aging from 4 months old (28), whereas extracellular Aβ is deposited from 8 to 12 months old (48) in Swedish 670/671 mutant APP-Tg mice (Tg2576) brain. However, cognitive impairment as memory loss in these mice was found to be present at ~6 months old (49). As shown in Fig. 6*A–C*, Aβ42 accumulation was found in some neurons in 3-, 6- and 15-month-old mice, respectively. Neurons positive for intracellular Aβ42 showed abnormal shapes that looked to be degenerating. In 15-month-old mice, in which marked extracellular Aβ42 deposition was observed (data not shown), we found sporadic intense Aβ42 immunoreactivity in neurons, which were similar in size and shape (Fig. 6*C*, *D*). Moreover, double staining revealed that p53 coincides with intense Aβ42 immunoreactivity (Fig. 6*F–H*) in the degenerating-shaped neurons (Fig. 6*E*, arrows). A similar linkage between intracellular Aβ42 and p53 was found in 17-month-old L286V mutant PS1-Tg mice brain (Fig. 6*I–L*), which we previously reported to have marked intracellular Aβ42 accumulation and apoptosis without plaque formation (19).

Subsequently, we checked p53 mRNA levels in Tg mice brain by semiquantitative RT-PCR. Before checking these Tg mice, we also checked 3-month-old APP-knockout (APP-KO) mice brain. As shown in Fig. 7*A*, p53 mRNA levels were similar in APP-KO, which lacked APP (Fig. 7*A*, upper), and control mice brain. Therefore, intracellular Aβ42 may not be obligatory for basic p53 mRNA expression. In contrast, p53 mRNA expression was stepwise elevated in 17-month-old non-Tg, wild-type PS1-Tg and L286V mutant PS1-Tg mice brain (Fig. 7*B*), while 32-kD N-terminal fragments of human PS1 were found in wild-type PS1-Tg and L286V mutant PS1-Tg but not in non-Tg mice brain (Fig. 7*B*, upper). Also, increased full-length APPs were found in wild-type and mutant APP-Tg mice brain (Fig. 7*C*, upper), and a similar increase in p53 mRNA was found in Tg2576. Interestingly, these increases were observed in 6- and 10-month-old mice, but little was found in 3-month-old mice, indicating that aging may alter the effect of APP mutation on p53 mRNA expression. In all RT-PCR studies in Fig. 7, β-actin mRNAs were used as the internal standards.

Intracellular Aβ42 accumulation associated with elevated p53 expression in AD brain

We then checked the connection between cytosolic/nuclear Aβ42 and increased p53 expression in AD brain. First, since accurate levels of p53 mRNA in human brain tissues could not be measured because of mRNA degradation (data not shown), we checked p53 protein expression levels. Consistent with a previous report (32), the p53 protein levels in AD frontal cortices were apparently elevated compared with age-matched control brains (Fig. 8*A*). Since neuron-specific enolase (NSE) bands were similarly seen in both AD and normal brains, p53 may be increased in areas where many neurons still remain. Next, we studied immunostaining of intracellular Aβ. We autoclaved the sections before staining to enhance intracellular immunoreactivity of both Aβ42 and p53 (compare Fig. 8*B*, *C*, left two panels); conventional 99% formic acid pretreatment enhanced senile plaques markedly but little intracellular Aβ42 (data not shown). Intracellular Aβ42 (BC-05), but not Aβ40 (BA-27, data not shown), was observed as cytosolic granular immunoreactivities in lots of neurons (Fig. 8*B*). Other anti-Aβ42 antibodies, i.e., polyclonal anti-Aβ35-42 (FCA3542), polyclonal anti-Aβ37-42 (Chemicon), and QCB42 (Biosource) also showed similar staining patterns (data not shown). Interestingly, neuronal Aβ42 immunoreactivities were sometimes variable (Fig. 8*B*, right two panels). Slightly positive (white arrows), cytosolic granular-positive (blue arrows), or markedly whole cell body-positive (red arrows) neurons were observed. Remarkably, the markedly whole cell body-positive neurons

looked to be degenerating. Consistent with a previous report (32), double immunostaining showed that p53 was positive in both neurons and astrocytes (data not shown). As shown in the right three panels of Fig. 8*C*, some apoptotic nuclei (arrows) were TUNEL-positive (green) and p53-positive (red) and show overlapping (yellow). Pre-embedded immuno-electron microscopic observation revealed no apparent amyloid fibrils in these immuno-labeled neurons (data not shown), which may be consistent with previous reports (21, 29). Finally, double immunostaining of p53 and Aβ42 (BC-05) showed some neurons strikingly positive for both antigens in putatively degenerating neurons (Fig. 8*D*, black arrows), phenomena similar to FAD mutant Tg mice brains (Fig. 6) and cultured neurons (Fig. 5*A*), while extracellular Aβ42 depositions were p53-negative (Fig. 8*D*, white arrow). The data appear consistent with our previous findings that TUNEL positivity was associated with intraneuronal Aβ42 accumulation in AD brain (25).

Relationships among the Aβ42 plaques, cytosolic Aβ42 positive neurons, and both nuclear and cytosolic Aβ42 positive neurons in AD brain

To further study the relative populations of Aβ42 plaques, cytosolic Aβ42 positive neurons and nuclear Aβ42 positive neurons, we counted their numbers in the CA1 sector of the hippocampus in six AD brains. As shown in the left panel of Table 1, numerous cytosolic Aβ42 positive neurons (C), but just a small number of both nuclear and cytosolic Aβ42 positive neurons (N), were noted in AD brains. The number of nuclear Aβ42 positive neurons was relatively stationary (13–24, average 20.7) compared with those of cytosolic Aβ42 positive neurons (91–273, average 192) and Aβ42 plaques (7–80, average 27.7). In contrast, apparently smaller numbers of cytosolic Aβ42 positive neurons (9–73, average 32.3) and only a few nuclear Aβ42 positive neurons and Aβ42 plaques were noted in age-matched control brain. Also, the intensity of cytosolic Aβ42 immunoreactivity in control brain was much less prominent than in AD brain (data not shown). Interestingly, there was a tendency for the number of Aβ42 plaques and cytosolic Aβ42-positive neurons to be correlated negatively with each other.

DISCUSSION

Our present study indicates a novel effect of intracellular Aβ42 like HSF (42). Support for this hypothesis comes from one of our major findings; that H_2O_2 treatment, an inducer for genomic DNA damage and expression of some heat-shock proteins (50), induced nuclear localization of Aβ42 and p53 mRNA expression in guinea-pig primary neurons. Because HSF binds HSE in a trimeric form and the carboxyl termini are important for binding DNA (42), the differences in effects of Aβ40 and Aβ42 on transcription may be due to the differences in their carboxyl termini and aggregative natures. The possibility that intranuclear Aβ42 induces direct DNA damage causing an indirect effect on the p53 promoter can be excluded by the following facts. We found 4 kD soluble Aβ42 but not putatively toxic oligomeric or fibrillar Aβ42 in the nucleus, Aβ42 bound and activated the p53 promoter in a sequence specific manner, and a novel cytosolic protein that may transport Aβ42 to the nucleus (described following). However, Aβ42 may not be an authentic HSF, but just a minor transcription co-factor. Because, Aβ42 may work with other nuclear proteins, a marked Aβ-independent decrease in p53 promoter activity was found when Aβ42 binding sequences were mutated, and p53 mRNA did not decrease in APP-KO mice. In fact, it was previously reported that the vulnerability of neurons against H_2O_2 treatment was

not altered in APP-KO mice (51). It is unclear whether Aβ has a nuclear localization signal like HSF (52). Nevertheless, as Johnstone et al. (53) suggested the possibility that Aβ may be actively transported to the nucleus. Although just preliminary, we have found that a novel cytosolic protein, which contains a nuclear localization motif, specifically binds Aβ in vitro and induces apoptosis when overexpressed with Aβ (unpublished data). Such a chaperon protein would regulate the Aβ42 effect on p53 mRNA levels. Primary neurons but not neuroblastoma cells have been shown to be vulnerable to injected Aβ42 (34), though our transfection may have produced more than enough Aβ42 to cause apoptosis even in neuroblastoma cells. The different effects of Aβ42 in various cells might be due to differences in such a regulating system.

Just how relevant cytosolic/nuclear Aβ42 is for AD is an important issue. Intraneuronal Aβ42 has been shown to have cytosolic granular immunoreactivities in AD or DS sections (14, 15, 17), and recent immunoelectron microscopic studies have shown that Aβ42 accumulates in multivesicular bodies (29, 54). However, no reports have shown an apparent intranuclear Aβ42 immunoreactivity. In this study, we found Aβ42 immunoreactivity in both cytosol and nuclei in some degenerating neurons in Tg mice and AD brain. However, careful observation is essential since it is difficult to find these neurons as they are soon cleared by microglias in brain. Also, we postulated two other reasons for our successful detection of such whole positive neurons in Tg mice and AD brain. First, we used fixed frozen human brain sections that preserve intracellular proteins much better than paraffin-embedded sections, which are often used for immunocytochemical studies (data not shown). Second, we autoclaved mice and human brain sections before immunostaining (55). Recent reports have demonstrated that heating pre-fixed frozen material greatly enhances immunoreactivity especially for nuclear proteins (56) and that heating pretreatment also enhances detection of p53 (57). In fact, we found nuclear Aβ42 positive neurons to be 6.2–20.9% of total Aβ42 positive neurons after autoclave treatment (see Table 1). Thus, our method seems an appropriate one to stain both intracellular Aβ42 and p53. Cytosolic granular Aβ42-accumulating neurons in AD brain may be at an early degenerating or proapoptotic stage, which corresponds to primary neurons at 6 h after H_2O_2 treatment in vitro (Fig. 5*A*). Consistent with this, apparent accumulation of p53 was found only in markedly Aβ42-accumulating and putatively degenerating neurons in Tg mice (Fig. 6), patterns, which were quite similar in AD brain (Fig. 8*B–D*). Moreover, cytosolic Aβ42 accumulation and extracellular Aβ42 deposits may be pathologically reciprocal phenomena (Table 1), while nuclear Aβ42 accumulation and apoptosis may occur continuously during the progression of AD.

It is unclear whether increased intracellular Aβ42 is simply neurotoxic or might potentially be protective against genotoxic damage. P53 induces cell-cycle arrest to repair DNA (30, 58, 59), protecting cells from genomic DNA damage; regulation of the p53 promoter may control p53 function (60, 61), as well as the widely known post-translational regulation of p53 protein. However, an overload of pathogenic stress as oxidative stress (62) or overproduction of Aβ42 due to FAD gene mutation may cause an inappropriate increase in cytosolic and nuclear Aβ42, resulting in enhancement of neuronal apoptosis in AD. Thus, intracellular Aβ42 may not be indispensable for p53 mRNA expression, but excessive Aβ42 accumulation in cytosol may increase the risk for p53-dependent apoptosis. At this cytosolic stage, synaptic function might be affected by Aβ42, as recently reported in triple-Tg mice (63).

Vast numbers of reports attribute neurodegeneration in AD to extracellular Aβ neurotoxicity (3). However, we suggest here a novel neurodegeneration pathway, one of multiple intracellular Aβ42 pathogeneses. Mitochondrial damage may be important for neuronal loss in AD (64) and DS (26) brains. Thus, it is of great interest that p53 may directly induce permeabilization of mitochondrial membrane and cytochrome *c* release (65–67). In addition, p53 is also reported to be associated with synaptic degeneration as well as mitochondrial dysfunction (68). Although a recent report has suggested a direct linkage between Aβ42 and mitochondrial toxicity (69), p53 might be involved in toxicity for mitochondria and synapses in AD. Moreover, neuronal stress increases p38 kinase (70), which mediates phosphorylation of p53 (71) and is activated in early stages of AD (72), indicating that p53 phosphorylation may be activated as well as p53 expression in AD. As well, it has very recently been reported that the p53 homologue p73 accumulates in nuclei and is located in dystrophic neuritis and tangles in AD hippocampus (73), which may imply a p53-like pathogenesis of p73 in AD neurons. Accordingly, appropriately combined treatments against Aβ pathogeneses, that is, Aβ neurotoxicity, inside and outside, may be promising for prevention of neuronal loss in AD, though the neurodegeneration process remains quite complicated (74). Indeed, protective strategies against neuronal apoptosis related to Aβ may be different between intracellular (75) and extracellular Aβ (76). Some p53 cascade-blocking drugs, which actually attenuate neuronal loss in a model of Parkinson's disease (77), could thus inhibit intracellular Aβ42-related neurodegeneration in AD brain. Alternatively, reducing cytosolic Aβ42 may be beneficial for neuronal survival. Although the lysosomal system may be associated with degradation of intracellular Aβ42 (15, 28), the ubiquitin-proteasome pathway may also degrade cytosolic Aβ42 (21, 78). Because the ubiquitin-proteasome system may be affected in AD brain (79) and contribute to neuronal degeneration (80), activation of Aβ42 degradation by proteasome would attenuate intracellular Aβ42 pathogenesis.

Interaction between FAD-related proteins and the intracellular Aβ42/p53 pathway remains to be elucidated. Wild-type, but not FAD mutant APP, prevents p53-dependent neuronal apoptosis by controlling p53 activation (81), and so a decrease in the anti-apoptotic effect of the FAD mutant APP might thus be mediated by an increase in intracellular Aβ42. In addition, FAD mutant PS1 and PS2 are known to increase Aβ42 generation (1); p53 was reported to inhibit PS1 expression (82), and PS2 reported to trigger p53-dependent apoptosis down-regulating PS1 expression (83). In AD model mice; FAD mutant PS1 (19), FAD mutant APP (28), PS1/APP double-Tg (84), and PS1/APP/tau triple-Tg mice (63), all showed intraneuronal Aβ42 accumulation without or before extracellular Aβ42 deposition, implying that neuronal loss may precede the accumulation of extracellular Aβ42. In support, in FAD mutant APP mice (Tg2576), intracellular Aβ accumulation at 4 months (28), cognitive impairment at ~6 months (49), and extracellular Aβ deposition at 8–12 months (48) are reported. Moreover, we have recently found enhanced generation of intracellular Aβ42 by FAD mutations in PS1 and PS2 in cultured cells (85). Thus, APP, Aβ42, PS1, PS2, and p53 may regulate each others level in neurons, and the disruption of their balance may increase the risk for p53-dependent apoptosis. Intracellular Aβ42, especially in cytosol and nuclei, may be an important target in the pathogenesis and therapeutics for FAD as well as sporadic AD.

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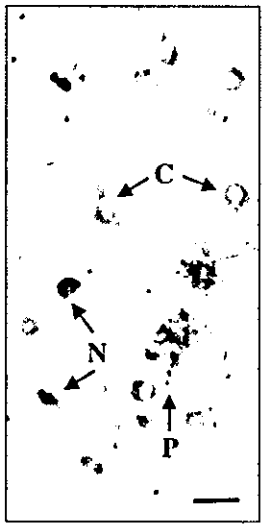
Table 1

Numbers of the Aβ42(+) plaque and neuron in human hippocampus (CA1)



case (years/sex)	plaque (P)	cytosol (C)	nucleus (N)	N/N+C (%)
A1 (89/ F)	80	91	24	20.9
A2 (84/ F)	17	194	23	10.6
A3 (90/M)	21	218	24	9.9
A4 (93/ F)	29	119	13	9.8
A5 (81/M)	12	257	22	7.9
A6 (86/ F)	7	273	18	6.2
C1 (83/M)	1	73	1	1.4
C2 (86/ F)	1	20	1	4.8
C3 (80/M)	0	9	0	0.0
C4 (90/ F)	2	27	2	7.4

Bar, 20 μm; A1-6, AD; C1-4, control; M, male; F, female; P, plaque; C, cytosol; N, nucleus

Fig. 1

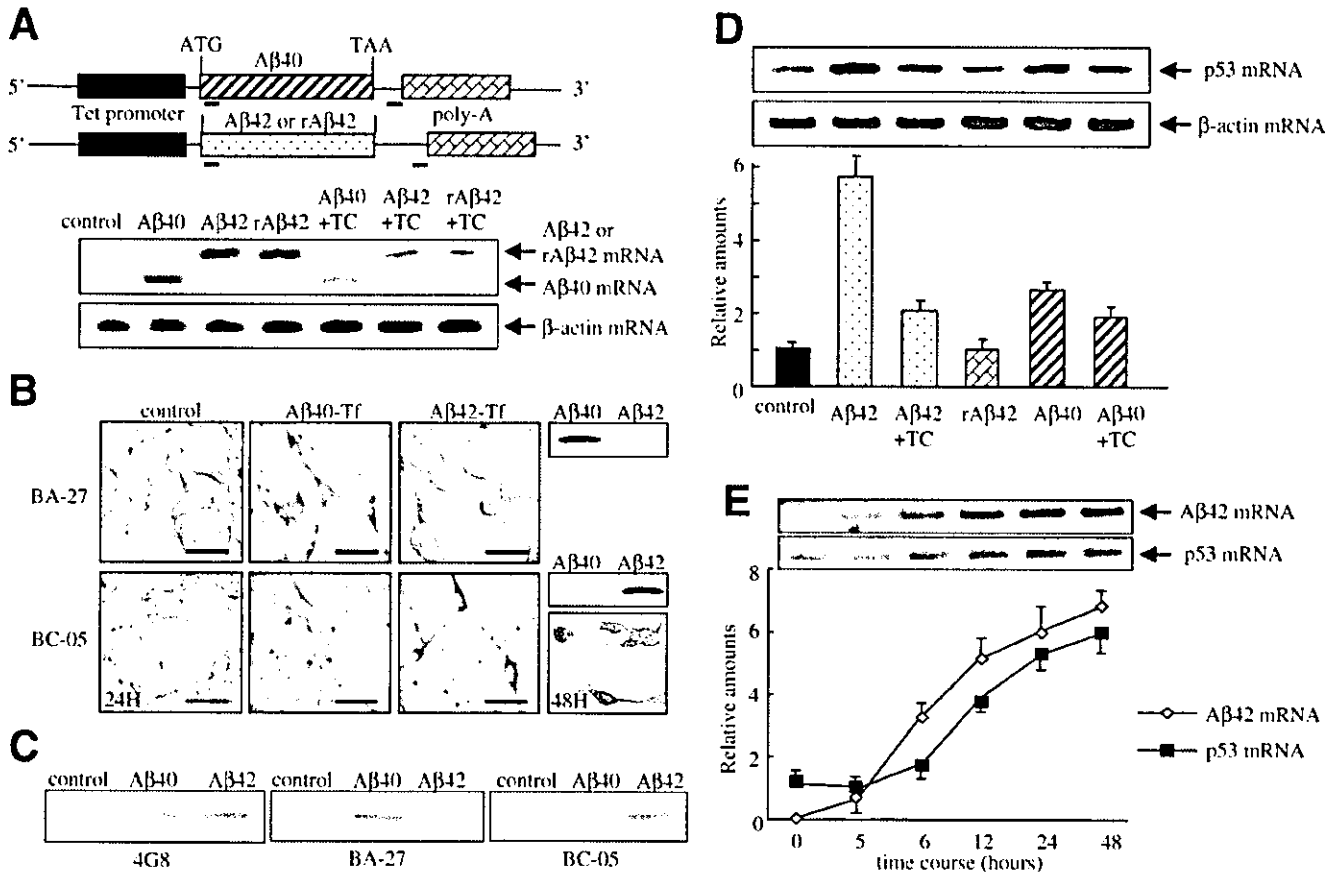


Figure 1. RT-PCR analysis of Aβ and p53 mRNA levels, immunoblotting and immunocytochemical staining of Aβ. ***A***) (***upper***) Constructs for transfection of Aβ40 (pTet-Aβ40), Aβ42 (pTet-Aβ42), or reverse Aβ42 (pTet-rAβ42). ATG and TAA indicate initiation (methionine) and stop codons, respectively. Bars indicate the primers for RT-PCR. (***lower***) RT-PCR of exogenous Aβ40, Aβ42, rAβ42, and β-actin mRNA 24 h after transfection. ***B***) (***left panel***) Immunocytochemical staining of cells transfected with Aβ40 (Aβ40-Tf) or Aβ42 (Aβ42-Tf) using anti-Aβ40 (BA-27) and anti-Aβ42 (BC-05) antibodies 24 h after transfection. (***right upper panel***) Immunoblotting of the respective antibodies for 20 pg each of synthetic Aβ40 and Aβ42. (***Right lower panel***) Immunostaining of Aβ42-Tf with BC-05 48 h after transfection. Scale bars, 20 μm. ***C***) Immunoblotting of NEP from transfected cells. After concentration of 3–10 kD proteins, 40 μg protein was electrophoresed in each lane. ***D***) Quantitative RT-PCR analysis of p53 mRNA with normalization by β-actin mRNA 24 h after transfection (n=3). ***E***) RT-PCR time course analysis of the expressions of Aβ42 and p53 mRNAs (n=3). Control cells were transfected with the pTet-splice vector only. TC was added at 1.0 μg/ml, a concentration at which cells remain healthy.

Fig. 2

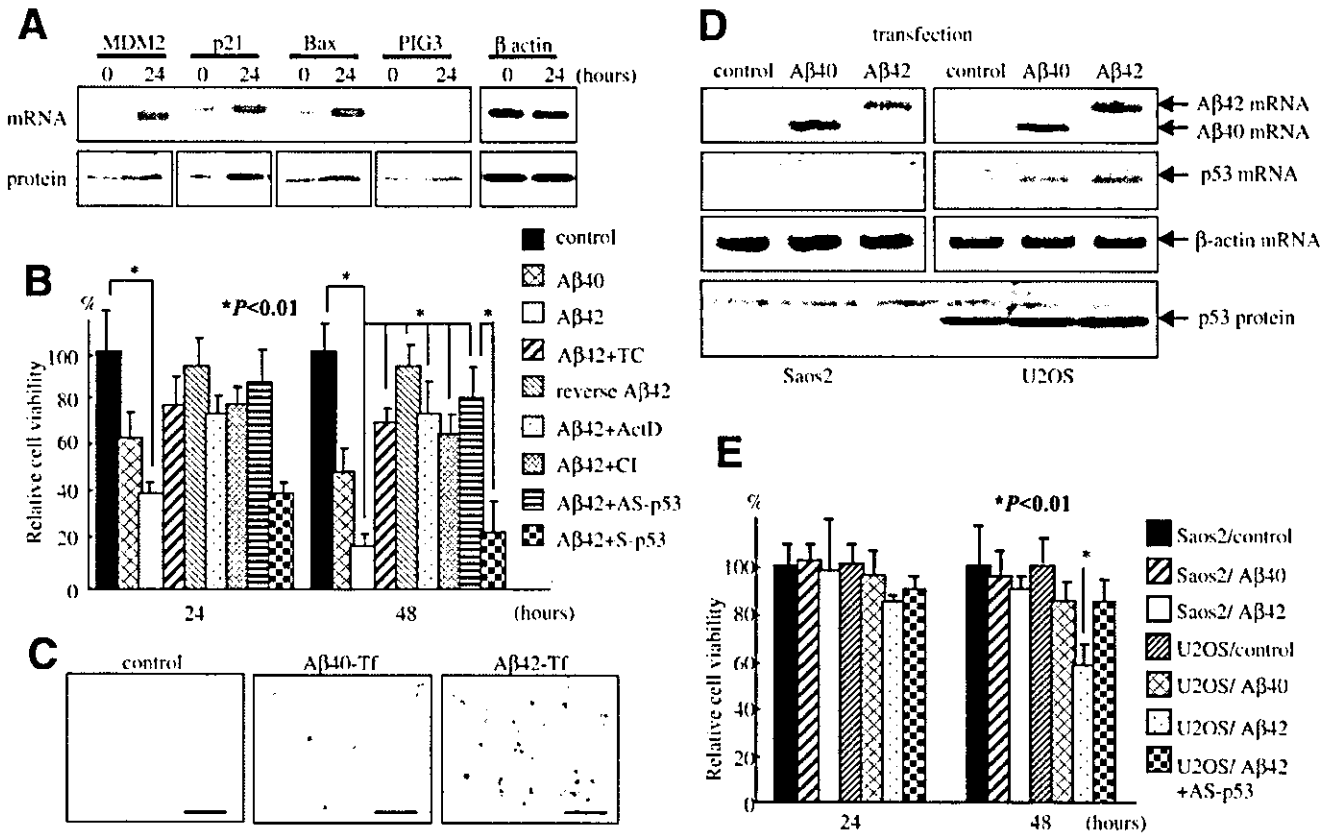


Figure 2. Analyses of immunoblotting, cell viability, TUNEL staining, and RT-PCR of neuroblastoma (SKN-SH) and osteosarcoma (Saos2 [p53–/–], U2OS [p53+/+]) cells transfected with Aβ40 or Aβ42. ***A***) RT-PCR (***upper***) and immunoblotting (***lower***) of MDM2, p21, Bax, PIG3, and β actin in Aβ42-transfected SKN-SH cells 0 and 24 h after transfection. ***B***) Relative cell viability of SKN-SH cells 24 and 48 h after transfection (n=5). TC, ActD, CI (Z-VAD-fmk), and sense/antisense DNA were added at 1.0 μg/ml and 5, 5, and 15 μM, respectively. ***C***) TUNEL staining 48 h after transfection. Scale bars, 50 μm. ***D***) RT-PCR and immunoblotting of p53 in osteosarcoma cells 48 h after transfection. ***E***) Relative cell viability of osteosarcoma cells 24 and 48 h after transfection (n=5).

Fig. 3

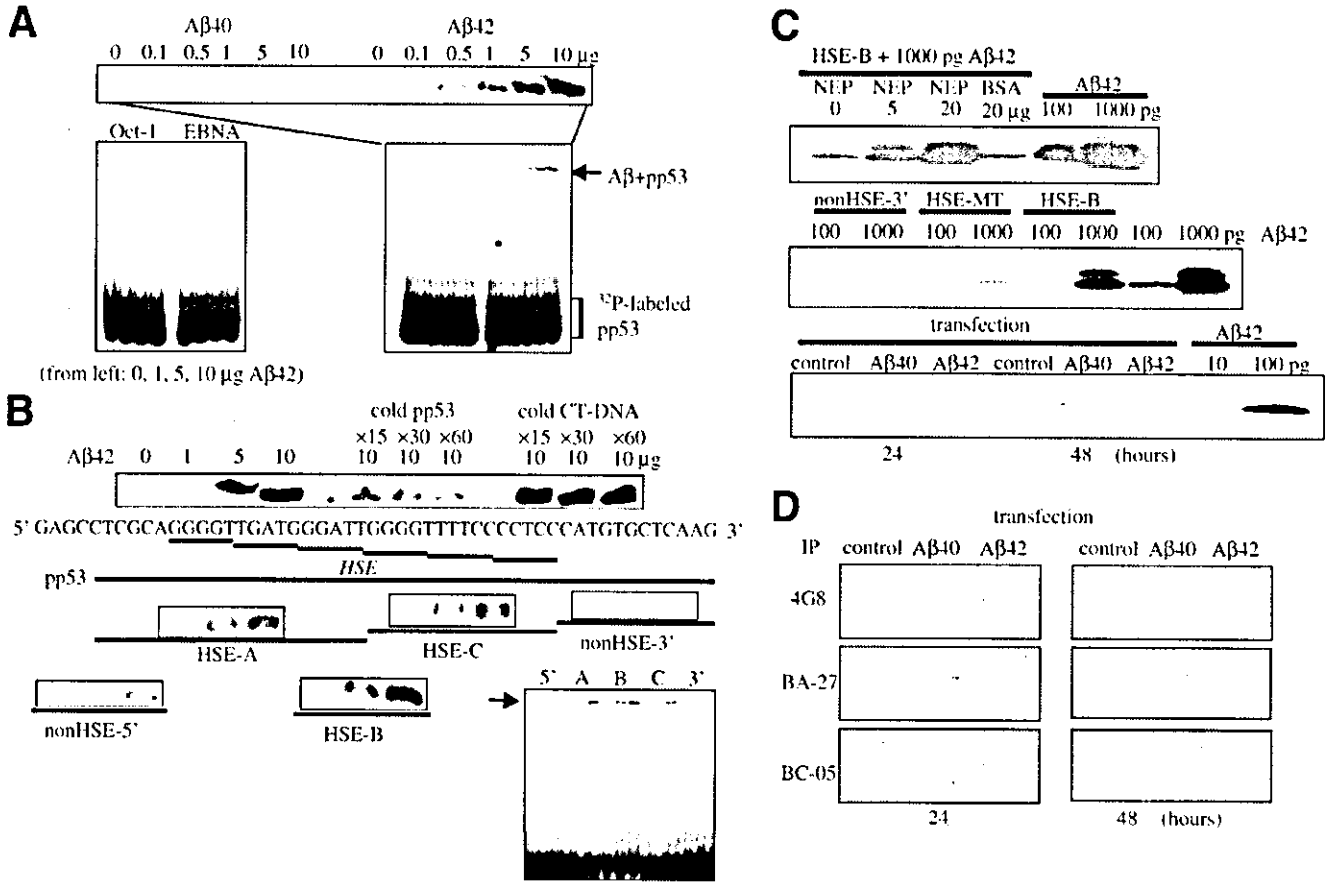


Figure 3. Gel mobility shift assays of the p53 promoter and Aβs and immunoblotting of Aβ collected by magnetic beads. ***A***) Gel mobility shift assay of synthetic Aβ40 or Aβ42 with labeled p53 promoter (pp53, ***right panel***), and with Oct-1/EBNA (***left panel***). ***B***) (***upper***) Binding of labeled pp53 and Aβ42 was inhibited by an excess of cold (unlabeled) pp53 but was not inhibited by cold calf thymus (CT) DNA. X15, X30, and X60 indicate the ratios of cold DNA to labeled pp53. (***lower***) Binding of labeled region-specific oligonucleotides (indicated as bars) and Aβ42. The gel shift images on the bars are isolated from the lower right panel (indicated by the arrow). ***C***) (***upper***) Immunoblotting detection of Aβ42 (BC-05) collected by biotinylated HSE-B and streptavidin-conjugated magnetic beads. Note that addition of nuclear extract proteins (NEP), but not bovine serum albumin (BSA), markedly increases the amount of Aβ42 recovered. (***middle***) Immunoblotting detection of 4-kD Aβ42 (BC-05) collected by oligonucleotides (nonHSE-3', HSE-MT, and HSE-B, see Figures ***3B*** and ***4A***). All samples contained 20 μg NEP during incubation. Note that HSE-B recovered Aβ42 much more efficiently than the other two oligonucleotides. (***Lower***) Immunoblotting detection of endogenous Aβ42 (BC-05) recovered by HSE-B from the NEP of transfected cells. Note that a small amount of Aβ42 is detectable in the NEP of Aβ42-transfected cells. ***D***) Chromatin immunoprecipitation (ChIP) assay of p53 promoter DNA using PCR. PCR detected p53 promoter DNA in the eluates immunoprecipitated with each specific anti-Aβ antibody (4G8, both Aβ40 and 42; BA-27, Aβ40; BC-05, Aβ42).

Fig. 4

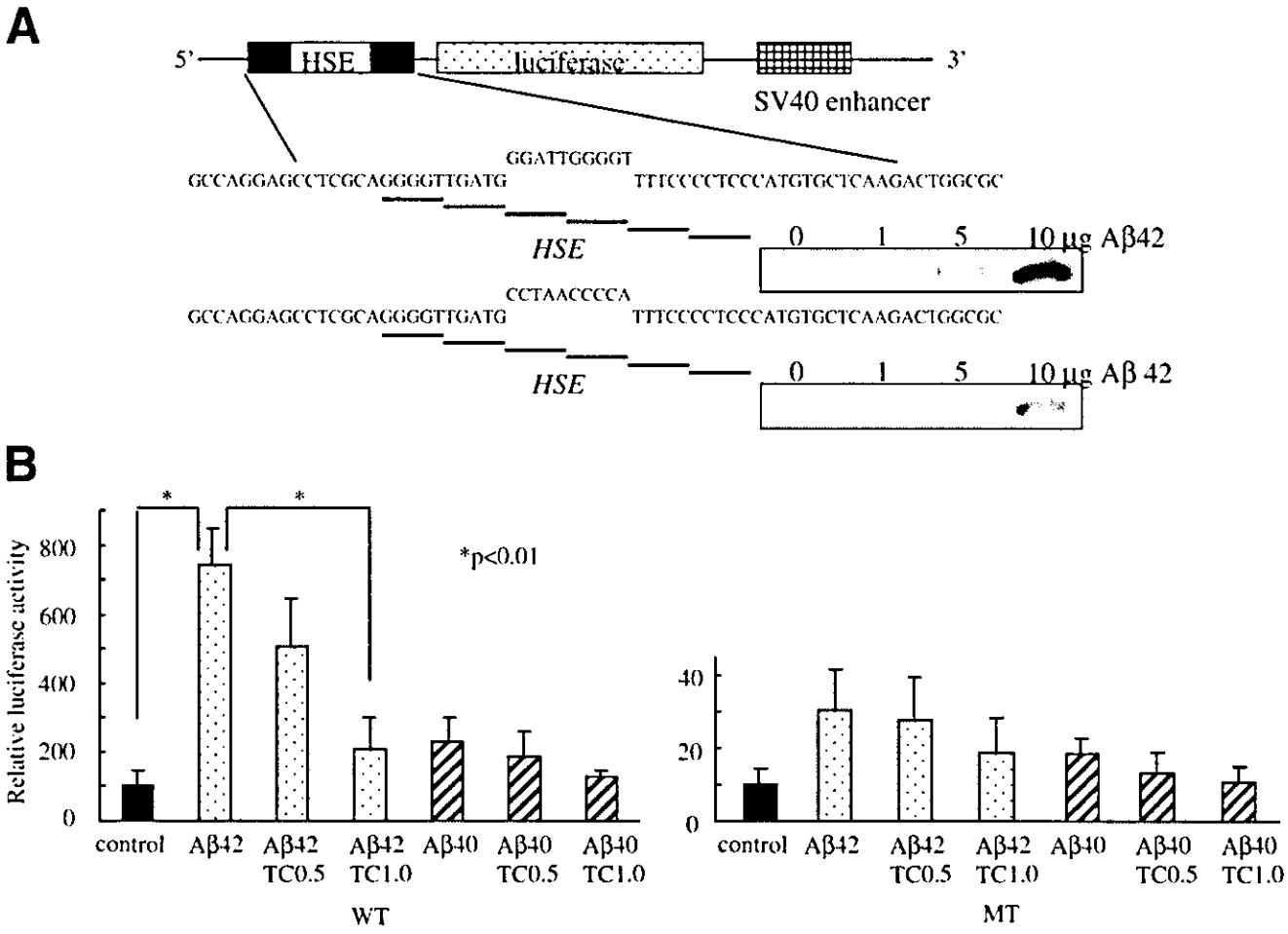


Figure 4. Luciferase assays of wild-type (WT) and mutant (MT) p53 Promoter activities in cells transfected with Aβ40 or Aβ42. *A*) Constructs for luciferase assays assessing the promoter activity of WT and MT pp53. The 10 nucleotides in the middle HSE of the MT pp53 were replaced with DNA in the reverse sequence. Each binding affinity is shown in the gel mobility shift assay presented under each construct. ***B***) Relative luciferase activities induced by WT (***left***) and MT (***right***) pp53 in transfected cells 48 h after transfection (n=5). TC was added at 0.5 or 1.0 μg/ml. The basal activity level of MT pp53 decreased to ~10% of WT pp53.

Fig. 5

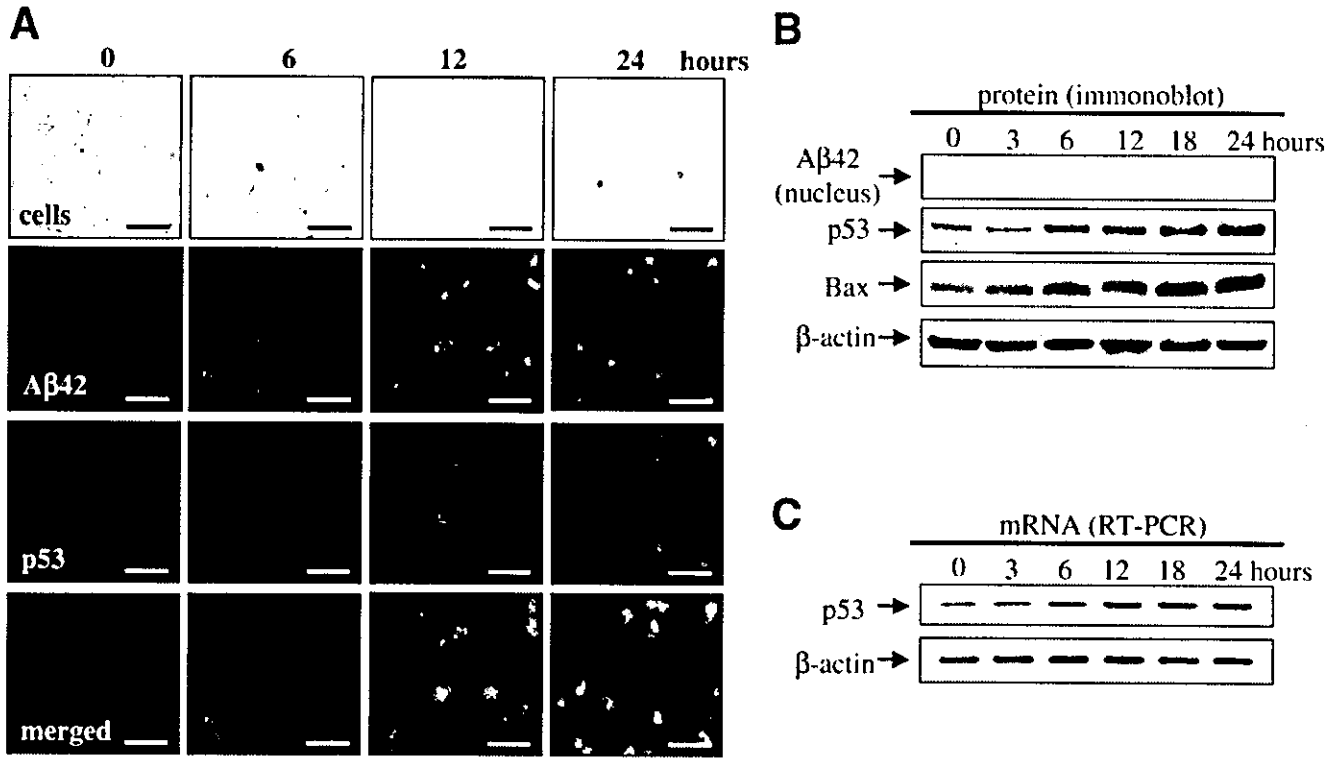


Figure 5. Time-course double immunostaining, immunoblotting, and RT-PCR analyses of 1 mM H_2O_2-treated primary cultured fetal guinea-pig brain cells. *A*) Double immunostaining of Aβ42 (BC-05, green) and p53 (FL393, red). Aβ42 becomes positive (green) in cytosol and nuclei at 6 h, then localizes to nuclei at 12 h. P53 becomes positive (red) in the whole cell body at 12 h. Scale bars, 20 μm. *B*) Immunoblotting of Aβ42 (BC-05) in NEP and intracellular p53, Bax, and β-actin. *C*) RT-PCR of p53 and β-actin. Consistent with the result of immunostaining in (*A*), p53 mRNA starts to increase at 6 h.

Fig. 6

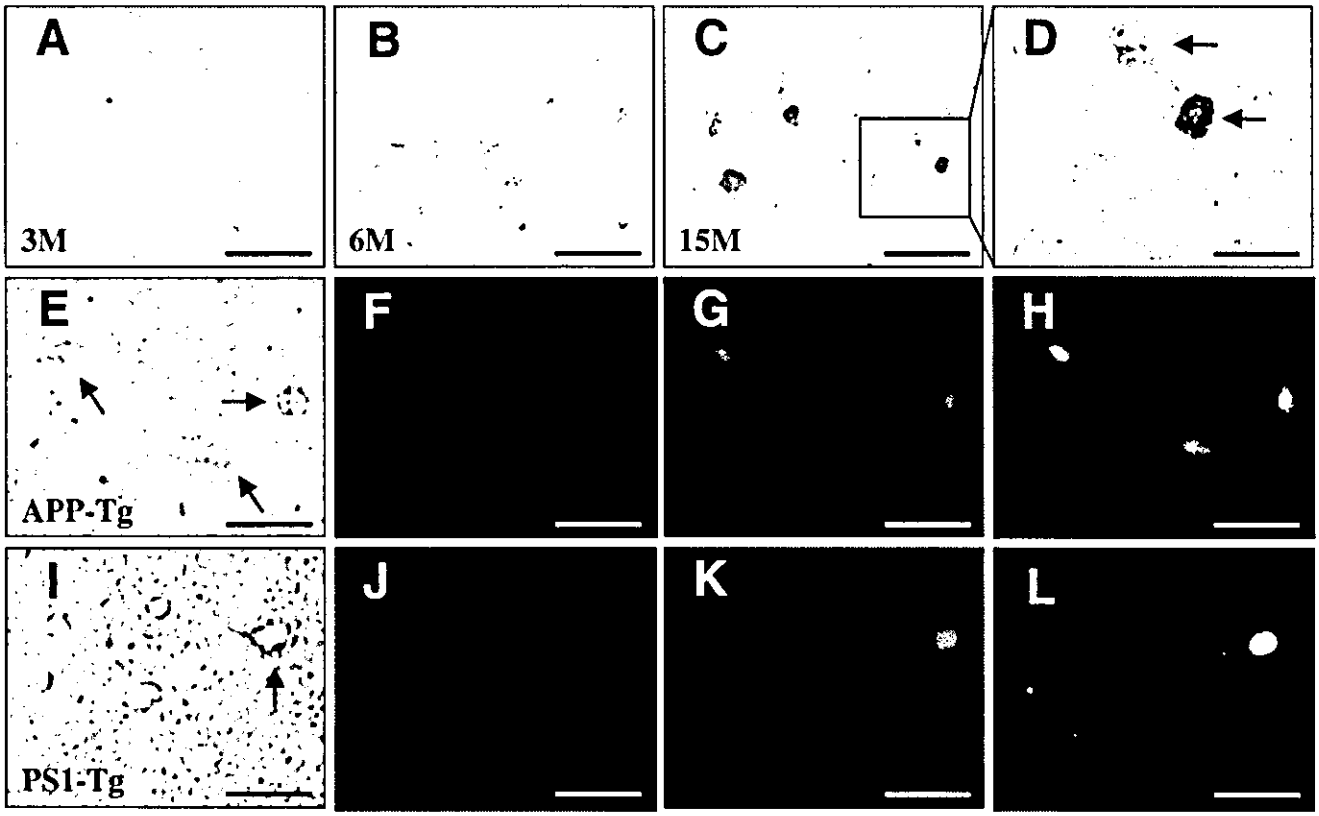


Figure 6. Immunocytochemical staining of Swedish 670/671 mutant APP-Tg mice (Tg2576, *A–H*) and L286V mutant PS1-Tg mice (*I–L*). *A*) Aβ42 staining (BC-05) in 3-month-old mice. *B*) Aβ42 staining (BC-05) in 6-month-old. *C*) Aβ42 staining (BC-05) in 15-month-old. *D*) Higher magnification of (*C*). Arrows indicate markedly positive cells that may originally have been neurons. *E*) Features of 6-month-old brain neurons. Arrows indicate both Aβ42 and p53 positive neurons that look to be degenerating. *F*) Aβ42 staining (BC-05, green) in 6-month-old. *G*) P53 staining (red) in 6-month-old. *H*) Merging of Aβ42 and p53 immunoreactivity in the 6-month-old; overlapping of green and red shows yellow. *I*) Features of the 17-month-old L286V mutant PS1-Tg mice brain neurons. Arrow indicates a neuron that is both Aβ42 and p53 positive and shows a degenerating shape. *J*) Aβ42 staining (BC-05, green) in 17-month-old. *K*) P53 staining (red) in 17-month-old. *L*) Merging of Aβ42 and p53 immunoreactivity in the 17-month-old. Overlapping of green and red shows yellow. Scale bars, 50 (*A–C*) and 20 μm (*D–L*).

Fig. 7

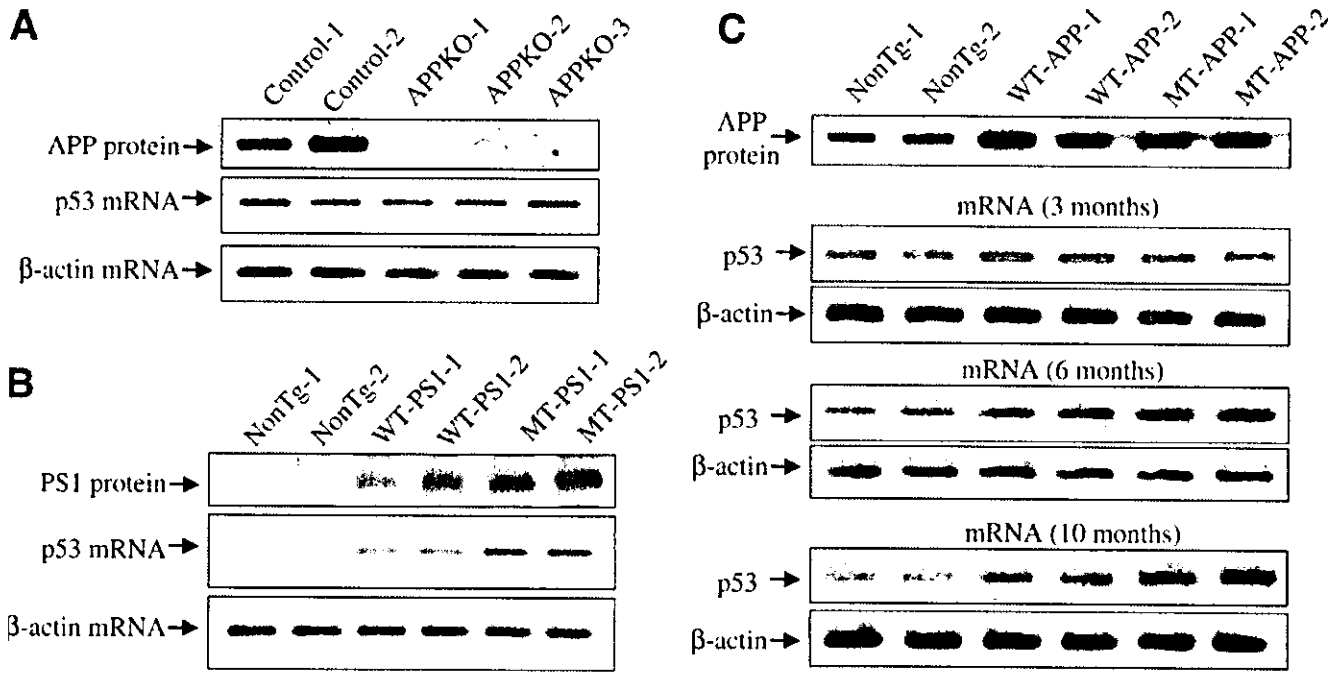


Figure 7. RT-PCR analyses of p53 mRNA in APP-KO (*A*), PS1-Tg (*B*), and APP-Tg mice (*C*). *A*) RT-PCR (p53 and β-actin mRNA) and immunoblotting (full-length APP) in 3-month-old control and APP-KO. *B*) RT-PCR (p53 and β-actin mRNA) and immunoblotting (32 kD N-terminal of human PS1) in 17-month-old non-Tg, WT PS1-Tg, and L286V mutant (MT) PS1-Tg. *C*) RT-PCR (p53 and β-actin mRNA) in 3-, 6-, and 10-month-old non-Tg, WT APP-Tg, and Tg2576 and immunoblotting (full-length APP) in 3-month-old mice.