

Commentary & View

Regulatory role of CCN3 in melanoma cell interaction with the extracellular matrix

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It is increasingly clear that melanoma cells modify their environment not only through the release of growth factors (GFs) and cytokines that have autocrine or paracrine effects and strongly modulate the immune response, but also by secreting proteins that become structural or transient components of the extracellular matrix (ECM). Melanoma cell secreted proteins play a significant role in cell-ECM interactions, helping tumor cells to invade neighbouring stroma, disseminate and survive in other tissue contexts. CCN3/NOV (nephroblastoma overexpressed) is a matricellular protein that belongs to the CCN family of proteins containing six members in humans. Its structure consists of modules related to functional domains previously identified in major regulatory proteins: insulin-like growth factor-binding protein (IGFBP), von Willebrand factor type C repeats (VWC), thrombospondin type 1 repeats, and secreted regulatory factors containing cysteine knot motifs. Extensive studies have indicated that the biological properties of CCN3 are dependent upon the cellular context, and its role in melanoma seems to recapitulate cell context functions.

Cutaneous melanoma has an extraordinary and largely unpredictable ability to metastasize and to progress to an untreatable disseminated disease. Surgical resection in its early stages is usually curative, but once it has spread beyond the skin to regional lymph nodes, metastatic melanoma is often incurable by the available treatment modalities. Among the genes that were highly expressed in metastatic melanoma cells compared to cells from the primary tumor excised from the same patient, we identified CCN3. CCN3 is a member of the CCN family of genes that comprises six secreted matricellular proteins characterized by a conserved multimodular organization, regulating cell growth and differentiation by modifying signalling of molecules associated with ECM, which are emerging as biologically significant in several tumor types.¹ No data about CCN proteins in human melanoma progression had been produced: only CCN1 gene expression was shown hypoxia-inducible in human

melanoma cells and constitutively expressed in cell lines with a high metastatic potential in immunosuppressed mice.²

When CCN3 protein expression was assayed in a large panel of melanoma metastases, high levels of expression were shown in visceral metastases and in nodal metastases of patients undergoing relapse and visceral metastatic disease. These results indicated that melanoma cells expressing CCN3 are prone to metastasize via the hematogenous route to visceral organs. In vivo assays of metastatic capacity confirmed a higher metastatic growth of melanoma cells expressing CCN3 after gene transfer when injected in the bloodstream of immunodeficient mice.³ In other studies, CCN3 expression was detected in the majority of nevi and primary melanoma samples tested with cell staining becoming weak in the dermal area; CCN3 expression decreased in thick melanoma lesions and appeared evident only in few metastatic melanomas. In vitro three-dimensional skin reconstruct models showed that CCN3 is important for the correct spatial localization of melanocytes to the basement membrane, and that CCN3 expression induced by gene transfer in an invasive melanoma cell line inhibited invasion and proliferation in the dermis.^{4,5}

Taken together, the functions of CCN3 in melanoma cells appear to be complex and dependent on different tissue contexts and possibly related to the different stages of disease. In the early steps of tumor growth, it appears that local invasion requires CCN3 to be downregulated, while for the growth of visceral metastasis CCN3 expression is upregulated. In melanocytes, endogenous CCN3 was shown to upregulate adhesion to collagen IV, a major constituent of basal lamina.⁵ Two melanoma cell lines expressing high levels of endogenous CCN3 were shown to be more adhesive to collagen IV, as well as to collagen I, fibronectin (FN) and laminin (LM),³ but overexpression of CCN3 by gene transfer in two melanoma cell lines did not increase adhesion to collagen IV,^{3,4} although adhesion to FN and LM was significantly increased.³ Some structural and functional characteristics of the CCN3 protein appear critical for the regulation of melanoma cell behavior and may explain these partly discordant findings. First, the structural issue: the different regulatory effects of CCN3 protein on melanoma cells at different disease stages may come from the expression of different CCN3 protein variants. In fact, CCN3 is found in melanoma cells as a cytoplasmic and secreted 46-kDa isoform as well as a 32-kDa amino-truncated form, which is found both in the culture medium and in the nucleus. This 'short-form' originates from post-translational processing of the 'full-length' CCN3 protein and lacks both IGFBP and VWC

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Table 1 Cellular functions regulated by CCN3 potentially involved in melanoma metastatic progression

Function	Mechanism	Cells/assays
Adhesion to ECM	Support ITG-dependent and independent cell adhesion	HUVEC, 1064SK, C2C12, VSMC
Migration	Induction of ITG-dependent cell migration	BACEC, 1064SK, G59, TC71/NOV
Cell survival	Increase proliferation and decrease of apoptosis	HUVEC, C2C12
Neovascularization	Induction of in vivo angiogenesis	rat corneal pocket assay
Regulation of intracellular calcium levels	Increase in intracellular calcium levels affecting cell growth, motility and spreading	G59, SK-N-SH
DNA synthesis	Enhancement of DNA synthesis induced by GFs	1064SK, C2C12
Regulation of genes involved in matrix remodeling	Upregulation of MMP-1, MMP-3, PAI and PDGFR α	1064SK, G59/NOV
Activation of intracellular signalling	Increase of FAK phosphorylation	HUVEC, C2C12
Multi drug resistance	CCN3 expression associates to MRP1 and MRP4 expression and to in vitro drug resistance	U-2OS/V, OST-SA

HUVEC, Human umbilical vein endothelial cells; 1064SK, Normal human skin fibroblasts; C2C12, Mouse myoblast cells; VSMC, Rat vascular smooth muscle cells; BACEC, Bovine adrenal capillary endothelial cell; G59, glioblastoma cells, G59/NOV stably transfected with CCN3 encoding vector; TC71/NOV, Ewing sarcoma cells stably transfected with CCN3 encoding vector; SK-N-SH, neuroblastoma cells; U-2OS/V, osteosarcoma cell variants resistant to methotrexate, doxorubicin or cisplatin; OST-SA, osteosarcoma specimens.

domains.⁶ The full-length secreted CCN3 acts for melanoma cells as an ECM protein itself and it mediates adhesion to other ECM proteins via integrin (ITG) receptors.³ Clearly CCN3 modifies the adhesive properties of melanoma cells, in line with results previously obtained in Ewing's sarcoma by Benini and coworkers.⁷ The functional effects of the truncated short form are less clearly defined and involve cell growth deregulation, possibly by the regulation of gene expression.^{8,9} CCN3 protein expression analysis in a large panel of short-term melanoma cell lines revealed a complexity of patterns and heterogeneity that include the expression of both protein forms or only one, with or without secretion of the full length protein. This indicates that in some melanoma cells only the short form is produced and suggests that the different CCN3 protein forms expressed may modulate function. Analysis of gene sequence at the transcript level has shown that the different patterns of expression of the protein are not due to sequence alterations or polymorphisms.³ Heterogeneous expression of CCN3 protein variants have also been reported in chronic leukemia¹⁰ and in Wilm tumor.¹¹

The expression of different CCN3 isoform patterns observed in melanoma cells may result from interaction with different protein partners. CCN3 multiple biological functions are exerted through various signalling pathways involving cell surface receptors such as ITGs, connexins, fibulins, Notch and calcium channels.¹ The multi-modular structure provides the basis for a wide range of interactions with different partners. CCN3, by acting as a scaffolding or adaptor protein, permits the interconnection between independent signalling pathways by associating with other proteins, and it may exert regulatory effects on the melanoma cells' microenvironment depending on which protein ligands are expressed. Moreover, melanoma cells produce an array of cytokines and growth factors (GFs), which have been associated with ability to metastasize.¹² The autocrine and paracrine GFs produced by melanoma cells, such as TGF β , may regulate CCN3 expression as shown in other cell types. In addition, CCN3 protein partners identified by two-hybrid screening have been shown to include, for example, interleukin-33,¹³ which may contribute to alter the tissue milieu in concert with other CCN3 functions, permitting metastatic deposits to grow. Table 1 lists the

available evidence linking CCN3 activities in different cell types with melanoma metastatic dissemination.

In particular, ITGs play a major role in cell adhesion and migration by integrating components of the ECM to the cellular cytoskeleton and by coupling to intracellular signaling pathways that control fundamental cellular processes, such as cell proliferation and survival.¹⁴ A wide variety of ITGs are expressed in melanoma cells, and the existing ITG pattern thus represents the second main trait of the cellular context shaping CCN3 functions. Our data indicate that in melanoma cells, CCN3 affects the expression and functions of ITG receptors by upregulating their expression and activation status. ITGs have been found to be deregulated in melanoma cells at different stages and upregulation of ITG expression is associated with the acquisition of a more metastatic phenotype. Modulation of ITG affinity, or ITG activation, has been shown to occur by activation of GFs receptors. Moreover, the binding of extracellular ligands determines ITG engagement within the ECM as well as the activation of signaling involving pro-survival pathways. It is conceivable that ITG-mediated signal pathways activated by CCN3 have a main role in promoting melanoma progression, by modulating melanoma cells behavior to enable the acquisition of a more aggressive phenotype, affecting cell growth as well as adhesive and migratory properties.

The level of expression of CCN3 protein was shown to be altered in different tumor types and high levels of CCN3 expression have been associated with either good prognosis or increased proliferative index and metastasis. In Wilm tumors, chondrosarcomas, neuroblastomas and chronic myeloid leukaemias increased CCN3 expression is associated with differentiation and/or good prognosis, while in renal and prostate carcinomas, Ewing tumors and osteosarcomas high CCN3 expression is associated with increased high proliferation and poor prognosis.¹⁵ Data describing such opposite effects also of the other CCN proteins in several tumor types have been reported.¹ These studies indicated that CCN3, as well as the other CCN proteins, exerts different functions that are strictly dependent upon the cellular context, tissues and tumor.¹⁵ Other melanoma secreted glycoproteins interacting with ECM and involved in tissue morphogenesis, with various effects reported in different tumor types, have

been recently associated with melanoma progression. Preliminary data suggest they can be regulated in concert with CCN3 in melanoma cells (unpublished observation). They share several common features with CCN3 and their expression appears to be similarly and tightly regulated. They present multiple isoforms with different functions generally resulting from proteolytic cleavage, which are detectable in serum, interact with ITG receptors and with other ECM proteins, are angiogenic stimulators, regulate cell adhesion, are regulated and function dependent upon cellular context, have Ca⁺⁺-binding properties and are regulated by TGFβ. The first group includes tenascin-C (TN-C), osteopontin (OPN) and osteonectin (SPARC) matricellular proteins.¹⁶ TN-C overexpression has been associated with tumor cell proliferation and migration/invasion¹⁷ and it regulates metalloproteinases (MMPs). Recently, TN-C has been pointed out as a possible prognostic marker predicting the propensity of primary melanomas to metastasize, as its staining correlated positively with metastasis to sentinel lymph nodes and its increased expression has been observed in invasive and metastatic melanomas.¹⁸ OPN is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family, promoting cell adhesion, motility and survival.¹⁹ OPN binds to CD44 isoforms and regulates MMPs as well EGF, HGF and other GFs. Several microarray studies have shown that OPN expression is higher in advanced stage melanomas with increased invasiveness compared to early stage tumors. Furthermore, Rangel and colleagues showed a significant correlation between OPN expression and recurrence-free survival.²⁰ SPARC has been shown both to promote tumor metastasis and to reduce tumor growth depending on the ITG types engaged.²¹ In melanoma cells, SPARC is induced by ectopic expression of ITG β3.¹⁶ SPARC binds to different GFs such as PDGF, VEGF, FGF and to ECM proteins such as collagens and vitronectin in a Ca-dependent fashion. Recently, it has been demonstrated that SPARC secreted by melanoma cells influences the in vivo cell growth capacity by causing stroma reorganization and increased angiogenesis.²² Other ECM-related proteins that can be added to this list are fibulins (FBLNs), a family of glycoproteins associated with basement membranes and elastic ECM fibers. FBLN1, the prototype member of this family, binds to CCN3 and to different ECM proteins, such as collagens and fibronectin, is expressed in several tumor types and implicated in cellular processes such as invasion, motility and tumor growth.²³ The *FBLN2* gene was identified as one of the 64 metastasis-associated genes in different tumor types.²⁴ In melanoma metastatic cells, we detected a higher expression of FBLN1 and *FBLN2* compared to autologous primary melanoma cells (unpublished observations).

Collectively, a profound ECM reorganization influencing tumor cell growth and dissemination and tumor-associated angiogenesis is induced by the secretion of these proteins by melanoma cells. Interestingly, such a remodelling of tumor microenvironment causes an alteration of cell-cell and cell-matrix interactions leading to the activation of survival signals, which help to confer to tumor cells resistance to drug-induced apoptosis.²⁵ In light of these considerations, it can be speculated that CCN3 regulates melanoma progression and dissemination not only through its pro-metastatic and pro-angiogenic activity, but also by cooperating with other matricellular proteins to organize the melanoma microenvironment in order to promote melanoma cell survival in the presence of cytotoxic drugs. In osteosarcomas, high expression of CCN3 correlated with worse

prognosis and shorter event-free survival; CCN3 was shown to be upregulated in doxorubicin-resistant osteosarcoma cell line variants, and high levels of CCN3 in parallel increased expression of MRP-1 and -4 of the ABC family of transporters associated to drug resistance.²⁶ In breast carcinoma, FBLN1 is induced by doxorubicin and its inhibition increased the pro-apoptotic effects of doxorubicin.²⁷ Preliminary data indicate that CCN3 expressed recombinantly confers melanoma cells resistance to the effect of different cytotoxic drugs in vitro (preliminary observations). Future studies will establish whether CCN3 and other secreted glycoproteins interacting with the ECM are regulated indirectly or directly by p53²⁸ and whether drug-resistant melanoma cells share stem cell properties, as recently described,²⁹ and CCN3 expression, as described for hematopoietic progenitor cells.³⁰

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