Investigating the effects of a TGF-β modifier locus on tumour metastasis

Bachelor Paper II

submitted at the

IMC Fachhochschule Krems

(University of Applied Sciences)

Bachelor Program

Medical and Pharmaceutical Biotechnology

By

Tanja Buchmann

External Supervisor: Eswari Dodagatta-Mari, PhD

Internal Supervisor: Dr. Elisabeth Hofmann

Acknowledgements

This thesis would have not been possible without the help Rosemary Akhurst, PhD and her whole lab team, who was not only giving me the chance to work in her lab but also guided me through my whole project. Especially I would like to thank, my external supervisor Eswari Dodagatta-Mari, PhD who made available her support in a number of ways.

I would also like to show my gratitude to the Biotechnology team at the University of Applied Sciences in Krems, especially to Dr. Harald Hundsberger, Dr. Barbara Entler and Dr. Christian Klein who supported me to help my internship place. And most importantly to my internal supervisor Dr. Elisabeth Hofmann, who helped me a lot with my thesis.

Lastly, I would like to thank the Marshall Plan foundation which gave me the financial support to do a 6 months internship in the US.

Abstract

Since several decades researchers study cancer as it is still one of the deadliest diseases in the world. One major reason is its ability to form metastases and thereby spread to distant organs. It is known that transforming growth factor beta (TGF-β) plays a dual role in cancer and an important role in angiogenesis and immunity. As a result genetic modifiers of the TGF-β pathway have gained interest of many research groups, as the modifier could be an important factor in tumour metastasis. Our focus was on the genetic modifier *Tgfbm3* located on chromosome 12 in mice (and chromosome 2 in humans), which has polymorphisms between the NIH in line 4 and the C57 in line 6. These polymorphisms lead to different enzymatic activity of the metalloproteinase Adam17 with its respect to the cell surface shedding of the TGF-β receptor type I. Consequently, the TGF-β pathway is upregulated in line 6 as the hypoactive form of ADAM17 cannot shed the receptor efficiently and therefore the signal is not down-regulated We suggested that, this variation ADAM17 may alter the number of tumor metastases and the extent of angiogenesis in line 6 compared to line 4 mice.

To find a possible answer to our hypothesis we genotyped F2 littermates generated from an F1 4A/6A intercross, in order to distinguish between homozygous 4A and 6A offspring from the same litters. Mice were injected with CarB mouse carcinoma cells into the tail vein. After 10 days, we harvested the lung tissue, which was inflated and fixed by 4% paraformaldehyde (PFA) overnight. Afterwards it was embedded into paraffin to create an FFPE tissue, of which 5µm sections were cut and stained by H&E. The parameters which we compared between the lines were the number of macrometastases, the number of micrometastases and the extent of angiogenesis inside of the lung.

When comparing the two lines there was no significant difference, however, we can conclude that there is a tendency towards more macrometastasis and higher extent of angiogenesis in line 4 compared to line 6. This conclusion is strengthened by comparing the data previously generated data from the lab. The conclusions are the opposite of our generated hypothesis, the reason could maybe be that Adam17 also

plays a role in shedding tumour-necrosis factor alpha (TNF-α), cell adhesion molecules and other cytokines.

Table of Contents

1 Introduction 1
1.1 TGF-β signalling pathway: 2
1.2 Role of TGF-β in cancer and metastasis: 6
1.3 Role of Adam17 in different Regulation of TGF-β: 8
1.3.1 Activation and Function of the Metalloproteinase Adam17: 9
1.3.2 Effects of Adam17 on CEPCs: 11
1.4 Hypothesis: 12
2 Materials and Methods: 13
2.1 F2 litter mates: 13
2.2 Hot Shot Tail Preparation and DNA extraction: 14
2.3 Identifying Adam17 polymorphisms: 14
2.3.1 PCR reaction: 14
2.4 Digest of the PCR product with XmnI: 15
2.5 Analysis with a 1.5% agarose gel 17
2.6 Process of making CarB LM cells 17
2.7 Tail vein injection into the mice 18
2.8 Harvesting of the lungs 18
2.9 Processing of the tumours for paraffin embedding 19
2.10 Analysing of the H&E stainings: 20
3 Results: 21
3.1 Genotyping of the F2 mice: 21
3.2 Part 1 – Experiment with the Non-litter mates: 23
3.3 Part 2 – Experiment with Litter mates: 27
3.4 Part 3 – Meta analysis of both experiments: 29
4 Discussion: 31
4.1 Genotyping and Evaluation of the number and extent of angiogenesis: 31
4.2 Experiment Part 1 – Litter mates: 33
4.3 Experiment 2: 34
4.4 Meta-analysis of both experiments: 35
4.5 Conclusion and Outlook: 36

List of Figures and Illustrations

Figure 1 – The structure of the type II receptor. 2
Figure 2 – The activation of the latent TGF-β complex. 4
Figure 3 – The SMAD dependent TGF-β signalling pathway in mammalians. **Fehler! Textmarke nicht definiert.**
Figure 4 – The difference in the Tgfbm3b locus on the mouse chromosome 12. 9
Figure 5 - Function of the metalloproteinase Adam17. 10
Figure 6 - Relative CEPC level after the addition of different inhibitors, named TMI-005 (inhibition of ADAM17) and Ly2109761(inhibition of TGF-β receptor type 1). 12
Figure 7 - Visualisation of the crossing of 4A and 6A WT mice. 13
Figure 8 - Restriction sites of the XmnI restriction enzyme. 17
Figure 9 - Examples of the harvested lung tissue. 19
Figure 10 - Examples of the Genotyping results. 21
Figure 11 - Example of an H&E staining slide of a 6A mouse. 22
Figure 12 - Example of an H&E staining slide of a 4A mouse. 22
Figure 13 - Results of the Number of metastasis present in the lungs of 4A and 6A mice. 23
Figure 14 - Results from the comparison between Female and Male mice (non-litter mates). 24
Figure 15 - Results from the percentage that the metastasis occupy from the total lung are. 25
Figure 16 - Results when comparing the number of metastasis between the oldest and the youngest mice in used in this experiment. 26
Figure 17 – Results when comparing the percentage that the metastasis occupy from the total lung area between the oldest and the youngest mice used in this experiment. 26
Figure 18 - Results of the number of metastasis of 4A and 6A mice (litter-mates). 27
Figure 19 - Results of the comparison between female and male mice (litter-mates). 28

Figure 20 - Results of the percentage that the metastasis occupy from the total lung area. 28
Figure 21 – Results of a semi-meta-analysis of the number of metastasis in 4A and 6A mice. 29
Figure 22 - Results of the percentage that the metastasis occupy from the total lung area. (4A vs 6A Non-litter and litter mates) 30
Figure 23 -Results the metastasis/lung percentage between the line 4A and line 6A by Dominique Meyer, PhD. 37

List of Abbreviations

- Acts …… Activins
- BMP …… Bone morphongenic protein
- CEPC …… Circulating endothelial progenitor cell
- CEPs …… Circulating endothelial progenitor cells
- EDTA …… Ethylenediaminetetraacetic acid
- EtOH …… Ethanol
- FFPE …… Formalin-fixed, Parafin-embedded
- H&E staining …… Hematoxylin and eosin staining of tissue and cell sections
- H_2O …… Dihydroxyoxygen
- I-SMADs …… Inhibitory SMADs
- LAP …… Latency associated peptide
- LLC …… Large Latent complex
- LTBP …… Latent TGF-β binding protein
- NaOH …… Sodium hydroxide
- PCR ……Polymerase Chain Reaction
- PFA …… Paraformaldehyde
- ROS …… Reactive Oxygen species
- SLC …… Small Latent Complex
- TGF-β …… Transforming Growth Factor β
- TNF-α …… Tumour necrosis factor alpha
- Tris-HCl …… Tris hydrochloride
- VEGF …… Vascular endothelial growth factors

1 Introduction

According, to the WHO 8.2 million people died from cancer in 2012, and the number rises from year to year. Additionally, the number in new incidences rose dramatically over the last years. Consequently, cancer is one of the leading causes of death worldwide. The most deadly aspect, about the disease is its ability to metastasize, which means that the tumour cells are able to spread via the lymphatics and blood-stream to distant bone tissue or vital organs. For example a tumour who grew inside of the lung can metastasize into the liver and vice versa. Furthermore, the tumour cells can modulate the micro environment to make it more conducive to tumour spread. Currently it is not established which somatic mutations and genomic rearrangements drive this effect on the tumour microenvironment, but it is proven that the TGF-β pathway is a major player in driving tumour progression and metastasis of most solid tumours.

Not only in metastasis, but in a lot of important processes inside of a cell, TGF-β is one of the major drivers. Examples include cell growth, differentiation, apoptosis, phagocytosis and cellular homeostasis. In addition, TGF-β is also required for proper development of several organs like heart and lung and regulates also the innate immune system and the process of wound healing.[1]

As mentioned before TGF-β mostly awoke the interest of many researchers, as it influences and controls many important mechanisms in cancer such as cancer cell metastasis. Consequently targeting its signalling pathway has promising effects for the treatment of cancer. However, potential drugs have to be highly specific as TGF-β has paradoxical effects in cancer. It is in early stages tumour suppressive and in later stages tumour promoting. Why and what causes this switch inside of a tumour cell, has not been fully understood so far. Only suggestions have been made, one is that a radical change is caused within the tumour environment which can be driven by the tumour itself.[2]

During my project, I was working with the genetic modifier *Tgfbm3* located on chromosome 12 in mice and on chromosome 2 in humans. The locus comprised several

genes of which I focused specially on Adam17, which has an effect on the regulation of the TGF-β pathway. Furthermore, the locus also influences on the number of circulating endothelial progenitors (CEPCs) which in turn may affect the extent of angiogenesis.

1.1 TGF-β signalling pathway:

The Transforming growth factor beta (TGF-β) molecule belongs to the family of dimeric polypeptide growth factors (TGF-β superfamily) which includes also bone morphogenic proteins (BMPs), Activins (Acts), Inhibins (Inhs) and growth and differentiation factors (GDFs). The BMPs regulate embryonic patterning, whereas the Acts and Inhs are known for their regulation of pituitary hormones. Müllerian inhibiting substance, is another member of the TGFβ superfamily that is involved in gender specific development of the sex organs, and myostatin, another superfamily member is a negative regulator of muscle growth.[1]

The TGF-β signalling pathway itself is mediated activated by three different ligands, named TGF-β1, -β2 and -β3, which bind to the two TGF-β receptors, type 1 and type 2. Both receptors show a similar structure and consist of a C-terminal protein kinase domain and a N-terminal ectodomain. The ectodomain consist of a fold nine-β-strands and one single helix which gets stabilized by six intra strand disulphide bonds. The folding of the receptor is clearly structured into five central stranded antiparallel beta sheets, eight residues a-long its centre and on top there is a second layer of antiparallel beta sheets.[1] (See Figure 1)

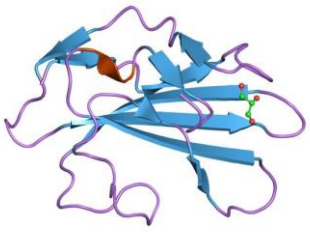

Figure 1 – The structure of TGF-β type II receptor. The folding of the receptor is clearly structured in five central stranded antiparallel beta sheets (blue arrows), eight residues a-

long its centre (green and red dots), which gets covered by a second layer of antiparallel beta sheets.[1]

Although the intracellular TGF-β signalling pathway has been highly characterized, only a little is known about how the ligands are activated, as this can be cell or tissue specific. The latent TGF-β activation can be mediated by integrins or other factors including proteases, pH and reactive oxygen species. The TGF-β molecule itself gets secreted in a latent form by a lot of different cells, for example macrophages. The bioactive dimer is encased within a complex of the latency-associated peptide (LAP), which is bound to TGF-β binding protein (LTBP).

The primary TGF-β polypeptide, consists of the NH2 terminal signal peptide (prere-gion), the latency associated peptide or LAP (proregion), the furin cleavage site and the carboxy terminal mature peptide. The LAP is covalently released from mature ligand dimer by furin cleavage, but the two remained non-covalently attached to each other in an inactive Small Latent Complex (SLC) (Fig. 2). The SLC gets se-creted after covalent binding to the Latent TGF-β Binding Protein (LTBP), which together form the Large Latent Complex (LLC). The TGF-β LAP nevertheless plays an important role in activation of the ligand, as it stays non-covalently encasing the mature ligand and covalently attached to the LLC within the extracellular matrix, waiting for its activation. Mutations of either the LAP or LTBP can result in improper activation of TGF-β signalling and leads to several severe diseases.[3]

Activation of TGF-β (i.e. release from the SLC or from the LLC) can be either be Integrin-dependent or Integrin-independent. Integrin-dependent activation can be performed with or without proteolytic cleavage. The activation by proteases includes the Matrix Metalloproteinases 2 and 9 which are brought with the help of Integrin αVβ36 and αVβ3 in close proximity with the latent TGF-β complex. In the next step the latent complex gets degraded and the TGF-β ligand is set free. The activation by conformational changes is mainly found in epithelial cells and does not include any Metalloproteinases. The αVβ6 integrin binds to the RGD motif which is present in LAP-β1 and its isoform LAP-β3 and induces adhesion-mediated mo-lecular forces that release the TGF-β molecule from its latent complex.[3]

The Integrin-independent activation pathway was mainly found by setting up different conditions during experiments in the lab, which would not represent natural conditions inside of the cell. Some examples would be the activation by pH, reactive oxygen species (ROS) and the activation by thromobospondin-1.[3]

An increased or decreased activation of the TGF-β molecule can lead to severe diseases like the Camurati-Engelmann disease (CED), where the patients suffer from thickening of the shafts of the long bones which leads to pain in both muscle and bone. It is caused by mutations in the prodomain of TGF-β1.[3]

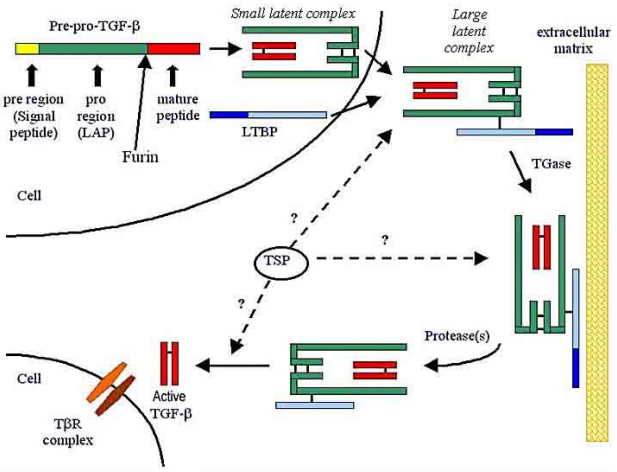

Figure 2 – The activation of the latent TGF-β complex . The pre-TGF-β molecule consists of the signal peptide (yellow bar), the pro region (or LAP, green), Furin and the mature peptide (red). It is located in the Small latent complex, which stays inside of the cell until it binds to the LTBP, which changes it then into the Large latent complex (LLC). The LLC stays in the extracellular matrix until protease(s) together with Integrins activate the TGF-β molecule by cleavage. The now activated TGF-β molecule can bind then to the type II receptor and thereby activate the pathway.[3]

After the activation of the ligand, it binds to the type II receptor, which gets thereby activated and forms a heterotetrameric complex with the type I receptor. Most of the time the two receptors are not in direct contact with each other but are brought into close proximity by the bound ligand. The kinase of the type II receptor is constantly active and phosphorylates the type I receptor, which leads to type I receptor activation. The signal gets than further transmitted over either the SMAD-dependent or SMAD-independent pathway into the nucleus. In the SMAD-dependent pathway the SMADS not only transduce the signal but also act directly on the target genes.[4]

The activation of the receptor type I leads to the recruitment of the receptor-regulated R-SMADs: SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8, which get phosphorylated on two Serine residues at their extreme C-termini. This leads to the formation of a complex with SMAD4, which travels from the cytoplasm into the nucleus where it has either positive or negative effects on the regulation of the target genes. SMAD4 is a weak DNA binding transcription factor, and binding of the SMAD complex to DNA is specified not only by which Smads are involved (SMAD-2,-3 in response to TGFβ and activins, and SMAD-1,-5,-8 in response to BMPs), but by SMAD-associated transcription factors, that confer specificity of DNA binding and transcriptional responses (whether positive or negative).[5]

The R-SMADs as well as SMAD4 have two highly conserved domains, named MH1 and MH2, which are separated by a less conserved Pro-rich linker region. With the help of the MH1 domain the R-SMADs bind directly but weakly to DNA, whereas the SMAD2 cannot directly bind as an extra exon disrupts its DNA-binding domain. The MH2 domain is responsible for SMAD-receptor interactions which include, SMAD-SMAD interactions and SMAD interactions with transcription factors, co-activators as well as corepressors. The SMADs themselves are not able to directly recruit the basal transcription machinery, but do so through other transcription factors. Recent data also suggest that they can remodel chromatin and modify histones which leads which can alter gene expression epigenetically. [5] (For an overview of the whole pathway see Figure 3).

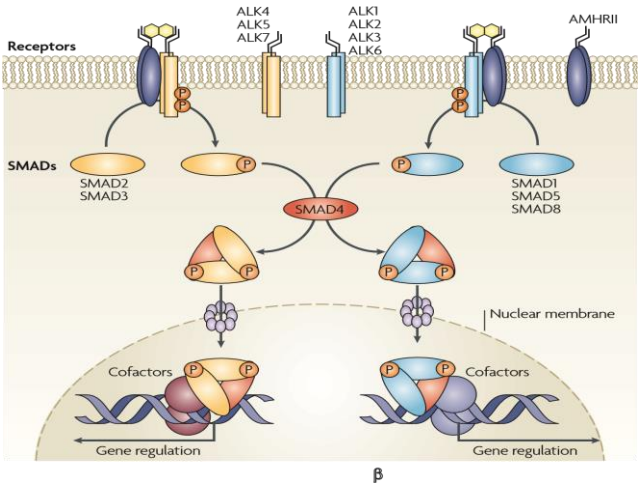

Figure 3 – The SMAD dependent TGF-β signalling pathway in mammalians. The activated TGF-β ligands bind to the type II receptor which gets thereby activated and formats a heterotetrameric complex with the type I receptor which causes its phosphorylation and thereby activation. The phosphorylated type I receptor recruits than SMAD2 and SMAD3 which from, after their phosphorylation and thereby activation, a compelx with SMAD4. The complex integrats then into the nucleus and binds there with different cofactors to the DNA, which leads to either up- or downregulation of the target genes.[4]

The SMAD-independent pathways are only a few in number which additionally regulate cellular functions. Those few get either activated by ligand-occupied receptors to intensify, weaken or regulated several cellular downstream processes including MAP kinases, Rho-like GTPases and phosphatidylinositol-3-kinases/AKTs.[6]

The inhibition of the TGF-β signalling pathway, which is also a termination of the pathway, are induced by a different type of SMADs, the inhibitory SMADs (shortly I-SMADs), SMAD6 and SMAD7. They inhibit the signalling pathway by direct or indirect mechanisms as for example, binding to the type I receptor to prevent the binding of the R-SMADs and thereby stop the transduction of the signal. As their role was not fully understood in physiological conditions most of the functions have been found based on overexpression studies, using RNA interference (RNAi).[4]

1.2 Role of TGF-β in cancer and metastasis:

Since cancer is still one of the deadliest diseases in the world, many research groups try to understand and make a full picture of the biology of cancer to develop potential drugs not only to treat but also to prevent cancer. As the TGF-β signalling

pathway is an important player in many different mechanism in the cell, therefore the dysregulation (either increased or decreased production of TGF-β) has fatally consequences. It can cause severe diseases like cancer, atherosclerosis and fibrotic diseases of the kidney, liver and lung. Additionally, mutations in the genes which express important proteins for the signalling protein or its receptors have been linked to diseases like hereditary haemorrhagic telangiectasia, or HHT.[7]

In healthy cells TGF-β signalling pathways purpose is to arrest the cell cycle at the G1 stage, which should on the one hand inhibit proliferation and on the other hand promote differentiation or induce apoptosis. In cancer cells, all the mutations which cause an altered TGF-β pathway, lead to resistance to growth inhibition of TGF-β, which consequently leads to uncontrolled proliferation of the cells.[8]

Although a lot is known about the TGF-β pathway there is only little known about its dual role in the context of cancer and metastasis. In early stages of tumour progression it has tumour suppressive characteristics, whereas in contrast in later stages it has tumour promoting characteristics. Why and when this change of its role happens is until now not fully understood. An example for its tumour suppressive characteristics would be the inactivation of the TGF-β type II receptor. It leads to tumours with little or no pathology in many different organs in different mouse models, which encourages the belief in its tumour suppressive effects in early stages. In contrast, it has also been shown that an overexpression of the inhibitor Smad-7 can induce carcinogenesis. Moreover, it has also been proven in different experiments that tumours can release compounds systematically which inhibit the TGF-β pathway and lead to loss of host-tumour interactions which were regulated by TGF-β, which includes not for example immune evasion.[9]

As mentioned above the deadliest characteristic of cancer is its ability to form metastasis and thereby spread throughout the body via the blood or lymphatic vessels. During metastasis, tumour cells must detach and get into the blood or lymphatic system by intravasation. They then travel through the body and extravasate into a distant organ parenchyma. There they have to avoid detection by the immune system in order to survive and grow. For this process a very important player is the

tumour microenvironment as it promotes metastasis and even promotes the formation of new blood vessels (vasculogenesis).

In various type of tumours, such as those of the gastrointestinal tract, , mutations of the TGF-β pathway have been found and this loss of signalling promotes cancer, whereas and in other tumor types, the signalling pathway is overexpressed. This is especially true in very aggressive and highly proliferating gliomas, a high expression level of TGF-β has been found in patients. It is also seen as a very important player which regulates inflammation by the immune cells and orchestrates the tumour microenvironment. In many types of cancer with an increase in tumour progression and tumour metastasis a deletion of the TGF-β type II receptor has been found. Moreover, deletions in SMAD4 have been found in a colon cancer model which had an increased number of CCR1+ myeloid cells (CD34+) which lead to a promotion of tumour invasion.[10]

1.3 Role of Adam17 in different Regulation of TGF-β:

The effects of the TGF-β signalling pathway are not only context dependent, as seen in cancer, but also differ due to germline genetic variations between individuals. The phenotype can either show an upregulated or downregulated pathway.

The genetic modifier locus, *Tgfbm3,* has effects on TGF-β which in turn have an effect on cancer susceptibility, especially angiogenesis. It has been proven that variations of the modifier locus *Tgfbm3*, which is located on the mouse chromosome 12, can determine the level of intracellular TGF-β signalling.[11] To evaluate this effect we compared the congenic NIH mice that inherit different extents of C57BL/6J DNA over the *Tgfbm3 locus.* In line 4A *Tgfbm3* is of the NIH haplotype, whereas in line 6A it is derived from C57. Genetic variations between C57 and NIH at *Tgfbm3* not only have an effect on the TGF-β signalling pathway but also on the number of circulating endothelial progenitor cells (CEPs). (See Figure 4 - A) Furthermore the *Tgfbm3b*^{C57} locus can suppress prenatal lethality of TGFβ1-/- embryos, as seen in Figure 4 – C, wherein the percentage of normal TGFβ1-/- alive and normal at birth is higher in line 6 compared to line 4.[11]

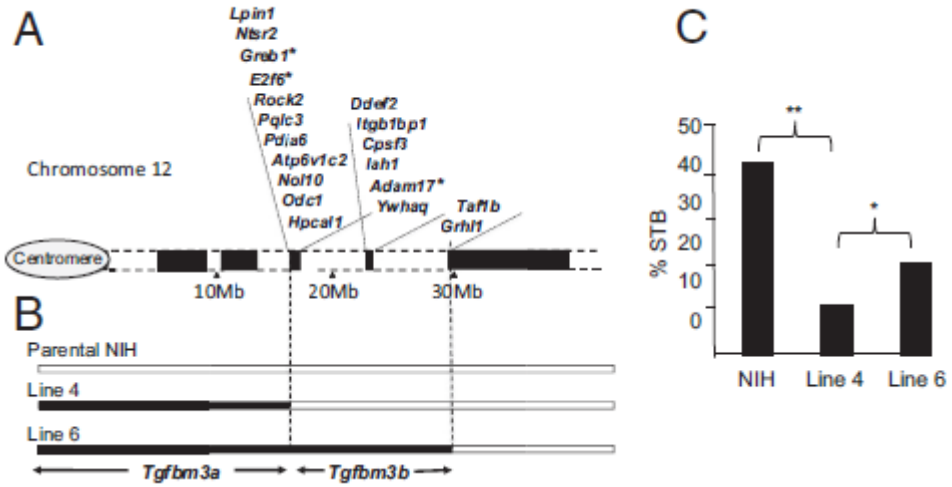

Figure 4 – (A) The difference in the Tgfbm3b locus on the mouse chromosome 12. On the mouse chromosome 12 the gene Adam17 is beside other genes like ASAP2, which encodes for Ddef2, located. The broken line represents repetitive DNA, whereas the dark spots are unique DNA. Bothe mice strands are NIH mice, whereas in the 4A the Tgfbm3b locus is absent and in the 6A the locus is bigger as it comes from the C57 mice.[11] (B) Due to the locus Tgfbm3b prenatal lethality of TGF1-/- gets suppressed, as seen in the graph as in line 6 the percentage of birth is much higher than in line 4.[12]

The variations on the locus lead to amino acid polymorphisms not only in the Adam17 gene but also in some other genes, such as *Asap2* which encodes DDEF2 proteins. Thus ADAM17 may not be the only variant protein that leads to the *Tgfbm3* modifier effect.

1.3.1 Activation and Function of the Metalloproteinase Adam17:

The gene *Adam17* encodes the metalloproteinase Adam17 (a distinegrin and metalloproteinase) which is a Zn^{2+} dependent modular cell surface protein. It belongs to the family of ADAM enzymes, which in turn belong to the family of adamalysin proteins. It consists of 824 amino acids and was discovered in 1997 by its activity in releasing soluble tumour-necrosis factor alpha (TNF-alpha) from its membrame bound precursor form. It is expressed at high levels in various tissue types and its expression is changing between embryonic development and adult life.[12]

The protein consists of multiple domains including the signal sequence (1-17aa), the prodomain (18-214aa) and a metalloenzyme, also called catalytic domain (215-473aa), a disintegrin domain (474-572aa), a cysteine-rich domain (603-671aa), a

transmembrane domain (672-694aa) and finally a cytoplasmic tail (695-824aa). It has very little in common with other ADAM proteins. Its closest relative is ADAM-10, and both of these proteases are inhibited by pharmacological inhibitors of ADAM17, despite the amino acid homology between the two being less than 30%.[12]

The most famous function of the catalytical activity of the ADAM proteins is the cleavage of ectodomains of various transmembrane proteins. Adam-17 for example can cleave the HB-EGF receptor which, consequently activates EGFR and initiates cell proliferation. However, another important function is the cleaving of receptors from the cell surfaces, since “ectodomain shedding” can actually stop the ligand-initiated signalling and therefore regulate cellular signalling such as TGF-beta.[12]

Adam-17 gets activated by the cleaving of the prodomain by furin, which is a pro-protein convertase present in the trans-Goldgi network. The prodomain serves as an inhibitor of ADAM17 during its translation intracellularly. Furin cleaves at the last 4 amino acids which precede the catalytic domain. Active Adam17 is then able to shed the TGF-beta receptor type I ectodomain which leads to its release into the extracellular space. This leads to the downregulation of the TGF-beta pathway as heterotetrameric receptor complex formation is lost and intracellular signalling is blunted. The hypoactive version of Adam17 (C57 Adam17) is not able to efficiently shed the receptor, therefore there is no ADAM17 feedback on the signal, which leads to upregulation of the TGF-beta pathway in the cell.[13](See Figure 5)

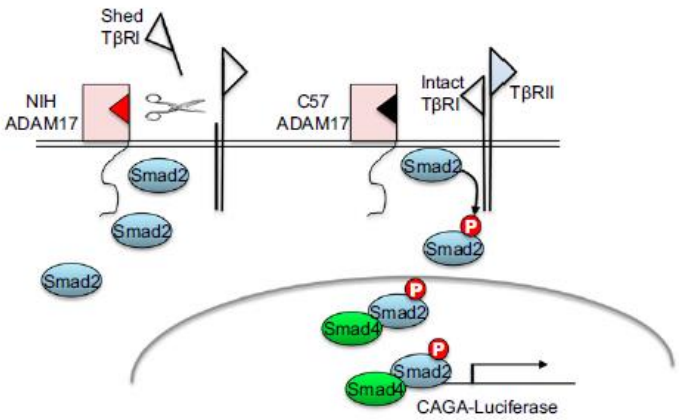

Figure 5 - Function of the metalloproteinase Adam17. The Metalloproteinase Adam17 sheds, in its active form (NIH ADAM17), the TGF-β type I receptor which leads to an inhibition of the transmittion of the signal. In the C57 Adam 17 form, it is hyperactive and as a

result not able to shed the type I receptor, which consequently leads to the transmittion of the signal and an upregulation of the pathway.[11]

Interestingly the closely related ADAM10 is also a sheddase for TGF-beta receptor type I. When using an ADAM10 inhibitor it was proven that the expression of TGF-beta receptor type I go increased on the cell surface and decreased in the extracellular space.[12]

Adam17 not only plays a role in the context of the TGF-β pathway and therefore in the role of cancer but also in brain repair, Alzheimer's disease, Multiple Sclerosis and heart diseases. As it is expressed by most mammalian cells and plays a role in so many diseases it is an enzyme worth investigation more deeply. Problematic is only the dual role of ADAM-17 as its effect can be on the one hand beneficial from some tissues or diseases and on the other hand harmful to others. Especially inhibitors have to be highly specific to block only ADAM-17 and not other metalloproteinases and moreover it should just block it in a specific context. [12]

1.3.2 Effects of Adam17 on CEPCs:

Furthermore, Adam17 has also effects on the number of circulating endothelial progenitor cells (CEPCs), which are in close relation with angiogenic potential.[11] Tumour vascularization is not only a result of angiogenesis, during which new blood vessel arise from already existing with the help of endothelial cells profileration and migration. It is also caused by vasculogenesis, during which CEPCs, which get secreted by the bone marrow, are differentiated into mature endothelial cells to form new blood vessels. The secretion of CEPCs is dependent on the activation of different metalloproteinases like Adam17 and a higher level of adhesion molecules, which is driven by vascular endothelial growth factors (VEGF). It has been for example proven that with the inhibition of Adam17 (with TMI-005) and/or the inhibition of the TGF-β type I receptor (LY2109761), the level of CEPC varies. In normal conditions the level of CEPC are relatively low in line 4 and line 6, although in line 6 slightly higher. But when adding both inhibitor the level is already increased. But the highest level of CEPC is reached when ADAm17 is blocked by TMI-005 but the type I receptor stays active, which consequently proves the relation between activity of Adam17 and number of CEPC present. (See Figure 6) Especially in lung cancer

CEPCs are present in a high number and correlate with the stage of the disease, as a higher number results in a higher development of angiogenesis.[14]

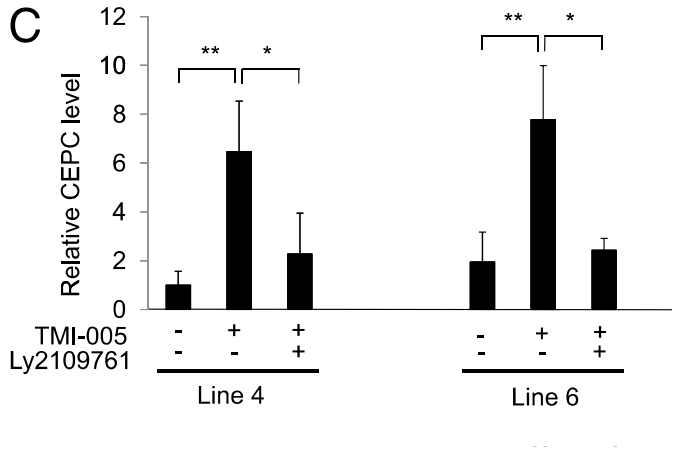

Figure 6 - Relative CEPC level after the addition of different inhibitors, named TMI-005 (inhibition of ADAM17) and Ly2109761 (inhibition of TGF-β receptor type 1). In both line is in normal conditions (absence of both inhibitors) the level of relative CEPC relatively low although it is slightly higher in line 6 than in line 4. But when adding both inhibitors it is already increased. What is fasctinating is the fact that when blocking only ADAM17 with TMI-005 the level of CEPC is the highest (again slightly higher in line 6 than in line4), which proves the connection between Adam17 and the number of CEPC present.

1.4 Hypothesis:

As both, the effect on TGF-β and the effect on the number CEPs, lead to the hypothesis, that due to the effects that the amino acid polymorphism between the lines have, the in the line 6A there must be an higher extent of angiogenesis and also when counting tumours the number must be higher in line 4.

2 Materials and Methods:

2.1 F2 litter mates:

The two strains which were compared in this project were the NIH in line 4A and the C57 in line 6A. The first part of the experiment was done with mice from two separate colonoes, on of homozygous 4A and one of homozygous 6A. To obtain more reliable and reproducible results, I also performed this experiment with the F2 generation of an 4A /6A F1 intercross to generate litter mates, that will be nearly genetically identical and exposed to the same prenatal and post-natal environment. All mice used were wildtype mice, differing only at the natural variants observed between C57 and NIH within the *Tgfbm3* locus.

F2 mice were generated by crossing first 4A and 6A wildtype mice to generate (4A/6A) F1 littermates that are heterozygous over the *Tgfbm3* locus. Those mice were then taken and intercrossed to generate a ratio of homozygous 4A/4A, F1.4A/6A and homozygous 6A/6A F2 mice. Theoretically the genotype ratios should be 1:2:1 according to the Mendelian Inheritance. (See Figure 7) Due to the identical appearance of these normal wild type mice, genotyping was an important method to distinguish between the genotypes.

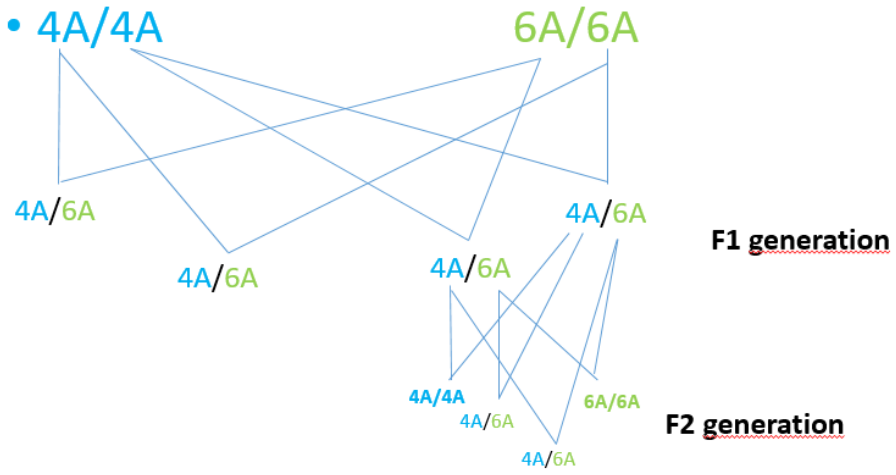

Figure 7 - Visualisation of the crossing of 4A and 6A WT mice. When Crossing 4A and 6A WTF mice, the F1 generation constists only of heterocygous mice (4A/6A). When crossing

then the F1 littermates with each other the F2 generation has the following genotypes 4A/4A, 4A/6A and 6A/6A in the ratio of 1:2:1.

2.2 Hot Shot Tail Preparation and DNA extraction:

For the genotyping of the mice, they were first put anesthetized with isoflurane and small pieces of the tail (approximately 2mm) cut and put into PCR tubes. It was very important to just take a very small piece of the tail in this method as the bigger the piece the more unnecessary material get extracted and can make the PCR impossible. 75µl of freshly prepared Alkaline Lysis buffer (5mL H_2O plus 7µl 50% NaOH plus 7µl of 0.5M EDTA) was added to each tube. Importantly, the tail is immersed in buffer with no air bubbles at the bottom of the tube. The tubes were then put into the PCR machine and removed promptly after 30 minutes incubation at 95°C followed by 15 minutes on ice (at 4°C). It was important to stop the lysis reaction after 30 minutes otherwise the DNA gets too degraded. To stop the reaction and avoiding the degradation of the DNA 75µl of the neutralization buffer (40mM Tris-HCl) was added to the tails and mixed briefly by using a sterile filter tip for each tail. The tail preps could then afterwards been used immediately for PCR.

2.3 Identifying Adam17 polymorphisms:

2.3.1 PCR reaction:

The set of primes which were used were specific to the polymorphism at amino acid 113 of the TACE protein, which is glutamine in NIH and arginine in C57. The specific PCR primers were TaceN113DF (5`ACC TCA GAT CCA GAG CCT CA-3`) and TaceN113DR (5´ - TGG GTG GCT ATT GTT CAT CTT – 3´). Those primers seemed to be most appropriate as they be could be used in combination with the Xmn1 restriction digest at a later step to distinguish between the Adam17 polymorphisms. An alternative set of primers would have been those that distinguish the polymorphism at amino acid 594, a valine to Isoleucine change. PCR primers: TaceV594IF (5´- GGG GCT ATC CAT CAC CAG – 3´) and the TaceV594IR (5´- CCT CCA GGG AAT AGC AGT GA – 3´) primers in combination with the NsiI restriction enzyme.

For the PCR reaction itself 5µl of the tail preps were used and 20µl of the PCR reaction mix which consisted of the following reagents.

10x Dream Taq Buffer (Lot00402477 – inclusive: 20mM $MgCl_2$)	2.5µl
dNTP (#N0447L – New England Biolabs – 10mM each dNTP)	0.5µl
Forward Primer (TaceN113DF)	0.5µl
Reverse Primer (TaceN113DR)	0.5µl
Taq Polymerase	0.2µl
dH_2O	15.3µl

After the adding of the reaction mix the following PCR conditions were used over 32 cycles:

1. 3 minutes at 94°C for the initialization step which is needed for DNA polymerases which require a hot-start PCR, so get activated over heat.
2. 30 seconds at 94°C, for the Denaturation step which causes the melting of the DNA during which the hydrogen bonds between complementary bases and single-stranded DNA molecules get destroyed.
3. 30 seconds at 60°C for the Annealing step which is used to allow the forward and reverse primer to anneal to the single-stranded DNA template.
4. 1 minute at 72°C for the extension or elongation steps. The temperature used depends on the type of enzyme used in the reaction, but the used Taq polymerase in the experiment is commonly used at 72°C.

After the 32 cycles the product should have stay for 10 minutes at 72°C. The final hold was set at 10°C to be able to store it for a short time but optimally it was used right away for the restriction digest to get good visible bands at the gel.

2.4 Digest of the PCR product with XmnI:

If a restriction digest is not performed the uncut PCR product would run at approximately 400 base pairs and one would not be able to distinguish the polymorphism

between 4A and 6A mice. We used the restriction digest Xmn1, as it worked very well in the combination with the primers used in the PCR reaction. The Digest reaction mix contained the following reagents and was put at 37° for 1 to 2 hours into the incubator:

XmnI (#R0194S – New England Biolabs – 20.000U/mL)	1µl
BSA 100x (#B9001S – New England Biolabs – 10mg/mL)	0.15µl
CutSmart (#B7204S – New England Biolabs – 10x Concentration)	1.5µl
H_2O	7.35µl

Theoretically, when the restriction digest did not work properly, we should get a band at 400nt or the 400nt band is still very distinctive. If the XMN1 is able to cut properly, the C57 allele (line 6A) should produce a product size of 136bp and 212bp. The NIH mice (line 4A) should have a product size of 136bp and 247bp, as the NIH include a single nucleotide polymorphism (SNP) that obliterates the XMN1 recognition site, thus the XMN1 cannot cut. The 400nt band can therefore be used as a control that both the PCR reaction and restriction digest worked. Positive controls of pure 4A, 6A, 4A/6A DNA were also included as well as a negative water only control. (See Figure 5)

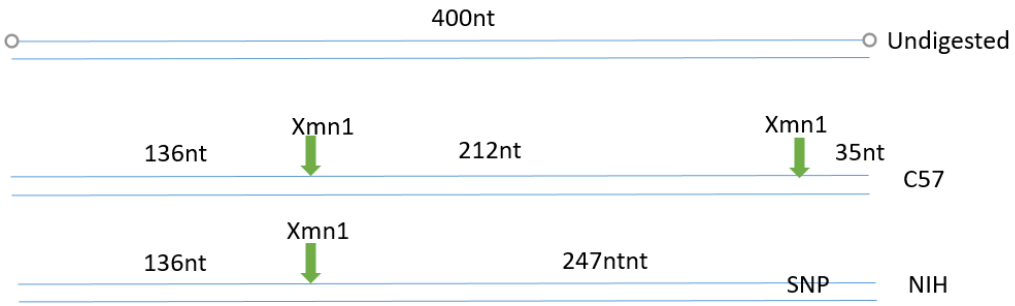

Figure 8 - Restriction sites of the XmnI restriction enzyme. The C57 mice will have a product 136bp and a 212bp, whereas in comparison the NIH mice will have a product size of 136bp and 247bp as due to the SNP the XMN1 is not able to bind. If the restriction digest was not working proberly only a band at 400nt or a very distinctive band at 400nt is pesent. The difference can be then clearly shown on a 1.5% agarose gel.

2.5 Analysis with a 1.5% agarose gel

To make the results from the PCR and restriction digest visible and to be able to differentiate better between the different genotypes a 1.5% agarose in 0.5x TBE (Tris/Borate/EDTA) buffer (1.5g/100mL/gel) was poured. As DNA stain GelRed was used, instead of Ethidium bromide as the latter is carcinogenic. For the loading of the gel we used, 8µl of the 100bp Ladder (#N32314 New England BioLabs – 500µg/mL) and 10µl of the restriction digest product, to which 1.5µl of purple Gel Loading Dye (#B7024S – New England Biolabs – 6x Concentrate) was added. For a good resolution the gel was run slowly at 120mV for about 1, 5 to 2 hours depending on the size of the gel.

2.6 Process of making CarB LM cells

The CarB-L mouse carcinoma lung metastasis cells which were used for my experiments, were generated by a former post-doc, Dr. Dominique Meyer, months prior to my joining the lab, by selection of CarB tumor cells that could undergo frequent lung metastasis. The CarB-L cells were then frozen down for later use.

CarB-L were generated by injecting parental CarB cells ($5*10^5$ cells/100µl), which rarely metastasize into ten mice via the tail vein. After several days the lung of the only sick mouse was harvested and the metastatic cells that grew out from the lung in culture were tested for metastasis by injection into 10 mice ($5*10^5$ lung metastasis cells/100µl) via the tail vein. All 10 mice got metastases in the lung with this CarB-L line, proving the metastatic ability of the cells.

2.7 Tail vein injection into the mice

To get vascular access into the mice and to make sure that the CarB mouse carcinoma lung metastasis cells reach and settle inside of the lung, Lateral Tail Vein injection has been performed. For this 28-30 gauge needles and a 300-500µl syringe have been used for the injection. Prior to anaesthesia the animal is put into a warming box to dilate the veins making them easier to find. Afterwards, the mouse was anesthetized, and $500*10^3$ cells in 100µl was injected into the tail vein. The vein is used in contrast to the artery to avoid local inflammation and to get the cells as fast as possible to their target, the lungs. As in the veins they get then directly pumped over the heart into the lungs where the intravasate and develop metastasis.

When preparing the cells for injection it was very important to minimize carryover of trypsin with the cells. On the one hand trypsin eases cell detachment form the plate, but on the other hand too much trypsin can cause the cells to clump and block the needle.

2.8 Harvesting of the lungs

On the 10^{th} day after the injection of the CarB LM cells, the lungs of the mice were harvested. The reagent for the fixation process was freshly made 4% paraformaldehyde out of a 16% paraformaldehyde stock. It was very important to work during this process in the chemical fume hood as paraformaldehyde is carcinogenic. It is mandatory to prepare it freshly as it can degrade quickly for formic acid when getting into contact with Oxygen.

The mice are anesthetized with isoflurane (not euthanized as it is important that the heart is still beating for the clearing of the lungs) and then pinned down, belly facing upwards. Afterwards the corpus gets opened up and the vein under the liver gets cut to have an exit during the clearing of the lungs. Afterwards one insert a butterfly needle over the right ventricle into the heart and inject two times 10mL PBS for washing (getting rid of the blood) and one time 10mL of 4%PFA to fix the lung tissue. As after this process the mouse is dead, one can remove the mask and cut the trachea free. Below the trachea a needle with a suture is thread through and a light knot tied around the trachea. With a normal syringe and needle approximately 10mL PBS were injected into the lung to inflate them. It is important that quickly after removing of the needle the knot gets drawn very fast to prevent liquid leakage out of the lungs. Afterwards the lungs were removed from the thorax and placed into 4% PFA for further fixation. The speed of the fixation depends on the type of tissue and the size, routinely the tissues are fixed between 24 and 28 hours. We decided to 24 hours at 4° while gentle agitation to equally distribute the 4%PFA on and into the tissue.

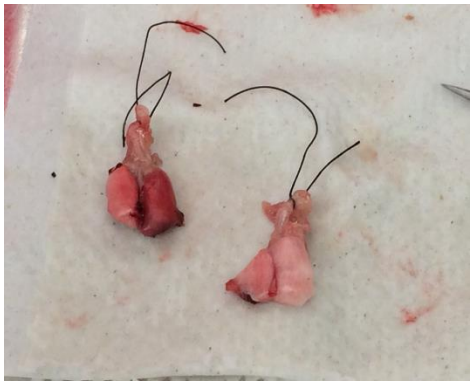

Figure 9 - Examples of the harvested lung tissue. In the picture above when can see the mice loungs after the harvesting and before the fixation step with 4% PFA. The lung on the left is much more bloody compare to the lung on the right would could be that because of the metastasis already inside of the lung the capillaries were knuckled and the neither the PBS nor the PFA were able to wash it.

2.9 Processing of the tumours for paraffin embedding

After 24 hours in 4% PFA the lung tissue is fully fixed and can be processed for the paraffin embedding. The tissue gets washed with PBS and then dehydrated through a series of 70% EtOH (30% in PBS, 50:50 in PBS, 50% in H_2O, 70% EtOH in H_2O),

about 5 minutes each with gentle agitation. Afterwards it can be either stored at 4°C or embedded into paraffin. For embedding in paraffin, heart and other tissue was removed from the five lobes of the lung, cut into separate parts to have a better embedding process and to have no overlaying when cutting sections out of the block.

After the embedding 5µm sections can be cut and stained with Hematoxylin and eosin staining (H&E staining) which is a widely used method for medical diagnosis. The combination of Hematoxylin and eosin produces blues, violets and reds.

2.10 Analysing of the H&E stainings:

After the H&E staining, the slides can be observed under a Dissecting Microscope and the number of metastasis determined. For the evaluation of the extent of angiogenesis high quality pictures of the slides have to be taken. With the help of ImageJ one can then determine the total lung area as well as the total area the metastasis. As we used the percentage for a comparison and not the are in mm^2 it was not important to determine the number of pixels which are equal one mm.

For the blotting of the Data we used the program prism and decided to draw a graph were every dot represents one dead mice for a better view of the distribution of either the number or the extent of angiogenesis.

3 Results:

3.1 Genotyping of the F2 mice:

By doing a restriction digest with the enzyme XMn1 after a PCR, we were able to distinguish the different genotypes of the F2 mice. As expected all of the 6A mice had a band at 136nt and 212nt (=4), whereas the 4A had a band at 246nt and 136nt (=3,5). The heterozygous ones had both bands present. (See Figure 5)

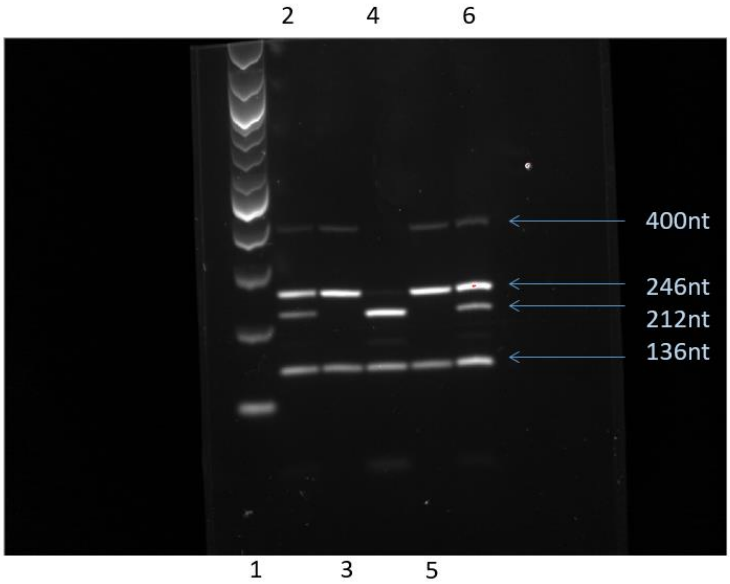

Figure 10 - Examples of the Genotyping results. (1)Maker. (2)4A/6A (3)4A (4)6A (5)4A (6)4A/6A

In total we used 30 wildtype mice of which 14 were 4A and 16 6A. Out of this 30 16 mice were female and 14 were male. For the second part of the experiment we used 27 F2 littermates of which 15 were 4A and 12 6A. Out of this 27, 17 were female and 10 were male.

After the injection of the cells into the mice, the lung tissue was harvested and embedded in paraffin to create FFPE tissue. Afterwards 5µm sections were cut onto microscope slides by a microtome and analysed by H and E staining. (See Figure 9) The following parameters were compared the number of Macrometastases, number of Micrometastases and the extent of the angiogenesis within metastasis (the percentage of area the metastasis occupy compared to the total are of the lung).

Furthermore, were not only 4A and 6A but also Female and Male without looking at the genotype, and different age groups compared (only in the WT mice).

There were not surface metastasis found on the lungs, but there were a lot of metastasis present inside of the lungs. When looking at Figure 9 and 10 one can clearly distinguish between the lung tissue (pink) and the metastasis (dark purple spots).

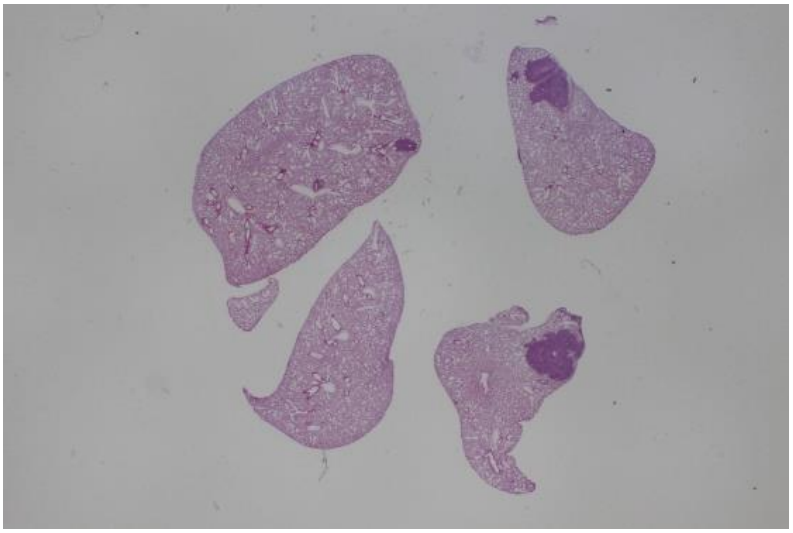

Figure 11 - Example of an H&E staining slide of a 6A mouse.

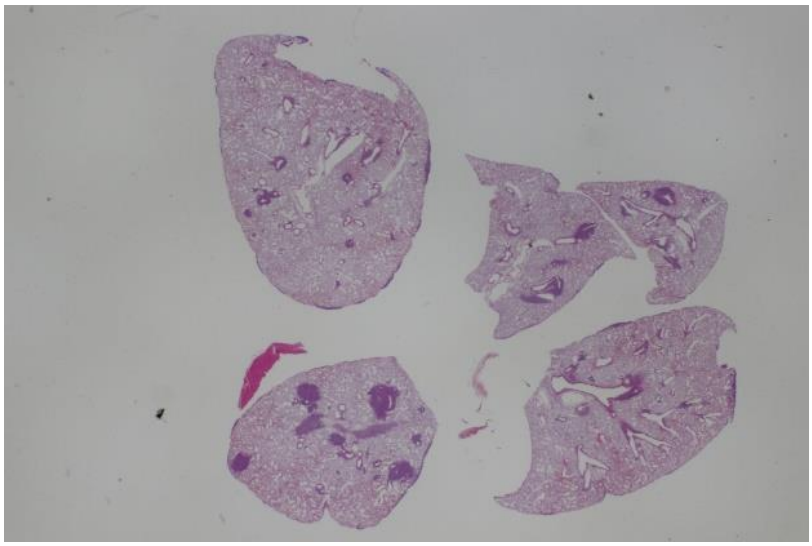

Figure 12 - Example of an H&E staining slide of a 4A mouse.

3.2 Part 1 – Experiment with the Non-litter mates:

In the first part of the experiment all injections were done in non-litter wildtype 4A and 6A mice. When comparing the number of metastases present inside of the lung it appears that the average is slightly higher in the 6A mice but in general the numbers are pretty similar. The only difference is that in the 4A mice 2 extremes with 14 and 17 tumours inside of the lung were present, whereas the average was around 5 per mouse. (See Figure 11)

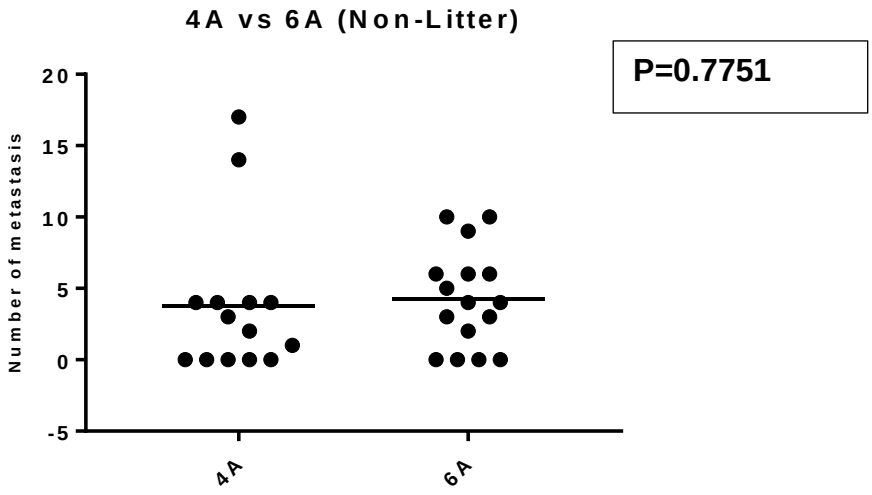

Figure 13 - Results of the Number of metastasis present in the lungs of 4A and 6A mice. Every dot in the graph represents one mouse, so one can compare the number of metastasis presents between 4A and 6A. Although the average is slightly higher in 6A (4.3 comapre to 3.7 in the 4A) there is no significant difference between 4A and 6A. The only slight difference is that the 4A mice have 2 extrems with 14 and 16 tumours in a lung where as the average value was 3,7 in the 4A mice.

When comparing female and male mice, we observed that there was a slight difference between the gender, as the female mice have a higher average (5.5 compared to 2.3) and furthermore they also have 5 outliers with 9, two times 10, 14 and 17 metastases. But nevertheless the numbers are not significant different.(see Figure 12)

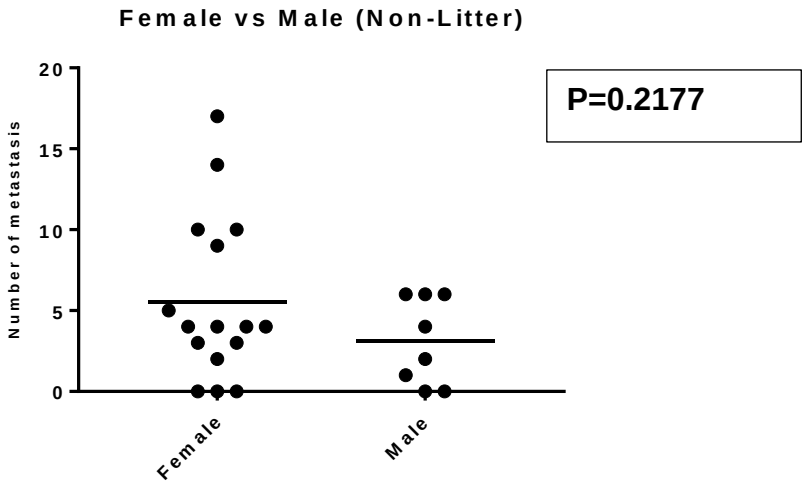

Figure 14 - Results from the comparison between Female and Male mice (non-litter mates). In this graph one dot represents one mice in the experiment and it was observed that the female mice, regardless of 4A or 6A mice have higher average (5.5) than the male mice (2.3). Furthermore, the 4A mice have five outliers (9,10,10,14,17 metastasis).

As the metastasis differ very much in size not just between 4A and 6A mice but also within the same lung, it was important to compare the difference of the percentage that the metastasis occupy of the total lung area, between the 4A and the 6A mice, as well as between female and male mice.

When comparing the 4A with the 6A mice in context of the percentage of lung that the metastasis occupied, there is no significant difference between the two, although the average of the 6A mice is smaller than the average of the 4A mice. (See figure 13).

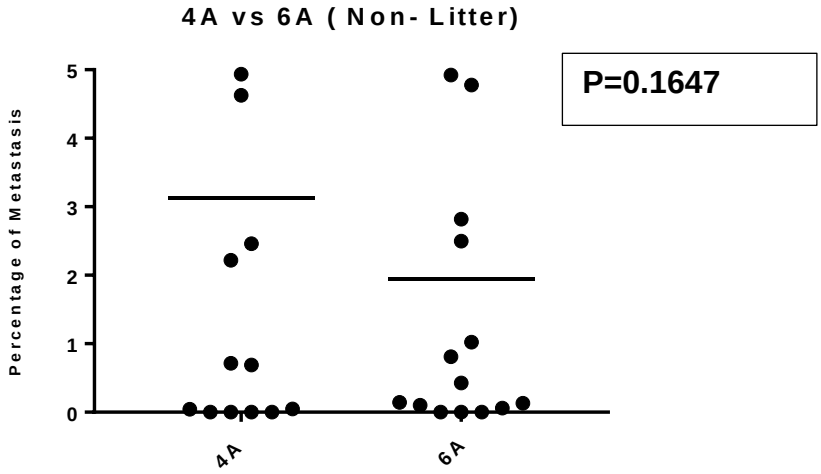

Figure 15 - Results from the percentage that the metastasis occupy from the total lung are. There is no significant difference between the percentage occupied by the metastasis between 4A and 6A mice although the average is higher in the 4A mice.

Another interesting point for comparison in this experiment was that, if the different age of the animals made a difference in the number of metastasis and in their size.

When comparing the number of metastasis present in the lung, between the oldest mice (07/20) and the youngest mice (09/16), it is visible that the younger ones had a higher average than the older ones. Especially in the older ones it is interesting to see that the numbers never got higher than 2.(See Figure 14) The difference between the youngest and the oldest mice got much smaller when looking at the percentage of area that the metastasis occupy from the total lung area. Here as already suggested by the low number in the oldest the percentage is nearly zero but also in the youngest ones the size was not big by barely more than 1%. (See Figure 15)

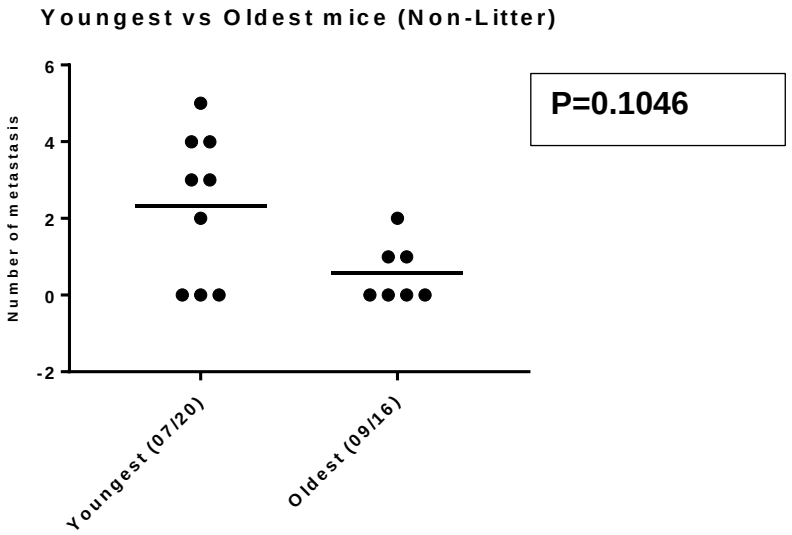

Figure 16 - Results when comparing the number of metastasis between the oldest and the youngest mice in used in this experiment. When looking at the number of metastassi present in the lung one can see that in the youngest mice were more metastasis present.

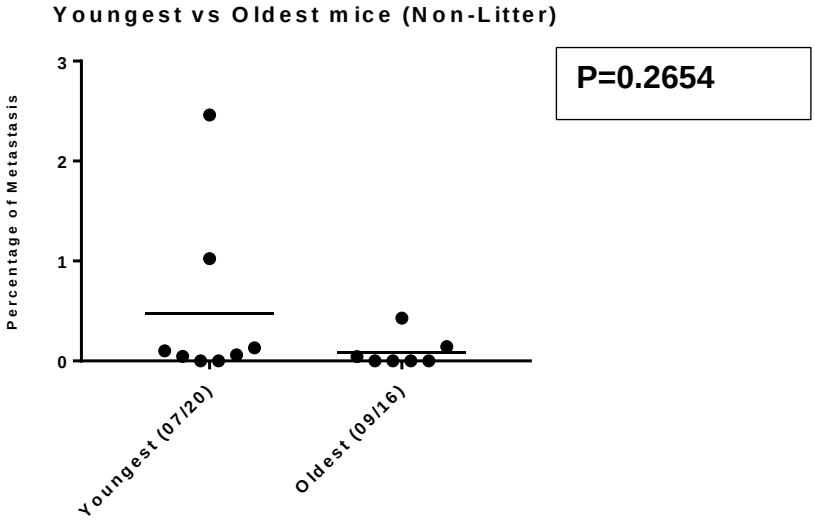

Figure 17 – Results when comparing the percentage that the metastasis occupy from the total lung area between the oldest and the youngest mice used in this experiment. Comparing the size of the metastasis the difference between the two age groups got smaller but still do the youngest ones have a higher mean as the oldest ones.

The comparison of the age was an interesting factor which was not performed in the littermates as they were all 5 weeks old.

3.3 Part 2 – Experiment with Litter mates:

In the second part of the experiment all injections were performed on F2 either homozygous 4A or homozygous 6A littermates. When comparing the difference in number of metastasis present inside of the lung, the 4A mice have a higher average than the 6A mice (6.6 vs. 3.1). Interestingly again there are 2 outlines in the 4A which had 18 and 20 tumours present inside of the lung. (See Figure 14)

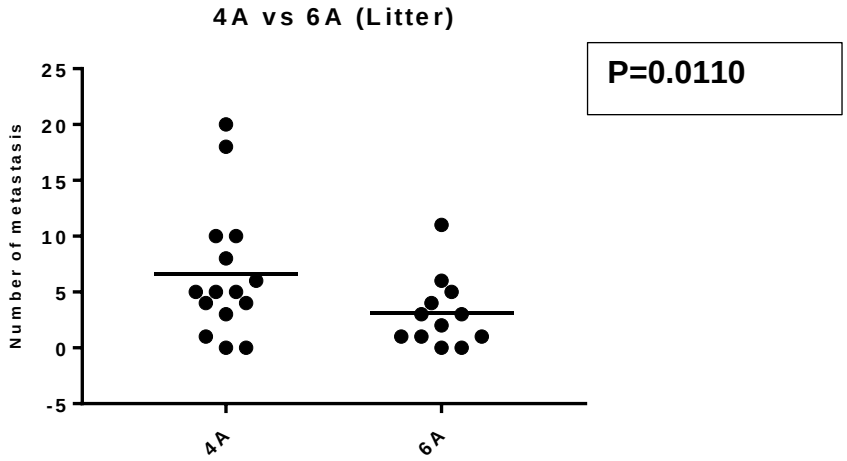

Figure 18 - Results of the number of metastasis of 4A and 6A mice (litter-mates). The average of the 4A mice is slightly higher compared to the one of the 6A mice (6.6 vs 3.1), but except the two outliers in the 4A mice (18 and 20 metastasis) there is no significat difference.

When comparing female and male mice, the male mice have a slightly higher average (5.7 compared to 3.8), but despite that there is not a big difference between female and male mice except that the male mice have two outliers (18 and 20 metastasis). (See Figure 15)

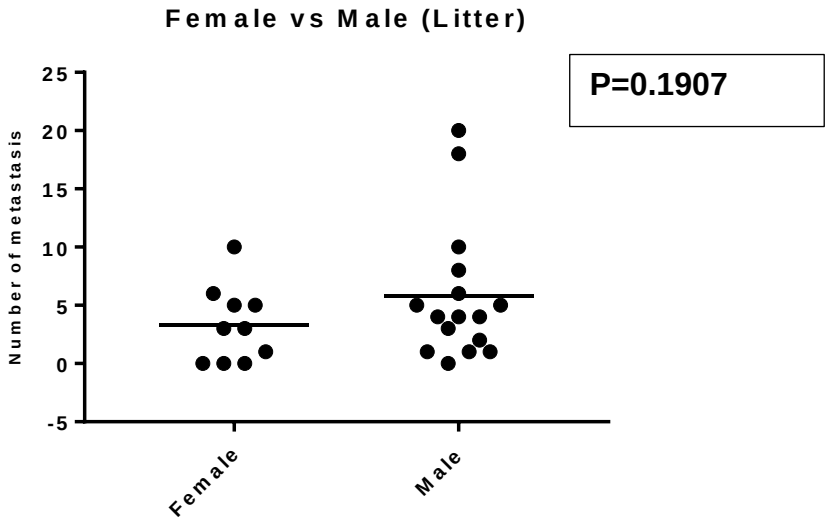

Figure 19 - Results of the comparison between female and male mice (litter-mates). There is no significant difference between female and male mice except that the male mice have a slightly higher average (5.7 vs 3.8) compare to the female and that the male have two ouliners (18 and 20 metastasis).

When comparing the percentage that the metastasis occupy of the total lung area it is clearly visible that the 4A have a higher average than the 6A and that the 4A have a very spread percentage number, although the 6A also have one outlier which has a higher percentage than the 4A mice outliers. (See Figure 16)

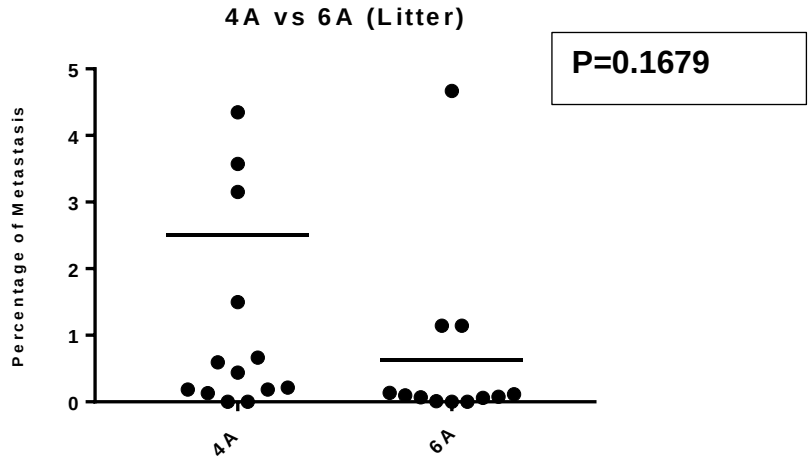

Figure 20 - Results of the percentage that the metastasis occupy from the total lung area. Clearly one can see that the average in the 4A mice is higher than in the 6A mice, although the 6A have one outlier which has a higher percentage than the 3 outlines of the 4A mice.

3.4 Part 3 – Meta analysis of both experiments:

When putting both data into one plot, to do a meta-analysis, one can see that 4A and the 6A mice have nearly the same average and that the numbers look very similar, except that the 4A have 4 extreme outliers that the 6A mice don't have. (See Figure 17)

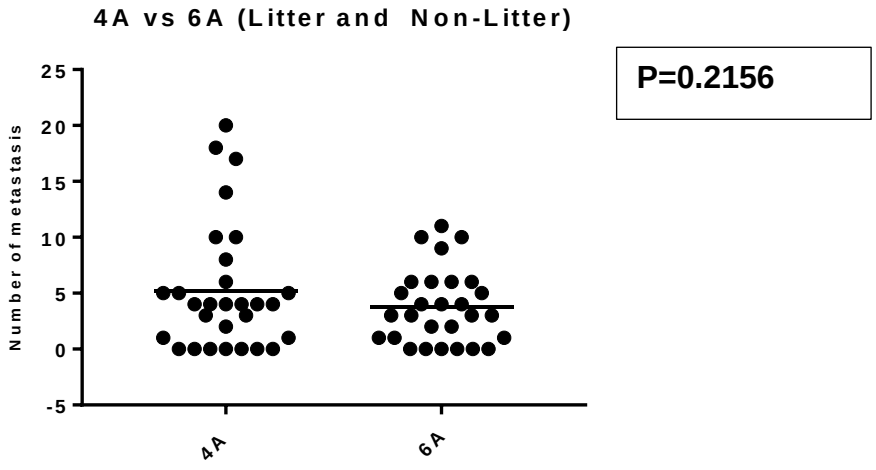

Figure 21 – Results of a semi-meta-analysis of the number of metastasis in 4A and 6A mice. Obviously there is no significant difference between the number of metastasis present in 4A compared to the 6A mice.

Concerning the percentage which the metastasis occupy compared to the total area of the lungs, the 4A mice have a higher average nevertheless is the distribution very similar. (See Figure 18)

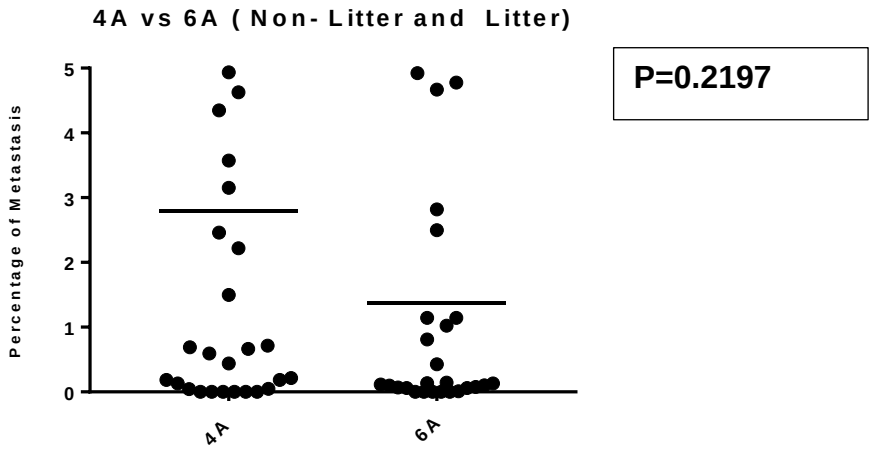

Figure 22 - Results of the percentage that the metastasis occupy from the total lung area. (4A vs 6A Non-litter and litter mates) The average of the the 4A mice is much higher than in the 6A mice, nevertheless there is not a big difference.

4 Discussion:

In this study, I wanted to show that the consequences of the two amino acid polymorphisms between the NIH in line 4 and the C57 in line 6 on the protein Adam17 and its consequent effects on the regulation of TGF-β signalling pathway and on the extent of angiogenesis. My hypothesis was that the line 6 may have a higher extent of angiogenesis due to the hypoactive form of ADAM17, which up regulates TGF-β signaling.

4.1 Genotyping and Evaluation of the number and extent of angiogenesis:

The most important step in the project for getting reliable results was the genotyping of the F2 generation. It is impossible to distinguish between the two lines with just looking at the mouse phenotype or using a simple PCR, we therefore used an additional restriction digest with the restriction enzyme XMN1 that can cut one allele but not the other. An indicator that that the restriction digest was working properly was not only the 4A, 6A and water control but also the absence or only slight presence of the 400nt uncut PCR band. In Figure 10, one can clearly see that the 247 and 216nt band are the strongest visible and that the 400nt band is only very weak visible, which indicates that the restriction digest was working properly. In case the 400nt band would have been strongly visible the genotyping would have had to be repeated as although there is a 4A band visible even that one is not fully digested and it could be 6A mice.

When breeding mice one cannot rely on the probability of obtaining exactly the ratio of 1:2:1 in the F2 generation. Indeed the genotype numbers varied between the line 4 and line 6 in both experiments. Especially when working with the F2 generation we wanted to have the same age for all mice, to get more reliable results and that is why we neglected not having the same number of mice in both groups. The same was also happening for the gender comparison. For the statistical comparison of the lines a difference of one or two mice is negligible.

Another important factor in the experiment was that we had to try to use the same or nearly the same passage of the CarB mouse carcinoma cells since it is proven that the longer the cell line is maintained in culture the greater is the risk of getting more changes in the genotype and phenotype and losing metastatic activity. Such changes can alter the behaviour and the characteristics of the CarB mouse carcinoma cells inside the lung tissue, a difference in the number and extent of angiogenesis could occur between the individuals of the same line.[15] A reason for using CarB mouse carcinoma lung metastasis cells and not the normal CarB mouse carcinoma cells was that we wanted to specifically compare the metastasis in the lung tissue and therefore we used cells which go back via the bloodstream to the tissue where they have been taken out.

To determine the best day for the harvesting of the lung we used some test mice before performing the experiment on the precious F2 matched littermates. We did some tests to evaluate not only which would be the best time to harvest the lung tissue but also to evaluate which number of cells for the injection would be appropriate. After injecting 500*10^3 and 100*10^^4 cells and harvesting after 7,10,12 and 14 days we decided that with 500*10^3 injected cells per mouse and harvesting after 10 days will be the best conditions for the experiment although there were not be any surface metastasis present. This could either be as we injected too less cells and additionally waited a too short time period till the harvesting. But we have chosen to neglect that as otherwise the determination of the number and extent of the angiogenesis would have not been that precise anymore. The metastasis would have been so big that it would have gotten impossible to distinguish between the individual metastasis. Especially the ratio between the metastasis is more important than the actual number of metastasis as less but bigger metastasis means a higher extent of angiogenesis as more and smaller metastasis.

For getting a precise percentage of the area that the metastasis occupy from the total lung area we used the program “ImageJ” and downloaded the extra “Fiji” as it was not so important to actually determine the size in mm^2 but to assess the area as a percentage of the total lung area between the mouse lines. It was important to

tell the program that that the difference in colour from the H&E staining (purple metastasis and pink lung tissue) means different tissue parts and to also not count in the white background in the picture.

4.2 Experiment Part 1 – Litter mates:

In experiment 1 we injected the CarB mouse carcinoma cells into 4A and 6A wildtype mice and what can be observed in figure 13 is that the average number of metastasis is slightly higher in the 6A than in the 4A but the numbers are not significant different. Eye-catching is that the 4A mice have in two extremes outliers present with higher numbers of metastasis (n=15) present in their lungs. A reason could be that maybe other mutations happened in the genotype of the mice which could harm the immune system or blocks/alters other pathways which made it more favourable of the metastasis to extravasate and grow. As the mice used where wildtype mice and not from the same litter it is nearly impossible to find an explanation for it.

In figure 14 we checked without taking into account the genotype of the mice if there is a difference in the number of metastasis in female and male mice. The male mice have a slightly higher average but again the numbers are not significant different. Eye-catching is again that the male have two outliers present, which are the 4A mice which were already in the comparison between line 4 and line 6 outliers. So we do not think that the gender plays a role in the number of metastasis presence but the different genotypes of the mice.

When looking at the percentage that the metastasis occupy from the total lung area we can clearly see that the average of the 4A mice is higher than in the 6A mice although the numbers are again not significant different. Which means that although the average in the 6A mice was higher when just considering metastatic numbers, the size of the metastases was larger in the 4A. Eye-catching is also that the two outliers in number of metastasis in line 4 are this time not outliers when it comes to the area of the metastasis.

Another important factor in the experiment comparing the 4A and 6A mice from separate homozygous colonies was their difference in age. We did the injections at the

beginning of October which means that the oldest mice were about 10 weeks old and the youngest about 5 weeks. When looking at figure 16 and figure 17 we can clearly see that the youngest mice have more and bigger metastasis than the oldest mice. As we used for all mice the same passage of CarB mouse carcinoma cells, it cannot be that other mutations could lead to this result. When comparing our results to several other studies performed in context of the effect of age on cancer we found controversial answers. On the one hand we found in the literature that it has been that the incidence of cancer is higher in aged patients than in younger ones as possible reasons could be somatic aging as it aging may increase the risk of getting cancer due to disturbances to the hormonal balance or a decline in the immune system with age. Another reason could be that the exposure to carcinogenic stimuli is higher and longer in aged people.[16] One the other hand it has also been proven that for example breast cancer is worse in patients younger than 40 years old and also poorer to diagnose. Reasons could be a higher tumour grad, a higher tumour proliferation, pronounces vessel-invading diseases, an increased expression of HER-2 and a reduced expression of both estrogen and progesterone receptor.[17] As a result we are still not sure how to explain the outcome of age differences in this experiment but a good suggestion would be to do further experiments and maybe inject into mice about a week apart and see if the graph decreases the more older the mice get or not.

4.3 Experiment 2:

In the second experiment, 27 littermates used for the injections, which limits the quantity of unreliable results as they are of more comparable genetic background as well as the same maternal environment. Furthermore, backcrossing is not required as the difference in genes were neutralized when the littermates were compared.[18]

When looking at Figure 18, apparently the 4A mice have a higher average in the number of metastasis present in the lung than the 6A mice. This is the opposite of the result in the 4A versus 6A colony comparison, however this could be due to

spontaneous variation, different age and different environmental factors in the different colonies. The littermates should have a more closely matched genetic and epigenetic background, therefore the results should be less variable and more reliable. Interestingly we have again two outliers in the 4A mice concerning the number of metastasis but we suggest that maybe in those mice there were some other mutations present which encouraged the formation of the tumours.

When looking at Figure 19, we have a higher average in the male mice concerning the number of metastasis than in the female mice. This time also in the WT different mutations could have happened which lead to the different result but it is difficult to say as in both experiment one of the gender was used more often than the other one which makes a statistical interpretation very difficult.

In the last Figure (Figure 20), we compare the percentage of the area that the metastasis occupied over the total lung area. This time definitely the line 4A have a higher average than the 6A mice, which we already saw in the same graph of the littermates. Nevertheless it is unfortunately not significant. But this strengthened the conclusion that the 4A mice have a higher extent of angiogenesis as the 6A mice, as their numbers in this figure are all very close to zero as in the 4A mice they are more spread. Interesting is that one particular mouse in the 6A line had an higher extent of angiogenesis than any mouse of the 4A mice. This could be due to a spontaneous mutation or a not complete function of the immune system, which can occur also in the littermates.

In the litter-mates the factor of age did not play a role as all of the mice were 5 weeks old and were injected with the same passage of CarB mouse carcinoma cells.

4.4 Meta-analysis of both experiments:

A widely used method in statistics is to combine results of two multiple experiments is meta-analysis. As we don’t have a statistically significant difference, we used meta-analysis to derive a pooled estimate which is closest to the null hypothesis. Furthermore, meta-analysis has the advantage to show the contrast of the results

from different studies but also identifies the similarities or other interesting relations between the results of different studies.

In figure **21,** one can see that the average of the number of metastasis is nearly the same although it is slightly higher in the 4A mice. Most of the values are very close together, however the outliers from the graphs described before are again outliers.

When looking at the gender the average of the female and male mice is nearly the same, as it was also very close together when looking at the two experiments separately. Which leads to the conclusion that there is no difference in the enzymatic activity of Adam17 when looking at the two genders.

Concerning the percentage of the metastasis line 4A has a higher average than line 6A which is due to the bigger extent of angiogenesis in line 4 in both experiments. But the data is not significantly different as the 6A mice have nearly the same distribution of the values as the 4A mice, which could be due to the higher number of metastasis present in the WT 6A mice.

Although the meta-analysis shows a certain direction of the experiments it is very questionable if one can mix the results of the WT mice with the results of the littermates as they differ in age, were injected different passages of the CarB mouse carcinoma cells and can have a different genetical background as well as different surrounding environment. Nevertheless, it was interessing to see that only when looking at the percentage area occupied from the metastasis the average is really different between the two lines. Which reinforced the argument that the extent of angiogenesis is a more precise factor for comparing the lines of mice.

4.5 Conclusion and Outlook:

Eventually, when looking at all the data on the different graphs one can only conclude that the Tgfbm3 locus may has an effect on tumour metastasis. As the data set was not large enough to exclude this possibility, but there is only a suggestion of the tendency of the results, which is that the 4A mice have controversial to our hypothesis a higher extent of angiogenesis and also more metastasis present in the lungs than the line 6A.

Strenghened is this conclusion by comparing the obtained results of the experiment prior to the data above. (See Figure 22)

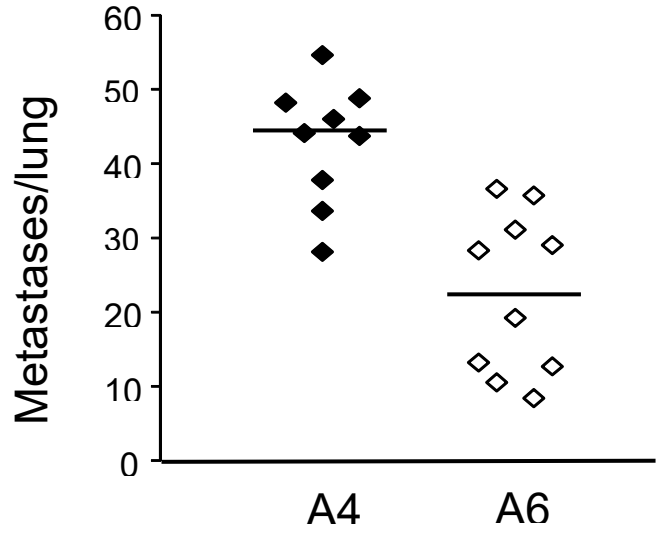

Figure 23 -Results the metastasis/lung percentage between the line 4A and line 6A by Dominique Meyer. In the graph above one can clearly see that the 4A have with a range from about 25% up to nearly 60% a much higher average than the 6A with a range from 10% to nearly 40%. The data is also significant as the P-value is 0.003. (p=0.003)

When looking at the results which Dominique Meyer, PhD, received when doing the experiment, one can clearly see that the 4A mice have a much higher average concerning the percentage that the metastasis occupy of the total lung area, than the 6A. Out of this data we also suggested that the number of metastases present in the lungs were much higher in the 4A than in the 6A, although it cannot precisely say as his metastases were so large that it was difficult to distinguish between single tumors. He injected in total 200*10^4 cells/10µl into each mice which is four times more than I did. Out of this high concentration he also harvested the lungs after 7 days as otherwise the mice would have died before he would be able to harvest the lung tissue. Nevertheless, we concluded that the results with the lower concentration and smaller size of the metastasis already points into the same direction as his. Especially the results of the littermates is directly comparable as also Dominique Meyer, PhD worked with F2 littermates which were 5 weeks old.

In the future the experiment has to be repeated at least a third time to see if there is the same result as we received in the runs before. An improvement could definitely be to focus only on the F2 littermates, as the WT mice are not genetically

comparable, and to inject instead of 500*10^3cells/^10µl , 100*10^4cells/10µl and still harvest after 10 days to have a higher extent of angiogenesis which would make the difference between the lines ever more distinctive. But it should still be in a range that single metastasis can be distinguish from each other to see if the number does play a role or not.

A full conclusion explaining why the results appear totally opposite to our hypothesis has not yet been found, but one suggestion is that Adam17 serves other functions, as for example the shedding of tumour-necrosis factor alpha (TNF-alpha) which results in its release of the membrane -bound precursor. Furthermore ADAM17 also processes cell adhesion proteins, cytokine and growth factor receptors and epidermal growth factor receptor ligands. [19] Furthermore it would be interesting to look if the polymorphism in the strains has not only an effect on the enzymatic activity of Adam17 on the TGF-β type II receptor but also on the shedding effect on, as mentioned before, TNF-alpha. As maybe the effect on the TGF-β could be tumor promotive the altered enzymatic activity could have tumour suppressive effects.

If there could be proven a link to the mutation on Adam17 and a different extent of angiogenesis between individuals this would improve the field of personalized medicine. As a genetic screening of the patient, as performed for example for BRCA1 and BRCA2 for breast cancer, could detect the presence or absence of the Tgfbm3b locus and therefore help to personalize the cancer therapy. Moreover it could also justify the hypothesis that the reason why some patients respond to certain drugs and others not is due to germline genetic variation between the individuals.

Annex

Annex 1 [Questionnaire].....40
Annex 2 [Further sources]..... **Fehler! Textmarke nicht definiert.**

Annex 1 [Questionnaire]

List of References

[1] Andrew P. Hinck, 'Structural Studies of TGFbetas and Their Receptors-Insights into Evolution of TGF-Superfamily', 2012, pp. 1860–70.

[2] Brian Bierie and Harold L. Moses, 'TUMOUR MICROENVIRONMENT TGF β : The Molecular Jekyll and Hyde of Cancer', *Nature Rev. Cancer*, 6.July (2006), 506–20 <http://dx.doi.org/10.1038/nrc1926>.

[3] Minlong Shi and others, 'Latent TGF-Beta Structure and Activation', 474.3 (2016), 343–49 <http://dx.doi.org/10.1038/nature10152.Latent>.

[4] Bernhard Schmierer and Caroline S Hill, 'REVIEWS TGF β – SMAD Signal Transduction : Molecular Specificity and Functional Flexibility', 8.december (2007), 970–82 <http://dx.doi.org/10.1038/nrm2297>.

[5] Minlong Shi and others, 'Latent TGF-Beta Structure and Activation', Nature 474.3, (2016), 343–49 <http://dx.doi.org/10.1038/nature10152.Latent>.

[6] Ye Zhang, 'Non-Smad Pathways in TGF-Beta Signaling', *Cell Research*, 19.1 (2009), 128–39 <http://dx.doi.org/10.1038/cr.2008.328.Non-Smad>.

[7] Gerad C. Blobe, William P. Schiemann, Harvey F. Lodish, 'Role of TGf-Beta in Different Human Diseases', New England Journal of Medicine, 342,2000,1350-158.

[8] Erik Meulmeester and Peter Dijke, 'The Dynamic Roles of TGF-β in Cancer', The Journal of Diseases', New England Journal of Medicine, 342,2000,1350-158

[9] Gareth J Inman, 'Switching TGF B from a Tumor Suppressor to a Tumor Promoter', *Current Opinion in Genetics & Development*, 21.1, 93–99 <http://dx.doi.org/10.1016/j.gde.2010.12.004>.

[10] Li Yang, 'TGF β and Cancer Metastasis : An Inflammation Link', 2010, 263–71 <http://dx.doi.org/10.1007/s10555-010-9226-3>.

[11] Kyoko Kawasaki and others, 'Genetic Variants of Adam17 Differentially Regulate TGF β Signaling to Modify Vascular Pathology in Mice and Humans', *Proceeding of the National Academy of Sciences*, 111.21 (2014), 7723–28 <http://dx.doi.org/10.1073/pnas.1318761111>.

[12] Monika Gooz, 'Adam-17 the Enzyme That Does It All.', *Critical Reviews in Biochemical and Molecular Biology*, 2011, 146–69 <http://dx.doi.org/10.3109/10409231003628015.ADAM-17>.

[13] Jennifer Lynn Elderbroom, "Ectodomain Shedding of TGF-beta receptors: Role in Signaling and Breast Cancer Biology", Departement of Pharmacology and Cancer Biology, Duke University, Published: 2013

[14] Kai Nowak and Neysan Rafat, 'Circulating Endothelial Progenitor Cells Are Increased in Human Lung Cancer and Correlate with Stage of Disease.pdf', *European Journal of Cardio-Thoracic Sugery*, 37 (2010), 758–63.

[15] Nature Protocols 2, 2276-2284 (2007), Published online: 13.September 2007 | http://www.nature.com/nprot/journal/v2/n9/full/nprot.2007.319.html (Date: 28.02.2017)

[16] Svetlana V. Ukranitseva, Anatoli I. Yashin, „Individual Aging and Cancer Risk: How are they related", Demographig research, Volume 9 (2003), Article 8, Pages 163-196

[17] Bharat A, Aft RL, Gao F, et al Patient and tumor characteristics associated with increased mortality in young women (<40 years) with breast cancer, J Surg. Oncol. (2009), 100:248-51

[18] Rikard Holmdahl, Bernard Malissen, „The need for littermates controls“, European Journal of Immunology, 42 (2012), 45-47

[19] NCBI – Adam 17 – Adam metallopeptidase domain 17 [homo sapiens], https://www.ncbi.nlm.nih.gov/gene/6868, update on 20.2.2017, (Accessed: 05.03.2017)