Tenascins – prominent molecules in tumor stroma

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Martin Degen

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Prof. Dr. Ruth Chiquet-Ehrismann

Prof. Dr. Nancy Hynes

Prof. Dr. Markus Rüegg

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Prof. Dr. Hans-Peter Hauri
Dekan

"What we know is a drop. What we don't know is an ocean.
If I have seen further it is by standing on the shoulders of giants."

Isaac Newton (1643 – 1727)

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SUMMARY

I. Summary

A very special connective tissue surrounds and interweaves solid tumors, the tumor stroma. Initially thought to only have a passive role, there is now more and more evidence accumulating that the tumor stroma plays an active function in the process of tumor progression as well as tumor initiation. Activated stromal cells express pro-proliferative paracrine signals to the epithelial cells, stimulate angiogenesis and can even show loss of tumor suppressor genes. Therefore, understanding the complex crosstalk between the epithelium and the tumor stroma might reveal novel therapeutic targets. Tenascin-C is known to be specifically expressed in the stroma of a variety of tumors triggering different stromal reactions required for tumorigenesis such as angiogenesis. We found in the stroma of mouse mammary tumors the induction of a second member of the tenascin family, tenascin-W. Moreover, we detected human tenascin-W in different human neoplasms, and could correlate its expression in breast cancers with tumor grade. Tenascin-W is enriched in low-grade breast cancers whereas the presence of tenascin-C does not correlate with tumor grade. Functionally, we could show that presence of tenascin-W does influence the cellular behavior of cancer cells. Fibroblasts adhere on tenascin-W in a β1-integrin-dependent way, and cancer cell migration towards fibronectin is stimulated by addition of tenascin-W.

In order to evaluate the significance of tenascin-W to act as tumor marker, we established a sensitive sandwich-ELISA to measure serum tenascin-W levels. By screening sera of healthy volunteers and sera from cancer patients, we could detect elevated serum tenascin-W levels in non-metastatic colorectal and non-metastatic breast cancer patients. However, not all cancer patients did show this increase. Furthermore, we found high tenascin-W expression in a large fraction of colorectal cancer extracts, but complete absence in normal colon mucosae. This suggests that tenascin-W is a better tumor marker for colorectal cancer than tenascin-C, which is also expressed in the normal colon mucosa. These observations warrant a follow-up study to evaluate the potential diagnostic or prognostic relevance of tenascin-W in colorectal cancer.

Glioblastomas are very aggressive human cancers. We found that glioblastomas frequently harbor amplifications at the *Notch2* locus and express high levels of Notch2 protein, which coincided with the presence of tenascin-C, an established prognostic marker for

glioblastomas. A conserved potential RBPJk binding motif was found in the tenascin-C promoter sequence, and by luciferase assays we could identify tenascin-C as a new RBPJk-dependent Notch-2 target. Furthermore, we could induce endogenous tenascin-C by transfecting fibroblasts with the intracellular Notch2 domain. These data imply a novel oncogenic function of Notch2 by inducing tenascin-C, a molecule enhancing cancer cell motility.

INTRODUCTION

II. Introduction

Formation and maintenance of multicellular structures requires a supporting framework to hold cells together. On the one hand, this is achieved by cell-cell junctions, which directly link neighboring epithelial cells and in addition to mechanical attachment serve many more purposes, such as force transmission to the cytoskeleton and generation of cell shape and polarity [1]. On the other hand, an essential part of the supporting framework is supplied by the extracellular matrix (ECM), a complex network of proteins which cells secrete and organize around themselves (Fig.II.1A). In the past the ECM was simply viewed as passive component functioning as inert scaffolding to stabilize the physical structure of tissues. During the last couple of years, however, it has become evident that the ECM is a very active and dynamic structure: it is subject to constant renewal; it has architectural roles during development and tissue repair; it regulates all the important cellular processes such as cell proliferation, differentiation, apoptosis, adhesion, and migration; it provides positional and environmental information essential for tissue maintenance and function; it harbors growth factors or growth factor-binding proteins, therefore playing an active role in their mobilization. Finally, there is strong evidence of its importance during a diversity of diseases, including cancer. The development and normal functioning of multicellular organisms depends on constant communication and interactions with molecules in the environment, including growth factors, cell adhesion molecules and various components of the ECM (for reviews see [2-4]). Therefore, careful analysis of the structure and function of specific components of different ECMs is a very important prerequisite to understand the molecular mechanisms of tissue interactions.

Connective and epithelial tissues represent two extremes of organization (Fig.II.1B). Polarized epithelial cells are tightly bound together via specialized cell junctions forming the epithelial sheets which line all surfaces and the internal cavities of the body. Underlying the basal surface of epithelial cells, there is a thin but dense layer of ECM, known as the basement membrane (BM) (Fig.II.1C/D). Its major functions include the separation of epithelial tissue from the interstitial matrix and the provision of adhesive sites for integrins expressed on epithelial cells thereby giving epithelial cells a polarized structure. The BM is assembled from proteins secreted largely by the epithelial cells. The main components include

type IV collagen, laminins, perlecan and nidogen [5]. In contrast to the BM in epithelial tissues, the ECM in connective tissue, which often carries mechanical load, takes up a large volume and the cells that produce this matrix, mainly fibroblasts, are sparsely distributed within it (Fig.II.1).

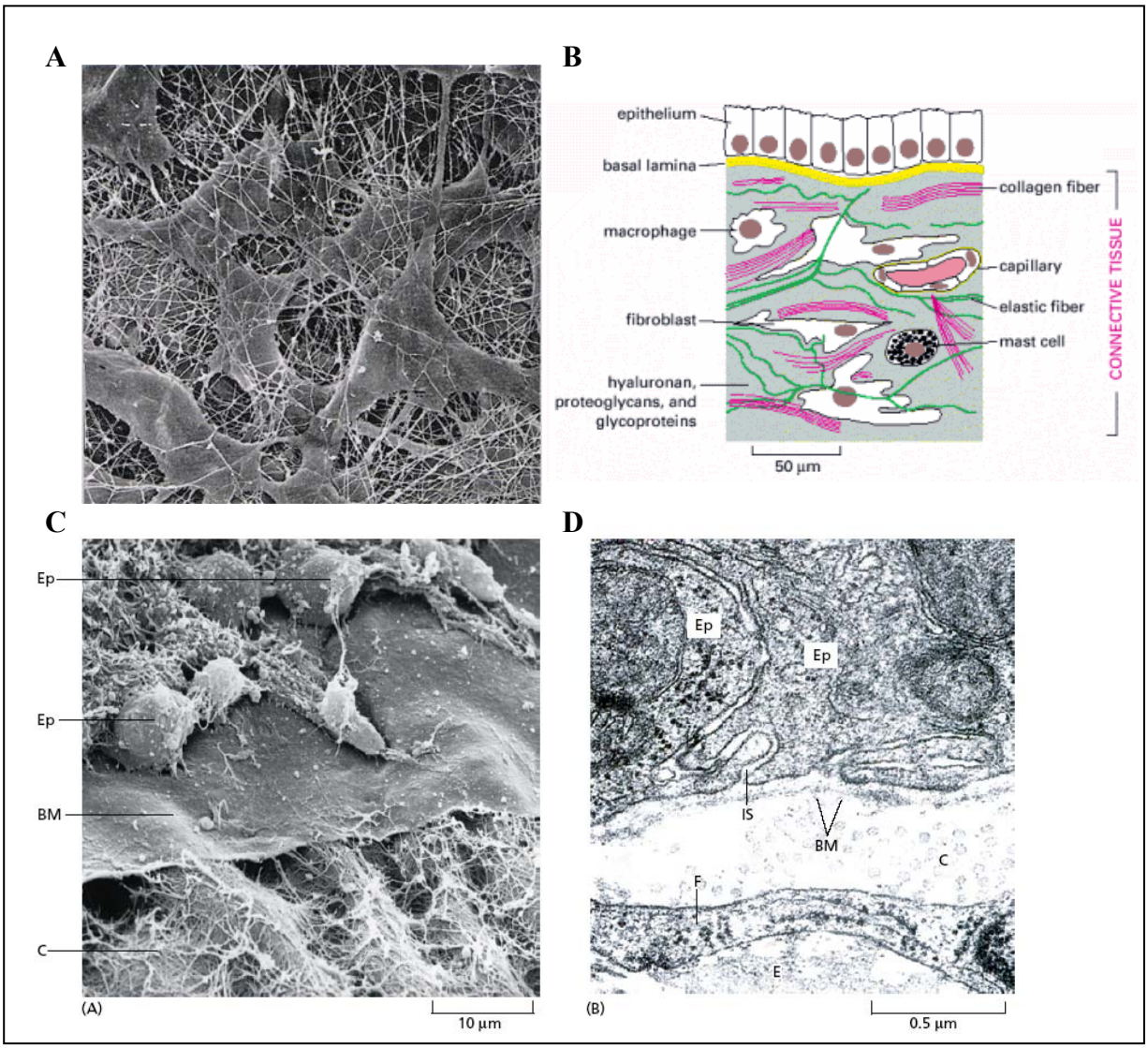

Figure II. 1: Epithelial and connective tissue

(A) Scanning electron micrograph shows fibroblasts embedded into a complex meshwork of proteins which they secrete and organize around themselves [6]. **(B)** Schematic representation of the epithelial cell sheet which is separated from the connective tissue by the basement lamina. Taken from [7]. **(C)** Scanning electron micrograph of a chick corneal epithelium. The basement membrane (BM) separates the epithelial cells (Ep) from the underlying connective tissue, which is composed of stromal cells and a network of collagen fibers (C). **(D)** Epithelium of a mouse trachea viewed at higher magnification through a transmission electron microscope. Epithelial cells (Ep) are located above the BM, while below are collagen fibrils (C), a fibroblast (F), and elastin fibers (E). The BM stays intact at the intercellular space (IS). Pictures (B) and (C) are taken from [8].

There is a considerable variation in the composition and amount of the ECM produced in different tissues, leading to an astonishing diversity in their physical properties: e.g. the BM in kidneys serving as a filtration barrier, resilient ECM of tendons, the cushioning, shock-absorbing ECM of cartilage in our joints, and the mineralized matrix of bones and teeth.

Essentially, there are three main classes of proteins that make up the ECM in mammals: (i) fibrous collagens, (ii) proteoglycans, and (iii) glycoproteins. Collagens, the most abundant proteins in mammals, form long triple-stranded helical structures and are packed together into thick fibers which provide tensile strength. Other collagens (e.g. type XII collagen), do not form fibrils, but associate with them and link them to one another and to other ECM components. Proteoglycans (e.g. aggrecan) are proteins to which different negatively charged polysaccharide chains, called glycosaminoglycans (GAGs), are covalently attached, thereby creating highly charged structures. This negative charge attracts osmoticly active cations leading to the recruitment of water. As a consequence, a swelling pressure is produced enabling the matrix to withstand compression (e.g. in cartilage). Glycoproteins (e.g. fibronectin, laminins, tenascins) fulfill cross-linking functions to other ECM molecules or to cells by means of their multiple binding domains.

To sum up, in order for each organ to successfully fulfill its characteristic functions within a given organism, all cells must be integrated into a framework which gives them structural and signaling input. Cells must know their exact position and environment and they must be able to send out and receive the correct signals at the right time and place. If successful, this leads to tissue homeostasis. Failures in generating homeostasis result in a wide spectrum of dysfunctions, including cancers. The focus of the first part of the introduction is on tumor development and progression with an emphasis on the tumor microenvironment rather than on the cancer cell itself. Thereafter, the tenascins, a family of ECM proteins, are introduced, which are well recognized to play an important role in the development of organisms as well as in different pathological situations [9].

II.1 Tumors and their microenvironment

Conventional knowledge states that carcinogenesis is a multistep process that reflects the acquisition and accumulation of mutations or epigenetic changes leading to genetic alterations (reviewed in [10-12]). In their landmark review, Hanahan and Weinberg (2000) enumerated six traits, probably shared by all types of human tumors that collectively dictate malignant growth. However, the order in which these capabilities are reached seems to be variable. These capabilities are (i) self-sufficiency in growth signals, (ii) insensitivity to anti-growth signals, (iii) resistance to apoptosis, (iv) limitless replicative potential, (v) sustained angiogenesis, and (vi) acquisition of properties required for tissue invasion and metastasis. Generally, there are three critical gene classes whose alterations are responsible for tumor progression: oncogenes, tumor suppressors, and caretaker (stability) genes. Mutations in oncogenes lead to the activation (or even to a constitutive activation) of the gene in conditions where the wild-type gene is normally not active. Such activation can arise from intragenic mutations, chromosomal translocations or gene amplifications. On the other hand, tumor suppressor genes show a reduced activity when altered, which renders them insufficient in counter-balancing the "growth-promoting" genes. Epigenetic silencing, miss-sense mutations, deletions or insertions can cause inactivation of these genes. Unlike oncogenes, tumor suppressors follow the "two-hit hypothesis" [13], which implies that both the maternal and paternal allele have to be mutated to manifest the phenotype. Genes that ensure the repair of subtle mistakes that may occur during DNA replication, belong to the class of caretakers or stability genes. Their function is to keep the general mutation rate in a cell to a minimum. As a consequence, mutations in caretaker genes result in an increased mutation rate, rendering oncogenes and tumor suppressors more prone to deleterious mutations.

A large amount of effort has been invested in the discovery and molecular analysis of genes belonging to any of these classes and the signaling pathways cancer cells are abusing for malignant growth (Fig.II.2). Research was clearly focused on the cancer cell itself and has resulted in considerable advances of our knowledge underlying the basic mechanisms for tumor formation and cancer therapies. However, recently it has become apparent that tumors are not just accumulations of carcinoma cells but represent very complex systems [14, 15]. In

carcinomas (solid, epithelial cell-derived tumors), neoplastic epithelial cells coexist with the surrounding connective tissue, the so-called tumor stroma which is composed of various cell types and is rich in ECM. During tumorigenesis, stromal components create a complex, tumor-permissive microenvironment that neoplasms require for their full manifestation [16, 17]. For a long time, it was believed that the tumor stroma only plays a passive role in the development of a tumor, even though the contribution of the microenvironment to tumor progression was already postulated in 1889 by Stephen Paget's "seed and soil" hypothesis [18]. By analyzing 735 cases of breast cancer, he found that metastases formed in the liver far more often than in any other organ. Hence, he proposed that breast tumor cells (seeds), although transported throughout the vasculature, only can live and grow where they find a permissive environment (soil).

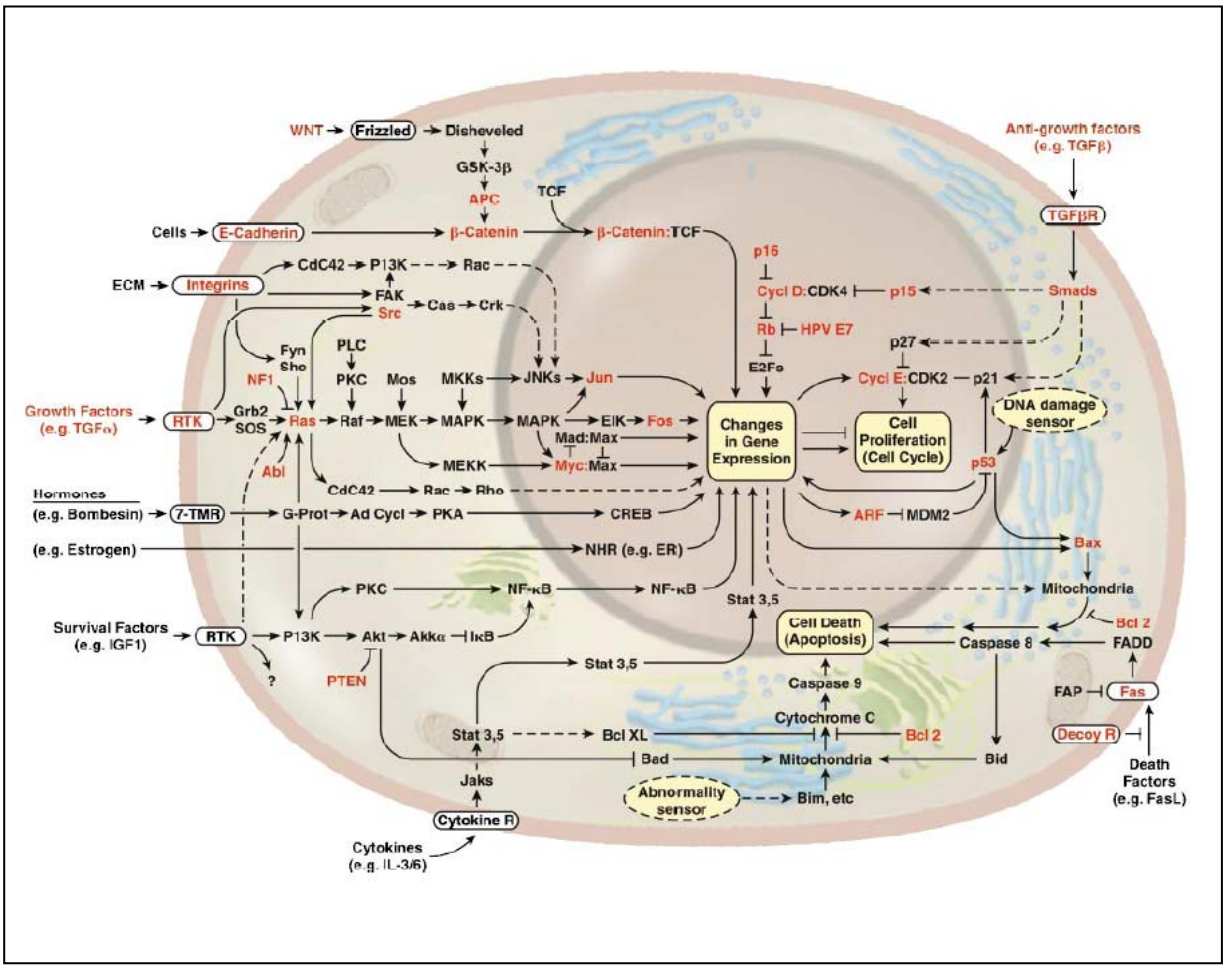

Figure II. 2: Signaling pathways within a cell

Emergent picture of the complex signaling pathways within a cell is shown. Genes that are known to be altered in cancer are highlighted in red [12].

As mentioned earlier, the stromal microenvironment is composed of a complex framework including a specific type of ECM, the tumor matrix, as well as several cell types such as those that are able to respond to inflammation (lymphocytes, macrophages, mast cells), those which are important for the vasculature (endothelial cells, pericytes, smooth muscle cells), and fibroblasts, which are the main producer of the ECM. The amount of stroma and its composition varies greatly from tumor to tumor, but does not correlate with the degree of malignancy. Nevertheless, it has become clear that each cell type present in the tumor stroma can have a variable influence upon tumor progression depending on tumor type (for reviews see [14, 16, 17, 19-23]).

II.1.1 The normal microenvironment acts as a tumor suppressor

The function of the stromal microenvironment under physiological conditions is to generate and maintain tissue homeostasis. Proper tissue architecture is established by the BM, a specialized matrix separating the epithelial cells from stromal cells, thereby suppressing inappropriate mixing of cells from different tissue types. Recently, the general notion about the BM has changed from simply being a structural barrier to represent a much more dynamic structure influencing cellular behavior. Therefore, the BM provides structural support and acts as a signaling substratum to orient cells through integrin-based adhesions, and it determines cellular apical and basal surfaces to the epithelium (reviewed in [5]). Proper signaling and communication with the surrounding ECM and neighboring cells keeps the cells constrained within one tissue compartment. Overall, the normal stromal microenvironment provides essential signals for a structured, differentiated epithelial phenotype as well as for proper adhesions (Fig.II.3A). In early-stage tumors, it can even act as a non-permissive barrier to block or delay tumorigenesis. For instance it was shown that disorganization and disruption of the periglandular BM and hemidesmosome structures in breast tissue correlates with malignant phenotype [24]. In *Drosophila*, loss of epithelial polarity induced by the absence of a protein called scribble, triggers neoplastic transformation [25, 26]. Therefore, it is hypothesized that as long as proper tissue architecture can be maintained, it is very difficult for a cancer cells to overcome this constraint [15]. Thus, an intact microenvironment has the capability to act as a powerful tumor suppressor, even in the presence of transformed epithelial cells. This tumor-suppressing property of the microenvironment was already

postulated a long time ago [27]. Subcutaneous injection of embryonal carcinoma cells into mice resulted in the formation of teratocarcinomas. In contrast, the same cancer cells injected into a blastocyst gave rise to phenotypical normal chimeric mice, indicating that, although pre-disposed to malignancy, the blastocyst environment might have acted as a tumor suppressor and kept the malignancy in check [27].

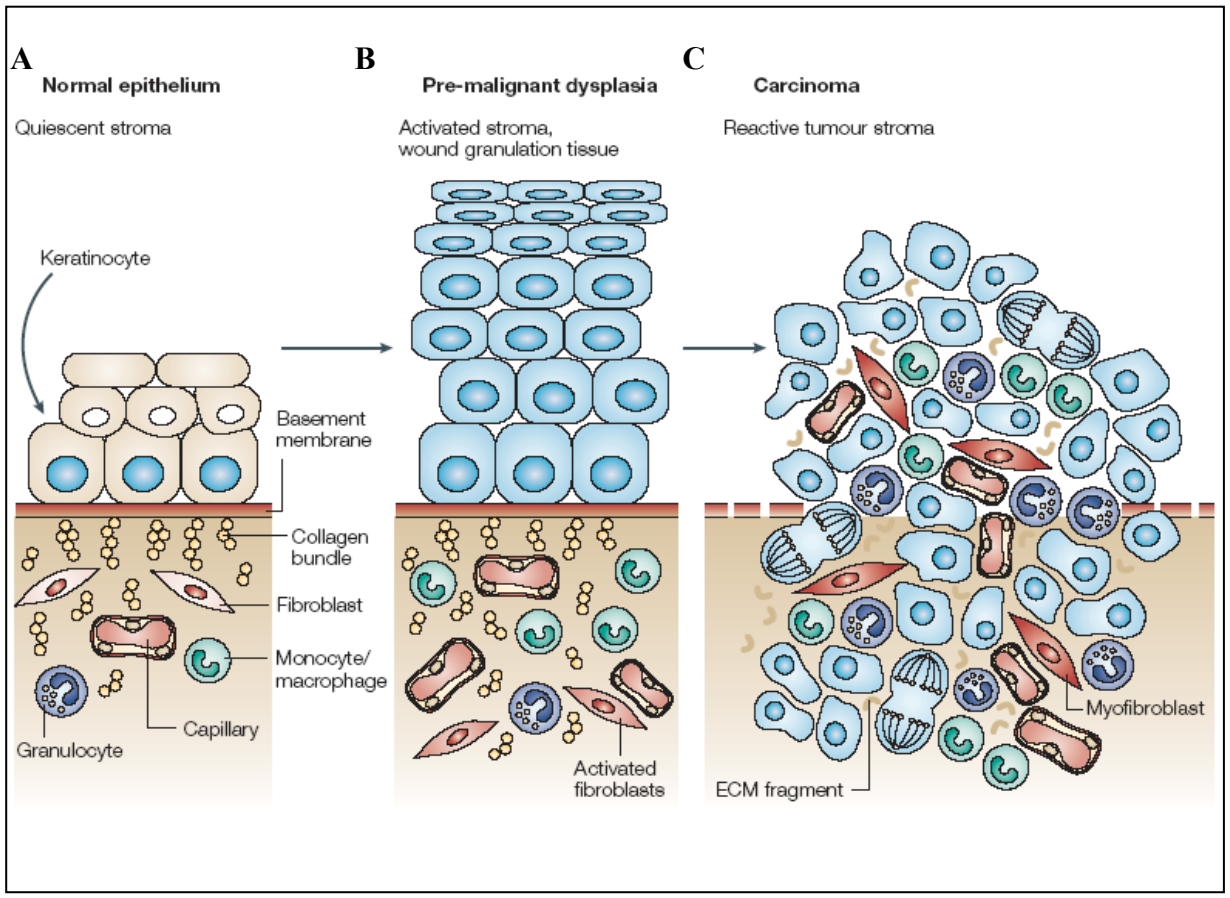

Figure II. 3: Transition from the normal to the reactive stroma

(A) The well-differentiated epithelium is separated by a BM from the underlying stromal compartment, which includes ECM proteins, fibroblasts, endothelial cells and leukocytes. **(B)** Pre-malignant dysplasia in the epithelium results in disturbed tissue homeostasis. Although the BM remains intact, fibroblasts are activated and stromal changes occur. **(C)** Cross-talk between the epithelium and the stroma through soluble effector molecules leads to secretion of proteolytic enzymes resulting in the breakdown of the BM, the differentiation of activated fibroblasts to myofibroblasts, and to ECM remodeling, thereby forming a congenial tumor microenvironment [16].

II.1.2 Activation of the stroma

Cancer cells possess the ability to alter their adjacent stroma to form a tumor permissive environment, called activated or primed stroma [14, 16, 17, 20, 23]. Therefore, it is not surprising that during the transition of a normal epithelium to pre-malignant conditions stromal changes occur, which have been initiated by soluble tumor cell-derived factors (Fig.II.3B). Initially, the BM remains intact, still separating the two compartments, one consisting of neoplastic epithelial cells and the other of stromal cells. However, cancer cells start to produce and secrete factors such as basic fibroblast growh factors (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor beta (TGFβ) or hepatocyte growth factor (HGF). These factors act alone or in concert to stimulate both stromal and tumor cells as paracrine and autocrine signals. Consequently, these factors trigger the induction of stromal reactions, including angiogenesis [28], inflammatory responses [21, 22], or activation of fibroblasts [19], finally leading to a congenial stroma for tumors.

Activation of fibroblasts eventually leads to their differentiation into myofibroblasts (Fig.II.3C) or so-called cancer-associated fibroblasts (CAFs). They possess highly increased contractibility and they produce smooth muscle α-actin which makes them distinguishable from normal fibroblasts (Fig.II.4A) [19]. CAFs start producing specific ECM components such as SPARC (secreted protein acidic and rich in cysteine), fibronectin containing an extra domain a, and tenascin-C. These proteins are usually not expressed or only to a limited amount under physiological conditions. Tenascin-C for instance influences many different stromal reactions including angiogenesis, immune and inflammatory responses and it provides oncogenic signals to the cancer cells leading to changes in their behavior (reviewed in [9] and see chapter II.2.3.1 “Tenascin-C and cancer”). Furthermore, CAFs secrete growth factors, among them the tumor-growth promoting and angiogenesis enhancing stromal-cell-derived factor 1 (SDF1) [29, 30], which acts by direct paracrine stimulation via the CXCR4 receptor expressed on carcinoma cells and on endothelial precursor cells (EPCs) [30]. Finally, CAFs produce cytokines, serine proteases and matrix metalloproteinases (MMPs), which mediate processes such as ECM remodeling, activation of the inflammatory response, and

stimulation of tumor angiogenesis by attracting EPCs. As a consequence, a congenial tumor microenvironment is formed, which facilitates tumor progression (Fig.II.4B). Activity of proteolytic enzymes leads to the breakdown of the BM and to the mixing of the cells from the epithelial compartment with stromal cells, and to the degradation of stromal ECM proteins. Thus the sustained presence of CAFs and other activated stromal cells in the tumor stroma has harmful consequences for tissue homeostasis, ultimately leading to an environment which enables tumor cell migration and invasion [19].

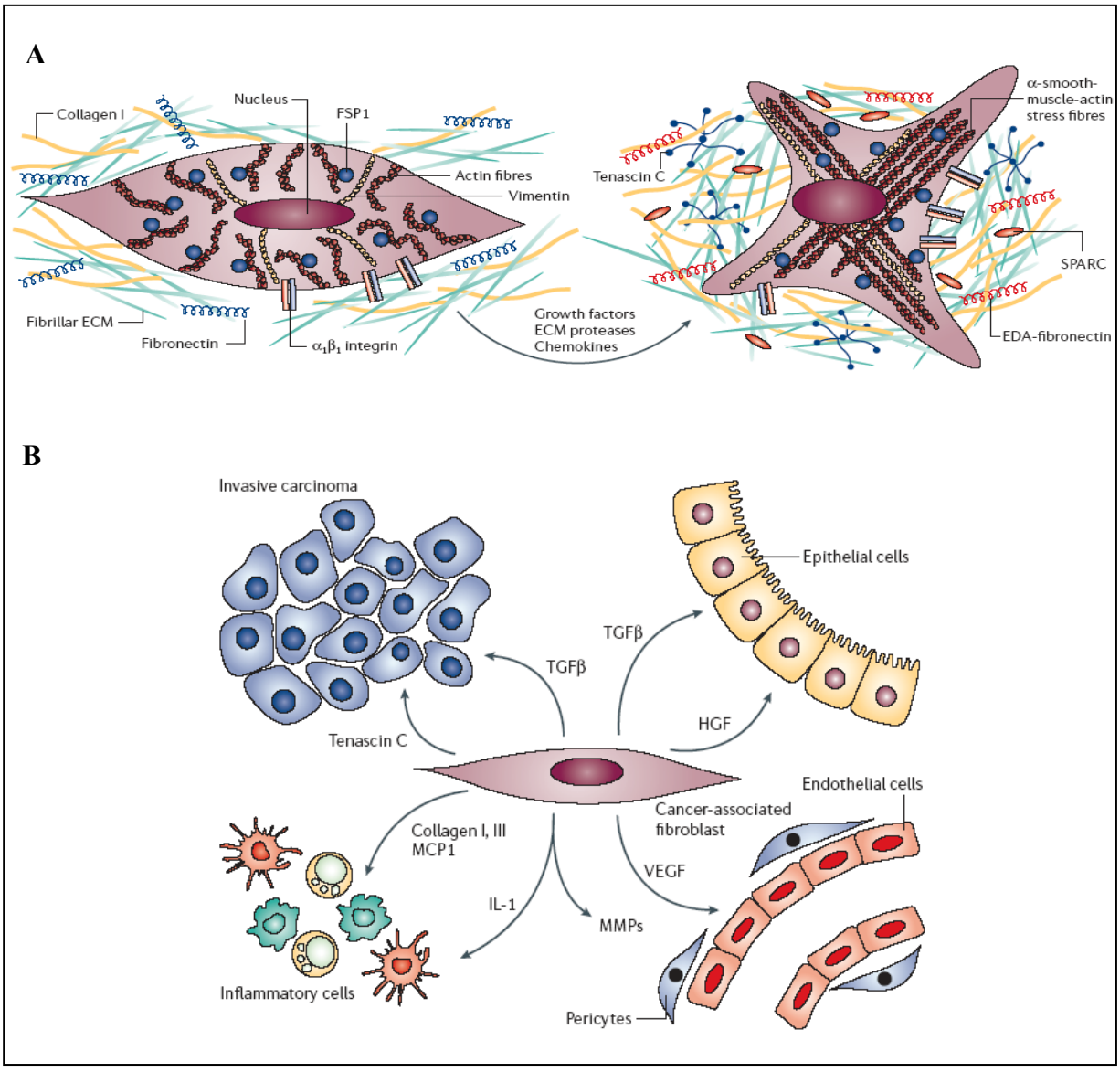

Figure II. 4: Cancer-associated fibroblasts

(A) By acquiring an activated phenotype (right), fibroblasts alter their genetic program and their microenvironment. **(B)** Activated fibroblasts influence different stromal reactions through soluble growth factors, cytokines, chemokines or ECM molecules such as tenascins [19].

Accompanying or preceding the stromal changes, cancer cells alter their morphology from a well-differentiated to a migratory and invasive phenotype. During this process of epithelial-mesenchymal transition (EMT), cells downregulate their epithelial-specific tight and adherens junction proteins and start expressing mesenchymal molecules instead. As a result, cells lose their cell-cell contacts which is required for cell invasion and motility (for reviews see [31-33]).

Normal stromal cells can acquire activated phenotypes also under non-tumorigenic conditions by various stimuli that arise when tissue injury occurs, when the tissue architecture is disrupted or in events of acute inflammation. Under these non-tumorigenic conditions, there is a discrete order of events. Usually, the injured epithelial cells are the source of the chemical signals that trigger and maintain a host response with the goal to heal the damaged tissue. These factors include the pro-inflammatory cytokines, tumor necrosis factor α (TNFα) or TGFβ1. As a consequence, resident mast cells, macrophages and dendritic cells are activated and recruited to the site of tissue damage. As a second step there is infiltration of effector immune cells (lymphocytes) which are responsible for specific immune responses. Once these cells are activated, they immediately release soluble mediators such as cytokines, chemokines, proteases and reactive oxygen species (ROS), which activate and recruit endothelial and mesenchymal cells to sites of tissue injury to form new blood vessels and collagen matrices (Fig.II.5A) (reviewed in [21, 22, 34]).

This process of stroma activation is successfully mimicked by cancer cells resulting in a striking histological and mechanistical similarity between an activated tumor stroma and the healing wound environment (Fig.II.5B). Moreover, epidemiological data indicate that more than 15% of human malignancies arise in association with chronic inflammation, viral or bacterial infections [21], which further highlights the contribution of the activated stroma to tumor progression. For example, *helicobacter pylori* infection has been linked to gastric cancer, hepatitis B virus to hepatocellular carcinoma, and chronic pancreatitis to pancreatic carcinomas [22]. However there are major differences between a non-cancerous activated stroma and the activated tumor stroma. In a healing wound, the activated state of the involved cells is reverted once the repair is completed. During carcinogenesis, however, the events occurring in the process of a healing wound become chaotically disorganized. The regulatory

mechanisms to revert the activated cell state back to normal are lost leading to the sustained presence of activated stromal cells. Therefore, a concept of tumors as "wounds that do not heal" was postulated [35].

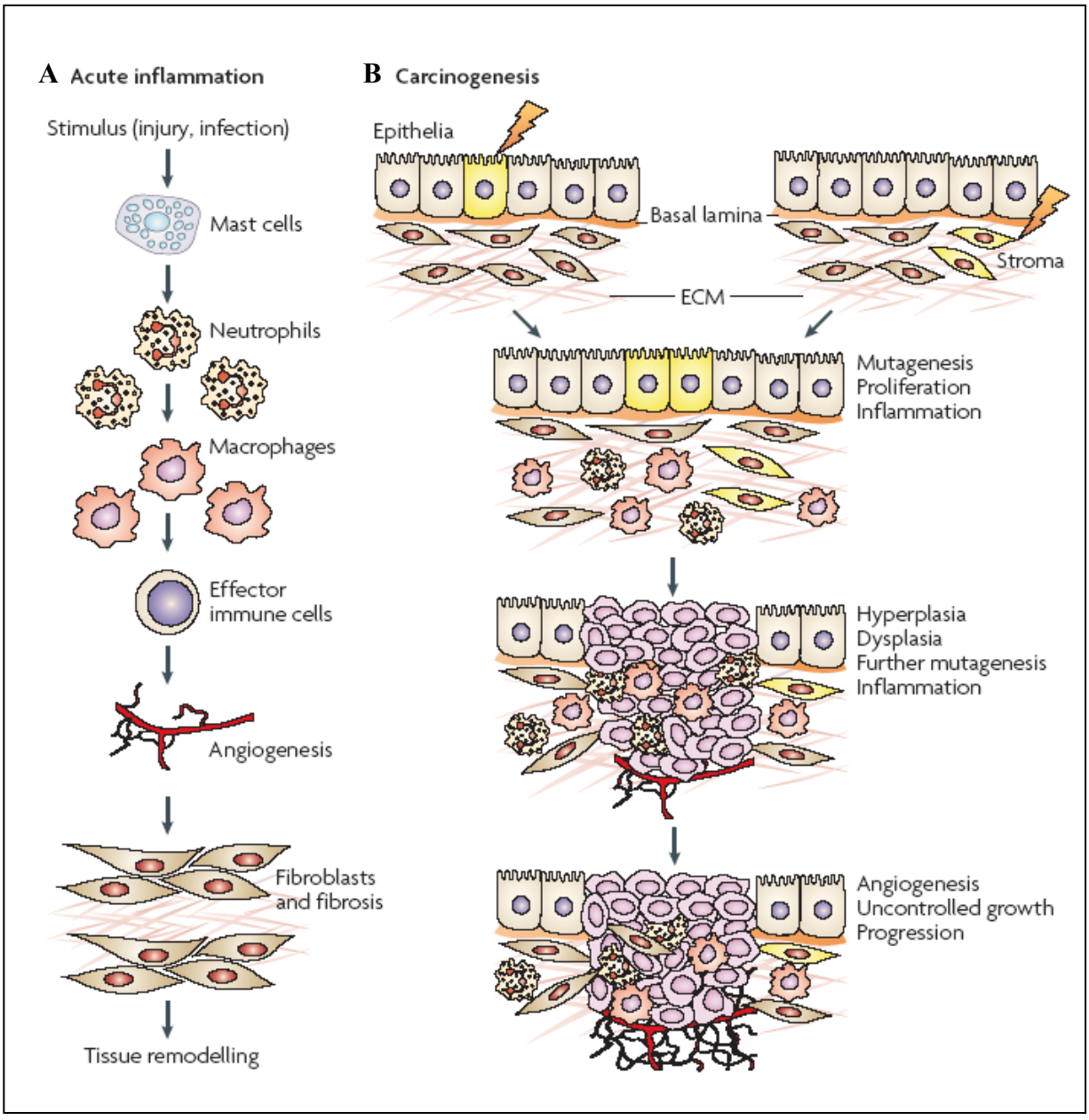

Figure II. 5: Similarities between inflammation and cancer

(A) Tissue injury leads to a discrete order of events: (i) activation of resident cells, (ii) infiltration of effector immune cells, (iii) activation and mobilization of endothelial cells and fibroblasts leading to the formation of new blood vessels and collagen matrices. **(B)** In cancer, the same events are disorganized and homeostasis is not achieved [36].

A large amount of attention is paid to CAFs and their contribution to cancer progression and initiation (reviewed in [19]). However, not much is known about the nature of the CAF's, their origin, and what distinguishes them from the normal fibroblasts. It is clear that CAF's are not identical to fibroblasts of normal adjacent tissue [37]. Unlike their normal counterparts which only marginally contribute to tumor growth [29], CAFs contain tumor-promoting properties. This was shown by comparing CAFs extracted from several human prostate carcinomas to normal fibroblasts isolated from non cancerous prostate gland. When mixed with non-tumorigenic human prostate epithelial cells and injected into immunodeficient host mice, only CAFs were able to stimulate the growth of the tumor mass [38]. Furthermore CAFs express proteins typical for fibroblasts (e.g. SDF-1 and FSP1), but also α-smooth muscle actin resembling myofibroblasts. Thus, they could be derived from fibroblasts, fibroblast-precursor cells, vascular smooth muscle cells or from carcinoma cells via EMT [39].

II.1.3 Activated stromal cells as new therapeutic targets

A major question remains: is the miss-function of the epithelium preceding the dysfunction of the stroma or *vice versa*? Although it was thought for a long time that the initial inductive trigger to drive tumor formation was provided by transformed epithelial cells, there is now increasing evidence that the stroma can have a more direct role in the progression of a tumor. A genetically unstable stroma might further increase the instability of the epithelium and therefore might act as a mutagen. In particular, stromal fibroblasts are thought to be potential inducers of certain carcinomas [19]. They directly influence the epithelium-mesenchyme cross-talk and epithelium transformation by secreting paracrine factors affecting normal epithelial cells as well as cancerous cells [19]. In addition, several unique chromosomal rearrangements were specifically found in stromal cells by PCR analysis of DNA extracts from stromal and epithelial tissues of mammary ductal carcinomas [40]. This implies that genetic alterations in stromal cells might occur without, or at least precede abnormalities in the epithelial cells. Furthermore, cells of the stromal compartment can even show loss of tumor suppressor genes [41]. Experiments using irradiation of stromal cells provided further evidence for an active role of these cells in cancer initiation and progression [42]. Injection of non-transformed mammary epithelial cells into irradiated cleared fat pads resulted in an

increase of breast tumor incidence compared with injections of similar epithelial cells in to fat pads containing non-irradiated fibroblasts. [42]. Senescent fibroblasts, which express similar sets of growth factors as CAFs, can also promote growth of epithelial cells *in vitro* and *in vivo* [43]. More recently the effect of TGFβ signaling in stromal cells was elucidated by conditionally knocking out the TGFβ type II receptor gene in fibroblasts [44]. This study showed that loss of the TGFβ pathway in fibroblasts stimulates growth and cancerogenesis of adjacent epithelia, which is probably due to dysfunctions in the HGF signaling pathway [45]. In summary there is now a lot of data suggesting that the activated stroma not only supports cancer progression, but also might play an active role in the initiation of a cancer by acting as a mutagen or by harboring mutations in tumor suppressor genes. Clearly, modifications of stromal fibroblasts can influence the malignant phenotype of adjacent epithelia (reviewed in [19]) .

All of these findings lead to a re-evaluation of the role of the tumor stroma in cancer initiation and progression and allows new strategies for cancer prevention and intervention (for reviews see [16, 36]). Normalization of an aberrant microenvironment could have the potential to reverse the tumorigenic phenotype or at least slow down tumor progression, even though the epithelial cells retain all their mutations. New strategies could include (i) removal or neutralization of the cancer-promoting features of CAFs by targeting specific cell surface marker of CAFs, for instance FSP-1 [46], which is not present in normal somatic tissues; (ii) prevention of tumor angiogenesis by blocking pro-angiogenic effector molecules or using anti-angiogenic molecules such as endostatin or tumstatin which are generated by proteolysis of collagen and other BM molecules (reviewed in [5]); or (iii) targeting mechanisms of tumoral immune tolerance or chronic inflammation which predisposes patients to cancer (reviewed in [47]). Hence, the activated stroma offers a lot of therapeutic targets, including activated cells, soluble effector molecules such as proteases or factors involved in the interaction between the epithelial and mesenchymal compartment. Beneficially and in contrast to cancer cells, stromal cells are generally more genetically stable and are therefore thought less likely to become resistant to therapy.

In summary, it is now well accepted that tumor progression needs malignant cells as well as an appropriate microenvironment and that it requires an extensive cross-talk between these two key components. This cross-talk leads to the recruitment of host cells and to their activation, which triggers pro-survival, proliferation and invasion pathways in both the cancer cells and their host (Fig.II.6) [48]. Full understanding of the defects in communication between malignant epithelial cells and host stromal cells could generate new promising targets for cancer treatment. Therefore, novel approaches for cancer therapy are being developed to target the oncogenic functions of the tumor stroma. A promising approach in treating cancers probably consists of a combination of conventional drugs, which target the highly proliferative cancer cell and anti-stromal agents, including enzyme and protease inhibitors (e.g. MMPs inhibitors), anti-adhesive molecules such as anti-integrin peptides or antibodies, signal modulators, and anti-fibrotic drugs. Ultimately, more information about the complex molecular and cellular cross-talk between the epithelium and the mesenchyme will lead to a better understanding of tumor development and progression and hence, should result in novel successful strategies for treatment or prevention of cancer.

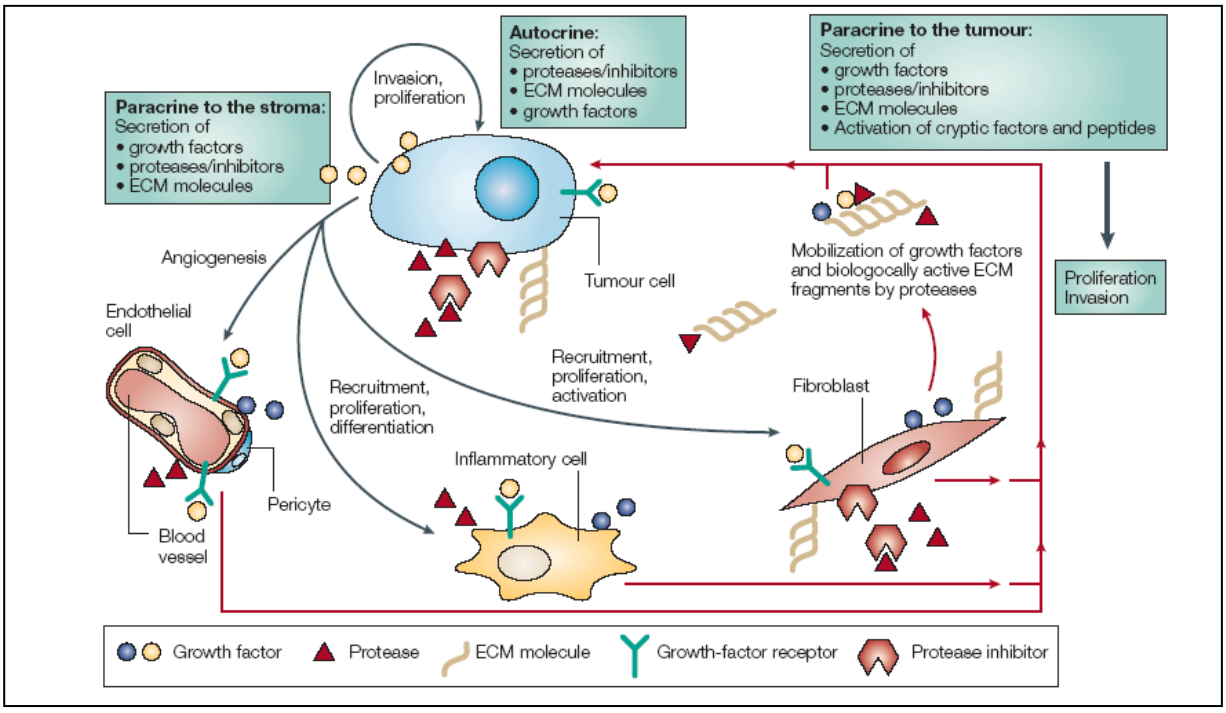

Figure II. 6: Cross-talk between tumor cells and activated stromal cells

Tumor cells activate and change their microenvironment by secreting growth factors and proteases, which can act in autocrine and paracrine manners to the tumor cells and/or to the stroma. Basically, all the activated stromal cells contribute to tumor progression [16].

II.2 Tenascins

The tenascins are a highly conserved family of large oligomeric glycoproteins present in many ECM's throughout the body (for reviews see [9, 49-51]). Whereas no tenascins have been identified in the *Caenorhabditis elegans* genome or in arthropod genomes, vertebrate genomes harbor four tenascin genes, which have been termed tenascin-C (TN-C), tenascin-R (TN-R), tenascin-X (TN-X), and tenascin-W (TN-W). ECM proteins such as tenascins are present during the development of an organism as well as in several different pathological situations, including tissue remodeling processes, inflammation and tumorigenesis [9, 52]. However, the functional impact of their presence is not very well understood so far. Nevertheless, it is suggested that tenascins act through interactions with specific cell surface receptors [49] as well as through binding to and blocking essential sites on other ECM molecules [53], thereby changing cellular behavior and signaling.

Although each tenascin family member has a very distinct expression pattern and shows discrete functional features, they share the characteristics of being tightly regulated during development and in the adult organism. Furthermore, they are all built from a common set of structural motifs, a typical feature of ECM proteins [54], in the same linear arrangement: amino-terminal heptad repeats, epidermal growth factor (EGF)-like repeats with the consensus sequence $X_4CX_3CX_5CX_4CXCX_8C$, fibronectin type III (FN III) domains, and a carboxyl-terminal globular domain shared with fibrinogens (Fig.II.7A). At their amino-terminus, each tenascin contains an oligomerization domain allowing the formation of trimeric structures and in the case of TN-C and TN-W to the formation of hexamers, so-called hexabrachions [55, 56], by the assembly of two trimers (Fig.II.7B). However, there is great variance in number and nature of EGF-like repeats and FN III domains between the different members of the tenascin family and also within specific tenascin orthologs [57]. Alternative splicing within the stretch of FN III domains has been described for TN-C and TN-R (Fig.II.7A). But only TN-C splice variants have been shown to be significantly expressed in specific tissues and to have distinct functions [9, 58-64].

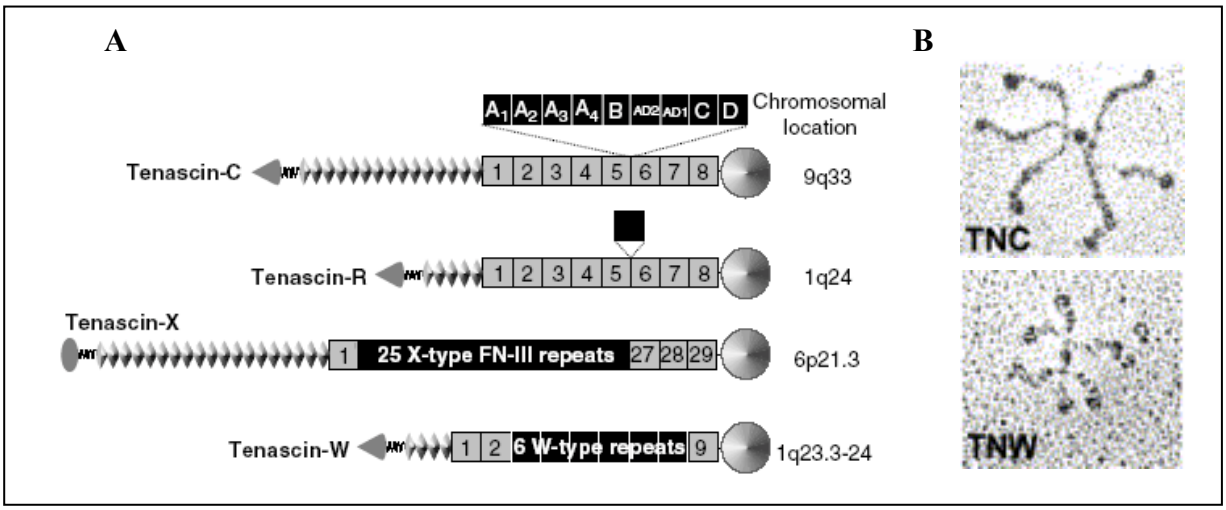

Figure II. 7: The tenascin family

(A) Schematic representation of one subunit of each of the four tenascin members. The following symbols have been used to identify the structural domains: heptad repeats (wavy line), EGF-like repeats (diamonds), constant fibronectin type III domains (grey boxes), fibronectin type III domains subject to alternative splicing (black boxes), fibrinogen globe (circle). **(B)** Electron micrograph of a tenascin-C and tenascin-W molecule after rotary shadowing shows their hexameric structure. Pictures are taken from [9, 56].

II.2.1 Tenascin-R

TN-R is predominantly expressed in the central nervous system (CNS) by oligodendrocytes [65], although there has been one report published showing TN-R expression in a peripheral nervous sytem-derived cell line [66]. Typical TN-R subunits consist of 4.5 EGF-like repeats and 8-9 FN III domains. Alternative splicing leads to two isoforms, however, their functional significance is not well understood so far [67, 68]. During the development of the CNS, TN-R expression shows partial overlap with TN-C, although TN-R appears at later time points. *In vitro* and *in vivo* studies have shown that TN-R on one hand induces actin-rich processes and branches along neurite shafts [69], promotes neuronal cell adhesion and migration, and on the other hand acts as a repellent guidance molecules for axons [70, 71]. TN-R knock-out mice are viable and fertile, and brain areas known to express TN-R in wild-type mice seem apparently normal [72]. Only recently, more detailed investigations revealed behavioral defects such as compromised motor coordination, decreased willingness to explore or increased anxiety in TN-R knock-out mice [72-74].

II.2.2 Tenascin-X

TN-X was identified as a "gene *X*" located in the major histocompatibility complex (MHC) class III gene locus [75]. Containing 18.5 EGF-like repeats and 32 FN III domains (human) it is the largest member of the tenascin family. A unique feature of TN-X is the interruption of the FN III domains by a proline-rich stretch of about 100 amino acids. It is widely expressed during development, but is limited to the connective tissue of heart and skeletal muscle as well as to the dermis of skin in the adult [76, 77]. *In vitro* studies revealed that TN-X mediates cell adhesion, but not cell spreading [78]. TN-X is the first tenascin whose deficiency has been clearly connected to a pathological syndrome in humans, the Ehler-Danlos Syndrome (EDS). *TN-X* is located in a group with the genes *RP*, *C4* and it overlaps in opposite direction with a gene called *CYP21* [79], the gene coding for steroid 21-hydroxylase, whose deletion results in congenital adrenal hyperplasia (CAH) [79]. This genetic unit occurs in tandemly repeated fashion, which makes it prone to numerous recombination events leading to diverse genetic diseases [80, 81]. First cases of human TN-X deficiencies were found in patients suffering from clinical signs of both CAH and EDS indicating a deletion of *CYP21* extending to the *TN-X* gene. EDS patients show symptoms which can be linked to ECM structural defects. They include poor wound healing, skin and joint hyper extensibility. These classical symptoms are caused by defects in collagen structure or collagen-processing enzymes. This implies a function of TN-X in either collagen fibril deposition into the ECM or regulation of the spacing between fibrils. Thereby, TN-X contributes to elasticity and strength of the dermis [82-84]. One possibility to achieve this would be through direct binding of TN-X to collagen fibrils, or to fibril-associated collagens such as collagen XII. Several lines of evidence strongly support the physical interaction between TN-X and collagens [85, 86].

II.2.3 Tenascin-C

TN-C is the founding member of the tenascin family. More than two decades ago, it was discovered as an ECM protein enriched in the stroma of gliomas [87] and as a myotendinous antigen [88]. Mammalian TN-C proteins normally contain 14.5 EGF-like repeats and 8 constant FN III domains, whereas 9 additional FN III domains can be included in a combinatorial manner by alternative splicing. This results in a great number and diversity in isoforms [89]. A prominent feature of TN-C is the assembly into hexamers, so-called hexabrachions (Fig.II.6B) [55].

TN-C together with proteins such as TN-X, SPARC, osteonectin, osteopontin, thrombospondin-1 and thrombospondin-2 belong to a class of extracellular matrix proteins which have been termed matricellular proteins [90, 91]. Matricellular proteins are defined by modulating cell-matrix interactions and cell function rather than having a direct structural role. TN-C expression is high during embryogenesis, but almost absent during normal postnatal life with some basal expression detectable in tendons and ligaments only. In adult life, TN-C is also expressed within the sub-ventricular zone (SVZ) in the CNS, a region that constitutes the neural stem cell niche [92]. A prominent feature of TN-C is its re-appearance in response to pathological situations such as infection, inflammation and tissue remodeling processes [93]. Another striking example of a pathological situation leading to the sharp re-expression of TN-C is the onset of tumorigenesis, where TN-C is specifically expressed in the activated tumor stroma [9].

Due to its prominent expression in tendons and embryonic ECMs, the gene family was named tenascin, which comes from *tenere* (to hold) and *nasci* (to be born) [94]. For most cells, TN-C does not support cell spreading and is therefore called anti-adhesive. Several receptors (e.g. intergrins α2β1, αvβ3 and α9β1, annexin II, and syndecan) and interactors (e.g. perlecan, fibronectin) have been described for TN-C which might account for the different effects attributed to TN-C (for a review see [95]). However, the finding that TN-C knock-out mice showed grossly normal phenotypes challenged the idea of TN-C as an important or essential protein which was assumed due to its highly regulated expression and its well conserved

presence in vertebrate genomes, both at DNA and protein levels [96]. However, more careful studies analyzing these mice revealed subtle abnormalities in behavior, wound healing, airway branching and angiogenesis [96-99].

II.2.3.1 Tenascin-C and cancer

TN-C is strongly expressed in the stroma of various cancers and has been reported to be associated with the invasive front of the tumors (for review see [100]). For cancers in the lung, colon, and brain, high TN-C expression correlates with poor prognosis, whereas in other cancer no clear correlation between TN-C and survival or malignancy exists [95]. Why this correlation applies to certain tumors only is not understood yet, but may reflect the fact that TN-C might have specific functions in different tumors. Some studies revealed the presence of specific TN-C isoforms in tumors, making it possible to get a more precise interpretation of the tumor physiology according to isoform expression [9, 59, 101-103]. In search for new diagnostic or prognostic tumor markers, TN-C levels have often been analyzed in sera of cancer patients and its potential value as a biomarker has been evaluated [104-108]. Although elevated TN-C serum levels have been found in certain cancers, it still remains a questionable tumor marker [104]. TN-C levels are scattered over a wide range with many cancer patients having normal TN-C concentrations and its expression strongly correlates with inflammation or infection [9].

TN-C can be induced by various stimuli: first, there are the pro- and anti-inflammatory cytokines such as different interleukins, TNFα or IFNγ and secondly, there are growth factors such as TGFβ, EGF or PDGF that are secreted either by transformed epithelial cells or by activated stromal cells [9, 109-111]. Furthermore, TN-C inducing stimuli include mechanical stress [112, 113], hypoxia [114, 115], and reactive oxygen species [116], factors or conditions which also might play a prominent role in tumors. It is astonishing that TN-C expression is very tightly regulated during development and in the adult organism despite this wide range of TN-C inducers.

By now it seems clear that TN-C is an ECM protein having anti-adhesive properties for cells when offered as substratum. Active inhibition of cell spreading by TN-C was further confirmed by mixing TN-C together with fibronectin, which is a classical adhesion protein

[117]. Whereas on fibronectin cells nicely spread, form focal contacts and actin stress fibers (Fig.II.8A), the same cells plated on a mixed fibronectin-TN-C substratum are not able to spread and do not form focal contacts and actin cables (Fig.II.8B).

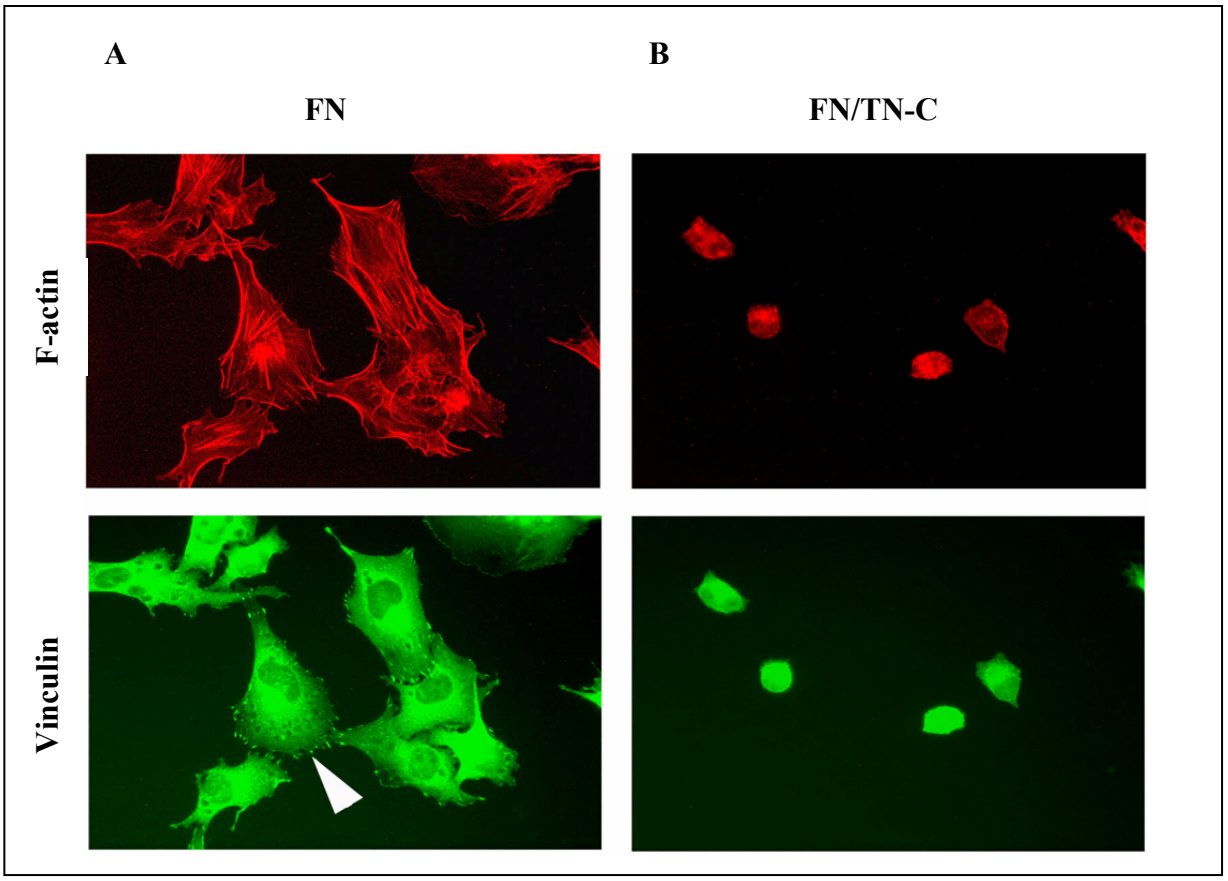

Figure II. 8: Tenascin-C inhibits cell spreading on fibronectin

(A) T98G glioblastoma cells plated on fibronectin form nice actin stress fibers and focal adhesions (arrowhead) as revealed by phalloidin and vinculin staining. **(B)** T98G cells plated on fibronectin/TN-C fail to form actin stress fibers and focal adhesions and keep a roundish morphology [53].

The molecular mechanism for this TN-C interference with fibronectin-mediated cancer cell spreading was only recently elucidated (Fig.II.9A/B) [53]. Cell spreading on fibronectin involves the classical fibronectin receptor integrin α5β1 in synergy with the transmembrane heparin sulfate proteoglycan syndecan-4 [118]. TN-C was shown to bind to the 13th FN III domain of fibronectin which is located within the heparin binding site II serving as ligand for syndecan-4. As a consequence of this competitive binding, the interaction of cells with fibronectin through syndecan-4 is prevented leading to the inhibition of cell spreading [53].

To find molecular changes in cells induced by TN-C, RNA profiling of glioblastoma cells revealed that presence of TN-C activates oncogenic signaling pathways such as EGFR [119], ERK/MAPK, and Wnt (Fig.II.9C) [120].

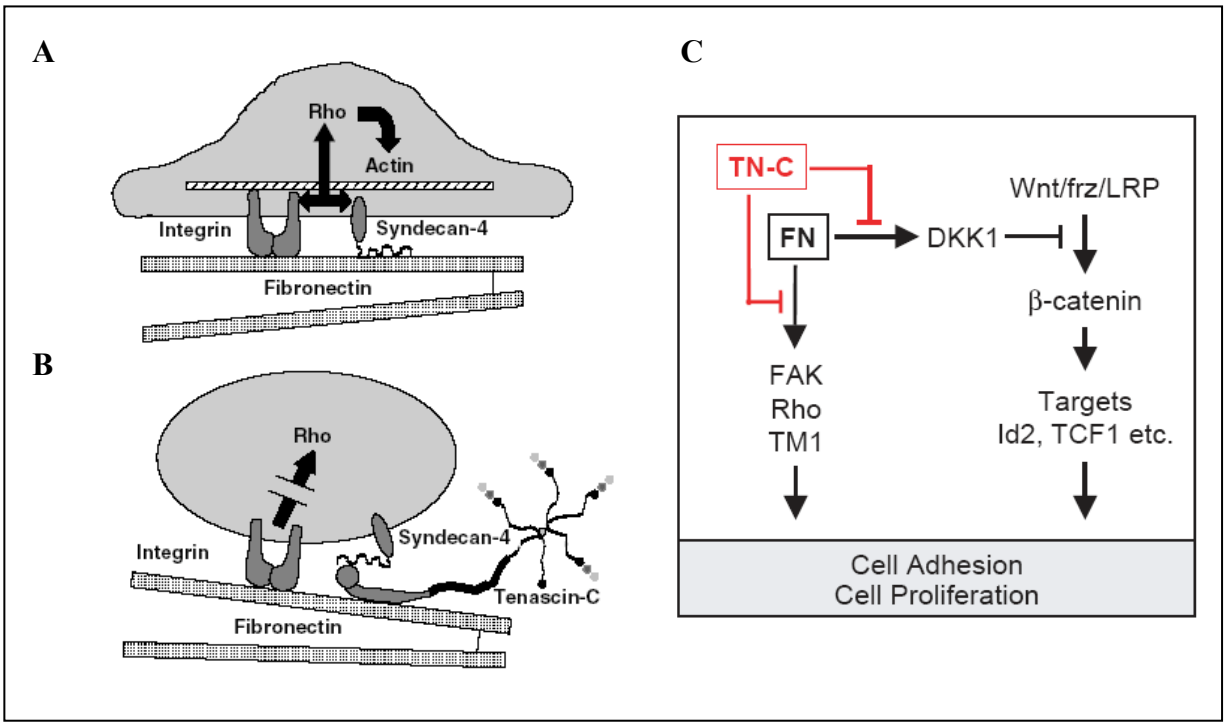

Figure II. 9: Model of tenascin-C action

(A) Full cell spreading on fibronectin needs integrin α5β1 and syndecan-4 binding. The synergistic binding activates Rho signaling, leading to the formation of stress fibers and cell spreading. **(B)** TN-C interferes with syndecan-4 binding to fibronectin, thereby disturbing the synergy between integrin α5β1 and syndecan-4. As a consequence, Rho is not activated anymore, actin stress fibers are disassembled and cell spreading is abolished. **(C)** Presence of TN-C with fibronectin leads to the activation of Wnt signaling by downregulation of DKK1, a Wnt inhibitor. On the other hand, TN-C mixed with fibronectin inhibits FAK, Rho and tropomyosin-1 (TM1) causing cell rounding. Pictures (A) and (B) were taken from [9], picture (C) from [95].

Overall, we can conclude that TN-C is a prominent ECM protein specifically expressed in the tumor stroma by activated stromal fibroblasts (see chapter II.1.2 “Activation of the stroma”). It influences several stromal reactions which are implicated in the progression of tumors. Similar to normal tissues, tumors reaching a certain size require an adequate supply of oxygen. Therefore, the process of tumor angiogenesis is essential for the expansion of a tumor mass [121, 122]. There is increasing evidence that TN-C is involved in this crucial process.

On one hand it was shown that TN-C is expressed around angiogenic vessels in many tumors [123-126] and on the other hand, it promotes and regulates angiogenesis *in vitro* and *in vivo* [97, 127]. Moreover, in glioma patients, clinical studies revealed an inhibition of tumor angiogenesis by applying antibodies directed against TN-C [128].

Tumor promoting activities of TN-C further include promotion of cancer cell proliferation [120], and enhanced glioma cell invasiveness in a 3-D collagen I matrix by up-regulating MMP-12 [129]. Furthermore, TN-C is part of the gene expression signature that identifies metastatic breast cancer cells preferentially metastasizing to the lung [130]. These observations imply a tumor promoting and oncogenic role for TN-C [100, 120, 129, 131, 132]. Since several molecules with demonstrated functions in maintaining genome stability and genome integrity were down-regulated in the presence of TN-C [120], it was hypothesized that TN-C might also influence genome stability, a further factor that can facilitate tumor formation (for review see [100]). TN-C expression in the neural stem cell niche [92, 133] as well as the observation that TN-C is important for stem cell number maintenance [134] may define a novel link to tumorigenesis. The recent realization that cancers may rely on cancer stem cells [135, 136] is supporting this hypothesis.

The question now is how to exploit this knowledge about TN-C expression and function in tumors for the benefit of patients. So far the only approach in cancer therapy involving TN-C is the use of monoclonal antibodies specific to TN-C. In glioblastoma and lymphoma patients radiolabeled antibodies specific to TN-C have been successfully used in the clinic for the treatment of these patients [137-139]. The aim of this method is to deliver therapeutic radionuclides or other bioactive molecules very specifically to the tumor site, which is rich in TN-C, using conjugated anti-tenascin monoclonal antibodies. This strategy allows a very local administration of the drug thereby preventing damage of the neighboring healthy tissue. This approach may interfere with tumor progression of glioblastomas and may even result in tumor regression [128, 140].

II.2.4 Tenascin-W

TN-W is the newest, and the last member of the tenascin family. It was initially identified in a screen for tenascin-related molecules by screening a zebrafish cDNA library constructed 20-28 hours after fertilization for the EGF-like repeats conserved in all tenascins [141]. Zebrafish TN-W contains 3.5 EGF-like repeats, followed by 5 FN III domains. The expression pattern was investigated by *in situ* hybridization in embryos and juvenile zebrafish. TN-W was found to be expressed in migrating cells of sclerotomal and neural crest origin partially overlapping with TN-C expression [141]. More recently, TN-W was identified and characterized in the mouse [56]. Similar to TN-C, electron microscopy after rotary shadowing of the purified TN-W showed the formation of hexamers (Fig.II.7B). Immunohistochemistry revealed prominent expression in the developing and adult metanephric kidney, developing and adult bones and transient expression in smooth muscles of the developing gut, often but not always overlapping with TN-C expression [56]. Another report of murine TN-W, referred to as tenascin-N [142], claims its expression in developing and adult brain. However, these findings could not be confirmed by others. Functional studies indicate that TN-W is an adhesive substratum for C2C12 cells and that their adhesion to TN-W is α8β1-integrin dependent. Furthermore, it was shown that TN-W expression can be induced in C2C12 cells by bone morphogenetic protein 2 (BMP-2) [56]. A gene and a putative human cDNA for TN-W have been annotated in the DNA databases (accession number # AL049689).

II.3 Aim of the work

The primary goal of this work was to characterize human TN-W by studying its expression pattern in different human tumors and to elucidate its biological function. To reach that aim, tools had to be generated, such as recombinant TN-W protein for functional studies and antibodies for the detection of TN-W in cells, tissues, and serum samples. The diagnostic or prognostic value of measuring serum TN-W levels was assessed by screening human sera from healthy volunteers and from tumor patients. As a side project, tools (antibodies, recombinant protein) for studying chicken TN-W functions during development were prepared as well.

Finally, I also focused on new aspects of TN-C presence in different human pathologies.

RESULTS

III. Results

III.1 Published papers

III.1.1 Tenascin-W is found in malignant mammary tumors, promotes alpha8 integrin-dependent motility and requires $p38^{MAPK}$ activity for BMP-2 and TNF-alpha induced expression *in vitro*

Arnaud Scherberich, Richard P.Tucker, Martin Degen, Marianne Brown-Luedi, Anne-Catherine Andres and Ruth Chiquet-Ehrismann

Oncogene, 2005, 24: 1525-32

My contribution: I studied the effect of α8-integrin expression on cell adhesion and cell migration of 3T3 cells on a murine tenascin-W substratum.

Oncogene (2005) 24, 1525–1532

www.nature.com/onc

Tenascin-W is found in malignant mammary tumors, promotes alpha8 integrin-dependent motility and requires p38^{MAPK} activity for BMP-2 and TNF-alpha induced expression *in vitro*

Arnaud Scherberich[1], Richard P Tucker[2], Martin Degen[1], Marianne Brown-Luedi[1], Anne-Catherine Andres[3] and Ruth Chiquet-Ehrismann*,[1]

[1]*Novartis Research Foundation, Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland;* [2]*Department of Cell Biology and Human Anatomy, University of California at Davis, Davis, CA 95616, USA;* [3]*Department of Clinical Research, Faculty of Medicine, University of Bern, Tiefenaustrasse 120, CH-3004 Bern, Switzerland*

Tenascins represent a family of extracellular matrix glycoproteins with distinctive expression patterns. Here we have analyzed the most recently described member, tenascin-W, in breast cancer. Mammary tumors isolated from transgenic mice expressing hormone-induced oncogenes reveal tenascin-W in the stroma around lesions with a high likelihood of metastasis. The presence of tenascin-W was correlated with the expression of its putative receptor, α8 integrin. HC11 cells derived from normal mammary epithelium do not express α8 integrin and fail to cross tenascin-W-coated filters. However, 4T1 mammary carcinoma cells do express α8 integrin and their migration is stimulated by tenascin-W. The expression of tenascin-W is induced by BMP-2 but not by TGF-β1, though the latter is a potent inducer of tenascin-C. The expression of tenascin-W is dependent on p38^{MAPK} and JNK signaling pathways. Since preinflammatory cytokines also act through p38^{MAPK} and JNK signaling pathways, the possible role of TNF-α in tenascin-W expression was also examined. TNF-α induced the expression of both tenascin-W and tenascin-C, and this induction was p38^{MAPK}- and cyclooxygenase-dependent. Our results show that tenascin-W may be a useful diagnostic marker for breast malignancies, and that the induction of tenascin-W in the tumor stroma may contribute to the invasive behavior of tumor cells.
Oncogene (2005) **24,** 1525–1532. doi:10.1038/sj.onc.1208342
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Keywords: tumor stroma; extracellular matrix; breast cancer; p38^{MAPK}; α8 integrin

Introduction

Extracellular matrix (ECM) not only provides a physical support to maintain normal tissue architecture but also regulates aspects of cell migration, growth, differentiation and survival. The tenascins are a family of ECM glycoproteins typically present in different connective tissues. Tenascins contribute to matrix structure and they influence the behavior of the cells in contact with the ECM (for reviews see Jones and Jones, 2000; Chiquet-Ehrismann and Chiquet, 2003; Chiquet-Ehrismann, 2004). In vertebrates the tenascin family is composed of four members: tenascin-C (TN-C), tenascin-R, tenascin-X (TN-X) and the most recently characterized member tenascin-W (TN-W) described in zebrafish (Weber *et al*., 1998) and mouse (Scherberich *et al*., 2004; also referred to as tenascin-N in Neidhardt *et al*., 2003). Searches of the genomic databases using sequence homologies indicate that TN-W is most likely the final member of the tenascin family.

In a previous study we cloned and characterized the murine TN-W (Scherberich *et al*., 2004). Rotary shadowing followed by electron microscopy showed that like TN-C, TN-W forms hexabrachions. During development TN-W is expressed during palate formation, osteogenesis and smooth muscle morphogenesis. In the adult, TN-W is found in the kidney, coronary ligament, corneal limbus and periosteum. TN-W and TN-C expression overlap in many of these areas. Bone morphogenic protein (BMP)-2 treated C2C12 cells secrete TN-W and are able to adhere to and to extend actin-rich processes on a TN-W substratum. *In vitro*, cells bind to TN-W in an RGD-dependent manner. This adhesion is increased by transfection of α8 integrin, which co-localizes with TN-W in the periosteum and kidney. The presence of the tenascins mainly in connective tissues suggests that they contribute to ECM structure (Chiquet-Ehrismann and Tucker, 2004) This is particularly clear for TN-X where patients with TN-X deficiencies suffer from an Ehlers Danlos phenotype with abnormalities of the elastic fibers and microfibrils in the dermis and a reduced dermal collagen content (Zweers *et al*., 2004).

TN-C and TN-X have been reported to be involved in general tumor malignancy (Mackie *et al*., 1987; Hasegawa *et al*., 1997; Chiquet-Ehrismann and Chiquet, 2003). Several lines of evidence have emphasized a role

*Correspondence: R Chiquet-Ehrismann;
E-mail: ruth.chiquet@fmi.ch
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for TN-C in breast cancer. In invasive mammary carcinomas, for example, TN-C expression correlates with lymph node status and tumor grade, and represents a good marker for negative prognostic value (Ioachim *et al*., 2002). In ductal carcinomas, large variants of TN-C correlate with the invasive phenotype of breast lesions (Adams *et al*., 2002; Tsunoda *et al*., 2003), and inversely correlate with the expression of the metastasis suppressor nm23-H1 (Kaya *et al*., 2002). In noninvasive and invasive ductal cancers, adenoses and fibroadenomas, TN-C is suitable for diagnosis of malignant disease and for the prediction of invasive potential of premalignant lesions (Goepel *et al*., 2000). In contrast, the expression of TN-X is downregulated in astrocytomas (Hasegawa *et al*., 1997) and malignant melanomas (Geffrotin *et al*., 2000) as the grade of malignancy progresses.

Here we studied the potential involvement of TN-W in mammary tumor formation and metastatic spreading. The expression of TN-W and its putative receptor α8 integrin was investigated in mammary tissues derived from transgenic mouse models with primary tumors of varying metastatic potential. In addition, we studied the role of TN-W in the migration and proliferation of murine mammary cell lines, as well as signal pathways relevant to breast cancer that govern the induction of TN-W *in vitro*. Our results show that TN-W may contribute to tumor metastasis, and that TN-W may prove to be a useful diagnostic marker.

Results

Expression of TN-W and alpha8 integrin in mammary tumors

To investigate the potential role of TN-W in breast cancer, we took advantage of well-characterized mouse mammary tumor models of transgenic mice transformed with the oncogenes c-myc, neuT (the rat homologue of c-erbB2), Ha-ras or from double transformations of neuT/EphB4 under MMTV-LTR or the mammary gland-specific promoter of whey acidic protein (Wap; Andres *et al*., 1987; Schoenenberger *et al*., 1988; Munarini *et al*., 2002). Immunohistochemistry with anti-TN-W and anti-TN-C antibodies was performed on frozen sections from mouse mammary glands. Immunoreactivity with the anti-TN-W antibody was not detected in the normal mammary glands of nontransgenic mice (data not shown). Similarly, no immunostaining for TN-W was found in mammary tumors from MMTV-neuT- and Wap-myc-transgenic mice (Figure 1). MMTV-neuT transgenic mice are known to develop isolated primary tumors but do not develop lung metastases (Munarini *et al*., 2002). Similarly, mice expressing the Wap-myc transgene develop well-differentiated, nonmetastatic breast tumors after several pregnancies (Andres *et al*., 1988). In Wap-ras- and MMTV-neuT/EphB4-transformed mice, which are known to develop undifferentiated, metastatic tumors (Li *et al*., 1994; Munarini *et al*., 2002), the tumor stroma was positive for TN-W (Figure 1). In contrast to the restricted labeling seen with anti-TN-W, staining with anti-TN-C was observed in all of the mammary tumors tested (Figure 1). As illustrated in the staining overlay (Figure 1), TN-W was often, but not always, co-localized with TN-C in the tumor stroma of the TN-W positive samples.

To confirm the induction of TN-W in tumors with the potential to metastasize, the expression of TN-W was further investigated by Western blot analysis of a larger series of samples. In normal mammary glands from virgin, pregnant or lactating mice, TN-W was not detectable by Western blotting (Table 1). Only two of the 15 different tumors isolated from mice expressing the MMTV-neuT- or Wap-myc-transgenes expressed detectable levels of TN-W. In contrast, TN-W expression was detected in 75% (12 of 16) of the Wap-ras- and MMTV-neuT/EphB4-induced mammary tumors.

Since α8 integrin was shown previously to serve as a potential receptor for TN-W, and often co-localized with TN-W (Scherberich *et al*., 2004), we performed a Western blot analysis of the same samples with an anti-α8 integrin antibody (Table 1). Normal mammary gland samples showed little or no expression of α8 integrin. Samples from MMTV-neuT- and Wap-myc-transgenic mice were negative as well, with the exception of a single MMTV-neuT sample. In samples from tumors with high metastatic potential, a strong induction of α8 integrin was observed except for Wap-ras-induced tumors where only a third of the samples were positive for α8 integrin. The localization of α8 integrin expression was then investigated by immunohistochemistry. α8 integrin immunostaining was absent from normal mammary gland (Figure 2a) but was seen in mammary tumors (Figure 2b). The expression was stronger at the periphery of the tumor and was absent in the normal tissue around the tumor. Figure 2c and d show that α8 integrin expression was prominent in the cells adjacent to the tumor stroma. We conclude that the chance to find α8 integrin expressing cancer cells surrounded by TN-W-positive tumor stroma increases in more malignant tumors.

TN-W promotes migration of mammary cancer cells

Since TN-W is present in the stroma surrounding some metastatic mammary cancers, it is important to investigate the effects TN-W might have on the neighboring cancer cells. Since TN-C was shown previously to promote the proliferation of tumor cells *in vitro* (Huang *et al*., 2001), a similar study was conducted with TN-W. In contrast to TN-C, TN-W had no effect on tumor cell proliferation (results not shown). Next, we investigated the effect of TN-W on the migration of mammary cells using a modified Boyden chamber migration assay. We compared the behavior of the normal mammary epithelial cell line HC11 to 4T1 mammary cancer cells. HC11 cells readily migrated across a filter coated with fibronectin, migrated slightly on type-I collagen, but failed to migrate across filters coated with either TN-W or TN-C (Figure 3a). Interestingly, the migration of 4T1 cells across the filter was stimulated by fibronectin,

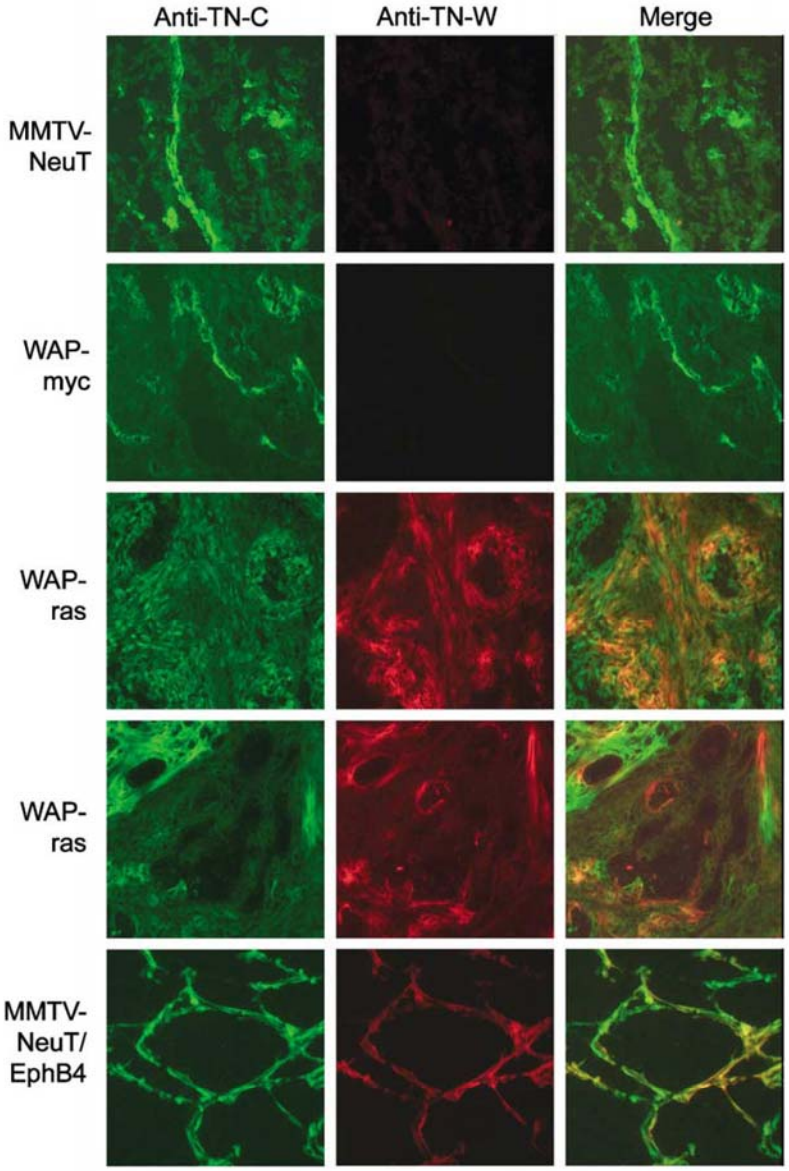

Figure 1 Double-label indirect immunohistochemical localization of TN-C and TN-W in mouse mammary tumors. Anti-TN-C (left column) stains the stroma of breast tumors from each of the transgenic mouse strains examined. The anti-TN-W labels the stroma of tumors isolated from Wap-ras and MMTV-neuT/EphB4 transgenic mice (middle column), but not the stroma of tumors from MMTV-neuT and Wap-myc animals. When the images are merged (right column), significant co-localization of TN-C and TN-W immunostaining is seen in the MMTV-neuT/EphB4 tumor, but the patterns only partially overlap in the stroma of tumors from Wap-ras transgenic mice

type-I collagen and TN-W, but not TN-C (Figure 3b). A possible explanation for the different behavior of these cells on TN-W-coated filters would be the expression of α8 integrin by 4T1 cells but not HC11 cells. This hypothesis was confirmed by Western blot analysis (Figure 3c). To demonstrate that the expression of α8 integrin is sufficient to promote adhesion to TN-W and migration across TN-W-coated filters, the ability of NIH/3T3 cells transfected with an expression vector encoding full-length α8 integrin to cross coated filters

Table 1 Expression of tenascin-W and α8 integrin in mouse mammary samples

	Tenascin-W		*α8 integrin*	
	+	–	++	– *or* +
Virgin mice ($n=2$)	0%	100%	0%	100%
Pregnant mice ($n=2$)	0%	100%	0%	100%
Lactating mice ($n=2$)	0%	100%	0%	100%
MMTV-neuT ($n=11$)	9%	91%	9%	91%
Wap-myc ($n=4$)	25%	75%	0%	100%
MMTV-neuT/EphB4 ($n=10$)	60%	40%	90%	10%
Wap-ras ($n=6$)	100%	0%	33%	67%

Approximately equal volumes of normal and tumor-containing mammary tissue samples dissected from control and transgenic mice were solubilized in SDS–PAGE sample buffer and subjected to a Western blot analysis with either anti-mouse TN-W or anti-mouse α8 integrin. For each sample, the positive or negative staining was determined and the results pooled by category. The percentage of samples with positive and negative staining is shown, as well as the number of samples in each category. For α8 integrin, a very faint band was sometimes present (+) but was considered with the negative samples, given the fact that positive bands (++) were much stronger

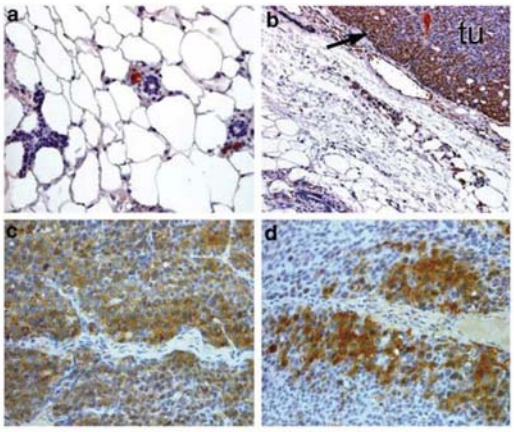

Figure 2 Immunohistochemical localization of α8 integrin in mouse mammary tissues. (**a**) A section through normal mammary tissue is not stained with the anti-α8 integrin antibody. (**b**) A section through mammary tissue from a Wap-ras transgenic mouse shows immunostaining for α8-integrin (arrow) at the periphery of a large tumor (tu). (**c** and **d**) Sections through the stroma of a mammary tumor from a Wap-ras transgenic mouse show intense α8-integrin staining in the cells adjacent to cords of stroma

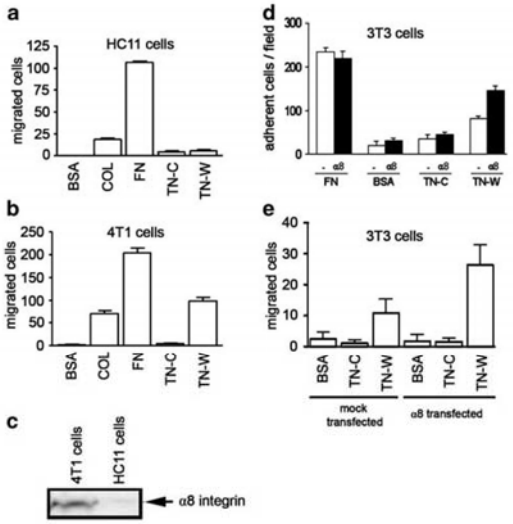

Figure 3 Cell migration on tenascin-W is depended upon α8 integrin expression. (**a**) The migration of HC11 mammary epithelial cells and (**b**) 4T1 mammary tumor cells across a filter coated with different substrata was assayed using a modified Boyden chamber. HC11 cells fail to migrate across control, TN-W- and TN-C-coated filters, but do migrate across filters coated with fibronectin (FN), and, to a lesser extent, across filters coated with collagen type I (COL). In contrast, 4T1 tumor cells use TN-W as a migratory substratum in addition to fibronectin and collagen type I, but fail to cross filters coated with TN-C. (**c**) Western blot analysis shows α8 integrin expression by 4T1 cells, but not HT11 cells. (**d**) 3T3 cells transiently transfected with α8 integrin (black bars) show a statistically significant ($P<0.001$) increase in their ability to adhere to TN-W, but do not show any difference in adhesion to fibronectin (FN), bovine serum albumin (BSA) or TN-C when compared with mock-transfected controls (white bars). (**e**) 3T3 cells transiently transfected with α8 integrin (black bars) show a statistically significant ($P<0.001$) increase in their ability to migrate across TN-W-coated filters when compared with mock-transfected controls. There was no difference in the behavior of the mock-transfected and experimental cells cultured on TN-C or BSA-coated filters

was compared with the behavior of cells transfected with the empty vector alone. Note that NIH/3T3 cells were selected for this experiment because of extremely low transfection efficiency encountered with HC11 cells and because we could not detect endogenous α8 integrin expression by Western blotting. Significantly more NIH/3T3 cells transfected with the α8 integrin expression vector adhered in short-term adhesion assays to TN-W coated dishes than control cells (Figure 3d). The same was true in migration assays. Vector only transfected cells do not migrate differently from control cells on bovine serum albumin (BSA) and TN-C-coated filters but α8 integrin overexpressing cells were stimulated to cross TN-W-coated filters (Figure 3e). Thus, TN-W in breast cancer stroma might contribute to the migratory behavior of tumor cells expressing α8 integrin.

BMP-2 induces the expression of TN-W in vitro

We have previously shown that BMP-2 can induce the expression of TN-W in C2C12 cells (Scherberich *et al.*, 2004). Here we show that primary mouse embryo fibroblasts also release high amounts of TN-W when stimulated by 100 or 200 ng/ml BMP-2 when compared with control, unstimulated cells (Figure 4a, upper blot). TGF-β1 was tested as well but failed to induce TN-W expression strongly, even at doses up to 50 ng/ml. The

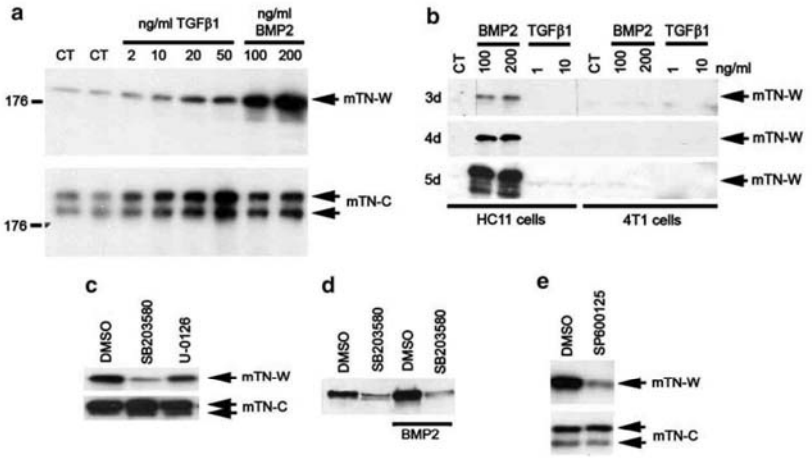

Figure 4 Pharmacological modulation of TN-W and TN-C expression induced by BMP-2 or by TGF-β1. (**a**) Supernatants from primary mouse fibroblasts cells with either TGF-β1 or BMP-2 were collected after 48 h and subjected to a Western blot analysis with an anti-mouse tenascin-W (mTN-W) antibody or an anti-mouse tenascin-C (mTN-C) antibody. TN-C appears as a doublet due to alternative splicing. There is a significant increase in TN-W expression following the addition of BMP-2 to the culture medium, and a significant increase in the expression of TN-C following the addition of TGF-β1. (**b**) Supernatants from HC11 and 4T1 cells treated with the indicated doses of BMP-2 or TGF-β1 for 3 days (3d), 4 days (4d) or 5 days (5d). BMP-2 induces the expression of TN-W (but not TN-C, results not shown) in HC11 cells, but not 4T1 cells. (**c**) Cell supernatants from primary mouse embryonic fibroblasts were subjected to a Western blot analysis with an anti-mouse TN-W (mTN-W) or an anti-mouse tenascin-C (mTN-C) 48 h following the addition SB203580 (an inhibitor of $p38^{MAPK}$), U-0126 (an inhibitor of ERK-1 and ERK-2), or the addition of DMSO alone (control). The endogenous expression of TN-W in these cells, but not TN-C, depends upon the $p38^{MAPK}$ pathway. (**d**) The endogenous expression (left two lanes) and the induction of TN-W expression by BMP-2 in mouse embryo fiblroblasts can be blocked by the $p38^{MAPK}$ inhibitor SB203580. (**e**) The endogenous expression of TN-W by primary mouse embryo fibroblasts, but not TN-C, is inhibited by the JNK inhibitor SP600125

opposite was true for the expression of TN-C (Figure 4a, lower blot). TN-C was strongly induced by TGF-β1, but its expression was induced to a lesser extent by BMP-2. Thus, BMP-2 not only induces TN-W expression in *in vitro* models of bone differentiation (Scherberich *et al*., 2004) but also in primary mouse fibroblasts. TN-C is regulated differently in these cells, and is particularly responsive to TGF-β1. We used embryonic fibroblasts as a model for the tumor stromal fibroblasts that are the source of TN-W in cancer. 4T1 mammary carcinoma cells themselves did not respond with TN-W production after BMP2 nor TGF-β1 addition (Figure 4b). In contrast, HC11 normal mammary epithelial cells could be stimulated to secrete TN-W after the addition of BMP2 (Figure 4b). To test the relevance of an induction of TN-W by BMP-2 in mammary cancer *in vivo*, we tested by Western blotting the expression of BMP-2 in homogenates of the normal and tumor samples described in Table 1 using an anti-BMP-2 antibody. BMP-2 was readily detected in only about 30% of the samples where TN-W was also expressed (results not shown). This indicates that BMP-2 may act to promote TN-W expression in some tumors, but that other factors are also likely to be responsible for the induction of TN-W.

$p38^{MAPK}$ and JNK regulate the expression of TN-W

BMP-2 can activate the $p38^{MAPK}$ and JNK signaling pathways to regulate osteoblast differentiation and gene expression (Gallea *et al*., 2001; Vinals *et al*., 2002; Guicheux *et al*., 2003). We therefore considered the potential involvement of $p38^{MAPK}$ and JNK in the regulation of TN-W expression. In the absence of BMP-2 stimulation, 10 μg/ml of a selective $p38^{MAPK}$ inhibitor (SB203580) reduced the endogenous expression of TN-W in primary mouse embryo fibroblasts (Figure 4c, upper panel). The expression of TN-C in these cells was not modified by this inhibitor (Figure 4c, lower panel). The same result was obtained with another $p38^{MAPK}$ inhibitor SB202190 (not shown). In contrast, an inhibitor of the ERK pathway (U-0126) had no effect on either TN-W or TN-C expression (Figure 4c). To show a role of $p38^{MAPK}$ in TN-W induction by BMP-2, SB203580 was added with BMP-2 to the cells. SB203580 inhibited the induction, as seen in Figure 4d. SP600125, an inhibitor of JNK, also reduced the expression of TN-W in primary mouse embryonic fibroblasts, but had no effect on TN-C expression (Figure 4e).

TNF-α induces TN-W expression in vitro

As the endogenous as well as the BMP-2 induced expression of TN-W by mouse embryo fibroblasts depend upon $p38^{MAPK}$ activity (see above), which are known to be triggered by various cytokines, we tested whether TNF-α could also induce the expression of TN-W. TNF-α stimulated the expression of both TN-W

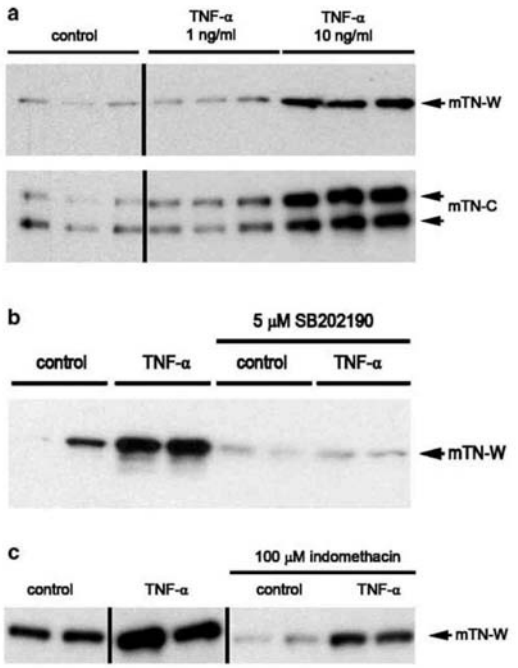

Figure 5 TN-W expression is also induced by TNF-α. (**a**) Western blot analysis of the medium conditioned by primary mouse embryo fibroblasts indicates that TNF-α can induce the expression of both TN-W and TN-C (triplicate results are shown). (**b**) The induction of TN-W by TNF-α in primary mouse embryo fibroblasts can be inhibited by the $p38^{MAPK}$ inhibitor SB202190 (duplicate results are shown). (**c**) The effect of indomethacin, a nonspecific inhibitor of cyclooxygenase, on the endogenous (control) and TNF-α-stimulated expression of TN-W in primary mouse embryo fibroblasts

and TN-C in primary mouse embryo fibroblasts (Figure 5a). Both the TNF-α-induced and the endogenous expression of TN-W by primary mouse embryo fibroblasts were inhibited by the specific inhibitor of $p38^{MAPK}$ SB202190 (Figure 5b), and by SB203580 (not shown). Finally, we showed that indomethacin, a nonspecific inhibitor of cyclooxygenases (COX), also inhibited the endogenous and TNF-α-induced expression of TN-W by primary mouse fibroblasts (Figure 5c). Thus, inflammatory cytokines are likely candidates responsible for the induction of TN-W in mammary tumors.

Discussion

Here we have characterized the potential role of TN-W in the development of mammary cancer. We found that this protein is more widely expressed in the mammary tumors of the mice known to develop metastases than in nonmetastatic tumors. Unlike TN-C, TN-W was not detected in normal mammary tissues. α8 integrin, which was previously shown to be a likely receptor for TN-W (Scherberich *et al*., 2004), was also mostly absent from healthy mammary tissue and in nonmetastatic tumors, but showed increased expression in the primary tumors with the greatest potential to metastasize. There is only one previous report showing an upregulation of α8 integrin in human tumors (Liu *et al*., 2002). In our study we show that the likelihood of stromal TN-W expression increases with the malignancy of a tumor and the incident of α8 integrin expression in the cancer cells themselves is also increased. Therefore, co-expression of these two proteins is more likely in malignant tumors. We postulate that if such a co-expression occurs the tumor cells are enabled to invade the TN-W expressing stoma. Such a mechanism is supported by our *in vitro* experiments. We found that TN-W can support the migration of breast cancer cells *in vitro*, but is not a favorable substratum for the migration of a cell line derived from normal mammary epithelium. This difference may be due to the expression of α8 integrin by the former but not the latter. Further support for a role of α8 integrin in cell adhesion to and migration on TN-W was obtained from 3T3 cells transfected with an α8 expression construct. Overexpression of α8 integrin increased cell adhesion to TN-W and promoted cell migration towards TN-W. We hypothesize that the presence of TN-W in the stroma of the tumor may help metastasizing cells that express α8 integrin to leave the tumor and invade the surrounding tissue. Unlike TN-C, which can stimulate the proliferation of tumor cells by blocking fibronectin-mediated adhesion signaling (Huang *et al*., 2001), TN-W does not have a significant effect on 4T1 cells in a proliferation assay.

In order to determine the mechanisms by which TN-W expression is induced in tumor tissue, we investigated how TN-W expression is regulated in cultured cells. We have shown in a previous report that BMP-2 is able to induce the expression of TN-W in C2C12 cells (Scherberich *et al*., 2004). BMP-2 and other members of the BMP family (Schwalbe *et al*., 2003) are interesting candidates for TN-W induction since they are known to be expressed in breast cancer cell lines and tumor samples (Clement *et al*., 1999; Pouliot *et al*., 2003). We confirmed here that BMP-2 is present in some but not all of the murine mammary tumors that also express TN-W. BMP-2, but not TGF-β1, could indeed induce the expression of TN-W in primary mouse embryo fibroblasts. Interestingly, just the opposite was observed for TN-C, which was induced by TGF-β1, but not BMP-2. The fact that TN-C and TN-W are independently regulated by TGF-β1 and BMP-2 could partly explain why they are not always co-localized during development (Scherberich *et al*., 2004).

Recently, several studies have shown that BMP-2 can activate $p38^{MAPK}$ and JNK, but not ERK/MAPK, in osteoblasts (Vinals *et al*., 2002; Guicheux *et al*., 2003), by a mechanism which is still largely unknown (Lemonnier *et al*., 2004). We therefore investigated the importance of these pathways on the endogenous expression of TN-W in fibroblasts and showed that the inhibition of $p38^{MAPK}$ or JNK dramatically inhibited the expression of TN-W, whereas an inhibitor of ERK/MAPK had no effect. None of these inhibitors affected

the expression of TN-C. The role of $p38^{MAPK}$ in the regulation of TN-W expression is particularly interesting since it was shown to be a promising potential target in the treatment of cancer (reviewed in Schultz, 2003). Activated $p38^{MAPK}$ appears to be expressed in a constitutive manner in a broad range of cancers including breast carcinoma (Chen *et al*., 2001; Xiong *et al*., 2001; Salh *et al*., 2002). We thus hypothesize that this activation of $p38^{MAPK}$ results, at least in part, in the induction of TN-W expression.

Several studies suggest that proinflammatory cytokines such as TNF-α and IL-1β have activities that contribute to metastatic tumor spread. For example, transfection of TNF-α in tumor cells confers a metastatic phenotype (Malik *et al*., 1990; Qin *et al*., 1993) and antibody neutralization of endogenous tumor-induced TNF-α leads to a significant decrease in the number of pulmonary metastases (Qin *et al*., 1993). Moreover, human clinical studies have similarly associated increased levels of TNF-α with metastatic disease (Ocvirk *et al*., 2000; Michalaki *et al*., 2004). Here we show that TNF-α is capable of inducing TN-W *in vitro*, and that its induction is dependent on $p38^{MAPK}$ and COX pathways. Thus, inflammatory cytokines including TNF-α are likely candidate molecules to induce TN-W expression in tumor stroma. The involvment of $p38^{MAPK}$ in TNF-α signaling is in contrast to a previous report on TNF-α induction of TN-C in keratinocytes, where the signal was dependent on JNK (Latijnhouwers *et al*., 1998).

TN-C is a useful prognostic marker of a number of types of tumors and appears to play a role in tumor metastasis. Here we show in a mouse breast cancer model that a recently discovered member of the tenascin family, TN-W, is more restricted in its expression than TN-C to tumors with the greatest potential to metastasize. TN-W can promote the motility of cells expressing α8 integrin, and this may contribute to the invasive spread of cancers. These observations point to future studies on the use of TN-W as a diagnostic tool as well as a potential target for cancer therapy.

Materials and methods

Antibodies and reagents

The rabbit polyclonal antibody to TN-W was developed in our laboratory and described previously (Scherberich *et al*., 2004). The rabbit polyclonal anti-α8 integrin antibody B11 was a generous gift from Dr U Müller (Basel, Switzerland). The mouse monoclonal antibody against BMP-2 was purchased from Santa Cruz Biotechnology. Recombinant human BMP-2 and mouse TNF-α, the rat monoclonal anti-TN-C antibody MTn-12, and the indomethacin were all from Sigma. Human TGF-β1 was purchased from R&D Systems. SB203580 and SP600125 were kindly provided by Dr Y Nagamine (Basel, Switzerland), and U-0126 was a gift from Dr V Biou (Basel, Switzerland). SB202190 was obtained from Sigma.

Cell culture

Primary mouse embryonic fibroblasts were kindly provided by Patrick Kopp (Basel, Switzerland), and cultured with DME medium supplemented with 10% fetal calf serum (FCS). The HC11 normal mouse mammary epithelial cells were provided by Dr N Hynes (Basel, Switzerland). They were cultured in RPMI 1640 medium supplemented with 10% FCS, 5 μg/ml insulin and 10 ng/ml EGF (Hynes *et al*., 1990). The 4T1 invasive mouse mammary tumor cells (CRL-2539) and the NIH/3T3 cells (CRL-1658) were obtained from The American Type Culture Collection and cultured in DME with 10% FCS.

Tumor samples

For protein analysis, mammary tissues from the different transgenic mice were removed, snap frozen in liquid nitrogen and stored at -70°C. For histochemistry mammary tissues were fixed, frozen and cryosectioned at 5 μm. The transgenic Wap-myc and Wap-ras mice have been described in detail previously (Andres *et al*., 1987, 1988; Schoenenberger *et al*., 1988), as have the transgenic neu-T and EphB4 mice (Munarini *et al*., 2002).

Immunohistochemistry

Immunostaining was performed on mouse mammary sections with antibodies against TN-W and TN-C as previously described (Scherberich *et al*., 2004). Appropriate Alexa red and green secondary antibodies (Molecular Probes) were diluted as recommended by the manufacturer. Images were acquired using a Zeiss fluorescence microscope, a Sony 3CCD camera and Scion Image software. Immunostaining with anti-α8 integrin was conducted using methods described in Nikolova *et al*. (1998).

Western blot analysis

Cells in culture, tissues or culture supernatants were directly solubilized for 5 min at 95°C in SDS–PAGE sample buffer, followed by electrophoresis on 6–10% polyacrylamide gels. Proteins were electro-transferred onto PVDF membranes (Millipore). After blocking for 1 h at room temperature in TBS with 0.05% Tween and 3% skim milk powder, membranes were incubated overnight with the appropriate primary antibody, washed, and then incubated for 1 h with the appropriate secondary antibody coupled to HRP (1/10 000). Specific staining was revealed using ECL kits (Amersham).

Adhesion and migration assay

Cell motility was tested in 8-μm-pore polycarbonate membrane Transwell chambers (Costar) essentially as described by the manufacturer. The underside of the polycarbonate membranes was coated with 40 μg/ml of rat tail collagen type I (Upstate Biotechnology), fibronectin (purified from horse serum as described in Fischer *et al*., 1997), recombinant mouse TN-W or recombinant mouse TN-C (both as described in Scherberich *et al*., 2004). 4T1 and HC11 cells were resuspended in serum-free medium and 100 000 cells were added to the top chamber. Serum-free medium was added to the bottom chamber, and cells were allowed to migrate for 20 h (HT11 and 4T1 cells) or 36 h (NIH/3T3 cells). Cells were scraped off the top of the membrane using a cotton swab. Cells that migrated across the membrane were fixed in 3.7% formaldehyde and stained with 1% crystal violet. Cells were counted under a microscope in four different high-power fields in duplicate wells in at least two independent experiments. *T*-tests to determine significant differences in the number of cells migrating across the filters were performed using Microsoft Excel.

NIH/3T3 cells were transfected with a full-length α8 integrin cDNA in pcDNAI kindly provided by Lynn Schnapp *et al.* (1998). After 1 day, α8-transfected and vector-only transfected cells were harvested and used for short-term adhesion assays as described before (Scherberich *et al.*, 2004). The same cells were also used for transfilter migration assays as described above.

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III.1.2 Avian tenascin-W: expression in smooth muscle and bone, and effects on calvarial cell spreading and adhesion in vitro

Caroline V Meloty-Kapella, Martin Degen, Ruth Chiquet-Ehrismann and Richard P Tucker

Developmental Dynamics, 2006, 235: 1532-1542

My contribution: cloning and sequencing of full-length chicken tenascin-W cDNA; expression and purification of chicken tenascin-W; production of the polyclonal anti chicken tenascin-W antibody

DEVELOPMENTAL DYNAMICS 235:1532–1542, 2006

RESEARCH ARTICLE

Avian Tenascin-W: Expression in Smooth Muscle and Bone, and Effects on Calvarial Cell Spreading and Adhesion In Vitro

Caroline V. Meloty-Kapella,[1] Martin Degen,[2] Ruth Chiquet-Ehrismann,[2] and Richard P. Tucker[1]*

Tenascins are glycoproteins found primarily in the embryonic extracellular matrix. Here we have characterized the fourth and final member of the tenascin family in birds: tenascin-W. Avian tenascin-W has 3.5 epidermal growth factor-like repeats, 6 fibronectin type III domains, and a C-terminal fibrinogen-related domain. Immunohistochemistry reveals that avian tenascin-W is expressed transiently in developing smooth muscle, tendons, and ligaments, but the primary site of tenascin-W expression during development is in the extracellular matrix of bone and the cellular periosteum. In bony matrix, tenascin-W-coated fibrils partly overlap with fibrils that contain tenascin-C. The anti-tenascin-W also labels fibrils in cultures of osteogenic embryonic chicken calvarial cells. Primary calvarial cells cultured on purified tenascin-W become rounded, and fewer of these cells spread on fibronectin when tenascin-W is added to the medium when compared with calvarial cells cultured on fibronectin alone. Moreover, tenascin-W reduces the adhesion of calvarial cells to collagen type I in a shear force assay. We conclude that tenascin-W is likely to play a phylogenetically conserved role in developing bone and that it shares some of the basic anti-adhesive and matrix modulatory properties as tenascin-C. *Developmental Dynamics 235:1532–1542, 2006.* © 2006 Wiley-Liss, Inc.

Key words: extracellular matrix; development; chicken; osteogenesis; osteoblast; immunohistochemistry; tenascin-C; tenascin-Y; tenascin-R

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INTRODUCTION

Tenascins are a family of extracellular matrix (ECM) glycoproteins (for reviews see Jones and Jones, 2000; Chiquet-Ehrismann and Chiquet, 2003). Each member of the tenascin family is composed of one or more epidermal growth factor (EGF)-like repeats, a variable number of fibronectin type III domains, and a C-terminal fibrinogen-related domain (FReD). Four members of the tenascin family have been identified in vertebrates: tenascin-C, tenascin-R, tenascin-X (known as tenascin-Y in birds), and tenascin-W (reviewed by Chiquet-Ehrismann and Tucker, 2004). Many functions have been postulated for the tenascins, including promoting cell motility, acting as selective barriers to migration, and modifying cellular responses to interactions with other ECM molecules, especially fibronectin (e.g., Orend et al., 2003; Midwood et al., 2004).

Tenascin-C is the original and most widely studied member of the family. In chicken embryos, tenascin-C is found in the developing central (CNS) and peripheral nervous systems (PNS), near migrating neural crest cells, at sites of epithelium-mesenchyme interactions, in smooth muscle, and in many connective tissues (e.g., Mackie et al., 1988; Tucker and McKay, 1991; Mackie and Tucker, 1992). When tenascin-C expression is knocked down using antisense morpholinos in the chicken embryo, neural crest cells fail to migrate from the surface of the neural tube (Tucker, 2001). Tenascin-C has a similar pattern of expression in mouse embryos, and tenascin-C knockout mice exhibit ab-

[1]Department of Cell Biology and Human Anatomy, University of California at Davis, Davis, California
[2]Friedrich Miescher Institute for Biomedical Research, Novartis Research Foundation, Basel, Switzerland
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***Correspondence to: R.P. Tucker, Department of Cell Biology and Human Anatomy, University of California at Davis, 1 Shields Avenue, Davis, CA 95616-8643. E-mail: rptucker@ucdavis.edu**

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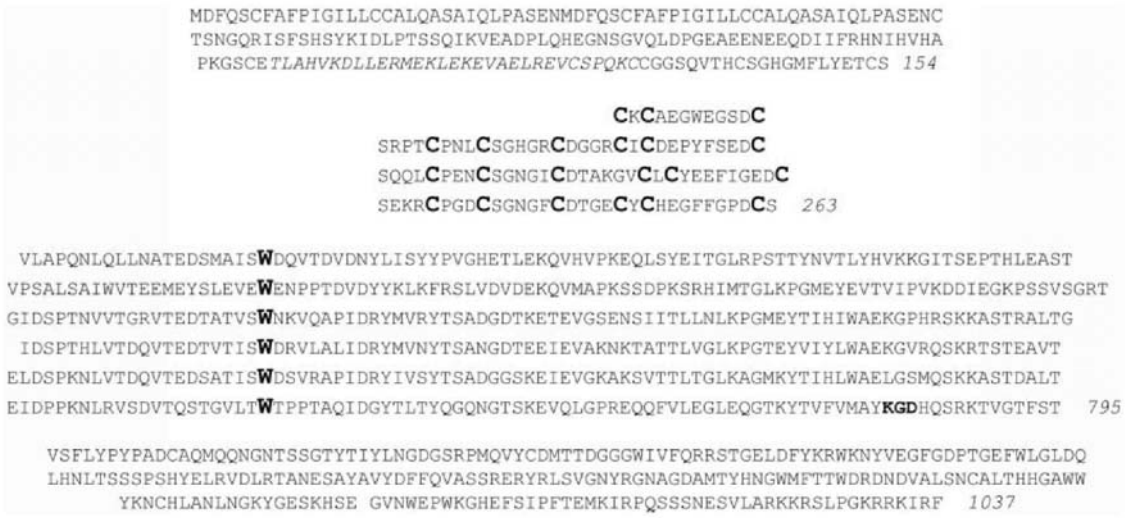


Fig. 1. Chicken tenascin-W. Amino acids 1–154 include predicted heptad repeats that are indicated in italics. The conserved cysteine residues of the EGF-like repeats (amino acids 155–263) are shown in bold. The conserved tryptophan residues found in the six fibronectin type III domains (amino acids 264–795) are aligned and shown in bold, as is a putative integrin-binding KGD motif in the sixth fibronectin type III domain. A fibrinogen related domain (amino acids 796–1,037) is found at the C-terminus of chicken tenascin-W.

normal behavior and responses to injury (reviewed by Mackie and Tucker, 1999). In contrast, tenascin-R expression is limited to the developing and adult CNS and PNS in both birds (Rathjen et al., 1991; Derr et al., 1998) and mice (Wintergerst et al., 1993). Tenascin-R knockout mice are viable and fertile, but have CNS abnormalities (Weber et al., 1999; Sykova et al., 2005). Tenascin-X and tenascin-Y are expressed primarily in epimysium and around blood vessels (Matsumoto et al., 1994; Hagios et al., 1996). In the chicken embryo, tenascin-Y is also found in the dorsal root entry zones and CNS floor and roof plates (Tucker et al., 2001). Tenascin-X knockout mice develop normally but eventually display connective tissue disorders similar to Ehlers-Danlos syndrome (Mao et al., 2002), which can also result from mutations of human tenascin-X (e.g., see Burch et al., 1997). Much less is known about tenascin-W. To date, it has only been described in zebrafish (Weber et al., 1998) and mouse (Scherberich et al., 2004). In developing zebrafish, tenascin-W transcripts are found in the somites and possibly in migrating neural crest cells, where it is co-expressed with tenascin-C (Weber et al., 1998). In the mouse, antibodies to tenascin-W show considerable overlap between the expression of tenascin-C and tenascin-W in the ECM of developing bone, smooth muscle, and the metanephric kidney (Scherberich et al., 2004). Scherberich et al. (2005) also found that tenascin-W is expressed in murine breast tumors with a high metastatic potential.

In order to understand more about the function and properties of tenascin-W, we have cloned the complete coding sequence of chicken tenascin-W, characterized its expression in the embryo and adult with immunological methods, and studied the effects of purified recombinant tenascin-W on primary cultures of cells derived from tissues that are rich in tenascin-W during development. Our results show both similarities and differences between the patterns of tenascin-W expression in birds and mammals, and demonstrate that tenascin-W, like tenascin-C, can modulate cell-ECM interactions in vitro.

RESULTS

The Structure of Avian Tenascin-W

The predicted amino acid sequence of chicken tenascin-W was found by BLASTP analysis of the chicken genome at GenBank. Using PCR and primers based on sequences found in the predicted protein (accession number XP 422277), a full-length complete chicken tenascin-W cDNA was constructed and sequenced (see Experimental Procedures section). The cDNA sequence varied from that of the predicted protein in the database in several ways, including the start site, a stretch of 32 amino acids between the EGF-like repeats and the fibronectin type III domains that is found in the predicted protein but not the cDNA sequence, and an exon encoding 30 amino acids in a fibronectin type III domain that is found in the cDNA, but not the predicted protein.

Figure 1 shows the complete predicted amino acid sequence of the chicken tenascin-W cDNA (accession number: AM231718). This cDNA encodes a protein with 1,037 amino acids. A region predicted to have heptad repeats capable of multimerization is found between amino acids 101 and 131. Like tenascin-W in the zebrafish and mouse, this is followed by one partial and three complete tenascin-type EGF-like repeats. Between amino acids 264 and 786 are 6 fibronectin type III domains. This is one more than reported in zebrafish tenascin-W and

three fewer than reported in the mouse. Unlike murine tenascin-W, the chicken sequence does not contain an RGD motif, but there is a KGD motif in a comparable region found in the sixth fibronectin type III domain. A FReD is found at the C-terminus of chicken tenascin-W (amino acids 796 to 1013).

The Expression of Chicken Tenascin-W During Development

An antiserum raised against a fragment of recombinant chicken tenascin-W was used to analyze tenascin-W expression in avian tissue homogenates by immunoblotting (Fig. 2). At all pre-hatching stages examined, the only tissue with detectable levels of tenascin-W was the mandible, where the antiserum recognized a single band with an M_r of approximately 175,000. Tenascin-W was not detected in homogenates of brain or crop from E8 (Fig. 2A), E10, E12, E14 (not shown), or E16 (Fig. 2B), nor was it found in homogenates from the E18 heart, kidney, telencephalon, optic tectum, or crop (Fig. 2C).

Double-label immunohistochemistry was performed on sections of chicken embryos at various developmental stages with the polyclonal antiserum to tenascin-W and a monoclonal anti-tenascin-C, and adjacent sections were incubated with a polyclonal antiserum to tenascin-Y and a monoclonal antibody to tenascin-R. Representative sections of regions that illustrate the appearance and disappearance of tenascin-W expression during development are shown in Figure 3. At E4, tenascin-C and tenascin-Y were found in the ECM in patterns consistent with prior publications, but there was no immunoreactivity detected with the anti-tenascin-W or the anti-tenascin-R (not shown). The earliest tenascin-W immunoreactivity was seen at E8 in the mandible, ribs, and developing calvarial bones (Fig. 3A) as well as in the smooth muscle of the proventriculus and gizzard (Fig. 3E). At each of these sites, the anti-tenascin-W labeled a subset of the ECM stained with the anti-tenascin-C. At no time during development was tenascin-W immunoreactivity found in the CNS, even though tenascin-C, tenascin-R, and tenascin-Y were present in the CNS from E8 through hatching in patterns that were described previously (e.g., Fig. 3A–D). At E12, the level of tenascin-W immunoreactivity in the smooth muscle of the digestive tract appeared to be reduced in comparison to the level seen at E8 (not shown), and by E17 tenascin-W immunoreactivity was found only in irregular patches in the innermost smooth muscle layers of the proventriculus (Fig. 3G). The anti-tenascin-W failed to stain the ECM of the small intestines at E17 (Fig. 3I), in contrast to the anti-tenascin-C and the anti-tenascin-Y (Fig. 3J). The tenascin-R antibody labeled the autonomic nervous system of the gut at all stages examined (e.g., Fig. 3H,J). Though lost from most smooth muscle at E17, tenascin-W immunoreactivity was seen in many connective tissues, including the fibrous cartilage of the heart (Fig. 3K), the central parts of large tendons (Fig. 3M), bone (Fig. 3O), and ligaments (Fig. 3Q). In each of these connective tissues, the tenascin-W immunoreactivity appeared to be a subset of the broader ECM labeled with the anti-tenascin-C, and in some (Fig. 3L, R) it overlapped with tenascin-Y-positive matrix. Note that tenascin-W was never detected in the developing PNS (Fig. 3Q,S), unlike tenascins -C, -R, and -Y (Figs 3Q–T). Tenascin-W immunoreactivity was not detected in lung, liver, kidney, or gonads at any time during development (not shown).

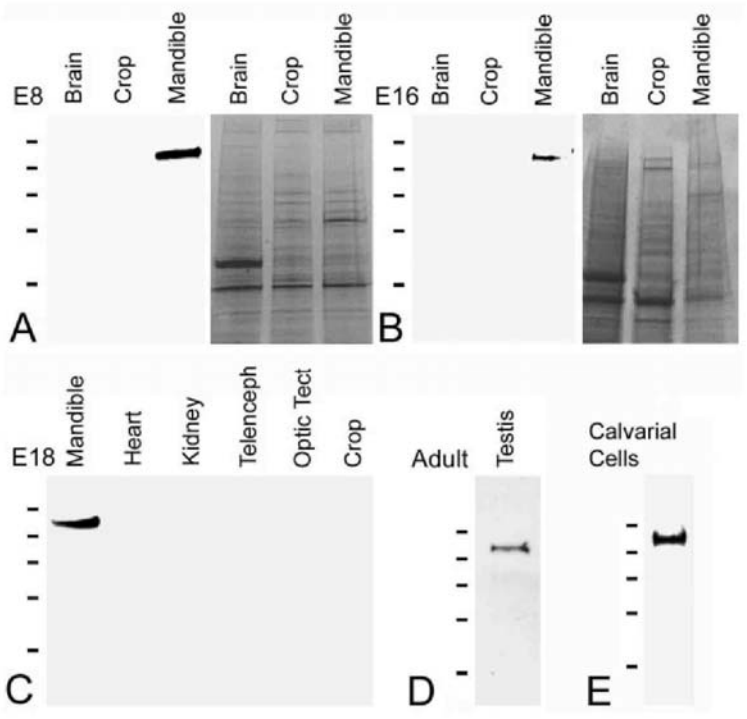


Fig. 2. The detection of tenascin-W in the developing and adult chicken by immunoblotting. **A,B:** The anti-tenascin-W recognizes a single band with an M_r of approximately 175,000 in homogenates of the E8 and E16 mandible, but not in homogenates of E8 and E16 brain or crop. Coomassie-stained gels shown to the right demonstrate the equal loading of protein on the gels. **C:** At E18, the anti-tenascin-W recognizes a single M_r 175,000 band in mandible homogenates, but not in homogenates of other tissues. **D:** In the adult, tenascin-W can be detected in testes homogenates. **E:** The presence of tenascin-W in cultures of primary calvarial cells was also confirmed by immunoblotting. The dashes indicate the approximate positions of size standards. From top to bottom: 250,000; 150,000; 100,000; 75,000; 50,000.

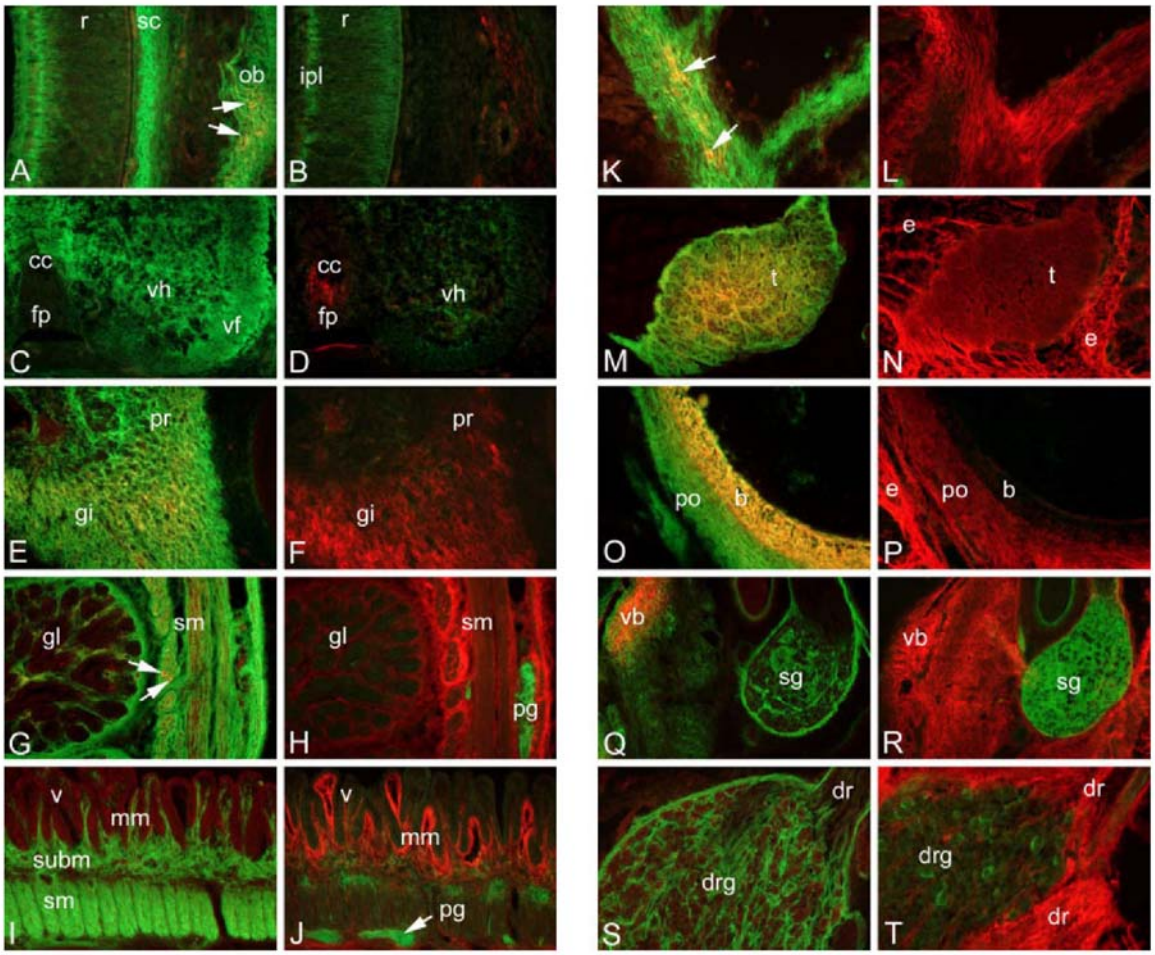


Fig. 3. Double-label immunohistochemical detection of tenascin-W (red) and tenascin-C (green; A, C, E, G, I, K, M, O, Q, S), and tenascin-Y (red) and tenascin-R (green; B, D, F, H, J, L, N, P, R, T) in embryonic chicken tissues. Co-localization appears yellow. **A:** One of the earliest sites of tenascin-W expression is at E8 in cranial bones forming by intramembranous ossification like the bones of the orbit (ob, arrows), where it is a subset of the ECM labeled with anti-tenascin-C. The cartilagenous sclera (sc) and inner parts of the retina (r) are positive for tenascin-C. **B:** Tenascin-Y is found in the ECM between the sclera and the orbital bone and tenascin-R is found in the developing inner plexiform layer (ipl) at E8. **C:** Tenascin-W was never detected in the developing CNS, unlike tenascin-C, which is found in the ventral horns (vh) and ventral funiculus (vf) of the E8 spinal cord. cc, central canal; fp, floor plate. **D:** Tenascin-Y is found in the floor plate of the E8 spinal cord, and tenascin-R is found in the ventral horns. **E:** Another early site of tenascin-W expression is in the developing smooth muscle of the E8 proventriculus (pr) and gizzard (gi), where it once again appears as a subset of the tenascin-C-positive ECM. **F:** Tenascin-Y is also present in the smooth muscle of the E8 proventriculus and gizzard. **G:** At E17, tenascin-W is still found in some of the smooth muscle (sm) of the proventriculus (arrows), but its labeling is faint and restricted when compared with tenascin-C. The latter is also seen in proventricular glands (gl). **H:** Tenascin-Y is found in the epimysium of the E17 proventriculus, and tenascin-R is seen in parasympathetic ganglia (pg). **I:** Tenascin-W was not detected in longitudinal sections of the small intestine at E17, even though tenascin-C is found in the ECM of smooth muscle, submucosa (subm), and in the muscularis mucosa (mm) underlying the villi (v). **J:** Tenascin-Y immunoreactivity is also seen in the muscularis mucosa at E17, while tenascin-R once again labels the parasympathetic nervous system (arrow). **K:** Tenascin-W is found in the core of the fibrous cartilage of the E17 heart (arrows) where it is co-localized with tenascin-C. **L:** Tenascin-Y is also found in the fibrous skeleton of the heart. Note the single autonomic neuron stained with the anti-tenascin-R in the lower left corner of the image. **M:** Tenascin-W immunoreactivity is seen in the core of a large scalene tendon (t) at E17 whereas tenascin-C is found throughout the tendon. **N:** Tenascin-Y is found near the periphery of the tendon and in the epimysium (e) of surrounding skeletal muscle. **O:** Tenascin-W and tenascin-C are co-localized in the bone (b) of the vertebral body at E17. Tenascin-C is also found throughout the fibrous periosteum (po). **P:** Tenascin-Y is not detected in bone, but is found in the periosteum and nearby epimysium. **Q:** Tenascin-W is abundant in a ligament binding adjacent vertebral bodies (vb) at E17. Tenascin-C immunoreactivity is seen near the edge of the ligament and in a nearby sympathetic ganglion (sg). **R:** Tenascin-Y is widely expressed in the loose connective tissue near the vertebral bodies at E17 while tenascin-R is limited to the sympathetic ganglion and rami communicantes. **S:** Tenascin-W is also missing from sensory ganglia at E17. Tenascin-C is found in both the dorsal root ganglion (drg) itself and surrounding the dorsal root (dr). **T:** Tenascin-Y is found in the dorsal rootlets of the dorsal root ganglion at E17, while tenascin-R is restricted to a subset of sensory neurons within the ganglion.

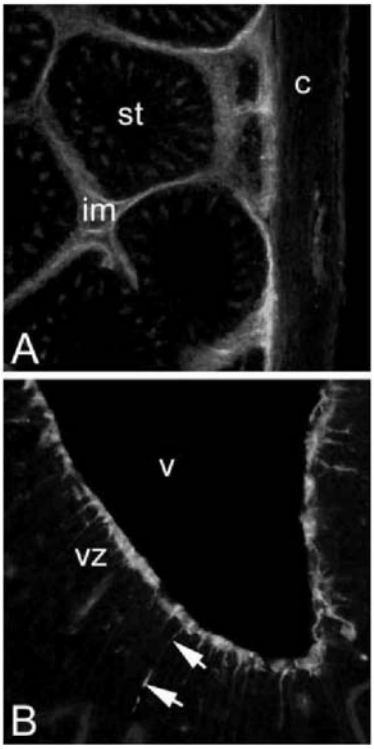


Fig. 4. Tenascin-W expression in the adult. **A:** Tenascin-W immunoreactivity is seen in the interstitial matrix (im) surrounding each seminiferous tubule (st) in the rooster testis. Tenascin-W is not seen in the capsular (c) tunica albuginea. **B:** Though never seen in the developing CNS or PNS, tenascin-W immunoreactivity was seen in the adult brain. Here the anti-tenascin-W labels the ventricular surface of the ventricular zone (vz) and fibers radiating from the ventricular zone a short distance into the optic tectum (arrows). v, ventricle.

Tenascin-W Expression in the Adult

Immunoblotting was used to assay the expression of tenascin-W in homogenates of numerous tissues dissected from a mature rooster. As in the embryo, tenascin-W had a restricted pattern of expression: homogenates of kidney, heart, lung, liver, spleen, and numerous brain regions were negative for tenascin-W (not shown), but the anti-tenascin-W recognized a single M_r 175,000 band in homogenates of adult testis (Fig. 2D).

The tissues used for immunoblotting were also fixed and processed for immunohistochemistry with the tenascin-W antiserum. Adult bones were too mineralized for standard cryohistology and were not examined. No tenascin-W immunoreactivity was found in the kidney, spleen, liver, lung, or heart, but tenascin-W was detected in sections of the testis and the CNS (Fig. 4). In the testis, tenascin-W was found in the interstitial matrix surrounding each seminiferous tubule. Though absent during development, tenascin-W immunoreactivity was found in the ECM of the adult rooster CNS. The immunostaining lined the ventricular and pial surfaces of each CNS region examined (hippocampus, optic tectum, and cerebellum), and was frequently observed to extend a short distance into the ventricular zone proper where it was associated with what appear to be the terminal segment and endfeet of radial glia. Anti-tenascin-W staining was not detected elsewhere in the CNS.

Tenascin-C and Tenascin-W Are Often Co-Localized in Developing Bone

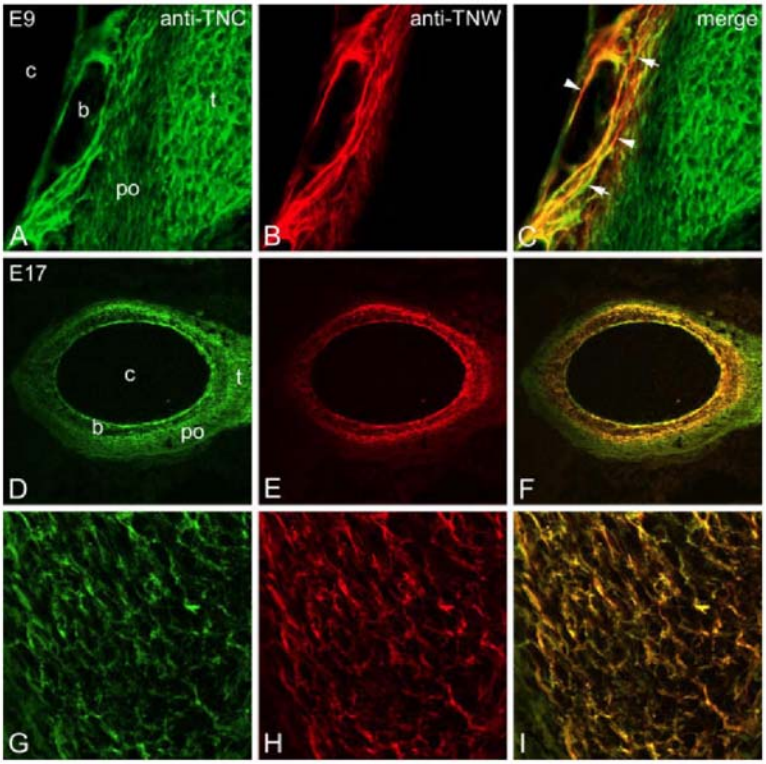
Throughout development, tenascin-W is most abundant in the ECM of bone. When examined at high resolution during the perichondral ossification of the E9 humerus, tenascin-W immunostaining appears as thick fibrils in the osteoid matrix near the cartilagenous model, and in thinner fibrils in the cambial or cellular layer of periosteum (Fig. 5B). Similar fibrils in the developing bone are immunostained with anti-tenascin-C (Fig. 5A), but merging the two images shows that some fibrils or regions along a fibril are only stained with the tenascin-W antiserum, others are only stained with the anti-tenascin-C, while others are immunostained with both (Fig. 5C). In the E17 rib, another bone that also undergoes perichondral ossification, tenascin-W immunostaining is also concentrated in the bony ring surrounding the cartilage where it overlaps extensively with the anti-tenascin-C labeling (Fig. 5D–F). At higher magnification, the tenascin-W and tenascin-C immunostaining within the osteogenic cellular periosteum appears to be identical. Thus, in developing bone, tenascin-C and tenascin-W can appear in different fibrillar systems, but they also can be extensively co-localized.

Tenascin-W Expression In Vitro

Primary cultures enriched for chicken osteoblasts were made from embryonic calvaria and cultured under conditions that promote differentiation and the deposition of ECM characteristic of bone. After several days in vitro, many foci that often contained phase-bright irregular deposits of mineralized matrix were seen in the cultures (Fig. 6A). The anti-tenascin-C labeled fibrillar matrix through-

Fig. 5. Partial co-localization of tenascin-W and tenascin-C in ECM fibrils during osteogenesis. **A–C:** Anti-tenascin-C (A) and anti-tenascin-W (B) label thick fibrils found in the bony matrix (b) surrounding the cartilage model (c) of the E9 chicken humerus. Both are also seen in thinner fibrils in the cellular periosteum (po), but only tenascin-C is detected in a nearby tendon (t). When the images are merged (C), it becomes apparent that the anti-tenascin-C and anti-tenascin-W staining patterns only partially overlap (yellow). Some fibrils are only labeled with anti-tenascin-C (arrows) and others are only labeled with anti-tenascin-W (arrowheads). **D–F:** Low-magnification overview of tenascin-C (D) and tenascin-W (E) expression in cross-sections through a rib at E17. Both tenascins are found in the bony matrix surrounding the disappearing cartilage model as well as in the part of the cellular periosteum lying adjacent to the bone. Tenascin-C is also seen in the surrounding fibrous periosteum and in a tendon. The merged image (F) shows extensive overlap in the expression pattern in bone. **G–I:** High-magnification images of the cellular periosteum bordering the bony matrix of the E17 rib illustrated in D–F immunostained with anti-tensacin-C (G) and anti-tenascin-W (H). The merged image (I) shows the nearly complete overlap of tenascin-C and tenascin-W in the cellular periosteum.

Fig. 6. The in vitro expression of tenascin-W. **A:** A phase contrast image of an osteogenic cell cluster in a culture of embryonic chicken calvarial cells. **B:** Anti-tenascin-C labels a fibrillar system throughout the cultures. **C:** Anti-tenascin-W labels fibrils concentrated under the osteogenic foci. The inset shows the punctate nature of the anti-tenascin-W staining. **D:** When the images in B and C are merged, one sees only partial overlap in the tenascin-C and tenascin-W immunostaining. The inset shows some co-localization (yellow and arrow), as well as other fibrils that are labeled with anti-tenascin-C only (arrowhead) or anti-tenascin-W only (red).



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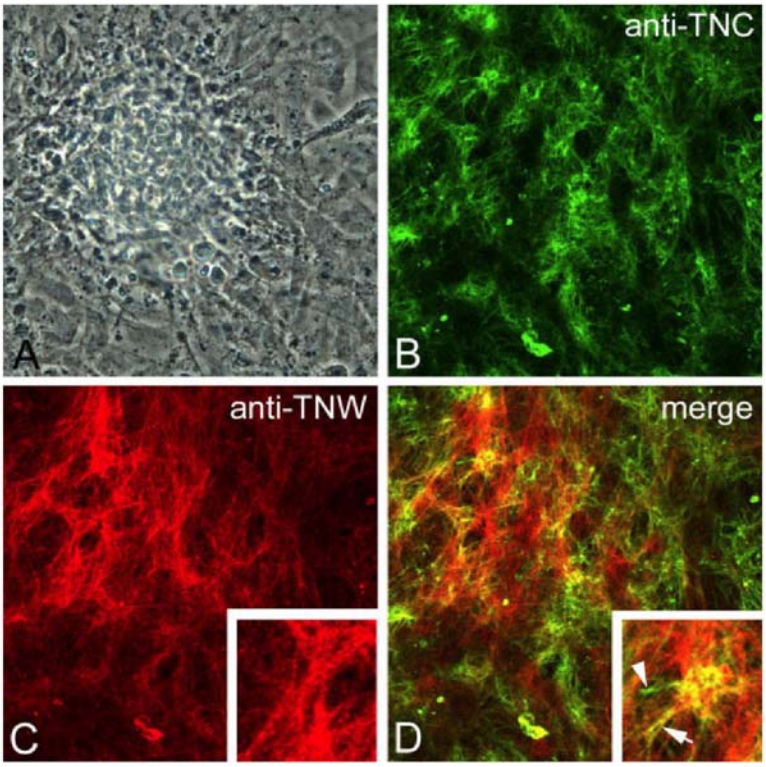

Fig. 6.

out the cultures, not only in the clusters of cells where the mineralized matrix was accumulating, but also under the surrounding monolayer of calvarial cells (Fig. 6B). The anti-tenascin-W immunostaining was different from that seen with anti-tenascin-C. Most of the staining appeared as fine dots that decorated fibrillar systems in and under the osteogenic foci (Fig. 6C). Numerous fine puncta were also seen unassociated with fibrils, but still concentrated near the sites of osteogenesis. Merging the anti-tenascin-C and anti-tenascin-W images shows that, as was observed in developing bone in situ, some fibrils are positive for tenascin-C alone, others for tenascin-W alone, and some are positive for both (Fig. 6D). The tenascin-W in the cultures was readily detected by immunoblotting, where a single band with an M_r of approximately 175,000 was seen (Fig. 2E). Thus, the deposition of tenascin-W during osteogenesis in vitro resembles in some ways the appearance of tenascin-W in situ: it is present as a single M_r 175,000 product that forms fibrils, and is partially co-localized with tenascin-C-rich ECM.

The Effects of Tenascin-W on Calvarial Cell Morphology and Adhesion in Vitro

Recombinant chicken tenascin-W was used as a substratum for primary cultures enriched for chicken osteoblasts. At very low coating concentrations (0.1 to 1 μg/ml), 65% of the cells formed phase-dark lamellae and contained phalloidin-positive stress fibers 3 hr after plating (Fig. 7A). These numbers were not significantly different from those seen in controls cultured on bovine serum albumin (BSA) alone. However, only 25% of the cells in the primary calvarial cultures formed lamellae when cultured on 10 μg/ml of tenascin-W. Most of the cells remained rounded or spread minimally without forming significant lamellae, resulting in a bipolar or triangular shape. Thus, in vitro tenascin-W appears to inhibit the spreading of the cell type that encounters tenascin-W in situ.

The functions of tenascins are re-

lated in part to their ability to modulate cell–ECM interactions. To test if tenascin-W shares this potential with other members of the tenascin family, primary calvarial cells were plated on tissue culture plastic coated with tenascin-W, fibronectin, or denatured collagen type I alone, or on tissue culture plastic coated first with fibronectin or collagen type I followed by incubation with tenascin-W. The concentration of tenascin-W used both to coat and to modify substrata (10 μg/ml) was the same concentration that had a significant impact on cell spreading (see above). After 30 min, the cultures were briefly placed on a rotary shaker, rinsed, fixed, and the numbers of remaining adherent cells were counted (Fig. 7C). Not surprisingly, significantly more cells adhered to fibronectin than to BSA alone. Incubation of the fibronectin with tenascin-W prior to the addition of the cells did not affect the number of cells that remained attached. In contrast, the addition of tenascin-W to collagen type I-coated plates resulted in a significant reduction in the numbers of primary calvarial cells that remained attached when compared with collagen type I alone (Fig. 7C). The effect of tenascin-W on the morphology of cells cultured on different substrata was also investigated. Significantly fewer cells in primary calvarial cultures formed lamellae on fibronectin when tenascin-W was present in the medium than on fibronectin alone (Fig. 7D). Tenascin-W did not have this effect on cells cultured on collagen type I, though these cells never spread significantly more than those cultured on BSA or on tenascin-W alone (Fig. 7D).

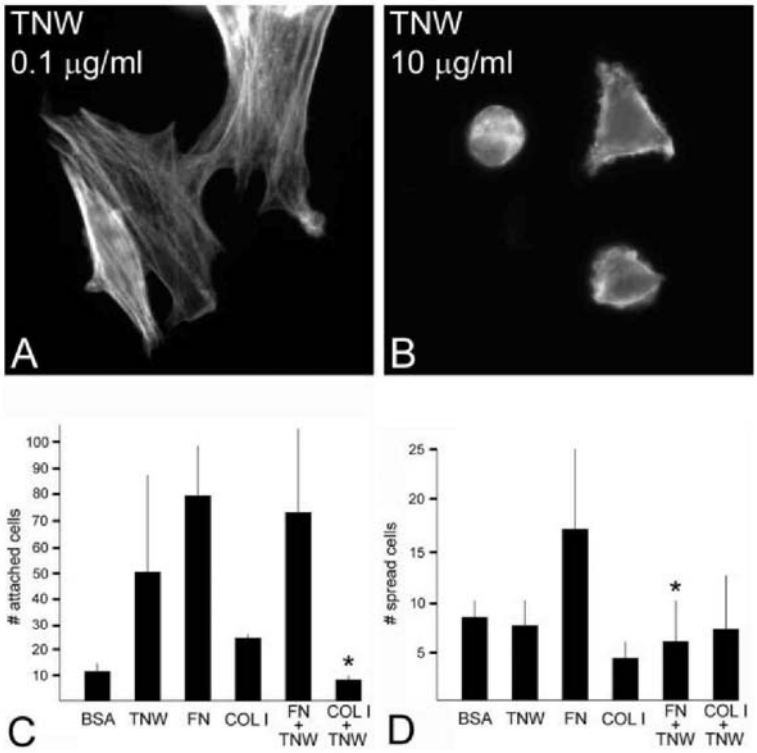

Fig. 7. The effects of recombinant tenascin-W on primary calvarial cells in vitro. **A:** Primary embryonic chicken calvarial cells spread and form rhodamine-phalloidin-positive stress fibers on low (0.1 μg/ml) concentrations of tenascin-W. **B:** When the same cells are cultured on high (10 μg/ml) concentrations of tenascin-W, they remain rounded. **C:** Fewer ($P < 0.05$) calvarial cells remain attached to a combination of collagen type I and tenascin-W than remain attached to collagen type I alone in a shear force-based adhesion assay. **D:** Fewer primary calvarial cells spread on fibronectin after tenascin-W as added to the medium than on fibronectin alone ($P < 0.05$).

DISCUSSION

Chicken tenascin-W contains heptad repeats, 3.5 EGF-like repeats, 6 fibronectin type III domains, and a C-terminal FReD. Other tenascins have one or more alternatively spliced variants, and these variants often have unique properties (e.g., Fischer et al., 1997; Mercado et al., 2004; Liu et al., 2005). Chicken tenascin-W, however, does not appear to have any major splice variants, as only a single band is detected by immunoblotting of homogenates of embryonic jaw, adult testis, and primary cultures containing osteoblasts.

Tenascin-W was described previously in the zebrafish and the mouse. Little is known about *Danio* tenascin-W except its cDNA sequence and the observation that it is co-expressed with tenascin-C by neural crest cells and somites (Weber et al., 1998). In the mouse, immunohistochemistry showed that tenascin-W is prominently expressed in the developing and adult metanephric kidney, developing and adult bone, and transiently in the smooth muscle of the developing gut (Scherberich et al., 2004). We show here that the latter patterns of expression are conserved in the chicken, though we saw no evidence of tenascin-W expression in the avian kidney. We also found tenascin-W in the interstitial matrix of the avian testis. This may reflect the expression of tenascin-W by myofibroblasts, which would be consistent with its transient expression during the early stages of smooth muscle differentiation. Interestingly, tenascin-W has also been detected in some murine breast tumors (Scherberich et al., 2005) where myofibroblasts may contribute to its deposition in the tumor stroma. Another report of tenascin-W in the mouse, which referred to this protein eponymously as tenascin-N (Neidhardt et al., 2003), described its expression not only in kidney but also throughout the developing and mature brain. This observation was not confirmed by Scherberich et al. (2004), and in the chicken tenascin-W was not detected in the developing brain by either immunoblotting or immunohistochemistry. However, tenascin-W is found in the adult avian brain near the pial and ventricular surfaces where it appears

to be associated with glial endfeet. Since this distinctive pattern of expression was not seen in the mouse, its significance is unclear.

The most prominent and conserved sites of tenascin-W expression in amniotes are osteoid and cellular periosteum. Tenascin-W is found in the ECM at sites undergoing intramembranous ossification as well as in thick fibrils of ECM at sites of perichondral ossification. In these fibrils, tenascin-W is sometimes co-localized with tenascin-C, but it can also be present without detectable levels of tenascin-C. Tenascin-W is also found in the cellular periosteal matrix adjacent to the site of bone formation where its expression overlaps extensively with that of tenascin-C. We show here that tenascin-W is occasionally found in tendons and ligaments where it is also a subset of the tenascin-C ECM. Overlap with tenascin-Y is occasionally observed, and the neuron-associated tenascin-R is never found in the same matrix as tenascin-W. Since tenascin-W is primarily expressed in developing bone in vivo, we chose to study its expression in cultures of embryonic avian calvarial cells that are enriched for osteoblasts. In these cultures, tenascin-W is expressed in ECM-rich nodules representing foci of osteogenesis. As in developing bone in vivo, tenascin-W in vitro appears in fibrils. However, instead of coating the fibrils evenly like tenascin-C, tenascin-W appears to decorate the fibrils as fine puncta. Tenascin-W is sometimes seen in the same fibrillar systems as tenascin-C, but usually the tenascin-W-coated ECM is distinct from that coated with tenascin-C. As was observed in bony matrix in vivo, tenascin-C expression in these cultures is more widespread than the expression of tenascin-W. We believe that these cultures will be useful in future studies of the regulation of tenascin-W expression in bone as well as studies of tenascin-W binding partners. For example, preliminary double-label immunocytochemical studies with antibodies to collagen type I and tenascin-W in these cultures indicate that some, but not all, of the tenascin-W labeling is associated with the collagen type I matrix.

Tenascin-C plays important roles in both chondrogenesis and osteogenesis in vitro (e.g., Mackie et al., 1987; Mackie and Murphy, 1998), but the tenascin-C knockout mouse does not have obvious connective tissue defects (reviewed by Mackie and Tucker, 1999). This leads to the hypothesis that tenascin-W may share some properties with tenascin-C that allow it to compensate for tenascin-C in the knockout mice. Tenascin-C is an anti-adhesive glycoprotein that causes cell rounding in vitro and promotes the detachment of cells from other ECM molecules (see reviews by Hsia and Schwarzbauer, 2005; Orend, 2005). Here we cultured cells that normally encounter tenascin-W in vivo on purified tenascin-W and show that like tenascin-C, tenascin-W can induce cell rounding. These cells do not completely detach from the tenascin-W substratum, however, since they remain attached during a simple shaking assay (Fig. 7C). In this same assay, significantly fewer calvarial cells adhered to a mixture of collagen type I and tenascin-W than to collagen type I alone, implying that a function of tenascin-W may be to modulate osteoblast-collagen type I adhesion. Tenascin-W also reduces the numbers of cells that spread on fibronectin-coated substrata, which is a property it shares with tenascin-C. The anti-adhesive and cell-rounding properties of tenascin-W are intriguing given that changes in osteoblast shape are associated with, and may help to regulate, the expression of genes associated with differentiation (e.g., see Gurlek and Kumar, 2001).

Tenascins can interact with integrins (see Jones and Jones, 2000, for review). Examination of the chicken tenascin-W amino acid sequence reveals multiple putative integrin recognition sequences, some of which are found in the exposed linker regions between the β strands of the fibronectin type III domains. For example, chicken tenascin-W has a KGD motif in the sixth fibronectin type III domain that is predicted to be exposed to integrin binding. The KGD motif is found in many disintegrins and is also a functional αVβ1 and α5β1integrin-binding motif in collagen type XVII (Nykvist et al., 2001). The IDG motif has been reported to be the recognition site for α9β1 integrin (Yokosaki et al., 1998), and this motif is also present in the sixth fibronectin type III domain of chicken tenascin-W. Finally, chicken tenascin-W has multiple copies of the IDSP and related motifs that are known to bind to α4β1 integrin in the exposed G to A linker sequences of fibronectin type III domains. Although α9β1 integrin has not been reported in bone, the other putative receptors listed above have been detected both in sections of bone and in cultured osteocytes (e.g., Bennett et al., 2001). Future studies with mutated protein and peptides should be directed toward understanding which of these potential motifs are functional in chicken tenascin-W, and whether they initiate signaling pathways similar to, or distinctive from, those used by tenascin-c.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Full-Length Chicken Tenascin-W

Full-length chicken tenascin-W was cloned from a cDNA that was obtained as described below. Primers for nested PCR were designed that amplified two separate, but overlapping, parts of chicken tenascin-W that we call part A and part B. The first reaction was performed with the primer sets 5′-CATGAATACCAGCCTTATGATCAC-3′/5′-CAGCTTGTAATAATCCACATC-3′ (for part A) and 5′-GAAAGCTTGTCACACTTTATCATGTGAAG-3′/5′-GTCCAAGTTGGTCAGCAGTTGCCTCC-3′ (for part B). The primer sets for the second reactions included *HindIII* and *EcoRI* restriction sites, respectively. Primer pair 5′-GAAAGCTTGTTTAGGGGTCAGTGCTTCCTCTTG-3′/5′-GAGAATTCCCTCCGTCACCCAGATGGCAC-3′ was used for part A, and primer pair 5′-GAAAGCTTGTCACACTTTATCATGTGAAG-3′/5′-GAGAATTCTCAGTGATGGTGATGGTGATGAAACCTGATCTTCCTCCTTTTTCC-3′ was used for part B. The two amplicons were put together using an internal *XbaI* restriction site found in the overlapping region of the two parts, resulting in a full-length chicken tenascin-W cDNA of 3,114 bp (GenBank accession number, AM231718). This full-length cDNA was cloned into the expression vector pCEP/Pu (provided by J. Engel,

Biozentrum, Basel). At the 3′ end of the tenascin-W cDNA, a 6×His-tag was inserted in front of the stop codon to allow the purification of full-length chicken tenascin-W protein expressed in mammalian cell culture. The full-length tenascin-W cDNA was transfected into EBNA293 cells (ATCC number CRL-10852) using FuGENE6 (Roche). Transfected cells were selected with puromycin and the serum-free medium containing the secreted recombinant chicken tenascin-W was collected. After ammonium sulphate precipitation (0–50%) and dialysis against PBS, the protein was passed over a gelatin-agarose column to remove fibronectin contamination in the preparation. Afterwards, the protein was affinity purified on a Ni-NTA matrix (Qiagen, Chatsworth, CA) in the presence of 0.5M urea, and after extensive washing of the column, the tenascin-W was eluted with 250 mM imidazole (pH 6.9) and finally dialyzed against PBS. The protein appeared pure on a Coomassie-stained acrylamide gel, and fibronectin could not be detected in the purified sample by immunoblotting.

Anti-Tenascin-W Antibody Production

cDNA was synthesized from total RNA that was extracted from a whole chicken head (E15) with Trizol (Invitrogen, La Jolla, CA). PCR was then performed using the E15 head cDNA as template and primers designed to amplify the sequences encoding the last two fibronectin type III domains with the Expand High Fidelity PCR System (Roche). The reaction was performed with the primer set 5′-GAGGATCCGAATTGGACAGCCCAAAGAATC-3′/5′-AGAAGCTTATGTTGAGAAGGTACCAACTGTC-3′. The primer set included a *BamHI* and a stop codon immediately followed by a *HindIII* restriction site to enable the directional cloning into the bacterial expression vector pQE30 (Qiagen), supplying a C-terminal His tag for the purification of the recombinant fragment. The recombinant fragment corresponding to fibronectin type III domains 5 and 6 was expressed and purified by affinity chromatography to a Ni-NTA matrix (Qiagen) following the supplier's instructions. Purification was performed under native conditions and elution by 250 mM imidazole (pH 6.9). The bacterially expressed fragment of tenascin-W was then used to raise polyclonal antisera in rabbits using standard immunization procedures. The antisera recognized an M_r 175,000 band on immunoblots of purified full-length tenascin-W (see below), but not similarly purified full-length recombinant tenascin-C. Recognition of purified tenascin-W with the tenascin-W antiserum was blocked by preincubation of the antiserum with the recombinant fragment used for the immunization.

Antibodies and Immunohistochemistry

This study was carried out using a rabbit polyclonal antiserum to chicken tenascin-W (see above), the mouse monoclonal anti-tenascin TnM1 (Chiquet and Fambrough, 1984), a mouse monoclonal anti-tenascin-R (antibody 619, a gift from M. Schachner), and a rabbit polyclonal antiserum specific to chicken tenascin-Y (Hagios et al., 1996). Timed (E3, E4, E8, E9, E10, E12, and E17) chicken embryos and parts dissected from an adult white leghorn rooster (culled from flocks maintained by the UC Davis Department of Avian Science) were fixed in 4% paraformaldehyde in PBS overnight, rinsed, and cryoprotected in 25% sucrose in PBS overnight, and embedded for cryosectioning. Frozen sections were cut at 14 μm in a Bright cryostat, collected on presubbed slides (Fisher), and allowed to air dry. Sections were rinsed in PBS, blocked in PBS with 0.5% BSA for 1 hr, and incubated at 4°C overnight in appropriate mixtures of diluted antibodies (see above), diluted preimmune serum (anti-tenascin-W only), or blocking solution alone. Following rinses in PBS, the sections were incubated in appropriate mixtures of secondary antibodies (1:500; Alexa-488 or Alexa 594-tagged goat anti-rabbit or goat anti-mouse, Molecular Probes, Eugene, OR) for 2 hr. The controls did not show background labeling. In addition, some sections were treated for various time periods with chondroitinase ABC (Sigma, St. Louis, MO; 0.7U) to reveal tenascin-W epitopes that may be masked by other matrix molecules. The immunostaining of chondroitinase-treated sections was brighter than that seen in untreated sections, but the overall pattern of tenascin-W immunostaining was the same. Photographs were taken using Elite Chrome (Kodak) slide film and a Nikon Optiphot epifluorescence microscope, a Q-Imaging digital camera, and a Nikon Eclipse photomicroscope, or an Olympus inverted confocal microscope.

Immunoblotting

Tissues were collected from chicken embryos (E8, E10, E12, E14, E16, and E18) and roosters and homogenized using a dounce homogenizer in 100 μl PBS containing protease inhibitor cocktail tablets (Roche). The homogenates were frozen and stored until further use. To study the expression of tenascin-W in culture, primary calvarial cells (see below) that had formed osteogenic nodules were scraped from their dishes and homogenized and stored as indicated for the tissues above. Equal protein concentrations of dissected tissue homogenates were solubilized for 2 min at 95°C in SDS-PAGE sample buffer, followed by electrophoresis on 7.5% polyacrylamide gels (Bio-Rad, Richmond, CA). Proteins were transferred onto nitrocellulose membrane (Bio-Rad) using a blotting apparatus (Bio-Rad). After transfer, the membrane was blocked for 1 hr at room temperature in PBS containing 0.05% Tween (TBS) and 5% skimmed milk powder. After blocking, the membrane was incubated overnight with anti-chicken-tenascin-W serum (1:250) and then for 1 hr with anti-rabbit IgG coupled to horseradish peroxidase (1:1,000; Pierce, Rockford, IL). Specific staining was revealed using an ECL enhanced chemiluminescence kit (Pierce). Coomassie blue staining was also performed to confirm appropriate sample loading.

Preparation of Cell Cultures and Immunocytochemistry

Primary cultures enriched for osteoblasts were cultured from the calvarial bones of 15-day chicken embryos according to the method of van der Plas et al. (1985) with minor modifica-

tions. Parietal bones were dissected free and the periosteum was removed. Isolated bones were treated with collagenase (Worthington, 180 U/ml) in 25 mM HEPES, 10 mM $NaHCO_3$, 100 mM NaCl, 3 mM K_2HPO_4, 1 mM $CaCl_2$, 30 mM KCl, 1 mg/ml BSA, and 5 mg/ml glucose for 10 min at 37°C. The dissociated cells were collected and the remaining bone fragments were incubated again in a fresh collagenase solution for 45 min. An equal volume of cold fetal calf serum (FCS) was added and the two digests were pooled together. The cells were resuspended in Eagle's minimum essential medium (MEM) containing 10% FCS, 50 μg/ml ascorbic acid, 1 mg/ml glucose, 200 μg/ml glutamine, 50 μg/ml gentamycin, and then seeded, grown to confluence, and passaged. The resulting mixture of cells was enriched with osteoblasts and their precursors but also contained some non-osteogenic cells. For that reason, they are referred to here as "primary calvarial cells." Primary chicken calvarial cells were cultured in Eagle's MEM supplemented with 10% FCS, glutamine, penicillin, and streptomycin. Cultures were maintained at 37°C in 7% CO_2. Cells were passaged by treatment with 100 μg/ml trypsin/0.02 % EDTA in PBS. Chicken embryo calvarial cells were then cultured on 8-chamber plastic slides (LabTek; Miles Laboratories, Inc., Elkhart, IN). Before staining, they were washed with PBS, fixed in 4% paraformaldehyde in PBS for 20 min, treated with 0.2% Triton X-100 in PBS for 5 min, and washed three times for 5 min with PBS/0.1% BSA. Cells were incubated with appropriate mixtures and dilutions of primary antibodies/sera or preimmune sera overnight. Following rinses, mixtures of Alexa anti-rabbit or anti-mouse secondary antibodies (Molecular Probes) were incubated on the slides for 1 hr. Results were recorded using an Olympus inverted confocal microscope.

Cell Morphology and Adhesion Assays

For the cell morphology assay, individual wells of microtiter plates (60 wells; Nunc) were coated for 1 hr at room temperature with 5 μl of recombinant chicken tenascin-W (0.1–10 μg/ml), human fibronectin (10 μg/ml; Chemicon, Temecula, CA), and chicken collagen type I (10 μg/ml; Chemicon). The wells were then blocked for 30 min with PBS/0.1% BSA and rinsed with calcium and magnesium-free PBS. Ten microliters of a chicken primary calvarial cell suspension containing 5×10^4 cells were then placed in each well. Mixed substrata were made as follows: after 30 min, tenascin-W (10 μg/ml) was added to some of the cells cultured on fibronectin or collagen type I. After 3 hr of incubation at 37°C, all cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and the number of cells with lamellae (i.e., spread) were counted. For some experiments, F-actin staining of primary calvarial cells was done using rhodamine-phalloidin (Molecular Probes) following the manufacturer's instructions.

The adhesion assays were performed essentially as described in Tucker et al. (1999). In brief, dots of BSA, 10 μg/ml chicken tenascin-W, 10 μg/ml of human fibronectin, 10 μg/ml chicken collagen type I, or combinations of these concentrations of fibronectin and collagen type I with tenascin-W, were made in a circular array near the periphery of a 35-mm polystyrene dish (Falcon, Lincoln Park, NJ) and incubated for 1 hr at room temperature. The plates were then blocked for 30 min at room temperature with PBS/0.1% BSA and rinsed. A suspension of 1 ml of primary chicken calvarial cells (1×10^5 cells) was then added to the dish, and after a short period of incubation (30 min) at 37°C weakly adherent cells were dislodged using a rotating shaker (1 min at 100 rpm) and discarded. Bound cells were then fixed in 4% paraformaldehyde, photographed, and counted. Statistical significance ($P < 0.05$) was determined using a Student's *t*-test.

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III.1.3 Tenascin-W Is a Novel Marker for Activated Tumor Stroma in Low-grade Human Breast Cancer and Influences Cell Behavior

Martin Degen, Florence Brellier, Renate Kain, Christian Ruiz, Luigi Terracciano, Gertraud Orend, and Ruth Chiquet-Ehrismann

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Research Article

Tenascin-W Is a Novel Marker for Activated Tumor Stroma in Low-grade Human Breast Cancer and Influences Cell Behavior

Martin Degen,[1] Florence Brellier,[1] Renate Kain,[4] Christian Ruiz,[2] Luigi Terracciano,[2] Gertraud Orend,[3] and Ruth Chiquet-Ehrismann[1]

[1]Friedrich Miescher Institute for Biomedical Research, Novartis Research Foundation; [2]Institute of Pathology and [3]Institute of Biochemistry and Genetics, Center for Biomedicine, University of Basel, Basel, Switzerland; and [4]Department of Clinical Pathology, University of Vienna, Vienna, Austria

Abstract

This is the first report about human tenascin-W, the fourth and final member of the extracellular matrix protein family of tenascins. Sixty-three human breast tumor extracts were analyzed by Western blotting for the presence of tenascin-W and compared with tenascin-C, an established marker of tumor stroma. Interestingly, we found tenascin-W expression in the majority of the tumor tissues, but no detectable expression in the normal mammary parenchyma. Eighty-one percent of the breast tumor samples were tenascin-W positive and 86% showed expression of tenascin-C. However, tenascin-W and tenascin-C amounts varied greatly between tumors and some contained either tenascin-W or tenascin-C exclusively, indicating independent mechanisms regulating their expression. Although there was no difference between high- or low-grade tumors with respect to the presence of tenascin-C, tenascin-W was more prominent in low-grade tumors. For 42 of the breast cancer tissues, a frozen tumor microarray was available to confirm the Western blot data by immunohistochemistry. Similar to tenascin-C, tenascin-W was detected in the tumor stroma. Fibroblasts adhered to tenascin-W in a β_1 integrin–dependent manner and spread with a distinctive morphology under conditions where they remained round on tenascin-C. CHOβ2 cells expressing $\alpha_v\beta_1$ or $\alpha_4\beta_1$ integrins were able to spread on tenascin-W. Furthermore, addition of tenascin-W to the culture medium increased migration of breast cancer cells toward a fibronectin substratum *in vitro*. These data imply that tenascin-W expression in the activated tumor stroma facilitates tumorigenesis by supporting the migratory behavior of breast cancer cells. [Cancer Res 2007;67(19):9169–79]

Introduction

Epithelial tissues depend on normal stromal cells and the basement membrane for maintenance of tissue homeostasis, cell adhesion to extracellular matrix (ECM) through integrins, and generation and preservation of epithelial cell polarity (1, 2). These interactions continue to be important in epithelial pathologies. During carcinogenesis, the stromal cells get activated by an initial trigger that usually comes from the cancer cell, leading to stromal changes and the formation of a tumor-permissive microenvironment (1). More and more evidence is accumulating that this aberrant tumor stroma influences cancer development and has an effect on the malignancy of a tumor (for reviews, see refs. 3–6). Tumor-associated cells of the stromal compartment express proproliferative paracrine signals for epithelial cells (7), stimulate angiogenesis (8, 9), and can even show loss of tumor-suppressor genes (10). Therefore, understanding the mechanisms of the complex crosstalk between the cancerous epithelial cells and the tumor stroma might lead to novel approaches for cancer therapies that target the functions of the activated stromal cells (for reviews, see refs. 3, 11–13).

Because of the newly recognized importance of the tumor stroma in cancer development, it is necessary to fully characterize this tissue compartment. A prominent ECM protein specifically present in tumor stroma is tenascin-C (ref. 14; for reviews, see refs. 15–17). Interestingly, tenascin-C was shown to be expressed around angiogenic vessels in many tumors (18–20) as well as to promote angiogenesis in cell culture studies (21). Furthermore, tenascin-C addition to a fibronectin substratum stimulated cancer cell growth in *in vitro* studies (22, 23). Therefore, tenascin-C is one of the potential candidate molecules mediating the protumorigenic effects of tumor stroma (for review, see ref. 15).

Recently, we found in the stroma of mouse mammary tumors the induction of a second member of the tenascin family of ECM proteins, tenascin-W (24). Because the human orthologue has never been analyzed, we cloned the human tenascin-W cDNA, expressed the protein, and raised antibodies against it. We determined the presence of tenascin-W in a large number of breast cancers where it was more prevalent in low-grade tumors. *In vitro*, tenascin-W did not interfere with cancer cell adhesion to fibronectin, but promoted migration of breast cancer cells toward fibronectin. Furthermore, fibroblasts were able to adhere to a tenascin-W substratum. Our data suggest tenascin-W as a marker for transformation of the normal physiologic stroma to an activated stroma in breast cancer, and that tenascin-W can influence cancer cell behavior.

Materials and Methods

Cloning, expression, and purification of full-length human tenascin-W and tenascin-C. cDNA was synthesized from total RNA extracted from the human osteosarcoma cell line KRIB with TRIzol (Invitrogen). PCR was done using the KRIB cDNA as template and primers for nested PCR were designed that amplified three separate but overlapping parts of human tenascin-W that we call part A, part B, and part C. The first reaction was done with the primer sets 5′-CAGGCATCCTGGAGGGTCTGCTCCC-3′/5′-GTGAGGGCATTGGTGTCAGCTTTC-3′ (for part A), 5′-GCGCTA-CACTTCTGCTGATG-3′/5′-CTGTGGAGAGGGTGGTGG-3′ (for part B), and

Requests for reprints: Ruth Chiquet-Ehrismann, Friedrich Miescher Institute for Biomedical Research, Novartis Research Foundation, Maulbeerstrasse 66, CH-4058 Basel, Switzerland. Phone: 41-61-697-2494; Fax: 41-61-697-3976; E-mail: chiquet@fmi.ch.

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5′-CGCAGTCTGGTGGCATATTG-3′/5′-CATGATTTGTTCTGCGGGC-3′ (for part C). Primer sets for the second reactions included *Xho*I and *Bam*HI restriction sites, respectively. Primer pair 5′-GACTCGAGCTTTCCAAGGATGAGTCTCCAGG-3′/5′-GAGGATCCCCTGGTTGCCCCTTTCAGCCC-3′ was used for part A, primer pair 5′-GACTCGAGTGCACAAGGATGAGAGCAG-3′/5′-GAGGATCCACCCTTAAAGGCAACAAGGG-3′ was used for part B, and primer pair 5′-GACTCGAGCGGCTACATTCTGACTTACC-3′/5′-GAGGATCCTCAGTGATGGTGATGGTGATGGAACGTTCGCAGCCTTCCTC-3′ was used for part C. The reverse primer for the second PCR of part C contained the sequence encoding a 6×His-tag followed by a stop codon. The first two fragments (A and B) were ligated using an internal *Acc*I restriction site present in the overlapping region, and the third fragment was added using an internal *Nar*I restriction site located in the overlap of part B and C. This resulted in a full-length human tenascin-W cDNA of 3,885 bp (corresponds to the sequence 16–3,900 of Genbank accession no. NM_022093). This full-length cDNA was cloned into the expression vector pCEP/Pu (provided by J. Engel, Biozentrum, Basel, Switzerland) and transfected into EBNA293 cells [American Type Culture Collection (ATCC)] using FuGENE6 (Roche). Transfected cells were selected with puromycin, and serum-free medium containing the secreted recombinant human tenascin-W was collected. After ammonium sulfate precipitation (0–50%) and dialysis against PBS, the protein was passed over a gelatin-agarose column to remove fibronectin followed by affinity purification on a Ni-NTA matrix (Qiagen) in the presence of 0.5 mol/L urea. After extensive washing of the column, tenascin-W was eluted with 250 mmol/L imidazole (pH 6.9) and finally dialyzed against PBS. The protein appeared as a single band on a Coomassie-stained acrylamide gel, and fibronectin could not be detected in the purified sample by immunoblotting or by ELISA.

A full-length human tenascin-C cDNA called HxBL was kindly obtained from H.P. Erickson (Department of Cell Biology, Duke University, Durham, NC). It was modified by the addition of a 6×His-tag at the COOH terminus and subcloned into pCEP/Pu for transfection into EBNA293 cells as described above for human tenascin-W (25). The same purification procedure as described above for tenascin-W was used for the isolation of human tenascin-C.

Anti–tenascin-W antibody production. To raise polyclonal antisera in rabbits, a recombinant fragment of human tenascin-W was cloned, bacterially expressed, and purified. To clone the recombinant fragment, specific primers were designed to amplify the sequence encoding the last two fibronectin type III domains (Fig. 1*A*) with the Expand High Fidelity PCR System (Roche). The cDNA of the full-length human tenascin-W (described above) was used as template and the PCR was done with the primer set 5′-GAGGATCCGAAATTGACGGCCCCAAAAACC-3′/5′-ATAAGCTTATGTGGAGAGGGTGGTGGA-3′. The forward primer included a *Bam*HI restriction site and the reverse primer a stop codon immediately followed by a *Hin*dIII restriction site to enable the directional cloning into the bacterial expression vector pQE30 (Qiagen), supplying a COOH-terminal His tag for the purification of the recombinant fragment. The recombinant fragment corresponding to fibronectin type III domains 3F/4 (Fig. 1*A*) was expressed and purified by affinity chromatography to a Ni-NTA matrix (Qiagen) following the supplier's instructions. Purification was done under native conditions and elution by 250 mmol/L imidazole (pH 6.9). The bacterially expressed fragment of tenascin-W was then used to raise polyclonal antisera in rabbits using standard immunization procedures.

To raise monoclonal antibodies (mAb) in mice, a recombinant fragment of human tenascin-W was cloned containing the last three fibronectin type III domains (Fig. 1*A*), bacterially expressed, and purified as described above. To clone the recombinant fragment, cDNA was synthesized from total RNA that was extracted from Saos-2 cells (ATCC) by TRIzol reagent (Invitrogen). Primers, amplifying the last three FN type III domains, were used for nested PCR reactions with the Expand High Fidelity System (Roche) using the Saos-2 cDNA as template. The first reaction was done with the primer set 5′-GGGAAGGAGCAGAGTAGCACTG-3′/5′-CCGCCTCTGGAAGACAATCC-3′, the second reaction with the primers 5′-AGGGATCCGACATTGACAGCCCC-CAAAACC-3′/5′-CTAAGCTTTCATGTGGAGAGGGTGGTGGATAC-3′. The forward primer for the second PCR included a *Bam*HI restriction site and the reverse primer a stop codon immediately followed by a *Hin*dIII restriction site to enable the directional cloning into the bacterial expression vector pQE30 (Qiagen), supplying a COOH-terminal His tag for the purification of the recombinant fragment. mAbs were obtained by immunizing female BALB/c mice with 34.6 μg of the purified recombinant tenascin-W fragment emulsified with STIMUNE (ID-Lelystad, Institute for Animal Sciences and Health). For boosting, mice were injected twice with a 4-week interval with 25 μg tenascin-W fragment. Splenic lymphocytes were fused with the myeloma cell line P3X63Ag8.653 (ATTC) and cultured according to standard protocols. The hybridoma supernatants were analyzed by ELISA and Western blot analysis of a tenascin-W fragment expressed in HEK293 cells using a construct containing the sequence of the last three FN III domains of tenascin-W fused to an NH_2-terminal fragment of chicken tenascin-C containing its secretion signal and the epitope for the well-characterized monoclonal anti-chicken tenascin-C antibody mAb60. IgGs from two mAb hybridoma clones were purified from conditioned medium by protein G Sepharose (Amersham) and clone 56O was used in this study.

Human tissue extracts and Western blot analysis. The following study was done in accordance with the guidelines of the ethical committee of the University of Basel. Fresh human tissue was frozen on dry ice immediately after surgery. For the processing of the tissue, it was thawed on ice, minced, and homogenized in lysis buffer [100 mmol/L phosphate buffer (pH 8.0), 300 mmol/L NaCl, 8 mol/L urea, 1% Triton X-100, 10 mmol/L β-mercaptoethanol, 50 mmol/L guanidinium hydrochloride, and complete protease inhibitor cocktail (Roche)]. Insoluble material was pelleted, and reducing SDS-PAGE sample buffer was added to the supernatant and boiled for 5 min at 95°C. After electrophoresis on 6% polyacrylamide gels, proteins were electrotransferred onto polyvinyldifluoride membranes (Millipore) using a semidry blotting apparatus (Millipore). After the transfer, membranes were stained with Amido Black to control equal protein loading. After blocking for 1 h at room temperature in TBS containing 0.05% Tween and 5% skim milk powder, membranes were incubated overnight with either the polyclonal tenascin-W antiserum (1:750), the mAb 56O raised against tenascin-W (1:1,000), the mAb B28-13 raised against tenascin-C (1:100; ref. 26), or the monoclonal antivinculin antibody (1:2,000; Sigma) followed by an incubation for 1 h with anti-rabbit IgG or anti-mouse IgG coupled to horseradish peroxidase (1:10,000), respectively. Blots were developed using SuperSignal (Pierce) and exposed to Kodak BioMax MR Films.

For Western blot quantification, the software Gene Tools from SynGene was used. Briefly, the quantity of 25 ng was assigned to the specific band obtained from 25 ng of the purified protein loaded on the same gel. By dividing the densitometric values of the bands of the tissue extracts by the value obtained with 25 ng of the purified protein loaded and developed on the same gel, a quantity for each band could be calculated and further normalized to vinculin.

Frozen tissue microarrays and immunohistochemistry. A frozen tissue microarray (TMA) was constructed from frozen tissue samples of 40 breast carcinomas and 2 fibroadenomas. Pathologic features of these samples are summarized in Table 1 (patients 1–42). Histologic grading of the breast carcinomas was done according to the Bloom, Richardson, Elston grading system. A second TMA was built from 10 frozen tissue samples of normal breast tissue. Both TMAs were constructed in frozen Tissue-Tek optimum cutting temperature compound (Miles Laboratories) as described previously (27). We optimized a commercial microarray device (Beecher Instruments) by using a 0.6-mm drill for recipient whole making instead of the conventional hollow needle.

All immunostainings were done using the Discovery XT automated stainer (Ventana Medical Systems), with 3,3′-diaminobenzidine Map detection kit (Ventana). Frozen TMA slides were dried 1 h at room temperature, fixed for 10 min at 4°C in acetone, and then introduced into the automate. No pretreatment was required for any staining. Slides were first blocked twice for 12 min with the AB Block reagent (Ventana). Afterward, they were incubated for 1 h at 37°C with mouse monoclonal anti–tenascin-C (B28-13; 1:2,500), rabbit polyclonal anti–tenascin-W (1:40), and mouse monoclonal anti–tenascin-W (clone 56O; 1:800). Slides were then treated for 32 min at 37°C with a biotinylated universal secondary antibody (Ventana) and counterstained with hematoxylin and bluing reagent (Ventana).

Cell culture. EBNA293 (ATCC), T47D (ATCC), MCF-7 (ATCC), MDA-MB-435 (ATCC), and Detroit 551 cells (ATCC) were grown in DMEM supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin and cultured under standard conditions. CHOB2 (28) and sublines CHOB2α27 (ref. 29; subclone 2C8), CHOB2α4β1 (30), and CHOB2v7 (31), expressing $\alpha_5\alpha_1$, $\alpha_4\beta_1$, and $\alpha_v\beta_1$, respectively, were grown in MEMα supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin and cultured under standard conditions.

Cell adhesion assays and cell morphology. Cell adhesion assays were done with the human mammary carcinoma cell lines T47D, MCF-7, MDA-MB-435; the integrin-deficient CHOB2 cells; CHOB2 cells expressing single integrins; and with human dermal fibroblasts Detroit 551. Sixty-well microtiter plates (Nunc) were coated for 1 h at room temperature with 5 μL per well PBS containing 0.01% Tween and 10 μg/mL of the indicated ECM protein and blocked for 30 min with PBS containing 1% bovine serum albumin (BSA). Cells for the adhesion assays were detached using trypsin-EDTA, resuspended in serum-free medium, and counted. Cells (1,500 per well) were plated on the different substrata for the indicated time at 37°C. Adherent cells were then fixed by the addition of 4% formaldehyde and stained with 0.1% crystal violet. Pictures of the entire wells were taken and cells were counted in triplicate wells in at least three independent experiments. Mixed substrata were prepared as follows: After coating the wells with a first ECM protein, they were washed with PBS and coated for another hour with the second ECM protein. The order of coating for the mixed substrata was not important as tested by ELISA.

To analyze cell morphology, 3.5-cm dishes containing four separate wells (Greiner) were coated as described above with the different ECM proteins. After 30 min of blocking in PBS/1% BSA, 10^3 Detroit 551 fibroblasts, resuspended in serum-free medium, were plated on the individual wells and incubated for 90 min at 37°C. To block β_1 integrin function, fibroblasts were plated in the presence of a function-blocking anti-integrin β_1 antibody (Chemicon, clone P5D2) at a concentration of 10 μg/mL. Fibroblasts were fixed by 4% paraformaldehyde in PBS for 30 min and permeabilized with PBS/0.1% Triton X-100 for 5 min followed by incubation with a monoclonal antivinculin antibody for 1 h at room temperature. Cells were washed thrice with PBS and incubated with a fluorescein-coupled anti-mouse IgG (Cappel, 1:1,000) and rhodamine-labeled phalloidin (Sigma; 1:500) for 1 h. After incubation with the secondary antibody, cells were washed thrice with PBS, once with H_2O, mounted using Mowiol (Calbiochem), examined, and photographed using an Axiophot microscope (Carl Zeiss MicroImaging) connected to a DFC480 camera (Leica).

Transwell migration assays. The transwell migration assay has been described previously (24). Briefly, the lower side of the membrane (Costar; porosity: 8 μm) was coated with 10 μg/mL ECM proteins for 2 h at 37°C. Serum-starved cells were trypsinized and resuspended in serum-free DMEM and a total of 10^5 cells were added to the upper chamber of each well coated on the lower side with 10 μg/mL ECM proteins. DMEM containing 10 μg/mL purified tenascin-W or tenascin-C was added to the bottom chamber and cells were allowed to migrate across the filter for 24 h at 37°C. Cells remaining on the upper side of the membrane were removed with a cotton swab and the cells that had migrated to the underside of the filter were fixed with 3.7% formaldehyde in PBS. Fixed cells were then stained with 0.1% crystal violet solution. Migration was quantified by counting cells per eight randomly selected fields of view using ×10 magnifications in triplicates in at least three independent experiments.

Results

Characterization of human tenascin-W. The gene encoding human tenascin-W is located on chromosome 1 in a tail-to-tail configuration next to the *tenascin-R* gene (32). The derived cDNA and amino acid sequences can be found under accession no. NM_022093. Because no data existed about the human tenascin-W protein, we decided to clone parts of tenascin-W for antibody production as well as the full-length protein for cell biological studies (described in Materials and Methods). The primary sequence of human tenascin-W encodes a protein of 1,294 amino acids. The structural motifs and their arrangement are shown in Fig. 1*A*. Tenascin-W contains an NH_2-terminal oligomerization domain, including heptad repeats followed by 3.5 epidermal growth

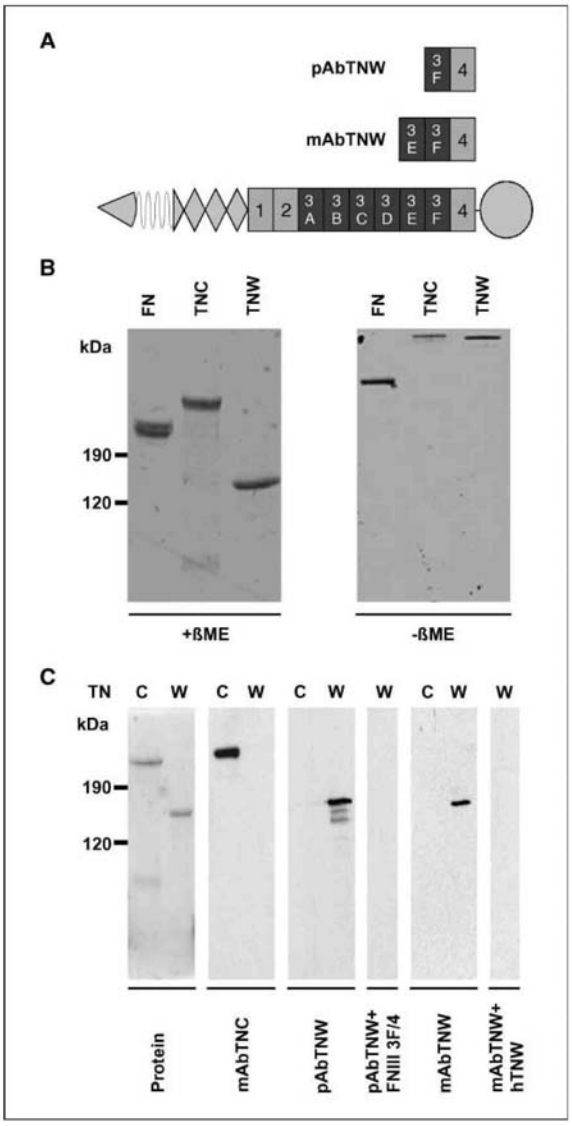

Figure 1. Tenascin-W protein and specific antibodies. *A*, schematic representation of human tenascin-W (*TNW*) and the recombinant fragments that served as antigens to raise polyclonal and mAbs. The following symbols have been used to identify the structural domains: heptad repeats (*wavy line*), EGF-like repeats (*diamonds*), FNIII domains (*boxes*), FNIII domains generated by duplication (*dark boxes*), fibrinogen globe (*circle*). *B*, SDS-PAGE analysis of the purified full-length human tenascin-W compared with purified fibronectin (*FN*) and tenascin-C (*TNC*) reveals a monomeric tenascin-W subunit of 160 kDa under reducing (*left*), and a hexameric structure under nonreducing (*right*), conditions. *βME*, β-mercaptoethanol. *C*, immunoblots showing the specificity of the antibodies used in this study. The anti–tenascin-W antibodies only recognize tenascin-W but not tenascin-C and vice versa, and their activities can be blocked by preincubation with the recombinant fragment or with purified full-length protein shown in *A*.

Table 1. Clinicopathologic features and tenascin levels

Patient	pT	pN	Grade	Type	TNW	TNC
1	pT_3	pN_1	G2	d.c.	n.d.	28.6
2	pT_2	pN_x	G2	d.c.	86.4	37.5
3	pT_2	pN_0	G2	d.c.	74.8	42.4
4	n.a.	n.a.	G2	d.c.	n.d.	13.5
5	pT_2	pN_0	G3	d.c.	16.9	40.8
6	pT_2	pN_1	G2	d.c.	44.8	22.2
7	pT_4	pN_1	G3	d.c.	n.d.	3.1
8	pT_2	pN_0	G3	d.c.	106.0	16.7
9	pT_2	pN_1	G3	d.c.	12.4	2.9*
10	pT_2	pN_1	G2	d.c.	39.2	8.2
11	pT_2	pN_0	G3	d.c.	32.8	75.5*
12	pT_2	pN_1	G3	d.c.	75.4	27.9
13	pT_2	pN_1	G2	d.c.	172.0	41.4
14	pT_4	pN_1	G3	d.c.	2.2	1.7
15	pT_3	pN_2	G3	d.c.	1.8	10.2
16	pT_2	pN_1	G2	d.c.	43.3	40.8
17	pT_2	pN_1	G2	l.c.	95.1	67.2
18	pT_2	pN_1	G2	d.c.	22.5	16.3
19	pT_2	pN_0	G2	l.c.	56.4	45.2
20	pT_4	pN_2	G2	l.c.	66.8	56.8
21	pT_2	pN_1	G1	d.c.	178.0	3.4
22	pT_2	pN_1	G2	d.c.	27.4	16.7
23	pT_2	pN_0	G2	d.c.	15.2	15.8
24	pT_3	pN_1	G3	d.c.	n.d.	6.0
25	pT_2	pN_1	G2	d.c.	1.8	27.3
26	pT_2	pN_0	G3	d.c.	n.d	5.8*
27	pT_4	pN_1	G3	d.c.	n.d.	48.7*
28	pT_3	pN_2	G2	l.c.	1.3	3.4
29	pT_4	pN_1	G2	d.c.	21.4	1.6
30	PT_2	pN_1	G3	d.c.	42.3	45.6
31	pT_4	pN_1	G3	l.c.	37.5	37.5
32	pT_2	pN_1	G2	d.c.	n.d.	n.d.
33				a.d.	162.0	41.1
34	pT_2	pN_1	G2	m.c.	25.2	n.d.
35				a.d.	86.9	41.3
36	pT_2	pN_0	G2	d.c.	22.3	12.3
37	pT_3	pN_1	G2	l.c.	88.6	5.9
38	pT_2	pN_1	G2	d.c.	64.8	31.2
39	pT_2	pN_1	G2	d.c.	7.2	44.1
40	pT_4	pN_1	G3	d.c.	n.d.	n.d.
41	pT_2	pN_1	G2	d.c.	3.6	n.d.
42	pT_2	pN_0	G2	d.c.	129.0	5.7
43	n.a.	n.a	n.a.	n.a.	28.6	3.9
44	n.a.	n.a.	n.a.	n.a.	52.2	1.0
45	n.a.	n.a.	n.a.	n.a.	54.1	10.2
46	n.a.	n.a.	n.a.	n.a.	n.d.	86.8
47	n.a.	n.a.	n.a.	n.a.	49.9	16.8
48	pT_1	pN_0	G3	d.c.	20.4	32.2
49				a.d.	93.4	73.3
50				a.d.	2.8	n.d.
51	pT_2	pN_1	G2	d.c.	48.8	70.5
52	pT_2	n.a.	n.a.	l.c.	n.d.	n.d.
53	pT_1	pN_0	G2	d.c.	66.9	62.8
54	n.a.	pN_0	n.a.	n.a.	18.6	n.d.
55	pT_1	pN_0	G2	d.c.	86.4	26.5
56	pT_4	pN_0	G2	d.c.	62.2	18.1
57	pT_2	pN_1	n.a.	m.c.	66.8	58.9
58	pT_2	pN_1	G3	d.c.	n.d.	n.d.
59	n.a.	n.a.	n.a.	n.a.	3.8	32.2
60	pT_2	pN_1	G2	l.c./d.c.	17.2	65.8
61	pT_1	pN_1	G1	d.c.	n.d.	n.d.
62				a.d.	26.0	38.0
63	pT_2	pN_0	G3	d.c.	18.8	56.7

Abbreviations: pT, tumor size; pN, palpable nodes; n.a., not available; n.d., not detectable; d.c., ductal carcinoma; lc., lobular carcinoma; m.c., medullary carcinoma; a.d., fibroadenoma; TNW, TNC, protein levels deduced by immunoblotting (Fig. 2).
*These tumors show stromal and epithelial tenascin-C staining.

factor (EGF)–like repeats, 9 fibronectin type III (FNIII) domains, and a COOH-terminal fibrinogen globe. Human tenascin-W shows high sequence conservation with mouse tenascin-W. There are, however, some noticeable differences. First, the mouse *tenascin-W* gene contains an RGD sequence located in the second FNIII domain, whereas the human *tenascin-W* does not. Interestingly, it is the opposite in the case of tenascin-C where the human protein contains an RGD sequence and the mouse orthologue does not. Second, the two tenascin-W orthologues differ in the number of FNIII domains (32). To analyze the structure and function of tenascin-W, we raised antibodies against bacterially expressed tenascin-W fragments consisting of FNIII domains (Fig. 1*A*). Full-length tenascin-W was purified from transfected EBNA293 cells. SDS-PAGE analysis revealed a molecular weight of 160 kDa per subunit under reducing conditions, which corresponds to the calculated size deduced from the cDNA sequence. Tenascin-W monomers are smaller than the monomeric forms of human tenascin-C and fibronectin (Fig. 1*B*). Under nonreducing conditions, tenascin-W migrates as a hexamer slightly faster than hexameric tenascin-C (Fig. 1*B*). To prove specificity of the tenascin-W antibodies, we did immunoblots of purified full-length tenascin-W and tenascin-C (Fig. 1*C*). The polyclonal as well as the monoclonal anti–tenascin-W antibodies specifically react with tenascin-W and show no cross-reactivity with tenascin-C. Furthermore, the immunoreactivity of both antibodies can be abolished by preincubation with either the bacterially expressed tenascin-W fragment or the full-length human tenascin-W protein (Fig. 1*C*).

Tenascin-W is present in extracts of human breast tumors but not in the corresponding normal tissue. To test whether tenascin-C shares its prominent expression in neoplasms with tenascin-W, we analyzed extracts from cancer tissues of 63 breast tumor patients (carcinomas as well as benign tumors; see Table 1 for details) compared with normal breast tissue. As shown by the immunoblots in Fig. 2, the majority of breast tumor tissue samples contained detectable levels of tenascin-C (54 of 63; 86%). Also, 81% of breast cancer samples (51 of 63) were positive for tenascin-W. For tenascin-C, we detected different isoforms due to alternative splicing as previously reported (33). In the case of tenascin-W, we only observed a single major band for all extracts. Thus, we do not have evidence for the existence of prominent alternatively spliced tenascin-W isoforms in breast cancer (Fig. 2). However, in some cases, the detected tenascin-W band migrated slightly differently

between patients, possibly representing differences in posttranslational modifications and in rare cases a very faint second lower band was detectable. We assume that the lower bands represent degradation products of tenascin-W, but we cannot exclude that alternative splicing might occur at very low levels. For patients 54 to 63, extracts from corresponding normal breast tissue were also available. In these normal tissues, neither tenascin-C nor tenascin-W could be detected, although most of the tumor extracts of the same patients revealed expression of tenascin proteins (Fig. 2). In summary, the majority of human breast tumors express both tenascins, but their relative amount varies largely between patients. In addition, some cancers express either tenascin-W or tenascin-C alone. This observation suggests that expression and/or deposition and degradation of tenascin-C and tenascin-W can be regulated differentially.

To confirm these results by immunohistochemistry and to localize the proteins within the tissues, we made use of frozen TMAs. The breast cancer TMA contained spots from the tumors of patients 1 to 42 (see Table 1; Fig. 2) and a second TMA contained spots from 10 normal mammary tissues. In 36 of the 42 cases, the staining revealed a very strong expression of tenascin-C in the tumor stroma surrounding the transformed epithelial cells but no staining in the normal tissues. In four cases, tenascin-C staining was also present within the epithelial compartment of the tumors (Table 1). These tumors all belong to high-grade G3 breast cancers. In contrast, tenascin-W was exclusively detected in the tumor stroma in 34 of the 42 patients and normal tissue was negative. Examples of the different staining patterns are shown in Fig. 3. Both monoclonal and polyclonal anti–tenascin-W antibodies gave the same staining patterns (Fig. 3*A*) and in most cases stromal staining for tenascin-C overlapped with tenascin-W staining (Fig. 3*B*). In patient 24, only very faint stromal staining is seen for either tenascin antibody consistent with the corresponding Western blots that were weakly positive for tenascin-C and negative for tenascin-W. In a rare case of ductal carcinoma (patient 27), tenascin-C staining was observed throughout the tumor, including the transformed epithelial cells, whereas tenascin-W is only expressed in the stromal compartment. Also, benign tumors were rich in tenascin-W and tenascin-C. Patient 33 gives an example of a fibroadenoma with strong staining by all antibodies throughout the tumor mass. The relative amounts of tenascin-W and tenascin-C in these benign tumors varied greatly (cf. Table 1) with mean tenascin-W levels of 74.2 $\pm$ 62 (Fig. 4*A*).

In summary, the immunohistochemical staining of the TMAs confirmed our results from the immunoblotting experiments and showed that tenascin-W is a specific marker for breast tumor stroma of benign as well as malignant lesions.

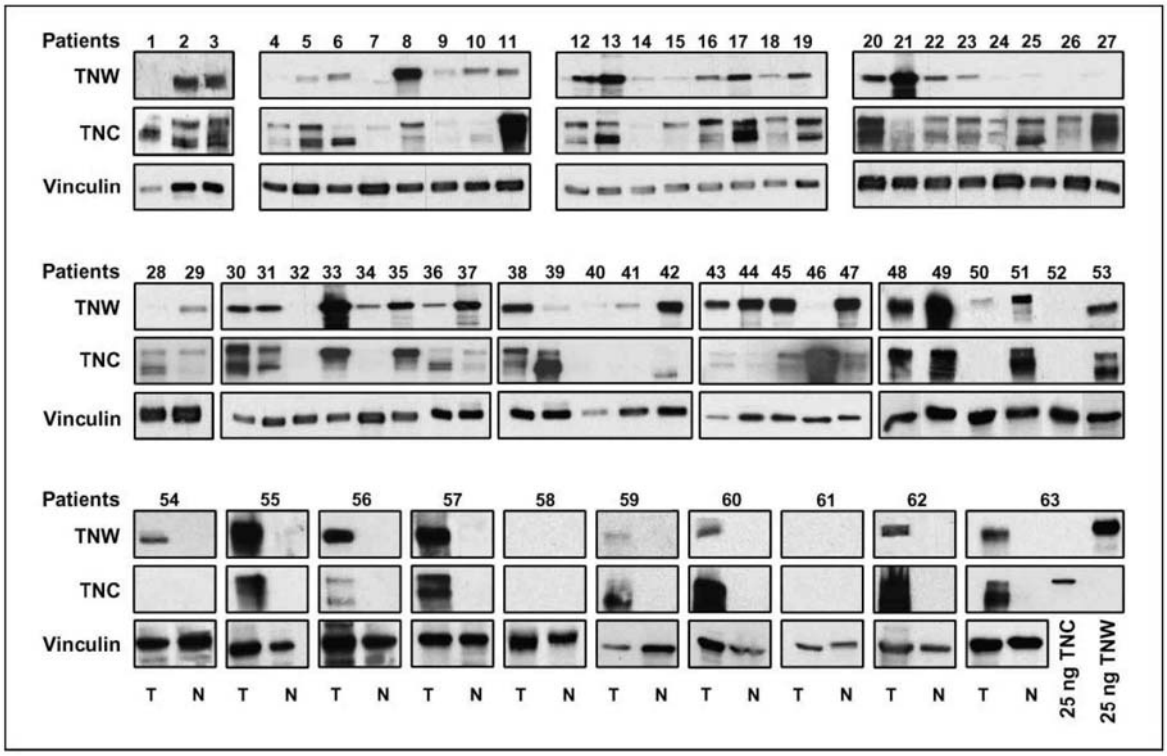

Figure 2. Tenascin-W is expressed in human mammary tumors but not in the corresponding normal tissue. Tumor extracts from 63 breast tumor patients were tested by Western blot for the presence of tenascin-W and tenascin-C. Analysis revealed high expression of tenascin-W and tenascin-C in the majority of the tested extracts. For patients 54 to 63, the corresponding normal breast tissue could also be analyzed and did not show any tenascin expression. To quantify the immunoblots (see Materials and Methods), 25 ng of the purified proteins were loaded on each gel (see patient 63) and vinculin detection was used to normalize for protein loading. *N*, normal tissue; *T*, tumor tissue.

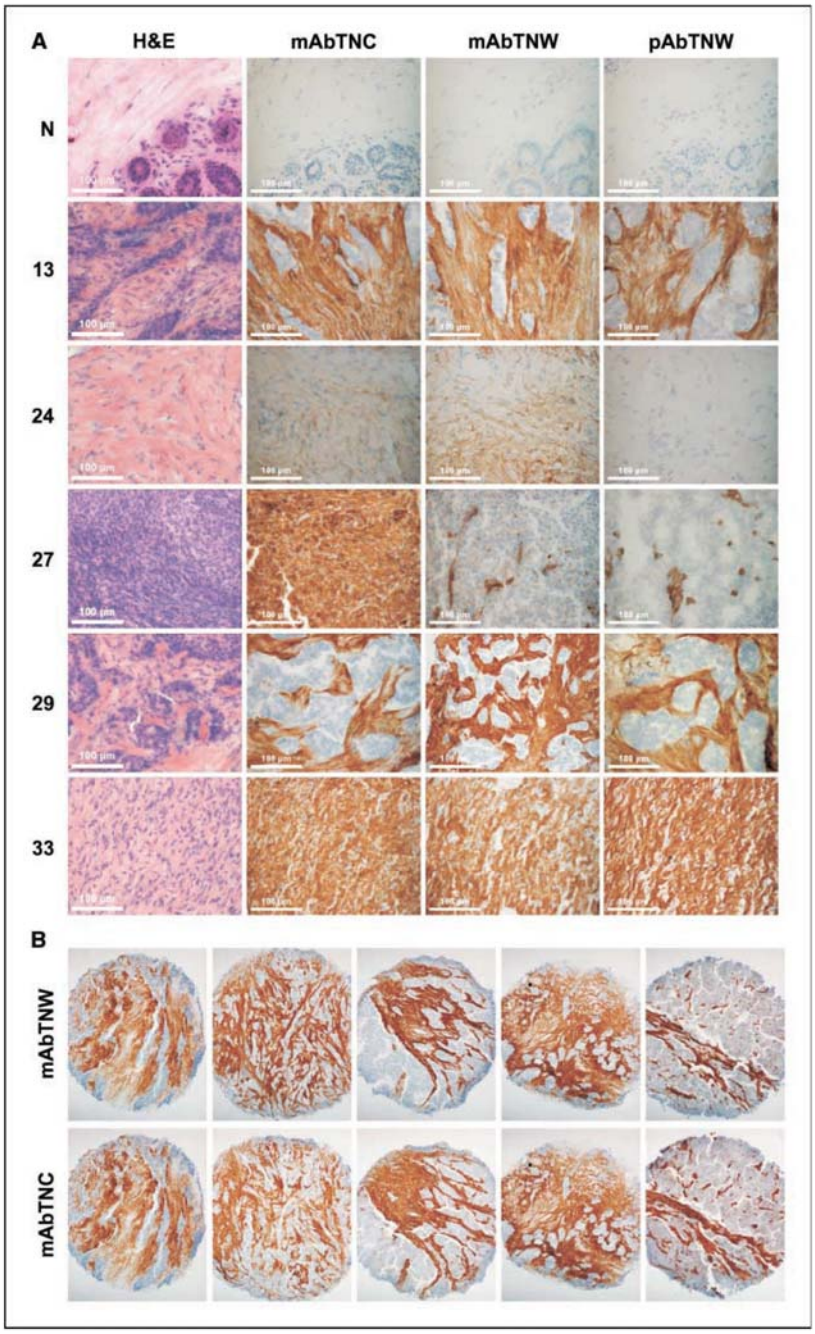

Figure 3. Immunohistochemical localization of tenascin-W and tenascin-C in human breast tumors. *A*, immunohistochemistry with a monoclonal anti–tenascin-C antibody (*mAbTNC*), a monoclonal anti–tenascin-W (*mAbTNW*), a polyclonal anti–tenascin-W antiserum (*pAbTNW*), and H&E-stained sections of frozen TMAs. Normal breast tissue is negative for both tenascins. Patient numbers are indicated on the left. In all of the patients (1–42) analyzed, it was found that if tenascin-W is present, it is exclusively localized in the tumor stroma and the polyclonal and the monoclonal anti–tenascin-W antibodies gave identical staining patterns. In contrast, there are some cases where tenascin-C besides stromal staining shows expression in the epithelial compartment (patient 27). A selection of ductal carcinomas with different patterns of tenascin expression is presented: both tenascins are highly expressed (patient 13); both tenascins are expressed at low levels (patient 24); tenascin-C is more prominent than tenascin-W (patient 27) and vice versa (patient 29). Not only carcinomas are highly positive for tenascins but also some of the fibroadenomas (patient 33). *B*, when both tenascins are present in the tumor stroma, the patterns of expression are highly similar (four times lower magnification than in *A*).

Tenascin-W is enriched in low-grade breast cancer. In most of the published work on breast cancer, no clear correlation of tenascin-C with malignancy or any other poor diagnostic or prognostic factors was observed (reviewed in ref. 34). We wanted to know whether this could be different for tenascin-W and whether expression of tenascin-W does correlate with any known diagnostic factor. When we compared the amount of tenascin-W in tumor lysates, as determined by immunoblotting (Table 1), to the histologic tumor grade, we realized that tenascin-W is significantly ($P < 0.03$) enriched in low-grade tumors (G1 and G2;

49.0 ± 47) compared with high-grade tumors (G3; 21.6 ± 30). The mean relative amount of tenascin-W in G1/G2 tumors is >2-fold higher than in G3 tumors (Fig. 4*A*). In contrast, there is no significant difference between high-grade (24.2 ± 23) and low-grade tumors (25.9 ± 22) with respect to tenascin-C expression (Fig. 4*B*). This correlation between higher tenascin-W expression and low-grade breast tumors could be confirmed in an independent RNA profiling study of different breast cancer patients done by van't Veer et al. (35), accessible in the ONCOMINE database (36). The heat maps presented in Fig. 4*C* show enrichment in tenascin-W transcripts in G1/G2 tumors compared with G3 tumors, whereas there is no obvious tendency visible in the same samples for the tenascin-C transcripts. In another study by Farmer et al. (37), transcript profiling was done of basal versus luminal breast carcinomas. Interestingly, tenascin-W is highly elevated in luminal compared with basal carcinomas, indicating that it may correlate with the estrogen receptor (ER) status as well (Fig. 4*D*).

Tenascin-W promotes fibroblast adhesion and stimulates cancer cell migration toward fibronectin. Because tenascin-W is present in the stroma surrounding tumors, we investigated its effects on stromal cells such as fibroblasts as well as on the neighboring cancer cells. Tenascin-C is an antiadhesive protein with adhesion-modulating effects leading to the expression of growth-promoting proteins (22, 23). To elucidate whether tenascin-W might act in a similar way, we first did cell adhesion assays using different breast cancer cell lines and normal human fibroblasts (Fig. 5*A*). The breast cancer cells T47D neither spread nor attached to a tenascin-W substratum, whereas they adhered to fibronectin or type I collagen. The same was the case for two other cell lines tested (MCF-7 and MDA-MB-435; not shown). Furthermore, we could confirm the adhesion-modulating effect by tenascin-C on breast cancer cell adhesion to fibronectin. In this respect, tenascin-W differed from tenascin-C because it did not affect tumor cell adhesion when it was offered as a mixed substratum with fibronectin or type I collagen. In contrast to tumor cells, fibroblasts did attach to a tenascin-W

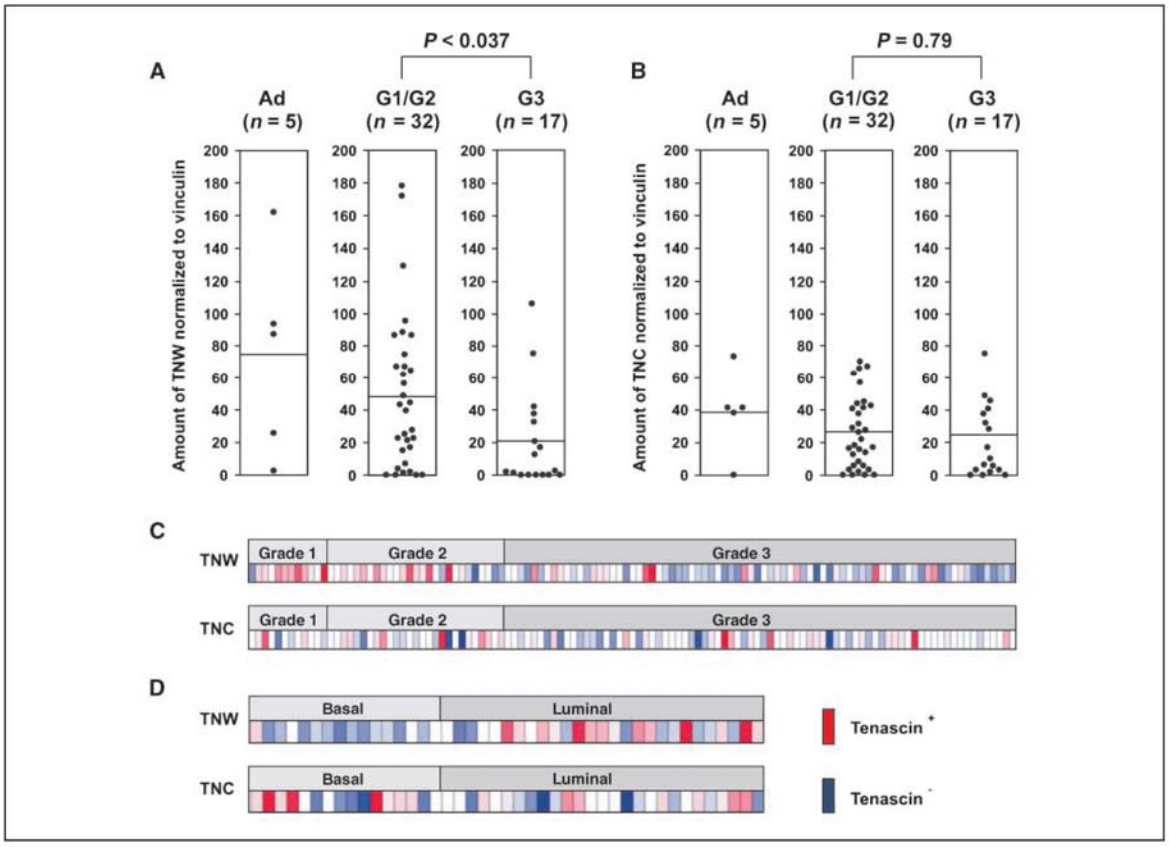

Figure 4. Tenascin-W is enriched in low-grade tumors. *A*, histograms showing the quantification of the immunoblot analysis of breast cancer extracts indicate a statistically significant higher mean tenascin-W expression in low-grade tumors (G1/G2; 49 ± 47) compared with its expression in high-grade tumors (G3; 21.6 ± 30). The mean tenascin-W amount in fibroadenomas (*Ad*; 74.2 ± 62) is similar to the mean value in low-grade tumors. Quantification was done as described in Materials and Methods. Below detection has been set to 0.1. *B*, in contrast to tenascin-W, there is no correlation between tenascin-C expression and tumor grade. *Bars*, mean expression value of the corresponding tumor grade. *C*, heat maps of tenascin-W and tenascin-C of an RNA profiling study of 117 breast cancer patients (35, 36). Enrichment of tenascin-W transcripts in low-grade tumors is confirmed in this study ($P = 4.8E10^{-5}$), whereas tenascin-C transcripts do not show any correlation with tumor grade. *D*, heat maps of tenascin-W and tenascin-C of an independent RNA profiling study of 43 breast cancer patient reveals that tenascin-W is almost exclusively found to be elevated in luminal breast cancers, whereas some of the basal breast cancers show high levels of tenascin-C (36, 37).

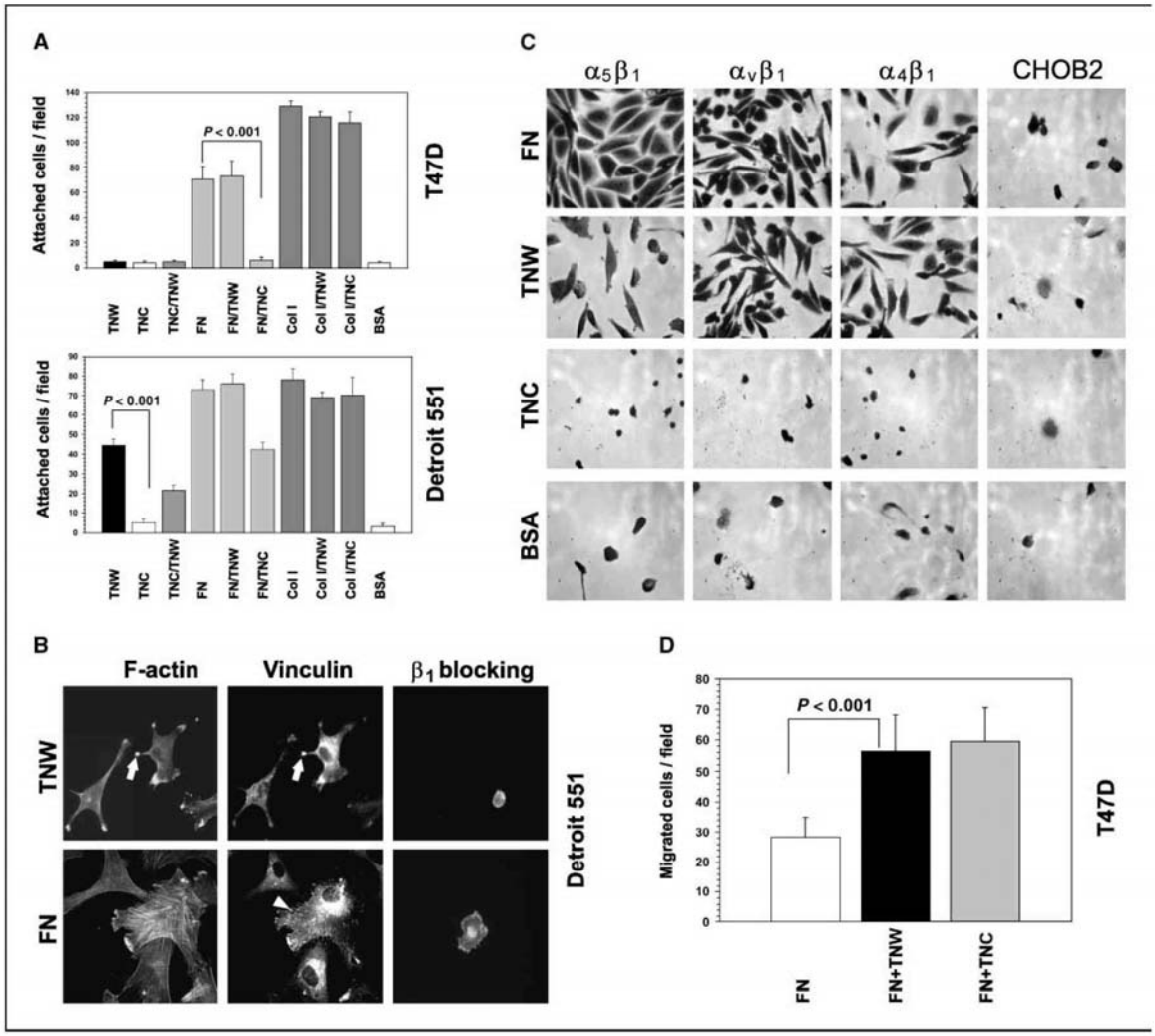

Figure 5. Tenascin-W promotes fibroblast adhesion and stimulates cancer cell migration. *A*, T47D breast cancer cells and Detroit 551 fibroblasts were plated for 2 h on the indicated single or mixed substrata before fixation, staining, and counting of adherent cells. Breast cancer cells (T47D) did not adhere to tenascin-W and tenascin-W does not have adhesion-modulating effects when mixed with fibronectin or collagen type I. In contrast, the adhesion-modulating effect of tenascin-C mixed with fibronectin was confirmed. Compared with T47D cells, Detroit 551 fibroblasts can attach to and partially spread on tenascin-W. However, an adhesion-modulating effect of tenascin-W was not observed when mixed with other ECM proteins. Again, addition of tenascin-C statistically significantly ($P < 0.002$) inhibits cell spreading on fibronectin. *B*, phalloidin and vinculin staining of Detroit 551 fibroblasts plated on tenascin-W or fibronectin. On fibronectin, fibroblasts formed long and thick actin cables and many focal contacts (*arrowhead*). On tenascin-W, actin fibers were absent and cells exhibited irregular shapes and formed long actin-rich protrusions (*arrow*) that were also positive for vinculin (*arrow*). Adhesion to tenascin-W is integrin mediated because addition of a function-blocking anti-integrin β_1 antibody (β_1 blocking) inhibited cell attachment and spreading. *C*, integrin-deficient CHOB2 cells and single integrin-expressing CHOB2 cells were plated for 20 h on the indicated ECM proteins before they were fixed, stained, and photographed. Integrin $\alpha_v\beta_1$- and $\alpha_4\beta_1$-expressing cells were able to adhere and spread on tenascin-W, whereas $\alpha_5\beta_1$-expressing cells preferentially attached and spread on fibronectin. None of the cells adhered to tenascin-C or BSA. *D*, transwell migration assay shows that addition of soluble tenascin-W or tenascin-C to the lower chamber leads to a statistically significant ($P < 0.001$) stimulation of T47D breast cancer cells across a fibronectin-coated filter.

substratum and partially spread (Fig. 5*A*). This property is unique for tenascin-W, because on tenascin-C fibroblasts remained round. When a mixed substratum of the two tenascin proteins was used, the antiadhesive effect of tenascin-C was slightly counteracted by tenascin-W. However, there is a distinct morphology of the spread fibroblasts on tenascin-W as revealed by phalloidin staining when compared with fibroblasts plated on fibronectin, type I collagen, or tenascin-C (Fig. 5*B*). Fibroblasts plated on fibronectin and type I collagen form actin stress fibers and a lot of focal contacts visualized by staining for vinculin (Fig. 5*B, arrowhead*). The same cells plated on tenascin-W appeared much more compact and irregularly shaped. They fail to form long actin cables, but instead produce many long actin-rich protrusions (Fig. 5*B, arrows*), which are also rich in vinculin (Fig. 5*B, arrows*). In contrast, the few fibroblasts that attached to

tenascin-C did not spread and remained with a round morphology (not shown). Adhesion to tenascin-W was integrin mediated, because addition of anti-β_1 integrin antibodies inhibited adhesion to tenascin-W (Fig. 5*B*). To find out which β_1 integrin(s) is able to mediate adhesion to tenascin-W, we made use of CHOB2 cells expressing single integrin α chains (29–31) and compared their morphology upon adhesion to tenascin-W, tenascin-C, or fibronectin (Fig. 5*C*). Integrin-deficient CHOB2 cells did not adhere to any substratum. Integrin $\alpha_5\beta_1$–expressing cells adhered and spread preferentially on fibronectin, whereas $\alpha_v\beta_1$- and $\alpha_4\beta_1$-expressing cells adhered to fibronectin and tenascin-W substrata with similar efficiency. Therefore, $\alpha_v\beta_1$ and $\alpha_4\beta_1$ can serve as receptors for cell adhesion to tenascin-W. None of the CHOB2 integrin-expressing cells tested adhered to tenascin-C– or BSA-coated plates (Fig. 5*C*).

Finally, we investigated the effect of tenascin-W on cell migration of breast cancer cells using transfilter migration chambers with the underside of the filters coated with fibronectin. Addition of soluble tenascin-W to the lower chamber stimulated T47D cell migration across the filters toward the fibronectin substratum (Fig. 5*D*). Migration toward fibronectin was enhanced 2-fold by the presence of tenascin-W in the culture medium, whereas cells did not migrate toward BSA. A similar migration-stimulatory effect was observed by the addition of soluble tenascin-C to the lower chamber of the transwell system (Fig. 5*D*). Therefore, the presence of tenascin-C as well as tenascin-W in tumor stroma may stimulate migration of cancer and cancer-associated cells.

Discussion

Here, we present the first report about human tenascin-W, the final member of the human tenascin family. We cloned the human tenascin-W full-length cDNA, expressed it in mammalian cell culture, and purified the protein. Human tenascin-W is built up from the same domain types as the other three known tenascins, tenascin-C, tenascin-R, and tenascin-X, namely heptad repeats, 3.5 EGF-like repeats, 9 FN III domains, and a COOH-terminal fibrinogen globe (see review in ref. 32). Although there are a lot of alternatively spliced variants in the other tenascins (38–41), which show different expression pattern and functions (42–44), there is no evidence for the existence of splice variants in tenascin-W, because we detected a single protein on immunoblots of tumor extracts.

Tenascin-W was originally identified in zebrafish, where it is coexpressed with tenascin-C in somites and by neural crest cells (45). More recently, murine (46) and chicken (47) tenascin-W have been described. In both species, tenascin-W is expressed in smooth muscle cells and bone. Chicken tenascin-W modulated adhesion and spreading of calvarial cells *in vitro* (47). In certain murine mammary tumors, tenascin-W was up-regulated and its presence appeared to correlate with the metastatic potential of the tumors (24).

In this report, we investigated the expression of tenascin-W in human breast cancer and found that 81% of the tumors tested expressed tenascin-W and 86% were positive for tenascin-C. However, the amount of tenascin-C and tenascin-W differed between samples, indicating independent mechanisms that regulate their expression. In our previous study on mouse tenascin-W in primary mouse embryo fibroblasts, we found that BMP-2 was a potent inducer of tenascin-W but not tenascin-C expression, and transforming growth factor-β showed the opposite effect and induced tenascin-C expression much more than that of tenascin-W (24). On the other hand, tumor necrosis factor-α strongly induced both proteins. It is possible that the relative amounts of these cytokines could account at least partially for the differential expression of the two tenascins in the tumor stroma of breast cancer.

We did not detect tenascin-W in normal human mammary tissue but could correlate tenascin-W expression levels with tumor grade. There is a statistically significant higher mean expression of tenascin-W in low-grade tumors (G1/G2) compared with high-grade tumors (G3). In contrast, in the present study, tenascin-C could not be correlated with tumor grade in mammary cancer. The differentially expressed tenascin-C isoforms could not be correlated with tumor grade either, although there is one report showing that some specific tenascin-C isoforms are only expressed in invasive breast carcinomas (48). However, in the transcript profiling study by Farmer et al. (37) extracted from ONCOMINE (36), only cases of basal cancers showed very high levels of tenascin-C, whereas tenascin-W was almost exclusively found to be elevated in luminal cancers. This indicates that tenascin-W might be elevated in ER-positive cancers, because ER-positive cancer cells tend to be enriched in luminal cancers. In contrast, high tenascin-C expression in basal cancers suggests a correlation with ER-negative cells enriched in basal cancers known to have a worse prognosis (49). In the literature, contradictory studies have been published on the value of tenascin-C as a prognostic marker in breast cancer, indicating that the correlations may not be very strong and may depend on the sampling (reviewed in ref. 34). In the case of tenascin-W, the data seem to be more consistent. In support of our findings, tenascin-W enrichment in low-grade breast cancers was independently confirmed in a different patient cohort by RNA profiling studies of breast cancer patients (35). These data are available from the ONCOMINE database. A good correlation between tenascin-W but not tenascin-C transcript levels and tumor grade could be found in this data set (cf. Fig. 4). Tenascin-C is, however, a useful prognostic marker for other types of tumors than breast cancer such as gliomas and lung cancer and in these cases seems to play a role in tumorigenesis (for review, see ref. 34). In many tumors, tenascin-C expression correlates with invasion and angiogenesis, whereas tenascin-C–deficient mice exhibit impaired angiogenesis (50, 51).

In the majority of breast cancers analyzed by immunohistochemistry, there was an almost perfect overlap between tenascin-C and tenascin-W expression in the tumor stroma, suggesting that both tenascins may serve similar but not identical functions. In contrast to tenascin-C, tenascin-W is an adhesive substratum for fibroblasts. They attached to tenascin-W and partially spread with an irregular cell shape that differed from fibroblasts on fibronectin or collagen type I where cells formed actin stress fibers and focal adhesions. The adhesion to tenascin-W was dependent on β_1 integrins and CHOB2 cells expressing either $\alpha_v\beta_1$ or $\alpha_4\beta_1$ adhered and spread on tenascin-W to a similar extent as to their known ECM ligand fibronectin. Thus, tenascin-W is a novel ligand for these two integrins.

Although both tenascins do not support breast cancer cell adhesion when they are used as a single substratum, they differ in their action when used as mixed substrata with fibronectin. Although tenascin-C inhibited cancer cell spreading on fibronectin, tenascin-W did not interfere with cell binding to fibronectin or type I collagen. Interestingly, both tenascins were able to induce cancer cell migration toward fibronectin. Because *in vivo*, the stromal ECM surrounding cancer cells contains a mixture of both tenascins together with many other ECM proteins, they may be part of an

activated tumor stroma promoting cancer cell migration and invasion. Because benign tumors can also exhibit high levels of stromal tenascin-W and tenascin-C, the presence of these two proteins is not sufficient to induce invasion. In fibroadenomas, the intact basement membrane separating the epithelial cells from the stromal compartment may protect them from the promigratory effect of the tenascins. The situation is different in carcinomas where additional factors lead to the breakdown of basement membranes and expose the epithelial cancer cells to the stromal environment.

For a long time, cancer research was mainly focused on the cancer cells alone. An amazing wealth of information about oncogenes and tumor suppressors was obtained, which led to the improvement of our understanding of the molecular events occurring in cancer cells and the proteins and signaling pathways affected represent promising new therapeutic targets. However, in recent years, it became clear that a cancer cell requires a permissive environment for progression and that carcinogenesis is accompanied by several changes in the stroma, which finally leads to an aberrant microenvironment that facilitates tumor growth and invasion (reviewed in refs. 4, 5). Because tenascin-W is expressed in lower-grade breast cancers and enhances cell migration, it might be an early marker of activated tumor stroma and thus a good antitumor target.

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III.1.4 Tenascin-W, a new of cancer stroma, is elevated in sera of colon and breast cancer patients

M. Degen, F. Brellier, S. Schenk, R. Driscoll, K. Zaman, R. Stupp, L. Tornillo, L. Terracciano, R. Chiquet-Ehrismann, C. Rüegg, and W. Seelentag

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Tenascin-W, a new marker of cancer stroma, is elevated in sera of colon and breast cancer patients

M. Degen[1], F. Brellier[1], S. Schenk[1], R. Driscoll[2], K. Zaman[3], R. Stupp[3], L. Tornillo[4], L. Terracciano[4], R. Chiquet-Ehrismann[1], C. Rüegg[2], and W. Seelentag[5]

[1] Friedrich Miescher Institute for Biomedical Research, Novartis Research Foundation, Basel, Switzerland
[2] Division of Experimental Oncology, Centre Pluridisciplinaire d'Oncologie, University of Lausanne, and Swiss Institute for Experimental Cancer Research, NCCR Molecular Oncology, Epalinges, Switzerland
[3] Centre Pluridisciplinaire d'Oncologie, University of Lausanne and Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland
[4] Institute of Pathology, University of Basel, Basel, Switzerland
[5] Institut de Pathologie, University of Lausanne and Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

Short title: Tenascin-W in human colorectal cancer

Keywords: extracellular matrix, tenascin, tumor biomarker, colorectal cancer, tumor stroma, serum

Abbreviations used: CEA, carcinoembryonic antigen; CRP, C-reactive protein; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-related kinase; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMA, tissue microarray; Wnt, wingless and int.

Statement of novelty and impact: This is one of the first descriptions of human tenascin-W and the first report on tenascin-W in colorectal cancer. In the present manuscript we show that serum levels of tenascin-W are significantly elevated in colorectal cancer patients and that tenascin-W is highly expressed in a large fraction of colorectal cancer tissue. These data are novel and will serve as the basis for many future studies on tenascin-W and its role in human cancer.

Corresponding author: Martin Degen
Friedrich Miescher Institute for Biomedical Research
Novartis Research Foundation
Maulbeerstrasse 66
CH-4058 Basel, Switzerland
e-mail: martin.degen@fmi.ch
Tel.: +41 61 697 85 90
Fax: +41 61 697 39 76

Abstract

Tenascins are extracellular matrix proteins present during the development of organisms as well as in pathological conditions. Tenascin-W, the fourth and last member of the tenascin family remains the least well-characterized one. Our study aimed to evaluate the potential significance of tenascin–W as cancer biomarker by monitoring its presence in the serum of colorectal and breast cancer patients and its expression in colorectal tumor tissues. To measure serum tenascin-W levels, a sensitive sandwich-ELISA was established. Mean tenascin-W concentration in sera of patients with non-metastatic colorectal cancer at time of diagnosis was highly increased compared to that of healthy volunteers. A similar tendency was observed for tenascin-C in the same patient cohort. However, the increase was much more striking for tenascin-W. We also detected elevated tenascin-W levels in sera of breast cancer patients. Furthermore, we could show a prominent expression of tenascin-W in extracts from colorectal tumor tissues by immunoblot analysis, whereas tenascin-W was not detectable in the corresponding normal colon mucosa. To confirm the western blot results, we performed immunohistochemistry of frozen sections of the same patients as well as of an additional, independently chosen collection of colorectal cancer tissues. In all cases, similarly to tenascin-C, tenascin-W was detected in the tumor stroma. Our results reveal a clear association between elevated levels of tenascin-W and the presence of cancer. These results warrant further studies to evaluate the potential value of serum and tissue tenascin-W levels as diagnostic, prognostic or monitoring biomarker in colorectal, breast and possibly other solid cancers.

Introduction

Tumor development and progression is not only dependent on the acquisition and accumulation of mutations leading to genetic alterations in cancer cells (for reviews see [1, 2]), but in addition depends on the cross-talk between the transformed epithelium and the tumor stroma, consisting of various cell types (e.g. activated fibroblasts, angiogenic endothelial cells, infiltrating inflammatory cells) and modified extracellular matrix (ECM) [3, 4]. In recent years it has been recognized that cancer cells are able to alter and activate their adjacent stroma by secreting soluble factors promoting the formation of a growth-permissive microenvironment required for full neoplastic manifestation [5-7].

The adhesion-modulating ECM molecule tenascin-C is expressed *de novo* by activated fibroblasts in most solid tumors [7-9]. Tumor promoting activities of tenascin-C include abolishment of cancer cell spreading on fibronectin through blocking of syndecan-4 [10, 11], promotion of cancer cell proliferation [10], induction of angiogenesis [12], and enhanced cell invasiveness by up-regulating MMP-12 [13]. Moreover, tenascin-C can trigger oncogenic signaling pathways such as EGFR [14, 15], ERK/MAPK and Wnt [16]. Importantly, tenascin-C is part of the gene expression signature that identifies metastatic breast cancer cells preferentially metastasizing to the lung [17]. Since tenascin-C is absent or expressed at greatly reduced levels in the adult organism, but gets re-expressed in tumors [18], it was reasonable to assume that increased tenascin-C expression in most cancers may be reflected in elevated tenascin-C levels in body fluids. Indeed, elevated levels of tenascin-C have been reported in serum of patients with different cancer types, including glioma, prostate or colorectal carcinomas, metatstatic melanoma, squamous cell carcinoma of head and neck, and non-small cell lung cancer [19-25]. However, the values for tenascin-C in serum of cancer patients were scattered over a wide range and a significant fraction of these patients had normal concentrations, resulting in a low sensitivity of tenascin-C measurement for detecting cancers. Moreover, high serum tenascin-C levels were clearly correlated with non-cancerous liver diseases including hepatitis and liver cirrhosis [26-29], and with elevated levels of C-reactive protein (CRP) [21], an acute phase protein expressed as a consequence of infection, tissue damage or other inflammatory conditions [30, 31].

Tenascin-W was originally identified in zebrafish where it was shown to be co-expressed with tenascin-C by neural crest cells and in somites [32]. More recently, murine [33] and chicken [34] tenascin-W have been described and in both of these animals tenascin-W was expressed in

developing and adult smooth muscle cells and bone. Chicken tenascin-W function includes modulation of calvarial cell adhesion and spreading *in vitro* [34]. So far only little is known about tenascin-W expression in cancer. The first study linking tenascin-W expression to the presence of a tumor was performed in mice. Using oncogene-induced mammary tumor models, it was shown that tenascin-W is highly expressed in the tumor stroma sharing its prominent expression with tenascin-C [35]. Functional studies identified mouse tenascin-W as a molecule promoting migration of mammary cancer cells [35]. Recently, we confirmed the presence of tenascin-W in the stroma of human breast cancer tissues [36].

Since little is known yet about human tenascin-W expression in health and disease, we decided to study its presence in serum of patients with colorectal or breast cancer and investigated its presence in tumor tissues.

Materials and Methods

Recombinant proteins

Human tenascin-W and tenascin-C were cloned, expressed and purified as described previously [36, 37]. Fibronectin was purified from filter-sterilized horse serum using a gelatin-agarose column. After washing of the column with PBS, fibronectin was eluted with 4 M Urea and finally dialyzed against PBS. Human fibrinogen was purchased from Sigma (Sigma, Buchs, Switzerland).

Anti-tenascin-W antibody production

Two polyclonal antisera recognizing human tenascin-W were generated. The first antiserum, called pAb (FL), was raised in rabbits against the purified full-length human tenascin-W [36] (Fig. 1a, indicated by the long line). To raise the second polyclonal antisera in rabbits, a recombinant fragment of human tenascin-W was cloned, bacterially expressed, and purified. To clone the recombinant fragment, specific primers were designed to amplify the sequence encoding the last two fibronectin type III domains with the Expand High Fidelity PCR System (Roche, Rotkreuz, Switzerland). The cDNA of the full-length human tenascin-W (described above) was used as template and the PCR was performed with the primer set 5'-GAGGATCCGAAATTGACGGCCCCAAAAACC-3'/5'-ATAAGCTTATGTGGAGAGGGTGGTGGA-3'. The forward primer included a *BamHI* restriction site and the reverse primer a stop codon immediately followed by a *HindIII* restriction site to enable the directional cloning into the bacterial expression vector pQE30 (Qiagen, Hilden, Germany), supplying a C-terminal His tag for the purification of the recombinant fragment. The recombinant fragment corresponding to fibronectin type III domains 3F/4 (Fig. 1a, short line below the molecule) was expressed and purified by affinity chromatography to a Ni-NTA matrix (Qiagen, Hilden, Germany) following the supplier's instructions. Purification was performed under native conditions and elution by 250 mM imidazole (pH 6.9). The bacterially expressed fragment of tenascin-W was then used to raise polyclonal antisera (pAb (3F/4)) in rabbits using standard immunization procedures.

To raise monoclonal antibodies (mAbs) in mice, a recombinant fragment of human tenascin-W containing the last three fibronectin type III domains (Fig. 1a, short line above the molecule) was cloned, bacterially expressed, and purified as described above. To clone the

recombinant fragment, cDNA was synthesized from total RNA that was extracted from Saos-2 cells (ATCC # HTB-85) by Trizol™ reagent (Invitrogen, Carlsbad, CA, USA). Primers amplifying the last three FN type III domains, were used for nested PCR with the Expand High Fidelity System (Roche, Rotkreuz, Switzerland) using the Saos-2 cDNA as template. The first reaction was performed with the primer set 5'-GGGAAGGAGCAGAGTAGCACTG-3' / 5'-CCGCCTCTGGAAGACAATCC-3', the second reaction with the primers 5'-AGGGATCCGACATTGACAGCCCCCAAAACC-3' / 5'-CTAAGCTTTCATGTGGAGAGGGTGGTGGATAC-3'. The forward primer for the second PCR included a *BamHI* restriction site and the reverse primer a stop codon immediately followed by a *HindIII* restriction site to enable the directional cloning into the bacterial expression vector pQE30 (Qiagen, Hilden, Germany), supplying a C-terminal His tag for the purification of the recombinant fragment. MAbs were obtained by immunizing female BALB/c mice with 34.6 µg of the purified recombinant tenascin-W fragment emulsified with STIMUNE (ID-Lelystad, Institute for Animal Sciences and Health, Lelystad, NL). For boosting, mice were injected twice with a 4-week interval with 25 µg tenascin-W fragment. Splenic lymphocytes were fused with the myeloma cell line P3X63Ag8.653 (ATTC # CRL-1580) and cultured according to standard protocols. The hybridoma supernatants were analyzed by ELISA and western blot analysis of a tenascin-W fragment expressed in HEK293 cells using a construct containing the sequence of the last three FN III domains of tenascin-W fused to an N-terminal fragment of chicken tenascin-C containing its secretion signal and the epitope for the well characterized anti-chicken tenascin-C antibody mAb 60 [38]. IgGs from two mAb hybridoma clones (mAb 29A and mAb 56O) were purified from conditioned medium by protein G sepharose (Amersham, Otelfingen, CH) and used in this study.

Sandwich-ELISA

Immunolon 1 flat-bottomed 96-well plates (Dynatech Laboratories, Chantilly, VA, USA) were coated with 50 µl of mAb 29A (capture antibody, 10 µg/ml in PBS) for 1 hour at 37°C and blocked with 4 % milk in PBS (blocking and washing solution) for another hour at room temperature. After washing the plate twice, 50 µl of serum samples or standard (purified human tenascin-W), both diluted in blocking solution, were incubated for 1 hour at 37°C. Each serum was assayed in a 2-fold dilution series ranging from 1:2 to 1:8. Samples were discarded and the plates were washed extensively four times with washing buffer. After

incubation with 50 µl of pAb (FL) (detection antibody, 1:500 in blocking solution, 1 hour at 37°C), the plates were washed again four times and incubated with peroxidase-labeled goat anti-rabbit IgG (1:2000 in blocking solution, 1 hour at 37°C). After washing the plates four times with washing buffer and twice with PBS, the enzymatic color reaction was carried out by adding 100 µl of reaction buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, 4 mM 1, 2-phenylendiamine, 0.003 % H_2O_2). The reaction was stopped with 50 µl of 4 M H_2SO_4 and the plates were read with a VERSAMAX Microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 490 nm against 620 nm.

To monitor serum tenascin-C, sandwich-ELISAs were performed as described previously [21] using the anti-human tenascin-C mAb B28.13 as capture antibody, a polyclonal chicken anti-human tenascin-C as detection antibody (1:500) and a peroxidase-labeled goat anti-chicken IgG (1:15,000), and with purified human tenascin-C as standard.

Patient population and serum preparation

Patients were between the age of 48 and 94 years (average age 70.3 years), presenting non-metastatic colorectal cancer (CRC) and scheduled for tumor resection. Exclusion criteria included patients with less than 10 g/dl hemoglobin, leucopenia, thrombocytopenia, deep venous thrombosis during the last three months, long term vascular catheter, chronic vascular or inflammatory disease, surgery and chemotherapy during the last 4 and 6 months respectively.

Healthy volunteers were gender mixed and between 30 and 76 years old, without history of cancer, chronic disease or medication other than hormonal contraception. Although the average age of 43 was lower than that of the cancer patients, there was no indication of an increase of tenascin-W levels with age. Ethical and scientific approvals were given by the local Ethics Committee of the Lausanne University Hospital. Written informed consent was obtained from all the patients and healthy volunteers.

For serum preparation, blood was collected in tubes without anticoagulant. After a 10 minute centrifugation at 1,000g, the serum was extracted and frozen at -80° C until use.

Histological diagnosis and grade of all tumors was reviewed by one pathologist (SW) according to the WHO Classification of Tumors [39]. Tumor stage was determined using the International Union Against Cancer (TNM classification of malignant tumors) [40].

Human tissue extracts and western blot analysis

Fresh human tissue was frozen on dry ice immediately after surgery. For the processing of the tissue, it was thawed on ice, minced and homogenized in lysis buffer (100 mM phosphate buffer pH 8.0, 300 mM NaCl, 8 M Urea, 1 % Triton-X-100, 10 mM beta-mercaptoethanol, 50 mM Guanidium Hydrochloride and complete protease inhibitor cocktail (Roche, Rotkreuz, Switzerland)). Insoluble material was pelleted and reducing SDS-PAGE sample buffer was added to the supernatant and boiled for 5 minutes at 95°C. After electrophoresis on 6 % polyacrylamide gels, proteins were electro-transferred onto polyvinyldifluoride membrane (Millipore, Bedford, MA, USA) using a semi-dry blotting apparatus (Millipore, Bedford, MA, USA). After the transfer, membranes were stained with amido black to control equal protein loading. After blocking for 1 hour at room temperature in TBS containing 0.05 % Tween and 5 % skim milk powder, membranes were incubated overnight with either the polyclonal tenascin-W antiserum (pAb (3F/4); 1:750), the mAb 56O raised against tenascin-W (1:1000), the mAb B28-13 raised against tenascin-C (1:100) [21], or the mAb against vinculin (1:2000; Sigma, Buchs, Switzerland) followed by an incubation for 1 hour with anti-rabbit IgG or anti-mouse IgG coupled to horseradish peroxidase (1:10,000), respectively. Blots were developed using Super Signal (Pierce, Rockford, IL, USA) and exposed to Kodak BioMax MR Films.

Immunohistochemistry and Frozen Tissue Microarray

A frozen tissue microarray (TMA) was constructed from frozen tissue samples of 32 colon carcinoma and 6 normal colon mucosae. Pathological features of these samples are summarized in Table III (patients 1-38). The TMA was constructed in frozen Tissue-Tek OCT compound (Miles Laboratories, Naperville, IL, USA) as described previously [41]. We optimized a commercial microarray device (Beecher Instruments, Sun Prairie, WI, USA) by using a 0.6 mm drill for recipient whole making instead of the conventional hollow needle.

All immunostainings were performed using the Discovery XT automated stainer (Ventana Medical Systems, Tucson, AZ, USA), with DAB Map detection kit (Ventana). Frozen tissue slides were dried for 1 hour at room temperature, fixed for 10 minutes at 4°C in acetone and

then introduced into the automate. No pretreatment was required for any staining. Slides were first blocked twice for 12 minutes with the AB Block reagent (Ventana). Then they were incubated for 1 hour at 37°C with mAb B28-13 against tenascin-C (1:2500), and mAb 56O against tenascin-W (1:800). Afterwards, slides were treated for 32 minutes at 37°C with a biotinylated universal secondary antibody (Ventana) and counterstained with hematoxylin and bluing reagent (Ventana).

Results

Precision and sensitivity of the sandwich-ELISA

Serum tenascin-W levels were analyzed by a sandwich-ELISA using mAb 29A as capture antibody and pAb (FL) as detection antibody (Fig. 1a). Specificity of the detection antibody was assessed by western blot analysis of tenascin-W conditioned medium, purified fibronectin, fibrinogen, tenascin-C, and tenascin-W (Fig. 1b). The anti-tenascin-W antiserum pAb (FL) specifically reacts with the human tenascin-W conditioned medium and with the purified tenascin-W, but did not show any cross-reactivity with tenascin-C or any of the other related proteins tested, which are known to be present in serum (Fig. 1b).

To further rule out that our detection antibody recognizes serum contaminants potentially binding to tenascin-W we used two distinct polyclonal anti-human tenascin-W antibodies pAb (FL) and pAb (3F/4) for tenascin-W detection. Table Ia shows that both polyclonal detection antibodies measure similar tenascin-W concentrations in the same serum samples making it highly unlikely that they cross-react with contaminating serum proteins possibly bound to tenascin-W. Furthermore, pAb (FL) recognizes different epitopes of tenascin-W than pAb (3F/4) since, in contrast to pAb (3F/4), its reactivity with tenascin-W can not be blocked by the addition of the bacterial expressed fragment used to raise pAb (3F/4) (Fig. 1c).

Testing a serial dilution of our purified tenascin-W, we concluded that with our newly established sandwich-ELISA, serum tenascin-W concentrations as low as 0.005 mg/l could be detected. A typical standard curve for tenascin-W is shown as log-log presentation in Figure 1d. When we tested a different capture antibody (mAb 56O) we obtained the same results as with mAb 29A (data not shown) and decided to use mAb 29A for all further sandwich-ELISAs. The precision of the assay was estimated by testing 4 serum samples three times in one assay (“within-run”, Table Ib) or in duplicates in two consecutive assays (“between-run”, Table Ic). The coefficient of variation (CV) was 5.6 +/- 1.3 % in the within-run and 6.52 +/- 4.9 % in the between-run. Therefore, we have established a sensitive, specific and precise sandwich-ELISA to detect tenascin-W in human serum samples.

Tenascin-W is up-regulated in the serum of colorectal and breast cancer patients

Since tenascin-C was shown to be elevated in serum samples of colorectal cancer patients [20, 25], we were interested to test whether tenascin-W shares this property with tenascin-C. Therefore, we measured tenascin-W levels in serum of 17 non-metastatic (at time of diagnosis) colorectal cancer patients and compared it to the levels monitored in the serum of 25 healthy volunteers. The mean value of tenascin-W in the control sera was 0.389 +/- 0.14 mg/l (Fig. 2a). This is about half of the published mean serum tenascin-C level in healthy individuals [21, 42]. Serum tenascin-W concentrations in colorectal cancer patients were significantly elevated compared to those in healthy individuals ($p < 0.01$) with a mean level of 0.794 +/- 0.38 mg/l (Fig. 2a). This corresponds to a 2-fold increase in comparison to healthy volunteers. Immunoprecipitation (IP) followed by immunoblot analysis of serum samples resulted in the detection of a single tenascin-W-specific band, corresponding to the size of the purified full-length tenascin-W (data not shown). This indicated that we were measuring intact mature tenascin-W protein in the serum. We also assayed the same sera for tenascin-C and obtained a mean tenascin-C level in healthy volunteers of 0.775 +/- 0.25 mg/l (Fig. 2b), which nicely matches the range described in the literature [21, 42]. The mean tenascin-C value in colorectal cancer sera was 0.98 +/- 0.24 mg/l and corresponds to a 1.2 fold increase compared to control sera (Fig. 2b). Clinicopathologic features of the colorectal cancer patients available for our study as well as their individually measured tenascin-W and tenascin-C levels are given in Table II. Although both tenascins showed the same tendency of increased levels in colorectal cancer patients, the difference in the mean value in cancer sera compared to volunteers was much more pronounced for tenascin-W. However, not every patient with a high tenascin-C serum level also contained high levels of tenascin-W (Table II), indicating that there is no strict correlation between serum levels of the two tenascins in colorectal cancer patients.Although, there was no overt correlation between elevated serum tenascin-W levels and staging of the colorectal tumors, it is worth noting that 4 out of 5 patients who developed tumor recurrence after surgery had high levels of tenascin-W. Mean serum tenascin-W level of recurrent patients (n=5) was 1.02 +/- 0.34 mg/l which corresponds to a 1.5-fold increase compared to non-recurrent colorectal cancer patients (n=12, mean=0.700 +/- 0.37 mg/l) and a 2.6-fold increase compared to healthy volunteers (Table II).

To test whether tenascin-W might be a specific serum marker for colorectal cancers or whether tenascin-W might have a broader spectrum of application, we analyzed tenascin-W in serum of 16 non-metastatic (at time of diagnosis) breast cancer patients and compared it to the levels monitored in healthy volunteers. The mean serum value of tenascin-W in the breast cancer cohort was 0.682 +/- 0.43 mg/l. This corresponds to a statistically significant ($p<0.02$) 1.75-fold increase compared to healthy volunteers (Fig. 2c). From these data we conclude that serum levels of tenascin-W are elevated in non-metastatic breast and colon cancer patients at time of first curative-intent surgery compared to healthy individulas.

Tenascin-W expression in normal colon and cancer tissue

To determine whether increased expression of tenascin-W in the tumor tissue could explain the elevated serum levels in colorectal cancer patients, we performed immunoblots on 11 colorectal tumor tissue extracts. As shown in Figure 3a, a large fraction of the tumor tissue samples contained detectable levels of tenascin-C (9/11; 82%). Furthermore, tenascin-W was also highly expressed in these tumor extracts (9/11; 82%). In two cases (patients E and F) tenascin-W and tenascin-C were absent or barely detectable while all other samples tested showed co-expression of the two tenascins (Fig. 3a). Besides tumor tissue, corresponding normal mucosa was available for patients K and L, and for patient J we obtained normal tissue only. In all normal colon mucosae tested, tenascin-W was not detectable even though the tumor tissue extracts of the same patients revealed strong tenascin-W expression (patients K and L). This is in contrast to tenascin-C which was also detectable in normal colon mucosae, but at a much reduced level compared to the corresponding tumor sample. For tenascin-C, we detected different isoforms, whereas for tenascin-W we mostly observed a single band in the extracts tested. However, there was a faint second lower tenascin-W band detectable in tumor extracts of patients B and I. This smaller band is likely to represent degradation products of tenascin-W, but we cannot exclude that it might be due to a low level of alternative splicing. Tenascin-C isoform expression differed between tumor and normal tissues. In normal colon mucosae, only the low molecular-weight isoform could be observed, whereas in tumor extracts low and high molecular-weight isoforms were detectable. In summary, the majority of human colorectal tumors express both tenascins, but their relative amount varies greatly between patients.

In order to confirm expression of tenascin-W and tenascin-C in tumor tissues and to localize their presence within the tissues, we stained frozen sections of normal colon mucosa and tumor tissue of patients K and L by immunohistochemistry. The staining pattern was the same for both patients. As shown for patient L (Fig. 3b) tenascin-C as well as tenascin-W staining revealed a very strong expression in the tumor stroma, the specialized connective tissue surrounding cancer cells. The normal colon mucosa was positive for tenascin-C but not tenascin-W consistent with the immunoblot findings. The tenascin-C expression was strong in the muscularis mucosa and was also present surrounding the glands. Taken together, immunohistochemical staining of frozen sections confirmed the result obtained by immunoblotting of the same colorectal tumor extracts and indicated that tenascin-W is a novel marker for activated tumor stroma in colorectal cancer.

To extend this analysis to a larger number of samples we stained a frozen colon tissue micro array (TMA) that contained 32 cancer spots and 6 spots of normal colon. Without exception all cancer tissue sections were stained for tenascin-W as well as tenascin-C, while all normal tissues were negative for tenascin-W, but positive for tenascin-C (Table III). Examples of the different staining patterns observed are shown in Fig. 4. Most of the tenascin-C positivity in normal colon tissue is seen in smooth muscle (Fig. 4, patient 36). In some of the tumors tenascin-W and tenascin-C stainings seemed to overlap entirely (Fig. 4, patients 4, 5), whereas in others the tenascin-W positive areas were a subset of the tenascin-C positive region (Fig. 4, patient 12). As can be observed in Fig. 4 (patient 15) the tenascin-C positive and tenascin-W negative area in tumors may also be due to the presence of smooth muscle, as can be seen at the boarder of a tumor and normal tissue (Fig. 4, patient 26). The extent of staining always overlapped with the stromal compartment of the tumor as judged from the H&E stained sections (Fig.4). In Table III patient data are summarized and the staining patterns are classified according to the area of the staining observed. This shows that tenascin-C has a slightly broader distribution than tenascin-W. Since tenascin-C is strongly expressed in smooth muscle of the colon, this may reflect the presence of smooth muscle cells within the cancer stroma.

Discussion

In 2006, colorectal cancer was the second major cause of cancer deaths in Europe, accountable for 207,400 deaths (12.2 % of total cancer deaths) [43]. Colorectal carcinomas develop in defined stages over several years [44]. Deaths from colorectal cancer may be prevented inmost cases if the tumor is detected at an early stage (polyp or non-invasive carcinoma) and removed by endoscopic polypectomy or surgery. In this context, effective screening tools are of utmost importance. Because of the low accuracy and sensitivity of the classical fecal occult blood test, several biochemical markers have been developed and explored over the years for colorectal cancer screening, including carcinoembryonic antigen (CEA) serum levels [45], galactose-N acetylgalactosamine fecal levels [46], and K-Ras mutations in human stool [47, 48] , but none is enough sensitive and specific for early cancer detection. Recently, the tumor M2-Pyruvate kinase, a dimeric form of the enzyme that catalyzes the last reaction within the glycolytic pathway was found to be up-regulated in both plasma and fecal samples of patients suffering from gastrointestinal cancers, including colorectal cancers [49-53]. Nevertheless, the most effective available method to prevent and detect colorectal cancer today is colonoscopy. However, this investigation is invasive, time-consuming, uncomfortable, and relatively expensive [54]. Moreover, the frequency of the colonoscopic exams every 3 to 5 years is limited by these factors and it is not rare to observe the development of cancer between two colonoscopies. Thus surrogate markers of colon cancer detectable in the blood may be a convenient method for screening and early cancer detection..
In search for new diagnostic or prognostic tumor markers, tenascin-C levels have often been analyzed in sera of cancer patients and its potential value as a biomarker has been evaluated [21-23, 55, 56]. Although elevated tenascin-C serum levels have been found in certain cancers, it still remains a questionable tumor marker [21]. Its levels are scattered over a wide range with many cancer patients having normal tenascin-C concentrations and its expression strongly correlates with sites of inflammation or infection [18].

In this report we evaluate tenascin-W for its potential to serve as biomarker in colorectal cancer. A sensitive, precise and tenascin-W-specific sandwich-ELISA was established using different anti-tenascin-W antibodies and the purified protein as a standard. We were able to detect and measure tenascin-W in the serum of healthy volunteers and colorectal cancer patients. The mean serum tenascin-W level in healthy individuals was 0.389 +/- 0.145 mg/l.

Homogeneously low levels in a control population is a strict requirement for a cancer biomarker. In comparison, serum levels of tenascin-C in controls were 2-fold higher and scattered over a broad range. Interestingly, we observed a clear increase in mean tenascin-W levels in colorectal cancer sera (0.794 +/- 0.38 mg/l). The histogram in Figure 2a (right), however, shows that within the cohort of the colorectal cancer patients, there are two subpopulations: one with elevated tenascin-W levels (approximately 2.5 times the mean of control values), the other with levels comparable to the controls. We could not identify any simple correlation between clinicopathologic features of the tumors, patients' characteristics and any of these patient groups. The meaning and relevance of these two subpopulations is not clear at this point. One plausible explanation is that not all tumors lead to an elevation of tenascin-W in serum due to differences in their cell biology or accompanying events (e.g. stromal reaction). Alternatively these groups may represent different kinetics of tenascin-W elevation in serum, whereby 'normal' levels would become elevated later during disease progression. Another important issue is whether the level of tenascin-W is associated with more unfavorable disease progression. In this regard, it is noteworthy that the follow-up studies of the colorectal cancer patients revealed that 4 out of 5 patients who developed tumor recurrence after treatment had high tenascin-W levels (i.e. above mean levels) in their sera (Table II). This suggests a potential value of tenascin-W levels in sera in predicting recurrence or progression, but the limited size of the patient cohort analyzed in this initial study is too small to draw any conclusions at this point. A study addressing this question is planned.
It is also important to consider that the presence of tenascin-W, or of any other biomarker in serum, could be influenced by any co-morbidity (e.g. cardiovascular diseases, inflammatory disease) or through indirect effects of the tumor on other organs (e.g. bone marrow, liver). For instance tenascin-C serum levels were shown to correlate with inflammation rather than tumorigenesis [21], but so far no correlation could be found linking tenascin-W to the presence of infection or inflammation (own unpublished observations).

We confirmed the tendency of tenascin-C to be present at higher levels in the serum of colorectal cancer patients (0.98 +/- 0.24 mg/l), however, the increase was much more moderate than for tenascin-W. We neither could observe a correlation between the levels of the two tenascins, nor with the classification and staging of the primary tumor. We also monitored a clear increase (1.75-fold) in the mean serum tenascin-W level in non-metastatic

breast cancer patients compared to that in healthy individuals. This indicates that tenascin-W is not a biomarker specific for adenocarcinomas of the colon, but might have a broader spectrum for possible applications. More studies are necessary to fully appreciate the expression of tenascin-W in different cancers.

To test for expression of tenascin-W in the tumor, we investigated tumor extracts of 11 colorectal cancer patients and for some of them, corresponding normal tissue by immunoblotting. We could show that tenascin-W as well as tenascin-C was detectable in the tumor tissue in 82 % of the patients. Since the immunoblotting analysis of tumor tissue was performed on different colorectal cancer patients we cannot make direct correlations between local tenascin-W expression in the tumor and corresponding circulating levels in the serum. Tenascin-W, however, was absent from normal colon mucosa, in contrast to tenascin-C, which is expressed (at reduced levels) in the normal tissue as well. Interestingly, in the normal tissue, only the low molecular-weight isoform of tenascin-C is present, whereas in the tumor tissue, low and/or high molecular-weight isoforms are over-expressed. This result implies differential tenascin-C isoform expression in normal and neoplastic human colon tissue. This is in agreement with previous reports showing expression of the small isoform in normal stable tissues [57], while larger variants are detectable in diseased tissues including neoplasia, wound healing or inflammation [58-61]. Immunohistochemical analysis of frozen sections of the same patients revealed prominent stromal staining for both tenascins. The stromal staining of tenascin-C and tenascin-W was confirmed on a frozen TMA containing 32 different colon cancer sections. This study revealed that all of the tumors were stained for both tenascins and that the staining correlated with the amount of tumor stroma present. Similar observations have been made in human breast tumors. Tenascin-W and tenascin-C were shown to be highly expressed in a large fraction of human breast tumors. In contrast to normal colon mucosae, which express tenascin-C, normal mammary parenchyma was negative for both tenascins [36].
To summarize, in our first exploratory study we measured a higher concentration of serum tenascin-W in samples from colorectal cancer patients and breast cancer patients compared to controls. However, not all cancer patients did show elevated tenascin-W levels. The fact that tenascin-W is highly expressed in the tumor tissue of most extracts, is detected in all cancer sections by immunohistochemistry but is completely absent in normal tissue, suggests that tenascin-W is a better candidate tumor marker for colorectal cancer than tenascin-C, which is

also expressed in normal colon mucosa. The positive correlation between elevated serum tenascin-W and the presence of colorectal or breast cancer raises the possibility that elevated tenascin-W levels in serum or tissue might be of clinical importance. These observations warrant a follow-up study to evaluate the potential diagnostic or prognostic relevance of tenascin-W in colorectal cancer and possibly to other cancer types.

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Figure Legends

Figure 1 – Overview of the antibodies raised against tenascin-W

(a) Schematic representation of the domain structure of human TNW. The recombinant full-length TNW and the fragments that served as antigens to raise polyclonal and monoclonal antibodies are indicated by lines. The following symbols have been used to identify the structural domains: heptad repeats (wavy line), EGF-like repeats (diamonds), FNIII domains (boxes), FNIII domains generated by duplication (dark boxes), fibrinogen globe (circle). **(b)** Coomassie-stained gel (lanes 1-5) and western blot (lanes 6-10). (1, 6) TNW conditioned medium 10 µl, (2, 7) fibronectin 2.5 µg**,** (3, 8) fibrinogen 2.5 µg, (4, 9) purified TNC 2.5 µg, (5) purified TNW 2.5 µg and (10) purified hTNW 500 ng. Immunoblot analysis shows that the detection antibody pAb (FL) specifically recognizes TNW and that it does not cross-react with the other tested proteins. **(c)** The reactivity of pAb (FL) with purified tenascin-W (W) can be blocked by incubation with 10 µg/ml of purified tenascin-W (FLhTNW), but not with 10 µg/ml of the bacterially expressed FNIII 3F/4 fragment, while the reactivity of pAb (3F/4) can be blocked by both tenascin-W preparations **(d)** Log-log presentation of a typical standard curve using mAb 29A as capture antibody, pAb (FL) as detection antibody, and a serial dilution of the purified TNW is shown. Detection limit was reached at TNW concentrations of about 0.005 mg/l.

Figure 2 – Tenascin-W serum levels

(a) TNW serum levels of healthy volunteers (n= 25, mean: 0.389 +/- 0.145 mg/l) and of non-metastatic colorectal cancer patients (n=17, mean: 0.794 +/- 0.38 mg/l) are shown. There is a statistically significant increase in mean tenascin-W level (bar) in colorectal cancer patients compared to that in volunteers. **(b)** Serum TNC levels of healthy individuals and of colorectal cancer patients is displayed. They show an increase of the mean serum TNC level (n=17, mean: 0.98 +/- 0.24 mg/l) compared to that in healthy individuals (n=13, mean: 0.798 +/- 0.24 mg/l). **(c)** Serum TNW levels in non-metastatic breast cancer patients (n=16, mean: 0.682 +/- 0.44 mg/l) and of healthy volunteers (n=25, mean: 0.389 +/- 0.145 mg/l) are shown. The bars indicate the mean tenascin values.

Figure 3 – Tenascin-W expression in colorectal cancer

(a) Eleven colorectal cancer extracts (T) and three normal colon tissues (N) were tested on immunoblots for the presence of TNW and TNC. Analysis revealed a basal expression of low molecular-weight isoform of TNC in the normal mucosa and up-regulation of mostly large TNC variants in the tumor tissue. The position of the recombinant high molecular weight TNC isoform loaded for comparison is indicated (arrowhead). TNW is detectable in a large fraction of tumoral tissues but not in normal colon mucosae. **(b)** Immunohistochemical analysis of frozen sections of patient L confirmed immunoblot results and showed strong staining in the tumor stroma for both tenascins. Whereas no staining was detectable in the normal tissue with respect to TNW, strong staining was observed for TNC in the muscularis mucosa. Calibration bar: 250 μm. N: normal; T: tumor.

Figure 4 – Frozen colon tissue microarray

Immunohistochemistry with a mAb against TNC, pAb (3F/4) against TNW and Hematoxylin and Eosin stained sections (H&E) of a frozen colon TMA of the patients indicated are shown (cf. Table III). Normal colon mucosa (patient 37) is negative for TNW, but positive for TNC. TNC is also strongly expressed in colon smooth muscle (patient 36). Patient 26 shows an example of a border of a tumor with the adjacent normal tissue. In the tumor patients 1-32 (Table III) analyzed it was found that TNW is exclusively localized in the tumor stroma. In some patients, TNW-positive areas seemed to be a subset of the TNC-positive region (patient 12). Patients 15 and 5 show large (+++) areas of tenascin positivity. Patients 4 and 13 display intermediate (++) to low (+) areas, respectively, of tenascin-W staining. In general, the staining correlates with the amount of tumor stroma present.

Table I

TABLE Ia - COMPARISON OF TWO DIFFERENT DETECTION ANTIBODIES

Sample	Tenascin-W (mg/l)	
	pAb(FL)	pAb(3F/4)
serum 1	0.339 +/- 0.04	0.396 +/- 0.08
serum 2	0.880 +/- 0.05	0.824 +/- 0.08
serum 3	0.184 +/- 0.05	0.176 +/- 0.01
serum 4	0.496 +/- 0.06	0.522 +/- 0.07

Serum TNW levels analyzed by sandwich-ELISA using two different detection antibodies pAb(FL) and pAb(3F/4). Serum TNW levels in all samples tested did not differ significantly between the two detection antibodies as revealed by student's t-test ($p>0.05$).

TABLE Ib - PRECISION OF THE ASSAY: WITHIN-RUN

Sample	Tenascin-W (mg/l)	
	mean +/- SD	CV (%)
serum 1	0.268 +/- 0.012	4.0
serum 2	0.288 +/- 0.023	6.9
serum 3	0.310 +/- 0.014	4.5
serum 4	0.452 +/- 0.028	6.2

Precision was estimated by assaying 4 serum samples 3 times in one assay (within run).
SD = standard deviation; CV (%) = coefficient of variation.

TABLE Ic - PRECISION OF THE ASSAY: BETWEEN-RUN

Sample	Tenascin-W (mg/l)	
	mean +/- SD	CV (%)
serum 1	0.268 +/- 0.001	0.4
serum 2	0.180 +/- 0.027	15.2
serum 3	0.194 +/- 0.014	7.4
serum 4	0.460 +/- 0.024	5.2

Precision was estimated by assaying 4 serum samples in duplicate in 2 consecutive assays (between run). SD = standard deviation; CV (%) = coefficient of variation.

Table II

Table II - CLINICOPATHOLOGICAL FEATURES AND TENASCIN LEVELS OF THE COLORECTAL CANCER PATIENTS

Patient	Age	Sex	Localization	Blood collection (dd/mm/yy)	Grade	TNM	TNW (mg/l)	TNC (mg/l)	Recurrence (May 2007)
1[a]	49	F	Rectum	n.a.	n.a.	T3N1M0	0.380	0.820	no
2	89	M	Left	n.a.	2	T4N1M0	0.330	0.810	no
3	48	F	Left	n.a.	2	T3N2M0	0.382	1.080	no
4	60	M	Left	13/02/03	1	T1N0M0	0.560	0.674	yes
5	83	M	Right	27/02/03	3	T3N0M0	1.100	0.920	yes
6	81	F	Rectum	17/04/03	1	T2N0M0	0.800	0.848	yes
7	62	F	Left	12/05/03	2	T3N2M0	1.230	1.230	yes
8	54	M	Left	03/07/03	2	T2N1M0	0.500	0.674	no
9	62	M	Left	10/07/03	2	T4N1M0	0.473	0.571	no
10	60	M	Right	11/08/03	2	T4N2M0	1.400	1.150	yes
11[b]	73	M	Left	15/08/03	2	T4N0M0	1.130	0.733	no
12	68	M	Right	15/08/03	2	T3N0M0	1.380	1.240	no
13	81	M	Left	08/10/03	2	T2N2M0	0.920	1.190	no
14	75	F	Left	n.a.	2	T3N0M0	1.270	0.908	no
15	67	F	Left	09/12/03	2	T3N0M0	0.560	0.915	no
16	89	F	Transverse	09/01/04	2	T2N0M0	0.563	1.620	no
17	94	M	Right	14/01/04	2	T4N1M0	0.520	1.280	no

n.a.: not available; Grade = tumor grade according to WHO Classification of Tumors, 2000; TNM (Tumor, Node,Metastasis) = tumor stage according to UICC Classification, 2002; TNW, TNC: protein levels inserum deduced by sandwich ELISA.

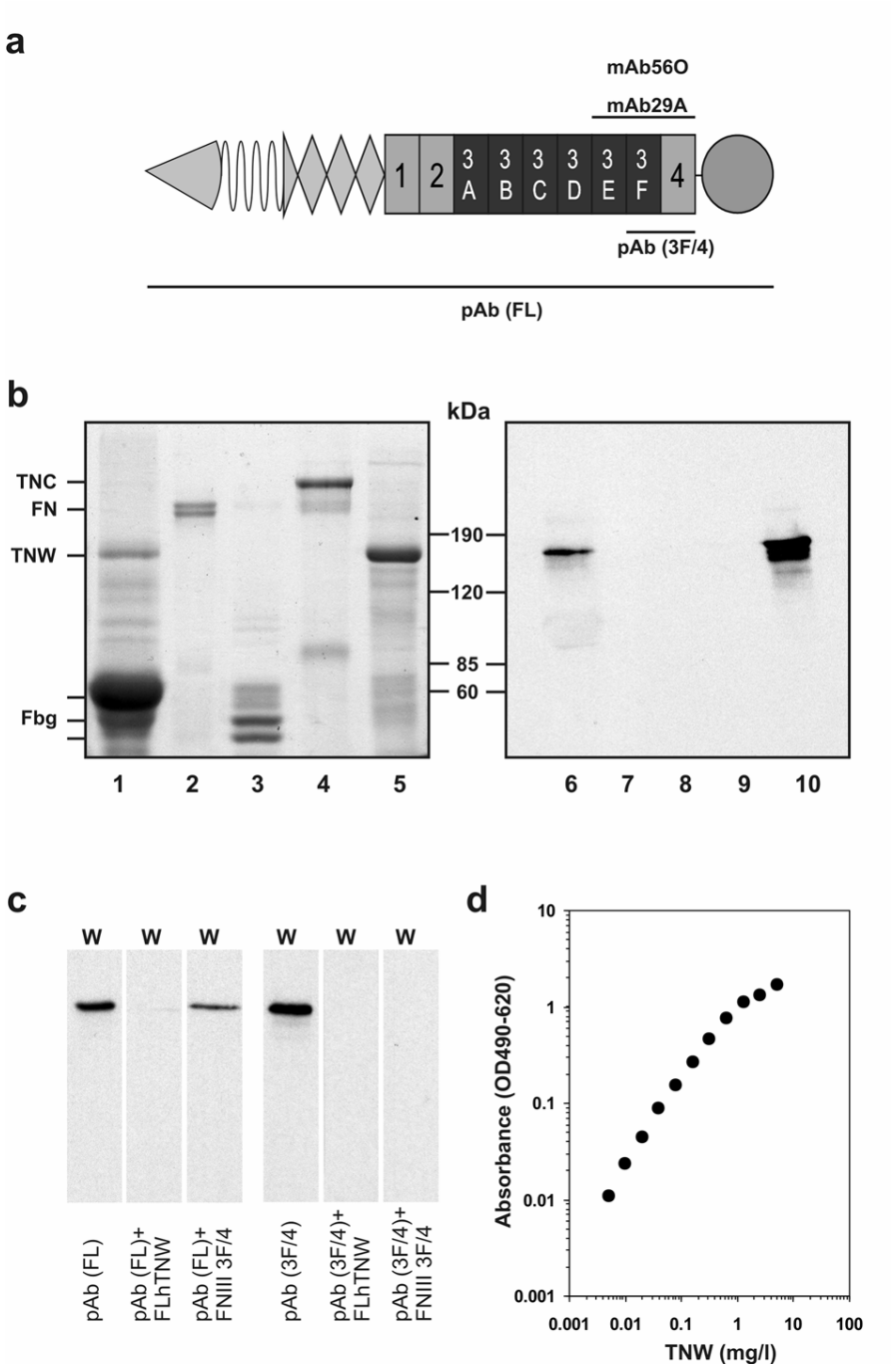
[a] Neoadjuvant radiotherapy (25/11/02 - 12/12/02)

[b] Adjuvant radiochemotherapy with 5-Fluorouracil (5-FU) (23/09/03 - 31/10/03)

Table III - CLINICOPATHOLOGIC FEATURES AND TENASCIN STAINING OF THE FROZEN TMA

Patient	Age	Localization	Grade	TNM	TNW staining	TNC staining
1	59	rectum	2	T3N0Mx	++	+++
2	71	left	3	T3N2Mx	++	+++
3	77	right	2	T2N0M1	++	++
4	66	rectum	2	T3N1Mx	++	++
5	31	right	2	T2N0M0	+++	+++
6	78	rectum	3	T4N1Mx	+	+
7	68	left	2	T2N1Mx	++	++
8	45	left	2	T3N2M1	++	++
9	79	left	2	T2N0Mx	+	+
10	80	left	2	T4N0Mx	+	+
11	83	left	2	T3N0Mx	+++	+++
12	82	right	3	T4N2M1	++	+++
13	78	right	2	T4N2M1	+	++
14	67	right	3	T3N1Mx	+	++
15	82	right	3	T4N2Mx	+++	+++
16	89	left	2	T4N0Mx	++	+
17	64	left	2	T3N0Mx	++	++
18	88	right	2	T3N1Mx	+++	+++
19	81	right	2	T3N2Mx	++	++
20	80	rectum	2	T2N1Mx	+++	+++
21	68	right	2	T2N0M0	++	++
22	66	right	2	T3N0Mx	+	+
23	54	left	3	T3N1Mx	+	+++
24	62	right	3	T3N2Mx	+	+
25	83	right	2	T3N0Mx	++	++
26	81	rectum	3	T2N1Mx	+	+
27	45	left	2	T4N1M1	++	++
28	74	left	2	T4N2M1	++	+++
29	85	rectum	2	T3N1Mx	+	++
30	65	left	2	T3N0Mx	+	++
31	87	rectum	2	T3N0Mx	+++	+++
32	46	left	2	T3N3M1	+++	+++
33	69			normal	-	+
34	93			normal	-	+
35	83			normal	-	+
36	70			normal	-	+++
37	93			normal	-	+
38	82			normal	-	+

Grade = tumor grade according to WHO Classification of Tumors, 2000; TNM (Tumor, Node, Metastasis) = tumor stage according to UICC Classification, 2002; Classification in categories: +++ >60% of tumor area is stained; ++ >20% of tumor area is stained; + <20% of tumor area is stained. See examples of each category in Figure 4.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pAb (FL)
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pAb (FL)+ FNIII 3F/4
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pAb (3F/4)+ FNIII 3F/4
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Absorbance (OD490-620)
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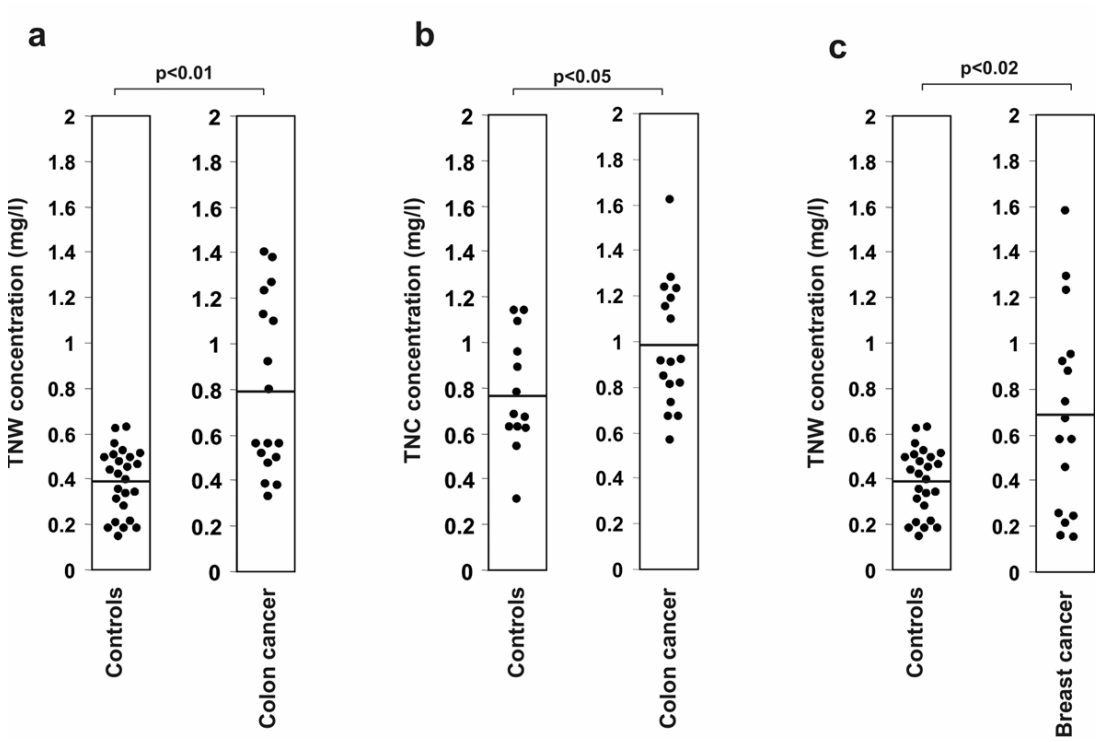
TNW (mg/l)

Figure 1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p<0.01
TNW concentration (mg/l)
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1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Controls
Colon cancer
b
p<0.05
TNC concentration (mg/l)
Controls
Colon cancer
c
p<0.02
TNW concentration (mg/l)
Controls
Breast cancer

Figure 2

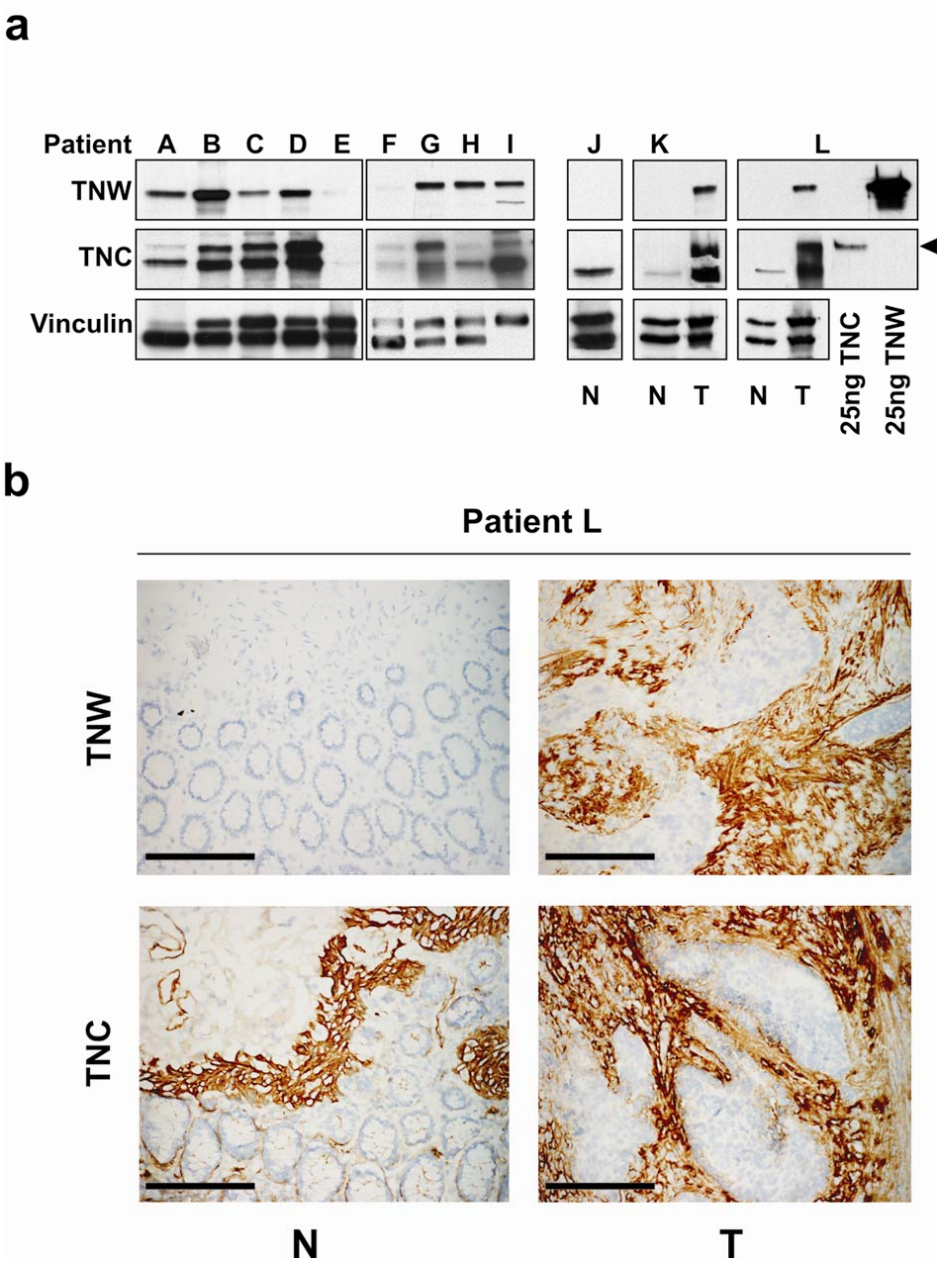
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Figure 3

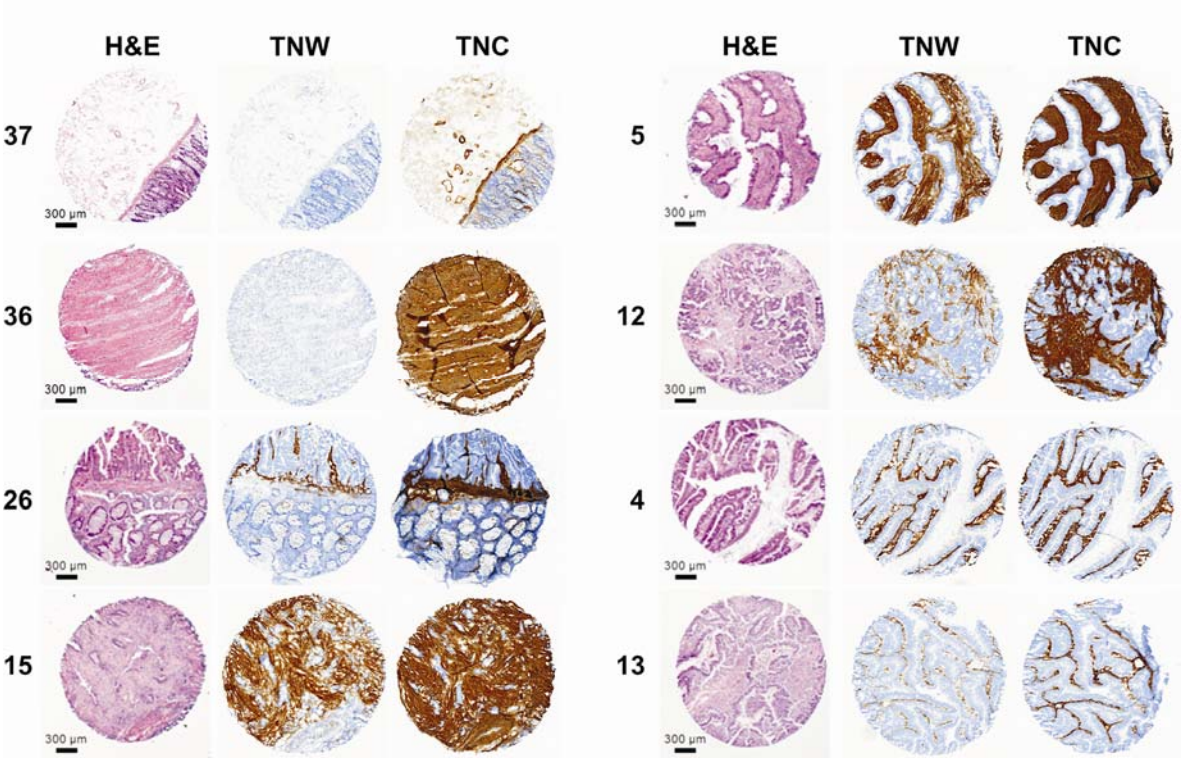

Figure 4

III.2 Submitted Manuscripts

III.2.1 Tenascin-C is a novel RBPJκ-induced target gene for Notch signaling in gliomas

B Sivasankaran[1†], M Degen[2†], A Ghaffari[1,3], ME Hegi[4] , M Ionescu[1], M Tolnay[5], S Mergenthaler[6], AR Miserez[7], R Kiss[8], MM Lino[1], R Chiquet-Ehrismann[2], A Merlo[1,3]* & JL Boulay[1*]

[1]Laboratory of Molecular Neuro-Oncology, Department of Research, University Hospital, CH-4031 Basel, Switzerland
[2]Friedrich Miescher Institute for Biomedical Research, Novartis Research Foundation, CH-4058 Basel, Switzerland
[3]Neurosurgical Clinic, University Hospital, CH-4031 Basel, Switzerland
[4]Laboratory of Tumor Biology and Genetics, Department of Neurosurgery, Centre Hospitalier Universitaire Vaudois and University of Lausanne, CH-1011 Lausanne and National Center of Competence in Research, Molecular Oncology, Swiss Institute of Experimental Cancer Research, CH-1066 Epalinges, Switzerland
[5]Institute of Pathology, University Hospital, CH-4031 Basel, Switzerland,
[6]Laboratory of Prenatal Medicine, Department of Research, University Hospital, CH-4031 Basel, Switzerland
[7]Research Laboratories, diagene inc., CH-4153 Reinach, Switzerland,
[8]Laboratory of Toxicology, Institute of Pharmacy, Free University, B-1070 Bruxelles, Belgium

†equal contributors

*Correspondence. AM: Neurosurgical Clinic, University Hospital, CH-4031 Basel, Switzerland. Tel.: +41.61.265.76.23; Fax: +41.61.265.71.38; E-mail:amerlo@uhbs.ch.
JLB: Laboratory of Molecular Neuro-Oncology, Department of Research, University Hospital, CH-4031 Basel, Switzerland.Tel.: +41.61.265.70.88; Fax: +41.61.265.90.60;
E-mail: jean-louis.boulay@unibas.ch.

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Abstract

Glioblastoma (GBM) are highly invasive and aggressive brain tumors that show limited response to conventional therapies. We observed in human GBM lines frequent *NOTCH2* locus amplification and high Notch2 protein levels to coincide with expression of the tumor progression marker tenascin-C. Furthermore, RBPJκ, the Notch signaling co-factor required for transcription activation of target genes, was significantly co-expressed with tenascin-C in GBM of a tissue microarray (P=0.02). In the tenascin-C-negative glioma line Hs683, we found a missense mutation (L1711M) in the conserved RBPJκ-interacting RAM domain of Notch2. Expression of the wild-type activated forms of Notch2 or Notch1 in glioma cells triggered RBPJκ-dependent induction of *tenascin-C* transcription that was abolished by the L1711M mutation. Furthermore, transfection of activated Notch2 or Notch1 increased endogenous tenascin-C expression. Since addition of increasing amounts of tenascin-C stimulated glioma cell migration, this may represent a mechanism underlying the invasive properties of glioma cells controlled by Notch signaling and defines a novel oncogenic pathway in gliomagenesis to be targeted for therapeutic intervention in GBM patients.

Introduction

Malignant gliomas are invasive tumors that can only transiently be controlled by surgery, radio-and chemotherapy (Louis et al., 2001; Maher et al., 2001; Merlo, 2003). Depending on histological subtype, median survival of glioma patients varies between 10 months in the prevalent glioblastoma (GBM) and 10 years in low-grade oligodendroglioma (OG) (Cairncross et al., 1998). This is reflected in distinct pathogenetic pathways operative in these different glioma entities associated with variable degrees of aggressiveness. Notch signaling is an evolutionary conserved pathway that controls cell fate and growth (Artavanis-Tsakonas et al., 1999). Ligand-dependent cleavage of the Notch transmembrane receptor releases the Notch intracellular domain (N-IC) into the cytoplasm. N-IC translocates to the nucleus, recruits and converts the transcriptional repressor RBPJκ/CBF1/Su(H)/CSL to induce expression of Notch targets such as *hairy/enhancer of split* (*HES*)-*1* and *HES-5* in humans (Allman et al., 2002; Artavanis-Tsakonas et al., 1999; Blaumueller et al., 1997; Jarriault et al., 1995; Wilson & Kovall, 2006). Notch2 is expressed during brain development in cerebellum external granule layer and subventricular zones, where it maintains proliferation and prevents neuronal precursor differentiation (Solecki et al., 2001). Notch2 expression in postnatal brain is restricted to ventricular germinal zones and dividing immature glial cells (Tanaka & Marunouchi, 2003). Conversely, *Notch2*-targeted mice show increased apoptosis in neural tissues, leading to embryonic lethality (Hamada et al., 1999). An oncogenic Notch2 function has been reported in human B cell leukemia (Hubmann et al., 2002), as well as in medulloblastoma (Fan et al., 2004). Although Notch1 oncogenic activity has also been shown in gliomas and other malignancies (Purow et al., 2005) it can also act as a tumor suppressor, depending on the cellular context (Radtke & Raj, 2003).

Tenascin-C (TN-C) is an extracellular matrix glycoprotein first identified in gliomas (Bourdon et al., 1983). High expression levels of perivascular TN-C correlates with shorter progression-free survival in glioma patients (Herold-Mende et al., 2002) and expression of stromal TN-C is increased in higher tumor grades with poor prognosis (Leins et al., 2003). In many cancers including brain tumors, TN-C expression correlates with invasiveness and formation of new vessels (for review see (Orend & Chiquet-Ehrismann, 2006)). In TN-C-deficient mice, maturation of oligodendrocyte precursors is accelerated (Garcion et al., 2001). Furthermore, cardiac neovascularization is impaired in TN-C-deficient mice (Ballard et al.,

2006). Thus, TN-C promotes cell migration, controls differentiation and is critical for angiogenesis.

We describe here that Notch signaling increases TN-C levels by gene *trans*activation, and that TN-C promotes migration of glioma cells. This indicates regulation of TN-C pro-migratory activity in invasive glioma by Notch signaling.

RESULTS

Notch2, Notch1, RBPJκ and tenascin-C expression in gliomas

Immunohistochemistry on glioma biopsies revealed strong Notch2 immunostaining in astrocytomas and GBM. In contrast, Notch2 protein was undetectable in OG and normal brain tissue [Figure 1a, left]. Real-time PCR-based genomic DNA dosage analysis revealed that 4/8 GBM lines had amplification at *NOTCH2* marker D1S2696, while LN18 exhibited local haploidy [Figure 1b]. Fluorescent *in situ* hybridization (FISH) showed that *NOTCH2* amplifications in U373 and U343 lines resulted from chromosome 1 trisomy [Supplementary Figure S1]. The glioma cell line expressing OG markers Hs683 (Branle et al., 2002) contained one *Notch2* gene copy per diploid genome, reminiscent of the prevalent OG chromosome 1p loss extending to *Notch2* (Boulay et al., 2007). Western blot analysis revealed concordance between genomic status and protein levels [Figure 1b]. Likewise, immunohistochemical analysis of TN-C in the same biopsies revealed high TN-C expression in GBM, and barely or not detectable levels in OG and normal white matter [Figure 1a, right]. Furthermore, in all cell lines tested, there was a striking coincidence between Notch2 and TN-C protein levels, particularly visible in Hs683 and LN18 cells with low Notch2 levels and absence of detectable TN-C [Figure 1c]. Finally, Notch1, Notch2, and RBPJκ protein levels were compared to Notch signaling activation assessed by *HES-1* transcript levels [Figure 1c]. RBPJκ was present in all GBM lines and decreased amounts of Notch1 or Notch2 were accompanied by lower *HES1* expression, as can be seen in Hs683, LN18 and LN401 cells [Figure 1c].

To extend our immunohistochemical analysis, we made use of GBM tissue microarrays revealing labeling frequencies of 83% (118/143) for Notch2, 59% (84/142) for Notch1, 37% (54/146) for RBPJκ and 55% (70/127) for TN-C. Thus, Notch2 was more frequently expressed than Notch1 and there was a significant association between RBPJκ and TN-C expression (P=0.02) [Table1; Figure 2]. This significant correlation suggested the possibility of a causal relationship and *trans*-activation of TN-C by Notch/RBPJκ signaling was considered.

The TN-C promoter contains a RBPJκ-responsive element

We analyzed the *TN-C* promoter sequences for the presence of a RBPJκ binding motif. Alignment of the upstream sequences of human and murine Notch target genes *HES1* and *HES5* with those of *TN-C* revealed the presence of a conserved RBPJκ binding motif GTGGGAA at the same distance from the TATA box in each of these genes [Figure 3a]. We, therefore, tested whether N2-IC and N1-IC were able to induce luciferase reporter gene expression driven by a 102 bp fragment of a human *TN-C* minimal promoter containing the RBPJκ binding motif [Figures 3a]. Co-transfection of Hs683 cells with a plasmid expressing either N2-IC or N1-IC both led to a two-fold increase compared to the basal luciferase activity in the presence of a control plasmid [Figure 3b, top].

To test whether this induction is dependent on RBPJκ function, point mutations, previously described to prevent RBPJκ binding (Tun et al., 1994) were introduced into the potential RBPJκ binding site of the *TN-C* promoter. Transfection of mutant promoter constructs *M1* and *M2* that prevent RBPJκ binding produced basal luciferase levels, but no longer allowed induction by N2-IC [Figure 3b, bottom]. Consistently, co-transfection of a plasmid expressing a dominant negative RBPJκ mutant (Chung et al., 1994) impaired N2-IC-mediated induction of the *TN-C* promoter [Figure 3c].

The tenascin-C negative line Hs683 contains a Notch2 mutation

In glioma cells, we sequenced *Notch2* fragments encoding N2-IC and covering mutation hotspots for gain- or loss-of-function described in *Drosophila Notch* (Brennan et al., 1997; Rebay et al., 1993), *NOTCH1* (Mansour et al., 2006) and *NOTCH2* (McDaniell et al., 2006). Interestingly, the OG line Hs683 had a C-to-A mutation in *Notch2* codon 1711 resulting in the substitution of leucine by methionine [Figure 4a]. Leucine 1711 is conserved throughout vertebrate Notch2 proteins [Figure 4b] and is located within the RBPJκ-interacting RAM domain (Tamura et al., 1995; Wilson & Kovall, 2006). Co-expression of the N2-IC*L1711M* mutant with the *TN-C* promoter construct no longer increased luciferase activity [Figure 4c]. Therefore, the Notch2 present in the HS683 cells seems to be non-functional.

Notch signaling increases endogenous tenascin-C levels

We further tested whether Notch signaling was able to induce the expression of endogenous TN-C protein. Since the abundant TN-C levels produced by GBM cell lines may mask any further increases, we focused on fibroblast lines Detroit 551 and MRC-5 that secrete moderate amounts of TN-C. Supernatants of fibroblasts transfected with N2-IC showed increased accumulation of TN-C in the medium compared to the controls [Figure 5a, top], while secreted fibronectin levels were similar [Figure 5a, bottom]. The same stimulation of tenascin-C expression was seen after transfection of N1-IC, but not with the mutant N2-IC*L1711M* construct [Figure 5b].

Tenascin-C enhances glioma cell migration

To test whether increased TN-C expression is able to alter the dynamics of cell migration, transfilter migration assays were performed with the TN-Cnegative Hs683 cells. Addition of increasing amounts of purified TN-C stimulated the migratory capacity of TN-C-negative Hs683 cells to an intermediate level to that of TN-C-positive LN319 cells [Figure 6].

In summary, we showed that RBPJκ-dependent Notch signaling transcriptionally activates TN-C expression, and that addition of purified TN-C in turn promotes cell migration. Taking these results together, they provide a potential mechanism for oncogenic Notch signaling in gliomagenesis.

DISCUSSION

Notch2 expression in GBM and OG

Notch2 is expressed in immature glial cells of ventricular germinal zones of normal brain (Irvin et al., 2001; Tanaka & Marunouchi, 2003; Wang & Barres, 2000), concordant with the hypothesis of an undifferentiated state of neoplastic cells in GBM. Consistently, the gene promoter of nestin, a marker for neural precursors and of GBM contains also a RBPJκ responsive element (Shih & Holland, 2006), implying that Notch2 may be involved in maintaining glioma cells in an undifferentiated state. However, the fact that RBPJκ target *HES-1* drives astrocytic cell fate (Wu et al., 2003) argues also for a role of Notch2 in astrocytic differentiation. It has been shown that primary GBM show high *HES-1* contents, while astrocytoma progression coincides with decreasing *HES-1* levels (Somasundaram et al., 2005). Since *HES-1* is under direct control of both Notch proteins, the question whether Notch1 plays redundant or complementary roles to Notch2 in glioma progression remains open.

Conversely, the Notch2 loss-of-function mutation in Hs683 cells provides an inactivation mechanism of Notch2 in OG, in addition to homozygous deletions detected in primary OG with 1p loss (Boulay et al., 2007). Thus, OG development appears dependent on Notch2 loss. Although both, Notch1 and Notch2 proteins expressed in the glial lineage are inducers of *TN-C*, Notch2 co-expression with *TN-C* seemed to be more common. This association may be based on the frequent Notch2 gene amplification and high protein expression we found in GBM.

A RBPJκ-responsive element in the TN-C gene promoter

The significant association between RBPJκ and TN-C expression in a GBM tissue microarray together with the identification of a RBPJκ-responsive element in a minimal *TN-C* promoter provide a novel mechanism of *TN-C trans*-activation.

Molecular cooperation between Notch2 and RBPJκ has been suggested in B cell development, where Notch2- and RBPJκ-targeted mice present a common phenotype both lacking a B cell subset (Saito et al., 2003; Tanigaki et al., 2002), and in B cell leukemia, where Notch2 together with RBPJκ upregulate *CD23a* transcription (Hubmann et al., 2002).

Nevertheless, half (31/62) of TN-C-positive GBM on the tissue microarray were RBPJκ-negative, suggesting RBPJκ-independent regulatory pathways. In human fibroblasts, the *TN-C* promoter is activated by platelet-derivate growth factor (PDGF) and transforming growth factor (TGF)-β (Jinnin et al., 2004; Jinnin et al., 2006). Since TGF-β signaling promotes PDGF-beta-dependent cell proliferation in glioma (Bruna et al., 2007), activation of the *TN-C* promoter by TGF-β signaling may also apply in glioma.

The Notch-Tenascin-C network

TN-C-deficient mice show compromised proliferation/migration of neural precursors, and accelerated oligodendrocyte differentiation (Garcion et al., 2001). Consistently, GBM lines and biopsies showed strong TN-C expression, while OG did not, although an OG subset has been described as moderately positive (McLendon et al., 2000). Strong TN-C expression is associated with the invasive front in many tumors types and is a diagnostic marker for glioma progression, implying a role for TN-C in tumor promotion. (for review see (Orend & Chiquet-Ehrismann, 2006). This supports the hypothesis of Notch2/RBPJκ/TN-C signaling to be operative in GBM, but not OG development.

From a clinical point of view, conventional therapeutic interventions based on tumor resection and radio- and chemotherapy have only moderately improved glioma patient survival over the past decades (Stupp et al., 2005). In addition to directly targeting TN-C (Merlo et al., 1997; Reardon et al., 2002), our data suggest the use of drugs blocking Notch signaling. We propose that the Notch/RBPJκ/TN-C pathway regulates tumor cell migration, a hallmark of invasive GBM. This molecular cascade provides a novel mechanism through which Notch acts in neoplastic transformation and possibly in normal development of the neuronal and glial cell lineages.

MATERIALS & METHODS

Cell lines and antibodies

Cells were grown in Dulbecco's Modified Eagles Medium with 10% fetal calf serum and 1% Glutamine. Immunoreactions were performed with monoclonal antibodies anti-Notch2 C651.6dbHN and anti-Notch1 bTAN20 (Zagouras et al., 1995) (Developmental Studies Hybridoma Bank, University of Iowa, USA), anti-TN-C B28-13 (Schenk et al., 1995), anti-RBPJκ (Institute of Immunology,Tokyo, Japan), anti-actin (Sigma, Saint-Louis MO, USA) and antiserum against fibronectin (Ehrismann et al., 1981).

Glioma biopsies and immunohistochemistry

Paraffin sections of formalin-fixed tissue samples were obtained from the University of Basel Department of Neuropathology. Tumors were diagnosed and graded according to the WHO Classification of Tumors of the Nervous System. Immunohistochemistry was performed with anti-Notch2 and anti-TNC using an overnight incubation at 4°C of a 1:50 dilution. Bound antigens were detected using avidin-biotin-peroxidase (Vectastain, Elite kit; Vector Laboratories) and sections were weakly counterstained with hematoxylin.

Nucleic acid extraction and analysis

Genomic DNA was extracted using the genomic DNA purification kit (Qiagen, Hilden, Germany). RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA), reverse-transcribed with the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed on an ABI Prism sequence 7700 detector (PE Applied Biosystems, Foster, CA, USA) at microsatellite marker D1S2696 using primers gaattacatcccaggcaatctga and cacacaacaggcccctaatca and probe FAM-agcccatgctcattcccactacactgg- TAMRA. GAPDH primers aatgggactgaggctcccac and ttatgggaaagccagtcccc and probe FAM-atccaagactggctcctccctgctg-TAMRA. *Notch2* cDNAs from glioma cells were sequenced at mutation hot spots, *i.e.* EGF repeats 11-14, 24-25, 29 and 32, Lin-12 domains and N2-IC. The Notch2 mutation L1711M found in Hs683 cDNA was confirmed by sequencing genomic DNA.

Western blot analysis

Cell extracts and conditioned media were separated by SDS-PAGE and electroblotted to PDVF membranes (Millipore, Bedford MA, USA). Proteins were detected with primary and secondary antibodies and blots developed using Super Signal (Pierce, Rockford IL, USA) on Kodak BioMax MR Films (Rochester, NY, USA). Protein bands were quantified using the software GeneTools (SynGene, Cambridge, UK).

Tissue microarray

The tissue micro array comprising 190 glioblastoma has been constructed from archived paraffin blocks at the University Hospital in Lausanne, Switzerland (Godard et al., 2003). Immunohistochemical determination for tenascin-C (dilution 1:2500), anti-RBPJκ (1:120), Notch2 (1:100) and Notch1 (Abcam, Cambridge, UK) (1:200) was performed according to standard procedures for paraffin sections using a high temperature epitope retrieval technique in citrate buffer (pH 6.0; pressure cooker, 3 to 5 minutes) and overnight incubation with the primary antibody. The immunostaining was scored semiquantitatively from low (score 0-1) *versus* high expression score (2-3) and was used for statistics by Fisher's exact test. Tumors were ordered by similarity of expression profiles (scores 0 to 3) using the SPIN software (Tsafrir et al., 2005).

Fluorescent in situ hybridization

Fluorescent *in situ* hybridization on metaphase chromosomes of cell lines was performed using the Nucleic Acid Labelling Systems kit (Q-BioGene, Illkirch, France).

Plasmids

Activated Notch2 (N2-IC, from nucleotides 5107 to 7425 of Notch2 cDNA sequence AF308601) was cloned into plasmid pcDNA3 (Invitrogen, Carlsbad, CA, USA). Mutations in the Notch2-IC (L1711M) and in the RBPJκ binding site (Tun et al., 1994) were obtained by site-directed mutagenesis (Stratagene, La Jolla, CA, USA). The Notch1 construct (Capobianco et al., 1997) was kindly provided by Dr. Radtke, and the dominant-negative RBPJκ plasmid (Chung et al., 1994), by Dr. Honjo. The *TN-C* promoter construct included 102bp 5'flanking sequence from the transcription start and 97bp of the first exon.

The reporter construct was cloned with the Expand High Fidelity PCR System (Roche, Rotkreuz, Switzerland) using the primer pair gagctagcctttaattcgccaactg / gactcgagctgcctttgtgcccacggag with genomic DNA from HEK293 cells as template and cloned into the pGL3 luciferase reporter vector (Promega, Madison WI, USA).

Transfections and reporter gene assays

Transfections were performed using Fugene (Roche, Rotkreuz, Switzerland). For the reporter gene assays, Hs683 cells were seeded in 6-well plates and transiently transfected with 1 µg TN-C luciferase reporter vector, 1 µg expression plasmid, and 0.1 µg *Renilla* Luciferase reporter vector phRL-TK for standardization (Promega, Madison WI, USA). Cells were harvested after 24 hours and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison WI, USA). Luciferase activity was measured from at least three independent experiments in a Mithras LB940 Luminometer (Berthold Technologies, Bad Wildbad, Germany). Western blots showing similar levels between N2-IC*WT* and N2-IC*L1711M* in transfected cells ruled out instability of N2-IC*L1711M*.

Transwell migration assays

Transwell migration assays were performed using modified Boyden chamber units with polycarbonate filters of 8µm porosity (Costar, Appleton Woods, Birmingham, UK). The lower side of the filter was coated with 10µg/ml fibronectin for 2 hours at room temperature. The bottom chamber was filled with serum-free DMEM containing 0.1% BSA plus/minus increasing amounts of purified human TN-C. Cells (104 cells/well in serum-free DMEM) were plated in the upper chamber and incubated for 16 hours at 37°C. After removal of the remaining cells from the upper surface of the filter, migrated cells at the bottom of the filter were fixed with 3.7% formaldehyde in PBS and stained with 0.1% crystal violet. For each condition, cells in five fields of three independent experiments were counted.

Statistical analyses

Kruskal-Wallis test was used for significance of genomic copy dosage. Significances of reporter gene expression and endogenous TN-C expression were established with the Student t test available at the web server of St. John's University, St. Joseph, MN (USA).

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LEGENDS TO FIGURES

Figure 1. Notch and tenascin-C expression in glioblastoma

(a) Immunostaining of normal white matter (top), oligodendroglioma (middle) and glioblastoma (bottom) biopsies with anti-Notch2 (left) and anti-TN-C (right). Notch2 and TN-C stainings were performed on sections of the same biopsies. (**b**) Detection of genomic amplifications at marker D1S2696 in glioma lines by real-time quantitative PCR. ΔCt value of 0 corresponds to normal diploidy. Notch2 Genomic status (*n*) is calculated as 2x2-ΔCt. All experiments were done in triplicate. Average values and standard deviations (error bars) are based on two independent experiments. *P*-value for comparison of all three groups (>2n; 2n; <2n): *P*=0.0002. (**c**) Notch2, TN-C, Notch1 and RBPJκ immunodetection in glioma cells. For each experiment, the corresponding actin standard is shown below. (**d**) Assessment of Notch signaling activity in glioma cells by real-time quantitative PCR of *HES-1* transcripts. ΔCt value of 0 corresponds to fetal brain level. *HES-1* amounts relative to fetal brain baseline calculated as 2ΔCt.

Figure 2. Visualization of Notch1, Notch2, RBPJκ and TN-C expression in 96 GBM

Protein expression was determined by immunohistochemistry on a GBM tissue microarray and scored 0 (indigo, no expression) to 3 (red, high expression). 96 0f the 190 spots could be classified for all four proteins. Data are ordered using the SPIN software (Tsafrir et al., 2005).

Figure 3. The *tenascin-C* promoter contains a functional RBPJκ responsive element

(**a**) Sequence alignment of human (h) and mouse (m) *HES5*, *HES1* and *TN-C* promoters. Conserved nucleotides are uppercase and marked by dots. TATA boxes and RBPJκ binding motifs are highlighted in black. (**b**) Notchdependent induction of luciferase driven by the human *TN-C* promoter occurs when the wild-type (*WT*) RBPJκ binding site is present, but not with *M1* or *M2* mutants. Average values and standard deviations (error bars) are based on three independent experiments. Significance of N2-IC induction on GTGGGAA *WT vs.* control pcDNA3 and *M1/M2* mutations: *P*<0.008. (**c**) A dominant-negative (*DN*) RBPJκ mutant impairs Notch2-dependent induction of *TN-C* transcription. Significance of N2-IC induction *vs.* pcDNA3 and RBPJκ*DN*: *P*<0.008 and of N1-IC vs. pcDNA3: P<0.035.

Figure 4. Notch2-IC but not the mutant Notch2-IC *trans*-activates the *tenascin-C* promoter
(**a**) Electrophoretogram of the Hs683 mutation L1711M. (**b**) Top. The N2-IC domains. Abbreviations: RAM, RBPJκ-binding site; NLS, nuclear localization signal; TAD, trans-activation domain; PEST, serine/threonine-rich domain. Bottom. Sequence alignment of the Hs683 Notch2 mutation with wild-type Notch2 RAM. Conserved aminoacids are uppercase, the mutation is highlighted. (**c**) Notch2-dependent induction of the *TN-C* promoter is impaired by mutation *L1711M*. Significance of N2-IC*WT* induction vs. pcDNA3 and N2- IC*L1711M*: $P<0.02$.

Figure 5. Notch signaling triggers endogenous tenascin-C production
(**a**) Time course of Notch2-dependent endogenous TN-C production. Detection of TN-C and fibronectin on western blots of supernatants from transfected Detroit 551 and MRC-5 fibroblasts collected 24 and 48 hrs after transfection with Notch2-IC or the control plasmid pcDNA3. Graphs show quantification of TN-C (top) and fibronectin (bottom) protein bands. (**b**) Transfection of wild-type Notch1-IC and Notch2-IC but not the mutated Notch2-IC*L1711M* lead to an increase of TN-C levels in fibroblasts.

Figure 6. Tenascin-C promotes glioma cell migration
Migration assays were performed in modified Boyden chambers with Hs683 (TN-C-) and LN319 (TN-C+) cells in the presence or absence of purified TN-C protein In the lower chamber. After 16 hrs, migrated cells were counted and numbers normalized to Hs683 cells in the absence of TN-C. Average values and standard deviations (error bars) are based on three independent experiments. Significance: Hs683 *vs.* Hs683 + 10µg/ml TN-C: $P<0.001$; Hs683 *vs.* LN319: $P< 0.001$.

Table

	RBPJk-high	RBPJk-low	Total	
TNC-high	31	31	62	
TNC-low	10	29	39	
Total	41	60	101	p=0.021[a]

[a] Fisher's exact test, 2-sided

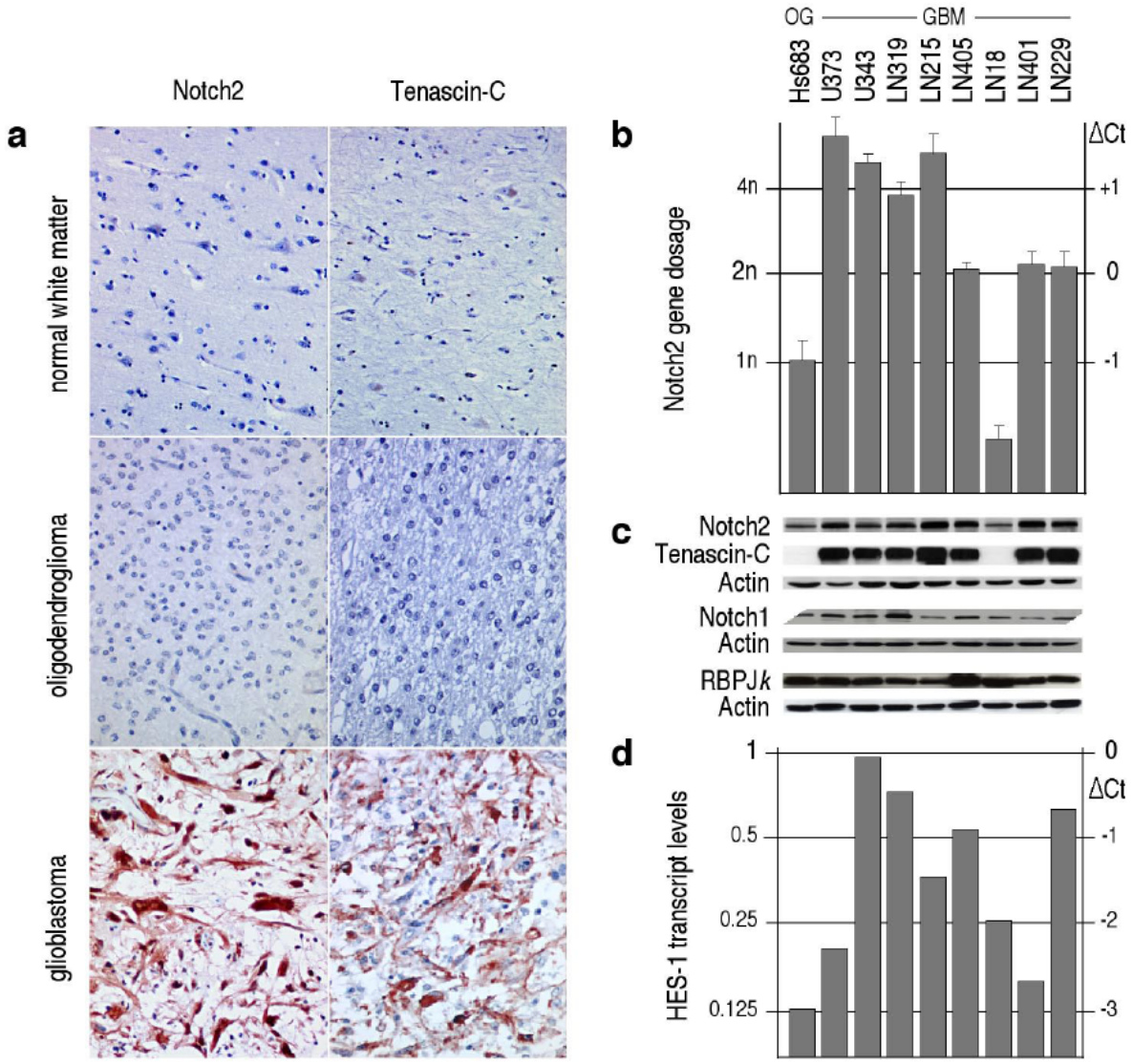


Figure 1

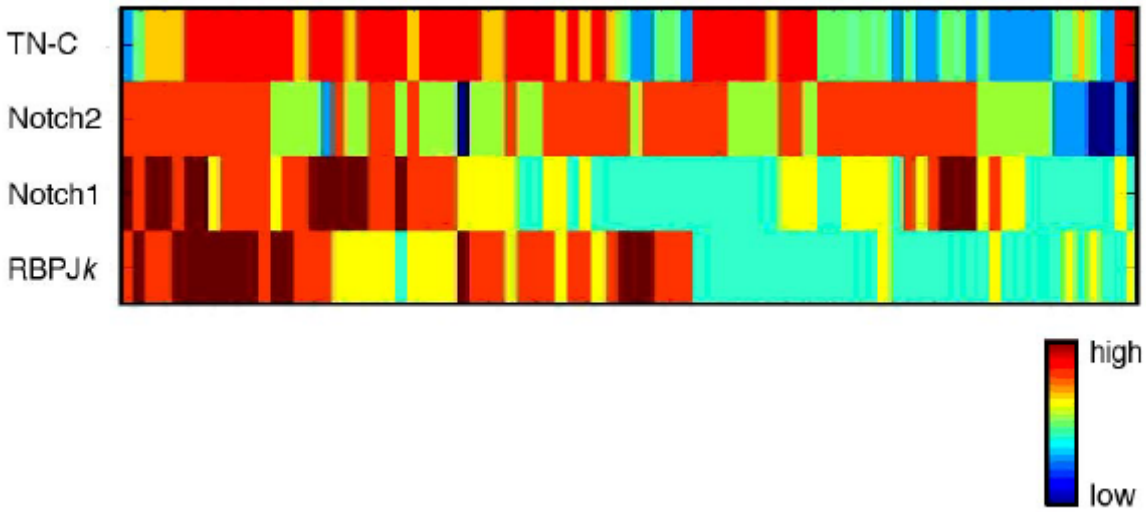


Figure 2

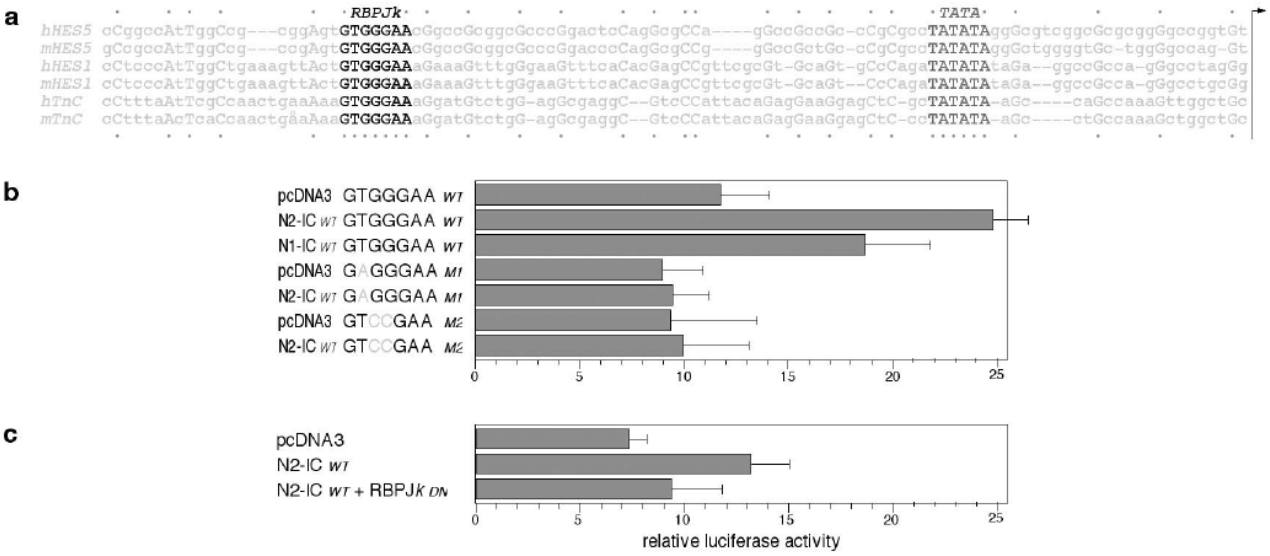


Figure 3

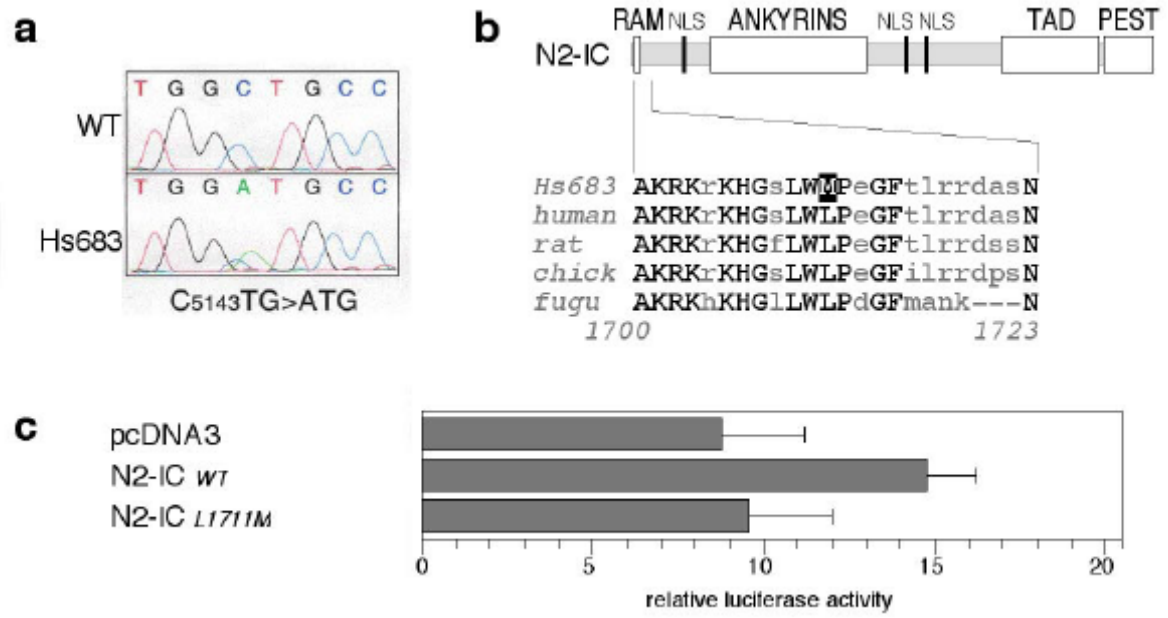


Figure 4

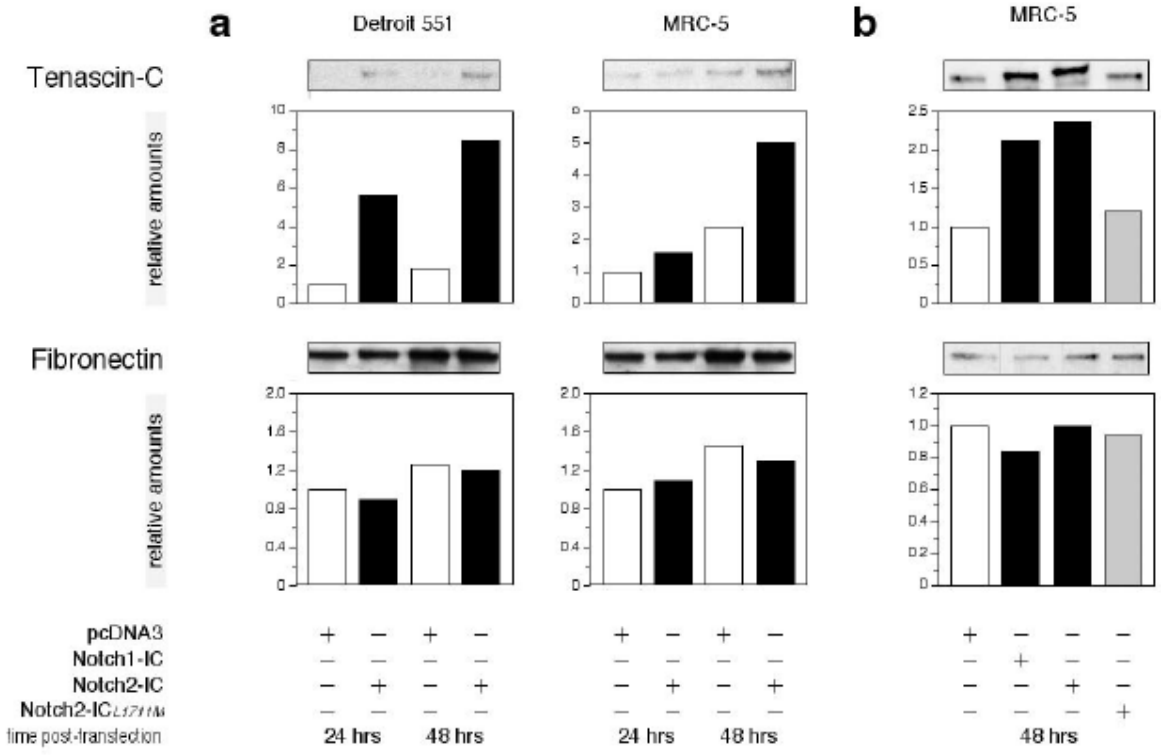


Figure 5

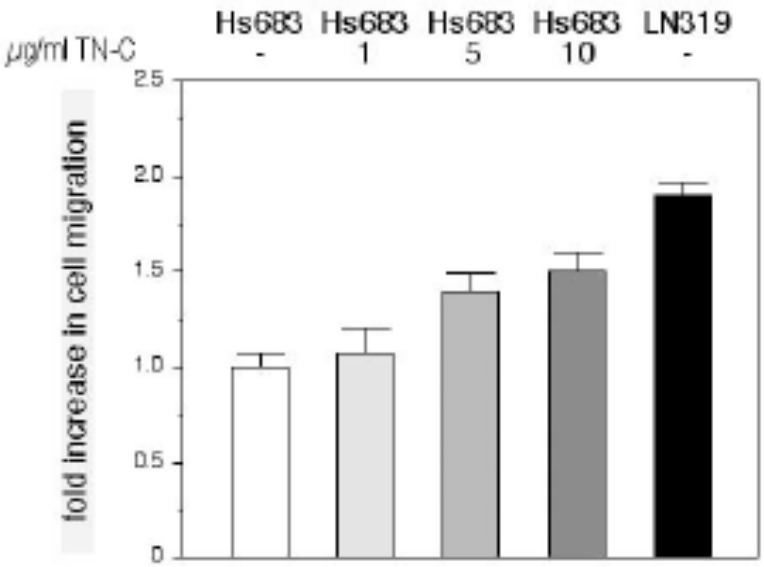
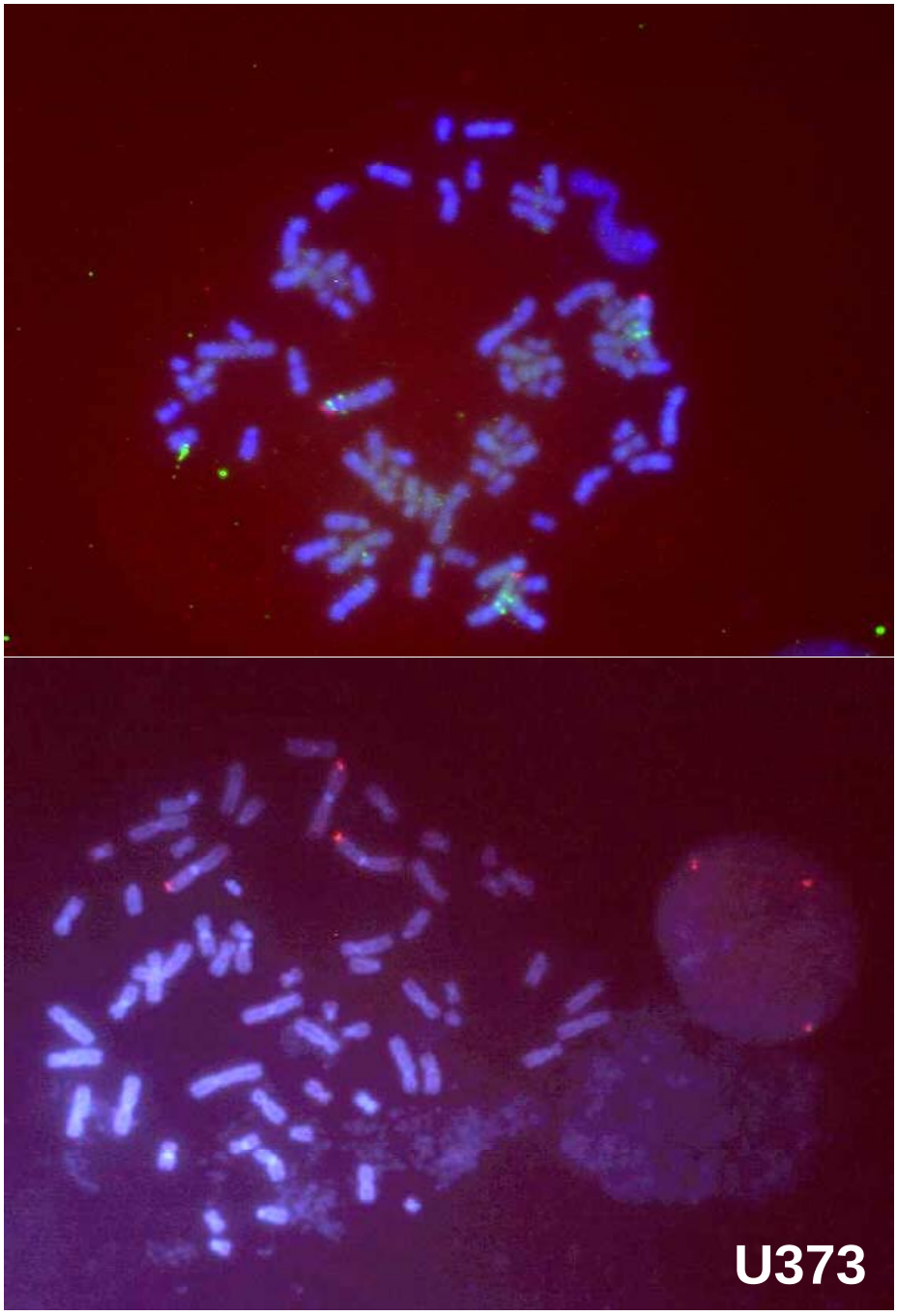

Hs683 Hs683 Hs683 Hs683 LN319
µg/ml TN-C - 1 5 10 -
fold increase in cell migration
2.5
2.0
1.5
1.0
0.5
0

Figure 6

Supplemental material



S1. Fluorescent in situ hybridization of dGreen labeled BAC clone RP5-1042I98 (GeneBank accession: AL359752) and rhodamine-labeled 1q telomeric probe D1S3739 on metaphase chromosomes of U373 and U343 cells. The BAC RP5-1042I98 labels the 11p11 area plus the 1q21.1 pericentric duplication.

III.2.2 Opposite effect of fluticasone and salmeterol on fibronectin and tenascin-C expression in primary human lung fibroblasts

M. Degen[a]*, S. Goulet[b]*, M. Roth[b,c], M. Tamm[b], and R. Chiquet-Ehrsmann[a]

[a]Friedrich Miescher Institute for Biomedical Research, Novartis Research Foundation, Basel, Switzerland
[b]Department of Research, Pulmonary Cell Research, University Hospital Basel, Basel, Switzerland
[c]The Woolcock Institute of Medical Rsearch, University of Sydney, New South Wales, Australia

* equal contribution

Corresponding author: Ruth Chiquet-Ehrismann
Friedrich Miescher Institute for Biomedical Research
Novartis Research Foundation
Maulbeerstrasse 66
CH-4058 Basel
Switzerland
e-mail: chiquet@fmi.ch

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Abstract

Airway remodeling is a key feature of asthma and chronic obstructive pulmonary disease (COPD). The remodeling process involves the deposition of extracellular matrix (ECM) proteins within the airways. Current therapies for asthma and COPD consist of inhaled corticosteroids and long-acting β_2-agonists. However, their effect on airway remodeling is not well understood so far. In this study we investigated the effect of fluticasone and salmeterol, either alone or in combination, on fibronectin and tenascin-C protein and mRNA levels in primary human lung fibroblasts. In our model, fibroblasts cultured in serum-free medium represented a non-inflammatory condition and stimulation with 5% FCS and/or TGF-β_1 mimicked an inflammatory environment with activation of tissue repair. In both conditions, fluticasone increased fibronectin transcript and protein levels, whereas it decreased those of tenascin-C. Salmeterol neither affected fibronectin and tenascin-C synthesis nor did it influence the effect of fluticasone when applied in combination. Furthermore, we found that treatment with fluticasone had an opposite effect on extra domain A- and B-containing fibronectin isoforms generated by alternative splicing compared to total fibronectin transcript levels, whereas tenascin-C isoforms were not differently modulated by fluticasone compared to total tenascin-C transcript levels. Our results indicate that standard therapies for inflammatory lung disorders influence ECM protein composition and relative expression levels.

Keywords: fibronectin, tenascin-C, human lung fibroblasts, asthma therapy

Abbreviations: COPD, chronic obstructive pulmonary disease; ECM, extracellular matrix; EDA/B, extra domain A/B; LABA, long-acting β_2-agonist; TGF-β_1, transforming growth factor β_1

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are among the world's most prelevant diseases. Both are characterized by chronic inflammation and tissue remodeling resulting in irreversible structural changes in the lung. Prominent features of the airway remodeling process include thickening of the lamina reticularis, enlargement of bronchial smooth muscle mass due to hyperplastic and hypertrophic smooth muscle cells, increase in vascularity, and increase of extracellular matrix (ECM) protein deposition within the airways (Roche, Beasley, Williams, & Holgate, 1989; Busse, Elias, Sheppard, & Banks-Schlegel, 1999; Bousquet, Jeffery, Busse, Johnson, & Vignola, 2000; Jeffery, 2001; Bergeron & Boulet, 2006). Whether pathological ECM deposition is the result or the cause for chronic airway inflammation is not yet clear. However, it is well established that the ECM is not only a passive component of tissues, but rather plays a very active and dynamic role in regulating cell proliferation, differentiation, apoptosis, adhesion, and migration. Furthermore, the ECM has architectural roles during development and tissue repair and provides positional and environmental information essential for tissue maintenance and function (Bosman & Stamenkovic, 2003). Therefore, it is not surprising that the ECM profile in the airways of healthy individuals differs both in quantity and composition from that of asthmatic and COPD subjects. Whereas deposition of collagen IV and elastin is decreased in asthmatic patients compared to healthy individuals, deposition of other specific ECM proteins, including collagen I, III, and V, fibronectin, tenascin, versican, and laminin is increased (Roche, Beasley, Williams, & Holgate, 1989; Bousquet et al., 1992; Altraja et al., 1996; Laitinen et al., 1997; Roberts & Burke, 1998).

Lung fibroblasts are important mediators of the lung tissue repair process as they are involved in cell proliferation and synthesis of ECM components. Among the various ECM proteins, lung fibroblasts secrete tenascin-C and fibronectin, two large multifunctional and multidomain ECM glycoproteins highly expressed during morphogenesis and tissue repair. Tenascin-C is the founding member of the tenascin family which includes three other members: tenascin-R, -X and –W (Chiquet-Ehrismann & Chiquet, 2003). All tenascins display complex, highly restricted, and dynamic patterns of expression. Tenascin-C, which is subjected to alternative splicing, shows high expression during embryogenesis and organogenesis, but reduced and restricted expression in adult organisms (Jones & Jones,

2000). However, there is a sharp re-expression of tenascin-C under pathological conditions such as inflammation, infection, tissue remodeling, wound healing, and tumorigenesis (Chiquet-Ehrismann & Chiquet, 2003). Tenascin-C expression in the healthy lung is either absent or weakly detected only in small vessels and basement membranes of large bronchi. In contrast, asthmatic lung tissue shows high tenascin-C expression in the subepithelial ECM (Laitinen et al., 1997; Amin et al., 2000; Laitinen, Altraja, Karjalainen, & Laitinen, 2000; Chiquet-Ehrismann & Chiquet, 2003; Karjalainen et al., 2003). Strong tenascin-C expression has also been reported in patients suffering from interstitial pneumonia and sarcoidosis (Kuhn & Mason, 1995; Kaarteenaho-Wiik, Mertaniemi, Sajanti, Soini, & Paakko, 1998). Recent data suggest that tenascin-C, which is particularly abundant at inflammation sites, might be involved in the pathogenesis of asthma (Nakahara et al., 2006).

Fibronectin is highly found in all major groups of vertebrates as insoluble component of many ECM and as soluble protein in many body fluids (Hynes, 1990). Although fibronectin is encoded by a single gene, it is subjected to alternative splicing resulting in 20 variants (Kornblihtt, Vibe-Pedersen, & Baralle, 1984; Kornblihtt, Umezawa, Vibe-Pedersen, & Baralle, 1985; Hynes, 1990; Pankov & Yamada, 2002). The most frequent splicing isoforms occur within the central set of type III repeats (EDA, EDB and IIICS). These splice variants are differentially expressed in development and disease (Ffrench-Constant & Hynes, 1989; Ffrench-Constant, Van de Water, Dvorak, & Hynes, 1989; Peters & Hynes, 1996) and are known to have various functions (Pankov & Yamada, 2002). EDA- or EDB-containing fibronectin are expressed at very low levels in adults (Peters, Chen, & Hynes, 1996), but their levels are markedly increased during pathological remodeling processes and cancer (Carnemolla et al., 1989; Matter et al., 2004; Trachsel, Kaspar, Bootz, Detmar, & Neri, 2007).

Several studies have shown increased fibronectin levels in body fluids as well as in the lung ECM of subjects with COPD, asthma or other interstitial lung diseases, claiming that fibronectin might contribute to the pathogenesis of lung abnormalities seen in these pathologies (Mattoli, Mattoso, Soloperto, Allegra, & Fasoli, 1991; Campbell et al., 1993; Gupta, Reinhart, & Bhalla, 1998; Meerschaert, Kelly, Mosher, Busse, & Jarjour, 1999; Trifilieff, El-Hashim, & Bertrand, 2000). Moreover, similarly to tenascin-C, fibronectin has been implicated in the regulation of cell proliferation, differentiation, spreading, and

migration (Pankov & Yamada, 2002). In asthma and COPD the proliferation and migration of mesenchymal cells is increased and contributes to the thickening of the airway walls (Bergeron & Boulet, 2006).

Current asthma and COPD therapies include inhaled corticosteroids and long-acting β_2-agonists (LABA) applied either alone or in combination. Corticosteroids have a well-described anti-inflammatory effect, however, their influence on airway remodeling remains controversial (Olivieri et al., 1997; Pauwels et al., 1997; Hoshino, Takahashi, Takai, & Sim, 1999; Boulet et al., 2000; Bergeron et al., 2005; Tomic, Lassiter, Ritzenthaler, Rivera, & Roman, 2005; Ward et al., 2005; Goulet, Bihl, Gambazzi, Tamm, & Roth, 2007). More than a decade ago, Pauwels et al. have shown that the addition of inhaled formoterol to budesonide resulted in better improvement of clinical outcome in asthma patients than higher dose of budesonide (Pauwels et al., 1997). However, the effect of combined corticosteroids and LABA on lung ECM synthesis and deposition remains to be fully elucidated.

In the present study, we investigated the effect of corticosteroids and LABA, alone or in combination, on tenascin-C and fibronectin expression in primary human lung fibroblasts. As previously described, we used two experimental conditions in our culture model: a serum-deprived medium representing a non-inflammatory environment and stimulation with 5% FCS and/or TGF-β_1 mimicking an early stage of inflammation with vessel leakage and activated tissue repair (Goulet, Bihl, Gambazzi, Tamm, & Roth, 2007).

MATERIALS AND METHODS

Cell culture

Primary human lung fibroblasts were established from non-diseased peripheral lung tissue samples obtained from patients undergoing lung resection following approval by the ethics committee (Faculty of Medicine, University Hospital Basel, Switzerland). Cells were grown in RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% FCS (GIBCO BRL Invitrogen, Basel, Switzerland) and 1% MEM-vitamins (Cambrex Bio Science). Fibroblasts were stimulated with recombinant human TGF-β_1 (2.5 ng/ml; Sigma, Schnelldorf, Germany) and treated with fluticasone and salmeterol (10^{-7} M; GlaxoSmithKline, London, UK). Culture conditions and optimal drug doses have been determined previously (Goulet, Bihl, Gambazzi, Tamm, & Roth, 2007). Prior to any treatment, cells were serum-deprived for 24 h in 0.3% human albumin. Fibroblasts between passages 3 and 6 were used for all experiments.

Reverse transcriptase-polymerase chain reaction

Confluent serum-deprived fibroblasts were treated with fluticasone and/or salmeterol (10^{-7} M) in 0.3 % albumin or 5% FCS for 48 h. Total RNA was extracted using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and reverse-transcribed using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). PCR was performed using the fibroblast cDNAs as templates and primers amplifying specific amplicons of GAPDH, FN, TN-C, TN-C isoforms, and the FN splicing isoforms EDA and EDB (Microsynth AG, Balgach, Switzerland; Table 1). PCR conditions were initial denaturation at 94˚C for 2 min then denaturation at 94˚C for 30 sec, primer annealing at 55˚C for 30 sec and extension at 72˚C for 1 min for 28 cycles. PCR amplicons were size fractionated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide stain under UV light. The intensity of each band was analyzed by densitometry using the software Gene Tools (SynGene, Cambridge, UK) and the relative mRNA expression of target gene was normalized to the GAPDH control. Results are expressed as relative to controls (without drug treatment) which were defined as 1.

Western Blotting and Quantification

30 µl of collected conditioned medium of primary human lung fibroblasts were mixed with 10 µl of 4x reducing SDS-PAGE sample buffer, boiled for 5 min at 95°C and loaded onto a 6% SDS-polyacrylamide gel. After electrophoresis, proteins were electro-transferred onto a polyvinyldifluoride membrane (Millipore, Billerica, MA, USA) using a semi-dry blotting apparatus (Millipore). Afterwards, membranes were stained with amido black to control equal protein loading. After blocking for 1 h at room temperature in TBS containing 0.05% Tween and 5% skim milk powder, membranes were incubated overnight at 4°C with either the monoclonal antibody B28-13 raised against human tenascin-C (1:100) (Schenk, Muser, Vollmer, & Chiquet-Ehrismann, 1995) or the monoclonal anti-human fibronectin antibody (1:1000) (Sigma, Buchs, Switzerland). Membranes were washed 3 times with TBS containing 0.05% Tween and then incubated with goat anti-mouse IgG coupled to horseradish peroxidase (1:10 000) (MP Biomedicals, Illkirch, France) for 1 h at room temperature. Blots were developed using Super Signal (Pierce, Rockford, IL, USA) and finally exposed on Kodak BioMax MR Films (Kodak, Rochester, NY, USA).

For Western blot quantification, the software Gene Tools was used. Results represent the quantification of three different blots using three different primary human lung fibroblasts cell lines. Graphs are expressed as relative to controls (without drug treatment) which were defined as 100%.

Statistical analysis

Data are expressed as means ± SEM. Statistical significances were established with the Student's t-test (available at the web server hosted by St. John's University, St. Joseph, MN, USA: www.physics.csbsju.edu/stats/t-test.html). Differences were considered significant if $p<0.05$ compared to control.

RESULTS

5% FCS and TGF-β_1 increase the expression of fibronectin and tenascin-C

Confluent fibroblasts cultured in serum-free medium, mimicking a non-inflammatory environment secreted a moderate constitutive level of fibronectin and tenascin-C detectable after 48 h by immunoblotting (Fig. 1). These control values were defined as 100% for further comparisons. Since we have shown previously that the addition of 5% FCS initiated a pro-inflammatory response in primary human lung fibroblasts (Goulet, Bihl, Gambazzi, Tamm, & Roth, 2007) we investigated the effect of FCS on fibronectin and tenascin-C synthesis. Stimulation with 5% FCS showed only a slight increase of fibronectin levels but did not reach significance compared to control ($p=0.06$) (Fig. 1A). In contrast, 5% FCS significantly increased tenascin-C levels by more than 2.5-fold compared to 0.3% albumin ($p<0.001$) (Fig. 1B). In order to investigate the effect of TGF-β_1 which can function either as a pro- or anti-inflammatory cytokine on inflammatory cells, we treated fibroblasts with TGF-β_1 under both experimental conditions. Addition of TGF-β_1 into serum-free medium significantly augmented fibronectin ($p<0.01$) and tenascin-C levels ($p<0.001$) compared to control. TGF-β_1 in the presence of 5% FCS significantly increased fibronectin levels by 3-fold compared to 0.3% albumin ($p<0.001$) and more than 2-fold compared to the presence of 5% FCS alone ($p<0.001$) (Fig. 1A). Since tenascin-C levels were already very high in the presence of 5% FCS, we did not detect a further increase by treatment with TGF-β_1 (Fig. 1B).

Fibronectin and tenascin-C are differently regulated by fluticasone and salmeterol in the presence or absence of 5% FCS and/or TGF-β_1

Next we evaluated by immunoblotting the effects of fluticasone and salmeterol, applied alone or in combination, in the presence or absence of 5% FCS on fbronectin and tenascin-C synthesis (Fig. 2). Under serum-free condition, fluticasone led to an increase in fibronectin expression compared to control ($p<0.01$), whereas salmeterol had no effect. Combined fluticasone and salmeterol increased fibronectin levels ($p<0.001$), similarly to fluticasone administration alone (Fig. 2A). Fluticasone had an opposite effect on tenascin-C levels, which were significantly decreased by 5-fold compared to control ($p<0.01$). Salmeterol alone did not modulate tenascin-C expression compared to control nor did it affect the decrease observed

with fluticasone in combination (Fig. 2B). Inflammatory conditions with 5% FCS stimulation did not influence the effects of both drugs on fibronectin and tenascin-C expression in comparison to the one observed in serum-deprived fibroblasts. Indeed, fluticasone augmented fibronectin levels ($p<0.01$), whereas it decreased tenascin-C levels secreted into the conditioned medium ($p<0.001$). Salmeterol administration either alone or in combination, did not show any effect on fibronectin and tenascin-C protein levels in the presence of 5% FCS (Fig. 2C, D). These effects of fluticasone and salmeterol on fibronectin and tenascin-C expression were not modulated by further stimulation with TGF-β_1 under both 0.3% albumin and 5% FCS (Fig. 3). We observed the same results when the primary human lung fibroblasts have been treated with other corticosteroids and/or LABAs such as budesonide, formoterol (AstraZeneca, Lund, Sweden) or dexamethasone (Calbiochem, Lucerne, Switzerland) (data not shown).

Modulation of fibronectin and tenascin-C mRNA expression by fluticasone and salmeterol

So far we investigated the effects of fluticasone and salmeterol alone or in combination on fibronectin and tenascin-C protein levels. In order to determine the drug's effect on fibronectin and tenascin-C transcript levels, we performed RT-PCR analysis on mRNA isolated from primary human lung fibroblasts cultured under both 0.3% albumin and 5% FCS. The agarose gels shown in Figure 4A confirm our immunoblot results. Expression of the transcript levels was analyzed by densitometry analysis and normalized to GAPDH and the relative gene expression from three cell lines is depicted in Figure 4B. Treatment with fluticasone under any experimental condition is associated with a significant increase in fibronectin mRNA accumulation ($p<0.05$), and with a significant reduction in tenascin-C transcript levels ($p<0.05$). On the other hand, administration of salmeterol applied alone or in combination with fluticasone did neither modulate fibronectin nor tenascin-C mRNA levels compared to controls (Fig. 4B).

To test the modulatory effects of fluticasone and salmeterol on the major splice variants of fibronectin and tenascin-C, we specifically designed primers which flank the sequences that are subjected to alternative splicing in the human fibronectin and tenascin-C cDNAs (Table 1, Fig. 5B top). Treatment with fluticasone in either 0.3% albumin or 5% FCS, but in the

absence of TGF-β_1 was associated with a down-regulation of the EDA isoform (EDA+) compared to non-treated cells, whereas salmeterol did not influence EDA-containing transcripts (Fig. 5A). This regulation of the EDA isoform is the exact opposite to total levels of fibronectin transcripts. However, in the presence of TGF-β_1, the changes in EDA+ mRNA expression are masked. The EDB+ isoform is regulated in the same manner as the EDA+ transcripts. However, the decrease of EDB+ by fluticasone was also observed in the presence of TGF-β_1 (Fig. 5A). In contrast to fibronectin, treatment with fluticasone and/or salmeterol did not differently modulate tenascin-C isoform expression generated by alternative splicing compared to total tenascin-C transcript levels (Fig. 5B, bottom).

Taken together, we show here that fluticasone not only regulate tenascin-C and fibronectin protein and mRNA levels in an opposite manner, but also has an opposite effect on total fibronectin versus EDA/EDB containing fibronectin isoform expression. This is in contrast to salmeterol which has no effect on fibronectin, fibronectin variants or tenascin-C levels under any conditions.

DISCUSSION

Asthma and COPD are among the world's most prevalent diseases. It is estimated that as many as 300 million people of all ages and ethnic origins suffer from asthma. Mortality due to asthma has now reached over 180 000 cases annually and its incidence is rising by 50% every decade, especially among children (National Institutes of Health, 2004). Furthermore, asthma is one of the leading causes of hospitalization in young children in industrialized countries. Thus, asthma represents a major human and economic burden for the society (Gergen, 2001).

Asthma is more or less effectively controlled in most patients with maintenance treatment. Control may be achieved with an inhaled corticosteroid or with a more effective approach of a combination inhaler containing a corticosteroid and a LABA. However, their exact mechanism of action and direct influence on airway remodeling are not well understood so far.

In our report, we studied the effect of fluticasone and salmeterol on tissue remodeling, a prominent characteristic of asthma and COPD. More specifically, we analyzed total fibronectin, tenascin-C, fibronectin- and tenascin-C isoform secretion by primary human lung fibroblasts. Although a recent paper using murine NIH-3T3 fibroblasts showed that fluticasone propionate inhibits fibronectin protein as well as mRNA levels (Tomic, Lassiter, Ritzenthaler, Rivera, & Roman, 2005), we could not confirm these results in our study. Rather, we show that fluticasone as well as other corticosteroids such as budesonide, dexamethasone (data not shown) increase fibronectin protein and mRNA levels. This is in agreement with other reports showing that corticosteroid treatment is associated with fibronectin accumulation (Furcht, Mosher, Wendelschafer-Crabb, Woodbridge, & Foidart, 1979; Oliver, Newby, Furcht, & Bourgeois, 1983; Talts, Weller, Timpl, Ekblom, & Ekblom, 1995; Pataki, Madarasz, & Kurucz, 2006). Down-regulation of tenascin-C by corticosteroids seems to be common to many cell types as has been shown in several reports (Ekblom, Fassler, Tomasini-Johansson, Nilsson, & Ekblom, 1993; Talts, Weller, Timpl, Ekblom, & Ekblom, 1995; Fassler, Sasaki, Timpl, Chu, & Werner, 1996; Laitinen et al., 1997; Zhao, 1999). Interestingly, our results show that even the presence of TGF-β_1, an inducer of tenascin-C production (Pearson, Pearson, Shibahara, Hofsteenge, & Chiquet-Ehrismann, 1988; Sakai, Kawakatsu, Ohta, & Saito, 1994) did not counteract the reducing effect of

fluticasone on tenascin-C levels. Thus, corticosteroids can down-regulate tenascin-C synthesis even in the presence of local stimulatory growth factors. On the other hand, salmeterol did not affect fibronectin or tenascin-C levels compared to control, nor did it attenuate the effect of fluticasone when applied in combination. This is the opposite of what we found previously for total ECM and collagen deposition by lung fibroblasts which was reduced by LABA (Goulet, Bihl, Gambazzi, Tamm, & Roth, 2007).

Many reports have shown that applying the current asthma therapy has an impact on airway remodeling. Here, we show that two ECM proteins, fibronectin and tenascin-C, are influenced in the opposite way by corticosteroids applied alone or in combination. This might have functional consequences, since it was shown that fibronectin and tenascin-C can directly interact with each other and that presence of tenascin-C results in abolishment of fibronectin-mediated cell spreading (Chiquet-Ehrismann, Kalla, Pearson, Beck, & Chiquet, 1988; Huang, Chiquet-Ehrismann, Moyano, Garcia-Pardo, & Orend, 2001). At least in tumor cells, fibronectin and tenascin-C interaction is associated with reduced cell adhesion and enhanced cell proliferation (Huang, Chiquet-Ehrismann, Moyano, Garcia-Pardo, & Orend, 2001; Ruiz et al., 2004). This might also be true in other pathological conditions such as asthma or COPD. Thus, indirect effects of corticosteroids may involve the modulation of cell-cell or cell-ECM interactions. In the lungs of asthmatic patients, corticosteroid's opposite regulation of tenascin-C and fibronectin might result in less interaction between the two proteins, which could decrease cell proliferation, but strengthen cell adhesion, thus normalizing the environment.

Alternative splicing in fibronectin which is dynamically regulated, both temporally and spatially, is a very prominent feature of this complex ECM protein (Ffrench-Constant & Hynes, 1989). Alternative splicing is clearly adding another level of complexity to the many functions ascribed to fibronectin. EDA- and EDB-fibronectin expressions are high during embryonic development and basically absent in the adult tissues. However, these extra domains get included into fibronectin during pathological conditions such as tumorigenesis, inflammation or tissue remodeling (Ffrench-Constant, Van de Water, Dvorak, & Hynes, 1989). More recently, the discrimination between EDA- and EDB-fibronectin expression in pathological conditions and normal tissues was used to specifically target or image sites of

tumor angiogenesis or chronic skin inflammation using EDA or EDB-specific human monoclonal antibodies (Rybak, Roesli, Kaspar, Villa, & Neri, 2007; Trachsel, Kaspar, Bootz, Detmar, & Neri, 2007). Therefore, EDA- and EDB-fibronectin have the potential to be used as marker for different pathological conditions such as a vascular marker of solid tumors and metastases, a marker for chronic skin inflammation or a marker for atherosclerotic plaques (Rybak, Roesli, Kaspar, Villa, & Neri, 2007; Trachsel, Kaspar, Bootz, Detmar, & Neri, 2007; von Lukowicz et al., 2007). In our report we show that treatment of primary human lung fibroblasts with fluticasone resulted in a down-regulation of EDA- and EDB-fibronectin mRNA, while transcript levels of total fibronectin were increased. Thus, EDA- and EDB-fibronectin show the same response to corticosteroid treatment as tenascin-C, which is the opposite to total fibronectin mRNA levels. This represents a further example of the co-expression of EDA- and EDB-containing fibronectin variants with tenascin-C expression, as has been observed during embryogenesis and in cancer. It implies that factors regulating alternative splicing of fibronectin seem to be co-regulated with tenascin-C expression. Tenascin-C is also subject to alternative splicing (Fig. 1-3). However, we do not have any evidence that tenascin-C splice variants are regulated in an opposite manner by corticosteroids and LABA compared to total tenascin-C levels as observed for fibronectin splice variants.

In summary, we analyzed the effect of corticosteroids and LABA on the expression levels of tenascin-C and fibronectin in primary human lung fibroblasts. Corticosteroid treatment negatively regulated expression of tenascin-C. The effect of corticosteroids on fibronectin was exactly the opposite: corticosteroids increased fibronectin deposition. LABA neither showed a prominent effect on tenascin-C or fibronectin expression nor modulated the effect of corticosteroids. However, since we have previously shown that total ECM and collagen deposition were decreased by LABA (Goulet, Bihl, Gambazzi, Tamm, & Roth, 2007), it becomes clear that the relative prelevance of specific ECM-proteins such as tenascin-C, fibronectin and its variants are affected by treatment with corticosteroids as well as LABA.

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FIGURE CAPTIONS

Fig. 1. Increased fibronectin and tenascin-C expression by 5% FCS and TGF-β1.

Confluent serum-deprived primary human lung fibroblasts were stimulated with 5% FCS and/or TGF-β1 (2.5 ng/ml) for 48 h. Supernatants were collected and fibronectin and tenascin-C were detected by immunoblotting. A representative immunoblot is shown for fibronectin (A, top) and for tenascin-C (B, top). Quantification of the immunoblots of three different primary cell lines is shown for fibronectin (A, bottom) and tenascin-C (B, bottom). Bars represent means ± SEM expressed as % of control, where 0.3% albumin was defined as 100%. *p<0.05, **p<0.01 and ***p<0.001. The p-values correspond to the comparisons to 0.3% albumin.

Fig. 2. Fibronectin and tenascin-C regulation by fluticasone and salmeterol.

Confluent serum-deprived primary human lung fibroblasts were treated with fluticasone (10^{-7} M) and/or salmeterol (10^{-7} M) in 0.3% albumin and 5% FCS for 48 h. Supernatants were subjected to immunoblot analysis for fibronectin and tenascin-C. Amido black staining is included to show equal protein concentrations. A representative immunoblot is shown for fibronectin (A, C, top) and for tenascin-C (B, D, top). The same membrane was used for (A) and (B) as well as for (C) and (D). Quantification of the immunoblots of three different primary cell lines is shown for fibronectin (A, C, bottom) and tenascin-C (B, D, bottom). Bars represent means ± SEM expressed as % of control, where 0.3% albumin and 5% FCS were defined as 100%. *p<0.05, **p<0.01 and ***p<0.001. The p-values correspond to the comparisons to control.

Fig. 3. Fibronectin and tenascin-C regulation by fluticasone and salmeterol in the presence of TGF-β_1.

Confluent serum-deprived primary human lung fibroblasts were treated with fluticasone (10^{-7} M) and/or salmeterol (10^{-7} M) in the presence of TGF-β_1 (2.5 ng/ml) in both 0.3% albumin and 5% FCS for 48 h. Supernatants were subjected to immunoblot analysis for fibronectin and tenascin-C. Amido black staining is included to show equal protein concentrations. A representative immunoblot is shown for fibronectin (A, C, top) and for tenascin-C (B, D, top).

The same membrane was used for (A) and (B) as well as for (C) and (D). Quantification of the immunoblots of three different primary cell lines is shown for fibronectin (A, C, bottom) and tenascin-C (B, D, bottom). Bars represent means ± SEM expressed as % of control, where 0.3% albumin and 5% FCS were defined as 100%. *p<0.05, **p<0.01 and ***p<0.001. The p-values correspond to the comparisons to control.

Fig. 4. Modulation of fibronectin and tenascin-C mRNA expression by fluticasone and salmeterol.

Confluent serum-deprived primary human lung fibroblasts were treated with fluticasone and/or salmeterol (10^{-7} M) in the absence or presence of TGF-β_1 (2.5 ng/ml) under both 0.3% albumin and 5% FCS. Total RNA was extracted after 48 h and cDNA was synthesized. Representative PCR gels are shown in (A) and quantification of three cell lines is shown in (B). Bars represent means ± SEM expressed as fold induction compared to control and are normalized to GAPDH. *p<0.05, **p<0.01 and ***p<0.001. The p-values correspond to the comparisons to 0.3% control.

Fig. 5. Modulation of fibronectin and tenascin-C isoform mRNA expression by fluticasone and salmeterol.

Confluent serum-deprived primary human lung fibroblasts were treated with fluticasone and/or salmeterol (10^{-7} M) in the absence or presence of TGF-β_1 (2.5 ng/ml) under both 0.3% albumin and 5% FCS. Total RNA was extracted after 48 h and cDNA was synthesized. (A) Representative PCR gels of the major fibronectin splice variants, where EDA+ and EDB+ indicate the EDA-containing fibronectin, or the EDB-containing firbronectin isoform respectively are shown. (B) Schematic representation of the 15 fibronectin type III repeats (white boxes: constant repeats; grey boxes: repeats subject to alternative splicing) present in tenascin-C and the primers used to detect the tenascin-C isoforms is shown (top). Representative PCR gels of the tenascin-C splice variants are shown (bottom). Seven TN-C isforms are detectable corresponding to the inclusion of the seven fibronectin type III repeats (grey boxes, top), each containing 276 bp. The major isoforms present in the primary human lung fibroblasts are highlighted by asterisks (*, **) and they give rise to the 250 kDa and 180 kDa protein respectively.

Table 1: Sequences of primers used for RT-PCR

Primers	Sequences	Amplicon size(s)
GAPDH forw	5'-GAAGGTGAAGGTCGGAGTC-3'	229bp
GAPDH rev	5'-GAAGATGGTGATGGGATTTC-3'	
FN forw	5'-CGAACAACATGAGAGCACACC-3'	401bp
FN rev	5'-TCACTGTGACAGCAGGAGCATC-3'	
TN-C forw	5'-GACACAGATTCAGCCATCACCA-3'	152bp
TN-C rev	5'-GAGTGTTCGTGGCCCTTCCAGT-3'	
EDA forw	5'-TTCTTTCATTGGTCCGGTCTT-3'	EDA+: 496bp
EDA rev	5'-GCAGCCCACAGTGGAGTATGT-3'	EDA-: 226bp
EDB forw	5'-CCCCTACAAACGGCCAGCAGGG-3'	EDB+: 731bp
EDB rev	5'-GCCAGTTGGGGAATCAAGACCTG-3'	EDB-: 458bp
TN-C isoforms forw	5'-ACCGCTACCGCCTCAATTACA-3'	**331bp**/607bp/883bp/1159bp/
TN-C isoforms rev	5'-GGTTCCGTCCACAGTTACCA-3'	1435bp/1711bp/**1969bp**[1]

[1]: Seven TN-C isoforms are detectable which corresponds to the inclusion of seven FNIII extra-domains, each consisting of 276bp (see Fig. 5B). The major isforms present in the primary human lung fibroblasts are highlighted in bold.

Table 1

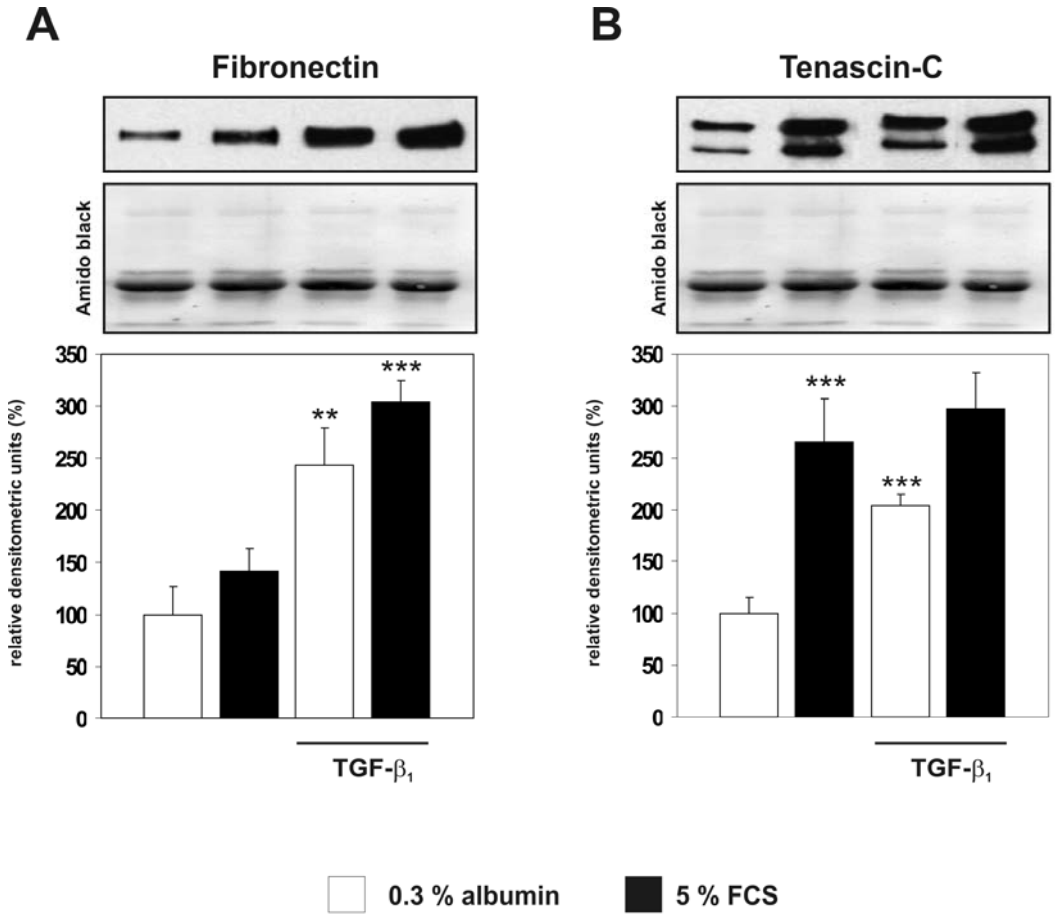

A
Fibronectin
Amido black
relative densitometric units (%)
350
300
250
200
150
100
50
0
**
***
TGF-β1
B
Tenascin-C
Amido black
relative densitometric units (%)
350
300
250
200
150
100
50
0
***
***
TGF-β1
0.3 % albumin
5 % FCS

Figure 1

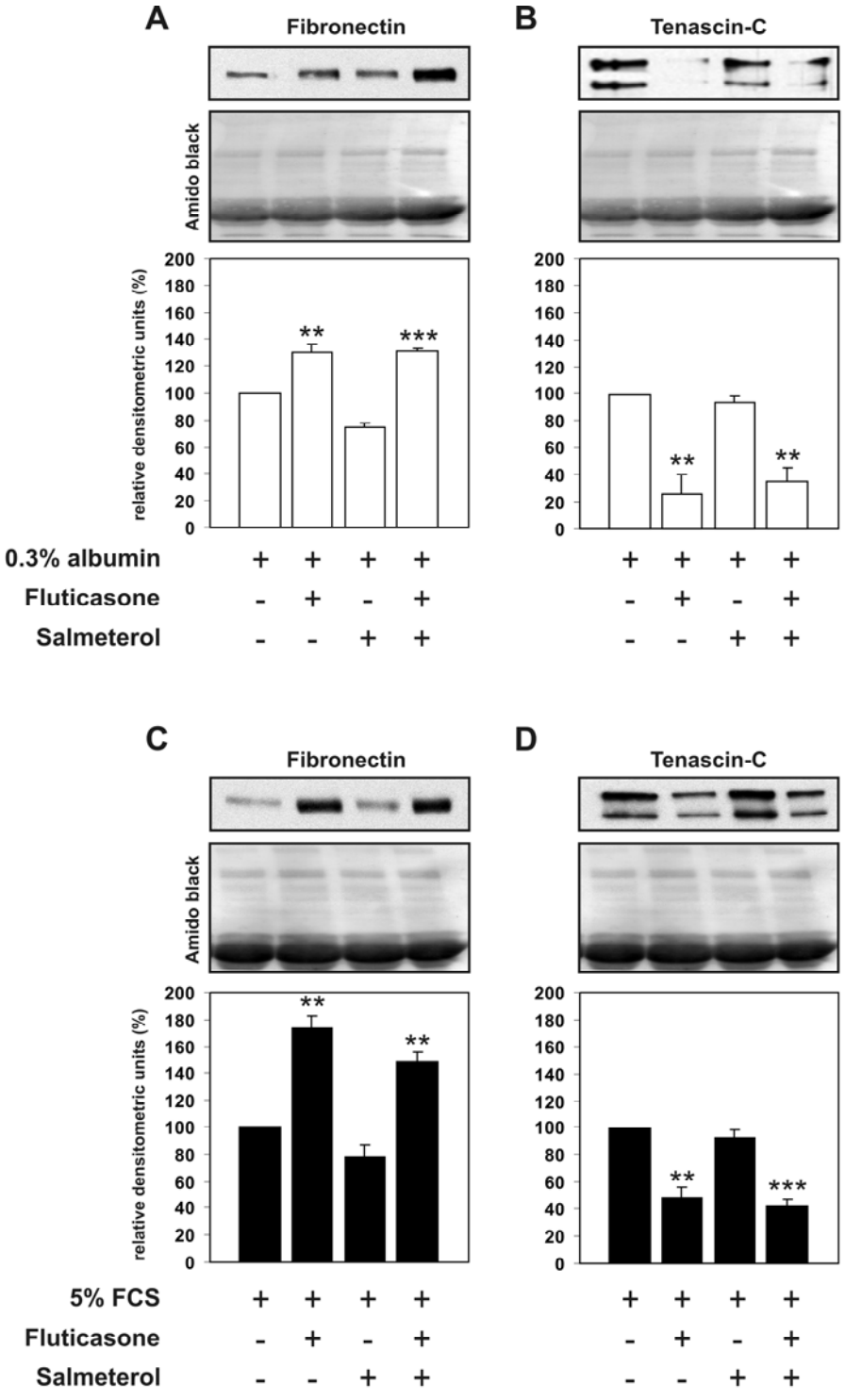

A
Fibronectin
B
Tenascin-C
Amido black
relative densitometric units (%)
200
180
160
140
120
100
80
60
40
20
0
**
***
**
**
0.3% albumin
Fluticasone
Salmeterol
+ + + +
- + - +
- - + +
C
Fibronectin
D
Tenascin-C
Amido black
relative densitometric units (%)
**
**
**
***
5% FCS
Fluticasone
Salmeterol
+ + + +
- + - +
- - + +

Figure 2

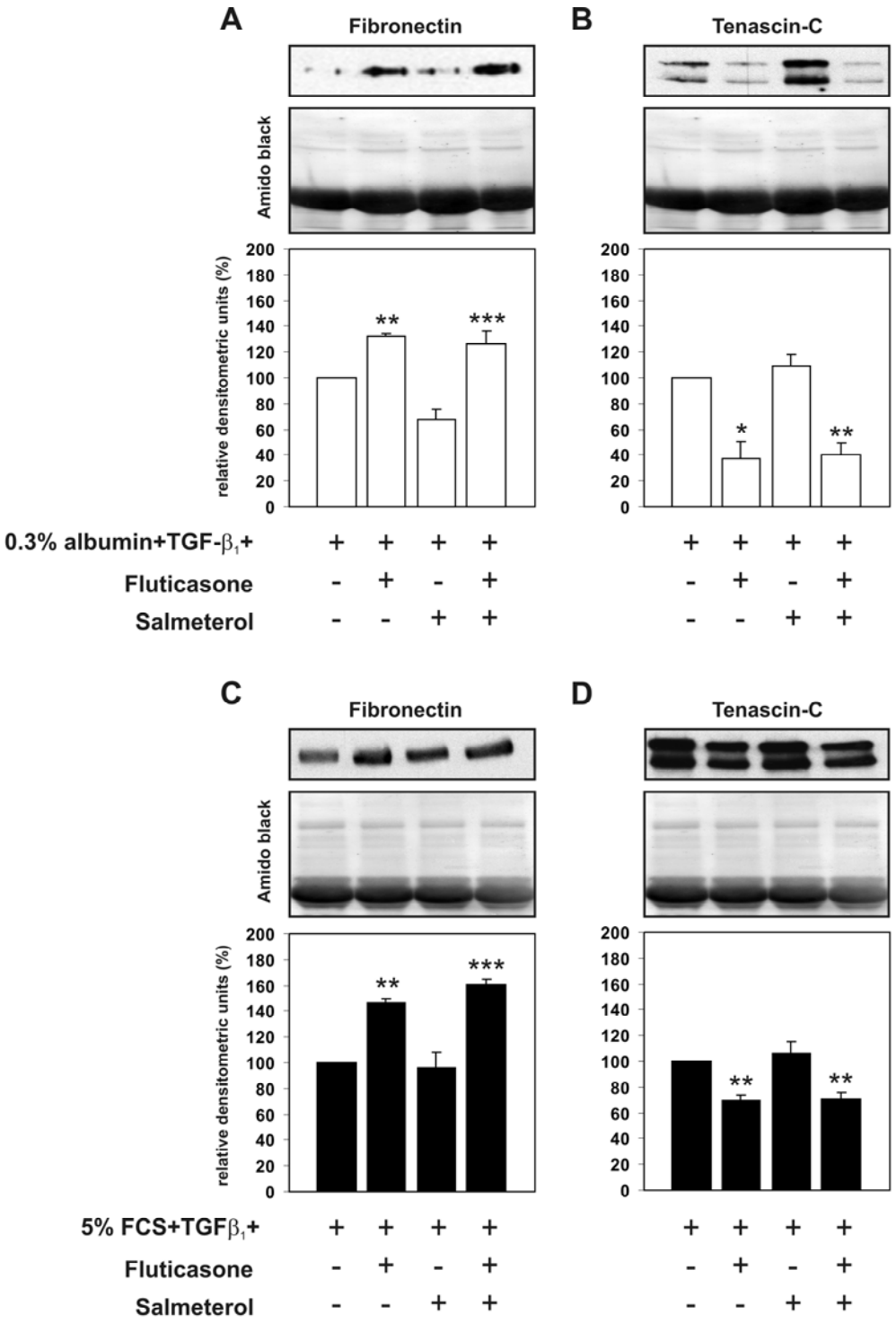

A
Fibronectin
B
Tenascin-C
Amido black
relative densitometric units (%)
200
180
160
140
120
100
80
60
40
20
0
**
***
*
**
0.3% albumin+TGF-β1+
Fluticasone
Salmeterol
C
Fibronectin
D
Tenascin-C
Amido black
relative densitometric units (%)
**
***
**
**
5% FCS+TGFβ1+
Fluticasone
Salmeterol

Figure 3

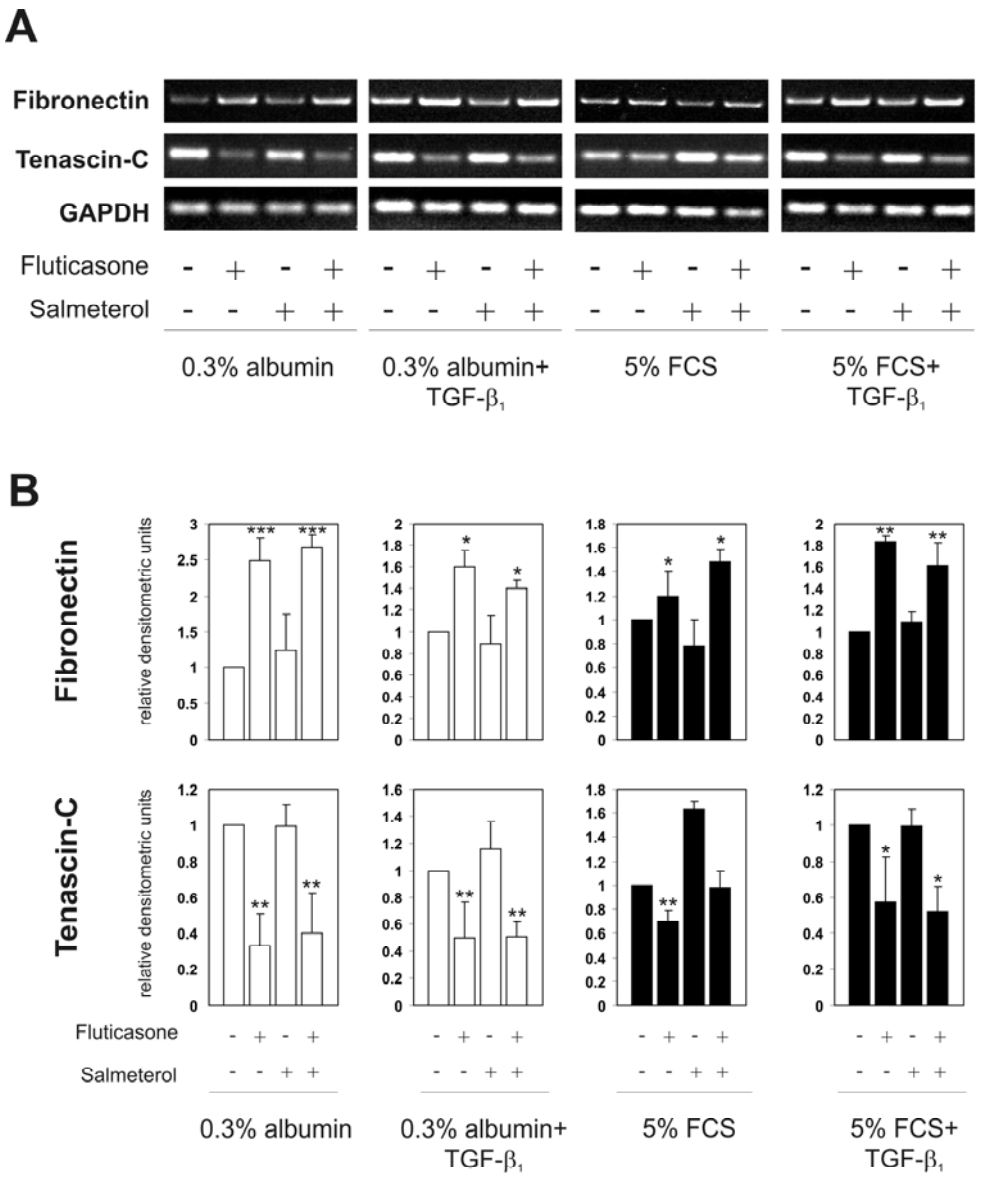

A
Fibronectin
Tenascin-C
GAPDH
Fluticasone
Salmeterol
0.3% albumin
0.3% albumin+ TGF-β1
5% FCS
5% FCS+ TGF-β1
B
Fibronectin
Tenascin-C
relative densitometric units
Fluticasone
Salmeterol
0.3% albumin
0.3% albumin+ TGF-β1
5% FCS
5% FCS+ TGF-β1

Figure 4

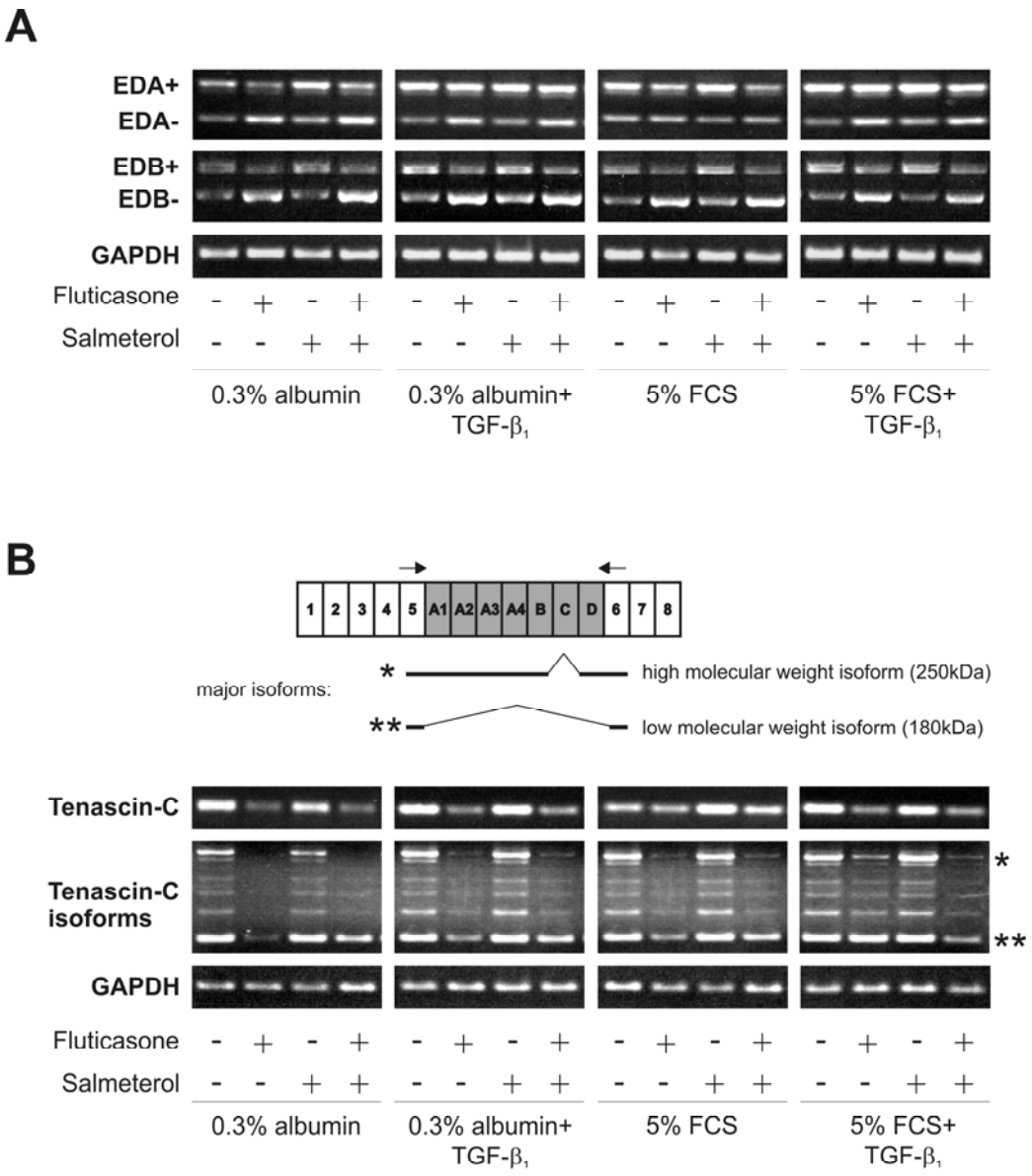

A
EDA+
EDA-
EDB+
EDB-
GAPDH
Fluticasone
Salmeterol
0.3% albumin
0.3% albumin+ TGF-β1
5% FCS
5% FCS+ TGF-β1
B
1 2 3 4 5 A1 A2 A3 A4 B C D 6 7 8
major isoforms:
high molecular weight isoform (250kDa)
low molecular weight isoform (180kDa)
Tenascin-C
Tenascin-C isoforms
GAPDH
Fluticasone
Salmeterol
0.3% albumin
0.3% albumin+ TGF-β1
5% FCS
5% FCS+ TGF-β1

Figure 5

III.3 Unpublished Results

III.3.1 Tenascin-W detection in human serum

In collaboration with **Curzio Rüegg**, Division of Experimental Oncology, Centre Pluridisciplinaire d'Oncologie, and Swiss Institute for Experimental Cancer Research (ISREC), NCCR Molecular Oncology, Epalinges, Switzerland.

After having established a sensitive sandwich-ELISA assay, we measured TN-W serum levels in 16 non-metastatic (at time of diagnosis) breast cancer patients, in 17 non-metastatic colon cancer patients and in 25 healthy volunteers. Serum before and 6 weeks after surgical tumor resection was available for each patient. The mean TN-W value of control sera was 0.389 +/- 0.14 mg/l (Fig.III.1A). In sera of breast cancer patients before surgery we measured an elevated mean TN-W level of 0.682 +/- 0.44 mg/l which corresponds to a 1.75-fold increase compared to control. However, the mean TN-W level in the same breast cancer patients six weeks after surgical tumor removals did not significantly decrease (0.631 +/- 0.45 mg/l) (Fig.III.1B). Details about the serum TN-W levels in breast cancer patients are given in table III.I.

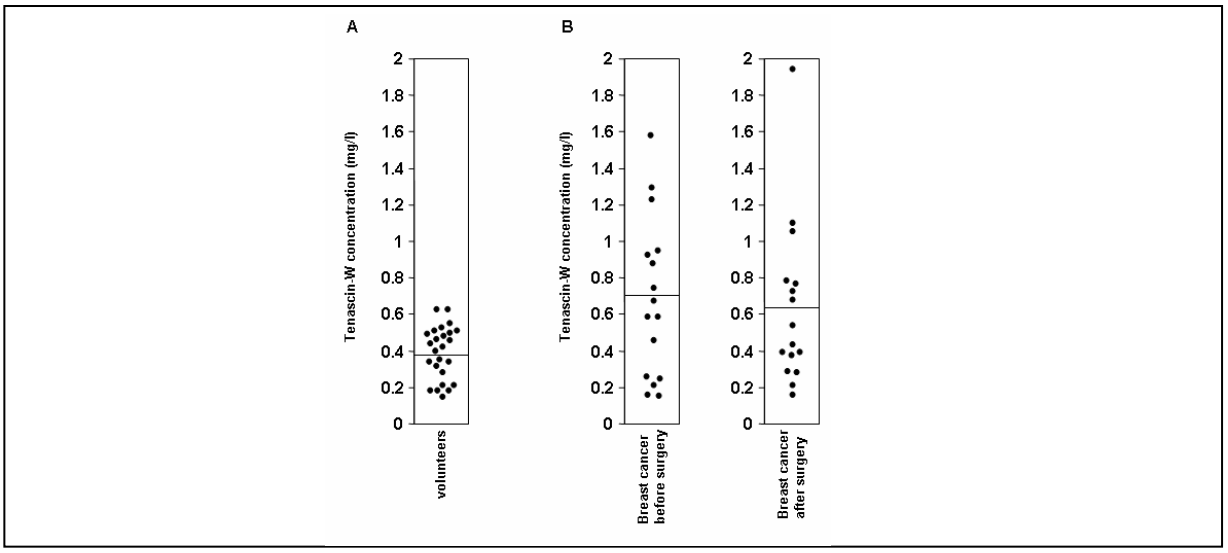


Figure III.1: Tenascin-W in sera of breast cancer patients

Histograms comparing the serum TN-W levels in healthy volunteers (n=25), breast cancer patients (n=16) before and after tumor removal. Mean TN-W levels: healthy individuals: 0.389 +/- 0.14 mg/l; breast cancer before surgery: 0.682 +/- 0.44 mg/l; breast cancer after surgery: 0.631 +/- 0.45 mg/l.

Table III.I: Tenascin-W levels in breast cancer serum

Patient	TNW (mg/l) before	TNW (mg/l) after
18	0.243	0.389
19	0.150	0.280
20	0.211	0.211
21	0.157	0.156
22	0.672	0.726
23	0.582	0.393
24	0.582	0.372
25	0.259	0.431
31	0.949	0.676
33	0.459	0.286
34	0.923	0.765
36	1.580	1.940
37	1.290	1.050
38	0.745	0.540
40	0.880	0.785
42	1.230	1.100
average	0.682 +/- 0.44	0.631 +/- 0.45

TNW: protein levels in serum deduced by sandwich-ELISA

Serum TN-W concentrations in non-metastatic colon cancer patients were significantly elevated compared to those in healthy volunteers ($p<0.005$) with a mean level of 0.794 +/- 0.38 mg/l (Fig.III.2A). This corresponds to a 2.04-fold increase in comparison to healthy individuals. Furthermore, we observed a reduction of the mean TN-W level in sera 6 weeks after tumor removal compared to the levels before (Fig.III.2A). However, not every patient followed this tendency. For some patients we monitored even increased TN-W levels after surgical tumor resection (Table III.II).

Since TN-C was shown to be elevated but scattered over a wide range in serum of patients suffering from different tumors [104-106, 143-146], we also monitored TN-C serum levels in the same cohort of colon cancer patients. We wanted to test whether TN-W might have a higher sensitivity for cancer detection than TN-C. In the few control samples that were available (n=13), we measured a mean TN-C level of 0.775 +/- 0.25 mg/l (Fig.III.2B), which is approximately twice as high as the control level of TN-W and which nicely fits to the literature [147]. The colon cancer patients showed elevated TN-C levels (mean: 0.98 +/- 0.26 mg/l) which is in agreement with the literature [144-146]. However, the increase (1.26-fold) was not as pronounced as that of TN-W. Mean concentration in serum after tumor resection only slightly decreased (0.902 +/- 0.23 mg/l), but did not reach the control value (Fig.III.2B). Similar to TN-W, some patient had even higher TN-C levels after tumor removal (Table III.II).

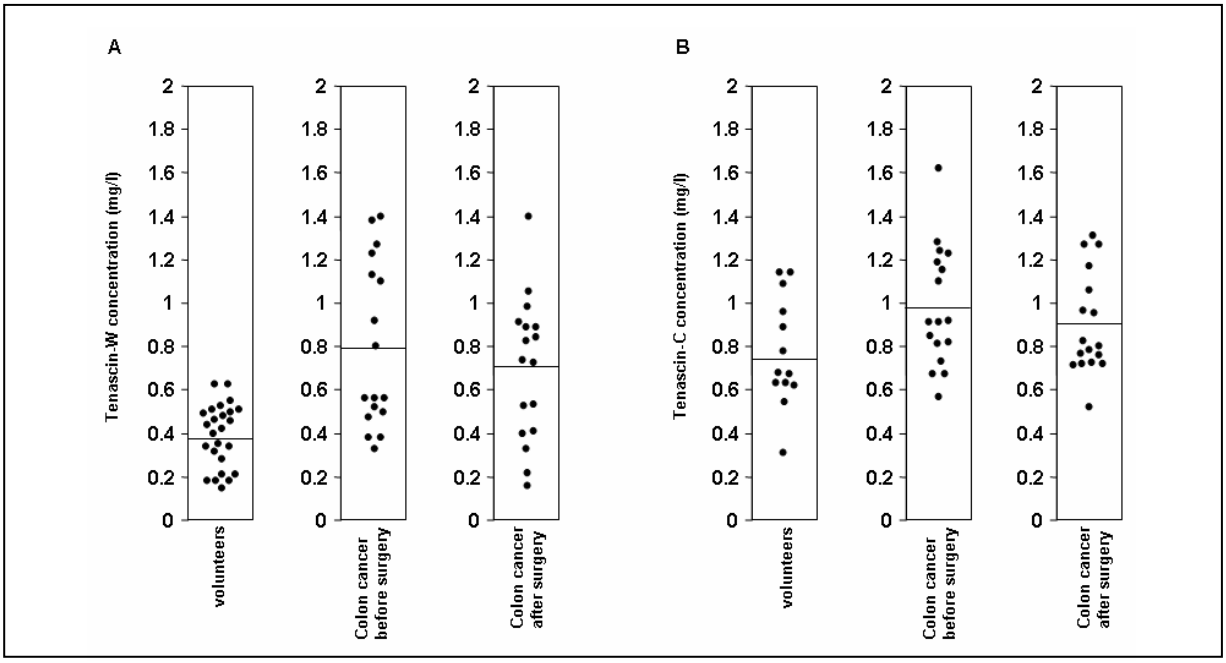


Figure III.2: Comparison between serum tenascin-W and tenascin-C levels in colon cancer patients
(A) Histograms of TN-W levels in colon cancer show a prominent increase ($p<0.05$) compared to them in volunteers. After tumor removal, TN-W levels slightly decreased ($p=0.348$). **(B)** Same tendency is true for TN-C however the increase in the colon cancer samples is more moderate.

Table III.II: Tenascin-W and tenascin-C levels in colon cancer serum before and 6 weeks after tumor removal

Patient	TNW (mg/l) before	TNW (mg/l) after	TNC (mg/l) before	TNC (mg/l) after
27	0.380	0.160	0.760	0.776
28	0.330	0.410	0.810	0.758
29	0.382	0.218	1.080	0.764
31	0.949	0.676	n.d.	n.d.
32	0.560	0.329	0.674	0.523
35	1.100	0.892	0.920	0.722
39	0.800	0.728	0.848	0.724
41	1.230	1.400	0.984	0.952
49	0.500	0.397	0.674	0.785
50	0.473	0.531	0.571	0.826
51	1.400	0.982	1.150	0.799
52	1.130	0.890	0.733	0.715
53	1.380	1.050	1.240	0.967
54	0.920	0.842	1.090	1.170
55	1.270	0.735	0.908	1.270
56	0.560	0.824	0.915	1.060
57	0.563	0.527	1.620	1.270
58	0.520	0.914	1.280	1.310
average	0.794 +/- 0.38	0.696 +/- 0.33	0.98 +/- 0.26	0.902 +/- 0.23

TNW, TNC: protein levels in serum deduced by sandwich-ELISA

Immunoprecipitation (IP) followed by immunoblotting of three different serum samples (control, colon cancer, and breast cancer) resulted in the detection of a single TN-W-specific band, corresponding to the size of the purified full-length TN-W (Fig.III.3). This indicated that we were measuring the intact mature TN-W protein in serum. Furthermore, we could confirm the tendency of TN-W towards higher expression in serum of colon cancer patients compared to control and breast cancer serum. Densitometric analysis (Fig.III.3, bottom) revealed a 1.9-fold increase in TN-W concentration in the colon cancer sample which nicely fits to the monitored increase by our sandwich-ELISA.

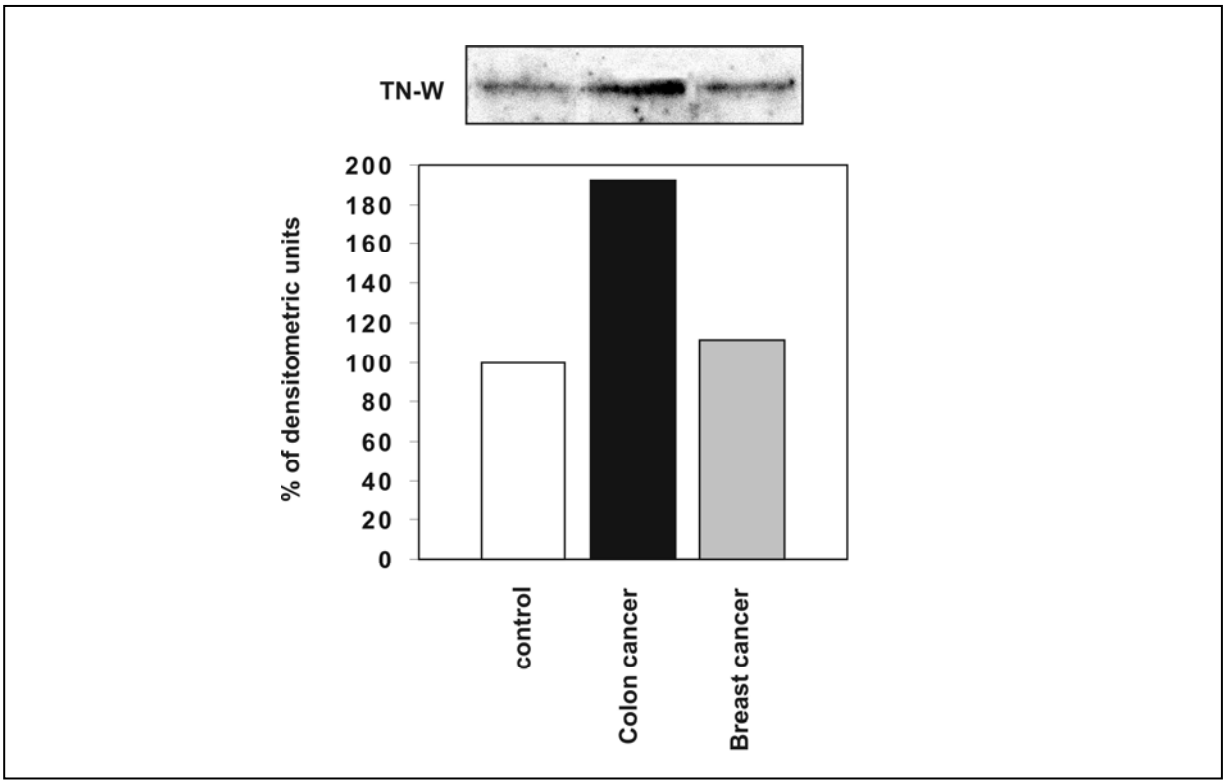


Figure III.3: Tenascin-W detection in serum by IP/western blot analysis

IP followed by western blot analysis reveals increased TN-W levels in colon cancer serum compared to the levels in control and breast cancer serum. Shown are the immunoblot (top) and the densitometric analysis (bottom).

In a last set of experiments with respect to serum tenascin levels, we analyzed TN-W and TN-C levels in serum from five melanoma patients. These patients have been treated with very high doses of TNFα and mephalan, a combination that was administered through isolated limb perfusion (ILP) [148-150]. Since TN-W was shown to be induced by TNFα [151], we were interested to measure serum TN-W levels in these patients at different time-points after ILP treatment (Table III.III).

Table III.III: TNW and TNC in sera of melanoma patients treated with ILP

Patient 1

hrs	TNW (mg/l)	TNC (mg/l)
0	0.84	1.07
2	0.3	4.05
8	0.45	4.27
24	0.38	7.71
48	1.45	4.63
72	1.04	5.7
96	1.08	8.13

Patient 2

hrs	TNW (mg/l)	TNC (mg/l)
0	1.4	1.14
2	1.37	1.3
8	0.97	1.23
24	0.92	12.52
48	2.2	13.47
72	2.2	7.64
96	1.7	7.04
120	2.3	7.33

Patient 3

hrs	TNW (mg/l)	TNC (mg/l)
0	0.654	1.46
2	0.29	1.2
8	1.06	2.31
24	0.999	9.38
72	1.08	2.25

Patient 4

hrs	TNW (mg/l)	TNC (mg/l)
2	1.05	0.34
8	0.359	0.66
48	0.194	4.77
72	0.687	3.88
96	1.51	7.06

Patient 5

hrs	TNW (mg/l)	TNC (mg/l)
0	2.85	0.17
2	2.31	0.4
8	1.76	0.48
24	1.73	1.74
72	2.51	1.64

TNW, TNC: protein levels in serum deduced by sandwich-ELISA

In all five melanoma patients we could detect prominently elevated serum TN-W as well as TN-C levels compared to that in healthy volunteers (Fig.III.2). Furthermore, all patients showed high TN-W levels at the early time-points (0, 2 hrs), which then transiently decreased, but finally increased again at later time-points after ILP. Except for patient 5 which showed highest TN-W concentration at time-point 0 hrs (patterned bar in Fig.III.4), all other patients displayed highest values for TN-W at late time-points. In contrast to TN-W, TN-C levels

mostly showed a steady increase with time reaching a maximum at late time-points. In general, we could not find any simple correlation between TN-C and TN-W levels in these patients, but observed a different response of the two tenascins to this ILP treatment (Fig.III.4).

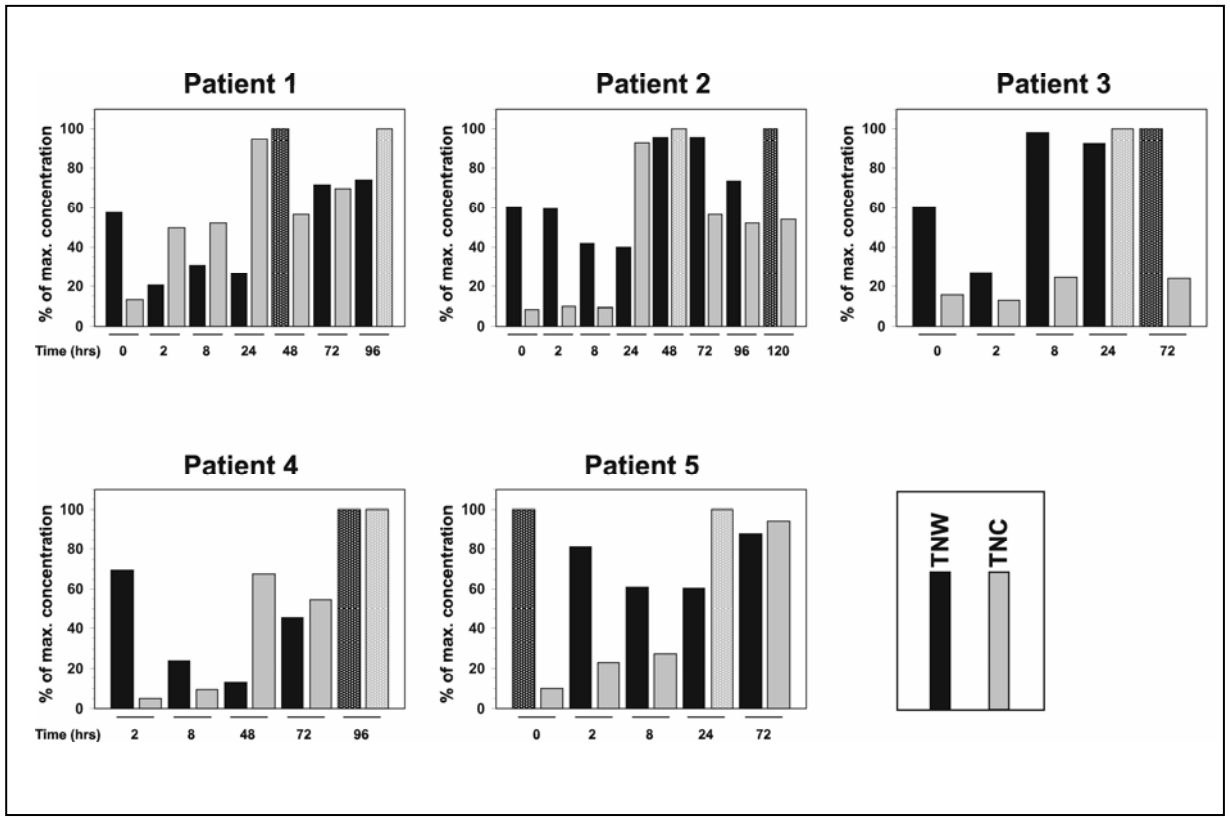


Figure III.4: Detection of tenascin-W and tenascin-W in ILP-treated patients

Patients were treated with high doses of TNFα in combination with Mephalan and their serum was collected at the indicated time points after ILP treatment and later analyzed by sandwich-ELISA. TN-W and TN-C show a different response to this treatment. Generally, TN-C levels are increasing with time, whereas TN-W levels are high at the beginning, followed by a decrease, but finally increase again. Highest tenascin concentration (patterend bar) was set to 100 % and all other concentrations are displayed relative to this maximal value.

III.3.2 Tenascin-W expression in cell cultures and tissues

III.3.2.1 Cell culture studies

Since no studies existed about human TN-W, we decided to analyze endogenous TN-W expression in human cell lines. Under standard cell culture conditions TN-W expression was detected in mouse embryonic fibroblasts (MEFs) only [151]. Its expression could be induced by bone morphogenetic protein 2 (BMP2) in C2C12, 4T1 cells and MEFs, and by TNFα in MEFs [56, 151]. These results together with the stromal staining patterns obtained by immunohistochemistry suggested stromal fibroblasts as cellular source for TN-W. Therefore we analyzed endogenous TN-W and TN-C expression in three different human fibroblast lines: Detroit 551 (skin), MRC5 (lung), and IMR90 (lung). We cultured them to a confluency of about 70 % in normal culture medium, switched them to starvation medium (1 % FCS) and added the indicated growth factors for another 72 hours. 500 µl of the cell supernatants of each condition was then subjected to IP followed by immunoblot analysis. As a positive control, we added 10 ng of purified TN-W to 500 µl medium. Immunoblots revealed that none of the fibroblasts under any condition expressed TN-W at detectable levels (Fig.III.5A). In contrast to TN-W, TN-C was expressed endogenously in all three fibroblasts (Fig.III.5B, PBS sample). Addition of growth factors was able to induce TN-C expression to different extents as indicated by the densitometric analyses (Fig.III.5B, bottom). There was a cell type-specific response to the stimuli. Nevertheless, in all three cell lines, TGFβ and TNFα were the most potent inducers for TN-C expression.

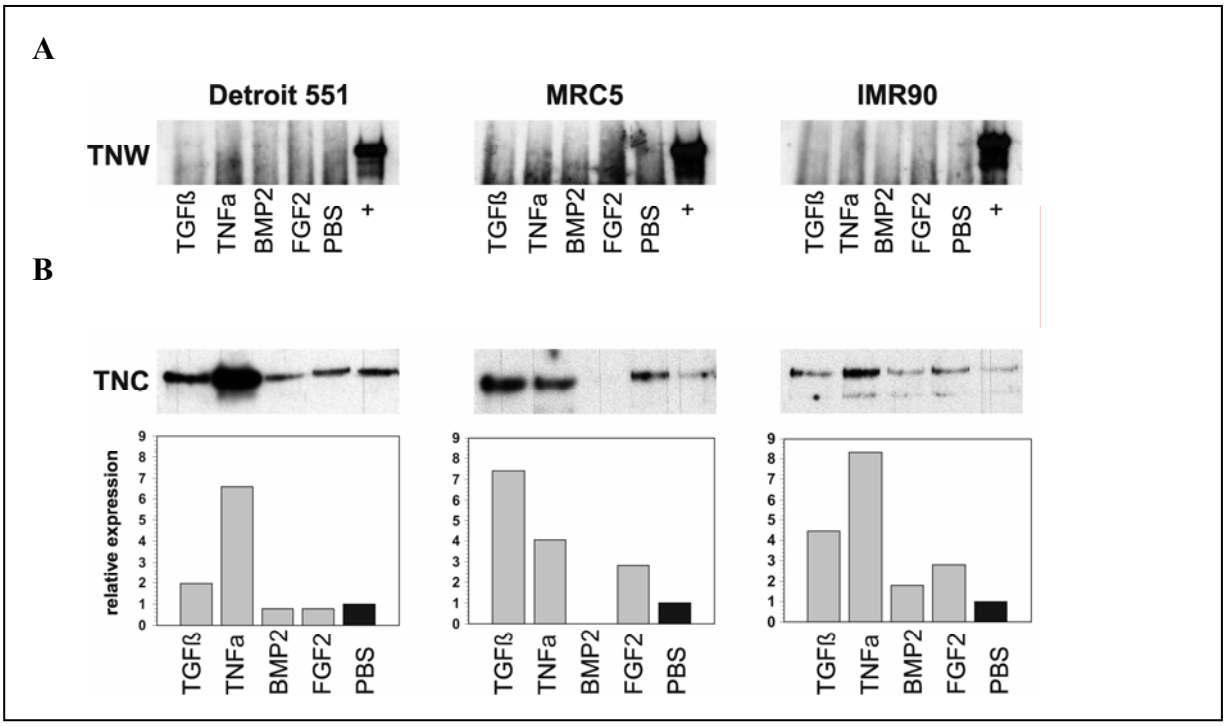


Figure III.5: Endogenous tenascin-W and tenascin-C expression in fibroblasts

Three different human fibroblasts lines were treated with different growth factors and their supernatants were analyzed for TN-W **(A)** and TN-C **(B)** presence by immunoblotting. TN-C blots were further quantified by densitometric analyses **(B, bottom)**. Concentrations of the growth factors used: TGFβ: 5 ng/ml; TNFα: 20 ng/ml; BMP2: 100 ng/ml; FGF2: 10 ng/ml.

Some reports indicate epithelial cancer cells as a potential source for TN-C production [152-154]. To test whether epithelial cancer cells could be a source for TN-W expression in tumors as well, we analyzed its production in different breast cancer cell lines (MDA-MB-435, MCF7, T47D). Breast cancer cells were chosen since we observed prominent TN-W presence in a different cohort of breast tumor patients by immunoblotting and immunohistochemistry. Cells were treated, and their supernatants analyzed as described before for fibroblasts. None of these cell lines tested expressed TN-W (Fig.III.6A, only the MDA-MB-435 cell line is shown) and only the MDA-MB-435 cell line produced endogenous TN-C (Fig.III.6B, PBS sample). TN-C expression could be further induced by addition of specific growth factors (Fig.III.6B). This was in contrast to TN-W (Fig.III.6A) whose expression could not be induced by any growth factor.

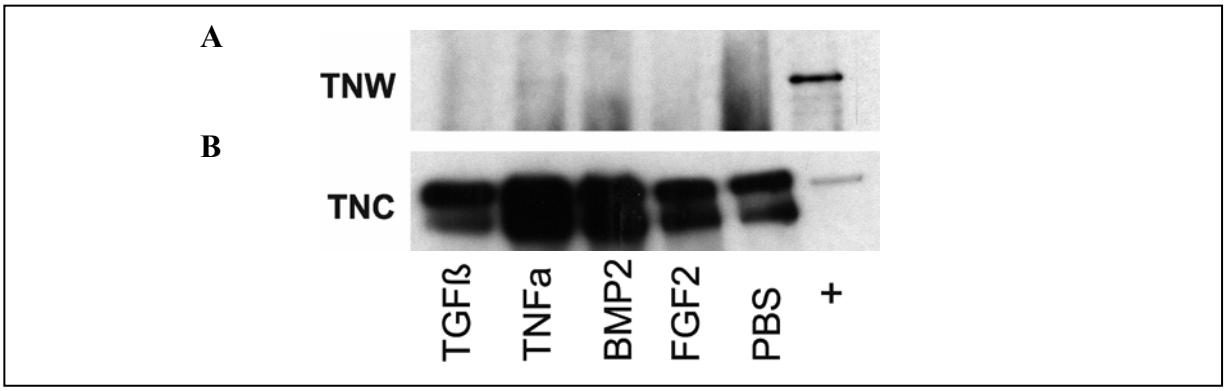


Figure III. 6: Endogenous tenascin-W and tenascin-C expression in breast cancer cells
MDA-MB-435 cells were treated with different growth factors and their supernatants were analyzed for TN-W **(A)** and TN-C **(B)** presence by immunoblotting.

Our own immunohistochemical studies as well as the literature revealed TN-W and TN-C expression in the activated tumor stroma [9, 151]. As described in the "Introduction", an activated stroma arises as a result of a complex cross-talk between epithelial and stromal cells [14, 16, 17, 20, 23]. Therefore, we tested whether we could induce TN-W expression by mimicking this *in vivo* situation *in vitro*. For that reason we performed co-culture assays. We cultured epithelial breast cancer cells together with fibroblasts for 72 hours, collected the conditioned medium and analyzed the medium by immunoblot analysis. However, we were not able to detect endogenous TN-W in these co-cultures either (data not shown).

To test several cell lines for the presence of TN-W mRNA, we performed RT PCR of 16 different cancer cell lines. With the primer pair 5'–AAATACAGAGGCACGGCAGG–3' / 5'– GTCCTTTCCAAGGCTCCCA – 3' a TN-W-specific fragment of 212 bp was amplified. In all cell lines tested a specific band of the expected size was detectable (Fig.III.7). This indicated that TN-W mRNA is present in all cell lines tested. Although mRNA was detectable, we were not able to detect the endogenous protein in the corresponding cell lines.

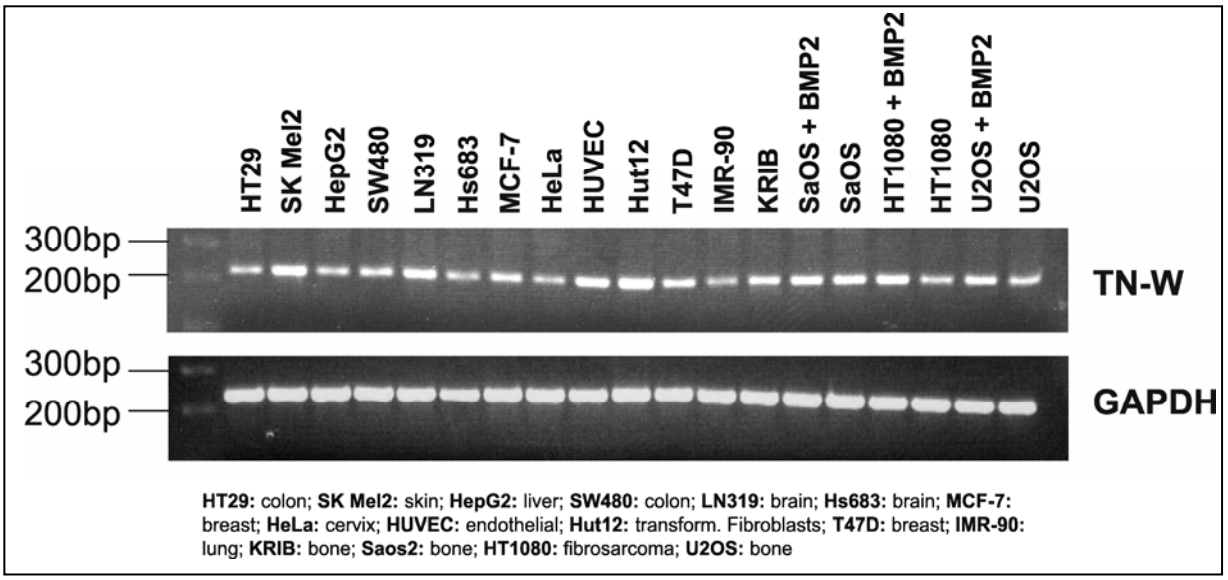


Figure III.7: Tenascin-W mRNA in different human cell lines

RT PCR of 16 different human cell lines reveals presence of TN-W mRNA in every cell line.

III.3.2.2 Tissue studies

In collaboration with **Luigi Terracciano**, Institute of Pathology, University of Basel, Basel, Switzerland

In order to analyze the endogenous TN-W expression in different adult tissues, we performed immunoblots of ten different normal organ extracts (Fig.III.8A). In the spleen and liver extracts a small amount of TN-W could be detected, whereas all other normal tissues tested were negative for TN-W. The same blots were probed with a monoclonal anti-TN-C antibody and revealed basal TN-C expression in all tissues, except for breast and heart. In pancreas, kidney and colon extracts only the low molecular-weight isoform of TN-C was produced (Fig.III.8A). To test whether TN-C shares its prominent expression in tumors with TN-W, we analyzed human extracts from pancreas (9 patients) and kidney (9 patients) tumors (Fig.III.8B). As expected, we detected TN-C expression in 7/9 (78 %) kidney cancer patients and 5/9 (56 %) pancreas cancer patients. Not only TN-C was highly up-regulated in these cancer tissue extracts, but also TN-W. 6/9 (67 %) kidney cancer samples and 7/9 (78 %) pancreas cancer samples were positive for TN-W. Similar to our previous findings in breast and colon cancer tissue extracts, there is great variance in the tenascin amounts produced in the different pancreas and kidney tumor extracts.

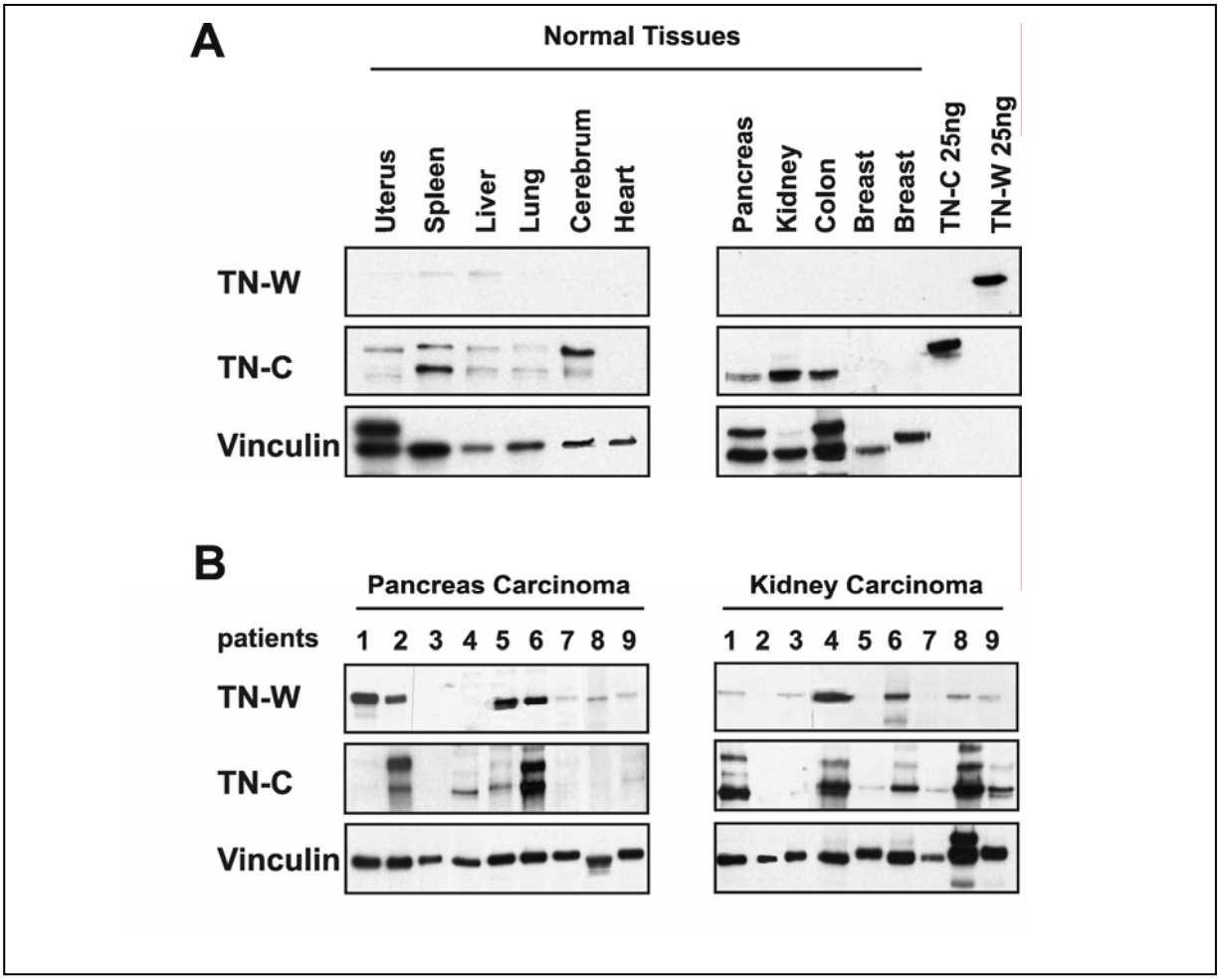


Figure III.8: Tenascin-W expression in normal and tumor tissues

(A) Western blot of ten normal adult tissues reveals low TN-W levels in spleen and liver, while all the other tissues tested are TN-W negative. In contrast, there is basal TN-C expression in the majority of the normal tissues tested. **(B)** In pancreas and kidney cancer patients, TN-W as well as TN-C are highly up-regulated.

III.3.3 Structure of the human *tenascin-W* gene and functional characterization of the promoter

Rapid amplification of cDNA ends (RACE) was performed to define and identify the 5' end of the human TN-W transcript. No band was detected on the agarose gel after the first PCR with the adapter specific outer primer (oligo dT-anchor primer) and the gene specific reverse primer hSP2 (Fig.III.8A; see appendix for primer sequences). However, after the second PCR with the adapter specific inner primer (PCR anchor primer) and the gene specific reverse primer hSP3 a band was detected on the gel (Fig.III.8A). Sequencing revealed that this fragment corresponded to the ATG-containing exon and a non-translated exon 1 of the human *TN-W* gene containing 78 bp (Fig.III.8B). Therefore, the most 5'- reaching cDNA was identified 128 bp from the translational start site present in exon 2. In the genomic DNA exon 1 is located 9448 bp upstream of exon 2 (Fig.III.9/10). Our experimental finding was confirmed by different database gene predictions (e.g.: www.ensemble.org).

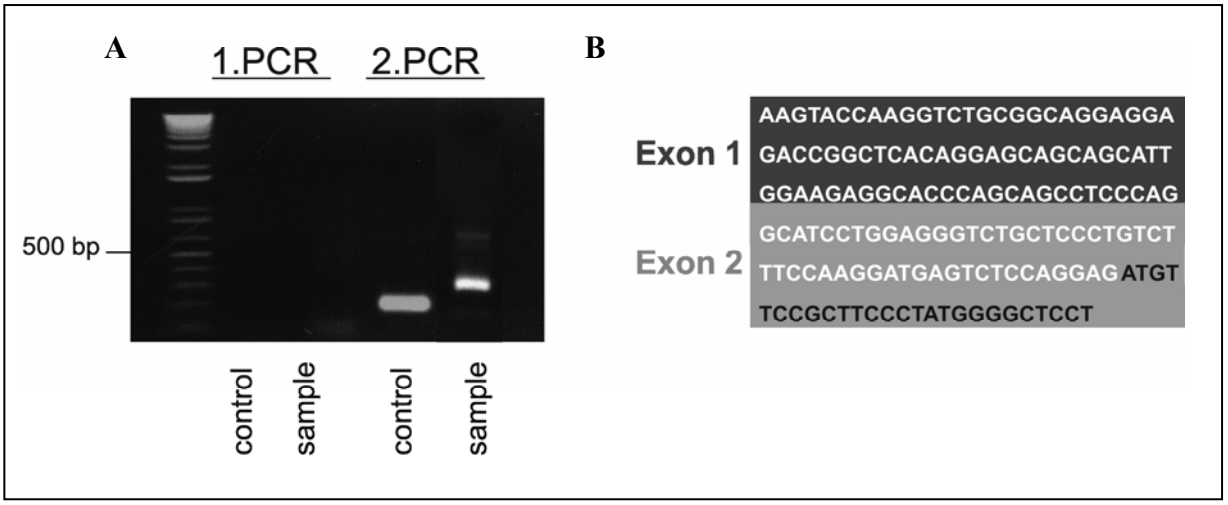
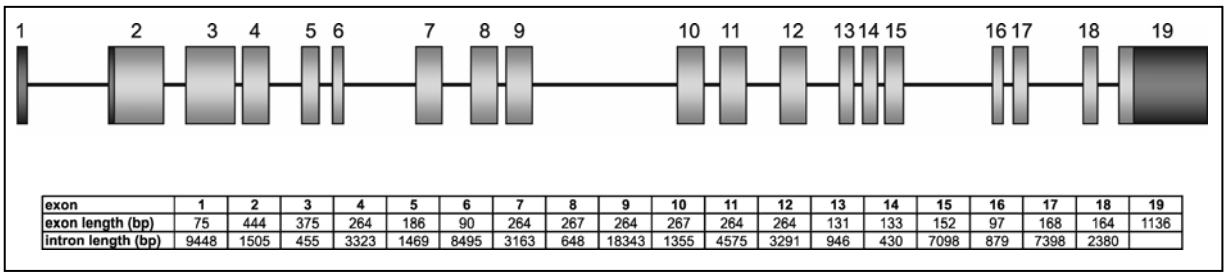


Figure III.8: 5' RACE

(A) 5'RACE PCR. 15 µl of the 25 µl PCR was separated on a 1.5% agarose gel. One microliter of the first PCR was used as template for the second PCR. Sample: 5'RACE probe. **(B)** Sequence of the first non-coding exon (black box) as found in the 5'RACE is shown. It contains 78 bp. Grey box indicates the second exon containing the translation initiation site (shown in black font). Black box shows the first non-translated exon of *TN-W*.

The human *TN-W* gene consists of a total of 19 exons spanning 80 kb of genomic DNA (Fig.III.9). The transcript starts with a non-coding exon followed by exon 2, which contains the start codon (ATG) for translation initiation.



exon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
exon length (bp)	75	444	375	264	186	90	264	267	264	267	264	264	131	133	152	97	168	164	1136
intron length (bp)	9448	1505	455	3323	1469	8495	3163	648	18343	1355	4575	3291	946	430	7098	879	7398	2380	

Figure III.9: Genomic organization of the human *tenascin-W* gene

The distribution of the 19 exons is shown in the upper part, whereas the lengths of exons and introns are indicated in the lower part.

Analysis of the genomic sequences of *TN-W* orthologs revealed conserved presence of a first non-coding exon upstream of the ATG-containing exon (Fig.III.10).

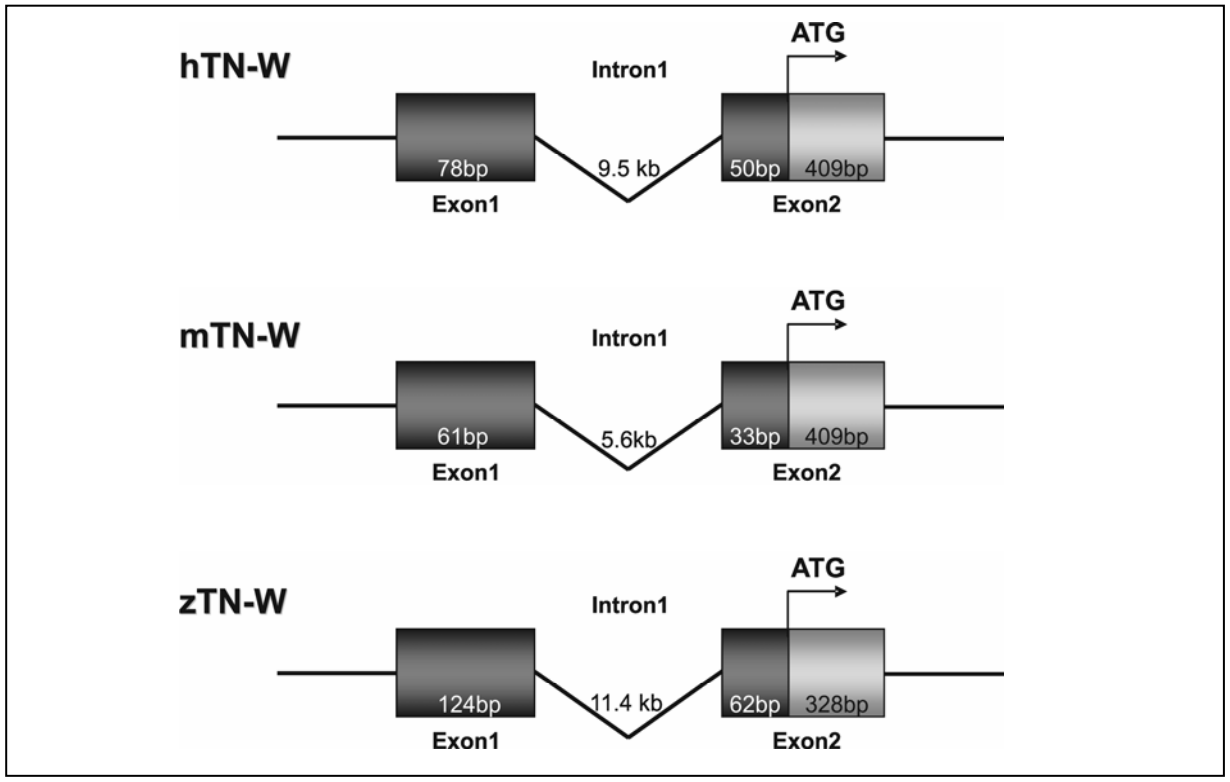


Figure III.10: Genomic distribution of the first two exons in the *tenascin-W* gene

Genomic distribution of the first two exons in different *TN-W* orthologs is shown. Non-translated regions are indicated in black. h: human; m: mouse; z: zebra fish

Sequence alignments [155] of the putative promoters of the human and mouse *TN-W* genes 500 bp upstream of the first exon and of exon 1 revealed a very high similarity as indicated by the black and yellow boxes (Fig.III.11).

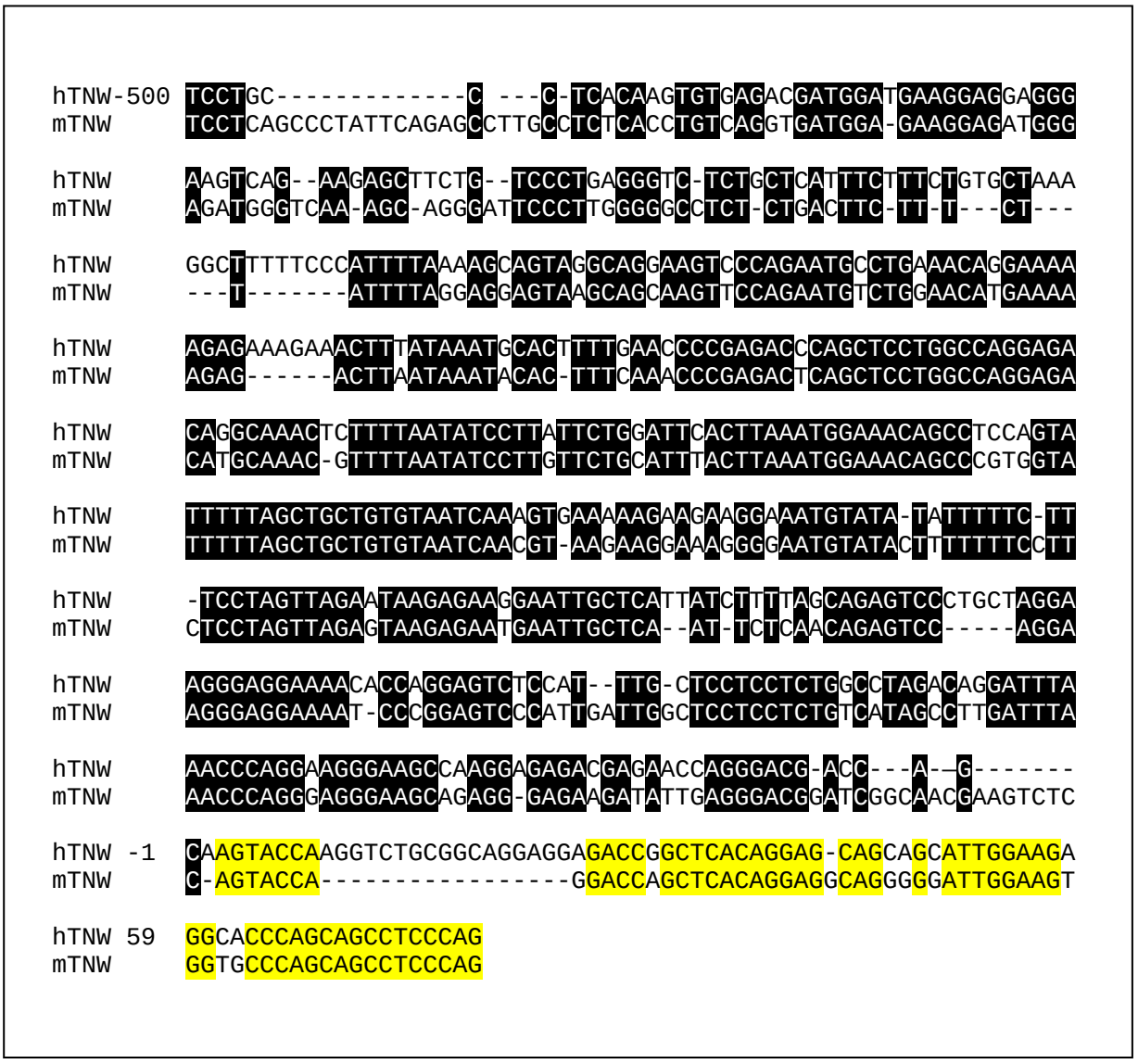


Figure III.11: Sequence alignment of the tenascin-W promoter regions

Sequence alignment of the 500 bp upstream of the first exon (black boxes) and of the first exon (yellow boxes) between mouse and human tenascin-W is shown.

To investigate the functionality of the TN-W promoter, we cloned different luciferase promoter constructs, all containing exon 1 and different lengths of the 5' flanking region (Fig.III.12A). To compare promoter activity of TN-W with that of TN-C, we cloned three TN-C luciferase constructs as well (Fig.III.12B).

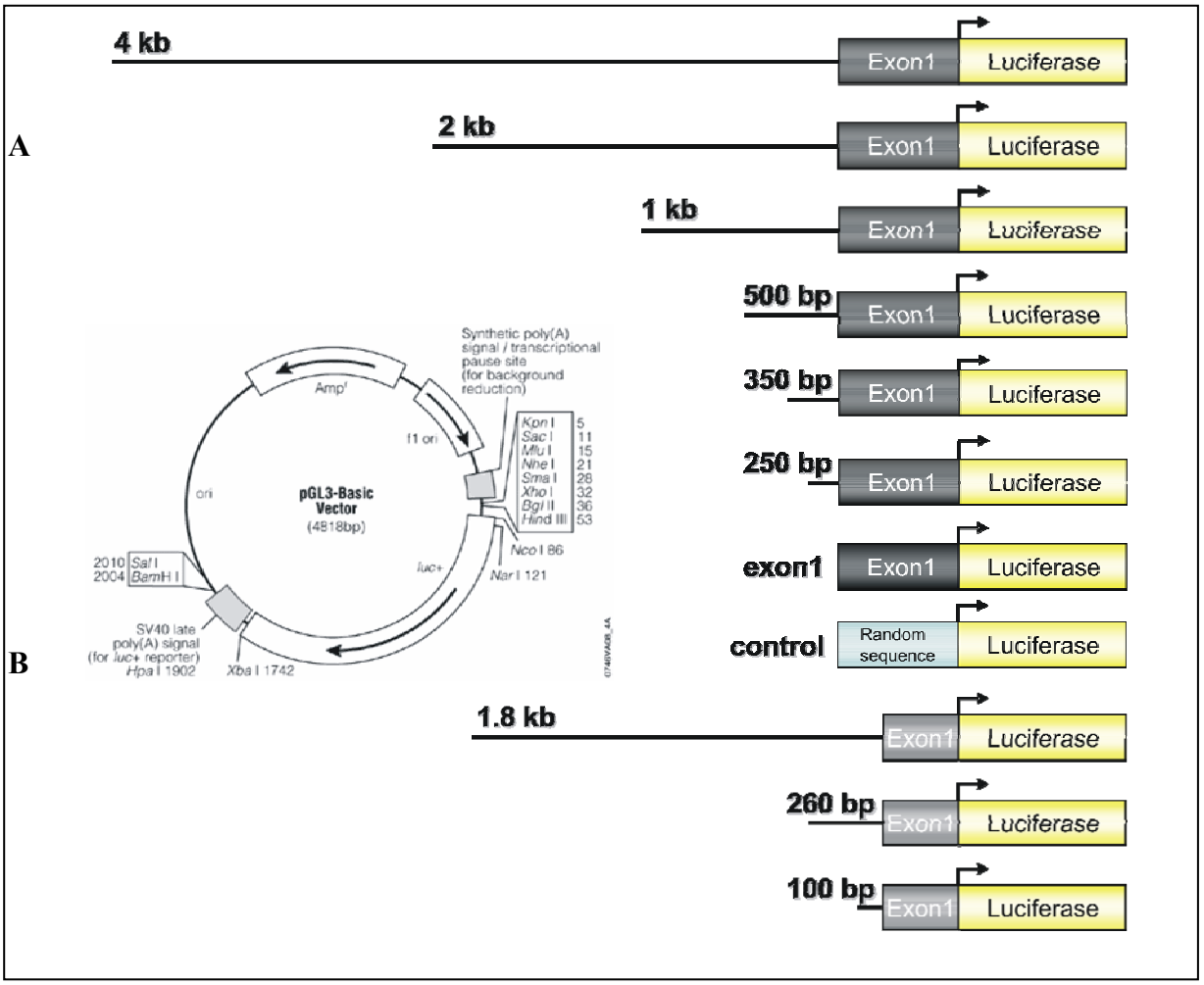


Figure III.12: Luciferase constructs

Shown are the TN-W **(A)** and TN-C **(B)** promoter constructs containing different lengths of the putative promoter regions that have been cloned in front of the *luciferase* gene present in the pGL3-basic vector.

This allowed a direct comparison between the regulatory mechanisms and the promoter strengths of TN-W and TN-C. The promoter constructs together with the β-Gal expression plasmid as internal standard for transfection efficiency were transiently transfected into different human cell lines. In a first try, we tested whether we get any luciferase activity with the TN-W specific constructs 4kb, 2kb, 1kb, 500bp and compared it to the activity obtained by either transfecting the empty pGL3-basic vector (negative control) or a SV40 promoter-containing expression vector (positive control). Indeed, in HT1080 (fibrosarcoma) as well as in Saos2 cells (osteosarcoma) all the promoter-containing luciferase constructs showed clear luciferase induction as compared to the negative control (Fig.III.13/14). Highest luciferase activity was obtained with the shortest (500 bp) construct in both cell lines (Fig.III.13/14) and reached about half the activity obtained by the strong SV40 promoter.

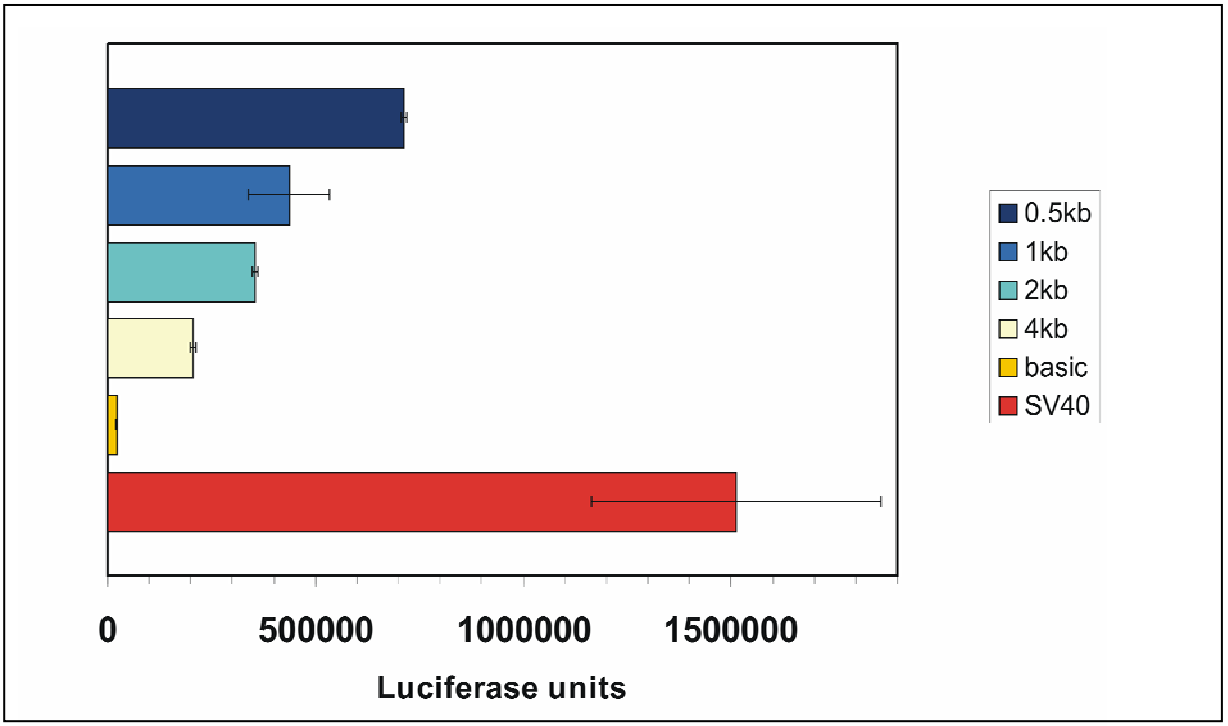


Figure III.13: Luciferase assay in HT1080 cells

Luciferase activity of different TN-W promoter constructs in HT1080 cells compared to a negative control (basic) and a positive control (SV40). Graph represents three independent experiments.

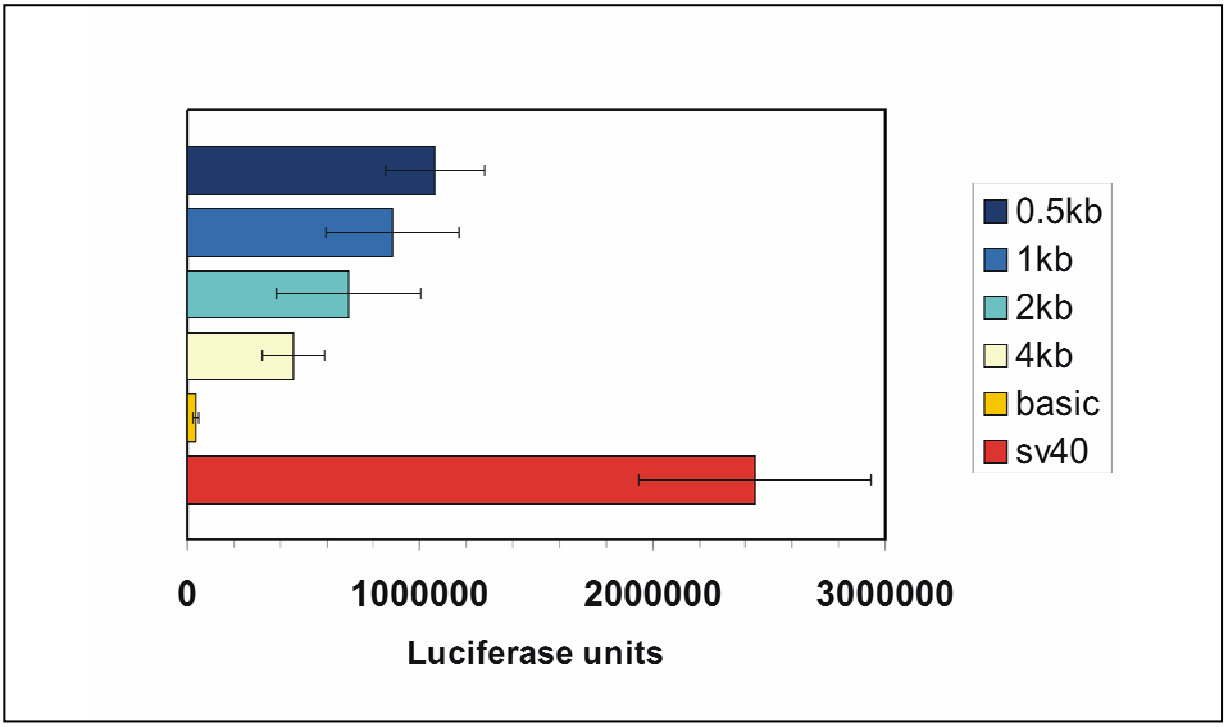


Figure III.14: Luciferase assays in Saos2 cells

Luciferase activity of different TN-W promoter constructs in Saos2 cells compared to a negative control (basic) and a positive control (SV40). Graph represents three independent experiments.

To identify the promoter regions important for transcription regulation, the constructs were further truncated. Figure III.15 shows that shortening the construct to 250 bp still gave optimal activity. Interestingly, there was still some activity in a construct containing the sequence of exon 1 only (Fig.III.15). This implies that the non-translated first exon contains regulatory elements as well. Furthermore, this study revealed that the TN-C promoter activity was equally strong as SV40 and about 2-fold stronger than that of TN-W (Fig.III.15). TN-C promoter activity was found to be highest in the 260 bp construct which is in agreement with previous publications [156].

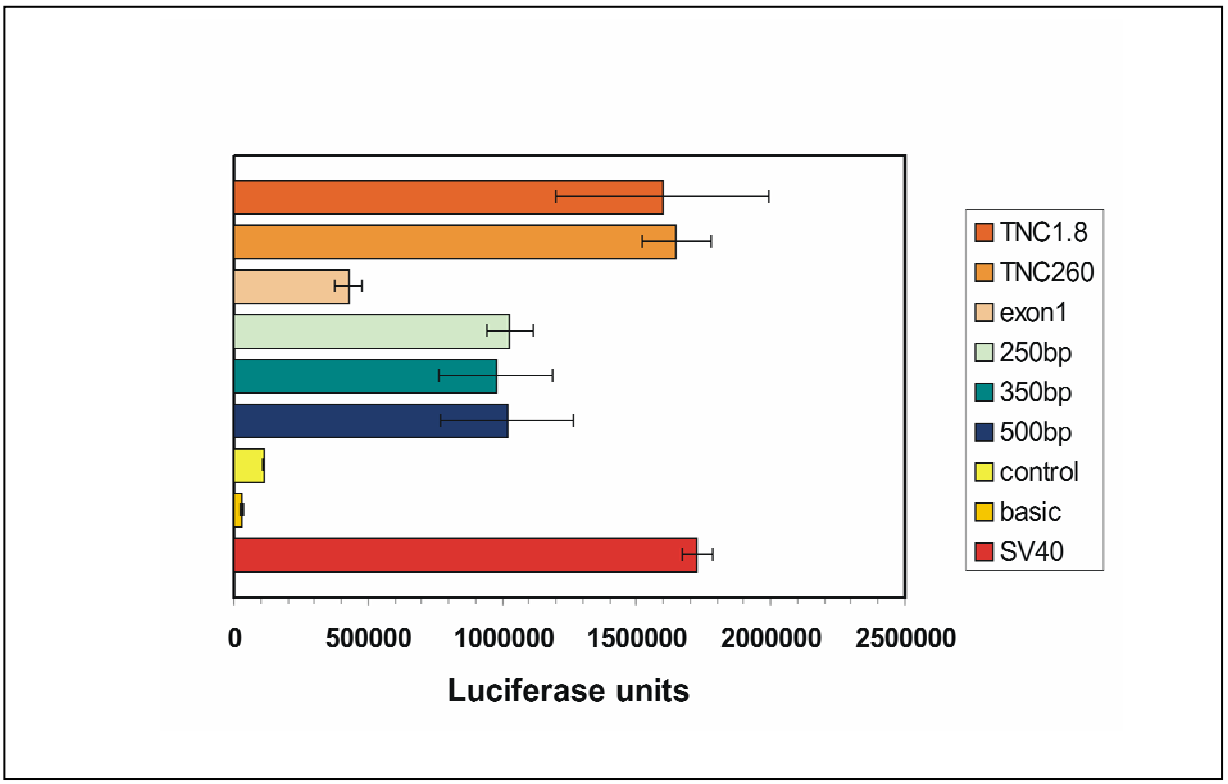
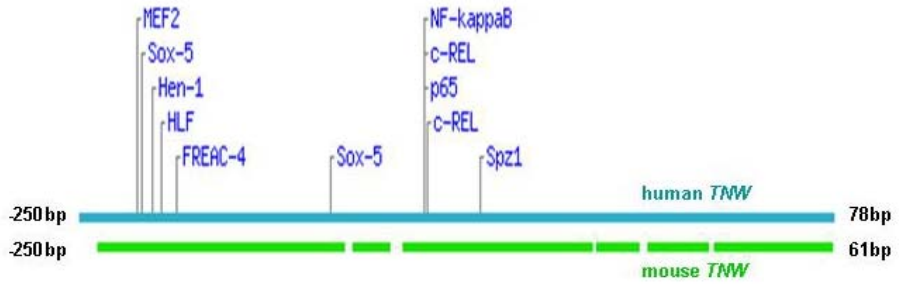


Figure III.15: Luciferase assay in HT1080 cells including TN-C constructs

Luciferase activity of TN-W promoter constructs in HT1080 cells compared to a negative control (basic) and a positive control (SV40). Graph represents three independent experiments.

Since we found highest TN-W promoter activity in the 250 bp construct, the first 250 bp upstream must contain important sites for *TN-W* gene regulation. Therefore, we performed transcription binding site predictions within these 250 bp using ConSite. Potential transcription factor binding sites, which are conserved between human and mouse *TN-W*, are shown in Figure III.16.



Transcription factor	human *TNW*				mouse *TNW*			
	Sequence	From	To	Strand	Sequence	From	To	Strand
MEF-2	GTATTTTTAG	-228	-219	+	GTATTTTTAG	-235	-226	+
Sox-5	ATTTTTA	-226	-220	-	ATTTTTA	-233	-227	-
Hen-1	TAGCTGCTGTGT	-221	-210	+	TAGCTGCTGTGT	-228	-217	+
HLF	TGCTGTGTAATC	-217	-206	-	TGCTGTGTAATC	-224	-213	-
FREAC-4	GTAATCAA	-211	-204	+	GTAATCAA	-218	-211	+
Sox-5	ATTGCTC	-144	-138	-	ATTGCTC	-149	-143	-
Nf-kappaB	GGAAAACACC	-103	-94	-	GGAAAATCCC	-116	-107	-
c-REL	GGAAAACACC	-103	-94	-	GGAAAATCCC	-116	-107	-
p65	GGAAAACACC	-103	-94	-	GGAAAATCCC	-116	-107	-
c-REL	GAAAACACCA	-102	-93	-	GGAAAATCCCG	-115	-106	-
Spz1	GCTCCTCCTCT	-79	-69	-	GCTCCTCCTCT	-90	-80	-

Figure III.16: Potential transcription factor sites in the first 250 bp

The potential transcription factor binding sites, which are conserved between mouse *TN-W* (green) and human *TN-W* (blue), are shown (top). Their specific binding motifs as well as their exact positions are indicated in the table (bottom).

DISCUSSION

IV. Discussion

This thesis is the result of a joint effort including many collaborators contributing materials, suggestions and ideas. Therefore, the discussion refers to "our" results.

IV.1 Our ambition

More than two decades ago, TN-C was identified as an ECM protein enriched in the stroma of gliomas [87] and as a myotendinous antigen [88]. The results of intensive studies on its role in cell and developmental biology as well as in different pathologies together with its high conservation in every vertebrate species [166] suggested a fundamentally important function for TN-C. Therefore, it was a huge surprise and disappointment to see that mice develop normally without TN-C [167]. Knock-out technology had not only failed to elucidate the function of TN-C, but it pointed to a redundant role for this protein [168]. However, this shock challenged people in the tenascin field to perform more thorough investigations. And upon more detailed inspection of the TN-C-deficient mice, more and more abnormalities appeared ranging from defects in behavior to impaired angiogenesis and deficiencies in stem cells (reviewed in [96]). In the meantime, three new tenascins have been discovered (for reviews see [9, 51]), the last one being TN-W [141]. TN-W represents the final member of the tenascin family.

TN-W was originally identified in zebra fish where it is co-expressed with TN-C by neural crest cells and somites [141]. More recently, murine TN-W was characterized and immunohistochemistry revealed prominent expression in the developing and adult kidney, developing and adult bone, and transiently in smooth muscle cells [56]. Despite the tight spatio-temporal regulation, a prominent feature common to all tenascins [58], TN-W expression often completely or at least partially overlapped with that of TN-C [56, 141, 151, 169]. Since TN-C is prominently and specifically expressed in the tumor stroma of a variety of cancers [9], it was evident to start studying TN-W expression and function in tumors.

Initially we studied TN-W function in murine tumor models, but then decided to switch to human TN-W which might be more relevant and of higher general interest. Our goal was to clone the gene, characterize the protein, and investigate its expression, its functions, and its involvement in tumor malignancies and compare it to TN-C, a known marker for activated tumor stroma [9].

Furthermore, we started collaborations to identify and describe new aspects of TN-C regulation during different human pathologies.

IV.2 Tenascin-W in the context of tumors

First data linking TN-W to the presence of tumor malignancies were obtained in mice [151]. Oncogene-induced mammary tumor models suggested that TN-W was preferentially expressed in primary tumors with the highest potential to metastasize. TN-W specifically localized to the tumor stroma, where it was, at least partially, co-localized with TN-C. In addition, its putative integrin receptor α8β1 [56] showed a similar tendency: its expression increased with the malignancy of a tumor. Functional studies revealed that TN-W can support migration of breast cancer cells *in vitro*, but not of normal mammary epithelial cells. Therefore, we proposed a model where presence of TN-W in the tumor stroma promotes motility of cancer cells expressing α8 integrin. Thereby TN-W contributes to the metastatic potential of cancers. Of course if these findings would hold true in human tumors, TN-W could have great potential as a diagnostic tool as well as a potential stromal target for cancer therapy.

Here, we have presented our first results on human TN-W. We investigated its function and determined its expression in tumors. For the expression analysis, we tested in total 97 different tumor tissue extracts (63 breast cancers, 9 kidney cancers, 9 pancreas cancers, and 16 colon cancers) for the presence of TN-W and TN-C. Out of these 97 different cancer tissues, 77 % (75/97) showed a prominent up-regulation of TN-W and 82 % (80/92) were TN-C positive (Table IV.I).

Table IV.I: Summary of TN-W and TN-C expression in tumors

tumor	n	TNW+ (%)	TNC+ (%)
breast	63	81	86
colon	16	69	88
kidney	9	67	78
pancreas	9	78	56

n: sample number

Despite the fact that a majority of the tested tumor extracts showed expression of TN-W and TN-C, their amounts differed greatly between patients. This implied independent regulation of their expression. Normal tissue was negative for TN-W, except for a slight expression in spleen and liver. This was in contrast to TN-C which showed basal expression in all tissues tested, except in breast and heart. For most of the 63 breast cancer extracts the clinicopathological features of the patients were available and we could correlate TN-W expression with tumor grade. We found a statistically significant enrichment of TN-W in low-grade breast tumors (G1/G2) although there were also high-grade (G3) patients having high TN-W levels and *vice versa*. But the mean value of TN-W in low-grade tumors was higher than that in high-grade tumors. This was in contrast to TN-C, whose expression could not be correlated with tumor grade in mammary tumors. This is in agreement with previous reports on TN-C in breast cancer, which showed no clear correlation between TN-C and patient prognosis. However, in other tumors such as in lung, colon and brain tumors, TN-C expression correlates with poor patient prognosis [95]. Although tenascins are known to be subject to alternative splicing [59], we do not have any evidence for the existence of TN-W splice variants. We always obtained a single band by immunoblot analysis of the different tissue extracts. However, we observed differential expression of TN-C isoforms in the different cancer patients. Although there is one report showing that some specific TN-C isoforms are only expressed in invasive breast carcinomas [101], we were not able to confirm this finding in our 63 breast cancer extracts.

In agreement with our findings, TN-W enrichment in low-grade breast cancer was independently confirmed in a different patient cohort by RNA profiling of breast cancer

patients [170]. This data set showed a good correlation between TN-W transcript levels and tumor grade. These results are accessible in the ONCOMINE database [171]. This correlation is probably tissue-specific since RNA profiling of prostate cancer patients in a different study revealed higher TN-W expression in metastasizing prostate carcinomas (high-grade) and lower expression in benign prostate tumors [172]. The opposite was the case for TN-C which displayed highest transcript levels in benign prostate tumors and lowest in metastasiszing cancers. It seems that at least in prostate cancer the two tenascins are expressed directly reverse to each other [172], which implies that they might have different functions during tumor progression. Experimental evidence for these observations in a different cohort of prostate cancer patients is lacking, but it seems likely that TN-W shows tissue specific expression and functions.

In tumor tissues expressing TN-W and TN-C, immunohistochemistry mostly revealed co-localization of the two proteins in the tumor stroma. TN-W was exclusively localized to the tumor stroma, whereas some rare tumors revealed TN-C expression in the epithelial compartment as well. Breast tumors with epithelial TN-C expression all belonged to high-grade (G3) tumors. This might suggest that initially, activated stromal fibroblasts are the cellular source for TN-C expression. However, during tumor progression, stromal cell-derived factors acting in a paracrine fashion to the epithelium induce synthesis of TN-C in the transformed epithelial cells. This would be in agreement with previous reports showing TN-C expression in certain cancerous epithelial cells [152-154]. Further evidence for our suggestion could be the fact that the highly aggressive breast cancer cell line MDA-MB-435 produces high amounts of TN-C, whereas the less-invasive MCF-7 and T47D breast cancer cell lines [173] do not express it (own observations). So far we can only speculate what the cellular source of TN-W is. We hypothesize that activated stromal fibroblasts produce and secrete TN-W. However we cannot exclude contribution of the transformed epithelium to TN-W deposition in the stroma. At least in mouse cells we could induce endogenous TN-W in MEFs and in 4T1 epithelial breast cancer cells by TNFα, exemplifying the two potential distinct cellular sources for TN-W.

For 11 breast cancer patients, follow-up data was available. Four out of these 11 patients showed tumor recurrence. Strikingly, we measured very high TN-C levels in the primary

tumor extracts of the corresponding patients by immunoblot analysis. This could indicate that high TN-C levels could identify those patients having higher probability to develop recurrent tumors. For TN-W, we could not observe a clear correlation between tumor relapse and high protein levels.

Co-localization of TN-W and TN-C in the activated tumor stroma in breast cancer suggested similar but not necessarily identical functions. We observed a similar effect of the two tenascins on adhesion when offered as single substratum to breast cancer cells: cells are not able to attach to and spread on tenascins. However, in contrast to TN-C, TN-W mixed with fibronectin did not abolish fibronectin-mediated cell-spreading. Furthermore, fibroblasts were able to attach to and partially spread on TN-W under conditions where they remained round on TN-C. Fibroblasts on TN-W changed their morphology, forming long actin-rich processes, and appeared much more contracted than the same cells plated on fibronectin. This morphology somehow resembled that described for CAFs [19]. Therefore, it would be interesting to know whether CAF's also show such adhesive properties on a TN-W substratum. Concerning the process of migration, TN-W and TN-C share the property of inducing cancer cell migration on an adhesion-mediating substratum such as fibronectin or type I collagen when added exogenously to the medium.

Taken together, our results imply an active role for TN-W in the process of tumor progression by influencing cancer cell and stromal cell behavior. We suggest the following model for TN-W action (Fig.IV.1): neoplastic epithelial cells alter their adjacent stroma by secreting soluble growth factors, cytokines or chemokines. As a response to the tumor-cell-derived factors, stromal cells are activated and they start to modulate their microenvironment by secreting autocrine as well as paracrine effector molecules. Among many others, they specifically produce TN-W and TN-C, which in combination with other ECM molecules influence cancer cell and stromal cell behavior.

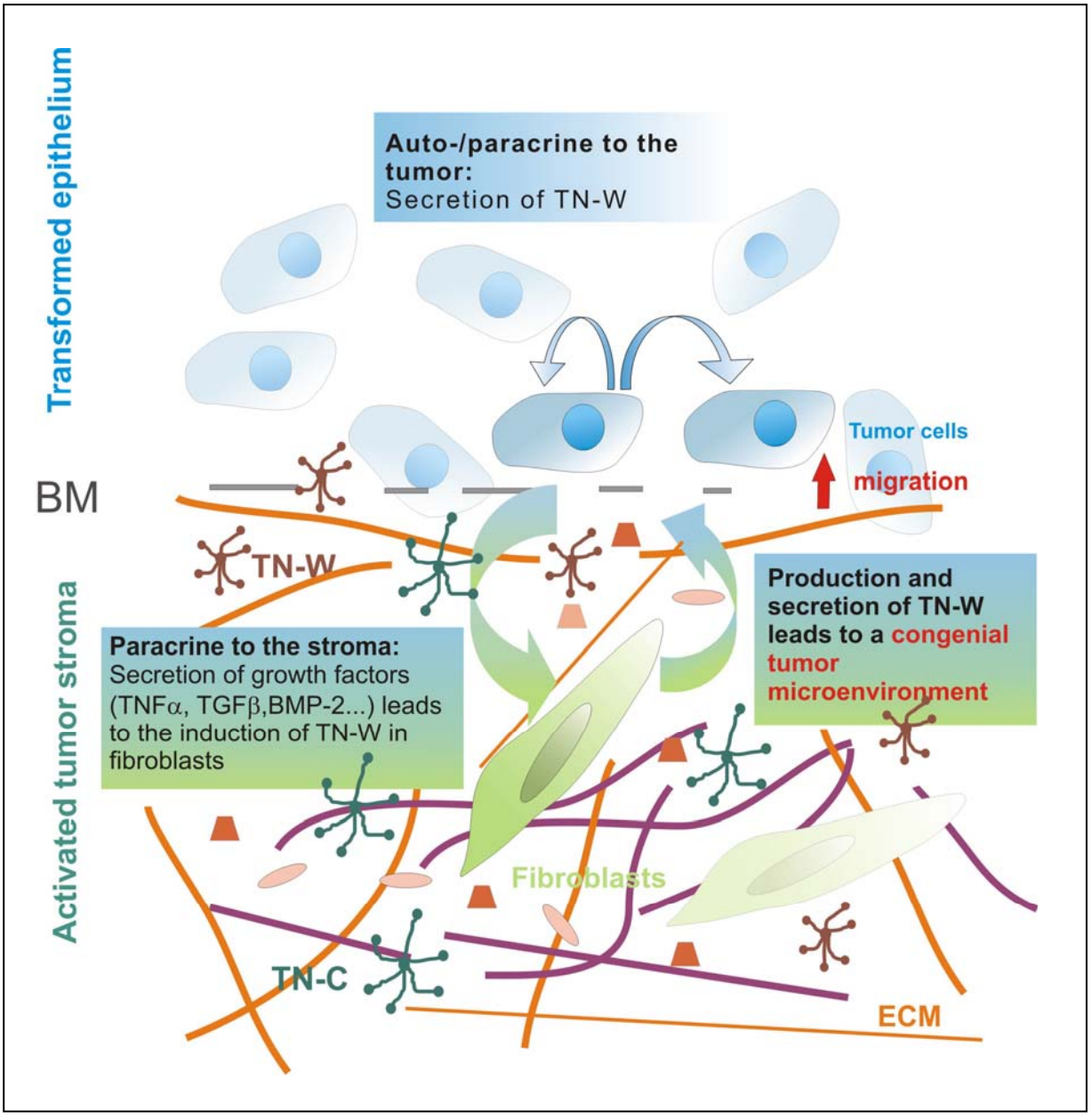


Figure IV.1: Model for tenascin-W action

Transformed epithelial cells (blue) secrete paracrine growth factors to the stromal fibroblast (green). As a response to these tumor cell-derived factors, fibroblasts are activated and change their expression profile. Among other effector molecules, TN-C and TN-W are produced. TN-W and TN-C in combination with other ECM molecules serve as a pro-migratory environment for cancer cells. Alternatively, cancer cell may be a source for TN-C and TN-W production themselves, thereby establishing their preferred, migration-facilitating environment. BM: basement membrane; ECM: extracellular matrix.

IV.3 Significance of tenascin-W as tumor marker

Up to now, we have discussed the fact that TN-W is highly expressed in a large fraction of different cancer patients, and that normal adult tissues do not show detectable TN-W levels. This implies that TN-W might have the potential to serve as tumor marker, since a clear difference between healthy and diseased tissue is a prerequisite for any biomarker. TN-C, which shows a similar expression pattern as TN-W, has been extensively analyzed in sera of different cancer patients and evaluated for its significance to act as tumor marker [104, 105, 143-145]. Most of the reports indicate increased TN-C serum levels in cancer patients. However, the values are usually scattered over a wide range, including cancer patients having normal TN-C levels. Hence, TN-C still remains a questionable tumor marker [104]. A further problem is that TN-C is not solely linked to neoplasms, but also to infections and inflammations [9]. Thus, a large fraction of TN-C in the serum could derive from such inflammatory pathological conditions rather than from the tumor mass.

In search for new diagnostic tumor markers, we monitored TN-W serum levels in cancer patients by a sensitive sandwich-ELISA assay. In the cohort of non-metastatic colon cancer as well as in the cohort of non-metastatic breast cancer patients we could detect a significant increase in mean TN-W levels compared to control. In sera of colorectal cancer patients we measured a 2.04-fold increase, whereas a 1.75-fold elevation could be detected in sera of breast cancer patients compared to volunteers. A useful tumor marker would have elevated levels at the time of diagnosis and reduced levels after tumor resection or during cancer treatment. This would allow to follow-up cancer patients after their therapy and the detection of a later potential recurrence as early as possible [174-176]. However, the levels in the same colon and breast cancer patients six weeks after surgical tumor resection did not drop significantly. Moreover, some patients showed even higher TN-W concentration after tumor removal. This of course raises the question whether the TN-W detectable in the serum is derived from the tumor mass itself or whether its levels are influenced by any co-morbidity (e.g. cardiovascular diseases, inflammatory disease) or through indirect effects of the tumor on other organs (e.g. bone marrow, liver). Another explanation could be that TN-W is a very stable protein and that it is still circulating in serum six weeks after tumor removal. This

question could be answered by analyzing serum TN-W levels of the same patients in longer follow-up studies.

To obtain evidence for a causal link between high TN-W serum levels and TN-W presence in the tumor, we investigated its expression in colon tumors by immunoblot analysis and immunohistochemistry. We were able to demonstrate that TN-W is highly expressed in the majority of colon cancer patients and that it is strictly localized to the tumor stroma, overlapping with TN-C expression. To really prove that the tumor mass is the source of serum TN-W, we would need to have serum and tissue samples from the same cancer patients. A clear correlation between high TN-W expression in tissue and serum would strongly support the concept of tumor-origin of serum TN-W.

We suggest that TN-W might have the better potential to serve as tumor marker than TN-C. This conclusion could be drawn based on three observations: (i) the difference of mean serum TN-W level between diseased and healthy individuals was much more pronounced than that of TN-C; (ii) normal colon mucosae are negative for TN-W, while there is some expression of TN-C; and (iii) TN-W is not known so far to be linked to other pathological situations such as infection or inflammation. Such secondary expression sites for sure influence serum levels of a given biomarker. Why TN-W levels in breast cancer sera did not change cannot be explained so far. Therefore, it would be important and necessary to study more cancer types to evaluate the clinical significance of TN-W. Our observations warrant follow-up studies to evaluate the potential diagnostic or prognostic relevance of TN-W in colorectal cancer and possibly in other cancers types.

IV.4 Regulation of tenascin-C and tenascin-W

Our extensive immunoblot studies of different tumor tissue extracts revealed that TN-W and TN-C amounts varied greatly between different patients. This implied that regulatory mechanisms exist that are unique to TN-W and such that are unique to TN-C. However, we cannot exclude regulatory mechanisms that have been conserved between the two tenascins. Several publications exist about the regulation of *TN-C* and its promoter sequence in several different species. Promoter regions from chicken, mouse, and human TN-C have been identified. In all these species, the proximal promoter region displayed remarkable similarities and several regulatory elements have been conserved [156, 177-180]. The optimal promoter activity of the region upstream of the TN-C transcription initiation site was found within 220 bp, in a region containing potential binding sites for PAX, NF-1 and Sp1 [156]. Studies in human fibroblasts revealed that Sp1 co-operated with Ets transcription factors in TN-C promoter activation of PDGF-stimulated cells and also with Smad3/Smad4 in TGFβ-stimulated cells [109, 111].

So far only little is known about the regulation of TN-W. The only known fact is the observation that TN-W can be induced by TNFα and/or BMP2 in MEFs, C2C12, and in 4T1 mouse breast cancer cells [56, 151]. Unfortunately, we did not succeed in repeating such experiments in different human cell lines.

To characterize the human TN-W promoter region, we performed 5'RACE and identified a non-coding first exon, which is located 9.48 kb upstream of the ATG-containing exon. This feature is common within the *TN-W* gene family. In contrast to the TN-C promoter region, the TN-W promoter does not contain any obvious promoter motifs such as CpG islands or a TATA box. By means of luciferase reporter constructs we identified the optimal TN-W promoter activity within the 250 bp upstream of the transcription initiation site. It would be interesting to use TN-W and TN-C promoter constructs to dissect their responsiveness to different growth factors or other stimuli. With this approach we could try to find common or specific stimuli for the two tenascins. Furthermore, we could identify the promoter sequences for the specific responses and link them to the involvement of particular transcription factors.

Results of such studies might explain the prominent expression of TN-W in tumors. Since we could not find a good cell line for TN-W induction, we did not apply this approach so far with human constructs. Maybe it would be easier to switch to the mouse TN-W promoter, since there, we have cell lines (C2C12, MEFs, 4T1) in which endogenous TN-W can be induced. Therefore, we know that in these cells all factors needed for TN-W expression are present.

For TN-C we discovered a complete novel signaling pathway that leads to induction of TN-C: the Notch2 signaling pathway. By aligning the sequences located upstream of the transcription initiation sites of the established human and murine Notch2 target genes HES1 (hairy/enhancer of split) and HES5 [181] with the TN-C promoter sequence, two conserved motifs were identified: the TATA box at -30 bp, and a RBPJk binding motif GTGGGAA at -80 bp. The striking conservation in sequence and position of the latter element prompted us to consider a functional RBPJk-responsive element in the TN-C promoter. Indeed, by luciferase reporter assays we could establish a molecular connection between Notch2/RBPJk signaling and TN-C expression.

Notch proteins can either act as oncogenes or as tumor suppressor genes [182] depending on the cellular context. We found *Notch2* gene amplification together with elevated protein levels in half of the glioblastoma (GBM) cell lines tested and identified *TN-C* as a Notch2 target gene. GBMs belong to the most aggressive human cancers [183, 184]. Their highly invasive, proliferative and angiogenic properties might be partly explained by the up-regulation of TN-C, which is known to be involved in these aspects of tumorigenesis (for reviews see [95, 100]). From a clinical point of view, the identification of the Notch2 pathway as novel oncogenic signaling event in gliomagenesis allows the development of new therapeutic approaches to interfere with cancer progression by targeting this pathway. For instance gamma-secretase inhibitors that impair Notch receptor processing, and therefore diminish sensitivity to ligand-induced signaling are promising drugs. They may be considered as potential anti-cancer drugs for Notch2-positive gliomas.

The connection between Notch2 and TN-C is not only interesting in the context of gliomagenesis, but also in the context of development. Normal brain development includes the action of Notch2 which is expressed in ventricular germinal zones [185, 186]. Notch

maintains proliferation and prevents differentiation of neuron precursors as well as of immature glial cells. Conversely, TN-C deficient mice show defects in proliferation and migration of neuronal precursors [133, 134]. This suggests that during normal brain development, the Notch2/RBPJk/TN-C signaling cascade could be responsible, at least in part, for maintaining the reservoir of neuronal precursor cells. This would also be in agreement with our findings that Notch2 and TN-C are more prominently expressed in the undifferentiated GBMs compared to well-differentiated oligodendrogliomas (OG) in which only a subset were moderately positive for TN-C [187].

The *Notch2* locus is located on chromosome arm 1p. This locus does not include the *TN-C* gene, which is found on chromosome 9. Thus, high TN-C expression associated with high Notch2 levels cannot be simply explained by amplification of the *Notch2* locus including the *TN-C* gene. However, the genes coding for TN-W and TN-R are adjacent to the *Notch2* gene [57]. Therefore, amplification of the *Notch2* locus could eventually lead to up-regulation of TN-W or TN-R. It would be of high interest to test whether Notch2 positive gliomas are also positive for TN-W or TN-R.

Our experiments have only addressed TN-C induction by Notch2. What about Notch1 which is also implicated in tumorigenesis and in important developmental decisions [188]? Since Notch1 signaling involves the same canonical signal cascade as Notch2, it will be reasonable to consider in the future whether TN-C can be regulated by Notch1 as well.

To summarize, we propose a novel pathway, the Notch2/RBPJk/TN-C cascade, which plays a critical role in gliomagenesis. Induction of TN-C might lead to enhanced tumor cell motility which is a hallmark of GBMs.

Moreover, we analyzed TN-C expression in primary human lung fibroblasts derived from asthmatic patients. Asthma is characterized by chronic airway inflammation and ECM remodeling. Under physiological conditions tissue homeostasis is maintained by the equilibrium between ECM synthesis and degradation. In asthma patients, however, this equilibrium is altered leading to structural changes. Fibroblasts belong to the main producer of ECM, and TGF-β is the most potent ECM inducing factor *in vitro*. Since ECM is important

for many cellular aspects, alterations in its composition and amount produced may influence and prolong the inflammatory and remodeling processes. Current therapies for asthma include inhalation of corticosteroids and long acting β2-agonists (LABA), but their effects on ECM remain only poorly understood. Here we have analyzed the effect of corticosteroids and LABA on the expression levels of TN-C and fibronectin using primary human lung fibroblasts. Corticosteroid treatment negatively regulated expression of TN-C. This is in agreement with previous reports [189]. The response of fibronectin to corticosteroids was exactly the opposite: corticosteroids increased fibronectin deposition. LABAs neither showed a prominent effect on TN-C or fibronectin expression nor modulated the effect of corticosteroids. Therefore, our findings might have implications for the short- and long-term treatment strategies in regard to airway remodeling in asthma and COPD.

IV.5 Perspectives

In recent years, it has become evident that tumors require a congenial microenvironment for progression and that carcinogenesis is accompanied by several stromal changes. This finally leads to an aberrant microenvironment that facilitates tumor growth and invasion. TN-C is known to be *de novo* expressed in the tumor stroma by activated fibroblasts [19]. Our studies in human mammary and colon tumors showed now the induction of a second tenascin family member, TN-W in the activated tumor stroma and identified TN-W as a novel marker for tumor stroma. Whether its presence directly promotes cancer progression needs to be further investigated.

Although several lines of evidence have emphasized a role for TN-C and TN-W in breast and in other tumors [9], their real function(s) may still be missing. 2-dimensional (2-D) *in vitro* systems have generated a first large amount of information about different aspects of tenascin function. However, it seems that tenascin research has reached a limit for new information with these 2-D studies. To gain more details about the real *in vivo* function of tenascins, we have to go a step further with our biological studies. In particular, we have to take into account that TN-C as well as TN-W expression may depend on epithelial-mesenchymal interactions. These conditions cannot be imitated in 2-D culture systems. There is a definite need for more appropriate 3-D models which mimic the *in vivo* situation as adequately as

possible and which can drastically change cancer cell behavior [190, 191]. With such systems, we can (re-)study the influence of TN-W and TN-C on complex processes such as cell adhesion, proliferation, and migration.

Another straight forward approach to elucidate tenascin function would be the generation of knock-out and knock-in mice. TN-C-deficient mice already exist for some time [167, 192]. However, they did not show any severe phenotypes [96]. The knock-out mouse published in 1992 [167] was among the first to be made by homologous recombination. Nowadays, new knock-out strategies (flox/cre system) allow the generation of conditional knock-outs, which are based on tissue-specific inactivation of the gene of interest. Probably this would be the appropriate system to study tenascin function in different tissues and at different developmental stages of an organism. Concerning TN-W, no knock-out mice exist so far. TN-W-deficient mice most likely would not show any severe phenotypes. However, they would be the prerequisite for the generation of the TN-C/TN-W double knock-out mice. These mice could then be analyzed for tumor development and tumor progression in various cancer aspects such as angiogenesis, metastasis, invasion, proliferation and cancer stem cell biology.

Tenascin researchers have hoped that deletion of the *TN-C* gene would produce more than just subtle phenotypes. However, a simple explanation for the lack of strong functional defects in these mice could be the compensatory expression by other molecules. The newly described TN-W could be an interesting and promising candidate for this compensatory function. During development and in tumors, TN-W shows very similar expression patterns to TN-C [56, 151, 169]. Therefore, we can hypothesize that at least in some specific tissues, TN-W could compensate for the lack of TN-C thereby hiding the real defects in TN-C-deficient mice.

TN-W is specifically expressed in the tumor stroma and may represent a good anti-tumor target. A possible application for TN-W would be the use of specific monoclonal antibodies which are coupled to bioactive molecules to deliver drugs specifically to the tumor mass [193]. This method is already applied using TN-C-coupled antibodies (see “Introduction”) to treat glioma and lymphoma patients [137-139]. However, we showed here that TN-W is even more exclusively expressed in tumor tissue than TN-C, with (so far) no expression in any

healthy tissues tested. This implies that antibodies specifically recognizing TN-W have great potential to localize to the tumor mass or to deliver chemotherapeutic reagents very precisely to the tumor site. If we cannot find or interfere with TN-W functions in the context of tumor progression, its prominent presence in tumors could be beneficial for patients in context of tumor prevention or treatment.

Here, we have presented the very first descriptions of human TN-W. Our data put forward TN-W as a marker for transformation of the normal physiological stroma to an activated stroma in breast and in colon cancer. Furthermore, we show that TN-W can influence cancer and stromal cell behavior. These data are novel and we are convinced that they will serve as the basis for many future studies on TN-W and its role in cancer.

APPENDIX

V. Appendix

V.1 Experimental procedures (unpublished data)

Luciferase constructs

Tenascin-W and tenascin-C promoter constructs were amplified by the Expand High Fidelity PCR System (Roche), using HEK293 genomic DNA as template. The primer sequences used for the nested PCR reactions are listed in table V.I and table V.II. The reverse primer2 (rev2) and the forward primers2 for the second reactions included *NheI* and *XhoI* restriction sites, respectively, enabling directional cloning into the pGL3-basic vector (Promega).

Table V.I: Sequences of the primers used for the TNW luciferase constructs

name	sequence (5' - 3')
rev 1	GAGACTAATTACACTGCCCGG
rev 2	GAAAGCTTGGGCAACTGGCACTCTTACCTGG
4 kb1	GCAAACTTGTCCTAAGGATCC
4 kb2	GAGCTAGCAGATCTCTTGGATGTGGAGATGGG
2 kb1	CTTGGAGAATATGAGTGAAGGG
2 kb2	GAGCTAGCACGCACTTCCATCTGCCCTCATCC
1 kb1	CAGACGAGTAAGCTGAGCCAC
1 kb2	GAGCTAGCGCAGACACAAGCTTCAGATCCAGG
500 bp1	GCCAGAAAGGGGCACGACTATTC
500 bp2	GAGCTAGCCCCACTTCAGAGTCCTGCCACTC
350 bp	GAGCTAGCGGATTCACTTAAATGGAAAC
250 bp	GAGCTAGCGAGAAGGAATTGCTCATTATC
exon1	GAGCTAGCCAAGTACCAAGGTCTGCGGCAGG
control 1	GAGCTAGCAGAACTCGACCCTCCCAG
control 2	GACTCGAGCTGTGGAGAGGGTGGTGG

First PCR reactions were performed with the primers labeled with 1, second PCR reactions with the primers labeled with 2.

Table V.II: Sequences of the primers used for the TNC luciferase constructs

name	sequence (5' - 3')
1.8 kb1	CCGATGGGCGAGAGACCTAG
1.8 kb2	GACTCGAGCTGCCTTTGTGCCCACGGAG
260 bp1	GGGCTGCGGATCCTACTCCC
260 bp2	GAGCTAGCGCAGGCGGGAATTCCTACTT
100 bp	GAGCTAGCCTTTAATTCGCCAACTG

5'RACE

To characterize the 5' end of human TN-W mRNA sequence, the 5' / 3' RACE Kit, 2nd Generation (Roche) was used according to the manufacturer's instructions. The primer sequences used for the 5'RACE are listed in table V.III. Primer sets for the second PCR reaction included *NotI* and *SalI* restriction sites, respectively. Total RNA was isolated from KRIB cells using Trizol (Invitrogen). cDNA was synthesized using the human tenascin-W specific primer hSP1. To identify the 5' end of the TN-W mRNA two gene specific primers (hSP2 and hSP3) were used. cDNA quality was tested by two control primers. The resulting PCR fragments were ligated into the pBluescript KS vector (Stratagene) and sequenced.

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

name	sequence (5' - 3')
hSP1	CTCCACCATCTCTTCCTCCAGC
hSP2	CGCAGTCCTTCTGTGGCGTC
hSP3	GAGCGGCCGCGATGTTCTGTTCCTCCCTGGCC

Luciferase assay

HT1080 (ATCC # CCL-121) and Saos2 cells (ATCC # HTB-85) were plated in 6-well dishes at a density of 80000 cells/well in the presence of 1%FCS. The next day at a cell-confluency of about 60-70 %, they were transfected with the luciferase constructs by FuGENE (Roche) and incubated for another 24 hours. Thereafter, cells were washed twice with PBS and lysed in 100 µl of 1x Reporter Lysis Buffer (Promega). The cell lysates were incubated on ice for 30 minutes, centrifuged and diluted in lysis buffer in serial dilutions (1/5, 1/10, 1/100). 50 µl of each sample was put into a 96 well Microlite 1 Flat Bottom Microtiter Plate (Catalys), and luciferase activity was measured in a Mithras LB 940 spectrometer (Berthold Technologies).
To normalize the transfection efficiency, a β-galctosidase construct was co-transfected. From the cell lysates described above, 1/2, 1/5, and 1/10 dilutions were made and 50 µl of each sample were transferred into a 96 well Test Plate (TPP). Afterwards, 50 µl of the β-galactosidase substrate was added into each well and after incubating the plate for 30 minutes at 37ºC, the reaction was stopped by adding 150 µl of 1M $Na_2CO_3^-$. Plates were read with a $VERSA_{MAX}$ Microplate reader (Molecular Devices) at 490 nm against 620 nm.

Buffers and solutions used for Luciferase assays

1 M $Na_2CO_3^-$	stop solution	
10xGlycin Buffer:	150 mM $MgSO_4$	
	250 mM Glycin	
	pH 7.8	
10xLuciferin:	2 mM in 1x glycin buffer	
	Sterile filtered	
	Stored at -20ºC	
ATP:	100 mM in H_2O, pH 7.8	
	Stored at -20ºC	
β-Gal substrate (2x):	0.2 M Na-phosphate buffer, pH 7.5	
	2 mM $MgCl_2$	
	5.5 mM ONPG	
	0.1 mM β-mercaptoethanol	
	Stored at -20ºC	
Luciferase substrate:	10x glycin buffer	150 µl
(for 1 ml)	Luciferin (10x)	150 µl
	ATP	75 µl
	H_2O	625 µl

V.2 List of Abbreviations

BM	basement membrane
BMP2	bone morphogenetic protein 2
bp	base pairs
BSA	bovine serum albumin
CAF	cancer-associated fibroblast
CAH	congenital adrenal hyperplasia
CEA	carcinoembryonic antigen
CNS	central nervous system
Col	collagen
COPD	chronic pulmonary disease
CRP	C-reactive protein
DKK1	dickkopf 1
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDS	Ehlers-Danlos syndrome
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
EPC	endothelial precursor cell
ERK	extracellular signal-related kinase
FAK	focal adhesion kinase
FCS	fetal calf serum
FGF	fibroblast growth factor
FN III	fibronectin type III
FN	fibronectin
FSP1	fibroblast specific protein 1
GAG	glycosaminoglycan
GBM	glioblastoma
HES	hairy/enhancer of split
HGF	hepatocyte growth factor
ILP	isolated limb perfusion
IP	immunoprecipitation
LABA	long-acting β2-agonists
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger RNA
OG	oligodendroglioma
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
ROS	reactive oxygen species
RTPCR	real-time polymerase chain reaction
SDF1	stromal-derived growth factor
SDS	sodium dodecyl sulfate
SPARC	secreted protein acidic and rich in cysteine
TBS	Tris-buffered saline
TGFβ	transforming growth factor β
TM1	tropomyosin 1
TN-C	tenascin-C
TNFα	tumor necrosis factor α
TN-R	tenascin-R
TN-W	tenascin-W
TN-X	tenascin-X
VEGF	vascular endothelial growth factor
Wnt	wingless/int
β-Gal	β-galactosidase

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V.4 Curriculum Vitae

Martin Degen
Mittlere Strasse 31
4056 Basel, Switzerland

Date of birth: 4th February 1978
Nationality: Swiss
Marital status: single

Educational background

Mar 2003 - June 2007	PhD Studies in Cell Biology at the University of Basel, Switzerland
Oct 1997 - Oct 2001	Diploma in Molecular Biology at the Univesity of Basel, Switzerland. Major: Cell biology
Aug 1989 - Jul 1997	Matura at the Gymnasium for Natural Science and Mathematics in Basel, Switzerland

Work experience

Mar 2003 – June 2007	International PhD program at the Friedrich Miescher Institute (FMI), Basel, Switzerland, Group of Dr. Ruth Chiquet-Ehrismann
Nov 2002 – Mar 2003	Trainee at the FMI, Basel, Switzerland, Group of Dr.Ruth Chiquet-Ehrismann
Apr 2002 – Oct 2002	Practical training at F. Hoffmann-La Roche AG, Basel, Switzerland, Department of Neuroscience, in the lab of Dr. Christophe Grundschober
Sep 2000 – Oct 2001	Diploma Thesis at the Biocenter, University of Basel, Switzerland, Group of Dr. Hans-Peter Hauri

Teaching experience

Oct 2004 – Sep 2005	Supervision of Diploma Student in the lab of Dr. Ruth Chiquet-Ehrismann
Oct 2003 – Mar 2004	Tutorial for biology students (Introduction in Biology), University of Basel, Switzerland
Mar 1997 – Sep 2001	Teaching for scholars in the subjects chemistry, physics, maths, and biology at in-tuition, Basel, Switzerland

International experience

Jul 1997 – Sep 1997	English Language Center, Boston, USA

Publications and scientific contributions

Posters

Gordon Research Conference: Fibronectin, Integrins and related Molecules, April 22-27, 2007, Il Ciocco, Italy

Federation of European Connective Tissue Societies,
July 1-5, 2006, Oulu Finland

45th Annual Meeting of the American Society for Cell Biology,
December 10-14, San Francisco, USA

Novartis Corporate Research Conference,
October 11-13, 2004, Boston, USA

Joint FMI-Novartis meeting, 2004, Fürigen, Switzerland

FMI annual meetings, 2003-2006, Switzerland

Talk

"The functions of tenascins in pathogenesis", FMI annual meeting,
September 16-19, 2005, Pontresina, Switzerland

Publications

Sivasankaran, B[1]., Degen, M[1]., Ghaffari, A., Hegi, ME., Ionescu, M., Tolnay, M., Mergenthaler, S., Miserez, AR., Kiss, R., Lino, MM., Chiquet-Ehrismann, R., Merlo, A., and Boulay, JL. Tenascin-C is a novel RBPJk-induced target gene for Notch signaling in gliomas (*manuscript submitted*). [1] equal contribution

Degen, M[1]., Goulet, S[1]., Roth, M., Tamm, M., and Chiquet-Ehrismann, R. Opposite effect of fluticasone and salmeterol on fibronectin and tenascin-C expression in primary human lung fibroblasts (*manuscript submitted*) [1] equal contribution

Degen, M., Brellier, F., Schenk, S., Driscoll, R., Zaman, K., Stupp, R., Tornillo, L. Terracciano, L., Chiquet-Ehrismann, R., Rüegg, C., and Seelentag, W. Tenascin-W, a new marker of cancer stroma, is elevated in sera of colon and breast cancer patients. *Int J Cancer.* 2008 (*in press*)

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Meloty-Kapella, CV., Degen, M., Chiquet-Ehrismann, R., and Tucker RP. Avian tenascin-W: Expression in smooth muscle and bone, and effects on clvarial cell spreading and adhesion in vitro. *Dev Dyn*. **235:** 1532-1542, 2006

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