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Modulation of Several Waves of Gene Expression During FGF-1 Induced Epithelial-Mesenchymal Transition of Carcinoma Cells

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Abstract During epithelial-mesenchymal transition (EMT), epithelial cells are converted into isolated motile and invasive mesenchymal cells. In model systems, EMT is induced most often by the activation of tyrosine kinase receptors through signaling pathways involving translational and post-translational regulation. In this study, we have used the NBT-II bladder carcinoma cell system to investigate in vitro Fibroblast Growth Factor-1 (FGF-1)-induced EMT. Transcriptome analyses were performed on NBT-II cells stimulated for 2, 6, 24, and 48 h with FGF-1. As some phenotypic changes occurred around 6 h post-stimulation, a supervised analysis was designed to identify transcript variations across defined time-periods. Our results clearly indicate that immediately after FGF-1 stimulation a set of genes assigned to transcriptional regulation (e.g., jun-B and v-ets) and to EMT induction (e.g., Notch 1) is transiently up-regulated. A set of genes involved in proteolytic systems (e.g., MMP-13 and uPAR) is immediately up-regulated but subsequently maintained throughout FGF-1 stimulation. Then follows a second wave of gene expression that includes a strong but transient up-regulation of ephrin B1 and arginase I. Finally, a third group of genes is stably modulated over 48 h which consists primarily of down-regulated genes specifically associated with the EMT-based loss of the epithelial phenotype and maintenance of the mesenchymal and invasive phenotype of carcinoma cells. Using genome-wide oligoarray technology, we have identified novel expressions of immediate, immediate-early and later EMT biomarkers that are specifically activated downstream of the FGF/FGFR pathway and which might be significant prognostic factors for tumor progression of carcinoma. J. Cell. Biochem. 104: 826–839, 2008.

Key words: EMT; carcinoma; FGF-1; arginase; MMP-13; oligoarray

Tumor invasion and carcinoma metastasis critically depend upon many cellular and molecular mechanisms such as proliferation, survival, adhesion and migration of malignant and stromal cells. Cell adhesion is regulated through the modulation of adhesion receptors and ligands during cell–cell and cell–matrix interactions.

Progression of carcinoma involves the disruption of junctional complexes, including tight junctions, adherens junctions and desmosomes. E-cadherin is a transmembrane protein that initially mediates adhesion between epithelial cells through transient intercellular contact with nectins [Gumbiner, 2000; Nakanishi and Takai, 2004]. These primary contacts mature rapidly into adherens junctions and contribute to the establishment of apico-basal polarity. β-catenin is a critical component of this maturation ensuring the connection to actin microfilaments absolutely required for the strengthening of adhesion over time [Chu et al., 2004].

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E-cadherin and β-catenin control a wide array of cellular behaviors, and alteration in their expression or mutation of the genes coding for them is correlated with a cellular dedifferentiation or the acquisition of an invasive cellular phenotype typically associated with increased oncogenicity [Nollet et al., 1999].

Epithelial-mesenchymal transition (EMT) is a process whereby epithelial cells loose polarity, cell–cell contacts and undergo a dramatic remodelling of the cytoskeleton. During EMT cells acquire a spindle-shaped, fibroblastic-like phenotype. Concurrent with a loss of epithelial cell adhesion and cytoskeleton components, cells undergoing EMT express mesenchymal components and acquire motile features. Investigations of EMT in tumor models have found that during the development of metastatic carcinomas, EMT, even partial and/or transient, is one of the initial and major events [Brabletz et al., 2005; Radisky, 2005; Christiansen and Rajasekaran, 2006]. The progression of carcinomas is complicated and multifaceted, and the identification of genes specifically involved in EMT can help in deciphering the initial, critical switch from benign to invasive carcinomas.

Several groups have developed microarray approaches to identify molecular players involved in EMT. In EpRas cells, a transformed mammary epithelial cell line, the use of an improved expression profiling method based on polysome-bound RNAs provided the opportunity to identify a small set of genes specifically regulated during TGF-β-induced EMT. Not surprisingly, these genes encode mesenchymal markers like vimentin and collagen type VI. Also identified were key regulators of cell proliferation, cell survival and invasiveness, all clearly relevant for tumor metastasis [Jechlinger et al., 2003]. Representational difference analysis (RDA) was applied to kidney epithelial cells immortalized with E1A. Those immortalized with wild type E1A retain their epithelial phenotype, whereas those lines immortalized with mutated E1A took on a fibroblastic-like phenotype. The 35 differentially expressed genes reported for the mutated E1A immortalized cells coded for growth factors, their cognate receptor tyrosine kinases, transcription factors and extracellular matrix proteins [Kiemer et al., 2001].

FGF/FGFR signaling regulates important intracellular signal-transduction pathways mediating cell proliferation, differentiation, survival and migration in a variety of cell types [Powers et al., 2000]. The multifunctional growth factors FGFs together with their tyrosine kinase receptors, may play a role in autocrine and paracrine growth control of malignant tumors. Their involvement in the pathogenesis of several human cancers has been reported, and enhanced FGF/FGFR signaling activity can be the result of either their overproduction or a constitutive FGFR activation [Ozen et al., 2001, 2004; Khnykin et al., 2006]. Rat bladder carcinoma NBT-II cells have previously been described to undergo EMT upon stimulation with different growth factors [Gavrilovic et al., 1990; Jouanneau et al., 1991; Bellusci et al., 1994; Billottet et al., 2004], and the mechanisms by which FGF-1 favors tumor progression of experimental carcinomas have been investigated [Billottet et al., 2002]. Furthermore, we have previously reported that FGF-1 signaling, through the FGFR, induces a series of cellular and molecular changes associated with EMT, thereby affecting the organization of several molecular complexes [Billottet et al., 2004].

NBT-II carcinoma cells are an established cell model used effectively to investigate the mechanisms of EMT induced by FGF-1 or other 'scattering factors' [Gavrilovic et al., 1990; Bellusci et al., 1994]. Specific targets of EMT are not well known, and the sequence of modulation over time of the identified genes resulting from FGFR activation in carcinoma cells is still uncertain. In an attempt to elucidate the molecular mechanisms responsible for EMT, we explored the global changes in gene expression profiles in epithelial NBT-II carcinoma cells stimulated over time by FGF-1. Comparative transcriptome analysis performed on epithelial carcinoma NBT-II cells stimulated over time with FGF-1 allowed the identification of immediate, immediate-early and late EMT gene targets.

MATERIALS AND METHODS

Cell Lines

NBT-II cells were derived from a chemically induced rat bladder carcinoma. These cells do not produce endogenous FGF-1 but express the FGFR2b high affinity FGF-1 receptor on their surface. Cells were cultured in Dulbecco's

Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium).

Reagents

FGF-1 was a gift from Dr. T. Maciag. Heparin was purchased from Choay. Monoclonal antibodies anti-arginase I (612621) and anti-β-actin (A5441) were purchased from BD Biosciences Pharmingen and Sigma, respectively. Polyclonal anti-ephrin B1 (AF473) was purchased from R&D Systems.

RT-PCR Amplifications and Real-Time PCR Amplifications

Total RNA was isolated from cell lines using the RNA PLUS™ extraction Kit (Bioprobes Systems). 1 μg of total RNA was reverse-transcribed into cDNA using the RNA PCR Kit (AMV-RT, Takara Biomedicals). The resulting cDNAs were amplified using specific primers for the genes of interest. Arginase I: Forward 5′-AAG GTC CCG CAG CAT TAA GG-3′; Backward: 5′-GGTCAGGGTGGACCCTGGCG-3′. MMP-13: Forward 5′-CTT GGC CAC TCC CTC GGT CT-3′; Backward 5′-CAG CAT CCA CAT GGT TGG GA-3′. Notch 1: Forward 5′-CAG CTC ATG CCC AAC GCC TGC-3′, Backward 5′-CAG TCC TCA CCA GTC CAG CCA-3′. We applied a real-time PCR method using the SYBR® Green PCR Master Mix as recommended by the manufacturer (PE Applied Biosystems, Inc., Foster City, CA). HPRT was used as an internal control, and the results were expressed in arbitrary units. HPRT: Forward 5′-GAC ACT GGA AAA CAA TGC AG-3′; Backward 5′-GGG TCC TTT TCA CCA GCA AG-3′. Negative controls lacking template RNA or reverse transcriptase were included in each experiment. The results presented are based on three independent experiments for Notch 1 and two independent experiments, each, for Arginase I and MMP-13.

Cell Lysis and Western Blot Analysis

Cell lysis and Western blot analysis were performed as previously described [Billottet et al., 2004].

Experimental Design of FGF Stimulation

NBT-II cells were trypsinized and 10 cm diameter culture dishes were seeded with 10^6 cells in complete medium. In all dishes, except for controls, recombinant FGF-1 was added at a concentration of 20 ng/ml together with 50 μg/ml heparin. Total RNA was prepared from control and stimulated cells at time 2, 6, 24, and 48 h for the Affymetrix procedure. The experiment was done in duplicate. In total, 10 RNAs were extracted.

Affymetrix GeneChip Hybridization

Hybridizations to the rat genome were performed as described in the Affymetrix Gene Chip® Expression Analysis Manual (Santa Clara, CA). All assays used the Rat Expression 230 A GeneChip® (RAE230 A), and the targets for the microarrays were prepared according to the manufacturer's suggested protocol. Ten Affymetrix GeneChip arrays were hybridized according to the above experimental design.

Statistical Analysis

Probes on the RAE230 A GeneChip® array code for 15,256 different sequences, most of them associated with a unique gene. Signal intensity for each probe set constituted the raw data (CEL files). CEL files were normalized and probe set information summarized using the Robust Multiarray Average (RMA) algorithm [Irizarry et al., 2003]. The normalized data have been deposited in the Gene Expression Omnibus (GEO) database, and the series record is GSE5664.

The expression of each gene represented in the RAE230 A GeneChip® array was analyzed for different time periods (TP) of the EMT experiment. Around 6 h, phenotypic changes of the EMT were clearly visible (data not shown). Considering that central time point, a supervised differential analysis was performed and each time period was chosen according to specific criteria. Additionally, the 2 h time point was chosen to identify immediate targets of the FGF-1-induced EMT, the 6 h time point to identify the earlier transient or maintained EMT targets, and the 24 and 48 h time points to identify the late transient or permanent EMT targets.

Results from six TP, each corresponding either to a punctual time or to an extended time frame, were compared to a reference TP (R). These six TP of interest are defined as follows. I: Immediate TP: 2 h; E: Early TP: 6 h; L: Late TP: 24 to 48 h; I & E: Immediate and Early TP: 2–6 h; E & L: Early and Late TP: 6–48 h; I & E & L: Immediate and Early and Late TP: 2–48 h

TABLE I. Definition of the Time Period of Interest

	Time				
Time period	0 min	2 h	6 h	24 h	48 h
I	R	TP	R	R	R
EP	R	R	TP	R	R
L	R	R	R	TP	TP
I & E	R	TP	TP	R	R
E & L	R	R	TP	TP	TP
I & E & L	R	TP	TP	TP	TP

Six Tp, corresponding to a unique time or a time group (Time Period Group or TP) were compared to another time or time group (Reference Group or R). I, Immediate TP, 2 h; EP, Early Punctual TP, 6 h; L, Late TP, 24–48 h; I & E, Immediate and Early TP, 2–6 h; E & L, Early and Late TP, 6–48 h I & E & L, Immediate and Early and Late TP, 2–48 h.

(Table I). For each TP and each probe set, the null hypothesis of no difference between the two groups (TP vs. R) was tested using the modified *t*-test as computed by significance analysis for microarray (SAM) Software [Tusher et al., 2001]. Analysis required the use of the same statistical *t*-test for each probe set. Accordingly, to avoid false positives, it was necessary to consider all pair-wise comparisons. To take into account the classical problems associated with such multiple comparisons, we selected subsets of modified genes based on the False Discovery Rate criterion [Benjamini and Hochberg, 1995]. The FDR was estimated using the SAM procedure for comparison of two classes of data. All analyses were performed using R software, version 2.4.1 (http://www.rproject.org), and Bioconductor libraries (http://www.bioconductor.org) [Gentleman et al., 2004].

Functional Annotation

In order to identify the molecular functions most represented in the identified gene lists, we used the integrative tool, DAVID, version 2.0 (http://apps1.niaid.nih.gov/david/) [Dennis et al., 2003], and more particularly, the web interface to the Gene Ontology Database (http://www.geneontology.org/). From the different possible groups of genes defined in Gene Ontology, we chose to analyze specifically the "cellular component" path, since EMT is dominated by strong morphological changes. Since the Gene Ontology database is organized using directed acyclic graph (DAG), we chose a medium precision level (level 3/5) of annotation, which is a good intermediate between specificity (the most detailed annotations are used in level 5, with the maximum number of categories) and coverage (representing the number of annotated genes) [Dennis et al., 2003]. We performed this analysis for two different groups of genes selected using SAM. The first group consisted of genes exhibiting a transient differential expression (corresponding to I, EP and I & E TP), and the second group consisted of genes selected for their maintained expression level across specific TP (L, E & L and I & E & L TP).

RESULTS

Modulation of the Cell Phenotype Over Time Periods Following FGF-1 Stimulation

Concomitant with internalization of their desmosomal components, NBT-II cells start to dissociate and move 6–7 h post-stimulation [Boyer et al., 1989]. The mesenchymal phenotype is fully achieved by 24–48 h, with the synthesis of the intermediate filament protein, vimentin, clearly visible after 2 or 3 days (Fig. 1). FGF-1 stimulated cells are motile in vitro at a rate of 30 μm/h, whereas unstimulated cells are essentially non-motile (rates less than 2 μm/h) [Jouanneau et al., 1991; Savagner et al., 1997].

Differential Microarray Analysis

Overall hybridization quality on RAE230A Genechip arrays was associated with high call rate values (from 58% to 62% of present calls detected depending on the arrays). Considering differential analysis, false discovery rate (FDR) estimated for sets of genes were low in all cases. Only 60% of genomic sequences represented on the RAE230A array are fully annotated. Among differentially expressed genes, 25% either showed poor homology to known proteins or corresponded to unknown transcribed sequences. These were not considered in the investigation. We decided to investigate annotated transcripts in the first 250th differentially expressed genes showing reasonnable FDR (lower than 15%) as a trade off between

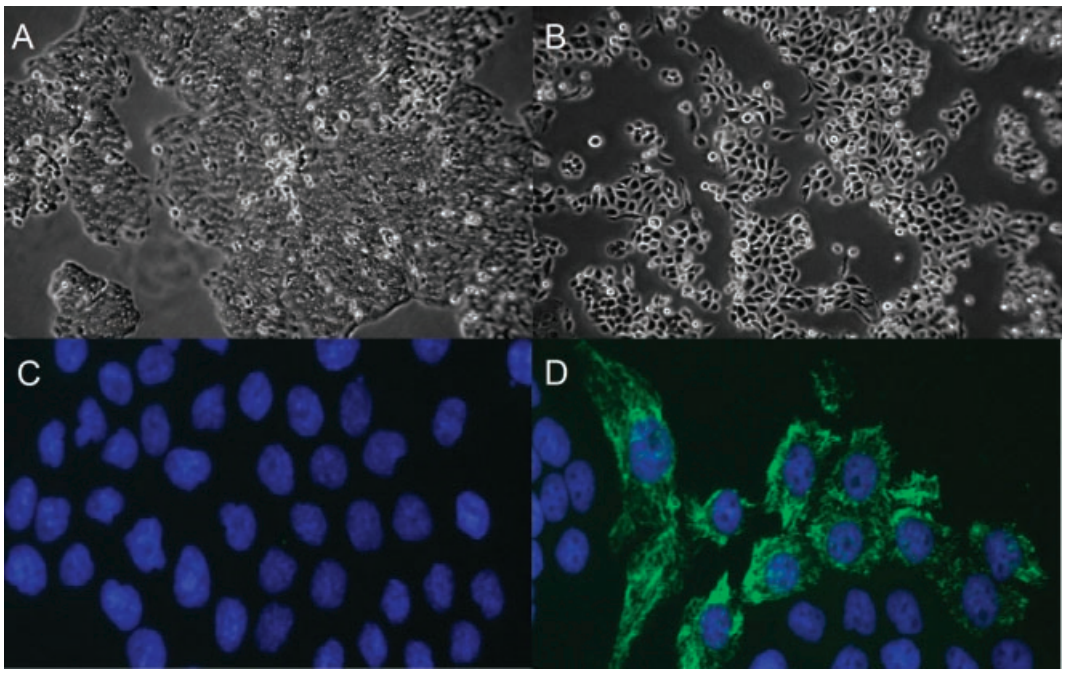

Fig. 1. FGF-1 induced EMT. NBT-II bladder carcinoma cells were either left unstimulated (**A**) or were stimulated (**B**) by FGF-1 (20 ng/ml) in the presence of heparin (50 μg/ml) during a 48 h period. Vimentin immunostaining (green) and nuclei stained with DAPI (blue) were performed on NBT-II cells in complete medium (**C**) or stimulated by FGF-1 (**D**), respectively. Photographs were taken at 400× magnification, using a fluorescence microscope coupled to a CCD camera.

stringency and sensitivity to new possibilities (supplementary online data). These genes were ranked according to their fold-change value in order to look for the most modified expressed sequences. This procedure allowed us to investigate subsets of genes with a high confidence in statistical significance. Genes previously known to be involved in EMT have been underlined in this study. Among the most modified genes, new transcripts of interest have been found and further investigated (Table II).

The Immediate Target Genes of FGF-1-Induced EMT

Upon FGF-1 stimulation of NBT-II cells, the expression of Immediate Target (IT, see Table I and supplemental Table I) genes was transient, dropping after 2 h back to the control level. Many IT genes coded either for transcription factors or for proteins otherwise involved in the regulation of transcription (Table II and Fig. 2A). For example, the proline rich two gene (or PNRC1) encodes a 35 kDa nuclear co-activator protein that modulates transcription [Zhou et al., 2000] and also binds Grb2, an adaptator of the Growth Factor/Ras pathway [Zhou et al., 2004]. PNRC1 undergoes a 3.3-fold increase in expression upon FGF-1 stimulation. Jun-B and Ets/Ets-related genes were likewise among the immediate targets of the FGF-1 signaling coding for transcription factors. After a 6 h stimulation, their fold change in expression dropped but was still significant (Table II and Fig. 2A). These results are consistent with our previous observations [Billottet et al., 2004].

Genes for several chemokines, as well as molecules otherwise involved in cell growth and signaling regulation, were also immediately and transiently up-regulated. One relevant IT gene, identified with two different probe sets, was that for the membrane bound Notch 1 (Table II and Fig. 2B). The activation of Notch signalling is of interest during EMT, as there is increasing evidence for a functional role of Notch signalling in mediating EMT during tumor metastasis and angiogenesis [Noseda et al., 2004; Timmerman et al., 2004]. Q-PCR showed that while Notch 1 is expressed at a basal level in the non-stimulated cells, it validated an immediate upregulation at 0.5 and 2 h (Fig. 3A). Another gene that was actively and transiently up regulated is that for the PTP non-receptor type 16, also named DUSP1 (dual specificity phosphatase-1) (Table II). This

TABLE II. Listing of New and Known Target Genes Involved in FGF-1-Induced EMT of NBT-II Cells

	Gene name	Probeset ID	Score(d)	Fold change	Q-value (%)	Expression profile
IT	Proline rich 2	1370381_at	8.0	3.3	0.0	
	Notch 1	1390426_at	5.5	3.4	0.8	
	Notch 1	1371491_at	5.7	4.1	0.8	
	Jun-B oncogene	1387788_at	4.8	2.8	1.5	
	v-ets oncogene	1372564_at	4.7	1.9	1.5	
	E74-like factor 1	1374137_at	5.0	1.8	1.5	
	Protein tyrosine phosphatase, non-receptor type 16	1368147_at	3.7	1.8	6.5	
EPT	Protein tyrosine phosphatase, receptor type, epsilon	1371115_at	5.7	2.3	2.0	
LT	Vimentin	1367574_at	3.1	3.3	5.3	
	Cyclin D1	1371150_at	10.7	1.9	0.0	
	Cyclin D1	1371643_at	5.8	1.8	0.0	
	Cyclin D1	1383075_at	3.6	1.7	3.2	
	Serine protease	1367478_at	5.2	1.3	0.0	
	Dynein light chain-2	1372612_at	4.4	1.5	1.5	
	Dynein light chain-2	1387782_at	3.3	1.6	3.7	
	Kinesin family member 3C	1369637_at	3.3	1.7	4.5	
	Kinesin family member 3C	1387657_at	4.5	1.6	1.5	
	FGFR2	1373829_at	−7.9	0.5	0.0	
	Junction plakoglobin	1387061_at	−4.1	0.6	1.9	
	PAI-2 type A	1368487_at	−3.6	0.5	3.4	
	LAR-PTP	368035_a_at	−3.3	0.6	6.1	
	Beta-catenin	1373067_at	−3.3	0.8	5.3	
	Keratin 5	1370863_at	−10.8	0.4	0.0	
	Cytokeratin 13	1388329_at	−4.6	0.4	1.3	
	Cytokeratin 15	1372153_at	−3.6	0.2	3.4	
	Keratin complex 1	1371895_at	−11.4	0.4	0.0	
	Type II keratin Kb1	1370868_at	−5.9	0.5	0.0	
	Keratin complex 2	1371530_at	−3.4	0.5	4.5	
IET	Arginase I	1368266_at	11.4	10.5	0.0	
	Diphtheria toxin receptor	1368983_at	6.5	6.2	0.0	
	Ephrin B1	1374403_at	10.6	1.9	0.0	
	Ephrin B1	1369476_at	5.1	2.0	0.0	
	uPA	1398287_at	10.2	5.9	0.0	
	UPA	1387675_at	9.1	6.4	0.0	
	Matrix metalloproteinase 14	367860_a_at	7.0	2.7	0.0	
	Matrix metalloproteinase 9	1398275_at	4.7	2.1	0.3	
	TIMP-1	1367712_at	6.7	1.9	0.0	
	Cathepsin C	1368280_at	5.2	2.3	0.0	
	Cathepsin C	1374778_at	4.7	2.6	0.3	
	Laminin 5 alpha 3	1370538_at	5.8	3.3	0.0	
	Fibronectin 1	1370234_at	4.8	2.0	0.3	
	Syndecan 1	1376062_at	18.0	2.4	0.0	
	Syndecan 1	1367849_at	9.6	2.3	0.0	
	Syndecan 4	1367721_at	4.8	1.7	0.0	
	CD44 antigen	1390659_at	4.7	1.8	0.3	
	v-rel oncogene	1372853_at	5.6	1.7	0.0	
	v-ets oncogene	1368851_at	5.6	1.9	0.0	
	v-ral oncogene	1387001_at	4.6	1.5	0.3	
ELT	Nidogen 2	1388618_at	5.7	2.2	0.0	
	Kinesin family member 3C	1369637_at	6.7	1.9	0.0	
	Kinesin family member 3C	1387657_at	4.5	1.7	2.3	
	ARP2/3 protein complex	1371511_at	3.9	1.2	0.0	
	Actin related 2/3 complex, subunit 1B	1386925_at	3.4	1.5	0.0	
IELT	Matrix metalloproteinase 13	1388204_at	8.0	21.7	0.0	
	uPAR	387269_s_at	4.5	6.2	11.2	

IT, Immediate Target, 2 h; EP, Early Punctual Target, 6 h; L, Late Target, 24–48 h; I & E, Immediate and Early Target, 2–6 h; E & L, Early and Late Target, 6–48 h I & E & L, Immediate and Early and Late Target, 2–48 h. The six waves of genes regulated during the FGF-1-induced EMT of NBT-II cells were identified using SAM software. The score (d) corresponds to the SAM modified T statistics. Q-value represents the lowest false positive rate for which the gene is called significant. Last column represents colored expression profiles for each transcript of interest, at the five time points (0, 2, 6, 24, and 48 h) (as described in Fig. 2). Intensity values were averaged for the time replicates in this graphical view. Those values were considered independently in the SAM analysis. The more expressed the transcript is, the darker the color (dark green).

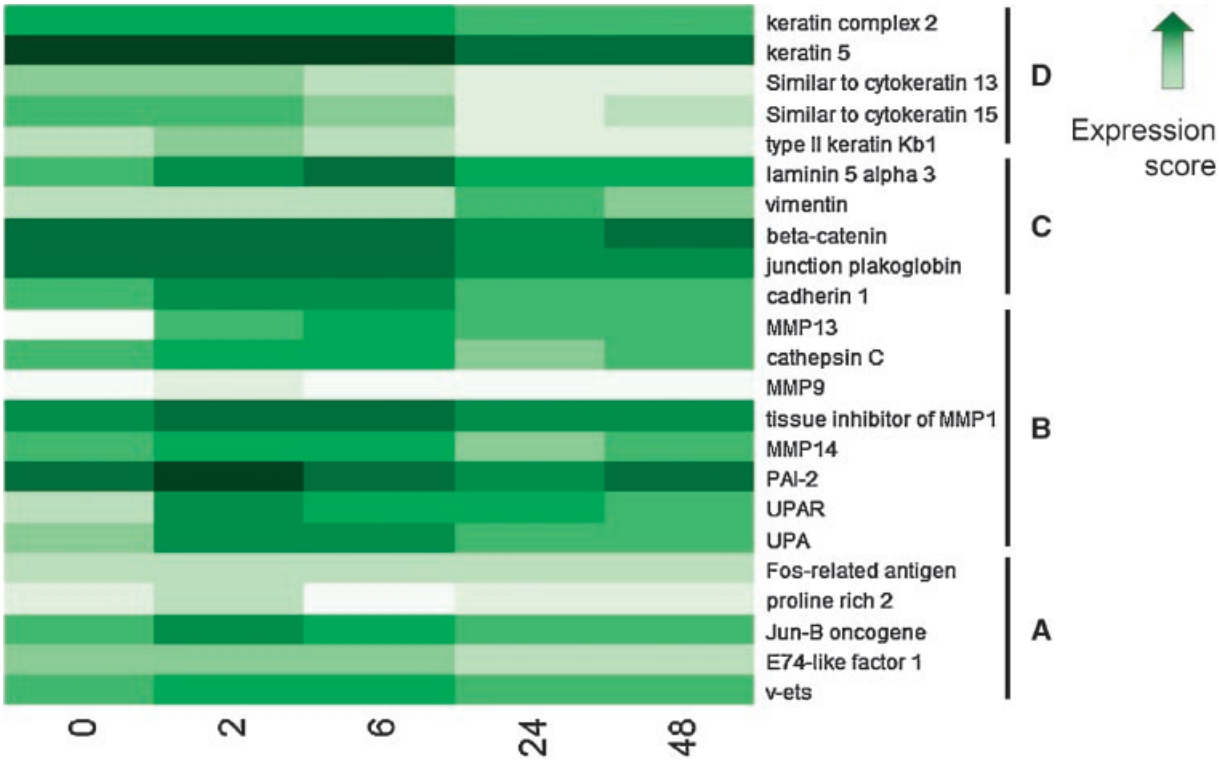

Fig. 2. Expression profiles of different groups of genes during FGF-1 induced EMT. Four groups of genes with different biological activities such as genes involved in transcription (group **A**), genes of proteases immediately and permanently over expressed (group **B**), genes associated with cell–cell contacts (group **C**) and keratin and cytokeratin-related genes (group **D**) have been represented. Log intensity of gene expression level (*y* axis) and time of stimulation by FGF-1 in hours (*x* axis). Gene expression levels are represented with a bar at time 0, 2, 6, 24, and 48 h. The expression score used here is calculated based on RMA preprocessing algorithm, and is averaged between the two replicates for each time point. The more expressed the transcript is, the darker the color (dark green).

enzyme has been shown to dephosphorylate activated Erk2 MAP kinase and could affect the MAPK pathway.

The Immediate Early Target Genes of FGF-1-Induced EMT

Upon FGF-1 activation some genes were up-regulated over a longer period of time (2–6 h) before their basal level of expression was restored. These are considered the Immediate&Early Target genes (IET, see Table I and supplemental Table II). In this category are a few genes that are not involved in the regulation of the transcription or in the cell cycle but which have highly up-regulated transcription levels (Table II).

For example, Arginase I, with a fold change of 10.5, is an enzyme that catalyses the hydrolysis of arginine to ornithine in arginine metabolism and thus regulates nitric oxyde (NO) production. L-Arginine is the unique substrate for nitric oxyde synthase (NOS) leading to the production of NO; as a result arginase and NOS compete for the same substrate. The

Fig. 3. Immediate and early induction of different genes during FGF-1-induced EMT. Immediate induction after 0.5 h of the Notch 1 gene transcript (**A**) validated by Q-PCR. Results represent the mean of three different experiments with the standard error of the mean. Early induction after 2 h of arginase I gene (**B**) and MMP-13 gene (**C**) transcripts validated by Q-PCR. TP in X coordinate (hours) and relative expression in Y coordinate (arbitrary unit, a.u.). Results are in arbitrary units relative to endogenous HPRT. Protein validation for arginase I and ephrin B1 by Western blot (**D**) on total cell lysates from NBT-II cells stimulated by FGF-1 from 0.5 to 6 h (H for hours). CL for control liver for the arginase I Western blot corresponds to a total cell lysate from a whole extract of mouse liver tissue. CB for control brain for the ephrin B1 Western blot corresponds to a total cell lysate from a whole extract of mouse brain tissue. β-Actin was used as a loading control.

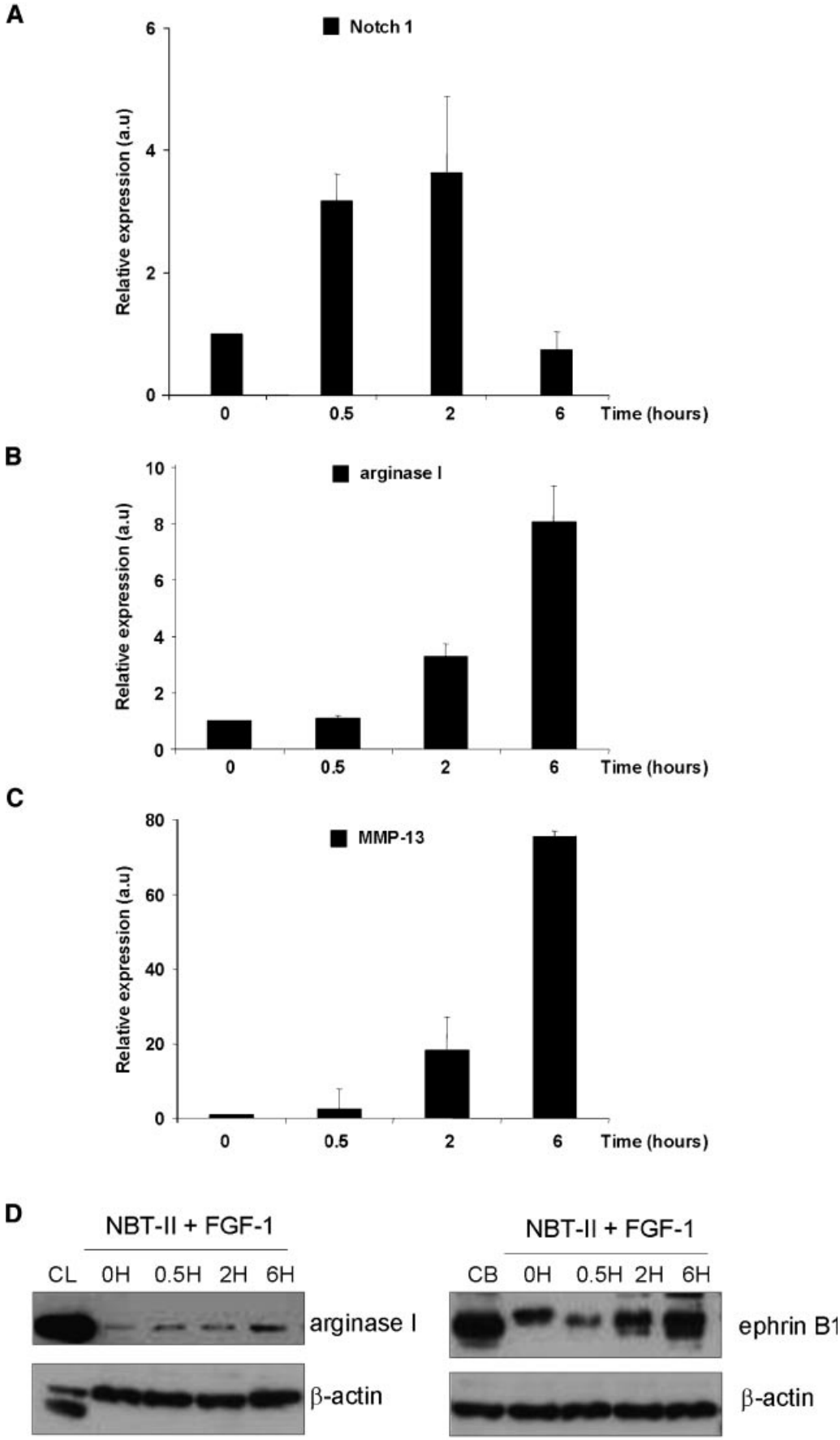
A
Notch 1
Relative expression (a.u)
6
4
2
0
0
0.5
2
6
Time (hours)
B
arginase I
Relative expression (a.u)
10
8
6
4
2
0
0
0.5
2
6
Time (hours)
C
MMP-13
Relative expression (a.u)
80
60
40
20
0
0
0.5
2
6
Time (hours)
D
NBT-II + FGF-1
CL
0H
0.5H
2H
6H
arginase I
β-actin
NBT-II + FGF-1
CB
0H
0.5H
2H
6H
ephrin B1
β-actin

Fig. 3.

upregulated arginase expression was confirmed by Q-PCR and Western blotting (Fig. 3B,D).

One relevant IET gene, identified with two different probe sets, was ephrin B1. A functional and physical interaction between FGFR and the ephrin B family of cell surface ligands has been shown [Chong et al., 2000], and there is increasing evidence that ephrin ligands and their receptor, Eph, contribute to oncogenesis by mediating cellular migration, dissemination and angiogenesis [Castellvi et al., 2006]. Our Western blotting analyses in NBT-II cells corroborate the immediate-early upregulation of Ephrin B1 (Fig. 3D). Another IET gene is Urokinase (uPA). Together with its receptor, uPAR, an immediate&early&late target gene (IELT), and its inhibitor, PAI, a late target (LT) (Table II and Fig. 2B) it constitutes a proteolytic system already shown to be an early target of the FGF-1 stimulation of the NBT-II carcinoma cells [Billottet et al., 2004]. Expression of these genes together with other proteases (i.e., MMPs) plays a major role in tumor invasion and metastasis. During EMT, uPA, cathepsin C (dipeptidyl peptidase 1), MMP-9 (a gelatinase), MMP-14 (a GPI anchored MMP) and TIMP-1 (an inhibitor of MMPs) were rapidly induced in our EMT model (Table II and Fig. 2B).

HB-EGF like, also named diphteria toxin receptor, undergoes a 6.1-fold up regulation in expression following FGF-1. It is involved with a MAPK signaling cascade and its elevated expression has been shown to be a response to epithelial tissue repair after injury [Ellis et al., 2001]. The gene for Growth Factor Binding Protein undergoes a 3.7-fold increase in expression. The corresponding protein is secreted and is reported to positively modulate FGF biological activities [Tassi et al., 2001].

Transcription factors such as v-rel, v-ets, v-ral were with fold change from 1.5 to 1.8. Genes corresponding to matrix proteins (such as laminin, fibronectin, syndecan 1, syndecan 4 and CD44 antigen), or to proteins that interact with the extracellular matrix and/or proteins (such as MMPs and osteopontin), also belong to this group of maintained targets. Such proteins are critical in remodeling the tumor microenvironment during invasion.

The Immediate Early and Late Maintained Target Genes of FGF-1-Induced EMT

Genes that maintained their altered level of expression over the entire period of FGF/FGFR signaling were described as Immediate&Early &Late maintained Target genes (IELT, see Table I and supplemental Table III). These genes are modulated immediately (either up- or down) upon FGF-1 stimulation, and are necessary for the maintenance of the mesenchymal phenotype. In this group were genes of several different proteolytic systems. For example, uPAR showed a 6.2-fold change in its expression. Even more dramatically, MMP-13 showed a 21.6-fold increase upon FGF-1 stimulation (Table II and Fig. 2B). Its sustained expression is critical for the maintenance of the invasive phenotype. As mentioned above, uPAR has already been reported to be modulated by FGF-1 in NBT-II cells [Billottet et al., 2004], and here the increased expression of the target MMP-13 was confrimed by Q-PCR (Fig. 3C).

The Early Punctual Target Genes of the FGF-1-Induced EMT

A few of the known targets (30% of the listed genes) were Early Punctual Target genes (EPT, see Table I and supplemental Table IV). These are specifically modulated around 6 h post-stimulation, the time period associated with appearance of phenotypical EMT characteristics. Among these, the receptor type protein tyrosine phosphatase ε (PTPrε) displayed a 2.3-fold increase (Table II). PTPrε is a physiological inhibitor of the ERK signaling [Toledano-Katchalski et al., 2003]. Its activity could be associated with the IT gene DUSP1 activity involved in the MAPK pathway.

The Early&Late Maintained Target Genes of the FGF-1-Induced EMT

Another series of genes which were activated at 6 h and whose expression was sustained for the duration of the FGF-1 stimulation of NBT-II carcinoma cells were designated Early&Late maintained Target genes (ELT, see Table I and supplemental Table V). Nidogen-2 protein had a 2.3-fold change in FGF-1 stimulated cells (Table II). Nidogen-2 may compensate for some but not all functional activities already ascribed to nidogen-1 [Kohfeldt et al., 1998] (Fig. 2B). Late activation of this gene corresponds to the remodeling of the matrix environment concomitantly with EMT. Kinesin and Arp2/3 complex genes (each with two probe sets smaller than 2) also belong to this group and play a major role in the regulation of the actin cytoskeleton.

The Late Target Genes of the FGF-1-Induced EMT

A further subset of genes that were activated even later (24–48 h) and whose expression was sustained over the entire FGF-1 stimulation were designated LT genes (see Table I and supplemental Table VI). These include genes affecting an array of cellular functions such as the metabolism of cholesterol, lipids, and diverse glycoproteins.

Of more direct interest here are genes associated with the EMT. Prominent among them is the gene for vimentin, a specific mesenchymal marker protein, which had a 3.3-fold increase upon FGF-1 stimulation (Table II and Fig. 2C). However, there are proteins potentially important for maintenance of the mesenchymal phenotype following EMT that undergo less dramatic activation (around twofold change). These include cyclin D1 (three probe sets with 1.9-, 1.8- and 1.6-fold changes), dynein (two probe sets with 1.6- and 1.5-fold change), and kinesin family member 3C (two probe sets with both 1.6-fold change).

At the same time, several proteins characteristic of the epithelial phenotype are clearly down regulated upon FGF-1 stimulation. For example, the protein tyrosine phosphatase receptor type F, or LAR-PTP, was down regulated after 6 h of stimulation (Table II). This phosphatase is known to be a FGF-1 target and its down regulation is correlated with the switch towards a mesenchymal phenotype [Billottet et al., 2004]. Similarly, a group of keratin genes encoding the cytokeratins and related protein (CK) (keratin 5, Keratin complex 1 and 2, CK-13 and -15) were significantly down-regulated (0.2–0.5-fold change) (Table II and Fig. 2D). The result is that these keratins, which are virtually signature for the epithelial-tissue type [Chu and Weiss, 2002], are partly lost after FGF-1 induced EMT. Correlatively, other epithelial markers involved in cell–cell junctions were lost after EMT. These include plakoglobin (a 0.6-fold change) and beta-catenin (a 0.8-fold change) (Table II and Fig. 2C).

Considering the modulation of genes typically involved in the maintaining of the phenotype of the cells, especially the genes involved in the cytoskeleton such as microfilaments, intermediate filaments and microtubules, the loss of epithelial cytokeratins is a key signature of the EMT.

Functional Evaluation of the FGF-1-Induced EMT Expression Profiles

Another way to interpret molecular modifications associated with EMT is the functional approach. Using the Gene Ontology database [Ashburner et al., 2000], we identified the most represented family of 'cellular components' for two different groups of genes. Taking into account the differential analysis results, we chose to consider gene targets belonging to the following two groups: Transient (IT + IET + EPT SAM results) and Maintained (IELT + ELT + LT SAM results).

As many ESTs were represented on the array, the functional annotation was clearly incomplete for each gene list. We chose the 'medium precision level' (level 3) and identified the cellular components under those conditions. The only cellular component highlighted by the transient targets, primarily transcription factors and DNA binding proteins, was the nucleus. being. Among the maintained group, two sets of annotated genes could be identified. The first set, up-regulated, corresponded to proteins of the cytoskeleton. The other set, down-regulated, corresponded to proteins of the cytoskeleton, including intermediate filaments, as well as proteins located in the intracellular and extracellular spaces.

DISCUSSION

In this study, gene expression profiles obtained from different TP during the FGF-1 induced carcinoma cell EMT were analyzed. Different sets of genes were characterized as either immediately up regulated (2 h following stimulation), immediately early regulated (2–6 h) or regulated later (24–48 h). All the different comparisons had a low FDR, supporting the reliability of the results. Moreover, some of the genes modulated during EMT (i.e., for IT: Notch 1, cyclin D1, uPA, Ephrin B1) were revealed with more than one probe set, and outcome further underscoring the reliability of the data. This redundancy can be interpretated as an internal biological control, since different genomic sequences representing the same transcript are differentially expressed during different TP.

For example, transcriptional factors such as AP1 Fos/Jun complexes and Ets/Ets-related factors were immediately and transiently up-regulated. On the other hand, known early or LTs of these transcription factors (such as cyclin

D1, uPA, uPAR, MMP-1, MMP-3, MMP-9, MMP-14, and Osteopontin) were up-regulated later. Other functional targets (i.e., E-cadherin, β-catenin, LAR-PTP, uPAR, and FRS2) modulated in the NBT-II EMT model were already known to be valid FGF-1 targets [Billottet et al., 2004] The changes in expression of these various genes upon FGF-1 stimulation lead us to consider their physiological relevance during EMT.

The Notch pathway is central to many developmental processes [Politi et al., 2004]. Recently, it has been shown that the Notch pathway, can induce EMT during cardiac valve development by inducing Snail and repressing VE-cadherin transcription. Similarly, overexpression of active alleles of Notch in immortalized endothelial cells resulted in an EMT due to Snail activation and VE-cadherin repression [Timmerman et al., 2004]. Noseda et al. [2004] reported the importance of the activation of Notch signaling during the endothelial to mesenchymal transformation. Taken together, these data suggest that Notch may have a fundamental role in tumorigenesis, specifically in inducing tumor metastasis. Very early in EMT there was a modest but significant over expression of the Notch 1 gene seemingly linked to EMT.

Ephrin B1 is a GPI anchored ligand that interacts with the EphB receptors. A functional and physical interaction between FGFR and the ephrin B family of cell surface ligands has been shown and is implicated in regulating cell adhesion [Chong et al., 2000]. Ephrins and their Eph receptors have been implicated in many biological processes, such as cell migration during axonal guidance, vasculogenesis and tumor proliferation [Surawska et al., 2004]. The ligand ephrin B1 transduces 'outside-in' signals in the neural system and endothelial cells, and recently, strong evidence supports its involvment in the cell–cell adhesion of epithelial and cancer cells [Huynh-Do et al., 2002; Tanaka et al., 2005]. In a cDNA microarray study, Schaner et al. [2003] showed that ephrin B1 is highly expressed in ovarian carcinoma. Also, there is increasing evidence for a fundamental role for ephrin B1 during tumor metastasis, as Eph receptors regulate actin dynamics through small GTPases of the Rho family. These latter GTPases control cell shape and movement by promoting the formation of stress fibers, lamellipodia or filopodia [Nobes and Hall, 1999]. In the NBT-II EMT model system, Ephrin B1 was immediately up-regulated upon FGF-1 stimulation above the basal level found in unstimulated cells. Despite of suggestions that Eph receptors/Ephrin interactions may play a role in tumor proliferation, these data indicate that their predominant role is the regulation of cell shape and movement rather than cell proliferation [Murai and Pasquale, 2003].

It is now widely accepted that inflammation is a critical factor in tumor progression and that reactive oxygen species (ROS) are the most likely factors to play a role in linking inflammation to carcinogenesis. Increasing evidence suggests that ROS act as second messengers in intracellular signaling cascades with the function to induce and to maintain the oncogenic phenotype of cancer cells [Wu, 2006]. Although low amounts of ROS are easily tolerated by the cell, abnormally high levels of ROS induce oxidative stress. According to recent reports, oxidative stress up-regulates the expression and activity of numerous molecules associated with the migratory behavior of epithelial cells [Radisky et al., 2005, 2007]. Mori et al. [2004] showed that in NMuMG mammary epithelial cells, oxidative stress induced the up-regulation of several MMPs, in particular, MMP-9 and MMP-13. Increased oxidative stress has also been associated with endothelial cell dysfunction, and among the upregulated genes reported here, arginase has been found to increase tumor growth by stimulating the angiogenic cascade [Davel et al., 2002; Thengchaisri et al., 2006]. Arginase I was also shown to be up-regulated in wound-derived fibroblasts [Witte et al., 2002], and in lung metastasis it can be activated to levels well above those found in either primary D122 tumors or B16 melanoma cells (with fold ratios of 22.5 and 4.9, respectively) [Margalit et al., 2003]. Tumor arginase activity has been proposed as a diagnostic marker for recurrent colorectal carcinoma as well as for colorectal liver metastasis [Porembska et al., 2002]. In NBT-II cells, over the 48 h period of the EMT experiment, arginase I and MMP-13 were two of the genes that underwent the most dramatic up-regulation (10.5 and 21.6 fold increases, respectively). Taken together, these results suggest that arginase I and MMP-13 could be important determinants in FGF-1-induced EMT and that long-term induction of these molecules may favor tumor growth. We thereferore propose

that FGF-1 stimulation, by way of mimicking an oxidative stress, may play a role in regulating the expression of specific genes such as arginase I and MMPs, thereby indirectly contributing to the EMT process.

Our results clearly show that FGF-1 induced EMT proceeds with early and late phases of gene target modulation. Importantly, many of the molecular and phenotypic changes associated with the EMT are also characteristic of aggressive metastatic tumor cells. During tumor progression, epithelial cells become less differentiated as they progressively lose their epithelial markers (such as E-cadherin, other transmembrane proteins and several of the epithelial cytokeratins). This loss of epithelial markers correlates with a significant reprogramming of gene expression and acquisition of a mesenchymal signature. In NBT-II carcinoma cells, FGF-1 behaves like an oncogene and induces phenomena related to those involved in tumor progression. Correlatively, several FGF-1 target genes coding to proteases known to be involved in cell invasion and metastasis (i.e., the plasmin system and different MMPs) were markedly up-regulated, and their expression level were maintained at high levels during the periods of time monitored for EMT activation.

The present study of expression profiles has proven to be useful for the analysis of oncogenic pathways. For example, Bild et al. have used the Myc, E2F3, Ras, Src, and β-catenin pathway signature to identify genes that in all probability constitute a comparable pathway in human cancer cells [Bild et al., 2006]. Similarly, the compilation analysis of our EMT target genes with the pathway signatures of human cancer cells has revealed a panel of common genes. All of the identified genes undergo significant regulation upon EMT, and interestingly all, except one (axin-2), belong to the Ras pathway. The common up-regulated genes interleukin 1 alpha, early growth response 1/2, Jun-B and chemokine (C-X-C motif) ligand 1 for IT, PTPε for EPT, Arginase, Ephrin B1, epiregulin, diphteria toxin receptor, MMP-14, TIMP-1 for IET and uPAR for IELT. Common down-regulated genes are LTs LT such as FGFR2. These results strike us as compelling, given the fact that Ras pathway is of central importance in FGF/FGFR signaling.

In summary, the use of microarray technology in the NBT-II carcinoma model provided us with new data that correlated very well with our previous observations [Billottet et al., 2004] and at the same time identified new candidate genes and signaling pathways modified by the FGF-1 stimulation. This study gives valuable insight into the molecular aspects of FGF-1-induced EMT and supports the concept that distinct biological changes underlie the phenotypic EMT characteristics. The microarray data allowed the distinction of several sets of genes that are differentially involved in the spatio-temporal aspect of FGF-1-induced EMT. These different genes work cooperatively within a network of cascading signals to determine the tumor progression of carcinoma cells. These results are encouraging for the strategy of using microarray experiments to find new target genes involved in signal-mediated phenotypic transitions, oncogenic or otherwise. The discovery of such target genes might provide new experimental strategies to inhibit tumor invasion and metastasis of carcinoma.

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