SPANDIDOS PUBLICATIONS

Fibronectin is distinctly downregulated in murine mammary adenocarcinoma cells with high metastatic potential

ALEJANDRO J. URTREGER[1*], SANTIAGO E. WERBAJH[2*], FRANK VERRECCHIA[3], ALAIN MAUVIEL[3], LYDIA I. PURICELLI[1], ALBERTO R. KORNBLIHTT[2] and ELISA D. BAL DE KIER JOFFÉ[1]

[1]Área Investigación, Instituto de Oncología 'Ángel H. Roffo', Universidad de Buenos Aires; [2]Laboratorio de Fisiología y Biología Molecular, Departamento de Fisiología, Biología Molecular y Celular, IFIByNE-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Buenos Aires, Argentina; [3]INSERM U697, Institut de Recherche Sur La Peau, Université Paris VII, Pavillon Equerre-Bazin, Hôpital Saint Louis, 75010 Paris, France

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Abstract. In the present work we used a murine mammary cancer model of two related adenocarcinomas with different lung metastasizing abilities, to compare their global gene expression profiles. Clontech Atlas mouse cDNA microarrays of primary cultured tumor cells were employed to identify genes that are modulated in the more metastatic variant MM3 relative to its parental tumor M3. A total of 88 from 1,176 genes were differentially expressed in MM3 primary cultures, most of them (n=86) were upregulated. Genes were grouped according to their functions as associated with signal transduction and transcription regulation (e.g. Stat1 and Zfp 92), with cell adhesion and motility (cadherin 1, fibronectin), with invasion and angiogenesis (uPA, 72 kDa MMP2), with the regulation of cell proliferation and cell death (cyclins G and A2, TNF), and also included growth factors and receptors, oncogenes and tumor suppressors genes (p107, TGFß2, TBR-I, PDGFR). Only 2 genes, TTF1 and fibronectin (FN), showed a significant downregulation. Notably FN expression, loss of which has been associated with a malignant phenotype, was reduced about 19-fold in the more metastatic MM3 cells. Previously known differences in expression patterns associated with the metastatic capacity of MM3 and M3 adenocarcinomas, including downregulation of FN or upregulated expression of TGFß and proteases, were confirmed by the array data. The fact that FN was one of the only two genes significantly down-regulated out of the 1,176 genes analyzed stresses the hypothesis that FN may behave as an important metastasis suppressor gene in mammary cancer.

Correspondence to: Dr Elisa D. Bal de Kier Joffé, Área Investigación, Instituto de Oncología 'Ángel H. Roffo', Av. San Martín 5481, (C1417DTB) Buenos Aires, Argentina
E-mail: elisabal@fmed.uba.ar

*Contributed equally

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Introduction

The major pathological effect of cancer is caused by the invasion of malignant cells to surrounding tissues and the subsequent metastasis to vital organs. The metastatic process involves the detachment and infiltration of the cells from the original primary tumor, intravascular invasion, transportation in the blood, arrest in the microvasculature, extravasation, and finally, proliferation at the target organ (1,2). This process involves the participation of numerous molecules with specific activities, such as growth factors and their specific receptors, cell adhesion molecules, cytoskeleton proteins, extracellular matrix components (ECM) as fibronectin (FN), and proteolytic enzymes such as plasminogen activators (uPA) and metalloproteinases (MMP) (3). Moreover, the molecular requirements for some of these steps may be tissue specific. In fact, the proclivity that some tumors have for specific organs, such as breast carcinomas for bone and lung, was described more than a century ago and is still a matter of analysis (4-6).

It is accepted that tumors are heterogeneous for many properties and contain subpopulations of more or less aggressive cells that differ in many of their biochemical and biological characteristics, such as growth rate, karyotype, expression of hormone receptors, and their invasive and metastatic capability (7). Taking advantage of this characteristic, many experimental models, useful to study critical determinants of tumor progression, have been developed (4,8). A mammary cancer model syngeneic in BALB/c of two related adenocarcinomas with different lung metastasizing abilities, was established by an *in vivo* procedure more than 20 years ago in our institute (9). MM3 adenocarcinoma variant was selected after 10 successive re-transplantations of M3 lung metastases into the flank of syngeneic mice, procedure that led to an enrichment of its lung metastatic potential. These tumors, together with their derived continuous cell lines (10), have

been extensively used to analyze critical molecular determinants of breast tumor growth and dissemination (10-15).

Although there is extensive knowledge of the changes that endow tumor cells with their metastatic functions, this is a subject of debate. Further insight into the complexity of the metastatic process can be approached employing the new technologies available to analyze the expression of hundreds of genes simultaneously. In the present work we compared the global gene expression profiles obtained by cDNA microarrays of M3 and MM3 primary cultures in order to identify those genes that are modulated in the more metastatic variant MM3 relative to its parental tumor M3. A total of 86 significantly upregulated genes (>2.7-fold) were identified in MM3 primary cultures, while the expression of only two genes was significantly downregulated (<0.33-fold). The expression level of FN, a plasma and extracellular matrix glycoprotein which loss has been associated with the acquisition of a malignant phenotype (16), was reduced about 19-fold (<0.05-fold) in the more metastatic MM3 cells, confirming our previous findings employing other methodologies (9-12,17). The fact that FN was one of the only two significantly downregulated genes among the 1,176 genes analyzed stresses the hypothesis that FN may behave as a metastasis suppressor gene in mammary cancer.

Materials and methods

Reagents and antibodies. Medium for cell culture (MEM, Cat. no. 41500) was from Gibco Life Technologies (Rockville, MD). Fetal calf serum (FCS) was from GENSA (Buenos Aires, Argentina). Triton X-100 was obtained from Dr J.T. Baker (Phillipsburg, NJ). All other reagents for polyacrylamide gel electrophoresis and zymography were obtained from Bio-Rad (Richmond, CA). Trypsin, pronase, DNase and anti-human fibronectin antibodies were obtained from Sigma Co. (St. Louis, MO). Anti-PDGFR2-α, anti-CD31, anti-retinoblastoma and anti-ß-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-E-cadherin and anti-Stat1 antibodies were from Transduction Laboratories (San Diego, CA). Horseradish peroxidase conjugated anti-rabbit and anti-mouse antibodies, Hybond-P membranes and chemiluminescence reagents (ECL) were from Amersham (Aylesbury, UK).

Tumors. An *in vivo* selected model of two related transplantable mammary adenocarcinomas with different lung metastatic potential was used in this study (9). Briefly, M3 tumor presents a latency of 6-8 days and has a 40% incidence of lung metastases. MM3 variant was previously obtained in our laboratory following successive subcutaneous (s.c.) trochar implants of M3 lung metastases into the flank of syngenic mice. Once MM3 achieved a stable growth and metastatic behavior, it was further maintained by grafts of s.c. tumor fragments. This variant presents a longer latency (12-14 days) and a 95% incidence of lung metastases (13). Both tumors were maintained by s.c. trocar implants into female BALB/c mice. Animals were maintained under the guidelines of the National Institutes of Health and approved protocols of institutional Animal Care and Use Committee of the Institute of Oncology 'Ángel H. Roffo'.

Primary culture. To reduce the risk of contaminating tumor parenchymal cells with stromal and/or other host cells, primary cultures of M3 and MM3 tumors were prepared, as previously described (13). Briefly, cell suspensions were obtained by enzymatic digestion of tumor fragments with 0.01% Pronase and 0.0035% DNase in MEM medium. Viable cells were counted employing trypan-blue exclusion test. Cells were cultured at 37˚C in plastic flasks in a humidified 5% CO_2 air atmosphere, in MEM supplemented with 10% FCS, 2 mM L-glutamine and 80 μg/ml gentamycin. M3 and MM3 cell monolayers were mostly composed of epithelioid polyhedric cells with less than 2% contaminating macrophages and fibroblast-like cells (13).

In vivo metastasis assay. To assay their spontaneous metastatic ability, M3 and MM3 cells, harvested from subconfluent primary cultures during the exponential growth phase by treatment with Trypsin-EDTA, were washed thoroughly with MEM and resuspended in the same medium. Non-anesthetized BALB/c female mice were inoculated s.c. into the left flank with $2x10^5$ viable cells of each tumor type, in a volume of 200 μl of serum-free MEM. Six weeks after inoculation animals were sacrificed and lungs were fixed in Bouin's solution. The number and size of surface lung nodules was determined under a dissecting microscope. Two independent experiments were performed.

RNA extraction

Differential hybridization of Atlas™ mouse cDNA expression arrays. Total RNA from M3 and MM3 cell monolayers was obtained using an RNeasy kit (Qiagen, Valencia, CA) and treated with DNase I to avoid genomic DNA contamination of reverse transcription reactions. Radioactive cDNA synthesis was carried out as described in the Atlas cDNA expression arrays user manual (Clontech, San Diego, CA). Equal amounts of ^{33}P-radiolabeled cDNAs (10^7 cpm) from M3 and MM3 RNA samples were hybridized in parallel to Atlas 1.2 mouse cDNA expression arrays (Clontech) for 18 h at 68˚C. Then filters were washed four times in 2X SSC and 1% SDS for 30 min at 68˚C and twice in 0.1X SSC and 0.5% SDS at 68˚C, according to the manufacturer's recommendation. Membranes were then exposed to Eastman Kodak Co. phospho-screens for 6 days. Hybridization signals were quantified with a Storm 840 phosphorimager using ImageQuant software (Amersham) and normalized against the mRNA levels of two house-keeping genes in the same samples: ß-actin and type-2A and protein phosphatase (PPA2). Significant modulation of gene expression was arbitrarily set to a ratio threshold of 2.7. Three independent experiments were performed.

Western blot analysis. Cell lysates were prepared by incubating the primary cultures in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1% Tween-20, 10 mM ß-glycerophosphate, 0.1 mM orthovanadate, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mM DTT, and 0.1 mM PMSF) on ice for 40 min. The lysates were cleared by centrifugation (12,000 rpm, 15 min at 4˚C). Total protein concentrations were determined using the Bradford Reagent (Bio-Rad). Cell extracts (60 μg protein) were separated on 7.5 or 10% SDS-polyacrylamide gel, and separated proteins were transferred

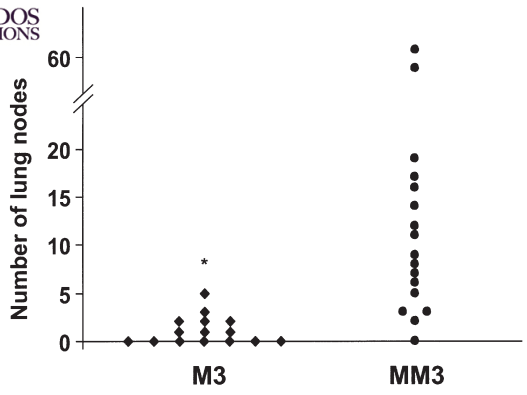

Figure 1. Spontaneous lung metastasizing ability of primary cultures derived from M3 and MM3 murine mammary adenocarcinomas. Cells were injected s.c. and 6 weeks after inoculation animals were sacrificed and the number of lung foci was recorded. The figure shows the results of at least 2 independent experiments. *p<0.01 vs. MM3 cells. Kruskal-Wallis test.

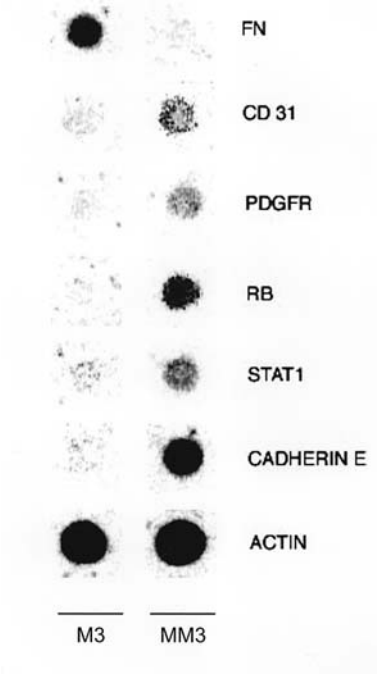

Figure 2. Representative spots of upregulated and downregulated genes in cDNA microarrays for M3 and MM3 cells. Total RNA from M3 and MM3 cell monolayers were obtained and ^{33}P-radiolabeled cDNAs were prepared and hybridized in an Atlas 1.2 mouse cDNA expression arrays.

onto Hybond-P membranes using semidry blotting (Bio-Rad). The membranes were blocked for 1 h with 5% skim-milk in TBST buffer (20 mM Tris-HCl, pH 7.6/137 mM NaCl/0.5% Tween-20), and incubated with the first antibodies overnight at 4˚C. Blots were washed three times with TBST buffer, then incubated for 1 h with a secondary antibody coupled to horseradish peroxidase. Detection was performed by chemiluminescence. Bands were quantified by scanning on a digital GS-700 densitometer and the resulting data processed with Molecular Analyst software (Bio-Rad). Membranes blotted with anti-ß-actin antibodies were used as loading and transfer control.

Results

In vivo metastatic behavior of M3 and MM3 primary cultures. Before initiating the cDNA microarray experiments, the spontaneous metastatic capacity of M3 and MM3 primary cultures was studied, in order to confirm that cultured cells did not lose the differences in their metastasizing ability shown by the non-cultured tumor variants (9).

MM3 primary cultured cells inoculated s.c. into syngeneic mice were significantly more metastatic than M3 ones. As shown in Fig. 1, 16/17 (95%) animals inoculated with MM3 cells developed spontaneous lung metastasis with a median number of 11 (range 0-62), while only 8/15 (53%) mice inoculated with M3 cells developed 1 (0-6) foci per mouse.

cDNA microarray analysis. The broad coverage Clontech Atlas 1.2 mouse cDNA arrays were used to profile the differential expression of genes involved in many crucial cellular pathways and functions associated with metastatic dissemination, employing our murine mammary cancer model of MM3 adenocarcinoma, highly metastatic to lung and its parental low metastatic mammary tumor M3.

It is important to note that cDNA was prepared with RNA extracted from primary cultures established from a pool of three s.c. tumors for each variant, in order to avoid sample contamination with RNA from stromal or migrating host cells.

Although there was certain variability in the magnitude of differential expression between experiments, only unidirectional consistent changes were taken into account for further analysis. Of the 1,176 genes analyzed, a set of 88 genes showed differential expression in a significant and consistent way in MM3 cells compared to M3 ones. Interestingly, most of them were upregulated. A list of these genes, grouped into several functional categories and ranked according to their ratio value, is presented in Table I. According to these results, the high metastatic phenotype of MM3 cells could involve the augmented expression of genes associated with the regulation of signal transduction and transcription (e.g. STAT1, Zfp 92, Zfp 36, interferon regulatory factors 1, 2, 7, and E2F transcription factor 5), with cell adhesion and motility (cadherin 1, α2 integrin, gap junction membrane channel proteins α1 and 3, kinesin family member 3A, thrombospondin, syndecans 1 and 3, keratin complex 1), with invasion and angiogenesis (uPA, 72 kDa MMP2, cathepsin W, granzyme C), with the regulation of cell proliferation and cell death [cyclins G and A2, CDKN1A, cyclin dependent kinase protein 1A (p21), TNF], and also including several growth factors and receptors, oncogenes and tumor suppressors (lung carcinoma myc related 1, p107 retinoblastoma like 1, proto-oncogene c-rel, C-CSF, TGFß2, TGFß receptor 1, PDGF receptor B). A large proportion of the upregulated genes in the highly metastatic MM3 cells are believed to be involved in signal transduction and gene expression regulation. Only two genes FN and TTF1 were significantly downregulated in MM3 cells with expression below the chosen cut-off threshold of 0.33.

Validation of differential expression. To confirm the differential expression results, a subset of six genes, five of them upregulated and FN as the main downregulated gene, were selected (Fig. 2) and their expression at protein level by Western blotting was analyzed in M3 and MM3 primary

Table I. List of upregulated (A) and downregulated (B) genes by microarray analysis (MM3/M3 primary cultures).

(A) Signal transduction and gene expression regulators

1.	X57621	Nuclease sensitive element binding protein 1	28.80
2.	S81932	Distal-less homeobox 3	24.89
3.	U41626	Split hand/foot deleted gene 1	23.83
4.	U47104	Zinc finger protein 92	22.71
5.	Y07836	Basic helix-loop-helix domain containing class B2	20.74
6.	M58566	Zinc finger protein 36. C3H type-like 1	18.29
7.	U58533	Est2 repressor factor	15.33
8.	L36435	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	12.91
9.	U69270	LIM domain binding	9.49
10.	U53925	Host cell factor C1	7.43
11.	U06924	Signal transducer and activator of transcription 1 (STAT1)	6.91
12.	M31042	Immediate early response 2	6.88
13.	M21065	Interferon regulatory factor 1	6.79
14.	X80339	Sine oculis-related homeobox 1 homolog (Drosophila)	6.42
15.	U36340	Kruppel-like factor 3 (basic)	6.32
16.	X61754	Heat shock factor 2	6.06
17.	U19119	Interferon inducible protein 1	5.94
18.	J03168	Interferon regulatory factor 2	5.80
19.	M20157	Early growth response 1	5.66
20.	L13204	Forkhead box J1	5.13
21.	U66918	Short stature homeobox 2	5.04
22.	X86925	E2F transcription factor 5	4.96
23.	D26046	AT motif binding factor 1	4.69
24.	X63507	Homeo box C10	4.64
25.	U73037	Interferon regulatory factor 7	4.46
26.	L35949	Forkhead box F1a	4.18
27.	AF013282	T-box 14	4.15
28.	X61753	Heat shock factor 1	3.94
29.	U09419	Nuclear receptor subfamily 1 group H member 2	3.80
30.	U52951	Enhancer of zeste homolog 2 (*Drosophila*)	3.74
31.	S74227	TEA domain family member 1	3.57
32.	U89876	RNA and export factor binding protein 1	3.43
33.	D13801	POU domain, class 6, transcription factor 1	3.26
34.	X73360	Transducin-like enhancer of split 3, Homolog of *Drosophila* E(spl)	3.26
35.	U41671	Zinc finger proliferation 1	3.13
36.	U47008	Ngfi-A binding protein 1	2.83

(A) Growth factors, cytokines, oncogenes and tumor suppressors

1.	L34169	Thrombopoietin	48.91
2.	U27177	Retinoblastoma-like 1 (p107)	22.99
3.	X15842	Reticuloendotheliosis oncogene	15.98
4.	X81580	Insulin-like growth factor binding protein 2	15.97
5.	M64292	Growth factor - B-cell translocation gene 2 anti-proliferative	13.81
6.	X56135	Prothymosin α	9.35

Table I. Continued.

(A) Growth factors, cytokines, oncogenes and tumor suppressors

7.	M86671	Interleukin 12b	8.03
8.	X04367	Platelet derived growth factor receptor, ß polypeptide	6.94
9.	X57413	Transforming growth factor ß 2 precursor (TGF-beta 2; TGFB2)	5.95
10.	Z11886	Notch gene homolog 1 (*Drosophila*)	5.30
11.	X82327	Myeloblastosis oncogene-like 1	4.39
12.	U66202	Fibroblast growth factor 13	4.18
13.	X05010	Colony stimulating factor 1 (macrophage)	4.17
14.	U37522	Tumor necrosis factor (ligand) superfamily member 10	3.86
15.	X13945	Lung carcinoma myc related oncogene 1	3.84
16.	AF039661	TGFß receptor type III (betaglycan)	3.77
17.	X70514	Nodal	3.69
18.	U21011	mutS homolog 2 (*E. coli*)	3.23
19.	D25540	Transforming growth factor ß receptor 1 (TGFß receptor 1; TGFR1)	3.10
20.	X77113	Growth differentiation factor 9	2.84

(A) Adhesion, motility and cytoskeleton proteins

1.	D12645	Kinesin family member 3A	18.06
2.	X06115	Cadherin 1	14.75
3.	S69407	Endoglin	11.99
4.	X12875	L1 cell adhesion molecule	8.25
5.	M28698	Keratin complex 1, acidic, gene 19	7.34
6.	M87276	Thrombospondin 1	7.04
7.	M63801	Gap junction membrane channel protein α1	6.60
8.	L06039	Platelet/endothelial cell adhesion molecule (PECAM1). CD31 antigen	4.63
9.	U44955	Gap junction membrane channel protein α3	4.65
10.	M34510	CD14 antigen	4.61
11.	D14340	Tight junction protein 1	3.98
12.	Z22532	Syndecan 1	3.74
13.	U52826	Syndecan 3	3.03
14.	X75427	Integrin α2	2.87

(A) Cell proliferation and cell death regulators

1.	AA289122	CDC28 protein kinase regulatory sub 2	27.78
2.	U09507	Cyclin-dependent kinase inhibitor 1A (P21)	8.41
3.	Z37110	Cyclin G1	7.49
4.	M22115	Homeo box A1	6.46
5.	Z26580	Cyclin A2	4.55
6.	L08235	Clusterin	3.90

(A) Invasion and angiogenesis

1.	AF014941	Cathepsin W	7.49
2.	X12822	Granzyme C	5.63
3.	M84324	72-kDa type IV collagenase type; 72-kDa gelatinase; gelatinase A; matrix metalloproteinase 2	4.23
4.	M75716	α1 protease inhibitor 2	2.75
5.	X02389	Urokinase type plasminogen activator	2.71

Continued.

(A) Other			
1.	J03752	Microsomal glutathione S-transferase 1	6.89
2.	AB001607	Prostaglandin I2 (prostacyclin) synthase	5.33
3.	U34920	ATP-binding cassette, sub-family G (WHITE), member 1	4.83
4.	M14757	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	4.31
5.	U20372	Calcium channel, voltage-dependent, ß3 sub	3.77
(B) Adhesion, motility and cytoskeleton proteins			
1.	X82402	Fibronectin 1	19.03
(B) Growth factors, cytokines, oncogenes and tumor suppressors			
1.	X83974	RNA polymerase I transcription termination factor 1 (TTF1)	5.65

Data are the average (mean) of two independent experiments between primary cultures of the highly metastatic MM3 mammary adenocarcinoma compared to the parental low metastatic M3 cells (MM3/M3). Values were corrected with the expression of two house-keeping genes (PPA2 and ACTIN) already included in the arrays. Only ratios >2.7- or <0.33-fold were considered significant.

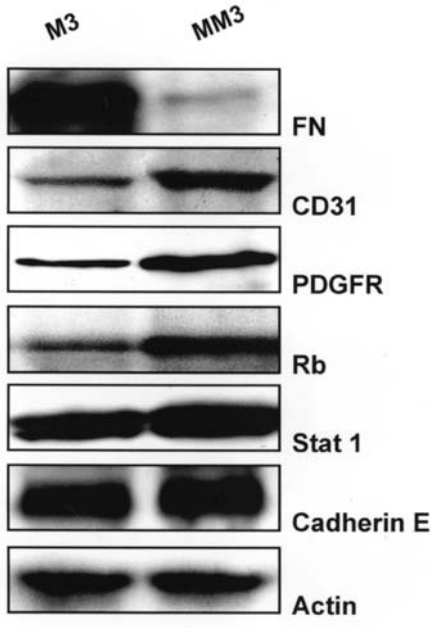

Figure 3. Validation of cDNA microarray results by Western blot analysis. The expression of 5 upregulated genes and the downregulated FN gene were selected to confirm Atlas 1.2 mouse cDNA expression array results. Actin expression was used as protein loading control.

culture lysates. As shown in Fig. 3 a very good correlation between both methodologies was observed, thus validating the cDNA microarray results.

Discussion

Tumor metastasis is a complex and dynamic process that is expected to involve alterations in many genes and transcriptional programs. However, it is unknown how and when metastasis genes are activated along the process of tumorigenesis and whether specific metastasis genes might have a function in primary tumor growth (4). The cDNA microarray methodology provides an interesting approach to gain insight into the molecular events that underlie metastasis dissemination of mammary cancer cells, as it allows analyzing as a conjunct a set of differentially expressed genes between primary tumors with distinct metastatic ability.

To avoid undesired contamination with non-parenchymatous cells, the cDNA microarray procedure was carried out in primary cultures of M3 and MM3 adenocarcinomas of almost only tumor epithelial cells (13). Before performing the molecular analysis we confirmed that primary cultures maintained the same spontaneous metastatic ability to the lungs as shown by the uncultured cells (9).

We identified a set of 88 genes, out of the 1,176 genes analyzed, that were differentially expressed in a significant and consistent way in the more metastatic MM3 mammary adenocarcinoma cells compared to the parental less metastatic M3 ones. These genes encode extracellular products including growth and survival factors and their receptors (TGFß2, FGF, PDGFR-B), chemokines (interleukin 12b), extracellular matrix components (FN) and proteases (uPA, MMP-2). They also include intracellular enzymes (prostaglandin 12 synthase) as well as transcriptional and signaling regulators (STAT1, E2F5, Zn finger proteins 92 and 36).

Some of these genes are known to be associated with proliferation, malignant transformation and metastasis in breast and other tissues, whereas, almost no studies have been reported, for addressing related functions for Zfp 92, Zfp 3611, Bhlhb2, Hcf1 and Ldb1 genes. For validation purposes we have chosen five genes upregulated in MM3 cells which potential relationship with tumor progression and the metastatic phenotype will be detailed below.

Relating to our observation that PDGFR-B gene expression was upregulated in MM3 cells, other authors have reported that the overexpression and/or activation of both PDGF receptors A and B are associated with migration and dissemination of malignant cells of different tissue origin (18,19).

STAT1 is a member of the family of signal transducers and activators of transcription that plays central roles in the responses of cells to cytokines and cell cycle regulation. Specifically STAT1 and STAT2 are intimately involved in the response of cells to interferons (20). Contradictory findings regarding its expression and/or activation status in tumors have been reported, and furthermore, whether STAT1 affects tumor growth and metastasis is still unclear (21,22). We found that STAT1 is about 6-fold upregulated in the more metastatic MM3 adenocarcinoma. This difference may be associated with the fact that several cycle regulators, such as cyclins G and A and CDKN1A (encoding p21), as well as a group of gene expression regulators involved in the response to interferon, namely interferon regulatory factors 1, 2 and 7

and interferon inducible protein 1, were also strongly upregulated. Although retinoblastoma protein expression (Rb105) has been inversely correlated with proliferation rate and metastatic potential in breast adenocarcinomas, this correlation has not always been observed either for the Rb105 or for the related proteins p107 and p130 in different tumors (23,24). In the present study we found a very strong upregulation of the retinoblastoma-like 1 (p107) gene in the more metastatic MM3 tumor, a finding that deserves further investigation.

The E-cadherin/ß-catenin cell adhesion system is often downregulated in epithelial tumors. This is thought to play an important role in cancer invasion and metastasis (25). Surprisingly, the cDNA microarray analysis revealed the E-cadherin expression was upregulated about 15-fold in the metastastic MM3 adenocarcinoma. Related to our finding, other authors have found a correlation between aberrant expression of E-cadherin or ß-catenin with lymph node metastasis, survival rate and survival length in breast cancer (26). To further support the notion that in some cases an aberrantly enhanced expression of this cell adhesion molecule may occur in cancer, Cavallaro and Christofori (27) have reported that metastatic prostate cancer shows strong E-cadherin expression while a transient downregulation of this molecule may occur in localized prostate cancer.

The platelet/endothelial cell adhesion molecule (CD31/PECAM1), expressed at the plasma membrane of platelets and endothelial cells, has been widely used as a marker of tumor angiogenesis and poor prognosis in cancer (28). Curiously, CD31 has also been detected in human, mouse and rat solid tumor lines (29). We found that MM3 cells overexpressed this endothelial cell marker about 5-fold relative to M3 parental tumor cells. In agreement with our finding, Righi *et al* (30) have recently reported that a subset of breast ductal *in situ* carcinomas express CD31, and demonstrated that CD31 overexpression reverted the undifferentiated morphology and aggressive behavior of MDA-MB-231 cells, indicating its active role in the morphogenesis of breast ductal *in situ* carcinomas. We still do not know the significance of CD31 expression in our murine mammary cancer model.

Previously known differences in expression patterns between the parental M3 and its highly metastatic variant MM3 were confirmed by the array data. We had previously found, in coincidence with the present results, that MM3 tumor secretes about 3- to 4-fold more uPA than M3 (14). Furthermore, the critical role of uPA in the invasive phenotype of this mammary tumor model was also demonstrated *in vivo* (31). The present analysis revealed that several members of the TGFßs/TßRs system [TGFß2, TGFß receptor 1 (TßR-1), TGFß receptor type III] were coordinately upregulated in MM3 adenocarcinoma. These results are validated by our previous studies reporting that MM3 showed a higher expression at protein level of the different isoforms of TGFß and its specific receptors, as compared with the parental M3 tumor, suggesting that the TGFß effects on cell proliferation and invasive capability would be favoring tumor progression (15).

While most of the differentially expressed genes were upregulated, only two genes showed a significant downregulation in the highly metastatic adenocarcinoma MM3. The expression of TTF-1, an RNA polymerase I transcription terminator factor, was reduced about 6-fold, and this fact could be impairing transcriptional regulation of ribosomal RNAs. Little is known about TTF-1 expression in cancer. Monitto *et al* (32) have found, in a murine adenocarcinoma model, that TTF-1 gene expression is decreased in a cell line that induces cachexia in NMRI nude mice, as compared with histologically similar cells that do not induce this paraneoplasic syndrome.

On the contrary, downregulation of FN is a well-known phenomenon associated with cancer (16,33). Our cDNA microarray analysis is distinctly and considerably (about 19-fold) downregulated in MM3 cells among 1,176 genes. Fibronectin is a plasma and extra-cellular matrix glycoprotein that plays key roles in the adhesive and migratory behavior of cells, being involved in processes such as embryonic development, hemostasis and wound healing as well as oncogenic transformation and metastasis (34,35). It has been demonstrated that some neoplastic cells synthesize FN at a lower rate than normal ones, or do not synthesize it at all, and are unable to deploy FN for an efficient adhesion or cannot retain FN on their surfaces (16,33). Conversely, reappearance of FN expression elicited by stable FN cDNA transfection or signaling activation has been shown to revert tumorigenic phenotypes (11,36). Furthermore, there is strong evidence indicating that the metastatic potential is inversely correlated with FN expression (37,38).

In previous work we found that the highly metastatic MM3 tumor variant did not express FN either at mRNA or at protein levels while the poorly metastatic parental tumor M3 expressed and deployed a FN network in the extracellular matrix. These results were confirmed by our present study, employing the cDNA microarray analysis and immunoblotting and immunocytochemistry validation. The relationship between FN loss and metastasis was further supported by our finding (17) that a continuous cell line derived from MM3 primary cultures (termed LMM3) (10), permanently transfected with constructs expressing full length FN cDNA under the control of a viral promoter, exhibited reduced spontaneous and experimental metastatic capacity, without modulation of the primary tumor growth. This less aggressive behavior was accompanied with an *in vitro* reduced migratory ability (17) and the deregulation of the uPA/uPAR system (39). In other work we have explored the molecular bases of the downregulation of FN expression in the highly metastatic MM3 cells. Using the very sensitive RT-PCR procedure we estimated that the levels of FN mRNA were about 40-fold lower in MM3 that in M3 cells and we attributed this difference to a reduced transcriptional activity involving the 220-bp proximal promoter region (11). The FN proximal promoter presents several SP1 sites (40). This could be related to the fact that the zinc finger protein 92 (Zfp 92), a transcription factor related to the SP1 family, was upregulated about 20-fold in MM3 cells compared to M3 ones (Table I). Since Zfp 92 may behave as a transcriptional repressor, its overexpression could cause the downregulation of the FN gene.

Contrary to our observations (37) it has been found that FN gene expression, as analyzed by cDNA microarrays, is downregulated in MDA-MB-435 human mammary carcinoma cell line stably transfected with the known metastasis suppressor gene NM23. In another epithelial tumor type, the

thyroid carcinoma, FN is markedly upregulated in on with normal thyroid tissue (41). On the other hand, and in agreement with us, Mackay *et al* (42) have also reported a significant downregulation of FN associated with ERBB2 overexpression, an established adverse prognostic factor in breast cancer, though in their model FN was one out of 25 significantly downregulated genes. Similar results were obtained analyzing the differential gene and protein expression in metastatic lymph nodes of primary breast cancers (43). Our present results, showing that the reduction of FN expression in the highly metastatic mammary tumor MM3 cells is a very rare event in an environment of up-regulated genes, places FN downregulation in a new context, and may help to better understand its role in tumor progression, supporting the hypothesis of FN as a main metastasis suppressor gene in mammary cancer.

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