Toxicol. Res.
Vol. 25, No. 4, pp. 189-193 (2009)

Official Journal of Korean Society of Toxicology

Available online at http://www.toxmut.or.kr

Establishment of a Stable Cell Line Expressing Green Fluorescence Protein-fused Hypoxia Inducible Factor-1α for Assessment of Carcinogenicity of Chemical Toxicants

Sung-Hye Kim[1,2], Hee-won Seo[1,2], Min-Ho Lee[1,2], Jin-Ho Chung[1], Byung Hoon Lee[1] and Mi-Ock Lee[1,2]

[1]College of Pharmacy, and [2]Bio-MAX Institute, Seoul National University, Seoul 151-742, Korea

(Received September 1, 2009; Revised November 13, 2009; Accepted November 14, 2009)

Hypoxia inducible factor 1α (HIF-1α) is a potential marker of carcicnogenesis since it is overexpresssed in many human cancers such as brain, breast, and uterus, and its role has implicated in tumor cell growth and metastasis. In this study, we established a stable cell line that express green fluorescence protein (GFP)-fused hypoxia inducible factor 1α (HIF-1α) and evaluated the potential use of this cell line for assessment of carcinogenicity of chemical toxicants. Western blot analysis as well as fluorescence measurements showed that protein-level of GFP-HIF-1α was significantly enhanced in a dose-dependent manner upon treatment of hypoxia mimicking agents such as dexferrioxamine and $CoCl_2$. Well-Known tumor promoters such as mitomycin and methyl methanesulfonate. significantly induced the fluorescence intensity of GFP-HIF-1α, whereas the known negative controls such as *o*-anthranilic acid and benzethonium chloride, did not. These results indicate that HIF-1α could be a biological parameter for detection of tumor initiators/promoters and suggest that the GFP-HIF-1α cell line is a useful system for screening of carcinogenic toxicants.

Key words: GFP-HIF-1α stable cell line, Chemical toxicants, Carcinogenicity

INTRODUCTION

Carcinogenesis is typically a multistage nature consisting of initiation, promotion, and progression. Initiation stage is thought to induce mutations in the critical genes which confer a selective growth advantage to specific cells. Promotion stage induces the tumor formation and a potential progression to malignancy by occurring the additional genetics or epigenetic changes (Trosko, 2001; Humble *et al.*, 2005). Though many *in vitro* assays have been developed with aim of detecting the carcinogenesis of chemical compounds, few of that satisfied in model for carcinogenesis testing.

Hypoxia inducible factor-1 (HIF-1) plays an essential role in cellular and systemic oxygen homeostasis. HIF-1 is a key transcriptional activator that is induced in hypoxia condition and regulates cell adaptation and survival to hypoxia. HIF-1 is a heterodimer that consists of a HIF-1α subunit and a constitutively expressed HIF-1β subunit (Machon *et al.*, 2001; Semenza, 2003). In normal condition, HIF-1α is rapidly degraded through the ubiquitin-proteasome pathway, while it is stabilized under hypoxia condition (Huang *et al.*, 1998). More than 60 putative direct HIF-1 target genes have been identified which are particularly involved in crucial aspects of cancer biology, including cancer angiogenesis, glucose metabolism, cell proliferation/survival and invasion/metastasis (Semenza 2003; Lee *et al.*, 2004). Recently, many studies revealed that HIF-1α is overexpresssed in many human cancers such as brain, breast, lung, cervix, ovary and uterus (Zagzag *et al.*, 2000; Biner *et al.*, 2001; Schindl *et al.*, 2002; Burri *et al.*, 2003; Swinson *et al.*, 2004). The association between HIF-1 and cancer was observed among tumors from most of patients (Zhong *et al.*, 1999). Together, these results indicate that HIF-1α is a potential marker of carcicnogenesis.

Previously we have developed a stable cell line of which chromosome containing a reporter encoding hypoxia response element that express luciferase in response to transcriptional activation of HIF-1. The benefit of this system included a high sensitivity and a good correlation with *in vivo* test (Hong *et al.*, 2007). In the present investigation, we established a new stable cell line that express green fluorescence protein (GFP)-

Correspondence to: Mi-Ock Lee, College of Pharmacy and Bio-MAX Institute, Seoul National University, San 56-1 Sillim-dong, Gwanak-gu, Seoul 151-742, Korea
E-mail: molee@snu.ac.kr

fused HIF-1α, to make an advance in the HIF-1 monitoring system in the aspects of handling process and experimental cost. The newly developed method could be easily applicable to an alternative screening method for tumor promoters, since this method requires only a simple process of fluorescence measurements.

MATERIALS AND METHODS

Cells and chemicals. The human cervical carcinoma cell line, HeLa (ATCC CCL-2) was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified eagle's medium containing 10% fetal bovine serum at 37°C in a 5% CO_2/95% air incubator. Hypoxia was induced chemically by treating cells with $CoCl_2$ (Sigma, St. Louis, MO) or dexferrioxamine (DFO) (Calbiochem, San Diego, CA). *o*-Anthranilic acid, benzethonium chloride, mitomycin (MMC), methyl methanesulfonate (MMS), methoxychlor, N-(1-naphthyl) ethylenediamine dichloride, griseofulvin and thioacetamide were purchased from Sigma.

Establishment of the GFP-HIF-1α stable cell line. HeLa cells were seeded in 60-cm^2 dishes (1×10^5 cells/dish) and incubated overnight. The cells were transfected with a 0.1 μg GFP-HIF-1α expression vector and pcDNA3.1/neo which is neomycin resistant gene using LipofectaminePlus (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Cells were cultured for 6 weeks in the presence of 800 μg/ml G418 and survival colonies are selected and maintained in media containing 600 μg/ml G418.

Fluorescence measurements. HeLa cells were seeded in 12-well plates (1×10^4 cells/plate) or 24-well plates (1×10^5 cells/plate) and incubated overnight. At 24 h after the treatment with DFO, $CoCl_2$, and other chemicals known as tumor promoters, fluorescence was detected by fluorescence microscopy.

Quantitative analysis for fluorescence of GFP-HIF-1α. HeLa cells were seeded in black 96-well plates (6×10^3 cells/plate) and incubated overnight. At 24 h after treatment of chemicals, the media were changed to phosphate buffered saline. Fluorescence intensity of plates was measured using Softmax software of High Throughput Screening (BiomekFX, Backman Coulter) with excitation at 488 nm and emission of 535 nm using bottom read. The quantitative data were shown as mean value of 10 replicates. MMC, MMS, griseofulvin and thioacetamide are known tumor iniciator/promoters whereas *o*-anthranilic acid, benzethonium chloride, methoxychlor and N-(1-naphthyl) ethylenediamine dichloride are negative controls (Haseman *et al.*, 1987; Spalding *et al.*, 1999).

Western blotting. Western blotting was performed as previously described using specific antibodies against GFP (Santa Cruz Biotechnology) and α-tubulin (Calbiochem) (Yoo *et al.*, 2004).

Statistical analysis. Experimental values were expressed as mean ± SEM. The significance of differences were determined by student's *t*-test and expressed as a probability value. Mean differences were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

Many *in vivo* and *in vitro* assays have been developed for detection and characterization of chemical toxicants and for detection and prevention of human cancer (Tennant *et al.*, 1987, 1988). Among these assays, in vitro transformation assay is not only regarded as a major method of the screen of potential carcinogens promoters, but is also an important approach for the study multistage carcinogenesis (Berwald and Sachs, 1963; Breheny *et al.*, 2005). However, this assay has not been exploited, since it requires a long period of cultivation, low transformation frequency, high cost, and high effort (Grover *et al.*, 1971; Fang *et al.*, 2001). Though this method was recently modified to make it more convenient, low transformation frequency is still not suitable for performing studies (Tsuchiya and Umeda, 1997; Custer *et al.*, 2000). We have concentrated efforts on development of *in vitro* system for rapid detection of carcinogenic agents. We have previously developed a stable cell line of which chromosome containing a reporter encoding hypoxia response element that express luciferase in response to transcriptional activation of HIF-1 (Hong *et al.*, 2007). The benefit of this system included sensitivity and a good correlation with the results obtained from *in vivo* test (Hong *et al.*, 2007). Here, we established a new stable cell line expressing GFP-HIF-1α, to make an advance in the HIF-1 monitoring system in the aspects of handling process and experimental cost.

To establish the stable cell line expressing GFP-fused HIF-1α, the human cervical carcinoma cell line, HeLa cells were co-transfected with a plasmid encoding GFP-HIF-1α and a vector encoding neomycin resistant gene, and cultured for 6 weeks in the presence of G418. Four survival colonies were obtained and one which responded sensitively to DFO was selected for further

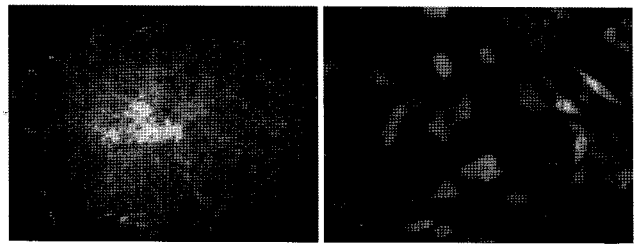

Fig. 1. Establishment of a stable cell line expressing GFP-HIF-1α. HeLa cells were transfected with a 0.1 μg GFP-HIF-1α expression vector and pcDNA3.1/neo which is neomycin resistant gene using LipofectaminePlus according to the manufacturer's instructions. Cells were cultured for 6 weeks in the presence of 800 μg/ml G418 and survival colonies are selected and maintained in media containing 600 μg/ml G418. Representative images for a colony formed (left, × 40) and stable cells expressing GFP-HIF-1α (right, × 400) taken by fluorescence microscopy were shown.

study (data not shown). Fig. 1 shows the colony formed and the established cell line that expressed GFP-HIF-1α protein. The response to hypoxia was examined after treatment of DFO or $CoCl_2$. After 24 h of treatment, the fluorescence of GFP-HIF-1α was significantly increased in a dose-dependent manner (Fig. 2), indicating that HIF-1α protein was stabilized in the presence of hypoxic stress. To confirm the increased expression of GFP-HIF-1. protein, we analyzed protein-level of GFP-HIF-1α by western blot analysis. As shown in Fig. 3A, the amount of GFP-HIF-1α protein was increased in a dose-dependent manner. To quantitate fluorescence of GFP-HIF-1α, the stable cells were seeded in 96-wll plate, treated with DFO or $CoCl_2$ and the fluorescence of the cells were directly measured using Softmax software of high throughput screening. Consistent with the western blotting results, fluorescence of the cells was increased dose-dependently (Fig. 3B). These results indicate that a stable cell line which expressed GFP-HIF-1α was successfully established.

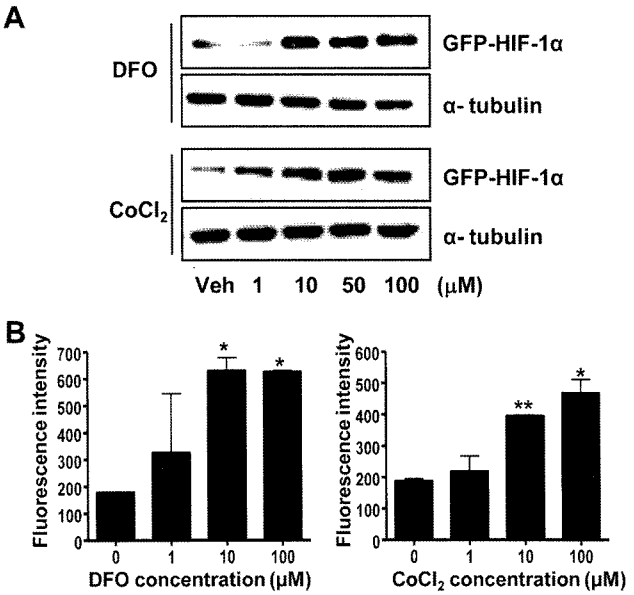

Fig. 3. Induction of GFP-HIF-1α protein after treatment with hypoxia mimicking chemicals in the GFP-HIF-1α stable cell line. (A) The GFP-HIF-1α stable cells were seeded in 60-mm^2 dishes (4×10^5 cells/dish) and incubated overnight. At 24 h after the treatment with indicated concentrations of DFO and $CoCl_2$, whole-cell lysates were prepared and the expression of proteins was analyzed using anti-GFP antibody by western blotting. (B) GFP-HIF-1α stable cells were seeded in the block 96-well plates (6×10^3 cells/plate) and incubated overnight. At 24 h after the treatment, fluorescence was measured using Softmax software of HTS. Each bar represents the mean ± SEM of at least three experiments. *$p < 0.05$ and **$p < 0.01$.

Next, we studied whether our stable cell line could serve as a useful system for assessment of carcinogenicity of chemical toxicants. We examined the expression of GFP-HIF-1α after treatment of four known carcinogens, *i.e.*, MMC, MMS, griseofulvin and thiaceta-

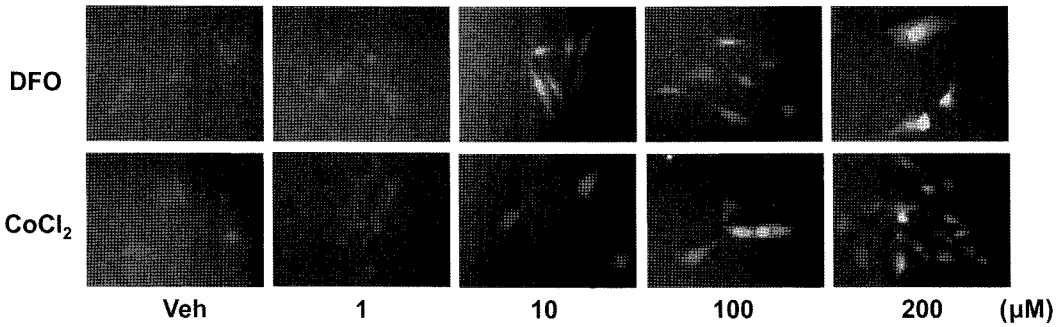

Fig. 2. Fluorescence of the GFP-HIF-1α stable cells induced by hypoxia mimicking agents. The GFP-HIF-1α stable cells were seeded in 24-well plates (1×10^5 cells/plate) and incubated overnight. At 24 hours after the treatment with indicated concentrations of DFO (A) and $CoCl_2$ (B), expression of GFP-HIF-1α was detected using fluorescence microscopy (× 400). Representative images obtained from at least three experiments were shown.

mide. MMC is a well-known mutagenic and clastogenic agent which induces base substitution, sister chromatid exchange, chromosomal aberration, and micronuclei formation *in vivo* and *in vitro* (Concepcion Becerril *et al.*, 1999; Tomasz *et al.*, 1974). MMS is a highly toxic DNA-alkylating agent, which methylates the DNA bases and causes DNA damage leading to strand breaks, chromosome breaks, micronucli formation, and cell death (Wyatt *et al.*, 2006; Lee *et al.*, 2007). Griseofulvin is a mycotoxin produced by various species of *Penicillium* and it has been used as a potent antifungal agent against dermatophyroses. Griseofulvin promoted skin tumor development in mice when given orally after methylcholanthrene initiation and affected a promotion stage of carcinogenesis (Vesselinovitch and Mihailovich, 1968; Labay *et al.*, 2001). Thioacetamide, originally used as a fungicide, is a potent hepatotoxin. Thioacetamide stimulates DNA synthesis and mitosis in the liver of rats at doses that produce limited necrosis (Mangipudy *et al.*, 1996, Chilakapati *et al.*, 2005). After 24 h of treatment with these toxicants, the fluorescence of GFP-HIF-1α was significantly increased at the dose ranges that are employed for carcinogenicity tests. The expression level of GFP-HIF-1α was enhanced 5 to 6 times in the presence of these toxicants (Fig. 4). In contrast, after 24 h of treatment with four known negative controls, the fluorescence of GFP-HIF-1α did not increased. These results demonstrate a good predictivity for carcinogeneicity and validate our GFP-HIF-1α stable cell line as a potential screening method. Since the newly developed GFP-HIF-1α stable cell line system could provide significant improvement in handling process and experimental costs, it is easily applicable to an alternative screening method for carcinogenic toxicants.

ACKNOWLEDGMENTS

This study was supported by grants from the Korea Food and Drug Administration for Studies on Standardization of Herbal Medicine (2009).

REFERENCES

Becerril, C., Ferrero, M., Sanz, F. and Castano, A. (1999). Detection of mitomycin C-induced genetic damage in fish cells by use of RAPD. *Mutagenesis*, **14**, 449-456.

Berwald, Y. and Sachs, L. (1963). *In vitro* cell transformation with chemical carcinogens. *Nature*, **200**, 1182-1184.

Biner, P., Schindl, M., Obermair, A., Breitenecker, G. and Oberhuber, G. (2001). Expression of hypoxia-inducible factor 1alpha in epithelial ovarian tumors: its impact on prognosis and on response to chemotherapy. *Clin. Cancer Res.*, **7**, 1661-1668.

Breheny, D., Zhang, J. and Massey, E.D. (2005). Application of a two-stage Syrian hamster embryo cell transformation assay to cigarette smoke particulate matter. *Mutat. Res.*, **572**, 45-57.

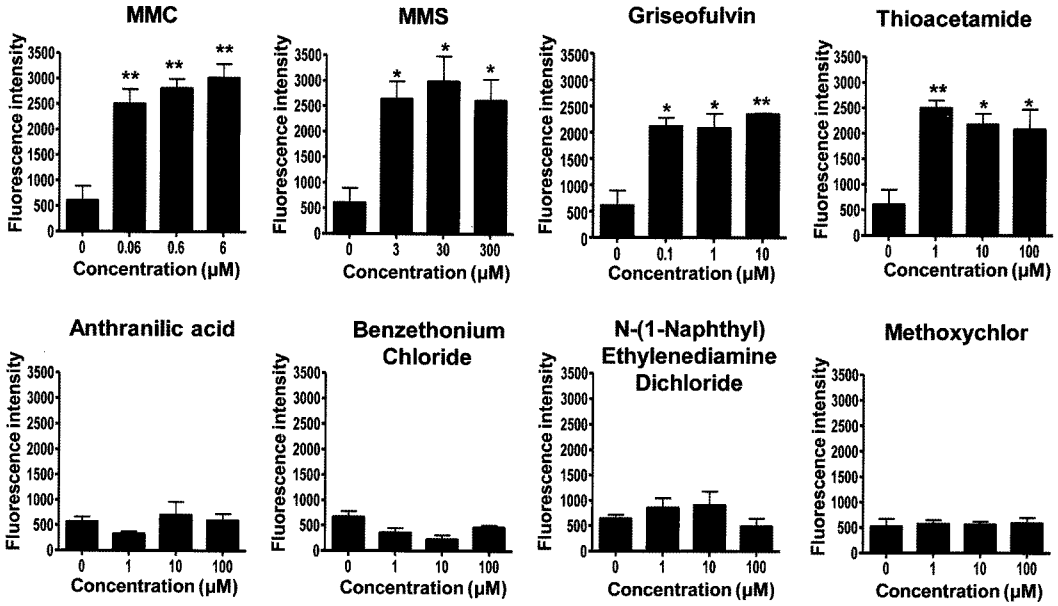

Fig. 4. Quantitative analysis of expression of GFP-HIF-1α after treatment with chemical toxicants. GFP-HIF-1α stable cells were seeded in the block 96-well plates (6 × 10 cells/plate) and incubated overnight. At 24 h after treatment of the indicated chemicals, fluorescence was measured using Softmax software of HTS. Each bar represents the mean ± SEM of at least three experiments. *$p < 0.05$ and **$p < 0.01$.

Burri, P., Djonov, V., Aebersold, D.M., Lindel, K., Studer, U., Altermatt, H.J., Mazzucchelli, L., Greiner, R.H. and Gruber, G. (2003). Significant correlation of hypoxia-inducible factor-1alpha with treatment outcome in cervical cancer treated with radical radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.*, **56**, 494-501.

Chilakapati, J., Shankar, K., Korrapati, M.C., Hill, R.A. and Mehendale, H.M. (2005). Saturation toxicokinetics of thioacetamide: role in initiation of liver injury. *Drug Metabo. Dispos.*, **33**, 1877-1885.

Custer, L., Gibson, D.P., Aardema, M.J. and LeBoeuf, R.A. (2000). A refined protocol for conducting the low pH 6.7 Syrian hamster embryo (SHE) cell transformation assay. *Mutat. Res.*, **455**, 129-139.

Fang, M.Z., Kim, D.Y., Lee, H.W. and Cho, M.H. (2001). Improvement of in vitro two-stage transformation assay and determination of the promotional effect of cadmium. *Toxicol. In Vitro*, **15**, 225-231.

Grover, P.L., Sins, P., Huberman, E., Marquardt, H., Kuroki, T. and Heidelberger, C. (1971). *In vitro* transformation of rodent cells by k-region derivatives of polycyclic hydrocarbons. *Proc. Nat. Acad. Sci. USA*, **68**, 1098-1101.

Haseman, J.K., Huff, J.E., Zeiger, E. and McConnell, E.E. (1987). Comparative results of 327 chemical carcinogenicity studies. *Environ. Health Perspect.*, **74**, 229-235.

Hong, I., Seo, H.W., Lee, M.H., Kim, J.W., Chung, J.H., Lee, B.H. and Lee, M.O. (2007). Establishment of reporter cell lines that monitor activities of hypoxia inducible factor1, P53 and Nur77 for assessment of carcinogenicity. *J. Toxicol. Pub. Health*, **23**, 231-238.

Huang, L.E., Gu, J., Sxhau, M. amd Bunn, H.F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 7987-7992.

Humble, N.M., Trempus, C.S., Spalding, J.W., Cannon, R.E. and Tennant, R.W. (2005). Biological, cellular, and molecular characteristics of an inducible transgenic skin tumor model: a review. *Oncogene*, **24**, 8217-8228.

Labay, K., Ould-Elhkim, M., Kles, V., Guffroy, M., Poul, J.M. and Sanders, P. (2001). Effects of griseofulvin in medium-term liver carcinogenesis assay and peripheral blood micronucleus test in rat. *Teratog. Carcinog. Mutagen.*, **21**, 441-451.

Lee, M.Y., Kim, M.A., Kim, H.J., Bae, Y.S., Park, J.I., Kwak, J.Y., Chung, J.H. and Yun, J. (2007). Alkylating agent methyl methanesulfonate (MMS) induces a wave of global protein hyperacetylation: Implications in cancer cell death. *Biochem. Biophys. Res. Commum.*, **360**, 483-489.

Lee, J.W., Bas, S.H., Jeong, J.W., Kim, S.H. and Kim, K.W. (2004). Hypoxia-inducible factor (HIF-1) alpha: its protein stability and biological functions. *Exp. Mol. Med.*, **36**, 1-12.

Machon, P.C., Hirota, K. and Semenza, G.L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev.*, **15**, 2675-2686.

Mangipudy, R.S., Rao, P.S. And Mehendale, H.M. (1996). Effect of an antimitotic agent colchicines on thioacetamide hepatotoxicity. *Environ. Health Perspect.*, **104**, 744-749.

Schindl, M., Schoppmann, S.F., Samonigg, H., Hausmaninger, H., Kwasny, W., Gnant, M., Jakesz, R., Kubista, E., Birner, P., Pberhuber, G., Austrian Breast and Colorectal Cancer Study Group. (2002). Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. *Clin. Cancer Res.*, **8**, 1831-1837.

Semenza, G.L. (2003). Targeting HIF-1 for cancer therapy. *Cancer*, **3**, 721-732.

Spalding, J.W., French, J.E., Tice, R.R. Furedi-Machacek, M., Haseman, J.K. and Tennant, R.W. (1999). Development of a transfenic mouse model for carcinogenesis bioassays: Evaluation of chemically induced skin tumors in Tg.AC mice. *Toxicol. Sci.*, **49**, 241-254.

Swinson, D.E., Jones, J.L., Cox, G., Richardson, D., Harris, A.L. and O'Byrne, K.J. (2004). Hypoxia-inducible factor-1 alpha in non small cell lung cancer: relation to growth factor, protease and apoptosis pathways. *Int. J. Cancer.*, **111**, 43-50.

Tennant, R.W., Margolin, B.H., Shelby, M.D., Zeiger E., Haseman, J.K. Spalding, J., Caspary, W., Resnck, M., Stasiewicz, S., Anderson, E. and Minor, R. (1987). Prediction of chemical carcinogenicity in rodents from *in vitro* genetic toxicity assays. *Science*, **236**, 933-941.

Tennant, R.W. (1988). Relationships between *in vitro* genetic toxicity and carcinogenisity studies in animals. *Ann. N. Y. Acad. Sci.*, **534**, 127-132.

Tomasz, M., Mercado, C.M., Olson, J. and Chatterjie, N. (1974). Mode of interaction of mitomycin C with deoxyribonucleic acid and other polynucleotides *in vitro*. *Biochemistry*, **13**, 4878-4887.

Trosko, J.E. (2001). Commentary: Is the concept of "tumor promotion" a useful paradigm? *Mol. Carcinog.*, **3**, 131-137.

Tsuchiya, T. and Umeda, M. (1997). Relationship between exposure to TPA and appearance of transformed cells in MNNG-initiated transformation of BALB/c3T3 cells. *Int. J. Cancer*, **73**, 271-276.

Vesselinovitch, S.D. and Mihailovich, N.T. (1968). The inhibitory effect of greseofulvin on the "promotion" of skin carcinogenesis. *Cancer Res.*, **28**, 2463-2465.

Wyatt, M.D. and Pittman, D.L. (2006). Methylating agents and DNA repair responses: Methylated bases and sources of strand breaks. *Chem. Res. Toxicol.*, **19**, 1580-1594.

Yoo, Y.G., Yeo, M.G., Kim, D.K., Park, H. and Lee, M.O. (2004). Novel function of orphan nuclear receptor Nur77 in stabilizing hypoxia-inducible factor-1 alpha. *J. Biol. Chem.*, **279**, 53365-53373.

Zagzag. D., Zhong, H., Scalzitti, J.M., Laughner, E., Simons, J.W. and Semenza, G. L. (2000). Expression of hypoxia-inducible factor 1α in brain tumors. *Cancer*, **88**, 2606-2618.

Zhong., H., De Marzo, A.M., Laughner, E., Lim, M., Hilton, D.A., Zagzag D., Buechler, P., Isaacs, W.B., Semenza, G.L. and Simons, J.W. (1999). Overexpression of hypoxiainducible factor 1α in commom human cancers and their metastases. *Cancer Res.*, **835**, 5830-5835.