Genetic Profiling of Epithelial Cells Expressing E-Cadherin Repressors Reveals a Distinct Role for Snail, Slug, and E47 Factors in Epithelial-Mesenchymal Transition

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Abstract

The transcription factors Snail, Slug, and bHLH E47 have been recently described as direct repressors of *E-cadherin* and inducers of epithelial-mesenchymal transition (EMT) and invasion when overexpressed in epithelial cells. Although a role of those factors in tumor progression and invasion has been proposed, whether the different repressors play distinct or redundant roles in the tumorigenic process has not been established. To further investigate this important issue, we have analyzed the gene expression profiling of Madin-Darby canine kidney (MDCK) epithelial cells expressing the different repressors (MDCK-Snail, MDCK-Slug, and MDCK-E47 cells) versus control MDCK cells by cDNA microarrays. A total of 243 clones (228 genes and 15 expressed sequence tags) were found to be differentially expressed between either of the three MDCK-derived cell lines and control MDCK cells. Twenty two of the candidate genes were validated by Northern blot, Western blot, immunofluorescence, and promoter analyses in cell lines and by immunohistochemistry in xenografted tumors. Gene clustering analysis indicated that about a third of the 243 candidate genes were common to MDCK cells expressing Snail, Slug, or E47 factors, whereas the rest of the genes were regulated in only one or two cell types. Differentially regulated genes include those related to EMT (45 genes), transcriptional regulation (18 genes), cell proliferation and signaling (54 genes), apoptosis (12 genes), and angiogenesis (9 genes). These results indicate that Snail, Slug, and E47 transcription factors induce common and specific genetic programs, supporting a differential role of the factors in tumor progression and invasion. (Cancer Res 2006; 66(19): 9543-56)

Introduction

The epithelial-mesenchymal transition (EMT) is a developmental process that is essential to establish the cell layers in the embryo and for the morphogenetic events that form various tissues (1–3). These fundamental biological processes rely on one of the hallmarks of EMT, the loss of epithelial polarity and homeostasis, which leads to the acquisition of a motile and migratory behavior. It has been proposed that similar EMT-like processes might occur during tumor progression in carcinomas. This might be particularly relevant at specific stages (i.e., invasion and metastasis) where tumor cells disassemble and migrate to tissue/organ sites away from the primary tumors (3, 4). Based on *in vitro* experiments, EMT is characterized by the loss of intercellular adhesion (E-cadherin), the down-regulation of epithelial makers (cytokeratin), the up-regulation of mesenchymal markers (vimentin), and the acquisition of a fibroblast-like motile and invasive phenotype (3, 5). In addition to the down-regulation of E-cadherin, the expression of mesenchymal cadherins like N-cadherin or cadherin-11 in carcinoma cell lines (a process known as "cadherin switching") has also been associated with the acquisition of a spindle-shape and an invasive phenotype (6). Whereas there is evidence that EMT is an important event in the progression of many carcinomas, this process may be transient and reversible (3, 7, 8).

An extensive genetic program must be set in motion for this phenotypic change to take place, altering the expression of an extensive array of molecules. However, the possibility exists that similar but distinct genetic programs of EMT might be at play in specific cell contexts, and that these may involve different aspects of epithelial plasticity (5, 8). Recent advances in our understanding of the regulation of EMT have benefited significantly from studies of development and tumor progression (2, 9). Indeed, recent studies on the molecular mechanisms underlying *E-cadherin* down-regulation in carcinomas, an event essential for EMT and tumor invasion, have proved to be particularly informative. Several transcriptional repressors of *E-cadherin* have recently been isolated, including the zinc finger factors Snail (10, 11) and Slug (12, 13), the two-handed zinc factors SIP-1 (ZEB-2) and δEF1 (ZEB-1; refs. 14, 15), and the bHLH factors E12/E47 and Twist (16, 17). The Snail, Slug, and E47 repressors all induce a similar phenotype when overexpressed in epithelial MDCK cells, eliciting complete EMT at both the morphologic and behavioral levels (11, 12, 16). The expression of Snail, Slug, and E47 in carcinoma cell lines with invasive and metastatic properties (11, 12, 16) and the expression of Snail in undifferentiated breast carcinomas and invasive hepatocarcinomas (18, 19) are evidence that these genes are also involved in tumor progression (reviewed in ref. 7). Moreover, it has recently been suggested that Snail is involved in the local recurrence of breast carcinomas (20). Furthermore, the differential expression of Snail, Slug, SIP1, and/or Twist

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-06-0479

has been observed in distinct types of gastric carcinomas (21) as well as in ovarian and breast, prostate, or esophageal squamous cell carcinomas (22–24).

The aim of the present study was to obtain additional insights into the role of different *E-cadherin* repressors in EMT. To this end, we analyzed the gene expression profile of the well-characterized MDCK-Snail, MDCK-Slug, and MDCK-E47 cell lines (11, 12, 16, 25) compared with control MDCK-CMV cells by cDNA microarrays and RNA arbitrarily primed-PCR (RAP-PCR). In this way, we were able to identify the existence of common and specific gene targets regulated by the expression of the three factors in the same genetic background. Validation of several selected markers *in vitro* and in xenografts supports the differential participation of the three repressors in EMT and tumor progression.

Materials and Methods

Cell Lines and Cultures

The generation, characterization, and culture conditions of MDCK-CMV (control), MDCK-Snail, MDCK-Slug, and MDCK-E47 cell lines has already been reported extensively (11, 12, 16). Briefly, stable transfectants expressing cDNAs for each of the mouse factors, as well as control mock transfectants, were obtained from parental MDCK-II cells after transfection with the corresponding pcDNA3 vector, selection with G418 antibiotic, and cloning to limiting dilution. At least 10 independent clones were isolated from each transfection. All the experiments were done with single clones of each transfectant cell type. Unless otherwise indicated, the results presented correspond to the following clones: MDCK-SnailB1, MDCK-SlugNC3, MDCK-E47B1, and MDCK-CMVA2. Cells were grown in DMEM containing 10% FCS and antibiotics as previously described (11, 12, 16). Human breast carcinoma cell lines (MCF7, MDA-MB-231, MDA-MB-435, and BT549) and melanoma A375P cells were cultured as described (11, 16).

Differential Expression by RAP-PCR

For RAP-PCR assays, total RNA from the different cell lines was extracted from 5×10^6 cells growing at low or high density using the isolation kit RNeasy Mini (Qiagen GmbH, Hilden, Germany). Fifty nanograms of RNA were reverse transcribed using an oligo-dT primer and MLV reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD); 2.5 μL of the reverse-transcribed mRNAs were used as templates in PCR reactions [1 unit of Taq polymerase (Boehringer Mannheim, Mannheim, Germany), 50 μmol/L of each deoxynucleotide triphosphate, and 1 μCi [α-^{32}P]dATP, 3.7 mmol/L $MgCl_2$] done with the arbitrary primers over 35 low-stringency cycles (94°C 30 seconds, 42°C 45 seconds, 72°C 1.5 minutes). Fingerprints were resolved on 6% denaturing acrylamide gels at 55 W for 3.5 hours or until the xylene cyanol front had reached the bottom of the gel. Gels were dried and the amplified products were visualized by autoradiography. All PCR reactions were done at least twice and the results were similar in all cases. The sequences of the primers used for reverse transcription-PCR (RT-PCR) and RAP-PCR are indicated in Supplementary Table S1. All combinations of primers used in this assay gave reproducible fingerprints that contained at least 50 bands. Fingerprints were compared and the differences in the intensity of the bands were evaluated from the scanned films using Phoretix 1D software (Newcastle-upon-Tyne, United Kingdom). Selected bands were cut out and identified by automated DNA sequencing after cloning into the pCR2.1 vector (Invitrogen, Inc., Carlsbad, CA). The sequences obtained were matched with those included in the GenBank using the BLAST software.[4] The origin of the bands was assessed by hybridization to the RAP-PCR gel after transfer to a blotting membrane (Qiabrane Nylon Plus, Qiagen, Chatsworth, CA). Hybridization was done at 65°C under high-stringency conditions to a probe that was generated from the cloned band and labeled with α-^{32}P by random primer method.

Differential Expression Profile Analysis

RNA extraction, amplification, labeling, microarray hybridization, and analysis. Total RNA from the different cell lines was extracted from duplicate 80% confluent cultures using the TRIZOL reagent following the instructions of the manufacturers (Life Technologies). Three micrograms of total RNA were used to synthesize the double-stranded cDNA as previously described (26) and 3 μg of amplified RNA were used to generate fluorescence antisense RNAs using the SuperScript enzyme protocol (Life Technologies).

The Oncochip microarray platform v1.1 contains 9,726 clones corresponding to 6,386 different genes, and it includes 2,489 duplicate clones that enable the reproducibility to be assessed. Duplicate samples from each of the MDCK transfectants were labeled with dUTP-Cy5 and hybridized against the dUTP-Cy3-labeled MDCK-CMV controls as previously described (26). Two additional hybridizations using the reciprocal fluorochrome labeling were done; thus, a total of four hybridizations were done for each condition. The slides were washed, dried, and then scanned in a ScanArray 5000 XL scanner (GSI Lumonics, Kanata, Ontario, Canada) to obtain 10-μm resolution images. These were then quantified using the GenePix Pro 6.0 program (Axon Instruments, Inc., Union City, CA). The array data were included in a GEO public repository (GSE4151).

The fluorescence intensity measurements of each array experiment were processed using the GenePix Pro 6.0 and MS Excel programs as described (26). For statistical analysis, we selected genes of which the expression differed by a factor of at least 2 with respect to the MDCK-CMV control. A hierarchical clustering method was applied to group the genes and samples on the basis of the similarities in expression, and the unsupervised analyses were visualized using the SOTA and TreeView software assuming euclidean distances between genes.[5] Genes with potentially significant changes in expression between the three transcription factors were identified using the POMELO program.[6] We tested the null hypothesis of equal means among the three groups by ANOVA, computing P values using a permutation test. To select differentially expressed genes, we adjusted for multiple testing using the false discovery rate method (27). Here we report differential expression in genes with a false discovery rate–adjusted $P < 0.05$.

Validation Studies

Northern blotting. To analyze the levels of gene expression, 15 to 30 μg of total RNA isolated by TRIZOL from the cell lines grown to 80% confluence were separated on 1% formaldehyde agarose gels, blotted onto nylon membranes (Schleicher & Schuell Bioscience, Keene, NH) by the capillary method, and UV cross-linked. The integrity and loading of RNA samples were assessed by methylene blue staining of the transferred RNA. The origin of the probes and specific hybridization conditions are described in Supplementary Methods.

For Northern blot analysis of RAP-PCR bands, RNA was prepared from either low-density or high-density cultures, as indicated, and the membranes were hybridized at high-stringency conditions using [α-^{32}P]cDNA from the cloned bands as probes.

RT-PCR analysis. Two micrograms of total RNA were used in RT-PCR experiments using specific primers for transcript detection of *MITF* (all isoforms): direct, 5′-GTGCAGACCCACCTGGAAAAC-3′, and reverse, 5′-AGTTAAGAGTGAGCATAGCCATAG-3′. Amplification was done over 40 cycles (94°C 30 seconds, 55°C 30 seconds, 72°C 2 minutes). RT-PCR for canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to control for the amount of cDNA and these amplifications were done as previously described (16).

In addition, to study mRNA of mouse and human Snail, Slug, and E47, we did a RT-PCR using as primers mouse E47 (forward, 5′-GTACAGATGAGGTGCTGTCCCT-3′; reverse, 5′-GACACCTTCTCCTCCTCCCTC-3′), human Slug (forward, 5′-CGCTCCTTCCTGGTCAAGA-3′; reverse, 5′-TTGCGTCACTCAGTGTGC-3′), and human E47 (forward, 5′-GAGGAGAAAGACCTGAGGGACC-3′; reverse, 5′-ACCTGACACCTTTTCCTCTTCTC-3′).

[4] http://www.ncbi.nlm.nih.gov/blast.

[5] http://bioinfo.cnio.es/cgi-bin/tools/clustering/sotarray.

[6] http://www.genoma.wi.mit.edu/MPR/software.

Primer sequences used for mouse Snail, mouse Slug, human Snail, and mouse and human GAPDH have previously been described (11, 12). PCR products were obtained after 30 cycles of amplification with an annealing temperature of 60°C to 65°C.

Immunofluorescence and Western blot analysis. For immunofluorescence, cells grown on coverslips were fixed in either methanol (−20°C) or 3.7% formaldehyde (for 30 minutes at room temperature) and then incubated with the primary and secondary antibodies as described elsewhere (11, 12, 28). For Western blot analysis, total cell extracts from the different cell lines were obtained in radioimmunoprecipitation assay buffer and analyzed as previously described (11, 16, 28). The list of primary and secondary antibodies used is provided in Supplementary Methods. Blots were developed using the enhanced chemiluminiscence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden).

Promoter analyses. The activity of *PAI-1* (29) and *cyclin D1* (30) promoters was done by detecting promoter driven luciferase activity as previously described for the *E-cadherin* promoter (12). The indicated cell lines were transfected with 200 ng of the reporter constructs and 20 ng of a TK-renilla construct as a control of transfection efficiency. Extracts were obtained 24 hours after transfection and luciferase and renilla activities were evaluated with a dual-luciferase reporter kit (Invitrogen). The activities were normalized to those of control MDCK-CMV cells and the experiments were done on duplicate samples at least twice for each experimental condition. Values represent the mean ± SD.

Induction of Xenografted Tumors and Immunohistochemistry

Xenografted tumors from the MDCK-Snail, MDCK-Slug, and MDCK-E47 cell lines were obtained following s.c. injection into 8-week-old female BALB/c nude mice as previously described (2×10^6 cells per flank: refs. 16, 25). Growing tumors in mice were measured every 2 days using calipers by determination of the two orthogonal external diameters and mice were killed when tumors reach a size of 0.5 cm^3. Tumors were surgically excised and processed for histology and immunohistochemistry. Mice were housed and maintained under specific pathogen-free conditions and used in accordance with institutional guidelines and approved by the Use Committee for Animal Care. A total of 8 to 12 tumors from each cell line were generated and at least four different tumors derived from each cell line were analyzed.

Immunohistochemical staining was done using the Envision method (Dako, Glostrup, Denmark) with a heat-induced antigen retrieval step where the sections were immersed in boiling 10 mmol/L sodium citrate (pH 6.5) for 2 minutes in a pressure cooker. Mouse anti-human antibodies E-cadherin (4A2C7 clone, Zymed, San Francisco, CA), N-cadherin (3B9 clone, Zymed), p120 (98 clone, BD Biosciences, San Jose, CA), and SPARC (15G12 clone, Novocastra, Newcastle, United Kingdom) were applied at 1:200, 1:25, 1:500, and 1:50 dilutions, respectively, and the rabbit anti-human antibody ID3 (Santa Cruz Biotechnology, Santa Cruz, CA) was applied at 1:50 dilution. The distribution of the different markers was scored as membranous, cytoplasmic, and/or nuclear depending on the marker analyzed.

Results

Experimental design. The stable expression of the E-cadherin repressors Snail, Slug, and E47 in MDCK cells induces a dramatic phenotypic change that corresponds to a complete EMT (11, 12, 16). To obtain further insight into the role of each factor in tumor progression, we compared the gene expression profiles induced by each transcription factor in the same genetic background. Because MDCK cells are of a canine origin, initial studies were done by RAP-PCR analysis, comparing the pattern of fragments generated from MDCK-Snail and MDCK-E47 cells to that from control MDCK-CMV cells. In the fingerprints obtained using several combinations of random primers (Supplementary Fig. S1), a series of 34 fragments were differentially expressed by MDCK-Snail and/or MDCK-E47 cells. Sequencing these differentially regulated bands identified 26 candidate genes, most of which were associated with EMT or cell signaling and similarly regulated in both cell types. In contrast, the expression of a few candidate genes was regulated specifically in MDCK-Snail or MDCK-E47 cells (Table 1). The differential expression of 11 of the 26 genes was confirmed by Northern blot analysis (Supplementary Fig. S2, and data not shown). Interestingly, BLAST analysis of the isolated canine cDNA sequences indicated greater homology (>90%) to the corresponding human cDNA sequences than to the mouse or rat orthologues. These similarities are in accordance with the high conservation between the dog and human genome recently shown, particularly in coding and potential regulatory sequences (31, 32). This prompted us to use microarrays based on human sequences in conjunction with the MDCK cell system.

Expression profiling of MDCK-Snail, MDCK-Slug, and MDCK-E47 cells. We chose a human cDNA microarray platform (26) to compare the gene expression profiles of MDCK cells stably transfected with Snail, Slug, or E47 factors. After processing, 243 clones (228 genes and 15 expressed sequence tags) were identified that displayed a >2-fold change in expression in at least one of the three MDCK cell lines with respect to the control MDCK-CMV cells. Using an unsupervised analysis, we found differences in the gene expression profiles associated with each transcription factor, and hierarchical clustering grouped together all the experimental replicates for each of the transcription factors (Fig. 1*A*). Interestingly, the cluster identifies two major branches, one containing Slug and E47 expression profiles and the other containing only those of Snail. Hence, the genetic programs triggered by Slug and E47 factors are more alike than those induced by Snail. Specific expression of each transgene was confirmed by RT-PCR analysis (Fig. 1*B*).

Functional grouping of the 228 modified genes (Fig. 1*C*) indicated that 20% of them (45 genes) were directly or indirectly related to EMT. A significant number of the candidate genes (54 genes; 23%) were related to the cell cycle, proliferation, and signaling pathways and 8% (18 genes) of them were involved in transcription (including transcription factors and chromatin modifying factors). Genes involved in other processes were also differentially expressed in either of the MDCK-derived cell lines, including genes that participate in metabolism and transport (22 genes), angiogenesis (9 genes), and apoptosis (12 genes). The rest of the candidate genes were primarily involved in basic cellular functions (31 genes) or correspond to genes with miscellaneous/unknown functions (37 genes; Fig. 1*C*).

Of the total number of candidate genes, 12 were down-regulated and 20 were up-regulated by expression of either of the factors (Fig. 1*D*), indicating the pleiotropic effects of the three transcriptional factors as repressors and activators of common primary or secondary targets. However, there were differences in the level of induction or repression of any selected gene by these factors, as witnessed for the SPARC (Table 2). Furthermore, the remaining genes showed a different expression pattern depending on the factor expressed. Indeed, some genes were down-regulated by one factor and up-regulated by the other two, or they were only regulated by one or two of the factors (Fig. 1*D*; Supplementary Table S2). Therefore, to better establish which genes might be differentially and specifically regulated by each transcription factor, we did a supervised analysis using the POMELO tool and ANOVA. Using this supervised approach, 100 genes were identified as differentially expressed with a significant association between

Table 1. Identification of genes differentially expressed in MDCK-E47 and MDCK-Snail cells (versus MDCK-CMV cells) by RAP-PCR

Gene	Name of the bands in fingerprints (referred to arbitrary primers)	GenBank		
		Human	Mouse	Dog*
In both E47- and Snail-expressing cells				
Reduced expression				
MHC class II	PLC511 3			AJ630365
Junctional adhesion molecule 1 (JAM-1)	PLC511 5	XM041917	MMU89915	*XM536132*
Keratin 7	TP53CA.1 3/5 †	NM005556	AF509888	*XM534795*
Desmocolin 2 (DSC2)	VEGF R+F 14	NM024422	X73885	CFAJ02299
Pleckstrin 2 (PLECK2)	ML18-B 12	BC00856	NM013738	*XM537486*
Tissue factor pathway inhibitor 2 (TFPI-2)	ML18-B 15	AY691946	NM009364	*XM532462*
Smad 4	ML 3	NM005359	NM011200	—
Proten tyrosin phosphatase IV A, member 1 (PTP4A1)	H12/B 7	NM003463	—	—
dJ347H13.4	H12/B 10	CR456458	—	*XM538350*
Increased expression				
Bone morphogenetic protein 1 (BMP-1)	PLC511 2	NM006129	BC063079	—
Protein KIAA1917	PLC511 6	AB067504	—	*XM540446*
Microtubule-associated protein 1B (MAP1B)	D12S77a+b 10	NM032010	BC037105	—
Vacuolar protein sorting 13A isoform A (VPS13A o CHAC)	VEGFR2 3	NM033305	NM173028	*XM533522*
Rad21 homologue	D4S912GT 1	NM0062565	NM009009	—
SPARC ‡	PLC511 A§	BC008011	NM009242.1	
In E47-expressing cells				
Increased expression				
Six transmembrane epithelial antigen of prostate (STEAP)	pU6 3	BC066301	NM027399	—
Aldolase	ML 5	XM372682	BC066149	*XM548671*
Chondroitin sulfate glycoprotein 2 (versican)	VEGFR2 4a	NM004385	D16263	*XM546039*
In Snail-expressing cells				
Reduced expression				
cdc 25B phosphatase (cdc25B)	ML18-B 4	BC009953	NM02317	—
Caveolin 2 (CAV2) ‡	ML 6	NM001233	NM016900	NM00101997
Nudix (nucleoside diphosphate linked moiety X)-type motif 13 (NUDT3)	H12/B 6	NM015901	BC0409948	*XM536385*
Increased expression				
Heat shock 70kDa protein 5 (glucose-regulated protein 78kDa) binding protein 1 (HSPA5BP1)	PLC511 1	NM178031	NM133804	—
AHNAK ribosomal	ML18-B 10	NM001620	NM009643	*XM540909*
RNAs 18S, 5.8S i 28S	ML18-B 19		J00623	—

*Numbers in italics indicate the prediction by GNOMON (http://www.ncbi.nlm.nih.gov/genome/guide/gnomon.html).
† Two different bands related to the same gene.
‡ Genes identified with the same expression pattern in the Oncochip analysis.
§ Hybridization of the fingerprint.

the factors (false discovery rate–adjusted $P < 0.05$; Table 2 and Supplementary Table S1, *asterisks*). These genes also showed relatively homogeneous expression between the replicates of each transcription factor when compared with the variation between the lines (see Fig. 1*A*). Together, the data provided by the cDNA array analysis suggest that the three transcription factors, Snail, Slug, and E47, induce common and specific gene expression programs that lead to EMT as well as conferring specific functional properties.

EMT-related genes. One of the major effects of Snail, Slug, or E47 expression is the induction of a mesenchymal and invasive phenotype in MDCK cells. This phenotypic transformation may involve alterations in the expression of a wide number of proteins involved in cell-to-cell and cell-to-extracellular matrix interactions, as well as in cytoskeletal reorganization and migration. Among the EMT-related genes detected in the microarray analysis, the epithelial markers (*E-cadherin, cytokeratin 19*, and *desmoglein 2*) and basal lamina components (*nidogen 2, laminin α3*, and *integrin β6* subunits) are down-regulated, whereas some mesenchymal markers (*SPARC, collagen type I, III*, and *V* subunits, and *integrin α5*), protease-related activities (*PAI-1* and *TIMP1*), and actin cytoskeleton associated proteins (*moesin, WASPIP*, and *caldesmon*) are up-regulated (Tables 2 and 3). Interestingly, some of these EMT-related genes were also detected in the RAP-PCR analysis in Snail- and E7-expressing cells (i.e., up-regulated *SPARC*; Table 1).

The strong repression of *E-cadherin* transcription in the three MDCK-derived cell lines was confirmed at the protein level (Figs. 2 and 3*A*), in accordance with the direct repression of promoter activity by either Snail, Slug, or E47 previously shown in these cells (11, 12, 16). The expression of the p120 catenin was also regulated by the three transcription factors (28), p120ctn being mainly localized in the cytoplasm of Snail-, Slug-, or E47-transfected

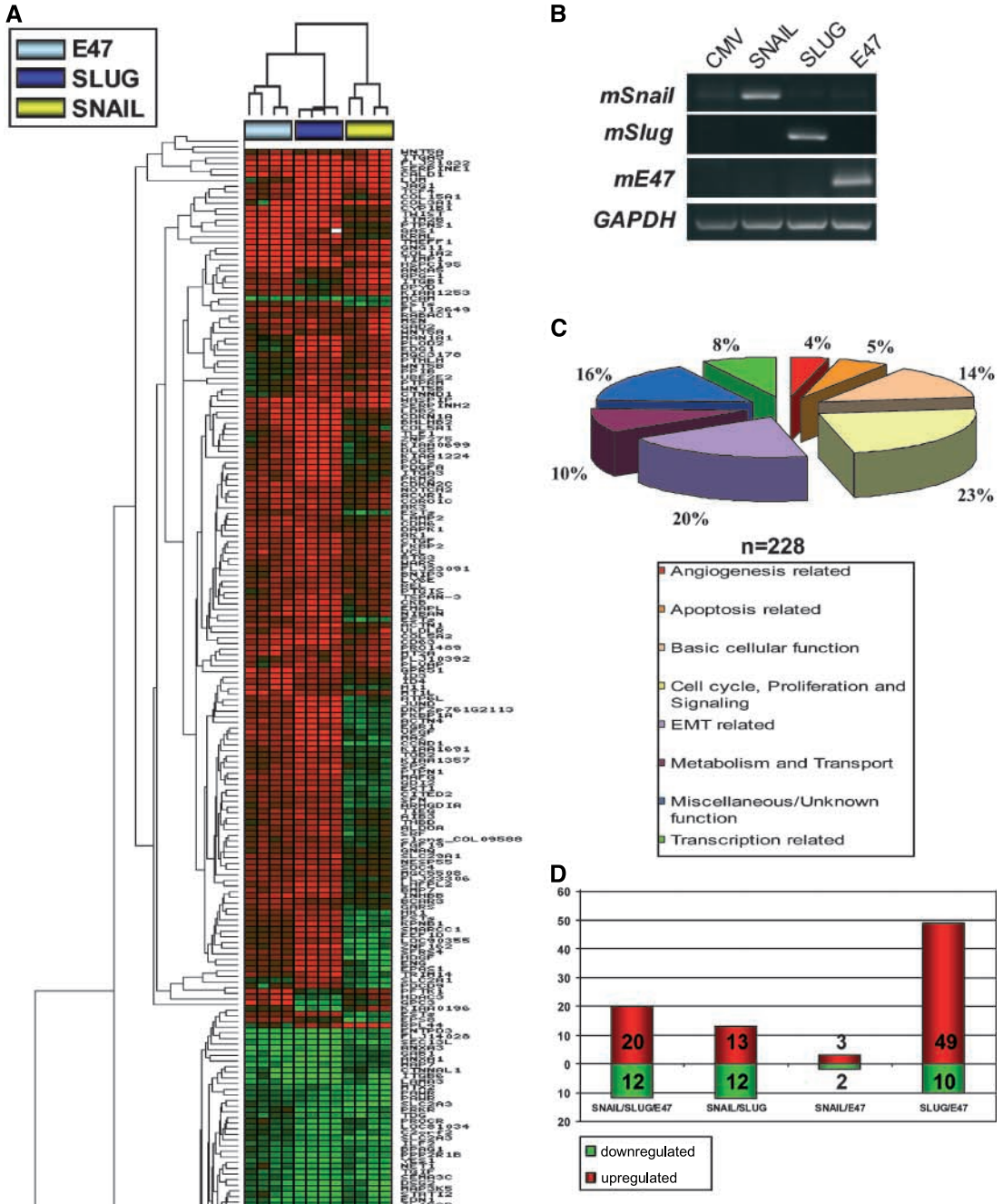

Figure 1. Snail, Slug, and E47 factors induce common and specific gene programs in MDCK cells. *A,* unsupervised cluster analysis of expression patterns detected in microarrays with MDCK cells expressing Snail (clone B1; *yellow*), Slug (clone NC3; *dark blue*), and E47 (clone B1; *light blue*) when compared with control MDCK-CMV cells. Only genes with >2-fold variation in at least one cell type were considered and four replicates of each cell type were presented. *B,* RT-PCR analysis for transgene expression in the indicated cell clones using mouse cDNA probes for detection of Snail (*top*), Slug (*top middle*), and E47 (*bottom middle*); GAPDH was used as control (*bottom*). *C,* functional grouping of the 228 genes differentially expressed in any MDCK-derived cell type. The specific functions are indicated using a color code and the numbers represent the percentage representation among all the candidate genes. *D,* changes in gene expression common to the indicated cell types (as compared with control CMV cells).

Table 2. Genes modified (at least 2-fold) in MDCK-Snail, -Slug, and/or -E47 compared with MDCK-CMV control cells

GenBank no.	MDCK-Snail	MDCK-E47	MDCK-Slug	Description
EMT related (n = 45)				
AA669042*	1.38	2.14	3.42	ACTN1, actinin, α1
R66605*	−1.61	2.34	3.46	ACTN4, actinin, α4
AA029597*	1.06	1.94	3.35	BMP7, bone morphogenetic protein 7
H44784	−1.70	−1.40	−2.30	BPAG1, bullous pemphigoid antigen 1
AA447737	7.19	5.12	14.67	CALD1, caldesmon 1
H97778	−16.60	−16.60	−16.60	CDH1, E-cadherin
AA421819	1.56	2.55	2.33	CDH6, cadherin 6, K-cadherin
AA464342*	1.35	1.91	11.10	COL15A1, collagen, type XV, α1
AA490172	4.02	8.56	3.21	COL1A2, collagen, type I, α2
T98612	24.60	2.58	14.28	COL3A1, collagen, type III, α1
R75635	1.33	2.06	5.23	COL5A1, collagen, type V, α1
AA962371	2.56	2.98	4.02	COL5A2, collagen, type V, α2
AA598794	1.63	2.09	3.18	CTGF, connective tissue growth factor
AA024656	−1.30	−2.30	−3.40	CTNNAL1, catenin α-like 1
AA024656*	3.44	1.10	3.12	CTNND1, catenin δ1, p120
W37448*	−2.10	−1.60	−2.90	DSG2, desmoglein 2
H90899	−5.60	−1.60	−3.10	DSP, desmoplakin I, II
AA487582*	−1.49	1.78	2.96	EXT1, exostoses (multiple) 1
AA775872*	1.10	3.89	−2.12	GPC3, glypican 3
AA936757	−4.50	−5	−5	HBP17, heparin-binding growth factor binding protein
AA424695*	1.06	1.90	4.30	ITGA3, integrin, α3
g757088	6.13	4.53	11.25	ITGA5, integrin, α5
W67174*	3.64	1.95	−1.08	ITGB1, integrin, β1
g2141721	−2.10	−2.10	−4	ITGB6, integrin β6
AA464250	−6.30	−4.30	−4.20	KRT19, keratin 19
AA001432	−2	−2.10	−3	LAMA3, laminin α3
vH74106	1.50	3.02	4.58	LDB2, LIM domain binding 2
AA005112	−2.10	−1.85	−1.90	LMO7, LIM domain only 7
AA453712*	3.95	1.20	15.49	LUM, lumican
R22977	2.78	2.32	2.40	MSN, moesin
W86202	−3.50	−5.20	−2.30	NET-6, tetraspan NET-6 protein
AA479199	−3.70	−4.50	−1.90	NID2, nidogen 2
N75719	14.22	2.90	19.09	PAI-1, plasminogen activator inhibitor type 1
AA278759	−2	−1.30	−4.20	PRG1, proteoglycan 1, secretory granule
AA148736*	1.14	1.99	2.93	SDC4, syndecan 4
AA461456	−2.4	−1.43	−2.7	SEMA3C, sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C
AA410517*	1.07	−1.61	−5.27	SERPINB6, serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6
R71440*	3.60	2.87	3.91	SERPINH2, serine (or cysteine) proteinase inhibitor, class H, member 2
g839914	41.39	26.68	16.24	SPARC, osteonectin
AA775616	−8.30	−7.70	−14.30	SPP1, osteopontin
g9888193	3.31	8.27	8.55	TIMP1, tissue inhibitor of metalloproteinase1
AA479202*	−4.20	−1.90	1.01	TIMP3, tissue inhibitor of metalloproteinase 3
AA287196	1.62	1.67	3.17	TSPAN-3, tetraspan 3
AA486728	1.75	2.44	3.06	VCL, vinculin
AA283699	3.89	3.64	4.75	WASPIP, Wiskott-Aldrich syndrome protein interacting protein
Transcription related (n = 18)				
AA864524*	−1.09	1.81	2.61	AIB3, nuclear receptor coactivator RAP250
AA026120*	1.01	2.64	5.23	bHLHB2, basic helix-loop-helix, class B2
AA115076*	−1.33	1.67	2.37	CITED2, Cbp/p300-interacting transactivator
AA973283*	1.50	2.99	−1.56	HDAC3, histone deacetylase 3
AA482119*	1.02	3.38	2.27	ID3, inhibitor helix-loop-helix protein 3
AA454080	1.14	4.29	2.08	ID4, inhibitor helix-loop-helix protein 4
T50121*	1.07	11.75	3.46	KRML, Kreisler maf-related leucine zipper
N21609*	−1.35	2.15	2.80	MAFG, v-maf musculoaponeurotic fibrosarcoma oncogene
N66177	−4.30	−3.10	−2.60	MITF, microphthalmia-associated transcription factor

(*Continued on the following page*)

Table 2. Genes modified (at least 2-fold) in MDCK-Snail, -Slug, and/or -E47 compared with MDCK-CMV control cells (Cont'd)

GenBank no.	MDCK-Snail	MDCK-E47	MDCK-Slug	Description
AA496787*	−2.32	1.70	2.67	SFRS4, splicing factor, arginine/serine-rich 4
AA133216*	−1.63	1.30	2.79	SMARCC1, SWI/SNF related, regulator of chromatin
T81155*	−1.03	1.68	3.20	SP2, Sp2 transcription factor
AA487973*	−1.16	1.75	2.77	SRF, serum response factor
AA669136*	2.42	2.04	8.77	TCF4, transcription factor 4, E2-2
AI268473	1.52	1.69	4.18	TLE1, transducin-like enhancer of split 1
AI220198	1.24	5.02	3.76	TWIST, Twist (*Drosophila*) homologue (acrocephalosyndactyly 3; Saethre-Chotzen syndrome)
AA454673*	−1.88	1.45	3.81	ZNF162, zinc finger protein 162
R08850	1.29	1.69	4.96	ZNF275, zinc finger protein 275
Cell cycle proliferation and signaling (n = 54)				
AA136910	1.88	2.59	2.49	ACVR1, activin A receptor, type I
AA775325*	1.84	2.35	2.87	AK1, adenylate kinase 1
AA991950	1.57	2.50	2.73	AK3, adenylate kinase 3
AA026354	−1.72	−1.35	−3.23	ARHGAP5, Rho GTPase activating protein 5
AA459400*	−1.49	1.52	2.62	ARHGDIA, Rho GDP dissociation
N74741	2.00	2.06	3.23	BTG3, BTG family protein 3
AA487700*	−2.17	2.33	4.43	CCND1, cyclin D1
g873830	1.76	2.55	4.07	CD63, CD63 antigen (melanoma 1 antigen)
R22625	−1.64	−1.43	−2.38	CDK7, cyclin-dependent kinase 7
g783509*	1.25	2.56	5.16	CDKN1A, cyclin-dependent kinase inhibitor 1A (p21, Cip1)
N72115*	1.48	2.77	2.90	CDKN2C, cyclin-dependent kinase inhibitor 2C (p18)
AA456063	1.72	2.19	2.86	CORO1C, Coronin, actin-binding protein, 1C
T77840*	1.06	2.01	4.68	DLG5, Discs large (*Drosophila*) homologue 5
N93476*	2.06	1.01	4.40	EDG1, sphingolipid G-protein coupled receptor 1
AA486628*	−1.49	2.02	3.39	EGR1, early growth response 1
H13623	−2.27	2.16	1.09	EPS8, epidermal growth factor receptor pathway substrate 8
g892048	−1.81	−2	−2.27	FGF13, fibroblast growth factor 13
g1839737*	1.07	1.79	2.28	FGF19, fibroblast growth factor 19
AI287541	−1.11	−1.41	−2.27	FZD4, frizzled (*Drosophila*) homologue 4
N68193*	−1.37	−2.27	−3.57	GAB1, GRB2-associated binding protein
R92806*	−1.49	2.03	2.44	GDI2, GDP dissociation inhibitor 2
AA454540	1.11	1.57	2.31	GNAQ, guanine nucleotide binding protein, q polypeptide
AA999901	2.07	6.48	2.54	GNG11, guanine nucleotide binding protein 11
AA775405	1.49	4.09	2.68	GPR51, G protein-coupled receptor 51
H57494*	−1.25	3.45	1.80	H11, protein kinase H11
AA453831*	−2.70	1.68	3.22	HDGF, hepatoma-derived growth factor
AA866115	−1.03	−1.68	−2.94	HELLS, helicase, lymphoid-specific
g1875225	3.95	7.03	15.72	IGFBP4, insulin-like growth factor-binding protein 4
AA431428*	−1.04	2.61	2.74	INHBB, inhibin β B
AA406020	−1.96	−1.81	−2.08	ISG15, IFN-stimulated protein
AI268273	−2.32	−1.49	−3.22	MAP3K5, mitogen-activated protein kinase kinase kinase 5
AA173453	−4.34	−2.70	−6.25	MST1R, c-met-related tyrosine kinase
R24543	−2.04	−1.20	−2.38	NET1, neuroepithelial cell transforming gene
AA630376	1.56	2.53	2.39	NOTCH2, Notch (*Drosophila*) homologue 2
AA701502	1.27	2.00	5.17	PDGFA, platelet-derived growth factor α
AA973492*	1.70	3.01	−1.09	PFTK1, PFTAIRE protein kinase 1
T47442	−1.52	−1.07	−2.44	PROCR, protein C receptor, endothelial (EPCR)
g5766201	1.67	1.57	2.57	PTGIS, prostaglandin I2 synthase
AA845432*	1.90	1.02	2.52	PTHLH, parathyroid hormone-like hormone
AA019459	−1.96	−1.89	−2.27	PTK9, protein tyrosine kinase 9
T57321	−1.28	2.10	3.35	PTPN1, protein tyrosine phosphatase, nonreceptor type 1
AA417279*	1.06	6.40	5.78	PTPNS1, protein tyrosine phosphatase, nonreceptor type substrate 1
H26426	4.51	1.22	3.24	PTPRM, protein tyrosine phosphatase, receptor type M
g1921092	1.55	1.94	2.48	REL, v-rel oncogene homologue
AI675670*	−10	−14.28	−5.88	RGS2, regulator of G-protein signaling 2
AI356363*	−1.35	1.80	2.06	SFN, stratifin
R23241	−1.96	−1.35	−2.86	STATI2, STAT-induced STAT inhibitor-2

(*Continued on the following page*)

Table 2. Genes modified (at least 2-fold) in MDCK-Snail, -Slug, and/or -E47 compared with MDCK-CMV control cells (Cont'd)

GenBank no.	MDCK-Snail	MDCK-E47	MDCK-Slug	Description
AI340883	−2	−2.04	−7.14	TACSTD1, tumor-associated calcium signal transducer 1
R83270	−2.50	−1.54	−2.63	TGIF, TGFB-induced factor (TALE family)
g1852393*	1.05	1.90	2.32	TIEG, TGFB inducible early growth response
AA486088*	−1.23	1.63	2.79	TOB2, transducer of ERBB2, 2
W93592	8.20	6.92	7.33	WNT5A, wingless-type, member 5A
W44518*	3.71	1.05	4.09	WNT5B, wingless-type, member 5B
N36882*	−1.89	−1.56	−3.23	YES1, v-yes-1

*Genes differentially expressed among Snail, Slug, and E47 with significant association ($P < 0,05$).

MDCK cells but not in control cells (Fig. 2). A proportion of p120 was membrane bound in the MDCK transfectants, probably reflecting the association of p120 with N-cadherin or another "classic" cadherin. Indeed, N-cadherin expression was slightly up-regulated by each of the transcription factors (Fig. 2), particularly in Snail- and Slug-expressing cells (Fig. 3*B*) as in other systems (33). The down-regulation of other epithelial genes, like *desmoplakin* and *α-catenin*, was also validated in Western blots (Fig. 3*A*).

With regards to mesenchymal and actin cytoskeletal markers, the up-regulation of SPARC, α5 integrin, vinculin, and caldesmon was validated at the protein level by immunofluorescence and/or Western blotting (Figs. 2 and 3*A*). In Northern blots, the up-regulation of *collagen 15A1* and *cadherin 6* transcripts was validated in the three cell types (Fig. 3*C*). The microarray data indicating that *collagen 3A1* was much more strongly induced in Snail- and Slug-expressing cells (24.6- and 14.3-fold, respectively) than in E47 cells (2.6-fold induction) were also confirmed in Northern blots (Fig. 3*C*). In contrast, the analysis of *CTGF* expression by Northern blot showed a strong expression in Snail cells, much lower levels in Slug cells, and its apparent absence in E47 cells (Fig. 3*D*), evidence that *CTGF* transcripts might be differentially expressed in MDCK-Snail cells. Importantly, a similar behavior in the expression of these various EMT markers was detected in several independent clones generated from each of the transfectant cell type and showing similar levels of the corresponding transgene (Supplementary Fig. S3*A* and *B*).

Table 3. Genes commonly up- (left) and down-regulated (right) by Snail, Slug, and E47

Up-regulated	Down-regulated
EMT related (n = 17)	
COL5A2	*CDH1*
MSN	*SPP1*
TIMP1	*KRT19*
SERPINH2	*HBP17*
WASPIP	*NET-6*
COL1A2	*ITGB6*
ITGA5	
CALD1	
SERPINE1	
COL3A1	
SPARC	
Transcription related (n = 2)	
TCF4	*MITF*
Cell proliferation and signaling (n = 5)	
WNT5	*MST1R*
AIGFBP4	*RGS2*
GNG11	
Angiogenesis related (n = 2)	
JAG1	*IL1RN*
Metabolism and transport (n = 1)	
	PLSCR1
Basic cellular function (n = 1)	
FKBP2	
Miscellaneous/unknown function (n = 4)	
FLJ21032	*SEC13L*
PRO1489	
TMEFF1	

Finally, the differential up-regulation of the protease-related activity PAI-1 by the three factors (strong in Slug and Snail cells and moderate in E47 cells) was validated by immunofluorescence (Fig. 3*F*) and promoter analysis. Indeed, the relative levels of *PAI-1* promoter activity in the three cell lines closely paralleled the microarray data (Fig. 3*E*, compare to PAI-1 data in Table 2).

Therefore, these results indicate that Snail, Slug, and E47 regulate the expression of common and specific pathways that may contribute to general and particular aspects of the EMT. They also indicate that the three factors can act both as repressors and activators of primary or secondary target genes required for EMT.

Transcription-related genes. About 8% of the genes identified are directly or indirectly related to transcriptional process (Fig. 1*C*; Table 2). Interestingly, only two of these seem to be regulated in a common fashion by Snail, Slug, and E47. The microphthalmia-associated transcription factor (*MITF*; ref. 34) was down-regulated by all three genes and the transcription factor 4 (*TCF4*; a bHLH factor also known as *E2-2*; ref. 35) was up-regulated in the three cell lines, although to a different extent (Tables 2 and 3). The expression of six transcription related genes was regulated in Slug and E47 cells, and these included members of the bHLH family (*BHLHB2* and *TWIST*) of the Id subfamily (*ID3* and *ID4*) and of the leucine zipper family (*MAFG* and *KRML*; Table 2). Interestingly, eight genes were only up-regulated in Slug cells, including several coactivators and modifiers of chromatin (*CITED2, AIB3*, and *SMARCC1*), proliferation response factors (*SRF* and *Sp2*), and zinc finger factors, whereas one gene only seemed to be up-regulated in E47 cells (*HDAC3*; Table 1). The differential up-regulation of *ID3, ID4, Twist*, and *KRML* mRNAs in E47 and Slug cells was validated in Northern blots, where *Twist* and *ID3* transcripts also seemed to be up-regulated, but to a lower extent, in Snail cells (Fig. 4*A*;

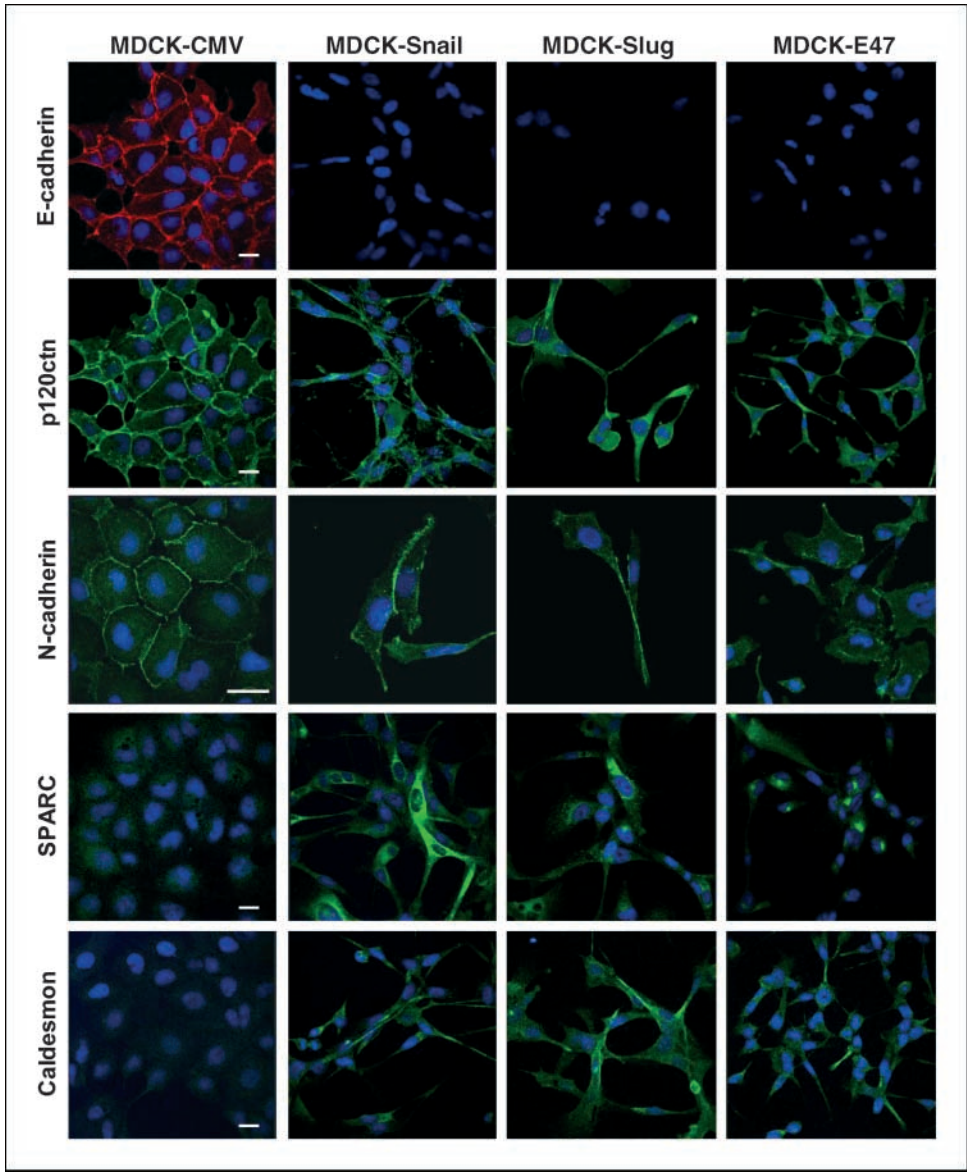

Figure 2. Immunofluorescence localization of EMT markers in MDCK cells expressing Snail, Slug, or E47. Confocal analysis of E-cadherin, p120 catenin, N-cadherin, SPARC, and caldesmon expression in the indicated cell lines. Bar, 25 μm. Images correspond to independent clones, MDCK-Snail (B1), MDCK-Slug (NC3), MDCK-E47 (B1), and control MDCK-CMV (A2).

Supplementary Fig. S3). Because of the relevance of the Id factors in tumor progression (36), we analyzed by Northern blot the expression of another two members of the Id family (*ID1* and *ID2*) that showed nonconfident results in the microarrays. Both *ID1* and *ID2* transcripts were strongly up-regulated in Slug- and E47-expressing cells (Fig. 4*A, top two rows*; Supplementary Fig. S3) and *ID1* mRNA was also induced in MDCK-Snail cells, albeit to a lesser extent. The differential regulation of *ID1* and *ID3* transcripts was also confirmed in independent clones from each transfectant cell type (Supplementary Fig. S3*C*). The alterations in *MITF* expression observed in the microarrays were validated by RT-PCR confirming that transcripts common to all described isoforms of *MITF* (34) were repressed in Snail-, Slug-, and E47-expressing cells, the stronger repression being detected in the latter cell line (Fig. 4*B*).

Cell cycle, proliferation, and signaling genes. Around 23% of the genes differentially expressed in Snail-, Slug-, and E47-transfected cells participate in processes related to proliferation, signaling, and the cell cycle. Again, some of these genes were affected in the three cell lines (6 genes) whereas others were only modified in Snail and Slug cells (6 genes), Slug and E47 cells (19 genes), or were specific to Slug-expressing cells (18 genes; Table 2). Among the genes of which the expression was altered by all three proteins, growth factors, receptor tyrosine kinases, and regulators of protein G signaling were found (Tables 2 and 3). Interestingly, *cyclin D1*, hepatoma-derived growth factor (*HDGF*), and GDP dissociation inhibitor 2 (*GDI2*) were differentially regulated in the three cell lines: down-regulated in Snail but up-regulated in Slug and E47 cells. Genes regulated by both Snail and Slug participate in several signaling pathways and include the nonreceptor tyrosine kinases (*v-yes*), RhoGAPs (*ARHGAP5*), tyrosine phosphatase receptors (*PTPRM*), and TGF-β-induced factor (*TGIF*). Most of the genes that were affected in Slug and E47 cells were up-regulated and, significantly, they included several components of the TGF-β/BMP/activin signaling pathways, including secreted factors (*BMP7* and *inhibin β B*) and TGF-β-induced genes (*TIEG*).

To gain further information about TGF-β-regulated genes, the expression of *TGF-β1* in Snail, Slug, and E47 cells was analyzed in Northern blots. *TGF-β1* transcripts were up-regulated in each

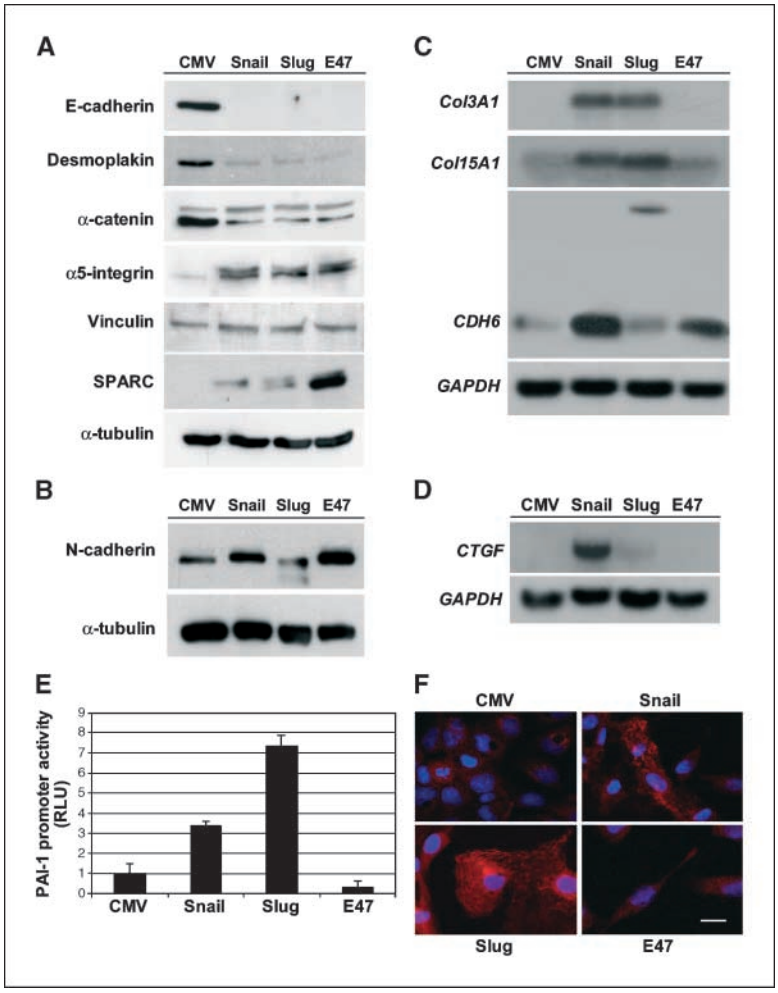

Figure 3. Validation of the expression of EMT-related markers. *A* and *B,* Western blots probed for the markers indicated in total extracts from the different cell types. Expression of α-tubulin was used as a loading control. *C* and *D,* Northern blot analysis of the genes indicated in the different cell types. GAPDH was used as a loading control. *E,* activity of the PAI-1 promoter in the cell lines. The relative light units (*RLU*) are normalized to those observed in control MDCK-CMV cells. *Columns,* mean of three independent experiments. *F,* immunofluorescence detection of PAI-1 in the indicated cell lines. Bar, 12.5 μm. Independent clones as in Fig. 2.

of the cell lines but with quantitative differences (Slug > Snail > E47; Fig. 4*C*), supporting the differential regulation of TGF-β-mediated signaling pathways by the three factors. On the other hand, two cyclin-dependent kinase inhibitors (*p21Cip* and *p18*) were up-regulated in Slug and E47 but no significant changes were observed in Snail-expressing cells. Promoter analysis of *cyclin D1* confirmed the microarray data for Slug- and E47-expressing cells and the expression of this gene was not significantly altered in Snail-expressing cells (Fig. 4*E*). A similar increase in the amounts of p21Cip protein was detected in the three cell lines when compared with control cells (Fig. 4*F*), indicating that additional posttranscriptional mechanisms influence p21Cip expression.

Genes related to other functions. The expression of genes that are involved in apoptosis was also altered, mainly in Slug- and E47-expressing cells. These included signaling and regulatory proteins of several apoptotic pathways, such as the up-regulation of *GAS1* and *MAZ* or the down-regulation of *CFLAR* (Supplementary Table S1). In addition, a few angiogenesis-related genes were seen to be differentially expressed in the three MDCK cell lines (Supplementary Table S1; Table 3). Whereas *Jagged 1* was up-regulated in each line, and particularly in Slug cells (18-fold induction), *VEGF* was up-regulated in Slug and E47 cells. The expression of other genes was only affected in Slug-expressing cells (i.e., *EPAS1, endoglin*, or *endothelin*). The increase in VEGF expression in Slug and E47 cells was validated in Northern and Western blots (Fig. 4*D*).

Finally, an important fraction of the genes identified were associated to metabolism and/or basic cellular functions (Fig. 1*C*), some common to the three cell types and others specific (Table 3; Supplementary Table S1). This further indicates that the regulation of metabolism might be an important function of these transcription factors, as recently suggested for Snail (37).

Histopathology and expression of EMT markers in xenografted tumor samples. To confirm the biological relevance of the identified gene targets and in particular of EMT-related genes, xenografted tumors induced by MDCK-Snail, MDCK-E47, and MDCK-Slug cells were subjected to histologic and immunohistochemical analysis. According to previous studies (25), tumors induced by MDCK-Snail and MDCK-E47 cells were highly undifferentiated spindle cell tumors without evidence of epithelial differentiation (Fig. 5, *left* and *right*). In contrast, tumors induced by MDCK-Slug cells grew slowly[7] and showed areas of glandular differentiation surrounded by proliferating malignant undifferentiated spindle cells (Fig. 5, *middle*). This phenotype is consistent with a diagnosis of carcinosarcoma. Thus, MDCK-Slug xenografts provided an interesting model for this tumor type, as well as provided evidence of the reversibility of the EMT phenotype.

[7] V. Bolós and A. Cano, unpublished data.

The expression of E-cadherin, N-cadherin, β-catenin, p120, vimentin, SPARC, and ID3 was analyzed by immunohistochemistry in xenografted tumors obtained from MDCK cells expressing Snail, E47, and Slug. The spindle cell component in all the tumors analyzed showed the same immunophenotype: absence of E-cadherin and β-catenin expression, intense N-cadherin, p120, vimentin, and SPARC expression, and strong nuclear expression of ID3 (Fig. 5, and data not shown). In contrast, the epithelial component of tumors induced by MDCK-Slug cells expressed membrane E-cadherin, β-catenin, and p120. The expression of N-cadherin and vimentin was focal and ID3 expression was less intense than in the spindle component. In addition, the epithelial cells of MDCK-Slug xenografts did not express SPARC (Fig. 5, *middle*). Similar results were obtained in xenografts induced by two independent clones derived from each transfectant cell type (data not shown). Taken together, these results validate *in vivo* the data obtained from the cell culture model and provide support for a role for identified EMT markers in tumor progression. In accordance with this, expression of *Snail, Slug*, and *E47*, as well as of *EMT* genes such as *CDH1* and *SPARC*, has been detected in several human cell lines representative of aggressive tumors and showing mesenchymal and/or invasive characteristics (Supplementary Fig. S4).

Discussion

The interest in understanding the molecular mechanisms responsible for EMT has increased in recent years. This is partially due to the fact that the role of EMT in human tumor progression or the influence that this process has on human malignancies remains an important matter of debate (38). Recent studies have identified several important genes that induce EMT, both in developmental and model systems. Interestingly, some of these were first characterized as transcriptional repressors of *E-cadherin*, an epithelial cell junction adhesion molecule of which the functional down-regulation is a hallmark of EMT (7). Completion of EMT requires the activation of a complex genetic program that, apart from repressing E-cadherin and other epithelial characteristics, also induces the expression of mesenchymal and migration-related proteins. Indeed, most of the *E-cadherin* repressors identified (Snail, Slug, E47, Twist, and δEF1/Zeb2) seem to fulfill this requirement, and they induce complete EMT when overexpressed in epithelial cell systems (10–12, 15–17). In addition, they have been functionally or causally implicated in developmental EMT processes, and their patterns of expression in carcinoma cell lines and tumors are fully compatible with their participation in tumor progression (7, 9). Nevertheless, it is still unclear whether different EMT programs, corresponding to particular states of epithelial plasticity, might be induced by different signals or factors (5, 8, 39). Moreover, it remains unclear whether the various inducers of EMT identified play a specific or redundant role in tumorigenesis.

To gain additional insight into the implication of Snail, Slug, and E47 in EMT and tumor progression, we analyzed the gene expression profile induced by each independent factor on the same genetic background. As such, we took advantage of the previously

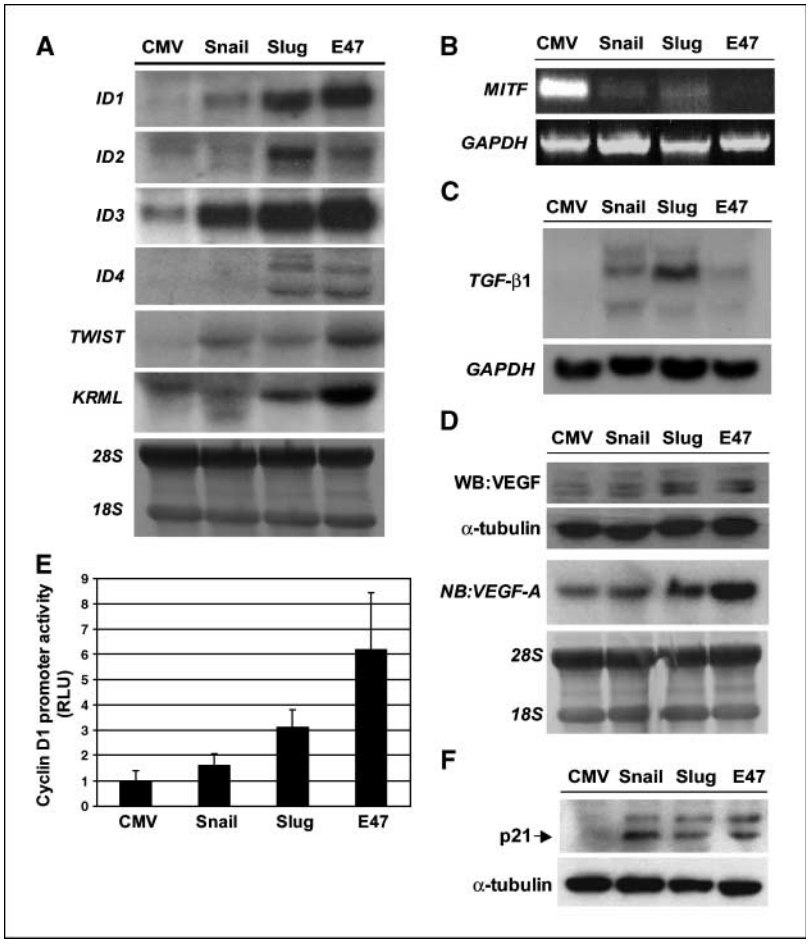

Figure 4. The Snail, Slug, and E47 transcription factors induce distinct transcription and cell signaling programs in MDCK cells. *A* and *B,* analysis of mRNA levels of the transcription factors indicated in the different cell lines by Northern blot (*A*) and RT-PCR (*B*). *C* to *F,* analysis of cell signaling and proliferation-related genes in the different cell lines. *C,* Northern blot analysis of TGF-β1 expression. *D,* VEGF detection at the protein (*top*) and mRNA level (*bottom*). *E,* activity of the cyclin D1 promoter in the cell lines indicated. The relative light units are normalized to that detected in control MDCK-CMV cells. *Columns,* mean of two independent experiments. *F,* Western blot analysis of p21Cip levels. α-Tubulin was used to control for protein loading (*D* and *F*). rRNA (*A* and *D*) or GAPDH (*B* and *C*) was used to control for RNA loading. Independent clones analyzed as in Fig. 2.

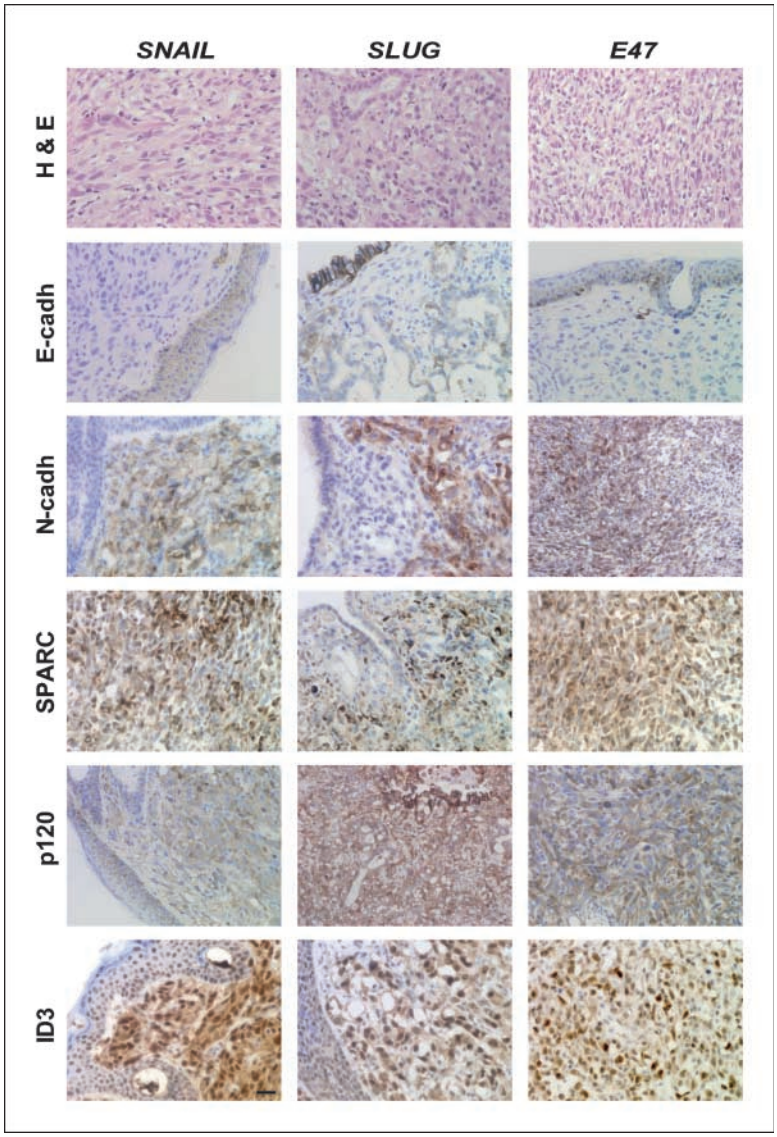

Figure 5. EMT markers are expressed in xenografts of MDCK-Snail, MDCK-Slug, and MDCK-E47 cells. Histologic (*top*) and immunohistochemical analysis (*second to bottom*) of E-cadherin, N-cadherin, SPARC, p120, and ID3 in the xenografts induced by Snail (clone B1; *left*), Slug (clone NC3; *middle*), and E47 (clone B1; *right*). See the mixed epithelial and spindle component of the Slug-xenografts. Bar, 150 μm. Note the positive control for E-cadherin stain in the adjacent epidermis of Snail and E47 xenografts.

characterized MDCK-Snail, MDCK-Slug, and MDCK-E47 cell lines stably expressing each of the transcription factors (11, 12, 16, 25), and compared gene expression in these cells with control mock-transfected MDCK cells using RAP-PCR and cDNA microarrays. Significantly, only 32 of the 228 (14%) candidate genes identified in the microarray analysis were regulated in a similar fashion (up- or down-regulated) by the three factors, whereas most of the genes were found to be regulated by different combinations of two of the factors or simply by just one of them. Therefore, these results indicate that Snail, Slug, and E47 might be involved in the regulation of common and specific pathways that contribute to general and particular aspects of EMT, as well as to other biological functions.

Unsupervised hierarchical clustering of the candidate genes showed that the genetic profile of MDCK-Slug and MDCK-E47 cells was more alike than that of MDCK-Snail cells. In principle, this was quite surprising because Snail and Slug are highly homologous members of the evolutionary conserved Snail gene family, sharing highly conserved DNA binding and transactivation domains (40). Indeed, these two genes are capable of mediating related developmental EMT events in distinct species (9). However, there are several lines of evidence indicating that these two members of the Snail family may play distinct roles, at least in mammals. For instance, whereas Snail is essential for early mouse development, being necessary for gastrulation to proceed correctly (41), Slug is dispensable during murine embryonic development but it is necessary in the adult for the differentiation and/or maintenance of specific cell lineages (42, 43). In addition, both factors have distinct patterns of expression in embryonic regions of EMT and/or in established mesenchymal tissues (9, 11, 44). The microarray data presented here fully support the differential implication of Snail and Slug in the regulation of specific gene programs, which, although sharing some common targets, are quite divergent. Thus, it seems likely that each factor is probably fulfilling specific functions in EMT and promoting distinct responses to external signals. In this context, the differential expression of some cell cycle mediators and effectors of apoptosis observed in Snail- and Slug-expressing cells might well be related to the different *in vivo* growth potentials observed for MDCK-Snail and MDCK-Slug xenografts.[7] The specific molecular mechanisms responsible for the differential

target regulation by Snail and Slug are currently unknown. The different affinities for specific E-boxes in gene promoters might account for the differential regulation of some genes, as shown for *E-cadherin* (12). We can also speculate that the recruitment of specific corepressors and coregulators by either factor could also be relevant (45). The studies necessary to clarify these important issues are presently under way.

In contrast, the strong similarity in the expression profiles of MDCK-Slug and MDCK-E47 cells, sharing 30% of the candidate genes, was somewhat unexpected. This uniformity might indicate that both factors converge on common primary or secondary target genes, thus regulating similar biological processes. Notably, the majority of genes common to Slug- and E47-expressing cells were up-regulated (49 of 59), suggesting that they may share common transcriptional regulators. Indeed, detailed inspection of the 18 transcription related genes shows that 6 of them are jointly up-regulated in MDCK-Slug and MDCK-E47 cells, including members of the HLH family and the leucine zipper family. The specific involvement of some of these transcription factors in the genetic programs elicited by Slug and E47 factors is presently under investigation. It will be particularly interesting to further understand the relationship and/or hierarchy between Slug/E47 and the Twist factors in specific stages of tumor progression like intravasation (17). In addition, the strong up-regulation of *ID1* and *ID2* transcripts observed in MDCK-Slug and MDCK-E47 cells, and of *ID3* in the three cell types (including MDCK-Snail cells), contrasted sharply with the down-regulation of *ID2* (and *ID3*) reported in EMT induced by TGF-β signals in other cell systems (46). Up-regulation of *ID3* mRNA by Snail, Slug, or E47 expression in MDCK cells was also confirmed at the protein level in the xenografts induced by the three cell lines. However, the striking differences in the global gene expression programs detected between MDCK-Snail and MDCK-E47 cells, particularly with respect to transcription and cell signaling genes, might explain the differential invasive and angiogenic behavior of both cell types *in vivo* (25). Together, these results provide evidence for the existence of distinct genetic programs underlying the EMT mediated by different signaling pathways and/or factors, in full agreement with the proposal of epithelial plasticity (5, 8, 39).

Remarkably, among the EMT-related genes detected in our microarray analysis, 16 (36% of *EMT* genes) were regulated in a common fashion by the expression of either Snail, Slug, or E47, whereas the rest seemed to be specifically regulated by only one or two of the factors. Analysis of the common regulated genes showed that 6 were down-regulated and 10 were significantly up-regulated (see Table 3). Importantly, most of them were validated in several independent clones from each transfectant cell type. It seems logical that these sets of genes should therefore participate in the acquisition of general functions common to the EMT process, such as the disruption of epithelial polarity and phenotype (E-cadherin and keratin 19), mesenchymal cell-extracellular matrix adhesion properties (collagen subunits Iα2 and IIIα1, integrin α5, and SPARC), remodeling of the extracellular matrix (PAI-1 and TIMP1), and dynamic actin cytoskeletal organization (caldesmon). In fact, some of these common genes have been functionally implicated in or associated with EMT (3, 5, 8, 9). Interestingly, some of these genes have recently been identified as targets of Snail or Slug in other cell systems: *SPARC* and *TIMP1* in melanocytes (47) or *cytokeratin 19* in breast cells (48). The other EMT-related genes that were seen to be differentially regulated by only one or two of the factors (like *CTGF* as a potential target of Snail) may contribute to specific aspects of the process that may only be required in some specific biological situations. Indeed, these genes may confer particular migratory and/or metastatic properties to tumor cells, as suggested for *CTGF* in bone metastasis of breast carcinomas (49). These important issues should be clarified in further studies. In this context, it also interesting to note that our analysis revealed few similarities with the genes regulated after transient expression of Snail in colon carcinoma cells (37). However, the important differences in the experimental design of both studies, including induced versus stable expression of Snail, absence of complete EMT in the colon system, and use of different microarray platforms, preclude a direct comparison.

Immunohistochemical analysis of the xenograft tumors generated by the MDCK transfectants confirmed the *in vivo* expression of related EMT markers. Indeed, this approach showed that the expression of Snail and E47 produced spindle cell tumors, whereas the expression of Slug induced tumors with mixed carcinomatous and sarcomatous components, consistent with the diagnoses of carcinosarcoma. These results further support the differential implication of Snail and Slug factors and also indicate the reversibility of the EMT phenotypes induced, supporting the previous proposal (3, 7) that EMT is a dynamic and transient process during tumor progression. Analysis of the expression levels of each factor in tumor biopsies should contribute to clarify the specific participation of the factors in tumor progression, something that awaits the availability of specific and reliable antibodies to each factor.

Acknowledgments

Received 2/9/2006; revised 6/2/2006; accepted 7/26/2006.

Grant support: Spanish Ministry of Education and Science grants SAF2001-02819, SAF2004-00361 (A. Cano), SAF2002-00817, SAF2005-03259 (A. Fabra), and SAF2004-08258 (J. Palacios); Instituto de Salud Carlos III grants PI050656 (A. Cano), PI051890 (J. Palacios), and RTICCC, FIS03, C03/10; BEFI fellowships 01/1695 and 01/9132 (E. Cubillo and D. Sarrió, respectively); and FPI fellowship (S. Villa) and postdoctoral contract (H. Peinado) from the Fundación Científica de la AECC.

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We thank Amalia Montes, Vanesa Santos, and Raquel Marcos for their excellent technical collaboration; Centro Nacional de Investigaciones Oncológicas Biotechnology Units (Immunohistochemistry, Comparative Pathology and Confocal Microscopy); and our colleagues who provided the reagents.

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