IN VIVO ANALYSIS OF THE ROLE OF TENASCIN-C IN TUMORIGENESIS

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Abbrevations

APC	**Adenomatous polyposis coli**
bFGF	**basic fibroblast growth factor**
BVEC	**Blood vessel endothelial cell**
α-SM	**α-smooth muscle**
CAM	**cell adhesion molecule**
CAF	**Cancer associated fibroblast**
CD31/(PECAM-1)	**platelet/endothelial cell adhesion molecule-1**
COL-IV	**Collagen-lV**
DAPI	**4',6-Diamidino-2-phenylindole**
eNOS	**endothelial nitric oxide synthase**
ECM	**Extracellular matrix**
EDNRA	**Endothelin receptor type A**
EGF	**Epidermal growth factor**
EMT	**Epithelial-mesenchymal transition**
FN	**Fibronectin**
FGF	**Fibroblast growth factor**
HGF	**Hepatocyte growth factor**
HSPG	**heparin sulfate glycoproteins**
IHC	**Immunohistochemistry**
IF	**Immunofluorescent**
IGF	**Insulin-like growth factor**

LEC	**Lymphatic endothelial cell**
MMP	**Matrix metalloproteinase**
TIMP	**Tissue inhibitors of metalloproteinase**
NCAM	**Neural cell adhesion molecule**
PAS	**Periodic acid-Schiff's reagent**
PCOL-1	**Procollagen-1**
PDGF	**Platelet-derived growth factor**
PIGF	**placental growth factor**
RT2	**Rip1Tag2**
SV40	**Simian virus 40**
TGF-β	**Transforming growth factor beta**
TNF-α	**Tumor-necrosis factor-α**
TNC	**Tenascin-C**
TNW	**Tenascin-W**
VE-cadherin	**Vascular Endothelial Cadherin**
VEGF	**Vascular endothelial growth factor**
VM	**Vasculogenic mimicry**
VSMC	**Vascular smooth muscle cell**

Summary

Tenascin-C is an adhesion-modulatory extracellular matrix (ECM) molecule that is highly expressed in most solid tumors. A high tenascin-C expression correlates with a bad survival prognosis of patients having glioma and breast carcinoma. This suggests an important role of tenascin-C in tumorigenesis. Results from cell culture experiments have shown a role of tenascin-C in enhancing tumor cell proliferation, promoting angiogenesis and inhibiting the immune system. However, the role of tenascin-C in tumorigenesis has not been conclusively addressed in an *in vivo* model system yet.

In this work, we produced transgenic RipTNC mice, in which the over-expression of tenascin-C is under the control of the rat insulin promoter. All transgenic RipTNC mice were healthy and fertile, and did not exhibit detectable alterations in tissue morphology of the pancreas or blood glucose levels, but displayed enhanced angiogenesis, indicating that ectopically expressed tenascin-C promotes angiogenesis in normal pancreatic tissues.

To investigate whether ectopically expressed tenascin-C affects tumorigenesis, we crossed the RipTNC mice with Rip1Tag2 (RT2) mice and generated double transgenic RT2/TNC mice. The RT2 mouse is a well-characterized model demonstrating the multistage carcinogenic process; these mice express oncogenic SV40 T antigen that induces hyperplasia, angiogenesis, and insulinoma formation in the pancreatic islets. Compared with RT2 mice, double transgenic RT2/TNC mice died earlier more frequently and showed an accelerated angiogenic and tumorigenic process. No difference in tumor onset was observed between these two genotypes. The overall number of carcinoma in RT2/TNC was higher than in RT2 mice at 10 weeks when double transgenic mice started to die. Accordingly, tumors from RT2/TNC mice exhibited an increased proliferation rate as shown by the staining for phosphorylated-histone-H3.

Immunohistochemical analysis showed that tenascin-C was expressed mainly by

cancer-associated fibroblasts (CAFs) within the tumor tissue of RT2 mice and was deposited into tube-like structures. In RT2/TNC transgenic mice the tubes were more pronounced: they were devoid of endothelial cells but accumulated tenascin-C together with laminin. Expression of β–catenin in tumors from RT2/TNC mice was much higher than in RT2 mice, and β–catenin was located in nuclei of carcinoma cells at the invading front, while in RT2 mice β–catenin was exclusively located at the cell membrane and cytoplasm. Further *in vitro* assay with MCF7 cells showed that tenascin-C induces epithelial-mesenchymal-transition (EMT).

We also investigated the potential effect of tenascin-C on tumor metastasis by immunofluorescent staining for insulin positive tumor cells in the lymph nodes and liver. We did not find any metastatic cells in the lymph nodes or liver of RT2 mice, but detected insulin-positive cells in both the regional lymph nodes and the liver of RT2/TNC mice, suggesting that tenascin-C triggered metastasis.

In summary, tenascin-C promoted several events in tumorigenesis causing malignancy. Induction of oncogenic signaling and in particular of Wnt and EDNRA signaling by tenascin-C may account for vasculogenic mimicry and carcinoma progression which potentially promotes tumor cell dissemination and metastasis. This model has a potential to be used for testing drugs to inhibit tenascin-C-induced metastasis.

In addition, in order to further study the role of tenascins in tumorigenesis, I also generated several lines of tenascin-W transgenic mice. Morphological characterization of the expressing founder lines and analysis of tumorigenesis in these double and triple transgenic mice are ongoing in Gertraud Orend’s laboratory.

I Introduction

1.1 The Rip1Tag2 tumor model

In patients, the molecular analysis of the multiple steps of tumorigenesis is hampered by the unavailability of tumor biopsies from all tumor stages. In contrast, mouse models of tumorigenesis allow the study of the multiple steps of tumorigenesis involving complex interactions between tumor cells and the tumor stroma. One approach of generating tumor-bearing mice is the introduction of highly oncogenic viral genes into the mouse genome. By use of tissue-specific promoters, expression of these oncogenes can be targeted to distinct organs, which are subsequently subject to malignant transformation.

Twenty years ago, Hanahan and colleagues (Hanahan, 1985) established the Rip1Tag2 (RT2) transgenic tumor mouse model by using the pancreas-specific rat insulin II promoter (Rip1) (**Fig.1**) to induce the expression of the oncogenic Simian Virus-40 (SV40) large tumor antigen (Tag) in β-cells of the pancreatic islets. These mice develop insulinomas and islet cell carcinomas (Hanahan, 1985). The SV40 Tag oncoprotein possesses a number of functional activities, including the ability to bind and thereby inactivate two tumor suppressor proteins, pRB and p53 (Ludlow, 1993). These activities induce malignant transformation of β-cells over a period of 12-14 weeks.

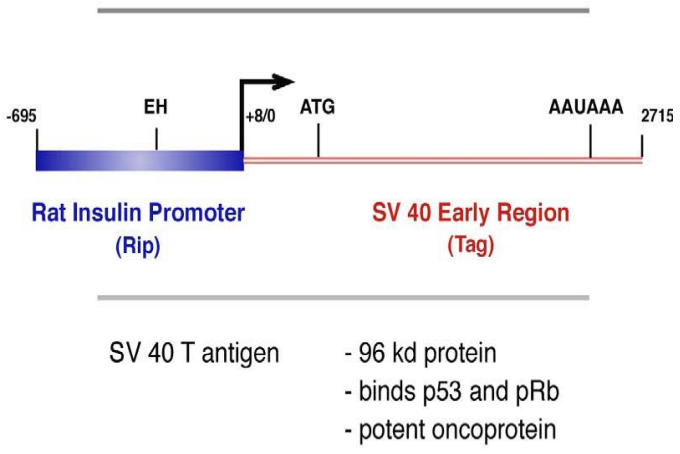


Fig. 1 Rip1Tag2 transgene. SV40 early region (Tag) is expressed under the control of the rat

insulin promoter (Rip1).

The pancreas has two functional components, the endocrine pancreas produces hormones that are essential for regulating the body's main energy source in the blood stream, consisting of the islets of Langhans, comprising only 1-2% of the mass of the whole pancreas; the exocrine pancreas produces pancreatic enzymes involved in food digestion.

The murine pancreas has approximately 400 islets of Langhans. A single islet consists of four major hormone producing cell types, α-, β-, δ- and PP cells, which synthesize the hormones glucagon, insulin, somatostatin and the pancreatic polypeptide. These cells constitute approximately 20%, 75%, 5%, and 1% of the islet cells, respectively. α cells are generally located around the periphery of the islet; β cells are distributed within the centre of the islet; δ cells are located around the periphery of the islet; the PP cells normally exist as single cells within the epithelium or are as small cell clusters associated with ducts.

The RT2 mouse presents a well-defined model in which the multiple stages of tumorigenesis occur in a reproducible manner (**Fig. 2**):
(i) "Normal stage", an initial phase of T antigen expression from embryonic day 8.5 to 3 weeks after birth, without apparent consequences at the cellular level (Alpert *et al*., 1988).
(ii) Hyperplastic/dysplastic stage, the emergence of hyperplastic pancreatic islets (50% all islets) at 4-6 weeks of age (focal activation of the expression of the growth/survival factor insulin-like growth factor II (IGF-II) (Christofori *et al*., 1994).
(iii) Angiogenic stage, approximately 20% of hyperplastic islets become angiogenic by 8-10 weeks of age. The angiogenic switch starts at 6 weeks of age, presumably due to inactivation of the angiogenic suppressor gene *Loh*2 on mouse chromosome 16 and concomitant induction of angiogenesis activators (Parangi *et al*., 1995).
(iv) Adenoma, about 1-2 % of angiogenic islets progress into solid tumors by 11-12 weeks of age; this presents a well-encapsulated, non-invasive benign tumor. Induction of Bcl-X_L expression blocks apoptosis (Parangi *et al*., 1995).
(v) Carcinoma, about 0.5 % of islets become dedifferentiated and developed into invasive tumors by 12-16 weeks of age. This is linked to the loss of E-cadherin expression (Perl *et al*., 1998).

Metastases are usually not found in these mice, probably because they succumb to hypoglycemia with increased tumor mass at 12-14 weeks of age (Parangi *et al*., 1996). In this model system overexpression of E-cadherin blocked invasion of islet carcinoma cells into substrata. However, overexpression of a dominant negative form of E-cadherin was not sufficient to induce development of metastatic tumors, but completely lost of E-cadherin can induce metastasis (Onder *et al*., 2008). In contrast, breeding the RT2 transgenic mouse into the N-CAM-/- background resulted in highly metastatic tumors by induction of lymphangiogenesis (Perl *et al*., 1999; Crnic and Christofori, 2004). In addition, lymph node and lung metastasis occurred also upon overexpression of the lymphangiogenic factors VEGF-C and VEGF-D under control of the rat insulin promoter in RT2/VEGF mice (Mandriota *et al*., 2001; Kopfstein *et al*., 2007).

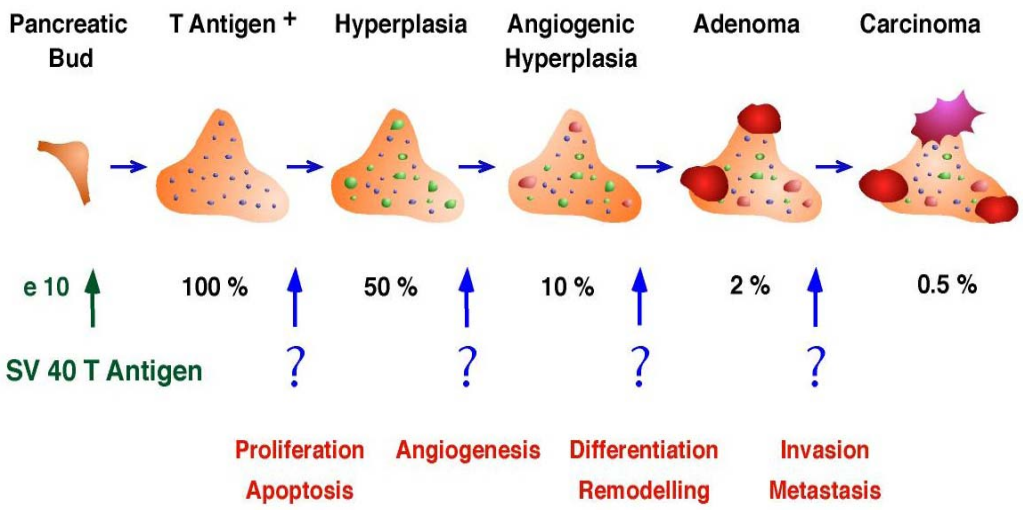


Fig. 2 Multistage tumorigenesis. Although all β-cells express the T antigen, only 50% of them become hyperplastic, 10% reach an angiogenic stage and 2% become adenomas. A small number of approximately 0.5-1% of islets become invasive carcinomas due to downregulation of E-cadherin (modified from Christofori *et al*., 1995).

Although insulinomas are rare in humans, the RT2 transgenic model presents an important tool for studying development and progression of common human epithelial cancers.

2. Invasion and metastasis

During metastasis cancer cells disseminate and establish secondary tumors at distant sites from the primary tumor. Metastasis is responsible for approximately 90% of all cancer deaths. The metastatic events involve detachment of cells from a primary tumor, local migration and invasion into stromal tissue, intravasation and transition through blood vessels, capillary bed arrest and extravasation, further local crawling and invasion, attachment, formation of micrometastases, survival, perhaps dormancy, and eventually further proliferation (Geho *et al*., 2005) (**Fig. 3**).

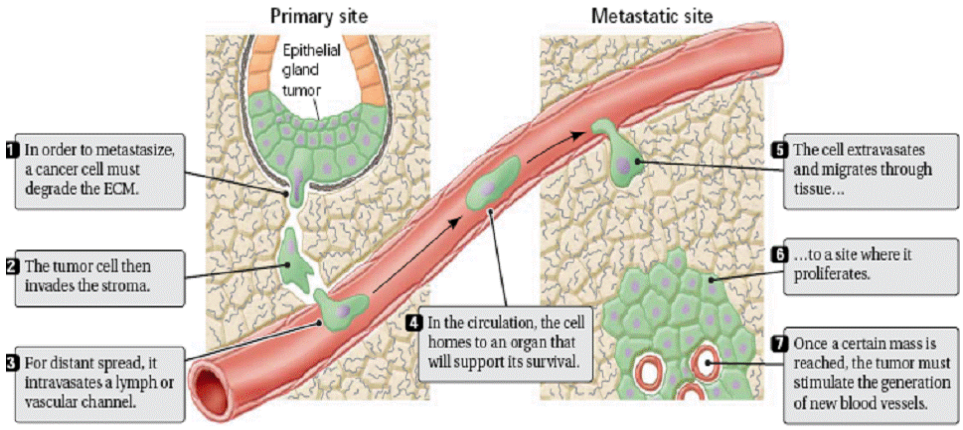


Fig. 3 The metastasis sequence (From Geho *et al.*, 2005).

2.1 Invasion and tumor cell migration

2.1.1 Epithelial - mesenchymal - transition (EMT)

Invasion is the first event leading to metastasis, but it is so far the least understood. It occurs by transferring of malignant cells from the primitive neoplastic focus into the surrounding host tissue and involves the acquisition of a motile phenotype (Christofori, 2006). For most carcinomas, progression toward malignancy is accompanied by loss of epithelial differentiation and a shift towards a mesenchymal phenotype, a process coined as epithelial- to- mesenchymal-transition (EMT). EMT allows stationary epithelial cells to become motile.

Epithelial cells are polarized and tightly connected to each other by intercellular junctions which prevent their motility. On the contrary, mesenchymal cells do not

establish stable intercellular contacts and have locomotive capabilities. During EMT epithelial cells lose intercellular junctions causing dissociation from surrounding cells, acquire mesenchymal-like characteristics and become able to migrate away from the original tissue. EMT is essential for correct organ development during embryogenesis by creating motile cells. Furthermore, molecular hallmarks of EMT include: E-cadherin down-regulation which is responsible for the loss of cell-cell adhesion; upregulation of matrix degrading proteases and mesenchymal-related proteins such as vimentin and N-cadherin; actin cytoskeleton reorganization mediated by Rho small GTPases to activate the motility machinery; upregulation and/or nuclear translocation of transcription factors underlying the specific gene program of EMT, such as β-catenin and members of the Snail, ZEB and basic helix-loop-helix families (Thiery and Sleeman, 2006). Factors acting at the tumour-stroma interface including growth factors and their receptors, ECM and related molecules (collagens, integrins, matrix-degrading proteases), as well as oncogenic signal transduction pathways (Ras, Src, Wnt) seem to play important roles in accomplishing EMT (**Fig.4** see next page). Matrix-degrading protease-mediated breakdown of the basement membrane would result in direct contact between carcinoma cells and the stromal microenvironment. Exposure to stromal-type collagens, with which epithelial cells would never come into contact under normal conditions, as well as to stromal growth factors, could initiate EMT (Guarino and Giordano, 1995; Shintani *et al*., 2006). Activation of Ras, Src, or Wnt pathways and alteration in the balance of the Rho GTPases Cdc42, Rac and Rho could provide further signals, eliciting the completion of cell dissociation and inducing the cytoskeleton remodeling required for movement.

EMT is believed to be a major mechanism by which cancer cells become migratory and invasive. Various cancer cells — both *in vivo* and *in vitro* — demonstrate features of epithelial-to-mesenchymal-like transition. However, there is evidence for metastasis without EMT. For example, while conditional ablation of p53 in the mammary gland causes expansive carcinoma development, conditional deletion of E-cadherin in the mammary glands of these mice results in the development of invasive lobular

carcinomas. These tumors are invasive in the absence of complete EMT, in spite of the loss of E-cadherin (Derksen *et al*., 2006). Furthermore, ectopic expression of podoplanin endows cells with invasive properties without loss of E-cadherin (Wicki *et al*., 2006). Moreover, time-lapse video-microscopy studies suggest that while EMT occurs independently in individual tumor cells, collective cell migration may be more frequent than the mesenchymal EMT-like migration *in vivo* (Berx *et al*., 2007).

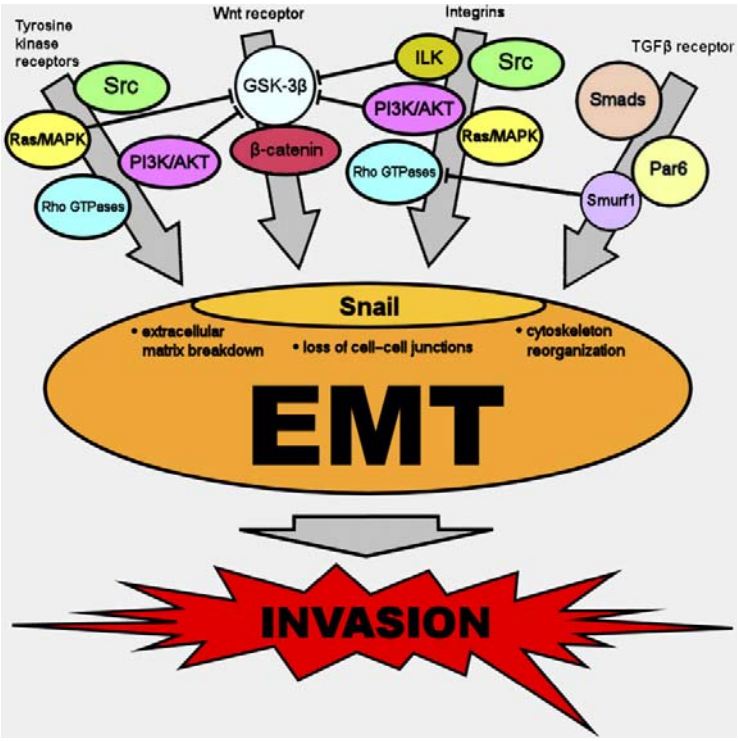


Fig. 4: Highly simplified diagram showing some of the better characterized transduction pathways involved in EMT. Tyrosine kinase receptors become activated and mediate downstream signaling after binding to various ligands including epidermal growth factor, fibroblast growth factor, insulin-like growth factor and hepatocyte growth factor. EMT occurs by hyperactivation of one pathway or, more probably, by simultaneous activation of more pathways, which either leads to Snail-mediated down-regulation of the E-cadherin gene, as Ras–Raf–MEK–MAPK, PI3K–Akt, TGFβ–Smads, ILK–Akt, and Wnt–β-catenin signalling do, or directly affects cell adhesion and/or the cytoskeletal dynamics, as accomplished by Src, TGFβ–Par6–Smurf1 and Rho GTPases. MAPK and β-catenin also up-regulate matrix-degrading proteases, while MAPK may, in addition, impact on motility through the direct activation of MLC kinase which generates phospho-myosin. Thereby, activation of a single molecular pathway can lead to one or more features of EMT, including loss of intercellular cohesiveness, cytoskeletal reorganization, increased motility and matrix degradation. Moreover, ILK and Akt stabilize β-catenin by phosphorylating GSK-3β, and thereby they can affect the expression of cell-cycleactivating molecules stimulated by the β-catenin signalling such as Myc and cyclin D1. In addition, Akt promotes cell survival by inhibiting pro-apoptotic proteins. Therefore, EMT could be coupled to changes in proliferation and/or to

survival-promoting responses that might be required to ensure the capability to survive as single cells in a foreign environment. Not included in this scheme are Notch, Hedgehog and NF-$_K$B signaling that have also been found to be involved in EMT (From Guarino *et al*., 2007).

2.1.2 Mechanism of cell migration

Once dislodged from the primary neoplastic tissue, post-EMT tumor cells must keep active the cytoplasmic machinery required for effective migration, thereby allowing local spreading and dissemination to secondary foci. Pro-motility environmental cues from ECM and growth factors are integrated and transduced to intracellular signals that drive migration through a cyclic sequence of protrusive, adhesive and contractile activities. Firstly, the tumor cell polarizes by establishing a leading edge and a rear end. At the leading edge a pseudopod protrusion forms as consequence of cortical actin polymerization and assembly into filaments which push the cell membrane forward. The growing pseudopod establishes new adhesions which connect ECM to integrins and the latter to the actin cytoskeleton, thus providing traction points for the subsequent translocation. Matrix-degrading proteases, that are upregulated in tumor cells, are recruited at the leading edge to effect local breakdown of the pericellular ECM, thereby creating a path for the advancing cell. Finally, the contractile shortening of membrane-anchored actin-myosin filaments leads to propulsion of the cell body forward whereas the cell rear detaches from the substrate and retracts, thus generating definite movement of the invading cell in the direction of migration (Friedl and Wolf, 2003). It is apparent that this sequence of protrusive – adhesive – contractile events is similar to those occurring in EMT, so that EMT and subsequent invasive migration could be regarded as parts of a continuum.

In addition, cellular phenotypic changes are likely plastic (Tarin *et al*., 2005; Christiansen and Rajasekaran, 2006). The changing characteristics of cancer cells during epithelial-mesenchymal transitions are likely associated with aberrant host microenvironment interactions that dictate the course of metastasis. Evidence continues to accumulate for the fact that cancer cells cannot act alone for the generation of metastasis.

2.1.3 Wnt/β-catenin signaling and invasion

In many cancer types, Wnt signaling is activated by mutations in a number of effector genes, including the genes encoding for adenomatous polyposis coli (APC), axin-1 and 2, and β-catenin, which predisposes to cancer. Besides their critical role in assembling the E- cadherin-mediated cell adhesion complex, β-catenin and γ-catenin also have important functions in the canonical Wnt-signaling pathway. Non-sequestered, free β- and γ-catenin are rapidly phosphorylated by GSK-3β in the APC/axin/GSK-3β complex and subsequently degraded by the ubiquitin-proteasome pathway. If the tumor suppressor APC is non-functional, as in many colon cancer cells, or if GSK-3β activity is blocked by the activated Wnt-signaling pathway, β-catenin accumulates at high levels in the cytoplasm. Subsequently, it translocates to the nucleus, where it binds to members of the Tcf/Lef-1 family of transcription factors and modulates the expression of Tcf/Lef-1-target genes, including c-Myc, cyclin D1, fibronectin, MMP-7, Id2, CD44, axin-2, Tcf- 1 and others, all genes implicated in cell proliferation, transformation and tumor progression. The dual function of β-catenin has motivated a multitude of experiments to assess whether the loss of E-cadherin function would subsequently lead to the activation of the Wnt-signaling pathway. In a number of cellular systems, it has been demonstrated that sequestration of β-catenin by E-cadherin can compete with the β-catenin/TCF-mediated transcriptional activity of the canonical Wnt-signaling pathway. The fact that E-cadherin does not completely deplete the cytoplasmic β-catenin suggests that β-catenin exists in different functional pools (Gottardi and Gumbiner, 2001; Stockinger *et al*., 2001). Interestingly, in breast and prostate carcinoma cell lines, E-cadherin suppresses tumor cell invasion by binding β-catenin without repressing β-catenin/TCF transcriptional activity, indicating that a novel, as yet unknown, additional function of β-catenin may be required for cellular invasiveness. Furthermore, activated Wnt signaling inhibits E-cadherin-mediated cell adhesion by inducing expression of Snail1, a transcriptional repressor of E-cadherin. Snail1 synergizes with the Wnt/β-catenin pathway by inducing Tcf expression, and β-catenin/Tcf can in turn repress E-cadherin transcription in cooperation with Snail1 (Jamora *et al*., 2003; Guaita *et al*., 2002).

2.2 Tumor angiogenesis and metastasis

Tumors have also been described as "wounds that never heal" (Dvorak, 1986) because blood vessels are constantly growing and are not stopped anymore. The formation of new blood vessels (angiogenesis) is crucial for the growth and persistence of primary solid tumors and their metastases. Furthermore, angiogenesis is required for metastatic dissemination since an increase in vascular density allows easier access of tumor cells to the circulation (Gannon *et al*., 2002). Without blood vessels, tumors can not grow beyond a critical size of 0.2 mm in diameter or metastasize to another organ. Hematogenous spreading of tumor cells from a primary tumor can be considered as a crucial step in the metastasis cascade leading eventually to the formation of clinically manifest metastases.

2.2.1 The angiogenic switch during tumorigenesis

Angiogenesis is a term that describes the formation of new capillaries from a pre-existing vasculature (Tonini *et al*., 2003). The vasculature is usually quiescent in the adult, but few adult tissues require ongoing angiogenesis including the female reproductive organs, organs that are undergoing physiological growth or upon injury (Bergers and Benjamin, 2003). Unregulated angiogenesis is seen in pathological conditions, such as psoriasis, diabetic retinopathy and cancer. During tumor growth, angiogenesis is required for proper nourishment and removal of metabolic wastes from tumor sites (Tonini *et al*., 2003).

Mammalian cells require oxygen and nutrients for their survival and are therefore located within 100 to 200 mm of blood vessels — the diffusion limit for oxygen. For tumors to grow beyond this size, they must recruit new blood vessels by vasculogenesis and angiogenesis (**Fig. 5**). This process is tightly regulated by a balance between pro- and anti-angiogenic molecules which was termed the 'angiogenic switch' (**Fig. 6**). It is now widely accepted that the 'angiogenic switch' is 'off ' when the effect of pro-angiogenic molecules is balanced by that of anti-angiogenic molecules, and is 'on' when the net balance is tipped in favour of angiogenesis (Hanahan and Weinberg, 2000). Various signals that trigger this switch have been discovered. These include

metabolic stress (low pO_2, low pH or hypoglycaemia), mechanical stress (pressure generated by proliferating cells), immune/inflammatory response (immune/ inflammatory cells that have infiltrated the tissue), and genetic mutations (for example, activation of oncogenes or deletion of tumour-suppressor genes that control production of angiogenesis regulators) (Carmeliet *et al.*, 2000).

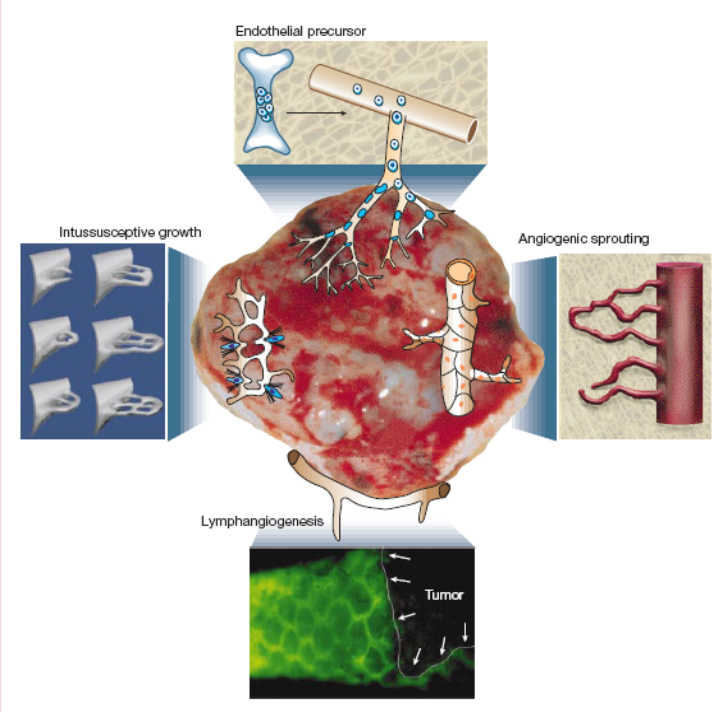


Figure.5: Cellular mechanisms of tumour (lymph) angiogenesis.
Tumour vessels grow by various mechanisms: (1) the host vascular network expands by budding of endothelial sprouts or formation of bridges (angiogenesis); (2) tumour vessels remodel and expand by the insertion of interstitial tissue columns into the lumen of pre-existing vessels (intussusception); and (3) endothelial cell precursors (angioblasts) home from the bone marrow or peripheral blood into tumours and contribute to the endothelial lining of tumour vessels (vasculogenesis). Lymphatic vessels around tumours drain the interstitial fluid and provide a gateway for metastasizing tumour cells (Adapted from Leu *et al.*, 2000).

Fig. 6: Balance of the angiogenic switch. Angiogenesis is regulated by variety of activators

and inhibitors-few of them on the list. When the switch is on quiescent endothelial cell will be activated to sprout new capillaries (angiogenesis) (From Eichhorn *et al*., 2007).

2.2.2 Formation of tumor blood vessels

In mature (non-growing) capillaries, the vessel wall is composed of an endothelial cell lining, a basement membrane, and a layer of cells called pericytes, which partially surround the endothelium. The pericytes are contained within the same basement membrane as the endothelial cells and occasionally make direct contact with them. Angiogenic factors produced by tumoral cells bind to endothelial cell receptors and initiate the sequence of angiogenesis. When the endothelial cells are stimulated to grow, they secrete proteases, heparanase, and other digestive enzymes that digest the basement membrane surrounding the vessel. Degradation of basement membrane and the extracellular matrix surrounding pre-existing capillaries, usually postcapillary venules, is a mechanism mediated by matrix metalloproteinases (MMPs), a family of metallo-endopeptidases secreted by the tumor cells and the supporting cells. The dissolution of extracellular matrix also allows the release of proangiogenic factors from the matrix (Bhushan *et al*., 2002). The junctions between endothelial cells become altered, cell projections pass through the space created, and the newly formed sprout grows toward the source of the stimulus. Endothelial cells invade the matrix and begin to migrate and proliferate into the tumor mass. In this location, newly formed endothelial cells organize into hollow tubes (canalization) and create new basement membrane for vascular stability. The fused and newly established blood vessels form the blood flow within the tumor. The formation of the lumen during canalization is driven by important interactions between cell-associated surface proteins and the ECM. Some of the surface proteins identified in this interaction are hybrid oligosaccharides, galectin-2, PECAM-1, and VE-cadherin (Gamble *et al*., 1999; Yang *et al*., 1999; Nangia-Makker *et al*., 2000). Different situations can provoke an unbalanced shift toward proangiogenic factors such as metabolic and mechanical stresses, hypoxia, and genetic mutations or altered expression of oncogenes or tumor suppressor genes which can stimulate blood vessels growth (Carmeliet *et al*., 1999; Kerbel, 2000).

Although tumors have the ability to induce new blood vessel growth by angiogenesis, the structure of tumor vessels and healthy blood vessels are fundamentally different:

(i) Chaotic architecture and blood flow: Tumor vessels are structurally and functionally abnormal. In contrast to normal vessels, tumor vasculature is highly disorganized, vessels are tortuous and dilated, with uneven diameter, excessive branching and shunts (**Fig. 7 c and d**). This may be due to an imbalance of angiogenic regulators, such as VEGF and angiopoietins. Consequently, tumour blood flow is chaotic and variable and leads to hypoxic and acidic regions in tumors. Although smooth muscle α-actin positive cells surround some tumor vessels, they do not function as normal contractile cells.

(ii) High vascular permeability: In terms of their ultrastructure, tumor vessels are also abnormal: their walls have numerous 'openings' (endothelial fenestrae, vesicles and transcellular holes), because of loss of adherence between endothelial junctions, and a discontinuous or absent basement membrane (**Fig. 7 c and d**). In addition, the endothelial cells are abnormal in shape, growing on top of each other and projecting into the lumen. These defects make tumor vessels leaky. Vascular permeability and angiogenesis depend on the type of tumor and the host organ where the tumor is growing, in part because each organ has different stromal cells which produce different pro- and anti-angiogenic molecules (Fukumura *et al*., 1998; Ellis and Fidler, 1995). Low-permeability tumors may overexpress Ang1 and/or underexpress VEGF or its homologue, placental growth factor (PlGF). Conversely, those with high permeability may lack Ang1 or overexpress its antagonist Ang2 (Jain and Munn, 2000). The induction of vascular permeability is mediated by the redistribution of platelet endothelial cell adhesion molecules (PECAM-1), and vascular endothelial cadherin (VE-cadherin). Some investigators have also revealed the involvement of Src kinases in this process (Carmeliet *et al*., 2000). Vascular permeability allows the extravasation of plasma proteins that constitute a momentary scaffold for migrating endothelial cells. Another very common feature in tumor blood vessels is the presence of focal hemorrhages that occur spontaneously if the tumor cells express VEGF121 or VEGF165 (Cheng *et al*., 1997). The structural aberrations described so far in tumor

vessels are also coupled to molecular and functional disorders such as the overexpression of growth factors, integrins, and the uptake of cationic liposomes (McDonald and Foss, 2000).

(iii) Mosaic vessels: tumor blood vessels may not only consist of blood vessel endothelial cells, they can also incorporate tumor cells onto the vessels. The presence of mosaic vessels has profound implications for metastasis (Chang *et al*., 2000). It has also been reported that tumor vessels can be produced by tumor cells, a process named vascular mimicry (Folberg *et al*., 2004).

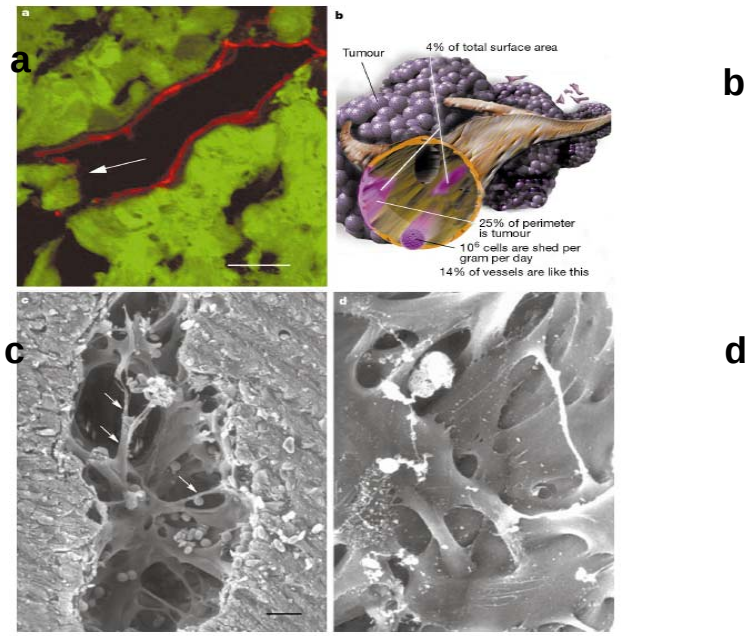


Fig. 7: Chaotic and mosaic vessels in tumours. a, Cancer cell in the lining of a tumour vessel, referred to as a mosaic vessel. Cellular components of the vascular wall in a human colon carcinoma xenograft:cancer cells in green , endothelial cells in red and lectin fluorescence to mark perfused vessels. The width of the endothelial gap exposing cancer cells to the vessel lumen is about 20 mm. (Adapted from Chang, 2000.). b, Mosaic vessels. In colon carcinoma ~15% of tumour vessels are mosaic in nature, and cancer cells occupy ~4% of the total vascular surface area. If each of these cells intravasate in 2 days, the tumour will shed about 10^6 cells per day per gram of tumour . c and d, Scanning electron microscopy of the luminal surface of a blood vessel in a murine mammary tumour showing various abnormalities. c,The abnormal endothelial cells that partition the lumen (arrowheads); d, multiple intercellular openings (arrows) of the order of 1–5 mm (Carmeliet *et al,*, 2000).

(iv) Non-uniform surface markers.

Cytokines and angiogenic molecules secreted by cancer and immune cells can modulate the expression of cellular adhesion molecules and other surface markers on

the tumor endothelium. For example: VEGF and tumor-necrosis factor-α (TNF-a) upregulate whereas basic fibroblast growth factor (bFGF) and transforming growth factor-β1 (TGF-β1) downregulate adhesion molecules (Jain, 1996). Chaotic blood supply coupled with non-uniform expression of adhesion molecules may explain why leukocyte-endothelial interaction is low in tumors and why activated lymphocytes adhere non-uniformly to tumor vessels. It is possible that tumor vessels express surface proteins that are absent or barely detectable in mature vessels. *In vivo* selection of phage display libraries has recently yielded peptides (amino acid sequences RGD and NGR) that preferentially recognize vessels in subcutaneous tumors in mice (Arap *et al*., 1998). These peptides can be used to target therapeutic agents to tumors. The challenge now is to discern how specific these 'vascular zip codes' are, as targeting drugs to the tumor vasculature has the potential to change the paradigm for cancer treatment.

It is known that the growth and metastasis of malignant tumors depends on neovascularization. It has also been suggested that the degree of tumor angiogenesis is related to clinical outcome in several tumor types. This is true for gastric carcinoma, where tumor angiogenesis is closely correlated with prognosis and hematogenous metastasis. It is an open question whether these vessels result from cancer cells invading the vessel lumen, from cancer cells mimicking endothelial cells ('vasculogenic mimicry'), from co-opted vessels or from the apoptosis of endothelial cells which exposes underlying cancer cells. Regardless of the mechanism involved, the presence of cancer cells in tumor vessels has significant implications for metastasis.

2.3 Lymphangiogenesis and metastasis

2.3.1 Lymphatic vessel formation

The lymphatic vascular system is widely distributed in nearly all tissues and organs with exceptions of the central nervous system, retina, bone marrow and placenta. The lymphatic capillaries consist of a thin layer of endothelium poorly coated with

pericytes/vascular smooth muscle cells (VSMCs) and with a discontinuous basement membrane, their physiological function is to collect extravasated fluid, macromolecules, and leukocytes to regional lymph nodes for immune surveillance, and finally transport them to the circulation (Kuchler *et al*., 2006). During embryonic development, the first lymphatic vessel is formed from the cardinal vein through differentiation of blood vessel endothelial cells (BVECs) into the lymphatic lineage. The lymphatic endothelial cells usually are organized as overlapping flaps that function as valves to prevent efflux of lymphatic fluids. These VSMCs lacking lymphatic capillaries are anchored to the extracellular matrix through the elastic fibers, controlling the drainage function. However, the larger conduit lymphatics could be coated with VSMCs and relatively elastic and resistant to permeability. Like blood vessel endothelial cells, lymphatic endothelial cells (LECs) also express endothelial nitric oxide synthase (eNOS), which might influence the contractile and transport activity.

2.3.2 The lymphangiogenic switch during tumorigenesis

Similar to blood vessels, lymphatic vasculatures in most adult tissues and organs remain quiescent under physiological conditions. But it seems likely that acquisition of new lymph vessels to supply the tumor is triggered at some point during the development of the tumor. Lymphangiogenesis, a complex process of sprouting of new lymphatic vessels, is regulated by multiple direct and indirect growth factors (**Fig. 8**). A ‘‘lymphangiogenic switch’’ in tumors might represent a mirror image of an angiogenic switch with imbalance of overproduction of lymphangiogenic factors and downregulation of lymphangiogenesis inhibitors (Cao *et al*., 2005).

Recently, a number of direct and indirect lymphangiogenic factors produced by tumor cells, stromal cells, or inflammatory cells have been identified. These include VEGF family factors such as VEGF-C, VEGF-D, VEGF-A and non-VEGF lymphangiogenic factors such as fibroblast growth factor 2 (FGF-2), platelet-derived growth factors (PDGFs), angiopoietin-1, angiopoietin-2, hepatocyte growth factor (HGF) and

insulin-like growth factor (IGF). The VEGF-C/VEGF-D–VEGFR3 pathway is the best characterized signaling system. It has a vital role in the budding of initial lymphatics from Prox1-expressing vein endothelium. Elimination of either VEGF-C or Prox1 genes in mice results in failure to form the initial lymphatic vasculature in embryos (Karkkainen *et al.*, 2004; Wigle *et al.*, 2002). These factors seem to have interdependent or collaborative roles with each other or with VEGFs in the establishment of functional lymphatics.

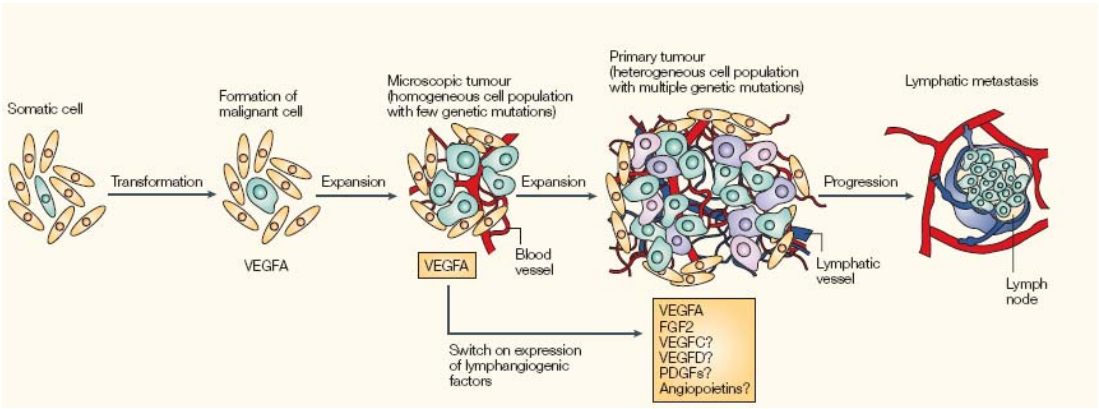


Fig. 8: The haemangiogenic switch and potential lymphangiogenic switch. Mutations of vital oncogenes and tumour-suppressor genes cause the transformation of a normal somatic cell into a tumor cell. At early stages of malignancy, a tumor at microscopic size contains a relatively homogenous cell population with a limited number of mutations in their genome. The tumor cells might only produce vascular endothelial growth factor A (VEGFA) as an angiogenic factor. However, during tumor progression, genomic instability of tumor cells often leads to the accumulation of genetic alterations that switch on the expression of multiple angiogenic and potentially lymphangiogenic factors and therefore promote cancer metastasis. Switching on of lymphangiogenesis during tumor progression is still a hypothesis. FGF2, fibroblast growth factor 2; PDGF, platelet-derived growth factor (Cao *et al.*, 2005).

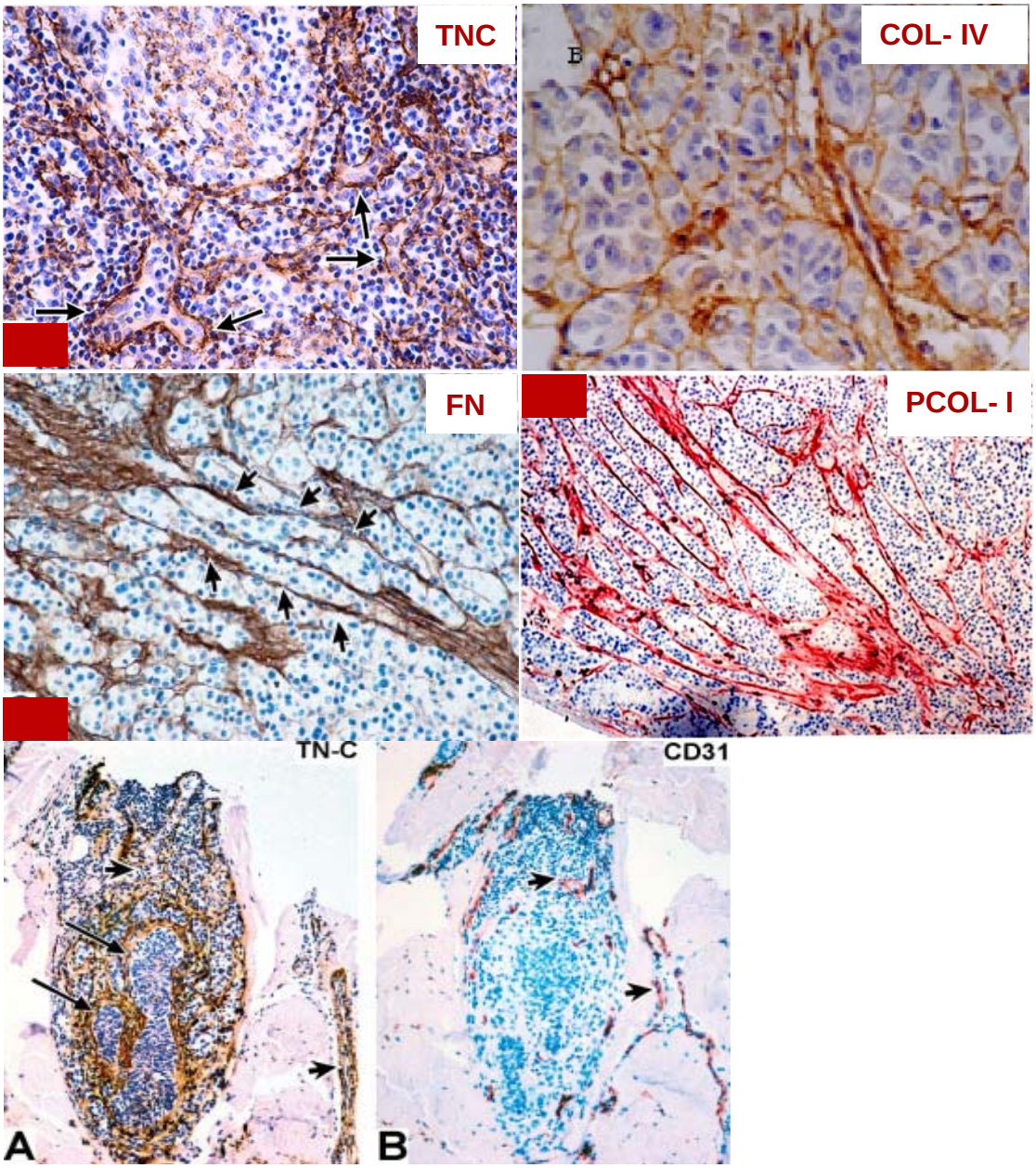
Metastasis of malignant tumors to regional lymph nodes is one of the early signs of cancer spread in patients, and it occurs at least as frequently as hematogenous metastasis. Particularly, in cancers, such as breast cancer, lymphatic metastasis is a predominant route for tumor spread. From the lymphatic system, cancer cells might enter the circulation and spread to distal organs/tissues via blood vessels. Dissemination of tumor cells from the primary sites to the lymphatic system is accomplished either by invasion into pre-existing lymphatic vessels in the surrounding tissues or by invasion into intratumoral lymphatic networks. Tumor-produced

lymphangiogenic factors are able to co-opt and dilate the pre-existing lymphatics surrounding the tumor tissue. Some of these factors are able to facilitate the transmigration of tumor cells through the lymphatic endothelium. In addition to facilitating dissemination of tumor cells from their primary sites, all known lymphangiogenic factors probably are able to stimulate the regrowth of tumors in the lymph nodes because they seem to simultaneously induce blood vessel growth (Cao *et al.*, 2007).

2.4 Vasculogenic mimicry

2.4.1 Concept of vasculogenic mimicry

In 1999, the term "vasculogenic mimicry" (VM) was introduced to describe the masquerade of tumor cells as endothelial cells. This process of cell plasticity occurs mainly in aggressive tumors in which tumor cells dedifferentiate to an endothelial phenotype and make tube-like structures. This mechanism provides tumor cells with a secondary circulation system of vasculogenic structures lined by tumor cells, independent of angiogenesis. This phenomenon was described for the first time in melanoma. Tissue sections of uveal and cutaneous melanomas and their respective liver metastases revealed patterned networks of interconnected loops of extracellular matrix, as identified by periodic acid-Schiff's reagent (PAS) staining. Importantly, the presence of PAS patterns was associated with worse patient outcome (Folberg *et al*., 1993). PAS-positive structures stained for tenascin-C, laminin, collagens IV and VI, mucopolysaccharide, and heparin sulfate glycoproteins (HSPG) (**Fig. 9).** Light microscopy, transmission EM, and immunohistochemical staining revealed that PAS-positive channels are lined externally by melanoma cells but have no inner lining of endothelial cells. The same patterned networks could be obtained *in vitro* in collagen and matrigel three-dimensional cultures with aggressive melanoma cell lines but not with poorly invasive melanoma cell lines (Maniotis *et al*., 1999).



(Adapted from Kääriäinen *et al*., 2006)

Fig. 9: Tubular channels in metastasizing melanoma: Stained for TN-C, collagen IV, fibronectin, and procollagen-I but not for CD31.

2.4.2 Molecular mechanisms underlying vasculogenic mimicry

Compared to less aggressive melanoma cells, highly aggressive melanoma cells express higher levels of matrix metalloproteinases (MMP-1, 2, 9, and 14) and laminin 5γ2, fibronectin, collagen IV α 2 and collagen I. This increased expression of MMPs and the presence of the laminin receptor on the surface of tumor cells can help cells to

adhere to laminin. Activated MMPs can cleave laminin into several short fragments and may eventually promote the formation of VM. PI3-kinase (PI3K) was shown to modulate the function of MMP-14 (MT1-MMP), which activates MMP-2 with the help of the tissue inhibitor of MMP-2 (TIMP2), and the activated MMP-2 then cleaves 5γ2 chain into $\gamma^{2'}$ and γ^{2X} chains. These two promigratory fragments in the ECM can promote formation of VM channels (Seftor *et al*., 2001).

2.4.3. Effect of the tumor microenvironment on the formation of tubular channels

The importance of the extracellular matrix, as a component of the microenvironment, in vasculogenic mimicry was demonstrated (Seftor *et al*, 2005). Normal epidermal melanocytes, exposed for 4 days to an extracellular matrix conditioned by metastatic cutaneous melanoma, were reprogrammed to express specific genes that were associated with the ability to form vasculogenic-like networks. Importantly, these changes in gene expression were only transient, because gene analysis after 7 to 21 days revealed a normal melanocyte phenotype. Recent findings suggested that another microenviromental component, oxygen, may be essential in melanocyte transformation. Low levels of oxygen or hypoxia, are known to promote melanoma cell invasion, metastasis and transformation (Rofstad *et al*., 2002). Moreover, hypoxia induces vasculogenic mimicry tube formation *in vitro* in a matrigel assay (Rybak *et al*., 2003). The role of several known tumor growth factors has also been studied, although with negative results. Several growth factors, such as basic fibroblast growth factor, vascular endothelial growth factor, transforming growth factor-β, platelet derived growth factor and tumor necrosis factor-α were found not to be able to induce formation of vascular networks when added to the poorly invasive melanoma cell lines (Maniotis *et al*., 1999). This indicates that angiogenesis and vasculogenic mimicry, in contrast to the previous described tumor vascularization types, are not sharing the same signaling pathways. Moreover, anti-angiogenic targeting strategies do not inhibit the process of vasculogenic mimicry and could even induce the formation of vasculogenic mimicry vessels as an escape mechanism of the tumor to keep on growing.

2.4.4 Vasculogenic mimicry and hematogeneous metastasis

The unique structure of VM channels facilitates the hematogeneous metastasis of tumor cells. Tumor cells, which line the inner surface of VM channels, are directly exposed to blood flow. Tumor cells that leak out can migrate through the bloodstream and metastasize to other organs. Furthermore, tumor cells that line the VM channel are highly malignant, poorly differentiated, and have high plasticity. These cells can degrade adjacent connective tissues and penetrate the basement membrane of blood vessels by secreting proteins that mediate tumor invasion and metastasis. Several studies (Maniotis *et al*., 1999; Sun *et al*., 2004) have demonstrated that VM is associated with poor clinical prognosis in patients. The 5-year survival rate of the cases with VM is close to 0%.

3. Cancer as a tissue disease — role of the tumor stroma in cancer

A "tumor" consists of more than a collection of cells, but also includes stroma — the extracellular and cellular tissue framework that surrounds and interacts with the embedded cells. The composition of tumor stroma can vary significantly from tumor type to tumor type and from location to location, suggesting that stroma formation depends on a complex set of interactions between cancer cells, tumor-associated cells and the ECM.

Strong evidence now exists that perturbations in the normal host compartment may drive tumorigenesis (Tlsty and Coussens, 2006). Stroma is associated with cancer cells at all stages of cancer progression (Kalluri and Zeisberg, 2006), and cancer development depends on the activation of stroma (**Fig. 10**). Indeed, neoplasia can be considered as a pathological imbalance of "tissue-cell societies" (Hanahan and Weinberg, 2000; Park *et al*., 2000). Malignancy is a state that emerges from a tumor-host microenvironment in which the host participates in the induction, selection and expansion of the neoplastic cells (Coussens and Werb, 2002; Bhowmick and Moses, 2005). Rather than being renegades, malignant tumor cells recruit vasculature and stroma through production and secretion of stimulatory growth factors and cytokines (Browne *et al*., 1999). The locally activated host microenvironment (both

cellular and extracellular elements) in turn modifies the proliferative and invasive behavior of the tumor cells. It has been shown that growth and malignant behavior of tumor cells can be regulated at the level of the tissue organization (Weaver *et al*., 1997; Maffini *et al*., 2005), i.e. the tissue structure determines the phenotype which in turn overrides the cellular phenotype (**Fig. 10**). So cancer can be considered as a tissue disease.

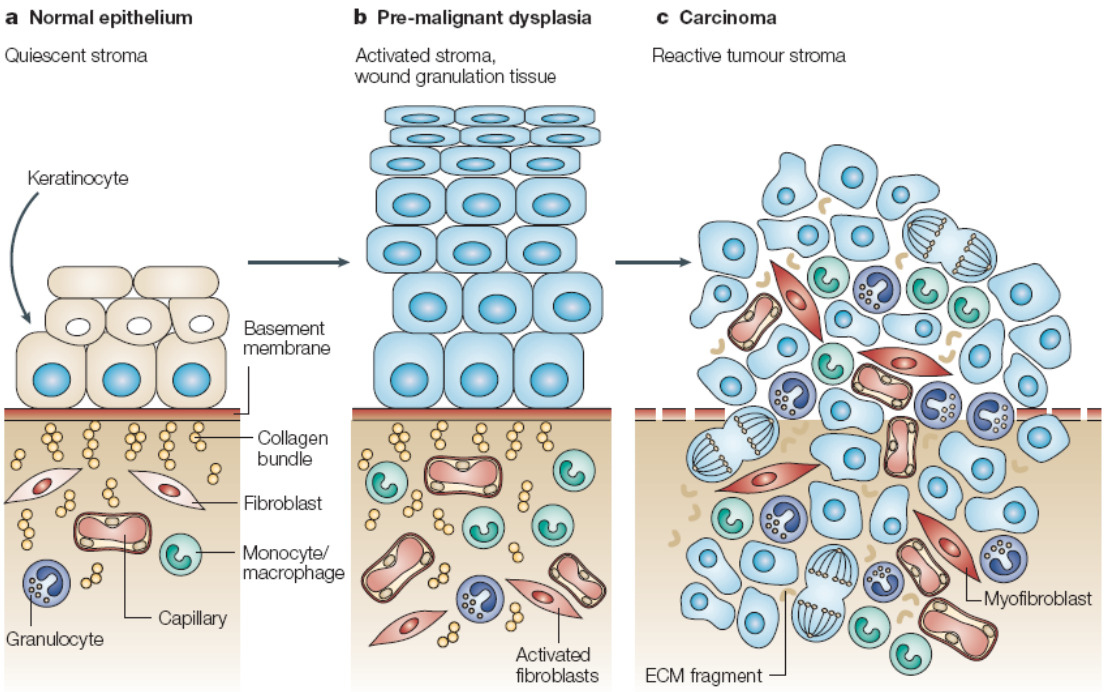


Fig. 10 Tumour stage depends on stromal activation. a**,** A normal well-differentiated stratified epithelium, made up of cells such as keratinocytes in the epidermis, is separated by a well-delineated basement membrane from the dermal or stromal compartment. This stromal compartment normally contains collagen bundles that surround resting fibroblasts, mature blood vessels encircled by an uninterrupted basement membrane (capillary), and a few resident leukocytes (monocytes and macrophages). b. During transition to pre-malignant dysplasia, differentiation of epithelial cells is disturbed, resulting in a hyperplastic epithelium (accumulation of blue cells). The basement membrane remains intact, separating the epithelium from a stromal compartment, which contains intact collagen bundles. Fibroblasts, however, become activated, and the number of macrophages increases. The transient angiogenesis that occurs initially during establishment of the transplant is followed by vessel maturation, resulting in a vasculature similar to the one seen with normal epithelia. c. Progression to a carcinoma is associated with proliferation of epithelial cells (mitotic cells) along with the development of an activated tumour stroma. In this case, ECM components such as collagen bundles are degraded, because of increased turnover. The number of inflammatory cells increases and fibroblasts differentiate into myofibroblasts, resulting in their expression of growth factors, matrix components and degrading proteases. Angiogenesis is maintained,

resulting in a high number of leaky tumour vessels. Following activation of a tumour stroma with persistent angiogenesis, invasion by tumour cells begins through the degraded basement membrane, and blood vessels infiltrate the tumour tissue (excerpted from Mueller and Fusenig, 2004).

As mentioned above, tumors consist of multiple cell types that signal to each other — cancer cells, vascular cells, stromal cells, inflammatory cells, and immunocytes, and all these different types of cells are meshed within an ECM that provides the scaffold tying them all together. The ECM is formed by a complex of self-assembling macromolecules, composed predominantly of collagens, non-collagenous glycol-proteins, elastin, hyaluronan and proteoglycans. Besides being a scaffold for the cells, the ECM serves as a reservoir for growth factors and cytokines and influences their activation status and turnover. As growth factors interact with ECM molecules, they may become sequestered from their signaling receptors and activated, e.g., by proteolytic processing, and they may be presented to the cells in a specific manner which alters their biological function. Beyond this, recent investigations show that extracellular matrix proteins may exert a direct signaling function, either by means of interactions with matrix receptors such as integrins or via direct interaction with growth factor receptors. This network of interactions has recently been investigated in detail for the group of ECM proteoglycans, and raises the discussion of ECM to a novel level of complexity.

3.1 ECM of cancer

It is getting more and more evident that ECM is a key "signaling molecule" crucial for the normal functioning of cells. That is, the ECM is one of the environmental factors (along with hormones) that communicate with a cell nucleus, modifying nuclear structures and leading to selective gene expression and thus determining the tissue phenotype (Lelievre *et al.*, 1998). This implies that alterations in the ECM or cellular responses to it could lead to malignancy. Using mammary gland development as a model for cancer progression, Bissell and her colleagues have demonstrated that ECM

modification could alter acinar formation and branching. Modification of cell-ECM interactions by an integrin blocking antibody can reverse the malignant phenotype of human breast cancer cells (Weaver *et al*., 1996) and modification by MMP-3 acts as a carcinogen because of its ability to induce EMT and genetic instability in mammary epithelial cells (Radisky *et al.*, 2005). In cancer the composition of ECM becomes aberrant, with expression of SPARC, osteopontin, fetal fibronectin, tenascin-W and tenascin-C amongst other ECM molecules.

3.2 Importance of the extracellular matrix in metastasis

Interactions governing cancer progression extends beyond simple cellular interactions. The emergence of fibroblasts as major players in cancer and metastasis highlights the role of extracellular matrix in these processes. Matrix components can be laid down by both cancer cells and various host cells, but fibroblasts are the prominent source of ECM in the body. Many of the effects fibroblasts have on cancer progression are likely to be mediated by its deposition of ECM and generation of growth factors. The increased proliferation of cancer-associated fibroblasts and the resulting change in matrix composition may have prominent effects on metastasis. ECM composition may determine whether a particular organ site is conducive to metastatic growth (Chung *et al*., 1988). For example, the establishment of premetastatic niches coincides with an increased deposition of fibronectin (Kaplan *et al*., 2005), suggesting that matrix composition may be one of the bookmarks recognized by the circulating cancer cells. Experiments have also demonstrated that metastasis is inhibited by the ectopic overexpression of tissue inhibitors of metalloproteinases (TIMPs) at sites of metastasis, demonstrating that changes in the matrix composition can block the metastatic potential (Kruger *et al*., 1997, 1998). The modulation of ECM by matrix metalloproteinases and its inhibitors plays an important role in cancer progression, as the expression level of specific matrix modulators has been observed to coincide with the metastatic potential of a cancer. However, different cancers appear to have varying dependencies on the palette of matrix modulators available at its disposal. The metastatic potential of transformed rat cell lines was found to correlate with the

expression levels of MMP-3 and -10 but not MMP-2 and -9 (Sreenath *et al*., 1992). The importance of host genetic background in cancer and metastasis is further supported by the degree of metastasis associated with polymorphisms in the matrix metalloproteinase promoters (Ye, 2000).

3.3 Tenascin-C

3.3.1 Tenascin- C and family members

Tenascin-C is the founding member of a family of extracellular matrix glycoproteins comprising tenascin-X, -R, -Y and -W in addition to tenascin-C (Chiquet-Ehrismann *et al*., 1994). It was discovered simultaneously in a number of laboratories in the 1980s as glioma mesenchymal extracellular matrix protein and as myotendinous antigen in the connective tissue. Its name has been created by Ruth Chiquet-Ehrismann in 1986 and represents a combination of the Latin verbs “tenere” (to hold) and “nasci” (to grow, develop, to be born), which provided the roots of English words “tendon” and “nascent” and reflected the location and developmental expression of the protein observed at that time. Tenascin-C is transiently expressed during fetal development and absent or greatly reduced in most adult tissues. However, it increases markedly in pathological conditions, including inflammation, wound healing and cancer (Chiquet-Ehrismamm and Chiquet, 2003).

3.3.2 Structure of tenascin-C

The tenascin-C protein, 2201 amino acids in length giving rise to a 190-300 kDa monomer, is encoded by 6603 bp of nucleotides, which is organized into at least 28 exons on chromosome 9q33. Tenascin-C is a modular molecule consisting of a N-terminal region containing a chaperone-like sequence forming coiled coil structures and interchain disulfide bonds that are essential for subunit to oligomerize into hexamers. Tenascin-C is comprised of 14.5 epidermal growth factor (EGF)-like repeats, 30-50 amino acids in length, which contain six cysteine residues involved in intrachain disulfide bonds. In tenascin-C, up to 17 fibronectin type III domains are present that are about 90 amino acids in length and that are composed of seven antiparallel β-strands arranged in two sheets. The nature and number of fibronectin

type III domains in tenascin-C is generated by alternative splicing that is modulated by the proliferative state of a cell, extracellular pH, and TGFβ1. At least nine different fibronectin type III domains are differentially included or excluded by RNA splicing. This can generate a considerable diversity among different cancers and can cause variable cell responses toward tenascin-C. The C-terminal fibrinogen globular domain resembling the β- and γ-chains of fibrinogen is 210 amino acids in length, and forms intrachain disulfide bonds (**Fig. 11**).

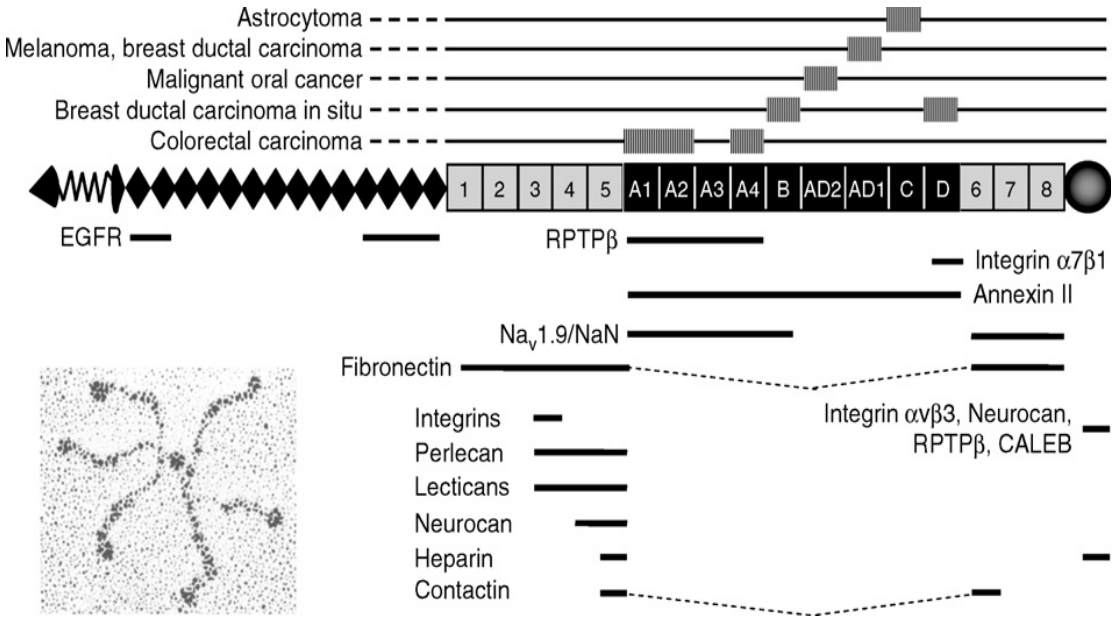


Fig. 11 Domain structure: binding partners and expression of tenascin-C in cancer tissue.

The N-terminal oligomerization, EGF-like, fibronectin type III and fibrinogen-like domains are schematically depicted as triangle, rhombomeres, boxes and circles, respectively. The alternatively spliced fibronectin type III domains A1-D are shown in black. An electromicrograph of a tenascin-C hexamer is shown at the left corner. Fibronectin type III domains specifically detected in certain cancers are highlighted above the model. EGFR, epidermal growth factor receptor; CALEB, chicken acidic leucine-rich EGF-like domain containing brain protein; RPTPβ, receptor protein tyrosine phosphatase-β/ζ; Nav1.9/NaN, sodium channel subunit β2 (Orend and Chiquet-Ehrismann, 2006).

3.3.3 Tenascin-C induction and interaction partners

Tenascin-C can be induced in a tumor by various pro- and anti-inflammatory cytokines and growth factors that are mostly secreted by stromal cells. In addition, hypoxia, reactive oxygen species, and mechanical stress, which are also present in tumor tissue,

induce tenascin-C expression. In contrast, glucocorticoids suppress tenascin-C expression. Signaling causing activation of transcription factors such as TCF/LEF, NfkB, c-Jun, Ets, SP1, and Prx-1 are involved in tenascin-C gene transcription. Tenascin-C is cleaved by matrix metalloproteinases and serine proteases, thus potentially releasing cryptic sites within the fibronectin type III domains of tenascin-C. Cell contact with tenascin-C also induces expression of matrix metalloproteases, thus presenting a positive feedback loop between induction of matrix metalloproteases by tenascin-C and cleavage of tenascin-C by these enzymes. Tenascin-C binds to extracellular matrix molecules such as fibronectin, perlecan, aggrecan, versican, and brevican (**Fig. 10**), thus potentially forming a tumor-specific ECM network. Cells can interact with tenascin-C via cell surface receptors including integrins α2β1, α7β1, α9β1, and αvβ3, syndecan, annexin II, and epidermal growth factor receptor (EGFR) amongst others (**Fig. 11**).

3.3.4 Tenascin-C and cancer

3.3.4.1 Tenascin-C expression in tumor stroma

Tenascin-C is one factor in the tumor-specific microenviroment and is expressed by both transformed epithelial cells and stromal cells. Tenascin-C is highly expressed in the majority of malignant solid tumors, including those arising in the brain, breast, uterus, ovaries, prostate, pancreas, colon, stomach, mouth, larynx, lung, liver, kidney, bladder, skin, bone, soft tissues, and in lymphomas (Orend and Chiquet-Ehrismann, 2006). As well as an increase in the overall level of tenascin-C in malignant tumor tissues, certain alternatively spliced fibronectin type III repeats are also expressed in a cancer tissue-specific manner. In cancers such as glioma, breast, colon and lung carcinoma, a high tenascin-C expression correlates with a low survival prognosis (Orend and Chiquet-Ehrismann, 2006).

3.3.4.2 Effect of tenascin-C on cell rounding and tumor cell proliferation

Tenascin-C has distinct effects on tumor cells, and tumor-associated cells such as carcinoma-associated fibroblasts, tumor-associated macrophages, and endothelial cells

within the tumor stroma based on as yet poorly understood cell type-specific responses toward tenascin-C splice variants. Tenascin-C contains adhesive and antiadhesive sequences that coexist in the native molecules. These opposing activities arise as a consequence of tenascin-C binding to extracellular matrix components and to cell surface receptors. One mechanism that induces cell rounding involves tenascin-C inhibition of cell adhesion to fibronectin. This occurs through competitive binding of tenascin-C to fibronectin, thus masking the binding site for the integrin $\alpha_5\beta_1$ coreceptor syndecan-4 (Huang *et al*., 2001). This blocks activation of the small GTPase RhoA and focal adhesion kinase. Activation of oncogenic Wnt signaling, endothelin receptor type A, and MAPK signaling induced by tenascin-C and elimination of G0 and G1 cell cycle transition control could contribute to enhanced tumor cell proliferation by tenascin-C (Orend, 2005).

3.3.4.3 Potential role of tenascin-C in metastasis

Tenascin-C is expressed around invasive carcinoma cells that have undergone EMT. Tenascin-C provides a substratum that supports migration of several cell types including glioma and laryngeal carcinoma cells (De Wever *et al.*, 2004). A mechanism by which tenascin-C supports colon carcinoma cell invasion involves secretion of tenascin-C by carcinoma-associated fibroblasts, activation of EGFR and, expression of hepatocyte growth factor and activation of its receptor c-Met. This triggered activation and inhibition of the small GTPases Rac and RhoA, respectively in the invading carcinoma cells. In addition to an EMT-associated migration, tenascin-C might also promote other forms of migration in cancer cells.

3.3.4.4 Potential role of tenascin-C in tumor angiogenesis

Tenascin-C plays a role in embryonic vascularization and promotes vascular sprouting. It is also expressed in the vascular smooth muscle cells during formation of new blood vessels in the adult as, e.g., in granulation tissue of wounds after myocardial infarction, in arthritis, and in neoplastic diseases (Mackie, 1994). In human gliomas, tenascin-C expression correlates with the degree of tumor neovascularization (Jallo *et al*., 1997).

Tenascin-C may promote angiogenesis by serving as chemo-attractant for endothelial cells, by initiating endothelial cell differentiation, survival, and proliferation (Schenk *et al*., 1999), events that involve integrin αvβ3 and vascular endothelial growth factor among not yet identified other molecules. It was suggested that tenascin-C has a positive effect on angiogenesis by stimulating VEGFA expression thus causing endothelial cell migration and proliferation and subsequently the formation of capillaries in the tumor. Studies with melanoma cells xenografted into tenascin-C knockout mice showed that tumor growth and angiogenesis was strongly reduced (Tanaka *et al*., 2004).

3.3.4.5 Tenascin-C modulates tumor-specific immunity

Established tumors can prevent clearance by suppressing endogenous immune mechanisms which allow for escape from tumor-specific immunity. Tenascin-C may be critical for immunosuppression observed in cancers since it is highly expressed in most solid tumors. Tenascin-C also has the ability to inhibit activation of T-lymphocytes by natural antigens which has been shown in vitro. This is done by modulating T-lymphocyte behavior via blocking of specific integrin-mediated cell adhesion to fibronectin. It is so far not clear how tenascin-C induces escape from tumor immunosurveillance but it may influence proliferation and survival of hematopoietic cells (Orend and Chiquet-Ehrismann, 2006).

4. Aim of the study

The objective of this study was to investigate whether ectopic expression of tenascin-C affects tumor progression. Here, we used the well characterized multistage carcinogenesis RT2 model. These mice reproducibly develop β cell tumors in a multistage tumorigenesis pathway involving islet hyperplasia, angiogenic hyperplasia and solid insulinoma formation due to ectopic expression of the SV40 large T antigen. Transgenic RipTNC mice with ectopic expression of human TNC in the pancreatic

β-cells under the control of the rat insulin promoter should be generated. The single transgenic RipTNC mice should be analyzed with regard to different parameters including transgene expression, tissue homeostasis, cell sorting and angiogenesis. Upon crossing with RT2 mice double transgenic RT2/TNC mice should be established. In RT2/TNC mice, it should be determined whether ectopically expressed TNC affects tumor cell proliferation, apoptosis, migration and invasion, as well as tumor incidence, tumor volume, angiogenesis and metastasis.

II Materials and Methods

1 Materials

1.1 Materials for preparation of the transgenic mice

Plasmids and vectors	**Sources and Provider**
pCEP-pu/huTNW	Chiquet-Ehrismann, FMI, Basel, Switzerland
PcDNA3.1/Hygro(-)	In Vitrogen
Rat insulin promoter 1 (Rip1) vector	Gerhard Christofori, DKBW, Basel, Switzerland
Kits	
QIAEXII gel extraction kit	Qiagen, Hombrechtikon, Switzerland
QuikChange II XL Site-Directed MutagenesisKit	Stratagene, La Jolla, CA, USA
BigDye® Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems, Foster City, CA, U.S.A.
Gene Clean Spin Kit	QBiogene, Luzern, Switzerland
lipofectamine ^{TM} 2000	Invitrogen, Grand Land, NY, USA
T4 DNA ligase	Boehringer Mannheim/Roche, Mannheim, Germany

1.2 Reagents used for immunohistochemistry:

Antigen	**Species**	**Dilution**	**Provider**
Primary antisera			
Anti-huTNC (B28-13)	Mouse	1:10	Chiquet-Ehrismann, FMI, Switzerland
Insulin	Guinea pig	1:100	Dakoytomation, Glostrup, Denmark
PECAM-1/CD31	Rat	1:40	PharMingen, Franklin Lakes, USA
E-cadherin	Rat	1:200	Zymed, South San Diego, CA, U.S.A.
β-catenin	Mouse	1:200	BD Transduction Laboratories, Germany
Secondary antisera and other chemicals			
Alexa 568	Goat anti-guinea pig	1:400	Molecular Probes, Eugene, OR, USA
Alexa 488	Goat anti- mouse	1:400	Molecular Probes, Eugene, OR, USA

Alexa 488	Goat anti- rat	1:400	Molecular Probes, Eugene, OR, USA
	Goat anti-rabbit	1:400	Molecular Probes, Eugene, OR, USA
Biotinlated antibody	Goat anti-mouse	1:200	Vector, Burlingame, CA, U.S.A
	Goat anti-rat	1:200	Vector, Burlingame, CA, U.S.A
	Goat anti-rabbit	1:200	Vector, Burlingame, CA, U.S.A
4`,6-Diamidino-2-phenylindole (DAPI)		1:1000	Invitrogen, Grand Land, NY, USA
ABC kit			Vector, Burlingame, CA, U.S.A
OCT compound Tissue-Tek			Torrance, CA, U.S.A.
3',3-Diaminobenzidine tetrahydrochloride (DAB)			Sigma chemical Co., St. Louis, MO, U.S.A.
3-amino-g-ethylcarrbazole (AEC)			Vector, Burlingame, USA
Entellan			Merck, Haar, Germany

2. Methods

2.1 Construction of the expression plasmid, generation of transgenic RipTNC mice and genotyping

The 7.5 kbp sequence of human TNC harboring a polyadenylation signal was removed from the HxBL.pBS plasmid by restriction cleavage with Not I and Kpn I and transferred into the intermediate pcDNA3.1/Hygro(-) vector. The resulting plasmid was cleaved with Xba I and Hind III before ligation behind the rat insulin II promoter into the corresponding cleavage sites of the 4.5 kbp RIP I vector containing an intron sequence (**Fig. 2.1**). Successful cloning was confirmed by restriction digestion and partial sequencing of 700 bp around the start and the stop codon. Expression and secretion of the transgene was determined upon 48 h transient transfection of the expression plasmid into βT2 cells by IF for human TNC with the B28.13 antibody in the absence of detergent (**Fig. 2.2**). Secretion of human TNC was also determined by sandwich ELISA on conditioned medium from βT2 cells 48 h after transfection using the B28.13 antibody for capturing and the TNC1.2.antibody for detection of bound

human TNC (**Fig.2.3**). The RipTNC expression vector was transferred into fertilized oocytes upon linearization with Sal I giving rise to 3 transgenic lines with stable expression and transmission of the transgene. Transgenic mice were identified by PCR with primers revP1 hTNC mice (5`-GAA AGA CAC CTG CCA ACA GC-3`) and RipES/VD: 5`-TAA TGG GAC AAA CAG CAA AG-3).

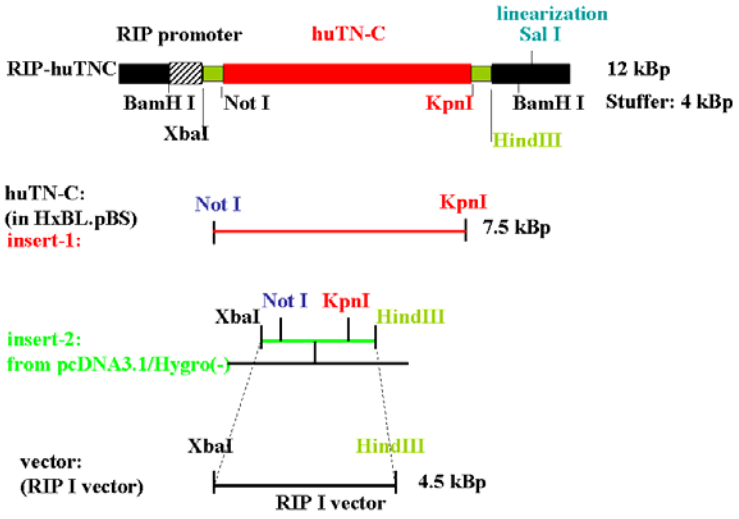


Fig. 2.1 Construction of Rip-huTNC.

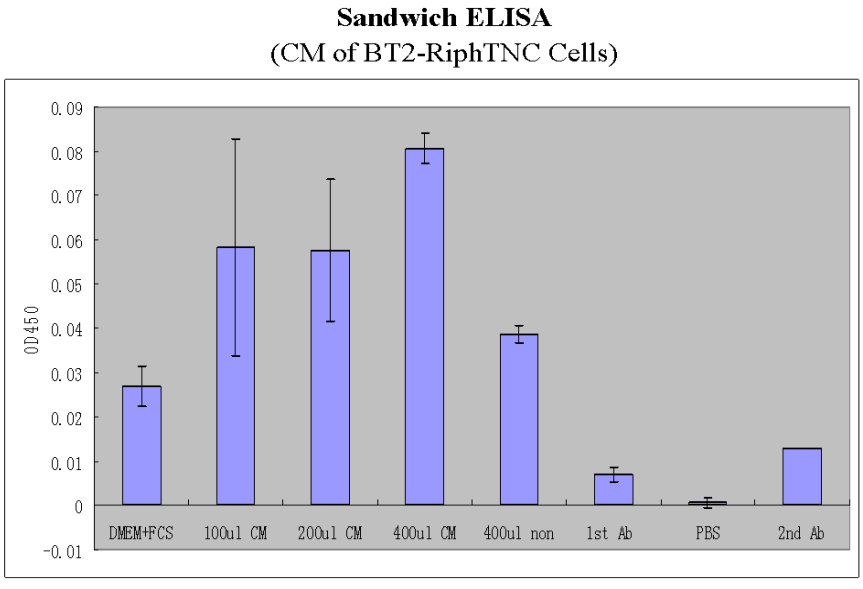


Fig. 2.2 Expression analysis of tenascin-C by Sandwich-ELISA.

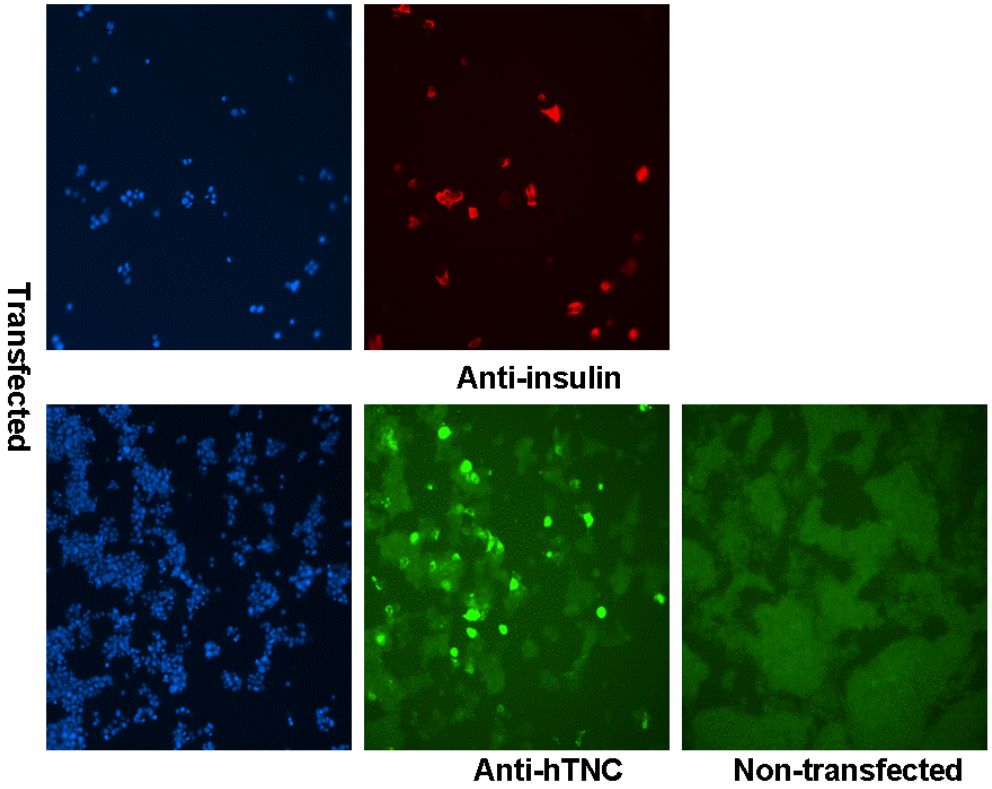


Fig. 2.3 Expression of tenascin-C in βT2 cells.

2.2 Mouse tissue processing

Animal care was taken in accordance with Swiss Animal Protection Ordinance issued by the Swiss Federal Veterinary Office. All mice were sacrificed by cervical dislocation, and pancreata were isolated and washed first in PBS. Tumors and pancreata were either embedded in OCT and freshly snap frozen in liquid nitrogen or fixed overnight in 4% paraformaldehyde in PBS, dehydrated in a Microm Spin Tissue Processor STP-120 and paraffin-embedded. 5 µm fresh frozen sections were methanol-fixed for 10 min at –20°C or fixed in 70% ethanol for 1 min at room temperature prior to storage at –80°C. 5 µm paraffin-embedded tissue sections were deparaffinized and rehydrated prior to usage according to standard procedures.

2.3. Histopathological analysis

Histologic sections were analyzed by hematoxylin and eosin staining, immunohistochemistry and immunofluorescence. The following antibodies were used for immunohistochemistry or immunofluorescence on paraffin and frozen sections:

mouse anti-human tenascin-C (B28-13), rat anti-mouse β-catenin, rat anti-mouse E-cadherin, guinea pig anti-insulin, rat anti-mouse CD31, FITC-conjugated goat anti-rabbit antibody, biotinylated goat anti-mouse antibody, biotinylated goat anti-rat antibody, and biotinylated goat anti-rabbit antibody. All biotinylated secondary antibodies for immunohistochemistry were used at a 1:200 dilution, and positive staining was visualized with the ABC horseradish peroxidase kit and DAB or AEC Peroxidase Substrate according to the manufacturer's recommendations. For analysis of tissue morphology, slides were briefly counterstained with hematoxylin or eosin. Alexa Fluor 568- and 488-labeled secondary antibodies diluted 1:400 were used for immunofluorescence analysis. 6-Diamidino-2-phenylindole (DAPI) diluted 1:1000 was used for nuclear staining. All paraffin-embedded sections were subject to antigen retrieval with 10 mM citrate buffer (microwave) except for insulin (10 min in 0.2% Triton X-100 in PBS), and CD31 (10 min 0.1% Proteinase K (Fluka, Switzerland) in PBS at 37°C). For each staining, sections of control mice or respective serial sections of transgenic mice without primary antibody incubation were used as negative control. Stained sections were viewed on a Axioskop 2 plus light microsope (Zeiss, Feldbach, Switzerland) using the axiovision 3.1 software (Zeiss, Feldbach, Switzerland) or a Nikon Diaphot 300 immunofluorescence microscope (Nikon, Egg, Switzerland) using the Openlab 3.1.7 software (Improvision, Coventry, England). The blood vessel diameter was measured on pictures of DAPI-stained slides, and microvessel density of islets as well as of RT2, RipTNC and RT2/TNC mice were determined upon immunofluorescence staining with anti-CD31 antibody and subsequent analysis with the ImageJ software (National Institutes of Health, Besthesda, MD). For islets of wildtype and RipTNC mice at least 10 pixels were counted as one event, and for tumors from RT2, RipTNC and RT2/TNC mice more than 50 pixels were counted as one event. A student's t-test was performed by using Graph Pad Prism 4 software.

2.4 Tumor incidence and volume:

The tumor incidence was determined by counting all apparent pancreatic tumors with a minimal size of 1 mm in diameter. The tumor volume was defined as total tumor

volume per mouse in mm^3, assuming a spherical tumor shape. The volumes were calculated based on the diameters and summed up for each mouse. The tumor volume formula is $V= 3/4\ \pi\ (d/2)^3$.

2.5 Clasification of tumor (islet) stages by size:

According to the manual of Hanahan's Lab islets of RT2 mice can be classified according to the diameter as normal islet (<0.2 mm), hyperplastic/dysplastic islet (0.2-0.5 mm), angiogenic islet (0.5-1mm), and the tumors (≥1mm). The ranges of tumors are from 1 mm to 8 mm in diameter. Tumors were classified by sizes as small (diameter:1 - 2.5 mm); middle(diameter 3.0 - 4.5 mm) and big (diameter 5.0 - 7.0 mm).

2.6 Tumor grading

All tumors on a pancreatic section were classified: normal/hyperplastic islet (including normal as well as enlarged islets), adenoma (bigger than 1 mm in diameter, well differentiated tumor cells, encapsulated tumor, no invasive tumor edges), carcinoma grade I (well differentiated and homogenous appearance of tumor cells, tumor capsule partially absent, one invasive tumor edge), carcinoma grade II (partially dedifferentiated and heterogenous appearance of tumor cells, tumor capsule largely absent, more than one invasive tumor edge), carcinoma grade III or anaplastic tumor (complete loss of tumor cell differentiation, very heterogenous tumor appearance).

2.7. Tumor hemorrhages:

Hematoxylin and Eosin (H&E) stained slides were used for determining severity of tumor hemorrhaging. Tumors with small and big fibrin-filled areas were classified as low and highly hemorrhagic tumor and were expressed as fraction of all tumors

III. Results:

Part A: Tenascin-C triggers metastasis involving nuclear translocation of β-catenin and extracellular matrix-rich tubes

Tenascin-C triggers metastasis involving nuclear translocation of β-catenin and extracellular matrix-rich tubes

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Abstract

The extracellular matrix molecule tenascin-C (TNC) is an important factor in tumor progression. In the multistage carcinogenesis Rip1Tag2 (RT2) model, in which the SV40 T antigen induces stochastically non-metastatic insulinomas we determined how ectopically expressed human TNC affects tumor progression. Transgenic RipTNC mice with rat insulin promoter driven ectopic expression of TNC in the pancreatic β-cells were generated. RipTNC mice did not exhibit detectable alterations in pancreas morphology and function, but displayed enhanced angiogenesis. A direct angiogenesis-promoting effect was demonstrated by purified TNC in the chicken chorioallantoic membrane assay. Upon crossing into the RT2 background enhanced tumor cell proliferation and increased angiogenesis with strong hemorrhages was detected in tumors of double transgenic RT2/TNC mice. Ectopically expressed TNC accelerated carcinoma progression with nuclear translocation of β-catenin. Nuclear translocation of β-catenin occurred also in MCF7 breast cancer cells in which TNC induced EMT. RT2/TNC tumor cells accumulated in tubes made of TNC and laminin that were not lined by endothelial cells. In contrast to RT2 mice, RT2/TNC littermates developed local lymph node and distant liver metastasis. In conclusion, in this first tumorigenesis model mimicking ectopic expression of TNC in cancer, TNC induced nuclear translocation of β-catenin that can explain TNC-driven angiogenesis, tumor cell proliferation, invasion and metastasis which may involve tumor cell dissemination by the TNC-containing tubes.

Introduction

Tenascin-C (TNC) is an important extracellular matrix (ECM) molecule of the malignant tumor microenvironment with a role in tumor progression (1) causally linked to lung metastasis in experimental breast cancer (2, 3). TNC is amongst the genes with predictive value in breast cancer metastasis (4). TNC affects the function of tumor suppressor genes, oncogenes and genome stability genes by acting on many cell types within a tumor thus, promoting uncontrolled proliferation, escape from immunosurveillance, angiogenesis and metastasis (5). But the underlying molecular mechanisms are not well understood. TNC causes cell rounding on fibronectin by induction of endothelin receptor type A (EDNRA) and blocking the integrin α5β1 coreceptor syndecan-4 involving inhibition of focal adhesion kinase (FAK), the small GTPase RhoA and tropomyosin-1 (TM1). TNC stimulated cell migration upon activation of the EGFR involving PLCγ (6) and, upon stimulation with the promigratory factors LPA/PDGF involving ROCK, PI3K and MEK (Lange et al., 2008, Cancer Res., *in press*). TNC also stimulated angiogenesis linked to VEGFA (7), integrin αvβ3 (8), FAK and Prx1 (9). In double transgenic mice with ectopic expression of c-Myc and VEGFA in the mammary gland, breast cancer cells metastasized to the lung in a tenascin-C-dependent manner (3). In cell culture TNC induced Wnt signaling by downregulation of the soluble inhibitor DKK1 which caused increased expression and nuclear localization of β-catenin and induction of several Wnt target genes in glioma cells (10). Induction of Wnt signaling by TNC might be relevant in colorectal carcinoma cell invasion since TNC surrounded carcinoma cells that exhibited nuclear β-catenin at the invasion front (11). In gliomas TNC is coexpressed with the Wnt target Id2 which correlated with malignancy (10).

Carcinoma-associated-fibroblasts (CAFs) play an important role in carcinoma cell invasion which involves remodeling of the ECM (12). CAFs secreted HGF and TNC, which together induced colorectal carcinoma cell invasion upon activation of c-Met, Rac and EGFR, and inhibition of RhoA (13). Recently, CAFs were shown to prepare a cue through the ECM dragging squamous carcinoma cell clusters behind, which depends on α3 and α5 integrins in the fibroblasts, and RhoA-mediated activation of myosin light chain. CAFs degraded the ECM in a MMP-dependent manner and deposited TNC amongst other ECM molecules (12). ECM-rich tracks that contained TNC and other ECM molecules were also observed in melanomas where they formed tubular channels, which is reminiscent of vasculogenic mimicry (VM) (14).

The formation of new blood vessels is an important step in tumor progression (15). Soluble growth factors such as VEGFA, PDGF, Wnt and their cognate receptors amongst other molecules trigger proliferation and differentiation of endothelial and vessel-associated smooth muscle cells. In addition, ECM molecules such as TNC (7), fibronectin (16, 17), laminins (18) and collagens (19) also regulate tumor angiogenesis. During metastasis tumor cells disseminate through hematogenic and lymphogenic vessels (20). In addition, VM also appears to contribute to metastasis several cancers (21-23). Inhibition of Cox-2, a downstream effector of EDNRA signaling, blocked experimental VM (24). Since TNC induced EDNRA signaling (10, 25), and is present in VM-associated ECM tubes it is possible that activation of EDNRA by TNC plays a role in VM.

The Rip1Tag2 (RT2) model is a well characterized multi-stage tumorigenesis model, where SV40 Tag is expressed under control of the rat insulin II promoter (26). SV40Tag inhibits the p53 and RB tumor suppressors, thus initiating transformation and hyperplasia which in turn triggers angiogenesis and insulinoma formation in a reproducible and sequential manner. Loss of E-cadherin is a hallmark of malignant progression of most epithelial cancer types which is linked to enhanced cell migration, invasion and metastasis (27, 28). Also in the RT2 model carcinoma formation and local lymph node metastasis depends on E-cadherin function (29). But RT2 mice do not develop distant metastasis which correlates with a lack of nuclear translocation of β-catenin (30). Nuclear β-catenin was proven to be instrumental in tumorigenesis since mice expressing a stabilized β-catenin in the gut developed multiple dysplastic lesions in the small intestine and the colon (31, 32).

We decided to use the RT2 model to investigate how ectopically expressed human TNC affects insulinoma formation and tumor progression. We observed that ectopic expression of TNC in the RT2 background accelerated proliferation, angiogenesis, VM and invasion thus, causing local lymph node and distant liver metastasis in RT2/TNC mice.

Results

Ectopic expression of human TNC in transgenic RipTNC mice

To mimic high expression of TNC in tumor tissue we generated double transgenic mice with ectopic expression of TNC in RT2 mice. Before injection of the expression-plasmid (**Supplemental Fig. 1A**) into fertilized oocytes induction of the transgene was confirmed by immunofluorescence (IF) (**Supplemental Fig. 1B**) and Sandwich-ELISA upon transfection into βT2 cells that were derived from a RT2 tumor (**Supplemental Fig. 1C**). First, three founders of transgenic RipTNC mice were generated which ectopically expressed the human TNC protein in β-cells of the pancreas under control of the rat insulin promoter (**Fig. 1A, Supplemental Fig. 2A**). Langerhans islets of wildtype mice did not expressed endogenous TNC (**Fig. 1A**). All transgenic RipTNC mice were healthy and fertile, and did not exhibit detectable alterations in tissue morphology of the pancreas which displayed α-glucagon positive cells around β-cells (**Supplemental Fig. 2B**). Mice also exhibited normal blood glucose levels (not shown).

Enhanced angiogenesis in RipTNC pancreatic islets and in the CAM assay

By quantification of CD31/PECAM-1 positive signals we determined angiogenesis in pancreatic islets of 12 week old RipTNC mice and observed that the endocrine pancreatic tissue of RipTNC mice was almost 2-fold more vascularized than that of wildtype littermates (**Fig. 1B**). To test, whether TNC directly induces angiogenesis, we used the chicken chorioallantoic membrane (CAM) assay where angiogenesis was analyzed upon addition of purified human TNC onto the CAM for 3 days. Addition of TNC showed a significant enhancement in blood vessel density and attraction of vessels to the TNC-soaked filter paper in comparison to the corresponding controls (**Fig. 1C**). Quantification of vessels bigger than 150 μm in diameter on and next to the filter (**Fig. 1D**) and of vessel density (**Fig. 1E**) revealed that TNC was similarly potent in attracting blood vessels to the CAM as PDGF-BB. Addition of PDGF-BB and TNC also exhibited some hemorraghing, demonstrating some abnormal vessel formation (**Fig. 1C**). These data show that TNC induces angiogenesis in normal tissue.

Reduced survival and accelerated tumorigenesis in RT2/TNC mice

We generated RT2/TNC mice by breeding RipTNC with RT2 mice. Tumors of double transgenic mice expressed human TNC as detected by IHC with the human TNC-specific antibody B28.13 (**Fig. 2A**). Islets and tumors from all three founders expressed human TNC (**Supplemental Fig. 2A**, data not shown). Since no difference in transgene expression and tumorigenesis was found between founders the data for all founders were pooled. As RT2 mice die between 12 – 14 weeks tumorigenesis in RT2/TNC mice was analyzed at 12 weeks of age. We realized that considerably more RT2/TNC mice died at this age than RT2 littermates (**Fig. 2B**). A possible explanation for an enhanced death rate in RT2/TNC mice could be an increased tumor number and/or tumor size. We observed that the number of tumors between genotypes was not different (**Supplemental Fig. 3**), suggesting that ectopically expressed TNC did not affect tumor onset. Next, we analyzed whether the islet size, which correlates with tumor progression (26), was different between RT2/TNC and RT2 mice at 10 and 12 weeks. The appearance of normal, hyperplastic, angiogenic and tumorigenic islets was determined according to the diameter of less than 0.2, more than 0.2 to 0.5, more than 0.5 to 1.0 and more than 1.0 mm, respectively (**Fig. 2C**). At 10 weeks, when tumor angiogenesis begins in RT2 mice (26), the number of angiogenic (47% versus 30%) and tumorigenic (7% versus 4%) islets was significantly higher in RT2/TNC mice than in RT2 littermates, respectively. In contrast, the number of hyperplastic islets was lower in RT2/TNC (**Fig. 2C**). This suggests that ectopically expressed tenascin-C accelerated tumorigenesis. At 12 weeks there was no difference detectable between the size of small, medium and big tumors from RT2 and RT2/TNC mice (**Supplemental Fig. 4**). RT2/TNC mice with big tumors presumably had died before analysis. Indeed, analyzed corpses displayed very big tumors (data not shown).

Increased proliferation in tumors of RT2/TNC mice

An accelerated tumorigenesis could be due to decreased apoptosis and/or enhanced proliferation. IF staining for cleaved caspase 3, a marker of apoptotic cells, did not reveal differences in apoptosis between tumors from 12 week old RT2 and RT2/TNC mice (data not shown). Upon staining for phosphorylated-histone-H3 (P-H3) as proliferation marker tumors from RT2/TNC mice exhibited statistically more cells than those from

RT2 tumors, both at 10 and 12 weeks of age. Some RT2/TNC tumors displayed an extraordinately high proliferation index (**Fig. 2D**). Thus, ectopically expressed TNC promoted tumor cell proliferation.

Enhanced angiogenesis and hemorrhaging in tumors of RT2/TNC mice

To address whether ectopically expressed TNC had an influence on tumor angiogenesis, we quantified CD31 positive signals in tumors of both genotypes at 10 and 12 weeks and observed that the angiogenic index was almost two-fold higher in tumors of RT2/TNC mice than in their RT2 littermates (**Fig. 3A**). We noticed that tumors from RT2/TNC mice often appeared highly hemorrhagic. Upon hematoxylin/eosin (H&E) staining, fibrin deposits were used to classify a tumor into little or highly hemorrhagic. Subsequent quantification showed that there was indeed more severe bleeding in tumors from RT2/TNC mice (**Fig. 3B**). Thus, ectopically expressed TNC enhanced tumor angiogenesis with leaky vessels as soon as 10 weeks of age.

TNC induces nuclear translocation of β-catenin, EMT and tumor progression

To investigate whether TNC potentiates carcinoma formation, we determined the grading of tumors which reflects tumor invasion (**Fig. 4A**). At 10 weeks the total number of tumors with up to multiple invasive fronts was higher in tumors of RT2/TNC (47%) than in RT2 (40%) mice. Invasive tumors grade 2 and 3, with two and multiple invasive fronts, respectively, were more abundant in RT2/TNC mice (**Fig. 4A**). This effect was not seen at 12 weeks, where only carcinomas grade 2 were more abundant in RT2/TNC mice than in RT2 controls (**Fig. 4A**). Mice with advanced tumors most likely had died before they could be analyzed (see above).

Since activation of Wnt signaling is a hallmark of tumor progression, we determined expression of E-cadherin and observed that dedifferentiated tumors had lost E-cadherin and insulin expression (**Fig. 4B**). Staining for β-catenin showed a profound difference between tumors of the two genotypes: expression of β-catenin was much higher in RT2/TNC tumors in comparison to RT2 tumors (**Fig. 4C**). Moreover, in tumors from RT2/TNC mice β-catenin was expressed in nuclei of cells especially at the invading front (**Fig. 4D**).

This was different to RT2 tumors that showed a weaker and exclusively cytoplasmic expression of β-catenin (**Fig. 4C**).

To determine whether TNC has the potential to directly activate Wnt signaling, we investigated EMT in the MCF7 breast cancer cell line that experiences EMT in culture upon growth on TNC or fibronectin for 7 days. Indeed, in contrast to fibronectin, TNC induced EMT in MCF7 cells which is evidenced by a switch toward a fibroblastoid morphology, a shift from E-cadherin to N-cadherin expression and nuclear translocation of β-catenin (**Fig. 4E**). Thus, TNC induced nuclear translocation of β-catenin and EMT, which may account for enhanced tumor cell invasion in RT2/TNC tumors.

TNC induces local and distant metastasis

Since TNC induced nuclear trasnlocation of β-catenin, promoted carcinoma formation and tissue invasion we searched for insulin positive metastatic tumor cells in RT2/TNC and control littermates that were sacrificed at 14 weeks of age. In 3 of 4 RT2/TNC mice we detected insulin positive cells as single cells or as cell clusters in the lymph nodes next to the pancreas (**Fig. 5A**). Moreover, in 2 of 4 RT2/TNC mice insulin positive cells showed up in the liver as clusters of about 10 cells, in particular next to the central vein (**Fig. 5B**), which is suggestive of tumor cells having traversed the central vein to home to liver tissue. This was different to RT2 mice, where we did not see any insulin positive tumor cells in the lymph nodes nor in the liver (0/5). Thus, ectopically expressed TNC triggered regional lymph node and distant liver metastasis.

TNC promotes formation of ECM-rich tubes in RT2 tumors

By IF we noticed that normal, hyperplastic, angiogenic and tumorigeneic islets exhibited some TNC expression in the RT2 mice. In tumors from 12 week old RT2 mice TNC was predominantly expressed in the hypoxic center independent of tumor size (**Fig. 6A**). Costaining for cancer-associated fibroblasts (CAFs) with antibodies against α-SM actin and vimentin, respectively together with TNC revealed that some CAFs, expressing either or both of the CAF markers, expressed TNC (**Fig. 6A, Supplemental Fig. 6**). But the absence of a complete overlay of signals for TNC and CAFs suggests that cells other than CAFs also contributed to expression of TNC (**Fig. 6A**). Costaining for CD31 and TNC showed only a limited overlay

of both signals which suggests that endothelial cells may only marginally contribute to TNC expression (**Fig. 6A**).

Upon staining for CD31 or insulin we realized that huge areas in the tumor centers lacked endothelial cells and exhibited huge holes that potentially presented VM (**Fig. 6B**). To investigate this possibility, we stained tumor tissue for laminin together with tenascin-C. In tumors of RT2 mice we observed costaining of TNC and laminin in tube-like structures (**Fig. 6B**). Laminin did not colocalize with TNC which was confirmed by confocal microscopy that revealed a close apposition of TNC and laminin, with laminin surrounding TNC (**Fig. 6B**). In RT2/TNC tumors the appearance of ECM-rich tubes of about 10 μm was more prominent. DAPI positive but CD31-negative (not shown) cells surrounded holes that were filled with TNC and laminin and DAPI (**Fig. 6B**). Moreover, DAPI positive cells were also found within the tubes in close association with laminin and TNC. These cells might be tumor cells that potentially migrate along the ECM tracks (**Fig. 6B**). Together, in the centers of RT2 and RT2/TNC tumors TNC and laminin formed tube-like structures, a process that was enhanced by ectopically expressed TNC. The prominent abundance of these ECM tubes in RT2/TNC tumors may have facilitated tumor cell dissemination thus, promoting metastasis.

Discussion

A high expression of TNC is found in many invasive cancers and in lymph node metastasis which suggests that TNC plays an important role in the events causing metastasis (1-3). Here, we phenocopied TNC actions in cancer by ectopically expressing TNC in insulinoma-prone RT2 mice and demonstrated that high levels of TNC trigger regional lymph node and distant liver metastasis. Moreover, TNC induced nuclear translocation of β-catenin which can explain enhanced angiogenesis, tumor cell proliferation and invasion, thus causing metastasis in RT2/TNC mice.

The number of tumors per mouse was not different between RT2/TNC and RT2 mice, which suggests that TNC does not affect tumor initiation which in turn exclusively depends on inhibition of p53 and RB by the SV40Tag. But in combination with SV40Tag, TNC enhanced proliferation of transformed cells. A proliferation-promoting effect of TNC was also described in cell culture and for several cancers (5). Since TNC activates growth promoting signaling linked to Wnt (10) and EDNRA (25) it is possible that

these pathways contribute to enhanced proliferation in cells with inactivated p53 and RB. Collaboration of TNC with mutated p53 and RB is highly relevant in TNC-expressing cancer tissue since these tumor suppressors are frequently inactivated in cancer (33, 34).

Ectopically expressed TNC accelerated tumor progression into highly invasive carcinomas. Activation of a motile genetic program is important to allow tumor cell invasion. Reduced adhesion to the ECM presents a prerequisite for cells to respond to signals that trigger migration. TNC blocked cell adhesion to fibronectin through activation of EDNRA (25) and inhibition of syndecan-4 (35). Moreover, in a TNC context the promigratory factors HGF (13) and LPA/PDGF (Lange et al., 2008, Cancer Res. *in press*) promoted colorectal carcinoma invasion and glioma cell migration, respectively. Loss of E-cadherin expression presents a first step toward a motile phenotype. Here, we showed that TNC inhibited E-cadherin expression in MCF7 breast cancer cells that experienced EMT on a TNC substratum. TNC also induced expression of MMP3 (10, 36), an important factor triggering EMT through induction of the E-cadherin repressor snail (37).

As previously described (30), dedifferentiated carcinomas from RT2 mice lack nuclear β-catenin despite the absence of E-cadherin. In contrast, carcinomas of RT2/TNC mice displayed nuclear β-catenin in cells at the invasion front, which suggests that TNC triggers nuclear trasnlocation of β-catenin to induce EMT and invasion. This possibility is supported by the observation of nuclear β-catenin together with high TNC at the invasive front in human colorectal carcinomas (11, 38). Activation of Wnt signaling by TNC might also account for high levels of the dominant negative regulator of basic-loop-helix proteins Id2 in malignant glioblastomas (10). Finally, Wnt signaling induces TNC expression through TCF/LEF1 binding sites within the TNC promoter (11). Hence, induction of TNC by Wnt signaling and accumulation of nuclear β-catenin by TNC presents an intriguing amplification loop that has the potential to trigger tumor cell invasion and metastasis. In the RT2 model, loss of E-cadherin function apparently is not sufficient for nuclear translocation of β-catenin, which might involve sequestration of β-catenin by N- or R-cadherin. How does tenascin-C trigger nuclear localization of β-catenin? Several possibilities leading to nuclear β-catenin can be considered that in addition to Wnt signaling may involve, c-Met, Fer or Fyn kinases (39), Rac-dependent activation of JNK (40), Rac1b-associated induction of snail involving MMP3 (37) and

PDGFRα-dependent activation of p68 RNA helicase (41). Since Wnt signaling (10) and Rac were activated in a TNC context (13), and MMP3 (36) and PDGFRα (10) were induced by TNC it is possible that the associated pathways play a role in nuclear translocation of β-catenin in RT2/TNC tumors, which needs to be addressed in the future. Thus, several pathways triggered by TNC may cause stabilization and nuclear translocation of β-catenin to induce an invasive and metastatic program.

TNC enhanced angiogenesis by attracting and stimulating growth of blood vessels in the CAM assay and in islets of single transgenic RipTNC mice. VEGFA, an important factor inducing angiogenesis, might be involved in TNC-stimulated angiogenesis in RT2/TNC tumors. This possibility is supported by data showing that VEGFA levels and angiogenesis dropped in melanoma-bearing nude mice lacking TNC expression (7). In addition, TNC induced multiple proangiogenic factors such as angiopoietin-1, semaphorin 3C/5A, Id2, VCAM1, PDGFRα (10), and EDNRA (25). Activation of Wnt signaling by TNC might also explain enhanced tumor angiogenesis in RT2/TNC tumors. In fact, lung vascularization was reduced in mice lacking TNC expression (42) and lung vascularization depended on Wnt signaling that was blocked by Dickkopf 1 (DKK1) (43). Since TNC activated Wnt signaling through repression of DKK1 (10), it is possible that regulation of DKK1 by TNC plays a crucial role in normal lung vascularization and in tumor angiogenesis.

In RT2 mice, TNC expression was independent on tumor size and stage but was prominent in the hypoxic tumor center, supporting the possibility that hypoxia was involved in inducing TNC expression (44). TNC was present in streaks that organized in tubular structures together with laminins. The formation of tenascin-C containing ECM tubes resembles VM (14, 21). Similar to VM in melanomas (14), we did not see endothelial cells surrounding these ECM tubes. TNC was expressed by CAFs in RT2 tumors, which resembles studies showing TNC expression by CAFs. It is possible that ectopically expressed TNC in cancer tissue turns on a genetic program with a blueprint for ECM-containing tubes. This possibility is supported by the existence of conduits in the thymus that are composed of TNC, fibrillar collagens, laminin-332 and fibrillin-1 (45). Moreover, tubular channels in metastatic melanoma also contained TNC in addition to fibronectin and procollagen-I (14). Finally, growth of glioma cells on a TNC substratum induced expression of several ECM molecules such as fibronectin, fibrillin and 6 different collagen types (α2 (I), α1 (IV),

α2 (IV), α2 (V), α2 (VI) and α1 (XVIII)) (10). Similarly, metastatic c-Myc/VEGF mammary gland tumors also displayed induction of multiple ECM molecules such as tenascin-C, fibronectin, laminin-332 (γ2), procollagens I, III, IV, V and XIV (3). In contrast to the thymic conduits with 2μm in diameter, where TNC surrounds a procollagen/fibrillin/laminin core structure (45), in RT2 tumors laminin wraps around a several fold bigger tube (10 μm) made of a TNC core. In RT2/TNC tumors the ECM tubes appeared more complex and often displayed a mixture of ECM molecules together with DAPI positive cells. It is conceivable that these cells that are not CAFs as they did not stain for vimentin nor αSM-actin are tumor cells, a situation that is similar to metastatic melanomas (14). The TNC-rich ECM tubes may offer a track for tumor cells as it has been demonstrated for squamous carcinoma cells *in vitro* (12). The presence of glycophorin-positive erythrocytes within the TNC tubes (data not shown) suggests a connection to blood vessels. Thus, our data suggest that blood vessels together with the TNC containing tubes enable transport of tumor cells to distant organs and promote metastasis formation. Our data further suggest that in addition to hematogenic and lymphogenic angiogenesis TNC-containing ECM tubes present a third route to facilitate tumor cell traveling to distant tissues in RT2/TNC tumors.

In summary, TNC stimulated and promoted several events causing metastasis. Nuclear translocation of β-catenin by TNC could explain enhanced angiogenesis, proliferation and invasion. TNC-rich tubular channels may promote tumor cell dissemination and metastasis (**Fig. 7**). In the future, the RT2/TNC tumor model ectopically expressing human TNC could be useful for testing drugs to inhibit TNC-induced metastasis.

Material and Methods

Construction of the TNC expression plasmid, generation of transgenic RipTNC mice and genotyping

The 7.5 kbp sequence of human TNC (accession number X78565) and harboring a polyadenylation signal was removed from the HxBL.pBS plasmid (46) by restriction enzyme cleavage with Not I and Kpn I and transferred into the intermediate pcDNA3.1/Hygro(-) vector (InVitrogen). The resulting plasmid was cleaved with Xba I and Hind III before ligation behind the rat insulin II promoter into the corresponding cleavage sites of the 4.5 kbp Rip1 vector containing an intron sequence (26). Successful cloning was confirmed by restriction digestion and partial sequencing of 700 bp around the start and the stop codon. Expression and secretion of the transgene was determined upon 48 h transient transfection of the expression plasmid into βT2 cells by IF for human TNC with the B28.13 antibody in the absence of detergent for detection of secreted TNC. Secretion of human TNC was also determined by sandwich ELISA on conditioned medium from βT2 cells 48 h after transfection using the B28.13 antibody for capturing and the TNC1.2. antibody (see below) for detection of bound human TNC. The RipTNC expression vector was used for injection into the pronucleus of fertilized C57BL/6 oocytes upon linearization with Sal I according to standard procedures (47), giving rise to 3 transgenic lines with stable expression and transmission of the transgene. Transgenic mice were identified by PCR with primers revP1 hTNC mice (5`-GAA AGA CAC CTG CCA ACA GC-3`) and RipES/VD: 5`-TAA TGG GAC AAA CAG CAA AG-3). For generation of double-transgenic RT2/TNC mice, single-transgenic RipTNC mice were crossed with RT2 mice (26). Mice were sacrificed between 9 and 14 weeks of age. Tumor incidence per mouse was determined by counting all macroscopically detectable pancreatic tumors with a minimal diameter of 1 mm. All experimental procedures involving mice and chicken were done according to the guidelines of the Swiss Federal Veterinary Office (SFVO), the regulations of the Cantonal Veterinary Office of Basel Stadt and the guidelines of Inserm.

Generation of rabbit anti-TNC antibodies

Recombinantly expressed his-tagged human TNC was purified as described (25) and was used for injection into 2 rabbits, which gave rise to antisera TNC1.2 and TNC2.2 recognizing human and murine TNC by western blotting, IF and IHC.

Histopathologic analysis and confocal microscopy

Pancreata from transgenic and control mice were isolated and fixed in 4% paraformaldehyde (PFA) overnight, dehydrated, and embedded in paraffin. Freshly isolated tissue was embedded in OCT compound (Tissue Tek) and snap frozen in liquid nitrogen. Histologic analysis was done on H&E-stained paraffin sections. Immunostaining was done on paraffin sections (5µm) or on cryosections (7 µm) as described (48). The following antibodies were used: rabbit against TNC (TNC1.2, TNC2.2, 1.200), total laminin (1: x,) and laminin α5 (1:x,), phospho-histone-H3 (), cleaved caspase 3 (), α−SM actin (), vimentin (), β-catenin (), E-cadherin (), N-cadherin (), rat antibodies against murine TNC (MTn12,), CD31 (), the guineapig antibody against insulin () and mouse antibody B28.13 (1: x, ref), xxx. Nuclei were stained with 4`,6-diamino-2-phenylindole (DAPI). The tumor diameter was measured on pictures of DAPI-stained tissue and intra-tumoral microvessel density was analyzed by counting CD31-positive signals using the Image J software (National Institute of Mental Health, Bethesda, MD). In islets of wildtype and RipTNC mice more than 10 pixels and in tumors of RT2 and RT2/TNC mice more than 50 pixels were counted as one event. Students t-test was performed by using the Graph Pad Prism 4 Demo software. Histologic staging and grading of tumors was done on H&E sections. All sections were analyzed with either an Axioskop2 plus light microscope using Axiovision 3.1 Software (Zeiss) or a Nikon Diaphot 300 immunofluorescence microscope (Nikon) using Openlab 3.1.7. Software (Improvision). Pancreatic islets were classified according to their size in diameter: normal islets (less than 0.2 mm), hyperplastic/dysplastic islets (0.2 – 0.5 mm), angiogenic islets (more than 0.5 – 1.0 mm) and tumorigeneic islets (more than 1 mm). Tumors were categorized according to size (in diameter) and morphology into normal/hyperplastic islets, including normal as well as enlarged islets; adenoma (more than 1 mm), well differentiated tumor cells, encapsulated tumor, no invasive tumor edges; carcinoma grade 1, well differentiated and homogenous appearance of tumor cells, tumor capsule partially absent, one invasive tumor edge; carcinoma grade 2, partially dedifferentiated and

heterogenous appearance of tumor cells, tumor capsule largely absent, more than one invasive tumor edge, carcinoma grade 3 or anaplastic tumor, complete loss of tumor cell differentiation, very heterogenous tumor appearance. H&E stained tissue was used for determination of the severity of tumor hemorrhaging. Tumors with fibrin-filled lacunae were classified as little or highly hemorrhagic.

DNA replication analysis

Phospho-histone-H3 positive nuclei were counted in squares of x μm2 (pixels) upon costaining with DAPI and insulin. Statistical analysis was done with Student`s T-test.

EMT assay

MCF7 breast cancer cells (ATCC) were plated onto fibronectin (10μg/ml) or TNC (10μg/ml) coated immunofluorescent dishes for 7 days in DMEM supplemented with 10% FCS. Medium was replenished every 2nd day. Cells were fixed with PFA, permeabilized and stained with the indicated antibodies.

CAM assay

Egg white (5 ml) was removed from chicken eggs at day 3 after fertilization and incubated for 8 days at 37 ° C in a humidied chamber. At day 8 whatman paper (0.5 cm2 in diameter, Whatman) soaked with 10 μl PDGF-BB (200 ng/ml, Sigma), purified human TNC (2 μg/ml) in 0.01% Tween-20 and controls I (PBS) and II (PBS, 0.01% Tween-20) was placed onto the CAM in a region devoid of blood vessels. At day 11 the CAMs were removed from the embryo and analyzed for blood vessel density and vessel morphology by light microscopy or by IHC for laminin upon PFA fixation and embedding in paraffin. The results derived from 3 independent experiments. Statistical analysis was done with Student`s T-test.

Figure legends

Fig. 1 TNC stimulates angiogenesis in pancreatic islets of RipTNC mice and in the CAM assay

A, Expression of ectopically expressed human TNC was determined by IHC with B28.13 in the pancreatic islet of a 9 week old RipTNC mouse. Islets of wildtype mice did not stain. Pancreatic islets of wildtype mice

did not stain with TNC1.2 which recognizes murine and human TNC. **B**, IF for CD31 and quantification of blood vessel density by the Image-J programme was done on CD31 positive signals in pancreatic islets of 12 week old wildtype (WT, 8 mice) and RipTNC mice (7 mice). **C**, CAM assay with 20 µl of PBS (9 CAMs), PBST (12), 100 ng/ml PDGF (10) and 100 µg/ml TNC (10), respectively. I, little vessels; II normal vessels with branching; III, hemorrhagic vessels; IV and V, normal and big vessels attracted by the sample, respectively. **D**, quantification of vessels with 150 µm in diameter found on top or next to the filter paper. **E**, The number of vessels in 20 squares of 115 pixel x 115 pixels (1 mm x 1mm) were used to quantify the vessel density around the sample. Scale bar represents x µm (**A**) and µm (**B**).

Fig. 2 Reduced survival, accelerated tumorigenesis and increased proliferation in islets of RT2/TNC mice

A, Expression of human TNC in tumors of RT2/TNC mice was detected by IHC with B28.13. Tumors of RT2 mice did not stain. **B**, spontaneous death incidence of RT2 and RT2/TNC mice is depicted in % of all animals at 10, 11 and 12 weeks of age (w). total: RT2, n = 36; RT2/TNC, n = 38. **C**, Numbers of normal (N), hyperplastic/dysplastic (H), angiogenic (A) and tumorigenic islets (T) is displayed for pancreata from RT2 and RT2/TNC mice at 10 weeks of age. Number of islets (mice), RT2, 89 (5) and RT2/TNC, 89 (7). Note, that RT2/TNC mice died earlier and more frequently than RT2 littermates. In addition, RT2/TNC mice exhibited more angiogenic and tumorigenic islets than RT2 mice. **D**, Proliferating cells in islets of 10 week old RT2 (n = 6) and RT2/TNC mice (n = 10) and 12 week RT2 (n = 4) and RT2/TNC mice (n = 5) were identified by immunostaining with a phospho-histone H3 (P-H3) specific antibody. The number of P-H3 positive nuclei was quantified per defined constant field. Number of islets (mice): 10 weeks, RT2 51 (6), RT2/TNC 100 (10), 12 weeks, RT2 99 (4), RT2/TNC 42 (5). **p = 0.16, * p = 0.08.

Fig. 3 Enhanced angiogenesis and hemorrhaging in tumors of RT2/TNC mice

A, Quantification of CD31 positive signals in tumors of 10 and 12 week old mice. Number of tumors (mice), 10 weeks; RT2, 13 (5), RT2/TNC, 19 (7), 12 weeks; RT2, 43 (7), RT2/TNC, 34 (7). **B,** Tumors with small and big fibrin-filled areas were classified as low (left panel) and highly (right panel) hemorraghic. The number of low and highly hemorraghic tumors was determined in insulinomas of RT2/TNC and RT2 tumors

in 10 week and 12 week old mice and is shown in % of all detectable tumors. Number of tumors (mice), 10 weeks; RT2, 47 (6), RT2/TNC, 112 (10), 12 weeks; RT2, 43 (4), RT2/TNC 51 (5). Note, that more tumors of double transgenic mice were highly hemorraghic at 10 and 12 weeks than tumors of RT2 mice. Accordingly, the number of low hemorraghic tumors is decreased in RT2/TNC tumors in comparison to tumors of RT2 mice.

Fig. 4 TNC promotes nuclear translocation of β-catenin, EMT and tumor progression

A, Carcinoma formation was determined as grade 1, 2 and 3 according to one, two and multiple invading fronts per tumor, respectively. The number of carcinoma with different grading is displayed in % of all tumors. Number of tumor (mice), 10 weeks; RT2, 47 (6), RT2/TNC, 112 (10), 12 weeks; RT2, 42 (4), RT2/TNC, 53 (5). **B, C**, **D** IF for insulin and IHC for E-cadherin and β-catenin in tumors of 14 week old RT2 and RT2/TNC mice. **E**, EMT assay. IF for E-cadherin, N-cadherin and β-catenin in MCF-7 cells that were grown on fibronectin (FN) or TNC (TNC) for 7 days in medium containing 10% FCS. Note that the TNC substratum induced EMT with nuclear β-catenin, which was in contrast to a fibronectin substratum with membrane localized β-catenin. Arrows point at nuclear β-catenin. Note that the β-catenin staining is stronger in tumors of RT2/TNC mice than in tumors of RT2 littermates with nuclear localization at the invading front of RT2/TNC tumors.

Fig. 5 TNC induces local and distant metastasis

IF staining for insulin positive tumor cells in regional lymph nodes and liver tissue. In 14 week old RT2/TNC mice insulin positive cells were found in regional lymph nodes (3/4) and liver tissue of 2/4. No insulin positive signal was detected in lymph nodes or liver tissue of RT2 mice (0/5).

Fig. 6 Expression of TNC in RT2 and RT2/TNC islets

A, IF for TNC, α-SM actin, vimentin and DAPI in islets of 12 week old RT2 mice. **B**, IF for total laminin and laminin α5 in tumors from 12 week old RT2 and RT2/TNC mice. TNC was stained with B28.13 (RT2/TNC) and TNC1.2 (RT2).

Fig. 7 Induction of nuclear translocation of β-catenin by TNC may trigger metastasis in RT2/TNC mice

Interaction of tumor cells with TNC induces nuclear translocation of β-catenin which may account for enhanced proliferation, migration, EMT, invasion and angiogenesis. In addition, vasculogenic mimicry may promote tumor cell dissemination and metastasis.

Supplemental Fig.1 Generation of the TNC expression vector and in vitro expression analysis

A, cloning of the sequence encoding human TNC into the Rip1 vector. **B,** Expression analysis of the pRipTNC vector by immunofluorescence upon transfection into βT2 cells. **C**, sandwich ELISA on conditioned medium (CM) from βT2 cells upon transfection of the pRipTNC vector for 48 h.

Supplemental Fig. 2 Expression of the transgene in RipTNC pancreatic islets and pancreas organization

A, expression analysis of human TNC (B28.13) in β-cells of the Langerhans islets in the pancreas of 9 week old wildtype mouse (WT) and RipTNC founders 2, 3 and 4. **B**, expression of α-glucagon and insulin in pancreata of RipTNC and wildtype littermates. Note, that ectopically expressed TNC does not disrupt pancreas organization.

Supplemental Fig. 3 Tumor number in 12 week old RT2 and RT2/TNC littermates

The total number of tumors (bigger than 1 mm in diameter) determined by macroscopical measurement is presented per mouse.

Supplemental Fig. 4 Tumor size in 12 week old RT2 and RT2/TNC mice

The number of tumors were classified according to size into small (1 – 2.5 mm), medium (>2.5 – 4.5 mm) and big (> 4.5 – 7.0 mm).

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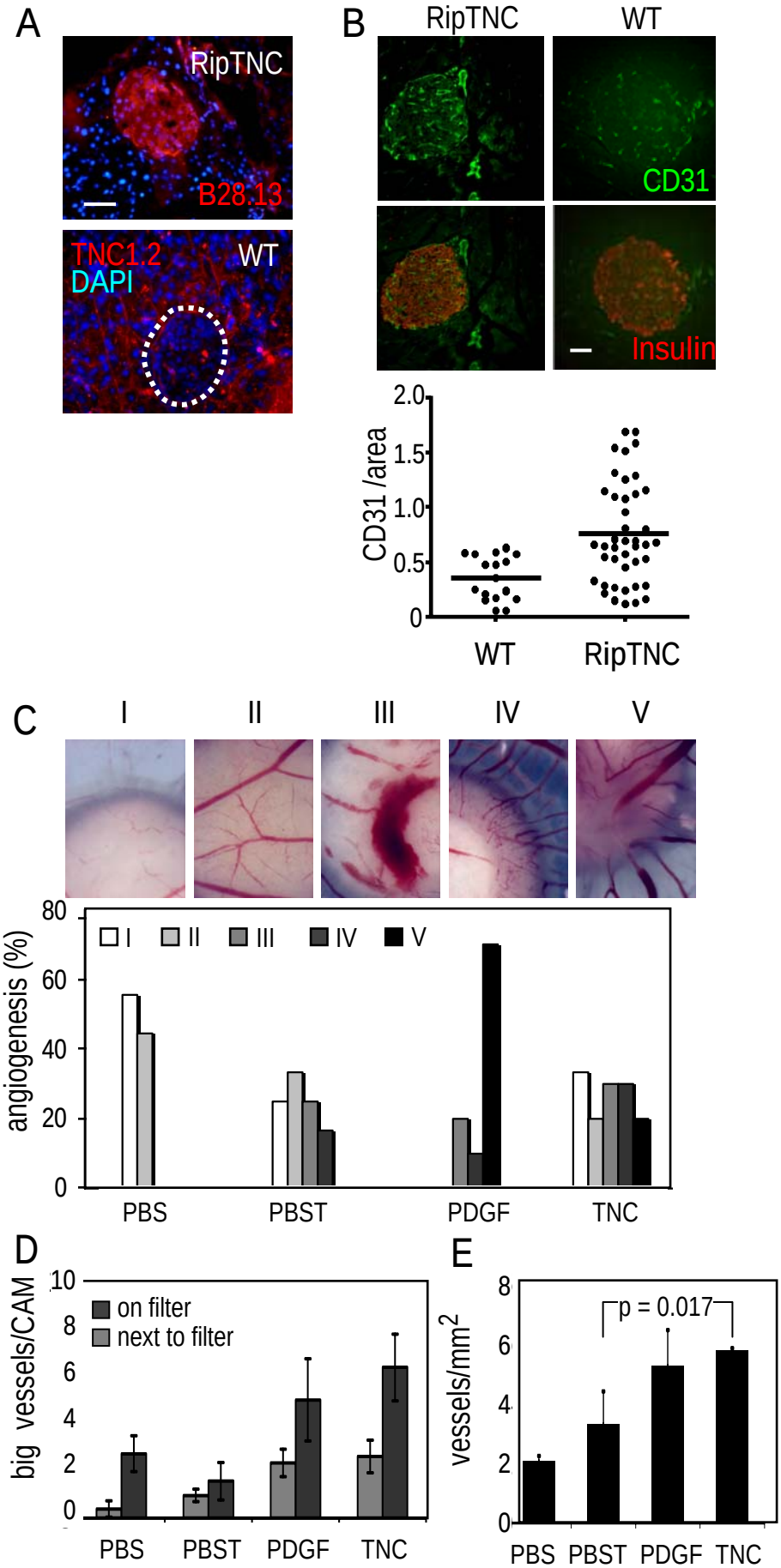
Fig. 1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RipTNC
B28.13
TNC1.2
DAPI
WT
B
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Insulin
CD31 /area
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1.5
1.0
0.5
0
WT
RipTNC
C
I
II
III
IV
V
angiogenesis (%)
80
60
40
20
0
PBS
PBST
PDGF
TNC
D
big vessels/CAM
on filter
next to filter
10
8
6
4
2
0
PBS
PBST
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TNC
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6
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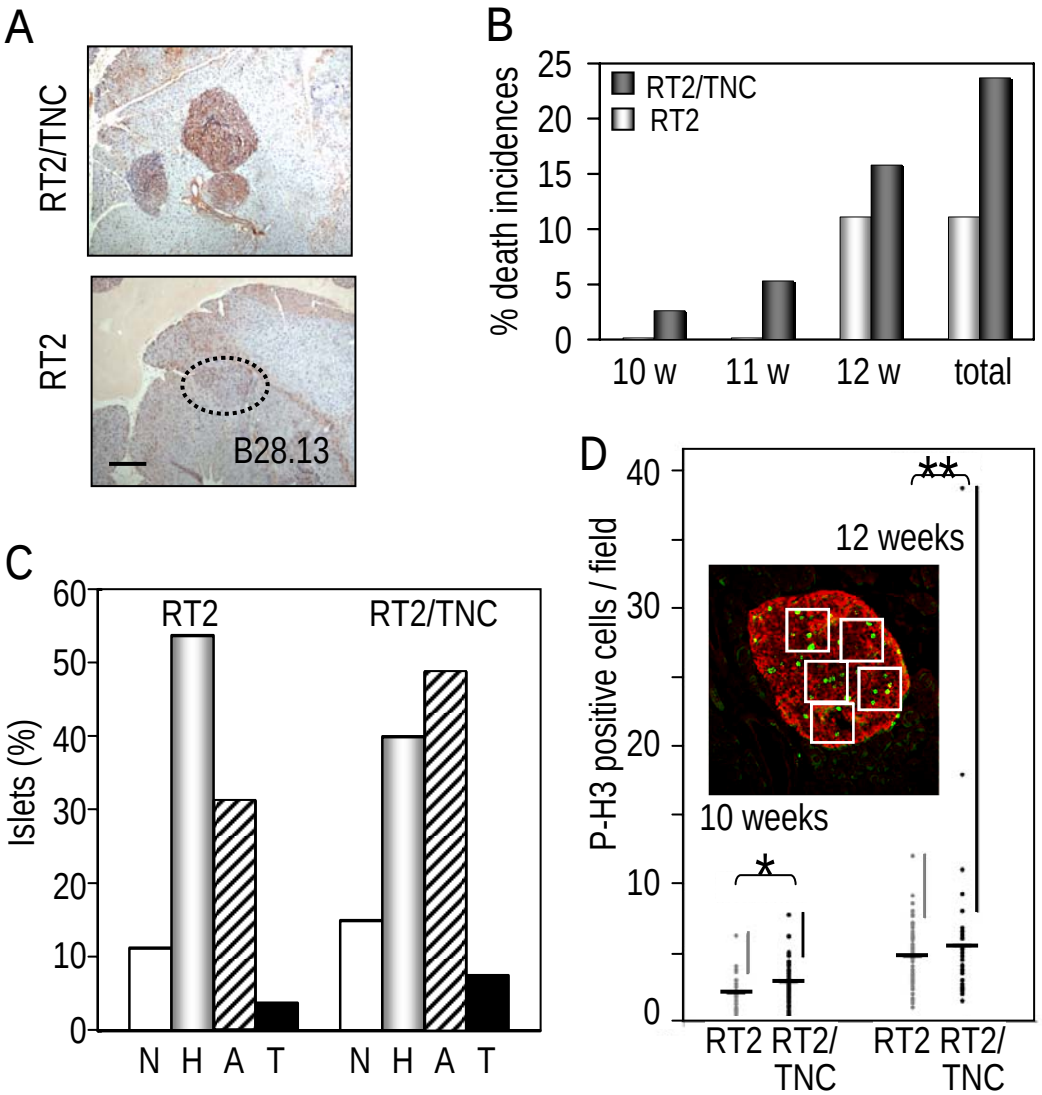
PDGF
TNC

Fig. 2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RT2/TNC
RT2
B28.13
B
% death incidences
25
20
15
10
5
0
RT2/TNC
RT2
10 w
11 w
12 w
total
C
Islets (%)
60
50
40
30
20
10
0
RT2
RT2/TNC
N H A T
N H A T
D
P-H3 positive cells / field
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30
20
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10 weeks
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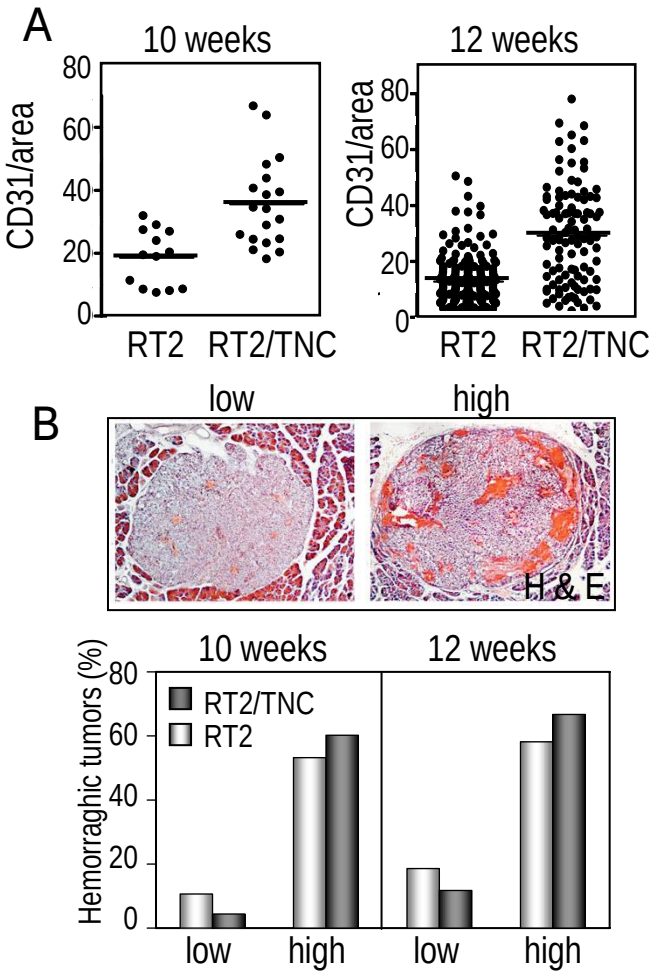
RT2
RT2/ TNC
RT2
RT2/ TNC

Fig. 3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12 weeks
CD31/area
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60
40
20
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RT2/TNC
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H & E
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12 weeks
Hemorraghic tumors (%)
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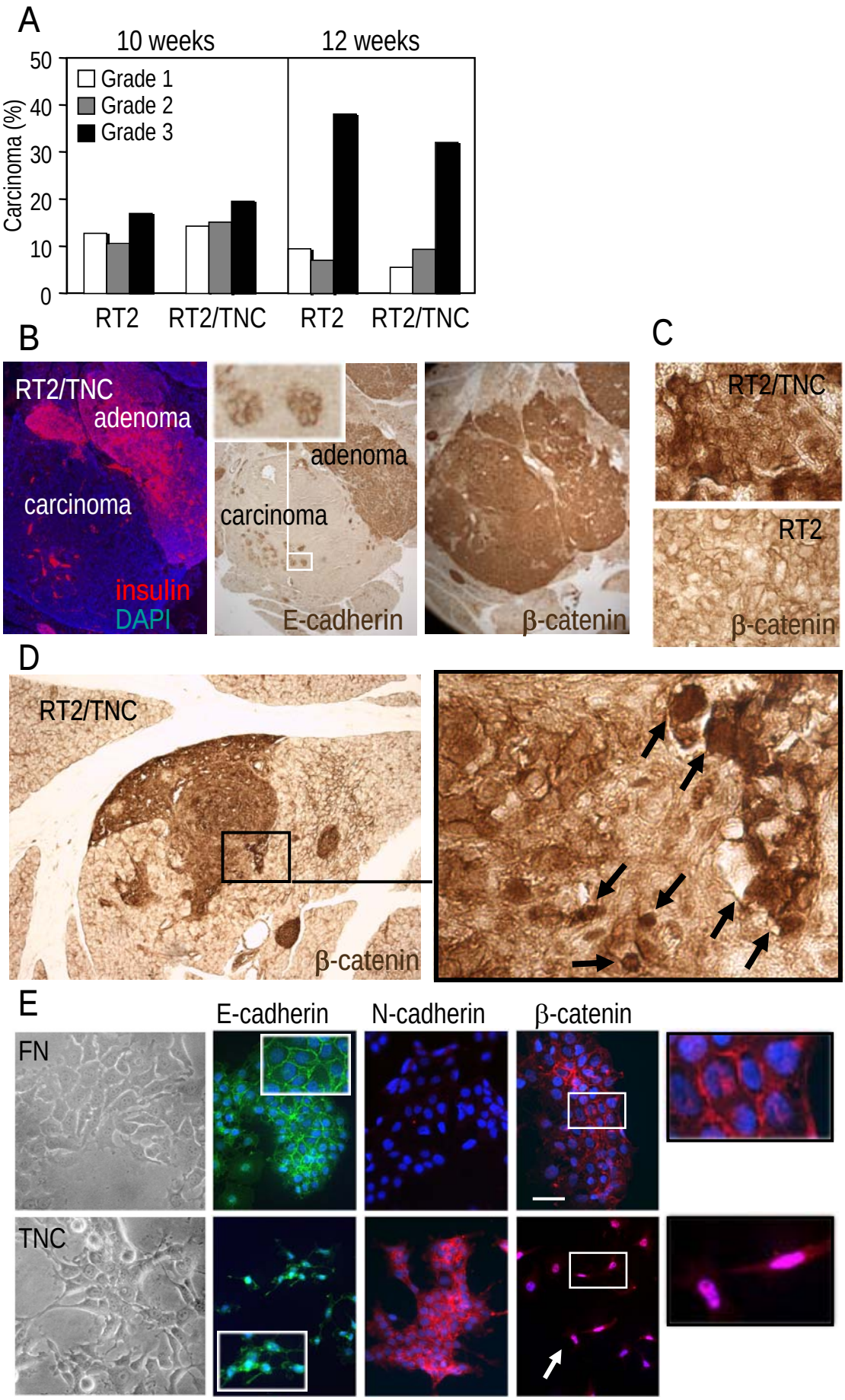
low
high
low
high

Fig. 4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12 weeks
Grade 1
Grade 2
Grade 3
Carcinoma (%)
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30
20
10
0
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RT2/TNC
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RT2/TNC
B
RT2/TNC
adenoma
carcinoma
insulin
DAPI
adenoma
carcinoma
E-cadherin
β-catenin
C
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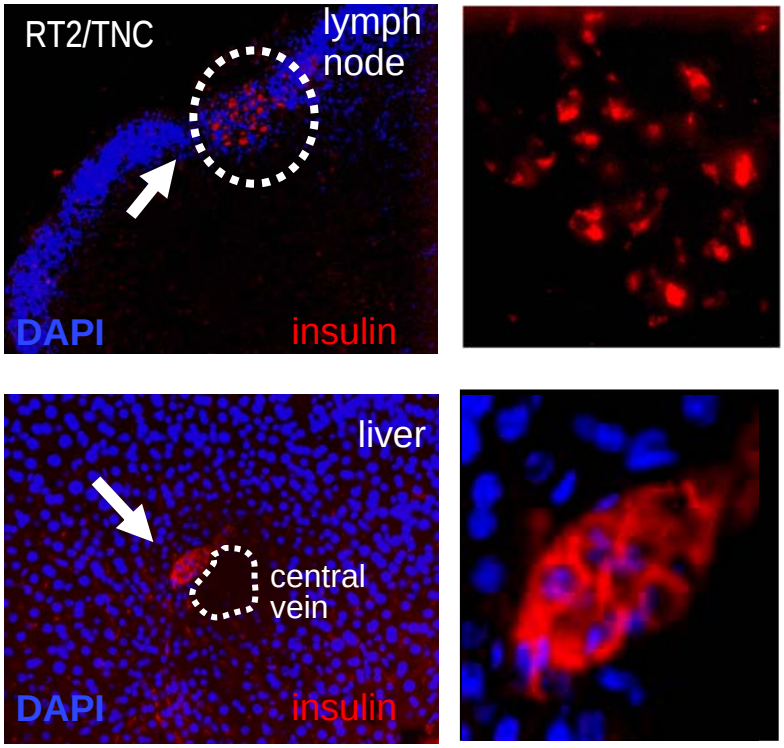
TNC

Fig. 5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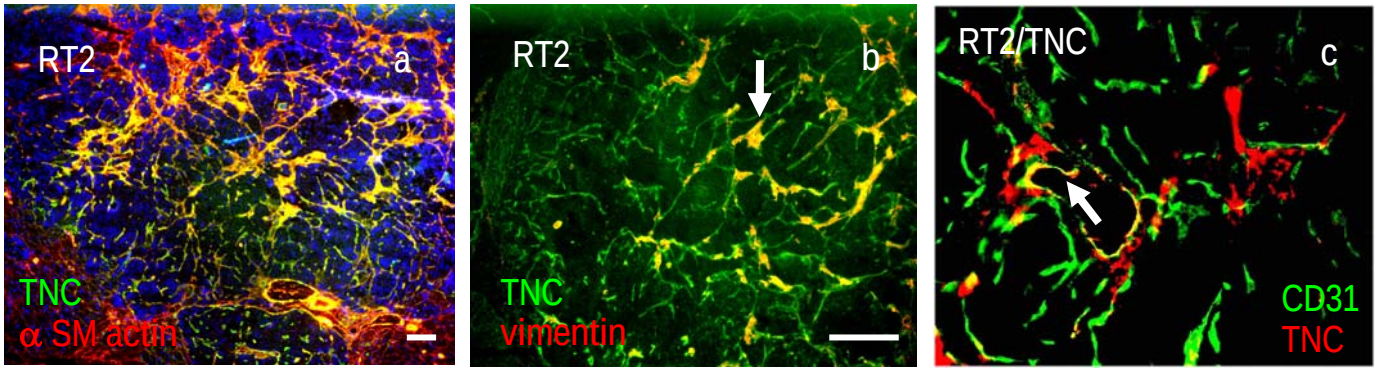
liver
central vein
DAPI
insulin

Fig. 6

Results:Part A

A



B

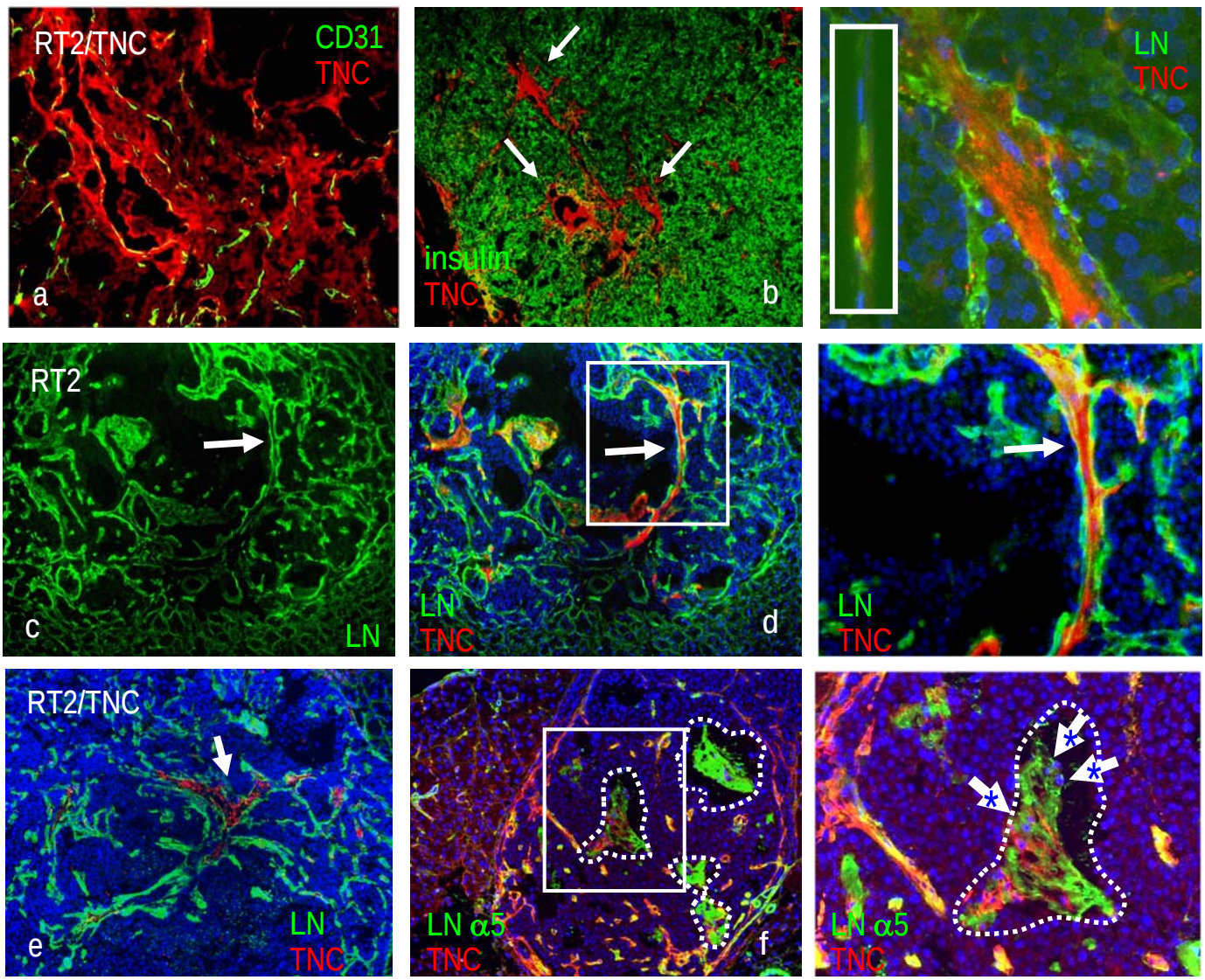
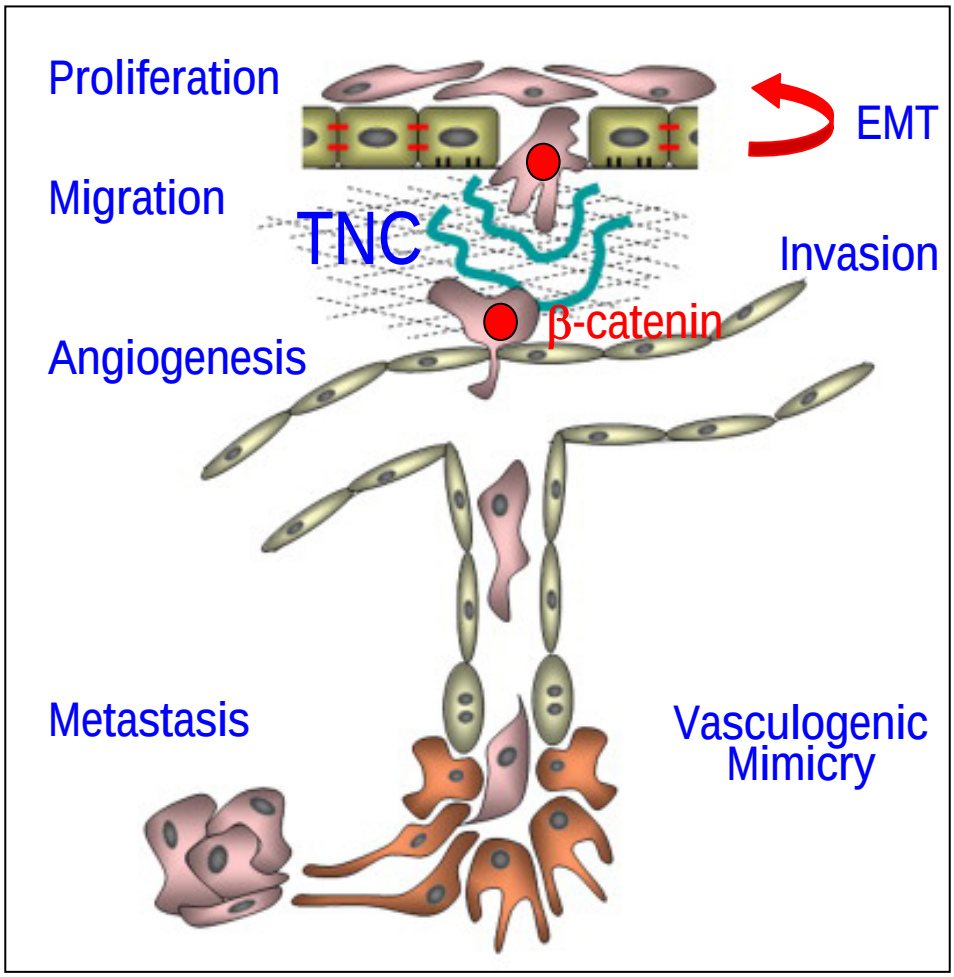


Fig. 7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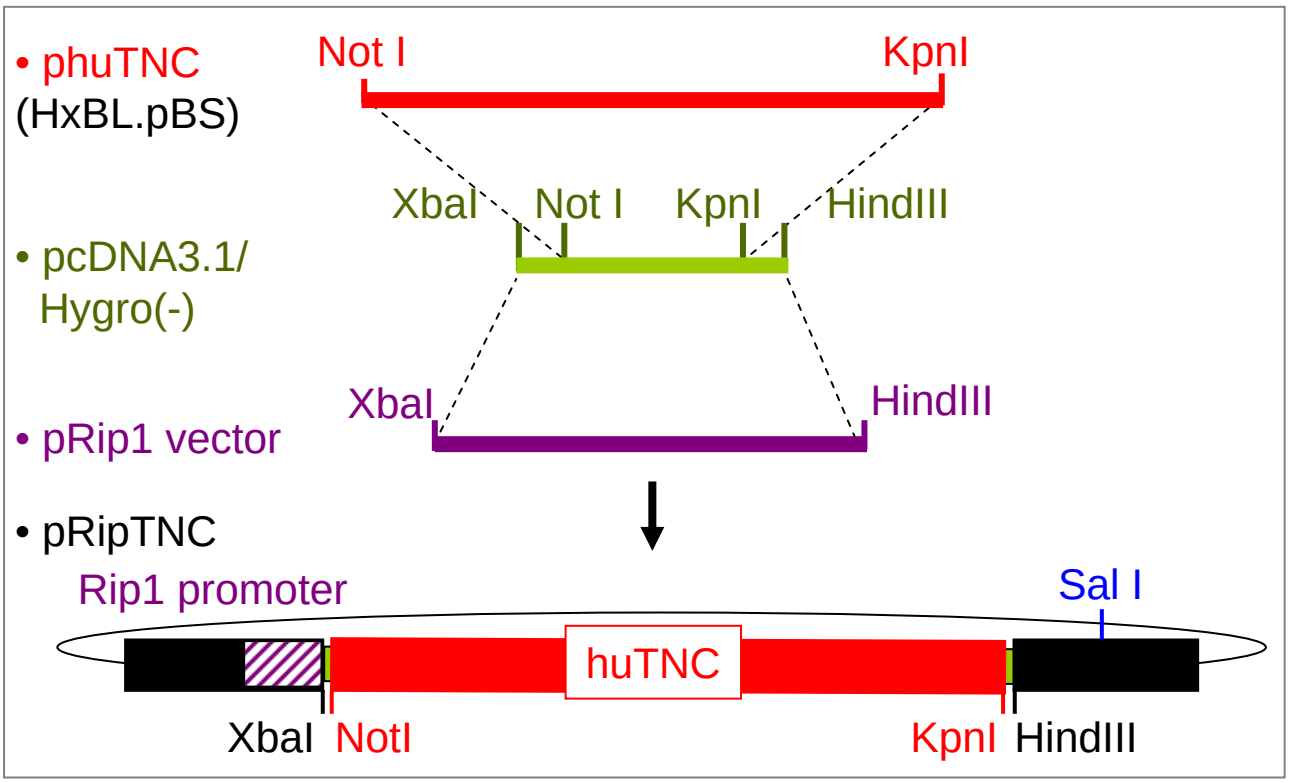
Metastasis
Vasculogenic
Mimicry

A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(HxBL.pBS)
Not I
KpnI
XbaI
Not I
KpnI
HindIII
• pcDNA3.1/
Hygro(-)
XbaI
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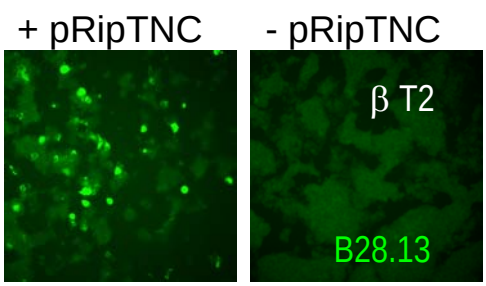
B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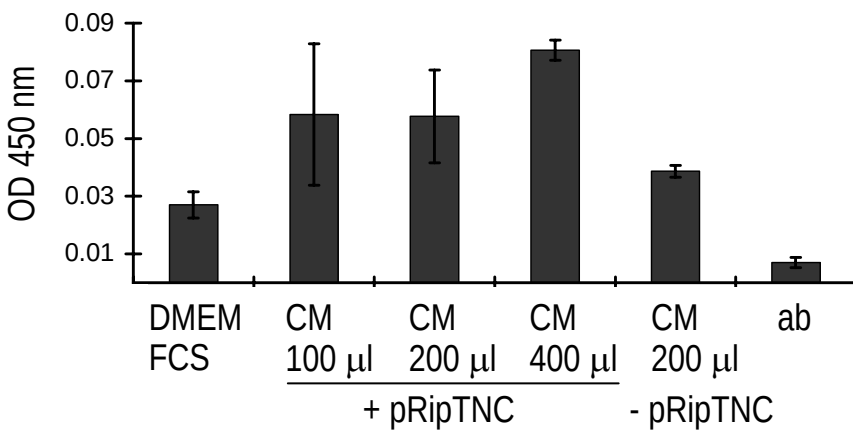
- pRipTNC
β T2
B28.13

C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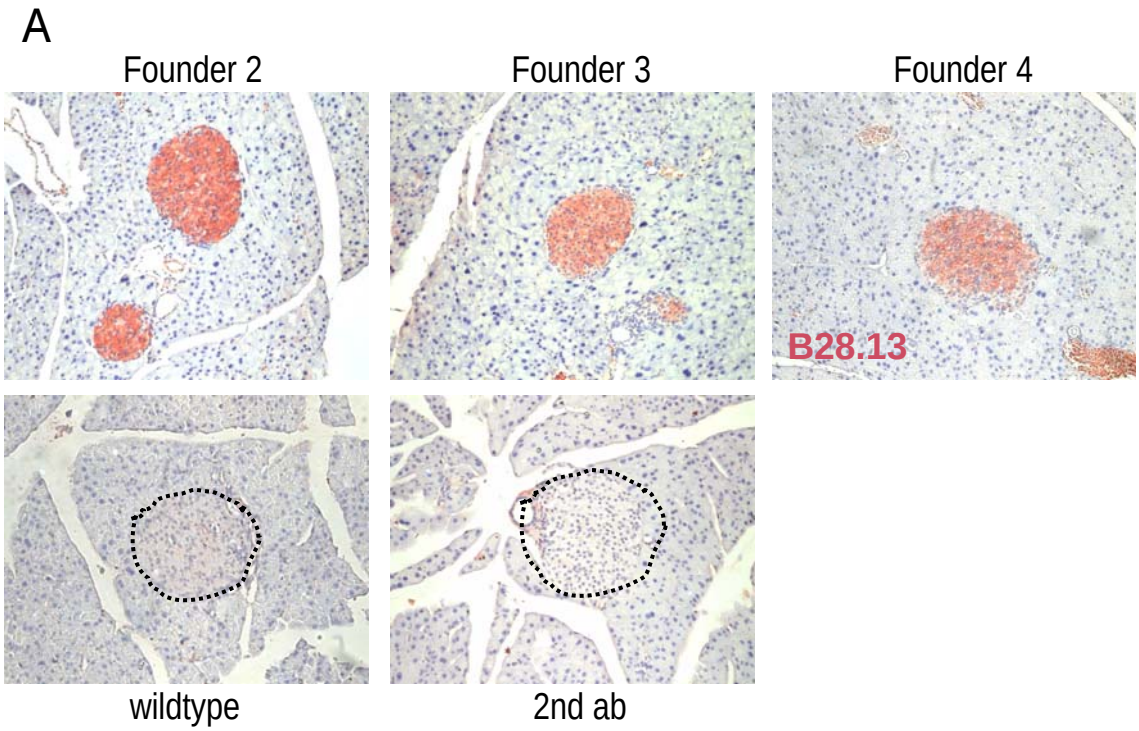
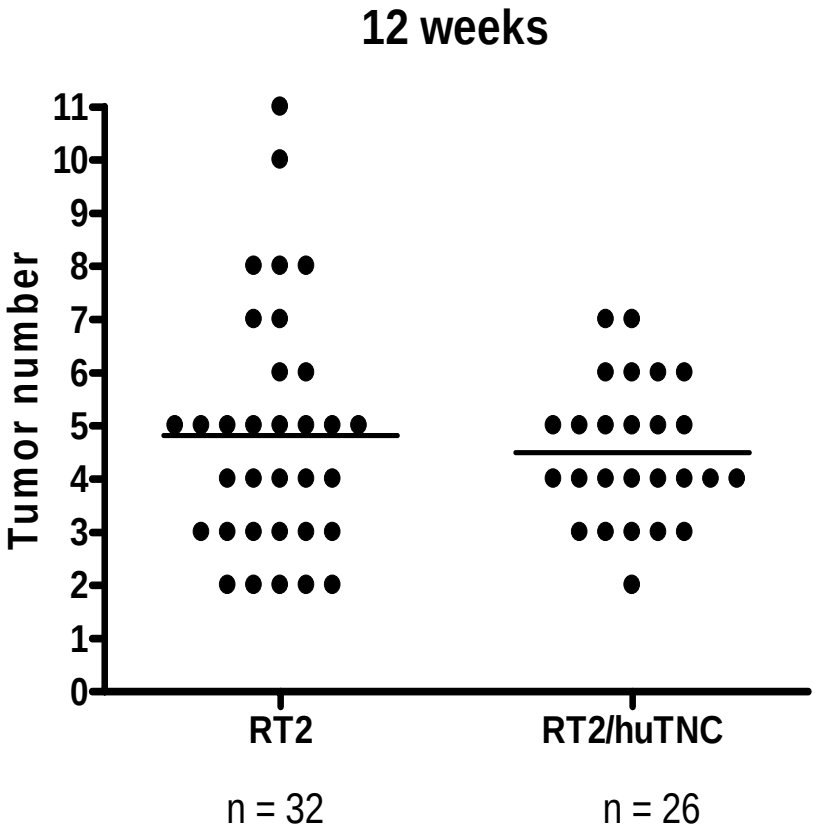
0.07
0.05
0.03
0.01
DMEM
FCS
CM
100 µl
CM
200 µl
CM
400 µl
CM
200 µl
ab
+ pRipTNC
- pRipTNC

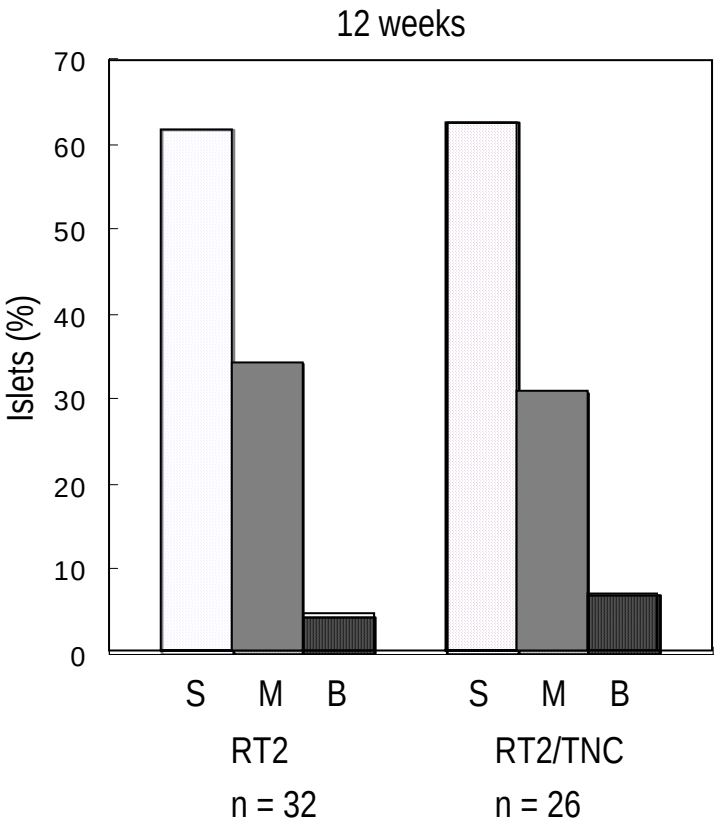
Supplemental Fig. 2



Supplemental Fig. 3



Supplemental Fig. 4



S, Small tumor: 1 - 2.5 mm
M, Medium tumor: > 2.5 - 4.5 mm
B, Big tumor: > 4.5 - 7.0 mm

III. Results:

Part B: In vivo analysis of the role of tenascin-W in tumorigenesis

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Abstract

Tenascins are a family of ECM glycoproteins typically present in different connective tissues, contributing to matrix structure and influencing the behavior of cells in contact with the ECM. Tenascin-W is the fourth and most recently described member of tenascins. Tenascin-W has been found to be expressed in tumor tissues but was not detectable in normal mammary glands, suggesting a potential role of tenascin-W in tumorigenesis. Here we report the generation of a transgenic mouse expressing human tenascin-W specifically in β-cells of pancreatic islets of Langerhans (RipTNW). We have also established a double RT2/TNW transgenic mouse by breeding the RipTNW with Rip1Tag2 (RT2) transgenic mice. Finally, a triple transgenic RT2/TNC/TNW mouse was produced to investigate a potential enhancing effect of both tenascins on tumorigenesis. Future studies of these double and triple transgenic mice will deepen our understanding of the role of tenascin-W in tumor onset, progression and metastasis.

Introduction

Tenascins are a family of glycoproteins characterized by a N-terminal globular domain and heptat repeats, which facilitates their multimerization, one or more tenascin-type epidermal growth factor (EGF)-like repeats with a consensus sequence $X_4CX_3CX_5CX_4CXCX_8C$, a series of fibronectin (FN) type III domains and a C-terminal fibrinogen-related domain. Diversity within the family exists at many levels. Each species of vertebrate examined to date has more than one tenascin gene, and the gene products themselves are frequently alternatively spliced (reviewed in Chiquent-Ehrismann, 2004). Tenascins are particularly expressed in the extracellular matrix during development but are also found in the adult during regeneration, wound healing and tumorigenesis. They act through interactions with cell surface receptors as well as by binding to and blocking sites on other extracellular matrix molecules (Huang *et al.*, 2001).

Tenascin-W is the fourth and most recently found tenascin member in human and most likely the final one according to the searching result of the whole human genome database (Weber *et al*., 1998). Like tenascin-C, tenascin-W forms hexamers (Scherberich *et al.,* 2004). During development, it 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 periosetum. TN-W and TN-C expression overlap in many of these areas. Like other members of tenascins, TN-W is also mainly present in the connective tissues that contribute to ECM structure (Chiquet-Ehrismann, 2004).

Recent studies have reported the detection of expression of tenascin-C and tenascin-W in breast cancer and colon cancer tissue by immunoblotting and immunohisto-chemistry and showed that many human mammary and colon cancer samples expressed tenascin-W alone or in combination with tenascin-C. Moreover, tenascin-W was not only expressed in the tumor stroma but also inside of epithelial cells (Scherberich *et al.*, 2004; Degen *et al.,* 2007, 2008). In mouse models of carcinogenesis tenascin-W was found to be expressed in metastasizing tumors (Scherberich *et al.,* 2005), thus potentially presenting a novel metastasis marker (Degen et al., 2007). In human breast cancer tenascin-W was expressed in early-stage tumors, suggesting that it may also play an early role in tumorigenesis (Degen *et al.,* 2008). Except integrin α8β1, α4β1and αvβ1 (Scherberich *et al.*, 2004; Degen *et al.,* 2008), receptors for tenascin-W, little is known about how cells interact with tenascin-W. Tenascin-W appears to act differently from tenascin-C since it did not inhibit cell spreading on fibronectin in cell culture (Degen *et al.,* 2007). Together these results suggest that tenascin-W is likely to play an overlapping yet non-redundant role from tenascin-C.

In order to understand the role of tenascin-W in tumorigenesis, in this study we established a transgenic mouse expressing human tenascin-W under the control of the rat insulin promoter (Rip1). Further breeding of this mouse with RT2 mice offers an

opportunity to examine whether ectopic expression of tenascin-W accelerates SV40 T-antigen-induced β-cell tumorigenesis. Since tenascin-W expression overlaps although not always with tenascin-C expression in tumor tissues (Degen *et al.*, 2007), to mimick coexpression of both tenascins triple transgenic RT2/TNC/TNW mice have been generated. Future phenotypical analysis is ongoing.

Materials and Methods

Construction of RiphuTNW expression plasmid

To generate RipTNW mice, plasmid pCEP-pu/huTNW which carries a cDNA fragment encoding the 3885 bp coding region of the full length human TNW (Acc. No. AL049689.1) was subcloned into pcDNA3.1. The resulted pCEP-pu/huTNW plasmid was cleaved with restriction enzymes XhoI and BamHI and ligated into the XhoI and BamHI sites of pcDNA3.1/Hygro(-) by T4 ligase to produce the pcDNA3.1/huTNW plasmid. The latter plasmid was digested by restriction enzymes XbaI and HindIII and cloned into the XbaI and HindIII sites of the Rip1 vector (Hanahan, 1985), establishing the Rip-huTNW$_{his}$ construct which contains 6xhis-tags at its 3'-terminus. The 6xhis-tag sequence was deleted by using the QuickChange II XL Site-Directed Mutagenesis Kit with primers causing deletion of the his-tag sequences in the Rip-huTNW$_{his}$ construct, warranting that the cDNA encoding the 3885 bp coding region of human TNW was cloned between the rat insulin II gene Rip1 promoter and a 436 bp fragment containing SV40 introns and polyadenylation signal sequences (**Fig. 3.1**).

Sequencing of RiphuTNW construction

The final construct was sequenced by using the BigDye® Terminator v1.1 Cycle Sequencing Kit with an ABI 310 sequencing machine. Almost the whole construct was sequenced except a short piece of 700 bp that could not be sequenced because of high sequence homology between the FNIII domains. By using PCR, we confirmed that the not-sequenced stretch of 700bp was present in the construct. The primers set for sequencing as well as the location of primers are shown in **Table 3.1**, and **Fig. 3.2**.

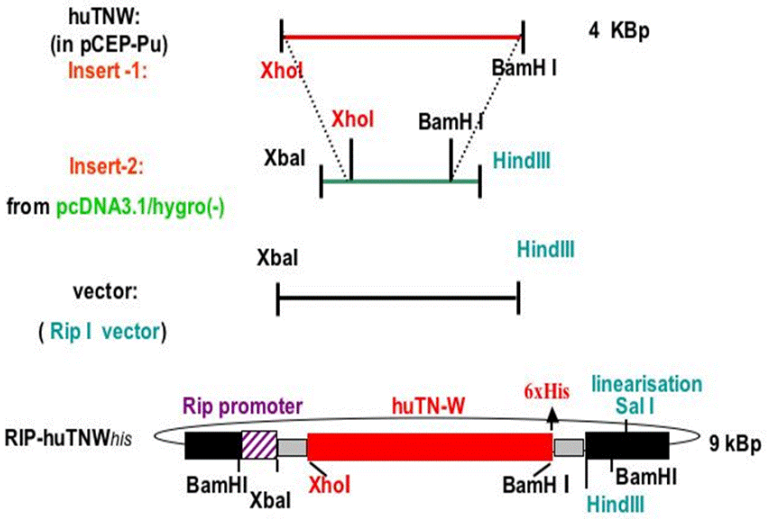

Fig. 3.1 Construction of RipTNW.

Table 3.1 Sequencing primers

Primers	locations	Sequences 5` to 3`
Rip ES/VD 5' fwd	-82bp (in Rip)	5'-TAA TGG GAC AAA CAG CAA AG-3'
huTNW LP1 fwd	269bp (In huTNW)	5'-CAC AGA AGG ACT GCG AGT TG-3'
huTNWLP2 fwd	649bp (In huTNW)	5'-ACT TCA TGT CGG AGG ACT GC-3'
huTNWLP3 fwd	1004bp (In huTNW)	5'-AGC AGC CCA CAG CAT CTA CT-3'
HuTNWFP2 fwd	1432 (In huTNW)	5'-GAC ACC AAG GAA ATG GCA GT-3'
huTNWP5 fwd	3039bp (In huTNW)	5'-CGC AGT CTG GTG GCA TAT TG-3'
3'end huTNW fwd	3667bp (In huTNW)	5'-ATC ATG GTG GCT GGT GGT AT-3'
huTNW rev P1 rev	351bp (In huTNW)	5'-ATC TCC ACC ATC TCT TCC TC-3'
$huTNW_SP4$ rev	3163bp (In huTNW)	5'-CTG TGG AGA GGG TGG TGG-3'
huTNW RP2 rev	3364bp (In huTNW)	5'-TCC ATC GCT TGA AGA AAT CC-3'
huTNW RP 1 rev	3816bp (In huTNW)	5'-ATG AGG GCG GAT TTT CAA CT-3'

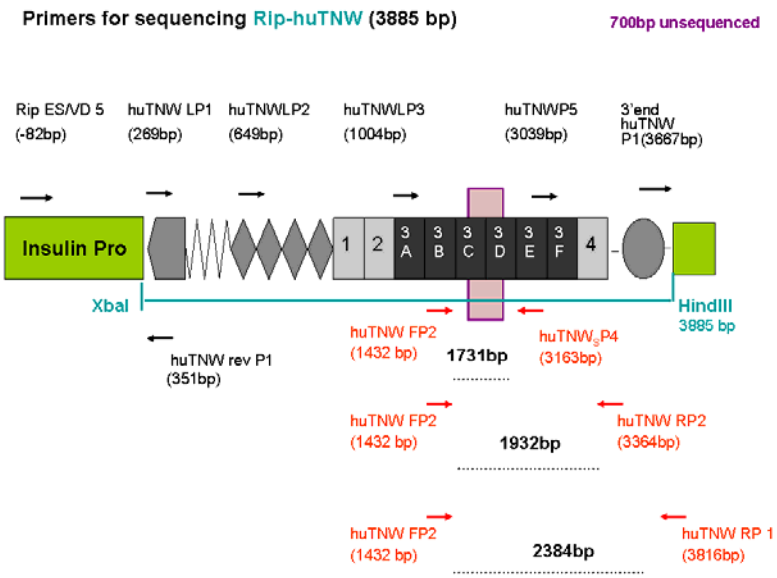

Fig. 3.2 Primers specific for huTNW sequencing and PCR

Expression analysis of tenascin-W plasmid

To further examine the expression and secretion of the transgene, the RipTNW vector was transfected into β T2 cells. Conditioned medium (CM) was collected after 3 days of transfection, and the expression of huTNW was determined by Sandwich- ELISA with a polyclonal anti-tenascin-W antibody as described (Scherberich *et al*, 2005) (**Fig. 3.3**).

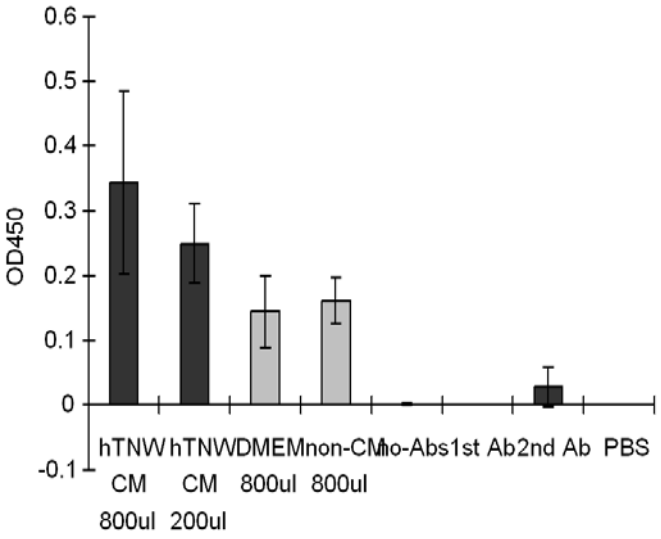

Fig. 3.3 Expression analysis of RipTNW by Sandwich-ELISA

Generation of Rip1TNW transgenic mice

100 µg of the RipTNW plasmid were digested with 10µl Sal I in a total volume of 450 µl for five hours and resolved on a 0.8% agarose gel. The ~ 8.5 kb band comprising the Rip1, the human TNW sequence, and the SV40 small T intron and polyadenylation signal sequences was excised from the gel and purified with the GENECLEAN SPIN kit (QBiogene, Luzern, Switzerland). The generation of transgenic mice was done at the Transgenic Mouse Core Facility TMCF, Biozentrum, Basel, according to standard procedures (Hogan *et al.*, 1994). The purified RiphuTNW fragment was injection into the male pronucleus of fertilized C57BL/6 zygotes. Surviving zygotes were implanted into pseudo-pregnant foster female mice.

Results:

Generation of RiphuTNW transgenic mice

To generate transgenic mice, specifically expressing human tenascin-W in β-cells of pancreatic islets of Langerhans, the cDNA comprising the full length coding sequence of 3885 bp human TNW was cloned between the rat insulin II gene promoter fragment (Rip 1) (Hanahan *et al.*, 1985) and a 436 bp fragment containing SV40 intron and polyadenylation signal sequences (**Fig. 3.4**). The final construct was sequenced. Almost the whole construct was sequenced. The sequences were checked by alignment to the sequences deposited for huTNW in the human genomic database (Acc. No.AL049689.1). No mutations were found in the sequences of the construct. We confirmed the presence of the unsequenced fragment of 700 bp in the construct by PCR. We further confirmed the expression in β T2 cells before microinjection in to zygotes by sandwich ELISA with a polyclonal anti-tenascin-W antibody (**Fig. 3.3**).

Pronuclear injection of the transgene into fertilized C57BL/6 oocytes resulted in five viable and fertile founder lines exhibiting stable germline transmission. All founder lines of transgenic mice were healthy and fertile.

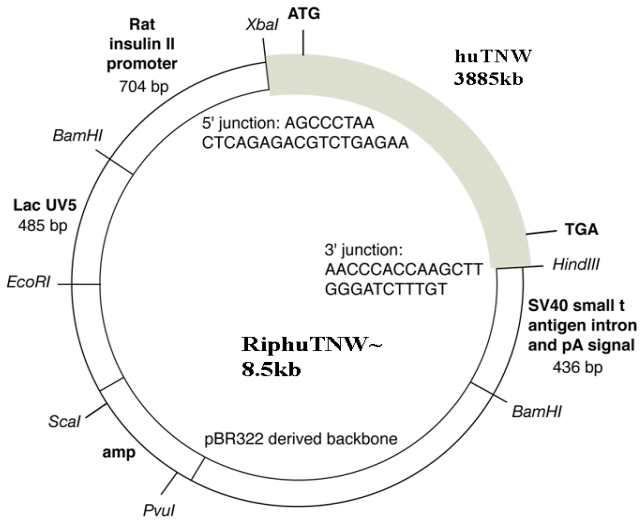

Fig. 3.4 Map of the huTNW construct in Rip vector

Genotyping of Rip1TNW transgenic mice

Genotypes were confirmed by PCR analysis of standard toe or tip genomic DNA preparations using a primer pair specific for the transgene (forward primer located in the Rip1 sequence: 5'-TCC GGA CTC GAC CTC TCG GAC; reverse primer located in the huTNW sequence: 5'-ATC TCC ACC ATC TCT TCC TC-3'). PCR cycles were: 95°C, 2 min (x1); 95°C, 1 min, 60°C, 2 min, and 72°C, 3 min (x35); and 72°C, 5 min (x1). PCR products were analyzed in 1.5% agarose gels.

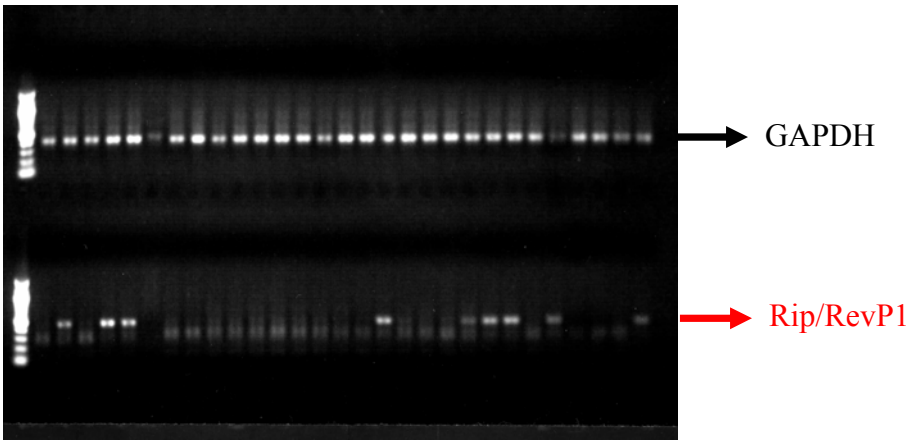

Fig. 3.5 Mouse genotyping: This figure shows a PCR gel for testing transgenic Rip1TNW mice. Visible bands indicate positive genotypes.

Expression analysis of TNW in the βcells of Langhans islets

Transgenic founder lines were tested for β-cell-specific expression of huTNW by immunohistochemical analysis using the full length rabbit anti-huTNW antibody. As shown in **Fig. 3.6**, the β cells from founder lines 10, 31, 32 expressed high level of TNW whereas in founder lines of 24 and 38 transgene it was only expressed in few β cells, in contrast islets of wild type littermates did not exhibit any detectable expression of TNW. The phenotypical characterization of these five expressed founder lines is currently ongoing in the laboratory.

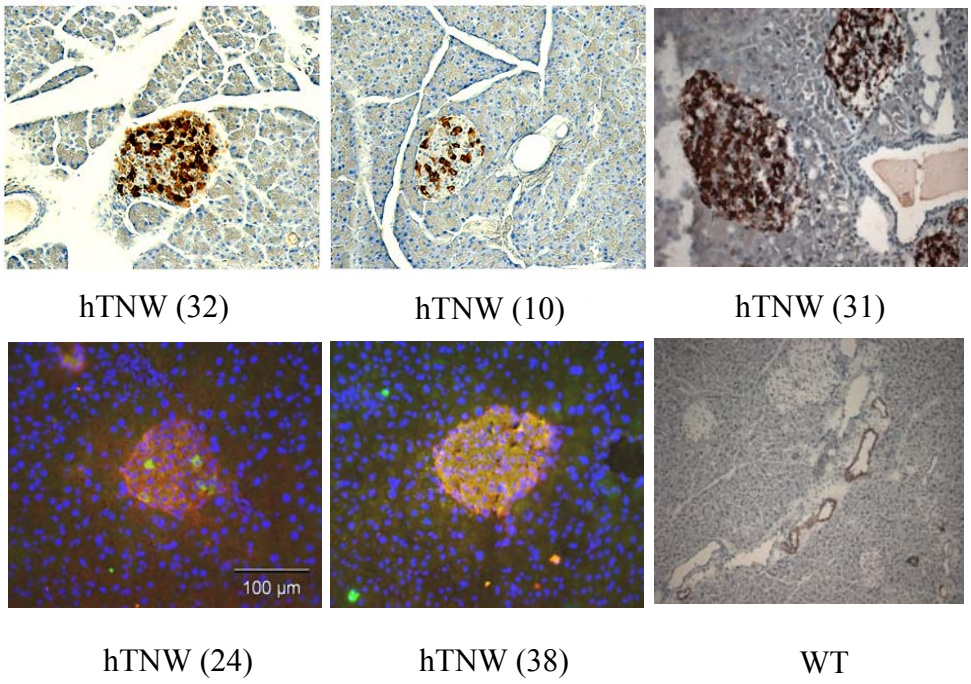

Fig. 3.6 Expression of human tenascin-W in the islets of transgenic RipTNW
The islets from mouse lines 10, 31, 32, 24, 38 were expressed transgene, but the islets of wildtype mice were negative for huTNW.

Generation of RT2/huTNW and RT2/TNC/TNW transgenic mice

I have also generated RT2/TNW and triple RT2/TNC/TNW transgenic mice by crossing RT2 with RipTNW and RiphuTNC transgenic mice, respectively. The double and triple transgenic mice have also been genotyped. The phenotypical characterization of the tenascin-expressing founder lines is currently ongoing in the laboratory of Gertraud Orend.

IV Discussion:

In this first *in vivo* approach to mimicking ectopic expression of tenascin-C in cancer, I have investigated the effects of transgenic expression of human tenascin-C in pancreatic β cells during islet development of RipTNC mice and during β cell carcinogenesis in double transgenic RT2/TNC mice. All transgenic RipTNC mice were healthy and fertile and did not exhibit detectable alterations in tissue morphology and function of the pancreas, but displayed enhanced angiogenesis, indicating that ectopically expressed tenascin-C promotes angiogenesis in normal pancreatic tissues.

Concomitant expression of SV40 T-antigen with tenascin-C caused an increased mortality in RT2/TNC mice in comparison to their RT2 littermates. As RT2 mice mostly die of hypoglycemia at the end of their lifespan, we also investigated whether the early death of double transgenic mice might be due to the aberrant glucose levels in RT2/TNC mice. However, I did not find any difference of blood glucose levels between RT2 and RT2/TNC double transgenic mice at 12 weeks of age, suggesting that the premature death of RT/TNC mice is not due to an increased hypoglycemia.

To determine why double transgenic mice died earlier than RT2 mice, I compared tumor incidence and tumor volume in RT2/TNC with that in RT2 mice at 10 and 12 weeks of age. The incidence of tumors per mouse was virtually of no difference between these two genotypes, which indicated that tumor initiation was independent of tenascin-C but completely dependent on inhibition of p53 and pRb due to expression of SV40Tag (Hanahan, 1985). A detailed analysis of the tumors in RT2/TNC mice at 10 weeks of age indicated that ectopic expression of tenascin-C accelerated tumor progression. Both at 10 and 12 weeks of age, tumors of RT2/TNC mice exhibited an extraordinary high proliferation rate. This pro-proliferation effect of tenascin-C has been recapitulated many times in several cancers and cell lines (Chiquet-Ehrismann, 1986; Huang *et al*., 2001). The mechanism underlying the pro-proliferation effect of tenascin-C might be due to the activation of growth promoting signaling which might be linked to EGFR (Swindle *et al.*, 2001), Wnt (Ruiz *et al*., 2004) and EDNRA (Lange

et al., 2007) (**Fig. 4.1**) together with inactivated p53 and pRb. Highly tenascin-C expressed tumors were frequently concomitant with mutated tumor supressors genes such as p53 and pRb (Ahuja *et al*., 2005)

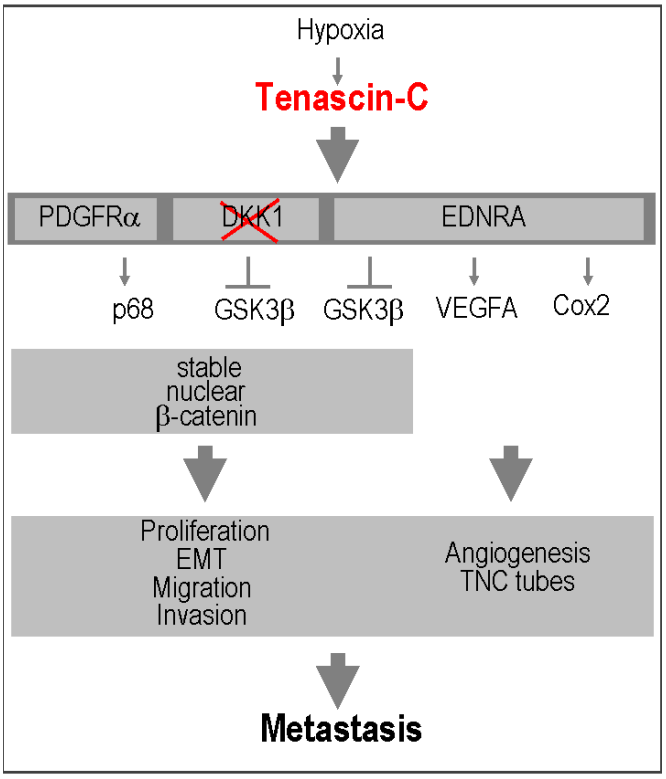

Fig. 4.1 Oncogenic signaling induced by tenascin-C.
Hypoxia induces tenascin-C expression. Highly expressed tenascin-C may activate PDGFRα, EDNRA and Wnt signaling which potentially cooperate in stabilization of β-catenin and its nuclear translocation. This may induce an invasive and metastatic program. Highly expressed tenascin-C may also induce angiogenesis and vasculogenic mimicry by inducing VEGFA and COX2.

Induction of metastasis by ectopically expressed tenascin-C

One of the major findings in my study is that ectopically expressed tenascin-C promotes tumor cell dissemination leading to regional lymph nodes and distant liver metastasis. The causal reason of the most cancer deaths is metastasis rather than the primary tumor. Therefore, metastasis might be the case for the premature death in RT2/TNC mice. In many invasive and metastatic human cancers, a high tenascin-C expression was especially detected at the invasive front of colorectal carcinoma (Beiter

et al., 2005) and metastatic melanomas (Kääriäinen *et al.,* 2006), but was not observed in non-invasive melanomas. In the later study, the intensity of tenascin-C staining correlated with metastasis to sentinel lymph nodes (Kääriäinen *et al.,* 2006). This indicated that tenascin-C plays an important role in metastasis. Metastasis is usually not found in RT2 mice (Hanahan 1985; Perl *et al.*, 1998; 1999) until 12 weeks of age. Of note, β-cell tumors that develop in RT2 mice are capable of local invasion but are not metastatic. To prolong the life of tumor mice and address the potential role of tenascin-C in metastasis, we fed tumor mice with 5% glucose to counteract hypoglycaemia. In this model, increased ectopic tenascin-C expression in double transgenic mice resulted in single or cell clusters of micro-metastasis observed in regional lymph nodes and livers but no metastatic cells were found in their RT2 littermates. These metastatic cells showed features of β-cells and were insulin positive which indicated that they were derived from primary β-cells tumors. It is possible that more metastatic cells were not detected in RT2/TNC mice because most metastasing tumor cells lost part of their differentiation status (Perl *et al.*, 1999). Recently, it was shown that knockdown of tenascin-C by using shRNA significantly diminished the invasive and metastatic ability of the breast cancer cells to the lung (Tavazoie *et al*, 2008). Together, these results indicate that tenascin-C plays an important role in metastasis.

Enhanced angiogenesis and hemorrhaging in RT2/TNC tumors

The growth and metastasis of malignant tumors depends on neovascularization and it has been demonstrated that the degree of tumor angiogenesis is related to clinical outcome in several tumor types (Orend and Chiquet-Ehrismann, 2006). I observed that ectopic expression of huTNC during β-cells carcinogenesis enhanced tumor blood vessel density in double-transgenic RT2/TNC mice with an increased number of CD31-positive endothelial cells as compared with single-transgenic RT2 littermates. Even single transgenic RipTNC mice already exhibited enhanced blood vessels density in the Langerhans islets. An *in vivo* CAM assay demonstrated that tenascin-C attracts neo-microvessel formation. Some of the tenacin-C-induced newly formed vessels were

apparently leaky. All together, these findings suggested that tenascin-C does promote angiogenesis. This is in accordance with previous studies showing that tenascin-C promotes angiogenesis both during development particularly in the lung (Zou *et al.*, 2005) and in pathological conditions such as wound healing, repair of myocardial infarction as well as in tumorigenesis. In human glioma, high tenascin-C expression correlates with the degree of tumor neovascularization (Herold-Mende *et al.*, 2002; Zagzag *et al.*, 1995; Higuchi *et al.*, 1993). CD31 and tenascin-C, however, were rarely co-localized in the double transgenic mice, suggesting an indirect pro-angiogenic effect of tenascin-C. Tenascin-C may exert its pro-angiogenic effect via several signaling pathways. For example, it has been reported that tenascin-C interacts with EGFR through its EGF-like repeats (Swindle *et al*., 2001) and that in the tumors of tenascin-C deficient mice the expression of VEGF and angiogenesis were simultaneously attenuated (Tanaka *et al.,* 2004). I found that the expression of β-catenin in the tumors from double transgenic mice was much higher than that in the single RT2 mice, indicating that tenascin-C activates the Wnt signaling pathway, which may be via downregulation of Dikkopf-1 (DDK-1) (Ruiz *et al.*, 2004). Activation of Wnt signaling is essential for formation of the vasculature in lung (De Langhe *et al.*, 2005), thus tenascin-C might promote tumor angiogenesis through the Wnt signaling pathway in RT2/TNC mice.

In addition, tenascin-C co-localized with the CAF markers α-smooth muscle actin (α-SMA) and vimentin in RT2 tumors. Myofibroblasts are known to foster angiogenesis (Kalluri and Zeisberg, 2006). This observation suggested that the pro-angiogenic effect of CAFs might be mediated by up-regulating tenascin-C expression. Tenascin-C expression may modify ECM composition and facilitate vascular sprouting and migration which are critical for angiogenesis. In tumors, we found that tenascin-C was mainly located close to the center of hypoxic areas, which is in accordance with the recent report that hypoxia induces tenascin-C expression (Gebb *et al,* 2003). The highly expressed tenascin-C in turn may act as a ligand of epidermal growth factor receptor (EGFR), annexin II, integins which are expressed on the surface

of cancer cells and endothelial cells, and may induce angiogenesis by indirect (increased expression of different angiogenic growth factors) and/or direct (endothelial cells) ways.

Tenascin-C promotes tumor progression

Invasion of cancer cells into the surrounding ECM and extravasation into lymphatic or angiogenic blood vessels, which are prerequisite for metastasis, is a complex process in which the ECM is remodeled and cancer cells become motile. E-cadherin-mediated cell-cell adhesion is frequently lost during the development of malignant epithelial cancers. Activation of EDNRA (Lange *et al,* 2007) and inhibition of syndecan-4 (Huang *et al*, 2004) by tenascin-C blocked cell adhesion to fibronectin, which allowed cancer cells to respond to a migratory signal. In this work we showed that tenascin-C induces the epithelial-to-mesenchymal transition of MCF7 breast cancer cells *in vitro*. Previous studies showed that E-cadherin suppression is the causal step for insulinoma transition to malignancy in the RT2 tumor model system but this is not sufficient to trigger distant metastasis (Perl *et al,* 1998). In addition, the β-catenin-mediated Wnt signaling pathway does not contribute to tumor progression in the RT2 model system (Perl *et al,* 1998; Herzig *et al*, 2007). This is probably one of the reasons why RT2 mice display rarely metastasis. In contrast, ectopically expressed tenascin-C led to increased invasive carcinoma numbers and accumulation of β-catenin in the nuclei of tumor cells at the invasion front which may have undergone EMT. These results suggest that in the double RT2/TNC transgenic mice ectopic expression of tenascin-C presumably induces EMT by activating canonical Wnt and/or other signaling. In support of this possibility, Beiter *et al.* (2005) reported that high tenascin-C expression was concomitant with strong nuclear β-catenin expression at the invasive border of colorectal carcinoma cells and revealed that tenascin-C is a target gene of Wnt signaling (Beiter *et al*., 2005). Activation of Wnt signaling by tenascin-C might also be linked to enhanced Id2 expression in the most malignant glioblastomas (Ruiz *et al*., 2004). Induction of tenascin-C by Wnt signaling and activation of Wnt signaling by tenascin-C may thus display a positive feedback loop which could initiate tumor cell

invasion and metastasis.

As mentioned above, tenascin-C expression co-localized with α-SMA and vimentin positive cells but not with CD31, which suggested that tenascin-C is mainly produced by CAFs but not by endothelial cells in RT2 tumors. This absence of a complete overlay of signals for tenascin-C and CAFs indicated that besides CAFs other cells also express tenascin-C. De Wever *et al* (2004) found that CAFs derived from TGFβ-treated normal fibroblasts and from tumors which secreted HGF and tenascin-C, amongst other factors. HGF and tenascin-C proved to be sufficient and essential for inducing an invasive phenotype in cocultured colon carcinoma cells. This involved activation of c-Met, EGFR and Rac and inhibition of RhoA (De Wever *et al*, 2004). This effect of tenascin-C is consistent with the postulated roles of these factors in cell migration, since inhibition of RhoA and activation of Rac are characteristic of migratory cells (Fryer *et al.*, 2005).

Activation of JNK through Rac1 controls nuclear localization of β-catenin (Wu *et al.*, 2008) and tenascin-C activated Rac (De Werver *et al.*, 2004; Orend and Chiquet-Ehrismann, 2006) to initiate colon carcinoma cell invasion, raising the possibility that Rac might play a role in nuclear translocation of β-catenin in RT2/TNC tumor cells. Alternatively, the PDGFRα pathway might be responsible for nuclear translocation of β-catenin by involving p68 RNA helicase (He *et al*, 2006). Since tenascin-C induced PDGFRα expression (Ruiz *et al,* 2004), it is possible that PDGFRα and Wnt signaling cooperate in stabilization of β-catenin and nuclear translocation, which in turn may induce invasion and metastasis.

It is now widely accepted that only a minor population of tumor cells can initiate and support the development of tumors (Dalerba *et al.*, 2007). This small population of tumor cells is presumably also responsible for seeding metastasis. It has been speculated that EMT in cancer stem cell populations maybe involved in the seeding and initiating the growth of metastasis (Brabletz *et al.,* 2005). Since tenascin-C is expressed in stem cell niches, it might play a role in cancer stem cell proliferation

(Orend, 2005). It is intriguing to speculate that those tumor cells at the invasive front exhibiting high expression of tenascin-C and β-catenin are cancer stem cells.

Potential role of tenascin-C in vasculogenic mimicry

Interestingly, analyzing the distribution of tenascin-C in tumor tissue of RT2 and RT2/TNC revealed that tenascin-C was not homogenously expressed but existed in a streak-like pattern that eventually accumulated in tubular channels. In RT2 mice, only few tumors expressed tenascin-C, whereas in double transgenic mice strongly enhanced tenascin-C positive components were deposited into tubular channels. We speculate that these tenascin-C positive components are immune cells, platelets or tenascin-C protein deposits. In RT2 mice the expression pattern of tenascin-C was independent of tumor size and stage, but high tenascin-C signals mainly appeared proximal to the hypoxic centers. A closer investigation showed that the ECM-rich tubular channels contained laminin α5 but no endothelial cells, which indicates that these channels are not blood vessels. Similar to our findings, ECM-rich tubular channels also have also been identified in human melanoma, in particular in those melanomas that were invasive and generated metastasis (Kääriäinen *et al.,* 2006). They were composed of tenascin-C, fibronectin as well as procollagen-I and were filled with melanoma cells. These tubes eventually connected to blood vessels (Kääriäinen *et al.,* 2006). Moreover, these tenascin-C-rich tubular channels in RT2 tumors resemble vasculogenic mimicry (VM, Maniotis *et al*., 1999): the ECM tubes stained for tenascin-C, laminin, collagens IV and VI, mucopolysaccharides, and heparin sulfate glycoproteins (HSPG) (Kääriäinen *et al.*, 2006). VM channels are lined by tumor cells, which are directly exposed to blood flow. This unique structure of VM eventually may facilitate tumor cells to disseminate into blood vessels or further migrate through the bloodstream and metastasize to distant organs. Several studies (Maniotis *et al*., 1999; Sun *et al*., 2004) have demonstrated that VM is associated with poor clinical prognosis in patients. In RT2/TNC tumors the ECM tubes appeared partially disorganized and frequently exhibited a mixture of ECM molecules together with DAPI positive cells. Interestingly, growth of glioma cells on a tenascin-C substratum induced expression of

several ECM molecules such as fibronectin, fibrillin and 6 different collagen molecules (collagen Iα2, collagen IVα1, collagen IVα2, collagen Vα2, collagen VIα2 and collagen XVIIIα1) (Kääriäinen *et al.,* 2006). Similarly, Gerd Klein`s group published the existence of tenascin-C containing tubes in the thymus which serve to transport small blood-borne molecules or chemokines (Drumea-Mirancea M *et al.*, 2005). In RT2 tumors, laminin is wrapped around a tenascin-C core forming a tubular channel with 10 μm of diameter instead of 2 μm in thymic conduits where a fibrillar/procollagen/laminin core is surrounded by tenascin-C. The tubular channels in melanomas, RT2 mice and thymus showed surprising similarity. All together, these findings gave rise to the hypothesis that ectopically expressed tenascin-C in cancer tissue may turn on a genetic program that establishes ECM-rich channels to facilitate tumor cells dissemination to distant organs and to seed metastasis. This hypothesis is supported by the demonstration of Gaggioli *et al.,* (2008) that tracks in the matrix made by fibroblasts are sufficient to enable cancer cell invasion and these tracks were also associated with the deposition of matrix components, such as fibronectin and tenascin-C.

Mouse models to study tumorigenesis and contribution to tumor therapy

Tenascin-C null mice have already been used for breeding with oncomice and for tumor xenograft transplantation assays. In the study of PyTag-induced mammary gland tumorigenesis with a tenascin-C negative background mice manifested that tenascin-C modulates tumor stroma and monocyte/macrophage recruitment but not tumor growth or metastasis (Talts *et al,* 1999). In contrast, Tanaka *et al*. (2004) observed a significantly reduced tumor angiogenesis in the tenascin-C knock-out background upon transplantation of human melanoma cells compared with nude mice expressing tenascin-C. This suggests that tenascin-C supports or enhances tumorigenesis *in vivo*.

Over the past 20 years cancer research has gained major insights into the molecular mechanisms due to the new technical development in transgenic mouse models. The RT2 mouse model offered a good opportunity to study the tumorigenic processes with

reproducible steps in the multiple stages of tumorigenesis. The fact that in this model, metastasis is rare, suggests another advantage, namely the possibility to analyze events triggering tumor metastasis. By taking these advantages of the RT2 mouse model, we created the first *in vivo* model to mimick ectopic expression of tenascin-C in cancer, which allowed us to causally link the function of tenascin-C with tumorigenic processes. We observed that tenascin-C promoted tumor progression by enhancing proliferation, promoting the angiogenic switch, inducing nuclear translocation of β-catenin to trigger invasion involving ECM-rich tubes that may facilitate cancer cell dissemination and metastasis (**Fig. 4.2**). Finally, the RT2/TNC model can be used for testing drugs to inhibit tenascin-C-induced metastasis and also contribute to identification and characterization of novel pathways involved in tenascin-C-induced metastasis.

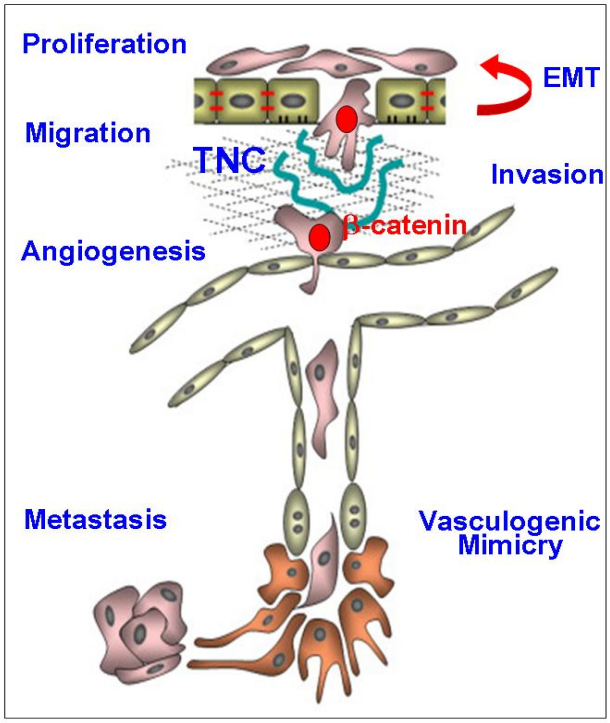

Fig. 4.2 Multiple effects of tenascin-C during tumorigenesis

Tenascin-C stimulated and promoted several events causing metastasis. Induction of nuclear translocation of β-catenin might account for enhanced angiogenesis, proliferation and invasion. TNC-rich tubular channels may promote tumor cell dissemination and metastasis.

How can we use our knowledge about the role of tenascin-C in tumorgenesis to combat cancer? Preventing tenascin-C action in a tumor, for example by restoration of syndecan-4 function in glioma cells, offers a new approach. Another possible approach is to use anti-tenascin-C directed antibody fragments, aptamers (Daniels *et al.*, 2003; Schmidt *et al*, 2004) or tenascin-C blocking small peptides with the aim to obstruct tenascin-C-induced metastasis. In glioblastoma, anti-tenascin-C directed antibody therapies have already been used in clinical trials and this approach seems to be effective (Akabani *et al,* 2005; Reardo *et al,* 2002). Alternatively, shRNA-mediated knock down of tenascin-C could be used to block tenascin-C-induced metastasis. The interference RNA specific targeting for tenascin-C was already used in glioblastomas and showed some efficacy (Zukiel *et al*., 2006).

In conclusion, tenascin-C appears to be a promising anti-cancer target. In the future, we need to increase our understanding of tenascin-C biology to provide more effective and safer anti-cancer therapies.

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VI Curriculum Vitae

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2005-present Ph.D student at the Institute of Biochemistry and Genetics, University of Basel, Switzerland research group of PD Dr. Gertraud Orend
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2004-2005 Visiting research scientist at University of Antwerp, Belgium.
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2003-2005 Doctor in Medicine (M.D), Department of Physiology, Medical school of Qindao University, China
M.D. Dissertation Title: Expression of growth hormone secretagogue receptor 1a in visceral vagal and spinal afferent pathways

2000-2003 Master in Medicine, Department of Physiology, Medical school of Qindao University, China
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1998-2000 Clinical doctor, Departmental of Gastrointestinal Diseases, People's hospital at Shangzhi, Heilongjiang, China

1993-1998	Bachelor in Medicine, Haerbin Medical University, Haerbin, China
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Expression of motilin in the hypothalamus and the effect of central erythromycin on gastric motility in diabetic rats. Yun-*dan,Jia,* Chang-qing Liu, Ming Tang, Zheng-yao Jiang. 2007. *Neuroscience Bulletin* 23(2):75-82.

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