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Increased skin carcinogenesis in a keratinocyte directed thioredoxin-1 transgenic mouse

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Thioredoxin-1 is a low molecular weight redox protein that protects cells against oxidant damage. Thioredoxin-1 levels are increased in the epidermal layer of sun-damaged human skin. Thioredoxin-1 levels are also increased in several human primary tumors where its expression is associated with increased tumor cell proliferation, decreased apoptosis and aggressive tumor growth. We have investigated whether increased thioredoxin-1 levels in skin can lead to increased tumor formation using transgenic mice with mouse thioredoxin-1 expressed in keratinocytes under the control of the keratinocyte-14 (K14) promoter. Thioredoxin-1 protein expression was increased 2-fold in the keratinocyte layer of the transgenic mice. The skin was macroscopically and histologically normal but in the two-stage model of carcinogenesis using topical dimethylbenzanthracene (DMBA) as an initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a promoting agent, there was a 6-fold increase in the number of papillomas per mouse and a 3-fold increase in papilloma size in the K14 thioredoxin-1 transgenic mice compared with non-transgenic littermates. Thus, increased thioredoxin-1 in keratinocytes acts as an enhancer of carcinogenesis in the DMBA/TPA two-stage model of skin carcinogenesis in mice.

Introduction

Thioredoxins are low molecular weight (10–12 kDa) redox proteins found in both prokaryotic and eukaryotic cells (for reviews see refs 1–4). The cysteine (Cys) residues at the conserved -Cys-Gly-Pro-Cys-Lys active site of thioredoxins undergo reversible oxidation–reduction catalyzed by NADPH-dependent flavoprotein thioredoxin reductases (5). Mammalian thioredoxin-1 is a predominantly cytoplasmic protein but is sometimes also found in the nucleus (6,7). Thioredoxin-1 has multiple effects in the cell (1–4) that includes the regulation of the DNA binding and *trans*-activating activity of redox sensitive transcription factors such as the glucocorticoid receptor (8), NF-κB (9), p53 (10), HIF-1 (11) and, indirectly through redox factor 1 (Ref-1/HAP1), AP-1 (Fos/Jun heterodimer) (12). Thioredoxin-1 binds in a redox-dependent manner to enzymes to regulate their activity including apoptosis signal-regulated kinase-1 (ASK-1) (13), protein kinases Cα, δ, ε and ζ (14) and the tumor suppressor PTEN (15). Thioredoxin-1 also provides reducing equivalents to cytoplasmic thioredoxin peroxidases that protect cells against oxidant-induced apoptosis by scavenging hydrogen peroxide and organic hydroperoxides (16).

Elevated thioredoxin-1 in cancer cells has been linked to increased cell proliferation and inhibited apoptosis. Stable transfection of mouse WEHI7.2 lymphoid cells with human thioredoxin-1 inhibited apoptosis induced by a variety of agents (17), while transfection with a redox-disabled thioredoxin-1 potentiated apoptosis (18). The thioredoxin-1 transfected WEHI-7.2 cells formed tumors in mice that grew faster than tumors formed by wild-type or vector transfected WEHI-7.2 cells (17). Human MCF-7 breast cancer cells stably transfected with thioredoxin-1 showed increased clonogenic growth while MCF-7 cells transfected with a redox-disabled mutant thioredoxin-1 had inhibited clonogenic growth and showed decreased tumor formation *in vivo* (19). Thioredoxin-1 levels are elevated in several human primary cancers (20–24) including squamous cell skin cancer (25). An increase in thioredoxin-1 has been associated with increased tumor proliferation and inhibited apoptosis in human primary gastric cancer (26), and decreased patient survival in human colon and non-small cell lung cancer (22,27).

Thioredoxin-1 is present in normal skin in the dividing cells of the outer root sheath of hair follicles, sebaceous glands and secreting components of apocrine and eccrine sweat units, but is not found in the interfollicular epidermis (25). Sun-damaged human skin with solar keratosis shows increased thioredoxin-1 expression in the epidermal layer (25). Ultraviolet A (UVA) and B (UVB) radiation increase thioredoxin-1 expression in human keratinocytes and skin fibroblasts (28–31), and increased expression of thioredoxin-1 protects human skin fibroblasts from UVA radiation-induced DNA damage (31). It has been proposed that antioxidants, such as thioredoxin-1, by preventing DNA damage protect against carcinogenesis in skin (32). However, thioredoxin-1 transfected human skin fibroblasts also show decreased apoptosis in response to UVA radiation (30). Apoptosis is a process that removes damaged cells from the body, thus, preventing malignantly transformed cells with damaged DNA developing into tumors (33). Thus, the increase in thioredoxin-1 in skin as a protective mechanism against UV light induced oxidant damage and apoptosis might, in the longer term, promote carcinogenesis.

To investigate whether an increase in thioredoxin-1 in the skin can lead to increased tumor formation we developed a transgenic mouse with mouse thioredoxin-1 under the control of the keratin-14 (K14) promoter to increase thioredoxin-1 expression in the keratinocyte layer of the skin. Using the two-stage model of skin carcinogenesis with topically applied dimethylbenzanthracene (DMBA) as an initator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a promoter we

Abbreviations: DMBA, dimethylbenzanthracene; K14, keratinocyte-14; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; UV, ultraviolet.

showed that the thioredoxin-1 transgenic mice have markedly increased tumor formation compared with control mice.

Materials and methods

Generation of transgenic mice

Mouse thioredoxin-1 cDNA from C57BL/6 mouse tissue was cloned into the human K14/bluescript vector (34) provided by Dr X.J.Wang, Baylor University, Texas. The vector was digested to produce a linear construct [K14/(m)thioredoxin-1] consisting of the human K14 skin promoter, a generic intron, the mouse thioredoxin-1 cDNA and a polyadenylation sequence. The DNA construct was injected into fertilized C57BL/6 × SJLF2 embryos and four founder mice expressing the mouse thioredoxin-1 transgene selected for breeding. Founders and K14/(m)thioredoxin-1 positive offspring were backcrossed with C57BL/6 wild-type mice (Harlan, Indianapolis, IN) for at least six generations. Mice were maintained on a 12 h light/dark cycle and provided food and water *ad libitum*. All breeding and experimental protocols were approved by the University of Arizona Institutional Animal Care and Use Committee.

Measurement of mouse thioredoxin-1 cDNA transgene and mRNA

DNA was extracted from mouse tail-tips utilizing the DNeasy Tissue kit (Qiagen, Valencia, CA) and the presence of the K14/(m)thioredoxin-1 transgene measured by PCR analysis using primers specific for the transgene. To confirm the expression of the transgene, RNA was extracted from ear punches using the ToTALLY RNA™ kit (Ambion, Austin, TX) and quantitative RT–PCR performed (TaqMan 48, Roche Diagnostics, Indianapolis, IN) utilizing primers that detected mouse thioredoxin-1 mRNA.

UVB-exposed mouse skin

Female SKH-1 hairless mice (Charles River Laboratories, Wilmington, MA) were irradiated three times weekly with Westinghouse FS-40 UVB lamps (National Biological, Twinsburg, OH) calibrated using a UVX digital radiometer with a UVX-31 sensor (UV Products, San Gabriel, CA). Lamp output was ~4% UVA radiation (320–400 nm), 80% UVB radiation (290–320 nm) and <1% UVC radiation (<290 nm) with the remainder of the output in the visible spectrum. Mice initially received 1.5 kJ/m^2 at each exposure in week 1, escalating weekly by 1.5–9.0 kJ/m^2 per exposure by week 6. Mice received 9.0 kJ/m^2 per exposure in a similar fashion during weeks 7–10 for a total cumulative exposure of 202.5 kJ/m^2. Dorsal skin was collected at week 10 from non-irradiated and irradiated mice, fixed overnight in 4% formaldehyde in phosphate-buffered saline, pH 7.4, followed by dehydration in 70% ethanol, embedded in paraffin and stained for thioredoxin-1.

Two-stage skin carcinogenesis study

The treatment groups consisted of 7–8-week-old K14/(m)thioredoxin-1 mice (eight female and eight male) and their non-transgenic littermates (eight female and eight male). The dorsal skin of the mice was shaved with electric clippers 2 days before the start of topical treatments. The mice were initiated with 400 nmol DMBA in 200 μl acetone applied topically under yellow lighting on days 1 and 10, and control mice were treated with 200 μl acetone. All the mice remained in the dark for 24 h following DMBA or acetone vehicle treatment before being transferred to clean cages. One week following the final DMBA treatment (day 17) twice weekly topical treatment with 10 μg TPA in 200 μl acetone, or 200 μl acetone alone was commenced and continued until 30 weeks. Mice were observed every 2 weeks for skin papillomas >2 mm in height. Mice were housed 3–4/cage until papillomas formed at which time they were housed individually to avoid injury to newly formed papillomas. One week following the end of treatment mice were killed, dorsal skin with and without papillomas removed, fixed and stained for thioredoxin-1.

TPA-induced increase in thioredoxin-1

The effects of TPA treatment on skin thioredoxin-1 levels were examined. The dorsal skin of K14/(m)thioredoxin-1 mice and their non-transgenic littermates, 4 mice/group (two male and two female), was shaved 2 days before being treated topically with 200 μl acetone or 10 μg TPA in 200 μl acetone, twice a week for 3 weeks. Twenty-four hours after the last treatment the mice were killed and the skin from the treated area fixed and stained for thioredoxin-1.

Immunohistochemistry

Paraffin-embedded skin from the mouse studies or human archival non-sun-exposed post-auricular skin and sun-exposed pre-auricular skin from a subject who had undergone cosmetic surgery, was cut into 4 μm sections and mounted on glass slides. The sections were heated at 60°C for 30 min, re-hydrated through xylene and graded alcohols before being blocked for 30 min in 4% milk, 1% goat serum, 0.1% thimerosal in phosphate-buffered saline. Endogenous peroxidase activity was quenched using a hydrogen peroxide-based inhibitor (DAB Basic Detection Kit, Ventana Medical Systems, Tucson, AZ) and endogenous biotin blocked using an AB Blocking Kit (Ventana Medical Systems). The slides were incubated for 32 min at 42°C using an ES Automated Slide Stainer (Ventana Medical Systems) with mouse monoclonal anti-human thioredoxin-1 antibody 5A3G5 at a 1:1000 dilution (27) for human skin, or with a rabbit polyclonal anti-mouse thioredoxin-1 antibody developed in our laboratory at a 1:1000 dilution for mouse skin, or with proliferating cell nuclear antigen (PCNA) monoclonal antibody (Novocastro, Newcastle upon Tyne, UK) at a 1:200 dilution. A biotinylated universal secondary antibody, which recognizes mouse IgG/IgM or rabbit IgG was then applied, followed by horseradish peroxidase-conjugated avidin, DAB/hydrogen peroxide and a copper enhancer. The slides were dehydrated through graded alcohols, toluene and xylene and cover slipped using Vectamount (Vector Laboratories, Burlingame, CA). Six 24-bit three-color images of each tumor section were captured in RGB TIFF format using a Nikon TE300 inverted microscope with 10× plan apo objective lens using a Coolsnap digital camera (Princeton Instruments, Trenton, NJ). Images were quantified using the Simple PCI digital image analysis software package (Compix Imaging Systems, Pittsburgh, PA). The low threshold for analysis was set using a tissue section stained with a non-reactive antibody. Proliferation was measured as the number of PCNA stained cells per field.

Results

Thioredoxin-1 in human and mouse skin

Thioredoxin-1 was expressed at low levels in non-sun-exposed post-auricular human skin (Figure 1A). Sun-exposed pre-auricular human skin showed hyperkeratosis, elastosis and had increased expression of thioredoxin-1 in the keratinocyte layer (Figure 1B). The thioredoxin-1 staining was almost exclusively cytoplasmic with little or no nuclear staining. Skin from SKH-1 hairless mice exposed to UVB for 10 weeks showed hyperplasia and increased thioredoxin-1 staining in the keratinocyte layer (Figure 1D) compared with skin from non-irradiated SKH-1 mice (Figure 1C). The thioredoxin-1 staining was predominantly cytoplasmic with no nuclear staining in either the non-irradiated or the irradiated mouse skin.

K14/(m)thioredoxin-1 transgenic mouse

To investigate the *in vivo* role of thioredoxin-1 in skin carcinogenesis a K14/(m)thioredoxin-1 C57BL/6 transgenic mouse with mouse thioredoxin-1 expression under the control of the K14 promoter was developed. Quantitative RT–PCR showed a 2.1-fold increase in mTrx-1 mRNA in the skin of K14/(m)thioredoxin-1 transgenic mice skin compared with non-transgenic mice. Thioredoxin-1 staining was increased in the keratinocyte layer of the K14/(m)thioredoxin-1 mice compared with non-transgenic mice and was primarily cytoplasmic (compare Figure 1F and E). Quantification of thioredoxin-1 staining in skin showed that thioredoxin-1 levels were increased (±SE) 2.2 ± 0.1-fold ($P < 0.01$) in the K14/(m)thioredoxin-1 mice compared with the non-transgenic mice (Figure 2A). The skin of the K14/(m)thioredoxin-1 mice was macroscopically and microscopically normal and mice kept for 2 years did not spontaneously form papillomas. Papillomas formed as a result of treatment with DMBA and TPA showed high levels of thioredoxin-1 staining in both the non-transgenic and the K14/(m)thioredoxin-1 mice (Figure 1G and H).

Effect of TPA on thioredoxin-1 in K14/(m)thioredoxin-1 transgenic mice

TPA treatment has been shown previously to acutely increase thioredoxin-1 in mouse skin keratinocytes (35). Twice weekly treatment with TPA for 3 weeks increased thioredoxin-1

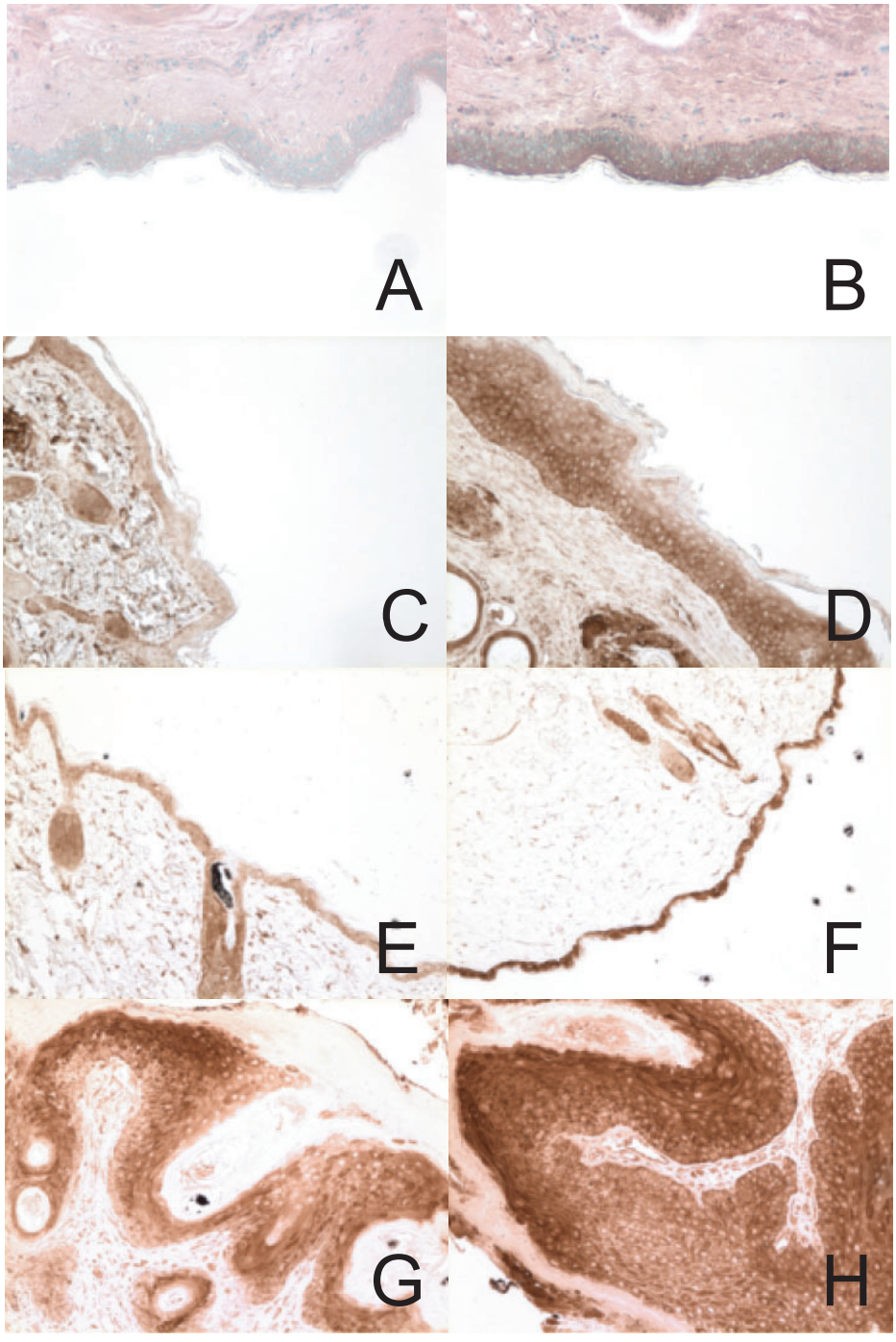

Fig. 1. Thioredoxin-1 expression in skin. Thioredoxin-1 was stained with antibodies that recognize either human thioredoxin-1 or mouse thioredoxin-1. (**A**) Human post-auricular non-sun-exposed skin showing thioredoxin-1 expression in the keratinocytes. (**B**) Human pre-auricular sun-exposed skin showing hyperkeratosis, elsastosis and increased thioredoxin-1 expression in the keratinocytes. (**C**) SKH-1 hairless mouse skin showing thioredoxin-1 expression in the keratinocytes. (**D**) Skin of a SKH-1 mouse exposed to UVB for 10 weeks showing hyperplasia and increased thioredoxin-1 expression. (**E**) C57BL/6 mouse skin from topical vehicle alone (acetone) treated non-transgenic mice showing thioredoxin-1 expression in the keratinocytes and the hair follicle. (**F**) Mouse skin from topical vehicle alone (acetone) treated K14/(m)thioredoxin mice showing increased thioredoxin-1 expression in the keratinocytes and the hair follicle. (**G**) Papilloma formed by topical DMBA/TPA treatment of non-transgenic mice showing thioredoxin-1 expressed throughout the papilloma. (**H**) Papilloma formed by topical DMBA/TPA treatment of K14/(m)thioredoxin mice also showing high levels of thioredoxin-1 expression.

staining in the keratinocyte layer of the skin of non-transgenic mice by 70% ($P < 0.01$) and K14/(m)thioredoxin-1 transgenic mice by 36.0% ($P < 0.01$) (Figure 2B). The subcellular location of thioredoxin-1 was not changed by TPA treatment in either the non-transgenic or K14/(m)thioredoxin-1 transgenic mice. The TPA treatment caused an increase in the number of PCNA-positive cells per field (±SE) from 52.0 ± 9.9 to 112.5 ± 10.7 ($P < 0.01$) in non-transgenic mouse skin and from 41.8 ± 4.9 to 97.7 ± 6.9 ($P < 0.01$) in K14/(m) thioredoxin-1 transgenic mouse skin.

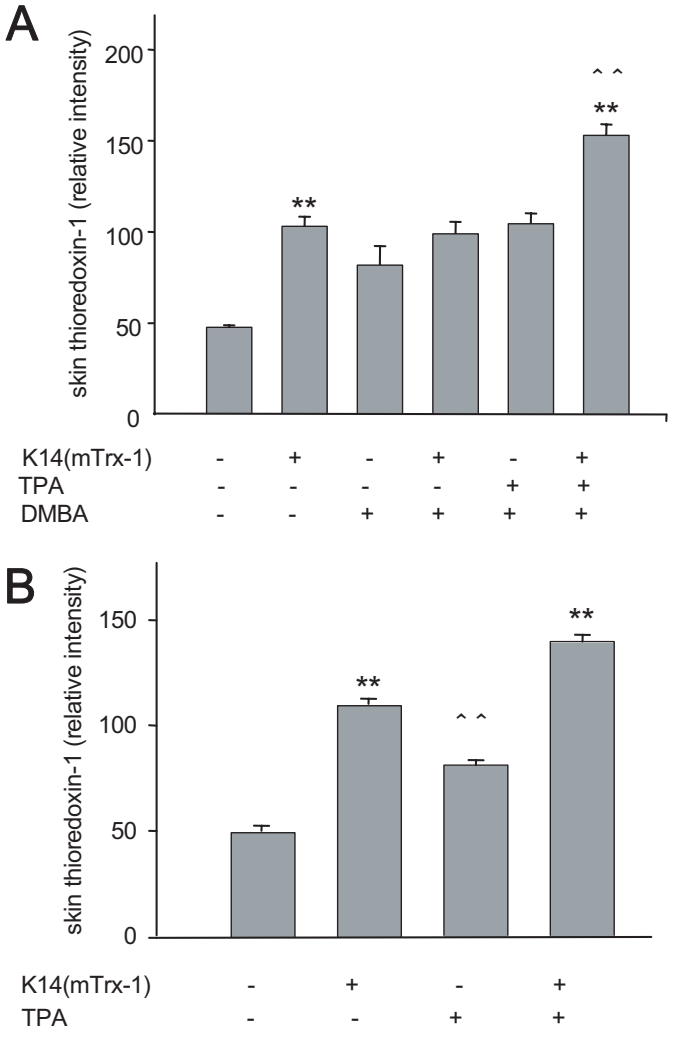

Fig. 2. Effect of DMBA and TPA treatment on thioredoxin-1 in skin of non-transgenic and K14/(m)thioredoxin-1 transgenic mice. Thioredoxin-1 was measured by quantitative immunohistochemistry. (**A**) Mice were treated topically with 200 μl acetone vehicle, with 400 nmol DMBA in 200 μl acetone on days 1 and 10, or with 400 nmol DMBA on days 1 and 10 and 10 μg TPA in 200 μl acetone twice a week until week 30. Values are the mean of eight mice per group and bars are SE. $^{**}P < 0.01$ for K14/(m)thioredoxin-1 transgenic mice compared with non-transgenic mice; $^{\wedge\wedge}P < 0.01$ for DMBA/TPA-treated K14/(m)thioredoxin-1 transgenic mice compared with vehicle alone treated non-transgenic mice. (**B**) Mice were treated topically with 200 μl acetone vehicle, or with 10 μg TPA in 200 μl acetone twice a week for 3 weeks. Values are the means of 4 mice/group and bars are SE. $^{**}P < 0.01$ for K14/(m)thioredoxin-1 transgenic mice compared with non-transgenic mice; $^{\wedge\wedge}P < 0.01$ for TPA treated non-transgenic compared with vehicle alone treated non-transgenic mice.

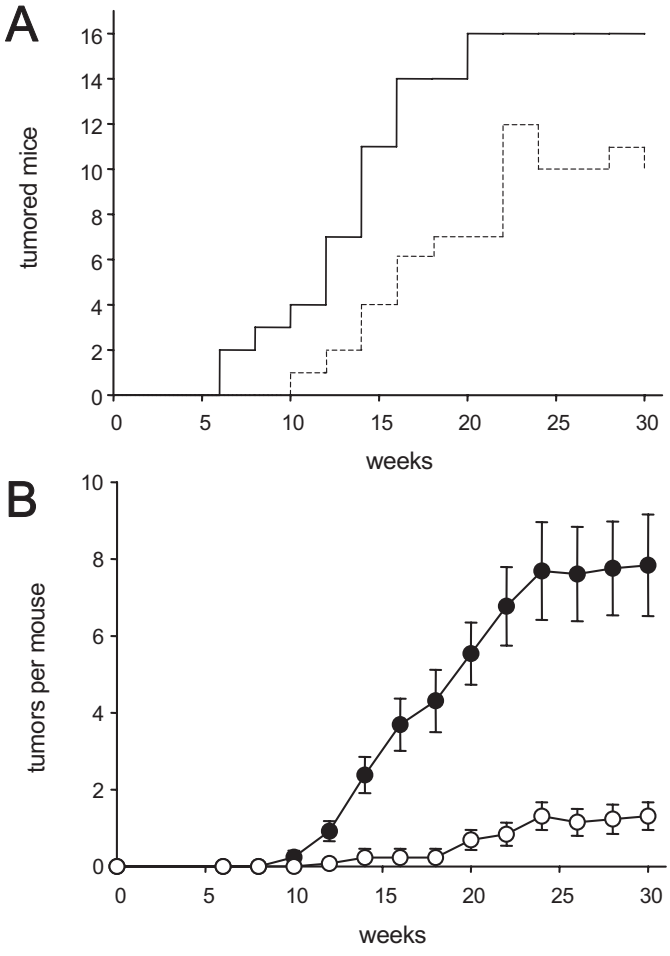

Fig. 3. Skin tumor formation by topical DMBA/TPA treatment in C57B/6 mice. Non-transgenic mice (○) and K14/(m)thioredoxin-1 transgenic mice (●) were initiated with 400 nmol DMBA applied topically to shaved skin on days 1 and 10. Twice weekly topical treatment with 10 μg TPA was commenced starting day 17 and continued until week 30. Mice were observed every two weeks for skin papillomas and papillomas >2 mm in height were recorded. (**A**) (- - -) Non-transgenic mice with tumors and (—) K14/(m)thioredoxin-1 transgenic mice with tumors. (**B**) Tumors per mouse. (○). Non-transgenic mice and (●) K14/(m)thioredoxin-1 transgenic mice. Values are the mean of eight mice per group and bars are SE.

Carcinogenesis in K14/(m)thioredoxin-1 transgenic mice

In order to determine if increased expression of thioredoxin-1 could act as a promoter, K14/(m)thioredoxin-1 transgenic mice and non-transgenic littermates were initiated with DMBA and then treated with acetone vehicle until week 30. At the end of the 30 weeks thioredoxin-1 staining in the skin of mice treated with DMBA alone had significantly increased by 1.7 ± 0.2 fold ($P < 0.01$) but did not further increase in the skin of K14/(m)thioredoxin-1 mice (Figure 2A). When DMBA treatment was followed by twice weekly TPA until week 30 there was a 2.2 ± 0.1-fold increase ($P < 0.01$) in thioredoxin-1 staining in the skin of the non-transgenic mice and a 1.5 ± 0.1 fold increase ($P < 0.01$) in the K14/(m)thioredoxin-1 transgenic mice. The cytoplasmic location of thioredoxin-1 was not changed by DMBA or TPA treatment, in either the non-transgenic or the K14/(m)thioredoxin-1 transgenic mice. The sex of the mice had no effect on the expression of thioredoxin-1 in the skin of either the non-transgenic or the K14/(m)thioredoxin-1 transgenic mice (data not shown).

None of the mice treated with DMBA alone developed papillomas irrespective of their phenotype indicating that increased keratinocyte thioredoxin-1, by itself, does not act as a tumor promoter in this model of carcinogenesis. When K14/(m)thioredoxin-1 transgenic mice were treated with DMBA followed by TPA the number of mice with papillomas was greater than for the non-transgenic mice (16 compared with 10 at the end of the study), and the papillomas first appeared at 6 weeks in the K14/(m)thioredoxin-1 transgenic mice compared with 10 weeks in the non-transgenic mice (Figure 3A). There was an increase in the multiplicity of papillomas in the K14/(m)thioredoxin-1 transgenic mice having reached (±SE) 7.8 ± 1.3/mouse at the end of the study

compared with 1.3 ± 0.4/mouse in the non-transgenic mice ($P < 0.01$) (Figure 3B). Some of the papillomas in K14/(m)thioredoxin-1 mice had progressed to sarcomas (~1/mouse) by the end of the study but there were no sarcomas in the non-transgenic mice. The sex of the mice had no effect on papilloma formation by either the non-transgenic or the K14/(m)thioredoxin-1 transgenic mice (data not shown). There were no significant differences in weight gain among the various treatment groups or between K14/(m)thioredoxin-1 positive mice and non-transgenic littermates (data not shown).

Discussion

Ultraviolet A and B radiation increase thioredoxin-1 expression in human keratinocytes and skin fibroblasts (28–31) and has been suggested to be a protective mechanism against oxidant-induced DNA damage (25). However, thioredoxin-1 transfected human skin fibroblasts also show decreased apoptosis in response to UVA radiation (30). Apoptosis is a process that allows the body to eliminate cells with damaged DNA so they will not develop into tumors (33). Resistance to apoptosis is a distinguishing feature of many forms of human cancer (36). Thioredoxin-1 is a potent inhibitor apoptosis (17,19) and human cancers with increased levels of thioredoxin-1 show decreased apoptosis (26). Thus, the elevation of thioredoxin-1 as in sun-damaged human skin might increase in the risk of developing skin cancer.

We tested the possibility that thioredoxin-1 might increase in the risk of developing skin cancer in the DMBA/TPA two-stage model of mouse skin carcinogenesis using C57Bl/6 mice, a strain that is relatively resistant to DMBA/TPA-induced skin carcinogenesis (37). Transgenic mice with thioredoxin-1 expressed in keratinocytes under the control of the K14 promoter showed an ~2-fold increase in thioredoxin-1 in the keratinocyte layer of skin compared with non-transgenic mice. The skin of the K14/(m)thioredoxin-1 transgenic mice appeared normal and when treated with DMBA alone did not develop papillomas showing that increased keratinocyte thioredoxin-1, by itself, is not a tumor promoter. However, when treated with DMBA followed by TPA the K14/(m)thioredoxin-1 transgenic mice showed a 6-fold increase in the number of papillomas per mouse compared with the non-transgenic mice. There was more rapid tumor progression in the K14/(m)thioredoxin-1 transgenic mice with papillomas appearing earlier, they were 3-fold larger and some progressed to carcinoma, which was not seen in the non-transgenic mice. Thus, skin tumor formation by DMBA is markedly increased in the K14/(m)thioredoxin-1 transgenic mice, but both the K14/(m)thioredoxin-1 transgenic mice and their non-transgenic littermates required TPA as promoter.

Topical TPA has been reported to increase the expression of thioredoxin-1 in the skin of mice, alone and after the topical application of DMBA (35). We confirmed this observation but found that the K14(m)thioredoxin-1 transgenic mice still had higher levels of keratinocyte thioredoxin-1 after TPA or DMBA/TPA treatment than the non-transgenic mice. Susceptibility to multistage carcinogenesis is determined primarily at the tumor promotion stage (38) and TPA is a relatively weak promoter in C57Bl/6 mice (39). Thus, the combined effects of increased thioredoxin-1 caused by topical TPA treatment and the increase in keratinocyte thioredoxin-1 in the skin of transgenic mice is most likely responsible for the increased tumor formation. TPA has been reported to cause the translocation of thioredoxin-1 from the cytoplasm to the nucleus in cultured cells (9). We observed primarily cytoplasmic thioredoxin-1 staining with very little nuclear thioredoxin-1 nuclear staining in the keratinocyte layer of the K14(m)thioredoxin-1 transgenic mice, or following TPA treatment of either transgenic or non-transgenic mouse skin. Thus, a difference in the subcellular localization of thioredoxin-1 by TPA or the K14 promoter in the transgenic mice does not appear to be the cause of the increased tumor formation in the K14(m)thioredoixn-1 transgenic mice.

There have been previous reports of thioredoxin-1 transgenic mice in which the mice show protection against acute oxidant stress. Transgenic mice with human thioredoxin-1 under the control of a β-actin promoter are functionally normal with no histological abnormalities (40). However, the thioredoxin-1 transgenic mice were protected against doxorubicin-induced cardiac damage and showed smaller infarct sizes after middle cerebral artery occlusion, both of which events are mediated by oxidant radical-induced tissue damage (41). Other studies showed that thioredoxin-1 transgenic mice are protected against hematotocity induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (42) and from retinal photoreceptor damage induced by intense light (43). Embryos from thioredoxin-1 transgenic mice are protected against oxidative DNA damage and developmental abnormalities induced by exposure to high oxygen concentrations (41). Thioredoxin-1 transgenic mice have also been reported to be more resistant than wild-type mice to influenza virus induced pneumonia suggesting a role for thioredoxin-1 in regulating the host inflammatory response (44) and impaired immunity during aging (45). There has been a report of the selective expression of human thioredoxin-1 under the control of a human insulin promoter in the β-islet cells of the pancreas that protected the mice against spontaneous diabetes and against streptozotocin-induced apoptosis, also an oxidant radical mediated event (46). Our finding of an increase in tumor formation in the skin of keratinocyte-directed K14(m)thioredoxin-1 transgenic mice is consistent with an acute protective effect of increased thioredoxin-1 against oxidant damage. However, the decrease in apoptosis caused by increased thioredoxin-1 is probably responsible for the increased tumor formation in the skin of the K14(m)thioredoxin-1 transgenic mice. The finding is important because of the increase in thioredoxin-1 in the skin of sun-exposed individuals.

It is noteworthy that two other antioxidant enzymes, intracellular glutathione peroxidase and Cu/Zn superoxide dismutase, that are also thought to protect skin cells against oxidant damage (32), when over-expressed either singly or together in C57BL/6 transgenic mice, increase tumor formation in the DMBA/TPA two-stage model of skin carcinogenesis (47). Both glutathione peroxidase (48) and superoxide dismutase (49) protect cells against oxidant-induced apoptosis. Thus, an effect of antioxidant enzymes such as thioredoxin-1, glutathione peroxidase and superoxide dismutase in preventing apoptosis may be as important for carcinogenesis as their antioxidant effects. Thioredoxin-1 is the antioxidant enzyme whose increased levels in human tumors (20–24,26) have been linked to aggressive tumor proliferation, decreased apoptosis (26) and to decreased patient survival (22,27).

In summary, the work shows that in transgenic mice with mouse thioredoxin-1 under the control of the K14 promoter, thioredoxin-1 is over-expressed in the keratinocyte layer of the skin. The skin of the thioredoxin-1 transgenic mice appears

normal but there is a marked increase in the formation of papillomas in the two-stage model of carcinogenesis using DMBA and TPA. Thioredoxin-1 appears to act as an enhancer of carcinogenesis that requires initiation by DMBA and promotion by TPA.

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References

1. Powis,G. and Montfort,WR. (2001) Properties and biological activities of thioredoxins. *Ann. Rev. Pharmacol. Toxicol*., **41**, 261–295.
2. Arner,E.S. and Holmgren,A. (2000) Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem*., **267**, 6102–6109.
3. Hirota,K., Nakamura,H., Masutani,H. and Yodoi,J. (2002) Thioredoxin superfamily and thioredoxin-inducing agents. *Ann. N. Y. Acad. Sci. USA*, **957**, 199.
4. Watson,W.H., Chen,Y. and Jones,D.P. (2003) Redox state of glutathione and thioredoxin in differentiation and apoptosis. *BioFactors*, **17**, 307–314.
5. Mustacich,D. and Powis,G. (2000) Thioredoxin reductase. *Biochem. J*., **346**, 1–8.
6. Matthews,J.R., Wakasugi,N., Virelizier,J.-L., Yodoi,J. and Hay,R.T. (1992) Thioredoxin regulates the DNA binding activity of NF-kappaB by reduction of a disulphide bond involving cysteine 62. *Nucleic Acids Res*., **20**, 3821–3830.
7. Gasdaska,P.Y., Oblong,J.E., Cotgreave,I.A. and Powis,G. (1994) The predicted amino acid sequence of human thioredoxin is identical to that of the autocrine growth factor human adult T-cell derived factor (ADF): thioredoxin mRNA is elevated in some human tumors. *Biochim. Biophys. Acta*, **1218**, 292–296.
8. Grippo,J.F., Tienrungroj,W., Dahmer,M.K., Housley,P.R. and Pratt,W.B. (1983) Evidence that the endogenous heat-stable glucocorticoid receptor-activating factor is thioredoxin. *J. Biol. Chem.*, **258**, 13658–13664.
9. Hirota,K., Matsui,M., Iwata,S., Nishiyama,A., Mori,K. and Yodoi,J. (1997) AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc. Natl Acad. Sci. USA*, **94**, 3633–3638.
10. Ueno,M., Masutani,H., Jun Arai,R., Yamauchi,A., Hirota,K., Sakai,T., Inamoto,T., Yamaoka,Y., Yodoi,J. and Nikaido,T. (2000) Thioredoxin-dependent redox regulation of p53-mediated p21 activation. *J. Biol. Chem*., **274**, 35809–35815.
11. Welsh,S.J., Bellamy,W.T., Briehl,M.M. and Powis,G. (2002) The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1alpha protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. *Cancer Res*., **62**, 5089–5095.
12. Abate,C., Patel,L., Rauscher III,F.J. and Curran,T. (1990) Redox regulation of fos and jun DNA-binding activity *in vitro*. *Science*, **249**, 1157–1161.
13. Saitoh,M., Nishitoh,H., Fujii,M., Takeda,K., Tobiume,K., Sawada,Y., Kawabata,M., Miyazono,K. and Ichijo,H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J*., **17**, 2596–2606.
14. Watson,J.A., Rumsby,M.G. and Wolowacz,R.G. (1999) Phage display identifies thioredoxin and superoxide dismutase as novel protein kinase C-interacting proteins: thioredoxin inhibits protein kinase C-mediated phosphorylation of histone. *Biochem. J*., **343**, 301–305.
15. Meuillet,E., Mahadevan,D., Berggren,M., Coon,A. and Powis,G. (2004) Thioredoxin-1 binds to the C2 domain of PTEN inhibiting PTEN's lipid phosphatase activity and membrane binding: a mechanism for the functional loss of PTEN's tumor suppressor activity. *Arch. Biocem. Biophys*., (in press).
16. Chae,H.Z., Kim,H.J., Kang,S.W. and Rhee,S.G. (1999) Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. *Diabetes Res. Clin. Pract*., **45**, 101–112.
17. Baker,A., Payne,C.M., Briehl,M.M. and Powis,G. (1997) Thioredoxin, a gene found overexpressed in human cancer, inhibitis apoptosis *in vitro* and *in vivo*. *Cancer Res*., **57**, 5162–5167.
18. Freemerman,A.J. and Powis,G. (2000) A redox-inactive thioredoxin reduces growth and enhances apoptosis in WEH17.2 cells. *Biochem. Biophys. Res. Commun*., **274**, 136–141.
19. Gallegos,A., Gasdaska,J.R., Taylor,C.W., Paine-Murrieta,G.D., Goodman,D., Gasdaska,P.Y., Berggren,M., Briehl,M.M. and Powis,G. (1996) Transfection with human thioredoxin increases cell proliferation and a dominant negative mutant thioredoxin reverses the transformed phenotype of breast cancer cells. *Cancer Res*., **56**, 5765–5770.
20. Fujii,S., Nanbu,Y., Nonogaki,H., Konishi,I., Mori,T., Masutani,H. and Yodoi,J. (1991) Coexpression of adult T-cell leukemia-derived factor, a human thioredoxin homologue, and human papillomavirus DNA in neoplastic cervical squamous epithelium. *Cancer*, **68**, 1583–1591.
21. Soini,Y., Kahlos,K., Napankangas,U., Kaarteenaho-Wiik,R., Saily,M., Koistinen,P., Paaakia,P., Holmgren,A. and Kinnula,V.L. (2001) Widespread expression of thioredoxin and thioredoxin reductase in non-small cell lung carcinoma. *Clin. Cancer Res*., **7**, 1750–1757.
22. Kakolyris,S., Giatromanolaki,A., Koukourakis,M., Powis,G., Souglakos,J., Sivridis,E., Gatter,K.C. and Harris,A.L. (2001) Thioredoxin expression is associated with lymph node status and prognosis in early operable non small cell lung cancer (NSCLC). *Clin. Cancer Res*., **7**, 3087–3091.
23. Kawahara,N., Tanaka,T., Yokomizo,A., Nanri,H., Ono,M., Wada,M., Kohno,K., Takenaka,K., Sugimachi,K. and Kuwano,M. (1996) Enhanced coexpression of thioredoxin and high mobility group protein 1 genes in human hepatocellular carcinoma and the possible association with decreased sensitivity to cisplatin. *Cancer Res*., **56**, 5330–5333.
24. Nakamura,H., Masutani,H., Tagaya,Y., Yamauchi,A., Inamoto,T., Nanbu,Y., Fujii,S., Ozawa,K. and Yodoi,J. (1992) Expression and growth-promoting effect of adult T-cell leukemia-derived factor. A human thioredoxin homologue in hepatocellular carcinoma. *Cancer*, **69**, 2091–2097.
25. Wakita,H., Yodoi,J., Masutani,H., Toda,K. and Takigawa,M. (1992) Immunohistochemical distribution of adult T-cell leukemia-derived factor/thioredoxin in epithelial components of normal and pathologic human skin conditions. *J. Invest. Dermatol*., **99**, 101–107.
26. Grogan,T.M., Fenoglio-Prieser,C., Zeheb,R. *et al*. (2000) Thioredoxin, a puntative oncogene product, is overexpressed in gastric carcinoma and associated with increased proliferation and increased cell survival. *Hum. Pathol*., **31**, 475–481.
27. Raffel,J., Bhattacharyya,A.K., Gallegos,A., Cui,H., Einspahr,J.G., Alberts,D.S. and Powis,G. (2002) Increased expression of thioredoxin-1 in human colorectal cancer is associated with decreased patient survival. *J. Lab. Clin. Med*., **142**, 46–51.
28. Sachi,Y., Hirota,K., Masutani,H., Toda,K., Takahashi,O., Takigawa,M. and Yodoi,J. (1995) Induction of ADF/TRX by oxidative stress in keratinocytes and lymphoid cells. *Immunol. Lett*., **44**, 189–193.
29. Funasaka,Y. and Ichihashi,M. (1997) The effect of ultraviolet B induced adult cell leukemia-derived factor/thioredoxin (ADF/TRXD) on survival and growth of human melanocytes. *Pigment Cell Res*., **10**, 68–73.
30. Didier,C., Kerblat,I., Drouet,C., Favier,A., Beani,J.C. and Richard,M.J. (2001) Induction of thioredoxin by ultraviolet-A radiation prevents oxidative-mediated cell death in human skin fibroblasts. *Free Rad. Biol. Med*., **31**, 585–598.
31. Didier,C., Pouget,J.P., Cadet,J., Favier,A., Beani,J.C. and Richard,M. (2001) Modulation of exogenous and endogenous levels of thioredoxin in human skin fibroblasts prevents DNA damaging effect of ultraviolet A radiation. *Free Rad. Biol. Med*., **30**, 537–546.
32. Trouba,K.J., Hamadeh,H.K., Amin,R.P. and Germolec,D.R. (2002) Oxidative stress and its role in skin disease. *Antiox. Redox Sign*., **4**, 665–673.
33. Raff,M.C. (1992) Social controls on cell survival and cell death. *Nature*, **356**, 397–400.
34. McGargill,MA., Mayerova,D., Stefanski,HE., Koehn,B., Parke,EA., Jameson,SC., Panoskaltsis-Mortari,A. and Hogquist,KA. (2002) A spontaneous CD8 T cell-dependent autoimmune disease to an antigen expressed under the human keratin 14 promoter. *J Immunol*., **169**, 2141–2147.
35. Kumar,S. and Holmgren,A. (1999) Induction of thioredoxin, thioredoxin reductase and glutaredoxin activity in mouse skin by TPA, a calcium ionophore and other tumor promoters. *Carcinogenesis*, **20**, 1761–1767.
36. Hanahan,D. and Weinberg,R.A. (2000) The hallmarks of cancer. *Cell*, **100**, 57–70.
37. DiGiovanni,J., Prichett,W.P., Decina,P.C. and Diamond,L. (1984) DBA/2 mice are as sensitive as SENCAR mice to skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Carcinogenesis*, **5**, 1493–1498.
38. Naito,M., Chenicek,K.J., Naito,Y. and DiGiovanni,J. (1988) Susceptibility to phorbol ester skin tumor promotion in (C57BL/6 × DBA/2)F1 mice is inherited as an incomplete dominant trait: evidence for multi-locus involvement. *Carcinogenesis*, **9**, 639–645.

39. Slaga,T.J. (1986) SENCAR mouse skin tumorigenesis model versus other strains and stocks of mice. *Environ. Health Perspect.*, **68**, 27–32.
40. Takagi,Y., Mitsui,A., Nishiyama,A., Nozaki,K., Sono,H., Gon,Y., Hashimoto,N. and Yodoi,J. (1999) Overexpression of thioredoxin in transgenic mice attenuates focal ischemic brain damage. *Proc. Natl Acad. Sci. USA*, **96**, 4131–4136.
41. Shioji,K., Kishimoto,C., Nakamura,H., Masutani,H., Yuan,Z., Oka,S. and Yodoi,J. (2002) Overexpression of thioredoxin-1 in transgenic mice attenuates adriamycin-induced cardiotoxicity. *Circulation*, **106**, 1403–1409.
42. Yoon,B.I., Hirabayashi,Y., Kaneko,T., Kodama,Y., Kim,D.Y. and Inoue,T. (2001) Transgene expression of thioredoxin (TRX/ADF) protects against 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced hematotoxicity. *Arch. Environ. Contamin. Toxi.*, **41**, 232–236.
43. Tanito,M., Masutani,H., Nakamura,H., Oka,S., Ohira,A. and Yodoi,J. (2002) Attenuation of retinal photooxidative damage in thioredoxin transgenic mice. *Neurosci. Lett.*, **326**, 142–146.
44. Nakamura,H., Tamura,S., Watanabe,I., Iwasaki,T. and Yodoi,J. (2002) Enhanced resistancy of thioredoxin-transgenic mice against influenza virus-induced pneumonia. *Immunol. Lett.*, **82**, 165–170.
45. Murata,Y., Amao,M., Yoneda,J. and Hamuro,J. (2002) Intracellular thiol redox status of macrophages directs the Th1 skewing in thioredoxin transgenic mice during aging. *Mol. Immunol.*, **38**, 747–757.
46. Hotta,M., Tashiro,F., Ikegami,H., Niwa,H., Ogihara,T., Yodoi,J. and Miyazaki,J. (1998) Pancreatic beta cell-specific expression of thioredoxin, an antioxidative and antiapoptotic protein, prevents autoimmune and streptozotocin-induced diabetes. *J. Exp. Med.*, **188**, 1445–1451.
47. Lu,Y.P., Lou,Y.R., Yen,P., Newmark,H.L., Mirochnitchenko,O.I., Inouye,M. and Huang,MT. (1997) Enhanced skin carcinogenesis in transgenic mice with high expression of glutathione peroxidase or both glutathione peroxidase and superoxide dismutase. *Cancer Res.*, **57**, 1468–1474.
48. Crack,P.J., Taylor,J.M., Flentjar,N.J., de Haan,J., Hertzog,P., Iannello,R.C. and Kola,I. (2001) Increased infarct size and exacerbated apoptosis in the glutathione peroxidase-1 (Gpx-1) knockout mouse brain in response to ischemia/reperfusion injury. *J. Neurochem.*, **78**, 1389–1399.
49. Lee,M., Hyun,D.H., Halliwell,B. and Jenner,P. (2001) Effect of overexpression of wild-type and mutant Cu/Zn-superoxide dismutases on oxidative stress and cell death induced by hydrogen peroxide, 4-hydroxynonenal or serum deprivation: potentiation of injury by ALS-related mutant superoxide dismutases and protection by Bcl-2. *J. Neurochem.*, **78**, 209–220.

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