Inactivation of p53 and life span extension of human diploid fibroblasts by mot-2

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Abstract Normal human lung fibroblasts were transfected with expression plasmids encoding mot-2, an hsp70 family member that is associated with the immortal phenotype. After the empty vector-transfected controls had become senescent and positive for senescence-associated β-galactosidase (SA-β-gal), the mot-2-expressing cells continued to proliferate for an additional 12–18 population doublings and showed a young cell morphology and much lower SA-β-gal activity. The tumor suppressor p53 was found to be transcriptionally inactivated in life span-extended cells. We have thus shown for the first time that overexpression of mot-2 in normal human cells is able to permit their temporary escape from senescence.

Key words: Normal human diploid fibroblast; Tumor suppressor p53; Senescence; mot-2; Life span extension

1. Introduction

Normal human cells undergo a finite number of cell divisions and ultimately enter a metabolically active state of permanent growth arrest called replicative senescence [1]. Cells can escape from the limits on their replicative capacity as a result of various genetic and epigenetic changes that are not completely understood. We originally cloned mortalin genes, mot-1 and mot-2, as hsp70 family members from normal and immortal murine cells, respectively [2,3]. Their proteins differ by only two amino acid residues but exhibit different subcellular localizations and have contrasting biological activity [2,3]. Normal and transformed human cells showed pancytosolic and four different kinds of non-pancytosolic distribution of the proteins [4,5]. Mortalin cDNAs isolated from two different human cell lines (HT1080 and HeLa) encode proteins with different staining patterns but are identical and, similar to mouse mot-2 cDNA, lead to malignant transformation of NIH 3T3 cells when transfected [6]. We recently demonstrated interaction between wild-type (wt) p53 and mot-2 protein in mouse and human transformed cells [7]. Transfection of COS7 cells with a mot-2 expression plasmid resulted in downregulation of p53-responsive genes, $p21^{SDI1/WAF1}$ and mdm-2. Furthermore, G1-associated nuclear translocation of p53 was abrogated by mot-2. These results demonstrated that mot-2 is able to interfere with p53 activity [7].

Loss of p53 activity has previously been shown to be an important step in the temporary escape of human cells from senescence (reviewed in [8]). This suggests that p53 is normally involved in the process of senescence. In support of this, there is evidence that the activity of p53 (both DNA binding activity and transcriptional activation) increases as cells near senescence [9,10]. The expression of the p53-induced cell cycle inhibitor $p21^{SDI1/WAF1}$ also increases at senescence [11]. Expression of a dominant negative p53 mutant in normal human diploid fibroblasts permitted them to continue dividing for a limited number of population doublings (PDs) beyond the point at which their normal counterparts became senescent [12,13]. Evidence that this effect was due to loss of wt p53 function and not due to a p53 gain-of-function mutation was provided by studies demonstrating that spontaneous loss of p53 function in fibroblasts from an individual with Li–Fraumeni syndrome resulted in a substantial, but finite, increase in proliferative capacity [14]. The great majority of these cells eventually entered a state of terminal proliferation arrest [14]. However, inactivation of the $pRb/p16^{INK4a}$ pathway in fibroblasts that have lost wt p53 function resulted in a further, but still finite, increase in proliferative potential [15,16].

There are thus at least two terminal proliferation arrest states beyond senescence that act as a barrier to unlimited proliferation of human fibroblasts (reviewed in [17]). The genes required for such proliferation barriers may be cell type-specific [18,19]. For cells to overcome the final barrier to unlimited proliferation (i.e. to become immortalized), activation of a telomere maintenance mechanism appears to be essential (reviewed in [20]). Although mechanisms of senescence [21,22] and telomere maintenance [20] have attracted a lot of attention, very little is known about the intermediate proliferation barriers including the terminal arrest that eventually occurs in cells in which p53 function has been inactivated. In addition, a substantial minority of immortalized cells and cancers do not contain any detectable abnormalities in the p53 gene. It is therefore of great interest to determine whether there are other genetic changes that have the same effect on proliferative life span as loss of wt p53 function.

Here we demonstrate for the first time that overexpression of mot-2 results in the temporary escape of normal human diploid fibroblasts from senescence. In addition to their increased proliferative capacity, the mot-2-expressing cells had the morphological characteristics of young fibroblasts and had reduced expression of senescence-associated β-galactosidase. The mot-2-expressing life span-extended cells exhibited

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decreased p53 function. These results show that overexpression of mot-2 has an effect on proliferative life span similar to that reported previously for loss of wt p53, and suggest that the effects of mot-2 in this regard are mediated via their interference with p53 function.

2. Materials and methods

2.1. Cell culture

Normal human lung fibroblasts, MRC-5, were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were serially passaged by 1:4 subculturing.

2.2. Transfections

Mortalin cDNAs encoding non-pancytosolic proteins were isolated from RS-4 (spontaneously immortalized clone from CD1-ICR mouse embryonic fibroblasts), HT1080 (human fibrosarcoma with activated N-*ras* oncogene) and HeLa (human cervical carcinoma) cells. These cDNAs (complete open reading frames) were cloned into the pSRα expression vector containing the hybrid SV40-human immunodeficiency virus promoter/enhancer and the neo^R gene and transfected into MRC-5 cells at PD 31 using LipofectAMINE™ (Life Technologies). Transfectants were selected in 50 μg/ml G418-supplemented growth medium (DMEM supplemented with 10% FBS). Clones were initially isolated by the ring isolation method and transferred into 3.5 cm diameter dishes and were subsequently subcultured at a 1:4 ratio in 6-cm dishes. Mot-2 expression was detected in the transfectants by Western blot analysis with anti-mortalin antibody [2].

2.3. Western blot analysis

The protein sample (10 μg) separated on an SDS–polyacrylamide gel was electroblotted onto a nitrocellulose membrane (BA85, Schleicher and Schuell) using a semidry transfer blotter (Biometra, Tokyo). Immunoassays were performed with anti-mortalin (mtHSP70, Affinity Bioreagents) or anti-actin (Boehringer Mannheim) antibodies. The immunocomplexes formed were visualized with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (ECL kit, Amersham Pharmacia Biotech).

2.4. Senescence-associated (SA) β-galactosidase staining

Detection of β-galactosidase activity was performed as described [23]. Cells were washed with phosphate-buffered saline (PBS, pH 7.2), fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS or 4% formaldehyde for 10 min at room temperature, and incubated at 37°C with a fresh staining solution consisting of 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside, 40 mM citric acid–sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$.

2.5. p53-mediated reporter assays

Cells were transfected with a p53-responsive luciferase reporter plasmid, pWWP-luc (containing $p21^{WAF1}$ promoter; kindly provided by Dr. Bert Vogelstein). Cotransfections of pRL-CMV were performed as an internal control to determine the efficiency of transfections. Forty-eight hours after transfections, luciferase assays (Dual-Luciferase™ Reporter Assay System) were performed. Luciferase activity values were calculated per microgram of the protein (to equalize for cell number) as determined by Bradford protein assay. To circumvent low transfection efficiency of late passage cells, microinjections of the p53-responsive β-gal reporter pRGCΔfos-lacZ (a kind gift from Dr. David Wynford-Thomas) were performed directly into the nuclei of cells growing on coverslips using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Control IgG was coinjected for the identification of the injected cells. After overnight incubation, cells were fixed with 4% formaldehyde for 10 min at room temperature, washed in PBS, permeabilized with PBS containing 0.1% Triton X-100 for 5 min on ice, washed three times with PBS and then stained with fluorescein isothiocyanate-conjugated secondary antibodies to detect injected IgG and β-galactosidase expression using the β-gal staining kit (Boehringer Mannheim). Cells were viewed using a Zeiss microscope. All cells showing any trace of blue staining were scored as positive for expression.

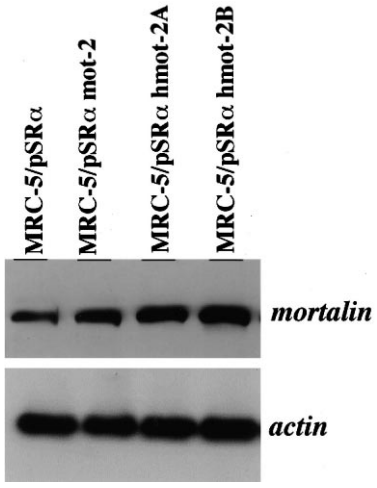

Fig. 1. Western blotting with anti-mortalin antibody in control and mot-2-transfected MRC-5 cells. A higher level of protein expression was detected in murine mot-2, hmot-2A and hmot-2B transfectants as compared to the untransfected and vector-transfected controls.

3. Results

3.1. Life span extension of MRC-5 cells

Expression plasmids containing cDNAs that encode mouse and human mot-2 proteins were transfected into MRC-5 normal human diploid fibroblasts at PD 31. Stable clones were isolated by selection in G418-containing medium. Clones containing about 200 cells were transferred into 3.5-cm dishes and then passaged serially in 6-cm dishes with a 1:4 subculture ratio. Expression of exogenous mot-2 cDNA was examined by RT-PCR using a forward primer from the expression vector and a reverse primer from the mot-2 sequence that yielded a ~500-bp DNA fragment (data not shown). The amplified DNA product was sequenced and confirmed to be mot-2. Two to three mot-2-expressing clones from each transfection were pooled to maintain adequately sized cultures for further analysis. Protein lysates from the vector-transfected control and mot-2-transfected cells were analyzed by Western blotting, which revealed increased expression of mot-2 in the latter (Fig. 1). Expression level was found to be higher in hmot-2B-derived clones. The hmot-2B construct contains a human mot-2 cDNA with a longer 3′ untranslated region than the hmot-2A plasmid [6]. The mot-2 protein expression level was higher in the hmot-2B transfectants than in those with hmot-2A or the murine mot-2 expression plasmid (Fig. 1).

Serial passaging was continued until the cells permanently stopped dividing. The cumulative PD level was calculated at each passage. The vector-transfected control cells underwent 28 PDs from the commencement of the study before ceasing cell division and exhibiting senescent morphology (Fig. 2A). The murine mot-2, hmot-2A and hmot-2B transfectants underwent 37, 38 and 45 PDs, i.e. their life span was extended by 9, 10 and 17 PDs, respectively (Fig. 2C). In contrast to control cells, the mot-2 transfectants had a young morphology after 24 PDs. Morphology of these cells at 40 PDs was comparable to the vector-transfected control cells at 26 PDs (Fig. 2A, compare upper and lower panels). In a replicate experiment vector-transfected control cells underwent 30 PDs whereas murine mot-2, hmot-2A and hmot-2B transfectants could proliferate for 44, 46 and 48 PDs, respectively. Thus in

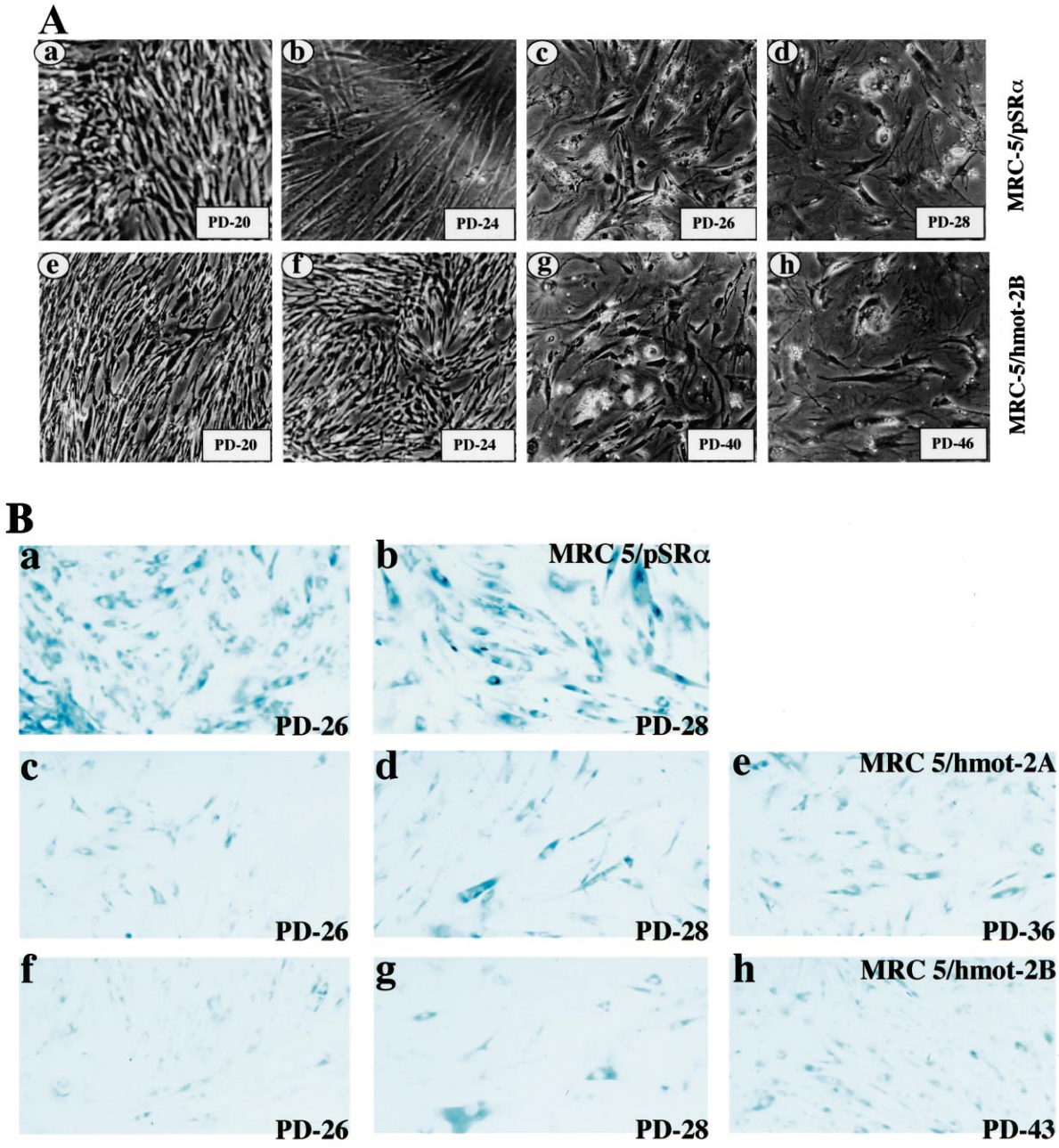

Fig. 2. A: Morphology of vector-transfected and hmot-2B-transfected MRC-5 cells during serial passaging. MRC-5/hmot-2B cells appeared young as compared to the MRC-5/pSRα (empty vector-transfected controls) at PD 24 (compare b and f). MRC-5/hmot-2B cells at PDs 40–46 appeared similar to MRC-5/pSRα cells at PDs 26–28 (compare c and d with g and h). B: SA-β-gal staining in MRC/pSRα, MRC-5/hmot-2A and MRC-5/hmot-2B cells. Decreased β-gal staining was detected in hmot-2A and hmot-2B cells as compared to the vector-transfected control at the same PDs (compare a and b of vector-transfected cells with c and d of hmot-2A, and f and g of hmot-2B-transfected cells). C: In vitro population doublings of MRC-5 cells transfected with empty vector (pSRα), murine mot-2 (pSRα/mot-2), hmot-2A (pSRα/hmot-2A) and hmot-2B (pSRα/hmot-2B) cDNAs. Cells were transfected at PD 31. G418-selected vector, murine mot-2, hmot-2A and hmot-2B clones underwent 28, 37, 38 and 45 PDs amounting to a total in vitro life span of 59, 68, 69 and 76 PDs, respectively.

the two experiments mot-2-transfected cells showed proliferative life span extension by 12–18 PDs.

The vector and mot-2 transfectants were stained for SA-β-gal that has been shown to be typical of senescent cells in culture [23]. Vector-transfected cells showed intense SA-β-gal staining at PDs 26 and 28 (Fig. 2B, a and b). However, murine mot-2 (not shown), hmot-2A (Fig. 2B, c and d) and hmot-2B (Fig. 2B, f and g) derivatives at comparable PDs had far fewer SA-β-gal-positive cells. An increase in the number of cells with SA-β-gal staining was observed from PDs 28 to 36 and from 28 to 43 in hmot-2A and hmot-2B derivatives, respectively, but the intensity of staining in the mot-2 transfectants was less than in the vector-transfected controls at all PDs analyzed.

3.2. *Life span-extended cells showed transcriptional inactivation of p53*

It is known that due to its low amounts and high turnover

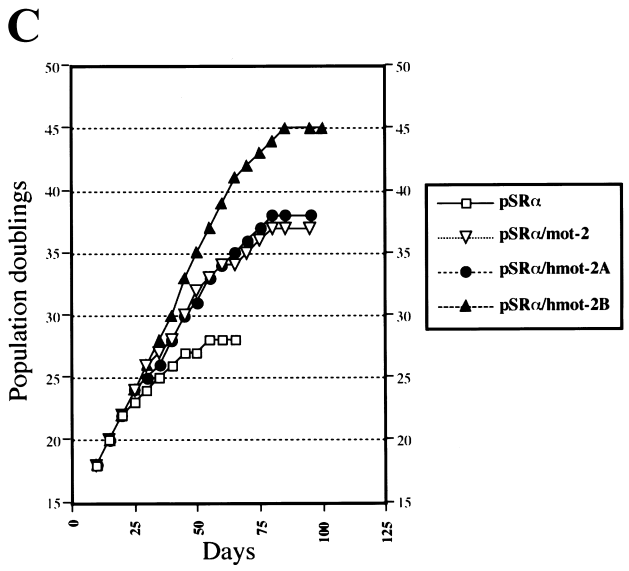

Fig. 2 (*continued*).

wt p53 protein in normal cells is not detected on Western blots. In contrast, the mutant p53 protein is easily detected due to its long half-life. Mock-, vector- and mot-transfected clones had undetectable levels of p53 on Western blotting (data not shown) suggesting its wild-type status and that the life span extension is not caused by mutational loss of p53 function. We next analyzed p53 activity by p53-responsive reporter assays. Vector- and mot-2-transfected cells at PD 21 were first transfected with pWWP-luc plasmid that contained the p21 promoter. The p53-dependent luciferase activity was nearly five-fold higher in the vector-transfected cells than in the cells transfected with murine mot-2, hmot-2A and hmot-2B (Fig. 3A). When a version of the luciferase reporter plasmid with both of the p53 binding sites deleted was used, the vector-transfected control and mot-2-transfected cells were seen to have comparable levels of p53-independent activity (Fig. 3B). Since normal cells had very low transfection efficiencies at later passages such as PD 25, we performed a similar experiment by microinjection of a p53-dependent β-gal reporter plasmid, pRGCΔfos-lacZ (containing 13 repeats of the p53 binding consensus sequence). About 200–250 cells were injected and analyzed for β-gal staining in two independent experiments. Such visualization of microinjected cells and β-gal staining revealed that whereas 90–95% of the untransfected and vector-transfected cells exhibited intense p53-responsive β-gal staining, only 8–10% of the mot-2 transfectants showed a comparable blue color (Fig. 3C). Young control cells when similarly microinjected were seen to have less intense staining (not shown). These data demonstrated increased p53 transactivation activity during senescence and its prevention by transfection and stable expression of mot-2.

4. Discussion

Mot-2 was originally cloned from mouse immortalized cells [2,3], but it was not clear whether expression of this hsp70 family member contributes to the immortal phenotype. Immortalization of human cells is usually a multistep process that may include a finite increase in proliferative life span associated with inactivation of the p53 and pRB/p16^{INK4a} tumor suppressor gene pathways [16]. Here we show a potential role for mot-2 in extension of proliferative life span by functional inactivation of p53.

Various viral oncoproteins are also able to extend proliferative life span in part by interfering with wt p53 function. In this regard the best known are the DNA virus oncoproteins, SV40 large T antigen and human papillomavirus E6, each of which binds to p53 and inactivates it (reviewed in [24]). Even after cells have become immortalized there is a requirement for continued inactivation of p53 and pRb [25,26]. The role of p53 in limiting proliferative life span has been demonstrated experimentally by showing life span extension following transduction of normal diploid fibroblasts by dominant negative p53 mutants [12] or microinjection of anti-p53 antibodies [27]. Inhibition of expression of p33^{ING1}, which is thought to be required for p53 function, was shown to result in extension of

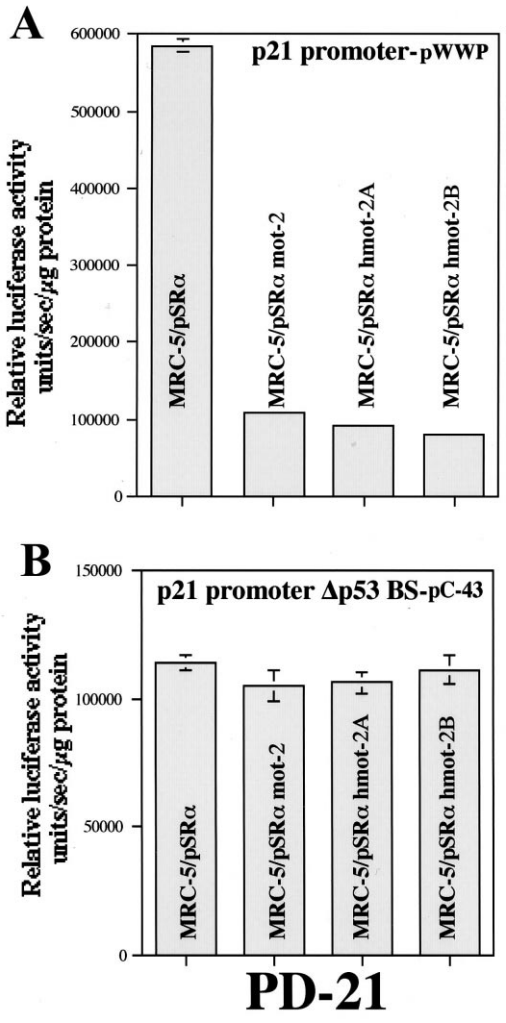

Fig. 3. Inactivation of p53 in life span-extended cells. p53-dependent (A) and -independent (B) luciferase reporter assay in vector-, murine mot-2-, hmot-2A- and hmot-2B-transfected MRC-5 cells at 21 PDs after transfection. Mot-2 transfectants showed about five-fold lower p53-dependent activity. C: p53-dependent β-galactosidase reporter assay in vector- and hmot-2B-transfected MRC-5 cells at PD 25 after transfection. The cells were microinjected with plasmid and rabbit IgG so that injected cells could be visualized by secondary staining with fluorescein isothiocyanate-conjugated rabbit IgG. The number of blue β-galactosidase-positive cells was significantly lower in hmot-2B transfectants than in the vector-transfected controls.

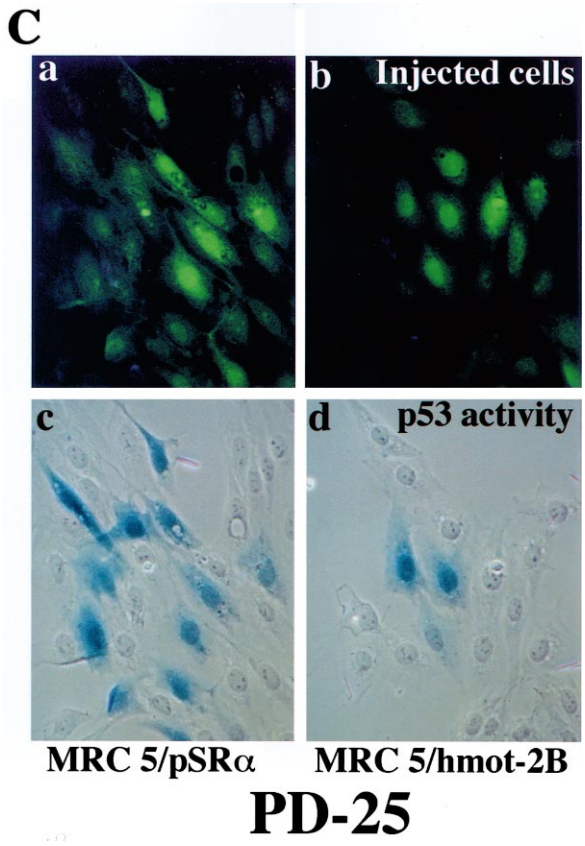

Fig. 3 (*continued*).

the proliferative life span of normal human fibroblasts by 7 PDs [28]. Indirect support for this role of p53 comes from the observation that targeting and thus inactivating both copies of the $p21^{SDI1/WAF1}$ gene, a key downstream effector of p53 function, resulted in a substantial extension of human fibroblast life span [29]. Mouse cells that are null for $p19^{ARF}$, an upstream activator of p53 that releases it from inhibition by MDM2 [30,31], fail to undergo senescence [32].

We have recently reported that mot-2 inhibits the transcriptional activation function of wt p53 by sequestering it in the cytoplasm [7]. Expression of mot-2 resulted in decreased expression of the $p21^{SDI1/WAF1}$ gene [7] that is normally transactivated by p53 [33] and upregulated at senescence [11]. Consistent with this finding, the p53 activity of the life span-extended MRC-5 cells was reduced five-fold (Fig. 3). The hmot-2B derivatives showed a higher level of expression of mot-2 compared to the murine mot-2 and hmot-2A transfectants. The life span extension for hmot-2B-transfected cells was 17 and 19 PDs in two experiments as compared to 9 and 14 PDs for murine mot-2 and 10 and 16 PDs for hmot-2A transfectants. This suggested that the life span extension is possibly related to the level of expression of mot-2 protein. In view of the key role of p53 in maintaining normal proliferative potential, it seems very likely that the life span extension effect of mot-2 is mediated through inhibition of p53 activity.

Overexpression of mot-2 in NIH 3T3 cells (that are already immortalized) causes malignant transformation [6]. In addition to loss of normal control of proliferative life span, loss of p53 function has other consequences (reviewed in [34,35]). It may therefore be expected that the effect of mot-2 expression will depend on cellular genetic context.

In summary, we have shown for the first time that expression of mot-2, a gene originally cloned on the basis of its upregulated expression in immortalized cells, is able to extend cellular proliferative life span. This is most likely due to the recently reported ability of mot-2 to sequester p53 in the cytoplasm and to inactivate it.

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