Extracellular Vesicles: An Emerging Mechanism Governing the Secretion and Biological Roles of Tenascin-C

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ECM composition and architecture are tightly regulated for tissue homeostasis. Different disorders have been associated to alterations in the levels of proteins such as collagens, fibronectin (FN) or tenascin-C (TnC). TnC emerges as a key regulator of multiple inflammatory processes, both during physiological tissue repair as well as pathological conditions ranging from tumor progression to cardiovascular disease. Importantly, our current understanding as to how TnC and other non-collagen ECM components are secreted has remained elusive. Extracellular vesicles (EVs) are small membrane-bound particles released to the extracellular space by most cell types, playing a key role in cell-cell communication. A broad range of cellular components can be transported by EVs (e.g. nucleic acids, lipids, signalling molecules and proteins). These cargoes can be transferred to target cells, potentially modulating their function. Recently, several extracellular matrix (ECM) proteins have been characterized as *bona fide* EV cargoes, exosomal secretion being particularly critical for TnC. EV-dependent ECM secretion might underpin diseases where ECM integrity is altered, establishing novel concepts in the field such as ECM nucleation over long distances, and highlighting novel opportunities for diagnostics and therapeutic intervention. Here, we review recent findings and standing questions on the molecular mechanisms governing EV-dependent ECM secretion and its potential relevance for disease, with a focus on TnC.

Keywords: tenascin C, extracellular matrix (ECM), exosomes, fibronectin (FN), tumor progression, cardiovascular disease, inflammation

INTRODUCTION

Multicellularity drove the emergence of cell differentiation and functional specialization, changing the continuous communication cells establish with their surrounding environment. A connective substance among tissues ensuring nurturing and functional coordination between cells evolved, giving rise to the extracellular matrix (ECM) (1, 2). In addition to providing a physical scaffold, the ECM actively participates of several biochemical and biomechanical processes related to morphogenesis, differentiation and homeostasis. A meshwork generally composed of water, proteins, glycoproteins and proteoglycans, the ECM exhibits tissue-specific matrix composition and architecture, which provide unique physicochemical properties (3, 4). Importantly, the ECM is constantly remodelled by cells to
maintain tissue and organismal homeostasis across conditions (5–7). Apart from the regulated secretion of specific structural components, ECM architectural remodeling is orchestrated by secreted modifying enzymes (metalloproteinases (MMPs) (8) and their inhibitors (TIMPs) (9), and other enzymes controlling ECM modification and crosslinking—such as lysyl oxidases (LOX) (10) or transglutaminases—, and a reciprocal biomechanical crosstalk with resident cells (11). Several growth factors and cytokines are bound to the ECM and modulate cell adhesion, differentiation, growth and migration (12) and its architecture and physical properties can modulate cell function (13). Conversely, cell proliferation, spatial arrangement and contractility drives ECM remodeling (14–16).

The broad functional relevance of the ECM is reflected by the numerous pathological conditions associated with ECM alteration or dysfunction. Some of these diseases are related to genetic abnormalities that imply a decrease in the expression, or post-translational modification, of certain ECM proteins (17–20). On the other hand, desmoplasia—an increase in bulk ECM deposition and/or dysregulated expression of certain ECM components—(21), causes architectural and biomechanical alterations driving different pathologies, including cardiovascular diseases, chronic inflammation or cancer.

Tenascins are a family of extracellular matrix (ECM) glycoproteins composed of five members (Tenascin-C (TnC), R, W, X and Y), TnC being the best characterized among them (22). TnC is a hexameric protein which contributes to regulate cell substrate adhesion through the modulation of focal adhesion (FA) binding to other ECM components such as fibronectin (FN) (23), and downstream events such as cell activation, apoptotic cascades, and migration. TnC is expressed abundantly during development, especially in the neural system. However, expression levels of TnC in adults are substantially reduced and its presence is virtually limited to stem cell niches and tendons. Increased TnC expression in adult, differentiated tissues is commonly associated with tissue damage and repair, as well as with pathological conditions such as dysregulated inflammation (as occurs, for example, in atherosclerotic lesions) or tumorigenesis (24–31).

Despite their physiopathological relevance, our understanding of the intracellular mechanisms regulating the trafficking and secretion of TnC and many other ECM components is limited (32). Notably, recent studies support that extracellular vesicles (EVs), including exosomes and microvesicles (MVs), can act as carriers of ECM components, including TnC and FN, a well-known, evolutionarily related partner of TnC (33, 34). Here, we review our current knowledge on the role of EVs on TnC secretion and ECM deposit, and their potential relevance for inflammation and disease.

**PHYSIOPATHOLOGICAL ROLES OF TnC AND THEIR MOLECULAR BASIS**

Certain features of tumor progression and metastasis are currently considered subverted, aberrant wound repair programs (35), where ECM deposit and remodeling by resident fibroblast is dysregulated. This altered stromal ECM can in turn promote several cancer hallmarks (36). For example, sustained proliferation requires cell adhesion to ECM and growth factor-dependent activation of Erk and PI3K, to promote G1/S transition. The ECM can also promote the induction of hypoxia-triggered angiogenesis acting as a reservoir of angiogenesis regulators, activate cell invasion through the regulation of cell adhesion and invadopodia formation, or modulate the immune response (13, 37). Several ECM components exhibiting differential expression and/or arrangement in tumors play relevant roles in the progression of the disease. Altered deposition of different collagen types can regulate cell growth, differentiation and cell migration. An excessive deposition of collagen I in many solid tumor types confers rigidity to the tumor stroma, and its altered assembly and crosslinking, mechanical properties and architectural features such as anisotropy, affect tumor cell biology (3, 5). Other key ECM components also exhibit altered expression in cancer. FN is considered a major building block in ECM fibre assembly and remodeling, and can bind to other molecules including heparin, collagens, tenascins or fibrin to modulate their assembly and their interaction with cells (38, 39).

During development, TnC is expressed robustly and contributes to physiological epithelial-to-mesenchymal transitions (EMT) and morphogenesis (25). In contrast, in normal adult tissues TnC expression is usually low, except for stem cell niches and tendons. Upon tissue damage, TnC can be rapidly upregulated and contributes to physiological inflammation and repair. Owing to its capacity to promote proinflammatory and activated states in different cell types, increased TnC deposition is associated with several pathological conditions. Persistent high levels of TnC can promote chronic inflammation and desmoplasia, driving pathological events such as fibrosis or oncogenesis.

TnC was initially characterized as a modulator of cell adhesion, either through its interaction with other ECM components (23) or through direct binding to specific cell receptors. Its binding to integrins such as α9β1, αVβ3, α8β1 and αVβ6 (27, 28) can induce EMT in several cancer models (40–42), modulate the dynamics of focal adhesions (43, 44) or reduce apoptosis. These characteristics support its potential as a marker of poor progosis, underpinned by its impact on cell motility and invasion, aberrant angiogenesis (45) and immunomodulation (31, 46, 47). Importantly, TnC modulates the activation state of immune cells such as macrophages and lymphocytes; this appears to be an important aspect of its contribution to both physiological tissue repair, as well as pathological conditions involving tissue remodeling (48–50).

Several mouse models reveal the importance of TnC in tumor progression and its implication in tumor cell survival, proliferation, invasion and metastasis (51, 52). TnC can influence fibroblasts and differentiation of epithelial cells onto myofibroblasts through the tumor growth factor-β (TGF-β) signalling pathway (53), regulate inflammatory signalling by an activation of Toll-like receptor 4 (TLR4) (54) or modulate epidermal growth factor (EGF)-receptor driven cell proliferation cell proliferation (55). As part of the AngioMatrix (56), TnC can participate in the angiogenic switch,
and generate an aberrant vasculature within tumours. Both as a result of this promotion of aberrant angiogenesis, as well as through direct modulation of immune cell populations, TnC is likely an important contributor to the emerging role of stromal ECM composition and architecture as central regulators of antitumor immunity (57–60). An intriguing feature that may be particularly relevant for the rationalization of TnC as a biomarker, or even therapeutic target, in the context of antitumor immunotherapy is its potential to selectively determine macrophage polarity towards M1-like, cytokine-releasing phenotypes (mainly through its interaction with α9β1, αVβ3 and TLR4 receptors); and promote an anergic state in T-cells (presumably by interfering with integrin signalling) (61, 62). Recent studies have shown the beneficial effects of targeting TnC in antitumor immunotherapy in breast (63) and oral squamous cell carcinoma (64) mouse models. Combinational therapy with monoclonal antibodies that inhibited TnC-mediated TLR4 activation and anti-PD-L1 treatment significantly reduced tumor growth and lung metastasis in vivo (63). In line with these results, ablation of TnC or its effector CCR7 implied inhibition of the lymphoid immune-suppressive stromal properties, reducing tumor progression and metastasis in oral squamous cell carcinoma (64), indicating a relevant approach in the therapy of head and neck tumors.

TnC has a prominent role in cardiovascular tissue remodeling. Almost invariably, TnC re-expression is associated with cardiovascular pathological processes coursing with inflammation, such as myocardial infarction, hypertensive cardiac fibrosis, myocarditis or dilated cardiomyopathy (65–67). Uprogulation of TnC is also a hallmark of the proatherogenic vessel remodeling, driving the progression of atherosclerotic disease (AS) (68–70); however, TnC deficiency in mouse models of genetic hypercholesterolemia exacerbate atherosclerosis and promote lesions prone to rupture, reflecting the delicate balance between the physiological roles of TnC in tissue homeostasis (71).

TnC can play a role in several diseases derived from a fibrotic state generated upon tissue damage (50, 52, 72, 73). For example, in neuroinflammation (29), brain injury (74) or glioma (59), where ECM deposition is enhanced, TnC accumulation is found associated with blood-brain barrier disruption, neuronal apoptosis and activation of inflammatory pathways (mitogen-activated protein kinases and NF-kB). Finally, TnC is implicated in other fibrotic diseases such as kidney and liver damage through orchestration of the fibrotic niche and is considered as a biomarker of poor prognosis (75, 76).

THE STANDING QUESTION OF TnC SECRETION

ECM component biogenesis, intracellular trafficking and export pathways are tightly controlled, but our current mechanistic understanding of these processes, particularly regarding non-collagen ECM components, is rather limited. Collagens, a family of large fibrillar ECM proteins, constitute over 30% of the total protein mass in multicellular organisms (77–79). These proteins are initially synthesized as an immature form, known as preprocollagen, in the endoplasmic reticulum (ER). These polypeptides undergo hydroxylation of proline and lysine residues and are assembled as triple helices, yielding procollagens (80). Procollagens must then be trafficked to the Golgi apparatus for further posttranslational modification. The coat complex type II (COPII) vesicle transport machinery facilitates the regulated transfer of proteins from the ER to the ER-Golgi intermediate compartment (ERGIC) and cis-Golgi (81, 82), and is strictly required for procollagen trafficking and secretion: mutation or genetic ablation of core COPII components such as SAR1B, SEC23A, SEC24A/C or SEC13 profoundly affect the secretion of collagens and lead to their accumulation in the ER (32). In contrast with smaller cargoes, procollagen units are too big (~300nm in length) to be incorporated into conventional ~80-nm COPII (83), and additional regulators (transport and Golgi organization protein 1 (TANGO1), cutaneous T-cell lymphoma-associated antigen 5 (cTAGE5), trafficking From ER To Golgi Regulator (TFG), or the KHLH12-cullin-3 ubiquitin E3 ligase complex) (84–87) have been identified as required for nascent COPII vesicles to accommodate and carry these rigid fibrillar molecules. Finally, procollagens are transported in tubular structures emanating from the Golgi to the plasma membrane (PM) and secreted to the extracellular space, where they will be cleaved to generate tropocollagens and assembled in crosslinked fibrils (Figure 1).

While this canonical route is relatively well characterized for collagens, several of its regulators, including core components of the COPII machinery, appear to be dispensable for the secretion of other ECM components. Indeed, the mechanisms governing the trafficking and secretion of a majority of non-collagen ECM proteins have remained puzzlingly elusive (32, 88). Soluble FN, which assembles in fibrillar structures upon secretion to the extracellular space and binding exposed integrins (38), is initially synthesized in the ER (32, 89). Current models describe its transport to the extracellular space through the secretory pathway (90–93), as it reaches the Golgi apparatus (94–98) to undergo further glycosylation (39). However, FN secretion seems to be unaffected by mutation or genetic ablation of core COPII components that severely impair collagen transport from the ER, such as SEC23A (99), SEC24D (100) or TANGO1 (32, 101), and its trafficking remains incompletely explored. Proteins such as peristin (89) and transmembrane P24 Trafficking Protein 2 (TMED2; the human homolog of emp24) (102) are proteins potentially associated with the export of FN from the ER.

TnC has a six arm-structure termed hexabrachion, consisting of six 320kDa monomers stabilized by amino-terminal disulphide bonds. In contrast to FN, oligomerization of TnC is a rapid process that takes place cotranslationally in the ER, and two models have been proposed. In one model, the six monomers are simultaneously assembled into a single hexabrachion, as suggested by pulse-chase approaches which found no apparent intermediate species (103). In the second model, oligomerization is a two-step process (104), whereby two intermediate trimers are first formed through the stabilization of alpha-helical coiled-coil interactions at their amino-terminal
domains. Then, hexabrachion assembly is favoured by an increase in homophylic binding affinity between the two trimers. Similar to FN, evidence supporting TnC transit through (105), and glycosylation at (24), the Golgi, suggests that TnC is trafficked from the ER to the Golgi apparatus. This transfer appears to be a rate-limiting step for secretion output (103) and is affected in cells treated with brefeldin-A, an inhibitor of ER-Golgi vesicle transport (106). However, like for many other ECM components, the precise mechanisms regulating TnC trafficking and secretion remain incompletely characterized. An unexpected, emerging mechanism for the secretion of these and other non-collagen components, is extracellular vesicle (EV) secretion.

**EV BIOGENESIS AND GENERAL FUNCTIONS**

Recent studies show that extracellular vesicles (EVs) can export ECM components to the extracellular environment (107), constituting alternative mechanisms for ECM secretion and deposition and implying specific regulatory principles for their trafficking (108, 109). EVs are a heterogeneous group of cell-derived membranous structures that include exosomes and MVs, which defer on their intracellular origin (110).

Exosome biogenesis takes place in the endosomal compartment through endosome membrane budding (111–113). Several mechanisms have been implicated in this process. One of the
most studied mechanisms is dependent on the ESCRT (Endosomal Sorting Complexes Required for Transport) machinery, whose four conserved complexes (ESCRT-0, -I, -II and -III) (114–116) assemble sequentially on the cytosolic surface of the endosomal membrane. Ubiquitylation is an important event not only for ESCRT-dependent vesicle formation, but also for the specification of cargo to be sorted onto exosomes (113, 117). Additionally, evidence for an ESCRT-independent mechanism for exosome biogenesis has been described (118). Specific lipid species such as ceramides (derived from sphingomyelinases-mediated hydrolysis of sphingomyelin) (119), LBPA (lyso-bis-phosphatidic acid) (120) or cholesterol, as well as proteins that modulate membrane organization, including tetraspanins (121) and caveolin-1 (108, 122, 123), have been recently identified as important regulators of ESCRT-independent endosome dynamics and exosome biogenesis.

On the other hand, MVs are derived from scission of small plasma membrane-derived vesicles (110). This process—termed ectocytosis—shares many similar steps to exosome formation. The ESCRT machinery, as well as cytoskeletal elements and their regulators, such as RHO family of GTPases and ROCK, are important for the formation of MVs together with other membrane-associated proteins, including tetraspanins and membrane cargos (124) (Figure 2).

Virtually every cell type can release EVs, and these structures are abundant in the extracellular space and body fluids such as plasma, urine and saliva. A broad spectrum of cargoes (e.g. nucleic acids, proteins or signalling molecules) can be sorted onto these vesicles and subsequently exported and transferred to target cells. Many cargoes have been related to the modulation of the biology of acceptor cells in multiple physiologic and pathologic scenarios.

Immune responses are exquisitely regulated to ensure defence from external pathogens or physicochemical insults as well as internal alterations such as tumor cell growth, while avoiding damage of the self. EVs are crucial in the intricate cell-cell communication involved. EVs are frequently described as pro-inflammatory mediators and participate in the propagation of inflammatory signals during infections and chronic inflammatory diseases among components of innate immunity. Mechanistically, several cargoes such as cytokines, receptors and microRNAs can modulate the activation state and function of macrophages, neutrophilic granulocytes and natural killer (NK) cells (125). EVs also participate of several steps of acquired immunity and antigen presentation. Antigen presenting cells (APCs), including B lymphocytes, dendritic cells (DCs) and macrophages can release major histocompatibility complex II (MHC-II) through exosomes enabling antigen presentation to CD4+ T lymphocytes at distance (126). EVs released by tumor cells or several pathogens can constitute a relevant source of antigens for APCs for their processing and presentation to CD4+ T lymphocytes (127, 128).

**Figure 2** Extracellular vesicle formation. (A) Extracellular vesicles are classified according to their origin. Microvesicles are formed from the PM in a process called ectocytosis that depends on the ESCRT machinery and the actin cytoskeleton. Exosomes are derived from the budding process on the membrane of the endosome/multivesicular body, and released to the extracellular space upon fusion of the endosome with the PM. (B) Diagram of the structure of the ESCRT machinery and of ESCRT-independent mechanisms implicated in exosome formation.
EVs also actively participate of the immune synapse between lymphocytes and APCs, and lymphocytes specifically reallocate multivesicular bodies (MVBs) towards the contact site, leading to a localized increase in exosome secretion and unidirectional transfer of microRNAs that modulate downstream responses. Highlighting the key role of EV communication in this process, inhibition of exosomes formation-secretion dysregulates gene expression in APCs (129) and reduces antibody production in activated B-cells (130, 131).

Immune cell-derived EVs are also involved in other inflammatory processes such as tissue fibrosis, where an increase in ECM deposition has been described to impact cell behaviour, including cell proliferation, migration and differentiation, and subsequently participating in the development of several pathologies. A prominent EVs profibrotic cargo is interleukin-1β (IL-1β) (132), which is released by DCs upon binding of ATP to P2X purinoceptor 7 (P2X7R) (133) and can act on several IL-1β receptor-expressing cell types (134, 135). IL-1β can, in turn, induce vesicular secretion of interleukin-6 (IL-6) in mast cells, amplifying inflammation (136). Other ligands that induce fibrosis such as TGF-β or TNFαt have also been described as EVs cargoes.

EVs-dependent secretion and inter-tissue communication is also involved in vascular physiopathology (137–140). EVs-mediated communication can be involved in either AS progression or lesion prevention. Krüppel-like factor 2 (KLF2)-expressing endothelial cells (ECs) (an atheroprotective hallmark) can load miR-143/145 in exosomes to control smooth muscle cell (SMC) activation and reduce AS lesion formation (141). In contrast, proinflammatory cues on ECs repress the presence of Ten-eleven translocation 2 (TET2) dioxygenase in exosomes, promoting plaque formation (142). SMCs can influence back endothelial function through EVs: SMC-derived E cargo miR-155 increases endothelial permeability (143). EVs also play a role in the development of an inflammatory environment in the progressing atherosclerotic plaque (144–146). EVs may also directly contribute to subendothelial matrix remodeling and lesion progression, either through recently discovered ECM deposition (see below), as well as spondomyelin phosphodiesterase 3 (SMPD3)-dependent calcification (147).

Myocardial injury engages mechanisms to repair and maintain cardiac function, including cardiac fibrosis by activation of resident fibroblasts through TGF-β, EDN-1, PDGF, CCN2 and AGTII ligands, which can be released through EVs derived from cardiomycocytes and ECs. Reflecting a role in events after myocardial injury, miRNAs cargo signatures on EVs (including miR-1, -208, -214) (148, 149) emerge as good biomarkers of myocardial infarction detection and prognosis from plasma samples.

Tumor cells (TCs) usually secrete large amounts of EVs, which can influence different aspects of tumor progression and behaviour, including tumour-associated fibroblast activation, angiogenesis, immunomodulation, matrix remodeling or the establishment of pre-metastatic niches. TC populations are heterogeneous (150–152). TCs communicate inside the tumor and can transfer part of their unique characteristics to other surrounding cancer cells. For example, tumour-derived EVs can modulate local growth via autocrine transfer of mutant KRAS proto-oncogene to wild type KRAS-expressing colon cancer cells (153). Similarly, glioblastoma microvesicles transport specific RNAs that promote neighbour proliferation (154). EVs can also transmit their capacity to adapt to the characteristic tumor stresses such as hypoxia, changes in pH and nutrient deprivation (155).

Tumor angiogenesis and abnormal vascularization determines its behaviour and response to therapy (156, 157). Many pro-angiogenic factors are tumoral EV cargoes, such as the vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), TGF-β, TNF-a or fibroblast growth factor (FGF) (158). Tumour-derived exosomes can also induce vascular permeability in distant organs in breast, melanoma and colorectal cancers (159–161).

Antitumor immunity and its suppression by tumors are another major focus of research and therapeutic intervention, and EVs also play a role in this process. DCs induce T-cell and NK cell activation in an EV-dependent manner to mount an antitumor response (126, 162–164). As the tumor progresses, TCs deploy mechanisms such as attenuation of NK cell cytotoxicity (block of NKG2D pathway), reduction of T-cell-mediated killing or activation of myeloid-derived suppressor cells (TC-derived EVs can contain PGE2, TGF-β and HSP72) (127).

Under physiological conditions, fibroblasts are in a quiescent state. Upon tissue damage, they can enter an activated state, whereby a “secretory phenotype”—to produce both paracrine signals and new ECM components—and contractile activity—for the biomechanical remodeling of tissue—are acquired. Dysregulated persistent activation is a hallmark of tumour-associated fibroblasts (TAFs) (165) and other pathological conditions coursing with fibrosis and desmoplasia. Tumour-derived EVs can induce fibroblast activation (166), by virtue of microRNA cargo subsets modulating motility, collagen contraction or proliferation (167). TC-derived exosomes can also induce secretion of specific ECM components, such as FN (168, 169), as well as ECM remodeling enzymes (170, 171).

Evidence suggests that EVs can actively participate of ECM sculpting (172, 173), through ECM remodeling cargoes such as MMPs (174) or lysyl oxidases (175, 176). Active MMPs such as MMP-1, -13, -2, -3 or -14 are detected on the surface of EVs (ADAM10 and ADAM17) have been described as EVs cargoes under physiological conditions, fibroblasts are in a quiescent state. Upon tissue damage, they can enter an activated state, whereby a “secretory phenotype”—to produce both paracrine signals and new ECM components—and contractile activity—for the biomechanical remodeling of tissue—are acquired. Dysregulated persistent activation is a hallmark of tumour-associated fibroblasts (TAFs) (165) and other pathological conditions coursing with fibrosis and desmoplasia. Tumour-derived EVs can induce fibroblast activation (166), by virtue of microRNA cargo subsets modulating motility, collagen contraction or proliferation (167). TC-derived exosomes can also induce secretion of specific ECM components, such as FN (168, 169), as well as ECM remodeling enzymes (170, 171).

Evidence suggests that EVs can actively participate of ECM sculpting (172, 173), through ECM remodeling cargoes such as MMPs (174) or lysyl oxidases (175, 176). Active MMPs such as MMP-1, -13, -2, -3 or -14 are detected on the surface of EVs derived from several tumor cell types. Moreover, ADAMs family (regulators of cell adhesion and migration) components and more specifically the two most notorious members of this family (ADAM10 and ADAM17) have been described as EVs cargoes (177, 178).

**EVs AS ECM CARRIERS: IMPLICATIONS IN ECM SECRETION**

Recent studies support that some ECM components are EVs cargoes themselves, implying that trafficking and export mechanisms could coexist with canonical secretion pathways, modulating ECM composition and architecture and...
subsequently impacting on cell behaviour. Additionally, EV function could also be linked to ECM remodelling in the sense that ECM fiber components might influence the retention of EVs at specific regions through discrete subsets of receptors in their surface (168), therefore contributing to their selectivity for cell type targeting and favouring a specific evolving composition and architecture of the ECM during its remodeling.

Early observations hinting at the involvement of EVs in matrix secretion and deposition described “matrix vesicles” (179, 180), as a relevant mechanism for osteoblast-mediated primary bone mineralization (181, 182). Secreted matrix vesicles initiate the nucleation of calcium phosphate crystals by an influx of Ca$^{2+}$ and PO$_4^{3-}$ through their membrane transporters and the action of several intraluminal enzymes such as tissue-nonspecific alkaline phosphatase (TNSALP), ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) or phosphoethanolamine/phosphocholine phosphatase 1 (PHOSPHO1) (182). Interestingly, a role for matrix vesicles has been also described in vascular SMC-driven calcification during AS progression (147).

Our understanding of the implication of EVs in the secretion and deposition of specific ECM components has since considerably lagged. Recent studies have shown that ECM proteins are exported and deposited by EVs (183, 184) (www.vesiclepedia.org, www.exocarta.org) and animal models in which exosome production has been abrogated through disruption of neutral sphingomyelinase (NSMase) activities show marked alterations in ECM deposition and architecture (185, 186). FN is a prominent ECM cargo in EVs from different cell types, and the blockade of exosome secretion partially alters, although does not completely impair, FN fiber deposit (108). Other groups have recently described that FN is transported by EVs. FN accumulates at the surface of exosomes through its binding to heparan-sulfate (187, 188) and that upon beta1 integrin endocytosis (189), FN can be redeposited from the endosomal compartment at the basal cell surface in epithelial cells (contactin-dependent) (190) and in epicardial cells (mediated by Bves and NDRG4) (191). Moreover, Weaver and colleagues suggested that exosome secretion plays a key role in autocrine deposition of FN at the leading edge of the cells: Golgi secreted FN would be in an inactive form previous to its assembly at the cell surface (38), exosomal FN, presumably sourced from the endosomal compartment (109, 189, 191) would constitute a rapid alternative pathway for competent adhesive substrate deposition (Figure 3).

FN-containing EVs have been associated with tumor progression. Certain features of tumor cells can be altered by the presence of FN-positive EVs in the media. Weaver and coworkers have characterized that exosome secretion in invadopodia is essential for FN resecretion, and regulates cell adhesion, directional motility and invasion in tumor cells (109, 192, 193). Invasiveness of fibroblasts is positively regulated by FN-positive EVs treatment (194). Exosomal FN can modulate other functional programmes such as proliferation (195), signal

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**FIGURE 3** | Structure and exosomal secretion of FN. (A) Structure of a FN dimer stabilised by a di-sulphide bond. Basic domains (FNI, II, and III) and the main binding sites to other ECM proteins and receptors are depicted. (B) Exosome-mediated FN secretion and cell migration. Caveolin-1-dependent beta1 integrin endocytosis is implicated in the internalization of extracellular FN. Upon endocytosis, FN is transported to endosomes, where exosomes are formed (stage I) (193). Exosomal FN is then released at invadopodia, and induces cell migration and invasion through its internalization (139) or via activation of integrin-mediated pathways (192) (stage II).

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transduction (138, 196), endocytosis (197) or cell survival (198). Finally, exosomal FN can modulate tumor immunity. Secretion of FN-containing EVs can be induced by tumour-associated leukocytes (199), but these FN pools can also induce pro-inflammatory IL-1β production by macrophages (200). FN interacts with acceptor cells through plasma membrane heparansulfate and α5 integrin receptor (109, 187, 200). However, the mechanism of action of exosomal FN seems to require its internalization (138). This apparent discrepancy may indicate that exosomal ECM could be activating several pathways depending on the mechanism by which they interact with acceptor cells (Table 1).

**EV SECRETION: AN INTEGRAL ASPECT OF TnC BIOLOGICAL ROLES**

Recent studies demonstrate that exosome secretion is strictly required for appropriate extracellular TnC deposition by both tumor cells and different fibroblast types (108, 122) (Figure 4). Circulating exosomes from cancer patients frequently carry TnC (24), and several cancer cell types secrete TnC in EVs *in vitro* (183, 184) (www.microvesicle.org, www.exocarta.org). Disruption of exosome secretion by pharmacological inhibition or RNAi-mediated depletion of NSMase 2 led to accumulation of TnC at the ER and decreased extracellular TnC fibre formation. These studies excluded internalization of extracellular TnC and established that exosome-secreted TnC is synthesized *de novo*. Mechanistically, caveolin-1 [Cav1; a pivotal regulator of membrane organization, mechanoadaptation, ECM remodeling and cholesterol efflux (16, 203–205)] is strictly required for the appropriate biogenesis of exosome subpopulations of different sizes, and the sorting onto them of specific ECM components, through the control of cholesterol content in endosomal compartments. Interestingly, this effect varies across ECM exosome cargoes, suggesting that the extent of dependency on different secretion routes may be specific for each ECM component; for example, in contrast with TnC, FN deposition is only partially decreased upon disruption of exosomal secretion. Cav1 deficiency, exogenous cholesterol loading or pharmacological inhibition of cholesterol trafficking from endosomes all markedly impaired exosomal secretion of TnC. Cholesterol homeostasis emerges as an as yet poorly understood mechanism by which membrane trafficking and metabolism potentially feed onto functions allocated at the endosomal compartment, including cell signalling regulation (206, 207) and exosome secretion (108).

The involvement of Cav1 as a central regulator of this process is not trivial. Cav1 is a central node simultaneously regulating the transduction of information on ECM composition and physical properties (204), and the coordinated remodeling of both aspects (16, 108, 208). This reciprocal crosstalk [first discussed by Bissell and Hall as stromal dynamic reciprocity (209)] is key to understand both physiological and pathological processes pertaining different tissues. Furthermore, collagens are not a class of ECM components correlating with TnC in their Cav1-dependent sorting onto exosomes; in fact, Cav1 might regulate oppositely COPII-dependent deposition of collagen, and exosome-mediated secretion of other ECM components (210). It remains to be studied whether other components of caveolae such as PTRF—which does appear to modulate exosome-mediated secretion (211)—also regulate the sorting of ECM components to exosomes. Cav1-dependent regulation of tissue architecture and cell function is relevant for several conditions in

| FN EVs origin | Target cell | Detection approach | Result | Ref. |
|---------------|-------------|--------------------|--------|-----|
| Myeloma RPMI-8226 and CA9 | Human bone marrow stroma (HS-5), Human umbilical vein endothelial cells | WB and light microscopy | Exosome-cell interaction and internalization | (187) |
| Fibrosarcoma (HT1080) | Fibrosarcoma (HT1080) | WB and sucrose gradient | Increased endothelial cell invasion | (192) |
| HIV-1 infected dendritic cells | T-lymphocyte | WB | Increased motility | (201) |
| Human trabecular meshwork cells | N/A | WB | Viral trans-infection | Increased IFN-γ, TNF-α, IL-1β and RANTES Activation of p38/Stat pathways | (185) |
| Fibrosarcoma (HT1080) | Fibrosarcoma (HT1080) | WB and sucrose gradient | Turnour cell migration | (109) |
| Transplantation patient serum | N/A | WB | Allograft rejection biomarker | (202) |
| Human trophoblast | Macrophage | WB | Increased IL-1β production | (200) |
| Mesenchymal stem cells | Bone marrow (SH-SYSY) | WB and Proteomics | Increased mitosis and growth factor secretion | (195) |
| Endothelial cells | Hepatic stellate cell | WB and electron microscopy | Increased AKT phosphorylation | (138) |
| Tumour-associated leukocytes | Breast cancer (AT-3), Colon cancer (4T1, CT26) | WB and FACS | Increased cell migration | (199) |
| Fibroblast (IMR90) | Fibroblast (IMR90) Proteomics, FACS | WB, proteomics, light microscopy | Increased exosomal FN | (199) |
| Primary melanocyte | Primary melanocyte | WB, proteomics, light microscopy | Increased tumour cell invasion | (194) |
| Microvascular endothelial cells | Oligodendrocyte precursor cell (OPC) Proteomics, enzyme-linked immunosorbent assay | | | (197) |
which TnC has a prominent role, such as tumor progression or cardiovascular remodeling (16, 212, 213). An additional standing question is whether Cav1 expression (both during exosome biogenesis as well as at destination) may determine the specificity of exosome-mediated communication, given the prominent role integrins appear to have in this process (168).

Exosome secretion appears to account for the major share (if not the totality) of TnC extracellular release and deposition (108); thus, virtually all biological/physiopathological roles of TnC should be framed by the specific features of exosomal communication. Exosomes enable the transport of cargoes across interorgan distances, and TnC-containing exosomes can nucleate ECM beds in different organs of TnCKO mice such as liver and lungs upon intravenous injection (108); these observations suggest that exosomal deposit of TnC and associated ECM components contributes significantly to pre-metastatic niche formation (169). These pools of exosomal TnC are fully functional and apart from fostering ECM fiber nucleation, efficiently induce proinflammatory states and features compatible with EMT in breast cancer cells in 2D and 3D culture models (108, 122). Exosomal TnC levels also correlate with invasiveness in pancreatic ductal adenocarcinoma (184, 214), and induce invasion through WNT/β-catenin signaling, a crucial pathway in EMT modulation, and activation of the NFκB pathway (214). Exosomes have also recently emerged as efficient platforms for immunomodulation in the tumor microenvironment and other tissue contexts (131, 215); it is likely that the prominent roles TnC has as a regulator of immune cell function (see first section) are exerted at least in part through exosomes. Interestingly, exosomes released by SARS-CoV2-infected cells are significantly enriched in TnC and could promote the propagation of inflammation to distant sites (216). Serum TnC levels have been explored as diagnostic/prognostic markers in different pathologies (24), but whether all circulating TnC is exclusively trafficked through EVs is yet to be determined. Other examples of paracrine secretion of TnC in exosomes include osteoblasts (217), airway epithelial cells (218) and several tumor cells (183, 184), where exosomal TnC has been associated to alterations of pre-existing ECM, impacting collagen and alkaline phosphatase activity. Yong and co-workers also described that brain tumour-initiating cells can secrete TnC in exosomes and suppress T-cell activation, enabling tumor progression and metastasis through the modulation of antitumor immunity (219). Mechanistically, TnC could inhibit T-cell activation and proliferation through the well-established TnC receptors a5β1 and αvβ6 integrins, reducing mTOR signaling (Figure 5 and Table 2).

Additionally, it may be considered that TnC fibers at a given ECM niche could act as efficient receptors for the homing of exosomes and thus be involved in the propagation of inflammation to distant organs and tissues (108).

**FIGURE 4** | Structure and exosomal secretion of TnC. (A) Structure of a trimer of TnC. Tenascin monomers bind via the tenascin assembly domain (TAD) located at the N-terminus. Basic domains (EGF-Like and FNIII-Like) and the main binding sites to other ECM proteins and receptors are depicted. (B) Models for TnC biosynthesis. In model 1, hexabrachions are formed in a very rapid co-translational process where six monomers are simultaneously assembled. In model 2, the hexabrachion assembly take place in two steps. First, monomers form an intermediary trimer through α-helical coiled-coil interactions in the TAD. Subsequently, two trimers assemble in a hexamer that is stabilized by disulphide bonds. (C) Exosome-mediated TnC secretion. After biosynthesis in the ER, TnC is transported to multivesicular bodies (MVBs) in a Cav1 dependent manner (Cav1+/+). The absence of Cav1 (Cav1−/−) increases the levels of cholesterol at MVBs and alters exosome formation, preventing the sorting of TnC onto exosomes and leading to the accumulation of TnC in the ER. Upon secretion, exosomal TnC can be locally deposited, or modulate the behavior of surrounding cells. On the other hand, exosomes can eventually reach the blood stream and generate new TnC nucleation points at distant organs and tissues (108).
exosome subsets exposing TnC-binding receptors, a mechanism that may contribute to ECM remodeling and its coordination with cell modulation. Finally, the consideration of features derived from exosomal secretion might be highly relevant for biomedical applications aiming at tissue repair and regeneration: exosomes would potentially enable for accurate "dosage" and target specificity (220), and might hold the key for leveraging on the tissue remodeling and repair activities of TnC (221) through

### TABLE 2 | Literature contributing evidence of TnC as an EV secreted cargo. EVs origin: cell type/tissue from which EVs containing TnC were detected; WB: western blotting.

| TnC EVs origin            | Target cell                          | Detection approach | Result                                                                 | Ref.   |
|---------------------------|--------------------------------------|--------------------|----------------------------------------------------------------------|--------|
| Fibroblast                | Breast cancer (MDA-MB-468)           | WB, sucrose gradient and proteomics | Matrix deposition in 2D, 3D and in vivo increased migration and invasion | (108)  |
| Breast cancer (MDA-MB-231)| Breast cancer (MDA-MB-231, T47-D)    | WB, proteomics     | Inhibition of mTOR signalling and inhibition of T-cell proliferation, activation and cytokine secretion | (122)  |
| Brain tumor-initiating cells | T-lymphocyte                         | WB                 | Bone mineralization                                                  | (217)  |
| Glioblastoma patients     | N/A                                  | WB                 | Increased migration and invasion                                      | (214)  |
| Osteoblast-like cells (SaOS2) | Pancreatic cancer (PC-1, PC-1.0, AsPC-1, Capan-2) | WB                  | Increased proliferation through activation of the NF/κB              | (183)  |
| Pancreatic cancer (PC-1, PC-1.0, AsPC-1, Capan-2) | N/A                                  | Proteomics         | Increased exosomal TnC in metastatic cell lines                      | (184)  |
| Metastatic colorectal cancer (SW480, SW620) | N/A                                  | Proteomics         | Increased exosomal TnC correlates with stromal TnC matrix           | (183)  |
| Pancreatic ductal adenocarcinoma patients (pancreatic duct fluid) | N/A                                  | Proteomics         | Increased exosomal TnC in metastatic cell lines                      | (184)  |

### FIGURE 5 | Roles of exosomal TnC in cancer progression and immunomodulation. Scheme of the main stages (I-IV) in carcinoma tumor progression. In stage I a normal epithelium is shown, composed by epithelial cells located on a basal membrane. Underneath, the interstitial matrix deposited by stromal cells provides support. Insults promote transformation of epithelial cells onto tumoral cells, which lose polarity and adhesion (Stage II). In stage III, continuously activated fibroblasts increase the production and secretion of ECM, including collagens, FN and TnC. Tumor cells start invading neighbouring tissues and degrading the basal membrane. Finally, in stage IV, a highly remodelled ECM favors tumor cell migration through the interstitial space towards blood and lymphatic vessels, to metastasize. The previously described roles of exosomal TnC in tumor progression are depicted (A–E). (A) Paracrine/autocrine secretion of TnC-loaded exosomes induce tumor cell proliferation and invasion (122). (B) Exosomal TnC derived from brain tumour-initiating cells suppresses mTOR activity and T-cell activity (219)(Mirzaei et al). Activated fibroblasts can also secrete exosomes carrying TnC that can (C) modulate tumor cells and/or deposit new TnC matrix (108) (D). Finally, TnC-positive exosomes can be released into the bloodstream to deposit TnC at distant organs (E). An increase in TnC in plasma has been proposed as poor prognosis marker in many cancers and inflammatory diseases (24).
very controlled time frames, bypassing uncontrolled chronic inflammation states.

CONCLUDING REMARKS AND PERSPECTIVES

The characterization of mechanisms driving ECM deposit and of antifibrotic agents (72, 222, 223) aiming at intervening or preventing diseases such as chronic hepatitis (224), kidney diseases (225), systemic sclerosis, pulmonary fibrosis (226, 227) or cancer and tumor progression (228) has been intensive. Throughout the past decade, the study of EV-associated ECM components has expanded our understanding of ECM biology. EVs have been suggested as integral components of stromal environments (172, 173), and enable the impact of ECM-secreting cell populations on distant organismal locations. These insights have opened several key questions. We do not know whether EV-mediated transport of certain ECM components specifies their function at destiny. Mechanistically, we have a very limited understanding as to how ECM components are routed for sorting onto exosomes, instead of being targeted for degradation at the endosomal compartment; whether and how cells use different potential mechanisms for the secretion of a given ECM component; and how these processes are integrated with the complex reciprocal regulation established between ECM and stromal cells. Finally, the principles by which target cell specificity (168) correlates with this ECM secretion activity remain unexplored. The potential interplay of EV-carried TnC with other cargoes regarding their impact on target cells is also a key question. Given the potential of EV-trafficked TnC levels as serum diagnosis/prognosis biomarkers, and the ability of EVs to nucleate novel ECM niches at specific organs, the biology of exosomal TnC secretion holds the promise to explore potential novel theranostic applications.

AUTHOR CONTRIBUTIONS

LA-A, MS-Á, and MP conceived and wrote the article. LA-A created all infographics, and led bibliographical revision with support from MS-Á. MP coordinated the review. All authors contributed to the article and approved the submitted manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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