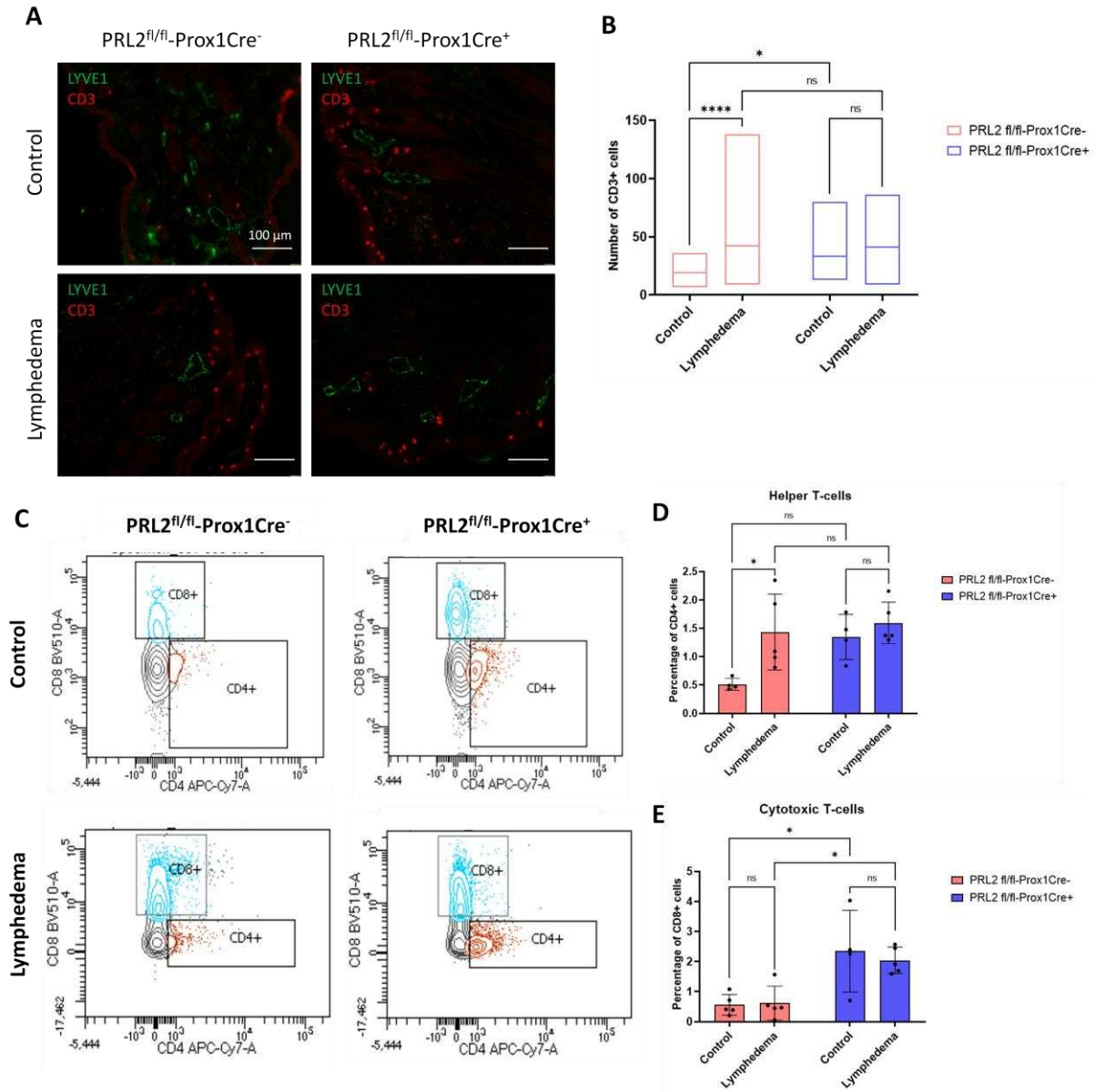


Figure 3.5: PRL2 deletion increases T-cells infiltration

(A) Representative images of LYVE1 and CD3 staining on skin from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice with or without lymphedema. (B) Quantification of CD3⁺ cells in skin from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice with or without lymphedema. (n=5 mice; Two-way ANOVA, Tukey's post-hoc test) (C-E) Flow cytometry analysis and quantification of helper and cytotoxic T cells infiltration in skin from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice with or without lymphedema. (n=5 mice; Two-way ANOVA, Tukey's post-hoc test). Graphs are represented in mean $\pm$ SD; ns=non-significant; * p<0.05; **** p<0.0001

3.3.5. Modification of lymph node structure following deletion of PRL2

Lymph nodes are the site of immune cell maturation. LECs are an integrated part of the lymph node structure, which could be affected by the absence of PRL2 expression. LYVE1 and VE-Cadherin staining were performed on inguinal lymph node to address this issue. We observed a reduced expression of LYVE1 and VE-Cadherin in the lymph node of PRL2^{fl/fl}-Prox1Cre⁺ mice, which could lead to a modification in the structure of lymph nodes and this could affect the maturation, activation and trafficking of immune cells (Figure 3.6A-C).

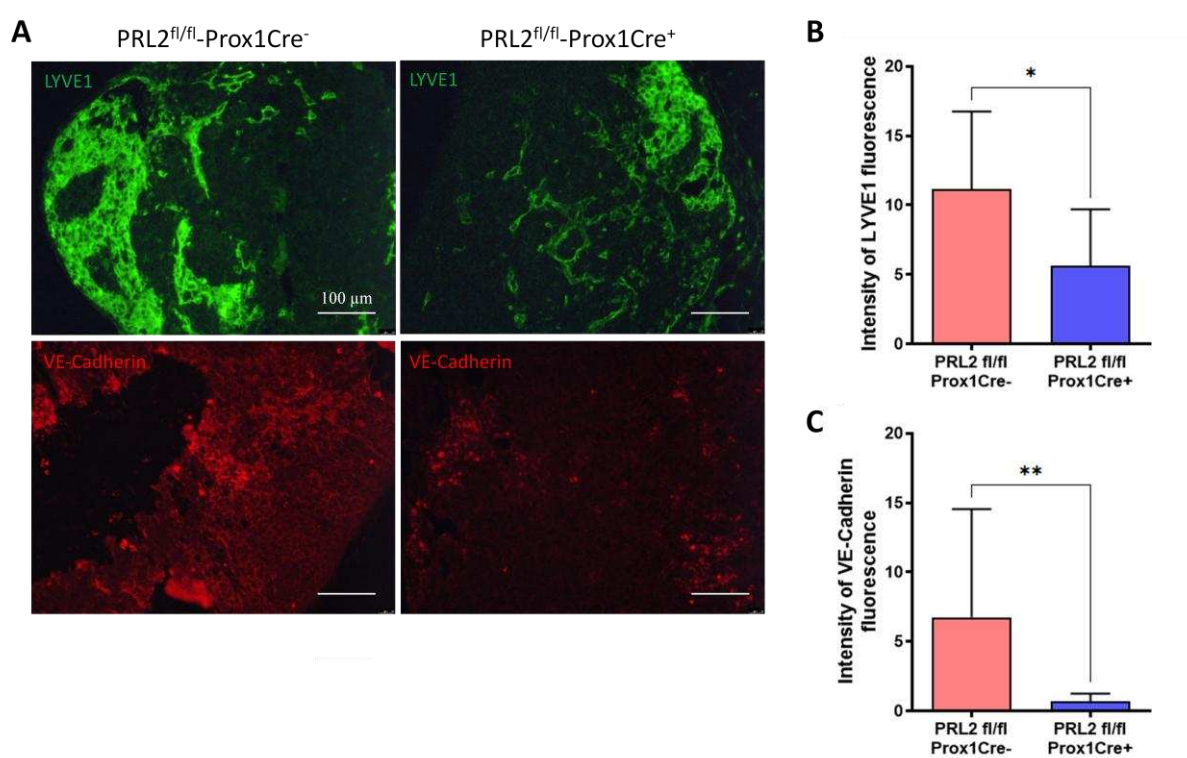


Figure 3.6: Lymph node structure is modified following PRL2 deletion

(A) Representative images of LYVE1 and VE-Cadherin immunostaining on inguinal lymph node from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice. (B-C) Quantification of fluorescence intensity for LYVE1 and VE-Cadherin. (n=4 mice; Student t-test for LYVE1 fluorescence and Mann Whitney test for VE-Cadherin fluorescence). Graphs are represented in mean $\pm$ SD; * p<0.05; ** p<0.01

3.4. Discussion

To understand the role of PRL2 in lymphatic vessels, we generated a mouse model with an inducible and specific deletion of PRL2 in LECs. PRL2 gene deletion after tamoxifen injection leads to a partial reduction of PRL2 expression close to 50%. This percentage of deletion could have been improved by a different schedule of tamoxifen injection or by using 4-hydroxytamoxifen, which is a major active metabolite of tamoxifen. However, even with this partial deletion, we obtained interesting results in this study.

There was no gross phenotype in LEC-PRL2-deleted mice and pre-existing lymphatic vessels seemed not to be affected. As LECs are in a quiescent stage in adults, it was not surprising that PRL2 deletion did not disturb the already formed lymphatic vasculature. Nevertheless, LECs from lacteals are in constant proliferation and this mechanism is in part regulated by DLL4⁹. In the previous chapter, we showed that the proliferation was decreased and DLL4 was downregulated after PRL2 deletion in LECs. Induction of PRL2 deletion in LECs in mice could have affected the renewal or the function of lacteals, but as PRL2^{fl/fl}-Prox1Cre⁺ mice kept on gaining weight the same way as the control mice, we assumed that the lacteals were functional. However, an analysis of adipose tissue would give us more insight into the effect of PRL2 deletion on lacteals, which could be interesting as lacteals are related to adipose tissue in physiology and pathology such as obesity^{42,88}.

Lymphedema is the accumulation of fluid in limb due to malfunction of lymphatic vessel following a developmental malformation or a surgery involving lymphatic system. Using a forelimb lymphedema model, we observed an accumulation of fluid in the limb of mice. Increased swelling of PRL2^{fl/fl}-Prox1Cre⁺ mice when compared to control mice indicates that PRL2 deletion could lead to aggravated edema. As lymphangiogenesis may help to reduce

edema, VEGF-C was proposed as a therapeutic agent for lymphedema patients⁸⁹⁻⁹². While VEGF-C improved lymphedema, it still leaves room for better and more efficient therapeutic treatment⁹³⁻⁹⁵. In PRL2^{fl/fl}-Prox1Cre⁺ mice with lymphedema, the number of lymphatic vessels was increased, but these vessels appeared not to be functional in their fluid uptake and might have a loss of vessel integrity due to the disorganization of cell-cell junction. This could be due to a modification in signaling pathways involved in the regulation of lymphatic junctions such as Akt and RhoA/ROCK1 signaling⁹⁶. While we already know that Akt phosphorylation was increased in absence of PRL2, which would favor the closing of junction between cells, we still have to investigate RhoA/ROCK1 activation. From the previous results *in vitro*, we could expect to have more activation of RhoA/ROCK1 as we had more focal adhesion formation in siPRL2 LECs and ROCK1 is necessary for FAK phosphorylation and focal adhesion formation⁹⁷.

Surprisingly, we saw no difference in lymphatic vessel formation between the two groups of mice. We expected less lymphangiogenesis in PRL2^{fl/fl}-Prox1Cre⁺ mice as this process seemed to be affected *in vitro* when PRL2 expression is silenced. However, it is to be noted that in the *in vitro* experiments, PRL2 expression was completely silenced, while in the mice we obtained around a 50% decrease in gene expression. This partial effect might not be enough to inhibit lymphangiogenesis; nevertheless, it could still affect vessel integrity. We speculate that there is possibly a dose-effect in relation to PRL2 gene expression. Thus, different levels of PRL2 expression in LECs could impact differently the phenotype and functions of lymphatic vessels. VEGFR-3 and Notch1 independent signaling pathways may also intervene and modify the response to PRL2 deletion^{2,23}.

Fibroadipose deposition is another problem in lymphedema. It is caused by fluid accumulation and inflammation. It is well known that macrophages and CD4⁺ T-cells are involved in fibrosis development in lymphedema^{98–101}. After *in vivo* PRL2 deletion in LECs, we observed smaller fibrotic area while the swelling was still important and immune cells were still accumulating in the tissue. There were even slightly more macrophages and a high number of T-cells at the basal level in PRL2^{fl/fl}-Prox1Cre⁺ mice, which indicates that PRL2 deletion modifies the ratio of immune cells present in the tissue, which could impact disease development. A more precise and detailed knowledge of each immune cell population in the lymphedema site will contribute to a better understanding of how PRL2 affects the microenvironment.

Polarization of macrophages in M1 or M2 could affect lymphedema development as M1 are pro-inflammatory while M2 are anti-inflammatory. The balance between M1 and M2 macrophages determines repair versus inflammation in the tissue. In lymphedema, M2 polarization is favored since M2 macrophages are VEGF-C-producing cells and thus, promote lymphangiogenesis^{100,102}. However, macrophages may play a more complex role in lymphedema as they can also interact with CD4⁺ T-cell to aggravate the pathology^{101,103}.

The most important CD4⁺ T-cell population in lymphedema is Th1, while Th2, Treg, CD8⁺ T-cells perform a minor role in this pathology¹⁰⁴. In PRL2^{fl/fl}-Prox1Cre⁺ mice, we already have a high number of both T-cells in the control limb and no further increase in cells in the lymphedema. The high number of T-cells in basal state could be explained by the previous observation that PRL2 is a mediator of self-renewal and proliferation of hematopoietic stem cells, in addition to being required for T-cell differentiation^{68,70}. In the present case, we hypothesized that in our animal model PRL2 indirectly affect T-cell differentiation and/or

activation as the lymph node structure was modified in PRL2^{fl/fl}-Prox1Cre⁺ mice. Since lymph nodes are immune cell activation and differentiation sites, this may affect T-cell activation and polarization.

In conclusion, the specific and partial deletion of PRL2 in LECs demonstrated an impairment of the fluid uptake and an increased number of immune cells that infiltrate the skin in a lymphedema model. This suggests that lymphatic PRL2 is involved in lymphedema, at least in part, by regulating immune cell infiltration and accumulation. Interestingly, PRL2 also seems to have an essential role in lymph node organization. Nevertheless, more investigations are needed to decipher the role of PRL2 in lymphedema pathogenesis.

Chapter 4: The importance of PRL2 expression in lymphatic vessels in a tumor context

4.1. Objectives

PRL2 is known to be overexpressed in several cancers and in metastatic lymph nodes. In the previous chapters, we demonstrated that PRL2 is involved in forming new lymphatic vessels which impacts the development of lymphedema. In this chapter, we determined if PRL2 expression in lymphatic vessels influences tumor development and metastatic spread.

4.2. Material and methods

4.2.1. List of antibodies

Table 4.1: List of antibodies used for western blot and immunostaining

Protein target	Antibody
CD3	Rabbit anti-CD3, Abcam #ab16669
F4/80	Rat anti-mouse F4/80, Biorad #MCA497G
ICAM-1	Rabbit anti-ICAM-1, CST #4915
LYVE1	Goat Anti-LYVE1, R&D #AF2125
NFκB (p65)	Rabbit anti- NFκB p65, CST #8242
Phospho-NFκB (p65)	Rabbit anti-phospho-NFκB p65 (Ser536), CST #3033)
PD-L1	Rabbit anti-PD-L1, R&D #MAB1562
Peripheral Node Addressin (PNAd)	Rat anti-mouse/human PNAd, Biolegend #120801
PRL 1/2	Mouse anti-PRL1/2, EMD Millipore #05-1583
α-Tubulin	Mouse anti-α-Tubulin, Sigma Aldrich #T5168
VCAM-1	Sheep anti-VCAM-1, R&D #AF809

4.2.2. Mice

For our experiment, we bred PRL2^{fl/fl}-Prox1Cre⁺ mice (heterozygous for Prox1-CreERT2 allele) with PRL2^{fl/fl}-Prox1Cre⁻ to obtain PRL2^{fl/fl}-Prox1Cre⁺ (also heterozygous for Prox1-CreERT2 allele) and their control littermates PRL2^{fl/fl}-Prox1Cre⁻. Only male mice were used for our experiment as B16-F10 cells are identified as male cells.

To induce the deletion of PRL2 gene, six-week-old mice received six intraperitoneal injections of tamoxifen (50µg/g; Sigma Aldrich, T5648) distributed over two weeks (approximately one injection every two days). Control littermates also received tamoxifen injections.

All protocols used on mice were approved by the Ethic Committee of Bordeaux (CE050, DAP38181).

4.2.3. Tumor implantation

Before the surgery, we applied a local analgesic to prevent pain in the site of injection. Then, mice were anesthetized with an intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg). The site of injection was sanitized with 70% ethanol before subcutaneously injecting a cell suspension of 300 000 B16-F10 cells in the right side of the animal.

Tumor development was followed for 21 days with a measure of the tumor size every two days, starting 7 days post-injection, using a caliper.

4.2.4. Immunostaining

Skin with tumor and inguinal lymph nodes from mice were embedded in OCT (Optimal Cutting Temperature) compound and stored at -80°C. 8 µm sections were cut with a cryostat. Slides were fixed in acetone stored at -20°C for 2 minutes before rehydrating the tissue in PBS 1X. Tissues were permeabilized with Triton 0.1% for 2 minutes and blocked with 5%

BSA for 1h. Primary antibodies were diluted in BSA 2% (1:100) and incubated for 1 hour at room temperature. Secondary antibodies Alexa Fluor were diluted in BSA 2% (1:500) and incubated for 1h at room temperature. Nuclei were visualized using DAPI (4',6-diamidino-2-phenylindole, Hello Bio, HB 0747). Coverslips were mounted on slides with ProLong Gold antifade mountant (Invitrogen #P36934). Images were taken using a fluorescent microscope (Leica, DMI8) and analyzed with ImageJ.

4.2.5. Hematoxylin and Eosin staining

Inguinal lymph nodes from mice were embedded in OCT (Optimal Cutting Temperature) compound and stored at -80°C. 8 µm sections were cut with a cryostat. Slides were fixed in acetone stored at -20°C for 2 minutes before rehydrating the tissue in PBS 1X. Nuclei were stained with Mayer's hematoxylin for 10 minutes. After washing the excess of hematoxylin, tissues were stained with eosin for 30 seconds and washed with ethanol 100% (3x5 minutes) and Histoclear (4 and 5 minutes). Coverslips were mounted on slides with Eukitt mounting medium (Sigma Aldrich #03989). Images were taken using a microscope (Leica, DMI8) and analyzed with ImageJ. Metastatic lesions were identified by the darker color of B16-F10 cells.

4.2.6. Cell culture

Human Dermal Lymphatic Endothelial Cells (LECs) were purchased from Promocell and cultured in Endothelial Cell Growth Medium MV2 (ECGM MV2, Promocell) on flasks or plates coated with 0.4% gelatin (PAN BIOTECH P06-25200). Cells were maintained in a humidified incubator with 5% CO₂ at 37°C and the media was renewed every 2-3 days. LECs were used between passage 4 and 7.

4.2.7. Transfection

siRNAs were purchased from Dharmacon : PTP4A2 (SMART Pool, M-009078-01-0005, GE Healthcare Europe GmbH) and negative control (SMART Pool, Non-targeting siRNA, D-001206-13-05, GE Healthcare Europe GmbH). Lipofectamine RNAiMAX kit (Invitrogen 13778075) was used for transfection, following manufacturer's protocol.

4.2.8. Stimulation with TNF α or IFN γ

LECs were starved overnight in Endothelial Cell Basal Medium MV2 (ECBM MV2 Promocell) supplemented with 0.5% fetal calf serum (FCS). Then, they were treated with 10 ng/ml of Tumor Necrosis Factor α (TNF α) or 10 ng/ml of interferon γ (IFN γ) for the indicated time.

4.2.9. Cytokines and chemokines quantification

LECs were treated with TNF α for 24h, and then media was collected for each condition to measure cytokines and chemokines production. This study used Luminex xMAP technology for multiplexed quantification of 71 Human cytokines, chemokines, and growth factors. The multiplexing analysis was performed using the Luminex™ 200 system by Eve Technologies Corp. (Calgary, Alberta). 71 markers were simultaneously measured in the samples using Eve Technologies' Human Cytokine 71-Plex Discovery Assay® which consists of two separate kits: one 48-plex and one 23-plex (MilliporeSigma). The assay was run according to the manufacturer's protocol. The 48-plex consisted of sCD40L, EGF, Eotaxin, FGF-2, FLT-3 Ligand, Fractalkine, G-CSF, GM-CSF, GRO α , IFN- α 2, IFN- γ , IL-1 α , IL-1 β , IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-18, IL-22, IL-27, IP-10, MCP-1, MCP-3, M-CSF, MDC, MIG/CXCL9, MIP-1 α , MIP-1 β , PDGF-AA, PDGF-AB/BB, RANTES, TGF α , TNF- α , TNF- β , and VEGF-A. The 23-plex consisted of 6CKine, BCA-1, CTACK, ENA-78, Eotaxin-2,

Eotaxin-3, I-309, IL-16, IL-20, IL-21, IL-23, IL-28A, IL-33, LIF, MCP-2, MCP-4, MIP-1 δ , SCF, SDF-1 α + β , TARC, TPO, TRAIL, and TSLP. Assay sensitivities of these markers range from 0.14 – 55.8 pg/mL for the 71-plex. Individual analyte sensitivity values are available in the MILLIPLEX® MAP protocol.

4.2.10. Western Blot

LECs were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche) for 30 min on ice, followed by a centrifugation at 10000 g for 10 minutes. Supernatants were collected and proteins were quantified following Pierce BCA protein assay kit protocol (Thermo Scientific #23225). Samples were prepared with 15 μ g proteins and Laemmli buffer (300 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.5 mg/ml bromophenol blue, 10% β -mercapto-ethanol), boiled for 5 minutes at 100°C, and then separated by SDS-PAGE. After migration, proteins were transferred on a PVDF membrane. Membranes were blocked with 5% milk for 1h at room temperature, then incubated with primary antibody diluted in antibody solution (PBS 1X supplemented with 0.5% Tween and 0.02% sodium azide) overnight at 4°C. HRP-conjugated secondary antibodies were diluted in antibody solution and incubated for 1h at room temperature. After washing, membranes were incubated with Clarity ECL (BioRad #1705061), and then imaged with ChemiDoc XRS+ (BioRad). Image analysis was performed using ImageLab software.

4.2.11. Statistical analysis

Statistical analyses were performed on GraphPad Prism 9 software. A Shapiro-Wilk test was performed to assess the normal distribution of the sample. Then, Student t-test (normal distribution) or Mann-Whitney test was used to compare two groups, while Two-way

ANOVA, followed by Sidak or Tukey's post-hoc test, was used to compare groups with more than one variable. The difference was statistically significant when the p-value was below 0.05.

4.3. Results

4.3.1. The absence of PRL2 increases lymph node metastasis

To study the effect of PRL2 expression in LECs on tumor development and metastasis, mice were subcutaneously injected with B16-F10 melanoma cells (Figure 4.1A). Tumor growth was monitored over 21 days: no significant difference was observed (Figure 4.1B). Tumor in PRL2^{fl/fl}-Prox1Cre⁺ mice had the tendency to grow faster compared to the implanted control mice. At the end of the experiment (21 days after tumor injection), no difference in tumor size was seen, although variability in PRL2^{fl/fl}-Prox1Cre⁺ mice was higher than in the control mice (Figure 4.1C). Thus, LEC-derived PRL2 is unlikely to play a significant role in the growth of primary tumor. However, when inguinal lymph nodes were analyzed, an increase in lymph node metastatic lesions was observed in PRL2^{fl/fl}-Prox1Cre⁺ mice (Figure 4.1D-E). All PRL2^{fl/fl}-Prox1Cre⁺ mice had metastatic lesions in the inguinal lymph nodes, while only a few PRL2^{fl/fl}-Prox1Cre⁻ mice exhibited lymph node metastasis. This result suggests that LEC-derived PRL2 modulates lymph node metastasis in a murine melanoma model.

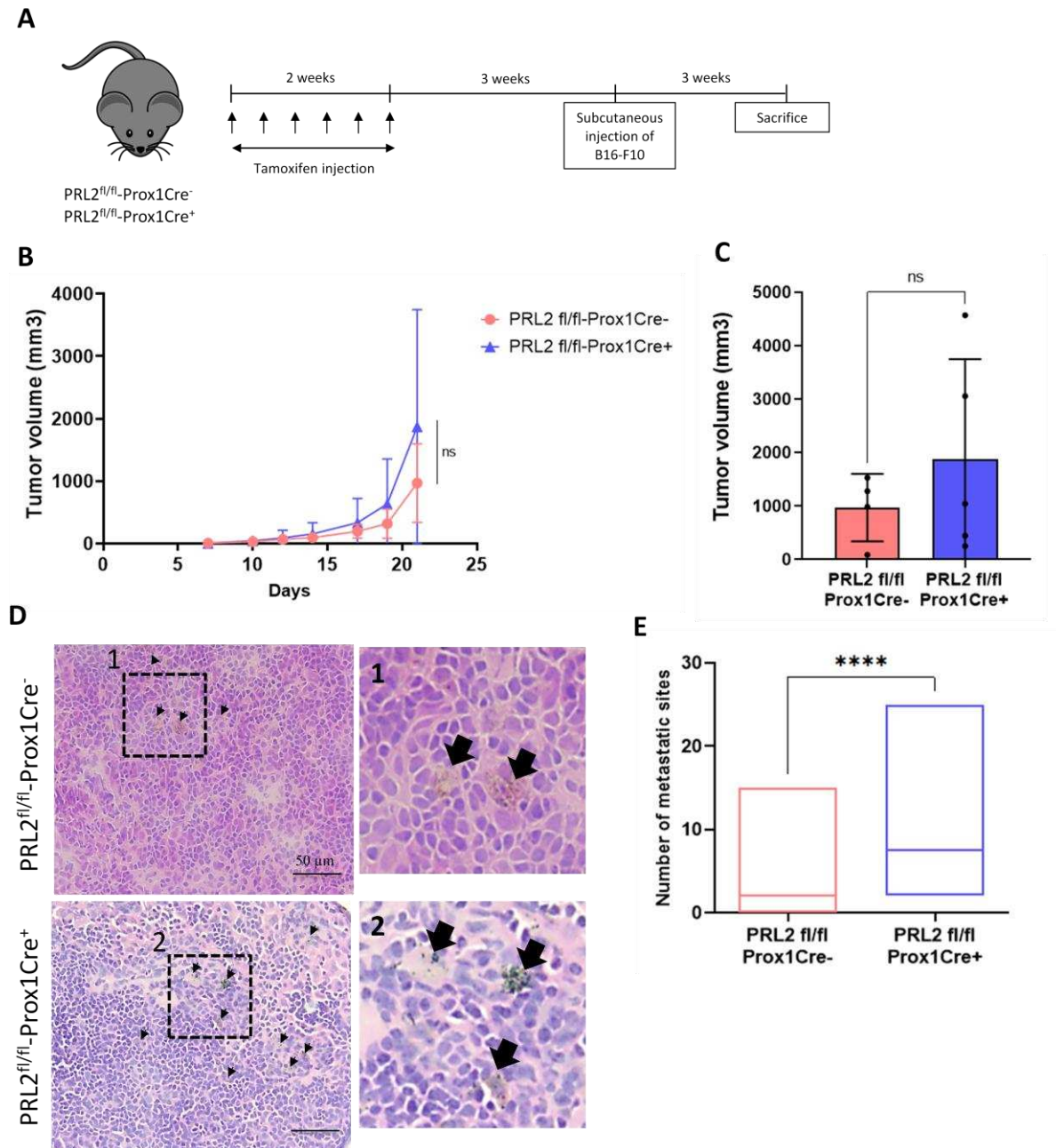


Figure 4.1: PRL2 deletion in LECs increased metastasis

(A) Timeline of tumor injection experiment. (B) B16 tumor growth in PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice. (n=5 mice; Two-way ANOVA, Sidak's post-hoc test) (C) Tumor volume 21 days after injection of B16-F10 in PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice. (D) Representative images of hematoxylin and eosin staining of inguinal lymph node 21 days after injection of B16-F10 in PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice. Black arrows show metastatic sites. (E) Quantification of metastatic sites (n=5 mice; Mann-Whitney test). Graphs are represented in mean $\pm$ SD; ns=non-significant; **** p<0.0001

4.3.2. PRL2 expression modifies sentinel lymph nodes

As more metastatic lesions were observed in lymph nodes of PRL2^{fl/fl}-Prox1Cre⁺ mice, tumor lymphatics should either be increased or functionally altered since they are the only route to reach peripheral lymph nodes. Tumor tissue slices were stained for LYVE1 to localize lymphatic vessels inside the tumor and the number of lymphatic vessels was quantified. After analysis, there was no significant difference between the two groups (Figure 4.2A-B). Therefore, increased lymph node metastasis is unlikely to be caused by increased lymphatic vessels at the tumor site.

Interestingly, when the inguinal lymph nodes were stained for LYVE1 and for Peripheral Node Addressin (PNAd), the intensity of LYVE1 staining was increased, and more vessels were PNAd⁺ in PRL2^{fl/fl}-Prox1Cre⁺ mice (Figure 4.2C-E). PNAd is a marker of High Endothelial Venules (HEVs) which are blood vessels specialized in lymphocytes trafficking to lymph nodes. These results demonstrate that more lymphatic vessels and HEVs are present in lymph nodes, which should impact tumor metastasis or immune cell trafficking.

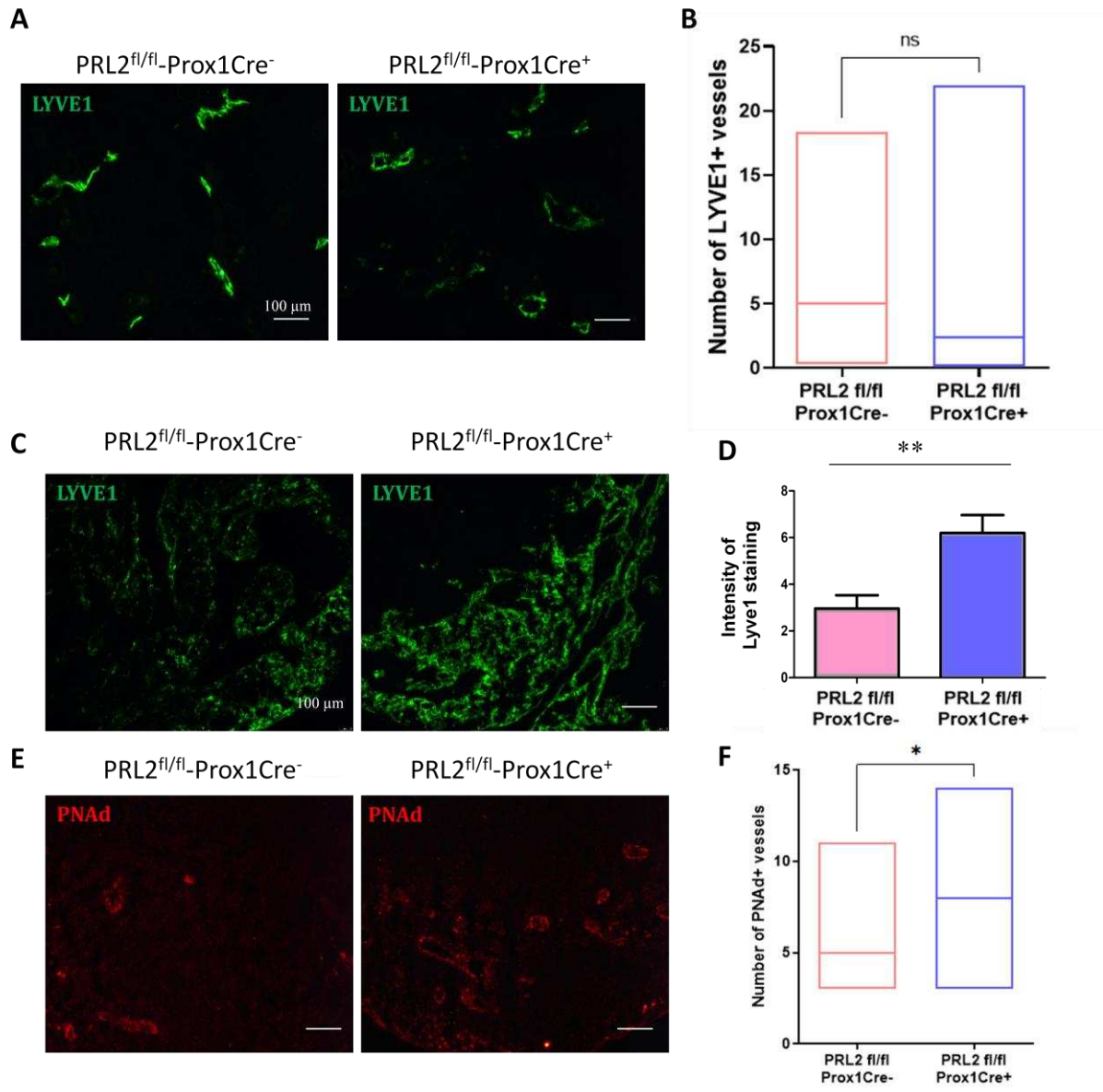


Figure 4.2: PRL2 deletion in LECs modified sentinel lymph node

(A) Representative images of LYVE1 staining on tumor from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice. (B) Quantification of LYVE⁺ vessels in the tumor (n=5 mice; Student t-test) (C) Representative images of LYVE1 staining on inguinal lymph node from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice. (D) Quantification of LYVE1 staining intensity inside inguinal lymph node. (n=5 mice; Student t-test) (E) Representative images of PNAd staining on inguinal lymph node from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice. (F) Quantification of PNAd⁺ vessels inside inguinal lymph node. (n=5 mice; Mann-Whitney test). Graphs are represented in mean ±SD; ns=non-significant; * p<0.05; ** p<0.01

4.3.3. PRL2 modulates immune cell infiltration in the tumor

Next, we hypothesized that an increase in lymphatic vessels and HEVs in lymph nodes leads to modifications in the composition of immune cells in PRL2^{fl/fl}-Prox1Cre⁺ mice when compared to control animals. To explore this hypothesis, immune cells were immuno-labeled and analyzed. Macrophages were labeled with anti-F4/80 antibodies and T-cells with anti-CD3 antibodies. Tissue from PRL2^{fl/fl}-Prox1Cre⁺ mice showed a significant increase in macrophages and T-cells at the tumor site compared to the one from PRL2^{fl/fl}-Prox1Cre⁻ mice (Figure 3). Macrophages also had different distributions inside the tissue of PRL2^{fl/fl}-Prox1Cre⁺ mice. While macrophages of control mice were more evenly distributed inside the tissue, macrophages from PRL2-deleted mice were mostly found at the tumor periphery. No difference in T-cell organization was observed. Overall, it seems that PRL2 deletion modifies the tumor microenvironment with an increase in immune cell infiltration (T-cells and macrophages) and differences in macrophage localization in the tumor.

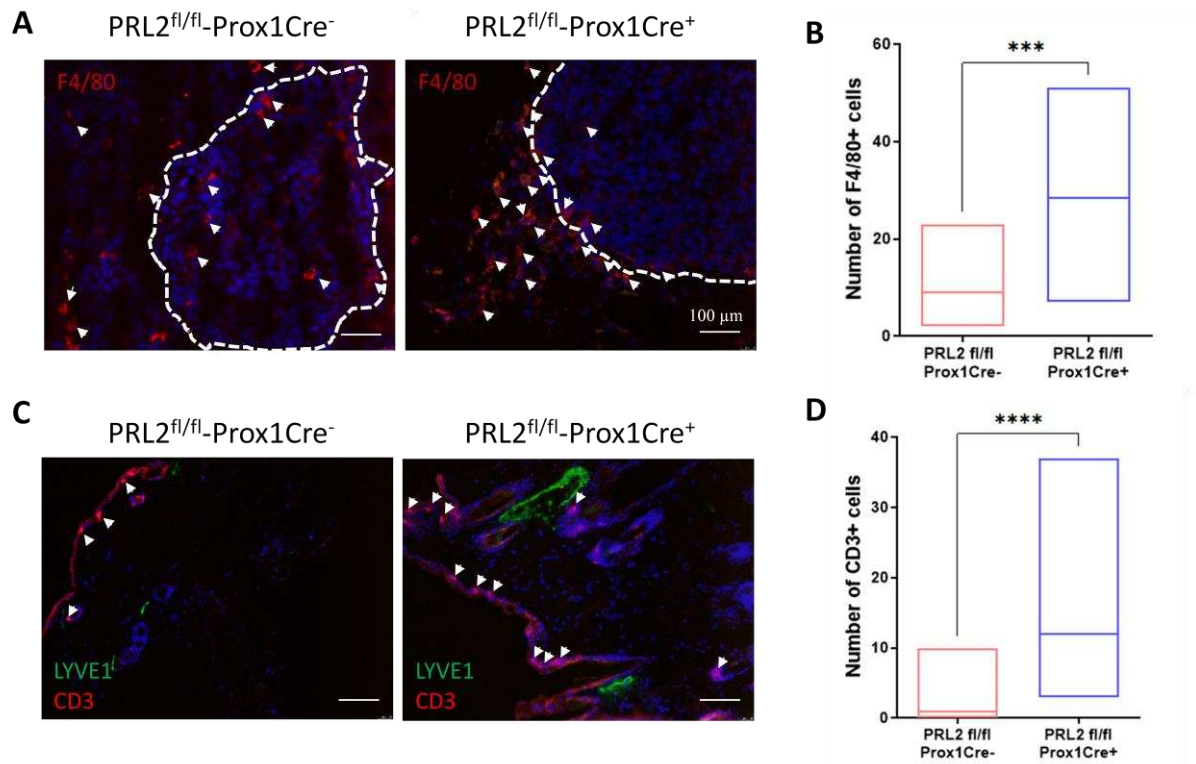


Figure 4.3: PRL2 deletion increased immune cell infiltration

(A) Representative images of F4/80 staining on tumor from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice. The tumor is delimited by the white dashed line. Nuclei were stained in blue. White arrowheads show macrophages. (B) Quantification of F4/80+ cells at the tumor site (n=5 mice; Student t-test) (C) Representative images of CD3 staining on tumor from PRL2^{fl/fl}-Prox1Cre⁻ mice and PRL2^{fl/fl}-Prox1Cre⁺ mice. Nuclei were stained in blue. White arrowheads show T-cells. (D) Quantification of CD3⁺ cells at the tumor site. (n=5 mice; Mann-Whitney test). Graphs are represented in mean $\pm$ SD; *** p<0.001; **** p<0.0001

4.3.4. Cytokines and chemokines production is modified upon PRL2 silencing in LECs

To understand how PRL2 deletion in LECs could affect the tumor microenvironment, especially the immune cell infiltration, cytokine and chemokine expression was analyzed. TNF α has been associated with the aggressiveness of melanoma¹⁰⁵, we therefore treated LECs *in vitro* with 10 ng/ml of TNF α . 24h later, we collected the media and quantified cytokines and chemokines released by LECs using a multiplex detecting 71 cytokines, chemokines and

growth factors (Figure 4). Following TNF α treatment in control LECs, several cytokines and chemokines were highly increased (more than 10-fold increase). Approximately, the expression of half of those cytokines/chemokines was modified by PRL2 silencing. The expression of 3 cytokines and 7 chemokines upon PRL2 silencing was significantly modified. Specifically, the production of the cytokines IL-1 β , IL-6, and IL-27 reduced by 38%, 45%, and 31%, respectively, in siPRL2 LECs compared to siCTL LECs (Figure 4A). Among the chemokines, 4 showed a decreased production: CX3CL1, CXCL9, CCL1, and CCL5 (Figure 4B). Specifically, CXCL9 and CCL1 display a higher level of reduction, with 44% and 58% reduction, with a higher level of 44% and 58% respectively. In addition, CX3CL1 production decreased by 26%, while CCL5 only decreased by 11%. In contrast, the chemokines CCL7, CCL11 and CCL22 showed increased production. CCL7 was increased by 44%, while CCL11 and CCL22 production was only moderately increased (23% and 15% respectively). These results indicate a modulatory role of PRL2 in the production of cytokines and chemokines by TNF α -stimulated LECs, thus modifying the inflammatory response.

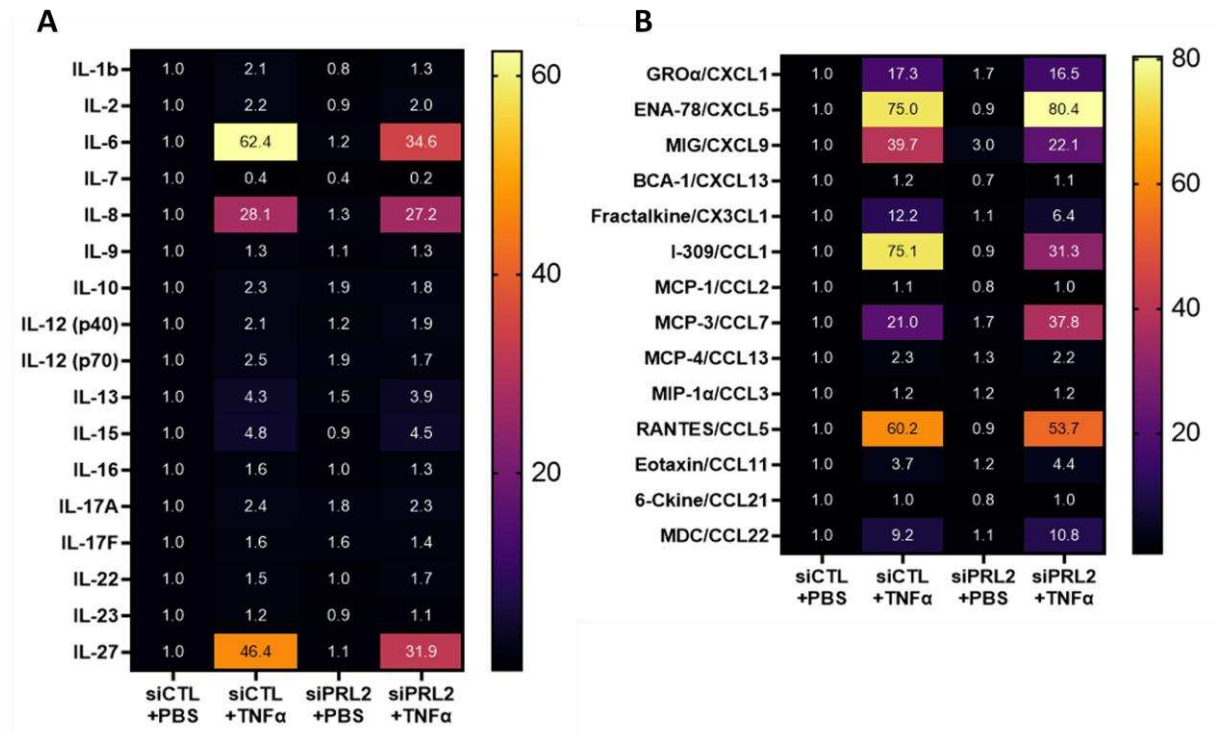


Figure 4.4: PRL2 modifies the profile of cytokines and chemokines released by LECs treated with TNFα

Heatmaps of the multiplex of cytokines (A) and chemokines (B) performed on supernatant from SiCTL LECs and SiPRL2 LECs treated with 10ng/ml of TNFα for 24h. (n=3)

4.3.5. NFκB signaling is affected by PRL2 silencing in LECs

Nuclear Factor Kappa B (NFκB) is a transcription factor that induces the production of pro-inflammatory cytokines/chemokines and regulates the immune response¹⁰⁶. Cytokine and chemokine expression was modulated by PRL2 in LECs, thus the NFκB signaling pathway was investigated following PRL2 silencing. NFκB phosphorylation stimulated by TNFα or IFNγ was impaired in PRL2-silenced cells (Figure 5A-B), which could explain some of the effects on cytokine/chemokine production.

NFκB regulates the expression of adhesion molecules to promote immune cell adhesion. Therefore, we analyzed the expression of Vascular Cell Adhesion Molecule-1 (VCAM-1) and Inter-Cellular Adhesion Molecule-1 (ICAM-1) upon 24h of TNFα treatment (Figure 5C). No difference in their expression was seen in siPRL2 LECs when compared to siCTL LECs.

However, we observed a drastic increase in Programmed Death-Ligand 1 (PD-L1), an inhibitor of immune response, in siPRL2 LECs. This observation suggests a role for PRL2 in the regulation of PD-L1 expression: the absence of PRL2 may promote the inhibition of the immune response while its presence should have the opposite effect.

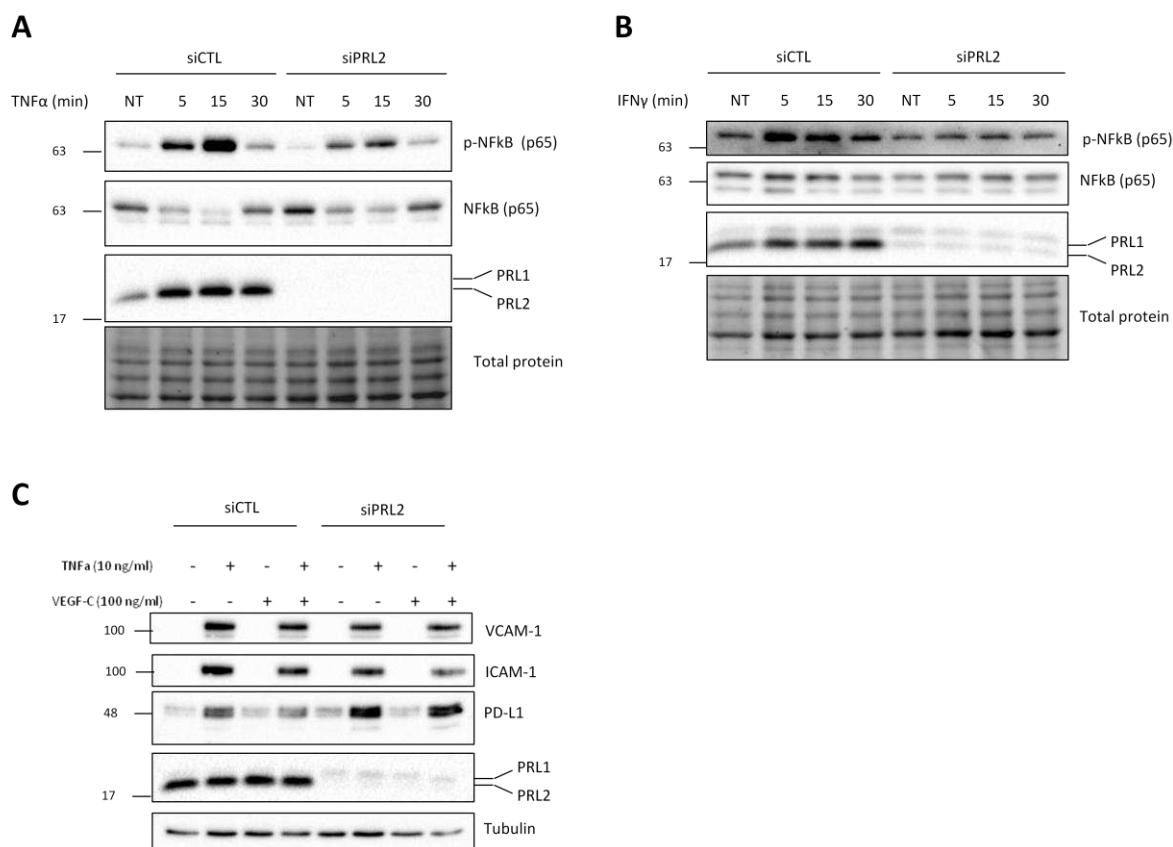


Figure 4.5: PRL2 can influence inflammation activation

(A-B) Western blot of p-NFkB and NFkB level in SiCTL LECs and SiPRL2 LECs treated with 10 ng/ml of TNFα (A) or 10 ng/ml of IFNγ (B) for the indicated time. (C) Western blot of VCAM1, ICAM1 and PD-L1 level in SiCTL LECs and SiPRL2 LECs treated with 10 ng/ml of TNFα and/or 100 ng/ml VEGF-C for 24h.

4.4. Discussion

In this chapter, we investigated how PRL2 deletion in LECs affects tumor development. Our *in vivo* results are not conclusive about the role of LEC-derived PRL2 on primary tumor growth due to the number of mice used. We suggest repeating the experiment with higher

number of mice. Nevertheless, tumor cell infiltration in the inguinal lymph nodes of PRL2^{fl/fl}-Prox1Cre⁺ mice was clearly documented. In addition, more immune cell infiltration at the tumor site of these mice was also observed. Interestingly this result is in line with the increased infiltration of immune cells observed in lymphedema. However, in the case of lymphedema the number of T cells was more affected than macrophages, while in the primary tumor, both macrophages and T-cells were significantly increased in PRL2-deleted mice. Currently we are performing more experiments to better characterize the type of T-cells and macrophages present in these tumors.

The cytokine and chemokine profile obtained in our *in vitro* experiments with TNF α stimulation could shed some light about the tumor microenvironment modulated by LEC-derived PRL2. When PRL2 is silenced, LECs produced less IL-1 β , IL-6 and IL-27. IL-1 β and IL-6 have been investigated in different types of cancer and, in several studies, they promote tumor progression and therapy resistance^{107–109}. However, some studies point a dual role for IL-1 β as it has a role in Th-1 activation in B cell lymphoma and myeloma and it shows an anti-tumor effect^{110–112}. Moreover, low levels of IL-6 have also been associated with metastatic prostate cancer¹¹³. So, in our case, the decrease in IL-1 β and IL-6 could also affect Th-1 activation and promote an immunosuppressive microenvironment that will favor metastasis. On the contrary, IL-27 has been shown to inhibit melanoma growth by inducing the expression of TNF-Related Apoptosis Inducing Ligand (TRAIL) on tumor cells¹¹⁴. Therefore, decrease in cytokine production in PRL2-deleted LECs could explain the increase of melanoma metastasis in mice.

Parallel to the reduced production of IL-1 β , IL-6 and IL-27, 4 chemokine expressions were also reduced: CX3CL1, CXCL9, CCL1 and CCL5. CX3CL1 and its receptor CX3CR1

display pro-tumor effects in several types of cancer such as breast¹¹⁵, colon¹¹⁶, ovarian¹¹⁷, prostate¹¹⁸, bladder¹¹⁹, pancreatic¹²⁰ and renal cell cancer¹²¹, although, CX3CL1 has also been associated with good prognosis in gastric adenocarcinoma¹²² and breast carcinoma¹²³ by increasing CD8⁺ T-cell and natural killer cell infiltration. Furthermore, both CXCL9 and CCL5 has been shown to promote CD8⁺ T-cell infiltration, especially that of effector CD8⁺ T-cells, in solid tumors, melanoma included^{124–129}. Effector T-cells have an anti-tumor effect. On the opposite, CCL1 has been associated with regulatory T-cell recruitment and metastasis^{130–132}. Regulatory T-cells are known to promote tumor progression by contributing to an immunosuppressive microenvironment. Decrease in the cytokines and chemokines listed above may affect the ratio of effector and regulatory T-cells and thus, promoting an immunosuppressive microenvironment that could lead to tumor growth and metastasis.

The cytokine and chemokine profile generated by LECs treated with TNF α also showed an increase in CCL7, CCL11 and CCL22. CCL7 has been associated with bad prognosis in gastric cancer¹³³ and migration/invasion of oral squamous cell carcinoma¹³⁴, while its inhibition prevents metastasis formation in colorectal cancer¹³⁵. Additionally, CCL11 and CCL22 have a role in attracting regulatory T-cells and contribute to an immunosuppressive microenvironment^{136–138}. High expression of CCL11 has also been related to impaired differentiation/maturation of dendritic cells and to an increase in Th2 immune response, resulting in a poor activation of effector CD8⁺ T-cell¹³⁹. So, taken all together, the increase in CCL7, CCL11 and CCL22 could favor an immunosuppressive microenvironment characterized by a more infiltration of regulatory T-cells, hence promoting tumor cell invasion.

Nevertheless, it is essential to indicate that these cytokines and chemokines mentioned above could display different function depending on the context which makes challenging the prediction of a specific outcome in *in vivo* model. Additionally, our *in vitro* experiments were performed with human LECs, hence differences with our murine model are also expected. Still, given the cytokine and chemokine profile in siPRL2 LECs, we hypothesized that PRL2 deletion could promote metastasis by modifying the ratio between regulatory and effector T-cells and thus, favoring an immunosuppressive microenvironment. The modification of the T-cell repertoire combined with an altered cytokine and chemokine profile could also affect macrophage polarization and favor an immunosuppressive macrophage subtype.

Upon TNF α treatment, we also observed a decrease in NF κ B activation in siPRL2 LECs. This decrease could lead to the downregulation of inflammatory cytokines and chemokines such as IL-1 β , IL-6 and CCL5¹⁰⁶. Moreover, IL-1 β has been shown to upregulate CXCL9 expression¹⁴⁰. Thus, the impaired NF κ B activation could also indirectly affect the production of CXCL9. Furthermore, TNF α also induced a higher expression of PD-L1 in absence of PRL2, which contributes to inhibit T-cell functions¹⁴¹. This result contradicts previous reports that PD-L1 expression is induced via NF κ B signaling pathway^{142–144}. Thus, in our case, the expression of PD-L1 in siPRL2 LECs might be regulated in an NF κ B-independent manner. More investigations should be done to understand the relation between PRL2 and the regulation of immune response in the tumor.

Like the previous chapter, we also observed a modification in the inguinal lymph node. While LYVE1 staining was lower in lymph nodes from healthy PRL2^{fl/fl}-Prox1Cre⁺ mice compared to control mice, LYVE1 staining was highly increased in lymph nodes in the metastasis model in PRL2^{fl/fl}-Prox1Cre⁺ mice. This difference could be explained by an increase in stress on the

lymph node. Tumor growth and metastasis formation add more signaling molecules outside and inside the lymph node, which can induce a remodeling¹⁴⁵. Moreover, the number of HEVs in the lymph node was also higher in mice with PRL2 deletion. LYVE1 and HEV increase could participate in increased immune cell trafficking. This could lead to the modification of the immune landscape and to tumor cell infiltration in the lymph nodes. This modification of the lymph node structure could also impact maturation and functions of immune cells.

Taken together, PRL2 deletion in LECs could affect the tumor/lymph node microenvironment by modifying cytokine and chemokine profile and promote lymph node metastasis. In this case, PRL2 deletion in LECs would be a factor for bad prognosis in melanoma as mice developed more lymph node metastasis and leaned towards an immunosuppressive tumor microenvironment.

Chapter 5: General Discussion

The role of phosphatases in lymphatic endothelial cells has yet to be well-investigated. With some exceptions, kinases such as VEGFRs have been the major focus in the vascular biology field and only few studies have explored the role of phosphatases in endothelial cells. For example, Deng et *al.* investigated the role of different PTPs in LECs and found that only the vascular endothelial PTP (VE-PTP, or PTPRB) inhibited ERK and Akt signaling downstream VEGF-C/VEGFR3 activation²⁴. PTPN11 (MIM176876; MIM163950) and PTPN14 (MIM603155; MIM613611) were found to be mutated in patients with lymphedema and Noonan Syndrome, a disease involving lymphatic malformation⁵⁴⁻⁵⁶. Both PTPs have been shown to modulate VEGFR3 signaling and thus, they could play an important function in lymphatic formation during development.

In this work, we proposed another PTP as a regulator of lymphatic functions: PRL2. It has been previously demonstrated that PRL2 is required for vasculature development in the mouse retina which is regulated by VEGFR-2 and Notch1 signaling pathways¹. As lymphatic vasculature mostly originates from veins, LECs may share phenotypic properties and signaling pathway with blood vascular endothelial cells¹⁴⁶ but striking differences between lymphatic and blood vascular endothelial cells with respect to PRL2 might also exist. Therefore, we explored the role of PRL2 in LECs. Similar to PRL2 silencing in HUVECs, we observed an inhibition of migration and sprouting in siPRL2 LECs. These cells also had a lower proliferation rate with a difference in the cell cycle when compared to their control counterpart. After analysis of the signaling pathways controlling these cellular processes, we noticed an increase in focal adhesion formation and Akt activation, but also a decrease of ERK 1/2 activation and Notch1 downstream signaling. A striking difference is that Notch1 cleavage is not affected after PRL2 silencing in LECs and that the affect is principally related to an inhibition in DLL4 expression.

While, PRL2 has already been associated with these signaling pathways in HUVECs¹, T-cells⁷⁰, germ cells⁶⁷, lymphoma cells⁷¹ and fibroblast⁸⁵, it remains to explain how PRL2 interacts with these signaling pathways in LECs and how it drastically decreases all the main cellular processes (proliferation, migration, sprouting). A possible mechanism would be its regulation of intracellular magnesium homeostasis through its interaction with CNNM3/TRPM7 complex⁶⁹. Magnesium has been shown to activate signaling pathways such as Notch1 and ERK 1/2^{147,148} and also to affect endothelial functions^{149–154}. Indeed, magnesium deficiency promotes a pro-inflammatory, pro-thrombotic and pro-atherogenic environment and thus, it increases the risk of developing diseases. Hypomagnesemia in clinic has been associated with sepsis, systemic inflammatory response syndrome, multiple organ failure and severe symptoms in COVID patients, but it is still not clear if the low levels of magnesium are the cause or the consequence of these pathologies¹⁵⁴. Interestingly, TRPM7, a transporter of magnesium, was also described to have an anti-inflammatory and anti-fibrotic effect in mouse¹⁵⁵. TRPM7-deficient mice had cardiovascular fibrosis and inflammation associated with more leukocyte transmigration and abnormal macrophage activation. Furthermore, TRPM7 plays an essential role in regulating immune functions such as the polarization of macrophages and development of function B- and T-cells^{156–158}, but it has also been shown to play an important role in endothelial function¹⁵⁹. These studies demonstrated that TRPM7 and its transport of magnesium are crucial for health and might be involved in several signaling pathways to regulate cell functions¹⁶⁰.

Here, we also showed that similar to TRMP7 and magnesium level modification, PRL2 expression in LECs might also modulate the inflammatory function of LECs. Following PRL2 ssilencing, less activation of NFκB was observed in LECs treated with inflammatory factors, which suggest a decrease of inflammatory response dependent on NFκB. However,

NFκB signaling pathway is not the only one regulating cytokine and chemokine production¹⁶¹. Akt signaling has been shown to modify cytokine and chemokine production in HUVECs, macrophages and monocyte^{162–164}. Notch signaling is also involved in the inflammatory response by modulating the cytokine and chemokine production in cancer cells or immune cells^{165–167}. In our *in vitro* experiment, we observed that both signaling pathways were modified following PRL2 silencing. Thus, associated with the decrease of NFκB activation, the modification of Notch1 and Akt signaling could explain the difference in cytokine and chemokine production of the host laboratories (Tremblay vs Bikfalvi), the role of PRL2 in immune cells is actively explored and it was shown that the deletion of PRL2 could affect the inflammatory response of macrophages and T-cells. For example, the expression of PRL2 in macrophages could play an important function in their polarization, favoring a M1 phenotype. Thus, similarly to TRPM7, PRL2 expression might be important for immune functions and its role might differ in each immune cell type and either promotes an anti- or a pro-inflammatory response. More investigations have to be done to determine whether regulation of the inflammatory response by PRL2 is related to TRPM7 activity.

Furthermore, while we did not see a difference in expression of adhesion molecules (VCAM-1 and ICAM-1), siPRL2 LECs expressed more PD-L1 independently of NFκB. This increase in PD-L1 could be related to the increase in Akt signaling we observed in absence of PRL2 as the activation of Akt signaling pathway has been shown to induce the expression of PD-L1 in breast cancer, prostate cancer¹⁶⁸ and lung cancer¹⁶⁹. SiPRL2 LECs also had a different cytokine and chemokine profile that might promote an immunosuppressive microenvironment. By modifying the microenvironment around them, LECs affect the activation and the recruitment of immune cells^{128,129,170}. In our mouse model with an inducible and specific deletion of PRL2 in LECs, PRL2^{fl/fl}-Prox1Cre⁺ mice, we observed an increase in

immune cell infiltration, especially macrophages and T-cells. However, the increased number of immune cells did not seem to improve the pathology and they might actually aggravate it as there was more metastasis in the lymph nodes from tumor-bearing PRL2^{fl/fl}-Prox1Cre⁺ mice. This result suggests that most immune cells in the microenvironment might not have an anti-tumor effect. Indeed, if PRL2-deficient-LEC LECs express more PD-L1, this could inhibit T-cell activation, especially CD8⁺ T-cells^{143,171,141}. Moreover, the decrease of CXCL9 and CLL5 released by PRL2-deficient-LEC LECs could result in a decrease of CD8⁺ T-cells in the tumor environment and the increase in CCL11 and CCL22 could increase the recruitment of regulatory T-cells. These results suggest a change in the T-cell subtype ratio, with more regulatory T-cells than cytotoxic T-cells. Consequently, the increase in regulatory T-cells can modify the immune cell population in the tumor microenvironment¹⁷². However, not only can LECs modulate the activation and recruitment of immune cells around the tumor, they can also affect the immune cells directly in lymph nodes as LECs in lymph nodes are crucial for antigen sorting, immune cell trafficking and immune cell tolerance¹⁷³. Thus, the deletion of PRL2 in LECs might also directly affect immune cells in lymph nodes.

In fact, we observed a difference of lymphatic network in lymph nodes from PRL2^{fl/fl}-Prox1Cre⁺ mice. In PRL2^{fl/fl}-Prox1Cre⁺ mice, lymph nodes had a decrease in LYVE1⁺ LECs as well as a reduced expression of VE-Cadherin, which suggest a modification in the lymph node structure and an increase in immune cell trafficking. As VE-Cadherin regulates junction between LECs, its reduced expression would decrease the tightness between cells and promote immune cell trafficking, which could explain the increasing number of T-cells in the skin. Interestingly, we also noticed a disorganization of VE-Cadherin following PRL2 silencing *in vitro*, which could increase the vasculature permeability. This disorganization of VE-Cadherin in the absence of PRL2 could be related to the disruption of Notch1 signaling.

The activation of Notch1 signaling can induce the expression of Ephrin B2 in LECs to promote lymphangiogenesis²⁵. Recently, Ephrin B2 was also associated with the maintenance of endothelial cell junction¹⁷⁴. Therefore, the disruption of Notch1 signaling could reduce the expression of Ephrin B2 and thus, decrease the cell junction stability and increase the permeability of lymphatic vessels.

In lymph nodes from tumor-bearing PRL2^{fl/fl}-Prox1Cre⁺ mice, we observed an increase in lymphatic vasculature and HEVs that might have promoted the formation of metastasis sites inside the lymph nodes. Our *in vivo* results suggested an inhibition of lymphangiogenesis in the absence of PRL2. Nevertheless, in the *in vivo* model of murine melanoma we did not observe differences in intra-tumoral lymphatic vessel formation between LEC-PRL2-deleted mice and control mice. Recently, it has been shown that cancer-derived extracellular vesicles promote cancer progression and lymph node metastasis^{175,176}. Extracellular vesicles are particles with a lipid membrane encapsulating various molecules, including proteins, nucleic acids, lipids and metabolites. In melanoma, these extracellular vesicles are transported to lymph nodes where they interact with LECs to promote their proliferation and facilitate metastasis formation¹⁷⁷. Extracellular vesicles also regulate immune cell activation by inhibiting CD8⁺ T-cells and polarizing macrophages towards an anti-inflammatory phenotype¹⁷⁶. Thus, the association of PRL2 deletion in LECs with the release of extracellular vesicles by melanoma cells could explain the development of an immunosuppressive microenvironment leading to the infiltration of tumor cells into lymph nodes to form metastasis.

It has been shown that in melanoma and other cancers, such as breast and prostate cancer, a subset of myeloid cells can express lymphatic markers and become LEC progenitors to integrate lymphatic vessels^{178–181}. These myeloid-LEC progenitors are hybrid cells with both

myeloid and lymphatic phenotypes. As they mainly derive from anti-inflammatory cells, namely M2 tumor-associated macrophages and myeloid-derived suppressive cells, they contribute to developing an immunosuppressive microenvironment and promote metastasis^{182,183}. Furthermore, by inhibiting VEGFR3 signaling, the main lymphatic signaling pathway, or by abolishing podoplanin expression in myeloid-LEC progenitors, a lymphatic marker, mouse survival was improved by preventing the accumulation of immunosuppressive cells and increasing the recruitment of CD8⁺ T-cells^{182,184}. Thus, LECs and cells expressing lymphatic markers play an important function in regulating immune cell activation, tumor progression and metastasis. Further experiments will help us to decipher whether macrophages accumulated around the tumor expressed some lymphatic markers and thus, contributed to the formation of lymphatic vessels inside the tumors and the lymph nodes.

Overall, in our mouse model, the specific deletion of PRL2 in LECs tends to worsen the pathology, namely lymphedema and melanoma as studied in this thesis. While the deletion was only partial in our mice, an effect on lymphatic vessels and microenvironment was clearly evidenced. We consider that a partial deletion could be more representative of drug effects in patients as it is hard to achieve a complete inhibition or deletion of a targeted protein inside cells. Consequently, our *in vivo* study may represent a good example of how a PRL2 inhibitor would work on the pathology.

In lymphedema, the partial deletion of PRL2 did not have drastic effects. PRL2 partial deletion resulted in a tendency for less vascular integrity compared to their control counterpart, which could impair the fluid transport and thus, affect the drainage of accumulated lymph in the limb. The partial deletion of PRL2 in LECs also resulted in a tendency for increased immune cell accumulation in the lymphedema site. Following PRL2 deletion, T-cells were already present in high numbers in the skin of limbs without

lymphedema, suggesting a deregulation of T-cell trafficking. This accumulation of immune cells might lead to chronic inflammation and in the long-term aggravation of symptoms. The only beneficial effect of PRL2 partial deletion in lymphedema is the delay of fibrotic area formation, but analysis at later times, more than two weeks post-surgery, would be necessary to conclude on this point firmly. Taken all together, PRL2 might be necessary to regulate lymphatic vessel drainage and immune cell trafficking in lymphedema.

The subcutaneous injection of melanoma cells did not show clear difference in tumor growth, but the partial deletion of PRL2 in LECs resulted in more metastasis formation in sentinel lymph nodes. PRL2 deletion in LECs also led to an increase in immune cell accumulation in the tumor microenvironment with a reorganization of macrophages around the tumor. These results are in favor of a modification of the tumor microenvironment dependent on PRL2 expression in LECs. Moreover, given the increase in metastasis formation and the *in vitro* results, the accumulation of immune cells probably leaned towards an immunosuppressive microenvironment, but more investigations are needed to identify specifically all the different populations of immune cells present in the tumor microenvironment. Therefore, in the tumor context, PRL2 might be essential to modulate the immune cell activation/polarization and thus, indirectly affect tumor progression.

The work in this thesis suggests that targeting PRL2 in pathology should be done carefully as inhibiting PRL2 in LECs leads to a worse prognosis, especially in cancer. Recently, drug development of phosphatase inhibitors increases due to the growing interest in the role of phosphatases in regulating cellular processes and thus, being involved in different diseases such as cancer and autoimmune diseases¹⁸⁵.

To this day, no PTP-targeting drugs are studied to treat lymphedema as little is known about PTPs in the pathogenesis of lymphedema. Furthermore, clinical trials treating lymphedema

involve gene therapy, cell therapy and lymph node transfer¹⁸⁶. Protein-targeting drug are still under investigation to find more candidates involved in pathological lymphatics related to lymphedema. While PRL2 deletion did not improve lymphedema, it affected fibrosis formation. In fact, PRL2 and its homolog PRL1 were already associated with TGF β -dependent fibrosis⁸⁵. Thus, it could be interesting for other pathologies to pursue the role of PRL2 in fibrosis.

In cancer, several PTP inhibitors are already studied to block tumor progression, but also metastasis formation¹⁸⁵. Kinase inhibitors are already widely used to target tumor cells and angiogenesis. More recently, these anti-angiogenic drugs are evaluated to be used as anti-lymphangiogenic drugs as both blood and lymphatic endothelial cells share similar kinase signaling pathways⁵¹. As these drugs are also already FDA-approved, anti-angiogenic therapies can be easily translated into anti-lymphangiogenic therapies to prevent metastasis through lymphatic vessels. For example, sorafenib, sunitinib and pazopanib are VEGFR inhibitors. They have been approved for their anti-angiogenic effect in cancer, but they also showed inhibitory effect on tumor lymphangiogenesis as they prevent VEGFR-3 phosphorylation. In addition to kinase inhibitors, several drugs targeting lymphangiogenic signaling are in clinical or pre-clinical trials and they showed promising results in inhibiting lymphangiogenesis and improving the survival. The main target is the VEGF-C/VEGFR-3 axis, the principal lymphangiogenic signaling pathway. However, drugs targeting other growth factors, such as angiopoietin 1/2 (Ang1/2) or hepatocyte growth factor (HGF), are also being investigated as promising candidates. Concerning PTP inhibitors, they have yet to be explored as anti-lymphangiogenic therapeutic agents.

Several studies have already shown that PRL2 inhibition can reduce tumor growth and improve survival in different cancer types, including breast cancer, lymphoma and

leukemia^{71,72,78,79,187}. However, the effect of PRL2 inhibition on lymphatic vessels in cancer has yet to be investigated. Contrary to PRL2, PRL3 has been well studied in cancer and it has also been correlated to lymph node metastasis⁵⁷⁻⁵⁹. Specifically, it has been shown that PRL3 is correlated to VEGF-C expression and lymphatic vessel density in lung cancer⁵⁷. Although, this study did not explore the regulation of VEGF-C expression by PRL3, it suggests that PRL3 could be indirectly related to lymphangiogenesis. Furthermore, PRL3 inhibition using a small molecule, JMS-053 or iminothienopyridinedione 13, showed an effect on endothelial cell permeability in addition to its anti-tumor effect¹⁸⁸. Thus, this inhibitor is presented as a good candidate for anti-tumor therapy, but, while JMS-053 is more sensitive to PRL3 ($IC_{50}=18$ nM), it also has an inhibitory effect on PRL1 and PRL2 ($IC_{50} = 50$ nM and 53 nM). However, our results on PRL2 deletion in LECs and melanoma suggest that this inhibitor could also lead to worsening outcome of the disease by inducing an immunosuppressive microenvironment, which results in aggravating metastasis. The use of JMS-053 in an anti-tumor therapy could depend on the type of tumor and its administration. Further investigations with this inhibitor should address more precisely the events occurring in lymphatic vessels and immune cells.

It is well-established that lymphatic vessels exhibit a dual role in cancer (1) promoting metastasis and (2) modulating the immune cell response. While lymphangiogenesis inhibition to prevent metastasis is extensively studied, little is known about the potential of lymphangiogenesis to modulate the tumor immune response. One study has explored the possibility to induce lymphangiogenesis for developing a stronger immunity against melanoma⁴⁷. They developed a vaccine using irradiated tumor cells overexpressing VEGF-C. After irradiation, tumor cells are unable to grow into a compact tumor when they were injected into mice, but they are still presenting tumor antigen at their surface. The

overexpression of VEGF-C induced lymphangiogenesis and increased the transport of antigen-presenting cells to lymph nodes. With this process, more immune cells, especially T-cells, are activated by tumor antigens to specifically target tumor cells and thus, resulting in improvement of survival. Therefore, this study underlines the importance of lymphatic vessels to potentiate the immune response against tumor cells. In our experiments, we observed an increase in immune cells at the tumor site with PRL2 partial deletion in LECs. Although these immune cells are supposedly more immunosuppressive, it would be interesting to investigate how the accumulation of immune cells impacts on tumor development and metastatic spread.

In this thesis, we have focused on the role of PRL2 in LECs and its implication in lymphedema and cancer. However, the lymphatic vasculature is involved in more diseases, as we have discussed in chapter 1. Namely, lymphatic vessels are also associated with obesity and atherosclerosis^{39,42}. We did not explore the role of lymphatics in fat absorption and exploring the effect of PRL2 deletion on lacteals would be interesting. Previously, it has been demonstrated that male mice with a full-body deletion of PRL2 have a decrease in adipose tissue⁶⁵. This phenotype could be related to lacteals defect in absorbing free fatty acid and transporting chylomicrons to blood circulation.

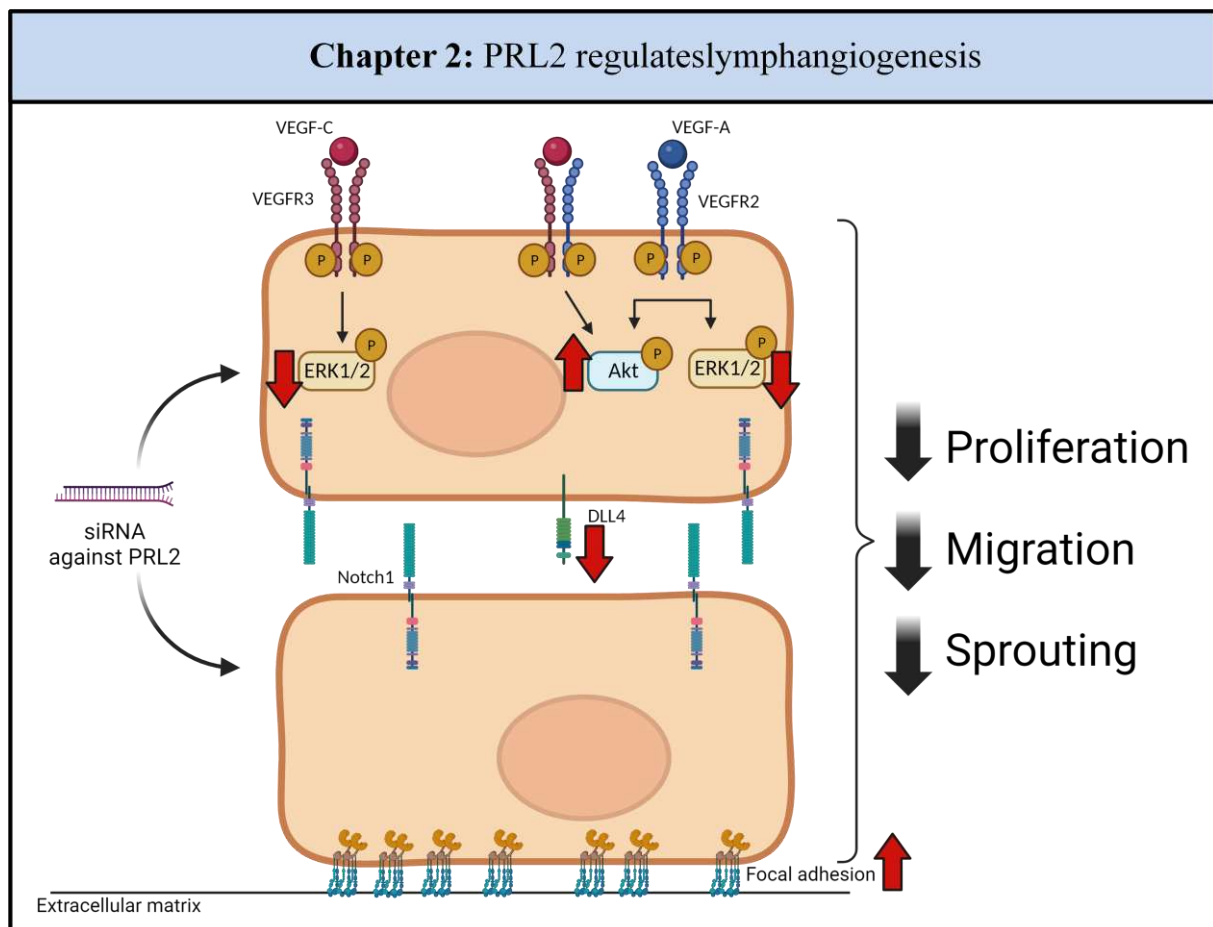
Conclusion

In this thesis work, we explored the role of PRL2 in LECs and its involvement in lymphatic-related diseases, namely lymphedema and melanoma (Figure 5.1). In chapter 2, we demonstrated that the silencing of PRL2 in LECs resulted in a decrease of proliferation, migration and sprouting of the cells. This phenotype was associated to an increase in focal adhesions and Akt phosphorylation, a decrease of ERK 1/2 activation and a disruption in Notch1 signaling. Taken together, these results suggested a defect in lymphangiogenesis.

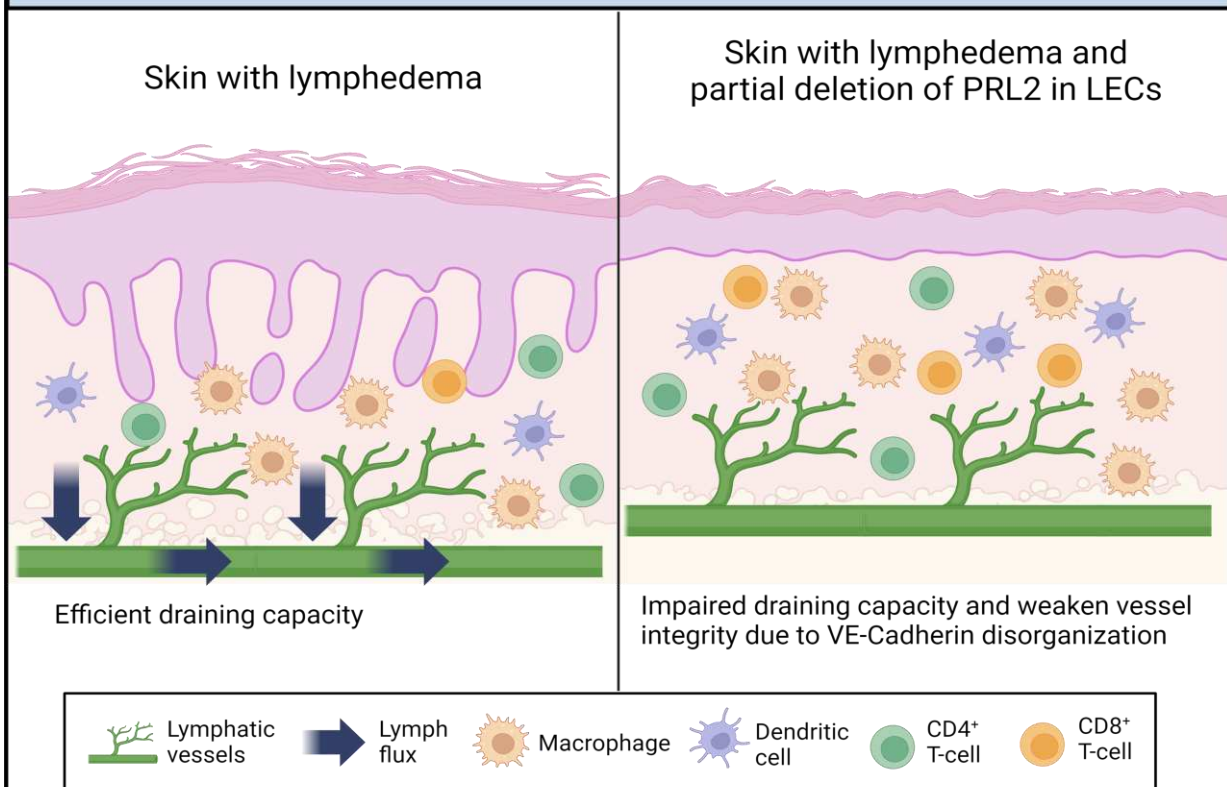
In chapter 3, we generated a mouse model with an inducible and specific deletion of PRL2 in LECs. Despite having only a partial deletion of PRL2 in our mice, we observed a difference when a secondary lymphedema was induced in these mice. With a partial PRL2 deletion, LECs were still able to form new lymphatic vessels, but these vessels have an impaired draining capacity as their vessel integrity was affected. Accumulation of immune cells tended to be more important, with the particularity of T-cell accumulation in the skin even without lymphedema. Thus, PRL2 might be necessary to regulate vessel integrity and lymphatic functions, namely fluid drainage and immune cell trafficking.

In chapter 4, we investigated the role of PRL2 in LECs in a tumor context by injecting melanoma cells in our mouse model. There was no significant difference in tumor growth between our PRL2-deficient-LEC mice and the control mice, but more lymph node metastasis were found in mice with PRL2 partial deletion. These mice also presented an increase in immune cells at the tumor site, macrophages and T-cells. By an *in vitro* analysis of cytokines and chemokines released, we determined that PRL2 deletion in LECs promotes an immunosuppressive environment around these cells. In absence of PRL2, NFκB activation was inhibited in LECs and more PD-L1 was expressed on the LEC surface, which is in favor of an inhibitory effect on inflammation. Consequently, PRL2 might influence the inflammatory response by modulating its surrounding environment and thus, directly or indirectly interacting with immune cells.

All along this thesis project, I contributed at deciphering the role of PRL2 in lymphatic vasculature with the aim to increase our knowledge of the role of PTPs in lymphatic biology. In summary, PRL2 is necessary for lymphatic vessel growth and functions, but also to modulate the inflammation surrounding the vasculature. However, there are still many questions that need to be addressed concerning PRL2 and lymphatic vessels. We must understand how PRL2 can control different cellular processes at the single cell level in LECs and how this is important in diseases. The role of PRL2 in lymph node structure and functions, but also in lacteals remains to be explored as it could constitute new avenues for studying additional PRL2 functions. This work, with its limitations, constitutes the first step for deciphering the role of PRL2 in lymphatic vasculature.



Chapter 3: PRL2 influences lymphatic draining function and immune cell trafficking



Chapter 4: PRL2 modulates inflammatory response and tumor development

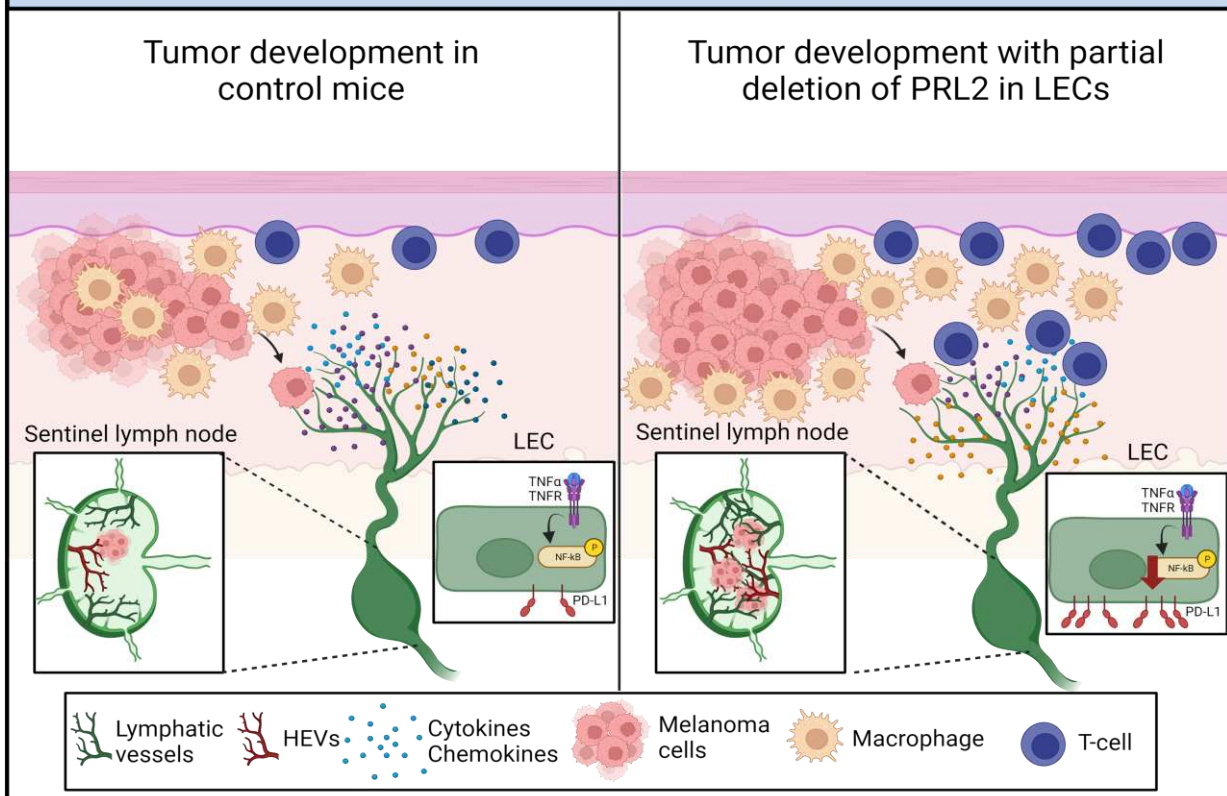


Figure 5.1: Schematic summary of this thesis.

In the chapter 2, PRL2 silencing was demonstrated to decrease lymphatic proliferation, migration and sprouting by affecting signaling pathways and focal adhesion formation in LECs. Thus, PRL2 might regulate lymphangiogenesis. In chapter 3, the partial deletion of PRL2 in LECs impaired the draining capacity of lymphatic vessels and deregulated the immune cell trafficking at the lymphedema site. The partial deletion of PRL2 also delayed the formation of fibrosis in the skin. In chapter 4, partial deletion of PRL2 in LECs resulted in an increase in immune cells, but also in lymph node metastasis. *In vitro* experiments demonstrated an increase in PD-L1 expression and a difference in cytokine and chemokines profile. These suggest that PRL2 can modulate the inflammatory response and the tumor development. (Created with BioRender.com)

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Appendices: Publications not discussed in this thesis

TGF- β promotes microtubule formation in glioblastoma through thrombospondin 1

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Abstract

Background. Microtubules (MTs), cytoplasmic extensions of glioma cells, are important cell communication structures promoting invasion and treatment resistance through network formation. MTs are abundant in chemoresistant gliomas, in particular, glioblastomas (GBMs), while they are uncommon in chemosensitive IDH-mutant and 1p/19q co-deleted oligodendrogliomas. The aim of this study was to identify potential signaling pathways involved in MT formation.

Methods. Bioinformatics analysis of TCGA was performed to analyze differences between GBM and oligodendroglioma. Patient-derived GBM stem cell lines were used to investigate MT formation under transforming growth factor-beta (TGF- β) stimulation and inhibition in vitro and in vivo in an orthotopic xenograft model. RNA sequencing and proteomics were performed to detect commonalities and differences between GBM cell lines stimulated with TGF- β .

Results. Analysis of TCGA data showed that the TGF- β pathway is highly activated in GBMs compared to oligodendroglial tumors. We demonstrated that TGF- β 1 stimulation of GBM cell lines promotes enhanced MT formation

and communication via calcium signaling. Inhibition of the TGF- β pathway significantly reduced MT formation and its associated invasion in vitro and in vivo. Downstream of TGF- β , we identified thrombospondin 1 (TSP1) as a potential mediator of MT formation in GBM through SMAD activation. TSP1 was upregulated upon TGF- β stimulation and enhanced MT formation, which was inhibited by TSP1 shRNAs in vitro and in vivo.

Conclusion. TGF- β and its downstream mediator TSP1 are important mediators of the MT network in GBM and blocking this pathway could potentially help to break the complex MT-driven invasion/resistance network.

Key Points

- TGF- β signaling is upregulated in GBM compared to chemosensitive oligodendroglioma.
- TGF- β promotes MT formation in GBM cells and increases calcium exchange and invasion.
- SMAD signaling and TSP1 are downstream mediators of TGF- β induced MT formation.

Importance of the Study

Microtubules (MTs) are important structural elements of GBM cells contributing to invasion and treatment resistance. Thus, identifying molecular pathways that drive MT formation is crucial in developing new targeted treatments. Our data show that TGF- β is a major pathway that distinguishes glioblastoma from chemosensitive oligodendroglioma. In vitro, TGF- β stimulation induced MT

formation in GBM cells by activating SMAD signaling and initiating Tsp1 expression. MT formation was inhibited in vivo by blocking either Tsp1 expression or TGF- β signaling. In conclusion, we identified a new function of TGF- β in tumor development and also characterized its specific downstream signaling which might open up new opportunities for GBM treatment.

Microtubules (MTs) are cytoplasmatic extensions of glioblastoma (GBM) cells that form a connective network leading to increased invasion and promoting resistance to radiotherapy.¹ The formation of MTs is heterogeneous and more pronounced in a subpopulation of invasive and stem-like GBM cells.^{1,2} Growth Associated Protein 43 (GAP43), a protein involved in neurite sprouting, was identified as an important mediator involved in the structural development of MTs. Intercellular calcium waves were detected as a way of communication through the MT network and Connexin 43, a major gap junction protein, was found to be functionally involved.¹ The MT network was observed extensively in GBM and also in astrocytic tumors in general, however, was scarce in oligodendroglioma. These 2 tumor types can be distinguished at the molecular level by IDH mutation and 1p/19q co-deletion, which are only present in oligodendroglioma.³ In contrast to GBMs and astrocytomas, oligodendroglioma patients show remarkable responses to radio- and chemotherapy and therefore have a much better prognosis.⁴ Thus, to identify mechanisms of MT formation in GBM and to unravel how MTs promote cell-to-cell communication might ultimately lead to a better understanding of therapeutic resistance and new ways of GBM treatment. Interestingly, a recent study showed that network formation

of MTs was enhanced in the resection cavity after neurosurgery in experimental models.⁵ MT formation in this context might partly explain why recurrent tumors often develop close to the resection site. Recently, it was shown that glioma cells and neurons are connected through so-called neuroglial synapses that are located on MTs indicating another layer of resistance through connection with normal cells.⁶ Molecular mediators driving the MT network identified so far such as GAP43 and tweety-homolog 1 (TTYH1)⁷ are also important in normal physiology of the CNS. Thus, the identification of more tumor-specific pathways that drive MT formation is of great interest in order to develop targeted therapies.

We recently showed by RNA sequencing of patient-derived xenograft (PDX) tissue from laser capture microdissected invasive and central tumor areas that the matricellular protein thrombospondin 1 (TSP1 or THBS1) was one of the most upregulated genes in infiltrative areas of GBM.⁸ TSP1 is a large trimeric calcium-binding molecule, which binds to diverse ligands and receptors.⁹ In patient biopsies, both TSP1 and TGF- β (transforming growth factor-beta) were expressed at high levels in GBM compared to low-grade gliomas (LGG).¹⁰ In addition, TSP1 is an unfavorable prognostic marker for

GBM patients.¹¹ The TGF- β 1⁸ but not the TGF- β 2¹² canonical pathway transcriptionally regulates TSP1 expression through SMAD3 binding to the *THBS1* promoter. TSP1 silencing inhibited cell invasion in vitro and in vivo, also in combination with anti-angiogenic therapy.⁸ Interestingly, TSP1 was also found to be regulated by calcium flux in several cancers.¹³

In the present study, we showed that TGF- β 1 stimulation of GBM stem-like cell cultures promotes MT formation and that TSP1 is an important mediator of MT formation downstream of TGF- β 1.

Materials and Methods

Ethics Statement

Patient material was obtained from surgeries performed at the Haukeland University Hospital (Bergen, Norway). Written consent was obtained from patients with procedures that were approved for the projects (project numbers 013.09 and 151825) by the Regional Ethics Committee (Bergen, Norway). In vivo experiments were conducted in accordance with the European Community for experimental animal use guidelines (L358-86/609EEC) with protocols approved by the Ethical Committee of INSERM (n° MESRI23570).

TCGA Data and GMT Files

For the “The Cancer Genome Atlas” (TCGA) dataset, RNAseqV2 median normalized data (RNA Seq V2 RSEM) and associated clinical data of the TCGA GBM and LGG cohorts were downloaded from cBioPortal (<https://www.cbioportal.org/>). Details are described in the [Supplementary Methods](#).

Cell Culture

The patient-derived GBM stem cell (GSC) lines P3,¹⁴ GG6 and GG16¹⁵ are all derived from IDH wild-type (wt) GBM patients. GG16 was a giant cell glioblastoma.¹⁶ Cell lines were cultured in Neurobasal Medium, B27 supplement, Glutamax (NBM; Life Technologies), FGF2 (20 ng/ml), and EGF (20 ng/ml; Peprotech) in non-treated culture flasks (Nunc). U87, U251, and 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% fetal calf serum (FCS) and 1% glutamine. Medium was changed twice a week. All cell lines were grown at 37°C in a humidified atmosphere of 5% CO₂.

In Vitro Experiments

2.5 × 10⁴ cells from GSC lines were seeded on coverslips. Cells were treated with growth factors (10 ng/ml) and/or inhibitors (10 μ M) for 48 hours. For MT analysis and quantification, 300 cells were counted in random microscopic fields.

Western Blots

Western blot of cultured cells was performed as described previously.⁸ Details are described in the [Supplementary Methods](#).

Immunofluorescence Staining

Immunofluorescence staining was performed as previously described.⁸ Details are described in the [Supplementary Methods](#).

Immunohistochemistry

Immunohistochemistry of paraffin-embedded formalin-fixed tissue sections was performed as described previously.⁸ Details are described in the [Supplementary Methods](#).

RNA Sequencing and Analysis

A total of 3 samples were assigned for each of the control (untreated) and treatment (TGF- β 48 hours) groups. Samples were processed for sequencing at Macrogen Inc, Korea where library preparation, sequencing, and quality control were performed. Details of the sample preparation and data analysis have been reported previously.¹⁷

GBM-Brain Organoid Co-Culture Ex Vivo Invasion Assay

For this assay, the preparation and culture of 18-day fetal brain organoids have been described in our previous work.¹⁸ For details, see [Supplementary Methods](#).

In Vitro Calcium Imaging

Calcium signal spread across cells was carried out as previously described.¹⁹ Fluo-3-AM intensity was determined using the ImageJ z-axis profile function. Mean fluorescence intensity was determined from 20 time lapses per condition.

In Vivo Experiments

Ragy2C^{-/-} mice were housed with free access to food and water in a 12-hour light/dark cycle. Craniotomy and GBM spheroid implantation were done as previously described.²⁰ LY2109761 was administered orally at 50 mg/kg twice daily (days 1-5 of each week) until the end of observation.

Statistics

All statistical analyses were performed on GraphPad Prism 8.1.2. Data are displayed as mean ± SD. To compare one variable between multiple groups, 1-way ANOVA with Tukey's post hoc test was used. To compare 2 or more variables

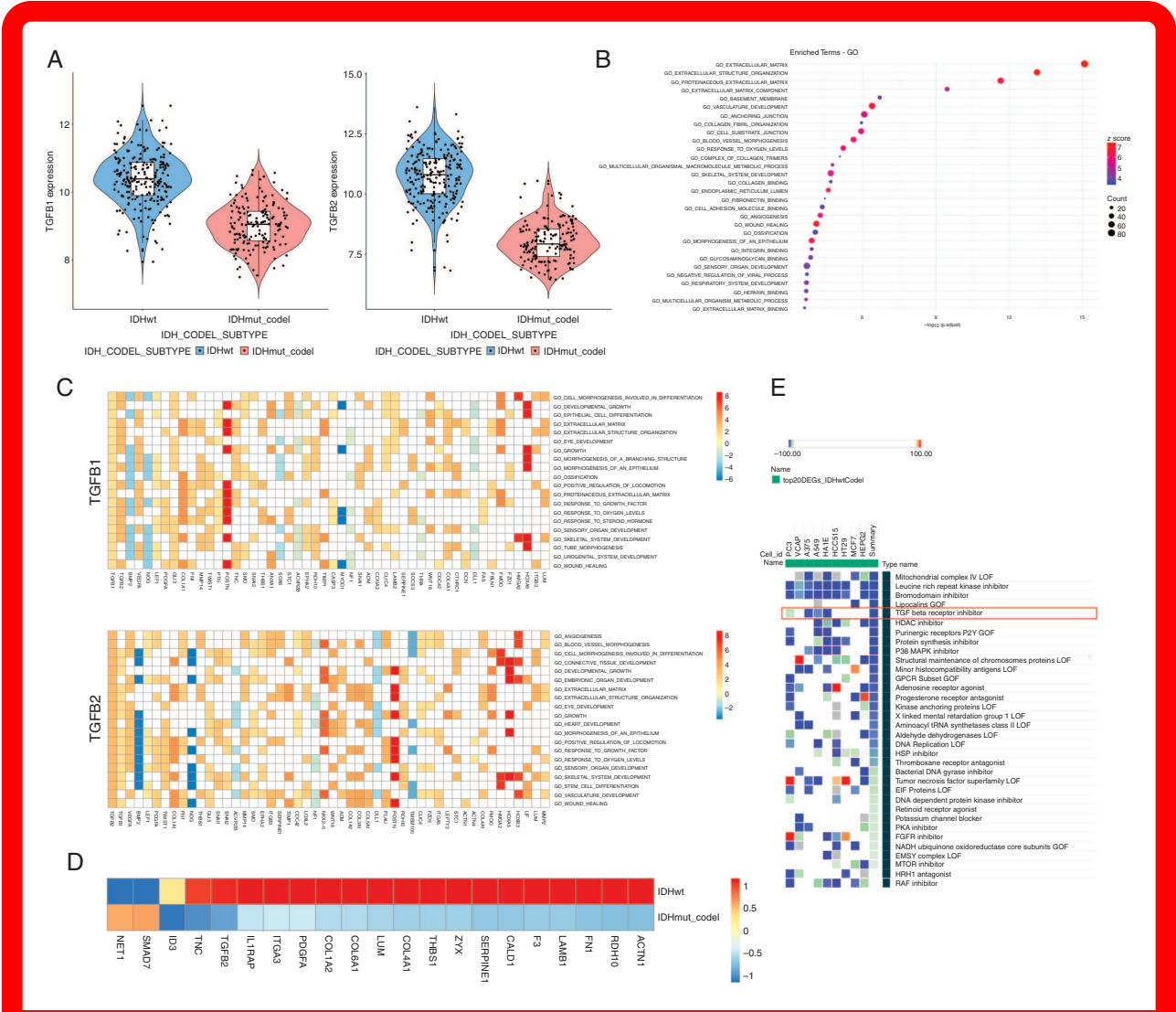
between multiple groups, 2-way ANOVA with Tukey's post hoc test was used. Differences were considered statistically significant when the *P*-value was below .05.

Results

TGF- β Signaling Is Upregulated in GBM Compared to 1p/19q Co-Deleted Oligodendroglioma

MTs are abundant in GBM, however uncommon in 1p/19q co-deleted oligodendroglioma. To identify new drivers of MT formation, we compared gene expression data from TCGA of IDH-wt tumors, the majority being GBM, to

IDH-mutant and 1p/19q co-deleted oligodendroglioma. We found that *TGFB1* and *TGFB2* were among the significantly differentially expressed genes (Supplementary Table 1). Both *TGFB1* and *TGFB2* are highly upregulated in IDH-wt tumors compared to IDH-mutant and 1p/19q co-deleted tumors (Figure 1a) and also upregulated in non-co-deleted (IDH-wt and IDH-mutant astrocytoma/GBM) vs co-deleted tumors (Supplementary Figure 1a). MT formation has been previously observed in particular at the invasive front of GBM.¹ As TGF- β is a known driver of GBM invasion, we performed more detailed analyses. Interestingly, *TGFB1* is located on chromosome 19q, which makes it an attractive candidate for MT formation. The differentially expressed genes of IDH-wt compared to IDH-mutant and 1p/19q co-deleted tumors (1.comparison) were then used to conduct a Gene Ontology (GO) term enrichment analysis.



TGF- β signaling is upregulated in GBM compared to IDH-mutant and 1p/19q co-deleted oligodendroglioma. Analysis of TCGA data comparing IDH-wt GBM and IDH-mutant and 1p/19q co-deleted oligodendroglioma. (a) *TGFB1* and *TGFB2* are upregulated in IDH-wt tumors. (b) GO term analysis reveals pathways related to extracellular matrix. (c) Heatmap of genes related to the top 20 GO terms where either *TGFB1* or *TGFB2* is present. (d) TGF- β responsive genes identified from the literature. (e) Analysis of drugs related to gene expression signature by connectivity map reveals that TGF- β inhibitors are one of the top candidate drugs that inhibit IDH-wt GBM signature. Abbreviations: GBM, glioblastoma; GO, Gene Ontology; IDH, isocitrate dehydrogenase; TCGA, The Cancer Genome Atlas; TGF- β , transforming growth factor-beta.

The top regulated pathways were related to extracellular matrix, which are important pathways in tumor invasion (Figure 1b). We performed the same GO term analysis for non-co-deleted vs co-deleted tumors (2.comparison) where extracellular matrix pathways were also found, however, below a number of immune-related pathways (Supplementary Figure 1b). We then analyzed the commonly regulated pathways from these 2 comparisons in a Venn diagram revealing that the top common pathways in both were related to extracellular matrix (Supplementary Figure 1c, d). As MT formation is also highly dependent on extracellular matrix reorganization this could indicate an important role of TGF- β in this process. When focusing on the top 20 GO terms where either *TGFB1* or *TGFB2* was present in the gene list, the majority of associated genes in these pathways were upregulated in IDH-wt compared to 1p/19q co-deleted tumors (Figure 1c). Next, we identified TGF- β responsive genes from the literature and showed that the majority of these genes are upregulated in IDH-wt compared to 1p/19q co-deleted tumors (Figure 1d). By using connectivity map from the Broad Institute,²¹ we analyzed drug candidates that would inhibit the expression signature of GBM (top 20 upregulated genes) compared to oligodendroglioma. One of the top drug candidates that came up in this screen were TGF- β inhibitors (Figure 1e) further highlighting an important role of the TGF- β pathway in promoting a GBM gene expression signature, when compared to chemosensitive oligodendroglioma.

TGF- β Promotes MT Formation in GBM Cell Lines In Vitro

To analyze the ability of different growth factors, including TGF- β , to induce cytoplasmic protrusions (MT-like structures), we stimulated P3 GBM cells with EGF, FGF, PDGFbb, TGF- β 1, or TGF- β 2. We observed that TGF- β 1 was most efficient in promoting cellular protrusions when analyzing the percentage of cells with protrusions, number of protrusions per cell, and length of protrusions (Supplementary Figure 2a). Next, we stimulated 2 different GSC lines (P3 and GG16) and 2 serum cultured GBM cell lines (U87 and U251) with TGF- β 1 and analyzed morphological changes of the cells. A clear promotion of cellular protrusions by TGF- β 1 was observed in all cell lines (Figure 2a; Supplementary Figure 2b, c). To verify that these protrusions are MTs we stained the cells for GAP43, a known driver for MT formation as well as nestin and actin to visualize the cytoskeleton. The protrusions were rich in GAP43 which colocalized with actin and nestin (Figure 2a; Supplementary Figure 2b). This indicated that the protrusions are MTs as described previously.^{1,2} To further verify that cells are interconnected through MTs upon TGF- β 1 stimulation, we performed Electron microscopy. We observed MTs extending from one cell and inserting into the membrane of a neighboring cell (Figure 2b). We analyzed and quantified the formation of MTs in detail by confocal microscopy using GAP43/ nestin or actin stainings. TGF- β 1 stimulation increased the number of cells connected through MTs and importantly, also the length of MTs increased significantly (Figure 2c; Supplementary Figure 2b, c). To block TGF- β signaling, we used the TGF- β inhibitor LY2157299, which significantly reduced pSMAD2 phosphorylation in P3 GBM cells

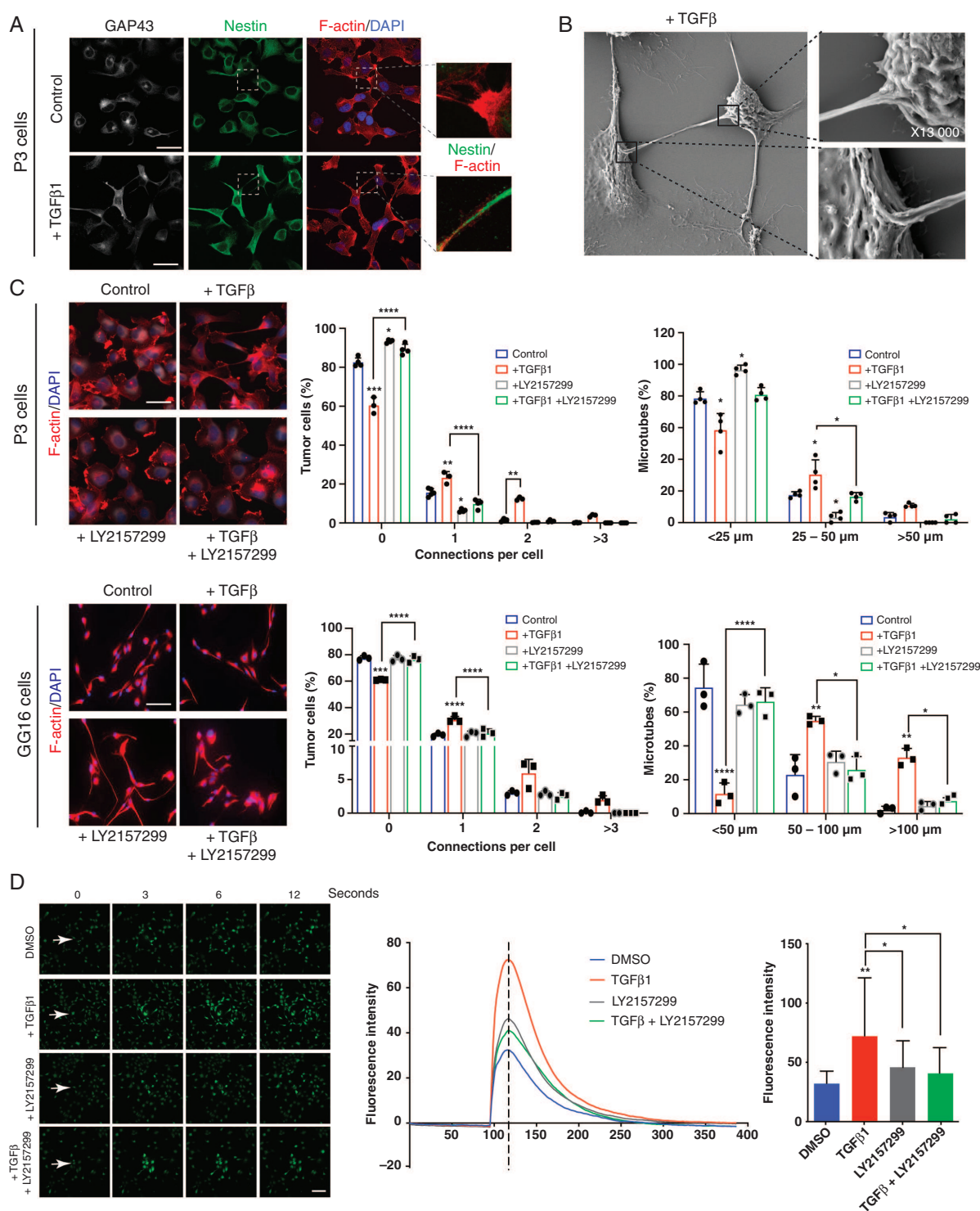
(Supplementary Figure 3a). MT formation was significantly inhibited by LY2157299: the number of MTs per cell, the number of cells with MTs, and the length of MTs decreased substantially under treatment with LY2157299 in both P3 and GG16 GBM cells (Figure 2c; Supplementary Figures 2c and 3b). In addition, we used an inducible shRNA to knock-down TGFBRII, as stable knockdown was lethal to the cells (data not shown). The inducible construct confirmed the inhibition of MT formation in P3 cells (Supplementary Figure 3c, d). As GAP43 has been shown to be a major structural element of MTs, we knocked down GAP43 with a shRNA to analyze if MT formation is blocked under TGF- β 1 stimulation. As expected, GAP43 knockdown prevented a significant increase in the number and length of MTs under TGF- β 1 stimulation (Supplementary Figure 4a, b) verifying that also in our culture system MT formation is dependent on GAP43 as described previously.¹ To demonstrate that there is a functional connectivity between the tumor cells through the MT network, we performed calcium imaging using fluorescence indicator of intracellular calcium 3 (Fluo-3).²² The intensity of the calcium wave was calculated for entire frames. Stimulation with TGF- β 1 resulted in increased calcium exchange between tumor cells through the MT network which was inhibited by LY2157299 (Figure 2d; Supplementary Figure 5; Movies 1–4).

TGF- β Induced MT Formation Is Associated With Invasion

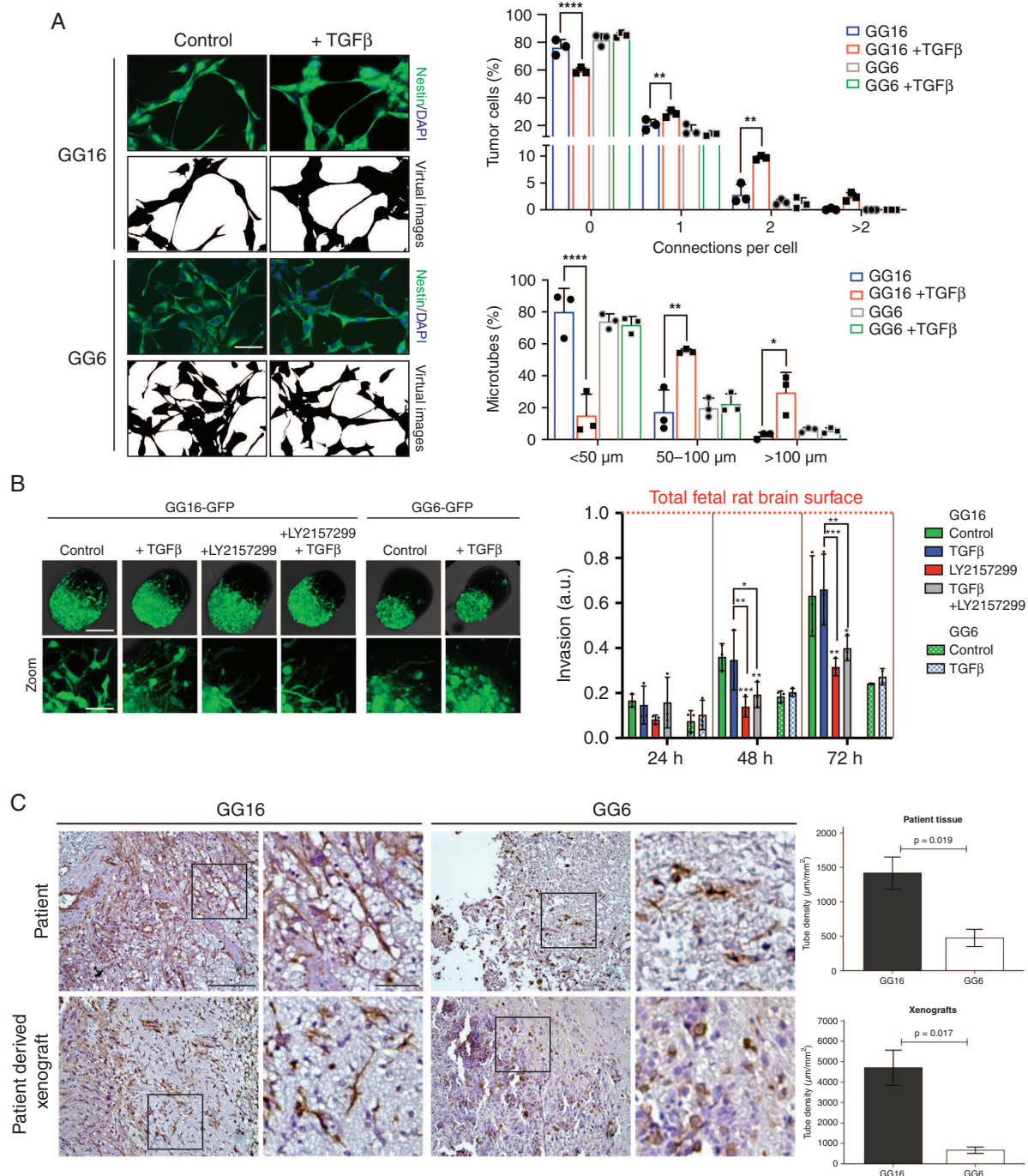
Among the GBM cell lines used in this work, we identified one GSC line, GG6, that did not show increase in MT formation upon TGF- β 1 stimulation. GG16, used as a control cell line, confirmed significant MT formation under stimulation (Figure 3a). As MT formation is associated with invasion, we aimed to analyze the invasiveness of the nonresponder cell line GG6 in comparison with the TGF- β 1 responding GBM cell line GG16. We used a co-culture system of tumor spheroids with brain organoids from rat fetal brains to mimic the invasive process as described previously.¹⁸ Invasion was quantified as indicated in methods. The nonresponding cell line GG6 showed no significant invasion over a time period of 72 hours when co-cultured with brain organoids. In contrast, the TGF- β 1 responding cell line GG16 showed substantial invasion into the organoid, which was not further increased by TGF- β 1 stimulation, however significantly reduced by TGF- β inhibition with LY2157299, indicating endogenous presence of TGF- β in the organoid microenvironment (Figure 3b). Upon implantation of both cell lines in vivo, we verified a less invasive phenotype of the nonresponder GG6 compared to the responder GG16 and a reduced MT density in nestin immunostained sections (Figure 3c). Importantly, we confirmed a reduced MT density in corresponding patient biopsies from GG6 compared to GG16 stained with nestin antibodies (Figure 3c).

SMAD Activation Is Important for MT Formation

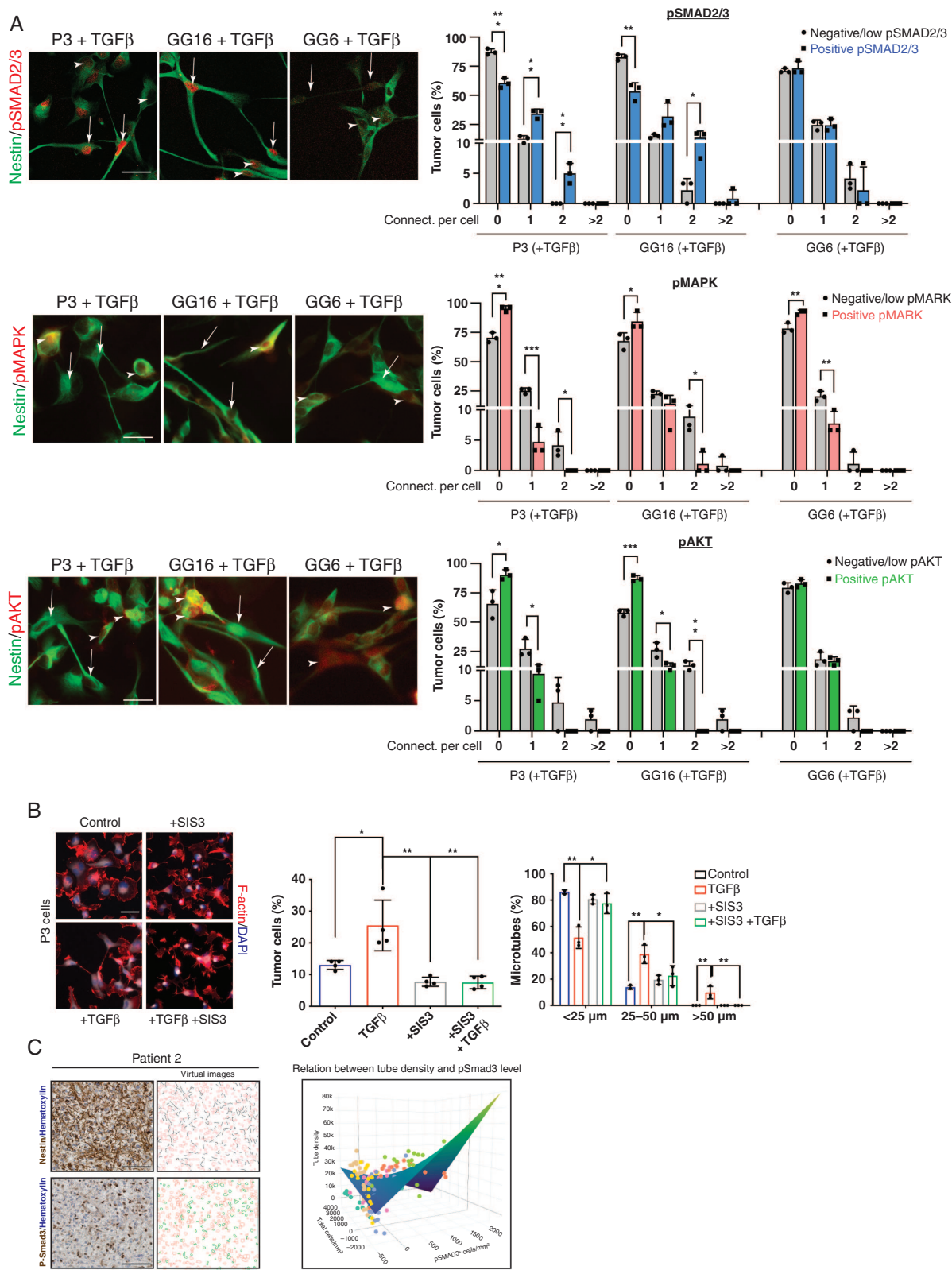
To identify major signaling mediators in TGF- β 1 induced MT formation, we analyzed activation of downstream pathways under stimulation with TGF- β 1 in P3, GG6, and



TGF- β promotes MT formation and communication via calcium signaling in GBM cells. (a) Cellular protrusions induced by TGF- β 1 in P3 GBM cells are identified as MTs due to the expression of GAP43 which co-localizes with the cytoskeleton protein nestin. Scale bar 20 μ m. (b) Scanning Electron Microscopy of P3 GBM cells shows that MTs connect 2 neighboring cells through cytoplasmic insertions. Higher magnifications of specific areas are provided as indicated. (c) TGF- β inhibitor LY2157299 inhibits MT formation in P3 and GG16 GBM cells. Immunofluorescence staining for F-actin is shown. Quantification of connections per cell and MT length is presented. Scale bar 10 μ m. Statistically significant differences of experimental groups compared to the control are shown on top of the respective bars. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$. (d) Calcium exchange between tumor cells is significantly increased upon TGF- β stimulation and inhibited by LY2157299 in P3 GBM cells. Fluorescence intensity represents the intensity of the calcium signal. The images were taken seconds following the laser injury as indicated. The bar graph represents the intensity at the time point as indicated by the dotted line in the curve diagram. Scale bar 30 μ m. * $P < .05$; ** $P < .01$. Abbreviations: GBM, glioblastoma; MT, microtube; TGF- β , transforming growth factor-beta.



TGF- β induced MT formation is associated with invasion. (a) GG6 GBM cells are not responding to TGF- β 1 stimulation with increased MT formation in contrast to GG16 GBM cells. Immunofluorescence stainings for nestin are shown. Black and white pictures are presented to better visualize the MT network. Quantification of connections per cell and MT length is presented. Scale bar 20 μ m. * P < .05; ** P < .01; *** P < .001; **** P < .0001. (b) Invasion of GG16 GBM cells into brain organoids is inhibited by LY2157299. TGF- β stimulation does not significantly increase invasion most likely due to the presence of TGF- β in the microenvironment. In contrast, nonresponder GG6 GBM cells do not show significant invasion into brain organoids. Immunofluorescence pictures of organoids after 72 h showing green fluorescent protein (GFP) signal (GBM cells). Higher magnifications of the invasive areas are provided below each organoid picture. Quantification of invasive cells is provided after 24, 48, and 72 hours. Scale bar 70 μ m (zoom 14 μ m). Statistically significant differences of experimental groups compared to the control are shown on top of the respective bars. * P < .05; ** P < .01; *** P < .001. (c) Immunohistochemical staining of GG6 and GG16 patient and xenograft GBM with nestin antibodies showing an extensive MT network in GG16, which is low to absent in GG6. Quantification of MT density is provided. Scale bar 100 μ m (zoom 10 μ m). Abbreviations: GBM, glioblastoma; MT, microtube; TGF- β , transforming growth factor-beta.



SMAD activation is important for MT formation. (a) Double immunofluorescence stainings of pSMAD2/3/nestin, pMAPK/nestin, and pAKT/nestin for P3, GG16, and GG6 cell lines are shown. Arrows indicate cells with MT formation. Arrowheads point to cells without MT formation. Quantification of connections per cell is presented. Scale bar 10 μ m. * P < .05; ** P < .01; *** P < .001. (b) SMAD inhibitor SIS3 inhibits MT formation

GG16 cell lines by double immunofluorescence stainings with nestin (Figure 4a). Canonical (SMAD2/3) signaling correlated with MT formation in P3 and GG16, however, not in GG6 where also pSMAD2/3 activation levels were lower. The non-canonical pathways (MAPK and PI3K/Akt) did not correlate with MT formation in all 3 cell lines (Figure 4a). Likewise, SMAD inhibition under TGF- β 1 stimulation using the SMAD inhibitor SIS3 significantly reduced MT formation (Figure 4b). To further substantiate the role of SMAD2/3 phosphorylation in MT formation, we immunostained consecutive sections from 7 GBM IDH-wt patient biopsies with pSMAD3 and nestin antibodies (Figure 4c; Supplementary Figure 6). Quantification of MT density in randomly selected pSMAD3+ and pSMAD3- areas was performed as described in the methods. For statistical analysis of the data, model selection was performed as indicated in the Supplementary Methods and Supplementary Table 2. The final model indicated a significant association between increased pSMAD3 expression and increased MT density when adjusting for overall cell density ($T = 3.80$, $P = .0002$).

Having demonstrated that pSMAD2/3 signaling is crucial for MT formation downstream of TGF- β , we performed co-immunoprecipitation of pSMAD2/3 signaling complexes in P3, GG6, and GG16 cell lines, followed by mass spectrometry to identify potential binding partners. Interestingly, P3 and GG16 samples clustered together, while GG6 samples were separated (Supplementary Figure 7a). Despite these differences, the proteins in common between all 3 cell lines were related to TGF- β /SMAD signaling as also revealed by KEGG pathway analysis and GO biological function (Supplementary Figure 7, Supplementary Tables 8, 10, and 11). However, the SMAD2/3 proteome was very different between P3/GG16 and GG6. While P3 and GG16 had 128 proteins in common, only 23 and 19 proteins were shared between GG6/P3 and GG6/GG16, respectively (Supplementary Figure 7, Supplementary Tables 3–9). The proteins in common between P3 and GG16 were related to a number of pathways/biological processes (Supplementary Tables 12 and 13), while none could be identified for proteins of GG6. These results further confirm the similarity in TGF- β /SMAD signaling between responders in contrast to the nonresponder GG6.

TSP1 Is a Candidate for MT Formation Downstream of TGF- β 1 and SMAD2/3

To identify targetable proteins involved in MT formation downstream of TGF- β 1 and SMAD activation, we performed RNA sequencing of the responder cell lines GG16 and P3 and the nonresponder cell line GG6 with and without TGF- β 1

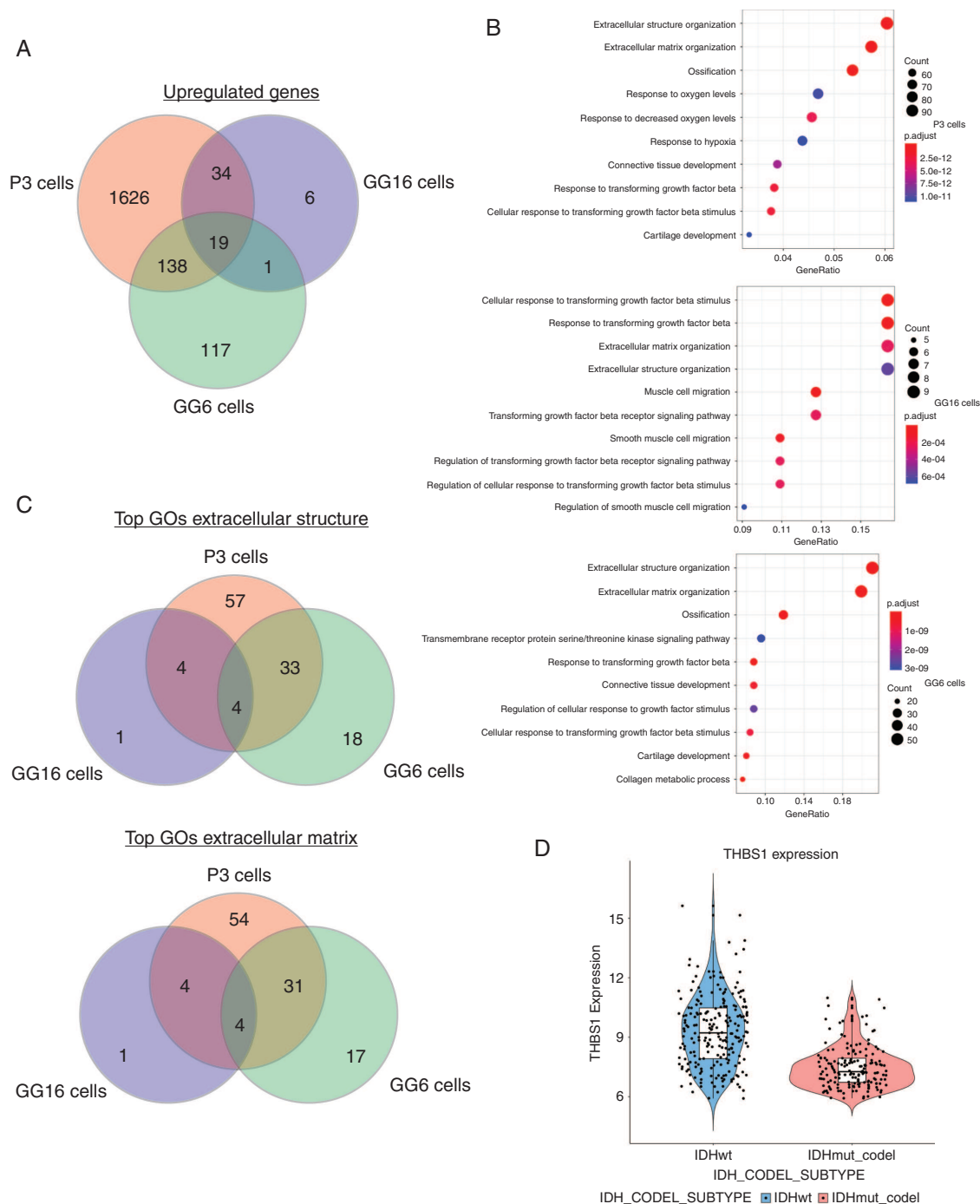
stimulation (48 hours) to identify differences in gene expression profiles that would indicate potential candidates for MT formation downstream of TGF- β 1/SMAD2/3 activation. The number of significantly upregulated genes under TGF- β 1 stimulation varied substantially between the cell lines (Supplementary Table 14). In particular, GG16 showed only 60 upregulated genes, however, 34 of these genes were shared with P3, while only 1 gene was shared with the nonresponder cell line GG6 (Figure 5a; Supplementary Table 14). 19 genes were commonly upregulated in all 3 cell lines. Gene enrichment analysis showed that in all 3 cell lines, extracellular structure organization and extracellular matrix organization were the top regulated GOs (Figure 5b). When analyzing these GOs for common genes between the responders P3 and GG16, only 4 genes were found, while also 4 genes were in common between all 3 cell lines (Figure 5c; Supplementary Tables 15 and 16). Among the 4 genes in common between the responders P3 and GG16, TSP1 (*THBS1*) was identified as an interesting candidate because (1) it also showed up in the previous analysis of TCGA data (Figure 1c, d); (2) it is upregulated in invasive tumor areas compared to the core in GBM xenografts,⁸ and (3) TSP1 is a downstream mediator of TGF- β 1 and SMAD3 signaling in GBM and important in tumor cell invasion.⁸ Analysis of TCGA data also confirmed upregulation of TSP1 in IDH-wt GBM compared to IDH-mutant and 1p/19q co-deleted oligodendroglioma (Figure 5d).

TSP1 Is Activated by TGF- β 1 and Mediates MT Formation

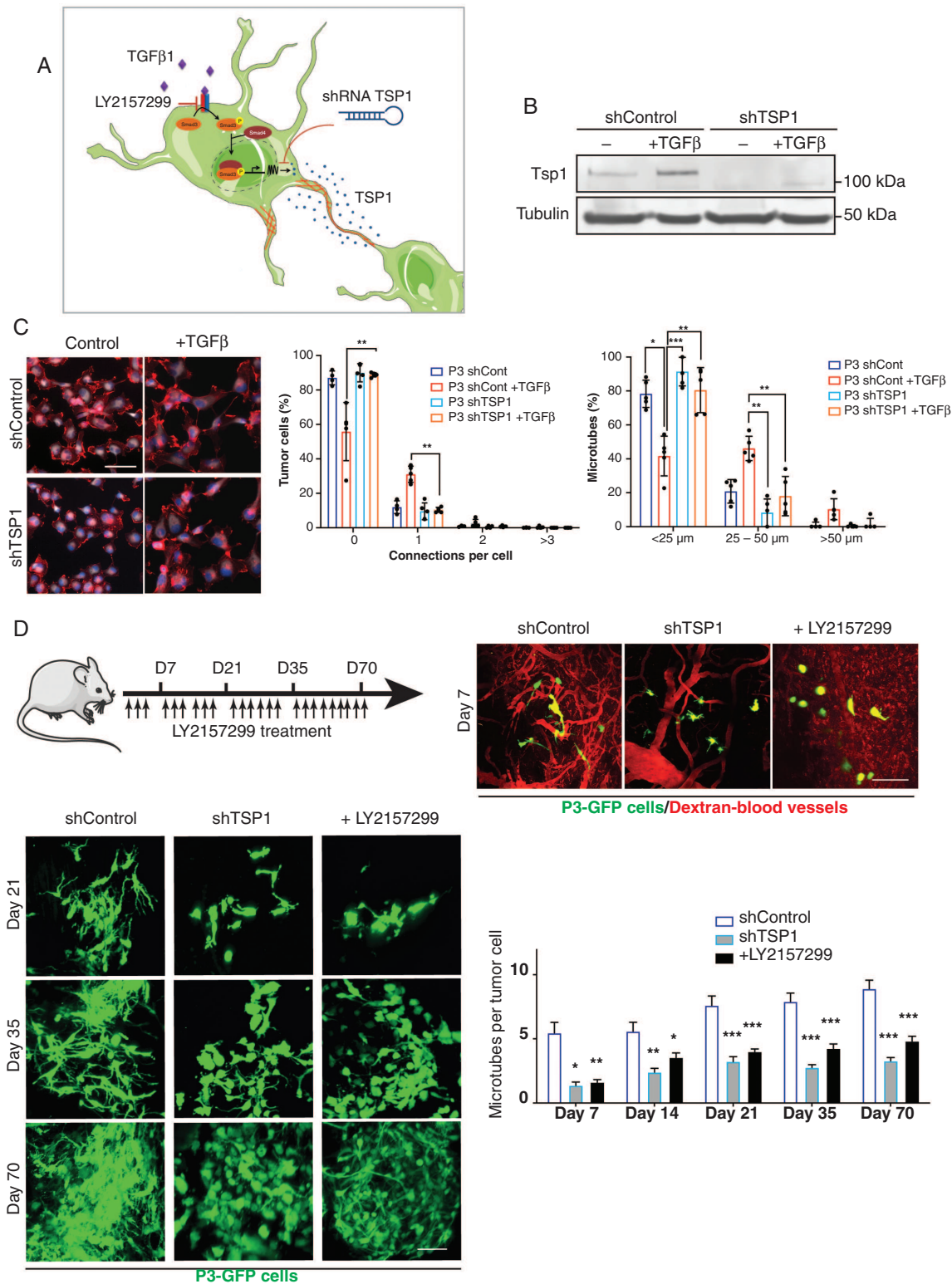
As described previously,⁸ Tsp1 expression is upregulated by TGF- β signaling through activation of the Tsp1 promoter by pSMAD3 (Figure 6a). Here, we confirmed that TSP1 protein expression was induced upon TGF- β 1 stimulation in the responder cell lines GG16 and P3, while it was absent in the nonresponder cell line GG6 (Figure 6b; Supplementary Figure 8). We used a shRNA to knock down expression of TSP1 and analyzed MT formation under TGF- β 1 stimulation. ShTsp1 significantly reduced MT formation in P3 cells (connections per cell and MT length) when compared to scrambled control (Figure 6c). Likewise, overexpression of TSP1 in P3 cells showed increase in MT formation compared to control cells (Supplementary Figure 9).

To verify that TGF- β and TSP1 are important players of MT formation in vivo, we performed intravital imaging of P3 cells implanted orthotopically in NOD/SCID mice. After 7–70 days, both inhibition of TGF- β by LY2157299 and knockdown of TSP1 by shRNA reduced the number of MTs per tumor cell in vivo compared to controls (Figure 6d).

under TGF- β 1 stimulation. Immunofluorescence staining for F-actin is shown. Quantification of cells with MTs and MT length is presented. Scale bar 10 μ m. * $P < .05$; ** $P < .01$. (c) SMAD3 phosphorylation correlates with MT formation in GBM patient biopsies. Quantification of MT length on consecutive pSMAD3 immunostained and nestin immunostained sections of 1 patient (patient 2) is shown (see the Supplementary Methods for details). Images in the left column show original image data, images in the right column show visualizations of the quantifications. Black lines mark MTs, red outlines denote nuclei, and green outlines pSMAD3-positive nuclei. The graph shows the correlation of pSMAD3 expression with MT length using a linear mixed model. The surface shows the predicted value based on the fixed parameters of the linear mixed model. The results of all patients are included in the graph (each color refers to 1 patient; single graphs are shown in Supplementary Figure 6b). Scale bar 100 μ m; $P = .0013$.



TSP1 is a candidate for MT formation downstream of TGF- β 1 and SMAD2/3. RNA sequencing data of P3, GG6, and GG16 GBM cells unstimulated and stimulated with TGF- β 1 for 48 hours. (a) Venn diagram showing number of common and unique upregulated genes upon TGF- β 1 stimulation. Hypergeometric test for all overlaps, $P < .0001$. (b) Gene enrichment analysis showing upregulation of pathways related to extracellular matrix/structure in all 3 cell lines. (c) Venn diagram showing the number of common and unique regulated genes among the 3 cell lines from extracellular matrix and extracellular structure GOs. Hypergeometric test for GG6/GG16 $P < .05$, for all other overlaps, $P < .0001$. (d) Comparison of TSP1 expression in TCGA data between IDH-wt and IDH-mutant, 1p/19q co-deleted tumors. Abbreviations: GBM, glioblastoma; GO, Gene Ontology; IDH, isocitrate dehydrogenase; MT, microtube; TCGA, The Cancer Genome Atlas; TGF- β , transforming growth factor-beta; TSP1, thrombospondin 1.



Inhibition of TSP1 reduces MT formation in vitro and in vivo. (a) Schematic figure showing upregulation of Tsp1 expression by TGF- β signaling through binding of pSMAD3 to the Tsp1 promoter. Tsp1 expression can be inhibited by LY2157299 or shRNA Tsp1. (b) Western blot of Tsp1 in P3 shControl and P3 shTsp1 with and without TGF- β stimulation. (c) MT formation is inhibited by shTsp1. Immunofluorescence staining for F-actin is shown. Quantification of connections per cell and MT length is presented. Scale bar 10 μ m. * P < .05; ** P < .01; *** P < .001. (d) Formation of MT network in vivo in the orthotopic P3-GFP GBM xenograft model was analyzed by intravital imaging. ShTsp1 and LY2157299 inhibit MT formation compared to shcontrol. Immunofluorescence pictures of intravital imaging are shown. Quantification of MT number per tumor cell is presented. Scale bar 150 μ m. * P < .05; ** P < .01; *** P < .001. Abbreviations: MT, microtubule; TGF- β , transforming growth factor-beta; TSP1, thrombospondin 1.

Discussion

In the present study, we identified a new molecular mechanism of MT formation in GBM. We showed that TGF- β 1, a multifunctional cytokine, promotes MT formation in vitro and in vivo. MTs have been defined as major drivers of GBM invasion and resistance to standard treatments such as radiation and chemotherapy.¹ Since this hallmark study, where the GAP43 protein was identified as a major structural protein of MTs, new and fundamental insights into detailed molecular mechanisms of MT formation are still lacking. In search of molecular drivers of MT formation, we performed a bioinformatics analysis comparing IDH-mutant 1p/19q co-deleted vs IDH-wt tumors. We chose this starting point because MT formation is abundant in IDH-wt tumors while it is low to absent in IDH-mutant and co-deleted tumors.¹ This analysis brought our attention to TGF- β . Among other gene candidates, TGFB1 and TGFB2 are expressed at significantly lower levels in co-deleted tumors. Importantly, *TGFB1* is located on chromosome 19q and very important in GBM invasion,²³ which are additional justifications for its potential role in MT formation. TGF- β is a cytokine with multiple functions which are dependent on the cell type and its associated microenvironment.²⁴ In GBM, TGF- β is well characterized as a cytokine that promotes invasion, angiogenesis as well as suppression of the immune system.²⁵ Thus, our results add an additional function of TGF- β to its divergent roles in GBM development. We demonstrated experimentally in different ways that TGF- β 1 is important in MT formation: (1) by stimulation with TGF- β 1; (2) by blocking of TGF- β activity using the inhibitor LY2157299 and an inducible shRNA. As our study is among the first to analyze MT formation in vitro, we had to verify that the structures we analyzed are corresponding to the MTs which have been shown in vivo.¹ We identified MTs in our culture system by demonstrating co-expression of major structural proteins of MTs such as nestin, actin, and GAP43. Nestin, a protein related to cellular stemness, has recently been identified as an important protein expressed in the GBM MT network in vivo.² Thus, we used this marker in our study to identify and quantify MTs. Knockdown of GAP43, which significantly reduced MT formation under TGF- β 1 stimulation, further corroborated that the structures we analyzed in vitro are very similar to the MTs formed in vivo.

MTs connect tumor cells with each other forming a communicating network. To show that network formation is promoted by TGF- β 1, we performed calcium imaging. As expected, calcium exchange increased upon TGF- β 1 stimulation and was inhibited by LY2157299. Network formation and increase in calcium exchange in GBM cells in vitro was also observed by da Silva et al using ROCK inhibition.¹⁹

The majority of our cell lines responded to TGF- β 1 with increase in MT formation. Interestingly, we identified one cell line (GG6), which did not respond and also showed very limited invasive capacity upon TGF- β stimulation using ex vivo brain organoid co-cultures and in vivo xenografts. These results highlight that MT formation and invasion are highly connected as also demonstrated previously.¹ Inhibition of TGF- β activity in GBM in a clinical

setting has been unsuccessful so far.^{26,27} Reasons for these disappointing results may be the dose limitations due to the important physiological roles of TGF- β in the body and also the BBB which often impairs drug penetration.²⁸ Thus, we aimed to further characterize downstream signaling of TGF- β inducing MT formation to reveal more cancer-specific downstream signaling. First, we identified the canonical SMAD2/3 signaling as being the most important downstream pathway and also showed in a patient setting that SMAD3 activation correlates with MT length in patient biopsies. Previously, SMAD2/3 signaling was shown to impact glioma invasion,^{15,29} whereas SMAD3 was defined as the main transcription factor regulating invasion and TSP1 expression in GBM cells.⁸ To further identify more specific downstream targets of TGF- β and SMAD signaling, we performed RNA sequencing and bioinformatics analysis of cell lines P3 and GG16 (responding to TGF- β with MT formation) and compared the data to the nonresponder cell line GG6. TSP1 came up as an interesting candidate which was absent in the nonresponder GG6 as also verified by western blotting. Interestingly, we have previously shown that TSP1 is activated by TGF- β signaling and specifically by SMAD3 through a SMAD3 binding site in the TSP1 promoter.⁸ We also demonstrated in this study that TSP1 promotes GBM invasion downstream of TGF- β and SMAD. Thus, as invasion and MT formation are linked to each other, we demonstrated that knockdown of TSP1 inhibited invasion and MT formation both in vitro and in vivo. TSP1 has a substantial interactome that is still not fully explored consisting of extracellular matrix components, matricellular proteins, different receptors, proteases, and growth factors.^{8,30} Thus, TSP1 likely involves a potential network of additional proteins in MT formation/invasion, which need to be identified in future studies.

In conclusion, we identified TGF- β as a new mediator of MT formation through SMAD and Tsp1 signaling. Blocking this pathway might be an attractive strategy to treat GBM in the future.

Supplementary Material

Supplementary material is available at *Neuro-Oncology* online.

Keywords

glioblastoma | microtubules | SMAD | TGF- β | Tsp1

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Tumor Cell Invasion and the Tumor Microenvironment: Special Focus on Brain Tumors



Thomas Daubon, Rarahu Magaut, and Andreas Bikfalvi

1 Introduction

Brain tumors are among the most aggressive tumors of the human body [45, 62]. Gliomas mainly form from neural stem cells, then getting characteristics from astrocytes but from oligodendrocytes [29], cells which support neuronal functions and activities [61]. Gliomas are also subclassified into four malignant grades (I to IV). Grades I and II are considered low-grade gliomas, with low proliferative rate and a better clinical outcome [20]. Grades III and IV are higher-grade gliomas, with grade IV also known as glioblastoma (GBM), which have a high proliferative rate and increased tumor cell invasion into the brain parenchyma [19] (Fig. 1). Transcriptomic and genetic profiling defines three glioblastoma subgroups which include proneural, mesenchymal, and classical subgroup [65]. The general classification of central nervous tumors has been recently revised, and tumors from astrocytic and oligodendrocytic lineages are now clearly distinguished [45]. With respect of astrocytomas, they are now subdivided into diffuse, anaplastic astrocytomas and glioblastomas.

GBM usually develops from neural stem cells, getting astrocytic characteristics, which undergo genetic mutations [48]. Low-grade gliomas exhibit IDH1 (isocitrate dehydrogenase 1) mutation, found in most of low-grade gliomas and in grade III. IDH1-mutated cells form also secondary glioblastomas. This mutation redirects energy metabolism and changes the function of the enzyme, causing them to produce 2-hydroxyglutarate, a potential oncometabolite, and NADPH [11]. The starting point of cell transformation is not well known in the case of GBM. One of the potential factors is exposure to ionizing radiations [35].

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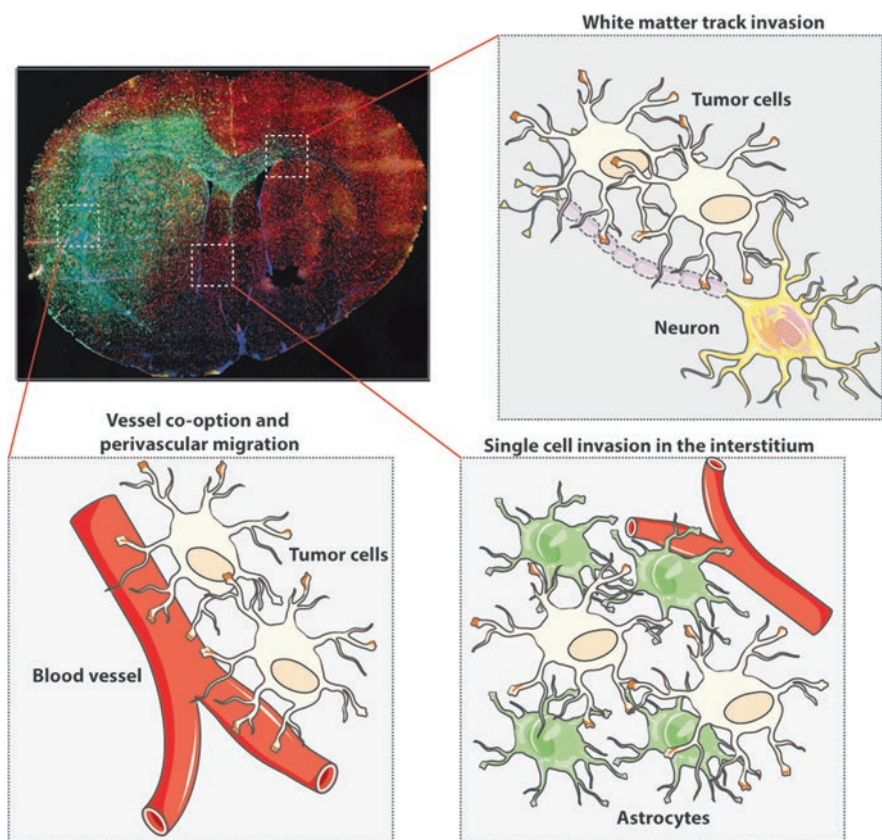


Fig. 1 The different modes of glioblastoma invasion (a) Section of a brain harboring a glioblastoma (tumor cells are in green, blood vessels in red, and nuclei in blue). (b) Invasion via white matter tracks (tumor cells are in light yellow). (c) Vessel co-option by tumor cells and perivascular invasion. (d) Invasion through the interstitial space

A high degree of invasion is one of the hallmarks of GBM, making the disease difficult to treat. Standard care consists of a large resection followed by adjuvant therapy (temozolomide) and local irradiation (Stupp protocol) which slows down tumor recurrence but only for a few extra months. Tumor recurrence occurs from invasive areas at the borders of the resection cavity. Recent advances in the understanding of GBM cell invasion emphasize the role of proteins related to the actin cytoskeleton, motility, or extracellular matrices (ECM) in this process.

This review summarizes the principal features of the invasive process in glioblastoma. The role of the tumor microenvironment will also be discussed with emphasis on its relation to the invasive process. Finally, blockade of tumor invasion, which will be shortly discussed at the end of this review, may be of therapeutic benefit for GBM patients.

2 General Features of the Invasive Process in Glioblastoma

At the contrary of all other types of cancers, metastases of glioblastoma cells are only observed in rare cases outside of the central nervous system (CNS). Indeed, some GBM nodules have been detected in the bone marrow of patients [44, 56]. GBMs have diffuse characteristics with tumor cells infiltrating the healthy surrounding brain tissue. GBM cells undergo a so-called epithelial-to-mesenchymal transition (EMT) or GMT (glioma-to-mesenchymal transition) to invade the micro-environment [39]. This transition is characterized by ECM remodeling, cytoskeleton re-patterning, and acquisition of stemlike features. Cells become migratory and invade the brain as single cells or via a process called collective migration [28]. Invading cells leave the tumor mass by loosening cell-to-cell contacts and by degrading the ECM via secretion of proteases such as MMPs (especially MMP-2 and MMP-9). After extension of a cytoplasmic protrusion and contraction of the cytoskeleton, the cell body moves toward the protrusion attachment. These protrusions resemble the membrane microtubules (MTs) described by Frank Winkler's group which were discovered to play a critical role in tumor cell invasion [52] (Fig. 2). Tumor cells connect via MT to each other at the leading tumor edge and use this to collectively invade the normal brain parenchyma [52]. The precise molecular interactions and signaling pathways for tumor cell invasion in GBM are not well understood. Rho GTPases play certainly a central role in these processes [32]. They regulate actin polymerization to form not only stress fibers, filopodia, and/or lamellipodia but also MTs or tunneling nanotubes (TNTs). Integrins are also involved to guide cell migration and invasion [17].

The tumor microenvironment critically impacts on the invasive phenotype of the tumor by triggering stimuli that modify the cellular phenotype which transition from a proliferative to a migratory state [67]. This transition is defined as the migration-proliferation dichotomy or the “Go-or-Grow” mechanism [51]. Cell metabolism may be one of the critical factors that favor a migratory phenotype. Indeed, the tumor mass is hypoxic, and acidification is present due to abnormal vascularization and a shift from oxidative to glycolytic metabolism [14]. Cells tend to evade these unfavorable conditions and invade healthy brain tissue. Another important parameter is the heterogeneity of the tumor which may select through a Darwinian process for highly migratory and invasive glioma cells [26].

Tumor cells invade the brain parenchyma via different routes defined as “secondary structures” by Scherer [60] which include white matter tracts, blood vessels, and the interstitial space between the cellular components (Fig. 1).

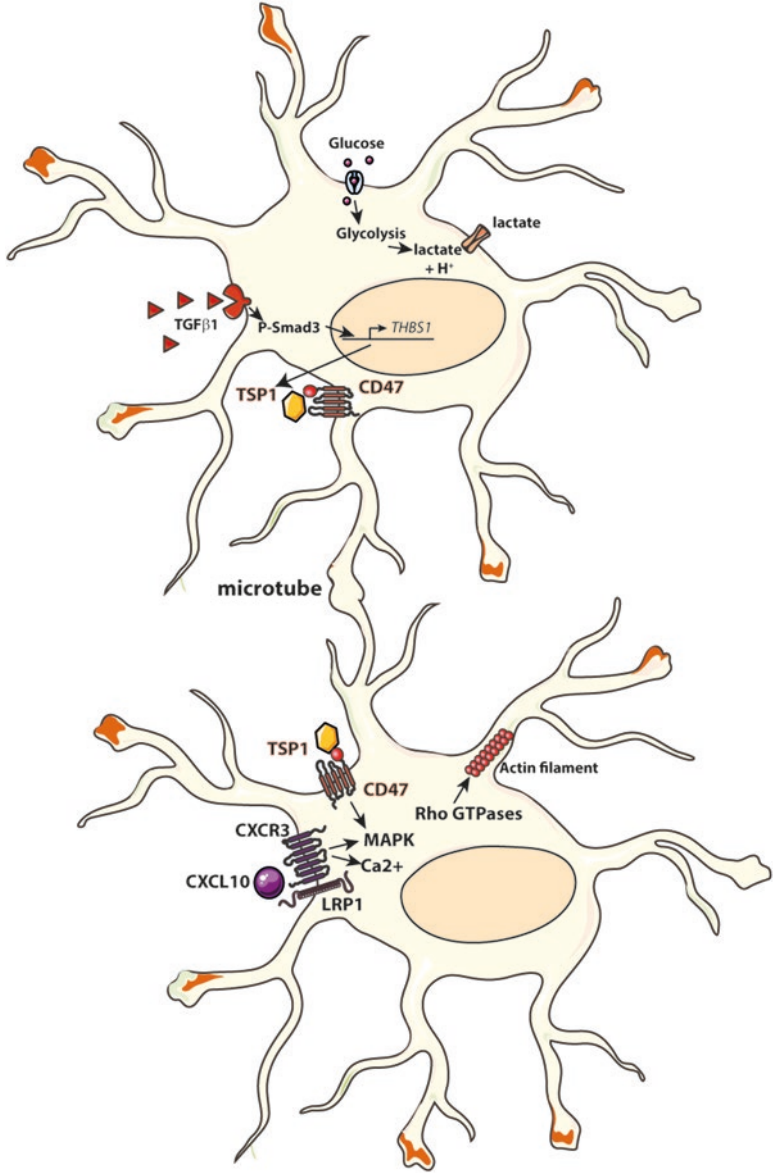


Fig. 2 Signaling mechanisms involved in glioblastoma invasion in tumor cells
The figure represents some of the major pathways. These include extracellular matrix (TSP1), chemokines (CXCL10/CXCR3), Rho GTPases and actin cytoskeleton remodeling, and cell metabolism (glycolysis and lactic acid)

3 Invasive Mechanisms at the Level of Tumor Cells

What are the molecular mechanisms operating at the level of tumor cells that promote tumor cell invasion? The chemokine network may contribute to tumor cell invasion. It has been shown in our laboratory that CXCR3 via CXCL10 binding is involved in promoting GBM invasion and that a cross-talk with LRP1 is involved [5] (Fig. 2). Furthermore, the CXCL12/CXCR4 axis seems also to contribute to invasion of GBM cells [15].

Another important player is the MET/VEGFR2 complex [46]. It has been shown that MET activation in glioblastoma promotes tumor cell invasion and is also linked to therapeutic resistance to angiogenic drugs. In presence of VEGF, c-MET is dephosphorylated and inactive. Inhibition of VEGF binding to VEGFR2 by bevacizumab, an anti-VEGF antibody, abrogates c-MET dephosphorylation and leads to full activation. Bevacizumab has also been described to modify tumor cell metabolism with a shift from oxidative metabolism to glycolysis which may promote tumor cell invasion [22, 42]. Other factors involved in the regulation of the invasive process via cell metabolism are glutamate and lactic acid. The neurotransmitter glutamate released from glioma cells into the extracellular space has been shown to induce, at high concentrations, neuronal cell death, which paves the way for cells to invade the healthy brain parenchyma [16, 63]. Inhibition of monocarboxylate transporters (MCTs), which impairs the efflux of lactic acid, was reported to interfere with glioblastoma invasion [12] (Fig. 2).

Wingless/Int1 (Wnt) signaling has also been shown to regulate glioblastoma invasion through the canonical β -catenin pathway, which is overexpressed in many GBMs [54]. Inhibition of the PI3K/Akt pathway, which is also linked to β -catenin activation, interferes with GBM invasion by decreasing MMP2 activity [31].

Rho GTPases play a central role in glioblastoma invasion, by regulating actin polymerization (Fig. 2). Activated Cdc42 determines the invasive phenotype of malignant gliomas mediated through its interaction with IQGAP1 (IQ motif-containing GTPase-activating protein 1) and phosphorylated FAK (focal adhesion kinase) [50]. In our laboratory, we demonstrated that a particular Rnd protein, known as RhoE, drives glioma cell invasion and apoptosis in a chicken chorioallantoic membrane model [9]. Microtubes are composed of actin filaments and microtubules which critically impact the glioblastoma cell phenotype and invasion [41, 52]. How microtubes influence tumor cell invasion is, at present, not known. One might speculate that these structures are required for the coordination of the invasive behavior of glioma cells at the tumor edge through exchanges of biochemical or electric signals. Recent articles showed that Rho GTPase effectors play a central role in tumor cell shape and motility. A mammalian Diaphanous (mDia)-related formin family inhibitor reduces invasion in a brain tumor neuro-sphere model by inducing an amoeboid morphology and by inhibiting microtube formation [53].

4 Brain Tumor Blood Vessels, Vessel Co-option, and Invasion

The brain tumor vasculature may also significantly impact the invasive process. Gliomas have a mature preexisting vasculature but also harbor aberrant immature newly formed vessels. Angiogenic brain tumor blood vessels are enlarged, tortuous, and leaky. Vessel branching is abnormal and arteriovenous shunts can be observed [27]. This leads to abnormal tumor perfusion and a high level of hypoxia in the tumor microenvironment. Gliomas initially grow along the native brain vasculature in a process called vascular co-option (Fig. 1). This is associated with an increase in angiopoietin-2 (ANG-2) levels in the brain vasculature [58]. ANG-2 induces vessel destabilization which results in tumor hypoxia with the direct consequence to increase of HIF1 α stabilization. This leads to upregulation of proangiogenic factors by glioma cells such as vascular endothelial growth factor (VEGF) [24]. Sprouting of new vessels, promoted by the interaction of VEGF and destabilizing factors (Ang2), then occurs. Another important factor is the stromal-derived factor-1 α (SDF-1 α) [25]. SDF-1 α is a chemoattractant for endothelial cells which also promotes capillary sprouting and branching. VEGF expression is not only dependent on HIF-1 regulation but also peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) through the estrogen-related receptor α (ERR- α) [64]. Growth factors such as transforming growth factor- β (TGF- β) or PDGFs have vascular tropism. TGF- β controls many other cellular pathways and induces changes in ECM composition [7, 34, 59]. Furthermore, TSP1 is found both vessel-associated and tumor cell-associated. Vessel-associated TSP1 seems to have an antiangiogenic function [1], whereas tumor-associated TSP1 regulates invasion [18] (for details of tumor cell-associated functions, see next paragraph).

In gliomas, two other alternative modes of neovascularization have been recognized: pseudotumor vessels made of tumor cells only (vascular mimicry) or vessels generated by trans-differentiation of tumor cells into endothelial cells or pericytes [2]. The reality of these two different vessel types is still matter of debate, but they may be observed in treatment-resistant high-grade glioma. How this is linked to tumor invasion remains to be determined.

The vasculature may intervene in tumor cell invasion via two independent routes. The first is through the action of preexisting blood vessels. Indeed, it has been shown that vascular cells can produce soluble mediators such as bradykinins to attract tumor cells [49]. The second is through the action of deficiency of newly formed blood vessels in specific tumor areas promoting hypoxia which indirectly impacts tumor cells by promoting their migratory behavior.

Taken together, the following conclusions of how the vasculature intervenes in tumor cell invasion can be drawn. First, the vasculature represents a scaffold on which from the beginning of tumor development tumor cells seed and migrate. Second, the defect in a functional vasculature increases hypoxia and the cellular stress response which favors cell migration. Third, at later stage vessels may be used as highways for tumor cell invasion into the healthy brain tissue. Fourth, the

vasculature provides trophic factors which attract tumor cells and favor their migratory potential.

5 Immune System and Tumor Cell Invasion

Bone marrow-derived tumor-associated macrophages (TAMs) are recruited by monocyte chemoattractant protein-1 (MCP-1 or CCL2) produced by glioma cells. TAMs, or more precisely GAMs (for glioma-associated macrophages), not only promote neovascularization, but they also act directly on tumor cells by modulating proliferation, invasion, and tumor immunity [33]. Inhibition of CSFR1 receptor activation in TAMs and microglia has been carried out in a mouse proneural GBM model [55]. This dramatically slowed down growth and invasion of intracranially implanted patient-derived glioma xenografts. The authors proposed the CSF-1R inhibition as a promising therapeutic strategy for proneural gliomas. Hypoxia promotes a pro-tumor TAM phenotype which leads to the secretion of extracellular matrix (ECM) degrading factors (metalloproteinases, MMPs) or angiogenic factors (VEGF, etc.) as well as of tumor cell-promoting factors such as transforming growth factor- β (TGF- β) or interleukin-10 (IL-10) [13].

6 Invasion of White Matter Tracts by Glioma Cells

Glioblastoma cells not only invade the perivascular space [29] and meninges but also migrate along white matter tracts (Fig. 1). Surprisingly, only little is known about invasive processes along white matter tracks [68]. In invasive glioblastomas, extensive demyelination is observed which is due to the invading tumor cells and to edema as a consequence of inflammation [66]. It is to mention that neurons provide input in the glioblastoma microenvironment via neuroligin-3 which promotes tumor growth and white matter tract invasion [40]. An additional support for white matter track invasion is provided by the ECM located in the vicinity of white matter tracts, which is composed of hyaluronic acid, tenascin R, and lecticans [57].

7 Role of Tumor-Associated Astrocytes in Glioblastoma Invasion

Astrocytes compose half of the total number of cells in mammalian brains and play an important physiological role in the central nervous system (CNS) [37]. Astrocytes support the neuronal network by dividing gray matter into distinct domains and increase the myelinating capacity of white matter [6]. It has been demonstrated that

astrocytes participate in the formation and the maintenance of neural circuits, commonly defined as tripartite synapses [10]. Astrocytes have a complex morphology, with the cell membrane constituted by fine processes with dimensions on the tens of nanometer scale and a high surface-to-volume ratios [70]. Astrocyte end-feet are part of the neurovascular unit (NVU) and promote cell-to-cell communication, by sensing the level of glutamatergic activity in the synapses [8]. Astrocytes are able to change their phenotype to become reactive astrocytes which are characterized by a higher proliferation rate, increase of cell size, and upregulation of nestin, vimentin, and GFAP [4, 30]. Moreover, activated astrocytes release chemokines, cytokines, and many other molecules, increasing the neuroinflammatory responses [30]. Reactive astrocytes are also found in the GBM microenvironment, adjacent to the tumor mass [36, 38]. They can interact directly with glioblastoma cells, through tunneling nanotubes (TNTs) [69] or via microtubes (MTs) [52]. Reactive astrocytes secrete growth factors and cytokines including interleukin-6 (IL-6), interleukin-19 (IL-19), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and transforming growth factor- β (TGF- β), as well as chemokines, such as CXCL10, which support tumor cell invasion and tumor growth [21, 38].

8 Role of the Extracellular Matrix in Tumor Invasion

ECM constitutes a noncellular component of the microenvironment and functions not only as a physical scaffold but also as a source of biochemical signals [67]. Quantity and quality of the composition of the ECM in the brain differs from other organs and represents around 20% of the tissue volume [71]. The ECM is composed of collagens, laminins, glycoproteins, and proteoglycans, as well as glycosaminoglycans [3]. In the brain parenchyma, the ECM consists more specifically in a complex arrangement of hyaluronic acid (HA), proteoglycans, and lecticans and also includes matricellular proteins such as thrombospondins, tenascins, or fibronectin [47].

The role of extracellular matrix (ECM) in glioblastoma invasion was recently studied by several groups including ours [18, 23, 43] (Fig. 2). Our work was focused on TSP1, which, initially, was primarily characterized as for its antiangiogenic properties [1]. However, we demonstrated that, in glioblastomas, tumor cell-associated TSP1 promotes contralateral invasion in mouse models. Inhibition of TSP1 expression or interaction with CD47 impairs tumor growth and invasion. Furthermore, downregulation of CD47 in tumor cells recapitulates this phenomenon [18]. This indicates that CD47 is not only important for inhibiting immune response (as a “don’t eat me signal”) but also directly plays an important role in tumor invasion via TSP1 binding.

9 Conclusion

The invasive behavior of glioblastoma is controlled by tumor cell intrinsic mechanisms such as activation of chemokine/cytokine receptors which stimulates cell motility through cytoskeleton-associated signaling molecules (Rho/Rac). Furthermore, there are several environmental cues that favor or modulate tumor cell invasion. These cues are derived from the vasculature, the extracellular matrix, or the surrounding cellular tumor environment which includes astrocytes, macrophages, and the microglia. This environment secretes an array of molecules including bradykinin (vascular-derived), CXCL10 (astrocytic-derived), CXCL12 (macrophage-derived), and many other growth factors and cytokines which then promote tumor cell invasion. Metabolism driven by tumor hypoxia will also select for GBM cells with increased migratory potential.

How this knowledge can be translationally applied in a therapeutic setting? It is clear that the first therapeutic intervention is surgery and radiotherapy. Anti-invasive therapy could be initiated already before surgery provided that adequate drug formulations exist that can reach the tumor when applied systemically. One can also block invasion by directly applying the drug inside the surgical cavity. However, suitable drug candidates with an adequate pharmacology have not yet been developed which should be the first aim to efficiently block tumor cell invasion in GBM.

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GAP43-dependent mitochondria transfer from astrocytes enhances glioblastoma tumorigenicity

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The transfer of intact mitochondria between heterogeneous cell types has been confirmed in various settings, including cancer. However, the functional implications of mitochondria transfer on tumor biology are poorly understood. Here we show that mitochondria transfer is a prevalent phenomenon in glioblastoma (GBM), the most frequent and malignant primary brain tumor. We identified horizontal mitochondria transfer from astrocytes as a mechanism that enhances tumorigenesis in GBM. This transfer is dependent on network-forming intercellular connections between GBM cells and astrocytes, which are facilitated by growth-associated protein 43 (GAP43), a protein involved in neuron axon regeneration and astrocyte reactivity. The acquisition of astrocyte mitochondria drives an increase in mitochondrial respiration and upregulation of metabolic pathways linked to proliferation and tumorigenicity. Functionally, uptake of astrocyte mitochondria promotes cell cycle progression to proliferative G2/M phases and enhances self-renewal and tumorigenicity of GBM. Collectively, our findings reveal a host–tumor interaction that drives proliferation and self-renewal of cancer cells, providing opportunities for therapeutic development.

Mitochondria are vital in cell metabolism through their role in generating ATP via oxidative phosphorylation; yet, they have also been implicated in numerous other cellular functions, including apoptotic cell death, inflammation, stem cell differentiation and autophagy^{1,2}. After the discovery of the Warburg effect identifying conversion of glucose to lactate in the presence of oxygen to fuel cancer growth³, aerobic glycolysis has been assumed to be the main energy pathway for cancer cells⁴, including in glioblastoma (GBM)⁵, the most common primary adult brain tumor. However, recent reports suggest a more complex situation where mitochondrial respiration is also an alternative energy source^{6,7}. The existence of multiple metabolic phenotypes is in line with the cellular heterogeneity of this disease^{8–10}.

While this heterogeneity may arise from variation in cell-intrinsic metabolic regulation, it may also arise via interaction with the tumor microenvironment (TME), namely by transfer of metabolic signals,

including mitochondria themselves, from the TME to tumor cells. In many contexts, dynamic microenvironmental interactions have been shown to be major drivers of tumor growth and therapeutic resistance in GBM^{11–13}, while the underlying direct cell–cell communication mechanisms remain poorly understood.

Transfer of mitochondria has been demonstrated between different cell types and via different routes. Mitochondria transfer by extracellular vesicles from astrocytes to neurons after stroke has been documented in vitro and in vivo¹⁴, identifying a CD38/cADP-dependent mechanism using calcium signaling. Extracellular vesicles as a mode of mitochondria transfer has also been shown for mesenchymal stem cells¹⁵, indicating that transfer by secretion is a viable route for this organelle. In other studies, mitochondria transfer mechanisms independent of secreted particles have been proposed^{16–18}. Intercellular membrane protrusions known as tunneling nanotubes have been

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implicated in organelle transport¹⁹ and more recently in mitochondria transfer.^{16–18} By connecting cells over distances that may exceed 100 μm (refs. 20,21), tunneling nanotubes are prime avenues for intercellular cross-talk²²; composed of components like actin and microtubules²², they maintain a close cytoskeletal relationship²².

Tumor microtubules (MTs) are similar structures; however, they are thicker and more stable than tunneling nanotubes and can reach lengths of more than 500 μm in vivo²³. MTs have so far only been described in glioma, particularly in GBM, and have been identified as an important mode of intercellular communication, invasion and therapy resistance^{24–27}. Growth-associated protein 43 (GAP43), a neuronal protein known for its important role in axon guidance²⁸, is a major structural protein of MTs. Knockdown of GAP43 in GBM cells reduced MT formation and tumorigenicity in vivo²⁴. This work highlighted the relevance of the interconnected network of GBM cells formed by GAP43⁺ MTs for tumor growth. In a recent study, mitochondria were reported to be exchanged in vitro among GBM cells interconnected via MTs²⁹.

Given the reports of horizontal mitochondria transfer in the brain across distinct cell types under pathologic conditions¹⁴, we hypothesized that GBM also acquires functional mitochondria from non-malignant cells in the TME, comprising an unexplored layer of metabolic heterogeneity. Here, we show that GBM cells acquire host mitochondria from astrocytes through a contact-dependent mechanism facilitated by GAP43⁺ structures consistent with MTs, resulting in enhanced metabolic activity and augmented tumorigenicity. Our findings identify mitochondria transfer from the TME as a fundamental protumorigenic mechanism in GBM.

Results

GBM acquires mitochondria from the microenvironment

To assess whether non-malignant host cells could transfer mitochondria to GBM in vivo, we orthotopically implanted green fluorescent protein (GFP)-expressing syngeneic mouse GBM models (SB28 and GL261) into transgenic C57BL/6 mice expressing a mitochondria-localized mKate2 fluorophore fused to the localization peptide of cytochrome c oxidase 8 (mito::mKate2 mice³⁰; Fig. 1a). Confocal microscopy of GBM tumors from mito::mKate2 mice revealed mKate2⁺ puncta within 15–60% of GFP⁺ GBM cells (Fig. 1b–f and Extended Data Fig. 1), demonstrating that host cell mitochondria were acquired in vivo by orthotopic GBM tumor cells. By co-staining tissue sections with wheat germ agglutinin (WGA) to highlight glycoprotein-rich structures³¹, we observed host mKate2⁺ mitochondria in transit at host–tumor interfaces along intercellular connections between GFP⁺ GBM cells and GFP⁺ host cells (Extended Data Fig. 1). To determine whether mitochondria transfer occurred in the context of human GBM in vivo, we first injected a high-titer mitoDsRed lentivirus into the brain of nude rats to transduce normal brain cells with a mitochondria fluorescent tag. After 7 d, P3 GFP⁺ human-derived GBM stem-like cells (GSCs) were injected in the same location (Fig. 1g). High-resolution confocal images verified mitochondria transfer from stromal cells to tumor cells, supporting our mouse in vivo data (Fig. 1g). When reconstructed in three dimensions, the mitoDsRed signal was present within acceptor cells positive for GFP. We verified

these results in a second human-derived GSC line GG16 transduced with mito-GFP and by immunostaining with a human-specific nestin antibody (Extended Data Fig. 2).

Having observed mitochondria transfer from the TME to mouse and human GBM models, we proceeded to interrogate the identity of the host mitochondria donor cells. GBM tumors in mouse models and humans are known to have substantial infiltration by both brain-resident glia and peripheral immune cells that transmigrate into the TME¹³. We established orthotopic GBM tumors in wild-type C57BL/6 mice that had first received lethal irradiation with subsequent bone marrow reconstitution from mito::mKate2 mice (mito::mKate2 $\rightarrow$ WT) to restrict mito::mKate2 expression to bone marrow-derived immune cells (Extended Data Fig. 3a). Analysis of single-cell suspensions by flow cytometry indicated negligible host mitochondria transfer to GFP⁺ GBM cells in mito::mKate2 $\rightarrow$ WT mice, while 20–60% of GFP⁺ GBM cells were mKate2⁺ in mito::mKate2 mice (Extended Data Fig. 3b,c). Taken together, these data suggest that brain-resident cells, not tumor-infiltrating immune cells, were the major donors of mitochondria to GBM cells in vivo.

To further elucidate the identity of the predominant mitochondria donor cell populations, we cocultured prevalent tumor-infiltrating cell types with GFP⁺ GBM cells at a 1:2 donor:recipient ratio. After 2 h, we assessed the percentage of mKate2⁺ cells as a marker of mitochondria transfer (schematized in Fig. 2a). Consistent with our in vivo observations, we found that brain-resident glia (astrocytes and microglia) donated significantly more mitochondria on a per-cell basis than bone marrow-derived macrophages, with astrocytes having higher transfer rates (Fig. 2b and Extended Data Fig. 4a). While further polarization of macrophages to an M2- or M1-like phenotype potentially favored increased mitochondria transfer, the degree of transfer was, on average, five- to tenfold less than that observed with the brain-resident glia in vitro (Extended Data Fig. 4b).

Given our observation that astrocytes displayed high rates of mitochondria transfer to mouse models of GBM and their previously described interconnected nature with GBM in vivo³², we focused our subsequent studies on this glial cell population. We began by coculturing either mito-mCherry or mitoDsRed immortalized human astrocytes with six different human-derived GSCs (L1, DI318, 3832, GG16, P3 and BG5) for 3–4 d to interrogate the applicability of our findings in human models of disease. As with mouse GBM models, we found that human-derived GSCs acquired mitochondria from astrocytes, with flow cytometry revealing a transfer rate of 5–40% (Fig. 2c,d and Extended Data Fig. 4c). We confirmed the internalization of astrocyte mitochondria by confocal microscopy of the GSCs, whereby the fluorescent protein-tagged mitochondria were visualized entirely within GSCs with cytoplasm prelabeled with CellTrace dye or expressing GFP (Extended Data Fig. 4d,e). We confirmed that astrocyte-to-human GBM transfer of mitochondria was relevant in vivo using the human orthotopic GBM model (P3) derived from Fig. 1g. We identified mitoDsRed⁺ astrocytes (glial fibrillary acidic protein (GFAP⁺) and GFP⁺) surrounding GFP⁺ GBM cells with transferred mitoDsRed⁺ mitochondria (Fig. 2e and Extended Data Fig. 4f). ImageStream analysis on a distinct set

Fig. 1 | GBM cells acquire host mitochondria from the TME. **a**, GFP-expressing GL261 and SB28 cells were implanted intracranially into wild-type (WT) and mito::mKate2 (mK) mice, and tumors were analyzed at humane endpoint. **b–e**, Single focal planes (xy) and z-stack orthogonal reconstructions (xz, yz) at areas of SB28 (**b,c**) and GL261 (**d,e**) tumor–host cell interfaces. Yellow arrowheads indicate host mKate2⁺ mitochondria (Mito) within recipient tumor cells. Cyan arrowheads indicate WGA-labeled tether-like structures connecting tumor and host cells. **f**, Three-dimensional confocal imaging segmentation-based estimation of mKate2⁺GFP⁺ GBM cell frequency from two to three visual fields from $n = 3$ (SB28) and $n = 2$ (GL261) WT and $n = 3$ (SB28) and $n = 4$ (GL261) mK mice; *** $P = 0.0003$ (SB28) and $P < 0.0001$ (GL261). Data were analyzed by two-tailed t -test. See also Extended Data Fig. 1 for additional data, including technical

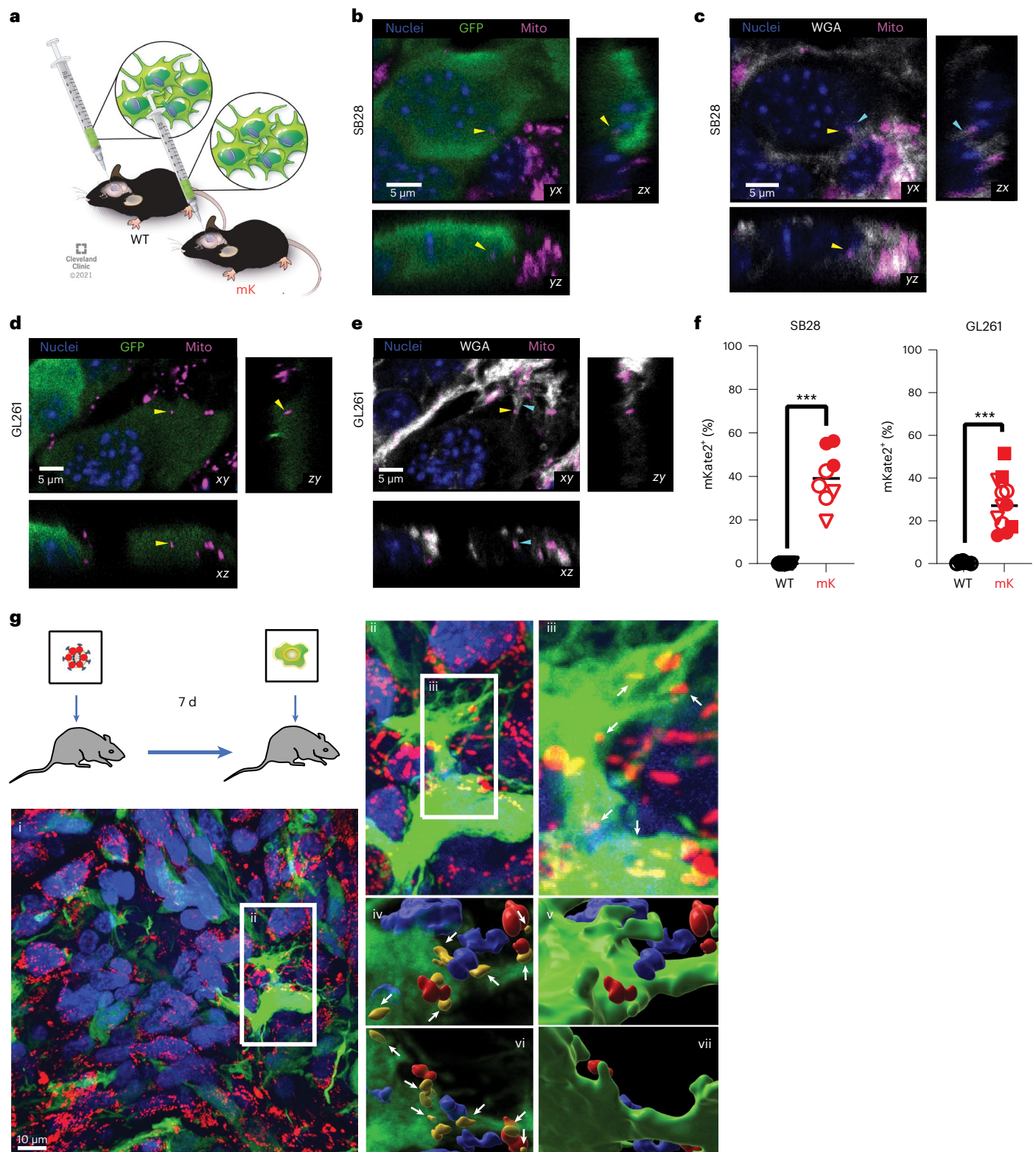
controls. **g**, Mitochondria transfer between the TME (mitoDsRed⁺) and human GBM cells (GFP⁺) in vivo. MitoDsRed lentivirus was injected into the brains of nude rats. Seven days later, human P3 GFP⁺ GSCs were injected into the same area. Confocal microscopy images of P3 GFP⁺ xenograft tumors are representative of at least six $\times 100$ images across three biologically independent animals. Details of the area along the tumor surface are shown in (i), with colocalization of GFP⁺ and mitoDsRed⁺ signal in (ii) and (iii). A 3D reconstruction of the mitoDsRed⁺ mitochondria (white arrows) seen from above, without (iv) and with (v) the GFP⁺ cell borders, is shown. From below, the mitoDsRed⁺ mitochondria are also visible in (vi) and reside within the cell in (vii). ii, $\times 1.5$ magnification; iii–vii, $\times 3$ magnification.

of human-derived co-culture models, where donor astrocytes were tagged with mito-GFP and GBM recipients with red-fluorescent protein (RFP), revealed similar results with internalized donor mitochondria in recipient cells (Extended Data Fig. 4g–i). Finally, we confirmed that transferred mitochondria labeled with mito-mCherry expressed the mitochondrial protein colocalizer of outer mitochondrial membrane 20 (TOMM20) by colocalization analysis, and the protein was distributed within the mitochondrial network of the receiving GBM

cell (Extended Data Fig. 5 and Supplementary Video 1 and 2). In summary, we observed mitochondria transfer from the TME to GBM *in vivo* and *in vitro* and identified astrocytes as a major mitochondria donor.

GAP43⁺ MTs facilitate mitochondria transfer

We considered that mitochondria transfer could occur via secretion¹⁴ and/or cell contact^{24,33}. To discriminate between the two mechanisms, we compared the mitochondria transfer rate of co-cultures to that of



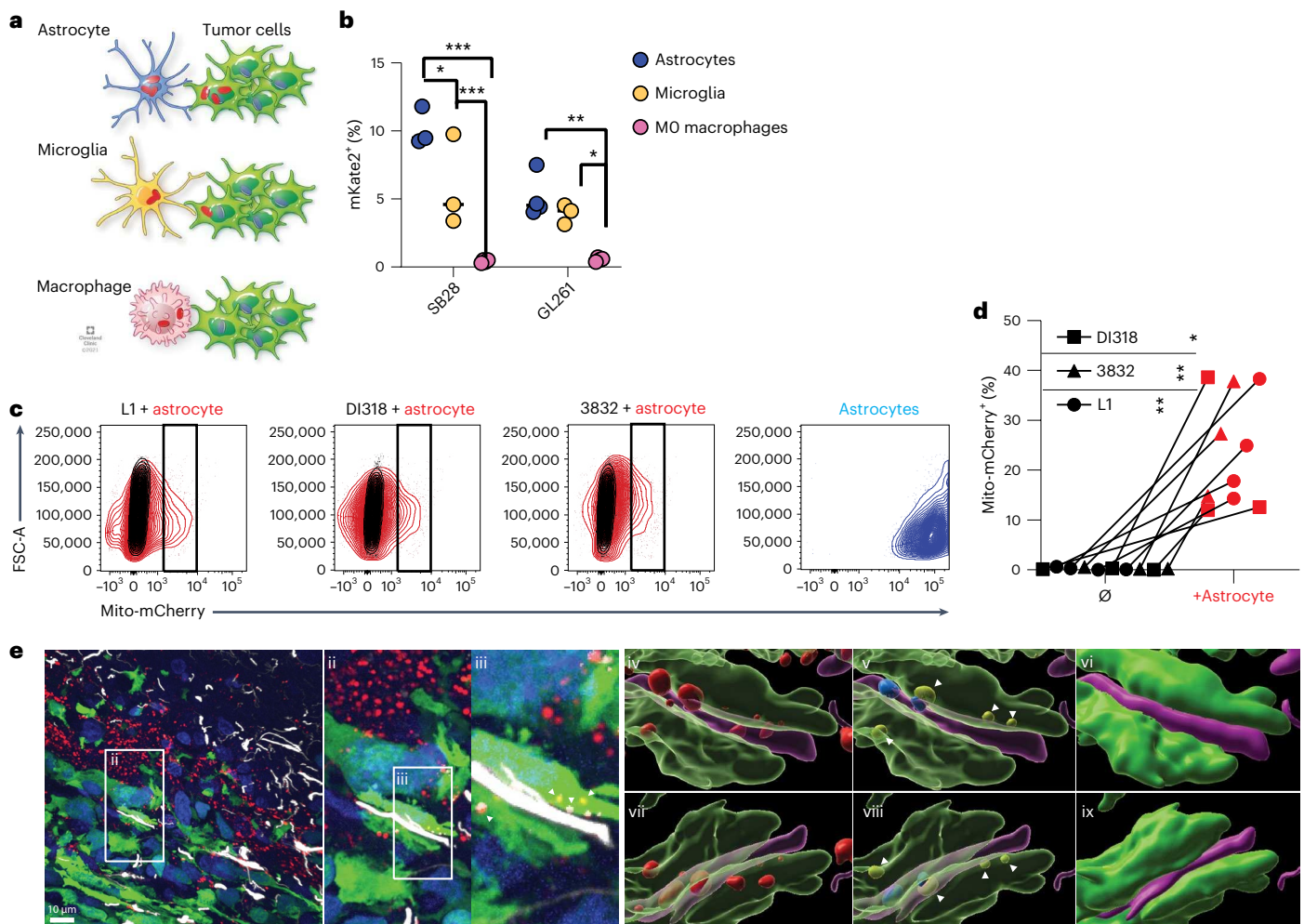


Fig. 2 | GBM cells acquire mitochondria from astrocytes. **a**, GBM cells were cocultured with astrocytes, microglia or macrophages from mK mice for 2 h, and mitochondria transfer was analyzed by flow cytometry. **b**, Relative frequency of mKate2⁺GFP⁺ GBM cells in co-cultures; $n = 3$ (SB28) and 4 (GL261) independent experiments; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Data were analyzed by two-way analysis of variance (ANOVA). **c**, **d**, Human-derived GSCs were cocultured with immortalized mito-mCherry⁺ human astrocytes for 4 d. Mito-mCherry⁺ GSCs (black rectangle gate) were quantified by flow cytometry (**c**), as summarized in **d**; $n = 3$ (3832), 3 (DI318) and 4 (L1) independent experiments. Data were analyzed by two-way ANOVA; $P = 0.006$ (L1), 0.03 (DI318) and 0.007 (3832). **e**, Mitochondria transfer between astrocytes (mitoDsRed⁺ and GFAP⁺) and human GBM cells (GFP⁺) in vivo. Confocal microscopy of a GFP⁺ P3 xenograft tumor

immunostained with antibodies to GFAP (white color) was used to visualize astrocytes. Images are representative of at least six $\times 100$ images across three biologically independent animals. Mitochondria transfer is highlighted in an invasive tumor area with colocalization of GFP⁺ and mitoDsRed⁺ signal in (i). Images in (ii) and (iii)–(ix) represent $\times 1.5$ and $\times 3$ magnifications, respectively. A 3D reconstruction of the mitoDsRed⁺ mitochondrial signal both within and around the GFP⁺ and GFAP⁺ surfaces is shown in (iv). The mitoDsRed⁺ mitochondria colocalized within the GFP⁺ tumor cell borders (yellow) and within the purple reconstructed GFAP⁺ astrocytic processes (blue), seen from above without (v) and with (vi) GFP⁺ and GFAP⁺ cell borders. From below, mitoDsRed⁺ mitochondria are also visible in (vii) and reside within the GFP⁺ and GFAP⁺ regions in (viii) and (ix).

transferred donor-conditioned medium (mouse models; Extended Data Fig. 6a,b) or cells separated by a 5- μ m porous transwell insert (human models; Fig. 3a). Regardless of the separation method, transfer primarily occurred when donor and recipient cells were in physical contact, and there was only low-level transfer by secretion that was near the limit of detection of our assays. Live confocal microscopy of mitochondria transfer from astrocytes to mouse GBM cells was also visualized in adjacent cells (Extended Data Fig. 6c and Supplementary Video 3). Transfer was abrogated in both mouse and human models when co-cultures were incubated at 4 °C (Extended Data Fig. 6d,e), suggesting that this was an active, energy-dependent process rather than a passive event. Taken together, these results indicate that active physical interaction of tumor and donor cells is required for effective mitochondria transfer, in line with imaging of in vivo brain tumor models (Figs. 1 and 2 and Extended Data Figs. 1 and 2).

MTs are established conduits of intercellular communication and network formation in GBM and, thus, are also potential mediators of mitochondria transfer^{24,32,34–36}. To visualize mitochondria within the interfaces of GBM cells, we performed confocal microscopy of co-cultures immunostained for TOMM20 and actin. In addition to a perinuclear localization, mitochondria were found in MT connections between tumor cells (P3 model; Fig. 3b), consistent with a previous study reporting that mitochondrial components localize in between astrocyte and GBM cell connections in vitro³⁷. Their intratubular location was confirmed by z stack. Importantly, confocal microscopy of mitoDsRed⁺ astrocytes and GFP⁺ P3 co-cultures immunostained with actin showed MTs with mitoDsRed⁺ mitochondria connecting both cell types. These were present in the vicinity of transferred mitochondria (Fig. 3c). We also found astrocytic mitoDsRed⁺ donor mitochondria shuttling between connected tumor acceptor cells, indicating that

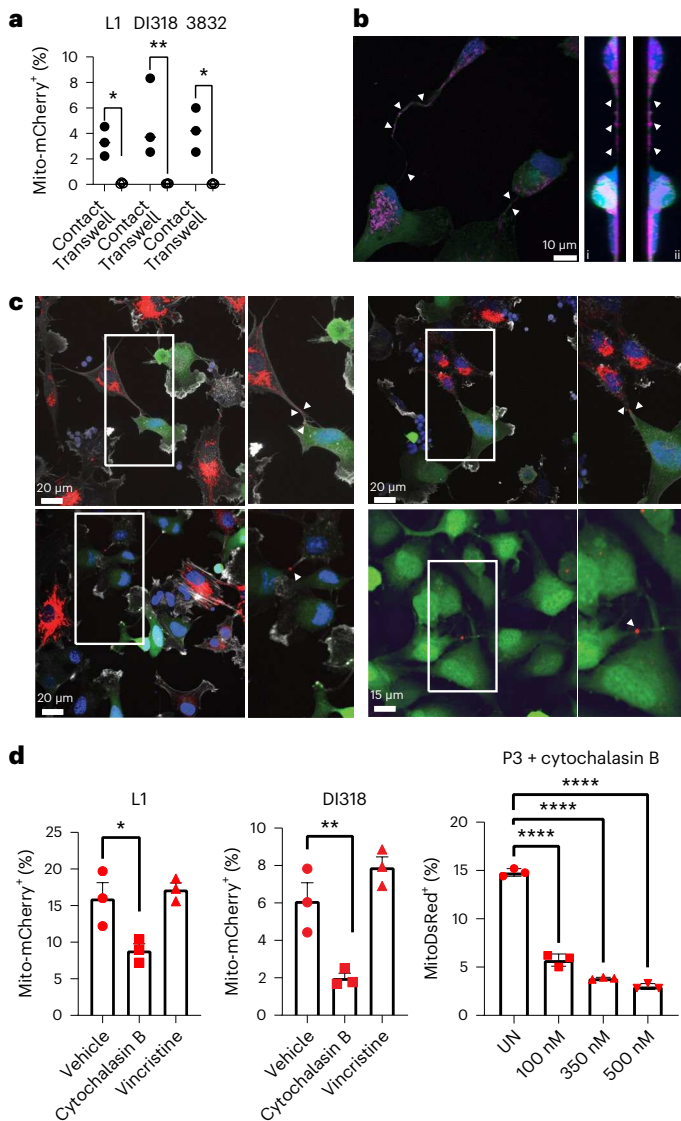


Fig. 3 | Astrocytes transfer mitochondria to GBM via actin-based intercellular connections. **a**, Human-derived GSCs (L1, DI318 and 3832; bottom) and astrocytes (top) were separated from each other with 5- μ m porous transwell inserts. Twenty-four hours later, mitochondria transfer was analyzed based on mito-mCherry signal; $n = 3$ independent experiments; $P = 0.02$ (L1), 0.007 (DI318) and 0.01 (3832). Data were analyzed by two-way ANOVA. **b**, Presence of mitochondria within MTs connecting P3 GFP⁺ cells. Immunostaining with TOMM20, representative of at least eight $\times 100$ images across three biologically independent co-cultures, is shown. Intratubular location is confirmed by z stacking, seen from the left in (i) and the right in (ii) side. Arrows indicate TOMM20⁺ signal. **c**, Co-culture between mitoDsRed⁺ astrocytes and GFP⁺ P3 cells immunostained with F-actin (white) to visualize membrane extensions connecting the two cell types, emphasized with high magnification. Images are representative of at least 20 $\times 60$ images across three biologically independent co-cultures. Arrows indicate mitoDsRed⁺ mitochondria in intercellular connections and transferred mitoDsRed⁺ mitochondria. MitoDsRed⁺ mitochondria are also observed in MT connections between two tumor cells, confirming the exchange of mitochondria within the whole network of tumor–tumor/tumor–astrocyte connections. Insets: $\times 1.5$ magnification. **d**, Human-derived GSCs were cocultured for 24 h with immortalized mito-mCherry⁺ and mitoDsRed⁺ human astrocytes in the presence of actin (cytochalasin B) or microtubule (vincristine) polymerization inhibitors or vehicle control (UN). The frequency of mito-mCherry⁺ or mitoDsRed⁺ GSCs was assessed by flow cytometry; $n = 3$ independent experiments. Data are presented as mean $\pm$ s.e.m. (L1 and DI318) and mean $\pm$ s.d. (P3); $^*P = 0.03$ (L1), $^{**}P = 0.01$ (DI318), $^{***}P < 0.0001$ (P3). Data were analyzed by one-way ANOVA with Holm–Sidak multiple comparison correction.

astrocytic mitochondria can be further exchanged within the complete network of tumor–tumor connections (Fig. 3c). After visualizing mitochondria in transit along intercellular connections containing actin filaments, we hypothesized that the actin cytoskeleton was critical in facilitating mitochondria transfer. Indeed, inhibition of actin polymerization by cytochalasin B resulted in a significant decrease in transfer rate, without an effect on cell viability (Fig. 3d and Extended Data Fig. 6f–h). By contrast, inhibition of tubulin polymerization with vincristine had no effect on mitochondria transfer (Fig. 3d).

Previous work showed that GAP43 facilitated the formation of an interconnected network of GBM cells in vivo, which enabled connexin 43-mediated propagation of calcium waves²⁴. GAP43 is an actin-associated protein that facilitates neurite outgrowth via growth cones^{38,39}. We visualized that GAP43 also localized to the cellular projections of GBM cells and astrocytes in co-culture (Fig. 4a). When we knocked down the expression of GAP43 in human-derived GSCs, we observed a decrease in the number of cellular projections (Fig. 4b,c and Extended Data Fig. 6i), as previously reported^{24,34}. Importantly, we found that mitochondria transfer from astrocytes was significantly decreased when GAP43 was knocked down in GSCs alone or in both GSCs and astrocytes (Fig. 4d,e and Extended Data Fig. 6i,j). These data identify a role for GAP43 in facilitating mitochondria transfer from astrocytes to GBM.

Astrocyte mitochondria metabolically reprogram GBM cells

We hypothesized that receiving entire organelles from astrocytes would have biologically important downstream functional sequelae in recipient GBM cells. We first interrogated how mitochondria transfer affected cells transcriptionally by bulk RNA sequencing (RNA-seq) of sorted mouse GBM cells with (mKate2⁺) or without (mKate2⁻) astrocyte mitochondria acquisition from co-cultures (Extended Data Fig. 7a). Unsupervised clustering of RNA-seq data revealed that tumor cells had a distinct gene expression profile compared to astrocytes, confirming that sorted mKate2⁺ SB28 cells were not meaningfully contaminated by astrocytes from the co-culture (Extended Data Fig. 7b–d). Genes that were upregulated >1.5 -fold in mKate2⁺ tumor cells compared to mKate2⁻ tumor cells (Extended Data Fig. 7e,f and Supplementary Table 1) were enriched within pathways related to mitochondrial biology, in particular electron transport and mitochondrial organization (Extended Data Fig. 7a).

Given the central role of mitochondria in ATP production and the results of our RNA-seq analysis, we investigated whether an increase in functional mitochondria through transfer causes a measurable change in metabolic parameters, in particular oxygen consumption rate. We performed a mitochondrial stress test on GFP⁺ acceptor cells (P3 model) with high and low mitochondria transfer from mitoDsRed⁺ astrocytes compared to control cells from the same co-cultures using the Seahorse system. After measuring the basal respiration rate, we sequentially added electron transport chain inhibitors to evaluate the changes in maximal respiration capacity. Interestingly, both basal respiration and maximal respiration rate were increased in accordance with mitochondria transfer (Fig. 5a–c). When dividing the cells into metabolic subgroups based on basal respiratory and glycolytic rates, cells with the highest mitochondria transfer were more aerobic and energetic than cells with lower transfer and controls (Fig. 5d). We further characterized the metabolic profile of a panel of GSCs after co-culture with human astrocytes by adapting a previously reported metabolic flow cytometry panel⁴⁰. The mitochondrial ATP synthase subunit ATP5A was among the most consistently upregulated metabolic proteins in recipient GSCs (Fig. 5e and Extended Data Fig. 8a) and in mouse models of GBM (Extended Data Fig. 8b,c). We evaluated whether these cells with higher respiratory capacity and ATP synthase levels generated more ATP. Across multiple human-derived GBM models, we verified that GBM cells that acquired mitochondria from astrocytes also had higher levels of ATP as measured by luminescence reporter assay (Fig. 5f,g).

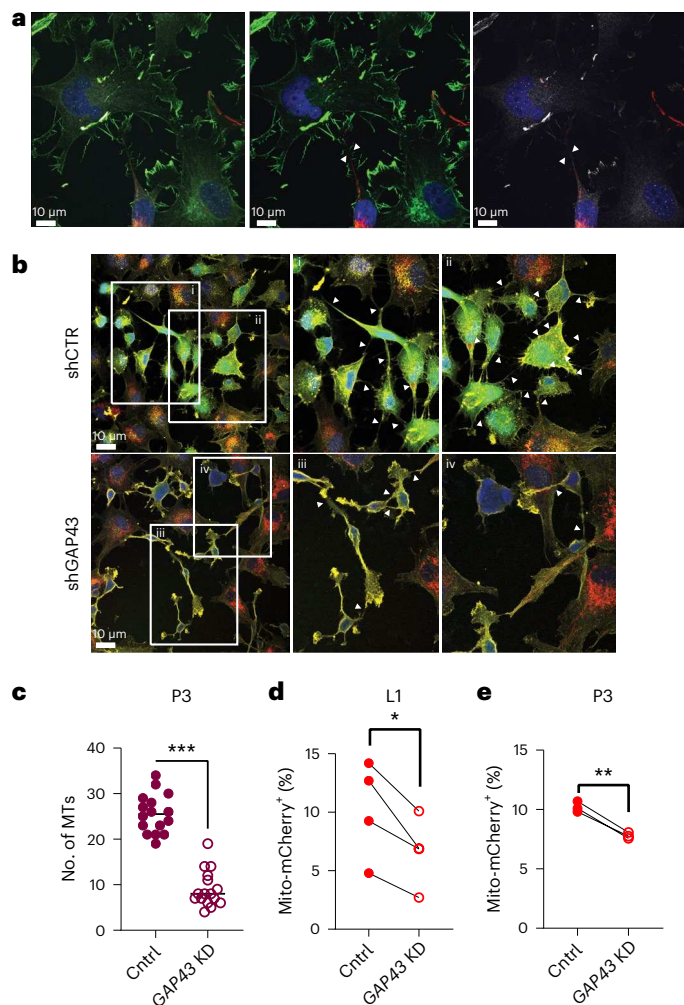


Fig. 4 | GAP43 facilitates mitochondria transfer via tumor-astrocyte MTs.

a, Presence of GAP43⁺ MT-like connections (arrows) between mitoDsRed⁺ astrocytes and P3 tumor cells. Immunostaining for GAP43 (white) and actin (green) is shown. Left: mitoDsRed (red), GAP43 and actin. Middle: mitoDsRed and actin. Right: mitoDsRed and GAP43. Images are representative of at least four ×100 images across three biologically independent co-cultures. **b**, Co-culture of mitoDsRed⁺ astrocytes and GFP⁺ P3 short hairpin GAP43 (shGAP43) cells indicates fewer membrane connections between donor and recipient cells than observed in GFP⁺ P3 short hairpin control (shCTR) cells. Immunofluorescence staining with F-actin (yellow) is shown and is further visualized at increased magnification (i–iv). Arrows indicate MTs. Insets: 2× magnification. **c**, Quantification of MTs from **b** across 16 independent ×60 images across $n = 4$ independent co-culture experiments per group. Data are shown as the mean ± s.d.; *** $P < 0.0001$. Data were analyzed by two-tailed t -test; Ctrl, wild type; KD, knockdown. **d**, Wild-type or GAP43-knockdown L1 human-derived GSCs were cocultured for 24 h with matching wild-type or GAP43-knockdown mito-mCherry⁺ astrocytes. Astrocyte-derived mitochondria transfer to GSCs was quantified by flow cytometry; $n = 4$ independent experiments; * $P = 0.01$. Data were analyzed by two-tailed t -test. **e**, Wild-type or GAP43-knockdown P3 human-derived GSCs were cocultured for 24 h with mitoDsRed human astrocytes. Astrocyte-derived mitochondria transfer to GSCs was quantified by flow cytometry; $n = 3$ independent experiments; ** $P = 0.001$. Data were analyzed by two-tailed t -test.

Collectively, these data show that transferred astrocyte mitochondria are bioactive and lead to augmented mitochondrial respiration and ATP production in recipient GBM cells.

Mitochondria can further influence cellular biology by modulating diverse metabolic pathways⁴¹, and there is increasing evidence that they influence intracellular phosphorylation signaling cascades^{42,43}. We thus performed a metabolite mass spectrometry assay and 584-site

protein phosphorylation array on sorted GBM cells from co-cultures with astrocytes to interrogate the functional consequence of mitochondria uptake. Metabolite enrichment analysis revealed multiple upregulated metabolic pathways in mKate2⁺ GBM cells shared between both GL261 and SB28 mouse models (Fig. 6a, Extended Data Fig. 8d and Supplementary Table 2). Among these pathways was amino acid and nucleotide metabolism, previously linked to proliferation, self-renewal and tumorigenicity in GBM^{44–46}. Metabolomic analysis of the sorted human-derived L1 cells showed that mCherry⁺ cells have higher amounts of glutamate, α -ketoglutarate, glutathione and essential amino acids than mCherry[−] cells (Fig. 6b and Extended Data Fig. 8e,f). Glutamate is one of three amino acids in glutathione, a major cellular antioxidant, and glutamate metabolism is a principal route for assimilation of nitrogen in de novo nucleotide synthesis, which promotes numerous oncogenic processes in gliomas^{47,48}. This suggests that transfer of mitochondria may be helping the GBM cells support proliferation and protect against oxidative stress. Our phosphoprotein array also revealed changes in the phosphorylation levels of numerous signaling and effector proteins in human-derived GSCs that received astrocyte mitochondria, many of which mapped to proliferation and cell cycle pathways (Fig. 6c and Supplementary Table 3). These findings are consistent with metabolic reprogramming of recipient cells beyond increased mitochondrial respiration, along with intracellular signaling with potential effects on cell cycle regulation and other processes.

Mitochondria transfer increases GBM tumorigenicity

To determine whether the metabolic and signaling changes that we observed in GBM cells that acquired astrocyte mitochondria resulted in altered cell cycle regulation, we performed cell cycle analysis in our models of mitochondria transfer by DNA staining and flow cytometry across multiple human-derived GSC models. We observed a consistent increase in the proportion of cells in the proliferative G2/M phases of the cell cycle following acquisition of astrocyte mitochondria (Fig. 7a,b and Extended Data Fig. 9a). This observation was consistent in vivo when analyzing mouse GBM models from orthotopic tumors established in mito::mKate2 mice (Fig. 7c,d). Moreover, orthotopic mouse GBM tumors originating from sorted cells that had acquired astrocyte mitochondria had a higher mitotic index (Extended Data Fig. 9b–e), suggesting that the increased proliferation phenotype was retained in vivo. Previous reports demonstrated that GSCs could take up isolated cell-free mitochondria added to cell culture medium⁴⁹. Addition of astrocyte-derived mitochondria to human-derived GSCs was sufficient to recapitulate the increased proportion of cells in G2/M phases of the cell cycle (Fig. 7e–g). These data suggest that the transfer of astrocyte mitochondria impacts cell cycle regulation in human GBM models.

To further assess the impact of mitochondria acquisition on self-renewal, we performed limiting dilution sphere formation assays using sorted GBM cells with and without astrocyte mitochondria. In both human and mouse GBM models, we found that cells that acquired astrocyte mitochondria had significantly higher self-renewal capacity, represented as a higher estimated stem cell frequency (Fig. 7h and Extended Data Fig. 10a). While human-derived GSC models expressed high levels of the pluripotency transcription factor SOX2, we found that GSCs that acquired astrocyte mitochondria further upregulated another self-renewal transcription factor, OCT4 (Extended Data Fig. 10b). These findings suggest that mitochondria transfer from astrocytes consists of a previously undescribed TME interaction that promotes GBM self-renewal.

Increased proliferative capacity and self-renewal are central hallmarks of cancer; we thus hypothesized that mitochondria transfer from astrocytes promotes tumorigenicity of GBM. We tested this hypothesis by assessing the lethality (time to death or humane endpoint) and tumor initiation capacity of human-derived GBM cells with and without acquisition of human astrocyte mitochondria by orthotopic implantation in immunocompromised mice. These studies revealed

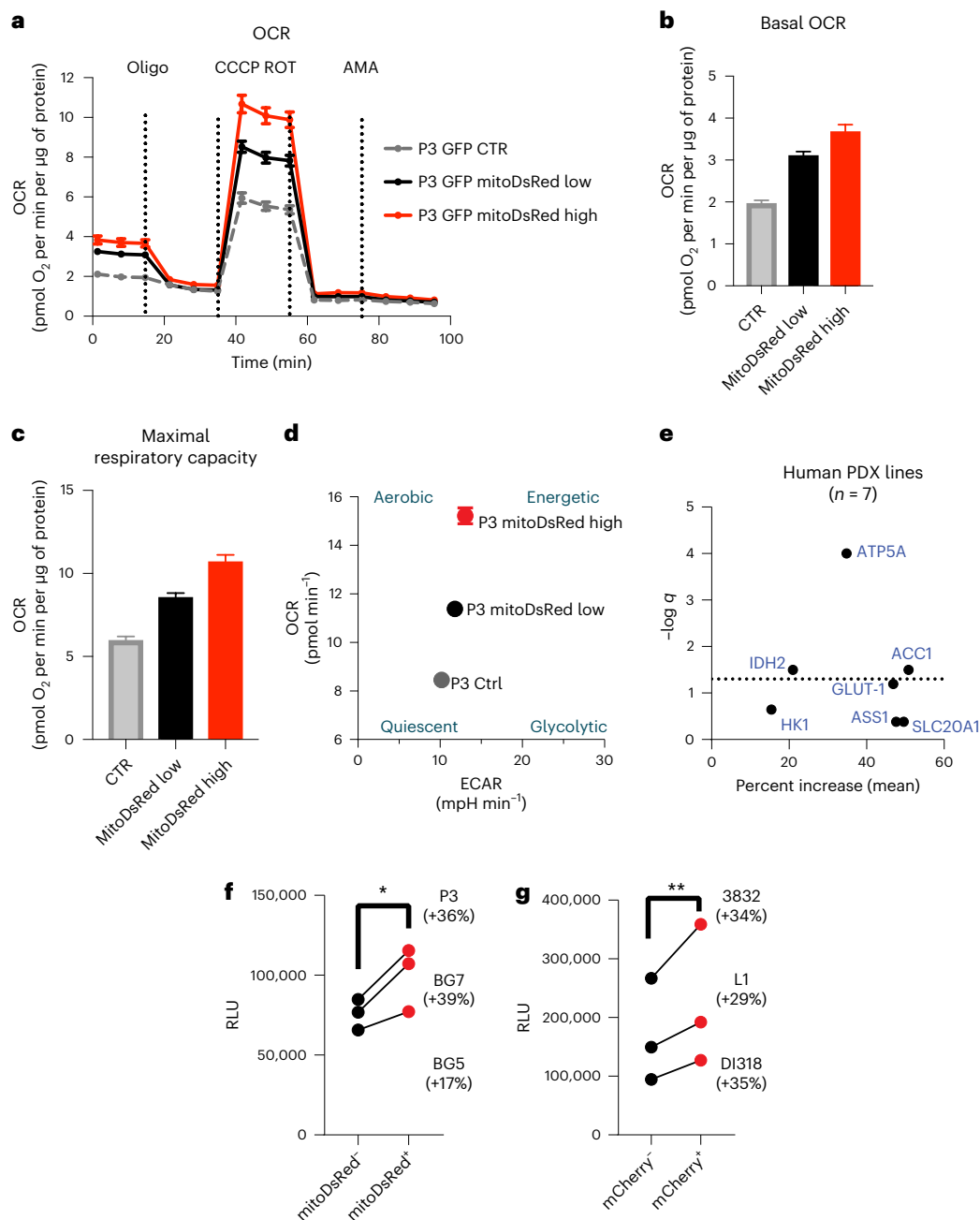


Fig. 5 | Acquisition of astrocyte mitochondria enhances ATP production by mitochondrial respiration in recipient GBM cells. a, Oxygen consumption rate (OCR) was measured in GFP⁺ P3 cells sorted after transfer with a high or low amount of mitoDsRed⁺ mitochondria from donor mitoDsRed⁺ astrocytes and was compared with that in sorted GFP⁺ mitoDsRed⁻ P3 cells from the same co-culture. Following readings of basal respiration, the stepwise addition of 3 mM oligomycin (Oligo) to measure leak respiration, 1.5 mM CCCP to quantify maximal and reserve capacity and 1 mM rotenone (ROT) followed by 1 mM antimycin A (AMA) to measure non-mitochondrial respiration was performed; CTR, control. **b**, Basal oxygen consumption gradually increased with cumulative mitochondrial content in GFP⁺ P3 cells. **c**, Maximal respiratory capacity gradually increased with cumulative mitochondria content in GFP⁺ P3 cells. **d**, Energy map indicating that GFP⁺ P3 cells with a higher degree of mitochondria transfer from astrocytes have a more aerobic and energetic phenotype than GFP⁺ P3 cells with less and no mitochondria transfer. Data in **a–d** are from a representative experiment from a total of three independent experiments. Data

shown as mean $\pm$ s.e.m.; $n = 18$ (control), 22 (mitoDsRed low) and 9 (mitoDsRed high) technical replicates; statistical comparison of technical replicates is not shown. ECAR, extracellular acidification rate; mpH, milli pH. **e**, Seven distinct human-derived GSCs were cocultured for 4 d with immortalized mito-mCherry⁺ human astrocytes and stained with antibodies recognizing key metabolic proteins for downstream flow cytometry quantification. Protein expression was compared between mito-mCherry⁺ and mito-mCherry⁻ cells by mixed-effects model analysis. The dotted line represents the statistical significance threshold (false-discovery rate (FDR) < 0.05). **f**, ATP levels in sorted mitoDsRed⁺ versus mitoDsRed⁻ cells from distinct human-derived GSCs, measured with the CellTiter-Glo luminescence assay; $n = 3$ independent experiments; * $P = 0.04$. Data were analyzed by two-tailed ratio paired t -test; RLU, relative light units. **g**, ATP levels in sorted mito-mCherry⁺ versus mito-mCherry⁻ cells from three distinct human-derived GSC lines, assessed by CellTiter-Glo luminescence assay; $n = 3$ independent experiments; ** $P = 0.003$. Data were analyzed by two-tailed t -test.

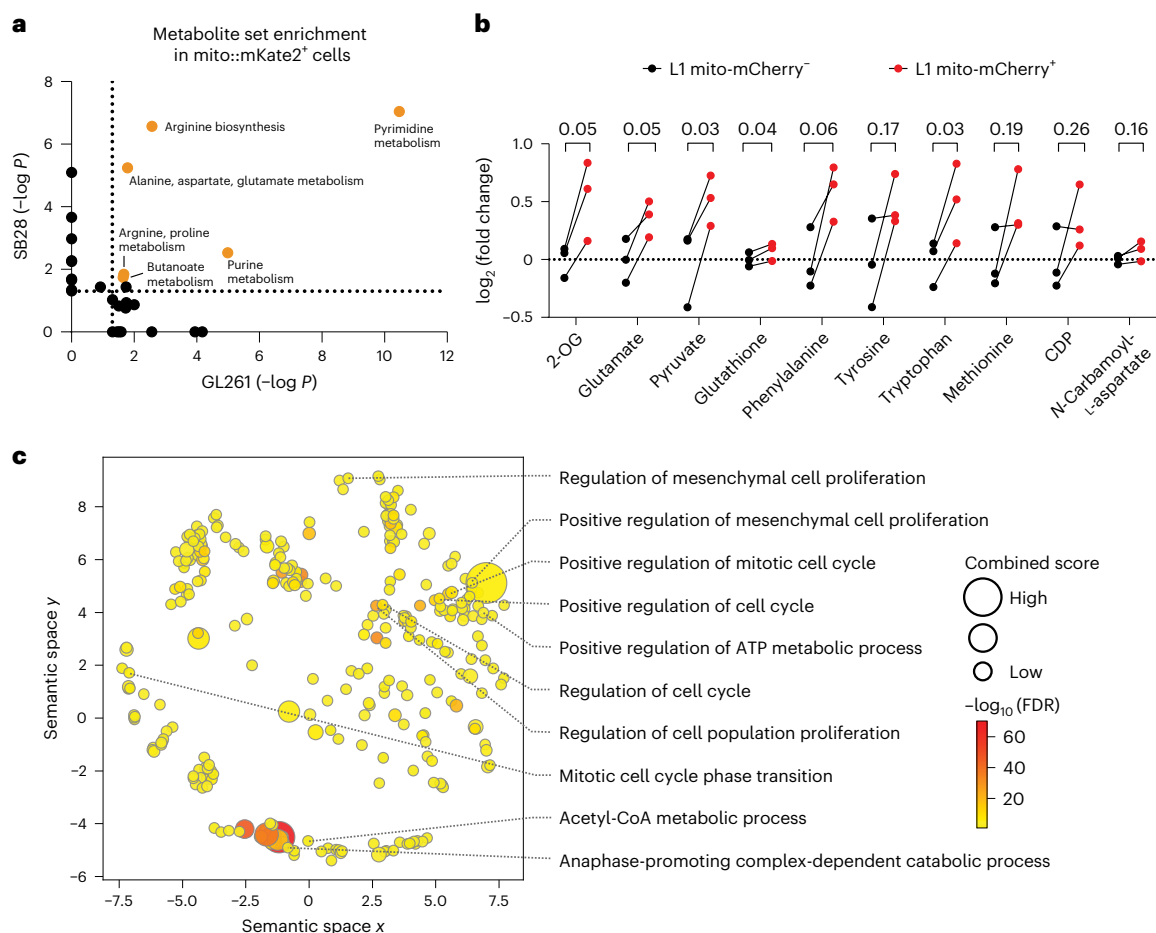


Fig. 6 | Mitochondria transfer from astrocytes reprograms GBM metabolism.

a, Metabolic pathway analysis (MetaboAnalyst) of metabolites enriched by >20% in mito::mKate2⁺ versus mito::mKate2⁻ mouse GBM cells. For each cell model, $n = 3$ independent co-culture experiments were pooled. Dotted lines represent the cutoff for statistical significance ($P < 0.05$). Pathways significantly enriched in both GBM models are highlighted with orange and are labeled with the pathway name. P values were calculated with the MetaboAnalyst 5.0 web tool using the one-tailed hypergeometric test for enrichment analysis (expected versus observed metabolites enriched in each pathway). **b**, L1 cells were cocultured with immortalized mito-mCherry⁺ human astrocytes for 4 d. Relative abundances

of metabolites in mito-mCherry⁺ versus mito-mCherry⁻ L1 cells indicate higher amino acid and glutathione metabolism in mito-mCherry⁺ L1 cells; $n = 3$ independent co-culture experiments. Data were analyzed by paired two-tailed t -test; 2-OG, α -ketoglutarate; CDP, cytidine-5'-diphosphate. **c**, Phospho-array pathway analysis (Enrichr) of protein phosphorylation sites upregulated in mito-mCherry⁺ versus mito-mCherry⁻ L1 cells, depicted with dimensionality reduction in semantic.xy space. Dots represent significantly upregulated pathways (FDR < 0.05). Selected pathways associated with cell metabolism and proliferation are labeled; $n = 3$ independent co-culture experiments were pooled and analyzed.

that tumors led to symptomatic or lethal disease much faster when originating from GSCs that had acquired mitochondria from astrocytes and had a significantly higher in vivo tumor initiation capacity (Fig. 7i,j and Extended Data Fig. 10c). This observation was similar in mouse GBM models (Extended Data Fig. 10d,e). Thus, beyond altering the phenotype of GBM cells in vitro, mitochondria transfer from astrocytes increases the in vivo tumorigenicity of these cells in animal models.

Discussion

Organelle transfer is an increasingly recognized biological process in models of GBM^{35–37} and other cancers^{50–52}. Most of the existing knowledge on mitochondria transfer in cancer relies on depicting mitochondrial exchange among tumor cells, in purely in vitro systems, and/or using tumor cells artificially depleted of mitochondria. Thus, the role of mitochondria transfer from the TME to cancer cells (and to GBM in particular) remains poorly understood. Specifically, there is a lack of understanding about the in vivo relevance of mitochondria transfer from the GBM microenvironment, the cell types involved, the mechanism of transfer and its downstream effects on cellular function and tumorigenicity in disease-relevant contexts.

We found that in the context of GBM, mitochondria transfer from the TME is a frequent in vivo event and involves brain-resident glial cell donors. Here, we focused mechanistically on astrocytes as donor cells given their abundance in the brain, and our data identify them as important mitochondrial donors. Our data support that astrocytes in the TME form physical actin-based connections with GBM cells that have strong similarity to the previously described MTs, which are network-forming connections between GBM cells. Our data also support an extension of the previously described interconnected network of GBM to non-malignant astrocytes that has recently been verified by exchange of calcium waves within this network in vivo^{24,32}. GAP43, typically found in neuronal projections and critical to GBM growth and the tumor cell network²⁴, mediates mitochondria transfer from astrocytes in a previously undescribed role for this actin-associated protein.

Our findings further demonstrate that mitochondria transfer from astrocytes to GBM cells drives metabolic reprogramming toward oxidative respiration with increased ATP production. Mitochondria transfer also leads to intracellular signaling via protein phosphorylation linked to cellular proliferation and cell cycle progression.

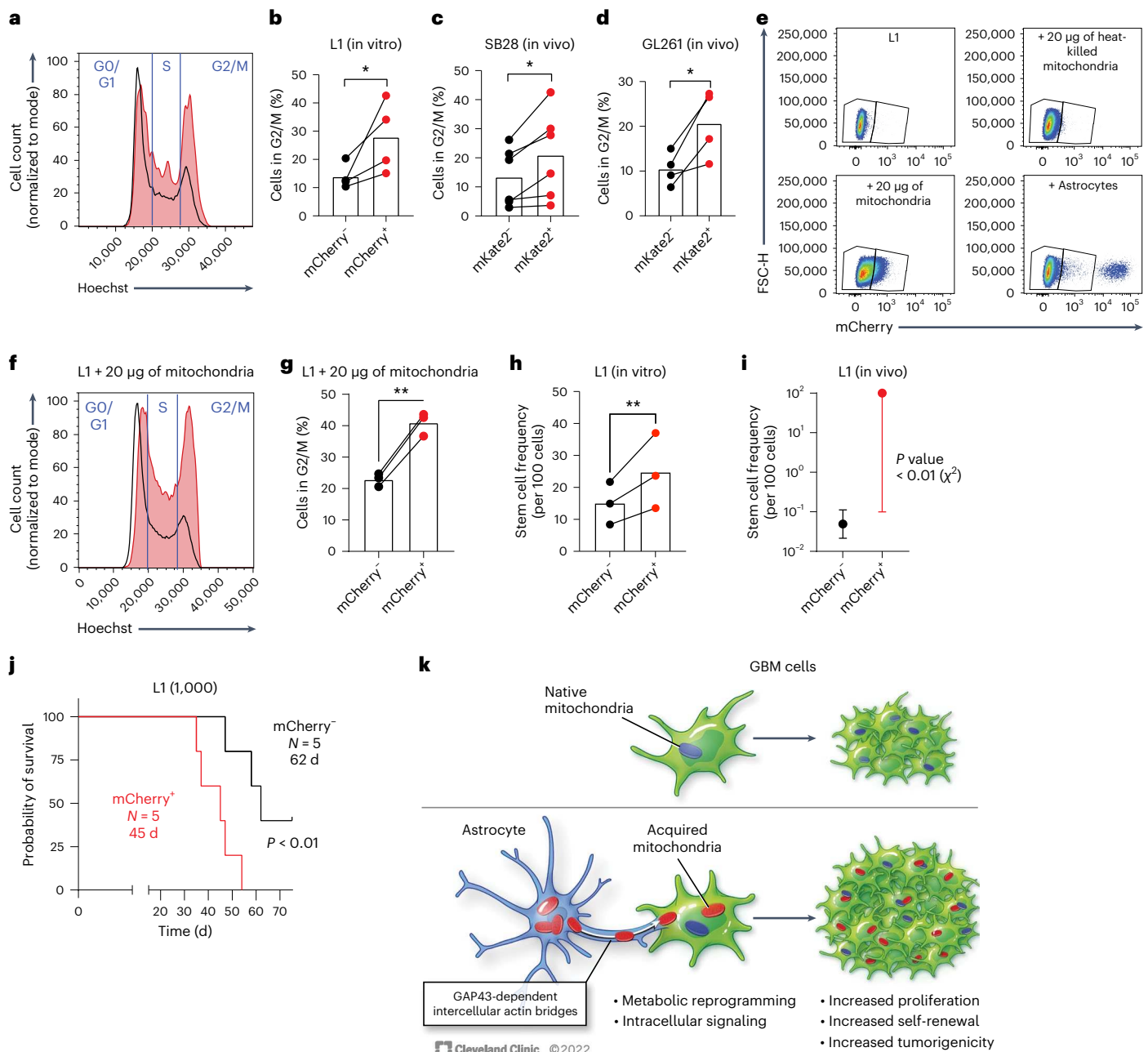


Fig. 7 | Mitochondria transfer from astrocytes drives GBM cell proliferation, self-renewal and tumorigenicity. **a, b**, Human-derived GSCs (L1) were cocultured with immortalized mito-mCherry⁺ human astrocytes. Representative histograms (**a**) and aggregate data (**b**) of $n = 4$ independent experiments depicting cell cycle analysis by flow cytometric DNA quantification in GSCs that acquired astrocyte mitochondria (mCherry⁺, red histogram/dots) versus those that did not (mCherry⁻, black histogram/dots) are shown; * $P = 0.04$. Data were analyzed by two-tailed t -test. **c, d**, Cell cycle analysis by ex vivo flow cytometry DNA quantification in GFP-expressing mouse GBM cells obtained from orthotopic tumors in mito::mKate2 mice; $n = 4$ –6 mice per tumor model and $N = 6$ (SB28; **c**) and 4 (GL261; **d**) mice per group; * $P = 0.02$ (SB28) and 0.03 (GL261). Data were analyzed by two-tailed paired t -test. **e–g**, Cell-free mito-mCherry⁺ astrocyte mitochondria (intact or heat killed) were added to an L1 culture; vehicle (PBS) or live mito-mCherry⁺ astrocytes were added to control wells. **e**, Representative dot plots depicting the identification of L1 cells that acquired cell-free mitochondria or mitochondria from cocultured astrocytes. In the +astrocyte condition, the mCherry^{hi} population outside of the gates is composed

of the mito-mCherry⁺ astrocytes. Representative histograms (**f**) and aggregate data (**g**) depicting cell cycle analysis of GSCs that acquired cell-free astrocyte mitochondria (mCherry⁺, red histogram/dots) versus those that did not (mCherry⁻, black histogram/dots) are shown; $n = 3$ independent experiments; ** $P = 0.003$. Data were analyzed by two-tailed paired t -test. **h, i**, Estimated stem cell frequency in mCherry⁺ versus mCherry⁻ human-derived GBM models sorted from astrocyte co-cultures and subjected to in vitro limiting dilution sphere formation assay (**h**) or in vivo orthotopic tumor initiation assay (**i**); ** $P = 0.002$, ratio paired t -test, $n = 3$ independent experiments (**h**); $P = 0.005$; compiled data from $n = 15$ NOD scid gamma (NSG) mice per group (distributed across three cell-dose levels). Data are shown as mean $\pm$ 95% confidence interval; χ^2 test with 1 degree of freedom analyzed by Extreme Limiting Dilution Analysis (ELDA; **i**). **j**, Survival of mice injected orthotopically with 1,000 sorted L1 GSCs per animal; $P = 0.009$. Data were analyzed by log-rank test. Survival analysis of other dose levels is presented in Extended Data Fig. 8. **k**, Schematic overview of findings.

The central finding of our study is that mitochondria transfer from a non-malignant cell of the TME promotes a highly tumorigenic cell phenotype characterized by both increased proliferative capacity and self-renewal (Fig. 7k). This manifested as higher penetrance and faster lethality of orthotopic tumors *in vivo*. Our findings suggest that the phenotype of increased proliferation and self-renewal driven by acquisition of astrocyte mitochondria before intracranial tumor implantation is sufficient to lead to increased tumorigenicity, while lacking this phenotype at the time of experimental tumor initiation leads to lower GBM cell proliferation. Thus, mitochondria transfer and the shift to oxidative metabolism comprises a fundamental protumorigenic interaction of GBM with its microenvironment. Our study adds a mechanistic understanding to this understudied process, forming the basis of future studies, which could also have broad applicability to tumors outside the central nervous system and other pathological contexts.

Methods

Ethics statements

Human material was obtained from surgeries performed at the Haukeland University Hospital (Bergen, Norway). Written consent was obtained from individuals with procedures that were approved for the projects (project numbers 013.09 and 151825) by the Regional Ethical Committee. Animal experiments were approved by the Institutional Animal Care and Use Committee of Cleveland Clinic and local ethical committee. Animals were treated in accordance with the Norwegian Animal Act.

Human cell culture

The GSC lines P3, GG16 (provided by F. Krut, University of Groningen), BG5 and BG7 (ref. 15) were all derived from *IDH* wild-type biopsy specimens from individuals with GBM; 3832 cells were provided by J. Rich (University of Pittsburgh Medical Center) and have been described previously⁵³. L1 cells were obtained from B. Reynolds (University of Florida) and have been described previously⁵⁴. DI318 cells were obtained from the Rose Ella Burkhardt Brain Tumor Center biorepository and have been described previously (Cleveland Clinic)⁵⁵.

Human-derived GBM cells were cultured in 'complete' Neurobasal medium (NBM): Neurobasal without phenol red (Gibco), supplemented with 2% B-27 supplement (Gibco), 1 mM sodium pyruvate (Gibco), 2 mM L-glutamine (Gibco), 1 U ml⁻¹ penicillin + 1 µg ml⁻¹ streptomycin (Cleveland Clinic Media Preparation Core), 20 ng ml⁻¹ epidermal growth factor (EGF) and 20 ng ml⁻¹ fibroblast growth factor 2 (FGF-2; R&D Systems). EGF was not added to P3 cells. Immortalized normal human astrocytes (provided by R. Pieper at University of California, San Francisco, and P. Øyvind Enger at Universitet i Bergen) were cultured in tissue culture vessels (adherent) in neural stem cell (NSC) medium (DMEM-F12 (Media Preparation Core), 1 U ml⁻¹ penicillin + 1 µg ml⁻¹ streptomycin, 5% fetal bovine serum (FBS), 1% N2 supplement (Thermo Fisher Scientific), 20 ng ml⁻¹ EGF and 20 ng ml⁻¹ FGF-2 (R&D Systems)). For experiments involving downstream *in vitro* limiting dilution assays with L1 and DI318 cell lines, human-derived cells were maintained in DMEM (Cleveland Clinic Media Preparation Core) supplemented with 10% FBS (Thermo Fisher), 1 U ml⁻¹ penicillin and 1 µg ml⁻¹ streptomycin (Cleveland Clinic Media Preparation Core) for at least 7 d before co-culture with astrocytes.

Human-derived xenograft D456 was provided by D. Bigner (Duke University), and the JX22 cell line was provided by J. Sarkaria (Mayo Clinic). DMEM-F12 containing 10 ng ml⁻¹ EGF, 10 ng ml⁻¹ FGF, 1% sodium pyruvate, 2% GEM21 (Gemini Bio) and 1 U ml⁻¹ penicillin + 1 µg ml⁻¹ streptomycin was used to culture the human-derived xenograft lines.

Lentiviral transductions of human cells

Human astrocytes were transduced with mito-mCherry lentivirus (Takara) and selected by fluorescence-activated cell sorting. GAP43

short hairpin RNA (shRNA) and non-target (control) lentiviruses were generated *in-house* according to the protocol described by Tisconia et al.⁵⁶. shRNA sequences and sources are described in Supplementary Table 4.

Lentiviral vectors encoding mitoDsRed (Addgene, 44386), enhanced GFP (eGFP)⁵⁷, mito-GFP (Addgene, 44385) and shRNA against *GAP43* (ref. 24) were prepared and titrated according to a protocol reported previously⁵⁸. The mitochondria donor cells were transduced with pLV-mitoDsRed. The acceptor cells were transduced with a lentiviral eGFP vector⁵⁷ or, for indicated *in vivo* experiments, with pLV-mito-GFP.

Human cell co-culture mitochondria transfer assessment by flow cytometry

L1, DI318 and 3832 cells were cultured in tissue culture vessels (adherent) precoated with Geltrex (Thermo Fisher Scientific; 1:250 in serum-free medium overnight) in 90% complete Neurobasal + 10% NSC medium for 4 d, unless otherwise indicated. For *GAP43*-knockdown experiments, co-culture time was reduced to 24 h to reduce the confounding effect of different growth rates in control versus knockdown GBM cells, and comparisons were made between co-cultures with similar astrocyte:tumor cell ratios. Growth factor-reduced Matrigel (Corning) was used as a coating reagent to attach P3, BG5, BG7 and GG16 GBM cells and normal human astrocytes.

The following inhibitors were used: cytochalasin B (Sigma, C6762) and vincristine sulfate (Sigma, V8388). An equivalent amount of vehicle (DMSO) was used as a control. Cell viability was assessed with a LIVE/DEAD Fixable Blue Dead Cell staining kit.

For viability testing of P3 in the presence of inhibitor, GBM cells were seeded at 5,000 cells per Matrigel-coated well of a 96-well plate in 150 µl of complete Neurobasal medium. After incubation with cytochalasin B for 24 h, cell proliferation reagent WST-1 (Roche, CELLPRO-RO) was added, and sample absorbance measurements at 450 nm were determined on a multiscan FC microplate photometer (Thermo Scientific).

For transwell experiments, human-derived GBM cells were plated on Geltrex-coated tissue culture wells. Under 'contact' conditions, human mito-mCherry astrocytes were simultaneously added to the culture well. Under 'transwell' conditions, an equal number of astrocytes was plated in a transwell insert (5-µm pore size, Corning), which was submerged in the culture medium of the underlying culture well.

Co-culture experiments were analyzed on a BD LSR Fortessa or BD FACSSymphony S6 (BD Biosciences) operated by BD FACSDiva software (v8.0 or v9.0). FlowJo software (BD Biosciences, v10.7.2 or 10.8.1) was used to analyze flow cytometry data. The gating strategy is described in Supplementary Fig. 1.

Mouse tumor cell maintenance and transduction

SB28 cells were a gift from H. Okada (University of California, San Francisco). GL261 cells were obtained from the Developmental Therapeutics Program, National Cancer Institute. All cell lines were treated with 1:100 MycoRemoval Agent (MP Biomedicals) after thawing and were routinely tested for *Mycoplasma* spp. (Lonza). Cells were maintained in RPMI 1640 (Media Preparation Core, Cleveland Clinic) supplemented with 10% FBS (Thermo Fisher Scientific) and 1 U ml⁻¹ penicillin + 1 µg ml⁻¹ streptomycin (Cleveland Clinic Media Preparation Core), that is, non-stem-promoting conditions. For the generation of GFP-expressing GL261 cells, parental GL261 cells were transduced with pReceiver-Lv207 (Genecopoeia) and were selected with 300 µg ml⁻¹ hygromycin B (Invitrogen). GFP expression was confirmed by flow cytometry.

Mice

Tg(CAG-mKate2)1Poche/J (mito::mKate2; stock 032188) mice were purchased from The Jackson Laboratory and were housed in the Cleveland Clinic Biological Research Unit. Both sexes of mito::mKate2 mice were

intracranially injected at 4–8 weeks of age with 10,000–20,000 SB28 or 100,000 GL261-GFP cells in 5 μ l of RPMI null medium into the left cerebral hemisphere 2 mm caudal to the coronal suture and 3 mm lateral to the sagittal suture at a 90° angle with the skull to a depth of 2.5 mm using a stereotaxis apparatus (Kopf). Male NSG mice were bred in-house by the Cleveland Clinic Biological Resources Unit, and tumors were established orthotopically as described above. Mice were fed standard chow (Teklad Global 18% Protein Rodent Diet, 2913, Envigo) and filtered water ad libitum and were housed in forced/filtered air isolator cages containing up to five mice. Mice were maintained on a 12-h light/12-h dark cycle, with a temperature of 20–26 °C and humidity of 30–70%.

Bone marrow transplantation

Four-week-old male mice were treated with 11 Gy radiation in two fractions 3–4 h apart. Reconstitution was achieved by retro-orbital injection of 2×10^6 bone marrow cells from mito::mKate2 mice. Drinking water was supplemented with Sulfatrim (trimethoprim–sulfamethoxazole; Pharmaceutical Associates) during the first 10 d, and mice were monitored for an additional 6 weeks for weight loss and symptoms of infection before tumor inoculation.

Orthotopic xenograft models in nude rats

Immunodeficient nude-RNU rats of both sexes, bred in-house, were fed a diet containing standard pellets (Sniff, V1536-000), had access to water ad libitum and were housed in filtered air isolator cages (Allentown type IV (Rat 1800), HEPA filter) with a 12-h light/12-h dark cycle at 21 °C and ~45% humidity. Stereotactical implantation of tumor cells into the brain has been described previously⁵⁷. To model mitochondria transfer from normal cells to tumor cells, a high-titer mitoDsRed lentivirus was injected into the brain followed by implantation of GFP⁺ or mito-GFP⁺ tumor acceptor cells after 7 d. Animals were euthanized with CO₂ and perfused with 0.9% NaCl.

Immunofluorescence

For immunofluorescence analysis, brains were fixed in 4% paraformaldehyde (PFA), dehydrated in a 30% sucrose solution, embedded in optimal cutting temperature compound and snap frozen. Sectioning (10 μ m thick) was performed on a Leica CM3050 S precooled to –20 °C before use. Sections were protected from light and frozen at –80 °C. For staining, tissue sections were incubated overnight at 4 °C with the following primary antibodies: monoclonal anti-GFAP, anti-human nestin, anti-F-actin, anti-GAP43, chicken anti-GFP, rabbit anti-phospho-histone H3 and rabbit anti-cleaved caspase-3. The following secondary antibodies were used: goat anti-mouse 647 at room temperature for 120 min, donkey anti-chicken Alexa Fluor 488 overnight at 4 °C and WGA Alexa Fluor 680 (4 μ g ml^{–1} in HBSS-T with magnesium and calcium) for 1 h at room temperature.

Cells on coverslips were fixed in 4% PFA and permeabilized with 0.1% Triton X-100 (Sigma) for 20 min at room temperature. Anti-TOMM20 or anti-nestin was incubated overnight at 4 °C. Goat anti-mouse 488 or goat anti-rabbit 647 were incubated for 60 min at room temperature.

Confocal microscopy and time-lapse imaging

Confocal microscopy of stained mouse tissue sections was performed using a Leica SP8. Still-image processing and z reconstructions were completed using LasX software (version 3.3; Leica). Image analysis for estimation of mito::mKate2 transfer to GBM cells in vivo was performed using Velocity software (version 6.3; PerkinElmer). The three-dimensional (3D) segmentation algorithms (Supplementary Note) were set to minimize the (1) detection of mKate2 channel noise (using wild-type tissue sections as a negative control) and (2) identification of GFP⁺ cells not morphologically compatible with tumor cells, likely the result of GFP phagocytosis in the TME (using a relevant size cutoff).

SB28 cells (40,000) were cocultured with 80,000 mito::mKate2 astrocytes in a glass-bottom 35-mm dish (Mat-tek) overnight. Growth medium was replaced with phenol red-free NSC medium. Multiple full-thickness z stacks were obtained every 10 min using a Leica SP8 microscope in a 37 °C chamber supplemented with 5% CO₂ and 95% humidity using a $\times 20/0.8$ -NA objective lens. Time-lapse frames were subsequently analyzed by LasX software.

L1 and DI318 cells were stained with CellTrace Green (Thermo Fisher) at 5 μ M in serum-free medium at 37 °C for 30 min and washed with complete Neurobasal medium before plating with human mito-mCherry astrocytes in ibidiTread 35-mm microscopy dishes (ibidi) that had been coated with Geltrex (Gibco). After 48 h, cells were washed gently with PBS, fixed with 4% PFA for 15 min at 4 °C and imaged by confocal microscopy.

For TOMM20 colocalization experiments, cells were plated as described above and cocultured for 72 h. Cells were then stained with primary antibody (anti-TOMM20; clone D8T4N) for 1 h at room temperature and with secondary anti-rabbit DyLight405 alpaca for 1 h at room temperature. Colocalization analysis (line profile quantification) was performed using Image-Pro Plus 10 (Media Cybernetics).

Confocal imaging for P3, BG5, BG7 and GG16 co-culture experiments was performed using a Leica TCS SP8 STED 3X (Leica Microsystems) run by LasX software (version 3.3, Leica, version LAS4.13). Image analyses were performed with ImageJ (v2.3.0/1.53f) and Imaris (v9.6).

Measurement of mitochondrial trafficking in vivo by flow cytometry

Resected tumors or the contralateral hemisphere were digested with 1 mg ml^{–1} collagenase IV (StemCell Technologies) and 1 mg ml^{–1} DNase I (Roche) for 15 min at 37 °C. Samples were strained through a 100- μ m strainer (Fisherbrand) and washed with PBS. Cells were stained with a LIVE/DEAD Fixable Blue Dead Cell Stain kit (Thermo Fisher Scientific) for 10 min on ice, treated with FcR blocking reagent (Miltenyi Biotec) diluted 1:50 for 15 min on ice and stained with 1:100 APC-conjugated anti-CD11b (BioLegend, clone M1/70) for 20 min to exclude phagocytic cells. Samples were fixed overnight with a eBioscience FoxP3 transcription factor fixation kit (Thermo Fisher Scientific) and analyzed with a BD LSRII Fortessa (BD Biosciences) in PBS.

Generation of mouse astrocytes and microglia and in vitro mouse mitochondria transfer assay

Brain-resident glial cell cultures were obtained, as previously described⁵⁹, by resecting the subventricular zone of brains from post-natal day 0–3 mice and culturing in NSC medium. Microglia were generated by culturing confluent monolayers of early passage (passage 2 or 3) glial cell cultures with microglia polarization medium (DMEM-F12, 1 U ml^{–1} penicillin + 1 μ g ml^{–1} streptomycin, 10% FBS and 20 ng ml^{–1} granulocyte–macrophage colony-stimulated factor (BioLegend)). After 5 d, microglia (loosely adherent) were obtained by agitation of the culture flask on an orbital shaker for 45 min.

A total of 20,000–40,000 astrocytes and microglia were separately cultured in a 96-well flat-bottom plate in NSC medium or microglia polarization medium, respectively. Forty-eight hours later, supernatants were collected and centrifuged at 400g for 5 min to remove residual cells; these supernatants consisted of the conditioned, cell-free culture medium. Tumor cells were added at a recipient: donor ratio of 2:1 to the adherent donor cell cultures to assess total mitochondria transfer (contact dependent and independent); separately, tumor cells alone were cultured with conditioned, cell-free medium described above to assess contact-independent (secreted) mitochondria transfer. Samples were incubated for 2 h and treated with Accutase to generate single-cell suspensions. Cells were transferred to 96-well U-bottom plates to stain with LIVE/DEAD dye. Exogenous mitochondria uptake by GFP⁺ tumor cells was assessed with a BD LSRII Fortessa.

Generation of mouse macrophages and in vitro mitochondria uptake assay

Bone marrow from the femurs and tibiae of 4- to 8-week-old male and female *mito::mKate2* mice was flushed with PBS using a 27-gauge needle. Eighty thousand cells were cultured in 24-well plates and treated with 50 ng ml⁻¹ recombinant mouse macrophage colony-stimulating factor (BioLegend) in IMDM (Media Preparation Core) supplemented with 1 U ml⁻¹ penicillin + 1 µg ml⁻¹ streptomycin and 20% FBS for 6 d. Interferon-γ or interleukin-4 (50 ng ml⁻¹; BioLegend) was added for 48 h to further induce polarization of macrophages to M1- or M2-like macrophages, respectively. Supernatants were collected and centrifuged at 400g for 5 min to remove residual cells and to generate conditioned, cell-free culture medium. Tumor cells were added at twofold abundance in technical duplicates for a 2-h incubation, as described above, to test contact-dependent versus contact-independent mitochondria transfer. Samples were incubated with Accutase for 5 min and transferred into 96-well U-bottom plates for staining with the viability dye and anti-CD11b as described above. Exogenous mitochondria uptake was analyzed in GFP⁺ tumor cells using a BD LSR II Fortessa.

Western blotting

Cells were washed twice with PBS and dissolved in lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, 1% Triton X-100 and 1 mM EDTA) supplemented with protease and phosphatase inhibitor cocktails (Roche). Protein concentration was quantified by Bradford assay (Euro-medex). Cell lysates were resuspended in Laemmli buffer (62.5 mM Tris (pH 6.8), 10% glycerol, 2.5% SDS and 2.5% β-mercaptoethanol). The primary antibody was anti-GAP43, the housekeeping protein vinculin was used as the loading control and the secondary antibody was goat anti-rabbit horseradish peroxidase (HRP; Invitrogen, 31462). Membranes were developed on a LAS 3000 (version 2.2; Fujifilm).

For GAP43 blots (Extended Data Fig. 6i,j) and stem cell transcription factors (Extended Data Fig. 10b), cells were lysed with ice-cold RIPA buffer. The following primary antibodies were used: anti-GAP43, anti-actin, anti-SOX2 and anti-OCT4. Secondary antibodies were goat anti-rabbit HRP or goat anti-mouse HRP.

Sorting of tumor cells from co-cultures

For mouse GBM models, astrocytes were collected from flasks by Accutase treatment and stained with a 1:1,000 dilution of CellTrace Violet cell proliferation dye in PBS at 37 °C for 20 min. Tumor cells and astrocytes were then cocultured at a 1:1 ratio for 48 h in NSC medium. Samples were sorted into RPMI with 20% FBS and cultured overnight in complete RPMI for subsequent functional assays.

For collection of double-positive or single-positive cells in co-cultures of P3, BG5 and GG16 GBM cells with immortalized mitoDsRed normal human astrocytes by cell sorting, 2 × 10⁶ cells of each cell line were seeded in Neurobasal medium on Matrigel (T75 flasks). For analyzing mitochondria transfer by flow cytometry, 1.5 × 10⁵ cells from each cell line were seeded in Neurobasal medium on Matrigel (T25 flasks). L1 and DI318 cells were sorted after 4 d of co-culture with immortalized mito-mCherry human astrocytes from Geltrex-coated flasks (seeded at 1:1 and 1:1.5 donor:recipient ratio, respectively, to adjust for differences in cell growth rate).

In vitro limiting dilution assay

Sorted tumor cells were cultured at decreasing cell densities over 12 technical replicates in complete Neurobasal medium, and the number of wells containing spheres was counted after 11–14 d. For the mouse cell line SB28, 400 to 25 cells per well of a 96-well plate were seeded; for human lines, this ranged from 100 to 1.25 cells per well. The online ELDA tool (<http://bioinf.wehi.edu.au/software/elda/>, 24 October 2014 version) was used to calculate stem cell frequency⁶⁰.

In vivo limiting dilution/tumor initiation assays

Sorted mouse/human GBM cells with and without the acquisition of astrocyte mitochondria in vitro were counted with trypan blue using a TC-20 cell counter (Bio-Rad) and were volume adjusted to achieve decreasing cell concentrations (18,000–1,000, as indicated) for intracranial implantation. Subsequently, C57BL/6 mice (mouse GBM models) or NSG mice (human GBM models) were intracranially implanted with equal numbers of tumor cells, as described above. The identity of the implanted cells was then blinded to investigators. Mice were euthanized at humane endpoints (neurological symptoms, weight loss, poor grooming or any other sign of distress).

Seahorse assay

For sorted P3 GFP mitoDsRed⁺ and mitoDsRed⁻ cells, mitochondrial respiration assays were performed using 3.0 mM oligomycin, 1.5 mM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 1.0 mM rotenone and 1.0 mM antimycin A in assay medium consisting of unbuffered, phenol red-free DMEM with 10 mM glucose, 2 mM sodium pyruvate and 4 mM L-glutamine with a pH of 7.4. Cells were incubated for 60 min in a Prep Station (Agilent) under non-CO₂ conditions at 37 °C and were subsequently placed in the Seahorse Xfe96 analyzer. The chemical compounds were serially injected to manipulate the cells and create metabolic flux reports. Data were analyzed using Agilent Seahorse Wave Controller v2.6.3, IDEAS software (version 6.2; EMD Millipore).

ATP quantification

CellTiter-Glo reagent (100 µl; Promega) was added to cells and incubated on an orbital shaker at room temperature for 20–30 min. ATP levels were quantified indirectly by relative luminescence intensity measured on a Victor 3 plate reader (PerkinElmer).

Metabolic protein flow cytometry analysis

For mouse cells, the metabolic protein flow cytometry panel was adapted from Ahl et al.⁴⁰. CellTrace Violet-stained astrocytes were cocultured with tumor cells overnight at a 1:1 ratio. Samples were fixed in eBioscience FoxP3 transcription factor fixation buffer for 30 min on ice and stained with the following antibodies in 1× permeabilization buffer for 30 min at room temperature: anti-argininosuccinate synthetase 1 (ASS1), anti-ATP synthase F1 subunit α (ATP5A), anti-glucose transporter 1 (GLUT1), anti-isocitrate dehydrogenase 2 (IDH2), anti-glucose 6 phosphate dehydrogenase (G6PD), anti-acetyl-CoA carboxylase (ACCI), anti-peroxiredoxin 2 (PRDX2), anti-hexokinase 1 (HK1), anti-carnitine palmitoyltransferase 1 (CPT1A), anti-SLC20A1, anti-mouse IgG1 (κ monoclonal), anti-mouse IgG2b (κ monoclonal) and rabbit IgG (monoclonal). Samples were washed and resuspended in 1× permeabilization buffer containing goat anti-mouse IgG H&L (Alexa Fluor 647) or donkey anti-rabbit IgG H&L (Alexa Fluor 647). After 30 min of incubation at room temperature, cells were washed with permeabilization buffer and resuspended in PBS for analysis with a BD LSR II Fortessa. Geometric mean fluorescence intensity was used to calculate expression levels after subtraction of the background levels from isotype control staining.

The same procedure was used for human cell metabolic flow, with minor modifications. Seven different human-derived cell lines were cocultured with human mito-mCherry astrocytes for 4 d. Most antibodies listed above cross-reacted with human antigens and were thus also used in these experiments, with the exception of using directly conjugated Alexa Fluor 647 antibodies for ATP5A and GLUT1.

Metabolomics analysis

Sorted mouse GBM cell pellets were resuspended in 80% ice-cold methanol for metabolite extraction and sent to an academic core facility for targeted metabolomics (Beth Israel Deaconess Mass Spectrometry Core Facility, Harvard Medical School). Data were normalized by peak count. Values of zero (below the limit of quantitation) were

substituted with the approximate limit of quantitation (2,000 peak count) to facilitate fold change analysis. Metabolites enriched by >20% in mKate2⁺ GBM cells were put into the metabolic pathway enrichment analysis algorithm MetaboAnalyst 5.0 web tool (www.metaboanalyst.ca)⁶¹. The following analysis settings were used: HMDB and KEGG compound names; feature type = metabolites; KEGG analysis.

For human GSCs, triplicate cell pellet samples were lysed in 80:20 methanol:water at dry ice temperature. The quantity of the metabolite fraction analyzed was adjusted to the cell count. Extracts were clarified by centrifugation, dried by nitrogen blower and reconstituted in equal volumes 50:50 methanol:water. Metabolite fractions were analyzed by targeted liquid chromatography–tandem mass spectrometry via dynamic multiple reaction monitoring. An Agilent Technologies Triple Quad 6470 liquid chromatography–tandem mass spectrometry system, consisting of the 1290 Infinity II LC flexible pump (Quaternary Pump), the 1290 Infinity II multisampler, the 1290 Infinity II multicolumn thermostat with six-port valve and the 6470 triple quad mass spectrometer, was used for analysis. Agilent MassHunter Workstation software LC/MS data acquisition for 6400 Series Triple Quadrupole MS with version B.08.02 was used for compound optimization and sample data acquisition. Studies were performed in negative ion acquisition mode with ion-pairing chromatography using an Agilent ZORBAX RRHD Extend-C18, 2.1 × 150 mm, 1.8 μm and ZORBAX Extend Fast Guards for UHPLC separation. Agilent MassHunter Workstation quantitative analysis for QQQ version 10.1, build 10.1.733.0, was used to integrate and quantitate metabolite peak areas. Liquid chromatography–mass spectrometry peaks corresponding to metabolites with coefficients of variation greater than 0.5 underwent manual inspection and integration. The data were normalized to the average sum of metabolites from all the samples and were analyzed using Morpheus to generate the heat map. Metaboanalyst was used to compare metabolites and metabolic pathways enriched in mito-mCherry⁺ L1 cells and mito-mCherry[−] L1 cells.

Phosphoprotein array

Human-derived GBM cells (L1) were cocultured with human mito-mCherry astrocytes for 4 d and flow sorted to obtain mCherry⁺ and mCherry[−] GBM cells. Sorted cell pellets were flash-frozen in liquid nitrogen and sent for a commercial microarray platform, antibody-based phosphoprotein array (Phospho Explorer array, FullMoon BioSystems). The complete antibody list can be found in Supplementary Table 3. Analysis was conducted using the phosphorylation ratio of each protein for samples tested (phosphorylated protein signal divided by corresponding total protein signal). We analyzed the functional enrichment of the upregulated phosphoproteins using Enrichr⁶² against the Gene Ontology (GO) biological process term data set. GO terms with FDR values of <0.05 were considered significantly enriched. We summarized the enriched terms using Revigo⁶³ to generate a graph-based view of the subdivisions of the terms.

ImageStream

A clone of HEK293T cells able to grow in serum-free medium (CSC293T) was generated and cultured as previously described⁶⁴. CSC293T cells were transfected with psPAX2, pCMV-VSVG and pLYSI-Mito-GFP or pCMV-RFP using FuGENE HD transfection reagent (Promega). Viral concentration was determined using the Lenti-X qRT-PCR titration kit (Takara Bio). Immortalized normal human astrocytes were transduced with mito-GFP lentivirus and selected for cells stably expressing mito-GFP using puromycin. D456 and JX22 cells were transduced with RFP lentivirus and selected using blasticidin S (Gibco) to select for stable RFP-expressing cells. Where indicated, cells were sorted for mito-GFP positivity or RFP positivity with assistance from the Flow Cytometry Core at the University of Alabama at Birmingham.

RFP-expressing D456 or JX22 cells were cocultured at a 1:1 ratio in the presence of mito-GFP⁺ astrocytes for 24 h. Samples were imaged at ×40 magnification with extended depth of field. Mito-GFP was acquired

on ch02, and RFP was acquired on ch04. Ch01 and ch09 were used for brightfield imaging, and ch12 was used for side scatter. Five thousand events were recorded, and relevant single-color and unstained controls were used. Data were analyzed using IDEAS software (version 6.2; EMD Millipore).

RNA-seq

mKate⁺ and mKate[−] SB28 cells and astrocytes from three distinct co-cultures (biological replicates) were sorted into multiple 1.5-ml DNA LoBind microtubes (Eppendorf), each containing 700 μl of RLT Plus lysis buffer (Qiagen) supplemented with 1% β-mercaptoethanol. RNA isolation was performed using the RNEasy Plus Micro kit (Qiagen).

RNA-seq and analysis were performed by GENEWIZ. Briefly, samples were sequenced using an Illumina HiSeq, with a 2 × 150 base pair configuration and ≥350 million raw paired-end reads. An average of 41.6 million paired-end reads was sequenced across nine samples. After Illumina universal adapters were trimmed, the reads were mapped to the *Mus musculus* GRCm38 reference genome using the STAR aligner v.2.5.2b. Unique gene hit counts were calculated by using featureCounts from the Subread package v.1.5.2.

For comparison of tumor cells with astrocytes, genes with an adjusted *P* value of <0.05 and absolute log₂ (fold change) of >1 were called as differentially expressed genes (DEGs) using DESeq2. For assessment of differentially upregulated pathways in mKate⁺ versus mKate[−] SB28 cells, genes that were upregulated >1.5-fold with a count number >50 and an unadjusted *P* value of <0.05 (Supplementary Table 1) were plugged into <https://maayanlab.cloud/Enrichr/>.

Protein–protein interactions and network visualization

Differential expression analysis was performed using edgeR 3.34 (ref. 65). Genes with a count per million greater than 1 in at least two samples were used for the analysis. *P* values of <0.05 were considered significant. The mouse DEGs were mapped to the human homologs using the NCBI HomoloGene database (<https://www.ncbi.nlm.nih.gov/homologene>). We then performed the enrichment analysis using Enrichr⁶² for the entire set of DEGs and for the up- and downregulated genes separately.

The protein–protein interactions among the DEGs were extracted using a human protein interactome we built previously⁶⁶ that contains 17,706 protein nodes and 351,444 protein–protein interaction edges. We then visualized this protein–protein interaction network using Cytoscape 3.8 (ref. 67). Genes that localize to mitochondria are indicated by a diamond node shape based on the Human MitoCarta2.0 database⁶⁸.

Cell cycle analysis

Single-cell suspensions (from mouse tumors or cultured cells) were fixed in PBS with 4% PFA for 1 h on ice and washed with permeabilization buffer (Foxp3/transcription factor staining buffer set; Ebioscience). Subsequently, cells were stained with Hoechst 33342 (Thermo Fisher) at 3.33 μg ml^{−1} in PBS for 1 h at room temperature, washed with PBS supplemented with 2% bovine serum albumin and assayed by flow cytometry.

Mitochondria isolation

Mitochondria were isolated from confluent immortalized human astrocyte cultures expressing mCherry by using the mitochondria isolation kit for cultured cells (Thermo Fisher Scientific), as per the manufacturer's instructions. Mitochondria were quantified by total protein using the Qubit protein assay kit (Thermo Fisher), according to the manufacturer's protocol.

Quantification of phospho-histone H3 and cleaved caspase-3 immunofluorescence

To quantify elements within the TME, tissue was examined from three animals per biological sex per mitochondrial transfer status. For a

given animal, 15 representative images were captured (5 images $\times$ 3 tumor-bearing sections). Cellular proliferation was quantified by counting each phosphorylated histone H3⁺ nucleus in each visual field. This count was divided by the fluorescence intensity of the GFP signal of the same field to account for variation in tumor size and cellular density to yield a mitotic index. The apoptotic cell death index was quantified by dividing the fluorescence intensity of the cleaved caspase-3 signal by the fluorescence intensity of the GFP signal in the same visual field.

MT quantifications

MT number was quantified manually using the NIH ImageJ software. DAPI and nestin-immunostained confocal images magnified $\times 200$ were used. For each experimental condition, a minimum of 80–120 MTs showing a direct connection between two tumor cells was included for measurement. All measured data were exported into Microsoft Excel and GraphPad Prism 8.1.2 for further calculation of statistical significance.

Statistics and reproducibility

For most in vitro experiments, a minimum of three biologically independent samples was used per experimental group; power was not calculated. For in vivo experiments not involving tumor initiation capacity, sample size was determined based on minimum utilization of vertebrate animals and high expected magnitude of effect without formal power evaluation. For in vivo experiments involving tumor initiation analysis, sample size was determined by prior experience and application of this assay in stem cell biology studies, for example, in Karunanithi et al.⁶⁹. No data were excluded from analyses. Key findings were replicated across time, by different institutions, using diverse models. For survival/tumor initiation studies, mice were randomized before tumor implantation, and investigators were blinded to the grouping. Data distribution was assumed to be normal, but this was not formally tested.

Data representation and analysis

Flow cytometry data were analyzed and generated using FlowJo software (BD Biosciences, v10.7.2). Graphs were generated and statistical analyses were performed using Excel (Microsoft Office, v16.52) or Prism (GraphPad, v9.2.0) software. All measurements shown represent distinct samples, unless otherwise indicated. All statistical tests are two tailed and corrected for multiple comparisons, unless otherwise indicated.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Sequencing files have been deposited to Gene Expression Omnibus under accession number [GSE183004](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE183004). Metabolic pathway analysis was based on the KEGG human metabolic pathways database (October 2019; <https://www.genome.jp/kegg/pathway.html#metabolism>). Protein phosphorylation array data were mapped to pathways based on the GO biological process term data set (<http://geneontology.org/>). RNA-seq reads of mouse cells were mapped to the *M. musculus* GRCm38 reference genome (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001635.20/). The RNA-seq inferred protein–protein interaction network was constructed by mapping to human homologs using the NCBI HomoloGene database (<https://www.ncbi.nlm.nih.gov/homologene>). Genes encoding mitochondria-localizing proteins were identified with MitoCarta2.0 (<https://www.broadinstitute.org/files/shared/metabolism/mitocarta/human.mitocarta2.0.html>). All other data are available in the main text or the Supplementary Information. Source data are provided with this paper.

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