응용약물학회지, The Journal of Applied Pharmacology 6, 345-350(1998)

Chronic Treatment of Ethanol Inhibits Proliferation of Normal Fibroblasts, but Not Oncogenic *ras*-Transformed Cells

Young-Hwa GU[1], Mi-Sun PARK[1,2] and Byung H. JHUN[1,2]*

[1]*Department of Pharmacy,*
[2]*College of Pharmacy, Research Institute of Oral Biotechnology,*
Pusan National University, Pusan, Korea 609-735

(Received October 15, 1998; accepted November 15, 1998)

Abstract – The adverse effects of ethanol on cell proliferation have been described for a variety of tissues and cells. In the present study, we investigated whether chronic ethanol intoxication impairs the cell proliferation and DNA synthesis induced by oncogenic H-*ras*^{V12}- and v-K-*ras*^{V12}-transformed cells. Ethanol treatment inhibited the cell proliferation and the DNA synthesis of control parental fibroblasts in a time- and dose-dependent manner. In contrast, ethanol did not suppress the proliferation of either oncogenic H-*ras*^{V12}- or v-K-*ras*^{V12}-transformed fibroblasts. Microinjection of oncogenic H-Ras^{V12} protein induces DNA synthesis and ethanol treatment did not interfere with the DNA synthesis. The antiproliferative toxicity of ethanol was rescued by antioxidants, such as N-acetylcysteine and 4-methlpyrazole. These results indicate that the antiproliferative action site of ethanol toxicity lies upstream or is independent of Ras and ethanol exerts its toxicity through a free radical formation.

Keywords □ Ethanol, ras, DNA synthesis, antioxidant

The proto-oncogene *ras* participates in various cellular processes, including proliferation, differentiation, and cytoskeletal organization (Bourne *et al.*, 1991; Vojtek and Der, 1998). Overexpression of *ras* and mutations of *ras* that render it constitutively active have been described in various human tumors, and mutated forms of *ras* genes at the position of 12, 13, and 61 residues are frequently associated with 30% of all human cancers (Bos, 1989). The mutated Ras protein causes constitutive, ligand independent activation of the proliferative signaling pathways, thereby promoting the abberrant growth of tumor cells.

Ethanol consumption has been reported to increase the risk of breast cancer (Singletary, 1997) and hepatocarcinoma (Ohnishi *et al.*, 1982). Ethanol acts as a co-carcinogen, which interacts with other carcinogens to cause tumorigenic response (Seitz and Simanowski, 1986). It enhances both the initiation and promotion stages of chemically induced rat mammary tumorigenesis. The International Agency for Research on Cancer (IARC) reviewed all of the literature in this field and concluded that there is sufficient evidence for the carcinogenicity of alcoholic beverage in human, whereas the evidence for the carcinogenicity of ethanol in animal is inadequate (IARC, 1987). Recently, Chen et al. (Chen *et al.*, 1996) reported that ethanol treatment of transgenic mice with an oncogenic H-*ras* oncogene exhibited a higher incidence of tumor than in control diet treatment, suggesting that ethanol, co-working with the oncogenic Ras protein, may play an important role in favoring tumorigenesis. Accurately defining the possible mechanisms for this cocarcinogenic effect of ethanol is required.

An adverse effect of ethanol on cellular proliferation has been described in the cerebral cortex (Miller and Nowakowski, 1991), Schwann cells (Miller and Nowakowski, 1991), lymphocytes (Jerrel *et al.*, 1986), osteoblasts (Klein and Carlos, 1995), rat hepatocytes (Carter *et al.*, 1988), and HL-60 myeloid leukemia cells (Cook *et al.*, 1990). Ethanol also interferes with the regeneration of hepatocytes following partial rat hepatectomy or chemical injury (Wands *et al.*, 1979 & 1980). Although the antiproliferative effects of ethanol are well documented, the exact action site at which ethanol acts has not been elucidated. In the present study, we investigated the relationship between the antiproliferative effect of ethanol and oncogenic Ras protein, by examining both the proliferation and DNA synthesis of fi-

*To whom correspondence should be addressed.

broblasts transformed with oncogenic H- and v-K-*ras*^{V12} oncogene. Our results indicate that the antiproliferative action site of ethanol does not lie downstream of Ras.

MATERIALS AND METHOD

Cell lines and materials

H-*ras*^{V12}-transformed Rat-2 (HO6) and v-K-*ras*^{V12}-transformed NIH3T3 (DT) were obtained from Dr. C.C. Kumar (Schering-Plough Research Institute) and maintained in DMEM containing 10% fetal bovine serum. Rat-2 and NIH3T3 fibroblasts were also maintained in the same medium. Rat-1 fibroblasts expressing human insulin receptors were maintained in Dulbecco's modified Eagles/F12 (DME/F12) medium supplemented with 10% fetal bovine serum as previously described (Jhun *et al.*, 1994). [^{3}H]-Thymidine, bromodeoxyuridine (BrdU), and mouse anti-BrdU antibody were purchased from Amersham. Rhodamine-conjugated anti-mouse IgG antibody and FITC -conjugated anti-rat IgG antibody were obtained from Jackson Lab. All other reagents were purchased from Sigma.

Cell proliferation assays

Cells were incubated with various concentrations of ethanol and refreshed everyday. To minimize the evaporative loss of ethanol, the cells were wrapped with parafilm and incubated at 37℃. The cells were then digested with trypsin and the cell number was counted with tryphan blue staining. The thymidine incorporation assay was performed as previously described (Jhun *et al.*, 1995). Ethanol-treated Rat-2 and HO6 cells were incubated with [^{3}H]-thymidine (0.5 uCi/well) for the final 16 hr and NIH3T3 and DT cells for 4hr. The cells were washed, dissolved in 1N NaOH, and counted in a β counter. Antioxidants, including N-acetylcysteine (1 mM), 4-methylpyrazole (1 mM), and vitamin E (25 mM), were treated for 30 min at 37℃ and then incubated with ethanol (100 mM). The effects of antioxidants on the antiproliferative effect of ethanol were examined as described above.

Microinjection and BrdU immunostaining

Single cell microinjection of recombinant oncogenic H-Ras^{V12} into Rat-1 fibroblasts expressing human insulin receptor and the DNA synthesis were examined as previously described (Jhun *et al.*, 1994). Briefly, cells were plated on coverslips and arrested cell cycle in serum-free DMEM. During cell cycle arrest, ethanol (100 mM) was treated for 4 hr before microinjection. The oncogenic H-Ras^{V12} protein (2 mg/ml) was then microinjected with rat IgG (4 mg/ml) to identify the microinjected cells. An average of 200 cells was injected in three coverslips per each condition and the experiment was performed in duplicate. The cells were incubated at 37℃ for 16 hr in the presence of BrdU and fixed with 90% of ethanol. The injected cells were immunostained with anti-BrdU antibody followed by TRITC-conjugated anti-mouse IgG antibody. To detect the injected cells, the cells were stain-

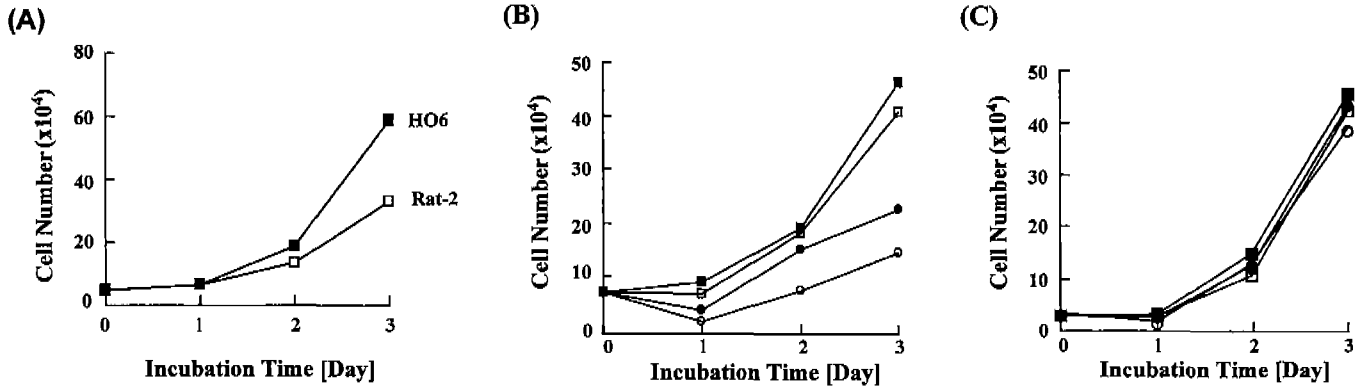

Fig. 1. Effects of chronic ethanol treatment on the proliferation of oncogenic H-*ras*^{V12}-transformed Rat-2 (HO6) and parental Rat-2 fibroblasts. (A) Proliferation of HO6 and Rat-2 cells. HO6 and Rat-2 cells (4×10^4 cell/well) were plated in 12 well-plate and the medium was changed everyday. The number of HO6 (■) and Rat-2 (□) cells was counted as described in the "Materials and Method". (B) Effects of ethanol on the proliferation of parental Rat-2 cells. Rat-2 cells were incubated in the presence of 0 mM (■), 1 mM (□), 10 mM (●), and 100 mM (○) of ethanol. The plates were tightly wrapped with parafilm, and media containing ethanol were refreshed everyday. The cell number was counted. (C) Effects of ethanol on the proliferation of HO6 cells. HO6 cells were incubated in the presence of 0 mM (■), 1 mM (□), 10 mM (●), and 100 mM (○) of ethanol. The plates were tightly wrapped with parafilm and media were refreshed everyday. The cell number was counted. The data of panels A, B, and C represent the means of two experiments and each was performed three times.

ed for co-injected rat IgG with FITC-labeled anti-rat IgG antibody. The injected cell was identified by cytoplasmic FITC staining and DNA synthesis was identified by nuclear TRITC staining. The cells were inspected and photographed with a Zeiss Axioplan fluorescence microscope.

RESULTS AND DISCUSSION

Ethanol inhibits the proliferation of normal fibrobalsts, but not oncogenic H- and v-K-*ras*^{V12}-transformed cells

Inhibition of cell proliferation by ethanol is a common observation in diverse tissues and cells (Miller and Nowakowski, 1991; Jerrels *et al.*, 1986; Klein and Carlos, 1995; Carter and Wands, 1988; Cook *et al.*, 1990; Wands *et al.*, 1979 & 1980), but its mechanism remains unknown. In the present study, we first examined the effects of ethanol on the growth rate of normal Rat-2 fibroblasts and oncogenic H-*ras*^{V12} transformed Rat-2 (HO 6) fibroblasts (Fig. 1). Rat-2 and HO6 cells proliferated exponentially up to 3 days and oncogenic *ras*^{V12}-transformed HO6 cells grew faster than parental Rat-2 cells (Fig. 1A). As seen in Fig. 1B, ethanol inhibited the proliferation of parental Rat-2 cells in a time- and dose-dependent manner consistent with the previous results of other cell lines. In contrast, the proliferation of oncogenic H-rasV12-transformed HO6 cells was normal, even in the presence of ethanol, indicating that the action site of ethanol toxicity does not lie downstream of the Ras protein.

In order to confirm the ethanol-independent proliferation of ras-transformed cells, the proliferation of oncogenic v-K-*ras*^{V12}-transformed cells was also examined (Fig. 2). Ethanol treatment inhibited the proliferation of parental NIH3T3 fibroblasts in a similar manner of Rat-2 cells (Fig. 2A) and did not inhibit that of v-K-*ras*^{V12}-transformed NIH3T3 cells (Fig. 2B), which was consistent with the results of H-*ras*^{V12} transformed HO6 cells. We also found the same antiproliferative effects of ethanol in HepG2 and PC12 cells (data not shown). These results suggest that the action site of antiproliferative effects of ethanol lies upstream or is independent of Ras protein.

Ethanol did not block DNA synthesis of oncogenic *ras*^{V12}-transformed fibroblasts and oncogenic Ras^{V12}-microinjected cells

We further examined the antiproliferative effects of ethanol by measuring DNA synthesis in normal and oncogenic *ras*^{V12}-transformed cell (Table 1). In parental fibroblasts such as Rat-2 and NIH3T3 fibroblasts, DNA synthesis in the ethanol treated cells was inhibited in a dose-dependent manner. 100 mM of ethanol treatment decreases DNA synthesis of Rat-2 and NIH3T3 by 45% and 26%, respectively. In contrast, the proliferation of oncogenic *ras*^{V12} transformed Rat-2 and NIH3T3 fibroblasts was not affected by ethanol treatment. DNA synthesis of *ras*^{V12}-transformed HO6 and DT cells somewhat increased in a low concentration of ethanol. These results

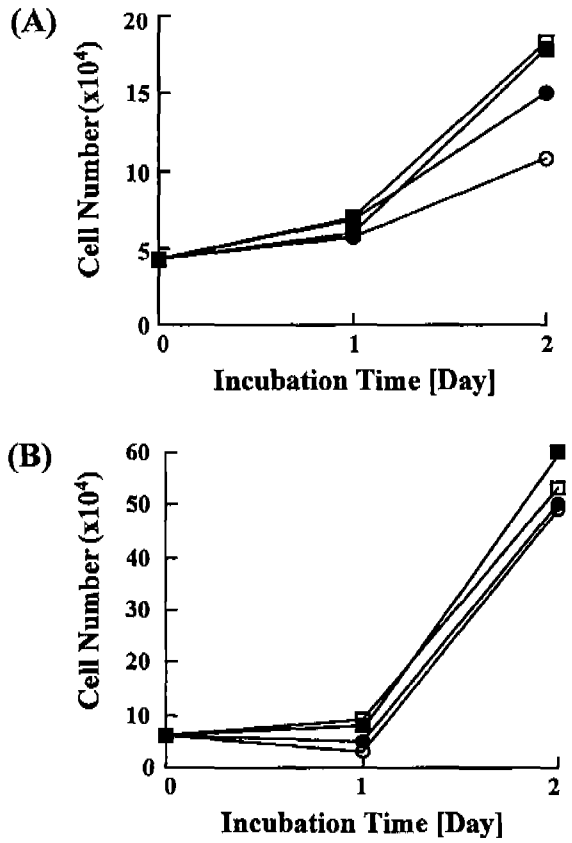

Fig. 2. Effects of chronic ethanol treatment on the proliferation of oncogenic v-K-*ras*^{V12}-transformed NIH3T3 (DT) and parental NIH3T3 fibroblasts. DT and NIH3T3 cells (4×10^4 cell/well) were grown in 12 well-plate up to 2 days, and then cell number was counted as described in the "Materials and Method". (A) Effects of ethanol on the proliferation of parental NIH3T3 cells. Rat-2 were incubated in the presence of 0 mM (■), 1 mM (□), 10 mM (●), and 100 mM (○) ethanol. The plates were tightly wrapped with parafilm, and media were refreshed everyday. The cell number was counted. (B) Effects of ethanol on the proliferation of DT cells. DT cells were incubated in the presence of 0 mM (■), 1 mM (□), 10 mM (●), and 100 mM (○) ethanol. The plates were tightly wrapped with parafilm and media were refreshed everyday. The cell number was counted. The data represent the means of two experiments and each was performed three times.

Table 1. Effects of ethanol on DNA synthesis in Rat-2, NIH 3T3, HO6, and DT cells. Cells (4×10^4 cells/plate) were incubated with the indicated concentrations of ethanol for 2 day, then 0.5uCi of [^{3}H]-thymidine was incubated for either 16hr (Rat-2 and HO6 cells) or 4 hr (NIH3T3 and DT cells). The cells were rinsed, dissolved in 1N NaOH, and counted in a β counter. Results are expressed as a percent of control, and the presented results are the means of two independent experiments and each was performed three times

Ethanol (mM)	Rat-2	HO6	NIH3T3	DT
0	100.0	100.0	100.0	100.0
1	93.3±16.2	128.0±12.2	92.5±11.3	109.1±13.2
10	79.7±12.7	97.9±11.0	94.8±7.2	141.2±13.7
50	57.1±8.6	94.6±3.0	81.0±11.1	107.5±9.0
100	54.9±5.1	87.1±4.3	73.8±1.7	101.5±11.5

again suggest that the action site of ethanol intoxication lies upstream of the Ras protein.

In order to confirm the relationship between oncogenic Ras^{V12} and ethanol action, we utilized a single cell microinjection technique. The cell cycle-arrested, quiescent Rat-1 fibroblasts were microinjected with the oncogenic H-Ras^{V12} protein and then the effect of ethanol on the DNA synthesis was examined. From our previous report (Jhun *et al.*, 1994), microinjection of oncogenic H-Ras^{V12} protein induced DNA synthesis in a dose-dependent manner. Cells were grown on glass coverslips, serum-starved, and trated with 100 mM of ethanol. The cells were then microinjected with Ras^{V12} (2 mg/ml). As shown in Fig. 3, approximately 10% of the uninjected cells on the same coverslip or control IgG-injected cells underwent DNA synthesis. In contrast, injection of oncogenic Ras^{V12} protein induced a number of cells underwent DNA synthesis, but ethanol did not affect the DNA synthesis in the oncogenic H-Ras^{V12}-injected cells. These results indicate that the site of antiproliferative action of ethanol lies upstream of the Ras protein in the signaling pathway of the Ras protein leading to the DNA synthesis and cell proliferation.

Antiproliferative effect of ethanol is mediated through free radical formation

It has been reported that free radical formation is important in the effect of ethanol (Reinke *et al.*, 1997; Rouch *et al.*, 1997). We next examined the effect of various free radical trapping agents on the antiproliferative effect of ethanol. Rat-2 fibroblasts were pretreated with

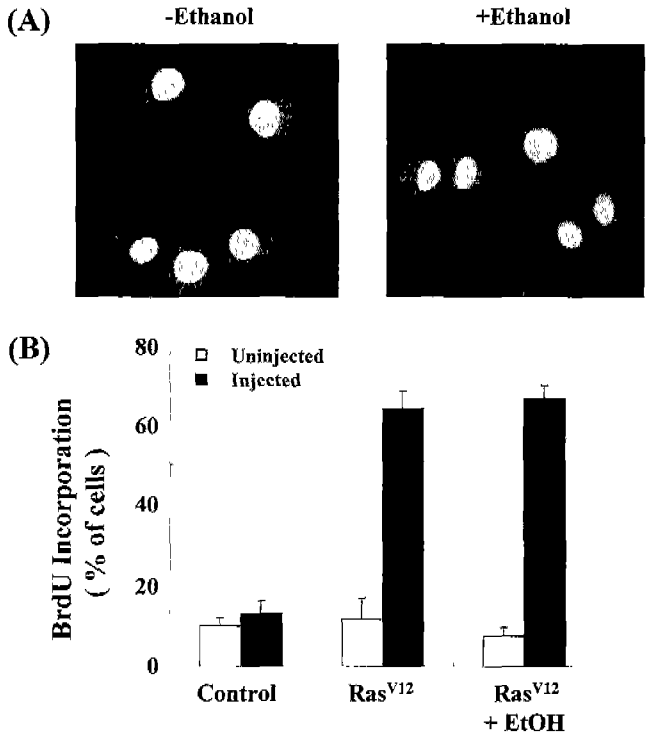

Fig. 3. Effects of ethanol on the DNA synthesis induced by microinjection of oncogenic H-RasV12 protein in Rat-1 fibroblasts overexpressing insulin receptor (HIRc-B). HIRc-B cells were starved for 24 hr and then injected with the oncogenic H-Ras^{V12} protein (4 mg/ml) containing rat IgG (5 mg/ml). Ethanol was treated 1hr before microinjection. After stabilization for 2hr, cells were incubated with BrdU for 16 hr at 37℃. They were then processed for double-label indirect immunofluorescence by sequential incubation of mouse anti-BrdU antibody, rhodamine-conjugated anti-mouse IgG antibody, and FITC-conjugated anti-rat IgG antibody. The injected cells were identified by cytoplasmic FITC staining, and DNA synthesis was identified by nuclear TRITC staining (*panel A*). An average of 167 control IgG-injected (range 152-187) cells, 156 H-Ras^{V12}-injected (range 145-188) cells, and 186 H-Ras^{V12}-injected and ethanol-treated (range 154-195) cells were counted (*panel B*). The results presented represent the means of two experiments and each was performed three times.

antioxidants such as N-acetylcysteine, a free radical scavenger, 4-methylpyrazole, a CYP2E1 inhibitor, and vitamin E, a lipid peroxidation inhibitor. The cells were pretreated with antioxidant and the ethanol-induced antiproliferative effect was examined (Fig. 4). N-acetylcycteine and 4-methylpyrazole effectively rescued the antiproliferative effects of ethanol, whereas vitamin E recovered ethanol intoxication with less potency. These results indicate that a free radical plays an important role in the antiproliferative effects of ethanol.

The presented data demonstrate that *ras*-induced on-

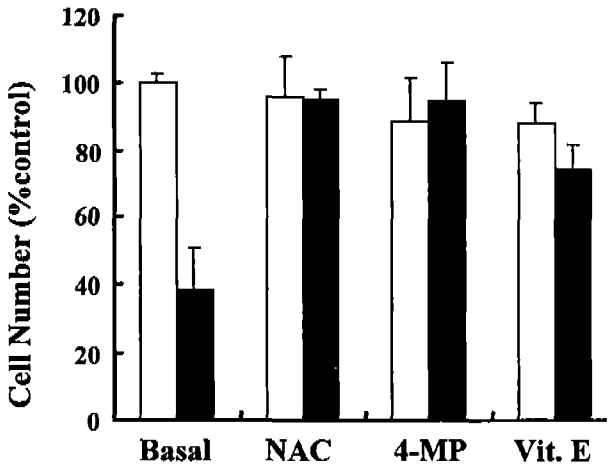

Fig. 4. Inhibition of antiproliferative effects of ethanol by antioxidants in Rat-2 cells. Rat-2 cells (4×10^4 cells/plate) were plated along with N-acetylcysteine (NAC) (1 mM), 4-methylpyrazole (4-MP) (1 mM), and vitamin E (Vit. E) (25 mM), and after 2 hr, the cells were incubated in the absence (filled bars) or presence (open bars) of 100mM ethanol. The cells were incubated for 2 days and cell numbers were determined with tryphan blue staining. The data represent the means of two experiments and each was performed three times.

cogenicity is not inhibited by chronic ethanol intoxication. The treatment of ethanol markedly reduced the proliferation and DNA synthesis of normal parental Rat-2 and NIH3T3 fibroblasts. Rat-2 fibroblasts, which proliferated at a slower rate, was affected more sensitively than NIH3T3 cells. In both oncogenic ras^{V12}-transformed HO6 and DT cells, cell growth and DNA synthesis was unaffected by ethanol. Single cell microinjection provides a direct means to determine whether an endogenous signaling molecule is required for a particular phenotype and whether a chemical has an effect on the role of signaling molecules (in this case, ethanol bioeffects). In the current study, we conducted a microinjection study with a constitutively active oncogenic Ras^{V12} protein and found that DNA synthesis was fully activated even in the presence of ethanol. These results indicate that the action site of the oncogenic Ras^{V12} protein is distal to the locus at which ethanol exerts its inhibitory effect.

There is ample evidence indicating that Ras is an important intermediate component in the growth factor-signaling pathway leading to DNA synthesis and cell proliferation (Bourne *et al.*, 1991; Vojtek and Der, 1998). Transfection or microinjection of oncogenically activated Ras mimics growth responses (Stacey *et al.*, 1987; Benot *et al.*, 1991). The *ras* proto-oncogene is frequently mutated in 30% of human tumors (Bos, 1989), making *ras* the most widely mutated human proto-oncogene. Whereas the signaling pathway and molecular mechanism underlying Ras-mediated cell cycle progression is well understood, there is no report elucidating the relationship between oncogenic ras^{V12}-induced oncogenicity and ethanol's cocarconogenic property. From our results, we conclude that the action site of ethanol lies upstream or is independent of Ras. The proliferation of normal cells is inhibited by ethanol, but that of *ras*-transformed cells is unaffected. Therefore, within the same tissue, transformed cells are continuously proliferating, even in the presence of ethanol, while normal cells stop proliferating. This disturbance of growth in normal cells caused by ethanol may favor tumorigenesis.

ACKNOWLEDGEMENT

This work was supported by '95 SPECIAL FUND for UNIVERSITY RESEARCH INSTITUTE, Korea Research Foundation.

REFERENCES

Benito, M., Porras, A., Nebreda, A. R. and Santos, E. (1991). Science **253**, 565-568.

Bos, J. L. (1989). Cancer Res. **49**, 4682-4689.

Bourne, H., Sanders, D. and McCormick, F. (1991). Nature **349**, 117-127.

Carter, E. A. and Wands, J. R. (1988). Alcohol. Clin. Exp. Res. **12**, 555-562.

Chen, G., Colombo, L. L., Lopez, M. C. and Watson, R. R. (1996) Int. J. Immunopharmacol. **18**, 251-258.

Cook, R. T., Keiner, J. A. and Yen, A. (1990) Alcohol. Clin. Exp. Res. **14**, 695-730.

IARC (1987) Monograph on the Evaluation of Carcinogenicity: An Update of IARC Monographs. Lyon: World Health Organization, International Agency of Research on Cancer, 1987, vols 1-42, Suppl. 7.

Jerrels, T. R., Smith, W., Eckardt, M. J. (1986). Alcohol. Clin. Exp. Res. **14**, 546-550.

Jhun, B. H., Haruta, T., Meinkoth, J. M., Leitner, J. W., Draznin, B., Saltiel, A. R., Pang, L., Sasaoka, T. and Olefsky, J. M. (1995). Biochemistry **34**, 7996-8004.

Jhun, B. H., Meinkoth, J. M., Leitner, J. W., Draznin, B. and Olefsky, J. M. (1994). J. Biol. Chem. **269**, 5699-5704.

Klein, R. F. and Carlos, A. S. (1995). Endocrinology **136**, 3406-3411.

Miller, M. W. and Nowakowski, R. S. (1991). Alcohol. Clin. Exp. Res. **15**, 229-232.

Ohnishi, K., Iida, S., Iwama, S., Goto, N., Nomura, F., Takashi, M., Mishima, A., Kono, K., Kimura, K., Musha, H.,

Kotota, K. and Okuda, K. (1982) Cancer **49**, 672-677.
Reinke, L. A., Moore, D. R. and McCay, P. B. (1997). Alcohol. Clin. Exp. Res. **21**, 642-646.
Rouch, H., Fataccioli, V., Gentil, M., Frech, S. W., Morimoto, M. and Nordmann, R. (1997) Hepatology **25**, 351-355.
Seitz, H. K. and Simanowski, U. A. (1986). Alcohol. Clin. Exp. Res. 10 (6 Suppl) 33S-40S.
Singletary, K. (1997). Alcohol. Clin. Exp. Res. **21**, 334-339.
Stacey, D. W., Watson, T., Kung, H.F. and Curran, T. (1987). Mol. Cell. Biol. 7, 523-527.
Vojtek, A. B. and Der, C. J. (1998) J. Biol. Chem. **273**, 19925 -19928.
Wands, J. R., Carter, E. A., Bucher, N. L. and Isselbacher, K. J. (1979). Gastroenterology **77**, 528-531.
Wands, J. R., Carter, E. A., Bucher, N. L. R. and Isselbacher, K. J. (1980) Adv. Exp. Med. Biol. **132**, 663-670.