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FUNCTIONAL LINKAGES BETWEEN THE TUMOR SUPPRESSOR PROTEIN p53 AND MnSOD

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Alteration in the content of pro- and anti-oxidant enzymes is a common feature of many spontaneous and experimentally induced tumors. This is particularly true with respect to the mitochondrial $O_2^{\cdot}$ scavenger manganese-dependent superoxide dismutase (MnSOD), an enzyme which catalyzes dismutation of superoxide into hydrogen peroxide in the mitochondrial matrix. This primary antioxidant was found decreased in many experimental tumors and tumor cell lines in comparison with the corresponding normal tissues. Since these initial reports, however, somehow conflicting evidence has been accumulating on this issue and in many cases cancer cells turned out to have higher rather than lower expression of MnSOD.

Speaking in particular about human cancers, a brief review of the publications appeared in the last twenty-five years (Fig. 1) reveals that reports on increased level of SOD2 in cancer cells are actually numerically prevalent over the ones showing the opposite alteration. Many of these papers have been published recently and involve very common neoplasms such as colorectal cancer, lung cancer and brain tumors (Landriscina, Remiddi, Ria, Palazzotti, De Leo, Iacoangeli, Rosselli, Scerrati & Galeotti, 1996; Nishida, Akai, Iwasaki, Hosokawa, Kusunoki, Suzuki, Taniguchi, Hashimoto & Tamura, 1993; Janssen, Bosman, Sier, Griffioen, Kubben, Lamers, van Krieken, van de Velde & Verspaget, 1998).

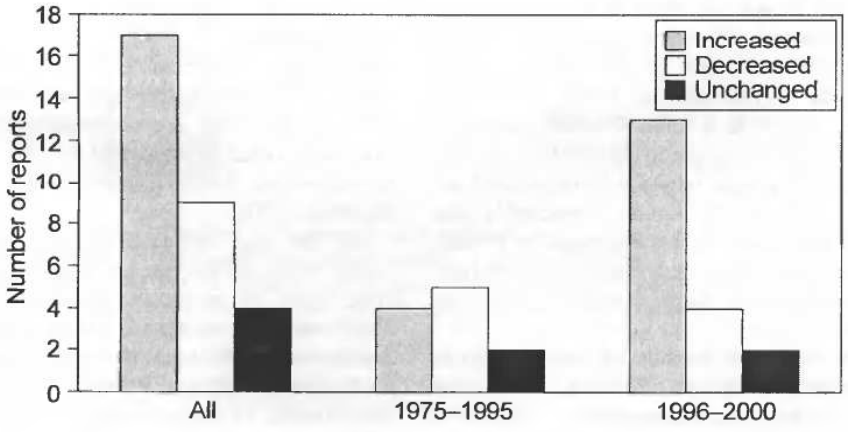

Fig. 1. MnSOD content in human cancer. An overview of the publications concerning MnSOD alteration in human cancer appeared in the past 25 years

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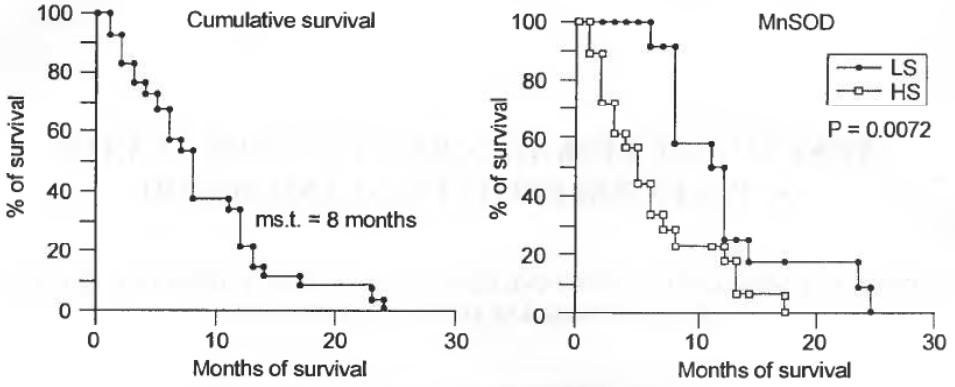

Fig. 2. Prognostic significance of MnSOD expression in primary brain tumors. Left panel: cumulative survival curve for patients with primary brain tumors. Right panel; High MnSOD expression in primary brain tumors is associated with a poorer prognosis. HS = High MnSOD content (immunohistochemical analysis); LS = Low MnSOD content

In a study published in 1996, Landriscina *et al.* has shown that MnSOD expression (activity, protein and mRNA) is increased in primary brain tumors in a fashion which correlates with tumor grade (Fig. 2, right panel).

Moreover, in this class of tumors MnSOD behaves as an independent prognostic factor for patients survival (i.e. the higher the content of MnSOD, the worse the prognosis) (Fig. 2, left panel).

This evidence challenges the common view that MnSOD is a tumor suppressor gene and rather suggests that, at least in some circumstances, this enzyme could favor tumor growth and expansion.

Which kind of selective advantages could MnSOD give to cancer cells?

It is well known that MnSOD can protect cells from deleterious effects of cytokines, oxidants, ionizing radiations and so on (Hirose, Longo, Oppenhaim & Matsushima, 1993). SOD2 can therefore operate as a survival protein, somehow similar to the antiapoptotic factor Bcl2, another mitochondrial protein with a well established oncogenic potential. It is thus conceivable that MnSOD takes a role in carcinogenesis by protecting cancer cells from death induced by immune factors and therapy, thereby increasing their malignancy.

Recent views on the role of mitochondria in apoptosis support the role of MnSOD as a survival protein. In fact, when cytochrome c (cyt. c) is released from mitochondria to activate cytosolic caspases, excess of superoxide anions is produced by the respiratory chain and these oxidants damage mitochondria and increase the release of proapoptotic factors thereby creating a vicious circle which pushes cells to die (Jacobson, 1996; Tan, Sagara, Liu, Maher & Schubert, 1998) (Fig. 3).

While Bcl2 acts by preventing cyt. c release (Yang, Liu, Bhalla, Kim, Ibrado, Cai, Peng, Jones & Wang,1997), MnSOD would exert its antiapoptotic effect by removing superoxide anion.

In agreement with this view we have observed that HeLa cells over-expressing MnSOD are much more resistant than their parental controls to a number of prooxidant insults relevant to tumor biology. These include anticancer drugs and as our group has recently demonstrated (Palazzotti, Pani, Colavitti, De Leo, Bedogni, Borrello & Galeotti, 1999), serum deprivation (Fig. 4). In this contest, MnSOD clearly behaves as an oncogene (i.e. as a gene whose product promotes carcinogenesis) rather than as a tumor suppressor gene, as previously suggested.

Interestingly, these stimuli, the resistance to which is increased by MnSOD, are powerful inducers of the tumor suppressor protein p53, and have been repeatedly reported to kill cells in a p53-dependent fashion (Lowe, Ruley, Jacks & Housman, 1993).

p53, the most well known tumor suppression protein exerts its function by inducing either cell cycle arrest or apoptosis. The latter effect is mainly achieved, as recently shown (Polyak, Xia, Zweier, Kinzler & Vogelstein, 1997), through the production of oxygen radicals and the induction of mitochondrial oxidative damage.

These latter observations suggest the existence of a functional antagonism between p53 and MnSOD. In this picture, while p53 kills cells by mitochondrial oxidative stress, MnSOD would prevent this process by removing mitochondrial superoxide. Another indirect evidence for a func-

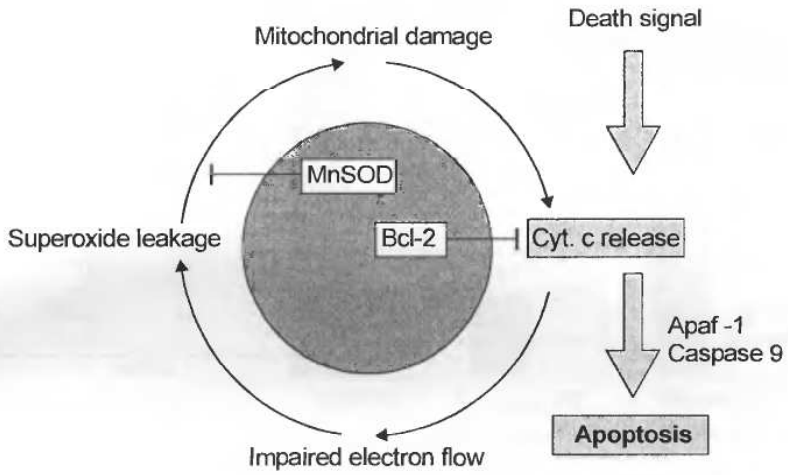

Fig. 3. The mitochondrial vicious circle in apoptosis. Cytochrome c release induces an impaired electron flow and consequent O_2^- production which can be counteracted by the MnSOD enzyme

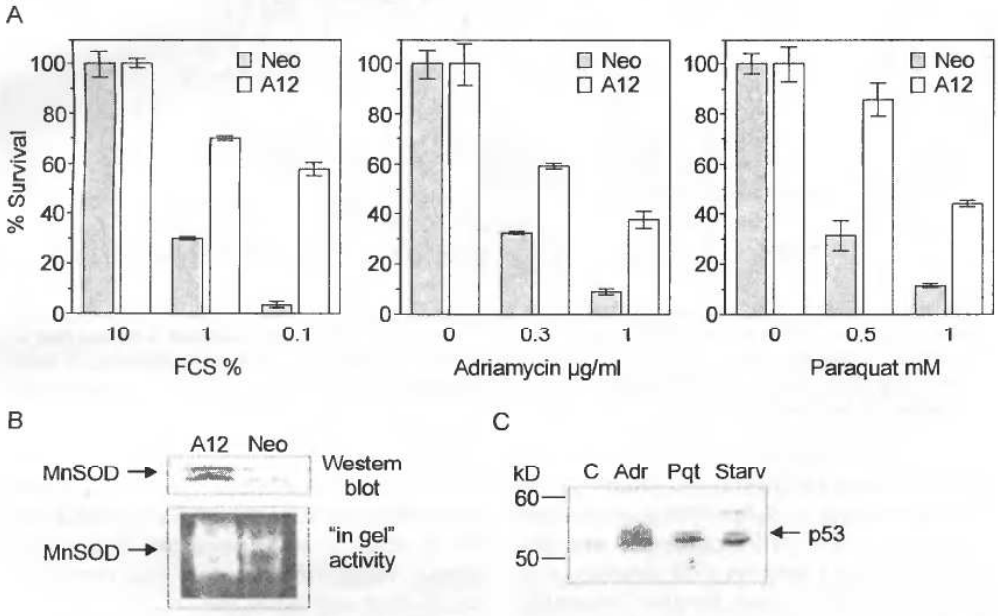

Fig. 4. Increased resistance to paraquat, adriamycin and serum deprivation of HeLa cells overexpressing MnSOD. A) Cytotoxicity of paraquat, adriamycin and serum deprivation on HeLa cells stably transfected with MnSOD (clone A12) or PcDNA3 empty vector (Neo) was evaluated by MTT reduction assay (Mossman, 1983). The MnSOD + clone A12 has been previously characterized (Palazzotti *et al.*, 1999). B) Increased levels of MnSOD activity (upper panel) and immunoreactive protein (lower panel) in A12 cells versus Neo control cells. C) p53 induction by pro-oxidant stimuli in HeLa cells. 5×10^5 cells were treated with 1 µg/ml adriamycin (24 hours), 500 µM paraquat (48 hours) or low serum (48 hours). Total lysates (80 µg) were immunoblotted with a cocktail of anti p53 Abs (DO-1 1:2000 + pAb240 1:2000) and immunocomplexes revealed by ECL. The p53 doublet band is indicated by arrow

tional linkage between MnSOD and p53 came from immuhistochemical analysis of primary brain tumors for these two proteins (Table 1). In particular, MnSOD expression increased with tumor grade and strikingly correlated with the appearance of cytosolic p53, an histochemical hallmark for mutated or inactivated p53 (Nakano, Oka & Taniguchi, 1996).

In order to directly assess the relationship between p53 functional status and superoxide dis-

Table 1. Immunohistochemical analysis of MnSOD and p53 expression on primary brain tumors MnSOD expression correlates, as a protein, with p53 cytosolic accumulation of the tumor suppresor protein p53, an hallmark of its functional inactivation

Tumor	Patients	p53	MnSOD
Meningioma	1	–	–
"	2	–/+	–
Astrocytoma II	3	–	+
"	4	+	+
"	5	–	–
"	6	–	+
Oligodendrogl.	7	–	–
Astrocytoma III	8	+++	++++
"	9	–	–
"	10	++	++
"	11	–	–

Tumor	Patients	p53	MnSOD
Glioblastoma	12	–/+	–/+
"	13	++++	++++
"	14	–	–
"	15	++++	++++
"	16	++	++
"	17	–	–
"	18	++++	++++
"	19	+++	+++

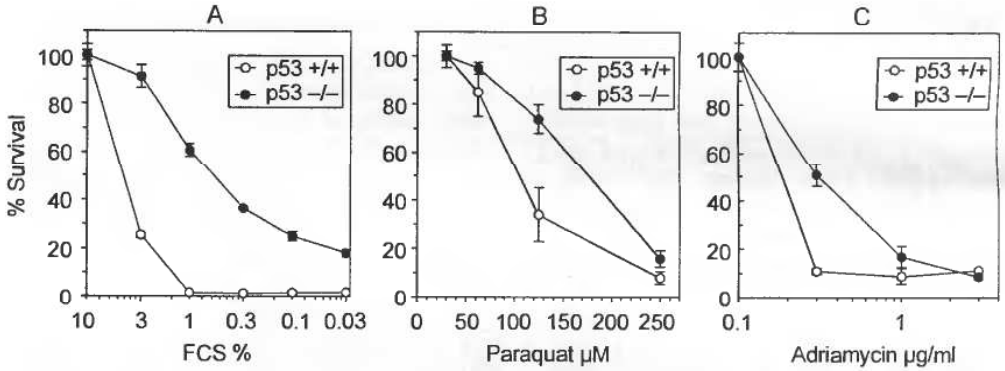

Fig. 5. Resistance of p53 deficient fibroblasts to cytotoxic effects of prooxidant stimuli. p53 +/+ (clone C8, open circles) and p53–/– (clone A9, closed circles) E1A/Ras transformed fibroblasts were seeded at 40000 cells/well in 96 well plate in presence of decreasing amounts of FCS (A) or increasing concentrations of either paraquat (B) or adriamycin (C). 48 hours later cytotoxicity was evaluated by MTT reduction test. Values are mean ± SD of triplicate cultures. Each panel is representative of at least two independent experiments

mutase, fibroblast cell lines transformed with Ras and either wild type or deficient for p53 were used (Lowe *et al.*, 1993). When challenged with prooxidant insults p53-deficient cells appeared more resistant than their controls therefore resembling cells overexpressing MnSOD (Fig. 5). This behavior of p53-deficient cells could depend either on a defective production of ROS or on the appearance of a specific resistance to the biological effects of ROS on tumor cells. The first possibility was ruled out by measuring ROS production in the two cell lines. This analysis revealed that absence of p53 does not results in any significant difference in the overall formation of oxidants as detected by the fluorescent probe DCF-DA (Fig. 6). This suggests that p53 deficient cells do produce oxygen radicals but have become resistant to them. In fact, p53 deficient cells express, at protein and activity level, much more MnSOD than the control cells whereas expression of CuZnSOD, which accounts for the most superoxide dismutase activity in cells, is fairly unchanged (Fig. 7, right panel). These differences are not restricted to transformed cells since they are also evident in normal untransformed hepatocytes (Fig. 7, left panel).

The above observations have important implications. First of all these data directly show that in cancer cells MnSOD content is modulated by functional status of p53. Second, p53 does not alter overall redox balance but rather selectively controls the antioxidant defense of mitochondria (the triggers of the apoptotic process).

Since p53 is a transcription factor it conceivably controls MnSOD expression at a transcriptional level.

HeLa cells, which express very low base level of p53, were used to prove it: These cells were tran-

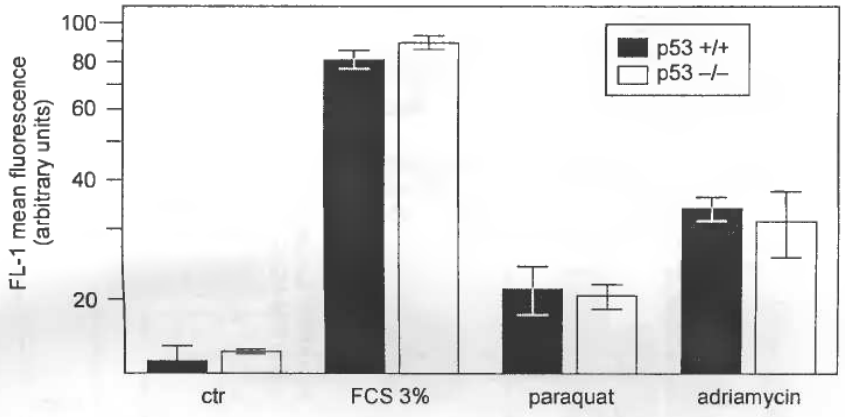

Fig. 6. Increased intracellular concentration of reactive oxygen species by serum starvation, paraquat and adriamycin. DCF-DA loaded p53 +/+ fibroblasts were tested for oxy-radicals production by FACS analysis. Cells (p53 +/+ gray columns, p53 –/– white columns) were exposed to the indicated stimuli (3% FCS, adriamycin 0.3 μg/ml, paraquat 125 μM) for 16 hours, followed by 30 minutes loading with 5 μg/ml DCF-DA, trypsinization and cytofluorimetric analysis. Values are mean ± error spreading of duplicate samples. Intracellular ROS increases following different treatments were all significant (at least $P < 0.05$ Student's "t" test), whereas differences between p53 +/+ and –/– cells where not

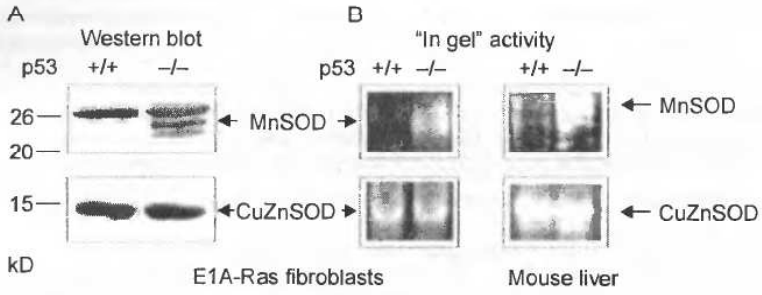

Fig. 7. Deregulated expression of MnSOD in p53 –/– cells. A) Anti MnSOD immunoblot of protein lysates from p53 +/+ and –/– fibroblasts. Mitochondria were purified as described in ref. 100 μgs of mitochondrial proteins were loaded in each lane. MnSOD appears as a doublet band immediately below the 26 kD marker, and is indicated by the arrow. The upper band, present in both +/+ and –/– samples, is non-specifically stained by rabbit IgG. Lower panel: anti CuZnSOD immunoblot analysis of total protein lysates (100 μg of proteins per lane). B) MnSOD activity in E1A/Ras fibroblasts (left panel) and mouse liver (right panel) protein extracts was assessed by "in gel" SOD assay according to Beauchamp and Fridovich (1971), in the presence of 1 mM $MnCl_2$. The white broad band corresponding to MnSOD is indicated by the arrow

siently transfected with wt p53 which appears over-expressed (Fig. 8, upper left panel) and functional (Fig. 8, lower left panel). In this condition steady state level of MnSOD mRNA is significantly decreased and so is its enzymatic activity (Fig. 8, right panel).

These experiments prove that p53 down-regulates the transcription of MnSOD gene; this has important patophysiological implications: in a later stage of carcinogenesis cancer cells lose p53 and become metastatic and resistant to chemotherapy. This could depend, at least in part, on the de-repression of the survival protein SOD2. But what is the physiological meaning of SOD2 down-regulation by p53? As it has been said, in order to kill cells, p53 has to induce an oxidative damage of mitochondria. MnSOD would interfere with this process and that is probably why p53, in order to fully exert its anticancer function, has to keep the enzyme at a low level. In order to prove this hypothesis and to show that MnSOD can interfere with the tumor suppression function of p53, HeLa cells were transfected with both genes. As one would expect, p53 alone (lane 4 versus lane 3, Fig. 9) greatly reduces colony recovery of transfected cells; interestingly, this effect is largely counteracted by simultaneous over-expression of MnSOD (lane 5 versus lane 4, Fig. 9).

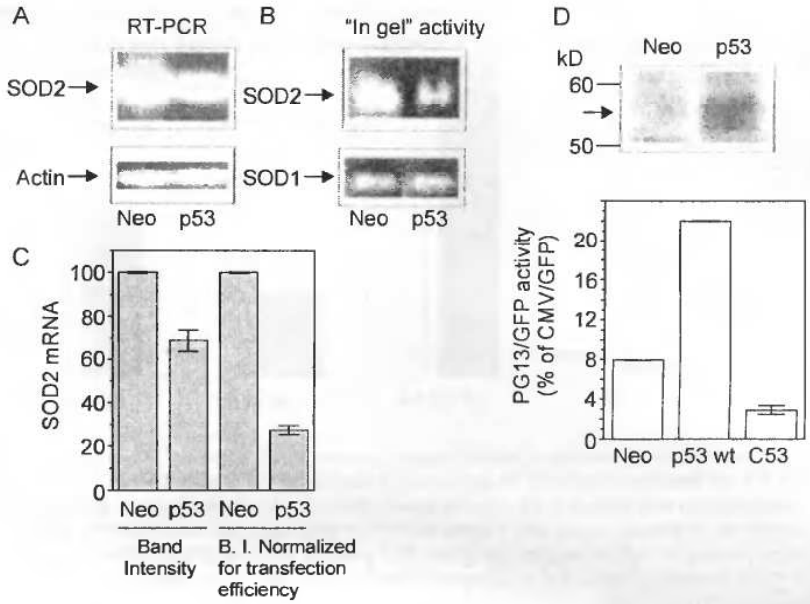

Fig. 8. MnSOD down-regulation by p53. HeLa cells (50–60 × 10^4/well in a 24 multiwell plate) were transiently transfected with either wild type p53 (200 ng) or empty vector (pCDNA3 200 ng), and MnSOD expression evaluated 48 hours later. Transfection efficiency was about 40% as assessed by GFP expression. A) RT-PCR of MnSOD transcript from mock and p53 transfected HeLa cells. The MnSOD band is 40% reduced in the p53 transfected sample. The actin band is indicated as RNA loading control. B) MnSOD activity in mock- and p53-transfected HeLa cells. SOD enzymogram was performed as in Fig. 7, except that $MnCl_2$ was omitted. The MnSOD and CuZnSOD activity bands are indicated. C) Densitometric analysis of MnSOD mRNA bands in neo and p53 transfected HeLa cells. Values are mean ± SD of two independent experiments. Normalization for efficiency of transfection was calculated as follows: $\frac{\%\text{ decrement of band intensity}}{\%\text{ of transfected cells}} \times 100$.

D) Expression of transfected p53 in HeLa cells. Upper panel: p53 immunodetection in total protein lysates from neo and p53/neo transfected cells. Lower panel: PG13-GFP reporter construct trans-activation by endogenous and transfected (wt = wild type; C53 = trascriptionally inactive "dominant negative") p53

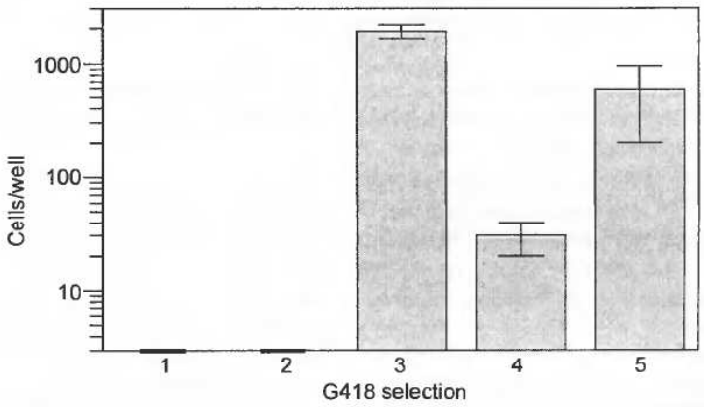

Fig. 9. MnSOD antagonizes the growth inhibitory effect of p53. Effects of p53 and MnSOD on HeLa cells growth in G418 containing selective medium. 48 hours after transfection with the indicated constructs cells were plated in selective medium (G418 800 μg/ml). 14 days later surviving cells were counted trypsinized and counted. Bar 1: no vector. Bar 2: MnSOD/zeo (zeocine resistance gene). Bar 3: pCDNA3/neo (neomycin resistance gene). Bar 4: wt p53 / neo + pCDNA3/zeo. (1:4); Bar 5: p53 /neo + MnSOD/zeo. (1:4) Note the increase in survival of p53/neo + MnSOD/zeo transfected cells versus the p53/neo + pCDNA3/zeo transfectants. Bars are mean ± SD of two independent transfections. Bars 3, 4 and 5 are significantly different by at least $P < 0.05$ (Student's "t" test)

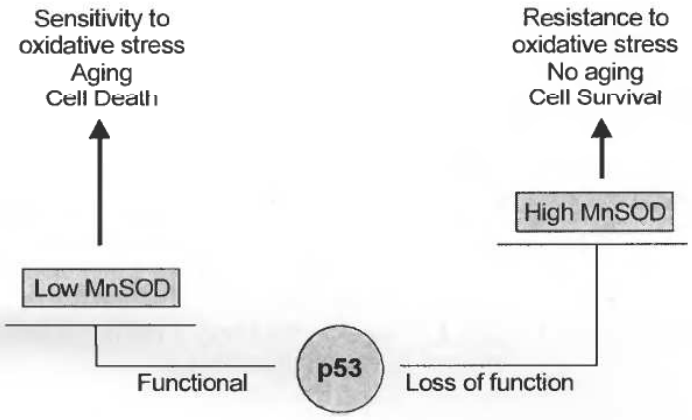

Fig. 10. A model. In this model deregulated MnSOD expression links loss of p53 function with increased resistance to oxidative stress and possibly with impaired apoptosis

All these observations come together into a model (Fig. 10) in which the cell content of MnSOD links p53 functional status to cancer cell malignancy. In fact, if p53 function is maintained in cancer cells they have low level of MnSOD and therefore they are sensitive to oxidative stress, they age and are responsive to chemotherapy. But when p53 is lost, high levels of MnSOD make cells resistant to oxidative stress and therapy and, in one word, more malignant.

Aknowledgemets

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