Downregulation of fibronectin transcription in highly metastatic adenocarcinoma cells

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Abstract Silencing of fibronectin (FN) expression seems to be one of the key mechanisms underlying metastatic behaviour. An inverse correlation exists between FN expression levels and the metastatic potential of two related murine mammary adenocarcinomas, M3 and MM3. Primary cultures of M3 tumour, which is moderately metastatic to lung (40% incidence), show a conspicuous FN extracellular matrix (ECM) and high levels of FN mRNA, while primary cultures of the highly metastatic MM3 tumour (95% lung incidence) are negative for FN in immunofluorescence and show at least 40-fold lower levels of FN mRNA, only detectable by RT-PCR, with a different pattern of alternatively spliced EDI isoforms compared to M3 cells. We show that the FN promoter sequence is not altered in MM3 cells. Transfection experiments with CAT constructs indicate that silencing occurs at the transcriptional level, involving the 220-bp proximal promoter region.

Key words: Fibronectin; Transcription; Metastasis

1. Introduction

Integrins and their ligands are involved in the malignant behaviour of tumour cells. Overexpression of α5β1 or α2β1 integrins suppresses tumorigenesis [1,2], while blocking of integrin function with either RGD-peptides or specific antibodies [3,4] reduces both tumorigenicity and metastasis. Fibronectin (FN) is the best characterised extracellular matrix (ECM) protein among integrin ligands. Loss of cell surface FN accompanies oncogenic transformation and it has been correlated with the acquisition of tumorigenic and metastatic potentials [5]. Conversely, reappearance of FN expression elicited by either FN cDNA transfection [6,7] or signalling activation [8,9] have been shown to revert tumorigenic and/or metastatic phenotypes. These findings confirm early observations that the addition of purified FN to transformed cells in culture restores normal morphology [10] and support the potential therapeutic use of FN, as recently shown by Pasqualini et al. [11], who found that a polymeric form of FN (superfibronectin) is able to act as a powerful antimetastatic agent against multiple tumour types.

We have previously described an inverse correlation between FN expression levels and the metastatic potential of two related murine mammary adenocarcinomas. Primary cultures of M3 tumours, moderately metastatic to lung, have a conspicuous FN ECM and high levels of FN mRNA, while primary cultures of the highly metastatic MM3 tumours, derived from M3 tumour by in vivo passage, show no FN ECM and undetectable FN mRNA levels by RNase mapping [12]. An established cell line derived from MM3 primary cultures (termed LMM3) [13], permanently transfected with constructs expressing full length FN cDNA under the control of a viral promoter exhibits reduced metastatic capacity compared to untransfected cells [6]. Though loss of FN in transformed cells was the original observation that led to the discovery and characterisation of FN [14,15] and remains the most remarkable regulatory event of this protein, little is known about the precise mechanism involved. This report explores the molecular bases of FN expression downregulation in the highly metastatic MM3 cells. We show that though the proximal FN promoter region has no sequence alterations, FN mRNA levels are at least 40-fold lower in MM3 cells than in M3 cells, revealing an inversion in the relative abundance of two alternatively spliced FN isoforms. By transient expression of FN promoter-CAT constructs we demonstrate that low FN mRNA levels are due to reduced transcription, involving the 220-bp proximal promoter region.

2. Materials and methods

2.1. Tumours

For all the experiments we used primary cultures of two BALB/c mammary adenocarcinomas with different lung metastatic potential. Briefly, M3 tumour presents a latency of 6–8 days and has a 40% incidence of lung metastases. The MM3 variant was obtained by Colombo et al. [16], following successive s.c. trocar implants of M3 lung metastases into the flank of syngenic mice. Once MM3 achieved a stable growth and metastatic behaviour, it was further maintained by grafts of s.c. tumour fragments. This variant presents a longer latency (12–14 days) and a 95% incidence of lung metastases [17]. Both tumours were maintained by subcutaneous trocar implants into female BALB/c mice.

2.2. Cell cultures

Primary cell suspensions of M3 or MM3 tumours were prepared by enzymatic digestion of tumour fragments with 0.01% pronase (Sigma) and 0.0035% DNase (Sigma) in MEM (41500, Gibco-BRL) medium. Cells were cultured in MEM supplemented with 10% heat inactivated foetal calf serum, 2 mM L-glutamine and 80 mg/ml gentamycin at 37°C. M3 and MM3 cell monolayers were mostly composed of epithelioid polyhedral cells with less than 2% contaminating macrophages and fibroblast-like cells. For some experiments we also used LM3 and LMM3 cell lines derived from M3 and MM3 tumours, respectively [13]. Both cell lines were maintained in MEM supplemented with 5% FCS plus L-glutamine and gentamycin as mentioned above.

2.3. Cell treatments

Cells were treated for 24 h in 1% (v/v) foetal calf serum with one of the following reagents: 10 nM okadaic acid (Gibco-BRL); 2.5 nM

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calyculin A (Gibco-BRL); 10 nM sodium vanadate; 50 nM phorbol (12,13-dibutyroyl) (PMA, Serva); 200 nM lovastatin; 10 μM forskolin (Sigma); 4 ng/ml transforming growth factor β1 (TGF β1). Serum treatment was performed by incubating the cells in 20% (v/v) foetal calf serum for 24 h after 17 h of serum starvation.

2.4. Immunofluorescence

Cells were fixed in 4% (v/v) formaldehyde in PBS for 20 min. Indirect immunofluorescence was performed using as first antibodies either FN-3E2 (Sigma), a mouse monoclonal against EDI epitopes, or a rabbit polyclonal against human FN (Gibco-BRL) that also recognises other vertebrate FNs.

2.5. Northern blot

Total RNA was extracted from cell monolayers using the acid guanidinium thiocyanate-phenol-chloroform procedure [18]. RNAs were electrophoresed in 1% agarose and 2.2 M formaldehyde gels, vacuum transferred to a nylon membrane (Hybond N) and hybridised at 68°C in 50% (v/v) formamide, 7% (w/v) SDS, 120 mM phosphate buffer (pH 7), 250 mM NaCl, 10% (v/v) PEG with an FN riboprobe [19].

2.6. RT-PCR amplification

One μg of total RNA was used as template for M-MLV reverse transcriptase (300 U) in a 20-μl reaction containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM Mg_2Cl, 10 mM DTT, 20 U of RNasin (Promega), 400 μM dNTPs, 3 mM pdT_{12-18} (Pharmacia), at 35°C for 1 h. PCR amplification of mouse FN cDNA was performed with 20 μM primers mFAdir (5′-CAGAAATGACCATTGAAGGT-3′) and mFArev (5′-ATGAGTCCTGACACAATCAC-3′), 1.5 mM $MgCl_2$, 200 μM dNTPs and 1.5 U of Taq DNA polymerase. PCR consisted of 30 cycles of 60 s at 94°C, 60 s at 50°C and 60 s at 72°C. RT-PCR products were electrophoresed in 1.8% agarose native gels and detected by ethidium bromide staining.

2.7. Single strand conformation analysis (SSCA)

SSCA was performed essentially as described by Sheffield et al. [20], using the following primers designed for the mouse FN promoter: forward, 5′-TTTGTTCGGGGCGAACCCAC-3′; reverse, 5′-CCTTTTCTCCCCCTGTGCAG-3′. These primers amplify a region comprised between positions −183 and +45. PCR was performed with 100 ng of genomic DNA, 1 μM primers, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (including 2 μCi of 3000 Ci/mmol [α-^{32}P]dATP per 50 μl reaction) and 1.5 U of Taq DNA polymerase. PCR consisted of 30 cycles of 45 s at 94°C, 30 s at 60°C and 45 s at 42°C. Five microliters of product were denatured in 95% (v/v) formamide at 95°C for 3 min, and loaded onto a 6% polyacrylamide non-denaturing, 0.5× TBE gel. Electrophoresis was performed at room temperature, at 2 W overnight. Gels were dried and autoradiographed.

2.8. Transfections and CAT assays

Segments of the human FN promoter (−224 to +64) were linked to the chloramphenicol acetyltransferase (CAT) gene. These constructs were used to transfect the M3 and MM3 cells. Cells were co-transfected with either cytomegalovirus (CMV)-β-galactosidase, Rous sarcoma virus (RSV)-β-galactosidase, or pCH110(SV40)-β-galactosidase reporter plasmids as measure of transfection efficiency. M3 and MM3 cells were transfected with 8 μl of lipofectamine (Gibco-BRL) and 2 μg of plasmid DNA in 35-mm tissue culture dishes. Cells were harvested 48 h post-transfection. Cell extracts and CAT assays were as previously described [21].

2.9. Quantification of autoradiograms

Bands in autoradiograms were scanned with a UMAX Scanner and quantitated densitometrically using the NHI image software.

3. Results

3.1. Downregulation of FN in MM3 cells

Previous to the investigation of FN transcriptional control, we applied procedures more sensitive than used before [12] to study the differences in FN and FN mRNA expression between M3 and MM3 cells. FN can be clearly detected in the extracellular matrix of M3 cultures by immunofluorescence, using either anti-total FN (Fig. 1A, a) or anti-EDI antibodies (Fig. 1A, b). The extra domain 1 (EDI, also termed EDA) is a FN internal segment encoded by an alternatively spliced exon, characteristic of proliferating cells, but absent from FNs of most resting adult tissues, including plasma FN [22]. In contrast, staining of MM3 primary cultures was completely negative (Fig. 1A, c and d) with both antibodies. These results correlated well with the lack of detection of full length FN mRNA in MM3 cells by Northern blot (Fig. 1B, lane 2) in comparison to the strong signal observed in M3 cells (Fig. 1B, lane 1). However, using the more sensitive RT-PCR procedure we were able to detect low amounts of FN mRNA in MM3 cells, which suggests that the FN gene is indeed transcribed in these cells. The use of RT-PCRs of increasing number of cycles and ribosomal RNA as internal control allowed us to estimate that the levels of FN mRNA are at least 40-fold lower in MM3 cells than in M3 cells (Fig. 1C). Noticeably, EDI^+/EDI^- FN mRNA ratio is 4-fold higher in the moderately metastatic M3 cells than in the highly metastatic MM3 cells, which suggests a change in alternative mRNA splicing.

3.2. Activation of various signal cascades has no effect on FN mRNA levels in MM3 cells

Assuming that the same regulatory factors were affecting negatively the endogenous and transfected FN promoters we performed a series of experiments aiming to activate the endogenous gene by affecting different signal transduction cascades. FN mRNA levels of MM3 cells, measured by Northern blot or RT-PCR, were not affected by treatment of cell monolayers with protein Ser/Thr phosphatase inhibitors such as okadaic acid or calyculin A; an inhibitor of protein Tyr phosphatases (vanadate); activators of protein kinase (PK) pathways such as PMA (PKC) and forskolin (PKA); an inhibitor of small GTP-binding protein isoprenylation (lovastatin); serum starvation followed by incubation with 20% (v/v) foetal calf serum; and a growth factor known to activate the FN gene in other cell types (TGF β1) [23] (results not shown, see Section 2 for treatment conditions).

3.3. Promoter sequence analysis

The proximal 220 bp of the FN gene promoters of human and rodents are highly conserved and contain most of the key *cis* regulatory elements: a cyclic AMP response element (CRE) at position −170, a CCAAT box at position −150, two AP-2 sites at positions −57 and −124, two Sp1 sites at positions −99 and −94 and a canonical TATA box at −25 (numbering corresponds to the human gene). Cooperativity between the neighbouring CRE and CCAAT sites has been extensively studied in our laboratory [24,25]. Introduction of disruptive point mutations into the CRE and CCAAT sites in the context of the proximal 220 bp reduces transcriptional activity by at least 80% in FN promoter-chloramphenicol acetyl transferase (FN-CAT) constructs transfected in various cell lines of either mesenchymal (NIH 3T3, HT1080) or epithelial (Hep3B, HepG2) origins (C.G. Pesce and A.R.K., manuscript submitted). So, this region fits the definition of promoter proposed by Blackwood and Kadonaga [26] and is a good candidate to investigate a putative negative transcriptional regulation in MM3 cells. To determine if the extremely low levels of FN mRNA in MM3 cells were due to disruptive mutations in the 220-bp proximal region of the endogenous FN gene we performed single strand conformation analysis

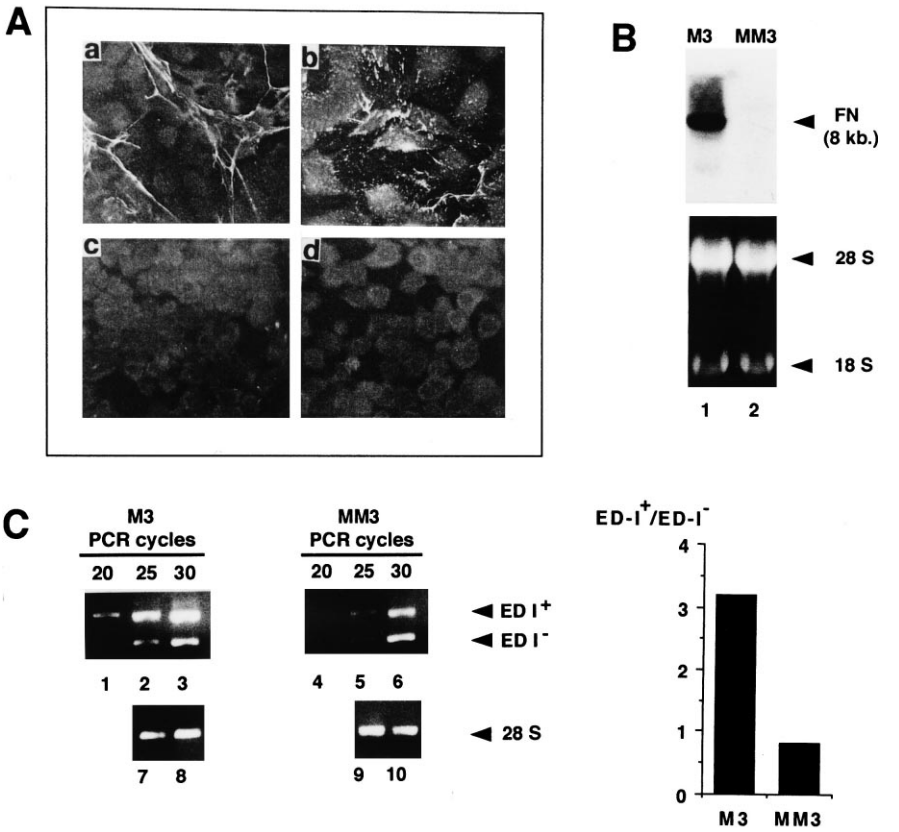

Fig. 1. A: Indirect immunofluorescence of M3 (a and b) and MM3 (c and d) cell monolayers with antibodies directed to total plasma FN (a and c) or the alternatively spliced EDI segment (b and d), characteristic of pericellular FN. B: Northern blot of total RNA from M3 (lane 1) and MM3 (lane 2) cells, hybridised with an FN riboprobe. The bottom panel shows ethidium bromide staining. C: RT-PCR of different numbers of cycles of total RNA from M3 (lanes 1–3, 7 and 8) or MM3 (lanes 4–6, 9 and 10) cells, using primers designed to detect EDI^+ and EDI^- splicing isoforms of mouse FN mRNA (lanes 1–6) or control 28S ribosomal RNA (lanes 7–10). Bars indicate EDI^+/EDI^- ratios resulting from quantification of bands in lanes 3 and 6.

(SSCA) of this region using genomic DNAs from normal mouse liver, M3 and MM3 cell cultures. Fig. 2A shows that the same band pattern is observed with templates from the three cell types. We then subcloned the same PCR products used for SSCA into a CAT expression vector (pUC19-CAT-0) and sequenced the inserts of 5 independent subclones for each cell type. All inserts showed the same sequence, which is identical to the published mouse sequence [27]. This indicates that downregulation of FN mRNA in MM3 cells cannot be attributed to structural defects of the FN promoter proximal region. A significant deletion or insertion outside this region has been ruled out previously [12].

3.4. Transient expression of FN-CAT constructs

The high degree of conservation between human and mouse promoter sequences allowed us to use indistinctly mouse FN-CAT or previously characterised human FN-CAT constructs [21,25] to investigate whether downregulation occurred at the transcriptional level. Since we found that MM3 cells have lower transfection efficiencies than M3 cells, we took special care in estimating them by co-transfecting FN-CAT constructs in separate experiments with plasmids in which the β-galactosidase (β-gal) reporter is under the control of different viral promoters. Fig. 3A shows that transcriptional activity of p-220-CAT is much higher in M3 cells (lanes 3, 5 and 7) than in MM3 cells (lanes 4, 6 and 8), independently of the promoter nature of the β-gal construct used for standardising transfection (Early Simian Virus 40 (SV40), lanes 3 and 4; Rous Sarcoma Virus Long Terminal Repeat (RSV), lanes 5 and 6; Immediate Early Cytomegalovirus (CMV), lanes 7 and 8)). Consistently, similar CAT activities were observed when

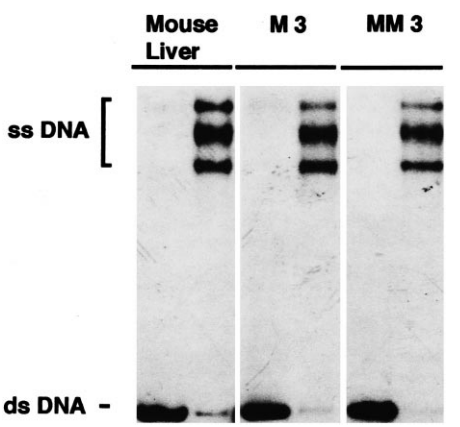

Fig. 2. Fibronectin promoter analysis in M3 and MM3 cells. A: Single-strand conformation analysis of PCR products obtained by amplification of the FN proximal promoter region of genomic DNA from mouse liver, M3 or MM3 cells. Left lanes of each pair correspond to controls in which DNA was not denatured prior to loading the non-denaturing polyacrylamide gel, and show the expected RT-PCR product of 228 bp.

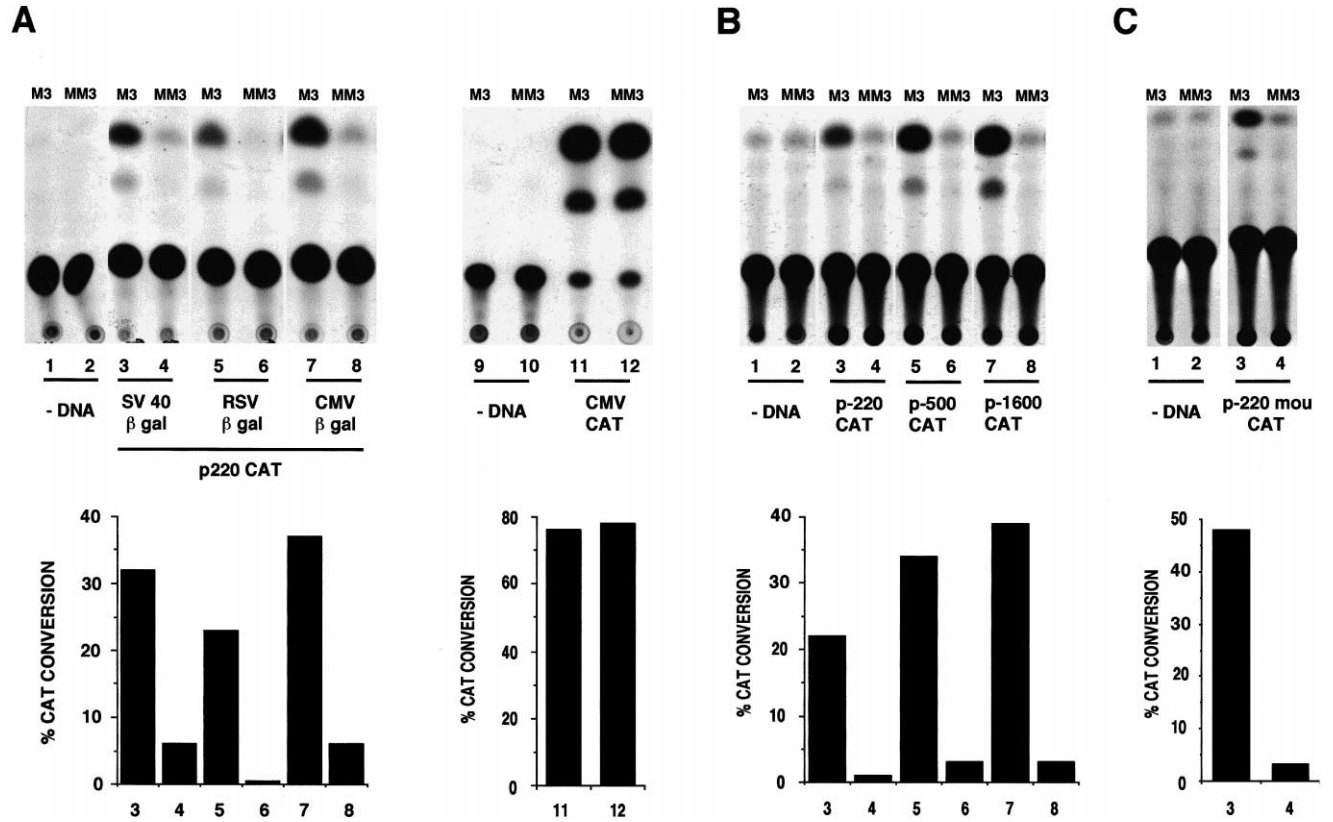

Fig. 3. Downregulation of FN in MM3 cells. Transient expression of FN-CAT constructs in M3 (odd numbered lanes) and MM3 (even numbered lanes) cells. A: Cells were transfected with p-220 CAT (lanes 3–8) by the lipofectamine method, cotransfecting with three different β-galactosidase expressing constructs as efficiency controls: SV40-βgal (lanes 3 and 4), RSV-βgal (lanes 5 and 6) and CMV-βgal (lanes 7 and 8). Control transfections with a CMV-CAT construct are shown in lanes 11 and 12. B: Cells were transfected with three FN-CAT constructs differing in the length of the promoter region: p-220 CAT (lanes 3 and 4), p-500 CAT (lanes 5 and 6) and p-1600 CAT (lanes 7 and 8). C: Cells were transfected with a 220-bp mouse FN promoter-CAT construct (lanes 3 and 4). In all panels lanes 1 and 2 are mock transfections with no DNA added. For experimental details see Section 2.

M3 or MM3 cells were transfected with a CMV-CAT construct (Fig. 3A, lanes 11 and 12), indicating that, unlike the FN promoter, the CMV promoter is equally active in both cell types. Similar results were obtained using the mouse FN promoter-CAT construct instead of human p-220-CAT (Fig. 3C). These results suggest that the very low transcriptional activity observed in MM3 cells involves *cis* elements located within the 220-bp proximal promoter region. Inclusion of upstream promoter sequences of up to −500 (Fig. 3B, lanes 5 and 6) or −1600 (lanes 7 and 8) in the CAT constructs increased transcriptional activity in M3 cells but did not override the low expression observed in MM3 cells.

4. Discussion

Several lines of evidence indicate that reduction of FN production in metastasising cells in comparison to their non-metastasising variants is not merely a circumstantial event. This idea is supported not only by the inverse correlation between FN production and metastatic potential observed in several tumour cell types [12,28] but most importantly by a set of independent experiments designed to perturb FN-integrin interactions both in vivo and in vitro. Injection of RGD-containing peptides [3] or anti-FN antibodies [4] into mice, treatment of metastatic cells with super-FN previously to injection into mice [11], intraperitoneal injection of super-FN into mice previously to challenge with metastatic cells [11] and transgenic overexpression of FN in highly metastatic cells [6] inhibit metastasis. On the contrary inhibition of FN synthesis by antisense RNA abrogates the suppression of malignancy induced by the hybridisation of melanoma cells with normal fibroblasts [29]. Similarly, FN mRNA levels were 8-fold lower in cells induced to metastasise by transfection with the v-*Ha-ras* oncogenes [28].

Several mechanisms could account for reduction in FN steady state levels in more metastatic cells. Impaired FN synthesis could be the result of selection of subpopulations of tumour cells in which the FN gene or any of its positive regulator genes have suffered disruptive mutations. Negative regulation of transcription, splicing or translation induced by viruses, oncogenes or external signals could affect directly the FN information flow or that of a positive regulatory factor. Alternatively, an increase in enzymes that degrade FN or alter FN mRNA stability would cause similar effects. It is quite plausible that two or more of these processes act in a concerted way in a particular cell type.

In this context, unravelling the inhibitory mechanisms becomes crucial for the development of therapeutic strategies to inhibit metastasis. The work of Pasqualini et al. [11] leads the way to a potential use of super-FN as a powerful antimetastatic agent with apparently no toxic effects on normal cells. Other strategies may include the development of drugs that

stimulate endogenous FN expression or prevent downregulation.

We show here that the loss of FN expression in MM3 cells is not caused by changes in the FN promoter sequence, but is the result of negative transcriptional regulation. We failed to revert this downregulation by treating MM3 cells with different agents that activate signal transduction cascades, including protein phosphatase inhibitors, protein kinase activators, small GTP-binding protein inhibitor, serum and TGF β1.

The observed decrease in transcription activity observed in FN-CAT transfections accounts for the 40-fold reduction in steady state levels of FN mRNA, leaving little room for post-transcriptional mechanisms as those caused by Ha-Ras in human osteosarcoma cells [31].

Adenovirus protein E1a, cyclic AMP and v-Src were reported to repress FN gene transcription. However, very little is known about these negative regulations. Expression of E1a causes oncogenic transformation and brings down FN transcription in 3Y1 rat fibroblasts [30]. Since both E1a splicing variants (13S and 12S) are equally able to repress the FN promoter, this effect is not related to the more characterised transcriptional activating role of E1a, which requires the domain that is absent in the 12S variant (named CR3), that acts through ATF/CRE sites. Oda and co-workers showed that E1a transformed 3Y1 cells possess a protein, termed G10-BP, that binds to G-rich sequences of the FN promoter, coincident with SP-1 sites. G10-BP might act as a transcriptional repressor by displacement of the ubiquitous activator SP-1 from its cognate site [32].

In the case of v-Src, a distal portion of the rat FN gene promoter (3.9 kb) has been implicated in mediating a 3–4-fold inhibition of FN transcription initiation caused by expression of $p60^{v-src}$ in NRK cells [33].

Fig. 3 shows that the proximal 220-bp region of either the human or mouse FN promoters are sufficient to mediate transcriptional downregulation in MM3 cells. Inclusion of additional upstream sequences does not override low transcriptional activity. Our results cannot rule out species-specific regulation for regions upstream of −220. Apart from the already mentioned SP-1 site, the 200-bp proximal promoter region contains the major CRE and an adjacent CCAAT motif. Cooperative interaction between the CRE and CCAAT sites has been reported in liver cells [24], involving the transcription factors ATF-2, NF-1 and CP-1 [25]. This interaction is also observed in cell types of non-hepatic origin, but it seems to be functionally important in liver cells since it mediates transcriptional activation by cAMP in the hepatoma cell line Hep3B, but not in fibroblasts (C.G. Pesce and A.R.K., manuscript submitted). We were not able to detect differences between M3 and MM3 nuclear extract binding to the SP1, CRE and CCAAT sites of the FN promoter, assessed by gel mobility shift or DNase I footprinting (data not shown). This indicates that the decrease in the FN promoter activity in MM3 cells must be the result of more subtle changes in the quality of *trans*-acting factors.

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