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Tumor suppressor p53 protein is a new target for the metastasis-associated Mts1/S100A4 protein: functional consequences of their interaction.

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Running title: Mts1/S100A4 binds p53 and modulates p53 function

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A physical and functional interaction between the Ca^{2+}-binding protein Mts1 (S100A4) and the tumor suppressor p53 protein is shown here for the first time. We demonstrate that Mts1 binds to the extreme end of the C-terminal regulatory domain of p53 by several *in vitro* and *in vivo* approaches; co-immunoprecipitation, affinity chromatography and Far Western blot analysis. The Mts1 protein *in vitro* inhibits phosphorylation of the full-length p53 and its C-terminal peptide by protein kinase C but not by CKII. The Mts1 binding to p53 interferes with the DNA–binding activity of p53 *in vitro* and reporter gene transactivation *in vivo* and this has a regulatory function. A differential modulation of the p53-target genes (p21/WAF, Bax, thrombospondin -1 and MDM2) transcription was observed upon Mts1 induction in tet-inducible cell lines expressing wild-type p53. Mts1 co-operates with wild type p53 in apoptosis induction. Our data implies that the ability of Mts1 to enhance p53-dependent apoptosis might accelerate the loss of wild type p53 function in tumors. In this way Mts1 can contribute to the development of a more aggressive phenotype during tumor progression.

SUMMARY

The *mts1/S100A4* gene has been isolated as a gene specifically expressed in murine and human metastatic tumor cells (1) Expression of *mts1* in nonmetastatic murine and human cell lines results in a more malignant phenotype (2).

Mts1/S100A4 is a small 11-kDa protein that belongs to the S100-family of Ca^{2+}-binding proteins. The existence of multiple S100 protein targets may explain the involvement of the

S100 proteins in a large group of cellular events such as neurite growth, cell-cell communication, cell growth, cell structure, energy metabolism, contraction, motility, intracellular signaling and cell division (3).

Mts1 participates in the regulation of cytoskeletal dynamics and cell motility by association with stress fibers, F-actin and tropomyosin (4,5). In our laboratory the heavy chain of nonmuscle myosin II (MHC) was identified as a target for Mts1 (6). The Mts1-MHC interaction results in the inhibition of the protein kinase C (PKC) -mediated phosphorylation of the MHC molecule and associates Mts1 with cell motility, which is one of the determinative functions in the metastatic disease (7).

Tumor suppressor p53 protein is a transcriptional factor, which regulates several cellular processes creating limitations for tumorigenic transformation. In response to "proliferation affecting" events (DNA damage, oncogenes, oxidative stress, etc), cells utilizing p53 pathways may switch to either growth arrest, programmed cell death or cell differentiation (8). The pathways of p53 regulation are only starting to emerge.

As a transcriptional regulator p53 modulates the expression of target genes by binding to specific p53-responsive sites. The majority of genes regulated by p53 are involved either in cell cycle control or apoptosis.

The p53 protein has three main functional domains. The N-terminal transactivation domain interacts with several components of the basal transcription machinery and provides a transcriptional regulation (9). The central part of the protein contains the specific DNA-binding domain, a hotspot for p53 mutations that affect its DNA -binding ability and lead to tumorigenic transformation of cells. The C-terminal part of p53 is a multifunctional domain,

responsible for oligomerization, nuclear translocation, and binding to damaged DNA. In addition, the C-terminus negatively regulates the specific DNA binding activity of the core domain. Binding to certain proteins, acetylation, or phosphorylation by PKC and casein kinase II (CKII) abolish the negative effect imposed by the C-terminus. The C-terminus regulates the conversion of p53 from inactive to active forms and vise versa, thereby modulating the transcriptional activity of p53 (10-11).

Here we show a physical link between Mts1 and amino acid residues 360-393 of the C-terminal domain of p53. This interaction affects the phosphorylation of p53 by PKC as well as its DNA-binding capacity *in vitro*. Moreover, Mts1 influences the transactivation of a reporter gene placed under the control of p53-binding elements *in vivo* and differentially modulates the transcription of p53 –regulated genes. Co-operation between wild type p53 and Mts1 may induce an apoptotic response depending on environment and cell type.

EXPERIMENTAL PROCEDURES

Plasmids

The coding region of *mts1* was cloned into pSK3 (Pharmacia AS, Copenhagen, Denmark) vector giving a construct pSV-mts1. pSVBcl2 was constructed by cloning the Bcl2 /XhoI insert from PEBS7-425 (gift of Dr. M. Jäättelä) into the pSK3 vector. The following mouse wild type p53 PCR products were designed: #1 – full size coding region (390 aa) was amplified using the primers: forward – CGGGATCC GACTGGATGACTGCCATGGA (including BamHI site) and reverse -CGAAGCTT CAGTCTGAGTCAGGCCCCACT

(including HindIII site); #2- N-terminal domain (106 aa): forward - same as the forward primer for #1, reverse: CGAAGTCTT GAAGCCATAGTTGCCCTGGTAAG (includes HindIII site); #3-DNA-binding domain (185 aa): forward –CGGGATCCCACCTGGGCTTCCTGCATGCT (includes BamHI site), reverse – CGAAGCTTGGACTTCCTTTTTGCGGAAATTTTC (includes HindIII site); #4-C-terminal domain (99 aa): forward – CGGGATCCCTTTGCCCTGAACTGCCCCCA (includes BamHI site), reverse- same as the reverse primer for #1. The PCR products were digested with BamHI/HindIII and cloned into the bacterial expression vector pQE30 (Qiagen, GmbH, Hilden, Germany). pSP65m65 plasmid DNA (gift of Dr. J. Skouv) was used for the amplification of mouse p53 and its domains. pC53-SN3 (human wild type p53) and pC53-SCX3 (human mutant p53 ,143$^{val}\rightarrow$ala) eukariotic expression plasmids were gifts of Dr. J. Skouv. For cloning into the tet-inducible, conventional expression system, *mts1* cDNA was excised, cloned into pUHD 10-3 and used for transfection of cell lines producing reverse tetracycline-controlled transactivator (pUHD172-neo) (Clontech Laboratories GmbH, Heidelberg, Germany). p21 -luc was constructed by cloning 13 copies of the p53 binding consensus element from the p21/WAF promoter, excised from pSK45-13-2 (provided by Dr.B.Vogelstein) into the pfLuc reporter construct containing the *Photinus pyralis* luciferase gene under the minimal *c-fos* promoter. For Bax-luc construction 5’ TCG ACA ATA TAG CCC ACG CCC AGG CTT GTC TC 3’ and 3’ G TCC GAA CAG AGA TTG AAC ACT CTA G 5’ oligonucleotides were annealed and the ends of double stranded DNA were extended with *Escherichia coli* DNA polymerase I Klenow fragment. Four copies of head-to-tail orientated fragments were cloned into the Hind III site of the pfLuc vector.

The β-galactosidase expression plasmid (pCMV-β-gal) was purchased from Clontech. pBabe-Hyg contains the Hygromicin-resistance gene and was a gift of Dr. J. Lukas. The pQE30 expression vector, producing recombinant Mts1-His (7) plasmids encoding human wild type and mutant p53 proteins fused with the GST protein, GST-p53 and GST-p53Δ30 (12) were used.

Cell lines transfection

Cells were transfected by electroporation: 1-3x10^6 cells in 100 μl of phosphate saline buffer were transferred into electroporation cuvette and single pulse of 250V and 250μFd was applied using Bio-Rad electroporation system. Clones were selected in the presence of 400μg of G-418. For the conventional clones double selection with G-418 and 200μg/ml Hygromycin was used. In the transient transfection experiments the efficiency of each transfection was monitored by use of co-transfection with a β-galactosidase expression vector, pCMV- β-gal. At 24-48 hours post-transfection, cells were lysed and the luciferase activity was measured using a luminometer (Promega, Madison, WI). Same lysates were tested for β-galactosidase activity by using *o*-nitrophenyl-β-galactopyranoside (Sigma, St.Louis, Missouri) as a chromogenic substrate (13).

Immunofluoresence analysis

Cells were grown on 10mm glasses and fixed with freshly prepared 4% paraformaldehyde in PBS 30 min on ice. 0.2% Triton X-100 was used to permeabilize the cells. Primary and secondary antibody were applied in DME culture medium with 10% FCS, either for 1 hour at

room temperature. The antibodies used: rabbit anti-mouse Mts1 affinity purified (1:500) and FITC-conjugated goat anti-rabbit IgG 1:100 (Zymed).

To evaluate the efficiency of transfection, the cells grown on one of the glasses were fixed with 0.5% glutaraldehyde in PBS for 5 min, washed 3 times with PBS and incubated with X-gal staining solution, 1mg/ml X-gal (5-bromo-4-chloro-3 indolyl β-D-galactopyranoside), 35 mM potassium ferricyanide, 35 mM potassium ferrocyanide and 2mM $MgCl_2$ in PBS.

Preparation of recombinant proteins

Histidine -tagged p53 and Mts1 proteins were expressed in XL-blue *E. coli* by induction with 0.2 mM isopropyl ²D-thiogalactopyranoside for 4 hours at 37°C. Protein isolation in denaturing conditions and the following renaturation were performed according the manufacturers protocol (Qiagen GmbH, Hilden, Germany).

Western blotting

Protein isolation and Western blotting were performed as described (6). Immunostaining and protein band visualization with ECL system SuperSignal ® (Pierce, Rockford, Il) was carried out according to the manufacturer's protocol.

Far Western (blot overlay)

Proteins were separated by SDS-PAGE and blotted to Immobilon-P (Millipore, Bedford, MA). After blotting the membranes were pre-incubated in the blocking buffer: 0.2M NaCl,

50 mM Tris HCl pH 7.5, 3% BSA, 0.1% PEG 8000 for 2h at room temperature. Overlay with 1 μg/ml of recombinant Mts1 protein was performed for 20 min at room temperature in 0.2M NaCl, 50 mM Tris pH 7.5, 12 mM β-Mercaptoethanol, 0.1% BSA, 1% PEG 8000 and 1mM $CaCl_2$. After three washings the membranes were immunoprobed with anti-Mts1 antibodies by standard Western blotting procedure.

Immunoprecipitation and in vitro pull-down assays

Cells were metabolically labeled for 4h in methionine- and cysteine-free medium supplemented with dialyzed and inactivated 10% FCS with 0.2mCi/ml [^{35}S]-methionine and –cysteine (Amersham Pharmacia Biothech, Hørsholm, Denmark). The cells were lysed in NP-40 buffer (150mM NaCl, 50mM Tris-HCl pH 7.6, 1% NP-40) supplemented with a protease inhibitor cocktail (1mM DTT, 10μg/ml leupeptin, 2μg/ml aprotonin, 0.1 mM PMSF, 1mM benzamidine) and pre-cleared on 50% protein A-Sepharose.

The lysates were then incubated for 2 hours with anti-p53, pAb421 monoclonal antibody, (gift of Dr. J. Bartek) or anti-Mts1 rabbit serum. The precipitated proteins were separated on gradient SDS-PAGE (4-20%) and detected by autoradiography.

For *in vitro* precipitation assay 1 ¼g recombinant Mts1 was mixed with either the recombinant full size p53 (#1) or its domain peptides (## 2, 3, and 4) in NP-40 buffer and pre-cleared on protein A-Sepharose, in the presence of protease inhibitors for 1 hour in the cold room. To the pre-cleared mixtures a fresh portion of the protein A-Sepharose and corresponding anti-p53 antibodies were added: pAb421 for full-size and C-terminal domain, pAb240 (gift of Dr. J. Bartek) - for DNA-binding core domain and pE-19 (Santa

Cruz Biotechnology Inc., Santa Cruz, CA.)– for the N-terminal domain, and incubated for 2 hours in a cold room. Immunoprecipitates were washed 5 times with NP-40 buffer, heated at 100°C – 5 min. Proteins were separated on 15% PAGE and transferred to an Immobilon –P membrane (Millipore, Bedford, MA). To detect the co-immunoprecipitated Mts1 protein, the membranes were probed with anti-Mts1 antibodies and developed by an ECL System. Recombinant human wild type GST-p53 and GST-p53-Δ30 (deletion mutant lacking aa 364-393) fusion proteins were used for pull down experiments with the Mts1 recombinant protein. 5μg of GST and GST-fusion proteins coupled with Glutathione–sepharose beads were incubated with 2μg of Mts1 in NP-40 - containing buffer for 2 h in a cold room with rotation. After 5 washings with NP-40 buffer proteins were isolated by boiling in protein-loading buffer for 5 min and analyzed using Western blotting followed by Mts1 immunoprobing.

Phosphorylation assays

Reaction were performed in a mixture (25μl) containing 50 mM Tris-HCl pH 7.6, 0.1 M NaCl, 10 mM $MgCl_2$, 4mM $CaCl_2$, 2 mM DTT, 50μM ATP, 2 μCi [γ-^{32}P]-ATP (>5 000 Ci/mmol, Amersham Pharmacia Biotech, Hørsholm, Denmark), 1μM recombinant wild type p53, or its protein fragments for 30 min at 30°C. The PKC assay was done in the presence of 3.75 μg of phosphatidylserine (Sigma, St.Louis, Missouri) by 0.016 μg PKC (Roche Molecular Biochemicals, Hvidorve, Denmark). CKII was purchased from (New England BioLabs Ltd, Hitchin, England) and 50 units were applied per each reaction. Recombinant Mts1 was used in concentrations of 0, 3, and 5 μM. Reactions were terminated by adding

equal volume of 2xSDS-loading buffer and proteins were separated in 15% SDS-PAGE. Gels were fixed in 5% trichloroacetic acid, dried and exposed to Kodak X-ray film. To quantify the intensity of the protein bands, a Molecular Dynamics computing densitometry (Sunnyvale, CA) with ImageQuant software was used.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as previously described (13). To perform EMSA, nuclear extracts were incubated with end-labeled oligonucleotides that contained binding sites for p53 or Oct-1 proteins. Oligonucleotide sequences were as follows: for Oct-1 – TGCGAATGCAAATCACTAGAA ; for p53 –GAACATGTCCCAACATGTTG, derived from the promoter of p21/WAF . The reactions were carried out in 10µl of the buffer containing 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 0.5 mg/ml BSA, 5% glycerol. To perform the gel supershift analysis, anti-p53 antibody pAb421 was added to the EMSA reaction mixtures. The incubation with antibody was carried out for 1 h at 4°C after the binding reaction was completed.

Northern blot analysis

CSML-0 conventional Mts1- tet-inducible cells were grown at low and dense conditions and induced with 2µg/ml Doxycyline for 0, 24, 48 and 72h. RNA was isolated as described (14). Gel electrophoresis and Northern blot analyses were performed as described (15). The same filter was sequentially hybridized with murine p21/WAF, Bax, Cyclin G1 and

thrombospondin-1 (THBS1) probes. The amounts of mRNA on the filters were calibrated by hybridization with γ-^{32}P-ATP-labeled poly (U) probe. To quantify the intensities of the bands membranes were scanned using a Molecular Dynamics computing densitometer (Sunnyvale, CA) with ImageQuant software.

RESULTS

Co-expression of Mts1 and wt-p53 leads to cell-specific apoptosis

Plasmid DNAs encoding *mts*1 and neomycin resistance were transfected into Mts1-negative CSML-0 and VMR-liv cells. Fig.1 shows that the clonal survival of the transfected cells evaluated after three weeks was four times lower compared to the mock-transfected ones (vector DNA). To investigate the cause of low clonal survival anti-apoptotic Bcl2 cDNAs, in sense and antisense orientations, were co-transfected. The data revealed that expression of the active (sense) Bcl2 acted as a rescue factor and increased the relative clonal survival up to control level, whereas the non-active (antisense) did not. Taken together, these results suggest that Mts1 expression induces programmed cell death, which could be prevented by Bcl2 expression.

Further experiments were designed to study the mechanism of the *mts1*-induced apoptosis. Since p53 is a key trigger of apoptosis, we addressed the question whether Mts1-induced apoptosis is dependent on functional p53. We analyzed the expression of Mts1 in a number of human and mouse tumor cell lines of various origins and in immortalized mouse fibroblasts with known p53 status. The data summarized in Table 1 show that all 7 tumor cell lines homozygous for wild type p53 displayed a Mts1-negative phenotype, while 16 cell lines out

of 18 with p53 abnormalities (mutated/deleted) are Mts1 positive. In immortalized fibroblasts (NIH 3T3, 10T½ and L) the expression of the Mts1 protein was abundant. Moreover, upon cultivation of primary mouse fibroblasts *in vitro* the expression of mts1 was increased after 2-3 passages (data not shown). The conversion of wild type p53 into mutant conformation during the immortalization described for 10T1/2 fibroblasts (16) might explain the coexistence of Mts1 and p53 in fibroblasts. Data obtained in tumor cell lines indicated a significant reverse correlation between wild type p53 and Mts1 expression in tumor cell lines. The expression of Mts1 is "allowed" when functional p53 is obstructed by mutation or other mechanisms. It appears that the expression of Mts1 and wt-p53 are mutually exclusive in the tumor cell lines tested.

To get insight into the role of p53 in Mts1-induced apoptosis, we tested the survival of clones after transfection with *mts1* in the presence or absence of p53 expression in two different cell lines. Murine non-metastatic adenocarcinoma CSML- 0 line, which contains wt-p53 and does not express Mts1, and p53-null human osteosarcoma line Saos-2, heterogeneous for the Mts1expression (~80% of Mts1-positive cells) were transfected with *mts1*, wt-p53, mt-p53, Mts1+wt-p53, Mts1+mt-p53 and vector DNA. The mutant p53 protein encoded by pC53-SCX3 is unable to bind DNA and to act as a transcriptional regulator. Moreover, it exhibits a dominant-negative effect and inhibits the activity of coexisting wt-p53 (17). The number of clones was estimated three weeks after transfection. The results presented in Fig. 2 demonstrate that the lowest clonal survival was seen when both Mts1 and wt-p53 were coexpressed (compare Saos-2 - bars 2, 4 and CSML-0 bars 1, 4). In CSML-0, which harbors wt-p53, the clonal survival was 5 times lower in *mts1*-

transfectants (bar1) than in the control transfection (bar 6). In Saos-2 cells where p53 is deleted, the survival of the *mts1*-transfected clones (bar 1) was comparable to the control transfection (bar 6). The transfection of mt-p53 did not affect clonal survival (bars 3 and 5), as well as transfection with the vector DNA (bar 6) in both cell lines.

To obtain more direct evidence of p53-dependent Mts1-induced apoptosis, we used two approaches based on transient p53 transfection experiments. First, Mts1-negative (#1109) and positive (#28) subclones of Saos-2 cells were transiently transfected with the wt- and mt-p53 expression vectors and the level of p53 protein was analyzed after 24, 48 and 72 hours using Western blot analysis. As shown in Fig. 3A, 24-72 hours post-transfection mt-p53 was readily expressed in Mts1-positive Saos-2.28 cells. In contrast, the level of wt-p53 expression was much lower as detected after 24 hours of the transfection. At 48-72 hours wt-p53 protein was practically undetectable. At the same time the expression of wt-p53 was abundant in Mts1-negative subclone #1109. In control experiments we were able to detect in the #28 subclone abundant expression of truncated p53 (N-terminal, core domain, C-terminal), but not full-length functional p53 (data not shown). These results indicate selective elimination of wt-p53 expressing Mts1-positive cells.

As an alternative approach we took advantage of stable clones expressing tet-inducible Mts1,which were derived from Mts1-negative Saos-2#1109 and CSML-0 cells. Upon transient transfection of wt-p53 in Saos-2#1109 expressing Mts1 (+Dox) (Fig.3B, panel c), cells bearing features of apoptosis were detected by DAPI staining 24 h post-transfection (Fig. 3B, panel a). mt-p53 did not initiate cell death program in Mts1-induced cells (data not shown). Parameters concerning cell transfection efficiency and apoptotic hallmarks 15 h after

transfection are illustrated in the Table 2. After wt-p53 transfection in Saos-2#1109 cells the number of nuclei with fragmented chromatin was three times higher in the presence of Mts1 expression (+Dox), than in its absence (÷Dox), reaching 15.6% and 5.1%, respectively (Fig. 3B, panel b and d). Similarly, 3 to 4 times higher number of apoptotic cells was detected upon tet-induced Mts1 expression in CSML-0 cells, as verified by TUNEL staining 24 hours after transfection with wt-p53 (Fig. 3C). Taken together, these data suggest that Mts1 co-operates with p53 in triggering apoptosis.

Mts1 physically interacts with p53

Next, we studied whether Mts1 co-operates directly with p53 via protein-protein interaction. To answer this question, we used immunoprecipitation (IP) and Far Western blot analysis.

Lysates from ^{35}S-metabolically-labeled CSML-100 (mt-p53 and Mts1-positive) and CSML-0 (wt-p53 and Mts1-negative) cells were used for IP with anti-p53 and anti-Mts1 antibodies. As shown in Fig.4A, an 11 kDa protein band corresponding to Mts1 was detected in anti-p53 immunoprecipitates from CSML-100, but not from CSML-0 cells. Conversely, a protein corresponding to p53 in anti-Mts1 immunoprecipitates was co-precipitated from CSML-100 cell lysate. A protein band corresponding to MHC, a known Mts1 target (7), was detected in anti-Mts1 immunoprecipitates but not in anti-p53 and control immunoprecipitates. This confirms that the anti-Mts1 antibodies used are able to co-precipitate Mts1 together with its target proteins. The amount of the Mts1 protein co-immunoprecipitated using anti-p53 antibody was lower than that of p53 in anti-p53 precipitates. This might indicate that only a minor portion of Mts1 is engaged in the

interaction with p53. Additionally, the specific radioactive activity per molecule of p53 is three times higher than that of Mts1.

To identify the region of p53, which interacts with Mts1, recombinant p53 peptides corresponding to the N-terminal (#2) aa 1-106, the core (#3) aa 104-288 and the C-terminal (#4) aa 289-390 of murine p53 were obtained. Full length p53 (#1) and the domain peptides were incubated with recombinant Mts1 protein and precipitated with anti-p53 antibodies followed by Western blot analysis using anti-Mts1 antibodies. As shown in Fig. 4B only full-length p53 and the C-terminal domain are able to interact with Mts1.

Far-Western blot analysis was used to further characterize the interaction between Mts1 and p53. Full-length p53 and its functional domains immobilized on a membrane were incubated with recombinant Mts1 in conditions permitting an interaction between the proteins. Mts1 bound to p53 was detected using anti-Mts1 antibodies. The data shown in Fig. 4C indicate that Mts1 physically interacts with full size p53 and the C-terminal domain of p53 (lanes 1, 4). As a positive control we used a recombinant fragment of non-muscle myosin, which is known to be Mts1 target (Fig. 4C, lane 5). Coomassie Blue stained gel, run in parallel, was used to verify the quantities of the proteins.

For more precise mapping of the Mts1 binding site on the C-terminal domain of p53, full-length GST-wt-p53 and GST- p53Δ30, lacking the last 30 C-terminal residues were used. The p53 fusion proteins as well as the GST protein alone were incubated with recombinant Mts1. The complexes were immobilized on Glutathione –Sepharose 4B beads. The Mts1 protein bound to p53 was analyzed by SDS-PAGE followed by Western blot analysis using anti-Mts1 antibodies. The data shown in Fig.4D demonstrate that Mts1 binds GST-p53Δ30

with much lower efficiency than to full-length p53. This indicates the importance of residues 364-393 of the C-terminal domain for the p53-Mts1 interaction.

Thus, our results taken together demonstrate a direct interaction between Mts1 and the C-terminal domain of p53.

The Mts1 protein inhibits phosphorylation of p53 by PKC

We showed previously that Mts1 interacts with MHC and inhibits the phosphorylation of myosin by PKC (7). Since the Mts1-binding site of p53 harbors both PKC and CKII phosphorylation sites, we investigated whether Mts1 affects p53 phosphorylation. Recombinant full-length p53 (#1) and its domain peptides (# 2, 3, and 4) were phosphorylated by PKC in the absence or presence of recombinant Mts1 and analyzed by SDS-PAGE. The data obtained in three independent experiments indicate a reliable 37% (SD± 2.6%) inhibition of the PKC-mediated phosphorylation of full-length p53 protein, and 56% (SD±8.1%) of its C-terminal fragment by Mts1 (Fig. 5A). Since Mts1 did not affect the phosphorylation of the N-terminal and DNA-binding domains of p53 as well as PKC autophosphorylation at the same conditions, we exclude the possibility that the recombinant Mts1 protein acts as a competitive substrate. Interestingly, no inhibition/activation of CKII mediated phosphorylation of full-length p53, or the C-terminal domain, was observed in the presence of Mts1 (Fig. 5B).

In conclusion, our results revealed that Mts1 specifically inhibits the phosphorylation of the C-terminal domain of p53 by PKC *in vitro*.

Modulation of p53 DNA-binding activity by Mts1

To assess the functional significance of the Mts1-p53 interaction, we investigated whether Mts1 affects the DNA-binding activity of p53 in EMSA. The data obtained in experiments using the p53-binding site from the p21/WAF promoter and nuclear extracts from CSML-0 cells are shown in Fig. 6. The presence of p53 in the complex was proven by a supershift of the complex by anti-p53 antibody pAb421 (lane 4), and the specificity of DNA binding was shown in competition experiments (lanes 2, 3 and 8). Incubation of nuclear extracts containing wt-p53 with the recombinant Mts1 protein prior to the addition of the labeled Oligonucleotide decreased DNA -binding activity of p53 (lanes 5, 6). The inhibition was less prominent when Mts1 was added after the formation of p53-DNA complex (data not shown). This indicates that preformed Mts1-p53 complexes had a lower capacity to bind DNA. The influence of Mts1 on p53-DNA binding capacity is strongly Ca^{2+}-dependent. Addition of EGTA to the reaction mixture completely abolished the effect of Mts1 on p53-DNA complex formation (lane 11) whereas in the presence of 0.5 mM Ca^{2+} Mts1 almost completely prevented binding of p53 with p21/WAF DNA (lanes 12, 13). Data obtained show that Mts1 does not influence Oct-DNA complex formation (lanes 14, 15). These experiments demonstrate that Mts1 is able to modulate the DNA-binding capacity of p53.

Mts1 affects p53 -dependent transcription

The results presented above suggest that Mts1 might be involved in the modulation of p53-

regulated gene expression. To test this idea we investigated the effect of Mts1 on the activity of p53 *in vivo*. Mts1 expression vector was co-transfected along with a luciferase reporter placed under the control of the synthetic p53 responsive elements from either p21/WAF (p21-luc) or Bax (Bax-luc) genes. Two different Mts1-negative cell lines, CSML-0 and VMR-liv were used in these experiments. The pCMV-β-gal plasmid was co-transfected for evaluation of the transfection efficiency. The results of several independent experiments are summarized in Fig.7. In both cell lines expression of Mts1 resulted in the inhibition of p21-luciferase activity, 60% in CSML-0 and 42% in VMR-liv. On the contrary, a slight (27-30%) increase of BAX-luc reporter transactivation was observed in both cell lines. No reporter gene expression was detected in p53-null Saos- 2 cells confirming p53-dependence of both p21-luc and Bax-luc expression (data not shown). Thus, Mts1 affects p53-mediated transcriptional activation in *vivo*. Interestingly, the effect of Mts1, inhibition or activation, depends on the particular response element.

Mts1 modulates the expression of p53-regulated genes

Next we examined whether Mts1 can modify the expression of endogenous p53-responsive genes. After induction of Mts1 expression by Dox in CSML-0/tet/*mts1* clone 13L total RNA was isolated from cells grown at low or high densities at different time points. The data shown in Fig.8 indicate that Mts1 induction in CSML-0 cells differentially influenced p53-dependent gene expression. Expression of p21/WAF was suppressed 25-30% 24-48 hours after Mts1 induction, in both sparse and dense cultures. In contrast, expression of the pro-apoptotic gene Bax was activated 1.5 fold by Mts1 in the sparse culture, and more than 3 fold

in the dense culture. The effect of Mts1 on Cyclin G1expression was minimal at both conditions. Modulation of the expression of THBS1 by Mts1 was remarkable and strongly dependent on the cell growth conditions. In sparse cultures the induction of Mts1 led to 1.3 fold up-regulation of THBS1 transcription, whereas in dense cultures Mts1 significantly, more than 65%, down-regulated THBS1 expression. The effect of Mts1 on *mdm-2* transcription was also dependent on the cell density. 72 hours post induction of Mts1 significant activation of Mdm-2 expression was observed in sparse cultures, whereas in dense cultures the effect of Mts1 was weaker.

Thus, our results show that Mts1is able to modulate the expression of several p53-regulated genes. The effect of Mts1 was dependent on the particular gene and cell growth conditions.

DISCUSSION

In the present study, we demonstrate a functional interaction between the small, Ca^{2+}-binding protein Mts1/S100A4 and p53 tumor suppressor protein and characterized the biological significance of this interaction.

It was initially observed that transfection of Mts1-negative tumor cells with *mts1* expression constructs results in intensive clonal death. Since co-transfection of the anti-apoptotic gene Bcl-2 was able to rescue the clones, we suggested that Mts1 induces cell death by apoptosis within 24-48 hours after transfection. Moreover, a direct co-operation of Mts1 and wt- p53 in the cell death was demonstrated by transfection of p53 expression constructs into Mts1-inducible cell lines. The data obtained demonstrate that the level of p53-dependent apoptosis

significantly increases upon Mts1 induction. These findings suggest that Mts1 and p53 co-operate in the promotion of apoptosis.

Analysis of 26 tumor-derived cell lines revealed a significant reverse correlation between the expression of wild type p53 and Mts1. This fact may, probably, explain our observation that Mts1 promotes p53-induced apoptosis. More likely, there is a selection against wt-p53 in Mts-1 expressing tumor cells *in vivo.* Indeed, a trend for more p53-positive human breast carcinomas to be positive for Mts1 compared to p53-negative ones in immunohistological studies was demonstrated (18, 19). Although the status of p53 in these tumor samples was not tested directly, the positive immunohistostaining indicates that p53 is likely mutated since the level of wt-p53 in most cells and tissues is at or below the level of detection in paraffin-imbedded samples. Though the association between p53 overexpression and the presence of Mts1 in above mentioned studies is not so striking compared to our data, it is nevertheless points to the similar conclusion.

Co-existence of wt-p53 and Mts1 in immortalized fibroblasts, probably, might be explained by conformational transformation of p53 as it was observed in the 10T½ cell line (16). The co-expression of Mts1 and elevated level of wt-p53 was shown in a dexamethasone-inducible clone of B16 melanoma cell line transfected with MMTV-*mts1* (20). We assume that there may be more than one interpretation of these data. This could be due to functional inactivation of p53 by glucocoticoid receptor (GR), since it was shown that GR forms a complex with p53 *in vivo*, resulting in cytoplasmic sequestration of both p53 and GR (21). Alternatively, dexamethasone-mediated pleiotrophic effect on gene regulation, cell cycle, apoptosis etc. in GR –positive cells might neutralize p53/Mts1 cooperation.

The most prominent biological effects elicited by p53 as a transcriptional activator are cell cycle arrest and apoptosis in response to diverse types of stress (22). A number of proteins that interact with p53 and modulate its functional activity by covalent and noncovalent modifications have been identified (9,8). The C-terminal basic domain of p53 was shown to negatively regulate the specific DNA binding activity of the protein (9). Modulators of p53, which bind or modify this region, dramatically affect the activity of the protein. Interaction with the C-terminal -specific antibody pAb421, single-stranded RNA and DNA (23) and deletion of this regulatory domain (24) abolish the negative regulatory role of the C-terminus thus activating p53 (9). Additionally, phosphorylation of 392^{Ser} by CKII or 371^{Ser}, 376^{Ser}, and 378^{Ser} by PKC results in protein activation (25). Therefore, it is logical that Mts1, which according to our data interacts with the C-terminal regulatory domain, is able to modulate p53 activity.

Using several approaches, we have demonstrated a physical interaction between p53 and Mts1. The Mts1- binding site is localized in the C-terminus of the p53 molecule, including aa 364-393. The interaction of Mts1 with p53 results in inhibition of p53 phosphorylation by PKC, but not by CKII. Moreover, binding of Mts1 to p53 leads to the inhibition of the complex formation between p53 and a p53-specific consensus oligonucleotide derived from the p21/WAF promoter. The interaction of p53 with another member of the S100-family, S100B, was shown previously. S100B physically interacts with the C-terminal domain of p53, inhibits its phosphorylation by PKC and influence p53 oligomerization and biological activity (26). However, a direct influence on the p53 transactivation activity is demonstrated only for Mts1 so far. Recently it was shown using NMR spectroscopy that a peptide from the

C-terminal domain of p53 (aa 367-388) does not have a regular structure in its native form. Binding of S100B in the presence of Ca^{2+} leads to conformational alterations of the p53-derived peptide. Three-dimensional structure of the complex reveals several hydrophobic and electrostatic interactions between S100B and p53, which result in a sterical block by S100B on the phosphorylation and acetylation sites on p53 (27). Such modifications can in turn alter the transcriptional activation capacity of p53 (induction and repression). It is conceivable that Mts1, similarly to S100B, can induce conformational changes in p53. However, this issue requires further elucidation.

Here we demonstrate that via interaction with p53, Mts1 differentially modulates the transactivation function of p53. Interestingly, the effect of Mts1 on p53 is dependent on the particular target gene and cellular environment. For instance, Mts1 inhibits the DNA-binding capacity of p53 to the p53-responsive element from the p21/WAF promoter *in vitro*. In accordance with that, the expression of Mts1 *in vivo* down regulates luciferase activity driven by the same p53-binding sequence. In contrast, more than 3 fold activation of the pro-apoptotic Bax gene expression occurs within 24-48 hours following Mts1 induction. Presumably, co-operation between p53 and Mts-1 in apoptosis induction observed in our experiments is at least partially due to the up-regulation of Bax gene expression.

In the reporter gene assay, the presence of Mts1 stimulates luciferase activity driven by the p53-responsive element from the Bax promoter only slightly. The disparity between the extent of RNA up-regulation and reporter gene transactivation is probably due to a requirement for additional regulatory elements present in the full length Bax gene promoter. An interesting pattern of gene expression was observed for THBS1, another p53-target. The

THBS1 gene is known to repress tumor progression since it is an inhibitor of angiogenesis. Repression of THBS1 promotes tumor vascularization, increasing the metastatic potential of tumor cells (28). We showed that Mts-1 strongly down-regulates THBS1 gene expression in dense cultures. This suggests that Mts1 might prevent the anti-angiogenic function of THBS1 *in vivo*. These results are in line with our observation that Mts1 is able to promote angiogenesis *in vivo* and *in vitro* (N.A., J. Klingelhofer, M.G., C.Christensen, M.K., E.T., G.Georgiev, V.Berezin, E.Bock, J.Rygaard, R.Cao, Y.Cao, E.L., submitted for publication). However, in sparse cultures Mts1 up-regulated THBS1 gene expression. This might indicate that the activity of Mts1 depends on a proliferation state of cells and cellular environment. Since the conditions inside the tumor rather resemble dense cultures, our results point to the possibility that down-regulation of THBS1 by Mts1 might play an important role in tumor angiogenesis.

The functional performance of the Mts1 and mutant p53 interaction in tumor cells is a matter of great interest since this combination is widely represented in malignant tumor cells as we have demonstrated. An inverse, oncogenic function is shown for several “gain of function” p53 mutants (29, 30). Some p53 mutants have oncogenic properties due to the ability of these mutants to regulate the expression of a new set of genes such as c-myc (31), EGFR, PCNA, MDR-1 (32), BAG-1 (33). The requirement of the C-terminal domain for the “gain of function” by p53 tumor-derived mutants was demonstrated (31, 32). Since we show that Mts1 binds to C-terminal regulatory region of p53, there is an intriguing possibility that Mts1 can also modulate the activity of mutant p53 proteins. We propose that Mts1 interacting with wild type p53 and stimulating apoptosis at early stages of tumor development may contribute

to the selection of the malignant phenotype. At later stages the Mts1 protein may be involved in the regulation of “gain of function” mutant p53 molecules and via modulation of the gene expression fulfill its “pro-metastatic” role and further advance tumor progression. Further studies elucidating the role of Mts1 in modulating the function of mutant p53 by C-terminal interaction might help to understand a mechanism of oncogenic potential of mutant p53.

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FOOTNOTES

Abbreviations:
BSA - bovine serum albumin
CKII – casein kinase II
DOX - doxycycline
DTT - dithiothreitol
DME – Dulbecco's Modified Eagles medium
EMSA – electrophoretic mobility shift assay

FCS – fetal calf serum
FITC – fluorescein isothyocianate
GST – glutathione S-transferase
MHC – heavy chain of nonmuscle myosin
PBS – phosphate saline buffer
PAGE – polyacrylamide gel electrophoresis
PKC – protein kinase C
PMSF – phenylmethylsulfonyl fluoride
PEG – polyethylene glycol
SDS – sodium dodecyl sulfate
TRITC – tetramethyl rhodamine isothiocianate

THBS1 – thrombospondin-1

FIGURE LEGENDS

Figure 1. Clonal survival of CSML-0 and VMR-liv cells transfected with mts1/S100A4.

Number of stable clones was evaluated three weeks after transfection with expression constructs, mts1 (pSV-mts1); mts1 + Bcl2-s (B-s); mts1 + Bcl2-a (B-a). In all transfections pSVneo was co-transfected for neomycin selection. Bcl2-s – the gene is in the sense orientation, Bcl2-a - the gene is in the antisense orientation.

Values shown represent mean ±SD of three (CSML-0) and two (VMR-liv) experiments.

Figure 2. Clonal survival of singly and doubly transfected Saos-2 and CSML-0 cell lines.

Saos-2 (p53-null and ~70-80% mts1-positive) and CSML-0 (wt-p53 and mts1-negative) cells were transfected with mts1, wild type (wt) and mutant (mt) p53 expression vectors. In three weeks the number of clones was estimated.

Values shown represent mean ±SD of two separate experiments in triplicate.

Figure 3. Simultaneous expression of Mts1 and wt-p53 in Saos-2 cell lines.

Mts1-positive Saos2#28 and Mts1-negative Saos2#1109 were transiently transfected with wt-and mt-p53 expression constructs. Proteins were isolated at 24, 48 and 72 hours and p53 protein was analyzed by Western blot following with immunodetection using anti-p53 antibodies. After 48-72 hours the expression of p53 in #28 clone is completely abolished in contrary to clone #1109. The band in C100 lane indicates the position of the endogenous p53. Immunofluorescence analysis of Mts1-inducible Saos-2.1109 cells transiently transfected with wt-p53. Cells, Dox (+) and Dox (÷), were transfected with wt-p53 expression vector. After 24 hours cells were fixed and stained with anti-Mts1 antibodies (c, d) and DAPI (a, b,). Hallmarks of apoptosis were easily detected in Mts1- induced cells upon the transient expression of wt-p53 (a) compared with non-induced cells (b). More than 25 fields were analyzed for each transfection in three independent experiments. Magnification x800. CSML-0/tet-mts1#13L cells Dox (+) and Dox (÷) were transiently transfected with wt-p53. After 24 hours cells were fixed and subjected to TUNEL -assay. Significant increase of the "apoptotic cells" is seen under the Mts1 activation.

Figure 4. Mts1 physically interacts with p53.

A - Immunoprecipitations of ^{35}S-labeled CSML-0 and CSML-100 cell lysates with anti-Mts1 and anti-p53 antibodies. Cell lysates were prepared using NP40-containing buffer and precipitated with anti-p53 monoclonal antibody pAb421; polyclonal affinity purified anti-

Mts1 rabbit serum and control serum. Proteins in the precipitates were separated in gradient PAGE (4-20%) and analyzed by autoradiography.

B - Pull-down of recombinant Mts1 protein by recombinant p53 (#1) and its domain peptides (#2, #3 and #4). P53 proteins were mixed with recombinant Mts1 and immunoprecipitated with control serum (control) and anti –p53 antibodies (Ab): #1 – pAb421; #2 – pE19; 3# -pAb240; #4 –pAb421. Immunoprecipitated proteins were separated by PAGE and Western blot was immunoprobed with anti- Mts1 antibody. Co-immunoprecipitation of Mts1 with p53 was observed with full size p53 (#1) and its C-terminal domain (#4), but not with DNA-binding domain (#3) and N-terminal domain (#2).

C - Blot- overlay (Far-western) of recombinant p53 and its peptides with the Mts1 protein. Full-size p53 (#1, lane 1), p53 domain peptides (#2, #3 and #4, lanes 2, 3, 4) and recombinant fragment of MHC (lane 5) were separated in SDS-PAGE, transferred on the membrane and incubated with recombinant Mts1. Mts1 bound to proteins was visualized by immunostaining with anti- Mts1 antibodies. On the parallel gel the same amounts of the recombinant proteins were loaded and stained by Commassie Blue.

D - Binding of Mts1 with GST-p53 fusion proteins.

GST-wt-p53, GST-p53Δ30 (deletion of AA 364-393) and GST proteins were bound to Glutathione- sepharose beads and incubated with recombinant Mts1 protein. Mts1 linked to protein-beads was recovered and analyzed by PAGE and Western blotting with anti-Mts1

antibodies.

Figure 5. Influence of Mts1 on phosphorylation of p53 and its domains *in vitro*.

Phosphorylation by PKC. #1 – wt-p53 was phosphorylated in the absence and presence of recombinant Mts1. #2, #3 and #4 – phosphorylation of p53 peptides (N-terminal, DNA-binding and C-terminal) at increasing concentrations of the Mts1 protein. Influence on PKC phosphorylation was observed with #1 and #4 p53 proteins, but not with #2 and #3. (B) Phosphorylation by CKII. #1 and #4 p53 proteins were phosphorylated in the absence and presence of the recombinant Mts1. No influence on CKII phosphorylation was detected.

> - position of the p53 derived polypeptides

Figure 6. Influence of Mts1 on p53 DNA-binding activity.

Nuclear extracts from CSML-0 and p53-binding element from p21/WAF promoter were used. A specificity of the p53-DNA complex is proven using specific- and nonspecific competitors (lanes 2, 3) and band supershift with anti -p53 pAb421 Ab (lane 4). 0.5-1 μg of the recombinant Mts1 protein was added to the DNA-binding reactions with specific oligonucleotide at 50μM Ca^{2+} (lanes 5, 6) and 0.5 -1mM Ca^{2+} (lanes 12, 13). 1μg of wt-Mts1 was added to the control oligonucleotide (Oct-1) (lane15). Addition of EGTA completely abolishes the Mts1 effect on p53-DNA binding (lane 11).

Figure 7. Influence of Mts1 on transactivation of p21/WAF- and BAX-luciferase

page * arabic19

reporter. CSML-0 and VMR-liv cells were transfected with p21-luc and BAX-luc constructs either alone or with Mts1 expression constructs. After 24 hours cells were harvested and luciferase activity was tested. pCMV-β-gal plasmid was co-transfected to evaluate the efficiency of transfection.

Values shown represent mean ±SD of three (p21-luc) and two (BAX-luc) separate experiments.

Figure 8. Influence of Mts1 on the transcription of p53-regulated genes.

Mts1-tet-inducible 13L CSML-0 clone was grown at low and high densities. Mts1 was induced by adding 2μg/ml of Doxycycline. Total RNA's were isolated at various time periods after induction and Northern blot analysis using p53-regulated gene probes was performed sequentially with the same filter. To normalize the amount of poly (A+) RNA on the membranes, hybridization with labeled poly (U) was performed. To quantify the intensities of bands and lanes, as in the case of poly (U), membranes were scanned using a Molecular Dynamics computing densitometer with Image Quant software.

Values showed represented means±SD of four (p21/WAF, THBS and BAX) and two (Cyclin G and MDM2) separate experiments.

Table 1

Cell lines	Type of tumor	Species	Status of p53	Status of Mts1	Ref. on p53 status
MDA-MB-157	breast cancer	human	null	++++	34
MDA-MB-231	breast cancer	human	mt	+++	34
MDA-MB-468	breast cancer	human	mt	++++	35
MDA-MB-453	breast cancer	human	*wt*	-	34
ZR-75-1	breast cancer	human	*wt*	-	*
SkBr-3	breast cancer	human	mt	++++	36
BT-20	breast cancer	human	mt	-	37
BT-549	breast cancer	human	mt	++++	38
MCF-7	breast cancer	human	*wt*	-	34
Saos-2	osteosarcoma	human	deleted	+++	39
HOS	osteosarcoma	human	mt	++++	40
Lovo	colon cancer	human	*wt*	-	41
CaCo-2	colon carcinoma	human	mt	+++	42
SW 480	colon carcinoma	human	mt	+++	35
SW 620	colon carcinoma	human	mt	++++	43
RD	rabdomyosarcoma	human	mt	++++	44
DU-145	prostate cancer	human	mt	+/-	45
AsPC	pancreas carcinoma	human	mt	++++	46
J82	bladder carcinoma	human	mt	-	47
CaSki	cervical carcinoma	human	*wt*	-	48
SVEC4-10	SV40-transformed endothelial cells	mouse	“mt “ (T-antigen)	++++	49
4T1	mammary carcinoma	mouse	null	+++	50
CSML-100	mammary carcinoma	mouse	mt	++++	*
VMR-liv	mammary carcinoma	mouse	*wt*	-	*
CSML-0	mammary carcinoma	mouse	*wt*	-	*
NIH 3T3	fibroblasts	mouse	wt	++++	51

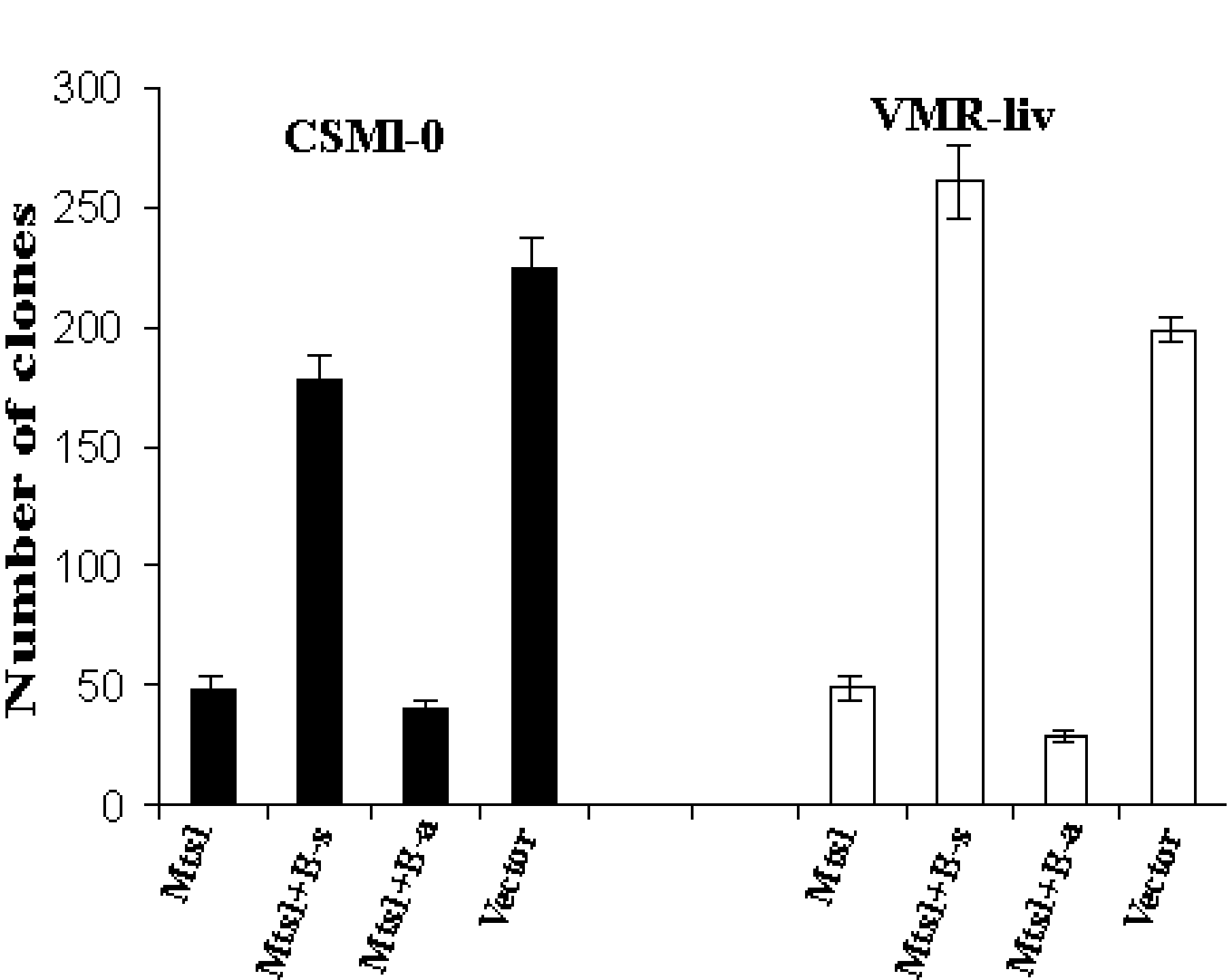
L	fibroblasts	mouse	wt	++++	52
10T½	fibroblasts	mouse	wt	++++	16

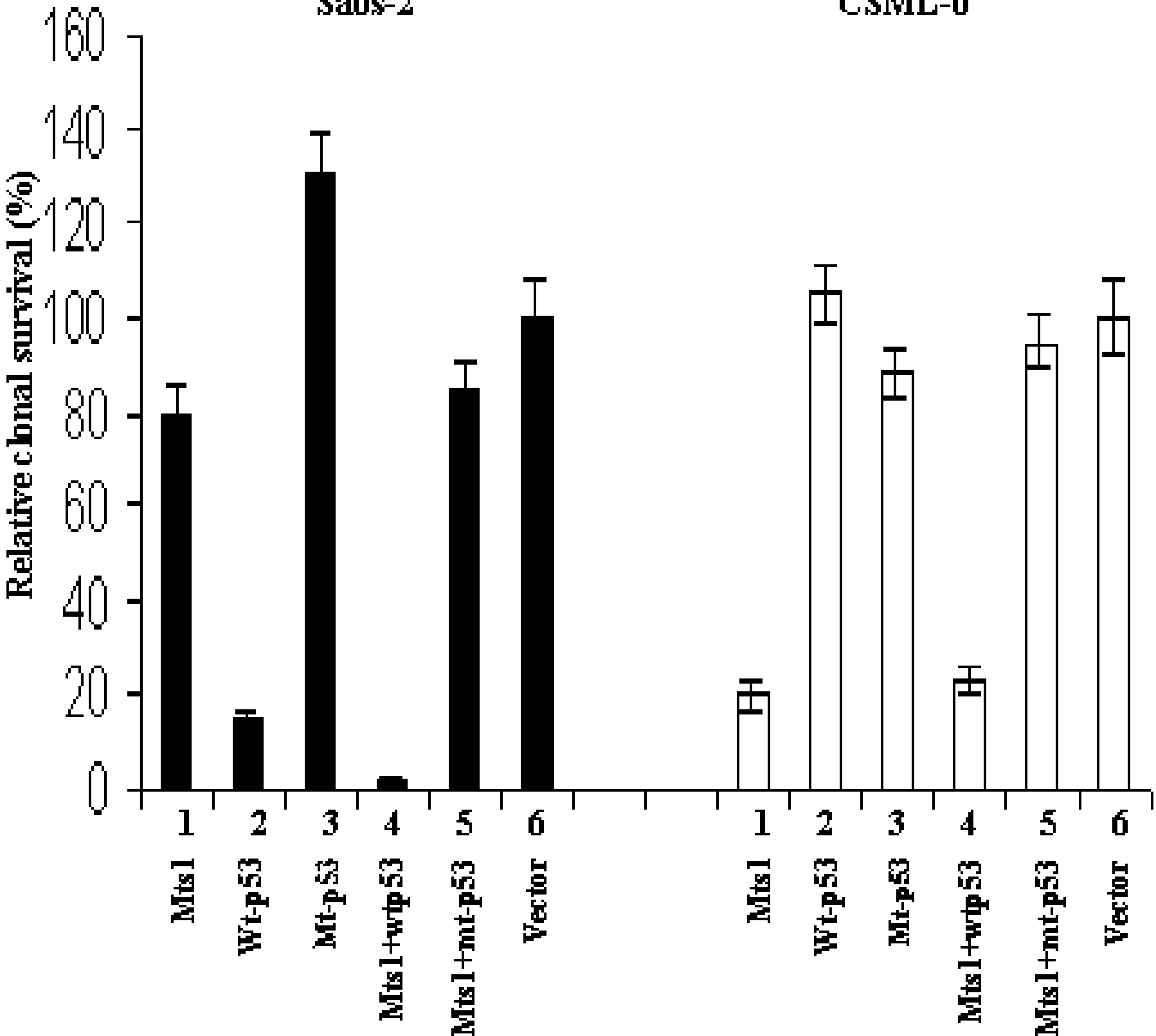
*Status of p53 was checked using immunoprecipitation with conformational antibodies (pAb1620 and pAb240).

Table 2*.

Parameters	***(-) DOX (average number and %)***	***(+) DOX (average number and %)***
Nuclei with the apoptotic hallmark	2,5 (**5,1%)**	6,125 (**15,6%**)
Nuclei (DAPI staining)	49,0	39,4
**Efficiency of the transfection	30,2%	32,6%

*Results of two independent experiments are summarized in the Table. The parameters were estimated as an average sum in ten random fields for each of triplicate glasses at magnification 800x.
**Evaluated by counting of the blue cells expressing β-galactosidase derived from co-transfected pCMV-βgal.


CSMI-0
VMR-liv
Number of clones
300
250
200
150
100
50
0
Msl
Msl+B-s
Msl+B-a
Vector
Msl
Msl+B-s
Msl+B-a
Vector


Saos-2
CSML-0
Relative clonal survival (%)
0
20
40
60
80
100
120
140
160
1
2
3
4
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6
Mtsl
Wt-p53
Mt-p53
Mtsl+wtp53
Mtsl+mt-p53
Vector

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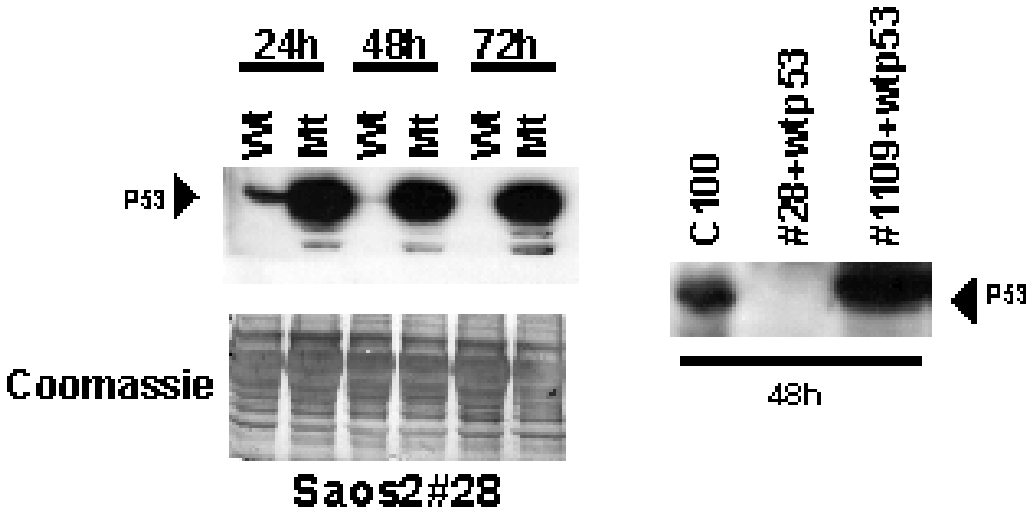
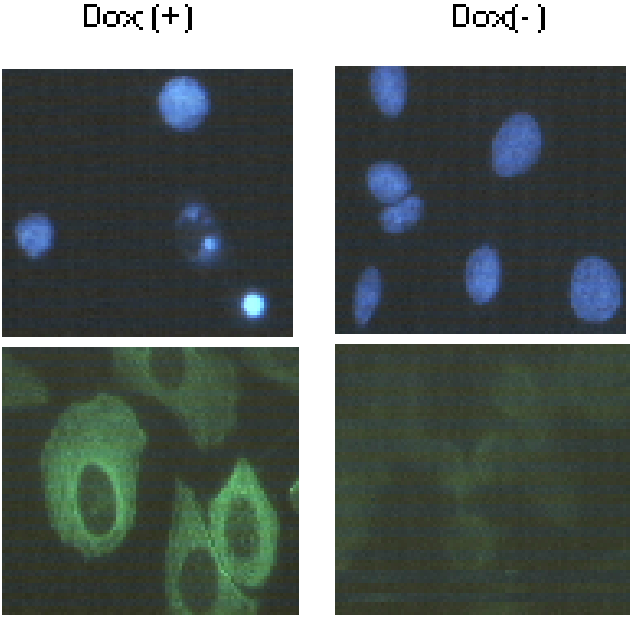


Saos2#28

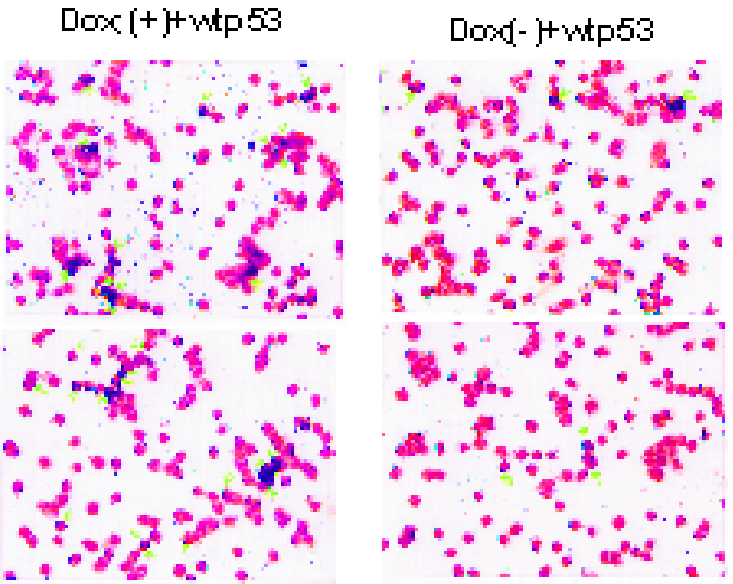
B



Saos2#1109/tet-*mts1*

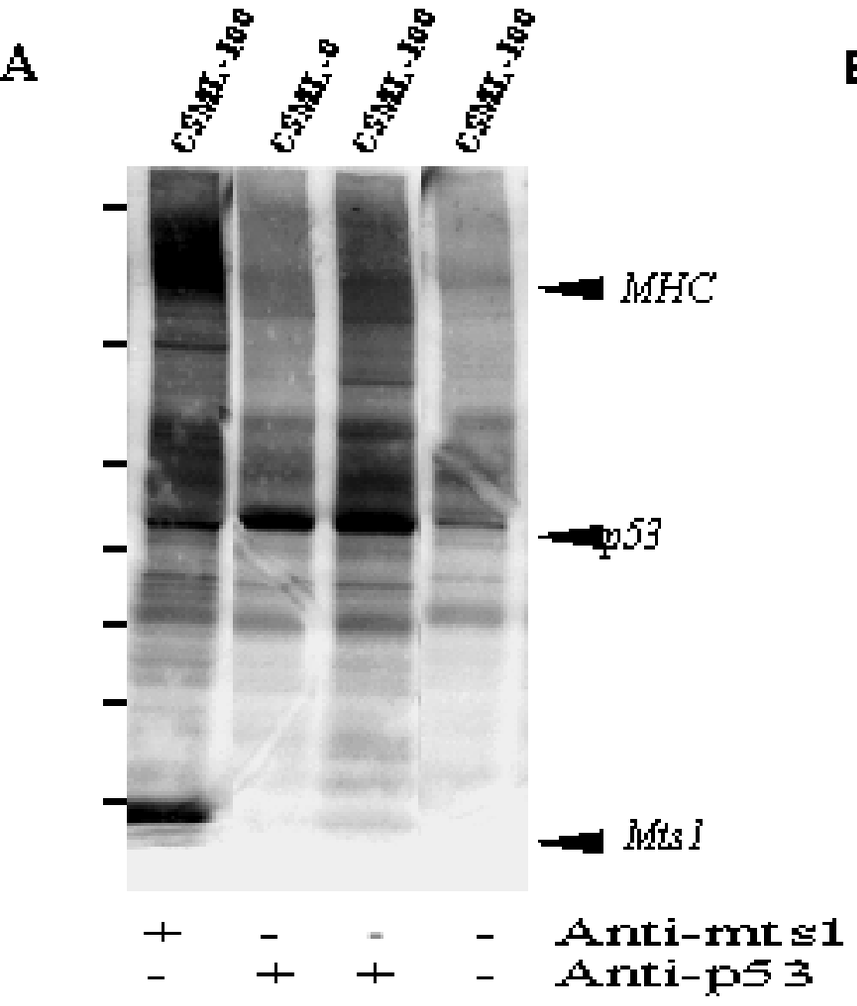
C

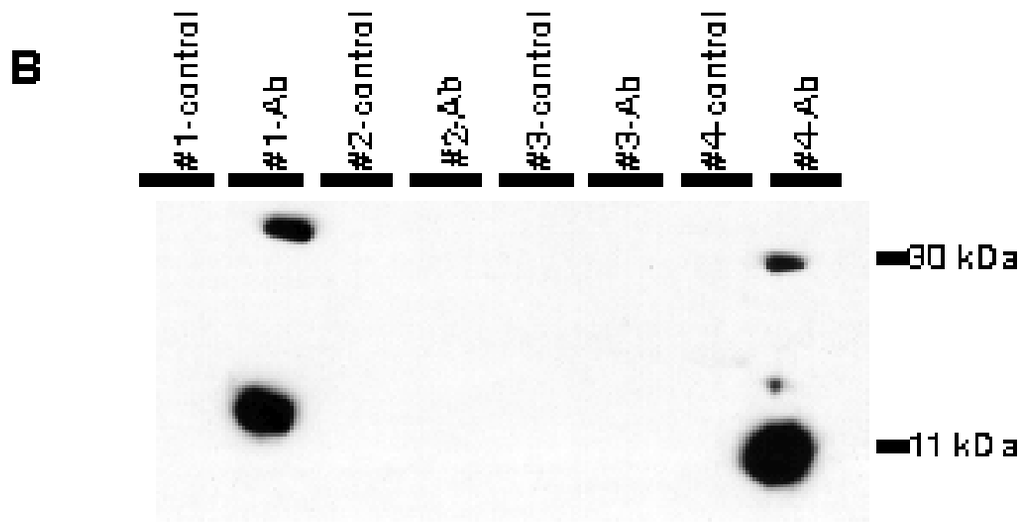
TUNEL

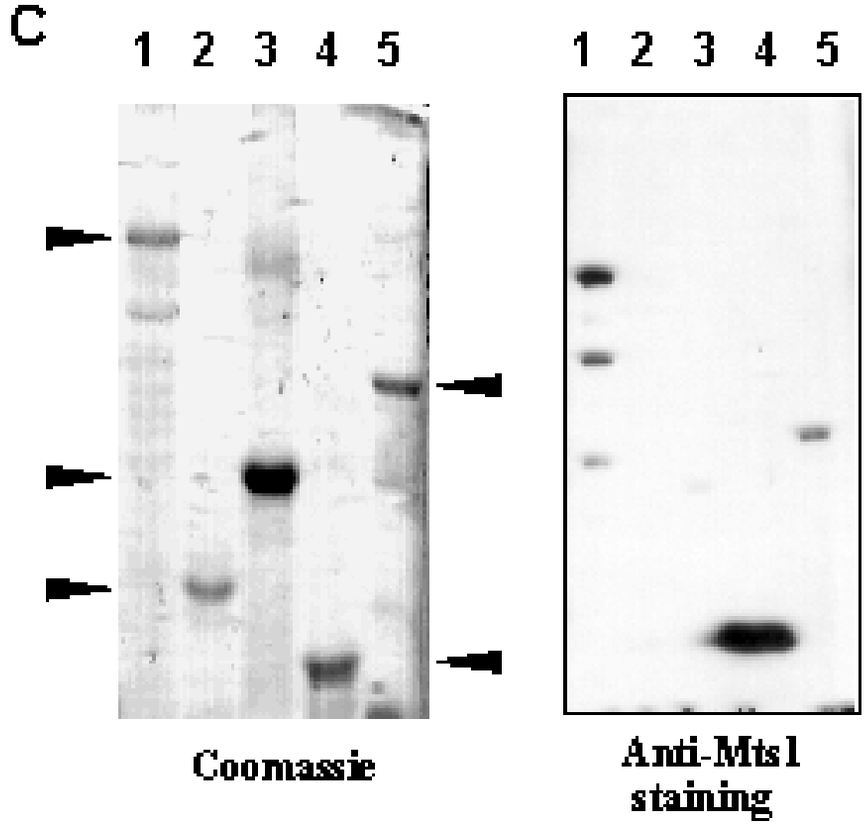


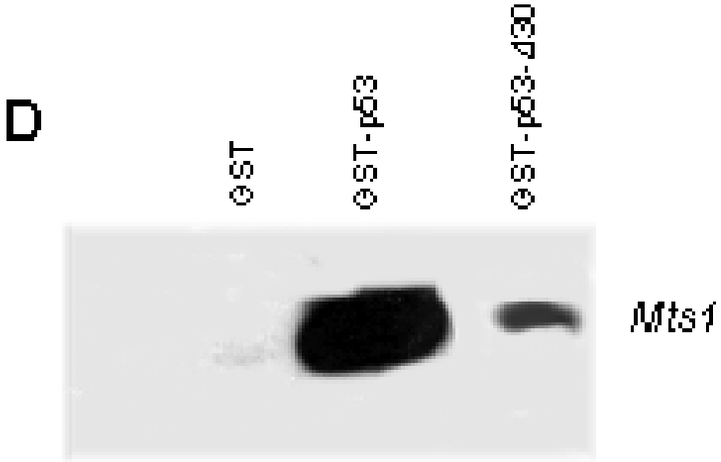
CSML-0/tet-mts1#13

Mts1 physically interacts with p53

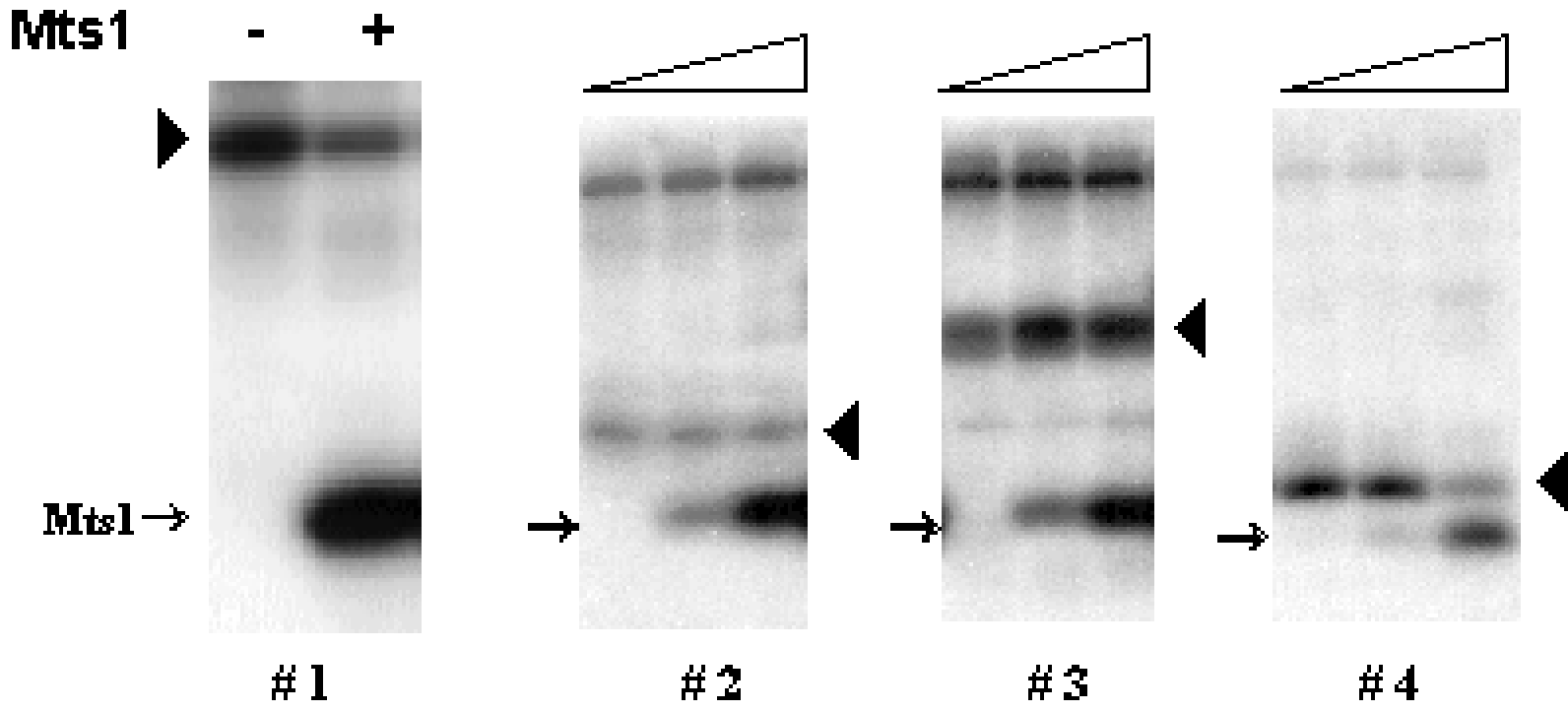




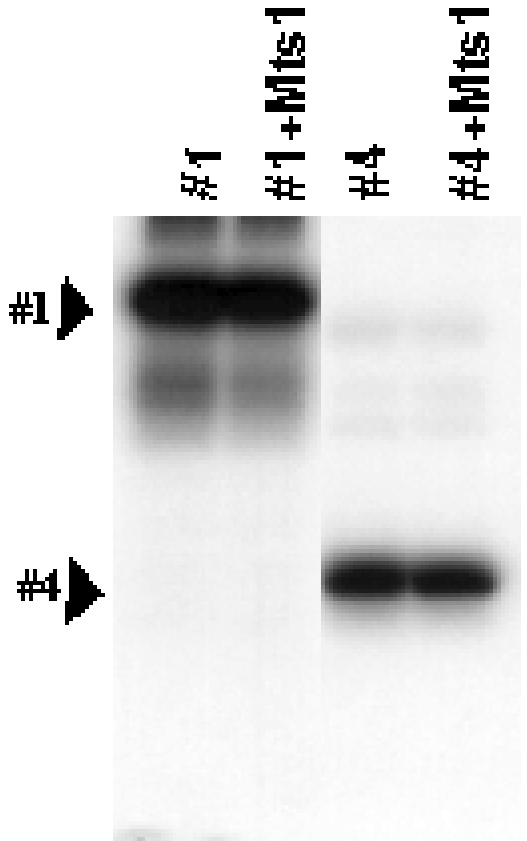


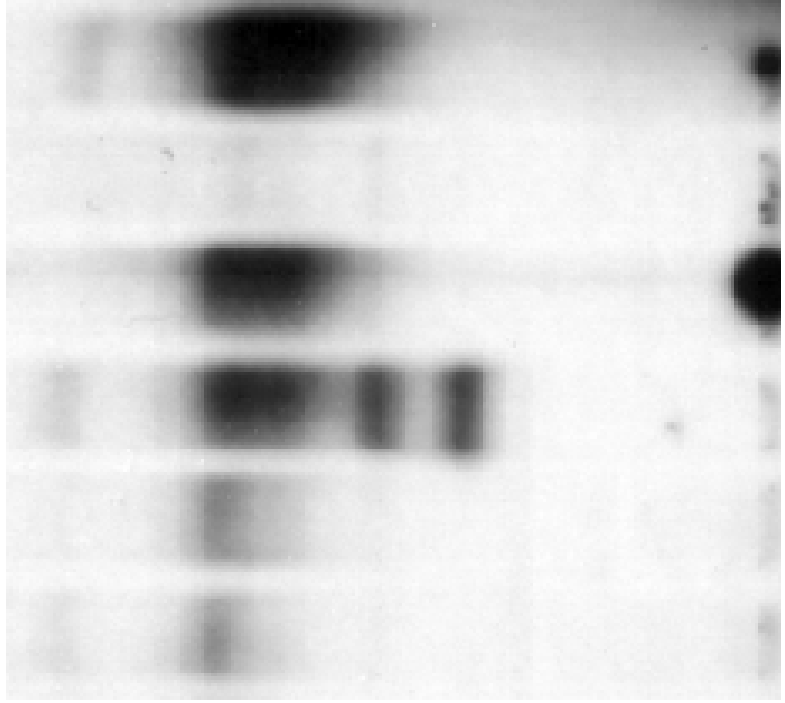


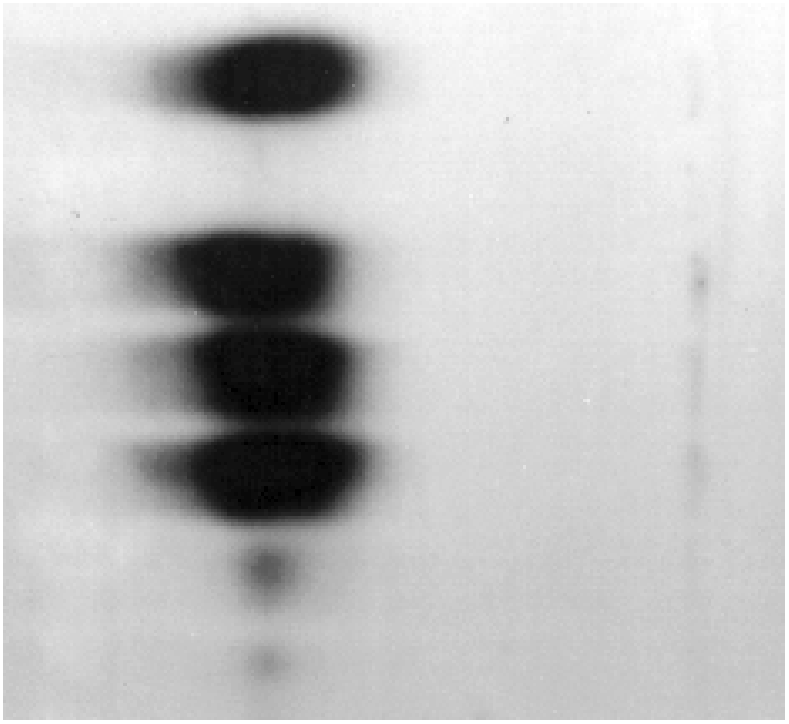
A (PKC)

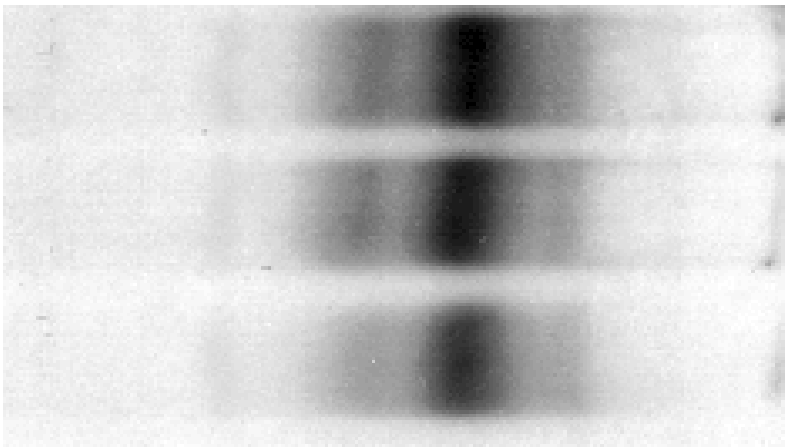


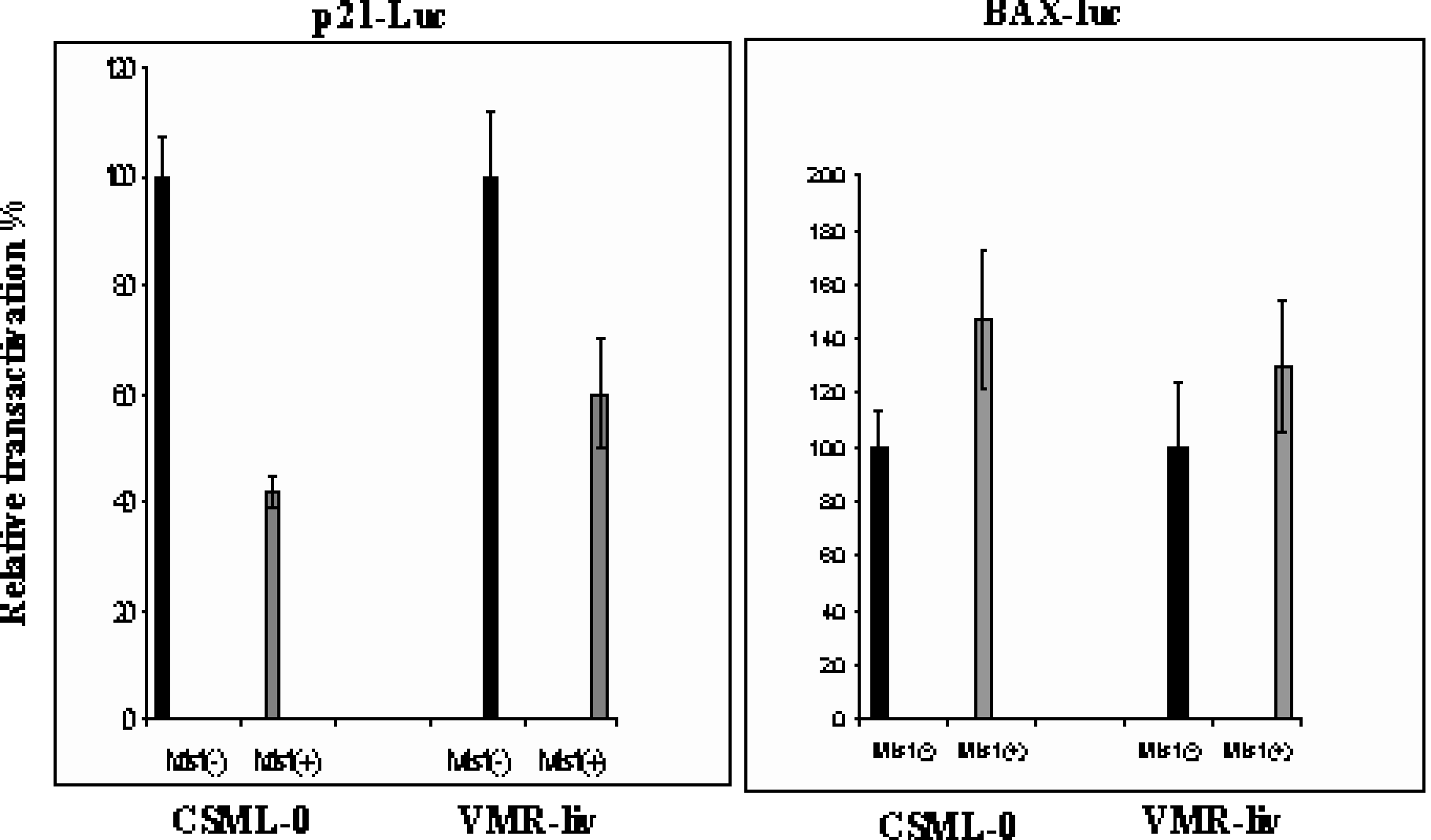
B (CKII)

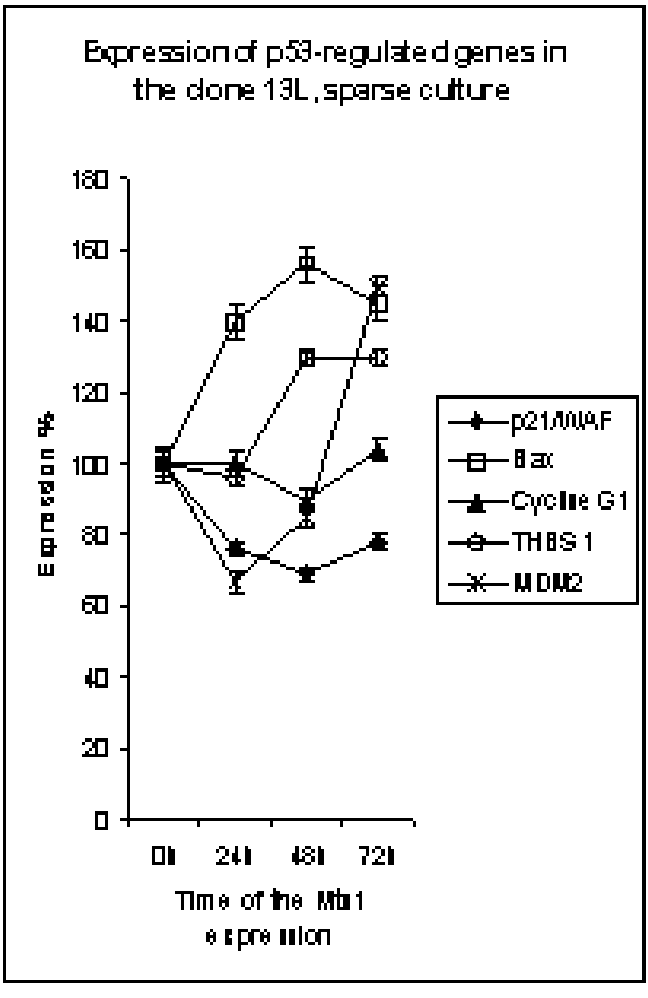


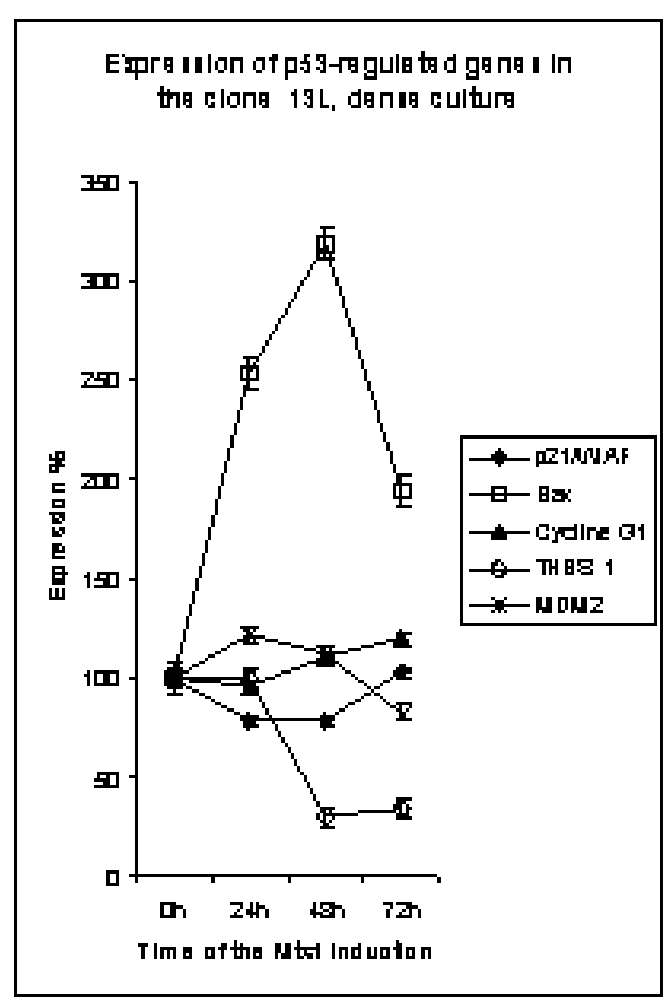

1
2
3
4
5
6
P53/p21 complex
+Specific competitor
+Non-specific competitor
+pAb421
+ 500ng Mbs1
+ 1000ng Mbs1

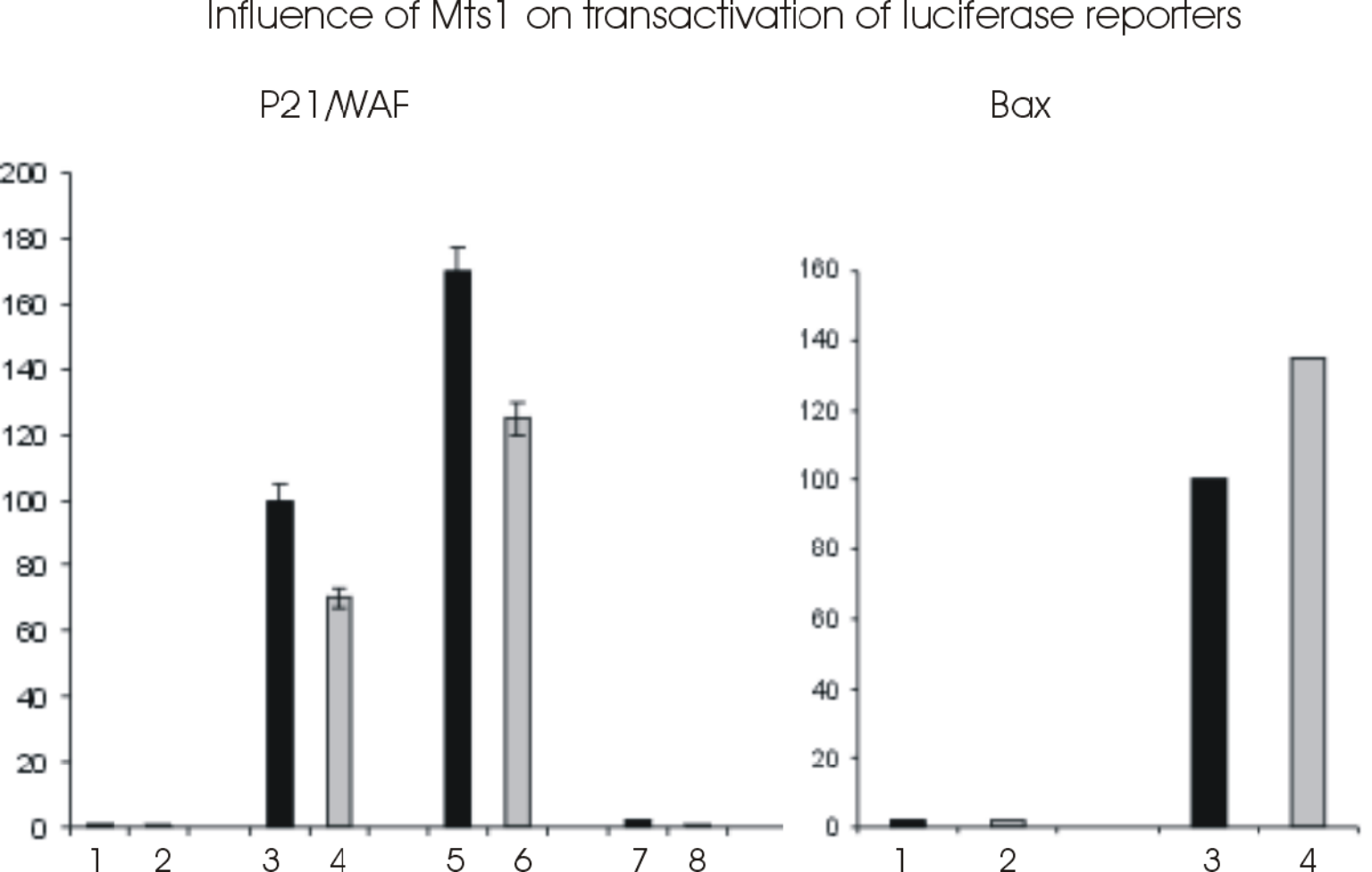

8
9
10
11
12
13
P53/p21 complex
+Specific competitor
+1mM Ca2+
+0.5mM EGTA
+Mbs1+0.5mM EGTA
+Mbs1+0.5mM Ca2+
+Mbs1+1mM Ca2+


14
15
16
Oct-1-complex
+Mbs1+1mM Ca2+
+pAb421


p21-Luc
BAX-luc
Relative transactivation %
0
20
40
60
80
100
120
140
160
180
200
Mst1(-)
Mst1(+)
Mst1(-)
Mst1(+)
Mst1⊖
Mst1⊕
Mst1⊖
Mst1⊕
CSML-0
VMR-liv
CSML-0
VMR-liv


Expression of p53-regulated genes in the clone 13L, sparse culture
Expression %
0
20
40
60
80
100
120
140
160
180
0h
24h
48h
72h
Time of the Mbd1 expression
p21/WAF
Bax
Cyclin G1
THBS 1
MDM2


Expression of p53-regulated genes in the clone 13L, dense culture
Expression %
0
50
100
150
200
250
300
350
0h
24h
48h
72h
Time of the Mbd1 induction
p21/WAF
Bax
Cyclin G1
THBS 1
MDM2


Influence of Mts1 on transactivation of luciferase reporters
P21/WAF
Bax
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
160
140
120
100
80
60
40
20
0
1
2
3
4

Tumor suppressor p53 protein is a new target for the metastasis-associated Mts1/S100A4 protein: functional consequences of their interaction

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