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In Ewing's sarcoma CCN3(NOV) inhibits proliferation while promoting migration and invasion of the same cell type

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Altered expression of CCN3 has been observed in a variety of musculoskeletal tumours, including Ewing's sarcoma (ES). Despite its widespread distribution, very little is known about its biological functions and molecular mechanisms of action. We transfected *CCN3* gene into a CCN3-negative ES cell line and analysed the *in vitro* and *in vivo* behaviours of stably transfected clones. Forced expression of CCN3 significantly reduced cell proliferation *in vitro*, growth in anchorage-independent conditions, and tumorigenicity in nude mice. Despite the antiproliferative effect, CCN3-transfected ES cells displayed increased migration and invasion of Matrigel. The decreased expression of $\alpha 2\beta 1$ integrin receptor and the increased amount of cell surface-associated matrix metalloproteinase (MMP)-9 following the expression of CCN3 may be the basis for the increased migratory abilities of transfected cells. Cells lacking $\alpha_2\beta_1$ are less facilitated to have stable anchorage since the predominant collagen extracted from ES tissue is indeed type I collagen, and proMMP-9 was recently found to provide a cellular switch between stationary and migratory ES cell phase. Our findings are in line with those recently obtained in glioblastoma. However, the underlying molecular mechanisms appear to be different, further highlighting the importance of the cellular context in the regulation of function of CCN proteins.
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Introduction

CCN3 gene (previously designated nephroblastoma overexpressed gene, *NOV*) maps on chromosome 8q24.1 and was originally identified as aberrantly expressed in avian nephroblastoma induced by myeloblastosis-associated virus (Joliot *et al*., 1992).

CCN3 belongs to the CCN family of genes, which comprises five other members: cystein-rich protein 61 (Cyr61), connective tissue growth factor (CTGF), Wnt-1-induced secreted protein (WISP)-1, WISP-2 and WISP-3 (Bork, 1993; Brigstock, 1999; Perbal, 2001). The CCN acronym was introduced a decade ago by P Bork, who recognized that the proteins encoded by Cyr61, CTGF and NOV were showing a common multimodular organization (Bork, 1993). The CCN genes encode secreted proteins of 35–48 kDa associated with the extracellular matrix (ECM) and cell membrane. These proteins contain 38 conserved cysteine residues and are organized into four distinct structural modules resembling insulin-like growth factor binding proteins, Von Willebrand type C factor, thrombospondin 1 and growth factors that contain a cysteine knot motif (including platelet-derived (PDGF), nerve growth factor and transforming growth factor-β). Their general properties have been extensively reviewed (Bork, 1993; Brigstock, 1999, 2003; Perbal, 2001, 2004; Planque and Perbal, 2003).

CCN proteins are involved in the regulation of various cellular functions, which include: proliferation, differentiation, survival, adhesion and migration. They are expressed in all derivatives of the three embryonic sheets and are implicated in the development of kidney, nervous system, muscle, bone marrow, cartilage and bone. During adulthood, they are recalled into function to heal wounds, especially bone fractures, to sustain angiogenesis and in some pathologies such as fibrosis, vascular afflictions and tumorigenesis. The mechanisms responsible for such a variety of biological functions are poorly understood. No specific cell surface receptors for these proteins have been identified to date, although recent studies have shown that CCN1, CCN2 and CCN3 can bind to cell surface integrins (Lau and Lam, 1999; Lin *et al*., 2003) and thereby may function through integrin-mediated signalling pathways. CCN proteins show strong affinity for heparin and are mainly localized on the cell surface or, associated with heparan sulphate proteoglycans, into the ECM.

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Compared with CCN1 and CCN2, CCN3 has been less studied and its biological role is even more elusive. CCN3 molecule is widely expressed, especially in the nervous and musculoskeletal systems as well as in the blood vessels. The functions of CCN3 protein among these different tissues might, however, be very different. Although CCN3 was originally described as antiproliferative (Joliot *et al*., 1992; Scholz *et al*., 1996) and its expression was associated with differentiation and growth arrest in Wilm's tumour, chondrosarcomas and rhabdomyosarcomas (Chevalier *et al*., 1998; Manara *et al*., 2002; Yu *et al*., 2003), more recent data correlate CCN3 with increased proliferative index of 3T3 fibroblast and tissue samples of prostate and renal carcinomas (Liu *et al*., 1999; Glukhova *et al*., 2001; Maillard *et al*., 2001). In osteosarcoma, it is inversely associated with expression of liver/bone/kidney alkaline phosphatase isoform (Manara *et al*., 2002), an early marker of osteoblastic differentiation (Stein *et al*., 1990). In Ewing's sarcoma (ES), expression of CCN3 was only sporadically observed (around 30% of primary tumours were found to be positive), but it was associated with a significantly higher risk of developing lung and bone metastasis (Manara *et al*., 2002). To study the role of CCN3 protein in the biology and malignancy of ES cells, we, therefore, transfected its gene in an ES cell line that lacked its expression. The effects of CCN3 on proliferation, cell adhesion, invasion and migration are described.

Results

Selection and characterization of CCN3-transfected clones

To analyse the role of *CCN3* gene in ES malignancy, we transfected the TC-71 ES cell line with the pCMV47 vector that encodes an epitope-tagged human CCN3 protein. Stable transfectants were selected in blasticidin (5 μg/ml) and characterized for CCN3 expression at mRNA and protein level by real-time PCR and Western blotting. Among the clones obtained, four were selected in relation to their differential level of CCN3 expression and studied in comparison with the parental TC-71 cell line and control cells transfected with the empty parental vector (TC/EmptyV5). To verify that the presence of a V5-His-tagged domain did not modify the functionality and biology of CCN3 in this system, TC-71 cells were also transfected with the pCMV82 vector expressing nontagged CCN3. The resulting TC/CCN3-82 control cells were selected in neomycin and pooled.

Figure 1 shows the relative expression of CCN3 for the selected clones either at mRNA (a) or protein level (b). All the TC/CCN3 transfectants showed high expression of the protein in conditioned medium obtained 48 h from cell seeding, whereas various amounts of CCN3 were detected in the cell lysates.

In vitro growth features of TC/CCN3-transfected clones

The effect of CCN3 expression on TC-71 ES cell growth was analysed either in monolayer or anchorage-independent condition, a hallmark of malignant transformation (Aaronson and Todaro, 1968). All TC/CCN3 clones showed a reduced cell growth in standard condition (Figure 2a); their doubling times were significantly lower than those of parental and TC/EmptyV5 control cells ($P<0.05$, Student's *t*-test), with the only exception of TC/CCN3-TAG26, which has the lowest expression of CCN3 (Table 1). The lower proliferative ability of CCN3-expressing cells seems to be due to increased apoptotic rate and delayed cell cycle, consisting of lower percentage of cells in the S phase (Table 1). Such antiproliferative effects were even more evident when anchorage-independent growth, that is colony formation in soft-agar, was tested. Cells expressing CCN3 showed a significant inhibition, ranging from 40 to 78%, of colony formation in soft agar compared to parental and TC/EmptyV5 cells (Figure 2b). Overall, the behaviour of CCN3-tagged clones is similar to that of TC/CCN3-82, indicating that the presence of a tag sequence does not modify protein functions.

To further confirm that the antiproliferative effect is directly due to CCN3 expression, two ponasterone-inducible clones (TC/IND47 and TC/IND82) were obtained after super-transfecting pVgRXR-transfected TC-71 cells with the pIND/47 and pIND/82 vectors. After exposure of the cells to 5 μM of ponasterone for 24 h, CCN3/NOV was produced at high levels in TC/IND47 and TC/IND82 but not in parental TC-71 cell supernatants (Figure 3a). In these clones, induced expression of CCN3 correlates with reduced cell growth both in monolayer (Figure 3b) and in soft-agar conditions (Figure 3c) in comparison to unstimulated cells. In addition, a further confirmation of the antiproliferative effect of CCN3 derives from exogenous CCN3. CCN3 protein was purified on antibody column and exposure of TC-71 cells to 1.5 μg/ml CCN3 induces a 35% growth inhibition after 72 h that is in line with the inhibition observed in monolayer conditions with transfectants.

Effects of CCN3 expression on the adhesive, migratory and invasive capabilities of TC-71 ES cells

CCN3 interacts with fibulin-1C, an ECM protein (Perbal *et al*., 1999), and promotes adhesion and migration of vascular smooth muscle cells and of glioblastoma cells (Ellis *et al*., 2000; Laurent *et al*., 2003). We observed induction of migratory and invasive ability of ES cells following transfection of CCN3. Figure 4a shows the significantly higher number of migrated TC/CCN3 cells with respect to parental TC-71 cell line and TC/EmptyV5, and Figure 4b shows that exposure of pIND cells to 5 μM of ponasterone promotes migration of TC/IND82 and TC/IND47 but not of parental TC-71 cells, consistently with the induced expression of CCN3 in these cells. By using

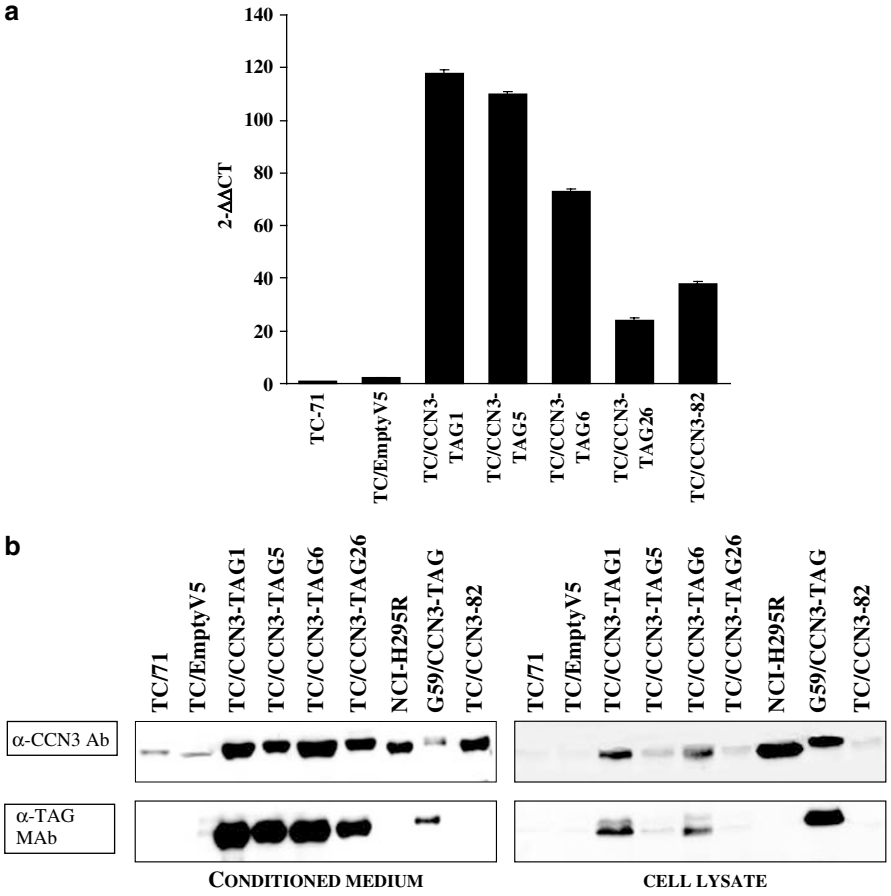

Figure 1 Expression of CCN3 in TC-71-transfected clones. (**a**) Relative expression of CCN3 mRNA in TC-71-transfected clones with respect to the parental cell line used as calibrator ($2^{-\Delta\Delta CT} = 1$), by real-time PCR. Triplicate for each sample were performed and results are shown as mean ± s.e. (**b**) Western blot analysis of CCN3 expression levels in TC-71-derived clones. Proteins from cell lysates and from the media conditioned by the same cells were electrophoresed on a 12% SDS polyacrylamide gel. CCN3 was identified by immunoblotting with the K19M antibody or with the anti-V5 antibody, which shows the presence of the V5 tag on CCN3 molecule secreted in the cell culture medium and contained in cell lysates. The adrenocortical carcinoma cell line NCI-H295R and the glioblastoma G59/CCN3-TAG transfected cells were used as positive controls

the Matrigel invasion chamber assay, we demonstrated that all three TC/CCN3 clones migrated twice more than TC-71 and TC/EmptyV5 cells (Figure 4c) and that ponasterone exposure enhanced invasion of inducible clones (Figure 4d).

The influence of CCN3 expression on the adhesive properties of TC-71 ES cells was analysed with respect to different ECM substrates, such as laminin, fibronectin, collagen I, collagen IV and vitronectin. Results, shown in Figure 5, indicate that TC/CCN3 clones did not differ from parental cells in the adhesion to the tested substrates, with the notable exception of collagen-I and -IV. Reduced adhesion of TC/CCN3 clones to collagen-I and -IV might be due to a decreased expression of $\alpha_2\beta_1$ (Figure 6a). Indeed, among the α-chains coupling β_1, only α_2 was found inhibited in CCN3-expressing cells. The CCN3-induced reduction in $\alpha_2\beta_1$ expression is due to a mechanism acting at transcriptional level, since quantitative evaluation of $\alpha_2\beta_1$ mRNAs indicated a significant decrease in their expression in TC/CCN clones (Figure 6b).

CCN3 was recently found to directly bind $\alpha_5\beta_1$ and $\alpha_v\beta_3$ (Lin *et al*., 2003). In our system, only $\alpha_5\beta_1$ may play a role in mediating CCN3 functions because no expression of $\alpha_v\beta_3$ was observed in our cells (Figure 6a). Similarly, although CCN3 was recently reported to increase the invasive ability of glioblastoma cells via platelet-derived growth factor receptor (PDGFR)-α-dependent mechanism (Laurent *et al*., 2003), we can exclude the involvement of the PDGFR-α and -β in our model since TC-71 cells and CCN3 transfectants did not express PDGFR-α and no variations were observed in PDGFR-β expression (Figure 7). Consistently, TC/CCN3 cells and controls showed similar migratory ability in the presence of PDGF-BB (data not shown). No modulations in the expression of other growth factor receptors (epidermal growth factor receptor; nerve growth factor receptor; and trk-A) were observed following CCN3/NOV expression (data not shown), apart from a moderate reduction in the expression of the insulin-like growth factor receptor (IGF-IR) (Figure 7).

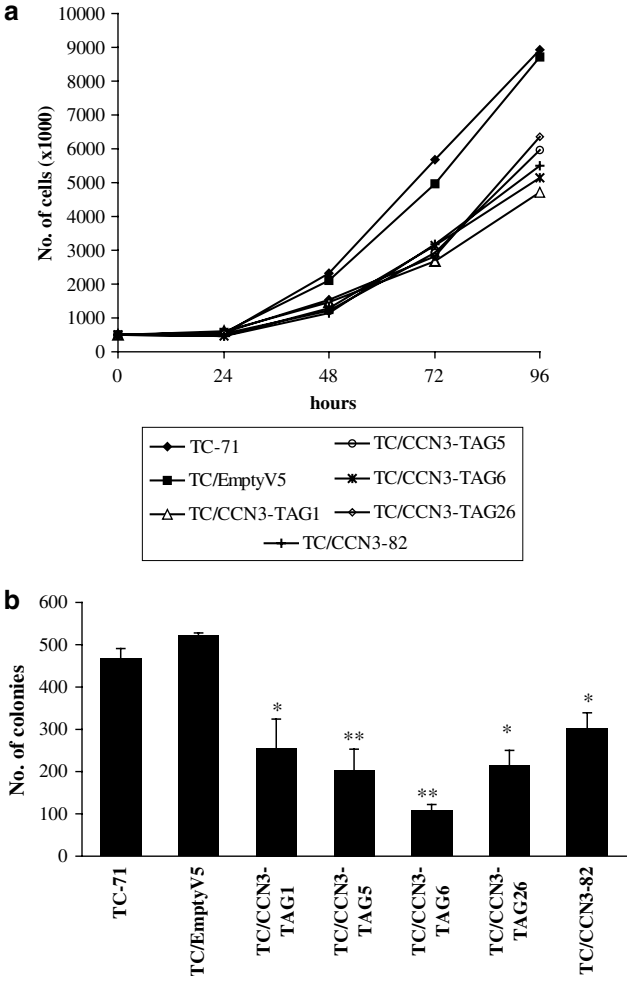

Figure 2 *In vitro* growth of TC-71-transfected clones. (**a**) Growth curves of TC-71 cells and derived clones in monolayer conditions. Results represent one experiment representative of three. (**b**) Growth of CCN3-expressing clones in anchorage-independent conditions. Cells were seeded at a concentration of 1000 cells and the number of colonies in duplicate plates was determined after 6 days of growth in IMDM 10% FBS. Data are expressed as means of 4–6 plates $\pm$s.e. $^{*}P<0.05$; $^{**}P<0.001$, Student's *t*-test, with respect to TC-71 parental cells

Effects of CCN3 on the expression and activity of matrix metalloproteines (MMPs)

CCN3 has recently been shown to modulate MMP-3 expression in glioblastoma cells (Laurent *et al*., 2003). To evaluate whether the invasive behaviour of TC/CCN3 cells might be due to metalloprotease (MMP) activities, we first identified which MMPs were expressed in TC-71 cell variants. In particular, mRNA expression of collagenase-1 (MMP-1) and -2 (MMP-8), gelatinase A (MMP-2) and B (MMP-9), and stromelysin-1 (MMP-3) was analysed by RT–PCR. Both TC-71 cells and their CCN3 derivate clones did not express MMP-1, -3 and -8 (data not shown) and showed similar expression of MMP-2 and -9 (Figure 8a). Activity of MMP-2 and -9 was also determined in TC/CCN3 cell culture supernatants. No remarkable variations were observed with respect to MMP-2, whereas a significant reduction was found for MMP-9 activity (Figure 8b). The decreased MMP-9 activity following CCN3 expression was confirmed in ponasterone-inducible clones (data not shown). At protein level we observed a similar expression of MMP-9 in cell lysates, whereas the protein in cell supernatants appeared to be reduced in TC/CCN3 transfectants (Figure 8c). It is conceivable that the lower expression and enzymatic activity of secreted MMP-9 observed in TC/CCN3 clones reflect higher amounts of proMMP-9 localized to their cell membrane. Consistently, we found an increase in the percentage of cells that express proMMP-9 on their surface in CCN3-transfected clones (Figure 8d).

Table 1 *In vitro* growth characteristics of TC/CCN3 transfectants

Cells	*Doubling time (h)* $\pm$*s.e.*	*BrdUrd LI*[a]	*Apoptosis*[b]
TC-71	14.5$\pm$1.1	59.0	1.7$\pm$0.6
TC/EmptyV5	14.7$\pm$0.3	60.6	2.1$\pm$0.8
TC/CCN3-TAG1	23.4$\pm$1.7[c]	43.6	5.2$\pm$1.7[c]
TC/CCN3-TAG5	19.9$\pm$2.1[c]	47.4	5.6$\pm$1.2[c]
TC/CCN3-TAG6	19.5$\pm$1.8[c]	49.4	6.2$\pm$0.7[c]
TC/CCN3-TAG26	18.2$\pm$0.2	53.8	4.5$\pm$1.2
TC/CCN3-82	18.8$\pm$1.3[c]	50.2	5.2$\pm$1.1[c]

[a]Bromodeoxyuridine labelling index. Data are from one representative experiment. [b]Percentage of apoptotic nuclei. Average$\pm$s.e. of three independent experiments. [c]$P<0.05$, Student's *t*-test

In vivo behaviour of TC/CCN3 clones

In vitro results showed a double, controversial effect of CCN3 in ES cells: its forced expression reduced cell growth rate, while increasing their invasive and migratory capabilities. Whether these contrasting effects might affect tumour growth *in vivo* was tested in a xenograft model. Mice injected with cells expressing CCN3 did not develop tumours (Figure 9), in agreement with the lower proliferative rate of TC/CCN3 cells *in vitro*.

Discussion

Expression of CCN3 in ES primary tumours was found to be associated with a higher risk to develop lung and/or bone metastases (Manara *et al*., 2002). Therefore, CCN3 may be one of the few molecular markers with prognostic relevance in this neoplasm. Indeed, although several clinical factors have been reported to have a prognostic value (Bacci *et al*., 2000), few genetic and molecular markers have clearly emerged so far (de Alava *et al*., 1998, 2000; Wei *et al*., 2000; Lopez-Guerrero *et al*., 2001; Ozaki *et al*., 2001; Perri *et al*., 2001; Zielenska *et al*., 2001; Hattinger *et al*., 2002; Manara *et al*., 2002; Ohali *et al*., 2003) and for none of them there is a general consensus since findings are often sporadic and in conflict. Moreover, the biological basis for the supposed prognostic impact of all these markers has not been clarified.

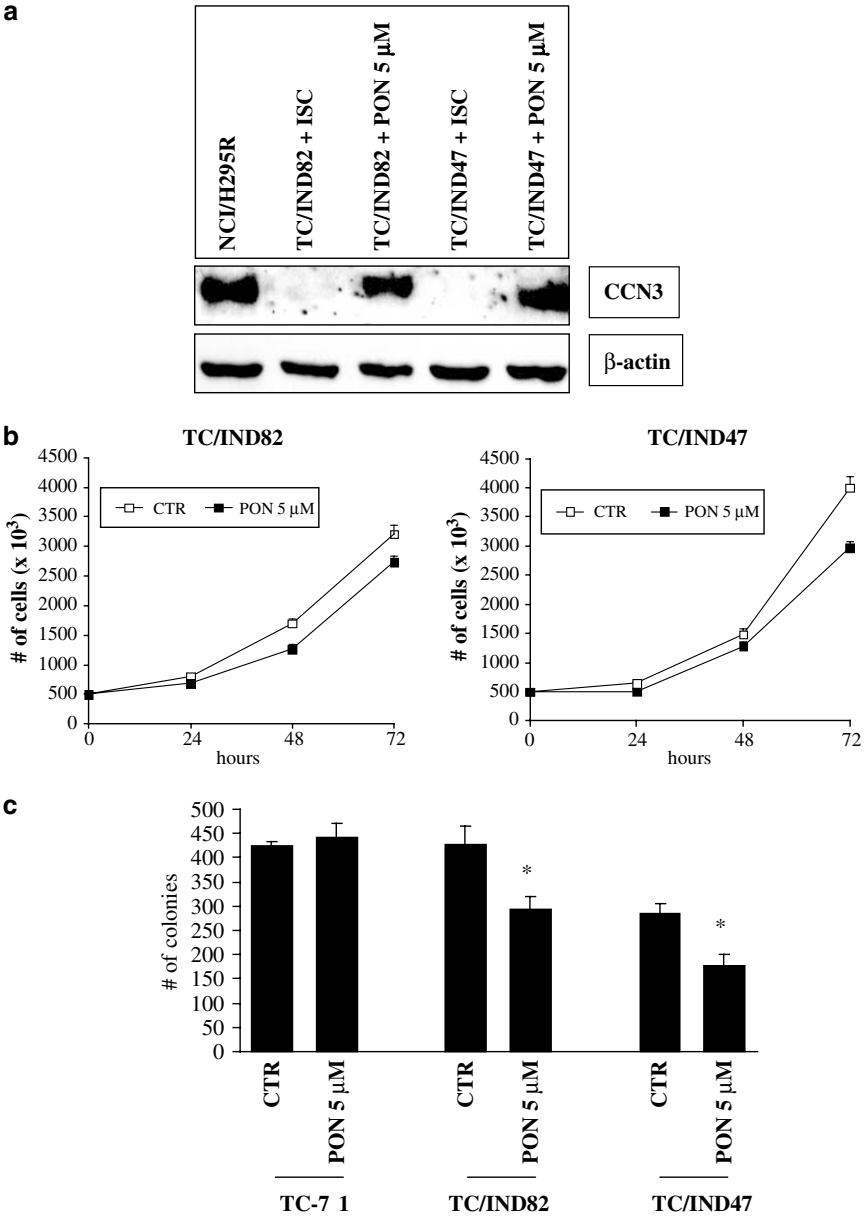

Figure 3 CCN3 expression and growth features in TC-71-inducible clones. (**a**) Western blot analysis of CCN3 expression levels in TC-71 inducible clones. Expression of CCN3 gene is under the control of a ponasterone-inducible promoter. Cells were exposed to 5 μM of ponasterone for 24 h before protein extraction. In the lower panel, the same blot was stripped and re-probed with the MAb for actin protein. (**b**) Growth curves of TC/IND82 and TC/IND47. Cells were exposed to 5 μM of ponasterone for 24 h before being seeded. (**c**) Growth of TC/IND82 and TC/IND47 in anchorage-independent conditions. Cells were exposed to 5 μM of ponasterone for 24 h before being seeded in soft agar. The number of colonies in duplicate plates was determined after 6 days of growth in IMDM 10% FBS. Data are expressed as means of 4–6 plates $\pm$ s.e. $^*P<0.05$, Student's *t*-test, with respect to TC-71 parental cells

In this study, we transfected the TC-71 ES cell line and obtained stable clones expressing *CCN3* gene under control of constitutive and inducible promoters to study its role in the biology of ES cells. CCN3 is still an obscure molecule and little is known about its biochemical activities and biological functions (Perbal, 2001). We show that ES cell production of CCN3 significantly reduces cell proliferation *in vitro*, and promotes ES cell migration and invasion, confirming that CCN proteins may have a variety of cellular functions.

Early studies on CCN3 indicated the antiproliferative effect of this protein: (i) expression of the full-length CCN3 protein in chicken embryo fibroblasts results in growth inhibition (Joliot *et al*., 1992), and (ii) expression of ccn3 is repressed by growth factors and is induced by serum deprivation or quiescence of fibroblasts (Scholz *et al*., 1996). In several types of human cancer, CCN3 expression has been associated with differentiation (Chevalier *et al*., 1998; Manara *et al*., 2002; Yu *et al*., 2003) and a marked antiproliferative effect of CCN3 has

been recently reported for glioblastoma (Gupta *et al*., 2001). Here, we demonstrate that CCN3 expression slows down the proliferative rate and increases the percentage of apoptosis of ES cells in basal conditions. The inhibitory effects on ES growth were more obvious when cells were prevented to adhere. The number of colonies in soft agar were significantly lower in clones expressing CCN3 constitutively and after induction by ponasterone exposure. Consistently with these antiproliferative *in vitro* effects, we found that expression of CCN3 abrogate tumorigenicity of ES cells in nude mice. Interestingly, ectopic expression of CCN3 resulted in abortive tumours that did not develop or regressed. Collectively, these results strongly suggest that CCN3 is involved in the negative control of ES cell growth. Our previous observations in primary ES tissue samples that showed a correlation between high levels of CCN3 expression and increased risk to develop metastases do not necessarily contrast with this report since besides cell growth inhibition, gene transfection induces cell migration and invasion in ES cells. Moreover, the prognostic value of CCN3 was determined in a series of patients who underwent chemotherapy. It is conceivable that primary ES cells that are not expressing CCN3 may be more easily destroyed by chemotherapeutic agents, thanks to their higher proliferative rate. On the contrary, CCN3-expressing cells by coupling a lower proliferative rate to a higher invasive ability may exhibit reduced chemosensitivity and increased chances to give metastases.

Our findings are perfectly in line with those previously obtained in glioblastoma. Expression of CCN3 by stable transfection significantly reduced proliferative activity of glioblastoma cells both *in vitro* and *in vivo* (Gupta *et al*., 2001), while increased cell migration and invasion through matrigel (Laurent *et al*., 2003). However, despite showing similar biological effects, molecular mechanisms underlying CCN3 activity in glioblastoma and ES cells appears to be different, further confirming the importance of the cellular context in regulating the activity of CCN proteins. In glioblastoma, microarray analyses and functional assays point to PDGFR-α and MMP-3 as key regulators of the CCN3-mediated cascade of events that activate glial cell migration (Laurent *et al*., 2003). We excluded the involvement of both proteins because ES cells do not express PDGFR-α or MMP-3. Nor did we observe consistent variations in the expression of PDGFR-β and, accordingly, exposure of cells to PDGF-BB did not differentially modulate the migratory ability of parental and CCN3-transfected cell variants. In ES, increased migration and invasion is

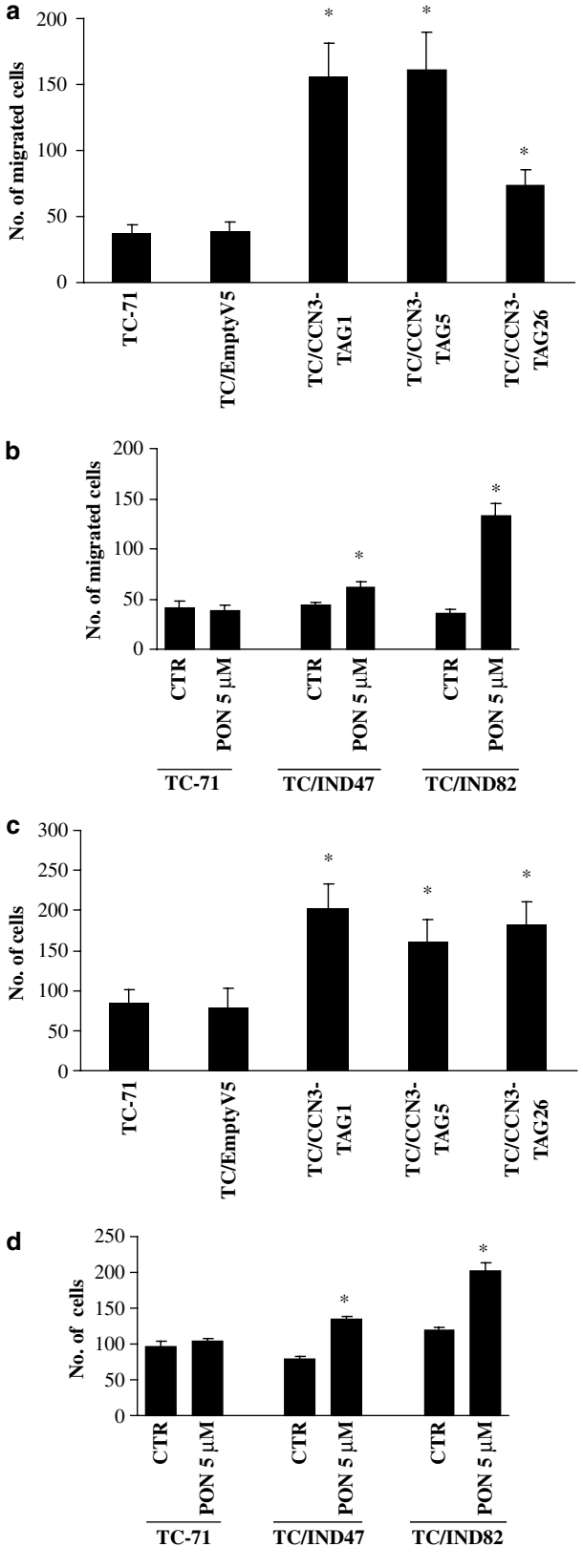

Figure 4 Migration and invasion ability of TC-71 cells and TC/CCN3 transfectants. (**a**, **b**) For migratory assays, cells were seeded in the upper compartment of a trans-well chamber. Inducible clones were pretreated with 5 μM of ponasterone for 24 h before being seeded in the trans-well chamber. In the lower compartment, IMDM 10% FBS was used as the source of chemoattractant. Each column represents the mean $\pm$ s.e. of three independent experiments. *$P = 0.05$, Student's *t*-test. (**c**, **d**). Invasion ability of TC-71 cells and derived clones through matrigel. In each experiment, each cell line was seeded in three wells and after 18 h cells in the lower compartment were stained with 1% toluidine blue and counted from 10 visual fields. Histograms represent the mean $\pm$ s.e. of three independent experiments. *$P = 0.05$, Student's *t*-test

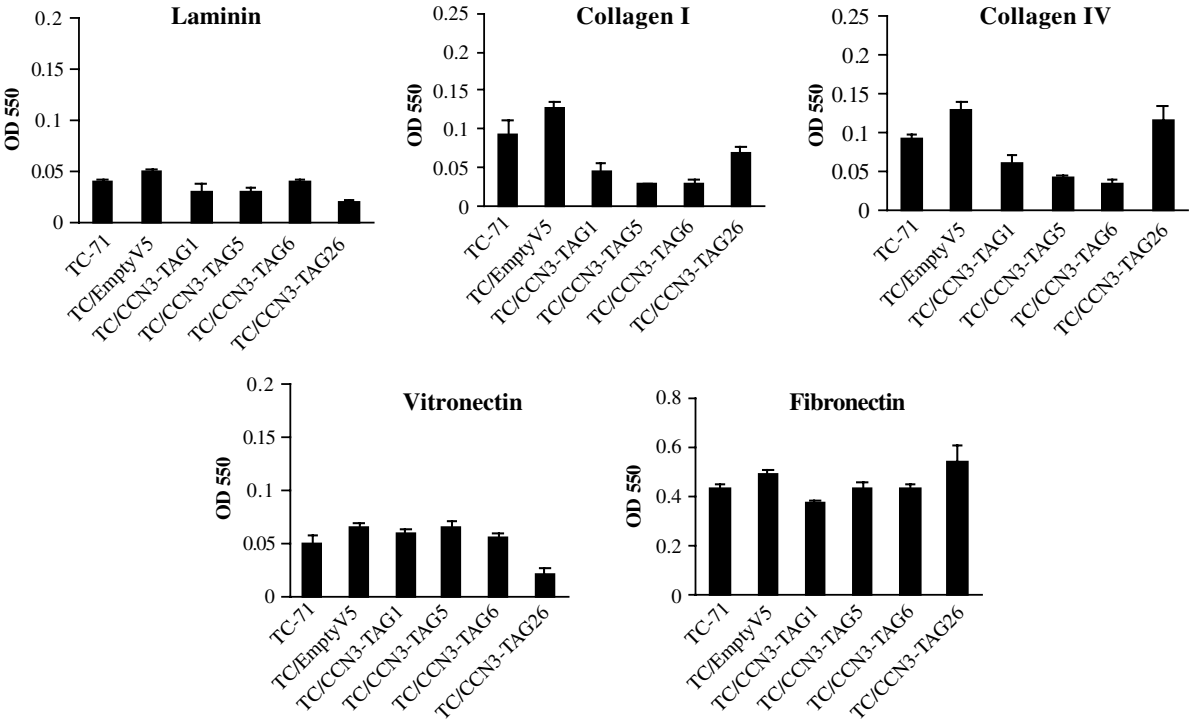

Figure 5 Effects of CCN3 expression on the adhesive properties of TC-71 ES cells. Cells were plated on wells coated with different ECM components accordingly to the manufacturer's instructions. After 1 h, the number of adherent cells was determined after incubation with crystal violet

associated with reduced cell adhesion to collagen I and IV, which might be due to reduced expression, both at mRNA and protein level, of its main receptor, the integrin $\alpha_2\beta_1$. ES cells are generally characterized by a high expression of $\alpha_5\beta_1$, moderate levels of $\alpha_6\beta_1$, heterogeneous expression of $\alpha_2\beta_1$ and null expression of $\alpha_3\beta_1$ (Scotlandi *et al*., 2000). Interestingly, in two different ES cell lines, derived from the same patients, $\alpha_2\beta_1$ was higher on primary cells compared with metastatic cells (vanValen *et al*., 1994). This may reflect the fact that the predominant collagen extracted from ES tissue and synthesized in short-term cultures is type I (Harvey *et al*., 1982). Therefore, cells lacking $\alpha_2\beta_1$ are less facilitated to have stable anchorage to the ECM in ES tissue and may have increased migratory abilities. Indeed, invasion and metastasis of tumour cells is a multiple process that depends on interactions between cells and their extracellular environment. These interactions are mediated directly by specific adhesion receptors and indirectly by extracellular proteases, such as MMPs, proteolytic enzymes that regulate various cell behaviours, including cell growth, differentiation, apoptosis, migration and invasion. CCN proteins were reported to modulate the expression of MMP-1, -2, or -3 in relation to their pro- or anti-motility effects on different cell types (Fan and Karnovsky, 2002; Lake and Castellot, 2003; Laurent *et al*., 2003; Soon *et al*., 2003). In this study, we have analysed the expression and/or the activity of MMP-1, -2, -3, -8 and -9. In agreement with previous data (Yabe *et al*., 2002), ES cells, either transduced or not with CCN3, did not express MMP-1, -3 and -8. Expression and activity of MMP-2 did not significantly change, whereas, surprisingly, we found a significant downmodulation of MMP-9 activity in the supernatants of CCN3-transfected cells. However, although highly malignant cells are thought less aggressive when MMP expression or activity is reduced, new functions for the MMPs in cancer progression have been recently described (Egeblad and Werb, 2002). In recent years, the classic view of MMPs in tumour progression in which the enzyme's major contribution to tumour invasion is to degrade the physical barriers that constitute ECM has been challenged and various studies have shown that MMPs can directly accomplish cleavage of cell adhesion molecules and growth factor receptors or release of growth factors. Secreted MMPs can also localize to the cell surface by binding to integrins or to CD44 (Brooks *et al*., 1996; Yu and Stamenkovic, 1999) and this localization appears to be necessary for invasive cell behaviour (Fridman *et al*., 2003). In ES, proMMP-9, independently from its enzymatic activity, was found to be critical for triggering the switch from a stationary to a migratory state, likely influencing cytoskeleton rearrangements through RhoA/paxillin/β-catenin signalling (Sanceau *et al*., 2003). Accordingly, in the more invasive TC/CCN3 cells, we found an increase in the percentage of cells expressing proMMP-9 on their surface. Whether CCN3 promotes a redistribution of MMP-9 by serving as a docking molecule to retain secreted MMP-9 to the cell surface will be further investigated. Our hypothesis is that CCN3 favours the association of the proenzyme to

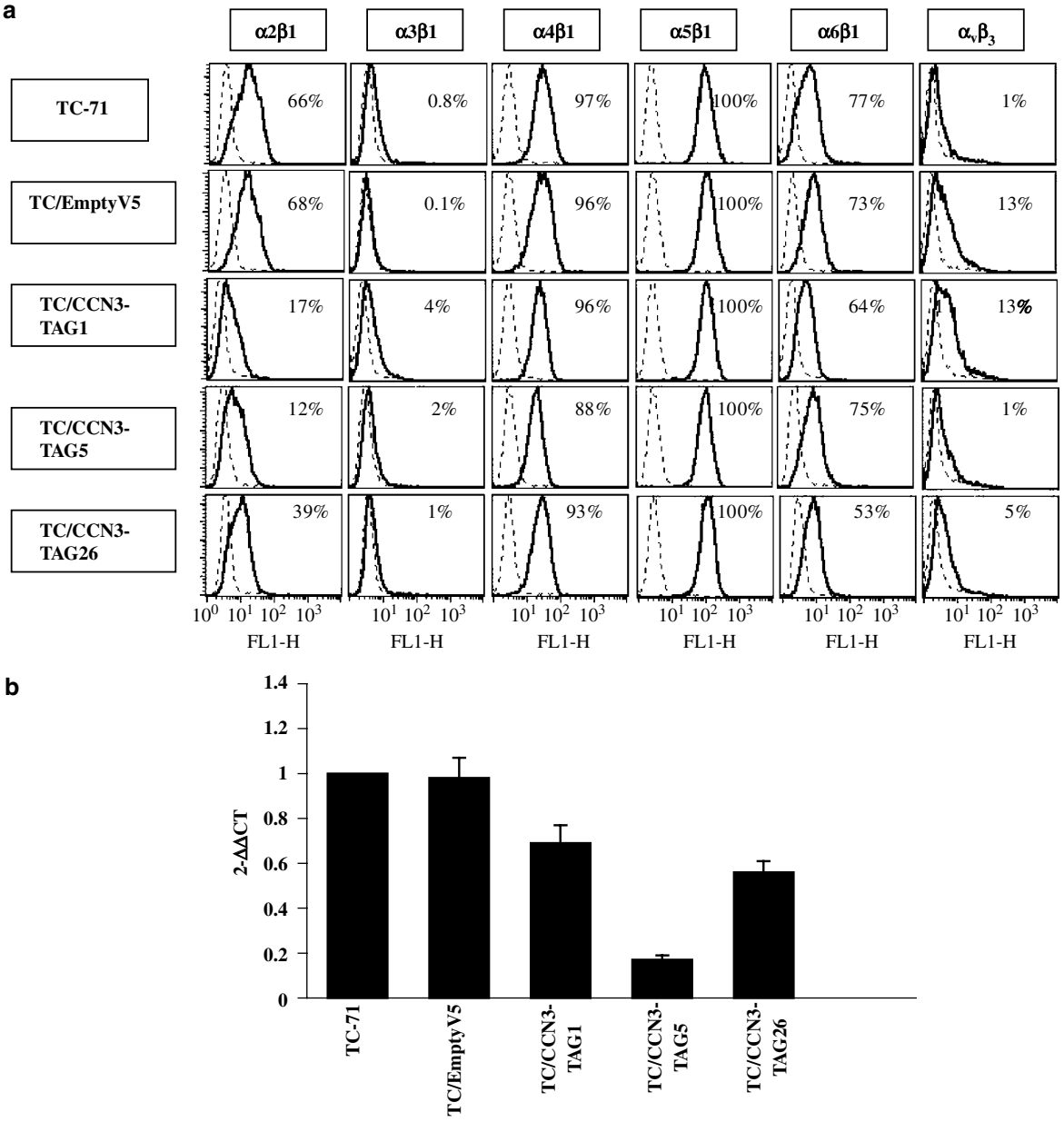

Figure 6 (**a**) Cytofluorometric analysis of integrin expression in TC/CCN3 transfectants. Open profile represents cells stained with secondary antibody alone; solid profile represents cells stained with the anti-CCN3 antibody. In each panel, the ordinate represents the number of cells. Data from an experiment representative of at least two similar experiments are shown. (**b**) Relative expression of $\alpha_2\beta_1$ mRNA in TC-71 transfected clones by real-time PCR. The parental cell line was used as calibrator ($2^{-\Delta\Delta CT} = 1$). Triplicate for each sample was performed and results are shown as mean $\pm$ s.e.

the cell matrix, inducing changes in cell shape and organization of the cytoskeleton that alter the migratory properties of ES cells, as already reported in the study of Sancéau (Sanceau *et al*., 2003).

In conclusion, we provide evidence that the expression of the matricellular protein CCN3 clearly serves important functions in ES cells. The molecule exhibits double-edged sword by triggering antiproliferative effects on one side, increasing migration and invasion on the other side. Further studies are clearly needed to clarify the mechanisms of action of this matricellular protein. New molecular clues may come from the genetic profile of TC/CCN3 clones, which is currently established in our laboratory, and from functional studies on MMP-9.

Materials and methods

Construction of expression vectors

The plasmid pcDNA6/V5-His (Invitrogen, Carlsbad, CA, USA) was used to construct the pCMV47 vector encoding a V5-6HIS-tagged human CCN3 protein. To insert the ccn3 coding sequence in frame with the V5-His tag pcDNA6, the

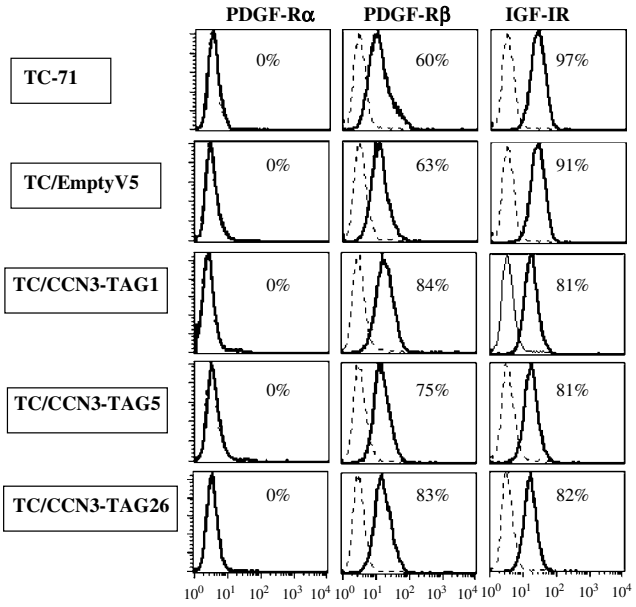

Figure 7 Cytofluorometric analysis of PDGF-Rα, PDGF-Rβ and IGF-IR expression in TC-71- and TC/CCN3-derived clones. Dotted profiles represent cells stained with secondary antibody alone; solid profiles represent cells stained with specific antibodies. Data from an experiment representative of at least two similar experiments are shown

ccn3 sequences spaning nucleotide 1–1143 from the pCMV82 CCN3 vector (Lin *et al*., 2003) were PCR amplified with 5′TATTGAATTCGGGAAGGCGAGCAGTGCCAATCTA and 3′CTGCGAATTCTCTAGACATTTTCCCTCTGGTA GTCTTCAG oligonucleotides that permitted to remove the TAA stop codon of ccn3. The PCR fragment was introduced in frame with the V5-6HIS tag at the *Eco*RI–*Xba*I site of the multiple cloning site (MCS) in pcDNA6 vector. Molecular cloning, plasmid amplification in *Escherichia coli* DH5α and sequencing of the recombinant clones were performed by using standard procedures (Perbal, 1988).

For nontagged CCN3 expression, a pcDNA3.1 vector (Invitrogen) containing neomycin resistance gene and ampicillin resistance was used to clone a full-length CCN3 cDNA in sense orientation after *Eco*RI digestion (Chevalier *et al*., 1998).

The pIND/V5-His and pVgRXR system was used for ecdysone-inducible expression of CCN3. The CCN3 sequences from pCMV47 was subcloned in frame with the V5-HIS tag at the *Eco*RI and *Xba*I sites of the pIND MCS to generate pIND47. Subcloning of the *Eco*RI fragment of pCMV82 into the same vector-generated pIND82. The pVgRXR vector was used to express the ecdysone receptor in cells co-transfected with pIND47. Induction of the CCN3 transcription in cells expressing the ecdysone receptor was achieved by exposure to ponasterone (5 μM, Invitrogen).

Amplified plasmids were purified through Qiagen columns (Santa Clarita, CA, USA) and sequenced prior to being use for transfections.

Cell lines and transfection

ES cell line TC71 was a generous gift from TJ Triche (Children Hospital, Los Angeles, CA, USA). Cells were routinely cultured in Iscove modified Dulbecco's medium (IMDM) (Invitrogen Life Technologies, Co., Carlsbad, CA, USA), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% inactivated fetal bovine serum (FBS) (Biowhittaker Europe, Verviers, Belgium) and maintained in 37°C in a humidified 5% CO_2 atmosphere.

Stable transfectants expressing CCN3 were obtained from TC-71 cells by using calcium-phosphate transfection. Cells transfected with the empty vector pcDNA6/V5-His were also used as negative control. The TC/CCN3-TAG, TC/EmtpyV5 and TC/CCN3-82 transfectants were selected and cultured in IMDM containing 10% FBS and 5 μg/ml blasticidin (Invitrogen) or 500 μg/ml neomycin (Sigma, St Louis, MO, USA), respectively. To establish stable inducible transfectants, TC-71 cell line was first transfected with pVgRXR vector by using the calcium-phosphate transfection procedure. Cells selected in IMDM plus 10% FBS plus 500 μg/ml zeocin (Invitrogen) were subsequently transfected with pIND/V5-His CCN3. The TC/IND47 and TC/IND82 transfectants were selected and cultured in IMDM containing 10% FBS, 500 μg/ml neomycin (Sigma, St Louis, MO, USA) and 500 μg/ml Zeocin (Invitrogen).

Western blot immunoassay

To determine the expression of CCN3, cells were washed twice with ice-cold PBS and cell lysates were prepared with a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 5 mM EDTA, 1% deoxycholate and protease inhibitors (1 mM phenylmethylsulphonyl flluoride, 1 mM sodium orthovanadate). The protein concentration of the samples was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Equivalent amounts of total cell lysate (50 μg) were separated by 12% SDS–polyacrylamide gel electrophoresis under denaturating conditions and transferred onto nitrocellulose membrane. The membranes were blocked for 1 h at 25°C with Tris-buffered saline-Tween 20 (TBST) containing 5% nonfat dry milk and incubated overnight with the primary K19M anti-CCN3 (dilution 1 : 1500) or the anti-tag V5 epitope (Invitrogen) antibody (dilution 1 : 5000). After washes with TBST, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Amersham Place, Buckinghamshire, UK) (dilution 1 : 3000), and revealed by ECL Western blotting detection reagents (Amersham).

To determine the expression of MMP-9, cell lysates and culture medium from parental and TC/CCN3 clones were separated by 10% SDS–polyacrylamide gel electrophoresis under nonreducing conditions and transferred onto nitrocellulose membrane and incubated overnight with the primary antibody anti-MMP-9 (dilution 1 : 1000) recognizing all MMP-9 forms (polyclonal antibody, Calbiochem S.Diego, CA, USA).

Membranes were then stripped in a buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS and 100 mM β-mercaptoethanol for 30 min at 50°C and re-probed with the primary monoclonal antibody (MAb) antiactin (dilution 1 : 2000) (Chemicon, Temecula, CA, USA) to verify the total proteins, as control.

Heparin-binding assay

Confluent TC-71 and transfected cells were cultured for 48 h in IMDM 10% FBS. TC/IND47 and TC/IND82 cells were pretreated for 24 h with 5 μM ponasterone A (Invitrogen). Conditioned media were collected and incubated overnight at 4°C with 200 μl of a 50% slurry of heparin-Sepharose beads (Amersham). After washing PBS, the beads were boiled and

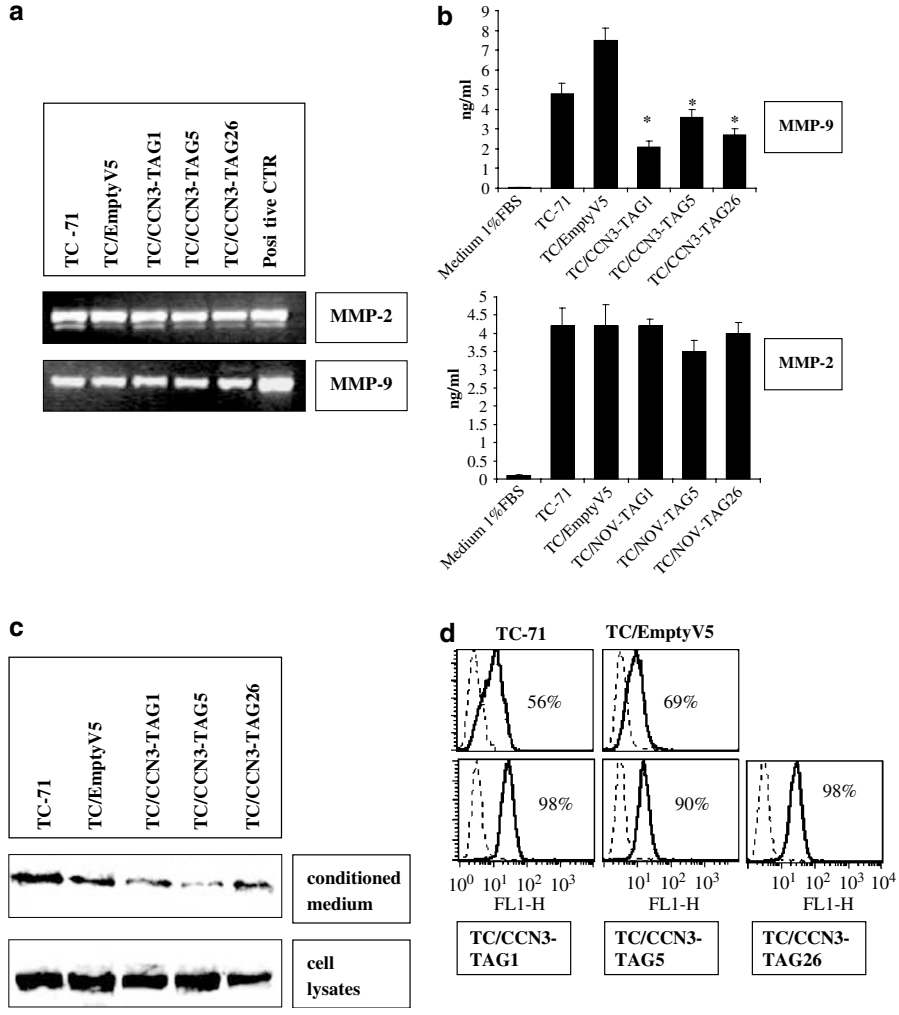

Figure 8 MMP-2 and -9 expression and activity of TC-71 cells and derived TC/CCN3 clones. (**a**) mRNA expression of MMP-2 and -9. Methods for reverse transcription of mRNA and PCR amplification of cDNA fragments were described in 'Material and methods'. Amplification products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. (**b**) MMP-2 and -9 activities in cell supernatants of parental and TC/CCN3 clones measured by a biotrak activity assay. $*P<0.05$; $**P<0.001$, Student's *t*-test, with respect to TC-71 parental cells. (**c**) Western blot analysis of MMP-9 expression in TC-71-derived clones. Proteins from cell lysates and from media conditioned by the same cells were electrophoresed on a 10% SDS polyacrylamide gel. (**d**) Cytofluorometric analysis of surface-associated MMP-9 expression in TC/CCN3 transfectants. Open profile represents cells stained with secondary antibody alone; solid profile represents cells stained with the anti-MMP-9 antibody

loaded directly in the wells. The presence of CCN3 protein was then analysed as described in the Western blot immunoassay section.

Purification of recombinant CCN3

K19M anti-CCN3 serum (5 ml) was precipitated with ammonium sulphate and dialysed against Na_2HCO_3 overnight at 4°C. In all, 1 g of CNBr-activated sepharose was washed with 220 ml of 1 mM HCl and left overnight at 4°C. Total protein from the antiserum (27 mg) were added to the swollen gel and rotated end-over-end for 2 h at room temperature (RT). The flow-through fraction was collected (5 ml: total protein content A280 of 6.5 mg). A volume of 5 ml of 0.1 M Tris-HCl, pH 8.0, was added to the gel and the column was rotated 90 min at RT. The column was washed with consecutive changes of 0.1 M acetate buffer plus 0.5 M NaCl and 0.1 Tris-HCl buffer plus 0.5 M NaCl. The column was washed with 6 ml of PBS and kept overnight at 4°C. Supernatant of the CCN3-producing cell line NCI-H295R was dialysed against PBS overnight at 4°C. A volume of 6 ml was passed through the absorbed column at 0.7 ml/min flow speed. The flow-through fraction (6 ml) was collected. The column was washed with PBS (no protein was detected at A280). Elution of bound proteins was carried out using 50 mM glycine-HCl, pH 2.7, in aliquots of 1 ml plus 0.1 ml of 1 M Tris, pH 8.0. Protein content of eluted fractions was measured by Bradford method and fractions were analysed by Western blotting.

Analysis of growth features in monolayer conditions

After transfection, cells were maintained in selective medium for a maximum of eight *in vitro* passages before the *in vitro* and *in vivo* characterization. Cells were analysed in IMDM plus 10% FBS without selective drug. TC/IND47 and TC/IND82 were treated with 5 μM of ponasterone A (Invitrogen) for 24 h

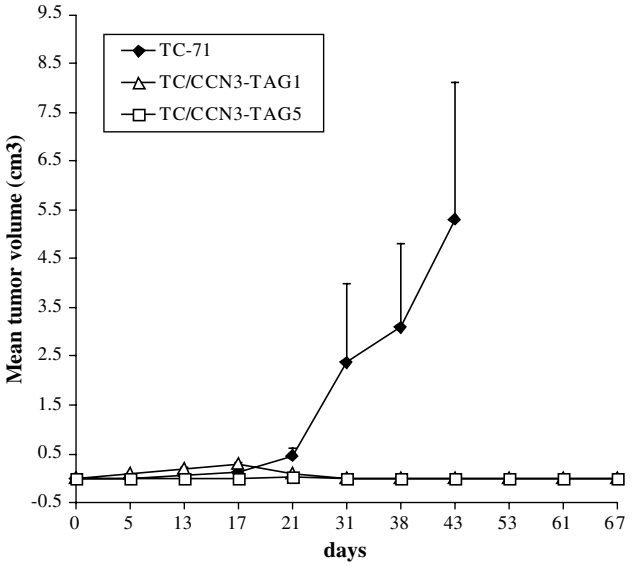

Figure 9 *In vivo* growth curves of tumors induced by TC-71 and TC/CCN3 transfectants. 5×10^6 cells were injected s.c. in nude mice. In each group, the mean size was determined by averaging measurements from the animals that grew tumors

before cell seeding. Doubling time was determined by daily harvesting of cells after seeding of 20 000 cells/cm^2. Cell viability was determined by trypan blue dye exclusion.

Soft-agar assay

Anchorage-independent growth was determined in 0.33% agarose (SeaPlaque, FMC BioProducts, Rockland, ME, USA) with a 0.5% agarose underlay. TC/IND47 and TC/IND82 were treated with 5 μM of ponasterone A (Invitrogen) for 24 h before cell seeding. Cell suspensions (1000–3300 cells per 60-mm dish) were plated in semi-solid medium (IMDM 10% FBS plus agar 0.33%) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Colonies were counted after 7 days.

Cell cycle analysis

For the evaluation of BrdUrd labelling index, 20 000 cells/cm^2 were seeded in IMDM plus 10% FBS. After 24–72 h, cell cultures were incubated with 10 μM BrdUrd (Sigma) for 1 h in a CO2 atmosphere at 37°C. Harvested cells were fixed in 70% ethanol for 30 min. After DNA denaturation with 2 N HCl for 30 min at room temperature, cells were washed with 0.1 M $Na_2B_4O_7$, pH 8.5. Then, 10^6 cells were processed for indirect immunofluorescence staining, using α-BrdUrd (Euro-Diagnostics, Milan, Italy) diluted 1 : 4 as a primary MAb, and analysed by flow cytometry (FACSCalibur, Becton Dickinson, Milan, Italy). For the cell cycle analysis, 70% ethanol-fixed cells were pretreated with 100 μg/ml of RNase for 30 min at 37°C and stained with 20 μg/ml of propidium iodide before flow-cytometric analysis.

Morphological assessment of apoptotic nuclei

In all, 5000 cells/cm^2 were seeded in IMDM plus 10% FBS. After 24–72 h, cells were fixed in methanol/acetic acid (3 : 1) and stained with 50 ng/ml Hoechst 33258 (Sigma). Cells with three or more chromatin fragments were considered as apoptotic. The percentage of apoptotic nuclei was evaluated out of 1000–2000 nuclei.

Motility assay

Motility assay was performed using Transwell chambers (Costar, Cambridge, MA, USA) with 8-μm pore size, polyvinylpyrrolidone-free, polycarbonate filters (Nucleopore, Pleasanton, CA, USA). In all, 10^5 cells in IMDM plus 10% FBS were seeded in the upper compartment, whereas IMDM plus 10% FBS alone or IMDM plus 10% FBS with PDGF-BB (50 ng/ml) (Upstate, Lake Placyd, NY, USA) were placed in the lower compartment of the chamber as chemoattractants. TC/IND47 and TC/IND82 were treated with 5 μM of ponasterone A (Invitrogen) for 24 h before cell seeding. Cells were incubated for 18 h at 37°C and the number of cells that migrated towards the filter to reach the lower chamber base was counted after fixation and Giemsa staining. All of the experiments were performed in triplicate.

Invasion assay

The invasive ability of TC-71- and CCN3-transfected cells was analysed by using Biocoat™ Matrigel™ invasion chambers (BD, Biosciences, Bedford, MA, USA). In all, 1.6×10^5 cells were seeded in the upper compartment of the chamber, whereas IMDM 10% FBS was placed in the lower compartment as a source of chemoattractant. Cells were allowed to migrate for 18 h at 37°C. After incubation, noninvasive cells and Matrigel were removed from the upper surface, whereas cells that had migrated to the lower side of the filter were fixed in methanol, stained with 1% toluidine blue and microscopically counted. All the experiments were performed in triplicate.

ECM adhesion assay

The adhesive ability of TC-71- and CCN3-transfected cells was analysed by using the CytoMatrix cell adhesion strips coated with human collagen type IV, vitronectin, fibronectin, laminin, or collagen type I (Chemicon International, Temecla, CA, USA). In all, 40 000 cells/well were seeded and incubated for 1 h at 37°C. Adherent cells were fixed and stained with 0.2% crystal violet 10% ethanol. After washing and solubilization with 5 mM NaH_2PO, pH 4.5, relative attachment was determined using absorbance readings at 550 nm on a microplate reader. All of the experiments were performed in triplicate.

Reverse transcription polymerase chain reaction (RT–PCR)

RT–PCR was performed to verify the constitutive expression of MMP-1, -2, -3, -8 and -9. Total RNA was extracted by TRIzol extraction kit (Life Technologies) from TC-71 cells and TC/CCN3-TAG clones. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide. The 18S and 28S rRNA bands were visualized under UV light. For RT–PCR, 1 μg of total RNA sample was denatured at 65°C for 10 min and then reverse-transcribed in a 100-μl reaction mixture containing 500 μM of each dNTP, 125 U of MultiScribe Reverse Transcriptase (PE Applied Biosystems), 40 U of RNase Inhibitor (PE Applied Biosystems), 2.5 μM oligo d(T), 1 × TaqMan RT buffer, 5 mM $MgCl_2$ at 48°C for 40 min. Reactions in which enzyme or RNA was omitted were used as negative controls. PCR was performed with a 60°C annealing temperature and for 35 amplification cycles. The PCR product was separated by electrophoresis on 2% agarose

gel. Specific primer pairs used are as follows: MMP-1: forward 5′-GGT GAT GAA GCA GCC CAG-3′; reverse 5′-CAG TAG AAT GGG AGA GTC-3′; product size 437 bp; MMP-2: forward 5′-CCA CGT GAC AAG CCC ATG GGG CCC-3′, reverse 5′-GCA GCC TAG CCA GTC GGA TTT GAT-3′; product size 486 bp; MMP-3: forward 5′-GTT AGG AGA AAG GAC AGT GGT CCT G-3, reverese'5′-GGC ATA GGC ATG GGC CAA AAC ATT-3′; product size 405 bp; MMP-8: forward *5′-AGC CAA ATG AGG AAA CTC TGG ACA-3′,* reverse *5′-TGA GTA GTT GCT GGT TTC CCT GAA A-3′*; product size 515 bp; MMP-9: forward *5′-GGT CCC CCC ACT GCT GGC CCT TCT ACG GCC-3*, reverse′ *5′-GTC CTC AGG GCA CTG CAG GAT GTC ATA GGT-3′*; product size 639 bp.

Quantitative real-time RT–PCR

Gene-specific primers were designed using Primer Express software (Applied Biosystems) (all 5′–3′ direction): CCN3 forward 5′-*CAC GGC GGT AGA GGG AGA TA*-3′, reverse 5′-*GGG TAA GGC CTC CCA GTG AA*-3′; GAPDH: forward 5′-*GAA GGT GAA GGT CGG AGT C*-3′, reverse 5′-*GAA GAT GGT GAT GGG ATT TC*-3′; product sizes of 224 and 251 bp, respectively. $\alpha 2\beta 1$ primers were purchased by Applied Biosystems (α2-subunit of VLA-2 receptor, Ref. Seq. NM_002203). SYBR Green PCR Master Mix (Applied Biosystems) was used with 1 ng of cDNA and with 200 nM of primers for the evaluation of GAPDH and CCN3 expression, respectively. Negative control without cDNA template was run with each assay. All PCR reactions were performed by using ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). All samples were run in triplicate. Reactions are characterized by the point when amplification of the PCR product is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The larger the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. The target gene mRNA is quantified by measuring C_T to determine the relative expression. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. In all experiments, the threshold value used to determine C_T during analysis was kept constant. Data were normalized to GAPDH. The relative mRNA expression of CCN3 was also normalized to a calibrator, consisting of the TC-71 mRNA, and was expressed as: $2^{-\Delta\Delta C_T}$, where $\Delta C_T = C_T$ target genes $- C_T$ GAPDH, and $\Delta\Delta C_T = \Delta C_T$ sample $- \Delta C_T$ calibrator. All samples were resolved in a 2% agarose gel to confirm the PCR specificity.

Phenotypic characteristics

The expression of specific membrane receptors, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_V\beta_3$ integrins, and MMP-9 was determined by flow cytometry after indirect immunofluorescence by using the following primary MAb: anti-IGF-IR (clone α IR3, Calbiochem-Novabiochem, Oncogene Research Products, S Diego, CA, USA), 1 : 10 dilution; anti-αPDGF-R (clone MM95 Calbiochem-Novabiochem) 1 : 20 dilution; anti-βPDGF-R (clone CM95 Calbiochem-Novabiochem) 1 : 20 dilution; antivitronectin receptor ($\alpha_V\beta_3$ clone LM609, Chemicon International) 1 : 50 dilution; CDw49b VLA2 (anti-alpha 2 chain, $\alpha_2\beta_1$, Immunotech SA), 1 : 10 dilution; P1B5 (anti-alpha 3 chain, $\alpha_3\beta_1$, Calbiochem-Novabiochem) 1 : 20 dilution; CDw49d VLA4 (anti-alpha 4 chain, $\alpha_4\beta_1$, Immunotech SA), 1 : 10 dilution; CDw49e VLA5 (anti-alpha 5 chain, $\alpha_5\beta_1$, Immunotech SA), 1 : 10 dilution; CDw49f VLA6 (anti-alpha 6 chain, $\alpha_6\beta_1$, Immunotech SA), 1 : 10 dilution; anti-MMP9 (polyclonal antibody, Calbiochem), 1 : 50 dilution.

MMP-2 and -9 activity

MMP-2 and -9 activity was evaluated on semiconfluent TC-71 cells and CCN3 transfectants by collecting low-serum cell supernatants. For MMP-9, supernatants were concentrated with a Centricon Plus-20 centrifugal filter device provided with an ultrafiltration membrane with a 30 000 nominal molecular weight cutoff limit (Millipore Corporation, Bedford, MA, USA). MMP-2 and -9 activity was determined by the ELISA test Biotrak MMP-2 or MMP-9 activity assay systems (Amersham Pharmacia Biotech, Milan, Italy). The test was performed according to the manufacturer's instructions. Briefly, standards and samples were incubated overnight at 4°C in microtiter wells precoated with anti-MMP-2 or -9 antibodies. After extensive washings, any bound MMP-2 or -9 was activated by adding aminophenylmercuric acetate. Active MMP-2 or -9 was then detected through the activation of a modified urokinase proenzyme and the subsequent cleavage of its chromogenic peptide substrate. The absorbance was read at 405 nm, and the concentration of active MMP-2 or -9 was determined by interpolation from the standard curve.

Tumorigenic and metastatic ability in athymic mice

Female athymic 6-week-old Crl:nu/nu (CD-1) BR mice (Charles River Italia, Como, Italy) were used. Tumorigenicity was determined after s.c. injection of 5×10^6 cells. Tumor growth was assessed once a week by measuring tumor volume, calculated as $\pi/6 \times [\sqrt{}(ab)]^3$, where a and b are the two major diameters. For ethical reasons, mice were killed and necropsied when the mean tumor volume exceeded 5 mm^3. The number of pulmonary metastases was determined by counting with a stereomicroscope after staining with black India ink. The experimental procedures were approved by the local ethical committee.

Statistical analysis

Differences among means were analysed using Student's *t*-test. Fisher's exact test was used for frequency data.

Abbreviations

NOV, nephroblastoma overexpressed gene; Cyr61, cystein-rich protein 61; CTGF, connective tissue growth factor; PDGF, platelet-derived growth factor; ECM, extracellular matrix; ES, Ewing's sarcoma; MMP, metalloprotease; PDGFR, platelet-derived growth factor receptor; MAPK, mitogen-activated protein kinase; IGF, insulin-like growth factor; IGF-IR, insulin-like growth factor receptor I; MAb, monoclonal antibody; IMDM, Iscove's modified Dulbecco's medium; FBS, fetal bovine serum; RT–PCR, reverse transcription polymerase chain reaction.

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