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Repression of *in vivo* growth of Myc/Ras transformed tumor cells by Mad1

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The Myc/Max/Mad network of transcriptional regulatory proteins plays an essential role in cell proliferation, growth, apoptosis, and differentiation. Whereas Myc proteins affect cell cycle progression positively, Mad proteins are negative regulators of cell proliferation. It has been shown in several *in vitro* systems that Mad proteins antagonize c-Myc functions. In this report we describe the inhibition of tumor cell outgrowth *in vivo* by Mad1 expression. Transformed cell lines were generated by co-transfection of c-*myc*, c-H-*ras*, and a chimeric *mad1ER* construct into primary rat embryo cells (MRMad1ER cells). Activation of Mad1 by 4-Hydroxy-Tamoxifen (OHT) resulted in abrogation of telomerase activity, reduced cloning efficiency, and decreased proportion of cells in S phase. Injection of MRMad1ER cells into syngenic rats induced aggressively growing tumors after a short latency period. This tumor growth was inhibited by OHT-treatment of animals, with the extent of inhibition correlating with the amount of OHT injected. No effect of OHT on tumor growth was observed with similarly transformed Myc/Ras cell lines which did not express Mad1ER. These data demonstrate that Mad1 is able to suppress Myc/Ras-mediated transformation under *in vivo* conditions.
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Introduction

The development and homeostasis of a multicellular organism is guided by four cellular processes: proliferation, growth (i.e. increase in cell mass), differentiation, and apoptosis. Compelling evidence indicates that the Myc/Max/Mad network plays a key role in the regulation of each of these (for review see Henriksson and Lüscher, 1996; Facchini and Penn, 1998; Johnson *et al*., 1999; Eilers, 1999; Grandori *et al*., 2000; Lüscher, 2001). The proteins of this network belong to the basic/helix–loop–helix/leucine-zipper (bHLHZip) class of transcription factors. Max plays a central role for network function because of its abundance and its versatility to form homodimers and to associate with Myc (c-, N-, and L-Myc), Mad (Mad 1, Mxil, Mad 3 and Mad 4), Mnt/Rox, and Mga (Blackwood and Eisenman, 1991; Ayer *et al*., 1993; Zervos *et al*., 1993; Hurlin *et al*., 1995, 1997, 1999; Meroni *et al*., 1997). The different dimers bind to E-box sequences (5′-CACGTG-3′) in promoters and regulate transcription (for review see Lüscher and Larsson, 1999; Grandori *et al*., 2000; Lüscher, 2001). Binding of Myc/Max contributes to gene activation through the N-terminal transactivation domain (TAD) of Myc whereas Mad/Max and Mnt/Max dimers repress transcription. Myc/Max heterodimers activate a number of genes that are directly or indirectly involved in cell cycle progression (for review see Cole and McMahon, 1999; Grandori *et al*., 2000; Amati *et al*., 2001; Lüscher, 2001). Among the increasing number of Myc-regulated genes, the recently discovered E-box sequences in the promoter of the gene encoding the catalytic component of human telomerase, hTERT (Wu *et al*., 1999; Greenberg *et al*., 1999), are of special interest since re-activation of telomerase is an obligatory finding in many human malignancies (for review see Shay and Bacchetti, 1997). It is an attractive hypothesis that overexpressed Myc promotes cellular immortalization at least in part through reactivation of hTERT (for review see Cerni, 2000). Indeed, *myc* genes are deregulated in a large number of human tumors and affect both the development and progression of hyperproliferations (see DePinho *et al*., 1991).

Myc stimulates the G1-S phase transition of the cell cycle by positively modulating cyclin D/CDK (cyclin dependent kinase) and cyclin E/CDK2 complexes and by negatively regulating the CDK inhibitors p21 and p27 (for review see Amati *et al*., 2001; Lüscher, 2001). Conversely, cell cycle exit and cellular differentiation are usually associated with downregulation of *myc*. While c-Myc normally is expressed in proliferating cells, Mad proteins are expressed in differentiating and resting cells, and terminal differentiation is closely linked to upregulation of *mad* genes (Ayer and

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Eisenman, 1993; Larsson *et al*., 1994, Hurlin *et al*., 1995; Västrik *et al*., 1995; Cultraro *et al*., 1997; Quéva *et al*., 1998; Pulverer *et al*., 2000). Mad proteins are negative regulators of cell growth and inhibit the proliferation of a variety of human and rodent cell lines and the transformation of rat fibroblasts by c-Myc/c-H-Ras and other combinations of oncoproteins (Cerni *et al*., 1995; Chen *et al*., 1995; Hurlin *et al*., 1995; Koskinen *et al*., 1995; Lahoz *et al*., 1994; Roussel *et al*., 1996; Roy and Reisman, 1995; Västrik *et al*., 1995; Quéva *et al*., 1999; Bejarano *et al*., 2000; Gehring *et al*., 2000). It has also been shown that Mad1 expressed from an adenoviral vector is able to inhibit the growth of a human tumor cell line transplanted into immunocompromised mice (Chen *et al*., 1995). In addition, Mad1 inhibits apoptosis induced in different cell systems and under different experimental conditions (Bejarano *et al*., 2000; Gehring *et al*., 2000). Furthermore, granulocytic progenitor cells of mice with a homozygous deletion of *mad1* display extra rounds of mitotic divisions prior to terminal differentiation. However the number of mature granulocytes is normal in these animals most likely due to enhanced apoptosis (Foley *et al*., 1998).

The repressive effect of Mad and Mnt is mediated by their N-terminal mSin3 interaction domains (SID). The mSin3 proteins in turn recruit corepressor molecules and histone deacetylases, and Mad and Mnt are therefore thought to exert their repressing activity at least in part by modification of chromatin structure (Alland *et al*., 1997; Hassig *et al*., 1997; Heinzel *et al*., 1997; Laherty *et al*., 1997; Sommer *et al*., 1997). The function of Myc has also been coupled to modulation of chromatin structure through its binding to the TRRAP protein which is part of a SAGA-like complex containing the histone acetyltransferase hGCN5 (McMahon *et al*., 1998, 2000; Bouchard *et al*., 2001). Since the Myc and Mad proteins are expressed during different growth conditions and have opposite effects on cell proliferation, it has been suggested that they constitute a molecular switch for proliferation versus growth arrest and/or differentiation (Ayer and Eisenman, 1993). According to this hypothesis, the abundance of Myc- versus Mad-containing heterodimers determines at least in part whether cells enter a differentiation pathway or remain in a proliferative state.

To investigate the effect of Mad1 on growth of tumorigenic cells we generated c-Myc/c-H-Ras transformed rat cell lines that express an inducible Mad1 protein. These cells were used to study the effect of Mad1 activation on proliferation of the tumorigenic cells *in vitro* and *in vivo*.

Results

Generation and characterization of Mad1ER cell lines

We transfected a construct encoding a chimeric Mad1-estrogen receptor ligand binding domain (Mad1ER) into rat embryo cells (REC) together with vectors encoding c-Myc (M) and c-H-Ras (R). The generation of transformed REC is strictly dependent on the coexpression of both c-Myc and an activated Ras (Land *et al*., 1983; Cerni *et al*., 1995). After G418 selection 50 colonies were picked and screened for Mad1 expression by immunofluorescence. Only two clones, MRMad1ER-5 and MRMad1ER-6, showed Mad1 fluorescence while the remaining 48 clones were negative (Figure 1a, lower panel and data not shown). In contrast, transformed control c-*myc*/c-H-*ras* cell lines (MR lines), that were not transfected with the *mad1ER* plasmid, did not stain with the Mad1-specific antibody (Figure 1a, upper panel and data not shown).

Northern blot analysis revealed that both MRMad1-ER clones expressed the chimeric *mad1ER* mRNA to a similar extent (Figure 1b). For comparison four transformed control cell lines, MR-1, -2, -3, and -6 were concomitantly analysed. In all four cell lines the expression levels of exogenous c-*myc* were similar while the levels of *ras* were significantly lower compared to the two Mad1ER lines (Figure 1b). Similarly, lower levels of *ras* were observed in 15 additional MR lines (data not shown), suggesting that the enhanced *ras* expression was specific for the MRMad1ER lines. As in MRMad1ER cells, addition of OHT to the transformed control cell lines altered neither *myc* nor *ras* expression (Figure 1b, and data not shown). Western blot analysis demonstrated that the size of the Mad1ER fusion protein, expressed in MRMad1ER cells, was identical to that expressed in transiently transfected COS-7 cells (Figure 1c).

Activation of Mad1ER results in repression of TERT activity

In order to examine the functionality of the activated Mad1ER fusion protein we analysed whether it would repress a known Myc target gene. It was recently found that the 5′ region of the human gene encoding the catalytic domain of telomerase, *hTERT*, contains an array of E-box sequences and that some of these are targeted by Myc/Max and Mad1/Max complexes (Greenberg *et al*., 1999; Wu *et al*., 1999; Xu *et al*., 2001). The TERT protein is the rate limiting factor for telomerase activity (Avilion *et al*., 1996; Ulaner *et al*., 1998), and Myc expression has been shown to induce hTERT expression and activity (Wang *et al*., 1998; Falcetti *et al*., 1999). Conversely, overexpressed Mad1 represses enzyme activity (Oh *et al*., 2000). We therefore analysed the activity of the rat *TERT* gene in the MRMad1ER-5 cells before and after OHT induction. Although the locus of the rat *TERT* is not characterized at present, conserved regulation of *TERT* genes by c-Myc can be deduced from the identical localization of relevant E-box sequences in the murine and human *TERT* promoters (Greenberg *et al*., 1999; Wu *et al*., 1999). Indeed, we have found that transfection of c-*myc*/c-H-*ras* into primary REC can induce high levels of telomerase activity (unpublished observation). The telomerase activity in MRMad1ER-5

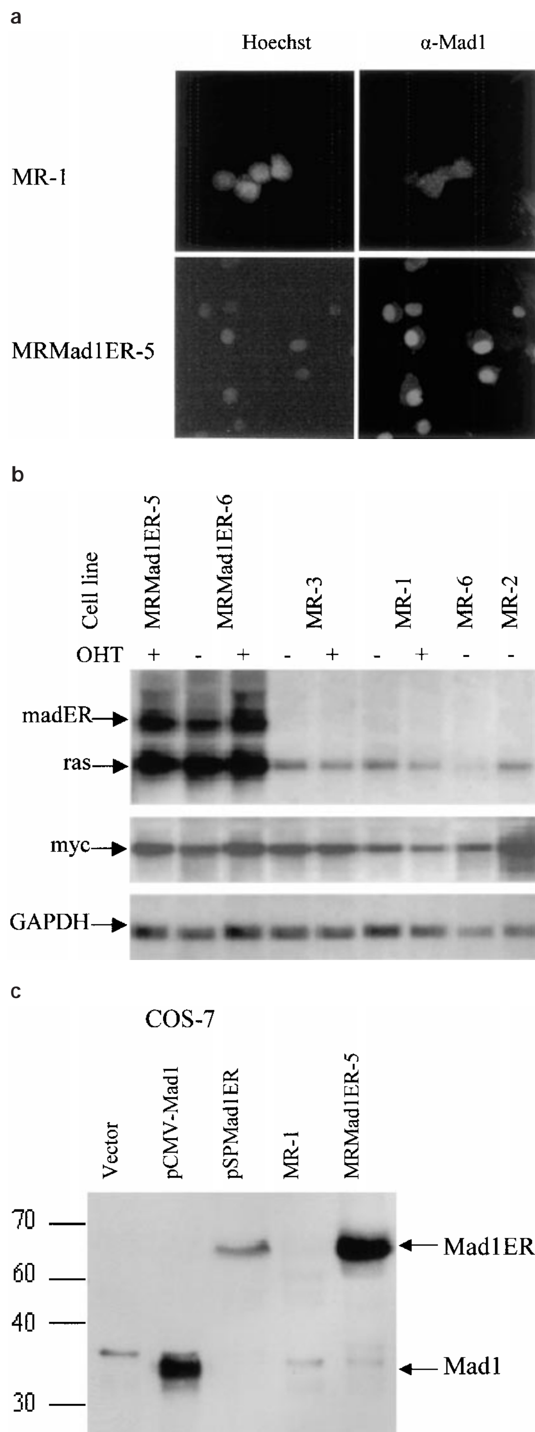

Figure 1 Expression of Mad1ER and the transgenes in Myc/Ras transformed cells. REC were transfected with plasmids encoding c-Myc, c-H-Ras, and Mad1ER together with a plasmid conferring neomycin resistance. (**a**) After G418 selection individual clones were screened for Mad1 expression by immunofluorescence in the presence of OHT. Nuclei were stained with Hoechst. A Myc/Ras transformed control cell line (MR-1) was negative for Mad1 expression (upper panel) whereas MRMad1ER-5 cells stained positive (lower panel). (**b**) MRMad1ER-5 and -6 cells and four MR cell lines were analysed by Northern blotting using probes for *mad1* and c-H-*ras* (upper panel), c-*myc*, and *GAPDH* (middle and lower panel). Where indicated cells were grown in the absence or presence of OHT for 4 days. (**c**) MR-1 and MRMad1ER-5 cells were grown in absence of OHT and analysed by Western blotting using a polyclonal antibody against Mad1. Extracts from COS-7 cells transfected with CMV vector, CMVmad1, or pSPmad1ER constructs were used as controls. Mad1ER and Mad1 are indicated

cells was approximately 20% of that observed in HeLa cells (Figure 2a,c). Similar results were obtained with cells that were kept for several days in ethanol, the solvent for OHT (Figure 2a,c, lanes 12–14). In contrast, OHT-treatment of the MRMad1ER-5 cells almost completely abolished telomerase activity (Figure 2a,c, lanes 2–6). Since MR-1 cells, used throughout this study as preferential control, had *a priori* only low levels of endogenous telomerase activity, we instead used MR-2 cells as control. As expected, presence of OHT had no effect on telomerase activity in MR-2 cells (Figure 2b,c). This finding corroborated our data that the OHT-induced Mad1ER inhibited TERT activity in the MRMad1ER-5 cells. Taken together, our results demonstrate that the transcriptional repressor function of Mad1ER is activated by OHT.

Inhibition of proliferation by Mad1ER in vitro

To examine the effect of Mad1 activation on cell proliferation, MRMad1ER cells were seeded at low cell numbers and incubated in the presence or absence of OHT for 10 days prior to staining with Giemsa. OHT-induction of Mad1 resulted in a significant reduction in both the number and the size of the colonies (Figure 3, lower panel). No influence of OHT on colony formation was observed with MR-1 cells (Figure 3, upper panel). The effect of Mad1 on cell growth was density-dependent since Mad1 did not significantly inhibit colony growth when cells were seeded at high densities (data not shown).

We then performed FACS analysis in order to analyse the effect of Mad1ER on cell cycle distribution. MRMad1ER-5 cells have a diploid karyotype despite their highly transformed morphology (Figure 4a), while MRMad1ER-6 cells are mainly tetraploid (Figure 4c). In such heteroploid cells, the overlap of the G2/M fraction of diploid cells with the G1 fraction of tetraploid cells restricts the cell cycle analysis to cells in S phase. The fraction of cells in S phase decreased from 51 to 25% and from 58 to 43% in clone MRMad1ER-5 and -6 cells, respectively, upon activation of Mad1ER (Figure 4a–d). A significant increase in the number of G0/G1 cells and a concomitant decrease in G2/M cells were seen in diploid MRMad1-ER-5 cells (Figure 4a,b). No effect of OHT was observed in MR-1 cells or in normal diploid REC (Figure 4e–h). Thus activation of Mad1ER resulted in a reduced fraction of cells in S phase in both cell lines but the effect was more pronounced in MRMad1ER-5 cells which also accumulated in G1 (Figure 4). The differences in S phase reduction in presence of OHT was also reflected in growth curves performed during 5 days of exponential growth, with MRMad1ER-5 cells

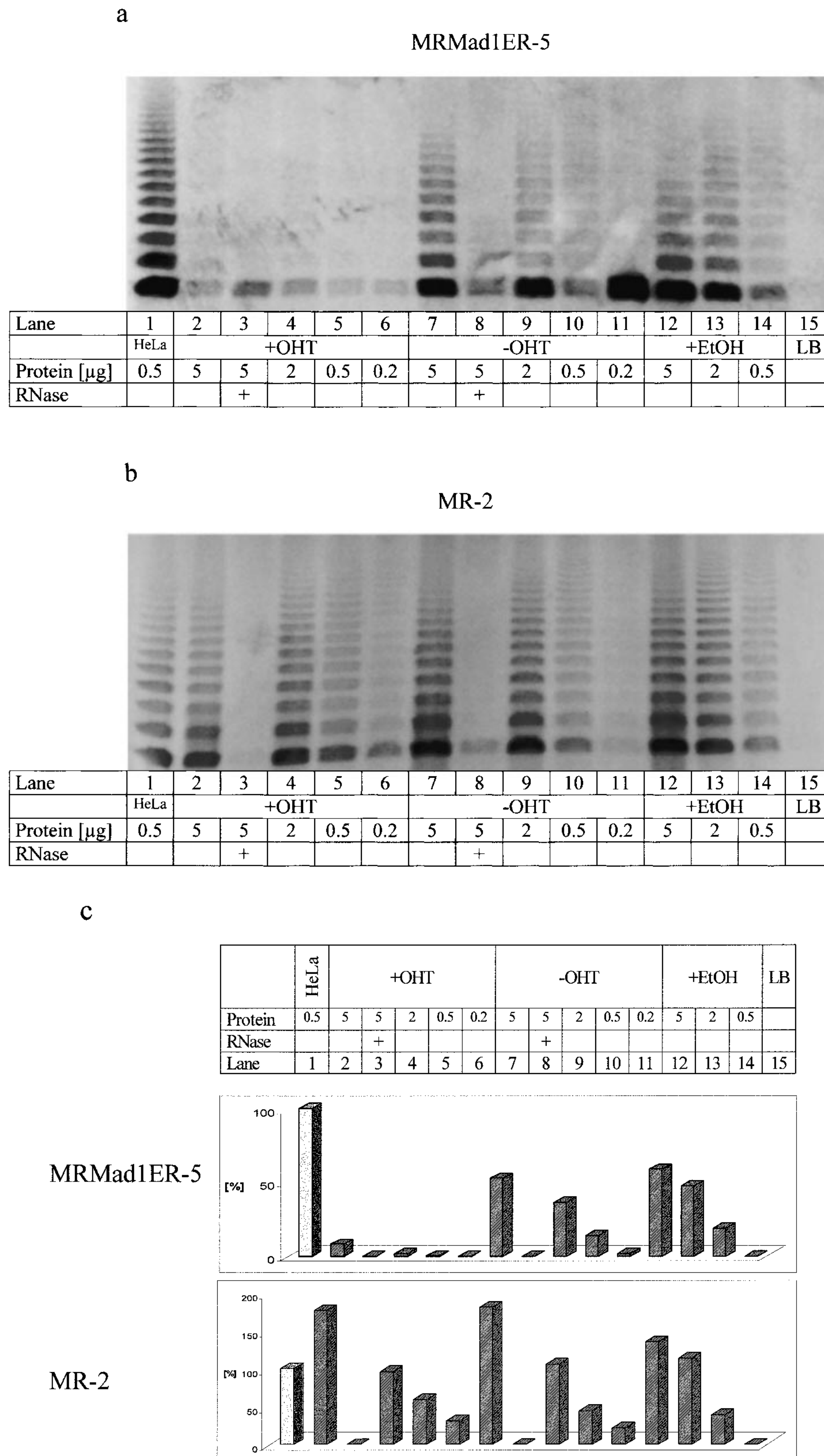

Figure 2 Activation of Mad1ER represses telomerase activity in Mad1ER-5 cells. Telomerase activity was determined in Mad1ER-5 (**a**) and MR-2 cells (**b**) and was semiquantitatively evaluated by ImageQuant 5.0 (**c**). Cells were cultured for 7 days in 2% FCS either in the absence or presence of OHT or supplemented with the appropriate concentration of ethanol (EtOH), the solvent for OHT. Various amounts of cell lysates were subjected to PCR-based TRAP assay. The amplification products were separated on a 10% nondenaturing polyacrylamide gel and stained with Vistra Green. 0.5 μg of HeLa cell extract was used for comparison. Lysis buffer (LB) served as negative control. One out of three independent experiments is shown

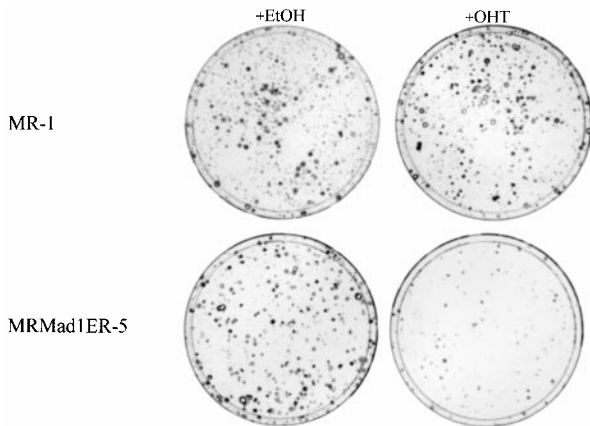

Figure 3 Inhibition of clonal growth by Mad1ER activation. MR-1 (upper panel) and MRMad1ER-5 cells (lower panel) were seeded in petri dishes and cultured in medium containing 10% FCS in the absence (left panel) or presence of OHT (right panel). After 10 days cell colonies were stained by Giemsa. One representative experiment out of six is shown

being more affected by OHT than MRMad1ER-6 or control cells (data not shown).

Activation of Mad1ER results in inhibition of Myc/Ras-dependent tumor growth

The Myc-antagonizing effects of Mad1 in the MRMad1-ER cells *in vitro* encouraged us to investigate whether activation of Mad1 could inhibit the *in vivo* growth of the cells. A small number (5×10^4) of MRMad1ER-5 cells was injected into syngenic 7 day old male Fisher rats. Concomitantly animals received intraperitoneally (i.p.) OHT (0.3, 1.2, and 2.4 mg/kg) or PBS injections that were repeated every other day throughout the experiment.

On day 5 after tumor cell injection the first small nodules were detected in the control, group A, that received PBS and group B that received 0.3 mg/kg OHT (Figure 5a,b). By day 12 all animals of these two groups had palpable tumors. The tumors grew rapidly reaching diameters of 20 mm and more after a few days. Of the seven animals in group C, which were treated with 1.2 mg/kg OHT, four rats developed tumors with a similar latency period as observed in the control and group B (Figure 5c). In three rats the first palpable tumors arose after a longer latency period. However, the growth kinetic of all tumors in group C was rapid and similar to those of the tumors in groups A and B. The doubling time of tumor size in groups A, B, and C was 2 to 3 days. In the animals of group D, that were treated with 2.4 mg/kg OHT, the first two tumors become detectable on day 12 after injection and only by day 19 small tumor nodules were palpable in the remainder (Figure 5d). Thus, the average latency period of tumor appearance in this group was doubled. The initially retarded outgrowth of injected MRMad1ER cells was followed by slow tumor growth. In all but one animal the doubling time of tumor size increased significantly to up to 2 weeks. Comparison of tumor growth in the four groups showed a clear dose dependent effect of OHT on the proliferation rate of MRMad1ER cells *in vivo*. Three slowly growing tumors of group D (animal No. 2, 4 and 6, respectively) started to grow fast after approximately 40 days which might indicate the outgrowth of a subpopulation of more aggressive tumor cells. Figure 5e summarizes the successive increase of mean tumor diameters in the various treatment groups. While low concentrations of OHT appeared rather to stimulate tumor growth, the highest OHT-concentration significantly inhibited the proliferation of MRMad1ER cells *in vivo*. The mean values of tumor diameters between groups A and D differed significantly ($P < 0.05$) on day 19 after tumor cell injection. The fact that the body mass of OHT-treated rats increased in line with solvent-treated animals (Figure 5f) and untreated rats (data not shown) argues against biasing side-effects of repeated i.p. injections or OHT-applications. When MRMad1ER-6 cells were injected into animals in an analogous experiment using 2.4 mg/kg OHT, an increase in latency period and a reduction of tumor growth were observed, albeit less dramatic than with the MRMad1ER-5 tumors (data not shown). This is consistent with the observed differences in reduction of S phase cells as determined by FACS analysis (Figure 4a–d).

It is known that tamoxifen, a clinically applied anti-estrogenic drug that is metabolized to OHT, can inhibit tumor cell growth both *in vivo* and *in vitro* in an unspecific manner, i.e. by mechanisms that are independent of the presence of estrogen receptors (Gundimeda *et al*., 1996; Treon *et al*., 1998; Lee *et al*., 2000). Although in previous *in vitro* experiments, a series of transformed c-*myc*/c-H-*ras* cell lines were found unresponsive to OHT application in culture as determined by growth curves and FACS analysis (Figure 4e,f and data not shown), we investigated the effect of the highest OHT-concentration on the *in vivo* growth of MR-1 cells (Figure 6). For comparison with the previous animal experiments, the number of injected cells was kept constant at 5×10^4 cells per rat although the low *ras* expression of MR-1 cells (Figure 1b) was suggestive of a probably reduced tumorigenicity. Indeed, one animal in each group, i.e. No. 5 in group E and No. 2 in group F, respectively, remained tumor-free. However, among the arising tumors, there was no difference between solvent- and OHT-treated rats neither with regard to tumor onset nor growth kinetics (Figure 6). Note that the *in vivo* proliferation of untreated MR-1 and MRMad1ER-5 cells was similar despite their different *ras* mRNA expression (Figure 1b).

Analysis of tumor-derived cell lines

We then examined whether the *in vivo* passage had altered the geno- and/or phenotype of MRMad1ER cells. Of nine isolated tumors from the different treatment groups, seven were successfully re-established

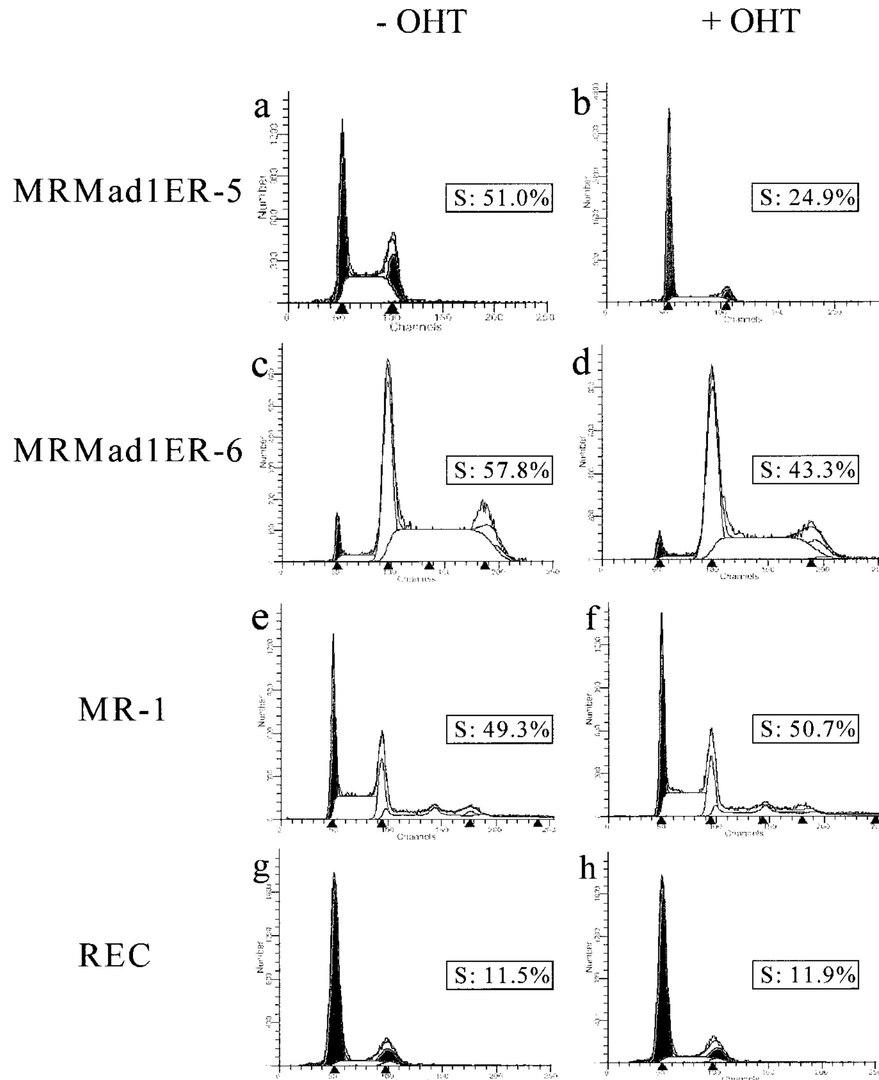

Figure 4 Cell cycle analysis of Mad1ER cells. MRMad1ER-5, MRMad1ER-6, MR-1, and REC were grown for 48 h in 10% FCS in the absence (**a,c,e,g**) or presence (**b,d,f,h**) of OHT. Cells were harvested, fixed, stained with propidium iodide and the DNA content was analysed by FACS. The percentage of cells in S phase is indicated. One experiment out of two is shown

in culture. All of them resembled the parental cells with regard to morphology (data not shown). Western blot analysis indicated that the tumor-derived (TD) lines still expressed the transfected c-*myc* and c-H-*ras* oncogenes and the chimeric *mad1ER* construct to a similar extent as the parental cells (Figure 7a,b). We observed that OHT reduced the expression of the Mad1ER protein in some of the cell clones. The reason for this is unclear at present. Since this was also found in the parental MRMad1ER-6 cells one may speculate that this could be part of a compensatory autoregulatory mechanism to limit Mad1 activity. We also performed FACS analysis and Table 1 shows the percentage of cells in S phase in the different TD lines. With exception of TD-A5, the overall percentage of cells in S phase was lower than in the parental cells. Three TD lines (TD-A5, TD-B2, and TD-C6) still responded to OHT as determined by the reduction in the number of S phase cells while the other three did not. We analysed the expression of some cell cycle-relevant proteins that could be responsible for the reduction of S phase cells in presence of OHT, with special interest on the still inducible TD-cell lines. However, Mad1 did not consistently affect the expression of p27, cyclin D1, or p21 (Figure 7b and data not shown). One cell line, TD-D2, deviated from the overall picture. These cells were derived from the tumor of animal No. 2 in group D, in which tumor growth was clearly accelerated after 37 days, indicating that some prominent genetic alterations had occurred. Indeed, addition of OHT resulted in a reduction of Mad1 expression (Figure 7a), induction of cyclin D1 (Figure 7b), and a concomitant increase of cells in S phase (Table 1). These data indicate that selective pressure against Mad1-mediated growth inhibition was exerted *in vivo*, with TD-D2 as a clear example.

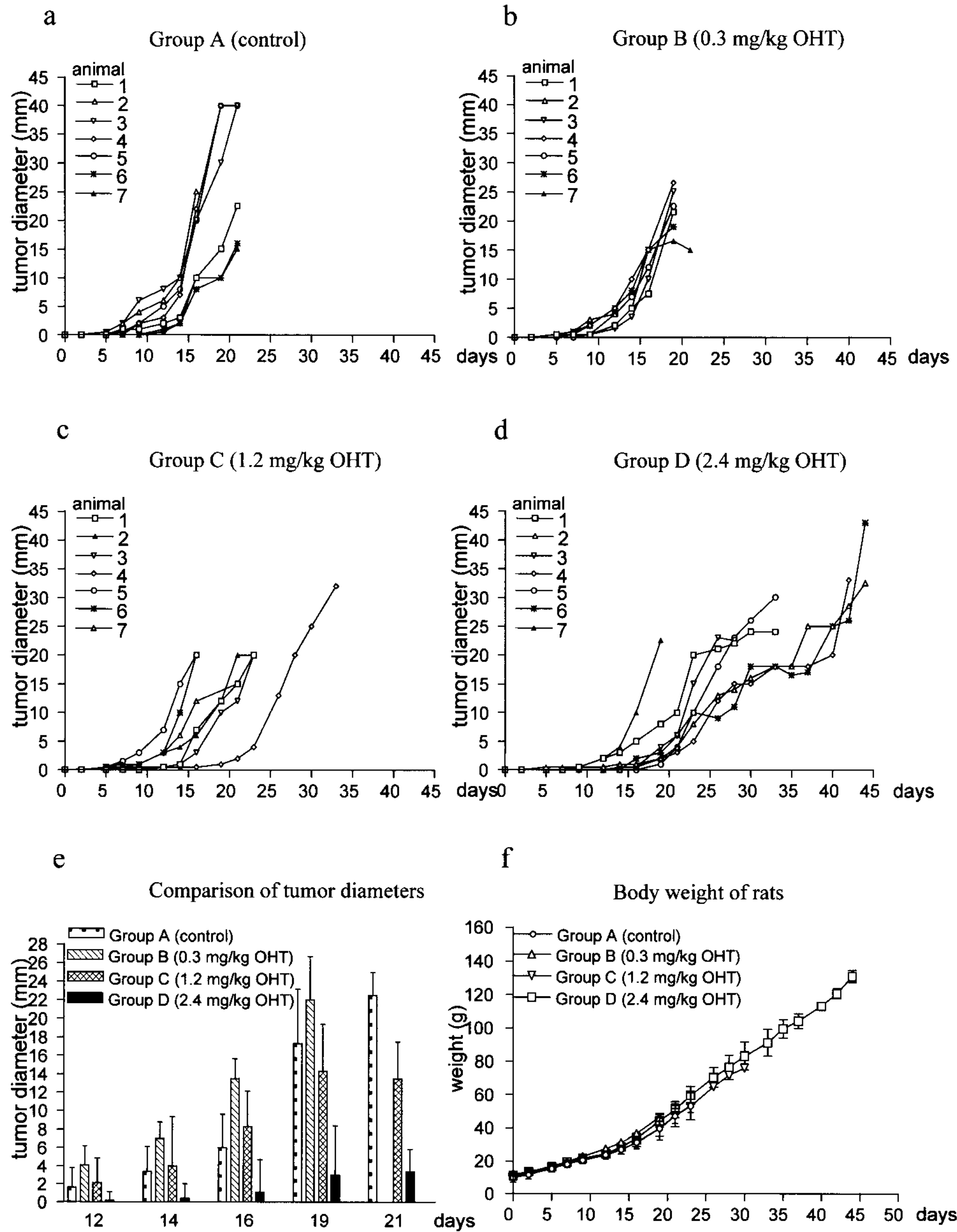

Figure 5 Inhibition of tumor growth of MRMad1ER-5 cells upon induction of Mad1ER. Seven days old, syngenic male rats were injected subcutaneously with 5×10^4 MRMad1ER-5 cells. Control animals received PBS with 4% ethanol intraperitoneally every other day (**a**). Group B received 0.3 mg/kg OHT (**b**), group C 1.2 mg/kg OHT (**c**) and group D 2.4 mg/kg OHT (**d**). Tumor appearance, tumor growth and animal weight was recorded regularly. Comparison of the average tumor diameters in the four treatment groups (**e**). There was no difference among the groups with regard to animal weight (**f**)

Discussion

We have generated transformed cell lines derived from primary rat embryo cells (REC) that express the human c-*myc* and c-H-*ras* genes together with a *mad1-estrogen receptor* fusion construct, *mad1ER*. Using these cells we have analysed the effect of activated Mad1ER on cell proliferation *in vitro* and *in vivo*. Our findings demonstrate that functional Mad1 can be expressed in REC concurrently with high-level expression of c-Myc and c-H-Ras and that activated Mad1 inhibited telomerase activity and proliferation of the transformed cells *in vitro*. Injection of the cells into syngenic rats induced aggressively growing tumors after a short latency period. In contrast, continuous activation of Mad1 prolonged the latency period of tumor appearance and reduced the growth rate of the tumors in an OHT-dose dependent manner. These effects were specific for Mad1 since no effect of OHT was observed with transformed MR-1 control cells. Taken together, our results provide further evidence

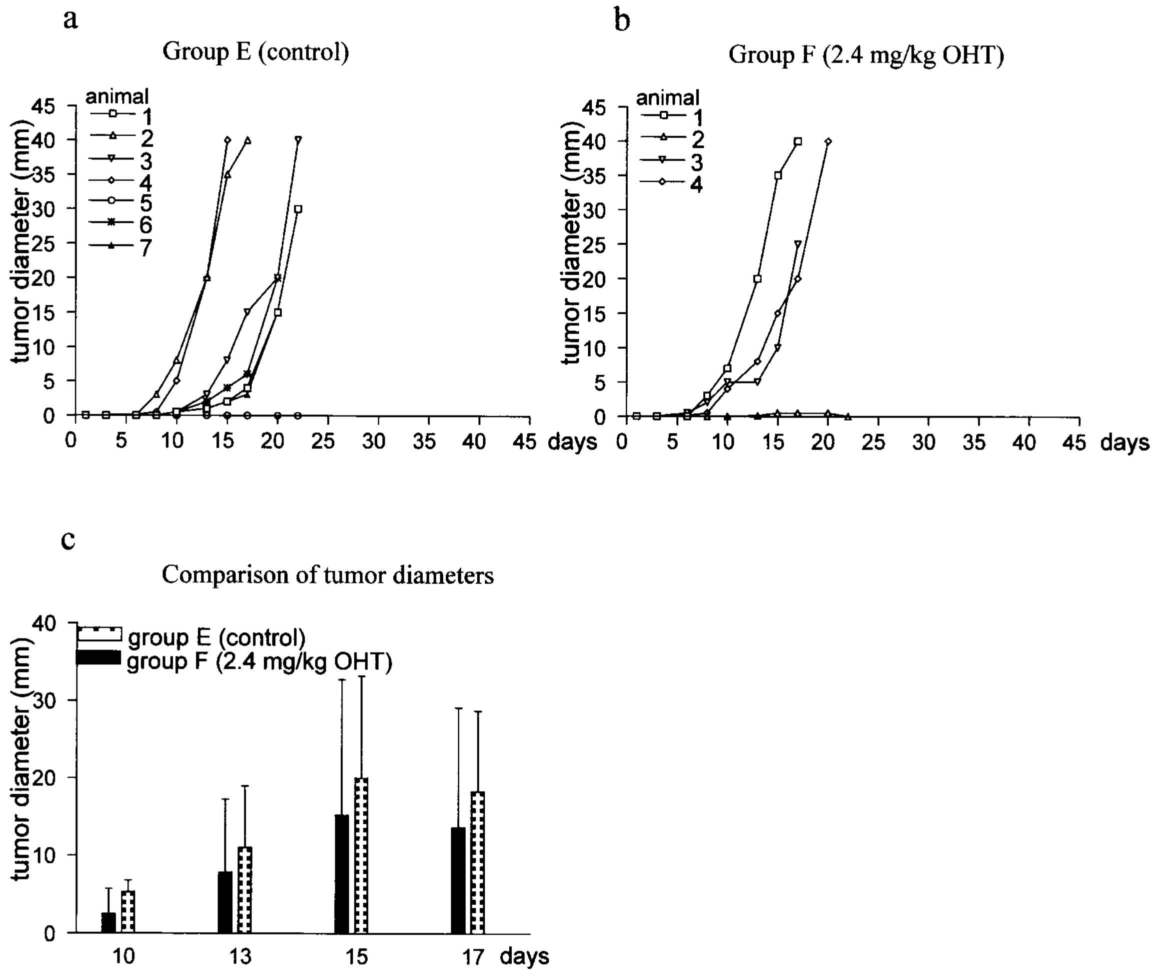

Figure 6 Treatment of syngenic animals with OHT does not inhibit *in vivo* growth of MR-1 cells. Transformed MR-1 cells were injected under the same conditions as MRMad1ER-5 cells. Animals received PBS with 4% ethanol (**a**) or 2.4 mg/kg OHT (**b**). There was neither an inhibitory effect of OHT administrations on tumor growth (**c**) nor on animal weight (data not shown)

for a growth inhibitory role of Mad1 and extend its function to the inhibition of *myc*-expressing tumors.

In our attempts to generate c-*myc*/c-H-*ras* transformed REC cell lines that express the chimeric *mad1ER* transgene, only two out of 50 clones analysed clearly stained positive for the Mad1ER protein. One possibility for the low yield is that the primary REC used as target cells represent a mixture of different cell types and it is feasible that the proliferation of some (or most) might be sensitive to even low amounts of Mad1. Although the transfected REC were kept in phenol red-free medium supplemented with low-estrogen or charcoal-absorbed serum, remaining traces of estrogen in the sera might have been sufficient to activate the Mad1ER fusion protein and consequently inhibit the outgrowth of potential founder cells. We have previously observed such inherent sensitivity toward low Mad1 levels in established cell lines (Gehring *et al*., 2000). Another possibility is that basal Mad1 levels can be tolerated only in the presence of high c-Myc levels. Abundant Myc/Max complexes might efficiently antagonize Mad/Max dimers, thereby allowing cell proliferation. In support of this hypothesis, Cultraro *et al*. (1997) reported that expression of Mad1 could only be obtained in the presence of c-Myc in mouse erythroleukemia cells. However, this does not seem to be the explanation in our cells since we found that the amount of c-*myc* mRNA in the two established MRMad1ER clones was similar to that expressed in four MR cell lines. In contrast, the former cells expressed unusually high levels of *ras* mRNA and protein. In this context, a recent study on the cooperation of oncogenic *ras* with endogenous c-*myc* is of interest. Bazarov *et al*. (2001) reported that high levels of a c-H-Ras mutant (G12V) could compensate for low c-Myc levels in anchorage-independent growth of Rat1a cells as evidenced by efficient cell growth in semi-solid medium. Thus, if low Mad1 levels in our cells compromised some of the c-Myc functions relevant for the transformed phenotype (which was the criterion for clonal isolation), then high c-H-Ras levels might have been the necessary prerequisite for clonal outgrowth. This assumption is not only supported by the high level of *ras* mRNA and protein

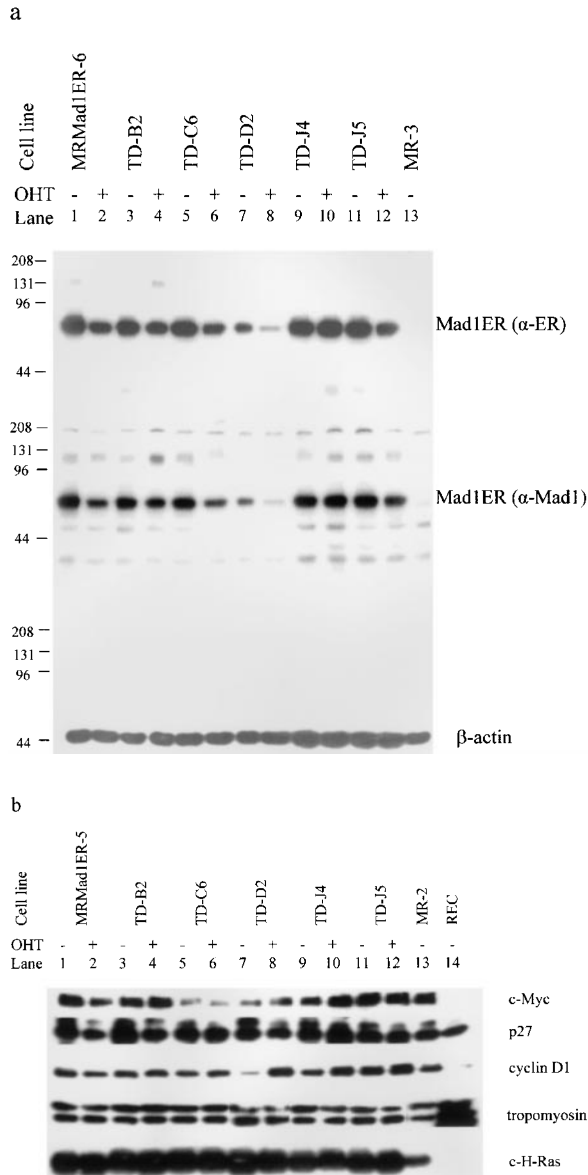

Figure 7 Expression of Mad1ER and cell cycle regulatory proteins in tumor-derived cell lines. Tumors from injected MRMad1ER-5 and -6 cells were excised and replated in culture in the absence or presence of OHT. Cell lysates were prepared and analysed for protein expression. (**a**) Two identical blots were incubated in parallel with an ER antibody (upper panel) and a Mad1 antibody (lower panel). (**b**) Cell lysates were analysed for expression c-Myc, p27, cyclin D1, tropomyosin, and c-H-Ras. TD-J4 and TD-J5 cells were re-established from MRMad1ER-6 tumors of rats treated with 2.4 mg/kg OHT

(Figures 1 and 7), but also by the stable morphology of the MRMad1ER cells and their tumor-derived cell lines, irrespective of Mad1 activation (data not shown). Furthermore, tropomyosin levels, which are high in normal, flat REC (Figure 7b, lane 14) and generally low in their transformed derivatives remained stable in the MRMad1ER cells and their TD-lines upon OHT treatment (Figure 7b and unpublished observation). The expression of tropomyosin has also been reported previously to decrease upon transformation (Prasad *et al*., 1999). Taken together, our data indicate that Mad1 does not alter the transformed morphology of our cell lines which might be due to the high level of Ras expression.

Analysis of cell cycle distribution of the MRMad1-ER cells revealed a reduction in the cell fraction in S phase upon Mad1 activation. The cells were however not arrested since they continued to cycle, albeit at a slower rate. A decrease in the number of S phase cells was also observed in response to Mad1 expression in astrocytoma and NIH3T3 cells (Chen *et al*., 1995; Roussel *et al*., 1996). In contrast to the modest effects during normal growth conditions, we found that Mad1 inhibited cell proliferation very efficiently when the MRMad1ER cells were plated at low cell density. When the number of plated cells was increased, this effect was no longer observed. We have made similar observations demonstrating more pronounced effects of Mad1 on proliferation during restrictive growth conditions in two other model systems (Bejarano *et al*., 2000; Gehring *et al*., 2000). These data suggest that Mad1 function is most clearly manifested under restrictive growth conditions.

To further characterize the consequences of Mad1-ER activation we studied the effect on rat telomerase activity. As anticipated, the telomerase activity of MRMad1ER-5 cells was reduced to almost undetectable levels upon addition of OHT, but remained high in the transformed control MR line under the same conditions. Similarly, we and others have recently reported reductions in telomerase expression and activity in two human tumor cell lines, U937 and HL60, in the course of drug-induced differentiation concomitantly with Mad1 upregulation (Günes *et al*., 2000; Xu *et al*., 2001). In addition, *hTERT* expression in normal and malignant human cells was found to have an inverse correlation with Mad1 expression (Cong and Bacchetti, 2000; Oh *et al*., 2000; Günes *et al*., 2000). The Mad1-induced repression of hTERT transcription is mediated by the N-terminal SID of Mad1 that recruits histone deacetylases to chromatin (Cong and Bacchetti, 2000). Furthermore, there is a switch from Myc/Max to Mad1/Max binding and a decrease in histone acetylation at the hTERT promoter during HL60 differentiation (Xu *et al*., 2001). This indicates that acetylation/deacetylation of histones contributes to the regulation of *hTERT* expression.

The inhibition of telomerase activity in response to Mad1 might contribute to the reduced *in vivo* growth of MRMad1ER-5 cells. REC are equipped with up to 150 kb ultralong telomeres that further increase by transfection of c-*myc* and c-H-*ras* (unpublished observation). Nevertheless, cell proliferation could well be affected by the lack of telomerase, probably even in case of long telomeres. Besides the effect of telomerase on telomere elongation, the enzyme exerts in addition a protective capping function on the telomeric ends that is independent from synthesis of telomeric repeats (Zhu *et al*., 1999). However, oncogene-transformed murine cells derived from animals without functional telomerase are as tumorigenic as their wild type counterparts,

Table 1 FACS analysis of tumor-derived cell lines

TD cell line	*Parental cell line*	*Animal group (animal No.)*	In vivo *treatment OHT* (mg/kg)	*% of cells in S phase* −*OHT* in vitro	+*OHT* in vitro	*Ratio*
TD-A4	MRMad1ER-5	Group A (No. 4)	PBS	45.0	43.6	0.97
TD-A5	MRMad1ER-5	Group A (No. 5)	PBS	50.6	24.0	0.48
TD-B2	MRMad1ER-5	Group B (No. 2)	0.3	30.4	20.8	0.68
TD-C6	MRMad1ER-5	Group C (No. 6)	1.2	23.4	16.0	0.69
TD-D2	MRMad1ER-5	Group D (No. 2)	2.4	32.0	38.4	1.20
TD-J5	MRMad1ER-6	Group J (No. 5)	2.4	21.8	21.4	0.98

Tumors from the various treatments were excised, minced and re-plated in culture in DMEM with 10% FCS. Tumor-derived (TD) cell lines were incubated *in vitro* for at least 48 h in the absence or presence of OHT before FACS analysis. The percentages of cells in S phase and their ratios are indicated

arguing against a contribution of telomerase activity for tumorigenic growth of rodent cells (Blasco *et al*., 1997). Thus at present it is unclear whether telomerase inhibition upon Mad1 induction *in vivo* contributes to the reduced tumor growth.

Considering the complexity of cellular requirements for autonomous growth *in vivo*, several possibilities are conceivable for Mad1-mediated tumor inhibition. Transformed cells will not give rise to progressively growing tumors unless their survival in the host organism is guaranteed by a sufficient supply of blood vessels. Since normal tissue generally produces more inhibitors than stimulators of neoangiogenesis, tumor cells must shift the balance in favor of angiogenic factors. Overexpression of c-Myc, a common finding in many different kinds of experimental and clinical malignant tumors, can suppress transcription of thrombospondin 1 (Tsp-1), a secreted inhibitor of angiogenesis (Good *et al*., 1990). It was recently shown that c-*myc*-transformed Rat-1a fibroblasts provoke neovascularization *in vivo* when injected into immunodeficient mice or immunprivileged sites (Ngo *et al*., 2000). It is likely that downregulation of angiogenic inhibitors such as Tsp-1 or similar factors by deregulated Myc contribute to the aggressive growth potential of *myc*-expressing tumor cells. Conversely, inhibition of c-Myc function by Mad1 could hamper the outgrowth of Myc expressing cells as a consequence of limited blood supply. Although determination of the nature and amount of angiogenic factors in the MRMad1ER cell lines is a future project, it is likely that the cells produce the potent angiogenic VEGF due to high c-H-*ras* expression (Rak *et al*., 1995; Larcher *et al*., 1996). Since neovascularization requires a coordinated array of a multitude of distinct proteins, upregulation of angiogenesis inhibitors such as for instance Tsp-1 by compromised c-Myc function could contribute to reduced tumor growth potential.

Mad1 might also oppose some c-Myc functions relevant for cell cycle progression. However, when we compared cell cycle distribution patterns of MRMad1-ER cells and protein data from the founder and the tumor-derived cell lines, no consistent picture emerged. One explanation for our results might be that the high amounts of c-H-Ras in MRMad1ER cell lines compensated some of the Mad1-compromised c-Myc functions, analogous to the reported attachment-independent growth of c-*myc* deficient, yet c-H-*ras* transformed Rat1a cells (Bazarov *et al*., 2001). Similar to the findings reported here, we could not detect any differences in expression of cell cycle regulatory molecules in Mad1-induced versus uninduced human tumor cells (Gehring *et al*., 2000). In contrast, Quéva *et al*. (1999) reported that mouse embryo fibroblasts derived from *mad1* transgenic mice displayed increased levels of p21 and p27 and reduced CDK2 and CDK4 activities. We also observed an increase in p27 expression and a reduction in CDK2 activity in Mad1-expressing NIH3T3 cells (Bejarano *et al*., 2000). These data support the notion that Mad1 exerts at least some of its functions through negative regulation of CDK activity. It remains to be determined if Ras oncoproteins are able to abolish or compensate the effects of Mad1 on the activity of CDK complexes.

Recently, a number of studies have identified Myc target genes involved in cell cycle regulation, cell death, immortality, matrix adhesion, protein synthesis, and metabolism (see Grandori *et al*., 2000; Coller *et al*., 2000; O'Hagan *et al*., 2000; Guo *et al*., 2001). The generation of Rat1-derived cell lines with homozygous deletion of c-*myc* allels revealed new insights into c-Myc functions. Comparison of gene expression between exponentially growing c-*myc*-null and wild type Rat1 cells identified 188 genes up-regulated and 95 genes down-regulated, directly or indirectly by endogenous c-Myc (Guo *et al*., 2001). However, when Rat1 cells over-expressing a c-*myc* transgene were analysed and compared to the wild type Rat1 cells, a smaller set of genes was found activated, with only partial overlap with the latter (Guo *et al*., 2001). These data therefore suggest that endogenous c-Myc might regulate a different set of genes than ectopically overexpressed c-Myc. It is possible that Mad1 interferes preferentially with the genes regulated by endogenous c-Myc rather than with those induced by overexpressed oncogenic Myc. A decrease, but not complete inhibition of cells in S phase would then be expected *in vitro* and, in turn, result in slower tumor growth *in vivo*, which is what we observed.

An inverse correlation between Myc- and Mad expression was found in a series of specimens of differently advanced human breast cancers. This correlated with the clinical stage, with high Mad1 in

benign or early diseases and high Myc levels in more advanced cases (Han *et al*., 2000). In invasive ductal carcinoma, Myc expression was prominent in cancerous tissue and proliferating cells, while Mad expression was sporadic and restricted to non-proliferating cells. In the more differentiated tumor cells, Mad expression was high and found in more cells, while it was reduced in less differentiated tumor cells. It was concluded that loss of Mad1 expression might contribute to malignant transformation of human mammary epithelial cells. These data, taken together with our results showing that activation of Mad1 in c-*myc*/c-H-*ras* transformed cells significantly retarded tumor appearance and reduced tumor growth, indicates that Mad1 might play a role in preventing tumorigenesis.

Materials and methods

Plasmids and antibodies

CMVmad1, pSPmyc and pSPmax have been described previously (Cerni *et al*., 1995). pSPmyc and pVZ1mad1 were obtained from L Kretzner and D Ayer, respectively. The mad1 insert of pVZ1mad1 was cloned into ERBS6 (a kind gift from J Lüscher-Firzlaff) generating mad1ER. The mad1ER insert was sequenced and cloned into pSP generating pSPmad1ER. From the plasmid pVEJB which expresses an activated c-H-*ras* oncogene and a *neo* resistance gene (Cerni *et al*., 1990), the *Bam*HI inserted *neo* gene was removed and the plasmid religated. pRSVneo was used as the neomycin resistance encoding plasmid (Gorman *et al*., 1983).

Polyclonal antibodies recognizing Mad1 (C-19), c-Myc (N-262), p27 (F-8), ER (F-10) were from Santa Cruz and the mouse monoclonal antibodies against Myc (Ab-2) and cyclin D1 (Ab-1) were obtained from Neomarkers. The 5C9 monoclonal antibody recognizing Mad1 has been described previously (Sommer *et al*., 1997). Anti-tropomyosin (TM311) was from Sigma and anti-human $p21^{ras}$ was obtained from Dako. DTAF- and Cy^3-labeled secondary antibodies were from Amersham Pharmacia Biotech, and anti-mouse and anti-rabbit IgG coupled to horseradish peroxidase were obtained from Calbiochem and Amersham Pharmacia Biotech, respectively.

Cell culture and transfection

Primary rat embryo cells (REC) were obtained from 15.5 gestation day old Fischer rat embryos by fractionated trypsinization as described (Cerni *et al*., 1990). REC were grown in phenol-red-free DMEM (Gibco) in 10% low-estrogen FCS (Bioconcept) and transfected with 2.5 μg pVEJB, 2.5 μg of pSPmyc, 5 μg of pSPmad1ER and 1 μg of pRSVneo per 60 mm petri dish using the calcium phosphate technique. In parallel REC were transfected with 2.5 μg pVEJB, 2.5 μg of pSPmyc and 1 μg of pRSVneo per 60 mm petri dish. Twenty hours after transfection the cells were trypsinized and replated at 3×10^5 per 60 mm dish. Two days later 200 μg/ml G418 (Geneticin, Gibco) was added and the cells were fed with fresh medium containing G418 every 2 to 3 days. After 14 days colonies were picked and checked for Mad1 expression using immunofluorescence. Two positive clones MRMad1ER-5 and -6 were identified, expanded and further characterized. Several Myc/Ras transformed clones (MR-1 *etc*) were also picked and expanded. The REC clones were maintained in phenol-red-free DMEM medium (Gibco) supplemented with 10% low estrogen FCS (Bioconcept) or charcoal-treated FCS (PAA, Austria) and penicillin/streptomycin (10 U/ml). 4-Hydroxy-Tamoxifen (OHT; Calbiochem) was added at a concentration of 1 μg/ml medium. HeLa cells were grown in DMEM supplemented with 10% FCS.

Immunofluorescence

Cells were grown on coverslips in the presence of OHT for 24 to 48 h, fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde, and permeabilized in PBS with 0.2% Triton X-100. Subsequently, cells were incubated in PBS containing 20% horse serum (blocking buffer). Antibody stainings were performed in blocking buffer. The DNA was stained with Hoechst 33258 (1 μg/ml in PBS) and the coverslips mounted with Moviol (Merck) in PBS containing 2.5% N-propylgallate (Sigma).

Northern blotting

Total RNA was isolated by Trizol (Gibco) according to the manufacturer's instructions. Ten μg of total RNA was separated on a glyoxal/dimethylsulphoxide agarose gel and blotted onto GeneScreen membranes (New England Nuclear), which were hybridized to random-primed cDNA probes of *mad1*, c-*myc*, c-H-*ras* and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*. Hybridizing mRNA was visualized by autoradiography.

Western blotting

Immunoblot analysis was performed as described previously (Bejarano *et al*., 2000).

Telomeric Repeat Amplification Protocol (TRAP) assay

Cell lysates were prepared using the CHAPS detergent lysis method (Kim *et al*., 1994). The protein concentration of lysates was measured using the BioRad (Bradford) protein assay. The PCR-based TRAP assay for detection of telomerase activity was performed according to Kim *et al*. (1994) with some modifications. 0.2–5 μg of lysates were subjected to 25 PCR cycles. The amplification products were separated on a 10% nondenaturing polyacrylamide gel, stained with Vistra Green (Amersham Life Science) and visualized in a FluorImager 595 (Molecular Dynamics) using ImageQuant 5.0. Semiquantitative densitometric evaluation was performed by ImageQuant 5.0.

Colony formation assays

Cells were seeded in petri dishes at low cell numbers (100 or 500 cells per 10 cm dish) and cultured in media containing 10% FCS in the absence or presence of OHT. After 10 days cell colonies were stained by Giemsa.

FACS analysis

Cells were seeded in 60 mm dishes in DMEM with 10% FCS and cultured for at least 24 h before induction. The cells were then incubated in FCS or charcoal-depleted FCS for at least 48 h in the absence or presence of OHT. Cells were harvested and fixed overnight in 70% ice-cold ethanol, washed in PBS and resuspended in 1 ml PBS containing 5 μg/ml propidium iodide (Sigma) and 100 μg/ml RNaseA (Sigma). Samples were incubated for 30 min at 37°C and placed at 4°C before

flow cytometry analysis of the DNA profile. All samples were analysed in a Becton Dickinson FACSCalibur system using CELLQuest software.

Animal experiments

Seven-day-old male Fisher rats received into the back subcutaneous injections of 5×10^4 transformed cells in PBS. OHT was dissolved in ethanol and diluted with PBS to concentrations of 3 μg/ml, 6 μg/ml, 12 μg/ml and 24 μg/ml. Since the latter contained 4% ethanol, the ethanol concentration was adjusted to 4% in the other OHT-dilutions to avoid a probably biasing variable. On day 0, animals were injected with the tumor cells followed by i.p. applications of 0.3, 0.6, 1.2, and 2.4 mg/kg OHT or PBS with 4% ethanol. Treatment was repeated every other day with freshly prepared OHT solutions. Animal weight and tumor size were monitored regularly. Animals were killed by cervical dislocation when tumor diameters were approximately 20 mm for younger and 40 mm for older rats or before an unfavorable tumor localization would have restricted rats' comfort. Mean tumor diameters were statistically compared by the Kruskal-Wallis-test using Graph Pad Prism. The animal experiment was performed according to the animal experiment approval GZ 66.009/98-Pr/4/00 given by the Austrian Ministry of Culture and Education.

Re-establishment of tumor-derived cell lines

Several tumors were excised, minced and replated in DMEM with 10% FCS without G418. A few days after explantation cells started to grow out from the tissue fragments. When small colonies had formed, cultures were carefully rinsed to remove abundant debris and floating tumor pieces. The morphology of attached, growing cells was indistinguishable from the initially injected cell lines. Since a first set of re-cultured tumors underwent apoptosis upon addition of G418 to the culture medium, we omitted the selection drug thereafter. Although the phenotype of these tumor-derived (TD) lines was clearly transformed, they had obviously lost the *neo* resistance gene in the course of the *in vivo* passage.

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