Characterization of Tenascin-W, an emerging player in the metastatic bone marrow niche

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I. Summary

Tumors are heterogeneous organ-like tissues including not only tumor cells themselves but also auxiliary cells such as, endothelial cells, fibroblasts, inflammatory cells, and bone marrow derived stem or stromal cells (BMSCs), which collectively create the surrounding microenvironment also referred to as stromal compartment. By now the active role of the tumor-stroma in driving the dissemination phase and the following engraftment of tumor cells in secondary organs is widely accepted. Indeed, the perpetual activation of stromal cells is extended beyond the local primary tumors and they can take part in preparing a permissive environment at distant anatomic sites by providing oxygen and nutrients essential for tumor growth and invasion.

Tenascin-W (TNW) is a matricellular protein with a dynamically changing pattern of expression during development and disease. Its pronounced presence in developing bones implies a function in osteogenesis. In adults, tenascin-W is mostly restricted to stem cell niches, and is also expressed in the microenvironment of solid cancers. These distinct expression patterns imply a complex regulation of tenascin-W gene expression at the transcriptional level. Here we analyzed tenascin-W expression in a xenograft model of breast cancer metastasis to the bone. Quantitative mRNA analysis revealed an upregulation of tenascin-W in mouse osteoblast populations sorted from bones harboring human breast cancer metastases. Long bone sections containing metastases exhibit expression of mouse tenascin-W protein proving that tenascin-W is supplied by the metastatic niche and not by the tumor cells. Transwell and co-culture studies show that bone marrow stem cells (BMSCs) express tenascin-W protein after exposure to factors secreted by MDA-MB231-1833 breast cancer cells. These findings prompted us to investigate the *cis* and *trans*-acting elements that drive tenascin-W gene transcription. 5'RACE analysis of mRNA from human breast cancer, glioblastoma, and bone tissue showed a single tenascin-W transcript with a transcription start site (TSS) at a non-coding first exon upstream of exon2, which contains the translation start codon (ATG). The promoter region between -957bp and -79bp influences transcription and the minimal promoter sequence is contained within 79bp from the TSS. Computational analysis shows the presence of Smad4 nuclear transcription factor binding site at -61bp from the TSS in proximity of a TATA box sequence. Site-directed mutagenesis of the Smad4-binding site strongly impaired the SEAP reporter gene expression driven by the basal promoter. Furthermore, we found three evolutionary conserved regions in the first intron harboring glucocorticoid response elements (GRE), which negatively affect

transcription initiation from the basal promoter (-79bp). Therefore, we assessed whether TGFβ1 and glucocorticoids (GCs) act on tenascin-W gene expression in the tumor context. We identified TGFβ1 as an important factor inducing human tenascin-W gene transcription in BMSCs through activation of ALK5. Preincubation of BMSCs with the ALK5 inhibitor, SB431542, abolished tenascin-W induction by TGFβ1. Moreover, GCs impaired tenascin-W mRNA expression in BMSCs. Finally, recombinant tenascin-W protein stimulated MDA-MB231-1833 cell proliferation and migration *in vitro* assays. Our experiments suggest that tenascin-W acts as a niche component for breast cancer metastasis to the bone by supporting cell migration and cell proliferation of the breast cancer cells.

The analysis of the tumor bed contribution to cancer progression is a new frontier to unravel. It will lead to novel approaches to interfere with mechanism implicated in drug resistance, tumor relapse and metastatic spread.

II. Introduction

Cellular pathways are controlled by a multitude of diverse intercellular communication processes. In multicellular organisms, the exchange of signals often depends on the compartment outside of cells, which provides a behavioral code to cells that interact with it. Indeed, different cell types such as fibroblasts, endothelial cells and immune cells within the tissue microenvironment are embedded in a complex meshwork of non-cellular components called extracellular matrix (ECM) made up from glycosaminoglycans (GAGs), collagens, glycoproteins, and proteoglycans (PGs). The ECM defines the fundamental spatial organization of the cell, contributing for instance to the apical-basal configuration of epithelial cells. Among several functions, the apico-basal orientation is pivotal for the asymmetric division of epithelial stem cells and lack of polarity contributes to the switch into symmetric divisions, resulting in stem cell-derived tumors (1). Cell-cell adhesion occurs through the lateral cell domain, which presents intercellular junctions, whereas hemidesmosomes anchor the cell to the extracellular matrix of the basal lamina. The latter is attached to the external, fibrillar reticular layer, together making up the basement membrane (BM), which includes several macromolecules such as type IV collagen, laminin, fibronectin, nidogen, agrin, perlecan and heparin sulfate proteoglycans (2-4). All these ECM ligands ensure a highly controlled communication with cells through the interaction with cell surface receptors such as integrins, a family of heterodimeric transmembrane glycoproteins (5, 6). The integrins are linked to the cytoskeleton by a large complex of different proteins including i.e. integrin-linked kinase (ILK), an adaptor protein that binds the cytoplasmatic tail of integrins, allowing transduction of signals from outside into the cell (7). Intracellular signal transduction is coupled with actin cytoskeleton remodeling leading to a variety of processes that control cell shape change, gene expression, proliferation, migration, differentiation, and survival. In addition to the common ECM integrin-binding motif, Arg-Gly-Asp (RGD) present in many ECM proteins, other sites are used for tethering to other cell surface-adhesion receptors, such as discoidin, syndecan, cadherin, Ig-CAM and selectin (8, 9). Yet other domains present in ECM proteins are necessary for binding growth factors. Binding sites for cytokines and growth factors have been found on glycosaminoglycans or small leucine-rich repeat proteoglycans (SLRPs) (10, 11). Additional proteoglycans, such as decorin, allow the access of TGFβ ligand to its receptor (12). A synergism has been shown between integrins and syndecan (13) and integrin occupation with growth factor signaling (14). For instance, receptor synergy between syndecan-4 and integrin is essential for the spreading of cells on fibronectin

(15). Different integrin receptor combinations in association with a multitude of ECM ligands can modulate adhesive events, thus, contributing to tissue properties during different developmental phases including the embryonic stage, or the inflammatory response and wound healing in adult tissue. Finally, the highly distinctive organization of ECM in tumors as compared to normal tissue reflects specific functions in disease. Therefore, it is of fundamental importance to explore not only the passive, space-filling contribution of ECM proteins but also their major active role in regulating tissue homeostasis.

II.1 The bright side of ECM during cellular processes and tissue repair

II. 1.1 Extracellular matrix: Platform of signal exchange with cells

Beyond the parameters which define ECM proteins as a physical scaffolding for cells and being in charge of attenuating external forces, such as hydrostatic pressure or tensional stress, more recently a more active ECM role in different cellular processes was proposed. The coexistence of several domains and motifs in the ECM proteins is important for the potential interaction with many factors (Fig.II.1) (16).

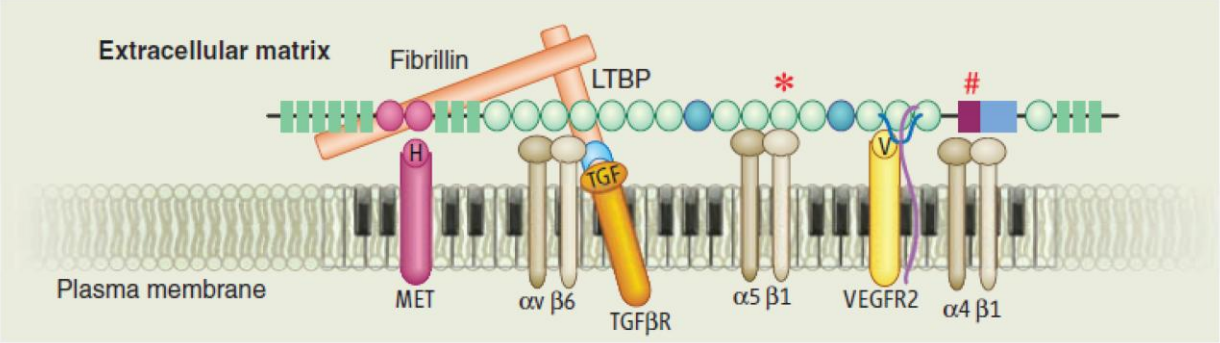

Figure II. 1: Multidomain interaction of ECM proteins with cells
Domains present on ECM proteins, such as fibronectin (green structure) are essential for the binding of integrins such as RGD (*) or LDV (#) as showed for $\alpha5\beta1$ and $\alpha4\beta1$. Growth factor VEGF binds two receptors: heparan sulfate chains of syndecan (purple/blue) and VEGRF2 (yellow). Integrin $\alpha5\beta6$ interacts with LAP protein, thus activating TGFβ. Through the N-terminal region of fibronectin the inactive TGFβ form can simultaneously interact with fibrillin. HGF binds the transmembrane HGF- receptor (MET, pink) and the integrin receptor linked to fibronectin (16).

Furthermore, such interactions can depend on alternative splicing mechanisms occurring in ECM protein domains or adhesion receptors leading to the inclusion or omission of specific binding motifs, thus influencing the association with certain molecules. An example of this is

fibronectin where the aberrant inclusion of the EDA domain was linked to fibrosis and thrombosis (17). Other mechanisms of creating ECM protein variants with different functions are the incomplete processing shown for type II procollagen. Two isoforms were shown to be differentially expressed during formation of the endochondral skeleton, with the isoform type IIA retaining the N-terminal propeptide displaying the specific capability to bind TGFβ and BMP2, two factors known to regulate chondrogenesis (18, 19). Diversity is further increased by multiple genes encoding different protein family members. This is the case for TGFβ, where many cells secrete precursors of different TGFβ isoforms in a latent form, associated with LAP (latency-associated peptide). This latent complex is in turn bound to latent binding protein (LTBP) (20). The cleavage of fibrillin or other ECM proteins from the LTBP complex by metalloproteases leads to the release and the subsequent activation of TGFβ. The interaction of integrin with LAP also leads to the activation of TGFβ (Fig.II.1). Sometimes juxtaposition of two receptors bound to the same ECM molecule is required for the activity of growth factors (Fig.II.1). For instance, the binding of vascular endothelial growth factor (VEGF) to the two receptors integrin α5β1 and VEGRF2 bound to the same fibronectin protein is essential in promoting endothelial cell proliferation and migration (21). In contrast, hepatocyte growth factor (HGF) requires the binding to two different ECM molecules, fibronectin and vitronectin, to exert its pro-angiogenic function (22). Therefore, ECM acts as a multidomain platform on which many factors converge to trigger a highly regulated response.

II. 1.2 Bidirectional communication between ECM and the stem cell niche

The hematopoietic niche in the adult bone marrow includes cells of different lineages, such as osteoblasts, vascular endothelial cells, neural cells and immune cells (Fig.II.2). Significant influences on the stem cell niche arise also from non-cellular elements, such as ECM components and secreted factors (23). In 1978, Schofield discovered the relevance of the surrounding environment in determining hematopoietic stem cell behavior (24). ECM proteins can act as a reservoir of growth factors (Fig.II.3). Therefore, they can influence the balance of stem cells between self-renewal and differentiation by modifying the biochemical composition of the cellular microenvironment (25). Furthermore, ECM can influence the localization of stem cells through cell-matrix interactions mediated by integrins (Fig.II.3) (26). In addition, mechanical tension can induce the release of specific factors by the ECM or the exposure of biologically active cryptic sites within ECM proteins following structural changes (27).

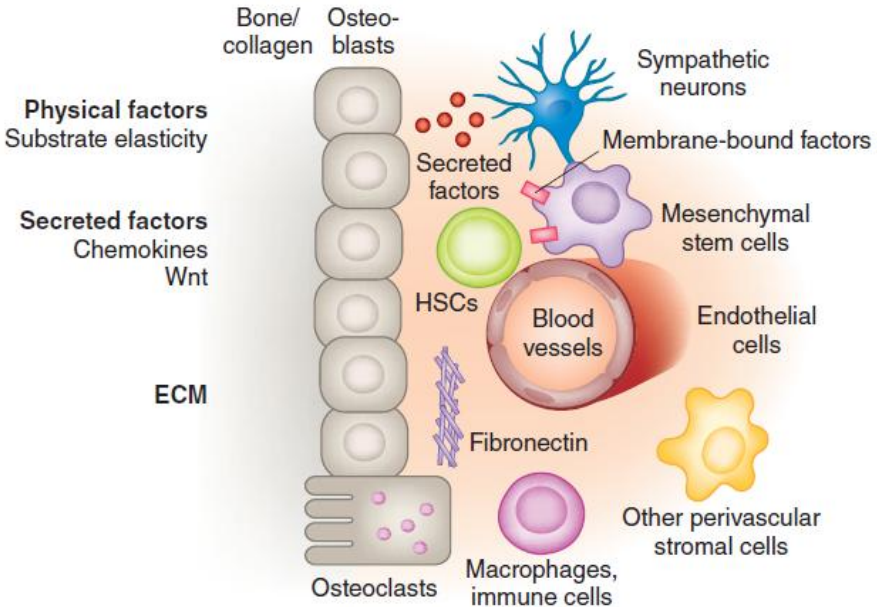

Figure II. 2: Stem cell niche
The stem cell niche includes structural components, such as ECM matrix and different types of cells: immune cells, endothelial cells and neurons. Secreted factors are involved in the regulation of the stem cell niche fate (23).

In turn, secreted factors can mediate the indirect communication between stem cells and the niche, thereby affecting the ECM composition. For instance, during the granulation stage in wound healing, various cytokines and growth factors act on the different fibroblastic cell subpopulations in the connective tissue. Besides local fibroblasts, this also includes mesenchymal stem cells (MSCs). Fibroblasts produce a large amount of ECM proteins during scar formation. However, other cell sources such as myofibroblasts can further sustain matrix deposition. One of the main fibrogenic mediators is TGFβ, a potent inducer of myofibroblastic differentiation (28). Myofibroblasts originate from either local fibroblasts through the epithelial-mesenchymal transition (EMT) or from local stem cells (29). Therefore, secreted factors are able to induce significant changes within the niche thereby indirectly targeting the stem cells.

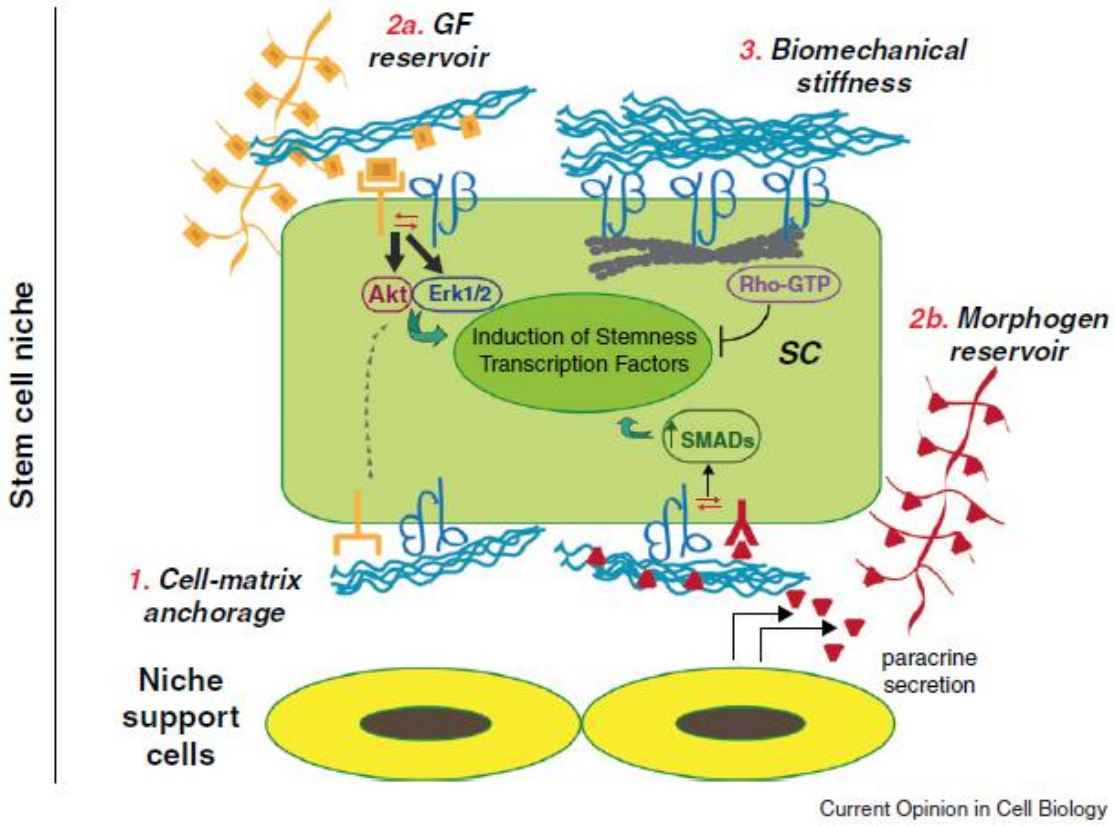

Figure II. 3: Cross-talk between stem cell niche and ECM
Stem cell niche includes structural components, such as ECM matrix. ECM proteins are a sink of growth factors, influencing stem cells behavior. ECM-integrin complex allows the anchorage of stem cells. ECM-physical properties contribute to changes in stem cells activity (27).

II. 1.3 Epithelial-mesenchymal transition and the role of TGFβ

Epithelial-mesenchymal transition (EMT) is a process which does not only affect differentiated adult cells but is also an important step required for the germ layer formation and the cellular motility and invasiveness during embryonic development (Fig.II.4) (30).

EMT transition consists in the acquisition of mesenchymal features with a migratory phenotype. Many EMT factors, such as Snail or Twist, are induced by different growth factors. Especially, Snail is a transcription factor that represses epithelial markers such as E-cadherin and its transcription is induced by TGFβ (31). Loss of this marker is associated with the disruption of adherens junction. Secretion of metalloproteases helps the degradation of the basement membrane and the compromised ECM structure leads to the loss of cell polarity (32). The

recruitment of MSCs at injury sites or, in a worse scenario, at tumor sites is initiated by the EMT process (Fig.II.4). Therefore, since EMT promotes cell invasion and migration, it is obvious to imagine how frequently this cell transition plays a crucial role in the development or tissue repair and in the onset of metastases.

Once that cells have migrated towards their destinations, due to their plasticity, they can revert back into an epithelial phenotype through a process named MET, for mesenchymal-epithelial transition (33).

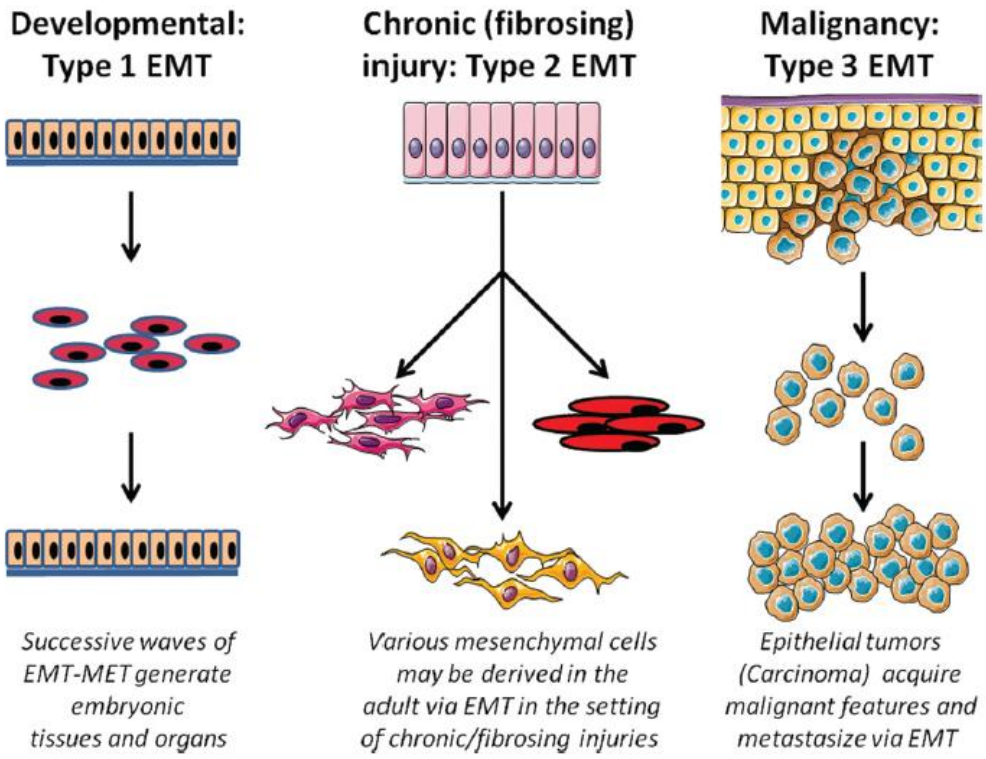

Figure II. 4: Epithelial-mesenchymal transition
Acquisition of a mesenchymal phenotype occurs during embryonic development and in adult stage, at injury sites and in metastatic tumors (34).

II. 1.4 Differential transcriptional regulation of growth factors involved in the EMT process

TGFβ is the prototypical EMT inducer. Members of the TGFβ superfamily include TGFβ itself, activins and bone morphogenetic proteins (BMPs). Like many other ubiquitous growth factors, TGFβ shows versatile functions that differ according to the physiological context or the degree of injury. Although TGFβ can act as a potent inhibitor of cell proliferation (35), in a pathological

scenario, the loss of important components of the TGFβ-mediated cell cycle arrest program, such as c-myc in cancer cells (36) or the genetic inactivation of specific TGFβ receptors (37) lead to the defective inhibition of cancer cell growth by TGFβ (38). BMPs are involved in regulating vascular cell proliferation and differentiation but, similarly to the dual role of TGFβ, defects in BMP signaling pathway generate hereditary vascular diseases (39).

BMP2 and TGFβ signaling pathways are mediated by transmembrane serine/threonine kinase type I and type II receptors. TGF-β1 binds to type I TGFβ receptor /ALK-5, whereas BMP to type I /Alk3. Once that type I receptors get phosphorylated by the type II receptors, Smad family intracellular proteins are activated. Smad2 and Smad3 mediate TGFβ and activin signalling, whereas Smad1, Smad5 and Smad8 act downstream of BMP receptors. The final common mediator is Smad4, which following its translocation to the nucleus regulates gene transcription by directly binding DNA or by interacting with other transcription factors, co-activators and co-repressor (Fig.II.5) (40-42). Non-canonical TGFβ-Smad signaling pathways are mediated by JNK and p38 MAPK.

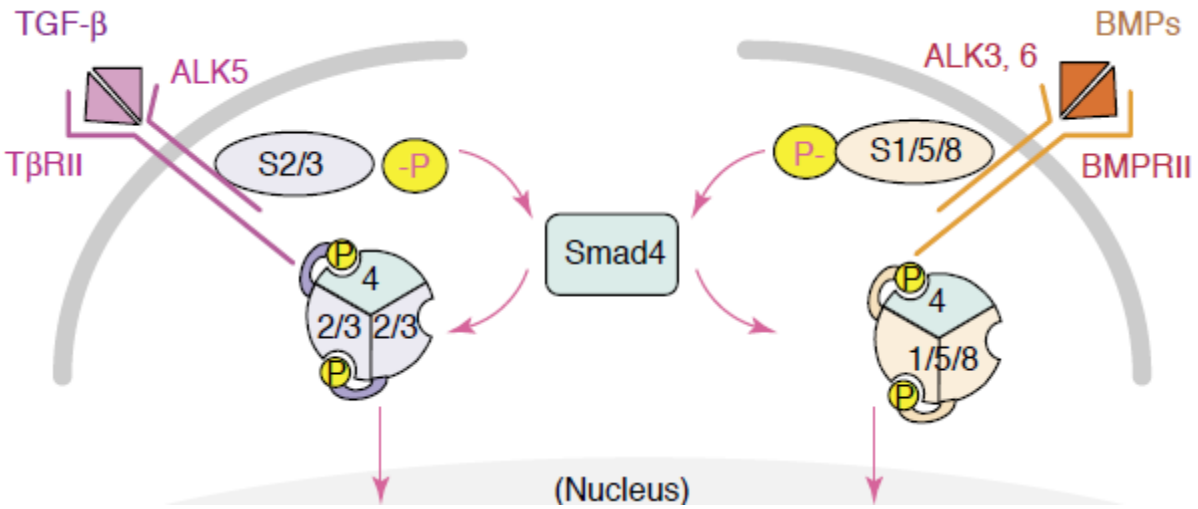

Figure II. 5: The canonical TGFβ superfamily Smad signaling pathway (42)

The importance of TGFβ ubiquity and versatility becomes evident in the recovery from liver injury. Through TGFβ stimulation, hepatic stellate cells (HSCs) undergo a mesenchymal-epithelial transition to transdifferentiate into liver progenitor cells to ensure liver regeneration following partial hepatectomy (PH) or chemical insults (43). Furthermore, TGFβ is also a major

inhibitor of hepatocyte proliferation (44). This explains why the conditioned medium collected from HSCs early after PH injury contains low levels of TGFβ, while it is enriched in hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) to promote hepatocyte proliferation. On the contrary, during terminal phases of liver regeneration HSCs produce high levels of TGFβ (45). This transient role of TGFβ in sustaining EMT and hence ECM deposition is regulated by differential phosphorylation of specific sites within Smad proteins. More precisely, TGFβ signaling through Smad2/Smad3 elicits cytostatic and EMT downstream signals, thus leading to the inhibition of HSCs proliferation and the enhancement of collagen synthesis. In turn, Smad7 induced by Smad3 interrupts the Smad phosphorylation cascade. In doing so, Smad7 induces a negative feedback mechanism in the fibrogenic activity of TGFβ (46).

II. 1.5 The physiological action of ECM proteins during the inflammation process

All sources of physiological insults such as infectious agents, tissue injuries or tumors cause a rearrangement of the ECM. ECM components represent the first barrier for the infiltration of inflammatory cells of the organism (47). During the process of extravasation, integrin-ECM interactions allow the adhesion of neutrophils to the vascular wall. Neutrophils constitute the first line of defense during infection, and contribute to the destruction of invading pathogens (48, 49). Importantly, removal of bacteria or oxidative burst by phagocytes is mediated by their adhesion to various ECM components (50). During the infection processes, Toll-like receptors (TLRs) do not only recognize exogenous danger signals such as bacterial lipopolysaccharide (LPS) but also endogenous factors (Fig.II.6). Interestingly, it has been shown that specific ECM domains, such as the fibrinogen-like globe of tenascin-C, are capable of inducing TLR4 activity in arthritic joint disease (51).

II. 1.6 The ECM and the control of wound healing

The hierarchical organization of fibrillar matrix arises from collagen crosslinking catalyzed by different enzymes, such as lysyl oxidase (LOX). This assembly of collagen molecules into fibrils results in a compact tissue important in preventing skin injury (10). In wound closure two physiological processes are essential: regeneration and tissue repair. In addition to the secretion of inflammatory mediators already mentioned above, several cell types participate in the granulation phase. Adjacent to the injury, healthy cells undergo mitosis to replace the lost cells. Repair of damaged tissue is provided by fibroblasts, endothelial cells and keratinocytes.

Fibroblasts secrete growth factors and induce new matrix deposition, which is responsible for the patching of harmed sites (Fig.II.6).

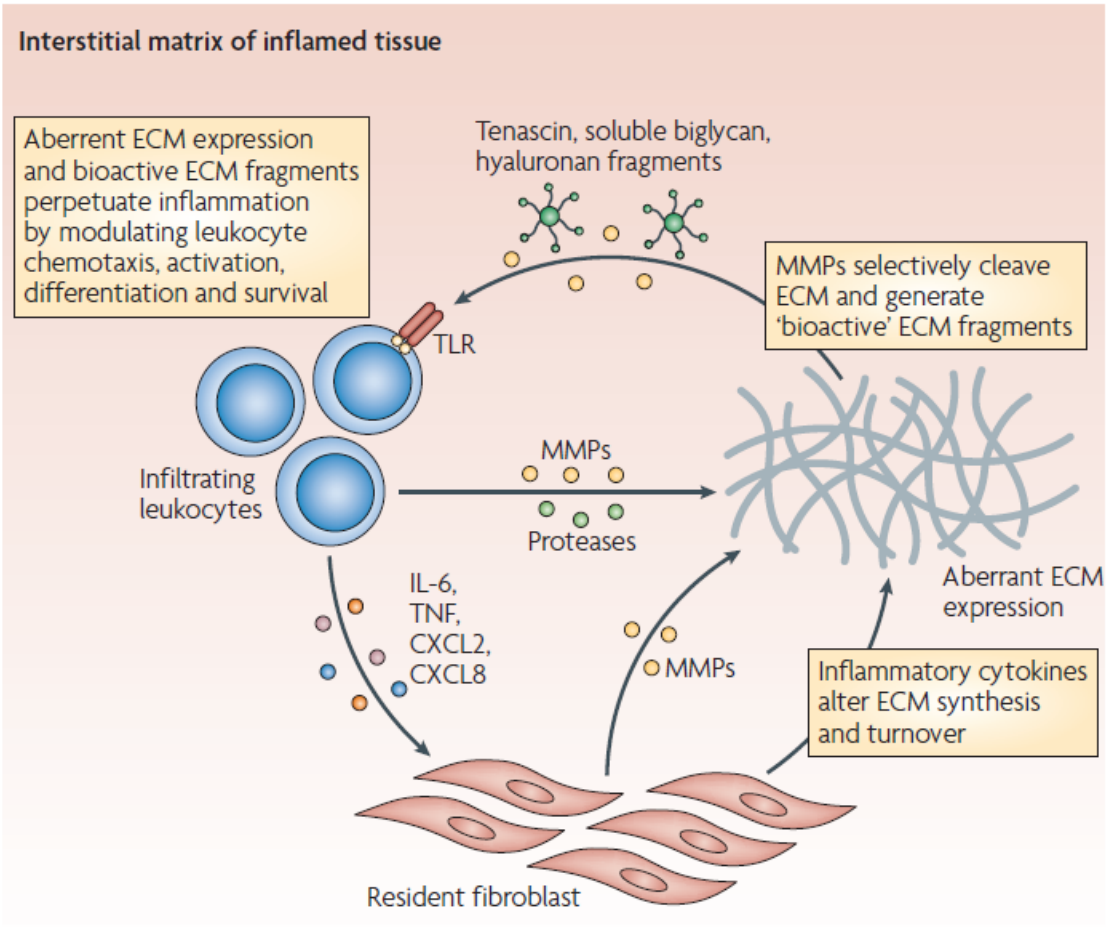

Figure II. 6: ECM proteins and activation of immune cells
Cleaved ECM proteins generate bioactive fragments which are responsible for the immune response mediated by Toll-like receptors. ECM deposition in tissue repair is controlled by resident fibroblasts and remodeling of the matrix is regulated by several metalloproteases (MMPs). Integrin-ECM axis mediates neutrophil infiltration in inflammation (47).

During wound healing, in order to balance the excessive matrix deposition by fibroblasts, which is the main cause of fibrosis, the extracellular matrix protein CCN1 promotes fibroblast senescence by inducing Nox1 and subsequently leading to an increased amount of ROS (56). For this, CCN1 requires the association with integrin $\alpha 6\beta 1$ and heparan sulphate proteoglycans (52). The deposition of ECM proteins, which occurs during fibrous scar formation, is also balanced through the activity of metalloproteases, which are tightly regulated by ROS in conditions of hypoxia following microvascular injury (53). Collagenase is an interstitial enzyme

expressed by keratinocytes. Its activity is important to remove the large amount of matrix proteins present during scar formation to allow cell migration (54). The ECM is also important for wound re-epithelialization. Keratinocyte migration into the wound bed is facilitated by focal adhesions dependent, integrin-mediated cell-ECM interactions (55). A number of external factors can affect this processes and lead to a delayed wound healing. Several conditions, such as obesity, nutrition, alcohol consumption, smoking, diabetes, administration of sex hormones, age and stress can alter the formation of the wound matrix, affect the availability of oxygen in the wound, or lead to decreased levels of pro-inflammatory factors (56). Moreover, it is well known that anti-inflammatory agents in therapeutic use, such as glucocorticoids (GCs), are associated with decreased proteoglycan synthesis (57).

II. 2 The dark side of ECM in unhealed wounds

II.2.1 Tumor stroma orchestrates the onset of cancer

The inflammatory response is the physiological reaction of the body which occurs following tissue damage. However, persistence of acute injury leads to a chronic inflammation which predisposes to cancer development. Cancer tissue is a heterocellular system consisting of cancer cells themselves and of non-transformed cells with non-cellular ECM components which are collectively referred to as stroma (Fig.II.7).

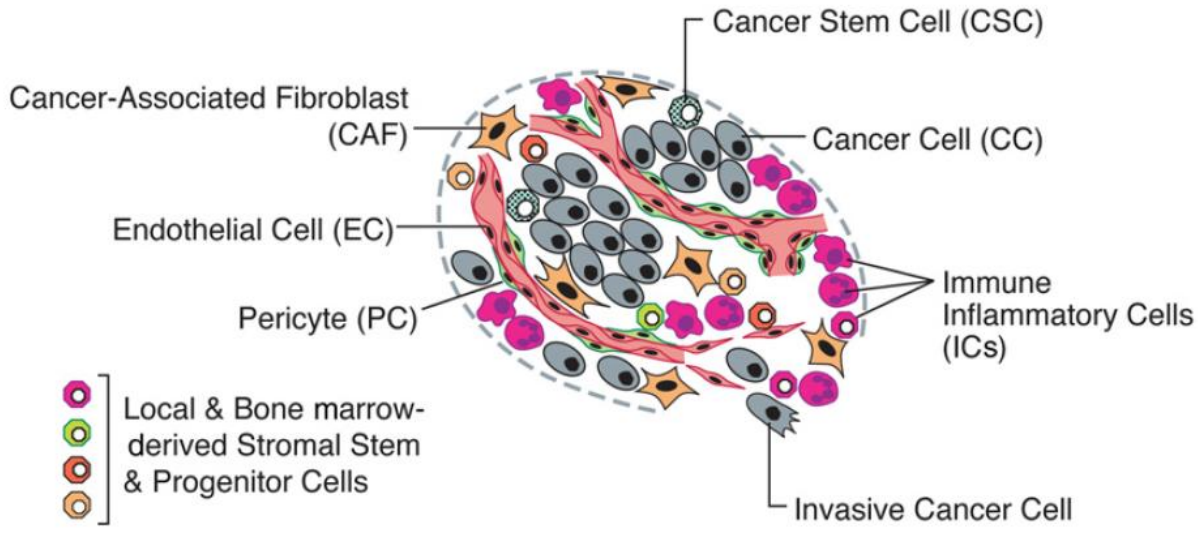

Figure II. 7: Tumor heterogeneity
Distinct cell types: stromal cells and cancer cells in a reciprocal interaction (58).

Cancer initiation is in most cases driven by genetic lesions such as point mutations, gene deletion/inactivation, chromosomal rearrangements and amplification, but the role of epigenetic modifications is also relevant in causing genetic alterations (59). Epigenetic regulation mechanisms are well known to control cellular differentiation. For instance, methylation of CpG islands within the genomic sequence leads to transcriptional silencing. Inactivation of the α-Smooth muscle actin (α-SMA) gene or of TGFβ by methylation was shown to impair myofibroblast differentiation (60). In turn, lack of epigenetic inactivation of these genes might be responsible of a constant myofibroblasts activity, leading to an increased ECM deposition in the stroma. Additional studies focusing on the importance of the tumor microenvironment in supporting the growth of cancer cells showed loss of tumor-suppressor p53 within the stroma (61). Furthermore, irradiated mammary stroma, which is hence affected by mutagenic events, was shown to drive the neoplastic behavior of normal, non-irradiated, injected mammary epithelial cells resulting in an increased tumor incidence (62).

II.2.2 Role of activated stromal components

Cancer stroma *per se* can be the bearer of genetic alterations that may cause spontaneous activation of normal fibroblasts in cancer associated fibroblasts (CAFs) (63). The already mentioned, the EMT process can occur in presence of growth factors, cytokines and other tumor secreted factors (41, 64) and as a consequence local fibroblast or cancer cells (65) can transdifferentiate in CAFs with the subsequent acquisition of markers such as α-SMA or vimentin and the loss of adhesion marker E-cadherin (66). Bone marrow derived progenitor cells can also contribute to the formation of CAFs (67). In addition, it was also shown that endothelial cells can assume a fibroblasts-like phenotype through endothelial to mesenchymal transition (EndMT) (68). The feature that most importantly distinguishes normal fibroblasts from the activated ones is the enhanced secretion of ECM proteins, growth factors and proteases (Fig.II.8).

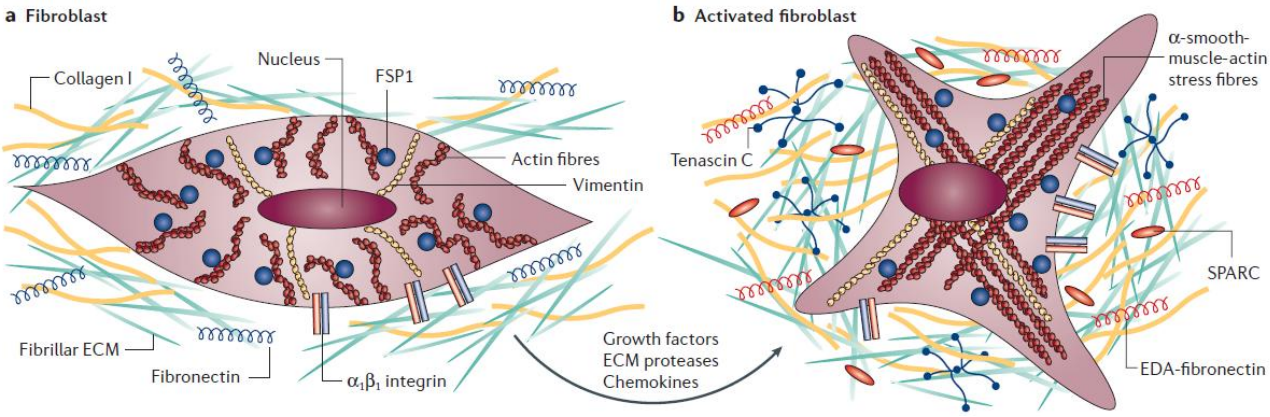

Figure II. 8: Activated fibroblasts
Physical and biochemical changes occur during the activation of local fibroblasts in a tumor microenvironment. Tenascin-C, SPARC, α-SMA and a wide range of others molecules are secreted during this transition (69).

An imbalance of any of these elements triggers irreversible cascades of events. Following a feedback mechanism, CAF derived factors can negatively influence adjacent cells within the stroma. Esophageal squamous cell carcinoma (ESCC) acquire enhanced invasive properties following the exposure to hepatocyte growth factors secreted by CAF cells (70). On the other hand, the injection of B16M melanoma cells in mice was shown to create a pre-metastatic niche by activating hepatic stellate cells (HSCs), which represent the liver-specific mesenchymal cells (71). After exposure to the B16M tumor cell conditioned medium, HSCs secrete matrix metalloproteinase-2 (MMP2), known to enhance B16M cell migration in vitro (72).

II.2.3 Recruitment of mesenchymal stromal cells

Thus far we have discussed the highly compromised tumor stromal microenvironment with an unbalanced secretion of several factors. Stromal or mesenchymal stem cell niches are affected in terms of impaired equilibrium between quiescence and proliferation/differentiation. Indeed, in primary tumors the features of the niche induces a shift of the mesenchymal stem cells (MSCs) from a quiescent to an activated state. In doing so, they represent one of the main regulators of cancer stem cells (73) with myofibroblast properties (74). Such MSCs are also actively involved in metastatic processes.

Indeed MSCs provide a permissive microenvironment for the engraftment of tumor cells arriving from distant primary sites. In response to several signals released into the circulation from the primary tumor, MSCs can generate a pre-metastatic niche already before the arrival of the

metastatic tumor cells. For instance, vitronectin and osteonectin produced by osteoblasts within the bone metastatic microenvironment have been shown to be strong chemoattractants for prostate and breast cancer cell lines (75, 76). Also, higher levels of TGFβ secreted by breast tumor cells contribute to tumor dissemination (77, 78). The use of neutralizing antibodies against TNF-α, TGFβ, and VEGF-A in mice injected with Lewis lung carcinoma cells (LLC) was able to impair the expression of the two specific pro-inflammatory mediators S100A8 and S100A9 in pre-metastatic lungs (79). On the other hand, MSCs can also be recruited from the circulation or from the surrounding normal tissue to the tumor-damaged tissue to support the primary tumor microenvironment (Fig.II.9) (80). The unique composition of a tumor stromal compartment and specific cytokines or growth factors secreted by tumor cells drive the metastatic tropism and the ability of cancer cells to spread to other organs.

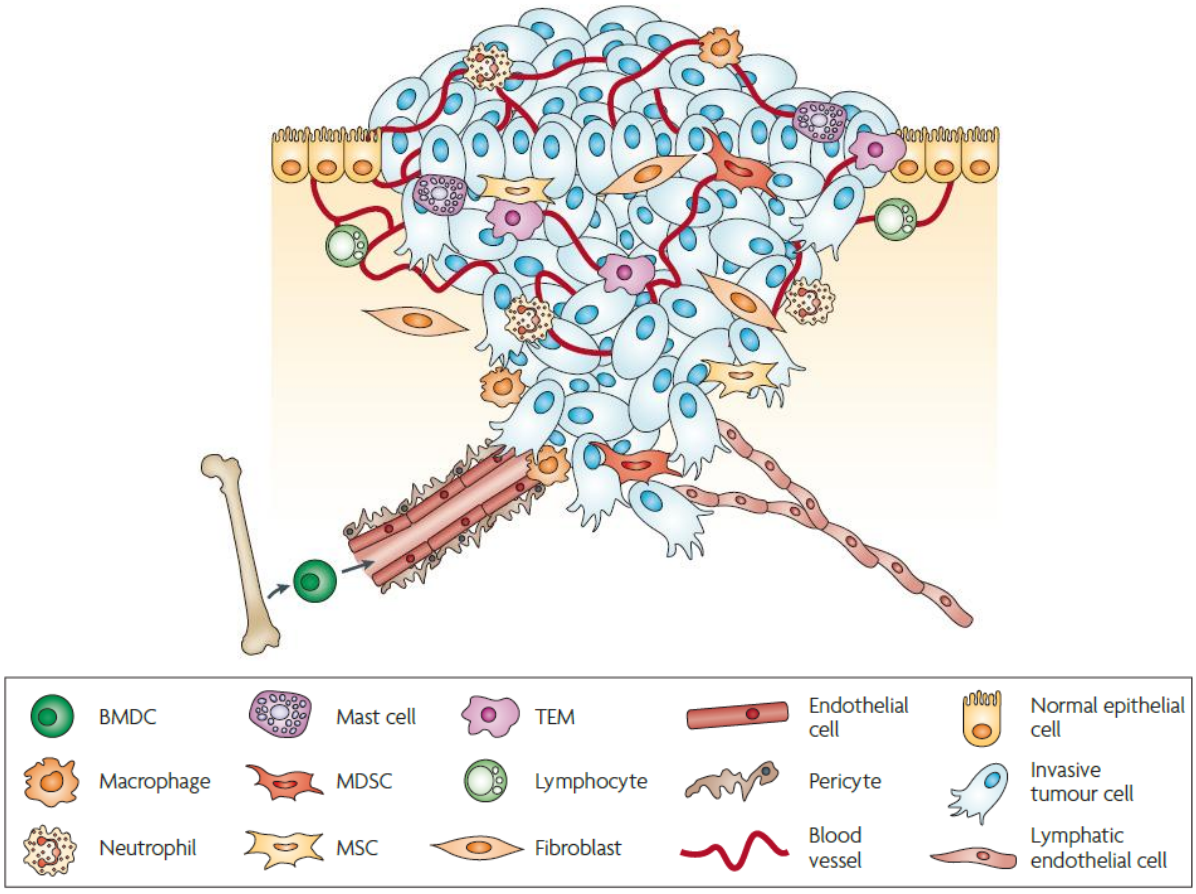

Figure II. 9: Recruitment of bone marrow stem cells to the primary tumor
Heterogeneity of a primary tumor microenvironment with many cell types arising from adjacent tissues (80).

II.2.4 Cancer: A chronic inflammatory state

The most prominent indicator for the presence of CAFs is the increase of ECM proteins in the stroma. For instance, the non-physiological continuous emergence of cells with a myofibroblast phenotype is associated with fibrosis in the liver, where the terminal phase of this process is cirrhosis. The increased amount of ECM proteins leads to the collapse of the hepatic parenchyma, which is replaced with an enriched collagen tissue (81). The distorted hepatic architecture impairs normal liver function. Downstream signaling in response to matrix stiffness precedes a functional linkage through which external stimuli are transduced in intracellular signals by the cytoskeleton axis. Matrix rigidity transmits forces which regulate signaling pathways involved for instance in the control of stem cell differentiation (82), cell migration (83) or also EMT-like processes (84). Among all factors that contribute to tissue stiffness hypoxia plays an important role. Indeed, previous reports have shown the association of low oxygen tension with an enhanced ECM production in dermal or in rat cardiac fibroblasts (85, 86). On the other hand, hypoxia-inducible factors (HIFs) can regulate the expression of matrix metalloproteinases, which regulate ECM degradation (87-89). New subfamilies of MMPs are the ADAMTS enzymes that unlike MMPs degrade aggrecan at different cleavage sites (90). MMPs control the equilibrium between ECM deposition and degradation. However their unbalanced expression leads to a continuous degradation of matrix barriers facilitating the invasive behavior of cells (89). Furthermore, MMPs are also involved in enhancing neovascularization. In particular, the membrane type I metalloprotease MT1-MMP was shown to induce VEGF expression (91). As was already mentioned, the ECM fragments released in the stroma after MMP-dependent ECM degradation can induce the expression of inflammatory mediators or act as chemoattractants for the recruitment of inflammatory cells. For instance specific cryptic sites exposed during the fragmentation of fibronectin increase secretion of cytokines such as IL-1, IL-6 or the tumor necrosis factor (TNF-α) (92). TNF-α and interleukins IL-1 or IL-6 normally suppress cell death, promote epithelial proliferation and activate stem cells during wound healing. However, in pathological conditions, the same cytokines have an anti-apoptotic effect on premalignant cells, and promote cancer cell proliferation (93). Also, elastin fragments from bovine ligaments induce the recruitment of monocytes in lungs of mice (94). ROS originating from the inflammatory response have a potential role in causing structural alterations to DNA (95) or deregulating methylation patterns (96). A supporting role of fibronectin, laminin, and collagen I in stimulating ROS production in human pancreatic adenocarcinoma through the

NADPH oxidative activity and the 5-lipoxygenase (5-LO) was also shown (97). ECM and ROS cooperation sustain pancreatic cancer cell survival. Collectively, following tissue damage, inflammatory mediators act on adjacent cells, which acquire a myofibroblastic phenotype. ECM remodeling supports the early stage of tissue repair. However, the incessant activity of all stromal components leads to onset of tumor formation (Fig.II.10).

As mentioned above, HIF not only controls ECM deposition but also processes such as angiogenesis, secretion of MMPs and cell survival in general. Therefore, the use of specific molecules, such as the topoisomerase I inhibitor topotecan (98) or cardiac glycoside digoxin (99) could impair processes triggered in the digoxin was shown to reduce tumor fibrosis by inhibiting the activation of the collagen cross-linking enzyme lysyl oxidase (LOX) mediated by HIF (99).

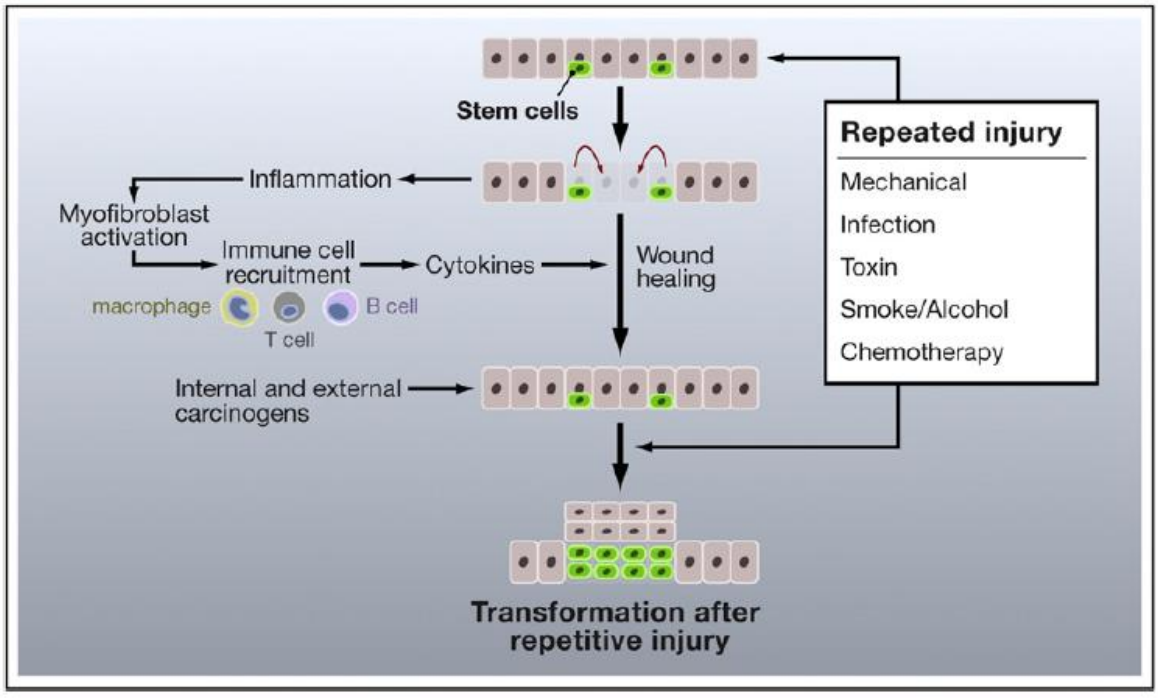

Figure II. 10: Tumorigenesis as result of chronic inflammation
Persistent acute inflammation leads to a failure of the physiological response. In such context, ECM proteins have shown an active role during the initiation and progression of cancer (93).

II.3 The tenascin family of ECM proteins

II.3.1 Tenascin proteins in cancer stroma

Tenascin family proteins are expressed in different types of connective tissues, and are composed of four members: tenascin-X, tenascin-C, tenascin-R and the last discovered member tenascin-W (100). All tenascin glycoproteins are characterized by a modular structure (Fig.II.11) which includes an N-terminal oligomerization region including heptad repeats, followed by epidermal growth factor (EGF)-like repeats, a variable number of fibronectin (FN) type III domains prone to alternative splicing mechanisms, and a C-terminal fibrinogen-related domain (FReD) (101). Via their N-terminal oligomerization domain, tenascin subunits form disulfide-linked homo-trimers (tenascin-R and -X) or -hexamers ("hexabrachions"; tenascin-C and -W), (Fig.II.11) (102, 103).

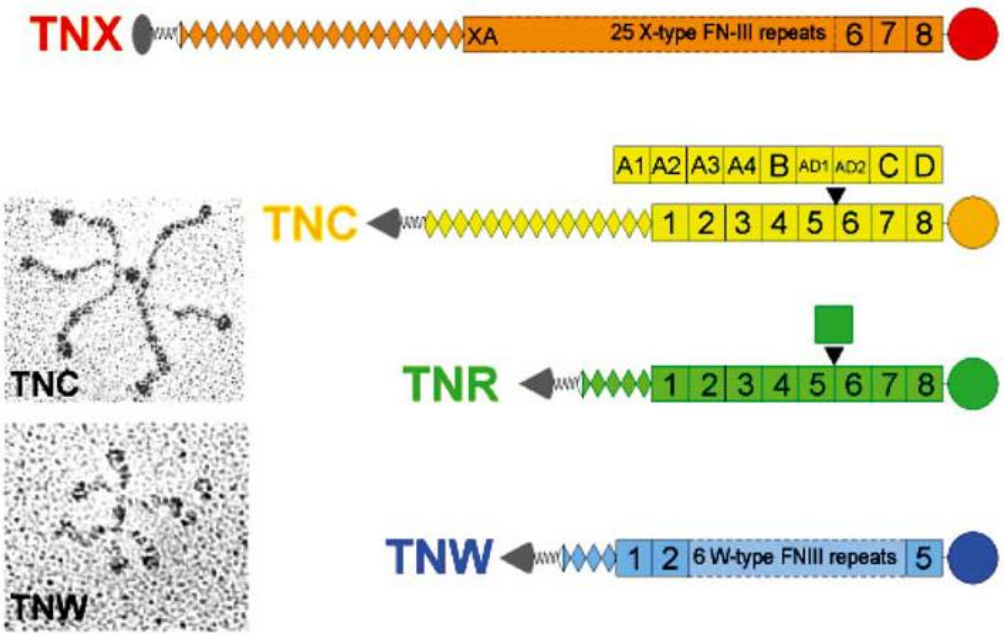

Figure II. 11: Graphical representation of the structure of tenascin proteins
The different tenascin domains were indicated as follows: heptad repeats (wavy line), EGF-like repeats (diamonds), fibronectin type III repeats (squares), and fibrinogen globe (circle). Site of splicing of fibronectin domains is highlighted by a black triangle. In addition, electronmicrographs shown on the left, display the hexameric structure of tenascin-C and tenascin-W (102).

Although in general the tissue distribution of tenascins is mutually exclusive, a significant overlapping expression pattern of tenascin-C and tenascin-W during embryonic development as well as in the adult stage has been observed (Fig.II.12).

In 2004, Scherberich and collaborators observed a co-expression of tenascin-C and tenascin-W in mouse embryos in smooth muscle cells in the stomach, in the periosteum of the ribs, mandible, palate and in teeth while tenascin-C alone was detected in lung, cartilage, liver, and brain at the embryonic stages analyzed. (104).

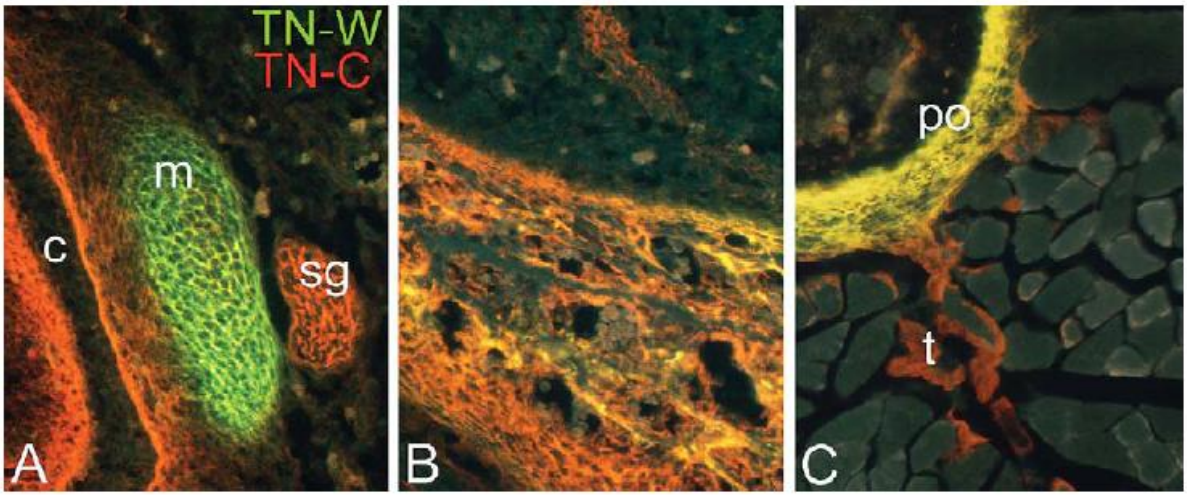

Figure II. 12: Co-expression of Tenascin-C and Tenascin-W in the connective tissue
Tenascin-C and tenascin-W in connective tissues. (A) Anti-tenascin-C (red) stains cartilage in the neurocranium (c) and a salivary gland (sg) in a coronal section through an embryonic mouse head (Day 16.5). Anti-tenascin-W (green) stains the connective tissue of a developing muscle of mastication (m). (B) Both tenascin-C (red) and tenascin-W (green, with co-localization appearing yellow) are found in the bony matrix of the embryonic mandible (Day 16.5). (C) In the adult mouse tenascin-C (red) and tenascin-W (co-localization with tenascin-C is yellow) are found in the periosteum (po) of the ribs. Only tenascin-C is detectable in intercostal tendon (t). (100).

The co-localization of tenascin-C and tenascin-W proteins during the embryonic development as well as in the adult may reflect overlapping functions. This could be related to their species-specific presence of the integrin binding site RGD in either tenascin-C or tenascin-W, but never in both proteins which could lead to a compensatory effect between them (101).

The Co-expression of tenascin-C and tenascin-W proteins has been thoroughly investigated also in pathological conditions, such as cancer. Both proteins were detected in breast and colon cancers. For tumors originating in epithelial organs, it was shown that the cellular sources of tenascin-C and tenascin-W were not the tumor cells themselves but cancer associated fibroblasts within the stromal cell compartment (Fig.II.13) (105, 106). Moreover, a more consistently elevated expression of tenascin-W rather than tenascin-C was detected in the sera of patients with colorectal and breast cancers (107). The presence of tenascin-W in the

bloodstream of cancer patients and its potential link with blood vessel structures was further investigated in 2010 by Martina and co-workers. They were able to observe co-staining of tenascin-W with von Willebrand factor in blood vessels of glioblastoma (108). Staining of tenascin-C was shown as well; however its localization was more uniformly distributed in the tumor tissue (Fig.II.14).

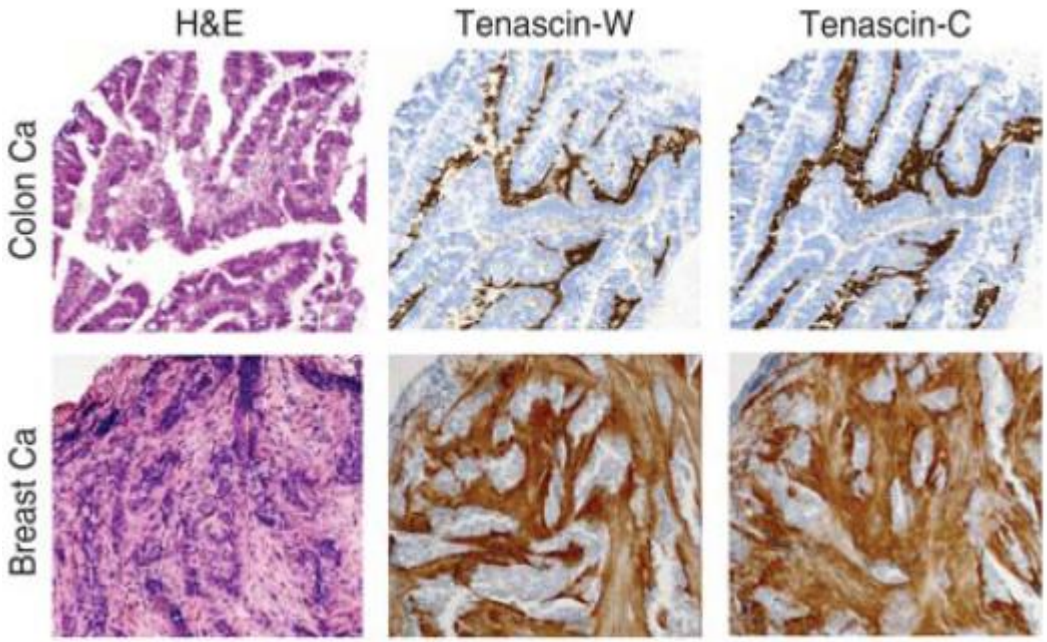

Figure II. 13: Tenascin-C and Tenascin-W in cancer stroma
Immunostaining of tenascin-C and tenascin-W in colon and breast cancers. H&E staining is shown on the left. In the adjacent panels, the brown stain reveals tenascin-C and tenascin-W localization in the tumor stroma (106).

In vitro studies showed an effect of tenascin-C and tenascin-W on endothelial cell behavior. Human umbilical vein endothelial cells (HUVECs) seeded on collagen substrata in presence of tenascin-W or tenascin-C showed an elongated cell shape, with thin protrusions, typical of migratory cells. Additional sprouting of HUVECs was observed when cells were embedded in collagen gels containing tenascin-W or tenascin-C (108).

Tenascin-W and tenascin-C proteins were also detected in many other human solid tumors: pancreas, kidney, lung and melanoma (109). Figure II.14 (B) shows the immunohistochemical analysis of tenascin-C and tenascin-W in lung tumor sections. Tenascin-C also appeared in normal lung tissue highlighting the fact that tenascin-W is a better tumor biomarker since it was exclusively expressed in the stroma of lung tumor sections and not in the healthy parts of the

lungs of the same patients. In addition, a wide number of other tumor types, such as kidney, colon, breast, ovary, prostate, and lung (Fig.II.14) showed a co-localization of tenascin-W with the endothelial marker CD31.

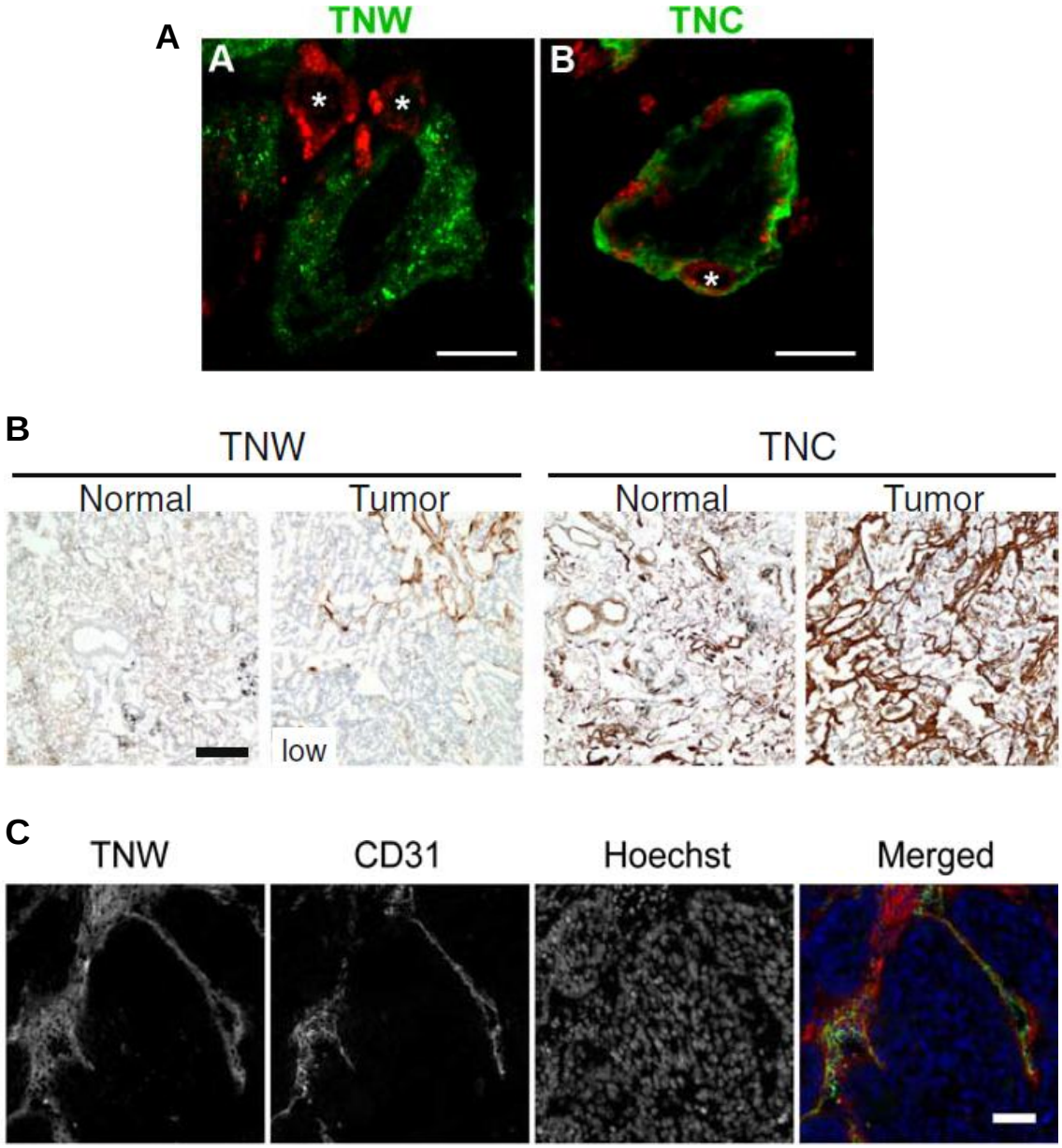

Figure II. 14: Tenascin-W involved in angiogenesis
(A) Immunofluorescence of tenascin-C and tenascin-W in glioblastoma (108); white stars mark desmin-positive pericytes. (B) Panel B shows the immunohistochemical analysis of tenascin-C and tenascin-W in normal and tumor lung sections. (C) Panel C shows cryosections of a lung tumor co-stained for tenascin-W (first panel; red) and for the endothelial marker CD31 (second panel; green) and nuclei (third panel). The merged picture is shown on the right (109).

Despite the existence of a considerable similarity between tenascin-C and tenascin-W, in general, the expression of tenascin proteins is tissue-specific and each of the tenascin family members is distinctly regulated by specific signaling pathways.

II.3.2 Transcriptional regulation of tenascin genes (submitted review article)

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Running title: Tenascin gene regulation

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The authors declare no conflicts of interest.

Abstract

Extracellular matrix proteins of the tenascin family resemble each other in their domain structure, and also share functions in modulating cell adhesion and cellular responses to growth factors. Despite of these common features, the four vertebrate tenascins exhibit vastly different expression patterns. Tenascin-R is specific to the central nervous system. Tenascin- C is an "oncofetal" protein controlled by many stimuli (growth factors, cytokines, mechanical stress), but with restricted occurrence in space and time. In contrast, tenascin-X is a constitutive component of connective tissues, and its level is barely affected by external factors. Finally, the expression of tenascin-W is similar to that of tenascin-C but even more limited. In accordance with their highly regulated expression, the promoters of the tenascin-C and -W genes contain TATA boxes, whereas those of the other two tenascins do not. This article summarizes what is currently known about the complex transcriptional regulation of the four tenascin genes.

1. Introduction: The tenascin gene family

Tenascins are a family of large, oligomeric, multi-domain extracellular matrix proteins (1). Four genes encoding tenascin-C, -R, -X, and -W proteins exist in higher vertebrates, and a single tenascin gene is found in cephalochordates whereas similar genes and proteins do not seem to exist in lower animals (2) (3, 4). Tenascins are characterized by their unique domain structure. Each monomeric unit comprises an N-terminus with heptad repeats flanked by cysteine residues. This N-terminal oligomerization region is followed by EGF-like repeats, and a variable number of fibronectin (FN) type III repeats as a result of alternative mRNA splicing. At the C-terminus, each subunit ends with a large C-terminal fibrinogen related domain (1). Via their N-terminal oligomerization domain, tenascin subunits form disulfide-linked homo-trimers (tenascin-R and -X) or -hexamers ("hexabrachions"; tenascin-C and -W). Rather than representing *bona fide* structural components of the extracellular matrix, three of the four tenascins are "matricellular" proteins (4) involved in modifying the interaction of cells with extracellular matrix and growth factors, and hence regulating cell adhesion, migration, growth and differentiation in a context-dependent manner (5); see other articles in this issue). The exception is tenascin-X, which helps to bridge collagen fibrils (6) and thereby organizes the fibers, as evidenced by human mutations in this gene that cause Ehlers-Danlos syndrome (7).

Except for tenascin-X, which has a widespread distribution like many ECM proteins, the other three tenascins show a very restricted pattern of expression during embryogenesis, tissue remodeling and tumor formation (8-11). Their specific patterns of localization, which are specific for each of the four family members, point to tightly controlled spatial and temporal expression, and are likely to reflect a complex gene regulation. This brief article reviews the expression patterns of the four tenascins in development, regeneration and disease, with a particular focus on the transcriptional regulation of the respective genes by growth factors, cytokines and mechanical stimuli.

2. Tenascin-C: Expression in organogenesis, inflammation and cancer

2.1. Expression patterns of tenascin-C in development and disease

Tenascin-C, the founding member of the protein family, received much attention after its discovery because of its highly specific and restricted expression patterns during vertebrate embryogenesis (12, 13). Whereas many other ECM proteins exhibit a ubiquitous distribution in the mesenchyme with only gradual differences, tenascin-C is often expressed in an all-or-none fashion both in space and time. Specifically, this protein is found in the mesenchyme around primordia of most epithelial organs right at the time of early morphogenesis (13). In addition, tenascin-C is associated primarily with development of the musculo-skeletal system, where it is an early marker of tendon, ligament and bone formation (12). Other prominent sources of the protein are neural crest cells in early embryos (14), neural crest derived Schwann cell precursors in developing peripheral nerves (15, 16) and vascular smooth muscle cells around developing arteries (17).

In the adult, tenascin-C is restricted to few tissues bearing high tensile stress (tendons, ligaments, periosteum, arterial walls) (18, 19), and interestingly to certain stem cell niches (20). However, the protein becomes prominently expressed de novo in practically every tissue upon chemical or mechanical injury, as well as in other pathological processes associated with inflammation and/or mechanical stress, not the least tumor and metastasis formation (1).

These findings indicate that the tenascin-C gene must undergo complex regulation encompassing 1. patterning genes in early morphogenesis, 2. paracrine growth factors regulating the communication between different cell types in organogenesis, 3. inflammatory mediators, and 4. mechanical stress. Published evidence is summarized in the following paragraphs.

2.2. Structure of the tenascin-C (TNC) gene

The human tenascin-C gene (gene ID: 3371) is found on chromosome 9q33; it contains 29 exons of which 9 (each coding for a FN3 domain) can be alternatively spliced (21-23). The transcript starts with a non-coding exon, separated by an intron >20 kb long, and followed by exon 2, which contains the ATG start codon for translation initiation. Tenascin-C mRNA from human fibroblasts and human melanoma cells analyzed by primer extension and S1 nuclease showed a single transcription start site (TSS) localized to the first exon. Sequencing of

approximatively 2300 bp of the tenascin-C gene 5'-flanking region has revealed several potential binding sites for transcription factors (see below) (21). The sequence of 220 bp upstream to the TSS was identified as region with high promoter activity; it contains a classical TATA box at -20 to -26 bp. A putative silencer sequence was localized to the fragment between -220 and -2300 bp (21). Similarly, primer extension analysis of mRNA isolated from brain tissue of mouse embryos showed a single TSS that lays 27 bp downstream of the TATA box (24). Moreover, the 230 bp proximal promoter sequence, which is conserved between species, was found to be highly active in driving reporter gene expression when transfected into both mouse and human fibroblasts (24). The chicken *TNC* (cytotactin) gene features a TATA box at a similar position as the mammalian counterparts (25) A comparison between the human, mouse and chicken *TNC* promoters has been summarized by Jones and Jones (2000) (19).

2.3. Tenascin-C gene regulation by patterning genes

During vertebrate embryogenesis, tenascin-C is often expressed in distinct spatial and temporal patterns associated with morphogenetic events, e.g. during somatogenesis, segmental nerve formation (16), mammary gland (26), tooth (27), kidney (28) and lung (29) development. It was therefore an obvious possibility that the *TNC* gene could be controlled by transcription factors encoded by segmentation and patterning genes. Indeed, some of the early publications on tenascin-C promoters from different species investigated their regulation by homeobox transcription factors. For example, the chick promoter was found to be strongly activated by co-transfection of fibroblasts with even-skipped homeobox 1 (Evx1), and by mutational analysis, an AP-1 element at -275 bp was identified that was essential for this response (30). The same AP-1 site was required for induction of the promoter by serum; Evx1 overexpression potentiated the effect of serum. Thus, Evx1 appears to activate the tenascin-C gene indirectly by synergizing with jun/fos transcription factors, which target the AP-1 site.

On the other hand, a homeobox transcription factor involved in anterior head formation, orthodenticle homolog 2 (Otx2), was shown to bind directly and with high affinity to a target sequence in the human tenascin-C promoter and to suppress its transcriptional activity (31, 32); the Otx2 target sequence is conserved at -550 bp in the mouse (but not chick) gene. Similarly, the POU-homeodomain transcription factor Pou3f2 (also called Brn2 or Oct-7) was demonstrated to interact directly with a reverse octamer sequence (ATGCAAAT) present at -200 bp in the mouse tenascin-C promoter, which is conserved in the human and also the chick gene.

Transfection with Pou3f2 (Brn2) expression vector stimulated transcription from the tenascin-C promoter in a neuroblastoma cell line, but had no effect in glioma cells (33).

In addition, the proximal promoter of the chick, mouse and human tenascin-C gene contains another conserved homeodomain binding sequence (HBS) at -45/-60 bp (19). Transcription factor paired-related homeobox 1 (Prrx1; formerly called Prx1 or Mhox) is often co-expressed with tenascin C, and overexpression of Prrx1 strongly induced a full-length mouse tenascin-C promoter construct in a vascular smooth muscle cell line (34). Later, Prrx1 was demonstrated to transactivate tenascin-C gene transcription in mouse pulmonary endothelial cells through direct interaction with the HBS located within the proximal promoter (35). Increased deposition of tenascin-C expression along the arterial wall in pulmonary vascular lesions of patients with mutated BMP type II receptors was highly associated with the expression of Prx1 (36).Tenascin-C is a prominent early marker for developing tendons, which form according to intricate patterns during trunk and limb morphogenesis (12). The basic helix-loop-helix transcription factor scleraxis (Scx) is also expressed specifically in all embryonic tendons, and was shown to be essential for development and differentiation primarily of long load-bearing tendons (37). Tenascin-C was therefore assumed to be a target gene of this tendon-specific transcription factor. Indeed, myocyte enhancer factor 2C (mef2c) and scleraxis were reported to synergize in inducing tenascin-C expression during tendon development in Xenopus, although the exact mechanism on the gene promoter level was not examined (38). However, scleraxis is not strictly required for tenascin-C production in developing tendons: In scleraxis null embryos, tenascin-C was still found to accumulate in condensing mesenchyme at positions where tendons normally develop (37). Therefore, although scleraxis might boost its expression, the tenascin-C gene appears to be controlled by additional factors that act even upstream of scleraxis during early tendon morphogenesis.

2.4. Tenascin-C gene regulation by growth factors and inflammatory mediators

TGF-β plays an important role as inducer of extracellular matrix protein expression during development and in pathologies (39, 40). Stimulation of tenascin-C synthesis has been detected after treatment of chick embryo fibroblasts with TGF-β1 (41), and this growth factor is also associated with the enrichment of tenascin-C in the stroma of malignant breast tumors (42, 43). SOX4 is a transcription factor overexpressed in many human tumors and tenascin-C was identified as a direct SOX4 target gene (44). Through gene set enrichment analysis it was found

that direct target genes of *TGFβ*–induced *SMAD3* are also enriched in the list of the SOX4 target genes. In the context of malignancies, this would suggest a cooperation between SOX4 and TGF-β1 in controlling tenascin-C expression. A direct role of TGF-β in promoting tenascin-C expression was observed in mammary epithelial cells (HC11) and in mouse embryo fibroblasts (45). For astrocytes, it was shown that the expression of tenascin-C is controlled by the canonical Smad-mediated TGF-β signaling pathway and by fibroblast growth factor (FGF) (46). Series of 5'-deletions of the human tenascin-C promoter reported the presence of a 5'-CAGA-3' motif capable of binding Smad2/Smad3 in the region between -248bp and +75 bp (47). In addition, it was shown that Smads interact with co-factors such as Sp1 or Ets1 and CBP/p300, which possess binding sites located within the same promoter region, in order to achieve proper tenascin-C gene transcription induced by TGF-β in human dermal fibroblasts (47).

In the same cells, platelet-derived growth factor (PDGF) regulates tenascin-C gene expression via PI3K/Akt signaling, which triggers the interaction of transcription factors Sp1 and Ets1/Ets2 in an active complex that recognizes Ets binding sites (EBS) in the promoter (48). Ets binding sites within the tenascin-C promoter were also shown to be the targets of EWS-ETS transcription factor. EWS-ETS is a chimeric gene found in several tumor types such as Ewing sarcoma and peripheral primitive neuroectodermal tumors (PNETs) (49). In a similar setting, oncogenic transformation of primary rat embryonic fibroblasts can be the consequence of the activity of transcription factor c-Jun in cooperation with an activated *ras* gene (50). The transitory expression of tenascin-C induced by c-Jun could facilitate the de-adhesion of fibroblasts from the extracellular matrix, thus promoting their transformation. The c-Jun transcription factor contains a bipartite DNA binding domain which recognizes GCN4/AP1 and NF-κB binding sequences, located in the tenascin-C promoter region from -220 to +79 (50).

Notch is a large transmembrane protein that acts as receptor for cell-bound ligands Delta and Jagged; upon activation, its intracellular domain is cleaved and translocates to the nucleus where it acts as transcriptional regulator through binding to RBPJk/CSL (51). A 102-bp sequence 5' from the transcription start site of the human tenascin-C gene includes an RBPJk/CSL binding sequence (GTGGGAA) responsible for Notch2-mediated transactivation in glioblastomas (52). Conversely, in lung metastases of breast cancer, tenascin-C expression has been implicated in supporting the Notch signaling pathway. Indeed, the enrichment in the metastastic stroma of musashi homolog 1 (MSl1), an adult stem cell marker induced by tenascin-C, acts as positive regulator of Notch (53). In addition to MSl1 expression, high levels

of JAG1 ligands mediate Notch signaling in human breast cancer (Michael Reedijk, et al.) Taking the two findings together, there may be a positive feedback between Notch and tenascin-C expression, which in turn will further amplify the Notch signaling pathway.

A number of cytokines has been shown to induce tenascin-C expression in various cell types; among them are both pro-inflammatory such as IL-1α (54) and IL-1β (55), as well as anti-inflammatory e.g. IL-4 (56) and IL-13 (57). Cytokines signal via various intracellular pathways, most notably Jak/Stat, MAPK, and NF-κB (58, 59). Pro-inflammatory cytokines such as IL-1 and TNF-α promote joint destruction leading to a chronic stage of rheumatoid arthritis (RA) (60), and high levels of these mediators are correlated with the upregulation of tenascin-C protein in RA synovium (61). Transcriptional regulation of the tenascin-C gene by interleukins has not been analyzed in detail yet, but it is reasonable to assume that some of the previously mentioned cis-acting elements (NFkB, AP1, Ets, AP1) in its promoter are involved. Along these lines, a high level of tenascin-C protein expression has also been shown in immune myeloid cells upon exposure to the bacterial cell wall component LPS, which in turn stimulates the pathogen-associated molecular pattern (PAMP) receptor, toll-like receptor-4 (TLR4) (62). TLR4 can also be activated by auto-antigens in the course of RA, and AKT/PI3K and NF-κB pathways triggered by TLR4 were demonstrated to induce transcription of tenascin-C in synovial fibroblasts and myeloid cells in this case (63). In addition to the known element in the proximal promoter, many additional potential binding sites for NF-κB were identified within conserved regions of the 5’ upstream sequence and in the first intron of the tenascin-C gene (63), but their exact role in the response to inflammatory mediators needs to be established.

Interestingly, based on a positive feedback mechanism, tenascin-C can in turn bind to and activate TLR4 through its fibrinogen-like globe (64). Therefore, tenascin-C can act directly as inflammatory stimulus, facilitating also inflammatory cell infiltration. Tenascin-C was also detected in the synovial fluid of osteoarthritic (OA) cartilage and its expression is correlated with proteoglycan loss from the articular cartilage (65). Following the activation of TLR4 by tenascin-C, a series of inflammatory mediators and proteases such as ADAMST4 promote matrix degradation in OA joints. Secreted tenascin-C protein was found in conditioned media of cartilage in culture upon IL-1a treatment (65). Tenascin-C is also involved in hepatic ischemia /reperfusion injury (IRI). In mice deficient for TLR4 and tenascin-C, a downregulation of MMP-9 and cytokines IL-6, IL-1b and CXCL2 was observed. Mice deficient in TLR4 and tenascin-C

were characterized by low liver damage and a significantly higher liver regeneration in this model (66).

Glucocorticoids are potent anti-inflammatory steroid hormones. They function by binding to nuclear receptors that act as transcription factors, but can also negatively regulate gene expression by inhibiting the activity of other factors like AP1 and NFkB (67). Glucocorticoids have also been described as important hormones involved in myelopoiesis, and they can directly act at the progenitor cell level or by modifying the expression of ECM components. Down-regulation of tenascin-C expression by glucocorticoids was shown in bone marrow stromal cells (68). These authors suggested that the different tenascin-C distribution between bone marrow of newborn and adult mice controlled by glucocorticoids might in part influence the hematopoiesis process. A putative binding site for glucocorticoid receptors is located at position -985 of the chicken tenascin-C promoter sequence (69), but its function in the hormone response has not been explored.

A further repressor motif was mapped to position -467 to 460 of the human tenascin-C promoter that was demonstrated to bind GATA6, a zinc finger transcription factor known to regulate the synthetic phenotype of vascular smooth muscle cells. Exogenous expression of GATA6 in dermal fibroblasts negatively modulated the level of tenascin-C protein, and inhibited its induction by IL-4 and TGF-β (70).

2.5. tenascin-C gene regulation by mechanical stress

Whereas tenascin-C is expressed transiently in many developing organs, it persists in the adult mainly in a few structures bearing high tensile stress, such as tendons, ligaments, and the smooth muscle walls of arteries (18, 19). It was therefore speculated early on that its gene might be regulated by mechanical forces. Indeed, tenascin-C expression was found to be induced in vivo e.g. by hypertension in the arterial walls of rats (71), or upon supra-physiological loading in skeletal muscle connective tissue of chick (72), rat (73) and human (74). Transduction of external mechanical stimuli requires integrins as bridges between ECM and the cytoskeleton (75). Depending on the precise nature of the stimulus, various integrin-dependent signaling pathways can then be triggered, such as Ca influx, activation of Erk1/2, NFkB, and RhoA/ROCK (76). An extensively studied mechanotransduction pathway concerns the rapid activation of the tenascin-C gene by cyclic strain (10%, 0.3 Hz for 1-6 h) in fibroblasts attached to elastic substrates, which depends on Rho/Rock signaling (77). Pericellular fibronectin, integrin α5β1

(78) and integrin-linked kinase (ILK) (79) were shown to be required for RhoA activation and tenascin-C induction in response to cyclic stretch in mouse fibroblasts. Strain-mediated Rho A activation triggered an increase in cellular actin assembly (80), which in turn lead to translocation of megakaryoblastic leukemia-1 (MKL1; also called MAL or MRTF-A) from the cytoplasm to the nucleus (79), where it is known to act as a trancriptional regulator (81). Accordingly, tenascin-C induction by cyclic strain was abolished by MKL1 knockdown in NIH3T3 fibroblasts (82). MKL1 regulates the transcriptional activity of serum response factor (SRF) (83). Indeed, the mouse tenascin-C promoter contains a serum response element (SRE; CArG-box) located 1414bp upstream from the transcription start site, which is in part involved in its activation. However, tenascin-C induction by cyclic strain was found to be independent of SRF but strictly dependent on the interaction of the SAP domain of MKL1, a putative DNA-binding domain, with the proximal tenascin-C promoter (82).

Among vascular diseases, hypertension is correlated with elevated tenascin-C abundance around vessels, concomitantly with an increase in wall stress. In human arterial smooth muscle cells, cyclic strain (13%, 0.5 Hz for 24 h) was found to control the expression and activity of nuclear factor of activated T cells 5 (NFAT5) in a JNK-dependent manner (84). Once translocated into the nucleus in response to strain, NFAT5 was able to induce tenascin-C gene expression. Five NFAT consensus sequences were found in the first 3512 bp of the human tenascin-C promoter sequence upstream of the transcription start site, and for the first of them (at -820 bp), cyclic strain-induced binding was demonstrated by ChIP analysis (84). Note that the Rho/MKL1 pathway described above directly activates the tenascin-C gene within 1-3 hours in response to strain, whereas the JNK/NFAT5 pathway requires prior synthesis of a transcription factor and takes 24 h for tenascin-C induction.

Yet a different mechanotransduction pathway was found to be responsible for tenascin-C induction by cyclic strain in rat cardiomyocytes. It is noteworthy that a similar strain amplitude (9-14%) but a higher frequency (1 Hz) was used (85). In this case, the response depended on release of reactive oxygen species and activation of NFkB. A consensus sequence for this transcription factor at -210 bp was required for mechanical activation of the tenascin-C promoter, and shown to bind the p50 subunit of NFkB in response to strain (85). Moreover, tenascin-C expression is not only regulated by dynamic (cyclic) strain, but also by static tensile stress. For example, tenascin-C expression by fibroblasts is high when they are embedded in an attached (stressed) collagen matrix, but diminished when the matrix is released from its

anchors (relaxed). A conserved DNA motif (GAGACC) at -550 bp in the the tenascin-C promoter was required for this response (69). This motif is present in the control regions of other mechanoresponsive genes where it is recognized by NFkB (86), but the factors binding to it in the tenascin-C promoter have not been identified. In any case, these examples show that depending on the cell type and on the exact mode and doses of mechanical stress, the tenascin-C gene appears to be regulated via distinct mechanotransduction pathways.

In chick embryo fibroblasts, PDGF and TGFβ growth factors were shown to act in an additive manner with tensile strain in promoting tenascin-C mRNA expression (77), and thus an increase in these factors might indirectly stimulate tenascin-C production in response to mechanical load. For example, tenascin-C accumulates in angiotensin II- induced perivascular fibrotic lesions in hypertensive mice. Angiotensin II was shown to trigger aldosterone-induced inflammation, which indirectly stimulated tenascin-C expression by upregulating PDGF-A/B, PDGF receptor a, and TGFβ1 in this model (87).

2.6. Posttranscriptional regulation of tenascin-C gene by microRNAs

Although this article focuses on transcriptional control of tenascin genes, it is important to note that other regulatory mechanisms are important as well. The role of microRNAs as regulators of post-transcriptional gene silencing is well documented (88, 89), and recent studies have shown that the repression of certain microRNAs corresponds to a more pronounced tumorigenesis (90). For example, downregulation of SOX4 and tenascin-C is controlled by miR-335, and loss of this microRNA in breast cancer was shown to induce metastasis in part by increased tenascin-C levels (91). Other findings show how tenascin-C promotes oncosphere formation by a metastasis-initiating breast cancer cell population for lung colonization, and in this context, GATA3 and miR-335 were downregulated (53). Conversely, the ectopic expression of some microRNAs turns out in a decreased metastatic potential. For instance, the rescued expression of miR-126 suppresses lung and bone metastasis by human breast cancer cells through targeting tenascin-C. Bioinformatic analysis has yielded target sequences for microRNAs binding at the 3’ UTRs of tenascin-C gene, explaining how the interaction can occur (91).

3. Tenascin-R: An ECM protein mainly restricted to the central nervous system

*3.1. Expression patterns of tenascin-R (*TNR*) in CNS development, injury and cancer*

Tenascin-R, originally designated as janusin in rodents and restrictin in chicken, is almost exclusively located to the central nervous system (92-95), but transiently appears also in Schwann cells during peripheral nerve development (96). Previous work has shown two tenascin-R molecular isoforms (splice variants) of 160 and 180 kDa expressed in the CNS by oligodendrocytes and a few neuronal cell types, but not by astrocytes or fibroblasts (97). In the developing human cortex, the spatiotemporal distribution of tenascin-R parallels neuronal migration (98). *In vitro* experiments have demonstrated that tenascin-R promotes adhesion and differentiation of oligodendrocytes and astrocytes by binding to sulfatides on cell surfaces (95, 99). Conversely, tenascin-R can inhibit neurite outgrowth either by interacting with adhesion molecule contactin 1 (F3/F11) or by interfering with integrin-dependent adhesion to fibronectin (reviewed in (100).

In a pathological context, activation of microglial cells after facial nerve axotomy in rats has been shown to downregulate tenascin-R protein expression with the subsequent loss of its anti-adhesive properties (101). On the other hand, tenascin-R is up-regulated in the injured visual pathway of the lizard that has the capacity to regenerate (102). In brain cancer, tenascin-R was reported to be overexpressed in pilocytic astrocytoma, oligodendroglioma and gangliоglioma, but not glioblastoma (103).

3.2. Structure of the tenascin-R (TNR) gene

The human *TNR* gene (gene ID: 7143) is located on chromosome 1q24 and has 23 exons. The human, rat and mouse genes show homologous sequences of exons 1, 2 and 3 of which the first two are noncoding and the latter contains the ATG start codon (104, 105). Exon 1 and 167 bp upstream of this exon were sufficient for full and cell type-specific activity of the tenascin-R promoter in cell culture (Leprini, Gherzi et al. 1998; Gherzi, Leprini et al. 1998; Putthoff, Akyuz et al. 2003). The TATA-less tenascin-R promoter displays canonical binding sites for potential regulators such as GATA-1, GATA-2, CREB, AP1 and p53 transcription factors as well as glucocorticoid receptors (105, 106). However, their functional relevance has not been addressed.

3.3. Tenascin-R gene regulation by growth factors

In mice, oligodendrocyte precursor cells synthesize most tenascin-R, whereas expression decreases with differentiation. In more mature oligodendrocytes, expression of TNR was stimulated by coculture with astrocytes or neurons, and was also induced by adding platelet-derived growth factor (PDGF) but not basic fibroblast growth factor (107). Rat pheochromocytoma cells (PC12) express high levels of *TNR* mRNA after nerve growth factor (NGF) treatment (92, 107). In contrast, oligodendrocytes treated with conditioned medium from activated microglia show a reduced *TNR* mRNA expression due to the release of injury factors such as TNF-α (101). Unfortunately, there are no studies yet how these growth factors and cytokines regulate the *TNR* gene on the promoter level.

4. Tenascin-X: A regulatory component of collagen fibers

4.1. Expression patterns of tenascin-X in collagen-rich tissues

Tenascin-X was discovered as a gene of the MHCIII locus overlapping with the *CYP21A2* gene, which encodes steroid 21-hydroxylase (7, 108). *CYP21A2* mutations cause congenital adrenal hyperplasia, but a fraction of cases with deletions in this genomic region is also deficient for tenascin-X; these patients suffer in addition from hyperextensible skin and joint laxity typical of Ehlers Danlos syndrome (109). Deletion of the tenascin-X (*Tnxb*) gene in mice was subsequently shown to phenocopy the connective tissue defects observed in affected human patients (110). Thus, in apparent contrast to the other tenascins, the tenascin-X protein has a clear structural role in connective tissue integrity by binding (indirectly) to collagen fibers and regulating collagen deposition in vivo (6, 111, 112). During embryonic development, *TNXB* mRNA is especially prominent in the epicardium, skeletal muscle connective tissue, and tendon primordia (113). In the adult, *TNXB* mRNA becomes widely and constitutively expressed in most connective tissues, but is present at higher levels in tendons, ligaments, and perineural sheaths (Geffrotin 1995). Despite of considerable overlap on the tissue level especially in the embryo, on a smaller scale the distribution of tenascin-X mRNA and protein was found to be distinct and often reciprocal to that of tenascin-C (9). Interestingly, also in the context of malignancy tenascin-X and tenascin-C appear to be regulated in opposite ways. For example, tenascin-X expression is prominent in normal skin but strongly suppressed in cutaneous melanoma, where tenascin-C is highly up-regulated (114). In contrast to tenascin-C, tenascin-X is not induced in breast and ovarian carcinomas, but has been reported to be a marker for malignant

mesothelioma (115). Also strikingly different from tenascin-C, there are no reports indicating an induction of tenascin-X expression in inflammation or wound healing.

4.2. Structure of the tenascin-X (TNXB) gene

In humans as well as rodents, there are two tenascin-X genes of which *TNXA* is a pseudogene. In the human genome *TNXB* (gene ID: 7148), the gene coding for TNX protein, is located on chromosome 6p21.3 in the neighborhood of *TNXA*. The *TNXB* gene counts 44 exons, and its structure appears to be unique among all tenascins since transcription can take place from three different promoters which lack TATA or CAAT boxes. In human fetal adrenal gland and muscle, the major transcript starts with the first untranslated exon located more than 10 kb upstream from the second exon containing the translation start codon (116). In contrast, in human NCI-H295 adrenocortical carcinoma cells *TNXB* expression is controlled by two additional promoters. *TNXB* transcripts can be initiated either from the same promoter as for an upstream gene, the Creb-related protein (CREB-RP; gene name *ATF6B*), or from a region at the 3' end of *ATF6B*. Both events produce transcripts with alternative first untranslated exons that are differentially spliced (116). Further analysis of 375 bp upstream of the noncoding first exon of the major transcript identified several putative binding sites for Sp1/Sp3 transcription factors, of which two were proven to be functional and required for driving *TNXB* expression in fibroblasts (117). Similarly, an Sp1 site 140 bp upstream of the major transcription start site of the mouse tenascin-X promoter was shown to play a critical role in expression of this gene (118).

4.3. Tenascin-X gene regulation by growth factors

Unlike for the other proteins belonging to the tenascin family, there are so far no reports indicating that tenascin-X is regulated by growth factors or cytokines. Thus, the signaling pathways that act on the Sp1/Sp3 sites described above in the tenascin-X promoter are at present unknown. Like tenascin-C and tenascin-W, however, tenascin-X is subjected to negative regulation by glucocorticoids (119), but again the mechanism has not been elucidated yet on the gene promoter level.

5. Tenascin-W: Expression in osteogenesis and tumorigenesis

*5.1. Expression patterns of tenascin-W (*TNW*) in bone formation and cancer*

Tenascin-C and tenascin-W proteins show a partially overlapping expression pattern in the developing and adult skeleton (120). Most of the research based on the regulation of TNW expression in a physiological context indicates its significant role in osteogenesis (10). In the adult organisms, TNW is predominantly expressed in cancer stroma of most solid tumors (Scherberich, Tucker et al. 2005; Degen, Brellier et al. 2007; Degen, Brellier et al. 2008; Martina, Chiquet-Ehrismann et al. 2010) and represents an even more specific tumor marker than tenascin-C (10, 11, 45, 121, 122).

5.2. Structure of the tenascin-W (TNN) gene

Tenascin-W was first discovered and cloned from zebrafish (123). Unfortunately, its mouse ortholog (124) was subsequently called tenascin-N (*Tnn*) (125), and this is now the official gene name in the NCBI data bank. The complete characterization of the human tenascin-W gene (TNN; gene ID: 63923) was carried out in 2007 (121). The human TN-W gene consists of a total of 19 exons spanning 80 kb of genomic DNA. The transcript starts with a non-coding exon. The transcription start site (TSS) is 79bp upstream the exon2, which contains the start codon ATG (126) The region comprised within -957 and -79bp from the transcription start site as well as a conserved region within the first intron are involved in transcription regulation, and specifically, the minimal basal promoter sequence is contained in the last 79bp fragment (126).

5.3. TNN gene regulation by growth factors

BMP2 is able to induce tenascin-W expression in periosteum during the endochondral bone formation in mice (127). An in vitro system of Kusa-A1 cells shows an increase of *TNN* transcript starting with the differentiation in osteoblasts (128). Mouse myoblasts (C2C12) differentiate into osteoblastic cells upon treatment with bone morphogenetic protein 2 (BMP2) and concomitantly they express tenascin-W (124). The induction of tenascin-W synthesis by BMP2 in MEF as well as in HC11 cells occurs via non-canonical p38 ^{MAPK} signaling pathway (45). Tenascin-W was also strongly induced by Bmp7 in embryonic cranial fibroblasts in vitro (129). Among other regulators of bone formation, Wnt5a signaling is indirectly involved in promoting TNW expression through p38 activation of an unknown TNW inducer, thus controlling bone density (130).

TGFβ1 and its receptor ALK5 were identified as important factors for human TNW gene transcription in human bone marrow stem cells (BMSCs) (126). Computational analysis shows the presence of a SMAD4 nuclear transcription factor binding site at -61bp from the TSS, in proximity of a TATA box sequence and site-directed mutagenesis of the SMAD4 binding site strongly impaired the SEAP reporter gene expression driven by the minimal basal promoter (126). Finally, similar to tenascin-C and tenascin-X, the expression of tenascin-W is also inhibited by glucocorticoids. In this case a negative glucocorticoid response element was identified in a conserved region of the first intron, displaying a glucocorticoid-dependent repressive action on the proximal tenascin-W promoter (126).

6. Conclusions and outlook

The four members of the vertebrate tenascin family are quite similar not only in their overall domain organization, but as typical "matricellular" proteins also appear to fulfill similar functions: There is increasing evidence that they all modulate cell adhesion and cellular responses to growth factors and cytokines in a context-dependent manner (1). In view of these structural and functional similarities, it is surprising that the four tenascins exhibit vastly different expression pattern in space and time. Tenascin-R is almost exclusively found in the central nervous system and its expression level is affected by just a few known growth factors. In contrast, tenascin-C is an "oncofetal" or "stress" protein that is controlled by many stimuli, can appear in almost any tissue and cell type in the embryo and the adult, however just at certain times and in specific locations. Tenascin-X is a constitutive component of most connective tissues and its level is barely influenced by growth factors, whereas the expression of tenascin-W is again more similar to that of tenascin-C, although it is even more restricted to developing/remodeling bone, certain stem cell niches (20), and to a subset of tumors. These observations point to very distinct mechanisms of regulation for the various family members. Fitting with a highly regulated versus a more constitutive expression, respectively, the gene promoters of tenascin-C and TNW have classical TATA boxes ca. 20-40 bp upstream of their transcription start sites, whereas the promoters of TNR and TNX are TATA-less. Tenascin-C and *TNR* genes have a first untranslated exon separated from the second ATG-containing exon by a very large intron, which is likely to be involved in gene regulation. *TNR* has two untranslated exons and the ATG translation start site is found in the third, whereas *TNX* even possesses three alternative promoters and non-coding first exons that are subjected to alternative splicing (see above).

Because the tenascin-C gene was the first of the family to be characterized, most is known about its regulation, although it turns out to be overwhelmingly complex. The responsiveness of the tenascin-C gene to segmentation genes, growth factors/cytokines and mechanical stress appears to be very similar in different vertebrate species, which is reflected in the high sequence similarity in parts of the promoter regions. Nevertheless, although many of the same cis-acting regulatory elements have been identified in the chick, mouse and human tenascin-C promoter, there appear to be differences in their arrangement and activity (19). Although TNW is quite distantly related to tenascin-C within this protein family, its expression pattern and the regulation of its gene appears to be most similar to that of tenascin-C, especially also in stem cell niches (131) and in cancer. Future research is likely to reveal more about similarities and differences in the control of these two genes e.g. by TGF-βs versus BMPs, or by various cytokines. Conversely, TNR is the closest family member of tenascin-C on the protein level, but it is regulated completely differently. It is remarkable that only about 200 bp of the proximal promoter and sequences in the first exon of the TNR gene are necessary and sufficient for its expression exclusively in neuronal cells (105). Thus, this gene appears to represent a relatively simple case of tissue-specific regulation, and it will be interesting to work out the exact mechanism. As for TNX, it exhibits a constitutive expression more like TNR, but in a reciprocal fashion since it is found in most tissues except the CNS. Nothing is known yet about the mechanism for tissue-specific expression of this gene, and the lack of regulation by growth factors and the relative scarcity of putative cis-acting elements in its promoter are noteworthy (117). For more meaningful comparisons between the genes of this family, it would be important learn more about the regulation of TNR, TNX and TNW genes in the future. In the case of tenascin-C, systems biology and computational approaches will probably be required to fully understand how a dozen or more signaling pathways converge to control its very complex gene promoter.

Why is it relevant to study the regulation of tenascins in even more detail? TNR and TNX, because of their largely constitutive expression, might perhaps be less interesting in this respect. Of course, TNX will remain in focus because of its important function in tissue integrity and its association with human disease, and TNR might be further investigated as a prime example for highly tissue and cell type-specific gene regulation. In case of the highly regulated tenascin-C and TNW, however, more and more evidence suggests that these two proteins are important regulators of cell division, migration and differentiation in adult stem cell niches (20) as well as in cancer (132). Moreover, because of their very localized and high expression in the

extracellular matrix, both tenascin-C and TNR are very well suited for targeting antibodies and drugs to certain types of tumors (133). For this therapeutic approach to work effectively, it is important to know how tenascin-C or TNW gene expression is affected e.g. by cytotoxic drugs in combined therapy, and what signaling pathways are involved. From a basic research point of view, the tenascin family provides an intriguing example for a vertebrate protein family of paralogues with similar structure and function, but with distinct expression patterns in space and time that are generated by different mechanisms of regulation of the respective genes.

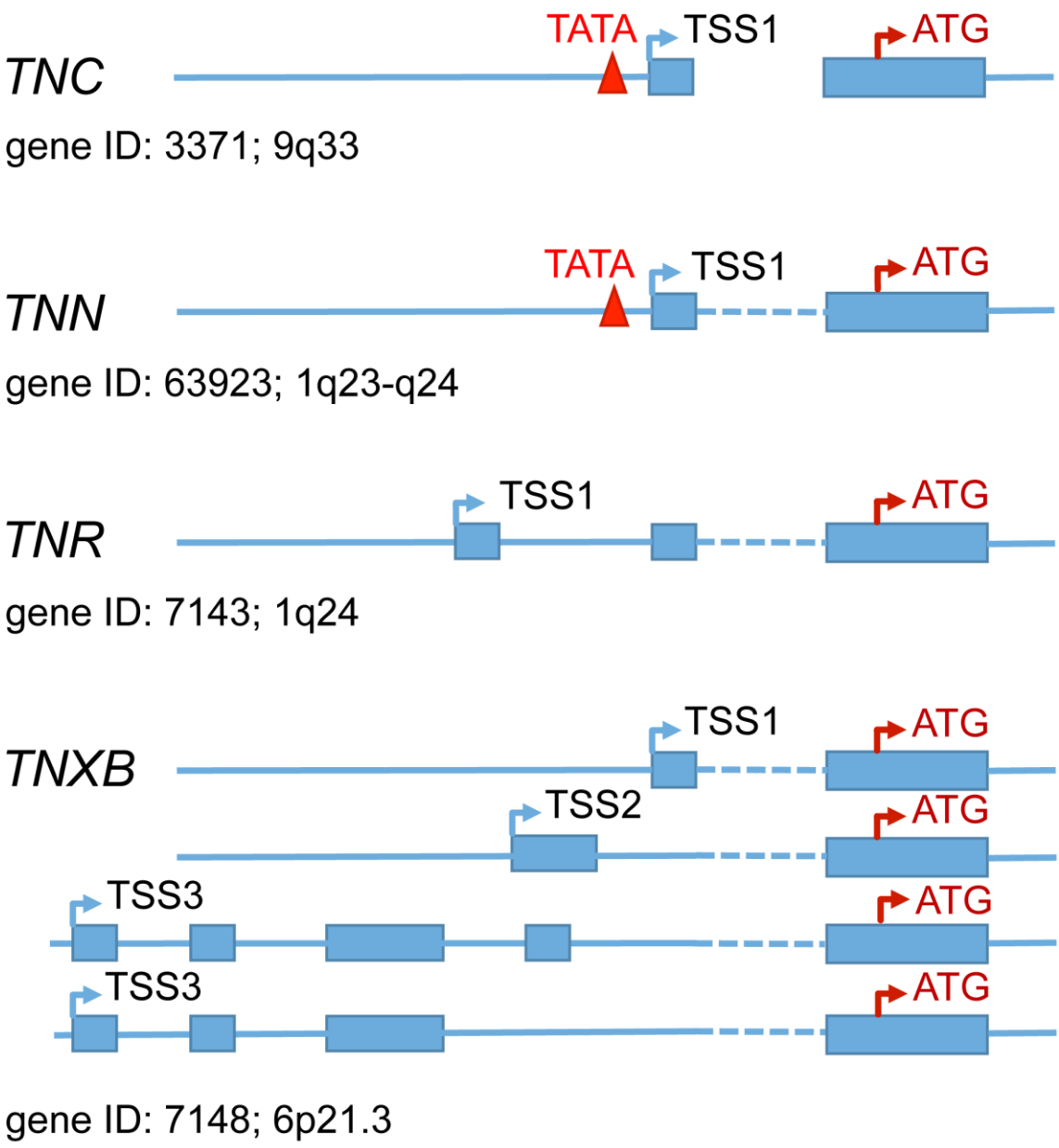

Figure Legend

Figure 1: Schematic representation of the transcription start sites and the first exons of the four human tenascin genes. *TNC*, tenascin-C, *TNN*, tenascin-W; *TNR*, tenascin-R; *TNXB*; tenascin-X. Note that the very large first introns are not drawn to scale (indicated by dotted lines); their length differs in the four genes. Note also that the *TNC* and *TNN* genes possess TATA boxes whereas the *TNR* and the *TNXB* genes do not. In contrast to *TNC*, *TNN* and *TNR* that have a single transcription start site (TSS1), the *TNXB* gene has three (TSS1, TSS2, TSS3), and it features several noncoding exons that are differentially spliced.

Table 1

	Growth factors/ Cytokines Other external signals	Signaling pathways / Transcription factors	
		Positive Regulators	Negative Regulators
TNC	Tissue Patterning	Evx1 [30] Otx2 [31,32] Pou3f2 [33] Prrx1 [34, 35, 36] Mef2c/Scleraxis [37, 38]	GATA3 [54] miR-126 [54] miR-335 [54] Glucocorticoids [69] GATA6 [71]
	TGFβ [41-45, 47] TGFβ/FGF [46]	SMADs/SP1/Ets1/CBP-p300 [47]	
	PDGF [48] PDGF /TGFβ [78, 88]	EWS-ETS [49] c-Jun/RAS/NFkB [50]	
		Notch2-RBPJk/CSL [53]	
	IL-α [55] IL-1β [56] IL-4 [57] IL-13 [58] IL-1/TNF-α [62] IL-1α [66] IL-6, IL-1b, CXCL2 [67]	TLR4/AKT/PI3K/NFkB [64] NFkB [86, 87]	
	Mechanical stress/Integrins	RhoA/Rock/MKL1 [77, 83] JNK/NFAT5 [85]	
TNR	PDFG [1] NGF [93, 108] TNF-α [102]	GATA 1/2, CREB, AP1, p53 [106, 107]	Glucocorticoids [106]
TNXB	N.D.	Sp1/Sp3 [118, 119]	Glucocorticoids [120]
TNN	TNF-α [45] TGFβ [127] BMP2 [45, 125, 128]	SMAD4/TGFβ1 [127]	Glucocorticoids [127]

Table 1: External signals, signaling pathways and transcription factors involved in the regulation of the four tenascin genes. *TNC*, tenascin-C; *TNR*, tenascin-R, *TNXB*, tenascin-X; *TNN*, tenascin-W. Numbers in superscript correspond to references according to the list. N.D., not determined.

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II.4 Aim of the work

It is well known that two of the four tenascin family members are significantly regulated by the tissue microenvironment: tenascin-C and tenascin-W (110). While a lot is known about tenascin-C, its regulation and gene structure, no information about these aspects of tenascin-W have been reported. In this work, the primary goals were to investigate the gene structure of tenascin-W and to explore its role and regulation in the bone metastatic niche of breast cancer. As catalyst for the exploration of the so far undefined tenascin-W gene structure and regulation, we performed computational as well as experimental analysis of the tenascin-W gene locus to identify the transcription start site(s), the promoter and its transcription factor binding sites as well as potential regulatory regions in the first intron. Since tenascin-W is known to be expressed in breast cancer stroma (105) and 80% of women with advanced breast cancer have bone metastases (111) we decided to investigate a potential role of tenascin-W in the metastatic spreading of breast cancer cells to bone.

III. Results

III.1 Submitted Manuscripts

III.1.1 Transcriptional regulation of tenascin-W by TGF-beta signaling in the bone metastatic niche of breast cancer cells

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Short title: Tenascin-W in bone metastases of breast cancer

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Abbreviations used: α-MEM: Minimal Essential Medium Alpha; BMSCs: bone marrow derived stromal cells; BrdU: 5-bromo-2-deoxyuridine; DCC: dextran-coated charcoal; ECM: extracellular matrix negative glucocorticoid response element; nGRE: L2G: Luc-2eGFP; qRT-PCR: quantitative real time PCR; SBE: Smad-binding element; SEAP: secreted embryonic alkaline phosphatase; TNC: tenascin-C; TNW: tenascin-W; TSS: transcription start site.

Article Category: Cancer Cell Biology

What`s new?
This is the first report describing a function for tenascin-W in metastasis as well as the first report on tenascin-W gene structure and transcriptional regulation by TGF-beta and glucocorticoids in human bone marrow stromal cells. Our results support a role for TNW as a niche component for breast cancer metastasis to bone by supporting cell migration and cell proliferation of the cancer cells.

Abstract

Tenascin-W is a matricellular protein with a dynamically changing expression pattern in development and disease. In adults, tenascin-W is mostly restricted to stem cell niches, and is also expressed in the stroma of solid cancers. Here we analyzed tenascin-W expression in the bone microenvironment of breast cancer metastasis. Osteoblasts were isolated from tumor-free or tumor-bearing bones of mice injected with MDA-MB231-1833 breast cancer cells. We found a 4-fold upregulation of tenascin-W in the osteoblast population of tumor-bearing mice compared to healthy mice, indicating that tenascin-W is supplied by the bone metastatic niche. Transwell and co-culture studies showed that human bone marrow stromal cells express tenascin-W protein after exposure to factors secreted by MDA-MB231-1833 breast cancer cells. To study tenascin-W gene regulation, we identified and analyzed the tenascin-W promoter as well as three evolutionary conserved regions in the first intron. 5'RACE analysis of mRNA from human breast cancer, glioblastoma, and bone tissue showed a single tenascin-W transcript with a transcription start site at a non-coding first exon followed by exon 2 containing the ATG translation start. Site-directed mutagenesis of a SMAD4 binding element in proximity of the TATA box strongly impaired promoter activity. TGFβ1 induces tenascin-W expression in human bone marrow stromal cells through activation of the TGFβ1 receptor ALK5, while glucocorticoids are inhibitory. Our experiments show that tenascin-W acts as a niche component for breast cancer metastasis to bone by supporting cell migration and cell proliferation of the cancer cells.

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Introduction

Despite great progress in the treatment of breast cancer, metastatic disease is not curable and the treatment options remain palliative. Bone is the most frequent site of metastatic lesions and occurs in 80% of women with advanced breast cancer.[1,2] Therefore, it is important to investigate the mechanism for this osteotropism as well as the interactions of the cancer cells with the bone microenvironment with the ultimate aim being to define new treatment options. The microenvironment, including the extracellular matrix (ECM) surrounding primary tumors as well as metastases, has been found to be an important factor determining tumor cell behavior.[3-6] Tenascin-C (TNC) and tenascin-W (TNW) are two ECM proteins that are highly expressed in the stroma of most solid tumors [7,8] and a crucial role for TNC in breast cancer metastasis to the lung has been demonstrated.[9,10] Currently there is no information on TNW expression or a potential role in breast cancer metastasis, the goal of the work described here.

Prominent expression of TNW has been reported in developing bone where it was shown to be particularly abundant in the stem cell niche of the cambium, the location of osteoblast progenitors [11]. Therefore, we decided to investigate TNW expression in the bone environment in the MDA-MB231 xenograft model of breast cancer metastasis.[12] We used MDA-MB231-1833 cells which have bone tropism following intracardial injection.[12] We found that MDA-MB231-1833 tumors induced TNW in situ in the bone stroma. Moreover, in a coculture model of MDA-MB231-1833 cells with human bone marrow derived stromal cells (BMSCs), we also observed increased levels of TNW. To provide mechanistic insight to this observation, we investigated the signaling pathways inducing TNW in BMSCs and characterized the gene structure of the human TNW gene. We identified a crucial effect of TGF-beta signaling in the regulation of TNW expression in human BMSCs which in turn will provide a congenial microenvironment for tumor cell growth.

Material and Methods

Bone metastasis model

The breast cancer cell line MDA-MB231-SCP1833 was kindly provided by Prof. J. Massagué (Memorial Sloan Kettering Cancer Center, New York). These cells were transduced with a lentiviral vector encoding Luc-2eGFP genes (L2G) as described in.[13] MDA-MB231-SCP1833 L2G cells were harvested from subconfluent cell culture plates, washed in PBS and injected into the left ventricle ($0.5x10^6$ in 100ul PBS) of 8-week old female NOD SCID mice. Successful injections were verified by the pumping of arterial blood into the syringe and imaging with a bioluminescence imager (Berthold technologies, NightOwl). Bone marrow metastases were monitored by *in vivo* imaging over 20 days after which long bones were excised for cell sorting or immunostaining.

Bone marrow cell suspensions from tumor free or tumor bearing mice (n=6-10 samples) were obtained by grinding the bone with mortar *and pestle* and digestion of the bone powder for 1h at 37ºC with 1mg/ml collagenase (Roche), 1mg/ml dispase (Roche) and 50KU/ml DNAse (Sigma) into a single cell suspension. Stromal cells and hematopoietic fractions were enriched via a discontinuous percoll density gradient separation using 1.065g/L and 1.115g/L (GE Healthcare). Remaining red blood cells were lysed (140mM NH_4Cl and 17mM Tris-base, pH7.4) and cells were stained and sorted directly into RNA extraction buffer (Qiagen) using a MoFlo cell sorter (Beckman Coulter). The osteoblast population was defined as $GFP^-TR119^-CD45^-SCA1^-CD51^+$ cells. RNA was extracted with *Pico Pure RNA Isolation Kit (Arcturus cat.KIT0204) and cDNA prepared with the* Ovation Pico Kit (NuGen cat.3302) following standard procedures and used for Real-Time Quantitative PCR (qRT-PCR, see below).

Cell Culture

Fibrosarcoma HT1080 cells (CCL-121, ATCC) and MDA-MB231-SCP1833 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS). Human bone marrow stromal cells (BMSCs) immortalized with the hTERT/GFP system were kindly provided by Dr. A. Scherberich (University Hospital of Basel).[14] BMSCs were cultured in Eagle's Minimal Essential Medium Alpha (α-MEM) with 2mM L-glutamine and 10% FBS. To

strip glucocorticoids from serum, 2.5g of dextran-coated charcoal (DCC; Sigma-Aldrich) was added to 125ml of serum and mixed gently overnight at 4°C. DCC was removed by centrifugation followed by sterile filtration.

For co-culture assays $3x10^3$ BMSCs and $6x10^3$ MDA-MB231-SCP1833 cells were seeded per $1cm^2$ into poly-L-lysine coated 8-well chamber slides (BD Falcon). In parallel each cell lines was cultured individually at a density of $3x10^3$ cells/cm^2. For transwell co-culture assays, cells were cultured in wells containing inserts separated by a polycarbonate membrane with 0.4 µm pores (Costar). MDA-MB231-SCP1833 or BMSCs cells were plated in the upper chamber ($5x10^3$ cells in 0.5ml medium) and BMSCs or MDA-MB231-SCP1833 ($5x10^4$ cells in 1.5ml) were cultured on 10mm round glass coverslips coated with fibronectin (5ug/ml, for 1h) placed in the bottom chamber. Cells were cultured in α-MEM/10% FBS and maintained for 7 days with medium changes every 2 days.

Immunostaining

Long bones were fixed for 48h in 10% formalin, decalcified in 0.5M EDTA (pH7.5) for 3 days at 4°C, dehydrated in 30% sucrose in PBS overnight and embedded in OCT. Antigen retrieval of 8µm sections was performed in citrate buffer 10mM pH6, 0.5% Tween20 (2 hours) followed by blocking with 3%BSA/0.2%Triton in PBS. Cultured cells were fixed with 4% formaldehyde in PBS for 15min at RT, permeabilized in cold 100% methanol for 2min at -20°, washed twice with PBS and blocked with 0.01% Tween/1% BSA in PBS. Slides with bone sections or cells were stained with rabbit-anti-mTNW [11] and the mouse monoclonal anti-hTNW56O [15] followed by secondary antibodies Alexa Fluor 568 (Invitrogen). Slides were mounted with ProLong Gold containing DAPI (Invitrogen) and images acquired using an Axio Imager Z2 LSM700 confocal microscope (Zeiss).

RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated from BMSCs treated with 5ng/ml TGFβ1 (R&D Systems), in α-MEM/0% FBS for 24h, with or without 10µM SB-431542 inhibitor (Sigma) added to the cell cultures 1h before the addition of TGFβ1. Total RNA was extracted from BMSCs using RNeasy Mini Kits and QIAshredder (Qiagen). RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies/Applied Biosystems) and qRT-PCR

analysis was performed with SYBR green PCR SuperMix-UDG/ROX (Invitrogen). The level of expressed genes was measured by Relative quantification (ΔΔCt method) or Relative Standard Curve quantification. Human TBP and mouse GAPDH genes were used as internal reference. Primers used are listed in additional supporting information file 1.

Cell proliferation and migration assays

TNW protein was purified as described.[11] For proliferation assays the 5-bromo-2-deoxyuridine (BrdU) chemiluminescent enzyme incorporation kit was used (Roche). Tumor cells were seeded in a 96-well black plate at a density of $1x10^4$ per well and incubated overnight. After the addition of different concentrations of TNW protein (0-10µg/ml) in serum-free DMEM cell proliferation was quantified 48h after plating and the addition of 10µM BrdU for 2h at 37°C. Cells were fixed and stained and the incorporated BrdU detected in a chemiluminescence microplate reader (Perkin Elmer).

Cell migration assays were performed using 24 transwell chambers (8 µm pore size; Costar). The underside of the polycarbonate membrane was pre-coated with different concentrations of TNW recombinant protein (0-10µg/ml) for 2h at 37°C and subsequently blocked for 30min at 37°C with 1% BSA/PBS. MDA-MB231-SCP1833 cells were cultured in the upper chamber in serum-free DMEM ($1x10^5$ cells in 1ml). Serum-free medium was added to the bottom chamber (0.6ml) and cells were allowed to migrate for 24h. Cells were scraped off the top of the membrane using cotton swabs. Cells that had migrated across the membrane were fixed in 4% formaldehyde and stained with 1% crystal violet and total cell-covered areas were quantified using ImageJ software as described before.[16]

Transcription Start Site Identification and cloning of the reporter constructs

5'RACE was performed using total RNA from normal bone (OriGene), breast cancer tissue (Clontech) and glioblastoma (OriGene) using the 5'/3' RACE Kit, (2^{nd} Generation, Roche). Briefly, 1µg of total RNA was reverse-transcribed into first-strand cDNA using the human tenascin-W specific primer hSP1. To identify the 5' end of the TNW mRNA two gene specific primers hSP2 and hSP3 were used (primers are listed in additional supporting information file 1). DNA products were cloned and sequenced.

Different promoter constructs, all containing exon 1 (77bp) preceded by different lengths of human tenascin-W promoter sequences were amplified from human genomic DNA isolated from HEK293 cells resulting in the following constructs: pSEAP-TNW (-1800bp), pSEAP-TNW (-957bp), pSEAP-TNW (-512bp), pSEAP-TNW (-320bp), pSEAP-TNW (-252bp), pSEAP-TNW (-148bp), pSEAP-TNW (-79bp), pSEAP-TNW (-59bp), pSEAP-TNW (-35bp), and pSEAP-TNW (+77bp). See additional supporting information file 1 for the list of primers used. The primers included NheI and XhoI restriction sites for directional cloning into the MCS of pSEAP2-basic (Clontech). The three conserved intronic DNA fragments were amplified from HEK293 genomic DNA as template and inserted into NheI/HindIII sites of the pSEAP-basic vector. Further deletions of the second conserved region were created. All primers used and their intronic locations are listed in additional supporting information file 1. The three intronic regions were also cloned into the NheI and MluI sites 5´ of pSEAP-TNW (-79bp) creating the plasmids pSEAPI/-79, pSEAPII/-79 and pSEAPIII/-79.

Site-directed mutagenesis of the SMAD4 binding site (gc**ctAGAC**agg) was performed using a scrambled sequence (AGAGTGATCA), which does not display any known *cis*-acting sequence. Overlapping primers used for PCR including the scrambled sequences underlined are listed in additional supporting information file 1. Following transformation of the DpnI-digested PCR, plasmid DNA was isolated and the mutations confirmed by sequencing.

Cell transfection and reporter gene assays

HT1080 cells were cultured in DMEM/10% FCS. For reporter assays cells were plated at $5x10^4$ cells/well in 12-well plates overnight to reach 60-70% confluence. Cells were transfected by jetPEI (Polyplus) with 0.6µg/well of total DNA (pSEAP reporter construct and pMetLuc for normalization mixed at 1:20 molar ratio) in DMEM/0.3% FCS. Reporter activity was measured 24h after transfection. Alternatively, cells were transiently transfected in 3% FBS or 3% DCC treated FBS for 24h. Secreted alkaline phosphatase (SEAP) activity in the culture medium was determined using the SEAP Reporter Gene Assay Kit (Roche) and for normalization the Ready-To-Glow secreted luciferase reporter system (Clontech) and measured in a luminometer (Mithras LB940; Berthold technologies). SEAP values were normalized to Luciferase values to control for transfection efficiency. The normalized luminescence values were then standardized by the background activity (empty vector). The *pSEAP-TNW-79bp* construct was included in all

experiments and was used to calibrate different experiments. Error bars represent the standard error of the mean (SEM) between all replicates of each experiment.

Statistical Analyses

Data are represented as means and SD or SEM as stated in the figure legends. Statistical analysis using a two-tailed *t*-test was carried out with SigmaPlot for Windows version 12.0. All the experiments shown are the means of 3 replicates. The difference between two groups was statistically significant when $P<0.05$.

In silico analyses

The genomic location of the human tenascin-W gene and its evolutionary conservation was examined using the UCSC genome browser (http://genome.ucsc.edu/). MatInspector (http://www.genomatix.de) was used to identify predicted transcription factor binding sites in the genomic sequences.

Results

Tenascin-W is induced in bone metastases of MDA-MB231-1833 breast cancer cells

We investigated the expression of TNW in the bone stroma of MDA-MB-231-SCP1883 tumor-bearing mice. Osteoblasts were isolated from tumor-free or tumor-bearing bones as described in Materials and Methods and RNA was extracted from this host cell population. Using quantitative real time PCR (qRT-PCR) mouse TNW mRNA levels were up-regulated 4-5 fold in osteoblasts isolated from bones harboring metastases compared with osteoblasts from non-tumor-bearing control bone (Fig.1A). Immunostaining of the long bones shows that TNW is expressed in the region surrounding the GFP-expressing tumor cells (Fig.1B). Using species-specific monoclonal antibodies, we verified that TNW is specifically unregulated in the host tissue since no human TNW was detectable. Thus, the MDA-MB231-1833 cells are not a source of TNW, but are surrounded by a host-derived TNW-rich extracellular matrix in the bone metastatic niche.

Breast cancer cells release factors that induce tenascin-W expression in human bone marrow stromal cells

Prompted by the striking correlation of TNW expression with breast cancer metastases, co-culture assays were established to investigate whether paracrine interaction between MDA-MB231-1833 and immortalized human BMSCs can induce TNW expression. Indeed, BMSCs are targets of homing signals and they represent a significant cellular source not only of osteoblasts but also of myofibroblasts within the tumor stroma supporting tumor cells.[17] Monocultures and co-cultures of the two cell lines were subjected to immunofluorescence staining to detect TNW protein expression. Interestingly, TNW was exclusively detectable in co-culture conditions and neither the tumor cells nor the BMSCs alone yielded any TNW staining (Fig.1C).

In order to examine whether the expression of TNW in co-cultures depends on direct cell-cell contacts, transwell co-culture assays were performed (Fig.2). Each cell type was cultured in the bottom well of transwell culture dishes (Fig.2A), either alone or with the other cell type cultured in an upper well (Fig.2B). Upper and lower compartments were separated by a polycarbonate membrane with 0.4 μm pores, to allow the diffusion of soluble factors, but not the transmigration of cells (Fig.2B). For each condition, the cells in the bottom well were analyzed

by immunostaining for TNW expression after 7days in culture. This confirmed that neither cell type alone produced TNW, but TNW was induced in BMSCs in the presence of tumor cells in the upper chamber and not vice versa. Thus, in this *in vitro* system, tumor cells can induce the expression of TNW in the bone-derived BMSCs and this depends on the release of a soluble factor (or factors) secreted by the tumor cells.

Transcriptional regulation of tenascin-W gene expression

To get a hint on the possible factor and signaling pathway inducing TNW expression in BMSCs, we decided to isolate and study the TNW gene promoter and to identify regulatory sequences present in the TNW gene. In the case of human TNW, the gene name in the databases is TNN. The 5`part of the TNN gene, as it is annotated in the UCSC browser, is shown in Fig.3A. It shows a first non-coding exon separated by a large intron from exon 2, which contains the ATG translation start. Underneath the sequence tracks, the evolutionary conservation reveals high conservation of the exon sequences and the putative promoter region as well as of three conserved regions in the first intron (Fig.3A). To experimentally confirm the predicted transcription start site (TSS) we performed 5'RACE experiments (Fig.3C). To assess potential transcript variants in different tissues that are known to express TNW, we used total RNA isolated from glioblastoma and breast cancer tissues which have high TNW expression levels [15,18] as well as RNA from healthy bone tissue, since TNW is known to be associated with the osteogenesis process.[19] PCR products were cloned and sequenced. From all RNA sources tested, we found a single TNW transcript with a TSS at the non-coding first exon. Thus, normal and cancer tissues tested here are using a common single TSS to initiate TNW transcription.

In order to identify regulatory regions present in the TNW promoter, promoterless SEAP (secreted embryonic alkaline phosphatase) reporter constructs containing exon 1 (+77) and different parts of the 5' flanking region up to a length of 2kb were cloned (Fig.3B). Reporter gene activity was examined using the human fibrosarcoma cell line HT1080. Transfection of the promoter constructs revealed that the main control region was contained within -512bp of the TSS since longer constructs gave similar results. Thus, the promoter region coincides with the UCSC Genome browser tracks ESPERR Regulatory Potential (Fig.3B; light blue) and the Vertebrate Multiple sequence Alignment & Conservation tracks (Fig.3B; dark blue). However, further shortening of the promoter constructs to -320, -252 and -148bp revealed a gradual loss of

reporter activity. This could be related to the loss of binding sites for factors that enhance the promoter activity in a cooperative manner. One such factor could be SMAD1/5, since by MatInspector analysis a GC-rich Smad-binding element (SBE) was detected at position -230. An increase of reporter activity was observed by the deletion of the sequence from -148 to -79, pointing to the presence of a negative regulatory site in this region. Using MatInspector, a GATA-binding element was detected in this region and may be responsible for the repression in a similar way as it was described for TNC.[20] Interestingly, the short -79bp minimal promoter exhibited the highest activity after transient transfection which was lost by the deletion of -79 to -59bp, indicating important transcription factor binding sites within these 20bp. Indeed, MatInspector analysis revealed a SMAD4 site at this location. The sequence of the TNW promoter and the first exon as well as potential transcription factor binding sites are given in additional supporting information file 2.

Analysis of the evolutionary conserved regions within the first intron.

Further inspection of the TNN genomic locus using the UCSC genome browser revealed striking patterns of histone modifications within the first intron of the TNN gene, namely an H3K4me1 enhancer chromatin signature, as well as H3K27 acetylation that defines nucleosome exclusion regions (Fig.4A). These epigenetic marks overlap with three evolutionarily conserved regions which we tested for a potential involvement in the regulation of TNW transcription (Fig.4B). We cloned the three intronic conserved modules I, II and III upstream of the SEAP reporter gene and tested their effects on reporter gene activity. While the first conserved region (+1178/+1909) appeared to act as a silencer, the second conserved region (+2920/+3447) strongly enhanced transcription when compared to the pSEAP basic vector. The third region did not seem to influence the reporter gene activity when tested on its own, but in conjunction with the active region II, both, region I as well as III seemed to silence the activity of region II (Fig.4B). Further dissection of the second conserved region showed that the sequence between +3299 and +3447 was mainly responsible for the strong activation (Fig.4B). Although we did not find any alternative TSS in the tissues analyzed, the possibility remains that this region might be an alternative promoter of the TNN gene.

Identification of negative and positive trans-acting elements in the TNW gene

After the experimental determination of the most important regions of the TNW gene affecting promoter activity, we experimentally tested the identified putative transcription factor binding sites within the promoter and first intron regions. Computational predictions showed the presence of a SMAD4 nuclear transcription factor binding site at -61bp from the TSS, next to a TATA box sequence located at -54bp upstream of exon 1. This SMAD4 binding site (gcctAGACagg) is located within the 20bp region found to be crucial for the high activity of the proximal TNW promoter. To test whether this binding site is responsible for the promoter activity, site-directed mutagenesis of the SMAD4 element was performed (Fig.5A). Indeed, the presence of the scrambled sequence (AGAGTGATCA) strongly impaired the SEAP reporter gene expression driven by the minimal basal promoter (-79bp). SMAD4 is an intracellular mediator of the TGF-β signaling pathway.[21] Therefore, we assessed whether TGF-β1 acts on endogenous TNW gene expression in BMSCs (Fig.5B). To examine the specificity of action of TGF-β1 on TNW gene transcription we evaluated the efficiency of SB-431542, a selective inhibitor of the TGF-β1 receptor/ALK5, to inhibit TNW induction (Fig.5B). Preincubation of BMSCs with the ALK5 inhibitor abolished induction of TNW transcripts as assessed by qRT-PCR. Thus, we identified TGF-β1 as a factor inducing human TNW gene expression in BMSCs through activation of ALK5. A similar result was obtained using conditioned medium of MDA-MB231-1833 instead of TGF-β1, which also led to an ALK5-dependent incease of TNW transcripts in BMSCs, confirming that TGF-β1 is one of the MDA-MB231-1833 secreted factors inducing TNW (Additional supporting information file 3).

Further sequence analysis of the intron showed a negative glucocorticoid response element (nGRE: tttttccaGGAGaga) located in the first conserved region. This prompted us to investigate whether or not glucocorticoids have an inhibitory effect on TNW transcription. For this purpose, we stripped endogenous steroids from the serum by using dextran-coated charcoal (DCC-FBS). Conserved intronic regions were cloned upstream of the -79bp proximal promoter construct and transfected into HT1080 cells in the presence of 3% untreated FBS or DCC-FBS for 24h (Fig.5C). Indeed the first intronic module (I./-79bp) containing the nGRE exerted transcriptional repression of the TNW promoter measured by a 2-fold lower activity in the cells grown in untreated versus steroid-stripped FBS. This was confirmed in BMSCs. In the presence of glucocorticoid-depleted FBS, BMSCs showed higher TNW transcript levels than in normal

FBS (Fig.5D). A model representing the main regulatory elements of the promoter and first intron of the TNW gene analyzed here is shown in Fig.5E.

Tenascin-W promotes breast cancer cell migration and proliferation *in vitro*

Since the metastatic tumor cells in vivo are surrounded by TNW we investigated a potential functional role of TNW in tumor progression, MDA-MB231-1833 cells were treated with different concentrations of human TNW recombinant protein (5-10µg/ml) and analyzed for proliferation and migration. There was a 3.5-fold increase in BrdU incorporation in MDA-MB231-1833 cells measured in the presence of TNW 48h after starting the cultures (Fig.6A). Cell migration was assessed using transwell chamber assays. The underside of the filters was coated with TNW to investigate its role as chemoattractant. There was a 3.5-fold increase in MDA-MB231-1833 cell migration towards TNW compared to control filters indicating a pro-migratory function of TNW for the MDA-MB231-1833 cells (Fig.6B).

Discussion

It is well known that paracrine signals released by breast cancer cells orchestrate the fate of stromal cells residing in the tumor microenvironment and vice versa.[4,22,23] Stromal cells are targeted by tumor-released factors and in response undergo myofibroblast-like differentiation to a so-called cancer associated fibroblast.[24] We propose that breast cancer cells, after homing to the bone marrow via blood circulation, secrete TGFβ1 which induces changes in the bone marrow niche, including the deposition of TNW in the extracellular matrix. Exposure of MDA-MB231-1833 cells to TNW supports their migration and proliferation establishing a "vicious cycle" for cancer progression by promoting the growth and further invasive behavior of the breast cancer metastases (Fig.6C). We showed that a crucial signaling pathway for the induction of TNW is the TGFβ1 secreted by the tumor cells which induces Smad4-dependent transcription of the TNW gene in the bone marrow stromal cells. Indeed, TGFβ1 has been found to be a major osteolytic factor secreted by MDA-MB231 cells.[25] Furthermore, downregulation of Smad4 in MDA-MB-231 cells strongly inhibited bone metastasis formation. [26] In contrast to the induction of TNW expression by TGFβ1, we found that glucocorticoids have a negative impact on TNW expression. A similar antagonistic effect has also been described for the regulation of the TNW family member TNC and TNX.[27,28] Interestingly, anti-inflammatory agents, including corticosteroids, are in use as cancer therapy.[29] It is likely that such treatments also affect the ECM composition of the cancer microenvironment.

The actual situation in vivo is of course more complicated and it is clear that also osteoclasts are involved in the metastatic process.[30,31] It is known that upon stimulation of osteoblasts by cytokines released by the tumor cells, osteoblasts release RANKL which in turn promotes differentiation and activation of osteoclasts. Activated osteoclasts resorb the bone matrix with the consequent release of cytokines including TGFβ.[32] These cytokines will act back on the osteoblasts as well as the tumor cells and in a vicious cycle keep the osteolytic lesions growing. Because of these findings, inhibition of osteoclasts by bisphosphonates as well as RANKL and TGFβ-targeting agents are presently tested as adjuvant therapy for the treatment of breast cancer patients with bone metastases.[32-36] The benefit of such treatments may include reduced TNW expression in the microenvironment of the metastatic cells and alleviate the tumor-promoting

effects exerted by TNW. Tumor-promoting effects of TNW may include promotion of growth and migration as well as pro-angiogenic effects, since TNW was found to be expressed around tumor blood vessels in several types of cancers and to stimulate endothelial sprouting in culture.[8,18] Furthermore, with its tumor and metastasis-specific expression, TNW may itself be a target for antibody-mediated drug targeting of the bone metastatic niche.

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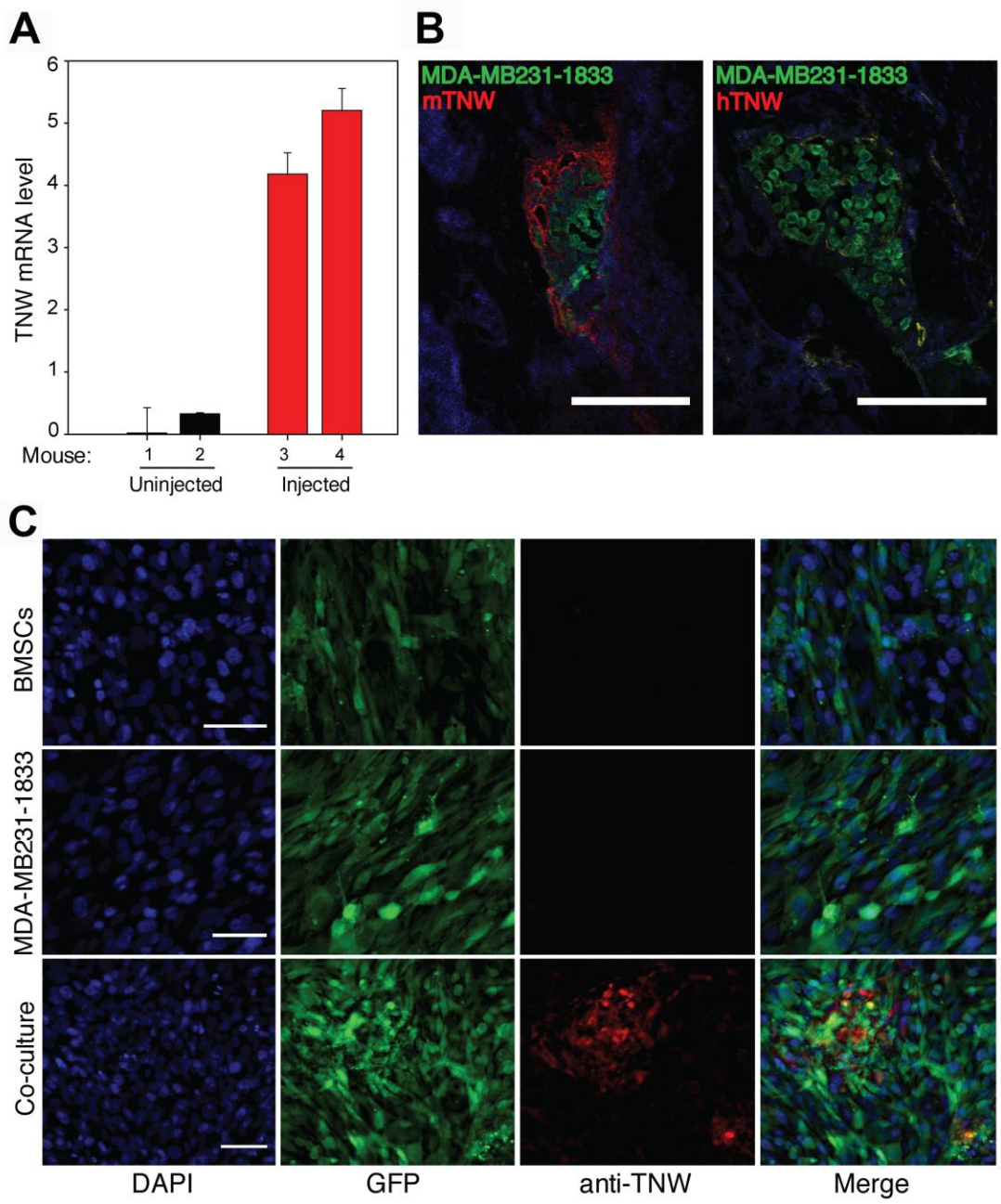
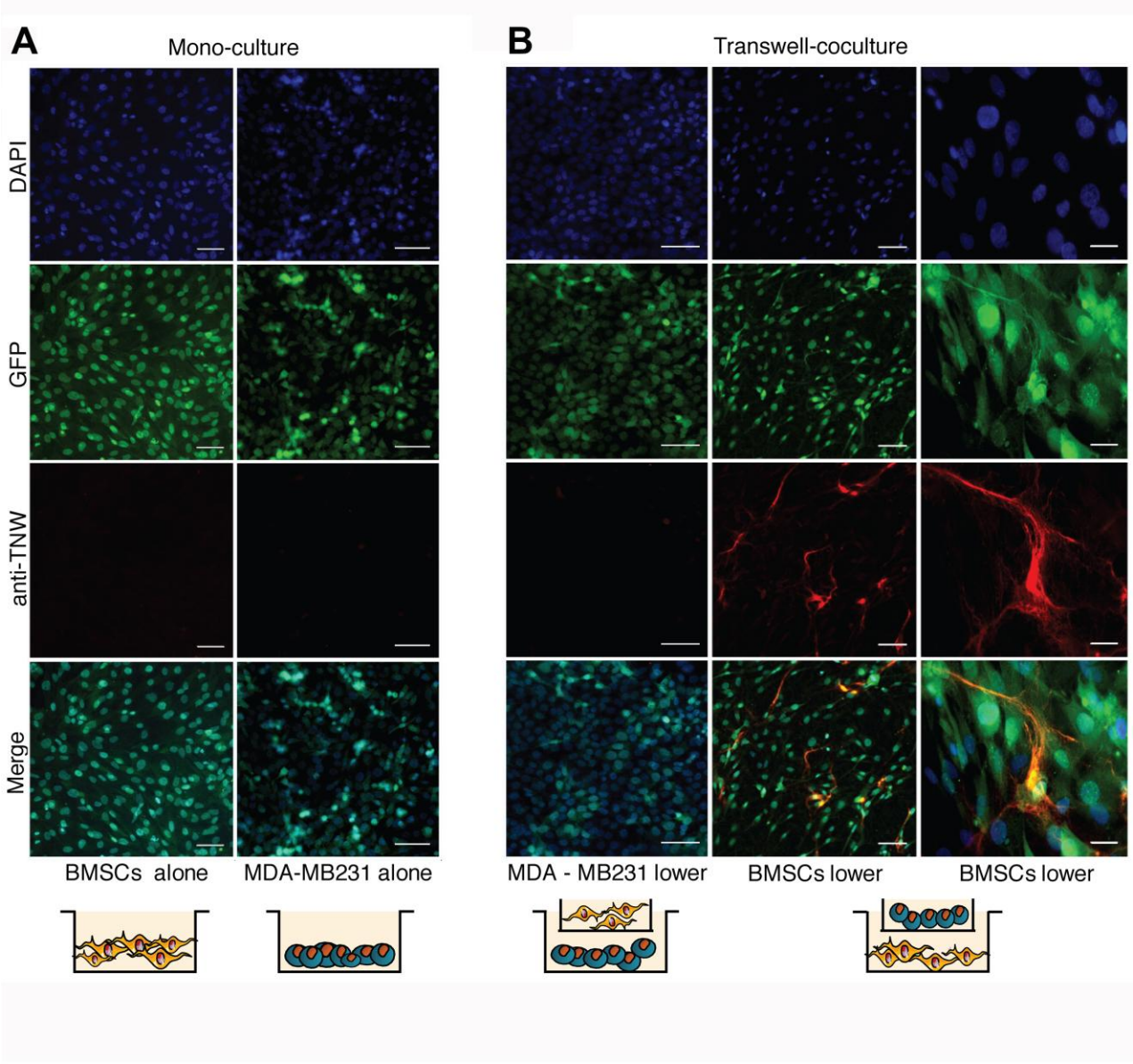

A
TNW mRNA level
0
1
2
3
4
5
6
Mouse:
1
2
3
4
Uninjected
Injected
B
MDA-MB231-1833
mTNW
MDA-MB231-1833
hTNW
C
BMSCs
MDA-MB231-1833
Co-culture
DAPI
GFP
anti-TNW
Merge

Figure 1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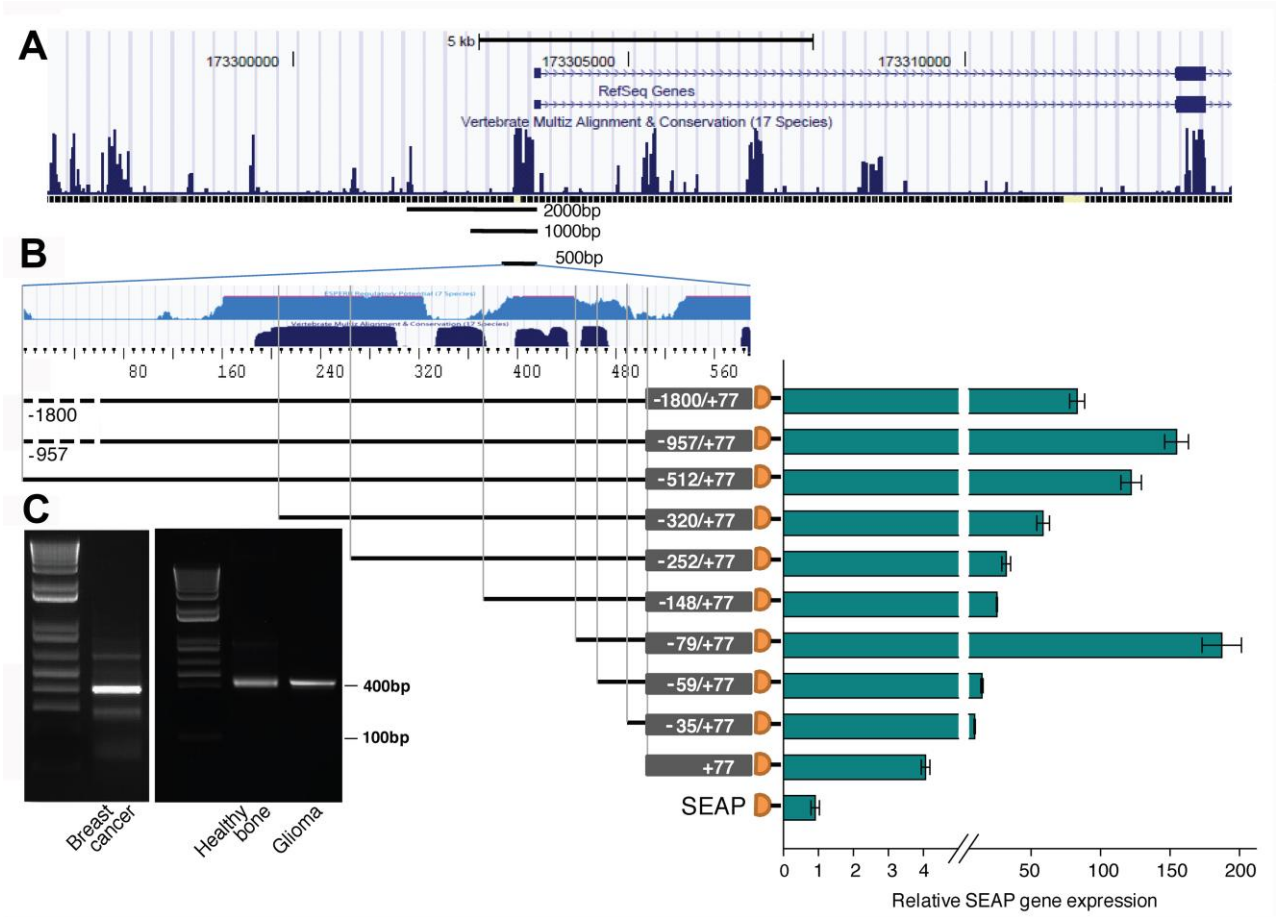
Mono-culture
B
Transwell-coculture
DAPI
GFP
anti-TNW
Merge
BMSCs alone
MDA-MB231 alone
MDA - MB231 lower
BMSCs lower
BMSCs lower

Figure 2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5 kb
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173305000
173310000
RefSeq Genes
Vertebrate Multiz Alignment & Conservation (17 Species)
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1000bp
500bp
B
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480
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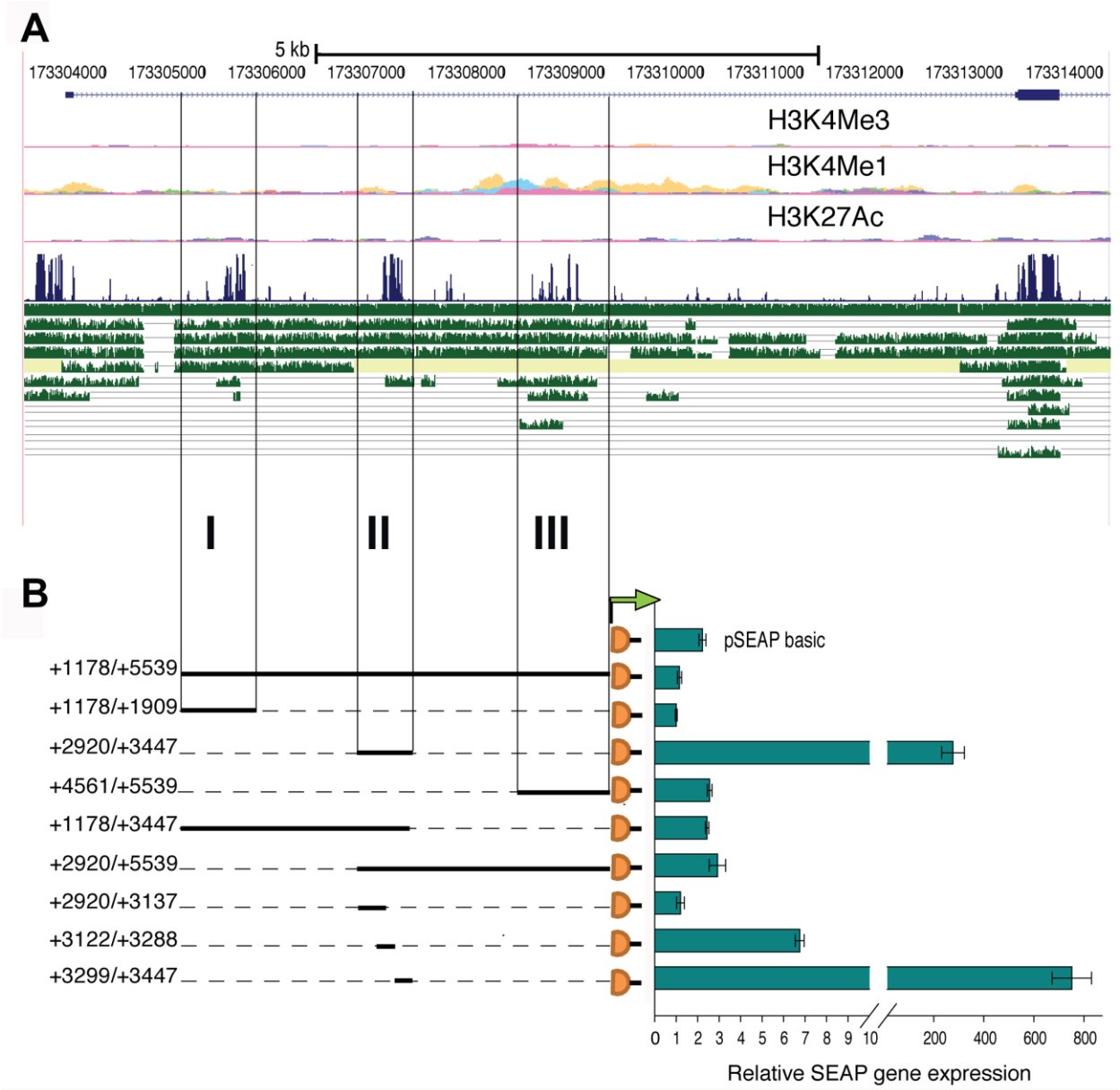
-957
-1800/+77
-957/+77
-512/+77
-320/+77
-252/+77
-148/+77
-79/+77
-59/+77
-35/+77
+77
SEAP
0
1
2
3
4
50
100
150
200
Relative SEAP gene expression
C
400bp
100bp
Breast cancer
Healthy bone
Glioma

Figure 3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II
III
B
pSEAP basic
+1178/+5539
+1178/+1909
+2920/+3447
+4561/+5539
+1178/+3447
+2920/+5539
+2920/+3137
+3122/+3288
+3299/+3447
0 1 2 3 4 5 6 7 8 9 10 200 400 600 800
Relative SEAP gene expression

Figure 4

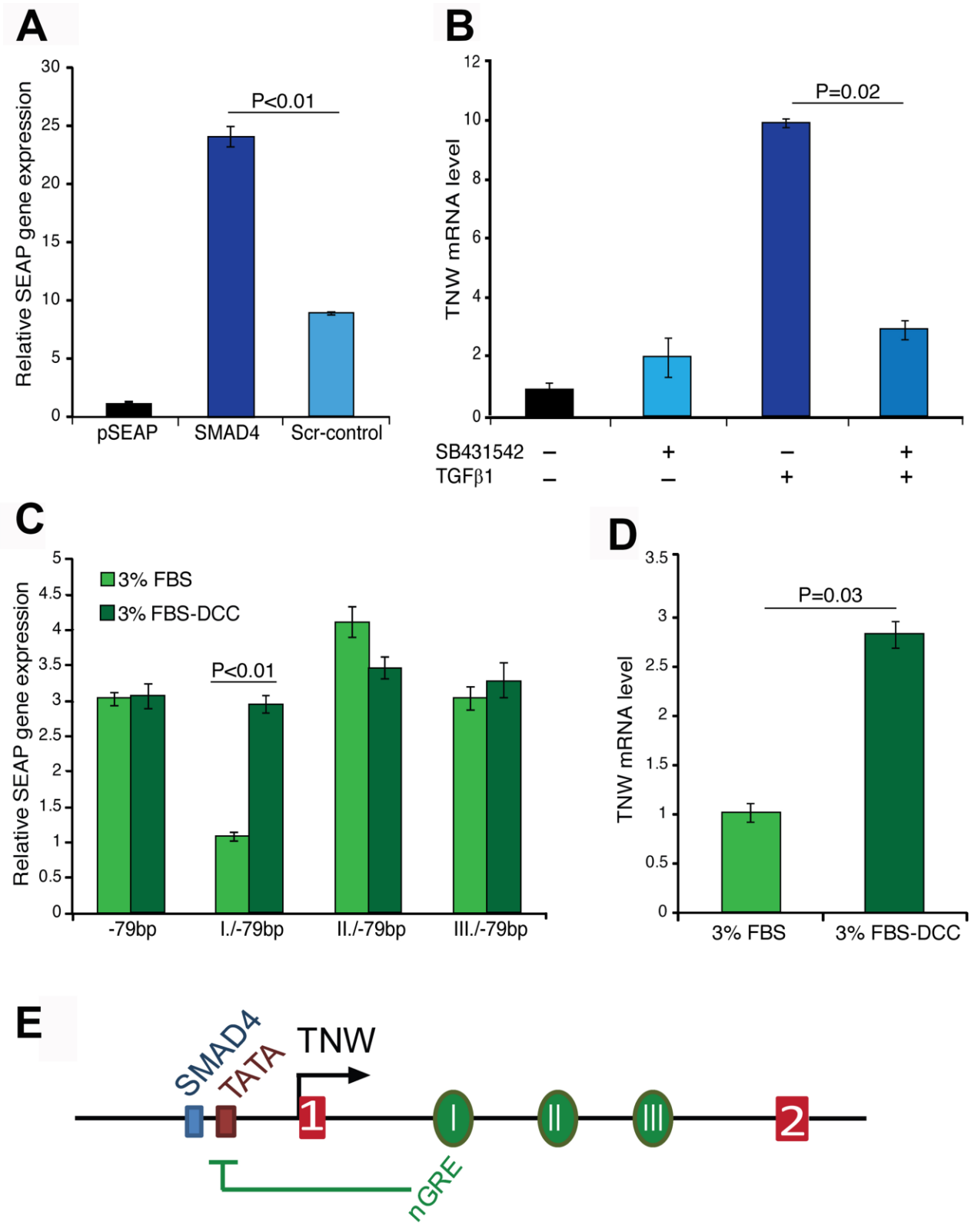

A
Relative SEAP gene expression
30
25
20
15
10
5
0
P<0.01
pSEAP
SMAD4
Scr-control
B
TNW mRNA level
12
10
8
6
4
2
0
P=0.02
SB431542 – + – +
TGFβ1 – – + +
C
Relative SEAP gene expression
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
3% FBS
3% FBS-DCC
P<0.01
-79bp
I./-79bp
II./-79bp
III./-79bp
D
TNW mRNA level
3.5
3
2.5
2
1.5
1
0.5
0
P=0.03
3% FBS
3% FBS-DCC
E
SMAD4
TATA
TNW
1
I
II
III
2
nGRE

Figure 5

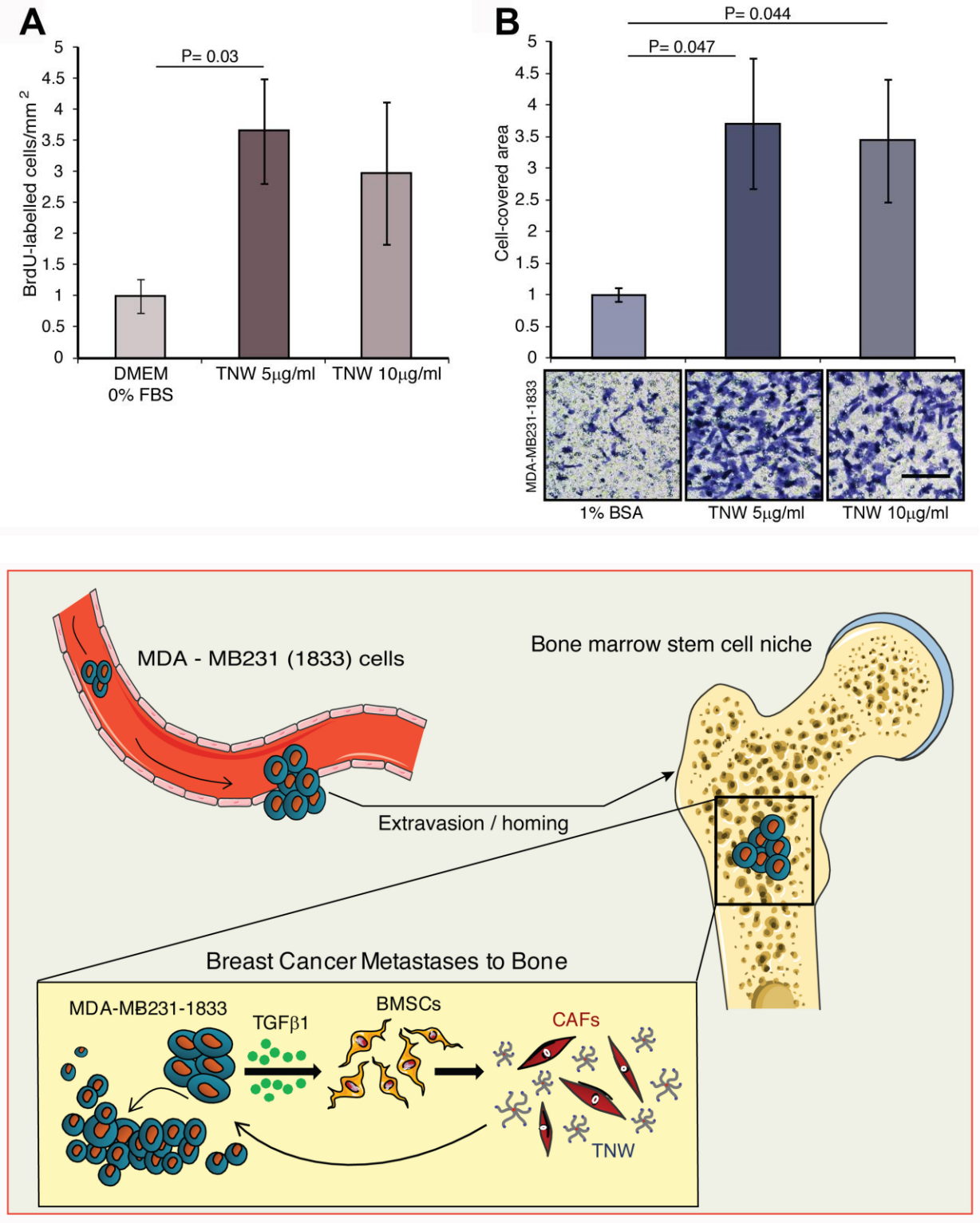

A
BrdU-labelled cells/mm 2
P= 0.03
DMEM
0% FBS
TNW 5µg/ml
TNW 10µg/ml
B
Cell-covered area
P= 0.044
P= 0.047
MDA-MB231-1833
1% BSA
TNW 5µg/ml
TNW 10µg/ml
MDA - MB231 (1833) cells
Bone marrow stem cell niche
Extravasion / homing
Breast Cancer Metastases to Bone
MDA-MB231-1833
TGFβ1
BMSCs
CAFs
TNW

Figure 6

Figure Legends:

Figure 1. **TNW is upregulated in bone metastasis of breast cancer.**

A, TNW transcript levels in RNA isolated from osteoblasts sorted from two individual tumor-free (1, 2, black) or two tumor-bearing mice (3, 4, red). Averages ± s.d. of two independent experiments in triplicate are shown. **B,** tissue sections of tibia show GFP-MDA-MB231-1833 metastases (green). Staining of TNW was detectable with anti-mouse TNW only (red, mTNW, left panel) but not with anti-human TNW (hTNW, right panel). Scale bar, 100μm. **C,** immunofluorescence staining for TNW of human bone marrow stromal cells (GFP-BMSCs) and human breast cancer cells (GFP-MDA-MB231- 1833) cultured alone, or in co-culture reveals TNW protein (red) expression after 7 days of co-culture. Nuclei were labeled with DAPI (blue). Scale bars, 50μm.

Figure 2. **Soluble factors secreted by MDA-MB231-1833 breast cancer cells stimulate TNW expression in BMSCs.**

A, monocultures of GFP-BMSCs (left panels) and GFP-MDA-MB231-1833 cells (right panels) maintained for 7 days in culture. Nuclei were stained with DAPI (blue). Staining with human anti-TNW monoclonal antibody does not detect any TNW expression. **B,** MDA-MB231-1833 cells or BMSCs were seeded in the bottom well of transwell chambers (MDA-MB231-1833 lower; BMSCs lower) and exposed to the other cell type in the upper chamber in an indirect co-culture system. Under these conditions TNW protein expression (red) was detected exclusively in BMSCs and not in MDA-MB231-1833 cells Scale bars, 100μm for all panels except for the magnification shown in the right panels representing 20 μm.

Figure 3. Experimental and computational analyses of the transcription start site and promoter activity of the TNW gene.

A, graphical representation of the two first exons of the TNW gene (marked in blue rectangles) using the UCSC Genome Browser. In addition to sequence conservation of the exons and promoter region, three regions of conservation among the vertebrate genomes are observed within the first intron. **B,** to characterize the TNW promoter, exon-1 (+ 77bp) and different lengths of the 5' flanking region as indicated were cloned upstream a promoterless SEAP vector.

The UCSC Genome browser tracks ESPERR Regulatory Potential (7 species; light blue) and Vertebrate Multiple sequence Alignment & Conservation (17 species; dark blue) of about 500bp upstream of the TSS are depicted above the promoter constructs. Plasmid DNA constructs were transiently transfected in HT1080 cells for 24 hours. SEAP activity is normalized to a co-transfected secreted luciferase plasmid and plotted relative to the promoterless SEAP vector control. Values are the average and SEM of three independent experiments. **c,** agarose gel electrophoresis shows the products resulting from 5'RACE of total RNA from breast cancer, healthy bone and glioma tissues as indicated. Cloning of the DNA bands (400bp) and the subsequent sequencing revealed a single transcription start site adding a 79bp first exon to exon 2 containing the ATG start codon.

Figure 4. Analysis of the evolutionary conserved regions within the first intron.

A, Multiple alignment of conserved genomic sequences using the UCSC Genome Browser is visualized in green lines and shows the conservation to human TNW from top to bottom of Rhesus, Mouse, Dog, Horse, Armadillo, Opossum, Platypus, Lizard, Chicken, X_tropicalis, and Stickleback orthologs. Chromatin signatures shown on top (H3K4Me3, H3K4Me1 and, H3K27Ac) overlap with the intronic modules. **B,** in order to assess potential transcriptional activities, each conserved region I, II and III, combinations thereof and truncated versions as indicated were cloned upstream of the SEAP reporter gene. Transient transfections of the constructs in HT1080 cells were analyzed for SEAP activity as described in Fig.3B. The experiment was performed in triplicates and repeated three times (error bars = SEM).

Figure 5. Regulation of TNW transcription by TGFβ1 and glucocorticoids.

A, The Smad4 binding site (gc**ctAGAC**agg) was mutated by site-directed mutagenesis and replaced with the scrambled sequence (Scr- AGAGTGAT). Construct -79/+77 containing the normal (SMAD4) or modified Smad4 sequence (Scr-control) was transfected in HT1080 cells and analyzed for SEAP activity in comparison to the empty pSEAP as described in Fig.3B. **B,** BMSCs were treated with SB-431542-DMSO (10µM) or DMSO only for 1h before the addition of TGFβ1 (5 ng/ml) for 24h. TNW transcript levels were then analyzed by qRT-PCR. **C,** Constructs with the three intronic regions (I./II./III.) cloned upstream the minimal TNW promoter (-79bp/+77) were transfected in HT1080 cells in medium containing 3% FBS (light

green bars) or glucocorticoid-depleted 3% DCC-FBS (dark green bars) for 24h. SEAP activity was analyzed as in Fig. 3 revealing that the intronic conserved region I inhibited the reporter activity in a glucocorticoid-dependent manner. **D,** BMSCs were cultured in untreated 3% FCS (light green bar) or in the presence of 3% charcoal/dextran treated FBS for 24h before measuring transcript levels of TNW by qRT-PCR. All experiments were repeated at least three times (error bars = SEM). **E**, Schematic model of the TNW gene regulation by a SMAD4 element preceding a TATA box in the proximal promoter region upstream of exon 1 (red box 1) and a negative glucocorticoid-response element (nGRE) in the first conserved region within the first intron (green oval I).

Figure 6. Effect of TNW on breast cancer cell proliferation and migration.

A, MDA-MB231-1833 cells were treated with different concentrations of recombinant human TNW protein (5-10 µg/ml) in serum-free medium. Proliferation was measured by BrdU incorporation 48h after exposure to TNW. **B,** MDA-MB231-1833 cells were seeded in the upper chambers of transwell migration dishes and allowed to migrate for 24 h towards TNW coated on the underside of the filters. Cells on the bottom side of the filters were stained and photographed (scale bar = 50 µm) and the cell-covered area was quantified (error bars = SD). Statistical analysis was assessed by paired Student's t-test. P values <0.05 are considered statistically significant. **C,** Model for the role of TNW in breast cancer metastasis to bone: After extravasion and homing to the bone MDA-MBA231 cells are releasing TGFβ1 which triggers BMSCs to differentiate into TNW secreting cancer associated fibroblasts (CAFs). TNW in turn is able to influence the growth and further invasion of the metastatic cancer cells.

Additional supporting Information

Transcriptional regulation of tenascin-W by TGF-beta signaling in the bone metastatic niche of breast cancer cells

Francesca Chiovaro[1,2], Enrico Martina[1,2], Alessia Bottos[1], Nancy E. Hynes[1,2] and Ruth Chiquet-Ehrismann[1,2]

Additional file 1. Primer list

Additional file 2. Promoter sequence and 5´end of the TNW gene.

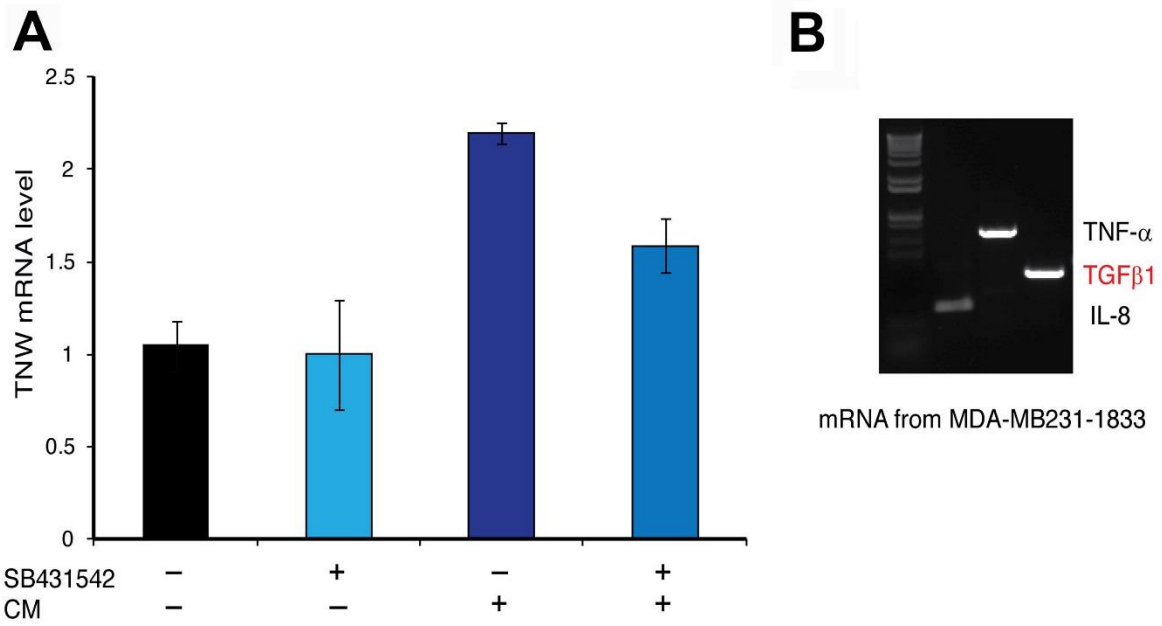
Additional file 3. Induction of TNW by MDA-MB231-1833 conditioned medium.

Supplemental Table 1. Primer List

Promoter primers			Size product
RW:	5'-CTGGGAGGCTGCTGGGTGCCTC-3'		end-exon 1 (+77)
FW:	5'-AAGTACCAAGGTCTGCGGCAGG-3'		start-exon 1(+77)
FW:	5'-ACGCACTTCCATCTGCCCTC-3'		-1800 bp
FW:	5'-GCAGACACAAGCTTCAGATC-3'		-957 bp
FW:	5'-CCCCACTTCAGAGTCCTGC-3'		-512 bp
FW:	5'-CACTTTTGAACCCCGAGACC-3'		-320 bp
FW:	5'-GGATTCACTTAAATGGAAAC-3'		-252 bp
FW:	5'-GAGAAGGAATTGCTCATT-3'		-148 bp
FW:	5'-ATTTGCTCCTCCTCTGGCC-3'		-79 bp
FW:	5'-GACAGGATTTAAACCCAG-3'		-59 bp
FW:	5'-AAGCCAAGGAGAGACGAG-3'		-35 bp
Intron primers			**Position**
FW:	5'-AACAGGCAGGGCTTCACTTA-3'		(+1178/+1909)
RV:	5'-TGAGTCCACTGTGGCAGAAG-3'		
FW:	5'-ATCGGTCCCCTAAGCCTCTA-3'		(+2920/+3447)
RV:	5'-TTCCCCAAAGAAGGACACAC-3'		
FW:	5'-ATGGGAAAATACCCCTCAGC-3'		(+4561/+5539)
RV:	5'-AATGAGTTGGAAGGCAGGTG-3'		
FW:	5'-ATCGGTCCCCTAAGCCTCTA-3'		(+2920/+3137)
RV:	5'-AAATGAGGGAGATGAGTGGCTA-3'		
FW:	5'-ACATTGATGCATCCCTAGCC-3'		(+3122/+3288)
RV:	5'-TCTCATCCTGCCTTGACAGTT-3'		
FW:	5'-CAGGATGAGATAATAAGGGA-3'		(+3299/+3447)
RV:	5'-TTCCCCAAAGAAGGACACAC-3'		
qPCR			
hTNW	FW:	5' ATGCCCTCACAGAAATTGACAG-3'	
	RV:	5'-TCTCTGGTCTCTTGGTCGTC-3'	
mTNW	FW:	5'-AGGTGAGGGCAGACTTACAGA-3'	
	RV:	5'-CGACAGCTTGTACCGCTCTTT-3'	
TGFb1	FW:	5'-GCCCTGGACACCAACTATTGC-3'	
	RV:	5'-GCTGCACTTGCAGGAGCGCAC-3'	
TNF-a	FW:	5'-GCCTGCTGCACTTTGGAGTG-3'	
	RV:	5'-TCGGGGTTCGAGAAGATGAT-3'	
IL-8	FW:	5'-TACTCCAAACCTTTCCACCC-3'	
	RV:	5'-AAAACTTCTCCACAACCCTC-3'	
5'RACE			
hSP1	5'-GTGGCGGCTTAGATCAGTGACTCCCTG-3'		
hSP2	5'-CGCTGGGCACTACACTGTTCCTTCATCT-3'		
hSP3	5'-GCCAAGAGCGAAGCCCCATCGTCA-3'		
Site-Directed Mutagenesis for SMAD4			
FW:	5'-TCCTCCTCTGTAGAGTGATCAATTTAAACCCAGGAAGGGAA-3'		
RV:	5'-GGGTTTAAATTGATCACTCTACAGAGGAGGAGCAAATGCTA-3'		

Supplemental Figure 1

Promoter sequence and 5´end of the TNW gene. The start of each promoter construct is designated above the sequence with the first bp in bold. The TSS is marked with +1 followed by the sequence of the first non-coding exon and the sequence of the second exon up to the translation start codon ATG in italic. The main transcription factor binding sites addressed in this paper are underlined (core sequences double underlined) and labeled above the sequence when present on the + strand and below, when present on the – strand.



Supplemental Figure 2

Induction of TNW by MDA-MB231-1833 conditioned medium. (**A**) BMSC cells were seeded at a density of 1 x 10^5 in 6 well plates overnight at 37°C to reach 70% confluence. After washing with PBS they were maintained in α-MEM serum free with or without the addition of 10 μM SB-431542 inhibitor for 1h (Sigma). Following SB-431542 treatment, cells were exposed to conditioned medium (conditioning time 48h) from MDA-MB231-1833 cells mixed in a ratio of 1:1 with fresh serum-free medium plus 0.2% BSA. Total RNA was extracted from BMSCs after 24h using and transcribed into cDNA. Samples were subjected to real time PCR analysis with human TBP as internal reference. (**B**) MDA-MB231-1833 cells were maintained for 24h in DMEM supplemented with 10% FBS and total RNA was isolated. RT-PCR reactions were analyzed by agarose gel electrophoresis to reveal the presence of TGFβ1 (336bp), IL-8 (160bp) and TNF-α (708bp) transcripts in MDA-MB231-1833 cells.

III. 2 Unpublished Results

III. 2. 1 An intronic positive transcription regulatory element

The high gene expression achieved by transfecting HT1080 cells with pSEAP vector containing the second conserved (+3299/+3447) region within the first intron (cf. Fig.4 of the manuscript Chiovaro et al., submitted), prompted us to investigate more closely the identity of this sequence. It is well known that the activity of enhancers is generally insensitive to the orientation and location, whereas changes in promoter position and orientation greatly impair its function (112). Therefore, cloning of the fragment (+3299/+3447) in opposite direction was carried out (Fig.III.1). The alteration in orientation was found to cause a strong decrease in SEAP gene expression. This suggested to us the presence of a second promoter in this region.

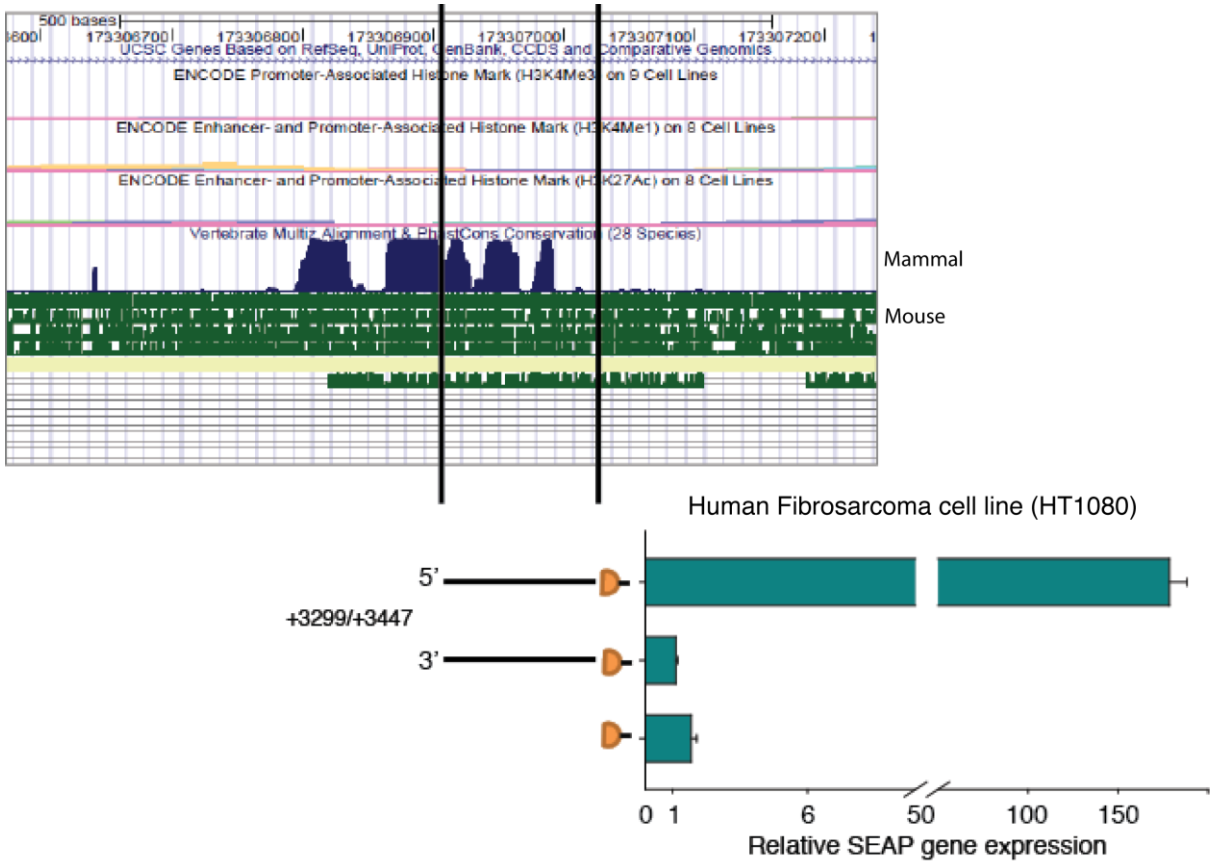


Figure III. 1: Graphical representation of the fragment (+3299/+3447) within the second conserved intronic region visualized by using the UCSC Genome Browser. Vertebrate Multiple sequence Alignment and Conservation for instance for human and mouse is indicated by green lines. The region (+3299/+3447) was cloned in opposite orientation (3'-5') into the plasmid containing the SEAP reporter gene (orange). SEAP activity of the TNW intronic constructs in HT1080 cells was compared to the negative control (pSEAPbasic). Experiment was performed in triplicates and repeated three times (error bars = SEM).

Most promoters harbor control elements for expression across a wide range of cell and tissue samples leading to a precise pattern of gene expression (113). However, another mechanism for tissue-specific gene regulation is the acquisition of alternative promoters for a single gene each leading to distinct tissue-specific expression (114) Although findings from our studies showed only one single tenascin-W transcript form generated by a TSS located upstream of the non-coding exon 1 in breast cancer, glioblastoma and bone tissues (Chiovaro et al., 2014), we cannot exclude the potential existence of an alternative tenascin-W promoter used in a different context. Recent reports have shown how enhancer sequences can act as alternative promoters to contribute to divergent transcription in an orientation-dependent manner (115), supporting our indication of an enhancing region within the +3299/+3447 fragment.

III. 2. 2 Functional dissection of the second conserved region

In order to narrow down the active sequence(s) within the intronic fragment +3299/+3447 of the second conserved region, further truncation constructs were cloned.

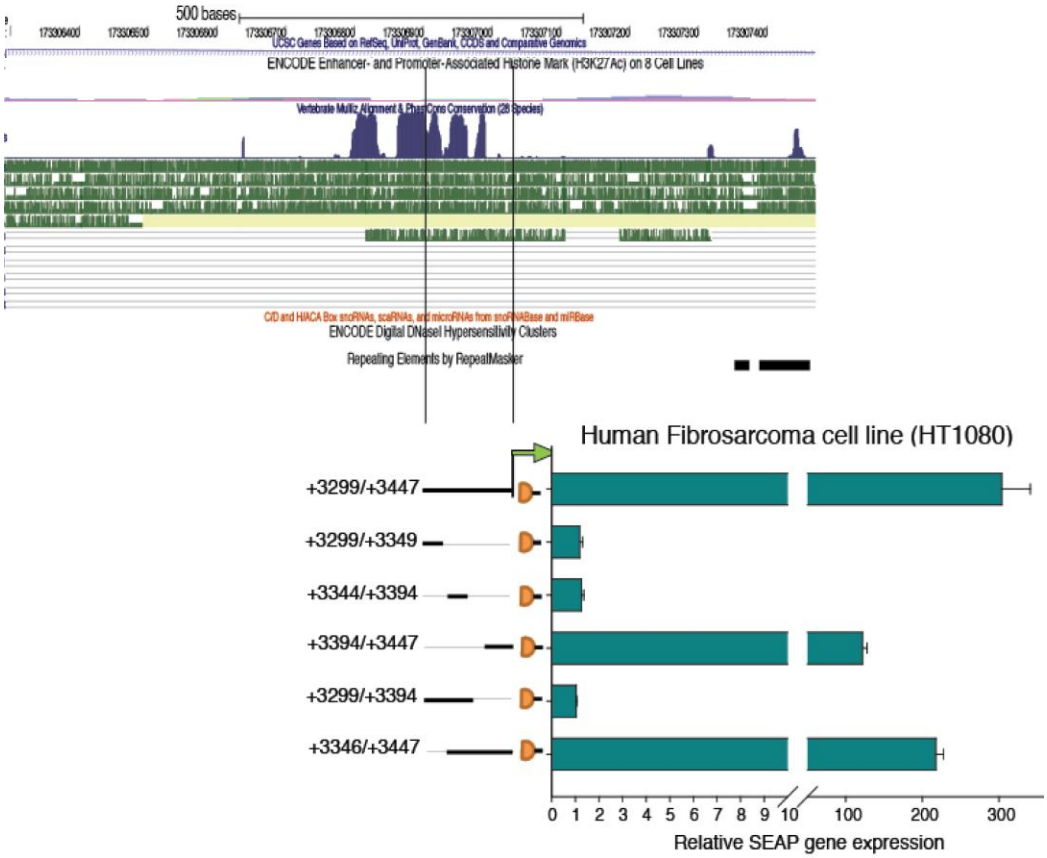


Figure III. 2: SEAP activity of different TNW truncated intronic constructs of the region (+3299/+3447) in HT1080 cells compared to the entire fragment (+3299/+3447). The experiment was performed in triplicates and repeated three times (error bars = SEM).

The fragment of 148bp was subdivided into three shorter fragments of roughly 50bp each (+3299/+3349; +3344/+3394; +3394/+3447) as well as combinations of them as indicated in Fig.III.2. We found that the 3'-end region (+3394/+3447) was responsible for the induction of the reporter gene expression. In order to identify the shortest nucleotide sequences with activity within this sequence, we performed site-directed mutagenesis (Fig.III.3). A scrambled sequence (AGAGTGATCA) that does not display any transcription factor binding sites was substituted for successive 10nt contained in the fragment of 53bp (+3394/+3447) as indicated in Fig. III.3. Constructs were transiently transfected in HT1080 cells for 24h. As shown in Fig. III.3, alteration of the central 20 bp in this sequence (segments III and IV) led to complete loss of SEAP expression, indicating that this region is essential for transcriptional activation.

All primers used for all promoter reporter constructions and their intronic locations are listed in table III.1. The sequences were cloned into the HindIII and NheI sites of the pSEAP vector. For the procedures for cell transfection and reporter gene assays, see Chiovaro et al., 2014.

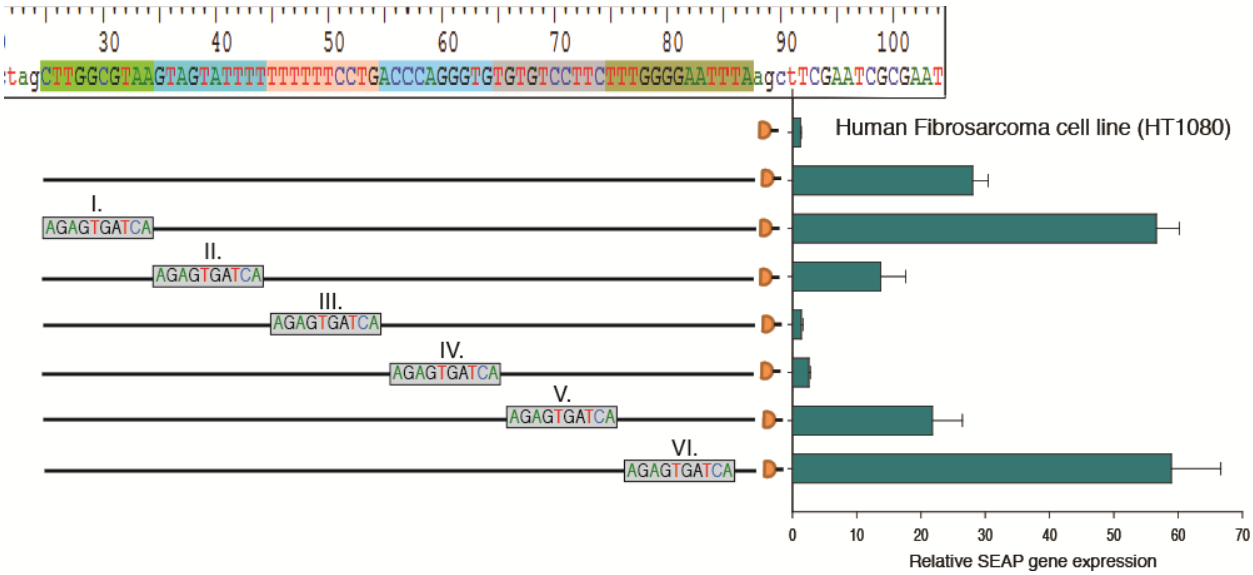


Figure III. 3: SEAP activity of different scramble-mutant +3394/+3447 constructs, containing the mutation at different position. Basic vector and the unchanged +3394/+3447 region used as controls. The experiment was performed in triplicates and repeated three times (error bars = SEM).

III. 2. 3 Computational analysis of the second conserved region

After the experimental determination of the active nucleotide sequences within the 53bp construct, MatInspector analysis revealed the presence of important transcription factor elements at this location (Table III.3). Binding sites for the transcription factors AP1, SP1 and a cAMP response element (CRE) well known to enhance gene expression, were found in region

IV (116, 117). Their replacement with the scrambled sequence showed a decreased SEAP reporter activity, proving their role as enhancer elements. In contrast to these positive regulators, in regions I and VI elements with potential transcriptional repression activity were identified. The proximal sequence element (PSE) of RNA polymerase III-transcribed genes is located in fragment I presenting a binding site for PSE-binding transcription factors (PTF), including co-activators as well as co-repressors (118). Moreover, a binding site for ETS1 is located in the last 10nt of the sequence. ETS1 belongs to the ETS-domain family of transcription factors and many co-regulatory partners, whether co-repressor or co-activator, can be recruited to ETS domain proteins (119).

Table III. 3: MatInspector analysis of TFs

Matrix Family	Matrix	Strand	Sequence
V$SNAP	V$PSE.02	+	cttgg**CGTA**agtagtattt
V$AP1R	V$BACH2.01	-	acaccc**TGGG**tcaggaaaaaa
V$SP1F	V$TIEG.01	+	accc**AGGG**tgtgtgtcc
V$CREB	V$TAXCREB.02	-	aaagaa**GGAC**acacaccctgg
V$ETSF	V$SPI1.03	+	ttctttgg**GGAA**tttaagctt

III. 2. 4 Influence of the first intron on human tenascin-W promoter activity

The region located 512bp upstream of exon 1 has been shown to be important for the regulation of transcription of the tenascin-W gene (Chiovaro et al., 2014). In the next set of experiments we aimed to investigate the influence of the three intronic conserved sequences on tenascin-W promoter activity. Therefore, intronic fragments were cloned upstream of the 512bp tenascin-W promoter preceding the TSS as well as upstream of the shorter 252bp and 79bp-long tenascin-W promoter construct (Fig.III.4), as diagrammed in Fig. III.5.

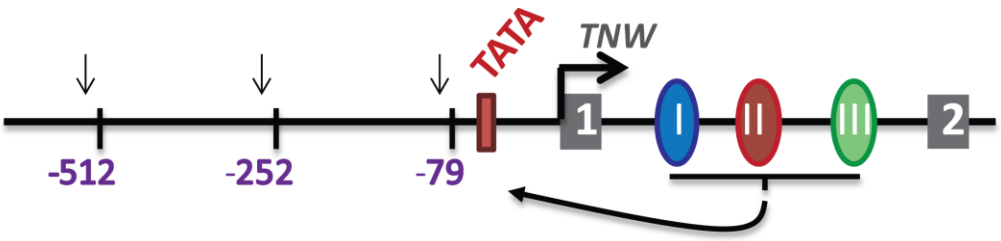


Figure III. 4: Schematic model of the three conserved regions within the first intron (oval shape; I.blue, II.red, III.green). TATA box sequence is located in the minimal promoter (-79), upstream the TSS of tenascin-W gene. Intronic fragments were cloned upstream different truncated forms of the promoter (-512, -252, -79).

We termed the three conserved intronic modules as I. (+1178/+1909), II. (+2920/+3447) and III. (+4561/+5539) (Chiovaro et al., 2014). Plasmid constructs were transiently transfected in HT1080 cells in media containing serum for 24h before SEAP activity was measured (Fig.III.5).

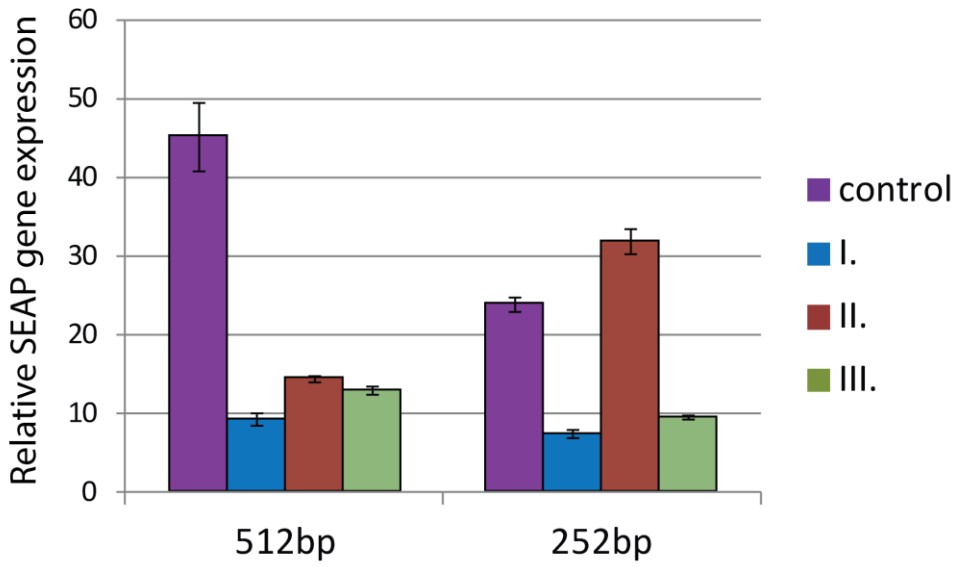


Figure III. 5: SEAP activity of intronic constructs (I., II., III.) located upstream of the regions with promoter activity (512bp and the shorter 252bp fragment). Intronic activity is compared with expression from single promoter regions, 512bp and 252bp (violet-control). The experiment was performed in triplicates and repeated three times (error bars = SEM).

The result was different depending on the length of the tenascin-W promoter used. For the long tenascin-W promoter, all intronic regions appeared to inhibit its activity, while region II had no effect on the shorter tenascin-W promoter. After exclusion of some transcription regulator binding sites from the 512bp fragment by truncation to 252bp, the promoter activity arising from the truncated region is no longer inhibited and even slightly enhanced by the presence of the second intronic module. In contrast, the first and third intronic modules negatively regulated the promoter activity regardless of the length of the promoter used.

Since we identified the minimal basal promoter of tenascin-W within 79bp upstream of the transcription start site (TSS) of the tenascin-W gene (Chiovaro et al., 2014) we also cloned the three conserved intronic regions upstream of this minimal promoter. To elaborate on possible differences between cell types and hence to assess how the cellular identity may influence the specificity of intronic activity on the promoter, we used human osteosarcoma cells (Krib), human

colon carcinoma cells (HCT116), human lung carcinoma cells (H1838) and HT1080 cells as model systems.

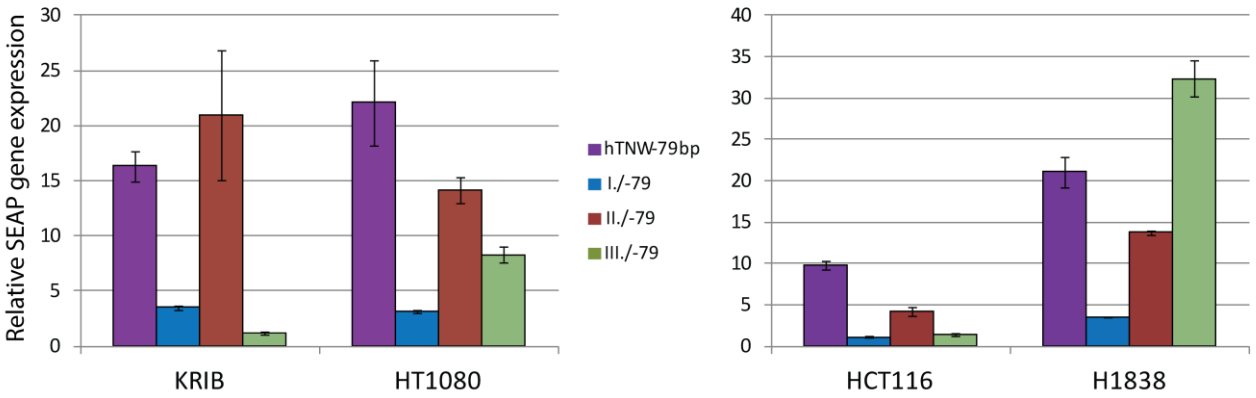


Figure III. 6: SEAP activity of intronic constructs (I.blue, +1178/+1909; II.red, +2920/+3447; III.green, +4561/+5539) located upstream the minimal promoter 79bp (violet). Intronic activity is compared with expression from single promoter region in the 79bp (hTNW-79bp). Experiments were performed in triplicates (error bars = SEM).

Tenascin-W mRNA expression was observed in HCT116 and H1838 cells (Hendaoui I., data unpublished), and given the association of tenascin-W with the osteogenesis process (120), Krib osteosarcoma cells were used. Results presented in Fig.III.6 show how the first conserved (blue) region exhibits a transcriptional repression of the minimal basal promoter (79bp) in all cell lines. The minimal promoter (79bp) by itself results in high levels of reporter expression in HT1080 and HCT116 cells and the presence of any of the three intronic regions upstream of the (79bp) minimal promoter leads to impaired induction of gene transcription. High induction of reporter activity by the third conserved region (green) is found in H1838 cells, whereas in Krib cells highest promoter activity is detected in presence of the second conserved region (red). Thus, the intronic regions seem to affect tenascin-W transcription in a cell type-specific manner and may thus contribute to the regulation of tissue-specific gene expression in vivo.

III. 2. 5 Intronic regions evolutionary conserved between human and mouse tenascin-W gene

Genomic sequences of tenascin-W orthologs share a common intron/exon organization with the same junction sites between different species (101).

To find possible local alignments of mouse and human intronic regions sequences were compared using the William Pearson's LALIGN program from the FASTA package (121).

Furthermore, the GC content was measured at the website http://tubic.tju.edu.cn/GC-Profile/ (Table III.3). The observed average identity of conserved sequences was 61.5 % (Table III.3). Changes in GC content reflect molecular evolution, giving suitable estimates of the sequence divergence among species (122). In addition, the content of CpG-islands positively correlates with gene expression levels (123). Evolutionary conservation of noncoding sequences can be indicative of transcriptional regulatory elements (124).

Table III.3. Features of human and mouse intron

Locus	Total Intron length	% GC content	% Identity Sequence
mTNW	5593 bp	45.38%	
hTNW	9448 bp	46%	61.5%

Interestingly, the sequence alignments produced by LALIGN program show a particularly high similarity (69.4%) between human and mouse loci in the intronic region +3290/+3450 (gray boxes) (Fig.III.7). This corresponds exactly to the second conserved region analyzed before and indicates a common regulatory function in mouse and man.

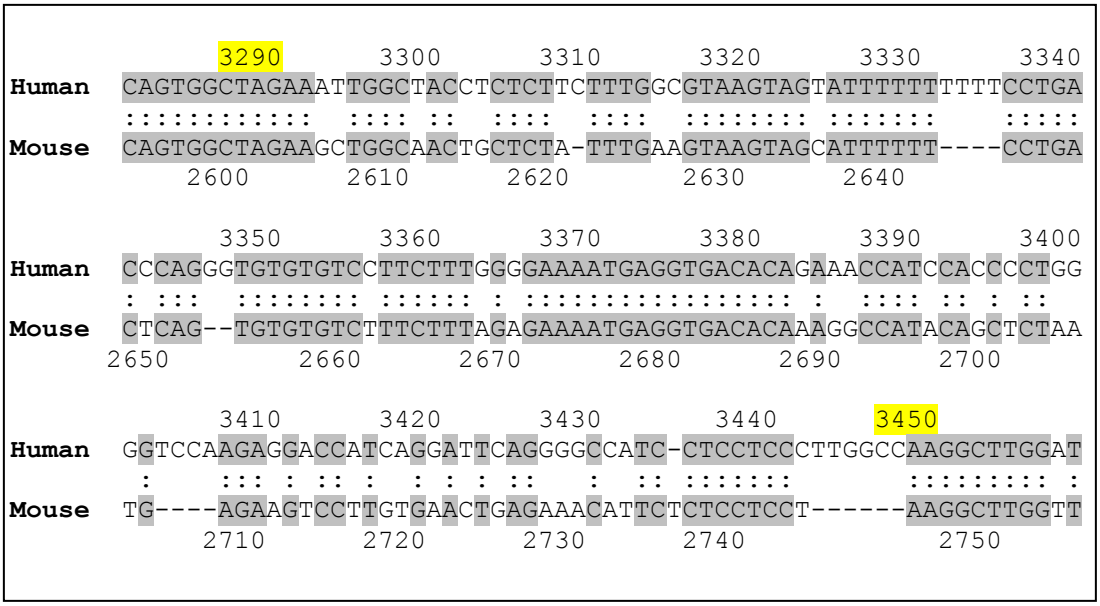


Figure III. 7: Sequence alignment of the +3290/+3450 region
Sequence alignment of the sequence (+3290/+3450) within the second intronic conserved region between human and mouse tenascin-W.

Due to the similarities between the human and mouse intronic sequences, we investigated the activity of the human intronic regions in mouse HC11 cells. Single intronic sequences or combinations thereof were cloned upstream of the SEAP reporter gene and these constructs were transiently cotransfected with a luciferase expression vector as internal standard for transfection efficiency. All values were compared to the negative control (pSEAP Basic, empty vector) and similarly to the results obtained in HT1080 (Chiovaro et al., 2014), the highest SEAP expression was observed with the shortest fragment within the second intronic conserved region (+3299/+3447). Thus, we conclude that the human intronic fragments show equal functional activity in a mouse in vitro-environment (Fig.III.8).

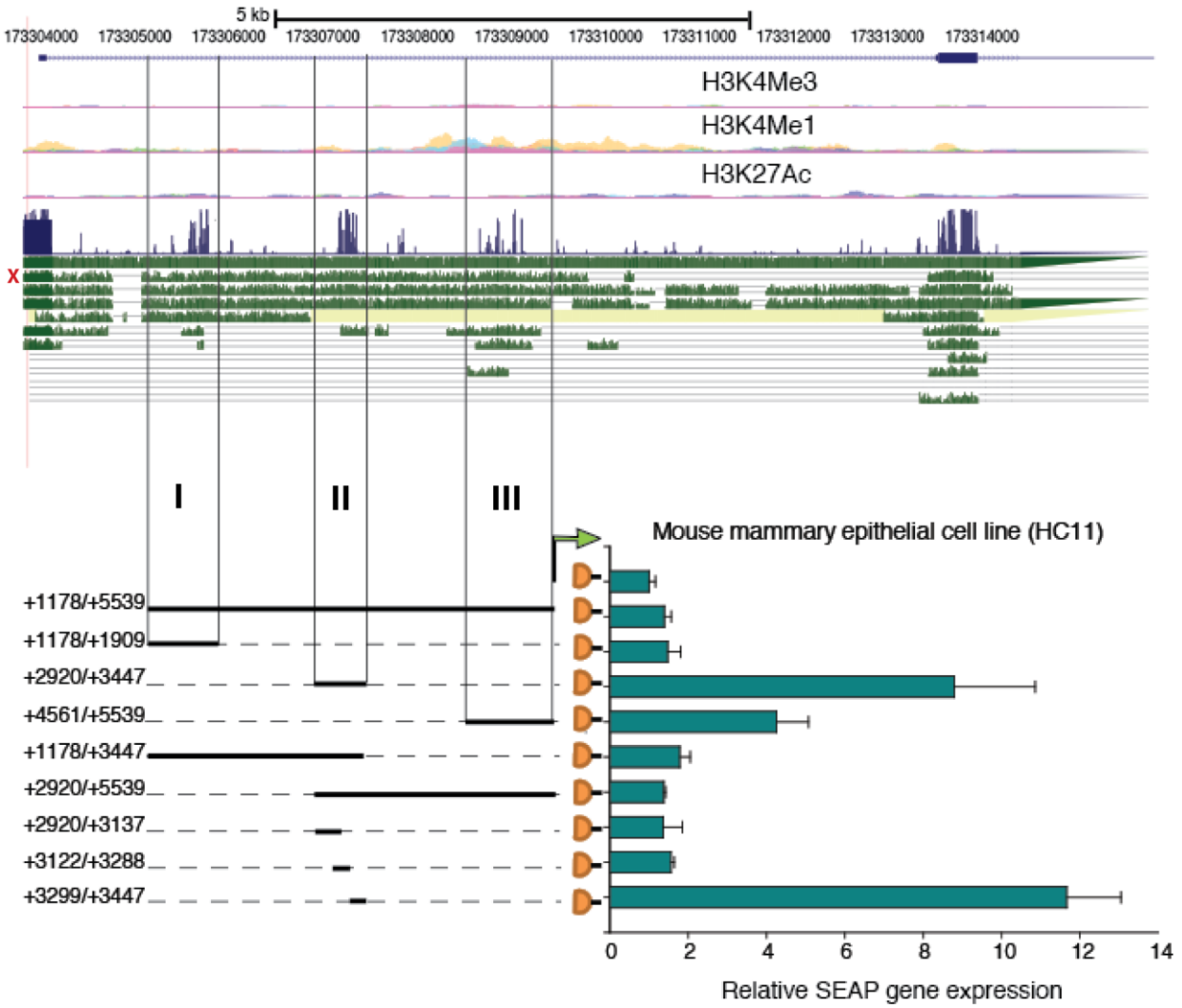
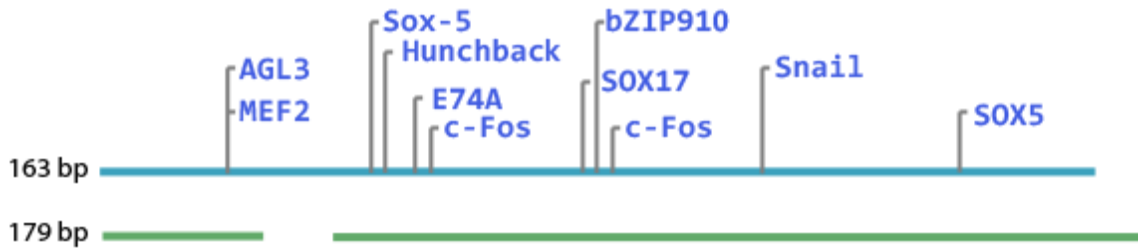


Figure III. 8: SEAP activity of different combination of human intronic regions transfected in HC11 cells. The second green line in the UCSC Genome Browser representation (indicated with a red cross, on the left) displays the evolutionary conservation track on the mouse. Basic vector is used as negative control. Experiment was performed in triplicates and repeated three times (error bars = SEM).

The in silico (Fig.III.7) and experimental (Fig.III.8) analysis of intronic regions show a very high functional similarity of the fragment +3299/+3447 in both mouse and human. Therefore, we performed transcriptional regulator binding site predictions using ConSite. Potential transcription factor binding sites, which are conserved between human and mouse tenascin-W gene, are shown in figure III.9.



Transcription factor	Human				Transcription factor	Mouse			
	Sequence	From	To	Strand		Sequence	From	To	Strand
AGL3	CTAGAAATTG	7	16	-	AGL3	CTATTTGAAG	26	35	-
MEF2	CTAGAAATTG	7	16	-	MEF2	CTATTTGAAG	26	35	+
Hunchback	ATTTTTTTTT	45	54	-	Hunchback	TTTTTTCCTG	45	54	-
Sox-5	ATTTTTT	45	51	-	Sox-5	ATTTTTT	44	50	-
E74A	TTTCCTG	53	59	-	E74A	TTTCCTG	48	54	-
c-FOS	CTGACCCA	57	64	+	c-FOS	TGACTCAG	53	60	-
SOX17	GAAAATGAG	86	94	-	SOX17	GAAAATGAG	79	87	-
bZIP910	ATGAGGT	90	96	+	bZIP910	ATGAGGT	83	89	+
Snail	GAGGTG	92	97	+	Snail	GAGGTG	92	97	+
c-FOS	GTGACACA	95	102	+	c-FOS	GTGACACA	85	90	+
Sox-5	AAACCAT	104	110	+	Sox-5	AAACCAT	136	142	+

Figure III. 9: Potential transcription factor binding sites in the second intronic conserved region
The potential transcription factor binding sites located in the region +3290/+3450, which are conserved between mouse tenascin-W (blue) and human tenascin-W (green) are shown. Their specific binding motif as well as their exact positions is indicated in the table (bottom).

III. 2. 5. 1 Conserved transcription factor binding sites in human and mouse tenascin-W gene orthologs

An overall profile of transcription factor binding sites displayed several elements associated with an enhancer-like activity. SOX binding sites are for instance found in the Nestin neural enhancer (125), and the the distal 5'-flanking region of the PDGF promoter contains an enhancer harboring an ELK/E74A motif (126), while AGL-3 factor drives BMP7 gene expression in kidney, eye, and limb by binding an enhancer region within its first intron (127), and finally Snail

negatively modulates the expression of E-cadherin, Claudins, Occludin and Mucin-1 genes by binding to the E-box enhancer sequence (128-130).

III. 2. 6 Transcription Start Site of the mouse tenascin-W gene

Expression of mouse tenascin-W following BMP2 treatment in HC11 cells (131), prompted us to use these cells to detect the TSS of the mouse tenascin-W gene. Rapid amplification of cDNA ends (RACE) was performed to identify the 5' end of the mouse tenascin-W transcript. Bands of 400bp were detected on the agarose gel after two rounds of PCR with the adapter specific inner primer (PCR anchor primer) and the nested gene specific reverse primers (Fig.III.10). Sequencing revealed that these fragments corresponded to the ATG-containing exon and a non-translated exon 1 consisting of 99bp. In the genomic DNA exon 1 is located 5593 bp upstream of exon 2.

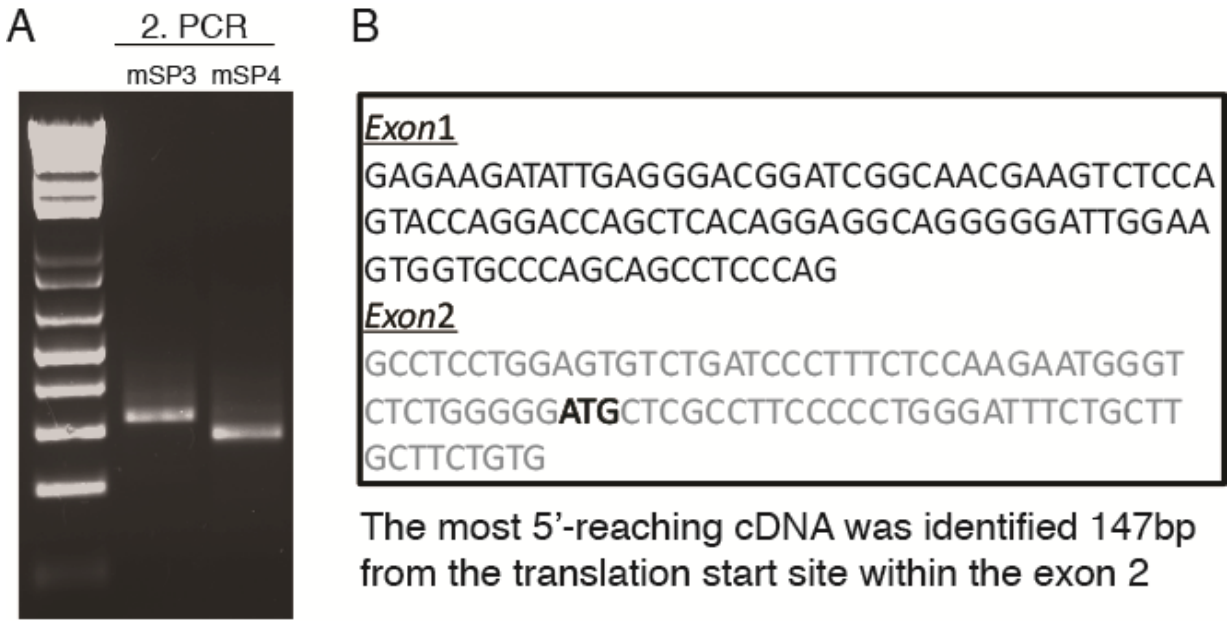


Figure III.10: 5'RACE PCR
(A) One microliter aliquot of a 1/20 dilution of the amplification product of dA-tailed cDNA was used as template for the second PCR. Second PCR was performed using a combination of two gene specific reverse primers mSP3 and mSP4. (B) Sequence of the first non-coding exon as found in 5'RACE is shown. It contains 99bp. Grey box indicates the second exon containing.

III. 2. 6. 1 Characterization of the mouse tenascin-W promoter

Our results allowed us to map the transcription start site upstream of the first non-coding exon of the tenascin-W gene in both human (Chiovaro et al., 2014) and mouse species. Prompted by the similarity between these two orthologous genes we decided to functionally characterize the mouse tenascin-W promoter region within 2kb upstream of the first exon as we already did for the human tenascin-W promoter (Fig.III.11). Various constructs containing this 2kb region and several truncated forms were transiently transfected in HC11 cells for 24h. The high SEAP reporter activity obtained from the construct including the 200bp upstream of the TSS implies that this fragment still contains the main sequence with promoter activity.

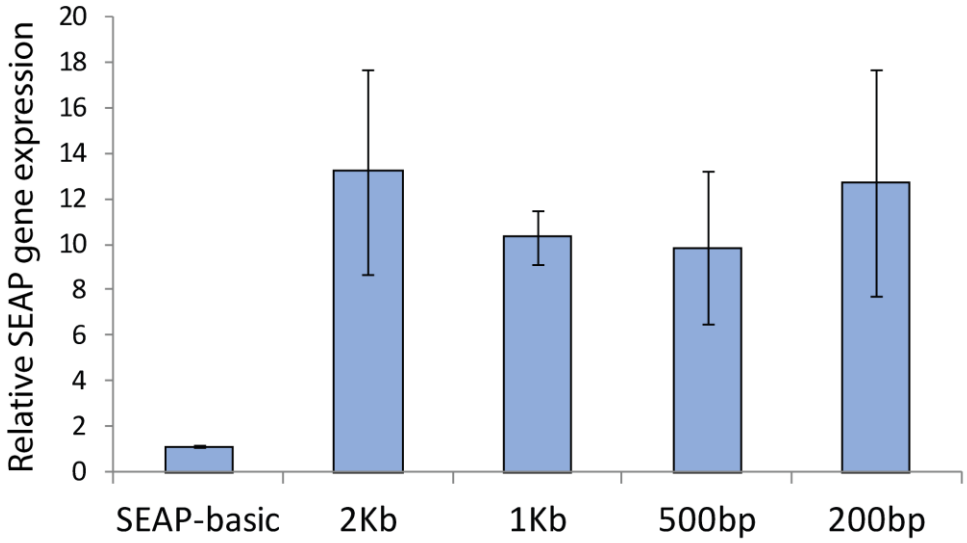


Figure III.11: SEAP activity of mouse tenascin-W promoter constructs. Gene expression is compared with the negative control (SEAP-basic). Experiment was performed in triplicates (error bars = SEM).

As expected, the sequence alignments generated by the LALIGN program between human and mouse promoter regions showed a high degree of conservation (76.7%) (Fig.III.12).In this figure, the sequences highlighted in gray show exon1. Comparison between human and mouse reveals the presence of a TATA box sequence in both species (dark blue). A conserved Smad binding site was observed 335bp (light green) upstream of the TSS in mouse, whereas, the human Smad motif (dark green) present in the minimal promoter (-79) (Chiovaro et al., 2014) did not show any alignment with the mouse sequence.

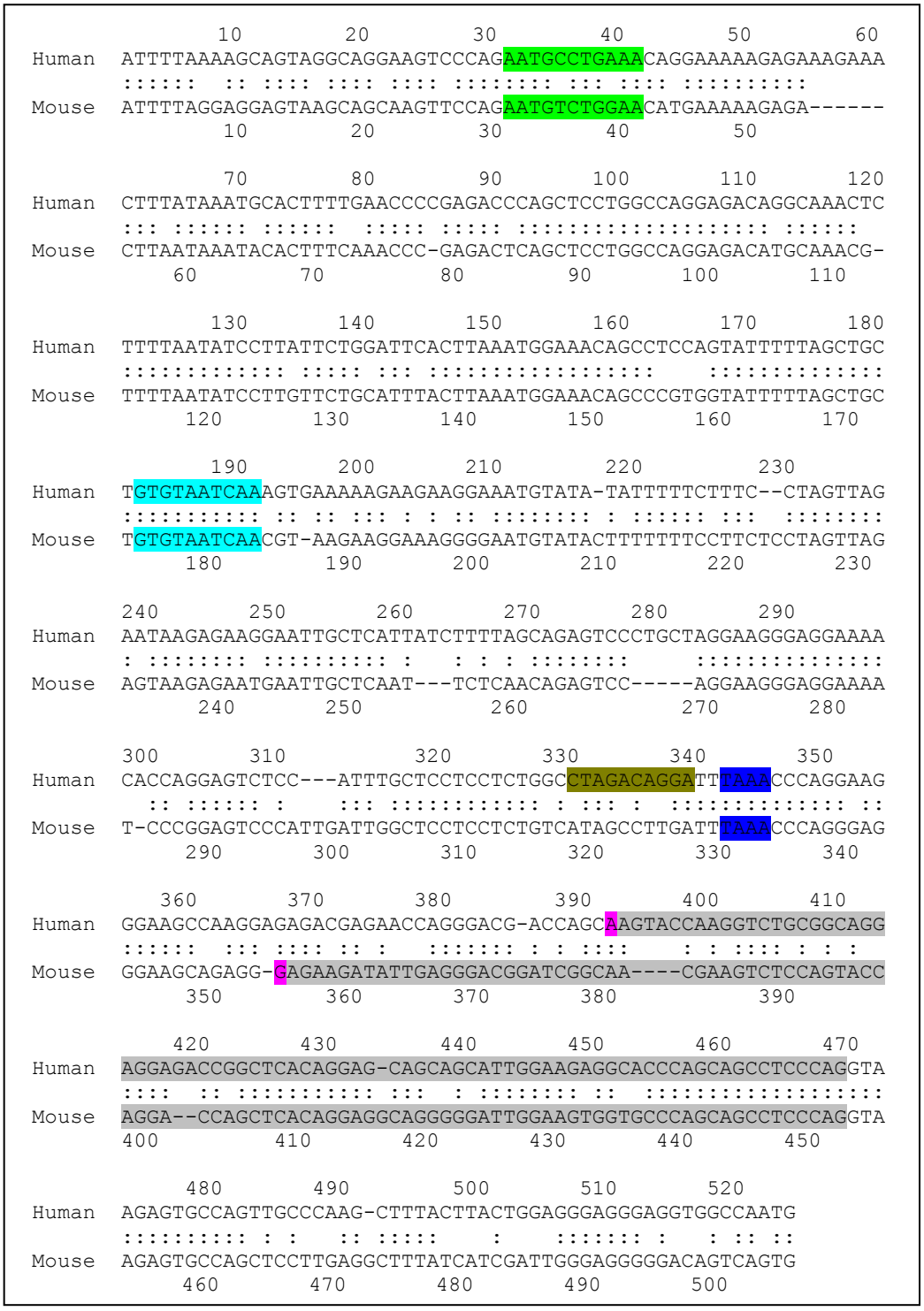


Figure III.12: Sequence alignment between human and mouse tenascin-W promoter regions (LALIGN program). Both human and mouse TNW gene are endowed by a TATA-box promoter (blue). In gray is underlined the exon 1. Conserved Smad motif (light green) in both species was detected 335bp from the mouse gene TSS (pink). The human Smad sequence for TGFβ signaling is indicated in dark green (Chiovaro et al., 2014). Bidirectional Hoxc-8 gene was found 193bp upstream of TSS in mouse tenascin-W promoter (light blue).

Moreover, we were able to identify a binding site for the transcription factor Hoxc-8 (light blue), placed 193bp upstream of the TSS of the mouse tenascin-W promoter. In this case the binding site is located on different DNA strands. In the mouse, the binding site for Hoxc-8 is located on the negative strand (TTGATTACAC), whereas, in human it is found on the positive strand (GTGTAATCAA). The different arrangement of binding sites could influence the binding preferences itself of transcription factor and in turn affect the gene regulation.

III. 2. 7 Tenascin-W expression in cell cultures

III. 2. 7. 1 Human osteosarcoma models

Previous studies in our lab aimed to detect endogenous human tenascin-W expression in human cell lines. Previous reports published in 2004 and 2005, have shown the expression of tenascin-W in primary mouse embryonic fibroblasts (MEFs) upon treatment with the growth factor BMP2 and the cytokine TNFα (104, 131). BMP2 also induced tenascin-W expression in mouse myoblast cells (C2C12), parallel to their differentiation into osteoblasts (104, 131). Although mouse embryo fibroblast cells were expressing tenascin-W, this was not the case for human fibroblast lines, such as Detroit (skin), MRC5 (lung) and IMR90 (lung), which did not show any detectable tenascin-W levels (Degen M., data unpublished). Given the well-known role of tenascin-W during embryonic bone development (120), we decided to investigate if human bone marrow stem cells (BMSCs) will express tenascin-W following their differentiation into osteoblasts. Indeed tenascin-W protein expression was detected in BMSCs treated with osteogenic medium (Martina E., data unpublished). Prompted by these results, we investigated human osteoblast models for tenascin-W expression. We tested whether the same osteogenic medium (OM) previously used for BMSCs could also induce tenascin-W expression in human osteosarcoma cell lines Saos-2 and U2OS.

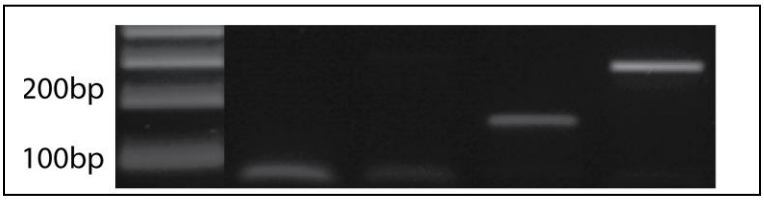


Figure III. 13: Detection of human tenascin-W mRNA in human osteosarcoma cell lines U2OS
The mRNA from untreated (first two columns) and OM-treated U2OS cells was isolated and tenascin-W expression was assessed by RT-PCR. TNW mRNA expression was shown by the bands at 149bp and 265bp.

The expression of tenascin-W in U2OS as well as in Saos-2 cell lines was assessed by RT-PCR. We were able to observe tenascin-W mRNA expression exclusively in U2OS cells after treatment with osteogenic medium (Fig.III.13). For this assay, we used two distinct pairs of primers for tenascin-W mRNA listed in table III.2. Moreover, tenascin-W protein expression was revealed by immunofluorescence staining of U2OS and Saos-2 cells kept in osteogenic medium for one week. In the first panel, (Fig.III.14-A) strong tenascin-W staining is displayed on the cell surfaces and as reticular fibers assembled around U2OS cells, whereas in Saos-2 cells the tenascin-W staining is weak and given the fact that no mRNA could be detected may represent background staining only (Fig.III.14-B).

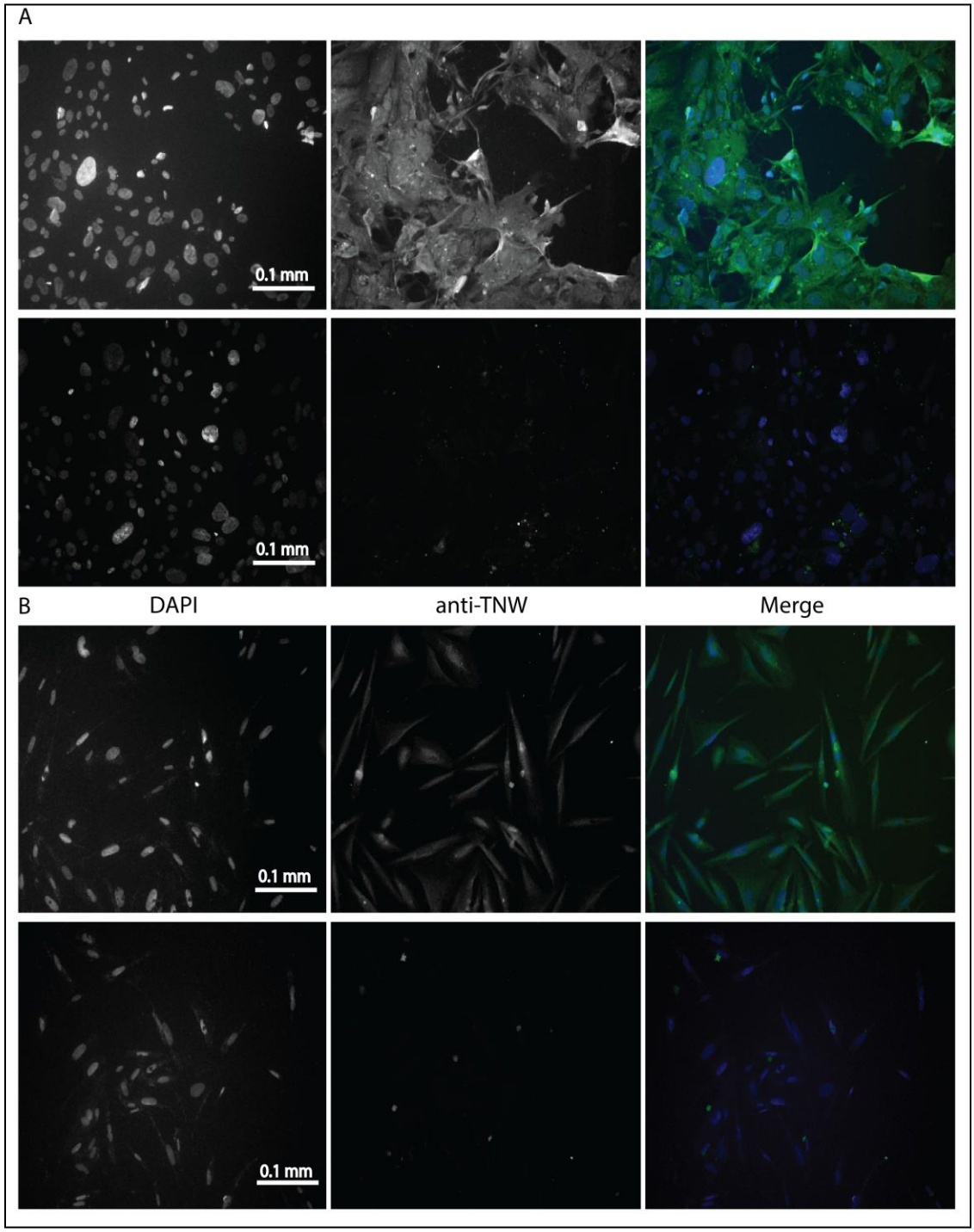


Figure III.14: Detection of human tenascin-W protein in human osteosarcoma cell lines
U2OS (A) and Saos-2 (B) cells were cultured in osteogenic medium (first and third row) or growth medium (second and fourth row) for one week. Cells were immunostained with anti-tenascin W monoclonal antibody and Alexa-488 secondary antibody for detection.

III. 2. 7. 2 Mouse and human mammary epithelial cells

The investigation of the role of tenascin-W in migration and proliferation of metastatic (4T1) or normal (HC11) murine mammary cell lines showed a specific role of BMP2 in inducing tenascin-W protein exclusively in HC11 cells (131). Therefore, we tested BMP2 as potential inducer of tenascin-W in mouse as well as human normal mammary epithelial cells.

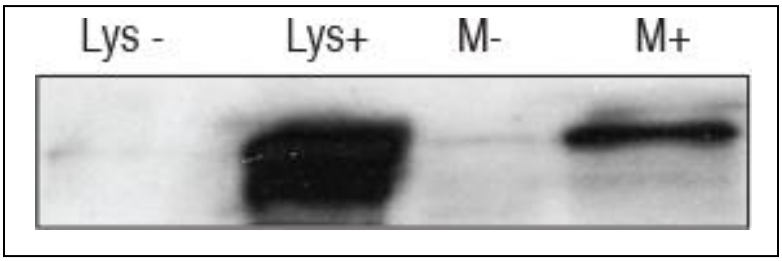


Figure III.15: Endogenous mouse tenascin-W protein expression in HC11 cells
HC11 cells were treated (+) or not (-) with BMP2 (100ng/ml) for 48h. Supernatants (M) and cell lysate (Lys) were analyzed for mouse tenascin-W expression.

We were able to detect tenascin-W protein both in cell extracts and conditioned medium of HC11 cells treated with BMP2 (Fig. II.15). In the case of the human mammary epithelial cells we analyzed the expression of tenascin-W in MCF10A cells treated or not with BMP2 by RT-PCR using cDNA from BMSC-derived osteoblasts (Martina E., unpublished data) as positive control (Fig.III.16). Indeed, the specific 149bp band was detected in BMP treated MCF10A cells by using TNW primers listed in table III.2.

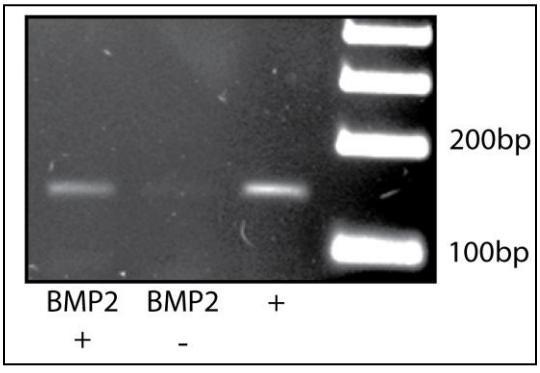


Figure III.16: Endogenous human tenascin-W mRNA expression in MCF10A cells
MCF10A cells were treated (+) or not (-) with BMP2 (100ng/ml) for 24h. cDNA from BMSCs differentiated in osteoblasts was used as positive control.

Although mRNA was detectable, we were not able to detect the endogenous protein in the corresponding cell line by immunoblotting.

III. 2. 8 Immunohistochemistry of bone metastases of breast cancer

Positive immunofluorescence stainings of mouse tenascin-W expression was performed on bone sections of mice harboring bone metastases of breast cancer (see Chiovaro et al., 2014). In addition, we investigated the distribution of tenascin-W protein in sections of metastatic bone by immunohistochemistry analysis using an antibody against mouse tenascin-W protein (Fig.III.17).

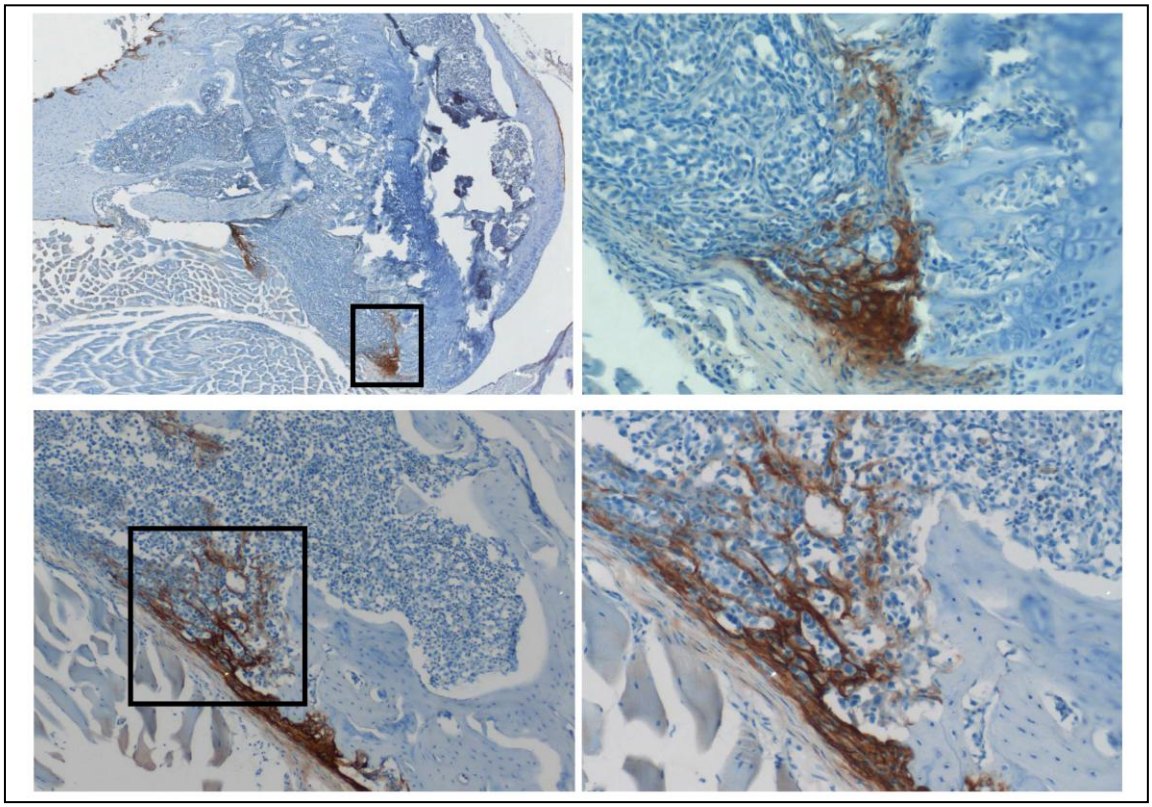


Figure III.17: Immunohistochemistry of sections of bone metastases of breast cancer
Immunohistochemical staining of mouse tenascin-W (brown). In the right panels a higher magnification (20X) of the section indicated by squares in the respective left panels is shown. Sections were counterstained with hematoxylin.

The tumor cells of the metastatic foci appear as a dense mass of cells exhibiting blue oval and round shapes. Tenascin-W protein, stained brown, seems to wrap and infiltrate the tumor nests. Staining of tenascin-W surrounding circular structures embedded in the tumor could suggest a potential role of tenascin-W in promoting the generation new blood vessels.

IV. Discussion

IV. 1 Tenascin-W as a component of adult stem cell niches in healthy and pathological conditions

Tenascin-W is the fourth and last discovered member of the tenascin family. Several reports have attributed to tenascin-W an important role in osteogenesis (120) and in periodontal ligament differentiation (132). Tenascin-W was also found in the adult stem cell niche of the aortic valve and in the periosteum of ribs (104). These different sites of expression, besides indicating the physiological conditions in which tenascin-W plays an active role, also highlight tenascin-W as a stem cell niche component involved in mechanisms of cellular differentiation (133). Although so far we have mentioned the bright side of tenascin-W, it is mostly cited for being a cancer biomarker in human solid tumors (109). The persistent tenascin-W expression in the stroma of colon and breast tumors and in gliomas, as well as the expression in the proximity of blood vessels has prompted us to explore tenascin-W in breast cancer metastasis to bone.

IV. 2 Tenascin-W expression in bone metastases of breast cancer: future directions of investigation

In our studies, we were able to identify tenascin-W as a component of the stromal niche hosting bone metastases of breast cancer. Our *in vitro* model tried to mimic what may occur when cancer cells located at a primary site start to metastasize. More precisely, circulating cancer cells, or also cancer cells still residing within the primary tumor site, can induce the establishment of a special distant niche to favor their subsequent metastatic engraftment by releasing a wide range of cytokines and growth factors into the bloodstream (134). As a consequence, the stromal compartment adopts an engraftment-permissive state following the arrival of the tumor-secreted factors. In particular, we observed the expression of tenascin-W protein following TGFβ-induced myofibroblastic differentiation of bone marrow stem cells. Moreover, we showed increased proliferation and migration rates of MDA-MB231-1833 breast cancer cells in the presence of recombinant tenascin-W protein. In our experiments, the role of tenascin-W on cancer cells was assessed independently of the stage of metastatic development. It would be interesting to investigate tenascin-W activity during each stage of breast cancer cell spreading in order to achieve a more refined framework of all different tenascin-W contributions in tumor and metastatic progression. Our experimental data may be oversimplified and in reality everything will be much more complicated, and a more holistic

observation would be needed to uncover specific cross-talks among several factors which are omitted in restricted *in vitro* systems.

IV. 3 How could tenascin-W influence the different developmental stages of cancer?

In our analysis of the transcriptional tenascin-W regulation, we have detected a promoter binding site for Smad4, which is a mediator of TGFβ signaling, as well as an intronic nGRE element. The roles of both of these regulatory sequences were experimentally investigated. Further analysis of the second intronic region (+3290/+3450) showed a binding site (E-box, GAGGTG) for the transcription factor Snail, which is conserved between human and mouse (Fig.III.9). Snail is transcriptionally controlled by signals including TGFβ, EGF, FGF, Wnt, TNF-α and other factors such as hypoxia or estrogen (135). It has been shown that Snail itself can form a complex with Smad2/3, acting as a repressor of the expression of cell-adhesion molecules, such as E-cadherin, occludin and claudin-3 (136). Moreover, Snail promotes the sprouting of endothelial cells *in vitro (137)* and the proliferation of tumor cells, for instance of melanoma cells, by inhibiting tumor suppressor CYLD (138).

In light of these data, it would be reasonable to speculate on one hand about an involvement of TGFβ in regulating the expression of both Snail and tenascin-W, and on the other hand about a potential enhancing/supportive role of Snail in the migration/proliferation-promoting activity of tenascin-W on breast cancer cells.

Interestingly, further immunohistochemistry of bone metastases of breast cancer sections revealed a remarkable tenascin-W protein expression surrounding the tumor mass, suggesting a possible involvement in blood vessel formation for the infiltration of tumor cells. This hypothesis is supported by many other previous studies which have detected a correlation between immunofluorescence staining of tenascin-W with von Willebrand factor in glioblastoma (108) and also a co-staining with CD31 in proximity to blood vessels in lung and kidney tumors (109).

Therefore, tenascin-W might sustain the dissemination and the subsequent engraftment of breast cancer cells to the bone by inducing angiogenesis. Following the initial promoting phase, TNW may regulate the proliferation of tumor cells during the metastatic process. This working model of tenascin-W activity, which follows a time-course of events in tumor development would need further investigation. It would be interesting to analyze whether the transcription factor

Snail acts synergistically with tenascin-W in the bone metastatic context by inducing tenascin-W transcription.

Thorough analysis of a role of tenascin-W in bone metastases of breast cancer could be performed by omitting or increasing its expression *in vivo*. However, it is well known that one issue regarding the knockout of ECM proteins is the compensation through other well-characterized matrix proteins. For instance, mice deficient for tenascin-C still present a normal skin wound healing and possibly tenascin-W has a compensatory role in inhibiting cell-adhesion, which is essential for tissue repairing (139). Also, within the matrilin protein family, a temporary regulation of matrilin-4 to compensate for the lack of collagen fibrillogenesis activity caused by the double knockout of the genes matrilin-1/3 was observed (140). Therefore, in both cases, no striking phenotype was detected. The ectopic expression of tenascin-W in normal tissue of transgenic mice could give some hints on its contributions during the appearance of a tumor and its metastatic progression. Alternatively, conditional mutants lacking the tenascin-W gene in the bone only, through Cre recombinase-mediated ablation, or the use of inducible siRNA/shRNA in mice harboring tumors could allow us to better monitor significant changes in tumor regression depending on the presence or absence of tenascin-W.

IV. 4 Targeting of TGFβ signaling pathway in bone metastases of breast cancer

Statistically, 70% of patients with advanced breast cancer display metastases preferentially in bones, which leads to clinical complications collectively referred to skeletal-related events, including significant tissue morbidity associated to osteolytic lesions, bone infiltration and pathological fractures (141). Bone tropism is also shown for prostate cancer; in this case however, unlike breast cancer, the tumor is responsible for an excessive osteoblast activity, which leads to atypical new bone formation (142, 143). The local breast tumor microenvironment contains the support for the metastatic spreading and it was shown to be enriched in TGFβ together with other factors (144-146). In our experiments we have shown a regulation of tenascin-W expression in the bone marrow metastatic niche by MDA-MB231-1833 cells through Smad4-mediated TGFβ signaling. Nevertheless, additional TGFβ can derive from osteoclastic bone resorption following the secretion of osteolytic factors from breast cancer cells (147). In turn, the release of TGFβ within the bone metastatic niche might indirectly support tumor cell proliferation through further induction of tenascin-W expression. Now the question to

address is how to keep this vicious cycle between breast cancer cells and osteoclasts under control.

Currently, the use of bisphosphonates has aimed to achieve a reduction in bone turnover and consequently to control bone resorption (148). Further potential interventions with the metastatic development have aimed at the possibility of interfering with the early stages of tumor progression. BMP7, unlike TGFβ, is an inducer of MET during for instance embryonic development (149) and it has been shown to counteract the ability of TGFβ to induce EMT in MDA-231 cancer cells (150). Moreover, BMP7 inhibits the invasive phenotype of MDA-231 cancer cells by interfering with TGFβ-induced integrin α5β3 expression (151). The direct targeting of TGFβ includes the use of the panTGFβ-neutralizing mouse monoclonal antibodies 1D11 and 2G7, or TGFβ receptor inhibitors impairing the kinase catalytic activity of TβRI/ALK5 including SB-431542, Ki26894 or LY364947 (147). In order to increase the efficiency of these molecules, it is possible to apply a combination therapy approach by using in addition various cytotoxic agents.

IV. 5 Counteracting the establishment of a stromal metastatic niche

One of the major problems associated with metastatic development is the inability to monitor it during its early stages of growth. This leads to the inefficacy of many drugs, which at advanced stages can no longer counteract the spread of cancer. The survival of tumor cells in the circulatory system is low and only a tiny fraction of cancer cell clones within the primary tumor is capable of colonizing a secondary organ (152). In addition, the migration out of the primary tumor site in order to find a permissive distant environment is the most delicate stage wherein cancer cells show their weakness and can be more sensitive to certain drugs conjugated with specific nanoparticles (153, 154). However, once arrived at the distant destination, what is really essential for the tumor cells is the interaction with the surrounding local stromal microenvironment, through which they can manifest their malignant features. Therefore, it is extremely valuable to take into account all possible strategies to handle the metastasis-promoting contributions arising from the metastatic niche.

Infiltrating tumor cells require oxygen and nutrients, which are supplied from newly formed blood vessels. Neovascularization in secondary sites is independent of tumor size and it can be modulated by tumor-secreted factors (155). In our experiments, immunohistochemical analysis

of bone metastases of breast cancer indicated a perivascular tenascin-W deposition pattern. This could suggest a role of tenascin-W in promoting tumor vascularization, which in turn would make tenascin-W a potential candidate for anti-angiogenic therapy. Moreover, as already mentioned, the increased ECM deposition in an altered stroma is caused by activated fibroblasts (CAFs). All the external signals are then transduced to the nuclear level by the ECM-integrin-cytoskeleton axis. In this regard, we have discussed the role of BMP7 in inhibiting TGFβ-induced integrin α5β3 expression (151) and, in addition, it has been shown that the blockade of α5β3 integrin receptors had a beneficial effect on breast cancer bone metastases by reducing the bone resorption (156). Treatment with the α5β3 integrin receptor inhibitor cilengitide showed a reduction in angiogenesis and in the localization of ECM proteins to blood vessels as well as an increased intratumoural vascular permeability, which collectively impaired osteoclast activity (156).

Vascular permeability highly influences the clinical outcome of drug delivery. For instance, the increased ECM deposition along the vessel walls with the consequent increased stiffness causes a high fluid pressure, which can impair drug penetration through tumor vessels. All current strategies based on the combination of anti-angiogenic and vascular normalization agents with traditional therapies aim to overcome and improve all those limits associated to the failure of chemo-radiotherapy.

IV. 6 Definition of human and mouse tenascin-W cellular sources

In summary our studies identified the tenascin-W protein as a component of the activated stem cell niche in the bone (working model, Chiovaro et al., 2014). Moreover, we sought for tenascin-W protein expression in human osteosarcoma cell lines Saos-2 and U2OS as well as in human mammary epithelial cells MCF10A. We were able to observe tenascin-W mRNA expression in U2OS and MCF10A cells (Fig. III, 13-16) and by immunofluorescence analysis tenascin-W was investigated in Saos-2 and U2OS cells (Fig. III.14). However, we did not take these cell lines into account as systems to explore tenascin-W regulation because of their low tenascin-W protein expression levels.

Previous studies have detected mouse tenascin-W protein expression in 4T1 epithelial breast cancer cells by TNFα, in MEFs and in HC11 mouse mammary epithelial cells by BMP2 (131). Therefore we used the HC11-expression system for the induction of tenascin-W by BMP2 for the isolation of mRNA to identify the transcription start site. This led to the identification of a TSS

at a first non-coding exon 147bp upstream from the ATG start codon present in exon2 (Fig. III.10). Promoter activity was located within 200bp upstream of the TSS (Fig. III.11), wherein, alignments produced by LALIGN program also showed a TATA box sequence (Fig. III.12). Further comparison analysis of mouse and human tenascin-W gene loci showed specific binding sites not only in the promoter but also in the first intron. Conserved repressive domains for glucocorticoid receptor (nGRE) located in the mouse intronic sequence could explain the similar negative modulation of gene expression driven by the first human conserved region transfected in HC11 cells (Fig.III.8). Many other transcription factor binding sites located in the region +3290/+3450 were conserved between mouse and human tenascin-W gene (Fig.III.9). Interestingly, the mouse promoter region was characterized by a unique exposure and rearrangements of binding sites for transcription factors. We observed a different placement (335bp upstream the TSS) for the mouse Smad motif (aatGTCTggaa) compared to the human one, which was located within the 79bp upstream the TSS. Moreover, in proximity of the mouse Smad motif we also observed a binding site for the homeobox gene Hoxc-8 (tacgttgATTAcacagcag). The Hoxc-8 transcription factor plays an important role in the mouse embryonic development wherein it is highly expressed in the limbs, backbone and spinal cord (157). Xingming Shi et al., have shown the direct interaction between Smad1 and Hoxc-8 detected by a yeast two-hybrid system and the direct control of the osteopontin gene transcription by BMP2 is mediated by a Hoxc-8 binding site (158). This could suggest a differential control of mouse tenascin-W by BMP2 signaling through Hoxc-8 and Smad1 depending on the context. Moreover, Hoxc-8 gene has been shown to play important roles in tumorigenesis and precisely, its depletion was associated with the inhibition of cell migration, invasion and metastasis of breast cancer cells (159). Given the tenascin-W expression in tumor stroma of breast cancer (105), we may speculate that Hoxc-8 could act as transcription regulator for mouse tenascin-W also in pathological conditions. The differential tissue-specific regulation of mouse as well as human tenascin-W may lead to a divergence of binding sites between human and mouse.

The differential tenascin-W gene transcription control can be dictated by species-specific physiology and behavior of human and mouse. It could be affected by the distribution and the different organization of transcription factor binding sites among orthologous promoters which in turn would imply the acquisition or loss of functional regulatory elements. Thereby, a thorough analysis of the evolutionary sequence conservation between species could have a significant impact for medical research.

V. Appendix

V.1 Experimental procedures (unpublished data)

Cell culture

Mouse mammary epithelial cells (HC11) were grown in RPM1640 medium supplemented with 10% FBS, 5ug/ml insulin and 10ng/ml EGF. Human mammary epithelial cells (MCF10A), kindly provided by Dr Mohamed Bentires-Alj (Basel, Switzerland) were cultured in DMEM/F12, 5% horse serum, 10ng/ml EGF, 0.5mg/ml Hydrocortisone, 100ng/ml cholera toxin and 10mg/ml insulin. Human osteosarcoma Saos-2, Krib and U2OS cells were cultured in α-MEM containing 10% FCS, 1% HEPES, 1% sodium pyruvate, 10 mM glutamine (control medium), supplemented with 5 ng/mL FGF2. Osteogenic differentiation medium consisted of control medium supplemented with 100 nM dexamethasone (cat #D-2915, Sigma), 10 mM β-glycerophosphate (cat #G-9891, Sigma), and 100 µM ascorbic acid 2-phosphate (cat #A-8960, Sigma). Colorectal carcinoma cells (HCT116) were grown in Mc Coy's 5A medium supplemented with 10% FBS. Human lung carcinoma cells (H1838) were cultured in RPMIB medium which was including RPMI 1640 with 1.5g/L sodium bicarbonate, 2mM glutamine, 1mM sodium pyruvate and 10mM HEPES.

Cloning of intronic reporter constructs upstream the human tenascin-W promoter

Intronic conserved regions were inserted into NheI/HindIII sites of the pSEAP-basic vector, whereas human tenascin-W promoter sequences had NheI/XhoI sites for directional cloning (Chiovaro et al., 2014). Promoter constructs pSEAP-TNW (-512bp), pSEAP-TNW (-252bp), and pSEAP-TNW (-79bp), were digested first with NheI enzyme and after 1h at 37^{o}C to inactivate the enzyme function the product was incubated at 72^{o}C for 15min. In order to convert the fragmented DNA into blunt-end, it was treated with DNA polymerase I, Klenow enzyme in the presence of dNTPs for 1h. Second digestion was performed with MluI restriction enzyme for 1h at 37^{o}C. To obtain intronic inserts, digestion with MluI and NruI enzymes was carried out. The restriction fragments were gel purified.

Immunofluorescence staining of human osteosarcoma cell lines

Saos-2 and U2OS cells were cultured on 8-chambers slide (BD Falcon) either in control or in osteogenic medium for one week. Cells were then fixed for 15 min in 4% formaldehyde at RT, rinsed with PBS and additionally fixed for 10 min in methanol at -20°C. After 30 min blocking at

RT in blocking buffer (PBS/0.01%Tween, 1% BSA), cells were incubated over night at 4°C in blocking buffer containing the primary antibody. After three washes in PBS, cells were incubated for one hour at RT in PBS/0.01%Tween containing the Alexa-488-conjugated secondary antibody (1:200) and Hoechst (1:2000). Cells were then washed three times with PBS and mounted in ProLong Gold Antifade reagent (Invitrogen). Pictures were acquired using an Axio Imager Z2 LSM700 confocal microscope (Zeiss) and processed with ImageJ.

Mouse tenascin-W protein detection

HC11 cells were seeded at a density of $7x10^4$ into 6 well with RPM1040 10% HS and incubated overnight. Subsequently, the medium was replaced with RPM1040 serum free, cointaing or not BMP2 (100ng/ml) for induction of mTNW. Following 48h from the treatment, supernatants and cell lysates were solubilized for 5min at 95°C in SDS-PAGE sample buffer, followed by electrophoresis on 6% gel polyacrilamide gels. Proteins were electro-transferred onto PVDF membranes (Millipore). After blocking for 1h at room temperature in TBS with 0.05% Tween and 5% skim milk powder, membranes were incubated overnight with the polyclonal rabbit anti-mouse TNW primary antibody, washed and then incubated for 1h with the appropriate secondary antibody coupled with HRP (1/10 000). Specific staining was revealed using ECL kits (Amersham). HC11 cells treated with BMP2 express mTNW.

5'RACE for transcription start site determination of mouse tenascin-W gene

To characterize the 5' end of mouse tenascin-W mRNA sequence, the 5'/3' RACE kit, 2^{nd} Generation (Roche) was used according to the manufacturer's instructions. mRNA was isolated from HC11 cells previously treated with BMP2 (100ng/ml) for 48h by using Dynabeads mRNA Direct Kit (Invitrogen). cDNA was synthesized from 1μg of mRNA using the mouse tenascin-W specific primer mSP1. To identify the 5' end of TNW mRNA gene specific primers were used. The resulting PCR fragments were ligated into pBluescript KS vector (Stratagene) and sequenced.

Table V. 1: Sequences of the primers used for the 5' RACE

5' RACE	
mSP1	5'-TGGCAGCAGCGGTTGGTATTGCAC-3'
mSP2	5'-GCGGTGTCTGAAGACGGATGTTGTG-3'
mSP3	5'-CCATCATCGCTGAGTGACTGTGGG-3'
mSP4	5'-GGCACGTCAATCTTGTAGGTGTGG-3'

Immunohistochemistry

Immunohistochemistry experiments were performed on Ventana DiscoveryXT instrument (Roche Diagnostics, Manheim) with the procedure Research IHC DAB Map XT. Frozen slides were thaw for 20 min on air and placed in the machine where they were covered with Reaction buffer (Roche Diagnostics) before starting the run. In the instrument, slides were fixed with Formal-Fixx 1:2 (Thermo scientific) for 12 minutes. Rabbit anti-mouseTNW was applied manually at a dilution of 1:100 and incubated for 1 hour at 37°C. For the detection we used ImmPRESS anti-rabbit Ig (peroxidase) polymer reagent (Vector laboratories MP-7401) which was manually applied and incubated for 32 minutes at 37°C. Sections were finally counterstained with Hematoxylin II and bluing reagent (Roche Diagnostics) for 4 min, dehydrated and mounted with Neo-Mount (Merck UN 1268).

Primer for +3299/+3447 deletion		Position
FW:	**5'-CAGGATGAGATAATAAGGGA-3'**	(+3299/+3447)
RV:	**5'-TTCCCCAAAGAAGGACACAC-3'**	
FW:	**5'-CAGGATGAGATAATAAGGGA-3'**	(+3299/+3349)
RV:	5'-CTTTATACTTAAATTCAGGG-3'	
FW:	5'-ATAAAGGTCTGTAACAGTGGCT-3'	(+3344/+3394)
RV:	5'-ACGCCAAAGAAGAGAGGTAG-3'	
FW:	5'-TTGGCGTAAGTAGTATTTTT-3'	(+3394/+3447)
RV:	**5'-TTCCCCAAAGAAGGACACAC-3'**	

Site-Directed Mutagenesis for +3394/+3447	
I.	FW:5'-GCGTGctag**AGAGTGATCA**GTAGTATTTTTTTTTTCCTG-3' RV:5'-AAAATACTAC**TGATCACTCT**ctagCACGCGTAAGAGCTC-3'
II.	FW:5'-gCTTGGCGTAA**AGAGTGATCA**TTTTTTTCCTGACCCAGGG-3' RV:5'-CAGGAAAAAA**TGATCACTCT**TTACGCCAAGctagCACGC-3'
III.	FW:5'-GTAGTATTTT**AGAGTGATCA**ACCCAGGGTGTGTGTCCTTC-3' RV:5'-CACCCTGGGT**TGATCACTCT**AAAATACTACTTACGCCAAGc-3'
IV.	FW:5'-TTTTTTCCTG**AGAGTGATCA**TGTGTCCTTCTTTGGGG-3' RV:5'-GAAGGACACA**TGATCACTCT**CAGGAAAAAAAAAATACTAC-3'
V.	FW:5'-GACCCAGGGTG**AGAGTGATCA**TTTGGGGAAAAGC-3' RV:5'-CTTTTCCCCAAA**TGATCACTCT**CACCCTGGGTCAGGAAAA-3'
VI.	FW:5'-TGTGTCCTTC**AGAGTGATCA**CTAagctTCGAATCGCGAATTCG-3' RV:5'-CGATTCGAagctTAG**TGATCACTCT**GAAGGACACACACCCTGGGTC-3'

Table III.1: Sequences of the primers used for the 5' RACE

RT-PCR for human TNW		Size bp
I.	FW: 5'- AAGCCAGGAGAGGCATACAAGG-3' RV: 5'CAGGAGATGGTGGCGGTATTCT-3'	149 bp
II.	FW:5'- AATGCCCTCACAGAAATTGACAG-3' RV:5'- GTGTCAGCCTTCTTGCTCTC-3'	265 bp

Table III.2: Sequences of the primers used for the RT-PCR

V.2 List of abbreviations

5-LO	5-lipoxygenase
α-SMA	α-Smooth muscle actin
BMP	Bone morphogenetic proteins
BMSCs	Bone marrow derived stem cells
CAFs	Cancer associated fibroblasts
DCC	Dextran-coated charcoal
E-box	Enhancer box sequence
EMT	Epithelial-mesenchymal transition
EndMT	Endothelial to mesenchymal transition
GAGs	Glycosaminoglycans
GCs	Glucocorticoids
HA	Hyaluronan
HGF	Hepatocyte growth factor
HIFs	Hypoxia-inducible factors
HPSE	Heparanase enzyme
HS	Heparan sulfate
HSCs	Hepatic stellate cells
ILK	Integrin-linked kinase
LOX	Lysyl oxidase
LPS	Lipopolysaccharide
mef2c	Myocyte enhancer factor 2C
MET	Mesenchymal-epithelial transition
MKL1	Megakaryoblastic leukemia-1
MMPs	Matrix metalloproteases
MSCs	Mesenchymal stem cells
MSl1	Musashi homolog 1
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NFAT5	Nuclear factor of activated T cells 5
NGF	Nerve growth factor
nGRE	Negative glucocorticoid response element
Nox1	NADPH oxidase 1
Otx2	Orthodenticle homolog 2
PDGF	Platelet-derived growth factor
PGs	Proteoglycans
PH	Partial hepatectomy
Prrx1	Paired-related homeobox 1
PSE	Proximal sequence element
RACE	Rapid amplification of cDNA ends
RGD	Arg-Gly-Asp
ROS	Reactive oxygen species
SBE	Smad-binding element
SLRPs	Small leucine-rich repeat proteoglycans
SRF	Serum response factor
TGFβ	Transforming growth factor beta
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor
TSS	Transcription start site
VEGF	Vascular endothelial growth factor

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V.4 Curriculum Vitae

Personal details

Name: **Francesca Chiovaro**

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Education

10.2014 – 03.2015 ***PhD*** at the Friedrich Miescher Institute for Biomedical Research (FMI), Basel, in the group of ***Prof. Dr Ruth Chiquet-Ehrismann***. Focus on ***extracellular matrix proteins in cancer.***

07.2010 Degree of Master of Science at the Department of Cell Biology and Development, Palermo, Italy. Specialization in **Molecular Biology.**

10.2008 – 06.2009 Master thesis with practical work at the Centre for Molecular Biology of the University of Heidelberg , **ZMBH**, in the group of ***Prof. Victor Sourjik*.**

09.2007 Faculty of Biology at the University of Palermo.
Diploma thesis in the group of ***Prof. Dr. Rainier Barbieri.***

1998 – 2002 Maturità, Liceo Statale, Socio-Psico-Pedagogico,
G. A De Cosmi. Palermo, Italy.

Languages

Italian Native language

English Good knowledge, reading and conversation

Technical expertise

- ✓ Molecular biology and biochemistry techniques:
    - DNA cloning, PCR, qPCR, Max-midi-mini prep, Site-Directed Mutagenesis, RNA-DNA Protein extraction/ purification, Trichloroacetic Acid (TCA) protein precipitation, Western blot, Northern blot.
- ✓ Bacterial cell culture
- ✓ Primer extension
- ✓ S1 mapping
- ✓ 5' Rapid amplification of cDNA ends (5'RACE)
- ✓ Reporter gene assay
- ✓ Animal Cell Culture of primary mouse cells, cell lines, ES cells
- ✓ In vitro culture techniques
    - Co-culture
    - Cell-conditioned medium experiments
    - Trans-well Assays
- ✓ BrdU Cell Proliferation Assay
- ✓ Cell Migration assay
- ✓ Immunofluorescence

Good command of:

- ✓ Fluorescence Activated Cell Sorting (FACS)
- ✓ Fluorescence microscopy and image acquisition

Computer skills

- Expert with ***PC***
- ***Electronic data analysis*** and ***Biostatistics*** software (Sigma Stat, Sigma Plot)
- ***Microsoft Office Suite*** software (Excel, Word, PowerPoint)
- ***Adobe Illustrator and Photoshop***, photo editing
- **Software for in-Silico Molecular Cloning, DNA analysis**, BioEdit, SerialCloner, PerlPrimer, Chromas lite
- Image processing and analysis (ImageJ, Axiovision)

Attended conferences and presentations

2014 *Speaker*: Matrix Biology Europe Conference, Rotterdam, Netherlands. Title of talk: Tenascin-W expression in bone metastases of breast cancer (June 21-24, 2014).

2013 *Poster Award*: FASEB, Science Research Conference on Matricellular Proteins in Development, Health, and Disease. Saxton River, USA (July 28-Aug.02, 2013).

2013 *Poster*, "Joint PhD Meeting Friedrich Miescher Institute for Biomedical Research-Wien Biocenter Campus. Wien, Austria (June 20-23, 2013).

2013 *Poster*, Annual Meeting of the German Society for Matrix Biology in Tübingen, (DGMB), Germany (March 07-09, 2013).

Publications

Francesca Chiovaro, Enrico Martina, Alessia Bottos, Arnaud Scherberich, Nancy E. Hynes and Ruth Chiquet-Ehrismann. "Transcriptional regulation of tenascin-W by TGF-beta signaling in the bone metastatic niche of breast cancer cells". *IJC* journal, 2015

Review article: Francesca Chiovaro, Ruth Chiquet-Ehrismann, Matthias Chiquet. "Transcriptional regulation of tenascin genes". *Cell adhesion & Migration* journal, 2015

References

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