

## Gene expression profile in fibroblast growth factor 2-transformed endothelial cells

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**Fibroblast growth factor-2 (FGF2) exerts paracrine and autocrine functions on endothelial cells. FGF2-over-expressing murine aortic endothelial cells (FGF2-T-MAE cells) induce opportunistic hemangioendothelioma-like tumors when inoculated in immunodeficient mice. To evaluate the impact of FGF2-mediated activation on gene expression profile in transformed endothelial cells, we performed subtractive suppression hybridization analysis between FGF2-T-MAE cells and parental MAE cells. The two cell populations were compared for differential gene expression also by gene macroarray hybridization with <sup>32</sup>P-labeled cDNAs. The two approaches allowed the identification of 27 transcripts whose expression was upregulated by FGF2 in endothelial cells. With the exception of one unknown gene, the differentially expressed transcripts encoded for proteins involved in the modulation of cell cycle, differentiation, and cell adhesion. Among them, the stress-inducible genes *A170*, *GADD45* and *GADD153* are upregulated by FGF2 transfection or recombinant growth factor treatment. Their expression was also induced in vascular tumors originated by parental or FGF2-transfected MAE cells in nude mice. This study extends the number of genes involved in tumor angiogenesis and/or endothelial cell transformation, a finding with possible implications for the discovery of novel targets for angiostatic therapy.** *Oncogene* (2002) 21, 2433–2440. DOI: 10.1038/sj/onc/1205301

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Angiogenesis is characterized by increased microvessel endothelial cell proliferation, production and/or activation of matrix degrading enzymes, migration in the subendothelial matrix, and differentiation into functional new blood capillaries (Risau, 1997). The local, uncontrolled release of angiogenic growth factors and/or alterations of the production of natural angiogenic inhibitors, with a consequent alteration of the angiogenic balance (Hanahan and Folkman, 1996), are

thought to be responsible for the uncontrolled endothelial cell proliferation that takes place during tumor neovascularization and in several angioproliferative diseases, including diabetic retinopathy, arthritis, Kaposi's sarcoma, and vascular tumors (Enzinger and Weiss, 1995; Folkman, 1995). Thus, the identification of selective targets in angiogenic and/or transformed endothelium may have significant implications for the development of anti-angiogenic therapies and for the treatment of vascular tumors, including Kaposi's sarcoma. In the last decade several targets for anti-angiogenic therapy have been discovered leading to the development of angiostatic compounds; some of them, including inhibitors of  $\alpha_v\beta_3$  integrin and of the vascular endothelial growth factor (VEGF) signaling pathway, are currently under test in clinical trials (for a review see Liekens *et al.*, 2001a). In this contest, gene expression profiles have been described for endothelial cells activated *in vitro* (Glienke *et al.*, 2000; Kahn *et al.*, 2000; Roland *et al.*, 2000; Wang *et al.*, 2000) or *in vivo* during tumor angiogenesis (St Croix *et al.*, 2000). The results have allowed the identification of several known and unknown gene transcripts upregulated in endothelial cells under these conditions.

FGF2 was one of the first angiogenic factors to be characterized and implicated in blood vessel growth in different physiological and pathological conditions (Klagsbrun and Baird, 1991). FGF2 may exert its effects on endothelial cells via a paracrine mode consequent to their release by tumor and stromal cells and/or by mobilization from the extracellular matrix. FGF2 may also play an autocrine role in endothelium, leading to the deregulation of endothelial cell behavior (Gualandris *et al.*, 1996). Accordingly, FGF2 has been implicated in the pathogenesis of lesions of endothelial cell origin, including Kaposi's sarcoma (Ensoli *et al.*, 1994a, b) and hemangiomas (Takahashi *et al.*, 1994).

Mouse aortic endothelial (MAE) cells express undetectable levels of FGF2 (Ribatti *et al.*, 1999). We generated FGF2-overexpressing pZipFGF2 MAE cells by stable transfection of parental MAE cells with a retroviral expression vector harboring a human FGF2 cDNA (Gualandris *et al.*, 1996). Transfectants are characterized by a transformed morphology, increased saturation density, and an invasive and morphogenic

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CLONE	NORTHERN MAE FGF2-T	BLAST IDENTIFICATION	ACCESSION NUMBER
A4		Prothymosin $\alpha$	X56135
A11		Prothymosin $\alpha$	X56135
C1		Lamin C	X14170
A8		Integrin $\alpha_3$	D13867
D9		Collagen $\alpha 2$ type I	X58251
B1		90.4% identity with rat Chaperone mt-GrpE	U62940
B6		A170	U40930
D11		A170	U40930
C2		87% identity with human HMG-I	M23614 J04239
G5		87% identity with human HMG-I	M23614 J04239
H6		Vimentin	M24849
F11		Vimentin	M24849
C4		NMB (osteoactivin)	AJ251685
C11		Ferritin heavy chain	X12812
E7		Capping protein $\alpha 2$ subunit	U16741
G6		Enolase $\alpha$	X52379
G9		89% identity with human p12 small subunit DNA polymerase $\delta$	AF179890
C9		Unknown	
H8		86% identity with human centaurin $\gamma 3$	XM_032107
E11		Moloney murine leukemia virus LTR	J02259
H10		Moloney murine leukemia virus LTR	J02261
		GAPDH	
		$\beta$ -actin	

**Figure 1** Identification of FGF2-induced genes by SSH. Parental BALB/c mouse aortic endothelial 22106 cells (MAE cells) and FGF2-T-MAE cells (Liekens *et al.*, 2001b) were grown in DMEM supplemented with 10% fetal calf serum in the absence or in the presence of 500  $\mu\text{g/ml}$  of G418 sulfate antibiotic (Life Technologies). Approximately  $2 \times 10^8$  subconfluent cells grown in complete medium were used for poly (A)<sup>+</sup> RNA extraction using FastTrack<sup>®</sup> 2.0 kit (Invitrogen) following manufacturer's instructions. Forward SSH (subtraction of FGF-T-MAE/MAE) and reverse SSH (subtraction of MAE/FGF-T-MAE) were performed with 2  $\mu\text{g}$  poly(A)<sup>+</sup> mRNA from the two cell populations, using the PCR-Select<sup>™</sup> cDNA Subtraction Kit (Clontech) according to manufacturer's instructions. PCR products from the secondary PCR of the forward subtracted SSH were directly ligated into pGEM<sup>®</sup>-T vector, using pGEM<sup>®</sup>-T vector System II (Promega) and transformed into DH5-competent *E. coli* cells. Bacteria were plated on ampicillin-containing agar plates, incubated overnight, and clones with cDNA inserts were identified by a white-blue screening using X-Gal solution and 0.1 M isopropyl-1-thio- $\beta$ -D-galactoside. Ninety-five clones and one blue colony (empty vector) were randomly chosen and arrayed onto a nylon membrane (GeneScreen, NEN) using a dot blot device (Life Technologies). This process was repeated to prepare four identical copies of each blot. Two nylon membranes were hybridized with radio-labelled  $\alpha$ -<sup>32</sup>P single-strand cDNA probes obtained by reverse transcription of the starting poly(A)<sup>+</sup> RNA from MAE and FGF-T-MAE cells. Reverse transcribed probes were synthesized in the presence of [ $\alpha$ -<sup>32</sup>P]dATP by moloney murine leukaemia virus (MMLV) reverse transcriptase (Life Technologies). In parallel, the other membranes were probed with the forward- and reverse-subtracted cDNAs according to PCR-Select<sup>™</sup> Differential Screening kit manual (Clontech). Equal amounts of heat-denatured <sup>32</sup>P-labeled cDNA probes ( $10^6$  c.p.m./ml hybridization solution) were added to the hybridization solution (ExpressHyb, Clontech) and the membranes incubated overnight at 68°C. The membranes were washed four times for 20 min with 2  $\times$  SSC, 0.5% sodium dodecyl sulphate (SDS) at 68°C and twice with 0.2  $\times$  SSC, 0.5% SDS at 68°C. Autoradiography was performed using a phospho-imaging system

behavior in three-dimensional gels. *In vivo*, they induce hemangiomas in the chick embryo and opportunistic vascular lesions in nude mice (Gualandris *et al.*, 1996; Ribatti *et al.*, 1999). A FGF2-overexpressing subclone (FGF2-*T*-MAE cells) was isolated from these lesions: it retained several of the *in vitro* properties of pZipFGF2-MAE cells but showed an higher tumorigenic capacity when re-injected in nude mice (Liekens *et al.*, 2001b). Thus, FGF2-*T*-MAE cells may represent an unique model in which FGF2-driven gene expression leads to a transformed, highly tumorigenic phenotype in endothelial cells. This model may help to elucidate the molecular determinants of endothelial cell activation during angiogenesis and vascular tumor progression.

To gain a molecular understanding of FGF2-triggered endothelial cell transformation, we compared the gene expression profiles between exponentially growing tumorigenic FGF2-*T*-MAE cells and parental MAE cells by subtractive suppression hybridization (SSH). Subtracted PCR products were ligated into a prokaryotic cloning vector and transformed into competent *E. coli* cells. Ninety-five randomly picked clones were arrayed on nylon membranes. Hybridization of the filters with <sup>32</sup>P-labeled subtracted cDNAs showed 58 positive spots. To confirm their putative differential expression, poly(A)<sup>+</sup> RNAs from MAE and FGF2-*T*-MAE cells were run in a formaldehyde-agarose gel, blotted on membrane, and hybridized with the inserts of the 58 clones. Filters were scanned with a phospho-imager analyser (FLA2000, Fuji), the signal intensities were normalized for expression of both GAPDH and  $\beta$ -actin mRNAs, and compared by computerized image analysis. Among the 58 clones, 21 transcripts showed an increased expression in FGF2-*T*-MAE *versus* MAE cells and were then sequenced. The results are summarized in Figure 1. Out of the 21 clones, five transcripts were present in duplicate, one of them encoding for the LTR sequence derived from the FGF2 retroviral expression vector. Thus, 15 murine genes were found to be upregulated in FGF2-*T*-MAE cells by the SSH approach. Among them, one encodes for a transcript of unknown function with 100% homology with murine EST AA791500. The other transcripts encode for proteins involved in various cell functions, including cell proliferation, stress response, cell adhesion, and cytoskeleton organization (Figure 1). In a second set of experiments, 48 newly analysed clones were found to encode for genes already identified during the first

screening, thus indicating the exhaustion of the subtracted library.

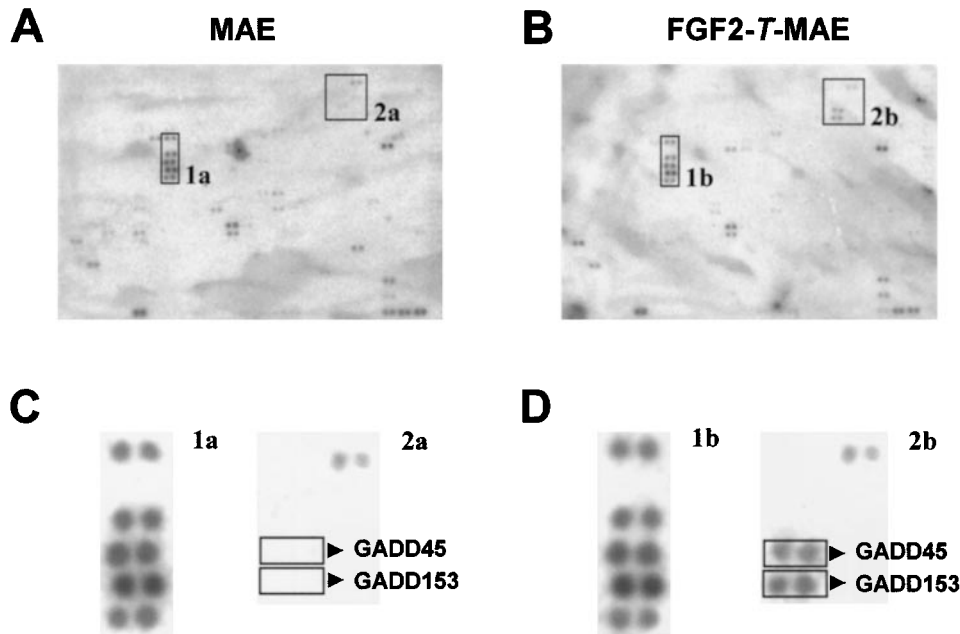
On this basis, in an attempt to detect additional differentially expressed genes, we analysed the same poly(A)<sup>+</sup> RNAs derived from MAE and FGF2-*T*-MAE cells by gene macroarray hybridization. To this purpose, reverse transcribed poly(A)<sup>+</sup> RNAs were <sup>32</sup>P-labeled and hybridized to Atlas<sup>TM</sup> Human 1.2 and Atlas<sup>TM</sup> Human Cancer 1.2 gene arrays (Clontech). With this alternative approach we were able to identify 12 additional genes that were overexpressed in FGF2-*T*-MAE cells when compared to parental cells (Figure 2 and Table 1). All the murine transcripts identified by gene array hybridization show a nucleotide identity higher than 80% compared to their human counterpart. Taken together, SSH and gene macro-array approaches allowed the identification of 27 transcripts whose expression was upregulated in FGF2-transformed endothelial cells when compared to normal cells (Table 2).

FGF2-*T*-MAE cells represent a subclone originated after *in vivo* selection of FGF2-overexpressing pZipFGF2-MAE cells. To confirm that the gene expression profile identified in FGF2-*T*-MAE cells is driven by FGF2 and does not merely reflect subclonal heterogeneity, the expression of randomly selected transcripts identified by SSH (*A170* and *High Mobility Group (HMG)-I*) and by gene array hybridization (*Growth Arrested DNA Damage (GADD)45* and *GADD153*) were investigated in parental MAE, pZipFGF2-MAE, and FGF2-*T*-MAE cells by Northern blotting. As shown in Figure 3A, the expression of the four genes tested was upregulated in both pZipFGF2-MAE and FGF2-*T*-MAE cells when compared to MAE cells. Also, Western blot analysis of the cell extracts confirmed that *GADD45* and *GADD153* upregulation was paralleled by a significant increase in the levels of the corresponding proteins (data not shown).

FGF2-overexpressing MAE cells release limited amounts of FGF2 that are responsible for the alterations in the biological behavior of these cells via an extracellular mechanism of autocrine stimulation (Ribatti *et al.*, 1999). On this basis, we evaluated the capacity of exogenous recombinant FGF2 to modulate *HMG-I*, *A170*, *GADD45* and *GADD153* mRNA levels in MAE cells. As illustrated in Figure 3B, recombinant FGF2 increases the levels of all the transcripts tested with similar kinetics, a significant stimulation being observed after 6 h of treatment.

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(FLA2000, Fuji). Clones hybridizing with cDNA from the forward subtracted library were considered differentially expressed and listed in the first column. 2.5 micrograms of poly(A)<sup>+</sup> RNA were separated by denaturing 1.2% agarose gel electrophoresis and transferred to nylon membrane using a TurboBlotter<sup>TM</sup> apparatus (Schleicher and Schuell). To obtain specific probes, 200 ng of plasmid DNA was subjected to 25 cycles of PCR reaction using nested primers provided with PCR-Select<sup>TM</sup> cDNA subtraction kit (Clontech); the reaction mixture was loaded on agarose gel and, after electrophoresis, the amplified products were isolated using Ultrafree<sup>®</sup>-DNA (Millipore). The recovered DNA was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using Ready-to-Go DNA labeling kit (Amersham Pharmacia Biotech). The hybridization procedure was the same described above and the results are shown in the second column. The cDNA inserts of differentially expressed clones were partially sequenced using DYEnamic Direct Cycle Sequencing kit (Amersham Pharmacia Biotech) exploiting the T7 promoter contained in pGEM<sup>®</sup>-T vector. Sequences were read with Applied Biosystems automated DNA sequencer and compared to GenBank. Homology or identity for each clone was determined based on the highest score using Advanced Blast 2.0 (Altschul and Lipman, 1990) and reported in the third and fourth columns



**Figure 2** Identification of FGF2-induced genes by gene array hybridization. cDNAs derived from exponentially growing FGF2-T-MAE cells and parental MAE cells were <sup>32</sup>P-labeled as described in legend to Figure 1 and hybridized with ATLAS™ Human 1.2 and ATLAS™ Human Cancer 1.2 arrays (Clontech). A typical result is reported in (A) and (B). Two regions of the filters are shown at higher magnification in (C) and (D). Detected genes are (from top to bottom): cytoke­ratin-6A, -7, and -8, vimentin (slightly upregulated in FGF2-T-MAE cells), and desmin (panels 1a and 1b); cytosolic superoxide-dismutase, GADD45, and GADD153 (panels 2a and/or 2b)

**Table 1** Summary of differentially expressed genes identified by gene array hybridization in FGF2-T-MAE cells vs parental MAE cells

FGF2 upregulated gene	Fold induction <sup>a</sup>
<i>fos</i> -related antigen-1	+
<i>c-jun</i> -proto-oncogene	++
Cytoplasmic dynein light chain	++
Defender against cell death 1 protein	++
DNA topoisomerase II $\alpha$	++
Inhibitor of differentiation-3	++
Thymosin $\beta$ -10	+
Transforming growth factor $\beta$ 2	++
Fibroblast growth factor receptor-1	+++
Growth arrest DNA damage-inducible protein 153	+++
Growth arrest DNA damage-inducible protein 45	+++
Macrophage migration inhibitory factor	++

<sup>a</sup>Relative expression of the transcript in FGF2-T-MAE cells vs MAE cells: +, 2–10-fold increase; ++, 10–20-fold increase; + + +, more than 20-fold increase

pZipFGF2-MAE cells cause the rapid growth of opportunistic vascular tumors when injected in nude mice (Gualandris *et al.*, 1996). In contrast, parental MAE cells are poorly tumorigenic, giving raise to highly differentiated, slow growing hemangiomas (Bastaki *et al.*, 1997). To validate the involvement of candidate genes in endothelial cell transformation and angiogenesis, we performed semi-quantitative reverse transcribed-PCR analysis of pZipFGF2-MAE and MAE xenografts. As shown in Figure 4A, all genes were upregulated in MAE tumors (lane 3) when compared to *in vitro* cultured cells (lane 1), possibly reflecting the presence of various endogenous cytokines, including

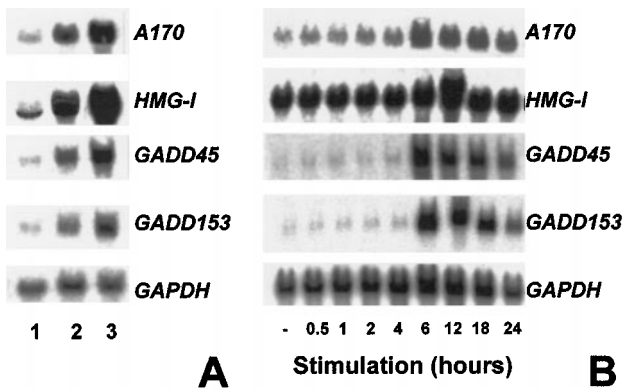
FGF2, in tumor microenvironment. The expression of *A170*, *GADD45* and *GADD153* genes was further increased in pZipFGF2-MAE xenografts (lane 4), similar to their *in vitro* counterpart (lane 2).

Finally, newly formed blood vessels invading FGF2-Matrigel plugs implanted s.c. in Balb/c mice were positive for GADD45 immunostaining (Figure 4B), further supporting the involvement of this protein in endothelial cell activation during angiogenesis.

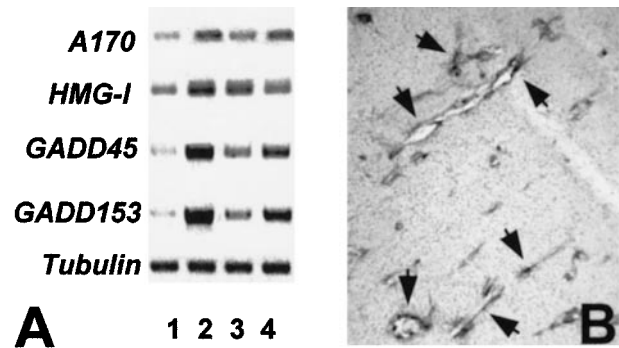
FGF2 interaction with non-signaling low affinity heparan sulphate proteoglycans is required for high affinity interaction with tyrosine-kinase FGF receptors (FGFRs; Klagsbrun and Baird, 1991). FGFR occupancy and autophosphorylation leads to the activation of various intracellular signal transduction pathways (for a review see Klint and Claesson-Welsh, 1999). In pZipFGF2-MAE cells, FGF2 accumulates intracellularly and is released in limited amounts in conditioned medium (Ribatti *et al.*, 1999). The released growth factor can interact with upregulated FGFRs (Table 1) present on the endothelial cell surface, thus activating the classical Ras/Raf-1/MEK/ERK pathway cascade (Besser *et al.*, 1995). Indeed, a constitutive increase in ERK<sub>1/2</sub> phosphorylation is observed in FGF2-T-MAE cells when compared to parental cells (data not shown). This can regulate the activity of different transcription factors including c-Myc, c-Fos, c-Jun and HMG-box proteins (Treisman, 1996). Both Fos and Myc proteins are essential for taking cells through the cell cycle at the G<sub>1</sub>/S border. Accordingly, we have identified *c-jun*, *fos* related antigen-1 (*Fra-1*), and the *c-myc*-induced genes *prothymosine- $\alpha$*  and *HMG-I* as differentially expressed in

**Table 2** Summary of differentially expressed genes identified by SSH or gene array hybridization in FGF2-*T*-MAE cells vs parental MAE cells

Biological function	FGF2-upregulated gene	Method of detection
Cell proliferation/differentiation	DNA polymerase $\delta$ p12 small subunit	SSH
	DNA topoisomerase II $\alpha$	Gene array
	FGFR-1	Gene array
	Prothymosin $\alpha$	SSH
Gene transcription	Transforming growth factor $\beta$ 2	Gene array
	Fos-related antigen-1 (Fra-1)	Gene array
	HMG-I	SSH
	Inhibitor of differentiation-3 (Id3)	Gene array
Stress/survival	c-Jun	Gene array
	A170 (Oxidative stress-induced protein)	SSH
	Defender against cell death 1 (Dad 1)	Gene array
	Enolase $\alpha$	SSH
	Ferritin heavy chain	SSH
	GADD45	Gene array
	GADD153	Gene array
	Mitochondrial chaperone GrpE	SSH
Cytoskeleton	Macrophage migration inhibitory factor (MIF)	Gene array
	Capping protein $\alpha$ 2 subunit	SSH
	Centaurin $\gamma$ 3	SSH
	Cytoplasmic dynein light chain	Gene array
	Lamin C	SSH
	Thymosin $\beta$ -10	Gene array
Cell adhesion	Vimentin	SSH
	$\alpha$ 3 integrin subunit	SSH
	Type I collagen $\alpha$ 2	SSH
Unknown	NMB (osteoactivin)	SSH
	EST AA791500	SSH



**Figure 3** FGF2-dependent modulation of *A170*, *HMG-I*, *GADD45* and *GADD153* expression. (A) Exponentially growing MAE (1), pZipFGF2-MAE (2), and FGF2-*T*-MAE (3) cells were washed twice with ice-cold PBS and RNA was extracted following the method described by Chomczynski and Sacchi (1987). (B) Exponentially growing MAE cells (-) were stimulated with 30 ng/ml of recombinant FGF2. RNA was extracted at different time points as indicated. In each lane, 20  $\mu$ g of total RNA were run on formaldehyde-agarose gel and analysed by Northern blotting, using the indicated probes



**Figure 4** *A170*, *HMG-I*, *GADD45* and *GADD153* expression in vascular tumors. (A) MAE and pZipFGF2-MAE cells ( $4 \times 10^6$ ) were injected s.c. in nude mice. Animals were sacrificed when lesions had reached an averaged size equal to 0.5 cm<sup>3</sup> (at 5 and 25 weeks for pZipFGF2-MAE and MAE xenografts, respectively) and tumors were snap frozen in liquid nitrogen. Total RNAs (2  $\mu$ g) extracted from MAE (3) and pZipFGF2-MAE (4) lesions and from subconfluent MAE (1) and pZipFGF2-MAE (2) cell cultures were retro-transcribed and 1/10th of the reaction were analysed by PCR using the following primers: *A170*, forward: TCGTTCACGGTGA-AGGCCTAT; reverse: AGTCCCCATCCTCATCGCGGTAGTG. *HMG-I*, forward: GAGTCGGGGCTATTCTGGTG; reverse: GCTCTTTCTGACTCCCGACCAGCG. *GADD45*, forward: CA-ACGTAGACCCCGATAACGTG; reverse: GTGGGAGTGA-CTGCTTGAGTAAC. *GADD153*, forward: CTGAAAGCAGAA-CCTGGTCCAC; reverse: GATGCCCACTGTTTCATGCTTG. *Tu-**bulin*, forward: TCACTGTCCTGAACTTACC; reverse: GGAA-CATAGCCCGTAAACTGC. (B) Matrigel plugs containing 500 ng/ml of FGF2 were implanted s.c. in Balb/c mice. After 5 days plugs were embedded in OCT compound, frozen, and 5- $\mu$ m sections were sequentially incubated with anti-GADD45 antibody, biotinylated secondary antibody, and avidin-biotin-peroxidase complex with no counterstaining. Arrowheads point to GADD45-positive blood vessels

FGF2-transformed endothelial cells. All these genes are considered as protooncogenes: high levels of phosphorylated c-Jun and Fra-1 correlate with malignant phenotypes in the multistage mouse skin carcinogenesis model (Zoumpourlis et al., 2000); an increased expression of HMG-I protein, as well as of prothymosin- $\alpha$ , leads to the neoplastic transformation of fibroblasts (Wood et al., 2000; Orre et al., 2001) and a HMG-I/Y

antisense construct abrogates transformation in Burkitt's lymphoma cells (Wood *et al.*, 2000). This suggests that their up-regulation may contribute to the capacity of FGF2-*T*-MAE xenografts to generate opportunistic vascular tumors (Liekens *et al.*, 2001b).

Inhibitor of differentiation-3 (*Id3*) mRNA is up-regulated in FGF2-*T*-MAE cells. Id proteins block the expression of muscle-specific genes. Indeed Id3 binds to ITF-2b, thus inhibiting ITF-2b/MyoD-mediated transcription (Chen and Lim, 1997). Consistent with its role as an inhibitor of differentiation, *Id3* mRNA has been detected in proliferating skeletal muscle cells, is further induced by FGF2, and is down-regulated in differentiated muscle cell cultures (Chen *et al.*, 1997). Recently, Id3 has been shown to be required also for neurogenesis, angiogenesis, and tumor xenograft vascularization (Lyden *et al.*, 1999). Id3 is expressed in endothelial cells during development and *Id3*<sup>-/-</sup> mice show vascular malformations and absence of blood vessel sprouting and branching (Lyden *et al.*, 1999). Our data indicate for the first time that *Id3* expression is regulated by an angiogenic growth factor in endothelial cells. Further experiments are required to define the role of this protein during neovascularization and vascular tumor progression.

Modulation of cell movement, invasion, and cell adhesion characterizes the angiogenesis process. This is paralleled by profound changes in cytoskeleton organization of the endothelial cell and in its ability to interact with the substratum. Accordingly, several cytoskeleton components are up-regulated in FGF2-*T*-MAE cells, including dynein light chain, actin capping protein  $\alpha 2$ , thymosin- $\beta 10$ , and the intermediate filament proteins lamin C and vimentin. Interestingly, we have identified the murine homologue to human *centaurin*  $\gamma 3$  as up-regulated in FGF2 transfectants. Centaurins, downstream mediators of phosphoinositide 3-kinase signaling cascades, have been proposed as involved in actin cytoskeletal organization (Jackson *et al.*, 2000). Also, FGF2-*T*-MAE cells express high levels of collagen  $\alpha 2$  type I and of the  $\alpha 3$  integrin receptor. Some of the identified genes have already been shown to be regulated in endothelial cells by FGF2, like integrin  $\alpha 3$  (Klein *et al.*, 1993) and vimentin (Carey and Zehner, 1995), or by VEGF (thymosin  $\beta 10$ , Vasile *et al.*, 2001). Also, the study of gene expression profiles during angiogenesis *in vitro* and *in vivo* has identified several cytoskeletal components, as well as extracellular matrix components and their receptors, as up-regulated in activated endothelial cells (Glielke *et al.*, 2000; Kahn *et al.*, 2000; St Croix *et al.*, 2000; Wang *et al.*, 2000). Relevant to this point is the observation that various angiogenesis inhibitors may exert their angiostatic activity by interacting with cytoskeleton (e.g. combretastatin, taxol, and endostatin) or by affecting integrin activity (e.g. cyclic RGD peptides and anti- $\alpha_v\beta_3$  integrin antibodies) (for a review see Liekens *et al.*, 2001a).

Defender against cell death 1 (*Dad1*) gene is up-regulated in FGF2-*T*-MAE cells. It encodes a mammalian oligosaccharyltransferase subunit with anti-apoptotic functions (Kelleher and Gilmore,

1997). Mice lacking *Dad1* express abnormal *N*-linked glycoproteins and undergo increased embryonic apoptosis paralleled by poor mesoderm development (Hong *et al.*, 2000). Relevant to this point, FGF-mediated signalling is required for mesodermal patterning during gastrulation (Yamaguchi *et al.*, 1994).

Interestingly, some of the FGF2-upregulated genes identified in this paper have been described previously as stress-inducible genes, including macrophage migration inhibitory factor (*MIF*), *A170*, *GADD45* and *GADD153* (Table 2). MIF is a cytokine that mediates host inflammatory and immune responses (for a review see Nishihira, 2000) and is up-regulated by transforming growth factor- $\beta$ , PDGF, and FGF2 (Takahashi *et al.*, 1998). Recently, immunoneutralization of MIF has been shown to inhibit tumor growth possibly by inhibiting endothelial cell proliferation (Ogawa *et al.*, 2000). *A170* was originally cloned as an oxidative stress-inducible gene (Ishii *et al.*, 1996). A170 protein contains a Zinc finger domain, two PEST sequences, and several potential phosphorylation sites for serine/threonine kinases. It has 90% homology with a human p62 protein that binds to the tyrosine kinase p56 (lck) SH2 domain, is induced by Nrf2 transcription factor (Ishii *et al.*, 2000), and is phosphorylated by casein kinase II (Yanagawa *et al.*, 1997). The meaning of FGF2-mediated induction of this gene has to be further evaluated. The two *GADD* genes are typically induced in response to DNA damaging agents. p53 upregulates *GADD45* that blocks cell cycle entry and acts in concert with *GADD153* to induce growth arrest (Zhan *et al.*, 1994). We have observed that upregulation of *A170*, *GADD45* and *GADD153* transcripts is not limited to FGF2-transfected endothelial cells but is observed also in normal MAE cells treated with the recombinant growth factor. Accordingly, *A170*, *GADD45* and *GADD153* transcripts are highly expressed in vascular tumors originated by MAE and pZipFGF2-MAE cells. Moreover, FGF2-mediated *GADD*s induction occurs also at the protein level as confirmed by Western blot analysis of the cell extracts and by immunostaining of newly formed blood vessels in s.c. FGF2-Matrigel plugs. In apparent contrast with these findings, FGF2-*T*-MAE cells show a high proliferation rate (M Belleri, unpublished observation) and FGF2 induces DNA synthesis when administered to MAE cell cultures (Bastaki *et al.*, 1997). Clearly, these observations point to a complex interplay among growth arresting genes and proto-oncogenes following FGF2 stimulation in endothelial cells.

FGF2 can be induced by hypoxia (Kuwabara *et al.*, 1995) and promotes angiogenesis under hypoxic conditions (Kuwabara *et al.*, 1995; Kroon *et al.*, 2001). Interestingly, some of the FGF2-responsive genes that we have identified are upregulated in endothelium also by hypoxic conditions. They include *vimentin*, *enolase*  $\alpha$  and *prothymosin*  $\alpha$  (Roland *et al.*, 2000). Moreover, VEGF expression can be induced by FGF2 as well as by hypoxia (Seghezzi *et al.*, 1998; Shweiki *et al.*, 1992). Thus, both stimuli appear to share, at least in part, common gene activation pathways in promoting angiogenesis.

In conclusion, our data extend the molecular fingerprint of the transcriptional profile of endothelial cells following activation by an angiogenic factor. Although some of the genes identified in the present work were already known to be involved in the angiogenesis process, other differentially expressed genes were unexpected. The understanding of their biological significance in endothelial cell activation may help to identify novel potential targets for angiostatic therapy.

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