universität wien

# DIPLOMARBEIT

Titel der Diplomarbeit

## The mechanism of ILEI cleavage and its physiological relevances

angestrebter akademischer Grad

Magister/Magistra der Naturwissenschaften (Mag. rer.nat)


| | |
|---|---|
| Verfasserin: | Betül ULUCA |
| Studienrichtung /Studienzweig (lt. Studienblatt): | A 441 Diplomstudium Genetik – Mikrobiologie (Stzw) UniStG |
| Betreuerin / Betreuer: | a.o. Univ. -Prof. Dr. Hartmut Beug |


Wien, im January 2011

## Acknowledgements

First of all, I want to thank Professor Hartmut Beug for giving me the opportunity to to do my thesis in his great lab. I am especially grateful to my advisor, Agnes Csiszar, for her support and encouragement throughout this year. I deeply appreciate her intelligence and patience that helped me to improve myself as a scientifist. This diploma thesis would have never been possible without her help and guidance.
Furthermore, I want to thank Gabi and Evi for their technical support and for answering all my questions. Thanks to my dear colleague, Ulrike, for her motivation and for sharing the hard times. Thanks to all my colleagues, Memo, Boris and Christina for the interesting discussions and for the enjoyable lunch times.
I express my gratitude to people and organizations from Turkey and Vienna, for their financial support and for providing me with accommodation.
During these hard years in Vienna, my parents have always supported me financially and emotionally. I am really indebted to them and also to my siblings for their faith and love. Thanks to all my friends in Vienna and Istanbul for being next to me all the good and hard times. Finally, my special thanks go to my husband Taceddin, for his love and for his company in the life.

**Table of contents**

## ACRONYMES and ABBREVIATIONS

| | |
|---|---|
| 1′ | 1 minute |
| CAF | carcinoma associated fibroblasts |
| C.M. | conditioned medium |
| DMSO | dimethylsulfoxid |
| DNA | deoxyribonucleic acid |
| Dox | doxycyclin |
| ECM | extracellular matrix |
| EDTA | ethylenediaminetetraacetic acid |
| EMT | epithelial to mesenchymal transition |
| EpC40 | EpH4 cells transformed with oncogenic ras that has a point mutation in effector loop C40 |
| EpH4 | mouse mammary epithelial cells |
| EpRAS | EpH4 cells transformed with oncogenic ras |
| ERK | extracellular signal regulated kinase |
| FCS | fetal calf serum |
| FGF | fibroblast growth factor |
| GAP | GTPase activating protein |
| GPI | glycosylphosphatidylinositol |
| GTP | Guanosine-5′-triphosphate |
| Hepes | 2-(4-(2-hydroxyethyl)- 1-piperazinyl)-ethansulfonacid |
| HGF | hepatocyte growth factor |
| IHC | immunohistochemistry |
| IP | immunoprecipitation |
| KO | knock out |
| ILEI | interleukin like EMT inducer |
| ILEI-ΔNRS | ILEI lacking of pro-peptide |
| ILEI-FD | cleavage mutant of ILEI |
| kda | kilo dalton |
| MAPK | mitogen-activated protein kinase |
| MEK | MAPK kinase |
| MET | mesenchymal to epithelial transition |
| MMP | matrix metalloproteinase |
| PAI | plasminogen activator inhibitor |
| PAR | protease activated receptor |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PDGF/R | platelet derived growth factor receptor |
| PDK1 | pyruvate dehydrogenase kinase 1 |
| PFA | paraformaldehyde |
| PI3K | phosphotidyl-inositol-3-kinase |
| PIP2 | phosphotidylinositol biphosphat |

| | |
|---|---|
| PIP3 | phosphotidylinositol triphosphat |
| Pln | plasmin |
| Plg | plasminogen |
| Raf | rapidly growing fibrosarcoma |
| Rpm | rounds per minute |
| RTK | receptor tyrosine kinase |
| rtTA | reverse tetracycline transactivator |
| SDS | sodium dodecylsulfate |
| SEM | standard error of means |
| SN | supernatant |
| TAE | Tris-acetat-EDTA |
| TBS-T | Tris buffered saline with Tween 20 |
| TE | Tris-EDTA |
| TetO(P) | Tet operator |
| TetR | tetracyclin repressor |
| TGFβ | Transforming growth factor β |
| TGFβR | Transforming growth factor β receptor |
| tPA | tissue plasminogen activator |
| tTA | tetracycline-controlled transactivator |
| uPA | urokinase plasminogen activator |
| uPAR | uPA receptor |
| XT | Ex-tumor cells |
| VEGF | vascular endothelial growth factor |
| ZO1 | Zona occludens 1 |

**ZUSAMMENFASSUNG**

Metastasierung ist ein sehr häufiger Prozess bei Krebserkrankungen und das entscheidende Kriterium für durch Krebs verursachte Todesfälle. Metastasierende Tumorzellen wandern aktiv im Gewebe, gehen ins Blut/Lymphe und werden invasiv, d.h. wandern in entfernte Organe aus, wo sie Tochtergeschwülste (Metastasen) bilden. Dies wird durch genomische Veränderungen, die Tumormikroumgebung mit erhöhter Protease-Aktivität und modifiziertem ECM und Stroma unterstützt. Infolgedessen brechen die Zellen durch Gewebebarrieren und Basalmembranen und es werden sekundäre Tumore in entfernten Organen gebildet. Bei den ersten Schritten der frühen Metastasierung, bevor sich die Zellen aus dem primären Tumor lösen und sich verbreiten, tritt ein Prozess namens Epitheliale mesenchymale Transition (EMT) auf, bei dem die Zellen ihre epitheliale Polarität und Fähigkeit zur Zellkontaktbildung verlieren. Sie weisen dann den Phänotyp von Bindegewebszellen (Fibroblasten) auf.

EMT erfordert eine kooperative Wirkung von aktiviertem Ras und TGFß Signalling. Eine Studie über Expressionsanalyse, um EMT spezifische , durch TGFß induzierte Targetgene in einem epithelialen Maus- Brustkrebs- Zellmodell zu finden , hat ein neues, Zytokin ähnliches Protein namens ILEI (Interleukin like EMT-Inducer) identfiziert. TGFß reguliert ILEI auf der translationalen Ebene hoch. Dauerhafte Überexpression von ILEI in einigen Zelllinien verursacht EMT und verstärkt Tumorwachstum und Metastasierung.

ILEI ist ein fakultativ sezerniertes Protein und seine sekretierte Form wird durch Serinproteasen wie Plasmin spezifisch an einer R / S Site am N-Terminus gespalten. Die physiologische Rolle der ILEI Spaltung in der Tumorgenese wurde bei tumorigenischen aber nicht metastatischen EpC40 Zellen, die verschiedene mutierte Formen des Proteins überexprimieren, analysiert. Das Protein wurde mutiert; entweder durch Einführung von Punktmutationen auf der Spaltstelle (cleavage mutant), welche das proteolytische Spalten durch Proteasen inhibieren, oder das Propeptid an dem N-Terminus wurde deletiert (Δ-pro peptid mutant), was zu einem konstitutiv gespaltenen, reifen ILEI Protein führt. (bei dem Protease-Spaltung nicht mehr benötigt wird.) Das Fehlen des Propeptides am N-Terminus gewährleistet.eine hohe Sekretion des Proteins. In vivo Tumorigenese Experimente haben demonstriert, dass die cleavage Mutante überexprimierte EpC40 Zelle kleine Tumoren und sehr wenige Metastasen im Vergleich zu den wt-ILEI überexprimierten Zellen gebildet haben. Daneben beschleunigten die Δ-pro Peptid ILEI überexprimierten Zellen die Tumorentwicklung sogar stärker als die wt-ILEI überexprimierten Zellen und wiesen erhöhte Metastasierung auf. Wir untersuchten die Notwendigkeit der ILEI Spaltung für ein verstärktes Tumorwachstum im Tumorigenese-Testen bei denen ein nicht spezifischer Proteaseninhibitor, Aprotinin verwendet wurde.

Wir erhielten eine signifikant verminderte Tumorwachstumsrate und Tumorgröße nur bei wt-ILEI überexprimierten Zellen im Vergleich zu den unbehandelten Tiere. Wie erwartet, hatte Aprotinin bei durch die Δ-pro peptid Mutante erzeugten, schnellwachsenden Tumoren keine Wirkung, da dieses Proteiun ja bereits gepalten ist. In vitro Experimente wießen darauf dass, die ILEI Sekretion durch exogenes Plasmin stimuliert werden kann.

In weiteren Analysen zur Erklärung der Ursache dieses Phänomenes, wurde getestet, ob die ILEI-Sekretion durch die Aktivierung von Plasmin – uPAR Signalling reguliert werden könnte und nicht durch einen „autocrine Feed-back" Mechanismus von ILEI, bei dem die gespaltene reife ILEI-Form seine eigene Sekretion ausgelöst haben könnte.

Im zweiten Teil dieser Diplomarbeit wurde ein doxycyclin induzierbarer (TET-ON) ILEI transgenischer Mausstamm in vitro und in vivo auf die Induzierbarkeit und die Expressions-Stärke des durch das ILEI Transgen kodierte Protein bestimmt. Zwei Transgene, TetrTA und tetO-ILEI, wurden durch homologe Rekombination in spezifische Loci (ROSA26 and Col1A1) des Mausgenoms eingeführt, um Nebenwirkungen von zufälliger Integrationen, wie Zerstörung von endogenen Genen, zu vermeiden.

Doxycyclin induzierte ILEI Expression wurde in allen untersuchten Geweben außer in der Lunge detektiert. Die höchsten Expression Levels wurden im Dünndarm und Dickdarm gefunden. Dieses induzierbare ILEI transgenische System ermöglicht das Studieren der physiologischen Funktion von ILEI und seiner Rolle in der Krebsentwicklung.

**ABSTRACT**

Metastasis is the main reason for cancer related deaths. Epithelial tumor cells become invasive and motile, aided by genomic alterations and the tumor microenvironment with increased protease activity and modified ECM and stroma. As a result, the cells break down tissue barriers and establish secondary tumors at distant organs. During the early steps of metastasis, when tumor cells start to disseminate from the primary cancer, a process called epithelial to mesenchymal transition (EMT) occurs, in which cells loosen cell-cell contacts and deregulate their epithelial polarity.
EMT requires a cooperative action of activated Ras and TGFβ signaling. Expression profiling studies to identify TGFβ-induced, EMT specific target genes in a murine mammary epithelial cell model has revealed a novel cytokine like protein, named ILEI (interleukin like EMT inducer). TGFβ upregulates ILEI at the translational level. Stable overexpression of ILEI in several cell lines caused EMT, elevated tumor growth and aspects of metastasis.
ILEI is a secreted protein and its secreted form is cleaved by serine-type proteases, like plasmin, specifically at an R/S site at its N terminus. The physiological role of ILEI processing in tumorigenesis was analyzed using tumorigenic but non-metastatic EpC40 cells overexpressing (OE) different mutant forms of the protein. The protein was mutated either by introducing point mutations at the cleavage site, which inhibited proteolytic cleavage by proteases, or the pro-peptide at the N-terminus was deleted, creating an artificially matured protein not requiring proteolytic cleavage. This lack of the pro-peptide at the N-terminus caused constitutive, abnormally high secretion of the protein. In vivo tumorigenesis experiments demonstrated that cleavage mutant overexpressing EpC40 cells formed small tumors and very few metastases compared to the wt-ILEI OE cells. In addition, Δ-pro peptide ILEI OE cells accelerated tumor development even more than the wt-ILEI OE cells and exhibited enhanced metastasis. We examined the necessity of ILEI cleavage for generating elevated tumor growth in tumorigenesis assay using a non-specific serine protease inhibitor, aprotinin. Mice treated with this inhibitor showed significantly decreased tumor growth rates and tumor sizes induced by wt-ILEI OE cells, as compared to animals not treated with inhibitor. In vitro assays indicated that ILEI secretion can be stimulated by exogenous plasmin. Further analyses in attempts to explain the cause of this phenomenon suggested that it might occur via the activation of plasmin - uPAR signaling, and not through an autocrine feedback mechanism of ILEI, where the processed form would trigger its own secretion.

In the second part of this diploma thesis, a doxycyclin inducible (TET-ON) ILEI transgenic mouse strain was characterized in vitro and in vivo for the inducibility and expression levels of the ILEI transgene. The two transgenes, TetrTA and tetO-ILEI were introduced into specific loci (ROSA26 and Col1A1) by homologous recombination, eliminating the side effects of random integration such as disruption of endogenous genes. Doxycyclin responsive ILEI expression was detected in all examined tissues except lung. Highest expression levels were found in small and large intestines. This inducible ILEI transgenic system allows studying the physiological role of ILEI and its role in cancer development.

# PART I
## MECHANISM OF ILEI CLEAVAGE and ITS PHYSIOLOGICAL RELEVANCES

## 1. INTRODUCTION

### 1.1 Through a malignant cell towards death: Cancer in multistep processes

Cancer is a major cause of mortality worldwide, leading around to 13% of all human deaths *(WHO),* despite improved therapeutic implications in the last five decades. The disease involves multiple steps, i.e various mutations alterations in the genome that cause the cells to become more and more malignant. Despite the highly diverse genetic changes that contribute to cancer, a common set of properties accounts for the generation of all types of human tumor cells. Indeed six acquired capabilities have been elucidated that are involved in this malignancy process. (a-f) *(Weinberg, Hanahan 2002)* (FIGURE 1)

**a) Self-sufficiency in growth signals**: Tumor cells produce their own growth signals. This capability decreases the dependence on exogenous signals from their microenvironment. Tumor cells escape from homeostatic mechanisms, which control normal cellular behaviour in a tissue.

**b) Insensitivity to growth-inhibitory signals:** Antigrowth signals can induce cellular quiescence (G0) and block active proliferation. Tumor cells must evade these cytostatic anti-growth signals using various strategies. Overexpression of c-myc oncoprotein or disruption of the pRb pathway disrupt the ability of cancer cells to undergo terminal differentiation.

**c) Evasion of apoptosis (programmed cell death):** Cancer cells acquire resistance towards apoptosis by altering molecular players that contribute to apoptosis signalling.

**d) Limitless replicative potential:** Beside independence on their environment and evading apoptosis, tumor cells must acquire unlimited replicative potential and circumvent cellular senescence. Normal cells have an intrinsic autonomous program of restricting proliferation after a fixed number of cell divisions, due to telomere shortening during consecutive divisions. Tumor cells evade also this limited proliferation capacity by maintaining telomere length through the constitutive upregulation of the telomerase enzyme.

**e) Sustained angiogenesis:** Neovascularisation – i.e formation of new blood vessels – is important for cancer cells. It provides oxygen and nutrients and thus enables cancer cells to form larger tumors. Tumor cells induce formation of new blood vessels by activating various angiogenic signals such as growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), cytokines or chemokines and counteracting inhibitors.

**f) Invasion and metastasis:** Tumor cells that have acquired all these five capabilities can disseminate from their primary tumor mass and invade surrounding tissues and blood vessels to establish new colonies at distant organs. The generation of epithelial tumors at secondary sites occurs in distinct steps and is called metastasis. During this process cells lose their epithelial polarity, gain anchorage independence and become invasive and motile. (FIGURE 2). In addition to other modifications, altered expression of extracellular proteases and surface molecules e.g. cadherins and integrins and changes in the tumor microenvironment give rise to this migratory phenotype, causing 90% of human cancer deaths. *(Sporn MB, 1969)*

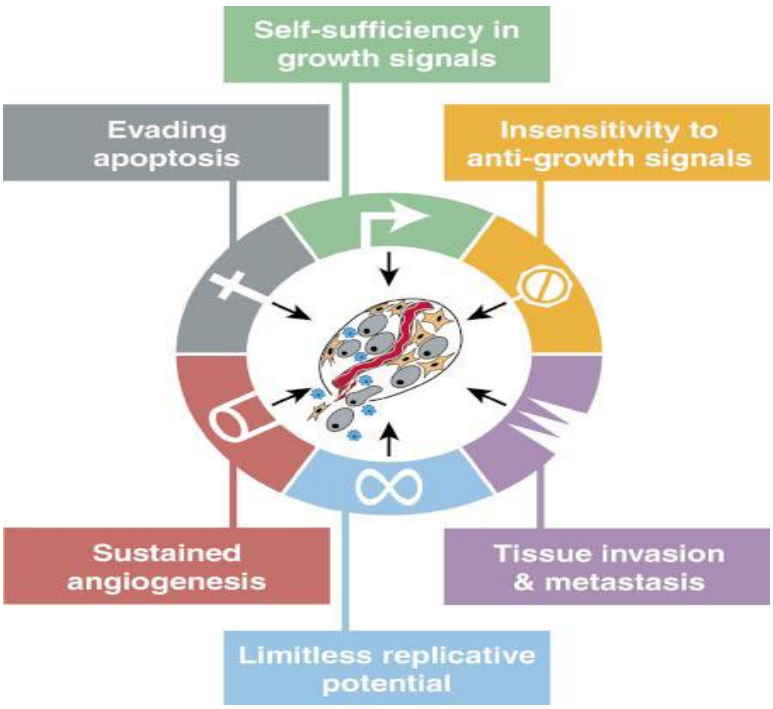



**FIGURE 1 | Hallmarks of cancer** ***(Hanahan and Weinberg, 2002)***
Most cancers have acquired the same set of functional capabilities during their development, through various mechanistic strategies.

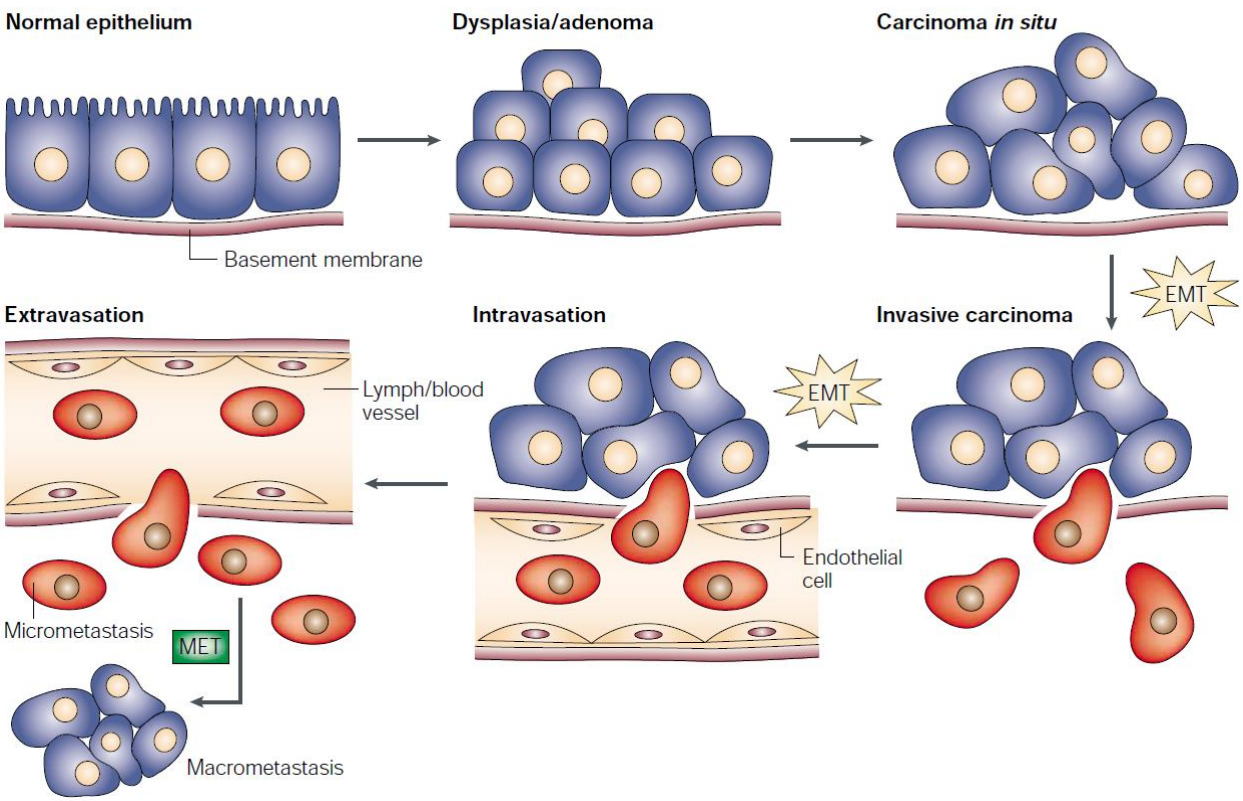



**FIGURE 2 |** Metastasis formation of epithelial derived cancer cells (Thiery JP. 2003)
EMT: epithelial to mesenchymal transition causes possibly the first step of local dissemination of carcinomas.

## 1.2 EMT

Two main cell types –epithelial and mesenchymal- were described on the basis of their shape and specialized membrane structures. Epithelial cells form monolayers, adhere to each other and to the extracellular matrix (ECM) by tight junctions, adherens junctions, desmosomes and gap junctions. Claudins and occludins are the major tight junction proteins, which maintain cell-cell interactions and join together the cytoskeletons of adjacent cells. Adherens junctions occur at cell-cell junctions and are composed of cadherins. Gap junctions built up by connexins directly connect the cytoplasms of two cells. Integrins are plasma membrane receptors for e.g. extracellular matrix proteins and the main players to mediate cell contact with surrounding tissues as well as with other cells or with the ECM. They can bind to and couple ECM components with the cytoskeleton of the cell. The organisation of these junctions in a basal plane, the distribution of the adherens and polarized organization of the actin cytoskeleton apical of the ECM are characterized as apico-basal polarity of epithelial cells, in which the movements of cells are limited to the two dimensional space and restricted by neighboring cells. On the contrary, mesenchymal cells form variable cell shapes with an anterior-posterior rather than apico-basal actin polarization. They interact with other cells only at focal adhesion sites and elongate with a leading edge enriched in integrins and matrix metalloproteinases (MMP). They are motile cells, indeed they can move in all three dimensions by actin reorganization. *(Berx G. 2007)*

EMT –epithelial to mesenchymal transition- occurs when cells lose their epithelial characteristics and gain mesenchymal features. This is actually a reversible process since it has been reported that these cells can regain a fully differentiated epithelial phenotype via mesenchymal-to-epithelial transition (MET). However, EMT and MET involve distinct signalling pathways depending on the cell type. EMT is first revealed during embryological processes, as a key process in early development. For example, in vertebrates it is associated with the establishment of the three-layer stage by gastrulation and later in development with the formation of some organs and tissues. In the adult, EMT-like processes contribute also in wound healing and in diseases such as fibrosis and tumour metastasis (FIGURE 3; *Thiery JP.; 2003, 2006)*

Epithelial and mesenchymal cells show large differences in the expression of specific markers. For example, E-cadherin, a transmembrane protein localized to the adherens junctions and basolateral membrane and organising cell-cell adhesion complexes is known as a typical marker of epithelial cells and usually downregulated during EMT. Another epithelial protein (zona occludens 1; ZO1) is expressed in tight junctions and interacts with components of tight junctions and also with the actin cytoskeleton. On the other hand, mesenchymal cells express intermediate filaments, a well-known example is vimentin. It helps maintaining cell integrity as a cytoskeletal component with a dynamic structure and provides flexibility to the cell. ECM components such as fibronectin and collagen precursors are expressed in mesenchymal cells as well. *(Thiery JP.,2006)*

In the context of tumorigenesis, cells acquire a spindle-shaped, highly motile and invasive fibroblastoid structure during EMT. Additionally they change their gene expression pattern through specific signalling molecules, i.e activation of mesenchymal markers combined with repression of tight- and adherens junction proteins. Thus they are able to migrate away from the primary tumor and to traverse through the basement membrane into the circulation, constituting early steps of metastasis. Loss of E-cadherin and gain of vimentin and ECM components are well-accepted markers of EMT. (FIGURE 3; *Thiery JP.;2003)*

Besides, at least five signalling pathways that are central players in early embryonic development also control EMT (WNT, TGFβ, Hedgehog, Notch and receptor tyrosine kinase (RTK)/Ras signalling pathways). In the context of cancer these pathways are generally disregulated, and drive cells towards EMT. *(Gruenert S., 2003; Huber MA., 2005; Berx G., 2007; Thiery JP. 2003; 2006)* Moreover gene expression profiling studies uncovered numerous transcription factors (e.g. Snail and Twist as repressors of E-cadherin) and signalling pathways (e.g. PDGF/R and NFκB signalling) that contribute to EMT. *(Yang, 2004; Huber, 2004; Jechlinger, 2006)*

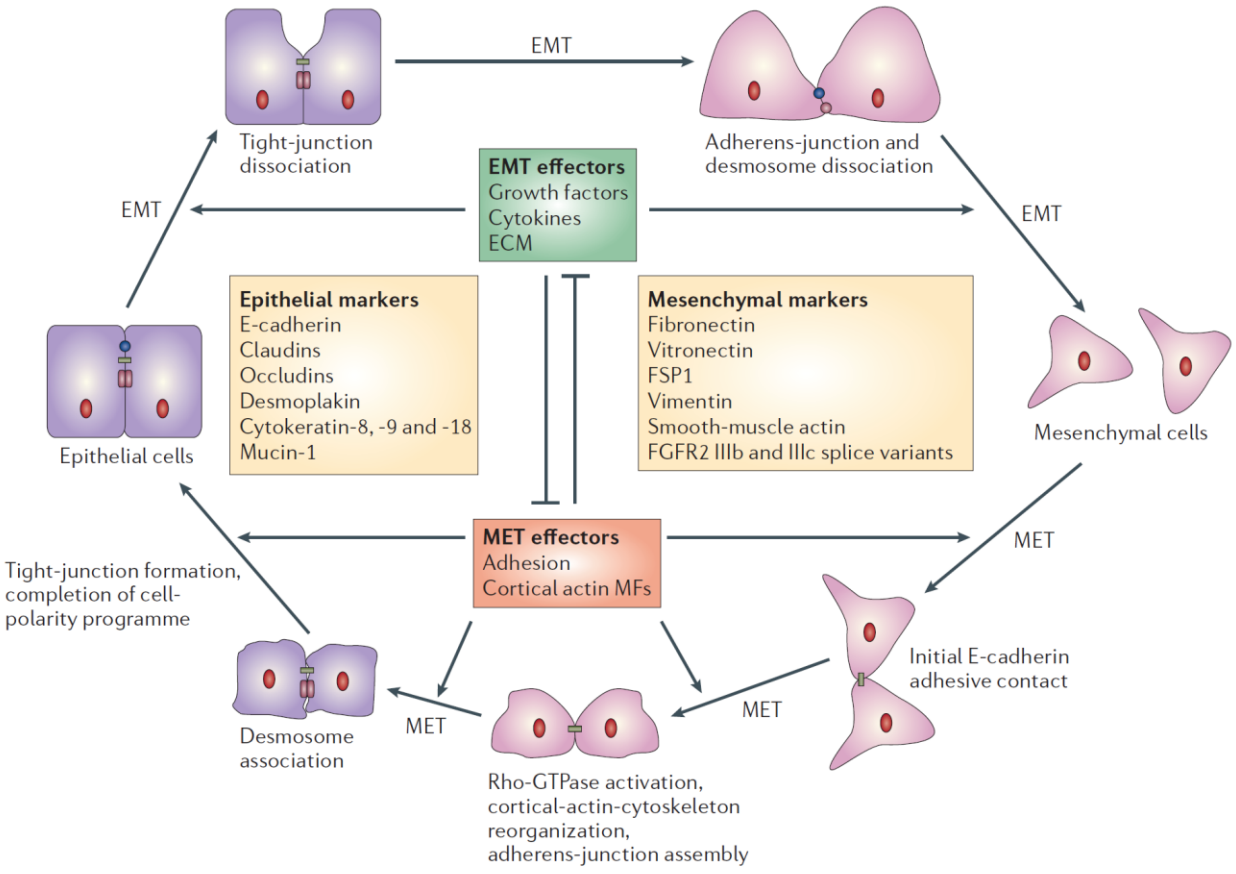



**FIGURE 3 | The cycle of epithelial cell plasticity (Thiery JP. 2006)**
E-cadherin, epithelial cadherin; ECM, extracellular matrix; FGFR2, fibroblast-growth factor receptor-2; FSP1, fibroblast-specific protein-1; MFs, microfilaments.

### 1.3 The dual face of TGFβ signalling on tumorigenesis

In mammals, the TGFβ family consists of three highly homologous genes encoding TGFβ-1,-2 and-3. They are secreted proteins and released to the ECM as latent growth factors, where they are activated by plasmin, matrix metalloproteinases or ECM proteins. Active TGFβ's bind to heteromeric signalling receptors. There are two types of transmembrane receptor serine threonine kinases forming the TGFβ receptor complex: TGFβ Receptor II (TβRII) functioning in ligand binding and TGFβ Receptor I (TβRI), representing the signalling receptor component. Once TGfβ binds to the receptor complex, TβRII phosphorylates TβRI, which then signals through distinct pathways: the well described SMAD signalling pathway and other pathways involving the mitogen-activated protein kinases (MAPKs), phosphoinositol-3 kinase (PI3K), and PP2A/p70s6K (FIGURE 4). TGFβs regulate diverse processes including cell proliferation, -development and -behaviour, wound healing, immune surveillance and angiogenesis. *(Wakefield LM.,2001; Massague, 1998)*

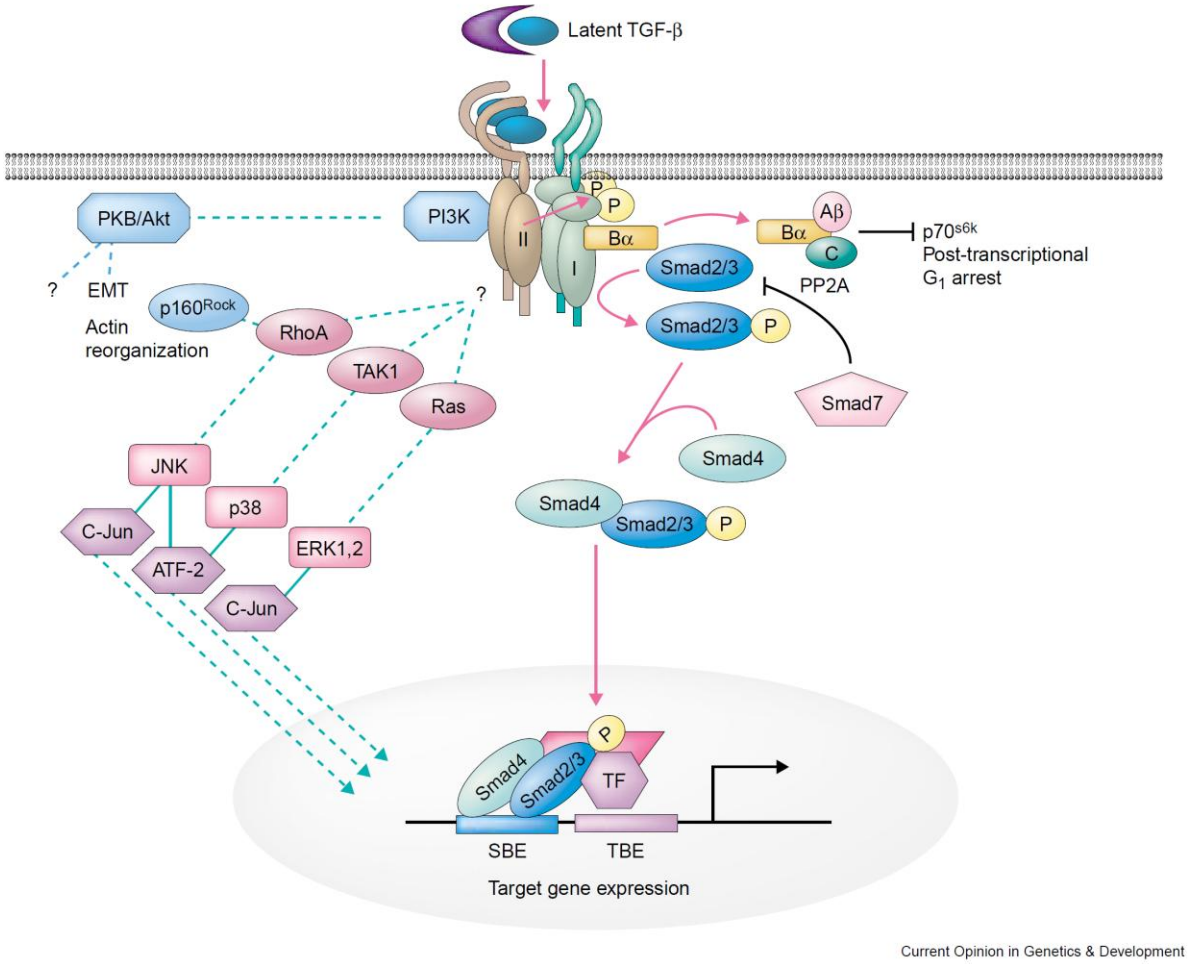



**FIGURE 4 | TGFβ signalling pathways (Wakefield LM. 2001)**
SBE, Smad-binding element; TBE, transcription factor binding element

In most epithelial cells, TGFβ inhibits cell proliferation through activation of SMAD protein complexes, which then lead to the upregulation of cyclin-dependent kinase inhibitors or to the repression of the c-myc promoter resulting in cell cycle arrest and often apoptosis. The ability to arrest cell growth identifies TGFβ as a potential tumor suppressor, this occurs, however, only during early stages of epithelial tumors. *(Cui W., 1996; Derynck R., 2001).* Interestingly, at later stages of tumor progression, almost all human carcinomas and carcinoma-derived cell lines overproduce TGFβ, since they have gained resistance to growth inhibition by TGFβ due to receptor mutations and defects in components of the TGFβ signalling machinery. *(Akhurst RJ., 2001; Derynck R., 2001; Wakefield LM.,2001)* Loss of growth inhibitory responses via diminished TβRII activity facilitates oncogenic effects of TGFβ on the tumor cell and tumor stroma. *(Oft M., 1996)* Further, TGFβ1 increased the metastatic potential of mammary tumors. *(Welch DR., 1990)* During tumorigenesis, cells most probably overcome the tumor suppressive activities of TGFβ switching towards its pro-oncogenic role and stimulating invasion, angiogenesis and metastasis of epithelial tumors (FIGURE 5). In breast cancer, the expression levels of TGFβ1 correlate positively with the disease state and metastatic potential. *(Gorsch SM., 1992)* Indeed, cell-autonomous TGFβ signalling via an autocrine loop is necessary for induction of in vitro invasiveness and support of metastasis at late stages of tumorigenesis. *(Oft M., 1998)* For skin carcinomas in mice the consequence of this cell autonomous TGFβ signalling is a more aggressive spindle cell phenotype and invasive behaviour, which is sufficient to enhance malignancy and metastatic capability of tumors. *(Portella G., 1998*) TGFβ has been shown in normal and transformed breast epithelial cells to be involved in epithelial to mesenchymal transition (EMT). EMT requires the cooperation of MAPK- and SMAD signalling, which are both target pathways of TGFβs. *(Oft M., 1996)*

In summary, TGFβs indirectly targeting the tumor stroma or directly affecting the tumor cell itself stimulate tumorigenesis as a result of its action in immunosuppression or in angiogenesis or of its direct affect in modulating cellular plasticity. (Derynck R. 2001)

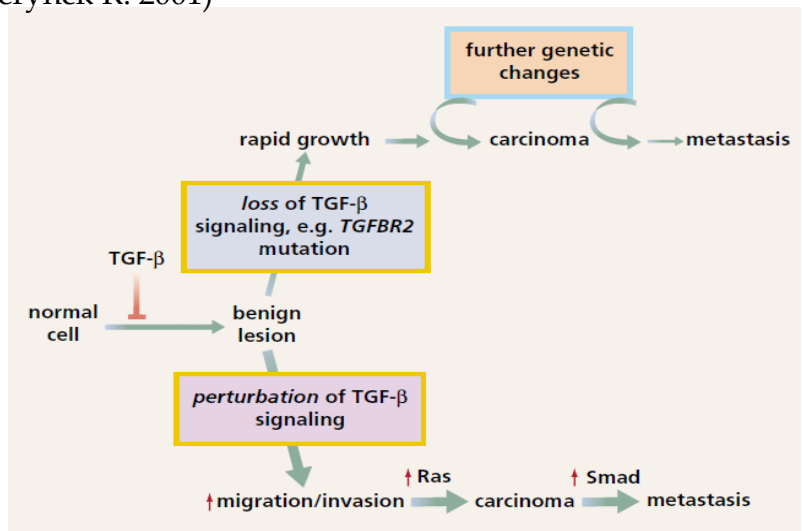



**FIGURE 5 | Alternative roles of TGFβ in tumorigenesis targeting the tumor cell. (Derynck R. 2001)**

## 1.4 RAS signalling

Ras proteins belong to a large family of small GTPases. There are three human ras genes, H-, N-, and K-ras. They associate with the inner face of plasma membrane where they mediate signalling through diverse extracellular stimuli transmitted to Ras via plasma membrane growth factor receptors and downstream signalling proteins. Ras activity relies on a switch mechanism between inactive GDP-bound and active GTP-bound protein conformations. GTP-bound Ras binds to and activate more than 10 different effector molecules that are characterized by their Ras binding domain. These effectors regulate complex signalling cascades, including cell proliferation, differentiation and survival The first discovered and best described Ras effector pathway is the Raf serin-threonin kinases-MEK-ERK cascade, and others Ral guanine nucleotide dissociation stimulator (RalGDS) and phosphatidylinositol 3-kinase (PI3K). (Rajalingam K., 2007, Schubbert S., 2007, Repasky GA., 2004)

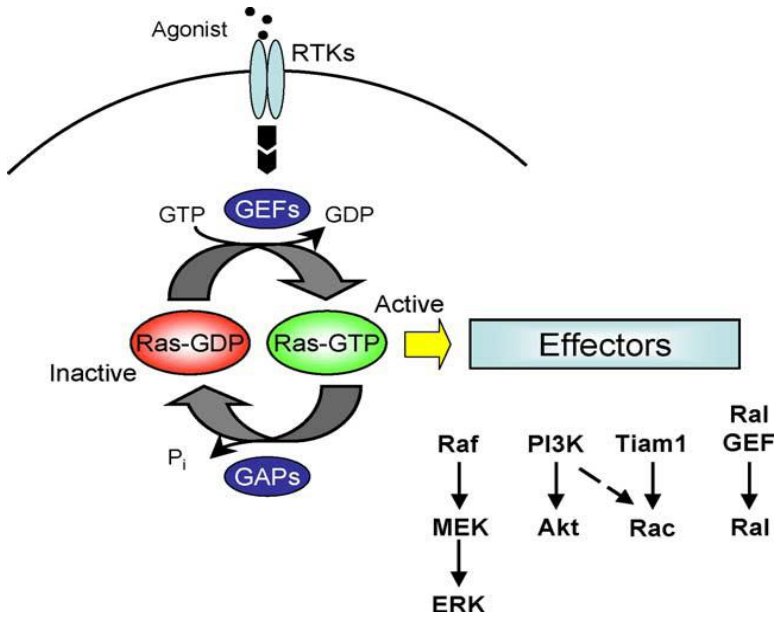



**FIGURE 6 | Ras upstream and downstream signalling Campbell PM., 2004**

Ras binds to and activates Raf, which in turn initiates a signalling cascade phosphorylating the MEK1 and MEK2 dual specificity kinases. These kinases then phosphorylate and activate ERK1 and ERK2 mitogen-activated protein kinases (MAPKs; FIGURE 6). Activated MAPKs can phosphorylate both cytosolic and nuclear substrates and activate transcriptional regulators that control gene expression and cell cycle progression. (Repasky GA., 2004)

PI3K consists of a regulatory p85 subunit and a catalytic p110 subunit. Activated Ras binds directly to the p110 subunit of PI3K and translocates it to the plasma membrane, mediating its binding to receptor tyrosine kinases (RTK) and activation.

Activated PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) by phosphorylation to the phosphatidylinositol-3,4,5-triphosphate (PIP3), which recruits a subset of signalling proteins including downstream kinases such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) and AKT, to the plasma membrane, where they are activated. These proteins then affect diverse cellular events controlling gene expression, cell metabolism, actin polymerization and cell survival. For example, Akt kinase phoshorylates several pro-apoptotic proteins, thereby inactivate them leading to survival and increased proliferation in many cell types. (Cantley LC., 2002)

Somatic mutations of the three Ras genes causing amino acid changes clustered at positions 12, 13 and 61 fail to be stimulated by GAPs and become constitutively active in a GTP-bound conformation. These activating mutations in the Ras genes are common in cancer and have been found in 30% of all human tumours. Additionally, Ras activation can be enhanced apart from mutations upon increased coupling to membrane receptors such as receptor tyrosine kinases, which are also overexpressed in most cancer cells. (Campbell PM., 2004) Oncogenic *Kras* expression promotes lung, pancreatic and haematological malignancies (Karnaub AE., 2008) and H-ras transformed cells form rapidly growing tumors and give rise to metastasis with an autocrine TGFβ signalling (Oft M., 1996). Aberrant activation of Ras proteins cause increased expression of ECM proteases that lead to degradation of the basement membrane as well. (Campbell PM., 2004) Thus, together with above mentioned roles of Ras effector molecules, Ras activation is an important mediator of tumor cell proliferation, transformation, invasion and metastasis.

### 1.4.1 Dissection of Ras effector pathways in the context of tumorigenesis

The role of a particular effector pathway of activated Ras in mediating invasive and proliferative capability of tumors has been searched for intensively. Dissection of Ras downstream signalling was determined by using both effector specific low molecular weight inhibitors and effector specific mutations in the core Ras effector domain resulting inefficient binding of Ras to different effectors. (Campbell PM.,2004) (FIGURE 7)

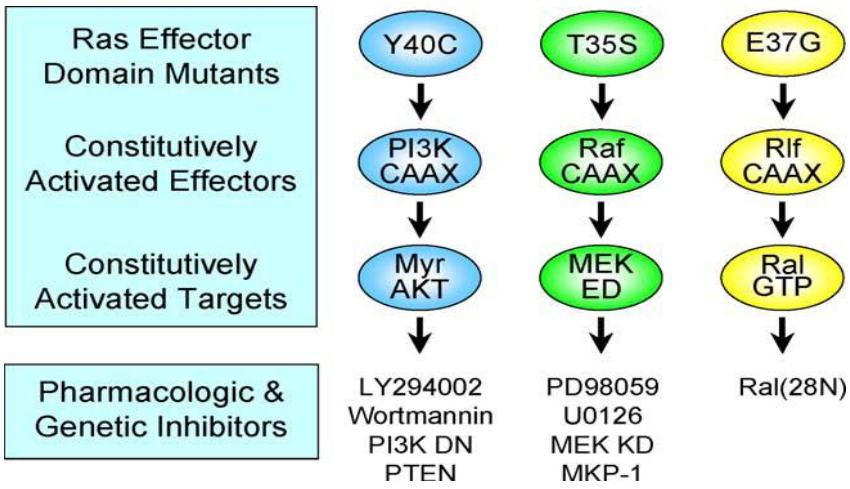



**FIGURE 7 | Experimental approaches to study Ras effector function. (Campbell PM., 2004)**

It has been found, that all effector mutants of activated H-ras were able to induce tumorigenesis in NIH3T3 cells, without respect to the specific pathway activated. However, only Raf/MAPK activated Ras-transformed cells could cause metastasis. (Webb CP., 1998). Additionally, oncogenic Ras transformed but still fully polarized EpH4 mammary epithelial cells require a hyperactive Raf/MAPK pathway for EMT in vitro and metastasis in vivo, whereas a hyperactive PI3K pathway alone was not sufficient to establish metastasis. In contrast, elevated PI3K, but not MAPK signalling in EpH4 cells causes cell hyperproliferation in vitro and rapid tumor growth in vivo, suggesting that MAPK mediated EMT/metastasis and PI3K induced high proliferation ability do not depend on the activation of each other. (Oft M., 1998; Janda E.,2002)

### 1.4.2 Cooperation of Ras with TGFβ

Since TGFβ signalling is required to drive metastasis in vivo (Oft M., 1998) and oncogenic Ras signalling is known to protect cells from TGFβ induced apoptosis after cell cycle arrest. (Oft M., 1996); it is suggested, that in diverse tumor cell types, oncogenic Ras cooperates with TGFβ to induce EMT and metastasis. Moreover, upon TGFβ exposure, oncogenic H-Ras transformed cells form spindle shaped structures with an invasive behaviour, maintain EMT and show autocrine TGFβ signalling. (Janda E., 2002) In summary, TGFβ needs a hyperactive Raf/MAPK pathway to stimulate EMT and metastasis. On the other hand activation of PI3K signalling is essential for the protection from TGFβ induced growth inhibition. (Janda E., 2002).

### 1.5 EpH4 derived cell systems

EpH4 cells are spontaneously immortalised, well-polarized and non-tumorigenic mammary epithelial cells. TGFβ induces apoptosis in these cells. In contrast, EpH4 cells overexpressing oncogenic Ha-ras (RasV12) (EpRAS cells) are tumorigenic and undergo EMT upon TGFβ stimulation in vitro and in vivo, forming fibroblastoid cells expressing mesenchymal markers at the expense of epithelial marker proteins. *(Oft M., 1996).* V12 Ras mutants generated by single amino acid substitutions in the effector loop of the Ras protein have been introduced in EpH4 cells. These oncogenic Ras mutants are not able to activate all effector pathways of Ras. They specifically signal either along the MAPK pathway, RasV12-S35 (EpS35), or the PI3K pathway, C40-V12–Ras (EpC40). Upon TGFβ induction in vitro, EpRAS as well as EpS35 cells form spindle-shaped cells expressing vimentin and no E-cadherin and maintain EMT via activated MAPK pathway as expected. In contrast, TGFβ-treated EpC40 cells show a migratory fibroblastoid phenotype and redistribution but not loss of plasma-membrane E-cadherin plus no upregulation of mesenchymal markes such as vimentin. This phenotypic change of EpC40 cells upon TGFβ induction is referred to as ‚scattering'. EpC40 cells, that can only hyperactivate PI3K pathway, are incapable to drive EMT by TGFβ signalling, due to the lack of an activated ERK/MAPK pathway. However, PI3K pathway stimulates hyperproliferation by protecting cells from TGFβ induced apoptosis; thus EpRAS and EpC40 cells grow in vitro rapidly, whereas EpS35 cells, which fail to signal through PI3K pathway, show slower proliferation rates compared to EpRAS and EpC40 cells by TGFβ. (FIGURE 8)

In vitro characterization of these cells correlates with the in vivo characterization of their ex-tumors, as well. EpRAS and EpS35 tumors are cytokeratin\vimentin positive and E-cadherin negativ, form fibroblastoid-like cells in culture; while EpC40 ex-tumors show expression of E-cadherin without vimentin staining and retain epitheloid phenotype in culture. Further, the in vivo metastatic capability of these cells correlates with in vitro EMT capability induced by TGFβ. Histological analyses of lungs of mice injected with EpRAS or EpS35 cells indicate multiple metastases in both cases, whereas EpC40 injected lungs remain free of metastases. *(Janda E., 2002)*

### 1.6 EMT specific gene profiling

EpH4-derived cell lines overexpressing different oncogenes in cooperation with external signals like TGFβ show diverse epithelial plasticity changes in culture and metastatic ability in vivo. An expression profiling study has been performed on different cell pairs before and after TGFβ induced EMT, including EpRAS, EpC40 and EpS35 cells, to identify specific genes regulated exclusively during scattering, EMT or metastasis. (Jechlinger M., 2003)

The characterization and identification of signalling pathways in tumorigenesis has been performed by using mRNA expression profiling so far. However, total mRNA levels only partly reflect the protein levels because of post-transcriptional/translational regulation. To overcome this limited correlation of transcript and protein levels, polysome-bound mRNA instead of total mRNA has been profiled in arrays yielding isolation of both translationally and transcriptionally controlled genes. (Mikulits W., 2002)

Applying this method to high density arrays, genes have been identified, which are translationally up- or downregulated specifically during cell polarity, invasiveness, EMT and metastasis. The candidate genes were selected due to changes in polysome bound mRNA expression versus free mRNA expression in cell pairs undergoing EMT, but not in those pairs undergoing other or no epithelial plasticity changes . As a result, more than 30 genes have been found to be specifically regulated during complete EMT and metastasis. (Jechlinger M., 2003)

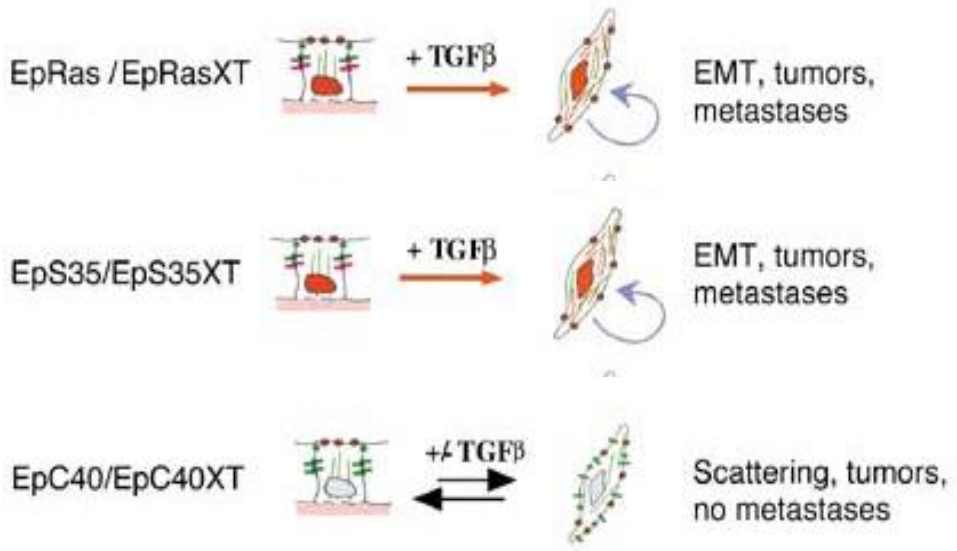



**FIGURE 8 | Different epithelial cell plasticity of EpH4-derived cells (Jechlinger M., 2003)**

apical markers, red dots; basolateral markers, green/pink squares; mesenchymal markers, red fibrils

## 1.7 Tumor stroma

Epithelial cells require stromal influences to control normal development and maintain cellular functions and tissue integrity. Stromal cells comprise fibroblasts, smooth muscle cells, adipocytes, inflammatory and endothelial cells. During carcinogenesis, cancer cells also induce changes in their microenvironment and alter the tumor associated stroma. At the same time modified ECM and stroma interact with tumor cells and contribute to tumor growth and metastasis. *(Liotta LA., 2001; Elenbaas and Weinberg, 2001)*

This tumor-stroma interaction functions bidirectionally and is dynamic. The participation of the tumor microenvironment in tumor progression and invasiveness is supported by fibroblast like cells, immune cells, ECM and angiogenic factors. As a consequence, all carcinomas become complex mixtures reflecting not only their own genomes, but also of the surrounding environment. Indeed, in many of the common carcinomas (like breast, colon, and pancreas) the majority of tumor mass, in some cases over 90%, is made up by the stroma. *(Elenbaas and Weinberg, 2001)*
The cross-talk between tumor and host involves heterotypic cell signalling and secreted molecules such as growth factors, cytokines, chemokines, matrix degrading enzymes, proteases and ECM proteins that provide cell motility. In turn, these molecules stimulate angiogenesis and inflammation; activate and modify surrounding tissues and cells such as fibroblasts, alter ECM, generating additional secretion of growth factors and proteases. *(Olivier De Wever and Marc Mareel, 2003; Mueller and Fusenig, 2004)*

Growth factors play an essential role in carcinomas; TGFβ induces metastasis and elevates platelet-derived growth factor receptor (PDGFR) signalling, which also enhances invasiveness of carcinomas *(Jechlinger M., 2006*). Angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) drive endothelial cell proliferation and motility, thereby supporting tumor progression and metastasis. *(Carmeliet P., 2000)* Overproduced cytokines recruit innate immune cells, such as lymphocytes, macrophages and neutrophils to the tumor. Infiltrating immune cells promote tumor growth and invasion by contributing to angiogenesis and ECM remodelling through expression of a range of proteases, chemokines and pro-angiogenic factors like VEGF, TGFβ or PDGF. *(Pollard JW., 2004; Fusenig and Mueller, 2004)*

An important modification of tumor stroma is the differentiation of fibroblasts into myofibroblasts via cancer cell derived cytokines like TGFβ, or PDGF, which stimulates their further proliferation. Myofibroblasts are also called carcinoma associated fibroblasts (CAFs). They have features intermediate between smooth muscle cells and fibroblasts. They are spindle shaped mesenchymal cells and are involved in tumor progression and metastasis by secreting pro-migratory ECM components, growth factors, cytokines and overexpressing serine proteases, matrix metalloproteinases (MMPs) that degrade and remodel the ECM. (*Olivier De Wever and Marc Mareel, 2003; Fusenig and Mueller, 2004)*

In many human carcinomas such as lung, breast, liver and colon, the presence of myofibroblasts correlate directly with the invasive and metastatic state of tumors. (*Olivier De Wever and Marc Mareel, 2003)*

Increased expression of serine- and cysteine proteases as well as MMPs secreted by inflammatory cells and CAFs lead to ECM remodelling, which is also required for angiogenesis and invasion of tumor cells. *(Elenbaas and Weinberg, 2001)* Hence, the tumorigenic and metastatic capacity of epithelial cell drived tumors depends strongly on the reactive stroma (FIGURE 9).

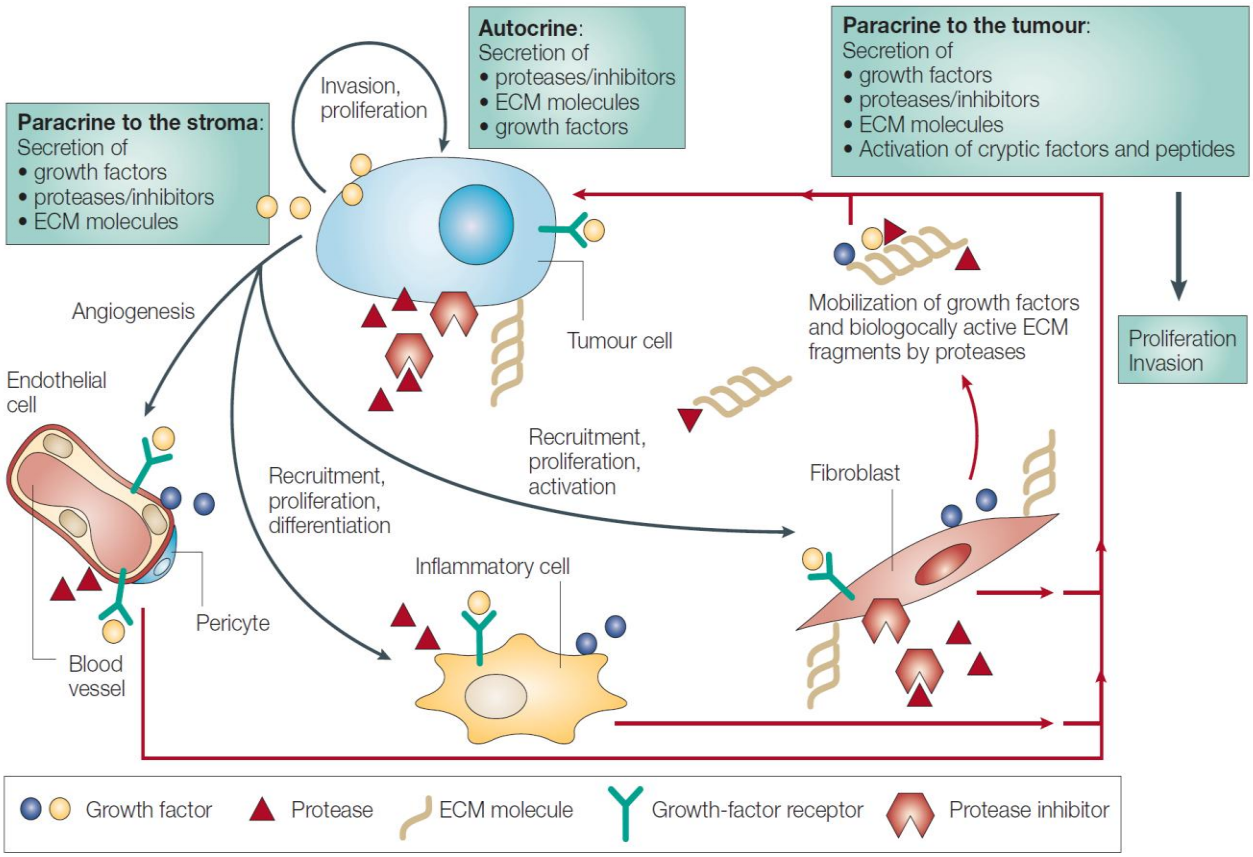



**FIGURE 9 | Heterotypic signalling between tumour cells and activated stroma**
**Mueller and Fusenig, 2004**

### 1.8 The effects of proteases on tumor cells and stroma

Proteases are highly abundant in the extracellular milieu of tumors. They include serine-, cystein-, aspartic acid- and metalloproteinases and are upregulated in different tumours. Beside tumor cells, diverse cell types in the tumor microenvironment such as myofibroblasts, inflammatory and blood vessel cells produce certain proteases as well. Proteases are able to modify tumor stroma and remodel ECM. Concerning their paracrine effects on carcinoma cells they cleave and activate a plethora of factors and ECM components, thereby generating reactive cleavage products and releasing many tumor promoting factors. Proteases are also involved in metastasis, due to their ability to break down ECM and basement membranes and thus allowing cells to invade surrounding tissues and enter the bloodstream. As mentioned, apart from ECM degradation, proteases have an important role in liberation and activation of growth factors and cytokines that are sequestered within the ECM scaffold. (*Fusenig and Mueller, 2004; Elenbaas and Weinberg, 2001)*

Another important target of proteases is the family of Protease activated receptors (PARs). PARs are involved in tumor growth, invasion and angiogenesis through activation of downstream signalling cascades in response to various proteases. PARs are overexpressed in several cancer types. High expression levels of PAR1 were found in highly metastatic breast carcinoma cell lines indicating a correlation with the invasive features of a tumor. (*Arora P, 2007; Yin yong-Jun, 2003)*

#### 1.8.1 Proteases that are involved in tumor – stroma communication

***Serine proteases:*** Most prominent members of the serine type protease family with known important function in tumor progression are those of the plasminogen system (PA-Plg system) - including Plasmin, uPA, tPA -, Kallikrein and Thrombin. They are described as enzymes cleaving and activating diverse latent growth factors. For instance, plasmin activates TGFβ, hepatocyte growth factor (HGF) and FGF, thereby stimulating tumor growth and invasiveness. (*Rakic JM., 2003)* Kallikrein shows also aberrant expression and dysregulated proteolytic activity in adenocarcinomas. (*Borgoño and Diamandis, 2004)* Kallikreins interact with PARs and determine tumor cell fate. Moreover, uPA, tPA and Kallikreins activate receptor-bound urokinase plasminogen activator, which activates latent growth factors. (*Borgoño and Diamandis, 2004;* PA-Plg system will be discussed in the next chapter in more detail.) Thrombin mediates cell migration and induces invasion and angiogenesis. MMP-1 and thrombin can cleave PAR1 and activate its downstream signalling pathways. *(Elzer KL., 2010)* In addition, the PA-Plg system induces tumor progression by activating MMPs. *(Stetler-Stevenson G.W., 2001)*

***MMPs*** are a family of >21 extracellular or transmembrane endopeptidases which require zinc metal ion for their activity. They are able to cleave highly diverse substrates, including ECM components, growth factors and some proteases like plasminogen. MMPs have distinct physiological roles in normal cells, such as wound healing or tissue remodelling and also in pathological conditions like tumorigenesis. *(McCawley L.J. and Matrisian L.M., 2001; Egeblad M. 2002)*

Many members of the MMP family contribute to tumor progression and invasion, while multiple other members have antitumorigenic effects. For instance, MMP-3, 7, 9 and 12 can process plasminogen in vitro to generate an anti-angiogenic molecule "angiostatin". *(McCawley L.J. and Matrisian L.M., 2001)* Together with this, MMPs have been shown to be implicated in the invasive activity of tumor cells and in endothelial cell formation as well. *(Mignatti and Rifkin, 1993)* Various studies involving loss of a single MMP resulted in reduction of tumor development and metastasis. For example, in the absence of MMP-9 and -2, tumor growth was attenuated and MMP-9 null mice also demonstrated decreased angiogenesis. *(Bergers G, 2000)* Unlike the PA/Plg system, which is activated in all cancer types, specific MMPs are selectively overproduced in various kinds and stages of cancer. Since there are MMPs with either activatory and inhibitory function on cancer development, the unexpected, unsuccessful outcome of clinical trials using MMP inhibitors active on several MMPs is understandable. (*Coussens L.M., 2002; Egeblad M. 2002)*

***Cathepsins*** belong to the aspartic and cystein type proteases localized in lysosomes, however in several human cancers they are secreted facultatively as a consequence of lysosomal dysfunction. Indeed altered expression and activity of multiple cystein cathepsins like B, L or D have been found in various malignant tumors. *(Jedeszko C., 2004)* For example, inhibition of cathepsin L in invasive human melanoma cell line prevented tumor growth and reduced metastasis. *(Frade R., 2008)*. Some cathepsins were also in the list of EMT-specific genes from the polysome-bound mRNA screen (Jechlinger, M, 2003)

## 1.9 The Plasminogen System

Activation of the fibrinolytic cascade and of many other proteolytic processes requires conversion of plasminogen to the plasmin. Plasminogen is secreted in inactive zymogene form and becomes activated after being cleaved between amino acid residues R560-V561 by urokinase or tissue type plasminogen activators (uPA and tPA). The active form, Plasmin, consists of two disulfide linked polypeptide chains and has a size of about 90kDa. Plasminogen is synthesized mainly in the liver. In blood, concentrations of approximately 2μM are measured, while 40% of plasminogen are present extravascularly. A wide/diverse range of cell surface molecules can serve as plasminogen receptors in eukaryotes. They are responsible for localized activation of plasminogen. The most frequent and sufficient criteria for plasminogen binding is the presence of surface exposed carboxyl terminal lysines (eg. α- enolase, annexin II) or without expression of terminal lysines when integrins are synthesized or even exposed at the cell surface (eg. αMβ2 on neutrophils). Plasminogen also binds to integrins αMβ2 and α5β1 in a lysine dependent manner. *(Castellino and Ploplis, 2005; Andreasen P.A., 2000)*

tPA and uPA, which convert plasminogen into plasmin, also belong to the serine protease family. Usually, tPA activates plasmin intravascularly for thrombolysis in a fibrin dependent manner, whereas uPA acts independently of fibrin, generating plasmin for pericellular proteolysis events including ECM degradation. Like plasmin, uPA is secreted as an inactive pro-enzyme, which binds to the urokinase receptor (uPAR), a cell surface protein tethered by a glycosylphosphatidylinositol (GPI) to the cell membrane. Hence, activation of plasminogen occurs at the cell surface. As a positive feed-back loop, plasmin catalyzes the cleavage of uPAR bound pro-uPA and thus, promotes the activation of receptor bound uPA. Kallikrein, Cathepsin B and blood coagulation factor XIIa have also been shown to activate pro-uPA in vitro. In addition to plasminogen, uPA can convert latent hepatocyte growth factor/scatter factor (HGF/SF) and macrophage-stimulating protein into their active forms. HGF/SF has been reported to affect cell motility and cellular levels of u-PA and u-PAR. *(Andreasen P.A., 2000)*

Independently of plasmin signalling, uPAR contributes to cell adhesion, proliferation and migration through intracellular signalling. Since GPI-anchored uPAR has no intracellular domain, it cannot propagate signal transduction alone, but utilises many different transmembrane molecules and receptors through protein/protein interaction. So far, uPAR has been proposed to interact with ECM protein vitronectin, integrins, G-protein coupled receptors and tyrosin kinase receptors like EGFR or PDGFR. *(Blasi and Carmeliet, 2002; Smith and Marshall, 2010)*

There are many physiological inhibitors and regulators of the PA-Plg system which ensure a fine tuned and sophisticated regulation of activity. PAI-1 and -2 are the inhibitors of plasminogen activator, while α2-antiplasmin (α2AP) directly inhibits plasmin. They are all members of the serpin superfamily of serine proteases inhibitors. *(Andreasen P.A., 2000)*

Binding of plasmin to its receptors via the lysine binding site, results in elevated activation of plasminogen and also preservation of the protein from circulating α2AP, which binds and inactivates only the unbound form of plasmin. As an opposite, aprotinin, a serine proteases inhibitor with a broad substrate specify, can inactivate both soluble and receptor bound plasmin through the association with its active site. (FIGURE 10; *Andreasen PA., 1997)*

Plasmin primarily has its function in the blood coagulation system, where it degrades fibrin-containing thrombi. Beside this, having a broad substrate specifity, it is also able to degrade many extracellular matrix proteins such as fibronectin, fibrin, laminin and vitronectin. Through this, plasmin intensively contributes to the degradation of cell-cell and cell-ECM barriers and thus facilitates cell migration. As a consequence, plasmin participates in important processes of tissue remodelling, e.g. during embryogenesis, angiogenesis, wound healing and pathological events like cancer dissemination. (*Castellino and Ploplis, 2005).* Plasmin can cleave and activate several metalloproteinases, cytokines and growth factors stored in the ECM such as TGFβ, VEGF, FGF, which all have a role in tumor growth, invasion and metastasis. *(Mignatti and Rifkin, 1993; Rakic JM., 2003; Andreasen PA., 1997)* Another potential mechanism how plasmin affects cellular processes and its downstream effector molecules may involve PAR1 activation and MAPK dependent signalling pathways. *(Pendurthi UR., 2002)*

Expression of uPA, plasminogen, uPAR and PAI-1 is enhanced during cell migration. Moreover, plasmin activity induces cell migration, and α2AP and other plasmin inhibitors, e.g. urinary- and bovine pancreas trypsin inhibitors prevent invasion. Besides, plasminogen activation needs uPAR bound uPA, to induce ECM degradation in vitro, indicating that cells require uPA catalyzed plasmin activity for a maximum invasion capability through the ECM. Thus, plasmin generation by uPA may be the rate-limiting factor for tumor invasion and metastasis formation. *(Andreasen PA., 2000, Rakic JM., 2003)*

In breast cancer, high expression levels of uPA, uPAR and PAI-1 in cancer cells and more strongly in fibroblasts correlate with malignancy, invasiveness and tumour size. *(Dublin E., 2000).* PAI-1, although preventing dissemination of cells at high levels, may be required for a controlled proteolytic activity of plasmin, whereas PAI-2 regularly inhibits invasion in vitro. *(Andreasen PA., 2000, Rakic JM., 2003)*

The essential role of plasminogen for tumor growth and metastasis has been shown in studies of Plg knockout mice. Plg-/- mice expressing Polyoma middle T antigen (PyMT) under the breast specific MMTV promoter resulted in decreased lung metastasis, while PyMT-driven breast carcinoma formation was not affected. *(Bugge TH., 1998)* In another study, fibrosarcoma cell transplanted Plg-/- and wt mice both developed sarcomas, but Plg deficient mice had a significantly reduced tumor burden. *(Curino A., 2002)* Thus, these reports indicate that plasminogen has an important role in tumor progression and metastasis.

However, the observation that metastasis was impaired but not absent in Plg deficient mice suggests that development and dissemination of tumors can be also influenced by other proteases that show partially overlapping functions with plasmin.

Aprotinin, a non-specific broad serine protease inhibitor, inhibits plasmin, plasminogen activator or kallikrein. Since proteases have tumor promoting roles, aprotinin is thought to be a good therapeutic tool to prevent tumor development, resection and metastasis. Moreover, it is been used to inhibit primary tumor growth of various cancer types in animals. However, inhibition of plasmin dependent fibrinolytic system increases thrombosis and blood coagulation factors. Many metastatic cells are associated with thrombi that actually have been demonstrated to enhance the survival of metastatic cells, allowing their retention in the secondary organs. Therefore aprotinin failed to be beneficial in metastatic cancer therapy. *(Turner G.A, 1981)* For instance, experimental metastasis assays of B16F10 murine melanoma cells showed increased metastases formation in aprotinin treated animals. *(Kirstein J.M., 2009)*

Since this study focuses on the mechanism of ILEI, which has been found to be processed by plasmin (see next chapter), data on the Plg/PA system supported our experiments and conclusions and were thus highly relevant in our studies.

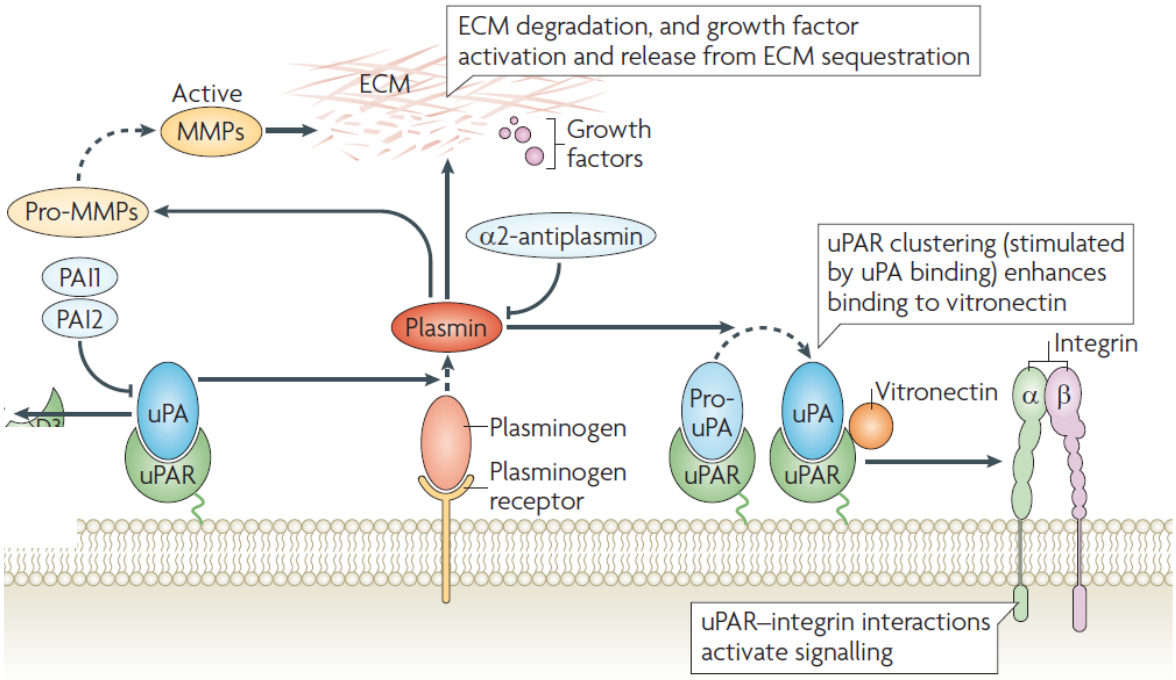



**FIGURE 10 | the plasmin/plasminogen activators system, its function and regulation (Smith WH. and Marshall CJ, 2010)**

## 1.10 ILEI

**I**nterleukin **l**ike **E**MT **i**nducer (ILEI) belongs to a novel cytokine gene family FAM3, which was identified by searching for novel four-α-helix-bundle cytokines in genomics databases (*Zhu and Xu, 2002).* Later structural analyses indicated, however, that the α-helices in ILEI did not really resemble the four-α-helix-bundle in typical cytokines. Four genes belong to the FAM family, i.e. FAM3A, FAM3B, FAM3C (which is ILEI), and FAM3D, each encoding a protein of 224-235 amino acids with a hydrophobic signal peptide. Northern blot analysis has indicated that FAM3D is expressed in high levels in the placenta, FAM3B in the pancreas, and FAM3A and FAM3C in almost all tissues. Additionally, FAM3C has been reported to be expressed in the inner ear and suggested a role in its development. *(Pilipenko and Reece, 2004)*

FAM3C has been identified and referred to as ILEI in a polysome bound expression profiling screen for EMT-specific genes. During EMT and metastasis, TGFβ upregulates ILEI exclusively at the translational level. *(Jechlinger M., 2003)* It is a facultatively secreted protein. Exogenous ILEI overexpression in non-tumorigenic (EpH4), tumorigenic but non-metastatic (EpC40) and tumorigenic and metastatic (EpRAS) mouse mammary epithelial cell lines has resulted in complete EMT, elevated tumor growth and metastasis. In EpH4 cells, however, ILEI alone caused a reversible EMT and weak metastasis. Full EMT induction by ILEI was dependent on oncogenic Ras signaling but independent of TGFβ signaling. It causes accelerated tumor growth as a consequence of stimulation of EMT without an effect on cell proliferation. Additionally, ILEI expression increases phosho-ERK levels in both EpH4 and EpC40 cells, which have only basal ERK/MAPK activation. Furthermore, ILEI stimulates upregulation of diverse cytokines, chemokines and growth factors and their receptors like PDGFR, which is known to be necessary in tumor progression. ILEI-induced EMT and -metastasis could be inhibited or reversed by RNAi-mediated knock down of ILEI in EpRAS- or mesenchymal EpRasXT cells (EpRas cells after TGFβ-mediated EMT), the latter failing to maintain EMT and regaining an epithelial cell phenotype. In human carcinogenesis, elevated ILEI levels and altered localization correlate with survival rates. (*Waerner T et al., 2006)*

In some epithelial tissues like uterus, prostate or lung, expression of ILEI protein was not detectable by immunohistochemistry, but in other secretory epithelia (duedonum, pancreas, colon) and in certain tumors, ILEI localization showed dot-like, suprabasal structures, most likely representing Golgi- or trans-Golgi-network (TGN) structures. However, in many metastatic tumors ILEI was found to be delocalized as small vesicles throughout the entire cytoplasm of tumor cells, which might reflect an enhanced secretion of the protein, which actually correlates with an enhanced translation and secretion levels of ILEI during EMT and metastasis. (*Waerner T et al., 2006).* TGFβ upregulates both intracellular and secreted ILEI levels in EpH4 and EpRAS cells, at least partially by increasing the translation of the protein, indicating that ILEI acts downstream of TGFβ during EMT (*Waerner T et al., 2006).*

A recent report has revealed the mechanism of TGFβ stimulated ILEI translation. TGFβ mediates phosphorylation and inactivation of hnRNP E1 protein. In its active form this protein binds to the 3' untranslated region of the ILEI transcript and represses its translation. TGFβ activated release of hnRNP E1 from ILEI mRNA results in increased translational activation of ILEI. *(Chaudhury A., 2010)*

### 1.10.1 Biochemical properties of ILEI protein

ILEI is a secreted protein. It consists of 227 amino acids (aa) with a signal peptide at its N terminus. (FIGURE 11) Western blot analyses showed that secreted ILEI is shorter than its intracellular form. (FIGURE 12 left part) Indeed, mass-spectrometry (MS) data confirmed that secreted ILEI lacks 17 amino acids at the N-terminus, which contains arginin/serine aminoacids as a potential cleavage site, in addition to the signal peptide. Thus, ILEI might be processed after synthesis.

Additionally, serum containing medium is sufficient to generate the short form of ILEI, whereas under serum free conditions ILEI is secreted but preferentially retains the MW of the full length form, indicating that the protein is processed extracellularly. In cell-free assays, serum alone is able to cleave ILEI. (FIGURE 12) Presumably, ILEI might be processed by serum proteases. In vitro, Aprotinin, a broad spectrum inhibitor of serine type proteases, efficiently inhibits ILEI cleavage (FIGURE 13). Finally, the serine type protease, plasmin, has been proven to be specifically able to cleave full length ILEI at the arginin/serine position. (FIGURE 14) (Agnes Csiszar, unpublished data)

Plasmin is widely overexpressed in tumors, it also has an important role in releasing growth factors and cytokines adhered to the ECM. (Detailed information about plasmin has been given in previous chapter) It remains to be clarified, whether plasmin is the only protease to generate the short form of ILEI, but pilot experiments suggest that closely related proteases also cleave ILEI in vitro.

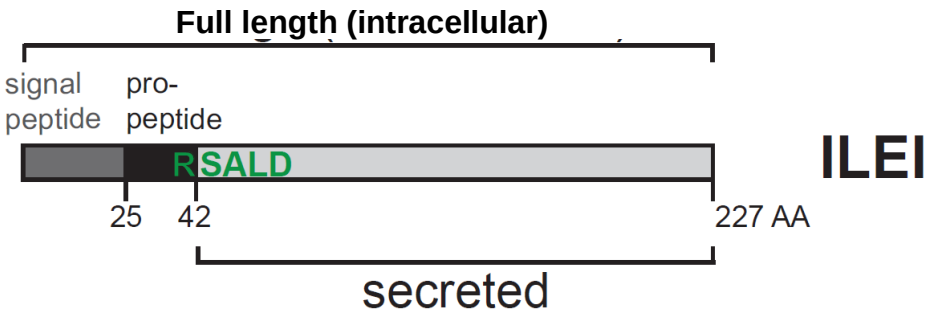



**FIGURE 11 | schematic drawing of ILEI structure**

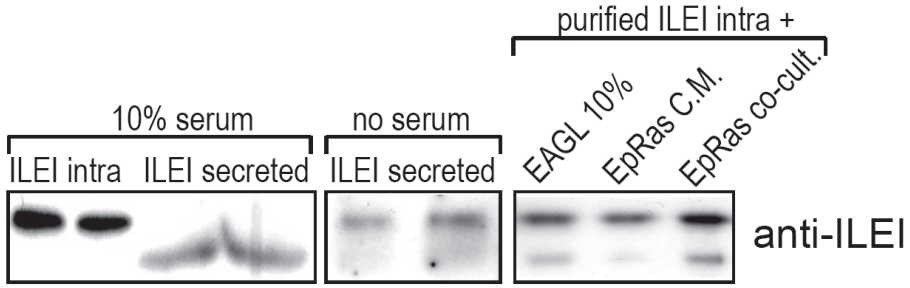



**FIGURE 12 | Serum alone is able to generate the short form of ILEI**
**C.M.; conditioned medium from respective cells**

| Inhibitor | Target protease |
|---|---|
| E64 | cystein and thiol proteases |
| CA-74 | Cathepsin D |
| Pepstatin A | acid proteases |
| GM6001 | MMPs |
| AEBSF | Serine proteases |
| Aprotinin | Serine proteases |

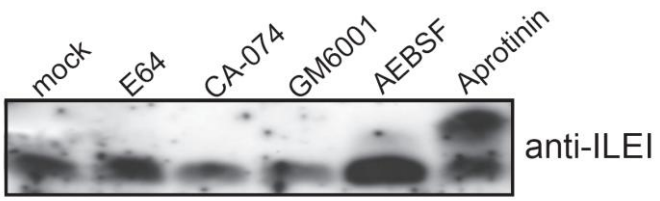



**FIGURE 13 | Proteases inhibitors with their targets (left) and inhibition of ILEI cleavage by aprotinin (right)**

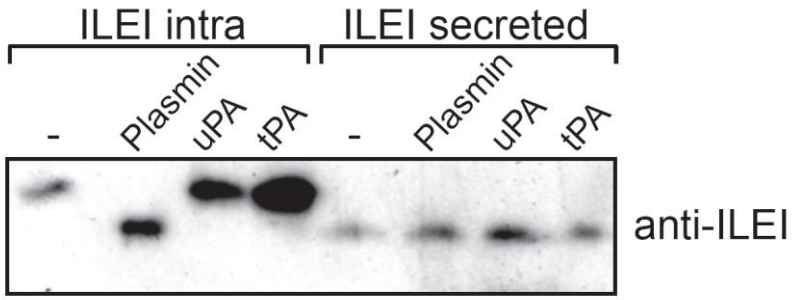



**FIGURE 14 | Plasmin is able to cleave ILEI.**

## 1. 11 Aim of this study

In the first part of my diploma thesis, I studied the mechanism of proteolytic cleavage of ILEI in vitro and the physiological relevance of ILEI cleavage in vivo. We questioned whether ILEI-directed tumor growth and metastasis depend on its cleavage. Furthermore, we studied potential connections between ILEI processing and secretion by addressing the question whether or not cleaved ILEI might stimulate its own expression and secretion directly in an autocrine manner via binding to putative ILEI receptors.

In the second part, I performed the initial characterisation of a doxycyclin inducible ILEI overexpressing transgenic mouse model using ex vivo and in vivo assays. The inducibility and tissue pattern of ILEI transgene expression were analysed in various organs of mice before and after induction.

# 2. RESULTS

## 2.1. ILEI CLEAVAGE ASSAYS

### 2.1.1 Cleavage test of ILEI by serum from Plasminogen knock out mice

To determine, whether plasmin is the only physiological protease responsible for ILEI cleavage, we made use in the availability of plasminogen knock out (Plg KO) mice. Blood sera were collected from Plg knockout mice and their wild type and heterozygous littermates. Purified full length ILEI-flag protein was incubated in 10% (v/v) serum for 8 hours at 37°C.

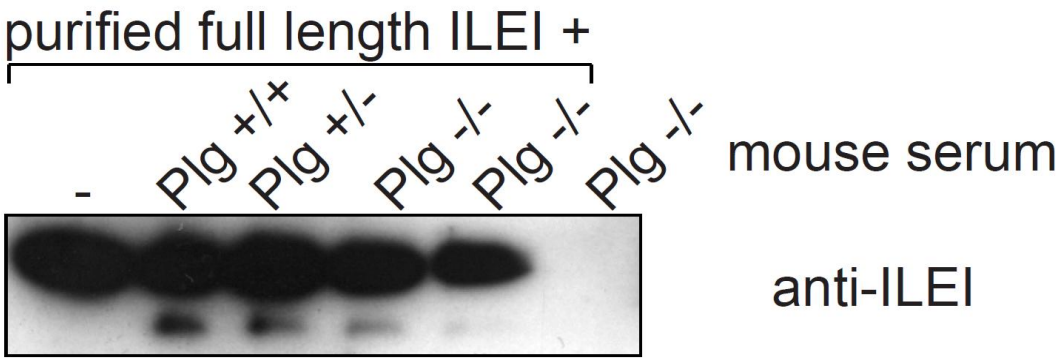



**FIGURE 15 | Plg-/- mouse serum able to cleave ILEI**

ILEI was cleaved in all types of mouse sera, including Plg KO mouse serum which cleaved less effectively, indicating that there should be other proteases with the ability of ILEI processing.

### 2.1.2 Cleavage test by other serine type proteases

We were wondering which other proteases might be able to process ILEI at the same site as plasmin does. As the plasmin type serine proteases thrombin and kallikrein have very similar cleavage recognition sites to plasmin, they were tested in in vitro cleavage assays. Together with the full length form of ILEI, the secreted protein (short form) was also purified and incubated with proteases. This would allow identifying only the proteases that process ILEI at its R/S site specifically generating a protein in ~19kDa size.

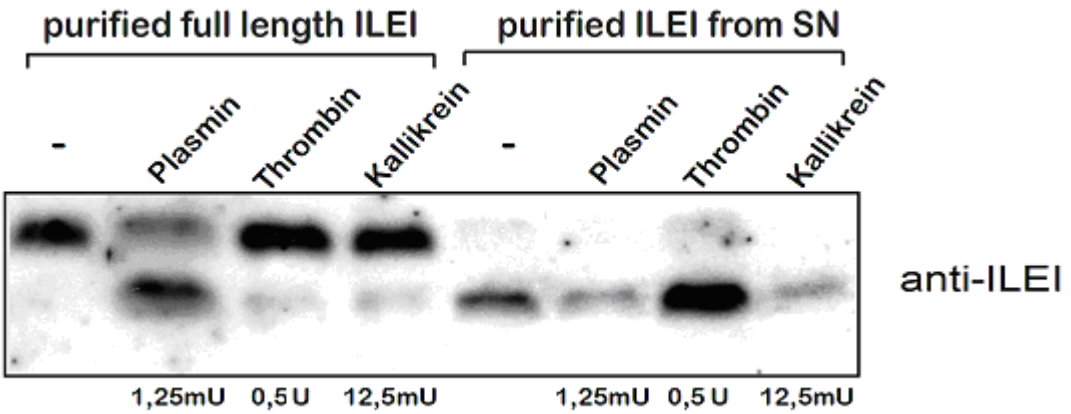



**FIGURE 16 | cleavage test of full length and secreted ILEI by other plasmin type proteases SN: supernatant**

Both, Kallikrein and thrombin were also able to cleave ILEI. However, much higher amounts of proteases were required to generate significant amounts of cleaved ILEI. In case of kallikrein and thrombin 5 times and 400 times more activity had to be added to the reaction, respectively, as compared to plasmin. Thus, in our tests plasmin was proven to be the most active protease for ILEI. All these proteases processed only full length ILEI, whereas secreted ILEI remained intact, suggesting that the cleavage occurs specifically at the propeptide region.

### 2.1.3 Generation of ILEI mutants

To investigate the physiological role of ILEI cleavage, a series of respective ILEI mutants were generated. Wild type (wt) and mutant ILEI proteins were C terminally fused to a Flag epitope tag to enable detection of the overexpressed protein separately from endogenous ILEI. Two different types of ILEI mutants were used for further analysis. One group contained ILEI cleavage mutants that had point mutations at the cleavage recognition site, where arginin (R) and serine (S) amino acids were substituted with the hydrophobic amino acid phenylalanine (F) and acidic aspartic acid (D), therefore this mutants are referred to as ILEI-FD or ILEI-DF. These mutants should not be substrates for the responsible proteases. The second group was the Δpro-peptide mutants that lacked the ‚pro-peptide', which has been proposed to be cleaved off after secretion of ILEI. The ΔN-RS-ILEI mutant was generated starting with RS amino acids, because they might be essential for the secretion of protein.

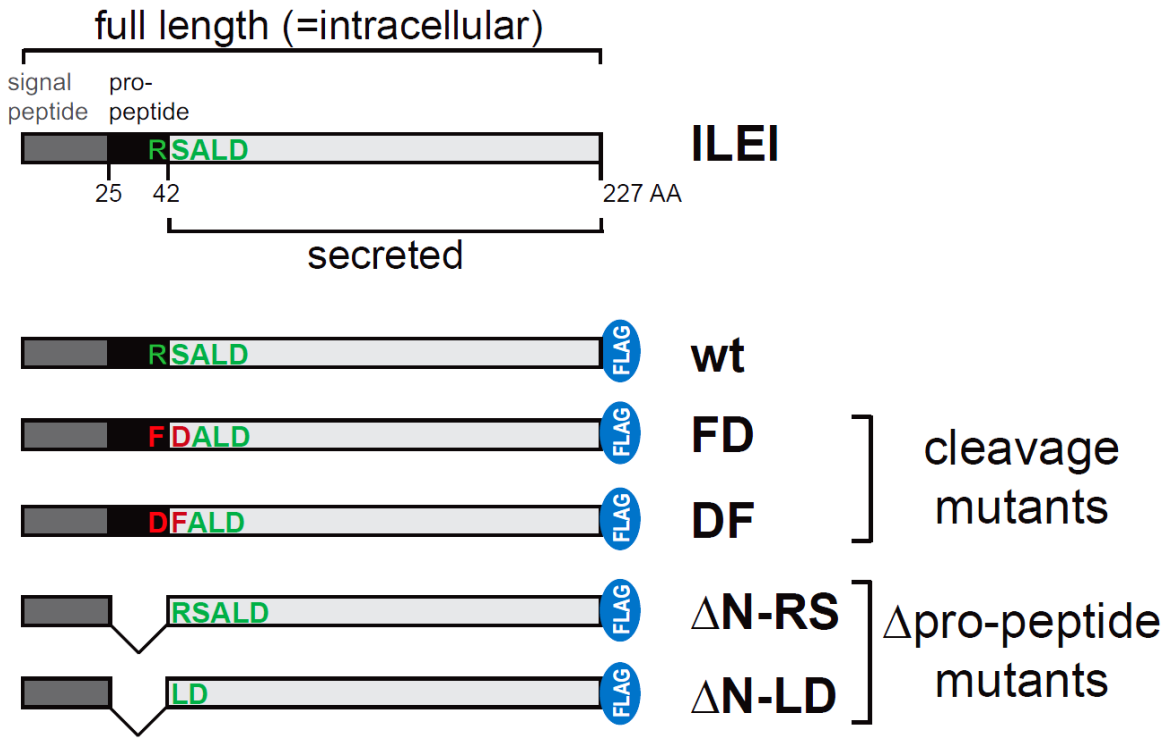



**FIGURE 17 | schematic representation of the ILEI protein and its mutant forms used in this study**

These wt and mutant ILEI proteins were overexpressed in EpRAS and EpC40 cell lines. First, we analyzed the intracellular and secreted ILEI levels of these cells by Western blotting.

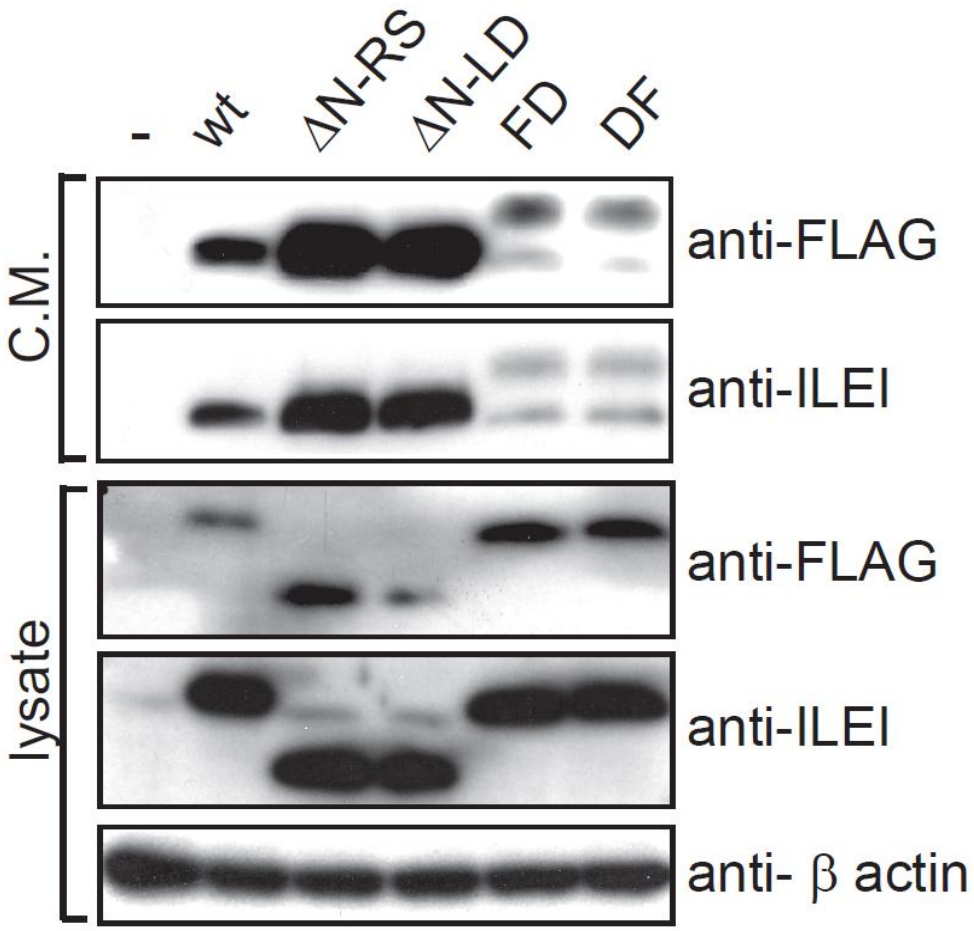



**FIGURE 18 | Intracellular and secreted ILEI levels of generated EpC40 cells over-expressing wt and mutant forms of protein.**
**C.M.: conditioned medium of cultivated cells.**

As expected, the secreted form of the cleavage mutants were indeed no more cleaved and run prefentially at higher molecular weights, and the intracellular forms of Δpro-peptide mutants were already shorter than wild type intracellular ILEI.

Secondly, we examined the cleavage of the generated wt and mutant forms of ILEI in vitro. For this, the proteins were purified from the overexpressing cells and the same cleavage assay was performed using the same proteases as described before for wt ILEI.

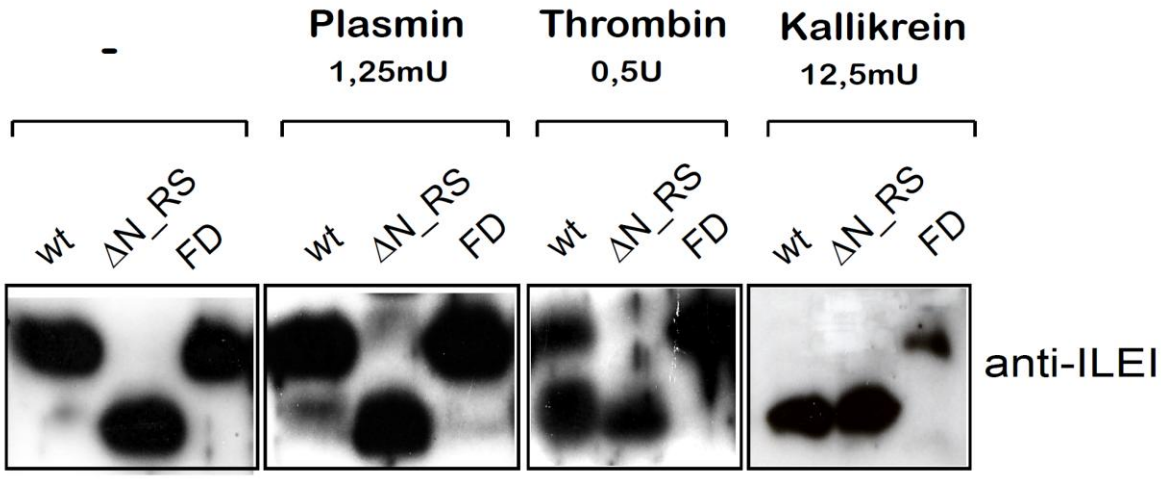



**FIGURE 19 | Cleavage test of mutant ILEI forms with plasmin, thrombin and kallikrein proteases**

All three proteases, plasmin, thrombin and kallikrein, were again able to cleave wt-ILEI. In contrast, the ILEI mutants indeed functioned no longer as efficient substrates of the proteases, showing that the mutants were generated successfully. However, we observed differences in the ratio of cleaved and non-cleaved ILEI compared to the previous cleavage assay, although the same amount of proteases were used. (compare Figure 16 and 19) Cleavage efficiency of the ILEI protein often depended on purification quality or pH of the purified protein. In the two experiments shown, separately purified proteins were used, which might be responsible for the slightly different results to some extent.
In these experiments, we also used higher amounts of the protein to overcome the loss by unspecific protein degradation, that was most prominent in the plasmin assay.

In addition, we observed different secretion levels of the different ILEI forms in the overexpressing cells. On the one hand, cells overexpressing the cleavage mutant forms secreted less ILEI than those with wild type ILEI. In contrast, cells overepressing the ILEI mutants lacking the propeptide (Δpro-peptide mutants), which bypass the need of proteolytic processing, secreted ILEI at even higher levels. Accordingly, it appeared that ILEI secretion levels might be linked to the cleavage of the protein.

### 2.1.4 In vivo tumorigenesis experiment

To analyse the physiological role of ILEI cleavage on tumorigenesis, we injected EpC40 cells overexpressing wt and mutant forms of the protein into nude mice. Third and fourth mammary gland fat pad of mice were injected with $2x10^5$ of these cells. As mentioned before (chapter 1.5 and 1.9.1), in the tumorigenic but non-metastatic EpC40 cells, overexpression of ILEI caused accelerated tumor growth and induced full EMT and metastasis.
The mice were sacrificed ~40 days after injection and mammary tumors were excised. A part of excised tumors were analysed in immunohistochemistry and the rest was recultivated for further experiments.

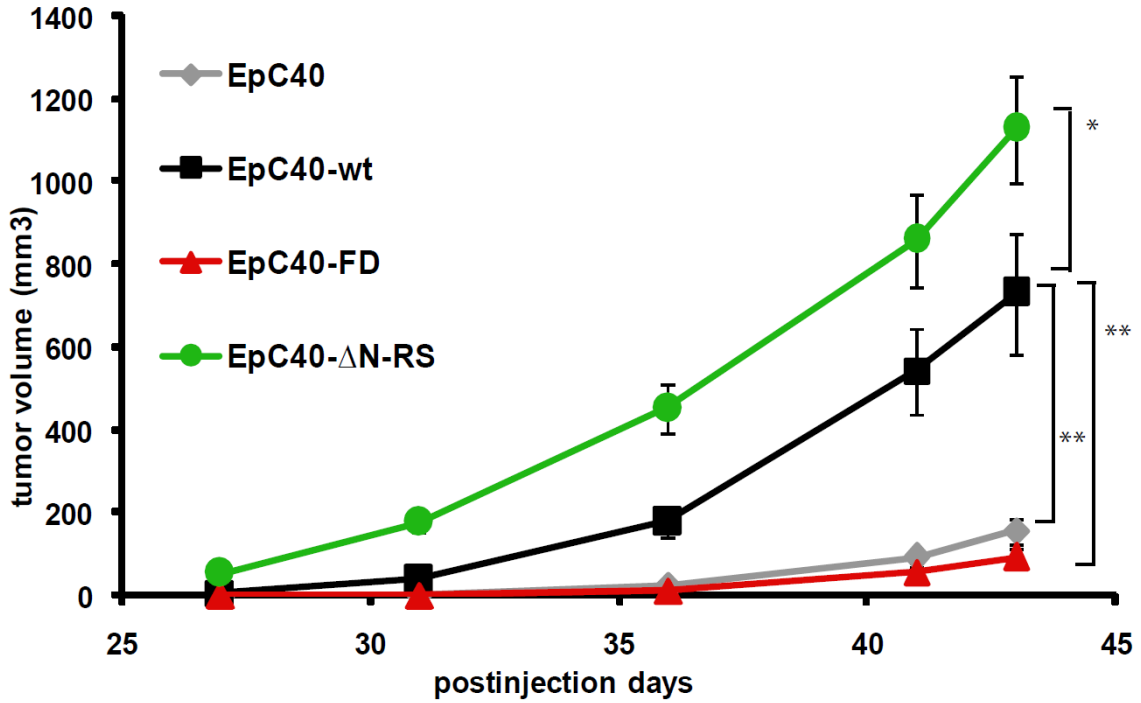



**FIGURE 20 | Tumor growth kinetics of EpC40 mammary tumors overexpressing wt and mutant forms of ILEI**
Error bars show standard error of means (SEM), * marks P<0,05 and ** P<0,002 calculated by two tailed student's t-test.

As expected, EpC40 wt-ILEI overexpressing cells showed significantly elevated tumor growth rates as compared to the control cells. Cleavage mutant overexpressing cells had very low tumor growth capacity, similar to control cells. Interestingly, the Δpro-peptide mutant overexpressing cells showed strongly accelerated tumor growth as compared to wt-ILEI overexpressing EpC40 cells.

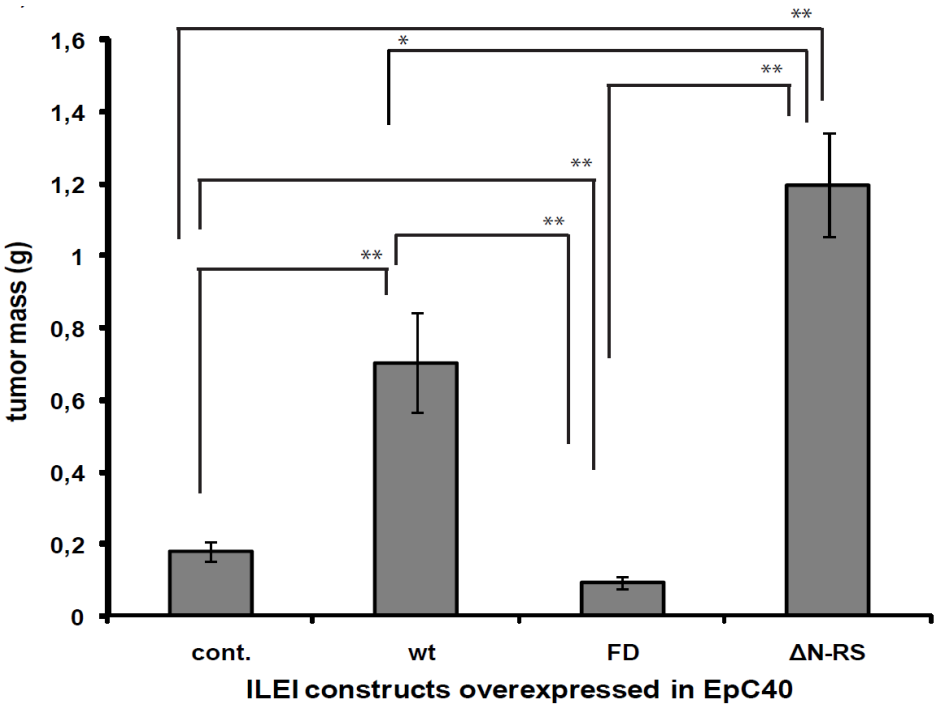



**FIGURE 21** **Tumor masses of different ILEI forms overexpressed in EpC40 mammary tumors** Error bars show standard error of the mean (SEM). *: P<0,02, **: P<0,007

Differences in tumor growth kinetics were also reflected in tumor weight, determined after sacrificing the mice and isolating the mammary tumors. Cleavage mutants and control cells formed small tumors. Wt-ILEI overexpressing cells formed bigger tumors, which were, however, still smaller than the tumors formed by cells overexpressing the Δpro-peptide mutants.

As a consequence of these results, we postulated that ILEI cleavage might play an important role for its function and the generation of the short form could be required for accelerated tumor growth and tumor burden. However, we could not exclude, that different tumor formation ability of these cells might have different reasons, such as different secretion levels of ILEI, which might be responsible for the different tumor growth rates observed. Therefore, we tested by inhibiting ILEI processing in vivo, whether the cleavage of the protein would be the main process required to stimulate tumor growth

### 2.1.5 In vitro aprotinin pilot experiment

Earlier in vitro cell free assays showed that the broad spectrum serin protease inhibitor aprotinin was able to prevent ILEI cleavage caused by fetal calf serum. (Chapter 1.9.2) Here, we first checked if aprotinin would be similarly efficient on mouse serum-induced ILEI cleavage. For this, purified wt and mutant ILEI proteins were incubated with mouse serum in the presence or absence of aprotinin.

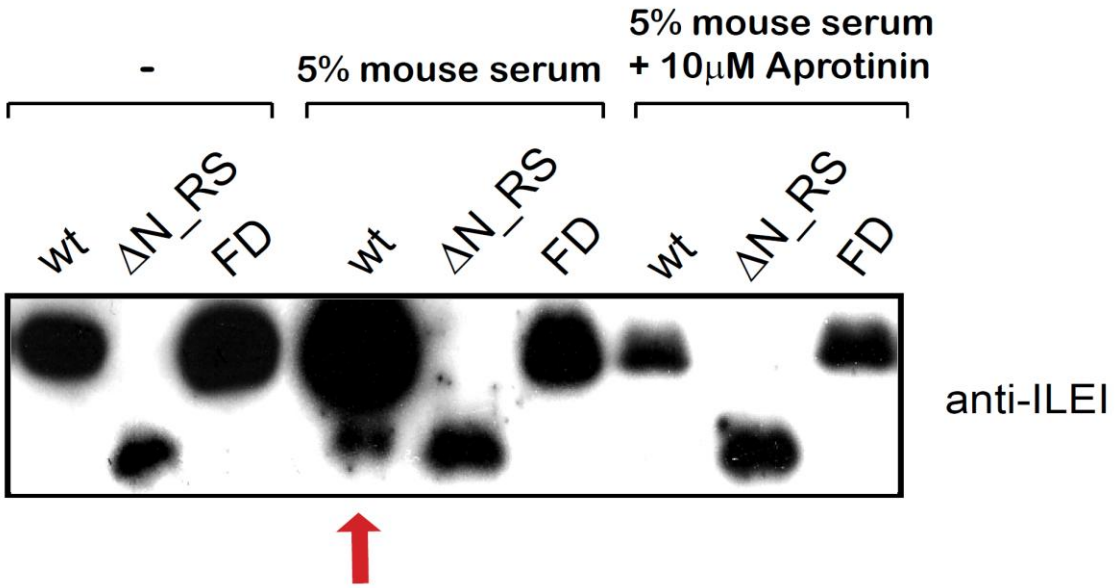


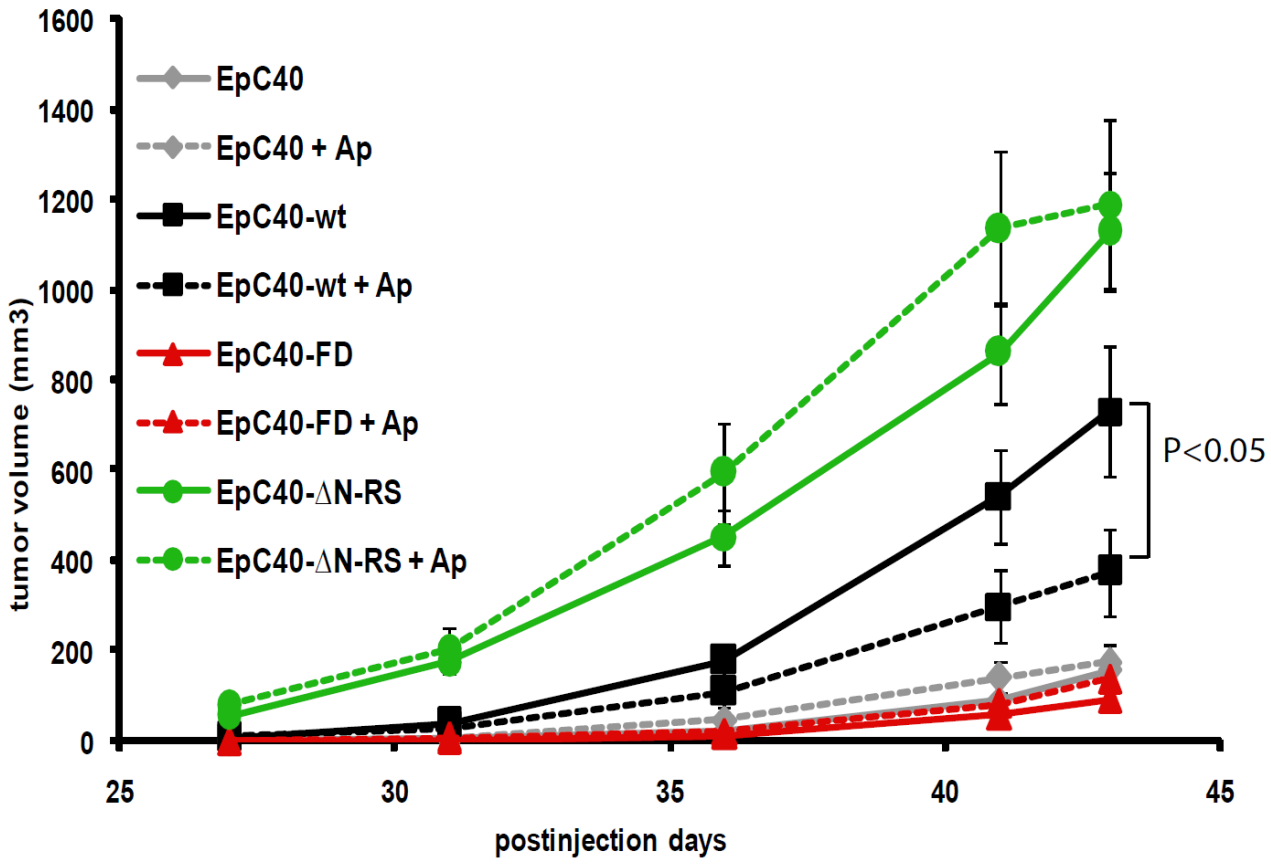

**FIGURE 22 | Cleavage of ILEI by mouse serum (red arrow) and its inhibition via aprotinin**

Wt ILEI was cleaved by mouse serum, whereas the mutants were not. Importantly, aprotinin could inhibit the cleavage of wt-ILEI by mouse serum (FIGURE 22). Thus, aprotinin appeared as a reasonable tool to inhibit ILEI cleavage in vivo . Since aprotinin has been used in mouse experiments and has already been used in clinical trials, a large body of information was available concerning dosage calculation and pharmacokinetics of this inhibitor.

### 2.1.6 In vivo tumorigenesis experiment with aprotinin treatment

An in vivo tumorigenesis experiment was performed as described above, except that in this experiment a subset of mice were treated with 4000 KIU of aprotinin by i. p. injection every day over the whole period of the experiment. This was necessary, since aprotinin fails to maintain its in vivo activity after 24 hours. Again, ~40 days after injection mice were sacrificed, mammary tumors were excised, weighed and fixed for immunohistochemical analysis (2.1.7)

Aprotinin treated mice injected with wt-ILEI overexpressing EpC40 tumor cells showed significantly decreased tumor growth rate (FIGURE 23 top) and tumor size, (FIGURE 23 bottom) as compared to control mice receiving the same cell injections, but no aprotinin treatment. Injection of all the other cell types caused tumors with similar, maybe even slightly increased growth rates and tumor sizes in aprotinin-treated mice as compared to non-treated animals. Thus, ILEI-induced increased tumor growth was repressed in case of wt-ILEI, but not in case of the delta pro-peptide ILEI mutants, indicating that an aprotinin-sensitive protease activity was important to generate the processed form of ILEI in vivo, which apparently is required for rapid tumor formation.



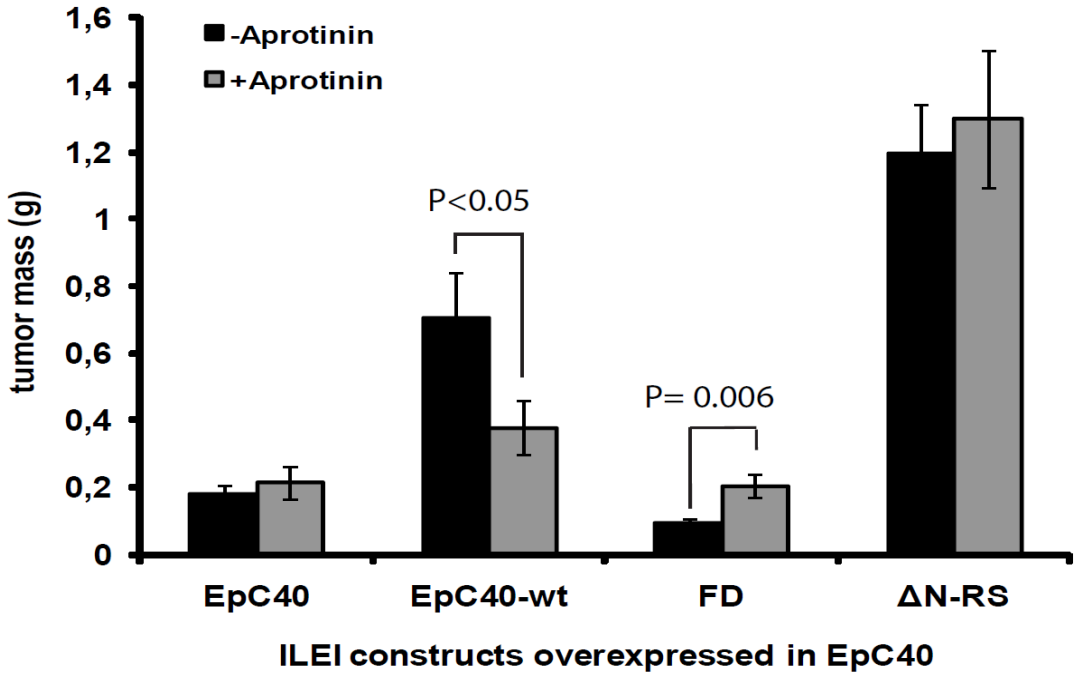



**FIGURE 23 Tumor growth rates (upper panel) and tumor weights (lower panel) of EpC40 mammary tumors overexpressing different forms of ILEI with or without aprotinin treatment.** Error bars show standard error of the mean (SEM).

### 2.1.7 In vivo characterization of excised mammary tumors by immunohistochemistry (IHC)

Mammary tumors from aprotinin-treated and non treated animals were immunostained for proliferation and apoptosis markers, E-cadherin /vimentin EMT marker proteins and ILEI/FLAG proteins. The stained sections were analysed for changes generated by the inhibitor treatment and for differences between the different cell lines

### 2.1.7.1 Ki67 (proliferation marker) and activated Caspase3 (apoptosis marker) stainings

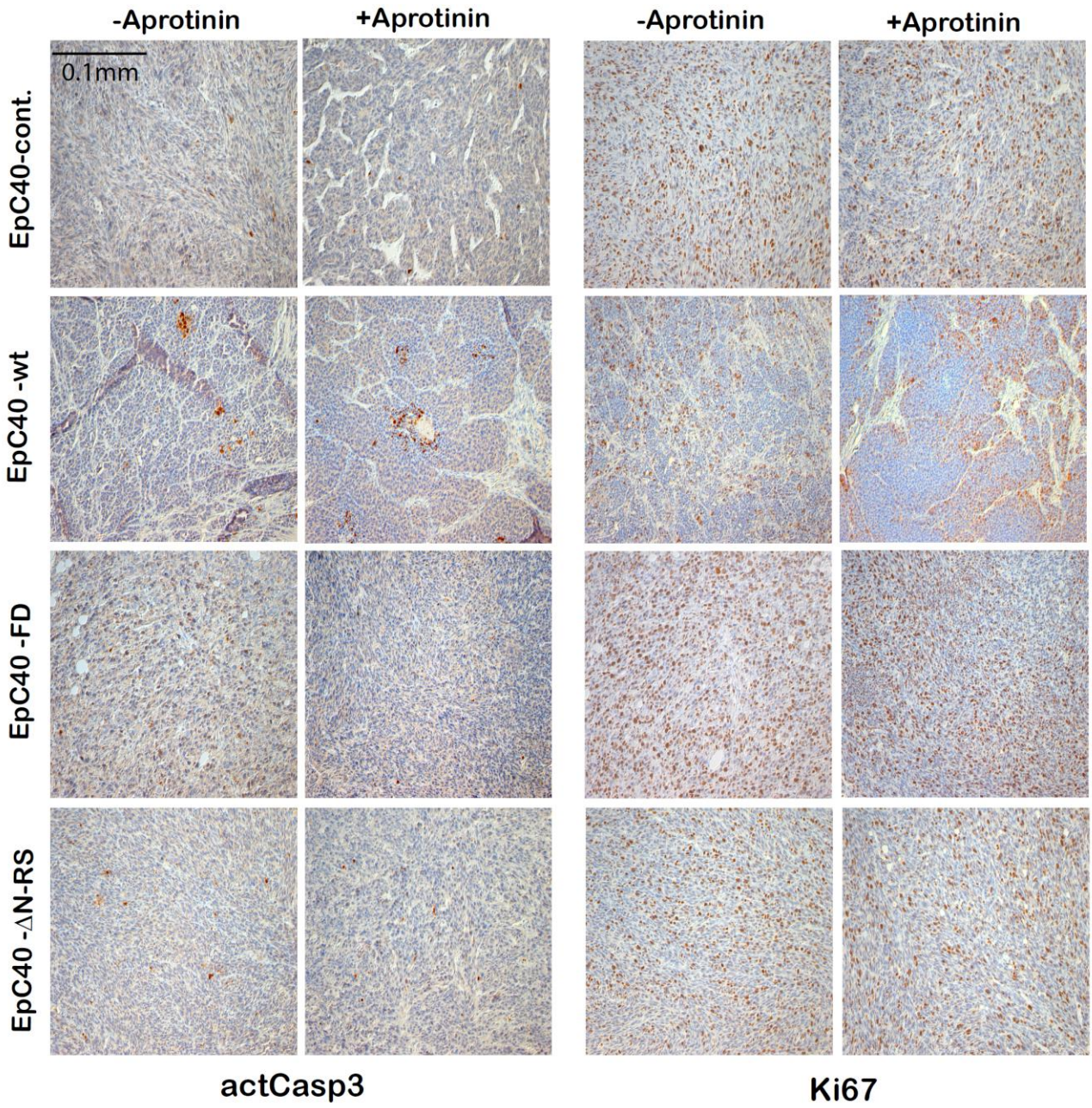



**FIGURE 24 | Ki67 and actCaspase3 IHC of EpC40 tumors**

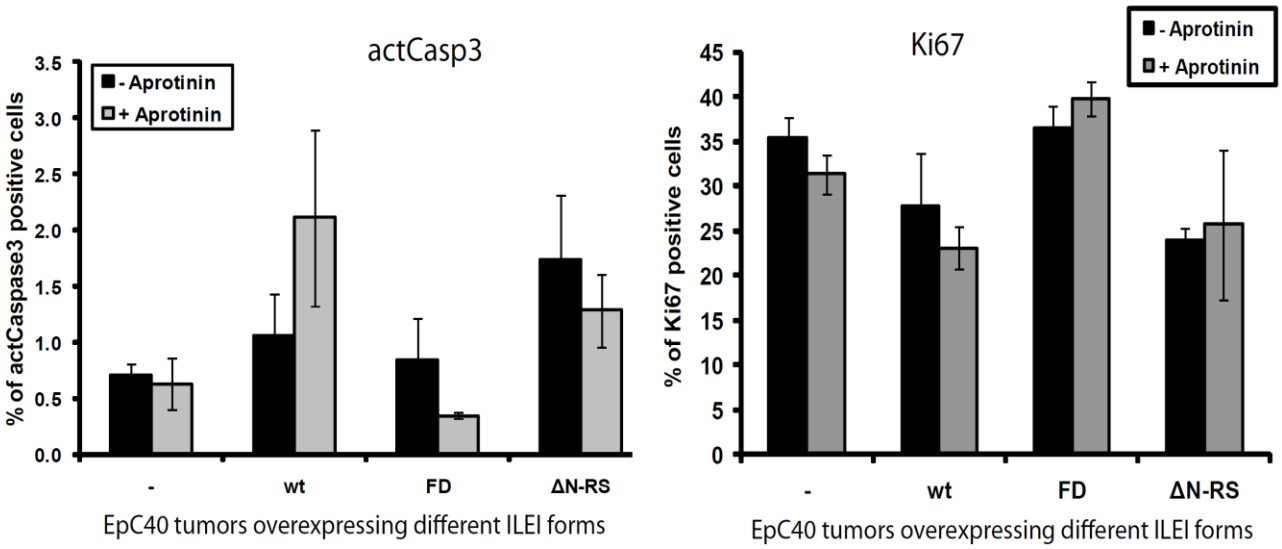



**FIGURE 25 | Quantification of Ki67 and actCaspase3 positive cells**
Error bars show standard error of the mean (SEM).

No significant changes in proliferation- and apoptotic marker expression of EpC40 tumors overexpressing wt and mutants of ILEI with/out aprotinin could be detected. Aprotinin might slightly decrease the apoptotic activity in all cells, except in wt-ILEI overexpressing EpC40 cells. However, enhanced apoptosis in some parts of this tumor was not significant. We concluded that apoptotic alterations and proliferation capacity were not involved in reduced tumor size of wt-ILEI tumors caused by aprotinin.

### 2.1.7.2 Immunostainings for E-cadherin and vimentin

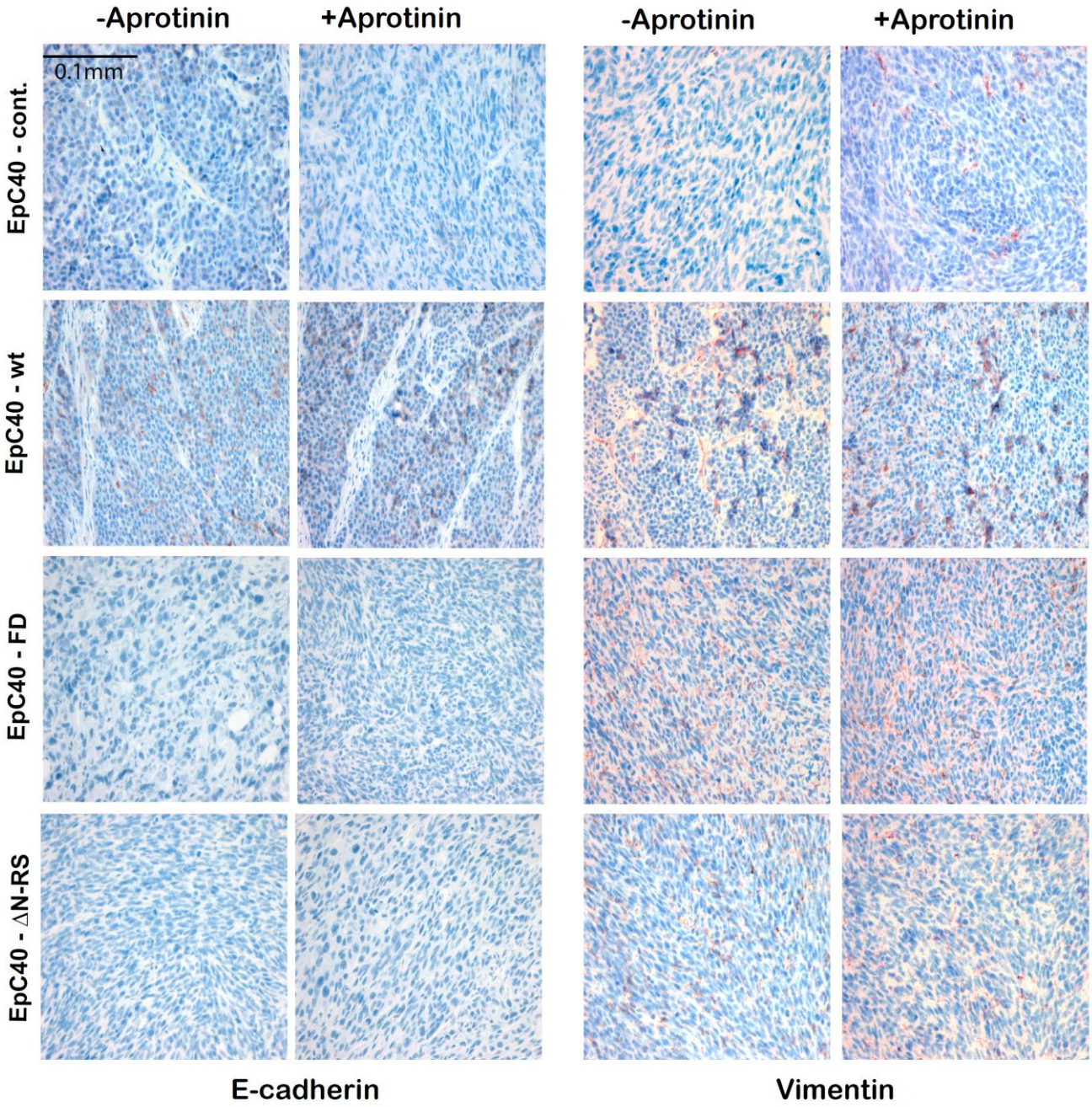



**FIGURE 26 IHC staining for E-cadherin and vimentin in ILEI overexpressing EpC40mammary tumors**

Although the staining intensity in all sections was very low, we observed loss of E-cadherin and upregulation of vimentin in these tumors. Only the control and wt-ILEI cells expressed some E-cadherin, but in case of wt-ILEI also vimentin. Cleavage- and Δpro-peptide mutant overexpressing EpC40 tumors showed the highest levels of vimentin without residual E-cadherin expression.

### 2.1.7.3 ILEI/FLAG stainings

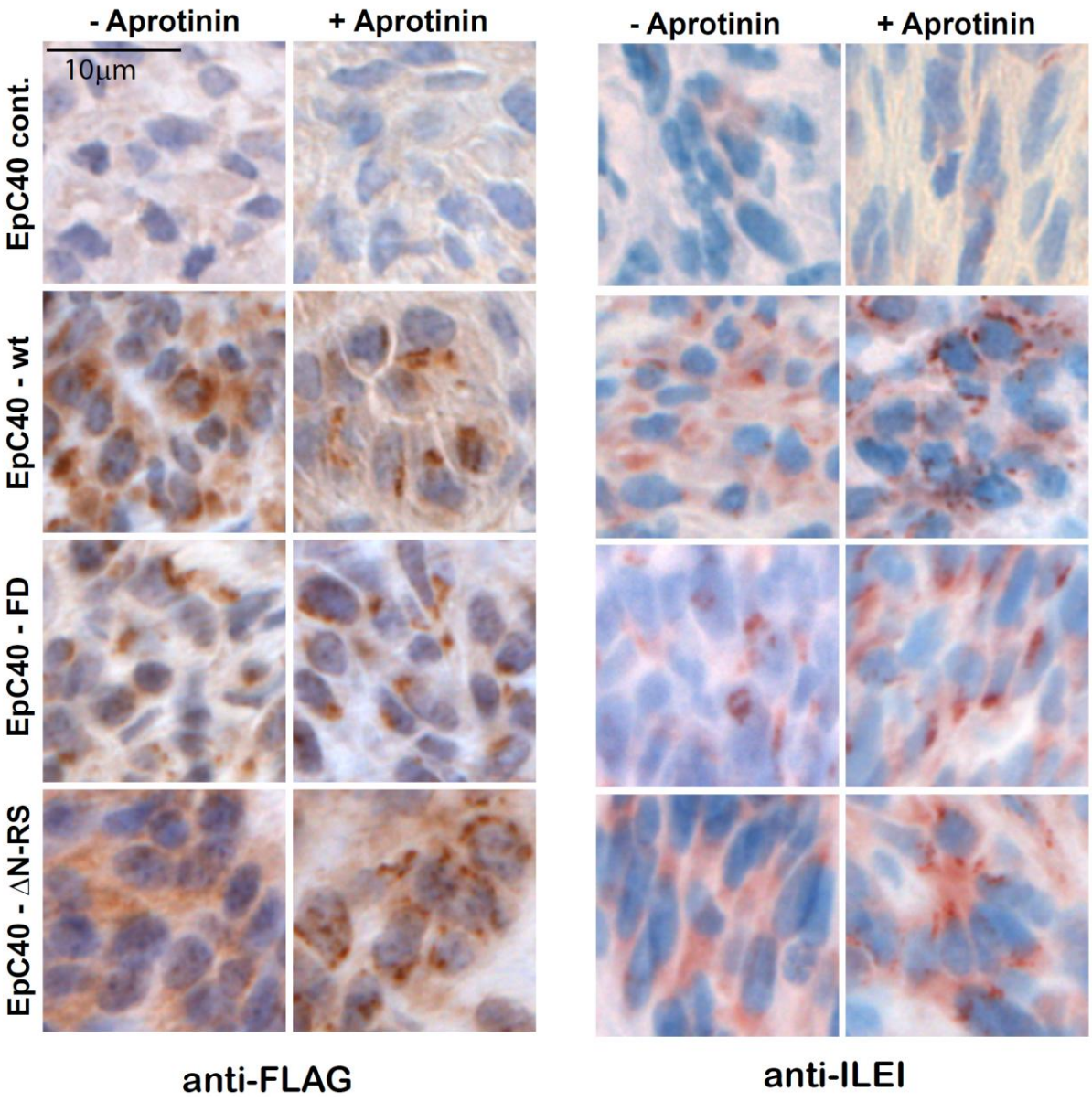



**FIGURE 27 | ILEI and Flag IHC of EpC40 mammary tumors**

EpC40 control cells expressed very low levels of endogenous ILEI and were negative for FLAG, as expected.
We have observed high levels of ILEI expression in all cells overexpressing the FLAG epitope tagged version of wt and mutant forms of the protein and could detect ILEI by both ILEI and FLAG antibodies.

In the absence of aprotinin treatment, localization of the ILEI protein in EpC40 and cleavage mutant-overexpressing tumors showed vesicular dot-like structures, while wt- and ΔN-RS ILEI tumors showed ILEI to be more delocalized to the cytoplasm of tumor cells.
In vitro characterization of these cells showed, that Δpro-peptide ILEI mutant overexpressing cells secrete the highest and cleavage mutant ILEI overexpressing cells the lowest levels of ILEI. The differences in secretion levels seem to correlate with the subcellular localization of ILEI in tumors formed by these cells. Increased secretion correlates with higher levels of diffuse cytoplasmic and lower levels of dot-like granular appearance of the protein. In addition, we observed a shift of cytoplasmic ILEI into dot-like granular structures upon aprotinin treatment, which is also in correlation with our in vitro findings, that ILEI secretion required higher extracellular plasmin levels. Lack of plasmin by using serum free medium resulted in lower secretion levels of ILEI, while addition of exogenous plasmin enhanced secretion; for details see Chapter 2.2). These results led us to hypothesize that ILEI delocalization to the cytoplasm might be due to enhanced secretion of the ILEI protein (Chapter 1.9.1).

### 2.1.8 In vitro characterization of ex-tumor cells

We were interested to elucidate the molecular mechanisms responsible for the observed differences in tumor growth caused by the different ILEI mutants. For this, cells of the above described mammary tumors were recultivated and analysed for their proliferation rate, morphology and for several cellular markers and activity of different signaling proteins, in comparison with the respective cells before injection into mice.

#### 2.1.8.1 $^{3}$H-incorporation in vitro proliferation test

We were wondering, whether differences on tumor formation would derive from differences in the proliferation capacity of these cells. We therefore compared the proliferation rates of the generated ILEI/ILEI-mutant-overexpressing EpC40 cells before injection and of recultivated cells from the respective tumors.

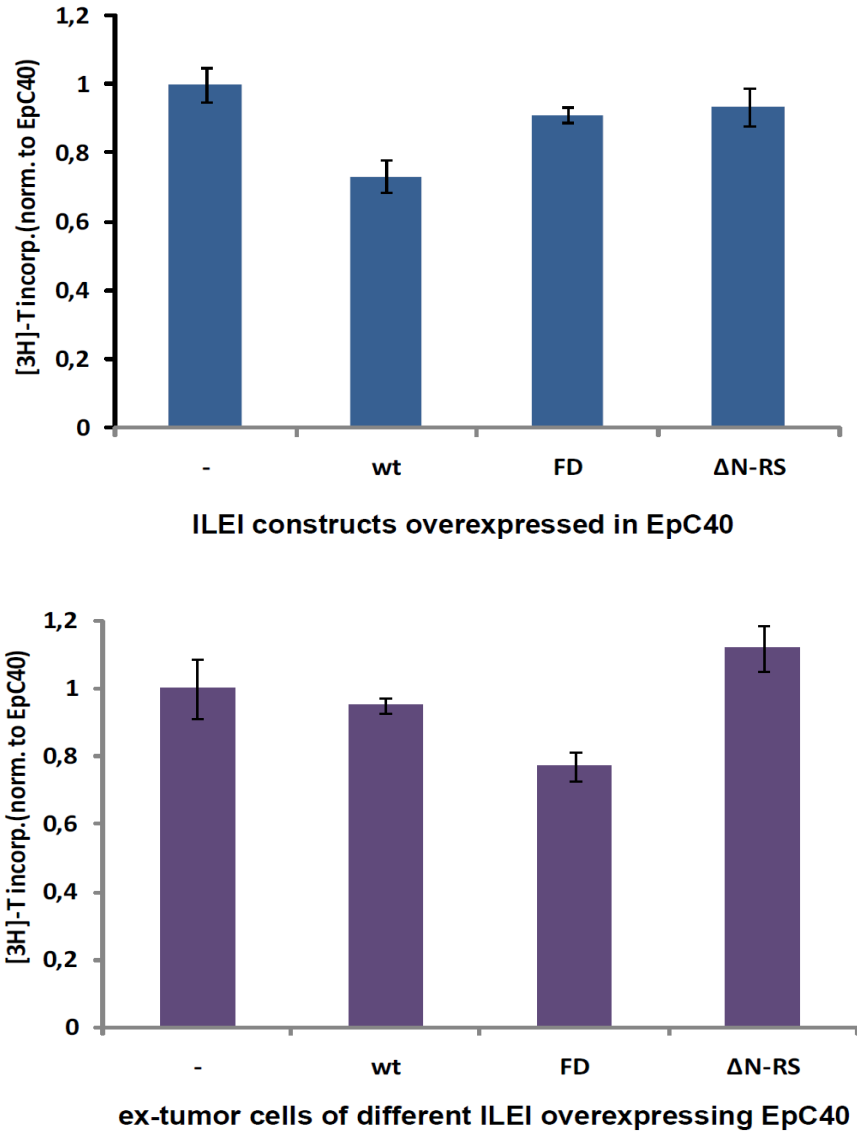



**FIGURE 28 | Proliferation rates of EpC40 cells harboring different ILEI forms before and after tumor formation in mice**
Error bars show standard error of the mean (SEM)., cells numbers were normalized to the EpC40 control cells (set to 1)

Proliferation rates of cells overexpressing wt and mutant forms of ILEI correlated neither before injection nor after re-cultivation with the differences in tumor growth and size that we observed. For instance, Δpro-peptide mutants were the ones that generated bigger tumors compared to the wt cells. Except that ex-tumor cells expressing this mutant proliferated somewhat faster than the other cells (FIGURE 28, bottom panel, not significant), there were no significant differences in the proliferation capacity of any of these cells, similar to what we observed by in vivo IHC characterization of these tumors. We concluded that alterations in proliferation capacity might not be involved in ILEI induced elevated tumor growth.

### 2.1.8.2 Morphology of recultivated tumor cells

Light-microscopic phase micrographs of the above ex-tumor cells in culture dishes were taken and are depicted in FIGURE 29.

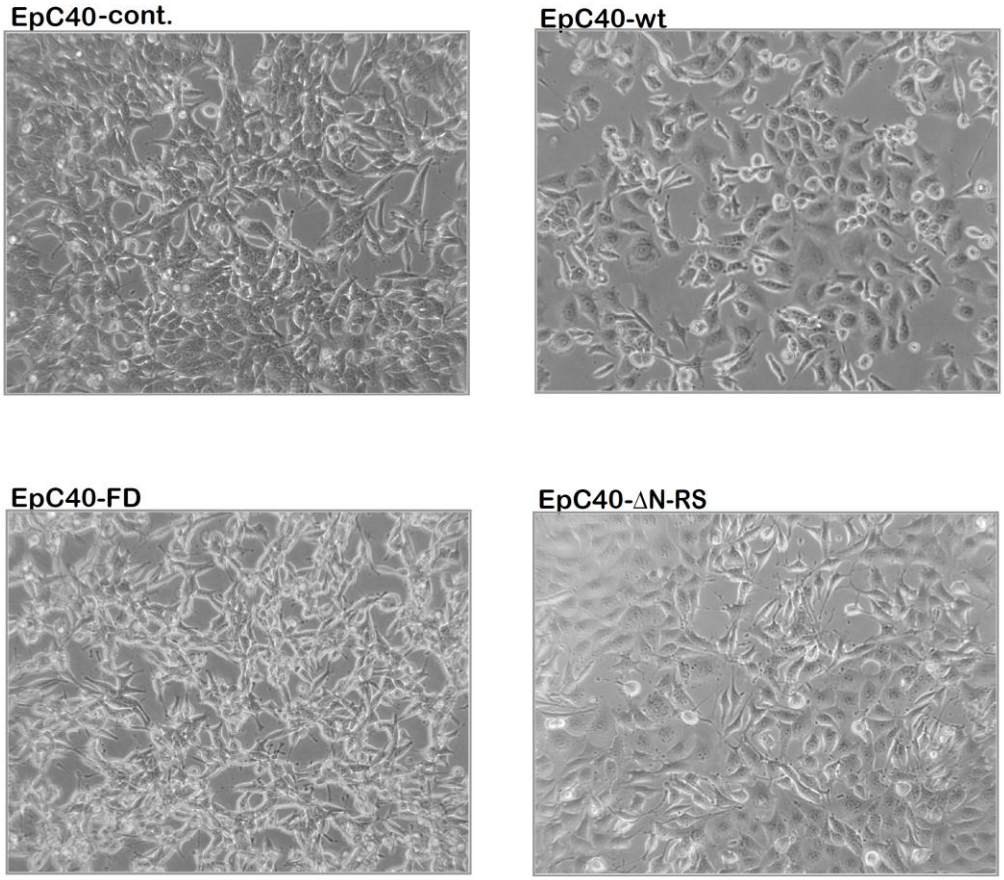



**FIGURE 29 | Morphology of re-cultivated tumor cells in culture dishes**

As mentioned before (chapter 1.5), EpC40 cells fail to induce EMT in vitro and form no metastases in vivo. Additionally, EpC40 ex-tumor cells after recultivation form epithelial cell like structures in culture.
Injected EpC40 control cells and wt-ILEI and Δpro-peptide mutant overexpressing cells have still partially kept their epitheloid phenotype even after they passed through a tumor stage. However, cleavage mutant ILEI overexpressing cells looked somewhat mesenchymal after recultivation from primary tumors, although this mutant had a similarly low tumor growth capacity as EpC40 control cells and formed the smallest tumors. However, the fast growing EpC40ΔN-RS cells also showed regions of mesenchymal looking cells.

### 2.1.8.3 Analysis of cellular markers and signalling proteins of recultivated cells

Recultivated mammary tumor cells were analysed for epithelial and mesenchymal marker proteins and for the activation status of some signaling molecules by Western blotting. EpH4 cells, from which EpC40 and EpRAS cells were generated, served as epithelial controls. Through this, we could distinguish the changes in markers and signaling caused by RasV12C40 oncogenic transformation from those generated by the overexpression of different types of ILEI. By testing the cell series before injection into mice and after recultivation from tumors, we could also examine the changes that occurred in vivo during tumorigenesis probably via the interactions of injected cells with their environment.

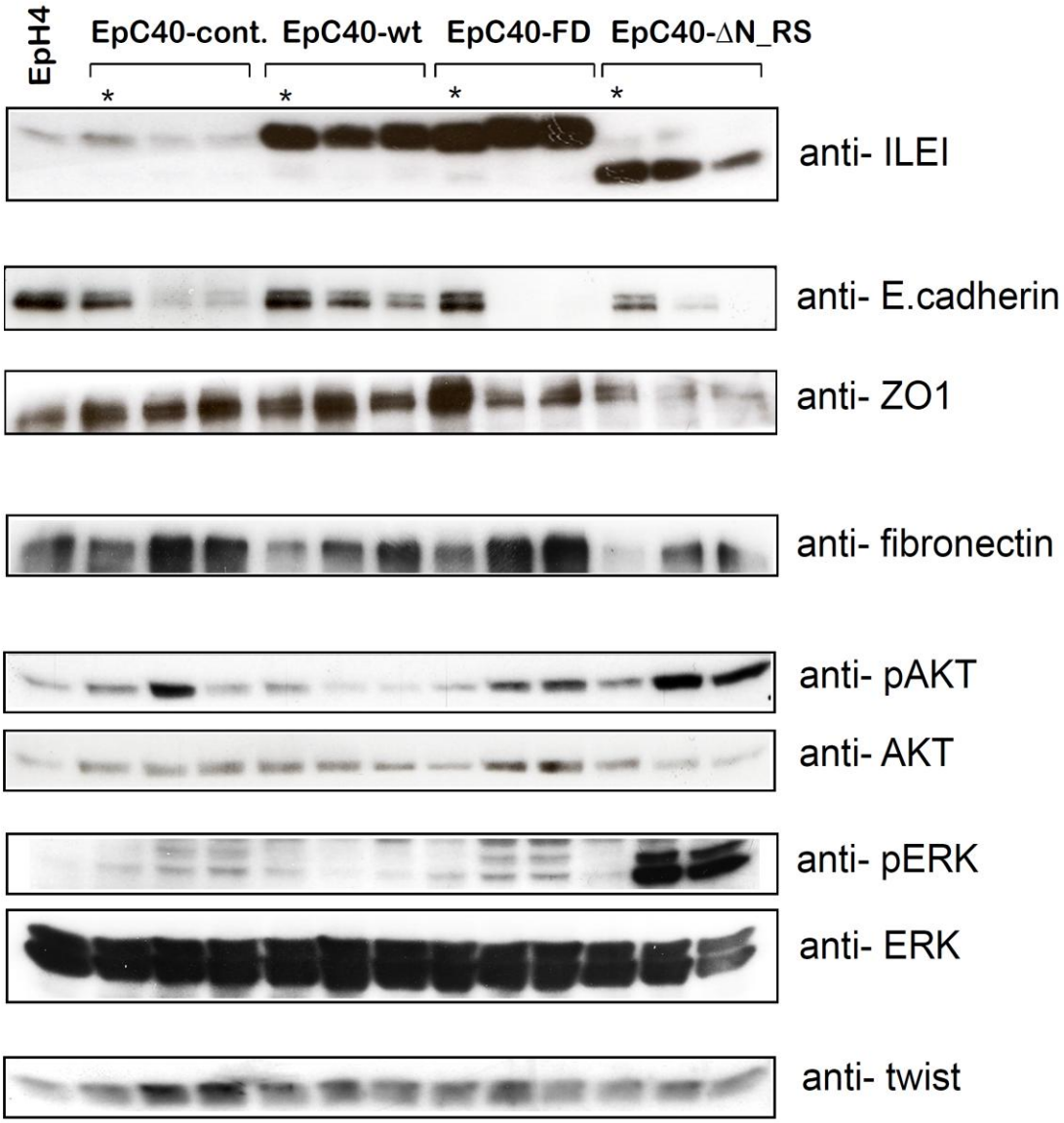



**FIGURE 30 | The regulation of some epithelial/mesenchymal marker and signalling proteins in EpC40 tumor cells overexpressing different ILEI forms**
**pERK: phosphorylated ERK and pAKT: phosphorylated AKT**

* marks whole cell lysate of cell lines before injection, the two following lanes are samples of recultivated cells from two independent tumors

As expected, intracellular ILEI levels of cells remained similar before and after injection. All injected and recultivated cells downregulated E-cadherin and ZO-1, most prominently in case of the cleavage and the Δpro-peptide mutant cells, while fibronectin was upregulated in these cell lines after tumor formation. These markers correlated positively with the morphology of recultivated cells and with the results of immunohistological characterization of the tumors, but do less well correlate with the differences in tumor growth rates and tumor weight. Indeed, wt-ILEI overexpressing cells were somewhat more epithelial than the others and here they still had relative high E-cadherin levels compared to the other cell lines. In contrast to this, they formed bigger tumors than control and cleavage mutant cells, which were more fibroblastoid-like cells in culture and had much lower tumor growth capacity.

pAKT, pERK and twist levels did not show any significant changes before and after injection or between the different cell types with the exception of Δpro-peptide mutant ex-tumor cells, which had elevated levels of pAKT and pERK signalling proteins. In fact, EpC40 cells have a mutated active Ras oncogene and are not able to signal through ERK/MAPK pathway and have only basal ERK levels. Thus, increased pERK signalling should be derived from elevated endogenous Ras signalling. Since enhanced pERK levels in Δpro-peptide mutant cells correlate with the elevated tumor growth of these cells, increased ERK signalling in these cells might be important and contribute to increased tumor growth rate.

On the other hand, the loss of epithelial markers like E-cadherin in EpC40 ex-tumor cells and the same pAKT levels of these cells as EpH4 cells were not in accordance with earlier published data on cultivated cells, that had not undergone a tumor passage. (Chapter 1.5)

### 2.1.9 In vivo lung colonization assay

So far, we studied the physiological role of ILEI cleavage only in primary tumor formation. EpC40 cells are incapable to enter the bloodstream from primary mammary tumors and also if these cells are injected directly into the circulation, they fail to extravasate and colonise to the lung. However, ILEI overexpression is sufficient to make these cells metastatic (*Waerner T et al., 2006)*. To test to what extent the different mutant forms of ILEI maintain this function of the protein, we injected $2x10^5$ of recultivated ex-tumor cells (mature cells) into the tail vein of nude mice to assay the metastasis formation capability of these cells. This experiment allows to analyse late steps of metastasis: survival in blood, capability of extravasation and homing and proliferation ability in the lung, from where tumor cells do not originate.

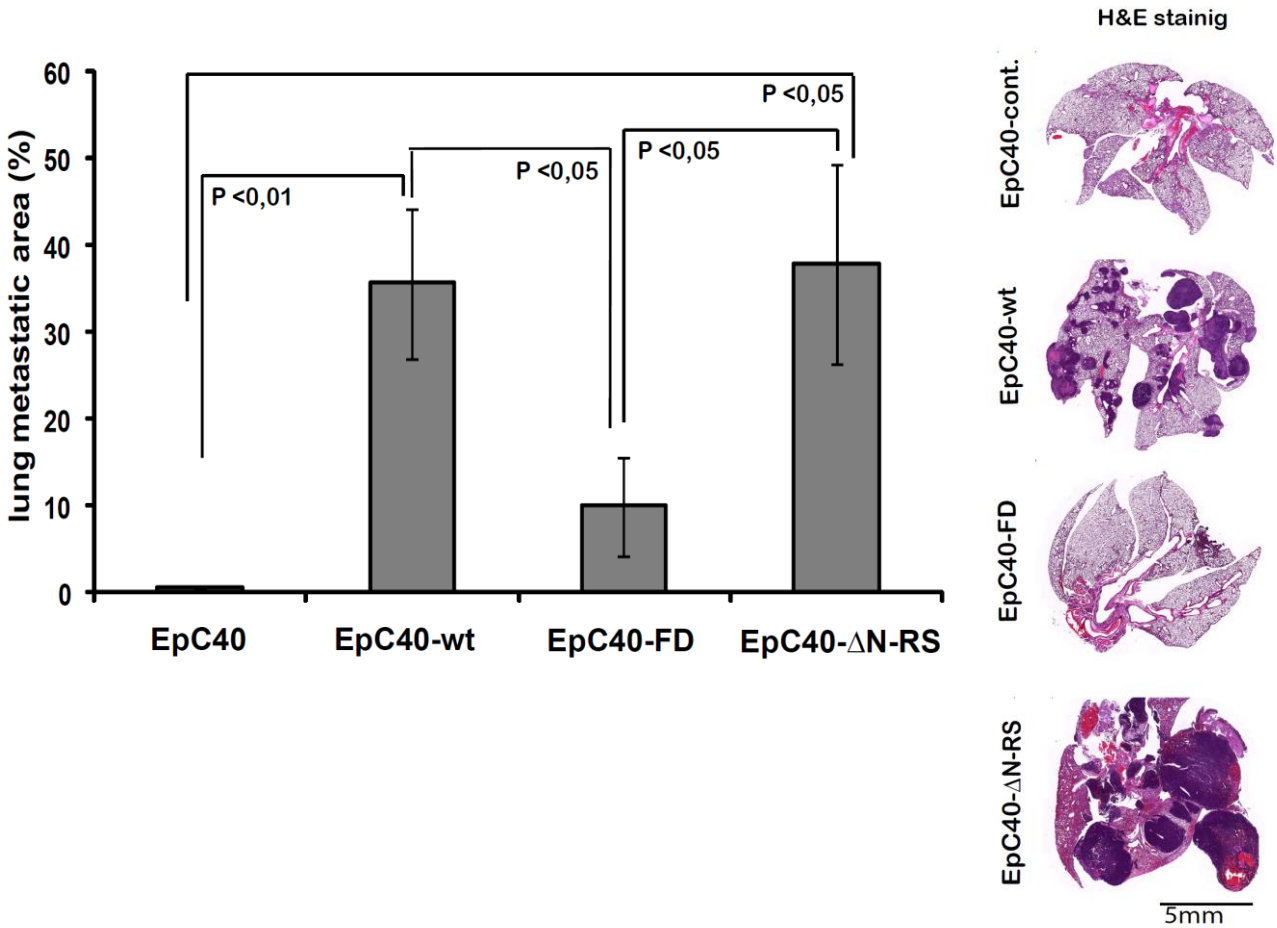



**FIGURE 31 | Lung colonization capability of EpC40 cells overexpressing different mutant ILEI forms**

**Percentage of lung metastatic area driven by EpC40 ex-tumor cells overexpressing different ILEI (left), Representative images of H&E stained lungs (right)**

Error bars show standard error of the mean (SEM); for calculation of lung metatstatic area, see chapter 5.7.2

As published earlier, EpC40 control cells fail to promote large metastases. We observed very small micro metastases (~1% of whole lung). wt- and ΔN-ILEI overexpressing tumor cells could form metastasis, as expected. Interestingly, by measuring total metastatic areas, the difference in the metastatic capacity between these cells was not significant, in contrast to primary tumor formation ability of these cells, where Δpro-peptide mutant cells caused much higher tumor growth rates and larger tumor sizes (FIGURE 31). From histological analysis of the lungs, it appeared, however, that the ΔN-ILEI mutant cells caused much larger metastases (FIGURE 31, right panels) Furthermore, cleavage mutant overexpressing tumor cells were also capable to induce some small metastases, but much less than wt cells (10% of whole lung), indicating that non-processed ILEI is also able to somewhat promote metastasis. It is obvious, however, that cells expressing the constitutively cleaved form ΔN-ILEI have strongly enhanced metastatic capacity.

## 2.2 ILEI secretion assays

Since constitutively cleaved, mature ILEI (ΔN-ILEI ) shows strongly enhanced secretion, there could be a positive feedback loop of mature ILEI stimulating its own secretion. We therefore addressed, whether or not the proteases that are responsible for its processing might have effects on its secretion as well.

The published data showed that TGFβ upregulated ILEI translation and secretion. (Chapter 1.9.1) After we confirmed that plasmin cleaves ILEI, we wanted to test the action of plasmin on the secretion of the protein. We did secretion assays in cell culture with and without serum containing medium.

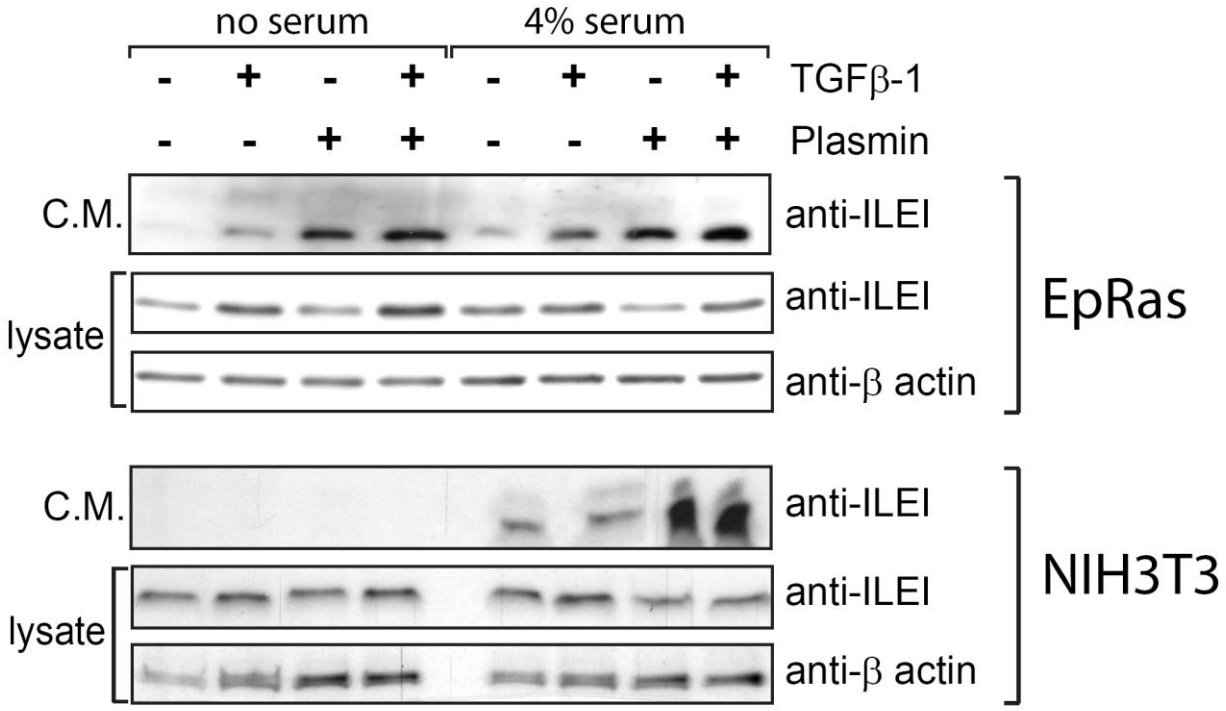



**FIGURE 32 Stimulation of ILEI secretion by TGFβ & plasmin in EpRAS and NIH3T3 cells** CM: conditioned medium from cells incubated with medium for 24 hours

Cells incubated with serum containing medium showed somewhat higher basal secretion levels of ILEI, this, however, did not influence the much higher stimulation of secretion obtained after factor/protease treatment (FIGURE 32).
TGFβ, indeed, increased both intracellular and secreted ILEI levels within 24 hours. Plasmin upregulated secretion levels of ILEI much stronger than TGFβ. Additionally, we observed a slight decrease in the intracellular levels of the ILEI protein after plasmin exposure, an effect observed also upon strong stimulation of secretion of other proteins.
The induction of secretion by plasmin was not specific for epithelial cells, since plasmin treatment of fibroblasts also enhanced ILEI secretion.

### 2.2.1 Stimulation of ILEI secretion: time course analysis

For the comparison of TGFβ and plasmin driven induction of ILEI secretion in more detail, we performed a time course analysis of ILEI secretion over 24 hours.

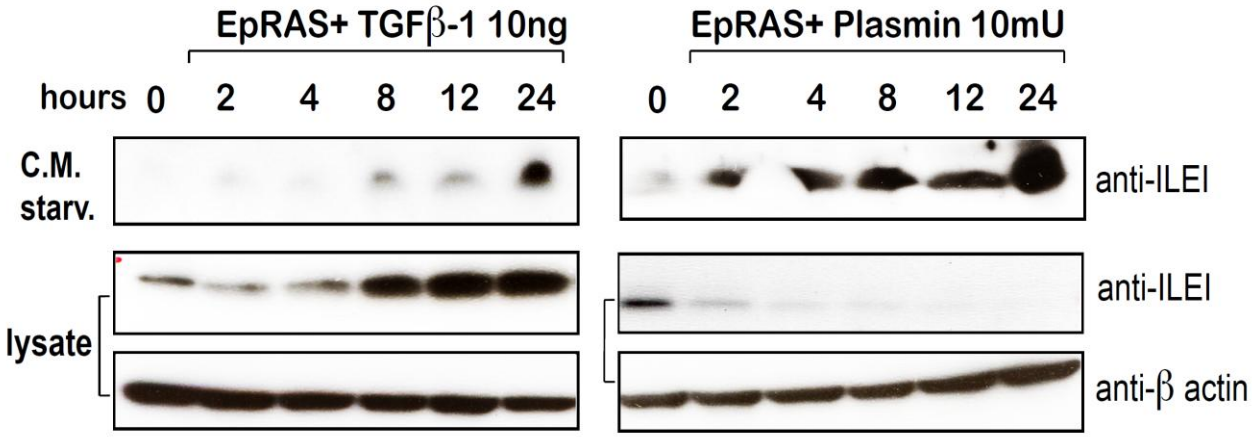



**FIGURE 33 Time course of Intracellular and secreted ILEI levels in EpRAS cells treated with TGFβ/Plasmin over 24 hours**
C.M. starv.: conditioned medium without serum (starvation medium)

TGFβ significantly upregulated intracellular ILEI only after ~8 hours, perhaps as a consequence of elevated secretion of the protein.
In constrast, plasmin induced ILEI secretion very rapidly, at the first time point taken (two hours) secretion was already almost maximal. At the same time, a strong, almost complete downregulation of the intracellular ILEI protein pool was evident after 4 hours. Repeating the time course analysis during the first two hours, ILEI secretion was completely turned on after 30 minutes, while nothing happened within 15 minutes (FIGURE 34)

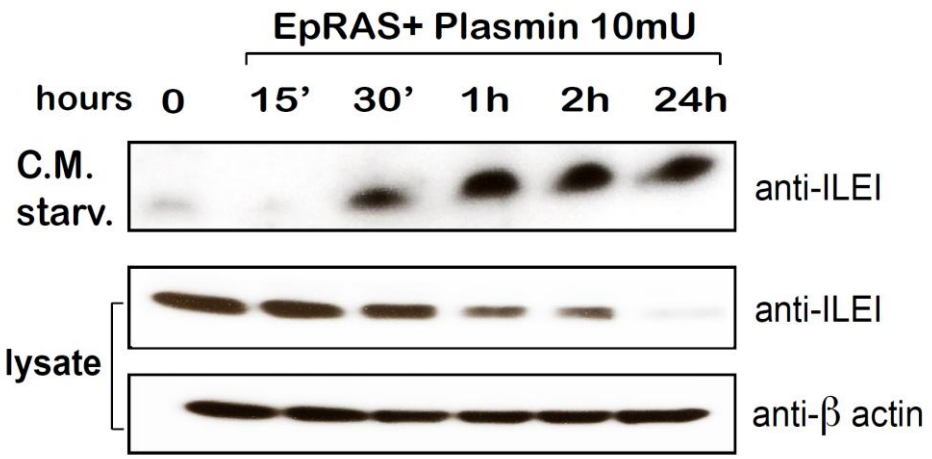



**FIGURE 34 | Induction of ILEI secretion via plasmin occurs within 1 hour.**

Thus, plasmin enhanced ILEI secretion within less than one hour and it was already combined with a decrease of intracellular protein, indicating that plasmin stimulates ILEI secretion at the expense of the intracellular ILEI pool. Most likely, therefore, plasmin-stimulated secretion occurred via a completely different regulatory mechanism as seen with TGFβ, where TGFβ primarily acts via upregulating ILEI translation, then leading to elevated secretion of the intracellular accumulating protein. (FIGURE 33-34)

### 2.2.2 Plasmin dependent ILEI secretion in human cell lines

Above, we showed that Plasmin drives the elevated secretion of ILEI in EpRAS and mouse fibroblast cells. This raised the question, if this mechanism might be also relevant in the human system and therefore tested several human tumor cell lines.

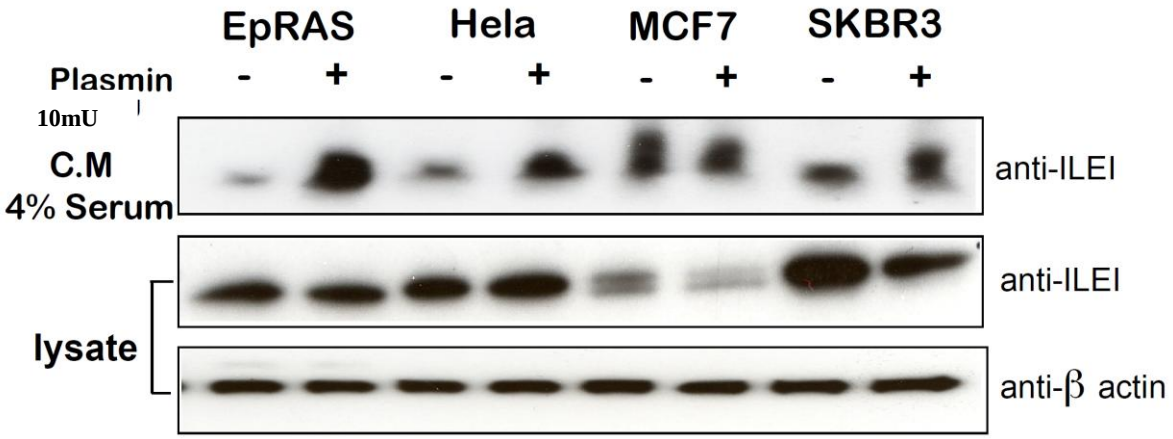



**FIGURE 35 Effects of plasmin on ILEI secretion in different human cell lines**
MCF7 and SKBR3 are human breast cancer cell lines and Hela is a human cervical cancer cell line.

In Hela and SKBR3 cell lines, plasmin had similar effects on ILEI secretion as in EpRAS cells, however increased secretion of the protein was not always tightly combined with reduced intracellular levels. No effect of plasmin was seen in MCF7 cells, which probably differ in the way they trigger ILEI signalling and enhance its secretion. In summary we could conclude that plasmin-induced upregulation of ILEI secretion occurs also in human cell lines, although to a different extent in the different cell lines.

### 2.2.3 Dose dependence of plasmin for elevated ILEI secretion

We determined the minimal concentration of plasmin required to stimulate ILEI secretion. All secretion experiments before were done with 10mU plasmin. For this, we treated cells with plasmin levels from 0,625mU to 10mU.

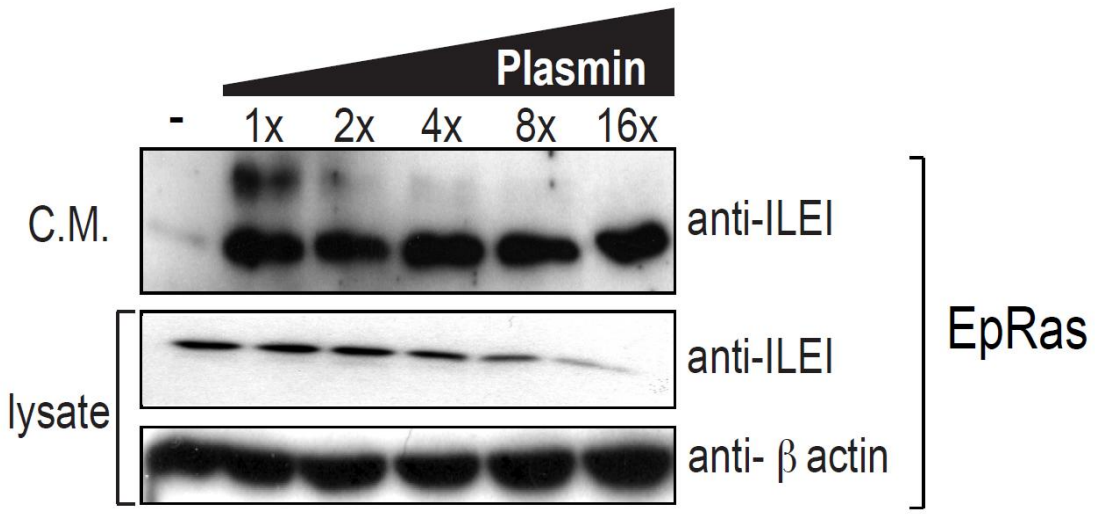



**FIGURE 36 Less than 1 mU of plasmin is sufficient to stimulate secretion of ILEI**

The lowest amount of plasmin added to the cells was aleady able to fully stimulate secretion of the ILEI protein, almost to the same extent as higher amounts, indicating that this amount of plasmin already saturates the ILEI secretory system. Interestingly, however, we detected uncleaved, full length ILEI in the conditioned medium (higher band on Western blot) of plasmin treated cells. The amount of uncleaved ILEI decreased stepwisely with the increasing amount of plasmin added to the cells. These results suggest that more plasmin might be required, to cleave secreted ILEI, than to trigger secretion of the protein. For the exact determination of the minimal plasmin amount needed to enhance secretion, one should repeat this experiment with much lower doses of the enzyme.

### 2.2.4 Protease-dependent ILEI secretion

There are several potential mechanisms how plasmin could lead to elevated ILEI secretion. One hypothesis is that elevated secretion of ILEI is a consequence of an autocrine positive feedback loop and plasmin is needed only to generate the cleaved, active form of ILEI to induce ILEI signaling. Alternatively, plasmin could play a specific role in ILEI secretion that is independent of ILEI cleavage and would act through plasmin signaling. To distinguish these two possibilities, we tested other enzymes that were able to generate the short form of ILEI, whether they would be able to enhance secretion of the protein in EpRas cells as well.

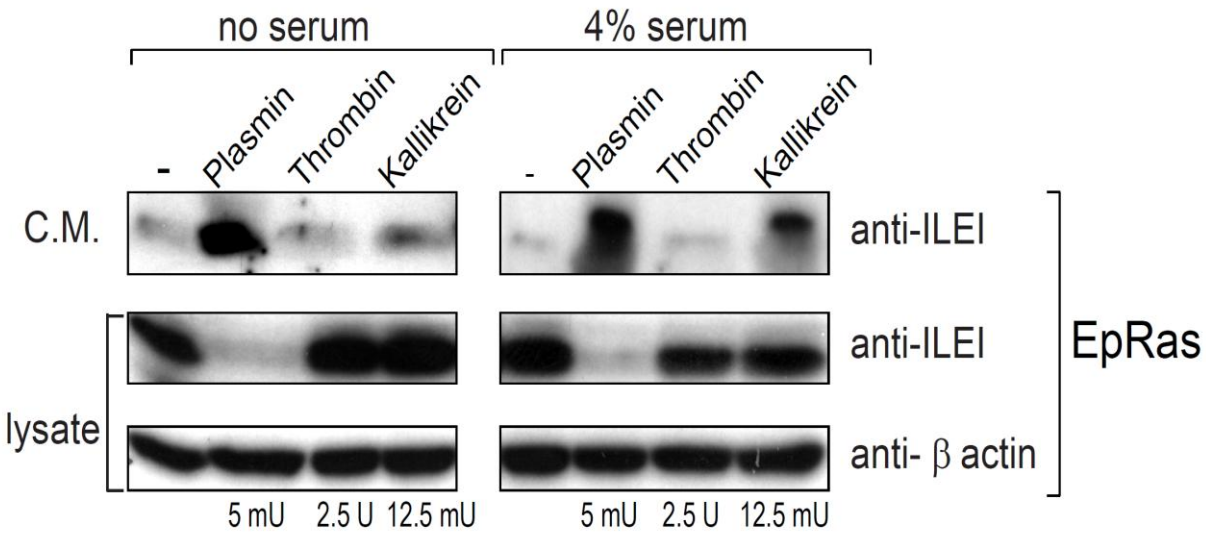



**FIGURE 37 | Intracellular and secreted ILEI levels in EpRAS cells subjected to Plasmin and other ILEI processing proteases for 24 hours**

Plasmin induced again ILEI secretion in both serum free and 4% serum containing medium in EpRas cells. In contrast, thrombin did not influence secreted and intracellular ILEI protein levels. Kallikrein was able to stimulate protein secretion, but only in serum containing medium, and to a clearly less extent than plasmin. Intracellular levels of the protein decreased upon enhanced secretion, clearly after plasmin and also slightly after kallikrein treatment.
This experiment showed that, cleaved ILEI is unlikely to trigger its own secretion directly, rather, Plasmin might induce secretion independently of ILEI cleavage. The fact that kallikrein became a potent inducer of secretion in the presence of serum is most probably due to its function as an activator of urokinase plasminogen activator (uPA) and plasminogen, since serum contains all the components of the plasminogen system. This would be in line with the assumption, that only plasmin could trigger elevated ILEI secretion.

### 2.2.5 Exogenous ILEI treatment

We wanted to verify the result we observed in the previous experiment , namely that enhanced secretion of ILEI might not depend on an autocrine feedback mechanism of the cleaved form which would trigger its own secretion, but plasmin signalling independently of ILEI might upregulate the secretion. For this, we treated the EpRAS cells with purified wt and mutant forms of ILEI-FLAG. After 24 hours incubation time conditioned media were collected and incubated with anti-Flag protein coupled beads to remove all exogenously added Flag tagged ILEI proteins. The flow through of these conditioned medium samples were loaded on an SDS-gel and analyzed for ILEI levels by Western-blotting.

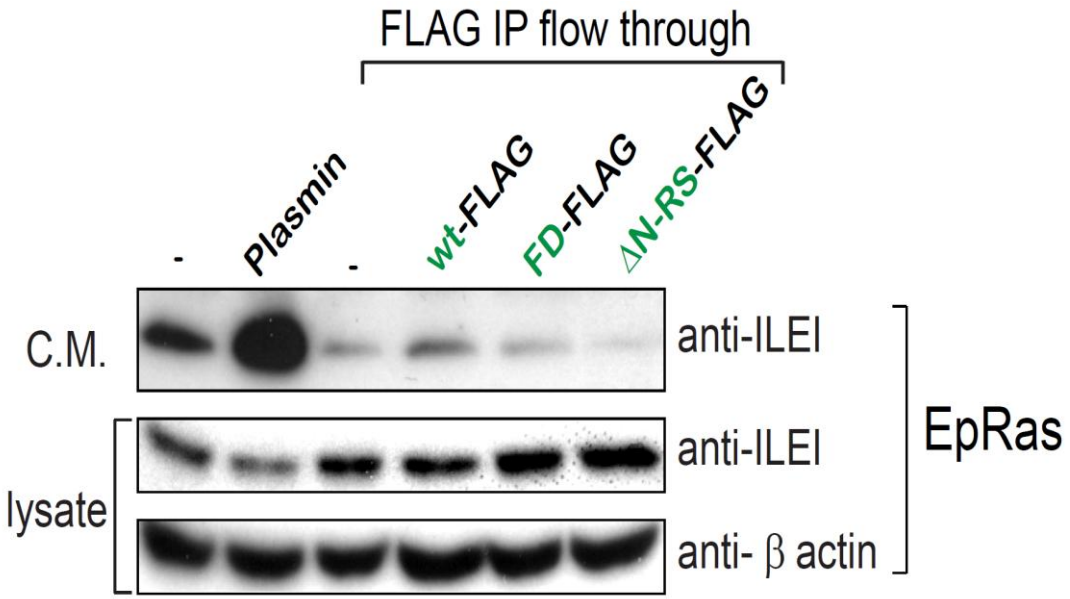



**FIGURE 38 | ILEI secretion levels in EpRAS cells exposed to exogenous wt and mutant forms of ILEI**

We did not observe any significant changes in secreted levels of ILEI, induced by any type of exogenously added purified forms of the protein, indicating that ILEI might not be able to initiate its own secretion. This result, however, would have to be confirmed by showing, that the added ILEI fractions had other biological activities

# 3. DISCUSSION

The first part of this diploma thesis aimed to dissect the mechanism of proteolytic processing of ILEI, its influences on the secretion of the protein and its physiological roles on tumorigenesis. Furthermore, we studied potential connections between ILEI processing and secretion by challenging the hypothesis if cleaved ILEI might stimulate its own expression and secretion directly in an autocrine manner via binding to putative ILEI receptors.

***Cleavage of ILEI:*** ILEI has been found to be cleaved by plasmin at a precise position at its N-terminus. Nevertheless, we show here, that plasminogen knock out mouse serum was also able to process ILEI, indicating that in addition to plasmin other serine type proteases could be also involved in ILEI cleavage. Indeed, kallikrein and thrombin were also able to generate the short form of the protein by specifically cutting at the same R/S site as plasmin, however with a much lower efficiency. Further work will be required to clarify to what extent these other proteases contribute in ILEI processing under physiological conditions or whether these observations are only a consequence of compensatory mechanisms in the lack of the adequate protease, plasmin.

***The physiological role of ILEI processing:*** To address this question, in vivo mouse tumor xenograft assays were performed. In these experiments, we used tumorigenic murine breast epithelial EpC40 cells overexpressing different mutant forms of ILEI that were previously generated. Beside empty vector expressing control and wild type ILEI overexpressing (OE) cells, two additional cell lines overexpressing different mutant forms of ILEI were included in this study. The first ILEI mutant (FD) was no longer cleavable (cleavage mutant) and the other represent the mature form of ILEI, already lacking the pro-peptide that normally has to be cleaved off (Δpro-peptide mutant, ΔN). These mutant forms allowed us to examine in detail the role and necessity of ILEI processing in tumorigenesis and metastasis. Primary tumour formation ability of these cells was analysed in nude mice. wt-ILEI OE EpC40 cells resulted in elevated tumor growth rate and size compared to the control cells, as published before. *(Waerner. T et al., 2006)* IHC analysis of mammary tumours indicated that elevated tumor growth and tumor masses by overexpression of ILEI did not involve any alterations of proliferation and apoptotic activities. We observed that cleavage mutant OE cells could generate only small tumors similar to the control cells and Δpro-peptide mutant OE cells formed even bigger tumors than wt-ILEI. Thus, ILEI derived accelerated tumour progression may require its processing. However, it is possible that different tumor formation abilities observed in these cells may occur for varying reasons, such as different secretion levels of ILEI. Indeed, in vitro characterization of these generated cells had differences at ILEI secretion levels, where cleavage mutant OE cells secreted less ILEI than wild type and Δpro-peptide mutant OE cells secreted the protein at higher levels.

Aprotinin, a broad spectrum serine protease inhibitor, was able to inhibit ILEI cleavage by mouse serum in vitro in earlier studies. Thus, we could evaluate the role of processed ILEI in vivo, presuming that aprotinin would prevent the cleavage in case of the wt-ILEI OE cells in vivo as well. Thus, we could examine whether the generation of this short form might be essential and sufficient for an elevated tumor growth. Aprotinin treated mice injected with wt-ILEI OE EpC40 showed a significantly decreased tumor growth rate and tumor mass in compared to mice not treated with aprotinin, without affecting apoptotic and proliferative activity of tumor cells. ILEI-induced increased tumor growth was repressed in case of wt-ILEI, but not of delta pro-peptide ILEI mutants, indicating that aprotinin-sensitive protease activity was important for ILEI to generate its processed, active form.

***Subcellular localisation of ILEI:*** IHC staining for ILEI revealed localisation of the protein in vesicular, dot-like structures and also throughout the cytoplasm in tumors with higher secretion levels, like in Δpro-peptide and wt-ILEI OE tumors. Increased secretion seemed to correlate with higher levels of diffuse cytoplasmic staining and lower levels of cells with dot-like, granular appearance of the protein. In addition, we observed a shift of cytoplasmic ILEI into dot-like granular structures upon aprotinin treatment. This may be a result of decreased secretion of the protein, however, we could not conclude, whether aprotinin could decrease ILEI secretion or the secretion could be diminished as a consequence of cleavage inhibition. Nevertheless, our finding that elevated secretion correlates with diffuse, cytoplasmic ILEI localization could be of major importance, because the same cytoplasmic subcellular localization of ILEI in a human breast cancer tissue array correlated with enhanced risk of metastasis formation and impaired survival (p-values $>10^6$ - $10^9$, Waerner, T. et al, 2006).

***In vitro characterization of EpC40 tumors:*** Morphology of recultivated tumor cells overexpressing different ILEI forms was in accordance with E-cadherin and vimentin levels of mammary tumors analysed by IHC and also marker protein analyses of recultivated tumor cells by immunoblotting. In culture, cells partially kept their epithelioid cell structure and some patches showed also spindle shaped, mesenchymal like cells with the exception of the cleavage mutant, which had the most fibroblastoid phenotype. All types of EpC40 cells upregulated mesencyhmal marker proteins like vimentin and fibronectin after tumor formation in mice and recultivation, and downregulated epithelial proteins such as E-cadherin and ZO-1. However the morphological and marker results did not mirror the differences in tumor formation capacity of these cells, considering the cleavage mutant overexpressing cells having the most mesenchymal morphology, formed small tumors comparable to control cells. We were able to detect elevated pERK and pAKT levels in Δpro-peptide mutant OE cells, which were able to generate accelerated tumor growth rate and tumor burden. EpC40 cells overexpress a mutated form of oncogenic Ras, which hyperactivates the PI3K pathway, but fails to hyperactivate the ERK pathway, leading to basal ERK levels in these cells. Thus, increased pERK signalling could result from endogenous Ras signalling that might be activated by the abundance of secreted ILEI. Consequently, enhanced ERK levels may be important for development of larger tumors and increased tumor growth rate.

EpC40 cells are known to signal through PI3K pathway hyperactivating pAKT expression and EpC40 tumors were found to retain E-cadherin expression and to lack the vimentin mesenchymal marker protein. (*Janda et al., 2002)* Nevertheless, in our study we found only basal pAKT expression in all EpC40 derived cells and downregulation of E-cadherin in EpC40 control mammary tumors was not consistent with this published data. Importantly, however, none of the earlier studies performed experiments using cells recultivated from tumors, one possibility to explain the observed discrepancies.

***Experimental metastasis assay:*** We tested the ability of metastasis formation of recultivated EpC40 ex-tumor cells overexpressing wt and mutant forms of ILEI in a lung colonisation assay after tail vein injection. Our data agreed with previous reports that EpC40- cells fail to induce EMT in vitro and metastasis in vivo. However, we also detected some micro metastases (1% of lung area) in control cells. As expected, wt and Δpro-peptide mutant OE cells could develop metastasis at high rates. However, the difference at the metastatic capacity between these cells was not significant in overall metastatic area measurements, but the Δpro-peptide mutant seemed to form fewer, but much larger metastases. In contrast, Δpro-peptide mutant OE cells significantly elevated tumor growth kinetics and tumor mass compared to wild type in primary tumor formation assay. On the other hand, cleavage mutant OE cells retained a weak metastasis forming capacity, much less than wt-ILEI OE cells (<10% of lung area). This suggests, that basal ILEI levels would not be enough to generate metastases in EpC40 cells, but elevated intracellular levels of ILEI without having enhanced secretion of the protein, might also be able to promote metastasis. In line with this, another ILEI cleavage mutant with two hydrophobic phenylalanines (FF) replacing the wt RS site, was still able to cause full EMT in EpRas cells (Csiszar, unpublished).

In theory, aprotinin could be a potential therapeutic tool to circumvent ILEI derived tumor and metastasis promotion. However, despite its ability to inhibit plasmin activity, aprotinin itself increases metastasis by increasing thrombosis and by retention of circulating cancer cells at the vascular endothelium. It has been published that aprotinin treatment enhances metastasis. *(Turner GA., Weiss L., 1981)* Ergo, long term exposure to aprotinin, as in ILEI-derived primary tumor formation experiment would be inefficient for lung colonisation assay because of its harmful side effects. Moreover, using of MMP inhibitors in cancer therapy had also disappointing outcomes. (*Coussens L.M., 2002)* Thus, to circumvent the risks of using broad spectrum inhibitors, crucial downstream signalling molecules of these proteases, which might be the optimal therapeutic targets, should be identified and characterised. Therefore, ILEI may promise such a potential target to prevent invasive carcinoma.

***In vitro secretion assays:*** Plasmin induces ILEI secretion in various cell lines; in EpRAS (tumorigenic and metastatic murine breast epithelial cells) and NIHT3T (mouse fibroblast cell line) and also in the human tumor cell lines HeLa and SKBR3. Plasmin induced ILEI secretion occurs very rapidly, within less than one hour, and at the same time causes a decrease of the amount of intracellular protein. Enhanced secretion on the cost of intracellular protein requires less than 1mU plasmin; a concentration which was unable to cleave all the secreted ILEI sequestered in the matrix. On the other hand, TGFβ was published as an inducer of ILEI secretion via upregulating ILEI translation. *(Waerner T. et al., 2006)* In agreement with these data, we found, that TGFβ-induced enhanced secretion occurred as a secondary step following enhanced translation of the protein, which took approximately 8 hours. Hence, plasmin's effect on ILEI occurs via a completely different regulatory mechanism than TGFβ-stimulated ILEI secretion.

Several hypotheses explaining the stimulation of the secretion are conceivable. It is possible that ILEI cleavage positively influences its own secretion, via autocrine signalling, and plasmin is needed only to generate the cleaved active form of ILEI. Another possibility is that plasmin may play a specific role in ILEI secretion, independent of ILEI cleavage, and acts through plasmin signalling. To discriminate between these possibilities, we tested whether plasmin, thrombin and kallikrein that were able to process ILEI, would enhance ILEI secretion due to the generation of the truncated protein. However, only plasmin triggered elevated secretion, supported by the finding that kallikrein failed to stimulate ILEI secretion in the absence of serum, while in the presence of serum, it activated plasminogen by activating the urokinase plasminogen activator, thus generating active plasmin to stimulate secretion. We conclude from these data that ILEI does not stimulate its own secretion via an autocrine feedback mechanism. We confirmed this idea in experiments treating cells with exogenous ILEI, in which none of the purified ILEI forms could stimulate self-secretion, not even the constitutively cleaved ΔN-ILEI.

Plasmin may upregulate ILEI secretion independently of ILEI cleavage via plasminogen (Plg)/plasminogen activators (PA) signalling. Indeed, plasmin can cause intracellular effects through uPA/uPAR signalling and, as recently showed, also through protease activated receptor 1 (PAR1) signalling *(Pendurthi U.R., 2002).* In our experiments, this may be irrelevant, because thrombin, an activator of PAR1, could not affect ILEI secretion. (For details in Plg/PA signaling, see chapter 1.8).

However, as suggested at the beginning, these data do not correlate with different secretion levels of different mutated ILEI forms being dependent on cleavability of the protein. One explanation may be that intracellular ILEI stimulates upregulation of the Plg/PA system via regulation of expression or localisation of the system components, leading to its increased secretion. There is supporting data from expression profiling experiments, indicating upregulation of tissue plasminogen activator (tPA) in EpRAS-XT and urokinase plasminogen activator receptor (uPAR) in EpRAS-ILEI cells. *(M. Jechlinger and H. Beug, unpublished).*

Another possibility may be that the cleavage site and pro-peptide play a role in the regulation of ILEI secretion. A resting cell might secrete less ILEI due to the presence of the pro-peptide, which could be an important retention signal for the protein to remain intracellular or sequestered in the extracellular matrix (*Bauskin A.R. et al.,2005),* and Plg/PA signalling could release this retention through unknown factors. This assumption would agree with our findings in ΔN-ILEI OE EpC40 cells, where lack of pro-peptide led to elevated secretion; this is, however insufficient to explain why cleavage mutant OE cells secreted less ILEI than the wt OE cells. Independent data showed the significance of cleavage site sequence for the secretion. Thus, mutation at the cleavage site of ILEI might cause diminished recognition of ILEI secretion, whereas other amino acid exchanges in this cleavage site (i.e. RS to FF) completely inhibited ILEI secretion. (data not shown). Since this mutant was still able to cause complete EMT in EpRas cells, the possibility remains, that ILEI also acts via intracellular mechanisms.

***Conclusions and future perspectives:*** Taken together, the plasmin and plg/PA systems, together with the surrounding tumor microenvironment, might be the rate limiting factors for ILEI-derived tumor and metastasis promotion. Plasmin is activated by urokinase plasminogen activator (uPA), but itself is also able to activate uPA receptor (uPAR) bound pro-uPA. The activated complex mediates cell signalling through binding to several surface proteins and integrins, which may affect cell adhesion and migration events. In various tumor cells, uPA/uPAR and also integrins, for instance αvβ3 integrin in breast cancer *(Liapis et al 1996*), were upregulated and correlated with tumor and metastasis progression. Therefore, plasmin dependent stimulation of ILEI secretion could be further analysed by inhibiting the urokinase receptor or binding sites of integrins by available antibodies to determine its upstream regulatory pathways. If uPAR and uPAR activated signalling pathways are not involved in this stimulation, it is possible that plasmin activates another signalling pathways via putative receptors.

Mechanisms explaining ILEI-dependent tumorigenesis remain still unknown. ILEI secretion levels correlated with tumor progression, since ΔN-ILEI with highest basal secretion levels generated bigger tumors and promote larger metastases. Furthermore, these tumors showed increased pAKT and pERK signalling, probably caused through ILEI-dependent activation of endogenous Ras effector pathways via binding to putative receptors activating MAPK signalling and leading to EMT and metastasis. Also, oncogene activation in a cell may lead to elevated expression of ECM proteases such as plasmin and plasminogen activators. These would then enhance ILEI secretion, which, perhaps through downstream signalling might positively affect ILEI-dependent Ras signalling, thus accelerating tumor growth and metastasis.

# 4. PART II

# DOXYCYCLIN-INDUCED ILEI EXPRESSION IN MICE

## 4.1 INTRODUCTION

In recent years, several transgenic mouse strains with conditional expression of a gene of interest by site specific recombinases or a tetracycline/doxycycline inducible gene expression system have been generated. They allow to analyse the function of various genes and their downstream signaling pathways, thereby studying physiological processes and modeling the pathogenesis of human diseases. Tetracyclin inducible systems can provide exact temporal and spatial control of gene expression. It bypasses the problem that sometimes the expression of a transgene during early stages of the development or in organs where it is normally not expressed can be detrimental because of lethality or toxicity. Further, the system is reversible and specifically induced by exogenous non-toxic agents in a dose dependent fashion, enabling an independent regulation of targeted gene from cellular pathways. *(Jaisser F., 2000)*

The tetracycline inducible system is based on the mechanism of tetracyclin resistance in *E.coli*. Tetracyclin repressor (TetR) binds on the regulatory elements of the tn10-specified tetracyclin-resistance operon of *E. coli* and negatively regulates the resistance-mediating genes. In the presence of the antibiotic tetracyclin or its derivatives (eg Doxocyclin; Dox) tetR cannot bind to its operators and transcription can occur. This system was described first by *Gossen and Bujard et al. (1992)*. Then they modified the original *E.coli* tet repressor (TetR) with the activating domain of virion protein 16 of herpes simplex virus and generated a tetracycline-controlled transactivator (tTA), which is expressed constitutively. Using random mutagenesis, a mutant form of modified tTA has been isolated. This mutant named reverse tTA (rtTA), is expressed upon exposure to the antibiotic, which is opposite to tTA in that it is functional in the absence of tetracyclin and its derivatives.

The system consists of two components: a tetracycline-controlled transactivator regulatory component (tTA or rtTA) and a response construct with a tTA/rtTA dependent promoter that regulates the expression of the gene of interest. In the presence of tetracycline or doxycycline (Dox), tTA cannot bind to the tet operator and transcription fails, therefore this system is termed as Tet-OFF. A mutant form of tTA termed rtTA binds to its operator after doxycycline induction, allowing transcriptional activation of a gene of interest only in response to varying concentrations of doxycycline. This system is termed as Tet-ON. *(Gossen and Bujard, 1995)*

For reduced basal activity and increased Doxcycline sensitivity, modifications and improvements has been made on the rtTA protein by *Urlinger et al. (2000)*. This has helped to overcome some of its limitations, like inefficient doxycycline response in some organs and residual affinity of rtTA to tetO in the non-induced state, which had originally led to enhanced background activities of the transgene.

Random integration of these elements into the genome may involve some disadvantages, like disruption of endogenous genes or position variegation effects. Therefore, these elements should be introduced by homologous recombination. *(Beard et. al., 2006)*

In this diploma thesis, a TET-ON mouse transgenics system was used, generated by the frt/flpase strategy in ES cells by the laboratory of Mathias Müller, VMU Vienna, adapted from *Caroline Beard et al.(2006)*. This system provides efficient targeting of inducible transgenes to a specific locus and is generated in two steps. First, site specific integration of a doxycycline responsive element TetOP controlling the gene of interest was targeted downstream of the collagen 1a1 locus, which has shown a high transgene expression profile in all cell types. This insertion element contains frt sites downstream of the promoter enabling the recombination and inducible expression of any desired genes after a flpase reaction. In the second step the modified M2 reverse tetracycline transactivator (M2-rtTA) is targeted into the ROSA26 locus (ROSA26::rtTA). High expression of M2rtTA from the endogenous ROSA26 promoter has been found in all tissues and cell types except in brain and testis, resulting in highly efficient and ubiquitous expression of the transactivator. *(Beard et. al., 2006)*

In this study transgenic mice were characterized where a C-terminally FLAG tagged version of the ILEI transgene was introduced into the Col1A1 locus. Two sets of mice were used. One group was engineered to express both the M2rtTA constitutively from the ROSA26 locus and a doxycyclin responsive element controlling ILEI-FLAG gene, targeted into the collagen 1A1 locus by frt/Flpase mediated site specific integration. I will refer to this group as double transgenics. In addition, single transgenic mice were taken as control animals, expressing only the M2rtTA and lacking the ILEI transgene to examine the background activity of the transactivator with doxycyclin.

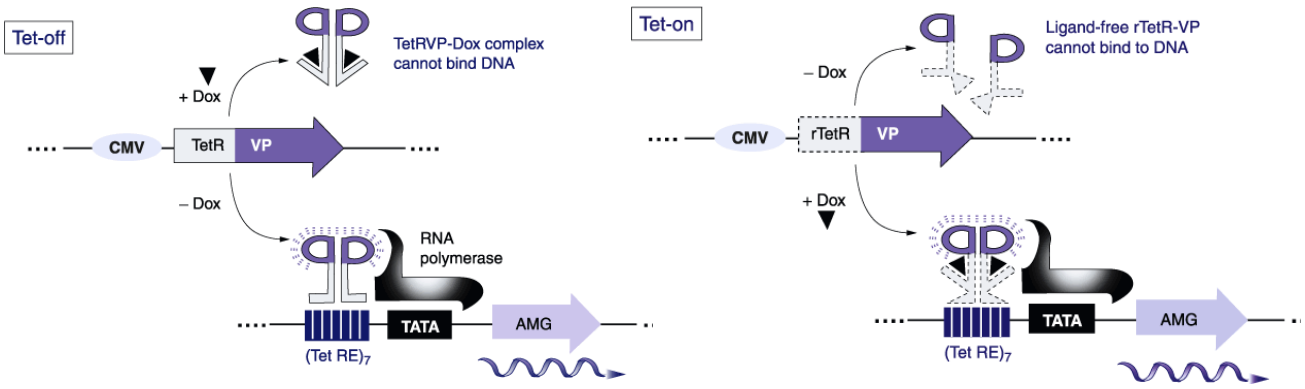



**FIGURE 39 Left panel: Repression of gene expression in the presence of the tetracycline analogue doxycyclin (TET-OFF)**
**Right panel: Gene expression in the presence of dox. by binding to the mutated Tet-on regulator (rTetR) (TET-ON)**
The VP16 transcriptional activation domain (VP) is derived from the herpes simplex virus gene encoding the VP16 protein
Ref.:http://www.biochem.arizona.edu/classes/bioc471/pages/Lecture15/Lecture15.html

## 4.2 RESULTS

Inducibility of ILEI upon doxycyclin exposure in our generated mice was tested in several organs. ILEI levels were analyzed by using Western blotting and immunohistochemistry. Doxocyclin was given to the mice in the drinking water.

### 4.2.1 ex-vivo Test of ILEI inducibility with doxycyclin

2,5x$10^6$ of primary bone marrow and spleen cells isolated from double and single transgenic mice were plated out and treated with different amount of doxycyclin for the time indicated (FIGURE 40). Thereafter, the cells were lysed and analyzed for ILEI expression levels by immunoblotting.

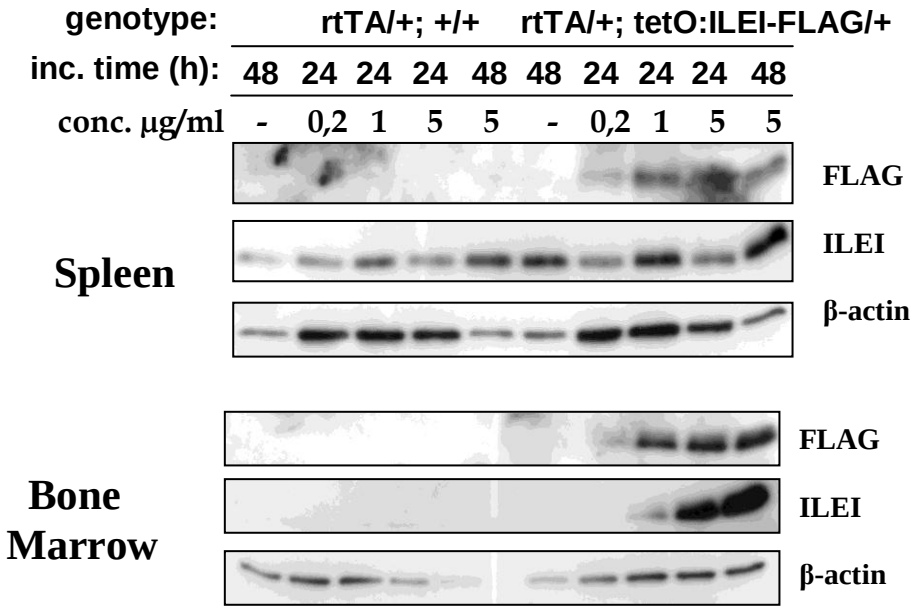



**FIGURE 40 Intracellular ILEI levels of spleen and bone marrow cells exposed or not exposed to doxycylin**

We have detected elevated ILEI expression levels both in spleen and bone marrow within 24 or 48 hours upon the addition of 1 to 5 µg/ml of dox. Additionally, we could specifically detect induced exogenous ILEI with α-Flag M2 antibody. We could observe endogenous ILEI expression in the spleen, but not in the bone marrow.

### 4.2.2 In vivo Test

We treated two double transgenic mice with 1 mg/ml of doxycyclin provided in the drinking water (supplemented with 5% sucrose) for 3 days. As controls, two additional double transgenic animals were kept on sucrose-supplemented drinking water without doxycyclin for the same time. After sacrificing the mice, we isolated several organs for further analysis. Spleen, bone marrow and blood sera were examined by immunoblotting for exogenous ILEI expression levels. Other organs, e.g. lung, intestines, liver, kidney and skin were fixed in 4% para-formaldehyde and analyzed by immunohistochemistry using ILEI and Flag antibodies.

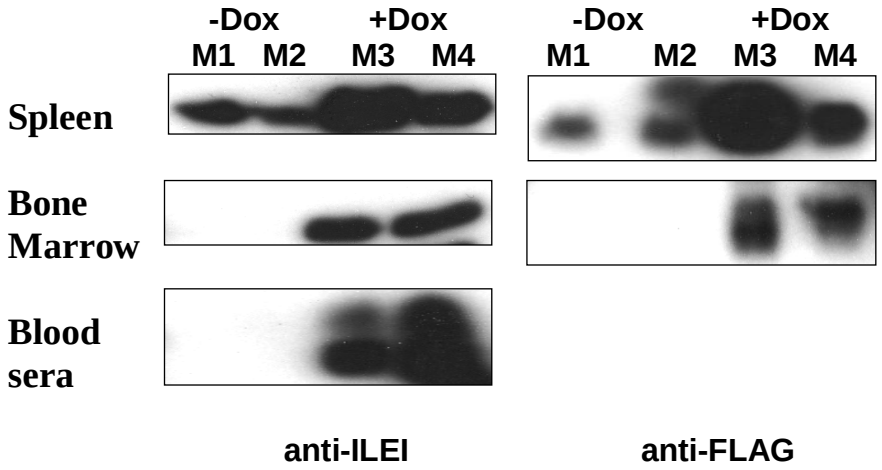



**FIGURE 41 | ILEI expression of spleen-, bone marrow- and peripheral blood cells from transgenic mice treated or not treated with Dox**

We obtained similar results as in the ex vivo pilot experiment. ILEI levels were increased both in spleen and bone marrow in mice treated with 1mg/ml dox. for 72 hours. Moreover we detected a high induction of ILEI expression in blood sera.
FLAG bands in –Dox spleen may be the result of tissue dependent unspecific binding of the antibody.
We have detected endogenous ILEI expression in small and large intestines and in kidney tubuli. Liver, skin and lung did not show any endogenous ILEI expression by immunostaining. Doxycyclin induced elevated ILEI expression in all investigated tissues except lung. In the lung, clara cells were detected as expressing ILEI after doxycyclin treatment. The epidermal layer of the skin and the cells of the hair follicles likewise showed ILEI expression upon doxycyclin induction (FIGURE 42). The highest ILEI levels after doxycyclin exposure were found in the villi of the large and small intestines. (FIGURE 43)

Interestingly, the exogenous, Dox-induced ILEI detected by anti FLAG antibodies showed the typical subcellular localization of ILEI in normal epithelial cells, i.e. rather large, granular structures apical of the nuclei, i.e. the position of the Golgi apparatus. This was true for kidney tubules (FIGURE 42) and small- and large intestine epithelia (FIGURE 43). It was remarkable, that tissues lacking expression of endogenous ILEI also expressed no or low levels of exogenous FLAG-ILEI, suggesting tight regulation of already synthesized ILEI by posttranslational mechanisms, e.g. protein degradation.

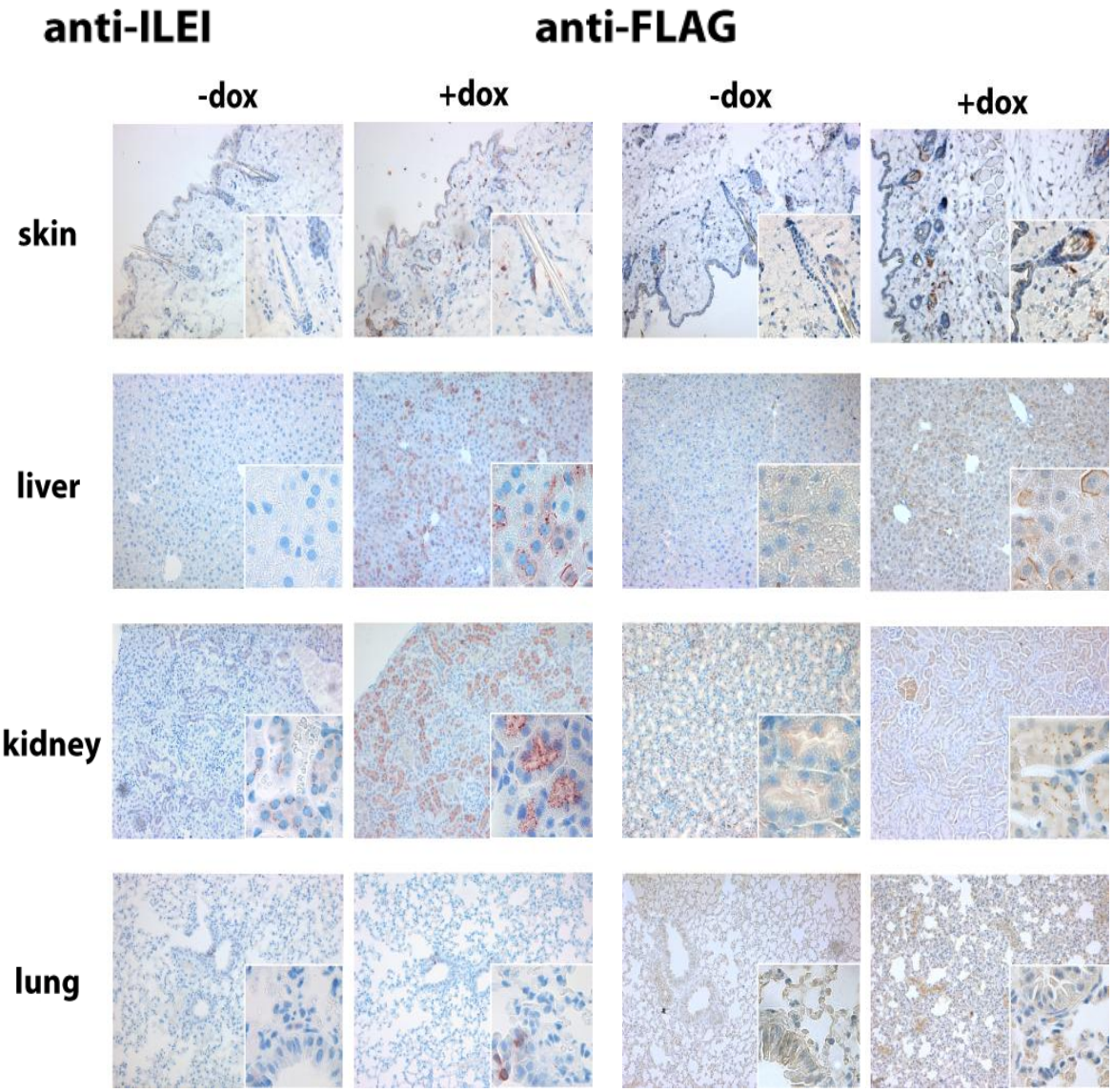



**FIGURE 42 | ILEI and Flag IHC staining of several organs with/out doxycyclin treatment**

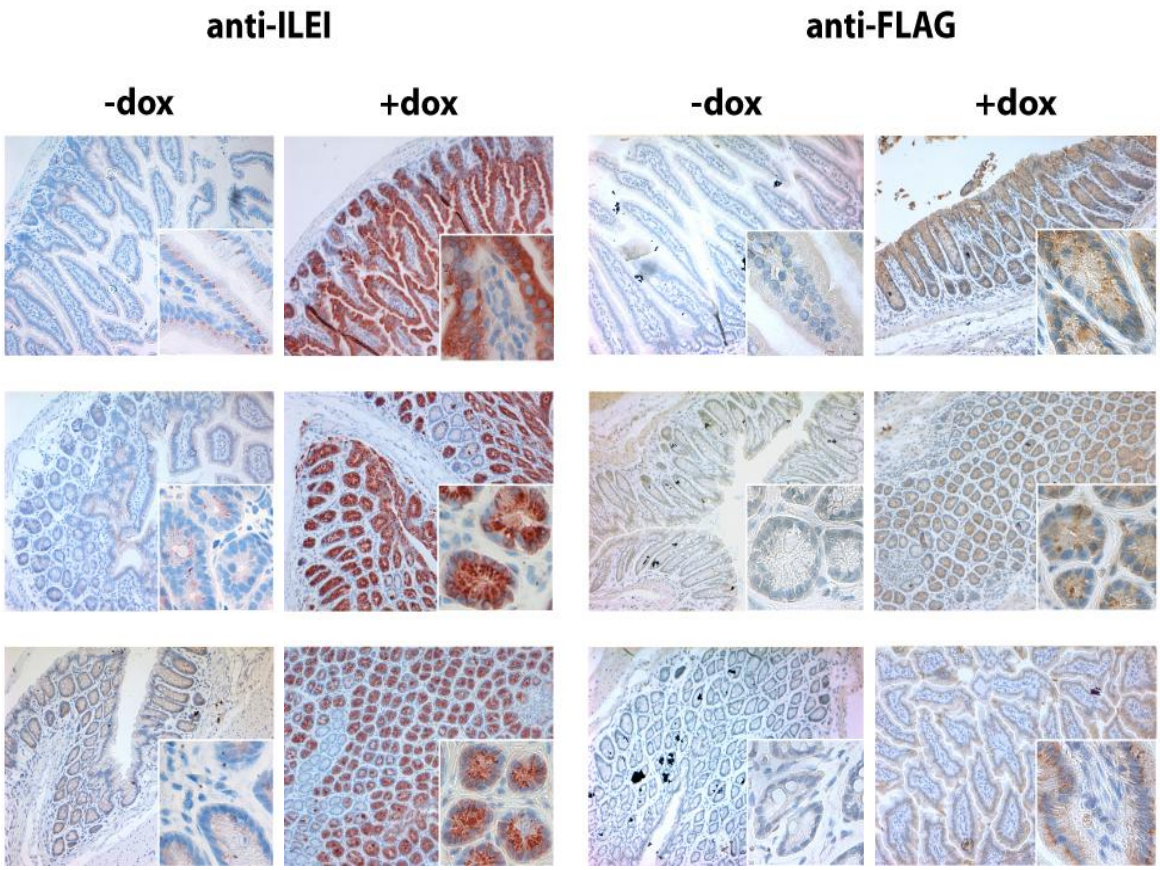



**FIGURE 43 | ILEI&Flag IHC staining of small and large intestines of mice**

## 4.3 DISCUSSION

An ILEI transgenic mouse strain by using the Tet-ON system inserted via the flp-in strategy has been generated successfully and these animals showed a specific, doxycyclin-responsive increase in ILEI expression in vivo without basal activity of the transgene in a non-induced state.

We could conclude that large and small intestines and kidney showed endogenous ILEI and elevated expression of the protein after induction as well. We detected good doxycyclin responsive expression in the skin and in the liver, however these tissues showed no endogenous ILEI expression. We did not observe any endogenous or induced ILEI in the lung, even after doxycyclin treatment.
Using the same transgenic strategy, which we adapted from Beard et al., these authors have observed EGFP expression in all tissues and all cell types, except for the absence in brain, testis and skeletal muscle. They suggested that the lack of expression in brain and testis might be due to the inability of doxycyclin to cross the blood-testis/brain barriers. In a separate study, using the same strategy, similar expression patterns of oct4 gene have been reported. *(Hochedlinger et. al., 2005)* Although the Rosa26 promoter results in ubiquitous and broad expression of transgene, we observed induced expression of ILEI not in all tested tissues. In addition, we detected lack of endogenous ILEI in the lung by immunostaining and in spleen by Western blotting. Nevertheless, ILEI (FAM3C) mRNA expression has been examined almost in all tissues, including lung and spleen by using Northern blot analysis *(Zhu et. al., 2002).* Since the translational control of ILEI expression is also known *(Waerner et. al., 2006),* tissue specific mRNA levels of ILEI after induction by RT-PCR or Northern blot should be checked, to clarify this contradiction.

This transgenic mouse model will now allow studying the physiological role of ILEI as well as its role on tumor progression and metastasis by the induction of stable ILEI expression in adult mice with or without other tumorigenic effectors in different tumor assays.

# 5. MATERIALS and METHODS

## 5.1 Cell Culture

| Cells used in this study | Cell Culture Medium |
|---|---|
| EpRAS, EpC40, SKBR3, Hela, MCF7 | Eagle (house made in media kitchen) supplemented with 10% FCS, 1U/ml Penicilin, 1µg/ml Streptomycin, 20mM HEPES and 2mM L-Glutamin |
| NIH3T3* | Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FCS, 1U/ml Penicilin, 1µg/ml Streptomycin, 20mM HEPES and 2mM L-Glutamin |
| | |

* NIH3T3 cells were treated with 1x Trypsin instead. 10xTrypsin was diluted with 1x PBS 1:10.

### 5.1.1 Generation and splitting of adherent cells

Cells were grown on plastic Petri dishes with indicated media at 37°C in cell culture incubators containing 5% $CO_2$. At confluency, they were splitted out to enable cells further growth. If they could not grow enough up to confluency, the media should be changed in every 2 days. For splitting, conditioned medium was removed, cells were washed once with 1x PBS and detached from dishes with 10x Trypsin (1 ml for a 10cm Petri dish) at 37°C for ~2-5'. Trypsin was inactivated with FCS containing medium, cells were resuspended and diluted in fresh medium appropriately to their proliferation capacity and seeded again in new dishes.

Cells were frozen to -80°C gradually and then kept at -270 in liquid nitrogen for long time. For freezing trypsinized cells were centrifuged for 5' at 1200rpm, pellets were suspended in FCS supplemented with 10% DMSO (1ml FCS for cells from a 10cm confluent Petri dish). Then they were transferred in cryogen micro tubes (Nalgene) and frozen. Thawing of these cells were performed quickly in a 37°C water bath, fresh media added to dilute DMSO and they were spinned down. After removing toxic DMSO, which is harmful for cell membranes, cells were given in fresh media and plated out.

### 5.1.2 ILEI Secretion Assay

400.000-700.000 cells were plated out with 3 ml of appropriate medium in 6-well plates. Once they were adherent medium was changed with 1 ml starvation* medium or medium with 4% FCS and exogenous treatments e.g. with TGFb or plasmin, if any, were administered. After 24 hour incubation time conditioned media

were collected and the cells were trypsinized, harvested and counted with cell chamber. Cells were centrifuged for 5' at 1200rpm and cell pellets were washed once with 1xPBS to remove rest of the medium.
* Starvation medium lacks FCS, as a replacement of high protein content the medium was supplemented with 0,1% BSA (sterile filtered).

### 5.1.3 Isolation and cultivation of primary spleen and bone marrow cells from mice

Dissected tibia and femurs of mice were cut off at both ends and the inner part of leg bones were rinsed out with a syringe with 1x PBS and the bone marrow was collected. Spleen were minced, suspended in PBS, filtered and centrifuged at 1200rpm for 5 min. PBS was removed and the cells were plated out in plastic culture dishes with Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FCS, 1U/ml Penicillin, 1µg/ml Streptomycin, 20mM HEPES and 2mM L-Glutamine. Indicated amounts of Doxycyclin were given to the culture medium for 24 or 48 hours.
Then they were suspended and collected and centrifuged for 5' at 1200rpm and the media were discarded. The cell pellets were washed again with 1xPBS to remove rest of the medium.

### 5.1.4 Isolation and re-cultivation of tumor cells

Excised tumors from mice were minced and incubated in EAGL 10% FCS supplemented with 2,5 mg/ml collegenase type I (Sigma) for at least 2h at 37°C. Digested tissues were suspended with pipette tips, filtered through nylon cell strainers (70µm, BD Falcon) to remove large particles, centrifuged and washed with 1x PBS. Then they were plated out for recultivation in Eagle 10% Medium treated with G418 sulphate antibody (Gibco Geneticin 800µg/ml) to select tumor cells and with amphotericin B (Biochrom AG stock 250µg/ml diluted in 5ml sterile aqua distilled) to protect cells from fungi and yeast.

### 5.1.5 In vitro $^{3}$H Thymidin proliferation assay

Cells were plated out as triplets in a 12 well/plate and incubated for 24 hours. Next day they were labeled for 2 hours by adding 30µCi/ml methyl $^{3}$H Thymidin (TRK 120) to the media. After removing radioactive media eloboratively, cells were treated with 30µl 10x Trypsin/well and 150µl 1xPBS was added. Samples were transferred then onto a filter mat (90x120mm) with Tomtec harvester. The filter was put wax on and dried in a microwave oven (max. 60°C) till the wax melt homogenously. The prepared samples were read in a 1450 Micro beta liquid scintillation counter (Wallac). Results were normalized to the cell numbers.

## 5.2 Protein Chemistry

### 5.2.1 Determination of protein concentration with Bradford reagent

BSA stock solution with 1mg/ml concentration was used to prepare a 2,5- 10µg/ml standard concentration series. The standards and 1µl of protein samples were mixed thoroughly in plastic cuvettes with 1ml Bio-Rad Bradford solution, which was pre-diluted 1:5 in distillated water. The extinction at 595nm was measured in spectrophotometer.

### 5.2.2 Preparing of whole cell lysates for SDS gels

The cell pellets were lysed with 60-100µl lysis buffer incubating them for at least 30′ on a rotator at 4°C and after that they were centrifuged at full speed for 20′ at 4°C. The supernatants (cell lysates) were collected in fresh tubes and protein concentrations were measured using Bradford assay.
Ingredients of the lyses buffer: 10% Glycerol
50 mM HEPES pH 7,5
150 mM NaCl
0,5% T-X100
1,5 mM MgCl2
1 mM EGTA
200µM Na3VO4
10 mM NaF

### 5.2.3 Concentration of supernatant (conditioned medium) for SDS-PAGE

Collected supernatants (~1ml) were concentrated (ultra-filtration) before running an SDS-PAGE. Supernatants were transferred into Ultracel YM-10 filters with a nominal weight limit of 10kDa, placed in Microcon centrifugal filter device and centrifuged for ~50′ at 14000g at 4°C. All proteins under 10kDa and medium were collected in the tube and discarded. The filter with concentrated proteins bigger than 10kDa (also ILEI 26kDa) were flipped up side down in a fresh tube and centrifuged for 2′ at 10000rpm. The concentrated volumes of supernatants were determined with a pipette and the amounts (conditioned Medium from ~1-2x$10^5$ cells) to be loaded onto an SDS gel were normalized to the cell numbers.

### 5.2.4 In vitro ILEI cleavage Assays

Cleavage of different mutant and wt ILEI proteins by proteases or sera was done using proteins purified from cells overexpressing these ILEI forms. Appropriate amount of proteins was put into a tube with indicated proteases or sera or protease inhibitor and incubated for 5-10 hours at 37°C in an incubator. They were loaded then on SDS-PAGE to detect cleaved and non-cleaved ILEI.

### 5.2.5 Immunoprecipitation (IP) of Flag epitope tagged proteins

Anti Flag M2 Agarose Beads (Sigma) were used for IP of different mutant ILEI-Flag proteins over expressing in EpRAS cells. The beads were already conjugated with Flag antibodies and there was no need for binding and cross linking of antibodies to the beads. The beads were pre-eluted 1x with 10 bed volumes (bv) 0,1 M glycin, pH 2 and washed 3x briefly with 10 bv 1xTBST* to re-equilibrate. We standardized amount of beads and proteins for an efficient binding and elution. Therefore 0,7mg protein from whole cell lysate (or supernatant) was incubated with 20µl of beads over night at 4°C on a rotator. Next day they were centrifuged for 2′ at 1000g at 4°C (beads are sensitive and should not be centrifuged higher than 1000g), supernatants were discarded. (They could be collected to see if the binding of antibodies to the proteins were sufficient). Pelleted beads were washed then 3x with 10 bv ice-cold IP-buffer** each for 10′ at 4°C. Then they were washed 2x with 150mM NaCl to remove pH-buffering agents and protease inhibitors. For the elution 25µl 0,1 M glycin (pH 2) were added to the beads, mixed for ~2′ and centrifuged 2′ at 1000g at 4°C and supernatant was transferred in a fresh tube. This elution step was repeated one more time. The beads should not be incubated with glycin pH 2 longer than 10′, therefore after elution they were washed 3x with 10 bv TBST to recover. To neutralise the eluate 1,5 M Tris-HCl, pH 9,2 ( 5µl Tris per 100µl eluate) was added and solution was tested with pH paper. (It should be approx. 7-8)

* TBST: 1x TBS + 0,04% Triton X-100
** IP-buffer: 20mM Tris-HCl, pH 7,5, 150mM NaCl, 10% glycerol, 2mM EDTA, 0,5mM TritonX-100
Plus phosphatase inhibitors: 20mM β-glycerophosphate, 10mM NaF, 5mM Na-pyrophosphate, 1mM Na-vanadate, 2mM EGTA
No protease inhibitor cocktail was added to the eluate because the eluates were then used in ILEI cleavage assays.

### 5.2.6 SDS PAGE

10% SDS Gel was prepared with 1 or 1,5mm thickness. 25µg/ml protein of each sample was mixed with 1x loading buffer, boiled 5′ at 95°C and loaded on 10% SDS gels.

| | **Stacking Gel: (10%)** | **Running Gel: (10%)** |
|---|---|---|
| | Stacking gel buffer (0.5 M Tris, pH 6.8 0.4% SDS) 625µl | Running gel buffer (1,5M Tris, pH 8.8) 2.5 ml |
| 30% AA/Bis Solution (BioRad) | 3.22 ml | 675 µl |
| dH2O | 2.89 ml | 3,7 ml |
| 10% AMPS | 55µl | 30µl |
| TEMED | 5,5µl | 12,5µl |

Composition of loading buffer (4x): 200mM TrisHCl, pH 6,8; 8% SDS, 0,4% Bromphenol Blue (BPB), 20% Glycerol, 10% 2-Mercaptoethanol
The gel was run with 1xPAGE running buffer (0.025 M Tris base, 0.2 M Glycine, 3,5 mM SDS) at 200V for 1 hour.

### 5.2.7 Semi-dry Western blot analysis

The proteins from SDS gel to PVDF membrane were transferred by semi-dry Western blotting. Thereby, two filter papers Watman 3MM and the gels were soaked into semi-dry transfer buffer, PVDF membrane was activated first in methanol then soaked into blotting buffer to equilibrate and then the blotting sandwich was put together from bottom-to-top Filter paper – membrane – gel – filter paper.
Blotting was performed at 60mA for each gel (with a size of 6,5cm x 8,5cm corresponding to 1mA/cm2) for at least 1,5 hours at room temperature. Then the membrane was blocked with 5% milk in 1xTBST* at room temperature for an hour or over night at 4°C . After blocking it was incubated with desired 1st antibodies for at least 1 hour at room temperature or over night at 4°C, washed 3x with 1xTBST and then incubated with horse radish peroxidase-coupled secondary antibodies (1:5000) for 1 hour at room temperature or over night at 4°C and washed again 3x with 1xTBST. For the detection of the desired protein the membrane was incubated with the ECL or ECL plus chemoluminescence reagents and a high performance chemoluminescence film was put on it in dark room. After an efficient lightening time the film was developed.
* TBST: 1x TBS (150 mM NaCl, 7,7mM TrisHCl); 0.1 % Tween20

### 5.2.8 Stripping of Western Blot membrane

To stain the same Western Blot membrane with another 1st antibody, the membrane was stripped for 20′ at 55°C shaking in stripping buffer*. It was washed then generously with 1xTBS-T and blocked again, afterwards the same western blot protocol was applied.
* Stripping buffer: 2% SDS, 62,5 mM Tris-HCl, 144 mM β Mercaptoethanol pH 6,7
* ILEI, Flag and phosphorylated antibodies were sensitive against stripping of blots.

## 5.3 Mouse work

### 5.3.1 Mice used in these experiments

The bloods were collected from Plg-/- and wild type mice with BL6 background. Doxycyclin induced ILEI overexpressing system was tested also in BL6 mice. Mammary gland fat pad and tail vein injections of tumor cells were performed in NMRI nude mice.

### 5.3.2 Mammary gland fat pad injection of EpC40 cells overexpressing different ILEI mutant forms

Mice were injected intraperitoneally with an anesthetic (0,2 ml per 10g of body mass) containing Ketamin (5 mg/ml) and Xylazine (0,8 mg/ml). Once they were anesthetized, $2*10^5$ of desired cells in 30µl 1xPBS were injected into 3rd and 4th pairs of the fat pads of mammary glands. Growing tumors were measured weekly with a caliper and the tumor volumes were calculated. (Tumor volume = (width$^2$ *length)/2)

### 5.3.3 Tail vein injection of re-cultivated tumor cells

Per mouse $2*10^5$ cells suspended in 200µl 1x PBS were injected into the tail vein to analyze the lung colonization capability of tumor cells overexpressed different mutant forms of ILEI.

### 5.3.4 Administration of Doxycyclin

1mg/ml Doxycyclin supplemented with 5% Sucrose were given into drinking water of mice.

### 5.3.5 In vivo Aprotinin treatment

100µl of 4000 KIU Aprotinin (Roth Karlsruhe) dissolved in 0,9 % NaCl (sterile filtered) was injected intraperitoneally into the mice every day.

### 5.3.6 Extraction of sera

Collected bloods from mice were centrifuged for 10′ at 6000rpm to pull down all blood components like white blood cells and erythrocytes. Then sera were taken out in fresh tubes.

## 5.4 DNA Techniques

### 5.4.1 Isolation of genomic DNA from mouse tail

2mm tail or fingers of ~2 weeks old mice were cut, 400µl tail buffer* and 0,5 mg/ml Proteinase K added and incubated ON at 55°C. Next day they were mixed for 5′ on Eppendorf mixer, added 133µl conc. NaCl (~6M), mixed again 5′ and centrifuged 10′ full speed. Upper part without top phase and pellet was given into a fresh tube and 350µl isopropanol was added, mixed 2′ on eppendorf mixer and spinned 2′ at full speed. Supernatant was discarded and pellet was washed with 300µl 70% Ethanol. Pellet was dried then in open tubes and dissolved in 50-150µl TE** incubating for 2 hours at 37°C with occasionally shaken on the Eppendorf mixer.

* Tail buffer: 50mM Tris-HCl pH8, 100mM EDTA, 100mM NaCl, 1% SDS
**TE buffer: 10mM Tris/HCl, 1mM EDTA pH8

### 5.4.2 Polymerase Chain Reaction (PCR)

25µl of reaction were prepared with 2µl DNA, 1µl primer mix (10mM each), 22µl ddH2O and PCR beads.
The program was started with initial denaturation step at 94°C for 3′.
In the first setup there were 19 cycles comprising of a short denaturation step at 94°C for 20′′, an annealing step at 64°C (-0,5° per cycle) for 30′′, an elongation step at 72°C for 35′′ (approx. 1′ for 1kb). The second setup of 24 cycles was performed as the following: 94°C for 20′′, 54°C annealing temperatur for 30′′ and 72°C for 35′′. Reaction was ended up with an elongation step at 72°C for 5′ and terminated at 4°C. Loading buffer (Orange G) was added to the samples and loaded onto an agarose gel.

### 5.4.3 Agarose gel electrophoresis

The size of separated DNA fragments depends on the Agarose concentration varying between 1-2%. Agarose was suspended in 1x TAE buffer and dissolved completely in microwave. After cooling down 10mg/ml ethidium bromide was added and the mix was poured into a gel tray and gelatinated. The gel was put then in a gel chamber containing 1x TAE and run at 100 mA.

## 5.5 Histology

### 5.5.1 Preparation of frozen tissue sections

Tumors from mice were washed once with 1xPBS, embedded in Tissue-Tec O.C.T. and frozen at -80°C. Frozen blocks were cut in 12µm sections with the Cryostat. Slides were dried for 30′ at RT and then stored at -20°C for IHC staining.

### 5.5.2 Preparation of PFA-fixed and paraffin embedded tissue sections

The dissected tissues were fixed in 4% PFA for at least 24 hours and dehydrated in Tissue-Tek VIP machine. Then they were embedded in hot paraffin. After they were solid 2µm sections were cut with a microtome and taken on super frost+ glass slides. Before staining, slides were dried at 55°C over night.

### 5.5.3 Staining of PFA-fixed and paraffin embedded tissue sections

#### 5.5.3.1 Hematoxylin and Eosin staining (H&E)

The staining was performed in the Thermo Scientific Microm HMS 740 Robot Stainer at RT.

#### 5.5.3.2 ILEI & FLAG immunohistochemistry

After slides were deparaffinizied they were boiled for 20′ in antigen retrieval buffer (DAKO, citrat buffer pH6) to unwind the antigen surfaces within the tissues. Then they were cooled down for 20′ in the buffer and washed with 1xPBS for 8′. The following drop reactions were performed in a wet chamber:
Blocking of unspecific binding sites with goat serum* for 15′
Addition of the first antibody diluted in 1% BSA/1xPBS for at least 1 hour
(anti rabbit anti ILEI 1:1000, pre-incubated with homologous peptides FAM3A and D 8ng/ml ON at 4°C)
Wash 3x with 1xPBS for 2′
Blocking of endogenous peroxidase with 0,3% H2O2 in 1xPBS for 20′
Wash 3x with 1xPBS 2′
Addition of secondary antibody in 1% BSA/1xPBS for 30′
(biotinylated goat anti rabbit 1:500) Wash 3x with 1xPBS 2′
Incubation with streptavidin-biotin complex for 30′ at RT (Vectastain Vector ABC Kit)
( 10µl solution A is mixed with 1ml 1xPBS then 10µl solution B added and this was incubated for 30′ at room temperature after that 1ml 1xPBS and 20µl goat serum was added in the reaction
Wash 3x with 1xPBS 2′

Color development in AEC** solution for 8'
Wash with H2O
Counterstain with hematoxylin (1g/L) for 3'', wash in H2O
Incubate in 0,16% HCl for 2'' for weakening strong signals
The slides were then dried at RT and mounted with Aquatex solution.

* goat serum blocking solution: 2% goat serum, 1% BSA, 0,1% cold fish gelatin, 0,1% TritonX-100, 0,05% Tween20, 0,05% sodium azide, 1xPBS, pH7,2; stored at 4°C
**AEC stock solution was 3,3,amino-9-ethyl carbozole dissolved in 100ml N,N-dimethyl form amid. For working solution 7ml of AEC stock solution were added in 200ml sodium-acetate buffer (20mM, pH=4,9) and filtered. Finally 22µl H2O2 (30%) were added.

#### 5.5.3.3 IHC Stainings for Casp3, Ki67 and F4/80

The stainings were done by Histolab similar to a standard IHC protocol except applying an instrument (IHC Kits) to strengthen and amplify the signal. IHC reagents to optimize the staining for each antibody are listed below:

| 1st Antibody | provider | Type | dilution | instrument | Antigen retrieval |
|---|---|---|---|---|---|
| Caspase 3 | Cell signaling | Rabbit monoclonal | 1:100 | Omni Map Dab anti-Rb | Dako |
| Ki67 | Novo castro | Rabbit monoclonal | 1:1000 | Dab Map | Dako |
| F4/80* | ABDSerotec | Rat antimouse | 1:100 | Dab Map | Citrat pH6 |

* After boiling in antigen retrieval Buffer, slides were incubated and blocked in 4% milk buffer for 1 hour. Slides were incubated with 1st and 2nd antibodies for 1 hour each.

#### 5.5.3.4 IHC stainings for E.cadherin, Vimentin and Flag

IHC stainings with anti-mouse antibodies on mouse tissues result high background staining, which then obscure the specific staining. This problem was eliminated by using M.O.M Immunodetection Kit (Vector). M.O.M Kit procedure was performed with the following mouse monoclonal antibodies:

| | provider | Dilution | Retrieval buffer |
|---|---|---|---|
| E.cadherin | BD Transduction | 1:800 | Dako |
| Vimentin | Sigma (clone 13.2) | 1:500 | Tris/EDTA* pH9 |
| Flag M2 | Sigma | 1:500 | Dako |

#### 5.5.3.5 IHC staining for CD31 antibody

Prepared frozen tissue sections were fixed 10′ at 4°C with acetone, washed 3x in 1xPBS. Then endogenous proxidase was inhibited 20′ with 0,3% H2O2/1xPBS. The sections were washed again 3x with 1xPBS and the standard IHC protocol, just described above, was followed. Slides were stained with 1st antibody CD31 ( rat anti mouse, BD Pharmingen, 1:50) and with 2nd antibody polyclonal biotinylated anti-rat antibody. (Dako)

## 5.6 Microscopy

### 5.6.1 Light microscopy

Pictures of IHC stained tissues were taken by Axioplan 2 imaging from a Zeiss microscope with Image Access software.
H&E stained lung tissues were scanned with a Zeiss scanner (Mirax) for lung metastasis analyses and representative images were taken with Zeiss Mirax viewer software.

## 5.7 Statistical Analysis

### 5.7.1 Statistical significance

It was evaluated by estimating two-tailed Student's T-test. P values under 0,05 were considered as significant.

### 5.7.2 Analysis of metastatic lung area

PFA fixed and paraffin embedded lungs were cut with a microtome, generating 2µm sections at 300µm intervals. ~9-11 sections per mouse were stained with Hematoxylin and Eosin. They were scanned then with the Zeiss Mirax scanner, whereby the actual analyses could be performed by Definiens Analyst software. All sections were worked on for metastatic and whole lung areas. Analysed areas from serial sections of one lung were added then and the percentage of metastatic area was calculated.

### 5.7.3 Evaluation of IHC Stainings

For evaluation of Ki67 and actCasp3 stainigs of primary tumor tissues, 3 different representative images per tumor were chosen, the numbers of positive and negative nuclei were counted and the percentage of stained positives cells were calculated.

# REFERENCES


1. Akhurst, R. J. and R. Derynck (2001). "TGF-beta signaling in cancer--a double-edged sword." Trends Cell Biol **11**(11): S44-51.
2. Andreasen, P. A., L. Kjoller, et al. (1997). "The urokinase-type plasminogen activator system in cancer metastasis: a review." Int J Cancer **72**(1): 1-22.
3. Andreasen, P. A., R. Egelund, et al. (2000). "The plasminogen activation system in tumor growth, invasion, and metastasis." Cell Mol Life Sci **57**(1): 25-40.
4. Arora, P., T. K. Ricks, et al. (2007). "Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer." J Cell Sci **120**(Pt 6): 921-8.
5. Bauskin, A. R., et al. (2005). "The propeptide mediates formation of stromal stores of PROMIC-1: role in determining prostate cancer outcome." Cancer Res. 15;**65**(6):2330-6.
6. Beard, C., K. Hochedlinger, et al. (2006). "Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells." Genesis **44**(1): 23-8.
7. Bergers, G. and L. M. Coussens (2000). "Extrinsic regulators of epithelial tumor progression: metalloproteinases." Curr Opin Genet Dev **10**(1): 120-7.
8. Berx, G., E. Raspe, et al. (2007). "Pre-EMTing metastasis? Recapitulation of morphogenetic processes in cancer." Clin Exp Metastasis **24**(8): 587-97.
9. Blasi, F. and P. Carmeliet (2002). "uPAR: a versatile signalling orchestrator." Nat Rev Mol Cell Biol **3**(12): 932-43.
10. Borgono, C. A. and E. P. Diamandis (2004). "The emerging roles of human tissue kallikreins in cancer." Nat Rev Cancer **4**(11): 876-90.
11. Bugge, T. H., L. R. Lund, et al. (1998). "Reduced metastasis of Polyoma virus middle T antigen-induced mammary cancer in plasminogen-deficient mice." Oncogene **16**(24): 3097-104.
12. Campbell, P. M. and C. J. Der (2004). "Oncogenic Ras and its role in tumor cell invasion and metastasis." Semin Cancer Biol **14**(2): 105-14.
13. Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science **296**(5573): 1655-7.
14. Carmeliet, P. and R. K. Jain (2000). "Angiogenesis in cancer and other diseases." Nature **407**(6801): 249-57.
15. Castellino, F. J. and V. A. Ploplis (2005). "Structure and function of the plasminogen/plasmin system." Thromb Haemost **93**(4): 647-54.
16. Chaudhury, A., G. S. Hussey, et al. (2010) "TGF-beta-mediated phosphorylation of hnRNP E1 induces EMT via transcript-selective translational induction of Dab2 and ILEI." Nat Cell Biol **12**(3): 286-93.
17. Coussens, L. M., B. Fingleton, et al. (2002). "Matrix metalloproteinase inhibitors and cancer: trials and tribulations." Science **295**(5564): 2387-92.
18. Cui, W., D. J. Fowlis, et al. (1996). "TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice." Cell **86**(4): 531-42.

19. Curino, A., D. J. Mitola, et al. (2002). "Plasminogen promotes sarcoma growth and suppresses the accumulation of tumor-infiltrating macrophages." Oncogene **21**(57): 8830-42.
20. De Wever, O. and M. Mareel (2003). "Role of tissue stroma in cancer cell invasion." J Pathol **200**(4): 429-47.
21. Derynck, R., R. J. Akhurst, et al. (2001). "TGF-beta signaling in tumor suppression and cancer progression." Nat Genet **29**(2): 117-29.
22. Dublin, E., A. Hanby, et al. (2000). "Immunohistochemical expression of uPA, uPAR, and PAI-1 in breast carcinoma. Fibroblastic expression has strong associations with tumor pathology." Am J Pathol **157**(4): 1219-27.
23. Egeblad, M. and Z. Werb (2002). "New functions for the matrix metalloproteinases in cancer progression." Nat Rev Cancer **2**(3): 161-74.
24. Elenbaas, B. and R. A. Weinberg (2001). "Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation." Exp Cell Res **264**(1): 169-84.
25. Elzer, K. L., D. A. Heitzman, et al. (2008). "Differential effects of serine proteases on the migration of normal and tumor cells: implications for tumor microenvironment." Integr Cancer Ther **7**(4): 282-94.
26. Frade, R., N. Rousselet, et al. (2008). "Intratumoral gene delivery of anti-cathepsin L single-chain variable fragment by lentiviral vector inhibits tumor progression induced by human melanoma cells." Cancer Gene Ther **15**(9): 591-604.
27. Gorsch, S. M., V. A. Memoli, et al. (1992). "Immunohistochemical staining for transforming growth factor beta 1 associates with disease progression in human breast cancer." Cancer Res **52**(24): 6949-52.
28. Gossen, M. and H. Bujard (1992). "Tight control of gene expression in mammalian cells by tetracycline-responsive promoters." Proc Natl Acad Sci U S A **89**(12): 5547-51.
29. Gossen, M. and H. Bujard (1995). "Efficacy of tetracycline-controlled gene expression is influenced by cell type: commentary." Biotechniques **19**(2): 213-6; discussion 216-7.
30. Grunert, S., M. Jechlinger, et al. (2003). "Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis." Nat Rev Mol Cell Biol **4**(8): 657-65.
31. Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.
32. Hochedlinger, K., Y. Yamada, et al. (2005). "Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues." Cell **121**(3): 465-77.
33. Huber, M. A., N. Azoitei, et al. (2004). "NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression." J Clin Invest **114**(4): 569-81.
34. Huber, M. A., N. Kraut, et al. (2005). "Molecular requirements for epithelial-mesenchymal transition during tumor progression." Curr Opin Cell Biol **17**(5): 548-58.

35. Jaisser, F. (2000). "Inducible gene expression and gene modification in transgenic mice." J Am Soc Nephrol **11 Suppl 16**: S95-S100.
36. Janda, E., K. Lehmann, et al. (2002). "Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways." J Cell Biol **156**(2): 299-313.
37. Jechlinger, M., A. Sommer, et al. (2006). "Autocrine PDGFR signaling promotes mammary cancer metastasis." J Clin Invest **116**(6): 1561-70.
38. Jechlinger, M., S. Grunert, et al. (2002). "Mechanisms in epithelial plasticity and metastasis: insights from 3D cultures and expression profiling." J Mammary Gland Biol Neoplasia **7**(4): 415-32.
39. Jechlinger, M., S. Grunert, et al. (2003). "Expression profiling of epithelial plasticity in tumor progression." Oncogene **22**(46): 7155-69.
40. Jedeszko, C. and B. F. Sloane (2004). "Cysteine cathepsins in human cancer." Biol Chem **385**(11): 1017-27.
41. Karnoub, A. E. and R. A. Weinberg (2008). "Ras oncogenes: split personalities." Nat Rev Mol Cell Biol **9**(7): 517-31.
42. Kirstein, J. M., K. C. Graham, et al. (2009). "Effect of anti-fibrinolytic therapy on experimental melanoma metastasis." Clin Exp Metastasis **26**(2): 121-31.
43. Liapis, H., A. Flath, et al. (1996). "Integrin alpha V beta 3 expression by bone-residing breast cancer metastases." Diagn Mol Pathol **5**(2): 127-35.
44. Liotta, L. A. and E. C. Kohn (2001). "The microenvironment of the tumour-host interface." Nature **411**(6835): 375-9.
45. Massague, J. (1998). "TGF-beta signal transduction." Annu Rev Biochem **67**: 753-91.
46. McCawley, L. J. and L. M. Matrisian (2001). "Matrix metalloproteinases: they're not just for matrix anymore!" Curr Opin Cell Biol **13**(5): 534-40.
47. Mignatti, P. and D. B. Rifkin (1993). "Biology and biochemistry of proteinases in tumor invasion." Physiol Rev **73**(1): 161-95.
48. Mikulits, W., B. Pradet-Balade, et al. (2000). "Isolation of translationally controlled mRNAs by differential screening." Faseb J **14**(11): 1641-52.
49. Mueller, M. M. and N. E. Fusenig (2004). "Friends or foes - bipolar effects of the tumour stroma in cancer." Nat Rev Cancer **4**(11): 839-49.
50. Oft, M., J. Peli, et al. (1996). "TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells." Genes Dev **10**(19): 2462-77.
51. Oft, M., K. H. Heider, et al. (1998). "TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis." Curr Biol **8**(23): 1243-52.
52. O. De Wever, M. Mareel, et at. (2003). "Role of tissue stroma in cancer cell invasion." J Pathol. **200**(4):429-4
53. Pendurthi, U. R., M. Ngyuen, et al. (2002). "Plasmin induces Cyr61 gene expression in fibroblasts via protease-activated receptor-1 and p44/42 mitogen-activated protein kinase-dependent signaling pathway." Arterioscler Thromb Vasc Biol **22**(9): 1421-6.
54. Pilipenko, V. V., A. Reece, et al. (2004). "Genomic organization and expression analysis of the murine Fam3c gene." Gene **335**: 159-68.

55. Pollard, J. W. (2004). "Tumour-educated macrophages promote tumour progression and metastasis." Nat Rev Cancer **4**(1): 71-8.
56. Portella, G., S. A. Cumming, et al. (1998). "Transforming growth factor beta is essential for spindle cell conversion of mouse skin carcinoma in vivo: implications for tumor invasion." Cell Growth Differ **9**(5): 393-404.
57. Rajalingam, K., R. Schreck, et al. (2007). "Ras oncogenes and their downstream targets." Biochim Biophys Acta **1773**(8): 1177-95.
58. Rakic, J. M., C. Maillard, et al. (2003). "Role of plasminogen activator-plasmin system in tumor angiogenesis." Cell Mol Life Sci **60**(3): 463-73.
59. Repasky, G. A., E. J. Chenette, et al. (2004). "Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis?" Trends Cell Biol **14**(11): 639-47.
60. Schubbert, S., K. Shannon, et al. (2007). "Hyperactive Ras in developmental disorders and cancer." Nat Rev Cancer **7**(4): 295-308.
61. Smith, H. W. and C. J. Marshall "Regulation of cell signalling by uPAR." Nat Rev Mol Cell Biol **11**(1): 23-36.
62. Sporn, M. B. (1996). "The war on cancer." Lancet **347**(9012): 1377-81.
63. Thiery, J. P. (2003). "Epithelial-mesenchymal transitions in development and pathologies." Curr Opin Cell Biol **15**(6): 740-6.
64. Thiery, J. P. and J. P. Sleeman (2006). "Complex networks orchestrate epithelial-mesenchymal transitions." Nat Rev Mol Cell Biol **7**(2): 131-42.
65. Turner, G. A. and L. Weiss (1981). "Analysis of aprotinin-induced enhancement of metastasis of Lewis lung tumors in mice." Cancer Res **41**(7): 2576-80.
66. Urlinger, S., U. Baron, et al. (2000). "Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity." Proc Natl Acad Sci U S A **97**(14): 7963-8.
67. Waerner, T., M. Alacakaptan, et al. (2006). "ILEI: a cytokine essential for EMT, tumor formation, and late events in metastasis in epithelial cells." Cancer Cell **10**(3): 227-39.
68. Wakefield, L. M. and A. B. Roberts (2002). "TGF-beta signaling: positive and negative effects on tumorigenesis." Curr Opin Genet Dev **12**(1): 22-9.
69. Webb, C. P., L. Van Aelst, et al. (1998). "Signaling pathways in Ras-mediated tumorigenicity and metastasis." Proc Natl Acad Sci U S A **95**(15): 8773-8.
70. Welch, D. R., A. Fabra, et al. (1990). "Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential." Proc Natl Acad Sci U S A **87**(19): 7678-82.
71. World Health Organization "Cancer" (February 2006) http://www.who.int/mediacentre/factsheets/fs297/en/. Retrieved 2007-06-25
72. Yang, J., S. A. Mani, et al. (2004). "Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis." Cell **117**(7): 927-39.
73. Yin, Y. J., Z. Salah, et al. (2003). "Oncogenic transformation induces tumor angiogenesis: a role for PAR1 activation." Faseb J **17**(2): 163-74.
74. Zhu, Y., G. Xu, et al. (2002). "Cloning, expression, and initial characterization of a novel cytokine-like gene family." Genomics **80**(2): 144-50.

## Curriculum Vitae

### Personal Details

Name : Betül ULUCA

Date of Birth : 19.06.1984

Place of Birth : Istanbul/Turkey

Nationality : Turkish

### Education

| | |
|---|---|
| 1999-2002 | Kadiköy Imam Hatip High School, Turkey |
| 2003-2004 | German Preparatory Course „Universitätslehrgang-Vorstudienlehrgang“, Vienna |
| 2004-2011 | Studies of Microbiology and Genetics, University of Vienna |