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ORIGINAL ARTICLE

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Gene expression profiling: cell cycle deregulation and aneuploidy do not cause breast cancer formation in WAP-SVT/t transgenic animals

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Abstract Microarray studies revealed that as a first hit the SV40 T/t antigen causes deregulation of 462 genes in mammary gland cells (ME cells) of WAP-SVT/t transgenic animals. The majority of deregulated genes are cell proliferation specific and Rb-E2F dependent, causing ME cell proliferation and gland hyperplasia but not breast cancer formation. In the breast tumor cells a further 207 genes are differentially expressed, most of them belonging to the cell communication category. In tissue culture breast tumor cells frequently switch off WAP-SVT/t transgene expression and regain the morphology and growth characteristics of normal ME cells, although the tumor-revertant cells are aneuploid and only 114 genes regain the expression level of normal ME cells. The profile of re-transformants shows that only 38 deregulated genes are tumor-specific, and that none of them is considered to be a typical breast cancer gene.

Keywords Breast cancer · Mammary gland epithelial cells · SV40 T/t antigen · Tumorigenesis · WAP-SVT/t transgene

Abbreviations *CA*: Correspondence analysis · *DMEM*: Dulcecco's modified Eagle medium · *IRF*: Interferon regulatory factor · *ME*: Mammary gland epithelial · *OAS*: 2′-5′-Oligoadenylate synthetase family members · *PBS*: Phosphate-buffered saline · *SV40*: Simian virus 40 · *WAP*: Whey acidic milk protein

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Introduction

Breast cancer is still one of the most frequent and fatal neoplasms in women in Western countries. The current concept proposes that tumorigenesis is a multistep event

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which includes different genetic and epigenetic alterations [1]. In most cases the primary hit remains unknown, and the subsequent molecular biological alterations leading from hyperplasia to neoplasia are not well understood. Alterations which affect cell-proliferation, cell-differentiation, and genomic stability are considered to be crucial for initiation and cancer progression. There are different genes which are frequently mutated or deregulated in breast cancer cells, including the gatekeeper p53 and cell cycle key regulators such as retinoblastoma protein [2], different cyclins (e.g., D), cyclin-dependent kinases and members of the signal transduction pathways such as Her/neu2 [3], and transcription factors such as cMyb [4]. Microarray studies have revealed that the gene expression profile among different breast tumors is variable, but differentiation between tumor-specific genes and secondary modifications is not conclusive [5].

To obtain more detailed information about the molecular mechanism leading to tumor formation different transgenic mice lines were established which express tumor genes in mammary gland epithelial cells (ME cells) such as the simian virus 40 (SV40) T/t antigen (T/t antigens are considered here as a unit). Although there is no indication that the T/t antigen is involved in human breast cancer formation, it is frequently used as a model system to study tumorigenesis. The SV40 T/t antigen is one of the strongest known tumor proteins, with a high mitogenic potential [6] which functionally inactivates the p53 and Rb [7] among others. However, loss of the p53 checkpoint function per se is not sufficient to initiate breast cancer formation, since $p53^{-/-}$ mice (strain C57BL/6) do not exhibit an increased sporadic breast cancer rate [8].

In contrast, all whey acidic milk protein (WAP)–SVT/t transgenic animals, which selectively synthesize the T/t antigen in ME cells, develop breast cancer after the first lactation [9]. Clearly T/t antigen synthesis is the initiating factor, but breast cancer formation requires further still unknown alterations. Considering the enormous number of T/t antigen positive ME cells present per female and the long latency period of 6–12 month before tumor formation occurs, malignant cell transformation is a rare event. However, in tissue culture SV40 T/t transforms ME cells at a low efficiency, and cells with tumor growth characteristics appear mostly around passage numbers 15–17 [10].

In this investigation we used microarray technology to characterize the gene expression profile of ME cells from NMRI animals, from WAP-SVT/t animals (NMRI is the founder mouse), and from breast tumor cells. These experiments revealed that as a first hit the SV40 T/t antigen alters the expression profile of 462 genes. The majority of the differentially expressed genes are cell cycle key regulators and are Rb-E2F related [11]. In the breast tumor cells an additional 207 genes are deregulated, the majority of them belonging to the cell communication category. Hence the expression profile of 669 genes is altered during the transition from normal to breast tumor cells. To differentiate between tumor-related and tumor-specific genes we generated tumor-revertant ME cell lines from breast tumors (revertant ME-B cells).

Although the revertant ME-B cells lost the growth characteristic of the breast tumor cells (e.g., growth in soft agar and in animals), only 114 genes regained the expression profile of the normal ME cells. This indicates that the SV40 T/t antigen deregulates a large number of genes, including the major cell cycle regulators, but that these alterations are not sufficient for the maintenance of the tumor state. Finally, the revertant ME-B cells were retransformed with SV40 T/t, and we found that of the 114 tumor-related genes only 38 were again differentially expressed as in the original breast cancer cells. This group includes genes that belong to the immune defense system (e.g., *H2-K1*, *OAS12*, *Irf7*), to the extracellular matrix (e.g., *Col11a1*, *Smoc1*), and to signal pathways (e.g., *Fgfrl1*, *Fzd9*).

Materials and Methods

Mammary gland biopsy

Inguinal mammary glands were isolated from NMRI (founder animal) and WAP-SVT/t transgenic mice. All WAP-SVT/t animals were heterozygous for the WAP-SVT/t transgene. WAP-SVT/t:$p53^{+/-}$ and WAP-SVT/t:$p53^{-/-}$ hybrid animals were generated by mating the WAP-SVT/t animals [9] with $p53^{-/-}$ animals (strain C57BL/6) [12] and identified by PCR analysis.

Histology

Tissue segments from breast tumors were fixed in 10% formaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Thin sections (4.5 nm) were stained with hematoxylin/eosin.

ME cell lines

To establish ME cell lines small mammary gland tissue segments were isolated from mammary glands and from breast tumors, transferred into tissue culture dishes, and cultivated in Dulcecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum [10]. To obtain the ME-BT/t cells the revertant ME-B cells were transfected with the pSVT/t DNA construct using the calcium phosphate method.

RNA preparation

RNA was isolated from 100 mg mammary gland tissue segments stored in liquid nitrogen from three normal NMRI and three WAP-SVT/t mice on the first day of lactation, from three WAP-SVT/t breast cancers (583 tumor 1, 585 tumor 2, 597 tumor 3), and from 2×10^7 cells of three distinct breast cancer derived cell lines (ME-A cells, revertant ME-B, cells and ME-B-T/t cells). The frozen tissue segments were ground to a fine powder using a liquid

nitrogen chilled mortar and pestle. Total RNA was extracted with 2 ml RNAzol reagent in accordance with the manufacturer's protocol (PeQLab, Biotechnology), and 100 µg of the isolated RNA was treated with 2 U RNase-free DNase (DNA-free Ambion, Austin, Tex., USA). The integrity of RNA was verified with the Agilent Bioanalyzer (Agilent Technologies) by the presence of prominent 28S and 18S bands on agarose gels and an A260/280 ratio in the range of 1.9–2.1.

Microarray analysis

Of total RNA 7 µg was used for production of biotinylated cRNA as described in the Affymetrix GeneChip analysis instruction manual (Affymetrix, Santa Clara, Calif., USA). In short, RNA was converted into cDNA using T7-oligo (dT) primer followed by the synthesis of double-stranded DNA with the double-stranded DNA synthesis kit (SuperScript Invitrogen, Life Technologies). The double-stranded DNA was transcribed in vitro into biotinylated cRNA by RNA Transcript labeling Kit (Enzo Life Sciences) and the synthesized cRNA was fragmented in 5× RNA fragmentation buffer (200 mM Tris-acetate, pH 8.2, 500 mM KOAc, 150 mM MgOAc) at 94°C for 35 min.

The Affymetrix GeneChip Mouse Expression Set 430A was then hybridized with the biotin-labeled cRNA fragments for 16 h at 45°C. Washing steps for the chip, staining with streptavidin-phycoerythrin, and signal amplification were performed according to the manufacturer's instructions (Affymetrix). Each hybridized Affymetrix GeneChip array was scanned with GeneChip Scanner 3000.

Data analysis

The raw experimental microarray data were normalized with the Affymetrix Microarray Suite (MAS 5.0) based on the housekeeping gene method. Expression values obtained were adjusted to the intensity of the corresponding expression value of 100 housekeeping genes. Transcripts that were found to be absent in all experiments were excluded from the data set. A subsequent data discrimination step was performed to exclude transcripts with average signal values lower than the minimum threshold of 20 in all experiments. The following groups were defined: normal animals (NMRI) on first day of lactation, transgenic animals (WAP-SVT/t) on first day of lactation, breast tumors (583 tumor 1, 585 tumor 2, 597 tumor 3), and tissue culture cell lines (ME-A cells, revertant ME-B cells, ME-B-T/t cells).

Differential expression

For ratio analysis, up- and downregulation were defined whenever each expression value of each group (three values) was higher or lower by factor 2.0 at least than each expression value of the other group.

Rescreening was carried out by correlation analysis. Transcripts were considered similar if the threshold of correlation (Pearson's correlation coefficient) was higher than 90%.

Visualization of the data set of the 12 independent experiments in a two-dimensional manner used the special numerical method of correspondence analysis (CA; the software *CorrXpression* was developed in our group). CA is based on methods described elsewhere [13, 14].

Immunofluorescence staining

ME cells of the corresponding lines were washed with PBS (4°C) and paraformaldehyde (4% in PBS) fixed for 15 min at room temperature, PBS washed and treated with 0.1% Triton ×100 (in PBS) for 20–30 min at room temperature and again washed with PBS. Cells were incubated with the corresponding antibody for 30 min at 37°C. The following antibodies were used: hamster anti-SV40 T/t antigen (Graessmann, Berlin), rabbit anti-cyclin A (H-432, Santa Cruz Biotechnology), rabbit anti-Steap (H-105, Santa Cruz Biotechnology), anti-H2-K1 (AF6-88.5, BD Biosciences Pharmingen), and anti-Mad2 (#610678, BD Biosciences Pharmingen). Rhodamine-conjugated sheep anti-mouse (Jackson Immuno Research 515-025-003), rhodamine, or Cy^{TM}2-conjugated goat anti-rabbit (Jackson Immuno Research 111-025-003, 11-225-144) and rhodamine conjugated rabbit anti-hamster (Jackson Immuno Research 309-025-003) were used as second antibody; incubation time 30 min at 37°C.

Chromosome preparation and in situ hybridization

ME-A and revertant ME-B cells were incubated for 1 h in DMEM with colcemid (0.15 µg/ml) at 37°C, centrifuged at 200 g for 6 min. The cell pellet was incubated in 4 ml hypotonic solution (60 mM KCL) for 6 min at room temperature and again centrifuged at 200 *g* for 6 min. The supernatant was removed, and cells were fixed (methanol: acetic acid 3:1) at room temperature for 15 min. For chromosome preparation three to five drops of the cell suspension were placed on a carefully cleaned glass slides (76×26 mm), air-dried, and the chromosome-slides were stored at 4°C for 5–7 days (aging). To denature the chromosomal DNA, the glass slides were dipped into denaturing solution (70% deion. formamide 2× sodium saline citrate) for 2 min at 70°C, washed in 2× sodium saline citrate, and alcohol-dehydrated at 4°C. For in situ hybridization T7-based biotinylated SV40 T antigen antisense RNA was prepared by in vitro transcription of the linearized pTyprs DNA [15]. We added 50 ng biotinylated RNA in 10 µl hybridization buffer (60% deion. formamide, 2× sodium saline citrate, 10 mM EDTA, 25 mM NaH_2PO_4 pH7.4, 5% dextran sulfate, 250 ng/µl sheered salmon sperm DNA) to the slide and cover with a cover slip. After overnight incubation at 42°C the cover slip was removed, and the slide was washed 2x for 5 min at room temperature

(50% deion. formamide 2× sodium saline citrate) and 2× at 42°C. After blocking for 30 min at 37°C (Blocking Reagent Roche 1096176, 0.5% in 0.1 M Tris, pH7.5, 0.1 M NaCl), 100 µl of the rhodamineavidin (Dianova) at a dilution 1:200 was added, and the slides were incubated for 30 min at 37°C and further processed for immunofluorescence microscopy (Zeiss Axioskop).

Results

Gene expression profile in ME cells of normal animals

To determine the gene expression profile of mammary gland epithelial cells (ME cells) on the first day of lactation mammary gland tissue segments were prepared from NMRI animals. RNA was extracted and 7 µg RNA were used to prepare T7-based biotinylated antisense RNA (cRNA) in accordance with the manufacturer's instructions (Affymetrix), as described briefly above. The biotinylated cRNA obtained was subjected to microarray analysis, using the Affymetrix MOE 430A ship which contains 22,626 mouse transcripts, representing 14,109 mouse-specific genes. The microarray data obtained were normalized with the Affymetrix Microarray Suite (MAS 5.0) based on the housekeeping gene expression profile. Expression values were adjusted to the intensity of the expression value of the 100 housekeeping genes. Transcripts that were found to be absent in the mammary glands of all three females analyzed on the first day of lactation were considered as nonessential and excluded from the data set. These experiments revealed that 8,742 genes are transcribed in the ME cells of the mammary gland on the first day of lactation. At this time the ME

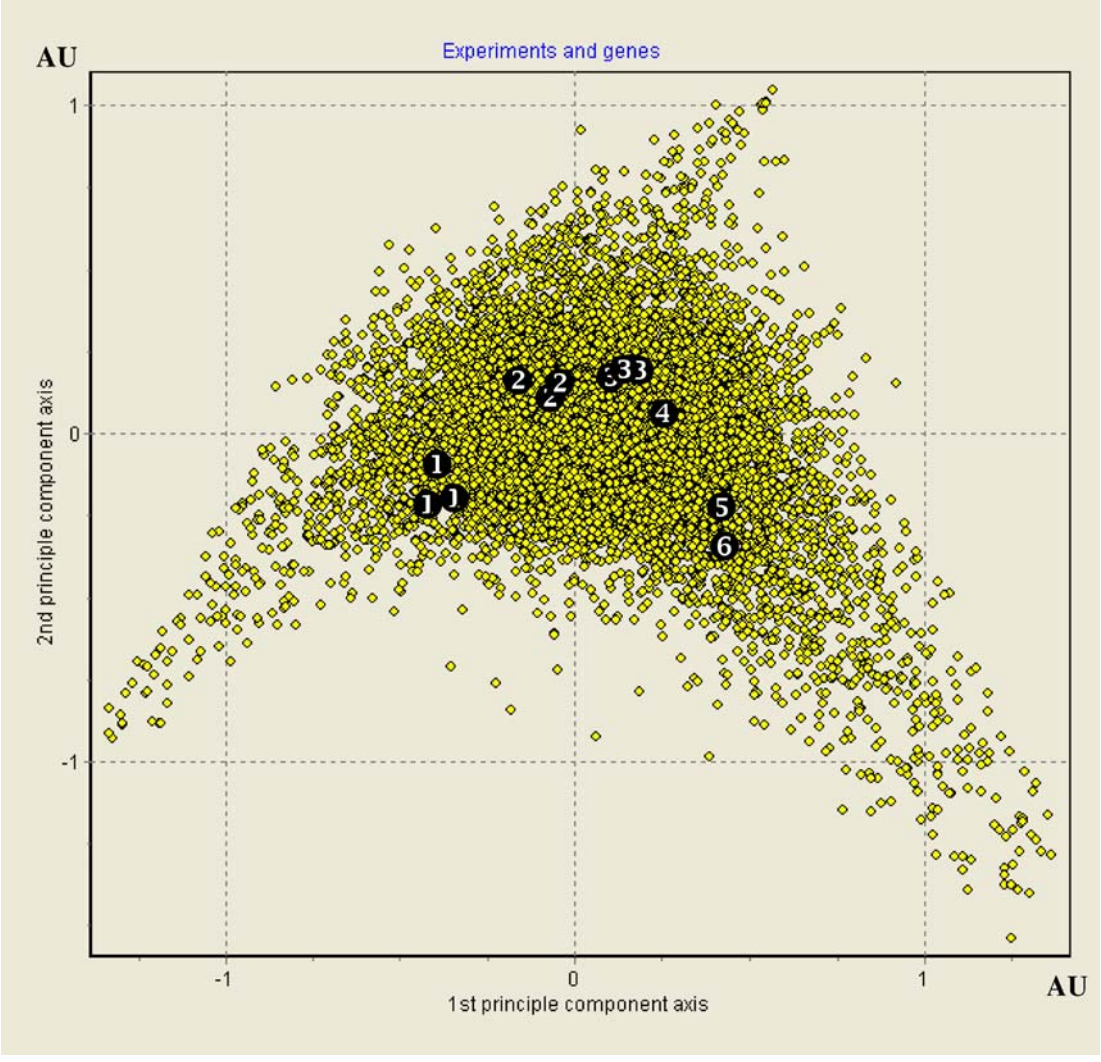

Fig. 1 Correspondence analysis. Presentation of all transcripts (*small cycles*) identified in the ME cells of the 12 analyzed samples in two-dimensional diagram. The relative position of the analyzed specimens in the diagram is indicated: *1* three normal animals on the first day of lactation; *2* three transgenic animals on the first day of lactation; *3* three breast tumors and three ME cell lines: *4* ME-A cells; *5* revertant ME-B cells; *6* ME-B-T/t cells. The expression profile of each transcript is presented at: http://www.medizin-fu-berlin.de/molbiochem/molbiol/tumbiol

cells are the dominant cell type of the mammary gland [9]. The correlation coefficient among the three analyzed lactating animals was between 0.95 and 0.96.

To further illustrate the close similarities of the expression profile of the three NMRI animals analyzed, the correspondence analysis (CA) was applied, which visualizes the transcripts in a two dimensional fashion. The CA diagram (Fig. 1) contains the 16,295 transcripts (10,452 genes) which represents the sum of the transcripts which were detected in the normal ME cells (NMRI animals), in the ME cells of the transgenic animals (WAP-SVT/t), in the breast tumor cells, and in the ME-tissue culture cell lines (see below). The black circles with the number 1 represent the position of the three NMRI animals, appearing as a cluster in this diagram.

Gene expression profile in ME cells of WAP-SVT/t transgenic animals

To determine the effect of the T/t antigen on the gene expression profile of ME cells mammary gland tissue segments were isolated from three WAP-SVT/t transgenic animals on the first day of lactation, and the extracted RNA was converted into antisense RNA and subjected to microarray analysis. As reported elsewhere, WAP-SVT/t transgenic animals selectively express the WAP-SVT/t transgene in the ME cells and initiation of T/t antigen synthesis occurs in synchrony with the endogenous WAP during the first pregnancy. In contrast to the WAP gene, WAP-SVT/t transgene expression is maintained at a high level in the ME cells after weaning [16].

These studies revealed that the gene expression profile of the ME cells was profoundly changed by the T/t antigen, and that 9,629 genes are transcribed in the mammary glands of the WAP-SVT/t animals vs. 8,742 genes in the mammary glands of NMRI animals. The correlation coefficient between the three animals analyzed was between 0.92–0.96. In the CA diagram (Fig. 1) the three WAP-SVT/t animals appear again as a cluster (2), separated from the position of the founder NMRI animals.

In further studies we considered only those genes that exhibited at least a twofold change in the expression level, and that were found to be differentially expressed in the ME cells (ME-T/t cells) of all three WAP-SVT/t animals analyzed. Under these conditions the T/t antigen changed

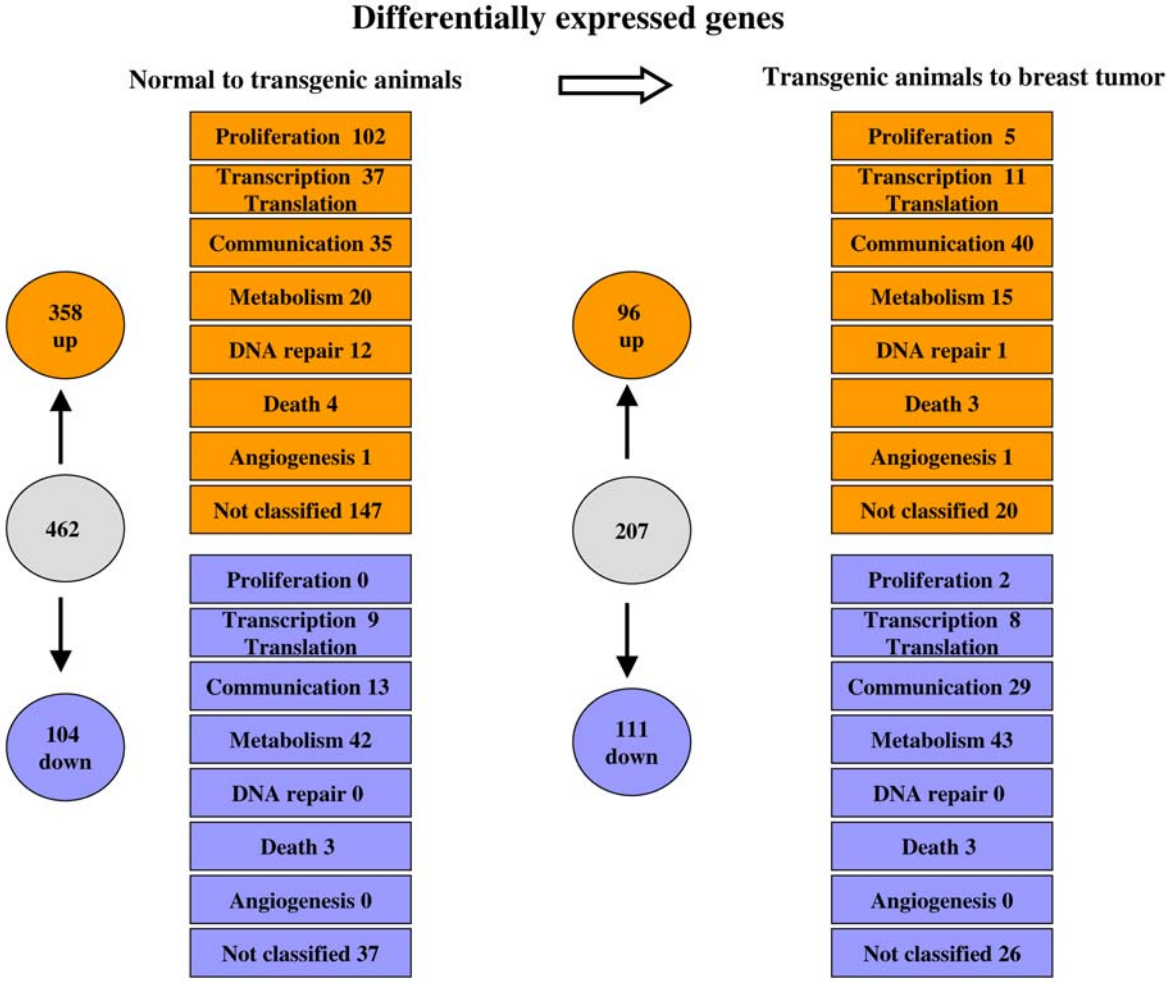

Fig. 2 Differentially expressed genes: from normal to transgenic animals and to breast cancer cells. The sum of the differentially expressed genes, the number of upregulated and downregulated genes, and the classified category are indicated

the expression profile of 462 genes. Of the 462 genes differentially expressed in the ME-T/t cells 358 are upregulated and 104 downregulated (see Electronic Supplementary Material 1). As shown in Fig. 2 we classified these genes into the following eight categories: proliferation, communication, transcription/translation, cell death, DNA repair, metabolism, angiogenesis, and not classified.

The category with the most differentially expressed genes is cell proliferation. Of the 358 genes upregulated 102 are cell cycle specific (see Electronic Supplementary Material 1). That the T/t antigen deregulates the expression level of cell cycle key regulators was not unexpected since the SV40 T antigen is known to be a strong mitogen [6] that also stimulates ME cell proliferation [9]. Of the 102 upregulated genes 40 are Rb-E2F related (e.g., cylin A, cylin B, cyclin E, *cdk2*, *cdk1*, *DHFR*, DNA polymerases α, δ), confirming that functional inactivation of the Rb is one of the early direct SV40 T antigen hits, causing ME cell proliferation. Upregulation of cyclin A and *cdk1* was further demonstrable by immunohistology (data not shown). Other key players of the cell cycle that are upregulated (e.g., *cdk4*, *cdc7*, *cdc25C*, cylin F, *chek1*), including the telomerase gene, are E2F independent and are presumably indirectly modulated by the T/t antigen. Telomerase upregulation was further verified by enzyme-linked immunosorbent assay (PCR-ELISA^{Plus}, Roche; data not shown).

Gene expression profile in ME cells of breast tumor

A further feature of the WAP-SVT/t transgenic animals is the high breast cancer rate. Clearly T/t antigen synthesis is the initiating hit since breast cancer formation in NRMI animals is a very rare event (<0.1%), and all WAP-SVT/t transgenic females analyzed (>600) developed breast cancer after the first lactation. However, T/t antigen synthesis per se is not sufficient to cause breast cancer formation. Although ME cells of all mammary glands synthesize the T/t antigen with similar efficiency, tumor formation occurs mostly in single glands of the inguinal or axilar region and in a clonal-like fashion after a latency period of several months [16]. This indicates that conversion of ME-T/t cells into tumor cells requires further hits. To typify the expression profile of ME tumor cells RNA was extracted from the ME cells of three independent

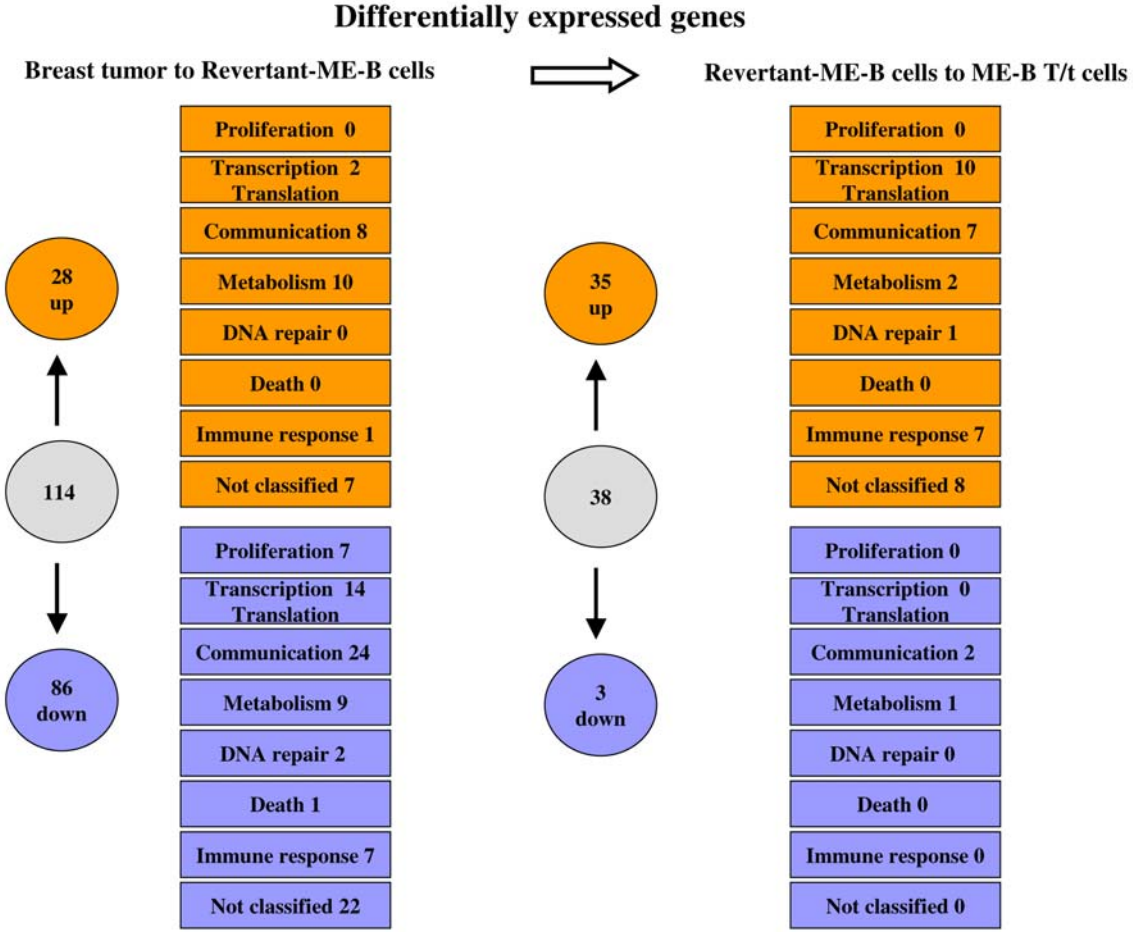

Fig. 3 Differentially expressed genes: from breast tumor to the revertant ME-B- cells and to retransformed ME-B-T/t cells. The sum of the differentially expressed genes, the number of upregulated and downregulated genes, and the classified category are indicated. Genes that are upregulated in the revertant ME-B cells were downregulated in the breast tumor cells or vice versa for the downregulated genes

breast tumors having a diameter of at least 1.5 cm (animal number: 583 tumor 1, 585 tumor 2, 597 tumor 3). The three tumors have a correlation coefficient between 0.92 and 0.93 and appear again as a cluster (3) in the CA diagram (Fig. 1).

Microarray studies revealed that 207 genes are further differentially expressed in the breast cancer cells; 96 genes are upregulated and 111 genes downregulated (Fig. 3; see Electronic Supplementary Material 2). As summarized in Fig. 3, the majority of the differentially expressed genes in the ME tumor cells belong to the cell communication category. Among the 40 upregulated genes 24 are part of the signal transduction pathway (e.g., *Efemp2*, *Igfbp3*, *Rap1ga1*), 15 are cell adhesion specific (e.g., *Mia*, *Mmp16*, procollagen IX α2), and one is involved in cell-cell signaling (*Gja1*). The 29 downregulated genes include 16 that encode for signal transduction proteins (e.g., *Camk2d*, *Pthr1*, *Sncg*) and 13 for cell adhesion proteins (e.g., *Ceacem10*, *CD36*, procollagen XVIIα1).

Reversion of the tumor cell status

The experiments completed to date reveal that 669 genes are differentially expressed in breast cancer vs. normal ME cells. However, this is not conclusive proof that all differentially expressed genes are essential for tumorigenesis. Tumor revertant ME cell lines were established (e.g., revertant ME-B cells) to identify which of these alterations are tumor-related and which are tumor-specific.

Since in the ME cells of the transgenic animals T/t antigen synthesis is under the transcriptional control of the WAP promoter, which is regulated by various lactotrophic hormones (e.g., estrogen, progesterone, prolactin, insulin, and glucocorticoids) and different local growth factors [17], WAP-SVT/t gene expression is frequently downregulated when breast tumor tissue segments are used to establish tissue culture cell lines.

Accordingly, cells that were formerly of tumor origin regain the morphology and growth characteristic of non-tumor cells. This transition occurs mostly within the first days after plating under standard tissue culture conditions (DMEM, 10% fetal calf serum). These T/t antigen negative ME cells fail to form colonies in soft agar and do not form tumors after inoculation into test animals. Less frequently, ME cell tissue culture cells lines can be established that show continuous T/t antigen synthesis and are permanently T/t antigen positive. These cell lines exhibit the morphology and growth characteristics of tumor cells [10].

To obtain revertant tumor cells small tissue segments from the tumor 1 (Fig. 4a) were isolated and used to establish ME cell lines. Although the ME cells of this tumor were T/t antigen positive at the time of isolation as shown by immunofluorescence staining (Fig. 4b) and reverse-transcription PCR analysis (data not shown), the vast majority of the ME tumor cells switched off WAP-SVT/t transgene expression during early passages in tissue culture (passages 2–3), and a T/t antigen negative cell line was established (revertant ME-B cells). The T/t antigen negative revertant ME-B cells (Fig. 4f) gained the flat morphology of normal ME cells (Fig. 4d) and failed to form colonies in soft agar (Fig. 4 h).

The second cell line obtained from tumor-1 segments are T/t antigen positive ME cells (ME-A cells). The ME-A cells exhibit the morphology (Fig. 4c) and the growth characteristics of breast cancer cells (Fig. 4g). The expression profile of the 669 tumor-related genes was not altered during establishment of the ME-A cell line and remained similar to the profile of the original breast tumor cells (data not shown). However, a change in the gene expression profile was demonstrable for the T/t antigen negative revertant ME-B cells. Microarray analysis revealed that of the 669 tumor-related genes 609 are still differentially expressed in the revertant ME-B cells. Only 60 genes regained the expression profile specific for the ME cells of the nontransgenic NMRI animals.

At this state we rescreened the entire microarray data by correlation analysis to identify further tumor-specific genes that were not characterized under the stringent conditions of the ratio analysis applied so far. In this way a further 54 genes were found that are differentially expressed in all breast tumor cells vs. the normal ME cells and the revertant ME-B cells. Therefore altogether 114 genes were considered to be tumor-specific (see Electronic Supplementary Material 3).

Proliferation

Of the 107 proliferation specific genes that are finally upregulated in the breast tumor cells 100 are still differentially expressed in the revertant ME-B cells, exhibiting a similar expression profile to the breast tumor or the T/t antigen positive ME-A cells. These include the majority of the Rb-E2F related genes, and this is shown for cyclin A2 and *Mad2* as examples (Fig. 5). Overexpression of these genes was also demonstrated by reverse transcription PCR analysis (data not shown) and immunofluorescence staining (Fig. 6a–d). As a consequence of this continuous gene upregulation revertant ME-B cells exhibit a similar proliferation rate to the ME-A cells (data not shown) but do not exhibit further growth characteristics of tumor cells such as growth in soft agar (Fig. 4h) or tumor formation in animals (data not shown). The seven cell proliferation specific genes which regained normal ME cell expression levels include the Rb-E2F related myeloblastosis oncogene (*cMyb*) and the replication factor C (*Rfc4*).

Transcription/translation

During the conversion of the breast tumor cells into the revertant ME-B cells 14 transcription/translation relevant genes are downregulated to the expression level of the normal ME cells (Fig. 3). Among these seven are regulators of transcription (e.g., *Hey1*), two are chromatin modulators (e.g., *H1f0*), and four are part of the ubiquitin protein degradation system (e.g., *Psmb9*). In the revertant

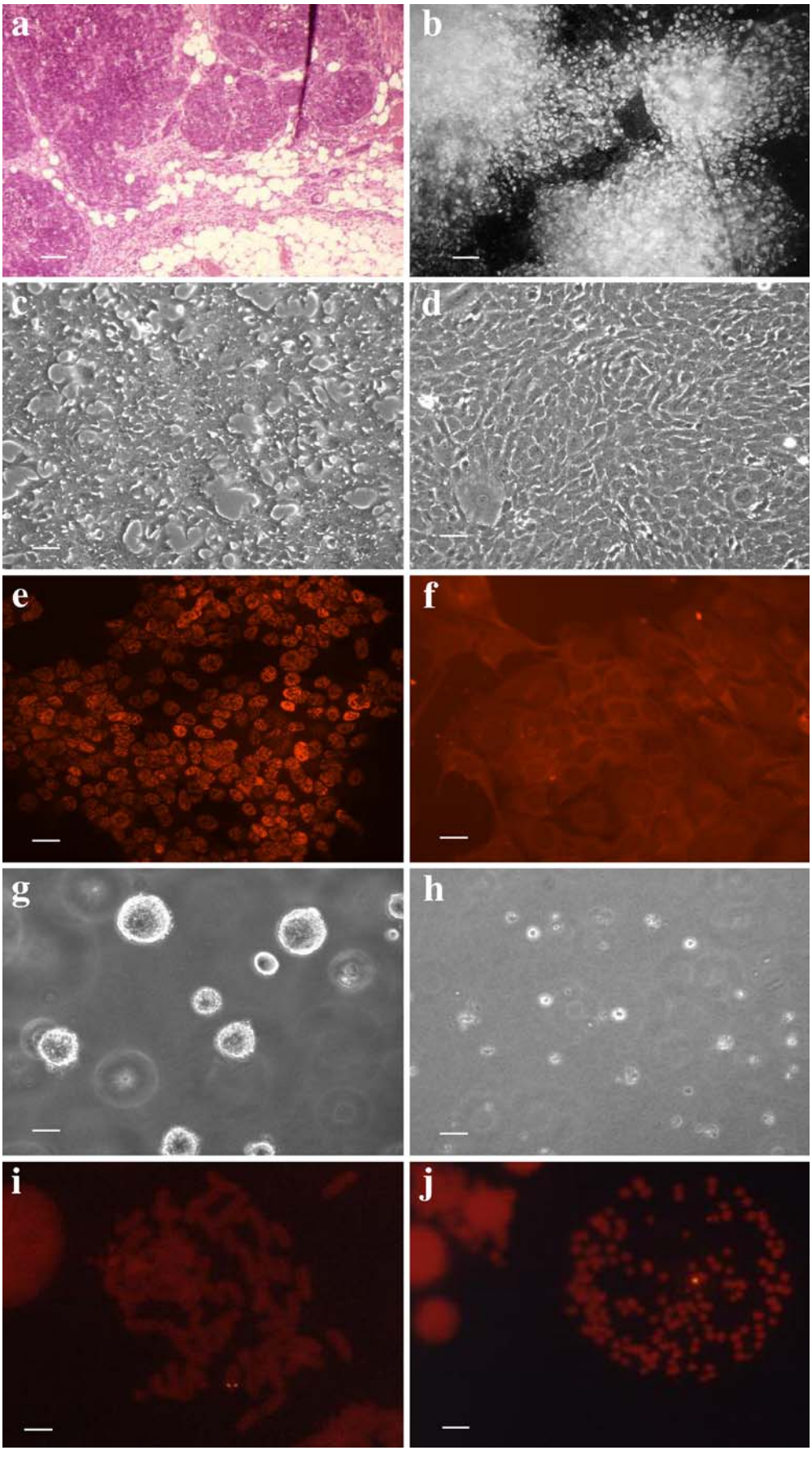

Fig. 4 Histology and immunofluorescence staining. **a** Histological section obtained from the breast tumor 583 tumor 1, hematoxilin/eosin stained. **b** Breast tumor 583 squeeze-preparation, fixed and stained with anti-SV40 T/t antigen antibody. **a**, **b** *Scale bars* 62.5 µm. **c** ME-A cells: phase contrast picture. **d** Revertant ME-B cells: phase contrast picture. **e** ME-A cells: stained with anti-SV40 T/t antigen antibody. **f** Revertant ME-B cells: stained with anti-SV40 T/t antigen antibody. **g** ME-A cells: growth in soft agar. **h** Revertant ME-B cells: growth in soft agar. **c**–**g** *Scale bars* 25 µm. **i** ME-A cells: chromosome preparation and in situ hybridization for SV40 DNA; cells are heterozygous for the WAP-SVT/t transgene. **j** Revertant ME-B cells: chromosome preparation and in situ hybridization for SV40 DNA; cells are heterozygous for the WAP-SVT/t transgene. **h**, **j** *Scale bars* 6.25 µm

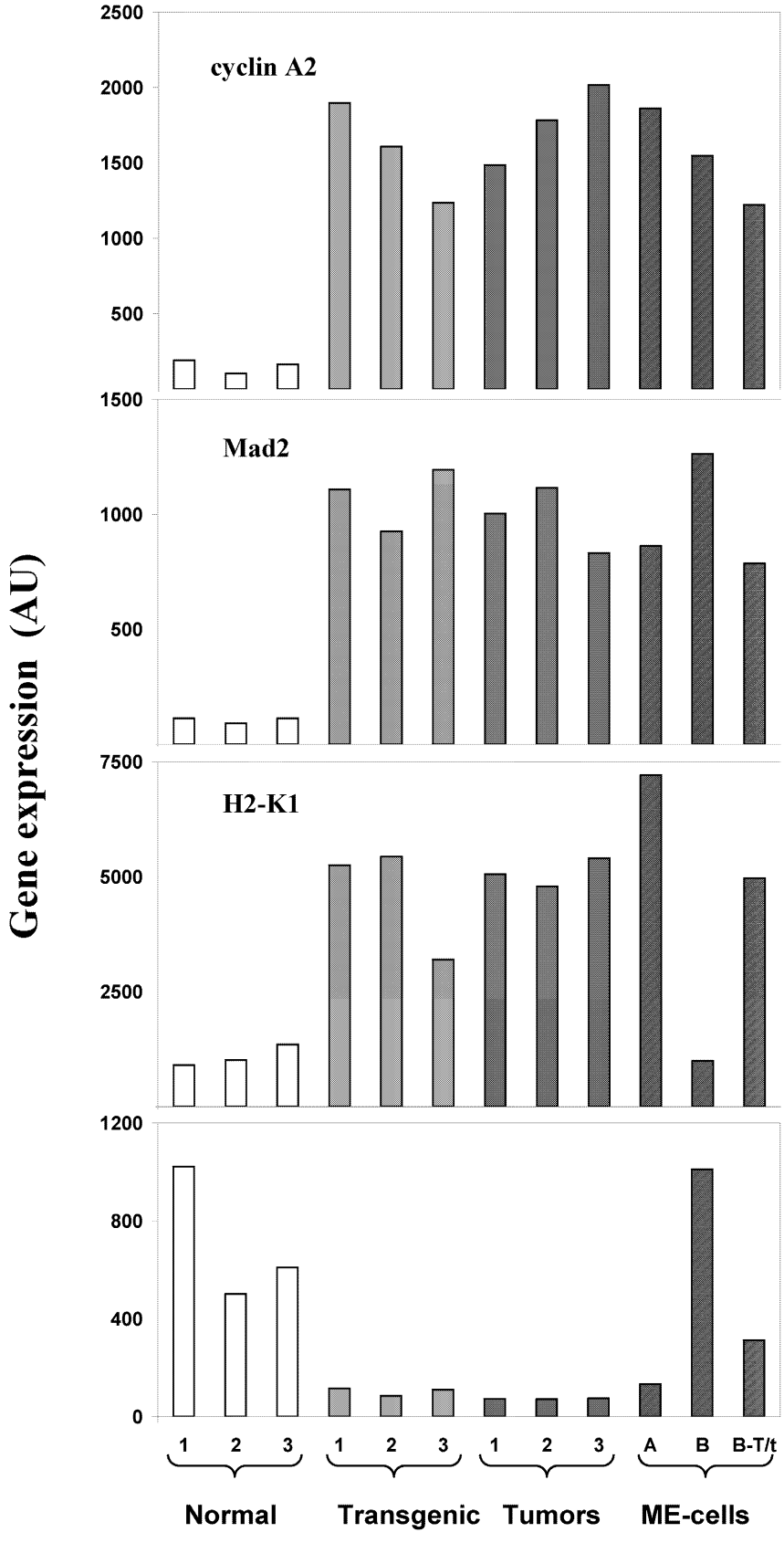

Fig. 5 Expression profile of four differentially regulated genes. Figure shows examples of the expression profile of cyclin A2, *Mad2*, *H2-K1*, *Steap* in the ME cells of three independent normal animals (*1*, *2*, *3*), three WAP-SVT/t transgenic animals (*1*, *2*, *3*) during lactation, of the three breast tumors (animal number: 583 tumor 1, 585 tumor 2, 597 tumor 3) in the ME-A cells (A), the revertant ME-B cells (*B*) and in the retransformed ME-B-T/t cells (*B-T/t*). The expression values are shown in arbitrary unit (*AU*)

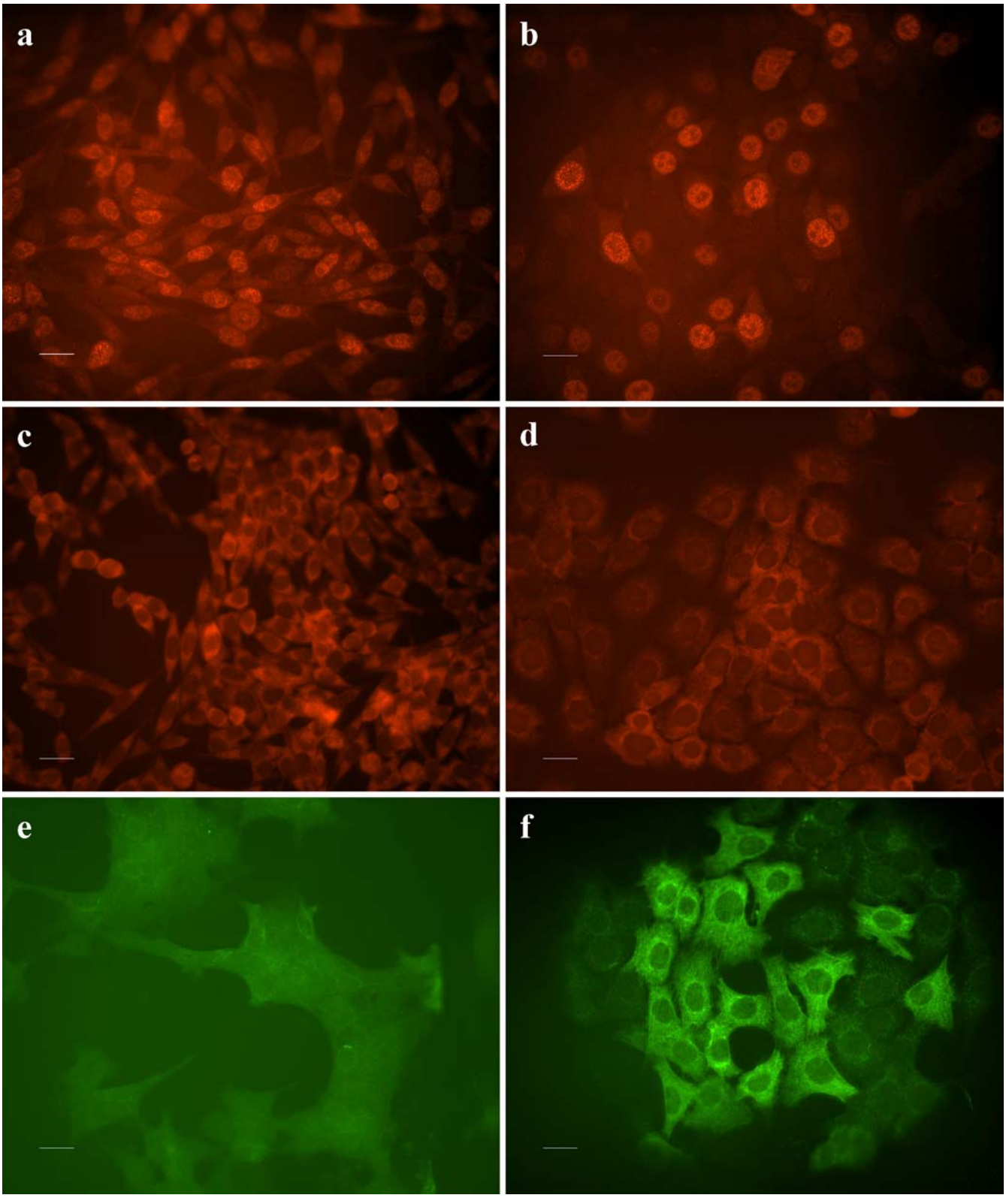

Fig. 6 Histology and immunofluorescence staining. **a** ME-A cells stained with anti-cyclin A antibody. **b** Revertant ME-B cells stained with anti-cyclin A antibody. **c** ME-A cells stained with anti-Mad2 antibody. **d** Revertant ME-B cells stained with anti Mad2 antibody. **e** ME-A cells: stained with anti-Steap antibody. **f** Revertant ME-B cells stained with anti-Steap antibody. **a**–**f** *Scale bar* 25 μm

ME-B cells two regulators of transcription are again upregulated (*Idb4*, *Trp63*).

Communication

Of the 114 genes classified as tumor specific 32 belong to the communication category, with 14 encoding for extracellular matrix (e.g., type XIα1 procollagen) and cell adhesion proteins, for example, the *six transmembrane epithelial antigen of prostate* (*Steap*, Fig. 5). Eighteen genes are involved in signal transduction (e.g., *Fgfrl1*).

Metabolism

Ten genes that were downregulated in breast tumor cells regained the expression levels for normal ME cells, and nine of the genes that were overexpressed in tumor cells were again downregulated (Fig. 3).

DNA repair

The loss of T/t antigen in the revertant ME-B cells caused the downregulation of two DNA-repair related genes (*Rad54l*, *Trpc2*).

Cell death

The Bcl2/adenovirus E1B interacting protein is the only cell death linked gene that is downregulated in the revertant ME-B cells.

Immune response

The microarray studies further revealed that SV40 T/t antigen synthesis is strongly correlated with upregulation of seven genes belonging to the immune defense system. This is the reason why the new immune response category was added (Fig. 3). The upregulated genes comprise three major histocompatibility complex I genes (*H2-K1*, *H2-T10*, *H2-T23*), the 2′-5′-oligoadenylate synthetase 1G and the 2′-5′-oligoadenylate synthetase-like2 in addition to the interferon regulatory factor 7 and interferon α inducible protein. The expression profile of *H2-K1* is illustrated in Fig. 5.

Not classified

Among the 29 genes in the nonclassified category the biological relevance of 21 is still unknown, two genes are involved in protein degradation (*Klk24*, *Serpinb6b*), and two are attributed to brain development (*Abr*) and epidermis development (*Cst6*). Further genes include coagulation factor III, prominin 1, RIKEN cDNA 1810017F10 gene and t complex testis expressed 1.

Retransformation of the revertant ME-B cells by SV40 T/t

To test whether it is possible to further diminish the number of tumor-specific genes the revertant ME-B cells were again reconverted into tumor cells by SV40 T/t. Since we failed to reactivate WAP-SVT/t transgene expression in the revertant ME-B cells by addition of various lactogenic hormones such as estrogen, progesterone, prolactin, insulin, and hydocortisone, the pSVT/t DNA construct was transfected into revertant ME-B cells and SV40 T/t antigen positive cell clones were G418 selected. These clones (>100) were collected and designated as ME-B-T/t cells. The ME-B-T/t cells regained the morphology and growth characteristic of the breast cancer cells (data not shown). RNA was extracted from the ME-B-T/t cells and subjected to microarray analysis. The data obtained were correlated with 114 tumor-specific genes. This experiment revealed that only 38 genes correlated with breast cancer formation, tumor cell reversion, and retransformation. The 35 upregulated genes are:

- Transcription translation 10
  - DNA segment, Chr 11, Lothar Hennighausen 2 (*D11Lgp2e*)
  - H_1 histone family, member 0 (*H1f0*)
  - Hairy/enhancer-of-split related with YRPW motif 1 (*Hey1*)
  - Proteosome (prosome, macropain) subunit, β type 9 (*Psmb9*)
  - RIKEN cDNA 1300010O06 gene
  - RIKEN cDNA 2310046K10 gene
  - RIKEN cDNA 2700084L22 gene
  - Sine oculis related homeobox 5 homolog (*Six5*)
  - Tripartite motif protein 34 (*Trim34*)
  - Zinc finger CCCH type domain containing 1 (*Zc3hdc1*)
- Communication 7
  - Agrin (*Agrn*)
  - Down syndrome critical region gene 1-like 2 (*Dscr1l2*)
  - Fibroblast growth factor receptor-like 1 (*Fgfrl1*)
  - Frizzled homolog 9 (*Fzd9*)
  - Procollagen, type XI, α1 (*Col11a1*)
  - SPARC-related modular calcium binding 1 (*Smoc1*)
  - Tribbles homolog 2 (*Trib2*)
- Metabolism 2
  - 2,3-Bisphosphoglycerate mutase (*Bpgm*)
  - Iduronidase, α-L (*Idua*)

- DNA repair 1
  - RAD54-like (*Rad54l*)
- Immune response 7
  - 2′-5′ oligoadenylate synthetase 1G (*Oas1g*)
  - 2′-5′ oligoadenylate synthetase-like 2 (*Oasl2*)
  - Histocompatibility 2, K1, K region (*H2-K1*)
  - Histocompatibility 2, T region locus 10 (*H2-T10*)
  - Histocompatibility 2, T region locus 23 (*H2-T23*)
  - Interferon regulatory factor 7 (*Irf7*)
  - Interferon α-inducible protein (*G1p2*)
- Not classified 8
  - cDNA sequence BC013672
  - Coagulation factor III (*F3*)
  - Sht-human, similar to Shb-like adapter protein
  - RIKEN cDNA 1110012M11 gene
  - RIKEN cDNA 2300006M17 gene
  - RIKEN cDNA 2310016F22 gene
  - RIKEN cDNA 2310047I15 gene
  - Transformed mouse 3T3 cell double minute 1

The three downregulated genes are:

- Glutaredoxin 1 (*Glrx1*)
- Parathyroid hormone-like peptide (*Pthlh*)
- Six transmembrane epithelial antigen of the prostate (*Steap*)

Discussion

The objective of this investigation was to characterize the genetic alterations that are critical for breast cancer formation. The gene expression profile was analyzed in normal ME cells, ME cells of transgenic animals, breast cancer cells, tumor-revertant cells (revertant ME-B cells), and retransformants (ME-B-T/t cells). Our microarray experiments revealed that as a first hit SV40 T/t antigen alters the expression profile of 462 genes, with 358 genes being up- and 104 downregulated (Fig. 2). The majority of the upregulated genes belong to the cell proliferation category, and 40 genes are Rb-E2F regulated. As a consequence ME cells start to proliferate early during pregnancy (days 14–16), and this affects the differentiation program of the mammary gland (9, 16). After weaning the remaining T/t antigen positive ME cells continue to proliferate, and eventually mammary gland hyperplasia occurs.

However, events such as functional inactivation of p53 and/or Rb and upregulation of key cell cycle regulators are not sufficient to cause breast cancer formation. This is further documented by the fact that ME cells from transgenic animals (WAP-SVBst-Bam), which synthesize N-terminal truncated T antigen molecules (T1/T2 antigen), exhibit a similar gene expression profile as the WAP-SVT/t transgenic animals during lactation (data not shown). The T1 antigen associates with p53 and Rb with a comparable efficiency as the wt T antigen. Although T1/T2 antigen synthesis leads to hyperplasia, breast cancer formation was not observed in more than 500 animals analyzed [18]. Also $p53^{-/-}$mice (strain C57BL/6) do not exhibit a higher spontaneous breast cancer rate than control animals, and the p53 status does not significantly affect breast tumor formation in hybrid WAP-SVT/t:$p53^{+/-}$ and WAP-SVT/t:$p53^{-/-}$ animals [8]. Therefore the 462 deregulated genes do not cause breast cancer formation. This requires further T/t antigen mediated alterations which then eventually lead to breast cancer formation.

Microarray experiments revealed that further 207 genes are differentially expressed, genes that were found to be additional deregulated in the three breast tumors analyzed. The majority of the deregulated genes belong to the cell communication category (Fig. 2). The expression profile of tumor-revertant cells (revertant ME-B cells) and of retransformants (ME-B-T/t cells) was analyzed in order to differentiate between tumor-related and tumor-specific genes. The loss of the T/t antigen in breast tumor cells under standard tissue culture conditions is frequent and associated with the conversion of breast tumor cells to nontumor cells.

However, it should be noted that we also established four T/t antigen negative ME cell lines from four different breast tumors, which remained highly tumorigenic. ME cells from three lines carry a p53 missense mutation at the codon 242, a mutation also frequently observed in human breast cancer cells (mouse codon 242 corresponds to human codon 245). The fourth cell line was obtained from a WAP-SVT/t:$p53^{-/-}$ tumor [10].

At present it is unclear which parameters determine WAP-SVT/t gene downregulation vs. continuous expression. In situ hybridization experiments (Fig. 4i, j), Southern blot analysis, and DNA sequencing experiments (data not shown) revealed that neither WAP-SVT/t mutations nor DNA rearrangement is the cause of the WAP-SVT/t transgene not being downregulated in the ME-A cells under standard tissue culture conditions (DMEM, 10% fetal calf serum).

We found that from the 723 tumor-related genes (669 by ratio analysis and 54 by correlation analysis) the expression level of 609 genes remained unchanged in the revertant ME-B cells and only 114 genes regained the expression level of the normal ME cells. In the T/t antigen positive ME-A cells the expression profile of the 723 tumor-related genes remained as in the original breast tumor cells. However, it should be pointed out that a large number of “nontumor” relevant genes were altered during the establishment of tissue culture cell lines. In the ME-A cells the expression profile of 1,189 genes was changed (351 up and 838 down) and 1,746 genes (600 up and 1,146 down) in the case of the revertant ME-B cells. This explains why the ME-A cells (4) and the revertant ME-B cells (5) shift away from the breast tumors in the CA diagram (Fig. 1).

However, different conclusions can be drawn from studies with the revertant ME-B cells: (a) The tumor status is reversible and occurs at a high frequency when T/t

antigen synthesis is blocked in the breast cancer cells. (b) Therefore it is unlikely that events such as gene mutation, aneuploidy, and sister chromatid exchange are the crucial alterations which lead to breast cancer formation. If so, reversion from tumor to nontumor cells would not occur at such a high frequency. Aneuploidy is frequent in human cancer cells, but it is still unclear whether chromosome instability is the cause or the consequence of the malignant phenotype. The fact that the revertant ME-B cells are still aneuploid (Fig. 4j) shows that chromosome instability is not the leading cause of breast cancer formation. Deregulation of the E2F transcription factor and *Mad2* overexpression is functionally related to aneuploidy [19]. Mad2 is a critical component of the spindle checkpoint and blocks activation of the APC/cyclosome complex necessary for sister chromatid separation. In the transgenic animals Mad2 and further sensor proteins (e.g., Bub1, Bub3) are upregulated even during the first lactation and remain overexpressed in breast tumor cells as well as in the T/t antigen positive and T/t antigen negative ME cell lines (Fig. 5). (c) Furthermore, the high mitotic rate of the ME cells is not only insufficient to initiate malignant cell transformation but is also not sufficient for the maintenance of the transformed state since the revertant ME-B cells have a similar high mitotic rate to the ME-A cells but exhibit the morphology and growth characteristic of normal ME cells. Of the 43 Rb-E2F (40 proliferation, 3 DNA repair) dependent genes upregulated after the onset of T/t antigen synthesis 41 remain upregulated in the T/t antigen negative revertant ME-B cells. At this stage we did not analyze how E2F is activated. Rb-phosphorylation by the upregulated cyclin-dependent kinases could be the regulatory step, but this would not explain why some Rb-E2F dependent genes are downregulated in the revertant ME-B cells.

As a further step we asked whether the 114 genes identified via the tumor revertant ME-B cells are indeed all tumor-specific. Therefore the revertant ME-B cells were again retransformed to tumor cells by the SV40 T/t antigen. The ME-B-T/t retransformants regained the morphology and growth characteristic of the original breast cancer cells and microarray experiments revealed that of the 114 genes only 38 fulfill the stringent parameter of being tumor-specific.

As listed above none of the key regulators of the cell cycle belong to genes that are finally considered as tumor-specific. This again demonstrates that ME cell proliferation is the important first hit and is presumably a prerequisite for malignant cell transformation, but it is not sufficient to convert the ME cells into breast cancer cells. In contrast, nine genes of the cell communication category fulfill the above condition of being tumor-specific. This includes the extracellular matrix protein collagen XIα1, the cell adhesion molecule SPARC-related modular calcium binding 1, and the fibroblast growth factor receptor-like 1. The observation that T/t antigen synthesis is correlated with upregulation of seven genes belonging to the immune defense system was unexpected. Among them are three histocompatibility class I genes (H2-K1, H2-T10, H2-T23). We can only speculate how T/t antigen mediates MHC gene upregulation. There is no indication for an activity of natural killer cells against the ME cells of the WAP-SVT/t animals, nor do the WAP-SVT/t females carry antibodies against the SV40 T/t antigen. This indicates that the SV40 T/t antigen is not considered as a foreign protein by the transgenic animals. In contrast, the SV40 T/t antigen is highly antigenic in the nontransgenic mice (NMRI). Whether the WAP-SVT/t transgene is transiently activated during pregnancy, mediating self-tolerance, remains to be analyzed.

In addition two members of the 2′-5′-oligoadenylate synthetase family (OAS) are correlated with T/t antigen synthesis. OAS are mainly interferon α stimulated and exhibit antiviral activity. OAS are activated by double-stranded RNA segments that catalyze the production of 2′-5′-oligonucleotides, which activate the RNase L [20, 21]. Furthermore, OAS also contain the BH3-only domain that is specific for proapoptotic proteins such as Bid, Bad, and Bik [22]. All T/t antigen positive ME cells exhibit a high spontaneous apoptosis rate, which starts with the initiation of WAP-SVT/t transgene expression during early pregnancy and is correlated with OAS upregulation. In contrast, normal ME cells and the T/t antigen negative revertant ME-B cells exhibit a low OAS expression level, and the apoptosis rate is minimal [23]. Therefore it is possible that the increased spontaneous apoptosis of the T/t antigen positive ME cells has a functional correlation with the upregulated OAS genes. This is specific for the ME cells since SV40 T/t antigen positive mouse kidney cells or fibroblasts do not exhibit this phenomenon (data not shown).

The interferon regulatory factor 7 is another upregulated gene (*Inf7*) which is three- to fourfold overexpressed in T/t antigen positive ME cells. IRF7 is not only an innate response protein but has been also attributed to apoptosis and tumorigenesis, mainly in lymphoid cells [24]. Viral IRF analogues complex with cellular IFR3 and IFR7 and to the transcriptional coactivator CBP/p300 as reported for the SV40 T antigen [25, 26, 27]. Whether T antigen also binds IRF7 remains to be analyzed.

The interferon α inducible protein (G1p2, human ortholog ISG15) shares amino acid sequences with ubiquitin and becomes conjugated to several intracellular proteins including Serpin2a, Jak1, Erk1, Stat1, indicating a possible role in the second messenger pathway and cellular responses [28, 29]. ISG15 is constitutively expressed, for example, in mouse embryo and human endometrium and can be induced in various tumor cells [30]. The ubiquitin pathway mediates rapid protein degradation and is considered as a crucial parameter for human breast cancer formation.

There are several genes encoding for proteins that are associated with tumorigenesis but exhibit an inverted correlation, such as the parathyroid hormone-like peptide (*Pthlh*) that is also associated with breast cancer [31]. Parathyroid hormone-like peptide expression is high in normal ME cells and the revertant ME-B cells but it is low in T/t antigen positive breast tumor cells.

The six-transmembrane epithelial antigen of prostate (*Steap*) is upregulated in multiple cancer cells lines (e.g., prostate, bladder, colon) and is considered a potential marker for advanced prostate cancer [32, 33]. However this gene is highly expressed in the normal ME cells and in the revertant ME-B cells and it becomes downregulated in all the SV40 T/t antigen positive ME cells (Fig. 5). Immunofluorescence staining confirmed that the level of six transmembrane epithelial antigen of prostate is high in the T/t antigen negative revertant ME-B cells and low in the T/t antigen positive ME-A cells (Fig. 6e, 6f).

Functional inactivation or loss of the p53 and Rb tumor suppressor proteins, upregulation of cell cycle key players, and aneuploidy are hallmarks of tumorigenesis. These alterations are enforced by the SV40 T/t antigen in the mammary gland epithelial cells of transgenic animals as a first hit, causing hyperplasia but not breast cancer formation, which requires further changes. Although all transgenic females develop breast cancer after the first lactation, only a small fraction of the T/t antigen positive cells show malignant transformation. Maintenance of the transformed state requires continuous T/t antigen synthesis. Once WAP-SVT/t gene expression is shut off, tumor cells regain the morphology and growth characteristic of normal cells unless they carry mutations in the p53 gene.

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