Transcriptional control of SV40 T-antigen expression allows a complete reversion of immortalization

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ABSTRACT

Conditional proliferation of mouse embryo fibroblasts was achieved with a novel autoregulatory vector for Tet-dependent expression of the SV40 T-antigen. The majority of cell clones that were isolated under induced conditions showed strict regulation of cell growth. Status switches were found to be fully reversible and highly reproducible with respect to gene expression characteristics. A consequence of T-antigen expression is a significant deregulation of >400 genes. Deinduced cells turn to rest in G0/G1 phase and exhibit a senescent phenotype. The cells are not oncogenic and no evidence for transformation was found after several months of cultivation. Conditional immortalization allows diverse studies including those on cellular activities without the influence of the immortalizing gene(s), senescence as well as secondary effects from T-antigen expression.

INTRODUCTION

Cell lines are an important tool for the elucidation of molecular processes. They are generated from tumors and from primary cells by selection of spontaneously immortalized cells or with the help of recombinant cellular or viral immortalizing genes. Spontaneously immortalized cells that were obtained by the 3T3 protocol show highly variable characteristics. In this respect, the transfer of specific immortalizing genes induces more clearly defined alterations. However, working with these cell lines still has limitations. The major disadvantage is that the immortalizing gene(s) interfere(s) with cellular processes, which might affect pathways to be studied. Fully reversible immortalization should overcome this type of problem as it avoids this interference. One of the frequently used genes for immortalization is the SV40 virus large T-antigen (TAg), which overcomes p53 and pRB dependent cell cycle arrest (1). A thermolabile mutant has been isolated (2) and used in the creation of conditionally immortalized cells (3,4). Cell lines created by this method represent a valuable resource. However, the interpretation of the generated data is complicated by the necessity to compare immortalized and reverted cells at two different non-physiological temperatures.

To overcome this problem, recombinase-mediated excision of the immortalizing gene was applied. Both the Cre/loxP and the Flp/FRT system have been used to revert the immortalization phenotype (5–8). However, to obtain reasonable cell numbers and a homogenous population of reverted cells, an efficient transfer of the recombinase is required. An alternative approach makes use of transcriptionally regulated expression of the immortalizing gene(s) by the Tet system (9). A technical issue with this is that it relies on the transduction of two expression units, which is not efficient in many primary cells.

We recently described a Tet-off based autoregulatory vector (10,11) that allows a 1000-fold regulation upon a single transduction step. We used the design of this vector as a basis for the construction of a Tet-on based reversible immortalization vector, which coexpresses TAg as the immortalizing gene together with the neomycin resistance gene and green fluorescent protein (GFP). All components that are needed for activation and selection are encoded on this plasmid, which supports a single step transduction. Reversion of the immortalizing activity is accomplished by Doxycycline (Dox) withdrawal. As a proof of principle, murine embryo fibroblasts (MEFs) were immortalized. The resulting clones show a highly regulated expression of the TAg that results in a strict proliferation control of the immortalized cell lines. Gene expression profiles of proliferating versus proliferation arrested cells revealed diverse alterations within the transcriptome, which are completely reversible.

MATERIALS AND METHODS

Vector description

In the vector pRITA (reversible immortalization with TAg), the bidirectional promoter P_{bitTA} that is derived from pBI-I (12) drives the expression of two mRNAs: one bicistronic mRNA encodes the rtTA2M2 transactivator that is derived from pUHrt62-1 (13) and a gene comprising the enhanced green fluorescent protein (EGFP) that is fused to the N-terminus of the selection marker neomycin. Both cistrons are linked by the encephalomyocarditis virus internal ribosome entry site and the mRNA is polyadenylated by the SV40 polyadenylation signal. The second mRNA encodes the SV40 TAg, and is terminated by the SV40 polyadenylation signal. The sequence and map of pRITA are available upon request.

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Generation and manipulation of immortalized cell lines

MEFs were obtained from 13.5-day-old embryos from Balb/c mice. The head and blood organs were removed and the remaining tissue was minced and dispersed in 0.1% trypsin (37°C, 30 min). The cells were plated on T75 flasks and maintained in DMEM, which contained 10% fetal calf serum, 2 mM L-glutamine, penicillin (10 U/ml), streptomycin (100 μg/ml), 1 mM nonessential amino acids and 0.1 mM β-mercaptoethanol.

Calcium phosphate coprecipitation of MEFs was done as described previously (14). Forty-eight hours after transfection, the cells were selected for G418 resistance (0.4 mg/ml). The resulting clones were selected by using a light microscope and expanded.

MEF derived cell lines (MBa10, MBa5, Balb/c 3T3) were maintained in the medium described above. Dox was added to a concentration of 2–4 μg/ml. The Balb/c 3T3 cell line was generated by using the 3T3 protocol (15). For this purpose, 3×10^5 cells were plated per 6 cm dish and passaged every 3 days.

Cell growth experiments

For determination of cell growth, 1×10^5 cells were cultivated in 6 cm dishes for growth curves at the indicated times. The cell number was determined with the CASY1 DT cell counter (Schaerfe Systems, Reutlingen, Germany). Growth kinetics were determined for three independent cultures of each cell line that were counted in duplicate. In the figures the resulting mean values are given.

For determination of clonogenicity 1×10^3 cells were plated in a 10 cm dish and cultivated until colony formation became evident (∼2 weeks). The resulting colonies were stained with crystal violet. Representative data obtained from three independent experiments are shown in the figures.

Anchorage-independent growth was determined by assessing clone formation of cells that were suspended in soft-agar. About 1×10^3 cells were plated in 50 μl of 0.3% agar in DME (overlay) in 96 well plates coated with 50 μl of 0.6% underlay agar in DME. Dox was added to the overlay agar to give a final concentration of 6 μg/ml. After 2 weeks pictures were taken (Nikon Coolpix 4500). In order to compare the size of the colonies from different experiments all pictures were taken at the same magnification.

The senescence associated β-galactosidase (SA-β-gal) staining was carried out as described previously (16).

FACS analysis of GFP and TAg expression

For the determination of EGFP expression, the cells were grown with and without Dox for three days. The cells were washed, trypsinized and stained with propidium iodide (50 μg/ml) to exclude dead cells from the analysis. The cells were analyzed by flow cytometry, by using a FACSCalibur (Becton Dickinson).

The immunostaining was performed by using a protocol without any fixation or washing steps. This procedure efficiently reduces the EGFP fluorescence level of the activated cells to the background fluorescence of control cells that were not stained with the TAg specific antibody. Cells were grown for 2 days at the indicated temperatures. The cells were then trypsinized and plated at a density of 3×10^5 per T25 flask and grown for an additional day under the same conditions. On day 3, the cells were trypsinized, washed with phosphate-buffered saline (PBS) and centrifuged. The pellet was resuspended in 0.5% Triton, 0.5 mM EDTA and 1% BSA in PBS at pH 7.2. The samples were incubated for 15 min on ice. The first antibody that recognizes TAg (Oncogene Research; mouse IgG) was added to the same buffer (10 μg/ml) for 30 min at room temperature. The fluorescein isothiocyanate (FITC) conjugated secondary antibody (Jackson Immunoresearch Laboratories; goat anti mouse IgG) was added to the same buffer (10 μg/ml) and incubated with the cells at room temperature for 30 min. The cells were analyzed by flow cytometry, by using a FACSCalibur (Becton Dickinson).

DNA microarray hybridization and analysis

Two independently immortalized cell lines (MBa10 and MBa5) from different batches and gender were used for the array analysis. Total RNA was isolated from 2×10^6 cells. The cells were trypsinized, washed and incubated with TRIZOL (Invitrogen). After two rounds of phenol/chloroform extraction, the RNA was precipitated with isopropanol. The pellet was resuspended in H_2O. The quality and integrity of the total RNA was confirmed by using the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). Biotin-labeled target synthesis was performed by using standard protocols that were supplied by the manufacturer (Affymetrix; Santa Clara, CA). Briefly, 5 μg of total RNA were converted to dsDNA by using 100 pmol of a T7T23V primer (Eurogentec; Seraing, Belgium) that contains a T7 promoter. The cDNA was then subjected to *in vitro* transcription in the presence of biotinylated nucleotides to generate biotin-labeled cRNA.

The concentration of biotin-labeled cRNA was determined by ultraviolet (UV) absorbance. About 12.5 μg of each biotinylated cRNA preparation were fragmented and placed in a hybridization cocktail that contained four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. All samples were hybridized to the same lot of Affymetrix MOE430A for 16 h.

After hybridization the GeneChips were washed, stained with streptavidin–phycoerythrin (SA–PE) and read by using an Affymetrix GeneChip fluidic station and scanner.

Data analysis

An analysis of microarray data was performed by using the Affymetrix Microarray Suite 5.0, Affymetrix MicroDB 3.0 and Affymetrix Data Mining Tool 3.0. For normalization, all array experiments were scaled to a target intensity of 150, otherwise using the default values of the Microarray suite. Filtering of the results was done as follows: genes were considered as regulated when their fold change is ⩾2 or less than or equal −2 (fold change 'A'), the statistical parameter for a significant change is <0.01 [change in *P*-value for changes called increased (I)] or >0.99 [change in *P*-value for changes called decreased (D)]. Additionally, the signal difference of a certain gene should be >200. Using these criteria, we found 271 probe sets that were upregulated and 216 that were downregulated. This accounts for 209 upregulated and 170 downregulated unigene clusters. The comparative analysis of expression data that uses the gene

ontology structured vocabulary was performed by using the GoSurfer analysis tool (http://www.gosurfer.org). The GO terms of these genes were compared for significant overrepresentation in one of the groups ($P < 0.05$). The cluster analysis was done with the program Genesis (17). The signals were normalized prior to clustering. Experiments were clustered into a hierarchical tree that uses the Euclidean distance measurement and average linkage algorithm. Genes were clustered by using k-means algorithm with Euclidean distance measurement.

RESULTS

Proliferation control from transcriptionally regulated TAg

For the reversible immortalization of primary cells, we generated an autoregulatory Tet-on vector pRITA, which allows

transcriptional regulation of TAg (Figure 1a). This vector immortalizes murine primary fibroblasts upon a single gene transfer step and confers G418 resistance under induced conditions (+Dox). All randomly selected and analyzed G418 resistant cell clones showed Dox dependent proliferation. Seven out of the 10 clones showed a strictly conditional proliferation, which we define as growth with absolutely no increase in cell number in the absence of Dox, and an exponential growth in the presence of Dox. Three out of the 10 clones were still regulatable albeit to a lower extent, which is manifested by some proliferation in the repressed state. The MBa10 represents a strictly regulated cell clone (Figure 1b). Although an exponential growth behavior is seen in the activated state, absolutely no proliferation is detectable after Dox withdrawal. This was confirmed by cell cycle analysis and BrdU staining (data not shown). A complete block of overall proliferation could be observed for at least 14 days. The clone
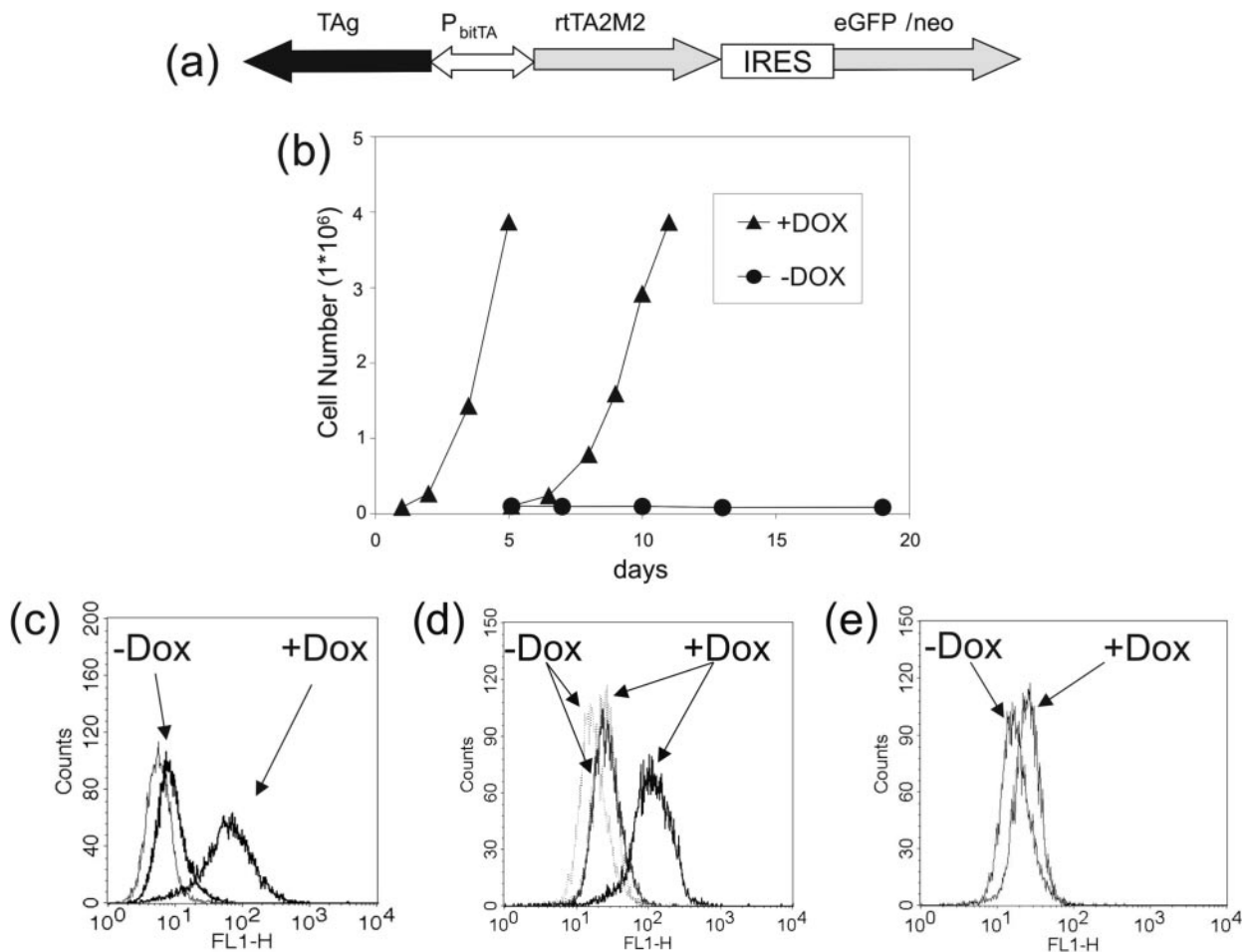

Figure 1. Regulation potential of the autoregulatory immortalization vector. MEFs were transfected with pRITA and screened for growth in G418 medium. A representative cell clone (MBa10) is shown for analysis of the regulation capacity of the vector. To this end the cells were cultivated for 3 days with (2 µg/ml) and without Dox. (**a**) Schematic presentation of the plasmid vector pRITA. The bidirectional Tet-dependent promoter P_{bitTA} (12) drives the expression of two mRNAs. One encodes the TAg, the other encodes the reverse transactivator rtTA2M2 (13), and a fusion protein of EGFP and neomycin. (**b**) Cells are usually cultivated with Dox (triangles) which results in an exponential growth behavior. For monitoring Dox dependent proliferation the cells were split and cultivated in parallel with (triangles) or without (circles) Dox, respectively. Three independent cultures were cultivated and counted in duplicate. Cell numbers are scaled to 1×10^6. (**c**) For GFP analysis the cells were washed and trypsinized followed by flow cytometry analysis. The arrows indicate the cultivation conditions. Balb/c 3T3 cells served as a control (light gray). (**d**) For determination of TAg levels the cells were permeabilized after trypsinization. Subsequently, indirect immunostaining of intracellular TAg was performed and cells were analyzed by flow cytometry. As controls, Balb/c 3T3 cells (not shown) and the secondary antibody alone with both activated and repressed cells (dotted lines) were analyzed [in (**e**) the controls are shown in a separate histogram].

was characterized for the expression of GFP and TAg protein (Figure 1c and d). Both proteins are strongly expressed in the presence of Dox whereas low levels of TAg and GFP are detectable in the repressed state. The integrity of the TAg as well as its regulation was confirmed by western blotting (data not shown). Propidium iodide staining confirmed that cell death was negligible for both the induced and the repressed state (+Dox: 2%; −Dox for 7 days: 2–5%). Thus, the strength of recombinant gene expression, in particular the immortalizing gene and the transactivator, is well tolerated.

Characterization of conditionally immortalized cells

TAg efficiently immortalizes primary rodent cells but leads to an oncogenic transformation only in rare cases (18). Anchorage-independent growth of MBa10 cells was determined in the presence and in the absence of Dox in soft agar cultures (Figure 2a). No growth was observed for cells that were cultivated in the repressed state. In the activated state only small colonies arose after 2 weeks. These colonies retained their size upon further cultivation. These results
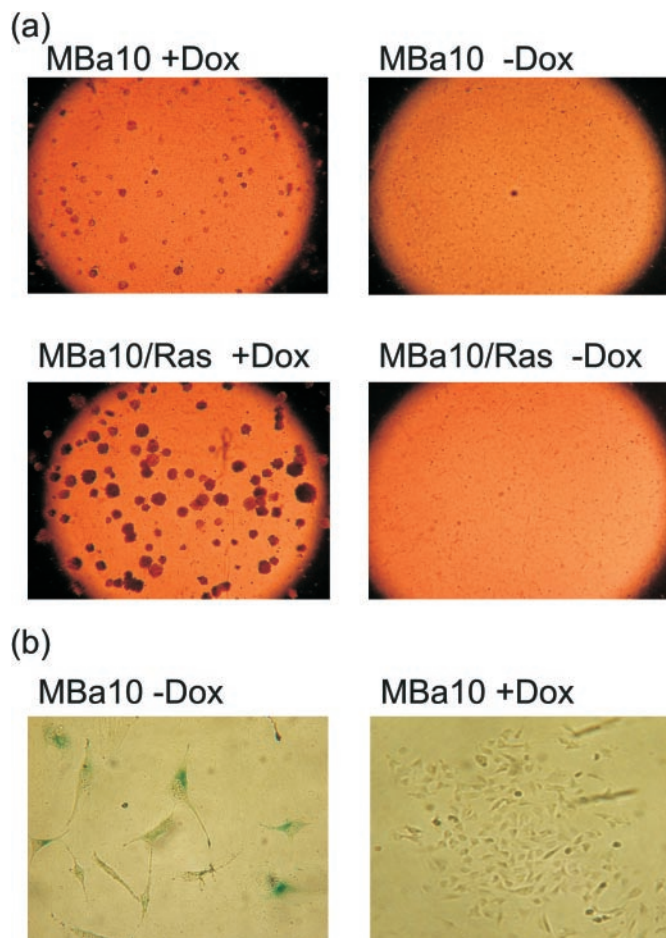

Figure 2. Cellular state of conditionally immortalized cells. (**a**) For discrimination between immortalization and transformation, the ability for anchorage-independent growth was determined. MBa10 cells and MBa10 cells stably transfected with constitutively expressed v-Ha-ras gene were grown in soft agar for two weeks with and without Dox. The pictures show representative cells of each experiment and were taken at the same magnification. (**b**) For SA-β-Gal staining (16) the cells were cultivated for 9 days without Dox.

were confirmed with several independently immortalized cell clones (data not shown), which indicates that pRITA-transfected cells are not transformed. To challenge for oncogenicity, MBa10 cells were transfected with a plasmid that conferred a constitutive expression of the v-Ha-ras oncogene. Cell clones generated thereof show a strong anchorage-independent growth in the induced state, but behave like the parental cells in the repressed state (Figure 2a). However, the constitutive activity of the ras oncogene was not sufficient to induce tumors in isogenic mice neither in the presence nor in the absence of Dox (data not shown).

About 4–5 days after Dox withdrawal MBa10 cells reveal morphological changes resembling a senescent phenotype: they appear enlarged and flattened (data not shown). After 9 days of cultivation without Dox they express SA-β-gal, a marker for senescence (16) (Figure 2b).

Importantly, these Dox-specific growth characteristics of clone MBa10 were not altered even after a cultivation period of 6 months (~70 passages) although, significant chromosomal rearrangements had occurred (data not shown).

Together these data indicate that the rather high TAg expression that is mediated by the autoregulatory vector immortalizes cells but does not induce tumorigenicity in primary mouse embryo fibroblasts. In the non-induced state these cells acquire a senescent-like phenotype.

Reanimation of proliferation blocked cells

In contrast to reversible immortalization systems that rely on the excision of the immortalizing genes by site-specific recombinases (5–8), the Tet-system supports multiple rounds of induction and repression. We speculated if reanimation of the proliferation blocked, senescent-like cells was possible. Such a cycle of reanimation was investigated by using MBa10 cells, thereby removing Dox for 7 days to stop proliferation and then again exposing the cells to Dox. After a short lag period the cells resumed exponential growth (Figure 3a) indicating that the block of proliferation is reversible. MBa10 cells were compared before, during and after proliferation arrest in a clonogenicity assay (Figure 3b). No colonies were detected in the repressed state whereas a large number of colonies arose when the cells were kept in the activated state ('controls' in Figure 3b). Importantly, cells reanimated after up to 9 days of repression, formed colonies as efficiently as before repression. When exposed to 12 and 20 days of inactivation prior to reanimation, a drop in the efficiency was observed ('reanimated samples' in Figure 3b). This implies that cells undergo irreversible alterations upon prolonged cultivation under repressed conditions.

TAg induced expression changes

Gene expression profiling was performed to generate a more detailed description of the molecular phenotype of proliferating versus proliferation-blocked cells. To further evaluate the reversibility of the system, RNA samples were generated from activated or repressed cells before and after the reanimation phase. Two independently immortalized cell lines, MBa10 and MBa5 (see Figure 4a for the experimental design) were used to learn about the clonal variation. Although phenotypically both cell lines display similar growth and regulation properties, they were derived from different batches of MEFs and the

embryos were of different genders. Additionally, MBa5 cells express a lower level of TAg (data not shown). The high biological variance of the samples should allow the elucidation of specific effects of TAg expression.

54–60% of the 22 600 probe sets specifically hybridized to the labeled RNA of the different samples. Uninduced versus induced samples were compared with respect to probes that were up- or downregulated at least 2-fold. As shown in Table 1 Dox removal alters the gene expression pattern dramatically, with 6–9% of the probe sets (758–1214) being differentially regulated (Table 1). Interestingly, the number of consistently regulated probe sets of a given cell line before and after a cycle
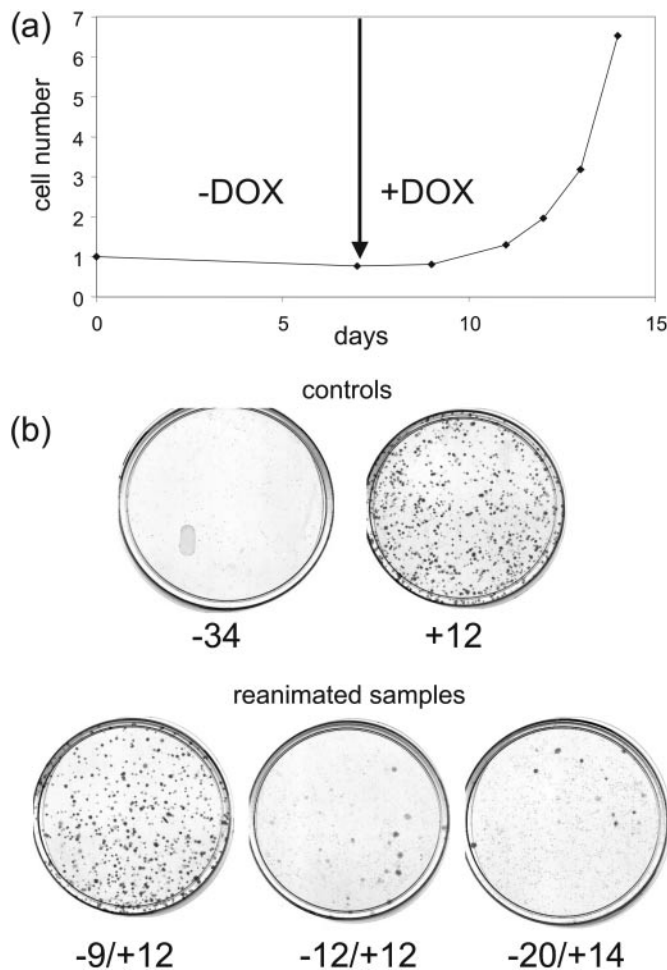

Figure 3. Reanimation of proliferation blocked cells. (**a**) For monitoring the growth behavior during reanimation, 1×10^5 cells were plated per 6 cm dish. Proliferation was blocked by the removal of Dox from the media. After 7 days reanimation was started by adding of 2 µg/ml of Dox to the media. Cells were counted at the indicated times. Cell numbers were determined for three independent cultures of each cell line and counted in duplicate. (**b**) To determine whether a longer proliferation block has an impact on the reanimation potential, the clonogenicity was measured. Therefore 1×10^3 cells were plated per 10 cm dish and grown under the conditions indicated. In the upper row control cells are shown that were cultivated in presence and absence of Dox. The days of cultivation are indicated. The lower row shows the clonogenicity of cells that were subjected to a proliferation arrest before reanimation. The number before the slash indicates the days of the proliferation block. The number after the slash indicates the reanimation period before cell staining.

of reanimation [Table 1(a)] is higher than the number of consistently expressed probe sets in the corresponding states of the different cell lines [Table 1(b)]. This reflects the variance between both cell lines. Thus, a certain number of differentially expressed probe sets can be ascribed to clonal variations within the two cell lines. However, 487 probe sets that corresponded to 379 genes were regulated consistently in all four comparisons [Table 1(c)]. These genes were further investigated since we assumed that they reflect TAg specific alterations of the transcriptome.

The 379 regulated genes were linked to the gene ontology (GO) hierarchy. We define genes as upregulated when expression is higher in the presence of Dox and vice versa. GO categories that are overrepresented in one of the groups were identified. The results are superimposed on the GO tree (Figure 4b and c). A significant number of downregulated genes are related to membrane and vacuolization processes (Figure 4b) whereas upregulated genes appeared mainly in nuclear processes. GO analysis of biological processes (Figure 4c) revealed that upregulated genes relate to 'cell proliferation' and 'nucleotide metabolism', the latter being mainly involved in RNA and DNA metabolism. Genes involved in cell proliferation contain cell cycle regulatory genes. These were analyzed in more detail by allocating the differentially expressed genes to known biological pathways by using the software GenMAPP (19). The differentially expressed genes fit well into the known schemes of proliferating versus resting cells (compare Discussion and see Supplementary Material S1).

High reproducibility of induced expression changes

Genes equivalent to the 487 differentially expressed probe sets from the two different clones were clustered. This clustering allows to identify genes that are expressed in a comparable manner. The dendogram in Figure 5a summarizes the results of the hierarchical cluster analysis by indicating the rate of similarity between the samples. As expected, samples generated before and after a cycle of reanimation are the closest neighbors, which indicate a high degree of identity. This is highlighted in Table 2, which documents that samples give rise to essentially identical expression profiles before and after a cycle of reanimation, since not a single probe set is changed in these comparisons. The high similarity was seen with regulated genes from the two different cell clones. Interestingly, the activated samples show a higher similarity than their inactivated counterparts. Figure 5b shows an alternative cluster analysis (*k*-means), which allows the identification of subclusters of comparably expressed genes. From the six clusters, the upper panel shows those clusters, which contain genes that are upregulated when TAg is activated. The lower panel shows the clusters, which contain genes that are downregulated when TAg is activated. Interestingly, the samples before and after a cycle of reanimation not only show similar expression patterns but also nearly identical relative expression levels as indicated by the distance from the base line (see also Supplementary Material S2 and S3). Quantitative differences between the independently derived cell clones probably reflect the different expression level of TAg. Together, these data indicate that cells that are immortalized with the autoregulatory vector pRITA show defined and highly reproducible
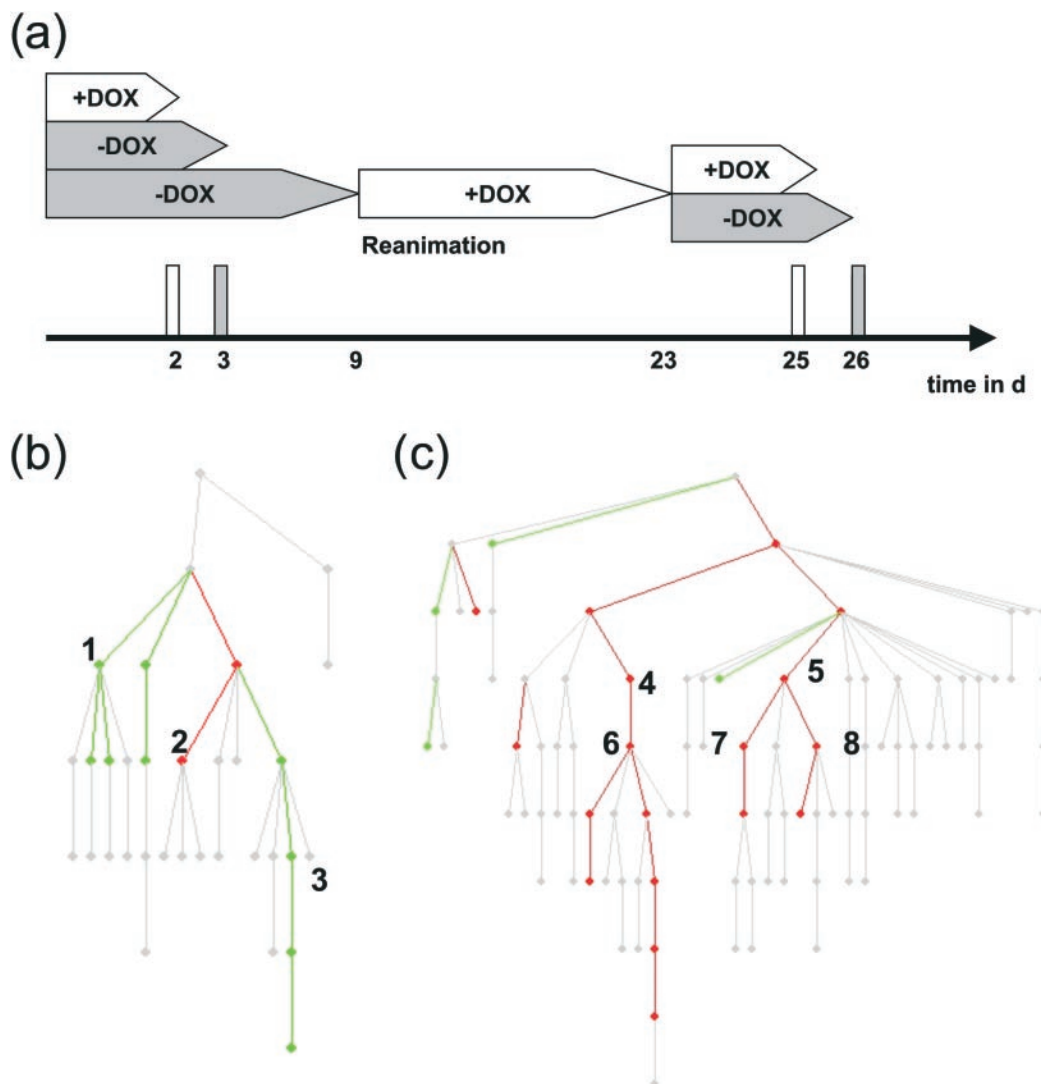
Figure 4. Influence of TAg on the gene expression. (**a**) Schematic presentation of the experimental setup. The cell lines MBa10 and MBa5 were cultivated in the presence (+ samples) and in the absence (− samples) of Dox (2 µg/ml) at the indicated times. In the reanimation experiment the cells of both cell lines were first cultivated for 9 days without Dox. Then reanimation was achieved by the addition of Dox. After 14 days of reanimation the cells were again grown with or without Dox ('R' samples). Analysis of the differentially expressed genes using the gene ontology hierarchy for cellular components (**b**) and biological processes (**c**). The tree represents those categories (dots) that matched at least three (cellular components) or five genes (biological processes) in one condition. Red categories are overrepresented ($P < 0.05$) in the activated cells, green in the repressed cells. Grey categories are not significantly altered upon TAg activation. The numbers are selected categories from this analysis. 1, Membrane; 2, nucleus; 3, vacuole; 4, cell proliferation; 5, nucleotide metabolism; 6, cell cycle; 7, RNA metabolism; 8, DNA metabolism.

Table 1. Differentially expressed probe sets in induced versus repressed samples

Comparison	Number of probe sets	Number of identical probe sets		
		(a)	(b)	(c)
MBa10+ versus MBa10−	1214	998	696	487
MBa10R+ versus MBa10R−	1203			
MBa5+ versus MBa5−	1012	633	562	
MBa5R+ versus MBa5R−	758			

Table 2. Differentially expressed probe sets before and after a cycle of reanimation

	Number of probe sets
MBa10+ versus MBa10R+	0
MBa10− versus MBa10R−	1
MBa5+ versus MBa5R+	1
MBa5− versus MBa5R−	22
In all comparisons changed	0

molecular alterations. Furthermore, the data documents that the generated cell lines are highly comparable before and after a cycle of reanimation, on a molecular level, thereby proving the reversibility of immortalization.

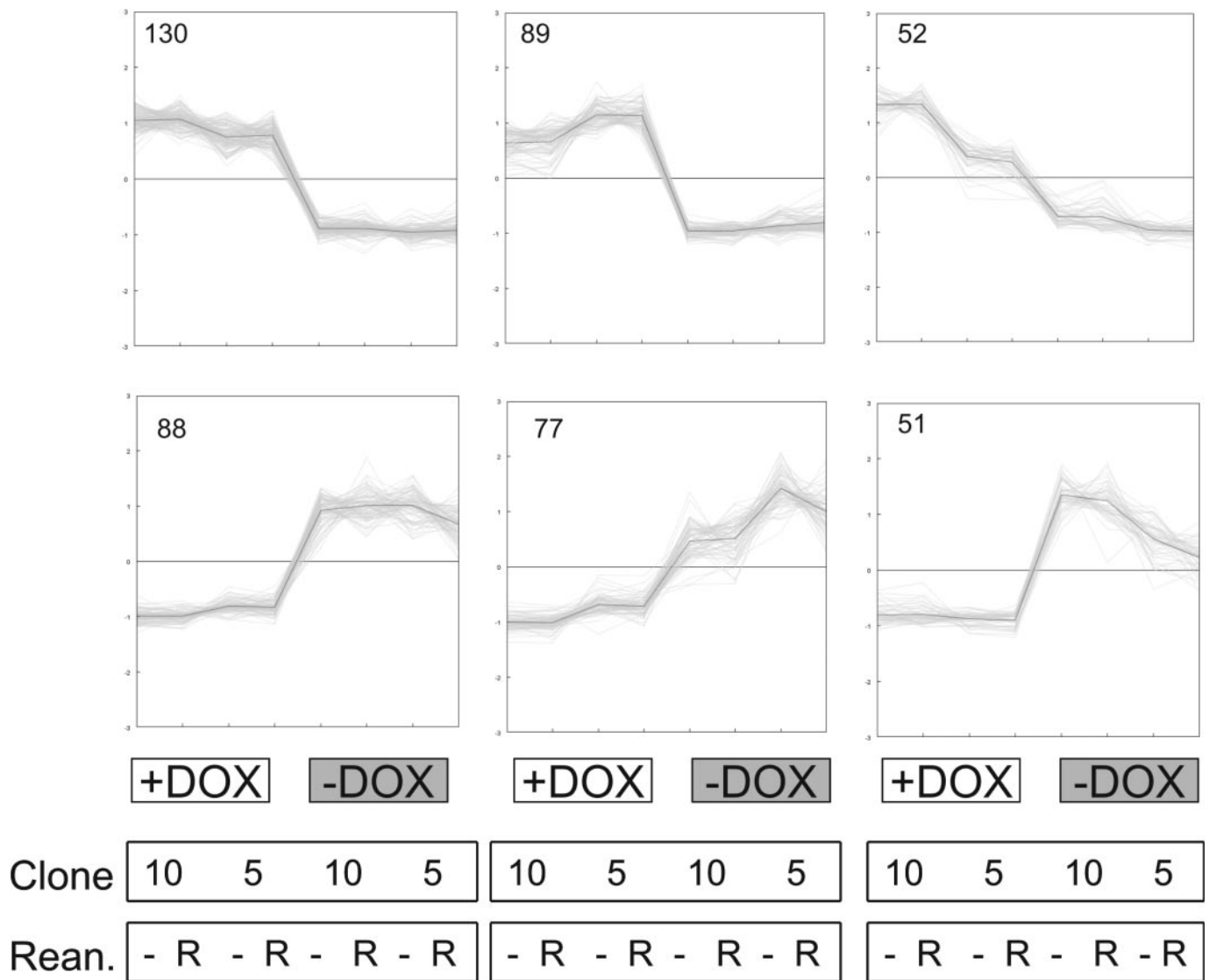**Figure 5.** Gene expression profile before and after a cycle of reanimation. (**a**) Cluster analysis over the experiments. The resulting dendogram is shown. Average linkage hierarchical clustering using Euclidean similarity was carried out for the differential expressed probe sets. (**b**) Cluster analysis over genes. *k*-means clustering using Euclidean similarity was carried out for the genes equivalent to the 487 differentially expressed probe sets. Genes were proposed to match to six clusters. The samples are plotted on the *x*-axis (from left to the right: MBa10+; MBa10R+; MBa5+; MBa5R+; MBa10−; MBa10R−; MBa5−; MBa5R−). On the *y*-axis the relative gene expression is plotted. Each probe set is represented by a grey line and the dark grey line is the mean of all probe sets present in one cluster. A lower number of clusters gave rise to a higher variance whereas more clusters led to very inhomogeneous groups.

DISCUSSION

Reversion of immortalization is a prerequisite to study continuous cell cultures without the influence of the immortalizing gene. In the past, approaches based on the recombinational excision of the immortalizing genes have been reported (5–8,20–22). In the current study, we developed a transcriptionally controlled system for conditional immortalization of cells. It is based on an autoregulatory Tet-dependent expression cassette (10,11) with both the immortalizing gene and the transactivator being coordinately regulated by Tet or its homologue Dox. Generally, the activation of autoregulatory cassettes requires a low basal expression of the transactivator in the repressed state, which corresponds to a low expression of the coregulated gene. Indeed, a low basal expression of the TAg in the absence of Dox was observed. Interestingly, this level is not sufficient to promote cell growth since the cells do not show any proliferation in the repressed state. This is in line with the view that a certain threshold expression is required for immortalization (1). The negligible amount of death cells demonstrates that the system is well tolerated, which confirms that the system can only drive the expression to a certain level. This is of importance, since it excludes that such a vector, once activated could amplify the transgene expression levels until cell destruction. Together, the autoregulated system represents a useful tool for strict and tight control of proliferation.

We show that TAg expression is sufficient to immortalize primary rodent cells, which is consistent with previous reports (18). TAg expressing cells are not transformed as proven from the failure of the cells to grow in soft agar. Correspondingly, no tumor formation has been observed upon injection of these cells into Dox fed mice (data not shown). A certain degree of oncogenic transformation could be achieved by additional expression of the v-Ha-ras oncogene that is known for its synergistic effects with TAg (23,24). Oncogenic transformation could have been expected by adventitious secondary mutations that could arise during cell arrest. Surprisingly, we did not observe colonies in the repressed state within 4 weeks. This indicates that both immortalization and transformation strictly depend on the activity of TAg, thereby excluding a strong selective pressure for accumulating additional mutations. This shows that in the immortalized cells, along with the ability to undergo senescence, the major safeguarding systems are still intact.

TAg is known to modulate the activity of a number of proteins. Among those, p53 and pRb are regarded as the most important ones. Binding of TAg to p53 inhibits p53 mediated growth control. It also interferes with the pRb mediated proliferation stop. A huge number of target genes are affected by TAg directly and indirectly. This is reflected by the high number of genes that were found to be differentially expressed in the proliferating and arrested cell cultures as analyzed here in detail. Table 3 lists genes that are found to be differentially expressed and described in the literature as known targets of either p53, E2F or TAg itself. The presented examples demonstrate the variety of biological processes that are influenced by TAg expression.

The GO analysis further confirmed that the induction of TAg leads to defined molecular alterations. As expected,

Table 3. Examples of differentially expressed genes

Gene	Induced by	Biological process
Cyclin A2	E2F1	S-phase entry (41)
Thymidine kinase	E2F1	DNA Synthesis (41)
Ribonucleotide reductase	E2F1	DNA Synthesis (41)
MCM genes	E2F1	Replication licensing (41)
Rad51	E2F1	DNA repair (42)
p21	p53	Cell cycle regulation [for review see (43)]
Mdm2	p53	Ubiquitination [for review see (43)]
Perp	p53	Apoptosis [for review see (43)]
Apaf1	p53	Apoptosis [for review see (43)]
Btg2	p53	DNA repair [for review see (43)]
Cyclin G1	p53	p53 stabilization [for review see (43)]
Nucleolin	TAg	DNA unwinding (44)
Dnmt1	TAg	DNA methylation (45)
Bub1	TAg	Spindle checkpoint (46)

for proliferating cells, gene categories associated with processes regarding nucleus, cell proliferation, DNA and RNA metabolism are significantly overrepresented.

In this respect, the reversible immortalization system should be a useful tool for unraveling signaling pathways of the TAg. It should even help to elucidate the complex networks associated with the TAg.

In contrast to systems that rely on excision of the immortalizing gene Tet-regulated expression of oncogenes enables repeated cycles of proliferation and arrest. In several mouse tumor models the effect of repeated activation of oncogenes was investigated (25–27). However, as these models are generated from two different transgenic mice (one harboring the transactivator, the other the oncogene) this approach is difficult to transfer to the cellular level. The autoregulated system allows an adoption of these strategies in *in vitro* culture, which is indispensable for the elucidation of the underlying molecular reactions. We compared cells before and after a cycle of reanimation to prove the feasibility of such an approach. Interestingly, only a handful of differentially expressed genes could be detected. This is also reflected by the cluster analysis, which revealed that, not only the expression pattern but also the expression level is nearly identical in samples before and after a cycle of reanimation. Only minor differences between the two independent cell lines are seen. Both clones show the same tendency but to different extents. This demonstrates (a) that the senescent state does not induce persisting alterations in the expression profile and (b) the high reproducibility of the switches.

When the TAg levels decrease, the cells stop proliferating and become senescent as indicated by the morphological changes and the SA-β-gal activity. This suggests that the cells have lost their endogenous replication potential during the selection process. Senescence can be induced by a variety of stimuli, such as oxidative stress, DNA damaging agents, oncogenic signaling and progressive telomere shortening (28,29). The extent to which these stimuli use the same or different effectors is not known. However, the major players, p53 and p16 are likely to be independent of the inducing stimulus. p53 exerts its activity through p21, which triggers senescence but is dispensable in the later stages (30). The gene

expression profiling shows that after Dox withdrawal, p21 is tremendously upregulated, which is due to the release of the p53 inactivation. GO analysis uncovered other markers of senescence. In absence of TAg, a number of genes are expressed, that are linked to membrane and vacuolization process. The latter is likely to account for the increased granularity (31,32) that is seen after Dox withdrawal (data not shown). It has also been reported that membrane bound proteins are highly expressed during senescence (33). Accordingly, we see an increase in the expression of membrane bound proteins after Dox release.

It has been recently described that senescent MEFs are efficiently reanimated through a lentivirally delivered siRNA directed against p53 (34). Another study in human cell lines revealed that the level of the p16 protein decides the revertability of senescence (35). There are hints that p16 triggers an epigenetic program that leads to heterochromatin condensation (36). It is not known if such irreversible epigenetic changes also occur in murine cells; however, the fact that in our experiments the efficiency of reanimation decreases with prolonged growth arrest argues toward this line.

The autoregulatable vector pRITA should be a valuable tool for the molecular elucidation of senescence and its reversion. It should allow the comparison of the transcriptome and the proteome of senescence in different cell types and/or in different genetic backgrounds. This is of particular interest as the senescence program seems to be highly cell-type specific (33,37).

Moreover, the vector can be employed for reversible expression of other immortalization genes, which might be useful for the determination of molecular fingerprints (38). Although the TAg immortalizes a variety of cell types (3,4) other immortalizing genes might be better suited to preferentially immortalize specific cell types. Examples are PymT for endothelial (39) and ΔNotch for hematopoetic cells (40). Conditional expression of these genes using the autoregulated vector principle might allow to establish novel reversibly immortalized cell types.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

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